

## Article

# The Impacts of Chlorine and Disinfection Byproducts on Antibiotic-Resistant Bacteria (ARB) and Their Conjugative Transfer

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**Abstract:** Antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are emerging contaminants leading to severe worldwide health problems. Chlorination, a widely used procedure, was extensively explored as one of the main methods to remove ARB and ARGs in recent years. In this study, to enrich the analyses of chlorination, several comprehensive effects of the chlorine disinfection system on ARB and their conjugative transfer ability were explored. The results presented that the low dose of chlorine (<3-log inactivation rate) had little influence on the survival of bacteria in terms of their capacity for conjugative transfer and antibiotic resistance. The high dose of chlorine (>3-log inactivation rate) triggered cell membrane changes, with little influence on the bacteria in terms of their antibiotic resistance. However, their capacity for conjugative transfer sharply decreased. Minor consumption of chloramphenicol was achieved with the chlorine dose applied in the disinfection system. Monochloroacetonitrile (MCAN) had limited effects on the frequency of conjugative transfer, indicating that the existence of MCAN would not aggravate the dissemination of ARGs by conjugative transfer. The overall impacts of the chlorine disinfection system with different containments on antibiotic resistance need further investigation.

**Keywords:** antibiotic resistance bacteria; conjugative transfer; chlorination; monochloroacetonitrile

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## 1. Introduction

The widespread use of antibiotics has saved millions of people from bacteria-related illnesses, but resulted in comparatively high environmental concentrations, as well as the emergence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs). ARB and ARGs, as emerging contaminants, have been found in different environments, including in surface water [1,2], groundwater [3], and drinking water systems [4], leading to severe worldwide health problems. Horizontal gene transfer (HGT) of ARGs, including conjugation within bacteria, natural transformation, and transduction, was considered the most critical driver for ARG transfer and deeply enhanced the spread of antibiotic resistance among communities of non-resistant bacteria [5–7]. Plasmid-based conjugative transfer widely occurred in the aquatic environment and laboratory conditions as the main method of conjugation within bacteria [5,8].

In particular, the risk of ARG transfer under the background of the COVID-19 pandemic should be paid further attention. Disinfection facilities, as the most significant barrier, were considered to ensure microbial safety [9,10]. However, Lu and Guo noted that antimicrobial resistance spread was accelerated by the increasing use of disinfectants due

to COVID-19 [11]. Therefore, the effects of disinfection on ARB and ARG transfer became more imperative to control the transmission of antibiotic resistance. Chlorination, as one of the most economical and convenient disinfection methods, was widely used in wastewater and drinking water treatment processes [12,13]. Chlorine-based disinfectants were common practices in water treatment plants to disinfect COVID-19-contaminated surfaces in many countries [14]. Chlorine had a sufficient effect on the inactivation of ARB and a certain impact on ARGs [15–19], and the inactivation rate of chlorination on ARB was drastically superior to that of ARGs [20]. However, few investigations paid attention to the effects of chlorine on decomposing antibiotics and simultaneously inactivating bacteria.

In recent years, the effects of chlorination on conjugation experiments have been investigated, with several different conclusions. Under a high dose of chlorine ( $>80$  mg Cl min/L, 8 mg/L active chlorine  $\times$  10 min), the frequency of conjugation was significantly repressed [12]. There were more reports on the effects of low doses of chlorine on conjugation experiments. Up to 40 mg Cl min/L (4 mg/L active chlorine  $\times$  10 min) disinfection generated chloramine, which stimulated the bacteria, improved the cell permeability, and significantly boosted the conjugation frequency (2–5 fold) [12]. Nevertheless, the conjugation environment with the minimum inhibitory concentration of chlorine (0.1–1 mg/L chlorine) significantly suppressed conjugation [13]. Meanwhile, 0.05–0.2 mg/L chlorine dosages limitedly influenced the conjugative transfer frequency, and 0.3–0.5 mg/L of chlorine significantly decreased the frequency of conjugative transfer [21]. Former research mainly focused on the conjugative transfer in a chlorine-existent environment, instead of concentrating on the conjugative transfer ability after chlorination and its derivatives. Disinfection byproducts (DBPs) generated by chlorine were one of the significant drawbacks of chlorination. The influences of four typical DBPs were explored, which enhanced bacterial antibiotic resistance towards ten individual and multiple antibiotics [22]. There is little research aiming at the effects of DBPs on conjugation experiments. Therefore, the effects of different chlorination and derived DBPs in conjugation experiments must be further studied.

Chloramphenicol, one of the most widely applied broad-spectrum antibiotics [23], was frequently detected in water [24–27]. Haloacetonitriles (HANs), one of the most common types of DBP in water, might influence conjugative transfer due to its genotoxicity and mutagenicity exhibited in vitro bioassays [28]. To comprehensively evaluate the safety concerns towards chlorination, the effects of the chlorine disinfection system on ARB conjugative transfer were explored from three aspects in this study: (1) the inactivation rates with or without antibiotics, the disinfection mechanism, and the changes in antibiotic resistance characteristics of ARB in a chlorine system were explored; (2) the changes in conjugative transfer ability in surviving ARB after chlorination were studied; (3) the influences of DBPs on the conjugative transfer frequency of ARB were investigated.

