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Measuring Antimicrobial Efficacy against Biofilms: A Meta-Analysis

Running Title: Measuring Antimicrobial Efficacy against Biofilms

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Abstract

Through a statistical meta-analysis of published data on antimicrobial efficacy against biofilms formed by two common bacterial species it was concluded that the particular experimental method used is the most important factor determining the outcome of the test. An expected dose-response behavior (greater killing with higher dose concentrations or longer treatment times) was observed for data sets derived from a single method, but was not observed when data from multiple studies using diverse methods were pooled. Method specific properties such as the surface area to volume ratio, areal biofilm cell density, and microbial species were shown to influence quantitative measurements of biofilm killing. A better appreciation of the method characteristics that affect anti-biofilm efficacy tests could aid decision-making related to investment in research and development and regulatory approvals for biofilm control strategies. These recommendations are offered to those working in research and development related to biofilm control: 1) report the log reduction, surface area to volume ratio, and biofilm areal cell density; 2) include data for a benchmark agent, making sure that this agent performs competitively at the dose tested; 3) measure a dose response, i.e., make measurements at multiple treatment concentrations or dose durations; and 4) use a standardized method in addition to research methods.

Introduction

Over the last several years there has been a flood of literature reports of new antimicrobial chemistries and strategies with efficacy against biofilms. These are important contributions because established biofilms are highly tolerant towards conventional antimicrobial agents (1,2) and there is a pressing demand for better approaches to controlling detrimental biofilms in medicine, dentistry, industry, and consumer environments. Molecules with anti-biofilm activity

span the chemical and biochemical spectrum and include novel synthetics, plant-derived agents, antimicrobial peptides, and a wide variety of nanotechnologies, photoactivated compounds, quorum-sensing inhibitors, and other creative strategies. The examples are too numerous to cite completely; we reference here a few recent reviews (3-7) and a few primary research articles to convey the possibility and excitement generated by this work (8-12).

Which of these approaches should be invested in? How can claims of antimicrobial activity against biofilms be evaluated and compared? Reliable and well-characterized *in vitro* methods are a critical enabling technology for answering these questions (13-16). There are few standardized methods for evaluating biofilm susceptibility to antimicrobials and it is not clear how the different methods in the literature compare or which factors govern outcomes. The purpose of the work reported in this article is to analyze literature data on antimicrobial efficacy against biofilms for patterns, determine the characteristics of the methods being used that are most important to the test outcome, and to make suggestions and recommendations for researchers and developers when turning to a biofilm antimicrobial test method.

The analysis pursued here was organized around the concept of an antimicrobial dose response (17, 18). In this simple version, an increase in biofilm killing (measured as a log reduction in viable cell counts, LR) is anticipated when the dose of antimicrobial agent (calculated as the product of dose concentration and dose duration, Ct_d) is increased.

Meta-Analysis

Data compilation and exclusions. Quantitative data on biofilm killing by antimicrobial agents was gathered from 35 published reports (19-53). These papers were identified by searching in the PubMed and Web of Science databases using keywords such as biofilm, antimicrobial,

disinfectant, biocide, and log reduction. Approximately one-third of the hits initially identified as containing quantitative data on killing also contained sufficient documentation of other parameters. The following criteria were required for a study to be included in the data set: 1) single species biofilm of either *Pseudomonas aeruginosa* or *Staphylococcus aureus*; 2) biofilm killing reported as a quantitative log reduction in viable cell numbers (or log reduction was readily calculable); 3) antimicrobial chemistry defined; 4) quantitative dose concentration, C , and dose duration, t_d , reported; 5) biofilm areal cell density, X_o , reported for untreated control in cfu cm^{-2} (or density was readily calculable); 6) surface area to volume ratio, A/V , of the biofilm treatment system reported or calculable (specifically, the biofilm-covered area divided by the total volume of antimicrobial treatment solution applied); 7) biofilm age at time of treatment reported. Data from any wild-type strain of *P. aeruginosa* or *S. aureus* were used. The data represent 13 different strains for *P. aeruginosa* and 16 different strains for *S. aureus*. Data from studies published until early 2017 were included. The resulting collection of data may not be comprehensive for the applied criteria. The data sets are tabulated in supplemental material Table S1.

