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Application of UV-LEDs for antibiotic resistance genes inactivation – Efficiency monitoring with qPCR and transformation

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ABSTRACT

A dose-response study on a plasmid vector containing ampicillin and kanamycin resistance genes was performed with UVC-LEDs at two different wavelengths (265 and 285 nm) using different UV fluences including 40 mJ/cm^2 and 186 cm^2, typically associated with 4-log inactivation of bacterial pathogens and viruses, respectively. DNA damage was assessed using qPCR and transformation assays: four different qPCR protocols targeting the resistance genes were designed to produce amplicons varying in lengths from 80 to 601 bp to quantify DNA damage. UV irradiation emitted at 265 nm was more efficient than 285 nm, as shown by the transformation and qPCR assays. The inactivation rate coefficients showed that the longer segments damaged faster compared with shorter ones for both 265 and 285 nm UV wavelengths. For the largest qPCR amplicon, UV fluence of 186 mJ/cm^2 using 285 nm wavelength led to a ~4.2-log quantification reduction whereas the corresponding reduction using 265 nm was ~6.5-log. Transformation assays confirmed these findings with 265 nm wavelength requiring lower UV fluence (100 mJ/cm^2) than with 285 nm (186 mJ/cm^2) to reach the no detection threshold for plasmid transformation and gene expression. Comparing rates of DNA damage were observed for these two methods; approximately 24 mJ/cm^2 was required for the loss of 1-log$_{10}$ of transformation efficiency using 285 nm. For qPCR, UV fluences required for the loss of 1-log$_{10}$ amplification were higher and inversely proportional to the amplicon length for both 265 and 285 nm.

1. Introduction

Antibiotic resistance has emerged as a global human and animal health challenge. Addressing the threat of antibiotic resistance bacteria (ARB) and associated antibiotic resistance genes (ARGs) is essential to help maintain effective antibiotic treatment in the future. Greater efforts are therefore being made to control their environmental spread and prevalence. For example, a global action plan by WHO was unveiled in 2015 to encourage international participants to take actions to control and monitor and address the occurrence and spread of antibiotic resistance [1].

The spread of ARGs may lead to antibiotic resistance transmission to both human and animal pathogens [2]. ARGs carried by mobile genetic elements (plasmids, integrons, and transposons) have the capability of spreading to recipient cells via horizontal gene transfer including conjugation (through direct cell to cell contact), transduction (involving a bacteriophage) and transformation (involving extracellular or free DNA) [3]. Notably, transformation does not require a viable donor cell or bacteriophage. Damaging free DNA is crucial in reducing the spread of ARGs considering free DNA could also originate from dead cells and released into the environment. Urban wastewater treatment plants serve as the critical control point for spread of contaminants but could lead to selective increase of certain bacteria possessing greater capacity to resist stress that could lead to enhanced emergence and spread of ARGs and ARBs [4]. Conventional water disinfection is primarily used for killing or inactivating pathogens to meet specific log inactivation regulations [5]. Chlorination, a widely used method for inactivating microorganisms may not efficiently destroy ARGs, leading to their discharge into the environment [6]. Moreover, biological treatment processes which are an integral part of current treatment schemes are known to promote resistance development and spread by creating potentially suitable selective conditions for bacteria due to continuous mixing of antibiotics at sub-inhibitory concentrations [7,8]. The need for a shift in approach from pathogenic inactivation to damaging ARGs has been highlighted in several investigations [6,9,10].

The second most widely used disinfection method involves UV irradiation. UV has been extensively investigated for water and wastewater disinfection with increasing number of practical applications worldwide

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particularly in Europe and North America. The impact of UV irradiation is wavelength dependent. Disinfection at 254 nm is the most investigated due to peak emission of low pressure (LP) mercury UV lamps at this wavelength. However, the most effective germicidal wavelengths are around 260–265 nm corresponding to the peak of the UV DNA absorption curve [11]. Direct germicidal effect on the DNA of microorganisms results in the formation of pyrimidine dimers [12–14]. Moreover, protein has absorbance maxima at 280 nm, which could assist in damaging repair enzymes, preventing DNA repair [20]. This has led to several authors supporting the application of a UV source emitting over a range encompassing 240–280 nm [15]. Since UV-LEDs allow emission over a narrow wavelength range covering various emission wavelengths, the use of 250–280 nm wavelengths is the most widely investigated for disinfection [16] considering the DNA and protein absorption spectra. Although UV radiation is absorbed by each of the five nucleobases, the pyrimidine nucleotides (TMP, UMP, and CMP) show the greatest sensitivity to UV damage [2] mainly by pyrimidine-pyrimidine dimerization [16,17]. The damage inflicted by UV irradiation is mainly dependent on the DNA sequence with CPDs mostly occurring at TT followed by TC, CT, and CC [18]. The damaged DNA can be repaired by photo-reactivation and dark repair [19]. DNA repair is dependent on the species of microorganism, their physiological state, UV fluence [20], and temperature. DNA repair may be avoided by damaging the repair enzymes which have been reported to be more vulnerable to high UV intensities [21].

Since UV-LEDs provide the opportunity to custom-select the emission wavelengths, they may be used to potentially enhance the disinfection efficiency and improve possibilities of full-scale implementation. In this study we compared the effect of irradiation at 265 ± 5 nm (near the relative peak for DNA UV absorbance) and 285 ± 5 nm (near the relative peak for protein/enzyme absorbance) on cell free DNA containing ARGs. Although 285 nm is selected to target enzymes which are not relevant for cell free DNA, the co-effect of DNA damage from irradiation at this wavelength might be significant and should be documented. Hence, even though our test set-up did not include exposure of enzymes or whole cells, this wavelength (285 ± 5 nm) might be considered for natural water treatment. It is therefore desired to document any contribution form this wavelength on the inactivation of ARGs directly.

