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Comparative removal of antibiotic resistance genes during chlorination, ozonation, and UV treatment

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ABSTRACT

Efficient treatment methods for the removal of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from drinking water are needed to reduce health risks. However, there is a lack of empirical data on ARB and ARG removal during conventional water disinfection processes. In this study, the removal of ARB and ARGs by three disinfection processes (chlorination, ozonation, and UV treatment) was investigated on a laboratory scale using *Escherichia coli* and *Enterococcus faecium* carrying ARGs. Bacterial inactivation was determined by plate count methods, and ARG damage was quantified using real-time PCR. Only for ozone treatment, similar inactivation rates for bacterial cells and ARGs were observed when 1 mg*L^{-1} of ozone, with a contact time of 5 min, was used, which resulted in a 5.0 log reduction of bacterial cells and a 4.3–4.6 log reduction of ARGs. For chlorine and UV, inactivation of bacterial cells was observed at lower doses than those needed for the decrease of ARG copy numbers. The use of 0.5 mg*L^{-1} free chlorine (30 min contact time) led to a 3.8–5.6 log reduction of the bacterial numbers and to a 0.8–2.8 log reduction of ARGs. Ultraviolet light irradiation with 600 J*m^{-2} resulted in a 4.8–5.5 log reduction of bacterial cells, but in a negligible reduction (0–1.0 log) of ARGs. Although UV and chlorine treatments were effective in the inactivation of bacterial cells, incomplete degradation of ARGs was observed. Therefore, plasmid-borne ARGs can potentially be transferred to other bacteria even after the disinfection process. Our results provide important insights into the fate of ARGs during drinking water disinfection processes.

1. Introduction

In the past few years, the spread of antibiotic-resistant bacteria (ARB) has gained global attention due a number of reports published by the World Health Organization (WHO, 2014 and 2015). Several studies have shown that ARB in hospital, domestic, and industrial wastewaters are only partially removed during wastewater treatment (Czekalski et al., 2014; Michael et al., 2013; Rizzo et al., 2013). The presence of ARB and antibiotic resistance genes (ARGs) has been reported in a number of environmental compartments throughout the world, including surface water (Adelowo et al., 2018; Jiang et al., 2013; Stange et al., 2016; Stoll et al., 2012), groundwater (Li et al., 2014), drinking water (Xi et al., 2009; Guo et al., 2014), and drinking water biofilms (Schwartz et al., 2003).

The release of clinically-relevant ARB and ARGs from anthropogenic sources, along with the excessive use of antibiotics in both human and veterinary medicine, contributes towards the rapid spread of ARB in the environment by providing a resistance gene pool and selection pressure. The global spread of ARB and the acquisition of ARGs by pathogenic and opportunistic bacteria have resulted in an increase in the extended hospitalization and high mortality rates among patients carrying ARB (WHO, 2014). Different environments like e.g. drinking water biofilms could be the source of pathogenic and opportunistic bacteria (Richards et al., 2015) and can also provide an ideal setting for horizontal gene transfer due to a high bacterial cell density, increased genetic competence, and the accumulation of mobile genetic elements (Fux et al., 2005; Gal-Mor and Finlay, 2006).

The dissemination of ARB and ARGs is facilitated by horizontal gene transfer, enabling the exchange of ARGs among different bacterial strains or species (Frost et al., 2005) and beyond the habitat of the original host (Moore and Lindsay, 2001). It is presumed that conjugation is the primary mechanism of horizontal gene transfer (von Wintersdorff et al., 2016), but also transduction (Brown-Jaque et al., 2015; Colomer-Lluch et al., 2011) and transformation (Lorenz and

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Wackernagel, 1994) have been observed. Wastewater treatment plants (WWTPs) provide an ideal setting for the exchange of mobile genetic elements carrying ARGs between pathogens, opportunistic pathogens, and environmental bacteria (Parsley et al., 2010). The high bacterial densities, biofilms, and stress caused by pollutant compounds such as antibiotics, heavy metals, surfactants, biocides, and pharmaceuticals can promote horizontal gene transfer in wastewaters (Aminov, 2011).

Sewage collection and treatment are essential for the protection of human and environmental health. The current WWTPs are designed to remove conventional pollutants, including suspended solids, nutrients (nitrogen and phosphorus), organic matter, and, to some extent, pathogens (Pruden et al., 2013). However, WWTPs are not designed for the removal of antibiotics, ARB, and ARGs and are therefore suspected to be critical nodes for the control of the global spread of antibiotic resistance. Within the European Union the quality of urban WWTP discharges is regulated by the Council Directive 91/271/EEC giving requirements for parameters like biological and chemical oxygen demand, total suspended solids, nitrogen and phosphorus. Microbiological parameters – and in particular ARB and ARGs – are not considered in the directive so far. Tertiary treatment of wastewater using chlorine, UV radiation, and ozone can effectively remove bacteria, but ARGs are more recalcitrant (Kim et al., 2010; McKinney and Pruden, 2012; Munir et al., 2011). Therefore, there is always a potential risk of transfer of ARGs through different mechanisms to pathogens and opportunistic pathogens in the environment after the discharge of treated effluent into freshwater (e.g. rivers and canals) or seawater (estuaries and coastal areas) environments. In this sense, recommendations of effective and economically sustainable treatment technologies at critical points within the wastewater stream are desperately needed (Berendonk et al., 2015).

