Ozone nanobubble treatment effectively reduced pathogenic Gram positive and negative bacteria in freshwater and safe for tilapia

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Running title: NB-O_3 reduces pathogenic bacteria in water

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Highlights

- Potential application of ozone nanobubble (NB-O_3) technology in freshwater aquaculture was explored in a laboratory setting.

- A water treatment protocol was established based on examination of nanobubble size, concentration, its disinfection property, and safety to the fish.

- A 10-min NB-O_3 treatment effectively reduced >96% of the bacteria (both pathogenic Gram positive and Gram negative bacteria) in water.

- A 10-min NB-O_3 treatment was safe for tilapia and did not negatively affect gill morphology of the fish.

- Nonchemical disinfection with NB-O_3 technology is promising for controlling bacterial diseases in freshwater aquaculture.

Abstract

High concentrations of pathogenic bacteria in water usually results in outbreaks of bacterial diseases in farmed fish. Here, we explored the potential application of an emerging nanobubble technology in freshwater aquaculture. Specifically, we aimed to determine if this technology was effective at reducing the concentration of pathogenic bacteria in the water, and to assess whether it was safe for fish. 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 with majority sizes less than 130 nm and ozone level of ~800 mV ORP. A single treatment with water contaminated with either *Streptococcus agalactiae* or *Aeromonas veronii* effectively reduced 96.11-97.92 % of the bacterial load. This same protocol was repeated 3 times with 99.93-99.99 % reduction in the bacterial concentration. In comparison, bacterial concentration the control tanks remained the same level during the experiments. In fish-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 i.e bacterial concentration was reduced by 42.94 %, 84.94 % and 99.27 % after the first, second and third treatments, respectively. To evaluate the safety of NB-O$_3$ to fish, juvenile Nile tilapia were exposed to NB-O$_3$ treatment for 10 minutes. 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 morphology. However, damage in the gill filaments was noticed in the fish receiving two or three consecutive exposures within the same day. Results of all the experiments conducted in this study suggest that NB-O$_3$ technology is promising for controlling pathogenic bacteria in aquaculture systems, and may be useful at reducing the risk of bacterial disease outbreaks in farmed fish.

Keywords: bacterial load, disinfection, NB-O$_3$, ozone nanobubble, tilapia

Introduction

The aquaculture sector has played a vital role in global food security. It supplies protein for approximately 4.5 billion peoples 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 (Watts et al., 2017; World Bank, 2014). Of particular importance to public health has been the increase in antimicrobial resistance (AMR). Alternatives for these products to control bacterial infections in all food production sectors have increased over the last few years (Reverter et al., 2020; Watts et al., 2017). In the aquaculture sector, previous and current approaches focus mainly on antibacterial compounds derived from natural products, probiotics, immunostimulants, and vaccines for prevention strategies (Reverter et al., 2020; Watts et al., 2017).

Other prevention strategies, usually used in closed recirculating systems to reduce the bacterial concentration that fish are exposed to, include water treatments with UV or Ozone. Both of these treatments have issues for the aquaculture industry. UV requires that water be very clean when it is exposed to the light source, which renders it less than ideal in pond culture. Ozone has a low dissolution property, rapid decomposition in water and can be lethal to fish (Huyben et al., 2018; Xia et al., 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 (Agarwal et al., 2011; Yamasaki et al., 2005) and recently being applied in aquaculture for the increasing concentration of dissolved oxygen in intensive aquaculture systems (Agarwal et al., 2011; Anzai et al., 2019; Mahasri et al., 2018; Rahmawati et al., 2020). This technology involves the injection of nano or ultrafine bubbles with a chosen gas into water (Agarwal et al., 2011; Anzai et al., 2019). Unlike macro- and microbubbles, these nanobubbles with a diameter less than 200 nm, have neutral buoyancy, thus remain in water for days (Agarwal et al., 2011; Takahashi et al., 2007).

Kurita et al. (2017) investigated the effect of exposing parasitic planktonic crustaceans to nanobubbles created from ozone (NB-O_3). They reported that a 25 min treatment with NB-O_3 successfully reduced 63% of the parasites compared to the untreated group. Most 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

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86   in Japanese aquaculture systems. In another study, Imaizumi et al. (2018) reported that NB-O_3

87   could be used for disinfection of *Vibrio parahaemolyticus*, a unique strain causing early mortality

88   syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) in whiteleg shrimp (*Penaeus*

89   *vannamei*). However, in their study NB-O_3 showed a negative effect on shrimp when administered

90   at a high level (970 mV ORP). When the NB-O_3 treated water was diluted by 50% and the results

91   revealed that all shrimp exposing to pathogenic *V. parahaemolyticus* survived from the bacterial

92   infection, while all shrimp died in the group without the NB-O_3 treatment (Imaizumi et al., 2018).

93   Preliminary results of NB-O_3 in marine aquaculture is promising. The impact of nanobubbles in

94   water of different salinity suggests that this technology may be even more effective in fresh water

95   (Li et al., 2013). However, there is a lack of studies on its effect on fresh water fish and their

96   pathogens. This study aims at the assess whether NB-O_3 can be used on fresh water fish pathogens

97   and is safe for tilapia.

