Doctoral Dissertation

A STUDY ON EFFECTIVE USE OF PRESSURIZED CARBON DIOXIDE FOR INACTIVATION OF PATHOGENS AS A NOVEL DISINFECTION METHOD FOR BALLAST WATER

(加圧二酸化炭素を効果的に利用したバラスト水中の病原微生物の新規殺菌方法に関する研究)

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A dissertation submitted to the Division of Environmental Science and Engineering of Yamaguchi University in partial fulfillment of the requirements for the degree of Doctor of Engineering (Dr. Eng.)

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ABSTRACT

Ballast water is routinely used onboard ships to adjust the manoeuvrability and stability at ocean but is now widely recognized as a serious environmental issue because of the risk of introducing alien species from ballast water discharge. Therefore, disinfection of ballast water plays an important role in preventing the spread of invasive species worldwide, and advanced water disinfection technologies that do not produce harmful by-products would be highly desirable. This dissertation presents results for the use of pressurized carbon dioxide (PCD) at less than 1.0 MPa and a liquid-film-forming apparatus for disinfection of seawater.

Escherichia coli, *Enterococcus* sp., and *Vibrio alginolyticus* were used as model microorganisms for examining the bactericidal performance of PCD. The sensitivity of three bacterial species to the PCD treatment was examined for various conditions of pressure, temperature, working volume ratio (WVR). Additionally, leakage of proteins and nucleic acids from cells was measured. Cell morphology of untreated cells and cells treated with PCD was assessed using scanning electron microscopy (SEM). PCD treatment affected all the bacterial species; however, *Enterococcus* sp. exhibited higher resistance to the PCD treatment than did *E. coli* and *V. alginolyticus.* Under the experimental treatment conditions (0.7 MPa, 70% WVR, 20 ± 1.0°C, and initial concentration of 5–6 log 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* and *V. alginolyticus* within 10 min and 3 min, respectively. Release of intracellular contents occurred during the treatment process and SEM images of *E. coli*, *Enterococcus* sp., and *V. alginolyticus* revealed that morphological changes had occurred after the treatment with PCD. These data indicated that PCD has potential applications for inactivating pathogens in ballast water.

To enhance the bactericidal activity of PCD, effects of sequences involving pressure cycling was employed. The key influences on frequency and magnitude of pressure cycling in enhancing *E. coli* and *Enterococcus* sp. inactivation are elucidated. The results revealed strong correlation between pressure cycling and inactivation efficiency (p<0.001). The results from linear regression analysis suggest that the model can explain about 91% of the *E. coli* inactivation efficiency (p<0.001). Approximately 5.3 log of the *E. coli* load was completely inactivated within 5 min by using PCD (100% CO_2, at 0.7 MPa, 20°C, 70% WVR) in the process involving pressure cycling (ΔP = 0.12 MPa, 18 cycles). As for *Enterococcus* sp. inactivation, the outcome of linear regression model analysis suggests that the model can

explain 93%, 85%, and 89% of the inactivation efficiency of (25% CO_2 + 75% N_2), (50% CO_2 + 50% N_2), and 100% CO_2, respectively. Under identical treatment conditions (pressure = 0.9 MPa, ΔP = 0.14 MPa, 70% WVR, and 20 ± 1°C), treatment with PCD (100% CO_2) resulted in complete inactivation 5.2 log of *Enterococcus* sp. after 70 cycles within 20 min. The *Enterococcus* sp. inactivation of PCD followed first-order reaction kinetics. The smallest *D*-value (largest *k*-value) was induced by PCD (100% CO_2) at 0.9 MPa, which was obtained at 3.85 min (0.5988 min^{-1}, $R^2 \geq 0.95$). The findings could provide an effective method for enhanced bactericidal performance of PCD.

High flow rate and large volumes of ballast water need to be treated according to the D-2 standard of the International Maritime Organization (IMO); however, space on a ship for such operations is typically limited. To improve the disinfection efficiency and reduce the treatment time, disinfection using PCD combined with a low-dosage of chlorine (NaOCl) was employed to inactivate of *Enterococcus* sp. in artificial seawater and bacteria (i.e. *E. coli*, enterococci, and vibrios) in natural seawater. Combined PCD/chlorine treatments resulted in greater disinfection efficiency than those for the two individual treatments. Synergy values were correlated with pressure and CO_2 concentrations ($p<0.001$). As for the disinfection of *Enterococcus* sp. in artificial seawater, combined treatment with PCD (100% CO_2, 0.3 MPa, 70% WVR, and 20°C) and chlorine (0.20 mg L^{-1}) achieved an average synergy value of 4.6 log and complete inactivation (5.2 log reduction) of *Enterococcus* sp. within 4 min. In contrast, when the two individual treatments (PCD and chlorine treatment) were used, only 3.7 and 1.8 log reductions were achieved after 25 min, respectively. On the other hand, combination of 0.3 MPa PCD (various CO_2 supply rates: 25% CO_2 + 75% N_2, 50% CO_2 + 50% N_2, 75% CO_2 + 25% N_2, and 100% CO_2) and chlorine (0.20 mg L^{-1}) substantially reduced bacterial viability in natural seawater. Specifically, the combined PCD/chlorine treatments reduced the number of *E. coli*, enterococci, and vibrios (include *Vibrio cholerae*) to below the IMO D-2 discharge standard within 3 min. These findings suggest that the combined PCD/chlorine treatment has synergistic benefits and provides a promising method for the disinfection of ballast water.

学位論文要旨

バラスト水は船舶の海洋における操作安定性のために恒常的に使われている。しかしながら、バラスト水に由来する外来生物の越境が問題となり、現在深刻な環境問題の 1 つとして認識されている。それゆえに、バラスト水の殺菌は外来生物の世界における広範な侵入を防ぐために重要な役割を果たす。また、有害な副生成物を生じない高度な殺菌技術の開発が求められている。この論文では、液膜生成装置を利用した 1 Mpa 以下の加圧二酸化炭素（PCD）による海水の殺菌に関して述べる。

PCD による殺菌実験に用いるモデル微生物として大腸菌（*Escherichia coli*）、腸球菌（*Enterococcus* sp.）、ビブリオ菌（*Vibrio alginolyticus*）を選定し、様々な圧力、温度、装置有効容積（WVR）について実験を行った。加えて、タンパク質と核酸について細胞からの溶出量を測定した。PCD による殺菌処理前後の細胞の様子を SEM により観察した。PCD による殺菌処理はすべての細菌に有効であったが、腸球菌（*Enterococcus* sp.）は大腸菌（*Escherichia coli*）やビブリオ菌（*Vibrio alginolyticus*）に比較して、高い抵抗性を示した。腸球菌（*Enterococcus* sp.初期菌数 5–6 $\log_{10}$ CFU mL^{-1}）について 4.1 log の殺菌効果を得るためには、圧力が 0.7 MPa、 WVR が 70%、 水温が 20 ± 1.0°C、処理時間が 25 分間必要であった。一方で、大腸菌（*Escherichia coli*）とビブリオ菌（*Vibrio alginolyticus*）に関しては、同じ設定条件でそれぞれ 10 分間、3 分間で完全な殺菌が可能であった。細胞内物質の溶出は処理中に生じており、大腸菌（*Escherichia coli*）、腸球菌（*Enterococcus* sp.）、ビブリオ菌（*Vibrio alginolyticus*）の SEM による観察で PCD 処理後にその形態的変化が生じていたことが確認された。以上のことは、PCD による殺菌がバラスト水中の病原性微生物の殺菌に有効である可能性を示すものと考えられる。

PCD の殺菌能力を増幅させるために、圧力サイクルに注目した。大腸菌（*Escherichia coli*）、腸球菌（*Enterococcus* sp.）の殺菌において、圧力サイクルの頻度（何回装置内を通過するか）とその程度（装置内に入る前後の圧力差）がキーとなることが示された。この結果は、圧力サイクルと殺菌効率との強い関係（$p<0.001$）を示している。線形回帰分析の結果から、このモデルによって約 91%の大腸菌（*Escherichia coli*）の殺菌効率を説明できる（$p<0.001$）ことが示唆された。おおよそ 5.3 log の大腸菌（*Escherichia coli*）を 5 分間で PCD（ガスとして 100%の CO_2 を用い、0.7MPa の圧力、20℃の水温、70%の WVR の条件下で、圧力サイクルの頻度が 18 サイクル、その際の圧力差ΔP = 0.12 MPa）により完全に殺菌できた。腸球菌（*Enterococcus* sp.）の殺菌の場合は、線形回帰分析の結果から、このモデルによって(25% CO_2 + 75% N_2)をガスとして用いた場合に 93%、 (50% CO_2 + 50% N_2) をガスとして用いた場合に 85%、(100% CO_2)をガスとして用いた場合に 89%の殺菌が可能であることが示唆された。理想的な条件下（圧力が 0.9 MPa、圧

力差ΔP = 0.14 MPa、70% の WVR、水温 20 ± 1°C）において、100％CO_2 を用いた PCD 処理（圧力サイクルの頻度が 70 サイクル、処理時間 20 分間）により、5.2 log の腸球菌（*Enterococcus* sp.）の完全殺菌が可能であった。腸球菌（*Enterococcus* sp.）の PCD 処理による殺菌は、１次反応にしたがうことがわかった。100％の CO_2 を用いた場合の PCD 処理（圧力 0.9 MPa、処理時間 3.85 分間）によって最小の *D* 値が導き出された。この結果は、PCD 処理による殺菌効率を向上させるための有効な方法を提供するものである。

高流量かつ極めて大きな体積を有するバラスト水は国際海事機構（IMO）の定める D-2 規格にしたがって処理されねばならない。しかしながら、船舶の限られた面積の中ではそれを満たす処理施設の設置は難しい。殺菌効率の改善とその処理に要する時間の大幅な削減を目指して、PCD 処理に少量の塩素処理（NaOCl）を組み合わせた殺菌方法を考案した。この方法により人工海水中での腸球菌（*Enterococcus* sp.）の殺菌、さらにはろ過後の自然海水中での大腸菌（*Escherichia coli*）、腸球菌（*Enterococcus* sp.）、ビブリオ菌（*Vibrio alginolyticus*）の殺菌実験を行った。実験結果から、この方法により個別に処理を行った場合より極めて大きな殺菌効率が得られた。相乗効果は圧力と CO_2 濃度とに相関があった（p<0.001）。人工海水中での腸球菌（*Enterococcus* sp.）の殺菌において、100％の CO_2 を用いた場合の PCD 処理（圧力 0.3 MPa、70％の WVR、水温 20℃）に塩素処理（0.20 mg L^{-1}）を組合せることで、完全殺菌（5.2 log reduction）を 4 分間で行うことができた。対照的に、それぞれ単独で処理した場合には 25 分間の処理により 3.7 log reductions と 1.8 log reductions しか得られなかった。一方で、0.3MPa の圧力下における PCD 処理（様々な CO_2 濃度：25% CO_2 + 75% N_2、50% CO_2 + 50% N_2,、75% CO_2 + 25% N_2 、100% CO_2）と塩素処理（0.20 mg L^{-1}）との組合せにより、自然海水中のバクテリアの実質的な増殖能力を削減できた。特筆すべきは、PCD 処理と塩素処理との組合せにより、わずか 3 分で大腸菌（*Escherichia coli*）、腸球菌（*Enterococcus* sp.）、ビブリオ菌（*Vibrio alginolyticus*）のそれぞれについて IMO の設定する D-2 の基準値以下まで殺菌を行うことができたことである。以上より、PCD 処理と塩素処理との組合せ殺菌方法は、バラスト水の殺菌方法として相乗効果を発揮することで十分な性能を有することが示されたと考えられる。

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LIST OF ABBREVIATIONS

ATCC:	American Type Culture Collection
BHI:	Brain Heart Infusion
BWTS:	Ballast water treatment system
CFU:	Colony forming units
CO_2:	Carbon dioxide
COD:	Chemical oxygen demand
DBPs:	Disinfection by-products
E. coli:	*Escherichia coli*
EPA:	Environmental Protection Agency
EtOH:	Ethanol
HOCl:	Hypochlorous acid
IMO:	International Maritime Organization
LB:	Luria-Bertani
Log:	logarithm
Log (N/N_0):	Log reduction ratio of microorganisms
NaOCl:	Sodium hypochlorite
N:	Number of colony counts after disinfection
N_0:	Number of colony counts before disinfection
N_2:	Nitrogen
NO_3-N:	nitrate-nitrogen
NO_2-N:	nitrite-nitrogen
NH_4-N:	ammonia-nitrogen
OD_{260}:	Absorbance at 260 nm wavelength (measured as nucleic acids in the supernatants)
OD_{280}:	Absorbance at 280 nm wavelength (measured as proteins in the supernatants)
OCl^-:	Hypochlorite ion
PBS:	Phosphate buffered saline
PCD:	Pressurized carbon dioxide
PO_4-P:	elemental phosphorus in the phosphate form

PN:	Pressurized nitrogen
PS:	Physiological saline
R^2:	Correlation coefficient
rpm:	Round per minute
SEM:	Scanning electron microscopy
t-BuOH:	Tert-Butanol
TCBS:	Thiosulfate citrate bile salts sucrose
THMs:	Trihalomethanes
TSS:	Total Suspended Solids
TP:	Total phosphorus
TN:	Total nitrogen
USCG:	United States Coast Guard
UV:	Ultra Violet
V. alginolyticus:	*Vibrio alginolyticus*
wt/vol:	Weight per volume
WVR:	Working volume ratio
MARPOL:	The Marine Pollution Convention for the prevention of air pollution from ships

GLOSSARY

1. **A low-dosage of chlorine:** is defined as less than the normal dosage required for sufficient inactivation by chlorine.
2. **Alien species:** means, with respect to a particular ecosystem, any species that is not native to that ecosystem.
3. **Aquatic invasive species:** includes phytoplankton, zooplankton, and aquatic pathogens that are not native and that may flourish in a new marine environment when introduced by various vectors, one of which is shipping. The presence of aquatic invasive species may cause ecosystem and infrastructure damage, economic losses and may pose risks to human health. Shipping related pathways for the transfer of aquatic invasive species include vessel ballast water.
4. **Bactericidal effect:** an antimicrobial that kills a bacterium is said to be bactericidal.
5. **Convention:** is the International Convention for the Control and Management of Ships' Ballast Water and Sediments.
6. **Disinfection:** disinfection means reducing the number of a viable microorganisms present in a sample.
7. **Disinfection by-products (DBPs):** disinfectants (i.e. chlorine) react with number of organic and inorganic compounds in water. Some of these by-products are dangerous to human health, while others are disinfectants.
8. **Invasive species:** is defined as alien species whose introduction does or is likely to cause economic or environmental harm or harm to human health.
9. **Pressurized CO_2:** CO_2 gas is dissolved in water in a pressurized condition.
10. **Pressure cycling:** is defined as a repetitive procedure that involves the decompression and compression of CO_2. 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. In this study, pressure cycling was conducted at low pressure (<1.0 MPa) and no discharge of CO_2 between each cycle of raised and lowered pressure.
11. **Sterilization:** sterilization is the killing of all microorganisms in source of water, a media, a material or on the surface of an object.

12. **Synergy value:** is calculated as the efficiency of the combined pressurized CO_2 and chlorine in compared with the two individual treatments.
13. **The D-2 standard:** Ballast water discharge standard by the International Maritime Organization.
14. **The decimal reduction time (*D*-values):** is the exposure time required for a 1-log reduction in the bacterial load.
15. **The USCG standard:** is the standard for living organisms in ships' ballast water discharged in United States waters.
16. **Vessel ballast water capacity:** is given in terms of volume of spaces that are available for ballasting expressed in m^3, and in terms of the ballast pump capacity expressed in m^3/h.
17. **Working volume ratio:** is defined as the ratio between the sample volume and apparatus volume.

CHAPTER I

INTRODUCTION

1.1 Problem statement

Interesting is growing on ballast water disinfection technologies. Ballast water is used to maintain safety and stability of ships during a voyage when ships are not laden with cargo or during cargo loading operations. Annually, about 3–5 billion tonnes of ballast water containing aquatic species is transferred among the world's oceans (GloBallast 2016), and if these organisms are released into new ecosystems that support their growth, they can become invasive species (Ruiz *et al*. 1997; David & Gollasch 2015). Invasive species pose threats to ecosystems and can even increase risks to human health (Ruiz *et al.* 1997). To avoid these problems, the International Maritime Organization (IMO) developed regulations for the control and management of ballast water (IMO 2004). The regulations require that the number of viable organisms in ballast water must be less than the level set out in the D-2 ballast water performance standard when discharged into the ocean (IMO 2004).

Thousands tonnes of ballast water need to be treated in as short as possible exposure time. In addition to the effectiveness of the treatment at inhibiting pathogens, other factors to consider when selecting a shipboard treatment method include the size of treatment equipment, cost-efficiency and environmentally safety concerns. Though there are many water treatment methods available, however, when applying those to ballast water disinfection purpose, no single treatment method can fulfilled these requirements (Tsolaki *et al*. 2010; David and Gollash 2015). 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 and inorganic compounds in seawater generates carcinogenic agents such as trihalomethanes, halogenic acetic acids, and bromate (Boorman *et al.* 1999; Fabbricino and Korshin 2005). Furthermore, some resistant microorganisms may only be inactivated with very high chlorine doses, which can exacerbate the formation of disinfection by-products (DBPs) (LeChevallier 2004). 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 (Von Gunten 2003; Werschkun *et al.* 2012). De-oxygenation by the injection of an inert gas (i.e. N_2, CO_2) is a cost effective disinfection method and can reduce corrosion of ballast tanks (Gregg *et al.*, 2009). However, this method may not be appropriate if the journey of the ship is short because it usually takes 1 to 4 days to reach acceptable discharge standards and asphyxiate organisms (Lloyd's Register, 2012). Besides, some organisms such as phytoplankton, cysts and spores, and anaerobic bacteria may adapt to such hypoxia, which makes the treatment more challenging (Gregg *et al.*, 2009; David and Gollasch, 2015). 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 (Werschkun *et al.* 2012). 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 (Werschkun *et al.* 2012). Taken together, each of the disinfection methods discussed here have their own advantages and disadvantages with regard to factors such as efficient at removing target organisms, cost, energy and space requirements, operational efficacy and environmentally-friendly perspective. Thus, it is necessary to develop new technologies for ballast water disinfection in a manner that exploits the advantages of current technology and minimise the disadvantages of the conventional methods. This study investigates the use of pressurized carbon dioxide (CO_2) and a liquid-film-forming apparatus for seawater disinfection.

Sterilization by using pressurized CO_2 (PCD) has been an active research field for decades (Haas *et al.* 1989; Garcia-Gonzalez *et al.* 2007). CO_2 has been used extensively to sterilize dried food and liquid products via a non-thermal sterilization method (Spilimbergo *et al.* 2002) because of its effectiveness in inactivating various pathogens, nontoxicity, and low cost (Zhang *et al.* 2006). Prior research on high-pressure CO_2 treatments have investigated the effects of several factors such as the pressure, temperature, type of microorganisms, agitation speed, decompression rate, and pressure cycling on the inactivation capacity of this method (Haas *et al.* 1989; Spilimbergo *et al.* 2002; Silva *et al.* 2013; Hong *et al.* 1997; Hong and Pyun 1999; Dillow *et al.* 1999; Fraser 1951). Most studies have reported that high-pressure operating conditions (4–50 MPa) are required to inactivate significant numbers of pathogens (Garcia-Gonzalez *et al.* 2007). 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 PCD 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 (Kobayashi *et al.* 2007, 2009; Cheng *et al.* 2011; Vo *et al.* 2013, 2014) largely because this method does not generate DBPs. Kobayashi *et al.* (2007, 2009) 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 to 55°C) requirements for effective inactivation (Kobayashi *et al.* 2007, 2009) are still high from a practical standpoint. Cheng *et al.* (2011) and Vo *et al.* (2013, 2014) have used low-pressure CO_2 treatments (0.2–1.0 MPa) based on technology that produces high amounts of dissolved gas in water to inactive *Escherichia coli* and bacteriophages in freshwater. Cheng *et al.* (2011) 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.* (2013) 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. This study investigated the use of PCD at less than 1.0 MPa for the sea ballast water disinfection purposes.

1.2 Objectives

The aim of this research is to develop an innovative method for ballast water disinfection. Large volumes of ballast water need to be treated according to the D-2 standard (IMO 2004); however, the space on a ship for such operations is typically limited. Ideally, a shipboard treatment method should be highly efficient at removing target organisms, quick to implement, low cost and free of problems related to residual toxicity. Thus, it would be desirable to develop new technologies for ballast water disinfection in a manner that exploits the advantages of current technology while minimising the disadvantages of the conventional methods. To accomplish these demands, sub-objectives of this dissertation were as follow:

- To evaluate the feasibility of using PCD (<1.0 MPa) for inactivating *Enterococcus* sp., *Escherichia coli* and *Vibrio alginolyticus* in seawater. Optimal conditions of pressure, temperature, working volume ratio was determined.

- To examine whether pressure cycling of PCD, which was conducted at low pressure (<1.0 MPa) without CO_2 release between each cycle of raised/lowered pressure, could be used to enhance the inactivation of bacteria in seawater.

- To evaluate whether the combinations of PCD (<1.0 MPa) and low-dosage chlorine could yield synergistic benefits and to assess the potential application of this method for ballast water treatment.

1.3 Structure of dissertation

The dissertation included six chapters and listed as follow:

Chapter I **Introduction**

states the problem, aims of the research, and structure of the dissertation.

Chapter II **Literature review**

The chapter reviews the literature of the previous studies and introduces the background knowledge associated with ballast water and PCD treatment.

Chapter III **Disinfection effect of pressurized carbon dioxide on *Escherichia coli*, *Vibrio alginolyticus*, and *Enterococcus* sp. in seawater**

The chapter presents the feasibility of using PCD to inactivate gram-negative bacteria, such as *Escherichia coli* and *Vibrio alginolyticus,* and gram-positive bacteria, such as *Enterococcus* sp., in seawater. The optimal pressure and temperature for inactivating these bacteria were evaluated. Moreover, the effect of PCD on bacterial cell morphology and intracellular material leakage are also presented in this chapter.

Chapter IV **Enhanced bactericidal performance of pressurized carbon dioxide by pressure and pressure cycling**

The findings of Chapter III indicate that PCD have a promising to inhibit bacteria in seawater. In order to improve the disinfection efficacy and reduce the treatment time, pressure cycling of PCD had been elucidated and optimized in this chapter. Theoretical explanation for inactivation mechanism involved turbulence caused by high-frequency counter-current agitation; collisions of microorganisms on

the surface shield; jets and shock waves formed by explosion of bubble; and CO_2 effectively penetrating into cells. Relationships among inactivation efficiency, pH, and dissolved CO_2 concentration were also indicated. Inactivation kinetics of PCD with various content rates are also presented in Chapter IV.

Chapter V **Synergistic effect of pressurized carbon dioxide and sodium hypochlorite on the inactivation of bacteria in seawater**

To enhance the disinfection efficacy and reduce the treatment time (for purposes of space savings and energy savings), the effect of combined treatment using PCD and low-dose chlorine on the inactivation of bacteria in seawater had been presented in this chapter. (1) Synergistic benefits of the combined PCD/chlorine treatment on the inactivation of an *Enterococcus* sp. in artificial seawater were highlighted; Optimum conditions for pressure, CO_2 content, and chlorine dose are presented. (2) Using the combined PCD/chlorine treatment to inactive *E. coli*, enterococci, and vibrios in natural seawater were studied.

Chapter VI **Conclusions and future works**

Summarizes the results of this study and mentions the scope for future research.

1.4 References

Boorman, G.A., Dellarco, V., Dunnick, J.K., Chapin, R.E., Hunter, S., Hauchman, F. 1999. Drinking water disinfection byproducts: review and approach to toxicity evaluation. Environ. Health Perspect., **107** (Suppl. 1), 207-217.

Cheng, X., Imai, T., Teeka, J., Yamaguchi, J., Hirose, M., Higuchi, T., Sekine, M. 2011. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. Applied Microbiology and Biotechnology, **90** (4), 1493-1500.

David, M., Gollasch, S. 2015. Global Maritime Transport and Ballast Water Management - Issues and Solutions. Invading Nature - Springer Series in Invasion Ecology 8. Springer: Dordrecht, The Netherlands.

Dillow, A.K., Dehghani, F., Hrkach, J.S., Foster, N.R., Langer, R. 1999. Bacterial inactivation by using near and supercritical carbon dioxide. Proc. Nati. Acad. Sci. U.S.A., **96** (18), 10344-10348.

Fabbricino, M., Korshin, G.V. 2005. Formation of disinfection by-products and applicability of differential absorbance spectroscopy to monitor halogenation in chlorinated coastal and deep ocean seawater. Desalination, **176**, 57-69.

Fraser, D. 1951 Bursting bacteria by release of gas pressure, Nature, **167** (4236), 33-34.

Garcia-Gonzalez, L., Geeraerd, A.H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Vanpe, J.F., Devlieghere, F., 2007. High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. International Journal of Food Microbiology, **117**, 1-28.

GloBallast 2016. The issue. http://globallast.imo.org/ballast-water-as-a-vector/ (accessed 29 May 2016).

Gregg, M., Rigby, G., Hallegraeff, G.M. 2009. Review of two decades of progress in the development of management options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water. Aquatic Invasions, **4**(3), 521-565.

Haas, G.J., Prescott, H.E., Dudley, E., Dik, R., Hintlian, C., Keane, L. 1989. Inactivation of microorganisms by carbon dioxide under pressure. Journal of Food Safety, **9**, 253-265.

Hong, S.I., Park, W.S., Pyun, Y.R. 1997. Inactivation of *Lactobacillus* sp. from kimchi by high pressure carbon dioxide. LWT-Food Sci. Technol., **30**, 681-685.

Hong, S.I. and Pyun, Y.R. 1999. Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. J. Food Sci., **64**, 728-733.

IMO 2004. International convention for the control and management of ship's ballast water and sediments. In: International Conference on Ballast Water Management for ships BWM/CONF/36, London.

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., Osajima, Y. 2007. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. Food Science and Technology Research, **13**, 20-22.

Kobayashi, F., Yamada, F., Ikeura, H., Hayata, Y., Muto, N., Osajima, Y. 2009. Inactivation of microorganisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. Journal of Water and Environment Technology, **7**, 241-250.

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. 112 p. DOI: 10.2166/9781780405858.

Lloyd's Register's 2012. Understanding Ballast Water Management series. Ballast water treatment technologies and current system availability. London, September 2012.

Ruiz, G.M., Carlton, J.T., Grosholz, E.D., Hines, A.H. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. American Zoologist, **37**, 621-632.

Silva, J.M., Rigo, A.A., Dalmolin, I.A., Debien, I., Cansian, R.L., Oliveira, J.V., Mazutti, M.A. 2013. Effect of pressure, depressurization rate and pressure cycling on the inactivation of *Escherichia coli* by supercritical carbon dioxide. Food Cont., **29** (1), 76-81.

Spilimbergo, S., Elvassore, N., Bertucco, A. 2002 Microbial inactivation by high-pressure. J. Supercrit. Fluid., **22** (1), 55-63.

Tsolaki, E., Diamadopoulos, E., 2010. Technologies for ballast water treatment: a review. Journal of Chemical Technology and Biotechnology, **85**, 19-32.

Vo, H.T., Imai, T., Teeka, J., Sekine, M., Kanno, A., Le, T.V., Higuchi, T., Phummala, K., Yamamoto, K. 2013a. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. Water Research, **47**, 4286-4293.

Vo, H.T., Imai, T., Ho, T.T., Sekine, M., Kanno, A., Higuchi, T. 2014. Inactivation effect of pressurized carbon dioxide on bacteriophage Qβ and ΦX174 as a novel disinfectant for water treatment. J. Environ. Sci., **26** (6), 497-505.

Von Gunten, U. 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water Research, **37**, 1469-1487.

Werschkun, B., Sommer, Y., Banerji, S. 2012. Disinfection by-products in ballast water treatment: An evaluation of regulatory data. Water Research, **46**, 4884-4901.

Zhang, J., Davids, T. A., Matthew, M. A., Drews, M. J., LaBerge, M., An, Y. H. 2006. Sterilization using high-pressure carbon dioxide. Journal Supercritical Fluids, **38**, 354–372.

CHAPTER II

LITERATURE REVIEW

2.1 Environmental issues of ballast water

2.1.1 Ballast water requirements on board ships

Nowadays, global shipping transports more than 90% of worldwide trade (IMO 2016a). Cargo ships carry ballast water to adjust their stability and trim during a voyage when a vessel is not fully laden. The ballast water is usually pumped into the ballast tank at the unloading port and discharged at the loading port.

Vessel ballast capacity can range from several cubic meters in sailing boats and fishing boats to hundreds of thousands of cubic meters in large cargo carriers. Large tankers or dry-bulk carriers can carry larger quantities ballast water (i.e. excess of 100,000 m^3 per vessel). Tankers and bulk carriers account for 76% (37% oil tankers and 39% bulk carriers) of the total amount of ballast water. The ballast water capacities for typical ship types are summarized in the table 2.1.

Table 2.1 Percentage of vessel ballast water capacity in relation to the vessel deadweight tonnage (DWT).

Vessel type	DWT	Ballast condition		References
		% of DWT	Tonnes	
Bulk carrier	250,000	30–45	75,000–112,500	AQIS (1993)
Bulk carrier	150,000	30–45	45,000–67,000	Suban *et al*. (2006)
Bulk carrier	70,000	30–45	21,000–31,500	Suban *et al*. (2006)
Bulk carrier	35,000	33–57	11,550–19,950	Suban *et al*. (2006)
Tanker	100,000	40–45	40,000–45,000	AQIS (1993)
Tanker	40,000	43	17,200	Suban *et al*. (2006)
Container	40,000	28–40	11,200–16,000	Suban *et al*. (2006)
Container	15,000	30	4,500	Suban *et al.* (2006)
General cargo	17,000	35	5,950	AQIS (1993)
General cargo	8,000	38	3,000	AQIS (1993)
Passenger/Ro-Ro	3,000	43	1,290	Suban *et al*. (2006)

The ballast water pump capacity is depend on the speed of loading or discharging cargo. In general, ballasting and deballasting operations takes place at flow rates between <1,000 m^3/h (i.e. container ships, car carriers) and 10,000 m^3/h or even faster (i.e. dry bulk carriers, tanker vessels).

Ships are getting larger, faster and the amount of ship traffic through the ocean will increase substantially in the future decades. This results in an increase in volume and transfer rate of ballast water worldwide, thereby increasing potential hazards to the marine environment due to the accidental introduction of alien species to areas where ballast water is discharged.

2.1.2 Impacts of ballast water

Ballast water is recognized as one of the principal vector responsible for the introduction of non-native aquatic species to marine ecosystems worldwide. Ballast water contains variety of living organisms including cysts, eggs and larvae of fish and zooplankton, macro-algae, phytoplankton, bacteria and other microbes. Annually, about 3–5 billion tons of ballast water containing aquatic species is transferred among the world's oceans (GloBallast 2016). It is estimated that about 7,000–10,000 different marine species are transferred among the world's oceans each day via ballast water (Carlton 1999). Although, not all these species can survive the voyage or establish viable populations once discharged in new environment, and not all introduced species are considered harmful. However, if these organisms are released into new ecosystems that support their growth, they can become invasive species (Ruiz *et al.* 1997; Molnar *et al.* 2008; David and Gollasch 2015). There have been many serious consequences of such transport (Table 2.2). Examples include the Asia clam (*Potamocorbula amurensis*) and European zebra mussel (*Dreissena polymorpha*) in North America, the Japanese kelp (*Undaria pinnatifida*) and Northen Pacific seastar (*Asterias amurensis*) in Tasmania - Australia, and the Atlantic comb jelly (*Mnemiopsis leidyi*) in the Mediterranean and Caspian seas. The threat posed by ballast water has been noticed on many aspects of human health, economy, and environment since the early 1990s.

Invasive species are recognized as a major threat to biodiversity (IMO 2016b). The introduction of invasive species into a new ecosystem can have negative impacts for ecological such as compete with native species for space and food, alternate habitat, alternate environmental conditions, displace native species, change community structure and food webs (Ruiz *et al.* 1997; Battle 2009; GloBallast 2016).

The spread of invasive species also have serious consequences for the economy. Alien species may reduce fisheries production (due to competition or displacement of the fishery species, altering environment); impact on aquaculture and tourism beaches (by algal blooms) (Battle 2009; GloBallast 2016).

On the other hand, the transfer of microbes via ballast water can even increase risks to human health (Ruiz *et al.* 1997). Bacteria and microalgae are major candidates for successful ballast water transport. They are small, universally abundant in marine and freshwater habitats, and are well capable of surviving hostile conditions posed by the ballast tank environment in the form of cysts, spores or other physiological resting stages (GloBallast 2016). Previous research so far evidenced that cholera epidemics can be related to ballast water discharges (Ruiz *et al*. 1997; Battle 2009; IMO 2016e). In addition, some toxic species of microalgae cause harmful algae blooms or "red tides" (i.e. dinoflagellate *Gymnodinium catenatum* in Tasmania, Australia, which is responsible for human illnesses such as paralytic shellfish poisoning) can also be transferred by ballast water (IMO 2016e). The recognition of human health risks has giving a driving force for preventing the introduction of non-indigenous organisms from ballast water discharge.

Table 2.2 Several undesirable organisms in ballast water and adverse impacts.

Invasive species	Native to	Introduced to	Impacts	Reference
Vibrio cholerae (various strains)	Cosmopolitan	Bengal, Peru, South and Central America, Gulf of Mexico, and other areas.	Some cholera pandemics are directly associated with ballast water. *V. cholerae* (O1 and O139) is the number one spot in the list of the most undesirable organisms in ballast water.	Battle (2009); IMO (2016e)
Toxic dinoflagellates and their cysts	Asia	Australia	Toxic "red tides" due to the worldwide transfer of dinoflagellates and their cysts in ship's ballast tanks. Can cause human illness and death by consumption of contaminated filter-feeding shellfish. Can foul beaches and impacts on tourism and recreation.	IMO (2016e)
Comb Jelly *Mnemiopsis leidyi*	America	Black, Azov, and Caspian seas	Reproduces rapidly in the new environment that supports their growth. Change food webs by reducing in zooplankton and predator fish species in Black, Azov, and Caspian seas. The local fisheries have suffered serious consequences as a result.	Battle (2009); GloBallast (2016); IMO (2016e)
Cladoceran Water Flea *Cercopagis pengoi*	Caspian Sea and Black Sea	Baltic Sea	Replaces the native zooplankton communities; causes clogging of trawls and fishing nets	IMO (2016e)
North Pacific Seastar *Asterias amurensis*	Northern Pacific	Southern Australia	Consummation of commercially valuable shellfish like oyster, scallop, and clam	Battle (2009); IMO (2016e)
Zebra mussels *Dreissina polymorpha*	Eastern Europe (Black sea)	the Great Lakes (North America); Western and Northern Europe	Reducing densities of planktonic organisms. Alters habitat, ecosystem and food web. This fouling species has become abundant in the Great Lakes system where it has been responsible for heavy fouling of intake pipes for hydroelectric schemes, with costly repercussions for the industry. It is estimated that eradication of the zebra mussels from hydro schemes in the Great Lakes could cost about $30 million.	Battle (2009); IMO (2016e)
European Green Crab *Carcinus maenus*	European Atlantic Coast	United States, Southern Australia, Japan and South Africa	Alters native crabs becoming a dominant species in invaded areas; displaces inter-tidal rocky shore ecosystems.	IMO (2016e)
Mitten Crab *Eiocheir sinensis*	Northern Asia	Baltic Sea, Western Europe, West Coast North America	Causes erosion and siltation of river banks and dikes; causes local extinctions by preying on native fish and invertebrate species.	IMO (2016e)

2.1.3 Ballast water discharge standard

2.1.3.1 The International Maritime Organization standards

To prevent the introduction and establishment of potentially invasive species via ballast water discharge, the International Maritime Organization (IMO) adopted the International Convention for the control and management of ships' ballast water and sediments (Convention) (IMO 2004). The Convention includes regulations for the discharge of ballast water. The regulations require that the number of viable organisms in ballast water must be less than the level set in the D-2 ballast water performance standard when the water is discharged into the ocean (Table 2.3; IMO 2004). The installation schedule in accordance with the IMO D-2 regulations is applied in a stepwise manner to ships undertaking international voyages in line with the year of building and the ballast tank capacity, with all ships required to adopt the standard by 2017 (Table 2.4). Once the Convention is ratified by 30 countries representing 35% of the global commercial shipping tonnage, it will enter into force 12 months after ratification. As at August 2016, 51 countries representing 34.87% of world tonnage have ratified the Convention, thus, the Convention has not yet in force (IMO 2016c).

2.1.3.2 The United States Coast Guard standards

As regards the significant matter of environmental concern, the United States (US) has implemented more stringent regulatory standards for discharging ballast water (Table 2.3). In the US, at the federal level ballast water discharges are under the jurisdiction of both the United State Coast Guard (USCG) and the US Environmental Protection Agency (EPA). Specifically, in August 2009, the United State established ballast water discharge standard in USCG Proposed Regulations. The USCG regulations have been divided into two phases. The Phase 1 standard is similar to the IMO D-2 and the implementation schedule is same as the IMO schedule. The phase 2 discharge standard requests must be met by new ships with a build date on or after 1 January 2016. For ships with a build date before 1 January 2016, the compliance date is the first drydocking after 1 January 2016 or five years after a Phase 1 system was installed, whichever is later. The installation schedule in accordance with USCG regulations is presented in Table 2.5.

In addition, 16 states in the US have specific ballast water management requirements. California and New York are considered to have the most stringent requirements.

2.1.3.3 The California standards

In January 2006, the California State Lands Commission enacted ballast water discharge standards in SB 497 (Table 2.3). The California standards are much more stringent than the IMO standards and similar (but not identical) to the Phase 2 standard recently proposed by the USCG standards. The California performance standard SB497 requires the following ballast water standards: No detectable discharges of organisms larger than 50 μm by vessels constructed on or after 2012, and all older vessels by 2016; The phased reduction of smaller organisms, bacteria and viruses discharged by all vessels 2016; California law currently sets a final discharge implementation date of 1 January 2020 that specifies zero detectable living organisms for all size ranges in the ballast discharge stream (California State Lands Commission 2014).

2.1.3.4 The New York standards

In February 2011, the state of New York's (NY) Water Quality Certification Agency issued a letter granting an extension of the implementation date for 'Condition 2' from 1 January 2012 until 13 August 2013 (Table 2.3). The NY standards is more stringent than the IMO and USCG standards (which are equivalent). Additionally, the NY performance standards go above and beyond the IMO and USCG (phase 1) standards by limiting the discharge of total living bacteria and viruses in ballast water in order to protect public health and the environment.

The USCG (phase 2), California, and NY (year 2013) performance standards for the total living bacteria and viruses are less than 1000 living bacteria per 100 mL and less than 10,000 living viruses per 100 mL, respectively. However, these standards are difficult to access because there currently are no widely accepted methods available to assess total living bacteria or virus concentrations in ballast water (California State Lands Commission 2014). Hence, no BWTS being tested for total living bacteria and viruses, thus, no shipboard BWTS can be proven to be efficacious with the USCG (phase 2)/California/NY performance standards (California State Lands Commission 2014).

Table 2.3 Ballast water discharge standards for maximum limits of viable organisms per defined volume of discharged ballast water.

