

Detachment characteristics of a mixed culture biofilm using particle size analysis

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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 analyzing 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 analyzed 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.

Keywords

Biofilm, detachment, erosion, sloughing, shear, size-analysis

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 generalized 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 extracellular network of *P. 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 recognizing 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 9mm^2 (hydraulic diameter of 3.39mm). 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 (2.44gL^{-1}), KH_2PO_4 (1.52gL^{-1}), $(\text{NH}_4)_2\text{SO}_4$ (0.50gL^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.20gL^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05gL^{-1}), EDTA (5.0mgL^{-1}), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (2.0m

g^L⁻¹), ZnSO₄·7H₂O (0.10m g^L⁻¹), MnCl₂·4H₂O (0.03m g^L⁻¹), H₃BO₃ (0.30m g^L⁻¹), CoCl₂·6H₂O (0.20m g^L⁻¹), CuCl₂·2H₂O (0.01m g^L⁻¹), NiCl₂·6H₂O (0.02 mg^L⁻¹), Na₂MoO₄·2H₂O (0.03 mg^L⁻¹) with sodium acetate as the carbon source at a concentration of 4.2 g^L⁻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 660nm) 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 summarizes 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 24h hours. 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 30min and subsequently raised again to flow regime #3 for another 30 min while samples were removed 5min-intervals during the regime #2 and #3. The optical density at a wavelength of 660nm 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 vacuum-filtered onto black polycarbonate filters ("Isopore", Millipore, UK, 0.22 µm pore size, 25 mm diameter) and observed using a fluorescence microscope (Olympus BX 51 with Mercury Arc Lamp and 40x lens). The images were collected using a digital camera (Olympus DP70) and the software "soft-imaging AnalySIS." Twenty images were taken per sample on arbitrary locations of the sample. The images were processed with MatLab (MathWorks Inc, MA, USA) to get a size distribution of clumps. This method was adapted from the method described by Wilson et al. [23] who also reported, that due to the vacuum filtration the clumps of cells flatten out on the filter. Therefore, it was assumed that the area of clumps is representative of the number of cells in one clump.

2.5. Computer analysis of filtered samples

The computer analysis of the microcopy images was separated into three scripts. The first script processed and enhanced the image and measured the sizes of clumps in the image. The second and third script uses this information to generate size distributions. In order to analyze the microscopy images, the images were prepared using standard methods of image processing that are implemented as functions of the "Image Toolbox" in MatLab (Math-Works, MA, USA). In particular the sequence of steps was; (i) Morphological opening: this operation removes small objects from the foreground. A

circle of a radius of 15 pixels was used as structuring element, so small objects below a radius of 15 pixels were discarded as foreground noise. (ii) Subtraction: the background was removed from the image to delete background noise (iii) Histogram equalization: the original distribution of grey values in the image was from #0 (black) to the white point, the brightest color-value in the image. In histogram equalization the distribution of grey values was recalculated to reach from #0 (black) to #255 (white). This step improves image contrast. (iv) Binarisation: this operation transfers a grey image with up to 256 grey values into a binary image with only two color values, black and white. For binarisation the automated Otsu's method was chosen, as manually selecting a threshold to assigning a grey value to black or white has proven difficult [24]. (v) Labeling: connected pixels were marked as belonging together to find shapes of objects (vi) Measuring the size of objects and returning the number of pixels (vii) Converting the size in pixels into m^2 by using the resolution of the camera attached (viii) Discarding every object below an equivalent diameter of $0.22 \mu m$ as noise. This diameter is the diameter of the pores of the polycarbonate filters used in the filtration step. (ix) Create size distribution of clumps. For each sample twenty images were taken on arbitrary locations of the filter area. The size distribution of the sample was then pooled from the analysis of twenty images.

2.6. Distinguishing between sloughing and erosion

In the biofilm literature the distinction between sloughing and erosion has only been made arbitrarily. In this study, the detachment data was split into two fractions to analyze the erosion and sloughing categories separately. Eroded clumps, were defined as particles of an area between $0.04 \mu m^2$ and $5.0 \mu m^2$, sloughed clumps were defined as having an area larger than $5.0 \mu m^2$. The lower size limit of eroded clumps was selected to be of an equivalent diameter of $0.22 \mu m$, the same diameter as the pores of the filter used for the microscopy analysis. Particles smaller than the pore diameter were regarded as noise. The threshold of $5.0 \mu m^2$, corresponding to an equivalent diameter of $2.5 \mu m$ was selected based a comparison of various thresholds taking into account the frequency of erosion events to sloughing events. This subject is covered in more detail in the discussion section below.

