Life under flow: A novel microfluidic device for the assessment of anti-biofilm technologies

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In the current study, we have developed and fabricated a novel lab-on-a-chip device for the investigation of biofilm responses, such as attachment kinetics and initial biofilm formation, to different hydrodynamic conditions. The microfluidic flow channels are designed using computational fluid dynamic simulations so as to have a pre-defined, homogeneous wall shear stress in the channels, ranging from 0.03 to 4.30 Pa, which are relevant to in-service conditions on a ship hull, as well as other man-made marine platforms. Temporal variations of biofilm formation in the microfluidic device were assessed using time-lapse microscopy, nucleic acid staining, and confocal laser scanning microscopy (CLSM). Differences in attachment kinetics were observed with increasing shear stress, i.e., with increasing shear stress there appeared to be a delay in bacterial attachment, i.e., at 55, 120, 150, and 155 min for 0.03, 0.60, 2.15, and 4.30 Pa, respectively. CLSM confirmed marked variations in colony architecture, i.e.,: (i) lower shear stresses resulted in biofilms with distinctive morphologies mainly characterised by mushroom-like structures, interstitial channels, and internal voids, and (ii) for the higher shear stresses compact clusters with large interspaces between them were formed. The key advantage of the developed microfluidic device is the combination of three architectural features in one device, i.e., an open-system design, channel replication, and multiple fully developed shear stresses. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4850796>]

I. INTRODUCTION

Marine biofilms are organised micro-communities of mixed bacterial and diatom species typically surrounded by extracellular polymeric substances, found attached on all submersed surfaces. Marine biofilms, also termed micro-fouling, constitute a major component of the overall biofouling since they are the first organisms to colonise an underwater surface within minutes following immersion. There is significant evidence to suggest that biofilms positively affect subsequent colonisation by larger organisms such as barnacles, seaweed, and tubeworms.¹ Overall, biofilm formation is a highly dynamic process that is influenced by physical and biochemical processes such as intra- and inter-species competition, water chemistry, and substrate properties (recently reviewed by Salta *et al.*¹).

All marine platforms are subject to hydrodynamic forces whether under tidal, wave, or propulsive motion. Several studies have described the effect of biofilms on the hydrodynamic performance of ship hulls (reviewed in Howell²). For instance, the formation of a 1 mm thick

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biofilm on a 23 m fleet tender resulted in an 80% increase in skin friction coefficient and a 15% loss in ship speed, when compared with the clean hull.³ In full-scale power trials for a naval frigate,⁴ it has been found that fouling, mainly in the form of biofilms, caused an increase of 8%–18% in drag. Schultz, Swain, and co-workers^{5–8} observed substantial fuel penalties resulting from increased surface roughness due to biofilms (in the range of 5 μm –1 mm, reviewed in Salta *et al.*⁹). Being part of a surface, biofilms will also be affected by these forces, and thus, it is imperative to better understand the effect of relevant hydrodynamic flow regimes¹⁰ on the biofilm processes (i.e., bacterial attachment and initial biofilm formation). Importantly, there is a lack of technical data associated with the initial colonisation and biofilm formation that occurs under slow flow conditions experienced by a docked ship. For instance, Southampton Water is tidally dominated with a maximum tidal flow of 1.2 knots.¹¹

Currently, the main antifouling technologies utilised are: (i) biocide based coatings where toxic compounds are released from the surface and (ii) silicone based coatings (foul release coatings, FRC) that depend on low surface free energy. FRCs facilitate the decrease in adhesion strength of fouling organisms and are reported to be successful for vessels that operate at high speeds (<20 knots).¹² The foul release system is considered to have the best antifouling efficacy against macro-organisms; however, marine biofilms tenaciously adhere on the hydrophobic FRC surfaces¹³ that operate in excess of 30 knots to 50 knots.¹⁴ Since FRCs depend on a vessel's speed, it is imperative to develop routine dynamic testing for technologies that rely on hydrodynamics to work against fouling.

There are a limited number of laboratory based biological assays specifically for marine antifouling surfaces, which are often conducted under static conditions.^{15,16} However, static assays cannot account for the influence of the overlying flow that directly affects the organisms' attachment. For dynamic testing, the majority of biofilm studies involve the use of macro-scale reactors and flow cells^{17,18} that have several limitations. However, more recently, there is increased awareness that microfluidic approaches could be more ideally suited to study biofilms (Table I). Ideally for antifouling, a microfluidic platform for biofilm studies should combine three key features: (i) on-chip replication to allow acquisition of statistically significant data; (ii) on-chip application of multiple and controllable wall shear stresses that allows simultaneous exposure of the bacteria to different hydrodynamic conditions; and (iii) an open-system design for testing of a range of different surfaces.

