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Ozone micron bubble pretreatment for antibiotic resistance genes reduction in hospital wastewater treatment

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Abstract

Ozone micron bubble (OMB) treatment offers a promising approach to effectively eliminate Antibiotic Resistance Genes (ARGs) from infectious medical wastewater and mitigate the threat of drug resistance transmission. This study evaluated the effectiveness of OMB treatment for reducing ARGs from infectious medical wastewater in laboratory and on-site pilot treatment setups. In part, the presence of antibiotic residues in a hospital wastewater treatment plant (WWTP) and the impact of hospital wastewater on the distribution of ARGs in a wastewater collection system were also investigated. The results of wastewater collection system survey revealed a high prevalence of ARGs in the system, particularly *mcr*-1, largely originating from medical wastewater discharges. Furthermore, analysis of antibiotic residues in the hospital wastewater treatment system showed significant accumulation, particularly of quinolone antibiotics, in the biomass of the biological oxidation tank, suggesting a potential risk of ARG proliferation within the system. Comparison of wastewater samples from domestic and hospital WWTPs revealed a relatively higher abundance of ARGs in the latter, with differences ranging from 2.2 to sixfold between corresponding locations in the treatment plants. Notably, the biological oxidation unit of both WWTPs exhibited a greater proportion of ARGs among all sampled points, indicating the potential proliferation of ARGs within the biomass of the treatment units. ARG degradation experiments showed that OMB treatment resulted in a significantly lower CT value (9.3 mg O_3 L^{-1} min) compared to ozone coarse bubble treatment (102 mg O_3 L^{-1} min) under identical test conditions. Moreover, the use of OMB on site significantly reduced the accumulation of ARGs in hospital wastewater, underscoring its potential as an effective solution for mitigating ARG spread.

Keywords Antibiotics, Antibiotic resistance genes (ARGs), Ozone micron bubble treatment, Advance oxidation process, Hospital wastewater

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1 Introduction

Conventional wastewater treatment plants (WWTPs) are ineffective in removing emerging contaminants and antibiotic-resistant bacteria (ARB) [1–4], which have garnered increased attention in recent years. As a result, these contaminants may be released into the aqueous environment [5–10].

Antibiotics, a crucial subgroup of emerging contaminants, have greatly advanced medical treatment, animal husbandry, and aquaculture. However, overuse, abuse, and improper disposal of antibiotics can lead to the emergence of ARB and the spread of antibiotic resistance genes (ARGs) through selection pressure and horizontal gene transfer [11–17]. Antibiotics that are not metabolized by humans and animals enter the wastewater through excrement. Ineffective removal of antibiotics, as well as the corresponding ARB, and ARGs from wastewater allows them to enter the environment via surface runoff [6, 10, 18]. Consequently, WWTPs, especially the ones practicing biological treatment processes, have become a critical link between humans, animals, and environmental bacteria, providing an ideal environment for the exchange or transfer of ARGs. This makes wastewater a hot spot for the emergence and spread of antibiotic resistance [19].

The hospitals, where pharmaceutical drugs are extensively used to treat inpatients with infectious diseases and other illnesses, have higher concentrations of antibiotics in their wastewater compared to domestic sewage. The concentrations in hospital wastewater can often exceed several hundred μg L^{-1}, while domestic sewage typically ranges from below detection limits to a few μg L^{-1} [20, 21]. Hospital wastewater also contain antibiotic metabolites and bacteria from patient excrement, making it a major contributor of antibiotics and ARGs in WWTPs and receiving waters [4, 22–24]. This poses a risk of spreading drug-resistant bacteria to other sewage and environmental water bodies. Inadequate treatment of infectious wastewater before discharge can lead to the spread of drug-resistance to other sewage or water bodies, such as lakes and rivers [25]. To mitigate this transmission risk, tertiary treatment processes, such as membrane bioreactors (MBR), ozone, material adsorption, membrane filtration, or combined treatments, should be used [22, 23, 26–28].

Ozone is a potent oxidant [29] that finds extensive use in drinking water and wastewater treatment [30, 31]. However, its low solubility, with a Henry's law solubility constant ranging from 1.0×10^{-6} – 1.3×10^{-4} mol m^{-3} Pa, and short half-life in water (20 min) limit its efficiency [32, 33]. The use of micro- and nanobubbles has been shown to enhance the solubility and stability of ozone, thereby making ozonation more effective than conventional aeration for persistent contaminants in wastewater [34–36]. Ultramicron bubbles have the ability to disperse in water for longer periods, increasing the likelihood of organic contaminants reacting with the reactive oxygen species produced on the surface of ozone micro- and nano bubbles [37–39]. Moreover, the high mass transfer rates associated with ultramicron bubbles enable lower chemical dosage, making the process more environmentally friendly compared to conventional methods. One specific area of study that has been gaining traction is the application of ozone micron bubbles (OMB) in enhancing the efficiency of ozone treatment in the specific context of hospital wastewater. This form of wastewater is unique in its high concentration of antibiotics and ARB, necessitating specialized treatment methods. Previous research, however sparse, has started to explore the use of OMB systems for this purpose, illuminating its potential benefits and highlighting areas for further study [40, 41]. Building on this existing body of research, our study seeks to delve further into this area.

The aim of this study is to investigate the impact of hospital wastewater on the spread of ARGs in the wastewater collection systems. Additionally, the prevalence of ARGs in both domestic and hospital WWTPs, as well as the presence of antibiotic residuals in the hospital wastewater treatment system, will be assessed. Furthermore, the effectiveness of using OMB as a treatment method for removing ARGs from hospital wastewater will be evaluated in both laboratory and on-site pilot setups. This approach combines the strong oxidizing power of ozone with the efficient mass transfer of micron bubbles in water. The study hypothesizes that OMB pretreatment of hospital wastewater can effectively reduce ARGs, decrease the proliferation of ARGs in the biomass during biological treatment, and reduce dissipation in sludge wastes. To the best of our knowledge, this study represents the first report on the application of OMB treatment, specifically at a pilot scale, for the reduction of ARGs in hospital wastewater.

