

Hydrogen peroxide, sodium dichloro-s-triazinetriones and quaternary alcohols significantly inactivate the dry-surface biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa* more than quaternary ammoniums

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Abstract

Globally, healthcare-associated infections (HAI) are the most frequent adverse outcome in healthcare delivery. Although bacterial biofilms contribute significantly to the incidence of HAI, few studies have investigated the efficacy of common disinfectants against dry-surface biofilms (DSB). The objective of this study was to evaluate the bactericidal efficacy of seven Environmental Protection Agency (EPA)-registered liquid disinfectants against DSB of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We hypothesized that overall, there will be significant differences among the bactericidal efficacies of tested disinfectants by product type and active ingredient class. We also hypothesized that depending on the species, higher bactericidal efficacies against DSB will be exhibited after 24 h of dehydration compared to 72 h. Wet-surface biofilms of *S. aureus* and *P. aeruginosa* were grown following EPA-MLB-SOP-MB-19 and dehydrated for 24 and 72 h to establish DSB. Seven EPA-registered disinfectants were tested against dehydrated DSB following EPA-MLB-SOP-MB-20. Overall, quaternary ammonium plus alcohol, sodium dichloro-s-triazinetrione and hydrogen peroxide products were more efficacious against DSB than quaternary ammoniums for both tested species. While there was no significant difference in the log₁₀ reductions between 24 and 72 h *S. aureus* biofilms, significantly higher log₁₀ reductions were observed when products were challenged with 24 h *P. aeruginosa* DSB compared to 72 h *P. aeruginosa* DSB. Species type, active ingredient class and dry time significantly impact disinfectant efficacy against DSB of *S. aureus* or *P. aeruginosa*.

INTRODUCTION

Healthcare-associated infections (HAI), a result of diverse interactions among modern healthcare practices, hospital environments and growing antibiotic resistance, among other factors, pose a crucial threat to human well-being [1]. Globally, the acquisition of HAI is the most frequent and adverse outcome in healthcare delivery [2]. In the USA, approximately 633 300 patients are affected by 687 200 HAI [3] with more than 72 000 deaths every year [4]. In Europe, about 4.5 million HAI occur yearly in acute care hospitals [5] with approximately 135 000 deaths [6]. In low- and middle-income countries (LMIC), the density of HAI in adult intensive care units is estimated at 47.9 per 1000 patient days, which is higher than rates in the USA and Europe [7]. Comparing HAI incidence rates in developed and LMIC, the pooled incidence is seven out of 100 patients in developed economies and ten out of 100 in LMIC [8].

The prevalence of HAI has been associated with biofilm formation by bacteria [9] as bacterial biofilms are more persistent and 'resistant' to disinfection than planktonic bacteria [10]; allowing biofilms to present an increased infection risk. Even higher infection risk is presented by dry-surface biofilms (DSB) [11], which are harder to inactivate than wet-surface biofilms (WSB) and planktonic bacteria [12], as desiccation induces the production of denser extracellular polymeric substances (EPS) [13]. The

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Abbreviations: ATCC, American Type Culture Collection; CDC, Centre for Disease Control; DSB, dry surface biofilms; EPA, Environmental Protection Agency; EPS, extracellular polymeric substances; HAI, healthcare-associated infection; PBS, phosphate buffered saline; TSB, tryptic soy broth; WSB, wet surface biofilms.

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Table 1. Active ingredients, concentrations, mode of use and contact times for disinfectants tested in this study

Disinfectant product*	Disinfectant active ingredient(s)†	Dilution at use	Active level at use‡	Label contact time (mins)§
CL	48.21% sodium dichloro-s-triazinetriene	RTU	4306 ppm	4
SH	0.39% sodium hypochlorite	RTU	0.39%	1
HP1	0.5% hydrogen peroxide	RTU	0.5%	1
HP2	0.5% hydrogen peroxide	RTU	0.5%	1
QA1¶	0.25% n-alkyl (68% C ₁₂ , 32% C ₁₄) dimethyl ethylbenzyl ammonium chloride 0.25% n-alkyl (60% C ₁₄ , 30% C ₁₆ , 5% C ₁₂ , 5% C ₁₈) dimethyl benzyl ammonium chloride 55% isopropanol	RTU	0.5%**+55%	2
QA2	0.76% didecyl dimethyl ammonium chloride 15% isopropanol 7.50% ethanol	RTU	0.76%**+22.5%	1
QT¶	0.14% n-alkyl (68% C ₁₂ , 32% C ₁₄) dimethyl ethylbenzyl ammonium chloride 0.14% n-alkyl (60% C ₁₄ , 30% C ₁₆ , 5% C ₁₂ , 5% C ₁₈) dimethyl benzyl ammonium chloride	RTU	0.28%	3

*Abbreviated naming scheme for commercially available EPA-registered disinfectants used in this study.

†Ready-to-use (RTU) concentration.

‡Active ingredient concentration after dilution.

§Defined label contact time.

||Tablet, RTU concentration prepared following manufacturer guidelines.

¶Liquid expensed from RTU disinfectant wipes.

**Total quaternary.

National Institute of Health (NIH) of the USA estimates that about 80% of all chronic infections are due to biofilm formation [9]. Bacterial biofilms are comprised of microbial cells adhered to a surface and to each other, forming a micro-colony encased in a polysaccharide dominant matrix [14]. In addition to the cells inhabiting the biofilm, DNA, proteins and biosurfactants are also prevalent [15]. Persistence of bacterial biofilms on environmental surfaces is due to their ability to adhere to common surfaces and produce EPS [16]. The EPS forms a matrix that presents a major barrier to removal from surfaces in healthcare facilities as it shields underlying bacterial cells from direct contact with disinfectants used for their inactivation [17, 18]. Biofilm thickness likely influences several factors such as the disinfectant ability to penetrate the biofilm matrix [19], the water-binding characteristic of the EPS matrix [14], and pH differences among various layers of biofilms [20]. While WSBs could vary in terms of thickness compared to artificially produced DSB or DSB collected from environmental samples, a depth of 31 µm of laboratory-developed DSB models represents a reasonable compromise between the thickness of biofilms collected from hard surfaces (i.e. 26.5 µm) and soft clinical surfaces (i.e. 45.6 µm) [21]. Considering biofilm thickness also influences biofilm architecture, this may result in aggregation of organic acids leading to the deactivation or reduced performance of disinfectants [20]. While these laboratory-grown models offer a closer representation of clinical biofilms, there is still limited work evaluating the efficacy of disinfectants in removing biofilm formed on dry clinical surfaces at different dehydration time points under near room-temperature conditions.