Table I summarises recently developed microfluidic systems for investigating biofilms, together with details on test organisms, device architecture, and materials used. It is evident that despite the majority of microfluidic devices being comprised of single-channel architecture, few studies have indeed reported multiple channel designs.^{17,19,20} However, in most cases, channels have a fixed cross-section, which impedes the simultaneous application of multiple wall shear stresses. An exception is the device reported by Rupprecht *et al.*,¹⁷ which has a tapered microfluidic channel with a linear increase of the wall shear stress along the fluid chamber. Moreover, only one study has reported an open-system microfluidic platform,²¹ whilst the majority of the devices are comprised of polydimethylsiloxane (PDMS) and glass layers permanently bonded together. Although these devices are considered to be cost-effective and easy to fabricate in large quantities, the bonding process could potentially alter the surface chemistry of the tested substrate. Although technologically advanced systems have been developed for biofilm studies (Table I), no current microfluidic device combines the three features which we consider important for antifouling applications within the same platform.

In the current work, we have developed a method to study bacterial adhesion/attachment and biofilm formation using a custom-built microfluidic device (μFD) with six parallel non-connected stepped channels. The design was optimised to bridge the advances made in the systems illustrated in Table I, and it crucially allows micro-fouling investigations under a range of well-defined shear stresses. Specifically, the shear stresses utilised in the current work are equivalent to those associated with low speed moving vessels and marine platforms exposed to tidal conditions²² where the initial biofilm formation is known to occur. For each shear stress, multiple channels are capable of being analysed providing statistically relevant data within a single experiment. Thus, the aim of the present study is to document and quantify the influence of hydrodynamics (adjusted and determined by

TABLE I. A summary of microfluidic designs and fabrication for the study of shear stresses on bacterial attachment and biofilm formation.

Authors	Organism	Materials	Shear stress (Pa) ^a	Channel height	Open system	On-chip replication	On-chip multiple shear stress (defined shear stress)	Comments
Richter <i>et al.</i> , 2007 (Ref. 24)	Fungal biofilms: <i>Candida albicans</i> , <i>Pichia pastoris</i>	Hybrid: PDMS glass	0.030–0.125	20 μm	No	No	No	24 h experiments
Lee <i>et al.</i> , 2008 (Ref. 25)	<i>Staphylococcus epidermidis</i>	PDMS	Device 1 0.024–0.041 Device 2 0.125	100 and 30 μm	No	No	No	120 h experiments
Janakiraman <i>et al.</i> , 2009 (Ref. 19)	<i>Pseudomonas aeruginosa</i>	Hybrid: PDMS glass	0.000006–0.000056	200 μm	No	Yes (two replicas)	No	48 h experiments
Bahar <i>et al.</i> , 2010 (Ref. 26)	<i>Acidovorax citrulli</i>	Hybrid: PDMS glass	0.125–32	50 μm	No	No	No	24 h experiments
Benoit <i>et al.</i> , 2010 (Ref. 27)	<i>Pseudomonas aeruginosa</i>	Hybrid: Polystyrene, glass	0.06, 0.17	70 μm	No	Yes (twelve replicas)	No	21 h experiments
Park <i>et al.</i> , 2011 (Ref. 28)	<i>Pseudomonas aeruginosa</i>	Hybrid: PDMS glass	0.008–0.104	40 μm	No	No	No	24 h experiments
Meyer <i>et al.</i> , 2011 (Ref. 21)	<i>Escherichia coli</i>	Hybrid: PDMS glass	0.0033	100 μm	Yes	No	No	72 h experiments
Rupprecht <i>et al.</i> , 2012 (Ref. 17)	Cell lines and amoeba <i>Dictyostelium</i>	Hybrid: PDMS glass	0.1–5	50, 100, and 150 μm	No	Yes (two replicas)	Yes	3 h experiment. Continuous variation of shear stress along the channel
Kim <i>et al.</i> , 2012 (Ref. 20)	<i>Escherichia coli</i>	Hybrid: PDMS glass	0.008	200 μm	No	Yes (eight replicas)	No	12 h experiments
Mbaye <i>et al.</i> , 2013 (Ref. 29)	<i>Pseudomonas putida</i>	Hybrid: PMMA, glass, vinyl	0.009–1	250 μm	No	No	No	4 h experiments
Salta <i>et al.</i> , current work	<i>Cobetia marina</i>	Hybrid: PMMA, glass, PDMS	0.03–4.3	1000–120 μm	Yes	Yes (six replicas)	Yes (four)	4 h experiments

^aWall shear stress was calculated using the analytical solution presented in Figure 1 and Eq. (3).

ASTM standard D4939-89²³) on the development and structure of a model biofilm-forming bacterium (*Cobetia marina*) using either on-chip *in-situ* monitoring and off-chip microscope image analysis. Although our device was originally designed for the application of marine structures, mainly, ship hulls, this work will demonstrate the proof-of-concept for a rapid assessment method that has the potential to be used for a broad range of biofilm studies (dental and biomedical devices), where the initial attachment and biofilm formation on substrates and under flow conditions is of primary importance.

