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Assessing a method for single- and multidomain biofilm growth in a novel biofilm reactor: shear stress, ruggedness, repeatability, and reproducibility

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ABSTRACT

Numerous lab-scale bioreactor systems exist to cultivate biofilms under desired growth conditions. However, existing systems have not been able to simultaneously deliver low shear stress, high gas transfer, and intermittent wetting. The industrial surfaces biofilm reactor (ISBR) was specifically developed to overcome these limitations by simultaneously providing these conditions. This study presents the first standard operating procedure (SOP) for the growth of a model biofilm-forming organism, *Pseudomonas aeruginosa*, within the ISBR, providing a reproducible framework for investigating biofilm development under conditions that closely mimic industrial environments. Statistical analyses confirmed the SOP's repeatability, ruggedness, and reproducibility using biofilm density and biovolume data, supported by an analytical shear stress model for ISBR coupons. Biofilm densities were rugged to recycle and rotation rate deviations but increased with influent feed rate. Conversely, biovolumes were rugged to influent feed and rotation rate changes but declined with higher recycle rates. Both metrics demonstrated excellent repeatability and reproducibility, underscoring the need to assess these characteristics for consistent biofilm formation. Additionally, as a proof-of-concept, two multidomain biofilms (a *Legionella pneumophila*-*Vermamoeba vermiformis* model and a *Rhodotorula mucilaginosa* yeast-*P. aeruginosa* bacterial coculture) were cultivated and analyzed. Both exhibited high repeatability, and the coculture remained stable over a month-long growth period.

1. Introduction

Biofilms, or communities of microorganisms living in a self-secreted polymeric matrix often attached to surfaces, can provide numerous benefits and detriments to human health and the environment. Widespread industrial and clinical problems, including microbially influenced corrosion, chronic wounds that require long-term medical attention, and various other infections can be linked with detrimental biofilm formation and growth [1,2]. Conversely, biofilms are essential for contaminant removal during wastewater treatment, nutrient uptake by plants and thus food production, ecosystem function, and

bioremediation efforts [2–5]. As such, harnessing or mitigating the effects of biofilms is a major research topic, and biofilm reactors are essential tools for in vitro cultivation and study of biofilms.

The ability to cultivate biofilms under diverse growth conditions in laboratory settings has been accomplished by developing and validating multiple bench-scale biofilm reactor models. For example, high fluid shear stresses and turbulent conditions can be modeled using a Center for Disease Control (CDC) biofilm or rotating annular bioreactors [6,7], whereas low or moderate shear conditions can be replicated using drip-flow or rotating-disk bioreactors, respectively [8,9]. Growth conditions and biofilm properties are inextricably linked, and fluid flow

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conditions are particularly influential on biofilm characteristics [10–12]. In short, simulating real-world biofilms in vitro requires bioreactors that faithfully emulate biofilm growth conditions.

Outdoor statues or architecture [13], household appliances (e.g., dishwashers [14], washing machines [15], showers [16], or toilets [17]), or industrial equipment (e.g., heat exchangers [18] or cooling towers [19]) are all environments with intermittently wet, low shear, high gas transfer conditions. Biofilms are also prevalent in these systems, but available bioreactors have, to date, failed to recapitulate these conditions. The industrial surfaces biofilm reactor (ISBR, BioSurface Technologies) was developed to fill this gap and specifically model cooling tower environments, which are known to foster biofilms containing the pathogen *Legionella pneumophila* [19,20]. Up to 84% of legionellosis outbreaks have been linked with aerosols from cooling towers [19]; thus, optimized biocide dosing strategies are needed and may be facilitated by ISBR-based testing. An ISBR image and process flow diagram are provided in Fig. 1. Briefly, the reactor consists of a glass vessel containing a spinning rotor with 18 coupons. Two ports at the bottom of the reactor maintain the fluid level approximately equal to the coupon rotor height, i.e., the coupons are not submerged. Influent medium is fed into the liquid at the bottom of the reactor. This liquid is then recycled and dripped onto the rotating coupons from the top. Coupon angles are adjustable in 10-degree increments between 80° and 110°. More details on reactor characteristics, operational procedures, and design can be found elsewhere [20,21].

Since the ISBR is a novel reactor and has yet to be employed in any published biofilm study, the overarching goals of this study were to develop a standard operating procedure (SOP) for biofilm growth of a model biofilm-forming organism (*Pseudomonas aeruginosa*) in the ISBR and to evaluate the method's repeatability, reproducibility, and ruggedness. A method is repeatable if repetition of the same experiment in the same lab gives similar outcomes and reproducible if the same experiment, conducted in different labs, gives similar outcomes [22]. A rugged method will produce similar outcomes despite minor operational changes [23]. To identify if small changes to SOP variables result in substantially different outcomes, SOP ruggedness was evaluated with a 2³ factorial experimental design in which rotation, recycle flow rate, and influent feed rates were varied by approximately 50–150% of SOP values. Variables were specifically selected because all are likely to deviate slightly from the SOP when implemented: for example, rotational speed is expected to vary slightly between different stir plates; likewise, fluid flow rates may shift due to pump calibration inconsistencies. Quantifying the effect of these operational variables is therefore essential for repeatable and reproducible biofilm growth and

testing as well as standard method development [22]. Additionally, to improve understanding of shear stress and fluid film thicknesses across ISBR coupons under standard operating conditions, a computational fluid dynamic (CFD) model of both parameters was developed.

Lastly, we demonstrated the ISBR's functional applicability and research utility through two distinct use cases. First, a method was established to repeatably grow model cooling tower biofilms containing *L. pneumophila* and *Vermamoeba vermiformis* and tested to confirm the ISBR could accurately cultivate and model cooling tower growth conditions. Second, a method for repeatable multidomain biofilm (MDB) growth was developed and tested with one fungal (*Rhodotorula mucilaginosa*) and one bacterial (*P. aeruginosa*) species. MDBs were cultivated and the stability of each population was assessed over time because, despite MDBs' prevalence in real-world environments [24,25], methods for sustained MDB cultivation in vitro are lacking in the literature. Both use cases are important examples of how the ISBR can be used to cultivate diverse biofilms. This work represents an important step in the ability to cultivate both single- and multidomain biofilms from low shear stress, high gas transfer, and intermittently wet environments.

2. Materials and methods

The following sections are organized by each type of biofilm cultivated in the ISBR, followed by a description of numerical modelling methods.

2.1. Standard operating procedure development and ruggedness tests

2.1.1. Inoculum preparation

All ruggedness tests were conducted with *P. aeruginosa* ATCC strain 15442. Before each test, frozen stocks (used no more than five times) were streaked onto R2A agar plates (BD Difco, prepared per manufacturer's instructions), followed by incubation at 35 °C for 24 h. A single colony was then transferred to 50 mL of sterile 3 g/L tryptic soy broth (TSB, MP Biomedicals) and shaken at 150 RPM for 24 h; the 24-h-old aqueous culture served as the inoculum for all ruggedness experiments. This process was repeated prior to all SOP and ruggedness tests.

2.1.2. Coupon and reactor cleaning procedure

Before each test, ISBRs were disassembled and washed with soap and water. Polycarbonate coupons (15.3 mm × 76.2 mm × 1 mm thick) were removed from the coupon rotor, and both coupons and rotor were sonicated in a 1% soap solution for 15 min. The coupons and rotor were then rinsed in tap water, and sonicated for another 15 min in fresh, soap-free tap water. ISBRs were then reassembled with clean tubing, and cleaned coupons were visually inspected for flaws before they were returned to the coupon rotor. To ensure surface irregularities did not influence biofilm growth distributions and thereby impact microscopy analyses, brand new coupons were placed in rotor slots 3, 6, 9, 12, 15, and 18 prior to each test, and only new coupons were sampled for the analyses described below. All coupons were set at an 80° angle from the horizontal (Fig. 1).

Cleaned ISBRs and tubing were autoclaved for 15 min prior to use. A diagram of flow directions within the ISBR system is shown in Fig. 1. Coupons and ISBRs were purchased from Biosurface Technologies Corp., Bozeman, MT.

