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ORIGINAL ARTICLE

Control of *Vibrio parahaemolyticus* (AHPND strain) and improvement of water quality using nanobubble technology

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Abstract

Nanobubble technology is used in wastewater treatment, but its disinfectant properties in aquaculture have not been clearly demonstrated. This study investigated the ability of nanobubbles to reduce *Vibrio parahaemolyticus* (AHPND strain) and to improve water quality. Two laboratory experiments were conducted over a one-week period, that is (a) assessing the effects of air and oxygen nanobubbles for 60 minutes per day and (b) comparing effects of ozone nanobubble treatments for 2, 4 and 6 minutes per day. Experiments were done in triplicate 100 L tanks with 15‰ saline water, inoculated with an initial bacterial concentration of 106 CFU/ml. At the end of experiment 1, the bacterial concentration of the air and oxygen nanobubble groups was counted for 69% and 46% of the control group respectively. At the end of experiment 2, the bacterial concentration of the 2-, 4- and 6-minute ozone nanobubble groups were counted for 23%, 2.2% and 0% of the control group respectively. Oxygen and ozone nanobubbles significantly increased oxygen reduction potential and oxygen values. Results indicate that under effective dosages nanobubbles can be used in the production farms to control *V.parahaemolyticus* and increase oxygen levels.

KEYWORDS
nanobubbles, oxygen reduction potential, shrimp farming, *Vibrio parahaemolyticus*

1 | INTRODUCTION

Acute hepatopancreatic necrosis disease (AHPND), formerly known as early mortality syndrome (EMS), is a disease that causes significant economic losses to the shrimp culture industry. This disease is caused by infection with Gram-negative bacteria, *Vibrio Parahaemolyticus* containing plasmids coding for a deadly Pir A and B toxins (Santos et al., 2020). Poor management of water quality, feed, biosecurity and microbial populations in ponds are risk factors for outbreaks of opportunistic *Vibrio*spp. bacteria and can be especially problematic for outbreaks of pathogenic *V.parahaemolyticus*(Dang et al., 2016). In Vietnam, the disease has been recorded since 2011 and estimates of the losses caused by AHPND in 2015 for the Mekong Delta alone were$ 26 million USD for white leg shrimp and $ 11 million USD for black tiger shrimp (FAO 2018). Currently, once ponds are affected, there are no effective control measures for *V.parahaemolyticus*outbreaks in farmed shrimp.

Nanobubbles (NBs) are defined as small gas bubbles with a diameter of less than 100–200 nm (Agarwal et al., 2011; Chaplin, 2019). In contrast to larger bubbles with diameters greater than 100 µm, which float to the surface of water at a speed of 6 mm/sec, smaller bubbles can exist in the water column for several weeks (Azevedo

et al., 2016; Parmar, 2013). Weijs and Lohse (2013) showed that surface NBs live for hours because of limited air diffusion in water, combined with effect of NBs clusters and 'pinned contact lines' of NBs, while Kirby (2010) mentioned that the stability of nanoparticles depends on zeta potential value; particles are more stable with magnitudes greater than 20 mV (Sjogreen et al. 2018). However, there is still a lack of empirical evidence explaining the high stability of NBs in water (Atkinson et al., 2019). There are several examples of application of NB in water treatment plants, medicine, agriculture and aquaculture. Cleaning characteristic of NBs was used to keep the pollutant from fouling (Zhu et al., 2016). Disinfection properties were applied because NBs attract negatively charged particles with their slightly positively charged outer surface, and they can generate oxidative free radicals when they implode on themselves (Gurung et al., 2016; Temesgen et al., 2017).

Oxygen NBs can be applied to closed or open circulating aquaculture systems to maintain high levels of dissolved oxygen, inhibit anaerobic and facultative bacterial growth and thereby help aquaculture species grow faster (Kaneo Chiba & Masayoshi Takahashi, 2007; Serizawa 2017). NBs have been shown to raise the dissolved oxygen and reduce nitrogen content (Moleaer, 2020), convert nitrite to nitrate, decompose nitrogen, carbon dioxide and hydrogen sulphide (Leilang, 2020). Ozone used in concentrations <0.4 ppm is converted into oxygen and is safe and environmental friendly (Nano Bubble Technologies, 2020). There are specific examples of improved growth of fish and shrimp raised with NB treatments. For example, NBs have been shown to optimize the dissolved oxygen utilization of white leg shrimp *(Litopenaeus vannamei)*(Galang et al., 2019). Sweet fish *(Plecoglossus altivelis)*and rainbow trout (*Oncorhynchus mykiss*) cultured in air NB treated water were reported by Ebina et al. (2013) to have better growth than those cultured in untreated water. There are also reports of koi fish *(Cyprinus carpio)*and red seabream (*Pagrus major*) benefiting from aeration with micro NBs (Henry Kasmanhadi Saputra et al., 2018; Stander, 2018).

The disinfection principle of ozone and ozone NBs is similar, both create oxygen free radicals which disrupt the permeability of cell membranes (Ikehata & Li, 2018). Ozone NBs have the same disinfectant properties as dissolved ozone and can persist in water for a long time due to the NBs characteristic (Anzaikantetsu, 2020). Ozone NBs stored frozen (−20°C) retained sufficient microbiocidal activity to kill *E.coli*W3110 within 5 min. after 1 month, 15 min. after 3 months and 60 min. after more than 1 year (Seki et al., 2017). Ozone NBs with ozone content of 3.5 mg/L and ORP of 960 mV killed the EMS/AHPND strain of *Vibrio parahaemolyticus*bacteria (Imaizumi et al., 2018). Delivering ozone via NBs is more efficient (and maybe safer), because it may require less gas to achieve the same disinfection effect and because less gas is lost to the air environment. Ozone will be converted to oxygen; therefore, it increases oxygen levels while disinfecting water (Nano Bubble Technologies, 2020). However, at certain levels ozone is toxic to fish so it is recommended that the ORP associated with ozone usage does not exceed 320 mV to avoid ozonation damage (Li et al. (2014). In this study, we evaluated whether air, oxygen and ozone NBs could reduce the concentration of *V.parahaemolyticus*(EMS/AHPND strain) and improve water quality under laboratory conditions.

2 | MATERIALS AND METHODS

2.1 | Laboratory set-up

Experiments were carried out in a bio-wet laboratory at the Centre for Environment and Disease Monitoring in Aquaculture, Research Institute for Aquaculture No1, North of Hanoi, Vietnam. Trials were conducted in 140 L cylindrical composite tanks with 100 L of 15‰ saline water, which is within the optimal range for *V.parahaemolyticus*(WHO 2011). Saline water was prepared from freshwater by adding Blue Treasures sea salt (Qingdao Sea-Salt Aquarium Technology, China). A NB generator model aQua+75MO (AquaPro Solutions, Singapore) with a 1 HP power (0.75 kW) with a water flow output rate of 1,000 L/h was used. The machine produces NBs with an average size of 168.9 ± 73.8 nm and a concentration of 1.04 x10^9± 2.6 × 10^8particles/ml (AquaPro Solutions, 2019). To create oxygen NBs, we attached an oxygen concentrator (model Yuwell 7F-10, Yuwell, China) set with a flow rate of 4 L/min flow rate to the nanobubbler (Figure 1). To create ozone NBs, the oxygen concentrator was attached to an ozone generator (model OM-Z10, OzoneMaxx, Vietnam) with an output flow rate of 15 L/min to produce 10 grams of ozone per hour to the nanobubbler (Figure 1). During the air and oxygen NB generator operation (1 hour), water temperature was found to increase at a rate of 2.5 °C/h, so in order to maintain the same temperature in all treatment tanks and control tanks, six 1-L ice bottles were added into the treatment tanks periodically.

