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Ozonation as an effective pretreatment for reducing antibiotic resistance selection potency in oxytetracycline production wastewater

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ABSTRACT

High concentrations of antibiotics in wastewater can promote antibiotic resistance during biological wastewater treatment. In this work, ozonation was evaluated regarding its removal efficiency of antibiotic, antimicrobial activity, and antibiotic resistance selection potency from oxytetracycline (OTC) production wastewater. Doses of 0.4 and 1.2 mg O_3 per mg of initial OTC permitted 100% and 92% OTC removal from OTC solution (OTC, 10 mg/L) and OTC production waste mother liquor (OTC, 702 mg/L), respectively. With the removal of OTC, the OTC solution lost its antibacterial activity against *Streptococcus aureus*. The antibiotic resistance selection potency of OTC before and after ozonation was also evaluated. When activated sludge was exposed to OTC (10 mg/L) solution for 18 d, the OTC-resistant bacteria ratio increased from 2.0% to 28.2%, and the relative abundances of tetracycline resistance genes (*tet*(A), *tet*(X)) and class 1 integron increased from 1.01% to 5.36% ($p < 0.05$). However, no significant change of OTC resistance was observed in activated sludge exposed to ozonated OTC solution, implying that the antibiotic resistance selection potency of OTC was alleviated by ozonation. This study demonstrated that ozonation can reduce antibiotic resistance selection potency from OTC production wastewater with a reasonable ozone dose.

Keywords: Ozonation; Oxytetracycline production wastewater; Antibacterial potency; Antibiotic resistance; Removal of antibiotic resistance selection potency

1. Introduction

Antibiotics have been found to be an important selective pressure for the occurrence and dissemination of antibiotic-resistant genes (ARGs) in the environment [1,2]. Recently, Bengtsson-Palme and Larsson [3] estimated the upper boundaries of the selective concentrations of resistant bacteria for 111 antibiotics, showing that predicted no effect concentrations for resistance selection ranged from 8 ng·L^{-1} to 64 μg·L^{-1} in water environment. During antibiotic production, waste mother liquor (WML) containing high concentrations of antibiotics (at mg/L level) is generated and is treated with biological wastewater treatment facilities after mixing with other waste streams (Fig. 1) [4,5]. The occurrence of antibiotic-resistant bacteria (ARBs) and ARGs in biological antibiotic production wastewater (APW) treatment systems has caused wide public health concerns [6–8]. Thus, it is critical to remove antibiotics from

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APW before biological treatment for the control of ARG proliferation.

Among many chemical oxidation methods that can effectively decompose antibiotics [9–11], ozonation selectively target unsaturated chemical bonds found in the structure of many antibiotics [12]. Although ozone effectively decomposes antibiotics and reduces ARGs from wastewater [13–16], the high concentrations of non-antibiotic organic pollutants consume ozone and necessitate high ozone concentrations, leading to high treatment cost. Ozonation can also generate oxidation intermediates prone to inducing antibiotic resistance in bacteria [12]. So it is important to make sure that ozone could selectively oxidize antibiotics in wastewater, and would not generate intermediates with a high ability to induce antibiotic resistance in bacteria.

This study aimed to evaluate the efficacy of ozonation on the removal of antibiotic resistance selection potency of oxytetracycline (OTC) from wastewater during biological wastewater treatment. The changes in OTC concentration and antimicrobial activity were followed over ozonation processes of wastewater with different matrix. The effectiveness of ozonation in reducing antibiotic resistance selection potency was evaluated using batch exposure tests with molecular and culture-based methods. The results of this study will provide a scientific basis for the control of ARG dissemination during biological APW treatment.

2. Materials and methods

2.1. Ozonation experiment

Ozone was generated from dried air by an ozone generator (Mitsubishi Electric, Japan). Ozonation was performed in a 2 L cylindrical glass reactor (diameter: 60 mm; height: 700 mm) by bubbling the ozone/air mixture through a ceramic diffuser fixed at the bottom at a flow rate of 10 L/h. The ozone concentrations at the inlet and outlet were determined by iodine titration, with the ozone decrease from inlet to outlet calculated as ozone consumption [17].