DSB are particularly widespread on surfaces in healthcare facilities [12, 22, 23]. In a recent study, Ledwoch *et al.* detected DSB on 95% (58/61) of environmental samples collected from hospitals in Wales [24]. The samples investigated included commodes, clipboards and sanitizing bottles [24]. DSB have also been detected on indwelling catheters [25]. Although multi-species DSB have been detected on a range of surfaces in healthcare facilities, major HAI pathogens as *S. aureus* [24] and *P. aeruginosa* are reportedly predominant [26, 27].

While DSB are widespread on surfaces in healthcare facilities, prolonged desiccation and starvation of bacteria in DSB leads to an overall increase in the percentage of protein content and slightly decreased carbohydrate content compared to WSB [21, 28]. Being the principal component of biofilms, this increase in the proportion of proteins may further contribute towards the reduced bactericidal efficacy of disinfectants against DSB [29]. Furthermore, metabolic changes resulting from cell-to-cell signaling, nutrient recycling within the matrix and transformation of lysed cells may contribute towards the prolonged survival of biofilms [30].

In the current protocol used by the Environmental Protection Agency (EPA) for biofilm claims on disinfectants, WSB established with species of *S. aureus* and *P. aeruginosa* are the required test pathogens [31]. Under real-world conditions such as in healthcare facilities, disinfectants are relied on to inactivate bacteria on dry surfaces to curb HAI incidences [32], which are usually in

the form of DSB [21]. Despite widespread evidence that bacteria in healthcare environments are more likely to be encased in DSB, the standard test for disinfectant efficacy testing and registration with the EPA are conducted using planktonic bacteria or bacteria in wet biofilms, and very few studies have demonstrated bactericidal efficacy of disinfectants against DSBs [12, 33]. To the best of our findings, no studies have evaluated the bactericidal efficacy of disinfectants against DSB of *S. aureus* and *P. aeruginosa* established at different dehydration time points consistent with routine cleaning and disinfection schedules (daily or three times/week) recommended by the CDC [34]. In a previous study by our group, Nkemngong *et al.* developed a rapid model for establishing DSB of *S. aureus* and *P. aeruginosa* at different time points (24–120 h) and at mean \log_{10} densities sufficient for disinfectant efficacy testing [13]. In this study, we evaluated the bactericidal efficacy of seven EPA-registered liquid disinfectants against DSB of *S. aureus* and *P. aeruginosa* after 24 and 72 h of dehydration following EPA-SOP-MB-20 [35]. We hypothesized that overall, hydrogen peroxide, sodium dichloro-s-triazinetrione and quaternary ammonium compounds plus alcohol (quaternary alcohol) disinfectants will be more bactericidal against DSB than quaternary ammonium disinfectants based on our prior work [36]. We also hypothesized that overall, we will observe higher bactericidal efficacies against 24 h DSB compared to their 72 h counterparts.

METHODS

Bacterial species and disinfectants tested in this study

DSB of *S. aureus* ATTC-6538 and *P. aeruginosa* ATCC-15442 were established on borosilicate glass coupons (1.27 ± 0.013 cm; Biosurface Tech) following Nkemngong *et al.* [13]. These species were selected as they are the standard bacterial species of choice for disinfectant efficacy testing [31]. They are also the standard EPA species for registering disinfectants with claims against WSB [35].

DSB establishment on borosilicate glass coupons

Initially WSB were established following EPA-MLB-SOP-MB-19 through batch and continuous stir tank reactor (CSTR) phases [35]. The batch medium was 3.0 g l^{-1} Tryptic Soy Broth (TSB) for *S. aureus* and 300 mg l^{-1} TSB for *P. aeruginosa*. A 500 ml batch medium held in a CDC biofilm reactor (Biosurfaces Technologies, Bozeman, MT, USA) was inoculated with 1 ml of an overnight culture of *S. aureus* or *P. aeruginosa*. The batch phase lasted 24 ± 2 h within the CDC biofilm reactor (Biosurfaces Technologies,

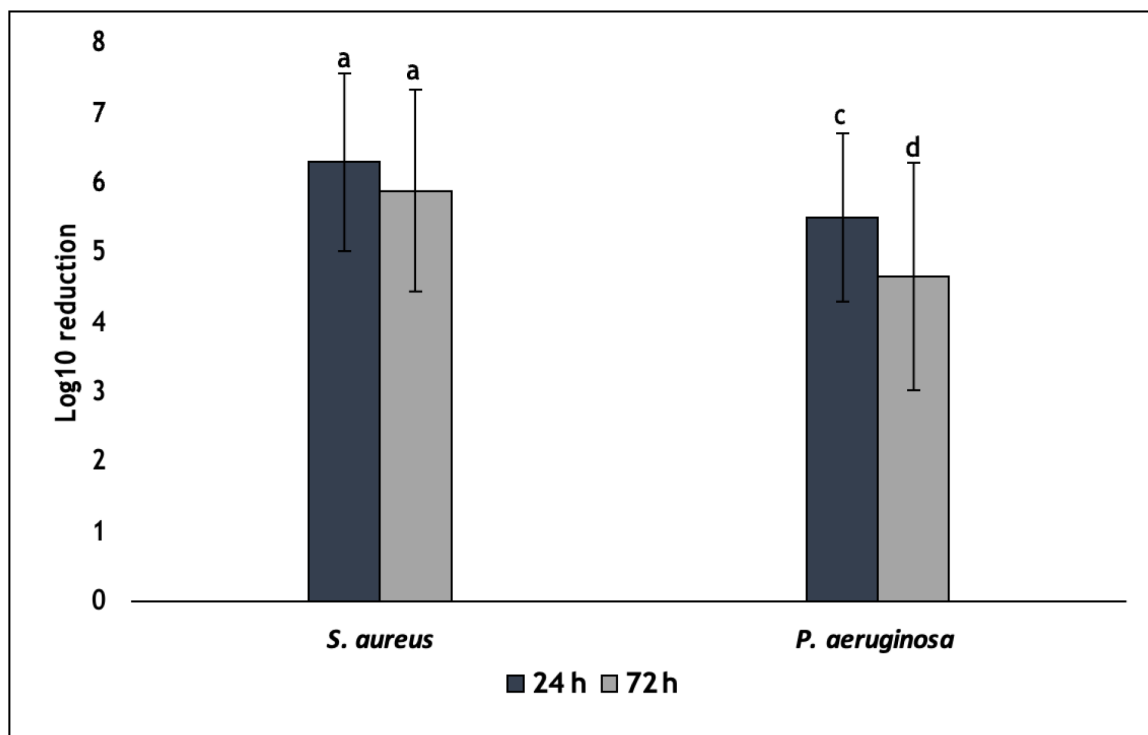


Fig. 1. Overall disinfectant efficacy against DSB of *S. aureus* and *P. aeruginosa* by dry times, i.e. 24 h (dark bars) and 72 h (light bars). Letter a Tukey grouping for disinfectant efficacy against *S. aureus* DSB only. Letters c, d Tukey grouping are for disinfectant efficacy against DSB of *P. aeruginosa* only. Bars with the same letters are not statistically different. Error bars represent standard deviation.

