Harvesting and Disaggregation: An Overlooked Step in Biofilm Methods Research

Kelli Buckingham-Meyer, Lindsey A. Miller, Albert E. Parker, Diane K. Walker, Paul Sturman, Ian Novak, Darla M. Goeres

TITLE:

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AUTHORS AND AFFILIATIONS:

Kelli Buckingham-Meyer[1], Lindsey A. Miller[1], Albert E. Parker[1], Diane K. Walker[1], Paul Sturman[1], Ian Novak[1], Darla M. Goeres[1]

[1]Center for Biofilm Engineering, Montana State University, Bozeman, MT

Email Addresses of Co-Authors:

Lindsey A.Miller: (lindsey.lorenz@montana.edu)
Albert E. Parker: (albert.parker@montana.edu)
Diane K. Walker: (dianew@montana.edu)
Paul Sturman: (paul_s@montana.edu)
Ian Novak: (iannovak@montana.edu)
Darla M. Goeres: (darla_g@montana.edu)

Email Address of corresponding author:
Kelli Buckingham-Meyer (kellib@montana.edu)

SUMMARY:

This paper details the methods that demonstrates three common biofilm harvesting and disaggregation techniques on two surface types, ruggedness testing of a harvesting method and minimum information to consider when choosing and optimizing harvesting and disaggregation techniques to increase reproducibility.

ABSTRACT:

Biofilm methods consist of four distinct steps, growing the biofilm in a relevant model, treating the mature biofilm, harvesting the biofilm from the surface and disaggregating the clumps, and analyzing the sample. Of the four steps, harvesting and disaggregation are the least studied but nonetheless critical when considering the potential for test bias. This article demonstrates commonly used harvesting and disaggregation techniques for biofilm grown on three different surfaces. The three biofilm harvesting and disaggregation techniques, gleaned from an extensive literature review, include vortexing and sonication, scraping and homogenization, and scraping, vortexing and sonication. Two surface types are considered: hard non-porous (polycarbonate and borosilicate glass) and porous (silicone). Additionally, we provide recommendations for the minimum information that should be included when reporting the harvesting technique followed and an accompanying method to check for bias.

INTRODUCTION:

The definition of biofilm has evolved over the last few decades and encompasses microbial association with a variety of biological and/or non-biological surfaces, inclusion of noncellular components[1] that display differing growth and genetic expression[2] within a matrix. Biofilm provides protection from environmental stresses such as drying and may render the action of

chemical disinfectants less effective resulting in the survival of microbes. The survivors within a biofilm can potentially provide a source of pathogenic microorganisms that are a public health concern[3].

Biofilm methods are comprised of four steps, growth, treatment, sampling (harvesting and disaggregation), and analysis. Growth, the first step, where the user determines the organism growth conditions, temperature, media, etc., is the most considered and reported upon in the biofilm literature[4,5,6,7]. The treatment step evaluates antimicrobials (e.g., disinfectants) to determine their efficacy either against a mature biofilm[3,8,9] or the antimicrobial may be incorporated into the surface to determine the ability of the product to prevent or reduce biofilm growth[10]. The third step, sampling, includes steps to harvest the biofilm from the surface on which it was growing and to disaggregate the removed clumps[3,8,11]. The fourth step, analysis, may include viable cell counts, microscopy, fluorescence measurements, molecular outcomes, and/or a matrix component assessment[8,9]. Assessment of data provides information about the outcome of an experiment. Of the four, sampling is often the most overlooked step because it presumes that the chosen biofilm harvesting and/or disaggregation technique is 100% effective, often without verification[11].

Planktonic suspensions of bacteria, often considered to be homogenous, require simple vortexing prior to analysis. Biofilms, however, are complex communities composed of microorganisms (prokaryotic and/or eukaryotic), exopolysaccharides, proteins, lipids, extracellular DNA and host cells[12]. Steps beyond traditional planktonic microbiological culture methods are needed in order to adequately harvest biofilm from a surface and then disaggregate it into a homogenous single cell suspension. An extensive literature review (information not included in this publication) demonstrated that the choice of the removal and disaggregation technique is dependent on a number of factors including species make up of the biofilm, surface that the biofilm is attached to (non-porous or porous), accessibility to growth surfaces (easily removable coupon or physical destruction of apparatus in which the biofilm is growing), surface geometry (area and shape), density of biofilm on growth surfaces, and available laboratory equipment.

