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Influence of shear stress, organic loading rates and HRT on the biofilm structure and on the competition between different biological aggregate morphotypes

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Abstract

This study aimed at understanding how growth conditions influence the biofilm structural properties and the competition between microbial aggregates (biofilms, streamers, suspended biomass). The biological aggregates were developed during more than one month under contrasted conditions in terms of shear stress, hydraulic retention time (HRT), apparent surface organic loading rate (SOLR) and COD/N ratio (fourteen conditions). At high HRT (absence of streamers) an increase of shear stress led to a decrease of the average biofilm thickness whereas it increased with the apparent SOLR. In addition, biofilm density increased with shear stress and with the apparent SOLR at high shear stress. The presence of streamers was observed only at low HRT (< 3h). This presence has been attributed to the residual concentration in the bulk and to the hydrodynamic conditions of growth. Turbulent conditions and low substrate concentration considerably favor the development of streamers which benefit from a higher external mass transfer compared to biofilms due to their flapping movement. In some

conditions, the mass of streamers in the reactor were close to the biofilm one's, highlighting the necessity to consider such morphotypes in further studies since they can have a significant impact on the global microbial activity.

Key-words: Biofilm structure; Streamers; Microbial competition; Mass transfer; Detachment.

Highlights: ► Biofilm structure depends on shear stress (τ_p) and organic loading rates (SOLR)
► The influence of SOLR on biofilm thickness and density depend on the τ_p applied ► An increase of τ_p leads to a decrease of thickness and an increase of density ► Competition between aggregate morphotypes is studied considering mass transfer and detachment ► Streamers presence was attributed to the low residual concentration in the bulk.

1 Introduction

In nature, in biological wastewater treatment plant (WWTP) or in industrial water pipes, most of the microorganisms are present in the form of microbial aggregates. Microbial aggregates (biofilms, flocs, etc.) are natural structures composed by microbial communities held together by a self-secreted matrix composed by extracellular polymeric substances (EPS) (Flemming et al., 2016; Flemming and Wingender, 2010; Seviour et al., 2019; Sheng et al., 2010). Intensive research in the last decades has revealed a wide variety in aggregate structures depending on the hydrodynamic, nutritional, and hydraulic conditions (Paul et al., 2012; Scheidweiler et al., 2019; Stoodley et al., 1998; van Loosdrecht et al., 1995). The physical and morphological properties of biological aggregates strongly influence the stability and performances of reactors used in WWTP (Aqeel et al., 2019; Filali et al., 2012; Laurenzi et al., 2019; Telgmann et al., 2004; van Loosdrecht et al., 2002) and the efficiency of industrial biofilm removal strategies (Gomes et al., 2021; Pechaud et al., 2012; Simões et al., 2010). These contrasted aggregates properties are associated to the “co-evolutionary” relationship between biological aggregates and their surrounding environment. Microbiological aggregates evolve in response to the physical and chemical properties of their environment and simultaneously change this environment by modifying its chemical gradients, local hydrodynamics, etc (Scheidweiler et al., 2019; Stoodley et al., 1998). Currently a main challenge is to predict how operational conditions influence biological aggregates properties and competition between morphotypes.

Biofilm physical and morphological properties are influenced by two main factors: the growth conditions (surface organic loading rates (SOLR), carbon sources, etc.) and the hydrodynamics (Aqeel et al., 2019; Catão et al., 2019; Paul et al., 2012; Pechaud et al., 2021; Rochex et al., 2008; Stoodley et al., 1998). The apparent SOLR controls the substrate flux available for bacteria and it now appears clear that biofilm thickness increases with SOLR

(Bassin et al., 2016; Garny et al., 2009; Kwok et al., 1998; Paul et al., 2012). In contrast, there is no consensus regarding the effect of the apparent SOLR on the density of biofilms (Characklis et al., 1982; Garny et al., 2009; Kwok et al., 1998; Wäsche et al., 2002; Wijeyekoon et al., 2004). Further experiences are thus required to bring a better knowledge about the influence of SOLR on biofilm structural properties. Moreover, a wide literature is available on the influence of hydrodynamic conditions on the physical and morphological structure of biofilms and planktonic aggregates ; high shear forces usually result in thinner, denser and stronger biofilms and flocs (Bassin et al., 2016; Chang et al., 2020; Coufort et al., 2008; Gerbersdorf et al., 2020; Lemos et al., 2015; Paul et al., 2012; Pechaud et al., 2021; Rochex et al., 2008; Stoodley et al., 1998; van Loosdrecht et al., 1995). However, only few data are available for shear stresses commonly encountered in industrial pipes and biofilm reactors (0.5; 10Pa) (Di Iaconi et al., 2005; Lelièvre et al., 2002; Nicoletta et al., 1996) and these results have a high variability. A first objective of the study was thus to better understand the link between SOLR, shear stress and biofilm physical properties in a large range of SOLR and shear stresses.