Organism Size/ Microbes	IMO	US Proposed Regulation[b]		California	NY (CWA 401)[d]	
	(Regulation D-2)	Phase 1 standard	Phase 2 standard	(SB 497)[c]	Year 2012	Year 2013 (new ship)
Plankton ≥50 µm in minimum dimension	<10 viable organisms/m^3	<10 viable organisms/m^3	<1 viable organisms/100 m^3	0	<1 viable organisms/10 m^3	0
10 ≤ plankton <50 µm in minimum dimension	<10 viable organisms/mL	<10 viable organisms/mL	<1 viable organisms/100 mL	<0.01 living organisms/mL	<1 viable organisms/10 mL	<0.01 living organisms/mL
Size <10 µm in minimum dimension	No limit	No limit	<10^3 bacteria/100 mL <10^4 viruses/100 mL	<10^3 bacteria/100 mL <10^4 viruses/100 mL	No limit	<10^3 bacteria/100 mL <10^4 viruses/100 mL
Toxicogenic *Vibrio cholerae* (O1 and O139)	<1 cfu[a]/100 mL or <1 cfu[a]/g wet weight zooplankton samples	<1 cfu[a]/100 mL	<1 cfu[a]/100 mL	<1 cfu[a]/100 mL or <1 cfu[a]/g wet weight zooplankton samples	<1 cfu[a]/100 mL	<1 cfu[a]/100 mL
Escherichia coli	<250 cfu[a]/100 mL	<250 cfu[a]/100 mL	<126 cfu[a]/100 mL	<126 cfu[a]/100 mL	<126 cfu[a]/100 mL	<126 cfu[a]/100 mL
Intestinal enterococci	<100 cfu[a]/100 mL	<100 cfu[a]/100 mL	<33 cfu[a]/100 mL	<33 cfu[a]/100 mL	< 33 cfu[a]/100 mL	<33 cfu[a]/100 mL

[a]Colony forming units – a measure of viable bacterial numbers.

[b]Ballast water discharge standards (BWDS) in USCG (United State Coast Guard) Proposed Regulation (August 2009).

[c]Final discharge standard for California, beginning January 1, 2020, is zero detectable living organisms for all organism size classes.

[d]New York imposed ballast water management requirements through the Clean Water Act section 401 (CWA 401).

Table 2.4 Installation schedule for the BWM systems in accordance with the IMO D-2 standard (IMO 2016b).

Ship's ballast water capacity	Date constructed	Ship's compliance date
≥ 1,500 m^3 but <5,000 m^3	Before 2009	By the first renewal survey of the International Oil Pollution Prevention (IOPP) Certificate following the date of entry into force of the Ballast Water Management Convention
<1,500 m^3 or ≥ 5000 m^3	Before 2009	
<5,000 m^3	During 2009 to the date of entry into force of the Convention	
≥ 5000 m^3	During 2009 but before 2012	
≥ 5000 m^3	During 2012 to the date of entry into force of the Convention	
All ships	On or after the date of entry into force of the Convention	By the completion date of the ship construction

Note: In case the Convention comes into effect not later than 31 December 2016

Table 2.5 Implementation schedule for US federal (USCG/EPA) ballast water discharge standards.

	Ship's ballast water capacity	Date constructed	Ship's compliance date
New ship	All	On or after 1 December 2013	On delivery
Existing ships	< 1,500 m^3	Before 1 December 2013	First scheduled dry-docking after 1 January 2016
	1,500 – 5,000 m^3	Before 1 December 2013	First scheduled dry-docking after 1 January 2014
	> 5,000 m^3	Before 1 December 2013	First scheduled dry-docking after 1 January 2016

2.1.4 Ballast water management system approval process

The Marine Environmental Protection Committee (MEPC) of the IMO had adopted guidelines for the uniform implementation of the International Water Convention. The MEPC takes into considerations if a BWTS is capable of treating ballast water according to the submitted type of approval. For the approval of BWTS, there are two kinds of BWTS,

one of which produce or utilize active substances and the other do not use any active substance. Treatment systems that use active substances have to go through both the "Procedure for Approval of BWTS that Make Use of Active Substances (G9)" and "Guidelines for Approval of Ballast Water Management System (G8)", while BWTS that do not use any active substance only have to go through "Guidelines for Approval of Ballast Water Management System (G8)" (IMO 2016b). There are two kinds of possible approvals for fitting systems on board ships: BWTSs that use active substances have to go through both the basic approval (pilot scale testing for toxicity) and final approval (land-based and ship test for biological efficiency testing), according to G8 and G9 guidelines. BWTSs that do not use active substances only have to go through the final approval according to G8 guidelines.

There are at least 160 shipboard BWT systems currently under development or available worldwide, many of which are undergoing testing to gain type approval under IMO and/or USCG type approval protocols (IMO 2016d). As at April 2016, fifty-five systems that make use of active substances have earned basic approval from the IMO; forty systems have received the IMO type final approval; sixty-five BWTS have received type approval certification by their respective administrations (IMO 2016d); while none have yet earned the USCG (phase 2) type approval for ballast water treatment technology.

2.1.5 Review of ballast water treatment technologies

2.1.5.1 Ballast water treatment platforms

Figure 2.1 represents the general platform types which have been explored for the development of BWTS (Tsolaki *et al.* 2010). Port based BWTS occurs at a port facility following transfer from a vessel. Meanwhile, shipboard BWTS are installed onboard vessels and integrated into a vessel's ballast water system. The ballast water may be treated in the pipe during uptake or discharge (in-line) or in the ballast tanks during the voyage (in tank). Shipboard systems are considered broadly applicable because they allow flexibility for the management of ballast water during normal operations.

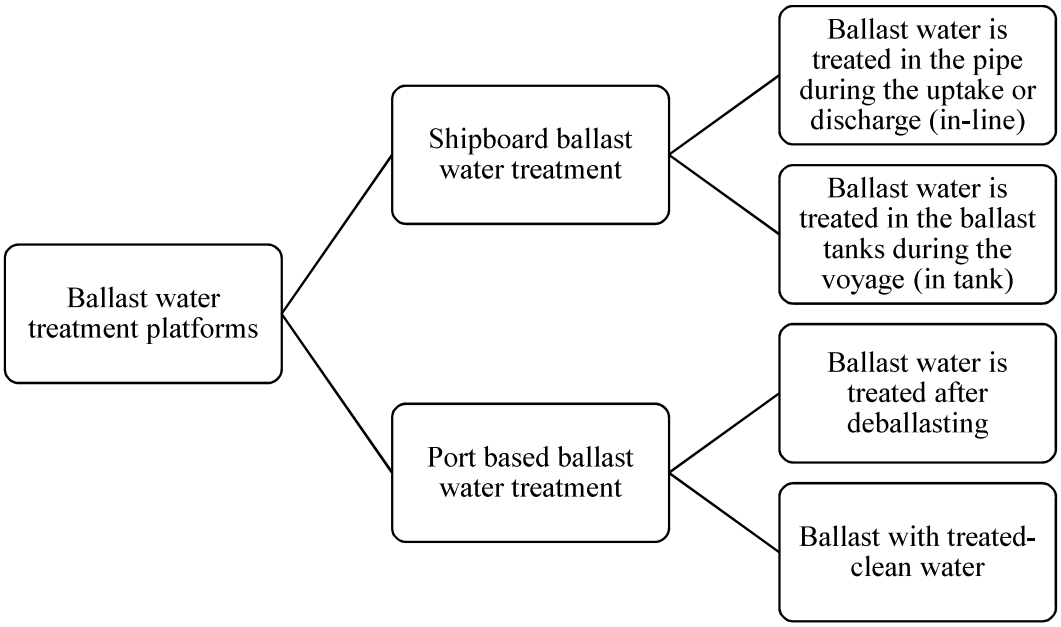

Figure 2.1 Ballast water treatment platforms.

2.1.5.2 Generic treatment process

The technologies used for treating ballast water are divided into two generic types, including solid-liquid separation process (pre-treatment) and disinfection process. Presently, most ballast water treatment systems (BWTS) use a two-stage approach involving solid-liquid separation process at the first stage and disinfection process at the second stage (Gregg *et al*. 2009; Tsolaki & Diamadopoulos 2010; Goncalves & Gagnon 2012; LIoyd's register 2012).

Solid-liquid separation (pre-treatment) is simply the removal of suspended solid and large organisms from ballast water by sedimentation or filtration. The separation process uses hydrocyclone or surface filtration, some system may combine with chemical (i.e., coagulation, flocculation) to enhance the treatment efficiency. The processes also produce a waste stream that comprises backwater water from the filtrating operations or underflow from a hydrocyclone separation (LIoyd's register 2012).

Disinfection process may use one or more of the following methods: chemical biocides and active substances, and physical methods such as heat treatment, UV radiation, ultra sound, cavitation, de-oxygenation (LIoyd's register 2012). Figure 2.2 represents a summary of various ballast water options.

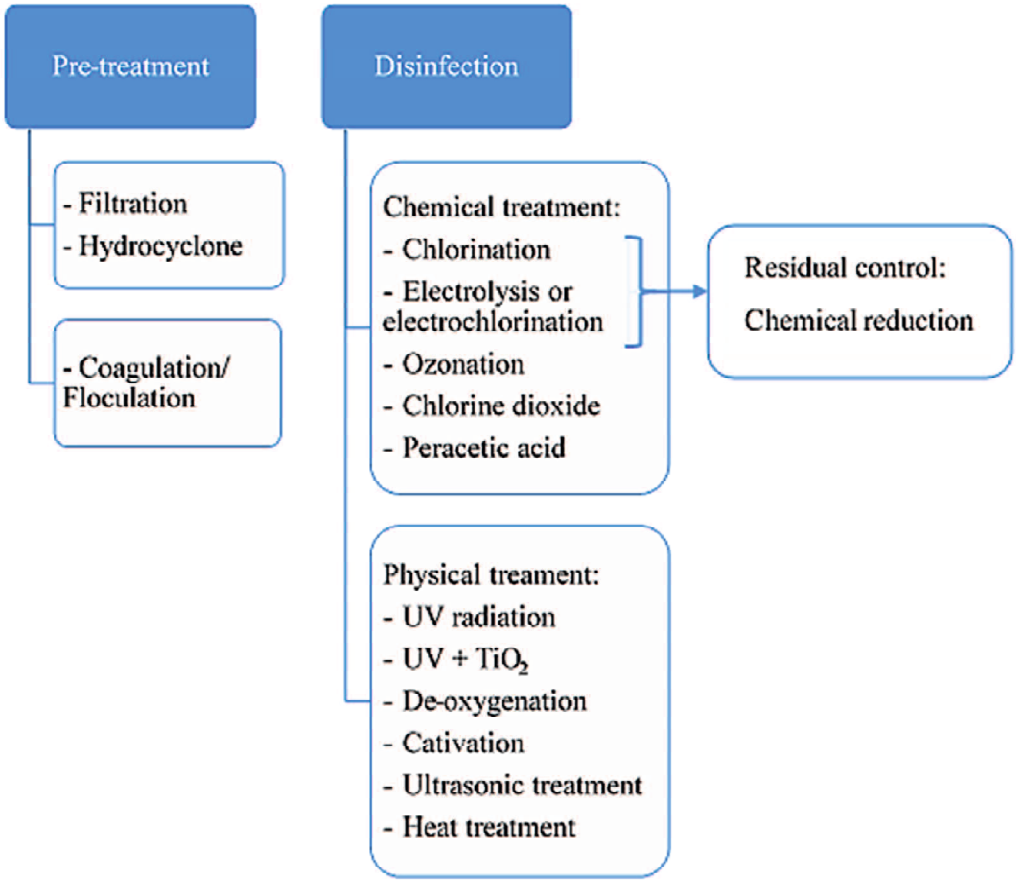

Figure 2.2 Ballast water treatment options.

Table 2.6 summarizes a comparison between the above described generic ballast water treatment methods. Each of the methods have their own advantages and disadvantages with regard to factors such as efficient at removing target organisms, cost, energy and space requirements, operational efficacy and environmentally-friendly perspective. Thus, it is necessary to develop new technologies for ballast water disinfection in a manner that exploits the advantages of current technology and minimise the disadvantages of the conventional methods.

Table 2.6 Generic ballast water treatment process.

Process	Method	Advantages	Disadvantages	Reference
Solid-liquid separation (Pre-treatment)				
Filtration	Using discs or fixed screens with automatic backwashing.	Removes mid-size and large particles including larger organisms. Limit the amount sediment.	Backwashing is required by maintaining flow with minimum pressure drop. Fine filtration systems are expensive to install and maintain.	LIoyd's register (2012)
Hydrocyclone	High velocity centrifugal rotation of water to separate particles.	Effect for removal of larger particles	Low effective with small particles	LIoyd's register (2012)
Coagulation	Pre-treatment prior to increase size of particles.	Enhance efficacy of filtration and hydrocyclone.	Long retention time.	LIoyd's register (2012)
Chemical disinfection				
Chlorination	Chlorine compound is added into seawater with a dose ~10 mg/L. The residual excess chlorine concentration in the treated water is neutralized to below 0.2 mg/L.	High oxidizing potential, low cost, and residual disinfectant properties to prevent microbial recontamination.	Disinfection by-products (DBPs). Some resistant microorganisms may only be inactivated with very high chlorine doses, which can exacerbate the formation of DBPs. Residual chlorine may pose an environmental risk due to its toxicity to aquatic organisms.	Gregg *et al.* (2009); Tsolaki & Diamadopoulos (2010); LIoyd's register (2012)
Electrochlorination	Directly creating electrolytic reaction in seawater.	High oxidizing potential and low cost. No need for storing and transporting chemical substances.	Only works in water with Cl^- (i.e., seawater, brackish). Disinfection by-products.	Gregg *et al.* (2009); LIoyd's register (2012)
Ozonation	Ozone gas (1-2 mg/L) is bubbled into the water.	Effective at inhibiting a variety of pathogens	High cost, forming bromate in water containing bromide.	Gregg *et al.* (2009); Tsolaki & Diamadopoulos (2010); LIoyd's register (2012)
Peracetic acid, hyrdrogen peroxide	Adding in water as oxidising biocide.	Produces few toxic by-products and relative stable.	Reagent is typically dosed at high levels, requires suitable storage facilities, and can be high cost. Discharging water laden with biocides may be hazardous for the crewmember and for native organisms.	Tsolaki & Diamadopoulos (2010); LIoyd's register (2012)

Table 2.6 (continued)

Process	Method	Advantages	Disadvantages	Reference
Physical disinfection				
UV radiation	Amalgam lamps surrounded by quartz sleeves produce UV light.	Do not produce DBPs or other problematic chemical residues.	Effectiveness depends on water quality. Both suspended solid and dissolved organic matter reduce UV light transmittance. Some organisms have repair mechanisms that can undo the damage caused by UV radiation and can regrowth.	Gregg *et al*. (2009); LIoyd's register (2012)
Cavitation	Using ultrasonic power or gas injection.	Do not produce DBPs or other problematic chemical residues.	High operational costs due to substantial energy consumption	Gregg *et al.* (2009);
Heat - Thermal treatment	Using waste heat produced by the ship's engines, or using heat created by backup boiler systems installed aboard the vessel. Minimum temperature required for disinfecting is over 40°C.	Do not produce DBPs or other problematic chemical residues.	Long periods of time is required for the effectiveness disinfection. High operational costs due to substantial energy consumption. Discharging of warm water potentially threaten for native estuarine organisms.	Gregg *et al*. (2009);
De-oxygenation	De-oxygenation with inert gas involves the exchange of gases; oxygen is stripped from the water whilst inert gas (i.e. N_2, CO_2) is introduced into water.	Do not produce DBPs or other problematic chemical residues. Reducing corrosion, thereby extending ship life.	The method requires a long treatment time (1 to 4 days) to sufficiently asphyxiate the organisms and thus may not be appropriate if the voyage of the ship is short. It is unlikely to eliminate some organisms (i.e. anaerobic bacteria, spores, and phytoplankton) that can survive in hypoxic conditions.	Tamburri *et al*. (2002); Gregg *et al.* (2009); LIoyd's register (2012)

Note: These treatment process may depend on the actual system.

2.1.5.3 Shipboard ballast water treatment systems

It is estimated that from the time the IMO ballast water management Convention enters into force and up to the closure of the compliance window by around 2021, more than 50,000 ships will have to be retrofitted with BWM system (Bimco 2016). Large volumes and high flow rates of ballast water need to be treated to meet the standard as set out in regulation D-2 before the water can discharge into the surrounding waters. In addition to the effectiveness of the treatment at inhibiting a wide range of organisms, other factors to consider when selecting a shipboard treatment method include the size of treatment equipment and cost-efficiency and environmentally safety concerns (IMO 2004; Tsolaki *et al.* 2010).

Figure 2.3 represents the summary of treatment technologies used for pre-treatment and disinfection. The information is based on 76 shipboard BWTSs (Table 2.7; California State Lands Commission 2014; modified). The review of shipboard BWTS efficacy is complicated because some data are missing (i.e. detailed technical data, species determination of observed organisms, unique research methodology). Also, since detailed data about the costs of installation and operation of the discussed systems are not available in the literature, a complete comparison of the BWT systems considering these criteria is impossible.

As shown in Figure 2.3a, some pre-treatment technology is used by 54 BWTSs (~69%), of these 53 systems use filtration, one system uses hydrocyclone; whereas 22 systems (~29%) do not have a pre-treatment step.

As shown in Figure 2.3b, most of the BWTS identified are regarded as BWTS that make use of an active substance (49 BWTS). The most commonly used technology for ballast water treatment is electrolysis/electrochlorination (28 system, ~31.5%), which is applied as a stand-alone method by 23 BWTS, and by 5 in combination with other disinfection methods. The remaining 21 BWTSs use dosing of different active substances (i.e. chlorine-based, Peraclean Ocean). UV radiation method ranges the second with 25 BWTSs (~28%), 18 of these use UV as a stand-alone method, whereas 7 systems use UV in combination with other methods (i.e. plasma, ozone, pressure vacuum reactor, photo-catalytic reaction, ultrasound, and TiO_2). Nine BWTSs (~10%) use ozonation, while five BWTSs (~6%) use de-oxygenation with inert gas and CO_2 in their operation. About 13.5% systems apply other treatments such as heat treatment, ultrasound, sonic energy, ferrate, non-oxidizing biocide, and non-chlorine chemical disinfection in their operation.

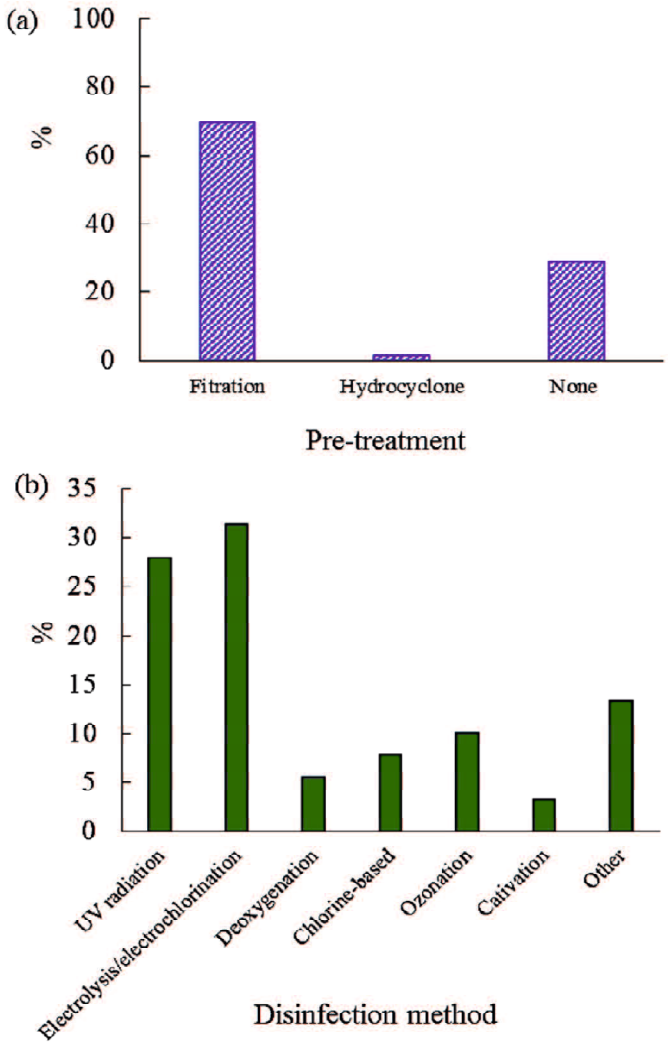

Figure 2.3 Summary of treatment technologies used for (a) pre-treatment and (b) disinfection. Note: one or more disinfection options may be used. "Other" treatments include heat treatment, ultrasound, sonic energy, ferrate, non-oxidizing biocide, and non-chlorine chemical disinfection. The information is based on 76 shipboard ballast water treatment systems (California State Lands Commission 2014).

Table 2.7 Shipboard ballast water treatment system

System name	Technology description	Approval
PureBallast 2.0/2.0 Ex	Filtration + advanced oxidation (UV + TiO2)	IMO Basic and Final, Type Approval (Norway)
PureBallast 3.0	Filtration + advanced oxidation (UV + TiO2)	Not approved
AquaStar™ BWMS	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic and Final, Type Approval (Korea)
AquaTriComb™	Filtration + ultrasound + UV	IMO Basic
ABWS	Filtration + electrolysis	Type Approval (Norway)
CrystalBallast®	Filtration + UV	
BIO-SEA BWTS	Filtration + UV	Type Approval (France)
BrillyantSea™	Electric pulse	
Coldharbour GLD™ (gas lift diffusion)	De-oxygenation + cavitation + ultrasound	Type Approval (United Kingdom)
Blue Ocean Shield	Hydrocyclone + filtration + UV	IMO Basic, Type Approval (China)
DESMI Ocean Guard OxyClean BWMS	Filtration + UV + ozone	IMO Basic and Final, Type Approval (Denmark)
RayClean™ BWTS	Filtration + UV	Type Approval (Denmark)
Dow-Pinnacle BWMS	Filtration + ozone + neutralization (sodium thiosulfate)	
Ecochlor® BWTS	Filtration + biocide (chlorine dioxide)	IMO Basic and Final, STEP[1], Type Approval (Germany)
BallaClean	De-oxygenation + hydrogen peroxide	
Model EL 1-3 B	Electrolytic generation of sodium hypochlorite	
BWDTS	Ozone + sonic energy	
BlueSeas BWMS	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic
BlueWorld BWMS	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic
ERMA FIRST BWTS	Filtration + hydrocyclone + electrolysis + neutralization (sodium bisulfite)	IMO Basic and Final, Type Approval (Greece)
Ferrator	Biocide (ferrate)	
BallastMaster ultraV	Filtration + UV	IMO Basic, Type Approval (Germany)
BallastMaster ecoP	Filtration + electrolysis + neutralization (sodium thiosulphate)	IMO Basic
EcoGuardian™	Filtration + electrochlorination + neutralization (sodium thiosulfate)	IMO Basic and Final
OceanGuard™ BWMS	Filtration + electrolysis + ultrasound	IMO Basic and Final, Type Approval (Norway)
SeaSafe-3	Heat treatment	New South Wales EPA
ClearBallast	Filtration +flocculation	IMO Basic and Final, Type Approval (Japan)

Table 2.7 Shipboard ballast water treatment system (continued)

System name	Technology description	Approval
HS-Ballast	Electrolysis + neutralization (sodium thiosulfate)	IMO Basic
HyCator® BWT Reactor System	Filtration + electrochlorination + neutralization (sodium thiosulfate)	
Hyde GUARDIAN Gold	Filtration + UV	STEP[1], IMO Basic, Type Approval (UK)
EcoBallast	Filtration + UV	IMO Basic and Final, Type Approval (Korea)
HiBallast	Filtration + electrolysis + neutralization	IMO Basic and Final, Type Approval (Korea)
JFE BallastAce	Filtration + biocide (sodium hypochlorite) + cavitation + neutralizing agent (sodium sulfite)	IMO Basic and Final, Type Approval (Japan)
JFE Ballast Ace with NeoChlor Marine™	Filtration + biocide (sodium hypochlorite) + neutralization (sodium sulfite)	IMO Basic and Final
OceanDoctor BWMS	Filtration + UV + photocatalytic reaction	IMO Basic and Final
SKY-SYSTEM®	Biocide (Peraclean® Ocean) + neutralization (sodium sulfite)	IMO Basic
KBAL BWMS	Pressure vacuum reactor + UV	Type Approval (Norway)
KTM-BWMS	Cavitation + electrolysis + neutralization (sodium thiosulfate)	IMO Basic
MICROFADE™ BWMS (formerly Kuraray BWMS)	Filtration + biocide (calcium hypochlorite) +neutralizing agent (sodium sulfite)	IMO Basic and Final, Type Approval (Japan)
BioVioletl	Filtration + UV	None
Ocean Protection System	Filtration + UV	IMO Basic and Final, Type Approval (Germany)
MARENCO BWTS	Filtration + UV	
MSI BWTS	Filtration + UV	
Mexel®	Non-oxidizing biocide	
MH BWT System	De-oxygenation (inert gas + CO_2)	
SPO-SYSTEM	Filtration + mechanical treatment + biocide (Peraclean Ocean)	IMO Basic (from Peraclean MEPC 54)
FineBallast MF	Pre-filtration + microfiltration (membrane)	
FineBallast® OZ (formerly SPHybrid BWMS Ozone)	Filtration + mechanical treatment + ozone + neutralization	IMO Basic and Final, Type Approval (Japan)
Venturi Oxygen Stripping (VOS)	De-oxygenation + cavitation	Type Approval (Liberia, Malta, Marshall Islands, Panama)
NK- 03 BlueBallast	Ozone	IMO Basic and Final, Type Approval (Korea)
Ballastmar	Filtration + electrochlorination + neutralization (sodium metabisulphite)	
SCX 2000, Mark III	Ozone	

Table 2.7 Shipboard ballast water treatment system (continued)

System name	Technology description	Approval
OceanSaver®	Filtration + cavitation + electrochemical disinfection + de-oxygenation	IMO Basic and Final, Type Approval (Norway)
OptiMarin Ballast System	Filtration + UV	Type Approval (Norway)
OceanSaver BWMS Mark II	Filtration + electrolysis	IMO Basic and Final, Type Approval (Norway), AMS.
GloEn-Saver™	Filtration + electrochlorination + neutralization (sodium thiosulfate)	IMO Basic
REDOX AS BWMS	Filtration + ozone + UV	IMO Basic
Resource BWTS	Cavitation + ozone + sodium hypochlorite	IMO Basic and Final, Type Approval (South Africa)
CleanBallast	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic and Final, Type Approval (Germany)
ARA Plasma BWTS	Filtration + plasma + UV	IMO Basic and Final, Type Approval (Korea)
Purimar™ BWMS	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic and Final, Type Approval (Korea)
Neo-Purimar™ BWMS	Filtration + electrolysis + neutralization (sodium thiosulfate)	IMO Basic and Final
INSITU BWMS	De-oxygenation + biological augmentation	
BALPURE® BP-500	Filtration + electrochlorination + neutralizing agent (sulfurbased reduction)	IMO Basic and Final, STEP[1], Type Approval (Ger.)
SiCure™	Filtration + electrochlorination	IMO Basic and Final
Cyeco™ BWMS	Filtration + UV	Type Approval (China)
BV Maritime Treatment System	Filtration + electrochlorination	
Smart Ballast BWMS	Electrolysis + neutralization (sodium thiosulfate)	IMO Basic and Final
Ecomarine™	Filtration + UV	Type Approval (Japan)
Blue Zone™ BWMS	Ozone + neutralization (thiosulfate)	IMO Basic
BalClor™ BWMS	Filtration + electrochlorination + neutralizing agent (sodium thiosulfate)	IMO Basic and Final, Type Approval (China)
Electro-Cleen™ System	Electrolysis + neutralizing agent (sodium thiosulfate)	IMO Basic and Final, Type Approval (Korea)
Van Oord BWMS	Chlorine + neutralization (sodium bisulfite)	IMO Basic
Marinex UV BWMS	Filtration + UV	
AQUARIUS® EC BWMS	Filtration + electrolysis + neutralization (sodium bisulfite)	IMO Basic and Final
AQUARIUS® UV	Filtration + UV	Type Approval (Netherlands)
BSKY™ BWMS	Filtration + UV	IMO Basic and Final, Type Approval (China)

2.1.5.4 The use of de-oxygenation with inert gas and carbon dioxide in ballast water treatment

Table 2.8 represents major advantages and disadvantages of several BWTS that use de-oxygenation with inert gas and carbon dioxide in their operation. In five BWT systems, de-oxygenation is applied as stand-alone method by one BWTS, and by 4 systems in combination with other technologies.

The MH BWT system uses de-oxygenation as stand-alone method (MH system 2016). Here, de-oxygenation with elevated CO_2 involves the exchange of gases; oxygen is stripped from the water whilst CO_2 is introduced into water (Husain *et al.* 2004; MH system 2016). This induces asphyxiation in organisms (hypoxia and hypercapnia) and reduces the pH of the water (~pH 6). Husain *et al.* (2004) reported that the majority of zooplanktons in ballast water were not alive after 15 min to 48 h, whereas about 99% (or 2 log reduction) of the *Vibrio cholerae* load was reduced within 24 h. De-oxygenation is a cost effective method; however, it is unlikely to eliminate some organisms (i.e. anaerobic bacteria, spores, and phytoplankton) that can survive in hypoxic conditions. Additionally, the method requires a long treatment time (1 to 4 days) to sufficiently asphyxiate the organisms and thus may not be appropriate if the voyage of the ship is short (Gregg *et al.* 2009; LIoyd's register 2012).

The Venturi Oxygen Stripping (VOS) uses inert gas (i.e. N_2, CO_2) injected into the ballast water with the help of a Venturi Injector in order to maintain a low level of dissolved oxygen in the ballast water tank. In the VOS system, hydrodynamic cavitation is used as a main (first) step of the treatment, and it happens in venturi tubes where the inert gas is introduced. Cavitation in venturi tubes destroys targeted organisms (NEI 2016). In this way, ballast water is sterilized, and the requirements of the IMO D-2 Standard are met. VOS is also considered to be an efficient system for the reduction of corrosion in ballast tanks (NEI 2016).

The Coldharbour GLD^{TM} is a BWTS that combine de-oxygenation, cavitation and ultrasound. The GLD^{TM} uses natural dynamics to stir the ballast water ensuring thorough treatment. Inert gas reduces the ballast water oxygen content while CO_2 reduces pH of the treated water. As gas is introduced to the GLD^{TM} it is made to generate an ultrasonic frequency that physically disrupts the cell walls of aquatic organisms while gas micro-bubbles amplify the ultrasonic effects. Thus organisms are killed in three ways: by hypoxia, by hypercapnia and by ultrasonic cell wall disruption (Coldharbour 2016).

The BallaClean® BWTS of EcologiQ LLC. (Michigan) uses a combination of de-oxygenation and hydrogen peroxide (H_2O_2) in their operation (GSI 2013). According to GSI (2013), the combination between de-oxygenation and H_2O_2 (100-200 mg L^{-1}) could reduce concentrations of *E. coli* and *Enterococcus faecalis* to a MPN of less than 20 within 48 h. However, H_2O_2 is dosed at high level (100−200 mg L^{-1}). Thus, this require suitable storage facilities and can be relatively expensive.

The OceanSaver® is a multi-component BWTS consisting of four main steps: (1) filtration by a mechanical back-flushing filter, (2) cavitation in a Closed Circuit Cavitation (C3T™) unit, (3) electrochemical disinfection unit, and (4) de-oxygenation with inert gas (Oceansaver 2016). In the BWTS, the filtration unit used is an automatic self-cleaning 50 μm wedge wire filter that operates during ballast intake returning trapped organisms and sediment back to the source location. The hydrodynamic cavitation unit involves the formation and implosion of cavitation bubbles which generate forces and shockwaves that affect organisms. The hydrodynamic cavitation is used as a main step of the treatment which happens inside a C3T™ unit induced by intense pressure pulses. The electrochemical disinfection unit produces active substances, while the de-oxygenation leads to hypoxic conditions in the ballast water. An advantage of this system is that it may be run in several configurations depending on the level of treatment required. However, disadvantages of this system are a complex system, and formation of by-products (Gregg *et al.* 2009).

Table 2.8 Several ballast water treatment technologies that use de-oxygenation with inert gas and carbon dioxide in their operation.

Name of the BWTS	Manufacturer	Technology description	Advantages	Disadvantages	Efficacy	Approvals	Reference
Coldharbour GLD™ (gas lift diffusion)	Coldharbour Marine Ltd.	De-oxygenation + cavitation + ultrasound	Reduction of corrosion. Storage or handling of chemical agents is not necessary.	Additional space is required for storage of inert gas.	NA	Type Approval (United Kingdom)	IMO (2016d); Coldharbour (2016)
Venturi Oxygen Stripping (VOS) -500 to VOS-6000	NEI Treatment Systems, LLC	De-oxygenation + cavitation	Reduction of corrosion. Storage or handling of chemical agents is not necessary.	Additional space is required for storage of inert gas. Long treatment period (4 day)	100% mortality of zooplankton within 120 h; 2.0 and 1.3 log reduction of *E. coli* and enterococci within 24 h, respectively.	Type Approval (the Netherlands)	NEI (2016); IMO (2016d)
MH BWT System	MH Systems	De-oxygenation	Reduction of corrosion. Storage or handling of chemical agents is not necessary. The system is simple in design, easy to operate.	Additional space is required for storage of inert gas. Long treatment period.	>95 % mortality of marine phytoplankton, zooplankton, macroalgae, and invertebrates within 48 h; and 2.0 log reduction of *Vibrio cholerae* within 24 h.		Husain *et al.* (2004); MH system (2016)
Oceansaver®	MetaFil AS (subsequently changed to OceanSaver AS)	Filtration + cavitation + electrochemical disinfection + de-oxygenation	The system is modular, thus it can be compatible with any pumping capacity.	Very complex system. Relatively high power consumption. Formation of by-products (i.e. THMs).	NA	IMO Basic and Final, Type Approval (Norway)	IMO (2016d); Oceansaver (2016)
BallaClean®	EcologiQ LLC.	De-oxygenation + hydrogen peroxide	Produces few harmful by-products and relatively stable.	H_2O_2 is dosed at a high level (100−200 mg L^{-1}). This requires suitable storage facilities and can be relatively expensive.	By the 48 h time period, both *E. coli* and *Enterococcus faecalis* were reduced to a MPN of less than 20.		GSI (2013); LIoyd's register (2012)

2.2 Potential application of pressurized carbon dioxide in treatment of ballast water

2.2.1 Pressurized carbon dioxide for sterilization

Pressurized carbon dioxide (CO_2) (PCD) has been used as a non-thermal sterilization technique in the food preservation industry (Garcia-Gonzalez *et al*. 2007), and potentially, it could be useful in many other applications. PCD has great potential for inhibiting various bacterial species present in both non-aqueous products (i.e. solid foodstuff, biomaterials, cotton, medical devices) and aqueous products (i.e. liquid foods, broth, water) (Isenschmid *et al.* 1995; Hong *et al.* 1997; Spilimbergo *et al*. 2003; Zhang *et al*. 2006; Kim *et al*. 2008; Fijan *et al*. 2011). In recent years, PCD has shown great potential as a sustainable disinfection technology in water and wastewater treatment applications (Kobayashi *et al.* 2007, 2009; Cheng *et al*. 2011; Vo *et al*. 2013a, b). The high bactericidal efficiency, nontoxic, inexpensive, and readily available character of CO_2 give it potential benefits over other sterilant agents (Zhang *et al*. 2006). For high-pressure CO_2 treatment, most studies have reported the influence of process parameters such as pressure, temperature, agitation speed, decompression rate, pressure cycling, bacterial concentration, and exposure time on the inactivation efficacy (Zhang *et al.* 2006; Garcia-Gonzalez *et al.* 2007).

2.2.2. Factors affecting to inactivation effect of pressurized carbon dioxide treatment

2.2.2.1 Influence of pressure and temperature

Pressure and temperature are important parameters influent the microbial inactivation of PCD. Rising pressure accelerates CO_2 solubilization rate as well as it penetrates into cell membranes (Isenschmid *et al.* 1995). While an increase in temperature may stimulate the diffusion of CO_2 into cells and may increase the fluidity of cell membranes. High pressure and temperature enables CO_2 to easily penetrate and modify the cell membrane and releases vital constituents from cells and cells membranes. Hence, by increasing pressure and/or temperature, a shorter exposure time is required to achieve the same log reduction (Hong *et al*. 1997; Zhang *et al*. 2006; Garcia-Gonzalez *et al*. 2007). However, the effect of pressure is limited by the saturation conditions of dissolved CO_2 in suspension, thus the exceed pressure does not substantially enhance the solubility of CO_2 (Spilimbergo *et al.* 2002; Zhang *et al.* 2006), but it increases substantially treatment cost. On the other hand, too high temperature reduces the dissolved CO_2 concentration in water. Therefore, to be more

attractive in terms of its economic feasibility, PCD treatment should not be implemented at too high pressure and/or above the critical temperature of CO_2.

Kobayashi *et al.* (2007, 2009) employed CO_2 microbubbles in the treatment of drinking water and succeeded in inhibiting *E. coli* within 13.3 min. However, the pressure (10 MPa) and temperature (35 to 55°C) requirements for effective inactivation (Kobayashi *et al.* 2007, 2009) are still high from a practical standpoint. Cheng *et al.* (2011) and Vo *et al.* (2013a, b) have used low-pressure CO_2 treatments (0.2–1.0 MPa) based on technology that produces high amounts of dissolved gas in water to inactive *E. coli* and bacteriophages in freshwater. These studies suggest that the use of PCD at less than 1.0 MPa and at ambient temperature may be applied in water treatment (Cheng *et al.* 2011; Vo *et al.* 2013a, b).

2.2.2.2 Influence of water content

CO_2 is hydro-lipophilic in nature, thus high water content helps CO_2 penetration to cell membrane easily. Microbicidal effect of PCD greatly improves with the high water content of cells suspension. Kamihira *et al.* (1987) reported that under treatment conditions (20 MPa, 35°C, and 120 min), only 0.3 log of *S. cerevisiae* and 1.2 log of *E. coli* were observed with low water content (2-10%), whereas the inactivation greatly increased (approximately 6 log and 4 log, respectively) with high water content (70-90%). Haas *et al.* (1989) found that when water content increased from 61% to 91%, inactivation efficacy of *E. coli* and *S. aureus* significantly increased from 75% to 99.96% for the former, and from 75% to 99.99% for the latter. Haas et al. (1989) also concluded that a PCD treatment would not be applicable to dry substances. The reason why pathogen in cells suspension are more strongly affected by PCD treatment than that in dry substances is probably related to an increased CO_2 solubility (Garcia-Gonzalez *et al*. 2007).

2.2.2.3 Influence of agitation

Agitation plays an important role in enhancing the contact efficacy between CO_2 and microbial cells in suspension. Lin *et al.* (1992) suggested that the inactivation efficiency of PCD against yeast cells was substantially decreased with the lack of agitation. Garcia-Gonzalez *et al.* (2009) found that stirring speed significantly improved the inactivation efficacy of high pressure CO_2 (at 13 MPa, 35°C during 20 min). Specifically, stirring speed at 200 and 400 min^{-1} resulted in completely inactivate *Pseudomonas* spp. and *Enterobacteriaceae*, whereas the cells were reduced to 2.0-D and 0.7-D after treatment at

100 min^{-1}, respectively (Garcia-Gonzalez *et al.* 2009). Strong agitation accelerates the CO_2 mass transfer in cell suspension as well as CO_2 solubility and diffusivity into microbial cells, thereby, strong agitation enhancing the antimicrobial performance of HPCD treatment (Lin *et al.* 1992; Hong *et al*. 1997).

2.2.2.4 Influence of depressurization rate and pressure cycling

Depressurization rate regards to sudden change of working pressure and this modifies physically to the psychology of cells leading to bacterial deaths or injure (Fraser *et al.* 1951). Enomoto *et al.* (1997) suggested that explosive depressurization with over 4 MPa has a strong effect to inhibition but not under 4 MPa. Cheng *et al.* (2011) considered that sudden discharge and resulting reduction of pressure led to mechanical cell rupture, which resulted in effective inactivation within 20 min by pressurized CO_2 at 0.3 MPa and room temperature.

