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Assessment of the ozonation against pathogenic bacteria in the effluent of the quarantine station

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Abstract This study investigated how ozone treatment can successfully inactivate pathogenic bacteria in both artificial seawater and effluents discharged from the fishery quarantine station in Pyeongtaek Port, Korea. *Vibrio* sp. and *Streptococcus* sp. were initially inoculated into the artificial seawater. All microbes were almost completely inactivated within 10 min and 30 min by injecting 6.4 mg/min and 2.0 mg/min of ozone, respectively. It was discovered that the water storing *Pleuronichthys*, *Pelteobagrus,* and *Cyprinus* imported from China contained the indicator bacteria, *Vibrio* sp., *Enterococcus* sp., total coliforms, and heterotrophic microorganisms. Compared to the control, three indicator bacteria were detected at two to six times higher concentrations. The water samples displayed a diverse microbial community, comprising the following four phyla: Bacteroidetes, Proteobacteria, Firmicutes, and Actinobacteria. Almost all indicator bacteria were inactivated in 5 min at 2.0 mg/min of ozonation; comparatively, 92.9%–98.2% of the less heterotrophic microorganisms were deactivated within the same time period. By increasing the dosage to 6.4 mg/min, 100% deactivation was achieved after 10 min. Despite the almost complete inactivation of most indicator bacteria at high doses after 10 min, several bacterial strains belonging to the Proteobacteria have still been found to be resistant under the given operational conditions.

Keywords : Ozonation; Quarantine effluent; Indicator bacteria; Fishery; Proteobacteria

1. Introduction

According to the United Nations Food and Agriculture Organization (FAO), there has been a dramatic increase in the production and consumer demand for aquaculture products since 1960 [1]. It has been estimated that the annual consumption of aquaculture products per capita has increased by 10 kg approximately in the last 50 years. Besides, population growth and improved distribution system have accelerated the expansion in the international trade dealing with aquatic products. Particularly, in developed countries, the consumption and trading markets for these aquatic products have been steadily increased, leading to an overwhelming increase in fishery imports in their markets. As a result, their international trades have been significantly increased by more than 500% from 1976 to 2014. Furthermore, reports on the global imports and exports of fishery products demonstrated that China, Norway, and Vietnam are the major exporters of fishery products, whereas the United States is the largest importer of fishery products, followed by Japan and China [1]. Korea is ranked tenth in the importing countries

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of fishery products as an effort of the Free Trade Agreement (FTA) [1,2].

However, such an expansion of international trades can often bring various ecological transboundary problems in the processes of storage and transport [3,4]. During the quarantine stage at importing countries, there has been an increased burden of seawater/freshwater of foreign origin to be discharged to the inland seashore. The water temporarily storing the imported aquatic products can serve as a breeding ground for many invasive microorganisms or pathogens, deteriorating or infecting the domestic offshore inhabitants [5].

The ballast water, having a similar characteristic with a quarantine effluent in terms of carrier of foreign organisms, has been emerged as a potential threat to the local habitats, resource, biodiversity, or human health [6]. However, the ballast water is disinfected before its discharge in an importing harbor or the near the coastal zone, according to the International Convention for the Control and Management of Ships` Ballast Water and Sediments (BWM) [7] established by the International Maritime Organization (IMO). Here, the criteria for the pathogenic bacteria are less than 1 CFU/100 mL, less than 250 CFU/100 mL, and less than 100 CFU/100 mL for *Vibrio cholerae*, *Escherichia coli,* and intestinal *Enterococci*, respectively. Recently, other types of aquatic bacteria and viruses have also been found in the ballast water [8-10]. On the contrary, the quarantine effluent has not been tested whether pathogenic or other invasive organisms are present or not. Furthermore, there is a notable lack of evidence on the possible risk of foreign microorganisms in quarantine effluents.