3. Results

3.1. Statistical analysis / Reproducibility of baseline data

After an initial cultivation of 96 hours, the flow cells were continued in operation at steady-state flow rate for a further 6 hours, referred to as the baseline period. Detachment rate was regularly assessed and the data was subjected to statistical tests for reproducibility. These tests aimed to test if the mean values of several datasets were equal (within tolerances). Several datasets were selected: optical density of samples, count of erosion events per sample, count of sloughing events per sample, cumulated area of eroded clumps, cumulated area of sloughed clumps, and total cumulated area. The cumulated area of clumps is the area of all clumps of one sample, i.e. all clumps that were found on all twenty images that were analyzed microscopically. It is therefore an integrative value that is an interpretation of the number of cells in one sample. Comparisons of these datasets in form of boxplots can be found in Figure 1

As the majority of the tested datasets was not normally distributed, it was necessary to employ alternative tests to ANOVA that do not require normal distribution of data. The Wilcoxon ranksum test for analysis of variance is an alternative. The test was implemented as the ranksum-function in MatLab. The Wilcoxon test compares pairs of two datasets to each other; the results of the Wilcoxon test for pairwise similarity of data are in table S1, an S2 in the Supplementary Information. The Wilcoxon test reveals that the majority of the pairs of datasets are reproducible, with the exception of the optical density, which has a limited reproducibility.

3.2. Detachment events

Dividing the detachment data into two fractions allowed comparison of the counts of detachment events per sample for sloughing and erosion separately. Figure 2 shows the number of detachment events per hour for sloughing and erosion over time on a logarithmic scale. It is apparent, that over the first six hours of the experiment the number of erosion events per hour is about three magnitudes higher than the average number of sloughing events. Indeed, the average number of sloughing events per hour is negligible during the six-hour baseline period. The raised fluid velocities at 360 min and 390 min lead to increased detachment. The short rise in detached clumps during flow regime 2 at 365 min is not significant - the number of sloughing events per sample briefly rises from one to two events within a 5 min sampling period and returns to the earlier established base line, while the number of erosion events per sample in fact is within the base-line during flow regime 1 and 2. Only after the flow velocity is increased to flow regime 3 at 390 min does the number of erosion and sloughing events per sample rise significantly, suggesting that a critical shear stress exists, below which the erosion rate is independent of shear stress. The number of sloughing events rises by three orders of magnitude from 1 h^{-1} at 380 min to 1320 h^{-1} at 410 min, while the number of erosion events rises from 4400 h^{-1} at 380 min to $2.3 \times 10^5 \text{ h}^{-1}$ at 410 min.

3.3. Mean size of detached clumps

Figure 3 shows the mean size of eroded clumps, i.e. clumps of an area less than $5.0 \mu\text{m}^2$, and figure 4 shows the mean size of sloughed clumps, i.e. clumps that are larger than the threshold. The size thereby is averaged from samples of three lines of the flow cell and error bars mark the standard deviation. It is apparent, that during the first flow regime a base line could be established. The mean size of eroded clumps was constant at around $0.4 - 0.5 \mu\text{m}^2$, corresponding to an equivalent diameter of $0.7 \mu\text{m}^2$ to $0.8 \mu\text{m}^2$. This corresponds to single cells. At the same time the mean size of sloughed clumps is around $5 - 6 \mu\text{m}^2$.

In the third flow regime the decreasing mean size of eroded clumps is apparent, while at the same time the size of sloughed clumps rapidly increased to up to $70 \mu\text{m}^2$. The simultaneous occurrence of these two events could be interpreted as very large clumps of biofilm breaking off leaving the lower layers of the biofilm susceptible to erosion. Due to nutrient starvation bacterial cells in the lower levels might be of a smaller size, hence the decreasing mean size of eroded clumps. Of further interest is the variation between the three lines of the flow cell. While the mean size of eroded clumps averaged from three lines exhibits a nearly constant standard deviation of 5-30%, the standard

deviation of the mean size of sloughed clumps rises from 5-30% during the flow regime 1 to 70-170% at flow regime 3, indicating that the sloughing process is a more erratic process than erosion.

3.4. Relationship of sloughing to erosion

The relationship between sloughing to erosion can be assessed based on two variables: the count of detachment events per sample, i. e. the number of detached clumps, or the cumulated area of clumps, i. e. the size of detached clumps. As shown above the count of detachment events per sample is different for sloughing and erosion by an order of at least two magnitudes, and a relationship based on the count of detachment events would inevitably lead to a meaningless value. Hence, it is better to calculate the relationship of sloughing to erosion based on the cumulated area of clumps that is attributed to sloughed or eroded clumps Figure 5 shows the trend of the fraction attributed to sloughed clumps over time.

It is apparent, that during the flow regimes 1 and 2 sloughed clumps only attributed 2 to 5% of the cumulated area of clumps, but this fraction rises steeply to 50 to 70% after changing to the third flow regime at 390 min. This trend can be explained as follows, the mean and the maximum size of sloughed clumps rises steeply during the third flow regime, and although the detachment counts per sample rise for both detachment and erosion, the area-based fraction of sloughed clumps increases. A fraction based on the count of detachment events per sample would not demonstrate this trend.