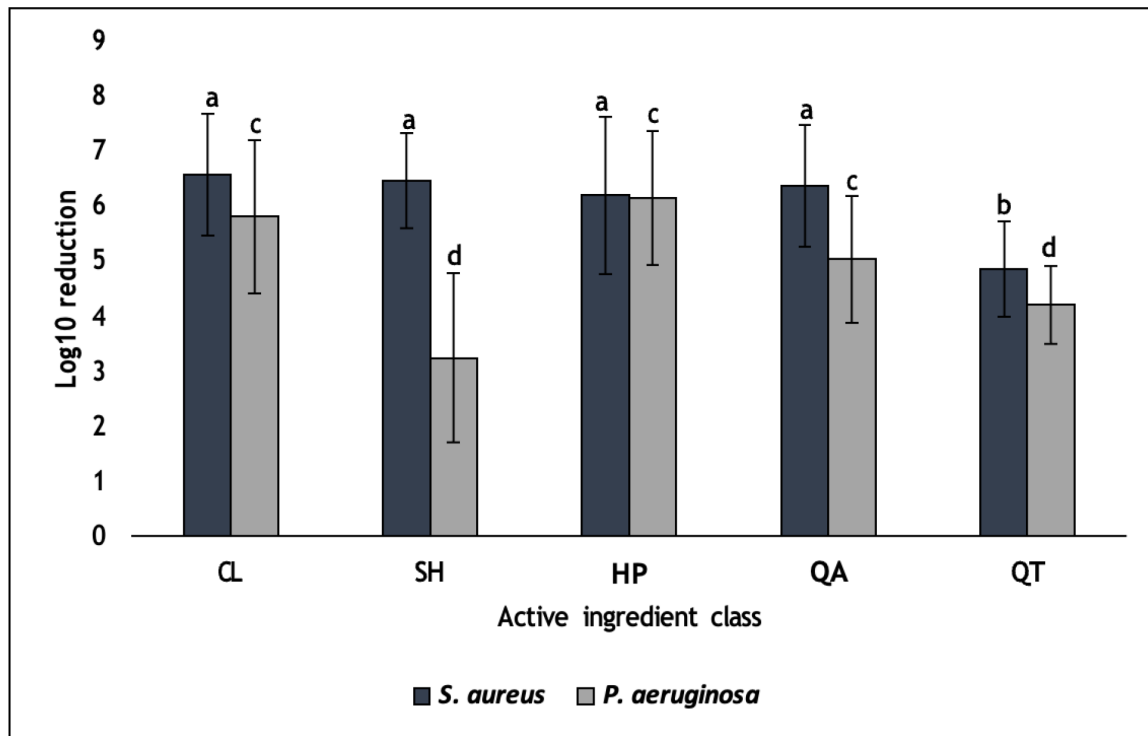


Fig. 2. Disinfectant efficacy against DSB of *S. aureus* (dark bars) and *P. aeruginosa* (light bars) by active ingredient class, i.e. CL, SH, HP, QA and QT. Letters a, b Tukey grouping are for disinfectant efficacy against *S. aureus* DSB only. Letters c, d Tukey grouping are for disinfectant efficacy against DSB of *P. aeruginosa* only. Bars with the same letters are not statistically different. Eight biological replicates were completed for QA and QT products and five biological replicates for CL, SH and HP products; three coupons ($n=3$) were used per control and treatment. Error bars represent standard deviation.

Bozeman, MT, USA) mounted on a magnetic hot plate stirrer (Talboys, Thorofare, NJ, USA) set at 60 ± 5 r.p.m. at 36 ± 1 °C for *S. aureus* or 125 ± 5 r.p.m. at 21 ± 2 °C for *P. aeruginosa*. CSTR medium in 20 l of sterile distilled water had a final concentration of 1.0 g l^{-1} TSB for *S. aureus* and 100 mg l^{-1} TSB for *P. aeruginosa*. CSTR medium was continuously pumped through the CDC biofilm reactor for 24 ± 2 h for both species.

After WSB were established through the batch and CSTR phases, rods from the CDC biofilm reactor; each holding three borosilicate glass coupons were dehydrated for 24 and 72 h at 25 or 21 °C for *S. aureus* and *P. aeruginosa*, respectively. Dry times and dehydration temperatures were informed by Nkemngong *et al.* [13].

Disinfectant efficacy testing against DSB of *S. aureus* and *P. aeruginosa*

At each dehydration time point (24 and 72 h), disinfectants (Table 1) were tested against DSB following EPA-MLB-SOP-MB-20 [35]. At each dry time and for each disinfectant product, three coupons with DSB of *S. aureus* or *P. aeruginosa* were harvested into sterile 50 ml conical tubes. Coupons ($n=3$) after 24 and 72 h of dehydration served as negative controls; controls were treated with 4 ml of PBS with a 1 min contact time. At each of the dry times, three harvested coupons (technical replicates) were independently treated with 4 ml of each disinfectant (Table 1). At the label-defined contact time of each disinfectant product and PBS, 36 ml of neutralizing buffer (1L H₂O+5.2 g Difco neutralizing buffer; Becton, Dickinson and Company Sparks, MD, USA) was added to each treated coupon to stop the disinfectant action. Treated coupons were vortexed and sonicated to release DSB from coupons into solution as described by Nkemngong *et al.* [13]. Post disinfectant treatment, DSB of *S. aureus* or *P. aeruginosa* were vacuum-filtered onto filter membranes (Pall Corporation, NY, USA), whereas negative controls were spread plated following EPA-MLB-SOP-MB-20 [35]. Eight biological replicates were completed for QA and QT products and five biological replicates for CL, SH and HP products as informed by Lineback *et al.* [36].

