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The influence of fluid shear on the structure and material properties of sulphate-reducing bacterial biofilms

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Biofilms of sulphate-reducing *Desulfovibrio* sp. EX265 were grown in square section glass capillary flow cells under a range of fluid flow velocities from 0.01 to 0.4 m/s (wall shear stress, τ_w, from 0.027 to 1.0 N/m^2). *In situ* image analysis and confocal scanning laser microscopy revealed biofilm characteristics similar to those reported for aerobic biofilms. Biofilms in both flow cells were patchy and consisted of cell clusters separated by voids. Length-to-width ratio measurements (l_c:w_c) of biofilm clusters demonstrated the formation of more ''streamlined'' biofilm clusters (l_c:w_c=3.03) at high-flow velocity (Reynolds number, Re, 1200), whereas at low-flow velocity (Re 120), the l_c:w_c of the clusters was approximately 1 (l_c:w_c of 1 indicates no elongation in the flow direction). Cell clusters grown under high flow were more rigid and had a higher yield point (the point at which the biofilm began to flow like a fluid) than those established at low flow and some biofilm cell aggregates were able to relocate within a cluster, by travelling in the direction of flow, before attaching more firmly downstream.
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Introduction

Sulphate-reducing bacteria (SRB) are a group of environmental anaerobic bacteria that reduce SO_4^{2-} to S^{2-} during dissimilatory anaerobic respiration. SRB are problematic in the oil industry where they have been associated with oil souring by H_2S (which is also explosive) and the pitting corrosion of iron and steel in pipelines and tanks [1,16]. A number of SRB readily form biofilms in laboratory reactors [19] and it is thought that biofilms accumulating at the injection sites used in enhanced oil recovery adversely affect formation permeability, and hence water and oil movement [1,6]. However, relatively little work has been conducted on the structure and rheology of SRB and other anaerobic biofilms, whereas confocal and time lapse microscopies have been used to study the hydrated structure of aerobic biofilms (*Pseudomonas aeruginosa* in particular) in detail. Aerobic biofilms are generally heterogeneous and consist of cell clusters separated by water channels. Hydrodynamics influences the structure of aerobic biofilms and a number of studies have shown that biofilms grown in turbulent flow tend to develop filamentous streamers that have been implicated with the onset of increased pressure drop [4,20,24]. Additionally, the deformation of cell clusters in response to variations in fluid shear in aerobically grown pure culture *P. aeruginosa* and mixed species biofilms has demonstrated that these biofilms were viscoelastic fluids [25] and appeared to move downstream over solid surfaces in a flowing motion [28]. However, little is known concerning the influence of hydrodynamics on the structure of SRB biofilms. In addition to considerations of pressure drop, the shape and distribution of cell clusters in SRB biofilms will also determine the localization of anodic zones, having a direct effect on pitting corrosion [16]. Information on the material properties of SRB biofilms will also be useful in predicting mechanisms of dissemination of these biofilms in pipes and oil reservoir formations through mechanisms such as detachment and surface transport. In this study, we employed methods previously used to investigate the influence of hydrodynamics on the structure and material properties of aerobic biofilms to determine if SRB biofilms behave in a similar manner. While specifically improving the characterization of SRB biofilms, this study will increase our understanding of biofilms in general.

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Materials and methods

Flow cell recirculating loop

Two pieces of square section glass tubing (3 mm×3 mm×20 cm length) (S-103; Camlab, Cambridge, UK) were incorporated in parallel into a recirculating flow loop with silicone tubing, giving a total volume of 175 ml (Figure 1). Nutrients and oxygen-free nitrogen were added to a mixing vessel, operating as a chemostat at 22°C (±0.2), which also included a septum for inoculation. A peristaltic pump controlled nutrient media flow at 10 ml/h^{-1}, giving a residence time of 17.5 h. The medium level in the mixing vessel was maintained by overflow. Nitrogen was bubbled through the media, having first been deoxygenated by a copper catalyst heated to 400°C in a tube furnace (Lindberg/Blue M, Ashville, NC, USA). The nitrogen flow rate was regulated at 8 ml/min^{-1}. The redox potential (E_h) of the effluent was −223 mV.