2.1.3. Ruggedness testing parameters

Standard operating protocol parameters for rotation rate and influent feed rate were based on those in the ISBR Operator's Manual provided by Biosurface Technologies [20,26]. The SOP recycle flow rate was set at 1 mL/min/coupon (i.e., 18 mL/min). Rotational speed was controlled with RT Touch Series Magnetic Stirrers (ThermoScientific), and recycle and influent media were pumped with Masterflex L/S Digital Drive peristaltic pumps (Avantor) pre-calibrated to the flow rates listed in Table 1. Influent media consisted of sterile deionized water and 3 g/L

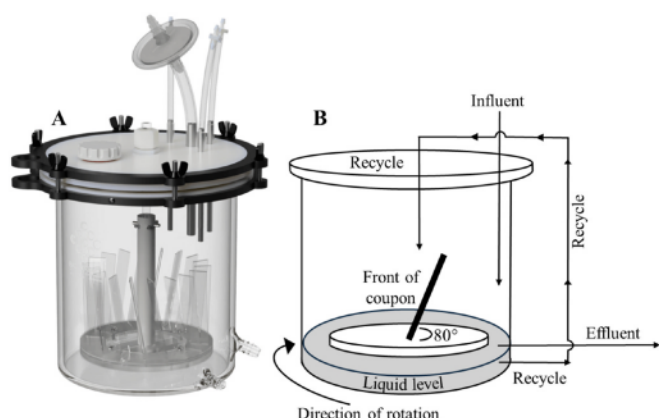


Fig. 1. Overview of ISBR Design and Hydrodynamics. A: Image of the ISBR provided by BioSurface Technologies [21]. B: Flow diagram for the ISBR. Note that influent medium is dripped directly to the liquid at the base of the reactor; it does not hit coupons as they rotate. Only recycle flow is fed onto the coupons directly. Diagram is not to scale.

Table 1
Ruggedness test parameters.

Operating Condition	Rotation rate (RPM)	Recycle rate (mL/min)	Influent feed rate (mL/min)		
			Water	3 g/L TSB	Total flow rate
Minimum	10	9	0.45	0.05	0.5
SOP	30	18	0.9	0.1	1
Maximum	50	27	1.8	0.2	2

TSB, fed to achieve a final influent TSB concentration of 300 mg/L. ISBRs were operated at room temperature ($21 \pm 1^\circ\text{C}$) on lab benchtops.

On day zero, effluent ISBR tubing was clamped shut and reactors were inoculated with 1 mL of the aqueous culture (Section 2.1.1) and 350 mL of sterile 3 g/L TSB via the sampling port. The optical density at 600 nm (OD_{600}) of the 24-h-old culture was measured prior to each inoculation and verified to be between 0.74 and 0.8 (corresponding to approximately 2.8×10^9 CFU/mL, drop plate method detailed in Section 2.1.4). Stir plates and recycle pumps were then turned on, and ISBRs were operated in batch phase for 24 h. Influent pumps were then turned on, and the effluent port was unclamped to begin the continuous phase. Sterile water and TSB medium for each operating condition were fed at the rates listed in Table 1. Rotation and recycle rates remained identical to those in the batch phase. The continuous phase lasted an additional four days, after which ISBRs were shut down and six coupons were sampled: three for biofilm density and three for biovolume (Sections 2.1.4 and 2.1.5).

SOP ruggedness to small changes in rotational speed, recycle flow rate, and influent feed rates was determined using a 2^3 factorial experimental design in which the test variables were purposefully varied by ~ 50 –150% of SOP values (Table 1). Each ruggedness test consisted of a unique combination of each variable at its minimum or maximum level, detailed in Table S1. Three independent ISBR runs under SOP conditions were also performed to assess the ISBR's repeatability variance across independent runs and the variability across coupons within the same run. All tests were randomly ordered as listed in Table S1.

2.1.4. Biofilm cell density enumeration

At the end of the continuous phase (day 5), ISBR pumps and rotation were turned off and aerosols were allowed to settle for at least 15 min prior to opening the reactor. A flame-sterilized hemostat was used to remove triplicate coupons from equally spaced rotor positions (3, 9 and 15). Once removed, coupons were gently rinsed once via immersion into sterile dilution water (0.0425 g/L KH_2PO_4 and 0.405 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, both Fisher). Rinsed coupons were then placed into new centrifuge tubes containing 40 mL sterile dilution water.

Following rinsing, biofilm cells were removed from coupons and disaggregated by a series of three 30-s vortex-sonication cycles [22]. Disaggregated samples (i.e., the 10^0 dilution) were then serially diluted (10^{-1} – 10^{-7}) with dilution water in a 96-well plate [27]. Five drops from each dilution were drop-plated onto R2A agar plates in duplicate. Plates were incubated at 35°C for 24 h, after which colonies were counted from the dilution with drops containing 3–30 colonies per drop. The arithmetic mean of colonies on duplicate plates was calculated, and the log density of biofilm coverage per coupon was calculated as detailed in Equation (1) [28]:

$$\log_{10}\left(\frac{\text{CFU}}{\text{cm}^2}\right) = \log_{10}\left[\left(\frac{X}{B}\right)\left(\frac{V}{A}\right)\left(\frac{1}{D}\right)\right] \quad (1)$$

where X = mean colony forming units (CFU) across all drops at the lowest countable dilution; B = drop volume plated; V = volume cells were disaggregated into (i.e. 40 mL); A = coupon surface area; and D = dilution factor (e.g., 10^{-5}). The area of one side of the coupon (11.62 cm^2) was used in this calculation.

2.1.5. Microscopy

Triplicate coupons from equally spaced rotor positions (6, 12, and 18) were sampled for biomass quantification via confocal scanning laser microscopy (CSLM). Following the initial rinse, coupons were placed biofilm-side up in sterile Petri dishes and stained with the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen) following the manufacturer's instructions for quantification of live and membrane-compromised cells. For imaging, three distinct regions of interest (ROIs) were identified (Fig. S1); ROI 1 at the droplet mark ("top"), ROI 2 at 2 cm below ("middle"), and ROI 3 at 4 cm below ("bottom"). For staining, coupons were incubated in the dark at room temperature for 15 min, then rinsed with sterile MilliQ water to remove excess stain before immediate imaging.

Stained biofilms were imaged using a Stellaris 8 DIVE upright CSLM with a $25\times$ Leica HCX APO L U-V-I water immersion objective (NA 0.90, WD 220 μm) and a digital zoom to achieve $63\times$ magnification. Fluorescent stain SYTO9 was excited at 488 nm and propidium iodide at 568 nm with an emission range of 498–550 and 578–650, respectively. For each coupon, three randomly selected $161 \mu\text{m} \times 160 \mu\text{m}$ fields of view (FOVs) were imaged per ROI. All images were acquired as z-stacks, spanning the full visible depth of the biofilm.

CSLM images were analyzed for live biofilm biovolume using Beer's analysis method, detailed elsewhere [29,30]. This method estimates the surface of the biofilm by fitting the 3D image intensity data with a regression model, then integrates under the surface to calculate biovolume.

2.1.6. Statistical methods

The ISBR's ruggedness in terms of biofilm log densities ($\log_{10}(\text{CFU}/\text{cm}^2)$) and biovolume (μm^3) was evaluated with respect to operational factors using separate linear mixed effects models (LMMs). Covariates in the LMMs included the operational factors rotation rate, influent rate, and recycle rate for both CFU and imaging data. The LMMs included experiment as a random block effect to account for triplicate coupons from each reactor run. The LMM for biovolumes also included ROI (i.e., the location of imaging data relative to the droplet mark on the coupons) as a covariate and coupon as a random effect to account for the three FOVs from each ROI on each coupon. All two-way and three-way interactions among the operational factors and ROI were considered. For all models, residuals were checked to verify assumptions of normality and homoscedasticity. Tukey post hoc tests were performed to maintain a family-wise significance level of 5%. Analyses were performed with Minitab v. 21. Correlation between biovolume and log density responses was assessed by averaging imaging and CFU data for each coupon and fitting a linear regression model to the resulting paired values.

The repeatability of the ISBR SOP in terms of log densities was assessed by fitting an LMM to the three SOP runs with a random effect for experiment. Repeatability is evaluated by independently repeating the same experiment in the same lab. Good repeatability, i.e. similar experimental results, is indicated by a small repeatability standard deviation (SD_r) [31]. The repeatability in terms of the biovolume was assessed by an LMM that had an additional random effect for coupon and a covariate for ROI [22].

Reproducibility is evaluated directly by performing the same method in different laboratories by different personnel. If similar results are observed across labs, the reproducibility standard deviation (SD_R) is also small [31]. The ISBR SOP was not conducted across multiple labs in this study. Instead, reproducibility was estimated by assuming that the nine unique factor level combinations (Table S1) represented nine faux labs. An LMM with nested random effects for faux lab and experiment were then used to estimate SD_R for the log density, and an LMM with an additional random effect for coupon to estimate SD_R for the biovolume.

