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Effect of ozone nanobubbles on the microbial ecology of pond water and safety for jade perch (*Scortum barcoo*)

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**ABSTRACT**

The microbial composition and diversity in aquaculture pond ecosystems are closely related to animal productivity and disease outbreaks. Interventions that alter the bacteria found in pond water can therefore affect the productivity of these systems. Ozone nanobubbles have recently been shown to reduce pathogens, improve dissolved oxygen, and influence fish innate immunity. However, little is known about the effect of nanobubble treatment on the microbial community of aquaculture ponds. This study investigated the impact of ozone macrobubbles (O3MB) and nanobubbles (O3NB) on the microbial ecology of pond water and fish health. We successfully eliminated between 90.9 and 99.4% of the heterotrophic bacteria and 90.1 to 95.2% of the bacterial productivity of these systems. Ozone nanobubbles have recently been shown to reduce pathogens, improve dissolved oxygen, and influence fish innate immunity. However, little is known about the effect of nanobubble treatment on the microbial community of aquaculture ponds. This study investigated the impact of ozone macrobubbles (O3MB) and nanobubbles (O3NB) on the microbial ecology of pond water and fish health. We successfully eliminated between 90.9 and 99.4% of the heterotrophic bacteria and 90.1 to 95.2% of the bacterial DNA in our small pond water ecosystems after treatment with 0.15 mg/L ozone. According to the shotgun metagenomic sequencing, ozone macro- and nanobubble treatments reduced the relative abundance of all bacteria in our water sample, including the dominant bacterial species, as well as Cyanobacteria. The top ten bacterial species in the community changed and were more evenly distributed within the water sample. The bacterial richness of the ozone-treated water samples declined slightly, but over 6000 species were still identified 24 h after the treatment. We also observed a rebound in the bacterial community 24 h after the ozone treatments. The advantage of the nanobubble delivery of ozone over macrobubble delivery of this gas was that the former took significantly less time to deliver the desired quantity of gas while it also greatly increased the dissolved oxygen in the water. Further, we assessed the impact of ozone nanobubbles on jade perch, and no effects were found on the fish at an exposure dose of 0.15 mg/L. This study provides preliminary information on potential applications of nanobubble technology for “resetting” microbial communities, which may be useful during disease outbreaks.

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**1. Introduction**

The increase in global demand for farmed aquatic food has led to an unprecedented increase in aquaculture production (FAO, 2020). This growth has come in the form of larger farms producing fish at higher densities, frequently within shared watersheds. The changes in the industry have posed challenges to the control of disease spread within and between farms. Increased disease outbreaks have led to higher consumption of antimicrobials (Jonas et al., 2017; Watts et al., 2017), which has increased the risk of antimicrobial resistance (AMR) in the aquatic ecosystem around farms (Jonas et al., 2017; Watts et al., 2017; Roca et al., 2015). As such, alternative products and technologies to...
prevent or control bacterial diseases have emerged over the last few years. In aquaculture, such products have included compounds derived from natural products, probiotics, immunostimulants, and vaccines (Watts et al., 2017; Reverter et al., 2020). Water disinfection strategies have also improved in an attempt to mitigate and control disease outbreaks. Good water quality and reducing pathogenic bacteria in the water column can help prevent diseases.

One of the new innovations that has emerged in the last decade to treat water is nanobubble technology. Nanobubble treatment involves injection of nano or ultra-fine bubbles created with a chosen gas into water (Agarwal et al., 2011; Anzai et al., 2019). Unlike macro- and microbubbles, these nanobubbles, with a diameter of <200 nm, have neutral buoyancy and thus remain in suspension for days (Agarwal et al., 2011). When they implode, they release free radicals that can kill bacteria (Takahashi et al., 2007; Agarwal et al., 2011; Mahassri et al., 2018; Anzai et al., 2019; Rahmawati et al., 2021). If these bubbles are created with ozone gas then their potential for disinfection is greatly increased (Pal et al., 2022). Ozone kills bacteria by generating oxygen free radicals, which damage bacterial cell walls and DNA strands (Ferng et al., 1997).

Ozone nanobubble technology has recently been shown to reduce the microbiological load in the water. Imaizumi et al. (2018) reported that ozone nanobubbles could be used for disinfection of Vibrio para-haemolyticus, the bacteria that causes acute hepatopancreatic necrosis disease in whiteleg shrimp (Penaeus vannamei). Also, Jhunkeaw et al. (2021) demonstrated that a 10-minute ozone nanobubble treatment effectively reduced over 96% Streptococcus agalactiae (Gram-positive bacteria) and Aeromonas veronii (Gram-negative bacteria) in freshwater systems. Furthermore, ozone nanobubbles were also found to be efficient in upregulating the innate immune system of fish, resulting in increased fish survival during pathogenic infections, as demonstrated by Dien et al. (2021) in their study of Nile tilapia infected with a pathogenic multidrug-resistant Aeromonas hydrophila.

In spite of these promising findings, there is a lack of information on the effect of ozone nanobubbles on the overall diversity and composition of the bacterial communities in aquaculture pond water. The water’s microbial community is of interest as it can be manipulated to mitigate emerging diseases and disease outbreaks in aquaculture (de Bruijn et al., 2018). Metagenomic sequencing technologies have increased our understanding of the role commensal microorganisms play in aquatic animal health. The bacterial community of aquatic animals is influenced by the composition of the bacterial community in their surrounding water, which serves as a microbe reservoir. Sehnal et al. (2021) recently examined the diversity of the microbiome from aquatic vertebrates and their functions within the hosts. They found that an imbalance in the environmental water microbiome may impact the inflammatory and immune responses of fish. Furthermore, when the aquatic environment is unbalanced (e.g., high levels of organic matter and increased numbers of harmful microbiota), interactions between microorganisms and farmed fish can increase stress, compromise fish immune competency, and reduce their ability to combat infections, leading to disease outbreaks (Kotob et al., 2017). This situation may be worsened when pathogenic bacteria dominate the bacterial community.