As the spread of ARGs is becoming increasingly alarming, the efficacies of both conventional processes and advanced oxidation processes (AOPs) for the elimination of ARB and ARGs have become a focal point of interest (Dodd, 2012). The methods include, inter alia, chlorination (Yoon et al., 2017; Zhang et al., 2015), UV treatment (Childress et al., 2014; McKinney and Pruden, 2012), ozonation (Alexander et al., 2016; Zhuang et al., 2015), the use of various oxidants (Oh et al., 2014), and photocatalysis with TiO_2 particles (van Aken and Lin, 2011). These treatment processes are intended to remove viable bacteria, but little is known about their efficacy in the removal of ARGs or mobile genetic elements carrying them.

The use of methods like chlorination, ozonation and UV irradiation is common practice throughout the developed and developing world to disinfect drinking water as part of the treatment process. The mechanisms of bacterial inactivation during these processes are well known, but there is a lack on empirical data on ARB and ARG removal during drinking water disinfection.

Chlorine kills bacterial pathogens by disrupting cell membranes, coagulating enzymes and nucleic acids (Dodd, 2012). Yuan et al. have investigated the fate of ARB and ARGs in wastewater treated by chlorination (0–300 $mg*Cl_2*min\ L^{-1}$) and reported effective inactivation of ARB, but only a limited reduction (40–80%) in erythromycin and tetracycline resistance genes (Yuan et al., 2015). Similarly, Xi et al. (2009) have used culture-dependent and quantitative molecular techniques to quantify ARB and ARGs in source water, drinking water treatment plants, and tap water from different cities in the United States. For water treatment including a chlorination step, a > 5 log reduction of the total heterotrophic bacteria was observed, whereas gene copy numbers of the 16S rRNA gene only decreased by approximately 1 log (Xi et al., 2009). The discrepancy between the reduction in heterotrophic bacteria and the reduction in the total 16S rRNA gene copy number is in accordance with the hypothesis that the final disinfection step effectively inactivated bacteria, but most of the dead or damaged cells were still present in the final drinking water (Xi et al., 2009).

Ozone has a high disinfection efficacy against bacteria as compared to chlorination due to its high oxidation potential. Ozone oxidizes cell components of the bacterial cell wall, followed by leakage of cellular constituents, breaking of aromatic structures of nucleic acids, and disruption of carbon-nitrogen bonds of proteins, leading to depolymerization (von Gunten, 2003). A recent study has reported 1.68 and 2.55 log removals of *sul1* and *tet*(G) genes, respectively, at an ozone concentration of 177.6 $mg*L^{-1}$ (Zhuang et al., 2015). In another study, erythromycin resistance gene (*ermB*) was reduced by two orders of magnitude after ozone treatment (0.9 ± 0.1 g ozone per 1 g DOC). However, increased prevalence of the clinically-relevant vancomycin resistance gene *vanA* and β-lactamase gene bla_{VIM} within the surviving bacterial population has also been reported (Alexander et al., 2016).

Chemical disinfectants kill bacteria by oxidative damage of cell compounds; therefore, a uniform concentration of the chemical disinfectant is required for an effective removal of ARB and ARGs from the water. Ultraviolet light can penetrate through the water and inactivate microorganisms; however, the germicidal effect is restricted to the area close to the UV source, and suspended particles in the water can increase microbial survival (Qualls et al., 1983). The mode of UV action primarily consists of dimer formation between pyrimidines (cytosine (C) and thymine (T)), but also of the generation of other photoproducts of nucleic acids and nucleic acid lesions (von Sonntag, 1987). In addition, inhibition of cell replication and transcription also prevent cell growth. A limited number of studies have focused on the fate of ARGs during UV disinfection, and a high variability in the removal of genes has been reported (Kim et al., 2010; McKinney and Pruden, 2012; Munir et al., 2011).

A number of studies have reported the presence of ARB in drinking water systems (Pruden et al., 2006; Schwartz et al., 2003; Xi et al., 2009). Therefore, the transfer of ARGs among bacteria in the drinking water networks after the disinfection process is a major public health concern. In this context, the main aim of this study was to investigate the efficacies of ozone, chlorine, and UV treatment for the removal of ARB and ARGs during drinking water treatment.

2. Material and methods

2.1. Bacterial strains used in the study

Escherichia coli K12 DH1 RU2945 (DSM 4960) was selected as a representative strain for Gram-negative bacteria. It carries a conjugative Kmr ts plasmid Rts1, including the naturally occurring transposon Tn1721. On the plasmid are, inter alia, the ARGs *tet*(A) and *ampC* located. The tetracycline resistance gene *tet*(A) encodes for a tetracycline efflux protein transporting the antibiotic out of the bacterial cell. The gene *ampC* encodes for class C β-lactamases, which breaks the β-lactam antibiotic ring and deactivates the antibacterial properties of the molecules.

