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Decreased efficacy of UV inactivation of *Staphylococcus aureus* after multiple exposure and growth cycles

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ABSTRACT

UV disinfection is a relatively simple and cost-efficient disinfection method, especially for in-home greywater treatment. In this study, a bench scale experiment was performed using a LED collimated UV-C beam with a peak wavelength of 256 nm to determine if potentially pathogenic bacteria such as *Staphylococcus aureus* may become enriched in a semi-recirculating greywater system with UV as the sole disinfection step. A statistically significant ($P < 0.001$) decreasing trend in UV-C efficacy was observed between the 1st and 6th UV exposure-growth cycles of *S. aureus* (ATCC 25923), resulting in a 1.5 decrease in $\log_{10}$ removal ($P < 0.00000$) by the 5th iteration. An eleven-point dose-response curve of the 7th iteration of *S. aureus* was estimated and compared to the dose-response curve of the original strain; due to a longer apparent shoulder period and a decay constant of lesser degree, the dose required for a 4-log reduction of the enriched *S. aureus* was estimated to be ~1.9 times greater (22.0 mJ·cm^{-2} versus 11.8 mJ·cm^{-2}). However, experimental results with *S. epidermidis* (ATCC 12228) and two wild strains, *S. aureus* and *S. warneri*, exhibited no trend of increased resistance. UV doses exceeding 20 mJ·cm^{-2} are generally sufficient in achieving a 4-log reduction of bacteria in drinking water systems; however, the results exhibited in this study suggest that when recirculation is involved, there may be a need for UV doses exceeding what is necessary for a 4-log reduction to suppress the enrichment of strains which could pose a public health risk.

1. Introduction

Increasing population growth in relatively water-scarce regions along with an increase in personal water consumption have greatly contributed to the urban water deficit faced around the world (Schiermeier, 2014). The concept of reusing greywater is becoming more popular; greywater, domestic household wastewater without input from the toilet, is a valuable commodity, which can be utilized to reduce domestic potable water usage. Typical greywater treatment systems may involve some form of pre-treatment with filtration, then secondary/tertiary treatment by chemical (e.g. chlorine disinfection) (Al-Gheethi et al., 2015), biological (e.g. membrane bioreactor) (Atasoy et al., 2007), or physical processes (e.g. ultra violet irradiation) (Friedler and Gilboa, 2010). This study focuses on some of the considerations of ultra violet (UV) irradiation as a disinfection process that is common for greywater treatment. UV irradiation is an attractive disinfection method due to its lack of disinfection bi-products and relatively low operational and maintenance requirements. However, as with most disinfection technologies, UV has its disadvantages; in particular, when bacteria are stressed they have the potential to adapt resistance to the stressor(s) (Friedler et al., 2011; Gayán et al., 2014; Jiang et al., 2016). Certain bacteria may exhibit various mechanisms, such as light and dark repair mechanisms for repairing damaged cell membranes and cellular components (Masschelein, 2002; Nebot Sanz et al., 2007). Additionally, damage to cell membrane (Pigeot-Rémy et al., 2012) may lead to mutagenesis resulting in vertical and subsequent horizontal gene transfer from resistant bacteria (Alcántara-Díaz et al., 2004; Rameš et al., 1997) and/or adaptation by upregulation of UV quenching molecules (Davies-Colley et al., 2007) from within an existing genome. Given the intrinsic resistance seen in other Gram-positive bacteria (Williams et al., 2007), potentially pathogenic staphylococci may be a public health concern in this context. With greywater reuse, especially in a semi-continuous loop system, adaptation is possible, such as DNA repair following damage caused by UV (Friedler et al., 2011). Adaptation is problematic, as pathogens may become more resistant to the disinfection method than the surrogates used to measure treatment performance, leading to an unrecognized increase in public health risk.