Three wastewater samples, including OTC-WML (pH 5.6), the influent (pH 7.0), and effluent (pH 7.4) from a full-scale APW treatment plant in North China Pharmaceutical Group Corporation, Shijiazhuang City, China (Fig. 1), were used for ozonation experiments after filtration with 0.22 μm membrane filters to remove bacteria (Millipore, USA). At the same time, synthetic OTC wastewater (OTC solution, 10 mg/L of OTC, pH 5.0) was prepared by dissolving analytical grade OTC hydrochloride (Sigma-Aldrich, USA) in ultrapure water (Millipore, USA) for ozonation and exposure experiments. The ozone reaction time was 60–200 min, depending on the pollutant concentration (60 min for synthetic OTC wastewater, the influent, and effluent; 200 min for OTC-WML). All experiments were conducted in triplicates and average values were reported.

2.2. Characterization of wastewater quality

Dissolved organic carbon (DOC) was determined using a Shimadzu DOC analyzer (Japan) after filtration with 0.22 μm filters and proper dilution (Table 1). OTC concentration and ozonation products were identified by ultra-performance liquid chromatography with tandem mass spectrometry (Waters ACQUITY UPLC/Quattro Premier XE, Waters, Ireland), using the multi-reaction monitoring and scan modes, respectively (Fig. 2). Conditions for liquid chromatography and mass detection can be found in a previous publication [4].

2.3. Antibacterial activity assay

Antibacterial potency was determined by the inhibition effect of tested samples to sensitive standard microorganism *Streptococcus aureus* using a turbidimetric method based on our previous study [18]. The antibacterial activities of water

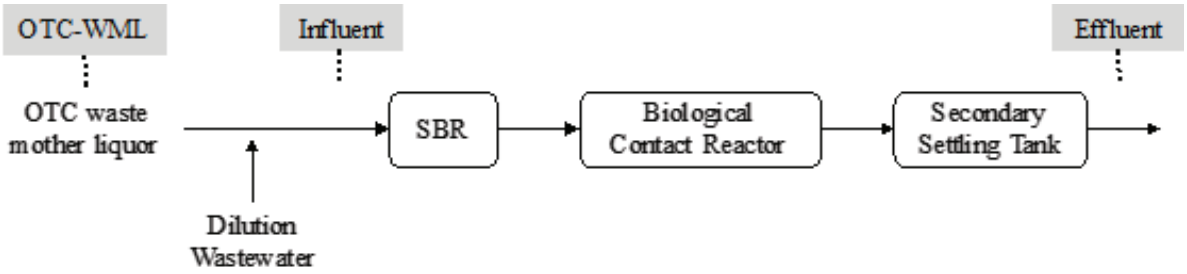

Fig. 1. Flow chart of OTC production wastewater treatment process and samples used for ozonation treatment efficiency evaluation. OTC-WML: OTC waste mother liquor; influent: influent after dilution; and effluent: effluent after secondary treatment.

Table 1
Chemistry characterizations of wastewater used for batch ozonation experiments

Sample	DOC (mg/L)	COD (mg/L)	OTC (mg/L)	OTC-associated DOC (mg/L)	pH
Synthetic wastewater	5.7 ± 0.4	17.7 ± 2.0	10.0	5.7	5.0
OTC-WML	3,541.7 ± 40.4	12,000.0 ± 280.8	702.1 ± 10.4	400.1	5.6
Influent	1,551.0 ± 30.8	5,000.0 ± 97.6	2.8 ± 0.2	1.6	7.0
Effluent	188.0 ± 11.6	600.0 ± 26.2	0.9 ± 0.1	0.5	7.4