In the meantime, various techniques (e.g., ozonation [11], electrolysis [11], ionization discharge [12], ultraviolet (UV) irradiation [13], and chlorination [14]) have been used to inactivate the pathogenic bacteria or invasive organisms contained in the ballast water [6]. Among them, ozonation is extensively used in a variety of industries due to its high disinfection efficiency and oxidation potential with a compact footprint. It has been applied to disinfect bacteria, fungi, and protozoa including pathogens in the fish farm [15] as well as oxidize a variety of organic matters and micro-solids produced from the manufacturing of pharmaceuticals [16] or pulps [17]. Additionally, ozone can improve the water quality by oxidizing ammonia into nitrite and nitrate together with reducing the dissolved organic carbon in the seawater [18].

Herein, this study was to compare the distribution of indicator bacteria (i.e., *Vibrio* sp., total coliforms, and *Enterococcus* sp.) and heterotrophic bacteria between quarantine effluents and control seawater, and to estimate the feasibility of ozonation as the effective disinfection technology for the quarantine effluent containing single or mixed microbial strains. It also investigated the change of bacterial community after the ozonation.

2. Materials and methods

2.1. Fishery quarantine effluent

2.1.1. Sampling of quarantine effluent

Several types of effluent samples discharged from the water tank in a fishery quarantine station located in Pyeongtaek Port, Korea, were collected in the Winter of 2019. They had come in with the importation of three different types of fish of *Cyprinus* (freshwater), *Peteobagrus* (freshwater), and *Pleuronichthys* (seawater) from China: which were abbreviated with WCP-F, WCC-F, and WCPL-S, respectively. In addition, a control seawater sample was taken offshore near the port. Field measurements were immediately performed to check the pH, electrical conductivity (EC), salinity, oxidation-reduction potential (ORP), dissolved oxygen (DO), and temperature using a portable multi-meter (YK-2001PHA, LUTRON Co., Taiwan) at each sampling location, while the concentrations of anions (i.e., F^-, SO_4^{2-}, NO_3^- and Cl^-) were determined using ion chromatography (ICS-3000, Dionex, USA) in the laboratory.

2.1.2. Indicator bacteria in the effluent

Indicator bacteria have the advantages of being inex-

pensive to culture and readily identifiable and quantifiable in the laboratory [19,20]. In this study, the population density of *Vibrio* sp., total coliforms, and *Enterococcus* sp. in the quarantine effluent were compared to those of control for warranting the introduction of proper treatment process before being discharged into the concerned coastal area. For that, three different selective media of Difco™ Thiosulfate-citrate-bile salts-sucrose (TCBS) Agar [21], Dicfo™ m Endo Agar LES [22,23], and Dicfo™ m Enterococcus Agar (BD, USA) [21] were prepared according to the manufacturer`s instruction. The diluted sample was first filtered through a 0.45 μm cellulose acetate membrane filter (ADVANTEC®, Japan) and then placed on each solidified selective medium in the petri dish (SPL Life Sciences, Korea). After that, they were cultivated at 35℃ in an incubator (VS3125Bi, Vision Scientific Co., Korea) for 24~48 h, and then the colonies were enumerated as the colony-forming unit (CFU)/100 mL. It was carried out in triplicate per samples where the mean population density and its standard deviation were calculated.