2.2. Use case 1: cooling tower biofilm cultivation

A detailed description of cooling tower biofilm inoculum

preparation, ISBR operation, and cell density enumeration can be found in Section S1.2 of the SI. In brief, *L. pneumophila* BAA-74 (ATCC strain F1724) and *Vermamoeba vermiformis* strain CDC-19 cultures were grown and maintained in ATCC 1034 medium [32]. *L. pneumophila* requires a eukaryotic host for replication outside of laboratory media; *V. vermiformis*, a wide-spread, free-living amoeba, was used for this purpose [33,34]. Both cultures were maintained as detailed in Section S1.2.1. ISBRs were cleaned and sterilized (Section 2.1.2), followed by inoculation with an undefined heterotrophic bacterial mixture from the effluent of a biologically activated carbon filter (Section S1.2.2). Biofilm was grown for three days to ensure sufficient biomass for *V. vermiformis* grazing. Following this period, *V. vermiformis* and *L. pneumophila* cultures were inoculated and the ISBR was returned to batch phase for 24 h (Section S1.2.2). The *V. vermiformis* and *L. pneumophila* representative cooling tower biofilms were then grown for an additional three days under continuous flow before coupons were sampled for enumeration (Section S1.2.3).

2.3. Use case 2: multidomain biofilm cultivation

Multidomain biofilms consisting of one fungal species (*Rhodotorula mucilaginosa* strain IF6SW-F1 [35]) and one bacterial species (*P. aeruginosa* ATCC strain 15442) were cultivated in the ISBR as a second use case demonstration. *Rhodotorula mucilaginosa* was the selected fungal species because it is commonly found in both human and environmental microbiomes [36,37] and known to cohabitate with *P. aeruginosa* [38,39]. *P. aeruginosa* was selected for further study because ruggedness tests demonstrated its fast and adaptable growth in the ISBR, and therefore we anticipated it would tolerate growth well with another species. Details on MDB inoculum preparation, ISBR operation, and biofilm enumeration are provided in Section S1.3. Briefly, ISBRs were first inoculated with *R. mucilaginosa* and operated with a 5-day batch phase followed by a 10-day continuous phase to establish fungal biofilms. *P. aeruginosa* was then added to the ISBR, and MDBs were grown for an additional 13 days (Section S1.3.2). Coupons were sampled for microscopy and cell density enumeration on days 5, 15, 21, and 28 (Section S1.3.3).

2.4. Analytical fluid film and shear stress model development

To support our experimental observations, we constructed a numerical model to simulate fluid flow within the ISBR. To accomplish this, the ISBR, was represented as a 40° pie-slice (as viewed from above, Fig. 2A), where the wedge encompasses two coupons and provides the smallest axisymmetric version of the reactor required to capture coupon rotation under the recycle port. The coupons, and the rotor that carries them, are further enclosed by a smaller cylindrical wedge enabling coupon rotation while the surrounding ISBR chamber and ports remain stationary. As the coupons advance in their rotation, the leading coupon (Coupon 1, Fig. 2A) transitions out of the wedge's front face and subsequently reenters through its back face.

For the CFD simulations, both the coupons and rotor were held stationary and coupons' location under the recycle port was varied. The justification for this design is three-fold: (i.) it allows droplets to run along each coupon's centerline, which improves comparability and model simplicity. Preliminary simulations showed negligible changes in the wall shear stress and only minor changes to the propagation path of droplets when droplets did not hit the center of coupons or when coupons were rotating. For this reason, including either parameter added unnecessary complexity to the model without significantly impacting model results, and therefore both were neglected. (ii.) Given that recycle flow rate is not guaranteed to synchronize with coupon rotation, it was not appropriate to model continuous coupon rotation without completing computationally prohibitive simulations; therefore, coupons were limited to discrete positions. (iii.) For model interpretation, the analysis was intentionally restricted as the intrinsically high spatial and

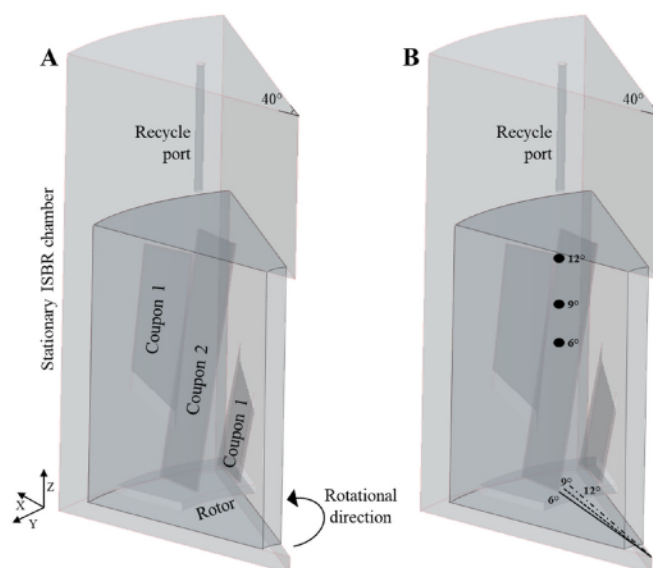


Fig. 2. Coupon orientations used in ISBR fluid dynamic modeling. A: The geometric representation of the ISBR used for CFD modeling. Coupon 1 is only partially shown in the figure because sections of its area are outside of the internal wedge. B: The three coupon positions analyzed in the model are indicated at 6°, 9°, and 12° about the origin by solid and dashed lines (not to scale). Black circles indicate approximately where droplets will impact coupons at each of the three coupon positions. Coupon 2 is pictured at the 6° location.

temporal complexity of the fluid motion became increasingly difficult to interpret as model details increased in specificity (e.g., contact angle or changing surface properties due to biofilm, evaporation, or fluid atomization). Restricting the analysis allowed major trends to be highlighted while avoiding overemphasizing potentially inaccurate outcomes driven by minor or uncertain aspects of the model setup.

To characterize the range of wall shear stress coupons experience during ISBR operation, our model examined coupons at three distinct positions under the recycle port. As coupons spin, the preceding coupon creates the equivalent of a “rain shadow” on the following coupon, thereby only allowing droplets to fall directly on the upper ~40% of each coupon. Coupons were therefore evaluated across this unshadowed portion to approximate the maximum spread in wall shear stress. Coupons were modeled at 6°, 9°, and 12° about the origin, or where water droplets hit each at approximately 30.5, 16, and 1.5 mm from the top, respectively (Fig. 2B). These positions represent the complete range over which recycle droplets may impact coupons and correspond to droplets travelling 45.7, 60.2, and 74.7 mm, respectively, down a coupon surface. The model was set at SOP conditions, i.e. 30 RPM rotation rate (equivalent to coupons passing under the recycle port every 0.0875 s) and 80° coupon angle.

CFD simulations were performed in Siemens' SimCenter Star-CCM+ (version 2502). Water (1000.0 kg/m³ density, 8.871E-4 Pa s viscosity) and air (1.18415 kg/m³ density, 1.85508E-5 Pa s viscosity) phases were treated as incompressible and laminar. The interface between the two fluids was captured with two multiphase formulations using the volume-of-fluid (VOF) method [40–42]. The thin film left as the droplet moved down the coupon was modeled with the fluid-film formulation [43,44] and coupled to the VOF method (0.072 N/m surface tension). The front and back of the modeled ISBR wedge were periodic, allowing water and air to move across both boundaries appropriately. Coupon surfaces were approximated as clean and smooth.

To maximize computational efficiency, the reactor was discretized with a polygonal mesh with an average element size of 1 mm. Regions under the recycle port and surrounding the coupon were further refined with an average element size of 0.175 mm to enhance model precision in areas experiencing bulk flow. To adequately resolve droplet shapes,

there were at least 10 elements per droplet diameter (per established practice [45,46]). A prismatic layer was applied to the coupon surface to improve resolution of droplet and fluid-film development. This configuration also ensured a uniform first-layer thickness adjacent to the surface, thereby reducing noise in the wall-shear-stress calculations. In total there were five prism layers growing with a rate of 110% away from the surface with a first layer thickness of approximately 0.049 mm. For the 6°, 9°, and 12° coupon positions, the total mesh had 3,102,510, 3,085,220, and 3,018,229 elements, respectively.

