A method for quantitative analysis of biofilm thickness variability

by Ricardo Murga

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
Montana State University

Abstract:
This thesis presents work done on developing an experimental method for measurement of biofilm thickness. This method involves embedding the biofilm, cross-sectioning it, and applying image analysis and mathematical techniques to reconstruct biofilm thickness profiles in order to characterize biofilm thickness variation. Quantitative data as well as qualitative observations obtained from Pseudomonas aeruginosa, Klebsiella pneumoniae and binary population biofilms revealed that the three biofilm systems are distinct and heterogeneous in their structure. Statistical analysis of biofilm thickness data showed that biofilm species composition influences structural characteristics. P. aeruginosa, K. pneumoniae and binary population biofilms appeared to have different mean thicknesses, different roughness values, and different spatial, distribution of biomass, voids and water channels. P. aeruginosa biofilms are relatively uniform and lack voids or water channels. They have an average thickness of 30 μn, and their roughness coefficient, Ra*, ranges between 0.1 and 0.2. Different sizes and lengths of water channels were observed in K. pneumoniae biofilms, but the most distinctive characteristic was large bare areas along the substratum. K. pneumoniae biofilms are patchy and their thickness varies drastically, having a mean thickness that ranged between 8 μn to almost 300 μm. The roughness coefficient for K. pneumoniae biofilms was the highest of the three biofilms systems, ranging between 0.8 to 1.3. The binary population biofilm was also full of water channels and voids. The mean thickness ranged between 300 and 600 μm, but its roughness coefficient only ranged between 0.2 and 0.3. The different ranges for the roughness coefficient values suggested that differences in biofilm thickness variation could be used as a distinctive characteristic for categorizing biofilms.

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APPROVAL

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Ricardo Murga

This thesis has been read by each member of the committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

This thesis presents work done on developing an experimental method for measurement of biofilm thickness. This method involves embedding the biofilm, cross-sectioning it, and applying image analysis and mathematical techniques to reconstruct biofilm thickness profiles in order to characterize biofilm thickness variation. Quantitative data as well as qualitative observations obtained from *Pseudomonas aeruginosa, Klebsiella pneumoniae* and binary population biofilms revealed that the three biofilm systems are distinct and heterogeneous in their structure. Statistical analysis of biofilm thickness data showed that biofilm species composition influences structural characteristics. *P. aeruginosa, K. pneumoniae* and binary population biofilms appeared to have different mean thicknesses, different roughness values, and different spatial distribution of biomass, voids and water channels. *P. aeruginosa* biofilms are relatively uniform and lack voids or water channels. They have an average thickness of 30 μm, and their roughness coefficient, Ra*, ranges between 0.1 and 0.2. Different sizes and lengths of water channels were observed in *K. pneumoniae* biofilms, but the most distinctive characteristic was large bare areas along the substratum. *K. pneumoniae* biofilms are patchy and their thickness varies drastically, having a mean thickness that ranged between 8 μm to almost 300 μm. The roughness coefficient for *K. pneumoniae* biofilms was the highest of the three biofilms systems, ranging between 0.8 to 1.3. The binary population biofilm was also full of water channels and voids. The mean thickness ranged between 300 and 600 μm, but its roughness coefficient only ranged between 0.2 and 0.3. The different ranges for the roughness coefficient values suggested that differences in biofilm thickness variation could be used as a distinctive characteristic for categorizing biofilms.

INTRODUCTION

The Problem

Microbial cells attach firmly to almost any surface submerged in an aquatic environment. The immobilized cells grow, reproduce, and produce extracellular polymers which help them form a complex matrix that provides structure to the biomass accumulation termed a *biofilm* (Characklis and Marshall, 1990). The adhesion to surfaces provides considerable advantages for bacteria within the biofilm. The biofilm matrix provides protection from antimicrobial agents, as well as facilitating the exchange of nutrients and genetic material due to close proximity of cells. While bacteria benefit from their close association with the surface, the physical presence of the biofilm compromises the function of the surface, reducing the efficiency of equipment in industry. This damage to the surface is referred to as biofouling. Some of the problems stemming from biofouling are summarized in the following paragraph.

Microbially induced corrosion (MIC) is initiated when clusters of bacteria form a patchy biofilm. These clusters establish microscale chemical gradients around the surface and thereby create both anodic and cathodic sites (Lee and Characklis, 1993., Geesey and Bremer, 1992., Videla and Characklis, 1992., Bremer and Geesey, 1991., Characklis et al. 1991, Geesey, 1990., Jelley et al., 1989., Characklis, 1989). Increase of fluid-frictional resistance in industrial

pipes results from the increase in biofilm thickness and roughness (Characklis, Zelver and Turkhia, 1981., Characklis and Turkhia, 1981., Picologlou et al., 1980., Kirkpatrick et al. 1980., Characklis, Zelver and Picologlou, 1978). Increase of heat transfer resistance results from the accretion of biomass and inorganic sediments (Turakhia and Characklis, 1984., Mussalli and Characklis, 1984., Characklis et al. 1984, 1983., Zelver et al., 1982., Characklis Nimmons and Picologlou, 1981., Characklis, Zelver and Turakhia, 1981). Bacterial colonization of a variety of medical implants and devices results in persistent infections (Costerton, 1982., Marrie et al., 1982., Marrie and Costerton, 1983., Marrie and Costerton, 1984). Detachment of microbial aggregates into the bulk liquid degrades products in the paper manufacturing and water distribution processes (Nix, et al. 1992., Block, 1992., Jung, et al. 1990., McFeters, 1990., van der Wende, et al. 1989., LeChevallier, et al. 1988., Camper, et al. 1986).

