Biofouling detection methods that are widely applicable and useful across disciplines: a mini-review

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July 2021

https://doi.org/10.1080/08927014.2021.1926998

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Left running head: A. M. CURTIN AND H. L. BUCKLEY

Short title : Biofouling

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Abstract

Biofouling, or the build-up of microorganisms in a biofilm at the solid-water or water-air interface, is an interdisciplinary problem. Biofouling causes various issues including clogging systems, contaminating devices, and creating infections that are extremely difficult to treat, to name but a few. Therefore, engineers, pharmacologists, microbiologists, wastewater treatment operators, chemists, food preservative formulators, home and personal care product formulators, and toxicologists all play a role in studying and have an interest in solving biofouling. High-throughput studies on biofilm prevention and removal can take the form of biofilm antimicrobial microdilution susceptibility (BAMS) tests. Due to vested interests of many disciplines, the results from these tests should be applicable and useful to each discipline. This critical review analyses the focuses, biological implications, and metrics required by each discipline. The possible detection methods that could satisfy each desired metric are then summarized. The detection methods were analysed in order to recommend two methods of biofilm detection, Crystal Violet stain and the LIVE/DEAD BacLight stain, which correspond with three metrics including total biomass, log reduction, and the MIC, BPC, MBIC, MBC, BBC, and/or MBEC values. Determining these three metrics for each BAMS test will allow this type of research to be widely applicable and useful across many disciplines.

Keywords: Biofouling; interdisciplinary; antimicrobial susceptibility tests; biofilm detection

Mitacs10.13039/501100004489W.E. Cowie Faculty Innovation AwardIESVic Clean Energy Systems Accelerator ProgramEcoCanadaUN Association of CanadaThis research was funded by Mitacs RTA, the W.E. Cowie Faculty Innovation Award, the IESVic Clean Energy Systems Accelerator Program, EcoCanada SWILP, NSERC USRA (M.C. Thibodeau), the UN Association of Canada Green Spaces Program, NSERC Discovery, CFI JELF, and BCKDF.

Introduction

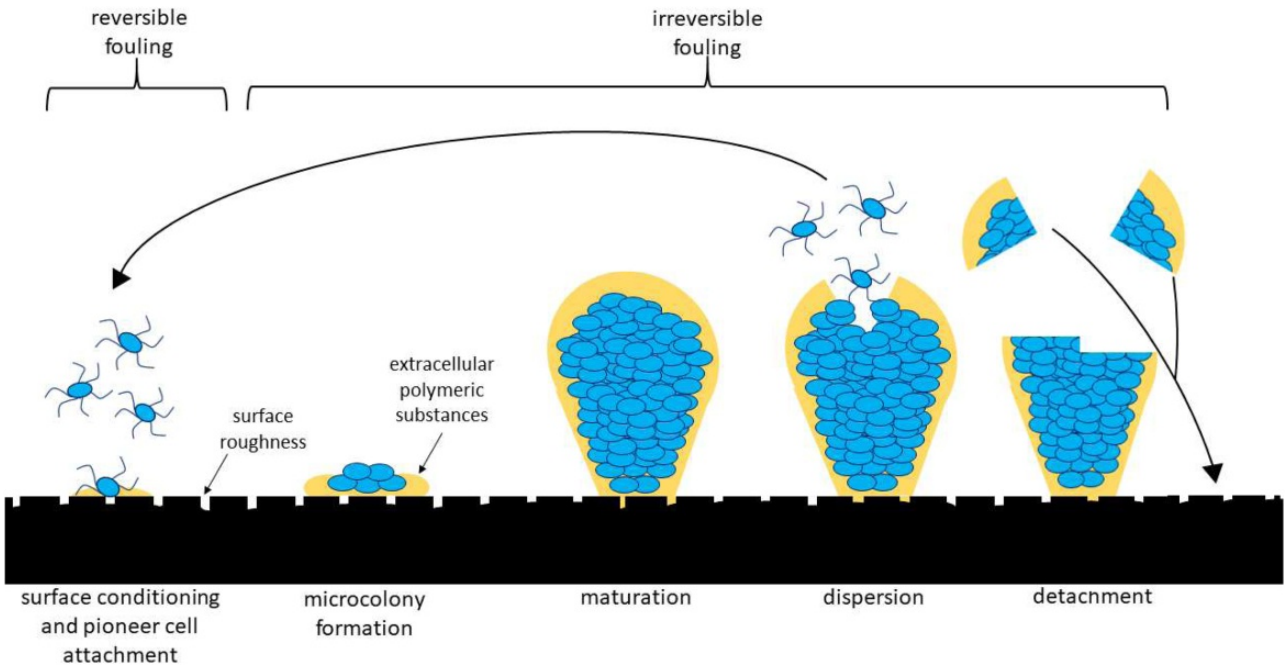
Research and practices that transcend inter-, multi-, and transdisciplinary boundaries are crucial to solving challenges that exist or are emergent in the twenty first century. Specifically, inconsistent communication styles, terminology, and reporting standards need to be standardized or made explicit. For example, Fruchter et al. (1996) highlight the difficulties associated with investigating and communicating building designs within a multidisciplinary team consisting of structural engineers and architects. The authors suggest that due to varying models for design, personal idioms from respective professions, and different media platforms for presenting designs, multidisciplinary building design

