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RELATION BETWEEN THE STRUCTURE OF AN AEROBIC BIOFILM AND TRANSPORT PHENOMENA

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ABSTRACT

An aerobic biofilm was characterized using confocal scanning laser microscopy (CSLM), O_2 micro-electrodes, particle tracking and microinjection of fluorescent dyes. The biofilms were found to consist of microbial clusters of cells and Extra-cellular Polymeric Substance (EPS) separated by interstitial voids. The cell clusters were ca 300 μm and the voids were ca 100 μm wide. The voids were open channels connected with the bulk fluid. Fluorescein micro-injection showed that liquid could flow through the voids, but was always stagnant in the cell clusters. Consequently, in voids both diffusion and convection may contribute to mass transfer, while in cell clusters transport is determined by diffusion only. Particle tracking with CSLM showed that flow velocity inside the biofilm was proportional to the bulk flow velocity. The importance of convective mass transport in biofilms was demonstrated by oxygen distribution measurements. At high flow velocities of the bulk liquid, the mass boundary layer followed the irregular biofilm surface. At lower velocities the mass boundary layer was parallel to the substratum. Mass transfer from voids to cell clusters increased with flow velocity, as result from convective mass transport from the bulk to the voids. Convective transport was insignificant at low flow velocities, but at high flow velocities it increased the total mass transport by 200-250%. The local diffusion coefficients in biofilms were measured using microinjection of fluorescent dyes and quantitative analysis of the subsequent plume formation using CSLM. The diffusion coefficient of small, non-binding molecules in cell clusters is close to that in water. Very large molecules were impeded in their diffusion through the biofilm matrix. It was calculated that the cell cluster matrix had the characteristics of a gel network with pore diameters of 80 nm.

KEYWORDS

cell clusters; confocal microscopy; convection; diffusion; microelectrodes; pores

INTRODUCTION

Surfaces immersed in water under physiological conditions are rapidly covered with biofilms, layers of microorganisms embedded in a matrix of Extra-cellular Polymeric Substance (EPS)(Characklis and Marshall, 1990). Microbial conversions in biofilms are controlled by growth kinetics and mass transport processes. Mathematical models proposed to describe the structure and microbial activity of biofilms describe biofilms as planar structures with homogeneous cell distribution (Characklis and Marshall, 1990; Rittmann and Manem, 1992; Wanner and Guyer, 1986). Mass transfer through the mass boundary layer and within the biofilm is assumed to be diffusional and perpendicular to the substratum to which it is attached. Mass transport inside biofilms is described using the effective diffusion coefficient (D_e). The D_e/D_w ratios for

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substrate molecules, such as oxygen, glucose, ammonium and nitrate, in biofilms and microbial aggregates are often assumed to be around 0.9 (Christensen and Characklis, 1990). However, microscopic observations indicate that biofilms are not flat. Instead, biofilms may form highly complex structures containing cell layers and clusters as well as voids or pores. Such structures have been found in many biofilms (Eigmy *et al,* 1983; Kugaprasatham *et al,* 1992; Lawrence *et al,* 1991; Robinson *et al,* 1984; Stewart *et al,* 1993). It has been speculated that the porous structure may enable liquid movement in the biofilm, increasing the mass transfer rate from the bulk liquid. To prove the possibility of convection, it was necessary to quantify and localize liquid flow within biofilms by direct measurements. Furthermore, if voids act as transport channels for substrates the substrate concentration in voids should be higher than that in adjacent biomass. For verification substrate concentrations in voids and cell clusters of physiologically active biofilms under flow conditions must be measured directly. Finally, to complete our understanding of mass transport processes a more precise insight of the diffusion properties of heterogeneous biofilms was necessary.

To achieve these goals we integrated CSLM, microelectrode and microinjection techniques to assess the relationship between the structure of biofilms and transport phenomena. CSLM enhances visualization of biofilm structures by eliminating the interference from out of focus objects (Caldwell *et al,* 1992; Wilson, 1990). No preparatory steps are needed, observations can be done under growth conditions, while the biofilm remains physiologically active. Microelectrodes allow local concentrations to be measured with minimal disturbance of the biofilm structure (de Beer *et al,* 1993a; Revsbech and Jorgensen, 1986). For qualitative but highly localized flow measurements a microinjection technique was used (de Beer, *et al.* 1994). For recording of velocity profiles near and within the biofilm a particle tracking technique using CSLM was developed (de Beer *et al,.* 1994, Stoodley *et al,* 1994). For diffusion studies a high spatial resolution technique was developed based on analysis of plume formation after microinjection of fluorescent dyes. With this technique diffusion coefficients can be measured in a volume element with a radius of 80 µm, sufficient to account for the scale of biofilm heterogeneity. Diffusion of macromolecules is impeded by biofilm matrices depending on the micro-structure of the matrices and the molecular size of the solute (Westrin, 1991). Conversely, information about the micro-structure of the biofilm matrix can be derived from its resistance to diffusion.

