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Comparative evaluation of biofilm disinfectant efficacy tests

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Abstract

Regulatory agencies are receiving registration applications for unprecedented, antibiofilm label claims for disinfectants. Reliable, practical, and relevant laboratory biofilm test methods are required to support such claims. This investigation describes the influence of fluid dynamics on the relevancy of a laboratory test. Several disinfectant formulations were tested using three different biofilm testing systems run side-by-side: the CDC biofilm reactor system that created turbulent flow (Reynolds number between 800 and 1850), the drip flow biofilm reactor system that created slow laminar flow (Reynolds number between 12 and 20), and the static biofilm system that involved no fluid flow. Each comparative experiment also included a dried surface carrier test and a dried biofilm test. All five disinfectant tests used glass coupons and followed the same steps for treatment, neutralization, viable cell counting, and calculating the log reduction (LR). Three different disinfectants, chlorine, a quaternary ammonium compound, and a phenolic, were each applied at two concentrations. Experiments were conducted separately with *Pseudomonas aeruginosa* and *Staphylococcus aureus* and every experiment was independently repeated. The results showed that biofilm grown in the CDC reactor produced the smallest LR, the static biofilm produced the largest LR, and biofilm grown in the drip flow reactor produced an intermediate LR. The differences were large enough to be of practical importance. The dried surface test often produced a significantly higher LR than the tests against hydrated or dried biofilm. The dried biofilm test produced LR values similar to those for the corresponding hydrated biofilm test. These results show that the efficacy of a disinfectant must be measured by using a laboratory method where biofilm is grown under fluid flow conditions similar to the environment where the disinfectant will be applied.

Keywords: Antimicrobials; Biofilm reactor; Fluid shear; Regulatory data; Relevant test

1. Introduction

Biofilm bacteria live in a self-organized, cooperative community of microorganisms attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, and exhibit altered phenotypes with respect to growth rate and gene transcription (Boles et al., 2004; Donlan and Costerton, 2002; Stoodley et al., 2002). Biofilms are prevalent in moist or aqueous environments, even if the surfaces are intermittently dehydrated. Bacteria predominantly exist as biofilm (Costerton et al., 1978; Costerton, 2004; Donlan and Costerton, 2002). Biofilm bacteria are different from their planktonic counterparts (Loo et al., 2000; Sauer et al., 2002; Sternberg et al., 1999).

Biofilm bacteria are notoriously tolerant to conventional chemical disinfectants (Donlan and Costerton, 2002; Stewart et al., 2000). These high tolerances may be caused by slow diffusion through the extracellular polymeric substance matrices, the existence of persister cells, development of resistant phenotypes, and adaptations to micro-environments (Spoering and Lewis, 2001; Stewart et al., 2000). Because detached biofilm clumps retain this increased resistance (Fux et al., 2004) and may contain enough bacteria to give an infective dose (Wilson et al., 2004), biofilm bacteria represent a potential health risk (Armon et al., 1997; Murga et al., 2001).

For these reasons, there is a recognized need for laboratory methods for testing the efficacy of chemical disinfectants against biofilm bacteria. The standard methods used to show

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potential antibiofilm activity of chemical disinfectants often employ the use of planktonic cells that have been dried on a hard surface carrier (ASTM International, 2003). However, a test against actual biofilm bacteria is required for relevancy to real-world applications (Bloomfield and Sims, 1996; Costerton, 2004; Costerton and Stewart, 2001; van Klingeren et al., 1998). Not only must a biofilm disinfection test method include all the biological, chemical, and analytical components of conventional suspension or dried surface tests, but the method also requires some engineered apparatus, such as a biofilm reactor, for growing a reproducible biofilm. Moreover, the laboratory biofilm should be grown so that it possesses the key attributes of the naturally-occurring biofilm where the disinfectant will be applied.

Fluid dynamics are an important consideration when designing a reactor to grow a relevant biofilm (Purevdorj and Stoodley, 2004). A biofilm will self-assemble into a characteristic architecture that depends upon the fluid shear conditions under which it grew. For example, biofilms formed under high shear, turbulent flow, are stronger, more stable, and more strongly attached than their low shear, laminar flow counterparts (Pereira et al., 2002; Purevdorj et al., 2002; Vieira et al., 1993). Biofilms grown in turbulent flow conditions have a greater mass, physiological activity, and total protein than biofilms grown in laminar flow (Simões et al., 2003a,b). Biofilms grown in turbulent flow are more dense than the fluffy biofilms grown in laminar flow (Pereira et al., 2002). It would be prudent to engineer fluid dynamics within the biofilm growth reactor that emulate the fluid dynamics in the target environment (Blanchard et al., 1998; Eginton et al., 1998; Simões et al., 2003a,b, 2005).