II. MATERIALS AND METHODS

A. Microfluidic device design and fabrication

The μ FD design, in terms of chamber depth, was performed by adopting a computational-based optimization approach (Fig. 1) in which the micro-channel architecture was iteratively refined in order to obtain the desired shear stress values on the substrate surface. A range of hydrodynamic shear stress values with relevance in marine antifouling coating applications was determined from ASTM standards.²³ Shear stress values corresponded to those that can occur on a ship hull, either during the tidal phase (i.e., vessel in the dock) or during vessel motion. For instance, typical fluid velocity regimes of tidal phases around the UK vicinity may range from 0.25 to 3.00 m s⁻¹. Whilst the device has been designed to achieve hydrodynamic conditions experienced during vessel operations (i.e., up to 15 knots), in this work, we report test

FIG. 1. The workflow for the μ FD design optimisation.

shear stresses found during tidal conditions (0.03–4.30 Pa) as this is the first step during which bacteria attach and further proliferate to produce the initial biofilm layer.

The overall μ FD design is illustrated in Fig. 2. The top plate was drawn in CAD software (AutoCAD, Autodesk, Inc.) and machined by micro milling on a PMMA (polymethyl methacrylate) layer with a micro-mill (Datron CAT3D-M6, Datron Dynamic, Inc.). The surface roughness of the milled channel was reduced by chloroform vapour treatment as previously reported.³⁰ The face to be processed was cleaned with detergent then rinsed with deionised water (18 M Ω cm purity water), ethanol and subsequently dried with an air stream. The PMMA layer was placed on metal stand (6 mm in height) in a flat bottom crystallizing dish, which was previously filled with chloroform in order to have the solvent level 2–3 mm below the layer surface (i.e., face to be treated). A lid was placed over the assembly to form a vapour exposure chamber. The surface was exposed for 6 min and subsequently left overnight in a water bath.

FIG. 2. The μ FD used to assess bacterial attachment. The cross section (left) and top view (right) of the four parts of the assembly are shown together with their dimensions where (a) top plate, (b) gasket, (c) experimental substrate (in this case glass), (d) bottom plate, and (e) cross-section of an individual channel with all the chambers (this is replicated 6 times).

The gasket was fabricated casting a PDMS (Sylgard 184, Dow Corning) layer with a thickness of 0.11 mm. The top part of the template for the cast, which contains the reliefs to produce the through cut features of the gasket, was fabricated on PMMA by micromilling and subsequently treated to reduce the surface roughness as reported above. The bottom part of the template consisted of the substrate layer to be included in the final device. To produce the PDMS gasket, PDMS monomer and curing agent were mixed in a ratio of 10:1 by weight, degassed under reduced pressure, injected into the casting template, and placed in the oven at 65 °C for 210 min.

B. Computational fluid dynamic (CFD) simulations

The fluid flow field within the microfluidic device was numerically simulated with a three-dimensional (3D) model using ANSYS Fluent 12.1.4 (ANSYS, Inc., Canonsburg, PA, USA). Computational fluid dynamics simulations based on the finite volume method (FVM) were performed on a geometry applying 1 200 000 cubic hexahedra (3D) elements. The flow field within the device was modelled using single phase 3D continuity and full Navier-Stokes equations for incompressible fluid flow

$$\nabla \cdot (\rho \vec{v}) = 0, \quad (1)$$

$$\rho \left(\frac{\partial \vec{v}}{\partial t} + \vec{v} \cdot \nabla \vec{v} \right) = -\nabla p + \mu \nabla^2 \vec{v} + \rho \vec{g}. \quad (2)$$

Mass conservation and momentum conservation equations were solved over the fluidic domain, where (v) is the fluid velocity, (ρ) is the density, (μ) is the dynamic viscosity, and (p) is the pressure. The parameters fluid density (ρ) and dynamic viscosity (μ) of 1024.75 kg m⁻³ and 1.08 × 10⁻³ Pa s, respectively, correspond to values of seawater at laboratory temperature of 20 °C.³¹ Velocity boundary condition was imposed at the flow inlet section, while a constant pressure (101 325 Pa) was imposed at the outlet boundary. Finally, no-slip conditions were applied at the solid walls of the modelled device.

The governing equations and their boundary conditions were solved iteratively until solution convergence was achieved. Note that under-relaxation technique was adopted to avoid divergence during the iterative solution procedure.