More recently, studies with fluidic devices have advanced our knowledge on the effect of flow on biofilm architecture (Coyte et al., 2017; Paul et al., 2012; Talvy et al., 2011; Wheeler et al., 2019; Yawata et al., 2016) and allowed to introduce a distinction between conventional biofilm (B) and streamers (S) (Ghosh et al., 2021; Scheidweiler et al., 2019; Stoodley et al., 1998; Taherzadeh et al., 2012). Streamers are structures consisting in a base attached to the support substratum and a tail elongated in the flow direction (Taherzadeh et al., 2012). These structures have been observed in microfluidic devices under turbulent flow (Stoodley et al., 1998), in secondary flows area (around corners) (Marty et al., 2012; Rusconi et al., 2011, 2010; Yawata et al., 2016) and in porous media under low Reynolds (Scheidweiler et al., 2019). If these studies highlighted the high architectural plasticity of biological aggregates, to date, the reason of the shift between biofilms and streamers still remains little understood and the

dynamics of streamers formation has been little studied (Ghosh et al., 2021; Scheidweiler et al., 2019; Taherzadeh et al., 2012). In addition, in biofilm systems, biofilms, streamers and flocs can co-exist but there is no study dealing with the competition between these three morphotypes. Thus a second objective of the present study was to better understand the reactor conditions that influence this competition and the dynamics of streamer formation and maturation.

We hypothesized that hydraulic retention time (HRT) can influence significantly this competition. HRT has been shown to have a significant impact on the competition between flocs and biofilm in biofilm systems (Aqeel et al., 2019; Cresson et al., 2008; di Biase et al., 2021; Laurenzi et al., 2019; Shao et al., 2017; Tijhuis et al., 1994; Wheeler et al., 2019). The HRT governs the dilution rate and thus the substrate concentration in the bulk liquid and influences the retention time of planktonic aggregates. The dilution rate of the system must be higher than the maximum specific growth rate (μ_{max}) of the microorganisms to wash out suspended bacteria and therefore enhance growth of fixed biomass. As HRT can influence the residual concentration in the bulk, it could have an effect on the competition between biofilms and streamers. Our hypothesis is that streamers, at low residual concentration in the bulk, could employ the viscoelastic material properties to their benefit by increasing their external mass transfer compared to biofilms.

The main purpose of this study was to evaluate how SOLR, hydrodynamic shear stress and HRT influence the physical properties of biofilms and the competition between biofilms, streamers and planktonic particles. More precisely, main objectives of the present study were: (i) to analyze the influence of the apparent SOLR and shear stress, over a wide range commonly encountered in biofilm systems, on the biofilm physical properties (in the absence of streamers), (ii) to better understand the reactor parameters that influence the competition between biofilms, planktonic particles and streamers and the underlying mechanisms and (iii) to analyze the

dynamics of streamers formation. The microbial aggregates were developed in a Couette-Taylor flow reactor under controlled hydrodynamic conditions during more than one month. Fourteen contrasted growth conditions in terms of shear stress, apparent SOLR, HRT and carbon source were compared. The physical properties of biofilms and streamers were monitored using image analysis. The analysis of the competition between biofilms, streamers and suspended biomass was based on the quantification of the organic mass of each morphotype category. Finally, the kinematic properties of streamers were monitored using image analysis from a fast camera acquisition.

2 Materials and Methods

2.1 Experimental scheme

2.1.1 Experimental setup

The reactors were inoculated with conventional activated sludge from a urban wastewater treatment plant facility of Toulouse vicinity (France). The microbial aggregates were grown in a Couette-Taylor flow reactor (Figure 1). The size of the gap between the inner and outer cylinders was 15 mm. Smooth rectangular plastic plates made of Polyethylene were used as substratum for the biofilms and streamers. The total surface available for attached biomass growth was 0.117 m² (26 plates). Plates were fixed to the reactor wall using screws. Every three days, reactor surfaces and tubing were mechanically cleaned to avoid biofilm growth outside of the plates; every fifteen days all the tubing was changed.

