Effects of chlorination and ultraviolet light on environmental tetracycline-resistant bacteria and *tet(W)* in water

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Abstract

The effectiveness of traditional water disinfection methods against both environmental antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) is still not well understood. The study objective was to evaluate two common methods for reducing concentrations of not only culturable tetracycline-resistant bacteria but also a resistance *tet(W)* gene fragment that has been frequently detected in the environment. Disinfection experiments were performed by exposing tetracycline-resistant isolates to various dosages of sodium hypochlorite or ultraviolet irradiation

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(UV). Concentrations of resistant bacteria and the *tet(W)* gene fragment were determined at 0 and 24 h after disinfection using traditional plating techniques and qPCR, respectively. Both the UV and chlorine disinfection methods were highly effective in initially reducing bacterial concentrations, and the chlorine disinfection method was more effective in preventing regrowth. However, both UV and chlorine disinfection were less consistent at reducing the concentration of the *tet(W)* gene fragment than for culturable resistant-bacteria at the dosages studied. Results indicated that after disinfection, resistance genes may remain detectable even if culturable resistant-bacteria are not, and that environmental ARBs and ARGs may survive these commonly used methods.

Keywords: Disinfection, antibiotic resistance genes, environmental resistance, qPCR

1. Introduction

Antibiotics are a critical tool used to prevent and treat human and veterinary infections. Approximately 100,000 to 200,000 tons of antibiotics are used worldwide yearly [1]. The effectiveness of antibiotics has decreased due to the development and dissemination of antibiotic resistance. The World Health Organization (WHO) has declared antibiotic resistance a serious threat to global public health [2]. Worldwide antibiotic resistance is commonly found in bacteria that cause common infections (urinary tract infections, pneumonia, bloodstream infections) [2]. Patients with infections caused by antibiotic resistant bacteria (ARB) are at an increased risk of illness and death [2]. WHO's 2014 report on global surveillance of antimicrobial resistance stated, "Without urgent, coordinated action, the world is heading towards a post-antibiotic era, in

which common infections and minor injuries, which have been treatable for decades, can once again kill” [2]. The spread of antibiotic resistance is mainly due to horizontal gene transfer, along with selective pressure from the environment, given that bacteria can transfer genetic material among a wide variety of bacterial species and genera [3-5].

Wastewater treatment plants (WWTPs) are considered ‘hotspots’ for development and spread of antibiotic resistance [6-9]. Influent to WWTP contains antibiotics, ARB, and ARGs creating an environment where non-resistant bacteria interact with resistant bacteria while experiencing selective pressure for antibiotic resistance [8,9]. These types of environments promote horizontal gene transfer and can increase the occurrence of not only ARB, but also ARGs. One study [10] evaluated the occurrence of ARB and ARGs in the effluent of various types of wastewater treatment including membrane biological reactors, conventional utilities (such as activated sludge, oxidative ditch, and rotatory biological contactors), and various sludge treatment processes (dewatering, gravity thickening, anaerobic digestion, and lime stabilization). The concentrations of ARB and ARGs in the final effluent was in the range from 5.00×10^2 to 6.10×10^5 CFU/100 mL and from non-detectable to 2.33×10^6 copies/100 mL, respectively. Another study [11] detected tetracycline-resistant bacteria (10^1 - 10^2 CFU/mL after UV, 10^1 - 10^3 CFU/mL after chlorination) and ARG concentration (10^4 - 10^5 copies/ μ L after UV, 10^6 - 10^7 copies/ μ L after chlorination) in the effluent of activated sludge WWTPs. Therefore, the effluent from WWTPs that is released into the environment can be a source for ARB and ARGs and alter the composition of the bacterial communities because of incomplete or ineffective removal during treatment [12-14].