4. DISCUSSION

4.1. Reproducibility of baseline data

The flow cell provides a defined and constant flow regime and is therefore an important cultivation system for the study of biofilm detachment. Under steady-state conditions the biofilm growth can be expected to be the same as the rate of biofilm detachment [25]. In the present study it was assumed that 96 hours of cultivation resulted in the attainment of a steady-state prior to the baseline period, during this initial period no major sloughing event occurred. Cultivation conditions were highly controlled and growth in each flow cell was from the same inoculum stock, moreover temperature and flow velocity was constant. The overall reproducibility of the baseline data, as measured by the Wilcoxon ranksum test for pairwise comparison of datasets found an overall reproducibility of 78% for the six analysed datasets. Reproducibility less than 100% is expected, and can have a number of reasons: In general, biological systems frequently lack satisfactorily reproducibility. Additionally, in this study a mixed culture biofilm was used, and although having used the same inoculum for all three lines of the flow cell, the exact microbial population might have differed slightly in each flow cell. Heydorn et al. analysed the reproducibility of *Pseudomonas aeruginosa* in three channels of a flow cell system in parallel, and they reported, spatial heterogeneity within the biofilm can depend on the nutrient levels [19]. They also found differences between parallel channels. Jackson et al. devised a process to grow biofilms that are reproducible with respect to viable cell numbers per surface area [20]. They introduced a “specific number of viable cells”, which is the number of viable cells per surface area per biomass. However, they still reported a standard deviation of 34% compared to 59% for the

number of viable cells per surface area[20]. In addition to biological reasons, the lack of reproducibility in the case of optical density could be related to the limitations of this method. The optical density relates the bacterial density in a solution to the light scattering by those bacteria (Beer-Lambert law). This relation is linear only in a limited range of bacterial densities, and at higher densities the optical densities do not correctly relate to the bacterial count in the sample[26]. In contrast, measurements based on the filtration method yielded higher reproducibility. However, this method also has some limitations in particular with respect to the image analysis where, e.g. the selection of images is influenced by the operator, or binarising the images can distort clump dimensions by falsely counting pixels on the edge of clumps. The notably high rate of reproducibility of the datasets of the cumulated area of clumps is due to the fact, that the cumulated area of clumps is an integral value and therefore is less susceptible to errors introduced during the analysis.

4.2. Definition of cell sizes

Different researchers used different methods to quantify detachment, including for example optical density of the spent medium [27], microscopy-based size analysis [23] and laser-based particle size analysis [28]. The advantage of microscopy based analysis is that it allows classification of particle sizes in addition to the potential to distinguish between viable and non-viable cells if fluorescent staining is used. In this article two size classes were used corresponding to erosion and sloughing. Bacteria are commonly thought to be in the size range of an equivalent diameter of approximately $1\mu\text{m}$. It was recognized in the 1950s that cell sizes of bacteria are dependent on the nutrient conditions during growth [29]. Sargent found cells of *Bacillus subtilis* growing in a batch culture to have almost doubled cell sizes in nutrient-rich medium compared to nutrient-poor conditions [30]. Further, cell size depends on the growth rate of bacteria and fast growing cells are generally larger than slow growing cells [29]. It has been shown that metabolic sensors are involved in delaying cell division during rapid growth to allow bacteria to become larger before dividing [31, 32]. This implies that under nutrient-poor conditions cells not only grow slower but also do not reach the same cell sizes as under favourable conditions. Foladori et al. analysed the size distribution of cells in activated sludge derived from a waste water treatment plant using flow cytometry and found single cells of an area of $0.35\mu\text{m}^2$ and an equivalent diameter of $0.67\mu\text{m}$ [33], while Sadr Ghayeni et al. found waste water bacteria to be small and rod shaped, and approximately $1\mu\text{m}$ by $0.5\mu\text{m}$ in size [34].

In terms of classification it is generally accepted that erosion implies particles of length scale greater than or equal to the size of individual bacteria. However there is no consensus on what the upper limit on the size of eroded particles should be. In the present study the size classification was chosen to fulfil the criteria that sloughing is a rare event under steady-state conditions, specifically less than one sloughed particle event per hour, averaged over three flow cells. This can be observed in Figure 2, which using a log scale, it can be seen that there is a three to four order of magnitude difference in the rate of erosion compared to sloughing. The selection of the threshold is arguably subjective, however a consistent threshold was used throughout in order to make allow a clear distinction to made between detached particles that or either of the length scale of bacteria or a larger length scale

4.3. Hydrodynamic considerations

During the whole length of the experiment the flow cell was operated in the laminar flow regime. This flow regime is characterised by a gradient of fluid velocity and shear stress over the diameter of the flow cell. Using magnetic resonance imaging in biofilm flow systems it has been shown that the local shear forces can be up to 3 times larger than the mean wall shear stress [35]. Ochoa and co-workers noted that in the biofilm literature, most studies used shear stresses that were calculated on the macroscopic level by using simple equations of flow mechanics and that those macroscopic shear stresses present only an average over the reactor volume. They suggested that detachment can be triggered by localised peaks of shear stress that can exceed a generalised shear stress and therefore the authors used computational fluid dynamics to model biofilm growth in a Couette-Taylor reactor and calculated shear stresses on a microscopic level [36]. In contrast to early beliefs of biofilms as a homogeneous layer of cells embedded in extracellular matrix, it is now known that biofilms comprise of a complex structure frequently incorporating stratified layers of biomass of different microbial populations, levels of microbial activity or different mechanical properties [37]. Coufort and co-workers used mixed-species wastewater biofilm and subjected them to erosion tests in Couette-Taylor reactors. It was found that the biofilm can be distinguished into three strata. A top layer closest to the bulk liquid was the most fragile and contributed a detached cell mass of roughly 60% of the initial biomass which could be detached at relatively low shear. An intermediate layer was detached at higher shear and made up roughly 20% of the initial biomass and a third residual layer remained on the surface that could only be detached at very high shear and made up roughly 20% of the initial biomass. Analysis of the basal layer showed that it constitutes a diverse microbial community that consisted of viable cells. It was argued that a high-strength basal layer of viable biofilm cells can initiate re-growth of bacterial biofilm even after the majority of bacterial cells detach due to chemical or mechanical cleaning processes. From conducting these tests with different substrata for biofilm adhesion and with both aerobic and anaerobic bacteria the authors concluded that this behaviour might be a general characteristic of biofilms [5, 38].

