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Microwave-Enhanced Photolysis of Rifampicin Resistant Bacteria

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Abstract. Antibiotic resistant bacteria (ARB) and antibiotic resistant gene (ARG), which pose great threaten to human health and ecological environment safety, have been widely recognized as new type of environmental pollutants. The microwave-enhanced photolysis process (MW/UV) was applied for the rifampicin resistant bacteria (RRB) disinfection in this paper. The effects of microwave power, irradiation time, initial concentration and pH of RRB were investigated, and the reactivation of the disinfected RRB in lake water, sea water and tap water was also studied. The RRB (40 mL, 3.8×10⁷ CFU/mL, pH = 7) disinfection rate could reach 100% after 60 s irradiation of 500 W microwave and 18.54 mW/cm² UV light. However, the RRB after MW/UV disinfection all experienced reactivation when discharged into the actual water matrices. The reactivation rate followed the sequence: Lake water > Sea water > Tap water. Results revealed that the MW/UV process was an effective RRB disinfection method, but further research on the change of rifampicin resistant genes in the MW/UV process was urgently needed.

1. Introduction

The production and consumption of antibiotics are huge in China. When large amounts of antibiotics enter into the environment, it will cause long-term selective pressure on microbes in the environment and lead to the generation of antibiotic resistant gene (ARG) and the increase of bacterial antibiotic resistance. Antibiotic resistant bacteria (ARB) and ARG, which were widely recognized as new environmental pollutants [1], have been frequently detected in various water matrices such as surface water, groundwater, drinking water, sewage [2,3] and pose great threat to human health and the safety of ecological environment [4]. However, ARB and ARG are unbiodegradable, replicable and have strong persistence, which make them more difficult to control than traditional chemical pollutants.

Disinfection was the main way to kill microorganisms in wastewater treatment plant. The most widely used disinfection methods were chlorine disinfection, ozone disinfection and ultraviolet disinfection. Among them, UV disinfection was highly recommended because it had high disinfection efficiency, broad-spectrum antimicrobial property, and would not produce toxic or harmful disinfection by-products. UV disinfection was also considered to be one of the most effective ways for the treatment of ARB and ARG [5]. When the DNA of the ARG matched with the UV emission spectrum, the UV light could effectively photolytic degrade the DNA fragments containing resistant gene [6]. However, when the UV dosage was insufficient, the DNA structure might be restored and the bacterial cells might be reactivated with the help of the bacterial reactivation enzyme [7].
Microwave is an electromagnetic wave with wavelength from 1mm to 1m. The thermal and non-thermal effect of microwave irradiation could cause the abnormal permeability of bacterial cell membrane and the degeneration of protein, resulting in bacterial metabolic abnormalities and irreversible damage of bacteria. The combination of microwave and UV disinfection could effectively make up the shortage of ultraviolet disinfection and improve the disinfection efficiency. Zhang et al. [8] studied the disinfection of the clarifier effluent in a wastewater treatment plant by microwave induced electrodeless UV irradiation. It was found that the coupling of microwave and ultraviolet light could take the advantages of both sides, and lead to rapid sterilization and irreversible damage to the bacteria.

Rifampicin is a broad-spectrum antibiotic, which has strong antibacterial property against Mycobacterium tuberculosis and is also effective for positive or negative gram bacteria and viruses. It was widely used in medical and pharmaceutical fields, and the rifampicin resistant bacteria (RRB) were also frequently detected in pharmaceutical factories and hospital wastewater. The microwave-enhanced photolysis process (MW/UV) was used for the disinfection of RRB in this paper. The effects of different operating parameters on the RRB disinfection were investigated and the operation conditions were optimized. The reactivation of the sterilized RRB in different actual water matrices was studied to explore the feasibility of the MW/UV disinfection of RRB.

2. Materials and methods

2.1. Materials

The RRB was provided by Institute of Urban Environment, Chinese Academy of Science. Rifampicin was purchased from Aladdin Reagent Co., Ltd. (Shanghai, China), while dimethylsulfoxide (DMSO), glycerinum and anhydrous ethanol were received from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The biochemical reagents tryptone and agar powder were also purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), while yeast extract powder was provided by Beijing double spin microbiological medium products factory (Beijing, China). All the chemicals were of analytical grade and used without further purification.

2.2. Experimental methods

2.2.1 Preparation of mediums. The LB solid medium was prepared by putting 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar into 800 mL distilled water, using 1 mol/L NaOH to adjust the pH to 7.0 ± 0.1, adding distilled water until the volume reaching 1 L, then packing the medium and sterilized under high pressure (121 °C, 20 min).

The antibiotic medium was prepared by adding sterilized rifampicin solution (3.2 mg/mL DMSO) into the cooled down LB solid medium (50-60 °C), and the final rifampicin concentration in the antibiotic medium was 4 μg/mL.