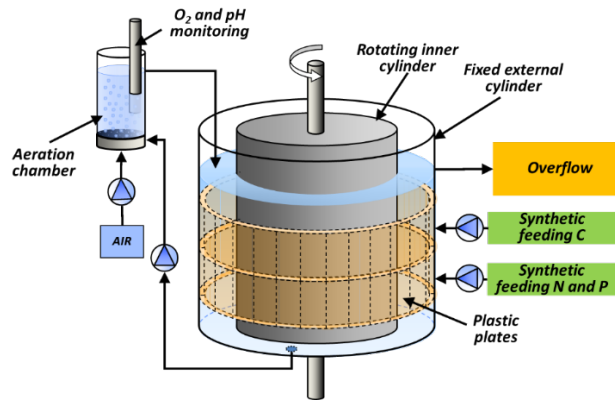


Figure 1 : Experimental setup: the reactor with Couette-Taylor hydrodynamic used for aggregates growth.

2.1.2 Growth conditions

Air was externally supplied by a recirculation loop to avoid the effect of bubbles on the aggregates and an oxygen concentration higher than $4 \text{ mgO}_2 \text{ l}^{-1}$ was maintained in the bulk liquid whatever the growth conditions. A COD/N ratio of 25 ± 5 was selected to limit the growth of autotrophic bacteria while providing enough nitrogen for heterotrophic growth. Phosphorus was in excess compared to growth requirements ($\text{COD/P}=30$). The nutrient solution contained $(\text{NH}_4)_2\text{SO}_4$ (200 mg l^{-1}), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (200 mg l^{-1}), KH_2PO_4 (67 mg l^{-1}), H_3BO_3 (0.06 mg l^{-1}), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.04 mg l^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 mg l^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.02 mg l^{-1}), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.02 mg l^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.02 mg l^{-1}), $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (0.02 mg l^{-1}), CaCl_2 (10 mg l^{-1}), Ammonium Iron Citrate (3.5 mg l^{-1}), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (30 mg l^{-1}).

The shear stress range tested has been selected to cover the wide range encountered in biofilm reactors and industrial pipes: (i) moving bed reactors [$0.01\text{-}1\text{ Pa}$] (Di Iaconi et al., 2005); fluidized bed reactors [$0.14\text{-}0.36 \text{ Pa}$] (Nicolella et al., 1996); annular reactors [$1.4\text{-}3 \text{ Pa}$] (Peyton, 1996); industrial pipes [$0.01\text{-}10 \text{ Pa}$] (Lelièvre et al., 2002). The shear stresses were controlled by the rotational speed of the inner cylinder (Derlon et al., 2013; Rochex et al., 2008).

In the shear stress range tested, Ta (Taylor number) was at least 40 times higher than Ta_{crit} (the critical Taylor number, minimum value for which vortices may be observed). For all the conditions tested, the flow regime was turbulent vortex flow (Coufort, 2004).

The HRT in biofilm systems are generally ranged between 2h and 24h (Calderón et al., 2012; Lariyah et al., 2016; Ødegaard, 2006). In order to have contrasted conditions commonly encountered in biofilm systems (MBBR, IFAS, etc.), HRTs of 3h and 20 h were selected in our study. In the same way the SOLR values were selected to study low SOLR ($<5 \text{ gCOD.m}^{-2}.\text{d}^{-1}$), moderate SOLR ($5\text{-}15 \text{ gCOD.m}^{-2}.\text{d}^{-1}$) and high SOLR ($>15 \text{ gCOD.m}^{-2}.\text{d}^{-1}$) encountered in biofilms systems ($1 \text{ and } 30 \text{ gCOD.m}^{-2}.\text{d}^{-1}$) (Bassin et al., 2016; Kwok et al., 1998; Li and Liu, 2019; Morgan-Sagastume, 2018; Ødegaard, 2006).

Table 1. Various conditions tested to evaluate the parameters (substrate load, shear stress, HRT, carbon source) influencing the biological morphotypes).