C. The μ FD experimental set-up

The experimental set-up of the μ FD is illustrated in Fig. 3. A peristaltic pump (Watson-Marlow series 323S) was used to convey the fluid in the microdevice, at a flow rate of 0.5 ml min⁻¹. For these proof-of-concept experiments, a single flow rate was applied; however, the device is designed to allow flow rates as high as 15 ml min⁻¹ that will result in shear stresses of up to 427 Pa (20.27 knots). The reservoir contained freshly prepared media, i.e., Sea Salts (S9883, Sigma Aldrich) plus 18 g l⁻¹ peptone (SeaSalts Peptone, SSP),³² and aeration was achieved through a sterile tube entering the reservoir allowing air exchange with the environment. A 0.22 μ m sterile filter was adjusted at the end of the tubing to prevent contamination from the surrounding environment during the aeration process. Differences in air-pressure within the medium reservoir created by the flow-through system, allowed air “bleeding” in the media. The SSP volume in the reservoir and recycling loop was approximately 500 ml. A damper was used to eliminate flow pulsatility. The components were connected by silicone rubber tubing (Masterflex, with a 3.1 mm internal diameter).

D. Bacterial attachment tests

The marine biofilm forming species used in this study was *Cobetia marina* ATCC 25 374. This species has previously been used as a model in studies on initial attachment of marine bacteria to surfaces.^{32,33} *C. marina* was revived from the original lyophilate and stored as frozen stock (cryopreservation) aliquots in SSP medium with the addition of 10% glycerol and

FIG. 3. Schematic illustrating the μ FD system, closed-loop, microscope set-up and a close-up of the μ FD. Arrows indicate the flow direction.

preserved at -80°C . The cryopreserved culture stocks were plated on Marine Agar (BD DifcoTM Marine Agar 2216) and left to grow until colonies formed (2 days). A single colony was then transferred in sterile SSP and the bacteria were left to grow overnight under agitation (80 rpm) at 28°C . *C. marina*'s growth was quantified using a microplate reader (FLUOstar Omega, BMG LABTECH, Offenburg, Germany) where absorbance was employed to measure the optical density at $\lambda = 595\text{ nm}$ (OD_{595}). The experiment initiated once the overnight culture of *C. marina* reached OD_{595} of 0.2. Prior to bacterial inoculation, the μ FD system was disinfected by circulating 70% EtOH for 15 min, immediately followed by three washes with sterile-filtered ($0.22\text{ }\mu\text{m}$) deionised water ($18\text{ M}\Omega\text{ cm}$ purity water) for a total of 1 h. Finally, the SSP medium was flown through the μ FD system for 1 h to allow conditioning of the channels. A sterile syringe (5 ml capacity) was used to inoculate *C. marina* while the μ FD was placed under a microscope for visualisation using the $10\times$ magnification transmitted objective lens (EVOS fl, AMG, Westover Scientific, Inc.). Bacterial cells were left to attach for 30 min under static conditions. The flow was then restarted (0.5 ml min^{-1}) and the attached cells were allowed to acclimatize to the flow for 30 min before image recording. The images were acquired using the microscope's built-in camera (a high sensitivity interline charge-coupled device camera). Following the microscopy imaging, the flow was stopped and tube clamps were applied to either side of the μ FD in order to ensure static conditions.

To assess attachment and biofilm formation, two measurement strategies were undertaken: (i) for temporal evaluation, time-lapse microscopy was performed for just one channel where for each chamber (i.e., shear stress) triplicate images were taken every 30 min for a total duration of 210 min, resulting in $N=12$ images per sampling time point; and (ii) corroborative observations were taken at time 30 min (t_{30}) and at the end point (210 min— t_{210}) using all μ FD flow chambers in triplicate, resulting in $N=48$ per sampling time point. All microscopy images were acquired under flow conditions and within a defined region of interest (ROI). The ROI width was $450\text{ }\mu\text{m}$ and centred at the channel axis, and thus sufficiently distant from the side walls to consider “corner effects” negligible. Furthermore, images were acquired only at regions where the flow was fully developed (i.e., at a sufficient distance from steplike connections between chambers). Biofilm surface coverage was calculated using ImageJ (MacBiophotonics ImageJ, USA) where the microscopy images were converted into a binary format (i.e., pixel value was either 0 or 255) and covered areas (i.e., surface areas with biofilm) were quantified.

E. Nucleic acid staining

The commercial kit LIVE/DEAD (FilmTracerTM LIVE/DEAD[®] Biofilm Viability Kit, Molecular Probes) was used, which contains two stains: SYTO9, a green fluorescent

intercalating membrane permeable stain, which is expected to stain all cells and, propidium iodide (PI), which is membrane impermeable and is known to stain cells with compromised membranes.³⁴ The LIVE/DEAD kit was freshly prepared according to manufacturer's instructions (i.e., 2 μ l of each stain component per mL of Phosphate Buffer Saline, pH 7.4), and the stains were introduced into the channels using a sterile syringe through the inoculation point. The flow was re-initiated (0.5 ml min⁻¹) for a few minutes to ensure stain circulation throughout the flow cell. Tube clamps were used to stop flow and the μ FD was then kept in the dark for 20 min at room temperature (20 °C). The stains were then flushed by initiating the flow (0.5 ml min⁻¹) for a total of 5 min to allow removal of excess staining.