4.4. Effect of increased fluid flow

During the first part of the experiment a baseline of data was established at flow regime #1 and subsequently the flow regime was raised to regime 2. Table 2 summarises the changes following the perturbation.

The wall shear stress was raised fourfold from 5.1 mPa to 21.8 mPa, which led to a slight increase of erosion events, but the number of sloughing events per hour, remained constant. The size of sloughed clumps during flow regime 2 cannot be taken into account, because during the comparatively short time of regime 2 (30 min at regime 2 compared to 6h at regime 1) not enough large clumps sloughed from the biofilm to justify comparing the averaged sizes. Based on the constant number of sloughing events and the nearly constant size of sloughed clumps it can be concluded that at the elevated shear stress during flow regime 2 sloughing remained a random event and the biofilm was able to retain its structure, at least for the analysed time span of 30 min.

The increased detachment of single cells is consistent with the works of Stoodley et al. who found increasing detachment of cells at a shear stress roughly two- to three-fold of the shear stress during growth [12]. In their work Stoodley and co-workers cultivated the biofilm at a shear stress of 30mPa, a value comparable to this study. Increased detachment at three-fold shear stresses was also reported by Coufort et al. [38] who found a 50%-reduction of remaining cells in a mixed-culture waste water biofilm studied with a Couette-Taylor reactor. Both studies of Stoodley et al. and Coufort et al. based the analysis on the calculation of residual biomass on the surface, not on the analysis of the effluent. However, Coufort et al. pointed out, that biofilms are organised in layers, whereby the top layer contributes roughly 60% of the biomass and is detached easily, while the intermediate and the base layer contribute 20% of the biomass each and are more strongly attached [38]. This is in line with findings of Stoodley et al. who reported, that with increasing shear stress the detachment rate increases[12], showing that not only the top layer of the biofilm but also the intermediate layer erode under shear.

An increasing detachment rate is clearly demonstrated in the second part of this experiment, where the detachment rate of erosion rises substantially while the wall shear stress is only doubled. In fact, during the second part of the experiment, the biofilm disintegrates on a large scale, demonstrated by the rising prevalence of sloughing events. While during the transition from flow regime 2 to regime 3 the shear stress was only doubled from 21.8 mPa to 43.6 mPa the number of erosion events increased 23-fold and the number of sloughing events 296-fold (Table 3). Table 3 clearly shows that not only the number of detachment events increased but also the size of sloughed clumps. Telgmann et al. reported that sloughing can increase the heterogeneity of biofilms and thus increase localised peaks of shear stress, which in turn can lead to more sloughing [39]. It is very likely that this happened in the flow cell during flow regime 3. Rupp et al. reported that clusters of biofilms can migrate along the surface of flow cells at elevated shear stresses [40]. The authors subjected *S. aureus* biofilms grown at 125mPa to shear stresses of 1.8Pa and found biofilm clusters rolling downstream. These clusters were attached to the surface with so called “tethers” which gradually stretched and eventually broke, allowing a downstream motion of the cluster. The authors reported, that these “tethers” can absorb shear variations elastically by deformation but over periods longer than the relaxation time, these “tethers” will flow and eventually break. In case of the analysed monoculture of *S. aureus* Rupp et al. reported a relaxation time of 12 min. It was impossible to monitor the biofilm behaviour using the technique of Rupp et al. in this study, so it could not be detected if the same mechanisms occurred in the analysed biofilm. It can be hypothesised that distinct “tethers” did not play a significant role, as the biofilm consisted of a mixed culture and therefore the detachment may have been influenced by the properties of each subculture.

In this study a steep increase of sloughing was observed immediately after the onset of the highest shear stress. Assuming subpopulations with a very short relaxation time, these subpopulations would slough off first, leaving “gaps” and heterogeneities in the biofilm. These heterogeneities lead to localised stress peaks in the biofilm and subsequent sloughing of other subpopulations, even if they were more rigid than others.

Another observation is that the mean size of eroded clumps decreases over the course of the experiment from $0.44\mu\text{m}^2$ to $0.35\mu\text{m}^2$ and finally $0.22\mu\text{m}^2$. As mentioned earlier, this size range is interpreted as single cells. The decreasing size of single cells during the experiment can be explained by the layered structure of biofilms: Cells in the top layer of biofilms have better access to nutrients from the bulk medium and thus can grow to a larger size than cells embedded in lower layers of the biofilm. These cells could be in dormant state due to nutrient starvation. Over the course of the experiment the top layer of cells is sheared off quite early, lower layers of the biofilm are subjected to erosion, and so the development of the mean size of eroded cells over time demonstrates a possible vertical size distribution of single cells within the biofilm, although conclusion in this regard are not possible when a mixed culture biofilm is used.