98

Materials and Methods

Concentration and size of nanobubbles

101   Two trials were carried out separately using the nanobubble generator (model: aQua+075MO;

102   maker: AquaPro Solutions Pte Ltd, Singapore) to determine the size of the air and oxygen

103   nanobubbles. The generator was operated in 100 L-fiberglass tanks containing 50 L distilled water

104   for 30 min, with either natural air or oxygen gas with a flow rate of 1 L/min. 50 mL of water was

105   sampled from each tank at 10, 15, 20, and 30 min. Water samples prior to the addition of

106   nanobubbles were used as baseline standards. The concentration and size of nanobubbles were

107   determined by NanoSight NS300 (Malvern Panalytical Ltd) with three replicates for each sample.

108   Ozone nanobubble measurement was not done due to its oxidation effect on the NanoSight

109   machine.

Effect of ozone nanobubbles (NB-O_3) treatment on water parameters

111   The experiment was performed in two separate tanks to evaluate the effect of NB-O_3 on water

112   parameters. Each tank contained 50 L of de-chlorinated tap water. Nanobubble generator was

113   operated for 10 min in each tank. The temperature in degree Celsius ($T^°$), dissolved oxygen (DO),

114   pH and oxidation reduction potential (ORP) were measured using a multi-parameter meter (YSI

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115   Professional Plus) every 1-2 min during 10 min-run and 15 min after stopping the nanobubble

116   generator.

Bacterial isolates and growth conditions

118   The Gram-positive bacterium *Streptococcus agalactiae* isolated from a tilapia farm which was

119   experiencing an outbreak of Streptococcosis, and Gram negative bacterium *Aeromonas veronii*

120   associated with hemorrhagic septicemia in tilapia (Dong et al., 2017) were used in this study. Prior

121   to experiments, the bacterial isolates were recovered from bacterial stocks stored at -80 $^\circ$C using

122   tryptic soy agar (TSA) medium, incubated at 30 $^\circ$C. To prepare bacterial inoculum, single bacterial

123   colonies were inoculated in 10 mL of tryptic soy broth (TSB) overnight at 30 $^\circ$C on a shaker

124   platform (150 rpm). Five mL of bacterial culture was then sub-cultured in 500 mL of TSB,

125   incubated with gentle shaking (150 rpm) at 30 ℃ until OD_{600} reached 0.8 (equivalent to ~10^8

126   CFU/mL). For subsequent trials, 100 mL of the bacterial culture was added into a tank containing

127   50 L de-chlorinated tap water.

Effect of treatment time on disinfection property of NB-O_3

129   An initial trial was carried out to investigate the effect of treatment time on the disinfection

130   property of NB-O_3. *S. agalactiae* was used as a representative bacterium in this time-course trial.

131   The experiment was performed in two 100 L fiberglass tanks containing 50 L of de-chlorinated

132   tap water each mixed with 100 mL bacterial culture ($OD_{600} = 0.8$). One tank was treated with NB-

133   O_3 while another tank was served as a control without NB-O_3. Water was sampled from the four

134   corners and the center of the tank (1 mL per spot). The samples were pooled together for

135   conventional plate count enumeration at different time points. Samples were collect prior to

136   inoculation (0 min), during treatment (5, 10 and 15 min) and after treatment (5, 10, and 15 min).

137   The samples were 10 fold-serially diluted with sterile saline solution (NaCl 0.85%) and 100 µL of

138   each dilution was spread on TSA in duplicates, incubated at 30 $^\circ$C for 36 h. Dilutions with a number

139   of colonies ranging from 30-300 were used for counting and mean bacterial colonies of two

140   replicate plates were calculated and expressed as CFU/mL. The percentage of bacterial reduction

141   was calculated based on the formula below.

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

143   We compared the reduction in bacterial concentration in the tank exposed to ozone and the control

144   tank for differences.

Effect of NB-O$_3$ on pathogenic Gram-positive and Gram-negative bacteria

146   To evaluate the effect of NB-O$_3$ on bacterial pathogens of tilapia, *S. agalactiae* and *A. veronii* were

147   used as representative Gram positive and Gram negative bacteria, respectively. Each set of

148   experiment comprised of 1 control tank (having normal aerator) and 3 treatment tanks (10 min-

149   treating with NB-O$_3$ 1 to 3 times at 15 min intervals). Each tank containing 50 L de-chlorinated

150   tap water was mixed with 100 mL of bacterial suspension (OD$_{600}$ = 0.8) as described above. Water

151   was sampled from control and treatment tanks before (0 min) and 15 min after the end of each

152   treatment to establish the bacterial concentration and the percentage of bacterial reduction.

153   Temperature, pH, DO and ORP were also recorded during the experiment.

Effect of NB-O$_3$ treatment on total bacteria in fish-culture water

155   Investigation of the disinfection property of NB-O$_3$ was also evaluated using "culture" water

156   (water from the fish-culture tanks which contained organic matter e.g. fish feces, mucus, left over

157   feed and unknown aquatic bacterial flora). Fish-culture water was taken from tanks containing

158   juvenile Nile tilapia (*Oreochromis niloticus*). A trial using three 10 min- NB-O3 exposure times

159   administered 15 minutes apart was applied in to three fiberglass tanks containing 50 L fish-cultured

160   water each. Water sampling scheme for total bacterial counts was conducted before and 15 min

161   after the end of each treatment. Water temperature, pH, DO and ORP were monitored.

