**Approaches to biofilm-associated infections: The need for standardized and relevant biofilm methods for clinical applications.**

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Keywords: Biofilm, standard methods, MBEC, effectiveness

**Abstract**

**Introduction:** The concept of biofilms in human health and disease is now widely accepted as cause of chronic infection. Typically, biofilms show remarkable tolerance to many forms of treatments and the host immune response. This has led to vast increase in research to identify new (and sometimes old) anti-biofilm strategies that demonstrate effectiveness against these tolerant phenotypes.

**Areas covered:** Unfortunately, a standardized methodological approach of biofilm models has not been adopted leading to a large disparity between testing conditions. This has made it almost impossible to compare data across multiple laboratories, leaving large gaps in the evidence. Furthermore, many biofilm models testing anti-biofilm strategies aimed at the medical arena have not considered the matter of relevance to an intended application. This may explain why some *in vitro* models based on methodological designs that do not consider relevance to an intended application fail when applied *in vivo* at the clinical level.

**Expert commentary:** This review will explore the issues that need to be considered in developing performance standards for anti-biofilm therapeutics and provide a rationale for the need to standardize models/methods that are clinically relevant. We also provide some rational as to why no standards currently exist.

**1. Introduction**

Since the early 1970’s, an explosion of research on the concept of biofilms and their involvement in human health and disease have appeared in the medical literature [1]. This new wealth of information, driven largely by advancements in emerging technologies and techniques applicable to the study of bacterial populations in situ, have advanced the understanding of “microbial biofilms”. The concept of biofilms in human health and disease is now universally accepted in chronic wounds [2, 3]periodontal disease and dental caries [4, 5], cystic fibrosis [6-8], in-dwelling medical device infection [9, 10], otitis media and other upper respiratory infections [11, 12], orthopaedic infections [13] and tuberculosis [14].

Current definitions have described biofilms as microbes attached to surfaces or to each other in aggregates or clumps. They encapsulate in a self-produced extracellular polymeric substance (EPS) or matrix, that can also contain host derived components. As such biofilms show extreme tolerance to antimicrobials and host defenses [15-18]. A plethora of *in vitro* biofilm models have elucidated that bacterial biofilms are more tolerant to antiseptics and disinfectants [19] as well as withstanding antimicrobial concentrations 100 to 1000 times higher then that of planktonic counterparts [20-23]. In spite of the wealth of research undertaken to identify biofilm tolerance to antimicrobials, no single causative mechanism has been identified. Instead it has been suggested that a likely combination of factors contributes to biofilm tolerance [24, 25] with several areas of interest including but not limited to; slow or incomplete permeation of antimicrobials through extracellular polymeric substance (EPS) [20, 26], altered microenvironment and niches within biofilms promoting slow growth rates and adaptive stress response [27, 28], efflux pumps [29], and the role of low frequency dormant “persister” cells [30].

Regardless of whether researchers fully uncover the answers to the biofilm riddle of tolerance, the practical implications are that individual patients suffer with prolonged chronic infections that often require multiple rounds of antibiotics. [31]. The current treatment strategy for chronic infections comes at a high cost to the healthcare system and, more importantly, to the patient, both economically and in the potential loss in there quality of life.

**2. Exploring the concept of what is a relevant reduction for medically relevant biofilms?**

Antimicrobial therapies for acute infections based on minimum inhibitory concentrations (MIC) (planktonic microorganisms susceptibility to antibiotics) target rapidly multiplying planktonic microorganisms with high efficacy. Therapies based on MIC results employed against biofilm phenotype microorganisms that differ markedly in both their physiology and activity, typically fail to eradicate the problem, leading to a chronic infection for the patient. For some patients with in-dwelling medical devices for example who have failed anti-biofilm strategies, the infection cannot be resolved until the material is completely removed [32].

Further clarity is required in understanding if this lack of correlation between conventional susceptibility test results, and therapeutic success in chronic infections maybe reflective of biofilm presence. A recent Cochrane review on standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis, identified that biofilm susceptibility testing was not superior to conventional antimicrobial susceptibility testing for biofilm [33]. In fact the Cochrane review suggests that biofilm antimicrobial susceptibility testing may be more appropriate in the development of newer, more effective formulations of drugs that can be tested in clinical trials.

This aside, researchers have been driven to evaluate the efficacy of anti-biofilm strategies, using susceptibility test results based on assays that identify the minimum biofilm eradication concentration (MBEC assay) through *in vitro* models such as the Calgary biofilm device [34].

In addition to antibiotics, various alternate agents have also been explored for anti-biofilm strategies. These have included peptides, antiseptics and oral and topical antimicrobials. How these agents are delivered to the biofilm have also varied greatly with mechanisms including coatings, drug eluting, wound gels, nanoparticles, irrigations and solutions, all being explored. To further complicate the picture several alternate techniques have been developed to quantify outcome measures of these agents *in vitro* Biofilm biomass has been explored most typically in 96-well microtiter plates and flow systems using staining methods (crystal violet, Syto9 staining) with optical density (ODnm) or confocal laser scanning microscopy (CLSM) to detect live/dead cells (expressed as percentages or ODnm) [35, 36]. Plate counts to enumerate viable cells that calculate antimicrobial efficacy expressed as cfu/ml, cfu/surface area, cfu/per mg tissue have also been utilised.