Sullivan and Karthikeyan [15] evaluated the influence of WWTP effluent in a subtropical watershed located in East Central Texas, establishing the presence of tetracycline ARB and ARGs. Sullivan et al. [16] characterized the tetracycline-resistant bacteria within the same watershed and found that the most frequently cultured tetracycline-resistant organisms from that watershed were Gram-negative and were closely related to potentially pathogenic species (*Acinetobacter* spp., *Chryseobacterium* spp., *Serratia* spp., *Pseudomonas* spp., *Aeromonas* spp. and *E. coli.*). Of the 14 tetracycline resistance genes also evaluated, *tet(W)* was detected the most frequently in the watershed [15]; moreover, *tet(W)* has been shown to persist after both drinking water and wastewater treatment [17]. These studies further support that wastewater treatment facilities contribute to the occurrence of resistant bacteria and resistance genes in water and aquatic sediments. As such, ARGs are readily detected globally in surface waters and aquatic sediments, especially in areas with anthropogenic influences [3,17]. Studies have suggested that the propensity of aquatic sediments for adsorption of some antibiotics could create important reservoirs for ARGs and promote horizontal gene transfer [18]. These reservoirs of ARGs are often in contact with source water for drinking water treatment plants [19]. Failure to remove ARGs during drinking water treatment could allow these ARGs to make contact with pathogenic bacteria furthering the risk to human health.

Prior to drinking water distribution or the discharge of wastewater effluent into the environment, disinfection is employed to kill potential pathogens and to reduce human health risks. The most common disinfection methods used in the U.S. are chlorination, ozonation, and ultraviolet light irradiation [20]. Many studies have shown that both chlorine and UV disinfection are effective in reducing concentrations of resistant bacteria [10,21-27]. One study [28] also suggested that UV

treatment followed by chlorine disinfection may provide enhanced reduction of ARB in wastewater effluent, albeit at significantly higher doses and contact times than are commonly employed. Another study suggests that concentration of certain types of ARB may actually be enriched after UV treatment [29]. However, many of these studies have focused on pathogenic species or indicator organisms; ARGs and environmental ARB were not the focus. These overlooked species can serve as transport vectors or reservoirs of resistance. Initial studies found that not only were ARGs detected in drinking water [19,30], but also that disinfection did not reduce gene concentration [10,11,31,32]. However, more recent studies [33,34] have indicated that chlorine may be effective in removing both ARB and ARGs in drinking water and wastewater.

1.1. Study objective

In order to prevent the spread of antibiotic resistance it is important to prevent ARB and ARGs from being introduced into natural environments. To further protect human health, it is important to prevent ARGs from surviving the drinking water treatment process. Therefore, it is critical to determine the effectiveness of disinfection methods for the removal of ARB and ARGs, specifically species that can act as reservoirs for resistance in the environment. The objective of this study was to evaluate the effectiveness of two common disinfection methods (chlorination and UV) at reducing the concentrations of both tetracycline-resistant bacteria isolates and a resistance gene *tet(W)* that has been frequently detected in the environment.

2. Materials and methods

2.1. Microbial strains and culture media

Six isolates were used to evaluate the disinfection effects; these included the resistant organisms that were cultured most frequently in a creek where baseline flow is maintained by treated wastewater effluent. These organisms, identified in Sullivan et al. [16], include *Aeromonas* sp. 14S232, *Acinetobacter* sp. 14W115, *Chryseobacterium* sp. 14S111, *E. coli* sp. 28W121, *Pseudomonas* sp. 28S434, and *Serratia* sp. 14S224 (GenBank, KC734024, KC734147, KC734003, KC734215, KC734127, and KC734021). All chosen isolates are known to carry the resistance gene *tet*(W). This resistance gene was the most frequently detected tetracycline resistance gene in water and sediment samples from the watershed described earlier [14]. Bacteria were grown by transferring 1 mL of frozen stock culture into 100 mL of Luria-Bertani (LB) broth (Difco, Franklin Lakes, NJ, USA) containing 0.056 mM tetracycline to ensure that isolates maintained gene function. The cultures were then incubated for 24 h at 37°C. Prior to the disinfection treatments, the prepared cultures were pelletized and rinsed twice to remove tetracycline and re-suspended in 0.2 g/L peptone water. Initial concentrations (C_0) of bacteria were determined in triplicate ($n=3$), using serial dilution and direct plate counts.

