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Research Article

Assessment of Ozone Nanobubble Technology to Reduce Freshwater Algae

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Algal blooms can lead to low dissolved oxygen and fluctuating pH, and the toxins produced by some species can be toxic to aquatic animals. In this study, we assessed the potential of ozone nanobubble technology for reducing a diatom species, *Nitzschia* sp., commonly found in freshwater ponds in Hong Kong. This study suggests that ozone at a low dose of 0.025 ± 0.003 ppm was sufficient to reduce algae by 66.4% within 5 minutes of treatment. An even higher killing effect (68.2%) was observed when ozone was delivered using nanobubbles for 9 minutes. A 24-hour delayed effect was also detected, with a further reduction of approximately 10% of the algae for both ozone treatments (macro and nanobubble delivery methods). In addition to controlling algae, applying ozone at a level that is not detrimental to fish may also benefit the dissolved oxygen levels in pond systems.

1. Introduction

Aquaculture production was globally valued at US$263.6 billion in 2018 [1]. Inland aquaculture makes up 62.5% of aquaculture production (51.3 million tonnes), and the most common type of inland aquaculture facility is earthen pond [1]. Freshwater algae play essential roles in this aquaculture system as they serve as part of the food chain and provide shade and oxygen [2]. However, the overgrowth of algae can be harmful to aquatic animals. Excessive nocturnal planktonic respiration and bacterial decomposition of decaying algal blooms can promote hypoxic or anoxic conditions and lead to fish kills [3]. Moreover, algal blooms often result in a fluctuating pH due to the changes in CO_2 concentrations through photosynthesis and respiration, especially when the water is not adequately buffered [2, 4]. Furthermore, toxins produced by some dinoflagellates and cyanobacteria can be toxic to fish [5].

Several strategies can be used to reduce algae in freshwater ponds. The most common algaecide used in freshwater ponds is copper, either copper sulphate or chelated copper [6]; however, copper has some disadvantages. For example, copper ions can accumulate in the food web, reach a toxic level, and harm aquatic animals [7]. More recently, attention has been given to the use of ozone for disinfecting pond water, including algae control [8]. In a recent study by Zhang et al. [9], ozone microbombs were successfully used to reduce 93% of *Microcystis aeruginosa*, a harmful algae species, in a lab-scale experiment.

Another new technology that may help reduce algal blooms is nanobubble technology. Nanobubble technology has been used for water treatment and to increase dissolved oxygen levels [10–12]. It has also been used to remove contaminants in sewage wastewater by flocculation [13]. Compared to larger bubbles, nanobubbles have a higher surface area per volume of gas. Therefore, they remain suspended in the water column for a long period of time and increase gas saturation [14]. In the study by Mauladani et al. [15], nanobubble technology was used to promote dissolved oxygen in aquaculture ponds with white-leg shrimp. Nanobubbles can be created from any gas, including ozone. It may be possible to disinfect water with relatively low levels of ozone by using nanobubble technology. The objective of our study was to compare the effects of nanobubble technology, using different gases, on algae and water quality.

2. Materials and Methods

2.1. Experimental Site and Setup. The study was conducted at the Au Tau Fisheries Office, Agriculture, Fisheries, and Conservation Department (AFCD), Hong Kong SAR, China. Water from a fishpond with jade perch was used to fill a mixing tank. 75 L of water was then distributed to each of the twelve experimental tanks housed at an outdoor facility. External filter pumps (EHEIM classic 350) were used to circulate the water in tanks at a flow rate of ~620 L/hr. Chillers (HQ-75, wattage: 75 W, Aqua One & KONG's, Australia) were used to maintain the water temperature between 18.5 and 24.1°C. The tanks were assigned to one of four treatment groups: (1) air macrobubbles, (2) air nanobubbles, (3) ozone macrobubbles, and (4) ozone nanobubbles. Air pumps (Dazs model AP-528, Hong Kong) with a flow of $4\,L{\cdot}min^{-1}$ were attached to air stones for the air macrobubble treatment. An air pump was attached to the nanobubbler (Model: aQua + 075M, AquaPro Solutions Pte Ltd., Singapore) to produce the air nanobubble treatment. An ozone generator (DNO-15G, Dino Purification Co., Ltd., China) with an ozone capacity of 15 g/h was attached to an air stone to deliver ozone macrobubbles to tanks in the third treatment group. Lastly, the ozone generator was attached to the nanobubbler to supply ozone nanobubbles to the fourth treatment group.