Adding to the conundrum is the absence of a “target” reference value required to ascertain the “effectiveness” of anti-biofilm strategies in aiding the host immune response to clear infective microorganisms is profound. This has important consequences at a treatment level where clinicians often seek guidance from laboratory-based studies (often due the lack of *in-vivo* data) in directing them to choose the most relevant and effective agent to reduce microbial colonization/infection. Granted these decisions have historically been based around managing infections using planktonic paradigms, further highlights the requirement for data on the efficacy of anti-biofilm strategies.

Importantly when deciphering what may be a “target reference” there are two sides of the fence to consider when posing questions around the performance standards of an agent that cites claims on “effectiveness” or “efficacy”. Firstly there is a regulatory perspective that looks to determine a “target reference” based on standardized approaches using statistical attributes in determining the repeatability standard deviation and type I and type II error associated with an agent [37, 38]. Undertaking this enhances statistical confidence in the outcome that an agent is efficacious.   Secondly, is how well *in vitro* or *ex vivo* results translate to clinical efficacy and if those target references correlate to improvements in clinical symptoms and resolution of chronic infections.

With respect to the consideration of what would be a potential target value, no suggestions in the literature have been cited, and there are no data to support what a reasonable figure would be. This question in itself is complex given that a target reference value may move depending on the type of infection, the infecting strain or the immune status of the patient. For example, data to support a reasonable target reference value for *in vitro* testing, must take into consideration, that any changes in infectivity when bacteria are expressing biofilm phenotype *in vivo* may alter drastically. Until a clear pathexists, then the most conservative approach is that the drug or device must demonstrate complete eradication of the biofilm in *in vitro* testing.”  The obvious approach to determine a reference value would be to transition from *in vitro* testing to *in vivo* clinical trails, as this would allow direct observations of what worked and what did not. In many cases however, it maybe not be possible (an unethical) to obtain biofilm data directly from patients.

How the United States Environmental Protection Agency (EPA) addressed this concern for human health biofilm disinfection claims is they are proposing a 6-log reduction in biofilm. The statistics tells us that if industry wants to be “highly confident that they will achieve this target log reduction, then they need to formulate the biocide to completely kill the biofilm.  What we are missing is data, and until that data is collected, the conservative approach would be to kill everything.

**3. How should we approach assessing the “effectiveness” of anti-biofilm therapies based on *in vitro* models in order to predict clinical results**

Through out this article the discussion of biofilm infections in patients has been purposefully over-simplified, given the complexity and breathes of discussing all concepts relating to how they contribute to human infection. Biofilms exist in many niches and vary significantly. This in it self, likely restricts the ability to develop an assay that closely mimics the exact architecture of an *in vivo* biofilm that could be used universally. Conversely, it is unlikely that an assay for every infection will be developed, given the large variation in biofilm architecture from *in vitro* to *in vivo*. Whether one is evaluating biocides for use against biofilm in toilets, or antibiotics to treat chronic wounds, it is virtually impossible to perfectly mimic an actual infection or environment in the laboratory.

A potential way forward for performance testing could be to develop a simplified biofilm assay that allows standardized adaptations (calibrated) to test parameters allowing the performance of a product to aid in predicting successful *in vivo* outcomes. Whilst no *in vitro* test will provide a direct answer to this, it does provide confidence to move forward with a very costly clinical trial.

Furthermore, it is not uncommon for researchers or testing laboratories to use standard methods beyond its original intended use.  For instance, a method designed and validated to test antimicrobial urinary catheters should not be used to study venous catheters without some significant modifications. This is similar to using a standard curve developed for chlorine to determine bromine concentrations – similar but not quite the same.  Sometimes labs use the method because that is all they have available, this may exemplified with 96 well-assays or the CDC reactor.  Once a laboratory starts using microtiter plates or CDC reactor, often they will just keep reapplying the same method to other applications. This raises questions how relevant the test is anymore.  As researchers, we do our best to model what are thought to be the most important parameters to gain insights into how the biocide or antibiotic will perform when actually used.  Ideally, there will be a menu of various methods designed for different application areas.  This takes time and money, and right now researchers are in the foundation stages of model development.

Various publications have stated the need for the standardization of methods for assessing the “effectiveness” of anti-biofilm therapies. A distinct problem however, facing anyone attempting to decipher the literature and or attempting to replicate biofilm models for new therapies has been the lack of standardized methods for experimentally studying biofilms. This has caused much confusion when attempting to compare results between different research groups, and has led to large discrepancies when attempting to replicate the same results between different laboratories. The lack of methods also means there is no pathway for companies to follow when attempting to register a new device and/or drug with a regulatory agency.

In the most applied sense, standard methods development is the creation of laboratory protocols for the purpose of comparison, both within a single laboratory and among various laboratories. Researchers choose to use a standard method for various reasons. For instance because every step of the laboratory process is exactly defined, a standard method is useful for teaching proper laboratory protocol or monitoring equipment performance. The impetus for the development of many microbial standard methods, though, is efficacy testing for product registration with a regulatory agency.

