Cohesion and detachment in biofilm systems for different electron acceptor and donors

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Abstract This work deals with the cohesion and detachment in biofilm systems for two electron acceptors and for two electron donors. Biofilms were developed on plates, under very low shear stress for one month and then subjected to an erosion test for two hours in a Couette-Taylor reactor. Biofilm was characterised in terms of average thickness and residual TOC mass. It was found that the biofilm structure is very heterogeneous and stratified. The top layer, which represents 60% of the biofilm mass, is very fragile and can be easily detached; the basal layer, which represents 20% of the biofilm mass, is very cohesive and can resist shear stresses up to 13 Pa. Between these two layers, a middle layer of intermediary cohesion represents 20% of the initial biofilm mass.

Keywords Biofilm; cohesion; detachment; hydrodynamics; shear stress

Introduction

In a large range of ecosystems microorganisms grow in the presence of surfaces, leading to the formation of biofilms. By corroding pipes, reducing heat transfer, contaminating drinking water or causing rejection of medical implants, biofilms can affect production quality or constitute a risk for human health. Conversely, biofilms represent an interesting potential for pollution treatment, especially in the case of wastewater. Consequently, among the wide range of biofilm applications and in order to control their development better, biofilms are still the subject of much research.

According to van Loosdrecht et al. (1995), biofilm structure and matrix composition are influenced by two main factors which are the hydrodynamics and the environmental conditions (loading rate, carbon source). Nevertheless, as these aspects are not always controlled, the comparison between results of different works is sometimes difficult to achieve properly. Hydrodynamics plays an important role in terms of transport of substrate and mechanical stress. Many studies (Vieira et al., 1993; Choi and Morgenroth, 2003; Horn et al., 2003) have dealt with the influence of shear stress on biofilms. The main conclusion is that an increase of shear stress leads to an increase of biofilm density. In addition, when the hydrodynamic force exceeds biofilm cohesion strength, detachment phenomena occur. Also, in industrial plants, a sudden increase of shear forces is generally used for removing an excess of biofilm from the system.

The relationship between environmental conditions and biofilm structure (especially biofilm heterogeneity) has been little studied. Recently, new observation methods (confocal scanning laser microscopy, computerised image analysis, etc.) have been used to analyse the complex nature of biofilm structure. Bishop and Rittman (1995) defined biofilm heterogeneity as spatial differences of any important parameter. These authors provided a few examples of biofilm heterogeneity: microbial diversity of species, diversity of substrates, variable presence of solids and physical properties of biofilms.
(thickness, surface roughness, biofilm density, porosity, viscosity). Measuring the diffusion coefficients within a biofilm, Beyenal and Lewandowski (2000) proposed the concept of stratified biofilms in terms of multilayered biofilms. In a further study (Beyenal and Lewandowski, 2005), they assessed this hypothesis using a mathematical model based on a cellular automata. Using the same type of modelling, Laspidou and Rittman (2004) showed that the stratification of a biofilm is directly linked to the matrix composition. The former is mainly made of extracellular polymeric substances (EPS) produced and released from the bacteria. In their numerical study, Laspidou and Rittman (2004) found that the top layer of the biofilm is quite “fluffy” and dominated by active biomass and EPS, while the bottom is dense and dominated by residual inert biomass. Xavier et al. (2005) used a representation of biomass that discriminates between active mass, inert biomass and EPS. Simulating the biofilm growth in an oxygen- and a substrate-limited regimen, they showed that the inert fraction increased towards the interior of the biofilm. In the oxygen-limited regimen, they reported that the outer regions were characterised by a very high ratio of active biomass and EPS. In the case of the oxygen-limited regimen and of high EPS production rate, cell density was very low at the biofilm bottom (Kreft and Wimpenny, 2001).

To study the link between biofilm cohesion and environmental conditions, it is necessary to use reactors in which the hydrodynamic conditions (especially the shear stress) are controlled and have already been studied. To that end, biofilms are developed in a low shear stress reactor until steady state and then are submitted to an erosion test in a Taylor-Couette reactor. Various environmental conditions are tested: two electron acceptors (oxygen and nitrate) and two electron donors (filtered wastewater and ethanol). The filtered wastewater is a complex substrate and consists mainly of colloidal matter, whereas ethanol is a more easily biodegradable carbon source. These large differences in environmental conditions lead to diverse types of biofilms. The results are analysed in terms of total organic carbon mass and are discussed in terms of detached mass and cohesion.