A variety of reactors and growth systems have been used successfully for research and/or disinfectant testing purposes (e.g., Ceri et al., 1999; Characklis, 1990; Charaf et al., 1999; Gilbert et al., 1998; Goeres et al., 2005; Kharazmi et al., 1999; Luppens et al., 2002; Pitts et al., 2003; Stoodley and Warwood, 2003; Wilson, 1999; Zelver et al., 1999). For the development and official registration of commercial disinfectants against biofilm bacteria, standardized laboratory reactors and associated standard operating procedures are required. The standardization process for biofilm tests has just begun. At present, only one biofilm reactor and operating procedure has been approved by the USA standard setting organization American Society for Testing and Materials International (ASTM), method #E 2196-02 (ASTM International, 2002). Tests of antibiofilm efficacy are based predominantly on ad hoc disinfectant testing methods.

The main goal of this study was to compare the efficacy results for three biofilm disinfectant tests, where each test utilized a different type of reactor fluid dynamics: turbulent flow, laminar flow, and no flow. Also included in the comparative study were disinfectant challenge tests against dried biofilm and a current hard surface carrier test method. Each biofilm growth reactor and associated test method chosen for this comparative study has potential for standardization, and consequently is a candidate method for the development, testing and registration of antibiofilm disinfectants. Therefore, the results include the statistical characteristics, such as the mean viable cell density on control carriers and the repeatability SD for LR values, for each of the individual tests.

2. Materials and methods

2.1. Bacterial species/strains and inoculum preparation

Pseudomonas aeruginosa ATCC 15442 were grown in 300 mg tryptic soy broth (TSB) l^{-1} and *Staphylococcus aureus* ATCC 6538 were grown in 30 g TSB l^{-1}. Both were incubated for 18–24 h in a 37 °C shaker. Bacteria were transferred no more than 5 times from the original culture stock.

2.2. Coupons and cleaning procedure

All five test methods used borosilicate glass coupons, which were disks having a diameter of 1.27 cm and a height of 0.4 cm (BioSurface Technologies, Corp., Bozeman, MT). Prior to use, the coupons were visibly inspected and discarded if flawed. They were cleaned according to ASTM E2196-02 (ASTM International, 2002).

2.3. CDC biofilm reactor method (CDC)

A high shear biofilm was grown in the CDC biofilm reactor (model CBR 90-1, BioSurface Technologies Corp., Bozeman, MT) as described by Goeres et al. (2005) and shown in Fig. 1. A 1 ml volume of *P. aeruginosa* or *S. aureus* inoculum was inoculated into the reactor containing 300 mg TSB l^{-1} or 30 g TSB l^{-1}, respectively. The reactor stood on a digital stir plate set at 180 rpm for *P. aeruginosa* or 125 rpm for *S. aureus*. The digital stir plate controlled the rotating baffle, which is that component of the reactor that produced fluid shear on the coupon surfaces. *P. aeruginosa* biofilm was grown at 23 ± 1 °C in batch conditions for 24 h, followed by a continuous flow of 300 mg TSB l^{-1} at a rate of 11.45 ml min^{-1} for an additional 24 h. The *S. aureus* biofilm was grown at 35 ± 2 °C in batch conditions for 24 h, followed by a continuous flow of 3 g TSB l^{-1} at a rate of 11.47 ml min^{-1} for an additional 24 h.

2.4. Drip flow biofilm reactor method (DF)

A low shear biofilm was grown in a modified drip flow reactor (model DF 202, BioSurface Technologies Corp., Bozeman, MT), as shown in Fig. 1. The previously described reactor system (Stewart et al., 2001) was modified to accommodate rubber sheeting machined to hold one coupon in each of the four channels. Each channel, containing 20 ml of 300 mg TSB l^{-1} or 30 g TSB l^{-1}, was inoculated with 1 ml of the *P. aeruginosa* or *S. aureus* inoculum, respectively. The *P. aeruginosa* biofilm was grown at 23 ± 1 °C and the *S. aureus* biofilm was grown at 35 ± 2 °C in batch for 24 h. Continuous flow was started immediately by placing the reactor on a stand with a 10 °C angle and pumping 300 mg TSB l^{-1} through at a flow rate of 0.82 ml min^{-1} per channel for *P. aeruginosa* or 3 g

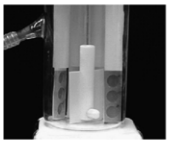
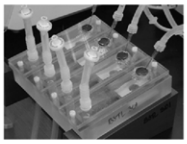
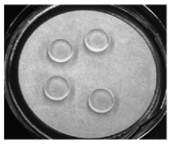
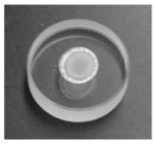
	(a) CDC reactor	(b) DF reactor	(c) SB system	(d) DS inoculum
				
