Designation: E2562 – 17

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor[1]

This standard is issued under the fixed designation E2562; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method specifies the operational parameters required to grow a reproducible (1)[2] *Pseudomonas aeruginosa* ATCC 700888 biofilm under high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one particular environment.

1.2 This test method uses the Centers for Disease Control and Prevention (CDC) Biofilm Reactor. The CDC Biofilm Reactor is a continuously stirred tank reactor (CSTR) with high wall shear. Although it was originally designed to model a potable water system for the evaluation of *Legionella pneumophila* (2), the reactor is versatile and may also be used for growing and/or characterizing biofilm of varying species (3-5).

1.3 This test method describes how to sample and analyze biofilm for viable cells. Biofilm population density is recorded as $\log_{10}$ colony forming units per surface area.

1.4 Basic microbiology training is required to perform this test method.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.7 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation according to Rice et al (6)

3. Terminology

3.1 *Definitions:*

3.1.1 *biofilm, n*—microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

3.1.1.1 *Discussion*—Biofilms may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a biofilm, including, but not limited to, population density, taxonomic diversity, thickness, chemical gradients, chemical composition, consistency, and other materials in the matrix that are not produced by the biofilm microorganisms, are controlled by the physicochemical environment in which it exists.

3.1.2 *coupon, n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a reproducible *Pseudomonas aeruginosa* ATCC 700888 biofilm in a CDC Biofilm Reactor. The biofilm is established by operating the reactor in batch mode (no flow of the nutrients) for 24 h. A steady state population is reached while the reactor operates for an additional 24 h with a continuous flow of the nutrients. The residence time of the nutrients in the reactor is set to select for

[1] This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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[2] The boldface numbers in parentheses refer to a list of references at the end of this standard.

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

biofilm growth, and is species and reactor parameter specific. During the entire 48 h, the biofilm is exposed to continuous fluid shear from the rotation of a baffled stir bar. Controlling the rate at which the baffle turns determines the intensity of the shear stress to which the coupons are exposed. At the end of the 48 h, biofilm accumulation is quantified by removing coupons from suspended rods, harvesting the biofilm from the coupon surface by scraping the biofilm from the coupon, homogenizing the removed biofilm to disaggregate the clumps, and diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in biofilms are phenotypically different from suspended cells of the same genotype. Research has shown that biofilm bacteria are more difficult to kill than suspended bacteria (**5**, **7**). Laboratory biofilms are engineered in growth reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. For example, research has shown that biofilm grown under high shear is more difficult to kill than biofilm grown under low shear (**5**, **8**). The purpose of this test method is to direct a user in the laboratory study of a *Pseudomonas aeruginosa* biofilm by clearly defining each system parameter. This test method will enable an investigator to grow, sample, and analyze a *Pseudomonas aeruginosa* biofilm grown under high shear. The biofilm generated in the CDC Biofilm Reactor is also suitable for efficacy testing. After the 48 h growth phase is complete, the user may add the treatment in situ or remove the coupons and treat them individually.

6. Apparatus

6.1 *Wooden Applicator Sticks*—sterile.

6.2 *Inoculating Loop.*

6.3 *Petri Dish*—100 by 15 mm, plastic, sterile, and empty to put beneath rod while sampling.

6.4 *Culture Tubes and Culture Tube Closures*—any with a volume capacity of 10 mL and a minimum diameter of 16 mm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.

6.5 *Pipette*—continuously adjustable pipetter with volume capacity of 1 mL.

6.6 *Vortex*—any vortex that will ensure proper agitation and mixing of culture tubes.

6.7 *Homogenizer*—any that can mix at 20 500 ± 5000 r/min in a 5 to 10 mL volume.

6.8 *Homogenizer Probe*—any that can mix at 20 500 ± 5000 r/min in a 5 to 10 mL volume and can withstand autoclaving or other means of sterilization.

6.9 *Sonicating Water Bath*—any cavitating sonicating bath that operates at 45 to 50 kHz for cleaning coupons.

6.10 *Bunsen Burner*—used to flame inoculating loop and other instruments.

6.11 *Stainless Steel Hemostat Clamp*—with curved tip.

NOTE 1—Alternatively, a coupon manipulating tool[4] may be used.

6.12 *Environmental Shaker*—that can maintain a temperature of 36 ± 2°C.

6.13 *Analytical Balance*—sensitive to 0.01 g.