Statistical analyses. Least-squares linear regression was used to examine the data from each study separately for the presence of a dose response and for the influence of individual method-specific parameters on the rate of biofilm killing. This analysis assumed that the rate of decrease of viable cells was proportional to both the antimicrobial agent concentration and the concentration of viable cells:

$$\frac{dX}{dt} = -kCX$$

Where X denotes biofilm areal cell density, t is time, k is a biofilm killing rate coefficient, and C is antimicrobial concentration. The solution to this equation, when C is constant, is

$$-\ln \frac{X}{X_o} = 2.303LR = kCt_d$$

where X_o is the initial biofilm areal cell density and $LR = \log_{10}\left[\frac{X}{X_o}\right]$. This result predicts that the log reduction should increase linearly with the dose Ct_d . To analyze for this dependence, log reductions were regressed against Ct_d on a linear scale and the coefficient of determination, R^2 , was calculated.

To evaluate the dependence of biofilm killing on features of the method used, the specific rate of killing calculated as $\log_{10}(LR/ Ct_d)$ was regressed against the $\log_{10}$ of identified methodological parameters (e.g., surface area to volume ratio) which yielded the coefficient of determination R^2 and slope. The slope on the log-log plot corresponds to the exponent in a power law dependence. For species and flow/static, an ANOVA was fit.

Data across multiple studies were analyzed by linear mixed effects models (lmm). These lmm's included a single predictor (as outlined above) and a random effect for study to estimate the variability of results across-studies. All lmm's were fit using the software R (54), package *lme4* (55). Model fit was assessed by residual plots. The conditional Akaike Information Criterion (AIC) was used to compare different lmm's (56) where smaller values indicate a better model. Also reported were the study reproducibility standard deviation was also reported (calculated as the square root of the sum of the among-study variance and the residual variance), and the conditional R^2 that is a measure of the variability of the response explained by both the predictor and the random effect in the model (e.g., Ct_d) (57, 58).

Results and Discussion

Antimicrobial dose-response against biofilm is method dependent. When biofilm killing (measured as a log reduction in viable cell counts) was plotted as a function of the product of antimicrobial concentration and exposure time (Ct_d), no coherent overall dose response was observed (Figure 1A). The coefficient of determination for a least squares linear regression of the data set graphed in Figure 1A (both microorganisms, all antimicrobial agents) is $R^2 = 0.106$. One way to interpret this value is that just 10.6% of the variation in log reduction can be attributed to the linear effect of antimicrobial dose. Separating out the data for the two microorganisms changes the correlations only slightly (Figure 1B for *P. aeruginosa*, $R^2 = 0.0378$; Figure 1C for *S. aureus*, $R^2 = 0.2561$).

Dose response is expected to be strongly influenced by the choice of antimicrobial agent, and it is reasonable to begin by inspecting the data for specific antimicrobial agents. Consider thus the data for quaternary ammonium compounds (QAC) for the *P. aeruginosa* data set (Figure 1B, blue symbols). No overall trend is evident in the nine data points from 4 studies ($R^2 = 0.2957$, $p = 0.1745$). Only when the data points from within an individual study (employing exactly the same method) are connected (dotted lines) does the expected dose response (larger log reduction with increasing dose) become visually evident. The two highlighted studies differ quantitatively, however: at a dose of approximately $1,000 \text{ mg min l}^{-1}$, the study of Campanac *et al.* (31) found a reduction about 17 times larger than the study of Buckingham-Meyer *et al.* (19). A similar picture and conclusion are arrived at by examining the *S. aureus* QAC data set over five studies (Figure 1C, blue symbols, $R^2 = 0.4197$). When data sets from a single study are inspected, they always display the expected dose response behavior (for example, see the data for thyme oil against *S. aureus* biofilm in Figure 1C, green symbols). Unfortunately, when comparing

independent studies of the same agent, quantitative results can range widely. To underscore this discrepancy, consider two investigations of the action of peracetic acid against *P. aeruginosa* biofilms (Figure 1B, magenta symbols). The two studies have internally consistent dose response behaviors. They differ dramatically, however, in their quantitative determination of efficacy. To achieve a 3-log reduction, the study of Blanchard *et al.* (48) indicates a dose of 16.5 mg min l⁻¹ whereas a dose of 84,000 mg min l⁻¹ is suggested by the data of Spoering and Lewis (24). This is a 5,000-fold difference - three orders of magnitude!

