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Hydrogen peroxide, chlorine, and peracetic acid disinfectants outperform quaternary ammonium compounds against *Staphylococcus aureus* and *Pseudomonas aeruginosa* wet and dry surface biofilms

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ABSTRACT

Background: Wet and dry surface biofilms (WSB and DSB), are a recognized source for healthcare-associated infections (HAI), and effective cleaning and disinfection regimens are critical to reducing HAI risk for patients. Disinfectant efficacy against biofilms on dry surfaces, however, remains poorly characterized relative to that against planktonic organisms.

Methods: The bactericidal efficacy of eight EPA-registered hospital-grade, one-step cleaner-disinfectants was assessed against WSB and DSB of *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442). Biofilms were grown in a CDC biofilm reactor following EPA-MLB-SOP-MB-19-06 and, for DSB formation, dehydrated at 25 and 21° C for *S. aureus* and *P. aeruginosa* respectively for 24, 48, and 72 h intervals to reflect recommended cleaning frequencies for healthcare surfaces. Biofilms were challenged with each disinfectant at its label-specified contact time following EPA-MLB-SOP-MB-20-04. Efficacy was expressed as $\log_{10}$ CFU/cm^2 reduction, and experiments were performed in triplicate.

Results: Hydrogen peroxide-, chlorine- and peracetic acid-based disinfectants demonstrated significantly greater efficacy than quaternary ammonium-based products against *S. aureus* biofilms ($P < 0.05$). Desiccation time significantly impacts bactericidal efficacy of *P. aeruginosa* biofilms ($P < 0.05$) unlike biofilms of *S. aureus*. There is greater disinfectant efficacy against *P. aeruginosa* biofilms with the addition of a solvent to a quaternary ammonium product.

Conclusions: Our findings indicate that current planktonic-based registration testing may overstate real-world disinfectant performance against the biofilm-embedded organisms healthcare facilities are realistically working to control, and that this gap varies by chemistry, organism, and how long a biofilm has been allowed to persist before disinfection.

Keywords:

Healthcare-associated infections (HAI)

Dry surface biofilm (DSB)

Staphylococcus aureus

Pseudomonas aeruginosa

Healthcare disinfectant

Background

Healthcare-associated infections (HAI) are caused by patient exposure to pathogens during treatment at medical facilities such as nursing homes, in-patient rehabilitation centers, dialysis centers, and hospitals [1]. In 2023, there were an estimated 518,000 HAI occurrences in the United States [2]. Effective use of disinfectants to control microbial growth, including biofilms, is a key tool for preventing HAI in medical environments [3].

The majority (65-80%) of microbial and chronic patient infections are associated with biofilms [4, 5]. Biofilms consist of an organized mass of microorganisms within a matrix of extracellular polymeric substances; after the initial attachment of microbes to a surface, biofilms form micro-colonies, mature and develop the biofilm architecture, and detach/disperse [4]. Biofilms offer microorganisms many advantages, including increased adhesion/cohesion capabilities, expanded nutritional sources, a metabolite exchange platform, and protection from a variety of environmental hazards such as environmental stresses, host immune attacks, shear forces, and antimicrobial chemicals [6].

Biofilm formation in healthcare settings is strongly associated with pathogens' persistence in the environment, ability to infect an individual, and resistance to antimicrobial interventions [7, 8]. In hospitals, biofilm formation commonly occurs in wet or damp locations, resulting in wet surface biofilms on medical devices and tubing on medical equipment [9]. However, without ready access to water, biofilms can also persist in a desiccated state called dry surface biofilms [9]. Despite being dehydrated, DSB are also capable of being dispersed to other surfaces [9, 10]. DSB are not necessarily permanently desiccated: ambient humidity and

incomplete removal of biofilms during routine cleaning and disinfection may periodically rehydrate the biofilm, potentially complicating disinfection [5]. Although not exclusively, DSB in healthcare settings have been found to be primarily formed by Gram-positive bacteria [11,12], which are generally tolerant of desiccation [13], whereas WSB are predominantly composed of Gram-negative bacteria [5, 14].

Staphylococcus aureus and *Pseudomonas aeruginosa* are among the most commonly reported HAI pathogens [15, 16]. In a National Healthcare Safety Network study analyzing HAI cases from 2018 to 2021, *S. aureus* and *P. aeruginosa* caused almost 20% of all recorded HAI [15]. Methicillin-resistant *S. aureus* (MRSA) account for a large portion of *S. aureus* HAI and many *P. aeruginosa* HAI involve antibiotic-resistant isolates [17, 18]. Both organisms readily form biofilms, and are known to exist in multi-species communities further complicating treatment [19, 20, 21].