6.14 *Sterilizer*—any steam sterilizer that can produce the conditions of sterilization is acceptable.

6.15 *Colony Counter*—any one of several types may be used, such as the Quebec, Buck, and Wolfhuegel. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.16 *Peristaltic Pump*—pump head that can hold tubing with inner diameter 3.1 mm and outer diameter 3.2 mm.

6.17 *Digital Magnetic Stir Plate*—top plate 10.16 × 10.16 cm, that can rotate at 125 ± 5 r/min.

6.18 *Silicone Tubing*—two sizes of tubing: one with inner diameter 3.1 mm and outer diameter 3.2 mm, and the other with inner diameter 7.9 mm and outer diameter 9.5 mm. Both sizes must withstand sterilization.

6.19 *Norprene*[5] Tubing—inner diameter 3.1 mm and outer diameter 3.2 mm.

6.20 *Glass Flow Break*—any that will connect with tubing of inner diameter 3.1 mm and withstand sterilization.

6.20.1 *Clamp*—Used to hold flow break, extension clamp with 0.5 cm minimum grip size.

6.20.2 *Clamp Stand*—height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

6.20.3 *Laboratory Screw Clamp*—used to clamp effluent tubing during batch growth.

6.21 *Reactor Components.*[6]

6.21.1 *Berzelius Borosilicate Glass Tall Beaker*—1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Barbed outlet spout added at 400 ± 20 mL mark. Angle the spout 30 to 45° to ensure drainage. Spout should accommodate flexible tubing with an inner diameter of 8 to 11 mm.

NOTE 2—The rods (see 6.21.3) and baffle (see 6.21.6) will displace approximately 50 mL of liquid when system is completely assembled. Therefore, an outlet spout at the 400 mL mark will result in approximately a 350 mL operating volume. The user should confirm the actual liquid volume in the reactor, when the rods and baffle are in place and the stir plate is turned on, before use. The measured operating volume is used to calculate an exact pump flow rate.

6.21.2 *Reactor Top*—Fig. 1. Ultra-high molecular weight (UHMW) polyethylene top (10.1 cm diameter tapering to 8.33

[4] The sole source of supply of the apparatus (coupon manipulating tool) known to the committee at this time is Biosurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the holder.

[5] Trademarked by the Saint-Gobain Performance Plastics Corporation.

[6] The sole source of supply of the apparatus (CDC Biofilm Reactor) known to the committee at this time is BioSurface Technologies, Corp. www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend. The user may also build the reactor.

