

Effects of vortical flow and shear stress on bacterial behavior and aggregation

By

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ABSTRACT

Microorganisms are always confronted with unique hydrodynamic situations involving high flow rates, high shear stresses and mixing. These factors affect their survival, their nutrient uptake and their competitiveness in a given environment. Previous studies have suggested that shear stress and vortical flow cause the formation of biofilms and aggregates but those studies were mostly performed on pure cultures of a single bacterium. This study is an attempt to investigate the effect of the same forces on a heterogeneous population of bacteria and determine characteristic behavior traits of different bacterium in order to study the manner in which bacteria have adapted to high flow situations.

Introduction

Bacteria are exposed to many different hydrodynamic conditions in nature – in fast flowing rivers, rapids, waterfalls, an open tap, a shower or even in medical tubing¹ yet there have been few studies considering how they are affected by their fluid environment and the modifications they have acquired in order to adapt to the fluid dynamic conditions around them. These conditions could affect their nutrient uptake², ability to compete with their neighbors and even their ability to survive. One empirically observed response to change in fluid dynamic conditions is the formation of biofilms or unattached microbial aggregates. Biofilms have been shown to form under vortical flow and shear stress.^{3,4}

It is still debated whether the formation of biofilms or aggregates is merely a stress response or whether it is in fact an adaptation to the changing environment, particularly the fluid surroundings, which gives the attached microbial community a singular advantage.⁵ However, there is plenty of evidence for engineered collective behavior as opposed to indeterminate clumping together. Some typical examples of beautifully regulated group behavior are the fruiting bodies formed by *Myxococcus xanthus* and the mixed communities of *Anabaena* during heterocyst development.^{6,7}

Another facet to this debate is requirement for surface attachment. Most of group behavior in microbial communities has been coincident with the presence of surface attachment. Yes, there are examples when microbes aggregate without the presence of a surface for initial settling.^{8,9}

In order to explore this question, it is imperative that we learn more about the conditions under which microbes aggregate and the kind of microbes which prefer to or are forced to aggregate. In this report, a natural flow system, Trunk River, was studied to elucidate the diversity of the bacteria in the flow systems and a heterogeneous population of microbes from nature was put under

conditions which induced aggregation and then their characteristics, including relative abundances of microbes within the aggregates, were studied.

MATERIALS AND METHODS

Sample collection

Trunk River was chosen as the natural flow system from which environmental samples would be collected. Two different sampling times were chosen, during the day and at night, such that flow conditions and bacterial community distributions would be different. Approximate flow rates were measured using a graduated cylinder and timer. The cylinder was placed in the direction of flow, tilted and just below the surface of water, such that only the tip of the cylinder was in contact with the flowing water. The water then trickles into the graduated cylinder at approximately the speed of the river current. Salinity and pH measurements were also taken at the different sampling sites. Several sites were chosen based on the flow as measured or approximated at the different sites. A few sites of stagnant and fast flowing water were identified and sampled from.

Laboratory-scale flow system set up

Three different hydrodynamic systems were set up in order to observe the difference in behavior of bacteria under different conditions. All the fluid circulating in the systems had been size fractionated using 2 μm membrane filters, thus removing any existing microbial aggregates that could have been suspended in the river water.

- a. Drip-flow cell - A rudimentary drip-flow cell was set up using a peristaltic pump rotating at 90 rpm. Filtered river water was circulated from the reservoir to a Tupperware container,

which was set up with a glass slide inclined at an angle of 60 to the horizontal plain. The water then dripped down the glass slide, through an opening in the Tupperware back to the reservoir. Samples were taken from the drip coming down from the slide and viewed under a microscope for aggregate formation.

