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Tracer measurements reveal experimental evidence of biofilm consolidation

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Running title: experimental evidence of biofilm consolidation

Summary

The ability to simultaneously measure both biofilm thickness and the mass transfer coefficient of an inert tracer through it provides a powerful method to study biofilm development. In this communication previously published data has been collated to interpret global trends in biofilm structure during the transition towards steady-state. It appears that sudden changes in biofilm structure (directly related to the rate of change of biofilm mass transfer resistance) may occur following transitions in rate of biomass production. These observations are consistent with the concept of consolidation, recently introduced into spatially structured biofilm mathematical models to account for structural realignment of the biofilm under dynamic conditions.

Keywords: biofilm; tracer; consolidation; mass-transfer

INTRODUCTION

Biofilm structure is determined by the spatial distribution of biomass components and must be considered a dynamic system under the influence of both physiological and physical mechanisms. A hypothesis postulated by van Loosdrecht *et al.* (1995 and 1997) and verified by Kwok et al (1998), that biofilm structure is determined by a balance between substrate surface loading (proportional to biomass surface production rate) and detachment force is now generally accepted and forms the basis for the assumptions in spatially structured mathematical models (Picioreanu et al, 1998; Kreft et al, 2001; Laspidou and Rittman, 2004). Impressive advances have been made in the application of these simulation techniques to the study of the interaction between biofilm and detachment (Picioreanu et al, 2001), hydrodynamics (Eberl et al, 2000) and population dynamics in multispecies biofilms (Picioreanu et al, 2004).

An emerging aspect in the area of biofilm simulation is the concept of consolidation which refers to possible structural realignment of the biofilm under dynamic conditions (Laspidou, 2004a). It has been suggested that, under certain conditions, for example following diminished biomass production or following biomass decay, hydrodynamic pressures forces cause the biofilm to undergo a structural realignment to form a higher density, lower porosity biofilm. Simulation studies by Laspidou (2004b), in which consolidation was explicitly modelled showed that when substrate loading was lowered the subsequent decrease in biofilm growth gave the biofilm more time to consolidate to an overall higher density. Simulation results from Xavier et al (2005) in which consolidation was not explicitly assumed suggested that consolidation may be an emergent property of developing biofilm under certain conditions; specifically, it was observed that total biomass increased while the biomass thickness remained constant. This was attributed to a reduction in porosity adjacent to the substratum associated with the filling of the void spaces by inert mass produced in the upper, more active region of the biofilm. More recently, Alpkvist et al (2006) included EPS decay in hybrid-continuum simulations to show the time dependent effect of consolidation.

If biofilm consolidation is defined as a temporal or permanent increase in biofilm density, whether uniform or localised, there are a number of possible contributory mechanisms;

(i) *Biomass production diverted to filling interstitial regions in the biofilm porous structure rather than to overall thickness increase*. This mechanism may be associated with transient events in the biofilm development process such as changes in the specific microbial growth rate or changes in detachment rate and is likely to have a timescale in the same order as specific growth rate, typically hours. A number of previous studies report data consistent with this mechanism. For example, Bakke et al al (1990) reported on the study of a monoculture biofilm of *P aeruginosa* where the

biofilm thickness reached a steady state within 24h to 48h, whereas the measured density continues to increase substantially beyond this time. Pereira et al 2002b reported an increasing trend in specific respiratory activity for laminar flow with a *P fluorescens* biofilm after a steady biofilm thickness had been attained.

(ii) *The accumulation of inerts in the biofilm which may accumulate predominantly adjacent to the substratum generating a density gradient*. This mechanism would have a rate comparable to that of cell endogenous processes, typically several hours or days. Several studies have been undertaken on the investigation of biofilm density gradients (Zhang and Bishop, 1994, Beyenal and Lewandowski, 2000). Zhang and Bishop (2001) extended this work to the investigation of gradients of EPS, phospholipids and density in 11 week old biofilms and determined that the deeper, more dense, region of the biofilm contained more nonviable cells and less EPS, these findings suggested that the density gradient could be attributable to the accumulation of inert material. This is consistent with other work (Matson and Characklis, 1976, Horn and Hempel 1997, Telgmann, 2004.) showing a decreasing (or at best steady) trend in substrate diffusion coefficients with increasing biomass age.