Previous studies investigating the effects of repeated UV exposure-growth cycles have used *E. coli*, which report some degree of decreased

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UV efficacy over the course of several cycles of UV exposure and growth (Alcántara-Díaz et al., 2004; Ewing, 1995; Wright and Hill, 1968). No study was identified that examined the effects of multiple exposures of UV-C towards Gram-positive wastewater bacteria (such as staphylococci or enterococci), however, data exists for increased resistance of *Enterococcus faecalis* following prolonged sunlight exposure (Hartke et al., 1998). Gram-positive cells tend to be more intrinsically resistant to UV stress (Gehr et al., 2003; Williams et al., 2007), and as such potential increases in resistance may be problematic for treatment systems using UV irradiation.

Each specific wastewater presents different contaminants, both chemical and biological, which require varying levels of reduction. Greywater presents particular challenges for treatment; unlike municipal wastewater, enteric pathogens are not the only organisms of concern (Zimmerman et al., 2014); skin bacteria such as staphylococci are documented as a consistently prevalent genus present in greywater (Gross et al., 2007; Linden et al., 2012; Zimmerman et al., 2014). *Staphylococcus aureus*, which colonizes some 30% of humans (Plano et al., 2011) is a potentially pathogenic *Staphylococcus* species capable of causing systemic infections including bacteremia, pneumonia, endocarditis, and osteomyelitis (Lowy, 1998). Most staphylococci grow on human skin; this makes the treatment and reuse of household greywater potentially problematic as skin-pathogenic bacteria may survive disinfection and return to the host (humans), where they may cause infection. Hence, the aim of this work was to investigate if a recirculating UV disinfection scenario may theoretically facilitate enrichment of UV resistant staphylococci which may be pathogenic or represent a model for other skin pathogens.

2. Materials and methods

2.1. Microbiological components

Two *Staphylococcus* strains obtained from the American Type Culture Collection (ATCC) and two wild *Staphylococcus* isolates obtained from a hand-rinse sample from one of the authors were used as test strains. *S. aureus* (ATCC 25923) and *Staphylococcus epidermidis* (ATCC 12228), as used in Shoults and Ashbolt (2017a), were selected as test organisms for this experiment based on their high skin prevalence on humans (Coates et al., 2014) and relatively high reporting of *S. aureus* and total staphylococci concentrations in raw greywater (Gross et al., 2007; Linden et al., 2012; Zimmerman et al., 2014).

A VITEK™ (2 COMPACT) was used to verify the genera and species of two suspected hand-rinse sample staphylococci isolates, which were confirmed to be *S. aureus* and *Staphylococcus warneri*. *S. aureus* (ATCC 25923) was confirmed by VITEK2. Upon VITEK2 phenotyping, *S. epidermidis* (ATCC 12228) was categorized as *Staphylococcus lentus*, as depicted in Shoults and Ashbolt (2017b); further confirmation by MALDI-MS VITEK confirmed the strain to be *S. epidermidis* (ATCC 12228). MS2 bacteriophage (ATCC 15597-B1) was used as a control specimen with *Escherichia coli* (ATCC 15597) as host cells.

2.2. Experimental setup

Overnight cultures of test strains were grown in tryptic soy broth (TSB). Once incubated, the cell broths were diluted to 1:100 in sterile deionized (DI) water within a sterile 60 mm Petri plate containing a sterile 5 mm × 2 mm stir bar and magnetic mixer operating at 400 rpm to facilitate mixing without a vortex forming. DI water was used in order to minimize potential contact with residual chlorine, which could have an inactivation effect on the cells (Zyara et al., 2016). A control using sterile DI water was performed to investigate any loss in viability due to osmotic pressure change. However, no significant difference in $\log_{10}$ CFU count was observed from 100 s after pipetting from the TSB liquid medium (the time needed to dilute the cells to a countable dilution) to 20 min (the estimated maximum time cells might be suspended in DI water throughout the experiment) (P = 0.286 & 0.289; for *S. aureus* and *S. epidermidis*, respectively). An AquaSense Pearl Beam collimated LED UV reactor (Florence, KY USA) with a peak wavelength of 256 nm and a half bandwidth of 11.5 nm was used to deliver 256 nm UV-C irradiation to target bacteria suspended in water using an adapted EPA protocol (United States Environmental Protection Agency, 2006). Equation (1) was used to calculate the effective intensity (E_{ave}) of the collimated beam based on measurable variables (NSF International, 2014):

$$E_{ave} = 0.98\left[\frac{E_0}{L}\left(\frac{(1-A)^L - 1}{\ln(1-A)}\right)\right] \quad (1)$$