teams often have to interpret, extract, and re-enter design information in the nomenclature of their own profession. This costs time and often decreases the quality of the final product (Fruchter et al. 1996). Like building design and construction, the challenge of addressing biofouling is a multisectoral problem, engaging researchers and practitioners from engineering (i.e. Yang et al. 2021), pharmacology (i.e. Güven and Kaynak Onurdağ2014), microbiology (i.e. Wood 2009), wastewater treatment (i.e. Schopf et al. 2018), chemistry (i.e. Saeki et al. 2021), food preservative formulation (i.e. Al-Shabib et al. 2016), home and personal care product formulation (Buckley et al. 2017), and toxicology (i.e. Niska et al. 2016). Thus, similar to Fruchter et al. (1996), this field relies on successful inter-, multi-, and transdisciplinary communication. In this paper the focuses and metrics required by relevant disciplines in regard to biofouling studies are highlighted and two biofilm detection methods are recommended that, if used in conjunction, can satisfy the needs of each discipline simultaneously.

Biofouling

Biofouling is the build up of microorganisms in the form of a biofilm at the water-solid, or water-air interface. Biofouling occurs *via* a four step process: (1) conditioning of the surface by pioneer organisms or non-biological material in the bulk water, (2) attachment of pioneer organisms to the conditioned surface, (3) formation of microcolonies, and (4) formation of a mature biofilm consisting of a community of organisms in a matrix of extracellular polymeric substances (EPS) (Watnick and Kolter2000; Bridier et al. 2011; Nguyen et al. 2012; Habimana et al. 2014; Vanysacker et al. 2014; Raval et al. 2018; Kucera 2019; Najjar et al. 2019) (Figure 1). Between □75% and 90% of the dry mass of a biofilm consists of the EPS matrix, with the remaining 10% to 25% consisting of the bacterial organisms (Costerton 1999; Kives et al. 2006; Flemming and Wingender 2010; Nguyen et al. 2012).

Figure 1. Biofouling begins by conditioning the surface with chemicals, usually proteins, in the bulk water followed by attachment of pioneer organisms to the conditioned surface. Next, a microcolony is formed by excretion of extracellular polymeric substances (EPS). Finally, a mature biofilm forms due to growth and diversification of the microcolony. The mature biofilm, therefore, consists of a community of organisms in a matrix of EPS. Organisms may disperse or detach from the biofilm based on environmental conditions (Watnick and Kolter 2000; Cockerill et al. 2012; Curtin et al. 2020). Before microcolony formation, biofouling is considered reversible fouling because it can be removed by shear force. However, after microcolony formation, biofilms are attached too strongly to the membrane to detach with increased shear force, therefore, requiring other treatment methods (i.e. chemical treatment).



One technology that is impacted by biofouling is reverse osmosis (RO). Biofouling tends to buildup on the membrane-water interfaces. It can clog RO membranes, resulting in an increased energy requirement to maintain flux across the membrane. Eventually, biofouling of RO membranes requires the membrane to be removed from the system and treated or replaced. Unfortunately, although common treatment methods, such as chlorine, are effective, they can damage the membrane (Antony et al. 2010). Increased energy requirements and decreased lifespan of RO membranes caused by biofouling cause RO to be a less efficient and cost-effective process. As such, methods for effectively removing or managing biofilms while in place in a RO system have potential for significant economic and humanitarian benefit.