F. Confocal laser scanning microscopy image acquisition and analysis

End-point images were taken at the centre of each chamber (from a total of two channels, i.e., a total of eight chambers) where at least three stacks were obtained, i.e., $N = 12$ per channel. Image acquisition was performed on a Leica TCS SP2 AOBS (Leica Microsystems, France) confocal laser-scanning microscope (CLSM). CLSM allowed the simultaneous 3D monitoring of PI and SYTO9 dyes. The excitation wavelength used for SYTO9 was $\lambda_{\text{EX}} = 488$ nm, and the emitted fluorescence was collected in the range of λ_{EM} : 500 to 600 nm. The red fluorescent nucleic acid stain PI was excited at $\lambda_{\text{EX}} = 594$ nm, and the emitted fluorescence was collected in the range of λ_{EM} : 650 to 700 nm. Images were collected using a 63 $\times$ magnification Leica water immersion objective. The z-stacks were constructed using the Volocity software (PerkinElmer, Inc., USA).

The CLSM images were analysed by COMSTAT,³⁵ which runs as a script within MatLab equipped with the Image Processing Tool. A fixed threshold value and connected volume filtration were used for all image stacks. The features calculated by COMSTAT in the current study, include: (i) bio-volume of each stack expressed as the volume of biomass per substratum area ($\mu\text{m}^3 \mu\text{m}^{-2}$), (ii) maximum thickness (μm) (iii) surface to volume ratio ($\mu\text{m}^2 \mu\text{m}^{-3}$), and (iv) the percentage area occupied by bacteria in each layer (this is the fraction of the area occupied by biomass in each image of a stack). Substratum coverage reflects how efficiently the substratum is colonized by bacteria of the population and provides the vertical distribution of the bio-film (from deeper to outer regions of the biofilm).

G. Statistical analysis

Differences in percentage coverage, and COMSTAT features between experimental datasets were assessed by applying one-way ANOVA. To establish the homogeneity of variances, Levene's test of equal variances was applied. Where homogeneity of variances was not met, the non-parametric Kruskal-Wallis test was applied. All conclusions are based on 5% level of confidence ($p < 0.05$). Statistical analysis was performed by using SPSS version 19.0 and Minitab version 16.

III. RESULTS AND DISCUSSION

A. CFD analysis of the μ FD performance criteria

During the optimization step, the well-known Poiseuille theory for the fluid shear stress field within a squared cross-section channel was employed to determine the initial chamber depth, which was used as an input for the CFD-based iterative optimization process (Fig. 1). The width of the chamber represents a compromise between a sufficiently large area of study and the requirement of keeping the total flow rate in a range easily achievable with standard laboratory peristaltic pumps. In this respect, channel width (w) was kept constant at 1 mm, while the local channel height (h_i) was determined from Eq. (3) for rectangular chambers,³⁶ as a function of the local shear stress (τ_i) and the volume flow rate at the device inlet (Q_{in}).

$$h_i = \sqrt{\frac{6 \cdot \mu \cdot Q_{in}}{\tau_i \cdot w}} \quad \text{for } i = 0, 1, 2, 3. \quad (3)$$

Q_{in} was set to 8.5 ml min^{-1} , which was within the range of volume flow rates provided by the peristaltic pump used for standard experimental procedures. Chamber height was adjusted with respect to the theoretical value to obtain good agreement between target and the computationally calculated values of the shear stress. The effect of this modified height (h_i^*) on the flow field characteristics (i.e., modified fluid shear stress, τ_i^*) was evaluated numerically by CFD simulations. The h_i^* values were subsequently refined in order to obtain satisfactory agreement between numerical shear stress values and ASTM³⁷ standards. This was achieved upon satisfaction of the following condition:

$$\frac{|\tau_i^* - \tau_i|}{\tau_i} < k = 0.05. \quad (4)$$

Equation (4) was verified for $i=0, 1, 2$, and 3 ; with a maximum per cent difference (k) between τ_i^* and τ_i of 4% within the chamber.

The results obtained from the iterative process for the optimization of the channel depths described above (see Sec. II A), suggested chambers depth of 2.00, 0.32, 0.16, and 0.11 mm, respectively, for a 1.00 mm width. Following the dimension optimisation, a series of CFD simulations was run controlling the inlet flow rate from 0.5 ml min^{-1} to 15 ml min^{-1} . The wall shear stresses obtained for each single chamber at different flow rates were interpolated to perform the calibration, see Table II.

Following the selection of the desired shear stress values to be generated within the device, a preliminary design of the flow cell was performed. Specifically, the design is characterised by a stairlike geometry that creates four chambers with different heights, as shown in Fig. 4(a). The stairlike geometry was proposed in order to obtain four different compartments each characterised by a different homogeneous wall shear stress on the substrate surface, while maintaining a constant flow rate. Fig. 4 shows the computed results of the shear stress at a flow rate of 0.5 ml min^{-1} of the channel inlet. Fig. 4(b) illustrates that four different regions with virtually homogeneous wall shear stress can be created using the current geometry. These results are further demonstrated by the plot of the shear stress distribution along both the cross-section of the chamber (see Fig. 4(c)) and the channel longitudinal direction (see Fig. 4(d)).