The focus of this study is the inactivation of cell free ARGs to prevent the spread of ARB associated with HGT by transformation. Several investigations have shown that UV-damaged ARGs were unable to function inside the host bacterial cell [2]. UV irradiation is therefore highly suitable for ARGs inactivation. To do so, a set of tools are needed to assess DNA damage and to understand the extent of DNA damage needed to neutralize transformation and gene expression. Quantitative real-time PCR (qPCR) may be used for monitoring and quantifying DNA damage. The likelihood of the presence of DNA damage and the possibility for PCR amplification interruption increases with increase in the size of the target amplicon [22,23]. Longer qPCR assays will also detect more biologically relevant DNA damage [24,25] and therefore shorter qPCR assays are considered more conservative. In this study four PCR primer pairs targeting various ARG areas of the pCR®II-TOPO® plasmid were used to amplify DNA fragments ranging from 80 to 601 bp. As qPCR monitoring of UV inflicted DNA damage does not provide direct measurement of DNA function loss [10], it should be supplemented with a biological end point to help understand the impact of treatment. Transformation assays may be used to benchmark qPCR DNA damage measurement. A biological benchmarking performed in the laboratory will provide a required fluence which may then be converted to the corresponding required specific qPCR $\log_{10}$ amplification reduction. Transformation is not a suitable environmental monitoring method since it is sensitive to contamination and reaction conditions requiring a competent receiving cell and a purified DNA vector to give a quantitative result. ARGs released from dead cells may be taken up by bacteria in natural environments by transformation. Hence, hindering transformation by damaging ARG by UV is possible to measure in controlled in-vitro laboratory experiments. However, in natural waters, there are generally too many uncontrolled factors and potentially too low concentrations for doing quantitative evaluations by looking for transformation of ARGs. Therefore, a more robust qPCR method has greater applicability for monitoring the efficiency of treatment at full scale. In this study, the UV-irradiated plasmids were transformed into *Escherichia coli* and the ability of the bacteria to express kanamycin and ampicillin resistance genes was measured to benchmark ARGs inactivation by UVC-LEDs.

Some investigations have reported the efficiency of 254 nm UV irradiation in damaging DNA assessed using qPCR, with [9] or without [10] transformation assays. Although UV-LEDs emitting at different wavelengths have been extensively investigated for conventional disinfection, their efficiency in damaging ARGs is rarely reported. UV-LEDs at selected wavelengths (265 ± 5 nm and 285 ± 5 nm) were used for the first time to investigate the effect that UV irradiation has on selected ARGs. According to the best of our knowledge, this is the first investigation looking at the effect of 265 and 285 nm UV irradiation on ARG inactivation measured by both qPCR, using a range of amplicons (80, 113, 217 and to 601 bp), and transformation efficiency testing. Furthermore, the impact of different UV fluences including those recommended for bacteria (40 mJ/cm^2) and virus inactivation (186 mJ/cm^2) was investigated for damaging ARGs. Although the established UV fluence standards are set for 253.7 nm, it is worth investigating how the equivalent UV fluence values compare with other wavelengths for damaging ARGs. Hence, we tested a wide UV fluence range including values recommended for 4-log bacterial and viral inactivation. Moreover, this study looked at the kinetics, and the extent of DNA damage to benchmark it to a biological endpoint for using qPCR DNA integrity assessment as a potential proxy for biological function loss.

2. Materials and methods

2.1. Model genes for antibiotic resistance

A plasmid (pCR™-II-TOPO® vector) containing genes for resistance to ampicillin and kanamycin was used as a test model in this study. The plasmid was obtained from a Topo™ TA™ cloning kit (Invitrogen). The plasmid was provided in a linearized formed and cyclized without input of DNA and transformed into Top 10 chemically competent *Escherichia coli* according to supplier's protocol to form blue colonies in the presence of x-gal, ampicillin and kanamycin on Lennox L Broth (LB) agar plates. Transformed *E. coli* were cultured for 16–20 h at 36 ℃ in LB Base (Invitrogen) with 50 μg/mL Kanamycine sulfate (Gibco). Plasmids were extracted with PureLink™ HiPure Plasmid Megaprep Kit (Thermo Fisher Scientific) according to the manufacture's protocol. The concentration of plasmid was measured with NanoDrop 2000 and diluted with MilliQ to a DNA concentration of 5 ng/μL.

2.2. UV irradiation

UV experiments were performed using a UVinaire™ UV-LED unit from AquiSense Technologies (Erlanger, KY). A schematic of the UV-LED set up is provided in Fig. 1. It consisted of a collimated beam apparatus having 265 and 285 nm UV-LEDs. A petri dish of 53 mm diameter was used to carry out experiments using a stirred suspension of plasmid (10 mL, 5 ng/μL DNA conc). The UV transmission (UVT) values of plasmid suspension were 85% and 92% at 265 and 285 nm, respectively, and were taken into account when calculating the UV fluence. The sample was gently mixed (~100 rpm) such that there was no vortex to minimize the uncontrolled reflections on the water surface. Irradiation was carried out at room temperature (22 ℃) by keeping UV-LEDs 7 mm from the end of the collimating tube (75 mm) for optimized UV exposure [26]. The average UV irradiance or fluence rate was measured by a radiometer (ILT2400) with sensor (SED270/QT5) and was recorded to

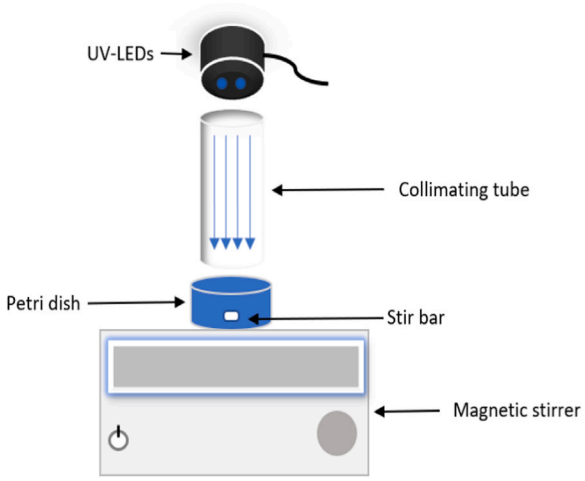

Fig. 1. Schematic of the UV-LED system used in this study.

be 0.1789 and 0.5945 mW/cm^2 for 265 and 285 nm, respectively. The radiometer was calibrated (SUV scanned calibration from 250 to 400 nm providing a calibration factor every 2 nm) by the supplier and the calibration factors provided were used calculate the fluence rate.