The incident intensity (E_0) was measured using a NSF certified radiometer (UVP Radiometer, Model UVX-25), calibrated for a peak wavelength of 254 nm. The water height (L) was measured to 1 cm (28.3 mL in a 60 mm cylindrical Petri dish), and a spectrophometer (Thermo Scientific Genesys 10S UV-VIS) was used to measure the absorbance (A_{256} nm) in the solution. The resulting E_{ave} was then multiplied by the exposure time (seconds) in order to calculate the resulting dosage in mJ·cm^{-2}. Doses varied slightly between exposure cycles; see supplementary information for absorbance, collimated beam intensity, and dosing data.

Prior to irradiating the sample, 100 µL of the sample was plated on tryptic soy agar (TSA) plates in triplicate at the appropriate dilution to effectively measure colony forming units (CFU) of the control. The collimated beam was placed over the Petri dish containing the 1:100 DI suspension of the target bacteria which was then irradiated to the specified dose; samples were diluted appropriately and 100 µL was plated on TSA plates in triplicate to assay the remaining CFU; with the $\log_{10}$ reduction based on the CFU per 100 µL count of the control at 0 mJ·cm^{-2} according to Equation (2):

$$\mathrm{Log}_{10}Reduction = Log\left(\frac{\frac{CFU\ Control}{100\,\mu L}}{\frac{CFU\ Dosage}{100\,\mu L}}\right) \quad (2)$$

This experimental sequence was performed as many as six cycles per strain. A consistent dose was used for each UV exposure-growth cycle to determine if there was a decreasing trend in $\log_{10}$ reductions. *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) were exposed to 11.8 mJ·cm^{-2} and 17.0 mJ·cm^{-2}, respectively, which are the estimated doses required for a 4-$\log_{10}$ reduction of each (Shoults and Ashbolt, 2017a). *S. aureus* (wild) and *S. warneri* (wild) were both exposed to subsequent doses of 15, 30, and 40 mJ·cm^{-2}; these doses were used to explore the potential effects of bacterial selection by UV doses that might be used in a treatment system, with 40 mJ·cm^{-2} and 16 mJ·cm^{-2} being the minimum UV doses for Class A and Class B UV treatment systems, respectively (NSF International, 2014). Following irradiation, a 200 µL aliquot of the irradiated sample was transferred to 5 mL TSB and incubated for 24 h to allow for growth of the surviving cells. The experiment was then repeated on a suspension containing the newly grown bacteria solution.

2.3. Dose-response curve

An 11-point test was performed on the 7th iteration of *S. aureus* (ATCC 25923) (denoted as *S. aureus*^{7th} from this point forth) in accordance with NSF's ultraviolet microbiological water treatment systems document (NSF International, 2014). Relative doses (0, 15, 30, 45, 60, 75, 90, 105, 120, 135, and 150%) based on the estimated dose required for a 4-log reduction (e.g. 15% of 21.3 mJ·cm^{-2} = 3.2 mJ·cm^{-2}) were assayed to determine a dose-response curve for *S. aureus*^{7th}; the dose response curve was compared to that of *S. aureus* (ATCC 25923).

2.4. Control experiments

Two additional control experiments were performed to ensure homogeneity with literature as well as consistency throughout the experiments. An 11-point dose-response curve using MS2 bacteriophage was performed using the above protocol and assayed appropriately (EPA, 2001); this curve was then compared to a 2006 UV-C study (Liu and Zhang, 2006) performed using MS2 and was found to be within the same $\log_{10}$ reduction range (See Figure S1 for graph comparison). To ensure absorbance did not change enough between experiments to significantly alter the doses and confound the results, a one-way ANOVA analysis was performed; the hypothesis that the average irradiances (using Equation (1) as a function of absorbance) differed between days was rejected ($P = 0.629$).