2.2. Treatments and Sample Collection. Treatments were applied to each tank on day 1, day 2, and day 4. On day 1, we applied 0.025 ± 0.003 ppm ozone concentration to the ozone macrobubble and nanobubble groups. This low dose was initially given because it was difficult to achieve a higher concentration initially with the high organic load in the water. Administration of ozone on days 2 and 4 was at a higher dose of 0.15 ± 0.015 ppm because this was the ultimate level we thought was needed for the reduction of algae. The ozone concentration was measured with a dissolved ozone meter (DOZ-30, Dino Purification Co., Ltd., China). The treatment was stopped once the target level of ozone was achieved. For each ozone treatment group, we measured the time it took to achieve the desired concentration of ozone. Injection of air during the air macrobubble treatments was conducted for the same duration as the ozone macrobubble treatments, while the air nanobubble treatment duration was similar to the average amount of time required to achieve the ozone concentration in the ozone nanobubble treatment tanks.

Water samples were collected before and after every treatment (day 1 pretreatment, day 1 posttreatment, day 2 pretreatment, day 2 posttreatment, day 4 pretreatment, and day 4 posttreatment). Samples were collected for algal counts and water quality measurements.

To quantify the algal cell counts, 50 ml of water was sampled from both the surface and the bottom of each tank and transported to the City University of Hong Kong laboratory for analysis. The samples were centrifuged at $3,200 \times g$ for 30 minutes and resuspended with PBS to make $100X$ concentrated samples. Lugol's iodine was added to the samples for a final concentration of 1% [16]. The samples were then stored at 4°C until they could be quantified. A hemocytometer (Neubauer Haemocytometry, Marien Field, Germany) was used to enumerate the algae cell counts under a light microscope (Primo Star KMAT, Zeiss, USA). Cells in 4 sets of 16 squares on the hemocytometer were counted. All species were included in our count; however, the diatom species *Nitzschia* sp. were the dominant algae in all our tanks (Figure 1).

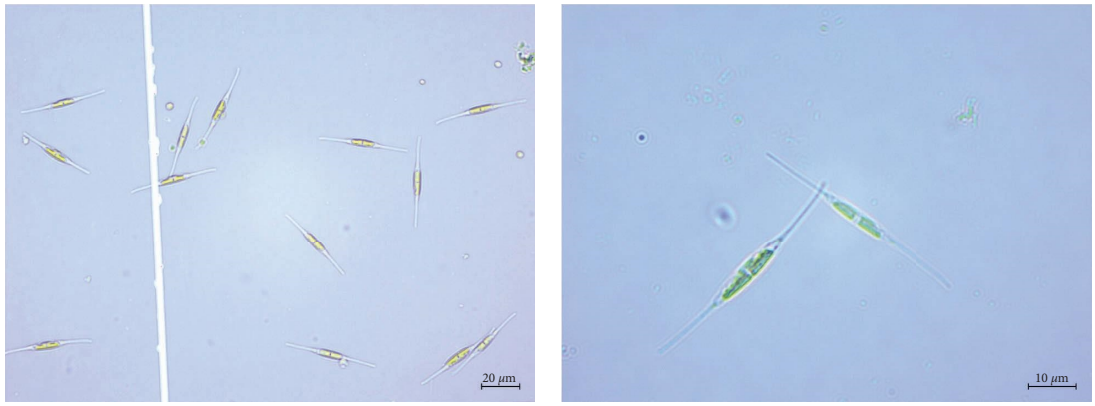
Nine water parameters were measured at six time points. Chlorophyll a concentration was assessed with a portable chlorophyll fluorometer (Model: ET1301, Shanghai Euro Tech Ltd., China); turbidity was measured with a water monitor probe (Aqua TROLL 500, In-Situ, Inc., USA); pH was measured with a pH meter (PH818, Smart Sensor, China); dissolved oxygen and temperature were measured with a handheld optical dissolved oxygen meter (model ProSolo with ODO probe, YSI, USA); and ammonia was tested with a portable parallel analyser using total ammonia Chemkey® reagents (SL1000—PPA, Hach, USA).