Biofouling efficacy tests

A simple and rapid technique used for studying the efficacy of biofilm treatment by chemical intervention is antimicrobial susceptibility tests. Standard protocols for suspension culture antimicrobial susceptibility (SCAS) tests can be found in CLSI document M07 (CLSI 2018). SCAS tests generally entail treating suspension cultures with a range of concentrations of a chemical treatment either in centrifuge/culture tubes (macrodilution) or in multi-well plates (microdilution). Data are collected as a result of assays for minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic that inhibits the visible growth of bacteria after overnight incubation (Macià et al. 2014).

In contrast with suspension culture tests, protocols for biofilm antimicrobial susceptibility tests are in their earlier stages, such as ASTM E2799 (2017) and ASTM E2871 (2019). In general, biofilm antimicrobial susceptibility tests can either focus on biofilm prevention or biofilm removal. The detection method for biofilms, however, varies and the selection of detection method determines the metric (i.e. living cells or EPS removal). It is therefore important to select biofilm detection method(s) based on the deliverables desired from the experiment and the application of the results.

Inherent difficulties detecting biofilms

Compounding the difficulty in selecting a detection method for anti-biofouling efficacy, there are underlying challenges in simply detecting and quantifying biofilms. In general, biofilms are complex communities of various organisms, both living and dead, integrated in varying types of EPS, therefore, developing a detection method that quantitatively determines all of these components is difficult (Stiefel et al. 2016b). Both Stiefel et al. (2016a) and Wilson et al. (2017) investigated various quantitative and qualitative detection methods for biofilms and they concluded that a combination of methods is ideal for accurately detecting biofilms. Additionally, up to 90% of a biofilm is made up of EPS matrix (Nguyen et al. 2012). Any detection methods that depend on interacting with bacteria or the entire EPS must have the time and ability to diffuse throughout the matrix. Lastly, some methods require homogenizing the biofilm before analysis *via* methods such as sonication, vortexing, and scraping the biofilms off the material on which they have formed. Completely removing and homogenizing the biofilm can be difficult, due to strongly adhered bacteria and EPS.

Limit of scope

The following critical review addresses metrics that pertain specifically to the growth and prevention of biofilms themselves, specifically with a focus on RO systems. This review does not address other metrics of RO membrane performance that are impacted by biofouling, including membrane flux, or energy requirement. Studies that utilize bench-scale or pilot-scale RO systems benefit from maintaining the previously stated metrics because they are performance indicators and more readily tracked in a functioning RO system. However, the experimental setup focused on in the critical review is intended to screen and provide recommendations of optimal antifouling chemistries for RO systems, and as such are performed as high-throughput antimicrobial susceptibility tests (i.e. biofilms grown in 96-well plates). High-throughput antimicrobial tests do not have the components of a bench-scale or pilot-scale RO system; therefore, the focus of this review is on biofilm detection methods, not RO system monitoring. Focusing on high-throughput antimicrobial susceptibility tests is useful because these types of studies provide relevant results across disciplines, however, this also leads to a limitation because the results may not be useful for drawing specific conclusions (Buckingham-Meyer et al. 2007; Manner et al. 2017).

Additionally, although this review is focused on RO systems, it should be acknowledged that biofouling is an issue across disciplines. One noteworthy technology that is also affected by biofouling is medical devices. For example, in 2011 it was found that 1 in 25 inpatient hospital admissions obtained a hospital acquired infection (HAI) and that >25% of the HAIs were directly associated with implanted medical devices (Magill et al. 2014). Another noteworthy technology that is negatively impacted by biofouling is membrane bioreactors (MBRs). The application of MBRs is limited due to biofouling for similar reasons as RO systems including flux decline and increased pressure drop across the membrane (Ramesh et al. 2006; Miura et al. 2007). However, the direct motivation of this review was anti-biofouling studies for RO membranes.

