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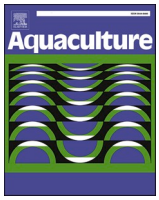

Ozone nanobubble treatment in freshwater effectively reduced pathogenic fish bacteria and is safe for Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

High concentrations of certain pathogenic bacteria in water usually results in outbreaks of bacterial diseases in farmed fish. Here, we explore the potential application of an emerging nanobubble technology in freshwater aquaculture, specifically aimed to reduce the concentrations of pathogenic fish bacteria in freshwater, and assess whether nanobubbles are safe for Nile tilapia (*Oreochromis niloticus*). An ozone nanobubble (NB-O_3) treatment protocol was established, based on examination of nanobubble size, concentration, disinfection property, and impact on fish health. A 10-min treatment with NB-O_3 in 50 L water generated approximately 2–3 $\times$ 10^7 bubbles/mL, with the majority of bubbles being less than 130 nm in diameter and an ozone level of 834 $\pm$ 22 mV oxidation-reduction potential (ORP). A single treatment with water spiked with either *Streptococcus agalactiae* or *Aeromonas veronii* effectively reduced the bacterial load by 26–48 fold or 96.11–97.92%. This same protocol was repeated three times. The result was a 22,058 to 109,978 fold reduction in bacteria or 99.93–99.99% decrease. In comparison, bacterial concentrations in the control tanks remained unchanged during the experiments. In Nile tilapia-cultured water with the presence of organic matter (e.g. mucus, feces, bacterial flora, feed, etc.), the disinfection property of NB-O_3 was reduced; however, we still observe a reduction of 59.63%, 87.25%, and 99.29% after the first, second, and third consecutive treatments, respectively. To evaluate the safety of NB-O_3 on fish, juvenile Nile tilapia were exposed to NB-O_3 treatment for 10 min. No mortality was observed during the treatment or 48 h post treatment. Gill histology examination revealed that a single NB-O_3 treatment caused no alteration in cell morphology. However, damage in the gill filaments, such as blood congestion, aggregates of basal cells at the secondary lamellae or loss of the secondary lamella was noticed in the fish receiving two or three consecutive exposures within the same day. Results of the experiments conducted in this study suggest that NB-O_3 technology is promising for reducing pathogenic bacteria in aquaculture systems and may be useful at reducing the risk of bacterial disease outbreaks in farmed fish.

1. Introduction

The aquaculture sector, especially in Asia, plays a vital role in global food security. It supplies protein to approximately 4.5 billion people and employs 19.3 million people worldwide (Béné et al., 2015; FAO, 2018). Similar to other food sectors, aquaculture has faced increasing challenges with infectious diseases. Control of these diseases has led to an increase in the use of antimicrobials (World Bank, 2014; Watts et al., 2017). Of particular importance to public health has been the increase in antimicrobial resistance (AMR). Alternatives to products to control

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bacterial infections in all food production sectors have increased over the last few years (Watts et al., 2017; Reverter et al., 2020). Previous and current approaches focus mainly on antibacterial compounds derived from natural products, probiotics, immunostimulants, and vaccines for prevention strategies (Watts et al., 2017; Reverter et al., 2020).

Other prevention strategies, usually used in closed recirculating systems to reduce the bacterial concentration in the aquatic environment, include water treatment with ultraviolet (UV) or ozone. Both of these treatments have logistical and economic issues for aquaculture industries. UV requires that water be very clean when it is exposed to the light source, which renders it less than ideal in pond culture systems commonly found in Asia. Ozone has a low dissolution property, rapid decomposition in water, and can be lethal to fish (Huyben et al., 2018; Xia and Hu, 2019). More effective non-chemical water treatment technology is needed to improve water quality for aquaculture systems such as intensive pond culture systems.

Nanobubble technology is an emerging technology for wastewater treatment (Yamasaki et al., 2005; Agarwal et al., 2011) and recently has been applied in aquaculture for increasing concentrations of dissolved oxygen in intensive aquaculture systems (Agarwal et al., 2011; Mahasri et al., 2018; Anzai et al., 2019; Rahmawati et al., 2020). This technology involves the injection of nano or ultrafine bubbles (<200 nm) with a chosen gas into water (Agarwal et al., 2011; Anzai et al., 2019). Unlike macro- and microbubbles, these nanobubbles have neutral buoyancy, and thus remain in water for days (Takahashi et al., 2007; Agarwal et al., 2011). The technology is highly efficient at dissolving gasses into the water column due to the bubbles' large surface area to volume ratio (Gurunga et al., 2016). The latter property may improve the efficiency of delivering oxygen or ozone to aquaculture systems.

Disinfection property of nanobubbles created from ozone (NB-O_3) on aquatic animal pathogens in marine water has been recently explored. Kurita et al. (2017) reported that a 25 min treatment with NB-O_3 successfully reduced 63% of the parasitic planktonic crustaceans compared to the untreated group. More importantly, this treatment condition was safe for both sea cucumbers (*Apostichopus japonicas*) and sea urchins (*Strongylocentrotus intermedius*), which are commonly infected with these pathogenic crustaceans in Japanese aquaculture systems. In another study, Imaizumi et al. (2018) reported that NB-O_3 could be used for disinfection of *Vibrio parahaemolyticus*, a unique strain causing early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in whiteleg shrimp (*Penaeus vannamei*). However, in their study, NB-O_3 showed a negative effect on shrimp when administered at a high level (970 mV ORP). When the NB-O_3 treated water was diluted by 50%, it reduced the toxic effect of ozone on the shrimp and appeared to improve the survival of the shrimp exposed to *V. parahaemolyticus,* compared to the positive control group without the NB-O_3 treatment, which caused 100% mortality (Imaizumi et al., 2018). These findings suggest that application of NB-O_3 at the appropriate concentrations may be useful at controlling infectious diseases in marine aquaculture.

There is limited scientific evidence of the benefits of NB-O_3 in freshwater aquaculture in terms of its disinfection effectiveness, its impact to water quality, and possible toxicity to fish. These knowledge gaps highlight the lack of and need for further understanding on potential applications of NB-O_3 in freshwater aquaculture. This study, therefore, evaluated the effect of NB-O_3 technology on concentrations of pathogenic bacteria in freshwater, water parameters, and the acute impact of NB-O_3 on fish under laboratory conditions.

