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The effect of tetracycline in the antibiotic resistance gene transfer before and after ozone disinfection

Junsik Oh[a], Carl Angelo Medriano[a], Sungpyo Kim[a,b,*]

[a]*Program in Environmental Technology and Policy, Korea University, 2511 Sejong-ro, Sejong 339-700, Korea, Tel. +82 418601457; Fax: +82 448601588; email: ub1905ub@korea.ac.kr (S. Kim)*
[b]*Department of Environmental Engineering, College of Science and Technology, Korea University, 2511 Sejong-ro, Sejong 339–700, Korea*

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ABSTRACT

Antibiotics are currently considered to be an emerging threat to the aquatic environment if left untreated. Persistence of antibiotic compounds has been detected in vast aquatic environments, including wastewater. This involves the development and increase of antibiotic resistance and bacterial presence in the aquatic communities. Several previous studies have been conducted for the removal of micro-contaminants by ozonation. The comprehensive study on residual antibiotics effect to antibiotic resistance transfer before and after oxidation has not yet been explored. Accordingly, the aim of this study is to (1) investigate the antibiotic resistance growth under the presence of tetracycline in short and long period without oxidation process and to (2) look into the amount of residual tetracycline that persists after oxidation and its possible effect in potentially influencing antibiotic resistance gene (ARG) transfer among bacteria. As shown in the results, a few ppm or few hundred ppb of tetracycline, regardless of ozonation, enhanced the ARG transfer compared to control. These results implied that ARG transfer can be enhanced by incomplete removal of micro-contaminants in the environment.

Keywords: Antibiotic resistance; Ozonation; Transconjugant; Tetracycline

1. Introduction

Antibiotics, however, used conveniently in various applications, are currently considered to be one of the emerging contaminants which have been discovered to pose various risks to the environment [1–3]. Persistence of antibiotic compounds had been detected in vast aquatic environments. It had also been found to be interacting with different communities even in very low concentrations [4–6]. Interactions of these compounds in the aquatic environment have been seen to pose different mechanisms and reactions from different species especially from micro-organisms [7,8]. The compounds are classified as emerging pollutants. These pollutants, aside from being a foreign substance not natural to aquatic environments, showed negative effects on vast number of organisms. One of the most pressing threats of these compounds is the promotion of antibiotic resistance. The presence of even low concentrations of these compounds could promote the spread of resistance among bacterial communities. Antibiotic resistance is a defense mechanism of micro-organisms against certain antibiotic compounds, which is expressed by certain genes. The spread of these

*Corresponding author.

genes would be a big problem especially when the genes are transferred to pathogens which could endanger health [9]. If pathogens become resistant to antibiotics, it would then be harder for these bacteria to be killed, making it more difficult to formulate more potent pharmaceutical products.

In many water and wastewater treatment plants, ozone disinfection is applied as a final step in removing organic contaminants, which includes micro-organisms. This is a type of advanced oxidation process which has a high efficacy in destroying various organic compounds and organisms thus, making it ideal for most plants [10]. Some studies have even considered ozone disinfection to have good efficiency in removing both antibiotic resistant bacteria and antibiotic resistance genes (ARGs) [11]. Also, a study has been able to map out the reaction of antibiotics with ozone [12]. Tetracycline, an antibiotic that is part of broad-spectrum antibiotics used in various applications, was seen to have high reactivity with ozone [13,14].

Although a number of studies have been conducted for parent antibiotic effect to the spread of antibiotic resistance in pathogens, this study focuses on the comparison of antibiotics effect to the ARG transfer before and after oxidation process. This study looks into two major concerns—first, the effect of the residual antibiotic after oxidation process to ARG transfer; second, the exploration of the threshold concentration of antibiotic to ARG transfer.

Therefore, the aims of this study are to (1) investigate the antibiotic resistance growth under the presence of tetracycline in short and long period without oxidation process and to (2) look into the amount of residual tetracycline that persists after oxidation and its possible effect in potentially influencing ARG transfer among bacteria. This study would be significant for both environmental and health aspects of antibiotics that persist in aquatic environments.