## 2. Materials and Methods

### 2.1. Bacterial Strains

*E. coli* HB101 and *E. coli* NK5449 were used in the experiments. *E. coli* HB101 (RP4), resistant to kanamycin, tetracycline, and ampicillin, was acquired from Professor Junwen Li of the Institute of Health and Environmental Medicine in China. *E. coli* NK5449, resistant to rifampicin and nalidixic acid, was purchased from the China General Microbiological Culture Collection Center. In the conjugation experiment, *E. coli* HB101 was used as the donor strain, while *E. coli* NK5449 was commonly used as the recipient strain [29]. The above strains were cultured in Luria–Bertani (LB) broth with appropriate representative antibiotics (*E. coli* HB101 with 50 mg/L kanamycin and *E. coli* NK5449 with 160 mg/L rifampicin) for 16 h with shaking at 150 rpm, 37 °C. The suspensions were centrifuged at 4000 rpm for 10 min. To prepare a bacterial suspension, the cells were washed twice and resuspended in 0.85% sterile saline. The suspensions before and after experiments were

appropriately diluted by gradient and then evenly mixed with LB agar plates. The LB agar plates were positioned in a 37 °C incubator for at least 24 h. Lastly, the number of colonies on the plates was counted. The amount was calculated as colony-forming units per milliliter (CFU/mL). All relevant instruments and solutions were sterilized.

## 2.2. Chemicals

Sodium hypochlorite (with 14% chlorine free radicals) and sodium thiosulfate (≥99%) were supplied by Shanghai Macklin Biochemical (China, Shanghai). Kanamycin, rifampicin, and chloramphenicol were obtained from Sangon Biotech (China, Shanghai).

The concentrations of chloramphenicol were examined by ultraviolet spectrophotometry using a microplate detector (DR2800, HACH, Loveland, CO, USA). Detection wavelength was 278 nm, and detection range varied from 0.5 to 10 mg/L (the standard curve:  $y = 31.129x - 0.0269$ ,  $R^2 = 0.9985$ ).

## 2.3. Chlorination Procedures

In this study, 100 mL bacterial suspensions with  $10^8$  CFU/mL initial bacteria number were carried out in 250-mL Erlenmeyer flasks with magnetic stir bars under room temperature (25 °C). Sodium hypochlorite was added to attain diverse initial concentrations of chlorine (2.5, 5, 10, 12.5, and 15 mg active Cl/L) for 10 min. Chlorine doses with values of 25, 50, 100, 125, and 150 mg Cl min/L were used, respectively. Excessive sodium thiosulfate solution (1.5%) was added as the quencher.

After plate counting of the disinfection procedure, one colony on the LB plates was selected by inoculating loop, and then cultured in LB broth. The samples were cultivated for 18 h at 37 °C, 150 rpm and then used for antibiotic susceptibility and conjugation experiments.

## 2.4. Determination of the Injury of Bacterial Membrane

The bacterial membrane damage was detected by the thiobarbituric acid method [30]. The concentration of malondialdehyde (MDA) of the bacterial membrane was examined following the procedures specified in a lipid peroxidation MDA assay kit (A003-1, Nanjing Jiancheng Bioengineering Institute, China, Nanjing) by a microplate detector (DR2800, HACH, USA, Colorado).

## 2.5. Antibiotic Susceptibility Testing

Ten µL bacteria suspension was prepared as described above, and one mL LB broth containing 0, 300, 500, 700, 1000, 1500, and 2000 mg/L kanamycin was added to a 1.5 mL centrifuge tube. After shaking for 18 h at 37 °C, 150 rpm, the OD<sub>600</sub> nm of the suspension was measured using ultraviolet spectrophotometry with a microplate reader (Epoch-2, Biotek, USA, Vermont).

## 2.6. Conjugative Transfer Experiment

The donor and recipient cells were cultivated in separate LB broth on a 150-rpm shaking incubator for 4–6 h at 37 °C to attain logarithmic stage bacteria. Then, suspensions were centrifuged at 4000 rpm for 10 min, resuspended by 0.85% sterile saline separately, and mixed in 1 mL (approximately  $10^7$  CFU/mL). The combinations were added to 100 mL LB broth and cultured at 37 °C, shaking at 150 rpm for 16–18 h before plate counting. The recipients obtaining the RP4 plasmid were recognized as transconjugants and were identified and calculated on LB plates containing both repetitive antibiotics of donor and recipient. The donor and recipient strains were cross-plated with no growth.

Monochloroacetonitrile (MCAN) is one of the nine species of brominated and/or chlorinated HANs [31] and was detected in drinking water [32] and was selected as the representative DBP in our report. In experiments testing the influences of MCAN on

conjugative transfer, the untreated donor and recipient bacteria were used, and 0, 10, 100, and 1000 µg/L MCAN were added into the conjugative environments, respectively.