B. Temporal variation of bacterial attachment and initial biofilm formation

Clear differences are apparent for the initial attachment of *C. marina* with time (Fig. 5). Specifically, under the lower shear stresses of 0.03 Pa and 0.60 Pa (Figs. 5(a) and 5(b)) the

TABLE II. The selected flow rate of 0.5 ml min^{-1} resulted in the following parameter matrix. The maximum Reynolds number (Re) is 14.25, i.e., the flow regime is characterised as laminar (1 knot is equivalent to 0.514 m s^{-1}).

Wall shear stress (τ_w)			
Shear 1 (Pa)	Shear 2 (Pa)	Shear 3 (Pa)	Shear 4 (Pa)
0.03	0.60	2.15	4.30
Reynolds numbers			
Re 1	Re 2	Re 3	Re 4
5.27	11.98	13.63	14.25
Velocity ^a			
v1 (knots)	v2 (knots)	v3 (knots)	v4 (knots)
0.72	1.75	2.24	3.18

^aFrom ASTM D4939, 2007.

FIG. 4. (a) Cross-section schematic of the micro-channel showing the dimensions of the four micro-chambers. The flow field modelling within the channel, using a flow rate of 0.5 ml min^{-1} at the inlet, (b) top-view colour map of the modelled wall shear stress on the substrate surface, (c) the wall shear stress along the cross section has been determined at the middle length of each chamber section, (d) wall shear stress at different positions along the channel, computed at half-width length of the channel sections showing the characteristic stairlike increase of the wall shear stress.

surface coverage of *C. marina* reached 56% and 59%, respectively. For the higher shear stresses, smaller surface coverage was observed for this species, i.e., 30% and 2.7% when exposed at 2.15 Pa and 4.3 Pa, respectively (Figs. 5(c) and 5(d)). In addition, under the higher shear stresses (2.15 Pa and 4.30 Pa), initial attachment is characterised by an exponential response (Figs. 5(c) and 5(d)). Overall, the current data illustrate distinctive initial attachment patterns between the four shear stress rates. Specifically, with increasing shear stress, there appears to be a delay in bacterial attachment, i.e., at 55, 120, 150, and 155 min for 0.03, 0.60, 2.15, and 4.30 Pa, respectively. The extended lag phase in attachment and biofilm kinetics for most of the observed shear stresses could be attributed to a possible adaptation period being necessary for cell acclimation on going from a static to a hydrodynamic environment.

FIG. 5. Hydrodynamic performance of *C. marina* attachment and initial biofilm formation (percentage of surface coverage) with time in single chambers under (a) 0.03 Pa, (b) 0.60 Pa, (c) 2.15 Pa, and (d) 4.30 Pa, Error bars $\pm$ SD. Figure (e) shows the *C. marina*'s surface coverage (%) at two time points, i.e., start of the experiment (t_{30}) and at the end point (t_{210}) for all channels ($N=48$). Error bars $\pm$ SE. All data were acquired under flow conditions and at the centre of each chamber.

In Fig. 5(e), the surface coverage of bacteria subject to the four shear stresses after 30 min and 210 min averaged for all the flow cells can be seen. Following 30 min of hydrodynamic exposure, bacterial attachment was significantly higher at the lowest shear stress, 0.03 Pa, when compared to the remaining shear stresses ($p < 0.001$). However, when comparing initial biofilm formation at the end point across the four shear stresses, there appears to be a transitional point beyond which initial biofilm formation is significantly lower. Specifically, at the end point, no significance in biofilm surface coverage was found between 0.03 Pa and 0.60 Pa; however, the attached bacterial colonies are significantly fewer when comparing the two lower and two higher shear stresses (0.03 Pa vs. 2.15 Pa and 4.30 Pa, $p < 0.001$ and $p < 0.001$; 0.60 Pa vs. 2.15 Pa and 4.30 Pa, $p < 0.007$ and $p < 0.001$). The greater variability in biofilm coverage at the lower shear stresses at t_{210} is due to localised large clusters or patches typically formed by this species (Fig. 5(e)). Overall, it has been shown that faster flow could bring more cells into contact with the surface due to better mixing, but the sticking efficiency (adhesion) may be reduced because of higher shear.³⁸

C. Morphological variation of initial biofilm formation

The 3D images obtained from the CLSM stacks at the end-point are illustrated in Figs. 6 and 7. It is clear that there is a distinct difference in morphology between young biofilms grown in low (0.03 Pa and 0.60 Pa) and high (2.15 Pa and 4.30 Pa) shear stress. Specifically, at

FIG. 6. Representative CLSM images of *C. marina* stained with SYTO9 and PI, grown in the μ FD and exposed under different shear stresses at the end-point (t_{210}), where Top: 0.03 Pa, Bottom: 0.60 Pa and (a) top view, (b) side view, and (c) 3D view. Each grid square is equivalent to $38 \mu\text{m}^2$, scale bars: $100 \mu\text{m}$; white arrows illustrate the direction of the flow.