2. Materials and methods

2.1. Bacterial culture

In this study, *E. coli* DH5α, containing the 64,508 bp nucleoide sequence of the Inc-P-1beta antibiotic resistance plasmid pB10, which has multiple resistance to a number of antibiotics [15], was used. Cultures were grown in Lysogeny Broth (LB) and stored in an incubated shaker at 37˚C and 150 rpm. A pure culture of gentamicin-resistant bacteria, *Pseudomonas aeruginosa PAKexoT* [16], was also grown using the same media and culture condition.

2.2. Ozone setup and experiment start-up

Ozone was generated with pure oxygen (99.9%) using an ozone generator (LAB 2B, Ozonia Korea). The flow rate of pure oxygen to the ozone generator was maintained at around 4–5 L/min. The ozone–oxygen mixture was introduced into the system at a constant rate at the reactor bottom via a porous gas diffuser and the concentration was measured by an ozone analyzer (Orbispheremodel 3600, Switzerland). The initial ozone concentrations used in experiments were 3 and 7 mg/L. As shown in Fig. 1, the experiments were done in a continuous system. At the start of each experiment, ozone was continuously introduced to a phosphate buffer solution for 60 min. After stabilization, 10 ppm tetracycline was added into reactor. Samples were taken after 5, 10, and 15 min.

2.3. Mating test

The pB10 plasmid transfer changes were evaluated by modified plasmid transfer mating technique. After harvesting, *E. coli* DH5α (donor) and *P. aeruginosa PAKexoT* (recipient) culture, each culture was centrifuged at 5,000 rpm for 15 min. After discarding most of supernatant, cultures (donor and recipient) were re-suspended in 1 mL supernatant. The donor and recipient cultures were then mixed together, poured on different concentration of antibiotic-supplemented agar plates, and incubated for 16 h at 37˚C. After incubation, the pellets were re-suspended with 1 mL LB broth and transferred into tubes and vortexed. The mixed culture was serially diluted and spread onto LB media plates, containing tetracycline (2 ppm) and gentamycin (10 ppm). After overnight incubation, the grown recipient colonies (transconjugant, *P. aeruginosa PAKexoT* (pB10)), were counted. The colony averages were calculated using duplicates plates.

2.4. High-performance liquid chromatography test

Each sample was measured for residual tetracycline using a high-performance liquid chromatography, Nanospace SI-2 (Shiseido Co., Japan). The separation was performed on a UK-C18 column (3 μm, 250 × 4.6 mm) with methanol–acetonitrile 0.01 M aqueous oxalic acid (1:2:7) [9] as the mobile phase at a flow rate of 1 mL/min with an injection volume of 20 μL and retention time of 20 min.

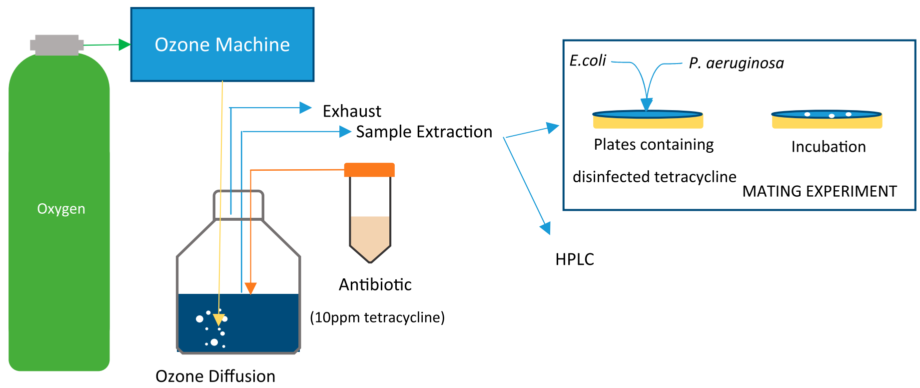

Fig. 1. Continuous ozonation process setup and mating experiment.