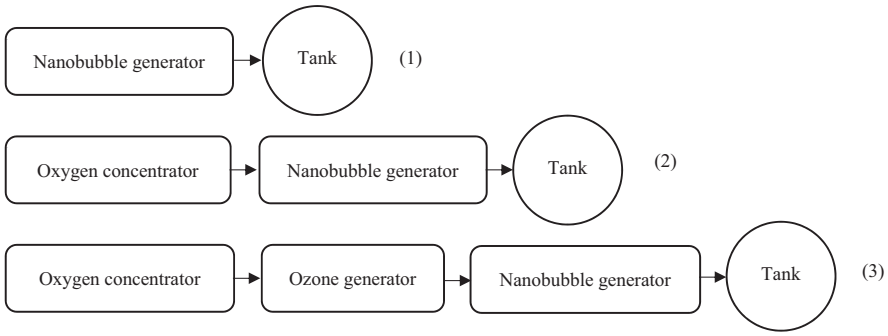

FIGURE 1 Nanobubble generator installation diagram for different gases: (1) for air nanobubble, (2) for oxygen nanobubble (3) for ozone nanobubble.

2.2 | Oxygen reduction potential pre-trials

In an operating water system, the measurement of ozone by titration or by kit is costly, requires many operational steps and is therefore difficult to use for continuous monitoring in a short time. The oxidation–reduction potential (ORP) is used, because it is easy to measure with handheld or automatic measuring devices, which can monitor fluctuations over short intervals. ORP is an index of the oxidation capacity of water at the measuring point. In water, oxidizers can be oxygen, chlorine, peroxide, ozone or any other oxidizing chemicals (Dramm Water, 2020). In clear waters, ozone content is directly proportional to the ORP values (Cefas, 2010). To gain a better understanding of how NBs increase the oxidation–reduction potential within our experimental set-up, we recorded ORP changes over one-minute intervals with a handheld ORP meter (Pro1020, YSI, USA). ORP values may serve as a simple, rapid and real-time proxy indicator for ozone concentration in water. ORP values may also serve as a simple, rapid and real-time proxy indicator for ozone concentration in water (Seki et al., 2017; Suslow, 2004) and is commonly used by aquaculture operators instead of ozone measurements through titration due to the complexity, cost and time required to process samples. Since contaminants in water (from salt, bacterial or Nutrient Broth media used in experiments) interfere with ORP readings, we conducted OPR reading on the NB generator in freshwater to eliminate biased ORP readings caused by 'dirty' water (Tantra et al., 2012). The pre-trials were set up with the three NB gases (air, oxygen, ozone), replicated three times and ran for 15 minutes each. Other pre-trials were carried out to test the NB dosages of different gases.

2.3 | Experimental design

Two separate experiments were designed to assess the capacity of air, oxygen and ozone NBs to reduce the concentration of *V.parahaemolyticus*from tank water. Experiment 1 consisted of three treatments each replicated three times, that is air and oxygen NB treatment and untreated control. Since three additional tanks were made available, they were used to set up three additional oxygen NB tanks; these additional tanks were set up three days after the first nine tanks, but the results are included in the analysis. All tanks were supplied with an aquarium air-stone operated continuously throughout the trials, while the air and oxygen NB tanks were treated with NBs for 60 minutes once per day for 7 days. Experiment 2 consisted of three treatment groups of 2, 4 and 6 min of ozone NB treatment per day and a negative control with no NBs. Each treatment group was also replicated three times, and all tanks were continuously aerated through an aquarium air-stone. Each tank was initially inoculated with *V.parahaemolyticus*targeted at a concentration 106 CFU/ml. Every 48 hours, 50 ml of Nutrient Broth culture medium was added to each tank to stimulate bacteria growth. Bacterial counts and water quality parameters including temperature, pH, oxygen, alkalinity, ORP were measured at 0, 6, 24, 48, 72, 96, 120, 144 and 168 hours from the start of the experiment. All tanks were stirred before sampling.

2.4 | *Vibrio parahaemolyticus*preparation and counts

The EMS/AHPND strain of *V.parahaemolyticus*was collected from the bacteria storage bank in Centre for Environment and Disease Monitoring in Aquaculture, Research Institute for Aquaculture No1 and was prepared as follows: (a) *V.parahaemolyticus*bacteria were thawed from ultra-low temperature freezer −80°C and defrosted at room temperature; (b) the bacteria were cultured in selective medium (Thiosulfate Citrate Bile Sucrose Agar-TCBS) in an incubator at 29°C. After 18–24 hours, single colonies were put into sterilized Erlenmeyer flask with Nutrient Broth added with 2% NaCl medium and shaken at 29°C for 18 hours; (c) the bacterial concentration was optically estimated ad OD600 nm with an Eppendorf Bio Spectrometer (Eppendorf, Germany) and checked again using dilution and quantification method on agar plate. Initial bacteria introduced to experiment tank were fixed at the same concentration 10^6colony-forming units per ml of water (CFU/ml); (d) during the experiments, 50 ml Nutrient Broth added with 2% NaCl medium solution was added into experimental tanks at 48 hour intervals to feed the bacteria.

Bacteria concentration in water samples collected from experimental tanks was quantified following Buller (2004). Collected water samples from experimental tanks were diluted 10-fold with 2% sodium chloride solution. A volume of 100 µl of dilution solution was inoculated on a TCBS agar plate and a glass page to evenly attach bacteria on the agar surface. Cultured agar plates were incubated in the incubator at 29.0 °C. After 24 hours, all colonies were counted, and the bacterial concentration was calculated using the following formula:

$$X = \frac{A}{V} xK$$

where: X is bacterial concentration in 1 ml sample (CFU/ml), A is the number of colonies growing on agar plates, V is the volume of water introduced into the culture (for example, if 100 µL is added, V = 0,1), $K$is the dilution factor (for example, at a concentration of 10–1, K = 10).

2.5 | Water quality parameters assessment

Water quality in each experimental tank was measured daily after each treatment. Temperature, oxygen, pH and ORP were measured by a Pro1020 Dissolved Oxygen and pH or ORP Instrument (YSI, USA). Alkalinity was analysed following the SMEWW 2302B: 2011 method (Baird & Bridgewater, 2017).

2.6 | Statistical analysis

Bacteria counts as $Log_{10}(x + 1)$ and the physical and chemical water quality parameters were compared among treatments and over time and the interaction between the two factors analysed using linear regression. For bacteria counts, time 0 was excluded from the analysis. Model assumptions were assessed using residual plots. Changes in the physicochemical parameters occur because of treatment, and these changes could be the cause of changes in bacteria counts. Since these parameters are not independent of one-another, a PCA was performed on temperature, pH, ORP, DO and alkalinity and the first two principal components scores were saved as variables which were then used as predictors of bacteria counts after adjusting for treatment and time. P-values <0.05 were considered significant. Analyses were carried out using XLSTAT statistical and data analysis solution (Addinsoft, 2020).