2. Materials and methods

2.1. Laboratory set up of NB-O_3 system

All experiments in this study were carried out using 50 L dechlorinated tap water in 100 L fiberglass tanks, 68 cm (L) × 49 cm (W) × 30 cm (D). The laboratory set up of NB-O_3 system in this study is shown in Fig. 1. The system is comprised of a nanobubble generator (model: aQua+075MO; maker: AquaPro Solutions Pte Ltd., Singapore), an oxygen concentrator (model: JAY-10; Longfian Scitech Co. Ltd., Hebei, China), and an ozone generator (model: CCba15D; Coco Technology Co. Ltd., Chonburi, Thailand). The system was connected to a tank containing 50 L water. Oxygen concentrated from the air was fed into ozone generator at a flow of 1 L/min. The generated ozone was then diffused with water inside the nanobubble generator to form NB-O_3 and returned to the tank. The system is flexible as it can generate different kinds of nanobubbles based on gas input, such as oxygen nanobubbles (NB-O_2) or air nanobubbles (NB-Air). To generate NB-O_2, the ozone generator was turned off to allow the feed of oxygen directly to the nanobubble generator. When the oxygen concentrator and ozone generator were disconnected with the nanobubble generator, only NB-Air was produced. Water parameters were measured using a multi-parameter water quality meter (YSI Professional Plus, YSI Incorporated, USA).

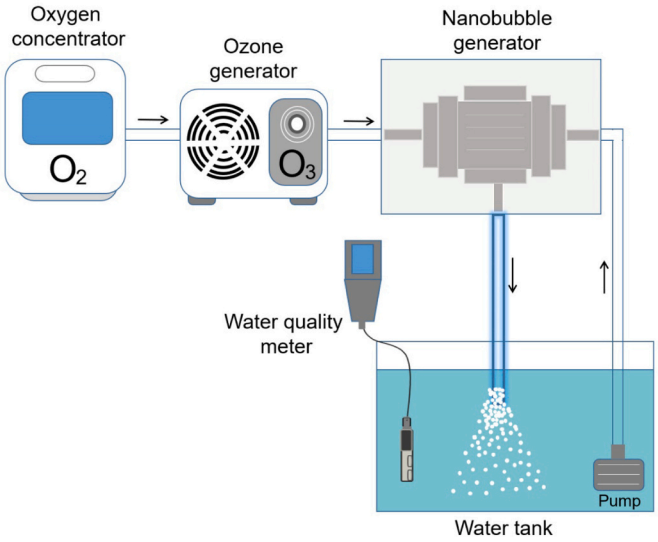

Fig. 1. Laboratory set up of NB-O_3 system in this study. Oxygen from the oxygen concentrator was fed into an ozone generator with a flow rate of 1 L/min. The generated ozone was then diffused with water inside the nanobubble generator and returned to the tank. Water parameters were measured using a multiprobe water quality meter.

2.2. Determining nanobubble concentration and size

Two trials were carried out separately using the nanobubble system described above to determine the sizes of the air and oxygen nanobubbles. The generator was operated in 100 L-fiberglass tanks containing 50 L distilled water for 30 min, with either natural air or oxygen gas at a flow rate of 1 L/min. 50 mL of water was sampled from each tank at 10, 15, 20, and 30 min. Water samples collected prior to the addition of nanobubbles were used as baseline standards. The concentration and size of nanobubbles were determined in triplicates (300 μL/sample) using a NanoSight NS300 (Malvern Panalytical Ltd). Ozone nanobubble measurement was not done due to its oxidation effect on the NanoSight machine.

2.3. Effect of NB-O_3 treatment on water parameters

The experiment was performed in two separate tanks to evaluate the effect of NB-O_3 on water parameters. Each tank contained 50 L of dechlorinated tap water, and the nanobubble generator was operated for 10 min in each tank. Temperature in degree Celsius (T°), dissolved oxygen (DO), pH, and oxidation reduction potential (ORP) were measured using a multi-parameter water quality meter (YSI Professional Plus, YSI Incorporated, USA) before the treatment, every 1–2 min during the 10 min run and 15 min after stopping the nanobubble generator.

2.4. Bacterial isolates and growth conditions

The Gram-positive bacterium *Streptococcus agalactiae* strain 2809, isolated from a tilapia farm which was experiencing an outbreak of streptococcosis in 2018 (laboratory strain, Centex Shrimp, Mahidol University), and Gram-negative bacterium *Aeromonas veronii* strain NT-03 associated with hemorrhagic septicemia in tilapia (Dong et al., 2017) were used in this study. Prior to the experiments, bacterial isolates were propagated from bacterial stocks stored at −80 °C, using tryptic soy agar (TSA) medium (Difco, Becton Dickinson, Sparks, USA), incubated at 30 °C. To prepare bacterial inoculum, single bacterial colonies were inoculated in 10 mL of tryptic soy broth (TSB) (Difco, Becton Dickinson, Sparks, USA) overnight at 30 °C on a shaker platform (150 rpm). Five mL of bacterial culture was then sub-cultured in 500 mL of TSB, incubated with gentle shaking (150 rpm) at 30 °C until OD_{600} reached 0.8 (equivalent to $\sim 10^8$ CFU/mL). For subsequent trials, 100 mL of the bacterial culture was added into a tank containing 50 L of de-chlorinated tap water.

2.5. Pilot study on effect of treatment time on disinfection property of NB-O_3

An initial trial was carried out to investigate the effect of treatment time on the disinfection property of NB-O_3. *Streptococcus agalactiae* was used as a representative bacterium in this time-course trial. The experiment was performed in two 100 L fiberglass tanks containing 50 L of de-chlorinated tap water, each mixed with 100 mL bacterial culture (OD_{600} = 0.8). One tank was treated with NB-O_3 while another tank served as a control without NB-O_3. Water was sampled from the four corners and the center of the tank (1 mL per spot). The samples were pooled together for conventional plate count enumeration at different time points. Samples were collected prior to inoculation (0 min), during treatment (5, 10, and 15 min), and after treatment (5, 10, and 15 min). The samples were 10-fold serially diluted with sterile saline solution (NaCl 0.85%), and 100 μL of each dilution was plated on TSA in duplicates and incubated at 30 °C for 36 h. Dilutions with a number of colonies ranging from 30 to 300 were used for enumeration (only whitish pinpoint colonies of *S. agalactiae*) and mean bacterial colonies of two replicate plates were calculated and expressed as CFU/mL. The percentage of bacterial reduction was calculated based on the formula below.

$$\%\text{reduction} = \left(\frac{\text{Mean bacterial } \frac{\text{CFU}}{\text{mL}} \text{ before treatment} - \text{Mean bacterial } \frac{\text{CFU}}{\text{mL}} \text{ after treatment}}{\text{Mean bacterial } \frac{\text{CFU}}{\text{mL}} \text{ before treatment}} \right) \times 100$$

The reduction in bacterial concentrations in the tank exposed to NB-O_3 and the control tank were compared.