When biofilm is harvested from a surface, the resulting cell suspension is heterogenous. If this nonuniform suspension is to be accurately enumerated, it must be disaggregated into individual cells. Viable plate counts assume that a colony forming unit originates from one bacterium. If aggregates of biofilm are placed on the growth medium, it is impossible to distinguish individual cells which could lead to inaccurate estimates. For example, during disinfectant efficacy testing, if a treatment removes biofilm very effectively from a surface compared to the control, the log reduction could appear artificially large compared to the control. On the other hand, a chemical disinfectant that fixes biofilm onto a surface compared to the control will appear to have a lower log reduction[11]. This type of scenario could lead to biased interpretation of experimental data.

In preparation for this publication a review of the literature determined that common approaches to harvesting and disaggregating biofilm include scraping, swabbing, sonication,

vortexing or a combination of these. Scraping is defined as physical removal of biofilm from surfaces with a sterile stick, spatula or other tool. Swabbing refers to removal of biofilm from surfaces with a cotton tipped stick or other fixed absorbent material. Sonication refers to disruption of biofilm from surfaces via ultrasonic waves distributed through water. Vortexing refers to the use of a mixer to achieve a liquid vortex of the sample inside a tube. Homogenization uses rotating blades to shear harvested biofilm clumps into a single cell suspension. In this paper, we present three harvesting and disaggregation methods for two different surface types, hard/non-porous and porous.

A list of recommended minimum information that researchers should include in the methods sections of publications is provided. We hope that inclusion of this information enables other researchers to reproduce their work. There is no perfect harvesting and disaggregation method, therefore, recommendations for how to check the technique are also provided.

Three common methods to harvest and disaggregate biofilm from common growth surfaces are demonstrated in this article. This information will enable researchers to better understand the overall precision and bias of a biofilm test method. Methods described are as follows: (1) A *Pseudomonas aeruginosa* biofilm grown on polycarbonate coupons (hard non-porous surface) under high fluid shear in the CDC Biofilm Reactor is harvested and disaggregated following a five step combination of vortexing and sonication to achieve biofilm harvest and disaggregation. (2) A *P. aeruginosa* biofilm grown on borosilicate glass coupons (hard non-porous surface) in the drip flow reactor under low fluid shear is harvested and disaggregated using scraping and homogenization. (3) An *Escherichia coli* biofilm grown in silicone tubing (porous surface) is harvested and disaggregated using scraping, followed by sonication and vortexing.

PROTOCOL:

1. Vortexing and sonication

1.1 Grow a mature *P. aeruginosa* ATCC 15442 biofilm grown according to ASTM Standard E2562[2].

1.2 At the end of the 48 h growth period, prepare to treat the biofilm and sample coupons according to ASTM Standard E2871[8]

1.3 Aseptically insert autoclaved splash guards into sterile 50 mL conical tubes using flame-sterilized forceps. Repeat for all tubes that will receive treatment. Tubes for control coupons do not need a splash guard.

1.4 Aseptically remove a randomly selected rod from the CDC Biofilm Reactor. Rinse coupons to remove loosely attached cells by gently dipping the rod into 30 mL sterile buffered water.

1.5 Hold the rod parallel to the bench top, over an empty, sterile 50 mL conical tube and using

a flame-sterilized Allen wrench, loosen set screw to drop a biofilm coated coupon into tube. Repeat for the desired number of coupons. Remove splash guards and place in a separate container for sterilization.

1.6 Using a 5 mL serological pipette, slowly pipette 4 mL of the treatment or control into the tubes so that the liquid flows down the inside of the wall of the tube.

1.7 Gently tap the bottom of the tube so that any air bubbles under the coupon are displaced. Allow 30 – 60 s between each addition.