Since most regulatory bodies specify a UV fluence of 40 mJ/cm^2 to ensure at least 4-log inactivation of pathogenic microorganisms [27], preliminary experiments were performed with UV fluences up to 40 mJ/cm^2. The U.S. Environmental Protection Agency Ultraviolet Disinfection Guidance Manual for the Final Long-term 2 Enhanced Surface Water Treatment Rule established guidelines aiming at inactivating the toughest pathogens [28]. These guidelines are based on UV fluence needed to inactivate viruses, *Giardia*, and *Cryptosporidium* with the maximum recommended UV fluence (253.7 nm) of 186 mJ/cm^2 resulting in 4-log inactivation of viruses [17]. This UV fluence was therefore also selected for both wavelengths. As 285 nm inflicted less damage to DNA compared with 265 nm in the preliminary experiments ($\leq$40 mJ/cm^2), a higher UV fluence (300 mJ/cm^2) was selected for 285 nm. All experiments were carried out at room temperature (22 ℃) in duplicates. Samples were collected after delivering the desired UV fluences for qPCR analysis and transformation assays.

2.3. Transformation experiments

Transformation was preformed according to supplier's protocol (Invitrogen) with Top 10 chemically competent *E. coli* and 4 μL (5 ng/μL) plasmid solution (UV treated or untreated) per tube of competent cells to quantify the capability of bacteria to obtain antibiotic resistance. Transformed *E. coli* was plated on LB agar plates with ampicillin (50 μg/mL) or kanamycin (50 μg/mL) supplement and incubated for 20–24 h at 36 ℃. Non-transformed *E. coli* was used as a control for the antibiotic supplement.

A preliminary study was preformed to assess the applicability of doing quantitative evaluation of the transformation results and establish the required plasmid concentration for UV-exposure experiments. Reproducibility of transformation reaction was investigated by preforming triplicate reactions, and by using a 10-fold serial dilution of plasmid in the reaction. The natural logarithmic of the concentration of transformed *E. coli* was plotted against natural logarithmic of initial plasmid concentration of the reaction and a linear regression was preformed assuming a first-order kinetics reaction in order to estimate "intact plasmids" after UV exposures based on concentration of successfully transformed *E. coli*.

Transformation of UV-irradiated plasmid was done without dilution or purification with one transformation reaction for each UV-exposure, with duplicate experimental replicates. The transformed cells were plated as a dilution series with triplicate LB plates with kanamycin or ampicillin for each dilution. Transformation of non-UV-exposed plasmid was used as positive transformation control.

2.4. qPCR analysis

For qPCR analyses, samples were directly analyzed without additional purification steps. Four primer pairs were designed using Oligo7 v7.60 [29] for targeting various ARG areas of the pCR®II-TOPO® plasmid, amplifying a range of DNA fragments from 80 to 601 bp (See Table 1) overlapping the kanamycin and ampicillin genes (See Supplementary Fig. S1).

Melt curve analysis was carried out from 65 to 95 ℃ using 0.2 ℃ increments. PCR amplification was conducted by a CFX96 thermocycler (BioRad, Hercules, CA, USA) using 10 μL reaction volume containing 5 μL SsoFast™ EvaGreen® (Bio-Rad), 0.4 μM of each primer (Eurofins MWG, Ebersberg, Germany) and 1.5 μL sample. Reaction volume was achieved using sterile deionised water. The optimal annealing temperature for PCR was determined by running a temperature gradient. The optimized two-step amplification for the four newly designed primer pairs was carried out under the following conditions: denaturing for 2 min at 98 ℃ followed by 40 cycles of 98 ℃ for 5 s and 61 ℃ for 20 s [30]. Melt curve analysis was carried out at temperature range of 65–95 ℃ using 0.2 ℃ increments [30]. A standard curve was included for every run and each primer pair for quantification of the tested samples. A calibrated 50 pg/μL plasmid solution was serially diluted in deionized water and analyzed in duplicates. Conversion from pg/μL to copy/mL was calculated from the MW of the plasmid deduced from its nucleotide composition (2.4 $\times 10^6$ g/M). LOD for the qPCR is 1 target copy per 1.5 μL reaction sample in the assay corresponding to 667 copy/mL sample.

3. Results and discussion

3.1. Determining plasmid concentration for transformation reactions

Testing of quantification of transformation reactions was carried out using a 10-fold dilution series of plasmid, and average of transformed *E. coli* from triplicate plate counts as well as triplicate reactions was determined for LB with ampicillin and triplicate reactions with single plate counts for kanamycin. It was concluded that the reproducibility of triplicate transformation reactions was acceptable as the percent standard deviation (%SD) of transformed *E. coli* was in the same range as for triplicate plate counts (see Supplementary Information Table S1 and S2). It was found that the transformation reaction did not produce transformed *E. coli* when the plasmid concentration was 5 $\times$ 10^6 (0.005 ng/μL) copies per reaction or less. Therefore, all DNA exposure experiments were done with highest possible plasmid concentration at 5 $\times$ 10^9 (5 ng/μL) to be able to detect up to 2-log reduction of transformation due to DNA damage.

3.2. Relationship between plasmid concentration and transformed E. coli

The number of colony forming units (CFU) of transformed *E. coli* on LB agar with added ampicillin or kanamycin in relation to number of plasmids (ARG) in transformation reaction is shown in Fig. 2. No significant difference between the two antibiotic supplements was observed and hence the average of colonies from both mediums were used for further correlation analysis. The transformation protocol involved 1-hour incubation step in neutral growth medium to allow expression of the genes in the acquired plasmid. Especially, kanamycin resistance gene needs 1 h incubation time for it to be expressed. However, the growth during this incubation period resulted in 2–4-fold increase of cells as counted by flow cytometry (data not shown) which added to the difficulty of correlating a successful transformation and the number of colonies on the agar plate. The other factor complicating the

Table 1
List of qPCR primers of kanamycin and ampicillin resistant genes used in this study.