FIG. 7. Representative CLSM images of *C. marina* stained with SYTO9 and PI, grown in the μ FD and exposed under different shear stresses at the end-point (t_{210}), where Top: 2.15 Pa, Bottom: 4.30 Pa and (a) top view, (b) side view, and (c) 3D view. Each grid square is equivalent to $38 \mu\text{m}^2$, scale bars: $100 \mu\text{m}$; white arrows illustrate the direction of the flow.

the low shear stresses (Fig. 6), internal channels appear to have formed. When comparing the overall morphologies, biofilms formed under 0.03 Pa exhibit a more homogenous and smooth texture when compared to the ones exposed under 0.60 Pa that appear to be comprised of more distinct interconnected patches. Conversely, under the higher shear stresses (2.15 Pa and 4.30 Pa), *C. marina* exhibited clustered formations with patchy and relatively rounded colonies (Fig. 7). It should be noted that both SYTO9 and PI stains were used; however, as seen in Figs. 6 and 7, it is clearly evident that there were almost no compromised cells (PI stained). For the end-point at t_{210} , it might be expected that there is negligible cell death for this slow growing species.

The data acquired from CLSM allowed for the quantification of additional parameters such as biofilm thickness, biovolume, surface to volume ratio, and vertical distribution of biofilm biomass. As seen in Fig. 8 where the biofilm thickness at the end point is colour-plotted, the initial formation of large biofilm patches can be seen in both low shear stresses. For the biofilm formed under 0.03 Pa, large internal pores/voids can be observed, while smaller voids can be seen for the biofilm developed under 0.60 Pa (Figs. 8(a) and 8(b)). Biofilm maximum thickness was found to be significantly different with shear stress ($p < 0.006$); Bonferroni post-hoc analysis revealed that exposure under the highest shear stress (4.30 Pa) produced significantly thinner biofilms when compared to biofilms exposed to 0.03 Pa and 2.15 Pa ($p < 0.006$ and $p < 0.05$, respectively). No significant difference was found in biofilms between 0.3 Pa and 0.60 Pa, see

FIG. 8. Representative intensity map reflecting biofilm thickness (scale in μm) grown in the μFD at the end-point. Each image represents a flow chamber for each shear stress, i.e., (a) 0.03 Pa, (b) 0.60 Pa, (c) 2.15 Pa, and (c) 4.30 Pa.

Table III. Additionally, there was a significant shear stress effect on biofilm biovolume ($p < 0.038$). Post-hoc comparisons showed that the effect was mainly between the lowest shear stress (0.03 Pa) and the two higher shear stresses (2.15 Pa and 4.30 Pa, $p < 0.05$ for both) (Table III). For surface to biovolume ratio, there is an apparent decrease with increasing shear stress; however, no significance was found, see Table III.

The biofilm vertical distribution from the glass surface to the fluid phase under the different shear stresses was determined from the 3D CLSM imaging data sets,³⁹ see Fig. 9. Interestingly, for the biofilms exposed at the lower shear stresses (0.03 Pa and 0.60 Pa), the bulk of the biofilm biomass appears to be distributed around $10\ \mu\text{m}$ from the surface, thus displaying a distinctive architecture characterised by mushroom-like structures,⁴⁰ see Fig. 9(a). Conversely, biofilms grown under the higher shear stresses (2.15 Pa and 4.30 Pa) display a very different morphology with bacterial biomass distributed in a more compact way, with a more homogeneous dispersion throughout the cluster, especially for the highest shear stress grown biofilms (Fig. 9(b)). In good agreement with the current work, van Loosdrecht *et al.*^{41,42} demonstrated

TABLE III. Maximum thickness, biovolume, and surface to volume ratio of *C. marina* under the four different shear stresses by COMSTAT analysis. $\pm$ Standard Error (SE), KW = Kruskal-Wallis, A = 1-Way ANOVA, NS = not significant. $N = 6$ per shear stress.