Goals, metrics, and challenges of measuring biofilms in various disciplines

Defining goals and metrics of various fields studying biofouling

Due to the negative impacts of biofouling on RO systems (i.e. Ferrera et al. 2015), hospital devices (i.e. Gomes et al. 2014), in industry (i.e. Bezek et al. 2019), and other systems and settings, researchers in various disciplines are investigating ways to mitigate biofouling. Biofouling treatment in these fields range from solubilized chemical treatments (i.e. linoleic acid (a plant fatty acid) (Kim et al. 2019)), surface modifications (i.e. silver nanoparticle-impregnation (Weber and Rutala 2013; Pan et al. 2019), and biological treatments (i.e. bacteriophages (McLean et al. 2011; Ma et al. 2018)). Due to the wide range of applications of biofouling treatment results, the results from biofouling treatment studies should be presented in a manner that is useful and accessible to all relevant disciplines.

Table 1 summarizes fields that are interested in biofouling treatment research deliverables, including RO engineers, pharmacologists and microbiologists, wastewater treatment plant operators, chemists, toxicologists, and preservative formulators (i.e. for food, home and personal care products). Each field has specific focuses for biofouling treatment research, which translate into microbiological implications and desired metrics for detection of efficacy.

Table 1. Disciplines interested in anti-biofouling research, their main focus in relation to biofouling, the biological implications they are interested in achieving through treatment methods, and what metric(s) the biological implications require.

Discipline	Focus	Biological implications	Metric(s)
Engineer (Energy Requirement of RO)	Maintaining flux through the RO process; minimizing energy requirement of the system	Prevent bacteria from forming biofilms; Remove both the bacteria on a membrane and the EPS on a membrane	Total biomass removal
Engineer (Permeate Water Quality Produced by RO)	Permeate water quality	Decreasing bacterial concentration to a recommended level	Log reduction
Pharmacologist/Microbiologist	Dose of antimicrobial required for successful treatment	The minimum amount of antimicrobial required to inhibit bacterial growth (planktonic and biofilm)	MIC, BPC, MBIC, MBC, BBC, and/or MBEC (Table 2)
Wastewater Treatment Plant Operator	Remove odor and discoloration from water; Remove pathogens	Decreasing bacterial concentration to a recommended level	Log reduction
Chemist	Mode of action	Generally depends on interests of collaborators	Varies; commonly accompanied by or complementary to metric of collaborator
Toxicologist	Safety	Generally depends on interests of the collaborators	Varies; commonly accompanied by or complementary to metric of collaborator
Food/Home and Personal Care Products Preservative Formulator	Preventing color and odor changes in shampoo, etc.	Minimizing growth of planktonic bacteria	MIC (Table 2)

For example, RO engineers are interested in minimizing the impacts of biofouling on the energy requirement of RO systems and creating high quality permeate water that meets regulatory standards for drinking or discharge. For the former interest, engineers need to know the total biomass remaining after biofouling treatment because the EPS matrix that bacteria reside in must be prevented or removed from the membrane in addition to inhibition or killing of microorganisms, to maintain or resume normal RO processing (Alasri et al. 1992; Meyer 2003; Sanawar et al. 2018, 2019; Jafari et al. 2020). Engineers need to know the number of organisms per liter (often calculated in laboratory studies as colony forming units (CFU) and the log reduction in bacterial organisms. Log reduction is a way to express

the relative number of microorganisms that were killed *via* disinfection. The World Health Organization Guidelines for Drinking-Water Quality present both maximum organisms per liter and log reduction recommendations for various organisms with associated health risks in drinking water (World Health Organization 2017). Thus, the figures of merit for RO engineers are total biomass and log reduction. In contrast, pharmacologists and microbiologists are generally interested in determining the viability of cells using cell count data from biofilm susceptibility tests that either focus on biofilm prevention or biofilm removal. Biofilm prevention tests are similar to suspension culture antimicrobial susceptibility tests, in that a suspension culture is treated with the chemical of interest and the biofilm prevention is monitored. The suspension culture in biofilm prevention tests is at a higher concentration than that used for antimicrobial susceptibility tests in order to encourage biofilm growth. For biofilm prevention, data can be collected in the form of biofilm prevention concentration (BPC) (Table 2). For biofilm removal, a biofilm is grown and then treated. Data can be collected as minimum biofilm inhibitory concentration (MBIC), minimum bactericidal concentration (MBC), biofilm bactericidal concentration (BBC), and minimal biofilm-eradication concentrations (MBEC), depending on the experimental setup (Macià et al.2014). The benefit of utilizing biofilm susceptibility protocols that lead to BPC, MBIC, MBC, BBC, and/or MBEC is that the data are generalized to biofilms in centrifuge/culture tubes or multi-well plates (similar to CLSI document M07 (CLSI, 2018)) or on pegs (Ceri et al1999) and can, therefore, be applied to multiple situations. For example, the MBIC of a chemical against a certain type of bacterium can be determined *via* a biofilm susceptibility test in order to provide information on biofilm removal from medical devices (Sandoe et al. 2006). However, those data can also be used to provide information inform on biofilm removal from RO membranes.