(iii) *Compaction of the biofilm due to an increase in fluid shear*. Ohl et al (2004) investigated the effect of varying fluid velocity on the density of biofilms grown for up to 80d. There was a strong relationship between the imposed velocity and measured density, an expected result based on prior reports (Vieira, 1993, Pereira et al, 2002a). However, the response time of the biofilm to increased or decreased velocities revealed insights into the consolidation process. After increases in Reynolds number biofilm density increased immediately, presumably due to the pressure induced compaction of the biofilm. However in a separate experiment where the

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Reynolds number was decreased after 30d, the biofilm density did not immediately

decrease, this was attributed to the earlier (cell lysis induced) consolidation process in

deeper layers of the biofilm preventing a quick transition to a lower density biofilm.

The objective of this short communication is to analyse collated data from published studies that

employed a particularly useful biofilm investigative methodology, the membrane attached

biofilm reactor, to interpret results in the context of current hypotheses on the development of

biofilm structure including consolidation. The cultivation biofilms on permeable membranes is a

useful approach since these experiments have the unique potential to provide useful

continuous/online data on biofilm structure if the mass transfer rate of an inert tracer through the

biofilm is continuously monitored without either affecting hydrodynamic conditions or requiring

inactivation ore removal of the biofilm (Zhang et al 1998; Casey et al, 2000a). If, in addition, the

biofilm thickness is measured, the dynamics of biofilm structural development can be assessed

quantitatively. A few studies are reported where all these parameters are measured over the full

course of biofilm development and in this communication the data is analysed in the context of

possible consolidation mechanisms.

Methods

Although experimental measurements of solute diffusion biofilms are commonly reported (for a

review see Stewart, 1998), for the purposes of the present study data was drawn only from

sources in which both biofilm thickness and the mass transfer rate of an inert tracer through the

biofilm were measured throughout the biofilm development. Furthermore, only experiments

where the biofilm reached or closely approached a steady-state thickness were included. It was

also necessary to exclude studies where the biofilm reportedly reached a steady state, but where

thickness was not recorded throughout the experiment (for example Lopez et al, 2003). Table I

compiles the pertinent details from each of the six selected experiments. In all experiments the substrate loading rate and the applied shear stress were maintained constant through each experiment. Biofilm thickness development for the six cases is shown in Figure 1. Cases 1, 3 and 4 and 6 show clear steady-state thicknesses, whereas cases 2 and 5 seem to be in close proximity to a steady state due to the substantial deceleration in average thickness. No trend in the final values of the thickness is inferred due to the variable growth and detachment conditions in each case.

The method described by Zhang *et al.* (1998), uses an inert tracer to elucidate the overall mass transfer coefficient (OMTC) of in a developing biofilm. It is assumed that the tracer does not in any way influence microbial physiology during the measurement. The measured OMTC can be subdivided in a resistances-in-series model to determine the individual mass transfer coefficients in the membrane, biofilm and boundary layer. In all cases, the overall mass transfer coefficient is composed of three resistances in series: membrane(k_M), biofilm(k_B) and hydrodynamic boundary layer at the biofilm liquid interface(k_L). In general k_M and k_L remain constant throughout an experiment, while k_B decreases as the biofilm develops. Although variation in k_L is possible due to the changing structure of the biofilm (Zhang et al, 1998), the dominant resistance at the biofilm steady state is the biofilm itself, and hydrodynamics plays an insignificant role in overall mass transfer resistance once the biofilm has developed beyond an arbitrary value of 50 μm.