Target	Primers	Sequences (5' – 3')	Amplicon size (bp)	Melting temp (°C)
Kanamycin	Kan385F18	GCTACCTGCCCATTCGAC	80	58.2
	Kan445R20	TGATCGACAAGACCGGCTTC		57.6
Ampicillin	Amp1198F17	ATAACACTGCGGCCAAC	113	52.8
	Amp1294R17	CTCCGGTTCCCAACGAT		55.2
	Amp1279F20	ATGTAACTCGCCTTGATCGT	267	55.3
	Amp1527R19	CAATGATACCGCGAGACCC		58.8
Kanamycin + Ampicillin	Kan-Amp710F17	CTGACCGCTTCCTCGTG	601	57.6
	Kan-Amp1294R17	CTCCGGTTCCCAACGAT		55.2

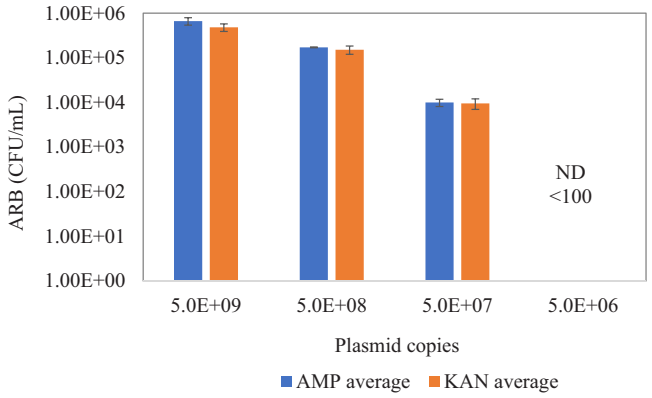

Fig. 2. Concentration of transformed E. coli (ARB) on LB plates with ampicillin (AMP) and kanamycin (KAN) in relation to number of plasmids (ARG) used in the transformation reaction. ND: Not detected; error bars represent 67% confidence interval.

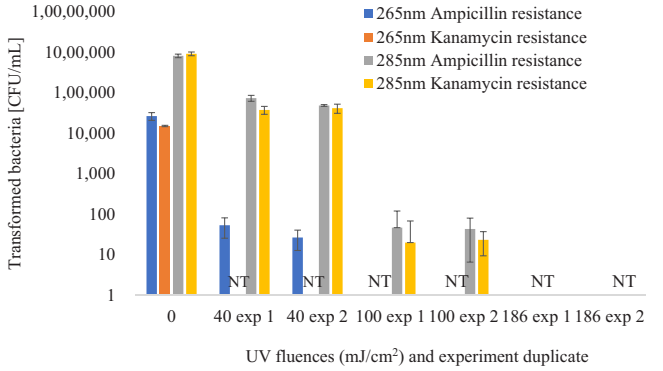

Fig. 3. Concentration of antibiotic resistant bacteria (Kanamycin and ampicillin resistance) after two parallel transformation experiments of UV-irradiated ARG at irradiation by 265 and 285 nm under various UV fluence conditions. NT: No Transformation; error bars represent 67% confidence interval.

correlation between intact plasmids and colony count is that each cell can take up several plasmids whereas only one is needed for successful expression. Thus, the initial dilution series experiment was done with a fixed cell concentration resulting in a cell to plasmid ratio of 50 plasmids per *E. coli* down to non-detect at 0.05 plasmids per *E. coli*. As expected, the ratio between the plasmid and the CFU was not linear. However, plotting the LN concentration of plasmid vs LN CFU gave a correlation resembling first order kinetics. The regression curve on the formula $y = 0.89x\text{-}7.32$ (where y is LN(transformed E. coli CFU/mL, x is LN (plasmid copies) and the fit is described by $R^2 = 0.95$) was used to back calculate the intact (or non-inactivated) plasmid concentration from CFU numbers of both AMP and KAN supplemented LB plates after UV exposure (Supplementary information Table S3).

3.3. Impact of UV fluence on transformation efficiency

Transformation may be used for measuring antibiotic resistance expression and therefore integrity of the coding material, albeit limited sensitivity precluding using it alone for monitoring purposes [9]. Preliminary experiments using fluences ranging between 1 and 10 mJ/cm^2 using both wavelengths showed no effect (results not shown) on reduction of transformation. An increase of the UV fluence to 40 mJ/cm^2 at 265 nm led to ~3-log reduction in transformation efficiency for ampicillin genes and no expression of kanamycin genes (~4 log reduction) (Fig. 3). The exposure experiment was repeated twice (exp 1 and exp 2 in Fig. 3) using the same non-exposed control. However, the decrease was smaller (~1.3 log) for 285 nm at the same UV fluence since 265 nm is closer to DNA absorption maximum than 285 nm. Even if the initial studies showed good repeatability of the transformation experiment (Fig. 2), the non-irradiated control for the 285 nm experiments had more than 1-log$_{10}$ higher concentration than the control for the 265 nm experiment (Fig. 3). There was little to no difference between ampicillin and kanamycin resistance for 285 nm irradiation. Increasing the UV fluence to 100 mJ/cm^2 resulted in no transformation (>3-log reduction) for 265 nm and ~2.5-log reduction for 285 nm. Detection threshold (no transformation) was reached at a UV fluence of 186 mJ/cm^2 for 285 nm.

The reduced number of transformed cells in transformations assays after UV exposure is a result of the DNA damage being too large for DNA repair and/or expression of the gene after DNA repair (failure in expression). Similarly, a positive transformation reaction could either mean the gene did not acquire any DNA damage, or that the DNA damage was repaired by the bacteria. Whether DNA-repair occurred in this study is not known. However, pre-incubation for 1 h in neutral medium (according to protocol) before plating on agar plates containing antibiotics was needed for expression of newly acquired genes that may have resulted in DNA repair.

3.4. UV irradiation effect on ARGs measured by qPCR analyses

Under UV fluence conditions of $\leq$ 10 mJ/cm^2 for 265 nm, the shortest 80 bp amplicon showed no reduction in quantification whereas the longest 601 bp amplicon had approximately 1-log reduction of ARG copy number (Supplementary Fig. S2a). Increasing the UV fluence further led to greater reduction in quantifiable DNA for the longest fragment with almost 2.5-log reduction at 40 mJ/cm^2 for 265 nm (Supplementary Fig. S2a). For 285 nm, however, there was very little effect with highest reduction of approximately 1-log for 40 mJ/cm^2 when using the longest DNA fragment (Supplementary Fig. S2b). Using a conventional mercury lamp emitting UV irradiation at 254 nm, Yoon et al. [31] reported comparable loss (1.8–2.6-log) using amplicons of 806 and 850 bp under similar UV fluence conditions (40 mJ/cm^2). Increasing DNA damage (4-log) with increasing UV fluence (100–140 mJ/cm^2) was reported by Yoon et al. which corroborates the findings of Chang et al. [9] where the authors reported similar correlation using 254 nm UV irradiation produced by LP mercury lamps.

