# Ozone nanobubble treatments improve survivability of Nile tilapia (*Oreochromis niloticus*) challenged with a pathogenic multidrug-resistant *Aeromonas hydrophila*

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## Highlights

- Multiple treatments of NB-$O_3$ in a modified recirculation system (MRS) were relatively safe for juvenile Nile tilapia
- NB-$O_3$ treatments in MRS significantly improved survivability of Nile tilapia challenged with multidrug-resistant (MDR) *A. hydrophila* with RPS of 64.7 - 66.7%
- Concentration of MDR *A. hydrophila* in MRS was reduced by 15.9 to 35.6% following each NB-$O_3$ treatment, and increased by 13.1 to 27.9 % in untreated control
- Surviving fish developed specific antibody IgM against MDR *A. hydrophila*
- NB-$O_3$ is a promising non-antibiotic approach to control diseases caused by MDR *A. hydrophila*

## Abstract

Multidrug-resistant (MDR) bacteria has rapidly increased in aquaculture, which highlights the risk of production losses due to diseases and potential public health concerns. Previously, we reported that ozone nanobubbles (NB-$O_3$) were effective at reducing concentrations of pathogenic bacteria in water and modulating fish immunity against pathogens; however, multiple treatments with direct NB-$O_3$ exposures caused alterations to the gills of exposed-fish. Here, we set up a modified recirculation system (MRS) assembled with an NB-$O_3$ device (MRS-NB-$O_3$) to investigate whether MRS-NB-$O_3$ were 1) safe for tilapia (*Oreochromis niloticus*), 2) effective at reducing bacterial load in rearing water, and 3) improved survivability of Nile tilapia following an immersion challenge with a lethal dose of MDR *Aeromonas hydrophila*. The results indicated no behavioral abnormalities or mortality of Nile tilapia during the 14 day study using the MRS-NB-$O_3$ system. In the immersion challenge, although high bacterial concentration (~$2 \times 10^7$ CFU/mL) was used, multiple NB-$O_3$ treatments in the first two days reduced the bacteria between 15.9% to 35.6% of bacterial load in water while bacterial concentration increased 13.1% to 27.9% in the untreated control. There was slight up-regulation of non-specific immune-related genes in the gills of the fish receiving NB-$O_3$ treatments. Most importantly, this treatment significantly improved survivability of Nile tilapia with relative percent survival (RPS) of 64.7 - 66.7% in treated fish and surviving fish developed specific antibody against MDR *A. hydrophila*. In summary, the result suggests that NB-$O_3$ is a promising alternative to antibiotics to control bacterial diseases, including MDR bacteria, and has high potential for application in recirculation aquaculture system (RAS).

**Keywords:** *Aeromonas hydrophila*, alternatives to antibiotics, antimicrobial resistance, multidrug resistance, ozone nanobubbles

## 1. Introduction

Motile *Aeromonas* septicemia (MAS) is one of the most important bacterial diseases responsible for the loss of millions of dollars in the global freshwater aquaculture industry (da Silva et al., 2012; Hossain et al., 2014; Peterman and Posadas, 2019; Pridgeon and Klesius, 2012). The control of bacterial diseases still depends heavily on antibiotics. In recent years, a global issue of concern is the increase in antimicrobial resistant (AMR) bacteria as the consequence of misuse of antibiotics (Cabello, 2006; Cantas and Suer, 2014; Malik and Bhattacharyya, 2019). The high levels of AMR in the aquatic environment and aquaculture products pose a negative impact to not only aquaculture production, but also public health and international trade, especially in low- and middle-income countries (LMICs) where aquaculture is highly concentrated (Ben et al., 2019; Heuer et al., 2009; Okocha et al., 2018; Reverter et al., 2020). Currently, there is a high proportion of pathogenic multidrug-resistant (MDR) bacteria strains causing diseases in aquaculture (Santos and Ramos, 2018). In the battle to combat AMR, apart from alternatives to antibiotics, there are efforts to explore novel approaches for reducing the risk of bacterial diseases in aquaculture systems e.g. bacteriophage and nanobubble technology.

Nanobubbles (NBs) are bubbles less than 200 nm in diameter filled with chosen gases, neutral buoyancy, and having long residence time in the liquid solutions (Agarwal et al., 2011; Tsuge, 2014). Oxygen nanobubbles (NB-$O_2$) have been used for improving dissolved oxygen (DO) in aquaculture systems, and promoting growth of Nile tilapia (*O. niloticus*) (Mahasri et al., 2018) and whiteleg shrimp (*Penaeus vannamei*) (Mauladani et al., 2020; Rahmawati et al., 2020). Recently, several studies have revealed that ozone nanobubbles (NB-$O_3$) show promise at reducing quantities of pathogenic bacteria and improving DO in water, as well as modulating the immune systems against bacterial infections (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Linh et al., 2021; Nghia et al., 2021).

Ozone is a powerful disinfectant that has been used to reduce concentrations of pathogens and improve water quality in both flow-through and recirculating aquaculture systems for many years (Powell and Scolding, 2018). However, low ozone solubility and poor stability are major reasons for low utilization efficiency. In addition, misuse of direct ozonation can critically impact aquatic organisms, resulting in behavioral abnormalities, changes in physiology, tissue damage, and mortality (Powell and Scolding, 2018). However, NBs technology has been reported to improve

gas dissolvability in water and promote rapid oxidation of organic substances (Gurung et al., 2016). Hence, NB-$O_3$ may enhance the solubility, stability, and efficacy of ozone in aquaculture systems (Fan et al., 2020). Kurita et al. (2017) reported that NB-$O_3$ treatment significantly reduced planktonic crustacean parasites (63%) in juvenile sea cucumbers (*Apostichopus japonicas*) and sea urchins (*Strongylocentrotus intermedius*). In another study, NB-$O_3$ demonstrated good disinfection of *Vibrio parahaemolyticus* in water, and prevention of acute hepatopancreatic necrosis disease (AHPND) in whiteleg shrimp (Imaizumi et al., 2018). We found that NB-$O_3$ treatment (1-2 × $10^7$ bubbles/mL) reduced the level of *Streptococcus agalactiae* and *Aeromonas veronii* in water by more than 97% and made it relatively safe for juvenile Nile tilapia (Jhunkeaw et al., 2021). Most recently, we also reported that NB-$O_3$ treatment modulated the innate immune defense system of Nile tilapia, and that pre-treatment of NB-$O_3$ improved survivability of fish challenged with *S. agalactiae* (relative percent of survival of 60 - 70%) (Linh et al., 2021). This finding suggests that NB-$O_3$ may be a promising non-antibiotic treatment to control pathogenic MDR bacteria in aquaculture.

The limitations of direct application of NB-$O_3$ with high level of ozone (3.5 mg/L, 970 mV ORP (oxidation reduction potential) is the tissue damage that this gas can cause to animals. Toxicity resulting in mortalities were reported for experimental shrimp in a study by Imaizumi et al. (2018). In our previous study on tilapia, we did not observe fish mortality but the fish gill morphology was damaged when fish were exposed directly to multiple NB-$O_3$ treatments with an ORP range between 860 ± 42 and 885 ± 15 mV (Jhunkeaw et al., 2021). In this study, we set up a modified recirculation system coupled with ozone nanobubbles (MRS-NB-$O_3$). Subsequently, we evaluated the system to determine if it was effective at suppressing pathogenic MDR *A. hydrophila* and the survivability of juvenile Nile tilapia.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

A laboratory strain of multidrug resistant *A. hydrophila* BT14, isolated from an outbreak of MAS in 2018, was used in this study. Briefly, this bacterial strain was identified by Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) and PCR-sequencing using *gyrB* housekeeping gene, following previous studies (Anand et al., 2016;

Navarro and Martínez-Murcia, 2018). Based on the method proposed by Magiorakos et al. (2012), *A. hydrophila* BT14 was identified as a multidrug-resistant bacterium due to the fact that it resisted at least three classes of antimicrobials, including Ampicillin 10 µg (Penicillins), Tetracycline 30 µg (Tetracyclines), and Sulfamethoxazole-Trimethoprim 23.75 - 1.25 µg (Folate pathway inhibitors) (Table S1). For the bacterial challenge test, MDR *A. hydrophila* BT14 was propagated in 1 L of TSB at 28 ˚C with 18 h shaking-culture at 150 rpm. The bacterial concentration was determined by conventional plate count method (Harrigan and McCance, 2014).

### 2.2. Experimental fish

Healthy Nile tilapia (3.92 ± 1.01 g) from a commercial tilapia hatchery in Thailand were acclimated in dechlorinated tap water for 2 weeks at 29 ± 1.0 ˚C before the experiments. Fish were fed with commercial tilapia feed (crude-protein 30%) at rate of about 3% of fish weight twice daily. Before starting the experiments, ten fish were randomly selected for bacterial isolation and found to be free of *A. hydrophila*. The experiments on animals were conducted with permission of Thai Institutional Animal Care and Use Committee (Approval no. MUSC62-039-503).