2.6. Effect of NB-O_3 on pathogenic Gram-positive and Gram-negative bacteria

To evaluate the effect of NB-O_3 on bacterial pathogens of tilapia, *S. agalactiae* and *A. veronii* were used as representative Gram-positive and Gram-negative bacteria, respectively. Each set of experiments was comprised of one control tank (having normal aeration) and three treatment tanks (10 min treatment with NB-O_3 one to three times at 15 min intervals). Note that the treatment time (10 min) was chosen based on the result of pilot study. Each tank containing 50 L de-chlorinated tap water was mixed with 100 mL of bacterial suspension (OD_{600} = 0.8), as described above. Water was sampled from control and treatment tanks before (0 min) and 15 min after the end of each treatment to determine the bacterial concentration and the percentage of bacterial reduction by plate count method. Colony counting was based on morphological characteristics of the bacteria. For example, *S. agalactiae* grows slower and forms whitish pinpoint colonies on agar plates after 36–48 h incubation. In contrast, *A. veronii* grows faster and forms cream-colored larger colonies on TSA after 18–24 h incubation. Water temperature, pH, DO, and ORP were also recorded during the experiment.

To investigate the ultrastructure of bacteria before and after treatment with NB-O_3, two experimental tanks were set up in the same manner as the treatment tanks: one tank contained *S. agalactiae* and the other contained *A. veronii*. Each tank was treated with NB-O_3 for 10 min. Water (200 mL) was collected before and 15 min after the NB-O_3 treatment, then pelleted by centrifugation at 6000 *g* for 10 min. The pellet was then resuspended in 0.5 mL of phosphate-buffered saline (PBS) solution. The bacterial suspension was smeared on coverslips coated with Poly-L-lysine (Sigma-Aldrich, Missouri, USA) and air-dried for 3 h. The samples were subsequently fixed with glutaraldehyde 2.5% and 1% osmium tetroxide before dehydration with ethanol, as described by Thanomsub et al. (2002). The ultrastructure of the bacteria was examined and photographed under a scanning electron microscope (SEM) (HITACHI SU8000, Tokyo, Japan) operated at 10 kV.

2.7. Effect of NB-O_3 treatment on total bacteria in culture water

Investigation of the disinfection property of NB-O_3 was also evaluated using "culture" water (water from the fish-culture tanks which contained organic matter e.g. fish feces, mucus, left over feed, and unknown aquatic bacterial flora). Fish-culture water was taken from tanks containing juvenile Nile tilapia (*O. niloticus*) that were cultured for five days without water exchange. A trial, using three of 10 min NB-O_3 exposures delivered 15 min apart, was conducted in three fiberglass tanks with 50 L of fish-cultured water. Water sampling for total bacterial counts was conducted before and 15 min after the end of each treatment. Water temperature, pH, DO, and ORP were also monitored.

2.8. Effect of NB-O_3 on fish health and gill morphology

The use of animals in this study was granted by the Thai Institutional Animal Care and Use Committee (Approval no. MUSC62–039-503). To investigate whether NB-O_3 treatment had negative effects on gill morphology and fish life, a trial was carried out which included two control and two treatment tanks, each tank containing 20 apparently healthy Nile tilapia juveniles of 6–8 g body weight. The 100 L fiberglass tanks each contained 50 L of de-chlorinated tap water. For the treatment tanks, 10 min NB-O_3 exposures were carried out three times at 15 min intervals. The control tanks were treated with normal aeration, and two fish from each tank were randomly sampled every 10 min treatment. The fish were euthanized by clove oil (200 ppm), and the gills were collected for wet-mount examination and histological study. The remaining fish were monitored for 48 h. For histological analysis, gill arches from one side of each fish were preserved in 10% neutral buffer formalin with a ratio of 1 sample/10 fixative (*v*/v) for 24 h before being placed in 70% ethanol for storage. The samples were then processed for routine histology and stained with hematoxylin and eosin (H&E). Fish behavior, and the gills of treated and untreated fish were compared visually. In this study, multiple NB-O_3 treatments were designed for