Table 2. Different parameters available for antimicrobial susceptibility tests (obtained from Macià et al. (2014)).

Parameter		Definition
MIC	Minimum inhibitory concentration	The lowest concentration of an antibiotic that inhibits the visible growth of a planktonic culture after overnight incubation
MBIC	Minimum biofilm inhibitory concentration	The lowest concentration of an antibiotic that resulted in an OD650 nm difference of ≤10% (1 log difference in growth after incubation for 6 h) of the mean of two positive control well readings
MBC	Minimum bactericidal concentration	The lowest concentration of an antibiotic producing a 99.9% CFU reduction of the initial inoculum of a planktonic culture
BBC	Biofilm bactericidal concentration	The lowest concentration of an antibiotic producing a 99.9% reduction of the CFUs recovered from a biofilm culture as compared to the growth control
MBEC	Minimal biofilm-eradication concentration	The lowest concentration of an antibiotic that prevents visible growth in the recovery medium used to collect biofilm cells
BPC	Biofilm bactericidal concentration	Same as the MBIC, but bacterial inoculation and antibiotic exposure occur simultaneously

Unfortunately, metrics including BPC, MBIC, MBC, BBC, and/or MBEC do not provide information directly on EPS removal, nor does total biomass directly indicate cell viability. Table 1 suggests that studies that focus on antimicrobial efficacy against biofouling should collect data and report data in three forms: total biomass reduction, log reduction, and one or more of BPC, MBIC, MBC, BBC, and/or MBEC to satisfy the metrics of all of the relevant

disciplines.

Available detection methods

In the following section, the biofilm detection methods that can satisfy the desired metrics including, total biomass, log reduction, and MIC/BPC/MBIC/MBC/BBC/MBEC, are investigated.

Total biomass

Total biomass is generally measured using dyes that bind to negatively charged molecules. These dyes can stain cells and EPS, however, they do not allow for differentiation between the two or determination of live and dead cell counts. After staining samples with the dyes, the absorbance is read. Crystal Violet, Safranin Red, and Congo Red are three types of stains that can be used for total biomass biofilm detection (Stiefel et al. 2016b) (Table 3). Each protocol is briefly discussed in Table 3.

Table 3. Summary of biofilm detection methods for total biomass (Stiefel et al. 2016b).

Total biomass detection				
Detection method name	Brief protocol description	Benefit(s)	Drawback(s)	Representative studies
Crystal Violet	Stain contents of wells (including cells and EPS); incubate for 30 min; rinse wells to remove excess stain; dissolve stain from well contents (i.e. *via* ethanol); read absorbance of well at 595 nm, compare with negative control	Inexpensive; highest absolute absorbance of the three	Sufficient biomass must be present to distinguish absorbance from the background noise	(Jennings et al. 2012; Haney et al. 2018; Wang et al. 2019)
Safranin Red	Similar to Crystal Violet, but absorbance read at 535 nm	Inexpensive	Sufficient biomass must be present to distinguish absorbance from the background noise	(Yoneda et al. 2013; Diogo et al. 2017)
Congo Red	Similar to Crystal Violet, but absorbance read at 500 nm, compare with negative control	Inexpensive	Sufficient biomass must be present to distinguish absorbance from the background noise	(Antoniani et al. 2010; Keelara et al. 2016)

Log reduction (living cells)

A simple, common way to determine log reduction is to spread plate the sample on an agar plate and count colonies before and after treatment (Biesta-Peters et al. 2010). This method, however, is time consuming and labor intensive, therefore alternative methods are often utilized.