Given the important role of the microbial ecology of aquaculture systems, the use of nanobubble technology in these systems requires a good understanding of its effect on the bacterial diversity and overall composition. This study aimed to characterize the diversity and microbial changes within the aquatic environment after the application of ozone nanobubbles. At the fish level, several stress indicators (cortisol, superoxide dismutase, and reactive oxygen species) were also studied to evaluate the physiological responses of jade perch (Scortum barcoo), a common species grown in earthen ponds in Hong Kong Special Administrative Region (SAR) of the People’s Republic of China (PR China), during and after water ozone nanobubble treatments.

2. Materials and methods

2.1. Effects of O3NB on the microbial community of pond water

2.1.1. Experimental design and sample collection

We conducted the experiments at Au Tau Fisheries Office, Agriculture, Fisheries and Conservation Department in Yuen Long, Hong Kong SAR, PR China. Pond water from a commercial pond which had recently been used to raise jade perch was pumped into a 600 L tank, mixed, and evenly divided into 12 tanks. The process was repeated until each experimental tank had 75 L of water. Water temperature in each tank was maintained at between 26°C and 28.7°C using commercial chillers (CI-280, Resun®). External filters (EHEIM classic 350) were used to circulate the water through the tank system at a flow rate of ~620 L/hr.

To determine the effect of ozone nanobubble technology on the microbial community, the following four groups were assigned to three replicate tanks: 1) Air macrobubbles (AirMB), 2) Air nanobubbles (AirNB), 3) Ozone macrobubbles (O3MB), and 4) Ozone nanobubbles (O3NB).

To generate ozone nanobubbles, we connected a nanobubbler (aQua-075MO, AquaPro Solutions Pte Ltd., Singapore) to an ozone generator (DNO-15G, Dino Purification Co., Ltd., China) with the flow rate of incoming oxygen stream at approximately 0.3 LPM. Water from the tanks was pumped into the nanobubbler then back to the tanks. The system was run until we achieved the desired ozone concentration of 0.15 mg/L in the three O3NB treated tanks. The time required to achieve this level of ozone was recorded for each tank. The ozone concentration was measured with a dissolved ozone meter (DOZ-30, Dino Purification Co., Ltd., China). The same ozone generator was attached to an air stone to deliver macrobubbles gaseous ozone to the three tanks in the O3MB group, where the water was treated until the same level of ozone (0.15 mg/L) was achieved. The time it took to attain this ozone level was recorded for each tank.

For the AirMB group, air pumps (Dazs model AP-528, Hong Kong) with a flow rate of 1.2 L/min were attached to air stones to provide macrobubble aeration, and these were run for the same amount of time as the pumps used in the O3MB treatment group. For the AirNB group, we used ambient air as the gas source in our nanobubbler to deliver aqueous dispersion of air nanobubbles to the tanks. The nanobubbler was run for the same duration of time used to achieve the desired level of ozone in the O3NB group.

Bacteriological culture and metagenomics analysis were conducted on each tank. A 400 mL water sample was collected before the treatment for the metagenomic analysis. Immediately after the treatment and 24 h post treatment, we collected 800 mL water samples from the ozone treated tanks and 400 mL from the air treated tanks to ensure we had sufficient DNA for shotgun metagenomic sequencing. Separate 50 mL samples were also collected before the treatments were applied, immediately after treatment, and 24 h after treatment for total bacterial culture. These were stored on ice until they could be submitted to the Australian Laboratory Services Technichem Pty Ltd. (ALS, HK) for analysis. No samples were stored longer than 3 h before submission.

2.1.2. Water quality

Dissolved oxygen and water temperature were measured with a handheld optical dissolved oxygen meter (model ProSolo with ODO probe, YSI, USA). Water pH was measured with a pH meter (PHB18, Small Sensor, China), and ammonia was tested with a portable HACH parallel analyzer using total ammonia Chemkey® reagents (SL1000 – PPA, Hach, USA). The latter was only tested at the start and end of the study. All other samples were collected at the same time as the water samples collected for the bacterial culture described above.

2.1.3. Bacteriology

To determine the heterotrophic plate counts, samples were submitted to ALS. They used the APHA 9215 B standard method (APHA, 1992)
to quantify the number of culturable aerobic bacteria in each sample.

2.1.4. Total DNA extraction

To determine the concentration of DNA and assess the biodiversity of the bacterial community in our samples, we concentrated the bacteria by vacuum-filtration using a polycarbonate membrane with a pore size of 1.2 μm (0.47 mm, Whatman 1822-047 GF/C Glass Microfiber Filters), followed by a second filtration through a 0.2 μm membrane (0.47 mm, Sartorius, Germany). To differentiate live from dead bacteria, the membranes were treated with 2 mL of 25 μM Propidium monoazide (PMA) in a petri dish. The protocol for PMA treatment is described in Jäger et al. (2018). Once this process was completed, we combined the two filters from the same sample and stored these at -80 °C.