Regulatory agencies require efficacy data when a product is registered to ensure the quality, safety, and efficacy of antimicrobials (biocides, disinfectants, sterilants) in circulation (in a particular country).For this purpose, standardized methods that are repeatable, reproducible, rugged and responsive are required [39]. A standard method should also be reasonable, meaning it should utilize equipment that is “typical” for a laboratory and it should not require an excessive amount of time, supplies or highly specialized training. Many biofilm research methods can uncover intriguing scientific insights even though the results are qualitative. However, regulatory authorities and standard setting organizations mostly prefer quantitative measures of efficacy.

In this manner, uniform test conditions permit comparison of results between products and laboratories. For a disinfectant to make a bactericidal claim for example, efficacy against planktonic *P. aeruginosa, Proteus vulgaris, E. coli* and *S. aureus* is required by the Australian Therapeutic Goods Administration (TGO 54) [40], with similar organisms being required by other regulatory organizations. All these organisms are good biofilm producers and are associated with clinically relevant biofilm infections so it seems reasonable to include anti-biofilm testing for these (or a subset of these) organisms. Unfortunately, standard methods only exist for biofilms formed by *P. aeruginosa* (ASTM Methods E2196, E2562, E2647, and E2799)*.*

Importantly, a relevant laboratory method should adequately emulate “real use” conditions so that a laboratory test is predictive of how well a device (or test product of interest) will perform *in vivo*. Highlighting the decontamination of equipment and clinical surfaces with disinfectants/ sterilants as an example, it is of importance for users (or clinicians) responsible for the decontamination of instruments or surfaces, to understand that the product they are using has been tested under conditions that best resemble the purpose they are to be applied, such as the hospital environment. Therefore, In terms of relevance, there are two basic strategies that researchers should strive to answer and that clinicians should strive to understand.

The first strategy is to engineer a biofilm in a laboratory test to have specific characteristics that emulates the biofilm *in vivo*, matching for example, the architecture, thickness, strength of attachment and host factors such as proteins or immune cells. This is because alterations in any of these parameters can lead to alterations in the test outcomes, for example the sensitivity of biofilm to disinfectants varies with both the age of the biofilm and the method of growth [41]. This was well demonstrated in a paper by Buckingham-Meyer where kill rate (log reduction) decreased as the amount of shear on the test biofilm during growth increased.

The ASTM standard biofilm methods were developed based upon this relevance strategy (Figure 1). By employing basic fluid dynamic concepts with regards to fluid shear and flow dynamics, the ASTM methods describe how to grow a biofilm that represents a general biofilm grown under high shear in a continuous stirred tank reactor (CSTR) (ASTM Method E2562) [42], in medium shear in a CSTR (ASTM Method E2196) [43], low shear in a plug flow reactor close to the air liquid interface (ASTM Method E2647) [44] and minimal shear in a batch reactor (ASTM Method E 2799) [45]. Others have recently reviewed the applicability of the biofilm reactors described in the ASTM Methods for various applications [46-48].

The second basic strategy in methods development involves using reactors that incorporate the most important physiochemical and biological characteristics in the environment of interest [49, 50]. An effective strategy that was followed for the development of the ASTM biofilm methods was to partition methods into sets of components. For testing the efficacy of disinfectants or antibiotics these components include: growing a repeatable and relevant biofilm, applying the antimicrobial treatment, harvesting a sample of the treated biofilm, and analyzing the sample for viable cells. To better visualize this concept Figure 2 shows a product testing and development guidance tree that outlines some of the numerous parameters under consideration for medically relevant biofilm standard methods.

In spite of the above, many researchers involved in biocide disinfection of a surface (not for medical devices or antimicrobial therapies) may still pose questions such as how well does the hydrated biofilm formed on a coupon or in the MBEC plate represent biofilm on a clinical surface which is in a semi-dehydrated state and encased in thickened EPS? It is also unlikely that a biofilm formed on coupons or in an MBEC device will present the same challenge to biocides as biofilm that has been subjected to multiple rounds of decontamination e.g. biofilm contaminating endoscope channels [51]. Biofilms form on all material types within the clinical environment, ranging from fabrics to plastics to stainless steel. Therefore, should research design questions be directed towards testing on different types of surfaces? For example, how relevant is a hard surface test as seen with current standards to killing biofilm on fabric? The CDC biofilm reactor (used in ASTM E2562) [42] uses removable coupons and thus has the capacity to compare different hard surface carriers with a range in free energy values and hydrophobicity e.g. glass, plastic, porcelain and steel. The premise of pushing the boundaries of any test condition and allowing researchers the “artistic” flexibility to mimic “real use” conditions often increases the test methodology’s in complexity. Typically, methods that try to exactly match every parameter of interest in this manner are complex and therefore, when the method is verified in an inter-laboratory study (or ring trial), they do not perform well.

**4. Expert Commentary**

Biofilm research as a whole has grown exponentially over the last two decades yet there is minimal data correlating *in vitro* results to clinical outcomes. In addition, whilst the medical community has a greater awareness of the role of biofilms in human health and disease, there are still many areas of confusion for clinicians, who in particular find it difficult to understand how *in vitro* methods translate to something of clinical relevance [52]. This begs the question, why are we not further along in the battle against biofilm associated infections? What is holding us back?