There are various other methods for determining log reduction. The BacTiter-Glo assay is used to quantify the amount of ATP in a sample, which is only produced and retained by live cells (Stiefel et al. 2016b). After the addition of the BacTiter-Glo reagent (Promega), the luminescence is measured and compared with a standard curve to determine live cell count. The turbidity threshold method entails the addition of growth medium to treated samples, incubation, and monitoring of optical density (OD). A standard curve can then be used to determine the number of live cells based on OD readings. For the tetrazolium salt assay, tetrazolium salt is added to a biofilm sample and incubated for two hours. In that time, the salt is crystalized by microorganisms in the form of formazan, which can be dissolved in DMSO and the absorbance can be read. Since the absorbance reading is based on formazan production,

which is converted by live microorganisms, it can lead to cell counts based on standard curves (Stiefel et al. 2016b). The LIVE/DEAD*Bac*Light Stain consists of fluorescent stains that intercalate with DNA. The stains label live cells green and dead cells red. The fluorescence can be read and compared to a standard curve or images of the biofilm can be taken and live cell counts can be determined by software such as ImageJ (Stiefel et al. 2016a; Wilson et al. 2017). Each protocol is briefly discussed in Table 4.

Table 4. Summary of biofilm detection methods for living cells, which can be translated to log reduction and minimum inhibitory concentration (MIC), biofilm prevention concentration (BPC), minimum biofilm inhibitory concentration (MBIC), minimum bactericidal concentration (MBC), biofilm bactericidal concentration (BBC), and minimal biofilm-eradication concentrations (MBEC) (Stiefel et al. 2016b).

Living cells: log reduction/MIC/BPC/MBIC/MBC/BBC/MBEC				
Detection method name	Brief protocol description	Benefit(s)	Drawback(s)	Representative studies
Log reduction *via* spread plates	Prevention: aliquots of the stock culture are spread plated to determine starting concentration of cells; after treatment of cells aliquots are plated to determine final concentrations of cells; log reduction is calculated between starting concentration and final concentration Removal: biofilms are grown; after treatment of cells aliquots are plated of both the experimental wells and the positive control; these cell counts are compared to calculate log reduction	Accurate	Time consuming; labor intensive	(Shukla et al. 2018; Ahmed Ali et al. 2019; Soleymani Lashkenari et al. 2019)
BacTiter Glo assay	Detach bacteria from multi-well plate; add BacTiter-Glo reagent, incubate for 5 min, read the luminescence with a plate reader with gain of 135, 1 s well-1	Accurate	Destructive sampling; expensive	(Jensen et al. 2014; Montgomery and Banerjee 2015; Stiefel et al. 2016a)
Turbidity Threshold assay	After treating biofilms, add broth; incubate plates on shaker incubator and measure OD at 600 nm every 30 min for 24 h; compare OD with cell count		Time consuming; labor intensive	(Uzer Celik et al. 2016)

Living cells: log reduction/MIC/BPC/MBIC/MBC/BBC/MBEC				
Tetrazolium Salt assay	Add 2-(4-iodophenyl)-3-(4-nitrophe-nyl)-5-phenyl-2H-tetrazolium chloride (INT) to each well; incubate the plate for 2 h; remove medium; add dimethyl sulfoxide to dissolve the dye from the biofilms; read absorbance at 470 nm	Metabolic activity detection	Concerns with consistency and false-positives and false-negatives	(Pitts et al. 2003; Nuryastuti et al. 2009; He et al. 2012; Sabaeifard et al. 2014)
LIVE/DEAD *Bac*Light Stain	Rinse wells; add stains solutions dissolved in 0.9% saline solution; incubate plates for 15 min while vortexing; read fluorescence with an excitation of 485 and emission of 528 and 640	Leads to live and dead cell count	Destructive; Issues with Gram-positive bacteria	(Karkhanechi et al. 2013; Ahmed Ali et al. 2019; Zhang et al. 2020)

Both of the previously discussed methods lead to live cell counts, however, they must be converted to log reduction. For biofilm prevention protocols, the live cell count is converted to log-reduction by comparing the final live cell count to the number of live cells inoculated at the beginning of the experiment. For biofilm removal protocols, the live cell count in treated wells is compared with the live cell count in positive control wells (Macià et al. 2014).

MIC/BPC/MBIC/MBC/BBC/MBEC (living cells)

As stated earlier, the MIC for cells in the planktonic phase is commonly determined by macro- or microdilution protocols(Cockerill et al. 2012). Both protocols entail growing organisms, treating the organisms with a range of concentration of the antimicrobial, and visually determining the MIC. However, BPC/MBIC/MBC/BBC/MBEC rely on determination of live cells (Macià et al. 2014). Detection methods that allow for the determination of BPC/MBIC/MBC/BBC/MBEC are similar to those for log-reduction. After determining live cells, the BPC/MBIC/MBC/BBC/MBEC are determined as values that are 90% lower than the positive controls (Macià et al. 2014). Each protocol is briefly discussed in Table 4.