2.1.3. Heterotrophic microbial community

The number of heterotrophic bacterial colonies was determined by the plate counting method and subsequently the type of heterotrophic microbial community was analyzed using 16S rRNA sequencing analysis. The aliquot was spread on the Difco™ Marine agar (BD, USA) and incubated at 30℃ in an incubator for 24 h. The colonies formed on the agar medium were individually isolated based on their shape and size, and then they were enriched in Difco™ Marine broth (BD, USA) at 30℃ shaking incubator (VS8480, Vision Scientific Co. Ltd., Korea) for 24 h. From that, total genomic DNA was extracted using a HiGene™ Genomic DNA Prep Kit (BioFACT, Korea) and stored at －20℃ in a freezer. DNA amplification was performed by mixing 3 μL of genomic DNA, 2.5 μL of 10X *Taq* reaction buffer, 0.3 μL of *Taq* DNA polymerase, 0.5 μL of dNTP mix (10 mM) (BioFACT, Korea), and 1 μL of each universal primer of 27F (5`-AGA GTT TGA TCC TGG CTC AG-3`) and 1492R (5`-TAC GGY TAC CTT GTT ACG ACT T-3`, 10 pmole/μL, Bioneer, Korea). The final volume of the mixture was then adjusted to 25 μL by adding sterile distilled water, and the PCR was implemented with an initial denaturation at 95℃ for 15 min, 30 cycles of denaturation (95℃, for 20 s), annealing (50℃ for 40 s) and extension (72℃ for 90 s), and a final extension at 72℃ for 5 min in the thermocycler (TProfessional Basic thermocycler, Biometra Ltd., Germany). The amplified product was purified using a HiGene™ PCR Purification Kit (BioFACT, Korea) and sequenced using an ABI 3730XL DNA Analyzer (Applied Biosystems™, USA). The NCBI (National Center for Biotechnology Information) database was used to identify the microbial species by comparing it to the reference nucleotide sequences listed on Gene Bank by employing BLAST (Basic Local Alignment Search Tool) and MEGA 7.0 (Molecular Evolutionary Genetics Analysis).

2.2. Experimental disinfection of artificial seawater

Prior to the implementation of ozonation for the quarantine effluent samples, the disinfection efficiency of pathogenic bacteria by ozonation was estimated using an artificial seawater containing six different pathogenic bacterial strains of *Vibrio harveyi*, *Vibrio ichthyoenteri*, *Photobacterium damselae*, *Streptococcus iniae*, *Lactococcus garvieae*, and *Edwardsiella tarda*, that cause the fishery diseases such as Vibriosis, Strepococcicosis, and Edwardsiellosis in the domestic aquatic animals [24]. These target bacteria were donated from the Korean Culture collection of Aquatic Microorganism (KoCAM) under the National Institute of Fisheries Science. They were inoculated on Bacto™ Tryptic Soy Broth (BD, USA) and then cultivated at 25℃ until reaching an optical density at 600 nm (OD_{600}) of 0.7. It was then harvested by centrifugation at 13,000 rpm for 5 min followed by washing twice with isotonic solution (0.85% (w/v) NaCl solution). Subsequently, they were added individually or mixed together into the artificial seawater made by dissolving 120 g of sea salt mixture (55% Cl^-, 31% Na^+, 8% SO_4^{2-},

4% Mg^{2+}, 1% K^{+} and 1% Ca^{2+} by weight, Sigma, USA) in 3 L of distilled water.

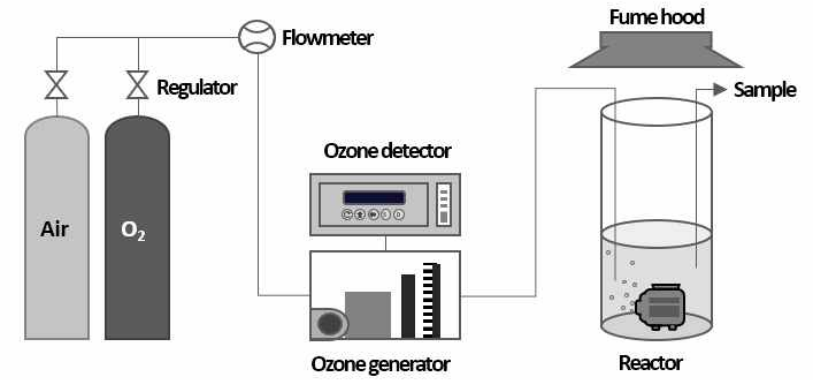

Figure 1. Schematic diagram of the laboratory-scale ozone treatment system.

