Colonization of a smooth surface by Pseudomanas aeruginosa : image analysis methods
by Andreas R Escher

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Civil Engineering
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Abstract:
Primary adsorption of bacteria to a clean substratum has generally been described by measuring net accumulation. Thus, the independent processes that contribute to the overall accumulation of biofilm, such as adsorption, desorption, cell multiplication, and erosion, cannot be considered separately to help to elucidate mechanisms of early colonization. With the use of image analysis techniques and additional software, these individual processes at the substratum in a continuous flow system have been measured directly. Additional parameters, such as cell movement and direction, orientation of the colony forming units (CFU), spatial distribution at the surface, and shape are also quantified with this technique. With the continuous flow system, the influences of operational parameters such as fluid shear stress, the bulk properties of the fluid, and the characteristic of the substratum can also be delineated in a fundamental manner. Two experimental variables, bulk CFU concentration and shear stress have been used to investigate early colonization under different conditions and to determine the rate controlling factor in biomass accumulation. In addition, a novel method for quantitative analysis of spatial distribution has been developed.

It was found that adsorption and desorption rates are independent of the surface concentration whereas growth and surface related processes are independent of bulk concentrations. At low surface concentration, P. aeruginosa tend to adsorb randomly. With increase in surface concentration the spatial distribution of adsorbing CFU becomes uniform indicating a formation of a repulsing area around adsorbed cells.

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BY <u>PSEUDOMONAS AERUGINOSA</u>:

IMAGE ANALYSIS METHODS

by

Andreas R. Escher

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ABSTRACT

Primary adsorption of bacteria to a clean substratum has generally been described by measuring <u>net accumulation</u>. Thus, the independent processes that contribute to the overall accumulation of biofilm, such as adsorption, desorption, cell multiplication, and erosion, cannot be considered separately to help to elucidate mechanisms of early colonization. With the use of image analysis techniques and additional software, these individual processes at the substratum in a continuous flow system have been measured directly. Additional parameters, such as cell movement and direction, orientation of the colony forming units (CFU), spatial distribution at the surface, and shape are also quantified with this technique. With the continuous flow system, the influences of operational parameters such as fluid shear stress, the bulk properties of the fluid, and the characteristic of the substratum can also be delineated in a fundamental manner. Two experimental variables, bulk CFU concentration and shear stress have been used to investigate early colonization under different conditions and to determine the rate controlling factor in biomass accumulation. In addition, a novel method for quantitative analysis of spatial distribution has been developed.

It was found that adsorption and desorption rates are independent of the surface concentration whereas growth and surface related processes are independent of bulk concentrations. At low surface concentration, <u>P. aeruginosa</u> tend to adsorb randomly. With increase in surface concentration the spatial distribution of adsorbing CFU becomes uniform indicating a formation of a repulsing area around adsorbed cells.

INTRODUCTION

Microbial cells attach firmly to almost any surface submerged in aquatic environments. The immobilized cells grow, reproduce, and produce extracellular polymers which extend from the cell forming a matrix of molecular fibers which provide structure to the assemblage termed a <u>biofilm</u>. Biofilms are sometimes distributed relatively evenly over the wetted surface and other times are quite "patchy" in appearance. Biofilms can consist of a monolayer of cells covering only a fraction of the substratum or can be 300-400 mm thick as observed in algal mats. Biofilms are generally heterogenous, frequently containing more than one distinct microbial environment. For example, biofilms with aerobic as well as anaerobic environments are frequently observed. Consequently, the term biofilm does not necessarily reflect a surface accumulation which is uniform in time and/or space.

Relevance of Biofilms

Biofilms serve beneficial purposes in the natural environment as well as in some modulated or engineered biological systems. Biofilms are responsible for removal of

contaminants from natural streams and in wastewater treatment plants. Biofilms in natural waters frequently control water quality by influencing dissolved oxygen levels and serve as a sink for many toxic and/or hazardous materials. Biofilm reactors are used in some common fermentation processes (e.g. "quick" vinegar process) and are being considered more frequently for biotechnological applications.