Statistical analysis

Log₁₀ reductions resulting from the treatment of coupons with DSB were calculated and used for statistical analyses. Specifically, mean bacterial log₁₀ densities per coupon were calculated for disinfectant and PBS-treated coupons. Mean log₁₀ densities per disinfectant-treated coupon were normalized against the mean log₁₀ densities of control coupons to determine log₁₀ reductions.

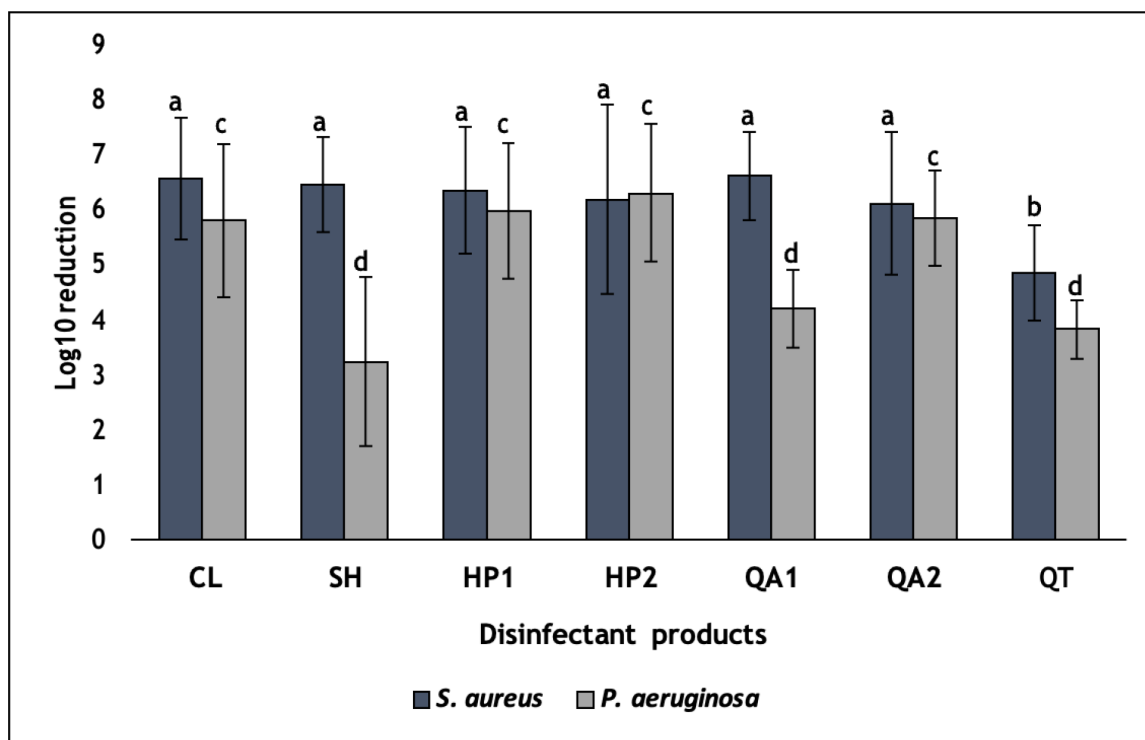


Fig. 3. Disinfectant efficacy against DSB of *S. aureus* (dark bars) and *P. aeruginosa* (light bars) by disinfectant product type (CL, SH, HP1, HP2, QA1, QA2 and QT). Letters a, b Tukey grouping are for disinfectant efficacy against *S. aureus* DSB only. Letters c, d Tukey grouping are for disinfectant efficacy against DSB of *P. aeruginosa* only. Bars with the same letters are not statistically different. Eight biological replicates were completed for QA and QT products and five biological replicates for CL, SH and HP products; three coupons ($n=3$) were used per control and treatment. Error bars represent standard deviation.

The least-squares method of the PROC GLIMMIX procedure was used to analyse and compare mean \log_{10} reductions ($n=70$ per species; $N=140$; $\alpha=0.05$) among the seven tested disinfectant products (CL, SH, HP1, HP2, QA1, QA2 and QT). The same test was used to statistically compare mean \log_{10} reductions by active ingredient class (CL, SH, HP, QA and QT), and dehydration time points (24 and 72 h). Pair-wise comparisons among products, species and dry times were completed with Tukey adjustments. All statistical procedures were completed using SAS version 9.4 (SAS Institute, Cary, NC).

RESULTS

Bactericidal efficacy against *S. aureus* DSB varied by product type and active ingredient class

The average \log_{10} densities of *S. aureus* DSB per coupon pre-treatment after 24 and 72 h dry times were 7.64 ± 0.76 and 7.0 ± 0.89 , respectively. Overall, there were significant differences among the product types and active ingredient classes ($P < 0.05$; Figs 1 and 2). Irrespective of the dry time (24 or 72 h), the \log_{10} reduction for 0.76% quaternary ammonium+22.5% alcohol (QA2; 6.11 ± 1.30), 0.5% H_2O_2 (HP2; 6.18 ± 1.72), 0.5% H_2O_2 (HP1; 6.34 ± 1.15), 0.39% NaOCl (SH; 6.45 ± 0.87), 0.4306% Na dichloro-s-triazinetriene (CL; 6.57 ± 1.11) and 0.5% quaternary ammonium+55% alcohol (QA1; 6.61 ± 0.80) were significantly higher than 0.28% quaternary ammonium (QT; 4.85 ± 0.86) ($P < 0.05$; Fig. 3). However, there were statistically insignificant differences among the mean \log_{10} reductions per coupon for QA1, QA2, CL, SH, HP1 and HP2 ($P > 0.05$; Fig. 3).

The mean \log_{10} reductions per coupon by active ingredient class were: 4.85 ± 0.86 for quaternary ammoniums (QT), 6.19 ± 1.43 for hydrogen peroxides (HP), 6.36 ± 1.10 for quaternary alcohols (QA), 6.45 ± 0.87 for sodium hypochlorite (SH), and 6.57 ± 1.11 for Na-dichloro-s-triazinetrienes (Fig. 2). Comparing the products by active ingredient class, QA, CL, SH and HP products were more bactericidal than the QT product ($P < 0.05$; Fig. 2). There were no statistically significant differences in the bactericidal efficacies between QA and CL, QA and SH, QA and HP; CL and SH, CL and HP; SH and HP, when disinfectants were challenged with *S. aureus* DSB ($P > 0.05$; Fig. 2).

