オゾン処理による下水中のアンピシリン耐性菌と細胞内遺伝子の除去効率に及ぼす温度の影響

Effect of Temperature on the Removal Efficiency of Ampicillin Antibiotic Resistant Bacteria and Intracellular Genes in Wastewater by Ozonation

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Abstract

The emergence and spread of antibiotic resistance have become a growing concern for human health. Domestic wastewater treatment plants, on the other hand, have been identified as key hotspots for the spread of antibiotic resistance in the environment. The capacity of conventional wastewater treatment to control and eliminate these micropollutants, particularly antibiotic resistance genes (ARGs), is limited. Here, the current study investigated the effect of temperature on the removal efficiency of ampicillin antibiotic resistant bacteria and intracellular genes by ozonation treatment in order to check the concrete impacts of an operating factors from the engineering point of views. Meanwhile, the variation of intracellular ARGs was determined as antibiotic resistant bacteria (ARB) are removed during treatment. In this study, *E. coli* DH5α competent cells carrying the ampicillin resistant gene bla_{TEM} were employed for ozonation removal experiment, in which the secondary effluent of wastewater was prepared as the matrix. Then the ARB and ARGs were quantified using a culture-based method, DNA extraction, and qPCR. As a result, it shows temperature has no significant effect on the removal efficiency of ARB and ARGs in wastewater by ozonation with ozone CT. However, for ozone injection time, the removal efficiency of ARGs is higher under lower temperature.

Keywords: Wastewater Treatment, Antibiotic resistance, Ozonation

1. Introduction

Antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) are becoming more prevalent in the environment as a result of their diversity and capacity to transfer across genera[1)]. Urban wastewater treatment plants have been identified as significant hotspots for the spread of antibiotic resistance in the environment over the last decade[2)]. As a result, the prevalence of ARB and ARGs in wastewater treatment plants (WWTPs) has recently attracted increased attention. Biological reactors in activated sludge systems, in particular, are regarded to be one of the most important compartments for containing both ARB and ARGs[2)]. Traditionally, before discharging into the environment, pathogens and fecal contamination in treated wastewater effluent can be controlled by disinfection methods such as chlorination, which is the most common treatment applied. However, in terms of recent studies, the capacity of chlorination to remove ARB and ARGs is restricted[3)]. Despite the fact that it efficiently reduces ARB, complete DNA remains may persist and transfer ARGs to downstream bacteria via transformation or transduction[3,4)]. In this case, ozonation has been widely used and researched considering its effective inactivation of ARB and

elimination of ARGs from wastewater effluent. A previous study mentioned that the ozone depletion rate varies with the increasing temperature[5]. However, it is still not clear how they affect the removal efficiency of ARGs.

2. Materials and Methods

2.1 Bacterial preparation and culture

E. coli DH5α competent cells were selected to be transformed by the plasmid pUC19 carrying the ampicillin resistant gene *bla*$_{TEM}$. The formed ampicillin resistant bacteria at the concentration of 10^8 CFU/mL can be used for the ozonation removal experiment further. The decrease in the number of viable *E. coli* colonies following ozonation was measured using a culture-based technique. Disinfected (for all the collection times) and nondisinfected samples containing *E. coli* DH5α (pUC19) were serially diluted with phosphate buffer to produce between 30 and 300 colonies on each XM-G agar medium plate containing ampicillin at the concentration of 32 μg/mL (minimum inhibitory concentration). After 18h-24h of incubation at 37 ℃, the colonies on the plates were counted. For each sample, duplicate plates were prepared, and the result was averaged.

2.2 Wastewater samples

All experiments were performed using actual wastewater effluent samples collected from the secondary clarifier of an WWTP located in Japan. This WWTP, serving a population equivalent of 10,000, has an average flow of 88400 m^3/day and employs conventional activated sludge as secondary treatment. In order to eliminate the impact of the bacteria existing in the wastewater originally, 1.5L secondary effluent sample for each experiment went through 0.45 μm pre-filtration. For each comparable set of experiments, the wastewater sample should be originated from the same sample. The qualitative parameters (pH, conductivity, TOC) of the secondary-treated wastewater samples used during the experimental period were determined via routine analysis. The pH of wastewater sample was adjusted to 7 for each experiment. 10 mL ampicillin-resistant *E. coli* transformed before was performed centrifugation at 2000 × g for 20 minutes so as to remove most of the medium components. The concentrate was added to 1.5 L of 0.45 μm filtered secondary effluent water to adjust the concentration of ampicillin-resistant E. coli in the sample to approximately 10^5 CFU/mL.