Reactor type	Continuous stirred tank reactor	Plug flow	Batch	Batch
Fluid shear	High	Low	None	None
Mixing	Well mixed	Dispersive	None	None
Growth media	Aqueous (liquid)	Aqueous (liquid)	Agar (solid)	Not applicable
Biofilm or planktonic bacteria	Biofilm	Biofilm	Biofilm	Planktonic

Fig. 1. Four systems were used to establish bacteria on the surfaces of disk-shaped glass coupons; each coupon was 1.27 cm diameter × 0.4 cm high. (a) Three coupons were held by each of 8 rods during biofilm growth in the CDC system. Each rod was adjusted so that each coupon face was perpendicular to the rotating baffle (white structure adjacent to the coupons in the photo) when the baffle passed by the coupon. The reactor cap and tubing are not shown in this photo. (b) In the four-channel DF system, each channel held one coupon, which appears in the photo as a light grey circle within a white, rectangular coupon holder under the needle and septum (upper right). The growth media dripped from the needle during biofilm growth. The white-topped vertical tubes are filtered air vents. (c) In the SB system, each coupon (four shown) rested on inoculated filter paper and the biofilm grew on the underside of the coupon, next to the paper through which the growth media diffused. (d) In the DS test, a 10 µl drop containing the inoculum and organic soil was placed on the face of the coupon. The drop was dehydrated to leave a circular dried organic film attached to the coupon.

TSB l^{-1} at a flow rate of 0.92 ml min^{-1} per channel for *S. aureus* for 24 h.

2.5. Static biofilm method (SB)

A biofilm was grown with no fluid shear according to a modification of the method described in Charaf et al. (1999), as shown in Fig. 1. Sterile filter paper (Whatman Qualitative Grade 2, 70-mm diameter; Whatman, Springfield Mill, UK) laying on R2A or tryptic soy agar (TSA) plates was inoculated with 1.5 ml of a 1:10 diluted *P. aeruginosa* or *S. aureus* inoculum, respectively. Sterile coupons were placed on top of the inoculated filter paper. The plate was incubated for 48 h at 37 °C in a humidified incubator. Halfway through the incubation period, the filter paper was remoistened with 1.5 ml of a 30 mg TSB l^{-1} for *P. aeruginosa* or 3 g TSB l^{-1} for *S. aureus*.

2.6. Dehydrated biofilm method (DB)

Four coupons were harvested from the CDC biofilm reactor and dried for 2 h according to ASTM Method E-2197-02 (ASTM International, 2003).

2.7. Dried surface test method (DS)

A modification of ASTM Method E-2197-02 (ASTM International, 2003) was used to establish planktonic bacteria on a coupon in a dried organic film. Approximately 16 ml of a 72 h planktonic culture of *P. aeruginosa* or *S. aureus* was centrifuged for 10 min at 2000 rpm The supernatant was removed and the cells restored to a final volume of 0.4 ml in sterile buffered dilution water. Then 0.34 ml of the concentrated cells were mixed with 0.16 ml of simulated organic soil consisting of 2500 mg bovine serum albumin Fraction V l^{-1}, 3500 mg tryptone l^{-1}, and 800 mg mucin l^{-1}. The cell mixture was vortexed and 10 µl volumes were pipetted onto sterile coupons. The inoculated coupons were dried for 2 h according to ASTM Method E-2197-02 (ASTM International, 2003).

2.8. Reynold's number calculations for CDC and DF

For these calculations, the bulk fluid was assumed to possess the properties of water at 20 °C when growing a *P. aeruginosa* biofilm or at 37 °C when growing a *S. aureus* biofilm. The Reynold's number at the interior coupon surface in the CDC reactor was estimated based upon fluid equations developed for two concentric cylinders where the inner cylinder rotates (Characklis et al., 1990). For the *P. aeruginosa* biofilm with the stir plate set at 180 rpm, the Reynold's number was approximately 1850. For the *S. aureus* biofilm with the stir plate set at 125 rpm, the Reynold's number was approximately 800. In the CDC biofilm reactor, flow changes from laminar to turbulent at a Reynold's number of approximately 700 (Bird et al., 2002; p. 92).

The Reynold's number in the DF reactor was estimated using equations developed for flow on an inclined plane (Bird et al., 2002; p. 46). A fluid velocity of 1.06 m min^{-1} was measured for a *P. aeruginosa* biofilm after 24 h of continuous flow. A fluid thickness of 175 µm was calculated based upon DF reactor

geometry and the measured velocity. The resulting Reynold's number was 12, which is considered laminar flow with negligible rippling (Bird et al., 2002; p. 46). A fluid velocity of 1.70 m min^{-1} was measured for a *S. aureus* biofilm after 24 h of continuous flow. The fluid thickness was calculated to equal 290 μm and the resulting Reynold's number was 20, which is at the boundary between laminar flow with negligible rippling and laminar flow with pronounced rippling.