2.3. Statistical Analysis. To determine whether there was a statistically significant treatment effect, the difference in algal cell counts before and after treatment for each tank was compared using linear mixed-effects models. The pretreatment algae counts were subtracted from the posttreatment algae count for each day. In our statistical analysis, tanks within a treatment were treated as a random effect to control for the tank effect. Statistically significant pairwise post hoc comparisons (p value ≤ 0.05) between treatment groups were estimated with Bonferroni correction. Mixed effect models were done using R version 4.0.3 (2020-10-10) (R Core Team, 2020) with the function lmer in the lme4 package [17]. Post hoc pairwise comparisons were performed with the emmeans function from the emmeans package, and plots were constructed using the ggplot2 package [18]. Statistical analysis of the water parameters was performed using a 2-way ANOVA with multiple comparisons between treatment groups with the software GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Algae Cell Count over Time. Algal counts on day 1 pretreatment were similar in all treatment groups ($p > 0.2503$). Algae in the air macrobubble and air nanobubble treatment groups did not change considerably over the course of the first two days of our study; however, there was an increase in algae in both these treatment groups on day 4 (Figure 2). At the end of the experiment (day 4 post treatment), the air macrobubble/nanobubble group had an algae count of 3800 ± 305 cell/ml and 4954 ± 312 cell/ml, respectively. This was significantly higher than the ozone treatment groups ($p < 0.0002$) (Figure 2).

The change in algae count before and after treatment at different time points was used to assess the effects of different treatments on algae. When comparing the difference



(a) (b)

FIGURE 1: *Nitzschia* sp. from the pond water samples; the samples were stained with 1% Lugol's iodine and observed under the light microscope. (a) 40*X*. (b) 100*X*.

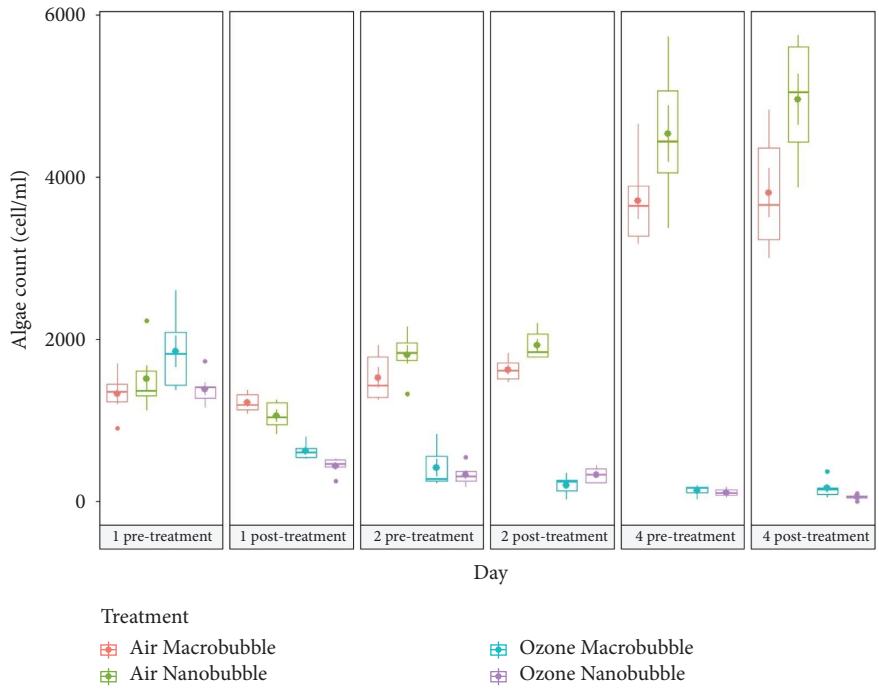


FIGURE 2: Algae count on day 1, day 2, and day 4, before and after the treatment during the experiment.