Using these combined experimental approaches we have constructed an overall concept of the transport processes in biofilms.

MATERIALS AND METHODS

Biofilm development Biofilms were developed in a continuous culture reactor system consisting of a flow cell (Fig. 1) attached to a recycle loop. The flow cell, connected to the support system with flexible tubing, could be mounted on the confocal microscope stage. The reactor was inoculated with a mixture of frozen stock cultures of *Klebsiella pneumonia, Pseudomonas fluorescens* and *Pseudomonas aeruginosa.* The start-up procedure, medium composition and reactor operation has been described earlier (de Beer *et al,* 1993b). The biofilms developed on an observation window were accessible for *in situ* microscopic examination and measurements with specially designed oxygen microelectrodes and microinjection capillaries. Experiments were performed on 150-200 µm thick biofilms, which were 4-5 days old.

Microelectrode measurements The oxygen microelectrodes used were cathode type, bent in a "J"-shape (Fig. 1). The "J" shape allowed the electrodes to be introduced into the flow cell with the tip raised to the coverslip, thus oxygen concentration profile measurements could be made in vertical direction. The microelectrode was positioned by a micromanipulator with a stepper motor. From oxygen profiles, measured at 50 to 80 µm intervals along transects traversing both pores and clusters, contour plots were made and the local gradients were calculated. Local oxygen diffusion fluxes were calculated from the gradients, assuming a diffusion coefficient of 2.27 x 10^{-9} m^2/s. The thickness of the hypothetical boundary layer (δ_b) was found by linear extrapolation of the substrate gradient at the biofilm surface to the bulk concentration (Kashe and Kuhlmann, 1980). In a rough biofilm the steepest part of the oxygen profiles may be found above the biofilm surface. In that case the top of the boundary layer is found by extrapolation of the steepest part of the oxygen profile to the bulk concentration.

Flow velocity measurements The occurrence of liquid flow in cell clusters and voids was determined by local injections of small volumes of fluorescein. With a nanolitre pump (Oriel A1400), supplied with a

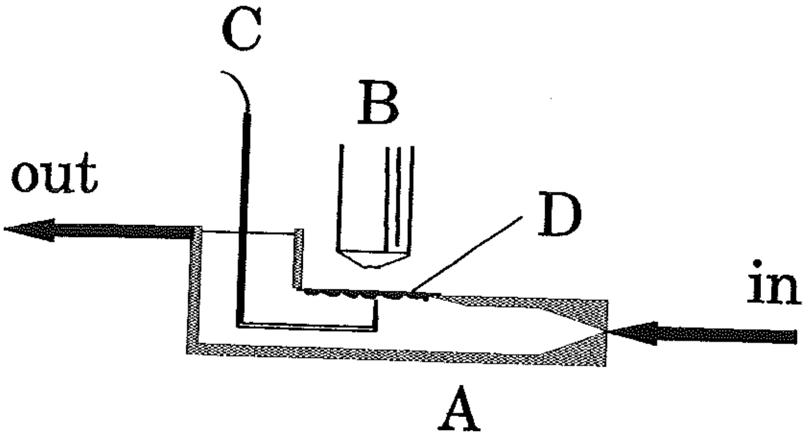


Fig. 1 The experimental set-up. A) Flow cell, B) microscope objective, C) "J" shaped microelectrode, D) observation window with biofilm.

"J"-shaped micropipet with a tip diameter of 5-10 μm, 3 mM fluorescein droplets were injected in pores or cell clusters. The plume, developing after the injection, was observed with CSLM. In stagnant medium the plume shape was spherical and in flowing medium elongated. Local liquid velocities in the flow cell were determined by tracking the velocity of suspended fluorescent beads (300 nm to 28 μm) with CSLM. The procedure is described in detail elsewhere (de Beer *et al*, 1994; Stoodley *et al*, 1994).