2.9. Treatment and control formulations

The control reagent was AOAC synthetic hard water (AOAC International, 1995) prepared with a total hardness of 400 mg l^{-1} and pH of 7.2±0.5. Treatment formulations, described in Table 1, were prepared in AOAC synthetic hard water with a total hardness of 400 mg l^{-1} and a pH adjusted to 7.2±0.5.

2.10. Preparation of neutralizers

A neutralizing agent, described in Table 1, was added to both the treatment and control vessels immediately following treatment. Every neutralizer used in this study passed the criteria stated in the ASTM Neutralization Assay E-1054-02 (ASTM International, 2004), data not reported.

2.11. Disinfectant concentration measurements

The pH was measured with a calibrated pH electrode (Denver Instruments, Denver, CO). Free available chlorine was measured according to the DPD Colorimetric Standard Method 4500-Cl G (APHA, 1995) using a standard curve prepared with potassium permanganate and Hach free chlorine packets (Cat. No. 1407799, Hach Co., Loveland, CO). Quaternary ammonium chloride (QAC) was measured according to the direct binary complex method (Hach Method 8337) using a standard curve prepared with CTAB and Hach QAC reagents (Cat. No. 24010-66 and 24012-68). Phenol was measured according to the 4-aminoantipyrine Standard Method 5530 D (APHA, 1995) using a standard curve prepared from reagent grade phenol and CHEMetric phenolic vacuvials (Cat. No. K-8003, CHEMetrics, Inc., Calverton, VA).

2.12. The typical experiment

In each experiment, a single concentration of one disinfectant was tested against one of the bacterial species by each of five test methods run in parallel (side-by-side) so that the comparisons of log reductions would be free of variability due to the uncontrolled factors (e.g., lab temperature, atmospheric pressure, humidity, etc.) that commonly cause disinfectant test outcomes to differ appreciably among repeats. For all five tests, the same techniques were used to apply the treatment, neutralize, count viable cells, and calculate the log reduction. The same technician conducted all the experiments. All tests utilized four coupons, two disinfected and two control, assigned at random.

The treat and analyze steps for the coupons were conducted as follows. Four coupons from the CDC, DF and SB systems were removed from their respective reactors and rinsed by gentle dipping in 10 ml of sterile buffered dilution water to remove loosely attached cells. Each coupon was placed in a separate, sterile 20 ml glass beaker. Each of four dehydrated DS coupons and four dehydrated CDC coupons was placed in a separate sterile 20 ml glass beaker. Then 10 ml of the appropriate treatment formulation was gently pipetted into each beaker. After a 10 min contact time, the neutralizer was immediately added to both the control and disinfected beakers. After 10 min in the neutralizer, the coupon was removed from the beaker.

Following methods described in Zelver et al. (2001), the bacteria were removed from the top surface of each coupon and the viable cell density for the suspension of removed bacteria was determined. In brief, cells were scraped into a dilution tube containing 10 ml of 1:10 diluted neutralizer, the suspension was homogenized to disaggregate biofilm clumps, serially diluted, and drop plated (Herigstad et al., 2001) using R2A agar for *P. aeruginosa* or TSA for *S. aureus*. Plates were incubated overnight at 37 °C, colony forming units (cfu) were enumerated, and the density of viable bacteria (cfu cm^{-2}) was calculated for each coupon. If no colonies grew at the first dilution, an artificial count of 0.5 cfu was substituted for the observed zero prior to calculating the density.

Table 1
Disinfectant treatments and neutralizers, with concentrations, used in the parallel evaluation of five test methods

Organism	Treatments		Concentration (mg l^{-1})	Neutralizers	
	Generic: trade name	Active ingredient(s)		Main ingredients	Concentration (g l^{-1})
P. aeruginosa	Chlorine: bleach	Sodium hypochlorite	100, 500	Sodium thiosulfate	11.2
	Quaternary ammonium chloride: Expose II 256	QAC, EDTA	100, 300	D/E neutralizing broth	19.5
	Phenolic: Divosan	*o*-benzyl-*p*-chlorophenol, *o*-phenylphenol	1000	Polysorbate 80, lecithin, TSB	5, 5, 15
			2000	Polysorbate 80, lecithin, TSB	10, 10, 15
S. aureus	Chlorine: bleach	Sodium hypochlorite	500, 1000	Sodium thiosulfate	11.2
	Quaternary ammonium chloride: Expose II 256	QAC, EDTA	200	Polysorbate 80, lecithin, TSB	10, 10, 15
			600	Polysorbate 80, lecithin, TSB	20, 20, 15
	Phenolic: Divosan	*o*-benzyl-*p*-chlorophenol, *o*-phenylphenol	1500	Polysorbate 80, lecithin, TSB	5, 5, 15
			2500	Polysorbate 80, lecithin, TSB	10, 10, 15

2.13. Experimental series

Two series of experiments were conducted, one with *P. aeruginosa* and the other with *S. aureus*. For each species, an experiment was conducted for each of two concentrations of each of three disinfectants. Every experiment was repeated at a different time.