The primary intervention for preventing HAI from forming biofilms is disinfection of environmental surfaces and patient care equipment [22, 23]. In the United States, for a disinfectant to be labeled for use in healthcare facilities, efficacy must be shown against vegetative forms of *S. aureus* and *P. aeruginosa* using EPA approved methods. However, the presence of these organisms in healthcare can also be due to the presence of biofilms. Both WSB and DSB have been associated with increased resistance to disinfectants, attributed to their slower growth and metabolism, modulation of stress response, changes in quorum sensing, and reduced penetration of a disinfectant's active ingredients [9]. The minimum inhibitory concentration of disinfectants is higher for cells in a biofilm environment compared to planktonic cells [3, 5, 24]. For this reason, the Environmental Protection Agency (EPA) should require

biofilm-specific efficacy testing with strains of *S. aureus* and *P. aeruginosa* for products to be labeled with claims against biofilms [25-27].

Initial studies to assess the efficacy of disinfectants against microorganism biofilms in both wet and dry surface phases indicate that biofilm resistance to disinfectants can vary based on the biofilm environment and a disinfectant's active ingredients [28, 29]. In this study, we evaluated the bactericidal efficacy of eight EPA-registered liquid disinfectants against *S. aureus* and *P. aeruginosa* as WSB and as DSB at 24-, 48-, and 72 h after dehydration. The purpose of the study was to identify differences in disinfectant efficacy a) for single-species *S. aureus* and *P. aeruginosa* biofilms across different timepoints of dehydration among eight different disinfectant products, and to b) contrast tested efficacy against EPA standard efficacy for registered disinfectants.

Methods

Bacterial strains and disinfectants

Eight EPA-registered, hospital-grade, one-step cleaner-disinfectants representing a range of common active ingredients (hydrogen peroxide [HP], isopropyl alcohol, peracetic acid [PA], quaternary ammonium [QA], sodium hypochlorite [Cl]) were tested against *S. aureus* (ATCC® 6538™; American Type Culture Collection) and *P. aeruginosa* (ATCC® 15442™; American Type Culture Collection, Manassas, VA) (Table 1) [25-27].

Table 1: Disinfectant active ingredients and contact times

Disinfectant product[a]	Active ingredient(s)	Dilution at use	Label contact time (min)
QA1	8.704% didecyl dimethyl ammonium chloride 8.190% n-alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride	1:256	10 min
QA2	2.40% alkyl (50% C_{14}, 40% C_{12}, 10% C_{16}) dimethyl benzyl ammonium chloride 1.80% octyl decyl dimethyl ammonium chloride 0.9% dioctyl dimethyl ammonium chloride 0.9% didecyl dimethyl ammonium chloride	1:64	10 min
QA/Alcohol	0.25% n-alkyl (68% C_{12}, 32% C_{14}) dimethyl ethylbenzyl ammonium chlorides 0.25% n-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chlorides 55% isopropyl alcohol	RTU[b]	2 min
QA/Solvent	0.105% n-alkyl (60% C_{14}, 30% C_{16}, 5% C_{12}, 5% C_{18}) dimethyl benzyl ammonium chlorides 0.105% n-alkyl (68% C_{12}, 32% C_{14}) dimethyl ethylbenzyl ammonium chlorides	RTU	2 min
HP1	0.5% hydrogen peroxide	RTU	1 min
HP2	0.5% hydrogen peroxide	RTU	1 min
Cl	0.65% sodium hypochlorite	RTU	3 min
PA	3.130% hydrogen peroxide 0.099% octanoic acid 0.050% peroxyacetic acid	RTU	3 min

[a]Abbreviated naming scheme for commercially available products used in this study;