2.2.2 MW/UV disinfection procedure. The static MW/UV disinfection experiments were carried out in an APEX microwave system produced by Preekem Scientific Instruments Co., Ltd. (Shanghai, China), and the UV light was a microwave discharged electrodeless lamp (MDEL) prepared by Shanghai Jiguang Special Illumination Instruments Factory (Shanghai, China). The MDEL had maximum wavelength at 254 nm, and the UV light intensity linearly increased with the microwave power. The relation was shown in equation (1).

\[ y = 0.0435x - 1.146, \quad R^2 = 0.96401 \]  

Where \( y \) was the UV light intensity, mW/cm\(^2\), \( x \) was the microwave power.

In the MW/UV disinfection procedure, 40 mL RRB solution was added into a 250 mL Erlenmeyer flask. The MDEL was inserted into the RRB solution, and then the whole flask was put in the center of the microwave oven. After certain time of irradiation, the RRB solution was withdrawn for analysis.
2.2.3 *RRB reactivation experiment.* Sea water (sampled from the sea near Xiamen Jiageng Park, China), Lake water (sampled from the Ligong Lake in Xiamen University of Technology, China), and Tap water (sampled from the lab of Xiamen University of Technology, China) were involved in the RRB reactivation experiments to investigate the RRB reactivation in different water matrices. The compositions of these three water samples were illustrated in Table 1.

**Table 1. The composition of the sampled water**

| Water sample | COD<sub>c</sub> (mg/L) | DO (mg/L) | Conductivity (mS/cm) | Temperature (℃) |
|--------------|------------------------|-----------|----------------------|-----------------|
| Lake water   | 397.72                 | 10.63     | 0.13                 | 22              |
| Sea water    | 696.00                 | 11.38     | 58.60                | 21              |
| Tap water    | 47.00                  | 10.80     | 0.07                 | 23              |

In the RRB reactivation experiments, different dosages of the MW/UV disinfected RRB solution were added into the three water samples, and separately cultivated under the same temperature with the sampled water matrices for 48 h. The Sea water and Lake water samples were cultivated in light, but the Tap water sample was cultivated in dark to simulating the dark water pipe environment.

2.3. *Analytical method*

2.3.1. *Determination of the bacteria population.* According to GB/T4789.2-2008, 100 μL of the MW/UV disinfected RRB solution was withdrawn using a sterilized pipette and gradually diluted to a proper concentration through ten-times dilution method. Then, two of the diluted 100 μL disinfected RRB solution was separately injected into the LB medium and rifampicin medium. After uniformly spreading, the mediums were cultivated in constant-temperature incubator at 28℃ for 48h. The number of the bacterial colonies on the LB medium was counted as the total bacteria amount (CFU/mL), and the number of the bacterial colonies on the rifampicin medium was counted as the total RRB amount (CFU/mL). Each experiment was conducted in triplicate, and only the result between 30-300 CFU/mL was regarded as effective.

2.3.2. *The calculation of the relative RRB abundance.* The relative RRB abundance was calculated by the total RRB amount divided by the total bacteria amount.

3. *Results and discussion*

3.1. *Effect of microwave power on the disinfection of RRB*

Microwave was the only energy source in the MW/UV process. Increasing the microwave power would linearly increase the UV light intensity (see in equation (1)). Therefore, the effect of microwave power on the disinfection of RRB was initially studied, and the results were presented in figure 1.

The disinfection rate of RRB was significantly enhanced when the microwave power increased. The main reason might be the improved UV light intensity caused by the increase of microwave power. The higher UV light intensity would provide more energy for the deconstruction of RRB [9]. On the other hand, the enhanced microwave power would accelerate the molecular movement in the RRB solution, which would induce the temperature rises and finally improve the disinfection of RRB. In all, the microwave and UV light worked together to achieve a higher reduction of RRB. Considering the energy consumption, 500 W was suggested to be a suitable microwave power for the MW/UV disinfection of RRB.
3.2. Effect of irradiation time on the disinfection of RRB
The irradiation time was then extended to investigate the effect of continuous irradiation on the disinfection of RRB. As shown in figure 2, extending the irradiation time improved the reduction of RRB. When the irradiation was prolonged to 120 s, the disinfection rate of RRB could reach 100%. The investigation revealed that the reduction of RRB had close relation with the accumulation of microwave and UV light irradiation. The more irradiation RRB received, the worse injury the cell and DNA got. Therefore, suitable irradiation time is critical to the reduction of RRB in the MW/UV process.

![Figure 1](image1.png)
**Figure 1.** The influence of microwave power on the disinfection of RRB (Experimental conditions: RRB volume, 40 mL; initial RRB concentration, 3.3×10⁹ CFU/mL; reaction time, 10 s)

![Figure 2](image2.png)
**Figure 2.** The influence of irradiation time on the disinfection of RRB (Experimental conditions: RRB volume, 40 mL; initial RRB concentration, 4×10⁹ CFU/mL; microwave power, 500 W; UV light intensity, 18.54 mW/cm²)

3.3. Effect of initial concentration on the disinfection of RRB
The initial concentration was another factor which might influence the removal of RRB. The raw RRB solution with the concentration of 3.77×10⁹ CFU/mL was diluted 10, 100, 1000 and 10000 times to study to effect of initial concentration on the removal of RRB. Results in figure 3 illustrated that the higher the initial concentration of RRB was, the lower the disinfection rate was gained. It was because the short wavelength UV lights were easy to be absorbed and attenuated when they were penetrating the RRB solution. The higher initial concentration meant more RRB cells existed, which would absorb more UV lights and block the penetration of UV lights [10]. Thus, more RRB couldn’t receive enough UV irradiation and couldn’t be effectively reduced. Besides, microwave irradiation was also prevented to a certain extent by the higher concentration RRB, which could be another reason for the drop of the disinfection rate under higher initial RRB concentration. In practical application, proper dilution would help the disinfection of RRB.