Case	A	B	C	D	E	F	G	H
Shear stress (Pa)		0.5			2		9	
Apparent SOLR (gCOD.m ⁻² .d ⁻¹)	2.5	4.5	25	4.5	8	25	4.5	25
Carbon sources				Acetate (50%) Ethanol (50%)				
HRT				20h				
Case	I	J	K	L		M		N
Shear stress (Pa)				1				
Apparent SOLR (gCOD.m ⁻² .d ⁻¹)	2.5	25	2.5	25		2.5		25
Carbon sources	Glucose (50%)	Glucose (50%)	Acetate (50%)	Acetate (50%)		Acetate (50%)		Acetate (50%) Ethanol (50%)

	Acetate (25%)	Acetate (25%)	Ethanol (50%)	Ethanol (50%)	Ethanol (50%)	
	Ethanol (25%)	Ethanol (25%)				
HRT	3	3	3	3	20	20

Fourteen growth conditions were tested. Experiments A to H were dedicated to study the influence of the apparent SOLR and shear stress on the structural properties of biofilm for a HRT value set at 20h. Experiments G to N allowed to study the influence of HRT, apparent SOLR and carbon source on the competition between the different biological morphotypes.

The monitoring of the biofilm physical properties, streamers and suspended biomass was performed once stable removal efficiencies (in terms of COD removal) and mean biofilm thickness were observed in the reactors (after more than 45 days of growth).

2.1.3 Growth characterization

The use of γ_{S,O_2} was introduced to evaluate the growth regime under aerobic conditions, as proposed by (Henze et al., 2001):

$$\gamma_{S,O_2} = (1 - Y_{OHO}) \frac{D_S}{D_{O_2}} \cdot \frac{S_{S,L_f}}{S_{O_2,L_f}} \quad (1)$$

Where D_S and D_{O_2} are respectively the diffusion coefficients in the biofilm of organic substrates and oxygen ($m\ s^{-2}$). S_{S,L_f} and S_{O_2,L_f} are the concentration of organic substrates and oxygen at the biofilm surface expressed respectively in $gCOD\ m^{-3}$ and $gO_2\ m^{-3}$. Y_{OHO} is the aerobic heterotrophic conversion yields expressed in $gCOD\ g^{-1}O_2$.

Soluble COD was daily measured in the inlet and in the outlet of the reactor (after filtration at 0.2 μm). In addition, N-NH_4^+ , N-NO_3^- and N-NO_2^- were monitored twice a week to check that nitrification did not occur. N-NH_4^+ concentration was measured using the Nessler method. N-NO_3^- and N-NO_2^- were measured by ionic chromatography (Dionex ICS-3000; pre-column Dionex AG11 and column IonPac AS11-HC; eluent KOH at 30°C; suppression system AERS 500 4 mm; Detection by conductivity). The COD was measured by oxidation with potassium dichromate using test tube reagent sets (Hanna Instruments).

2.2 Biomass characterization

2.2.1 Accumulated mass of the biological morphotypes

A distinction is introduced in the present paper between biofilm (B), streamers (S) and planktonic aggregates (P).

In brief we sampled biofilms and streamers using tweezers and needles. The quantification of the organic mass of biofilms, streamers and planktonic aggregates were carried out by measuring the COD or VSS measurements. The biofilms were scraped off and then introduced into a 50 ml tube. The surface A of each plate was 45 cm^2 . The accumulated mass $m_{VSS \text{ or } COD}$ was calculated as follow:

$$m_{VSS \text{ or } COD} (gCOD \text{ or } VSS.m^{-2}) = \frac{VSS (gVSS) \text{ or } COD (gCOD)}{A(\text{cm}^2)} \quad (2)$$

After rinsing the plate, the tweezer and needle, then collecting the rinsing water in a sample tube, this tube was completed to the gauge line by adding distilled water. Then, a sonication step was performed in order to homogenize the mixture (1 min, 500 W, 20 kHz) prior COD quantification. The use of this method has shown good repeatability with a standard deviation of less than 5%.

The mass of streamers was quantified using the same methods as for biofilms. The major experimental error when measuring the mass of streamers lied in the sampling of streamers. Indeed, it was difficult to know precisely the limit between the base of the streamer and the surrounding biofilm. However, since mature streamers were relatively long ($L_s > 20\text{mm}$), the error associated with their collection has been estimated to be less than 20% of their total mass.

The COD mass of planktonic aggregates (P) consisted in measuring the particulate COD concentration in the suspension and to multiply this concentration by the liquid volume in the reactor. The particulate COD was obtained from the subtraction of the total COD by the soluble COD (of the filtered ($0.2\ \mu\text{m}$) mixed liquor).