2 Materials and methods

2.1 Survey of ARGs in wastewater collection system—sample collection and pretreatment

Wastewater grab samples were collected from a total of 15 sites, comprising 9 samples taken directly from a wastewater collection system in northern Taiwan, 3 samples from a domestic WWTP (referred to as TS), and 3 samples from an in-hospital WWTP (referred to as YD). The 9 samples collected from the wastewater collection system were labeled as L1 to L9, as shown in Fig. 1a. Sample site L1 was connected to a local hospital, while L9 was connected to the inflow of TS. Samples taken from the three sites at each WWTP were

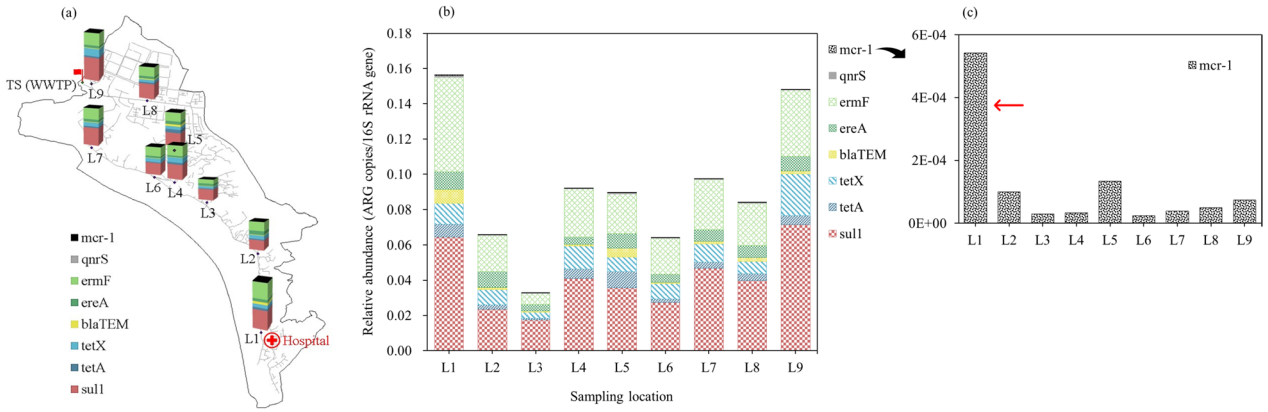

Fig. 1 **a** Prevalence of ARGs in all sampled locations in the community wastewater network in Tamsui area of Taiwan; **b** Relative concentration of ARGs at each sampled location; **c** Relative concentration of *mcr*-1 at each sampled location. (L1 – L9—Sampled locations 1 – 9 in the wastewater network; WWTP – Local wastewater treatment plant in Tamsui area)

as follows: the influent pumping station (TS_Inf), the mixed liquor from the aerated biological oxidation tank (TS_BO_Sludge), and the disinfected discharge water (TS_Eff) from TS, and the infectious wastewater inflow (YD_Inf), the mixed liquor from the biological contact oxidation tank (YD_BO_Sludge), and effluent water after disinfection (YD_Eff) from YD. Details regarding the sampling sites and sample pretreatment conditions are provided in Text S1 in the Supplementary Materials. Their corresponding locations within the wastewater collection system, as well as in the TS and YD WWTPs, are depicted in Figs. S1, S2, and S3, respectively.

The collected samples were analyzed for various basic water quality parameters, including pH, dissolved oxygen (DO), chemical oxygen demand (COD), and ammonia nitrogen (NH_3-N). The pH and DO measurements were conducted using the Orion Star™ A111 Benchtop pH Meter (Thermo Scientific, USA) and the Orion Star™ A329 Portable Multiparameter Meter (Thermo Scientific, USA), respectively. The COD was determined using the Dichromate/H_2SO_4 method with the Lovibond® COD Vario Tube Test MR reagent (0–1500 mg L^{-1}) and the Lovibond® MD610 photometer with a detectable range of 0–1500 mg L^{-1} COD, provided by Tintometer GmbH, Germany. For ammonia quantification, the Salicylate method was employed using the Lovibond® Vario AM Tube Test Reagent Set HR (0–50 mg L^{-1} NH_3-N) with the Lovibond® MD610 Photometer at 660 nm. The data obtained for pH, DO, COD, and ammonia nitrogen from the wastewater collection system, as well as the YD and TS samples, are presented in Tables S1, S2, and S3, respectively.

2.2 Quantification of ARGs

The nucleic acids were extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, Germany), with liquid and solid samples processed separately in PowerBead Pro Tubes, following the manufacturer's instructions (refer to Text S2 for a detailed description of the extraction steps). The purity and concentration of the extracted DNA were confirmed using a NanoDrop spectrophotometer (ND-1000, Thermo Fisher Scientific, USA) and Qubit DNA assay (Qubit 2, Thermo Fisher Scientific, USA).

The ARG and 16S-rRNA gene copy number quantification were performed using the QIAcuity digital PCR (dPCR) system (QIAcuity One, QIAGEN, Germany) with the QIAcuity Software Suite. Prior to dPCR, quantitative PCR (qPCR) was conducted to estimate the sample dilution factor necessary for accurate digital nucleic acid quantification. The qPCR process utilized the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) and was integral in obtaining the cycle threshold value required for the subsequent dPCR stage. Details of the PCR reaction mixtures are provided in Text S3, while the corresponding primer sequences and operating conditions for the qPCR and dPCR processes are presented in Tables S4, S5, and S6, respectively.