5. Conclusions

Mixed culture biofilms were cultivated in flow cells to study detachment behaviour. The focus of the study was not on the effect of biofilm properties or cultivation time on detachment as is extensively reported recently [41-43]. Rather, the results established that reproducibility with respect to detachment was possible, provided appropriate methods were used to classify detached particles. This conclusion was established by undertaking frequent sampling over a six hour base-line period following 4 days of cultivation at a wall shear stress of 5.1 mPa, biofilms in flow cell triplicates. During this period sloughing events were negligible and erosion dominated. It was found that at a shear stress of 21.8 mPa the frequency of erosion and sloughing remained constant. However at 43.6 mPa the frequency of erosion events increased 57-fold and the frequency of sloughing events increased 250-fold. The step increases in shear stress was associated with sharp increase in the average size of sloughed particles. The results emphasize the importance of particle size analysis in the study of biofilm detachment.

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TABLES

Table 1. Summary of flow conditions for each regime. Regime 1 lasted for 6 hours while regime 2 and 3 were of 30 minutes 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

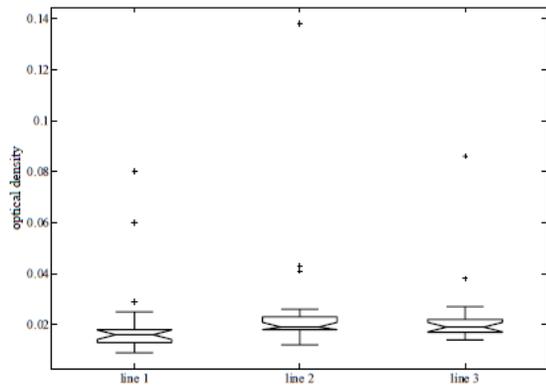
Table 2: Changes of size parameters during the transition form regime 1 to 2

Parameter	Unit	Regime #1	Regime #2	Relative change
Erosion events	hr^{-1}	2100 ± 1383	5200 ± 1270	2.5-fold increase
Sloughing events	hr^{-1}	3 ± 3	5 ± 8	Constant
Mean size/erosion	μm^2	0.44 ± 0.06	0.35 ± 0.06	20% reduction
Mean size sloughing	μm^2	3.67 ± 3.2	(2.02 ± 2.3)	(reduction)*
Maximum size/sloughing	μm^2	4.40 ± 4.3	(2.63 ± 3.3)	(reduction)*

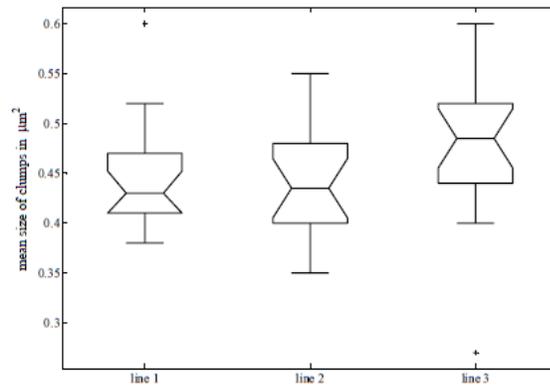
* the size of sloughed clumps is based on too few samples to be representative for regime #2

Table 3: Changes of size parameters during the transition from regime 2 to 3.

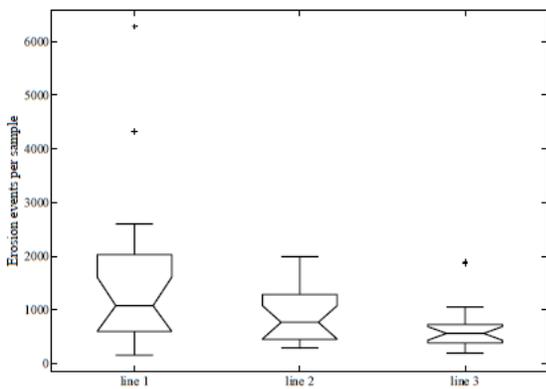
Parameter	Unit	Regime #2	Regime #3	Relative change
Erosion events	hr^{-1}	5200 ± 1270	120,000 ± 76,600	23-fold increase
Sloughing events	hr^{-1}	5 ± 8	800 ± 469	296 fold increase
Mean size/erosion	μm^2	0.35 ± 0.06	0.24 ± 0.06	31% reduction
Mean size sloughing	μm^2	(2.02 ± 2.3)	21.6 ± 26	10 fold increase
Maximum size/sloughing	μm^2	(2.63 ± 3.3)	381 ± 499	145 fold increase