2.2.2 Physical properties of biofilm

Biofilm thickness was measured using the same protocol than in Paul et al., 2012 and Pechaud et al., 2012. Briefly, images of the biofilms sampled from the reactor were obtained using a high resolution video camera (Photron ultima APX $1024 \times 1024\ \text{p}^2$) mounted on a stereoscopic microscope ($100\ \text{pixel}.\text{mm}^{-1}$). For all images, the grey-level image was converted into a binary image using VISILOG[®] software. Image analysis led to the determination of the mean biofilm thickness of a colonized plate.

To determine biofilm density ρ_f , the biofilm was considered as a homogeneous structure with constant thickness (Wäsche et al., 2002):

$$\rho_f = \frac{m_{VSS\ or\ COD}}{L_f} \quad (3)$$

Where $m_{MS\ or\ COD}$ is the accumulated mass expressed in $\text{mgCOD}.\text{m}^{-2}$ or $\text{mgVSS}.\text{m}^{-2}$ and L_f is the mean biofilm thickness (m).

2.2.3 Physical properties and kinematic properties of streamers

The length (L_s) and diameter (d_s) of the streamers were measured from an image analysis of the streamers. Top view and side view images were taken to quantify the length of the streamers (resolution 35 pixels / mm). The mean diameter of the streamers was evaluated from several pictures taken at different angles (front, top and side) (resolution of 100 pixels / mm). From these measurements, the Finess ratio (L_s/d_s) was calculated.

The frequency and amplitude of oscillation were measured by a Photron Fastcam SA3 fast camera (Photron USA, Inc., San Diego, USA) with a field of 1024×1024 pixels² and equipped with a 60 mm Nikon lens. The camera was placed at a distance of 30 cm from the tank. Focusing was achieved using a graduated staff located at a distance of 10 cm from the wall with a resolution of 100 pixels mm⁻¹. The average oscillation frequency was evaluated from an acquisition of 10 s. The maximum oscillation frequency of the streamers measured being 30Hz, a number of 1000 images / s was used to obtain at least 30 images per period.

From frequency measurements, the dimensionless Strouhal number which characterizes the periodic flow around an object can be quantified (Taherzadeh et al., 2012; Vogel, 1996) and was calculated as follows:

$$St = \frac{f d_s}{U_{mean}} \quad (4)$$

Where f is the frequency of flow oscillations, d_s is the characteristic length (here the base diameter), and U_{mean} is the mean velocity of fluid passing the body (Fig SM-1). This mean velocity was estimated from Particle Image Velocimetry (PIV) measurements. For a shear stress close to 1 Pa (conditions I to N), U_{mean} was close to 0.4 m.s⁻¹ (Coufort, 2004).

3 Results

3.1 Influence of loading rates and shear stress on biofilm thickness and density

The mean biofilm thicknesses (L_f) measured for the growth conditions A to H, where no streamers were observed, are shown on Figure 2. The different environmental growth parameters influenced directly L_f . The “steady-state” L_f (after more than 45 days of growth) ranged between 250 μm and 4200 μm . The highest L_f was achieved at the highest SOLR (25 $\text{gCOD}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) and the lowest τ_p (0.5 Pa). Inversely, the thinner biofilms were observed for the lowest SOLR (4.5 $\text{gCOD}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) and the highest τ_p (9 Pa). Moreover, an increase in τ_p resulted in a smaller slope of the $L_f - \text{SOLR}$ curve. The data from Figure 2 were used to formulate an empirical function to describe the influence of SOLR and τ_p on L_f :

$$L_f = 220 \cdot \text{SOLR}^{0.8/\tau_p^{0.3}} \quad (5)$$

Such formalism allows describing the increase of L_f with SOLR, balanced by the τ_p applied. It must be stressed that Eq 5 has been estimated only for the experimental conditions used in the present study and should be checked for others.