In this study, we focused on quantifying resistance genes associated with six prominent types of antibiotics due to their reported clinical relevance and observed prevalence in wastewater systems. The selected ARGs included those associated with sulfonamides (*sul*1), tetracyclines (*tet*A, *tet*X), β-lactamides (bla_{TEM}), streptavidin combinations of MLSB resistance genes (*ere*A, *erm*F), quinolones (*qnr*S), and colistin (*mcr*-1). Additionally, we also measured the total bacterial 16S rRNA gene (V3

region) to provide a broader perspective on bacterial presence.

2.3 Quantification of antibiotics

Samples were analyzed for target antibiotics using a solid phase extraction method in combination with liquid chromatography-tandem mass spectrometry (LC–MS/MS). The equipment employed was the Sciex API 4000 from Applied Biosystems in Foster City, CA, with positive and negative electrospray ionization interfaces. In alignment with our focus on the ARGs, we measured the levels of selected antibiotics most closely associated with those ARGs. The antibiotics analyzed included sulfonamides (sulfamethoxazole, sulfadiazine, and sulfaquinoxaline), tetracycline, quinolones (ciprofloxacin, ofloxacin, nalidixic acid, oxolinic acid), and macrolides (erythromycin, clarithromycin). Detailed information on the methods and processes for analyzing the compounds, as well as the types of compounds analyzed, can be found in Text S4. Corresponding information on the LC gradient conditions, operational parameters, and the internal standards of the investigated compounds is included in Tables S7, S8, and S9, respectively. Prior to sample analysis, method validation was conducted, and the control procedures and quality assurance for the 11 compounds analyzed in this study adhered to the guidelines established in others [42–46].

2.4 Batch ARG degradation experiment using OMB treatment

The degradation of intracellular ARGs using OMB was investigated in a batch system. The experiments were divided into three groups: Group A, which was treated with air micron bubbles (10–30 μm) only; Group B, which employed ozone coarse bubbles (1–3 mm); and Group C, which was treated with OMB (10–30 μm). Groups A and B served as control groups, while Group C was the experimental group used to evaluate the degradation effect (log reduction) of the target ARGs. The experimental setup is illustrated in Fig. S4. The treatment target for this study was *Escherichia coli* carrying the pWH1266 plasmid, which contains two ARGs, *tet*A and $bla_{\text{TEM-1}}$. For further details on the pWH1266 plasmid and the *E. coli* culture harboring the plasmid, please refer to Text S5 (including Fig. S5) and Text S6, respectively. According to the definition provided in ISO20480-1:2017, bubbles that have a diameter of less than 100 μm are classified as "fine bubbles". Within the fine bubble category, bubbles that range in size from 1 to 100 μm are referred to as "micron bubbles", while those that range from 1 nm to 1 μm are known as "ultrafine bubbles". This study was conducted with a micron bubbler with dimeter ranging from 10 to 30 μm. Further details on the production of ozone gas and detection of ozone gas concentration can be found in Text S7.

The gas supply rate of air/O_3 for all three test groups was maintained at 0.5 L min^{-1} using a flow meter. The experiments utilized a fixed ozone concentration of 75 g O_3 m^{-3}, and the test medium consisted of 10 L of 2 mM sterile phosphate buffer solution (Table S10). The test organism, *E. coli*, was introduced to the test medium at an initial concentration of approximately 10^5–10^6 CFU mL^{-1}, and a magnetic stirrer was used to keep the culture in suspension during the experiment. The apparatus was started, and the operation time was recorded, with 10 mL samples periodically taken at predetermined intervals. In the two ozone treatment groups, i.e., ozone coarse bubbles and OMB, 75 μL of sodium thiosulfate solution was added to each sample as reducing agent to stop residual ozone reaction. Following sampling, 3 mL of the sample was used for plasmid extraction, as detailed in Text S8. The relative quantification of the two genes was conducted using qPCR, as outlined in Section 2.2, with the primer sequences and reaction conditions provided in Tables S11 and S12, respectively. Both the full-length (long amplicon) and partial fragments (short amplicon) of the genes were quantified separately.

2.5 Pilot study: OMB pretreatment of hospital medical wastewater for ARG degradation

The pilot system for OMB pretreatment of medical wastewater at the YD hospital operates in conjunction with the existing wastewater treatment system. The experimental design is described in detail in Text S1-1 (including Fig. S3). Due to limitations of the pilot setup, the degradation of ARGs in the infectious wastewater was assessed by measuring the ARGs in the biofilm from the contact aeration tank after OMB pretreatment. To achieve operational stability, the pilot system was operated for two months using air micron bubble pretreatment to cultivate the biomass in the biological oxidation tank, followed by five months of OMB pretreatment. The experimental observation began at the time of cessation of ozone supply to the micron bubble pretreatment unit. The first sample was taken shortly before the ozone supply was shut off, while the second and third samples were collected 30 and 60 days after ozone supply was discontinued and only air micron bubble pretreatment was applied to the medical wastewater before the biological oxidation process. The collected samples were then processed and quantified for the target ARGs, as described in Sections 2.1 and 2.2.

3 Results and discussion

3.1 Prevalence and distribution of ARGs in wastewater collection system

The prevalence of eight ARGs (*sul*1, *tet*A, *tet*X, bla_{TEM}, *ere*A, *erm*F, *qnr*S, and *mcr*-1) across the nine sampled locations (L1–L9) in the wastewater collection network is presented in Fig. 1a. The relative concentrations of ARGs were normalized by the copy number of 16S rRNA in the samples. The absolute concentrations of 16S rRNA in the samples are provided in Fig. S6, while the relative concentrations of ARGs for each of the nine sampled sites are illustrated in Fig. 1b.