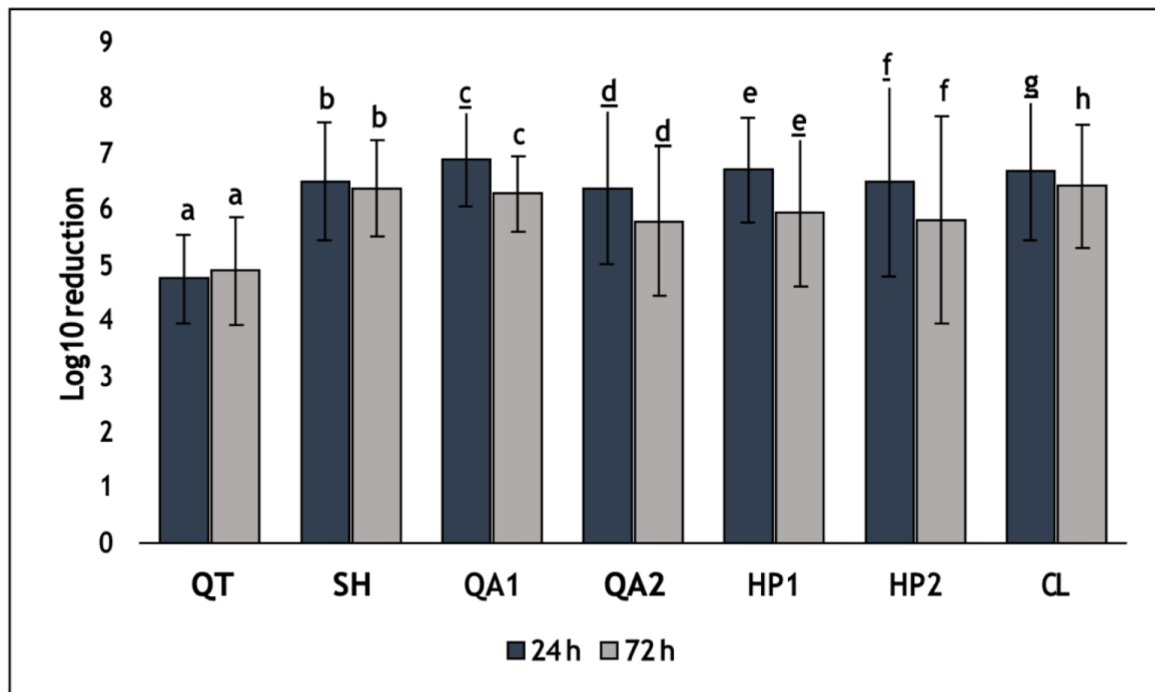


Fig. 4. Disinfectant efficacy against DSB of *S. aureus* by product type (QT, SH, QA1, QA2, HP1, HP2 and CL) at 24 h (dark bars) and 72 h (light bars) dehydration time points. Letters a, b Tukey groupings are for each product type only. Bars with same letters are not statistically different. Coupons ($n=3$) with DSB of *S. aureus* were dehydrated for 24 and 72 h at 25 °C. Error bars represent standard deviation.

Mean log₁₀ reductions for *P. aeruginosa* DSB were higher for oxidizing agents compared to quaternary ammonium products

The mean log₁₀ density of *P. aeruginosa* DSB per coupon pre-treatment was 7.08 ± 0.75 after dehydration for 24 h and 72 h. Overall, there were significant variations in log₁₀ reductions by product type and active ingredient class ($P < 0.0001$; Figs 2 and 3). Regardless of the dry time (24 or 72 h), there were significantly higher log₁₀ reductions for HP1 (5.97 ± 1.23) and HP2 (6.30 ± 1.26) as compared to QA1 (4.20 ± 0.71), QT (3.82 ± 0.53) and SH (3.23 ± 1.53) ($P < 0.05$; Fig. 1). Similarly, CL (5.79 ± 1.40) and QA2 (5.85 ± 0.87) had significantly higher log₁₀ reductions against *P. aeruginosa* DSB than QA1, QT and SH ($P < 0.05$; Fig. 3). However, there were no statistically significant differences among QA1, QT and SH ($P > 0.05$; Fig. 3). There were also no statistically significant differences in the bactericidal efficacies of HP1, HP2, CL and QA2 ($P > 0.05$; Fig. 3).

Among the active ingredient classes investigated, there were statistically significant differences among CL, HP, SH, QA and QT ($P < 0.0001$; Fig. 2). Overall, HP products resulted in a significantly higher bactericidal efficacy than QT and SH products ($P < 0.05$; Fig. 2). Similarly, CL and QA products had significantly higher mean log₁₀ reductions than QT and SH products ($P < 0.05$; Fig. 2). However, there were no differences between QT and SH, CL and HP, HP and QA, and QA and SH products ($P > 0.05$; Fig. 2).

Dry time does not significantly impact disinfectant efficacy against *S. aureus* DSB, unlike *P. aeruginosa* DSB

Mean log₁₀ densities of *S. aureus* DSB per control coupon after 24 and 72 h of dehydration were 7.64 ± 0.76 and 7.00 ± 0.89 , respectively. Regardless of the bacteria species, the mean log₁₀ reductions per coupon for 24 h old DSB were 6.91 ± 0.83 (QA1), 6.73 ± 0.94 (HP1), 6.70 ± 1.23 (CL), 6.52 ± 1.70 (HP2), 6.52 ± 1.06 (SH), 6.41 ± 1.38 (QA2) and 4.77 ± 0.80 (QT; Fig. 4). However, the average log₁₀ reductions per coupon for 72 h old DSB were 6.44 ± 1.10 (CL), 6.39 ± 0.86 (SH), 6.30 ± 0.67 (QA1), 5.95 ± 1.31 (HP1), 5.83 ± 1.87 (HP2), 5.80 ± 1.35 (QA2) and 4.92 ± 0.97 (QT; Fig. 4). On average, the bactericidal efficacies for all tested disinfectants against *S. aureus* DSB at 24 and 72 h dehydration time points were 6.30 ± 1.27 and 5.89 ± 1.21 , respectively. There was no significant difference between the average log₁₀ reductions per coupon of *S. aureus* DSB at 24 h compared to 72 h ($P > 0.05$; Fig. 1).