Considering the low DNA damage observed in our preliminary

experiments, the UV fluence was increased to enhance DNA damage and to enable a detailed and meaningful evaluation of qPCR and transformation results under identical UV fluence conditions. Therefore, in the second set of experiments, UV fluence was increased up to 186 mJ/cm^2 for 265 nm, and 300 mJ/cm^2 for 285 nm. Increasing UV fluences therefore led to enhanced damage to all amplicons as shown by qPCR quantification. Overall, increased DNA damage was observed giving less amplification with 265 nm compared with 285 nm, concurring with the low UV fluence experimental results. Increasing the UV fluence to 100 mJ/cm^2 gave 4.6-log loss of DNA in this study (Fig. 4a) compared with 4-log for 60–90 mJ/cm^2 reported by Yoon et al. [31]. The largest amplicon (601 bp) was the most sensitive with amplification reduced by 7-log when using 265 nm for UV fluence of 186 mJ/cm^2 and 6-log for 285 nm at UV fluence of 300 mJ/cm^2 (Fig. 4b). These results are further discussed along with transformation results using the same UV irradiated ARGs (Section 3.5). The second largest amplicon length (267 bp) exhibited much lower DNA damage (<3-log) for both wavelengths at the maximum UV fluences tested for each wavelength indicating the importance of amplicon length in DNA damage susceptibility. Suss et al. [33] used different amplicon lengths (100, 500, and 900 bp) and demonstrated that detection of DNA damage increased with increase of the amplicon size. According to another study by McKinney and Pruden [10], a 3- to 4-log damage to extracellular ARGs (*mec*A, *van*A, *tet*A, *amp*C) with target lengths around 1000 bp required UV fluences of ~200 mJ/cm^2 at 254 nm irradiation using LP mercury lamp. This is about 3-log less than what we have reported in this study using 186 mJ/cm^2 at 265 nm targeting the largest amplicon (601 bp), albeit using UV-LEDs.

Reaction kinetics for UV fluence over the entire tested range (0–186 mJ/cm^2) using 265 nm followed first-order kinetics for all amplicon lengths with R^2 value ranging from 0.94 to 0.98 (See Supplementary Fig. S3). Similar first-order kinetics were observed using 285 nm at UV fluence range of 0–300 mJ/cm^2 for all amplicon lengths, with R^2 values ranging from 0.94 to 0.98. The first-order kinetics were consistent with Yoon et al. [31] who used ampicillin and kanamycin resistance gene with amplicon size of 850 and 806 bp respectively, for evaluation of UV treatment. Similarly, Chang et al. [9] found that the loss of the short amplicon (~200 bp) followed first-order kinetics for investigated range of UV fluence (0–400 mJ/cm^2). The long amplicon (800–1200 bp), however, exhibited deviation from first-order kinetics under similar range of UV fluence while reporting > 90% reductions.

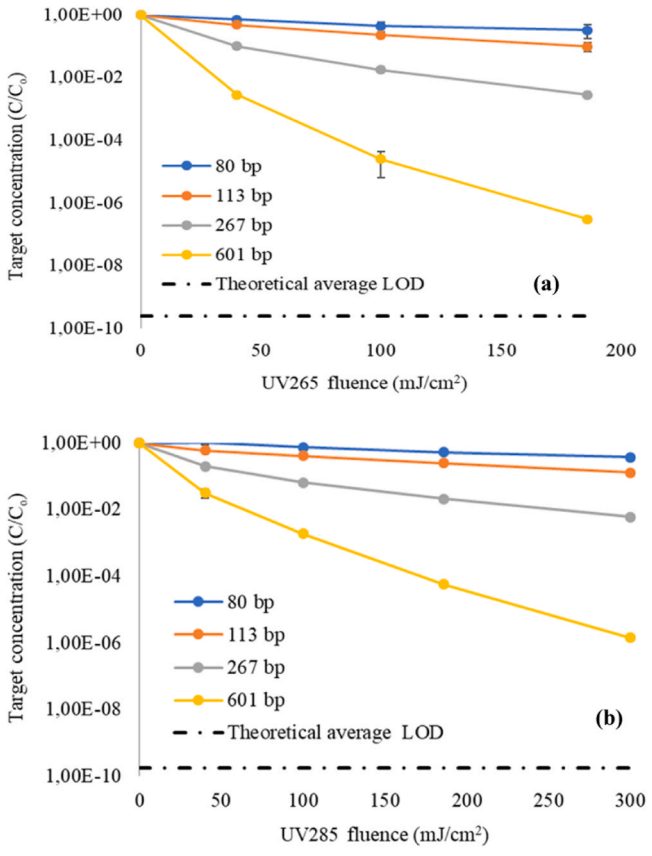

Fig. 4. Concentration of amplifiable target before and after 265 nm (a) and 285 nm (b) UV irradiation for different amplicon lengths during qPCR analysis; error bars represent 67% confidence interval.

As DNA damage kinetics are first-order both for transformation and qPCR assays, rates of required UV fluences for 1-log$_{10}$ DNA damage were calculated to enable overall comparison independently of initial DNA load (Table 2). The inactivation rate coefficients (k_{UV}) given in Table 2 demonstrates that the longer segments damaged faster compared with shorter ones for both 265 and 285 nm UV wavelengths. Furthermore, the inactivation rate coefficients were higher for 265 nm than for 285 nm indicating that the damage per unit of energy was greater using 265 nm than when using 285 nm.

When looking at the inactivation coefficients per segment length, the 601 bp target amplicon appears to be proportionally more sensitive to degradation than the 267 bp target amplicon. This might be explained by a higher adjacent pyrimidine count as well as a higher proportion of the most sensitive pyrimidine type, TT, as shown in Table 3. As mentioned earlier, the damage inflicted by UV irradiation is most frequent at TT dipyrimidines [16]. In fact, Chang et al. [9] found that the reactivity of genome with UV was most dependent on the TT base count than the size of the DNA. Therefore, the greater DNA damage for the largest amplicon is attributed to the greater number of the adjacent TT pyramidines (Table 3).

The impact of UV irradiation on ARGs has been reported to be reduced by a factor of ~1.7 ($p < 0.05$) for intracellular versus extracellular ARGs, showing that cellular components can play a protective role [31]. Similar conclusions were reached in another study in which intracellular *amp*C in *Pseudomonas aeruginosa* was detectable at UV fluences of 1000 mJ/cm^2 [10]. The same study also found a positive correlation between the number of adjacent pyrimidines and sensitivity to UV damage. Nonetheless, the authors [10] found no significant effect (statistical significance was defined as *p*-value ≤0.10) of aqueous matrices (PBS and filtered wastewater (TOC 4.61 mg/L)) on either ARB inactivation or ARG damage. It is important to note that the wastewater was filtered out for turbidity which could seriously reduce the effectivity of UV treatment.