Enterococcus faecium (DSM 13590) was used as a representative for Gram-positive bacteria; the selected strain is positive for the ARGs *vanA* and *ermB*. The *vanA* gene is part of a gene cluster responsible for the synthesis of peptidoglycan precursors containing a depsipeptide instead of the usual D-alanyl-D-alanine terminus, which reduces the vancomycin binding affinity. It is associated with both vancomycin and teicoplanin resistance. The *ermB* gene encodes for methylase 23S rRNA, which is responsible for the methylation of erythromycin and clindamycin receptor sites in ribosomes. The phenotypic expression of the according macrolide-lincosamide-streptogramin B resistance can be either constitutive or inducible.

2.2. Bacterial culture and water sample collection

To prepare bacterial suspensions for the experiments, frozen bacterial stocks (30% glycerol, −70 °C) were streaked onto ChromoCult® Coliform agar (*E. coli*, Merck) or Slanetz and Bartley agar (*E. faecium*, Oxoid). After 24 h (*E. coli*) or 48 h (*E. faecium*) of incubation at 36 °C, the purity of the culture was verified and bacterial colonies were rinsed off with sterile 0.9% NaCl solution. The optical density of the bacterial suspension was measured (600 nm) using a nanophotometer (Implen) and adjusted to 1.

Local drinking water was used for the disinfection experiments. This water is routinely monitored for indicator bacteria (once a month) according to the German Drinking Water Ordinance. The drinking water was spiked with *E. coli* and *E. faecium* by adding 1 mL each per 100 mL of the water sample. The dissolved organic carbon and pH values of the water samples were measured after seeding both bacteria. In addition, water samples not spiked with bacteria were analyzed using culture methods and quantitative real-time PCR (qPCR).

2.3. Chlorine treatment

For the chlorination experiment, the water samples seeded with *E. coli* and *E. faecium* were transferred to sterile 250-mL bottles. Sodium hypochlorite was added from a standardized stock solution to establish different concentrations (0–1.5 $mg*L^{-1}$, in duplicates) of free chlorine. The samples were gently mixed by hand and incubated at room temperature for 30 min. The concentrations of free and total chlorine were measured using a NANOCOLOR® tube test (Macherey-Nagel). After a contact time of 30 min, sodium thiosulfate solution (1.5%) was added to terminate the chlorination process.

2.4. Ozone treatment

Ozonation experiments were performed as batch experiments in 250-mL glass vessels filled with drinking water. The ozone stock solution was prepared by sparging ozone gas through a cooled reactor column (5 °C) filled with ultrapure water. The ozone concentration (20–30 $mg*L^{-1}$, in duplicates) was measured by the indigo method (Bader and Hoigné, 1981) before adding the solution to the batch vessels in final concentrations between 0 and 13 $mg*L^{-1}$. After an incubation time of 5 min, the reaction was stopped by adding sodium thiosulfate (1.5%).

2.5. UV treatment

For the UV treatment, low-pressure collimated beam irradiation at 253.7 nm was conducted according to a standardized protocol by Bolton et al. (2015). The UV device contained a low-pressure mercury lamp, mounted horizontally within an enclosure. The bottom side of the enclosure had a circular opening, or aperture, allowing UV light to be transmitted to the sample located below the opening. Of the sample, 10 mL were filled into Petri dishes (94 mm) and placed on a magnetic stirrer under the opening of the UV device. The fluence rate (irradiance) was determined using the reference radiometer IL Metronic MUV 2.4 (Sensortechnik GmbH). The dose delivered to the suspension was calculated using an equation that takes into account a number of measurable factors, including incident irradiance, uniformity of the irradiance field, ultraviolet transmittance of the sample, reflection off the sample surface, and divergence of the beam upon entering the sample. Various irradiation energies (0–800 $J*m^{-2}$, in duplicates) were adjusted by the application of different exposure times. After irradiation, the samples were stored at room temperature in the dark until further processing.

2.6. Analytical methods

Dissolved organic carbon (DOC) was measured with a vario TOC cube (Elementar Analysensyteme GmbH). Ten milliliters of the samples were filtered (0.45 µm, Pall Life Science), acidified (two drops of semi-concentrated hydrochloric acid) and stored at 4 °C until analysis. The pH was determined with a WTW multimeter.

2.7. Bacterial culturing techniques

For *E. coli*, 1 or 10 mL of processed water and its serial dilutions were filtered through sterile membranes (cellulose nitrate, 0.45 µm pore size, 47 mm diameter, Millipore). For *E. coli* enumeration, the membranes were placed on ChromoCult Coliform agar (Merck). After incubation at 37 °C for 20–24 h, dark-blue to violet colonies were counted. For *E. faecium* enumeration, membrane filters with appropriate dilutions were incubated on Slanetz and Bartley agar for 40–48 h at 37 °C, followed by incubation on Bile Aesculin agar (Merck) for 1 h at 44 °C. Red, pink, and reddish-brown colored colonies with positive aesculin reaction were counted.

2.8. Analysis of antibiotic resistance genes

After chlorine and ozone treatment, 100 mL of each approach were filtered through a 0.2-µm Supor®-200 membrane (47 mm diameter, Pall Life Science). The membranes were stored at −20 °C until DNA extraction and analysis. Total DNA was extracted directly from the membranes by using the Fast DNA® SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's instructions. Because of the limited process volume for UV irradiation, only 10 mL of each sample were centrifuged to pellet the bacteria before proceeding with DNA extraction, using the BacterialXpress™ Nucleic Acid Extraction Kit (Merck Millipore).