1.8 At the end of the specified contact time, pipette 36 mL of neutralizer into the tubes in the same order that the treatment (or control) was applied.

NOTE: The final volume of combined treatment and neutralizer is important for accurately determining biofilm log density.

1.9 Vortex each tube on the highest setting for 30 ± 5 s. Ensure that a complete vortex is achieved.

NOTE: Caution should be exercised when vortexing heavy coupons such as stainless steel in glass vials where breakage could occur.

1.10 Determine optimal number of tubes per bath and placement within the test tube rack prior to processing actual samples. If processing multiple samples, confirm that the water temperature in the sonicating bath is 21 ± 2 °C.

1.11 Place tubes in tube rack suspended in degassed sonicator such that water level in bath is equal to liquid level in tubes. Sonicate at 45 kHz, 100% power and Normal function for 30 ± 5 s. Repeat vortex and sonication cycles and then end with a final vortex (5 cycles total).

NOTE: These tubes with the harvested and disaggregated biofilm are the 10° dilution.

1.12 Serially dilute sample in buffered water. Plate on R2A agar using appropriate plating method. Incubate at 36 ± 2 °C for 24 h. Count colonies as appropriate to plating method used and record data.

2. Scraping and homogenization

2.1 Grow a mature *P. aeruginosa* ATCC 15442 biofilm according to the ASTM Standard E2647[13].

2.2 Set up the sampling station to include sampling board, 95% ethanol in a beaker, alcohol burner, hemostats, coupon removal tool, beakers with sterile dilution water and dilution tubes for rinsing the coupons.

2.3 Turn off the pump. Remove a channel cover and use sterile coupon removal tool and hemostats to remove the coupon, being careful not to disturb the biofilm.

2.4 Rinse the coupon by gently immersing, with a fluid motion, in 45 mL of sterile dilution water (contained in a 50 mL centrifuge tube). Immediately reverse the motion to remove the coupon.

2.5 Place the coupon into a beaker containing 45 mL of sterile dilution water. Scrape the biofilm-covered coupon surface in a downward direction for approximately 15 s, using a sterile spatula or scraper. Rinse the spatula or scraper by stirring it in the beaker. Repeat the scraping and rinsing process 3-4 times, ensuring full coverage of the coupon surface.

2.6 Rinse the coupon by holding it at a 60° angle over the sterile beaker and pipetting 1 mL of sterile dilution water over the surface of the coupon. Repeat for a total of 5 rinses. The final volume in the beaker is 50 mL.

NOTE: The final volume of combined treatment and neutralizer is important for accurately determining biofilm log density.

2.7 Replace each channel cover as the coupons are removed.

2.8 Working in the biosafety cabinet, homogenize the scraped biofilm sample. Attach a sterile homogenizer probe to the homogenizer, place the probe tip in the liquid, turn the homogenizer on and ramp up to 20,500 rpm.

2.9 Homogenize the sample for 30 s. Turn down the RPMs and switch the homogenizer off.

2.10 Sanitize the probe between biofilm samples by homogenizing a 9 mL of sterile dilution blank at 20,500 rpm for 30 s as described above. Homogenize a 9 mL tube of 70% ethanol for 30 s, detach the probe and let stand in the ethanol tube for 1 min. Homogenize two additional dilution blanks.

NOTE: A disposable homogenizer probe may be used for each sample.

2.11 Serially dilute the samples in buffered water. Plate on R2A agar using the appropriate plating method. Incubate plates at 36 ± 2°C for 24 h, count colonies as appropriate to plating method used and record data.

3. Scraping, vortexing and sonication

3.1 Grow a mature *Escherichia coli* ATCC 53498 biofilm in silicone catheter tubing[10].

3.2 Prepare sampling materials: rinse tubes, sterile centrifuge tube, empty sterile Petri dish, flame sterilized stainless steel hemostat and scissors, timer, and ruler.

3.3 With pump paused, use 70% ethanol to clean the outside of the tubing. Measure 2 cm from the end, avoiding the area attached to the connector, and mark the tubing to determine cutting locations.