## 2.7. Data Analysis

### 2.7.1. Dose–Response Curve of Bacteria Resistant to Kanamycin

The dose–response curves of bacteria to kanamycin were fit by Origin, using the model of the general form of a four-parameter logistic function, as shown in Equation (1) [33].

$$\text{Survival (\%)} = \frac{A_1 - A_2}{1 + \left(\frac{x}{x_0}\right)^p} + A_2 \quad (1)$$

Survival (%) is calculated as the proportion between the bacterial counts (OD<sub>600</sub>) with and without different concentrations of kanamycin. A<sub>1</sub> is the OD<sub>600</sub> without antibiotics spiked during antibiotic exposure with a primary value of ‘100’. A<sub>2</sub> is the OD<sub>600</sub> for a ‘limitless’ dose with the original value ‘0’. x<sub>0</sub> is the 50% inhibitory concentration (IC<sub>50</sub>) (mg/L). x is the kanamycin concentration (mg/L). p is a ‘slope factor’, which defines the steepness.

### 2.7.2. Stage Survival Rate

The bacterial survival rate of kanamycin was calculated from Equation (2).

$$\text{Stage survival (\%)} = \text{survival}_x (\%) - \text{survival}_y (\%) \quad (2)$$

Survival<sub>x</sub> (%) is the survival rate (%) of bacteria at x mg/L kanamycin. Survival<sub>y</sub> (%) is the survival rate (%) of bacteria at y mg/L kanamycin.

### 2.7.3. Frequency of Conjugative Transfer

As the recipient was excessive in the experiments, the frequency of conjugative transfer was calculated, as shown in Equation (3) [34].

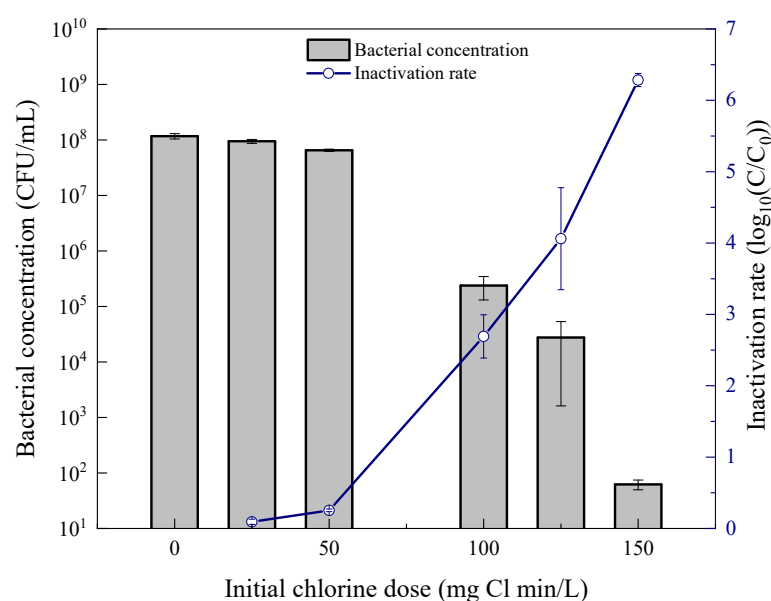
$$\text{Frequency of conjugative transfer} = \frac{\text{number of transconjugants} \left(\frac{\text{CFU}}{\text{mL}}\right)}{\text{number of donor cells} \left(\frac{\text{CFU}}{\text{mL}}\right)} \quad (3)$$

## 3. Results and Discussion

### 3.1. Inactivation of ARB under Chlorine System

#### 3.1.1. Inactivation of ARB under Different Concentrations of Chlorine

The inactivation effects of chlorination on *E. coli* HB101 are presented in Figure 1. Chlorine had little inactivation effect on *E. coli* HB101 with a low initial chlorine dose. When the disinfection dose was below 50 mg Cl min/L, the inactivation rate was proximate to 0.25-log. The inactivation rate was greatly amplified with the increase in the initial chlorine dose. When the chlorine dose value reached approximately 100 mg Cl min/L, the inactivation rate extended to around 3-log, and a significant reduction in bacterial concentration was detected. The inactivation rate rose to around 6-log with 150 mg Cl min/L initial chlorine dose, and only 10<sup>2</sup> CFU/mL bacteria remained alive in the suspension.