The data reveals that *sul*1 is highly prevalent in all sampling points. A statistically significant positive correlation ($r = 0.899$, p-value $= 0.001$) was observed between the relative abundance of *sul*1 and *int*I1. This association likely arises from the sulfonamide selection pressure and *sul*1's location on a conserved fragment of a class 1 integrase gene, enhancing its transmission capacity and prevalence [47]. The *tet*X, which is resistant to tetracycline and tigecycline—a backline antibiotic for multidrug resistant infections in hospitals [48], showed a significant increase in proportion when effluent from all collection lines combined at the treatment plant, possibly due to the transposon-mediated horizontal gene transfer in wastewaters. The prevalence of bla_{TEM}, *ere*A, *erm*F, *qnr*S, and *mcr*-1 genes was found to be highest at location L1, with *mcr*-1 posing the highest risk to human health among the measured ARGs, as it is a colistin gene located in a plasmid that is easily transmitted. Colistin is a regulated antibiotic exclusively used in hospitals for treating multi-drug resistant infections. The prevalence of *mcr*-1 was specifically noted to be the highest at location L1 (Fig. 1c), which is in close proximity to a hospital, indicating the potential impact of hospital discharge on the wastewater environment. This finding is consistent with the study by Hembach et al. [6] that suggested *mcr*-1 may be present and transmitted in the sewage environment, indicating that hospital wastewater containing high concentrations of *mcr*-1 could pose a downstream watershed transmission risk.

Location L1 also exhibited the highest proportion of the eight ARGs among all samples, which could be attributed to the collection of hospital wastewater, implying that hospital wastewater may significantly contribute to the presence of ARGs and serve as a primary source of ARGs in the wastewater collection system. Furthermore, the hydraulic conditions in pipes, selection pressure, adsorption and biodegradation during wastewater convergence to the treatment plant could affect the fate of ARGs, potentially leading to further changes and increased percentages of ARGs upon reaching the treatment plant at L9. A high proportion of ARGs was also noted at locations L4 and L5. These results are consistent with observations made in earlier studies [24, 49], highlighting the persistence and widespread nature of this ARG in hospital wastewater discharges. Additionally, the findings suggest an urgent need for further investigations and interventions in hospital discharge procedures to mitigate the risks associated with the spread of ARGs in wastewater collection systems.

3.2 Abundance and removal of ARGs in conventional WWTPs

The study also assessed the removal efficiency of ARGs in the conventional biological treatment process of TS domestic and YD hospital WWTPs. To ensure accurate assessment of the abundance of ARGs in all samples, the relative concentration of each gene was standardized using the absolute concentration of the 16S rRNA gene. Figure 2 presents the standardized results of the relative concentrations of ARGs.

It was observed that the proportion of *sul*1, *tet*X, bla_{TEM}, *ere*A, and *erm*F increased in YD effluent compared to YD influent. Similarly, the proportions of *tet*A, *tet*X, and *erm*F increased in the effluent from TS. These results suggest that the activated sludge process in conventional biological treatment can affect the bacterial population, potentially leading to the retention or amplification of ARGs. The significantly higher presence of ARGs in the biomass of the biological treatment process of both the domestic and hospital WWTPs, i.e., TS_BO_Sludge and YD_BO_Sludge, indicates that the nutrient-rich and biologically dense environment in the biological oxidation tank may facilitate the proliferation of ARGs. YD hospital showed a notably higher proportion of ARGs, potentially due to its smaller treatment facility or the presence of conditions favorable for the transmission of ARGs, such as high concentration of antibiotics, disinfectants, heavy metals, iodinated X-ray contrast agents, and other chemicals [50, 51]. Despite the similar hydraulic retention time for the biological oxidation units at YD and TS treatment plants (ranging from 0.85 to 2 h), the TS treatment plant receives substantially higher domestic wastewater influent (56,000 CMD) compared to the YD plant (1,500 CMD). The presence of ARGs in domestic wastewater may be diluted through the addition of non-toilet flushing waters, potentially leading to a lower occurrence of ARGs in fecal matter. On the contrary, the YD hospital influent, comprising a population predominantly composed of ill individuals subjected to relatively higher antibiotic usage, could contribute to the heightened occurrence of ARGs in the influents. With higher selection pressure in the YD treatment plant (due to the elevated presence of disinfectants and antibiotics in the

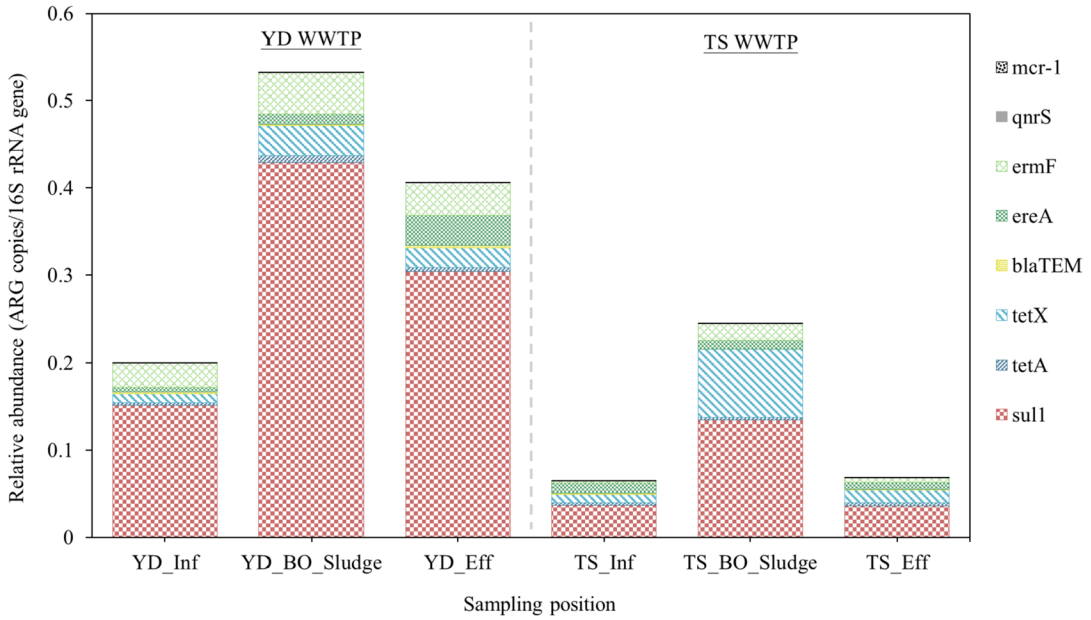

Fig. 2 Relative concentration of ARGs in the samples from YD hospital and TS domestic WWTPs. (Inf – Influent wastewater, BO_Sludge – Suspended sludge from biological oxidation process, Eff – Effluent water)