3. Methodological exceptions

The experimental procedure for *S. aureus* (wild) and *S. warneri* (wild) had the following difference from the protocol above: overnight TSB cultures were centrifuged to form pellets which were re-suspended in a sterile 0.85% NaCl buffer, vortexed, then pipetted into a petri dish containing NaCl buffer for UV irradiation. For each trial, 100 µL samples were taken to undergo serial dilutions and CFU assays before and after exposure. The rational for making this procedural adjustment was to remove the TSB to have a higher concentration of suspended cells without the presence of TSB increasing the turbidity.

3.1. Statistical analyses

SigmaPlot (Version 13.0, Systat Software, Inc., San Jose, CA) was used for all statistical analysis. Paired t-tests were used for comparing mean data, and two-tailed p-values are reported. Linear regression analysis was used to analyze the presence of potential resistance trends. All reported statistics passed Shapiro-Wilk normality test.

3.2. Considerations for future UV protocols

Equation (1), which is intended for monochromatic UV lamps, does not account for other factors (such as divergence and petri factors) which may contribute to a lower/higher effective dose (Bolton and Linden, 2003). There is a collective need for collimated beam studies to use one protocol to maintain consistency between studies (Bolton and Linden, 2003).

4. Results and discussion

The following results are a theoretical account of an in-home water recirculation scenario. While this study was not conducted using real greywater, the results are important to consider when designing household water treatment systems. However, without running a wet study examining greywater from recirculating greywater systems over the course of several weeks, it is difficult to determine the extent to which the following results should be considered when designing such systems.

4.1. Iterative UV-C inactivation of ATCC strains S. aureus and S. epidermidis

The results of UV irradiation delivered to *S. aureus* at a dose of $11.8\ mJ{\cdot}cm^{-2}$ over six UV exposure-growth cycles are displayed in Fig. 1a. A regression analysis indicated a declining trend in $\log_{10}$ reduction ($P < 0.001$). While it is difficult to say what the mechanism(s) causing *S. aureus* resistance may be, it is important to note that Gayan et al. (Gayán et al., 2014) reported intraspecific variation in UV resistance amongst *S. aureus* strains of 1.3 fold when exposed to UV-C at 254 nm. Given the single strain (ATCC 25923) used in the current