Local diffusion coefficients. The local diffusion coefficients in cell clusters and voids were determined by microinjection of fluorescent dye (2 mM fluorescein or 4 g/l phycoerythrin). The same set-up was used as for the flow measurements, described in the previous section. After injection in stagnant medium the fluorescent dye diffused radially away from the point of origin forming a fluorescent plume. The distribution of a substance diffusing from an instantaneous spherical source in an infinite medium is described by the analytical equation found by Crank (1975). Sequences of images of the fluorescent plume developing around the point of origin were recorded at equal time intervals using CSLM. The fluorescence intensity at a defined radius from the point of origin was plotted against time. These plots show an initial increase of fluorescence followed by a decay. The peak-times were fitted with the equation of Crank (1975) to determine the local effective diffusion coefficient (D_e in cell clusters, D_w in medium or voids).

RESULTS AND DISCUSSION

Biofilm structure. The biofilm structure and the microscopic techniques employed are extensively described previously (de Beer *et al*, 1993). In short, it consisted of cell clusters with a thickness of 150-200 μm and a diameter of 200-300 μm, covering ca 50% of the substratum. The clusters were separated by voids 50-100 μm wide. The voids had open connections with the bulk liquid. Cells and EPS were exclusively present in the cell clusters and not in the voids. These observations lead to a new conceptual model of biofilms - a conglomerate of cell clusters attached to the substratum. This model has profound consequences for the understanding of substrate- and product transport.

Liquid flow. Injection of 3 mM fluorescein resulted in the formation of a fluorescent plume, which was spherical in stagnant medium and elongated in flowing medium, the length to width ratio depending on the flow velocity. With microscopic guidance the micro-pipet tip could be positioned in the

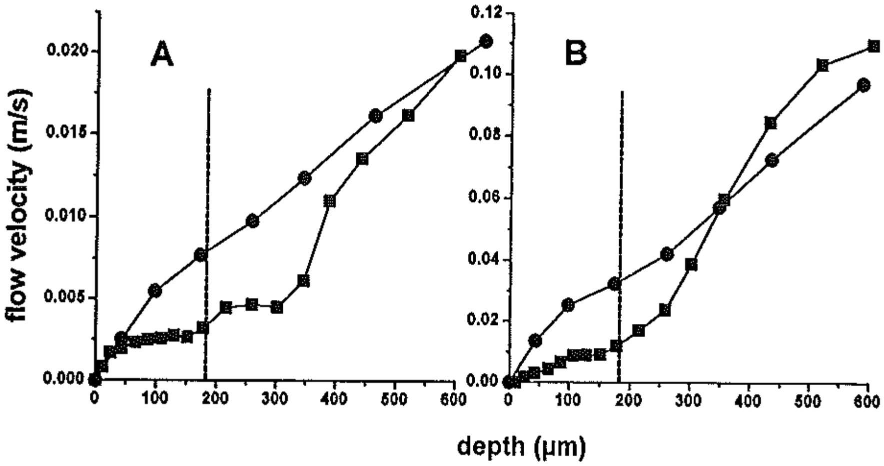
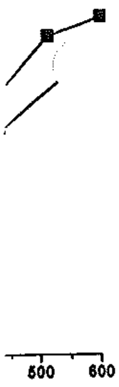


Fig. 2 Liquid velocity profiles near the wall of the flow cell with (■) and without (+) biofilm, flow velocity was (A) 0.034 m/s and (B) 0.14 m/s.

voids or into the cell clusters. Using this technique we could show that in voids flow was possible but in cell clusters the liquid was always stagnant. Consequently, in cell clusters diffusion is the only mechanism for mass transport, while in voids both diffusion and convection may take place.

Quantification of intra-biofilm liquid flow was done by following fluorescent bead movement using CSLM. These beads do not penetrate cell clusters, so only the velocity in voids could be monitored. In the presence of biofilm two linear regions could be distinguished, one in the biofilm and another above the biofilm surface (Fig. 2). The velocities at the biofilm-bulk liquid interface ranged from 0.002-0.015 m/s. Since the void diameter was approximately 100 µm, *Re* in the voids was lower than 1.5, indicating that the flow regime in the voids was laminar. The measurements showed unambiguously that the liquid movement in biofilms could take place through the voids, and that the flow velocity in the voids was proportional with the bulk liquid velocity. Whether the intra-biofilm flow contributed to increased substrate transport was further investigated with oxygen microelectrode measurements.