[b]RTU- Ready-to-use

Establishing wet surface biofilms

WSB were established following EPA-MLB-SOP-MB-19-06 [25] through batch and continuous phases on borosilicate glass coupons (1.27 ± 0.013 cm; BioSurface Technologies Corporation). The batch phase medium consisted of 3.0 g/L tryptic soy broth (TSB) for *S. aureus* and 300 mg/L TSB for *P. aeruginosa*. Five hundred mL of batch medium was held in a CDC biofilm reactor (Biosurface Technologies, Inc., Bozeman, MT) and inoculated with 1mL of an overnight culture of at least 10^7 CFU/mL of either *S. aureus* or *P. aeruginosa* and placed on a

magnetic hot plate stirrer (Talbays, Thorofare, NJ) set at 60 ± 5 rpm at 35 ± 1°C (*S. aureus*) or 125 ± 5 rpm at 20 ± 2°C (*P. aeruginosa*). The batch phase lasted 24 ± 2 h. Continuous medium was prepared by adding 500 mL of 40g/L TSB to 20 L of sterile distilled water for a final concentration of 1.0 g/L TSB for *S. aureus* and 50 mL of 40g/L TSB to 20 L of sterile distilled water for a final concentration of 100 mg/L TSB for *P. aeruginosa.* Continuous medium was then pumped through the CDC biofilm reactor for 24 ± 2 h for both strains.

Dry surface biofilm establishment

DSB were established following the methodology developed by Nkemngong et al. [10]. After WSB were developed, rods containing coupons from the CDC biofilm reactor were dehydrated for 24-, 48-, and 72 h at 25°C or 21°C for *S. aureus* and *P. aeruginosa*, respectively.

Disinfectant efficacy testing

Disinfectants (Table 1) were tested against *S. aureus* and *P. aeruginosa* biofilms following EPA-MLB-SOP-MB-20-04 [26]. WSB were tested at the end of biofilm development, and DSB were tested at each dehydration timepoint (24, 48 and 72 h). At each timepoint, and for each disinfectant product and organism, six coupons were harvested from rods into sterile 50 mL conical tubes: three were treated with 4 mL of disinfectant and three served as negative controls, treated with 4 mL of phosphate-buffered saline (PBS) for the same contact time. Each coupon was treated independently. Contact times were those specified on each product label (Table 1). At the end of the contact time, 36 mL of neutralizing buffer (5.2 g Difco neutralizing buffer per 1 L H_2O; Becton, Dickinson and Company, Sparks, MD) were added to each treated and control coupon. Coupons were vortexed and sonicated (30 s each) to release the biofilm into suspension as described by EPA MB-20-04 [26] and tenfold serial dilutions were performed. Suspensions were vacuum-filtered onto 0.22 µm membranes (47 mm grid, individually sterile-packed;

Millipore Sigma-Aldrich, St. Louis, MO), and membranes were aseptically transferred to Tryptic Soy Agar (TSA; Fisher Scientific, Hanover Park, IL) following EPA-MLB-SOP-MB-19 and incubated overnight at 37 °C [25]. All experiments were performed in triplicate.

Statistical analysis

Mean bacterial $\log_{10}$ densities per coupon were calculated for treated coupons (exposed to disinfectant) and control coupons (treated with PBS) averaging the three technical replicates within each condition. Mean $\log_{10}$ densities per disinfectant-treated coupon were normalized against the mean $\log_{10}$ densities of control coupons to determine reductions. Mean $\log_{10}$ reductions were analyzed using fixed-effects ANOVA models across all figures (n = 3 replicate coupons per condition; 96 observations per species; N = 192; α=0.05). Replicate coupons were obtained across multiple reactor runs conducted on separate days using two CDC biofilm reactors.

Separate one-way ANOVAs were performed at each biofilm dry time to compare disinfectant products, with Tukey's HSD applied to LSMeans within each time point for determining significance. Average $\log_{10}$ reductions across products were normalized against mean control coupon densities and compared between dehydration timepoints using one-way ANOVA with Tukey's HSD. Statistical analyses and all figures were conducted in SAS 9.4.

Declaration of generative AI and AI-assisted technologies

During the preparation of this work the authors used Claude (claude-sonnet-4-6, Anthropic, San Francisco, CA, USA) to generate code for plotting the figures. The AI tool was not used for data collection, statistical analysis, or interpretation of results. The resulting figures were reviewed, verified for accuracy against the data, and edited as needed by the authors. The authors take full responsibility for the accuracy and integrity of the content presented.

Results

Efficacy was evaluated both as mean $\log_{10}$ CFU/cm^2 reduction and, where relevant, in reference to the six-log reduction EPA requires for registration against planktonic cells of each organism for disinfectant claims in the United States.