What this qualitative analysis by inspection suggests so far is that the overwhelming factor determining a dose response for an antimicrobial biofilm test is the particular method used to make the measurement. This interpretation is borne out by statistical analysis of data for individual antimicrobial agents (Table 1). Within an individual study, log reduction correlates positively and strongly with Ct_d . When data from two or more studies are combined, the correlation usually vanishes. The loss of dose response correlation when combining data from studies using different methods is made apparent in Figure 2. We conclude that antimicrobial dose-response against biofilm is highly method-dependent.

Predictors influencing outcome of an antimicrobial biofilm test. One can gain some insight into the features of a method that are most important by briefly digressing to consider the methodological differences between the Blanchard and Spoering systems. Again, these two studies both investigated the effect of peracetic acid against *P. aeruginosa* biofilms but arrived at quite different results. Blanchard used a modified Robbins device under continuous flow. Biofilm covered coupons grown in this device were transferred to a clean device and treated with a once-through flow. This resulted in a surface area to volume ratio of approximately 0.002 cm⁻¹.¹ Spoering employed the Calgary biofilm device (now marketed as the Innovotech MBEC

assay) in which biofilm grows on pegs immersed in the wells of a 96 well plate (59). This is a static system with a much, much higher surface area to volume ratio, approximately 2 cm^{-1} . The surface density of bacteria was approximately 25 times higher in the Spoering system (10^8 cfu cm^{-2}) than in the Blanchard apparatus ($4 \times 10^6 \text{ cfu cm}^{-2}$). The biofilms in these two systems were challenged at similar biofilm ages. This comparison suggests that flow condition, biofilm areal density, or surface area to volume ratio might be critical parameters in an antimicrobial biofilm test.

We identified the following five model-dependent parameters that could influence the outcome of a biofilm antimicrobial efficacy test: microbial species, surface area to volume ratio, biofilm areal cell density, static or flow conditions, and biofilm age. The choice of antimicrobial agent also influences the outcome, as already discussed above. Linear regressions were computed to assess the relative importance of these factors individually in determining the killing rate $\log_{10}(\text{LR}/C_{td})$; results are tabulated in Table 2 with additional statistical results contained in Table S2.

The method parameter that most consistently and strongly influenced the rate of antimicrobial killing in a biofilm ($\log_{10}(\text{LR}/C_{td})$) was the surface area to volume ratio of the test system (Table 2; for data set combining both species, $R^2 = 0.611$, $\text{AIC}=253$, $p = 0.0002$). For the combined data set and also for the two species individually (*P. aeruginosa* $R^2 = 0.673$, $\text{AIC}=127$, $p = 0.0081$; *S. aureus* ($R^2 = 0.329$, $\text{AIC}=132$, $p = 0.016$), the slopes of the regressed lines were close to -1, indicating a reciprocal dependence. In other words, doubling the surface area to volume ratio approximately halved the killing rate. The effect of flow rate and species were the next most important factors for the combined data set. Age and initial biofilm areal cell density did not have large influences on the killing rate for either species.

Although biofilm age did not emerge in this analysis as an influential predictor, multiple groups have reported a clear effect of biofilm age within a given study (1). The absence of correlation with other method-dependent parameters noted does not prove that these parameters are not important. The inherent variability associated with diverse experimental methods makes it difficult to detect the contribution of individual method features. Further research to understand the additional factors that influence antimicrobial susceptibility of biofilm is warranted. Some of the potentially important factors that come to mind include biofilm age, biofilm thickness (as a proxy for diffusive path length), growth rate, extracellular polymeric substances content, and temperature. The number of factors involved is a reminder to scientists working in the biofilm field to report as many of these factors as possible when describing their work (60).

For each of the *P. aeruginosa* and *S. aureus* data sets we discovered that a combination of surface area to volume ratio and biofilm areal cell density, X_oA/V , which was termed biomass density, yielded the strongest effect. This relationship for *P. aeruginosa* is graphed in Figure 3A ($R^2 = 0.672$, AIC = 132, $p = 0.005$); the relationship for *S. aureus* is graphed in Figure 3B ($R^2 = 0.335$, AIC = 129, $p = 0.017$).

Identifying superior antibiofilm agents. We return now to the question of how to compare agents so as to identify the most effective antimicrobial for a biofilm application. It will be helpful to consider a couple of specific papers to illuminate the issues.