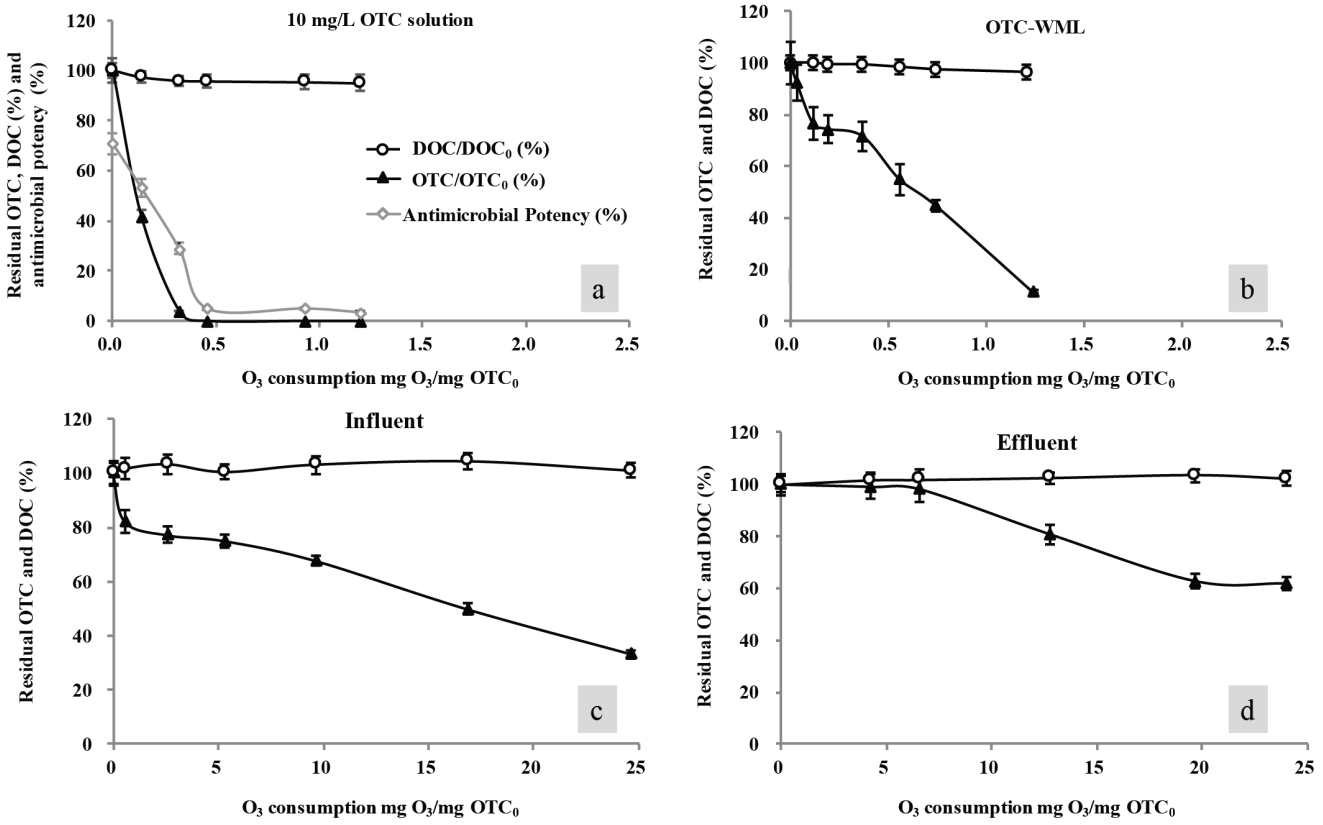

Fig. 2. Changes in OTC concentration, DOC, antimicrobial activity (in terms of the inhibition rate against *Staphylococcus aureus*) during ozonation of OTC solution (OTC_0: 10 mg/L) (a). Changes in OTC concentration and DOC during ozonation of OTC-containing wastewater, including (b) OTC production waste mother liquor (OTC-WML), (c) the influent from full-scale OTC wastewater treatment plant (influent), and (d) the final effluent from the full-scale OTC wastewater treatment plant (effluent).

samples were evaluated on a microbiological turbidimetric apparatus (WBS-100, Beijing Xianqu Weifeng Technology Development Co., China) following the United States Pharmacopeia [19,20]. Briefly, *S. aureus* was reactivated and grown by spiking 1 mL of frozen stock culture (–80°C) into 100 mL of nutrient broth. *S. aureus* was cultured to an optical density (OD_{580nm}) of 1.0. Each sample (1 mL) was mixed with 9 mL of *S. aureus* in antibiotic medium No. 3 (Dingguo, China). The mixture was incubated shaking in dark at 37°C for 3 h, with OD_{580nm} measured automatically for every 30 min. The percentage reduction of OD_{580nm} during the logarithmic growth phase in tested samples compared with the control solution (phosphorus buffer saline, 0.1 M, pH 7.2) was applied to evaluate the antimicrobial activity of each sample against *S. aureus* (Fig. 2).