Experimental procedure

The experiment was divided into two parts. Primarily, the two flow cells — one with a high flow (mean velocity of 0.4 m/s, corresponding Reynolds number, Re, 1200, and theoretical wall

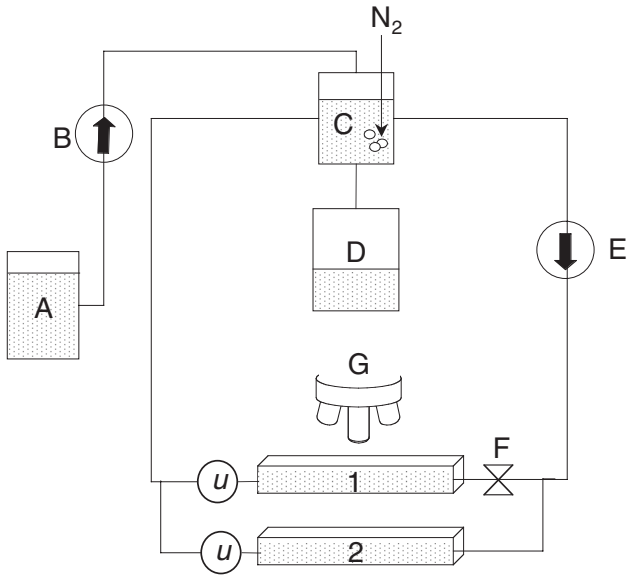

Figure 1 Schematic of the biofilm reactor loop showing; nutrient feed (A); peristaltic pump (B); nitrogen fed reactor vessel (C); effluent overflow (D); vane head pump (E); two glass flow cells 1 and 2 at high and low flow, respectively; clamp (F); microscope objective (G); and sample ports (*u*).

shear stress, τ_w 1.0 N/m^2) and one with a low flow (0.04 m/s, Re 120, τ_w 0.1 N/m^2) — were analysed *in situ* by image analysis for 7 days (Re was based on the hydraulic diameter of the flow cell and the theoretical wall shear stress was estimated after Stoodley *et al* [29]. These flow cells were replaced aseptically by two newly sterilised flow cells. Flow cells with established biofilm were clamped off and taken for analysis by confocal microscopy (CM). New biofilm from the same planktonic population was then allowed to attach and grow over 7 days for strain measurement. The planktonic population is defined as the bacteria in suspension in the fluid phase throughout the reactor loop, while the biofilm population is defined as the bacteria associated with the glass surface in the flow cell. The flow velocities were set lower in this part of the experiment to allow latitude for increases in flow rate within the constraints of the pump specification. However, a 10-fold difference in flow rate was maintained between high- and low-flow cells. Low flow was at 0.01 m/s (Re 30, τ_w 0.027 N/m^2) and high flow at 0.1 m/s (Re 300, τ_w 0.27 N/m^2).

Culture and medium

For these studies, we used the SRB *Desulfovibrio* sp. EX265. This strain was previously isolated from a North Sea oil production facility and identified using 16*S* rDNA sequencing and analysis. The sequence has been submitted to Genbank under accession no. AF234891. Bacteria were cultured in anaerobic artificial seawater medium (ASW) [2] composed of (g/l): Instant Ocean™, a convenient powder allowing reproducible seawater makeup (Aquarium Systems, Mentor, OH) (33), yeast extract (0.1), ascorbic acid (0.1), and thioglycolate (0.1) with sodium acetate and sodium pyruvate as the principal carbon sources. To prepare the inoculum, batch cultures were grown in ASW with 0.7 g/l sodium acetate and 6 g/l sodium pyruvate. To grow the biofilms, we used ASW with 0.07 g/l sodium acetate and 0.6 g/l sodium pyruvate. All media were prepared anaerobically. Deaeration by heating to 105°C for 20 min was followed by nitrogen sparging for 20 min. The pH was adjusted to 6.5 before final sterilisation at 121°C for 30 min, together with the entire flow reactor set-up.