### 2.3. MRS-NB-$O_3$ system setup and water parameter measurement

The ozone nanobubble system consisted of an oxygen concentrator (Model: VH5-B, Shenyang Canta Medical Technology Company Limited, Liaoning, China) connected to an ozone generator (Model: CCba15D, Coco Technology Company Limited, Chonburi, Thailand) and a nanobubble generator (Model: aQua+075MO, AquaPro Solutions Private Limited Company, Singapore). The NB-$O_3$ system was attached to a modified recirculation system (MRS) which contained two 100 L-fiberglass tanks (50 L dechlorinated tap water in each tank) that exchanged water by water pumps. One tank received the NB-$O_3$, the other tank housed the fish (Figure 1). All water quality parameters were measured in triplicate in the MRS-NB-$O_3$. Water temperature, pH, dissolved oxygen (DO), and oxidation reduction potential (ORP) were measured and compared from both tanks using a multi-parameter meter (YSI Professional Plus, YSI Incorporated, USA). During the application of the NBs, water samples were collected at 0 min, 5 min, 10 min of NB-$O_3$ treatment and 30 min post-treatment for measurement of dissolved ozone (ppm-mg/L) using K-7434 Ozone Vacu-vials Kit (Oxidation Technologies, USA).

### 2.4. Effect of MRS-NB-$O_3$ on fish safety

To evaluate the safety of Nile tilapia juveniles cultured in MRS-NB-$O_3$ system, 136 fish were divided into four tanks (50L dechlorinated tap water per tank) consisting of two replicate groups (controls and MRS-NB-$O_3$) with 34 fish per tank. The treatment group was treated with NB-$O_3$ (oxygen input 2 L per min) 7 times (10 min/time) at 1, 12, 24, 36, 48, 60, and 72 h from the start time of the experiment. Aeration was provided one hour after each treatment. The control group was treated with normal aeration instead of NB-$O_3$. Fish were observed every 12 h for behavioral abnormality and mortality over a 14-day period. The water parameters including temperature, pH, DO, and ORP were measured before and during treatment. After every treatment, two fish in each tank were randomly collected and preserved for gill histology examination. Formalin preserved samples (n = 28) were subjected to routine histology. The histopathological changes were observed under the Leica DM1000 digital microscope equipped with a digital camera DFC450 (Leica, Singapore).

### 2.5. Immersion challenge trial for MDR *A. hydrophila* BT14

To establish the immersion challenge dose, 80 fish were divided into four 50 L tanks, each tank containing 20 fish. Three tanks were challenged with MDR *A. hydrophila* BT14 by adding 1 L of bacterial culture ( approx. $8 \times 10^6$, $8 \times 10^7$, and $8 \times 10^8$ CFU/mL) to each tank to reach the final concentrations of $2 \times 10^5$, $2 \times 10^6$, and $2 \times 10^7$ CFU/mL, respectively. A total 1 L of culture medium without bacteria was added to a negative control tank. Air-stones were used in all tanks for air supply and approximate 50% of the water was changed after 48 h. Clinical signs of MAS and mortalities were recorded every 12 h for 14 days. The representative dead or moribund fish were subjected to bacterial re-isolation using selective medium Rimler Shotts (RS, Himedia, India) supplemented with Novobiocin (Oxoid, UK).

### 2.6 Effect of multiple NB-$O_3$ treatments in MRS on Nile tilapia challenged with MDR *A. hydrophila*

#### *Fish survivability, gill collection, and water collection*

Two trials were conducted to test the effect of our MRS NB-$O_3$ treatments. In the first trial, 128 fish were randomly divided into four groups (32 fish per tank): Group 1 was exposed to culture medium without NB-$O_3$ treatment (no Ah + no NB-$O_3$); Group 2 was exposed to bacteria without NB-$O_3$ (Ah + no NB-$O_3$); Group 3 was exposed to culture media only and treated with NB-$O_3$ (no Ah + NB-$O_3$); Group 4 was challenged with *A. hydrophila* and treated with NB-$O_3$ (Ah + NB-$O_3$).

In bacterial challenge groups 2 and 4, 1 L of MDR *A. hydrophila* BT14 (approx. $8 \times 10^8$ CFU/mL) was added to 50 L water to reach a final concentration of approx. $2 \times 10^7$ CFU/mL. The fish were maintained at 29 ± 1 °C with aeration for 3 h. Afterwards, fish in groups 3 and 4 were treated for 10 min with NB-$O_3$ at 1, 12, 24, 36, and 48 h post-challenge, while group 1 and group 2 were treated with normal aeration. In order to investigate the effect of NB-$O_3$ treatments on the fish immune response in our MRS, the gills from 4 fish were randomly sampled at 3 h after the 1st, 2nd, and 3rd NB-$O_3$ treatments and preserved in 200 µL of Trizol reagent (Invitrogen, USA) for immune genes analysis. The remaining fish were observed daily for 14 days and mortality was recorded. Representative moribund or freshly dead fish were collected for bacterial re-isolation using Rimler Shotts (RS) medium plus Novobiocin as described above. The relative percent survival (RPS) was calculated according to the formula described by Ellis (1988): RPS = [1 - (% mortality in challenge/ % mortality in control)] × 100. In parallel, water samples from groups 2 and 4 (challenged with *A. hydrophila*) were evaluated for bacterial enumeration using conventional plate count method (Harrigan and McCance, 2014). The percentage of bacterial fluctuation was calculated based on bacterial concentration (CFU/mL) before and after NB-$O_3$ treatment.

In the second trial, the experiment was repeated in the same manner as the first with the exception that 20 fish were used for each group and this experiment focused mainly on monitoring survival rate and bacterial enumeration. This experiment was repeated to confirm our initial survival results in the first trial.

***Visualization of live and dead bacteria before and after treatment with NB-$O_3$***

A volume of 25 mL water in group 4 (Ah + NB-$O_3$) was sampled before and after the first NB-$O_3$ treatment for assessment of the viability of *A. hydrophila*. A bacterial suspension was prepared and stained following the protocol of LIVE/DEAD *Bac*light Bacterial Viability Kit (Cat. No. L7012, Thermo-Fisher Scientific, USA). In brief, the bacterial suspension were centrifuged at 10,000 x *g* for 10 min at 4°C. The pellets were collected and re-suspended in 2 mL of sterile normal saline buffer, incubated at room temperature for 1 h, mixing every 15 min. Bacteria were washed two times by centrifugation at 10,000 x *g* for 10 min at 4°C and pellet resuspension was done in 20 mL and 10 mL of sterile normal saline buffer for the first and second time of washing. Staining processes were conducted by mixing 1.5 µL of SYTO®9, 1.5 µL of Propidium Iodine (PI), and 1 mL of bacterial suspension in a microtube. The mixture was incubated at room temperature in the

dark for 15 min. After that, 5 µL mixtures were pipetted onto glass slides, covered with a coverslip and examined under a confocal laser scanning microscope CLSM (Model: DM1000, Leica Microsystem Private Limited Company, Singapore) assembled with incident light fluorescence to visualize live and dead bacteria. Five random fields from each slide were imaged. Fluorescence signals were counted in ImageJ software based-on Watershed algorithm.

***Expressions of innate immune-related genes***

To investigate expression of innate immune-related genes, total RNA of gill samples was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The first complementary DNA (cDNA) strand was synthesized from 2.0 µg of the total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad, USA) according to the procedure described in the product manual. Quantitative real-time PCR (qPCR) using SYBR green reagent (iTaq™ Universal SYBR™ green Supermix, Bio-Rad, Hercules, CA, USA) was carried out using primers specific for 3 immune genes (Table 1). The qPCR amplification cycles were performed using a CFX Connect™ Real-time System (Bio-Rad, USA). Cycling conditions were 94 °C for 15 s, 40 cycles of denaturation at 95 °C for 30 s, annealing at the optimal temperature of each primer as indicated for 30 s, and a final extension at 72 °C for 30 s. Melting curves were obtained in the 55 to 85°C range with 0.1 °C increments per second to evaluate for the specificity of all qPCR products. The qPCR data will be analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001). The transcript levels of each target gene were obtained as $C_q$ values and normalized to that the *EF-1a* as an internal reference.

***Determination of serum antibody by the enzyme-linked immunosorbent assay (ELISA)***

In order to determine whether surviving fish at day 14 post challenge develop specific antibodies (IgM) against *A. hydrophila*, blood samples were collected from fish in the first trial (four from Ah + no NB-$O_3$ group and five from each of the other groups) . Blood samples were kept at room temperature for 1 h before being centrifuged at 8.000 x g for 15 min. The collected fish sera were stored at -20$^{o}$C until used. An ELISA was carried out following the protocol described by Linh et al. (2021) with minor modification. In brief, 96 well EIA/RIA plates (Costar®, Corning Inc., USA) were coated with formalin-killed *A. hydrophila* whole-cell antigen ($OD_{600nm}$ = 1.0). Fish sera (dilution 1:256), anti-Tilapia IgM secondary antibody (1:200) (Soonthonsrima et al., 2019), and commercial goat anti mouse antibody horseradish peroxidase (HRP) conjugate (1:3000) were used

for the ELISA assay in this study and samples were read at an absorbance of 450 nm using a SpectraMax® iD5 Multi-Mode Microplate Reader (Molecular Devices, USA).