2.14. Statistical methods

The density recorded for each coupon was $\log_{10}$-transformed. All statistical calculations were performed on the log density values. For each test, the log densities were converted into a log reduction (LR) measure of efficacy. The LR is the mean log density for control coupons minus the mean log density for the corresponding treated coupons (Zelver et al., 2001).

Statistical analyses were conducted to compare the five test methods, to calculate the mean and repeatability standard deviation of log density values for control coupons for each method, and to calculate the mean LR and repeatability SD of LR values for each method. Because each concentration x disinfectant constitutes a separate "treatment," there were six treatments in all. For each species, there were at least 12 independent experiments, two replicates of each of six treatments. Each experiment produced one LR for each test method. All the LR values were plotted for quality control purposes and for visualizing the concentration-response effects for each disinfectant for each test method (graphs not shown). The *P. aeruginosa* and *S. aureus* data were analyzed separately.

To compare methods, the mean difference between the LR values for each pair of test methods was calculated and tested for statistical significance using a two-sided *t*-test. The control coupon log density values for each test method were separately analyzed using a one-factor, random effects analysis of variance (ANOVA; Neter et al., 1996). The output was the overall mean control log density and the repeatability SD of the mean across two control coupons. The ANOVA showed the percentage of the repeatability variance that was attributable to within-experiment (different log densities among coupons) and between-experiment sources.

Preliminary analysis of the LR values indicated that, for each test method, the variance among replicates for stronger treatments was different than the variance for weaker treatments, but the variances were about the same for treatments of the same strength. Consequently, two separate ANOVAs were conducted for each method, one using all LR values for the weaker treatments (treatments for which at least one of the