Inoculation procedure and monitoring

A 1% inoculum of culture (10^9 cells/ml by total cell count) was injected directly into the reactor (Figure 1) to give an initial concentration of cells in the reactor vessel of 10^6 cells/ml. Planktonic bacterial enumeration was carried out by diluting 1 ml of sample in series, staining with Live/Dead *Bac*light according to the manufacturer's instructions (Molecular Probes, Eugene, OR), and then filtering through a 0.2-μm Millipore polycarbonate membrane before total cell count using epifluorescence. Three further 50-ml samples of effluent were each filtered through a 0.2-μm pore size membrane at the termination of the experiment. DNA was extracted from these membranes [21] and PCR amplification of the 16*S* rDNA gene was then performed using primers A and H [9]. Restriction fragment length polymorphism analysis was then done with the restriction enzyme *Rsa*I to confirm culture purity [14].

Hydrodynamic condition measurement

The flow rate through the recycle loop was maintained by a vane head pump (Millipore, Bedford, MA). The flow velocity through each of the flow cells was controlled independently by constricting clamps on the inlet tubing. Flow velocity was calibrated against pump setting by timing the movement of an injected nitrogen bubble (1 ml was sufficient volume to fill the tube lumen) between two points, a known distance apart, below each flow cell. The hydrodynamics of these glass capillary flow cells has been characterised elsewhere and it was found that the transition between laminar and turbulent flows occurred at a Reynolds number of approximately 1200 [23].

Image analysis

Image analysis was carried out using a closed circuit television camera (CCTV) linked to a Power Macintosh 7200/90 equipped with Scion Image (NIH Image modified for windows by Scion, Maryland 21703, USA). Ten images along the length of the flow cell were captured periodically using a 5× objective. The percentage surface area covered by cell clusters in each field was measured by setting a threshold so that the cell clusters were black and the surrounding voids white [26]. For strain measurement, five fields of view, in proximity to the centre of the flow cell, were arbitrarily selected by moving along the flow cell in 1-mm increments and all colonies above a certain diameter were measured after 40 h. (With our microscope settings, we estimated that we could only confidently resolve distinct cell clusters with areas down to 140 μm^2, 13 μm in diameter, and this was taken as our limit of resolution. Cell clusters with areas smaller than this were not included in the data set.) Their lengths (l_c) (length dimension parallel to flow) and widths (w_c) (length dimension perpendicular to flow) were measured perpendicular to the flow direction and a mean length-to-width ratio (l_c:w_c) was then calculated for biofilm clusters grown at high- and low-flow velocity.

Confocal microscopy (CM)

The flow cells were clamped off and aseptically removed after 7 days. The biofilm was stained with the *Bac*light (Molecular Probes) Live/Dead kit (5 μl/ml), which was added to the flow cell

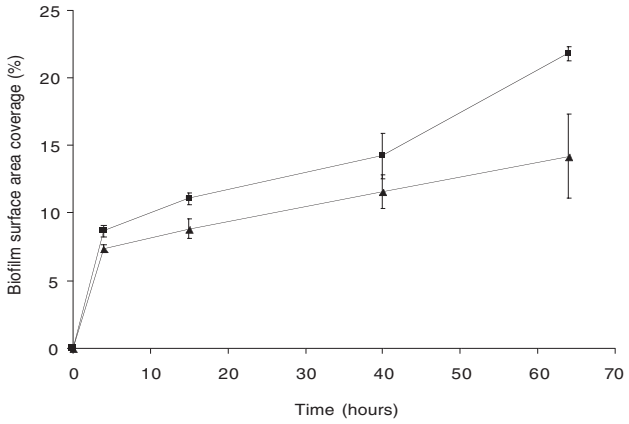

Figure 2 Increase in surface area cover by *Desulfovibrio* sp. EX265 biofilm. Biofilm established on the lower surface of a glass flow cell under high (■) flow (Re, 1200) and low (▲) fluid flow (Re, 120). Each data point represents the average of measurements from five fields of view. Error bars represent standard error of the mean.

by syringe. The flow cells were incubated for 1 h in the dark, at 5°C. A series of vertical sections was captured on a Leica TCS/NT CM using an excitation wavelength of 488 under a 63× water objective.