Pressure cycling, a repetitive procedure of release and compression of CO_2, is a promising means to increase inactivation efficacy (Zhang *et al.* 2006; Silva *et al.* 2013). Theories explaining the inactivating mechanism of pressure cycling involve explosive cell rupture and mass transfer rate, in which compression intensifies the mass transfer of CO_2 across cell membranes (Hong *et al.* 1997; Dillow *et al.* 1999) and decompression enhances the cell rupture (Fraser *et al.* 1951). Dillow *et al.* (1999) found that the inactivation was substantially increased from 3 log to 9 log of the reduction ratio, corresponding to an increase of pressure cycling from three to six cycles with treatment conditions 20.5 MPa and 34°C within 0.6 hour. In addition, Spilimbergo *et al.* (2002) observed that an approximate 3.5 log reduction of *Bacillus subtilis* spores was achieved after 15 cycles at 8.0 MPa and 36°C for 30 min, but without pressure cycling, only a 0.5 log reduction was obtained under treatment conditions 7.5 MPa and 36°C for 24 hours. Ferreira *et al*. (2009) suggested that the use of pressure cycles was more effective than sustained high pressures to inhibit *Byssochlamys nivea*; a greater than 3.0 log reduction was observed after five cycles compared with a nearly 1.0 log reduction after one cycle in treatment conditions 550 MPa and 20°C for 15 min. Silva *et al*. (2013) reported that number of pressure cycles and system pressure were two significant parameters for the inactivation of *Escherichia coli* with supercritical CO_2. An 8.0 log bacterial load was reduced with five cycles and 8 MPa after 140 min of treatment, whereas a 5.0 log reduction was obtained with 8 MPa and one cycle within 28 min (Silva *et al*. 2013). Hence, pressure cycling shows promising results for inhibiting pathogens in the field of liquid food preservation. However, high pressure operation (> 4 MPa) and release of

CO_2 between each cycle of compression and decompression reported in the previous works is less interesting from an economic standpoint.

2.2.2.5 Effect of additives and combination treatments

Practical studies of the high-pressure CO_2 (>4 MPa) method have shown that the inactivation effect could be improved by the use of additives or by combining PCD with other methods (Zhang *et al.* 2006; Garcia-Gonzalez *et al.* 2007). Spilimbergo *et al.* (2003) found that only 2.5 log reductions of *E. coli* in glycerol solution were obtained by PCD (20 MPa, 34°C, 10 min) treatment alone, whereas more than 7 log reductions were achieved by sequenced treatments with pulsed electric fields (10 pulsed at 25 KV/cm) and PCD (20 MPa, 34°C, 10 min). Kim *et al.* (2008) suggested that *L. monocytogenes* inactivation by PCD was substantially accelerated by adding a small amount of surfactant to the cell suspension; specifically, a treatment period of 15 min was achieved by PCD (10 MPa, 35°C) in the presence of sucrose monolaurate (0.1%, w/v) compared with a 30 min period by PCD alone. Fijan *et al.* (2011) reported that about 3.1 log reductions of *Enterococcus faecium* were observed after a 25 min treatment with PCD (6 MPa, 20°C) and hydrogen peroxide (H_2O_2, 10%), but without H_2O_2 addition, only 0.3 log reductions were achieved. Hence, the use additives with PCD or the combination of PCD with other methods offer promising opportunities for improving the inactivation efficiency. Nevertheless, the high-pressures (6–20 MPa) required to effectively inactivate pathogens and the demands involved with this purpose (i.e. heavy-duty pressure equipment, substantial power consumption) are less interesting from both economic and implementation viewpoints.

2.3 Conclusions and future outlook

In recent years, PCD has been investigated as an innovative disinfection technology for water and wastewater treatment because of its inactivation efficiency, safety, and lack of problems associated with residual toxicity (Kobayashi *et al.* 2007, 2009; Cheng *et al.* 2011; Vo *et al.* 2013a,b 2015).

Previous research has shown that pressure cycling is a potential means to improve bacterial inactivation during PCD treatments (Dillow *et al.* 1999; Spilimbergo *et al.* 2002; Zhang *et al.* 2006; Ferreira *et al.* 2009; Silva *et al.* 2013); nevertheless, the inactivation mechanism is still unknown for this process. 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 (Dillow *et al.* 1999; Spilimbergo *et al.* 2002; Ferreira *et al.* 2009; Silva *et al.* 2013). Despite the good bactericidal performance of PCD technology enhanced by pressure cycling (Hong *et al.* 1997; Dillow *et al.* 1999; Ferreira *et al.* 2009), 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, it would be desirable to improve the bactericidal performance of pressure cycling in a manner that conducts at low pressures and with no release of CO_2 between each cycle of raised/lowered pressure.

On the other hand, practical studies of the high-pressure CO_2 (>4 MPa) method have shown that the use additives with PCD or the combination of PCD with other methods offer promising opportunities for improving the inactivation efficiency (Zhang *et al.* 2006; Garcia-Gonzalez *et al.* 2007). Furthermore, practical studies of the de-oxygenation with elevated CO_2 for ballast water treatment have shown that the disinfection effect could be improved by the incorporation of elevated CO_2 into electro-chlorination (Cha *et al.* 2015), or by combining CO_2 with other methods such as cavitation (NEI 2016; Coldharbour 2016), ultrasound (Coldharbour 2016), and hydrogen peroxide (GSI 2013). However, it is not clear from the existing research literature whether PCD (0.2–0.9 MPa) combined with other treatment methods such as chlorination would be able enhance the disinfection efficacy and reduce the treatment time.

2.4 References

AQIS. 1993. Ballast water management. Ballast water research series report no 4. Australian Quarantine and Inspection Service, AGPS Canberra.

Bimco 2016. Ballast water management. www.bimco.org/About/Viewpoint/02_Ballast_Water_Management.aspx (accessed 2 September 2016).

Battle, J. 2009. Silent Invasion–the spread of marine invasive species via ships'ballast water. WWF International, Gland.

California State lands commission 2014. Assessment of the efficacy, availability, and environmental impacts of ballast water treatment technologies for use in California waters. Produced for the California State legislature.

Carlton, J.T. 1999. Molluscan invasions in marine and estuarine communities. Malacologia, **41**(2), 439-454

Cha, H.-G., Seo, M.-H., Lee, H.-Y., Lee, J.-H., Lee, D.-S., Shin, K., Choi, K.-H. 2015. Enhancing the efficacy of electrolytic chlorination for ballast water treatment by adding carbon dioxide. Marine Pollution Bulletin, **95**, 315-323.

Cheng, X., Imai, T., Teeka, J., Yamaguchi, J., Hirose, M., Higuchi, T., Sekine, M. 2011. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. Applied Microbiology and Biotechnology, **90** (4), 1493-1500.

Coldharbour 2016. Ballast water treatment systems. http://www.coldharbourmarine.com/ballast-water-treatment (accessed 21 September 2016).

David, M. and Gollasch, S. 2015. Global Maritime Transport and Ballast Water Management - Issues and Solutions. Invading Nature - Springer Series in Invasion Ecology 8. Springer: Dordrecht, The Netherlands.

Dillow, A.K., Dehghani, F., Hrkach, J.S., Foster, N.R., Langer, R. 1999. Bacterial inactivation by using near and supercritical carbon dioxide. Proc. Nati. Acad. Sci. U.S.A., **96** (18), 10344-10348.

Ferreira, E.H.d.R., Rosenthal, A., Calado, V., Saraiva, J., Mendo, S. 2009. *Byssochlamys nivea* inactivation in pineapple juice and nectar using high pressure cycles. J. Food Eng., **95**, 664-669.

Fijan, S., Skerget, M., Knez, Z., Sostar-Turk, S., Neral, B. 2011. Determining the disinfection of textiles in compressed carbon dioxide using various indicator microbes. Journal of Applied Microbiology, **112**, 475-484.

Fraser, D. 1951. Bursting bacteria by release of gas pressure, Nature, **167** (4236), 33-34.

Garcia-Gonzalez, L., Geeraerd, A.H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Vanpe, J.F., Devlieghere, F. 2007. High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. International Journal of Food Microbiology, **117**, 1-28.

Garcia-Gonzalez, L., Geeraerd, A.H., Elst, K., Van Ginneken, L., Van Impe, J.F, Devlieghere, F. 2009. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. Journal of Supercritical Fluids, **51**, 74-82.

GloBallast 2016. The issue. http://globallast.imo.org/ballast-water-as-a-vector/ (accessed 29 May 2016).

Goncalves, A.A., Gagnon, G.A. 2012. Recent technologies for ballast water treatment. *Ozone: Science and Engineering*, 34, 174-195.

Gregg, M., Rigby, G., Hallegraeff, G.M. 2009. Review of two decades of progress in the development of management options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water. Aquatic Invasions, **4**(3), 521-565.

GSI 2013. Great ships initiative bench-scale test findings. http://www.greatshipsinitiative.org/GSI-BS-P-TR-4.pdf (accessed 15 October 2016).

Haas, G.J., Prescott, H.E., Dudley, E., Dik, R., Hintlian, C., Keane, L. 1989. Inactivation of microorganisms by carbon dioxide under pressure. Journal of Food Safety, 9, 253-265.

Hong, S.-I., Park, W.-S., and Pyun, Y.-R. 1997. Inactivation of *Lactobacillus* sp. from kimchi by high pressure carbon dioxide. LWT-Food Science and Technology, **30**, 681-685.

Husain, M., Felbeck, H., Apple, R., Altshuller, D., Quirmbach, C. 2004. Ballast water treatment by de-oxygenation with elevated CO_2 for a shipboard installation – a potential affordable solution. In: Matheickal, J.T., Raaymakers, S. (eds), 2nd International Ballast Water Treatment R&D Symposium, IMO, London, UK, 21-23 July 2003: proceedings. GloBallast Monograph Series No. 15, IMO London, 48-64.

IMO 2004 *International convention for the control and management of ship's ballast water and sediments*. International Conference on Ballast Water Management for ships BWM/CONF/36, London, February 2004.

IMO 2016a. Introduction to IMO. http://www.imo.org/en/About/Pages/Default.aspx. Last accessed on July 25, 2016.

IMO 2016b. Ballast water management – the control of harmful invasive species. http://www.imo.org/en/MediaCentre/HotTopics/BWM/Pages/default.aspx. (Accessed 01 September 2016).

IMO 2016c. Status of Conventions. http://www.imo.org/en/About/Conventions/StatusOfConventions/Pages/Default.aspx. (Accessed 01 September 2016).

IMO 2016d. BWM technologies. http://www.imo.org/en/OurWork/Environment/BallastWaterManagement/Pages/BWMTechnologies.aspx. (Accessed 01 September 2016).

IMO 2016e. Aquatic Invasive Species (AIS). http://www.imo.org/OurWork/Environment/BallastWaterManagement/Pages/AquaticInvasiveSpecies%28AIS%29.aspx. (Accessed 24 September 2016)

Isenschmid, A., Marison, I.W., von Stockar, U. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. Journal of Biotechnology, **39**, 229-237.

Kamihira, M., Taniguchi, M., Kobayashi, T. 1987. Sterilization of microorganisms with supercritical carbon dioxide. Agricultural and Biological Chemistry, **51**, 407–412.

Kim, SR., Park, H.J., Yim, D.S., Kim, H.T., Choi, I.G., Kim, K.H. 2008. Analysis of survival rates and cellular fatty acid profiles of *Listeria monocytogenes* treated with supercritical carbon dioxide under the influence of cosolvents. Journal of Microbiology Methods, **75**, 47-54.

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., Osajima, Y. 2007. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. Food Science and Technology Research, **13**, 20-22.

Kobayashi, F., Yamada, F., Ikeura, H., Hayata, Y., Muto, N., Osajima, Y. 2009. Inactivation of microorganisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. Journal of Water and Environment Technology, **7**, 241-250.

Lin, H.-M., Yang, Z.Y., Chen, L.-F. 1992. Inactivation of *Saccharomyces cerevisiae* by supercritical and subcritical carbon dioxide. Biotechnology Progress, **8**, 458–461.

Lloyd's Register's 2012. Understanding Ballast Water Management series. Ballast water treatment technologies and current system availability. London, September 2012.

MH system 2016. Ballast water treatment system – MH system. http://www.ballastwatersolution.com/images/BWT_by_De-Oxygenation_With_Elevated_CO2.pdf (accessed 15 October 2016).

Molnar, J.L., Gamboa, R.L., Revenga, C., Spalding, M.D. 2008. Assessing the global threat of invasive species to marine biodiversity. Frontiers in Ecology and the Environment, **6**, doi: 10.1890/070064.

NEI Marine 2016. N.E.I. Treatment Systems, Test data. http://www.nei-marine.com/images/pdf/NEI_Test_Data.pdf (accessed 21 September 2016).

Oceansaver 2016. How the system works. http://www.oceansaver.com/ (accessed 21 September 2016).

Ruiz, G.M., Carlton, J.T., Grosholz, E.D., Hines, A.H. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. American Zoologist, **37**, 621-632.

Silva, J.M., Rigo, A.A., Dalmolin, I.A., Debien, I., Cansian, R.L., Oliveira, J.V., Mazutti, M.A. 2013. Effect of pressure, depressurization rate and pressure cycling on the inactivation of *Escherichia coli* by supercritical carbon dioxide. Food Control, **29** (1), 76-81.

Spilimbergo, S., Elvassore, N., Bertucco, A. 2002 Microbial inactivation by high-pressure. J. Supercrit. Fluid., **22** (1), 55-63.

Spilimbergo, S., Dehghani, F., Bertucco, A., Foster, N.R. 2003. Inactivation of bacteria and spores by pulse electric field and high pressure CO_2 at low temperature. Biotechnolology and Bioengineering, **82** (1), 118-125.

Suban, V., David, M., Perkovič, M. 2006. Model for the assessment of the quantity of ballast water discharges using ships' traffic data. In: Conference proceedings. 8. International conference maritime transport and infrastructure, Riga, pp 69–75.

Tamburri, M.N., Wasson, K., Matsuda, M. 2002. Ballast water deoxygenation can prevent aquatic introductions while reducing ship corrosion. Biological Conservation, **103**, 331-341.

Tsolaki, E., Diamadopoulos, E. 2010. Technologies for ballast water treatment: a review. Journal of Chemical Technology and Biotechnology, **85**, 19-32.

Vo, H.T., Imai, T., Teeka, J., Sekine, M., Kanno, A., Le, T.V., Higuchi, T., Phummala, K., Yamamoto, K. 2013a. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. Water Research, **47**, 4286-4293.

Vo, H.T., Imai, T., Yamamoto, H., Le, V.T., Higuchi, T., Kanno, A., Yamamoto, K., Sekine, M. 2013b. Disinfection using pressurized carbon dioxide microbubbles to inactivate *Escherichia coli,* bacteriophase MS2 and T4. Journal of Water Environment Technology, **11**(6), 497-505.

Vo, H.T., Imai, T., Ho, T.T., Dang, T.-L.T., Hoang, S.A. 2015. Potential application of high pressure carbon dioxide in treated wastewater and water disinfection: Recent overview and further trends. Journal of Environmental Sciences, **36**, 38-47.

Zhang, J., Davids, T.A., Matthew, M.A., Drews, M.J., LaBerge, M., An., Y.H. 2006. Sterilization using high-pressure carbon dioxide. Journal Supercritical Fluids, **38**, 354-372.

CHAPTER III

DISINFECTION EFFECT OF PRESSURIZED CARBON DIOXIDE ON *Escherichia coli, Vibrio alginolyticus* AND *Enterococcus* sp. IN SEAWATER

3.1 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). Following the regulatory regimes, ships are required to limit the number of viable organisms in ballast water to meet the D-2 ballast water performance standard before it can be discharged into the sea (IMO 2004). The discharge limit must not exceed 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 ballast water. Chlorine or ozone has been commonly used for inactivating microorganisms in water owing to the high bactericidal efficiency of the treatments. However, toxic by-products generated during disinfection treatments remain in the water and the use of such treatments can be disadvantageous (Von Gunten *et al.* 2003; Fabbricino *et al.* 2005; 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 (Tsolaki *et al.* 2010; Werschkun *et al*. 2014).

High-pressure carbon dioxide (HPCD) has been widely used to sterilize food (Zhang *et al.* 2006; Garcia–Gonzalez *et al*. 2007) and to disinfect medical textiles under dry conditions (Fijan *et al.* 2012). The potential benefits of CO_2 as a sterilizing agent over other agents include its high bactericidal efficiency, nontoxicity, inexpensiveness, and availability (Zhang *et al.* 2006). However, the requirement for high pressure (> 4 MPa), as reported in previous work, is a disadvantage from an economic and logistics standpoint. Recently, HPCD was reported to effectively inactivate pathogens in water and wastewater (Kobayashi *et al.* 2007, 2009; Cheng *et al.* 2011; Vo *et al.* 2013a, b). Kobayashi *et al.* (2007, 2009) reported that at a pressure of 10 MPa and temperature of 35°C, supercritical CO_2 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 CO_2 (below 1.0 MPa) at room temperature. Previous studies on HPCD treatment were conducted using distilled water or water with low salinity (≤ 9‰ salinity) as the suspension medium. The efficacy of HPCD treatment for disinfecting seawater (~34‰ salinity) has not yet been studied.

In the present study, we examined the bactericidal effect of pressurized CO_2 (0.2–0.9 MPa) for disinfecting seawater (34‰ salinity). *Enterococcus* sp. (ATCC 202155), *E. coli* (ATCC 11303) and *V. alginolyticus* (ATCC 17749) were used as representative gram-positive and gram-negative bacteria, respectively, in our study. The effects of pressure, temperature, and WVR (defined as the ratio between the sample volume and apparatus volume) on the efficacy of pressurized CO_2 to disinfect seawater were assessed. In addition, the release of bacterial intracellular contents and changes in cell morphology after pressurized CO_2 treatment were evaluated to characterize the bacterial inactivation efficacy of pressurized CO_2 against *Enterococcus* sp., *E. coli* and *V. alginolyticus* in seawater. In general, the research objective was to determine whether CO_2 at low pressure (below 1.0 MPa) could be used to inactivate bacteria present in sea ballast water. The findings of this study could be useful for the development of a sustainable technology for disinfecting ship ballast water.

3.2 Materials and methods

3.2.1 Microorganism preparation and enumeration

The bacterial inoculums for *E. coli* (ATCC 11303), *V. alginolyticus* (ATCC 17749) 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), marine broth (Wako, 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 hours 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 8000 × *g* at room temperature) in a CF15D2 centrifuge (Hitachi, Japan). The pellet was re-suspended in 100 mL saline solution. Permanent stocks were maintained in 20% glycerol at −80°C.

All *E. coli*, *V. alginolyticus*, 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 34‰ salinity, and 100 µL of either a diluted or undiluted sample was plated on LB agar (Wako, Japan) for *E. coli*, on thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Wako, Japan) for *V. alginolyticus*, 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.

3.2.2 Seawater samples 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 bacterial 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).

3.2.3 Apparatus and procedure for disinfection

The experiment apparatus for disinfection was a stainless steel pressurized chamber with an internal volume of 10 L (Figure 3.1). The pressurization apparatus was designed to include a small nozzle and a shield to enable vigorous agitation of the influent for creating bubbles (Figure 3.2). 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 CO_2 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. High-pressurized water stream was introduced into the main chamber through a nozzle such that it collided with a bubble-generating shield to promote CO_2 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 CO_2 treatment was determined by varying the CO_2 pressures (0.2–0.9 MPa) applied for a 25 min treatment period. 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. The 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 number of circulation cycles performed in 25 min was calculated in relation to the treatment time and hydraulic retention time (HRT), wherein HRT = sample volume / flow rate. HRT values were 0.36, 0.43, 0.50, and 0.57 min, corresponding to WVR values of 50%, 60%, 70% and 80%, respectively.

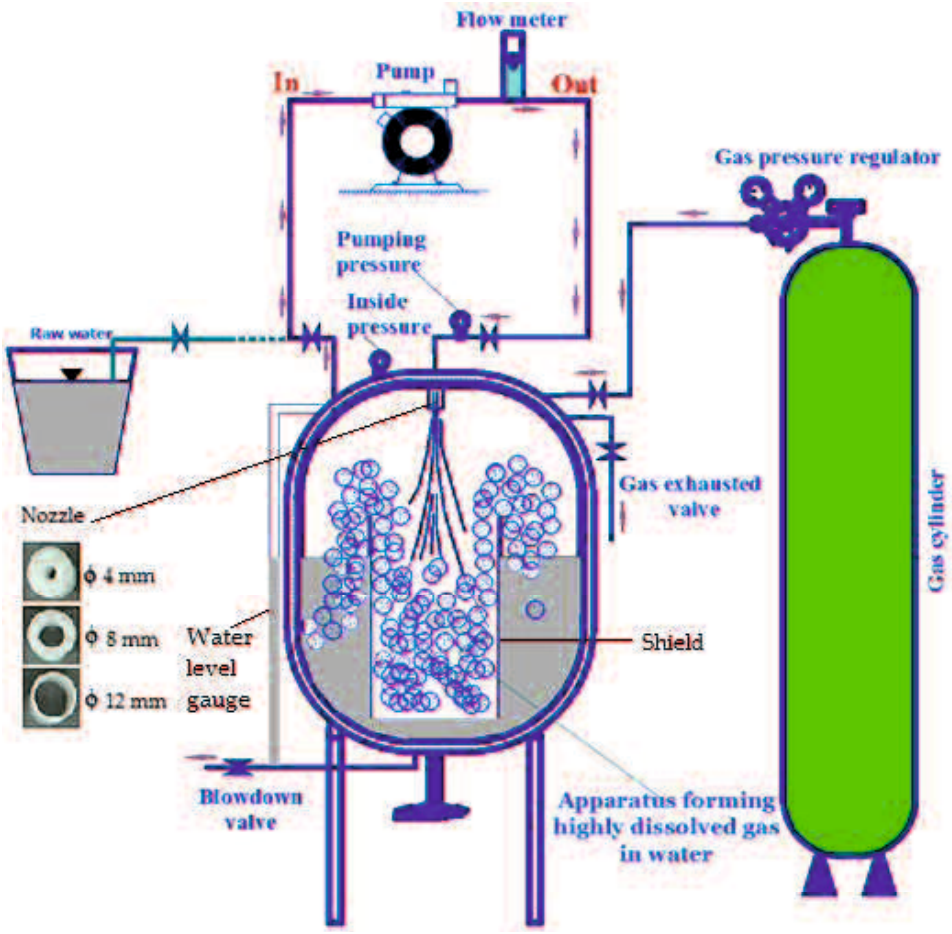

Figure 3.1 Setup of the water treatment apparatus.

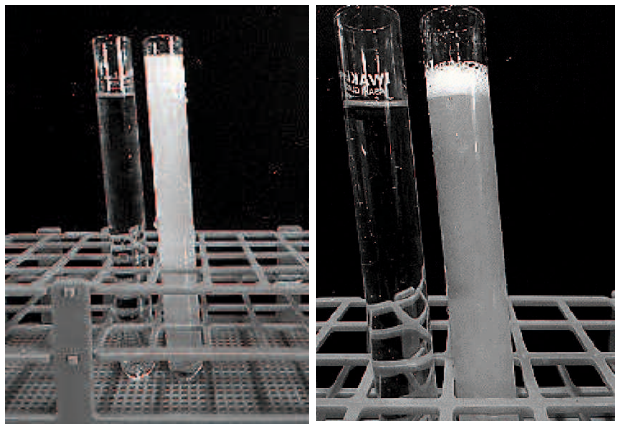

Figure 3.2 Pictures of an untreated sample and a CO_2-treated sample (the latter contains many small bubbles).

3.2.4 Measurement of intracellular material leakage

To quantify intracellular material released from *E. coli*, *V. alginolyticus*, and *Enterococcus* sp. cells, pressurized CO_2-treated and untreated samples were centrifuged at 1000 × *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.

3.2.5 Scanning electron microscopy

Changes in cell morphology after pressurized CO_2 treatment were assessed by using SEM The pellets of *E. coli*, *V. alginolyticus*, and *Enterococcus* sp. were immobilized with 2.5% glutaraldehyde (Wako, Japan) in 0.2 M Millonig's phosphate buffer saline pH 7.4 (PBS) for 3 hours 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 concentration of ethanol solutions (50%, 70%, 80%, 90%, 95%, and 100%), followed by ethanol/t-butyl alcohol (v/v = 1:1) treatment for 30 minutes. The prepared cells were then soaked in t-butyl alcohol two times for 1 hour, freeze-dried for 2 hours (JEE 4X vacuum evaporator, JEOL, Japan), and sputter coated with gold-palladium. Finally, the cells were examined using a scanning electron microscope (Quanta^{TM} 3D, FEI Co., USA) at 20 kV.

3.2.6 Inactivation kinetics assessment

The inactivation rate for *E. coli* and *Enterococcus* sp. was estimated by the following equation for a conventional first-order inactivation or linearized model (Erkmen *et al*. 2001).

$$log_{10}\frac{N}{N_0} = \frac{-kt}{2.303} \tag{3.1}$$

Here, N is number of colonies at time t (CFU mL^{-1}), N_0 is the number of colonies at time zero (CFU mL^{-1}), k is an inactivation rate constant (min^{-1}) calculated from the slope (= -k/2.303) of the reduction curve, and t is the exposure time (min); The decimal reduction time (D-value) is the exposure time required for a 1-log reduction in the bacterial load. The D-

value was obtained as the negative reciprocal slope of the $\log_{10}(N_t/N_0)$ versus time and was thus calculated by

$$D = \frac{2.303}{k} \tag{3.2}$$

3.3 Results and discussion

3.3.1 Bactericidal performance of pressurized CO_2 and pressurized air against *E. coli* in artificial seawater and filtered 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 3.3). 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. 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 3.3a).

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 3.3b). It has been hypothesized that the decrease in pH caused by pressurized CO_2 is probably a major factor driving the bacterial inactivation process (Hutkins and Nannen 1993; Hong and Pyun 1999; Vo *et al*. 2013a, b). 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.

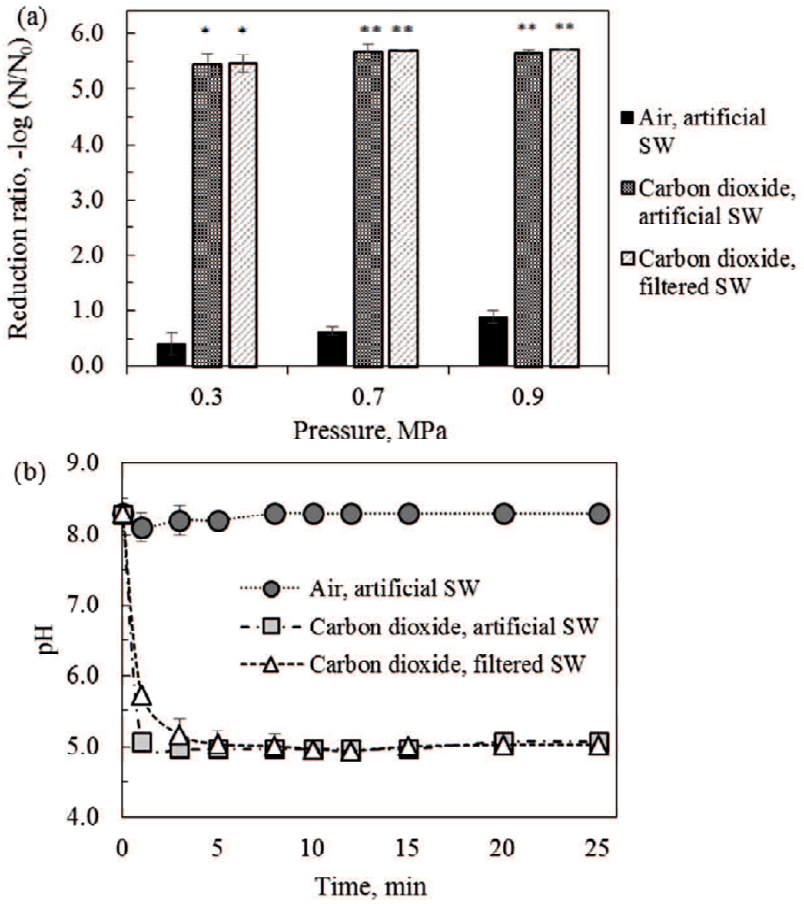

Figure 3.3 Effect of pressurized CO_2 and pressurized air on (a) *E. coli* inactivation and (b) the pH of seawater (SW). Operating conditions: 0.3–0.9 MPa, 20 ± 1°C, and a working volume ratio (WVR) of 70%. Asterisks (*) and (**) indicate that the *E. coli* load was completely inactivated after 25 and 10 min, respectively.

3.3.2 Effect of pressure on bactericidal effect of pressurized CO_2

The effect of various pressure conditions (range: 0.2–0.9 MPa) on the inactivation of *E. coli*, *V. alginolyticus*, and *Enterococcus* sp. is shown in (Figure 3.4). In general, the bactericidal activity of CO_2 on both bacterial species increased with increasing pressure, and higher pressure required shorter exposure times to achieve the same level of log reduction. The reduction of bacterial load was 5.3–5.7 log for *E. coli* (Figure 3.4a), 2.9–4.3 log for *Enterococcus* sp. (Figure 3.4b), and 5.1–5.7 log for *V. alginolyticus* (Figure 3.4c)

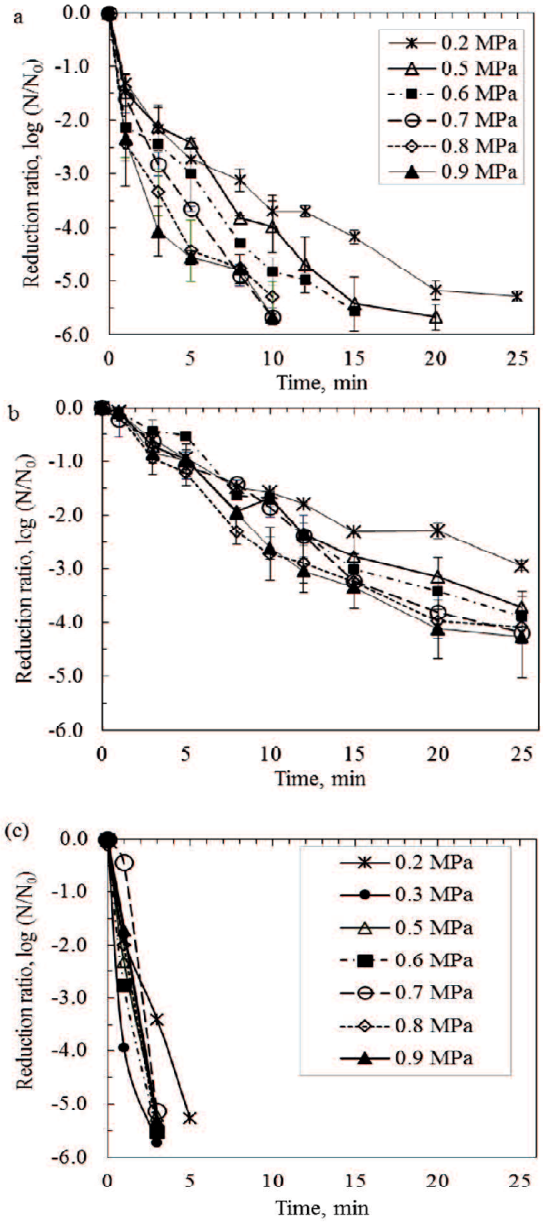

Figure 3.4 Effect of various pressure conditions (0.2–0.9 MPa) on (a) *E. coli*, (b) *Enterococcus* sp., and (c) *V. alginolyticus* inactivation in response to pressurized CO_2 at 20 ± 1.0°C and 70% WVR. Initial bacterial concentrations was 5–6 $\log_{10}$ CFU mL^{-1}.

At higher CO_2 pressures, shorter treatment times were required to inactivate *V. alginolyticus* and *E. coli.* For example, 25 min of 0.2 MPa CO_2 treatment was required to reduce the *E. coli* load by approximately 5.0 log, whereas only 20 and 15 min of 0.5 MPa and 0.6 MPa CO_2, respectively, were required to reduce the *E. coli* load to a similar extent. The treatment period could be reduced to 10 min with pressures between 0.7–0.9 MPa.

However, *E. coli* inactivation was not significantly enhanced by pressures exceeding 0.7 MPa (i.e. 0.8 MPa and 0.9 MPa). A similar relationship between CO_2 pressure and the efficacy of bacterial inactivation was observed with *Enterococcus* sp. (Figure 3.4b). With pressure ranging from 0.2 to 0.9 MPa, 2.9 to 4.3 log reduction of *Enterococcus* sp. was achieved. Nevertheless, the *Enterococcus* sp. inactivation was not significantly enhanced by pressures exceeding 0.7 MPa (i.e. 0.8 MPa and 0.9 MPa). 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 CO_2 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 CO_2 treatment (Figure 3.4b) was lower than that of *E. coli* (Figure 3.4a) and *V. alginolyticus* (Figure 3.4c). Under the experimental treatment conditions (0.7 MPa, 70% WVR, 20 ± 1.0°C, and initial concentration of 5–6 $\log_{10}$ 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* and *V. alginolyticus* within 10 min and 3 min, respectively. The differential sensitivity of *Enterococcus* sp., *V. alginolyticus*, and *E. coli* to pressurized CO_2 is likely due to differences in the structure of their cell walls. Compared to gram-negative bacteria, such as *V. alginolyticus* and *E. coli*, which have a thin peptidoglycan layer (Figure 3.5), gram-positive bacteria, such as *Enterococcus* sp., have a thick peptidoglycan layer that likely make them more resistant to inactivation by pressure (Zhang *et al.* 2006).

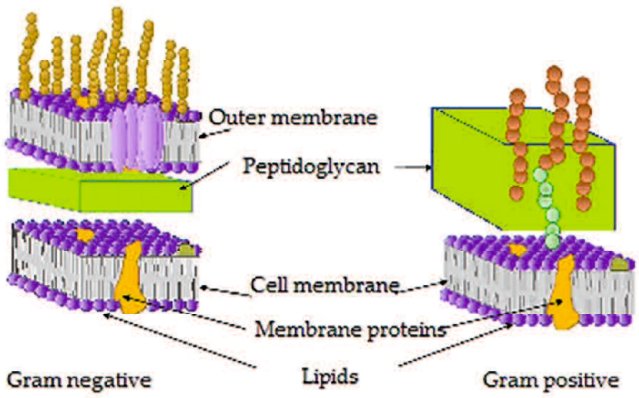

Figure 3.5 Comparison of gram-positive and gram-negative bacterial cell walls (peptidoglycan) (Maier *et al.* 2000)

3.3.3 Effect of temperature on bactericidal effect of pressurized CO_2

Figure 3.6 shows the bacterial inactivation efficiency of pressurized CO_2 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 3.6a), 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 3.6b), 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.

Thus, the disinfection efficiency of pressurized CO_2 increased with increasing temperatures for both *Enterococcus* sp. and *E. coli*. The disinfection efficiency could also be increased by enabling better contact between CO_2 and seawater in the liquid-film-forming apparatus to improve the solubility of CO_2 in seawater. Since CO_2 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 CO_2 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 CO_2 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 CO_2 microbubbles at 0.7 MPa and 26.6 ± 0.4°C. Despite same pressurized CO_2, the treatment time obtained in the present study (10 min for *E. coli*) was shorter than that obtained by Vo *et al.* (2013b).

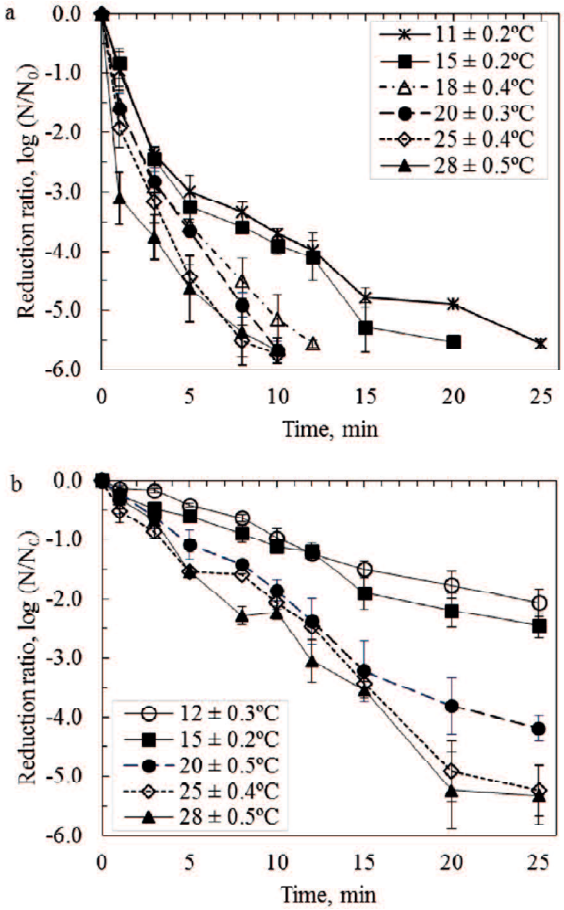

Figure 3.6 Inactivation of (a) *E. coli* and (b) *Enterococcus* sp. in artificial seawater by pressurized CO_2 treatment at different temperatures (ж, 11°C; ○, 12°C; ■, 15°C; Δ, 18°C; ●, 20°C; ◊, 25°C; and ▲, 28°C). All tests were performed using pressurized CO_2 at 0.7 MPa, and at 70% WVR, and 5–6 $\log_{10}$ CFU mL^{-1} initial bacterial concentration.

The inactivation of *E. coli* and *Enterococcus* sp. by pressurized CO_2 treatment followed a first-order kinetic model (Table 3.1). The inactivation kinetic rate constant *k* increased with temperature from 11 to 28°C under treatment conditions (0.7 MPa and 70% WVR within 25 min). Accordingly, high temperature led to small *D*-values (Table 3.1). Specifically, 5.08 to 2.17 min was required for 1-log reduction of *E. coli* load with pressurized CO_2 at ambient temperature in the range of 11 to 28°C. These *D*-values were 11.40 to 4.42 min ($R^2 \geq 0.97$) for *Enterococcus* sp. corresponding to temperature in the range of 12 to 28 °C, respectively. This suggests that the increase in temperature enhanced disinfection efficiency, producing smaller *D*-values.

Table 3.1 Effect of temperature on inactivation constant and decimal reduction time *D*, obtained by pressurized CO_2 at 0.7 MPa against *E. coli* and *Enterococcus* sp. in seawater.

Microorganism	Temperature	k (min^{-1})		D (min)		R^2
	°C	$\bar{X}$	± SD	$\bar{X}$	± SD	
E. coli	11	0.4532	0.0073	5.08	0.08	0.85
	15	0.5972	0.0360	3.86	0.23	0.88
	18	0.9698	0.0797	2.73	0.19	0.92
	20	1.1985	0.0232	1.92	0.04	0.95
	25	1.2356	0.1437	1.86	0.17	0.90
	28	1.0628	0.2280	2.17	0.35	0.77
Enterococcus sp.	12	0.2020	0.0248	11.40	1.52	0.98
	15	0.2308	0.0332	9.98	1.45	0.98
	20	0.4104	0.0225	5.61	0.32	0.98
	25	0.4905	0.0270	4.69	0.26	0.98
	28	0.5212	0.0625	4.42	0.58	0.97

$\bar{X}$ = means, SD = standard error from at least two determinations, and R^2 = regression coefficient.

3.3.4 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 3.7). 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 3.7c shows an increase in the water level (11 to 22 cm) and a decrease in the cycle number (72 to 44 cycles) as a consequence of increase in WVR from 50% to 80%.

Remarkably, the disinfection efficacy of pressurized CO_2 against both *E. coli* and *Enterococcus* sp. greatly increased with decreasing WVR. Thus, the bacterial load was reduced by 5.0 log for *E. coli* within 5 min of treatment at 50% (± 1%) WVR, whereas 15 min of treatment was required at 80% (± 1%) WVR (Figure 3.7a). 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 3.7b).