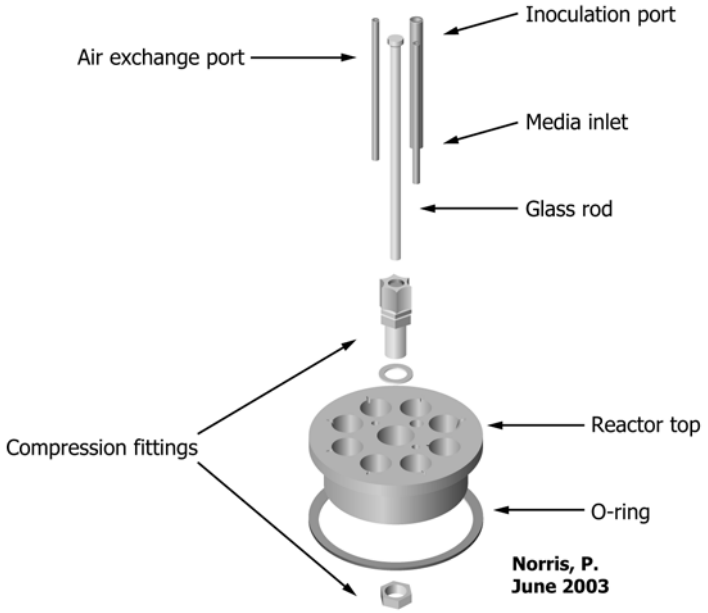

FIG. 1 Expanded Schematic of Reactor Top

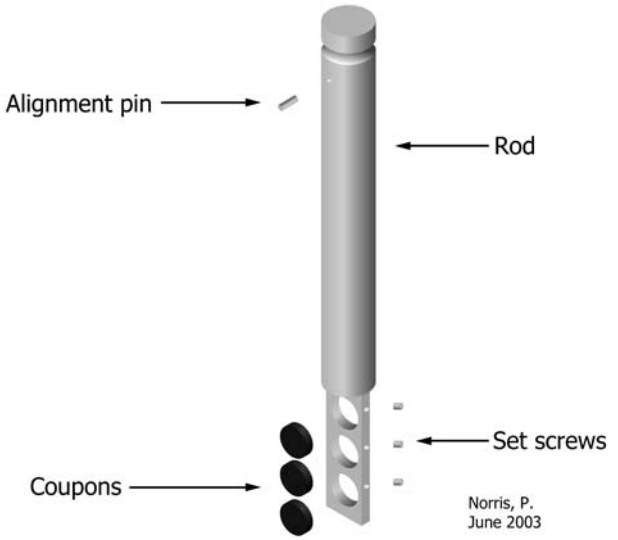

FIG. 2 Expanded Schematic of Rod and Coupons

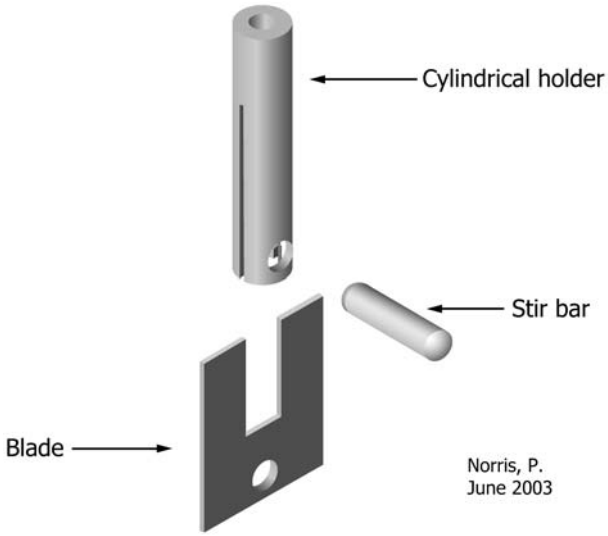

FIG. 3 Expanded Schematic of Baffled Stir Bar

cm) equipped with a minimum of three holes accommodating 10 cm pieces of stainless steel or other rigid autoclavable tubing with outside diameter of 5 to 8 mm for media inlet, air exchange, and inoculation port. Center hole, 1.27 cm diameter, to accommodate the glass rod used to support the baffle assembly. Eight rod holes, 1.905 cm diameter, notched to accommodate stainless steel rod alignment pin (0.236 cm outside diameter).

6.21.3 *Polypropylene Rods*—Fig. 2. Eight polypropylene rods, 21.08 cm long, machined to hold three coupons (see 6.21.4) at the immersed end. Three 316 stainless steel set screws imbedded in side to hold coupons in place. Rods fit into holes in reactor top and lock into preformed notches with alignment pin.

6.21.4 *Twenty-four Cylindrical Polycarbonate Coupons*—with a diameter of 1.27 ± 0.013 cm, thickness of approximately 3.0 mm.

6.21.5 *Small Allen Wrench*—for loosening set screws.

6.21.6 *Stir Blade Assembly (Baffled Stir Bar)*—Fig. 3. PTFE blade (5.61 cm) fitted into cylindrical PTFE holder (8.13 cm) and held in place with a magnetic stir bar (2.54 cm). PTFE holder fits onto a glass rod (15.8 cm), fitted into the reactor top. The glass rod is held in place with a compression fitting and acts as a support for the moving blade assembly.

6.22 *Carboys*—two 20 L autoclavable carboys, to be used for waste and nutrients.

6.22.1 *Two Carboy Lids*—One carboy lid with at least two barbed fittings to accommodate tubing ID 3.1 mm (one for nutrient line and one for bacterial air vent). One carboy lid with at least two 1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).

NOTE 3—Carboy tops can be purchased with fittings.

6.22.2 *Bacterial Air Vent (Filter)*—autoclavable, 0.2 µm pore size, to be spliced into tubing on waste carboy, nutrient carboy, and reactor top; recommended diameter 37 mm.

6.23 Fig. 4 illustrates a schematic of the assembled system.

7. Reagents and Materials

7.1 *Purity of Water*—All reference to water as diluent or reagent shall mean distilled water or water of equal purity.

7.2 *Culture Media:*

7.2.1 *Bacterial Liquid Growth Broth*—Tryptic Soy Broth (TSB) is recommended.