between day 1 pretreatment and day 1 posttreatment, there was a significantly higher (p value <0.05) algae count in the air macrobubble tanks than that in both of the two ozone treatments (Figure 3). The ozone macrobubble tanks had a change in algae count of -1229 ± 271 cell/ml (66.4% reduction); the ozone nanobubble group had a change in algae count of -946 ± 69 cell/ml (68.2% reduction), the air macrobubble group had a change in algae count of only -113 ± 106 cell/ml (8.5% reduction), and the air nanobubble group had a change in algae count of -450 ± 14 cell/ml (29.8% reduction). The decrease in algae for the ozone macrobubble group was statistically lower than its control group (air macrobubbles) (p value = 0.0051), but the difference in the counts on the first day for the ozone nanobubble group and its control group (air nanobubble) was not statistically significant (p value = 0.3047) (Figure 3). However, after 24 hours, the pretreatment algae levels in both ozone treatments were significantly different from their respective controls (Figure 4). The ozone macrobubble group had a change in algae count of -1433 ± 296 cell/ml (77.5% reduction) (p value = 0.0022), while the ozone nanobubble group had a change of -1058 ± 87 cell/ml (76.3% reduction) (p value = 0.0073). Algae counts in the air macrobubble and air nanobubble groups actually increased

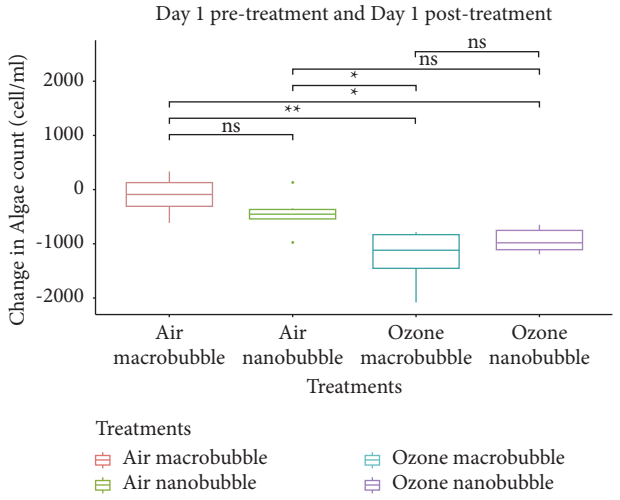


FIGURE 3: Change in algae count between day 1 pretreatment and day 1 posttreatment.

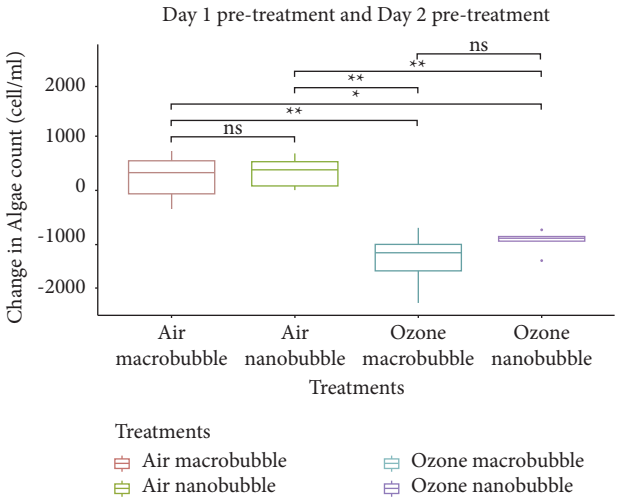


FIGURE 4: Change in algae count between day 1 pretreatment and day 2 pretreatment.