- b. Stir plate vortexer - A glass beaker was placed inside a larger glass beaker with sand holding the small beaker in place. River water was then poured to a height above the inner beaker. The stir bar was placed in the inner beaker and the whole set up was placed on a stir plate and turned on. The vortex generated in the inside beaker contrasts with the inertial forces on the outside leading to a gradient of mixing in the annular space.
- c. Shake tube - 50ml Falcon tubes with river water were incubated horizontally in a shaker incubator set to 30 C and shaking at 220rpm. A slide was placed in the center of the tube and so were rocks at the bottom of the tube, and a control tube without rocks. The rocks were autoclaved to remove existing communities of microorganisms and cleaned with 3% H₂O₂ overnight to remove organic matter. (NOTE: Removal of organic matter was previously shown to be incomplete with H₂O₂.)

Monitoring growth of cells and aggregates

The flow devices were monitored for growth every day by taking 20 µl samples for light microscopy and looking for increase in the number of cells and growth of aggregates.

CARD-FISH

Samples taken from the different flow cells and from the site directly were filtered onto 2µM GTTP membrane filters (Millipore) and the inherent communities and aggregates were visualized by CARD-FISH. An existing protocol¹⁰ was adopted and used with the following probes - ALF968,

BET42a, GAM42a, DELTA495a, EPSY914, ALT1413, CF319a, ARCH915, EUB338 and NON338. Cell counting was performed on free-living microorganisms on the filtrate of Trunk River water and afterwards, the River water was incubated for 2 days in the flow devices.

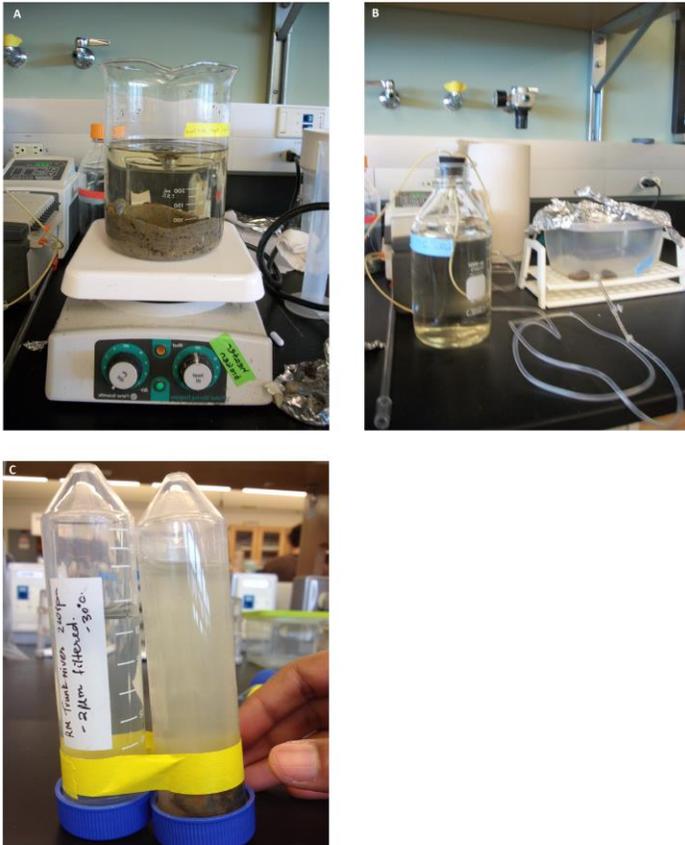


Figure 1. Photographic images of the three flow devices. A, Stir-plate vortexer B, Drip-flow cell and C, Shake tubes, with and without rocks.

RESULTS

Growth of bacteria under different flow regimes

It was observed that the bacteria grew much better in the shake tubes than in the stir-plate vortexer or drip-flow cell. It was also evident that the bacteria grew much faster and to denser OD in the shake tube with rocks than they did in the shake tubes without rocks. The final OD_{600nm} of the shake tube with rocks was shown to be 1.4, after six days, whereas the OD_{600nm} of the shake tube without rocks was less than 0.1. It was also seen that bacteria grew better in the stir-plate vortexer than the drip-flow cell.