On the other hand, average log₁₀ densities of *P. aeruginosa* DSB per coupon pre-treatment were 7.40 ± 0.75 and 6.77 ± 0.61 after 24 and 72 h dry times, respectively. While the mean log₁₀ reductions per coupon for all tested disinfectants after 24 and 72 h were 5.50 ± 1.45 and 4.65 ± 1.63 , respectively, there were no significant differences between the average log₁₀ densities per coupon after 24 h and 72 h of dehydration ($P > 0.005$). Mean log₁₀ reductions for each product after 24 and 72 h of drying, respectively, were as follows: QA2 (6.55 ± 0.94 , 5.50 ± 0.62), HP2 (6.53 ± 1.40 , 6.06 ± 1.20), HP1 (6.36 ± 1.12 , 5.59 ± 1.30), CL (6.20 ± 1.08 , 5.39 ± 1.67), QT

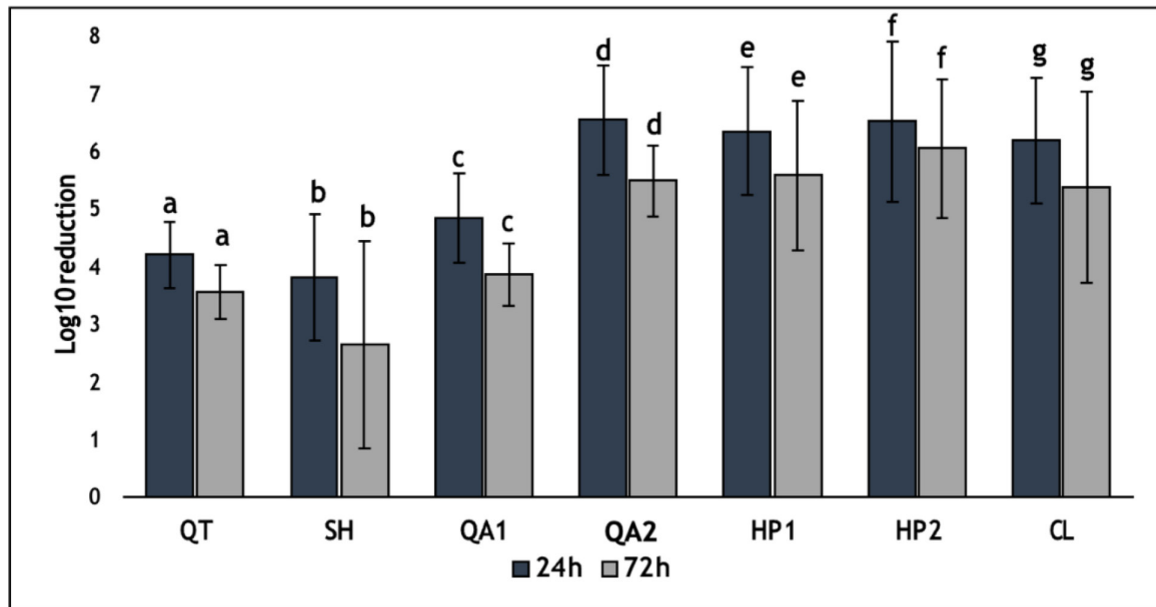


Fig. 5. Disinfectant efficacy against DSB of *P. aeruginosa* by product type (QT, SH, QA1, QA2, HP1, HP2 and CL) at 24 h (dark bars) and 72 h (light bars) dehydration time points. Letters a, b Tukey groupings are for individual products only. Bars with the same letters are not statistically different. Coupons ($n=3$) with DSB of *P. aeruginosa* were dehydrated for 24 and 72 h at 21 °C. Error bars represent standard deviation.

(4.21 ± 0.58 , 3.56 ± 0.46), QA1 (4.85 ± 0.77 , 3.86 ± 0.54) and SH (3.82 ± 1.09 , 2.65 ± 1.80) (Fig. 5). Overall and regardless of the product type or active ingredient class, significantly higher bactericidal efficacies against DSB of *P. aeruginosa* were recorded after 24 h as compared to 72 h of dehydration ($P < 0.05$; Fig. 1).

Higher bactericidal efficacy against *S. aureus* DSB than *P. aeruginosa* DSB

Overall, there were statistically significant differences in mean \log_{10} reductions between *S. aureus* and *P. aeruginosa* ($P < 0.05$; Fig. 2). Regardless of drying time and product type, the mean \log_{10} reductions for *S. aureus* and *P. aeruginosa* were 6.096 ± 1.251 and 4.941 ± 1.505 , respectively. Although product and active ingredient dependent, significantly higher \log_{10} reductions were observed when some of the tested disinfectants were challenged with *S. aureus* compared to *P. aeruginosa* ($P < 0.05$; Figs 2 and 3).

DISCUSSION

In this study, we employed a rapid DSB model previously developed by our group for disinfectant efficacy testing [13] and evaluated the bactericidal efficacy of seven EPA-registered disinfectants against 24 and 72 h old DSB of *S. aureus* and *P. aeruginosa*. Although there are few studies that have developed a rapid *in vitro* DSB model [21, 23], there is no clear standard for how many days or the conditions to establish a DSB. A study by Ledwoch *et al.*, utilized a sedimentation protocol to develop DSB based on alternating hydrated and desiccation phases over a period of 12 days [23]. However, desiccation temperatures as high as 37 °C utilized by this study may not represent clinical settings. Additionally, dynamic systems such as a CDC biofilm reactor are increasingly investigated for their ability to introduce fluid shear and thus affecting the distribution and utilization of nutrients and oxygen into the depths of biofilms compared to the static systems such as well assays. A similar study by Almatroudi *et al.*, developed semi-dehydrated model of *S. aureus* resulting in an average of \log_{10} 7.13 ± 0.04 c.f.u./coupon on polycarbonate coupons using a CDC biofilm reactor [21]. However, this study also utilized temperatures (i.e. 35 °C) at which micro-organisms readily colonize surfaces and form biofilms. Keeping in consideration, we established DSB of *S. aureus* and *P. aeruginosa* at 25 and 21 °C, respectively, to more closely represent conditions for the formation of DSB on dry contaminated hard non-porous surfaces in healthcare facilities. In addition, our study evaluated the bactericidal efficacy of disinfectants against DSB of *S. aureus* and *P. aeruginosa* established at different dehydration time points consistent with routine cleaning and disinfection schedules (daily or three times/week) recommended by the CDC.