Once all samples were processed, they were thawed, and each filter was transferred into a 2 mL homogenization tube (Precellys lysing kit CKMix, Bertin Technologies) containing 1 mL InhibitEX buffer provided by the QIAamp Fast DNA Stool Mini Kit (Qiagen™). The membranes were homogenized with the Precellys 24Dual homogenizer (Bertin Technologies, France) for 90 s at 4500 rpm speed with a 15-s pause between the homogenization steps. The total DNA was then isolated using QiAamp Fast DNA Stool Mini Kit (Qiagen™) according to the manufacturer’s instructions. Lysis was completed at a temperature of 95 °C for 5 min. During the final DNA elution step, 40 μL of elution buffer (10 mM Tris-Cl, pH 8.0) was used. After checking the purity and concentration of the extracted DNA by Qubit 4 Fluorometer (Thermo Fisher Scientific), we retained 10 μL of DNA for quantitative PCR analysis. The remainder of the samples were sent to Novogene (Novogene HK Company Limited, Hong Kong) for shotgun metagenomic sequencing. The following formulas were used to standardize the DNA amounts for different volumes of water samples.

\[
\text{DNA amounts (ng/100 mL)} = \left( \frac{\text{DNA concentration (ng/mL) \times 40 \mu L}}{\text{total volume of water samples (mL)}} \times 100 \right) \times 100 \%
\]

2.1.5. Quantitative PCR analysis (qPCR)

To determine the quantity of 16S genetic material, SYBR Green qPCR assays were performed using a Bio-Rad Cycler CFX96 (CFX96 Touch™ Deep Well Real-Time PCR Detection System, Bio-Rad, Munich, Germany). The results were analysed using the manufacturer’s software (Bio-Rad CFX Manager Software). In brief, the reaction mixture consisting of 1 μL template DNA, 1 μL Primer FW (10 mM), 1 μL Primer Rev. (10 mM), and 10 μL PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific). Distilled water (Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water, Thermo Fisher Scientific) was added to adjust the total volume to 20 μL. The primers used are described in Muyzer et al. (1993). The thermocycler profile consisted of 1 cycle at 95 °C for 3 min for DNA polymerase activation, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s for primer annealing and elongation. All qPCR assays were performed in triplicate. Cycle threshold (Ct) values from the triplicates were averaged and used to determine the 16S gene (V3) copy numbers. Melting curves and non-template controls were used to assess reliability.

To determine the copy number, we used the methodology described by Jian et al. (2020). A standard curve ranging from 10^2 to 10^7 copies was produced using Escherichia coli to quantify the 16S gene. Copy numbers were expressed per mL of sampled water.

2.1.6. Shotgun metagenomics analysis

To characterize the composition of the microbiome in our samples at the species level, shotgun metagenomic sequencing was performed. Library preparation and sequencing were done by Novogene Company (Hong Kong, China). Quality and quantity of the prepared library were checked by an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and quantified by real-time PCR (to meet the criteria of 3 nM). Sequencing was performed on an Illumina NovaSeq 6000 platform to generate paired-end (PE) 2 × 150 bp sequencing reads. Quality control of raw reads was also carried out by Novogene.

The Kraken2 standard database (https://ceb.jhu.edu/software/kraken2/index.shtml) was used for taxonomic classification. Clean reads were taxonomically classified with a standard database, and the k-mer based lowest common ancestor (LCA) approach was used in the Kraken2 taxonomic sequence classification system, as described in Wood and Salzberg (2014). To avoid the interference of rare reads, operational taxonomic units (OTUs) with fewer than ten reads were dropped. The resulting file was used for further data analysis downstream. The bioinformatics processing was performed on the Compute Canada Cluster (https://docs.computecanada.ca/wiki/Cedar).

2.1.7. Statistics analysis

All our water quality, bacterial counts and DNA data are depicted graphically. These data were expressed as mean ± standard deviation. Differences between the pre and the immediate post-treatment values, as well as the difference between the immediate post-treatment and the 24 h post-treatment values, were calculated and compared between the treatment groups using ANOVA followed by pairwise comparisons. We also compared the absolute values of different parameters between treatment groups within the same time period using a Kruskal Wallis test followed by pairwise multiple comparisons using a Dunn’s test (Dinno and Dinno, 2017). To evaluate whether there was a significant change in the absolute values within a treatment group over time, we log-transformed the data and performed a one-way repeated measures ANOVA test. This test could only be used for the comparison within the control groups, as the assumptions of this parametric test were not met with the ozone-treated groups. For the latter, we compared data between time points using paired t-tests on transformed data. The statistical significance was set at p-values below 0.05. All analyses were performed using R Statistical Software (v4.1.2) (R Core Team, 2021).

For the genomic data, once we completed the classification step, an alpha-rarefaction curve was generated to determine if sequencing depth was sufficient to describe the microbial communities. The dataset was rarified to the lowest sequencing depth to remove batch effects. Operational taxonomic units classified as Cyanobacteria phylum were removed from our analysis. To determine diversity within the community (alpha diversity), we calculated two specific metrics, Pielou’s evenness and Chao1. These two metrics estimated the evenness and richness of the community, respectively. We calculated these metrics based on 100% of the bacteria identified in the samples, as well as for the top 80% and the top 30% most abundant bacteria. Venn diagrams were generated to illustrate the number of bacterial OTUs shared between time points from the same treatment groups.

We compared the richness and evenness of 100%, top 80% and 30% of the microbial communities pre and 24 h post-treatment within each treatment group using paired t-tests. When the model residuals did not meet the assumptions of normality and homogeneity of variance, we conducted a Tukey’s Ladder of Powers transformation before we analysed the data. We also calculated the differences between the pre and the 24 h post-treatment values for evenness and richness across the different treatment groups and compared these using ANOVA followed by pairwise comparisons.

In addition, we constructed an unweighted principle coordinate analysis (PCoA) with Bray-Curtis distance using the Phylosqes package to assess the differences in microbial composition between treatment groups (beta-diversity) (McMurdie and Holmes, 2013). The entire bacterial community, excluding Cyanobacteria, was used for this analysis. A PERMANOVA test was conducted to assess the statistical differences between the clusters (Oksanen et al., 2019).