### 2.7. Statistical analysis

Cumulative mortality and percent survival data from the challenge experiments were analyzed by the Kaplan-Meier method and differences among groups were tested using a log-rank test, *p*-values of 0.05 or less were considered statistically significant. Fish innate immune-related gene expression was analyzed by ANOVA, *p*-values of 0.05 or less were considered statistically significant. Duncan's post-hoc test was used to measure specific differences between pairs of mean. The $OD_{450nm}$ readings from our indirect ELISA assay were analyzed using a Kruskal-Wallis test, *p*-values of 0.05 or less were considered statistically significant. Multiple comparison analyses were performed by Bonferroni test. All statistical analyses were performed using SPSS Software ver22.0 (IBM Corp., USA).

## 3. Results

### 3.1. Effect of MRS-NB-$O_3$ on water parameters

For the 10 min NB-$O_3$ treatment in the MRS, the change of water parameters, including temperature, pH, DO, and ORP, are displayed in Figure 2. Temperature and pH values appeared stable over time in both the NB-$O_3$ treated tank and the culture tank (which did not have fish for this investigation). The DO increased significantly after 10 min NB-$O_3$ treatments in both tanks. The DO level in the culture tank increased from 5.07 ± 1.61 to 13.97 ± 0.84 mg/L (increase of 8.9 mg/L), while there was an higher increase in NB-$O_3$ tank (from 6.84 ± 1.08 to 19.74 ± 1.28 mg/L). The significantly different trend of ORP value was observed in the NB-$O_3$ treated tank and culture tank. The ORP decreased slightly from 424.9 ± 24 to 396 ± 61.9 mV in fish culture tank, whereas the ORP in NB-$O_3$ tank increased rapidly from 417.7 ± 23.6 to 791.7 ± 71.5 mV after 5 min NB-$O_3$ treatment and reached 870.1 ± 12.4 mV after 10 min. During NB-$O_3$ treatment, dissolved ozone concentration at 0 min, 5 min, and 10 min in treated tank were 0.02, 1.16, and 1.37 mg/L respectively, whereas significantly lower values, 0.03, 0.06, and 0.14 mg/L were recorded in system's fish culture tank at the same time points. At 30 min post-treatment, dissolved ozone concentration in NB-$O_3$ treated and fish culture tanks decreased to 0.05 and 0.03 mg/L respectively.

### 3.2. Effect of MRS-NB-$O_3$ on fish safety

No mortality or behavioral abnormalities in fish were observed in either the control and NB-$O_3$ treated groups during and after treatments. All fish survived the 14 day study period. Histologically, there were no differences in gill morphology in control and treatment groups after five NB-$O_3$ treatments. However, alterations were observed in the gill filaments after the $6^{th}$ and $7^{th}$ treatments (Figure S1). The fluctuation of water parameters was consistently similar during every treatment (Table S2), and similar to the trend in the previous experiment without fish (Figure 2). Temperature and pH increased slightly in both groups during treatment. Dissolved oxygen in the fish culture tanks of the MRS-NB-$O_3$ increased significantly from 4.98 - 6.97 mg/L (before each treatment) to 12.26 - 15.33 mg/L (at each 10 min of treatment) and dropped to 9.28 - 12.69 mg/L after the 10 min treatment. ORP values in fish culture tanks did not increase and remained relatively stable in control and NB-$O_3$ treated groups.

### 3.3 Immersion challenge trial for MDR *A. hydrophila* BT14

The cumulative mortality of Nile tilapia challenged with three different doses of MDR *A. hydrophila* BT14 by immersion was dose-dependent (Figure 3). The fish challenged with $2 \times 10^7$ CFU/mL (high dose) had a 75% mortality rate, and death occurred mainly in the first 4 days of the experiment. In the 10-fold lower dose, there was only 25% mortality and most fish died from days 4 to 9. There was no mortality in the group challenged with $2 \times 10^5$ CFU/mL or the control group (Figure 3). The clinically sick fish showed lethargy, loss of appetite, and tended to swim at the surface, but did not reveal significant external or internal symptoms except pale livers. Bacterial isolation from representative dead fish (n = 5) revealed dominant colonies of bacteria, morphologically resembling *A. hydrophia* on selective medium. From this result, the dose of $2 \times 10^7$ CFU/mL was used for subsequent challenge assays.

### 3.4 MRS-NB-$O_3$ improved survivability of Nile tilapia challenged with the MDR *A. hydrophila* BT14

The results of the challenge tests were consistent between replicates (Figure 4). The group challenged with *A. hydrophila* followed by NB-$O_3$ treatments (Ah + NB-$O_3$) had 70 and 75% survival compared to 15 and 25% in the group challenged with bacteria receiving no NB-$O_3$ treatment (Ah + no NB-$O_3$). This difference was statistically significant ($p = 0.001$) in both trials. No mortality was observed in the negative control group (no Ah + no NB-$O_3$) during the 14 day

study period. However, there were 5 and 15 % mortality in the control groups treated with NB-$O_3$ without a precedent bacterial challenge (no Ah + NB-$O_3$). However this was not statistically significant to the negative control group in either trials ($p$ = 0.317 in trial 1 and $p$ = 0.075 in trial 2 (Figure 4)). The relative percent survival (RPS) of NB-$O_3$ treatments in the 2 replicate treatment groups were 64.7 and 66.7%, respectively.

The moribund fish in challenge groups showed pale liver and behavioral abnormalities, including lethargy, loss of appetite, and surface swimming. The typical colonies of *A. hydrophila* were consistently recovered from internal organs (i.e. liver, kidney) of representative dead fish using RS medium supplemented with Novobiocin.

In parallel, bacterial concentration in the water column was monitored in two groups challenged with *A. hydrophila*. In the group Ah + NB-$O_3$, bacterial load in fish culture tanks after the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ treatments were reduced by 35.6, 23.3, and 20.2%, respectively in the first trial, and by 23.9, 21.1, and 15.9%, respectively in the second trial (Figure 5). By contrast, bacterial load in the Ah + no NB-$O_3$ increased by 13.4, 13.1, and 27.1% in the first trial, and by 15.6%, 19.8, and 27.9 % during the same time period in the second trial. Representative photomicrographs of comparative visualization of live and dead bacteria before and after treatment with NB-$O_3$ are illustrated in Figure 6. Before NB-$O_3$ treatment, the majority of bacterial cells appeared to be alive (i.e. stained fluorescent green), with very few dead cells (i.e. red color) (Figure 6A-C). However, after 10 min NB-$O_3$ treatment, the density of dead cells (red staining cells) increased considerably (17.45%) per microscopic field.

### 3.5 Expressions of innate immune-related genes

The expression levels of innate immune genes from different groups after each NB-$O_3$ treatment are shown in Figure 7. Although not statistically significant, the overall expression levels of immune genes *LYZ, HSP90,* and *TNF-a* in the gills of the fish exposed to NB-$O_3$ treatments tended to be slightly higher than that of the untreated control, except for the first treatment. Specifically, the trends included *LYZ* expression in treated group with or without *A. hydrophila* challenge which rose after the 2$^{nd}$ and 3$^{rd}$ treatment compared to that in the negative control group. The highest expression level (approx. 2.2 folds) was recorded in NB-$O_3$ treated group with *A. hydrophila* at the 3$^{rd}$ treatment. Expression of *HSP90* had different patterns for different experiments. The expressions in NB-$O_3$ treated group with or without *A. hydrophila* challenge increased at the 2$^{nd}$

treatment but decreased similar to the levels in the control group for the 3rd treatment. The relative transcription level of *TNF-a* increased slightly (1.4 fold) with the highest expression level in NB-$O_3$ treated group.

### 3.6. Specific antibody (IgM) response post-challenge

All surviving fish in both groups challenged with MDR *A. hydrophila* had significantly higher levels of specific antibody (IgM) compared to the two unchallenged control groups ($p < 0.05$) as measured by indirect ELISA (Kruskal-Wallis test: H (3) = 15.542, $p = 0.001$). The serum from fish in the Ah + NB-$O_3$ group had the highest $OD_{450}$ readings (0.44 ± 0.076), followed by OD readings of serum in Ah + no NB-$O_3$ group (0.42 ± 0.06). In contrast, the lowest level (0.06 ± 0.004) was recorded in the negative control (no Ah + no NB-$O_3$). A higher level but not statistically significant difference with negative control was shown in group no Ah + NB-$O_3$ (0.1 ± 0.013) (Figure 8).