We found that mean \log_{10} densities per coupon from this study were comparable to the ranges previously reported by Nkemngong *et al.* [13]. We also found that irrespective of drying time, CL, SH, HP and QA disinfectants were significantly more bactericidal against DSB of *S. aureus* than QT disinfectants. Moreover, when DSB of *P. aeruginosa* were challenged with disinfectants, CL and HP were significantly more bactericidal than SH and QT disinfectants. Overall, we demonstrated that prolonged dehydration

had varied effects on the bactericidal efficacy of disinfectants against DSB of *S. aureus* or *P. aeruginosa*. Specifically, we found that there were no significant differences in the bactericidal efficacies of disinfectants against 24 and 72 h DSB of *S. aureus*. There was however, a significantly lower \log_{10} reduction against 72 h DSB of *P. aeruginosa* compared to 24 h DSB of the same species.

Product type and active ingredient class significantly impact disinfectant efficacy against *S. aureus* DSB

There were significant differences among products, with QA1, QA2, CL, SH, HP1 and HP2 being more bactericidal than QT. In a related study against *S. aureus* WSB, Lineback *et al.*, demonstrated that a sodium hypochlorite and five hydrogen peroxide disinfectant products were significantly more bactericidal than two quaternary ammonium compounds [36]. This could be explained by the production of reactive oxygen species (ROS) by hydrogen peroxide disinfectants. The production of ROS results in more necrotic death compared to quaternary ammonium compounds due to DNA damage [37]. Comparatively, quaternary ammonium compounds mainly rely on a positively charged N-atom to bind to cell membranes, creating 'pores' for n-alkyl side chains to transverse the cell membrane resulting in lysis and leakage of cytoplasmic contents [38, 39]. Considering the presence of a denser EPS in DSB compared to WSB, the EPS in DSB may present a significant barrier for quaternary ammonium products compared to sodium dichloro-s-triazinetrione, sodium hypochlorite and hydrogen peroxides with an 'oxidative' mode of action against bacteria. Moreover, strong oxidizers such as sodium dichloro-s-triazinetrione, sodium hypochlorite and hydrogen peroxides have low molecular weight active ingredients that when compared to larger molecules such as quaternary ammonium, can more easily bypass the cell membrane to damage internal cellular components [37]. Quaternary alcohol products may have resulted in significantly higher bactericidal efficacies owing to the 'rapid' bactericidal mode of action of alcohol [40].

We also found that the mean \log_{10} reductions between HP1 and HP2; QA1 and QA2 were comparable when disinfectants were challenged with *S. aureus* DSB. Our findings are consistent with Lineback *et al.*, who reported no significant differences among the bactericidal efficacies of five hydrogen peroxide products tested against *S. aureus* WSB [36]. Similarly, in a recent study that evaluated the bactericidal efficacies of six disinfectant wipes against *S. aureus* ATTC-6538 inoculated on hard-non-porous surfaces, Voorn *et al.*, reported no significant differences in the bactericidal efficacies among three hydrogen peroxide products or three quaternary alcohol products [41]. However, we found that quaternary alcohol products were overall more bactericidal than quaternary ammonium products without alcohol. This suggest that the defined percentage of alcohol added to quaternary ammonium compounds influences the bactericidal efficacy as alcohol confers a rapid and more potent (tuberculocidal) action against bacteria [40].

HP and CL products are more bactericidal against *P. aeruginosa* DSB than SH, QT and QA products

Overall, CL, QA2, HP1 and HP2 had significantly higher \log_{10} reductions against *P. aeruginosa* DSB than QA1, QT and SH. Our findings are similar to those of West *et al.* who demonstrated that hydrogen peroxide-based disinfectants are overall, more bactericidal against *P. aeruginosa* allowed to dry on a Formica disc than quaternary ammonium disinfectants [42]. In another study, Tote *et al.* found that hydrogen peroxides had a stronger antibiofilm activity against 1 day old *P. aeruginosa* biofilms as they were biologically active against both viable *P. aeruginosa* cells and their EPS matrix unlike isopropanol disinfectants [43]. The high efficacy of HP1 and HP2 compared to SH against DSB could be explained by the relatively low concentration (0.39%) of sodium hypochlorite in SH as in a 2018 study, Lineback *et al.* compared the bactericidal efficacies of 0.5% hydrogen peroxide and 1.312% sodium hypochlorite disinfectants against WSB of *P. aeruginosa* and found no difference in their efficacies [36]. The same intrinsic factor of a relatively low sodium hypochlorite concentration in SH may also account for the higher bactericidal efficacy of CL compared to SH as demonstrated in a study by Tiwari *et al.*, 0.60% sodium hypochlorite resulted in a superior bactericidal efficacy against clinical isolates of *S. aureus* biofilms [44]. These reports suggest that although sodium hypochlorite is generally more bactericidal than quaternary ammoniums owing to their mode of action, the degree of disinfection is largely concentration dependent.

Although QA2 had a higher quaternary ammonium and lower alcohol content (0.76% quat+22.5% alcohol) than QA1 (0.5% quat+55% alcohol) (Table 1), QA2 demonstrated a significantly higher kill against *P. aeruginosa* DSB than QA1. This suggests that the synergistic effect of quaternary ammonium compounds and alcohol in QA1 formulation may not be sufficient alone, as in the case of QA1. Moreover, in a 2018 study by Wesgate *et al.*, the authors reported that quaternary ammonium formulations with side alkyl chains in the C_{12-16} range, as is the case for QA1, were more adsorbed to different wipe material types than other formulations [45]. Consequently, and considering that wipes were 'wringed' to dispense disinfectant liquid from QA1, the quaternary ammonium compound in QA1 may have been more adsorbed to the wipe material than QA2, resulting in an overall lower final disinfectant liquid concentration in QA1 than QA2 [45].