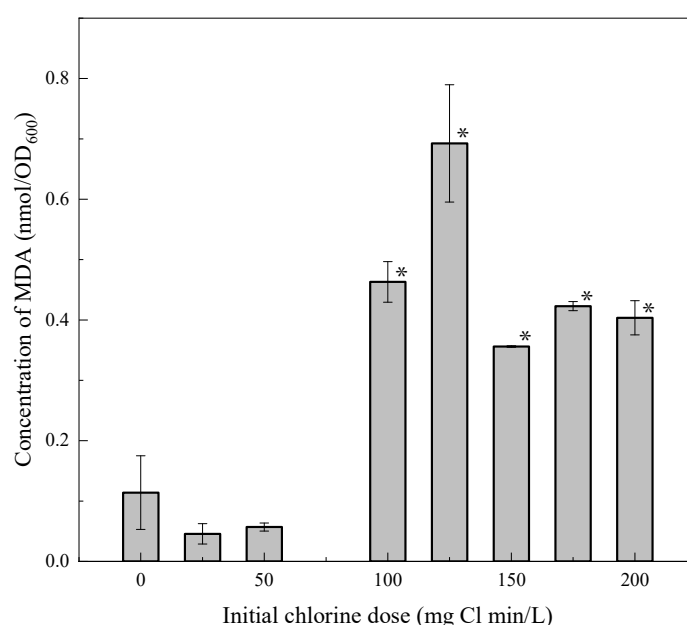
**Figure 1.** The inactivation rates and bacterial survival counts with the different initial concentrations of chlorine. Temperature = 25 °C; inactivation time = 15 min; the error lines represent the standard deviation of 3 replicate experiments.

Different correlations between chlorine doses and inactivation rates were testified. For instance, more than 450 mg Cl min/L (30 mg Cl<sub>2</sub> mg/L × 15 min) was necessary for removing over 90% (1-log inactivation rate) of ARB *E. coli* DH5 $\alpha$  [35]. On the contrary, only 20 mg Cl min/L (20 Cl<sub>2</sub> mg/L × 1 min) achieved 6-log inactivation of *Pseudomonas* HLS-6 [19]. In the correlation of these two reports, a chlorine dose of 60 mg min/L (60 mg Cl<sub>2</sub> × 1 min) achieved 3.72 and 4.89-log inactivation of *Morganella morganii* and *Enterococcus faecalis*, respectively [36]. Meanwhile, several studies had demonstrated that higher doses of chlorine exerted a much more powerful inactivation effect on ARB [12,17]. It was discovered that even the same chlorine dose might result in different inactivation rates. Therefore, when comparing the effects of chlorination, the inactivation rate might be more accurate than the chlorine dose.

The mechanism of chlorination has been studied recently, and the damage by cell permeability dislocation, nucleic acids, and enzymes was discovered [37]. Meanwhile, it was reported that cell permeability was injured by less than 5 mg/L chlorination with less than 2-log inactivation and a small lesion in DNA was noted at a high dose of chlorine (>5 mg/L) treatment with 6-log inactivation [38]. Hence, the cell permeability and the change in ARB's antibiotic resistance characteristics after chlorination were detected.

### 3.1.2. Changes in Cell Permeability after Chlorination

Since MDA is a typical product of lipid oxidation [39], MDA was tested after chlorine disinfection, as shown in Figure 2, to investigate the change in cell permeability triggered by chlorine. The concentrations of MDA in the bacterial suspension after chlorination had the same trend as the disinfection curve; the more significant the inactivation rate was, the higher the MDA concentration was. Under less than 100 mg Cl min/L chlorination with less than 3-log inactivation, the concentration of MDA in ARB remained at the same level (around 0.1 nmol/OD<sub>600</sub>) as the blank control, indicating a slight alteration in the cell membrane. It was discovered that a higher dose of chlorination (>100 mg Cl min/L, >3-log inactivation) drastically amplified the MDA release. After contact with 200 mg Cl min/L, the MDA concentration in suspension rose to around 0.4 nmol/OD<sub>600</sub>, four-fold that of the untreated sample, indicating that the cell permeability was drastically reduced after a high dose of chlorine treatment (>3-log inactivation).



**Figure 2.** The concentrations of MDA in the bacterial suspension after chlorination. The error lines represent the standard deviation of 3 replicate experiments; the “\*” indicates a significant difference between the experimental and control groups.