## 4. Disscussion

Several studies have reported potential applications of NB-$O_3$ for pathogen disinfection in aquaculture water to reduce the risk of infectious diseases in both fish and shrimp (Imaizumi et al., 2018; Jhunkeaw et al., 2021; Kurita et al., 2017). We recently reported an additional benefit of NB-$O_3$ in modulating of the innate immune defense system in Nile tilapia to fight against *S. agalactiae* (Linh et al., 2021). However, all the precedent studies exposed the animals directly to NB-$O_3$ (NB-$O_3$ was exposed directly into the tank containing fish or shrimp) and this resulted in mild to severe health impacts on the exposed animals. High dose of ozone (960 mV ORP) were toxic to shrimp (Imaizumi et al., 2018), or caused gills alteration in tilapia after repeated exposures to NB-$O_3$ (~860 mV ORP) (Jhunkeaw et al., 2021). Therefore, we modify a NB-$O_3$ system on a laboratory scale to better understand this technology and overcome this drawback.

Ozone is an unstable molecule, even in the form of nanobubbles, which degrades relatively quickly (Jhunkeaw et al., 2021). Based on this characteristic, we set up a modified recirculation system coupled with NB-$O_3$ technology (MRS-NB-$O_3$), which separated the NB-$O_3$ treatment tank from the culture tank containing fish to reduce direct exposure of the fish to high level of ozone. Interestingly, during treatment, ozone level increased rapidly in the NB-$O_3$ treatment tank but did not increase in the fish culture tank, as indicated by ORP values (870.1 ± 12.4 vs. 396 ± 61.9 mV

ORP) and dissolved ozone concentrations (1.37 vs. 0.14 mg/L). Several studies suggested that ORP levels in the range from 300 to 425 mV ORP were safe for fish, crustaceans, and molluscs (Li et al., 2014; Powell and Scolding, 2018; Stiller et al., 2020). In the MRS-NB-$O_3$ set up, multiple treatments (up to seven 10 min treatments) in this study appeared to be relatively safe for juvenile Nile tilapia, with no mortality over a 14 day period. We also noticed that the MRS-NB-$O_3$ system could avoid excess DO level in the culture tank that commonly occurred when the NB-$O_3$ treatments were applied directly to the fish tanks (Jhunkeaw et al., 2021).

This study revealed that multiple NB-$O_3$ treatments in our MRS-NB-$O_3$ system improved survivability of Nile tilapia (*O. niloticus*) challenged with a pathogenic multidrug-resistant *A. hydrophila*. Motile Aeromonads have been reported as one of the most common pathogens in freshwater aquaculture (Hayatgheib et al., 2020). *A. hydrophila* can cause between 35-100% mortality during disease outbreaks (Baumgartner et al., 2018; Pridgeon and Klesius, 2011; Rasmussen-Ivey et al., 2016). Under experimental conditions, *A. hydrophila* can cause between 50 to 80% mortality in Nile tilapia (Abass et al., 2018; Dawood et al., 2020; Suprayudi et al., 2017). In the present study, relatively high mortality (75 - 85%) was observed in immersion challenges with a MDR *A. hydrophila*. Interestingly, multiple NB-$O_3$ treatments were effective with RPS of 64.7 - 66.7%. The RPS value in this study was similar or higher than several studies using antibiotics for Aeromonads control in Nile tilapia e.g. RPS of 60% in orally administered with Oxytetracycline 4g/kg/feed per day (Abraham et al., 2017) or RPS 25.9 % in orally fed with Oxytetracycline 60 mg/kg biomass per day (Julinta et al., 2017).

Compared to other alternatives to antibiotics, NB-$O_3$ offered comparable protective efficacy to some probiotic-based products against *Aeromonas* sp. AC9804 infection such as *Lactobacillus rhamnosus* which reported RPS values of 66.7% (Ngamkala et al., 2010) and *L. plantarum* with an RPS of 64% (Dawood et al., 2020). The results of this study were also comparable to some plant-based products used to control *A. hydrophila*, with reported RPS around 71% for Indian ginseng, *Withania somnifera* powder (Zahran et al., 2018), 35.3% for American ginseng, *Panax quinquefolius* (Abdel-Tawwab, 2012), and 58.7% for ginger, *Zingiber offcinale* (Payung et al., 2017). Our finding suggests that NB-$O_3$ treatments could be considered a potential non-antibiotic approach or an "alternative to antibiotics" to control bacterial disease in aquaculture.

Ozone is among the most powerful oxidant known with oxidative potential of 2.07 volts, nearly twice of chlorine (Hugo et al., 1999). Further, aqueous ozone can generate hydroxyl radicals ($OH^-$) with higher oxidative potential (2.83 volts) than ozone (Qingshi et al., 1989). Ozone ruptures cells by destroying the glycoproteins and glycolipids on the cell membranes. Moreover, ozone attacks the sulfhydryl groups of enzymes results in disruption of normal cellular enzymatic activity and loss of function. Lastly, ozone can directly damage the purine and pyrimidine bases of nucleic acids (Megahed et al., 2018). When NBs collapse, they generate shock waves that consequently lead to the formation of hydroxyl radicals (Fan et al., 2020; Takahashi et al., 2007). Thus, NB-$O_3$ may enhance the disinfectant efficacy of ozone in aquaculture systems.

Although the differences in bacterial concentration in the Ah + NB-$O_3$ group were only 1.0 to 1.6 fold lower than the Ah + no NB-$O_3$ group after each treatment, clear differences in survivability of the fish were observed in these groups. It is also possible although not statistically significant on an individual basis the overall upregulation of innate immune genes and stimulation of humoral immune response for fish in the NB-$O_3$ treatment group partially contributed to better survival rates after bacterial challenges. This has been reported by others as well (Linh et al., 2021). The stimulation of innate immunity is the first line of defense against invading pathogens and leads to improvements in health conditions and resistance to pathogens of fish (Magnadóttir, 2006). Pro-inflammatory cytokines, particularly *TNF-a* is an important macrophage-activating factor produced by leukocytes (Whyte, 2007), while lysozyme is a vital defense molecule of fish immune system due to make the demolition of bacterial cell wall (Saurabh and Sahoo, 2008). In addition, heat-shock proteins have a function in the development of specific and non-specific immune response to infections (Roberts et al., 2010).

Another factor which may also have improved survival of fish in this experiment was the DO in treated groups. Higher level of DO in NB-$O_3$ treated groups during and after treatments may improve fish health by maintaining or improving normal physiological functions. Previous studies suggested that high level of oxygen improved the immunocompetence in fish (Bowden, 2008; Cecchini and Saroglia, 2002). Romano et al. (2017) revealed that 12 - 13 mg/L oxygen increased immune response performance of sea bass (*Dicentrarchus labrax*).

One of the limitations of this study was our small sample size which could account for the non-significant difference in the up-regulation of innate immune genes between groups. Further, due

411 to the limited facilities, we were unable to compare effectiveness of different forms of ozone
412 bubbles (macro-, micro- and nanobubbles) in reducing bacterial loads and improving fish survival
413 rate upon bacterial infection. Further studies should explore these issues to gain better
414 understanding of this promising technology. In addition, the MRS-NB-$O_3$ system need to be scaled
415 up to be utilizable in aquaculture systems.

416 Despite these limitations, this study reported a MRS coupled with NB-$O_3$ technology was
417 successful at reducing mortality in fish and not exposing fish to high levels of ozone. It may be
418 possible to scale this system up for use in hatcheries and commercial farms that use RAS systems.
419 Our MRS-NB-$O_3$ allowed multiple NB-$O_3$ treatments without obvious negative impacts on the
420 fish. This system not only suppressed MDR bacterial loads in the culture tanks, but also improved
421 fish survivability. Application of NB-$O_3$ may be a promising non-antibiotic method of reducing
422 the risk of infectious diseases caused by bacteria, including MDR bacterial strains.

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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 there are no conflicts of interest.

## CRediT authorship contribution statement

**Le Thanh Dien**: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Nguyen Vu Linh**: Investigation, Methodology. **Pattiya Sangpo**: Investigation. **Saengchan Senapin**: Data curation, review & editing, **Sophie St-Hilaire**: Conceptualization, review & editing, Funding acquisition, **Channarong Rodkhum**: Supervision, Validation, review & editing. **Ha Thanh Dong**: Conceptualization, Data curation, Writing - review & editing, Supervision, Validation, Funding acquisition, Project administration.