Effect of NB-O$_3$ on fish health and gill morphology

163   Animal use protocol in this study was granted by the Thai Institutional Animal Care and Use

164   Committee (MUSC62-039-503). To investigate whether NB-O$_3$ treatment had negative effects on

165   gill morphology and fish life, we carried out a trial which included 2 control and 2 treatment tanks,

166   each tank containing 20 apparently healthy *O. niloticus* juveniles of 6-8 g body weight. The 100 L

167   fiberglass tanks had 50 L of de-chlorinated tap water. For the treatment tanks, NB-O$_3$ was applied

168   at 15 minute intervals 3 times for 10 minutes. The control tanks were treated with normal aeration.

169   Two fish from each tank were randomly sampled after every treatment for wet-mount examination

170   and histological study of the gills and the remaining fish were monitored for 48 h. For histological

171   analysis, gill arches from one side of each fish were preserved in 10% neutral buffer formalin with

172   a ratio of 1 sample/10 fixative (v/v) for 24 h before being placed in 70% ethanol for storage. The

173   samples were then processed for routine histology and stained with hematoxylin and eosin (H&E).

174   Gill morphology of the experimental fish was examined under a microscope equipped with a

175   digital camera. We compared fish behavior, the gills of treated and untreated fish visually. Fish

176   were also monitored for mortality over a period of 48 hours post treatment.

177

178   ## Results

179   **Concentration and size of nanobubbles**

180   The results of NanoSight readings from the air nanobubbles (NB-Air) (Fig. 1A) and the oxygen

181   nanobubbles (NB-O_2) (Fig. 1B) were similar. Majority of nanobubbles (or particles) were less than

182   130 nn in size. The concentration of these bubbles after a 10 min treatment was of $2.39 \times 10^7 \pm$

183   1.01×10^7 particles/mL for NB-Air and $3.03 \times 10^7 \pm 1.11 \times 10^6$ particles/mL for NB-O_2. Increasing

184   treatment times (15, 20 and 30 min) generated larger bubbles with quantity in the same order of

185   magnitude (Fig. 1). The result confirmed that the nanobubbler used in this study produced

186   nanobubbles and 10 min operation in 50 L of water generated the most uniform nano-sizes. Thus

187   this scheme was also applied to generate ozone nanobubbles (NB-O_3).

188   **Effect of NB-O_3 treatment on water parameters**

189   Changes of water parameters (T°, DO, pH and ORP) during and after treatment with NB-O_3 were

190   consistently similar between the two trials (Fig. 2). Significant changes were observed in DO and

191   ORP values while T^o increased slightly (~2°C) and pH remained relatively stable during and after

192   NB-O_3 treatment. With respect to DO, the value increased rapidly reaching to 23-25 mg/L after 10

193   min treatment and reduced slowly to ~20 mg/L 15 min post treatment. By contrast, ORP increased

194   quickly, reaching over 700 mV within 6 min and ~800 mV within 10 min and dropped back to the

195   starting level (~300 mV) 15 min post treatment.

196   **A 10-min NB-O_3 treatment reduced >90% bacterial loads in water**

197   As shown in Fig. 3, similar bacterial loads (*S. agalactiae*) at the starting point were used in the

198   control tank (1.17×10^6/mL) and treatment tank (1.83×10^6/mL). However, upon NB-O_3

199   treatment, bacterial density reduced quickly during exposure time in the treatment tank. The

200   percentage drop in concentration in the treated group during the treatment at 5, 10 and 15 min were

201 62.30%, 97.76% and 99.40%, respectively, indicating that disinfection occurred rapidly during the

202 treatment process. Bacterial concentration remained low 15 min after treatment. In contrast,

203 bacterial concentration in the control tank remained stable at ~10^6 CFU/mL during the same time

204 period (Fig. 3). With respect to water quality, changes were observed only in the treatment tank.

205 DO increased from 6.2 mg/L (before treatment) to 21.8 mg/L (at 5 min), 25.8 mg/L (at 10 min)

206 and 27.9 mg/L (at 15 min) and dropped to 23.3 mg/L at 15 min post treatment. Water temperature

207 increased approximately 1 $^{\circ}$C every 5 min of the treatment, from 26.5 $^{\circ}$C (before treatment) to 29.2

208 $^{\circ}$C (at 15 min) and remained at this temperature 15 min post treatment. Relatively no change was

209 observed in pH (7.6-7.7) and ORP (293-306 mV) during the experiment.

210 **NB-O₃ treatment effectively reduced both pathogenic Gram positive and negative bacteria**

211 The trial with *S. agalactiae* started with similar bacterial loads; 1.17×10^6 CFU/mL in the control

212 tank and 3.45×10^6 CFU/mL in treatment tanks (Fig. 4A). A single 10-min treatment with NB-O₃

213 effectively reduced 96.11% bacterial load in the tank. When the same protocol was repeated for

214 the second and third time, 99.93% and 99.99% bacteria were inactivated, respectively. The

215 bacterial concentration in the control tank (without the NB-O₃ treatment) was maintained at 10^6

216 CFU/mL (Fig. 4A). Similar patterns were also observed in the trials with the Gram negative

217 bacterium *A. veronii*. Average initial bacterial counts of *A. veronii* for control and treatment tanks