3. Results and discussion

3.1. Cultured with different volumes of N-limited medium

Before tetracycline oxidation by ozonation, the effect of tetracycline on the variation of transconjugant (*P. aeruginosa PAKexoT* (pB10)) concentration, which took the pB10 plasmid from *E. coli*, grown on liquid phase, were monitored. In this experiment, tetracycline (2 ppm, final concentration) was added in liquid medium, LB broth, it was then inoculated with the same amounts of donor and recipient (10^4 CFU/mL). Since the culture was shaken in the 150 rpm, the culture was assumed in the aerobic condition. As shown Fig. 2, the transconjugant concentrations in tetracycline added samples were higher than the transconjugant concentrations in control.

Some studies found that antibiotic resistance is conjugating well in anaerobic system [17,18]. So in anaerobic system like ground water, well and even leachate, antibiotic resistance can transfer to environmental recipients easily. Accordingly authors conducted similar tests above, but under anaerobic condition. For maintaining anaerobic condition, nitrogen gas was bubbled in the mixed culture to meet 0.1 mg/L of dissolved oxygen and fill the head space as nitrogen gas. As shown in Fig. 3, the time profile of transconjugant concentrations in anaerobic system under different concentrations of tetracycline (0, 10 ppb, 2 ppm) were monitored during 7 d. Higher transconjugant concentrations were observed under the presence of tetracycline even with 10 ppb compared to control as shown in Fig. 2.

Based on these studies (Figs. 2 and 3), we observed that tetracycline might enhance the pB10 plasmid transfer (vertical growth) and/or stimulate transconjugants to grow (horizontal growth) in short-aerobic or long-anaerobic conditions although these cannot be distinguished in this experiment. This result implied that the pharmaceutical compounds such as antibiotic in aquatic environment should be removed for controlling the potential enhanced spread of antibiotic resistance. However, at the same time, we observed that even very low concentrations of tetracycline (ppb) could help to increase the transconjugants in the experiment (Fig. 3).

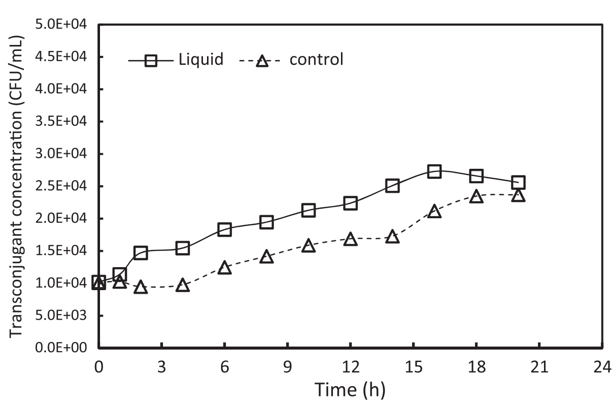

Fig. 2. Transconjugant concentration grown in aerobic system.

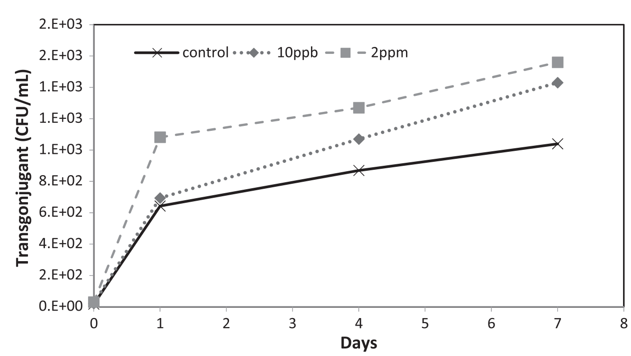

Fig. 3. Transconjugant concentration grown in anaerobic system.