Hydrogen peroxide-, chlorine- and peracetic acid-based disinfectants demonstrated significantly greater efficacy than quaternary ammonium-based products against *S. aureus* biofilms

Overall, quaternary ammonium-based products achieved meaningfully lower $\log_{10}$ reductions in *S. aureus* and *P. aeruginosa* biofilms compared to hydrogen peroxide-, chlorine-, and peracetic acid-based products. For *S. aureus* biofilms, all quaternary ammonium-based products (QA1, QA2, QA/Alcohol, and QA/Solvent) were significantly less effective than HP1, HP2, and PA across all stages of biofilm dehydration maturity ($P < 0.05$, Fig.1.a-d). The chlorine-based disinfectant (Cl) exhibited statistically greater efficacy than the quaternary ammonium-based products against WSB, 48 h DSB, and 72 h DSB, but was only statistically different from QA1 against 24h DSB ($P < 0.05$, Fig.1.b).

For *P. aeruginosa* biofilms, three quaternary ammonium-based products (QA1, QA2, and QA/Alcohol) were significantly less efficacious compared to HP, Cl, and PA products ($P < 0.05$, Fig. 2.a-d). Treated *S. aureus* and *P. aeruginosa* biofilms that recovered no viable counts (*S. aureus*: WSB- HP1, HP2; 24 h DSB- HP1, HP2, Cl, PA; 48 h DSB- HP1, HP2, PA; 72 h DSB- HP2, Cl, PA; *P. aeruginosa*: 24 h DSB- HP1, Cl, PA; 48 h DSB- HP1, Cl, PA; 72 h DSB- HP1, Cl) were reported as 0.50 CFU/cm^2 per EPA MLB SOP MB-20 as the reported detection limit when no cells are recovered, thus these columns contain no calculable standard deviation (Fig.1.a-d and Fig. 2.a-d).

In contrast to active ingredient, dehydration time had a limited effect on overall efficacy. Although drying reduced control densities of both organisms from WSB to 72h DSB *S. aureus*: 7.41 ± 0.40 to 5.87 ± 0.47 $\log_{10}$ CFU/cm²; *P. aeruginosa*: 8.62 ± 0.28 to 7.20 ± 0.58 $\log_{10}$ CFU/cm²), $\log_{10}$ reductions averaged across active ingredients did not differ among timepoints for *S. aureus* ($P > 0.05$). For *P. aeruginosa*, they differed only between 24 h and 72 h DSB ($P < 0.05$; Supplemental data).

Active ingredient chemistry of quaternary ammonium with solvent exhibits greater efficacy against *P. aeruginosa* biofilms than other QA products

The QA/Solvent disinfectant proved significantly greater $\log_{10}$ reductions than the other quaternary ammonium-based products against *P. aeruginosa* biofilms with reductions of 3.87±0.75, 5.09±0.64, 5.28±0.68, and 5.42±1.15 CFU/cm^2 as WSB and after 24-,48-, and 72 h respectively ($P < 0.05$, Fig. 2) On the other hand, QA/Solvent was less efficacious against biofilms of *S. aureus,* performing similarly to the other quaternary ammonium products tested (Fig.1).

Discussion

EPA-registered disinfectants for use in healthcare facilities are currently required to demonstrate efficacy only against planktonic forms of *S. aureus* and *P. aeruginosa*, despite the prevalence of biofilm-embedded populations in the healthcare environment. However, current standards do not account for differences in biofilm maturity or hydration state that likely occur on healthcare surfaces. It is well documented that both *S. aureus* and *P. aeruginosa* have the ability to persist on dry hospital surfaces for weeks to months, a perspective that planktonic

testing does not address [32]. This study is among the first to compare the efficacy of a broad panel of EPA-registered disinfectants against WSB and DSB, better representing realistic healthcare challenges.

The comparatively poor performance of QA-based products against biofilms of both organisms is consistent with known limitations of cationic surfactants when met with biofilm matrix [22, 28]. The extracellular polymeric substance (EPS) that encases biofilm cells is predominantly negatively charged and can bind positively charged quaternary ammonium molecules at the surface, reducing the efficacy against the embedded cells [18, 29, 30]. This effect is compounded by the increased density and reduced hydration of the EPS in DSB, reducing pore size and restricting penetration of larger or charged molecules [9, 10]. Oxidizing agents such as hydrogen peroxide, chlorine, and peracetic acid act by damaging essential cell proteins, lipids, and nucleic acids [35]. Cl and PA chemistries exhibit further capabilities of directly degrading EPS matrix integrity as they diffuse [35], plausibly explaining why these chemistries demonstrated greater bactericidal efficacy than the QA products.