Consider first the synthetic anti-staphylococcal pyrrolomycins reported by Yang *et al.* (8). Biofilm killing was measured in a microtiter plate assay and compared to vancomycin, a well-known antibiotic. Killing was reported in the article as a percent reduction in viable cell numbers. As it is preferable to work in terms of log reduction, we have made this conversion. A 48-h treatment of *S. aureus* biofilm with 32 $\mu\text{g/ml}$ vancomycin (22 μM) produced a 0.2 log

reduction whereas a 48-h exposure to 4 µg/ml pyrrolomycin 4 (11 µM) produced a 2.0 log reduction. Thus, the new compound outperforms the benchmark antibiotic. Examining the dose-response relationship for vancomycin (Figure 4), it can be seen that vancomycin is consistently ineffective (log reductions against biofilm cells of less than 0.5) when relatively short doses are used. Vancomycin works well when prolonged exposures are evaluated (41). In other words, beating vancomycin in the Ct_d range of 10^4 to 10^5 mg min l⁻¹ is not a particularly impressive outcome. Comparing the performance of pyrrolomycin 4 to other antimicrobials, its efficacy was respectable but not outstanding (Figure 4). This leads to the suggestion to choose a benchmark agent that exhibits competitive efficacy in the dose range of interest.

Another novel anti-biofilm drug was reported by Morrow *et al.* (40). Here the benchmark agent was the antibiotic ciprofloxacin, which falls in the middle of the pack on the overall dose-response plot for *S. aureus* (Figure 4) with a log reduction of 1.1 at a Ct_d value of 8,640 mg min l⁻¹. The new fluoroquinolone JNJ-Q2 clearly outperforms the benchmark at the same dose (Figure 4) with a log reduction of 4.1. The model used by Morrow *et al.* was robust in that it involved a four-day old biofilm, a high biofilm areal cell density, and a surface area to volume ratio above the median.

Next, we undertake to rank the potency of oxidizing biocides against *P. aeruginosa* biofilm. We take biomass density into account because we know this parameter to be important. As displayed graphically in Figure 5, biofilm killing rate ranks in this order: monochloramine > chlorine and chlorine dioxide > peracetic acid > hydrogen peroxide. Such a ranking of biofilm potency based on measurements from multiple investigations has not been previously reported.

It is worth remembering that relative potency is by no means the only or most important property of an antimicrobial agent. In practical application, cost, environmental impact, materials

compatibility, cytotoxicity, and safety are all important considerations in the selection of an antimicrobial chemistry.

One solution to the problem of comparative testing of antibiofilm agents would be for the research community to adopt a common method. Ideally this common method will have been through a rigorous standardization process such as that afforded by the American Society for Testing Materials (ASTM). There are two candidate standard methods for measuring biofilm killing. In 2017, the US Environmental Agency (EPA) issued guidance for testing the efficacy of antimicrobial products against *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilms grown in the CDC biofilm reactor (61, 62). The mature biofilm grown in this high shear system is used to test antimicrobials using the Single Tube Method (ASTM Method E2871). By design, the Single Tube Method only measures the log reduction in viable biofilm bacteria, in other words, removal is not assessed. The second candidate method is the MBEC biofilm disinfection efficacy test. Formally known as the Calgary Biofilm Device, the MBEC grows biofilm in a low shear environment on pegs molded to the top of a microtiter plate lid (59). The mature biofilm is then exposed simultaneously to multiple concentrations of biocide by placing the lid on a new microtiter plate bottom. The MBEC biofilm disinfection test has been standardized and validated as ASTM Method E2799 (63). Both of these methods have been validated in interlaboratory studies.

In summary, we performed a meta-analysis of published data on antimicrobial efficacy against biofilms formed by two common bacterial species. We conclude that the particular experimental method used is the most important factor determining the outcome of the test and that different methods can produce extremely different results even for the same antimicrobial agent. Method specific properties such as the surface area to volume ratio, areal biofilm cell density, and

microbial species can influence quantitative measurements of biofilm killing. These recommendations are offered to those working in research and development related to biofilm control: 1) report log reduction, surface area to volume ratio, and biofilm areal cell density; 2) include data for a benchmark agent, making sure that this agent performs competitively at the dose tested; 3) measure a dose response, i.e., make measurements at multiple treatment concentrations or dose durations; and 4) use a standardized method in addition to research methods.