On the negative side biofilms result in fouling. Fouling is the accumulation of a deposit on equipment surfaces which result in decreased performance and/or reduced equipment lifetime. Biofilms have been observed to increase fluid frictional resistance in water conduits and on ship hull surfaces. Microbial (as opposed to macrobial) films can significantly increase drag of a ship. Biofilm accumulation in pipes has been observed to reduce the flow rate by as much as 50% even when the film thickness was 0.1% of the pipe diameter (Characklis, 1973). Biofouling deposits decrease heat transfer in power plant condensers on shipboard as well as in land based power plants (Turakhia and Characklis, 1984). As a result, the power plant consumes more fuel to produce the same amount of power. The accumulation of biofilms has also been linked with accelerated corrosion of metallic surfaces, deterioration of

wooden structures, and degradation of concrete structures. Reduced performance may be observed in many other ways.

For a better understanding of biofilms it is essential not only to study fully developed biofilms but also the mechanisms of the buildup, i.e. the early colonization of surfaces by bacteria, the separation between adsorption and desorption, and between other, growth related processes.

Previous Research

Much of the emphasis on adsorption of microbial cells has concentrated on biological and chemical aspects of mechanisms with little emphasis on physical factors in the environment or concern about the rate of adsorption. Nor has much consideration been given to the influence of the initial events on the extent of subsequent biofilm accumulation or the biofilm composition.

In most, if not all reported research on microbial cell adsorption, net cell accumulation at the substratum is observed (Figure 1). Most of the microbial adsorption research has been conducted in quiescent (i.e., fluid velocity equals zero), closed (i.e., no input or output flows) systems. Such systems create a significant number of
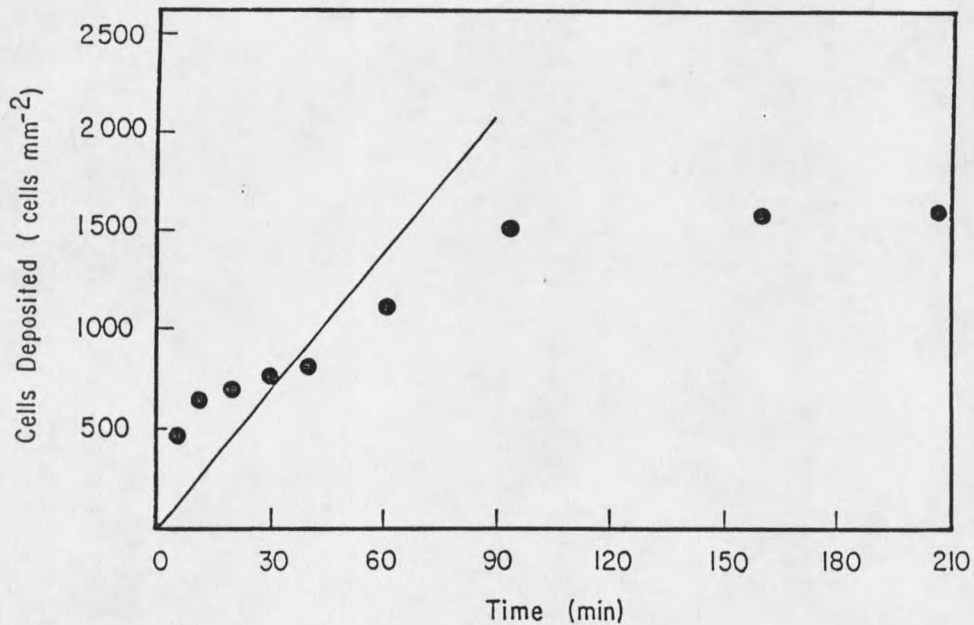
Figure 1. Experimental data from Powell and Slater (1983) for adsorption of <u>Bacillus cereus</u> to the surface of clean glass at a shear stress of 0.075 N m^{-2} and temperature of 53° C. The straight line represents the theoretical adsorption rate for non-motile cells calculated from Bowen <u>et al</u>. (1979).

experimental artifacts leading to rate data which are irrelevant to environmental and/or technical applications. Thus, open, continuous flow experimental systems will be described. The initial events in the accumulation of cells at the substratum consist of the following steps, each occurring with a characteristic rate:

1. Transport of the cell from the liquid phase to the substratum.

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2. Cell adsorption to the substratum, a direct substratum-particle interaction.

3. Desorption of some of the adsorbed cells and their reentrainment in the liquid phase.

4. In some cases, microbial reproduction may contribute to the initial events.

These processes occur either in parallel or in series and, thus, the overall rate of cell accumulation at the substratum will be determined by the combination of the different rates. Variables such as fluid shear stress, liquid phase cell density, and nutrient concentration influence each individual process rate to a different extent and, therefore, influence net cell accumulation. Consequently, the process for cell accumulation in a tube enclosing turbulent flow will be very different from the one for a glass slide immersed in quiescent water, even though the net rate of accumulation may appear to be equal in both cases. Therefore, a closer look at each process is essential to develop a useful, predictive model for cell adsorption.