3 | RESULTS AND DISCUSSION

3.1 | Pre-trials

The results of the oxidation–reduction potential (ORP) pre-trials showed that in the ozone NB tanks with initial ORP value of approximately 240 mV the ORP rose to 351 ± 78 mV, 644 ± 71 mV 830 ± 70 mV after two, four and six minutes respectively and remained stable at over 900 mV once the machine was operating for 10 minutes or more. When we ran oxygen NBs, the ORP gradually increased to maximum 355 ± 15 mV after 15 minutes of operation. In the air NB tanks, ORP also increased gradually for 15 minutes reaching a maximum of 315 mV (Figure 2). The results of this pre-trial were used as reference for our experimental design with bacteria. Results showed that air NB cannot kill bacteria, but it can improve water quality, while oxygen and ozone NB can kill bacteria. Oxygen NB treatment, however, requires longer treatment time than ozone NB treatment. The experiment was designed to last for 7 days with the assumption that this would be long enough to see the change of bacteria concentration and realistic to apply to disease shrimp farms in the future.

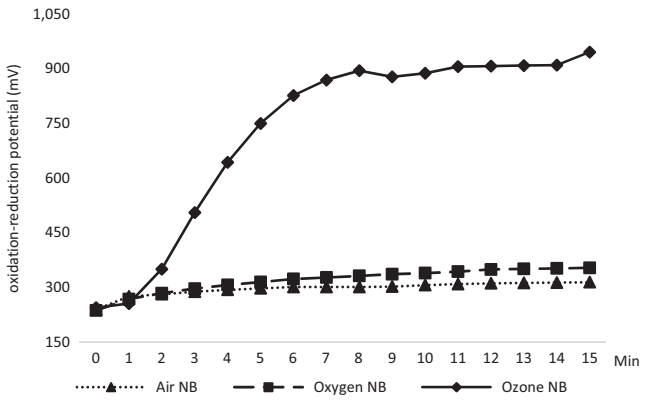

FIGURE 2 Mean value of oxidation–reduction potential (ORP) of air, oxygen and ozone nanobubble treated tanks. Values are means of three replicate tanks per sampling time in each group.

3.2 | Effects nanobubbles to bacterial count

In all three treatments of the experiment 1, there was some bacterial growth during the first 48–72 hours followed by a marked decline thereafter (Figure 3). Bacteria counts in the oxygen NBs treatment were generally lower than those in the control and air NBs treatments, and the decline in counts in the oxygen NBs treatment started earlier than in the other two treatments. Counts in the air NBs were similar to those in the control during the first 24 hours of the experiment, but after this time period counts were lower in air NBs treatment than in the control (Figure 3). Differences among the three treatments were most marked from 72 hours to 120 hours (Figure 3) while thereafter differences diminished. At the end of the trial, the original bacterial concentration are follows: $1.6x10^3±8.7x10^2$, $1.1x10^3±9.9x10^2$, and $7.3x10^2±6.2x102$ CFU/ml respectively for control, air NBs, and oxygen NBs groups (Table 1). Thus, at the end of experiment 1, the bacterial concentration of the air and oxygen NBs groups was counted for 69% and 46% of the control group respectively. The interaction between treatment and time, however, was not significant. The main effect of time was significant ($p < 0.001$) and so was the main effect of treatment ($p < 0.001$). The average difference between the NBs treatments and the control on a log-scale was −0.3198 ($p < 0.01$) and −0.7235 ($p < 0.001$) and on the original scale, counts in the air NB treatment were 47.9% of that in the control and the similar value for the oxygen NBs was 18.9%. The difference between the two NBs treatment was significant ($p < 0.001$).

In the experiment 2, bacteria density increased in the control tanks up to 24 h then started to decline. At 48 h, density was still higher than that originally inoculated and from 96 h until the end of the study the density continued to reduce to $1.0x10^2±5.4x101$ CFU/ml at the end of the experiment (Figure 4). Treatment with ozone for 2 min did not prevent bacteria growth, but density was lower than in control tanks and bacteria density started to decline from 48 h after start until 144 h, but at the end of the trial (168 h) there was a slight resurgence in density ($2.3x10^1±2.7x101$ CFU/ml) (Table 2). For the 4 min ozone treatment, there was a slight growth after 6 h, where afterwards density declined to 0 CFU/ml after 144 h. Also, in this group, there was a slight resurgence at the end of the trial ($2.2x10^0±1.9x100$ CFU/ml) (Table 2). Treatment 3 (6 min) resulted in marked decline in density 1 hour after treatment, but after a small resurgence at 6 h, density declined rapidly to 0 CFU/ml at 96 hours and there were no bacteria recorded thereafter (Table 2). Differences among the 4 groups at 1 h were not statistically significant, but the interaction between treatment and time was significant ($p < 0.001$). At the end of the trial, the original bacterial counts are as follows: $1.0x10^2±5.4x10^1$, $2.3x10^1±2.7x10^1$, $2.2x10^0±1.9x10^0$and 0 CFU/ml respectively for control, 2-, 4- and 6-minute ozone NBs groups (Table 2). Thus, the bacterial concentration of the 2-, 4- and