The ozone treatment system was composed of the ozone generator (Ozone Tech, Korea), ozone gas detector (H1-Ozone analyzer, IN USA™, USA), and the cylindrical reactor equipped with the underwater pump (UP500, Hyupsin, Korea) as shown in Figure 1. To estimate the deactivation effect, ozone was injected for 5 min into the reactor with a dose of 0.5 mg/min with air, 2.0 mg/min with 100 mL/min of pure oxygen gas, or 6.4 mg/min with 200 mL/min of pure oxygen gas into the artificial seawater under the presence of pathogenic bacteria. Then, the water was continuously stirred for an additional 55 min using an underwater pump without any further injection of ozone. The disinfection efficiency was determined by comparing the number of colonies formed on the Bacto™ Tryptic Soy Agar medium after disinfection against the non-disinfected sample.

In addition, the dissolved ozone concentration in the artificial seawater was analyzed using Ozone AccuVac ® Ampules (HR, measurable range: 0-150 mg O_3/L, Hach, USA) with a Hach DR 2800 (Hach, USA), and simultaneously ORP was measured by the portable multi-meter (YK-2001PHA, LUTRON Co., Taiwan), in the absence of microbes.

2.3. Disinfection of real quarantine effluents

To evaluate the feasibility of the ozone treatment system for disinfection of indicator and heterotrophic bacteria present in the actual fishery quarantine effluents, the ozonation was performed at the dose of 2.0 mg/min and 6.4 mg/min. Herein, the viable bacterial population density was determined using the selective media and Difco™ Marine Agar medium, in the same manner as mentioned in the section *2.1.2* and *2.1.3*, respectively. Furthermore, some microbes tolerant to the ozone disinfection were characterized with 16S rRNA sequencing in the quarantine effluent of WCPL-S.

3. Results and discussion

3.1. Fishery quarantine effluent

3.1.1. Water quality of quarantine effluent

Three different fishery quarantine effluents and control seawater taken in the quarantine station and coast area near the Pyeongtaek Port were analyzed, and their water quality result is listed in Table 1.

Table 1. Water quality parameters of fishery quarantine effluents and the control seawater sample

	Control	Fishery quarantine effluents		
	Seawater	WCC-F	WCP-F	WCPL-S
pH	7.52	7.16	7.13	6.88
EC (mS)	37.7	32.6	36.4	38.0
Salinity (%)	36.7	11.7	13.6	34.5
ORP (mV)	236	162	178	245
DO (mg O_2/L)	8.5	7.15	7.21	6.86
Temp. (℃)	19.2	18.1	17.9	16.2
F^- (mg/L)	16.7	14.0	22.1	N.D.*
SO_4^{2-} (mg/L)	1700.9	97.24	98.96	859.7

NO_3^- (mg/L)	27.9	76.99	52.43	63.3
Cl^- (mg/L)	12,423.4	102.2	100.7	8,625.6

*. N.D.: Not detected.

It exhibited a good correlation between the salinity level and Cl^- concentration, showing that the samples containing seawater had a higher concentration than those of freshwater. Cl^- is a dominant controlling factor that is directly related to the decay rate for total residual oxidant (TRO) after ozonated [25,26]. However, their correlation has not yet been completely verified. Apart from this, DO concentration was the highest at the control, whereas it was lower in the fishery effluents. Concentration of SO_4^{2-} is an important parameter if the water is under a reducing environment because it can be transferred into H_2S when foodstuff residuals and feces accumulate on the bottom of the reservoir. This not only imposes a toxic effect on living organisms but also cause eutrophication [27]. However, in this study, these effects were neglected due to the oxic condition of them with a relatively high level of DO and ORP. On the other hand, NO_3^- can be produced by the oxidation of ammonia and nitrites originating from residual fishery feed and feces. As expected, the lowest level of NO_3^- was found in the control while the quarantine effluents demonstrated a higher level. It suggests that the additional treatment system will be facilitated to mitigate the pollutant loaded from these effluents.