Goal of Research

The goal of the research is to determine the influence of different independent parameters, such as fluid dynamics,

biomass concentration in the bulk flow, surface characteristics (interface free energy), and cell physiology on early colonization of surfaces.

Objectives of Research

The specific objectives of the research related to early colonization of substrata are as follows:

1. Develop a method to directly measure the rate of different processes contributing to colonization.

2. Develop a method to observe individual "behavioral"[1] characteristics of the organisms at the substratum for elucidating mechanisms contributing to early colonization of surfaces.

3. Derive a mathematical model describing the accumulation of biomass during the early stage of biofilms accumulation.

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[1] "Behavioral" characteristics include all the characteristics which can be observed but are not part of the kinetic system, such as shape, orientation, gliding at the substratum, and more.

BACKGROUND

Process Analysis

During the early events of fouling, biomass can be expressed as colony forming units (CFU), or cells. This distinction is essential since cells can adsorb in groups or as single cells. Moreover, not all the cells accumulate at the surface through transport alone. Cells can be produced at the substratum (growth), the number of cells per colony can change with time, or cells may glide away from their colony of origin to form a new colony. The following four processes must be distinguished (Figure 2) in terms of CFU:

Transport: This process is responsible for carrying the CFU to a point adjacent to the substratum. This step does not include the adsorption process.

Adsorption: This process is defined as the linking of the CFU with the substratum. The cell is adsorbed to the substratum only if it has a linkage to it and, hence, becomes immobilized for a finite time.

Desorption: Desorption is the breaking of the linkage of the CFU and its complete removal from the substratum. Desorption is the reverse of adsorption.

CFU - Separation: A CFU with more than one cell can split into two independent CFU. This process forms a new CFU at the substratum and, hence, contributes to the accumulation of CFU at the substratum. CFU-separation is a growth related process.
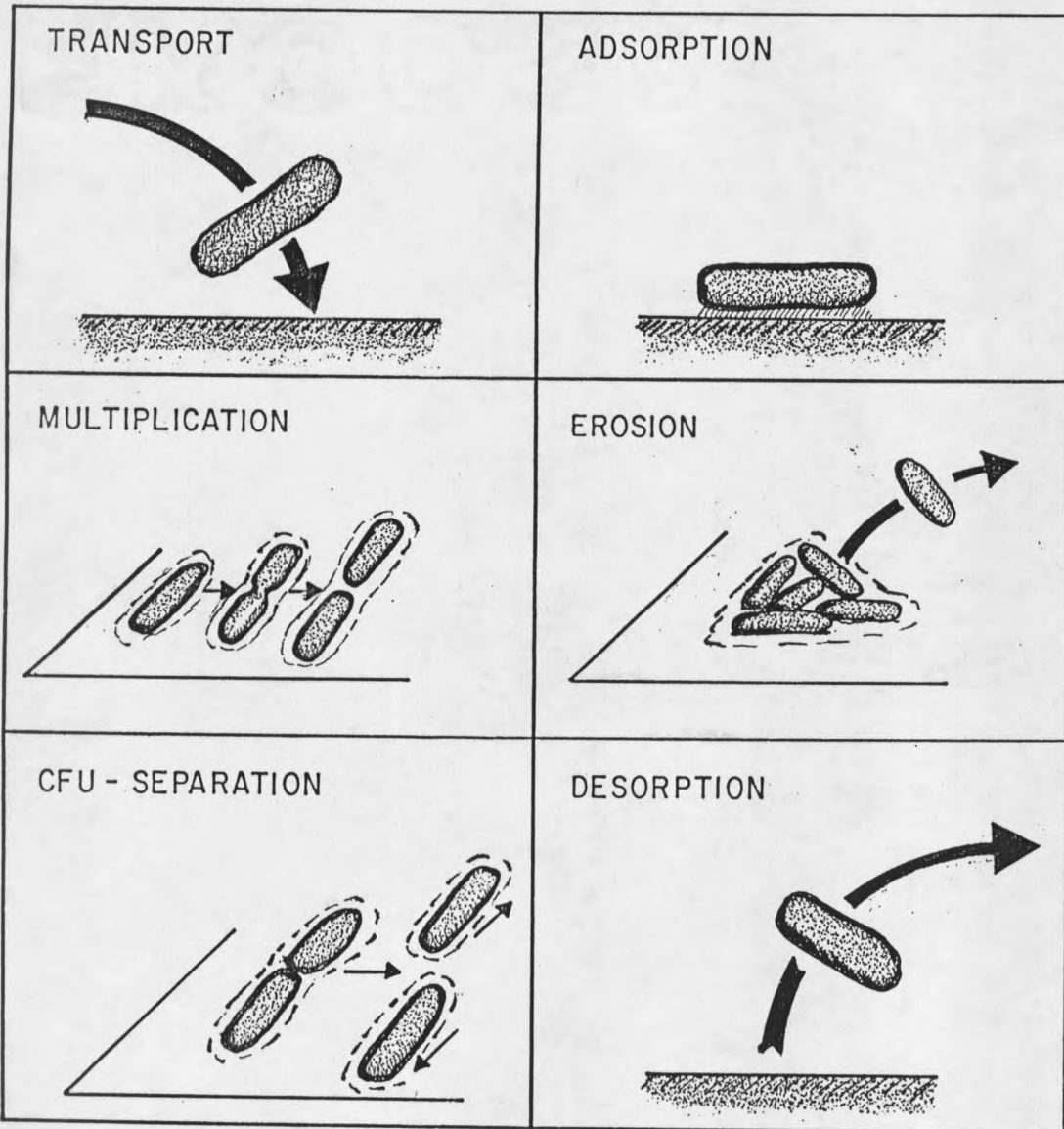
Figure 2. Definition of processes during early colonization of a substratum.