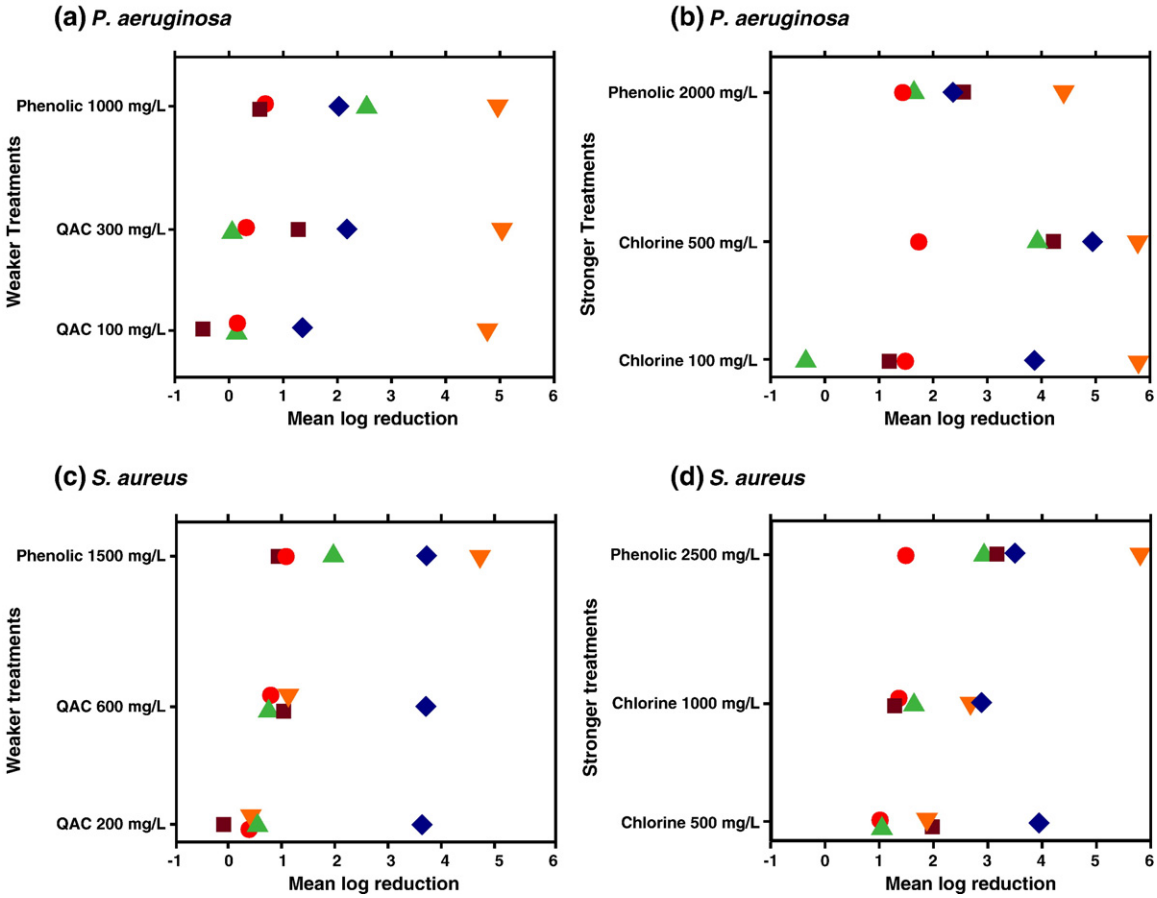


Fig. 2. Mean LR for each test method, averaged over replicate experiments at each combination of concentration, disinfectant, and species. Panels (a) and (b) are for tests against *Pseudomonas aeruginosa* and panels (c) and (d) are for tests against *Staphylococcus aureus*. Panels (a) and (c) show the results for the weaker treatments. Panels (b) and (d) show results for the stronger treatments. In each panel, ● = CDC, ■ = DF, ♦ = SB, ▲ = DB, and ▼ = DS.

hydrated biofilm methods, CDC, DF, or SB, produced a LR less than or equal to 1.0) and the other using all LR values for the stronger treatments (treatments for which all of the methods CDC, DF, and SB produced LRs greater than 1.0). Each ANOVA was based on a one-factor, fixed effects model for treatments (Neter et al., 1996). The estimated repeatability SD of LR for a method and treatment class was the square root of the mean squared error produced by the ANOVA.

Because extra replicate experiments were conducted for selected species x treatment combinations and because laboratory errors invalidated one SB experiment, one DS experiment, and a very few individual coupon counts, the actual numbers of coupons, samples, etc., that are shown in the final summary tables differ slightly from the design presented above.

3. Results

3.1. Comparing LR values among the five test methods

The mean LR values for each of the five test methods for each of the six treatments for each species are shown in Fig. 2. In each panel of Fig. 2, the spread of LR values among test methods covers a range of at least 4 logs.

3.1.1. Comparisons among hydrated biofilm methods

For the *P. aeruginosa* biofilm, the LRs were inversely related to fluid shear. This trend was evident for the *S. aureus* biofilm also, but mainly because of the high LRs observed for the SB method. The mean differences and *p*-values for comparing each pair of hydrated biofilm tests are shown in Table 2. The SB method produced significantly higher LRs than the CDC and DF methods, although the difference between SB and DF was not statistically significant for the *P. aeruginosa* tests. For the stronger treatments, the DF test produced larger LR values than the CDC test, but the differences did not achieve statistical significance.

Table 2
Comparison of the three hydrated biofilm test methods

Test methods	Weaker treatments	Stronger treatments
	Pseudomonas aeruginosa	
	quat, 100 & 300 mg l^{-1}; phenol, 1000 mg l^{-1}	chlorine, 100 & 500 mg l^{-1}; phenol, 2000 mg l^{-1}
DF minus CDC	0.08 (0.87)	1.12 (0.10)
SB minus CDC	1.49 [a] (0.01)	2.20 [b] (0.03)
SB minus DF	1.41 (0.10)	1.07 (0.18)
	Staphylococcus aureus	
	quat, 200 & 600 mg l^{-1}; phenol, 1500 mg l^{-1}	chlorine, 500 & 1100 mg l^{-1}; phenol, 2500 mg l^{-1}
DF minus CDC	−0.06 (0.90)	0.85 (0.13)
SB minus CDC	2.92 [a] (<0.01)	2.33 [a] (<0.01)
SB minus DF	3.02 [b] (0.03)	1.47 [a] (<0.01)

Each entry is the mean of the difference between LR values, averaged over experiments and disinfectant treatments, summarized separately for the weaker treatments and the stronger treatments. In parentheses after each entry is the two-tailed *p*-value for the null hypothesis that the true mean difference is zero.
[a] Statistically significant at the 0.01 level.
[b] Statistically significant at the 0.05 level.

Table 3
Comparison of the dried surface (DS) LRs to the hydrated and dried biofilm test methods

Test methods	Weaker treatments	Stronger treatments
	Pseudomonas aeruginosa	
DS minus CDC	4.57 [a] (<0.01)	3.97 [a] (<0.01)
DS minus DF	4.49 [a] (<0.01)	2.83 [b] (0.02)
DS minus SB	3.09 [a] (<0.01)	1.34 [b] (0.03)
DS minus DB	4.02 [a] (<0.01)	3.52 (0.07)
	Staphylococcus aureus	
DS minus CDC	1.30 (0.14)	2.00 [b] (0.02)
DS minus DF	1.64 (0.18)	0.96 (0.11)
DS minus SB	−2.16 [b] (0.04)	−0.51 (0.52)
DS minus DB	0.99 (0.16)	1.48 [a] (0.01)

Each entry is the mean of the difference between LR values, averaged over experiments and disinfectant treatments, summarized separately for the weaker treatments and the stronger treatments. In parentheses after each entry is the two-tailed p-value for the null hypothesis that the true mean difference is zero. Weaker and stronger treatments are as identified in Table 2.
[a] Statistically significant at the 0.01 level.
[b] Statistically significant at the 0.05 level.