2.4. Batch exposure tests of OTC solution before and after ozonation

Three cylindrical sequencing batch reactors (SBRs) with effective volumes of 3 L were operated under aeration for 18 d. The reactors were fed with synthetic wastewater (C:N:P = 100:5:1, glucose 360 mg/L, protein 80 mg/L, $NaHCO_3$ 24 mg/L, KH_2PO_4 14 mg/L, NH_4Cl 60 mg/L, $CaCl_2$ 18 mg/L, $MgSO_4$ 24 mg/L). Mixed liquor suspended solids was maintained at 3,000 mg/L. Hydraulic retention time was 24 h. The inoculated sludge was collected from the aeration tank of a full-scale sewage treatment plant in Beijing, China, of which the background tetracycline level in water phase ranged from several to 100 ng/L [21]. Among the three SBRs, one was spiked with 10 mg/L of synthetic OTC wastewater (OTC system), one was spiked with 10 mg/L of OTC after ozonation when OTC was below the detection limit (1 μg/L) (O_3 system), and the last was supplied with no OTC (control system). Sludge samples were collected for further analysis on Day 0, 7, and 18.

2.5. Determination of bacterial OTC resistance ratio by culture-based method, tetracycline resistance genes, and class 1 integron by real-time polymerase chain reaction

The OTC resistance ratio of bacteria in activated sludge exposed to different solutions was measured by culture-based method with selective media as previously described [6]. The ratio of colony forming units on plates with OTC (64 mg/L) to those on plates without OTC was defined as the bacterial OTC resistance ratio (Table 2).

FastDNA Spin Kit (MpBio, USA) was used to extract genomic DNA from sludge exposed to different solutions and the inoculum. Two typical tetracycline resistance genes (efflux protein genes *tet*(A), enzymatic modification gene *tet*(X)), which have been commonly reported in activated

Table 2
Bacteria resistance ratios to OTC in activated sludge exposed to synthetic wastewater without OTC (control), 10 mg/L of OTC (OTC), and ozonation products (O_3 product)

Exposure conditions	Resistance ratios (%) to OTC		
	Day 0	Day 7	Day 18
Control	2.0 ± 0.4	2.3 ± 0.5	1.6 ± 0.2
OTC	2.0 ± 0.4	6.7 ± 0.6	28.2 ± 1.4
O_3 product	2.0 ± 0.4	1.7 ± 0.2	2.5 ± 0.6

sludge [22], the most frequently detected mobile element (class 1 integron, *intI1*), and universal bacterial 16S rRNA genes were quantified using Sybr-Green real-time polymerase chain reaction [4]. The relative abundances of *tet*(A), *tet*(X), and *intI1* were calculated by normalizing their abundances to those of the bacterial 16S rRNA genes.

2.6. Statistical analysis

Student's *t*-test was used to test the significant differences between samples. Principal component analysis was performed using R Version 3.2.3 after Bartlett's test of sphericity, Kaiser-Meyer-Olkin test of sampling adequacy, and data normalization (Tables S1 and S2).

3. Results and discussion

3.1. Changes in OTC concentration, DOC, and ozonation products during ozonation

To investigate OTC removal efficiency under different matrix conditions, ozonation of synthetic OTC solution and wastewater from the APW treatment plant (OTC-WML, the influent and effluent) was performed. The initial water quality parameters of these samples are shown in Table 1. Changes in OTC and DOC over different ozone doses are shown in Fig. 2. OTC in the synthetic solution was efficiently reduced to below the limit of detection (1.0 μg/L) at an ozone consumption of 0.4 mg O_3/mg OTC_0 (Fig. 2(a)). However, much higher ozone doses were required for OTC destruction in the three wastewater samples. For example, 0.63 and 16.5 mg O_3/mg OTC_0 were required for 50% OTC removal from OTC-WML (Fig. 2(b)) and the influent (Fig. 2(c)), respectively, while 19.6 mg O_3/mg OTC_0 consumption only led to 40% OTC removal for the effluent (Fig. 2(d)). Ozonation could selectively oxidize OTC in OTC-WML, achieving 92% removal at an ozone dose of 1.2 mg O_3/mg OTC_0. By comparison, the OTC removal efficiency for OTC-WML was much higher than those for the influent and effluent.