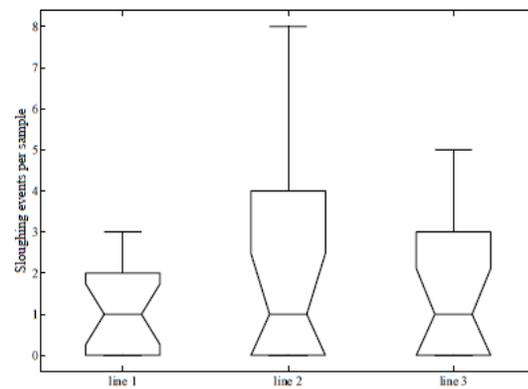
(a) optical density



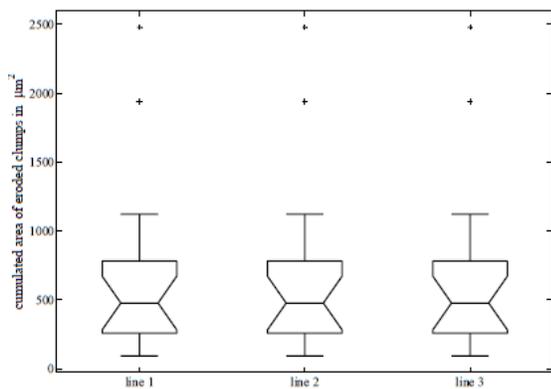
(b) mean size of clumps - erosion



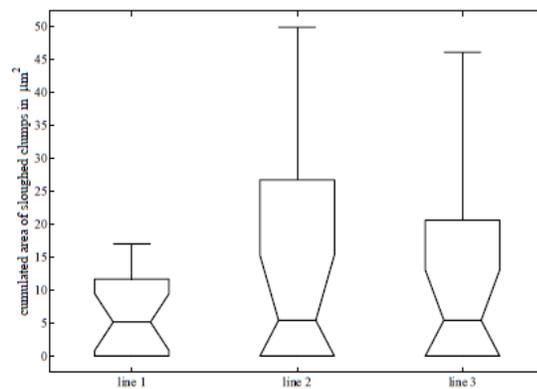
(c) number of erosion events



(d) number of sloughing events



(e) cumulated area - erosion



(f) cumulated area - sloughing

Figure 1. Representative box-plots showing each parameter used for statistical analysis of flow cell detachment experiments. Lines 1, 2 and 3 refer to the three replicate flow cells.

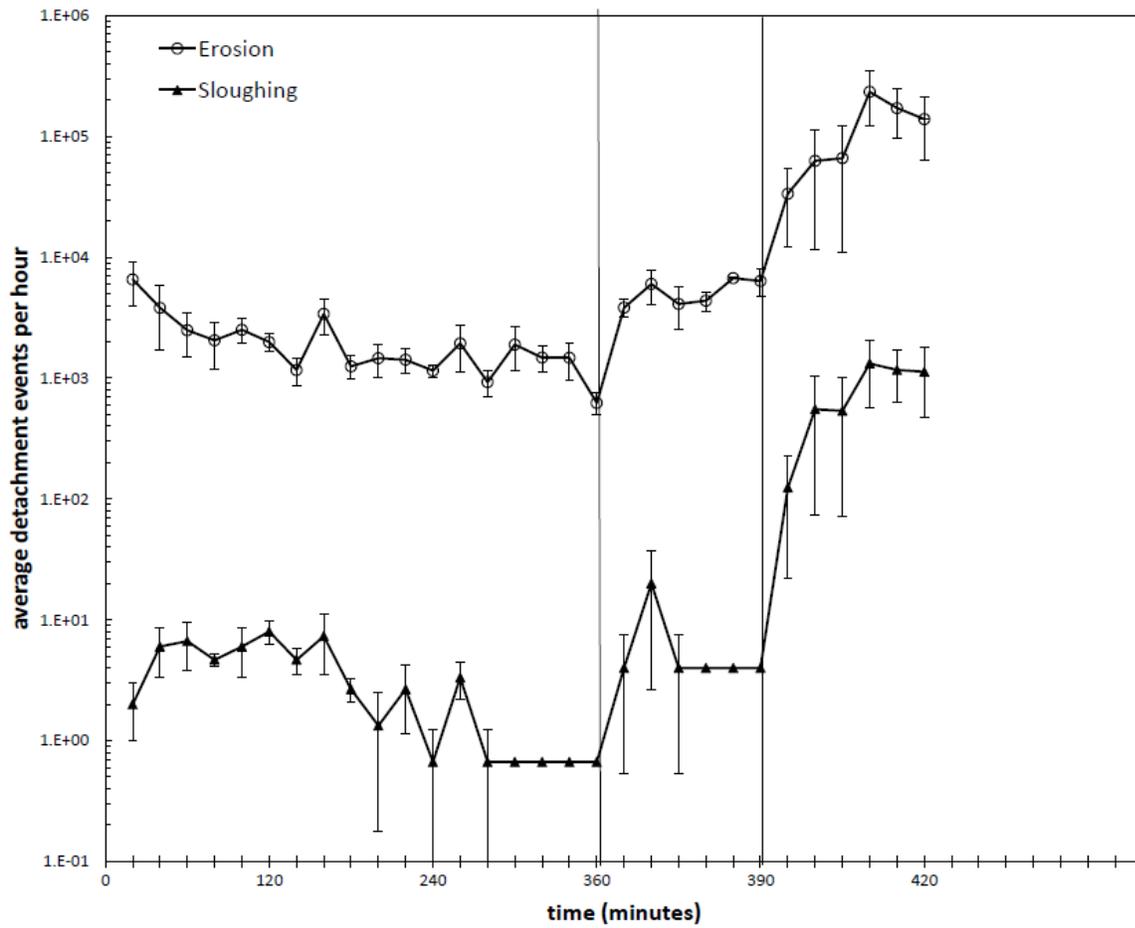


Figure 2. Number of detachment events over time for erosion and sloughing; the vertical lines mark the change in flow regime at 360 min and 390 min.