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626

627 ## Tables and Figures

628

629 **Table 1.** Primers used to quantify relative gene expression in this study

630

| Target gene | Oligo sequence (5′ -3′) | Genbank Acession No. | Product size | Annealing temperature | References |
|---|---|---|---|---|---|
| *TNF-α* | F: CTTCCCATAGACTCTGAGTAGCG<br>R: GAGGCCAACAAAATCATCATCCC | NM_001279533 | 161 bp | 60 ºC | Liu et al. (2011) |
| *HSP90* | F: ATTGCTCAGCTGATGTCCCT<br>R: GTGGGATCCGTCAAGCTTTC | Unpublished | 128 bp | 56 ºC | Linh et al. (2021) |
| *LYZ* | F: AAGGGAAGCAGCAGCAGTTGTG<br>R: CGTCCATGCCGTTAGCCTTGAG | XM_003460550.2 | 151 bp | 63 ºC | Qiang et al. (2016) |
| *EF-1a* | F: CTACAGCCAGGCTCGTTTCG<br>R: CTTGTCACTGGTCTCCAGCA | AB075952 | 139 bp | 60 ºC | Velázquez et al. (2018) |

F: Forward primer, R: Reverse primers, bp: base pair

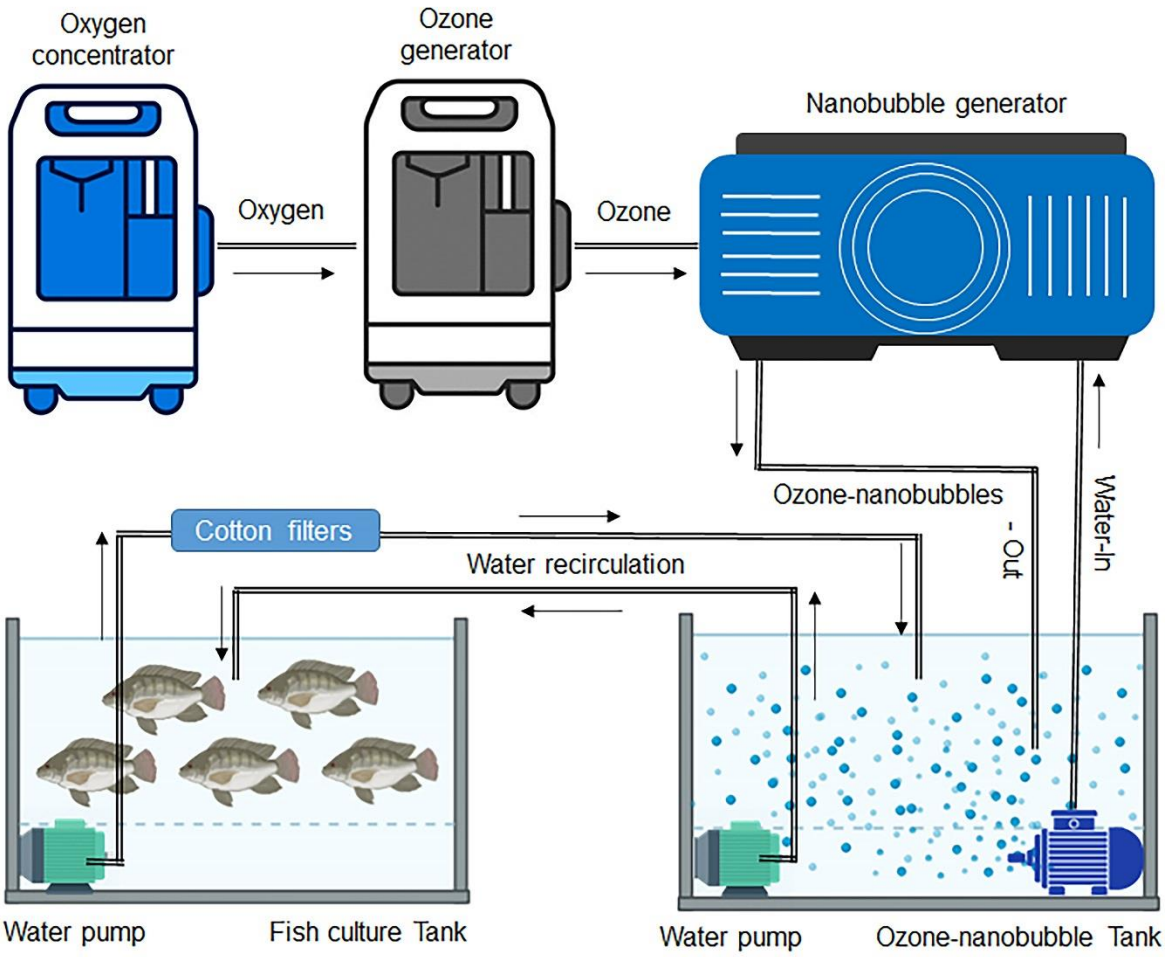
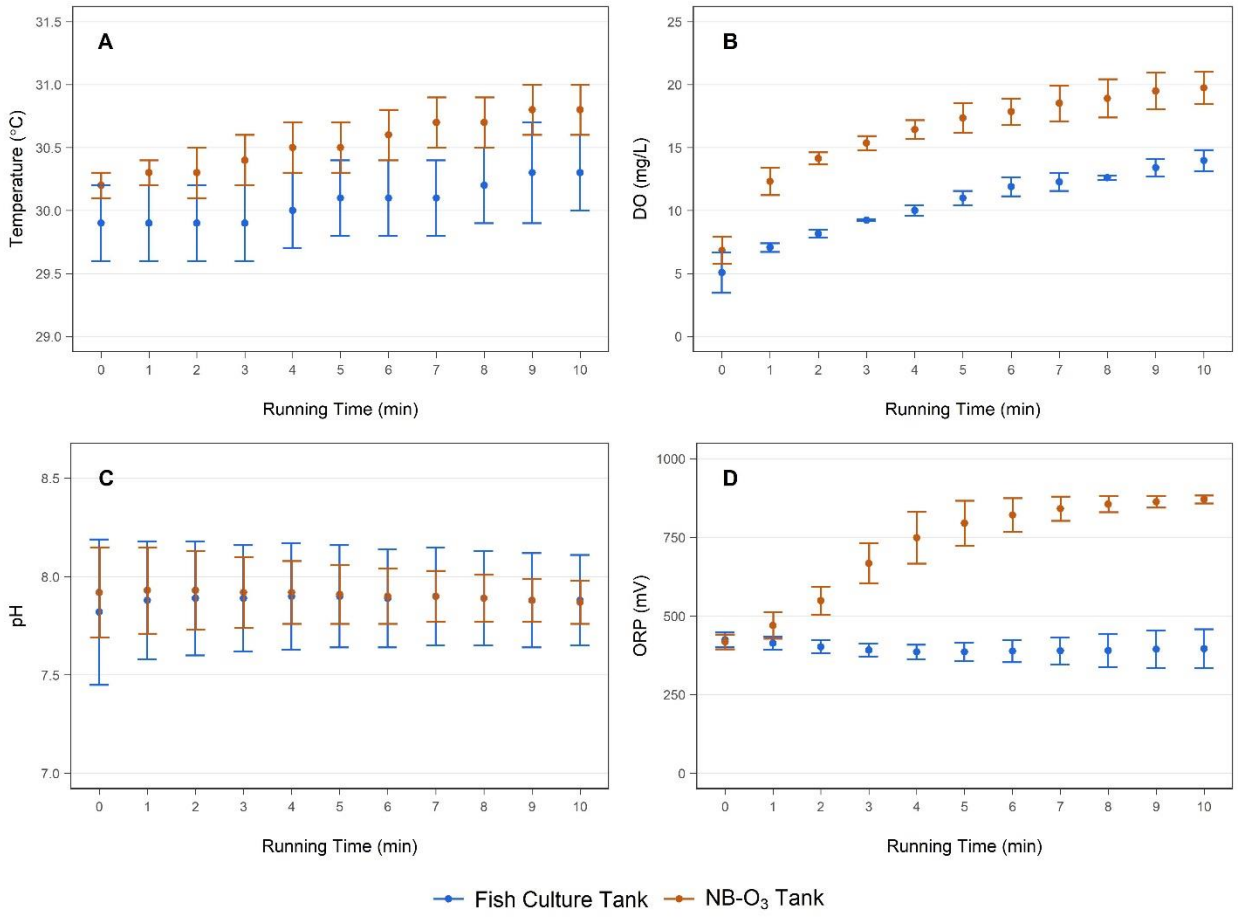


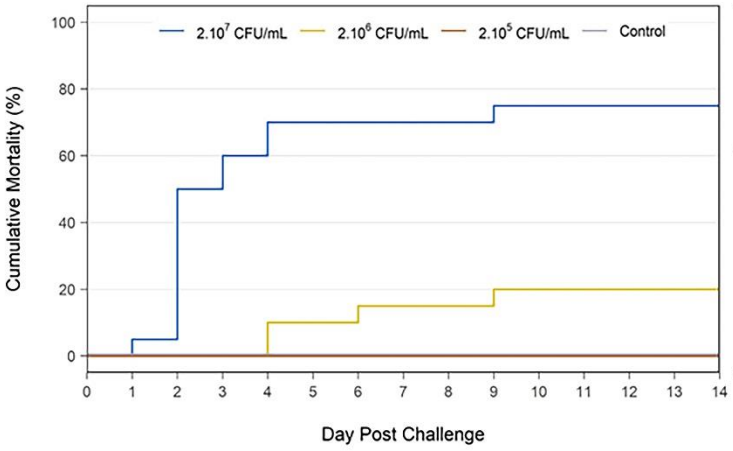

**Figure 1.** Experimental set-up of MRS-NB-$O_3$. Oxygen concentrator releases oxygen as a material to synthesize ozone using ozone generator. Ozone was lead to nanobubble generator. Inside the system, ozone was diffused in nanobubble water and released to ozone-nanobubble tank. Thereafter, NB-$O_3$ water was pumped to fish culture tank. The rearing water were recirculated between NB-$O_3$ tank and fish culture tank via a pump system assembled to cotton filter box to absorb fish feces and leftover feed.