Measurement of strain on biofilm

The physical strain on individual biofilm clusters was measured by a comparison of cluster length before and after each incremental change in flow velocity. During these tests, time lapse image sequences were captured to observe any detachment phenomenon that may occur at the elevated flow velocities. Fields of view containing at least two clusters (for duplicate measurements) were chosen at random from both the high- and low-flow velocity flow cells. After initial cluster length measurements, flow velocity was incrementally increased for low-low biofilm from 0.01 to 0.27 m/s and for high-flow biofilm from 0.1 to 0.28 m/s at 10-s intervals, before similar flow decrease. Length was measured at each increment for calculation of engineering strain (ε) [25]:

$$\varepsilon = \Delta l_c / l_c$$

where l_c is the original microcolony length measured in the direction of the bulk liquid flow and Δl_c is the change in length. Three cycles of strain loading were undertaken and values averaged. One minute was allowed for biofilm recovery between each cycle. The stress–strain curves were used to determine an apparent elasticity modulus (E_{app}) of the clusters [30], the residual strain (ε_r). E_{app} is analogous to Young's modulus (E), which is a measure of stiffness or resistance to deformation and was found from linear regression of the loading portion of the stress–strain curves. One-way analysis of variance (ANOVA) tests were performed to compare E_{app} and ε_r of biofilm clusters grown at different τ_g. Differences were considered significant when $P<0.05$. When the Δl_c of the clusters was so small that no strain could be measured, a "greater than" value was calculated based on a detection limit of a change in length of three pixels divided by the original length of the monitored cluster. For statistical analysis, the "greater than" value was used to give the most conservative estimate of significant difference at the 95% level. The strain was related to the fluid shear at the flow cell wall to determine the longitudinal elastic modulus of the cell cluster [25,27].

Results

Bacterial population

Monitoring of the bacterial population showed 10^7 cells/ml in the effluent on day 7, while 16*S* rDNA fragments obtained by digestion with the restriction enzyme were consistent before and after the experiment, indicating culture purity (data not shown).

Effect of flow on biofilm accumulation and development

In both the high- and low-flow regimes, cells rapidly attached to the surfaces of the flow cells. These cells then began to divide to form cell clusters (dense aggregates of cells), which were evident within 15 h. The percentage cover of cell clusters increased from 0% to a peak of 14% in the low-flow cell between 0 and 64 h (Figure 2). Over the same period in the high-flow cell, the percentage cover rose from 0% to 22%. This difference was significant at 64 h ($P<0.05$).

Effect of flow on biofilm morphology

A distinct difference in biofilm morphology and coverage pattern was observed between clusters growing in high- and low-flow

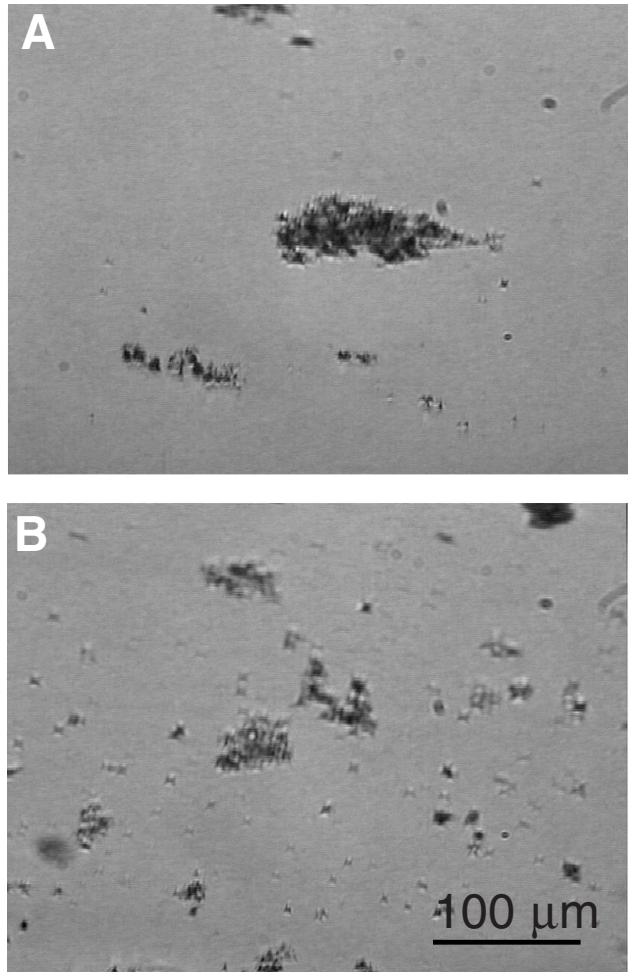

Figure 3 Difference in *Desulfovibrio* sp. EX265 biofilm cell clusters at time=60 h, established under high (A) flow (Re, 1200) and low (B) flow (Re, 120). Real time images were captured of bacterial biofilm growing *in situ*. Direction of flow in all images →.