**Materials and methods**

**Experimental setups**

Two pilot plants were used: a low shear stress reactor (LSSR) for the biofilm growth and a modified Couette-Taylor reactor (CTR) for the erosion test. The LSSR has a volume of 15 L. It consists of two perpendicular axes mounted on a rotating shaft and supporting plates on which biofilm grows (Figure 1a). To guarantee a low hydrodynamic stress during biofilm growth, a very low rotation speed ($N = 4.5$ rpm) was applied, leading to a shear stress of approximately 0.1 Pa. Temperature was kept constant at 20°C. In the case of the aerobic experiments, a recirculation loop in which air is injected provided oxygen in the bulk liquid.
at a concentration of 3–4 mg/L. A peristaltic pump allowed nutrient injection. Details of reactor systems have been described previously (Ochoa et al., submitted).

After one month of growth in the LSSR, an erosion test was realised in the CTR (Figure 1(b)). The CTR is composed of a pair of concentric cylinders, the inner one rotating and the outer being fixed. The inner cylinder has a radius Ri of 125 mm and the outer has a radius Re of 150 mm. After one month of growth in the LSSR, colonised plates with mature biofilms were fixed on the external cylinder wall. Thus, the presence of the plate locally reduced the gap between the inner and the outer cylinder (gap of respectively 23 or 17 mm for clean plates of 2 or 8 mm thick).

Plates
Smooth rectangular plastic plates of polyethylene were used as a support for the biofilm (L × H: 50 × 100 mm). The plate thickness was either 2 or 8 mm. Plates were characterised by a rather smooth surface (roughness less than 1% before colonisation). Plates were divided into five segments of 10 mm width to allow a local measure of residual biofilm mass as shown in Figure 1(c). To avoid biofilm formation on the back of the plate, on the upper part and on the leading edge, an adhesive band covered them during experiments and was removed before biofilm analysis. The total surface available for biofilm formation was 45 cm² for each plate.

Operating growth conditions
Two nutrient solutions were used in oxic or anoxic conditions: ethanol and a more complex one which is a filtered domestic wastewater (WW). Wastewater mean characteristics were the following: total chemical oxygen demand: 200 ± 30 mg COD.L⁻¹, total Kjeldahl nitrogen: 60 ± 10 mgN.L⁻¹, suspended solids: 80 ± 20 mgSS.L⁻¹. To avoid biofilm abrasion by large suspended particles, wastewater supply was filtered at 75 μm, before being used to feed the reactors. Easily biodegradable organic matter concentration during biofilm formation was crucial in mass accumulation. This easily biodegradable COD consisted of relatively simple molecules that may be consumed directly by heterotrophic bacteria and used for growth of biomass. In the urban wastewater used in this work, this fraction was low and variable (Spérandio et al., 2000). For anoxic growth, nitrate concentration was kept constant at 20 mgN.L⁻¹ (COD/N: 9–10) and oxygen concentration was kept lower than 0.1 mgO₂.L⁻¹ by nitrogen gassing in the airtight Couette-Taylor reactor. For aerobic growth, dissolved oxygen concentration was kept in a range of 3–4 mgO₂.L⁻¹. To prevent unwanted biofilm formation (for example on the internal and external cylinders), the reactor surfaces and tubes were mechanically and chemically cleaned twice a week.

Biofilm thickness steady state was reached after 15 days but plates were sampled only after 30 days of operation. Under controlled hydrodynamic conditions, biofilm growth is strongly related to the substrate concentration of the bulk liquid. Consequently, substrate-loading rate was kept constant during each experiment at 4 gCOD.m⁻²d⁻¹.

In steady-state conditions, the easily biodegradable fraction was rapidly degraded and, consequently, COD concentrations were low (<50 mg COD.L⁻¹).

Hydrodynamics
The use of CTR allowed the control of hydrodynamics both by the rotation speed of the inner cylinder and by the gap between the concentric cylinders. Local characterisation of the flow generated by the presence of the plates was performed by both experimental and numerical studies. These experiments were achieved before biofilm growth. Numerical simulations were performed on a two-dimensional domain with Fluent® code (Fluent 6.1). The shear stress distribution on the plate was thus calculated. By using plates of...
different thicknesses (2 and 8 mm), one can obtain shear stresses varying from 0.2 to 13 Pa. Detailed information about the hydrodynamic study can be found in Ochoa et al. (submitted).

**Erosion test**

Erosion tests were performed after one month of growth. For the erosion tests, plates were sampled and transferred into the RTC. The erosion test consisted of submitting the biofilm for two hours to a constant rotational speed of the internal cylinder. Shear stresses were controlled both by the rotational speed of the inner cylinder \( N = 20, 60, 65, 180 \) and 190 rpm) and the size of the gap between the two cylinders. Finally, the plate was sampled and the TOC of the residual biofilm mass was measured.

**Biofilm characterisation**

Average biofilm thickness was measured using image analysis. Plates were sampled from the reactor, put in a dedicated box assembled on the lens of a microscope; then pictures of the biofilm (side view) were recorded using a high resolution photo camera (Olympus C7070) mounted on a microscope (Leica DMLB 100T). Two pictures of biofilm are recorded for each plate. Thickness was measured in different locations of the biofilm and then an average thickness was estimated using image analyser software.