2.3 Ozone treatment experiment set-up and procedure

This study carried out the ozone treatment experiment using the cylindrical semi batch reactor. The capacity of the reaction tank is 2.0 L. Ozone gas is generated from ozone generator and flows into the reaction tank. The ozone gas concentration entering the reaction tank is measured by an input ozone gas monitor, and the flow rate of the input ozone gas is measured using a flowmeter. The ozone gas concentration expelled from the reaction tank is determined by an output ozone gas monitor. The temperature of the reaction tank can be kept at a certain temperature during the reaction period using a constant temperature tank. The water sample in the reaction tank is stirred by magnetic stirrer.

The temperature was set at 10, 20 and 30 ℃. The input ozone gas concentration was maintained at 1.0 g/m^3 approximately. The acquired samples were transferred into 10 mL tubes containing 0.1 mL 1% $Na_2S_2O_3$ solution and 50 mL glass tubes including 5mL indigo solution in order to remove any residual oxidants. The ozone concentration in the liquid phase was measured by the indigo colorimetric method. The experiment was performed in triplicate and average values are quoted as results.

2.4 DNA extraction and qPCR

DNA was extracted using the Fast DNA™ Spin Kit for soil following the protocol of the manufacturer. Single DNA extraction was performed for each sample. The concentration and quality of the extracted DNA were measured by spectrophotometry. The extracted DNA samples will be stored at −20 °C until quantification analysis.

Antibiotic resistant genes in ozone-treated water samples were quantified by Real-time PCR using TaqMan probes. The genes examined to this study is plasmid pUC19 with the ampicillin resistance gene *bla*TEM. For the quantification of pUC19, standard DNA for calibration curve was prepared. As the standard DNA, pUC19 extracted by NucleoBond Xtra Midi Plus from the ampicillin-resistant Escherichia coli culture solution was used. Quantification of standard DNA processed using a Qubit 2.0 Fluorometer, and was converted to concentration (copies/μL). The DNA for preparing the calibration curve was prepared to 10^2 to 10^6 copies/μL by 10-fold serial dilution, and each dilution was subjected to the determination operation in triplicate.

3. Results and Discussion

Figure 1 and 2 show the increasing removal rate of ARGs and ARB as the CT value increased under different temperature. As can be seen, the removal rate of ARGs and ARB with each CT value under 10°C, 20°C, 30°C intermingles together and there is no significant difference under different temperature, implying that temperature has no significant impact on the removal rate of ARB and ARGs with ozone CT value. Generally, the inactivation rate constant (k) for ARB and ARGs by ozone inactivation will be calculated using the first-order Chick−Watson expression $\ln (C_t/C_0) = -kCT$. According to previous study[6], the inactivation rate constant k of ARGs can approach 10^2 L/(mg·min) approximately in clean systems. However, based on our results using real wastewater effluent, the inactivation rate constant k of 20°C is 4.12 L/(mg·min). The discrepancy between the clean system and real wastewater effluent indicates that inactivation of antibiotic resistance genes in real wastewater effluent is relatively ineffective.

Figure 3 and 4 show the increasing removal rate of ARGs and ARB as the time increased under different temperature. In contrast, the removal rate of ARGs shows a significant increase with the decreasing temperature, which can be attributed to 1) Given that ozone decomposition rate increases with the increasing temperature[5], the ozone concentration would be lower under higher temperature 2) Considering the dissolvability of gas, dissolved ozone concentration increases a lot