In trying to understand why the pursuit of new anti-biofilm therapies has been lethargic in some areas of medically relevant biofilm research, potential explanations are: 1. A lack of standardized methods for testing anti-biofilm models that is clinically applicable (to be discussed in the next section) [53, 54]. 2. The lack of regulatory guidance for setting performance standards for biofilm related product claims in the medical device arena. 3. A poor understanding of what defines “effectiveness” when applied to anti-biofilm strategies. 4. The slow response of industry in pursuing new anti-biofilm therapeutics, perhaps due to the lack of regulatory guidance, standard methods, the cost of research and development and the cost of appropriate human clinical trials. These factors inadvertently force the industry to test their potential anti-biofilm therapies using methods that do not correlate to clinical outcomes. 5. Lack of funding resources to support the development of standard methods. 6. The slow progression in translating anti-biofilm research and therapeutics to clinically relevant information [47].

With the explosion in evidence detailing most aspects of biofilm involvement in human health and disease, clinicians and regulatory agencies have been hesitant to accept and pursue anti-biofilm treatment strategies. In contrast, the chemical disinfection world for example, has lobbied hard for anti-biofilm claims on products and these efforts have led to the development, validation and approval of standard methods for testing of anti-biofilm products. Examples of these are five ASTM standard test methods (E2196, E2562, E2647, E2799 and E2871) (Table 1). The culmination of working towards developing standardized approaches that industry can utilise has meant that within the next two years, we may well see products with “kills biofilm” claims.

However, the overall the lack of advancement in anti-biofilms strategies from industry that include medical device / biocide companies, maybe explained in their haste to scramble towards testing their current therapeutics. Historically promoted for use against planktonic microorganisms in acute infections, the drive to ascertain if they now have an action against biofilms may explain why industry are not diversifying away from traditional antimicrobials that have a high efficacy against planktonic microorganisms, and move towards new research and development specifically targeting anti-biofilm strategies. A major contributor for this is most likely the significant investment costs required to develop new therapies utilising evidence from *in vitro* through to *in vivo*. The experimental designs in human studies for example would likely need to include a large number of patients for a statistically relevant conclusion to be reached. An example of this could be the bioengineering approaches to medical devices such as catheters and the lengthy processes required to bring a new device to market.

In tandem with a lack of investment from industry is the ever-increasing challenge to find funding to support the development of standard methods. The other point to consider is the time it takes to develop a standard method.  Once a standard operating procedure is written, the method needs to go through rigorous intra-laboratory testing to accumulate sufficient data that supports that the method is repeatable, responsive and rugged.  This process may take one to two years, depending upon how compatible for standardization the research method is.  The method is then taken to a standard setting organization where each step is critically reviewed and discussed, which may also a few years.  Finally, the method goes through a multi-lab collaborative study to determine the reproducibility of the method. Assuming the method performs well, the process is complete.  But, if the method does not do well, it goes back to a standard setting organization (i.e. ASTM group) and is modified, extending the cost and time associated with standardizing it.

What is startling is why clinicians haven’t demanded the same development of anti-biofilm therapeutics? Or why medical device companies have been slow to pursue new therapeutics. One reason to explain this slow progress is when clinicians come across a new drug and/or device, the regulations on the wording of the claim/documentation is focused on curing or preventing infection. Biofilm does not become part of the discussion. This may seem to be a case of semantics, but simply not having biofilm be part of the discussion means generally it is not included as part of the clinician’s decision making in terms of infection management. With regards to an appropriate outcome, clinicians would also need to understand what “effectiveness” of a product meant, whether biofilm was reduced (if so, by how much?) or if a 100% kill was achieved. Importantly, any reductions or killing of a biofilm would need to be associated with a reduction of infective symptoms and improved patient outcome.

For a change to happen, clinicians need to start asking if the patient has a chronic biofilm or an acute infection. In orthopaedic-device, catheter or cardiac valve related infections, clinicians are fully aware of the presence of biofilm. In fact treatment is directed at biofilm with well-documented anti-biofilm activity such as rifampin or fluroquinolones. what happens when clinicians identify biofilm as being the driver of infection, institute anti-biofilm strategies (such as the aforementioned antibiotics) yet fail to eradicate or control biofilm. This translates into demand for new strategies/treatments to cure biofilm infections.

In defence of clinicians, there are no diagnostic tools or biomarkers to help identify when biofilm is the driver of infection [55, 56]. In the age of science based medicine, how can clinicians be expected to deviate from standard measures of treating planktonic infections based on antimicrobial stewardships and make decisions to treat the infection as a biofilm infection, if there is no way to verify it?

When medical devices companies decide to pursue anti-biofilm strategies they are faced with the barriers of navigating the minefield of regulatory standards. In this instance regulatory agencies want clinical data that demonstrates a new drug or device’s ability to decrease infection rates in patients. Historically, the regulatory tests to make these claims have been based on the minimum inhibitory concentration for planktonic microorganisms (Clinical laboratory standards institute (CLSI), M02-A12, M07-A10, M100 –S26). This is different than showing that a device prevents and/or reduces biofilm. Although logically, a person cannot develop a biofilm-based infection if no biofilm forms, but this is not the outcome that is being regulated or monitored by clinicians.