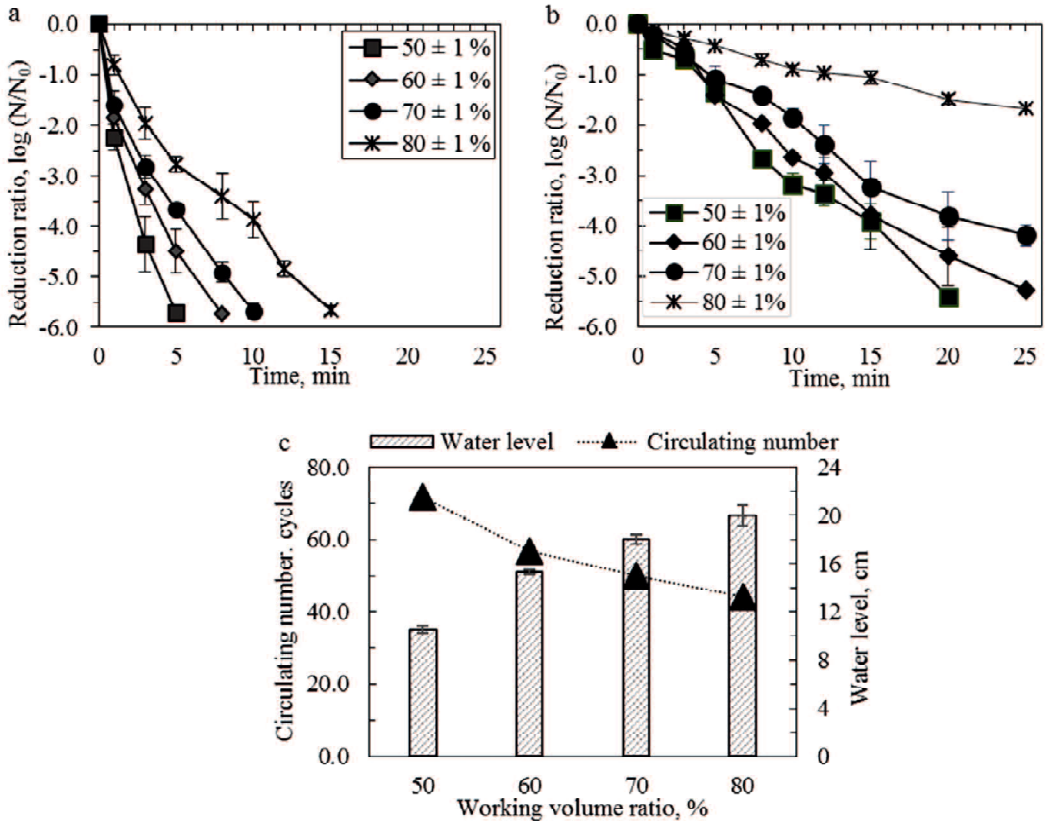

Figure 3.7 Effect of WVR (50%–80%) on inactivating (a) *E. coli* and (b) *Enterococcus* sp. in seawater by pressurized CO_2 at 0.7 MPa and 20 ± 1°C with flow rate 14 L min^{-1}, and initial bacterial concentration 5–6 log_{10} CFU mL^{-1}. (c) Influence of WVR on water level in main chamber and circulation number required for disinfection.

In general, operating at a smaller WVR results in a higher inactivation rate, which is related to the influence of the mass transfer rate of CO_2 in water (Lin *et al.* 1993; Hong *et al.* 1997; Garcia-Gonzalez *et al.* 2009). In this study, the disinfection efficacy decreased dramatically when operated at 80% WVR, indicating that under identical treatment conditions, the transfer rate of CO_2 was limited at 80% WVR. The reduced disinfection efficiency at 80% WVR may be related to a reduction in the number of circulations completed (44 cycles/25 min) or an increase in the water level (22 cm, Figure 3.7c). In particular, 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 CO_2 mass transfer. Operating at a low WVR provides a larger space to generate CO_2 bubbles and increases the number of circulation cycles (50 to 72 cycles within 25 min, respectively;

Figure 3.7c). In addition, a pump was used to apply a higher pressure (0.12 MPa) than that inside the main chamber. Therefore, circulation cycles were characterized by repetitive of raised and lowered pressure. Thus, an increase in the circulation number, results in more rapid repetitions of raised and lowered pressure, which may in turn enhance the solubility of CO_2 in seawater and probably increase the bactericidal efficiency. Hence, further research on the effects of pressure cycling on disinfection efficacy is necessary.

Table 3.2 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 CO_2, 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 M KH_2PO_4, 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 CO_2 (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 *E. petroleum* within 5 min and 20 min, respectively. These findings demonstrate the excellent bactericidal activity of pressurized CO_2, and suggest that this method could be further developed as a sustainable technology for disinfecting ship ballast water.

Table 3.2 Disinfection times for several different pathogenic microorganisms with chlorinated water compared to pressurized CO_2

Disinfectant	Experimental conditions	Microorganism (initial concentration)	Time, min	References
Monochloramine (0.5 mg L^{-1})	Pure culture in 0.05 M KH_2PO_4, pH 7.0, at 5°C	*E. coli* (6.2 $\log_{10}$ CFU mL^{-1})[d]	30[a]	Rice *et al.* (1993)
		E. faecium (5.3 $\log_{10}$ CFU mL^{-1})[d]	60[b]	
Chlorine (0.5 mg L^{-1})	Natural seawater, 3.5% salinity, 20–25°C	*E. coli* (6.0 $\log_{10}$ CFU mL^{-1})	12[c*]	Azanza *et al.* (2001)
CO_2	Artificial seawater, salinity = 3.4%, pressure = 0.7 MPa, WVR = 50%, at 20°C	*E. coli* (5.7 $\log_{10}$ CFU mL^{-1})	5*	This study
		E. petroleum (5.4 $\log_{10}$ CFU mL^{-1})	20*	

[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).
*no viable microorganism was observed.

3.3.5 Leakage of intracellular contents after pressurized CO_2 treatment

The amount of UV-absorbing substances released from *V. alginolyticus*, *E. coli*, and *Enterococcus* sp. cells increased steadily during the pressurized CO_2 treatment process (Figure 3.8). The result demonstrates that *V. alginolyticus*, *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 *V. alginolyticus* and *E. coli* supernatants that were measured using 260 nm wavelength (Figure 3.8a) and 280 nm wavelength (Figure 3.8b) 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 *V. alginolyticus* and *E. coli*, gram-negative bacterium. These data support the findings from previous section that showed that gram-positive bacteria such as *Enterococcus* sp. were less susceptible to pressurized CO_2 treatment owing to the presence of a thicker peptidoglycan layer (Zhang *et al.* 2006).

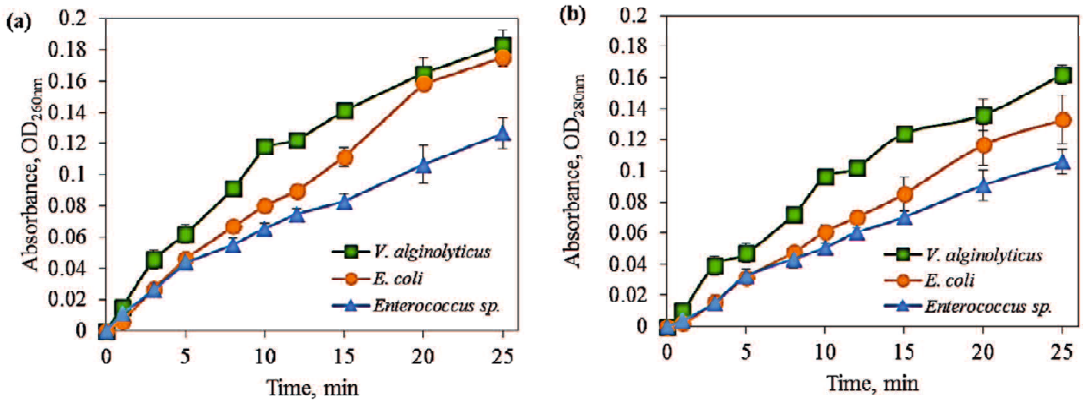

Figure 3.8 Quantitation of proteins and nucleic acids that leaked out of *V. alginolyticus*, *E. coli*, and *Enterococcus* sp. after pressurized CO_2 treatment (at 0.7 MPa, 20 ± 0.3°C, and 70% WVR). The UV absorbance values of supernatants obtained from pressurized CO_2-treated samples were measured at 260 nm for determining nucleic acid content (a) and at 280 nm for determining protein content (b). OD is optical density.

3.3.6 SEM analyses

To examine the effect of pressurized CO_2 treatment on bacterial morphology, SEM assessment was performed using *E. coli* (Figure 3.9a, b), *Enterococcus* sp. (Figure 3.9c, d) and *V. alginolyticus* (Figure 3.9e, f) samples treated with pressurized CO_2 at the determined optimal conditions (0.7 MPa, 20°C, and 50% WVR) for 25 minutes. Comparative SEM images of untreated samples and samples treated with pressurized CO_2 did not reveal dramatic changes in the cell shape of *Enterococcus* sp.; however, some *E. coli* and *V. alginolyticus* cells that were treated with pressurized CO_2 did not retain the original shape and appeared to be lysed. Notably, the treated cells of three species had several small vesicles on the cell surface, whereas the untreated cells did not present such structures on the cell surface.

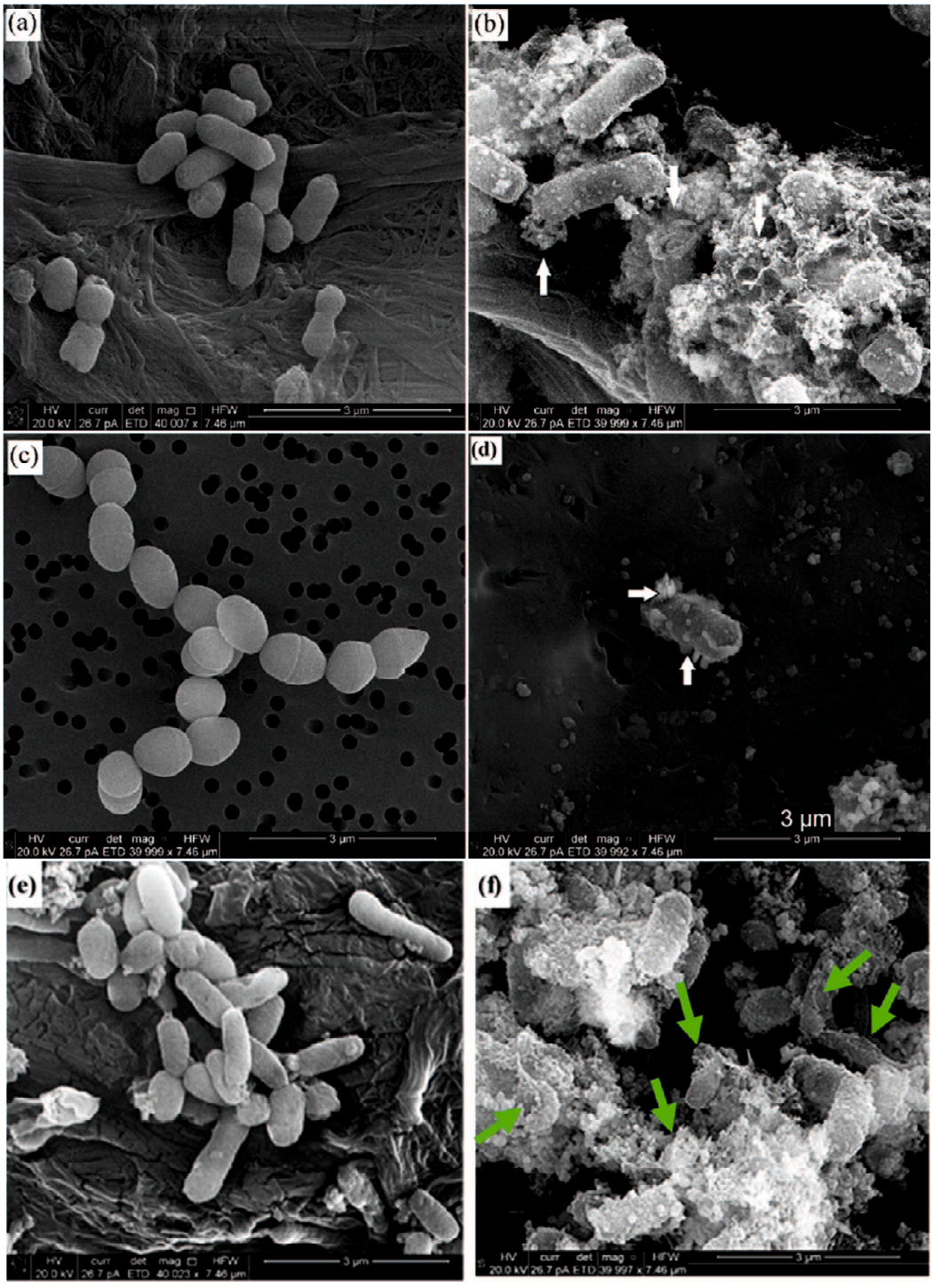

Figure 3.9 Representative SEM images of (a) untreated *E. coli* cells, (b) PCD treated *E. coli* cells, (c) untreated *Enterococcus* sp. cells, (d) PCD treated *Enterococcus* sp. cells, (e) untreated *V. alginolyticus* cells, and (f) PCD treated *V. alginolyticus* cells. The PCD treated cells were exposed to pressurized CO_2 by using pressure of 0.7 MPa, at 20°C, and 50% WVR within 25 min.

Previous studies have used SEM and transmission electron microscope imaging to show that *Salmonella typhimurium* and *E. coli* cells treated with supercritical CO_2 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 CO_2 (0.7 MPa and 20°C), this study showed that the formation of several extracellular small vesicles was not only observed in *E. coli* and *V. alginolyticus*, 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 that shows that the leakage of intracellular materials occurred during the treatment period. The data also affirmed the superior performance of pressurized CO_2 treatment.

3.4 Conclusions

Pressurized CO_2 treatment can be used to eliminate *V. alginolyticus*, *E. coli*, and *Enterococcus* sp. from seawater. The gram-positive bacterial species, *Enterococcus* sp., had lower susceptibility to pressurized CO_2 treatment than did the gram-negative bacterial species, *V. alginolyticus* and *E. coli.* The seawater disinfection efficiency can be considerably improved by enhancing the solubility of CO_2 into seawater to increase penetration of CO_2 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 5.1 log reduction of *V. alginolyticus*, 5.7 log reduction of *E. coli*, and 5.4 log reduction of *Enterococcus* sp. within 3 min, 5 min and 20 min, respectively. Taken together, these data indicate that pressurized CO_2 could be potentially used for treatment of ballast water. Further research is required to elucidate the effects of pH and pressure cycling on bactericidal activity of pressurized CO_2.

3.5 References

Azanza, M.P.V., Azanza, R.V., Gedaria, A.I, Sententa, H.G., Idjao, M.V. 2001. Decimal reduction times of *Pyrodinium bahamense* var. *compressum* and *Escherichia coli* in chlorine- and ultraviolet-treated seawater. *Letters in Applied Microbiology* **33**, 371-376.

Cheng, X., Imai, T., Teeka, J., Yamaguchi, J., Hirose, M., Higuchi, T., Sekine, M. 2011. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. *Applied Microbiology and Biotechnology* **90** (4), 1493-1500.

Erkmen, O., Kamaran, H. 2001. Kinetic studies on the high pressure carbon dioxide inactivation of *Samonella typhimurium*. *Journal of Food Engineering* **50**, 25-28.

Fabbricino, M., Korshin, G.V. 2005. Formation of disinfection by-products and applicability of differential absorbance spectroscopy to monitor halogenation in chlorinated coastal and deep ocean seawater. *Desalination* **176**, 57-69.

Garcia-Gonzalez, L., Geeraerd, A.H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Vanpe, J.F., Devlieghere, F. 2007. High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. *International Journal of Food Microbiology* **117**, 1-28.

Garcia-Gonzalez, L., Geeraerd, A.H., Elst, K., Van Ginneken, L., Van Impe, J.F, Devlieghere, F. 2009. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. *Journal of Supercritical Fluids* **51**, 74-82.

Garcia-Gonzalez, L., Geeraerd, A.H., Mast, J., Briers, Y., Elst, K., Van Ginneken, L., Van Impe, J.F, Devlieghere, F. 2010. Membrane permeabilization and cellular death of *Escherichia coli*, *Listeria monocytogenes* and *Saccharomyces cerevisiae* as induced by high pressure carbon dioxide treatment. *Food Microbiology* **27**, 541-549.

Hong, S.-I., Park, W.-S., and Pyun, Y.-R. 1997. *Inactivation of Lactobacillus sp. from kimchi by high pressure carbon dioxide. LWT-Food Science and Technology* **30**, 681-685.

IMO 2004. *International convention for the control and management of ship's ballast water and sediments*. International Conference on Ballast Water Management for ships BWM/CONF/36, London, February 2004.

Isenschmid, A., Marison, I.W., von Stockar, U. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. *Journal of Biotechnology* **39**, 229-237.

Kim, S.R., Rhee, M.S., Kim, B.C., Lee, H., Kim, K.H. 2007. Modeling of the inactivation of *Salmonella typhimurium* by supercritical carbon dioxide in physiological saline and phosphate-buffered saline. *Journal of Microbiology Methods* **70**, 132-141.

Kim, SR., Park, H.J., Yim, D.S., Kim, H.T., Choi, I.G., Kim, K.H. 2008. Analysis of survival rates and cellular fatty acid profiles of *Listeria monocytogenes* treated with

supercritical carbon dioxide under the influence of cosolvents. *Journal of Microbiology Methods* **75**, 47-54.

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., Osajima, Y. 2007. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. *Food Science and Technology Research* **13**, 20-22.

Kobayashi, F., Yamada, F., Ikeura, H., Hayata, Y., Muto, N., Osajima, Y. 2009. Inactivation of microorganisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. *Journal of Water and Environment Technology* **7**, 241-250.

Lin, H.M., Yang, Z., Chen, L.F. 1993. Inactivation of *Leuconostoc dextranicum* with carbon dioxide under pressure. *Chemical Engineering Journal* **52** (1), B29-B34.

Molnar, J.L., Gamboa, R.L., Revenga, C., Spalding, M.D. 2008. Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment* **6**, doi: 10.1890/070064.

Oulé M.K., Tano K., Bernier A.M., Arul J. 2006. *Escherichia coli* inactivation mechanism by pressurized CO_2. *Canadian Journal of Microbiology* **52**, 1208-1217.

Rice, E.W., Covert, T.C., Wild, D.K., Berman, D., Johnson, S.A., Johnson, C.H. 1993. Comparative resistance of *Escherichia coli* and enterococci to chlorination. *Journal of Environmental Science and Health, part A: Toxic/Hazardous substances and Environmental Engineering* **28** (1), 89-97.

Ruiz, G.M., Carlton, J.T., Grosholz, E.D., Hines, A.H. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist* **37**, 621-632.

Tsolaki, E., Diamadopoulos, E. 2010. Technologies for ballast water treatment: a review. *Journal of Chemical Technology and Biotechnology* **85**, 19-32.

Vo, H.T., Imai, T., Teeka, J., Sekine, M., Kanno, A., Le, T.V., Higuchi, T., Phummala, K., Yamamoto, K. 2013a. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. *Water Research* **47**, 4286-4293.

Vo, H.T., Imai, T., Yamamoto, H., Le, V.T., Higuchi, T., Kanno, A., Yamamoto, K., Sekine, M. 2013b. Disinfection using pressurized carbon dioxide microbubbles to inactivate *Escherichia coli,* bacteriophase MS2 and T4. *Journal of Water Environment Technology* **11**(6), 497-505.

Von Gunten, U. 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. *Water Research* **37**, 1469-1487.

Werschkun, B., Sommer, Y., Banerji, S. 2012. Disinfection by-products in ballast water treatment: An evaluation of regulatory data. *Water Research* **46**, 4884-4901.

Werschkun, B., Banerji, S., Basurko, O.C., David, M., Fuhr, F., Gollasch, S., Grummt, T., Haarich, M., Jha, A.N., Kacan, S., Kehser, A., Linders, J., Mesbahi, E., Pughiuc, D., Richardson, S.D., Schwarz-schulz, B., shah, A., Theobald, N., Gunten, U.V., Wieck, S., Höfer, T. 2014. Emerging risks from ballast water treatment: the run-up to the International Ballast Water Management Convention. *Chemosphere* **112**, 256-266.

Zhang, J., Davids, T.A., Matthew, M.A., Drews, M.J., LaBerge, M., An., Y.H. 2006. Sterilization using high-pressure carbon dioxide. *Journal Supercritical Fluids* **38**, 354-372.

CHAPTER IV

ENHANCED BACTERICIDAL PERFORMANCE OF PRESSURIZED CARBON DIOXIDE BY PRESSURE AND PRESSURE CYCLING

4.1 Introduction

Chlorination is the most common method for water disinfection. It has the advantages of high bactericidal efficiency, low cost, and residual disinfectant. However, chlorine can combine with organic compounds in water to produce carcinogenic agents such as trihalomethanes and halogenic acetic acids (Boorman *et al.* 1999; Fabbricino *et al.* 2005). Therefore, growing concerns about the potential hazards associated with disinfection byproducts have boosted efforts to develop alternative methods of water disinfection. Another method, ozonation, is effective in inhibiting pathogens, and is considered a capable alternative to chlorination. However, disadvantages of ozone disinfection are high cost, lack of residual disinfectant, special operations, and the formation of disinfection byproducts in water containing bromine (Von Gunten *et al.* 2003). Other water treatment methods such as ultraviolet (UV) radiation, ultrasound, cavitation, or heat application can be used for mechanical disruption and inactivation of organisms. Although mechanical disruption methods do not have problems of residual toxicity, they have high operational costs because of their substantial power requirements. In addition, the disinfecting efficiency of UV light is reduced for waters with high turbidity or high concentration of dissolved organic matter (Werschkun *et al.* 2012).

Pressurized CO_2 has been used for eradicating pathogens in food as a non-thermal sterilization method (Garcia-Gonzalez *et al.* 2007). The high bactericidal efficiency, nontoxic, inexpensive, and readily available character of CO_2 give it potential benefits over other sterilant agents (Zhang *et al.* 2006). For high-pressure CO_2 treatment, most studies have reported the influence of process parameters such as pressure, temperature, agitation speed, decompression rate, pressure cycling, bacterial concentration, and exposure time on the inactivation of bacteria. Among these parameters, pressure cycling, a repetitive procedure of release and compression of CO_2, is a promising means to increase inactivation efficacy (Zhang *et al.* 2006; Silva *et al.* 2013). Theories explaining the inactivating mechanism of pressure cycling involve explosive cell rupture and mass transfer rate, in

which compression intensifies the mass transfer of CO_2 across cell membranes (Hong *et al.* 1997; Hong and Pyun 1999; Dillow *et al.* 1999) and decompression enhances the cell rupture (Fraser *et al.* 1951). Dillow *et al.* (1999) found that the inactivation was substantially increased from 3 log to 9 log of the reduction ratio, corresponding to an increase of pressure cycling from three to six cycles with treatment conditions 20.5 MPa and 34°C within 0.6 hour. In addition, Spilimbergo *et al.* (2002) observed that an approximate 3.5 log reduction of *Bacillus subtilis* spores was achieved after 15 cycles at 8.0 MPa and 36°C for 30 min, but without pressure cycling, only a 0.5 log reduction was obtained under treatment conditions 7.5 MPa and 36°C for 24 hours. Ferreira *et al.* (2009) suggested that the use of pressure cycles was more effective than sustained high pressures to inhibit *Byssochlamys nivea*; a greater than 3.0 log reduction was observed after five cycles compared with a nearly 1.0 log reduction after one cycle in treatment conditions 550 MPa and 20°C for 15 min. Silva *et al.* (2013) reported that number of pressure cycles and system pressure were two significant parameters for the inactivation of *Escherichia coli* with supercritical CO_2. An 8.0 log bacterial load was reduced with five cycles and 8 MPa after 140 min of treatment, whereas a 5.0 log reduction was obtained with 8 MPa and one cycle within 28 min (Silva *et al.* 2013). Hence, pressure cycling shows promising results for inhibiting pathogens in the field of liquid food preservation. However, high pressure operation (> 4 MPa) and release of CO_2 between each cycle of compression and decompression reported in the previous works is less interesting from an economic standpoint.

Recently, pressurized CO_2 has seen renewed interest in the field of water and wastewater treatment, owing to its high inactivation efficiency, safe use, and lack of disinfection byproduct problems (Kobayashi *et al.* 2007, 2009a,b; Cheng *et al.* 2011; Vo *et al.* 2013, 2014). The first related work was published by Kobayashi *et al.* (2007), who applied supercritical CO_2 to treatment of wastewater and succeeded in eradicating *E. coli* within 13 min. However, they required very high pressure (up to 10 MPa) and high temperature 55 °C (Kobayashi *et al.* 2007). Another works was conducted using low-pressurized CO_2 based on a gas bubbles method to inactivate *E. coli* and bacteriophages in water (Cheng *et al.* 2011; Vo *et al.* 2013, 2014). Cheng *et al.* (2011) believed that sudden discharge and resulting reduction of pressure led to mechanical cell rupture, which resulted in effective inactivation within 20 min by pressurized CO_2 at 0.3 MPa and room temperature. Vo *et al.* (2013, 2014) claimed that acidified water and cellular lipid extraction caused by pressurized CO_2 at 0.7 MPa and room temperature were major factors for changes of cell

membrane structure and efficient disinfection within 25-min treatment. Although these studies suggest that pressure cycling was an important parameter in pressurized CO_2 inactivation, no data were presented.

This study examined the effects of pressure cycling on bactericidal activity of CO_2 at low pressure (< 1 MPa) and no release of CO_2 between each cycle of raised/lowered pressure. The inactivation performance of pressurized CO_2 against *E. coli* and *Enterococcus* sp. were examined for various conditions of pressure, temperature, flow rates, and working volume ratios (WVRs, defined as the ratio between the sample volume and apparatus volume, was in the range 50%−80%). The sensitivity of gram-positive *Enterococcus* sp. to the pressurized gases was evaluated for various conditions of CO_2 content rate (100%, 50%, 25%, and 0%). Relationships of bactericidal effect to CO_2 concentration and pH of treated water were also evaluated. We also investigated the influence of pressure on inactivation kinetics of *Enterococcus* sp. in artificial seawater resulting from the pressurized CO_2 treatment.

4.2 Materials and methods

4.2.1 Microorganism preparation and enumeration

Bacteria *Enterococcus* sp. (ATCC 202155) was cultivated in brain heart infusion broth (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 35 g L^{-1} sodium chloride. *E. coli* (ATCC 11303) was cultivated in LB broth (Wako, Japan), which was supplemented with sodium chloride to obtain a final concentration of 30 g L^{-1}. Bacterial cultures were incubated for 18 hours at 37°C using a reciprocal shaker rotating at 150 rpm. Permanent stock was maintained in 20% glycerol at -80°C.

The concentrations of *Enterococcus* sp. and *E. coli* were determined by plating aliquots of the culture onto brain heart infusion agar and LB agar, respectively. For samples with a low number of viable cells, 1 mL of the undiluted sample was poured into agar maintained at 45°C. The plates were incubated at 37°C for 24 hours. Colonies of bacteria were then counted on plates that contained 30−300 CFUs/plate. Each sample was analyzed in triplicate.

4.2.2 Seawater sample preparation

Artificial seawater was prepared by adding artificial sea salt (GEX, Osaka, Japan) to distilled water to obtain a final salinity of 3.4% as measured by a salinity meter (YK-31 SA, Lutron Electronic Enterprise Co. Ltd., Taiwan). For all experiments, the bacterial preparations were diluted in the artificial seawater to obtain a bacterial concentration of 10^5

CFU mL^{-1}, which was used as the initial concentration for all experiments. pH and temperature of the samples were measured with a pH meter (Horiba D-51, Horiba Co. Ltd., Japan). The concentration of CO_2 in seawater was measured by a CO_2 meter (CGP-31, TOA-DKK, Japan).

4.2.3 Apparatus and procedure for disinfection

The experiment apparatus for disinfection was a stainless steel pressurized chamber with an internal volume of 10 L and tolerance up to 1.0 MPa. The pressurization apparatus was designed to include a solid stream nozzle and shield to enable vigorous agitation of the influent to create bubbles (Figure 4.1). Sample water was pumped into the device through a small nozzle such that the pressurized water stream collided with a shield to generate bubbles under high pressure (Figure 4.2). The main chamber was soaked in a water bath to maintain the stability of initial temperature of the sample during the treatment period.

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 (Dillow *et al.* 1999; Spilimbergo *et al.* 2002; Ferreira *et al.* 2009; Silva *et al.* 2013). 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 bacterial 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, a variety of nozzle sizes (15 mm height × 4, 5, 6, 7, 8, 10, 12, and 15 mm diameters) and two pumps (0.20 and 0.75 kW of pumping power) were used to change the flow rate and pressure power of the input, in which nozzle diameter (15 mm × 15 mm) equaled the diameter of the pipeline inlet (15 mm). Seven liters of seawater was pumped in one shot into the device as influent. Following the first influx of water, pressurized gas was also injected into the main chamber, and system pressure was adjusted by a gas pressure regulator to 0.7 MPa. The water sample was then circulated by pumping inside the system for 25 min. Here, pressure cycling alternately raised and lowered pressure without the release of gas out from the reactor. Pumping pressure and system pressure were measured by pressure gauges. The pressure difference ΔP = pressure caused by pump suction (MPa) − pressure inside main chamber (0.7 MPa). Water flow rate was measured by a flow meter (GPI, Nippon Flow Cell Co., Ltd., Japan). The circulation number

was calculated in relation to exposure time and hydraulic retention time (HRT), where HRT = sample volume / flow rate.

To investigate the effect of pressure, the sensitivity of *Enterococcus* sp. to pressurized CO_2 treatment was determined using various pressures (0.3, 0.5, 0.7 and 0.9 MPa). 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%). Water level was measured by a gauge to evaluate the effects of WVR on the shield inside the main chamber. Each experiment was conducted in triplicate.

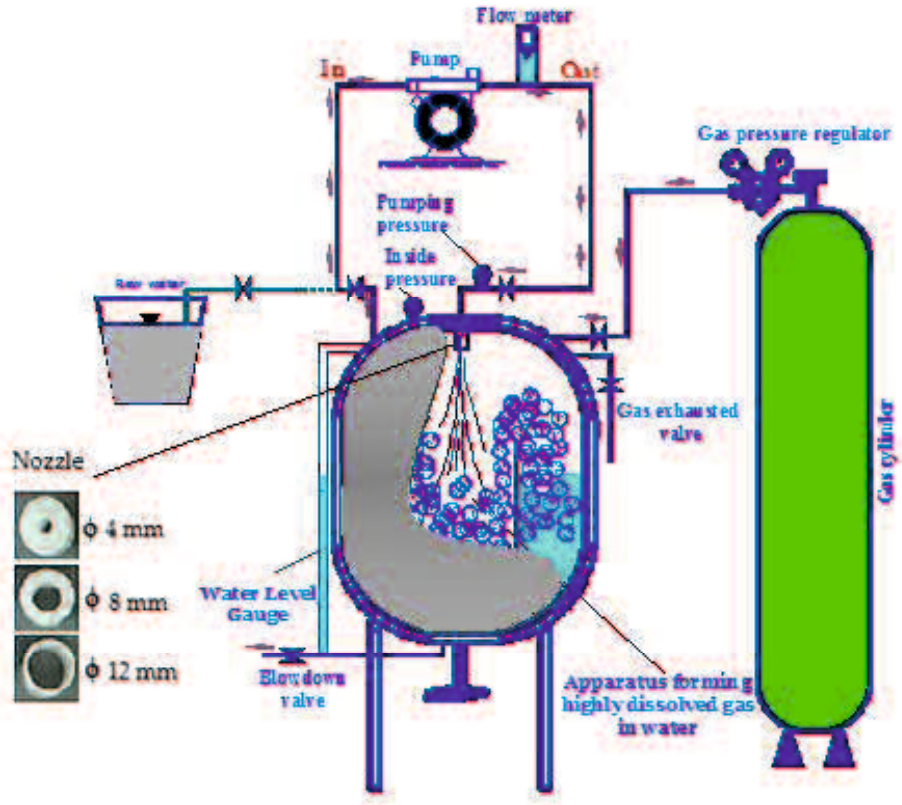

Figure 4.1 Water treatment apparatus setup. Water is pumped into liquid-film-forming chamber through a small nozzle that allows pressure control. Pressurized gas is also pumped into main chamber. A highly pressurized water stream is introduced in main chamber such that it collides with a bubble-generating shield that promotes gas diffusion in the water.

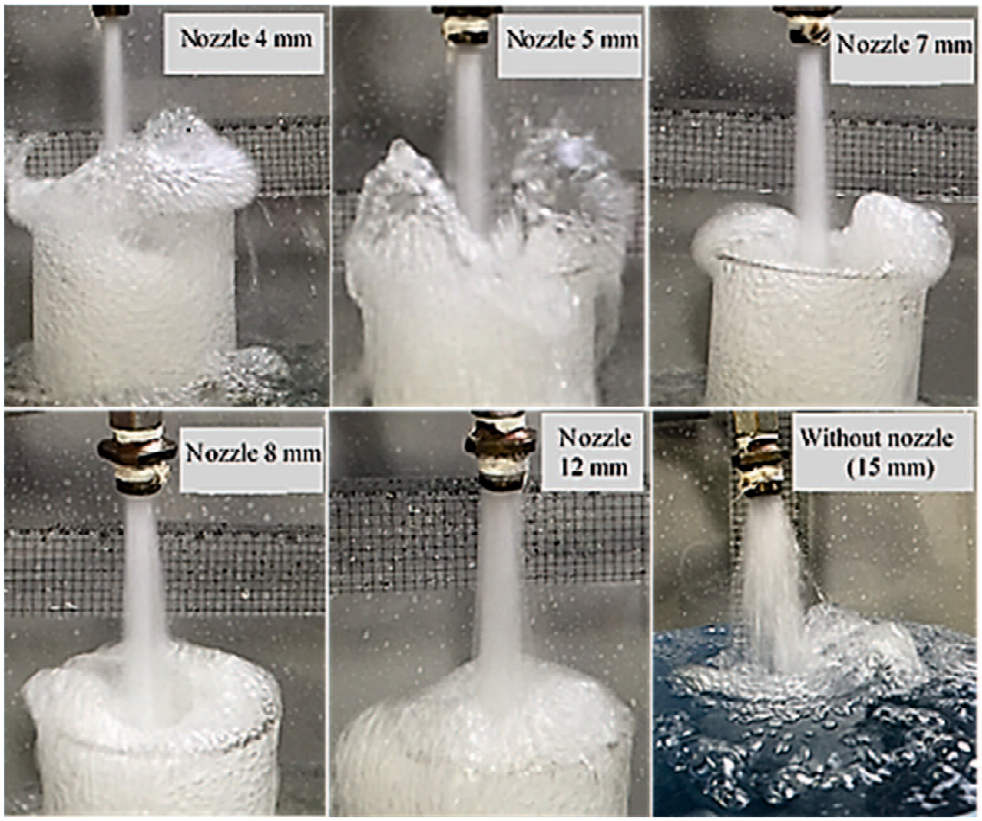

Figure 4.1 Representative pictures of liquid film formation with various nozzle diameters at a normal pressure in the pipeline.

4.2.4 Inactivation kinetics assessment

The inactivation rate for *Enterococcus* sp. was estimated by the following equation for a conventional first-order inactivation or linearized model (Erkmen *et al*. 2001).

$$log_{10}\frac{N}{N_0} = \frac{-kt}{2.303} \tag{4.1}$$

Here, N is number of colonies at time t (CFU mL^{-1}), N_0 is the number of colonies at time zero (CFU mL^{-1}), k is an inactivation rate constant (min^{-1}) calculated from the slope (= -k/2.303) of the reduction curve, and t is the exposure time (min); The decimal reduction time (D-value) is the exposure time required for a 1-log reduction in the bacterial load. The D-value was obtained as the negative reciprocal slope of the $\log_{10}(N_t/N_0)$ versus time and was thus calculated by

$$D = \frac{2.303}{k} \tag{4.2}$$

4.2.5 Statistical analysis

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

$$y_i = \beta_0 + \sum \beta_i x_i \quad (4.3)$$

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.

4.3 Results and discussion

4.3.1 Effect of pressure cycling on bacterial inactivation

4.3.1.1 Effect of pressure cycling on *Enterococcus* sp. inactivation

To investigate the effect of pressure cycling on the inactivation efficiency of pressurized CO_2, two pumps (0.20 and 0.75 kW), a variety of nozzle diameters (4–15 mm) were used to change the flow rate of the input. Here, disinfecting was done with 0.7 MPa of pressurized CO_2, 20 ± 0.5°C, and 70% WVR within 25 min (Figure 4.3). As shown in Figure 4.3b, a larger nozzle diameter increased the cycle number but decreased ΔP. For every nozzle diameter, stronger pumping power enhanced not only the frequency but also the magnitude of pressure cycling. Operation with a 0.75-kW power pump increased the disinfecting efficiency over that with a 0.20-kW power pump, for every nozzle diameter (Figure 4.3a).

Pressure cycling raises inactivation efficacy by providing a driving force for mass transfer penetration of CO_2 into the cell membrane (Dillow *et al.* 1999; Zhang *et al.* 2006). Hence, it is hypothesized that an increase in cycle number augments bactericidal efficiency. However, our results indicate a lower disinfection efficacy associated with number of circulations (Figure 4.3). For 0.20 kW pumping power, inactivation efficacy significantly decreased from 5.2 to 0.9 log reduction (Figure 4.3a), corresponding to an increase of circulation number from 53 to 74 cycles, respectively (Figure 4.3b). A similar association between the frequency of pressure cycling and bacterial inactivation was found in the case of 0.75 kW pumping power. The reduction in bacterial load decreased from 5.0 to 2.4 log with increase of circulation number between 75 and 96 cycles, respectively. These data

indicate that the inactivation effect of pressure cycling could not be simply attributed to the cycle number alone.

In contrast, *Enterococcus* sp. inactivation dramatically dropped from 5.0 log to 0.9 log reduction with decrease of ΔP from 0.25 to 0.05 MPa, respectively (Figure 4.3). A plausible explanation of weakened disinfection by more frequent pressure cycling is related to a synergistic reduction of ΔP corresponding to increasing nozzle diameter (Figure 4.3). This suggests that bactericidal performance of pressure cycling does not simply depend on the cycle number but also on ΔP.