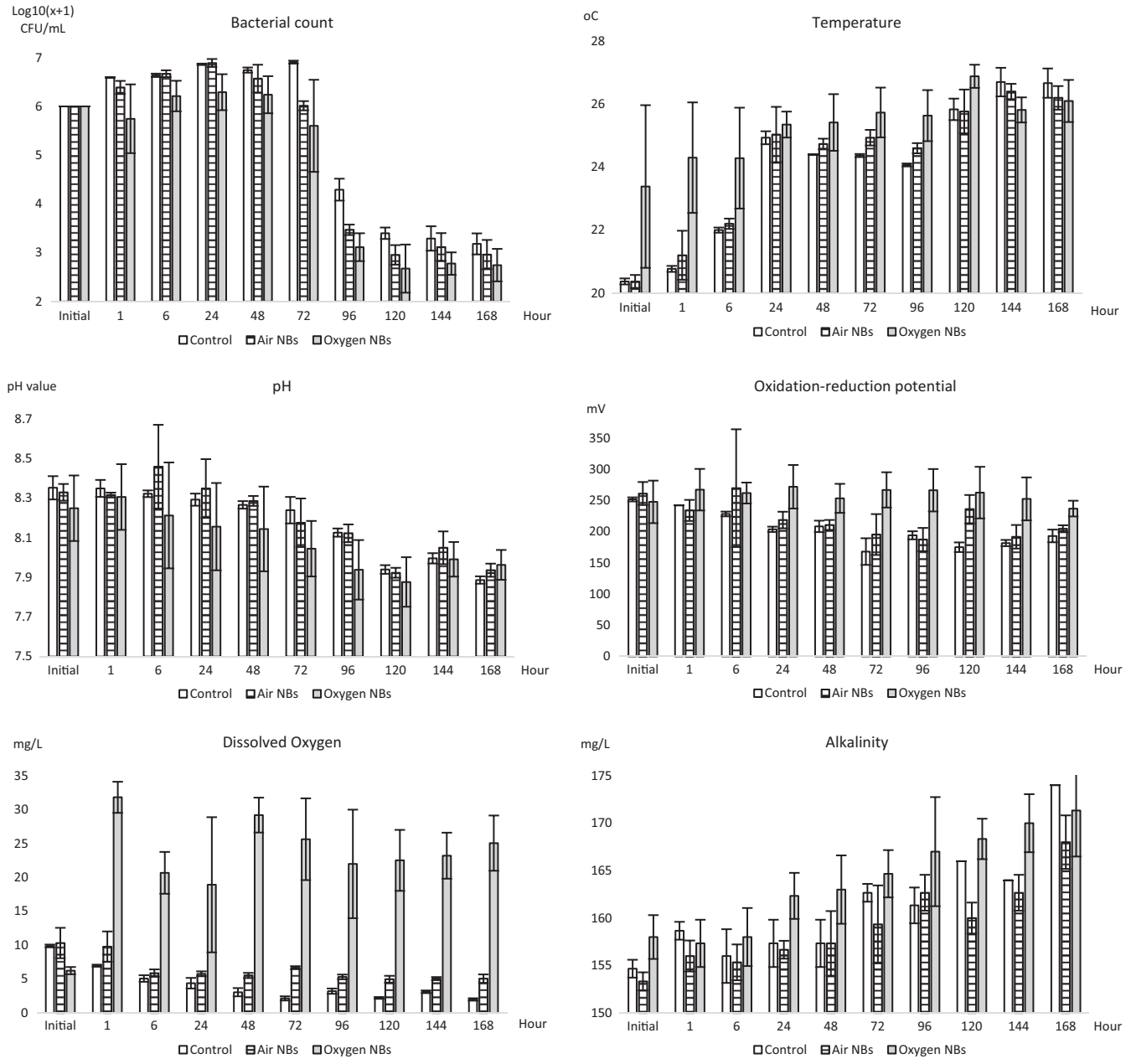

FIGURE 3 Changes of bacteria count, temperature, pH, ORP, dissolved oxygen, alkalinity in the three treatments over time.

6-minute ozone NBs groups were counted for 23%, 2.2% and 0% of the control group respectively. Our results indicated that one ozone NB treatment a day, even as short as 2 minutes per 100L, may reduce the bacterial density sufficiently to reduce the risk of infection. Treatments of 4 or 6 minutes a day were even more effective. Within 4 days of adding ozone NBs to 100 L tanks, which increased ORP value to 425.8 ± 187 mV, we could no longer detect *V. parahaemolyticus*.

Except for the effects of different NB treatments, and additional nutrient broth media, *V. parahaemolyicus*counts in all tanks followed the five bacterial growth phases (lag, exponential, stationary, death and long-term stationary phases) (Givskov et al., 1994; Jenkins et al., 1990; Navarro Llorens et al., 2010; Pin & Baranyi, 2008). It was noticeable that the decrease in bacterial concentration in the control group started a day later than the air and oxygen NB treatment groups. NB treatment groups had lower overall bacteria count compared with the control group in both experiments. Ozone NB treatment showed a much stronger bactericidal effect than air and oxygen NBs relative to the controls. Adding an ozone nanobubbles for 6 minutes in 100 L of water for 2 days appeared to reduce bacterial counts from 106 CFU/ml to below 103 CFU/ml. The latter is the threshold *Vibrio*sp. concentration in shrimp aquaculture considered problematic for disease outbreaks (Anand Ganesh et al., 2010). Interestingly, the application of air NBs also had a decline in the *V. parahaemolyticus*concentration; however, the reduction in bacteria may not be sufficient to reach the desired threshold for reducing

TABLE 1 Mean values of bacterial count, temperature and chemical factors at start (0 h) and end (168 h) of the control, air and oxygen nanobubble treatment groups

Parameters	Time	Control (n = 3)	Air NB (n = 3)	Oxygen NB (n = 6)
Bacterial count (CFU/ml)	Start	$1.0 \times 10^6 \pm 0.0$	$1.0 \times 10^6 \pm 0.0$	$1.0 \times 10^6 \pm 0.0$
	End	$1.6 \times 10^3 \pm 8.7 \times 10^2$	$1.1 \times 10^3 \pm 9.9 \times 10^2$	$7.3 \times 10^2 \pm 6.2 \times 10^2$
Temperature (°C)	Start	20.4 (20.3–20.5)	20.4 (20.1–20.6)	23.4 (20.0–26.0)
	End	26.7 (26.2–27.3)	26.2 (25.7–26.6)	26.1 (25.3–27.0)
pH	Start	8.35 (8.27–8.40)	8.33 (8.27–8.36)	8.25 (8.05–8.53)
	End	7.89 (7.86–7.90)	7.94 (7.90–7.98)	7.96 (7.82–8.03)
ORP (mV)	Start	252 (248–256)	261 (248–287)	248 (196–297)
	End	193 (183–207)	205 (197–210)	237 (226–259)
DO (mg/L)	Start	9.89 (9.60–10.10)	10.31 (7.29–12.65)	6.25 (5.23–6.95)
	End	2.00 (1.76–2.17)	5.06 (4.29–5.82)	25.06 (17.12–29.30)
Alkalinity (mg/L)	Start	155 (154–156)	153 (152–154)	158 (154–160)
	End	174 (174–174)	168 (164–170)	171 (164–176)

disease outbreaks in shrimp culture systems. The results of the present study are consistent with previous publications regarding control bacteria and micro-organisms using ozone (Feng et al., 2018; Gerba & Pepper, 2019; Summerfelt et al., 2009; Suslow, 2004) and ozone NB treatments (Cefas, 2010; Gurung et al., 2016; S. Saijai et al., 2018; Seki et al., 2017; Serizawa 2017; Tekile et al., 2017; Temesgen et al., 2017; Tsuge, 2014). ORP or ozone values of the diluted water of treatments were not mentioned in Imaizumi et al. (2018); however, diluting water with an ORP of 980 mV would be similar to the ORP that we measured when we ran our nanobubbler for 2 minutes. It is possible that NB treatments employed here somehow interfered with the mechanisms of bacteria reproduction by disrupting their cell walls through oxidization from free radicals (ORP) and inhibiting their division process (Kanunnikova et al. 2017). Circulating shrimp pond water and raising ORP to a suitable level maybe therefore have an effective disinfecting effect in the field; however, trials under commercial culture conditions are warranted. These results suggest that oxygen and ozone NBs may contribute to lower levels of *V.parahaemolyticus*in brackish waters. For safety, these findings need to be applied to a setting with shrimp to determine whether the animals can tolerate these levels of ozone over time.

3.3 | Effects of nanobubbles to water physicochemical parameters

Several water quality parameters changed when we used NBs in brackish water. The changes observed were sometimes related to the type of gas used and should be taken into consideration when considering using NBs in shrimp culture. In the experiment 1, since the chemical factors are correlated, changes in water quality would be best illustrated by analysis of all factors jointly using principal component scores on all treatments and time points combined (n = 120). The first PC explained 49.69% of the variation and the second PC 29.93% and in total 79.62%. Temperature and alkalinity loaded positively on PC1 while pH loaded negatively. Dissolved oxygen and ORP both loaded positively on PC2 (Figure 5). At start of the trial, there was considerable overlap among the three groups; there was, however, a pronounced difference within the oxygen NBs treatment, that is the last three tanks had considerable higher scores for PC1, and this was mainly due to the higher temperatures at start in these tanks. After 24 hours, there was overlap between the control and air NBs on PC1, while oxygen NBs all had higher scores than the other two treatments for PC1, while along PC2 the oxygen NBs treatment overlapped with both the control and the air NBs treatment. There was no overlap between the control and the air NBs treatment along PC2. After 72 hours, the oxygen NBs treatment tanks were separated from both other treatments both on PC1 and PC2, while air NBs and the control overlapped on both PC1 and pc2. After 168 hours, there was overlap between the three treatments on PC1, while there was no overlap on PC2. PC1 increased over time ($p < 0.001$) in all three groups and the interaction term was not significant. The main effect of treatment was significant; while air NBs did not differ from the control, the oxygen NBs treatment had higher values than the control (b = 1.6194, $p < 0.001$). The difference between the two NBs treatment was also significant ($p < 0.001$). For PC2, there was a significant interaction between treatment and time ($p < 0.001$), but differences at start were not different. PC2 decreased somewhat over time in all three groups, but the oxygen NBs groups had higher scores than the other two treatments. Both PC1 and PC2 are significant predictors of bacteria counts, that is increase in both is negatively associated with a decrease in bacteria count when adjusting for treatment and time, but then the oxygen NBs treatment was not significant (due to collinearity).