Notably, QA/Solvent was the only quaternary ammonium product to show significantly greater efficacy against *P. aeruginosa* than the other QA formulations, despite performing similarly to them against *S. aureus* ($P < 0.05$). This organism-specific advantage may relate to the solvent component, which could enhance penetration through the outer membrane of *P. aeruginosa*, a structure absent in Gram-positive *S. aureus* [36]. Solvents can disrupt lipid packing and increase membrane fluidity, potentially facilitating QA access to the cell envelope in a species where it may otherwise be more restricted [36]. The poorer performance of the other QA formulations is likely multifactorial. *P. aeruginosa* is intrinsically tolerant of QA compounds, impacting its low outer membrane permeability in addition to efflux pumps which

actively remove these compounds from the cell and are induced by exposure to benzalkonium chloride [37,38]. Exposure can also alter the outer membrane proteins that govern uptake, and sub-lethal benzalkonium chloride has been shown to increase biofilm formation in this species [38,39].

Our study noted additional species-specific differences in the overall bactericidal efficacy against DSB of *S. aureus* and *P. aeruginosa* after prolonged dehydration for 24- and 72 h. *P. aeruginosa* biofilms demonstrated significantly lower reductions at 72 h than at 24 h of dehydration which is consistent with previous findings which demonstrated that older DSB contain greater encasing of EPS [10], a major factor for reduced bactericidal efficacy [28].

In experiments that mimicked the temperature conditions and disinfection schedules in a healthcare environment, QA products were overall significantly less effective in reducing *S. aureus* and *P. aeruginosa* biofilm populations compared to products containing HP, Cl, and PA. These results mirror previous studies which examined the efficacy of different active ingredients against *S. aureus* and *P. aeruginosa* biofilm populations [28, 33, 40]. The EPA threshold for disinfectant efficacy against both *S. aureus* and *P. aeruginosa* biofilm populations is a minimum mean six-log reduction; among QA products, none met this standard [27]. Notably, a product falling short of six-log reduction against WSB or DSB may not meet the performance standard set by EPA registration requirements for healthcare facility disinfection.

Limitations of the study

This was a small-scale study conducted with single reference strains of *S. aureus* and *P. aeruginosa.* Wild-type isolates may differ in their susceptibility to the disinfectants tested, either positively or negatively, and so there is scope for more extensive testing across a wider panel of strains. Efficacy was assessed by viable cell count (CFU) recovery, which does not capture

viable-but-non-culturable (VBNC) cells that may persist after both desiccation and treatment, potentially underestimating initial density in addition to true residual following disinfection. Additionally, dehydration times and temperatures were selected to represent CDC-recommended schedules and general healthcare conditions, but real-world surfaces and environments vary in temperature, humidity, and soil load, all of which can further affect bactericidal efficacy. Future studies should evaluate viability-based detection methods, alongside trials incorporating varying relative humidities and soil loads to better understand pathogen persistence in an array of environmental settings.

Conclusions

Biofilms pose a challenge to ensuring the safety of healthcare environments, and an effective and informed cleaning and disinfection regimen is necessary to provide a safe healthcare environment. Our study demonstrates that QAs were significantly less effective than HPs, Cls, and PAs in reducing *S. aureus* and *P. aeruginosa* in both wet and dry surface biofilms, underscoring a potential gap between registration testing and real-world efficacy against biofilm-embedded organisms. These findings indicate that current planktonic-based registration testing may overstate real-world disinfectant performance against the biofilm-embedded organisms healthcare facilities are realistically working to control, and that this gap varies by chemistry, organism, and how long a biofilm has been allowed to persist before disinfection. We recommend that EPA efficacy testing incorporate validated wet and dry surface biofilm models, such as those used here, so that healthcare facilities can select disinfectants based on performance under realistic conditions.

Abbreviations

ATCC: American Type Culture Collection

CDC: Centers for Disease Control and Prevention

CFU: Colony-forming units

DSB: Dry surface biofilms

EPA: (US) Environmental Protection Agency

HAI: Healthcare-associated infections

MRSA: Methicillin-resistant *Staphylococcus aureus*

PBS: Phosphate-buffered saline

RTU: Ready-to-use

TSA*:* Tryptic soy agar

TSB*:* Tryptic soy broth

VBNC*:* Viable but non-culturable

WSB: Wet surface biofilms

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

PT is an employee of Diversey, a Solenis company, which funded this study. The other authors declare no competing interests.