638

639 **Figure 2.** Measurement of water parameters including temperature (**A**), DO (**B**), pH (**C**), and ORP
640 (**D**) during 10 min NB-$O_3$ treatment with 2 L/min oxygen input in MRS. Value of water parameters
641 are mean ± SD (n = 3).

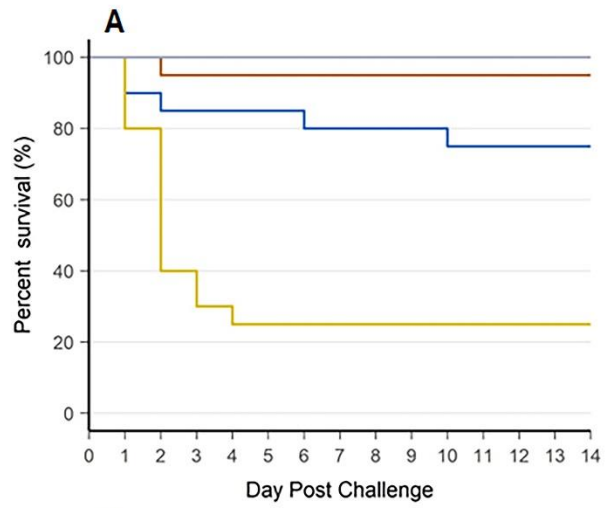
A




B

| Groups | Significance | | |
|---|---|---|---|
| | $2.10^5$ CFU/mL | $2.10^6$ CFU/mL | $2.10^7$ CFU/mL |
| Control | ns | 0.037 * | 0.000 * |
| $2.10^5$ CFU/mL | | 0.037 * | 0.000 * |
| $2.10^6$ CFU/mL | | | 0.000 * |

**Figure 3.** Kaplan-Meier analysis of cumulative mortality of Nile tilapia (n = 20) challenged with 3 doses of MDR *A. hydrophila* BT14 by immersion method (A). Control was exposed to culture medium without bacteria. Differences between groups were tested using log-rank test shown in (B). "*" denotes significant difference ($p < 0.05$), "ns" means not significant.



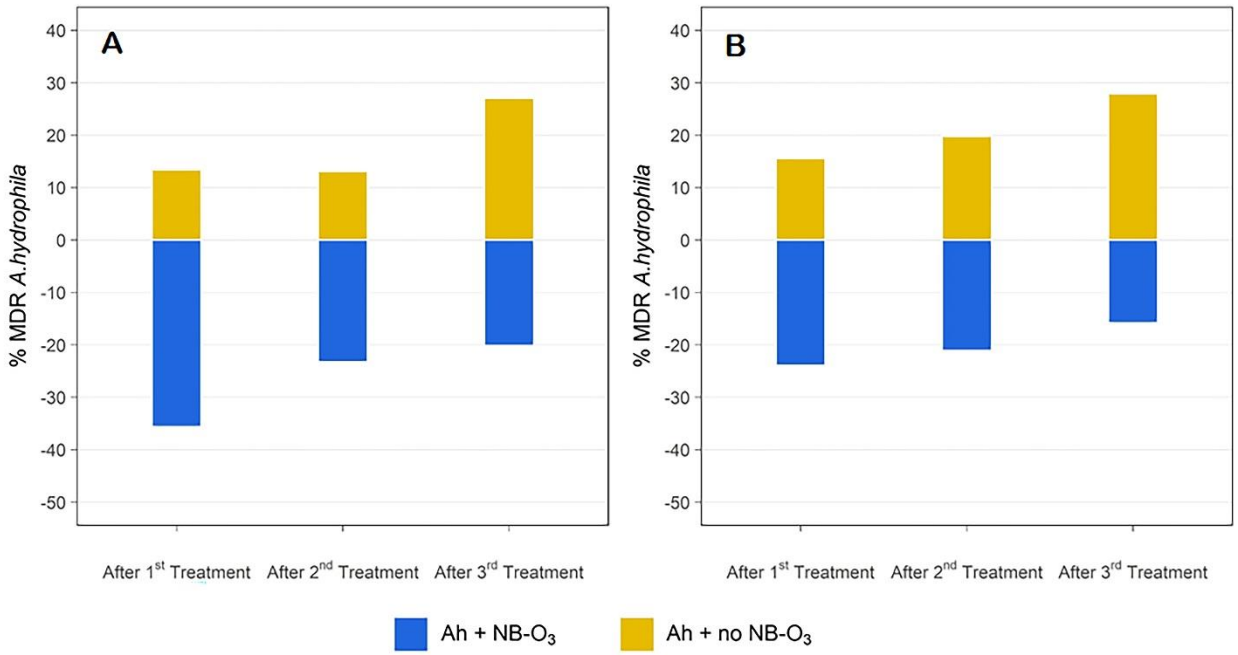

**B**

| Groups | Significance | | |
|---|---|---|---|
| | Ah no NB-$O_3$ | no Ah NB-$O_3$ | Ah NB-$O_3$ |
| no Ah no NB-$O_3$ | 0.000 * | 0.317 ns | 0.018 * |
| Ah no NB-$O_3$ | | 0.000 * | 0.001 * |
| no Ah NB-$O_3$ | | | 0.08 ns |

**D**

| Groups | Significance | | |
|---|---|---|---|
| | Ah no NB-$O_3$ | no Ah NB-$O_3$ | Ah NB-$O_3$ |
| no Ah no NB-$O_3$ | 0.000 * | 0.075 ns | 0.009 * |
| Ah no NB-$O_3$ | | 0.000 * | 0.001 * |
| no Ah NB-$O_3$ | | | 0.243 ns |

**Figure 4.** Kaplan - Meier analysis of percentage survival of Nile tilapia (n = 20) challenged with MDR *A. hydrophila* BT14. The experiment was done in two independent trials, trial 1 (A) and trial 2 (C). Differences between groups in each trial were tested using log-rank test shown in (B) and (D) respectively. "*" denotes significant difference ($p < 0.05$), "ns" means not significant.




652

653 **Figure 5.** Concentration of MDR *A. hydrophila* BT14 in rearing water between un-treated and
654 treated by 10 min NB-$O_3$ groups after the 1st, 2nd, and 3rd treatment. A, trial 1; B, trial 2.

