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Detachment characteristics of a mixed culture biofilm using particle size analysis

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HIGHLIGHTS

- Mixed culture biofilms were grown in three parallel flow cells.
- Detached particles were enumerated and classified by size using fluorescence microscopy.
- Over a 6 h period, detachment was reproducible under steady flow conditions.
- Step changes in shear stress resulted in significant changes to the particle size distribution.

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ABSTRACT

Detachment is a critically important aspect of biofilm processes; it impacts not only on the characteristics of the biofilm itself but also has general implications for the dissemination of pathogenic bacteria and the operation of biofilm reactors. The mechanisms of biofilm detachment are of fundamental importance in the analysis of biofilm processes. However the complexity of biofilm detachment creates difficulties in performing and analysing experiments. It is necessary to identify if, under steady conditions, biofilms experiments are reproducible with respect to detachment. In this study mixed culture biofilms were cultivated under low shear conditions over four days in glass flow cells in triplicate under non-recirculation conditions. Detached particles were regularly sampled, were stained, filtered and analysed using a fluorescence microscope to establish size distributions of detached cells and cell clumps. This study has shown that, despite the existence of a complex particle size distribution, reproducibility is possible in four day old mixed culture biofilms. This has important implications for the study of active or passive detachment in biofilm systems. This study also distinguished between erosion and sloughing following step increases in shear stress.

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1. Introduction

Biofilms are dynamic, structurally complex communities of surface-adhering microorganisms that are embedded within an extracellular polymeric matrix [1]. The steps involved in biofilm development are; initial attachment of the bacterial cells to the surface, irreversible binding and the growth-dependent accumulation to form multilayered cell clusters surrounded by an extracellular polymeric substances (EPS) matrix, biofilm maturation with the development of a characteristic morphology, and detachment, a generalised term used to describe the release of cells (either individually or in aggregates) from a biofilm. Detachment is of general importance in the context of public health particularly with regard to the dissemination of pathogenic bacteria associated with device related infection [2] and in water distribution systems [3]. Detachment also has important implications for the optimum performance of biofilm reactors in wastewater treatment [4].

Detachment processes are frequently distinguished into three different physical mechanisms: sloughing, erosion and abrasion [5]. Sloughing is the apparently random loss of large pieces of biofilm. Erosion refers to the continuous loss of single cells or small cell clumps due to physical forces or cell cycle mediated events [6]. Abrasion is the removal of biofilm due to the collision of particles on the biofilm surface [5].

Traditionally, biofilm detachment has been studied in flowing systems where the dominant mechanism was believed to be the result of local shear forces acting on the biofilm exceed the cohesiveness of the biofilm [4] However it is emerging that there are a range of contributory mechanisms including; quorum sensing [7], the action of matrix degrading enzymes [8] increased expression of flagella and down-regulation of twitching motility [9]. Moreover hydrodynamic shear may play a role in a phenotypic response [10]. Boyd and Chakrabarty [11] reported that the extracel-

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lular network of *Pseudomonas aeruginosa* was important in determining the degree of cell detachment. They found that non-mucoid strains detached up to 50-fold more cells than mucoid strains and concluded that the extracellular network anchors the bacteria to the surface and greatly influences the rate of detachment. Stoodley and co-workers [12] used flow cells to perform detachment experiments with various *P. aeruginosa* strains grown under different levels of fluid shear. They found biofilms grown under low shear conditions to be less dense and to detach at low fluid shear, while biofilms grown under high shear conditions detached at higher fluid shear. From their experiments it was found that the onset of detachment occurred at a shear stress of roughly twice the shear stress during growth. Growth conditions of biofilms strongly affect the detachment process: Applegate and Bryers [13] reported that the nutrient conditions during biofilm growth affect the detachment rates: biofilms grown under carbon substrate-limited conditions contained less extracellular polymer per cell, bound less calcium and exhibited a higher detachment rate than oxygen-limited biofilms while almost never sloughing, even when subjected to prolonged periods of nutrient starvation. Conversely, biofilms cultivated under oxygen limitation showed little detachment but a high and repeatable tendency to slough. Hunt et al. found starvation to trigger detachment in *P. aeruginosa* biofilms [14]. Thormann et al. added that oxygen depletion triggers the detachment of *Shewanella oneidensis* MR-1 as early as five minutes after the oxygen supply was cut off. It was concluded that detachment was a consequence of the bacteria not being able to adapt quickly enough to the changed conditions in the surroundings [15].

The mechanisms of biofilm detachment are poorly understood and yet are of fundamental importance in the analysis of biofilm processes. It is critically important to be able to quantify baseline levels of detachment during steady-state cultivation in order to be able to study the effect of perturbations (for example increased shear, introduction of disinfection agents) on biofilm detachment particularly in the period of cultivation prior to the introduction of such perturbations. Previous studies have shown that detachment occurs throughout biofilm development and it is incorrect to assume that detachment occurs only after the biofilm has matured [16,17]. This suggests that that biofilm formation is a mechanism for proliferation in addition to the role in survival typically mentioned in the literature [18].