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

PSS conceived the meta-analysis, compiled data, performed preliminary statistical analyses, and wrote the manuscript. AEP designed the statistical models, performed the statistical analyses and wrote and edited sections of the manuscript.

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Figure Legends

Figure 1. Lack of overall dose response for biofilm killing by antimicrobial agents. A, combined data for *P. aeruginosa* and *S. aureus*. B, *P. aeruginosa*; C, *S. aureus*. Dashed lines connect data points from a single study.

Figure 2. Dose response correlations for biofilm killing by specific antimicrobial agents diminish as data from multiple studies are aggregated.

Figure 3. Rate of killing of *P. aeruginosa* biofilms depends on the biomass density, X_0A/V , in the test system (A); rate of killing of *S. aureus* biofilms depends on surface area to volume ratio, A/V , in the test system (B).

Figure 4. An appropriate benchmark agent aids in comparing antimicrobial activity against biofilms.

Figure 5. Relative potency of oxidizing biocides against *P. aeruginosa* biofilms becomes apparent when biomass density is taken into account.

Table 1. Dose-response relationship statistics for antimicrobial agents tested against biofilms show strong correlation for data from a single study and mostly poor correlations when data from multiples studies are aggregated. This analysis used data sets when 3 or more data points were available from a single study, 5 or more points combined for two studies, or 3 or more points for 3 or more studies. Boldface indicates a negative slope for the regressed line, contrary to the expected positive dose response. The column SD(LR) indicates the study reproducibility SD of LRs when $M > 1$; for single studies ($M = 1$), the column indicates only the within-study SD of the LR.

Agent	Source	M	N	slope (LR/mg min ⁻¹)	p	R^2	AIC	SD(LR)
<i>P. aeruginosa</i>								
QAC	(31)	1	3	3.946	0.237	0.8676	11.9	1.12
Bismuth thiol	(38)	1	3	3.316	0.0599	0.9912	0.04	0.16
Peracetic acid	(24)	1	5	12.0	0.0009	0.9839	5	0.29
Peracetic acid	(48)	1	4	3.263	0.0692	0.8664	11.3	0.66
Chlorine dioxide	(51)	1	11	5.525	< 0.0001	0.8712	33.9	0.95
Monochloramine	(53)	1	3	13.2	0.305	0.7881	13.5	1.46
Peracetic acid	(24, 38)	2	9	4.3	0.1969	0.000	37.7	11.4
Hydrogen peroxide	(22, 25, 26, 49, 50)	4	4	-0.3125	0.643	0.1278	9	0.51

Chlorine	(19, 38, 50)	4	7	1.9377	0.0049	0.8213	27	1.06
QAC	(19, 31, 46)	4	9	1.72	0.1745	0.2957	36	1.99
Fluoroquinolone	(20-22, 38, 47)	5	5	-0.375	0.718	0.0498	23	1.51
Aminoglycoside	(20-22, 28, 38, 47)	6	6	0.013	0.994	1.5×10^{-5}	31	2.23
All	(19-22, 24-26, 28, 31, 38, 46-53)	18	63	0.195	0.4867	0.0378	284	2.31
<i>S. aureus</i>								
Thyme oil	(34)	1	8	1.7163	< 0.0001	0.9873	-5.9	0.13
Bismuth thiol	(38)	1	3	3.897	0.117	0.9667	7.8	0.57
biguanide/pyridine	(32, 36, 37)	3	3	0.1609	0.937	0.0098	15.8	2.15
vancomycin	(23, 41, 43)	3	4	2.4771	0.1356	0.9237	13	0.94
chlorine	(19, 32, 33)	4	6	0.0686	0.8765	0.000	13	0.55

QAC	(19, 27, 30-32)	5	8	2.280	0.0263	0.4197	23	1.85
All	(19, 23, 27, 29-45)	21	55	0.7825	<0.0001	0.2561	200	1.48

M – number of studies (Buckingham-Meyer *et al.* (19) counts as 2, one for CDC one for DF)

N – total number of data points

Table 2. Rate of biofilm killing ($\log_{10}(\text{LR}/C_{td})$) depends on surface area to volume ratio, microbial species, and biofilm areal cell density. See Table S2 for additional statistical results of this analysis.