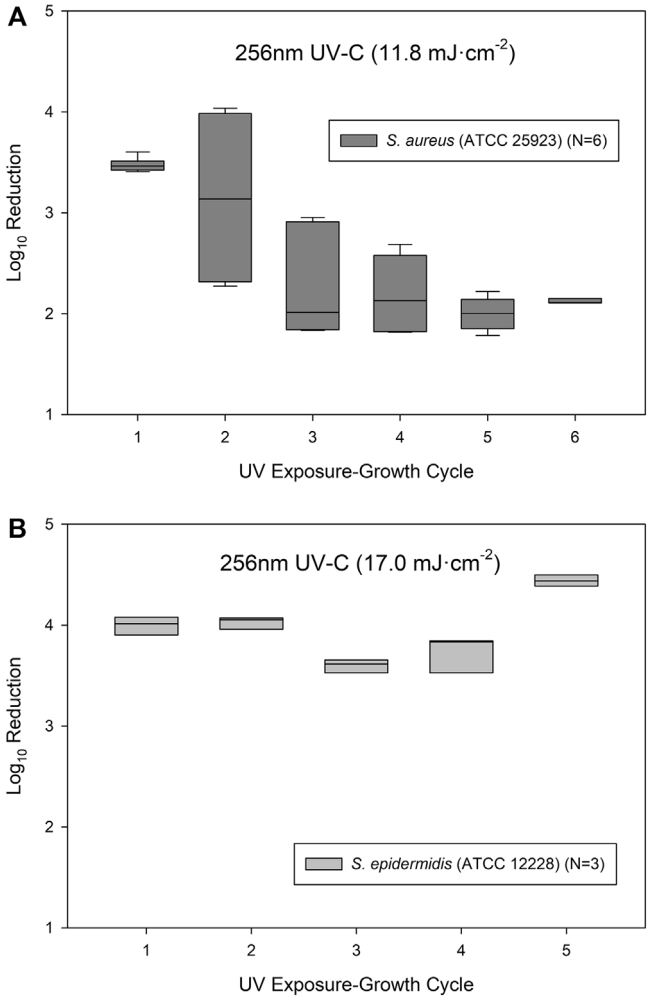

Fig. 1. Cyclic growth-irradiation of bacteria using 256 nm UV-C (**a**) *S. aureus* (ATCC 25923) at $11.8\ mJ{\cdot}cm^{-2}$ ($N = 6$); (**b**) *S. epidermidis* at $17.0\ mJ{\cdot}cm^{-2}$ ($N = 3$).

study, however, there appears to be a significant trend of increasing UV resistance within the surviving population. The higher variability of the second and third exposure iteration shown in Fig. 1a can be explained by the two separate runs of the experiment. The first experimental trial yielded a significant difference in $\log_{10}$ reduction between the first and second exposure iteration while in the second trial a similar change in reduction was not observed until the third iteration, resulting in the large variation in the combined data in Fig. 1a. The variation in results between the two trials suggests a randomization effect may play a role in the adaptation of cells. While there was no significant change in reduction between the third and sixth cycle ($P = 0.260$), Fig. 1a depicts a narrowing margin of the results as cycles continue; this may suggest an increasing homogeneity of the bacteria as more cycles are completed. The results of UV irradiation delivered to *S. epidermidis* at a dose of $17.0\ mJ{\cdot}cm^{-2}$ are displayed in Fig. 1b; a regression analysis rejected the hypothesis that a declining trend in $\log_{10}$ reduction existed over the five iterations of the experiment ($P = 0.315$). While it cannot be concluded that *S. epidermidis* lacks the adaptive mechanisms that *S. aureus* appears to exhibit, *S. aureus* appeared to be more readily enrichment for UV-resistance.

This study emphasizes the need for sufficient UV dosing to ensure adequate reduction of bacteria; the United States Environmental Protection Agency (2012) suggests a dose of $100\ mJ{\cdot}cm^{-2}$ for UV inactivation suitable for water reuse. With such doses, the increased resistance exhibited by *S. aureus* (ATCC 25923) may not be a factor;