Equation 1 describes the components of the OMTC for the tracer. The term representing the biofilm resistance is of particular interest, because together with biofilm thickness, the average effective diffusion coefficient can be determined.

$$\frac{1}{k_O} = \frac{r_M \, ln\left(\dfrac{r_i}{r_M}\right)}{P_{M,t} H_t} + \frac{r_M \, ln\left(\dfrac{r_i + \delta}{r_i}\right)}{D_{e,t}} + \frac{r_M}{(r_i + \delta) k_{L,t}} \qquad (1)$$

Eq. 1 can be rearranged to give:

$$\gamma = \frac{\varepsilon}{D_{e,t}} + \frac{1}{k_{L,t}}$$ (2)

Where

$$\gamma = \left(\frac{1}{k_O} - \frac{r_M \ln\left(\frac{r_i}{r_M}\right)}{P_{M,t}H_t} \right) \frac{(r_i + \delta)}{r_M}$$ (3)

and

$$\varepsilon = (r_i + \delta) \ln\left(\frac{r_i + \delta}{r_i}\right)$$ (4)

Linear regression using the variables γ and ε determines the effective diffusion coefficient and the mass transfer coefficient of the tracer in the boundary layer at the biofilm liquid interface. The studies reported by Zhang et al (1998) and Casey (2000a and b) used tubular membranes, cylindrical coordinates were used and the mass transfer coefficients were defined with respect to either the inner or outer tubular area. In this communication, in the interests of clarity, only the biofilm component of the OMTC ($1/k_B$) is plotted against ε.

Results and Discussion

In the analysis of the mass transfer measurements from membrane-attached biofilm experiments it is important to highlight the unique characteristics of such systems with respect to the indirect role of biofilm thickness on reaction rate; the region of biofilm growth is not necessarily located adjacent to the biofilm-liquid interface and stratified regions of the biofilm which are depleted of one or more substrates act as diffusional resistances to other substrates. Experimental verification of this concept was demonstrated by Cole et al, 2004 and La Para et al, 2005. It is not uncommon

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to encounter a situation where progressive increases in thickness result in dual limitation by co-limiting substrates supplied form opposite sides of the biofilm (Debus and Wanner, 1992; Casey et al 1999; Nicolella and Livingston, 2000; Semmens and Essila, 2001). Eventually, a critical biofilm thickness is reached, at which point the concentration one of the limiting substrates declines to a value approaching the Monod constant and a rapid decline in reaction rate and biofilm growth is observed. Figure 2 summarises the available data from cases 1 to 4; reaction rate data was not available for cases 5 and 6. It is clear that progressive increases in biofilm thicknesses follow a trend of diminishing substrate utilisation rate under constant loading conditions. However, cases 1 and 2 show a dramatic decrease in reaction rate as the biofilm thickness approaches its steady-state value, the transitions are shown as dashed lines. In these two cases it seems that the biofilm thickness reached a level where severe diffusional limitation of the carbon substrate (acetate) results.

Figure 3 plots the biofilm mass transfer resistance ($1/k_B$) in each case against ε, the linearised biofilm thickness. As would be expected, the mass transfer resistance increases with biofilm thickness. For a given biofilm thickness the value of k_B is dependent on the mass transfer characteristics of each biofilm, which is in turn dependent on the structural properties of the biofilm and the hydrodynamic conditions. It is possible that the heterogeneous structure of biofilms facilitates mass transport by convection as well as by diffusion into the biofilm pores (Rasmussen and Lewandowski, 1998). It is clear that a general linear relationship exists between ε and $1/k_{B,i}$, suggesting that the biofilm structure remains reasonably consistent during the accumulation of biofilm. However, in some experiments (cases 1, 2 and 5), at a point when the biofilm thickness approaches its steady-state it appears that the trajectory in the data plotted in Figure 3 increases, corresponding to a decrease in the biofilm effective diffusion coefficient,

inflection points are annotated on figures 2 and 3 accordingly. Based on this data, it appears that the structural transition occurs approximately at the point where severe substrate limitation is first manifested and it is postulated that this result is consistent with the concept of consolidation. The most likely explanation for this effect is that when the reaction rate declines sharply (cases 1 and 2), the internal pressure created by biomass production (the net force being outward) subsides and hydrodynamic pressures forces (net force inward) gradually force the biofilm to structurally realign. This transition may impact on the biofilm porosity or on the average thickness or both. In the experiments examined here, it seems that porosity is decreased and the rate of biofilm accumulation decreases. These results are in accordance with the investigations of Wasche et al (2002) and Ohl et al, (2004) who have shown that biofilm density increased with increasing flow velocity and decreasing substrate supply. Overall, it appears that transitions in biofilm density (assumed to be proportional to the overall mass transfer resistance) are determined by the interrelationship between hydrodynamics and the rate of biomass production.