Wall shear stress	Max. thickness (μm)	Biovolume ($\mu\text{m}^3\ \mu\text{m}^{-2}$)	Surface to volume ratio ($\mu\text{m}^2\ \mu\text{m}^{-3}$)
0.03 Pa	31.7 ± 2.3	6.4 ± 4.4	0.9 ± 0.2
0.60 Pa	23.0 ± 3.0	3.8 ± 3.0	0.7 ± 0.4
2.15 Pa	27.4 ± 4.0	0.23 ± 0.10	0.6 ± 0.02
4.30 Pa	18.2 ± 3.5	0.09 ± 0.02	0.4 ± 0.12
p value	$p_A < 0.006$	$p_{\text{KW}} < 0.038$	NS

FIG. 9. Representative profiles of bacterial vertical distribution from the glass surface to the fluid phase determined from 3D CLSM imaging data sets of newly formed biofilms grown in the μ FD and under four different shear stresses, i.e., (a) 0.03 and 0.60 Pa and (b) 2.15 and 4.30 Pa.

that, at higher shear forces biofilm morphology was characterised by compact structures with less growth occurring in the outer filamentous biofilm and more in the base of the biofilm. It was suggested that at high shear stresses, the filamentous structures and protrusions are detached before they proliferate out.

Computational simulations have also shown that under low shear stresses (and subsequently low nutrient transport rates), porous and even filamentous biofilms can be formed.⁴³ In terms of biofilm porosity and nutrient (or any substrate) transport, it was found that only at high flow rates convection (as opposed to diffusion) will dominate media transport and biofilm will adapt by becoming less porous and smoother, in a way that mass transfer is buffered.⁴³ Studies on biofilm mechanical properties have established a link between hydrodynamics, biofilm structure, and strength.^{44,45} In general, the higher the flow velocities the greater the nutrient flux towards the biofilm due to a diminished external resistance to the boundary layer.⁴⁶ Computational simulations revealed that at $Re = 13.3$, the biofilms remained thin, suggesting that the higher the Re the stronger the forces that the biofilm must resist. On the contrary, lower $Re = 6.7$ produced bulkier colonies/biofilms.⁴⁶

Interestingly, Rieu *et al.*⁴⁷ investigated the biofilm structure of *Listeria monocytogenes* under flow conditions, and they found that following initial adhesion as single cells, the developed biofilm consisted of dense, ball-shaped microcolonies separated by poorly colonized zones

(16 h exposure). The cell morphology of *L. monocytogenes* was also affected by the growth conditions, i.e., under static conditions bacteria were characterised by short rods while under dynamic conditions, long cells created a network of knitted chains.⁴⁷ They also showed that flow conditions (dynamic and static), besides biofilm structure of *L. monocytogenes*, deeply influenced the temporal patterns of gene expression.

In a comprehensive review by Kovarik *et al.*⁴⁸ for lab-on-a-chip technologies on cell biology, it was highlighted that most of the devices often remain within research laboratories and that the scientific community is lacking simplicity, robustness, and continuity with currently acceptable protocols. For instance, Kovarik *et al.*⁴⁸ reported that device fabrication using PDMS, which is often used due to its low cost, is not a well-established method for cell work when compared to glass that is routinely used by biologists. Furthermore, glass is known to have higher chemical affinity and more stable surface chemistry. It was concluded that devices mimicking the natural processes and environmental conditions will be largely beneficial towards a better understanding of how biological systems operate. Indeed, the principal driver for the current work was to create a more representative environment where marine species are found since we believe that hydrodynamics can affect the overall response of the organisms to treatments. It is encouraging that hydrodynamic tests are increasingly being included in biofilm studies through microfluidic technologies;^{21,49–52} however, our work attempts to combine in a single device, statistical relevance (6 channels), the option to utilize different surfaces (e.g., glass), and the simultaneous application of four shear stresses, which to our knowledge is first reported here. Also, it would be very interesting to assess the biofilm's mechanical properties (using techniques such as cantilever-based indentation and “conventional” indentation) at the end-point to test whether bacterial clusters formed under higher shear stresses would result in stiffer structures.

D. General considerations

A limitation of the microfluidic approach for the assessment of biofilm processes is the risk of chamber vertical confinement due to excessive biofilm growth. This may lead to overall changes in local pressure differences created within the chambers. Biofilm confinement (i.e., ratio between biofilm height and channel height) could potentially influence biofilm growth. Notably, confinement effects have been demonstrated for other biological systems.⁵³ However, to the best of our knowledge, this topic has yet to be investigated for biofilm work, as illustrated by the large variability in channel heights utilized in this field (see Table I). The current study focused on the short timescales typically associated with bacterial attachment and initial biofilm formation where the effect of confinement is negligible.

IV. CONCLUSIONS

In the current work, a novel microfluidic device that allows parallel *in-situ* observations of bacterial attachment and biofilm initial formation under four shear stresses was developed. Overall, it was found that the two lower shear stresses resulted in biofilms with distinctive morphologies characterised by mushroomlike structures, interstitial channels (similar to river banks), and internal voids. For the two higher shear stresses, compact clusters with large inter-spaces were formed. The simple μ FD developed here has the capability to provide a greater understanding of the processes occurring during biofilm formation providing good controllability, replication and relevance to realistic hydrodynamic conditions. We believe our device has a wide spectrum of potential applications ranging from the marine and the medical fields (e.g., biomedical devices and dental research).

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