### 3.1.1.3. Changes in Antibiotic Resistance Characteristics of Survival ARB

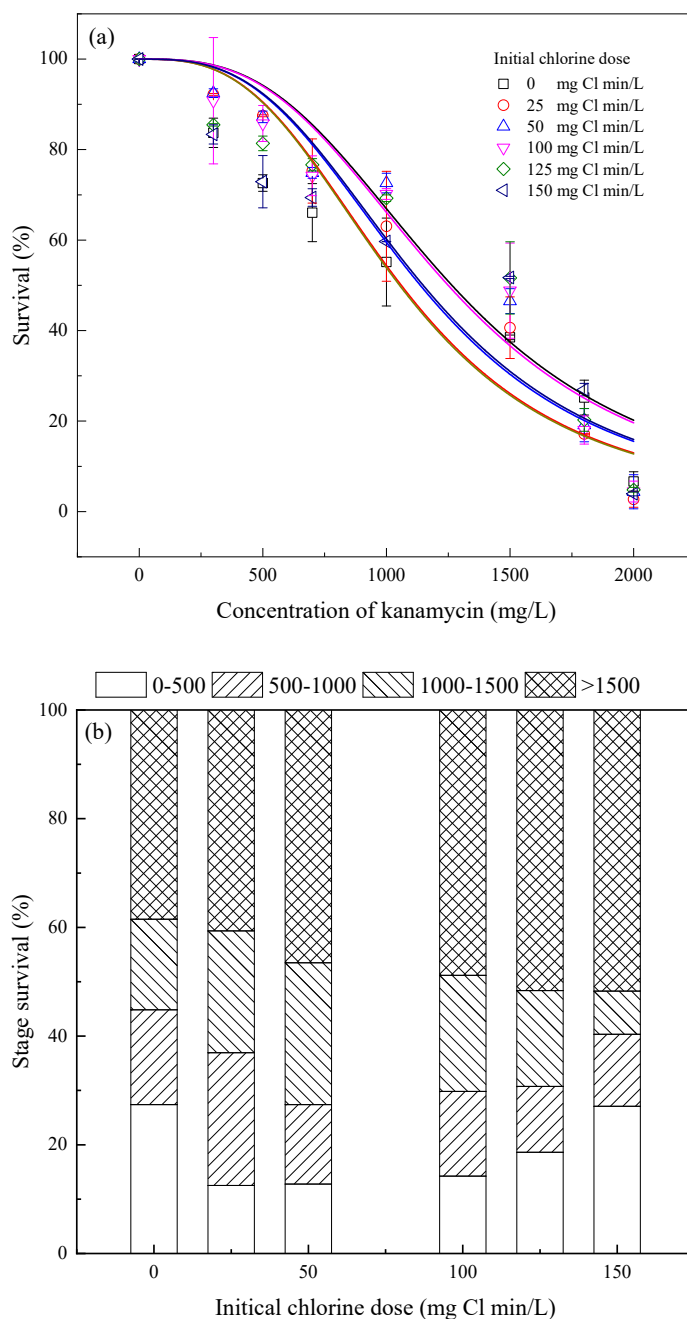
Under a specific concentration of kanamycin, a higher survival rate suggested that the bacteria had a higher tolerance level for kanamycin. The antibiotic-resistant characteristic of bacteria could represent the expression or the concentration of the corresponding iARG.

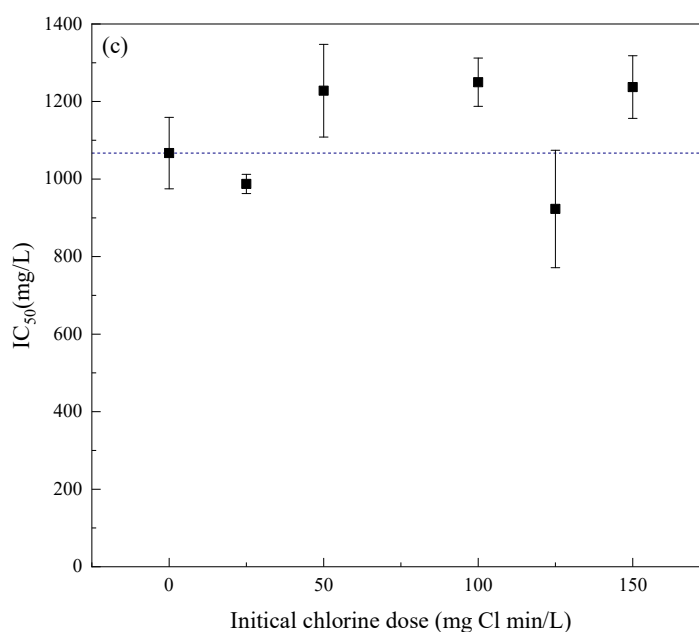
The dose–response curve after chlorination could reveal the tolerance level of *E. coli* HB101 to kanamycin. There was little difference between the curves before and after the reaction with chlorine occurred, as shown in Figure 3a, indicating that the bacteria had the same tolerance level as the control. Figure 3b compares the stage survival rates, where the kanamycin concentration was separated into a plurality gradient. The bacteria that could survive under 0–500, 500–1000, 1000–1500, and >1500 mg/L kanamycin were characterized as low-, common-, high-, and extremely high-resistance bacteria, respectively. However, the four plurality gradients showed limited alterations during the increase in chlorine concentration. IC<sub>50</sub> is the kanamycin concentration at the 50% survival rate of *E. coli* HB101. The higher the IC<sub>50</sub>, the more excellent the resistance of *E. coli* HB101 to kanamycin. Figure 3c indicates that with the amplification of the initial concentration of chlorine, IC<sub>50</sub> of kanamycin to the bacteria had no significant difference and remained at the concentration of around 1200 mg/L. The outcomes presented that chlorine had a limited effect on the antibiotic resistance characteristics of survival ARB and their offspring, indicating that the effect that chlorine imposed on the corresponding iARG was minor.