Biological characterisation was realised by the measure of the mass of the TOC. A destructive method by alkaline solubilisation and TOC measurement was chosen. Biofilm was solubilised in a 1 N NaOH solution (pH 14) at 80°C for 45 minutes (Ochoa et al., 1999). Samples were homogenised and TOC was then measured using a TOCmeter (Dohrmann DC 180) as described by French Standards NFT 90-102 (Afnor, 1994).

In order to assess the polyethylene influence on the alkaline matrix in the final TOC value, a blank without biofilm was systematically performed for each experiment. A constant value of 0.04 ± 0.01 mgTOC/cm² was found and was subtracted from the final biofilm TOC value.

The evaluation of the overall technique repeatability was performed, using ten colonised plates for different biofilm TOC concentrations. For high concentrations, the averaged TOC was 0.55 mgTOC/cm² with a standard deviation of 7%; for the lowest concentrations, the averaged TOC was 0.075 mgTOC/cm² with a standard deviation of 27%.

**Results and discussion**

Biofilms were developed on plastic plates under a low shear stress for one month to reach steady-state conditions. The plates were then sampled and submitted to an erosion test in a Taylor-Couette reactor as described in the previous section.

**Biofilm characteristics before erosion test**

*Figure 2* shows pictures of biofilms grown for 30 days, under anoxic conditions in the low shear stress reactor.

As can be seen in *Figure 2*, whatever the environmental condition, biofilm surface was particularly rough. The roughness was more pronounced in the case of biofilms grown under anoxic conditions and with ethanol as the carbon source than with wastewater.

However, it should be noticed that the roughness concept is strongly related to the thickness. For biofilms grown under oxic conditions, pictures are not presented here but the effect of carbon source leads to the same comments concerning the roughness.

*Table 1* shows biofilm thicknesses and TOC mass at steady state (after 30 days in the low shear stress reactor).

Between 15 and 30 days, the biofilm thickness did not vary, which indicates that the steady state was reached. Biofilm average thickness increased up to 2,000 μm for biofilms...
developed in anoxic–ethanol conditions and remained less than 1,400 µm for other environments. For biofilms developed in oxic conditions, even if the final average thickness is quite different, they were characterised by a similar TOC₀ value of 0.55–0.6 mgTOC.cm⁻². For anoxic biofilms TOC₀ values were higher: 0.85 mgTOC.cm⁻² for anoxic–ethanol conditions and 0.76 mgTOC.cm⁻² for anoxic–WW conditions. The fact that biofilms grown under anoxic conditions are thicker than biofilms developed under aerobic conditions is in accordance with the work of Ohashi et al. (1999). Indeed, the authors measured a thickness of less than 1,000 µm for oxic biofilms whereas anoxic biofilms reached more than 2,000 µm. The accumulated thickness of the biofilm mass varies with the electron donor or acceptor and significant TOC masses were observed.

**Erosion test results**

As previously mentioned in the Material and methods section, during the erosion test, plates colonised by biofilm were submitted to different levels of shear stress ranging from 0.1 to 13 Pa. In Figure 3, pictures of a biofilm grown under anoxic conditions and with ethanol are presented before and after the erosion test.

As can be seen in Figure 3, only a slight amount of biofilm remained at the end of the erosion test. The average thickness of the initial biofilm was 2,000 µm whereas after the erosion test the average thickness of the residual biofilm was measured to approximately 200 µm.

For each environmental condition, the residual biofilm mass was plotted versus the shear stress. Figure 4 shows the resulting profiles for oxic conditions.

Three different layers were highlighted in this graph of the evolution of the residual biofilm mass versus the shear stress. The first one corresponded to low shear stresses (between 0.1 and 0.3 Pa), the second one corresponded to shear stresses (τ_p) between 0.3 and 2 Pa and the last one corresponded to shear stresses higher than 2 Pa.

For shear stresses ranging from 0.1 to 0.3 Pa, the residual biofilm mass decreased strongly and sharply from 0.55 to 0.28 mgTOC.cm⁻². A surface detachment rate corresponding to the slope of the curve, for shear stresses between 0.1 and 0.3 Pa, was defined. The surface detachment rate in this zone was 2.2 mgTOC.cm⁻².Pa⁻¹. For shear stresses ranging from 0.3 to 2 Pa, the residual biofilm mass decreases again, but less

**Table 1** Biofilm TOC mass and thickness after 30 days in the inoculum reactor

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<tr>
<th></th>
<th>Oxic conditions</th>
<th>Anoxic conditions</th>
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<tbody>
<tr>
<td></td>
<td>WW</td>
<td>Eth</td>
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<tr>
<td>TOC₀ (mg.cm⁻²)</td>
<td>0.55±0.06</td>
<td>0.6±0.06</td>
</tr>
<tr>
<td>Average thickness (µm)</td>
<td>1,000±150</td>
<td>1,400±200</td>
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strongly from 0.28 to 0.1 mgTOC.cm\(^{-2}\) (giving a surface detachment rate of approximately 0.09 mgTOC.cm\(^{-2}\).Pa\(^{-1}\)). For shear stresses higher than 2 Pa, TOC values were stable and close to the limit of quantification.