2.2. Chlorination

A 20 mL volume of the re-suspended isolate culture was transferred to a petri plate and thoroughly mixed using a magnetic stirrer housed in a polyvinyl chloride (PVC) box of dimension 81 x 61 x 51 cm (L x B x H) to shield the experiments from light. Sodium

hypochlorite was added to achieve 0, 0.05, 0.5, 1, and 2 mg/L of available chlorine. The experimental dosages were chosen based on previous studies and levels commonly employed in water treatment processes [22,25]. Each treatment was expected to immediately achieve at least a 4-log bacteria reduction ($t = 0$ h) for all isolates studied. Each treatment was conducted in triplicate ($n=3$). The chlorine remained in contact with the microorganisms for 10 min at room temperature (25°C). Then sodium thiosulphate was added (0, 0.08, 0.8, 1.6, and 3.2 mg/L) to terminate chlorination. After disinfection, the samples were stored in the dark for 24 h to minimize photoreactivation.

2.3. UV disinfection

A 20 mL volume of the re-suspended isolate culture was transferred to a petri plate and mixed using a magnetic stirrer. The plates were housed in a PVC box of dimension 81 x 61 x 51 cm (L x B x H), then exposed to a horizontally mounted UV-C low pressure mercury germicidal lamp (Bryant energy, USA) with a wavelength of 254 nm. The average fluence rate (irradiance) measured using a radiometer before sample exposure was 0.23 mW/cm^2 , which corresponds to dosage times (and fluence) of 0 min (Control), 5 min (69.8 mJ/cm^2), 10 min (140 mJ/cm^2), and 20 min (279 mJ/cm^2). The experiments were conducted in triplicate ($n=3$) at room temperature (25°C). After disinfection, the samples were stored in the dark for 24 h to shield the experiments from light and minimize photoreactivation. The experimental dosages were chosen based on previous studies and levels typically used for treatment of wastewater effluent [24,31]. Each dose was expected to achieve at least a 4-log bacteria reduction immediately after treatment ($t = 0$ h) for all isolates studied.

2.4. Enumeration of microorganisms *following treatment*

In order to evaluate disinfection and regrowth, the samples were plated twice: once immediately after disinfection (0 h) and again after being stored in the dark for 24 h (minimizing regrowth due to photoreactivation). Samples were serially diluted in sterilized DI water and spread-plated on nutrient agar (Difco, Franklin Lakes, NJ, USA) containing 0.056 mM tetracycline hydrochloride, the concentration initially used for isolation of the resistant organisms [13]. The plates were incubated at 37°C for 24 h and shielded from light to prevent antibiotic degradation. Final concentrations (C_t) were determined from direct plate counts following incubation. Surviving culturable fraction is reported as C_t/C_o .

2.5. Concentration of *tet(W)* using DNA extraction and qPCR

Total DNA was extracted from 1.8 mL of the peptone water culture before and after disinfection using a MO BIO UltraClean[®] Microbial DNA Isolation kit according to the protocol provided by the manufacturer (MoBio Laboratories Inc., Carlsbad, CA, USA). The DNA concentration was determined using UV spectroscopy (NanoDrop[®], Wilmington, DE, USA).

The both initial (C_o) and final (C_t) concentrations of *tet(W)* were determined by quantitative PCR (qPCR) using Eppendorf Mastercycler Realplex2 thermocycler (Eppendorf, Westbury, NY, USA). Forward and reverse primers described by Aminov, et al. [35] were used creating a 168

bp amplicon. The 5PRIME qPCR Master Mix (5PRIME Inc., Gaithersburg, MD, USA) consisted of Mg^{2+} buffer, dNTPs, Taq DNA polymerase, and SYBR Green. The temperature program consisted of initial denaturing at 95°C, followed by 40 cycles of 15 s at 95°C, annealing for 30 s at 64°C, and a final extension of 15 s at 72°C. Reactions were performed in triplicate (n=3) for all initial and final (0 and 24 h after treatment) concentrations. Detected fraction of *tet(W)* is reported as C_t/C_o .