3.4 With flame sterilized scissors, cut the tubing on the 2 cm mark and place segment in empty sterile Petri dish. Wipe the tubing with 70% ethanol and reconnect the distal end to waste tubing.

3.5 Rinse tubing segment to remove planktonic cells. With flame sterilized forceps, gently immerse tubing segment into 20 mL of sterile dilution water then immediately remove. Place the segment into 10 mL of neutralizer.

3.6 With flame sterilized forceps, hold the tubing segment and scrape with sterile wooden applicator stick until all inner areas of the tubing have been scraped. Occasionally rinse the stick in the 10 mL of neutralizer and place the segment back into the sample tube. The scraped tubing segment is the 10^0 or 0 dilution.

NOTE: The final volume of combined treatment and neutralizer is important for accurately determining biofilm log density.

3.7 Vortex each tube on the highest setting for 30 ± 5 s. Place the tube in tube rack suspended in sonicator such that water level in bath is equal to liquid level in tubes. Sonicate at 45 kHz, 100% power and Normal function for 30 ± 5 seconds. Repeat vortex and sonication cycles then end with a final vortex.

NOTE: This tube with the harvested and disaggregated biofilm is the 10^0 dilution.

3.8 Serially dilute the samples in buffered water. Plate on Tryptic Soy Agar using the appropriate plating method.

3.9 Incubate plates at 36 ± 2 °C for 24 h. Count colonies as appropriate to plating method used, record data and calculate the arithmetic mean.

REPRESENTATIVE RESULTS:

Validation/Confirmation of a Harvesting Method

Several studies that were conducted in our laboratory examined the ability of vortexing and sonication to effectively harvest biofilm grown in the biofilm reactor (ASTM E2562)[2] using the Single Tube Method (ASTM E2871)[8].

A *P. aeruginosa* ATCC 15442 biofilm was grown according to ASTM E2562[2] on borosilicate glass coupons. After 48 hours, four coupons were placed into vials, “treated” with 4 mL sterile

buffered water and neutralized with 36 mL of 2x D/E Neutralizing Broth. The initial sonication setting of 45 kHz, 10% power, Sweep setting, 30 +/- 5 s was used to harvest and disaggregate the biofilm from three of the four coupons. Upon completion of the vortex and sonication cycle, each coupon was stained with crystal violet and photographed. **Figure 1** demonstrates the amount of biofilm remaining on the three coupons after vortexing and sonication as compared to the control.

To test this further, a *P. aeruginosa* ATCC 15442 biofilm was grown as described previously and two sonication settings were compared: 1) 45 kHz, 10% power, Sweep setting, 30 +/- 5 seconds and 2) 45 kHz, 100% power, Normal setting, 30 +/- 5 seconds. One coupon from each set of the three was stained with BacLight Live/Dead stain and imaged using confocal microscopy (CM). The remaining two coupons from each set were diluted, plated and enumerated for viable cells. The viable plate count results were 9.230 Log10 CFU/coupon +/- 0.007 (SD_R) for setting 1 and 9.272 Log10 CFU/coupon +/- 0.066 (SD_R) for sonication setting 2. This data corroborated a 2015 EPA Single Tube Method Collaborative Study where 9.03 Log10 CFU/coupon +/- 0.272 (SD_R) was achieved[9]. According to the viable plate counts, it appears that all three means are similar enough to not warrant further investigation into differences between the two harvesting methods. However, the microscopic images shown in **Figure 2** may suggest that more biofilm remained after use of setting 1 than setting 2. While we observe that biofilm remaining on the coupons appears dead (red in color), interpretation of viability when using BacLight Live/Dead stain is difficult[14, 15]. Rather than focusing on viability implications of the stained biofilm, we used this stain to visualize biofilm remaining on the surfaces. Additionally, while we acknowledge that sonication could be deleterious to bacterial viability, a 2007 publication by Kobayashi et al.[16] demonstrated that increased sonication time beyond 5 minues resulted in decreased viable plate counts. Since our study used a total of 1 minute sonication, we are confident that few cells were killed via sonication as shown by the >9.2 LOG10 CFU/coupons for the two sonication parameters. It is interesting that a complete harvest of the biofilm from the surface was not achieved by either method. This finding demonstrates that viable plate counts alone are not adequate to determine harvesting and disaggregation bias and therefore must be paired with an additional method, microscopy, for example.