2.9. qPCR analysis

Gene copy numbers of the 16S ribosomal DNA and the resistance genes were measured by qPCR using previously published primer sets (Table 1), allowing the amplification of short amplicons (160–420 base pairs).

All qPCRs were performed using a Rotor-Gene 6000 cycler (Corbett) with a SensiMix SYBR No-Rox Kit (Bioline). The temperature profile for the SensiMix was as follows: 10 min 95 °C (initial phase), 45 cycles of 15 s at 94 °C (denaturation), 20 s at 55–68 °C (annealing), and 15–25 s at 72 °C (elongation). Melting curves were measured immediately after the run by stepwise temperature increases (1 °C/5 s) from the annealing temperature up to 99 °C.

All samples and standards were analyzed in duplicates. The presence of PCR inhibitors was excluded by analyzing dilutions of the DNA samples, demonstrating that observed decreases in quantification cycles or copy numbers are consistent with the anticipated results. The qPCR standards were prepared from the serial dilutions of known quantities of linearized plasmid-containing target genes. The limit of quantification (LOQ) was determined by the lowest DNA plasmid concentration that the assay could quantify and maintain in the linear portion of the standard curve, showing a PCR efficiency between 90 and 105% and R^2 values above 0.980, as well as visually limited dispersion around the curve. The overall limit of quantification was 10 copies per µL. Amplification products were verified via Melting curve analysis and the QIAxcel® Advanced system (Qiagen).

2.10. Long-amplicon PCR

In addition, we performed qPCR analysis amplifying long fragments of 1070–1120 base pairs. The primer sets used in the study are listed in Table 1. The basic principle was the same as that for the short-amplicon PCR, but for larger amplicons, we used SsoFast EvaGreen (BioRad). The initial incubation step was set to 3 min, and the elongation time was increased (Table 1).

3. Results

Prior to each treatment, the pH and DOC values for all water samples were determined. All pH values were in the neutral range

Table 1
Primer sequences.

Gene	Sequence (5′-3′)	Amplicon in bp	Reference	Annealing temperature, elongation time
sul1	F: CGCACCGGAAACATCGCTGCAC R: TGAAGTTCCGCCGCAAGGCTCG	160	Pei et al. (2006)	68 °C 20 s
sul2	F: TCCGGTGGAGGCCGGTATCTGG R: CGGGAATGCCATCTGCCTTGAG	190	Pei et al. (2006)	60 °C 15 s
ermB	F: CATTTAACGACGAAACTGGC R: GGAACATCTGTGGTATGGCG	420	Gevers et al. (2003)	63 °C 25 s
ampC	F: TTCTATCAAMACTGGCARCC R: CCYTTTTATGTACCCAYGA	550	Schwartz et al. (2003)	55 °C 25 s
tet(A)	F: CCTGCGCGATCTGGTTCACT R: GCCAGCGAGACGAGCAAGA	160	Stanton and Humphrey (2003)	55 °C 30 s
*van*A	F: ATGAATAGATAAAAGTTGCAATAC R. GGAGTAGCTATCCCAGCATT	375	Klein et al. (1998)	55 °C 15 s
ermB	F: CATTTAACGACGAAACTGGC R: GGAACATCTGTGGTATGGCG	420	Gevers et al. (2003)	63 °C 25 s
16S rRNA	F: CCTACGGGAGGCAGCAG R: ATTACCGCGGCTGCTGGC	160	Smits et al. (2004)	68 °C 20 s
vanA	F: CTCTGCAATAGAGATAGCCGC R: CCCCTTTAACGCTAATACGATCCA	1120	Clark et al. (1993)	60 °C 30 s
tet(A)	F: GTAATTCTGAGCACTGTCGC R: CATAGATCGCCGTGAAGAGG	1054	McKinney and Pruden (2012)	55 °C 30 s

(7.2–7.6). The DOC values were less than 2 mg*L^{-1}; therefore, the loss of free chlorine or ozone by DOC can be neglected.

3.1. Chlorine

Chlorine treatment resulted in successful inactivation of the model bacterium *E. coli* (Fig. 1, top graph). However, we observed clear differences between the behaviors of cultivable bacteria and ARGs. With increasing concentrations of free chlorine, the number of cultivable bacteria was reduced more rapidly than that of the ARG copy numbers. This was particularly obvious with a chlorine dose of 0.25 mg*L^{-1} in the experiment with *E. coli*. At this concentration, the bacterial count showed a reduction of 5.1 log (over 99.999%), whereas the gene copy numbers decreased by only 0.4–0.5 log (57.11–65.81%). Higher chlorine doses resulted in a more pronounced decline in ARG concentrations. At a maximum of 1.03 mg of free chlorine *L^{-1}, the qPCR signals were below the limit of quantification; similar results were obtained with *E. faecium* (Fig. 1, bottom graph). At a dosage of 0.25 mg of free chlorine*L^{-1}, a log reduction of 2.9 (99.89%) was achieved for the bacterial counts, while the ARG copy number decreased by 0.9 log (*ermB*, 87.40%) or 0.8 log (*vanA*, 84.12%). However, the reduction of *E. coli* at low chlorine doses was more distinct than that of *E. faecium*.