In addition to modeling the baseline shear stress, or that occurring under SOP recycle flow rates (18 mL/min), the shear stresses occurring at the minimum (9 mL/min) and maximum (27 mL/min) recycle flow rates was also modeled to aid interpretation of ruggedness results. Changing rotation rates were not evaluated, as preliminary simulations showed that rotation negligibly impacted shear. Instead, rotation mainly impacted the “rain shadow” between coupons, with shadows declining with lower rotation rates. Likewise, since influent flow droplets do not hit the coupons, changing influent flows were not modeled.

In total, six simulations were performed that evaluated each coupon position under the SOP recycle flow rate. As the coupon with the longest droplet flow path, the 12° coupon was also evaluated under the minimum and maximum recycle flow rates to assess the relationship between recycle rate and shear. All simulations were run with a time step of 2E-4 s to 0.9 s, except for those at the minimum recycle rate, which were run to 1.46 s. Data was collected every five timesteps, or 0.001 s. Data from the bottom 7.5 mm of the coupon are not presented here, as droplets pooled on the rotor in this region and therefore did not contribute to the bulk of the shear stress and fluid dynamics investigated.

3. Results

3.1. Ruggedness assessment

Fig. 3 shows biofilm log densities in each ruggedness experiment versus varied operational parameters, with experiment number corresponding to the varied rotational speed, recycle flow rate, and feed rate conditions in Table S1. Biofilm densities ranged from 7.06 to 8.51 $\log_{10}(\text{CFU}/\text{cm}^2)$ across all 11 experiments, with an overall mean of 7.83 $\log_{10}(\text{CFU}/\text{cm}^2)$. A multiple regression model fit to the ruggedness data indicates that biofilm log density (LD) was related to the operational variables Rotation (RPM), Recycle (mL/min) and Influent (mL/min) as follows:

$$LD = 7.73 + 0.0015(\text{Rotation} - 30) + 0.008(\text{Recycle} - 18) + 0.574(\text{Influent} - 1) \quad (2)$$

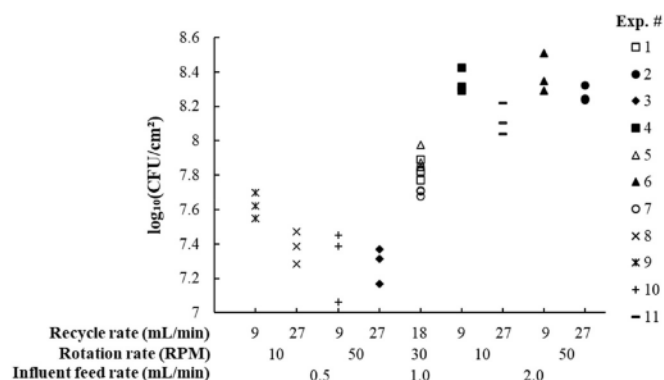


Fig. 3. *P. aeruginosa* biofilm log densities per coupon versus varied operational factors. Experiment number is included to indicate how experimental conditions varied in each ruggedness test. Open symbols indicate results under SOP conditions (i.e., experiments 1, 5, and 7); all other points show results for each non-SOP combination of variables. Experiment number and conditions are defined in Table S1.

Variables in Equation (2) were centered at each of the respective SOP values; therefore, the constant term 7.73 is interpretable as the mean biofilm log density averaged over all 11 experiments. As shown in Fig. 3 and confirmed by statistical analyses of log density data from all experiments (Table 2), influent feed rate was the only parameter to significantly impact the mean log density ($p < 0.0005$). For a 1 mL/min increase in feed rate over the interval tested (0.5–2 mL/min), the mean log density was predicted to increase by 0.57 $\log_{10}(\text{CFU}/\text{cm}^2)$, or nearly four times more CFU/cm². Conversely, neither rotation rate nor recycle rate significantly impacted biofilm density ($p \geq 0.14$, Table 2). Two-way interactions between all variables were also assessed (Fig. S2 and Table S4); however, no statistically significant interactions were identified and thus Equation (2) does not account for any interactions between variables. The lack of two-way interactions indicates that, although influent feed rate had a significant impact on log density, its effect was neither enhanced nor depressed when varied at the same time as rotational speed or recycle rates.

Altogether, statistical analyses indicate that the SOP is rugged with respect to rotation rate changes between 10 and 50 RPM and recycle rate changes between 9 and 27 mL/min. However, the SOP is not rugged for influent feed rate changes between 0.5 and 2 mL/min.

Biovolumes for individual ROIs in each ruggedness experiment are shown in Fig. 4. Across all 11 experiments, total log biovolumes in the 161 $\mu\text{m} \times 161 \mu\text{m}$ FOV ranged from 4.2 to 5.4 $\log_{10}(\mu\text{m}^3)$. Multiple regression analysis of the ruggedness data showed that log biovolume (LB) was associated with the operational variables as follows:

$$LB = 4.85 + 0.0065(\text{Rotation} - 30) + 0.014(\text{Recycle} - 18) + 0.025(\text{Influent} - 1) + 0.013(\text{ROI} - 2) + 0.004[(\text{Recycle} - 18) \times (\text{ROI} - 2)] \quad (3)$$

Similar to log density analyses, operational factors in Equation (3) were centered at each of their SOP values; therefore, the constant term 4.85 is the mean log biovolume averaged over all 11 experiments. Results from Equation (3) show that increasing the recycle rate results in reduced median biovolume, but the interaction term shows that the extent of this reduction varied by ROI ($p < 0.05$). Equation (3) predicts that increasing the recycle rate from 9 to 27 mL/min will decrease median log biovolume by 43% at the top the coupon (ROI = 1). This effect diminishes to 34% reduction at the middle (ROI = 2) and 24% at the bottom (ROI = 3) of the coupon. Neither rotational speed nor influent rate impacted the biovolume ($p > 0.05$). These results demonstrate that biovolume and its spatial distribution across the coupon are sensitive to changes in recycle rate between 9 and 27 mL/min. In contrast, the biovolume generated by the SOP appears rugged over rotation rates from 10 to 50 RPM and influent flow rates from 0.5 to 2 mL/min.

3.2. SOP variance assessment

Log density and biovolume results from the three SOP runs were assessed to evaluate the ISBR's repeatability. *P. aeruginosa* densities under SOP conditions ranged from 7.68 to 7.98 $\log_{10}(\text{CFU}/\text{cm}^2)$ with an overall mean of 7.81 $\log_{10}(\text{CFU}/\text{cm}^2)$ and SD_r equal to 0.11. This value indicates that when the ISBR SOP described herein is implemented by

Table 2
Summary statistics for the regression equation (Equation (2)), which quantifies the effect of operational variables on biofilm log density.

Term	Coefficient	Standard Error	95% Confidence Interval	P-value
Constant	7.73	0.0396	[7.63, 7.82]	<0.0005
[Rotation – 30]	0.00146	0.00224	[-0.00675, 0.00384]	0.536
[Recycle – 18]	0.00826	0.00497	[-0.020, 0.0035]	0.141
[Influent – 1]	0.574	0.0588	[0.435, 0.713]	<0.0005