Measuring microbial diversity in Trunk River using CARD-FISH

Trunk River water, filtered down to 2 μ m particles was subjected to CARD-FISH and studied under 10 different probes, as listed earlier. The diversity of the system was studied by counting the number of different cells stained by specific probes versus the number of cells stained by DAPI. The diversity is described in the bar chart in Figure 2a. δ -proteobacteria were shown to be the most abundant microbes, at 28.3 % abundance while γ - and ϵ -proteobacteria were shown to have relative abundances of 21.2% and 18% respectively. The EUB probe that targets all bacteria was shown to stain 87% of the visualized microbial cells, indicating the presence of a number of archaea or eukaryotes in the FISH sample.

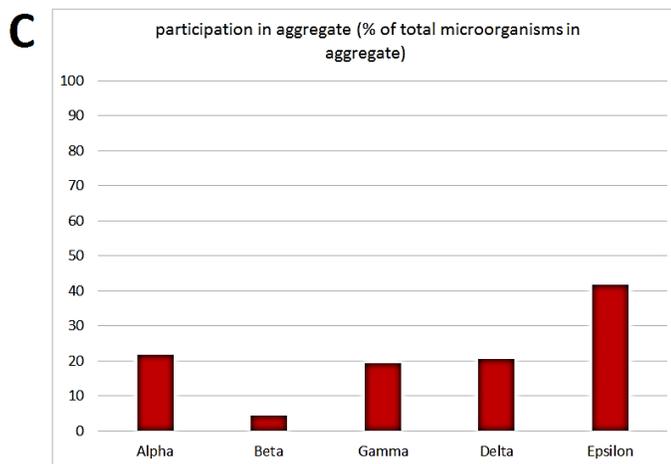
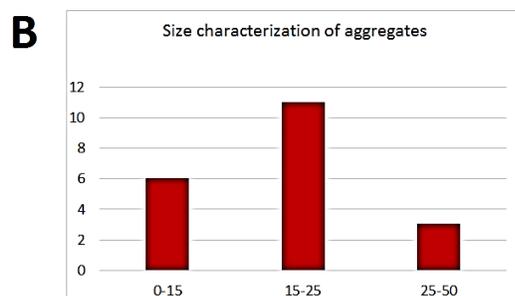
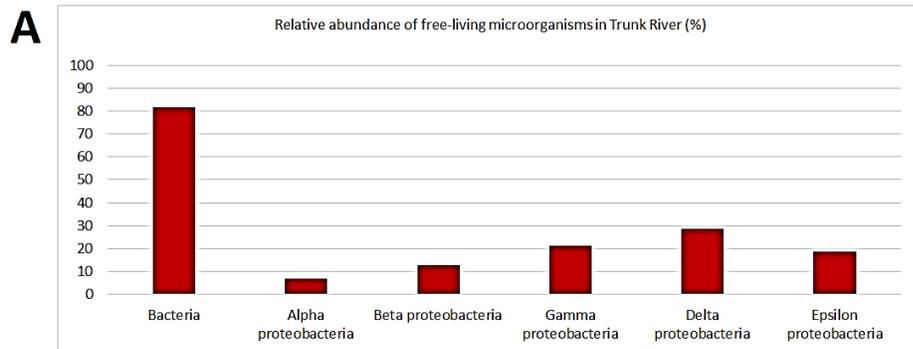


Figure 2 Relative abundance of different microbes by FISH A. Relative abundance of free-living microorganisms in Trunk River B. Size distribution of aggregates found in the shake tubes C. Relative abundance of microorganisms in aggregates.