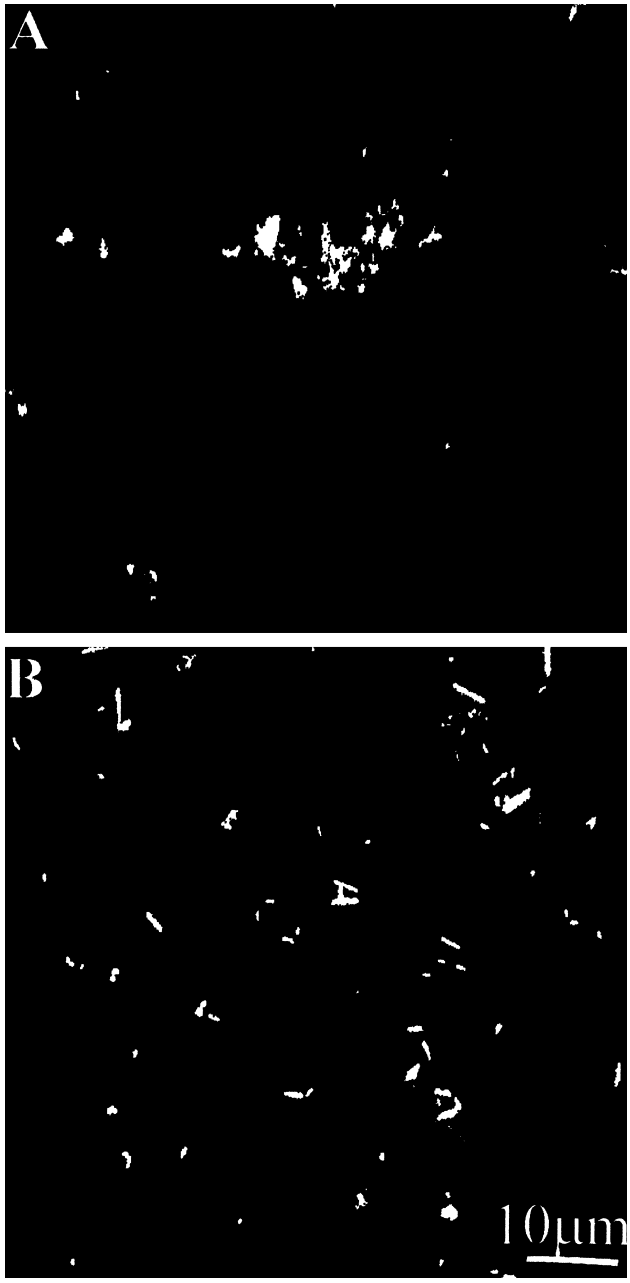

Figure 4 Difference in *Desulfovibrio* sp. EX265 biofilm cell clusters at time=70 h, established under high (A) flow (Re, 1200) and low (B) flow (Re, 120). Confocal scanning laser microscopic images (63× objective) obtained of fully hydrated biofilm stained *in situ* in flow cells using the Live/Dead *Bac*light system; both live and dead fractions are shown together. Direction of flow in all images →.

conditions. Under high-flow conditions, "streamlined" clusters predominated (clusters that were longer than they were wide), orientated in parallel with the direction of flow (Figure 3A; time=60 h). These clusters consisted of a wider "head" facing the oncoming flow, narrowing towards a "tail end," and were interspersed with large voids. In contrast, cluster morphology in the biofilm grown under low flow was more variable in height and width with less directional alignment (Figure 3B; time=60 h). The mean length-to-width ratio (l_c:w_c) of the biofilm cell clusters established at higher flow was 3.03±0.84 (mean±SD, n=24 microcolonies), while the mean l_c:w_c of the biofilm microcolonies established at lower flow was 1.3±0.67 (n=23). One-way ANOVA confirmed that the l_c:w_c between clusters grown at high and low flows was significantly different at the 99% level (P<0.005).