Chlorine injury caused by 4 mg/L sodium hypochlorite was reported to enhance antibiotic resistance against different antibiotics by 1.4–5.6 fold in *P. aeruginosa*, and this change was not heritable [40]. These results are consistent with our study, as the ARB were collected and cultured from the survival bacteria after chlorination. The mechanism of chlorine was mainly a change in membrane permeability and only a tiny lesion in DNA [38], and chlorination was not a complete means to eliminate the ARGs [41]; hence, it was reasonable that no heritable injury of iARGs was detected in this experiment.

However, the influences of chlorine on the antibiotic resistance characteristic of the bacterial group might need further investigation. Some found that chlorination treatment could select for tetracycline-resistant ARB [42,43], and the percentage of the bacteria

resistant to chloramphenicol, cephalothin, and trimethoprim increased [44]. These results indicated that chlorine was one of the factors that contributed to the shifting of the bacterial community and resistome adjustment. On the contrary, the percentage of resistance in *E. coli* strains isolated from wastewater was not significantly affected by chlorination [45]. These divergences might indicate safety concerns in the application of chlorination disinfection.

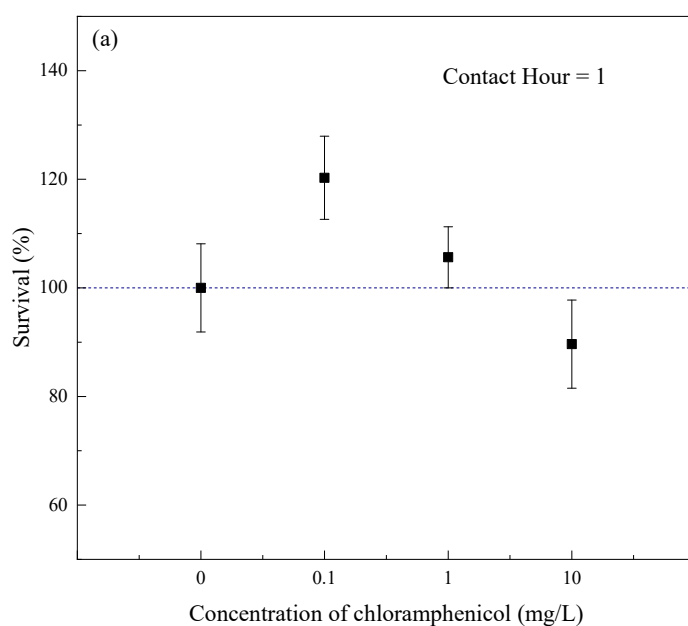




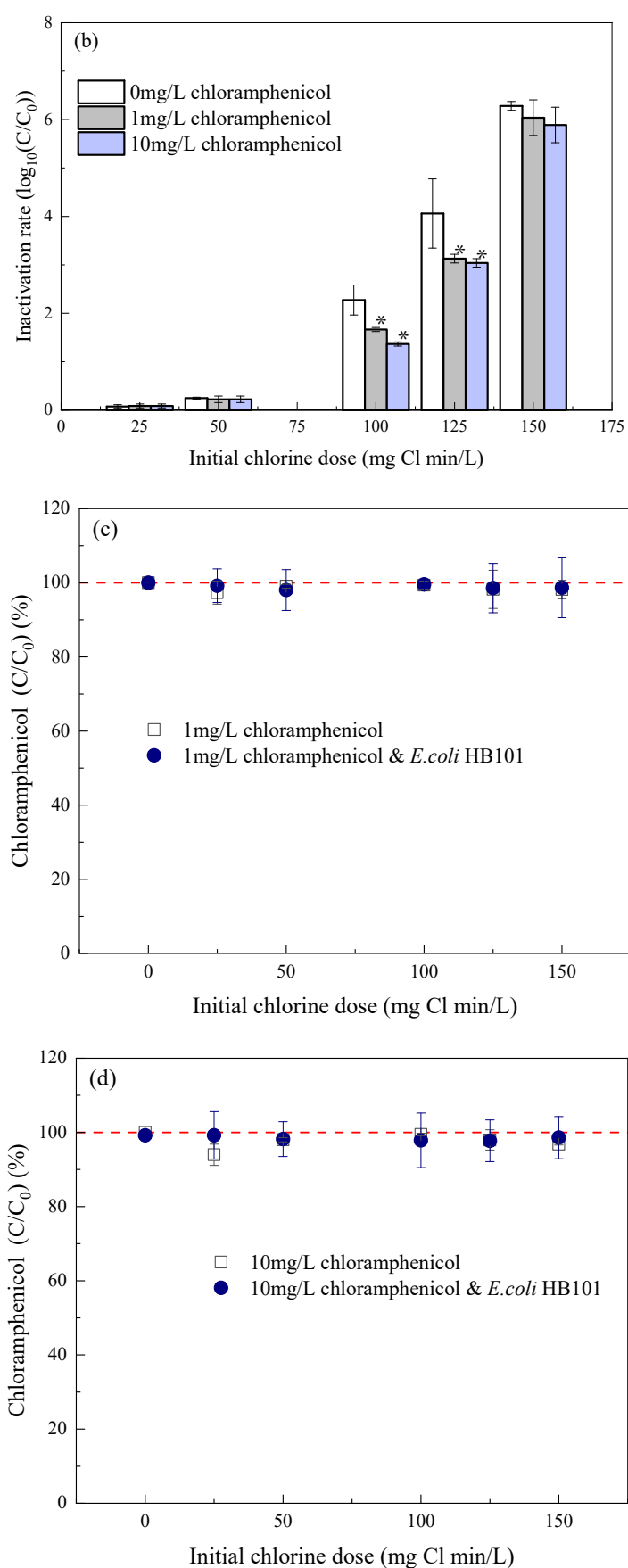
**Figure 3.** Effects of chlorination on antibiotic resistance characteristics of ARB (*E. coli* HB101). (a) Dose–response curve of ARB (*E. coli* HB101). (b) Stage survival of ARB (*E. coli* HB101). (c) IC<sub>50</sub> of ARB (*E. coli* HB101). The error lines represent the standard deviation of 3 replicate experiments.