3.1.2. Indicator bacteria in the effluent

Three types of indicator bacteria were commonly found in all of the samples (i.e., control and the quarantine effluents of WCP-F, WCC-F, and WCPL-S) as shown in Figure 2. The mean population density of them in the control was determined with 300±16 CFU/100 mL, 980±16 CFU/100 mL, and 70±17 CFU/100 mL for *Vibrio* sp., total coliforms, and *Enterococcus* sp., respectively. Of them, total coliforms can be found at the highest population density due to their faster growth rates regardless of significant variations in oxygen or temperature. In comparison, *Vibrio* sp. and *Enterococcus* sp. had a relatively lower concentration since their growth might be dependent on the temperature, attributing to the poor metabolism in the winter season. On the other hand, there is a significant difference in the population density of these indicator microorganisms between the control and quarantine effluents (t-test, $p < 0.05$), except for the case of *Vibrio* sp. in the freshwater-based effluents (i.e., WCP-F and WCC-F). In particular, the WCPL-S had the highest abundance for these indicator bacteria among the collected quarantine effluents. It was in agreement with the lowest DO concentration and highest NO_3^- concentration in it, as previously delineated. Taking account of these, the proper disinfection of these effluents should be implemented before the products are traded into the domestic market or effluent is discharged near offshore.

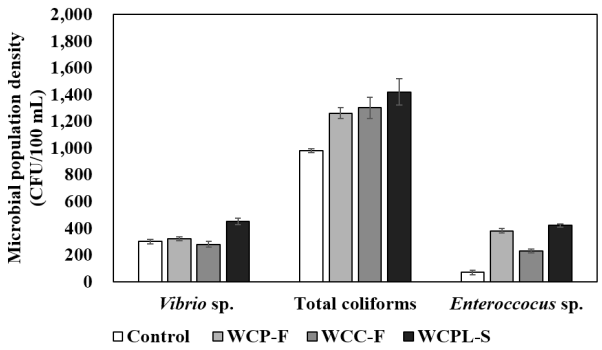

Figure 2. Distribution of indicator bacteria in the real effluent samples (WCP-F, WCC-F, and WCPL-S) and control seawater.

3.1.3. Heterotrophic microbial community

After inoculating the samples on the Dicfo™ Marine Agar medium and incubating them, the heterotrophic microbial strains forming the colonies were enumerated as of CFU/mL, and some of them were individually identified. As a result, the microbial population was more abundant in the quarantine effluents with a level of 6.1×10^5 CFU/mL (for WCP-F), 5.2×10^5 CFU/mL (WCC-F), and 5.6×10^5 CFU/mL (WCPL-S) as compared to the control (4.7×10^5 CFU/mL). Furthermore, the bacterial community was also found to be diverse in the quarantine effluents because they have various habitat, feeding, and behavior characteristics depending

on the types of fish species. There were very disparate patterns of microbial types. In other words, *Cyclobacterium* spp., *Microbacterium* spp., and *Planococcus* spp. were dominantly measured in the WCPL-S, whereas the control sample contained *Micrococcus* spp., *Staphylococcus* spp., and *Bacillus* spp. In addition, *Dietzia* spp., *Aeromicrobium* spp., *Paenarthrobacter* spp., *Exiguobacterium* spp., and *Pseudomonas* spp. were mainly observed in WCP-F, while *Kocuria* spp., *Paracoccus* spp., *Microbacterium* spp., and *Rhodococcus* spp. were found in WCC-F.

3.2. Ozonation of pathogens in artificial seawater

Before the ozone disinfection of marine pathogenic bacteria, the level of dissolved ozone concentrations and ORP were monitored in the absence of microbes (Figure 3).