218 were 1.03×10^6 CFU/mL and 1.65×10^6 CFU/mL, respectively. Following the 1st, 2nd and 3rd NB-

219 O₃ exposure, bacterial loads were reduced by 97.92, 99.99 and 99.99%, respectively (Fig. 4B). No

220 significant changes in bacterial counts were observed in the control tank during the experiment

221 (Fig. 4B). Changes in water quality were shown in Table 1. Temperature changes in the NB-O₃

222 treatment tanks were 1.9-2.6 °C after the 1st treatment, and 4.3-4.7 °C after 3rd treatment, whereas

223 pH values were relatively stable at 7.4 to 8.0. Notably, DO increased sharply (from 3.9-4.4 to 26.4-

224 29.9 mg/L) and was maintained at this high level after every treatment, while ORP values did not

225 increase as much as seen in the water study without bacteria (Fig. 2).

226 **Effect of NB-O₃ treatment on total bacterial counts in fish-cultured water**

227 In this trial, the bacterial load was compared before and after treatment. Before treatment, the total

228 bacterial concentration in the fish-cultured water was $6.93 \times 10^5 \pm 7.81 \times 10^5$ CFU/mL (Fig. 5).

229 After exposure to NB-O₃ for 10 min, 42.94% of the bacteria was inactivated. When the same

230 protocol was repeated, 84.94% and 99.27% bacteria were reduced in these treatments (Fig. 5).

231 During the experiment, DO increased sharply from very low at the beginning 0.6 ± 0.1 mg/L to

232 27.7 ± 0.6 mg/L after the first 10 min treatment. The DO was 30.8 ± 7.7 mg/L after the second 10

233 min treatment, and 28.7 ± 7.6 mg/L after the third treatment. Water temperature was increased

234 slightly from 26.7 ± 0.3 to 28.3 ± 0.4, 29.8 ± 0.3 and 31.2 ± 0.2 $^{\circ}$C after the 1st, 2nd and 3rd treatment,

235 respectively. In contrast, pH and ORP were stable during the experiment (7.5-7.6 for pH, 210-250

236 mV for ORP).

Effect of NB-O₃ on fish health and gill morphology

238 No Fish died during the NB-O₃ treatments or up to 48 h post treatment when we stopped the

239 experiment. However, abnormal signs were observed in the gills in all fish examined after

240 receiving the second and third treatments. These signs included reddening at the base of the fins,

241 erratic swimming, and the attachment of bubbles to the body surface. These bubbles disappeared

242 after several minutes of fish movement.

243 The wet-mount examination of the gills revealed no significant difference between control and

244 treatments at any of the treatment times (Fig. 6A-D). There were no gross clinical signs of gas

245 bubble disease. H&E stained sections of the gills showed the normal structure of the gills in the

246 first treatment group (Fig. 6F) compared to the control group (Fig. 6E). However, abnormal

247 changes were observed in the fish exposed to the second treatment. Aggregates of basal cells at

248 the base of the secondary lamellae were apparent with increasing severity corresponding to the

249 dose of ozone exposure (Fig. 6G, arrows). Gills in the third experiment had some loss of the

250 secondary lamella (Fig. 6H, arrows) and infiltration of red blood cells (blood congestion) (Fig.

251 6H).

252 During the treatment, water parameter (T$^{\circ}$, DO and pH) fluctuations were similar (Table 2) to the

253 experiment with clean water spiked with *S. agalactiae* or *A. veronii* and NB-03 with the exception

254 that tanks exposed to ozone had ORP levels of 860-885 mV after each 10 min treatment.

Discussion

256 Application of ozone gas using nanobubble technology is relatively new to aquaculture. A previous

257 study reported the sterilization efficacy of NB-O₃ against pathogenic *V. parahaemolyticus,* a Gram

258 negative bacteria causing disease in marine shrimp (Imaizumi et al., 2018). In this study, we first

259   revealed that NB-O$_3$ has disinfection property against two common bacterial pathogens of

260   freshwater farmed tilapia, *S. agalactiae* and *A. veronii*.

261   The disinfection effectiveness of NB-O$_3$ likely depended on the organic load in the water. In clean

262   de-chlorinated tap water spiked with a known concentration of either *S. agalactiae* or *A. veronii*,

263   a single treatment (10 min) with NB-03 could successfully reduce more than 96% of the bacteria.

264   However, the same protocol applied to water that was taken from a tilapia-cultured tank, resulted

265   in a reduction in the disinfection potential by roughly half. Ozone is known as a strong oxidizing

266   agent (Powell et al., 2016; Summerfelt, 2003); thus, it was possible that organic matter (e.g. feces,

267   mucus, etc.) in the dirty tank water competed for the oxidation potential of the NB-03 thus slowing

268   down the speed of disinfection. This finding suggests that increased treatment time or increasing

269   the frequency of treatments, as was evaluated in this study, may be required for water with

270   abundant organic matter.

271   Interestingly, we also noticed that when bacteria (organic matter) was were added to water,

272   oxidation reaction potential (ORP) value did not increase as seen in the treatment with clean water

273   that did not have bacteria. Similarly, ORP did not increase during treatment with the fish-cultured

274   water (rich of organic matter). This indicated that the measurement of ORP as an indicator of O$_3$

275   level administered by the nanobubbler is not reliable in the presence of organic matter. It was

276   probably due to the rapid oxidation and degradation of O$_3$ molecules when contacting organic

277   matters. Therefore, to accurately measure ORP in NB-O$_3$ water, clean water without organic

278   matters is required. In clean water, ORP dropped relatively quick and returned to normal after we

279   ceased to introduce NB-O$_3$ (Fig. 2), indicating that O$_3$ molecules might be unstable even in the

280   form of nanobubbles. This is consistent with the high levels of DO maintained after treatment (Fig.