In addition to the sonicator settings, we investigated other important factors that affect sonication. These included the volume of liquid in the vials (10 or 40 mL), the type of liquid in the vial (buffered water or 2X D/E Neutralizing Broth) and the number of vials placed in the bath at the same time (3 or 12 vials)[9].

P. aeruginosa biofilms on CDC Biofilm Reactor coupons were sonicated using sonicator setting 2 (45 kHz, 100% power, Normal setting, 30 +/- 5 seconds). All samples were vortexed either 3 at a time or 6 at a time using a vortexer fitted with a 6-place tube attachment then sonicated as described in **Figure 3**. One coupon from each of the following categories was imaged using CM (**Figure 3**).

In the second study where sonication parameters were investigated, the microscopic images

(**Figure 3**) suggest that minimizing the volume in the tubes, reducing the number of tubes processed at once and use of D/E Neutralizing Broth (which contains surfactant) all contribute to enhanced biofilm harvesting from the coupons.

Biocides may positively or negatively enhance harvesting and disaggregation. Similar to confirming that a neutralizer effectively stops the active while not increasing kill prior to performing an efficacy test, it is important to confirm that a biocide does not differentially impact harvesting and disaggregation. For efficacy testing, bias results if and only if there is differential removal for the control vs treated biofilm samples[11].

FIGURE LEGENDS

Figure 1: Photos of coupons stained with crystal violet demonstrating residual biofilm. A) Coupon removed from the reactor and stained with crystal violet. B, C, D) Three replicate coupons were separately "treated" with sterile buffered water then neutralized. To harvest and disaggregate, the coupons were vortexed (30 +/- 5 seconds) and sonicated (45 kHz, 10% power, Sweep setting, 30 +/- 5 seconds) twice then received a final vortex. Images courtesy of Danielle Orr and Blaine Fritz.

Figure 2: Confocal microscopy images of coupons comparing two different sonication settings. Coupons (12.5X magnification) processed via sonication setting 1 (45 kHz, 10% power, Sweep setting) on the left or sonication setting 2 (45 kHz, 100% power, Normal setting) on the right.

Figure 3. Confocal microscopy images of coupons comparing volumes, sonication liquid and number of tubes. Coupons (12.5X magnification) processed in 10 or 40 mL volumes, in dilution water (DW) or D/E Neutralizing Broth (D/E), 3 or 12 tubes at a time with optimized sonication setting (45 kHz, 100% power, Normal setting).

Supplementary File 1: Key parameters of importance for harvesting and disaggagregation.

DISCUSSION:

Minimum Information for Harvesting and Disaggregation Methods

To create reproducible biofilm data across the scientific community, it is imperative that authors include as much detail as possible regarding each of the growth, treatment, sampling and analysis steps of a biofilm method. The standardization of biofilm methods has aided in this endeavor as it allows the researcher to reference a specific method and any relevant modifications. However, many papers include only a sentence or two to describe biofilm harvesting and disaggregation. For better reproducibility, we recommend that minimum information for biofilm harvesting be included in publications. This builds on the Minimum Information About a Biofilm Experiment (MIABiE) initiative presented by Lourenco et al.[17]. In the case of using sonication for biofilm harvesting and disaggregation parameters the information should include: position of tubes within the bath (manufacturers'

recommendations to avoid damaging the transducers), number of tubes sonicated at the same time, tube material, volume and type of liquid in the tube, presence of surfactant, position of liquid in tubes relative to liquid level of ultrasonic bath, device used to hold the tubes in the bath (glass beakers vs. test tube rack), degassing of water bath prior to sonication of samples (if degassing is not a manufacturer option, simple operation of the bath before inserting the samples will help remove some dissolved gasses from the bath liquid), temperature of water bath (temperatures can rise after long periods of sonication), frequency (25 kHz or 45 kHz, for example), bath function (sweep or normal, for example) and power range delivered to transducers (10 – 100%, for example). These settings should be optimized for the biofilm being studied, the system used to grow the biofilm and the specific make/model of ultrasonic bath[18].