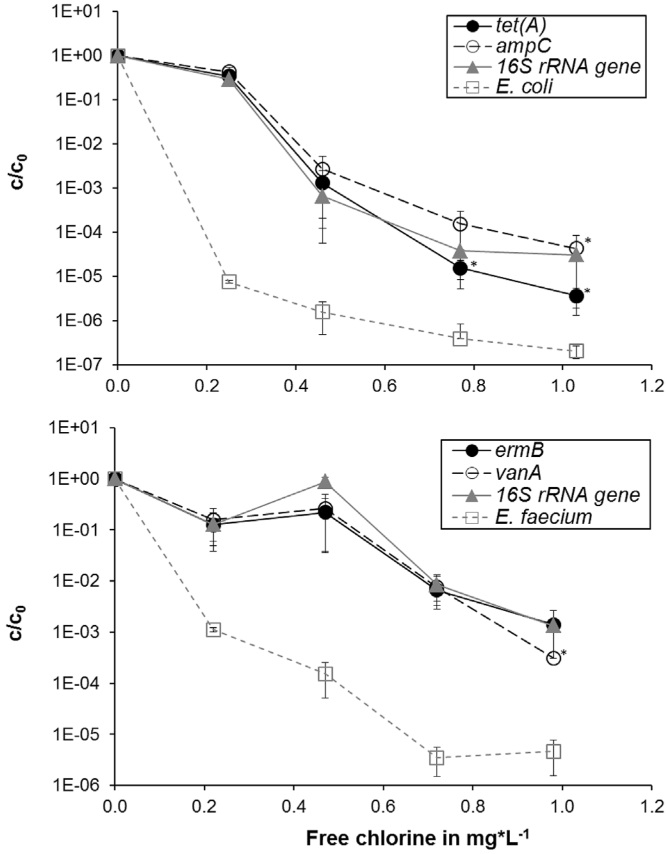

Fig. 1. Bacterial counts and qPCR results for chlorine-treated *E. coli* (top graph) and *E. faecium* (bottom graph). Error bars represent standard deviation. Values calculated with gene copy numbers under the limit of quantification are marked with an asterisk.

3.2. Ozone

The reduction of cultivable *E. coli* and *E. faecium* bacteria correlated with the decrease in copy numbers of ARGs (Fig. 2). At a concentration of about 1 mg*L^{-1}, cultivable bacteria and ARB were reduced by 5.0 log (over 99.999%), while for ARGs, a reduction rate of at least 4.3 log (over 99.995%) was observed; the qPCR signals were below the quantification limit (see Fig. 3).

3.3. UV

Cultures of *E. coli* and *E. faecium* were irradiated with UV doses from 0 to 600 J*m^{-2}. In terms of bacterial counts, an inactivation at 100 J*m^{-2} was observed for both strains (2.5–4.2 log, over 99.7%). For *E. faecium*, maximum reduction rates (6.1 log, 99.99992%) were observed after UV irradiation with 150 J*m^{-2}. Maximum *E. coli* elimination was achieved using a dose of 300 J*m^{-2} (4.7 log, 99.998%). However, it should be noted that the determined elimination rates for all UV doses over 100 J*m^{-2} were within the same range (99.989–99.998%). For the UV treatment, large differences were observed between the cultivable bacterial numbers and the gene copy numbers. While bacterial numbers decreased more rapidly with increasing irradiation doses, the gene copy numbers in conventional short-amplicon PCR decreased only slightly. For the experiments with *E. faecium,* the highest UV dose (600 J*m^{-2}) implicated a reduction of the ARGs by only 0.33 log (53.5%). Using *E. coli* as model bacterial strain, no removal was found for the tetracycline resistance gene *tet*(A),