Confocal microscope analysis revealed isolated aggregates of cells in the high-flow regime (Figure 4A; time=70 h). High-flow clusters exhibited a noticeable streamlined alignment with flow. In the low-flow cell, there was a greater dispersion of cells over the surface. More single cells and fewer associated aggregates of cells were observed (Figure 4B; time=70 h). The proportions of "live":"dead" cells were 6:1 (low-flow cell) and 19:1 (high-flow cells).

Biofilm behaviour under high shear

A selected biofilm cell cluster developed under high flow was observed with time lapse imaging to measure alteration in shape

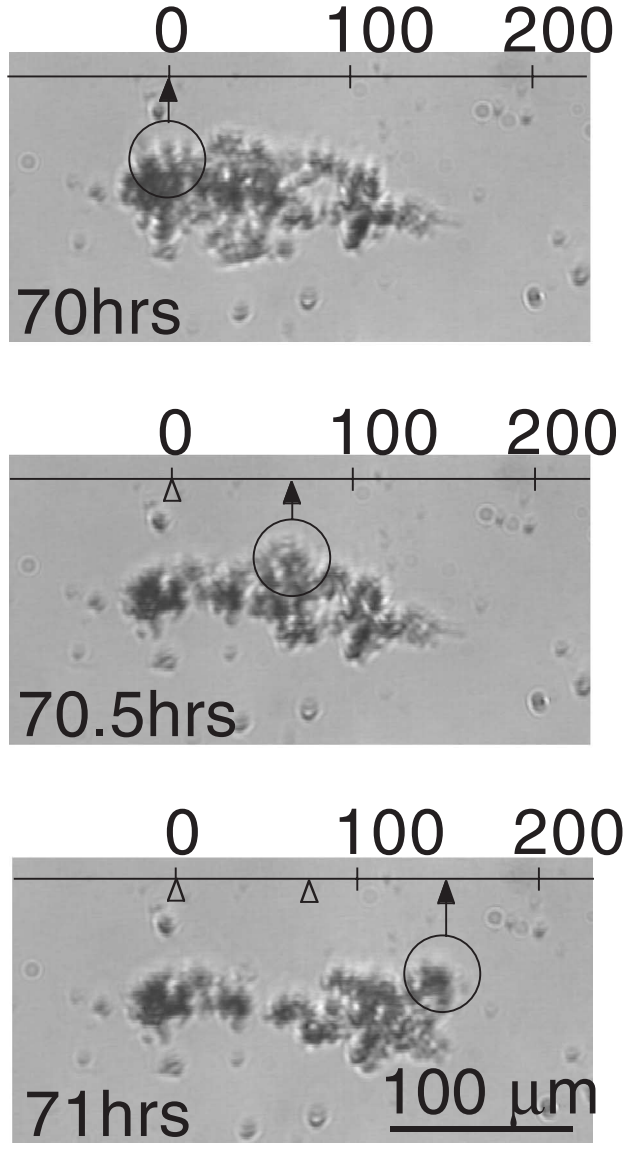

Figure 5 *Desulfovibrio* sp. EX265 cells moving within a cell cluster established under high flow (Re, 1200). A cell aggregate was observed to "peel" and move downstream without detachment to the liquid phase.

with time (Figure 5). Over a 60-min period, a part of the cluster from the head of the streamer moved along the length of the main body of the streamer downstream towards the streamer tail. The moving cell aggregate travelled over the substratum at a velocity of 160 μm/h but did not detach. Downstream movement and detachment were observed in several other cell clusters.

Effect of flow on biofilm rheology

For biofilm clusters grown at high-flow velocity, an increase in the mean flow velocity from 0.1 to 0.28 m/s increased the strain imposed on the biofilm from 0 to a maximum around 0.1. The strain on the high-flow biofilm was restored to 0 as flow velocity was reduced (Figure 6). The loading–unloading curve showed no hysteresis (the loading and unloading portions of the curve followed the same paths). Lack of hysteresis is indicative of a solid-like linear elastic response.