To further understand how bacterial composition changed over time and between treatments, the proportion of rarefied OTU counts was determined (absolute counts/sum of counts per sample) to estimate the relative abundance of each species. The top 10 abundant species were visualized using a stacked bar graph. All analyses were performed using R Statistical Software (v4.1.2) (R Core Team, 2021) and the statistical significance was set at p values below 0.05.
2.2. Effect of O3NB exposure on Jade Perch (Scortum barcoo)

2.2.1. Animals and sample collection

The jade perch (4.4 g to 17.8 g, average = 11.1 g) used in this study were purchased from a commercial farm in Hong Kong. Fish were maintained in a 360-L tank on a recirculating system for one week prior to the start of the experiment. Fish were fed once daily at a rate of 0.5% body weight. Approximately 1/2 of the water was changed daily to maintain water quality. Total ammonia nitrogen and nitrite were tested daily, with water exchanges performed to maintain total ammonia nitrogen below 0.1 ppm. Mortality, swimming, and feeding behavior were monitored prior to the treatment while the fish were acclimating, and for 24 h after the ozone treatment. The procedures in this study were approved by the Animal Ethics Committee at City University of Hong Kong and the Government of Hong Kong Special Administrative Region Department of Health (Reference No.: (20-Kong and the Government of Hong Kong Special Administrative Region Department of Health (Reference No.: (20

After acclimation, 20 fish were placed in six 75 L tanks, including three control tanks (AirMB), and three treatment tanks (O3NB). Ozone was delivered to the treatment tanks until the water ozone concentration reached 0.15 mg/L. This gas was measured continuously during application using a dissolved ozone meter (DOZ-30, Dino Purification Co., Ltd., China). The time required to achieve the optimal level was recorded and used to define the time for the air macro-bubble delivery in the control tanks.

Three fish from each tank were randomly sampled one day before treatment, immediately after treatment, and one day after treatment. Fish were euthanized with an overdose of Tricaine Methanesulfonate (Syndel, MS-222). Blood was collected from a caudal vessel immediately after the opercular movement had ceased, for serum cortisol testing. After the opercular movement had ceased, blood was collected from a caudal vessel immediately after the treatment for serum cortisol testing. Samples were placed into 2 mL sterilized tubes containing AllProTECTTM (Byeotime, China) prior to being frozen at -80°C for later analysis. cortisol levels were determined using a Fish Cortisol ELISA Kit (Cat: SL0014Fi, SunLong Biotech Co., LTD) according to kit instructions. After bleeding, cervical dislocation was performed, and liver and gill samples were collected for enzyme analysis. Samples were placed into 2 mL sterilized tubes containing AllProTECTTM (Byeotime, China) prior to being frozen at -80°C for determination of superoxide dismutase (SOD) and reactive oxygen species (ROS) levels.

2.2.2. Biochemical analysis of oxidative stress

Liver and gill tissue samples from ozone nanobubble- and air-treated fish were thawed and transferred to 2 mL tubes (Precellys lysing kit CK28R, Bertin Technologies) containing 1 mL of saline solution. The tissues were then homogenized with the Precellys 24-Dual homogenizer (Bertin Technologies, France) for 15 s at 6500 rpm three times. To avoid possible thermal effects on the enzymes, the cell suspensions were cooled down for 1 min in an ice bath between the homogenization steps, followed by centrifuging at 10,000 g for 10 min at 4°C. The supernatant was used for superoxide dismutase (SOD) and reactive oxygen species (ROS) levels. SOD activity was determined using a total superoxide dismutase assay kit following the NBT method (S0109, Beyotime Institute of Biotechnology, China) (Beyer Jr and Fridovich, 1987). Intracellular ROS levels were measured with a 2,7′Dichlorodihydrofluorescein diacetate probe (H2DCFDA, MedChemExpress, HY-D0940). In brief, supernatants were incubated with 10 μM H2DCFDA diluted in DMSO for 30 min at 37°C in the dark. Fluorescence was then immediately detected at 485/525 nm (excitation/emission) wavelength using a microplate reader.

2.2.3. Statistics analysis

Serum cortisol, SOD activity and ROS level were expressed as mean ± standard deviation and graphed. To determine if there were significant differences between values for the control and O3NB-exposed fish at each time point, Wilcoxon-Mann-Whitney rank-sum tests were performed on the original data. Statistical analyses were performed using R Statistical Software (v4.1.2) (R Core Team, 2021).

3. Results

3.1. Effects of O3NB on the microbial composition of pond water

3.1.1. Water quality and ozone exposure

A significantly longer time was required to achieve 0.15 mg/L ozone using a traditional air stone to deliver the gas than using a nanobubbler delivery system (Fig. 1). In some tanks, it took >1.3 h to achieve this level of ozone when using macrobubbles. In contrast, it took an average of 4.1 min to achieve 0.15 mg/L ozone using the nanobubbler delivery system (Fig. 1). Therefore, a more efficient transfer of ozone was achieved using nanobubbles.

Dissolved oxygen (DO) levels increased after the ozone treatments, from an average of 8.25 ± 0.03 mg/L to 14.75 ± 0.63 mg/L in the O3MB tanks, and from 8.22 ± 0.02 mg/L to 26.03 ± 1.60 mg/L in the O3NB group (Fig. 2a). These increases were significantly higher compared to the AirMB groups, which never increased by >0.42 mg/L (Table S1). The increase in the oxygen was temporary, with DO concentrations returning to their pre-treatment levels within 24 h (Fig. 2a). Although statistically significant differences were also found in DO levels among groups 24 h after treatments (p = 0.034, Table S2), the actual difference was never >0.25 mg/L.

The pH values in tanks ranged from 7.32 to 7.80 over the course of the study. A significant change in pH was observed immediately after the treatment in the O3MB and O3NB groups (Table S3); however, this decrease was only by 0.046, which may not have been biologically significant (Fig. 2b). No significant differences in pH values between groups were found for either the pre-treatment (p = 0.75) or the 24 h post-treatment samples (p = 0.38) (Tables S2 and S4).