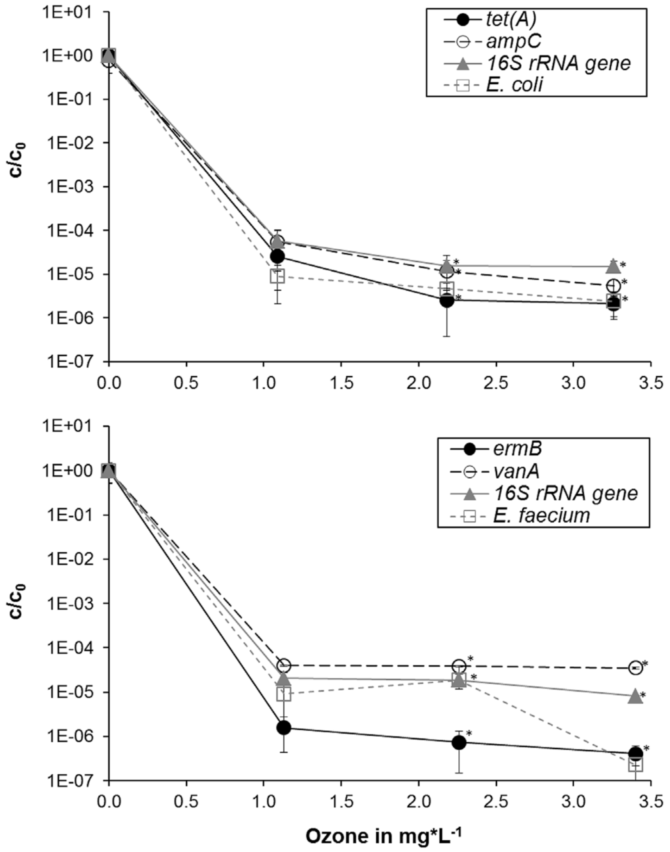

Fig. 2. Bacterial counts and qPCR results for ozone-treated *E. coli* (top graph) and *E. faecium* (bottom graph). Error bars represent standard deviation. Values calculated with gene copy numbers under the limit of quantification are marked with an asterisk.

and a low reduction rate of 1.0 log (90%) was reported for β-lactam ARG *ampC*.

3.4. Long-amplicon PCR

Drinking water spiked with the model bacteria *E. coli* and *E. faecium* was irradiated with various doses of chlorine, ozone, and UV and analyzed using qPCR with different amplicon sizes. For chlorination, qPCR with short amplicons (< 550 bp) and long amplicons (> 1000 bp) gave the same results (Fig. 4, top graphs). The results of the UV irradiation show a higher log reduction with long-amplicon PCR (Fig. 4, bottom graphs). For ozonation, the gene copy numbers for all analyzed ozone concentrations (1 mg*L^{-1} and higher) were below the quantification limit. For this reason, the results of the long-amplicon PCR are not shown.

4. Discussion

4.1. Chlorination

The mechanisms of action of chlorine on bacteria have been widely investigated (Venkobachar et al., 1997; Virto et al., 2005). When sodium hypochlorite or calcium hypochlorite are added to water, hypochlorous acid (HOCl) is formed. Proteins and peptidoglycan bound to the cell wall and the cell membrane are the initial points of attack for chlorine disinfection (Dodd, 2012; Venkobachar et al., 1997). After damage of the cell wall and membrane, HOCl can penetrate the cell wall and reach the cytoplasm, followed by reactions with purine and pyrimidine bases of the nucleic acids, whereby thymidine, deoxy guanosine, and uridine monophosphate are preferred targets (Dodd, 2012).

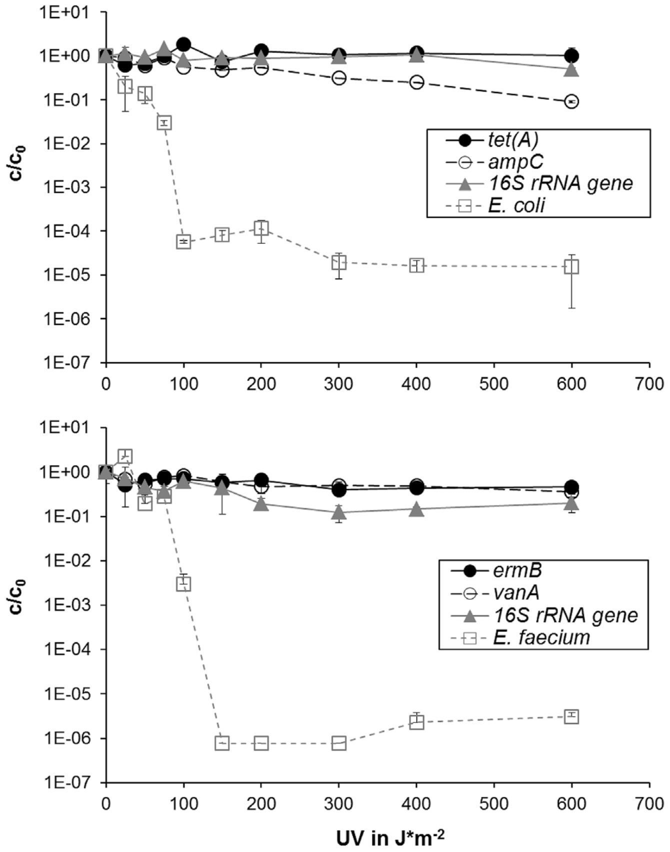

Fig. 3. Bacterial counts and qPCR results for UV-treated *E. coli* (top graph) and *E. faecium* (bottom graph). Error bars represent standard deviation.