NOTE 4—Two different TSB concentrations are used in the test method, 300 mg/L for the inoculum and batch reactor operation, and 100 mg/L for the continuous flow reactor operation.

7.2.2 *Bacterial Plating Medium*—R2A Agar is recommended.

7.3 *Buffered Water*—0.0425 g/L KH_2PO_4 distilled water, filter sterilized, and 0.405 g/L $MgCl{\cdot}6H_2O$ distilled water, filter sterilized (prepared according to Method 9050 C.1.a(**6**)).

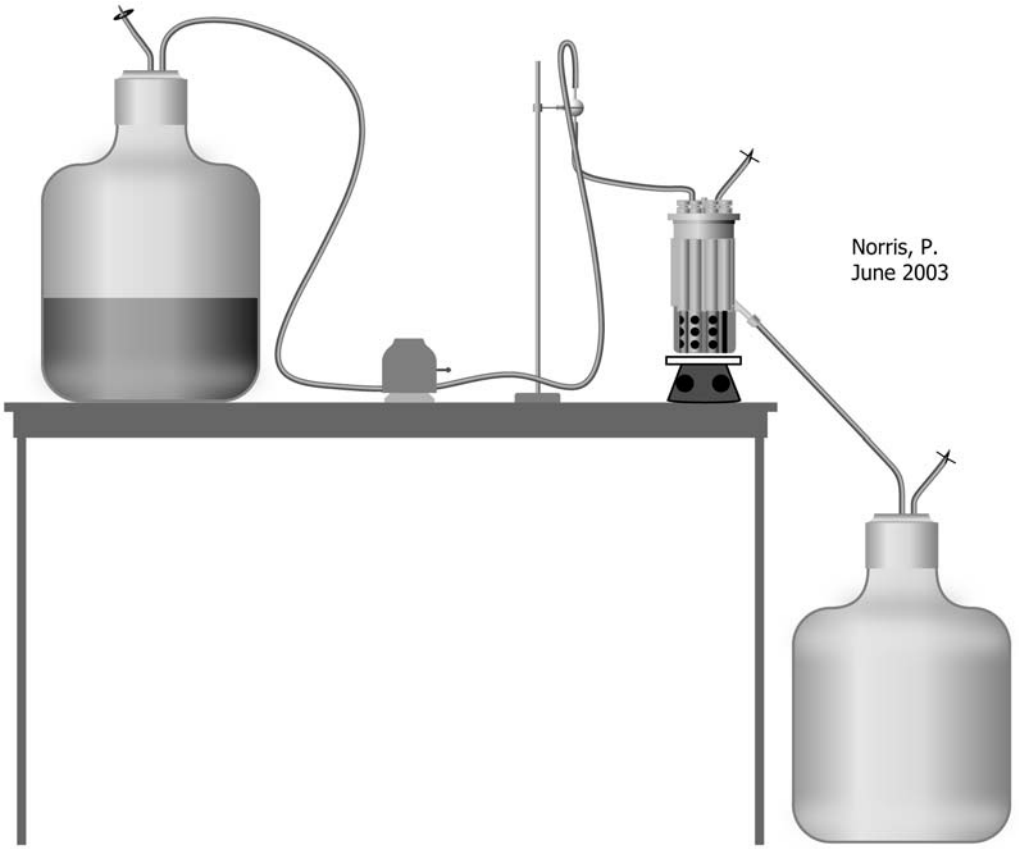

FIG. 4 Schematic of Completely Assembled Reactor System

8. Culture Preparation

8.1 *Pseudomonas aeruginosa* ATCC 700888 is the organism used in this test. Aseptically remove an isolated colony from an R2A plate and place into 100 mL of sterile TSB (300 mg/L). Incubate bacterial suspension in an environmental shaker at 36 ± 2°C for 22 ± 2 h. Viable bacterial density should equal 10^8 CFU/mL, and may be checked by serial dilution and plating.

9. Reactor Preparation

9.1 *Preparation of Polycarbonate Coupons:*

NOTE 5—Coupons can be used once and discarded or used repeatedly with proper cleaning and sterilization between each use. Check each coupon for scratching, chipping, other damage, or accumulated debris before each use by screening under a dissecting microscope at a magnification of at least 20×. Discard those with visible damage to surface topography.