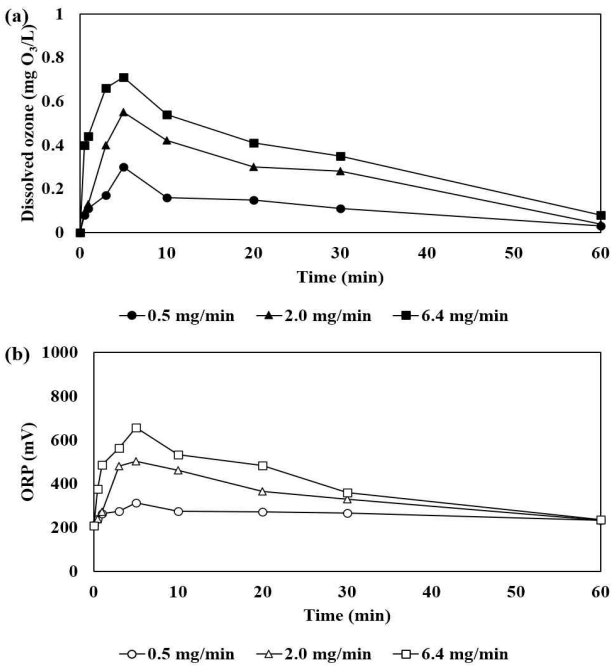

Figure 3. Temporal variation of the (a) dissolved ozone concentration and (b) ORP of the artificial seawater.

Dissolved ozone concentration was rapidly increased while the ozone was supplied into the reactor, and then they were slowly decreased after stopping the ozone injection (Figure 3 (a)). The variation of ORP has corresponded to that of the dissolved ozone concentration (Figure 3 (b)) with a high correlation coefficient (R) of 0.951, 0.957, and 0.942 when ozone was injected with 0.5, 2.0, and 6.4 mg/min, respectively.

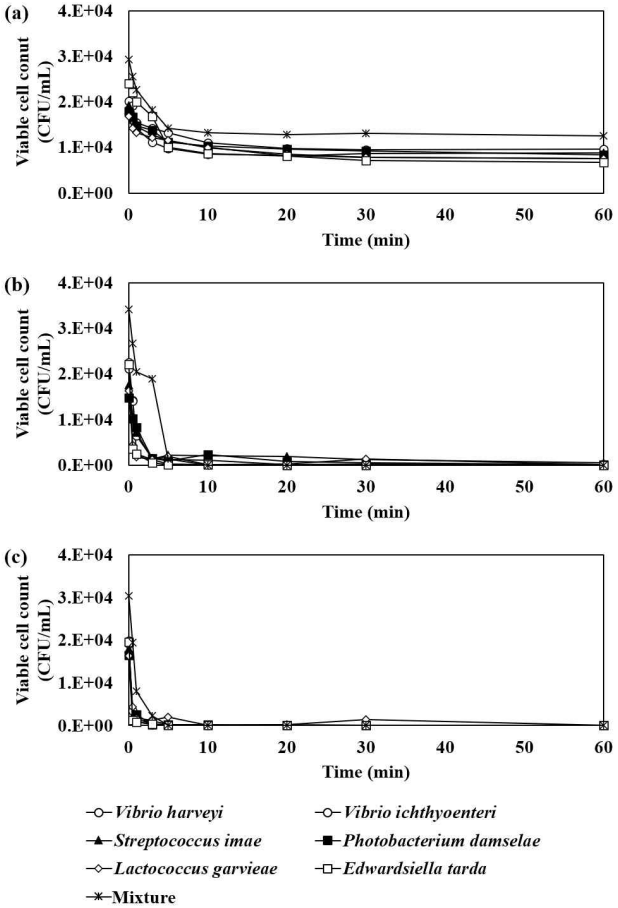

Figure 4. Ozonation of artificial seawater either containing six different microbes or mixture of them with various ozone injection volumes of (a) 0.5 mg/min, (b) 2.0 mg/min, and (c) 6.4 mg/min.

After that, ozonation was carried out in the artificial seawater intentionally contaminated with six different pathogenic bacteria, as presented in Figure 4. Even though these pathogenic bacteria were found in neither actual fishery quarantine effluents nor the control seawater, it was imperative to examine the operational condition and its disinfection behavior for the different types of microbes. First, at the level of 0.5 mg/min of ozone, there was no notable inactivation observed, remaining only 50% disinfection occurred (Figure 4 (a)).