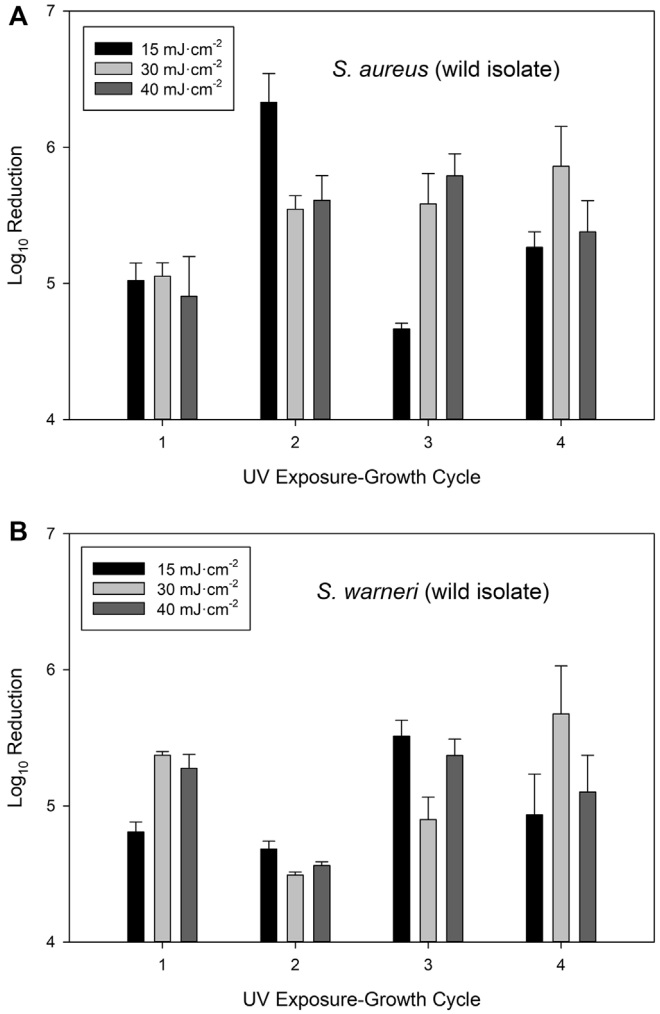

Fig. 2. Cyclic growth-irradiation of bacteria at 256 nm UV-C doses of 15, 30, and 40 mJ·cm^{-2} (**a**) *S. aureus* (wild isolate) (N = 3); (**b**) *S. warneri* (wild isolate).

however, it is important to remain vigilant as we progress into an era where water reuse becomes more available and necessary. It is important to consider the implication of semi-continuous water cycles, as might be experienced in a recirculating shower for example, and how treatment needs may differ from conventional water treatment.

4.2. Iterative UV-C inactivation of wild S. aureus and S. warneri isolates

The two wild staphylococci, *S. aureus* and *S. warneri* were examined to determine if the resistance trend exhibited in Fig. 1a would be consistent with wild isolates. As shown in Fig. 2, neither the wild *S. aureus* nor *S. warneri* exhibited increased resistance after four UV exposure-growth cycles when exposed to 15, 30, or 40 mJ·cm^{-2}. The differing results between *S. aureus* (ATCC 25923) and the wild *S. aureus* isolate may be explained by varying UV stress exposure in parent generations. Silverman and Nelson (2016) reported that bacteria of wastewater origin were significantly more resistant to sunlight UV inactivation when compared to laboratory-grown strains. Although a previous study by Shoults and Ashbolt (2017b) yielded no significant difference in inactivation kinetics between *S. aureus* (ATCC 25923) and *S. aureus* (wild), it is possible *S. aureus* (wild) may have previously developed UV-resistance not present in *S. aureus* (ATCC 25923); frequent exposure to UV sun rays, having been isolated from human skin may have caused a plateau in the ability of *S. aureus* (wild) to increase in resistance. However, caution should be taken when analyzing the data shown in this Fig. 2, as four cycles may not have been enough to exhibit a trend. Certain bacteria may require more generations of reproduction than others to exhibit environmental adaptation; hence more UV exposure-growth cycles may be necessary to show a significant change in UV resistance, as reported by Alcántara-Díaz et al. (2004).

The lack of any significant inactivation response between 15, 30, and 40 mJ·cm^{-2} for the two wild isolates depicted in Fig. 2 was surprising; while the reason in this study is unknown, the apparent tailing effect from 15 mJ·cm^{-2} to 40 mJ·cm^{-2} may be the result of bacterial aggregation, as the presence of particle shielding can contribute to a multilinear dose response (Mamane-Gravetz and Linden, 2005; Winward et al., 2008). There is little reason to suspect shielding from non-bacterial particles occurred in this study, given the experimental setup; cell shielding by aggregation seems most likely, however, this was not investigated or verified. Additionally, staphylococci are often arranged in pairs and tetrads, which may contribute to shielding effects (Kloos and Schleifer, 1975); additionally, *S. aureus* can naturally aggregate into clusters which may further contribute to shielding effects (Crosby et al., 2016).