Low-flow biofilm clusters were subjected to an increase in mean flow velocity from 0.01 to 0.27 m/s. The strain on this biofilm increased from 0 to 0.25. When the flow velocity was reduced, the strain did not decrease at the same rate as it had increased. In addition, a residual strain of between 0.07 and 0.1 remained. The hysteresis and residual strain indicated that the biofilm was irreversibly deformed and, in this case, the curve resembled the characteristic response of a viscoelastic fluid. The large hysteresis in the curve of the biofilm grown at 0.01 m/s indicates that the yield point was exceeded below 0.27 m/s and the biofilm was behaving like a viscous liquid. The strain imposed on a *Desulfovibrio* sp. EX265 biofilm cluster established at high flow was half that imposed on a low-flow cluster when subjected to the same cycles of increased flow velocity. Indeed, the yield point of the biofilm grown at 0.15 m/s was not exceeded when u was increased to 0.28 m/s, demonstrating that the biofilm cell clusters grown at high flow were more resistant to strain than those grown at low flow. Thus, when a cluster was grown under high flow, its material properties were altered as well as its shape. The elastic moduli of the two biofilm phenotypes differ accordingly. The strain was related to the fluid shear at the flow cell wall to estimate the longitudinal elastic modulus of the cell cluster after Stoodley *et al* [25,27]. An elastic modulus of 2.5 was determined when biofilm was grown at 0.03 N/m^2 of growth shear (low flow). An elastic modulus of 5 was calculated for high-flow biofilm clusters with a growth shear of 0.47 N/m^2.

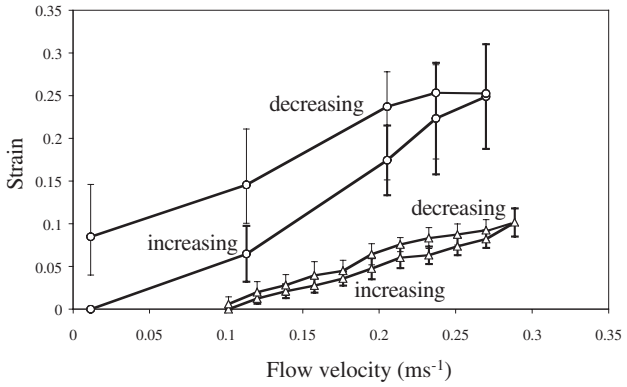

Figure 6 The strain (change in length, [l_c]) of cell clusters as a function of increasing and decreasing flow velocity. This is shown for biofilm clusters grown at flow velocities (u_g) of 0.01 [○] and 0.1 [△] m/s. Bars represent one standard error calculated from triplicate measurements from each of two cell clusters. Heavier bars indicate the standard error of data on the increasing curves.

Discussion

Using approaches developed for aerobic biofilms, the influence of fluid velocity on structure and material properties of an anaerobic biofilm was studied. We were interested in determining whether phenomena observed for a limited number of aerobic biofilms would exist in sulphate-reducing biofilms. While providing new knowledge on the previously unstudied structure and properties of an SRB biofilm, such information also gives more confidence to conclusions drawn in general about biofilm structure and behaviour. It was anticipated that biofilm formed by a species of SRB might differ from that formed by aerobic species. Extracellular polymeric substances (EPS) are crucial to the structure of biofilms [11] and the composition of EPS from biofilms has been shown to vary widely even within the SRB functional group [10]. Indeed, the SRB studied here were isolated from the production fluids of a North Sea oil reservoir, a very disparate environment from the drinking water systems where many previously studied aerobic biofilm species originated. However, the findings from this work are discussed in the light of previous results for aerobic biofilms because these are the only studies that characterise structure and rheology of biofilms.

Influence of fluid velocity on biofilm structure

Based on cluster surface area cover, the high-flow cell had a greater biofilm accumulation rate after 60 h. This faster rate of accumulation was possibly due to increased mass transport that occurs under high-flow velocities [8]. Thus, a higher growth rate of cells deep in the biofilm cluster may have resulted in the high-flow cell compared to cells deep in the biofilm in the low-flow cell. The proportions of live/dead bacteria showed more "live" cells in the biofilm grown under high-flow rate, supporting this conclusion.

Streamlined SRB clusters predominated in the high-flow cell and the figures reported here compare closely with the average length-to-width ratios at 8 days (1.08 and 2.18 for low- and high-flow aerobic biofilm clusters, respectively) reported previously [24,26]. Streamlined clusters have been described in many aerobic biofilm systems [4,13,17,22,24,26]. Streamers generally form in higher shear flows presumably as the developing biofilm clusters are stretched out in the downstream direction by the fluid shear exerted on the structure [15,24,26].