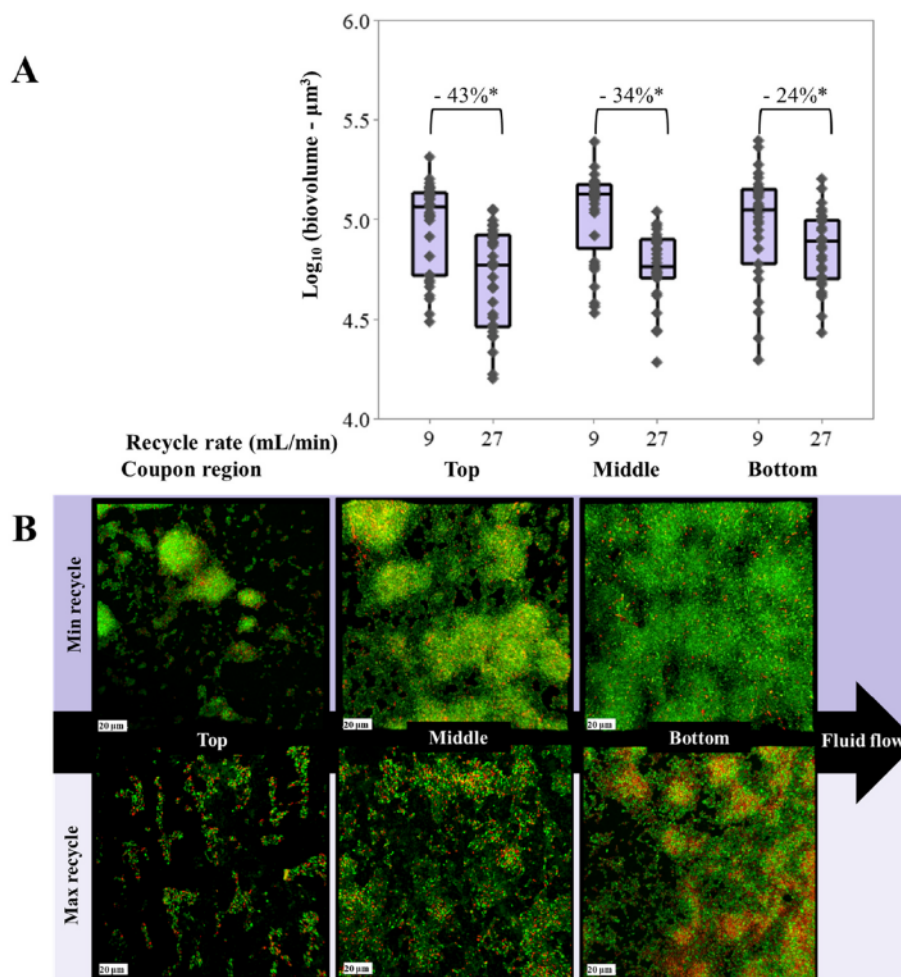


Fig. 4. The effect of recycle flow rate on biofilm biovolume. (A) Increasing recycle rate from 9 to 27 mL/min reduced the overall log biovolume, with impact most pronounced at the top of the coupon. *Indicates statistically significant differences ($p < 0.05$). (B) 3D confocal images over a $161 \mu\text{m} \times 161 \mu\text{m}$ FOV show *P. aeruginosa* biofilms grown in the ISBR under minimum and maximum recycle conditions, collected from the top, middle, and bottom of the coupons. Green and red indicate live and dead cells, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

other laboratories, they are predicted to generate a biofilm log density on a single coupon within 0.11 of the true mean log density for that lab. Approximately 77% of the variance could be attributed to between-experiment sources, such as differences in inoculum preparation or lab temperature; the remaining 23% was attributable to within-experiment sources such as differing environmental conditions in the reactor between coupons.

P. aeruginosa biovolumes under ISBR SOP conditions ranged from 4.20 to 5.30 $\text{log}_{10}(\mu\text{m}^3)$ with an overall mean of 4.78 $\text{log}_{10}(\mu\text{m}^3)$ and SD_T equal to 0.25. Approximately 43% of the variance was attributed to between-experiment sources; the remaining 57% was due to within-experiment variation.

As described in Section 2.1.6, the ISBR's reproducibility was roughly estimated by assuming that results from each of the nine unique ruggedness tests (Table S1) represented those from nine different laboratories. The SD_R was equal to 0.46, which indicates that if a new lab were to run the ISBR SOP in a single experiment, the log density of *P. aeruginosa* on one coupon is predicted to be 0.46 away from the true mean log density. For log biovolumes, the SD_R was equal to 0.27.

3.3. Use case 1: cooling tower MDB growth

Multidomain biofilms consisting of heterotrophic bacteria, *L. pneumophila*, and *V. vermiformis* were successfully cultivated in triplicate model cooling tower experiments. Log cell densities per coupon

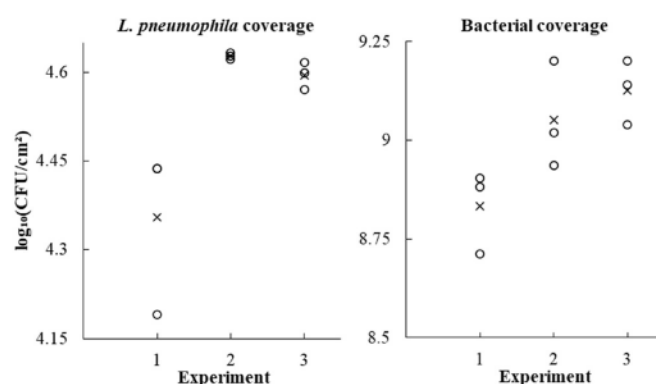


Fig. 5. Cooling tower MDB log cell densities. Cell densities were measured after seven days of biofilm growth. *L. pneumophila* coverage is shown on the left; bacterial coverage is shown on the right. "X" points are the mean log density values for each experiment, "O" points are the log density values per coupon. Note that y-axis scales differ and some points overlay each other.

were consistent across experiments: the *L. pneumophila* density per coupon ranged from 4.36 to 4.63 $\text{log}_{10}(\text{CFU}/\text{cm}^2)$ and mean bacterial densities ranged from 8.83 to 9.13 $\text{log}_{10}(\text{CFU}/\text{cm}^2)$ ("X" points, Fig. 5). The overall mean *L. pneumophila* and total bacterial log densities,

respectively, were 4.53 and 9.00 $\log_{10}(\text{CFU}/\text{cm}^2)$. The SD_r was 0.15 for both *L. pneumophila* and mixed heterotrophic bacterial log densities. Lastly, 11% of the repeatability variance for *L. pneumophila* density was attributable to within-experiment sources and 89% was attributable to between-experiment sources. Altogether, statistical analyses demonstrate that this method exhibits excellent repeatability with consistent densities of both bacteria and *L. pneumophila*.

3.4. Use case 2: yeast-bacterial MDB growth

Population-stable yeast-bacterial MDBs were also cultivated in the ISBR (Fig. 6). *R. mucilaginosa* yeast log densities ranged from 4.23 to 5.11 $\log_{10}(\text{CFU}/\text{cm}^2)$; *P. aeruginosa* bacterial LDs ranged from 7.50 to 8.33 $\log_{10}(\text{CFU}/\text{cm}^2)$. Neither domain showed a significant change in LD with time in any replicate experiment ($p \geq 0.7$), except for *P. aeruginosa* in experiment 2, which declined between day 22 and 28 for unknown reasons. Unfortunately, we could not evaluate if bacterial populations in experiment 2 recovered after day 28, as all coupons had been sampled at that point. Despite this potential outlier, SD_r values calculated from mean LDs at each time point in the experiment were less than 0.15 and 0.35 for fungal and bacterial densities, respectively. Both values indicate that this method provides excellent repeatability throughout the entire experimental duration, with consistent, stable MDB densities.

Confocal microscopy was used to support log density analyses and confirmed the presence of both yeast and bacteria in the ISBR. Fig. 7A shows yeast cells at the end of the continuous phase, prior to bacterial addition (day 15). Fig. 7B shows yeast cells and filaments as well as bacterial cells and was taken on the final day of the experiment (day 28).

3.5. Fluid film and shear stress model

Simulation of the fluid mechanics in the ISBR indicated a highly dynamic system. After droplets hit each coupon, they changed shape, meandered, split, coalesced, and left a residual film that influenced subsequent droplets. Although the bulk behavior of all droplets was similar, spatial and temporal changes persisted, and droplets did not behave consistently across locations or time. Fig. 8 shows simulated droplet flow across the 12° coupon under SOP conditions (18 mL/min recycle). Of note are the small secondary droplets that remain attached to the surface and do not move at the same speed as the primary droplet. Additionally, changes occur in the fluid film beneath the primary droplet as it entrains the existing film and leaves new, localized preferential wetting paths.

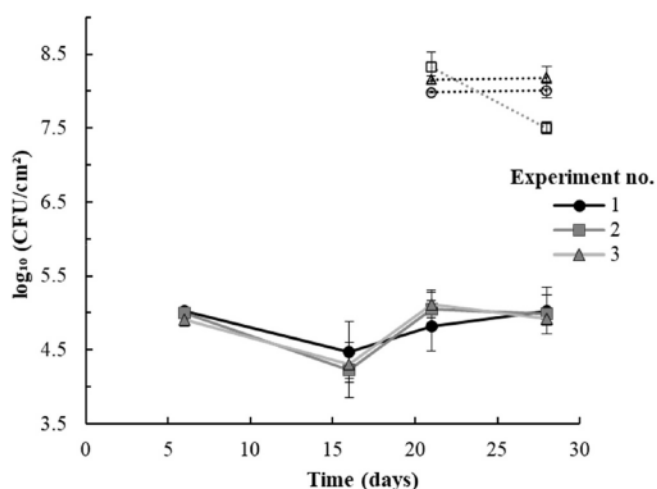


Fig. 6. Mean bacterial (dotted lines and open symbols) and fungal (solid lines and symbols) log densities over time in triplicate ISBR runs (experiments 1–3). Error bars indicate the standard deviation of triplicate coupon samples and at times are smaller than sample points.