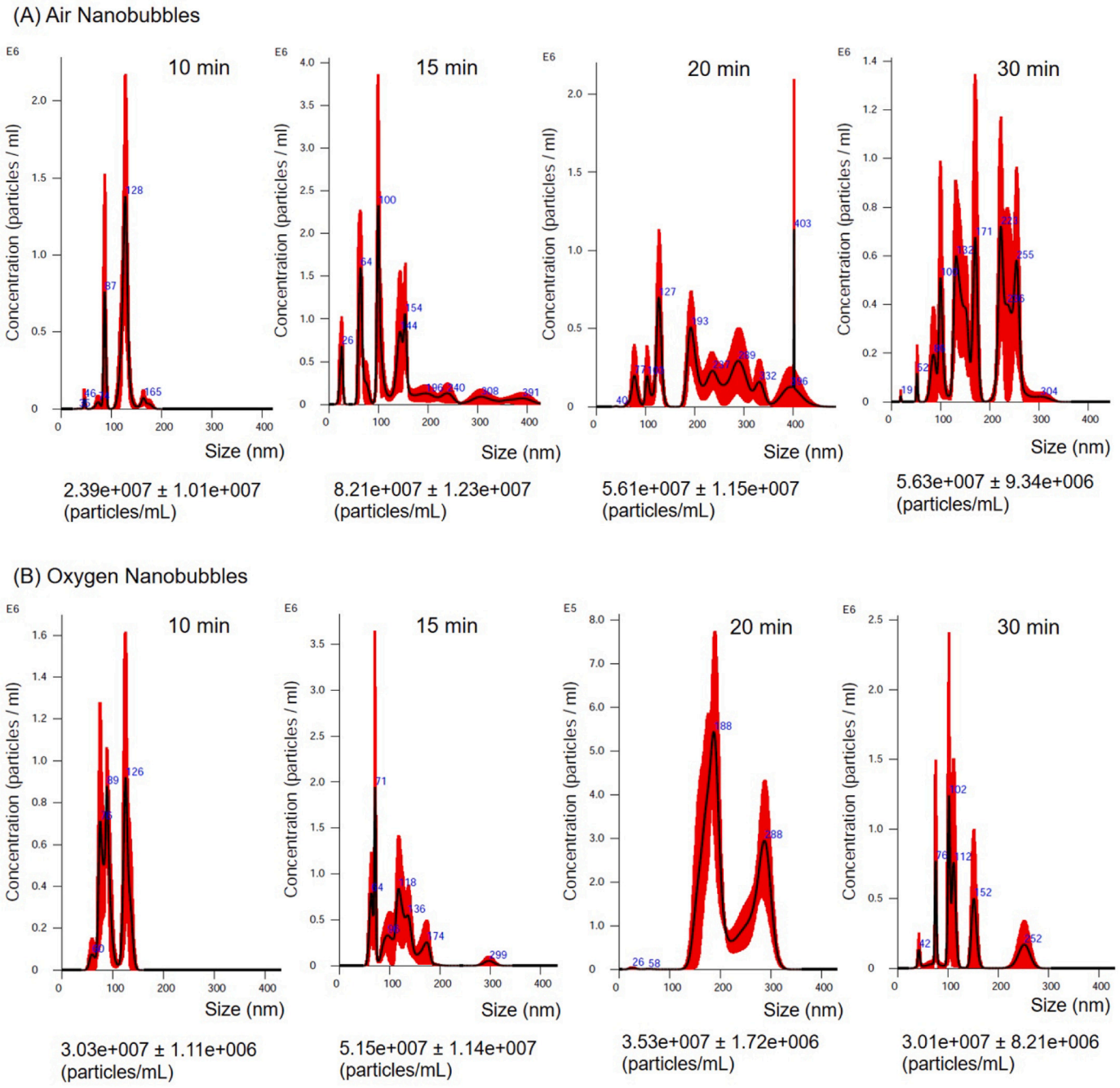

Fig. 2. Concentration and size of bubbles generated using air (A) or oxygen (B) following treatment for 10, 15, 20, and 30 min. Peaks represent the concentration of dominant bubbles with similar sizes and blue numbers indicate the bubble sizes. Total concentrations of bubbles are shown at the bottom of each graph. Values were calculated from 3 replicated experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

qualitative assessment of histological changes. We aimed to determine when the lesions start to appear. Then, introduction of NB-O_3 should be ceased before that point.

3. Results

3.1. Nanobubble concentration and size

The results of NanoSight readings from the air nanobubbles (NB-Air) (Fig. 2A) and the oxygen nanobubbles (NB-O_2) (Fig. 2B) were similar. The majority of nanobubbles (or particles) were less than 130 nn in size. The concentration of these bubbles after a 10 min treatment was of $2.39 \times 10^7 \pm 1.01 \times 10^7$ particles/mL for NB-Air and $3.03 \times 10^7 \pm 1.11 \times 10^6$ particles/mL for NB-O_2. Increasing treatment times (15, 20, and 30 min) generated larger bubbles with quantities in the same order of magnitude (Fig. 2). The results confirmed that the nanobubbler used in this study produced suitable nanobubbles, and that a 10 min operation in 50 L of water generated the most uniform nano-sizes. Thus, this scheme was also applied to generate ozone nanobubbles (NB-O_3).

3.2. Effect of NB-O_3 treatment on water parameters with no fish or bacteria

Changes of water parameters (T°, DO, pH, and ORP) during and after treatment with NB-O_3 were consistently similar between trials (Fig. 3). Significant changes were observed in DO and ORP values, while T^{o}

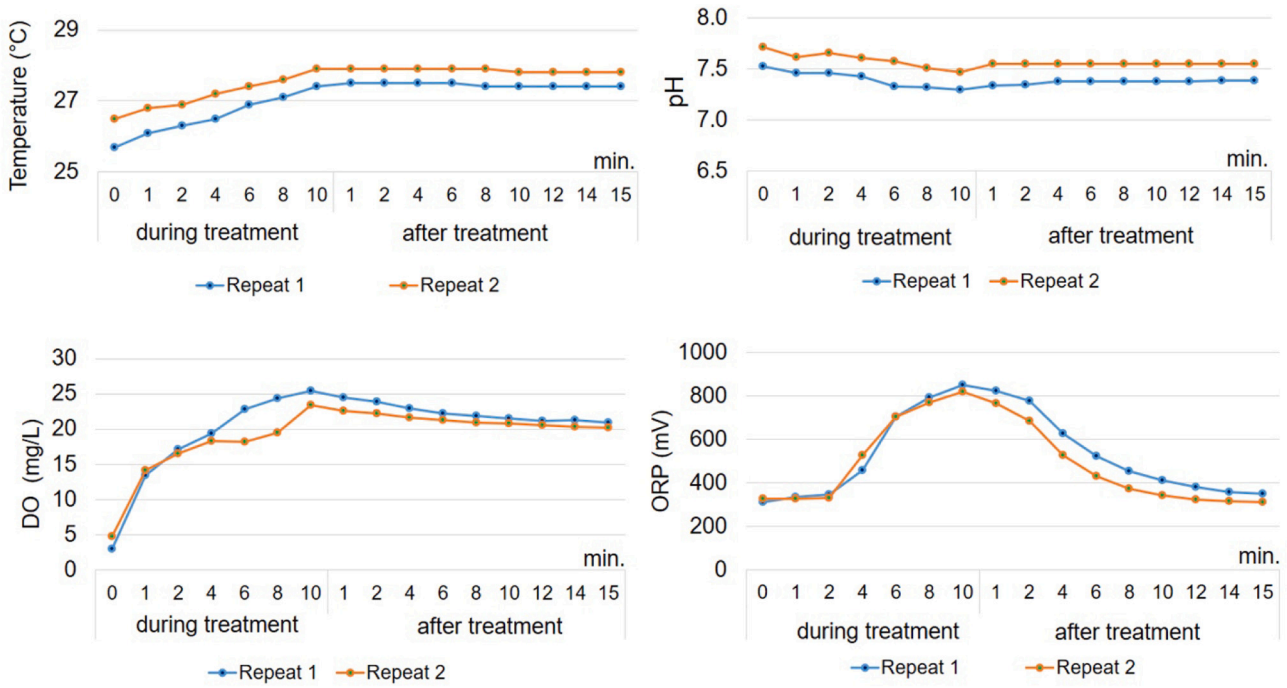

Fig. 3. Water parameters (temperature, pH, DO and ORP) during 10 min treatment and 15 min after exposure to ozone nanobubbles. The experiment was carried out in 2 replicates.

increased considerably (~2 °C) and pH remained relatively stable during and after NB-O_3 treatment. With respect to DO, the value increased rapidly, reaching 23–25 mg/L after a 10 min treatment, and reduced slowly to ~20 mg/L at 15 min post treatment. By contrast, ORP increased quickly, reaching over 700 mV within 6 min and 834 ± 22 mV within 10 min, and dropped back to the starting level (318 ± 12 mV) at 15 min post treatment.