waters), a higher abundance of ARGs from the biological oxidation unit can be expected.

The observed increase in certain ARG proportions in the effluent aligns with the findings of Stalder et al. [52], Ory et al. [53], and Manoharan et al. [54], showing that conventional biological treatment processes may inadvertently favor the growth or retention of ARG-bearing bacteria. Furthermore, the higher proportion of ARGs in the YD hospital wastewater suggests the need for implementing advanced treatment processes to mitigate this specific problem.

3.3 Prevalence of antibiotics in hospital WWTP

Table 1 presents the analysis of residues of antibiotics in the influent, biological oxidation tank, and effluent of the YD hospital wastewater treatment system. The total concentration ranges (ng L^{-1}) of four major antibiotic classes, including sulfonamides, quinolones, tetracyclines, and macrolides, were determined for the influent. The concentration ranges were 6.5–533 ng L^{-1} for sulfonamides, 2.8–97.1 ng L^{-1} for quinolones, 14.1–45.8 ng L^{-1} for macrolides, and 36.9 ng L^{-1} for tetracycline. In the biological oxidation tank, the corresponding values were 8.4–3022 ng L^{-1} for sulfonamides, 2.3–20427 ng L^{-1} for quinolones, 65.3–105 ng L^{-1} for macrolides, and 44.4 ng L^{-1} for tetracycline. For the effluent, the corresponding values were 9.5–756 ng L^{-1} for sulfonamides, 7.5–16360 ng L^{-1} for quinolones, 55.5–86.1 ng L^{-1} for macrolides, and below the detection limit for tetracycline. The relative concentrations of all antibiotics analyzed, except for tetracycline, showed an increase in the effluent compared to the influent, indicating that the biological oxidation process was ineffective in removing these antibiotics. The increase was particularly significant for the quinolone antibiotics, including Ciprofloxacin, Ofloxacin, and Nalidixic. Additionally, the concentrations

Table 1 Residual concentration of antibiotics in the wastewater treatment system at YD hospital wastewater treatment plant

Antibiotic compounds	Influent	Biological oxidation (contact aeration) tank	Effluent
Sulfonamide antibiotics			
Sulfamethoxazole	533	3022	756
Sulfadiazine	6.5	8.4	9.5
Sulfaquinoxaline	160	70.4	165
Quinolone antibiotics			
Ciprofloxacin	*N.D*	64.1	352
Ofloxacin	*N.D*	20427	16360
Nalidixic acid	97.1	4029	319.5
Oxolinic acid	2.8	2.3	7.5
Macrolide antibiotics			
Erythromycin	45.8	65.3	86.1
Clarithromycin	14.1	105	55.5
Tetracycline antibiotics			
Tetracycline	36.9	44.4	*< M.D.L*

Units ng L^{-1}, *ND* Not Detected, *MDL* Minimum Detection Limit

of sulfamethoxazole, ofloxacin, nalidixic, and clarithromycin were found to be higher in the biological oxidation tank, suggesting that these compounds accumulated in the biomass.

Our observations are consistent with the findings from previous literature. Sahar et al. [55] reported that biomass, especially in MBR systems, exhibited high sorption potential for antibiotics. In their batch experiments, sorption to both suspended and membrane-attached biomass was recognized as a significant removal mechanism, with > 82% for sulfonamides and > 92% for macrolides at varied mixed liquor suspended solids concentrations. Their results indicated that the biomass demonstrated a substantial potential for bioaccumulation. Li et al. [56] also highlighted the possibility of sorption playing a crucial role in the accumulation of antibiotics in treatment processes. They pointed out that antibiotics might be adsorbed or sorbed onto the suspended solids or sludge and could then be released into the water body during treatment, potentially leading to increased levels in the effluent. Furthermore, studies by Yang et al. [57] supported that sulfonamide antibiotics were majorly removed from the water column through a combination of adsorption and biodegradation by the activated sludge. Adsorption occurred initially, and biodegradation of antibiotic compounds commenced after a delay, suggesting the critical role of adsorption in the early stages.

3.4 ARG degradation experiment

3.4.1 Degradation and reaction rate constants of ARGs in phosphate buffer saline with OMB and ozone coarse bubbles

Air micron bubble degradation The degradation results for both long and short amplicons of the two ARGs are depicted in Fig. 3a and b. In the air micron bubble group, ARG exhibited no degradation for either amplicon length. The results indicated that the use of air micron bubbles did not have any effect on the ARG degradation.

Takahashi et al. [58] noted that the sudden collapse of fine bubbles produces free radicals, primarily •OH, dissipating the chemical potential of high-density ions on the bubble surface. The pH of a solution can influence the surface potential (zeta potential) of fine bubbles and, consequently, the production of •OH. The phosphate buffer test medium used in this study, adjusted to a neutral pH 7.0, may not have facilitated gene degradation, potentially due to this pH effect.