Conclusions

From the experimental data compiled for this study, it is clear that in the mass transfer resistance of the biofilm follows a linear relationship with thickness, at least during the initial stages of biofilm development. However, it is apparent that dramatic changes in biofilm structure (directly related to the rate of change of biofilm mass transfer resistance) may occur under certain conditions. Based on the limited available data it has been shown that there may be a link between major transitions in rate of biomass production rate (directly related to the reaction rate of a limiting substrate) and biofilm structure. Although further experimental investigation is required, the observations reported in this communication are consistent with the concept of consolidation, recently introduced into spatially structured biofilm mathematical models.

The cultivation of biofilms on permeable membranes, though not a new technique, provides a powerful method in the study biofilm development when used in conjunction with an inert tracer. Although the data compiled for this communication was sourced from experiments where dual limitation from substrates supplied from opposite sides of the biofilm was possible, the technique is not restricted to such configurations and can easily be applied investigations where the membrane exists solely for the tracer measurements. In the application of the technique developed by Zhang et al (1998) it is recommended that any inflection points observed in the plots of biofilm mass transfer resistance against thickness are interpreted in conjunction with measurements of the rate of biomass production or a related variable.

Notation

De diffusion coefficient in biofilm, $m^2 s^{-1}$

H Henry's law constant, $Pa\ m^3\ mol^{-1}$

k_L mass transfer coefficient at biofilm–liquid interface, ms^{-1}

k_O overall mass transfer coefficient, ms^{-1}

P_M membrane permeability, $mol\ m^{-1} s^{-1} Pa^{-1}$

r_i inner radius of membrane, m

r_M outer radius of membrane, m

δ biofilm thickness, m

ε parameter defined in Eq. (4)

γ parameter defined in Eq. (2)

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Figure Captions

Figure 1. Biofilm thickness development plotted against elapsed time for Case 1 (◯), Case 2 (▢), Case 3 (△), Case 4 (▽), Case 5 (◇), Case 6 (●).

Figure 2. Measured acetate removal rates plotted against biofilm thickness for Case 1 (◯), Case 2 (▢), Case 3 (△), Case 4 (▽). Reaction rate data was not available for cases 5 and 6. Vertical dashed lines indicate transitions from dual limitation to acetate limitation as a result of diffusional limitation through an increasing layer of oxygen depleted biofilm

Figure 3. Biomass mass transfer resistances plotted against linearised biofilm thicknesses for Cases 1 to 6.

Table I Summary experimental details of each case

Case	Reference	Conditions	Organism/Substrate	Tracer
1	Casey et al (2000a)	Single tube MABR at 12.5 kPa intra-membrane pressure and 2 cm/s liquid flow velocity	*V natriegens* with acetate as sole carbon source	Nitrogen
2	Casey et al (2000a)	Single tube MABR at 12.5 kPa intra-membrane pressure and 6 cm/s liquid flow velocity	*V natriegens* with acetate as sole carbon source	Nitrogen
3	Casey et al (2000a)	Single tube MABR at 12.5 kPa intra-membrane pressure and 12 cm/s liquid flow velocity	*V natriegens* with acetate as sole carbon source	Nitrogen
4	Casey et al (2000b)	Single tube MABR at 50 kPa intra-membrane pressure and 6 cm/s liquid flow velocity	*V natriegens* with acetate as sole carbon source	Nitrogen
5	Zhang et al, (1998)	Single tube EMB	*Pseudomonas* JS150 with monochlorobenzene	TCE
6	Splendiani et al, (2006)	Single tube EMB	*Burkholderia sp* JS150 with monochlorobenzene	TCE

case 3