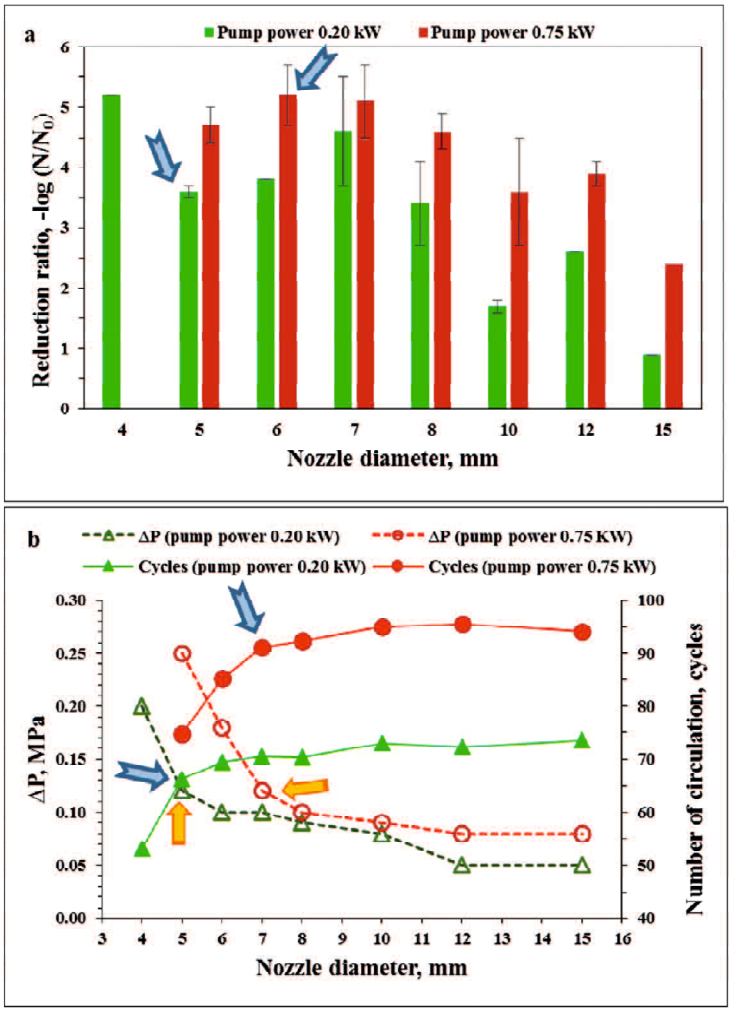

Figure 4.3 Effects of pressure cycling on inactivating *Enterococcus* sp. in seawater. (a) Effect of various nozzle diameters and pump powers on inactivation of pressurized CO_2. (b) Influence of nozzle diameter and pump power on pressure difference ΔP (dotted line) between pump and main chamber interior, along with circulation number (solid lines)

required for disinfection. Operating conditions: 0.7 MPa, 20 ± 0.5°C, salinity 3.4%, initial bacterial concentration 5–6 $\log_{10}$ CFU mL^{-1}, 70% WVR within 25 min of treatment.

Remarkably, for the same ΔP, an increase in the number of pressure cycling improved *Enterococcus* sp. inactivation. For example, at the same ΔP of 0.12 MPa (generated by a 5-mm nozzle and 0.20 kW pump, and 7-mm nozzle and 0.75 kW pump), the disinfection effect had a significant increase from 3.6 log (after 66 cycles) to 5.1 log (after 91 cycles) (Figure 4.3). A similar tendency was found at ΔP = 0.10 MPa; about a 3.8-log reduction was attained after 70 cycles (6-mm nozzle and 0.20 kW pump), whereas an approximate 4.6-log reduction was achieved after 92 cycles (8-mm nozzle and 0.75 kW pump). The findings affirm the influence of pressure cycling on the inactivation of *Enterococcus* sp.

Based on Pearson matrix correlation, four components, nozzle diameter, ΔP, pump power, and number of circulations were identified as significantly affecting inactivation efficiency of pressure cycling (Table 4.1). The strongest positive relationship (r = 0.85, p-value < 0.001) was obtained between the cycle number and inactivation efficiency, followed by ΔP (r = 0.39, p-value < 0.001). Pump power showed weak correlation with inactivation efficiency (r = 0.23, p-value = 0.005). Pearson regression tests indicated an inverse correlation (r = −0.20) between nozzle diameter and disinfection efficiency (p-value < 0.05). Taken together, these data indicate that operation with a high-power pump, high ΔP, larger circulation number, and small nozzle diameter produced effective inactivation (p-value < 0.05). Nevertheless, regarding the use of small nozzle diameter toward effective inactivation, it was found that the requirement for operating at high ΔP and low flow rate may be much more complex and less interesting from an economic standpoint. More than 5.0-log reduction of *Enterococcus* sp. was achieved within 25 min using the 7-mm nozzle and 0.75 kW pump; disinfection efficiency was nearly equal to that using smaller nozzles (Figure 4.2). Hence, that nozzle diameter and pump power were used for subsequent experiments, because a greater processing capacity is of greater commercial interest.

Table 4.1. Coefficient of correlation of operating parameters on inactivation efficiency

Factor	Unit	r	R^2	p_value
Nozzle diameter	mm	-0.20	0.04	0.017*
Pressure difference ΔP	MPa	0.39	0.15	6.191e-07*
Pump power	kW	0.23	0.05	0.005*
Number of circulation	Cycles	0.85	0.76	< 2.2e-16*

p _value <0.05 (significant for 95% confidence level)

4.3.1.2 Effect of pressure cycling on *E. coli* inactivation

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 ± 1°C with a WVR of 70% for a duration of 25 min (Figure 4.4). In general, larger nozzle diameters led to higher flow rates (Figure 4.4c) and faster fluid recycling in the treatment system (Figure 4.4d). In contrast, increases in the nozzle diameter reduced the pressure difference ΔP (Figure 4.4c). 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 4.4c). At every nozzle diameter, operation of the pump with 0.75 kW of power (Figure 4.4b) yielded greater inactivation efficiencies than those with 0.20 kW of power (Figure 4.4a).

It is hypothesized that pressure cycling enhances the inactivation efficiency by facilitating the mass transfer of CO_2 into bacterial cell membranes (Dillow *et al.* 1999; Zhang *et al.* 2006). 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 4.4a), 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 to 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 4.4 b); 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 4.4). 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.

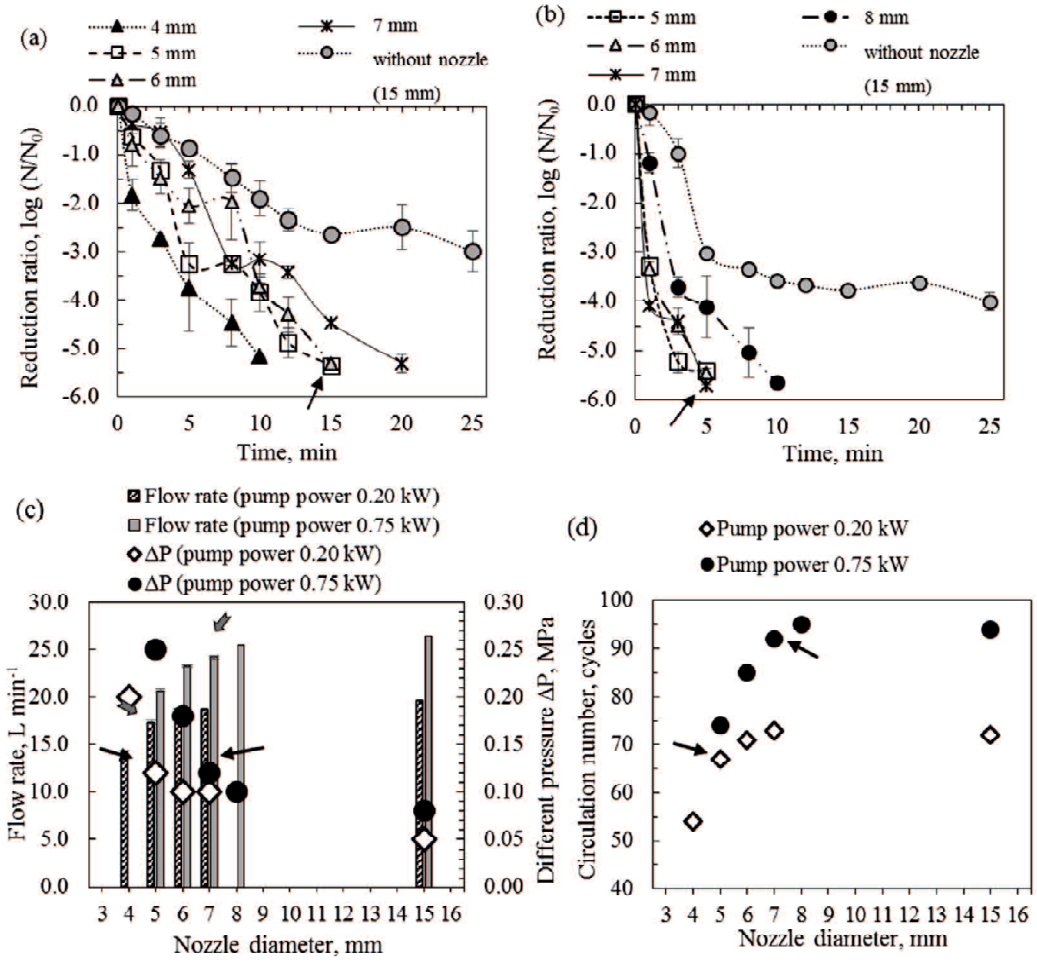

Figure 4.4 Effect of pressure cycling on the inactivation of *E. coli* in seawater. Effect of (a) 0.20 kW pump power and (b) 0.75 kW pump power along with various nozzle diameters on the inactivation with pressurized CO_2. Influence of different pump powers and nozzle diameters on the (c) flow rate and pressure difference ΔP and (d) the circulation number. Operating conditions: 0.7 MPa, 20 ± 1°C, and a working volume ratio (WVR) of 70% within a duration of 25 min.

Noticeably, at the same ΔP value, a faster frequency of circulation substantially augmented the *E. coli* inactivation efficiency (Figure 4.4). 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 $\Delta P = 0.10$ 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 min 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 4.2 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.

Regression coefficients, *t*-values, and *p*-values were analyzed for the four factors as shown in Table 4.3. 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 to 7 mm nozzle diameters and the 0.75 kW pump were used (Figure 4.4b). Since a large processing

capacity is of great commercial interest, the 7 mm nozzle and 0.75 kW pump were used for subsequent experiments.

Table 4.2 Correlation coefficients among various operating parameters associated with pressure cycling and the *E. coli* inactivation efficiency.

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 4.3 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%).

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.

4.3.2 Effect of pressure on disinfection of *Enterococcus* sp. in seawater using pressurized CO_2 with various content rates

Enterococcus sp. was disinfected in four pressure conditions (0.3, 0.5, 0.7, and 0.9 MPa) at 20°C and 70% WVR during 25 min of treatment (Figure 4.5a). Here, four inert gases of 100% CO_2, (50% CO_2 + 50% N_2), (25% CO_2 + 75% N_2), and 100% N_2 were used as disinfectants to investigate bactericidal performance. Overall, *Enterococcus* sp. inactivation was significantly increased with increasing pressure and CO_2 concentration (Figure 4.5a), whereas the solubilization of CO_2 in seawater was strongly affected by pressure and the percentage of CO_2 content in the gases (Figure 4.5b).

As shown in Figure 4.5b, CO_2 solubilization in artificial seawater rose with increasing pressure from 0.3 to 0.9 MPa. Accordingly, CO_2 concentrations in water were 1607−2020 mg L^{-1} for pure CO_2, 1040−1747 mg L^{-1} for (50% CO_2 + 50% N_2), and 795−1507 mg L^{-1} for (25% CO_2 + 75% N_2). The data also indicate that a large percentage of CO_2 content facilitated high solubility of CO_2 in seawater.

Noticeably, at every operating pressure, using gas with a high percentage of CO_2 greatly enhanced bactericidal performance (Figure 4.5a). The reduction in bacterial load caused by 100% CO_2 ranked first with 3.7−5.5 log, followed by (50% CO_2 + 50% N_2) with 1.1−3.3 log, and (25% CO_2 + 75% N_2) with 0.9−1.8 log. Conversely, pure pressurized N_2 treatment yielded the poorest bactericidal performance at every pressure, with 0.1−0.8 log reduction. The *Enterococcus* sp. inactivation by pressurized CO_2 also greatly increased with pressure from 0.3 to 0.9 MPa (Figure 4.5a). Remarkably, the greatest reduction of bacterial load was for pressure application at 0.9 MPa. An approximate 1.8-log and 3.3-log reduction was obtained after 25 min using (25% CO_2 + 75% N_2) and (50% CO_2 + 50% N_2), respectively, whereas a more than 5.0-log reduction resulted in complete inactivation within 20 min when 100% CO_2 was used. Hence, a pressure of 0.9 MPa was chosen for subsequent experiments.

As shown in Figure 4.5c, although sample pH remained near 8.0 for pure N_2 treatment, pressurized CO_2 with various content rates (25%−100%) acidified the treated water after the first minute of exposure. Accordingly, pH dropped from 8.2 to 5.1−5.0 via 100% CO_2 application, whereas the end-point pH caused by (25% CO_2 + 75% N_2) and (50% CO_2 + 50% N_2) was 5.4 to 5.1, corresponding to pressures of 0.3 to 0.9 MPa, respectively.

One theory suggests that low external pH caused by pressurized CO_2 reduces bacterial resistance to achieve inactivation (Hutkins *et al.* 1993; Hong *et al.* 1999; Vo *et al.* 2013, 2015). Compared with the N_2-treated sample (pH 8.0), the low acidity caused by CO_2

treatment was probably a major cause of the bactericidal effect, as suggested by Vo *et al.* (2013). Nevertheless, the data showed that despite the same reduction of pH (pH 5.1 at 0.9 MPa; Figure 4.5c), a higher percentage of CO_2 content in the gases led to a stronger inactivation effect. An approximate 5.0-log reduction was achieved within 20 min by CO_2 (100% purity), whereas about 3.3- and 1.8-log reductions were obtained within 25 min by (50% CO_2 + 50% N_2) and (25% CO_2 + 75% N_2), respectively (Figure 4.5a). This suggests that the low external pH alone is not the main reason for the bactericidal effect. Probably, with the simultaneous presence of pressure and CO_2 concentration, the low external acidity helped cells become more permeable, facilitating cellular penetration by CO_2.

The low inactivation efficacy caused by N_2 treatment affirmed that N_2 has poor solubility in water, and that pressurized N_2 of 0.3−0.9 MPa by itself was insufficient for inhibiting *Enterococcus* sp. In contrast, CO_2 is hydrophilic and lipophilic in nature (Isenschmid *et al.* 1995; Kim *et al.* 2007, 2008; Vo *et al.* 2015). Hence, CO_2 easily penetrated the phospholipid bilayer of the cell membrane, and accumulated there (Isenschmid *et al.* 1995). In our study, the fluid was mixed well by a counter-current agitated (mixed by fluid recirculation) to enable high solubility of CO_2 in water. Thus, operating at high pressure promoted highly dissolved CO_2 in water and may have enhanced its diffusion into the cells. As soon as too much CO_2 penetrates the phospholipid layer, it can damage cell membrane, such as disordering of cell cytoplasm (Kim *et al.* 2007, 2008), leakage of intracellular substance, or modification of the membrane surface (Vo *et al.* 2013). Other theory assumes that a large number of CO_2 molecules penetrating the membrane can reduce intracellular pH so as to exceed the buffering capacity of cytoplasm, killing cells (Hong and Pyun 1999; Garcia-Gonzalez *et al.* 2007; Vo *et al.* 2015).

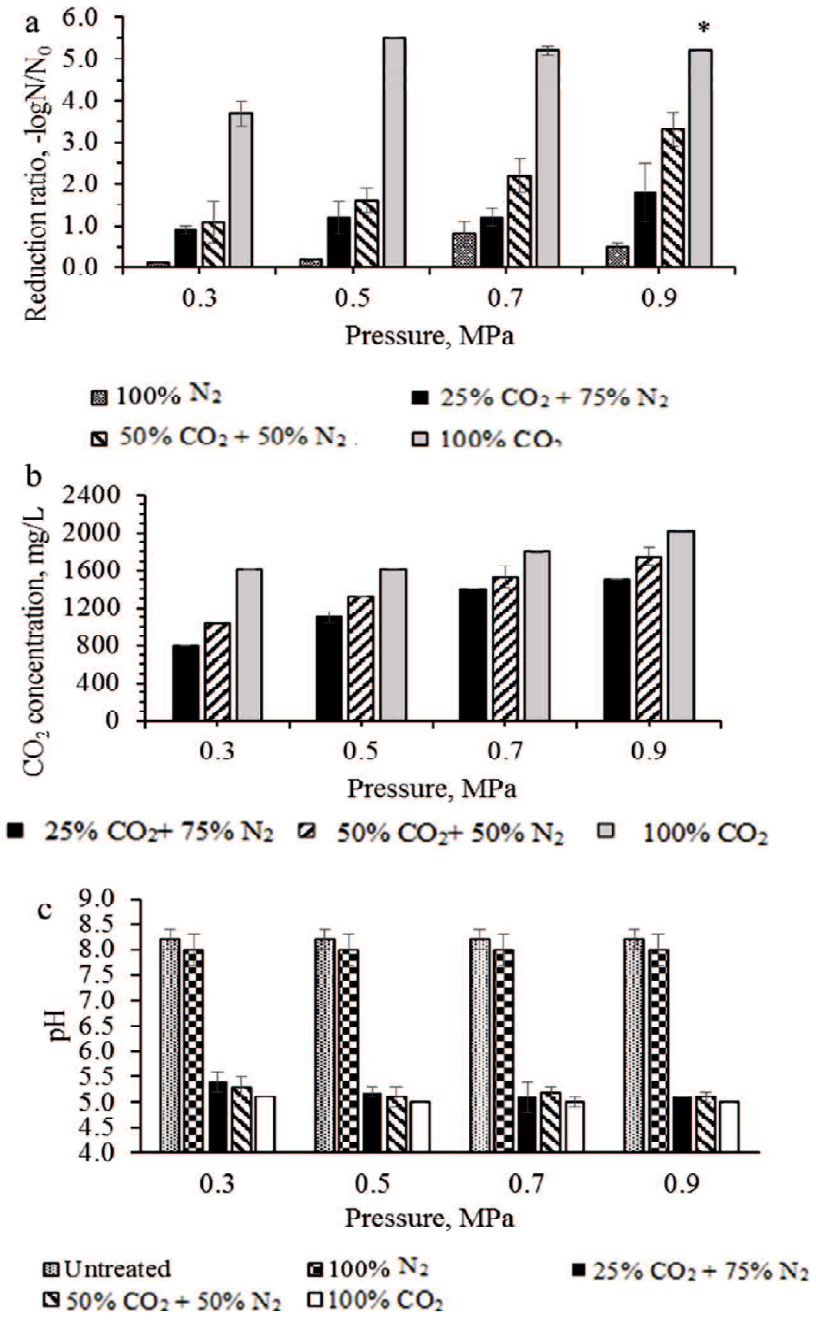

Figure 4.5 Effect of various pressure conditions (0.3−0.9 MPa) on (a) *Enterococcus* sp. inactivation, (b) CO_2 concentration, and (c) pH of seawater (3.4%) in response to pressurized inert gases of 100% CO_2, (50% CO_2 + 50% N_2), (25% CO_2+ 75% N_2) and 100% N_2 at 20 ± 1.0°C and 70% WVR, within 25 min. Asterisk (*) indicates bacterial load was completely inactivated after 20 min. Initial bacterial concentration at start of experiment was 5–6 $\log_{10}$ CFU mL^{-1}.

4.3.3 Effect of working volume ratio on bacterial inactivation

4.3.3.1 Effect of working volume ratio on *Enterococcus* sp. inactivation

The effect of WVR was investigated using four ratios (50%, 60%, 70%, and 80%) and four pressurized gases (100% CO_2; 50% CO_2 + 50% N_2; 25% CO_2 + 75% N_2; and 100% N_2) at 0.9 MPa and 20 ± 1.0°C, with flow rates 24–26 L min^{-1} for 25 min (Figure 4.6). Overall, *Enterococcus* sp. inactivation increased with decreasing WVR. Moreover, increasing WVR from 50% to 80% reduced pressure cycling (124 to 76 cycles, respectively) and increased the water level (11 to 22 cm, respectively) (Figure 4.6e).

Remarkably, although most gases showed weaker *Enterococcus* sp. inactivation at 80% WVR, the disinfection efficacy of pressurized CO_2 against *Enterococcus* sp. greatly increased with further decrease of WVR from 70% to 50% (Figure 4.6). At every WVR, a high CO_2 content rate improved inactivation efficacy (Figures 4.6a, b and c), while N_2 had little inactivation effect (Figure 4.6d). When 100% CO_2 was used, the bacterial load was reduced by ~5.0 log within 20 min with 50% to 70% WVR, whereas only a 3.5-log reduction was attained at 80% WVR within 25 min (Figure 4.6a). Bactericidal performance of (50% CO_2 + 50% N_2) ranked second with a 3.0- to 3.9-log reduction (Figure 4.6b), followed by (25% CO_2 + 75% N_2) with a 0.8- to 3.0-log reduction within 25 min, corresponding to WVRs of 80% to 50%, respectively (Figure 4.6c).

It is hypothesized that operation at a smaller WVR enhances the mass transfer rate of CO_2, producing a greater inactivation efficacy (Lin *et al.* 1993; Hong *et al.* 1997; Garcia-Gonzalez *et al.* 2009). In our study, most gases showed weak inactivation when 80% WVR was used, suggesting that CO_2 mass transfer was limited at that WVR. The low disinfection efficacy with large WVR may be related to a reduction of circulation number (76 cycles/25 min) and increase in water level (20 to 22 cm; Figure 4.6e). In particular, a high water level (20 to 22 cm at 80% WVR) submerged the shield inside the apparatus, which might have reduced bubble formation via shield interaction and limitation of CO_2 mass transfer. In contrast, smaller WVRs (70% to 50%) led to faster pressure cycling (87 to 124 cycles within 25 min, respectively) but no change of ΔP (0.14 MPa). The higher-frequency pressure cycling facilitated the solubilization of CO_2 and probably promoted its penetration into the cells, thereby accelerating *Enterococcus* sp. inactivation, as discussed in the previous section.

Regarding the effect of pressure cycling as a function of WVR, Pearson regression tests showed strong correlation between circulation number and disinfection efficiency (*p*-

value <0.001). Coefficients of correlation were 0.97, 0.92 and 0.94, corresponding to (25% CO_2 + 75% N_2), (50% CO_2 + 50% N_2) and pure CO_2, respectively. Moreover, according to regression analysis (Table 4.1), the experimental results fit the first-order model as shown by the following equations.

$$y_1 = -0.04 + 0.02 \times x \quad (4.4)$$

$$y_2 = 0.33 + 0.04 \times x \quad (4.5)$$

$$y_3 = 0.40 + 0.07 \times x \quad (4.6)$$

Here, x is the pressure cycling number (cycles); y_1, y_2, and y_3 are reduction ratios ($-\log N/N_0$) of *Enterococcus* sp. caused by (25% CO_2 + 75% N_2), (50% CO_2 + 50% N_2) and CO_2 (100% purity), respectively.

As shown in Table 4.4, the *t*-statistic of the regression model was 19.89 for (25% CO_2 + 75% N_2), 12.63 for (50% CO_2 + 50% N_2) and 14.02 for 100% CO_2, with *p*-value ≤ 0.001 in all cases, indicating that the model result was significant ($p < 0.05$). The coefficient of determination (R^2 = 0.93, 0.85, 0.89; p <0.001) revealed that pressure cycling alone contributed 93%, 85% and 89% of the inactivation efficacy of (25% CO_2 + 75% N_2), (50% CO_2 + 50% N_2), and 100% CO_2, respectively. Predicted values of inactivation efficacy were calculated based on Equations 4.4, 4.5 and 4.6 and are given in Table 4.5, along with experimental results. The predicted values were close to the experimental results, suggesting that the model had a strong correlation with the bactericidal performance of pressure cycling (p <0.05). Taken together, the results indicate that at constant ΔP, more frequent pressure cycling greatly accelerated inactivation efficiency. These findings suggest that pressure cycling with low-pressurized CO_2 (0.9 MPa) and without CO_2 leakage is a useful method to raise inactivation efficiency, overcoming problems associated with high-pressure CO_2 and its leakage shown by previous research.

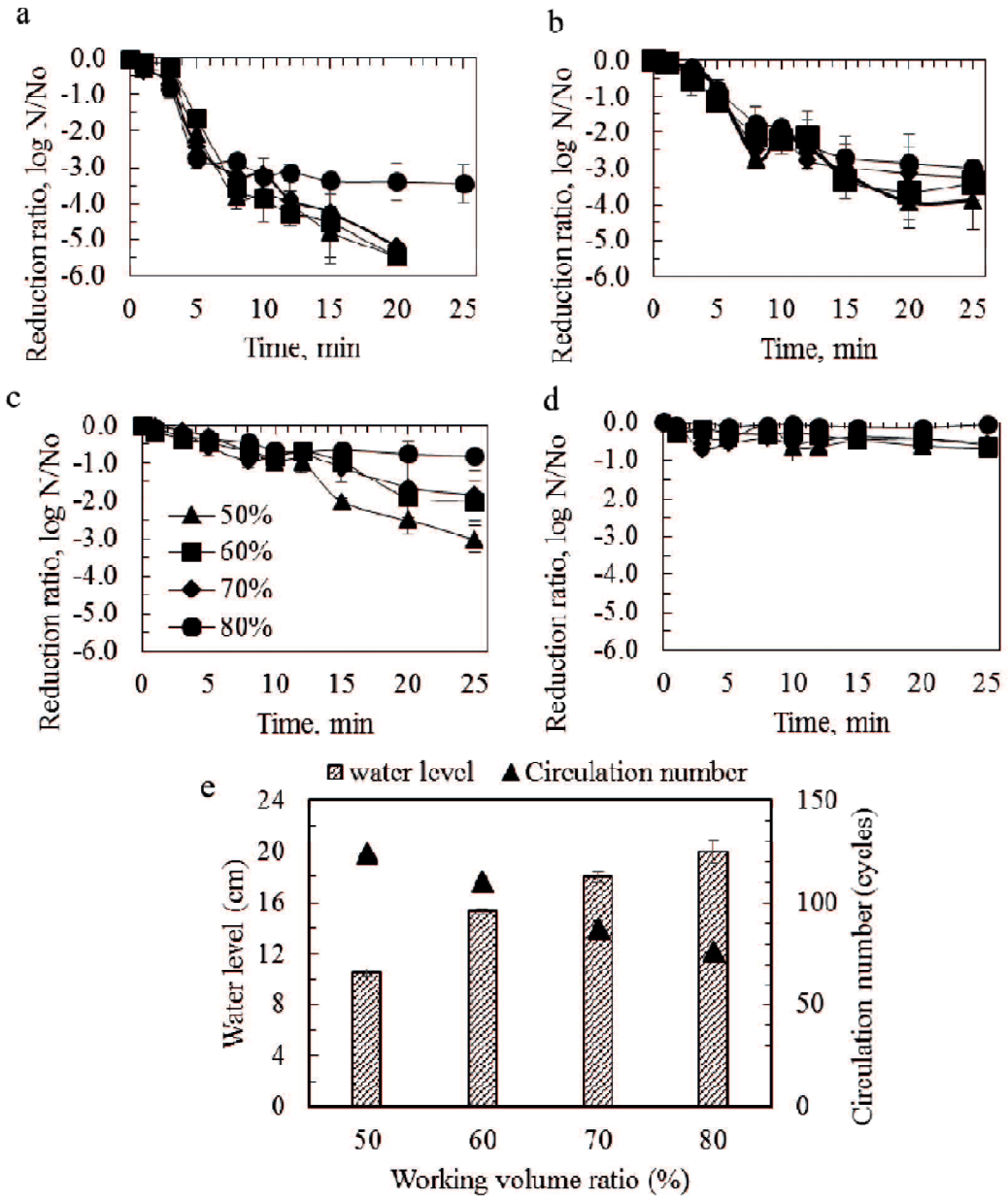

Figure 4.6 Effect of WVR (50%−80%) on inactivating *Enterococcus* sp. in seawater by pressurized gases: (a) 100% CO_2; (b) 50% CO_2 + 50% N_2; (c) 25% CO_2 + 75% N_2; (d) 100% N_2 at 0.9 MPa and 20 ± 1°C with flow rates 24−26 L min^{-1}, and initial bacterial concentration 5–6 log_{10} CFU mL^{-1}. (e) Influence of WVR on water level in main chamber and frequency of pressure cycling required for disinfection.

Table 4.4 Regression results showing influence of pressure cycling on *Enterococcus* sp. inactivation efficiency (at 20 ± 1°C, working pressure = 0.9 MPa, ΔP = 0.14 MPa, water flow rates = 24–26 L min^{-1}, and initial bacterial concentration = 5–6 log_{10} CFU mL^{-1}).

Source	Coefficients	Estimate	Standard Error	t-Statistic	*p*-value	R^2
25% CO_2 + 75% N_2	Intercept	−0.04	0.060	−0.62	0.548	
	Cycle number	0.02	0.001	19.89	<0.001*	0.93
50% CO_2 + 50% N_2	Intercept	0.33	0.156	2.10	0.045*	
	Cycle number	0.04	0.003	12.63	<0.001*	0.85
100% CO_2	Intercept	0.40	0.206	1.94	0.064	
	Cycle number	0.07	0.004	14.02	<0.001*	0.88

** 95% confidence level*

Table 4.5 Validation of model regression of pressure cycling response to *Enterococcus* sp. inactivation efficiency as function of WVR (at 20 ± 1°C, pressure = 0.9 MPa, ΔP = 0.14 MPa, water flow rates = 24–26 L min^{-1}, and initial bacterial concentration = 5–6 log_{10} CFU mL^{-1}).

Gas	Variables		Responses	
	WVR, %	x_i: Pressure cycling, cycles	y_i: Reduction ratio, $-\log(N_t/N_0)$	
			Experiment	Predicted
25% CO_2 + 75% N_2	50	124 [d]	3.0 ± 0.3	2.7 [a]
25% CO_2 + 75% N_2	60	106 [d]	2.0 ± 0.5	2.3 [a]
25% CO_2 + 75% N_2	70	87 [d]	1.8 ± 0.7	1.9 [a]
50% CO_2 + 50% N_2	50	124 [d]	3.9 ± 0.8	4.9 [b]
50% CO_2 + 50% N_2	60	106 [d]	3.4 ± 0.5	4.2 [b]
50% CO_2 + 50% N_2	70	87 [d]	3.3 ± 0.4	3.5 [b]
100% CO_2	50	99 [e]	5.5 ± 0.0	6.9 [c]
100% CO_2	60	86 [e]	5.5 ± 0.0	6.0 [c]
100% CO_2	70	70 [e]	5.2 ± 0.0	5.0 [c]

[a, b, c] predicted values calculated based on Equations 4.4, 4.5 and 4.6, respectively.
[d] Treatment time = 25 min.
[e] Exposure time was 20 minutes, when bacteria were completely inactivated.

4.3.3.2 Effect of working volume ratio on *E. coli* inactivation

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 L min^{-1} and 25 L min^{-1}) for 25 min (Figure 4.7). As shown in Figure 4.7c, decreasing WVR from 80% to 50% resulted in a decrease in the water level (22 to 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 4.7). 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 4.7a). A similar tendency was found in the case of the 25 L min^{-1} flow rate (Figure 4.7b). 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 (Hong *et al.* 1997; Hong and Pyun 1999; Dillow *et al* 1999; Zhang *et al.* 2006; Silva *et al.* 2013). 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 (Hong *et al.* 1997; Lin *et al.* 1993; Garcia-Gonzalez *et al.* 2009). 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 4.7c), which led to submergence of the shield inside the device; this may have in turn decreased bubble formation via shield interactions. 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.

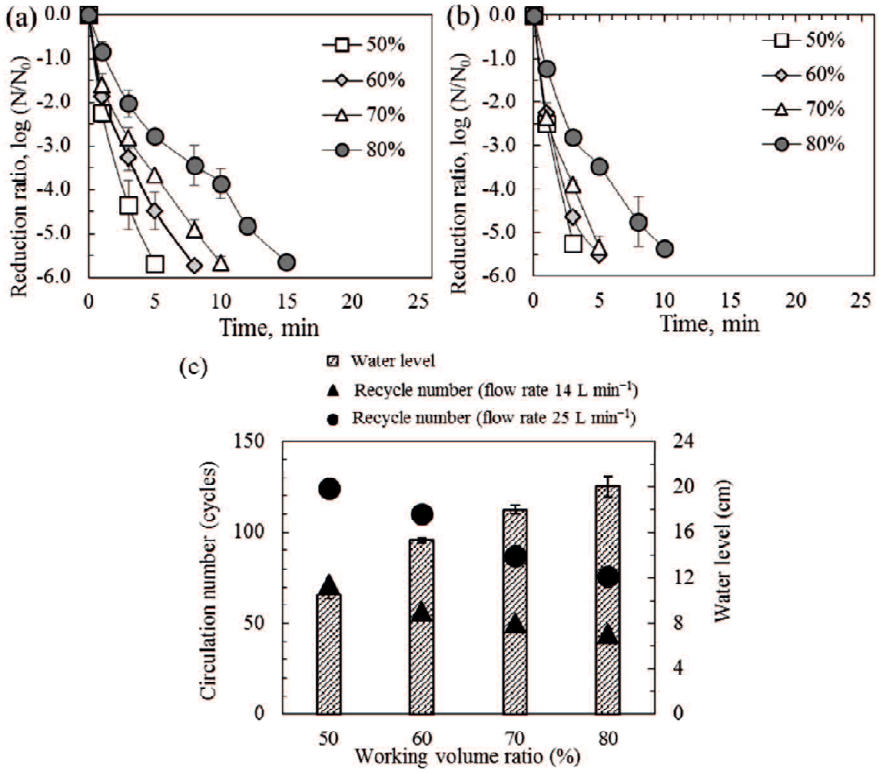

Figure 4.7 Effect of the working volume ratio (WVR) on the inactivation of *E. coli* in seawater by pressurized CO_2 at 0.7 MPa and 20 ± 1°C with (a) a flow rate of 14 L min^{-1} and (b) a flow rate of 25 L min^{-1}. (c) Influence of the WVR on the circulation number and water level in the main chamber.

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 4.6). According to the regression analysis, the experimental results fit the linear model shown in the following equation:

$$y_4 = 0.736 + 0.285 \times x_4 \quad (4.7)$$

Here, x_4 is the recycle number (cycles), and y_4 is reduction ratio (-log N/N_0) of *E. coli* caused by pressurized CO_2.

As shown in Table 4.6, 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 (ΔP = 0.12 MPa, flow rate = 14–25 L min^{-1}). Predicted values of *E. coli* reduction ratios were calculated based on Equation 4.7, and the data are summarized in Table 4.7 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*. (1999) reported that an increase of pressure cycling from three to six 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.* (2013) found that an 8.0 log reduction could be achieved with pressure cycling (five cycles/140 min) and supercritical CO_2 at 8 MPa, whereas a 5.0 log reduction was observed with one 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.

Table 4.6 Regression results showing the influence of pressure cycling on *E. coli* inactivation efficiency (at 20 ± 1°C, system pressure = 0.7 MPa, ΔP = 0.12 MPa, flow rate = 14 to 25 L min^{-1}, and initial bacterial concentration = 5–6 log_{10} CFU mL^{-1}).

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 4.7 Validation of model regression for *E. coli* 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}). HRT, hydraulic retention time.

		Variables		Responses y_4: Reduction ratio, -log(N_t/N_0)	
Flow rate, L min^{-1}	HRT, min	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*

*Predicted values calculated based on Equation 4.7.

[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.

4.3.4 Kinetic evaluation of inactivation of *Enterococcus* sp. with pressurized CO_2

Enterococcus sp. inactivation by pressurized CO_2 treatment followed a first-order kinetic model with large correlation coefficient ($R^2 \geq 0.90$ in all cases; Table 4.8). The inactivation kinetic rate constant k increased with pressure from 0.3 to 0.9 MPa under treatment conditions (20 ± 1°C and 70% WVR within 25 min). Accordingly, high system pressure led to small D-values (Table 4.8).

Despite constant pressure, D-values significantly decreased with increasing CO_2 percentage ($p < 0.01$; Table 4.8). These values were 30.77 to 13.28 min for (25% CO_2 + 75% N_2) application and 23.58 to 6.93 min for (50% CO_2 + 50% N_2) treatment, corresponding to pressures of 0.3 to 0.9 MPa, respectively. Interestingly, the smallest D-values were induced by CO_2 (100% purity) and were obtained at 6.35 to 3.85 min ($R^2 \geq 0.95$). This suggests that the increase in pressure and CO_2 concentration enhanced disinfection efficiency, producing smaller D-values. Silva *et al.* (2013) reported that 5.35 min was required for 1-log reduction of *E. coli* load with supercritical CO_2 at 8 MPa. Despite lower pressurized CO_2 (0.9 MPa of

pure CO_2), the *D*-value obtained in the present study (3.85 min) was smaller than that obtained by Silva *et al.* (2013). Gram-positive bacterial strains such as *Enterococcus* sp. are more resistant to pressurized CO_2 than gram-negative *E. coli* (Zhang *et al.* 2006). These findings affirm the superior performance of pressurized CO_2 treatment.

Table 4.8 Effect of pressure (0.3−0.9 MPa) on inactivation constant and decimal reduction time *D*, obtained by pressurized CO_2 with variable content rates (25%−100%) against *Enterococcus* sp. in seawater at 20 ± 1°C.

Gas	Pressure (MPa)	k (min^{-1})		D (min)		R^2
		$\bar{X}$	± SD	$\bar{X}$	± SD	
25% CO_2 + 75% N_2	0.3	0.0748	0.0079	30.77	3.16	0.92
	0.5	0.1142	0.0183	20.16	3.43	0.92
	0.7	0.1112	0.0138	20.70	2.57	0.93
	0.9	0.1734	0.0647	13.28	5.67	0.98
50% CO_2 + 50% N_2	0.3	0.0976	0.0211	23.58	5.35	0.92
	0.5	0.1310	0.0335	17.57	4.67	0.96
	0.7	0.1886	0.2720	12.21	1.94	0.94
	0.9	0.3326	0.0574	6.93	1.31	0.91
100% CO_2	0.3	0.3625	0.0054	6.35	0.09	0.95
	0.5	0.4917	0.0418	4.68	0.37	0.99
	0.7	0.4873	0.0158	4.73	0.15	0.99
	0.9	0.5988	0.0184	3.85	0.12	0.95

$\bar{X}$ = means, SD = standard error from at least two determinations, and R^2 = regression coefficient.

4.4 Conclusions

This study successfully enhanced bactericidal performance of pressurized CO_2 via pressure cycling, which was conducted at low pressure (0.9 MPa) with no CO_2 discharge during treatment. The bacterial inactivation was concomitantly influenced by two key elements associated with the frequency and magnitude of pressure cycling. At constant ΔP, faster pressure cycling improved disinfection efficiency (p-value <0.001). In addition, disinfection substantially increased with increased pressure (0.3 to 0.9 MPa) and CO_2 content rate (0% to 100%). The first-order death kinetics model described the *Enterococcus* sp. inactivation rate of pressurized CO_2, with a large correlation coefficient (R^2 = 0.91 to 0.99).

The smallest *D*-values were produced at 0.9 MPa, and were 3.85, 6.93, and 13.28 min for 100% CO_2, (50% CO_2 + 50% N_2), and (25% CO_2 + 75% N_2) treatments, respectively. We succeeded in inactivating *Enterococcus* sp. by 5.2 log in seawater within 20 min, using sequences involving pressure cycling (ΔP = 0.14 MPa, 70 cycles) and pressurized CO_2 (100% purity) at 0.9 MPa, 20 ± 1°C, and 70% WVR. 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 (ΔP = 0.12 MPa, 15 cycles). The findings suggest that disinfection by pressurized CO_2 could be helpful in inhibiting waterborne pathogens.

4.5 References

Boorman, G.A., Dellarco, V., Dunnick, J.K., Chapin, R.E., Hunter, S., Hauchman, F. 1999. Drinking water disinfection byproducts: review and approach to toxicity evaluation. Environ. Health Perspect., **107** (Suppl. 1), 207-217.

Cheng, X., Imai, T., Teeka, J., Yamaguchi, J., Hirose, M., Higuchi, T., Sekine, M. 2011. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. Appl. Microbiol. Biotechnol., **90** (4), 1493-1500.

Dillow, A.K., Dehghani, F., Hrkach, J.S., Foster, N.R., Langer, R. 1999. Bacterial inactivation by using near and supercritical carbon dioxide. Proc. Nati. Acad. Sci. U.S.A., **96** (18), 10344-10348.