281   2), most likely derived from the degradation of O$_3$ into O$_2$ molecules (Batakliev et al., 2014). If

282   this is the case, the treatment of NB-O$_3$ in aquaculture farms could have dual benefits: disinfection

283   of bacteria and improvement of DO.

284   In this study, extreme treatment conditions (repeating treatments 3 times at 15 minute intervals)

285   was designed to evaluate the acute effect of NB-O$_3$ on the fish. Although multiple NB-O$_3$

286   treatments were not harmful to fish life, increased exposure caused damage to the fish gills. In fact,

287   a single treatment with 10-min NB-O$_3$ is enough to effectively reduce bacterial loads in water, and

288   it was safe for fish. If more than one 10-minute treatment of NB-03 was used there was some

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289   evidence of irritation to the gills. In reality, if this technology is applied in fish ponds, chances of

290   contact between fish and NB-O_3 will inevitably be low. However, given the evidence of gill

291   damage after 3 consecutive treatments more in-depth investigations are required prior to scaling

292   up NB-O_3 technology for commercial applications.

293   One of the limitations of this study was the limited sample size with the experiments. Our tank

294   numbers were limited by the number of nanobubble generators we had. Also we could not include

295   a normal ozone air-stone treatment group due to the personnel safety issue in our laboratory.

296   However, when we consider all the experiments together there is strong evidence to suggest that

297   NB-O_3 technology is not only a promising disinfection method but also enriches dissolved oxygen

298   in freshwater aquaculture and in low dose it is not harmful to the fish. As a disease prevention tool,

299   NB-O_3 treatment might be a novel approach to controlling overgrowth of pathogenic bacteria in

300   water, thus reducing the risk of bacterial diseases. This nonchemical disinfection technology may

301   be a promising alternatives to antibiotics as a means of reducing antibiotic use in aquaculture, and

302   possibly inadvertently reducing the risk of AMR. We are currently investigating the effect of NB-

303   O_3 on fish immunity and stress response, microbiome, plankton profiles and growth performance.

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Acknowledgements

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Disclaimers

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

312 **Tables and Figures**

313 **Table 1**: Comparative water parameters in control and NB-O_3 treatment groups with the presence

314 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 ± 0.3	27.5	25.9 ± 0.8
	10 min (1st)	26.9	29.8 ± 1.3	27.4	27.8 ± 0.6
	10 min (2nd)	27.0	30.4 ± 0.2	27.3	29.3 ± 0.6
	10 min (3rd)	27.0	31.5 ± 0.3	27.4	30.6 ± 0.5
DO	Before treatment	4.3	3.9 ± 0.5	4.7	4.4 ± 0.2
	10 min (1st)	4.3	27.8 ± 1.6	4.6	30.3 ± 2.4
	10 min (2nd)	4.2	26.9 ± 0.4	4.6	29.9 ± 0.1
	10 min (3rd)	4.2	26.4 ± 0.6	4.5	29.5 ± 1.0
pH	Before treatment	7.8	7.6 ± 0.2	7.8	8.0 ± 0.1
	10 min (1st)	7.8	7.5 ± 0.0	8.0	7.8 ± 0.1
	10 min (2nd)	7.8	7.4 ± 0.0	7.9	7.7 ± 0.1
	10 min (3rd)	7.8	7.4 ± 0.0	7.9	7.6 ± 0.0
ORP	Before treatment	325	290 ± 16	279	294 ± 6
	10 min (1st)	314	281 ± 7	289	271 ± 8
	10 min (2nd)	306	275 ± 4	261	270 ± 6
	10 min (3rd)	304	273 ± 3	265	272 ± 4

315 T^o, temperature in degree Celsius; DO, dissolved oxygen; ORP, oxidation reduction potential.

316 Values in the NB-O_3 treatment are expressed as mean $\pm$ SD from 3 replicates.

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317 **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^0	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	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	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^*	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

318 $T^{º}$, temperature in degree Celsius; DO, dissolved oxygen; ORP, oxidation reduction potential; ND,

319 not done. Values are expressed as mean $\pm$ SD from 3 replicates. *ORP dropped to normal (~330

320 mV) after 15 min of every treatment time.