Li et al. [59] reached a similar conclusion in their study on phenol degradation using air micron bubbles, observing no degradation when the pH exceeded 6.4. Therefore, the pH plays a significant role in the generation of free radicals by the collapse of fine bubbles. Given that the pH of

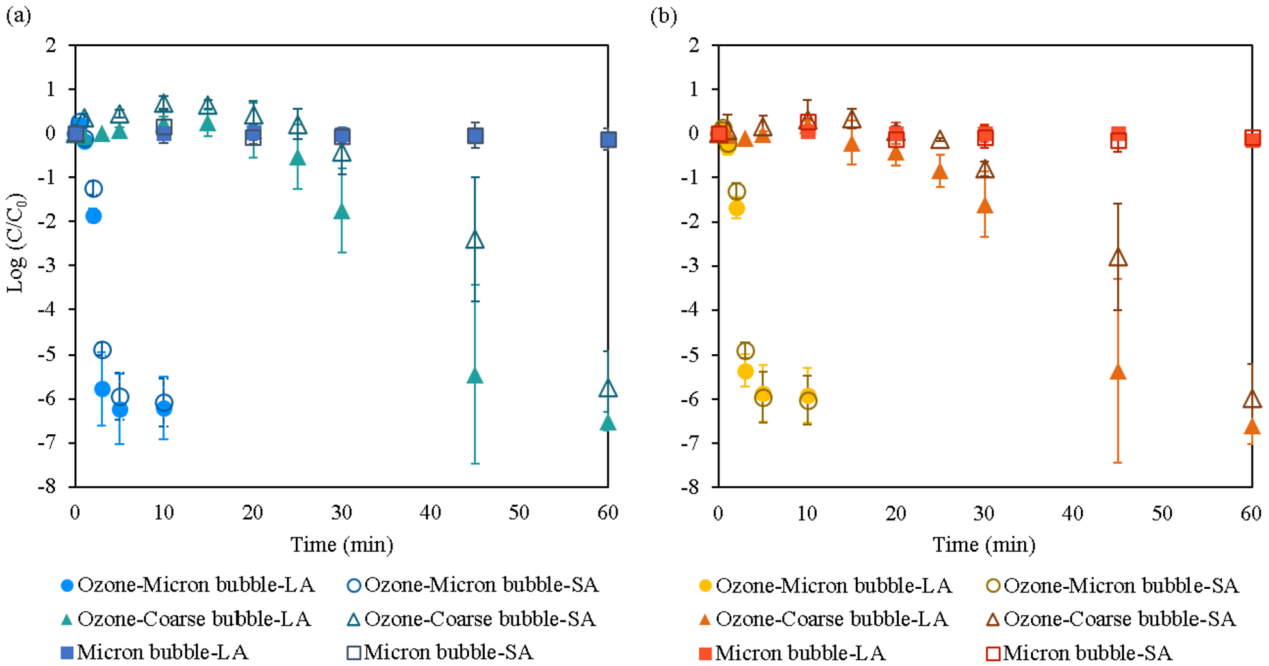

Fig. 3 Degradation curves of three groups of treatment methods for **a** *tet*A long and short amplicons, **b** bla_{TEM-1} long and short amplicons. (LA – long amplicon, SA – short amplicon). The C_0 ranged from 5.3–5.8 × 10^6 copies $mL.^{-1}$

general wastewater usually falls between 6–9, air micron bubbles might not be optimal for gene degradation.

Ozone coarse bubble degradation In the ozone coarse bubble treatment, degradation of long amplicons for ARGs was found to increase rapidly after 25 min. Degradation rates were 6.51 ± 0.21 log for *tet*A and 6.60 ± 0.41 log for $bla_{\text{TEM-1}}$ within a span of 45–60 min. In contrast, short amplicons in the ozone coarse bubble group necessitated a longer duration—60 min to attain a degradation efficiency comparable to the long amplicons, averaging at 5.86 log. The diminished reaction sites on the short amplicon, which represents a smaller pollutant, contrast with the long amplicon's greater number of reaction sites for ozone [60]. Consequently, the initial stages might experience an inadequate ozone dosage, attributed to its limited solubility, thereby extending the degradation time. By the 60-min mark, the similar degradation rates suggest a more thorough oxidation of both amplicon lengths.

DNA's alkaline pairs are constituted by double hydrogen bonds between A-T and triple hydrogen bonds between G-C. As a result, genes with a high GC content, due to their heightened resilience against degradation, might undergo varied oxidation effects. Previous research suggests that ozone oxidation may favor the proliferation of genes or strains with a high GC-content, possibly due to their increased stability against ozone impacts, as observed in findings from Alexander et al. [61]. Table S13 shows the GC content of the target genes used in this study. The GC content of the *tet*A long amplicon was higher at 61.4% compared to the short amplicon at 57.9%. For $bla_{\text{TEM-1}}$, the long amplicon had a lower GC content at 49.4% compared to the short amplicon at 51.7%. Both the size and GC content of the target gene influence the degradation efficiency, resulting in slower degradation of genes with short fragments and high GC content. The degradation efficiency, ranked from highest to lowest, is as follows: $bla_{\text{TEM-1}}$ long amplicon (49.4%) > *tet*A long amplicon (61.4%) > $bla_{\text{TEM-1}}$ short amplicon (51.7%) > *tet*A short amplicon (57.9%).

OMB degradation For the long amplicons within the OMB group, both genes exhibited an average degradation efficiency of 6.07 log within a span of 3–5 min. When examining the short amplicons of the two genes, the results were similar to those of the long amplicons. Specifically, the OMB group demonstrated an average degradation efficiency of 6.06 log in 5 min. This group emerged as the most efficient in degrading ARGs. In contrast to ozone coarse bubbles, the degradation curves for OMB were consistent across both long and short amplicons of the two genes. By the 5-min mark, comparable degradation levels were observed for both amplicon lengths. These findings suggest that while the length of the target gene fragment influenced the degradation curves, the GC content did not. As such, the degradation efficiency of the gene fragments, when ranked from the highest to lowest efficiency, is as follows: *tet*A long amplicon (1200 bp) > $bla_{\text{TEM-1}}$ long amplicon (861 bp) > *tet*A short amplicon (216 bp) ≒ $bla_{\text{TEM-1}}$ short amplicon (209 bp).