Even though researchers have demonstrated that biofilm is the root cause of many chronic infections there is limited clinical biofilm data because clinicians do not routinely collect samples for biofilm specific diagnostics. Granted this would be extremely challenging, but with advancements in new non-invasive technologies, the possibility certainly exists that a mechanism for collecting these samples will exist in the future. This can be exemplified in chronic non-healing wounds complicated by biofilm, where in general practice the clinician does not collect a swab or tissue sample of the wound bed to quantify the biofilm in order to direct antimicrobial therapy to treat the infection. Based upon data from industrial research, bacterial counts in the process water do not necessarily correlate to counts on the pipe’s surface. This could also hold true in the human body. A low count in the urine does not mean that no biofilm is present; it just means that the biofilm has not grown to the point where the body is showing signs of infection. And of course it would be unethical to do a study where catheters are removed over time to record the biofilm that forms, and correlate this number to when the “typical” person begins to show signs of an infection (which is what occurs in industrial models for biofilm testing).

However, it is only useful to develop biofilm specific sampling if clinical microbiology has the tools for appropriate diagnostics. Currently confocal microscopy is considered the most direct way of demonstrating biofilms in clinical specimens [57] but these methods are time consuming and require highly specialized training.

This leads to a very important question. We do not know what the necessary log reduction in biofilm bacteria is that will ultimately cure the infection. For testing measures pertaining to the performance standards of an antimicrobial against planktonic microorganisms, the necessary reduction in microorganism counts has been defined as a greater than 3 log reduction (If the reproducibility standard deviation is 1 log10 then the antimicrobial must achieve a greater than 4 log10 reduction) [58]. Without knowing what this reference value is for biofilm-based infections, a conservative approach would be for the regulatory agencies to require that the antibiotic/device must kill everything.

**5. 5-year view**

Is there a clear path towards the direction of standardized approaches to biofilm strategies?Many examples outlined in this review article highlight the biofilm specific issues that need to be addressed in order to help provide better guidance to clinicians managing biofilm associated infections. When the performance of an anti-biofilm strategy relates to the clinical care of patients, there is a need to achieve a standardized biofilm methods “utopia”. This will provide pharmaceutical / device manufacturers all the experimental parameters required so that a collaborative study may be done. From a regulatory perspective, this would also allow for the method’s reproducibility standard deviation (SD) to be determined. This requirement is highly relevant for clinicians to appreciate, who may read a paper on a new technology that performed fabulously in a one laboratory study, did fine in an animal model, but failed miserably in a clinical trial. If an appropriate statistical analysis had been performed the probability of failure would have been predicted.  In general, a large percentage of experiments may lack the statistical attributes that are required of a standard method, and without statistics, there is no statistical confidence in the outcome.

In the same instance their needs to be delineation between absolute standard methods and research methods, with the latter affording the flexibility for researchers to advance new therapeutic strategies towards biofilm-associated infections. Roberts and colleagues made reference to this notion suggesting that researchers should not be afraid of undertaking “preliminary experiments” (non-standardized experiments), in doing so this may actually enhance the capability to better understand biofilm associated infections better (Roberts et al 2016). However, Roberts and colleagues make the same conclusion as we would, which is the most relevant system should be used based upon the questions being asked. Although preliminary experiments will allow researchers to make advances in our basic understanding of these biofilm infections, regulatory agencies require data collected with methods that have been statistically validated, which generally means the method has gone through a standardization process. Perhaps it is the reluctance of medical researchers to use standard methods that has provided a roadblock and explains why the medical field lags behind the biocide/industrial field with regards to biocide claims.

6. Key Issues

Biofilms show remarkable tolerance to many forms of treatments and the host immune response.

The lack of correlation between conventional susceptibility test results and therapeutic success in chronic infections maybe reflective of biofilm presence.

The absence of a “target” reference value required to ascertain the “effectiveness” of anti-biofilm strategies to clear infective microorganisms suggests complete eradication is required.

A potential way forward for performance testing could be to develop a simplified biofilm assay that allows standardized adaptations (calibrated) to test parameters allowing the performance of a product to aid in predicting successful *in vivo* outcomes.

No *in vitro* test provides a prediction on how well a product will work *in vivo*, but it does provide confidence to move forward onto animal models or costly clinical *in vivo* trials.

Many areas of confusion regarding anti-biofilm strategies still exist for clinicians who are caught either; 1. Finding it difficult to understand how *in vitro* methods translate to something of clinical relevance or 2. Think successful *in vitro* outcomes will provide similar results in vivo.