Oxygen distribution. Oxygen profiles were measured in pores and cell clusters. Under growth conditions they were significantly different (Fig. 3B). In the cell cluster oxygen penetrated 30 µm and the lower 50 µm was anaerobic, in the pore oxygen penetrated to the substratum. At a given distance from the substratum the oxygen concentration in the pore was higher than in the cell cluster, inducing a diffusional flux from the void to the cell clusters. At very low velocities no such concentration differences were observed (Fig 3A). To get a more complete representation of the oxygen distribution 2-D contour plots were contructed. Contour plots were measured at flow velocities in the range of 0.008-0.165 m/s. The velocity of the bulk liquid had a strong influence on the oxygen concentrations and distribution patterns in the biofilm (Fig. 4). At flow velocity higher than 0.04 m/s the contour lines followed the solid biofilm surface. Oxygen penetrated through the voids to the substratum and up to 50 µm into the cell clusters. The vectors representing the local oxygen gradients indicated that oxygen was supplied to the cell clusters both directly from the bulk liquid as well as from the voids. At flow velocities lower than 0.04 m/s the contour lines became parallel with the substratum. Oxygen did not penetrate deeper in the voids than in the cell clusters. The gradients in the contour plots were mainly oriented perpendicular to the substratum and the largest were present at the top of the cell clusters. Thus the fluxes towards the biofilm were vertically directed from the bulk to the cell clusters, and no lateral diffusion from the voids to the cell clusters occurred. The increase in concentration difference between the cell clusters and the voids with increasing flow velocity reflects the increase in convectional transport.



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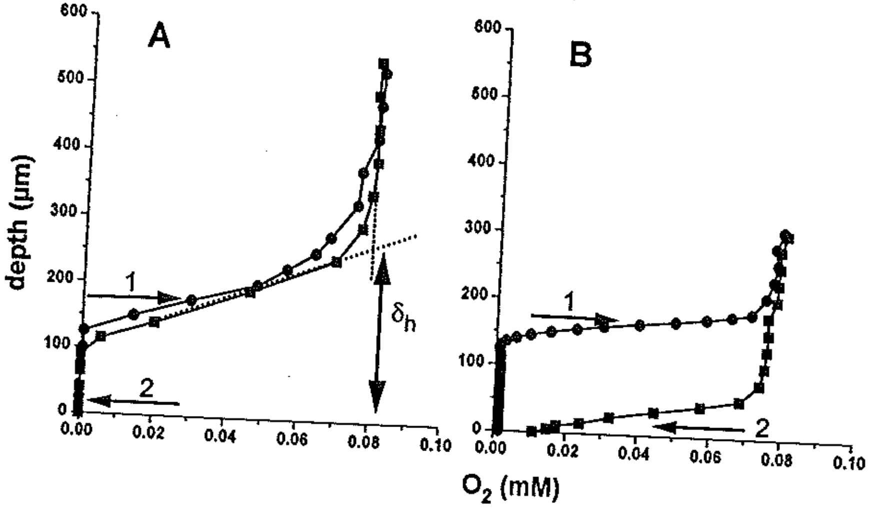

Fig. 3 Oxygen profiles at flow velocity of 0.0078 m/s (A) and 0.064 m/s (B), above cell clusters (+) and voids (■). The dotted lines indicate extrapolations from the steepest gradient and the bulk concentration, the dashed arrow is δ_h. The solid arrows indicate the solid biofilm surface of the cell clusters (1) and the base film in between the cell clusters (2).

The oxygen profiles show the steepest gradients at the surface of the solid biofilm parts if flow velocity > 0.04 m/s, both in voids and above cell clusters. At lower flow velocities the steepest part of the profiles in voids were found away from the surface, up to 150 μm (Fig. 3A). For such flow velocities δ_h was determined by linear extrapolation of the largest gradient to the bulk concentration as shown in Fig. 3A, instead of using the gradient at the solid biofilm parts-liquid interface. The upper limit of the mass boundary layer followed the solid biofilm surface closely if flow velocity was higher than 0.04 m/s. At lower flow velocity it separated from the solid biofilm surface and became parallel with the substratum surface. Convection in voids was also demonstrated from the shape of the mass boundary layer. At high flow velocity the mass boundary layer followed the irregular biofilm surface, while at low flow velocity it was planar. This demonstrates that only at higher flow velocity the renewal of liquid in the voids with bulk liquid was sufficient to maintain the substrate concentration in the voids significantly above that in the cell clusters. The upper limit of the mass boundary layer approximates the effective exchange area, which is approximately 2.5 times the substratum area (assuming cell clusters to be cylinders with a diameter of 300 μm, 175 μm high, covering 50% of the substratum). Substrate transport increases with flow velocity, because δ_h decreases and because the effective exchange area is enlarged.