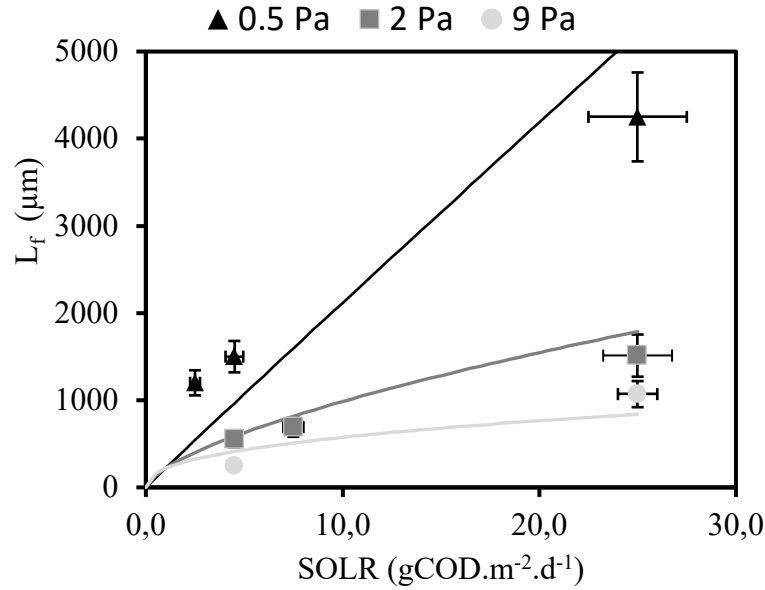


Figure 2 : Influence of apparent SOLR and shear stress on biofilm thickness (L_f) for biofilms developed under growth conditions A to H of Table 1: 0.5 Pa (\blacktriangle); 2 Pa (\blacksquare); 9 Pa (\bullet). Markers represent experimental values; continuous lines represent values obtained with eq 5.

Figure 3 shows the influence of SOLR at a fixed τ_p of 2 Pa (Fig 3A) and the influence of τ_p at a fixed SOLR of 4.5 gCOD.m⁻².d⁻¹ (Fig 3B) on biofilm density (ρ_f). Biofilm densities were ranged between 4.5 and 15 kgVS.m⁻³ and increased with τ_p . At low τ_p (between 0.5 and 2 Pa), an increase of τ_p induced a more pronounced increase of ρ_f than at high τ_p (between 2 and 9 Pa). For biofilms developed at a τ_p of 2 Pa, an increase of SOLR also resulted in an increase of ρ_f . From these results the following empirical relations were obtained:

$$\rho_f = 6.14\tau_p^{0.34} \text{ for fixed SOLR}=4.5 \text{ gCOD.d}^{-1}.\text{m}^{-2} \quad (6)$$

$$\rho_f = 3.8\text{SOLR}^{0.3} \text{ for fixed } \tau_p=2 \text{ Pa} \quad (7)$$

It should be noted that the parameters of the power function are very similar on the influence of τ_p and SOLR on ρ_f in these conditions ranges.

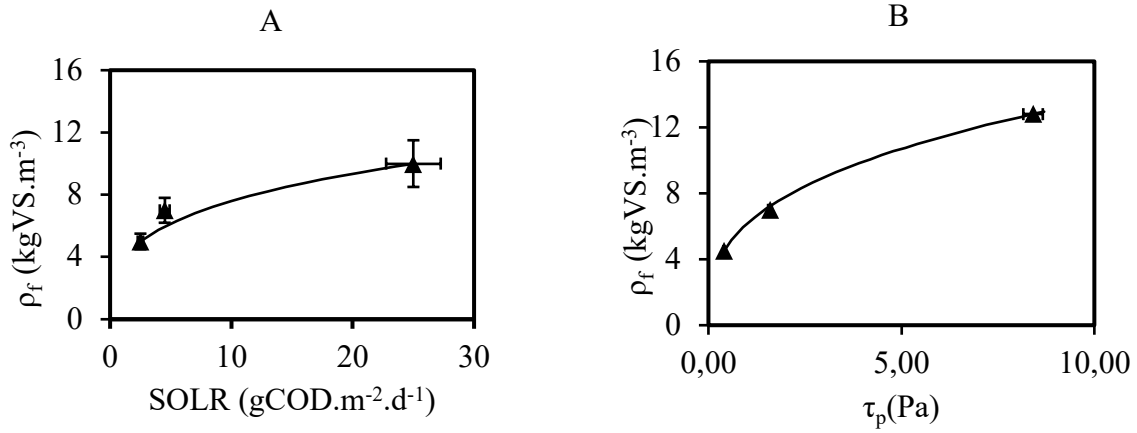


Figure 3: A) Influence of the apparent SOLR on biofilm density for biofilms developed at a constant shear stress of 2 Pa. B) Influence of shear stress on biofilm density for biofilms developed at a constant SOLR of 4.5 gCOD.m⁻².d⁻¹. Marker represents density measurements whereas continuous lines represent calculated densities with our empirical relations: $\rho_f = 6.14\tau_p^{0.34}$ for fixed SOLR=4.5 gCOD.d⁻¹.m⁻² (panel A); $\rho_f = 3.8SOLR^{0.3}$ for fixed $\tau_p=2$ Pa (panel B).