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Authors' contributions

CH performed disinfectant efficacy testing and wrote the original manuscript. HS contributed formal analysis, interpreted data generated, and writing review and editing. AG was a contributor in writing and editing the manuscript. PT provided testing materials, industry experience, and was a contributor in writing and editing the manuscript. HO-J served as the principal investigator for the study and was a contributor in writing and editing the manuscript. All authors read and approved the final manuscript.

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Figure Titles and Captions

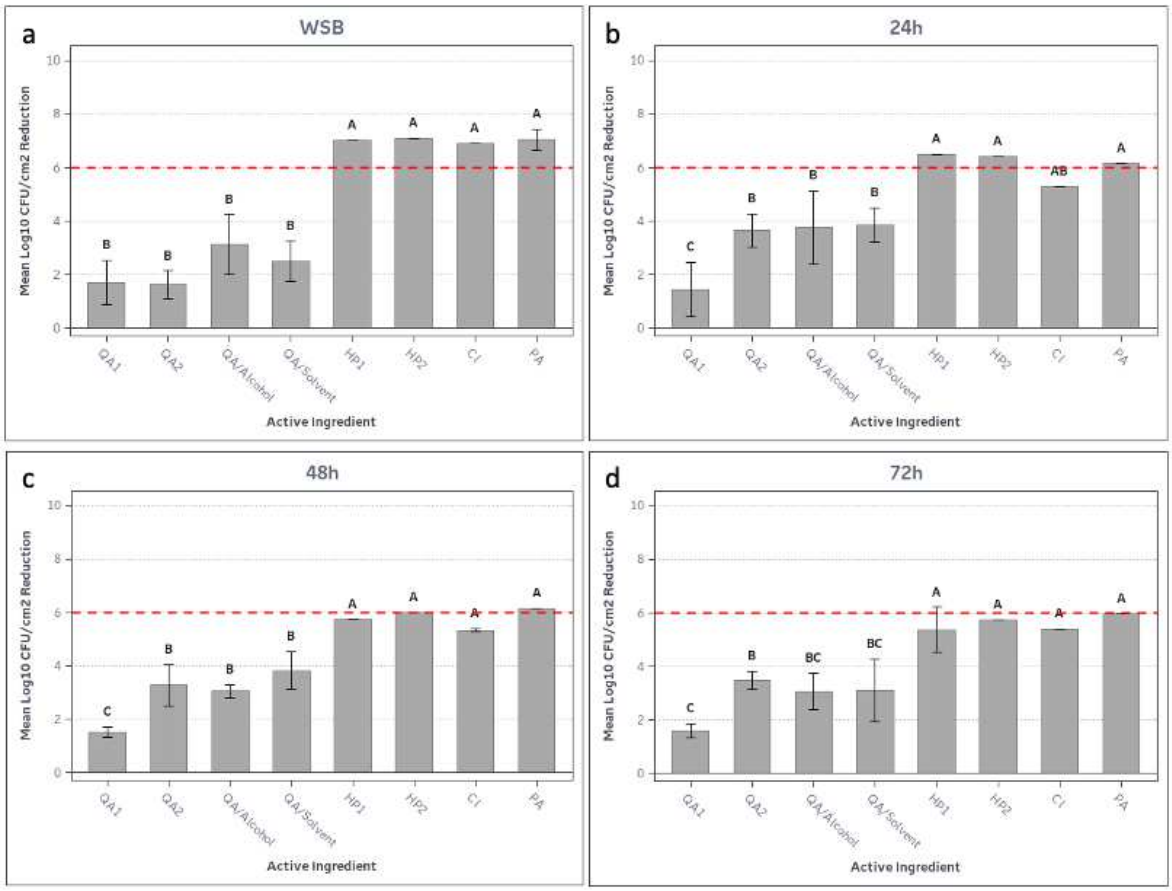
Fig. 1. Efficacy of disinfectants against *S. aureus* biofilms

Fig. 1. Mean $\log_{10}$ CFU/cm^2 reduction from treated *S. aureus* biofilms by active ingredient for each dehydration maturity: (a) WSB, (b) 24h, (c) 48h, and (d) 72h. The dashed line indicates the

six-log reduction threshold required for classification as a biofilm disinfectant in the United States by the Environmental Protection Agency (EPA). Different letters above columns indicate significant differences between products ($P < 0.05$).