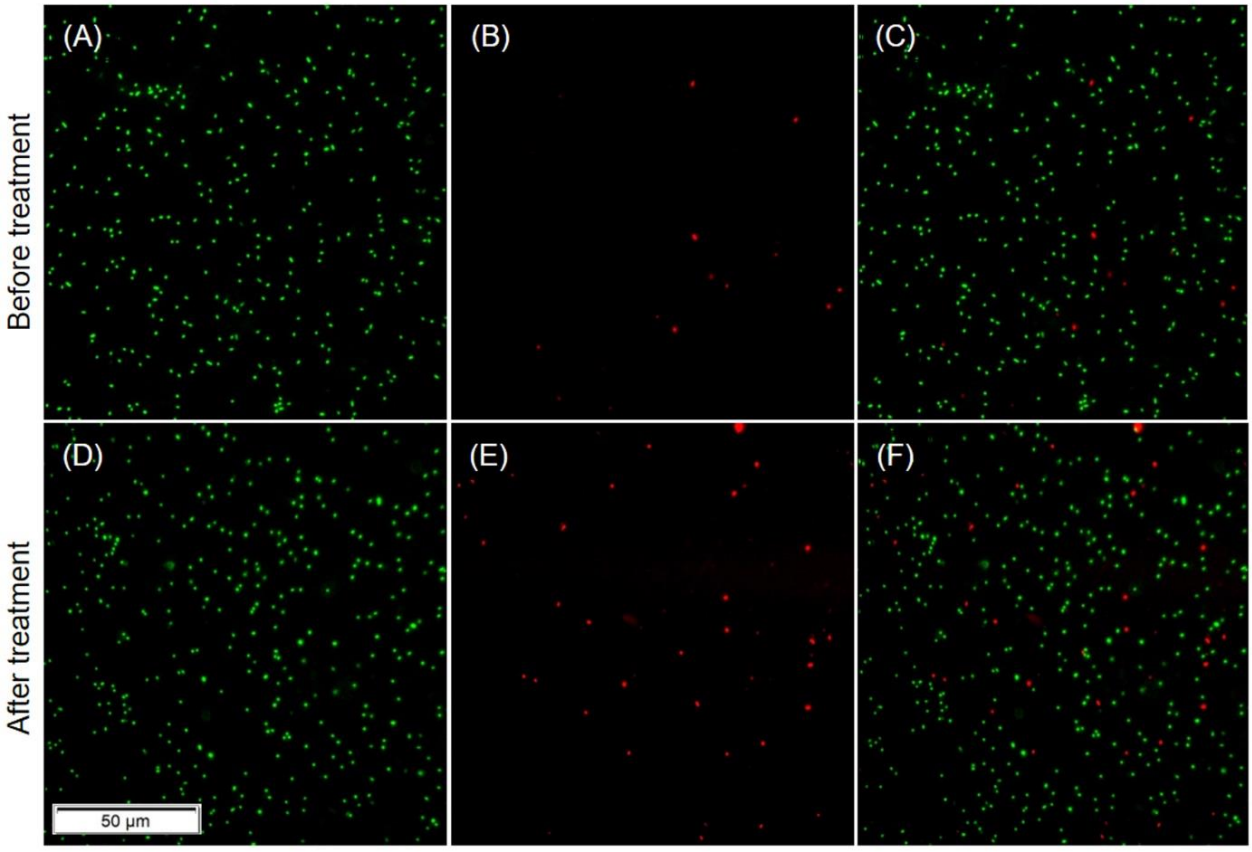



**Figure 6.** Confocal scanning laser microscope image of MDR *A. hydrophila* BT14 viability following the 1st treatment with 10 min NB-$O_3$ (A-C: before 1st treatment and D-F: after 1st treatment). Figure C is merged by A and B whereas figure F is merged by D and E. Green fluorescent indicates live bacterial cells and red fluorescent indicates dead bacterial cells using LIVE/DEAD *Bac*light Bacterial Viability Kit with two staining reagents SYTO®9 and PI.

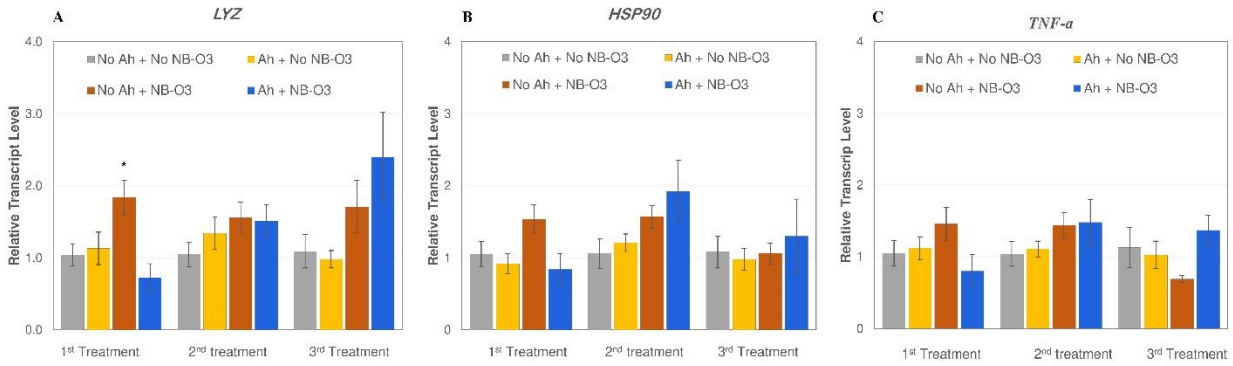



**Figure 7.** Relative expression of *LYZ* (**A**), *HSP90* (**B**) and *TNF-a* (**C**) in fish gills in 4 groups: no Ah + no $NB\text{-}O_3$, Ah + no $NB\text{-}O_3$, no Ah + $NB\text{-}O_3$ and Ah + $NB\text{-}O_3$ after 1st, 2nd and 3rd treatment with $NB\text{-}O_3$. The expression of target genes was normalized using *EF-1a*. Value of relative transcript level are mean ± a standard error of the mean (SEM) bar (n = 4) and "*" above the bar indicates significant difference between groups ($p < 0.05$).

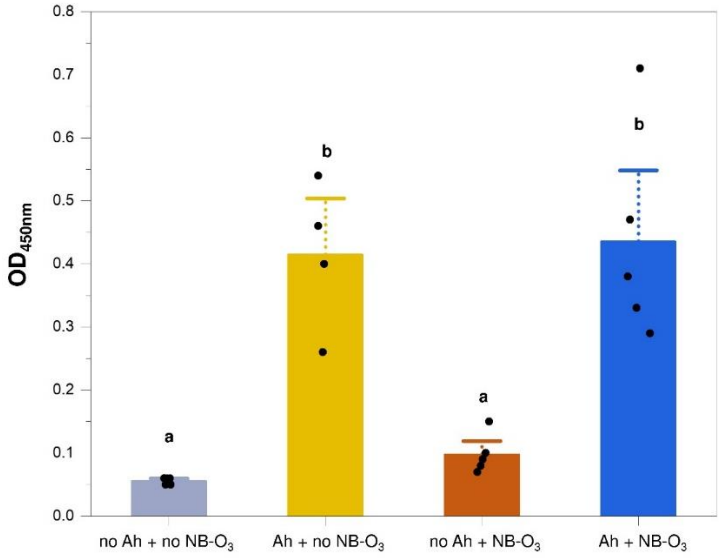



**Figure 8.** Indirect ELISA analysis of *A. hydrophila* specific IgM antibody. Fish sera were collected on day 14 and 1:256 dilutions were used to test for antigen specific IgM. Data were expressed as mean absorbance at $OD_{450nm}$ with a SEM bar. One dot represents one biological replicate (n = 4 in group Ah + no NB-$O_3$, n = 5 in other groups). Different letters above the bar indicate significant difference between groups ($p < 0.05$).

## Table S1. Identification and antibiogram of *A. hydrophila* BT14

**Identification using MALDI-TOF MS**

| Sample name | Organism (best match) | Score value | Organism (second-best match) | Score value | Identification |
|---|---|---|---|---|---|
| BT14 | *Aeromonas hydrophila* | 2.15 | *Aeromonas hydrophila* | 2.08 | *Aeromonas hydrophila* BT14 |

**DNA gyrase subunit B (*gyr*B) sequence (1030 bp) of *A. hydrophila* BT14 (99.03% identity to *A. hydrophila* 2TS54 strain (accession number MT371989.1)**

**ATCAGGGTGCCCACCTCCTGGGAGGAGATCATCTTGTCGAAACGGGCCTTCTCCACGTTCAGGATCTTGCCCTTGAGCGGCAGGATGGCCTGGTTCTTCCGGT**
**TGCGACCCTGCTTGGCGGAACCGCCAGCAGAGTCCCCTTCCACTATGTAGAGTTCGGAGAGCGCCGGGTCTTTTTCCTGACAGTCGGCCAGCTTGCCGGGCAG**
**ACCGGCAATATCCAGCGCGCCTTTGCGGCGGGTCAGTTCGCGAGCCTTGCGGGCCGCTTCACGGGCACGGGCCGCATCGATGATCTTGTTGACCACGATCTTG**
**GCATCGCCCGGGTTTTCCAGCAGGAAGTCGGCCAGCTTCTCGCCCATCGCCTGTTCGACTGCGGTCTTCACTTCGGAAGAGACCAGCTTGTCCTTGGTCTGGG**
**AGGAGAACTTAGGGTCAGGCACCTTGACGGAGATAACGGCAATCAGACCTTCACGCACGTCGTCGCCACTGGCGGCAGACTTGGCCTTCTTGCTGTAGTCCTC**
**TTTGTCCATGTAGGAGTTGAGGGTACGGGTCAGCGCGGTACGGAAGCCCACCAGGTGGGTGCCGCCATCACGCTGGGGAATGTTGTTGGTGAAGCAGTAGACC**
**CCTTCCTGATAGGCGTCGTTCCACTGCATCGCCACTTCGACGCCAATGCCGTCCTGCTCGGTGGTGAAGTGGAACACCTTCGGGTGGATCGGGGTCTTGTTCT**
**GGTTCAGGTACTCGACGAACGCCTTAATGCCGCCTTCGTAGCAAAAGTGCGCCTCGCGGCCGTCACGCTCGTCCATCAGACGGATGGAGACGCCGGAATTGAG**
**GAAGGAGAGCTCGCGCAGACACTTGGCCAGGATCTCGTAGTGGAACAGGGTGTCGCTGAAGATGGTCGGGCTCGGCCAGAAACGCACTTCGGTCCCGGTGGTG**
**GTGGCATCGCCCATCTGCTTGAGCGGCGCCTGCGGCTCACCCAGGTGATAGGTCTGCTCGTAAAAATGACCGTTGCCACGGAAGGTCAGCAACAACTTGTCAA**