In the experiment 2, the first component explained 75.69% of the variation and the second component 13.86%, that is total 89.55%. Most factors loaded similarly on component 1 possibly because the main factor affected by the treatments was the dissolved oxygen. At start, the was overlap between groups, both along PC1 and PC2 (Figure 6), while after 24 hours, the control was

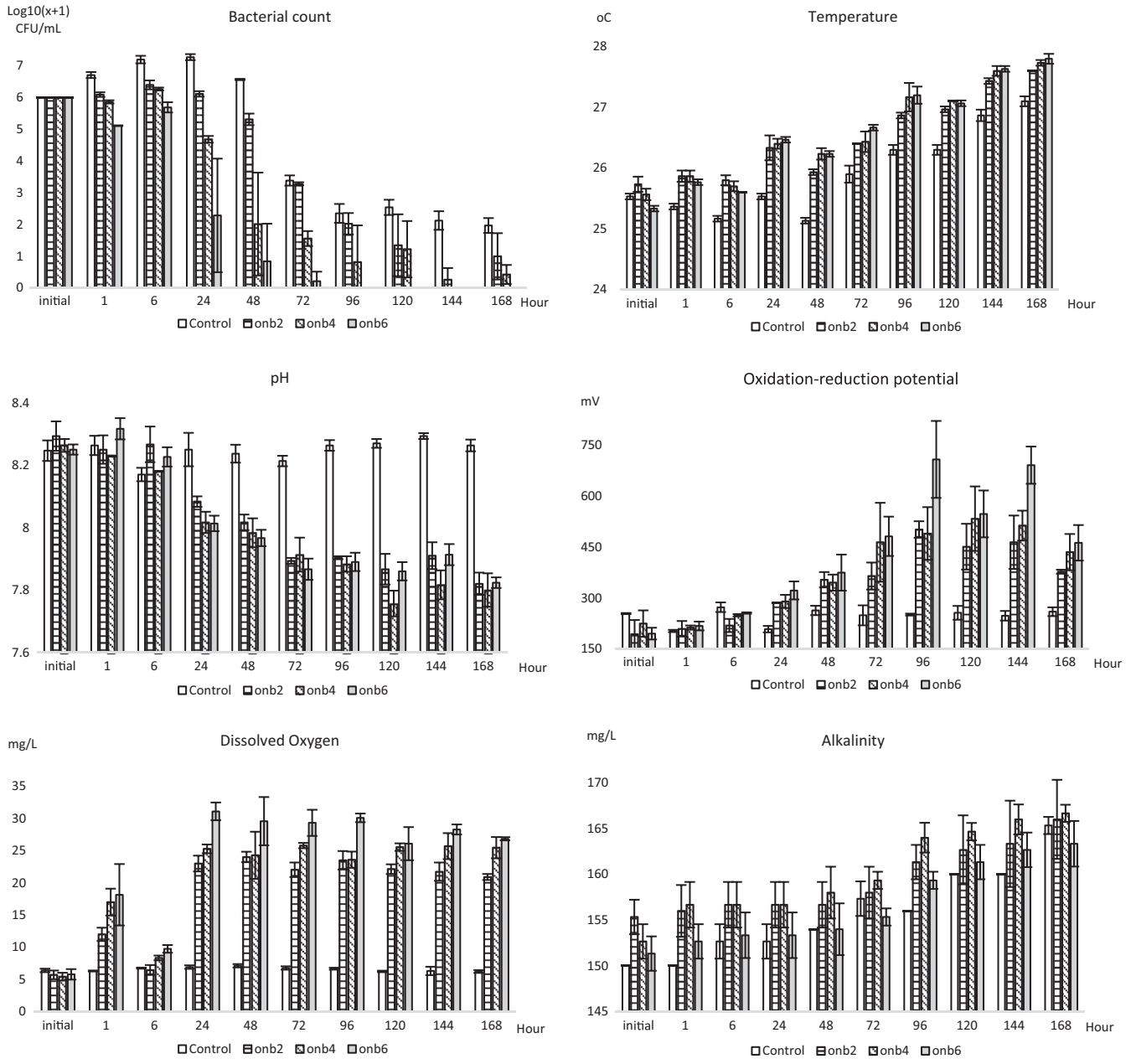

FIGURE 4 Changes of bacterial counts, temperature, pH, dissolved oxygen, ORP, alkalinity in the daily ozone nanobubble treatments of 2, 4 and 6 minutes (onb2, onb4, onb6 respectively) and control groups.

very different along PC1 from the three ozone treated groups. The three ozone treated groups overlapped to some extent with the control along PC2 after 24 hours. After 72 hours, there was complete separation between the control and the three ozone treated groups combined, both along PC1 and PC2. Furthermore, the 6-minute ozone treatment had no overlap with the 2- and 4-minute treatment groups along PC2. At the end of the experiment, the pattern was basically maintained although there is more overlap. PC1 increased over time in all treatments, but to a lesser extent in the control group than in the three ozone treatments. There was a significant interaction between time and treatment ($p < 0.001$). PC2 increased in the control group over time, and after 48 h, it also increased in the 3 NBs treatments.

Temperature in the experiment 1 increased in all treatments over time ($p < 0.001$) (Figure 3). After 24 hours, the differences between the three treatments were not significant. From 24 hours until the end of the trial, temperature variation over time differed among treatments, that is the interaction term was significant ($p < 0.01$). The temperature increase was caused by changes in room temperature, and somewhat due to the operation of the nanobubbler. Temperature in the experiment 2 increased in all groups towards the end of the experiment (Figure 4) but the temperature in the control group was lower than in the three treatment groups. At time 0, temperature was slightly lower in treatment 3 than in the other 3 groups ($p < 0.001$) and the interaction between treatment and time was significant ($p < 0.001$).