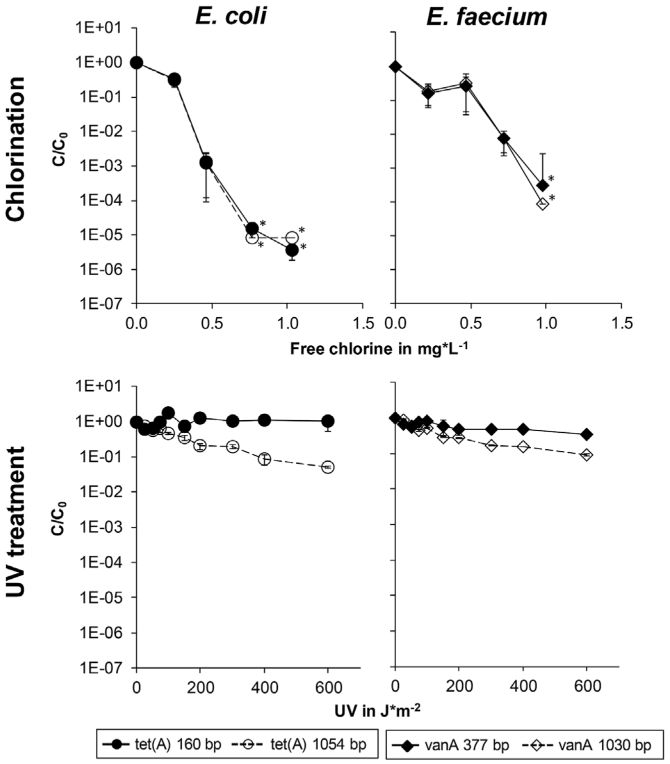

Fig. 4. qPCR and long-amplicon PCR results for *E. coli* (left side) and *E. faecium* (right side). Error bars represent standard deviation. Values calculated with gene copy numbers under the limit of quantification are marked with an asterisk.

As shown in Fig. 1, there was a noticable difference in the reduction of cultivable *E. coli* compared to *E. faecium*. This is most likely due to morphological differences in the structure of the cell walls. Gram-negative microorganisms are more sensitive to chlorine disinfection than Gram-positive microorganisms (Virto et al., 2005). The observed low effect on nucleic acids at low HOCl concentrations is due to its inability to reach the cytoplasm and to cause damage to the DNA (Fig. 1). In contrast, at higher chlorine concentrations, a larger impairment of the cell wall and membrane was caused, enabling HOCl to penetrate the cell wall and to react with the nucleic acids. This resulted in high-level DNA damage to the bacterial cells, consequently impeding DNA amplification, even for short amplicons. In this study, *E. coli* numbers were reduced by 3 log at a free chlorine dose of 0.15 $mg*L^{-1}$, whereas considerably higher concentrations of chlorine (0.5–0.6 $mg*L^{-1}$) were required to decrease the ARG numbers by the same extent. For the Gram-positive bacterium *E. faecium*, the concentration to achieve a 3-log reduction of the ARGs was even higher (0.9–1.05 $mg*L^{-1}$ free chlorine). This observation is in agreement with the previously published study by Yuan et al. (2015), who showed an effective inactivation of ARB, but only a limited reduction of ARGs using chlorine during wastewater treatment. This leads us to infer that a higher dose of chlorine is required to achieve the same log reduction for the ARG copy numbers compared to the cultivable bacterial numbers.

4.2. Ozone

Similar to chlorine, ozone also reacts with similar functional groups such as amines, amino acids, activated aromatic compounds, and reduced sulfur residues (von Gunten, 2003). In addition, ozone also reacts with unsaturated bonds of phospholipids, proteins, peptidoglycan, and liposaccharides in the cell envelope (McNair Scott and Lesher, 1963).

Based on the data from the ozone experiment with *E. coli* and *E. faecium,* even at a low concentration of 1 $mg*L^{-1}$, ozone resulted in > 99% removal of ARB and ARGs. Similarly, in another study with multi-drug resistant *E. coli* (strain DH5 α) in synthetic wastewater, 3 $mg*L^{-1}$ of ozone were required for a 90% reduction in bacterial numbers and ARGs (Oh et al., 2014). In addition, ozonation has also been reported to be more efficient than chlorination. Conversely, in a previous study, a considerably higher chlorine dose (160 $mg*L^{-1}$) was more effective than ozone (178 $mg*L^{-1}$) in the removal of ARGs during the treatment of urban wastewater (Zhuang et al., 2015). The present study demonstrates that ozone treatment of drinking water is effective in the elimination of both ARB and ARGs at a low dose of 1 $mg*L^{-1}$. However, additional studies with different water turbidity conditions are required to validate the findings of this study.