3.3. A 10-min NB-O_3 treatment reduced > 90% bacterial loads in water

As shown in Fig. S1, similar bacterial loads (*S. agalactiae*) at the starting point were used in the control tank (1.17 × 10^6/mL) and treatment tank (1.83 × 10^6/mL). However, upon NB-O_3 treatment, bacterial density reduced rapidly during exposure. The bacterial concentration in the treated group at 5, 10, and 15-min was reduced by 62.30, 97.76 and 99.40%, respectively, indicating that disinfection occurred during the treatment process. This amounted to a 141 fold reduction in bacterial concentration in the treatment tank. In contrast, bacterial concentration in the control tank remained stable at ~10^6 CFU/mL during the same time period (Fig. S1). With respect to water quality, changes were observed only in the treatment tank. DO increased from 6.2 mg/L (before treatment) to 21.8 mg/L (at 5 min), 25.8 mg/L (at 10 min) and 27.9 mg/L (at 15 min) and dropped to 23.3 mg/L at 15 min post treatment. Water temperature increased approximately 1 °C every 5 min of the treatment, from 26.5 °C (before treatment) to 29.2 °C (at 15 min) and remained at this temperature 15 min post treatment. Relatively no change was observed in pH (7.6–7.7) and ORP (293–306 mV) during the experiment.

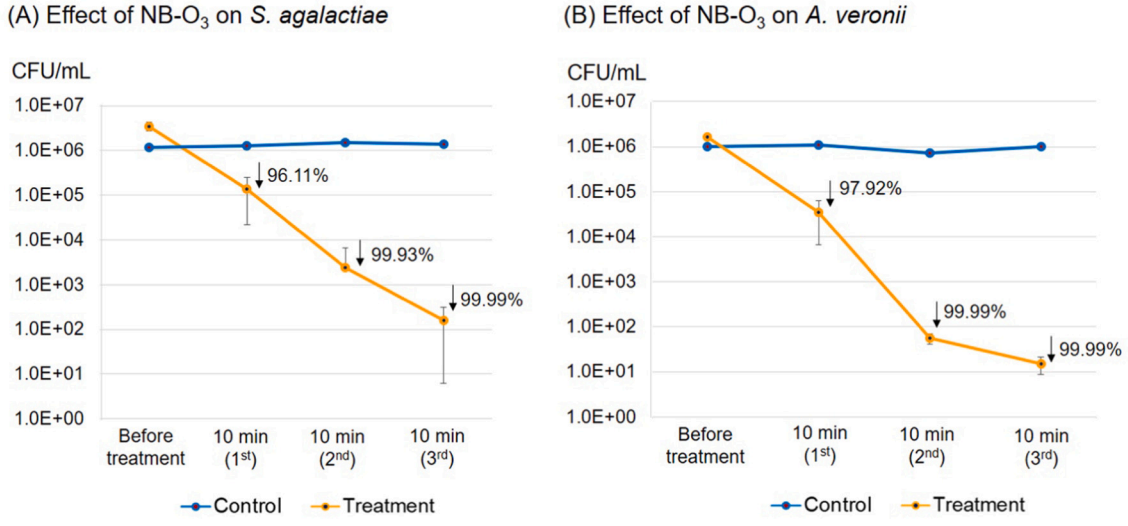

Fig. 4. Bacterial counts of *S. agalactiae* (A) and *A. veronii* (B) upon exposure to NB-O_3 for 10 min, three times (orange lines), compared to that of the control water without NB-O_3 (blue lines). Arrows indicated % reduction of bacterial loads compared to the starting bacterial concentration. Bars represent standard deviation from 3 replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. NB-O_3 treatment effectively reduced both pathogenic Gram-positive and negative bacteria

The trial with *S. agalactiae* started with similar bacterial loads: 1.17 $\times$ 10^6 CFU/mL in the control tank and 3.45 $\times$ 10^6 CFU/mL in treatment tanks (Fig. 4A). A single 10 min treatment with NB-O_3 effectively reduced 26 fold or 96.11% of the bacterial load in the tank. When the same protocol was repeated for the second and third time, bacterial concentrations were reduced 1415 and 22,058 fold or 99.93 and 99.99%, respectively. The bacterial concentration in the control tank (without the NB-O_3 treatment) maintained at ~10^6 CFU/mL (Fig. 4A). Similar patterns were also observed in the trials with the Gram-negative bacterium *A. veronii*. Average initial bacterial counts of *A. veronii* for control and treatment tanks were 1.03 $\times$ 10^6 CFU/mL and 1.65 $\times$ 10^6 CFU/mL, respectively. Following the 1st, 2nd, and 3rd NB-O_3 exposures, bacterial loads were reduced 48, 29,176, and 109,978 fold to 3.44 $\times$ 10^4 $\pm$ 2.78 $\times$ 10^4, 56 $\pm$ 15, and 15 $\pm$ 6 CFU/mL (equivalent to 97.92, 99.99 and 99.99% reduction), respectively (Fig. 4B). No significant changes in bacterial counts were observed in the control tank during the experiment (Fig. 4B).

Changes in water quality are shown in Table 1. Temperature changes in the NB-O_3 treatment tanks were 1.9–2.6 °C after the 1st treatment, and 4.3–4.7 °C after the 3rd treatment, whereas pH values were relatively stable at 7.4 to 8.0. Notably, DO increased sharply (from 3.9–4.4 to 26.4–29.9 mg/L) and was maintained at this high level after every treatment, while ORP values did not increase as much as seen in the water study without bacteria (Fig. 3).

Ultrastructural examination of the bacterial surface by SEM revealed that the majority of bacterial cells (both *S. agalactiae* and *A. veronii*) were collapsed and destroyed after treatment with NB-O_3 for 10 min compared to the normal intact surface structure of bacteria before treatment (Fig. 5).

3.5. Effect of NB-O_3 treatment on total bacterial counts in water from fish-culture tanks

In this trial, the bacterial load was compared before and after treatment. Before treatment, the total bacterial concentration in the fish-cultured water was 8.18 $\times$ 10^5 $\pm$ 6.77 $\times$ 10^5 CFU/mL (Fig. 6). After exposure to NB-O_3 for 10 min, 59.63% of the bacteria were inactivated. When the same protocol was repeated, 87.25 and 99.29% bacteria were reduced in these treatments (i.e. a 141-fold reduction from 8.18 $\times$ 10^5 $\pm$ 6.77 $\times$ 10^5 to 5.80 $\times$ 10^3 $\pm$ 5.20 $\times$ 10^3 CFU/mL) (Fig. 6).

During the experiment, DO increased sharply, from very low at the beginning 0.6 $\pm$ 0.1 mg/L to 27.7 $\pm$ 0.6 mg/L after the first 10 min treatment. The DO was 30.8 $\pm$ 7.7 mg/L after the second 10 min treatment, and 28.7 $\pm$ 7.6 mg/L after the third NB-O_3 treatment. Water temperature increased slightly from 26.7 $\pm$ 0.3 to 28.3 $\pm$ 0.4, 29.8 $\pm$ 0.3 and 31.2 $\pm$ 0.2 °C after the 1st, 2nd, and 3rd treatments, respectively. In contrast, pH and ORP were stable during the experiment (7.5–7.6 for pH, 210–250 mV for ORP).