Analysis of reaction rate constants The gene degradation in relation to the increasing ozone concentration over time followed a second-order reaction pattern. To deduce the reaction rate constant (k), the experiments were plotted as pseudo first-order reactions, with plots based on degradation rate versus the CT value (ozone concentration × time). Graphical representations of the degradation rates versus CT values for both *tet*A and $bla_{\text{TEM-1}}$, encompassing long and short amplicons in ozone coarse bubbles and OMB, are illustrated in Figs. S7 and S8, respectively. The derived k values are tabulated in Table S14. The findings indicated an augmentation in k when using micron bubbles for both *tet*A and $bla_{\text{TEM-1}}$. Specifically, the long and short amplicons of *tet*A reflected a 9.8-fold and 10.4-fold surge in k, respectively. As for $bla_{\text{TEM-1}}$, the k values for long and short amplicons increased by 8.3 and 11.1 times, respectively. This implies that the use of micron bubbles accelerated the reaction rate by a factor of 8 to 11, subsequently diminishing the CT value of ozone. In practical terms, the mean CT value essential for achieving 6 log gene degradation was reduced to 9.3 (mg O_3 L^{-1} min) with micron bubbles, a stark contrast to the 102 (mg O_3 L^{-1} min) necessitated with coarse bubbles.

In conclusion, micron bubble technology augments the solubility of ozone and increases the contact area, thereby enhancing the oxidation efficiency and facilitating the selective targeting of ARGs. While the degradation efficiency of genes is influenced by gene size and GC content under ozone coarse bubbles, the OMB system enhances this efficiency, irrespective of the GC content. This results in faster and more thorough oxidative damage. Furthermore, micron bubbles significantly increase the reaction rate constants for both long and short amplicons, expediting the degradation process.

3.4.2 Degradation of ARGs in hospital wastewater

The degradation of target genes in wastewater may be impacted by competition with other organic matter, micro-organisms, and background substrates present in the water. Hence, OMB experiments were conducted using actual hospital wastewater collected from YD.

The removal efficiencies of four common genes (*sul*1, *tet*A, bla_{TEM-1}, and *mcr*-1) in the wastewater were analyzed (Fig. 4). Under the same operating conditions as in the batch experiments, the initial 10 min showed no significant degradation of the four genes, with an average removal efficiency of 0.73 ± 0.22 log at 10 min. However, after 10 min, the removal efficiency significantly increased due to the rise in dissolved ozone concentration in water and the oxidation of competing substances, resulting in an average removal efficiency of 3.57 ± 0.40 log and 4.85 log for *mcr*-1 at 20 min.

Consequently, based on the results for the medium tested, it was concluded that a CT value of at least 132 mg O_3 L^{-1} min (15 min response time) is required to achieve a significant removal effect. An average removal efficiency of 3.82 ± 0.67 log of ARGs can be achieved at a CT value of 171 mg O_3 L^{-1} min (20 min response time).

3.5 Pilot study results: effect of OMB pretreatment on ARG abundance in medical wastewater.

The impact of OMB pretreatment on the degradation of ARGs in infectious hospital wastewater was investigated under on-site operating conditions using a pilot treatment setup. The results, shown in Fig. 5, revealed that the relative abundance of ARGs in the carrier sludge in the biological oxidation tank

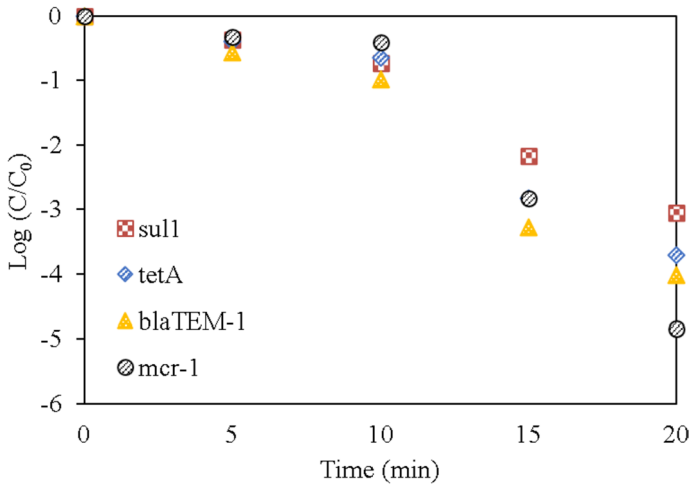

Fig. 4 Degradation curves of OMB treatment for four ARGs in hospital wastewater (The C/C_0 were obtained from compare the amplification cycle threshold to the time zero sample in qPCR)