4.3. UV

In contrast to the use of chemical disinfectants, UV treatment results in the physical damage to the nucleic acids. The high energy associated with short-wavelength UV energy, primarily at 254 nm, is absorbed by cellular RNA and DNA. In this study, an increase in the UV dose resulted in an increased reduction of the cultivable bacteria; however, no detectable decrease or only a slight decrease in ARG copy numbers was observed with the short amplicons. Using long-amplicon qPCR, a reduction of 1–2 log was observed. However, even for long PCR products, only a minor reduction was achieved in comparison to the elimination of bacteria. This means that long gene sections withstand UV irradiation unharmed, while the bacteria are inactivated by DNA degradation. A comparison of the genome of the used bacterial strains showed that the genome of *E. coli* (5.1 Mb) is larger than that of *E. faecium* (2.8 Mb), and in accordance, the number of dimer formation sites (CC, TT, CT, TC) is noticeably higher in *E. coli* (about $1.2*10^6$ sites) compared to *E. faecium* (about $7.2*10^5$ sites). This is reflected in the observed higher sensitivity of the *E. coli* strain to UV irradiation compared to *E. faecium*. The outcomes of this study are in agreement with recent studies on the efficacy of UV disinfection for the removal of ARGs. In a previous study, UV disinfection was an effective technology to reduce tetracycline-resistant heterotrophic bacteria and *E. coli* in WWTP effluent (Childress et al., 2014). However, two Chinese studies have reported that UV treatment of wastewater resulted in a low removal of ARGs by 0.36–0.60 log (2495 $J*m^{-2}$) and 2.48–2.74 log (124,770 $J*m^{-2}$), respectively (Zhang et al., 2015; Zhuang et al., 2015). In comparison, the UV doses used in these studies were in a considerably higher range than those used in this study (maximum 800 $J*m^{-2}$), which was most likely due to the high turbidity of wastewater, suggesting that water turbidity is a major factor influencing the efficacy of UV in the removal of ARGs. Another study has investigated the potential of UV disinfection for the removal of four different ARGs (*mecA*, *vanA*, *tet*(A), and *ampC*) – in extracellular and intercellular form – under controlled laboratory conditions (McKinney and Pruden, 2012). As model organisms, the methicillin-resistant *Staphylococcus aureus* and the vancomycin-resistant *E. faecium*, *E. coli* SMS-3-5, and *Pseudomonas aeruginosa* 01 were used. According to the results, the Gram-negative bacteria were more sensitive to UV disinfection than the Gram-positive bacteria. However, the two Gram-positive organisms had smaller total genome sizes, which could have reduced their susceptibility to UV treatment. In general, higher UV doses are necessary for the reduction of ARGs (2000–4000 $J*m^{-2}$ for 3–4 log decrease) than for the inactivation of ARB (100–200 $J*m^{-2}$ for 4–5 log decrease) (McKinney and Pruden, 2012). In contrast to the study of McKinney and Pruden (2012), a maximum fluence of 600 $J*m^{-2}$ was applied in this study. However, the results for the UV doses up to 600 $J*m^{-2}$ are in accordance.

Yoon et al. (2017) have performed UV experiments with phosphate-buffered solutions or wastewater matrices spiked with about $5*10^5$ CFU per mL *E. coli* carrying plasmid-encoded ARGs. At a UV fluence of 400 $J*m^{-2}$, the reduction of intercellular ARGs was 1.1–1.6 log (Yoon et al., 2017), which was slightly higher compared to the reduction achieved in our study. Most likely, this is due to the lower initial concentrations of bacteria. To achieve significant ARG removal (~4 log), the authors reported considerably higher doses of 1000–1400 $J*m^{-2}$ (Yoon et al., 2017).

Ultraviolet light primarily damages the nucleic acids. A few strand breaks can result in bacterial inactivation, but large sections of genomic DNA may remain intact. These intact sections of DNA carrying ARGs are still detectable using qPCR methods, as these assays are typically designed for amplicon lengths of only 75–500 bp. The short amplicon length limits the potential to capture DNA damage (Ho et al., 2016). For this reason, long-amplicon qPCR assays were used to enhance the capture of DNA damage events to a DNA segment covering nearly the complete gene sequences. With the detection of larger amplicons, the signal reduction after UV treatment was higher. However, ARGs were more prevalent as compared to viable bacterial cells. Overall, the results show that UV treatment is limited in its potential to reduce ARGs in water and wastewater.

5. Conclusions

The potential of chlorine, ozone, and UV disinfection to eliminate ARGs varies (summarized in Fig. 5). Only for ozone, similar elimination rates were observed in terms of cultivable bacteria and ARGs. For chlorine and UV, inactivation of bacteria was observed at lower doses than those needed for the decrease in resistance gene copy numbers. Obviously, these disinfection methods do not completely eliminate ARGs, potentially allowing gene transfer even after water treatment. Overall, advanced water treatment technologies for the removal of ARB and ARGs are becoming increasingly available, but additional research is needed to determine the effectiveness of the newer advanced treatment processes and to characterize the associated microbiological risks. Recommendations for effective and economically viable options for the removal of ARGs at critical points are urgently needed.

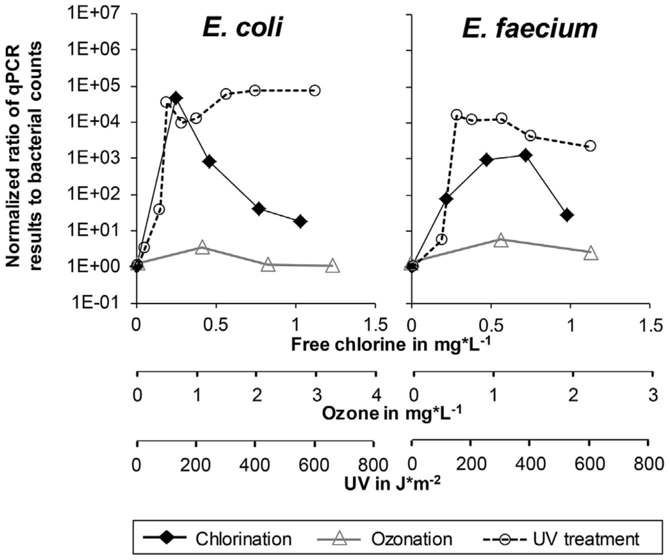

Fig. 5. Normalized ratio of qPCR results (*tet*(A) or *vanA*, short amplicon) to bacterial counts as function of dose for *E. coli* (left side) and *E. faecium* (right side).

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