Parameter	Combined			<i>P. aeruginosa</i>			<i>S. aureus</i>		
	R^2	p	slope ^{1,2}	R^2	p	slope ²	R^2	p	slope ²
Species	0.568	0.0995	0.304						
$\log_{10}(A/V)$	0.611	0.0002	-0.825	0.673	0.0081	-0.882	0.329	0.016	-0.754
$\log_{10}(X_0)$	0.560	0.4681	-0.091	0.627	0.4536	-0.186	0.304	0.053	-0.299
Flow vs Static	0.569	0.0935	0.648	0.637	0.1503	0.966	0.246	0.951	0.026
Age	0.559	0.612	-0.001	0.624	0.7294	0.0011	0.253	0.462	-0.002
$\log_{10}(X_0 A/V)$	0.582	0.011	-0.277	0.672	0.0052	-0.562	0.335	0.016	-0.291

¹For the categorical factor Species, the difference in means between *P. aeruginosa* and *S. aureus* is reported.

²For the categorical factor “Flow vs Static”, the difference in means between Flow and Static is reported.

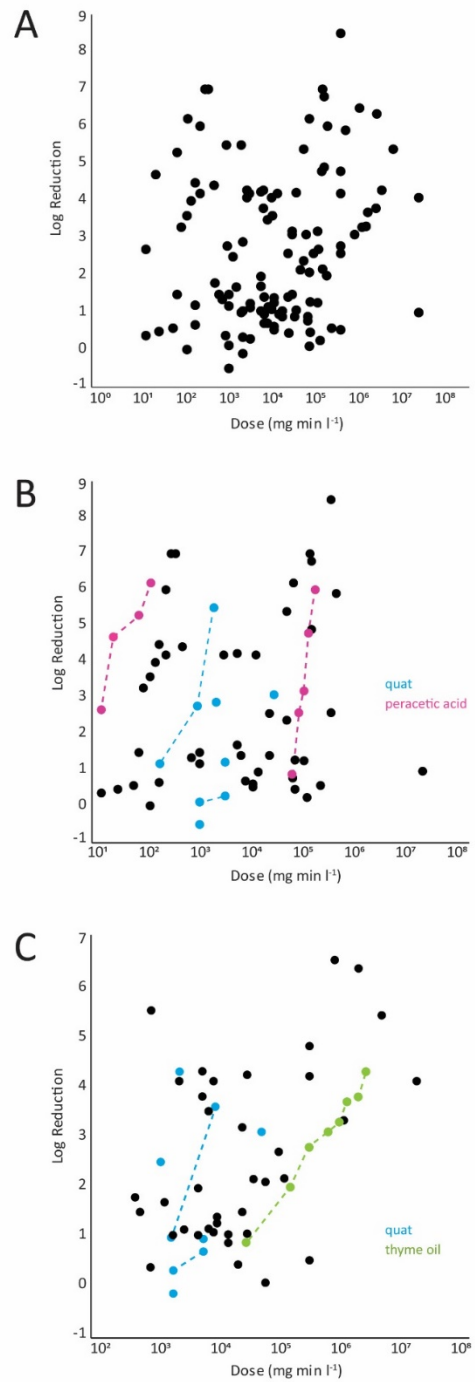


Figure 1. Lack of overall dose response for biofilm killing by antimicrobial agents. A, combined data for *P. aeruginosa* and *S. aureus*. B, *P. aeruginosa*; C, *S. aureus*. Dashed lines connect data points from a single study.