Research goals

The aim of this review is to select specific anti-biofouling efficacy detection methods to maximize inter-, multi-, and transdisciplinary applications of the results of biofouling treatment studies (Table 1). Studies should therefore measure total biomass, log reduction, and BPC/MBIC/MBC/BBC/MBEC. Additionally, detection methods should be applicable to high-throughput antimicrobial susceptibility tests, therefore, the selected detection methods need to be completed in a timely manner and be cost effective.

The chosen detection methods

After reviewing the literature, two methods for determining anti-biofouling efficacy including the Crystal Violet stain (CV) and the LIVE/DEAD *Bac*Light Stain are recommended for use in antimicrobial susceptibility tests. Using both of these methods will allow for the determination of both total biomass, *via* CV, and viability of cells (log reduction and BPC/MBIC/MBC/BBC/MBEC), *via* the LIVE/DEAD *Bac*Light Stain.

Crystal violet stain

CV binds to negative charges, including bacteria and EPS. The protocol begins with adding CV to each well of the 96-

well plate. The plate is then incubated for 30 min. Afterward, the contents of each well are aspirated out and each well is washed three times with sterile saline. Lastly, ethanol is added to each well to dissolve the biofilm-bound CV and the absorbance is read at 595 nm (Stiefel et al. 2016b). Total biomass removal is determined as a well with an absorbance reading that is not statistically different from the negative controls.

Why crystal violet stain?

CV is recommended for detecting total biomass instead of Safranin Red and Congo Red (Table 3). CV is recommended largely because it is a relatively simple and quick method for detecting biofilms (Stiefel et al. 2016b; Wilson et al. 2017). Additionally, researchers have found that CV could detect up to a 98.7% reduction in *Pseudomonas aeruginosa* biofilms, which is higher than the detectability for Safranin Red and Congo Red, two other total biomass stains (Stiefel et al. 2016b). Stiefel et al. (2016a) identify one drawback common to total biomass detection methods, including CV; a high amount of biomass must be present to allow for distinction from background noise. However, Stiefel et al. (2016a) found that CV was the best at detecting these changes because it has a higher absolute absorbance than other stains like Safranin Red and Congo Red, which makes it easier to differentiate from the background noise.

LIVE/DEAD BacLight stain protocol

The LIVE/DEAD*Bac*Light Stain consists of two fluorescent nucleic acid dyes, SYTO9 and propidium iodide (PI). SYTO9 can permeate through the cell membrane and intercalate with the DNA of live and dead cells. SYTO9 fluoresces green. PI cannot permeate an intact cell membrane, therefore, it only intercalates with the DNA of dead cells. PI fluoresces red. It has a higher affinity to DNA than SYTO9 therefore it replaces any DNA of dead cells intercalated with SYTO9 (Stocks 2004).

The protocol for the LIVE/DEAD*Bac*Light Stain starts by aspirating out the contents of the 96-well plate. Next, the wells are rinsed with sterile saline to remove any remaining planktonic cells. The stain is then added to the wells and incubated for 15 min while vortexing. Common practice is to read the fluorescence with a plate reader twice, both with an excitation of 585 nm and an emission of 528 nm for SYTO9 and 645 nm for PI (Stiefel et al.2016b). Fluorescence readings can be compared with a standard curve for SYTO9 and PI fluorescence*vs* cell counting to determine the cell viability/cell count in experimental samples. The cell viability determined by the standard curve can be compared with the original inoculum amount or to the positive controls for prevention studies and removal studies, respectively. Other sources suggest imaging the biofilm *via* a confocal laser scanning microscope (CLSM) and counting the fluorescent cells with Image J software, which is a useful technique because it does not require disturbing the biofilm, or a flow cytometer to count cells (i.e. Hirsch et al. 2019; Wen et al. 2019).