A similar profile of the relationship between the residual biofilm mass and the shear stress was obtained using ethanol as carbon source and under oxic conditions (Figure 4(b)). It was still characterised by three layers: a first layer characterised by a strong and sharp decrease of the residual biofilm mass as soon as a shear stress, even low, is applied. Then a second layer characterised by a slower decrease and, finally, a third layer characterised by a nearly constant residual biofilm mass value that did not detach. In that case, the residual biofilm mass remaining under applied \(\tau_p\) ranging from 1.8 to 8.5 Pa is higher than the limit of quantification. The base of the biofilm (near the support) could thus resist a high shear stress of at least 9 Pa.

Figure 5 presents the results for biofilms grown under anoxic conditions using wastewater (Figure 5(a)) or ethanol (Figure 5(b)) as the electron donor. Again, for both substrate conditions, there was a strong and sharp decrease of the residual biofilm mass as soon as a slight increase of the shear stress was applied, followed by a moderate decrease of the residual biofilm mass as \(\tau_p\) was increased up to 2.5 Pa. For \(\tau_p\) ranging from 2.5 to 13 Pa, the residual biofilm mass remained constant.

The previous results show that whatever the environmental conditions (in terms of electron donors or electrons acceptors), similar behaviours are highlighted. All these previous data can be gathered together in the same graph as shown in Figure 6(a).

Before the erosion test, the TOC biofilm mass accumulated was always larger for biofilms grown under anoxic conditions than for biofilms grown under oxic conditions.
After primary and secondary detachment, this trend was always observed. In the same way, the TOC biofilm mass accumulated was always larger for biofilms grown with ethanol as carbon source than with wastewater, before and after erosion tests (Figure 6(c)).

According to the previous observations, the strong and sharp decrease of the residual biofilm mass found for a small increase of the shear stress was related to a primary detachment. Whatever the electron donor or acceptor, the quantity of detached biofilm is quite important and corresponds to a detached mass fraction of approximately 60% of the initial biofilm mass, as shown in Figure 6(b). For intermediate shear stress values (between 0.3 and approximately 2 Pa), the detachment was less pronounced than the previous one, observed for \( \tau_p \) smaller than 0.3 Pa. This secondary detachment was related to a loss of 20% of the initial biofilm mass. For shear stresses larger than 2 Pa, the detachment phenomena were low or almost null, leading to a constant residual biofilm mass that represented approximately 20% of the initial biofilm mass.

Concerning the biofilm structure, and thus the biofilm cohesion, the top layer, closest to the bulk liquid, is the most fragile since an important mass fraction detached under a low shear stress. Conversely, the bottom layer is very cohesive since it can resist relatively high values of the shear stress (up to 13 Pa for the biofilms obtained in the anoxic conditions). Between the two layers, an intermediary layer can be identified. This layer appears to be more resistant than the top layer but weaker than the bottom layer.

A general biofilm behaviour, relative to erosion tests, was found. Indeed, detached mass fractions are quite constant whatever the type of biofilm. Nonetheless, one must keep in mind that, firstly, the resulting biofilms are different: the initial TOC mass and the thickness are related to the electron donor and acceptor nature (Figure 6(c)). Moreover, the detached TOC mass is significant and proportional to the initial biofilm state.
Secondly, it should be noticed that even if biofilms behave in the same way during erosion tests in terms of detached mass fraction, the involved detachment mechanisms are unknown and probably different.

**Conclusion**

The aim of our study was to understand better the link between biofilm cohesion and environmental conditions. To that end, biofilms were developed under low shear stress and were then submitted to an erosion test in a Taylor-Couette reactor. Different environmental growth conditions were tested by changing the electron acceptor and donor. Whatever the environmental conditions, the biofilms appeared to have a stratified structure. This structure could be divided into three layers that were characterised by different surface detachment rates. The top layer has a high detachment rate, whereas the bottom layer has an almost zero detachment rate. In addition, it was found that the cohesion of the layers increases with depth. Indeed, the bottom layer is very cohesive and can resist shear stress up to 13 Pa. Finally, even if the biofilms were of variable nature and the mechanisms involved were probably different, it appeared that all the biofilms behave in the same way in terms of detached mass fraction.

**References**