These four processes can be expressed in terms of cells by using the number of cells in each individual CFU. However, CFU - separation does not contribute to the accumulation in terms of cells. Two additional processes can be defined (after transport, adsorption, and desorption) when cell accumulation is considered:

Multiplication: Multiplication is related to cellular growth, but only refers to those daughter cells which remain within the same CFU. Cells within a CFU multiply and increase the number of cells within this CFU. This does not change the accumulated number of CFU but does change the accumulated number of cells.

CFU - Erosion: Cells within a CFU can "detach" and, hence, reduce the cell number of the CFU. This process is the reverse of multiplication.

Therefore, processes of early colonization can be separated into sorption- and growth-related which then can be expressed as rates. With these rates, mass balances for accumulation both in terms of CFU and cells can be accomplished:

$$\frac{d\ CFU}{dt} = + K_{ads} - K_{des} + K_{sep} \qquad 2.1$$

Accumulation   Adsorption   Desorption   Separation
CFU   CFU   CFU   CFU

$$\frac{d \text{ cell}}{dt} = + K_{ads} - K_{des} + K_{mul} - K_{ero} \qquad 2.2$$

Accumu-   Adsorption   Desorption   Multipli-   Erosion
lation   cells   cells   cation   cells
cells   cells

Equations 2.1 and 2.2 demonstrate the importance of measuring the different process rates for a better understanding of surface colonization mechanisms, since they reveal whether growth- or sorption-related processes dominate the accumulation.

Transport

For a better understanding of adsorption processes and comparison between adsorbed and suspended biomass, the transport process must be understood very well, both in terms of transport of biomass to the substratum and the transport of nutrients to the adsorbed biomass.

Currently, most of the information about kinetics of cellular adsorption and desorption has been derived from stagnant systems with no shear stress. The absence of flow or shear forces in natural and engineered systems is not common and, therefore, not of great practical interest. To define transport in quiescent systems is not an easy task,

since it depends on parameters , i.e. diffusivity, which can not be measured directly. Additionally, adsorption kinetics in stagnant systems are subject to artifacts produced by the combination of sedimentation and active adsorption rates. Some studies in flow systems have been published, focusing on net accumulation rates at the substratum. From the literature, it appears that it is essential to measure both adsorption and desorption rates independently. Figure 1 (Powell and Slater, 1983) illustrates the difficulty in determining adsorption rates from the accumulation alone. The data fit the theoretical calculated rates poorly. Measuring accumulation rates instead of adsorption can lead to artifacts since accumulation can include other processes, such as cellular multiplication at the substratum, which are not related to adsorption itself but which can be a major contribution to an increased accumulation.