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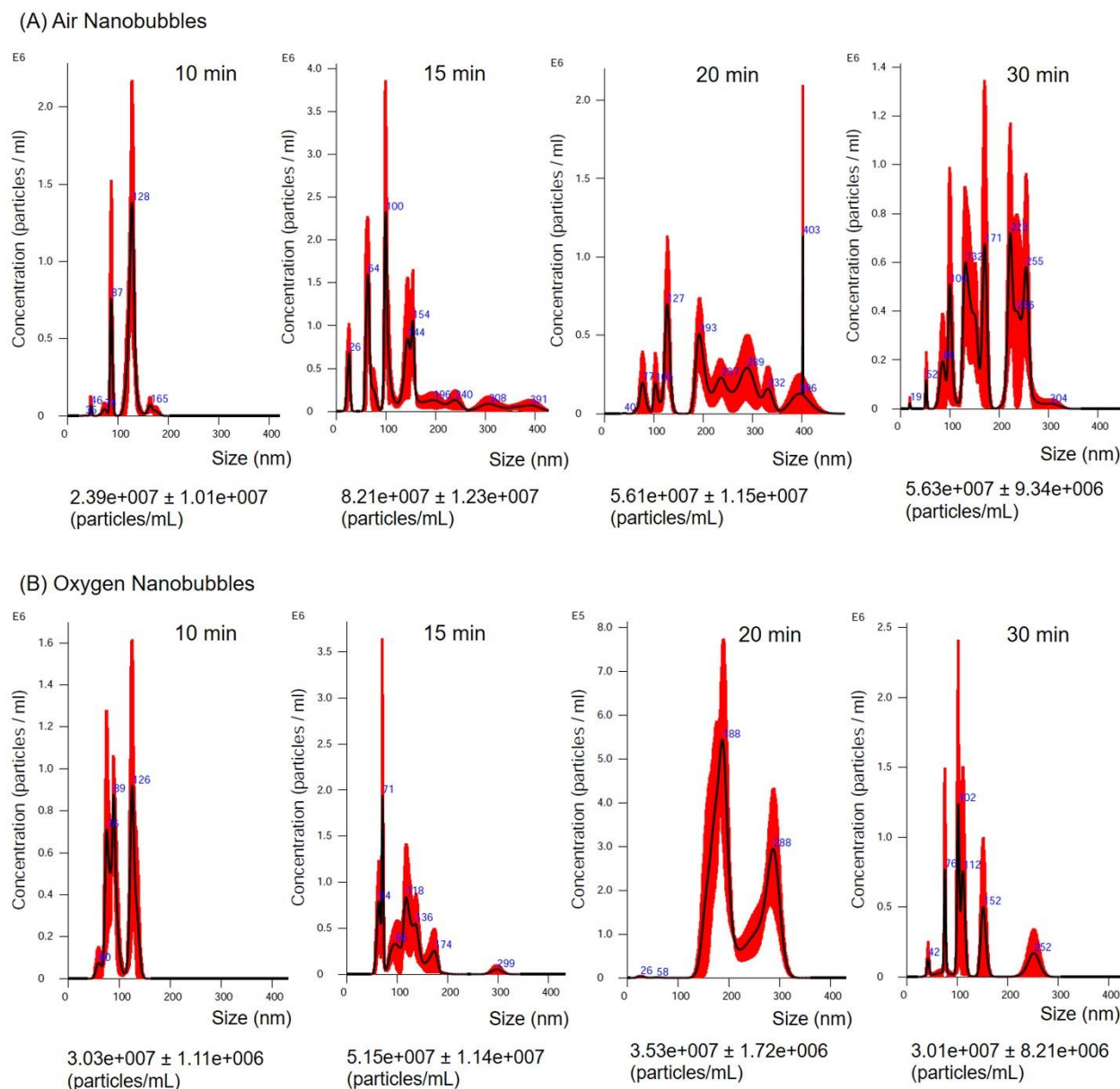
Figure 1: Concentration and size of bubbles generated using air (A) or oxygen (B) following treatment for 10, 15, 20 and 30 min. Peaks represent the concentration of dominant bubbles with similar sizes and blue numbers indicate the bubble sizes. Total concentrations of bubbles are shown at the bottom of each graph. Values were calculated from 3 replicate experiments.
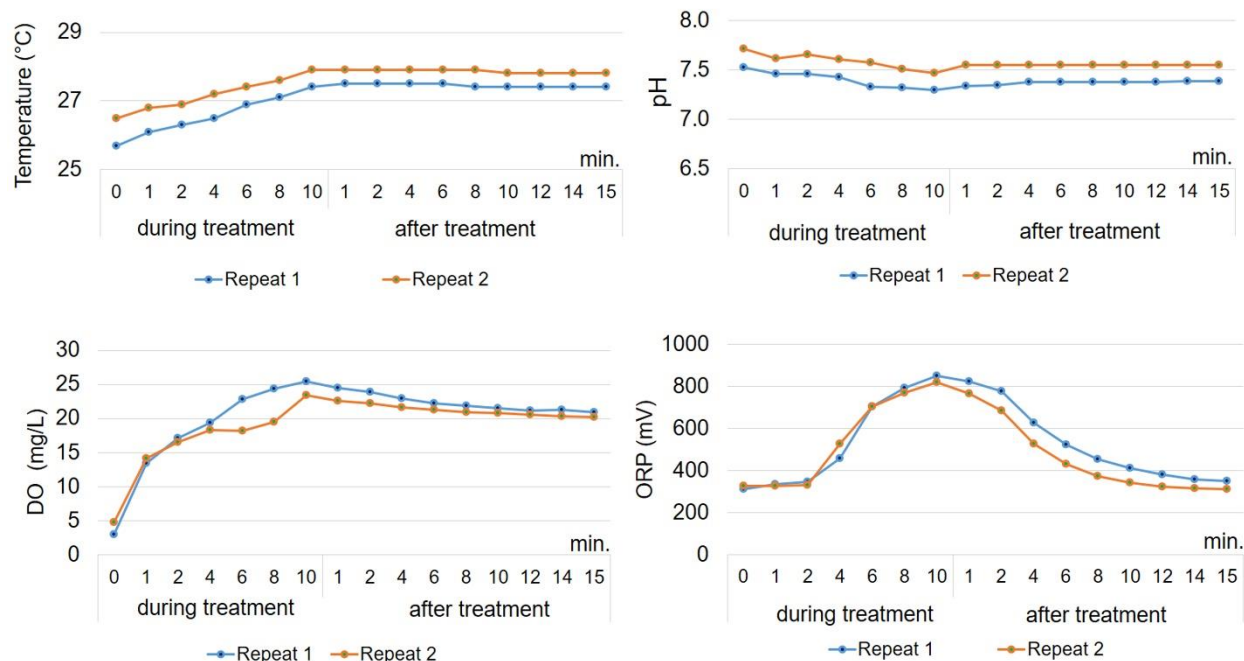
Figure 2: Water parameters (temperature, pH, DO and ORP) during 10 min treatment and 15 min after exposure to ozone nanobubbles. The experiment was carried out in 2 replicates.