During the study, water temperatures ranged from 26.0°C and 28.7°C, with a slight increase (1°C) after the treatment in all groups (Fig. 2c). A higher ammonia concentration was observed in the O3NB group 24 h after treatment (Fig. 3d), but this was not statistically significant in comparison with other groups (p = 0.14), and the level (i.e. <2 mg/L) was well within the safety level for fish (Benli et al., 2008; Meade, 1985; Person-Le Ruyet et al., 1995).
3.1.2. Bacteriology
Plate counts suggested the application of ozone macrobubbles and nanobubbles produced similar reductions in total heterotrophic bacterial counts, with approximately a one thousand-fold reduction in culturable bacteria between pre and immediate post-treatment samples. There was minimal change in total bacterial counts in the AirMB groups between pre and post-treatment samples (Fig. 3). We observed a large increase in total heterotrophic bacterial counts in the ozone-treated groups 24 h after treatment, which was significantly higher than those in the AirMB group ($p = 0.027$ for O3MB; $p = 0.006$ for O3NB) (Fig. 3; Table S2). The increases in bacterial counts from immediate post-treatment samples to 24 h post-treatment were significantly less in the air control group (AirMB & AirNB) compared to the ozone treatment (O3MB & O3NB) groups (Fig. 3; Table S5).

3.1.3. Total DNA measured using Qubit
The total DNA/100 mL in the samples collected immediately after the O3MB and O3NB treatments yielded an average of $4.51 \pm 1.12$ ng and $7.91 \pm 3.87$ ng, respectively, while the AirMB and AirNB immediate post-treatment groups yielded an average of $117.73 \pm 36.60$ ng and $169.67 \pm 36.61$ ng (Fig. 4). The difference in DNA amount between pre and immediate post-treatment for the O3MB and O3NB groups was significant (Table S3): DNA from samples treated with ozone decreased significantly while the controls were consistent over time. There was a drop in the DNA amount in the air-treated groups (AirMB & AirNB) 24 h after treatment, though values from these two groups throughout the three time periods were not statistically significant ($p = 0.59$ for AirMB, $p = 0.079$ for AirNB). In contrast, there was a significant increase in the DNA amount in the ozone-treated groups between immediate and 24 h.

Fig. 2. Dissolved oxygen (a), pH (b), temperature (c) and ammonia (d) measured in the pond study across time in each treatment and control group. Values are given as mean ± SD. X-axis represents time points.

Fig. 3. Total heterotrophic bacterial counts for the four treatment groups at the three sampling time points. Values are given as mean ± SD. X-axis represents time points.
post-treatment levels of DNA (Table S3), but these values were still lower than the initial DNA quantities (Fig. 4).

3.1.4. Quantitative PCR analysis (qPCR)

Copy numbers of the V3 16S gene, determined by the standard curve for the three replicate tanks in our four treatment groups, are shown in Fig. 5. The copy numbers of 16S gene detected by qPCR in all groups were not significantly different pre-treatment ($p = 0.39$; Table S4). The quantity of 16S gene in both O3MB and O3NB groups had significant reductions immediately after the treatment compared with the changes in AirMB and AirNB groups (Fig. 5; Table S1). However, the reduction effect did not last for 24 h. The amount of 16S genes rebounded to the initial abundance, which was consistent with the change in total heterotrophic bacterial counts.

3.1.5. Shotgun metagenomics analysis

3.1.5.1. Data processing and descriptive analyses. Detailed output of the number of reads at each quality control step in the bioinformatics procedure for shotgun metagenomics analysis is provided in Table S6. We successfully processed 30 samples out of 36 samples using shotgun metagenomics analysis. The immediate post-ozone treatment samples ($n = 6$) did not have sufficient DNA to conduct this analysis and were, therefore, not processed or analysed. All other samples were sequenced to a minimum depth of 41,854,554 paired-end reads, averaging 43,673,320 ± 260,619 paired-end reads per sample. Very few reads were discarded during the quality trimming process (0.2%); the post-quality control (QC) data had 43,584,916 ± 261,566 paired-end reads per sample, generating 21,792,458 ± 130,783 merged reads. The rate of OTU classification (number of mapped reads/number of cleaned reads) was, on average, 27.0% ± 3.0% across all samples. A higher proportion of classified reads were found in the 24 h post-treatment samples in the ozone treatment groups compared to the air treatment groups (50.9% ± 3.0% vs. 14.4% ± 0.6% reads, respectively). The O3MB group had higher proportions of OTU assigned reads in the 24 h post-treatment samples (57.5% ± 1.2%) compared to the O3NB group (44.4% ± 0.4%).

Fig. 4. Quantity of DNA (ng) per 100 mL water samples determined by Qubit. Values are given as mean ± SD. X-axis represents time points.

Fig. 5. Quantification by qPCR of bacterial 16S rRNA gene copies in samples obtained from four groups (AirMB, AirNB, O3MB and O3NB) at three time points (pre-treatment, immediate and 24 h post-treatment). Values indicate the average of three replications and are given as mean ± SD. X-axis represents time points.
The rarefaction of samples to 2,099,248 reads was sufficient to obtain a good representation of the OTUs in the microbial community (Fig. 6). Overall, 9286 species-level OTUs were assigned after rarefaction, among which 6737 OTUs belonged to the bacteria kingdom. After removing the rare counts (<10 reads) and Cyanobacteria, a total of 388 OTUs were lost from our samples, representing 5.8% of the total number of bacterial species. Around 99.0% of the OTUs in the bacterial population pre- and 24 h post-treatment were shared in all groups. When we included only the top 80% of the bacterial OTUs, approximately 70% of the species were shared between the two time points within the AirMB and AirNB groups (i.e. 71.9% and 68.8%, respectively), and even fewer species were shared between these two points in the O3MB and O3NB groups (i.e. 18.7% and 23.5%, respectively) (Fig. 7). When we focused our analysis on the top 30% OTUs in the bacterial population, we observed a large decline in the number of species after 24 h of the treatments across all groups. Interestingly, there were a few species in the O3MB and O3NB groups that emerged in the top 30% after the ozone treatments (Fig. 7).