These results show a complex relation between flow velocity and transport in a heterogeneous biofilm system. At low flow velocities the mass boundary layer was parallel with the substratum and no transport occurred from the voids to the cell clusters. Under such conditions heterogeneous biofilms behave as though they were planar structures, with homogeneous cell distribution and diffusion as the principal internal mass transport mechanism. At high velocities the boundary layer followed the solid biofilm surface and oxygen transport through the voids became significant. In that case the biofilm heterogeneity was important, since the exchange surface with the bulk liquid was increased. Consequently, channels in biofilms do not always facilitate mass transport. Enhanced mass transport occurs if the mass boundary layer follows the solid biofilm surface, *i.e.* if the flow velocity is high enough to reduce δ_h to well below the thickness of the biofilm. Then voids act as transport channels for substrates and increasingly so at higher flow velocities.

Diffusion coefficient and cluster microstructure. Since inside cell clusters diffusion governs mass transport

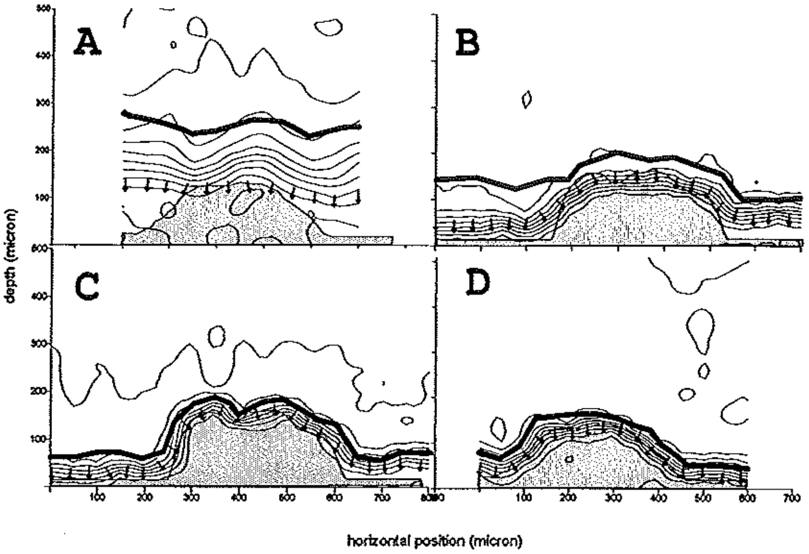

Fig. 4 Oxygen contour plots, measured at flow velocity of 0.0078 m/s (A), 0.0174 m/s (B), 0.064 m/s (C) and 0.115 m/s (D). The outline of the solid biofilm parts are indicated as shaded area. The thin lines represent the oxygen contours, drawn at equal intervals of oxygen concentration. The numbers in the figure and on the right margin indicate local oxygen concentrations (mol/m^3). The thick line indicates the upper limit of the mass boundary layer.

it was necessary to investigate the diffusional resistance of the cell cluster matrix. After a pulse injection of fluorescein and phycoerythrin in medium, voids and cell clusters, the fluorescent plume initially expanded to a maximum diameter and then gradually faded away due to dilution of the dye. Fluorescein or phycoerythrin did not bind to the biofilm matrix and the fluorescence intensities decreased eventually to background levels after microinjection. Calculated values of diffusion coefficients in voids, cell clusters and sterile medium are presented in Table 1. The measured diffusion coefficients of fluorescein in cell clusters, voids and medium were very close. It was concluded that the diffusivities of other compounds with molecular weights in the same order of that of fluorescein are not decreased in cell clusters. This is the case for most nutrients.