Characterizing microbial aggregates using CARD-FISH and DAPI staining

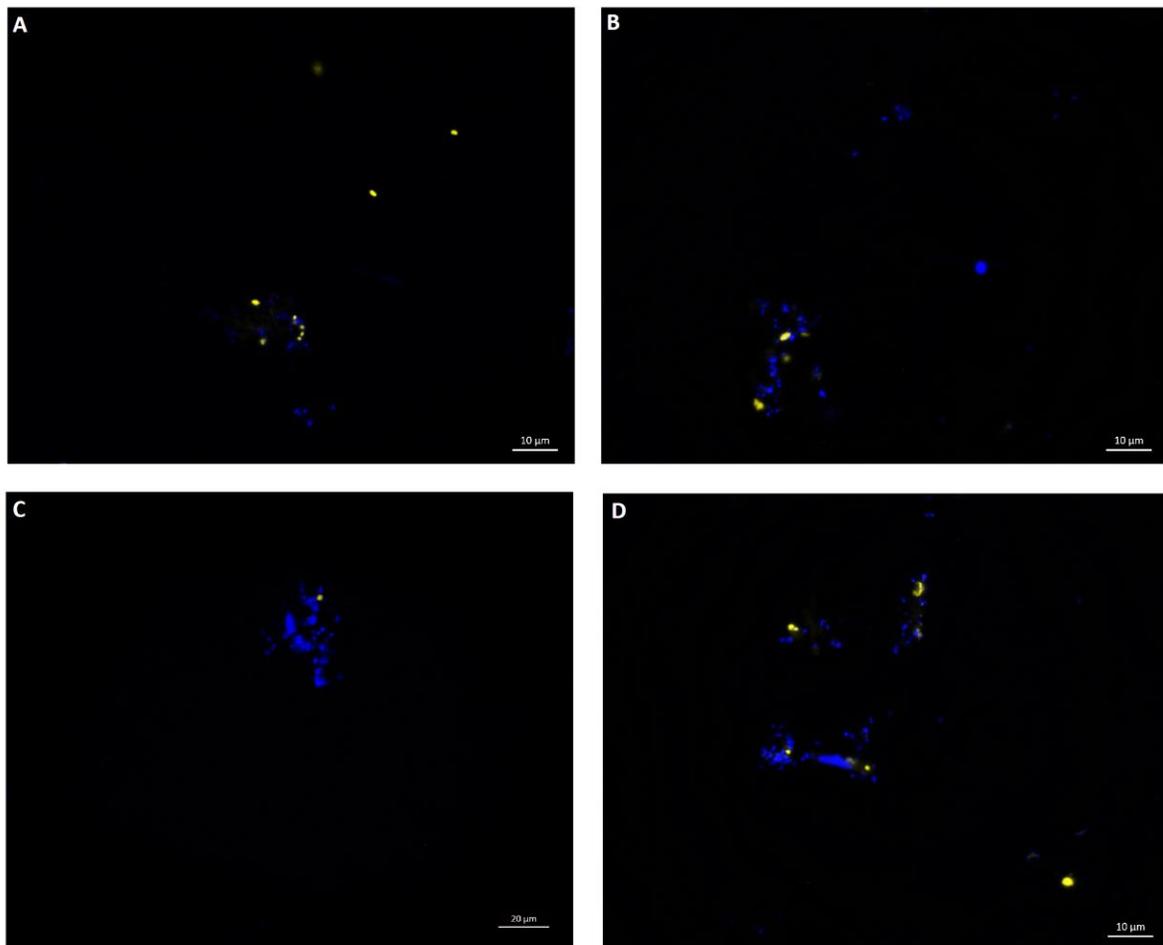


Figure 3 Characterization of bacterial aggregates by juxtaposing images with FISH probe signal on top of images with DAPI signal. A. DAPI with ALF-968 signal B. DAPI with GAM-42a. C. DAPI with BETA-42a and D. DAPI with GAM42a

The microbial aggregates were first analyzed by size, taking the longest side or widest normal to be the size of the aggregate. The distribution of aggregates is represented in Figure 2b. Thereafter, the dominance of any one microbe in an aggregate was documented by counting the number of

cells stained by a particular probe in an aggregate versus the number of cells stained by DAPI. The results of the counting are represented in Figure 2c as the average participation of bacteria of a given clade in the aggregates of which they are a part.

Bacteria of the clade α -proteobacteria and ϵ -proteobacteria were found most often in aggregates (representative images are shown in Figure 3a and 4a,b respectively). Proteobacteria of the clade, γ and δ , were also found to be in aggregates, constituting 20% of the aggregates on average. (Representative images are shown in 3b,d) β -proteobacteria were rarely found in aggregates, and when found, only contributed to 5% of the population of the aggregate.