3.4. Effect of pH on the disinfection of RRB
Microbes need a suitable pH environment for growth, so the pH of the raw RRB solution was adjusted to 3, 5, 7, 9, 11 to investigate the effect of pH on the disinfection of RRB in the MW/UV process. Under the experimental conditions, the lowest disinfection rate occurred at pH = 5, and the removals of RRB under more acidic and alkali environment were much more effective (shown in figure 4). That was because the most suitable pH for the growth of the *Escherichia. coli* was around 5.5. When the pH of the water matrice was around 5.5, the RRB cultivated from *Escherichia. coli* had the strongest resistance to the unfavorable external conditions and was most likely to survive. On the contrary, when the water matrice went more acidic or alkali, the acid-base balance of RRB was destroyed and the RRB was more easily to be damaged in the MW/UV process. Therefore, adjusting the pH properly would improve the disinfection of RRB in practical application.
Figure 3. The influence of initial concentration on the disinfection of RRB (Experimental conditions: RRB volume, 40 mL; microwave power, 500 W; UV light intensity, 18.54 mW/cm²; irradiation time, 60 s)

Figure 4. The influence of pH on the disinfection of RRB (Experimental conditions: RRB volume, 40 mL; initial RRB concentration, 3.8×10⁷ CFU/mL microwave power, 500 W; UV light intensity, 18.54 mW/cm²; irradiation time, 60 s)

3.5. The reactivation of the RRB in different water matrices

Although RRB could be disinfected under short time irradiation of microwave and UV light, it was still not clear whether the antibiotic resistance in *Escherichia coli* was completely removed. It was not sure whether the RRB would be resurrected under appropriate natural environment. Based on this consideration, simulated resurrection experiments were conducted by cultivating the disinfected RRB solution in different collected water samples. For the Lake water and Sea water case, the samples were cultivated under light and constant temperature for 48 h. For the Tap water case, the sample was cultivated in dark to simulate the dark environment in water pipe. The specific cultivation conditions and the composition of different water samples were presented in section 2.2.3.

Figure 5. Effect of the disinfected RRB dose on the resurrection of RRB in different water samples. (a) Total resurrected RRB counts; (b) Relative resurrected RRB abundance

Figure 5 demonstrated the RRB resurrection in different water samples when accepted different doses of the MW/UV disinfected RRB solution. After 48 h cultivation, RRB resurrection occurred in all the three water samples, and both the total RRB counts and the relative abundance increased with the increase of the disinfected RRB dose. Those meant the rifampicin resistant gene was not thoroughly degraded after the short time MW/UV irradiation. The RRB might be damaged, and the rifampicin resistant gene might be released from the cell. When the outside environment was suitable, the rifampicin resistant genes would be expressed again.

Comparatively, the resurrected RRB in Lake water was more serious than other two water samples. It might be ascribed to the higher RRB counts and relative abundance in the raw Lake water. On the other side, the presence of large amounts salt in Sea water might inhibit the growth of RRB. As for the Tap water condition, it almost had no RRB in the raw Tap water, and the Tap water sample was
cultivated in dark after injected with the disinfected RRB. The water samples cultivated in light, such as the Lake water and Sea water cases, might experience photo-reactivation, while the Tap water sample cultivated in dark might occur dark-reactivation. In photo-reactivation, a photo-reactivating enzyme formed in the bacteria, which restored the replicative ability of the cell and finally increased the RRB amounts. Meanwhile, the repair in the dark-reactivation belonged to the excision repair, which might take a long time to recover, so the RRB reactivation in Tap water was not significant.

Considering the resurrection of the disinfected RRB, further research on the change of rifampicin resistant gene in the MW/UV process must be carried out to evaluate the ecological risk.

4. Conclusions
RRB could be effectively reduced by the coupled irradiation of microwave and UV light. When the microwave power was 500 W, UV light intensity was 18.54 mW/cm², RRB volume was 40 mL, initial RRB concentration was 3.8×10⁷ CFU/mL, and pH was 7, the RRB disinfection rate could reach 100% after 60 s reaction. Enhancing the microwave power, diluting the RRB solution, and adjusting the RRB solution to acidic and alkali condition would all improve the disinfection of RRB. But unfortunately, the RRB after MW/UV disinfection all experienced reactivation when discharged into Lake water, Sea water and Tap water samples. The reactivation rate followed the sequence: Lake water > Sea water > Tap water. The MW/UV process was an effective RRB disinfection method, but further research on the change of rifampicin resistant genes in the MW/UV process was urgently needed.

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