Preliminary analyses indicated that wall shear stresses were elevated for the first droplet hitting a clean, unwetted coupon, but quickly reached steady state with the impact of a second or third droplet. For that reason, shear analyses are focused on coupons at steady state. Fig. 9 shows the steady state wall shear stress and droplet height profile over time along the vertical centerline of coupons in the 6°, 9°, and 12° positions. Regardless of coupon position, shear stress on the coupon surface was mainly concentrated under the bulk recycle droplet, though smaller droplets or the moving fluid film also had elevated shear levels (>2 Pa, indicated by smaller black peaks in Fig. 9 outside of the bulk droplet). Areas of the coupon that were not exposed to the droplet dynamics were solely influenced by shearing stress from moving air (near 0 Pa).

Although the lowest impact location (6° coupon) is hit by droplets with the longest free fall and thus highest momentum, the starting wall shear stress was not substantially larger for 6° coupons than it was for 9° or 12° coupons (~ 25 Pa). Likewise, regardless of coupon position or droplets' position along coupons, wall shear stress was greatest at droplets' leading edge where the droplet height was tallest and around the contact line between the droplet and coupon. Shear was also elevated at the tail end of droplets and remained between 10 and 20 Pa for the 6° and 9° coupons throughout their 0.06–0.08 s travel times. Interestingly, for the 12° coupon, shear stress at the tail end of the droplet was less than 10 Pa within 0.01 s of droplet travel, perhaps due to the difference in droplet shape for the 12° coupon compared to the 6° and 9° coupons. As shown in Fig. 9, droplets on 6° and 9° coupons quickly become teardrop-shaped, whereas droplets on 12° coupons maintain their roundness for at least 0.04 s. This behavior might also be caused by the speed at which flow transitioned from droplet-dominated to film-dominated for 12° coupons: shear stress was lower in film dominated areas; therefore, fluid film encountered by the tail end of the primary droplet might be expected to decline faster in these well-wetted zones.

Although shear stress at the leading droplet edge was not affected by where droplets landed on coupons, the average wall shear stress under droplets was strongly affected by droplets' impact location (Fig. 10). Droplets that fell further prior to impact provided higher and more variable shear stress than those that fell a shorter distance. For example, for each droplet that hit the 6° coupon under SOP recycle flow rates, average wall shear stress peaked at 20 Pa and sharply declined to 5 Pa within 0.05 s (Fig. 10). Conversely, for droplets hitting the 12° coupon, average shear peaked at approximately 10 Pa and remained relatively constant before dropping to 5 Pa after 0.11 s.

The effects of changing recycle flow rates on wall shear stress were also investigated for the 12° coupon (Fig. 10). Droplet volumes and impact frequencies increased with recycle rate (Table 4). Recycle flow increases from the minimum to SOP rate resulted in a 1% increase in droplet volume and 51% increase in impact frequency, whereas increases from the SOP to maximum recycle flow rate resulted in a 20% increase in droplet volume and 24% increase in impact frequency. Despite these significant changes, the maximum average wall shear stress experienced by 12° coupons was not significantly different across different recycle rates (~ 10 Pa, Fig. 10). Instead, maximum shear simply occurred more frequently, which is logical given the faster rate of droplet impact. Investigating changing recycle rate impacts on the 6° and 9° coupons were beyond the scope of this study; however, based on results for the 12° coupon, we anticipate that increased recycle flow will similarly increase the frequency of elevated shear on coupons in these positions without increasing maximum average shear stress.

It is important to note that the impact frequencies listed in Table 4 apply only to the simplified CFD model and do not reflect those in the ISBR experiments described herein. Although recycle droplets will fall at the frequencies listed in Table 4, they will not always fall on the same coupon, since coupons are rotating. Instead, approximately one droplet will hit one coupon per rotation under SOP conditions. The lower frequency at which droplets impact the same coupon may decrease the fluid film remaining on coupons and thereby increase wall shear stress.

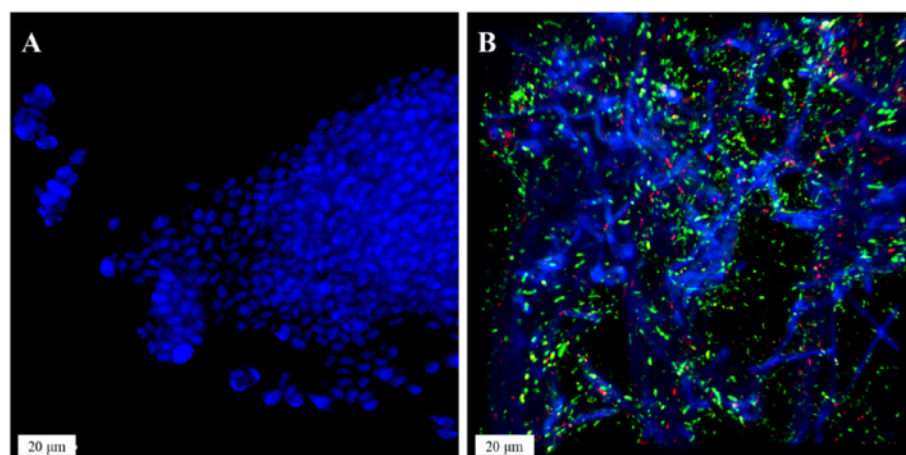


Fig. 7. Yeast-bacterial biofilms over time. (A) *R. mucilaginosa* yeast (blue) at the end of the first continuous phase, before bacterial addition. (B) Co-presence of both *R. mucilaginosa* (blue) and *P. aeruginosa* (live cells in green; dead in red) after 12 days of multidomain growth (day 28). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

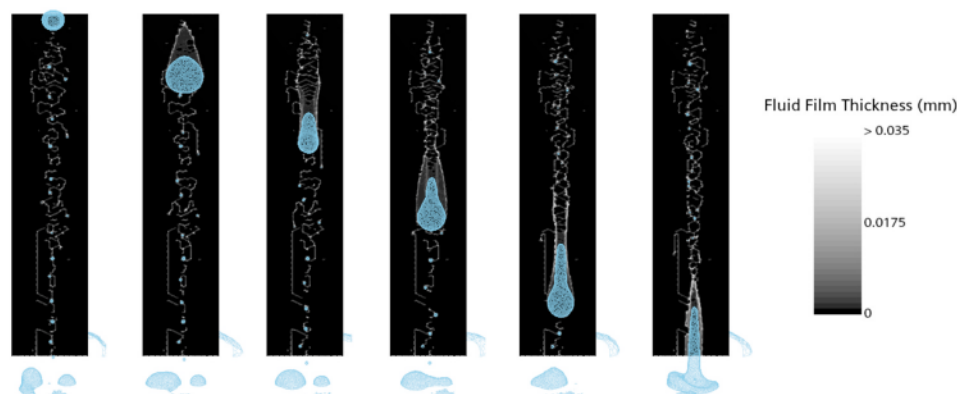


Fig. 8. Simulated progression of a bulk recycle droplet (light blue) and fluid film (grayscale) across a coupon (black) over time under SOP recycle flow. The 12° coupon position is shown here (i.e., 1.5 mm bulk droplet impact position). Time increases left to right in 0.02 s increments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

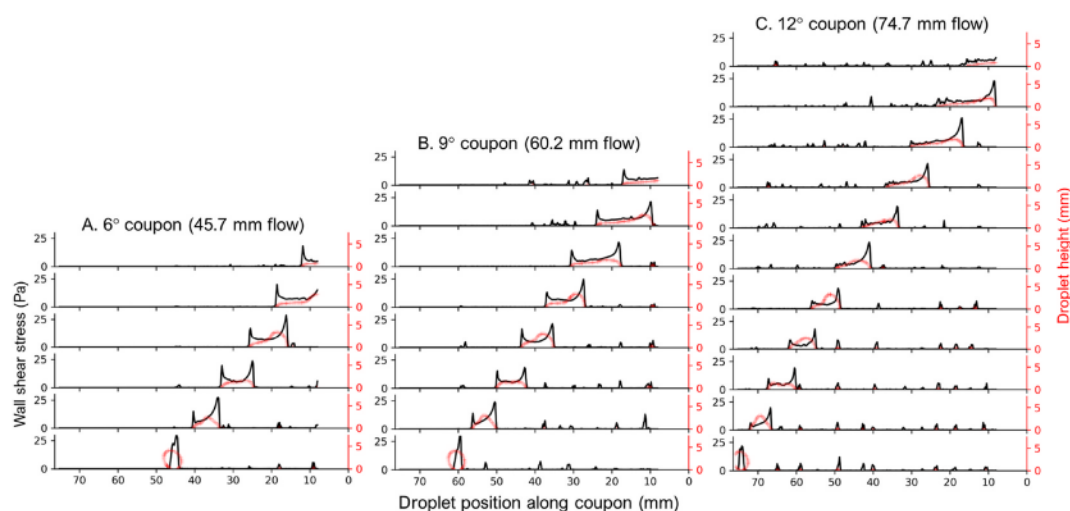


Fig. 9. Time history of wall shear stress (in black) and droplet height (in red) along the vertical centerline of coupons. All three positions modeled (6°, 9°, 12°) are shown in Figures A-C, respectively. Flow distances are the length the droplet travels from its point of impact to the bottom of the coupon (0 mm coupon location). The top of the coupon occurs at 76.2 mm or the leftmost edge of all plots. Recycle was set to the SOP rate of 18 mL/min in this analysis. Time progresses upward vertically in 0.01 s increments for each plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