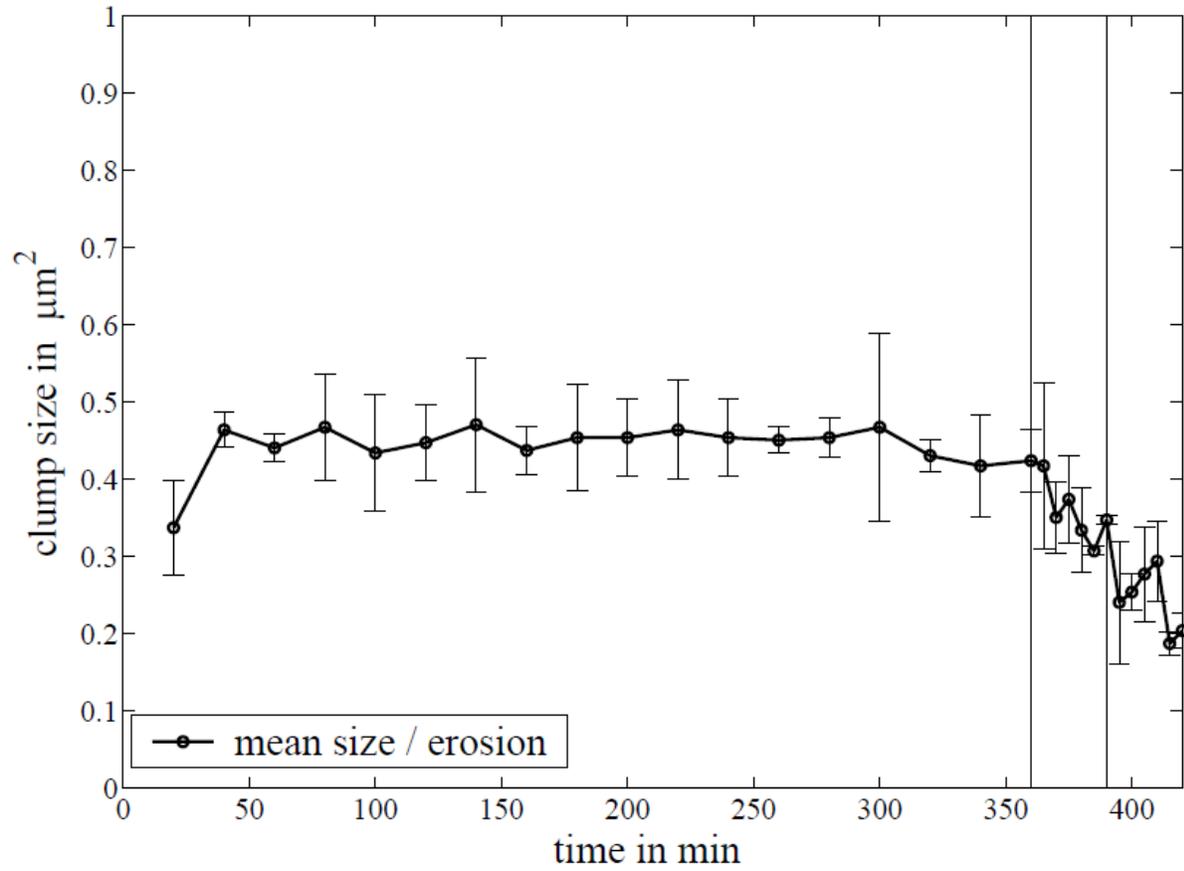


Figure 3. Mean size of eroded clumps; the vertical lines mark the change in flow regime at 360 min and 390 min.