3.6. Effect of NB-O_3 on fish health and gill morphology

No fish died during the NB-O_3 treatments or within 48 h post treatment. However, abnormal signs were observed in the gills in all fish examined after receiving the second and third treatments. The predominant signs included reddening at the base of the fins, erratic swimming, and the attachment of bubbles to the body surface. These bubbles disappeared after several minutes of fish movement.

The wet-mount examination of the fish gills revealed no observable difference between control (n = 4) and first treatment (n = 4) (Fig. 7A-B). However, mild congestion (4/4) was observed in the gill filaments when the treatment was repeated (Fig. 7C-D). There were no gross clinical signs of gas bubble disease. H&E stained sections of the gills showed the normal gill structure in the fish sampled after the first treatment (Fig. 7F) and control group (Fig. 7E). However, abnormal changes were observed in the fish exposed to the second treatment. Aggregates of basal cells at the base of the secondary lamellae (4/4) were apparent with increasing severity corresponding to the dose of ozone exposure (Fig. 7G). The gills of 3 fish out of 4 fish exposed to the third NB-O_3 treatment had mild loss of the secondary lamella (Fig. 7H) and infiltration of red blood cells (blood congestion) (Fig. 7H).

During the treatment, water parameters (T°, DO and pH) fluctuations were similar (Table 2) to the experiment with clean water spiked with *S. agalactiae* or *A. veronii* and NB-O_3 (Table 1), with the exception that tanks exposed to ozone had ORP levels of 860–885 mV after each10 min treatment.

4. Discussion

Application of ozone gas in nanobubble technology is relatively new to aquaculture. A previous study reported the sterilization efficacy of NB-O_3 against pathogenic *V. parahaemolyticus,* a Gram-negative bacterium causing disease in marine shrimp (Imaizumi et al., 2018). In this study, we first revealed that NB-O_3 treatment has a similar disinfection

Table 1
Comparative water parameters in control and NB-O_3 treatment groups with the presence of either *S. agalactiae* or *A. veronii* in the water.

Parameter	Measurement time	*S. agalactiae*		*A. veronii*	
		Control	NB-O_3 treatment	Control	NB-O_3 treatment
T^0	Before treatment	26.9	27.2 $\pm$ 0.3	27.5	25.9 $\pm$ 0.8
	10 min (1st)	26.9	29.8 $\pm$ 1.3	27.4	27.8 $\pm$ 0.6
	10 min (2nd)	27.0	30.4 $\pm$ 0.2	27.3	29.3 $\pm$ 0.6
	10 min (3rd)	27.0	31.5 $\pm$ 0.3	27.4	30.6 $\pm$ 0.5
DO (mg/L)	Before treatment	4.3	3.9 $\pm$ 0.5	4.7	4.4 $\pm$ 0.2
	10 min (1st)	4.3	27.8 $\pm$ 1.6	4.6	30.3 $\pm$ 2.4
	10 min (2nd)	4.2	26.9 $\pm$ 0.4	4.6	29.9 $\pm$ 0.1
	10 min (3rd)	4.2	26.4 $\pm$ 0.6	4.5	29.5 $\pm$ 1.0
pH	Before treatment	7.8	7.6 $\pm$ 0.2	7.8	8.0 $\pm$ 0.1
	10 min (1st)	7.8	7.5 $\pm$ 0.0	8.0	7.8 $\pm$ 0.1
	10 min (2nd)	7.8	7.4 $\pm$ 0.0	7.9	7.7 $\pm$ 0.1
	10 min (3rd)	7.8	7.4 $\pm$ 0.0	7.9	7.6 $\pm$ 0.0
ORP (mV)	Before treatment	325	290 $\pm$ 16	279	294 $\pm$ 6
	10 min (1st)	314	281 $\pm$ 7	289	271 $\pm$ 8
	10 min (2nd)	306	275 $\pm$ 4	261	270 $\pm$ 6
	10 min (3rd)	304	273 $\pm$ 3	265	272 $\pm$ 4

T°, temperature in degree Celsius; DO, dissolved oxygen; ORP, oxidation reduction potential. Values in the NB-O_3 treatment are expressed as mean $\pm$ SD from 3 replicates.

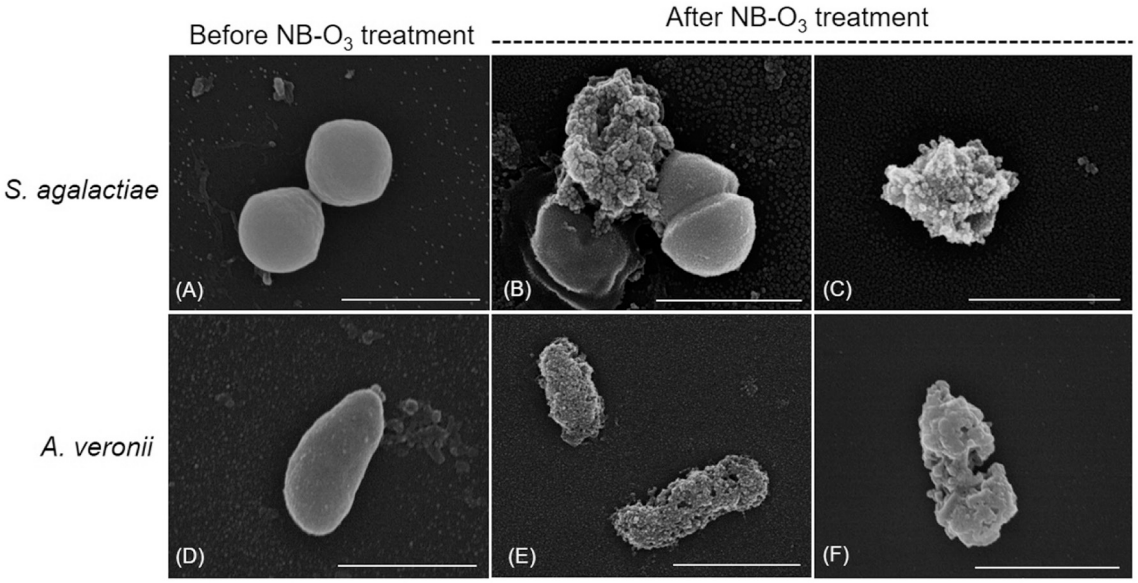

Fig. 5. Scanning electron micrographs of *S. agalactiae* (A-C) and *A. veronii* (D-F) before and after treatment with NB-O_3 for 10 min. Bacterial morphology was normal before treatment while cell destruction was observed after treatment with NB-O_3. Scale bar, 1 μm.

efficiency against both pathogenic Gram-positive (*S. agalactiae*) and Gram-negative (*A. veronii*) bacteria in freshwater, and the disinfection mechanism appears to destroy the bacterial cell wall, as revealed by SEM. Further, we discovered a short exposure to NB-O_3 (10 min, ORP reached 860 ± 42 mV) did not cause acute effect to the fish and was adequate to reduce bacteria concentration by 26 to 48 fold (>96%). Although NB-O_3 treatment did not eliminate bacteria in the water completely, 26 to 48-fold reduction of pathogenic bacteria may be useful to prevent disease outbreaks. Importantly, we determined that our 10-min treatment protocol applied in this study produced nanobubbles (< 200 nm) with the concentration of approximately 2–3 × 10^7 bubbles/mL, and the majority of bubbles were less than 130 nm in diameter. Our findings indicate that NB-O_3 technology has the potential to reduce pathogenic organisms in not only marine, but also freshwater aquaculture systems.