The standard curve was developed using a positive control created from *tet(W)* PCR product. The PCR product was inserted into a pGEM-T[®] Vector using pGEM-T[®] cloning kit utilizing the JM109 High Efficiency Competent Cells provided with the kit according to the manufacturer's protocol (Promega, Madison, WI, USA). Plasmid DNA containing the *tet(W)* PCR product was extracted using a MO BIO plasmid extraction kit according to the manufacturer's protocol (MoBio Laboratories Inc., Carlsbad, CA, USA). The DNA concentration was determined using UV spectroscopy and diluted to achieve working concentrations varying between 2.90×10^2 and 2.90×10^8 copies/ μ L (NanoDrop®, Wilmington, DE, USA). A negative control consisting of PCR grade water was also included in all PCR reactions. The limit of quantification (LOQ) was set as the lowest dilution that consistently amplified for all qPCR runs, which was 290 copies/reaction.

2.6. Statistical analysis

Statistical analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC). Samples that fell below the LOQ were set as ½ the LOQ [36] for statistical analysis. Significance was defined

as p-values ≤ 0.05 . One-way ANOVA (PROC GLM) was used to generate population means, standard deviation, 95% confidence intervals (CI), and significant difference for bacteria and gene concentrations. The least significant difference (LSD) test within PROC GLM was also used for comparison of population means for categorizing significant differences between treatments.

3. Results and discussion

3.1. Disinfection of tetracycline-resistant bacteria

The reduction of culturable bacteria concentrations for all isolates immediately (0 h) and 24 h after chlorine and UV disinfection are shown in Tables 1 and 2, respectively. Surviving culturable fraction (C_t/C_0) for each isolate are presented in Figures 1a-f.

Table 1. Average log reduction of tetracycline-resistant bacteria 0 h after disinfection.

	Chlorine 0.05 mg/L	Chlorine 0.5 mg/L	Chlorine 1 mg/L	Chlorine 2 mg/L	UV 5 min	UV 10 min	UV 20 min
<i>Acinetobacter</i>	4.30	> 5	> 5	> 5	> 5	> 5	> 5
<i>Aeromonas</i>	4.22	> 5	> 5	> 5	4.30	4.52	5.00
<i>Chryseobacterium</i>	NA	> 5	> 5	> 5	4.52	> 5	4.70
<i>E. coli</i>	NA	> 5	> 5	> 5	4.70	5.00	5.00
<i>Pseudomonas</i>	> 5	> 5	> 5	> 5	4.70	> 5	> 5
<i>Serratia</i>	NA	> 5	> 5	> 5	4.15	> 5	> 5

NA - Experiments were not conducted at the given dosage
Averages based on three replicates (n=3)

Table 2. Average log reduction of tetracycline-resistant bacteria 24 h after disinfection.

	Chlorine 0.05 mg/L	Chlorine 0.5 mg/L	Chlorine 1 mg/L	Chlorine 2 mg/L	UV 5 min	UV 10 min	UV 20 min
<i>Acinetobacter</i>	> 5	> 5	> 5	> 5	> 5	> 5	> 5
<i>Aeromonas</i>	4.52	> 5	> 5	> 5	2.16	3.72	4.15
<i>Chryseobacterium</i>	NA	> 5	> 5	> 5	1.26	> 5	2.91
<i>E. coli</i>	NA	> 5	> 5	> 5	2.51	2.30	2.15
<i>Pseudomonas</i>	4.70	> 5	> 5	> 5	1.89	1.87	2.94
<i>Serratia</i>	NA	> 5	> 5	> 5	3.05	5.00	> 5

NA - Experiments were not conducted at the given dosage

Averages based on three replicates (n=3)

Chlorine disinfection was highly effective in reducing the culturable concentration of tetracycline-resistant bacteria for all isolates tested. The majority of the chlorine treatments achieved greater than a 5-log₁₀ reduction in culturable tetracycline-resistant bacteria concentration for all isolates immediately after disinfection (Table 1). Twenty-four hours after chlorine disinfection, most treatments and tested bacteria maintained or achieved 100% reduction in culturable tetracycline-resistant bacteria concentration. Two isolates (*Acinetobacter* and *Aeromonas*) exposed to the lowest chlorine treatment of 0.05 mg/L had an increase in inactivation after 24 h (Figures 1a and 1b). The *Pseudomonas* isolate had a slight increase in culturable concentration (Figure 1c) at the lowest chlorine dosage, but it was not significantly higher than the concentration directly after disinfection.