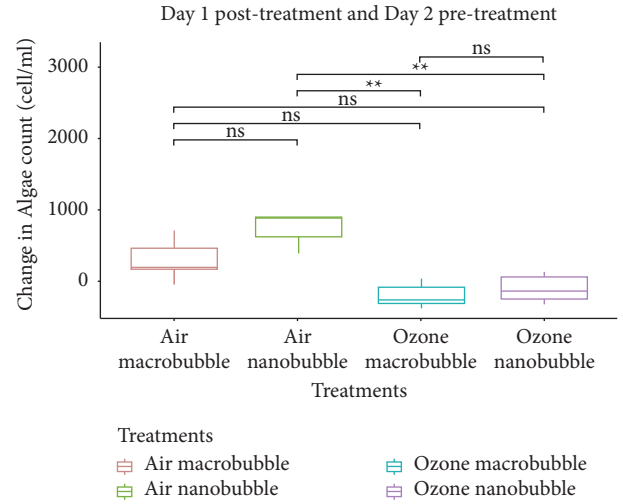


FIGURE 5: Change in algae count between day 1 posttreatment and day 2 pretreatment.

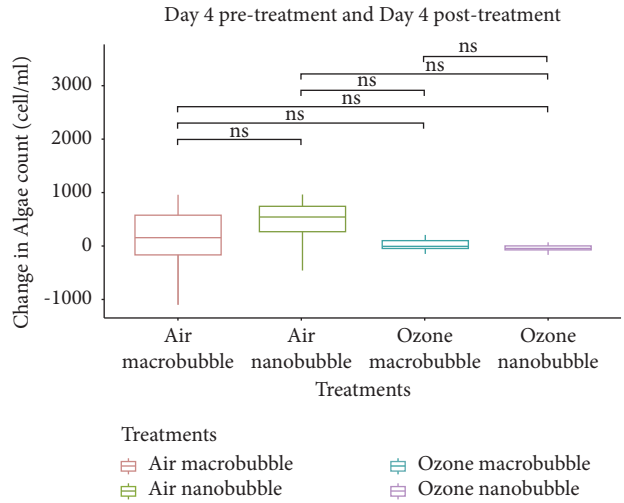


FIGURE 6: Change in algae count between day 4 pretreatment and day 4 posttreatment.

over 24 hours, i.e., 200 ± 232 cell/ml (15% growth) for the air macrobubble group and 300 ± 79.4 cell/ml (19.9% growth) for the air nanobubble group (Figure 5).

After the second and third treatments on days 2 and 4, respectively, there were no statistically significant differences (p value always >0.9207) between pre and posttreatment counts across all groups (Figure6). The algae in the ozone treatment groups were quite low. At the end of the experiment, the algae count of both ozone treatment groups was below 2×10^2 cell/ml, while that of the air treatment groups increased to above 3×10^3 cell/ml (Figure 2). The difference between day 1 pretreatment and day 4 pretreatment was significantly lower for the ozone treatment groups compared to their control groups (Figure 7).

3.2. Water Quality. Dissolved oxygen (DO) levels increased after the treatment with ozone nanobubbles and ozone macrobubbles (Figure 8). During the first treatment, the DO increased from 8.49 ± 0.06 mg·L^{-1} to 11.55 ± 0.24 mg·L^{-1} in the ozone macrobubble group (Figure 8). This was significantly different compared to its control group ($p = 0.0145$). In the ozone nanobubble group, the DO greatly increased from 8.27 ± 0.22 mg·L^{-1} to 24.51 ± 1.10 mg·L^{-1} ($p = 0.0097$) after treatment with similar increases in DO found after the 2nd and 3rd treatments (Figure 8). However, no long-lasting effect of oxygen was found in this experiment as the dissolved oxygen values decreased to their original levels within 24 hours (Figure 8).

The pH values ranged from 7.64 to 8.02 throughout the study groups. The largest change in pH was observed after the treatment on day 2 in the ozone macrobubble group, with a small drop in pH from 7.91 ± 0.035 to 7.70 ± 0.055 (Figure 9). No significant differences in the pH value were found in the ozone treatment groups compared to their respective control groups.