The disinfection effectiveness of NB-O_3 likely depends on the organic load in the water. In clean de-chlorinated tap water spiked with a known concentration of either *S. agalactiae* or *A. veronii*, a single treatment (10 min) with NB-O_3 successfully reduced more than 96% of the bacteria (killed 1.62 × 10^6 to 3.31 × 10^6 CFU/mL). However, the same protocol

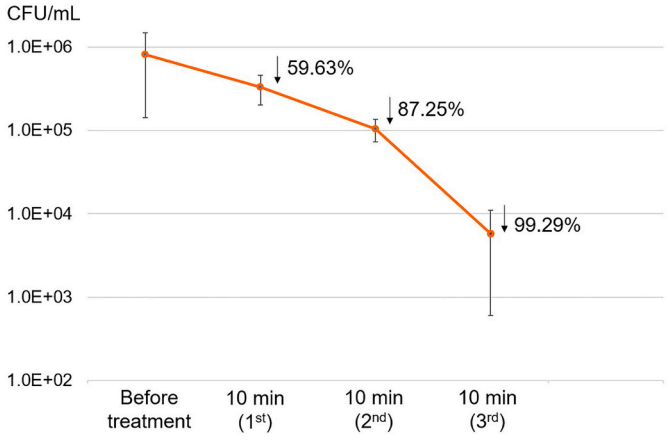

Fig. 6. Total bacterial counts from fish-cultured water upon exposure to NB-O_3. Arrows indicated % reduction of bacterial loads compared to the starting bacterial concentration. Bars represent standard deviation from 3 replicates.

applied to water that was taken from a tilapia-cultured tank, resulted in a reduction in the disinfection potential by roughly 1.6 times. Ozone is known as a strong oxidizing agent (Summerfelt, 2003; Powell and Scolding, 2016); thus, it was possible that organic matter (e.g. feces, mucus, etc.) in the dirty tank water competed for the oxidation potential of the NB-O_3, slowing down the efficacy of disinfection. This finding suggests that increased treatment time or increased frequency of treatments, as was evaluated in this study, may be required for water with abundant organic matter.

Compared to the previous study published by Imaizumi et al. (2018), the sterilization rate of NB-O_3 in this study was lower. The devices and experimental set up of two studies could account for some of the differences. In this study, the trials were conducted in freshwater and the tested bacteria were spiked directly into the tank (50 L) before treatment, while Imaizumi et al. (2018) tested NB-O_3 in marine water, and the disinfection experiments were done by incubation of the treated water and bacteria in a relatively small volume (500 mL). Another possibility is that the initial concentration of bacteria used in this study was approximately 10-fold higher than in the published study, making the organic load higher and sterilization more difficult.

Due to instability of O_3, direct and accurate measurement of the concentration of ozone in water is difficult. Therefore, the oxidation-reaction potential (ORP) is commonly used for indirect measurement of the ozone in the water (Suantica et al., 2001; Hess-Erga et al., 2008; Imaizumi et al., 2018). Interestingly, we also noticed that when bacteria (organic matter) were added to water, the ORP value did not increase when ozone was added to the system, as was observed in the initial treatment without bacteria. Similarly, ORP did not increase during the treatment with the fish-cultured water (rich in organic matter). This was probably due to the rapid oxidation and degradation of O_3 molecules in the presence of organic matter. In clean water, ORP dropped relatively quickly and returned to normal after we ceased to introduce NB-O_3, indicating that O_3 molecules oxidize relatively quickly. This was consistent with the high levels of DO maintained after NB-O_3 treatment, most likely derived from the degradation of O_3 into O_2 molecules (Batakliev et al., 2014). If this is the case, the treatment of NB-O_3 in aquaculture farms could have dual benefits: disinfection and improvement of DO in the culture systems. Exposure to extended periods of time at high levels of DO may have a detrimental impact on fish and needs to be further explored before the technology is used commercially. In this study, repeating treatments was designed to evaluate the acute effect of