Even worse, after 10 min, there was no longer any disinfection progress in the reactor because the dissolved ozone was nearly exhausted after 5 min of retention contact time. In contrast, with an increase in the ozone dose to 2.0 mg/min, the number of viable bacterial colonies was rapidly decreased within early 5 min after the ozone injection (Figure 4 (b)). After 10 min, the reactor reached an equilibrium with a high disinfection efficiency of more than 99%, although a small number of colonies were still viable in the reactor. Increasing the ozone dose to 6.4 mg/min could allowably accomplish nearly 100% disinfection efficiency within 5 min where the microbes were present either solely or mixed (Figure 4 (c)).

3.3. Ozone disinfection of actual quarantine effluents

Ozonation was performed to disinfect three different types of quarantine effluents; ozone was provided at 2.0 and 6.4 mg/min for 5 min followed by gently stirring for a subsequent 55 min. During the test period, the viability of indicator bacteria and heterotrophic microorganisms were tested by plate counting techniques. With 2.0 mg/min of ozone, *Vibrio* sp., total coliforms, and *Enterococcus* sp. had been inactivated within 20 min (Figure 5 (a-c)). On the contrary, some heterotrophic microorganisms were still viable, showing a lower disinfection efficiency from 92.9% to 98.2% at the end of the treatment process. The lowest value was observed in dealing with the WCPL-S effluents. Increasing up to the ozone dosage of 6.4 mg/min, indicator bacteria had been more efficiently inactivated with a higher disinfection efficiency of more than 99% in a shorter time. In the long run, they were completely inactivated after an extended time of contact by the residual ozone in the aqueous phase, as detected in Figure 3. Likewise, the heterotrophic microbial consortium in WCPL-S much faster reached the equilibrium after 5 min of injection of the higher level of ozone dose, as compared to that of the lower one (Figure 5 (d)).

Concurrently, Table 2 demonstrated that ozone disinfection can alter the microbial community through the identification of the heterotrophic bacterial strains that still remained viable after the treatment. When ozone was injected at 2.0 mg/min, *Ulvibacter* spp., *Albirhodobacter* spp., *Psychrobacter* spp. and *Bacillus* spp. were still viable after 60 min of the test period.