Sonicator settings may be changed to optimize desired harvesting effects. Degassing removes dissolved air within the bath liquid thereby enhancing cleaning power. Frequency can be adjusted to a low or high setting. A low setting such as 25 kHz, for example, would aid in harvesting tenacious samples while a higher setting of 45 kHz would be more appropriate for sensitive samples. A bath function of sweep or normal allows for distribution of cavitation. The sweep function creates a continuous shifting of the sound pressure maxima. The normal function allows the transducers to operate in double half wave mode which may result in dead zones, thereby making the sonication less efficacious. Power ranges can be altered from 10 – 100% of the power delivered to the transducers[19]. It is known that sonication can detrimentally effect bacterial viability. A 2008 study by Stamper et al.[20] exposed bacterial cultures to increasing ultrasonice energy over time to create bacterial kill curves. We recommend that users confirm that a particular combination of sonication settings does not cause a decrease in viable bacteria[20].

There is no one perfect method to harvest and disaggregate biofilm, but particular methods do work better for some surfaces/microbe combinations than others. We advocate for the reader to determine which parameters are important for their particular biofilm scenario. Key parameters of importance for harvesting and disaggregation are included in **Supplementary File 1**.

For the sonication studies done to assess harvesting and disaggregation bias, we found that sonication is convenient, efficient and able to be standardized for harvesting and disaggregating biofilm from surfaces. Placement of coupons in vials minimizes technician to technician variability that would be encountered in methods where coupons are physically scraped by laboratory personnel, for example. Although it seems simple enough to place vials in a sonicating water bath, there are many parameters that need to be considered to achieve optimal harvesting of biofilm.

Two types of sonication equipment are available, ultrasonic baths and ultrasonic probes. This paper focuses primarily on ultrasonic baths where ultrasonic energy is generated in the range of high (20 – 45 kHz) to normal (40 – 60 Hz) frequency.

Three main processes are at play when using an ultrasonic device to clean a surface. Electrical

energy is converted to acoustic energy when a high frequency current is sent to a piezoelectric or magnetostrictive transducer that oscillates in response to the current. The oscillation generates compression (rarefaction) waves in the liquid. Cavitation bubbles form due to negative pressure during rarefaction. The bubbles grow until they reach an unstable size and collapse, creating a water jet that cleans surfaces[21].

Three harvesting and disaggregation approaches are demonstrated in this article: sonication and vortexing methods for harvesting and disaggregating biofilm when grown on polycarbonate coupons in the CDC Biofilm Reactor according to the Single Tube Method. Scraping and homogenization methods for harvesting and disaggregating biofilm are shown when grown on glass coupons using the Drip Flow Biofilm Reactor. Scraping, sonication and vortexing methods for harvesting and disaggregating biofilm are shown when grown in silicone tubing.

There is no perfect method to harvest and disaggregate the biofilm, but some approaches are better for different surfaces and/or applications. What is important is to take the time to validate the method used. In this paper, we discussed the use of crystal violet and microscopy, but other choices exist depending on the sensitivity required. If research includes efficacy testing, then it is critical to confirm the validity of the approach in the presence of the antimicrobial[6]. All equipment is slightly different, so even if the harvesting and disaggregation method have been standardized, it is still prudent to confirm the process for the equipment used. Harvesting and disaggregation methods are specific for surface associated and biofilm bacteria. Research has demonstrated that improper choices may lead to biased test results. Nonetheless, harvesting and disaggregation are the least studied of the four steps in biofilm methods. It is generally also the unvalidated step (taken for granted as working) with the least information present in published paper making it challenging to reproduce the process in a different lab. This paper and accompanying video show three common approaches for two surface types and suggests how to validate a method for an individual lab. This information will help researchers make more informed decisions on which method to use and provides guidance on what to report to improve reproducibility.

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