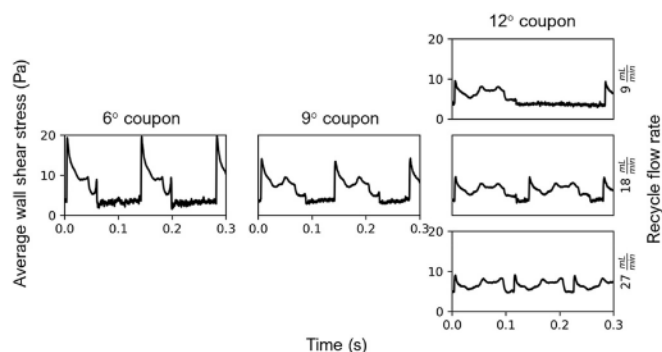


Fig. 10. Average wall shear stress under bulk recycle droplets over time. All coupon positions were analyzed under SOP conditions (18 mL/min recycle flow). The 12° coupon was also analyzed across the range of recycle flows used in ruggedness tests. All analyses were conducted at steady state (i.e., after coupons had been impacted by at least four droplets). Note that the period between droplet peaks is artificially short due to simplifications made for the model and does not reflect the actual time gap between droplets on the same coupon.

Table 3
Summary statistics for the log biovolume regression equation (Equation (3)).

Term	Coefficient	Standard Error	95% Confidence Interval	P-value
Constant	4.85	0.057	[4.72, 4.99]	<0.0005
[Rotation – 30]	0.0065	0.003	[-0.00099, 0.01388]	0.079
[Recycle – 18]	0.0140	0.007	[-0.03017, 0.00312]	0.098
[Influent – 1]	0.0254	0.083	[-0.22073, 0.16990]	0.767
[ROI – 2]	0.0132	0.014	[-0.01341, 0.03987]	0.329
[Recycle – 18] * [ROI – 2]	0.0035	0.002	[0.00005, 0.00699]	0.047

Table 4
Droplet volumes and impact frequencies at the three recycle flow rates evaluated in ruggedness tests.

Recycle rate (mL/min)	Volume (μL)	Frequency (s ⁻¹)
9 (minimum)	40.91	3.58
18 (SOP)	41.41	7.25
27 (maximum)	49.75	9.01

However, measurements taken during preliminary SOP experiments indicated 80–90% humidity in the ISBR, and therefore we anticipate that coupons remain sufficiently wet between droplet impacts to maintain some level of fluid film. For that reason, we expect that the wall shear stresses depicted in Figs. 9 and 10 accurately approximate shear under bulk recycle droplets but overestimate the frequency that droplets impact the same coupon.

Further discussion of the relationship between recycle rate, shear, and biofilm characteristics is provided in Section 4.2.

4. Discussion

4.1. The ISBR versus ASTM-standardized biofilm reactor systems

As a novel reactor system, the ISBR was not previously validated. Development of a standard operating procedure was thus a prerequisite for its broader application in biofilm research. In this study, we developed the first ISBR SOP that accounts for key operational factors (influent, recycle, and rotation rates) and demonstrated that the ISBR

Table 5

Repeatability and reproducibility standard deviation values for log densities of *P. aeruginosa* grown in the ISBR versus those for ASTM-standardized reactors. *Faux refers to the nine unique factor level combinations used in ruggedness tests (Table S1), which approximated results from nine “faux labs”.

	Repeatability (SD _r)	Reproducibility (SD _R)	Source
ISBR SOP (this study)	0.11	0.46 (9-lab faux*)	
Drip flow reactor	0.23	0.27 (10-lab study)	[28,47]
CDC biofilm reactor	0.22	0.24 (6-lab study)	[22,48]
MBEC reactor	0.33	0.67 (8-lab study)	[49,50]

reliably supports biofilm cultivation. Repeatability, reproducibility, and ruggedness for ISBR biofilm densities were comparable to values for ASTM-standardized systems, summarized in Table 5, and indicative of excellent repeatability and reproducibility [28,48,49]. To facilitate accurate comparisons, only those reactors with standard methods for *P. aeruginosa* biofilm growth that were also ruggedness tested are listed.

Similarly, repeatability and reproducibility values for biovolume measurements (0.25 and 0.27, respectively) were within the range of SD values listed in Table 5, which may indicate acceptable biovolume repeatability for the developed SOP. The biovolume repeatability SD was also comparable to that for *P. aeruginosa* biovolumes in the CDC reactor (SD_r = 0.19) [30]. However, Parker et al. (2022) [30] is the only study to our knowledge to analyze biovolume repeatability, and therefore an adequate SD threshold for biovolume repeatability is essentially unknown. Log density repeatability and reproducibility values of 1 or less are generally considered adequate for ASTM standardization of biofilm growth methods [28,48,49,51–54]. Since recycle rate changes significantly impacted biovolume, but did not impact log density (Figs. 3 and 4), we suggest that the conventional SD threshold of 1 is insufficient for biovolume repeatability or reproducibility. Findings herein suggest that biovolume repeatability SD values over 0.25 may indicate biofilms with non-repeatable or non-reproducible morphologies.

Although log density results indicated the ISBR is a rugged system for variations in rotation and recycle rate, increased influent feed rate was correlated with more biomass. This trend between increased influent feed rate and log density was also documented in ruggedness testing of the drip flow reactor [8], though it was not observed in CDC reactor ruggedness tests [6]. Flow is also droplet-based in the drip flow reactor, which may explain why this trend was shared between the two systems. Unfortunately, ruggedness test results for the different bioreactors in Table 5 could not be compared directly since media concentrations and growth periods differed. It was also curious that increased influent feed rates did not correspond to increased biovolumes, despite increases in log density. The reasons for this are unclear, likely because biovolume analyses are missing from ruggedness studies. Log density has been observed to poorly correspond to biovolume [30], which may partially explain this discrepancy. Likewise, nutrient limitations have been shown to stimulate EPS production [55], which may explain why biofilms maintained their biovolumes under low influent feed conditions. Altogether, of the three parameters tested, influent feeding rates must be most carefully controlled by ISBR users to obtain *P. aeruginosa* biofilms with log densities at the levels measured herein.

Additionally, ruggedness testing on biovolumes showed that increased recycle rates reduced biofilm biovolume but in a spatially-dependent manner, with the largest reductions occurring at the top of the coupon and diminishing toward the middle and bottom regions. This spatial differentiation implies that differences in local biofilm thickness and morphology across the coupon surface are affected by shear, since average wall shear stress was found to vary spatially with droplet impact location (Fig. 10). Further discussion of the relationship between shear and biovolume continues in Section 4.2. Importantly, biofilm thickness and morphology influence key physicochemical properties including substrate diffusion [56], extracellular polymeric substance composition and distribution [57], hydraulic resistance [58], and porosity [59].

Thus, biovolume changes with recycle flow rate likely indicate biofilms with significantly different characteristics, and log density measurements alone will not capture this change. We therefore argue that both biomass and morphological assessment are necessary to accurately assess a method's ruggedness, ensuring that standardized procedures capture not only microbial abundance but also structural features that influence function and stability of biofilms.