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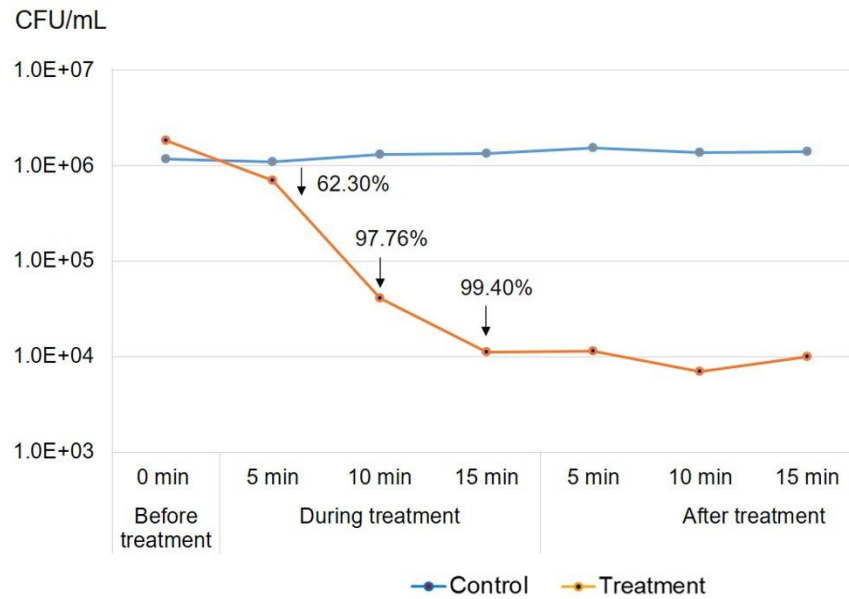
Figure 3: Number of *S. agalactiae* colony counts from the water with and without NB-O_3 exposure (treatment and control group, respectively). NB-O_3 treatment was performed for 15 min and stopped for 15 min. The water sample was collected from both the control and treatment groups every 5 min for plate count. Arrows indicated significant % reduction of bacterial counts compared to the starting point of the NB-O_3 treatment group

(A) Effect of NB-O_3 on *S. agalactiae*
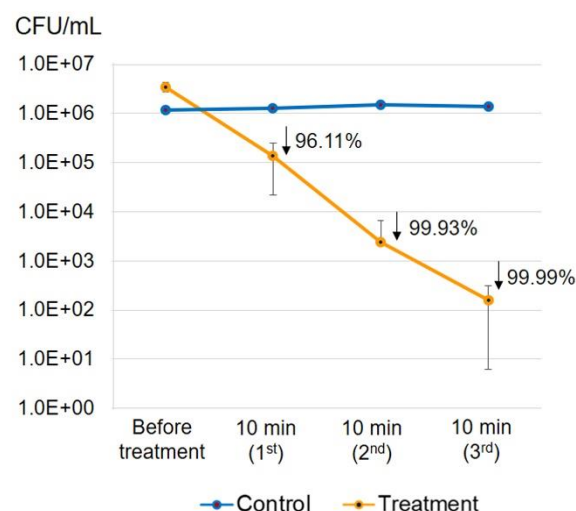
(B) Effect of NB-O_3 on *A. veronii*
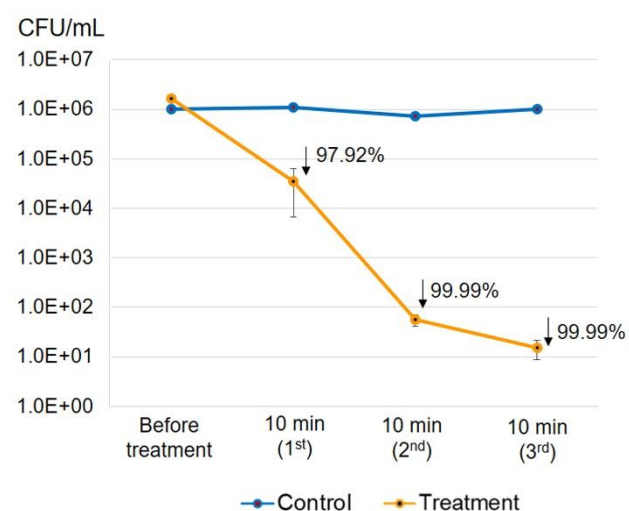
Figure 4: Bacterial counts of *S. agalactiae* (A) and *A. veronii* (B) upon exposure to NB-O_3 10 min three times continuously (orange lines) compared to that of the control water without NB-O_3 (blue lines). Arrows indicated % reduction of bacterial loads compared to the starting bacterial concentration. Bars represent standard deviation from 3 replicates.