Transport and Adsorption to the Substratum

Diffusivity: Under laminar flow conditions, particles are transported to the substratum by diffusion perpendicular to the flow. Microorganisms with a size of 1 to 4 μm^3 have a very small Brownian motion and, hence, a small Brownian diffusivity. Therefore, motility is of considerable importance during the processes of transport but has often

been neglected in adsorption studies. Jang and Yen (1985) calculated the non-Brownian diffusivity (motility) for different microorganisms to be in the range of $0.4 \cdot 10^{-3}$ to $5.6 \cdot 10^{-3}$ mm² s⁻¹, compared to the Brownian diffusivity of $50 \cdot 10^{-6}$ mm² t⁻¹. They used the following equation to calculate non-Brownian diffusion:

$$D_c = \frac{v_r \cdot d_r}{3 \cdot (1 - \alpha)} \qquad 2.3$$

D_c : diffusivity [L² t⁻¹]
v_r : velocity of motility [L t⁻¹]
d_r : free length of random run [L]
α : main cosine of angle of turn [-]

Under condition of no chemotaxis, α can be assumed to be zero. If motility is not considered as a contributing factor in the process of transport to the substratum, the transport rate might be underestimated 20 to 50 times. The non-Brownian diffusivity can be calculated from the velocity and the mean free path length. Vaituzi and Doetsch (1969) measured speeds up to 55.8 μm s⁻¹ for Pseudomonas aeruginosa with "track photography". Their results suggest a mean free path length of random run in the range of 50 to 85 μm. These values yield a non-Brownian diffusivity (Equation 2.3) of 10^{-3} mm² s⁻¹ for P aeruginosa.

Transport Rate to the Substratum: Good information is available for the transport and adsorption of inert particles to different substrata.   Bowen et al. (1976) proposed an analysis with a first-order-reaction approximation for the surface-particle capture rate, which leads to an expanded Graetz solution.   This solution converges well for relatively large Peclet numbers and proved to be accurate for inert particles adsorbing to charged surfaces.   The resulting equation has the following form:

$$
K_{CFU} = \frac{c_0 \cdot D_c}{h} \cdot \left[\frac{\left[\frac{2}{9K_1} \right]^{1/3}}{\Gamma \left[\frac{4}{3} \right] + \left[\frac{1}{\epsilon} \right] \cdot \left[\frac{2}{9K_1} \right]^{1/3}} \right]
$$

2.4

K_{CFU} : Adsorption rate of CFU to the substratum   [# L^{-2} t^{-1}]
c_0 : CFU concentration in bulk liquid [# L^{-3}]
D_c : Diffusivity of CFU   [L^{-2} t^{-1}]
h   : Half-thickness of channel   [L]
K_1 : Dimensionless distance from channel inlet   [-]
ϵ : Surface-particle capture factor   [-]
Γ : Gamma function [Γ ($4/3$) = 0.89338]

In the special case where the surface-particle capture rate ϵ approaches infinity ($\epsilon = \infty$), Equation 2.4 becomes:

$$N_{CFU} = \frac{c_0 \cdot D_c}{h} \cdot \left[\frac{\left[\dfrac{2}{9K_1} \right]^{1/3}}{\Gamma \left[\dfrac{4}{3} \right]} \right]$$

2.5

N_{CFU} :   Flux of CFU to Substratum $[\# \ L^{-2} \ t^{-1}]$

The strength of this solution is that one can estimate the flux of particles to the substratum ($\in = \infty$, Eq. 2.5) and obtain a discrete value for the surface-particle capture factor, independent of concentration and motility, for different substrata. In addition, a "sticking efficiency" can be calculated with Equations 2.4 and 2.5 by dividing adsorption rate by the CFU flux to the substratum.

Assay for Substratum Properties: The use of Equations 2.4 and 2.5 to describe transport and adsorption processes, especially the dimensionless factor $\in$, is a good analysis for studying the effects of different degrees of hydrophobicity of substrata (independent of concentration and diffusivity).

Fletcher and Marshall (1982) show an increase of accumulation with an increased hydrophobicity. They use the double-layer theory (DLVO) of the colloidal chemistry to explain these mechanisms, based on the calculated charge of the substratum measured with the bubble contact angle method

and the overall charge of the organisms. Van Pelt et al. (1985) showed that there is statistically seen a very poor correlation between the free surface energy and extent of accumulation. They propose as possible reasons for this poor correlation that accumulation as the measured value is not the most appropriate parameter to describe adsorption, that the free surface energy during the experiment is not the same as that previously measured, or that the mechanisms of adsorption are different depending on the free surface energy.