3.2 Influence of HRT, apparent SOLR and carbon source on the competition between morphotypes

To characterize the growth regime under aerobic conditions, values of γ_{S/O_2} were calculated for different environmental growth conditions (G to N). These values are given together with the residual bulk COD concentrations and the mass of the different biological morphotypes at steady state in Table 2 and the relative masses of each morphotypes (biofilms (B), streamers (S), planktonic aggregates (P)) are given in Figure 4.

The effect of HRT can be assessed by comparing cases K and M and cases L and N of Table 2. Low HRT led to significantly lower dissolved COD concentration (C_s) but lower COD removal yield. The biofilm mass accumulated was 2.5 to 4 times higher for high HRT (cases M and N),

corresponding to lower nutrients limitations, than for low HRT (cases K and L). In the same way, planktonic particles mass was significantly higher for the high HRT (6 to 17 times higher). On the contrary, streamers were only observed at low HRT with the relative biomass ranging from 9% (case M) to more than 25% (case J and K). In addition, the total biomass (sum of B, S and P mass) was significantly higher at high HRT growth conditions (more than 10 times higher at low SOLR and more than 5 times higher at high SOLR). It must also be stressed that, though DO in the bulk was always higher than $4 \text{ mgO}_2\cdot\text{l}^{-1}$, changing the HRT induced a shift from an oxygen growth limitation ($\gamma_{S/O_2} < 1$) (high HRT) to electron donor growth limitation ($\gamma_{S/O_2} > 1$) (Table 2).

One can also evaluate the effect of SOLR at low HRT by comparing I and J, and, K and L cases in Table 2. Although the removal efficiency increased (10% increase from low to high SOLR values), increasing SOLR led to higher soluble COD concentrations in the reactor. For all morphotypes, a notable accumulation of biomasses with SOLR was observed. Concerning the relative proportion between morphotypes, no straightforward conclusion can be drawn about the effect of SOLR at low HRT. Inverse trends were observed when comparing cases I and J and K and L. In the same way than at low HRT, an increase of the accumulation of biomasses with SOLR was observed at high HRT except that no formation of S was detected (cases M and N).

The influence of the nature of the carbon source can be assessed by comparing cases I and K, and J and L in Table 2. The COD removal yield was slightly higher (about 10%) when glucose was not used in the carbon substrate mixture. The mean biofilm (B) mass was similar whatever the C-source for biofilms developed under low SOLR but twice for biofilms developed on glucose at high SOLR. Suspended biomass was also slightly higher when glucose was used (comparing cases J with L). The influence of the carbon source nature on the presence of streamers was not straightforward. Depending on the SOLR the presence of glucose in the feed

led to opposite trend in terms of streamer biomass accumulation (comparing cases I with K, and cases J with L).

Table 2 : Influence of growth conditions on the growth regime parameters, bulk COD concentrations in the bulk and respective COD mass of the three morphotypes considered at steady-state (shear stress : 1 Pa).

Cases		I	J	K	L	M	N
Growth conditions	SOLR (gCOD.m ⁻² .d ⁻¹)	2.5	25	2.5	25	2.5	25
	Carbon source	Gl, Ac, Et	Gl, Ac, Et	Ace, Eth	Ace, Eth	Ace, Eth	Ace, Eth
	HRT (h)	3h	3h	3h	3h	20h	20h
	γ_{S/O_2}	0.33±0.13	0.90±0.09	0.29 ± 0.1	0.67±0.15	1.2±0.3	1.8 ± 0.3
CODs	Soluble COD concentration (mgCOD.l ⁻¹)	5.7 ± 4.6	21.7 ± 5.1	4.3 ± 3.7	16.2 ± 3.4	19.5 ± 5.2	40.2 ± 6.5
	CODs removal (%)	53 ± 4	82 ± 6	66 ± 3	88 ± 7	76.5 ± 4	96 ± 1
Morphotypes mass	Biofilm (B) mass (mgCOD.l ⁻¹)	8.6 ± 3.5	30.4 ± 3.9	8.6 ± 0.8	15.6 ± 1.9	18.3 ± 2.7	78.1 ± 7.8
	Streamer (S) mass (mgCOD.l ⁻¹)	1.2 ± 0.8	21.2 ±13.2	3.5 ± 3.2	5.1 ± 0.6	No streamers	No streamers
	Suspended biomass (P) in the bulk (mgCOD.l ⁻¹)	2.5 ± 1.5	22 ± 9	3.5 ± 2.1	18 ± 5	31 ± 7	152 ± 20
	Total Biomass (mgCOD.l ⁻¹)	12.3 ± 3.5	71 ± 8	16 ± 2	39 ± 5	49 ± 6	230 ± 15