9.1.1 Sonicate coupons for 30 s in a 1+99 dilution of laboratory soap and tap water. The soapy water must completely cover the coupons.

9.1.2 Rinse coupons with reagent grade water and sonicate for 30 s in reagent grade water.

9.1.3 Repeat rinsing and sonication with reagent grade water until no soap is left on the coupons. Once the coupons are clean, care must be taken to prevent oils and other residue from contaminating the surface.

NOTE 6—Coupons may be made out of alternative materials such as glass or stainless steel. The user should adjust the cleaning procedure so that it is appropriate for the coupon material being used.

9.1.4 Place a coupon into each hole in the reactor rods, leaving the top of the coupon flush with the inside rod surface. Tighten set screw.

9.1.5 Place rods into reactor top loosely (not yet fitted into notches).

9.2 *Preparation of Reactor Top:*

9.2.1 Invert the reactor top and place baffle onto glass rod positioned in the center of the reactor top.

9.2.2 Invert the reactor beaker and place onto the assembled top. Turn the reactor over so that the reactor top is upright.

NOTE 7—The baffle was designed to allow it to rotate freely.

9.2.3 Connect the bacterial air vent by fitting the vent to a small section of appropriately sized tubing, and attach to one of the rigid tubes on the reactor top.

9.2.4 The glass flow break is spliced into the nutrient tubing line near the reactor top.

NOTE 8—The other ports on the reactor top may be adapted for anaerobic use, dilution water, or treatment solutions as necessary. If these ports are not used, cover with aluminum foil or other autoclavable material to maintain reactor sterility.

9.3 *Sterilization of the Reactor System:*

9.3.1 Place the reactor top securely on the beaker before sterilization. To allow for pressure escape, do not set rod alignment pins in notches during sterilization.

9.3.2 Cover the end of the nutrient tubing that connects to the nutrient carboy and the end of the overflow (waste) tubing

with aluminum foil. Cover any extra openings on the reactor top with aluminum foil. This is to maintain sterility after autoclaving.

9.3.3 Prepare batch culture medium by dissolving bacterial liquid growth medium (300 mg/L TSB) in 500 mL reagent grade water in an autoclavable container.

9.3.4 Sterilize the reactor system and separate batch culture medium for 20 min on the liquid cycle of a steam sterilizer.

10. Procedure

10.1 *Batch Phase:*

10.1.1 With the overflow (waste) line clamped, aseptically add the cooled batch culture medium to the cooled reactor.

10.1.2 Place reactor onto a stir plate.

10.1.3 Clamp flow break in upright position; leave other tubing clamped and foiled.

10.1.4 Secure the rod alignment pins into the reactor top notches.

10.1.5 Inoculate the reactor with 1 mL of bacteria from the culture prepared previously (see Section 8.1): Aseptically pipette the inoculum into the reactor through one of the available rigid reactor top tubes.

10.1.6 Turn on the magnetic stir plate. Set the rotational speed to 125 ± 5 r/min. The reactor system incubates in batch mode at room temperature (21 ± 2°C) for 24 h.

NOTE 9—The rotational speed of the baffled stir bar directly determines the amount of shear stress that the biofilm experiences. Ruggedness testing showed that biofilm accumulation on the coupons is sensitive to changes in the baffle's rotational speed. The baffle rotational speed is a critical factor that must be controlled.

10.2 *Continuous Flow Operation (CSTR Mode):*

10.2.1 Prepare 100 mg/L TSB continuous flow nutrient broth. Dissolve and sterilize the broth in a smaller volume to prevent caramelization. Aseptically pour the concentrated broth into a carboy of sterile reagent[7] grade water to make a total of 20 L.

10.2.2 Aseptically connect the nutrient tubing line to the carboy containing the continuous flow nutrient broth.

10.2.3 Pump a continuous flow of nutrients into the reactor at a flow rate determined by dividing the reactor volume by a 30 minute residence time, see Note 10. Attach tubing from the drain spout to a waste carboy and remove clamp. The drain spout on the beaker allows overflow to occur, maintaining a constant bacterial liquid growth broth concentration of 100 mg/L in the reactor during CSTR (continuously stirred tank reactor) mode.