Lower oxidation efficiency in real wastewater was attributed to the complex matrices, such as suspending solids, dissolved organic compounds (e.g., humic acid), etc. [23]. While OTC was the only target for ozonation in the synthetic OTC solution, the matrices in real wastewater streams competed with OTC in consuming ozone. As shown in Table 1, OTC accounted for 11% of the total DOC in OTC-WML. However, OTC only accounted for 0.1% and 0.3% of the total DOC in the influent and effluent, respectively. This result was supported by the positive

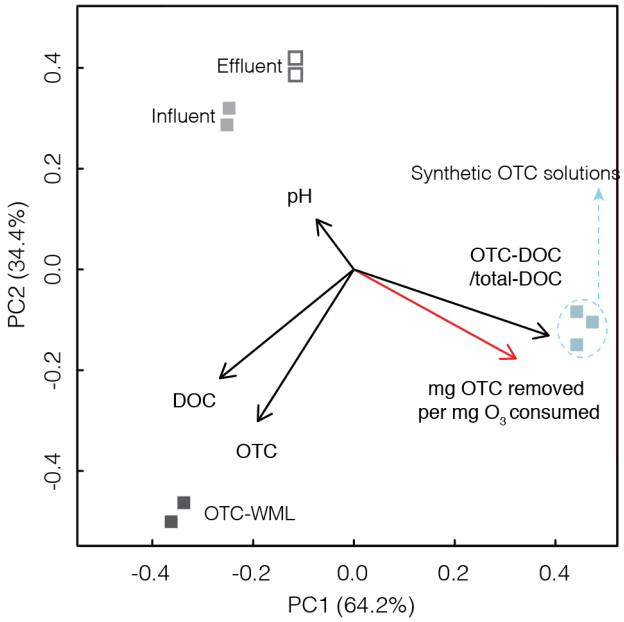

Fig. 3. Principal component analysis of various parameters.

correlation between ozonation efficiency (mg OTC removed/mg O_3 consumed) and the proportion of OTC associated DOC in total DOC (OTC-DOC/total-DOC), according to principal component analysis (Fig. 3). From the perspective of ozonation cost, it was calculated that 1.35, 36.9, and 51.5 mg of ozone would be required to remove 1 mg OTC from OTC-WML, the influent, and the effluent, respectively. The high ratio of OTC-related DOC and lower matrices level in OTC-WML favored the selective ozonation of OTC, suggesting it is more cost-efficient to use ozonation as a pretreatment for OTC-WMS before biological treatment.

Though the OTC concentration decreased throughout ozonation in all OTC containing wastewater, the DOC concentration showed no decrease (Fig. 2). A new mass spectrum peak (retention time, 6.2 min) occurred and gradually increased with the decrease in OTC (retention time, 7.3 min) during ozonation (Fig. 4). The ozonation product with a mass-to-charge ratio of 477.5 (OTC: 462.5) might be a mono-oxidation product, consistent with previous reports that an oxygen atom could be added on the C11a position of tetracycline during ozonation [24].

3.2. Changes in antibacterial potency during ozonation

As shown in Fig. 2(a), the antibacterial potency (in terms of inhibition to *S. aureus*) decreased quickly from 85.6% to 8.1% with the decrease in OTC concentration from 10 mg/L to below 1 μg/L, when 0.44 mg O_3/mg OTC_0 was consumed after 60 min of ozonation. This result suggested that the ozonation products of OTC might have little residual antibacterial potency against *S. aureus*, consistent with previous report that antimicrobial activity of OTC against *Escherichia coli* and *Bacillus subtilis* decreased after ozonation [25]. The linearly fused tetracycle, A–B ring junctions, dimethylamino group positions, and the keto–enol system (position C11, C11a, C12, and C12a) are important to maintain the antibacterial activity

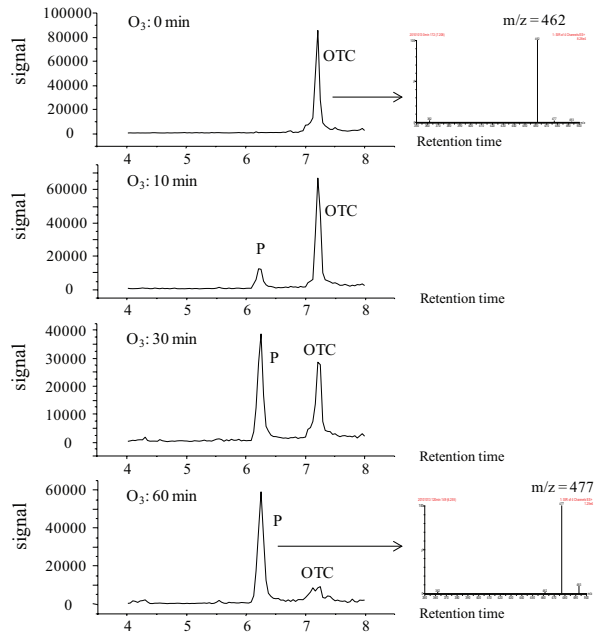

Fig. 4. Chromatogram of OTC solution during ozonation and spectrum of OTC and its main ozonation product.