The most surprising result was the apparent decrease in resistance exhibited by *S. aureus* (wild) when exposed to 15 mJ·cm^{-2} on the second cycle and *S. warneri* when exposed to 30 mJ·cm^{-2} between the second and fourth cycle. While it's possible this could be attributed to some unknown experimental error, Alcántara-Díaz et al. (2004) reported a similar trend in their experimental trials with *E. coli*; two out of five trials resulted in decreased UV resistance between the 50th and 80th cycles. Mutated strains are known to be favoured over non-mutated strains under some environmental stresses (Blázquerz, 2003; Chopra et al., 2003), however, mutated genes may be deleterious and persist (Charlesworth, 2012). For this reason, it is possible that for a time, deleterious mutants may persist in subsequent generations, decreasing the UV resistance of the strain.

4.3. UV dose-response curve for S. aureus

An 11-point dose-response curve was generated for progeny from the 7th iteration of *S. aureus* (ATCC 25923) (*S. aureus*^{7th}) and was compared to the 11-point dose response curve of the parental *S. aureus* ATCC 25923 strain (to be referred as *S. aureus*^{1st}) generated in a previous study by (Shoults and Ashbolt, 2017a); the estimated dose required for a 4-log reduction of *S. aureus*^{7th} was 22.0 mJ·cm^{-2} which is ~1.9 times higher than the dose required for a 4-log$_{10}$ reduction of *S. aureus*^{1st} (11.8 mJ·cm^{-2}). The first order decay coefficients (k) of the linear portions of the UV dose-response curves for *S. aureus*^{1st} and *S. aureus*^{7th} depicted in Fig. 3 were estimated to be $k_{1st} = -0.45$ ($R^2 = 0.990$) and $k_{7th} = -0.24$ ($R^2 = 0.997$), respectively. *S. aureus*^{7th} had a longer apparent shoulder period, as well as a lower k-value. Firstly, the shoulder effect can likely be attributed to the action of DNA repair mechanisms, requiring multiple hits on a single organism for cell death (Gayán et al., 2014). Secondly, the lower magnitude of the k-value of *S. aureus*^{7th} suggests it is intrinsically more resistant to UV-C (256 nm) irradiation than *S. aureus*^{1st}.

4.4. Mechanisms of UV-induced mutagenesis

Previous UV resistance studies have examined in partial, the adaptive mechanisms present in enriched cells (Alcántara-Díaz et al., 2004); while this study did not attempt to identify such mechanisms, better understanding such mechanisms is important when considering UV irradiation. As with the evolution of all life, many factors play a role in cell adaptation and DNA mutagenesis; thus identifying the exact cause for adaptation from UV stress is difficult. Upon several UV exposure-growth cycles, Alcántara-Díaz et al. (2004) found that *E. coli* had divergent results among five different trials, each totalling 80 cycles, all starting from the same parent strain. While each of the five trials

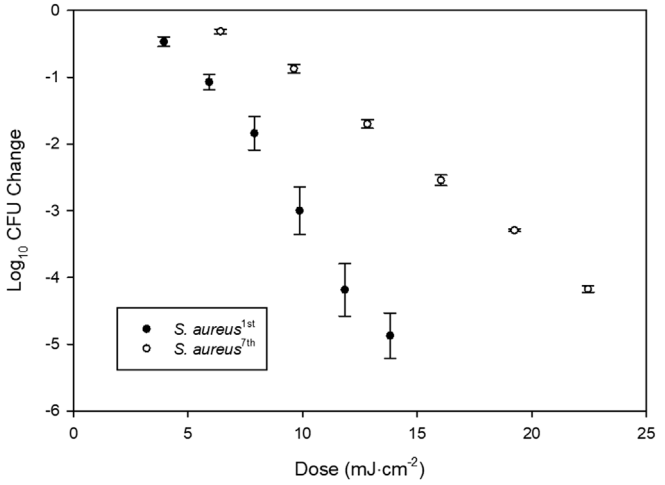

Fig. 3. 11-point dose response curves of *S. aureus*^{1st} and *S. aureus*^{7th} when exposed to 256 nm UV irradiation.