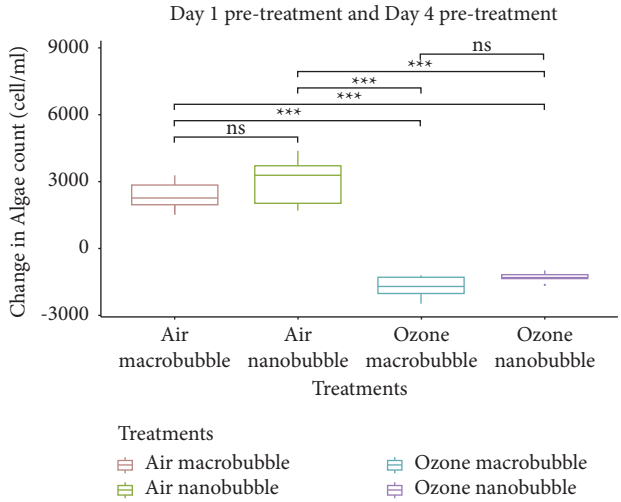


Figure 7: Change in algae count between day 1 pretreatment and day 4 pretreatment.

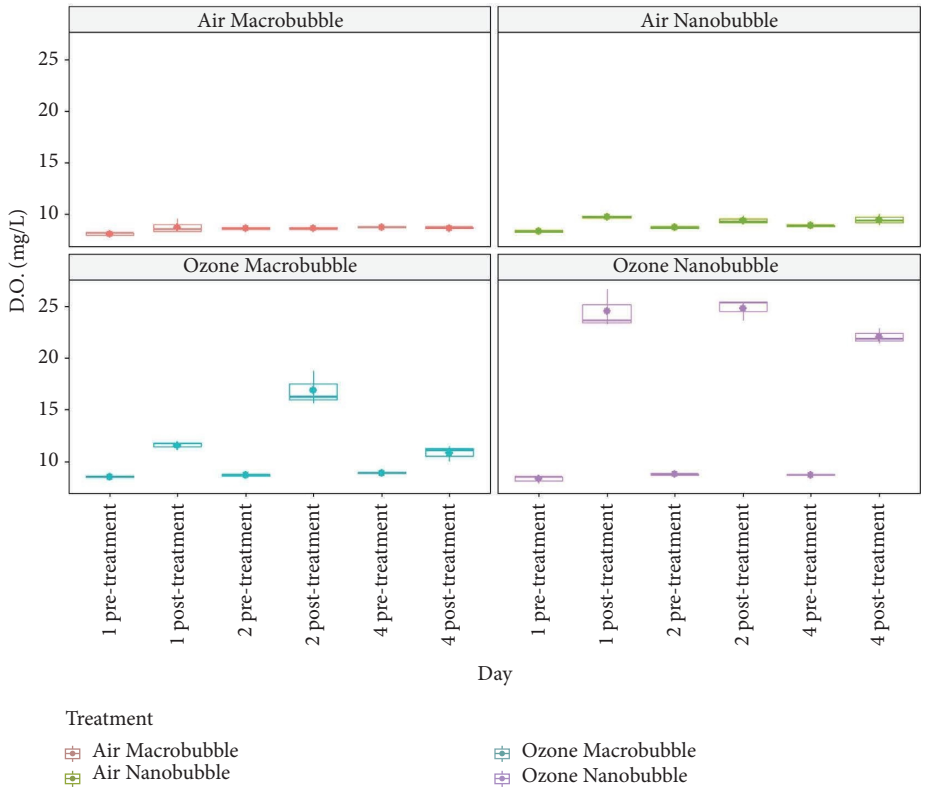


Figure 8: Dissolved oxygen level at each time point for the four treatment groups.

4. Discussion

The algae counts in our tanks were significantly reduced 24 hours after a low dose of ozone. We treated our tanks three times, but the main effect of ozone was observed after the first dose. Although the ozone killed algae relatively quickly, with over 66% of the total algae dying shortly after the ozone was administered, there was an additional delayed effect after 24 hours after treatment (an additional 10% decline). Overall, after 3 treatments, we killed 91.2% of the algae in the ozone macrobubble group and 96.1% in the ozone nanobubble group. These findings suggest a potential mitigation strategy for algae control.