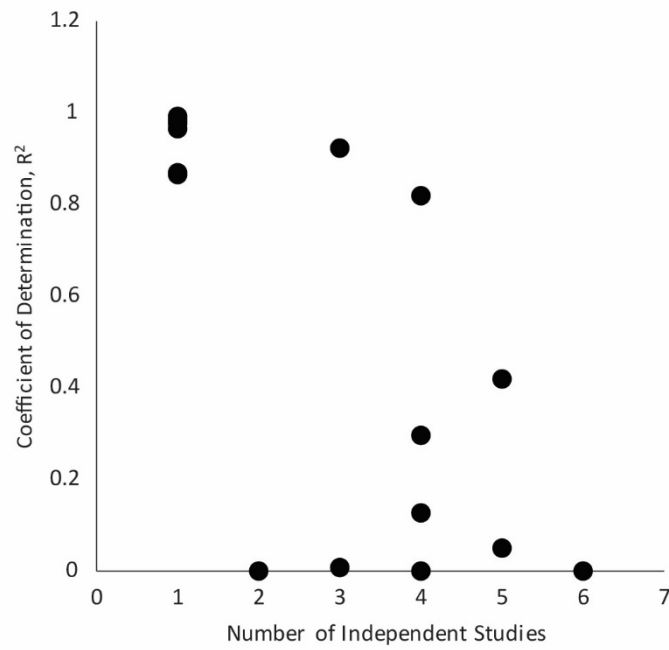


Figure 2. Dose response correlations for biofilm killing by specific antimicrobial agents diminish as data from multiple studies are aggregated.

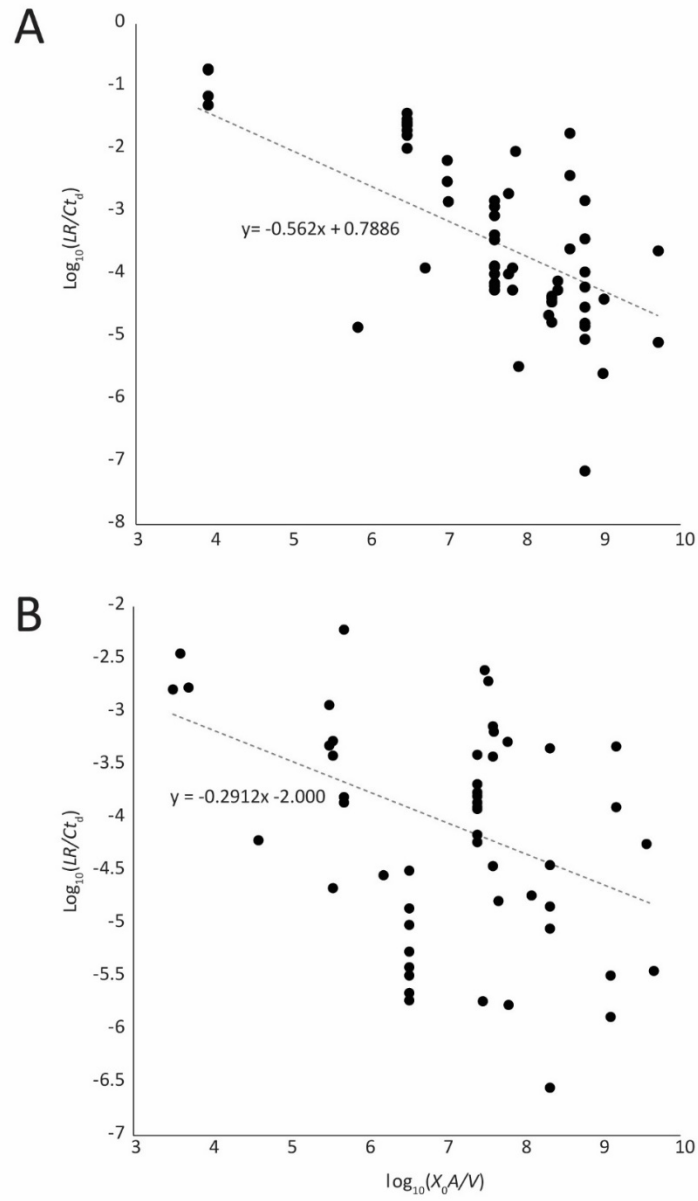


Figure 3. Rate of killing of *P. aeruginosa* biofilms depends on the biomass density, X_0A/V , in the test system (A); rate of killing of *S. aureus* biofilms depends on surface area to volume ratio, A/V , in the test system (B).