### 3.1.5.2. Alpha diversity

When we included data on all the bacterial community, excluding Cyanobacteria, the Pielou evenness index was significantly reduced 24 h after the treatments in all groups (Fig. 8). When considering only the bacteria in the top 30% most abundant species, the reduction in evenness was less pronounced in the air-treated groups, and in fact, for the ozone-treated groups, there appeared to be an increase in evenness (Fig. 8). This change in evenness between pre and 24 h post-treatment was only statistically significant for the O3MB group.

A detailed summary of the pairwise comparison of the reduction in the evenness index after the treatments between groups is provided in Table S7: the only statistically significant reduction was between the AirMB samples and the ozone-treated groups. This was the case for both the top 30% and the entire microbial community (Table S7).

In contrast to the evenness, when the entire microbial community was included in our analysis the richness (Chao1) index was not greatly affected after treatment for the air-treated groups, but in the ozone-treated groups, the richness of the bacterial population significantly decreased between pre and 24 h post-treatment samples (Fig. 8). However, when we analysed the richness of the top 30% most abundant bacteria, we observed a significant reduction in this index in the air-treated groups. Interestingly, the drop in richness after treatment in the O3NB group was no longer significant when we only looked at the top 30% most abundant bacteria (Fig. 8).

When we considered the entire microbial community, the ozone treatment groups all had a significant drop in richness compared to their respective air control groups (Table S7). Furthermore, the change in richness between pre and 24 h post-treatment samples was significantly greater in the O3MB group compared to the O3NB group (Fig. 8 and Table S7). For the top 30% bacterial community, the Chao1 indices in all groups declined, and none were statistically different from one another (S7).

### 3.1.5.3. Composition of bacterial community

*Anabaena variabilis* (12.1%) was the most abundant species in the pre-treatment samples in all groups. This species increased in relative abundance after 24 h in both air-treated tanks (i.e. AirMB and AirNB groups), accounting for the relative abundance of 22.4% and 21.4%, respectively (Fig. 9). However, in the O3MB and O3NB samples, this species dropped from the top 10 species (Fig. 9) and was reduced to a relative abundance of 0.003% and 0.6%, respectively. Overall, there was a shift in the top 10 bacterial species in our ozone-treated groups, where the evenness of bacteria became more apparent, and there was a change in the species composition. In the case of the air-treated groups, we did not observe such a shift. Most species in the top ten remained present in the post-treatment sample, although they shifted slightly in relative abundance (Fig. 9).

### 3.1.5.4. Beta diversity

The Principal Co-ordinates Analysis (PCoA) plot of the Bray-Curtis dissimilarity matrix showed that the microbial composition of all pre-treatment samples clustered together (Fig. 10; PERMANOVA $R^2 = 0.25$, $p = 0.61$, $Df = 3$) and the 24 h post-treatment samples clustered separately based on the type of treatment received (air vs. ozone; PERMANOVA $R^2 = 0.81$, $p = 0.02$, $Df = 1$).

### 3.2. Effect of O3NB on jade perch

#### 3.2.1. Water quality and ozone depletion in fish tanks

Changes in water quality and ozone depletion for our fish tanks were similar to those reported in the pond water study mentioned earlier (Table S8). The ozone concentration levels quickly decreased after treatment (Fig. 11).

#### 3.2.2. Fish survival and behaviors

No fish mortalities were recorded during the experiment. The fish did not go off feed nor showed abnormal behaviors during the course of the experiment.

#### 3.2.3. Physiological responses

A significant difference in the SOD activity was observed between AirMB- and O3NB-treated fish one day after the treatment (Fig. 12) with SOD activity higher in the O3NB-treated fish (i.e. 8.0 ± 0.9 units) compared to the AirMB-treated fish (4.5 ± 0.8 units) (Wilcoxon-Mann-Whitney rank-sum tests; $p = 0.02$). The SOD values for the treated fish returned to their initial levels two days post-treatment (i.e. 4.5 ± 0.6 units).

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**Fig. 6.** Rarefaction curve of 30 samples from shotgun data after rarefying to the minimum read depth of 41,854,554 paired-end reads per sample. The x-axis represents the sequencing depth in the number of reads, and the y-axis is the estimation of the OTU richness detected at the species level.
The results suggest that ozone treatments exhibited bactericidal effects very rapidly after application, regardless of whether this gas was delivered via nanobubbles or macrobubbles. Low doses of ozone (0.15 mg/L) killed 90.9 to 99.4% of the total heterotrophic bacteria and reduced 90.1 to 95.2% of the total DNA in our samples. These results were consistent with what was expected given the known disinfection effect of ozone (Czekalski et al., 2016; Imazumi et al., 2018; Wang et al., 2018). Ozone has been shown to destroy both bacterial cell structures, as well as damage DNA strands (Feng et al., 1997; Duan et al., 2021). However, what was surprising in our study was that many bacterial species remained in the pond water after ozone treatments, and there was regrowth of a diverse bacterial community within 24 h of the treatments. This new community appeared to comprise of a near-identical population of bacteria based on the metagenomics data (Fig. 7), but the proportion of each bacterial species was vastly different (Fig. 9). Overall, ozone seemed to balance out the population of bacteria by reducing the dominant species. Aerobic or facultative anaerobic bacterial species were the most prominent once the population recovered from the ozone treatments after 24 h. Our findings suggest that applying ozone to pond water at levels that should be safe for freshwater fish (Powell and Scolding, 2018) could help reduce dominant bacterial species. This management strategy may be useful when controlling bacterial disease outbreaks where there can be a sudden increase and shift in the abundance of one or two pathogenic bacterial species.