Diffusion of large molecules may be impeded by the cell cluster matrix, as indicated by the D_e/D_w ratio of phycoerythrin. Gel matrices can reduce the diffusivity of molecules in at least two ways: by physical obstruction or molecular binding. It should be emphasized that with the technique of microinjection the diffusion coefficient is measured under transient conditions. This may be different from the diffusion of substrates and products under steady state conditions, when the rate of substrate transport into the biofilm is equal to the conversion rate. Binding is inherently a transient process. Only after binding has stopped (e.g. when all binding sites are occupied) steady state is possible, during which no further binding occurs. Consequently, transient state measurements underestimate the diffusion coefficients for steady state transport if the compound can bind to the matrix (Libicki *et al*, 1988). Phycoerythrins do not bind to cells (Glazer, 1988), which makes it an excellent compound to study purely diffusional phenomena of large molecules in gels and biofilms. From diffusion studies information can be derived about the cell cluster matrix. With the Stokes-Einstein equation (Westrin, 1989), using the measured D_w for phycoerythrin, a value for the molecular radius of 5.5 nm was calculated. Thus the pore diameter of the cell cluster matrix must be at least 11 nm to allow diffusion of phycoerythrin. We also showed that 300 nm BSA treated latex beads do not penetrate cell clusters (de Beer *et al*, 1994). Consequently, the pore diameter of the cell cluster matrix is between 10 to 300 nm, but a more precise estimation is possible. Applying the modified Renkin equation (Satterfield *et al*, 1973) a value for the pore diameter of 80 nm was calculated. This suggests that only particles smaller than 80 nm can diffuse through the biofilm matrix, while bigger particles, such as cells, are at the least strongly restricted in their mobility.

TABLE 1 DIFFUSION COEFFICIENTS

	medium	void	cell cluster	D_e/D_w
fluorescein (MW 332)	5.5(±0.4)	6(±0.8)	5.8(±1)	0.97
phycoerytrin (MW 240000)	nd	0.39(±0.04)	0.23(±0.03)	0.59

Diffusion coefficients ($x10^{10}$ $m^{-2}.s$) in a heterogenous biofilm, determined by microinjection. De/Dw is the ratio of diffusion coefficients in cell clusters and voids.

CONCLUDING REMARKS

An important question is whether biofilm heterogeneity is a universal phenomenon, or only a characteristic of the laboratory biofilm used in this study. A certainly not comprehensive study in our laboratory learned that heterogeneity is indeed common: biofilms grown on a glass slide under a running tap, nitrifying biofilms on concrete slabs, biofilms from a waste gas treating trickling filter and a biofilm growing on stainless steel in water from a mountain stream consisted of clusters and voids (data not shown). Recently reports were published by different authors addressing heterogeneity of biofilms from various origins. Nitrifying biofilms from a wastewater plant showed an irregular surface (Kugaprasatham *et al*, 1992). Biofilms from bioreactors treating petroleum contaminated groundwater showed a comparable biofilm architecture (Massol-Deya *et al*, 1994; Massol-Deya *et al*, 1995). Other CSLM studies on relatively thin biofilms (10-40μm) showed cell clusters and voids (Korber *et al*,1994; Lawrence *et al*, 1991). Some biofilms seem to deviate: bacterial aggregates from waste water treatment plants can form rather smooth spheres of 2-3 mm diameter, through which no convection is possible Tijhuis, 1994). Their smooth appearance may be caused by erosion due to continuous collisions between the particles during their development. This leads to the intriguing question that remains to be answered: what is the nature of the morphogenetic factors that cause heterogeneous biofilm architecture. Further research must be done to answer this, it may supply powerful tools for biofilm control.

The actual biofilm structure is complex and seems well designed. The increased biofilm roughness may lead to increased resistance to flow and increased turbulence. The architecture seems specially designed for exchange of substrates and products with the environment. The increased surface area and liquid flow through the biofilm allows efficient substrate transport and high microbial activity. The cell cluster microstructure warrants the maintenance of the integrity without compromising the diffusion of substrate and products. The poresize of the cell cluster matrix restricts the mobility of large molecules and cells, but allows unimpeded diffusion of substrates and products. Our data undermine the conventional conceptual model of biofilm structure, used for mathematical modelling of biofilm activity and growth. Biofilms are not flat homogeneous layers of cells, in which transport is exclusively diffusional. The transport of metabolites involves both convection and diffusion, with a relative importance depending on the hydraulic regime. Future biofilm modelling attempts should be preceded by a thorough check of the assumptions.

The research was conducted at the Center for Biofilm Engineering, Bozeman, USA.

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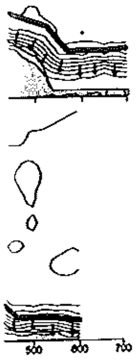

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