Nanobubbles have been used to remove contaminants in sewage wastewater by flocculation [13]. It was expected that this technology would reduce algae in our pond water tanks regardless of the type of gas used to create the nanobubbles; however, this was not the case. Our air nanobubble-treated

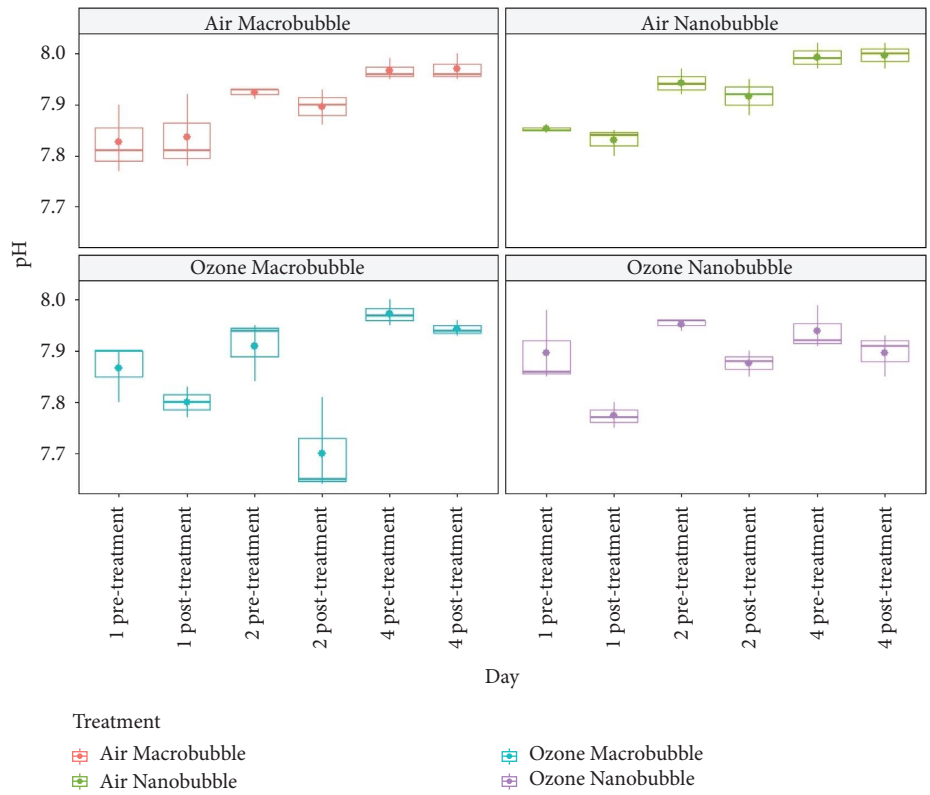


Figure 9: pH value at each time point for the four treatment groups.

tanks only had a slight reduction in algae compared to the air macrobubble treatment group, and this difference was not significant. It would appear that the kill effect in our study was predominantly due to the ozone and not nanobubbles. In fact, we observed a similar reduction in algae between the ozone macrobubble and the ozone nanobubble groups. These results suggest that it may not be necessary to administer ozone through nanobubbles as long as the dose is adequate. Delivering ozone via nanobubbler technology may be more efficient than using an air stone as it takes less operating time to reach the same dose; however, the cost of the machine may not warrant its purchase if it is only used for algae control.

An added benefit that we found using nanobubble technology to deliver ozone, which may justify the expense, was the increase in dissolved oxygen. The DO in our ozone nanobubble groups was considerably higher than that in all other treatment groups, including the tanks where the ozone was delivered with an air stone (Figure 8). Therefore, applying ozone nanobubbles in ponds at night may have the additional advantage of reducing the drop in oxygen that can occur with high levels of algae when respiration exceeds the reduced photosynthetic activity [19].