Although conventional mercury lamps remain the main source of UV irradiation, studies on the use of UV-LEDs for ARG inactivation are expected to increase in coming years. A recent study by Shen et al. [32] used 268 nm and 275 nm UV-LEDs for the inactivation of two gram-positive tetracycline resistant bacteria (TRB-3 and TRB-5) from *Bacillus* species. A complete (5.4-log) inactivation of TRB-3 was seen at UV fluence of 15.36 mJ/cm^2 with both 268 nm and 275 nm whereas the UV fluences needed for complete (5.7-log) inactivation of TRB-5 were greater, i.e., 23 and 30.72 mJ/cm^2, for 268 nm and 275 nm, respectively. The authors also analyzed the *tet* determinant (*tet*(L)) by qPCR although no details on amplicon length were provided. The UV fluences used for damaging *tet*(L) were much lower (38.4 and 46 mJ/cm^2) than

Table 2
Inactivation rate coefficients (k_{UV}) for 1-log$_{10}$ damage to ARGs segments.

265 nm		
ARG segment length (bp)	k_{UV} (cm^2/mJ)	k_{UV} per segment length (cm^2/mJ)
267	0.0131	4.9×10^{-5}
601	0.033	5.5×10^{-5}
	285 nm	
267	0.0069	2.6×10^{-5}
601	0.0187	3.1×10^{-5}

Table 3
Adjacent pyrimidine counts for qPCR amplicons (both strands).

qPCR product (bp)	Dimers (count)					Dimers/amplicon length (%)					Dimers/total dimers (%)			
	CC	CT	TC	TT	Total	CC	CT	TC	TT	Total	CC	CT	TC	TT
601	61	74	84	97	316	10.2	12.3	14.0	16.1	52.6	19.4	23.4	26.6	30.6
267	33	32	29	34	128	12.3	12.0	10.9	12.7	47.9	25.7	25.1	22.7	26.5
113	14	12	15	17	58	12.4	10.6	13.3	15.0	51.3	24.1	20.7	25.9	29.3
80	9	8	14	6	37	11.2	10.0	17.5	7.5	46.2	24.3	21.6	37.9	16.2

that used by others [9,31] that resulted in DNA damage of 0.93 and 0.95-fold, respectively, using 268 nm UV-LEDs.

3.5. Relationship between DNA damage and gene inactivation

ARG transformation assays by traditional culture-based methods are laborious and material-intensive [34,35], and are difficult to use with environmental samples. However, there is a framework available for correlating qPCR data with genome damage in several organisms [24, 33,36]. Our approach assumes that it is the UV-inflicted DNA damage that causes transformation and qPCR to fail. Based on this assumption, transformation will fail when there is a certain level of DNA damage within the genes whereas qPCR will fail when DNA-damage occurs within the targeted amplicon. For qPCR, the probability of DNA damage within target amplicon increases with increased amplicon length. It is therefore possible to find a relationship between the DNA damage needed for stopping transformation and that for giving a failure in qPCR with a certain amplicon size. Benchmarking DNA damage to a biological endpoint for using qPCR DNA integrity assessment as a proxy for biological function loss would be useful for several applications. It is worth investigating if qPCR could be used for quantification of ARG integrity as a proxy for expression in transformed cells [2]. In this study, qPCR measurements and transformation results were combined to investigate whether loss in ARG expression after transformation may be correlated to loss in qPCR amplifications. The two largest fragments (267 and 601 bp) showed the maximum susceptibility to DNA damage during the qPCR assay and were therefore selected to be compared with the transformation results based on ampicillin resistance in Fig. 5. The number of plasmids that resulted in successful transformation and expression of ampicillin resistance was calculated from the CFU concentration of transformed *E. coli* using the equation described in Section 3.2 and in Supplementary Information (Table S3). Irradiating the plasmid solution using 265 nm UV-LEDs up to UV fluence of 40 mJ/cm^2 reduced the amount of functional transformed plasmids. Increasing the UV fluence to 100 mJ/cm^2 resulted in reaching no detection threshold as indicated by the dotted red line in Fig. 5(a), exhibiting a total reduction of at least 3.8-log.

For, 285 nm, however, higher UV fluences were needed to reach the LOD for ARG transformation and expression (Fig. 5b). More than 4-log reduction was achieved at 100 mJ/cm^2, reaching no detection threshold for transformation at UV fluence of 186 mJ/cm^2 which corresponds to a reduction of $\geq$ 5.6 log. The greater reduction of plasmid expression using 285 nm compared with 265 nm irradiation was due to the greater initial concentration (about ~1.8-log difference). Consistent with qPCR, first-order kinetics (R^2 = 0.99) were noted during transformation experiments (Supplementary Fig. S2) for 285 nm whereas it was not possible to determine the kinetics for 265 nm due to reaching no detection threshold at 100 mJ/cm^2 giving only two data points. With the initial DNA loads used in these experiments, transformation ability was lost with 100 and 186 mJ/cm^2 using 265 nm and 285 nm respectively. However, although qPCR amplification ability was incrementally reduced, the smallest amplicon size assay (267 bp) remained above detection limit even after the maximum UV fluences of 186 and 300 mJ/cm^2 for 265 nm and 285 nm, respectively.

For qPCR, fluences required for loss of 1-log$_{10}$ amplification were higher than transformation varying from 54 mJ/cm^2 for 601 bp to 576 mJ/cm^2 for 80 bp, and 30 mJ/cm^2 for 601 bp to 384 mJ/cm^2 for 80 bp for 285 nm and 265 nm, respectively (Fig. 6). Results for transformation experiments post 265 nm UV irradiation could not be plotted since there was only two data point above detection limit for calculation,