NOTE 10—Calculating the flowrate: For example, if the reactor volume is equal to 350 mL (see Note 2), then for a 30 minute residence time divide 350 by 30 minutes for a flowrate equal to 11.7 mL/min

10.2.4 Operate the reactor in CSTR mode for 24 h.

10.3 *Sampling the Biofilm:*

10.3.1 Prepare sampling materials: vortex, homogenizer, culture tubes, pipettes, empty sterile petri dish, wooden applicator sticks, and flame-sterilized stainless steel hemostat or coupon holder.

10.3.2 Remove one of the rods containing coupons by pulling it straight up firmly. Hold over petri dish while carrying to sampling area.

10.3.3 Remove a randomly chosen coupon from the rod by loosening the set screw and centering the coupon over a sterile support that is smaller in diameter than the coupon (for instance, an inverted carboy barbed fitting cover). The support is sitting on a surface that has been disinfected. Gently push down on the rod to free the coupon. Secure the coupon by holding it with a flame-sterilized hemostat or coupon holder. Care should be given to not disturb the top coupon surface in any way until it is scraped. If other coupons are to be sampled, replace rod into the reactor to prevent the biofilm from drying. Keep track of which coupon and rod were sampled.

10.3.4 Rinse the coupon to remove planktonic cells: Orient the coupon at a 45° angle over a beaker that contains 20 mL sterile buffered water. Gently immerse coupon with a continuous motion into the buffered water then immediately remove the coupon.

10.3.5 Remove the biofilm from the coupon: Scrape the top coupon surface for approximately 15 s using the flat end of a wooden applicator stick held perpendicular to the coupon surface. Rinse the stick by stirring it in the dilution buffer in the culture tube. Repeat the scraping and rinsing process 3–4 times, ensuring full coverage of the coupon surface.

10.3.6 Hold the coupon at a 60° angle over the culture tube and pipette 1 mL of sterile dilution water over the top surface of the coupon to rinse off any remaining cells. The final volume in the culture tube is now 10 mL.

NOTE 11—Each culture tube originally contains 9 mL of buffered water.

10.4 *Analyze the Biofilm Sample:*

10.4.1 Homogenize the scraped biofilm sample at 20 500 ± 5000 r/min for 30 s to disaggregate the biofilm clumps into a homogeneous cell suspension. If more than one biofilm sample is taken, rinse the homogenizer probe between each new sample: Homogenize a dilution blank for 30 s at 20 500 ± 5000 r/min, homogenize a tube containing 10 mL 70 % ethanol for 15 s, then remove the probe and let it sit in the ethanol tube for 1 minute. Shake any remaining ethanol off the probe, reattach it and homogenize a dilution blank for 30 s. Homogenize a second dilution blank as final rinse. The homogenizer probe is then ready for the next sample tube.

10.4.2 Serially dilute the sample.

10.4.3 Culture each dilution in duplicate for colony growth using an accepted plating technique such as drop (**9**), spread or spiral plating (see Practice D5465).

10.4.4 Incubate the plates for 24 ± 2 h at 36 ± 2°C.

10.5 *Cell Enumeration:*

10.5.1 Count the appropriate number of colonies according to the plating method used.

10.5.2 Calculate the arithmetic mean of the replicate samples plated.

[7] *Reagent Chemicals, American Chemical Society Specifications*, American Chemical Society, Washington, DC. For Suggestions on the testing of reagents not listed by the American Chemical Society, see *Annual Standards for Laboratory Chemicals,* BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary,* U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, MD.

10.6 The $\log_{10}$ density for one coupon is calculated as follows:

$$LOG_{10}(CFU/cm^2) = LOG_{10}[(X/B)(V/A)(D)]$$

where:

X = mean CFU,
B = volume plated,
V = volume scraped into,
A = surface area scraped, and
D = dilution.

NOTE 12—The equation in 10.6 is accurate only if the culture tube the biofilm was scraped into is referred to as the 10^0 dilution.

10.7 Calculate the overall biofilm accumulation by taking the mean of the $\log_{10}$ densities calculated in 10.6.

11. Precision and Bias

11.1 Randomization is used whenever possible to reduce the potential for systematic bias.

11.2 The internal reproducibility standard deviation for this protocol, when the biofilm density is based on a single coupon, was calculated to equal 0.59 (**4**). The sources of variability were:

53 % attributable to within-experiment sources,
47 % attributable to between-experiments sources.

12. Keywords

12.1 biofilm; coupon; growth reactor; *Pseudomonas aeruginosa*; reactor; sampling shear

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