TABLE 2 Mean values of bacterial count, temperature and chemical factors at start (0 h) and end (168 h) of control and ozone nanobubble treatment groups

Parameters	Time	Control (n = 3)	Treat 2 (n = 3)	Treat 3 (n = 3)	Treat 4 (n = 3)
Bacterial count (CFU/ml)	Start	$1.0 \times 10^6 \pm 0.0$	$1.0 \times 10^6 \pm 0.0$	$1.0 \times 10^6 \pm 0.0$	$1.0 \times 10^6 \pm 0.0$
	End	$1.0 \times 10^2 \pm 5.4 \times 10^1$	$2.3 \times 10^1 \pm 2.7 \times 10^1$	$2.2 \times 10^0 \pm 1.9 \times 10^0$	0
Temperature (°C)	Start	25.5 (25.5–25.6)	25.7 (25.6–25.9)	25.6 (25.5–25.7)	25.3 (25.3 –25.4)
	End	27.1 (27.0–27.2)	27.6 (27.6–27.6)	27.7 (27.7–27.8)	27.8 (27.7–27.9)
pH	Start	8.25 (8.20–8.27)	8.29 (8.26–8.36)	8.26 (8.24–8.29)	8.25 (8.23–8.27)
	End	8.26 (8.25–8.29)	7.82 (7.79–7.87)	7.80 (7.73–7.86)	7.82 (7.80–7.84)
ORP (mV)	Start	254 (252–256)	194 (150–250)	225 (170–255)	196 (181–220)
	End	260 (242–272)	378 (373–386)	435 (362–487)	463 (400–528)
DO (mg/L)	Start	6.39 (6.06–6.72)	5.70 (4.80–6.43)	5.43 (4.91–6.28)	5.79 (4.63–6.42)
	End	6.23 (5.93–6.40)	20.92 (20.36 –21.50)	25.48 (23.30–27.30)	26.86 (26.50–27.06)
Alkalinity (mg/L)	Start	150 (150–150)	155 (154–158)	153 (150–154)	151 (150–154)
	End	165 (164–166)	166 (162–172)	167 (166–168)	163 (160–166)

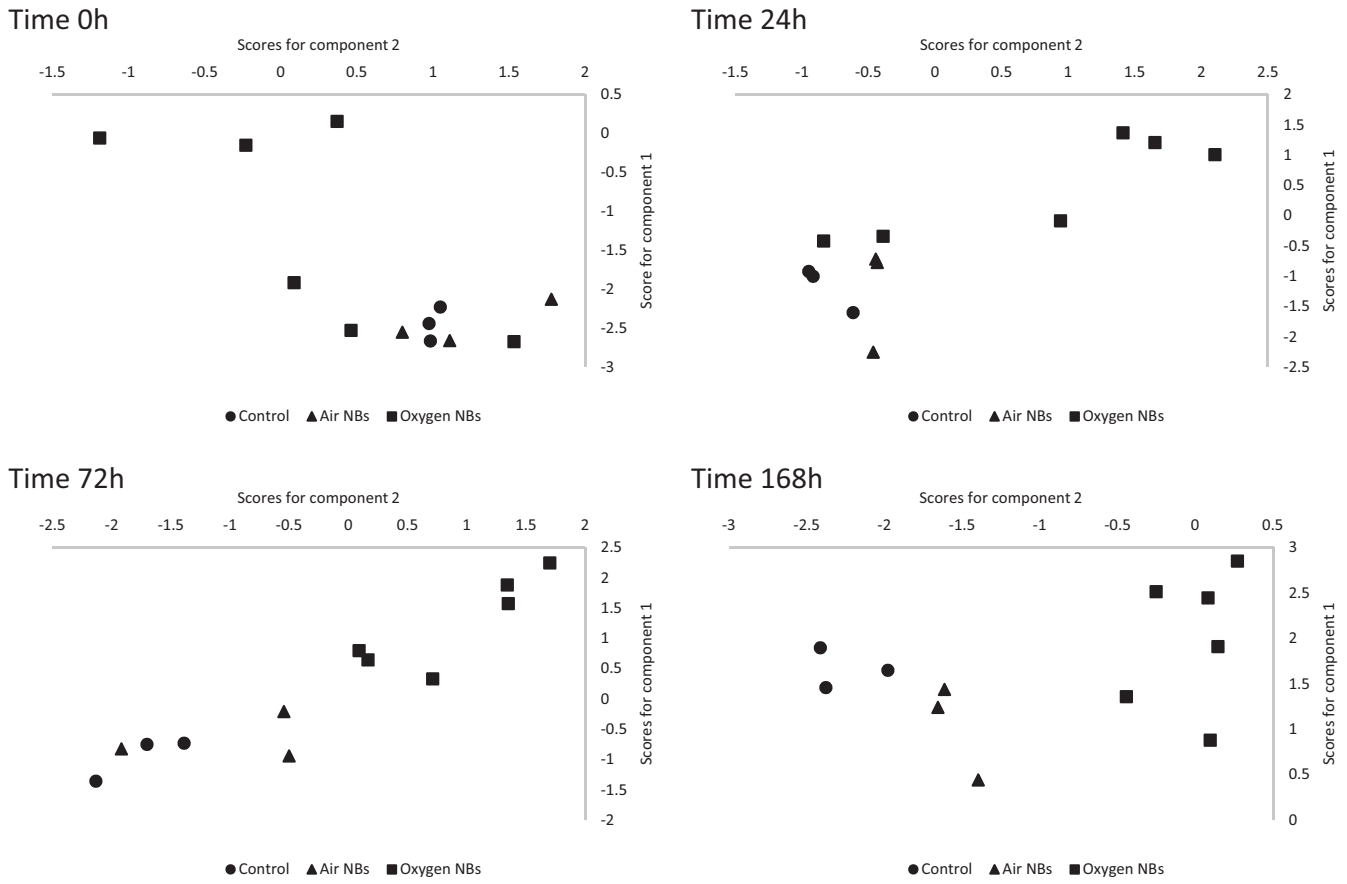

FIGURE 5 PC1 and PC2 scores of the control, air nanobubbles and oxygen nanobubbles treatments at selected time points.

Regardless of the gas used to create the NBs, the water temperature may increase significantly over time, which could be detrimental to the shrimp if the bubbler is run for an extended period of time in a small tank or body of water. Temperature may affect bubble diameter and concentration. Higher temperature decease zeta potential and increase bubble size (Meegoda et al., 2018; Thompson et al., 2017). The larger the bubble size, the shorter their life in the water column (Smirnov & Berry, 2015). Also, higher temperature cause bubbles to collapse (Serizawa 2017) and thus lower NB concentration. This result suggests that temperature should be considered when applying NB technology to shrimp farming. For the most effective treatment, we should generate NBs at as low a water temperature as possible. We observed an increase in water temperature with the use of the nanobubbler, especially for long-time machine running such as air or