Figure 1a. Surviving culturable fraction after treatment for *Acinetobacter*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 4.33E+5 CFU/mL.

Figure 1b. Surviving culturable fraction after treatment for *Aeromonas*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 1.36E+6 CFU/mL.

Figure 1c. Surviving culturable fraction after treatment for *Pseudomonas*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of $2.61E+7$ CFU/mL.

Ultraviolet disinfection did not appear to be as effective as chlorine for disinfection of most isolates. However, the lowest UV dosage had greater than 4- $\log_{10}$ reduction for all isolates and the *Acinetobacter* isolate was the most susceptible to UV disinfection, wherein the lowest UV dosage had greater than a 5- $\log_{10}$ reduction. In general, increasing the UV dose increased the amount of bacterial inactivation for all isolates, but the reduction was not large enough to be statistically significant for most isolates ($p > 0.05$).

Twenty-four hours after UV disinfection, most of the treatments and tested bacteria experienced regrowth and reactivation (Table 2). The *Acinetobacter* isolate was the only species tested that did not appear to experience regrowth or reactivation, and was highly susceptible to both UV and chlorine treatments. The *Serratia* isolate was also highly susceptible to UV and chlorine treatment (Figure 1d), and only the UV treatments at 5 and 10 min exposure demonstrated

regrowth and reactivation. The *Chryseobacterium* isolate had over a 3-log₁₀ increase in culturable bacteria concentration after 24 h at the 5 min (69.8 mJ/cm²) UV treatment, but increasing the UV dose from 5 min (69.8 mJ/cm²) to 10 min (140 mJ/cm²) appeared to inhibit regrowth and reactivation, resulting in less than 1-log₁₀ increase in concentration (Figure 1e). The *E. coli* (Figure 1f) and *Pseudomonas* isolates displayed the highest amounts of regrowth and reactivation of the isolates evaluated. The *Pseudomonas* isolate had a 3-log₁₀ increase in bacteria concentration 24 h after the 5 min (69.8 mJ/cm²) treatment, and increasing the UV dose increased the amount of regrowth and reactivation. This pattern was more evident in *E. coli* treatments.

Figure 1d. Surviving culturable fraction after treatment for *Serratia*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C₀ = initial concentration of 1.24E+6 CFU/mL.

Figure 1e. Surviving culturable fraction after treatment for *Chryseobacterium*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 3.51×10^6 CFU/mL.

Figure 1f. Surviving culturable fraction after treatment for *E. coli*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 1.39×10^7 CFU/mL.

The level of disinfection observed in this study (greater than 5- $\log_{10}$ reduction) is higher than those seen in other studies (0.28-3.1 $\log_{10}$ reduction) [22,23,25]. However, studies by Macauley et al. [23] and Huang et al. [25] focused on wastewater that contained higher concentrations of mixed cultures and organic matter, which could cause variation in the disinfection efficacy of the chlorine. Moreover, Koivunen and Heinonen-Tanski [22] specifically chose a chlorine dosage that would reduce the concentration of the isolates evaluated by 0-3 $\log_{10}$ in order to compare various treatments. All of the isolates evaluated were susceptible to chlorine, which indicates that under the conditions of this study the isolates were not co-resistance to tetracycline and chlorine, which is supported by the findings of Huang et al. [25]. Results from Huang et al. [25] showed that exposing wastewater to chlorine did not select for tetracycline-resistant bacteria, and that the tetracycline-resistant population behaved similarly to the total heterotroph population.