Erkmen, O., Kamaran, H. 2001. Kinetic studies on the high pressure carbon dioxide inactivation of *Samonella typhimurium*. J. Food Eng., **50**, 25-28.

Fabbricino, M., Korshin, G.V. 2005. Formation of disinfection by-products and applicability of differential absorbance spectroscopy to monitor halogenation in chlorinated coastal and deep ocean seawater. Desalin., **176**, 57-69.

Ferreira, E.H.d.R., Rosenthal, A., Calado, V., Saraiva, J., Mendo, S. 2009 *Byssochlamys nivea* inactivation in pineapple juice and nectar using high pressure cycles. J. Food Eng., **95**, 664-669.

Fraser, D. 1951 Bursting bacteria by release of gas pressure, Nature, **167** (4236), 33-34.

Garcia-Gonzalez, L., Geeraerd, A.H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Van Impe, J., Devlieghere, F. 2007. High pressure carbon dioxide inactivation of microorganisms in foods: the past, the present and the future. Int. J. Food Microbiol., **117** (1), 1-28.

Garcia-Gonzalez, L., Geeraerd, A.H.; Elst, K., Van Ginneken, L., Van Impe, J.F, Devlieghere, F. 2009. Inactivation of naturally occurring microorganisms in liquid whole egg using high pressure carbon dioxide processing as an alternative to heat pasteurization. J. Supercrit. Fluid., **51**, 74-82.

Hong, S.I., Park, W.S., Pyun, Y.R. 1997. Inactivation of *Lactobacillus* sp. from kimchi by high pressure carbon dioxide. LWT-Food Sci. Technol., **30**, 681-685.

Hong, S.I. and Pyun, Y.R. 1999. Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. J. Food Sci., **64**, 728-733.

Hutkins, R.W. and Nannen, N.L. 1993. pH homeostasis in lactic-acid bacteria. J. Dairy Sci., **76**, 2354-2365.

Isenschmid, A., Marison, I.W., von Stockar, U. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. J. Biotechnol., **39**, 229-237.

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., Osajima, Y. 2007. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. Food Sci. Technol. Res., **13** (1), 20-22.

Kobayashi, F., Yamaza, F., Ikeura, H., Hayata, Y., Muto, N., Osajima, Y. 2009. Inactivation of microorganisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. J. Water Environ. Technol., **7** (4), 241-250.

Kobayashi, F., Hayata, Y., Ikeura, H., Tamaki, M., Muto, N., Osajima, Y. 2009. Inactivation of *Escherichia coli* by CO_2 microbubbles at a lower pressure and near room temperature. Trans. ASABE, **52** (5), 1621-1626.

Kim, S.R., Rhee, M.S., Kim, B.C., Lee, H., Kim, K.H. 2007. Modeling of the inactivation of *Salmonella typhimurium* by supercritical carbon dioxide in physiological saline and phosphate-buffered saline. J. Microbiol. Methods, **70**, 132-141.

Kim, SR., Park, H.J., Yim, D.S., Kim, H.T., Choi, I.-G., Kim, K.H. 2008. Analysis of survival rates and cellular fatty acid profiles of *Listeria monocytogenes* treated with supercritical carbon dioxide under the influence of cosolvents. J. Microbiol. Methods, **75**, 47-54.

Lin, H.M., Yang, Z., Chen, L.F. 1993. Inactivation of *Leuconostoc dextranicum* with carbon dioxide under pressure. Chem. Eng. J., **52** (1), B29-B34.

Silva, J.M., Rigo, A.A., Dalmolin, I.A., Debien, I., Cansian, R.L., Oliveira, J.V., Mazutti, M.A. 2013. Effect of pressure, depressurization rate and pressure cycling on the

inactivation of *Escherichia coli* by supercritical carbon dioxide. Food Cont., **29** (1), 76-81.

Spilimbergo, S., Elvassore, N., Bertucco, A. 2002 Microbial inactivation by high-pressure. J. Supercrit. Fluid., **22** (1), 55-63.

Vo, H.T., Imai, T., Teeka, J., Sekine, M., Kanno, A., Le, T.V., Higuchi, T., Phummala, K., Yamamoto, K. 2013. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. Water Res., **47** (13), 4286-4293.

Vo, H.T., Imai, T., Ho, T.T., Sekine, M., Kanno, A., Higuchi, T. 2014. Inactivation effect of pressurized carbon dioxide on bacteriophage Qβ and ΦX174 as a novel disinfectant for water treatment. J. Environ. Sci., **26** (6), 497-505.

Vo, H.T., Imai, T., Ho, T.T., Dang, T.-L.T., Hoang, S.A. 2015. Potential application of high pressure carbon dioxide in treated wastewater and water disinfection: Recent overview and further trends. J. Environ. Sci., **36**, 38-47.

Von Gunten, U. 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. Water Res., **37**, 1469-1487.

Werschkun, B., Sommer, Y., Banerji, S. 2012. Disinfection by-products in ballast water treatment: An evaluation of regulatory data. Water Res., **46**, 4884-4901.

Zhang, J., Davis, T.A., Matthews, M.A., Drews, M.J., LaBerge, M., An, Y.H. 2006. Sterilization using high-pressure carbon dioxide. J. Supercrit. Fluid., **38** (3), 354-372.

CHAPTER V

SYNERGISTIC EFFECT OF PRESSURIZED CARBON DIOXIDE AND SODIUM HYPOCHLORITE ON THE INACTIVATION OF BACTERIA IN SEAWATER

5.1 Introduction

Ballast water is used to improve the manoeuvrability and stability of ships during a voyage when ships are not laden with cargo or during cargo loading operations. Annually, about 3–5 billion tons of ballast water containing aquatic species is transferred among the world's oceans (GloBallast 2016), and if these organisms are released into new ecosystems that support their growth, they can become invasive species (Ruiz *et al.* 1997; David & Gollasch 2015). Invasive species pose threats to ecosystems and can even increase risks to human health (Ruiz *et al.* 1997). To avoid these problems, the International Maritime Organization (IMO) developed regulations for the control and management of ballast water (IMO 2004). The regulations require that the number of viable organisms in ballast water must be less than the level set in the D-2 ballast water performance standard when the water is discharged into the ocean (IMO 2004).

So far, several technologies have been developed for ballast water treatment. Chlorine has been commonly used for water disinfection owing to its wide antimicrobial range, rapid bactericidal effect, low costs, and simplicity of use (Fukuzaki 2006; Gregg *et al.* 2009). Chlorine can be added to water as sodium hypochlorite (NaOCl), or it can be directly generated on board via electrolysis reactions in seawater. The majority of ballast water treatment systems that employ chlorine disinfection use a dose of about 10 mg L^{-1} of chlorine (David and Gollasch 2015). Unfortunately, the chemical reactions between chlorine and the organic and inorganic compounds in seawater generate potentially carcinogenic agents such as trihalomethanes (THMs), halogenic acetic acids (HAAs), and bromate (Fabbricino and Korshin 2005; Werschkun *et al.* 2012). Although high chlorine doses may yield greater disinfection efficiency, they can also exacerbate the formation of those toxic disinfection by-products (DBPs) (Fabbricino and Korshin 2005; Tsolaki and Diamadopoulos 2010). Therefore, advanced technologies that do not produce DPBs or that minimise the amount of DPBs produced are highly desirable. Ozonation, for example, is effective in treating water, but it has some disadvantages including high costs, special operation requirements, and the

formation of bromate as a by-product in waters containing bromide (Von Gunten 2003; Werschkun *et al.* 2012). Although ultraviolet (UV) radiation disinfection does not generate toxic by-products (Werschkun *et al.* 2012), this method requires large amounts of energy and frequent equipment maintenance including the replacement of UV lamps (Lloyd's Register, 2012). Additionally, the disinfection efficiencies of UV light and chlorination are relatively low in waters with high turbidity (Tsolaki and Diamadopoulos 2010). De-oxygenation by the injection of an inert gas (i.e. N_2, CO_2) is a cost effective disinfection method and can reduce corrosion of ballast tanks (Gregg *et al.* 2009). However, this method may not be appropriate if the journey of the ship is short because it usually takes 1 to 4 days to reach acceptable discharge standards and asphyxiate organisms (Lloyd's Register 2012). Besides, some organisms such as phytoplankton, cysts and spores, and anaerobic bacteria may adapt to such hypoxia, which makes the treatment more challenging (Gregg *et al.* 2009; David and Gollasch 2015). Heat treatments and ultrasound or electric pulse technology can also be applied to inactivate many organisms, but these methods require large amounts of energy and have high operational costs (Tsolaki and Diamadopoulos 2010).

Large volumes of ballast water need to be treated according to the D-2 standard (IMO 2004); however, the space on a ship for such operations is typically limited. Ideally, a shipboard treatment method should be highly efficient at removing target organisms, quick to implement, and free of problems related to residual toxicity (Tsolaki and Diamadopoulos 2010). Thus, it would be desirable to develop new technologies for ballast water disinfection in a manner that exploits the advantages of current technology while minimising the disadvantages of the conventional methods. This study investigates the synergistic benefits of combined treatment methods that employ pressurized carbon dioxide (PCD) and NaOCl for seawater disinfection.

Pressurized carbon dioxide has been used as a non-thermal sterilization technique in the food preservation industry (Garcia-Gonzalez *et al.* 2007), and it could potentially be useful in many other applications. The PCD method has shown great potential for inhibiting various pathogens present in both non-aqueous products (i.e. solid foodstuff, biomaterials, cotton, medical devices) and aqueous products (i.e. liquid foods, broth, water) (Isenschmid *et al.* 1995; Hong and Pyun 1999; Zhang *et al.* 2006; Kim *et al.* 2008). However, high-pressures (4–50 MPa) are required to inactivate the pathogens effectively and the demands associated with this requirement (i.e. heavy-duty pressure equipment, substantial power consumption) make PCD not desirable from both economic and implementation viewpoints.

In recent years, PCD has been investigated as an innovative disinfection technology for water and wastewater treatment because of its inactivation efficiency, safety, and lack of problems associated with residual toxicity (Kobayashi *et al.* 2007, 2009; Cheng *et al.* 2011; Vo *et al.* 2013, 2015). Kobayashi *et al.* (2007) reported that a 13.3 min CO_2 microbubble treatment is sufficient for eradicating *E. coli* and coliform bacteria in drinking water; however, this treatment required supercritical conditions (at 10 MPa and 35°C) for efficient disinfection. Vo *et al.* (2013) found that PCD treatments at 0.7 MPa could reduce (4.7–5.2 log reductions) *E. coli* in distilled water within 25 min, and they claimed that the low pH caused by the PCD treatment is probably the major factor responsible for the bactericidal effect. Dang *et al.* (2016a) boosted the bactericidal performance of PCD by using sequences involving pressure cycling and succeeded in eradicating *Enterococcus* sp. in seawater within 20 min; the treatment conditions included PCD at 0.9 MPa (20 ± 1°C, 70% working volume ratio) and pressure cycling (at ΔP = 0.14 MPa, 70 cycles). It is not clear from the existing research literature whether PCD (0.2–0.9 MPa) combined with other treatment methods such as chlorination would be able enhance the disinfection efficacy and reduce the treatment time.

Chlorine hydrolyses in water to become free chlorine, which exists mainly in two forms, hypochlorous acid (HOCl) and hypochlorite ions ($^-$OCl). The proportion of these forms depends on the pH of the solution. HOCl predominantly exists at low pH levels of 4–6, while $^-$OCl exists at pH levels of 8.5–10 (Fukuzaki 2006). In seawater, HOCl rapidly oxidizes bromide ions to form hypobromous acid (HOBr) and/or hypobromite ions ($^-$OBr) (IMO 2010). It is hypothesised that a reduction of pH caused by CO_2 (Vo *et al.* 2013) may help increase the proportion of HOCl and HOBr in the water. The bactericidal activity of HOCl is stronger than that of $^-$OCl (Fukuzaki 2006); therefore, by combining CO_2 and chlorine, the disinfection efficacy might be increased (Cha *et al.* 2015). Cha *et al.* (2015) reported that an approximate 1.8-log reduction of *Artemia franciscana* in seawater was achieved after a 5-day post-treatment incubation with a combination of electro-chlorination at 6 mg L^{-1} and CO_2 addition. Without CO_2, only a 1.2-log reduction was obtained at 6 mg L^{-1} of total residual oxidants (TRO). Hence, the incorporation of CO_2 into electro-chlorination shows promising results for inhibiting zooplankton in ballast water; however, high concentrations of DPBs such as THMs (9.12 mg L^{-1}) and HAAs (36.2 mg L^{-1}) generated in the 6 mg L^{-1} + CO_2 treated brackish water (Cha *et al.* 2015) are undesirable from an environmental perspective.

This study investigated the use of PCD combined (0.2–0.9 MPa) with a low-dosage of chlorine (low dosage was defined as less than the normal dosage required for sufficient inactivation by chlorine alone) for seawater disinfection applications such as ballast water treatment. The sensitivity of bacteria to the combined PCD/chlorine treatment was examined under various conditions of chlorine dosages, pressures, and CO_2 supply rates. The bactericidal effects of PCD and chlorine both as the combination and as two individual treatments were evaluated and compared. Relationships of the disinfection efficiency with the chlorine dosage, dissolved CO_2 concentrations, and pH were also elucidated. The overall aims of this study were to evaluate whether the combinations of PCD (<1.0 MPa) and low-dosage chlorine could yield synergistic benefits and to assess the potential application of this method for ballast water treatment.

5.2 Materials and methods

5.2.1 Microorganism preparation and enumeration

5.2.1.1 Microorganism preparation

The bacterial inoculums for *E. coli* (ATCC 11303), *V. alginolyticus* (ATCC 17749) and *Enterococcus* sp. (ATCC 202155) from stock cultures (American Type Culture Collection, Manassas, VA, USA) were prepared by inoculation of 100 μL of bacterial glycerol stock into 100 mL of Luria-Bertani (LB) broth (Wako, Japan), marine broth (Wako, 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 24 hours at 37°C with continuous shaking at 150 rpm. The permanent stock was preserved in 20% glycerol and -80°C.

For each disinfection experiment, 100 μL of bacterial glycerol stock was transferred into 100 mL of the broths. The inoculum was incubated at 37°C and shaken at 150 rpm for 24 h. Cells were then harvested and rinsed with 0.9% (w/v) saline solution three times followed by centrifugation (10 min at 10,000 rpm at 4°C) in a refrigerated centrifuge (H-15FR, Kokusan Co. Ltd., Japan). The pellet was re-suspended in 100 mL saline solution.

5.2.1.2 Microorganism enumeration

In regard to the artificial seawater disinfection tests, the colonies of the *Enterococcus* sp. were enumerated using the plate count technique. Specifically, a series of ten-fold

dilutions was performed by using autoclaved artificial seawater at 3.4% salinity. One hundred micro litre of a diluted or an undiluted sample was spread out on BHI agar (Wako, Japan) plates. After incubating the plates for 24 h at 37°C, the number of colonies was counted on each plate containing 30–300 CFUs. For samples with a low number of viable cells (below 30 CFUs/plate, in case of the undiluted sample), 1 mL of the undiluted sample was poured into the agar maintained at 45°C. The CFUs on each plate were counted after incubating the plates for 24 h at 37°C. Each sample was analysed in triplicates.

In regard to the natural seawater disinfection tests, both *E. coli* and enterococci were enumerated by using the membrane filtration technique. Specifically, the concentrations of *E. coli* and enterococci were measured by membrane filtration following the U.S. Environmental Protection Agency (USEPA) methods 1603 and 1600, respectively. Briefly, 100 mL of either a diluted (with phosphate buffered saline water) or undiluted sample was passed through a 0.45 μm cellulose acetate membrane filter (Advantec Toyo Roshi Kaisha Ltd., Tokyo, Japan). Then, for *E. coli* enumeration, the membrane filters were placed on M-TEC HiCrome agar (Sigma-Aldrich Chemie GmbH. Industriestrasse, Buchs, Switzerland) and the plates were incubated at 35°C for 2 h followed by 44.5°C for 22–24 h. As for enterococci enumeration, the membrane filters were placed on modified Enterococcus (M Enterococcus) agar plates (Sigma-Aldrich, Switzerland) and incubated at 41°C for 48 h; after the incubations, the filters were transferred to Esculin Iron agar plates and incubated for a further 20 min at 41°C. Colonies were counted on plates that contained 20–60 colonies on the membrane surface (i.e., to select the most accurate dilution for the analyses), and data were reported as CFU 100 mL^{-1}. Each sample was analysed in triplicate.

The cell concentrations of vibrios and heterotrophic bacteria were determined by plating 100 μL of either a diluted or undiluted sample onto TCBS agar (Wako, Japan) plates and marine agar (Wako, Japan) plates, respectively. The CFUs on each plate were counted after incubating the plates overnight at 37°C, and data were reported as CFU mL^{-1}. Each sample was analysed in triplicate.

5.2.2 Seawater sample preparation

5.2.2.1 Artificial seawater sample preparation

Artificial seawater was prepared by adding artificial sea salt (UMIJIO, Kamihata Fish Industries Ltd., Hokkaido, Japan) to distilled water to obtain a final salinity of 3.4% and pH

of 8.3. For all experiments, the prepared *Enterococcus* sp. culture was added into the seawater to obtain a bacterial concentration of 5–6 $\log_{10}$ CFU mL^{-1}, which represents the initial concentration. The salinity was measured with a salinity meter (YK-31SA, Lutron Electronic Enterprice Co. Ltd., Taiwan). The pH and temperature of the samples were measured with a pH meter (Horiba D-51, Japan). The concentration of CO_2 in seawater was measured by a CO_2 meter (CGP-31, TOA-DKK, Japan).

5.2.2.2 Natural seawater sampling and analysis

Seawater was collected from the port of Ube in the Yamaguchi prefecture, Japan (longitude: 131° 14' 25" E and latitude: 33° 56'18" N) (Figure 5.1). The seawater samples were collected on September 10–19, 2014 and on August 6-22, 2016. The pH and temperature of the samples were measured with a pH meter (D-51 Horiba), salinity was measured with a salinity meter (YK–31 SA, Lutron), and conductivity was measured with a conductivity meter ES-14 (Horiba). Seawater samples were analysed for such parameters as TSS, NO_3-N, NO_2-N, NH_4-N, PO_4-P, TN, and TP. Analyses were performed by using the standard methods described by the APHA (1999). In these samples, the bacterial concentrations (i.e. *E. coli*, vibrios, enterococci, and heterotrophic bacteria) were also determined.

5.2.2.3 Natural seawater sample preparation

The seawater collected at Ube harbour was immediately used for disinfection experiments. In these seawater samples, the concentrations of *E. coli* and enterococci, and vibrios were relatively low (10^1–10^3 CFU 100 mL^{-1}; and 10^3 CFU mL^{-1}, respectively). In order to establish the biological efficacy of the disinfection method, tests had to be conducted with water containing a high concentration of microorganisms. Therefore, microorganisms from the prepared bacterial cultures of *E. coli* (ATCC 11303), *Enterococcus* sp., and *V. alginolyticus* were added into the natural seawater to obtain an initial concentration of *E. coli* and enterococci in the range of 10^4–10^5 CFU 100 mL^{-1}, and an initial concentration of vibrios in the range of 10^4–10^5 CFU mL^{-1}.

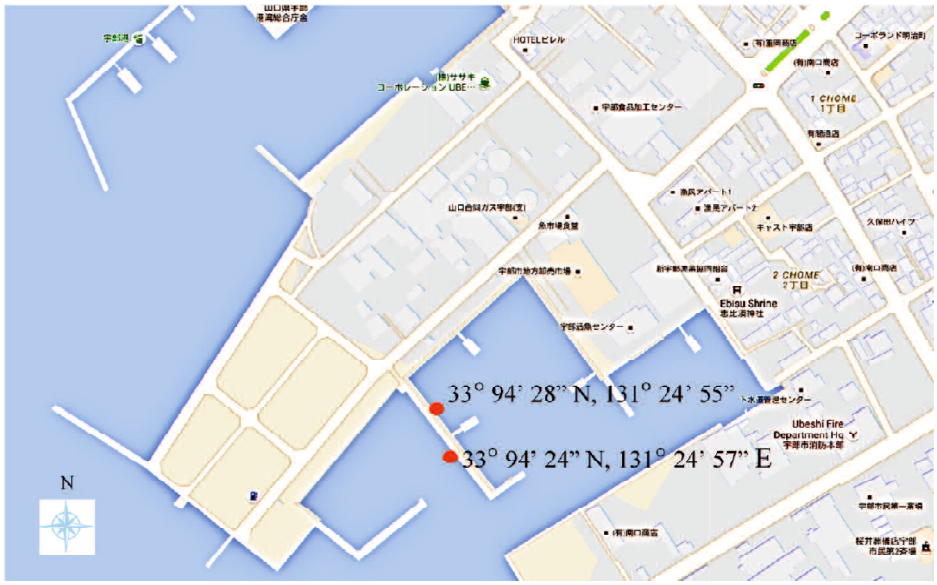

Figure 5.1 Sampling points of seawater at the port of Ube (Suo-nada Sea) in the Yamaguchi prefecture, Japan.

5.2.3 Experimental design for seawater disinfection by chlorination

A sodium hypochlorite solution (NaOCl with 5.0% available chlorine content; Wako, Japan) was used for testing the bactericidal effect of chlorine. The stock solution was diluted in chlorine-demand-free water to attain various chlorine concentrations for disinfection experiments. Quantitative amounts of NaOCl were then added into beakers containing 1000 mL of seawater inoculated with *Enterococcus* sp. (5–6 $\log_{10}$ CFU mL^{-1}) and the initial concentration of chlorine was immediately measured. The beakers were wrapped with aluminium foil to avoid photo-degradation and the solution was magnetically stirred at ambient temperature (20 ± 1°C) for 25 min. After a certain exposure time, two samples were taken from the solution, one of which was used to determine residual chlorine and the other was used to analyse bacterial viability. The concentrations of chlorine (as mg Cl_2 L^{-1}) were immediately determined by the *N,N*-diethyl-*p*-phenylenediamine (DPD; Sansyo Co. Ltd., Japan) colorimetric method with an ion-specific meter (SCH400, Sansyo Co. Ltd., Romania). For determining the number of viable bacteria, residual chlorine was quenched immediately with a 0.02 M solution of sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$; Wako, Japan) at a ratio of 5 moles of $Na_2S_2O_3 \cdot 5H_2O$ for every 8 moles of NaOCl in the samples and bacterial

concentrations were enumerated as described above. Each experiment was conducted in triplicate.

5.2.4 Apparatus and procedure for PCD and PCD/chlorine disinfection experiments

The reactor for disinfection was designed based on highly dissolved gas water technology as described previously. Briefly, a solid stream nozzle and shield was setup inside the device to generate bubbles under various pressure conditions. During the treatment process, the fluid was mixed well by counter-current agitation to facilitate gas diffusion into water (Figure 5.2). In PCD disinfection experiments, 7 L of seawater was pumped in one shot into the device as influent, and the gas was then fed into the main chamber. The pressure inside the device was adjusted by a gas regulator and an exhaust valve. Following the first influx of water, the fluid was circulated inside the system at a flow rate of 25 L min^{-1} by using a pump (0.75 kW, 32 × 32 mm SUP-324 M, Toshiba, Japan). The sensitivity of bacteria to PCD treatment was determined at various pressures (0.2–0.9 MPa) and CO_2 supply rates (0%, 25%, 50%, and 100%), which were applied for 25 min. At different time steps (0, 1, 2, 3, 4, 5, 8, 10, 12, 15, 20, and 25 min), the treated water was collected from the bottom valve of the reactor.

In combined PCD/chlorine treatments, the appropriate chlorine dosage was added to the seawater before the PCD. The seawater was mixed well and immediately pumped in one shot into the device as influent. The rest of the experiments followed the PCD method described above. The PCD treatments were started after chlorination (~30 s) to capitalize on the benefits of mixing by the circulation pump, which likely accelerated the contact between disinfectants and bacterial cells and might have enhanced synergistic disinfection effects. At different time steps (0, 1, 2, 3, 4, 5, 8, 10, 12, 15, 20, and 25 min), two samples were collected; one was used to determine residual chlorine concentrations and the other was used to analyse bacterial viability (viability was assessed on quenched samples). Each experiment was conducted in triplicate.

To investigate the effect of pH, the synergy effect of the PCD/chlorine treatment (at pH 8.3) was compared with that of pressurized nitrogen (PN)/chlorine treatments at two pH levels (5.0 and 8.3). The pH 5.0 seawater was adjusted with hydrochloric acid 0.1 M (HCl; Wako, Japan) prior to the PN/chlorine and PN treatments.

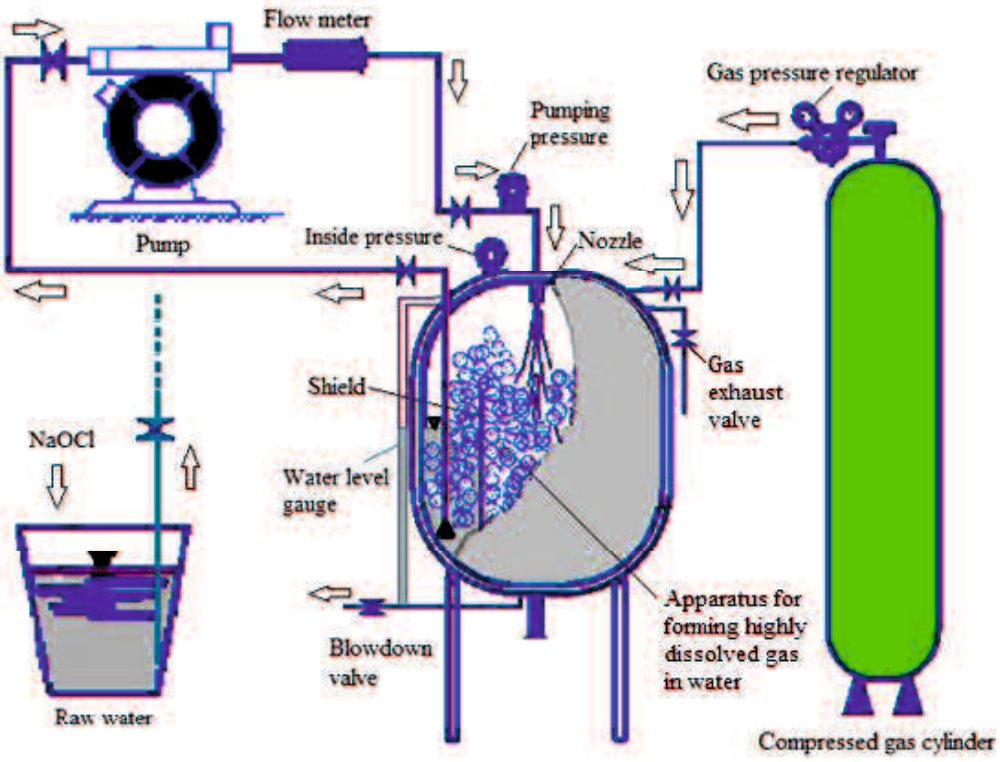

Figure 5.2 Setup of the water treatment apparatus.

5.2.5 Presentation of results and statistical analysis

Disinfection efficiency was evaluated by the $\log_{10}$ of the reduction ratio from the number of colonies before and after treatments. The synergistic disinfection effect of the combined PCD/chlorine treatment was calculated by the following equation (Koivunen & Heinonen-Tanski 2005):

Synergy value (log units) = Reduction ratio caused by combined PCD/chlorine – (Reduction ratio caused by chlorine alone + Reduction ratio caused by PCD alone).

According to this equation, a positive value means that the efficiency of the combined PCD/chlorine treatment is greater than the sum of the two individual treatments, i.e. a synergistic benefit exists. Meanwhile, a negative value reveals an antagonistic interaction. A value of zero indicates that the disinfection efficiency of the combination treatment is equal to the summed efficiency of the two individual treatments, i.e. the combined treatment was not synergistic.

Statistical analyses were performed by using the computing environment R (version 3.2.2, available at http://cran.R-project.org). Pearson's correlation coefficients were used to

assess the relationships between synergy values and other variables such as pressure and CO_2 concentrations at a 5% significance level (p <0.05).

5.3 Results and discussion

5.3.1 Synergistic effect of PCD and chlorine on the inactivation of *Enterococcus* sp. in artificial seawater

5.3.1.1 Bactericidal effect of chlorine

The disinfection activity of the chlorine treatment alone against *Enterococcus* sp. in artificial seawater was studied at various dosages (0.1 to 0.8 mg L^{-1}) (Figure 5.3). In general, bacterial inactivation significantly increased with the chlorine dosage (R^2 = 0.97, p = 0.002).

As shown in Figure 5.3a, a 0.43 mg L^{-1} chlorine dosage for a treatment period of 25 min was sufficient to reduce approximately 5.1 log of the bacterial load, whereas only 15 min of a 0.51 mg L^{-1} chlorine dosage was required to reduce the bacterial load to a similar extent. Since 0.43 mg L^{-1} was deemed a reasonable chlorine dosage for *Enterococcus* sp. inactivation, lower chlorine levels (i.e. 0.1 to 0.3 mg L^{-1}) were used to determine the synergistic effect of the combined PCD and chlorine disinfection treatment.

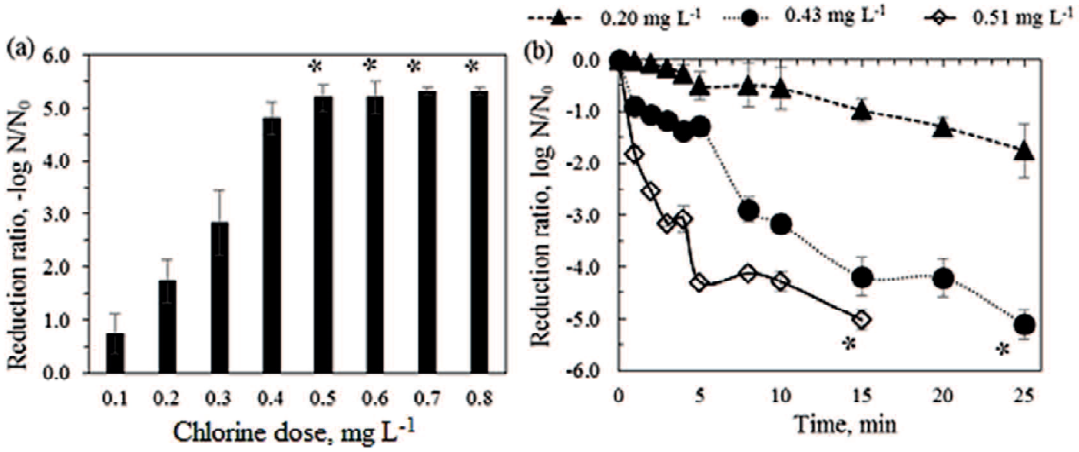

Figure 5.3 Bactericidal effect of chlorine with different dosages (0.1–0.8 mg L^{-1}) for treatment of *Enterococcus* sp. in artificial seawater (pH = 8.3, salinity = 3.4%) at 20 ± 1 °C for 25 min. The initial bacterial concentration was 5–6 $\log_{10}$ CFU mL^{-1}. Asterisks (*) indicate that no colonies were detected. The error bars represent the standard deviation from the mean.

5.3.1.2 Effect of pressure on *Enterococcus* sp. inactivation of the combined PCD/chlorine treatment

The effect of pressure was investigated at five pressure conditions (0.2, 0.3, 0.5, 0.7, and 0.9 MPa) (Figure 5.4); here, PCD (25% CO_2 + 75% N_2) and chlorine (0.20 mg L^{-1}) were used to promote bactericidal activity in both individual and combination treatments. In general, bactericidal activity of the combined treatments significantly increased with increasing pressure, and higher pressures required shorter exposure times to reach the same log reduction values for *Enterococcus* sp. (Figure 5.4b). At every operating pressure, the *Enterococcus* sp. inactivation efficiency associated with the combined PCD/chlorine treatment was greater than that of the PCD treatment alone. Specifically, the reduction of the bacterial load was 3.6–5.4 log for the former treatment (Figure 5.4b) and 0.6–1.8 log for the latter treatment (Figure 5.4a) in the pressure range of 0.2 to 0.9 MPa.

Remarkably, higher pressures with the combined PCD/chlorine treatment required shorter treatment times for inhibiting *Enterococcus* sp. (Figure 5.4b). For instance, an approximate 3.6 log reduction was achieved with 0.2 MPa within 25 min, and bacterial inactivation further increased to 5.3–5.2 log reductions with pressures between 0.3–0.5 MPa within 25 min; conversely, only 8 and 3 min at 0.7 and 0.9 MPa, respectively, were required to reduce the bacterial load to a similar extent. These data demonstrate the superior bactericidal activity of the combined PCD/chlorine treatment and suggest that combination treatments involving PCD in the range of 0.3 to 0.9 MPa and low-dosage chlorine (~47% of a normal dosage) could be used to inhibit *Enterococcus* sp. in seawater effectively.

It is noteworthy that most synergistic assessment values resulting from the combined PCD/chlorine treatment were positive, which is indicative of synergy, i.e. the benefits of the combined PCD/chlorine disinfection treatment were greater than those of the sum of the two individual treatments. Pearson correlation tests revealed a positive correlation (r = 0.62, p <0.001) between pressure and synergy values. Pressures of 0.2, 0.3, and 0.5 MPa during a treatment period of 25 min were required to produce approximately 1.2, 2.6, and 2.2 log synergistic values, respectively, whereas a 3.9 log synergistic value was attained with a pressure of 0.7 MPa within 8 min. In particular, the highest synergy values reached 4.9 log within 3 min with 0.9 MPa during the combined PCD/chlorine disinfection treatment. These results suggest that higher pressures resulted in greater synergistic values and shorter treatment times for sufficient *Enterococcus* sp. inactivation.

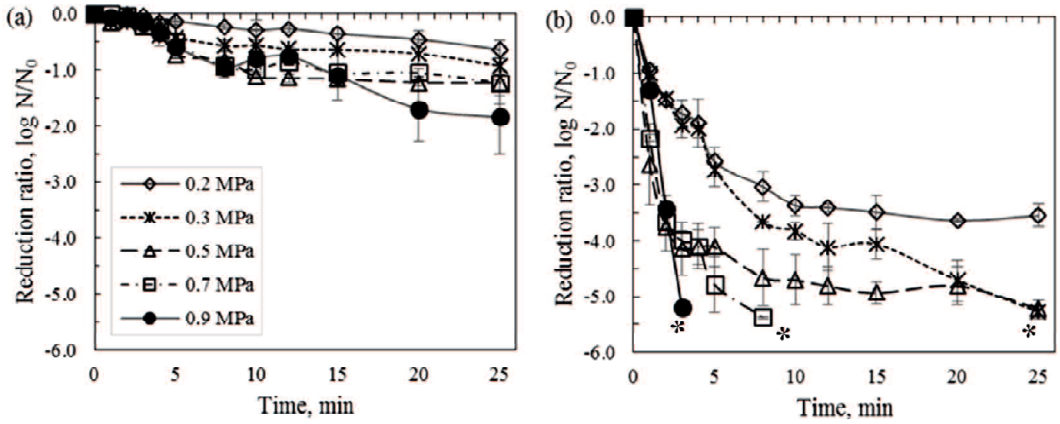

Figure 5.4 Effect of various pressure conditions (0.2–0.9 MPa) on *Enterococcus* sp. inactivation by (a) pressurized carbon dioxide (PCD) (25% CO_2 + 75% N_2) alone and (b) the combined PCD (25% CO_2 + 75% N_2)/chlorine (0.20 mg L^{-1}) treatment. The initial bacterial concentration was 5–6 $\log_{10}$ CFU mL^{-1}. Operating temperature: 20 ± 1.0 °C. Asterisks (*) indicate that no colonies were detected. The error bars represent the standard deviation from the mean.

5.3.1.3 Effect of pH

The relationship between the *Enterococcus* sp. inactivation and pH was investigated at two pH levels of 8.3 (Figure 5.5a and c) and 5.0 (Figure 5.5b and d) and at 0.9 MPa, 20 ± 1.0°C. Here, PCD (25% CO_2 + 75% N_2), PN (100% N_2), and chlorine (0.20 mg L^{-1}) were used to compare bactericidal effect in both individual and combination treatments. In general, the synergy effect of the combined PCD/chlorine treatment was always greater than that of the combined PN/chlorine and combined PN/chlorine/HCl treatments. The bacterial inactivation efficiency of the combined PN and chlorine treatment at pH 5.0 was higher (~3.4 times) than that at pH 8.3.

Sample pH was unchanged during pure N_2 treatment, while PCD (25% CO_2 + 75% N_2) acidified the treated seawater (Figure 5.5a and d). The pH remained near 8.3 and 5.0 for the PN and PN/HCl treatments, respectively. Meanwhile, the pH of PCD-treated samples dropped from 8.3 to 5.1 after the first few minutes of exposure time (Figure 5.5c).

At any pH level, PN alone yielded the lowest *Enterococcus* sp. inactivation, i.e. approximately 0.5 and 0.6 log reductions were achieved within 25 min at pH 8.3 (Figure 5.5a) and pH 5.0 (Figure 5.5b), respectively. The PCD treatment alone reduced the bacterial load by approximately 1.8 log within 25 min, which was 3 times higher than the effect caused

by PN. These data suggest that neither pH (range 5.0–8.3) nor PN of 0.9 MPa by itself was sufficient to inhibit *Enterococcus* sp. Perhaps the concomitant presence of CO_2 and pressure prompted the bacterial cells to become more susceptible to the PCD (25% CO_2 + 75% N_2) treatment.

In contrast, bactericidal activity of the combined PN/chlorine treatment significantly increased with decreases in the solution pH. An approximately 4.4 log reduction was achieved within 25 min at pH 5.0 (Figure 5.5b), whereas only a 1.3 log reduction was achieved at pH 8.3 (Figure 5.5a). Recall that the bactericidal activity of chlorine depends on the proportion of HOCl, which is the predominant form at low pH levels (Fukuzaki 2006). Thus, the increasing bactericidal activity at pH 5.0 may be attributed to the increase in the proportion of HOCl and HOBr.

Remarkably, despite the same end-point pH of the treated water (pH = 5.0 ± 0.1 at 0.9 MPa), the synergy effect of the combined PCD/chlorine treatment (Figure 5.5a) was greater than that of the combined PN/chlorine/HCl treatment (Figure 5.5b). An approximate 5.2 log reduction was achieved within 3 min by the former, whereas a 4.4 log reduction was achieved within 25 min by the latter. Accordingly, the average synergy value reached 4.9 and 2.0 log for the PCD/chlorine and PN/chlorine/HCl treatments, respectively. This suggests that the synergy effect of the combined PCD/chlorine treatment does not simply depend on the predominant of HOCl and HOBr at pH 5.0 but also on the dissolved CO_2 in seawater.