Biofilm movement

Evidence described here suggests that SRB within a biofilm do not have to detach in order to migrate under higher shear flows. Importantly, previous work has demonstrated that aerobic biofilms can exhibit viscous flow [11,27,28]. Major constituents of biofilms are EPS that surround the bacterial cells. In polymers, viscous flow is ascribed to the breaking of weak intermolecular bonds between polymer chains, allowing them to pull apart and slip past each other [18]. Here, a *Desulfovibrio* sp. EX265 cluster grew in size until the shear stress acting on it exceeded the attachment strength, causing the cell aggregates to peel away and migrate with the flow, towards the tail end of the cluster where they subsequently reattached. A similar phenomenon describing the movement of biofilm ripples and clusters along a surface has been reported in mixed species

aerobic biofilms [23,27]. These data confirm that biofilm is able to relocate without detachment to the planktonic phase. This may be an important consideration in the control of bacterial dispersion since biofilm cells can be 500 times more resistant to antibacterial agents than their planktonic counterparts [3,5].

Influence of fluid velocity on biofilm rheology

Viscoelastic properties of biofilms are important as they influence the pressure drop of pipe fluid flow [20]. We have shown that the viscoelastic properties of similar biofilms are influenced directly by the flow velocity at which the biofilm was established. High-flow biofilm clusters had a higher elastic modulus and a greater propensity to return to the original shape after strain induced by increased flow velocity. The low-flow biofilm by comparison was less rigid with irreversible damage to structure upon exertion of strain. Previously, contraction in biofilm cluster length was demonstrated using a mixed culture aerobic biofilm when flow was decreased to give a growth shear of 0.12 N/m^2 from 5.09 N/m^2 [25,27]. Moreover, it was concluded that biofilm microcolonies behaved elastically below the yield point but flowed when the shear stress was increased beyond the yield point [25,27]. Here, SRB biofilm behaved similarly to those mixed culture and pure culture *P. aeruginosa* aerobic biofilms.

Wider implications

Frictional resistance and reduced flow capacity can result from biofilm growth [4]. The physical properties described for *Desulfovibrio* sp. EX265 biofilm varied widely, depending on the flow velocity under which the biofilm was established. Such variation in biofilm structure and rheology would influence the magnitude of flow reduction. This is an important observation for many industrial and medical situations where reduced flow is undesirable. Examples include pipelines [4], closed conduit reactors [15], and urinary catheters [12]. Fluid flow through porous media is also affected by pressure drop. Bacterial biofilm plugging of porous rock in oil fields can be beneficial if the bacteria are preferentially situated to improve water sweep by diverting water through oil-saturated areas of rock [1]. However, biofilm presence is often detrimental, causing plugging near injection well bores, preventing water injection [7]. A greater understanding of the structure and rheology of biofilm and its effect on hydrodynamics may assist our predictions of water sweep through certain areas of reservoir rock, therefore improving oil recovery. This will only come from fundamental research on *in situ* growing biofilms.

Conclusions

1. Flow velocity had an influence on both the structure and material properties of the SRB biofilms.
2. The biofilm cell clusters grown at higher flow (0.47 m/s) were more streamlined than those grown at lower flow (0.03 m/s).
3. The biofilm cell clusters grown at higher flow (0.1 m/s) were stronger (had a higher yield stress) than those grown at lower flows (0.01 m/s).
4. The SRB biofilms behaved like viscoelastic fluids exhibiting an elastic response at low fluid shears but like viscous liquids when the shear stress was increased above the yield point.
5. Phenomena observed here for SRB biofilms compared closely to those observed previously for aerobic biofilms.

Nomenclature

ε	Strain (dimensionless)
ε_r	Residual strain (dimensionless)
E	Young's modulus of elasticity (N/m^2)
E_{app}	Apparent longitudinal elastic modulus (N/m^2)
l_c	Length of biofilm cluster, measured perpendicular to flow direction (m)
Re	Reynolds number (dimensionless)
τ_w	Wall shear stress (N/m^2)
τ_g	Wall shear stress at which biofilm was grown (N/m^2)
u	Average flow velocity (m/s)
v	Kinematic viscosity (m^2/s)
w_c	Width of the biofilm cluster, measured perpendicular to flow direction (m)

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