*Why the LIVE/DEAD Bac*Light *stain?*

The LIVE/DEAD*Bac*Light Stain is recommended for determining log reduction and cell viability (Table 4). The LIVE/DEAD*Bac*Light stain protocol is advantageous because it can determine cell viability. It can also be detected with various devices including a plate reader, a confocal laser scanning microscope, and a flow cytometer. In the case of utilizing a plate reader for fluorescence measurements, a limitation related to the use of the LIVE/DEAD*Bac*Light Stain is utilizing a vortex to homogenize the biofilms during stain incubation. In preliminary research (not shown here), it was found that vortexing may not sufficiently disturb biofilms for a single-point fluorescence read, therefore an area scan is required which is quite time consuming (about 40 min per 96-well plate). To continue to make this an effective high-throughput detection method, a more homogenous solution needs to be made, for example by sonicating the biofilms (i.e. Li et al. 2019).

An additional limitation of this protocol is issues staining Gram-negative cells (Stiefel et al. 2015; Rosenberg et al. 2019). For example, Stiefel et al. (2015) found that samples that contained 100% dead cells had 2.7-fold higher SYTO9 fluorescence than samples with 0% dead cells (100% living cells). In other words, Stiefel et al. (2015) found that samples of 100% dead Gram-negative cells contained more living cells (stained by SYTO9) than samples containing 100% live cells. In contrast, for *Staphylococcus aureus,* a Gram-positive bacterium, there was a 9-fold weaker SYTO9 signal for 100% dead cells compared with 0% dead cells (100% living cells), which is more in line with expected results. Researchers suggest this may be related to difficulties of SYTO9 crossing the two cell membranes of Gram-negative bacteria, therefore, when the cell is dead and has damaged cell membranes, it can more easily reach the DNA and is not be replaced by PI quickly enough, leading to artificially high SYTO9 fluorescence readings for dead cells (Stiefel et al. 2015). However, preliminary results obtained by the authors (not provided) and other research including Berney et al. (2007), Venkata Nancharaiah et al. (2012), and Lambadi et al. (2015), were not affected by this relationship as strongly as in Stiefel et al. (2015). However, caution is recommended when utilizing the LIVE/DEAD*Bac*Light Stain with Gram-negative bacteria, including utilizing another detection method for comparison.

The BacTiter-Glo assay is not recommended because, although it is very accurate, it is prohibitively expensive for a high-throughput assay (Stiefel et al. 2015). The turbidity threshold assay is not recommended because it is considered time consuming (Stiefel et al. 2015). Unlike the LIVE/DEAD*Bac*Light Stain, which can be improved by implementing sonicat, the turbidity threshold assay cannot be shortened (Macià et al. 2014). Lastly, the tetrazolium salt assay is not recommended because there are concerns with its consistency and false-negatives and false-positives due to background interference (Fajardo et al. 2018).

Conclusion

Biofouling is a concern in various disciplines, therefore, biofouling treatment results should be widely applicable so that they are useful across disciplines. In this critical review, it was determined that assays aimed at measuring the effect of chemical interventions (antifoulants) on biofouling will best serve a range of researchers and practitioners if the studies detect both total biomass removal and cell viability/cell counts, which can be translated to log reduction, and BPC/MBIC/MBC/BBC/MBEC (as applicable). After analyzing possible methods for biofilm detection of total biomass and cell viability/cell count, guided by Stiefel et al. (2016a), a combination of two detection methods is recommended: the LIVE/DEAD*Bac*Light Stain and the CV stain. However, it is important to use caution with the LIVE/DEAD BacLight Stain with Gram-negative bacteria, but utilizing an additional detection method for comparison may negate this concern. Both methods are well aligned with a high-throughput protocol and the combination will lead to both total biomass and viability counts, which can lead to log reduction and BPC/MBIC/MBC/BBC/MBEC measurements. Other biofilm detection protocols discussed had drawbacks, such as consistency, cost, and duration that make them less useful for biofilm detection *via* high-throughput susceptibility assays. By detecting and reporting both cell viability or cell counts and total biomass, results from biofouling treatment research can be useful across disciplines. However, results from high-throughput analyses are useful for screening purposes, but not necessarily direct application to specific anti-biofouling situations.

Authors' contributions

A.M. Curtin performed this critical review with guidance by H.L. Buckley. A.M. Curtin lead manuscript preparation. H.L. Buckley provided feedback and revised the manuscript. H.L. Buckley secured funding for this project.

Disclosure statement

The authors declare no conflict of interest.

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