UV disinfection was also highly effective in reducing the concentration of tetracycline-resistant bacteria (4 to 5+ $\log_{10}$ reduction). These reduction amounts are greater than those achieved in a study by Macauley et al. [23] who used UV to disinfect swine wastewater, but the reduction amounts of *E. coli* were similar to those found by Kaur et al. [37] with and without titanium dioxide. In general, increasing the amount of time that the isolates were exposed to UV caused a greater reduction in tetracycline-resistant bacteria concentration. However, increasing the UV dose from 5 min (69.8 mJ/cm^2) to 10 min (140 mJ/cm^2), while providing a significant reduction ($p > 0.05$) in bacteria concentration, would increase operating costs and would not be a feasible solution for improving disinfection of tetracycline-resistant organisms.

Both chlorine and UV were effective in initially reducing the concentration of culturable tetracycline-resistant bacteria. However, 24 h after chlorine disinfection none of the isolates experienced any significant amounts of regrowth and repair, whereas a majority of the isolates experienced regrowth and repair 24 h after UV disinfection. Residual chlorine in the drinking water distribution systems would decrease the potential regrowth of ARB. The results, which are similar to those found by Huang et al. [32], indicate that chlorine treatment will prevent regrowth and reactivation of bacteria after disinfection. The log reduction of the *Chryseobacterium* isolate 0 h after disinfection was 4.52 and then reduced to 1.26 after 24 h for the 5 min UV treatment (69.8 mJ/cm²). *Pseudomonas* isolates also showed significant regrowth and repair and had a 2-_{log₁₀} increase or greater in concentration for both the 10 and 20 min UV treatments. *E. coli* isolates exposed to increased UV doses also experienced increased regrowth and repair after 24 h. This suggests that *E. coli* has effective regrowth and repair mechanisms. The regrowth of *E. coli* could be attributed to an increase in available carbon since the viable cells could utilize the carbon stored in the dead cells. Also, exposure to UV irradiation has been known to trigger an SOS response, which leads to an increase in transcription of genes used to repair DNA damage [38]; this response could be responsible for the increase in regrowth and repair of the *E. coli* isolate as the UV dose was increased. Previous studies have also shown that *E. coli* is capable of regrowth and repair after UV exposure [24,37]. The amounts of regrowth seen in this study were higher than those seen by Quek and Hu [24] but were similar to those seen by Kaur et al [37]. Few studies have evaluated the amount of regrowth and repair of the other species used in this study since they are not frank pathogens and are not used as indicators of water quality. The results indicate that chlorine disinfection is more effective than UV at maintaining low

concentrations of the tetracycline-resistant organisms used in this study at concentrations and intensities typically used at WWTPs.

3.2. Removal of tetracycline resistance gene *tet(W)*

The reduction of the *tet(W)* gene fragment for all isolates immediately (0 h) and 24 h after chlorine and UV disinfection are shown in Tables 3 and 4, respectively. The detected *tet(W)* fraction (C_t/C_o) for each isolate are presented in Figures 2a-f.

Table 3. Average log reduction of *tet(W)* 0 h after disinfection.

	Chlorine 0.05 mg/L	Chlorine 0.5 mg/L	Chlorine 1 mg/L	Chlorine 2 mg/L	UV 5 min	UV 10 min	UV 20 min
<i>Acinetobacter</i>	1.77	1.77	1.77	1.66	0.03	1.72	1.65
<i>Aeromonas</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Chryseobacterium</i>	NA	4.00	4.00	4.00	0.02	0.02	0.05
<i>E. coli</i>	NA	0.87	0.87	0.92	0.54	0.92	0.92
<i>Pseudomonas</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Serratia</i>	NA	0.14	0.46	0.40	0.00	0.00	0.00

NA - Experiments were not conducted at the given dosage
Averages based on three replicates (n=3)

Table 4. Average log reduction of *tet(W)* 24 h after disinfection.