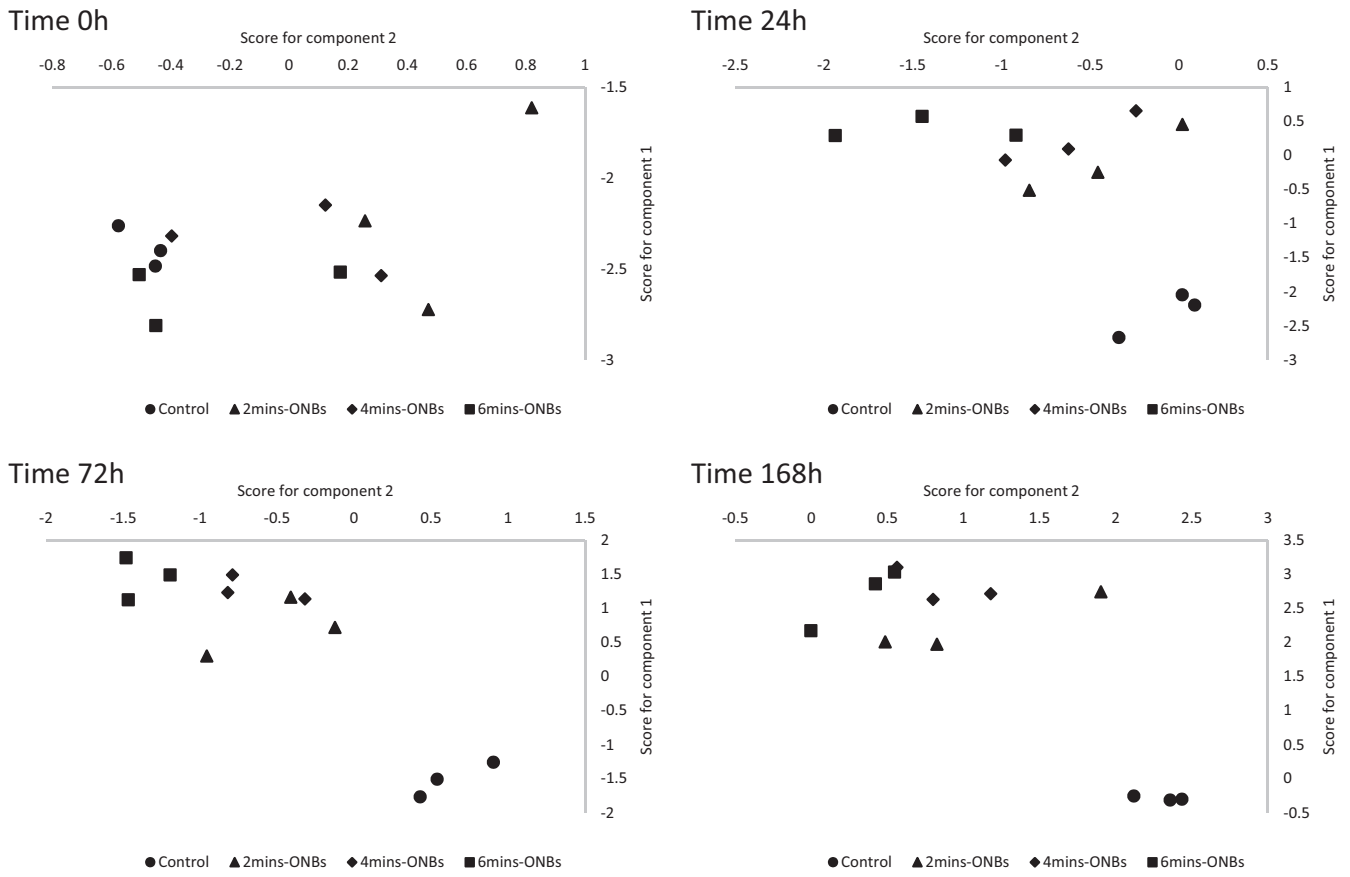

FIGURE 6 PC1 and PC2 scores of the control and ozone nanobubbles treatments of 2, 4 and 6 minutes at selected time points.

oxygen NBs. It may be an important factor if the nanobubbler is used for an extensive period of time in small culture volumes if there are animals in the system, as a sudden change in water temperature can be problematic for survival.

Dissolved oxygen(DO) in the experiment 1 decreased gradually from about 10 mg/L at the beginning to about 2 mg/L for control group and 5 mg/L for the air NB group at the end in the experiment 1 (Figure 3). Dissolved oxygen did not differ among treatments at start, and the level in the control group declined up to 72 h, where after it remained relatively stable. The interaction term between treatment and time was significant ($p < 0.001$). DO level in the oxygen NB group increased from about 6 mg/L to more than 31 mg/L after the first treatment with NBs and throughout the remaining time DO was considerably higher ($p < 0.001$) in the oxygen NBs treatment than in the other two treatments. Differences between the control and air NBs were less marked but still significant, that is on average from 1 h to the end of the experiment the DO in the air NBs treatment was 2.43 mg/L higher ($p < 0.001$) than in the control. DO in the experiment 2 of the control group remained relatively stable throughout the experiment (Figure 4). After the first treatment, DO level increased in all ozone NB treatment groups (i.e. at 1 hour) and declined again after 6 hours, but after the second ozone treatment, DO in all three treatment groups increased and remained at that level until the end of the experiment (Figure 4). Differences among the 4 groups at start were not significant, but the interaction term between time and treatment was significant ($p < 0.001$). Comparing only the ozone-treated groups showed that they followed the same pattern over time (interaction not significant). The main effect of treatment showed that both the 6-minute and the 4-minute groups had higher DO level than the 2-minute treatment, that is b = 5.3563 ($p < 0.001$) and b = 2.4980 ($p < 0.001$) respectively.

Changes of DO significantly happened over time, and oxygen NB treatment can elevate and maintain dissolved oxygen at a high level as others also have reported (Ebina et al., 2013; Wang et al., 2018). Oxygen NB change the electromagnetic field and this leads to a slight decrease in the concentration of metal dissolved ions and the treatment also made oxygen saturation over 2 to 3.5 times higher in comparison with the saturation threshold of its content in natural water (Kanunnikova et al. 2017). Similar results for DO levels were spiked and maintained at a high level (Wang et al., 2018). Maintaining high DO levels is very important in shrimp farming, not only to provide oxygen for the shrimp, but also to permit denitrifying bacteria to convert ammonia and nitrite (Hargreaves, 1998). Hyperoxia by oxygen NB may contribute to elevated metabolism, higher food intake and promoted growth of the farmed shrimp (Ebina et al., 2013). We were able to increase the oxygen levels in the tanks even when we ran the ozone nanobubbler for only 2 minutes per day. In water, ozone will readily degrade back to oxygen, and during this transition a free oxygen atom, or free radical form. Adding ozone NBs may be an efficient way to reduce bacteria and increase oxygen in shrimp

tanks. The NBs may maintain the gas in the water column for a longer period of time, so it is more readily available for use. DO reached high level after short treatment time of ozone NB. It is known that if DO level exceed 20 mg/L the saturation rate is more than 300%. In aquaculture practice, supersaturation of dissolved oxygen is potentially harmful to fish and shrimp (Boyd & Fast, 1992). This condition can result in gas bubble disease (gas bubble trauma), which has been described in a wide variety of fish and invertebrates. Weitkamp and Katz (1980) reported that bubbles were observed in the gills of dying fish as well as between fin rays and under scales when dissolved oxygen levels are over 300% saturation. Numerous marine fish died when dissolved oxygen concentrations were above 250% of saturation in Galveston Bay (Weitkamp & Katz, 1980). However, other study indicated that supersaturation of a single gas such as oxygen may not produce gas bubble trauma (Colt, 1986). To our best knowledge, effect of different supersaturated levels of dissolved oxygen on shrimp is not well understood and needs to be clarify especially if ozone and oxygen NBs are used to reduce bacteria in the water systems.

pH levels were at about 8.3 for the control and the two treatment groups at the beginning of the experiment, and pH gradually decreased to less than 8 at the end in the experiment 2 (Figure 3). At the beginning of the trial, differences among treatments were not significant and the interaction term between treatment and time was not significant. The air NBs did not differ significantly from the control while the oxygen NBs had pH values that were on average 0.089 units lower than those in the controls ($p < 0.01$) and it was also lower than that of the air NBs treatment by 0.11 units ($p < 0.01$). pH level remained rather stable over time in the control group while pH in the ozone treated group decreased towards the end in the experiment 2 (Figure 4). Differences among the four treatments at the start of the experiment were not statistically significant, but there was a significant interaction between treatment and time ($p < 0.001$), and this is mainly due to difference in changes over time between the control group and three treatment groups. Comparing only the three treatment group showed that the three group followed a similar pattern, that is the interaction term was not significant. In the latter model, significant differences were indicated among the three groups, that is treatment 2 (4 min) had slightly lower pH than the other two treatments.