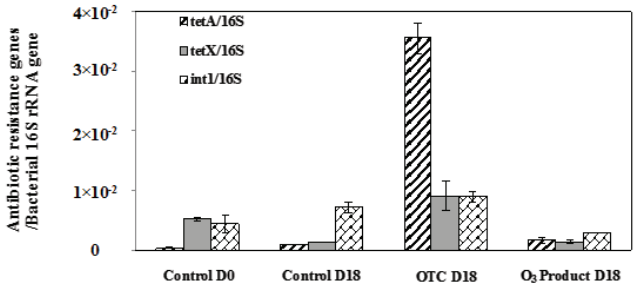

Fig. 5. Relative abundances of *tet*(A), *tet*(X), and *intI1* in activated sludge exposed to synthetic wastewater without OTC (control), 10 mg/L of OTC (OTC), and ozonation products (O_3 product). D0: seed sludge on Day 0; D18: sludge collected on Day 18 under the abovementioned three exposure conditions.

of OTC [26]. Perhaps mono-oxidation on C11a of OTC was responsible for the sharp decrease in antibacterial activity.

3.3. Antibiotic resistance profiles under different exposure conditions

The antibiotic resistance profiles of activated sludge from the three SBR reactors exposed to three conditions: (1) control system: exposed wastewater without OTC; (2) OTC system: spiked with 10 mg/L of synthetic OTC wastewater; and (3) O_3 system: spiked with ozonated OTC wastewater when OTC decreased from 10 mg/L to below 1 μg/L were shown in Fig. 5. Representative *tet* genes, including ribosomal protective protein *tet*(A), enzymatic *tet*(X), and the most frequently reported mobile element *intI1* were followed. The presence of *intI1* is not only an indicator of anthropology pollution, but also an indicator of ARGs' wide spread [27,28]. Our previous study has shown that *intI1* was positively correlated with the occurrence of *tet*(A), *tet*(G), and *tet*(X) in an OTC production wastewater treatment system [4]. The relative abundances of *tet*(A), *tet*(X), and *intI1* to 16S rRNA genes were 3.8×10^{-4}, 5.2×10^{-3}, and 4.5×10^{-3}, respectively, in the inoculated sludge (control, Day 0), and 9.2×10^{-4}, 1.4×10^{-3}, and 7.3×10^{-3}, respectively, in the control sludge (control, Day 18), showing little change in these resistance genes ($p = 0.93$) with the absence of OTC. These results are similar with those reported for activated sludge from 15 municipal wastewater treatment plants in different countries [29].

Significant increases in the relative abundances of *tet*(A), *tet*(X), and *intI1* (3.6×10^{-2}, 9.1×10^{-3}, and 9.1×10^{-3}, respectively) were observed in sludge exposed to 10 mg/L OTC-spiked synthetic wastewater (OTC, Day 18) ($p = 0.02$), showing that OTC posed a selective pressure on bacteria, which increased the risk of releasing ARGs and mobile elements to the receiving environment. Previous study has also showed that exposure to antibiotics for 10 d could result in multi-drug resistance [1]. However, the relative gene abundances of *tet*(A), *tet*(X), and *intI1* (1.7×10^{-3}, 1.4×10^{-3}, and 2.8×10^{-3}, respectively) in the sludge samples exposed to the ozonated OTC solution (O_3 product, Day 18) showed no significant differences from those in the inoculated sludge ($p = 0.12$) or control ($p = 0.18$), suggesting that the antibiotic resistance selection potency of OTC was reduced by ozonation.