4.2. Fluid dynamics in the ISBR

Hydrodynamic shear stresses are well known to strongly impact biofilm development and persistence, with community diversity, density, thickness, adhesion, and cohesion highly dependent on the magnitude and spatial distribution of fluid forces [60–63]. Given the influence of this parameter, we sought to characterize the shear conditions in the ISBR to improve understanding of its growth environment and compare ISBR growth conditions to those in other common biofilm reactors. Computational fluid dynamic modeling demonstrated that fluid behavior and wall shear stresses across ISBR coupons were highly variable. Average shear stress was strongly dependent on droplets' impact location (Fig. 10), and since droplets may fall anywhere on the upper 40% of coupons, coupons are likely to experience wide variations in shear throughout multiday experiments. Furthermore, shear varies both spatially and temporally across coupons (Fig. 9), with areas of extremely high shear (25 Pa) concentrated under bulk droplets, areas of moderate shear (~2 Pa) created by smaller droplets and fluid film movement, and areas completely free of any fluid-driven shear outside of the main fluid film (black regions in Fig. 8).

Wall shear stress distributions like those across ISBR coupons do not appear to occur in any other common biofilm reactor. For example, in a modeling study by Johnson et al. (2021), shear across CDC reactor coupons was shown to range from 0 to 1 Pa [64]. Shear has been estimated at 7.3 Pa in rotating annular reactors [65], 0.32 Pa in tissue culture plate reactors [42], and 0.29 Pa in drip flow reactors [10]. Of course, wall shear stress in all cases depends on multiple factors (e.g., fluid flow rates, rotational speeds, biofilm properties, etc.), and therefore the aforementioned systems may provide higher wall shear stresses than those listed here. Regardless, the ISBR provides a unique, wide-ranging mixture of shear conditions, with some coupon areas experiencing extremely high shear and others near zero. ISBR shear conditions appear most similar to those in a cooling tower, as originally intended by the ISBR designers [20], but may recapitulate conditions in any system wherein flow is predominantly droplet-based. Biofilm growth was mainly concentrated under the droplet pathway (Fig. S1) and therefore the extremely high shear provided by the bulk droplet does not appear to have significantly impeded biofilm growth, though future studies should assess if growth patterns change over longer (>5 day) growth periods.

CFD results also helped interpret ruggedness results, particularly regarding biofilm biovolume. We initially interpreted the recycle rate-dependent changes in biovolume (Fig. 4) as potentially resulting from increased average wall shear stress at higher recycle flow rates. However, results in Fig. 10 contradict this interpretation: the average shear under bulk recycle droplets is consistent, regardless of flow rate. Instead, droplet impact frequency appears to be the major driver of declining biovolume. This suggests that the temporal pattern of shear exposure, rather than shear magnitude alone, is important for decreases in biovolume. Since log densities were unaffected by recycle rate changes, we conclude that a higher frequency in localized shear stress is consistent with thinner, more compact biofilm structures rather than biomass loss. Additionally, the decrease in biofilm biovolume under high recycle was more pronounced at the top of the coupon than at the center (Fig. 4), which contrasts with model predictions indicating that average shear stresses were substantially higher when droplets struck the center of the coupon (6° coupon) than the top (12° coupon; Fig. 10). Therefore, as droplets fall more frequently, the center of coupons will more often

experience 20 Pa wall shear stress compared to 10 Pa at the top. However, the CFD model captures peak shear events and impact frequency, not the duration or cumulative shear exposure experienced by the biofilm. This discrepancy may suggest that peak shear stress magnitude alone does not govern biovolume response in the ISBR, particularly given the intermittently wetted, droplet-driven nature of this system. Multiple studies have shown that higher shear stresses cause biofilms to decrease in thickness while increasing in cell density [11,66], however, these studies are largely based on steady or continuous flow systems. Results presented herein indicate that such relationships may not translate directly to intermittently wetted, droplet-driven reactors like the ISBR, and biomass biovolume might be governed by shear timing, intermittency, and exposure patterns rather than average shear stress magnitude alone.

The SOP's ruggedness to changing rotation rates was also verified by the CFD model, as preliminary results showed negligible changes in shear stress with changing rotation rates. Since increasing influent feed rates were correlated with higher log density (Fig. 3), but had no impact on shear stress or biovolume (Table 3), we can conclude that greater nutrient availability leads to overall higher log densities but shear stress remains the driving force for biofilm biovolume characteristics.

Lastly, modeling the presence of biofilm was beyond the scope of our CFD model, despite its likely substantial impact on fluid dynamics. Biofilm may redirect primary recycle droplets along new flow paths, exposing different regions of the coupon to higher shear stresses and previously inaccessible nutrients. In addition, biofilm elasticity and porosity are likely to dampen wall shear stress on the outer biofilm-fluid interface, potentially creating areas of low- and high-density biofilm within this boundary region.

4.3. The ISBR as a multidomain biofilm cultivation system

Multidomain biofilms are ubiquitous in the real world but are rarely represented in lab environments, in part because it is difficult to maintain stable populations from multiple domains over long growth periods [67–69]. As a result, most laboratory studies grow MDBs in batch systems, such as multiwell plates, for sub-10-day periods [69–72]. However, biofilm morphology, extracellular polymeric substances (EPS), populations, resistance to antimicrobials, and many other characteristics differ as biofilms increase in age or experience fluid flow [73–78], and all will impact experimental conclusions. For example, Pan et al. (2022) observed that biofilms growing under turbulent fluid flow had higher EPS-associated protein concentrations than biofilms growing in transitional or laminar flow regimes [78]. EPS-associated proteins are linked with antimicrobial protection, resource capture, and enzymatic activity, among other functions [79], and therefore shifts in their concentration may monumentally impact biofilm characteristics. Furthermore, microorganisms in older, established biofilms will express different genes than those in younger, developing biofilms [80]. This is also certain to impact experimental outcomes and may also affect population stability [81]. In short, flow conditions and biofilm age are important factors in MDB studies, and the method detailed herein, as well as the unique growth conditions in the ISBR, provide a valuable springboard for development of longer-term MDB growth methods in flow-through systems.

As an MDB case use, we grew *P. aeruginosa* and *R. mucilaginosa* for an approximately month-long period in the ISBR and demonstrated that both yeast and bacterial populations were stable over testing period (Fig. 6). Although this timeframe is longer than that in most MDB studies [69–72], it is still short relative to the lifetimes of most real-world MDBs. Future work should therefore evaluate cross-domain population stability in the ISBR over longer timeframes, as there is evidence that “engineered MDBs”—i.e., MDBs cultivated from pre-defined species, like the cooling tower and yeast-bacterial MDBs described herein—are less stable over time than “natural MDBs”, or those cultivated from naturally-established MDB samples [82,83]. Despite this, engineered

MDBs often have more repeatable and reproducible characteristics than natural MDBs [84]. Our results also support this trend: both MDBs had highly repeatable log densities across multiple domains and time points (Figs. 5 and 6). Furthermore, engineered MDBs may still recapitulate valuable characteristics of natural MDBs. For example, *L. pneumophila* densities on ISBR coupons (Fig. 5) were similar to those of biofilms in real cooling tower systems [85,86]. Altogether, MDBs grown via the methods detailed herein may not exhibit the same nuances in morphology or communities as natural MDBs, but exhibit the robust repeatability expected for engineered biofilms with the added benefit of longer population stability than that of most other engineered MDBs.

5. Conclusions

In this study, a standard operating procedure for *P. aeruginosa* biofilm growth in a novel bioreactor was developed and ruggedness tested. The ISBR SOP had excellent repeatability in a single lab and was rugged for rotation and recycle rate changes in terms of log density, which suggested excellent reproducibility across multiple laboratories. However, biofilm morphology analyses indicated that the SOP was not rugged for recycle rate changes over the range tested, as higher recycle rates resulted in thinner biofilms. A wall shear stress CFD model corroborated this finding, as shear stress was elevated more frequently across coupons at higher recycle rates. These findings highlight the importance of evaluating biofilm morphology in addition to log density, as log density analyses alone would have falsely indicated that recycle rate changes did not impact the biofilm. Lastly, two methods for growth of unique multidomain biofilms in the ISBR were developed. The repeatability of both methods was excellent, and both yeast and bacterial populations remained stable over an approximately month-long growth period. Altogether, this study establishes the ISBR as a robust platform for biofilm cultivation.

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CRediT authorship contribution statement

Kylie B. Bodle: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ghazal Vahidi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Erick Johnson:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. **Diane Walker:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Al Parker:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Christopher Jones:** Conceptualization, Data curation, Methodology, Project administration, Validation, Writing – review & editing. **Darla M. Goeres:** Conceptualization, Data curation, Methodology, Project administration, Validation, Writing – review & editing. **Brent M. Peyton:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Heidi J. Smith:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Brent Peyton reports financial support was provided by US Department of Defense. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biofilm.2026.100372>.

Data availability

Data will be made available on request.

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