	Chlorine 0.05 mg/L	Chlorine 0.5 mg/L	Chlorine 1 mg/L	Chlorine 2 mg/L	UV 5 min	UV 10 min	UV 20 min
<i>Acinetobacter</i>	1.66	1.77	1.77	1.77	1.77	1.77	1.77
<i>Aeromonas</i>	0.00	0.00	0.00	0.00	0.00	0.00	0.00
<i>Chryseobacterium</i>	NA	1.84	1.85	1.69	1.34	1.86	1.89
<i>E. coli</i>	NA	0.43	0.55	0.48	0.45	0.56	0.41
<i>Pseudomonas</i>	0.00	0.05	0.00	0.00	1.40	0.59	0.00
<i>Serratia</i>	NA	0.65	0.51	0.49	0.45	0.32	0.51

A - Experiments were not conducted at the given dosage
Averages based on three replicates (n=3)

The initial concentrations of *tet(W)* varied greatly among the isolates evaluated, with *Chryseobacterium*, *Pseudomonas*, and *Serratia* isolates having higher concentrations than *Acinetobacter*, *Aeromonas*, and *E. coli* isolates. The occurrence of *tet(W)* immediately after disinfection, as shown in Table 3, indicated that for *Aeromonas* (Figure 2a) and *Pseudomonas* (Figure 2b) there was no reduction in the concentration of the *tet(W)* gene fraction immediately after any disinfection treatments, with the survival fraction of approximately 1 for those isolates. The survival fraction of *Pseudomonas* isolate was greater than 1, indicating that concentration of the *tet(W)* gene fragment increased after both UV and chlorine treatment.

Figure 2a. Detected fraction of *tet(W)* after treatment for *Aeromonas*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C₀ = initial concentration of 145 copies/μL.

Figure 2b. Detected fraction of tet(W) after treatment for *Pseudomonas*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of $7.11E+3$ copies/ μ L.

The *Acinetobacter* and *E. coli* isolates (Figures 2c and 2d) both showed a larger reduction of *tet*(W) using chlorination than using UV, though increasing the UV dose did increase the reduction of *tet*(W). The *Chryseobacterium* isolate (Table 3) appeared to be the most susceptible to chlorine disinfection, and all chlorine treatments achieved a 4- $\log_{10}$ reduction in the *tet*(W) gene fragment concentration. UV disinfection of the *Chryseobacterium* isolate was not as effective as chlorine disinfection, but increasing the exposure from 5 min (69.8 mJ/cm^2) to 20 min (279 mJ/cm^2) slightly increased the average log reduction from 0.02 to 0.05 (or 3.41% to 9.99% percent reduction).

Figure 2c. Detected fraction of tet(W) after treatment for *Acinetobacter*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 8546 copies/ μ L.

Figure 2d. Detected fraction of tet(W) after treatment for *E. coli*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 1200 copies/ μ L.

Table 4 shows the log reduction for all isolates tested 24 h after chlorine and UV disinfection. A majority of the isolates tested (*Acinetobacter*, *Aeromonas*, *E. coli*, and *Serratia* – Figure 2e) had no significant change in concentration from 0 h to 24 h after chlorine disinfection for most treatment doses. The *Chryseobacterium* isolate had a significant increase in *tet(W)* concentration for all chlorine treatments (Figure 2f), while the *Pseudomonas* isolate had a significant reduction in concentration for all treatments ($p \leq 0.05$) after 24 h. The *Aeromonas* isolates had no reduction of the *tet(W)* gene fragment concentration for any disinfection treatments after 0 h and 24 h.

Figure 2e. Detected fraction of *tet(W)* after treatment for *Serratia*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_o = initial concentration of $5.30E+6$ copies/ μ L.

Figure 2f. Detected fraction of tet(W) after treatment for *Chryseobacterium*. Positive error bars represent standard deviation based on three replicates (n=3). C_t = final concentration, C_0 = initial concentration of 1.48E+6 copies/ μ L.