3.1.2. DS compared to CDC, DF, SB and DB

The mean difference of LR values between the DS test and each of the other four tests are compared in Table 3. For the *P. aeruginosa* tests, the DS LR values were significantly higher than for the CDC, DF, and SB hydrated biofilm methods. For the *S. aureus* tests, however, the DS method produced LR values that were smaller than the SB method, and the DS LRs were only 1.0 to 2.0 larger than those for the CDC and DF tests.

For *P. aeruginosa*, bacteria in the dried biofilm were much more difficult to kill than planktonic bacteria inoculated and dried on a surface. The mean difference (DS minus DB) in LRs was approximately 4.0. For *S. aureus*, the greater stringency of the DB test relative to the DS test was evident, but not so pronounced.

Table 4
Statistical characteristics of the control coupon log densities for each of the five test methods and both bacterial species

Test	Mean log density (cfu cm^{-2})	Two coupon repeatability SD	Percentage contribution	
			Within experiment (%)	Between experiment (%)
Pseudomonas aeruginosa (12 experiments, 2 control coupons per experiment)				
CDC	8.5	0.211	10	90
DF	8.5	0.788	18	82
SB	6.0	0.208	29	71
DB	6.6	0.548	33	67
DS	5.3	0.435	16	84
Staphylococcus aureus (13 experiments, 2 control coupons per experiment)				
CDC	8.3	0.224	36	64
DF	8.6	0.586	89	11
SB	6.6	0.330	29	71
DB	8.3	0.322	32	68
DS	7.9	0.157	29	71

Table 5
Repeatability SD (degrees of freedom) of LR values for each combination of test method, relative treatment strength, and microbial species

Test methods	Weaker treatments		Stronger treatments	
	P. aeruginosa	*S. aureus*	*P. aeruginosa*	*S. aureus*
CDC	0.11 (3)	0.31 (3)	0.39 (3)	0.36 (4)
DF	1.12 (3)	1.34 (2)	0.19 (3)	1.27 (5)
SB	1.05 (3)	0.48 (2)	1.50 (3)	0.95 (5)
DB	0.49 (3)	0.26 (3)	2.94[a] (3)	0.45 (4)
DS	0.58 (3)	0.46 (3)	0.70 (2)	0.79 (5)

Weaker and stronger treatments are as in Table 2.
[a] Large SD due to an unusually large LR in one experiment.

3.1.3. DB compared to CDC, DF, and SB

The DB method was quite variable compared to the three hydrated biofilm methods (data not shown). The DB test produced the lowest mean LR among all the biofilm methods for the low concentration of chlorine and *P. aeruginosa* and the highest LR for the low concentration of the phenolic and *P. aeruginosa* (Fig. 2).

3.2. Control coupon log densities

For the log densities on control coupons, Table 4 displays the mean, the repeatability SD, and the relative contribution of the between-experiment variance. The CDC method had a low repeatability SD for both species. The DF method had the highest repeatability SD. With the exception of the *S. aureus* DF data, the between-experiments variance was always a larger component of the repeatability SD than was the within-experiment variance.

The CDC and DF methods grew both *P. aeruginosa* and *S. aureus* biofilm to a mean log density of approximately 8.5 whereas the SB method achieved a mean log density between 6.0 and 6.6. For the dried biofilm and dried surface tests, the drying step reduced the numbers of *P. aeruginosa* cells, although it did not much affect the *S. aureus* viable cell counts. For *P. aeruginosa*, the DB mean log density was about 2.0 less than the CDC mean log density. For *S. aureus*, both the DB and CDC mean log densities were 8.3. The means of control log densities for the DS method were 7.9 for *S. aureus* and 5.3 for *P. aeruginosa*.

3.3. Repeatability SD of LR values

The repeatability SD of LR for each combination of the test methods, species, and treatment strength are listed in Table 5. A literature review (Tilt and Hamilton, 1999) showed that, for a selection of standard suspension and dried surface antimicrobial tests, the repeatability SD for LR ranged from 0.2 to 1.2, with a median of 0.5. These values can be used as rough guidelines for evaluating an antimicrobial test method. From this point of view, the CDC and DS methods exhibited good repeatability SDs, ranging from 0.11 to 0.39 for CDC and from 0.46 to 0.79 for DS. The DB method also exhibited good repeatability, except for one large repeatability SD due to a suspicious, outlying LR value observed when testing the high chlorine concentration against *P. aeruginosa*.

4. Discussion

The challenge when estimating "real-world" efficacy in the laboratory is deciding which factors most influence the outcome, then choosing a method that incorporates those factors as part of the test. Biofilms grow in diverse and dynamic environments where factors such as fluid shear are important. The results of this investigation convincingly demonstrated that biofilm reactor choice is critical. Biofilm grown under high fluid shear conditions (CDC) produced the smallest LR, biofilm grown in the absence of fluid shear (SB) produced the largest LR, and biofilm grown under low fluid shear conditions (DF) produced an intermediate LR.