The objective of this article was to study biofilm reproducibility in steady-state mixed-culture biofilm operation with a particular emphasis on detachment rate. Previous studies in this area have focused on the reproducibility, as quantified by biofilm structural parameters [19–21]. In particular it was suggested that sloughing events influence the development of biofilm structure. Accordingly, reproducibility, as measured by structural parameters, is likely to decrease over cultivation time due to the stochastic nature of sloughing events [21,22]. The novel aspect of the present study is the development of a framework to assess reproducibility in the context of detachment rate but recognising that detached particles have a highly variable size distribution. A primary objective was to quantify the size distribution and detachment rate during steady-state (baseline) biofilm cultivation in flow cell replicates and assess reproducibility. A second objective was to examine the characteristics of detachment following changes in shear conditions.

2. Methods

2.1. Biofilm cultivation system

Biofilms were grown in glass flow cells of square cross-section 20 cm length with 3- by 3-mm sides, BioSurface Technologies (Montana, USA), giving a cross-sectional area of 9 mm^2 (hydraulic diameter of 3.39 mm). Three capillaries were operated in parallel, using a collective feed tank and waste tank but three separate pumps and effluent collection tanks. All experiments were undertaken in an incubator at 30 °C. Dilution rate was set to exceed the maximum specific growth rate of the mixed culture in order to ensure that biomass in the spent medium represented detached cells rather than suspended growth.

2.2. Medium and microorganism

The mixed culture inoculum used in the experiments was obtained from a biofilm reactor used to treat high strength wastewater at a pharmaceutical production plant (Schering-Plough, Rathdrum, Ireland). Frozen stocks of the inoculum were grown overnight in shake flasks at 30 °C. These overnight cultures were diluted with growth medium to an optical density of OD660 to be used as inoculum. The minimum salts medium was composed of Na_2HPO_4 (2.44 gL^{-1}), KH_2PO_4 (1.52 gL^{-1}), $(NH_4)_2SO_4$ (0.50 gL^{-1}), $MgSO_4{\cdot}7H_2O$ (0.20 gL^{-1}), $CaCl_2{\cdot}2H_2O$ (0.05 gL^{-1}), EDTA (5.0 m gL^{-1}), $FeSO_4{\cdot}7H_2O$ (2.0 m gL^{-1}), $ZnSO_4{\cdot}7H_2O$ (0.10 m gL^{-1}), $MnCl_2{\cdot}4H_2O$ (0.03 m gL^{-1}), H_3BO_3 (0.30 m gL^{-1}), $CoCl_2{\cdot}6H_2O$ (0.20 m gL^{-1}), $CuCl_2{\cdot}2H_2O$ (0.01 m gL^{-1}), $NiCl_2{\cdot}6H_2O$ (0.02 m gL^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (0.03 m gL^{-1}) with sodium acetate as the carbon source at a concentration of 4.2 gL^{-1}.The salts were dissolved in deionised water prior to setting the pH-value to 6.9 and autoclaving. While growing the culture in shake flasks the growth rate of a fresh subculture was determined by measuring the optical density (at 660 nm) of samples as a function of time.

2.3. Growth protocol

The experiment was conducted in three glass capillaries in parallel. The flow cells were operated at three different flow rates. Table 1 summarises flow rates, flow velocities and Reynolds' number for the empty flow cell, calculated using the hydraulic diameter of the flow cell and material properties of water. An inoculum of 3 mL was injected into the glass flow cell and allowed to attach for the initial 24 h. Subsequently, fresh medium was pumped through the flow cell at flow regime #1 for 96 h prior to the experiment. During this time the effluents of all three lines were routed to a collective waste tank. During the experiment the initial flow regime #1 was maintained for 360 min while samples were removed at 20 min-intervals to establish a base line of data. Subsequently the flow rate was raised (flow regime #2) for 30 min and subsequently raised again to flow regime #3 for another 30 min while samples were removed 5 min-intervals during the regime #2 and #3. The optical density at a wavelength of 660 nm was measured immediately after collecting the sample. The sample was then diluted and filtered for microscopy assessment.

2.4. Microscopy assessment of filtered samples

A volume of 1 mL of the effluent was diluted in Ringers solution (quarter strength) to a dilution of 1:10 and stained with acridine orange at a concentration of 100 μL/mL. The stained samples were

Table 1
Summary of flow conditions for each regime. Regime 1 lasted for 6 h while regimes 2 and 3 were of 30 min each.

Regime	Flow rate (L/h)	Reynolds number (–)	Velocity (mm/s)	Wall shear stress (mPa)
#1	0.07	7.32	2.16	5.1
#2	0.3	31.4	9.26	21.8
#3	0.6	62.8	18.5	43.6

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