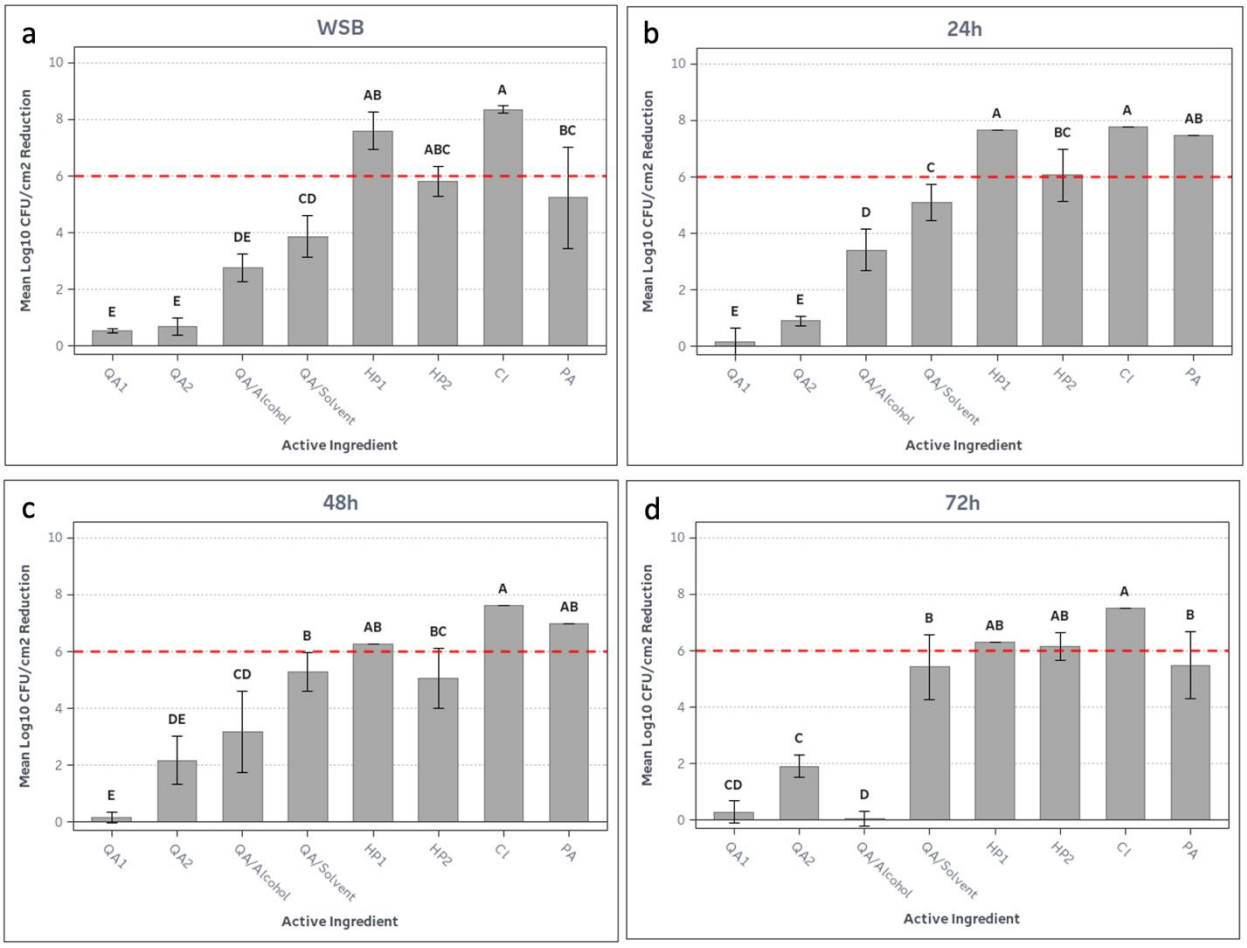
Fig. 2. Efficacy of disinfectants against *P. aeruginosa* biofilms

Fig. 2. Mean $\log_{10}$ CFU/cm^2 reduction from treated *P. aeruginosa* biofilms by active ingredient for each dehydration maturity: (a) WSB, (b) 24h, (c) 48h, and (d) 72h. The dashed line indicates the six-log reduction threshold required for classification as a biofilm disinfectant in the United States by the Environmental Protection Agency (EPA). Different letters above columns indicate significant differences between products ($P < 0.05$).



441

442 Figure 1



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444 Figure 2

Supplementary Files

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