Bactericidal efficacy varies by species after prolonged dehydration

Our study found differences in the overall bactericidal efficacy of disinfectants against DSB of *S. aureus* and *P. aeruginosa* after prolonged dehydration for 24 and 72 h. While there was no significant difference in \log_{10} reductions between 24 and 72 h DSB of *S. aureus*, the reverse was true for DSB of *P. aeruginosa* as 72 h DSB of *P. aeruginosa* were harder to kill than their 24 h counterparts. In a previous study by our group, we found that 100% of *P. aeruginosa* DSB established at a dehydration temperature of 21 °C

were encased in EPS while this was true for only 92% of *S. aureus* DSB established at 25 °C [13]. The consistent presence of EPS on DSB of *P. aeruginosa* at various dehydration time points from 24 to 120 h, as previously demonstrated by our group, suggested that older DSB of *P. aeruginosa* developed using our model may be encased in more EPS; making them harder to kill [13]. This is consistent with previous studies that have demonstrated the presence of a thick EPS matrix as a major factor for reduced bactericidal efficacy in biofilms compared to planktonic bacteria [29]. Moreover, previous studies [21, 46] have also suggested that unfavourable conditions such as dehydration may trigger bacterial biofilms to produce more EPS. While this may be true for *P. aeruginosa* DSB as evidenced in our previous study [13], the same may not be the case for *S. aureus* DSB. However, the presence of planktonic bacteria on the surface of biofilms does not 'disqualify' them from being biofilms considering that mature bacterial biofilms shed planktonic cells, and this could explain the occasional lack of a visible EPS in 8% of the coupons as imaged by our previous study by Nkemngong *et al.* [13]. More EPS production translates into a thicker barrier for disinfectants to bypass before contact with underlying bacteria. Additionally, a thicker EPS matrix may also result in a pH gradient, which can impact bactericidal efficacy [20]. These factors could account for the reduced bactericidal efficacy against 72 h DSB of *P. aeruginosa* compared to 72 h DSB of *S. aureus*.

***P. aeruginosa* DSB are harder to inactivate than *S. aureus* DSB**

Our data delineate statistically significant higher average \log_{10} reductions when disinfectants were treated against *S. aureus* DSB compared to *P. aeruginosa* DSB. Overall, the low bactericidal efficacy of disinfectants against biofilms is often linked to the EPS matrix [47]. The reduced efficacy of disinfectants, regardless of the product type, observed with Gram-negative *P. aeruginosa* can be partially explained by the presence of alginate [48], Psl, Pel [49] and extracellular DNA (eDNA) [50] as important components of the biofilm matrix characteristic of *P. aeruginosa*. Specifically, the overproduction of alginates by *P. aeruginosa* mutants results in the formation of larger microcolonies than wild-type species [51]. This suggests the role for alginates in decreased susceptibility to antimicrobials [52] compared to non-alginate-producing bacteria such as *S. aureus* [49]. Pel, on the other hand, plays a vital role in cell-to-cell interactions within these biofilms [53] and in the biofilm maturation [50]. A spike in alginate and carbohydrate production during biofilm formation and maturation confers an overall increase in the net negative charge of the EPS matrix, enhancing the electrostatic attractions between the EPS matrix and positively charged antimicrobials as quaternary ammonium compounds [47]. This limits the diffusion of cationic antimicrobials through the EPS matrix, thus shielding the underlying bacteria from direct antimicrobial contact [47]. However, the cell wall of Gram-positive bacteria such as *S. aureus* is essentially composed of peptidoglycan and teichoic acid and substances with high molecular weight can traverse the cell wall [54]. This may explain the higher \log_{10} reductions observed against *S. aureus* DSB compared to *P. aeruginosa* DSB exposed to quaternary alcohol and quaternary ammonium products.

Our results suggest that comparatively higher mean \log_{10} reductions are achieved when sodium hypochlorite was challenged with *S. aureus* compared to *P. aeruginosa* DSB. This could be due to the act that negatively charged disinfectants as sodium hypochlorite destroy the cellular activity of bacterial proteins [55] and are capable of increased penetration of outer cell layers even in an unionized state [54]. Similarly, hydroxyl free radicals from HP-based products specifically target sulfhydryl groups, double bonds [56] and destroy bacterial lipids, proteins, and DNA.

Our results support previous findings that DSB are harder to kill than planktonic bacteria; all the products tested in this study are EPA registered, indicating high levels of efficacy against planktonic bacteria of *S. aureus* and *P. aeruginosa*. To reduce patient safety risks in healthcare facilities, it is critical to conduct baseline disinfectant efficacy testing for product registration using bacteria biofilms as DSB that best mimic healthcare environments.

We acknowledge that the scope of our study is limited as we did not investigate the bactericidal efficacy of the tested products against mixed culture bacterial biofilms common on dry contaminated hard-non-porous surfaces in healthcare facilities. We also acknowledge that our study is limited to reference strains recommended by US EPA for disinfectant efficacy testing. Inclusion of clinical isolates in our future work will expand existing knowledge about biofilms found in these clinical settings and their response to disinfection. We also acknowledge that our study did not specifically investigate disinfectant efficacy against DSB of *S. aureus* and *P. aeruginosa* subjected to longer hours of dehydration as this could impact the efficacy levels of commonly used disinfectants. A wider range of disinfectant active ingredients could have also been investigated. We also acknowledge that the surface material plays a major role in bacterial cell attachment, biofilm formation and EPS production. We selected borosilicate glass coupons as this is the representative surface material type recommended by the US EPA for biofilm development. Our future goals are to conduct similar studies utilizing wider range of surface material types found. However, we also acknowledge that our study has set a solid foundation for future investigations of DSB of *S. aureus* and *P. aeruginosa*.

CONCLUSION

Although it is generally agreed that DSB pose a severe challenge for the disinfection of hard non-porous surfaces in healthcare facilities and are a significant contributor to the incidence of HAI, the success of any disinfection regime is dependent on multiple intrinsic and extrinsic factors. Our study definitively demonstrated that significant kill levels of the DSB of major healthcare

pathogens that cause HAI can be achieved although this is highly dependent on the choice of disinfectant as hydrogen peroxides, and Na dichloro-*s*-triazinetrione were more bactericidal than quaternary ammoniums without alcohol. We also demonstrated that for a less hardy pathogen such as *S. aureus*, the age of the DSB does not present a challenge for the overall efficacy of disinfectants unlike for *P. aeruginosa* where 72 h DSB were harder to inactivate than their 24 h counterparts. It is therefore critical for healthcare stakeholders to consider these factors in efforts to reduce HAI rates.

Availability of data and material

All quantitative data generated or analysed during this study are included in this published article.

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Author contributions

C.A.N., G.K.C.: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing - original draft preparation, writing - review and editing, visualization, project management; P.T. and X.L.: conceptualization, methodology, investigation, writing - review and editing, funding acquisition; H.F.O.: conceptualization, methodology, writing - review and editing, visualization, project administration, supervision, funding acquisition.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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