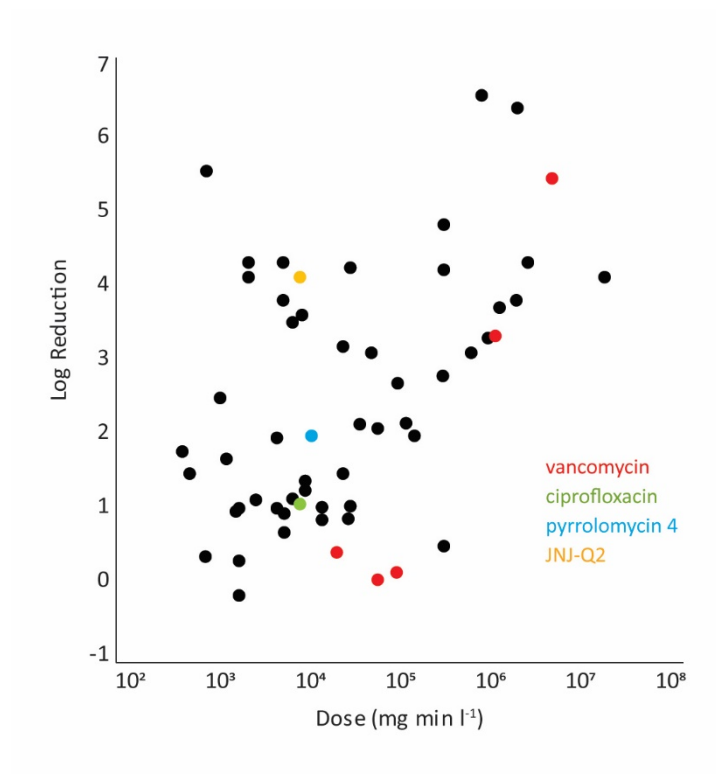


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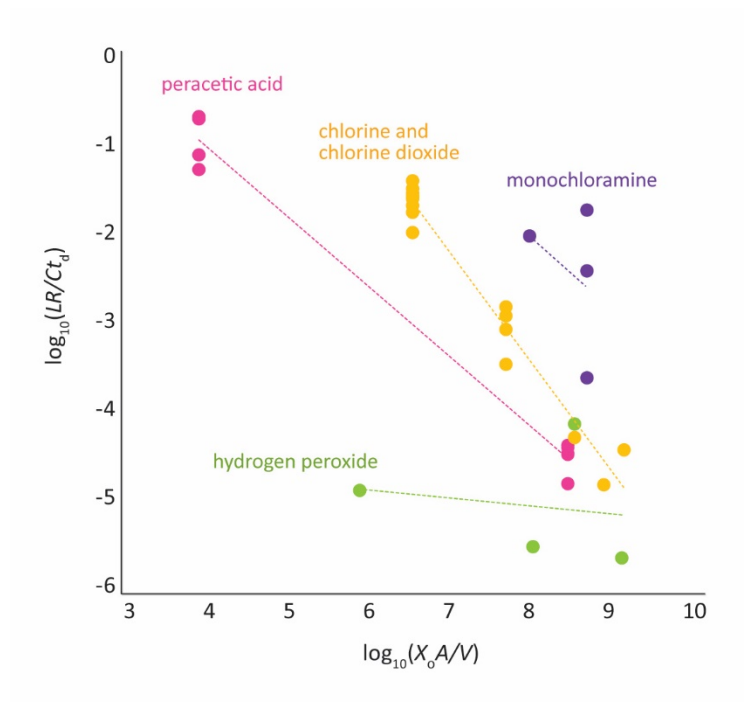


Figure 5. Relative potency of oxidizing biocides against *P. aeruginosa* biofilms becomes apparent when biomass density is taken into account.

Table S2. Additional statistical results in support of those presented in Table 2: Rate of biofilm killing ($\log_{10}(\text{LR}/C_{td})$) depends on surface area to volume ratio, microbial species, and biofilm areal cell density. The column SD(LR) indicates the study reproducibility SD of LR's when $M > 1$; for single studies ($M = 1$), the column indicates only the within-study SD of the LR. AIC denotes the conditional Akaike Information Criterion.

Parameter	Combined		<i>P. aeruginosa</i>		<i>S. aureus</i>	
	<i>AIC</i>	<i>SD(LR)</i>	<i>AIC</i>	<i>SD(LR)</i>	<i>AIC</i>	<i>SD(LR)</i>
Species	257	1.20				
$\log_{10}(A/V)$	253	1.05	127	1.18	132	0.92
$\log_{10}(X_o)$	258	1.22	133	1.29	129	0.99
Flow vs Static	257	1.19	131	1.29	132	1.04
Age	257	1.24	130	1.37	131	1.05
$\log_{10}(X_o A/V)$	258	1.13	132	1.08	129	0.94