Comparing ozone to other treatments for algae control, there appear to be both benefits and disadvantages to its use. For example, copper sulphate has been commonly used to control algal blooms. However, it cannot be applicable in crustacean culturing ponds due to its toxicity to most species [20]. Copper sulphate could also potentially cause environmental problems as it can accumulate in the food chain or in the soil [7, 21]. Hydrogen peroxide can also be used to mitigate algal bloom, but it generally targets cyanobacteria species [22], which could limit its use. Physical methods of controlling algae, such as ultrasonication, cause the algae to sink to the pond's bottom and eventually die from lack of light. This technology requires a certain depth of pond (e.g., over 0.8–1 m) to be viable [23]. Ozone, on the other hand, does not have this restriction. This study suggests that ozone macrobubbles, at a dose of 0.025 ppm, are sufficient to control algae in pond water, while nanobubbles might not be necessary and could add extra cost for the treatment. However, some farmers may want to have a nanobubbler for other reasons, such as to increase oxygenation [10–12]. In these circumstances, it would simply be a matter of switching the input gas from oxygen to ozone to control algal blooms.

Even though we found ozone to be effective in algal control, it has limitations when used in pond water. Given ozone's characteristics as an unstable gas, it was difficult for us to maintain its concentration, and it took time to achieve the desired dissolved ozone concentration. Achieving such a concentration can vary according to the temperature, pH, and other water parameters [24], and the administration time to reach the desired concentration may vary for different water bodies.

One of the water quality parameters that may be affected by ozone treatments is pH. In general, we observed a slight reduction in pH after our treatments with ozone. We are not sure why this difference occurred, but a fluctuation in the pH value might impact the culturing organism. A possible reason could be the reaction between the hydroxyl radical produced during ozonation and carbonate/bicarbonate in

the water [25, 26]. To prevent stress on pH-sensitive species, applying a buffer such as calcium carbonate before or during the treatment could be beneficial.

Ozone itself could be toxic to aquatic animals if it is used in too high a concentration. Our study suggests that very low levels of ozone are required to control algae, and at this level (i.e., 0.025 ± 0.003 ppm), ozone is well below the lethal limit for common carp (*Cyprinus carpio* L.) [27] and Nile tilapia (*Oreochromis niloticus*) [28]. In the study of Al-Shammari et al. [27], common carp had a high survival rate after the ozone treatment at 0.50 mg/L. Nile tilapias were treated with ozone nanobubble at an ozone concentration of 0.14 mg/L with no mortality over a 14-day period [28].

One of the limitations of this study was that the initial algae counts in the water were relatively low, so it may be difficult to extrapolate the results of this study when the counts are high. In the latter situation, perhaps a second and third treatment would be important, and perhaps a higher level of ozone may be necessary. Another limitation of this study was that it was conducted in 75 L tanks. The required ozone concentration for an aquaculture pond is likely to be much higher, given the organic loading in most ponds [29]. In the event that higher concentrations of ozone are needed to reduce algae, the safety of these concentrations should be evaluated on the species in question.

5. Conclusions

Despite these limitations, this study provides preliminary data to suggest that ozone was very effective at killing algae when the concentration of cells was around 10^3 cells per ml. This study suggests that direct ozone injection at a dose of 0.025 ppm could be sufficient to provide more than a 65% algae reduction in pond water. The applied ozone concentration is likely not harmful to common carp [27] and Nile tilapia [28]. Additional studies are required to better understand the minimum concentration of ozone needed for algae control in earthen ponds. Nanobubbles could boost the killing effect of ozone slightly, but in this study, the improvement was not significant over the macrobubble application of ozone. However, the added benefit of the ozone nanobubble treatment was an increase in the DO level. In conclusion, the ozone treatments could be a possible mitigation strategy to reduce pH and DO fluctuations associated with algal blooms as well as to remove toxin-producing algae in fish ponds.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Supplementary Materials

Water parameters during the nanobubble experiment: (A.1) ammonia concentration during the experimental period; readings are collected at 1 pretreatment, 4 pretreatment, and 4 posttreatment; (A.2) ozone concentration during the experimental period; (A.3) water temperature during the experimental period; (A.4) turbidity during the experimental period; (A.5) chlorophyll a concentration during the experimental period; (A.6) chlorophyll a viability during the experimental period; (A.7) viable chlorophyll a concentration during the experimental period. (*Supplementary Materials*)

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