At the same time, the ratios of OTC resistance bacteria in different exposure systems obtained by culture-based method are shown in Table 2. The bacterial OTC resistance ratio in inoculated sewage sludge was as low as 2.0%, which remained almost unchanged throughout the 18 d of exposure experiment for the control system (no OTC exposure). After being exposed to 10 mg/L of OTC for 7 and 18 d (OTC system), the bacterial OTC resistance ratios increased to 6.7% ± 0.6% and 28.2% ± 1.4%, respectively, suggesting that OTC-resistant bacteria were selected in the presence of OTC. Previous research found that the bacterial OTC resistance ratios increased in activated sludge exposed to 250 μg/L of tetracycline [30]. Based on metagenomic analysis, *tet* genes in municipal activated sludge increased from 0.78% to 6.99% after exposure to 20 mg/L of tetracycline for 6 d [31]. However, no significant increase of the bacterial OTC resistance ratio was observed in the system exposed to ozonated OTC, further proving that the antibiotic resistance selection potency of OTC was significantly reduced by ozonation.

Control of ARGs has encountered many difficulties because they are self-reproducible and transferable [32]. Previous studies have demonstrated that oxidation processes, such as chlorination, ozonation, and UV oxidation, are effective in removing ARGs at high consumption of oxidants [33,34]. The oxidant doses required to destroy ARGs are much higher than those required to oxidize antibiotics or sterilize ARBs [26,35,36]. In addition, some oxidation processes, including chlorination and UV irradiation, might co-select for antibiotic resistance when applied in secondary sewage, and drinking water treatment [34,37–39]. Our study showed that even short-term exposure of activated sludge to OTC wastewater resulted in an increase in the ARG and ARB ratios, while exposure to ozonated OTC wastewater did not. Therefore, it is more suitable to remove antibiotics before biological treatment for the control of ARG proliferation.

4. Conclusions

Ozonation could selectively oxidize OTC in OTC-WML, achieving 92% removal at an ozone dose of 1.2 mg O_3/mg OTC_0. The ozonation product exhibited significantly lower antibacterial potency against *S. aureus* compared with that of the parent compound. Bench-scale wastewater treatment experiments showed that both OTC-resistant bacteria and tetracycline resistance genes were selected because of exposure to OTC. However, exposure of activated sludge to ozonated OTC solutions did not show significant increase of OTC resistant bacteria or tetracycline resistance genes. Therefore, ozonation could be applied as an effective source control strategy to alleviate the occurrence of antibiotic resistance in OTC production wastewater treatment systems by removing OTC before biological treatment. However, it should be noted that long-term exposure experiments with products of various oxidation strategies still need to be investigated in the future.

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Supporting information

Table S1
Chemical parameters and ozonation efficiency of all tested samples

Sample_name	mg OTC removed/ mg O_3 consumed	DOC (mg/L)	OTC (mg/L)	OTC-DOC/ total-DOC[a]	pH
Synthetic 10 mg/L OTC	2.270	5.7	10.0	1.0000	5.0
Synthetic 1 mg/L OTC	2.500	0.57	1.0	1.0000	5.0
Synthetic 100 mg/L OTC	2.270	57.0	100.0	1.0000	5.0
OTC-WML_1	0.730	3,582.1	712.0	0.1130	5.6
OTC-WML_2	0.750	3,501.3	692.0	0.1130	5.5
Influent_1	0.028	1,580.0	2.6	0.0009	7.0
Influent_2	0.026	1,518.0	3.0	0.0011	7.1
Effluent_1	0.019	200.0	1.0	0.0029	7.4
Effluent_2	0.020	176.0	0.8	0.0026	7.4

[a]OTC-DOC/total-DOC: the proportion of OTC-associated DOC in total DOC.

Table S2
Normalized variants of all tested samples for principal component analysis

Sample_name	mg OTC removed/ mg O_3 consumed	DOC (mg/L)	OTC (mg/L)	OTC-DOC/ total-DOC	pH
Synthetic 10 mg/L OTC	0.9080	0.0016	0.0140	1.0000	0.68
Synthetic 1 mg/L OTC	1.0000	0.0002	0.0014	1.0000	0.68
Synthetic 100 mg/L OTC	0.9080	0.0159	0.1404	1.0000	0.68
OTC-WML_1	0.2920	1.0000	1.0000	0.1130	0.76
OTC-WML_2	0.3000	0.9774	0.9719	0.1130	0.74
Influent_1	0.0112	0.4411	0.0037	0.0009	0.95
Influent_2	0.0104	0.4238	0.0042	0.0011	0.96
Effluent_1	0.0076	0.0558	0.0014	0.0029	1.00
Effluent_2	0.0080	0.0491	0.0011	0.0026	1.00

Note: Each data were normalized to the highest value of the variant among all samples.