**Declaration of interest**

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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| --- | --- | --- | --- |
| **Biofilm model** | **Method**  | **Nutrient availability**  | **Potential applications and relevance**  |
| Rotating disc reactor (annular reactor)(ATSM E2196 – approved 2002) | This test method is used for growing a reproducible *P. aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions.  | Open systemDynamicContinuous flow | Rotating disc reactors are designed for laboratory evaluations of biocide efficacy, biofilm removal, and performance of anti-fouling materials. Example is to model a toilet bowl [59]. It is important to note that the rotating disk and CDC reactor were not originally designed to study medically relevant biofilms. |
| Drip flow reactor (ATSM E2647 – approved 2008) | This test method is to grow, sample, and analyze a *P. aeruginosa* biofilm under low fluid shear and close to the air/liquid interface. | Open systemDynamicBatch or continuous flow  | DFR are employed for growing biofilms for direct *in situ* visualization. The DFR can model environments such as food-processing conveyor belts, catheters, and the oral cavity [60] [61]. |
| CDC biofilm reactor(ATSM E2562 – approved 2007) | This test method is used for growing*P. aeruginosa* biofilm under moderate to high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one particular environment. | Open systemDynamicBatch or continuous flow | Studies that utilized this reactor showed that it could be used for detecting biofilm formation, characterizing biofilm structure [62] and assessing the effect of antimicrobial agents on the biofilm (Note there is a large body of literature on how researchers are using the CDC, DFR and MBEC for various research applications.) |
| MBEC assay / microtiter plates.(ASTM E2799 – approved 2011) | This test method specifies the operational parameters required to grow and treat a P. biofilm in a high throughput-screening assay. | Closed systemLow shear (the reactor sits on a shaker)Batch | MBEC assay allow rapid throughput of multiple samples of anti-biofilm therapeutics such as antibiotics, antiseptics, compounds and peptides [63]. |
| Single tube disinfection (ATSM 2871- approved 2013) | Standard test method for evaluating disinfectant efficacy against *P. aeruginosa* biofilm grown in the CDC biofilm reactor using the single tube method. | The single tube method is only an efficacy test. Biocides are tested in a batch system, with no mixing at room temperature. | This test was originally designed to determine the efficacy of liquid biocides against biofilm (bleach, quats, hydrogen peroxide blends, etc). Although it has been optimized using biofilm grown in the CDC reactor, the original intent was that the biofilm could originate from any biofilm reactor, as long as the appropriate controls were carried along. |

Figure 1. Commonly employed laboratory models for biofilm investigation.



Figure 2. Product testing and development guidance. The decision making process begins with understanding the mechanism of action (MOA) of the of the anti-biofilm technology. The technology then determines the regulatory claim. For instance, an antimicrobial surface would most likely be associated with a “prevents initial attachment” or “reduces biofilm accumulation” claim, whereas a biocide manufacturer would most likely pursue a “kills” or “removes” biofilm claim. The claim then determines the necessary test output that will provide the necessary data to support the claim. For instance, a test that measures the log reduction in viable biofilm bacteria would provide the relevant data for “kill” claim. The next step is to determine which laboratory growth and treatment methods best mimic the real world application. Various parameters of particular concern for biofilm methods are included in this figure, but it is important to note that the list is not exhaustive. The growth and treatment will often determine how the laboratory biofilm will be harvested and analysed. For instance, biofilm grown in microtitier plates is often not harvested, but stained directly and placed into a plate reader. Finally, every standard method must meet the statistical attributes listed in the figure. The text highlighted in red demonstrates the standardization path taken to measure the efficacy (kill) of biocides against biofilm. In this case, a single species biofilm is grown under high shear in the CDC reactor. The mature biofilm is removed from the reactor and tested under static conditions for a contact time specified by the biocide manufacture. Appropriate controls are always included. Sonication and vortexing is used to harvest the biofilm and the viable cells are enumerated using viable cells counts. Finally, the proposed method has undergone a collaborative study to verify that it meets the required statistical attributes. The text highlighted in purple demonstrates a potential strategy for testing antimicrobial surfaces engineered to prevent biofilm attachment.

References:

1. Geesey, G.G., et al., *Sessile bacteria: An important component of the microbial population in small mountain streams 1.* Limnology and Oceanography, 1978. **23**(6): p. 1214-1223.

2. James, G., et al., *Biofilms in chronic wounds.* Wound Repair Regen, 2008. **16**(1): p. 37 - 44.

3. Bjarnsholt, T., et al., *Why chronic wounds will not heal: a novel hypothesis.* Wound Repair and Regeneration, 2008. **16**(1): p. 2-10.

4. Marsh, P.D. and D.J. Bradshaw, *Dental plaque as a biofilm.* Journal of Industrial Microbiology, 1995. **15**(3): p. 169-175.

5. Marsh, P.D., *Microbiology of Dental Plaque Biofilms and Their Role in Oral Health and Caries.* Dental Clinics of North America, 2010. **54**(3): p. 441-454.

6. Lam, J., et al., *Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis.* Infection and Immunity, 1980. **28**(2): p. 546-556.

7. Costerton, J.W., *Cystic fibrosis pathogenesis and the role of biofilms in persistent infection.* Trends in Microbiology, 2001. **9**(2): p. 50-52.

8. Høiby, N., O. Ciofu, and T. Bjarnsholt, *Pseudomonas aeruginosa biofilms in cystic fibrosis.* Future Microbiology, 2010. **5**(11): p. 1663-1674.

9. Cole, S.J., et al., *Catheter-Associated Urinary Tract Infection by Pseudomonas aeruginosa Is Mediated by Exopolysaccharide-Independent Biofilms.* Infection and Immunity, 2014. **82**(5): p. 2048-2058.

10. Hola, V., T. Peroutkova, and F. Ruzicka, *Virulence factors in bacteria from biofilm communities of catheter-associated urinary tract infections.* FEMS Immunology & Medical Microbiology, 2012. **65**(2): p. 343.