exhibited significant increases in UV resistance, the degree of resistance varied considerably between trials, showing UV-induced mutagenesis is aleatory (Alcántara-Díaz et al., 2004). Alcántara-Díaz et al. (2004) showed that the concept of divergent mutagenesis is possible, and that multiple adaptations are possible from the same stressing agent. While many explanations for UV-induced mutagenesis are possible, a likely explanation can be found in the *recA* protein; Gascón (1995) found that *E. coli recA*$^{+}$ exhibited significantly higher resistance to UV (254 nm) when compared to *E. coli recA*$^{-}$. This is of little surprise, given the function of *recA* in SOS induction when cells face DNA damage (Matic et al., 1995). Since different UV wavelengths cause different stress in microorganisms (Eischeid and Linden, 2011), polychromatic UV sources may cause multiple DNA mutagenesis responses.

As previously mentioned, it is well documented that particle shielding, both from non-bacterial particles and aggregation of bacteria, can significantly reduce the inactivation effects of UV irradiation. The observed increase in resistance of *S. aureus* (ATCC 25923) may be attributed to a UV-induced aggregation of the bacteria examined; Kollu and Ormeci (2015) observed an increase in particle size following UV exposure, which was attributed to UV-induced self-aggregation of *E. coli*. Here we postulate the possibility of a decreased bacterial response time and/or a greater degree of UV-induced aggregation upon cyclic UV exposure-growth cycles.

4.5. Total staphylococci as UV-treatment surrogate for greywater

As we have previously discussed (Shoults and Ashbolt, 2017a), total staphylococci provide several useful attributes as endogenous treatment surrogates for greywater performance testing. Although the results in Shoults and Ashbolt (2017a) indicated total staphylococci was more susceptive to UV-C irradiation than other traditional faecal indicator microorganisms, they may still serve as a suitable surrogate due to their consistently high concentrations relative to traditional faecal indicator bacteria (FIB) in greywaters (Casanova et al., 2001; Gross et al., 2007; Linden et al., 2012; Zimmerman et al., 2014). In addition, the data analyzed in the current work shows that one important pathogenic member, *S. aureus* may be a more conservative surrogate for representing pathogenic risk than previously thought, due to the potential for enrichment in a recirculating UV disinfection system. When compared to the dose required for a 4-$\log_{10}$ reduction of *S. aureus*^{1st} (11.8 mJ·cm^{-2}), the dose required for a 4-$\log_{10}$ reduction of *S. aureus*^{7th} (21.8 mJ·cm^{-2}) was more similar to the doses required for a 4-$\log_{10}$ reduction of *E. coli* (ATCC 13115), *Enterococcus faecalis* (ATCC 29212), and *Enterococcus casseliflavus* (ATCC 9199), which were 20.4, 25.6, and 22.1 mJ·cm^{-2} respectively (Shoults and Ashbolt, 2017a). In addition to total staphylococci being reported as 3 to 5-$\log_{10}$ higher in abundance than traditional FIB in untreated laundry greywater (Zimmerman et al., 2014), the potential of increased resistance of *S. aureus* as described by the current study may cause total staphylococci to be a more conservative surrogate for treatment performance than previously thought. However, this increased resistance may be confounded by the potential selection of other enteric bacteria with some capability of growth within the greywater system (Friedler and Gilboa, 2010; Jahne et al., 2017) which have been consistently shown to be able to adapt over several UV exposure-growth cycles (Alcántara-Díaz et al., 2004; Ewing, 1995; Wright and Hill, 1968).

5. Conclusions

Given *S. aureus* can theoretically become enriched in a circulating system with UV as the disinfection process, further research is needed to determine the scale to which the results shown in this study may apply in practice, and particularly so in greywater treatment recirculating systems. To better understand the effects of staphylococci as a surrogate for treatment performance in a greywater reuse system, there is a need to better understand the levels of adapted bacteria (both staphylococci and enteric bacteria such as *E. coli*) being reintroduced into the cycle. There is also a collective need to better understand the mutagenic mechanisms present in bacteria which make the observed trend possible.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijheh.2018.08.007.

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