#### 3.1.4. Simultaneous Degradation of Chloramphenicol

The survival rates of bacteria in the presence of chloramphenicol remained at the same levels as in the control group, as shown in Figure 4a, implying that chloramphenicol had little impact on the bactericidal activity of *E. coli* HB101. The bacterial survival rates were slightly increased to 120.3% in the presence of 0.1 mg/L chloramphenicol, and with the increase in the chloramphenicol concentration, the bactericidal activity declined in comparison; the water sample with 10 mg/L chloramphenicol displayed the lowest survival rate (89.6%).







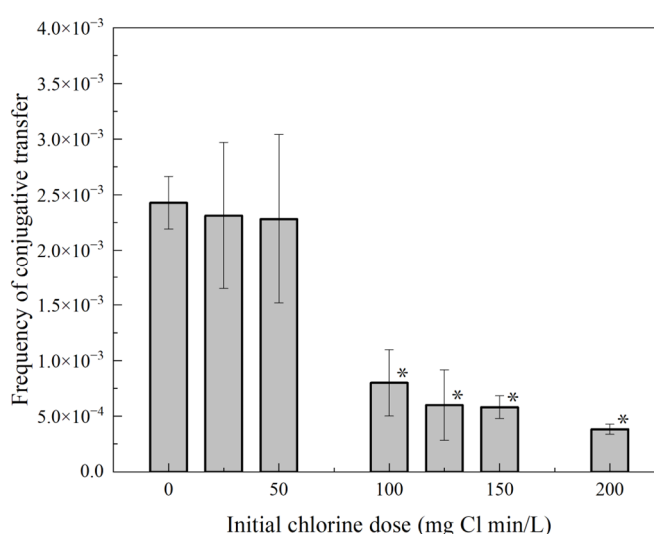
**Figure 4.** The removal effects on chloramphenicol and ARB of chlorine disinfection system. (a) The survival rate of *E. coli* HB101 under different concentrations of chloramphenicol. (b) Inactivation

rate of chlorine disinfection system with or without chloramphenicol. (c) The removal effects on 1 mg/L chloramphenicol with the chlorine disinfection system with or without ARB. (d) The removal effects on 10 mg/L chloramphenicol with the chlorine disinfection system with or without ARB. The error lines represent the standard deviation of 3 replicate experiments; the ‘\*’ indicates a significant difference between the experimental and control groups.

Conversely, after adding chloramphenicol, the inactivation rate was lower than the that of control sample, as shown in Figure 4b. With 100 and 125 mg Cl min/L initial chlorine doses, significant reductions were detected, suggesting that the chloramphenicol might consume chlorine and cause the inactivate rate to decrease. To confirm the consumption of chloramphenicol in the chlorine disinfection system, the chloramphenicol concentration was detected, as shown in Figure 4c,d. As the chemical is not the best removal method of antibiotics [46], minor consumption of chloramphenicol was achieved in the ‘synchronal removal’ system, indicating that the chlorine dose applied in the disinfection system was not sufficient for the chloramphenicol system. Moreover, a 100 mg/L chlorine dose needed 2.5 h to remove five types of sulfonamides and bacteria [47], confirming that the disinfection system did not have an adequate chlorine dose to degrade the antibiotics simultaneously. More advanced water treatment procedures might need to be investigated to achieve the degradation of these antibiotics considering antibiotic-resistant pollution.

### 3.2. Conjugative Transfer Ability of Bacteria Surviving from Chlorine Disinfection

A large amount of untreated recipient *E. coli* NK5449 cells was added to LB broth with the survival donor *E. coli* HB101 cells, as seen in Figure 5. An initial chlorine dose lower than 50 mg Cl min/L (<1-log inactivation rate) had a limited influence on the conjugation frequency, which showed a homologous transfer ability with the control. The frequency of conjugative transfer after 100 mg Cl min/L treatment significantly decreased and reached  $6.0 \times 10^{-4}$ , around 25% that of the control group, as the disinfection rate sharply increased to around 3-log. With the increase in the initial chlorine dose, the transfer frequency was maintained at the same level, suggesting that a chlorine dose with a higher inactivation rate also weakened the conjugative transfer ability of donor cells, and these types of changes were heritable.