Our analysis of the microbial community was based on both the heterotrophic bacterial counts and the shotgun metagenomics analysis of extracted DNA from water samples. Both methods provided us with similar conclusions, but the shotgun metagenomics analysis allowed us to examine the entire bacterial community. It is possible that we detected DNA from non-viable cells in our genetic analysis, which we tried to reduce by using a PMA stain to bind the DNA from dead cells in our samples before the extraction step. Given our lack of DNA using Qubit and the high Ct values on our 16S qPCR analysis in the immediate post-ozone treatment samples (Figs. 4 and 5), we do not think there was much DNA from non-living bacteria after the PMA staining step. The diverse number of species in our 24 h post-treatment samples likely came from live bacteria and suggested that many of the bacterial species survived the low dose (0.15 mg/L) ozone treatments at least in small quantities.

Although there was a statistically significant reduction in the richness index in the ozone-treated groups (Paired t-test; p = 0.003 for O3MB; p = 0.027 for O3NB), this amounted to only a few hundred bacterial species lost relative to >7000 species identified in the initial water sample. There are several explanations for the survival of almost all bacterial species after our ozone treatments. Firstly, we did not use a high level of ozone to treat the water, rather we used a level of ozone that was safe for many fish species (Powell and Scolding, 2018) and that was known to be lethal to several known pathogenic bacteria (Liltved et al., 1995); however, some species, such as Pseudomonas spp., are not as susceptible as others bacteria to this concentration of ozone (Sidiqui and Khan, 2017). These bacterial species have been shown to develop various levels of resistance to ozone through the generation of intracellular antioxidant enzymes (e.g., superoxide dismutase and catalase) (Feng et al., 2018). It is also possible that these bacteria interacted together to form biofilms (Zhu et al., 2020; Zhu et al., 2021) which protect colonies of bacteria from several disinfectants (Bezirtzoglou et al., 2008). Bacteria embedded within particulate matter or clumped in bacterial aggregates can be shielded from oxidation (Sharrer and Summerfelt, 2007), which is the primary mechanism for ozone disinfection (Pell et al., 1997). Supporting this hypothesis was the finding that Rhineheimera sp. F8 was proportionally higher after treatment, and this bacteria is known to be a biofilm-forming gamma-proteobacterium (Tang et al., 2013). Despite the shift in the top ten bacterial species found after our treatment, we still observed most species in our samples and the bacterial population was relatively even. It would appear that if ozone is only given as a low dose one-time treatment to a mix bacterial species. This management strategy may be useful when controlling bacterial disease outbreaks where there can be a sudden increase and shift in the abundance of one or two pathogenic bacterial species.
Fig. 8. Alpha-diversity indices for the total population of bacteria as well as the top 80% & 30% bacterial population in our samples. Richness index (Observed OTUs) and evenness index (equitability or Pielou’s index) per sample for shotgun metagenomics data. Each bar represents the mean ± SD (*p < 0.05, **p < 0.01, ***p < 0.001).

AirMB: air macrobubble group; AirNB: air nanobubble group; O3MB: ozone macrobubble group; O3NB: ozone nanobubble group.
aquatic community, the dominant species are reduced without removing too many minor species from the environment.

When we restricted our microbial community analysis to the top 30% of the bacterial population in our samples, we observed an increase in the evenness of this bacterial community after the ozone treatments, which was not observed in the untreated tanks (Fig. 8). This phenomenon was likely due to the large reduction in the dominant species after ozone treatments. Interestingly, when we included all the bacterial species in our analysis, the evenness in all groups appeared to decrease. This was because the bottom 20% of the bacterial community in the ozone treatment groups remained very low in abundance compared to the top 80%, driving the evenness index down. The evenness in the bacterial populations in the control tanks also decreased; however, this phenomenon appeared to be driven by the dominant species becoming more prevalent. It is possible that this occurred in our control tanks because the nutrients were depleted, and some bacterial species propagated better under these oligotrophic circumstances (Hugoni et al., 2017; Osman and Dubow, 2019).

The rebound of bacteria after the ozone treatments was likely associated with the large increase in oxygen, which resulted from the chemical reaction between $O_3 + H_2O$ (Sotelo et al., 1987; Von Sonntag and Von Gunten, 2012). This appeared to select for aerobic and facultative anaerobic species such as Hydrogenophaga sp. BA0156, Pseudomonas alcaligenes, and Acidovorax sp. KKS102 (Fig. 9). Interestingly, the dissolved oxygen was particularly high in the nanobubble groups, suggesting the oxygen in these tiny bubbles remained in solution for longer.

**Fig. 9.** Compositions of the bacterial community on the species level in all experimental tanks across different time points, showing only the top 10 species.

**Fig. 10.** Principal coordinate analysis (PCoA) ordination of a Bray-Curtis dissimilarity matrix for the entire microbial community. Plots illustrate distances between communities (24 water samples in four groups). Colours were used to distinguish between three clusters.
than when the gas was delivered via macrobubbles where it is degassed relatively quickly (Meegoda et al., 2018). The other stimulus for the regrowth of the bacterial community in our study may have been the high nitrogen content after the initial killing of the microbial community. Even after 24 h, the ammonia appeared to be higher in the ozone groups, especially the ozone nanobubble group (Fig. 2d). The rapid die-off of algae and the dead bacteria detected immediately after the treatment would have provided a nutrient-rich community for the revival of bacterial species. Although we did not measure the organic matter in our tanks, other researchers have shown an increase in dissolved organic carbon (DOC) and assimilable organic carbon (AOC) associated with high levels of algae die off after ozonation (Hammes et al., 2007). Further, the initial competition from the dominant species that existed in high numbers before the treatment would no longer be

Fig. 11. Ozone concentration curve for three fish tanks. The grey line indicates when we stopped the administration of ozone.