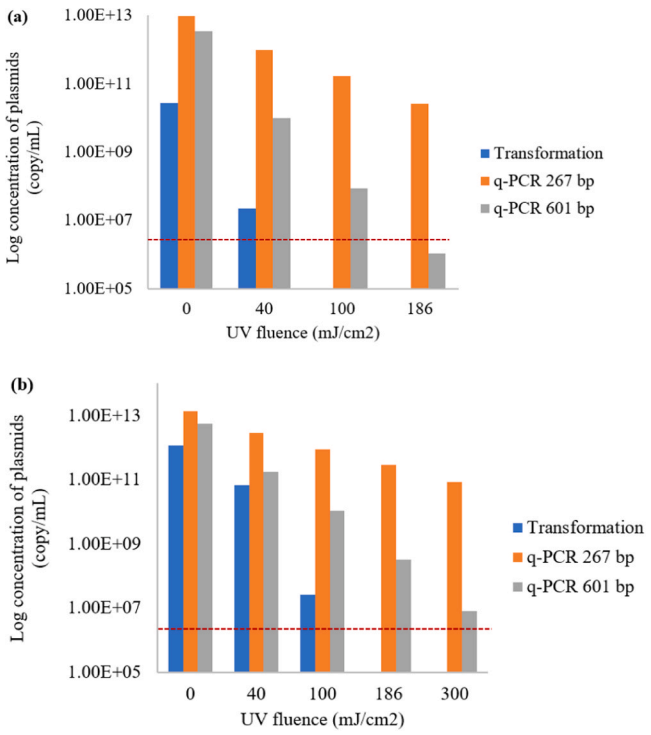

Fig. 5. Comparison of plasmid concentration before and after 265 nm (a) and 285 nm (b) UV irradiation under different UV fluences during transformation (Amp resistance), and qPCR experiments (dotted red line represents the no detection threshold for transformation assays, error bars represent 67% confidence interval). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

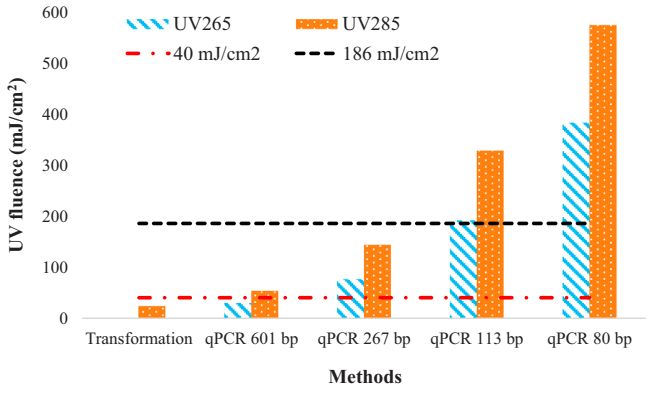

Fig. 6. Required UV fluences for damaging 1-log$_{10}$ of DNA as measured with transformation and qPCR assays.

showing stronger reduction of transformation than with 285 nm (Fig. 3). Results for transformation experiments post 285 nm UV irradiation showed that a UV fluence of 24 mJ/cm^2 was sufficient for 1-log$_{10}$ reduction of transformation for ampicillin resistance. Overall, in this study using a maximum target amplicon size of 601 bp, qPCR underestimated the loss of plasmid transformability and was therefore more conservative for evaluating DNA damage which agrees with the previous findings of Chang et al. [9]. The smallest qPCR amplicon (80 bp) required 12.8 and 10.3-fold more UV fluence than for the 601 bp amplicon for damaging 1-log$_{10}$ DNA with UV265 and UV285, respectively. With 285 nm UV-LEDs, the larger qPCR amplicon (601 bp) produced the closest results to the transformation with a measured degradation rate 2.3-fold less than with transformation, which is in the same range as reported by Chang et al. [9] who used 254 nm UV irradiation using 800–1200 bp qPCR protocols. Hence, the smaller the amplicon (which corresponds to lower number of nucleotide bps and thus reaction sites) the greater would be the underestimation of the loss of transformation efficiency [37]. It is therefore recommended to cover the entire gene sequence where possible in which case the ability of the gene to be transformed would be much better assessed than when only a fragment of gene is monitored with qPCR [9]. Independently of the specific ARG used in the transformation assay, an increase of the qPCR assay amplicon size will increase its sensitivity to UV damage. Using a larger amplicon than 601 bp will eventually match or exceed the sensitivity of the transformation assay.

First-order DNA degradation kinetics measured with transformation and qPCR enabled calibration of a qPCR assay against the desired transformation fluence. For instance, the UV285 transformation results showed a requirement of 24 mJ/cm^2 per Log$_{10}$ reduction of ampicillin resistance which may be used as biological reference for desired level of treatment. This fluence requirement may then be converted specifically for each qPCR system in order to measure the treatment effect using a qPCR assay instead of a transformation assay. For instance, using 265 nm, for UV fluence of 40 mJ/cm^2, the 601 bp qPCR assay will show a damage of 1.3-Log$_{10}$ whereas that for the 267 bp assay will be 0.5-Log$_{10}$. Likewise, the 285 nm would show 0.75- and 0.28-Log$_{10}$ damage using 601 and 267 bp, respectively. Based on these findings, it would seem possible to extrapolate this approach to in-situ UV treatment, although further assessment is warranted.

In our study, the same UV-irradiated plasmid ARGs were used directly into the PCR and transformation reactions for direct comparisons between these methods without any further processing steps (such as DNA purification) that could influence the plasmid concentration. However, the PCR uses only one enzyme (DNA polymerase) for DNA copying, while the transformation reaction is a complex biological process involving a living cell, *E. coli*, and various proteins in connection to plasmid uptake, repair and expression. The size of the plasmid (about 4 kb) compared with the largest qPCR amplicon (about 0.6 kb) increases the susceptibility of the plasmid to UV irradiation although it may be counterbalanced by the presence of bacterial DNA repair mechanisms. Recent work showed that extrapolating qPCR results to an amplicon similar to the size of the plasmid used in the transformation experiments would result in a more accurate detection of loss than the transformation assay [9].

In agreement with Chang et al. [9], kinetics of loss in amplicon could not be correlated directly with measures in the loss of ARGs' biological function (e.g., loss of transformation). However, these results demonstrate that qPCR method successfully quantifies UV-induced DNA damage which is proportional to the size of the amplicon. Another approach using qPCR could involve targeting a universal bacterial marker expected to be present in the influent, instead of ARG, in order to better monitor the damage to DNA present in the effluent. This would help enable direct in-situ monitoring of DNA degradation even though ARG may be in low concentrations or even absent from the sample.