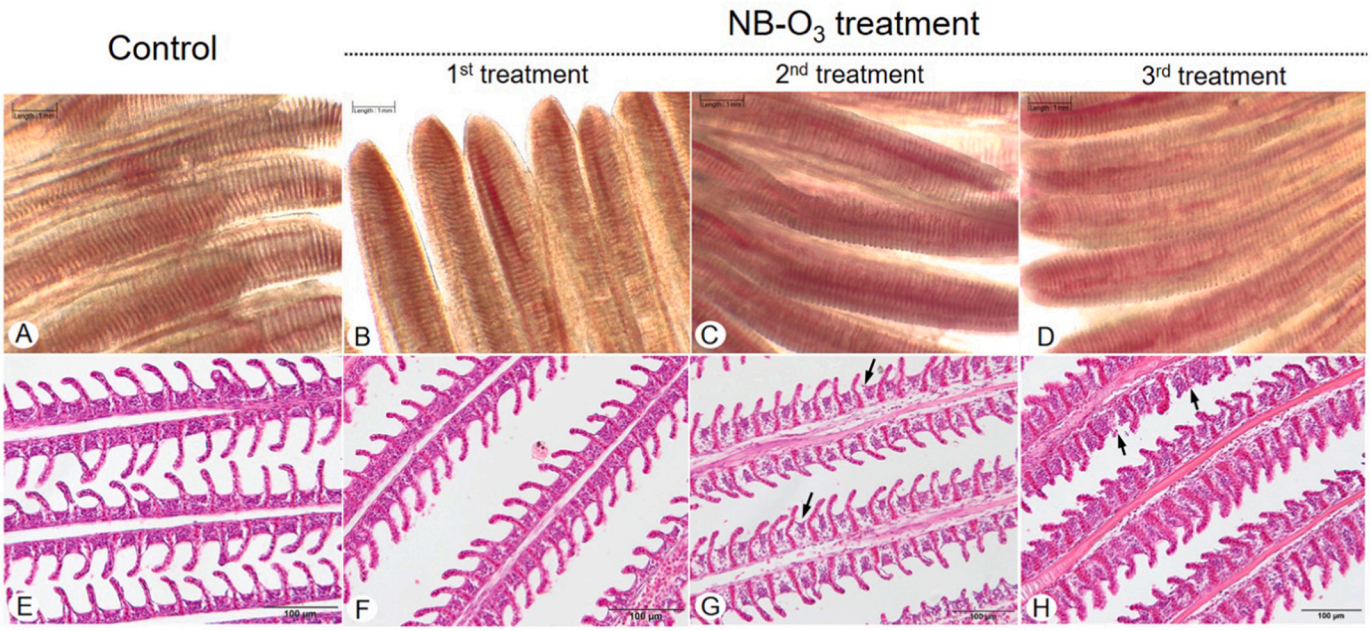

Fig. 7. Photomicrographs of wet-mount (A-D) and H&E stained sections (*E*-H) of the gills of tilapia from control and NB-O_3 treatment. No observable difference in gill morphology by wet-mount between control (A) and first treatment (B) groups. Mild congestion in the gill filaments was observed in the fish receiving second (C) and third (D) treatments. H&E staining revealed the normal structure of the gill filaments in both control (E) and the first treatment with NB-O_3 (F). Slight damage and shrinking of the basal lamellae (arrows) and mild blood congestion were observed in the fish receiving second exposure (G), and increasing damage of the gill filaments, loss of some secondary lamella (arrows), and more severe blood congestion in the secondary lamellae were observed in the fish that received the third exposure (H).

NB-O_3 on the fish. Although multiple NB-O_3 treatments did not kill the fish, increased exposure caused damage to the fish gills. If more than one 10 min treatment of NB-O_3 was used there was some evidence of irritation to the gills, but no acute mortality. The damage to the gills was likely from exposure to ozone, based on other studies (Wedemeyer et al., 1979; Good et al., 2011), but it may have also been exacerbated by high levels of oxygen (i.e. 26.9–28.5 mg/L) in the water. In an earlier experiment with a similar set up, fish were treated with oxygen nanobubbles for 10 min per day over a 26-day period, and no mortality was observed. Further, the treated fish had the same growth performance as non-treated control (unpublished data), suggesting the trauma to the gills observed in this study was likely from repeated exposure to ozone.

Table 2
Water parameter fluctuation in fish tanks with and without and NB-O_3 treatment.

Parameter	Measurement time	Control	NB-O_3 treatment
T°	Before treatment	28.7 ± 0.1	28.8 ± 0.0
	10 min (1st)	ND	29.6 ± 0.5
	10 min (2nd)	ND	30.7 ± 0.4
	10 min (3rd)	26.7 ± 0.1	31.6 ± 0.3
DO (mg/L)	Before treatment	4.9 ± 0.1	4.6 ± 0.1
	10 min (1st)	ND	28.2 ± 0.1
	10 min (2nd)	ND	28.5 ± 0.6
	10 min (3rd)	5.1 ± 0.0	26.9 ± 0.2
pH (1–14)	Before treatment	8.0 ± 0.0	8.0 ± 0.0
	10 min (1st)	ND	7.6 ± 0.1
	10 min (2nd)	ND	7.6 ± 0.1
	10 min (3rd)	7.15 ± 0.2	7.3 ± 0.0
ORP* (mV)	Before treatment	314 ± 13	337 ± 6
	10 min (1st)	ND	860 ± 42
	10 min (2nd)	ND	875 ± 18
	10 min (3rd)	313 ± 12	885 ± 15

T°, temperature in degree Celsius; DO, dissolved oxygen; ORP, oxidation reduction potential; ND, not done. Values are expressed as mean ± SD from 3 replicates. *ORP dropped to normal (~330 mV) after 15 min of every treatment time.

Taken together, the findings suggest that a single10 min exposure to NB-O_3, with ORP level reaching 860 ± 42 mV, is safe for fish.

It is notable that water temperature increased considerably during multiple nanobubble treatments in a relatively small volume of water (50 L). During the operation, the nanobubble generator produces a considerable amount of heat, which is transferred to the water. Increasing temperature may also cause stressful for the experimental fish. However, in fish ponds with large volumes of water, this might not be a problem due to rapid interchange of temperature between the water body and the surrounding air.

If this technology is applied in fish ponds, the concentrations of nanobubbles used would have to be much lower than what we applied in this study so the impact on the gills and fish health would be minimized. However, our findings of disinfection efficacy in water with abundant organic matter suggest that farmers may also have to apply the treatment more frequently to achieve the same level of bacterial reduction. Further research under field conditions is required to establish the optimal dosing of NB-O_3 in different size ponds to achieve a desirable reduction in bacterial concentration and to ensure this dose is not detrimental to fish health. If so, periodic treatment with appropriate dosage, during culture periods with high risk of bacterial disease outbreaks, might be useful for disease prevention. Nevertheless, more in-depth investigations are required prior to scaling up NB-O_3 technology for commercial applications, particularly with regard to the effects of NB-O_3 on fish immunity and stress response, microbiome, and growth performance.

One of the limitations of this study was the limited sample size in our experiments. Our tank numbers were limited by the number of nanobubble generators we had. Also, we could not include a normal ozone air-stone treatment group due to the personnel safety issue in our laboratory. However, when we consider all the experiments together there is strong evidence to suggest that NB-O_3 technology is not only a promising disinfection method, but also enriches dissolved oxygen in freshwater aquaculture, and in low doses (i.e. 10 min at 1 L/min in a 50 L tank) it was not harmful to the fish. As a disease prevention tool, NB-O_3

treatment might be a promising technology to control overgrowth of pathogenic bacteria in water, thus reducing the risk of bacterial diseases. This nonchemical disinfection technology may be a promising alternative to antibiotics as a means of reducing antibiotic use in aquaculture, and possibly inadvertently reduce the risk of AMR. Further research is required to investigate if NB-O_3 can be used as a treatment during bacterial disease outbreaks.

In summary, this study provides initial evidence to support that NB-O_3 are effective at reducing the concentration of the bacterial pathogens *S. agalactiae* and *A. veronii* in freshwater and they are relatively safe for tilapia. These findings should prompt the industry to further investigation this technologies application under commercial field conditions. Although commercially available devices were used in this study, several technical issues with the assessment of the technology remain. For example, direct measurement of the ozone dose and residue in water, energy efficiency, ozone utilization ratio, ozone decomposition dynamic and its disinfection time, potential toxic by-products, as well as the ozone mass transfer during NB-O_3 disinfection. These issues should be explored in further studies for a more comprehensive understanding of NB-O_3 technology.

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Disclaimers

The views expressed herein do not necessarily represent those of IDRC or its Board of Governors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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