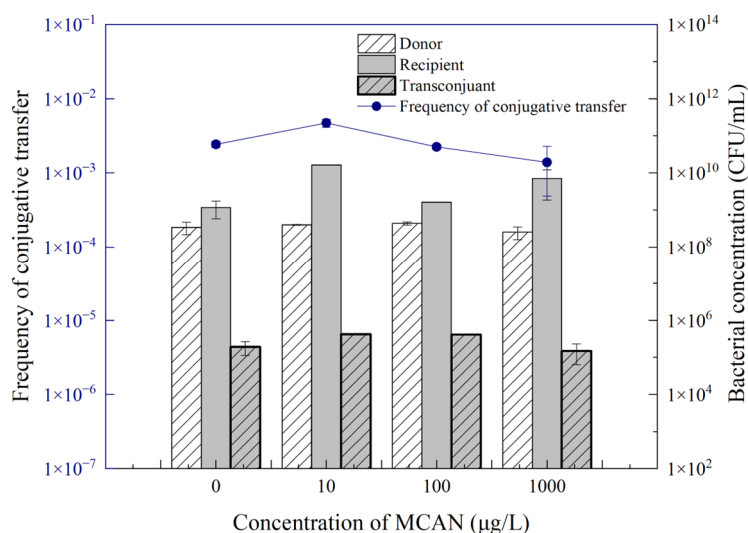


**Figure 5.** After survival following chlorination, the conjugative transfer frequency of ARB (*E. coli* HB101) was assessed. The error lines represent the standard deviation of 3 replicate experiments; the ‘\*’ indicates a significant difference between the experimental and control groups.

According to our previous study [12], lower doses of free chlorine increased the conjugation frequency by 2–5 fold in experiments through the generation of chloramine, which stimulated cell permeability. On the contrary, the LB broth was used as the conjugative environment, rather than wastewater, which would generate chloramine, and the donor cells employed in the conjugative transfer were obtained and re-cultured after chlorination in this study, rather than the donor cells, which were directly acquired from the test suspension in the previous research. In the mentioned study, the frequency of conjugative transfer was significantly suppressed under more than 80 mg Cl min/L chlorination, confirming that high-dose chlorine disinfection (>3-log inactivation rate) weakens the conjugative transfer ability of ARB.

### 3.3. The Effects of MCAN on the Frequency of Conjugative Transfer

As shown in Figure 6, different concentrations of MCAN had no significant influence on the donor and recipient survival rates. When 10 µg/L MCAN was added to the system, the frequency of conjugative transfer was amplified to  $4.72 \times 10^{-3}$  (two-fold that of the untreated group). Conversely, under 100 and 1000 µg/L MCAN, the frequency marginally dropped to  $2.24 \times 10^{-3}$  and  $1.39 \times 10^{-3}$ , respectively, indicating that these concentrations of MCAN had a limited effect on the promotion of conjugative transfer. The above results revealed that the existence of MCAN in an aquatic environment would not significantly aggravate the ARG transfer. It seemed to reduce the health risks of DBPs. However, two common DBPs, chlorite and iodoacetic acid, were reported to evolute ARB under both near-MIC and sub-MIC concentrations through antibiotic-like effects, which amplifies the health risks of chlorination [48]. As there are a number of unexplored DBPs, the unknown health concerns of antibiotic resistance still need to be investigated in the future.



**Figure 6.** Influence of MCAN on conjugative transfer frequency of ARG (*E. coli* HB101). The error lines represent the standard deviation of 3 replicate experiments.

## 4. Conclusions

The effects of a chlorine disinfection system with different components on antibiotic resistance showed that chlorination presented fewer safety concerns in these aspects, especially in the procedure using a high dose of chlorine; DBPs generated by disinfection would not aggravate the spread of ARGs by conjugative transfer.

(1) There was little influence on the bacteria in terms of their capacity for conjugative transfer and their antibiotic resistance characteristics after a low dose of chlorine

disinfection (<3-log inactivation rate). Meanwhile, after high-dose chlorine disinfection (>3-log inactivation rate), changes in the cell membrane were detected, and little influence occurred on the bacteria in terms of their antibiotic resistance characteristics. Nonetheless, their conjugative transfer ability sharply decreased, indicating the highly efficient effects on ARB and conjugation.

(2) The inactivate rate was reduced with the addition of chloramphenicol. Minor consumption of chloramphenicol was achieved in the ‘synchronal removal’ system, specifying that the chlorine dose applied in the disinfection system was not sufficient for chloramphenicol.

(3) MCAN had a limited promotional effect on the frequency of conjugative transfer, indicating that the existence of MCAN would not aggravate the spread of ARGs by conjugative transfer.

The overall effects of the chlorine disinfection system with different components on antibiotic resistance need further investigation.

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