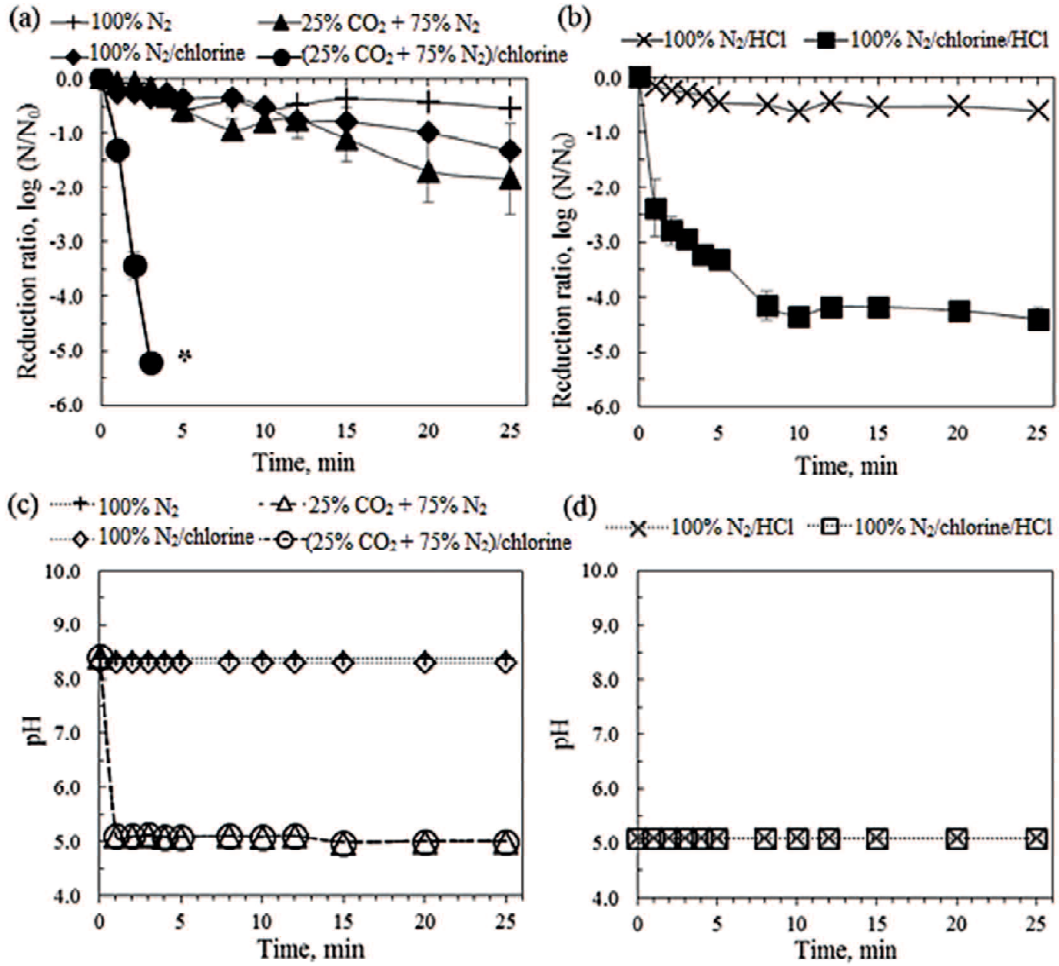

Figure 5.5 *Enterococcus* sp. inactivation in seawater at (a) pH 8.3 and (b) pH 5.0. The pH changes during the treatment period are shown for the treatments that started with initial pH values of (c) 8.3 and (d) 5.0. Chlorine dosage = 0.20 mg L^{-1}. Operating conditions: 0.9 MPa, 20 ± 1.0 °C, and 5–6 log_{10} CFU mL^{-1}. Asterisk (*) indicates that no colonies were detected. The error bars represent the standard deviation from the mean.

5.3.1.4 *Enterococcus* sp. inactivation of the combined PCD (with different supply rates)/chlorine treatments

The *Enterococcus* sp. inactivation tests with the combination PCD (different supply rates: 25% CO_2 + 75% N_2, 50% CO_2 + 50% N_2, and 100% CO_2) and low-dosage chlorine (0.20 mg L^{-1}) treatments were conducted at two pressures (0.3 and 0.9 MPa) and 20 ± 1.0°C. In general, *Enterococcus* sp. inactivation by both the combined PCD/chlorine treatment and PCD alone significantly increased with increases in the CO_2 supply rate (Figure 5.6a, b);

CO_2 concentrations in seawater were strongly affected by the pressure and CO_2 supply rates (Figure 5.6c).

As shown in Figure 5.6c, the higher pressure promoted higher CO_2 solubilization in artificial seawater (3.4% salinity, pH 8.3). The data also show that the larger CO_2 percentages resulted in greater CO_2 concentrations dissolved in seawater. The measured CO_2 concentrations were 706–1462 mg L^{-1} for 25% CO_2 + 75% N_2, 852–1609 mg L^{-1} for 50% CO_2 + 50% N_2, and 1587–2020 mg L^{-1} for 100% CO_2, with the ranges corresponding to pressures from 0.3 to 0.9 MPa, respectively.

As for PCD alone, *Enterococcus* sp. inactivation greatly increased with the higher pressure and higher CO_2 supply rate (Figure 5.6a, b). Pearson correlation tests revealed a positive correlation between the disinfection efficiency and CO_2 concentration in seawater ($r = 0.61$, $p < 0.001$). These findings affirm both the influence of dissolved CO_2 and pressure on the *Enterococcus* sp. inactivation as mentioned in the previous section.

Noticeably, *Enterococcus* sp. inactivation of the combined PCD/chlorine treatment was concomitantly influenced by both the pressure and CO_2 supply rate (Figure 5.6a, b). Synergy values were correlated with CO_2 concentrations ($r = 0.64$, $p < 0.001$). When treatments proceeded at 0.3 MPa, use of gas with higher CO_2 percentages required shorter exposure times for efficient disinfection (Figure 5.6a). For instance, 25 min with 25% CO_2 + 75% N_2/chlorine was required to completely inactivate the approximately 5.3 log initial bacterial load, whereas only 20 and 4 min of 50% CO_2 + 50% N_2/chlorine and 100% CO_2/chlorine, respectively, were required to reduce the bacterial load to a similar extent. Accordingly, 2.6, 3.1, and 4.6 log average synergy values were obtained for the 25% CO_2 + 75% N_2/chlorine, 50% CO_2 + 50% N_2/chlorine, and 100% CO_2/chlorine treatments, respectively (Table 5.1). Meanwhile, the highest inactivation efficiency and synergistic benefits were observed for the pressure application at 0.9 MPa (Figure 5.6b). Nevertheless, the *Enterococcus* sp. inactivation efficiency of PCD/chlorine at 0.9 MPa was not significantly increased by the CO_2 percentages (i.e. 25%, 75%, and 100%). Accordingly, the treatment period could be reduced to 3 min (Figure 5.6b), and average synergy values were attained in the range of 4.4 to 5.2 log (Table 5.1). Taken together, these findings suggest that CO_2 concentrations around and above 1500 mg L^{-1} (Figure 5.6c) are probably optimal to achieve the synergistic disinfection effect; higher CO_2 supply rates with lower pressures or lower CO_2 supply rates with higher pressures could also be chosen.

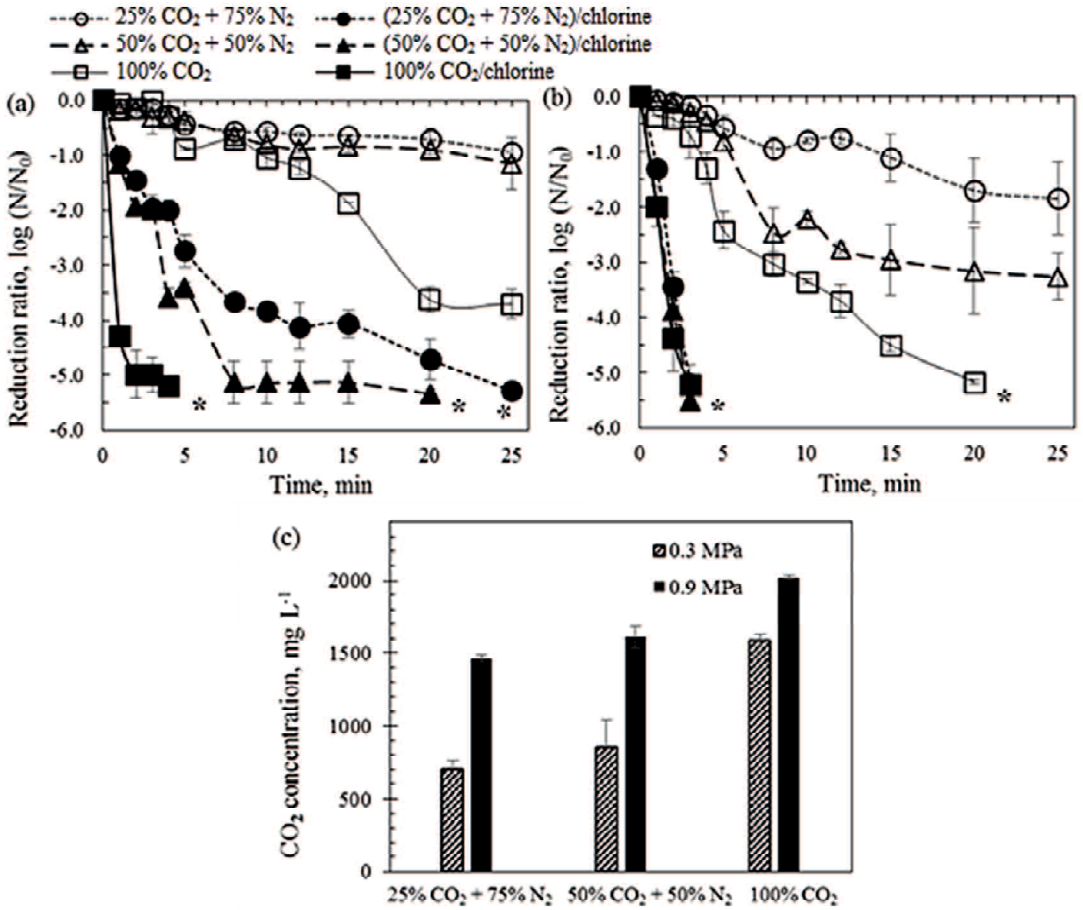

Figure 5.6 *Enterococcus* sp. inactivation by pressurized carbon dioxide (PCD) (CO_2: ○ 25%, Δ 50%, and □ 100%) alone and by the combined PCD (CO_2: ● 25%, ▲ 50%, and ■ 100%)/chlorine (0.20 mg L^{-1}) treatment at (a) 0.3 MPa and (b) 0.9 MPa. (c) CO_2 concentrations in the pressurized treatments consisting of 25% CO_2 + 75% N_2, 50% CO_2 + 50% N_2, and 100% CO_2 in seawater at two pressure levels 0.3 and 0.9 MPa. Operating conditions: 20 ± 1.0 °C and 5–6 $\log_{10}$ CFU mL^{-1}. Asterisks (*) indicate that no colonies were detected. The error bars represent the standard deviation from the mean.

The potential mechanisms for synergistic disinfection with PCD/chlorine technology remain to be elucidated; nevertheless, there are several reasonable hypotheses (Figure 5.7). CO_2 is both hydrophilic and lipophilic in nature; it can easily penetrate the phospholipid bilayer of cell membranes (Isenschmid *et al.* 1995). In addition, low pH caused by CO_2 could facilitate the predominance of the HOCl form of chlorine. HOCl can also penetrate the lipid bilayer, whereby it will attack cells from outside and inside the cellular environment

(Fukuzaki 2006). In this study, the contact efficiency between disinfectant and bacterial cells may have been improved by fluid recirculation in the liquid-film-forming apparatus, thus enabling HOCl and CO_2 to efficiently penetrate into the cells. The mechanism of the bactericidal activity of HOCl may be related to its ability to inhibit enzyme activity, cause damage to cell membranes and DNA, and impair the transport capacity of cell membranes (Fukuzaki 2006). Besides, once large numbers of CO_2 molecules penetrate the phospholipid layer, this can disorder the cell cytoplasm (Kim *et al*. 2008) and lead to releases of intracellular materials (Vo *et al*. 2013; Dang *et al.* in press); intracellular pH may even be reduced if the influx exceeds the buffering capacity of the cytoplasm, which will lead to cell death (Hong & Pyun 1999; Garcia-Gonzalez *et al*. 2007; Vo *et al*. 2015). Probably, with the simultaneous effects of pressure, CO_2 concentrations, low pH caused by the CO_2, and HOCl presence, cells of *Enterococcus* sp. become much more susceptible to the combined PCD/chlorine treatment, thereby enhancing the synergy effect.

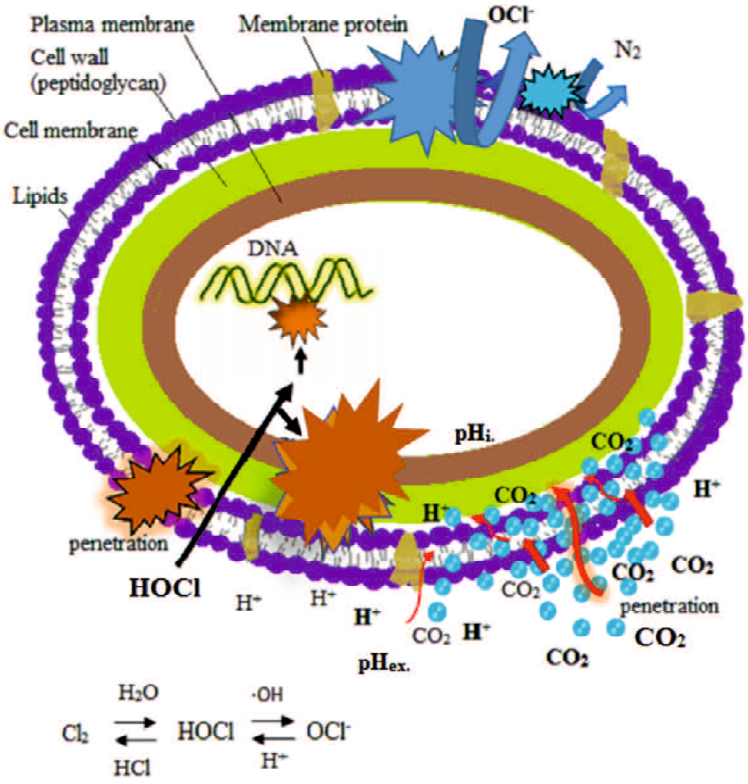

Figure 5.7 A model illustrating mechanisms of synergistic inactivation.

5.3.1.5 Effect of chlorine dosage on *Enterococcus* sp. inactivation of the combined PCD/chlorine disinfection

Enterococcus sp. disinfection was evaluated at various dosages of chlorine (0.11, 0.16, 0.22, 0.33, and 0.39 mg L^{-1}) and PCD (100% purity, 0.3 MPa) in both the two individual treatments (Figure 5.8a) and the combined treatment (Figure 5.8b) at 20 ± 1.0°C for 25 min. In general, *Enterococcus* sp. inactivation was achieved by both the combined PCD/FC treatment and chlorine alone, whereby inactivation increased with increasing chlorine dosages in both treatments, and the inactivation efficiency caused by the combined PCD/chlorine treatment was higher than that of the two individual treatments. When 0.11 to 0.39 mg L^{-1} chlorine dosages were used, the combined PCD/chlorine treatment caused 5.0 to 5.6 log reductions, respectively, in terms of the bacterial load. Noticeably, the greatest log reduction occurred during a treatment period of 3–5 min (Figure 5.8b).

The bactericidal activity of the combined PCD/chlorine disinfection treatment significantly increased with increases in the chlorine dosage (0.11–0.39 mg L^{-1}; Figure 5.8b). The *Enterococcus* sp. load was reduced by a 5.0 log reduction within 25 min by chlorine (0.11 mg L^{-1})/PCD, whereas 15 min with chlorine (0.16 mg L^{-1})/PCD was required to reduce the load by a 5.4 log reduction. The treatment period could be reduced to 4 min with chlorine dosages between 0.22–0.39 mg L^{-1} to achieve 5.3–5.6 log reductions. However, *Enterococcus* sp. inactivation efficiency was not significantly enhanced by the chlorine dosages exceeding 0.22 mg L^{-1} (Figure 5.8b). These data indicate that ~0.20–0.22 mg L^{-1} is probably the optimal chlorine dosage for the combined PCD/chlorine treatment.

In addition, there existed a synergistic effect in the combined PCD/chlorine disinfection with any chlorine dosage (0.11–0.39 mg L^{-1}). For example, 0.2 and 2.6 log synergy values were obtained by 25 min with chlorine (0.11 mg L^{-1})/PCD and by 15 min with chlorine (0.16 mg L^{-1})/PCD, respectively. Average synergy values reached 4.6, 3.7, and 4.0 log within 4 min with chlorine dosages of 0.22, 0.33, and 0.39 mg L^{-1}, respectively. Taken together, these results suggest that higher chlorine dosages with shorter exposure times or lower chlorine dosages with longer treatment times could be applied to enhance the bactericidal activity of PCD.

Moreover, at any chlorine dosage (0.11–0.39 mg L^{-1}), the combined PDC/chlorine treatment resulted in residual chlorine concentrations below the detection limit (0.05 mg L^{-1}) after the first min, while chlorine residues were evident at ~0.08–0.28 mg L^{-1}, respectively, after 25 min with the chlorine alone treatment. David & Gollasch (2015) reported that most

chlorination systems used a dose of about 10 mg L^{-1} chlorine for ballast water disinfection, and free excess chlorine needs to be neutralized to less than 0.2 mg L^{-1} residual chlorine by an obligatory neutralization process before discharging ballast water. As demonstrated in the present study by the low-dosage of chlorine (~0.20–0.22 mg L^{-1}) used and the lack of residual chlorine in the treated water, the problem of by-products may be minimized in the combined PCD/chlorine treatment. Nevertheless, further research on potential problems related to the by-products during treatment is needed.

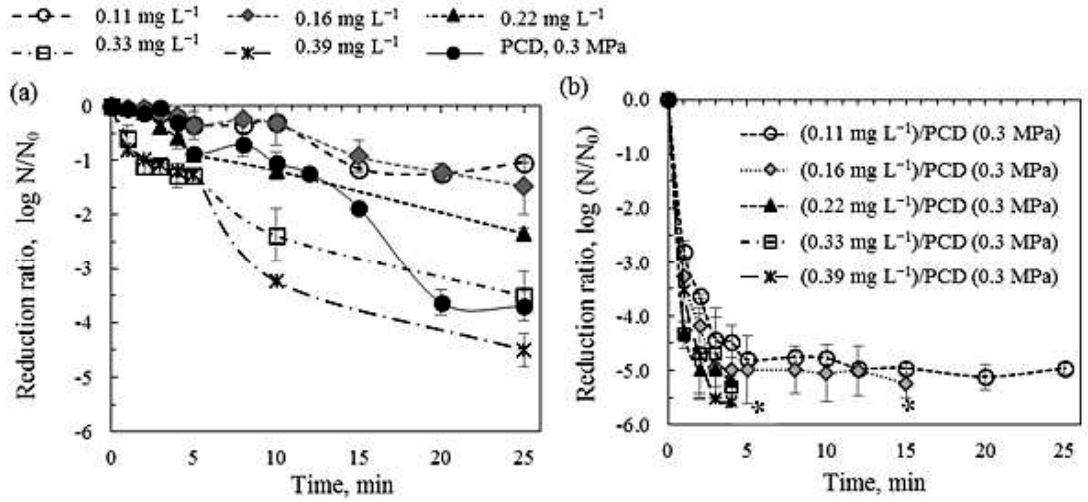

Figure 5.8 Bactericidal effect of chlorine (dosage: 0.11–0.39 mg L^{-1}) and pressurized carbon dioxide (PCD) (100% CO_2, 0.3 MPa) on *Enterococcus* sp. in seawater; results shown are for (a) the two individual treatments and (b) the combined treatment. Operating conditions: 20 ± 1.0°C and 5–6 $\log_{10}$ CFU mL^{-1}. Asterisks (*) indicate that no colonies were detected. The error bars represent the standard deviation from the mean.

Table 5.1 summarizes the average synergy values obtained by the combined PCD/chlorine treatments under various conditions of pressure, CO_2 supply rate, and chlorine dosage. Synergistic benefits were observed with all the tested samples. Spilimbergo *et al.* (2003) reported that a 7 log reduction of *E. coli* in glycerol solution could be achieved by pretreatment with a pulsed electric field (10 pulses at 25 KV cm^{-1}) followed by a supercritical CO_2 treatment (20 MPa, 34°C) within 10 min. Fijan *et al.* (2011) observed that 25 min of a combined treatment with hydrogen peroxide (10%) and PCD (6 MPa, 20°C) led to 4.6 and 3.1 log reductions of *Enterobacter aerogenes* and *Enterococcus faecium*, respectively. Despite the lower pressure of PCD, the exposure time in the present study (i.e. 3 to 4 min, Table 5.1) was shorter than that of Spilimbergo *et al.* (2003) and Fijan *et al.* (2011). These

findings affirm the synergistic benefits of the combined PCD/chlorine treatment and suggest that this method could potentially be useful for ballast water treatment.

Table 5.1 Average synergy values obtained in the combined pressurized carbon dioxide (PCD)/chlorine treatments for various pressures (0.2–0.9 MPa), CO_2 supply rates (25%–100%), and chlorine dosages (0.11–0.39 mg L^{-1}) at 20 ± 1°C.

Experimental conditions				Log_{10} reductions caused by			Synergy values, log[d]
PCD		Chlorine dosage, mg L^{-1}	Exposure time, min	PCD alone[b]	Chlorine alone[b]	Combined PCD/chlorine[b]	
Gas	Pressure, MPa						
25% CO_2 + 75% N_2	0.2	0.20	25	0.6 ± 0.2	1.8 ± 0.5	3.6 ± 0.2	1.2
25% CO_2 + 75% N_2	0.3	0.20	25[a]	0.9 ± 0.1	1.8 ± 0.5	5.3 ± 0.1[c]	2.6
25% CO_2 + 75% N_2	0.5	0.20	25[a]	1.2 ± 0.4	1.8 ± 0.5	5.2 ± 0.1[c]	2.2
25% CO_2 + 75% N_2	0.7	0.20	8[a]	0.9 ± 0.0	0.5 ± 0.4	5.4 ± 0.0[c]	3.9
25% CO_2 + 75% N_2	0.9	0.20	3[a]	0.2 ± 0.1	0.2 ± 0.1	5.2 ± 0.1[c]	4.9
50% CO_2 + 50% N_2	0.3	0.20	20[a]	0.9 ± 0.1	1.3 ± 0.2	5.3 ± 0.1[c]	3.1
50% CO_2 + 50% N_2	0.9	0.20	3[a]	0.3 ± 0.0	0.2 ± 0.1	5.7 ± 0.1[c]	5.2
100% CO_2	0.3	0.20	4[a]	0.3 ± 0.1	0.3 ± 0.2	5.2 ± 0.1[c]	4.6
100% CO_2	0.9	0.20	3[a]	0.7 ± 0.4	0.2 ± 0.1	5.2 ± 0.4[c]	4.4
100% CO_2	0.3	0.11	25	3.7 ± 0.3	1.0 ± 0.0	5.0 ± 0.1	0.2
100% CO_2	0.3	0.16	15[a]	1.9 ± 0.0	0.9 ± 0.3	5.4 ± 0.3[c]	2.6
100% CO_2	0.3	0.22	4[a]	0.3 ± 0.1	0.6 ± 0.2	5.5 ± 0.0[c]	4.6
100% CO_2	0.3	0.33	4[a]	0.3 ± 0.1	1.3 ± 0.3	5.3 ± 0.4[c]	3.7
100% CO_2	0.3	0.39	4[a]	0.3 ± 0.1	1.2 ± 0.1	5.6 ± 0.1[c]	4.1

[a]The treatment period when no viable bacteria were detected after disinfection by the combined PCD/chlorine treatment.

[b]Log_{10} reductions (average ± standard deviation) of *Enterococcus* sp. detected after the treatment period. The results are based on three parallel experiments.

[c]*Enterococcus* sp. load was completely inactivated.

[d]Average synergy values = log reduction by combined PCD/chlorine disinfection – (log reduction by PCD disinfection + log reduction by chlorine disinfection).

Table 5.2 compares the disinfection efficacy of several different ballast water treatment methods such as de-oxygenation, and electro-chlorination plus CO_2, as previously reported by several researchers (Husain *et al.* 2004; Tamburri and Ruiz 2005; NEI marine 2016; Cha *et al.* 2015), and the combined PCD/chlorine treatment, as reported in this study. Husain *et al.* (2004) observed that de-oxygenation induced with mixed gases (2% O_2 + 12% CO_2 + 86% N_2) eliminated more than 95% of zooplankton within 48 h, whereas only a 2 log reduction of *Vibrio cholerae* was achieved after 24 h of treatment. De-oxygenation induced with the

Venturi Oxygen Stripping™ system was capable of eliminating 100% of zooplankton after 120 of treatment, whereas approximate 2.0 and 1.3 log reductions of *E. coli* and enterococci were achieved after 24 h of treatment, respectively (Tamburri and Ruiz 2005; NEI marine 2016). Meanwhile, PCD (at 0.7 MPa, 20°C, and 50% working volume ratio (WVR)) could completely inactivate *E. coli* and *Enterococcus petroleum* in seawater within 5 min and 20 min, respectively (Dang *et al*. 2016b). Also, PCD (at 0.9 MPa, 20°C, and 70% WVR) resulted in complete inactivation of the *Enterococcus* sp. following a 5.2 log reduction within 20 min. These findings demonstrate the excellent bactericidal effect of PCD. Recently, some ballast water treatment systems (BWTS) that use a combination of de-oxygenation with other methods have been shown to be effective in treating ballast water (Gregg *et al*. 2009; Lloyd's Register 2012). For example, the Oceansaver® BWTS system uses a combination of treatments including filtration, cavitation, electrochemical disinfection, and de-oxygenation, and it has received basic and final approval from the IMO (Lloyd's Register, 2012). The Coldharbour GLD™ (gas lift diffusion) BWTS system of ColdHarbour Marine Ltd. uses a combination of de-oxygenation, cavitation, and ultrasound to treat ballast water, and it has received approval from the United Kingdom Maritime and Coastguard Agency – Lloyds Register (IMO, 2016).

Remarkably, the combined PCD (0.3 MPa, 100% CO_2)/chlorine (0.20 mg L^{-1}) treatment resulted in complete inactivation of the *Enterococcus* sp. within 4 min. Despite the lower pressure of PCD (0.3 MPa), with chlorine added at 47%–51% of a normal chlorine dose, the exposure time of the combined treatment in the present study was 5 times shorter than that of PCD (at 0.9 MPa) alone. Compared to the *Enterococcus* sp., *E. coli* was more susceptible to PCD (Dang *et al*., 2016b); therefore, it is expected that the combined PCD/chlorine treatment would be able to rapidly reduce the *E. coli* load to below the limit value (<250 CFU 100 mL^{-1}) of the D-2 standard.

In the present study, the apparatus was designed to include a solid stream nozzle and a shield inside. In the apparatus, when water is introduced in the main chamber through a small nozzle, the highly pressurized fluid stream strongly collides with the shield and may cause physical damage to organisms. In this study, this process was repeated at high frequency and this would have accelerated the physical damage to organisms. Tamburri *et al*. (2004) observed that many larger zooplankton (mostly copepods) were damaged and killed under the impacts of cavitation and turbulence posed by the Venturi injector. The combined PCD/chorine treatment was probably capable of eliminating organisms larger than 10 μm.

Because organisms vary greatly in their resistance to different levels of disinfectants and pressures, further research on the applicability of this disinfection method to other organisms is warranted. Further research is also needed to study the effects of the other factors present in natural seawater (i.e. organic compounds, turbidity, and temperature) on this disinfection method.

Table 5.2 Disinfection efficacies for several different organisms and microorganisms in seawater with various disinfection methods compared to the combined pressurized carbon dioxide (PCD)/chlorine treatments.

Treatment option	Organism, microorganism	Treatment conditions	Efficacy	Reference
De-oxygenation (12% CO_2 + 86% N_2 + 2% O_2)	Zooplankton	nondetectable O_2, 48 h	>95% mortality	Husain *et al.* (2004)
	Vibio cholerae	nondetectable O_2, 24 h	2.0 log reduction	
De-oxygenation (Venturi Oxygen Stripping™)	Zooplankton	0.27 to <1 mg L^{-1} O_2, 120 h	100% mortality	Tamburri & Ruiz (2005); NEI marine (2016)
	E. coli	<1 mg L^{-1} O_2, 24 h	2.0 log reduction[c]	
	Enterococci	<1 mg L^{-1} O_2, 24 h	1.3 log reduction[d]	
Electrolytic chlorine + CO_2	*Artemia franciscana*	6 mg L^{-1} TRO, CO_2 injection rate at 100 mL min^{-1}, 5 day	1.8 log reduction	Cha *et al.* (2015)
	Heterotrophic bacteria		1.2–1.9 log reduction[b]	
PCD (100% CO_2)	*E. coli*	0.7 MPa, 50% WVR, 5 min	5.7 log reduction[a]	Dang *et al.* (2016b)
	Enterococcus sp.	0.7 MPa, 50% WVR, 20 min	5.2 log reduction[a]	
PCD (100% CO_2)	*Enterococcus* sp.	0.9 MPa, 70% WVR, 20 min	5.2 log reduction[a]	Dang *et al.* (2016a); This study[e]
PCD (100% CO_2)/chlorine	*Enterococcus* sp.	0.3 MPa, 0.20 mg L^{-1}, 4 min	5.2 log reduction[a]	This study[e]
PCD (100% CO_2)/chlorine	*Enterococcus* sp.	0.9 MPa, 0.20 mg L^{-1}, 3 min	5.2 log reduction[a]	This study[e]
PCD (50% CO_2 + 50% N_2)/chlorine	*Enterococcus* sp.	0.9 MPa, 0.20 mg L^{-1}, 3 min	5.7 log reduction[a]	This study[e]
PCD (25% CO_2 + 75% N_2)/chlorine	*Enterococcus* sp.	0.9 MPa, 0.20 mg L^{-1}, 3 min	5.2 log reduction[a]	This study[e]

[a]Bacterial load was completely inactivated.

[b]Values were calculated from 2.1–2.8 log_{10} CFU mL^{-1} in the treated samples and initial bacterial concentrations of >4.0 log_{10} CFU mL^{-1} in the control samples.

[c]Values were calculated from approximately 8 CFU 100 mL^{-1} after treatment for 24 h and >800 CFU 100 mL^{-1} for the initial concentration.

[d]Values were calculated from approximately 38 CFU 100 mL^{-1} after treatment for 24 h and >800 CFU 100 mL^{-1} for the initial concentration.

[e]In the present study, all the disinfection experiments involving PCD and PCD/chlorine were conducted at a WVR of 70%.

5.3.2 Synergistic effect of PCD and chlorine on the inactivation of pathogens in natural seawater

5.3.2.1 Characteristics of seawater from Ube port

Analytical results for basic parameters of seawater collected from Ube port are shown in Table 5.3. The concentrations of TN, TP, *E. coli*, enterococci, and vibrios in seawater from Ube harbour were relatively low. Besides, the concentration of COD in the seawater was in the range of 2 to 3 mg L^{-1} (Ube City 2015).

Table 5.3 Characteristics of seawater collected from Ube harbour.

Parameter	Unit	Range	Mean ± standard deviation (n = 6)
Temperature	°C	24.1 – 29.9	27.7 ± 2.5
Salinity	%	2.9 – 3.4	3.3 ± 0.2
pH		8.0 – 8.4	8.2 ± 0.1
Conductivity	mS cm^{-1}	46.2 – 49.6	47.9 ± 1.4
TSS	mg L^{-1}	3 – 34	22.2 ± 14.2
NO_3-N	mg L^{-1}	0.04 – 0.16	0.09 ± 0.04
NO_2-N	mg L^{-1}	0.0	0.0 ± 0.0
NH_4-N	mg L^{-1}	0.20 – 0.56	0.35 ± 0.14
PO_4-P	mg L^{-1}	0.10	0.10 ± 0.00
TN	mg L^{-1}	1.57 – 1.71	1.6 ± 0.1[a]
TP	mg L^{-1}	0.07 – 0.11	0.09 ± 0.02[a]
E. coli	CFU 100 mL^{-1}	111 – 3,030	1,839 ± 1,095
Enterococci	CFU 100 mL^{-1}	233 – 830	529 ± 231
Vibrios	CFU mL^{-1}	2,440 – 5,200	4,027 ± 1,426
Heterotrophic bacteria	CFU mL^{-1}	5,120 – 18,400	7,583 ± 3,999

[a]n = 3

5.3.2.2 Bactericidal effect of PCD against bacteria in natural seawater

Table 5.4 shows the averages and ranges of the relevant environmental variables during tests with the PCD treatment. The bactericidal effect of PCD against *E. coli*, enterococci, and vibrios in natural seawater was examined at 0.3 MPa, at ambient temperature 29 ± 1.0°C, and with different CO_2 supply rates (25% CO_2 + 75% N_2, 100% CO_2; Figure 5.9). In general, bacterial inactivation greatly increased with the higher CO_2 supply rate (Figure 5.9).

Specifically, PCD reduced the *E. coli,* enterococci, and vibrios concentrations to below the IMO D-2 and USCG discharge standards within 3 and 1 min, corresponding to 25% CO_2 + 75% N_2 and 100% CO_2, respectively.

Moreover, higher PCD with higher CO_2 supply rate required shorter exposure time for disinfection. For example, PCD (25% CO_2 + 75% N_2) completely inactivated *E. coli* (by approximately 3.4 $\log_{10}$ CFU 100 mL^{-1}), enterococci (by approximately 2.8 $\log_{10}$ CFU 100 mL^{-1}), and vibrios (by approximately 3.6 $\log_{10}$ CFU mL^{-1}) within 25, 20 and 3 min, respectively; whereas only 20, 8 and 1 min of PCD (100% CO_2) were required to reduce loads of *E. coli,* enterococci, and vibrios to a similar extent, respectively. These findings affirm the bactericidal performance of PCD.

Table 5.4 Averages and ranges of relevant environmental variables during tests with the pressurized carbon dioxide (PCD) treatment.

Variable	Unit	Range	Mean ± standard deviation (n = 3)
Temperature	°C	29.6 – 29.9	29.8 ± 0.1
Salinity	%	2.9 – 3.3	3.1 ± 0.3
pH		8.0 – 8.2	8.1 ± 0.1
TSS	mg L^{-1}	32 – 34	33 ± 1.4
E. coli	CFU 100 mL^{-1}	1,900 – 3,030[a]	2,397 ± 429[a]
Enterococci	CFU 100 mL^{-1}	490 – 830[a]	660 ± 139[a]
Vibrios	CFU mL^{-1}	2,440 –5,200[a]	4,027 ± 1,426[a]

[a]Concentrations of the indicator microbes were detected from natural seawater (without the addition of *E. coli* (ATCC 11303), *Enterococcus* sp., and *V. alginolyticus*).

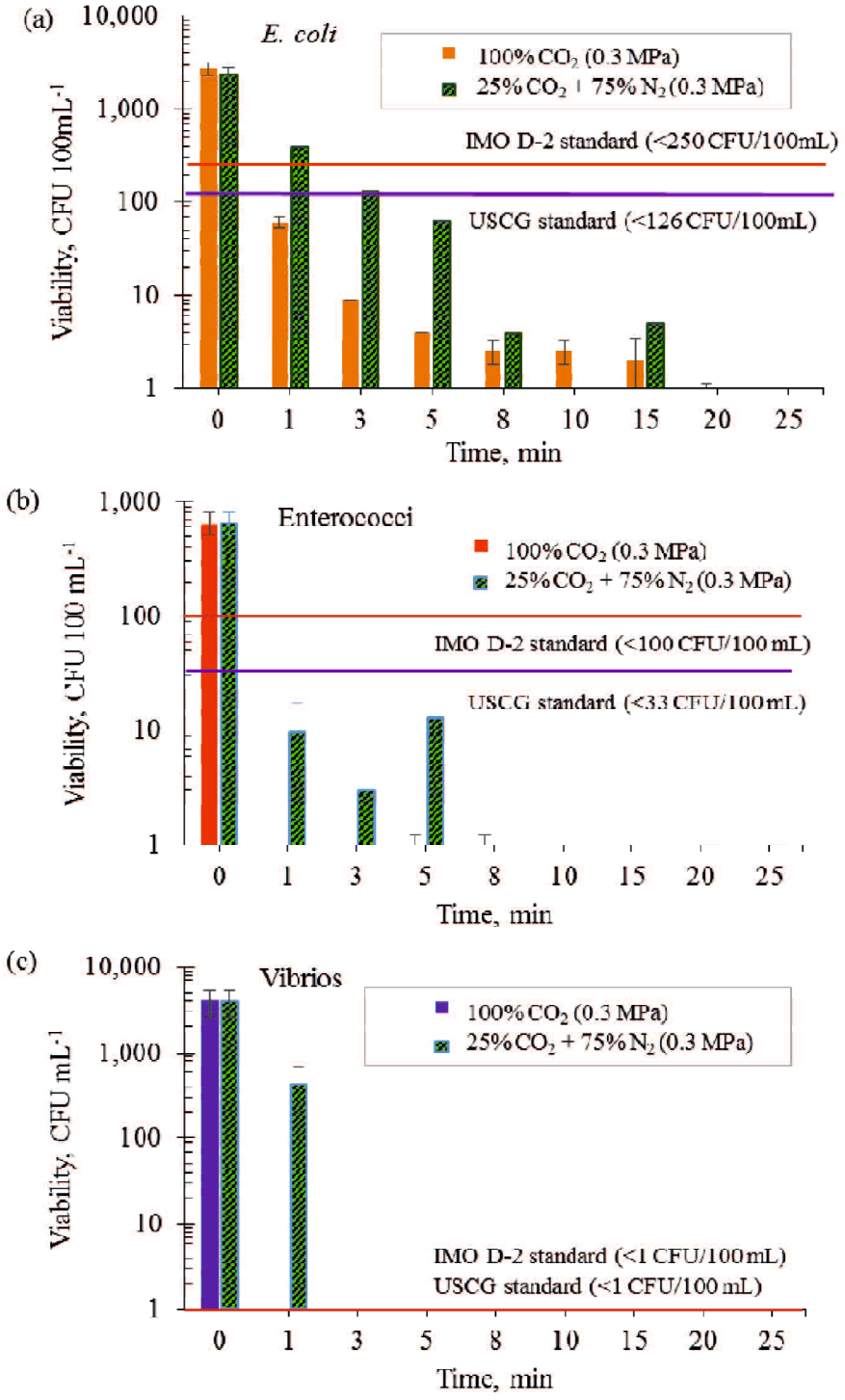

Figure 5.9 Bactericidal effect of pressurized carbon dioxide on (a) *E. coli*, (b) enterococci, and (c) vibrios in natural seawater.

5.3.2.3 Bactericidal effect of the combined PCD/chlorine treatments against bacteria in natural seawater

Table 5.5 shows the averages and ranges of the relevant environmental variables during tests with the combined PCD/chlorine treatments.

Table 5.5 Averages and ranges of relevant environmental variables during tests with the combined PCD/chlorine treatments.

Variable	Unit	Range	Mean ± standard deviation (n = 5)
Temperature	°C	29.6 – 29.9	29.8 ± 0.1
Salinity	%	2.9 – 3.3	3.1 ± 0.3
pH		8.0 – 8.2	8.1 ± 0.1
TSS	mg L^{-1}	32 – 34	33 ± 1.4
E. coli	CFU 100 mL^{-1}	$1.9 \times 10^4 - 3.7 \times 10^{4a}$	$2.8 \times 10^4 \pm 8.1 \times 10^{3a}$
Enterococci	CFU 100 mL^{-1}	$6.8 \times 10^4 - 4.9 \times 10^{5a}$	$1.8 \times 10^5 \pm 1.8 \times 10^{5a}$
Vibrios	CFU mL^{-1}	$4.6 \times 10^4 - 1.1 \times 10^{5a}$	$8.8 \times 10^4 \pm 2.7 \times 10^{4a}$

[a]Natural seawater was added to *E. coli* (ATCC 11303), *V. alginolyticus*, and *Enterococcus* sp.

Bacterial inactivation tests with the combination PCD (different supply rates: 25% CO_2 + 75% N_2, 50% CO_2 + 50% N_2, 75% CO_2 + 25% N_2, and 100% CO_2) and low-dosage chlorine (0.20 mg L^{-1}) treatments were conducted at two pressures (0.3 and 0.9 MPa) and an ambient temperature of 29 ± 1.0°C (Figure 5.10). In general, the combined PCD/chlorine treatment substantially reduced the viability microbes in seawater (Figure 5.10); and the disinfection efficiency increased with increases in the pressure and CO_2 supply rate.