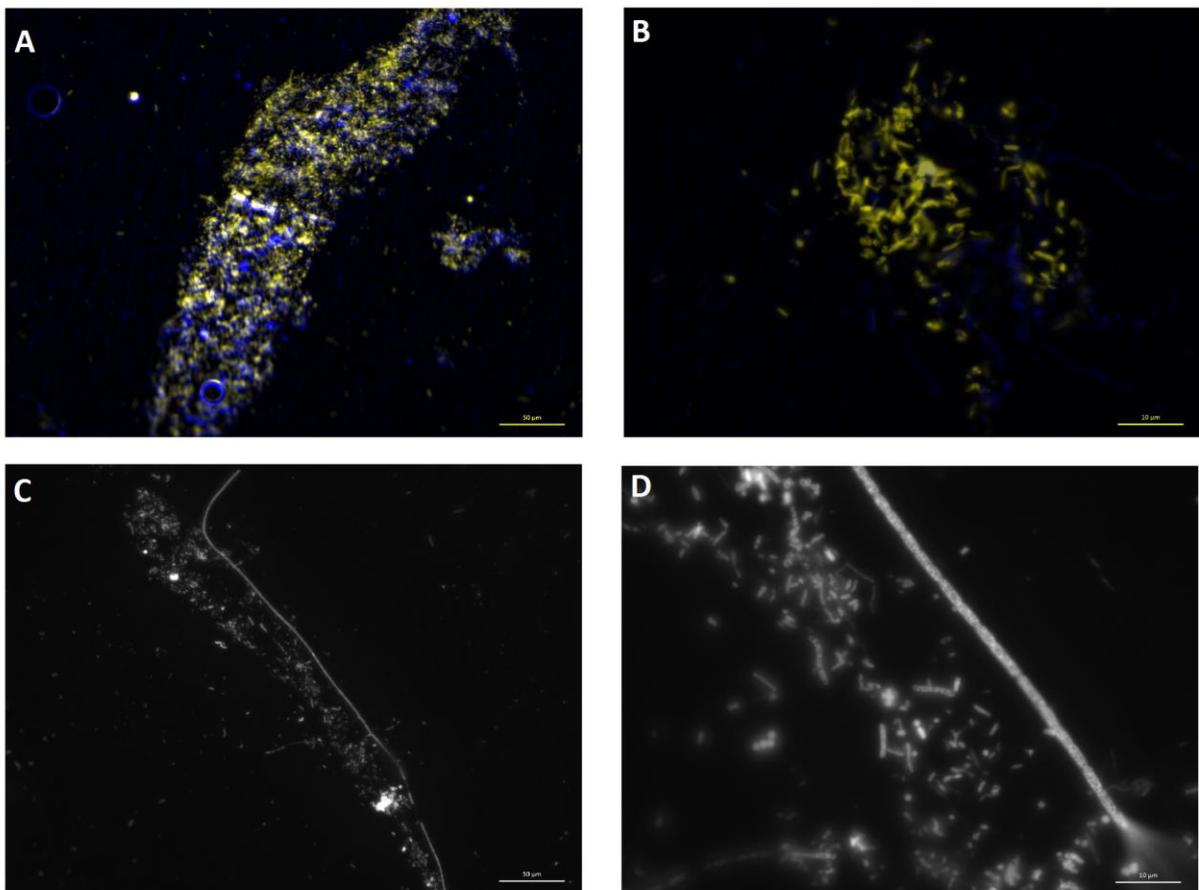


Figure 4 Non-typical structures formed by ϵ -proteobacteria A,B. Aggregate of ϵ -

proteobacteria with a filament that fluoresces in the chlorophyll region. C,D- Thick ϵ -proteobacterial filaments with more ϵ -proteobacteria surrounding them.