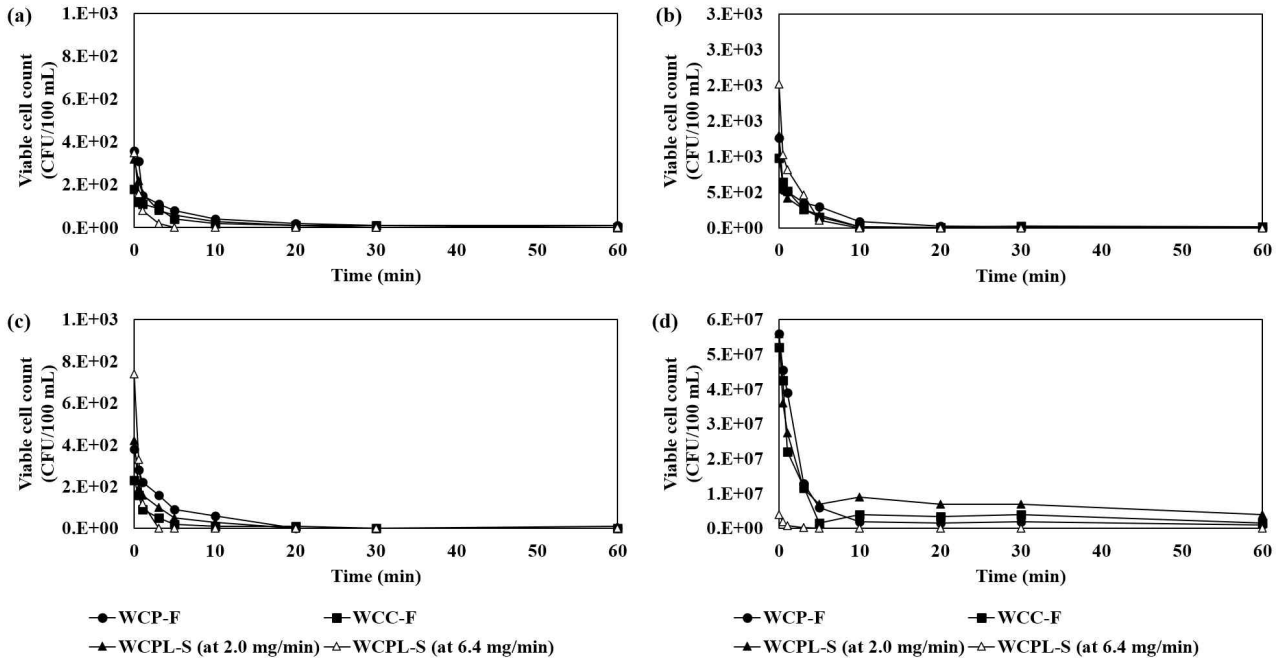

Figure 5. Ozone disinfection of fishery quarantine effluents at the different level of ozone concentrations for three pathogenic bacteria of (a) *Vibrio* sp., (b) total coliforms and (c) *Enterococcus* sp. and (d) heterotrophic bacteria, respectively.

Table 2. Comparison of residual bacterial species before and after disinfection in the effluent of WCPL-S

Phylum	Bacterial species	Raw sample	Ozone disinfection	
			2.0 mg/min for 60 min	6.4 mg/min for 5 min
Bacteroidetes	*Ulvibacter litoralis* (NR_025731.1)	+	+	-
	Ulvibacter antarcticus (NR_044279.1)	+	+	-
Proteobacteria	*Albirhodobacter confluentis* (NR_159309.1)	+	+	+
	Albirhodobacter marinus (NR_126203.1)	+	+	-
	Ahrensia kielensis (NR_113807.1)	-	-	+
	Ahrensia marina (NR_148642.1)	-	-	+
	Sulfitobacter donghicola (NR_044164.1)	+	-	-
	Pseudoseohaicola caenipelagi (NR_135874.1)	+	-	-
	Psychrobacter adeliensis (NR_117632.1)	-	-	+
	Psychrobacter aquimaris (NR_025731.1)	+	+	-
	Psychrobacter muriicola (NR_114669.1)	+	+	-
	Psychrobacter nivimaris (NR_028948.1)	-	-	+
Actinobacteria	*Salinibacterium amurskyens* (NR_043140.1)	+	-	-
	Leifsonia rubra (NR_028012.1)	+	-	-
Firmicutes	*Bacillus drentensis* (NR_118438.1)	+	+	-
	Bacillus firmus (NR_112635.1)	+	+	-

+: Detected; -: Non-detected in the raw and disinfected samples

However, when the ozone dose was increased up to 6.4 mg/min, few colonies were formed on the Marine Agar plate and they were identified as *Albirhodobacter* sp., *Ahernsia* spp., and *Psycobacter* spp., which belongs to the phylum of *Proteobacteria*. It has been previously demonstrated that disinfection processes can rearrange the microbiological community leading to be predominant by specified species, mainly belonging to the Proteobacteria [28-30]. They have a strong adaptability to overcome a wide range of variable metabolism conditions, which can induce their unchangeable rapid growth [29]. In this context, future study should suplementarily explore to investigate on more diverse microorganisms in the quarantine effluents and their deactivation behavior by ozonation and other disinfection techniques.

4. Conclusions

This study was the first to report to analyze the bacterial culture in the domestic fishery quarantine effluents and to implement the laboratory-scale disinfection process using ozone. A comparative analysis has shown that there was higher concentration of indicator bacteria in the effluents, indicating the need for the implementation of proper treatment process before discharge of these effluents into the coastal zone. For this reason, we tried to implement the ozonation of three different quarantine effluents, and reliable disinfection efficiency was obtained for both indicator and heterotrophic bacteria. Moreover, some bacteria belonging to the phyla of Proteobacteria have survived against high level of ozone injection than other bacterial species so that their deactivation behavior would be further studied with various disinfection techniques on the real quarantined seawater.

Acknowledgements

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Culture collection of Aquatic Microorganisms (KoCAM) under the National Institute of Fisheries Science.

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