As shown in Figure 5.10a, *E. coli* inactivation substantially increased with the higher CO_2 content. The combination of most gas concentrations (CO_2 content: 25% to 100%) and chlorine reduced the number of viability *E. coli* to less than the IMO D-2 standard after a duration of 1 min. Additionally, the method reduced the *E. coli* load to below the limit value of the USCG standard within 1 and 3 min, depending on the CO_2 supply rates (i.e. 25%, 50%, 75%, and 100%). Noticeably, the combined 100% CO_2/chlorine treatment yielded the greatest reduction of the *E. coli* load. Specifically, an approximate loading amounting to 5.0 log_{10} CFU 100 mL^{-1} was completely eradicated within 10 min by the combined 100% CO_2/chlorine treatment at 0.3 MPa. When a pressure of 0.9 MPa was used, the *E. coli* load was reduced by approximately 5.3 log_{10} CFU 100 mL^{-1} with a treatment period of 5 min.

A similar relationship between the CO_2 supply rate and the disinfection efficacy was observed with enterococci (Figure 5.10b). Disinfection results obtained at 0.3 MPa with PCD/chlorine revealed that the number of viable enterococci were less than the permitted limit according to the IMO D-2 standard, and the acceptable reductions were achieved within 8 min for the CO_2 supply rates of (25% CO_2 + 75% N_2) and (50% CO_2 + 50% N_2), and within 3 min for the CO_2 supply rate of (75% CO_2 + 25% N_2) and 100% CO_2 (Figure 5.11b). Additionally, the method reduced the enterococci load to below the limit value of the USCG standard within 10, 8, and 3 min for the CO_2 supply rates of 25%, (50% and 75%), and 100%, respectively. Remarkably, the combined 100% CO_2/chlorine treatment at 0.3 MPa completely inactivated enterococci (by 4.9 $\log_{10}$ CFU 100 mL^{-1}) in seawater within 10 min, whereas only 5 min of the combined treatment at 0.9 MPa was required to reduce the enterococci load by 5.6 $\log_{10}$ CFU 100 mL^{-1} (Figure 5.10b).

Vibrios were more susceptible to the combined PCD/chlorine treatments than *E. coli* and enterococci. No viabile Vibrio species were detected within 3 and 1 min at 0.3 and 0.9 MPa, respectively (Figure 5.10c). Taken together, these findings affirm that the combined PCD/chlorine treatment could successfully eliminate pathogens in seawater; furthermore, use of gas with higher CO_2 contents and pressure required shorter exposure times for efficient disinfection.

After disinfection and decompression, the combined PCD/chlorine 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*, enterococci, and vibrios in the treated samples had not increased, i.e. no regrowth of bacteria was observed.

Pressurized CO_2 decreased the pH of the treated seawater to approximately 5.0. When the PCD treated sample was placed at normal condition and ambient temperature, the pH gradually increased from 5.0 to neutral pH 6.6 after 5 days. The pH would be recovered to the initial pH value of 8.0 after 8 days.

In the present study, commercial compressed gases were used for the disinfection experiments. If this disinfection method is applied to treat ballast water in tanks during the ballast voyage, CO_2 could be supplied from the emissions of fuel combustion. Before discharging ballast water, the pH of the treated water can be raised to neutral levels by diluting it with the receiving water in the harbour at least 5 times. The final discharged water will have little effect on aquatic organisms.

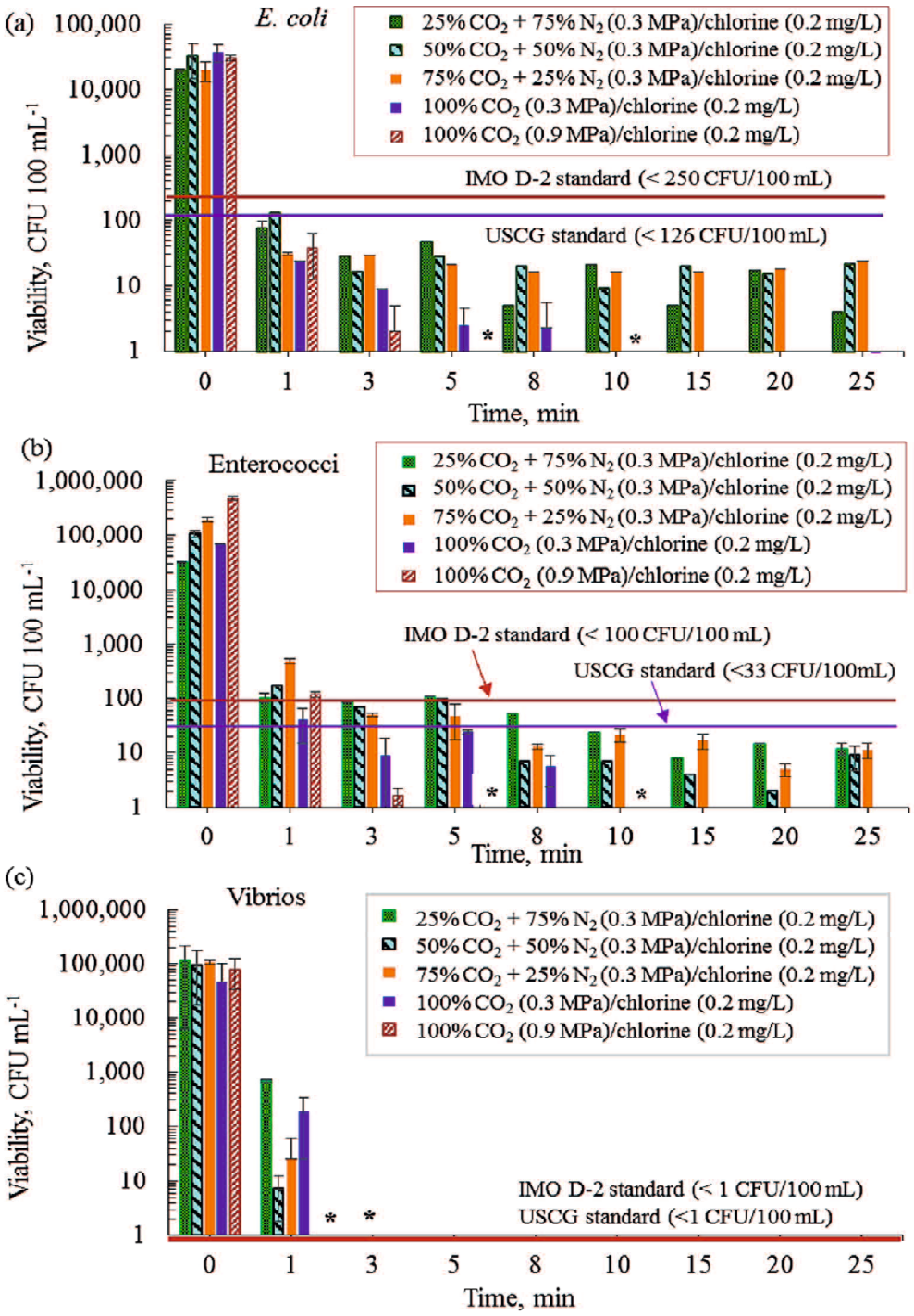

Figure 5.10 Bactericidal effect of the combined pressurized carbon dioxide (pressure: 0.3–0.9 MPa, CO_2 content: 25%, 50%, 75%, and 100%)/chlorine (0.2 mg L^{-1}) treatment on (a) *E. coli*, (b) enterococci, and (c) vibrios in seawater. Asterisks (*) indicate that no colonies were detected.

5.3.3 Cost benefit analysis of the disinfection process

It is expected that the advancement ballast water treatment systems should be designed in possible ways to improve treatment efficacy, to reduce treatment and system costs, and to minimize disinfection by-products.

5.3.3.1. Identification of relevant costs and benefits of intervention

- Disinfection efficiency (in compared with the discharged standards of IMO D-2, USCG, and California (Jan. 1st, 2020));
- Disinfection time (which directly influence requirements of space, circulation pumping);
- Disinfection by-products;
- Cost of energy consumption;
- Cost of chemical consumptions (i.e. compressed CO_2 and N_2, NaOCl, neutralization for the residual control stage);
- System costs (device, gas generator, pressure pipe, valves, and pumps);
- Other system operating parameters.

5.3.3.2. Valuation of costs and benefits

The operating costs include for energy (electricity), compressed CO_2 and N_2, NaOCl solution. The cost estimates are based on unit energy cost of ¥18-20/kWh of electricity, ¥216/kg of compressed CO_2; ¥93/kg of compressed N_2; and ¥631/L of NaOCl solution.

- Average cost for refilling a 30 kg cylinder CO_2 (99.9% purity) is ¥6480, and 1kg CO_2 = ¥216.
- Average cost for refilling a 47L cylinder N_2 (99.995% purity) is ¥3240, and 1kg N_2 = ¥93.
- Electricity price of 1kWh is ¥18-20 (http://www.energia.co.jp/).
- Sodium hypochlorite price of 1L NaOCl (with 6% available chlorine content) is ¥631 (http://flier.monotaro.com/69136/pageview/pageview.html#page_num=44, assessed 14 December 2016).

The solubility of N_2 in water follow Henry's law ("the amount of air dissolved in a fluid is proportional with the pressure of the system"). The dissolved gas concentration was calculated by the following equation:

Dissolved gas concentration (g/L) = Volume of gas dissolved in water (at 1atm and depend on temperature, cm^3/cm^3) × Pressure (atm) × Molecular weight (g/mol)/22.4.
For example, at pressure of 0.9 MPa and temperature of 20°C, N_2 solubility in water is approximately 0.18 g/L (= 0.016 × 8.88 × 28/22.4).

Table 5.6 shows the operation cost of the combined PCD/chlorine treatment process. The cost estimates for the treatment of ballast water were calculated as cost per m^3 of water treated. Highest operation costs are for power and CO_2. The major contributor to energy consumption cost was 23.4% of total, which was used for circulation pumping requirements. Meanwhile, the chemical consumption costs were mainly attributed by compressed CO_2 (72.3%), N_2 (3.9%), and NaOCl (0.5%). Operation cost was 436 ¥/m^3 ballast water.

However, in the present study, commercial compressed gases were used for the disinfection experiments. If this disinfection method is applied to treat ballast water in tanks during the ballast voyage, CO_2 could be supplied from the emissions of fuel combustion. According to the Intergovernmental Panel on Climate Change (IPCC), when polluters release CO_2 into the atmosphere, emitters of CO_2 pay a price in relation to the amount emitted. Specifically, the emission fee was about \$10/ton-CO_2 from 2015, \$100/ton-CO_2 from 2050, and \$1000/ton-CO_2 from 2100 (IPCC, 2014). Hence, the price of CO_2 from commercially purchased in the future may be relatively low. At the same time, emitters of CO_2 require for CO_2 capture and storage or pay for users that employ CO_2 for other purposes such as water disinfection in this study. It means that the operation cost of the combined PCD/chlorine treatment would be reduced.

Table 5.6 Operation cost of the combined pressurized carbon dioxide (25% CO_2 + 75% N_2)/chlorine treatment (at 0.9 MPa, 1462 mg CO_2 L^{-1}, 0.2 mg Cl_2 L^{-1}, 3 min)

Unit operation	CO_2 (kg)	N_2 (kg)	Power (kWh)	NaOCl (L)
Consumption per m^3 ballast water	1.46	0.18	5.36	0.003
Cost (¥/m^3 ballast water)	315	17	102	2
Total cost (¥/m^3 ballast water)		436		

Electricity cost at 18–20 ¥/kWh, CO_2 cost at 216 ¥/kg, N_2 cost at 93 ¥/kg, sodium hypochlorite (with 6% available chlorine content) cost at 631 ¥/L.

Table 5.7 compares the operation cost between PCD/chlorine treatment and other methods such as electrolyzing, and electro-chlorination + CO_2. The operation cost of PCD/chlorine treatment was relatively higher than that of electrolyzing and electro-chlorination + CO_2. Nevertheless, the operation cost of PCD/chlorine treatment can continue to reduce (i.e. ~104 ¥/m^3) by using CO_2 from the emission of fuel combustion as mentioned above. In addition, disinfection cannot be based on cost comparisons alone. While a combination of electro-chlorination at 6 mg L^{-1} TRO and CO_2 addition reduced approximately 1.2 to 1.9 log of heterotrophic bacteria, the treatment method produced high concentrations of DPBs such as THMs (9.12 mg L^{-1}) and HAAs (36.2 mg L^{-1}) (Cha *et al.* 2015). The majority of ballast water treatment systems that employ chlorine disinfection use a dose of about 10 mg L^{-1} of chlorine, and free excess chlorine needs to be neutralized to less than 0.2 mg L^{-1} residual chlorine by an obligatory neutralization process before discharging ballast water (David & Gollasch 2015). In the present study, lower levels of chlorine dosage used, shorter treatment time, and no residual chlorine were the highlights of the combined PCD/chlorine treatment, therefore, disinfection by-products would be less.

Table 5.7 Cost analysis of several disinfection methods compared to the combined pressurized carbon dioxide (PCD)/chlorine treatments.

Treatment option	Treatment conditions	Efficacy	Operation cost (¥/m^3)	Reference
Electro-chlorination	200 mg L^{-1} TRO[#], 255 A/m^2 (~8.6 kWh/m^3), 45 min	100% mortality of *Artemia salina* and *E. coli*	163 ¥ + cost of chemical such as sodium thiosulfate consumption per m^3, which was used for the residual control stage.	Lacasa *et al.* (2013)
Electrolytic chlorine + CO_2	6 mg L^{-1} TRO[#] (~6.5 kWh/m^3), CO_2 injection rate at 100 mL min^{-1}, 5 day	1.8 log reduction *Artemia franciscana*, 1.2–1.9 log reduction heterotrophic bacteria	124 ¥ + cost of CO_2 consumption per m^3 ballast water treated	Cha *et al.* (2015)
PCD/chlorine	0.9 MPa, 0.20 mg Cl_2 L^{-1}, 3 min	5.2 log reduction *Enterococcus* sp.	104 ¥ + cost of CO_2 and N_2 consumption per m^3 ballast water treated (332 ¥)	This study

[#]Total residual oxidants (TRO)

5.4 Conclusions

The combined disinfection treatment with PCD and low-levels of chlorine substantially improved the disinfection efficiency and resulted in significant synergistic benefits (p <0.001) in regard to bacterial inactivation in seawater. The main findings are as follows:

- Disinfection substantially increased with increased pressures and CO_2 supply rates.
- CO_2 concentrations around and above 1500 mg L^{-1} are probably optimal to achieve the synergistic disinfection effect. Moreover, chlorine dosages of 0.20–0.22 mg L^{-1} are probably optimal for the combined PCD/ chlorine treatment.
- This study succeeded in inactivating *Enterococcus* sp. by 5.2–5.5 log in artificial seawater within 4 min by using the combined treatment with PCD (0.3 MPa of pure CO_2) and chlorine (~47%–51% of the normal dosage) at 20 ± 1.0°C.
- The concentrations of TN, TP, *E. coli*, enterococci and vibrios in seawater collected from Ube harbour were relatively low. The initial concentration of *E. coli*, enterococci, and vibrios in seawater were approximately 2.4×10^3 CFU 100 mL^{-1}, 6.6×10^2 CFU 100 mL^{-1}, and 4.0×10^3 CFU mL^{-1}, respectively. Hence, PCD (0.3 MPa, 100% CO_2) was capable of completely inactivating *E. coli,* enterococci, and vibrios within 20, 8 and 1 min, respectively. The PCD at 0.3 MPa reduced the concentrations of *E. coli,* enterococci, and vibrios to below the IMO D-2 and USCG discharge standards within 3 and 1 min, corresponding to 25% CO_2 + 75% N_2 and 100% CO_2, respectively.
- When the initial concentrations of *E. coli*, enterococci, and vibrios in seawater were in the range of $1.9 \times 10^4 - 3.7 \times 10^4$ CFU 100 mL^{-1}, 6.8×10^4–4.9×10^5 CFU 100 mL^{-1}, and 4.6×10^4–1.1×10^5 CFU mL^{-1}, respectively, the combined PCD (0.3 MPa of pure CO_2)/chlorine (0.2 mg L^{-1}) treatment was capable of completely inactivating *E. coli*, enterococci, and vibrios within 10, 10, and 3 min, respectively. When a pressure of 0.9 MPa was used, the exposure time of the combined treatment was 2 times shorter than that of the combined treatment at 0.3 MPa. The treatment reduced the number of viable microbes to less than the IMO D-2 and USCG standards after a duration of 3 min.

Overall, the findings of this study highlight the synergistic benefits of combined PCD/chlorine disinfection technology and suggest that this novel approach could provide a promising method for ballast water disinfection. Further research is required to fully assess

the disinfection efficacy of the combined PCD/chlorine treatment for other organisms (e.g. zooplankton and phytoplankton).

5.5 References

Cha, H.-G., Seo, M.-H., Lee, H.-Y., Lee, J.-H., Lee, D.-S., Shin, K., Choi, K.-H., 2015. Enhancing the efficacy of electrolytic chlorination for ballast water treatment by adding carbon dioxide. Marine Pollution Bulletin 95, 315-323.

APHA, AWWA, WEF, 1999. Standard Methods for the examination of water and wastewater,

20th Edition, Washington DC, USA.

Cheng, X., Imai, T., Teeka, J., Yamaguchi, J., Hirose, M., Higuchi, T., Sekine, M. 2011. Inactivation of *Escherichia coli* and bacteriophage T4 by high levels of dissolved CO_2. *Applied Microbiology and Biotechnology* 90 (4), 1493-1500.

Dang, T.-L.T., Imai, T., Le, T.V., Vo, H.T., Higuchi, T., Yamamoto, K., Kanno, A., Sekine, M. 2016. Disinfection effect of pressurized carbon dioxide on *Escherichia coli* and *Enterococcus* sp. in seawater. *Water Science and Technology: Water Supply (in press),* doi: 10.2166/ws.2016.086

David, M. and Gollasch, S. 2015. Global Maritime Transport and Ballast Water Management - Issues and Solutions. Invading Nature - Springer Series in Invasion Ecology 8. Springer: Dordrecht, The Netherlands.

Fabbricino, M. and Korshin, G.V. 2005. Formation of disinfection by-products and applicability of differential absorbance spectroscopy to monitor halogenation in chlorinated coastal and deep ocean seawater. *Desalination* 176, 57-69.

Fijan, S., Skerget, M., Knez, Z., Sostar-Turk, S., Neral, B. 2011. Determining the disinfection of textiles in compressed carbon dioxide using various indicator microbes. *Journal of Applied Microbiology* 112, 475-484.

Fukuzaki, S. 2006. Mechanisms of actions of sodium hypochlorite in cleaning and disinfection processes. *Biocontrol Science* 11 (4), 147-157.

Garcia-Gonzalez, L., Geeraerd, A.H., Spilimbergo, S., Elst, K., Van Ginneken, L., Debevere, J., Vanpe, J.F., Devlieghere, F. 2007. High pressure carbon dioxide inactivation of microorganisms in foods: The past, the present and the future. *International Journal of Food Microbiology* 117, 1-28.

GloBallast 2016. The issue. http://globallast.imo.org/ballast-water-as-a-vector/ (accessed 29 May 2016).

Gregg, M., Rigby, G., Hallegraeff, G.M., 2009. Review of two decades of progress in the development of management options for reducing or eradicating phytoplankton, zooplankton and bacteria in ship's ballast water. Aquatic Invasions 4 (3), 521-565.

Hong, S.-I. and Pyun, Y.R. 1999. Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. *Journal of Food Science* 64, 728-733.

Husain, M., Felbeck, H., Apple, R., Altshuller, D., Quirmbach, C., 2004. Ballast water treatment by de-oxygenation with elevated CO_2 for a shipboard installation – a potential affordable solution. In: Matheickal, J.T., Raaymakers, S. (eds), 2nd International Ballast Water Treatment R&D Symposium, IMO, London, UK, 21-23 July 2003: proceedings. GloBallast Monograph Series No. 15, IMO London, 48-64.

IMO 2004. International convention for the control and management of ship's ballast water and sediments. In: *International Conference on Ballast Water Management for ships BWM/CONF/36,* London, February 2004.

IMO, 2010. Harmful aquatic organisms in ballast water – Application for final approval of the Ecochlor ballast water management system submitted by Germany. In: Marine Environment Protection Committee MEPC 61/2/8.

IMO, 2016. BWM technologies. http://www.imo.org/en/OurWork/Environment/BallastWaterManagement/Pages/BWMTechnologies.aspx (accessed 15 September 2016).

Isenschmid, A., Marison, I.W., von Stockar, U. 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast cells. *Journal of Biotechnology* 39, 229-237.

Kim, SR., Park, H.J., Yim, D.S., Kim, H.T., Choi, I.-G., Kim, K.H. 2008. Analysis of survival rates and cellular fatty acid profiles of *Listeria monocytogenes* treated with supercritical carbon dioxide under the influence of cosolvents. *Journal of Microbiological Methods* **75**, 47-54.

Kobayashi, F., Hayata, Y., Kohara, K., Muto, N., Osajima, Y. 2007. Application of supercritical CO_2 bubbling to inactivate *E. coli* and coliform bacteria in drinking water. *Food Science and Technology Research* 13, 20-22.

Kobayashi, F., Yamada, F., Ikeura, H., Hayata, Y., Muto, N., Osajima, Y. 2009. Inactivation of microorganisms in untreated water by a continuous flow system with supercritical CO_2 bubbling. *Journal of Water and Environment Technology* 7, 241-250.

Koivunen, J. and Heinonen-Tanski, H. 2005. Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Water research* 39, 1519-1526.

Lacasa, E., Tsolaki, E., Sbokou, Z., Rodrigo, M. A., Mantzavinos, D., Diamadopoulos, E. 2013. Electrochemical disinfection of simulated ballast water on conductive diamond electrodes. *Chemical Engineering Journal* 223, 516-523.

Lloyd's Register, 2012. Ballast water treatment technologies and current system availability. Understanding Ballast Water Management series.

NEI Marine, 2016. N.E.I. Treatment Systems, Test data. http://www.nei-marine.com/images/pdf/NEI_Test_Data.pdf (accessed 21 September 2016).

Ruiz, G.M., Carlton, J.T., Grosholz, E.D., Hines, A.H. 1997. Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist* 37, 621-632.

Spilimbergo, S., Dehghani, F., Bertucco, A., Foster, N.R. 2003. Inactivation of bacteria and spores by pulse electric field and high pressure CO_2 at low temperature. *Biotechnolology and Bioengineering* 82 (1), 118-125.

Tamburri, M.N., Little, B.J., Ruiz, G.M., Lee, J.S., McNulty, P.D., 2004. Evaluations of Venturi Oxygen Stripping™ as a ballast water treatment to prevent aquatic invasions and ship corrosion. In: Matheickal, J.T., Raaymakers, S. (eds), 2nd International Ballast Water Treatment R&D Symposium, IMO, London, UK, 21-23 July 2003: proceedings. GloBallast Monograph Series No. 15, IMO London, 34-47.

Tamburri, M.N., Ruiz, G.M., 2005. Evaluations of a ballast water treatment to stop invasive species and tank corrosion. The Society of Naval Architects and Marine Engineers (SNAME) Maritime Technology Conference & Expo and Ship Production Symposium, Houston, TX.

Tsolaki, E. and Diamadopoulos, E. 2010. Technologies for ballast water treatment: a review. *Journal of Chemical Technology and Biotechnology* 85, 19-32.

Ube City 2015. Environment of Ube city (2015). http://www.city.ube.yamaguchi.jp/machizukuri/kankyouhozen/kankyoukihonkeikaku/documents/h27main2.pdf (accessed 9 October 2016).

Vo, H.T., Imai, T., Teeka, J., Sekine, M., Kanno, A., Le, T.V., Higuchi, T., Phummala, K., Yamamoto, K. 2013. Comparison of disinfection effect of pressurized gases of CO_2, N_2O, and N_2 on *Escherichia coli*. *Water Research* 47, 4286-4293.

Vo, H.T., Imai, T., Ho, T.T., Dang, T.-L.T., Hoang, S.A. 2015. Potential application of high pressure carbon dioxide in treated wastewater and water disinfection: Recent overview and further trends. *Journal Environmental Sciences* 36, 38-47.

Von Gunten, U. 2003. Ozonation of drinking water: Part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. *Water Research* 37, 1469-1487.

Werschkun, B., Sommer, Y., Banerji, S. 2012. Disinfection by-products in ballast water treatment: An evaluation of regulatory data. *Water Research* 46, 4884-4901.

Zhang, J., Davis, T.A., Matthews, M.A., Drews, M.J., LaBerge, M., An, Y.H., 2006. Sterilization using high-pressure carbon dioxide. The Journal of Supercritical Fluids 38, 354-372.

CHAPTER VI

CONCLUSIONS AND FUTURE WORKS

6.1 Conclusions

Pressurized carbon dioxide (PCD) treatment can be used to eliminate pathogens from seawater. Table 6.1 summarizes the log reduction obtained by the PCD and the combined PCD/chlorine treatments under various conditions of pressure, WVR, CO_2 supply rate, and chlorine dosage. The main findings are as follows:

- Disinfection substantially increased with increased pressure and temperature. Conversely, the bactericidal efficiency increased with decreasing WVR. The gram-positive bacterial species, *Enterococcus* sp., had lower susceptibility to PCD treatment than did the gram-negative bacterial species, *V. alginolyticus* and *E. coli.*
- Inactivation mechanism of PCD involved multiple impacts of (1) turbulence caused by the high-frequency recirculation of the fluid; (2) collisions of microorganisms on the surface shield; (3) jets and shock waves formed by explosion of bubble; (4) CO_2 effectively penetrating into cells. Specifically, PCD (0.7 MPa) cause the leakage of intracellular materials during the treatment process. Comparative SEM images of untreated samples and samples treated with PCD did not reveal dramatic changes in the cell shape of *Enterococcus* sp.; however, some *E. coli* and *V. alginolyticus* cells that were treated with PCD did not retain the original shape and appeared to be lysed.
- The results reveal strong correlation between pressure cycling of PCD and inactivation efficiency (p-value <0.001). The bacterial inactivation was concomitantly influenced by two key elements associated with the frequency and magnitude of pressure cycling. At constant ΔP, faster pressure cycling improved disinfection efficiency (p <0.001). Specifically, the outcome of linear regression model analysis suggests that the model can explain 91% and 85%–93% of the inactivation efficiency of *E. coli* and *Enterococcus* sp., respectively, with p-value <0.001 in all cases.
- Disinfection efficiency substantially increased with increased pressure and CO_2 supply rate. The *Enterococcus* sp. inactivation rate of PCD was described by the first-order death kinetics model (R^2 = 0.91 to 0.99), and the smallest D-values were produced at 0.9

MPa. At constant pressure, the *D*-values significantly decreased with increasing CO_2 content (p <0.01). Specifically, the exposure time required for 1-log reduction of *Enterococcus* sp. load with PCD at 0.9 MPa were 13.28, 6.93, and 3.85 min, corresponding to (25% CO_2 + 75% N_2), (50% CO_2 + 50% N_2) and 100% CO_2, respectively. PCD (at 0.9 MPa, 20°C, and 70% WVR) resulted in complete inactivation of the *Enterococcus* sp. following a 5.2 log reduction within 20 min.

- The combined disinfection treatment with PCD and low-levels of chlorine substantially improved the disinfection efficiency and resulted in significant synergistic benefits (p <0.001) for bacterial inactivation in seawater. Most synergy values resulting from the combined PCD/chlorine treatment were positive. Disinfection substantially increased with increased pressures and CO_2 supply rates. CO_2 concentrations around and above 1,500 mg L^{-1} are probably optimal to achieve the synergistic disinfection effect. Moreover, chlorine dosages of 0.20–0.22 mg L^{-1} are probably optimal for the combined PCD/chlorine treatment. Remarkably, the combined PCD (0.3 MPa, 100% CO_2)/chlorine (0.20 mg L^{-1}) treatment inactivated *Enterococcus* sp. by 5.2–5.5 log in artificial seawater within 4 min. Despite the lower pressure of PCD (0.3 MPa), with chlorine added at 47%–51% of a normal chlorine dose, the exposure time of the combined treatment in the present study was 5 times shorter than that of PCD (at 0.9 MPa) alone.

- The concentrations of TN, TP, COD, *E. coli*, enterococci, and vibrios in seawater collected from Ube harbour were relatively low. The following values were detected: salinity = 3.3 ± 0.2%, pH = 8.2 ± 0.1, TSS = 22.2 ± 14.2 mg L^{-1}, TN = 1.6 ± 0.1 mg L^{-1}, TP = 0.09 ± 0.02 mg L^{-1}, COD = 2–3 mg L^{-1} (Ube City 2015), *E. coli* = 1.1 × 10^2 to 3.0 × 10^3 CFU 100 mL^{-1}, enterococci = 2.3 × 10^2 to 8.3 × 10^2 CFU 100 mL^{-1}, and vibrios = 2.4 × 10^3 to 5.2 × 10^3 CFU mL^{-1}. Under treatment conditions (0.3 MPa and 28 ± 1.0°C), PCD reduced the concentrations of *E. coli*, enterococci, and vibrios to below the IMO D-2 and USCG discharge standards within 3 and 1 min, corresponding to 25% CO_2 + 75% N_2 and 100% CO_2, respectively.

- When the initial concentrations of *E. coli*, enterococci, and vibrios in seawater were in the range of 1.9 × 10^4–3.7 × 10^4 CFU 100 mL^{-1}, 6.8 × 10^4–4.9 × 10^5 CFU 100 mL^{-1}, and 4.6 × 10^4–1.1 × 10^5 CFU mL^{-1}, respectively, the combined PCD (0.3 MPa of pure CO_2)/chlorine (0.2 mg L^{-1}) treatment was capable of completely inactivating *E. coli*, enterococci, and vibrios within 10, 10, and 3 min, respectively. When a pressure of 0.9 MPa was used, the exposure time of the combined treatment was 2 times shorter than that of the

combined treatment at 0.3 MPa. The treatment reduced the number of viable microbes to less than the IMO D-2 and USCG standards after a duration of 3 min.

- When the PCD treated sample was placed at normal condition and ambient temperature, the pH gradually increased from 5.0 to pH 6.6 after 5 days. The pH would be recovered to the initial pH value of 8.0 after 8 days. Before discharging ballast water, the pH of the treated water can be raised to neutral levels by diluting it with the receiving water in the harbour at least 5 times. The final discharged water will have little effect on aquatic organisms.

Overall, this study successfully enhanced the bactericidal performance of PCD via pressure cycling. Also, the findings of this study highlight the synergistic benefits of combined PCD/chlorine disinfection technology and suggest that this novel approach could provide a promising method for ballast water disinfection.

6.2 Future works

- Further research is required to fully assess the disinfection efficacy of the PCD and the combined PCD/chlorine treatments for other organisms (e.g. planktons, organisms <10 μm, and viruses).
- By the low-dosage of chlorine (0.20 mg L^{-1}) used, the problem of by-products may be minimized in the combined PCD/chlorine treatment. Nevertheless, further research on potential problems related to the by-products during treatment is needed.
- The shipping industry need to reduce emissions of CO_2 and other gases such as NO_x and SO_x according to the regulations of the Marine Pollution Convention (MARPOL) for the prevention of air pollution from ships. In the present study, commercial compressed gases were used for the disinfection experiments. If this disinfection method is applied to treat ballast water in tanks during the ballast voyage, CO_2 could be supplied from the emissions of fuel combustion (other gases such as NO_x and SO_x need also to be removed prior).
- On the other hand, the method for forming highly dissolved CO_2 in water in the present study could potentially be useful in other applications (i.e. aeroponics technology, hydroponics technology).

Table 6.1 Log reduction obtained in the pressurized carbon dioxide (PCD) and the combined PCD/chlorine treatments for various treatment conditions.

Treatment system	Treatment option	Microorganism	Treatment conditions				Exposure time, min	Log_{10} reductions	Cost, (¥/m^3)		Target of treatment		
			Pressure, MPa	Pressure cycling, cycle	Temp., °C	Chlorine dosages, mg L^{-1}			Main	Gases (CO_2, N_2)	IMO-D2 standard	USCG standard	California standard (Jan., 2020)
Pump power: 0.20 kW, Nozzle diameter: 5 mm, Flow rate: 14 L min^{-1} WVR: 70% HRT: 0.50 min	PCD (100% CO_2)	*V. alginolyticus*[b]	0.7	6	20 ± 1	0.0	3[a]	5.1	102	389	○	○	○
		E. coli[b]	0.7–0.9	20			10[a]	5.7	339	389	○	○	○
		Enterococcus sp.[b]	0.7–0.9	50			25	4.1–4.3	848	389	×	×	×
Pump power: 0.75 kW, Nozzle diameter: 7 mm, Flow rate: ~25 L min^{-1}, WVR: 70% HRT: ~0.28 min	PCD (25% CO_2 + 75% N_2)	*Enterococcus* sp.[b]	0.9	87	20 ± 1	0.0	25	1.9	848	332	×	×	×
	PCD (50% CO_2 + 50% N_2)	*Enterococcus* sp.[b]	0.9	87			25	3.3	848	365	×	×	×
	PCD (100% CO_2)	*Enterococcus* sp.[b]	0.9	70			20[a]	5.2	679	436	○	○	○
	PCD (25% CO_2 + 75% N_2)/chlorine	*Enterococcus* sp.[b]	0.9	11	20 ± 1	0.20	3[a]	5.2	104	332	○	○	○
	PCD (50% CO_2 + 50% N_2)/chlorine	*Enterococcus* sp.[b]	0.9	11			3[a]	5.7	104	365	○	○	○
	PCD (100% CO_2)/chlorine	*Enterococcus* sp.[b]	0.9	11			3[a]	5.2	104	436	○	○	○
	PCD (100% CO_2)/chlorine	*Enterococcus* sp.[b]	0.3	14			4[a]	5.2	138	367	○	○	○
	PCD (100% CO_2)/chlorine	*E. coli*[c]	0.9	18	28 ± 1	0.2	5[a]	4.5	172	436	○	○	○
		Enterococci[c]	0.9	18			5[a]	5.6	172	436	○	○	○
		Vibrios[c]	0.9	4			1[a]	4.9	36	436	○	○	○

"○" indicate that the number of viable microbes was less than the permitted limit according to the ballast water discharge standard, whereas "×" reveal that the treatment does not fulfill the discharge standard.

[a]The treatment period when no viable bacteria were detected after disinfection.

[b]Artificial seawater, initial concentration of bacteria were in the range of 5-6 log_{10} CFU mL^{-1}.

[c]Natural seawater was added to *E. coli* (ATCC 11303), *Enterococcus* sp., and *V. alginolyticus*. The initial concentrations of *E. coli*, enterococci, and vibrios in seawater were in the range of 1.9×10^4 – 3.7×10^4 CFU 100 mL^{-1}, 6.8×10^4–4.9×10^5 CFU 100 mL^{-1}, and 4.6×10^4–1.1×10^5 CFU mL^{-1}, respectively. The treatment fulfilled the IMO-D2 and USCG discharge standards after 3 min.

APPENDIX

LIST OF PUBLICATIONS, PRESENTATIONS, AND AWARDS

LIST OF PUBLICATIONS

PART OF THE THESIS HAS BEEN SUBMITTED FOR PUBLICATION

1. **Thanh-Loc T Dang**, Tsuyoshi Imai, Tuan V Le, Diem-Mai K Nguyen, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, Masahiko Sekine. (2016). Synergistic effect of pressurized carbon dioxide and sodium hypochlorite on the inactivation of *Enterococcus* sp. in seawater. *Water Research,* Vol. 106, 204-213. DOI: 10.1016/j.watres.2016.10.003
2. **Thanh-Loc Thi Dang**, Tsuyoshi Imai, Tuan Van Le, Huy Thanh Vo, Takaya Higuchi, Koichi Yamamoto, Ariyo Kanno, Masahiko Sekine. (2016). Disinfection effect of pressurized carbon dioxide on *Escherichia coli* and *Enterococcus* sp. in seawater. *Water Science and Technology: Water Supply,* Vol. 16(6), 1735-1744. DOI: 10.2166/ws.2016.086
3. **Loc T.T. Dang**, Tsuyoshi Imai, Tuan V. Le, Satoshi Nishihara, Takaya Higuchi, Mai K.D. Nguyen, Ariyo Kanno, Koichi Yamamoto, and Masahiko Sekine. (2016). Effects 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 (Toxic/Hazardous Substance & Environmental Engineering),* Vol. 51(11), 930-937. DOI:10.1080/10934529.2016.1191309
4. **Dang-Thi Thanh-Loc,** Tsuyoshi Imai, Takaya Higuchi, Le-Van Tuan, Vo-Thanh Huy. (2015). Disinfection of *Escherichia coli* in seawater using pressurized carbon dioxide. *Journal of Science and Technology – Vietnam Academy of Science and Technology* (ISSN 0866-708x), Vol. 53(3A), 91-96.

PUBLICATIONS IN CO-AUTHOR

5. Le Van Tuan, Huynh Xuan Toan, Nguyen T. Thao Nguyen, **Dang T. Thanh Loc**. (2016). Performance of H_2O_2 – aerated biofilters in treatment of wastewater containing humic acid. *Journal of Science and Technology*, Vol. 54(2A), 149-155.
6. Huy Thanh Vo, Tsuyoshi Imai, Truc Thanh Ho, **Thanh-Loc Thi Dang**, Son Anh Hoang. (2015). Potential application of high pressure carbon dioxide in treated wastewater and

water disinfection: Recent overview and further trends. *Journal of Environmental Sciences*, Vol. 36, 38-47.

7. Tuan Van Le, Tsuyoshi Imai, Daisuke Ayukawa, Hiroaki Fujinaga, Huy Thanh Vo, Tung Quy Truong, **Thanh-Loc Thi Dang**, Yatnanta Padma Devia. (2014). Application of microbubbles ozonation enhanced by coarse bubbles in treatment of oil-in-water emulsions and humid acid mixture. *Journal of Science and Technology – Vietnam Academy of Science and Technology*, Vol. 52(3A), 96-103.
8. Tuan Van Le, Tsuyoshi Imai, Daisuke Ayukawa, Hiroaki Fujinaga, Huy Thanh Vo, Takaya Higuchi, **Thanh-Loc Thi Dang**, Yatnanta Padma Devia. (2014). Application of tiny microbubbles ozonation enhanced by coarse bubbles on treatment of oil-in-water emulsions presented humid acid. *Proceedings of the International Water Association (IWA) specialist conference on Advances in particles science and separation: from mm to nm scale and beyond.* Sapporo, Japan. June 15-18, 2014. p. 371-378.

LIST OF PRESENTATIONS

1. **Dang-Thi Thanh-Loc,** Tsuyoshi Imai, Takaya Higuchi, Le-Van Tuan, Vo-Thanh Huy. Disinfection of *Escherichia coli* in seawater by using pressurized carbon dioxide. *The 5th International Forum on Green Technology & Management.* Hue, Vietnam. July 28th - 30th, 2015. (Oral presentation)
2. **Thanh-Loc Thi Dang**, Tsuyoshi Imai, Satoshi Nishihara, Kim Diem Mai Nguyen, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, Masahiko Sekine. Effect of pressure cycling on inactivation of *Enterococcus* sp. and *Escherichia coli* in seawater using pressurized carbon dioxide. *Water and Environment Technology conference 2016.* Tokyo, Japan. August 27th – 28th, 2016. (Oral and poster presentation)
3. Mai Kim Diem Nguyen, Tsuyoshi Imai, Wataru Yoshida, **Loc Thi Thanh Dang**, Takaya Higuchi, Ariyo Kanno, Koichi Yamamoto, Masahiko Sekine. Removal of carbon dioxide from the mixed gas by using water absorption process advanced with the forming of fine bubbles. *Water and Environment Technology conference 2016.* Tokyo, Japan. August 27th – 28th, 2016. (CO-AUTHOR)

AWARDS

1. "**WET excellent presentation award**" was given by Japan Society of Water Environment (JSWE) in 2016.