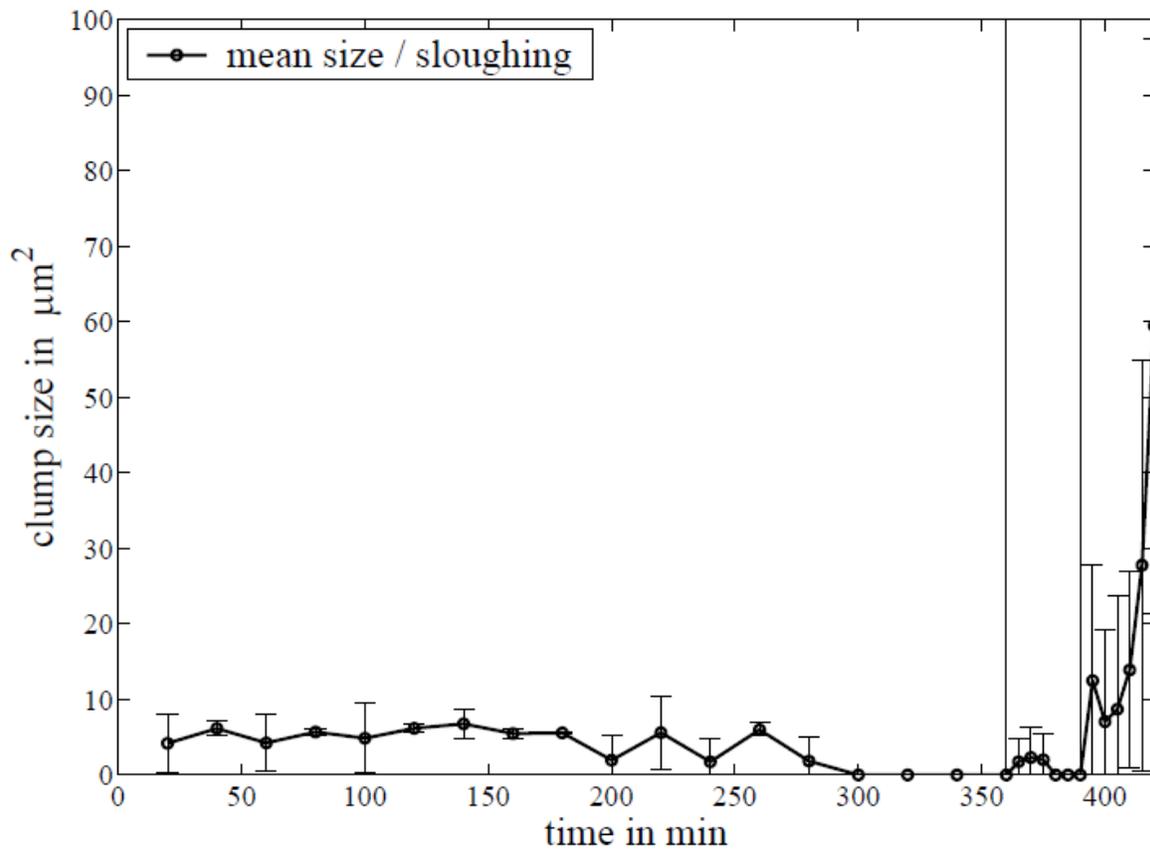


Figure 4. Mean size of sloughed clumps; the vertical lines mark the change in flow regime at 360 min and 390 min.

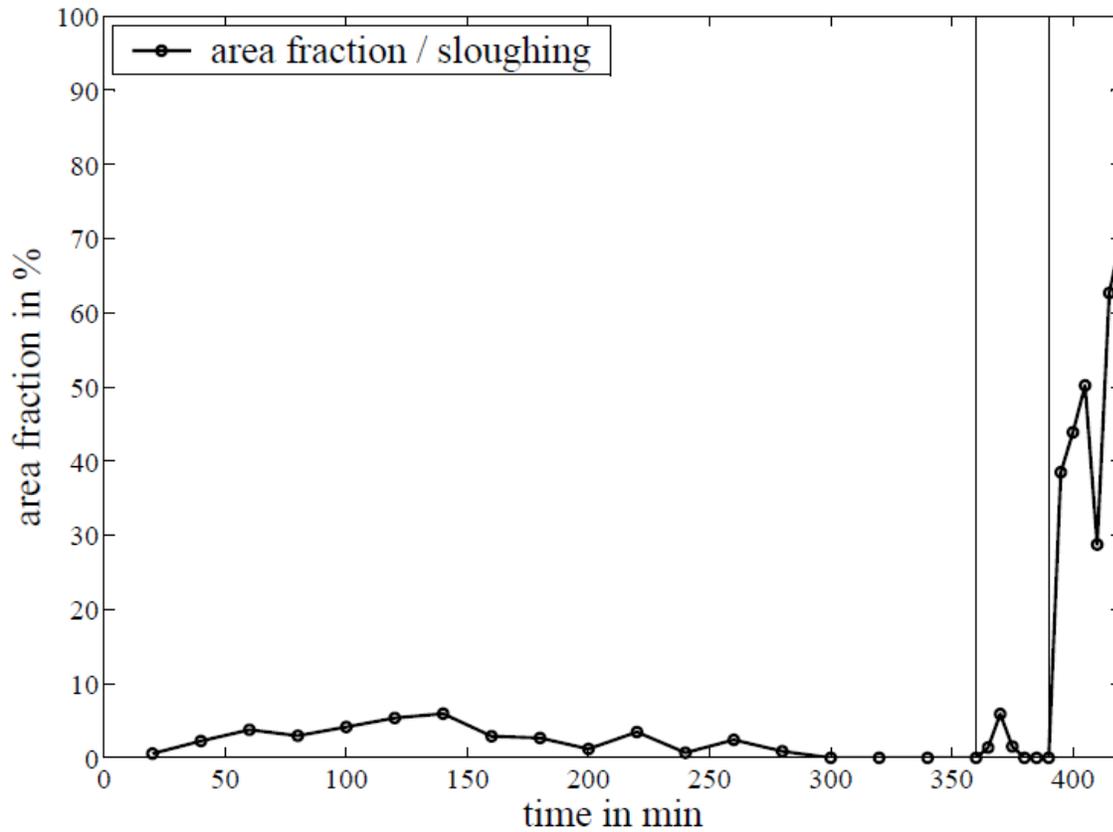


Figure 5. Fraction of the cumulated area of clumps attributed to sloughing over time; the cumulated area is averaged over three lines of the flow cell. The vertical lines mark the change in flow regime at 360 min and 390 min.

6. References

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