Biofilm development and activity is the result of several physical, chemical and biological rate processes. Such processes include transport of cells to the substratum, adsorption of cells onto the substratum, growth and other metabolic processes within the biofilm, and detachment of portions of the biofilm. These processes are influenced by the biofilm structure and the properties of the environment around the biofilm. Surface roughness, for example, significantly influences transport rates and microbial attachment for several reasons including the following: 1) increased convective mass transport near the surface, 2) shelter from shear forces for small particles, and 3)

increased surface area for attachment (Characklis, W.G., 1981). Surface roughness is one of the many aspects that reflect the structural heterogeneity of the biofilm matrix. This structural heterogeneity should therefore be studied and quantitatively described in order to better understand, predict and solve the problems caused by biofouling. Obtaining quantitative information on surface roughness and structural heterogeneity remains a challenge to biofilm researchers.

Goal and Objectives

The long term goal of this research is to understand how biofilm structural heterogeneity impacts biofilm function. The broad objective of this thesis was to develop experimental methods and apply mathematical techniques to characterize biofilm thickness variation. The specific objectives were: 1) to develop an experimental method suitable for measurement of biofilm thickness profiles, 2) to construct 1-dimensional thickness profiles of steady state pure culture biofilms of *P. aeruginosa*, *K. pneumoniae*, and the binary population combination, 3) to calculate a roughness index (R_a^*) and to perform a spatial correlation analysis for each profile, 4) to obtain photomicrographs of particles in the effluent of pure culture and binary population biofilm reactors, 5) to document temporal changes in biofilm thickness characteristics during development of binary population biofilms by obtaining biofilm thickness profiles, R_a^* values, and spatial correlation analyses in time series.

LITERATURE REVIEW

Evidence of Biofilm Structural Heterogeneity

Recent publications provide evidence of structural heterogeneity in biofilms. Table 1 shows a few examples of the types of evidence described in publications from the last decade alone. Several techniques have been used to qualitatively describe biofilm structure including light microscopy, atomic force microscopy (AFM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM), and Fourier transmission infrared spectroscopy (FTIR). The systems in which these observations were made range from laboratory reactors to medical and industrial systems.

Many factors may contribute to the structural heterogeneity of biofilms such as the uneven distribution of microbial species, the presence of different shapes of bacteria buried in amorphous extracellular polymers that constitute the biofilm matrix, nitrogen bubble formation in some cases, shear and hydrodynamic forces, deposits of sediments, and encrustations on pipe walls. All these contribute to different forms of structural heterogeneity in biofilms due to the disruption of the microbial matrix and biomass detachment (sometimes leaving entire bare areas), helping to the formation of patchy biofilms. Most heterogeneous biofilms have water channels, pores, microtowers, ridges, and large variations in biofilm thickness and surface roughness.

Table 1. References for evidence of heterogeneity.

Author	Ref	Microorganism	Reactor Design	Evidence
Allen et al, 1980.	1	Mixed population.	Wat. dist. system	Electron micrographs revealed a hard but porous surface.
Bremer et al, 1992.	7	CCI#8 bacterium.	Copper coupons submerged in flasks with culture medium.	Atomic Force Microscopy (AFM) showed biofilm "several cells thick in places, distributed heterogeneously over coupon surface with considerable variation in surface contour.
Bryers, 1991.	9	*P. putida.*	Annular reactor.	Observed sloughing events due to nitrogen bubble formation that lead to disruption of the microbial matrix.
Bryers and Characklis, 1981.	10	Undefined mixed population from sewage.	Tubular reactor system running as CSTR	Microscopically, biofilms appeared as discrete fibers randomly distributed, 1.5 to 1500 μm long.
Capdeville et al, 1990.	12	Heterotrophes.	Annular reactor	SEM and photographs showed observations of thickness profile and top view of rough, filamentous and patchy surface.
Christensen et al, 1988.	24	Denitrifying biofilm from wastewater.	Annular reactor	Pictures show heterogeneity in biofilm accumulation due to nitrogen bubble formation.

Table 1 (cont.)

Eighmy et al, 1983.	29	Undefined biofilm from wastewater.	Inert substratum and supporting structure suitable for submersion in waste-stream, bacterial adhesion and subsequent examination by TEM and SEM.	SEM and TEM micrographs showed filamentous non-smooth surface.
Gujer, 1987.	34		A model of segregation of biomass in biofilms.	Describes a biofilm as an inhomogeneous microbial matrix due to uneven distributions of microbial species.
Hamoda and Abd-el-bary, 1987.	35	Mixed culture from activated sludge.	Aerated submerged fixed-film (ASFF) bioreactor.	Microscopic observation of spongy biofilms with thickness of up to 2.3 mm.
Harremoes et al, 1980.	37	Denitrifying biofilm.	Annular reactor.	Extreme heterogeneity in biofilms due to nitrogen bubbles formation that detached big particles of biofilm leaving large bare sections of the wall visible.
Howell and Atkinson, 1976.	38		Trickling filter biofilm model.	Computer output showed film thickness profiles to have a varied irregular pattern.