The goal of this paper was to evaluate and compare different methods that could be used for efficacy testing of antibiofilm treatments. There was no intent to evaluate and compare the disinfectants, to compare the species, or to address other basic biological and disinfection issues of interest when developing antibiofilm formulations. Nothing should be inferred from the partitioning of the results into "weaker" and "stronger" treatments where the breakpoint was LR = 1. The weaker/stronger terminology was just a convenient way to describe the partitioning of the data into two groups, so that there was homogeneous statistical variability within each group. That partitioning led to a more powerful statistical comparison of the test methods.

The strategy of running the five test methods side-by-side was the main feature that provided sufficient power to detect differences among the methods. The side-by-side differences were free of the day-to-day sources of variability that can greatly affect antimicrobial tests. Such factors as the disinfectant formulation, the dilution water, and the lab temperature were exactly the same for all five methods. The investigators believe that the experiments described in this paper are the first side-by-side (parallel) comparisons of disinfectant tests against biofilm grown under different fluid shear conditions, the first parallel comparisons of a dried surface test to biofilm tests, and the first experiments to compare dried biofilm tests to hydrated biofilm tests and to dried surface tests (dried planktonic bacteria). Because experiments were conducted against each of two bacterial species (the gram-negative *P. aeruginosa* and the gram-positive *S. aureus*), for two concentrations of each of three different disinfectants, and each experiment was replicated, there is some assurance that the observed differences between methods were consistent and repeatable.

Simões et al. (2003a,b, 2005) found that biofilm formed under laminar flow was more susceptible to disinfection than biofilm formed under turbulent flow. Eginton et al. (1998) found that, when testing disinfectants against *P. aeruginosa* biofilm, the most firmly attached cells were the least susceptible to the treatments, the loosely attached cells were of intermediate susceptibility, and the most susceptible were the planktonic cells. Blanchard et al. (1998) conducted a single *P. aeruginosa* biofilm test of peroxygen and found a LR of 3.5 when the biofilm was grown in a low shear stress environment and 0.8 for

a no shear environment. This difference is opposite the results presented in this paper, however, the Blanchard tests were not run in parallel nor were there replicate experiments.

One might wonder whether the observation that the LR decreased as the fluid shear stress increased was simply due to using different biofilm reactors for each shear stress. To check this possibility, some further *P. aeruginosa* experiments were conducted in the CDC reactor, testing low and high concentrations of chlorine. Two CDC reactors were run in parallel, one set to a lower shear stress and one set to a higher shear stress. The experiments were replicated. The results showed that the LR achieved against biofilm from the high shear stress environment was consistently, significantly lower than the corresponding LR from the low shear stress environment (data not shown).

The results showed that a DS test was not a suitable substitute for tests against hydrated or dried biofilm if the biofilm was grown under fluid shear conditions because the DS test produced significantly higher LR values. When testing against *P. aeruginosa*, the dried surface test (DS) consistently produced a higher LR than the biofilm tests. When testing against *S. aureus*, however, the LR values for the DS test were similar to those produced by the SB test. The DS results are in agreement with Luppens et al. (2002). Luppens noted that, when testing chlorine against *S. aureus*, a dried surface test required 20 times more disinfectant to achieve the same LR as in a suspension test but a biofilm test required 600 times more disinfectant than in the suspension test. It should be noted that the developers of dried surface tests have never suggested that the tests should be used for assessing antibiofilm efficacy.

The results showed that the DB produced LR values that were similar to those for the corresponding hydrated biofilm test (CDC); i.e., dried biofilm bacteria are as tolerant to disinfection as are hydrated biofilm bacteria. The DB test may be relevant to a situation where dehydration is intermittent and the hydrated intervals supply the biofilm with nutrients for growth.

This work focused on the manner in which the bacteria became associated with a surface. The various test methods were adapted to use the same glass coupons and to follow the same steps for sampling, treating, neutralizing, and analyzing the coupons. For this reason, all of the methods are adaptations of published methods and do not explicitly correspond to a published procedure. The protocols should be examined carefully before extrapolating these results to an established method.

To provide a convincing measure of efficacy for biofilm bacteria, the laboratory test method must use a biofilm that is at least as tolerant to disinfectants as in the real-world application. Although there are many different definitions of biofilm, including the working definition that began this paper, both the CDC and drip flow reactor biofilms satisfy every definition known to the authors. Of the systems evaluated, the CDC reactor consistently provided the most conservative estimate of disinfectant efficacy.

These results show that the efficacy of a biofilm disinfectant must be measured by using a laboratory method where biofilm is grown under fluid flow conditions similar to the environment where the disinfectant will be applied.

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