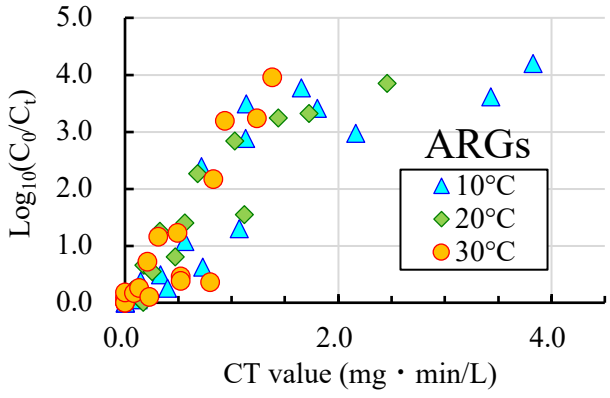

Figure 1: removal rate of ARGs with CT value

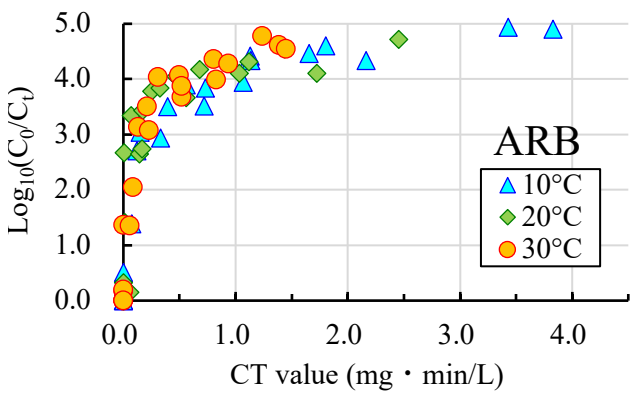

Figure 2: removal rate of ARB with CT value

under the lower temperature. Combined with these two factors, the dissolved ozone concentration increased with lower temperature simultaneously, which facilitated the reaction further. In figure 4, the removal rate of ARB shows a tiny increase as the temperature is lower as well.

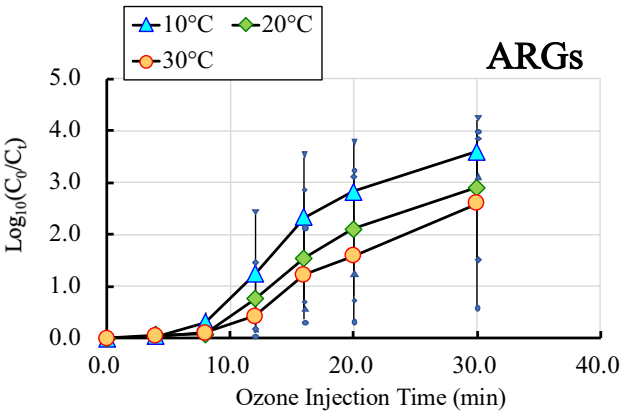

Figure 3: removal rate of ARGs with Time

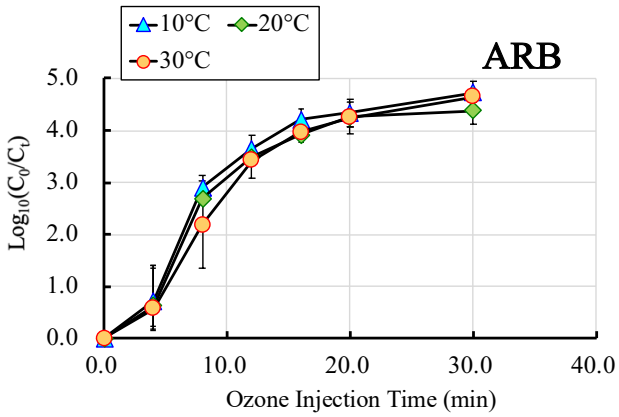

Figure 4: removal rate of ARB with Time

4. Conclusion

1) Temperature has no significant effect on the removal efficiency of ampicillin antibiotic resistant bacteria (ARB) and intracellular genes (ARGs) in secondary effluent of wastewater (pH7.0, TOC 6.9 mg/L) by ozonation with ozone CT value. However, for ozone injection time, the removal efficiency of ARGs is higher under lower temperature.
2) 1 mg・min/L and 0.2 mg・min/L of CT may be required to inactivate ARGs and ARB sufficiently (2-log reduction) in wastewater under 20°C and 1.0 g/m^3 ozone gas concentration.
3) It is suggested that ozonation can be applied to wastewater treatment plants relatively more efficiently in cold regions in order to achieve better removal of ARGs and get the most out of ozonation.

Acknowledgement

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5. Reference

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