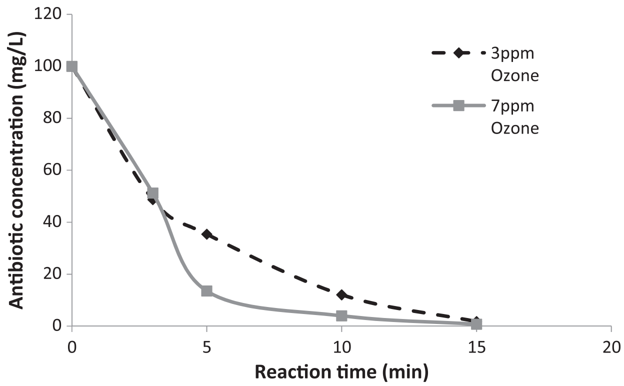

Fig. 4. Decreasing concentrations of antibiotic during ozonation.

Therefore, we further studied to reveal (1) whether residual tetracycline after ozonation could enhance the pB10 transfer and (2) if yes, what is the threshold concentration to pB10 transfer? For this purpose, two different concentrations of ozone (3 and 7 ppm) were used to disinfect the tetracycline (10 ppm) in this study. Residual tetracycline was monitored as shown on Fig. 4. After 15 min of oxidation, the concentration was reduced about 60–200 ppb level (Fig. 4).

In the following experiment, ozonated tetracycline sample under different reaction times (5, 10, and 15 min) via 3 ppm or 7 ppm ozone exposure was added to the plates that were used for the pB10 transfer mating experiment. In each experiment, tetracycline concentration was delineated in Fig. 4. As shown in Fig. 5, transconjugant concentrations were lower compared to control (no tetracycline added plated) when mating experiment was conducted with 1 ppm of residual tetracycline (about 10 min ozonation). Higher concentration of tetracycline (over 3.5 ppm) might significantly reduce the recipient concentration and resulted in the lower transconjugants compared to control. However, higher transconjugant population (14–35% higher than control) was found to be grown

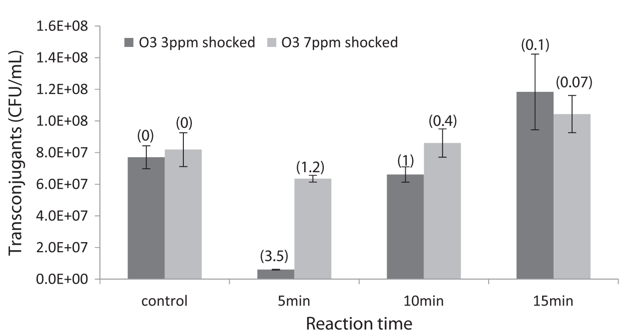

Fig. 5. Transconjugant population on plates with different antibiotic concentrations after ozonation.

below 1 ppm tetracycline supplemented plates (about 15 min ozonation).

In environment, a number of different pharmaceutical compounds are present and the concentrations are around ppm levels such as in the livestock waste [9]. Ozone, although a very powerful oxidant which causes high removal of organic contaminants and high efficacy in microbial disinfection, showed incomplete degradation of ppm level of tetracycline even with 15 min oxidation time in this study. As shown in Fig. 5, our results indicated that incomplete removal of micro-contaminants might attribute in possibly increasing transfer rate of antibiotic resistance at residual concentrations. This finding confirmed previous studies on how antibiotics could act as a promoter in increasing transfer rate of resistance genes [5]. If such low concentrations would be able to persist after ozone treatment, this might then entail to increasing the risk of treated wastewater, moreover potentially showing a risk for continuous propagation of certain micro-bacteria and the re-use of these treated wastewater.

4. Conclusions

In this study, 2 ppm of tetracycline could induce the increase of transconjugant (*P. aeruginosa PAKexoT* (pB10)) in the liquid media. Even with the high efficiency of ozone in treating organic compounds, the study on residual antibiotics after oxidation has been shown to persist through the process. With the presence of ppb level of residual tetracycline after ozone oxidation, higher facilitation of gene transfer was observed which implies the greater significance of the removal of these micro-pollutants. More intensive and comprehensive study for the effect of micro-pollutant after oxidation process to ecosystem is necessary.

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