DISCUSSION

Effect of different flow regimes on bacterial behavior

The three different flow regimes can be differentiated by the amount of shear stress and vortical flow in the three different flow devices. The amount of mixing and shear stress is the highest in the shake tube with rocks and it was hypothesized therefore that this would have the greatest aggregate formation and this has been shown to be true. A rudimentary scheme of mixing and shear forces in the shake tube is presented in Figure 5. 5 A and B visualize the two different systems in the shake tubes with and without rocks. The orbital shaker is assumed to rotate in a clockwise direction, with the center of mass rotating in a clockwise direction about an axis at a distance, a . The orbital frequency, f , in this case is 220 rpm. A scalar model exists for the shear forces in an orbital shaker but it is found to be insufficient in the case of an irregular shape in the xz plane.¹¹ The shear profile is instead marked in the clock-wise direction, opposite in sign to the velocity profile. In addition to the shear forces in the xz plane, there are shear forces in the y plane due to fluid contact with the glass slide in the center of the shake tube as well as the rocks in the bottom surface. The irregularity of the rock surface as well as the reduced distance between the glass slide and the top surface of the rocks, leads to much greater shear force in the shake tube with rocks. Moreover, the spaces between the irregular rock surfaces leads to a lot of mixing similar to the case reported for rocks as obstacles in the river. A complete mathematical model for the system was found to be beyond the scope of this work, and perhaps, beyond the training of the author.

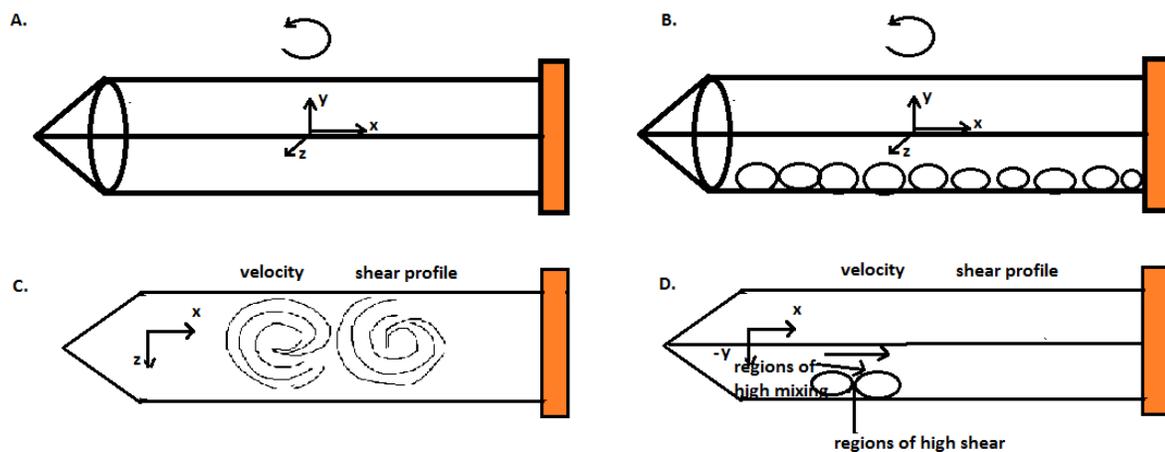


Figure 5. A, B- Comparison of shake tubes with and without rocks. 5 C- velocity and shear profiles in the xz plane. 5D. Regions of high shear and mixing, caused by the presence of the rocks.

Tendency to aggregate

Under the conditions of this experiment, the ϵ -proteobacteria showed a marked tendency to aggregate or form other non-typical structures. (Figure 4) It was observed that they particularly congregated around filaments that fluoresces in the same region as chlorophyll and it was hypothesized that the ϵ -proteobacteria and cyanobacteria may co-exist in some closely interacting microbial community. Figure 4d also shows a unique thick filamentous organism that has not yet been identified. Proteobacteria of the ϵ - clade have previously been shown to have the ability to withstand high temperatures and pressures in the deep sea hydrothermal vents and it would be interesting to see if they have also developed an ability to adapt to high shear and high flow environments.

Conclusions

In this work, a heterogenous population of microbes taken directly from nature was used to study the bacterial aggregates under typical conditions as well as bacterial aggregates that tend to form under high flow and high shear conditions. ϵ -proteobacteria were identified as possible dominant players in such conditions but further work is required to understand the unique structures they form as well as the advantages they gain in forming them.

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