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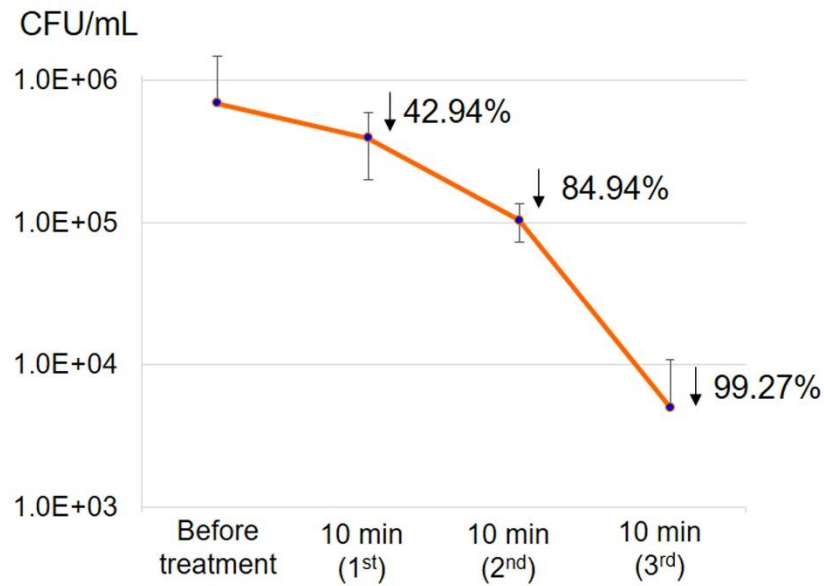
Figure 5: Total bacterial counts from fish-cultured water upon exposure to NB-O_3 10 min three times continuously. Arrows indicated % reduction of bacterial loads compared to the starting bacterial concentration. Bars represent standard deviation from 3 replicates.
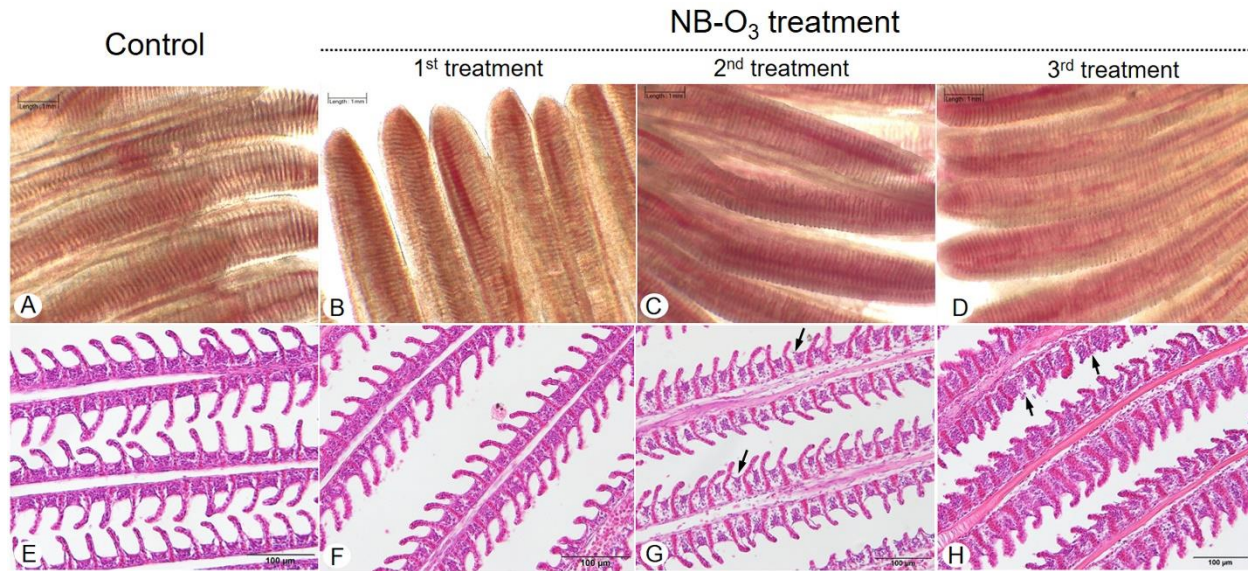
Figure 6: Photomicrographs of wet-mount (A-D) and H&E stained sections (E-H) of the gills of tilapia from control and NB-O_3 treatment. No significant difference in gill morphology by wet-mount between control (A) and treatment (B-D) groups. H&E staining revealed the normal structure of the gill filaments in both control (E) and the first treatment with NB-O_3 (F). Slight damage and shrunken of the basal lamellae (arrows) were observed in the fish received second exposure (G) and increasing damage of the gill filaments, loss of some secondary lamella (arrows) and severe blood congestion in the secondary lamellae were observed in the fish received the third exposure (H).

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