Fig. 12. ROS and SOD measurements in fish study. Each bar represents the mean ± SD; the difference between air treated group and the ozone nanobubble-treated group was tested by the Mann-Whitney rank sum test.
present, allowing minor species to establish themselves. These changes would not have been present in the control tanks. In fact, the control tanks may have seen a depletion in nutrients, which may have driven the increase in species that propagate better under low-nutrient environments.

The data from this study suggests that we can effectively reset the bacterial community with a low dose of ozone. Further applications of this technology could include controlling disease outbreaks when dominant pathogenic bacteria dominate the aquatic environment. It may also be possible to use this technology to improve the efficacy of probiotics consisting of aerobic bacteria. The colonization of these specific bacteria could be enhanced if ozone nanobubbles are used preemptively to help remove the dominant species in a pond. Without resetting the pond’s microbial community, it may be difficult to apply probiotics successfully, as it would require a large number of bacteria to shift the complex microbial ecology of a pond. Future research should investigate the potential for applying these two strategies (i.e. nanobubbles and probiotics) synchronously to maximize their impact and control bacterial disease outbreaks. It may also be important to re-seed ponds after ozone nanobubble treatments with bacteria more favourable to fish.

Another finding from this study was the similar shifts in bacterial communities regardless of whether we delivered ozone via nanobubbles or macrobubbles. The advantage of using nanobubbles was that it drastically reduced the amount of time required to achieve the desired concentration of ozone in our study tanks. It took >1.3 h to achieve 0.15 mg/L ozone with macrobubbles, while it took <5 min to reach the same concentration using nanobubbles. It is likely that nanobubbles remained in the water column longer as they do not float to the surface due to their small size (Zhou et al., 2021). In contrast, ozone delivered via macrobubbles rapidly escaped into the air, making it more difficult to accurately measure this gas and delaying the time to achieve the desired concentration of this gas. The longer time required to achieve the concentration of ozone in the macrobubble group may have led to the significantly higher loss in richness in this group compared to the nanobubble group (Table S7; O3MB vs. O3NB in the entire microbial community), as the killing effect of ozone is dependent on both the concentration and contact time with the product (Haas et al., 1996).

Overall, the effects of these two methods of ozone delivery on the microbial community were not biologically significant, but the time required for the treatment had practical implications. Ultimately, adding ozone to commercial ponds using traditional macrobubbles is likely to be impractical due to the time constraint, but it may be feasible using nanobubbles.

Interestingly, the nanobubbles themselves did not appear to have an effect on the bacterial community. Several researchers have speculated that when nanobubbles implode, they release free radicals at a concentration and contact time with the product (Haas et al., 1996). However, this speculation may have been impractical due to the time constraint, but it may be feasible using nanobubbles.

Another interesting finding from this study was the effect of ozone on the Cyanobacteria (blue-green algae) population in our water samples after treatment. We initially removed the sequences that belonged to this phylum from our dataset because these organisms are different from other bacterial species in that they undergo photosynthesis (Moestrup, 1996; Knoll, 2008; Hilborn and Beasley, 2015), and this phylum greatly dominated our taxonomic dataset. *Fonsebacter ubiquis* and *Methyl*...
research team is currently working on establishing the flow rate and equipment required to shift the microbial ecosystem of a commercial size pond. The fact that several aquaculture companies in Malaysia and Indonesia are using this technology to increase dissolved oxygen in commercial settings (Musa et al., 2020; Myo Thant et al., 2022) suggests that it may be possible to achieve a short ozone treatment in this type of environment. However, given the limitations of ozone treatments in warm and nutrient-rich environments, there may be some seasonal limitations to the use of this type of treatment. Further, research should investigate if lower concentrations of ozone than those used in this study can still affect the pond ecosystem. Lower concentrations may be easier to attain in a large pond and may have less impact on fish and algae.

Another limitation of this study was that we could not evaluate the technology during disease outbreaks. Although there were dominant species in our initial water samples, these may not respond to ozone in the same manner as pathogenic bacteria, so it is difficult to extrapolate the outcome of using this technology in a disease situation. Further research is required to evaluate this technology under disease outbreaks. In addition, the economic benefits of applying ozone through nanobubbles should be assessed; however, given that some industries have already embraced the use of nanobubbles for distributing oxygen to their ponds, the anecdotal data suggests it may be economically viable.

5. Conclusion

Despite the limitations of this study, the data provides preliminary information that suggests ozone nanobubble treatments were effective at killing bacteria, and the effect was more pronounced in the dominant bacterial species in the microbial community: a dose of 0.15 mg/L reduced the culturable bacteria by approximately 90.9 to 99.4%, the quantity of bacterial 16S gene by 90.1 to 95.2% immediately after the treatment. The regrowth of the bacterial community was more even than before the treatment, at least in the top 80% of the bacterial species, which may indicate a more balanced ecosystem. The use of this strategy to shift the microbial population had the added benefit of increasing the dissolved oxygen level in the water and did not negatively affect juvenile jade perch. Ozone delivery through nanobubbles was significantly faster than using traditional air-stone macrobubbles and this technology may provide a tool to treat aquaculture ponds during disease outbreaks or when the pond’s microbial community needs to be reset with or without probiotics.

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CRediT authorship contribution statement


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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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