We believe that NB and live bacteria maintained a pH higher than 8. The reason pH value decreased to below 8 in both experiments is likely because bacteria died in the systems (Ratzke & Gore, 2018). Meegoda et al. (2018) suggested that NBs added to water with a higher pH value produces smaller bubble diameter, and higher zeta potential. It is indicated that higher pH value in the solution produces smaller bubble diameter and higher zeta potential. In the case of ozone NBs, the higher the pH, the more bubbles are required to achieve the same ORP (Suslow, 2004).

ORP levelsin the experiment 1 at start were about 250 mV for all groups. ORP at the beginning of the trial did not differ among treatments (Figure 3), and the interaction between treatment and time was not significant. The initial ORP level was maintained to the end of the experiment for the oxygen NB group, while it declined in the other two treatments. The mean level of ORP over time in the oxygen NBs treatment was 60.57 mV higher than in the control ($p < 0.001$), while the air NBs treatment did not differ significantly from the control group (b = 17.11). Differences between the two NBs treatment were also significant ($p < 0.01$). In the experiment 2, ORP level ranged from 200 to 250 mV initially for all groups. This level remained stable in the control group throughout the experiment, while in the three ozone NB treatment group ORP was higher than that in the control from 24 h after the experiment started (Figure 4). Differences between the 4 groups were not statistically significant at start, but the interaction term between treatment and time was significant ($p < 0.001$). When comparing only the three ozone NB treatment groups, the interaction between treatment and time was not significant, but the main effect of treatment showed that the 6-minute treatment had higher ORP than the 2-minute treatment ($p < 0.001$) and the 4-minute treatment ($p < 0.04$).

ORP reading is influenced by organic material in the water (Suslow, 2004). As such, it may explain why the ORP decreased as the bacterial count decreased in experiment 1 in the groups that did not have oxygen added to them. Nanobubbles can increase ORP, and when ORP value reaches about 350 mV, it starts killing bacteria, so the result can be explained with *V.parahaemolyticus*as a facultatively anaerobic bacteria (Anses, 2012). That means *V.parahaemolyticus*can live under aerobic conditions and adapt to quite high ORP levels. This is also consistent with the study of Robles et al. (2013) in which *V.parahaemolyticus*was isolated from water covering the entire observed ORP range (73.8–301.5 mV). Suslow (2004) mentioned the higher the pH, the higher treatment dose is needed to achieve the same ORP, so longer NB generator running times are needed. Each species of bacteria fits an optimal range of ORP values, the greater this value, the more inhibition of bacterial growth is observed (Kimbrough et al., 2006). YSI (2008) and Cefas (2010) recommend the best ORP range for shrimp pond is 150–250 mV because at this level, the bacterium does not grow too fast and this is a favourable condition for biochemical reactions such as nitrification, BOD degradation with free molecular oxygen and biological removal of phosphorus. In experiment 2, there were higher ORP levels in treated groups due to the strong oxidization effects of ozone. The pre-trials showed that ORP values in fresh (tap) water increased to higher levels when compared to ORP values in experiment (containing bacteria and nutrient broth in brackish water) if treated at the same NB dose. It suggests that ozone NBs can increase ORP in contaminated water but at lower level compare to clean fresh water, even so, it retains its bactericidal properties (Nghi et al., 2018). The ORP electrode is, however, affected by particulate and organic matter present in the water. Any substance on the electrode—whether organic or inorganic, visible or invisible, alive or not—can lead to misrepresentative readings. The measurements will reflect the ORP of the contaminant rather than the tank water. For this reason, it is important to keep the sensor clean and its surface smooth (OTT HYDROMET, 2015), and it is thought that in our study the lower ORP values in experiment water than in clean fresh water were because of organics and

suspended particles present in the tanks. (Cefas, 2010); Nghi et al. (2018) reported that ORP is positively correlated with ozone content in water. Using Cefas (2010) data, mean value of ozone concentration in the treatment tanks was calculated, and it falls in the range from 0.2 to 0.6 mg/L. However, further studies are needed to identify the optimum and safe ORP levels when ozone NBs are used for shrimp farmed under commercial culture conditions.

Alkalinity in the experiment 1 started at about 155 mg/ml at the beginning of the experiment for all groups. These values gradually increased to about 170 mg/L at the end of the experiment (Figure 3). Differences in alkalinity among treatments at start were not significant. Alkalinity over time was higher in the oxygen NBs treatment than in the control (b = 2.7407, $p < 0.01$) while alkalinity in the air NBs was slightly lower than the control (b = −2.1481, $p < 0.05$). Alkalinity in the experiment 3 increased for all groups, from about 150–155 mg/L at the beginning of the experiment to about 165 mg/L at the end of the experiment (Figure 4). The 4 groups did not differ significantly at start of the trial, and the interaction between treatment and time was not significant. The final model without the interaction showed that the three ozone treated groups differed from the control group, that is b = 3.4667 ($p < 0.001$), b = 4.3333 ($p < 0.001$) and b = 0.8667 (p was not significant) for the 2-, 4- and 6-minute treatment respectively.

Alkalinity had an increasing trend in both control and treatment groups the two experiments. Elevation of alkalinity may be caused by increase in temperature which shifts the equation $HCO3^{-} \Leftrightarrow CO3^{2-} + H^{+}$to the right, slightly increasing the carbonate to bicarbonate ratio. This means that a warmer tanks water can have better buffering capacity (alkalinity) but lower pH (AWC, 2020). Nanobubbles are more stable in the neutral or higher alkaline environment than in acidic environment. Hence, DO, ORP, zeta potential value would be more stable with NB treatment in alkaline environment (Meegoda et al., 2018). This is an advantage of NBs treatment application for shrimp farming because brackish water is usually a high alkaline environment. It is suggested that that alkalinity was independent of the ozone NB treatment. However, alkalinity can affect the NBs because higher alkalinity level results in a lower decay ozone rate (Gardoni et al., 2012). In the other word, higher alkalinity likely maintains ozone NBs concentration in water, which is also supported by Meegoda et al. (2018). The strong correlation between temperature and alkalinity in the control and all treatment groups supports the idea that alkalinity is temperature dependent (Scholze et al. 1992).

4 | CONCLUSION

The study evaluated the ability of NB gases to reduce the level of *V.parahaemolyticus*(AHPND strain) in an experimental setting. With an initial bacterial concentration of 106 CFU/ml, after one-week period, bacteria counts in the air and oxygen NB treatments were 69% and 46% of that in the control group. Bacteria counts in the 2, 4 and 6 minutes of ozone NBs treatments experiment were 23%, 2.2% and 0% of that in the control group. The use of NB treatments demonstrated the reduction in bacterial growth, especially ozone NB treatments. In addition, oxygen and ozone NBs significantly increased dissolved oxygen levels to more than 20 mg/L, which is beneficial for aquatic animals if this technology is used in aquaculture. From the present results, we propose to further investigate the safety of using oxygen NB and short exposure times with ozone NBs on live shrimp to determine whether it can be used to control disease outbreaks. We also caution that water quality parameters such as temperature and pH should be closely monitored when NBs are applied in systems with relatively small volumes.

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AUTHOR CONTRIBUTIONS

We declare that all listed authors meet the following criteria: Substantial contributions to conception and design of, or acquisition of data or analysis and interpretation of data. Drafting the article or revising it critically for important intellectual content. Final approval of the version to be published. We declare that nobody who qualifies for authorship has been excluded from the list of authors.

ETHICS STATEMENT

The research is met the ethical requirement in research which certified by The Scientific Committee of the Research Institute for Aquaculture No1.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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