Results from this study suggest that neither chlorine nor UV disinfection methods, at the studied dosages, are consistent in reducing the concentration of the *tet(W)* gene fragment from the isolates evaluated. Other studies [10,11] found similar results and indicated that disinfection did not significantly contribute to the reduction of resistance genes. However, Zhang et al. [34] achieved a maximum removal of 1.30-1.49 log using a free chlorine dosage of 30 mg/L, which is a much higher dosage than used in this study. Zhang et al. [34] achieved a maximum removal of 0.6 log for the 16Ss rRNA genes at a fluence of 249.5 mJ/cm². It should be noted that a small gene fragment (189 bp) was used for this study and previous studies have found that a short amplicon may not effectively capture UV damage [31]. The varied concentrations of *tet(W)* between isolates could be attributed to variability in extraction or amplification, cell features, or differences in copy numbers of *tet(W)* among the species evaluated. In addition, the effectiveness of disinfection varied among isolates evaluated, suggesting that reduction of *tet(W)*

concentration is highly species-dependent most likely due to variability of cell morphology. The disinfection experiments conducted in this study used pure cultures and focused only on Gram-negative bacteria. In contrast, wastewater and drinking water influent is composed of mixed cultures which would further complicate the disinfection of ARGs, therefore it is important to look at both the resistant bacteria population and the gene concentration to understand the potential risk of resistance.

Most of the isolates evaluated had very little change in *tet(W)* concentration 24 h after disinfection, while the *Chryseobacterium* isolate had a significant increase and the *Pseudomonas* isolate had a significant decrease ($p \leq 0.05$). Regrowth and repair of both culturable and viable-but-non-culturable tetracycline-resistant bacteria after the UV disinfection could explain the increased concentration of *tet(W)* after UV disinfection, though the short amplicon size used in this study may not accurately capture DNA damage after UV disinfection. However, *Chryseobacterium* did not experience any regrowth or reactivation after chlorine disinfection. It is possible that the length, sequence, and conformation of the plasmid carrying *tet(W)* in *Chryseobacterium* caused it to react differently than the other types of free DNA and might have provided protection from degradation [39], while the plasmid carrying *tet(W)* in *Pseudomonas* might have been more susceptible to nuclease activity and degraded more quickly.

At the given dosages, the chlorine treatments were generally more effective in reducing the concentration of the *tet(W)* gene fragment than the UV treatments. Similar results were seen for the disinfection of culturable tetracycline-resistant bacteria. However, none of the treatments achieved 100% reduction of *tet(W)* gene fragment. If the *tet(W)* gene behaves similarly to the

gene fragment used in this study then the gene could remain intact and functional, increasing the risk of species acquiring *tet*(W) through horizontal gene transfer [19].

4. Conclusion

The results from this study indicate that both UV and chlorine disinfection, at the dosages studied, are highly effective in initially reducing the concentration of culturable tetracycline-resistant bacteria. Moreover, chlorine disinfection maintained a 5-log₁₀ reduction in culturable tetracycline-resistant bacteria concentration after 24 h, but isolates exposed to the UV treatments experienced regrowth and repair. According to the methods applied in this study, neither the chlorination nor the UV disinfection consistently reduced the concentrations of *tet*(W), and the results varied between bacterial isolates. Within the same disinfection treatment, the log reduction ranged from 0 to 2.0 (or 0% to 99%) depending on the isolate evaluated. In general, chlorine treatment was more effective than UV for reducing the concentration of the gene fragment of *tet*(W). However, it is important to note that the small amplicon size used in this study may not efficiently capture DNA damage after UV disinfection.

While these results indicate that traditional disinfection methods are effective at reducing culturable tetracycline-resistant bacteria concentrations, they are less effective in reducing the concentration of the *tet*(W) resistance gene fragment. As such, disinfected water could still contribute to the occurrence of resistance genes even while reducing culturable resistant bacteria. The gene fragment detected in this study could also have been present as free DNA or within viable-but-non-culturable bacteria, both of which are still capable of spreading antibiotic

resistance via horizontal gene transfer. **Therefore**, further research is needed to evaluate whether the *tet(W)* gene detected following disinfection remained functional and if it could undergo horizontal gene transfer.

This study illustrated some of the challenges in addressing antibiotic resistance since each of the bacteria species reacted differently when exposed to disinfection methods. Therefore, there is no simple solution for preventing ARB and ARG from entering the environment and posing health risks.

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