**Antibiogram of *A. hydrophila* BT14**

| Antimicrobials | Antimicrobial class | Concentration (µg) | Zone diameter (mm) | Zone diameter (mm) interpretive criteria | | | Result | Standard protocol |
|---|---|---|---|---|---|---|---|---|
| | | | | Resistant | Intermediate | Susceptible | | |
| Ampicillin | Penicillins | 10 | 0 | ≤ 13 | 14 - 16 | ≥ 17 | Resistant | CLSI M100 |
| Tetracycline | Tetracyclines | 30 | 11 | ≤ 11 | 12 - 14 | ≥ 15 | Resistant | CLSI M45-A3 |
| Sulfamethoxazole-Trimethoprim | Folate pathway inhibitors | 23.75 - 1.25 | 0 | ≤ 10 | 11 - 15 | ≥ 16 | Resistant | CLSI M45-A3 |
| Ciprofloxacin | Flouroquinolones | 5 | 30 | ≤ 15 | 16 - 20 | ≥ 21 | Susceptible | CLSI M45-A3 |
| Chloramphenicol | Phenicols | 30 | 15 | ≤ 12 | 13 - 17 | ≥ 18 | Susceptible | CLSI M45-A3 |

**Table S2.** Water parameters in Nile tilapia culture tank during 10 min NB-$O_3$ treatment in MRS

| Treatment | Measurement time | Temperature (°C) | | pH | | DO (mg/L) | | ORP (mV) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | NB-$O_3$ treatment | Control | NB-$O_3$ treatment | Control | NB-$O_3$ treatment | Control | NB-$O_3$ treatment |
| 1st | Before treatment | 28.1 ± 0.2 | 27.7 ± 0.0 | 7.76 ± 0.03 | 6.64 ± 0.42 | 6.58 ± 0.08 | 6.97 ± 2.21 | 470.4 ± 3.1 | 421.3 ± 1.0 |
| | 10 min treatment | 28.1 ± 0.3 | 28.3 ± 0.0 | 8.06 ± 0.06 | 8.03 ± 0.05 | 6.57 ± 0.04 | 15.33 ± 1.76 | 412.8 ± 0.1 | 367.8 ± 7.5 |
| | 10 min post treatment | ND | 28.6 ± 0.0 | ND | 7.98 ± 0.03 | ND | 12.69 ± 0.68 | ND | 328.4 ± 6.1 |
| 3rd | Before treatment | 30.3 ± 0.0 | 28.6 ± 0.1 | 7.89 ± 0.03 | 7.63 ± 0.08 | 4.98 ± 0.42 | 5.82 ± 0.04 | 326.7 ± 3.8 | 465.5 ± 0.7 |
| | 10 min treatment | 30.5 ± 0.1 | 29.2 ± 0.1 | 7.98 ± 0.08 | 7.92 ± 0.17 | 4.82 ± 0.40 | 12.29 ± 0.88 | 323.8 ± 1.8 | 387.2 ± 8.2 |
| | 10 min post treatment | ND | 29.3 ± 0.1 | ND | 7.97 ± 0.16 | ND | 9.55 ± 1.24 | ND | 350.6 ± 11 |
| 5th | Before treatment | 29.8 ± 0.1 | 29.8 ± 0.6 | 7.59 ± 0.28 | 7.82 ± 0.00 | 4.72 ± 1.25 | 4.89 ± 0.31 | 433.8 ± 1.2 | 432.8 ± 0.8 |
| | 10 min treatment | 29.5 ± 0.1 | 30.0 ± 0.4 | 7.98 ± 0.21 | 8.08 ± 0.04 | 5.12 ± 0.40 | 12.7 ± 0.32 | 405 ± 10.3 | 400.3 ± 9.0 |
| | 10 min post treatment | ND | 30.3 ± 0.2 | ND | 8.14 ± 0.02 | ND | 9.28 ± 0.80 | ND | 386.2 ± 1.1 |
| 7th | Before treatment | 29.1 ± 0.1 | 29.4 ± 0.3 | 7.91 ± 0.03 | 7.95 ± 0.06 | 4.91 ± 0.12 | 5.39 ± 0.08 | 296.7 ± 9.3 | 309.9 ± 1.4 |
| | 10 min treatment | 29.1 ± 0.1 | 29.7 ± 0.3 | 7.92 ± 0.06 | 8.01 ± 0.04 | 5.19 ± 0.30 | 12.26 ± 2.25 | 290.4 ± 4.6 | 310.3 ± 4.7 |
| | 10 min post treatment | ND | 29.8 ± 0.4 | ND | 8.06 ± 0.04 | ND | 9.66 ± 1.70 | ND | 310.1 ± 4.9 |

DO: Dissolve Oxygen, ORP: Oxidation Reduction Potential, OB-$O_3$: ozone-nanobubbles, ND: Not done

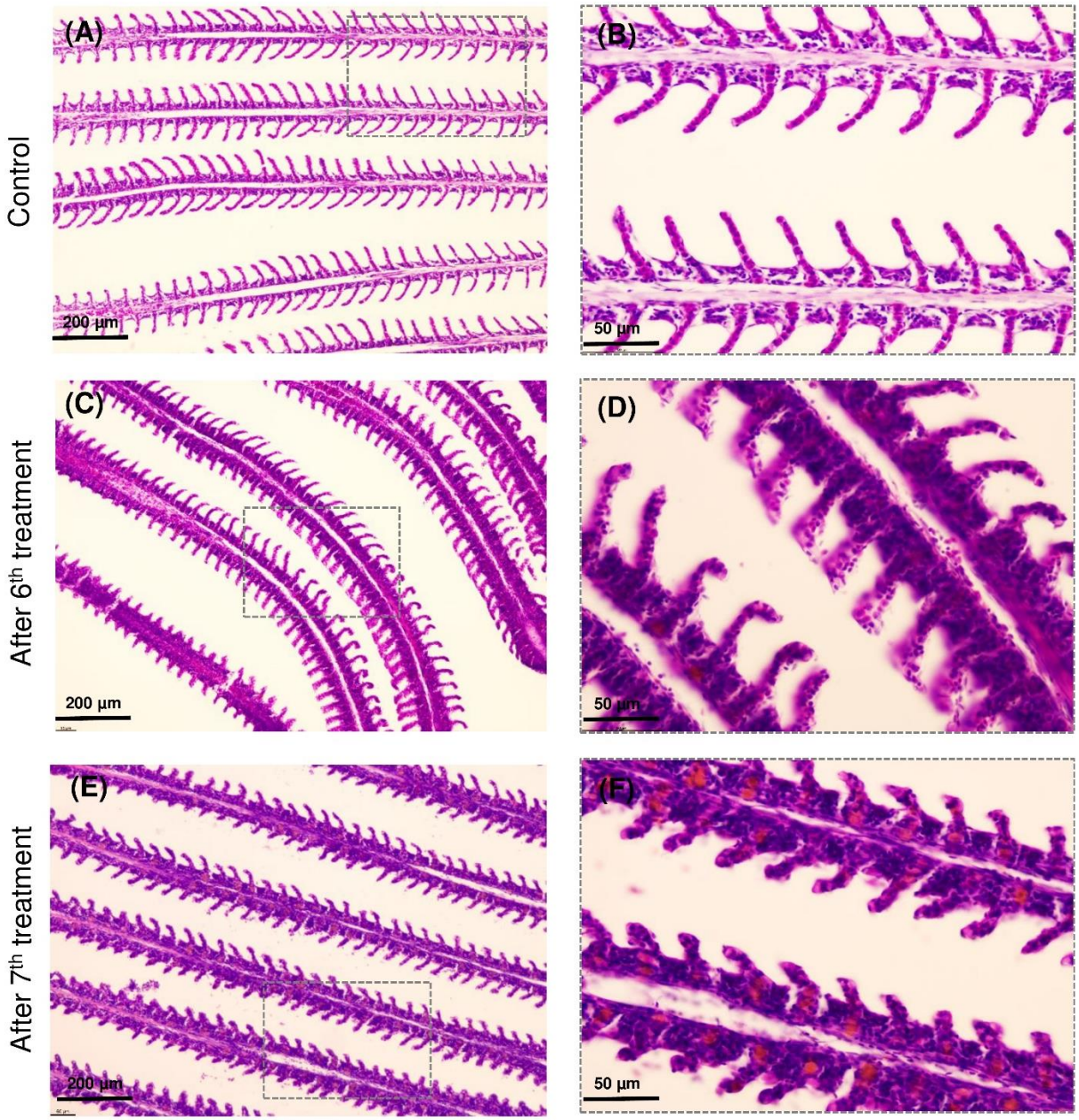



**Figure S1.** Representative photomicrographs of H&E stained sections of the gills taken at low and high magnifications. A, B, normal gill morphology from fish in control group. C, D, slight alterations in the gill lamella observed after 6$^{th}$ treatment. E, F, alteration and increasing melanin containing cells in the gill filaments after 7$^{th}$ treatment.