Van Pelt's first proposition is that the change in accumulation or the net adsorption is the result of adsorption minus desorption and, therefore, measurements of accumulation does not reflect the primary occurring process.

The second proposition is in accordance with the observation of the "conditioning film". This change of well defined surfaces due to exposure to water containing organic macro molecules has been described by Loeb and Neihof (1975), Baier and Weiss (1975), Abbott et al. (1983), and Little and Zsolnay (1986). They summarize the effect of a conditioning film that a solid surface in contact with liquids containing diverse organic macromolecules alter due to the formation of a monolayer of adsorbed macromolecules. This results in the following: hydrophobic surfaces become

hydrophilic, positively- and negatively-charged surfaces acquire a net negative charge and Zeta potentials, contact potentials, and critical surface tensions are increased.

The third proposition is that depending on the free surface energy cells use a different mechanism for adsorption. Fletcher and Marshall (1982) indicated the importance of proteins in the processes of adsorption. By adding Pronase they reduced the increase of accumulation. Paul and Jeffrey (1985) showed that for hydrophobic substrata, such as polystyrene, the protein linking is essential, whereas for hydrophilic substrata other adsorptive mechanisms dominate. Their result indicates that, indeed, different mechanisms of adsorption dominate depending on the free energy of the substratum.

Growth-Related Processes

Very little work has been done in regard to growth-related processes during early colonization of surfaces. Only two groups of studies are available:

1. The early phase of biofilm accumulation (Trulear, 1983; Turakhia, 1986).

2. Growth measurements with Image Analysis (Caldwell and Lawrence, 1986,).

Trulear and Turakhia determined growth rates in biofilms grown under constant shear stress and turbulent flow. During the first or second day after exposure of acrylic plastic surfaces to P. aeruginosa, the adsorbed biomass had a growth rate ($\mu \geq 0.65$ h^{-1}) exceeding the maximum growth rate in suspension ($\mu_{max} \approx 0.5$ h^{-1}). After this initial time, the measured overall growth rates were reduced to levels below the maximum rate in suspension. Their results do not indicate whether this increased initial activity is due to prior physiologic or genetic phenomena or due to analytical variations after adsorption. Data suggest that the process of adsorption is very selective for the "more active" organisms (cells with a high motility have a greater diffusivity).

Caldwell and Lawrence (1986) measured "behavior" of adsorbed P. fluorescens under laminar flow conditions. They observed different patterns of adsorption and subsequent growth at the substratum. Unfortunately, they do not state the shear stress of the liquid on the wall but only an average velocity. From the publication, shear stress cannot be calculated since the geometry is unknown. The growth rates measured do not answer all questions since the substrate concentration used were unusually high (1 g glucose l^{-1} and 100 mg glucose l^{-1}). The cells were grown in batch cultures and washed twice before being used in the

adsorption study. The measured specific growth rates are in the range of 0.42 h^{-1}.

Spatial Distribution

The spatial distribution of the CFU during adsorption can be of great value since it can determine whether the process is controlled by the adsorbing cells alone or a cell - substratum interaction (including the existing colonization). If the process is due to cells only, without any influence by the substratum's present state of colonization, a random distribution would be expected. If conditioning and occupation of the surface positively influences adsorption, then an aggregated distribution can be anticipated. A uniform distribution, conversely, results when the area around an existing CFU is blocked for adsorption indicating a negative influence. Classic Nearest Neighborhood Analysis cannot be used for this analysis since only distance to the single nearest neighbor is used ignoring the other adsorbed cells. An analysis is needed which accounts for the overall population density. An extensive description of a unique solution for spatial distribution analysis is presented in Appendix A.

Fluid Dynamics

Defined fluid dynamics is essential for adsorption studies under constant shear stress. Hydrodynamics control both transport and adsorption: Transport is a function of the loading rate and adsorption depends on shear stress. Due to this relationship, it is difficult to compare experimental results obtained under different hydrodynamic conditions without their precise definition. Two different flow patterns must be considered:

1. Turbulent flow
2. Laminar flow

Turbulent Flow

The nature of turbulent flow does not allow an easy determination of the velocity gradients near the wall. Depending on the Reynolds number of the flow and the roughness of the wall, a wide variety of velocity gradients and, thus, shear stresses are possible. Using the theory of the "viscous sublayer", within its limitations, i.e. smooth surface, it is possible to estimate the shear forces.