11. Hall-Stoodley, L., et al., *Direct Detection of Bacterial Biofilms on the Middle-Ear Mucosa of Children With Chronic Otitis Media.* JAMA : the journal of the American Medical Association, 2006. **296**(2): p. 202-211.

12. Boase, S., et al., *The microbiome of chronic rhinosinusitis: culture, molecular diagnostics and biofilm detection.* BMC Infectious Diseases, 2013. **13**(1): p. 1-9.

13. McConoughey, S.J., et al., *Biofilms in periprosthetic orthopedic infections.* Future microbiology, 2014. **9**(8): p. 987-1007.

14. Ojha, A.K., et al., *Growth of Mycobacterium tuberculosis biofilms containing free mycolic acids and harbouring drug-tolerant bacteria.* Molecular Microbiology, 2008. **69**(1): p. 164-174.

15. Carpentier, B. and O. Cerf, *Biofilms and their consequences, with particular reference to hygiene in the food industry.* Journal of Applied Bacteriology, 1993. **75**(6): p. 499-511.

16. Costerton, J.W., et al., *Microbial Biofilms.* Annual Review of Microbiology, 1995. **49**(1): p. 711-745.

17. Elder, M.J., et al., *Biofilm-related infections in ophthalmology.* Eye, 1995. **9**(1): p. 102-109.

18. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the Natural environment to infectious diseases.* Nat Rev Micro, 2004. **2**(2): p. 95-108.

19. Otter, J.A., et al., *Surface-attached cells, biofilms and biocide susceptibility: implications for hospital cleaning and&#xa0;disinfection.* Journal of Hospital Infection. **89**(1): p. 16-27.

20. Walters, M.C., et al., *Contributions of Antibiotic Penetration, Oxygen Limitation, and Low Metabolic Activity to Tolerance of Pseudomonas aeruginosa Biofilms to Ciprofloxacin and Tobramycin.* Antimicrobial Agents and Chemotherapy, 2003. **47**(1): p. 317-323.

21. Anwar, H., et al., *Interaction of biofilm bacteria with antibiotics in a novel in vitro chemostat system.* Antimicrobial Agents and Chemotherapy, 1989. **33**(10): p. 1824-1826.

22. Machado, I., et al., *Antimicrobial Pressure of Ciprofloxacin and Gentamicin on Biofilm Development by an Endoscope-Isolated Pseudomonas aeruginosa.* ISRN Biotechnology, 2013. **2013**: p. 10.

23. Stewart, P.S. and J. William Costerton, *Antibiotic resistance of bacteria in biofilms.* The Lancet. **358**(9276): p. 135-138.

24. Olsen, I., *Biofilm-specific antibiotic tolerance and resistance.* European Journal of Clinical Microbiology & Infectious Diseases, 2015. **34**(5): p. 877-886.

25. Lewis, K., *Persister cells and the riddle of biofilm survival.* Biochemistry (Moscow), 2005. **70**(2): p. 267-274.

26. Tseng, B.S., et al., *The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin.* Environmental microbiology, 2013. **15**(10): p. 2865-2878.

27. de Beer, D., et al., *Effects of biofilm structures on oxygen distribution and mass transport.* Biotechnology and Bioengineering, 1994. **43**(11): p. 1131-1138.

28. James, G.A., et al., *Microsensor and transcriptomic signatures of oxygen depletion in biofilms associated with chronic wounds.* Wound Repair and Regeneration, 2016: p. n/a-n/a.

29. Kvist, M., V. Hancock, and P. Klemm, *Inactivation of Efflux Pumps Abolishes Bacterial Biofilm Formation.* Applied and Environmental Microbiology, 2008. **74**(23): p. 7376-7382.

30. Brooun, A., S. Liu, and K. Lewis, *A Dose-Response Study of Antibiotic Resistance inPseudomonas aeruginosa Biofilms.* Antimicrobial Agents and Chemotherapy, 2000. **44**(3): p. 640-646.

31. Rhoads, D.D., R.D. Wolcott, and S.L. Percival, *Biofilms in wounds: management strategies.* Journal of Wound Care, 2008. **17**(11): p. 502-508.

32. Kathju, S., et al., *Bacterial Biofilms on Implanted Suture Material Are a Cause of Surgical Site Infection.* Surgical Infections, 2014. **15**(5): p. 592-600.

33. Waters, V. and F. Ratjen, *Standard versus biofilm antimicrobial susceptibility testing to guide antibiotic therapy in cystic fibrosis.* Cochrane Database of Systematic Reviews, 2015(3).

34. Ceri, H., et al., *The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms.* Journal of Clinical Microbiology, 1999. **37**(6): p. 1771-1776.

35. Skogman, M.E., P.M. Vuorela, and A. Fallarero, *Combining biofilm matrix measurements with biomass and viability assays in susceptibility assessments of antimicrobials against Staphylococcus aureus biofilms.* J Antibiot, 2012. **65**(9): p. 453-459.

36. Peeters, E., H.J. Nelis, and T. Coenye, *Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates.* Journal of Microbiological Methods, 2008. **72**(2): p. 157-165.

37. Parker A, H.M., Tomasino SF, *A Statistical Model for Assessing Performance Standards for Quantitative and Semiquantitative Disinfectant Test Methods.* Journal of AOAC International, 2014. **97**(1): p. 58-67.