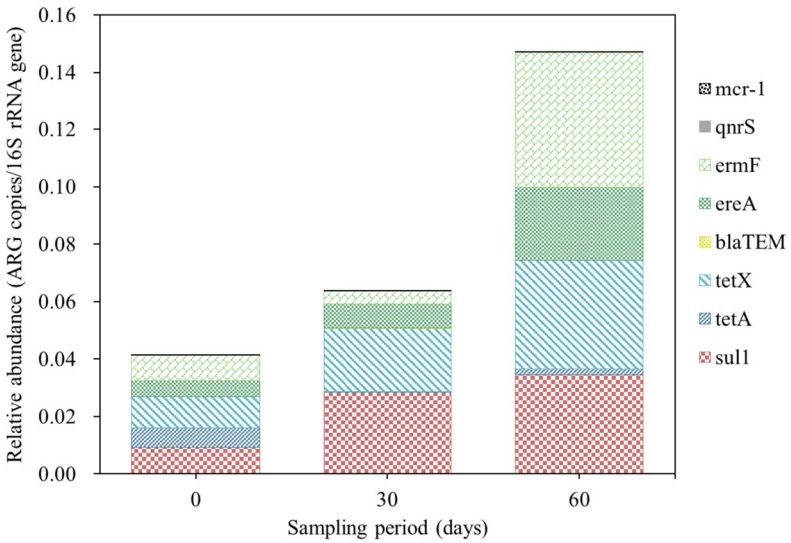

Fig. 5 Relative concentration of ARGs in the carrier biomass in contact aeration tank under micron bubble only pretreatment

increased significantly after the cessation of ozone gas to the micron bubble pretreatment unit during the 60-d period. This increase in ARG abundance in the reactor biomass can be attributed to the absence of oxidative damage from OMB. As observed earlier in this study (Section 3.1), the genes *sul*1, *tet*X, *ere*A, and *erm*F were found to have a higher relative abundance among the analyzed ARGs.

Previous literature has shown that biofilms act as active sinks for various contaminants, including intracellular and extracellular ARGs [62]. Similarly, the persistence and transfer of ARGs were noted to increase in attached biofilm systems [63]. The steady increase in the relative abundance of ARGs over 30 and 60 days in this experiment can be attributed to the lack of OMB capacity to degrade ARGs in the wastewater pretreatment. On the other hand, the low relative abundance of ARGs at the start of the experiment (i.e. day 0) suggests a low prevalence of resistance genes in the biomass due to the degradative effect of the OMB pretreatment.

4 Conclusions

Our study suggests that hospital wastewater discharge into the wastewater collection system may enhance the abundance of specific ARGs downstream, notably the *mcr*-1 gene. In a comparative analysis of wastewater samples from domestic and hospital WWTPs, the hospital samples showed up to a sixfold higher abundance of certain ARGs across corresponding locations. However, it is essential to highlight that the eight ARGs we focused on were chosen as markers and might not represent the entire range of ARGs. Hospital WWTP, notably, exhibited a significant presence of ARGs within the biomass in biological oxidation tank. The detected accumulation of antibiotics in the hospital wastewater treatment system's biomass might contribute to the growth of particular ARGs, stressing the need for efficient wastewater treatment strategies to mitigate ARG proliferation. Our laboratory and on-site pilot studies indicate that OMB treatment could be a crucial component in these strategies. The OMB treatment displayed superior ARG degradation efficiency, surpassing ozone coarse bubble treatment with a significantly lower CT value (9.3 vs 102 mg O_3 L^{-1} min) under comparable conditions. Our on-field pilot findings suggest that, in the absence of OMB's degrading capabilities, specific ARGs could accumulate in medical wastewater's biomass, indicating OMB pretreatment's potential advantages. Future studies should explore the capabilities of OMB further, not only for ARG degradation but also to boost wastewater treatment efficiency, especially for hospital discharges.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42834-023-00203-9.

Additional file 1: Text S1. Sample description and record of basic physicochemical parameters. **Text S1-1.** Pilot setup at YD. **Text S2.** Nucleic acid extraction using DNeasy PowerSoil Pro Kit. **Text S3.** Real-time quantitative polymerase chain reaction. **Text S4.** Target analyte extraction for antibiotics. **Text S5.** pWH1266 plasmid. **Text S6.** *E. coli* culture. **Text S7.** Production of ozone micron bubbles and detection of ozone gas concentration. **Text S8.** Plasmid extraction. **Table S1.** Sampling record of the wastewater collection system in Tamsui area. **Table S2.** Sampling record of YD hospital WWTP. **Table S3.** Sampling record of TS domestic WWTP. **Table S4.** qPCR and dPCR primer sequences. **Table S5.** qPCR reaction conditions. **Table S6.** dPCR reaction conditions. **Table S7.** LC gradients conditions. **Table S8.** The investigated compounds and their LC–MS/MS parameters. **Table S9.** The internal standards of investigated antibiotics and their LC–MS/MS parameters. **Table S10.** Phosphate buffer solution - for batch ARG degradation experiment. **Table S11.** qPCR primer sequences of tetA and $blaT_{EM-1}$. **Table S12.** qPCR reaction conditions of tetA and $blaT_{EM-1}$. **Table S13.** Fragment length and GC content of the target genes. **Table S14.** Reaction rate constants of ozone coarse bubble and ozone micron bubble treatment of target genes. **Fig. S1.** Sampling sites in the wastewater collection network and the corresponding collection basins. **Fig. S2.** Process flow diagram of TS domestic WWTP. **Fig. S3.** Process flow diagram of the YD hospital WWTP and the experimental design of the pilot reactor. **Fig. S4.** Schematic diagram of batch experimental setup. **Fig. S5.** Plasmid pWH1266 [8]. **Fig. S6.** Absolute concentration of 16S-rRNA gene at each sampling point of the wastewater collection system and the wastewater treatment center. **Fig. S7.** Reaction of tetA long and short amplicons with ozone coarse bubbles and ozone micron bubbles. **Fig. S8.** Reaction of blaTEM-1 long and short amplicons with ozone coarse bubbles and ozone micron bubbles.

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Authors' contributions
Shui-Shu Hsiao & Chia-Yu Hsu provided conceptualization, validation, conduct experiments and writing. Balamurugan Ananthakrishnan conduct experiments and writing. Ming-Hao Hsu provide experimental design and supervise onsite experiments. Yu-Ting Chien conducted experiments. Li-Pang Wang & Hsin-Hsin Tung provided research design, writing, reviewing and editing. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study will be made available upon request from the corresponding authors.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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