As UV fluence of 186 mJ/cm^2 was sufficient to obtain loss of transformation both with 265 and 285 nm UV irradiation and that up to 6–7 log DNA reduction was achieved with qPCR 601 bp, the 186 mJ/cm^2 standard for achieving 4-log viral inactivation would seem suitable for UV disinfection applications aiming at significant loss or damage to ARGs. Moreover, 1-log$_{10}$ reduction of qPCR using 113 bp for UV265 nm treatment may be used to trace the effect of 186 mJ/cm^2 (Fig. 5). However, it should be noted that 40 and 186 mJ/cm^2 are recommended UV fluences for 253.7 nm radiation whereas 265 and 285 nm UV-LEDs were used in this study. Similar UV fluences would therefore correspond to a different number of photons when using wavelengths other than 253.7 nm. In this study, the commonly used concept of UV fluence was used. Bolton et al. [38], introduced the concept of photon fluence (the ratio of the photon fluence to UV fluence is proportional to λ at a given wavelength). Based the second law of photochemistry, the authors established that photon fluence-based units are recommended to be used for determining kinetic of photochemical and photobiological processes using both mono- and polychromatic UV since all photochemical events should be independent with the rate of such events being proportional to the rate of photon absorption. Although the use of fluence-response is also correct, considering "action spectrum is a plot of a relative biological or chemical photoresponse ($=\Delta y$) per number of incident (prior to absorption) photons, vs wavelength" [38], the use of photon fluence is recommended in future investigations. The possibility of using 285 nm rather than 265 or 254 nm is also important considering the greater energy efficiency for higher wavelengths but remains to be better evaluated considering the higher UV fluence requirements.

Although there is not much work done on the inactivation of viruses at 265 and 285 nm, these results provide interesting insight for future research aimed at simultaneous control of environmental spread of ARGs and inactivation of viruses. Overall, concentration of plasmids as measured by qPCR assays never reached LOD at 666.6 copies/mL (corresponding to 1 copy/1.5 μL) for 265 nm and 285 nm. The greatest reduction was observed using 265 nm for the 601 bp qPCR assay reducing from 3.5×10^{12} to 1.1×10^{6} copies/mL. Transformation, on the other hand, reached no detection threshold after 100 and 186 mJ/cm^2, for 265 and 285 nm respectively, albeit different initial plasmid concentration. Since a direct comparison was not possible, the plotted DNA damaging rates as shown in Fig. 6 to enable comparison of treatment efficiency independently of initial DNA load.

It must be noted that the damage inflicted by LP mercury UV lamps emitting radiation at 254 nm is specific to DNA since these are the only classes of biomolecules that absorb most UV at this wavelength [2]. Since the absorption spectrum of protein peaks around 280 nm, this wavelength could prevent DNA repair by destroying the repair enzymes. Contrary to the repair of DNA damage associated with LP mercury UV lamps [39], it has been demonstrated that the damage induced by high intensity polychromatic medium pressure UV lamps is difficult to repair [40]. Although medium pressure lamps are polychromatic, their peak intensities are dependent on the emission properties of mercury [12] and therefore allow limited control over the selection of emission wavelength(s). An exceptional feature of UV-LEDs compared with conventional UV lamps is that UV-LED-based systems can incorporate LED array(s) of different UV wavelengths that enable designing custom built systems to achieve optimum effect for a specific and/or for a wide-ranging target [12]. For example, considering the unique advantage of combination different UV wavelengths, a careful selection of wavelength combinations could be highly useful for achieving greater and irreversible DNA damage. A review article by Song [16] have reported the efficacy of different wavelength UV-LED for conventional disinfection indicating the need to comprehensively investigate microbial response to different wavelengths and wavelength combinations to achieve maximum advantage of their unique features. Likewise, it is important to investigate other UV-LED wavelengths including wavelength combinations for the inactivation of ARGs with a special focus on both reducing repair potential and energy requirements.

4. Historical developments in UV-LED technology and future recommendations

Significant improvements in output power and external quantum efficiency (EQE) (ratio of the number of photons emitted from the LED to the number of electrons passing through the device) as well as reduced capital cost, which are some of the major factors restricting the large-scale implementation, have occurred over the last decade. For example, the EQE of UVC-LEDs have increased to ˃10% from a much lower value of ˂1% in 2007 [41]. Moreover, considerable improvements in optimization of the reactor design have been made resulting in increased UV-LEDs applications although primarily for point-of-use applications. A first full-scale application (3.15 L) of UV-LEDs (275 nm) was recently reported for MS2 inactivation with levels of inactivation (0.5–3.9-log) comparable to mercury-based UV lamps [42]. The authors recommended identifying specific UV-LEDs disinfection applications to encourage innovation and development in this field. Damaging ARGs by 265 nm and 285 nm UV-LEDs has been demonstrated in this investigation. Within this perspective, a greater focus on the use of combination of specific and most effective wavelengths, improved reactor design and improved energy efficiency is recommended. The developments in the UV-LED technology over the last several years are encouraging both in terms of economic viability and process efficiency paving the way for large-scale UV-LED applications that may initially be limited for very specific purposes. Considering these rapid developments, the technology is expected to be economically viable by 2023 [43]. A detailed outlook of the future of UV-LEDs can be found in published review articles [5,43].

5. Conclusions

UV-LEDs, a mercury free source of UV irradiation, demonstrated a quicker degradation of DNA with 265 nm compared with 285 nm, as measured with transformation as well as with qPCR analysis. The largest amplicon showed the greatest DNA damage for both wavelengths. A 3.3-log reduction in the transformation required 40 mJ/cm^2 using 265 nm whereas only 1.3-log was achieved using 285 nm. Increasing the fluence to 100 mJ/cm^2 led to $>$ 4-log reduction in transformation for 285 nm whereas no detection threshold was reached for 265 nm UV-LEDs. Using a UV fluence of 186 mJ/cm^2, more than 4-log reduction in both qPCR (for 601 bp) and transformation assays was achieved with both 265 nm and 285 nm UV-LED wavelengths. It can therefore be inferred that this UV fluence could be used to simultaneously achieve 4-log reduction of ARGs in addition to viruses and parasites for which this norm was set – a finding of great practical importance. The damage to DNA quantified by qPCR was 2.3-fold lower than the loss in transformation efficiency even for the largest studied amplicon. This knowledge may be used for using qPCR in routine analysis to determine UV treatment effect. Overall, the findings of this study demonstrate the potential of UV-LEDs, both at 265 nm and 285 nm, in successfully damaging the DNA for controlling the environmental transformation of ARGs. However, future research on how the water matrix impact the efficiency of UV-LEDs in damaging DNA need to be conducted. Moreover, further work on the combination of low and high UV-LED wavelengths should be carried out to investigate the potential of repairable DNA damage and improve UV treatment protocols.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.105260.

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