38. Goeres, D.M., et al., *Statistical assessment of a laboratory method for growing biofilms.* Microbiology, 2005. **151**(3): p. 757-762.

39. Hamilton, M.A.H., Gordon Cord; Goeres, Darla M.; Parker, Albert E., *Guidelines for the Statistical Analysis of a Collaborative Study of a Laboratory Method for Testing Disinfectant Product Performance.* Journal of AOAC International, 2013. **96**(5): p. 1138 - 1151.

40. Administration, T.G. *Guidelines for the evaluation of sterilants and disinfectants*. 1998; Available from: <https://www.tga.gov.au/node/5327>

41. Stojicic, S., Y. Shen, and M. Haapasalo, *Effect of the Source of Biofilm Bacteria, Level of Biofilm Maturation, and Type of Disinfecting Agent on the Susceptibility of Biofilm Bacteria to Antibacterial Agents.* Journal of Endodontics, 2013. **39**(4): p. 473-477.

42. International, A. *ASTM E2562-12, Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor*. 2012; Available from: <http://www.astm.org/cgi-bin/resolver.cgi?E2562-12>.

43. International, A. *Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown with Medium Shear and Continuous Flow Using Rotating Disk Reactor*. 2012; Available from: <http://www.astm.org/cgi-bin/resolver.cgi?E2196-12>.

44. International, A. *Standard Test Method for Quantification of Pseudomonas aeruginosa Biofilm Grown Using Drip Flow Biofilm Reactor with Low Shear and Continuous Flow*. 2013; Available from: <http://www.astm.org/cgi-bin/resolver.cgi?E2647-13>.

45. International, A. *Standard Test Method for Testing Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay*. 2012; Available from: <http://www.astm.org/cgi-bin/resolver.cgi?E2799-12>.

46. Coenye, T. and H.J. Nelis, *In vitro and in vivo model systems to study microbial biofilm formation.* Journal of Microbiological Methods, 2010. **83**(2): p. 89-105.

47. Roberts, A.E.L., et al., *The Limitations of In Vitro Experimentation in Understanding Biofilms and Chronic Infection.* Journal of Molecular Biology, 2015. **427**(23): p. 3646-3661.

48. Gomes, I.B., M. Simões, and L.C. Simões, *An overview on the reactors to study drinking water biofilms.* Water Research, 2014. **62**: p. 63-87.

49. Goeres, D.M., et al., *Evaluation of disinfectant efficacy against biofilm and suspended bacteria in a laboratory swimming pool model.* Water Research, 2004. **38**(13): p. 3103-3109.

50. Goeres, D.M., L.R. Loetterle, and M.A. Hamilton, *A laboratory hot tub model for disinfectant efficacy evaluation.* Journal of Microbiological Methods, 2007. **68**(1): p. 184-192.

51. Pajkos, A., K. Vickery, and Y. Cossart, *Is biofilm accumulation on endoscope tubing a contributor to the failure of cleaning and decontamination?* Journal of Hospital Infection. **58**(3): p. 224-229.

52. Hall-Stoodley, L. and P. Stoodley, *Evolving concepts in biofilm infections.* Cellular Microbiology, 2009. **11**(7): p. 1034-1043.

53. Peterson, S.B., et al., *Different Methods for Culturing Biofilms In Vitro*, in *Biofilm Infections*, T. Bjarnsholt, et al., Editors. 2011, Springer New York: New York, NY. p. 251-266.

54. Buckingham-Meyer, K., D.M. Goeres, and M.A. Hamilton, *Comparative evaluation of biofilm disinfectant efficacy tests.* Journal of Microbiological Methods, 2007. **70**(2): p. 236-244.

55. Høiby, N., et al., *ESCMID∗ guideline for the diagnosis and treatment of biofilm infections 2014.* Clinical Microbiology and Infection, 2015. **21, Supplement 1**: p. S1-S25.

56. Parsek, M.R. and P.K. Singh, *Bacterial Biofilms: An Emerging Link to Disease Pathogenesis.* Annual Review of Microbiology, 2003. **57**(1): p. 677-701.

57. Bjarnsholt, T., et al., *The in vivo biofilm.* Trends in Microbiology. **21**(9): p. 466-474.

58. Tomasino, S.F., *Development and assessment of disinfectant efficacy test methods for regulatory purposes.* American Journal of Infection Control. **41**(5): p. S72-S76.

59. Zelver, N., et al., *[45] Measuring antimicrobial effects on biofilm bacteria: From laboratory to field*, in *Methods in Enzymology*. 1999, Academic Press. p. 608-628.

60. Goeres, D.M., et al., *A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor.* Nat. Protocols, 2009. **4**(5): p. 783-788.

61. Woods, J., et al., *Development and Application of a Polymicrobial in vitro Wound Biofilm Model.* Journal of Applied Microbiology, 2012. **112**(5): p. 998-1006.

62. Donlan, R.M., et al., *Model System for Growing and Quantifying Streptococcus pneumoniae Biofilms In Situ and in Real Time.* Applied and Environmental Microbiology, 2004. **70**(8): p. 4980-4988.

63. Harrison, J.J., et al., *Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening.* Nat. Protocols, 2010. **5**(7): p. 1236-1254.