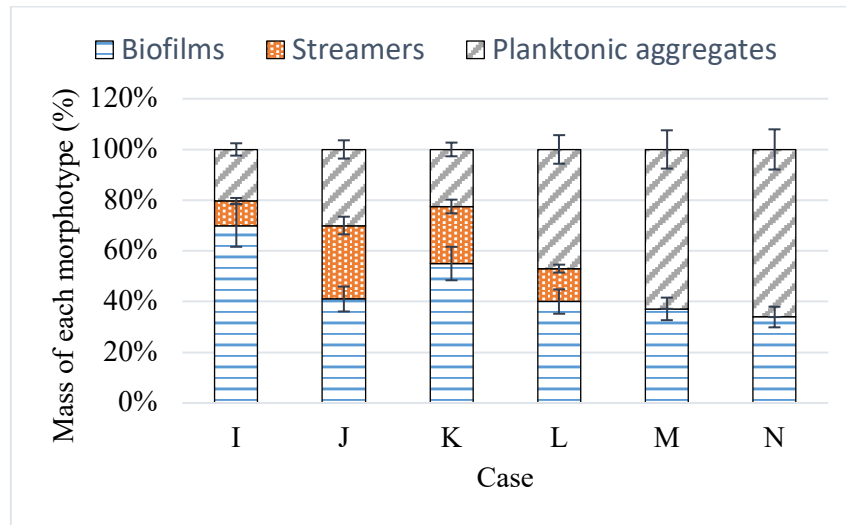





Figure 4 : Influence of growth conditions on the respective mass percentage of each morphotype :  biofilm (B);  streamers (S);  suspended biomass (P) in the bulk.

3.3 Dynamics of streamer formation

To study the dynamics of streamers formation, several morphological parameters were measured over time for the growth condition J.

During the first days of growth, many thin and long streamers were attached to the plates with a Fineness ratio between 15 and 35 (Figure 5 and Figure SM-2). After 7 days, streamers were more distributed at the biofilm surface and looked like fluffy feathers with fineness ratios between 18 and 26. After 35 days, more compact streamers were formed with fineness ratios between 4 and 8 (Figure 5 and Figure SM-2). Microscopic images of streamers are presented after respectively 3 days and 35 days of growth in Figure SM-2. During the first days, streamers were composed with mainly filamentous micro-organisms (panels A and B) whereas after 35 days of growth, a mixture of bacilli morphotypes and some rotifers protozoa were observed (panels C and D). In the biofilms attached to the substratum, non-filamentous bacteria predominated both at day 3 and day 35 (panels E and F).

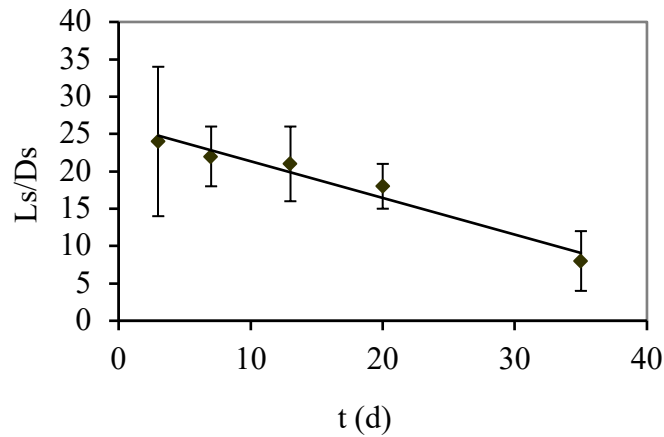


Figure 5 : Dynamic evolution of the Fineness ratio (L_s/D_s) over time ($n=3$) for condition I.

Oscillation frequencies, streamers length, oscillation amplitude and Strouhal numbers are presented in Table 3. The oscillation frequency was quantified only for young streamers since the amplitude was not sufficient to quantify a mean period between oscillation events for older streamers. The oscillation amplitude was significantly higher for the young streamers (10 days) than for the older ones (35 days). In addition, Strouhal values (St) for young streamers were ranged between 0.17 and 0.38.

Table 3 : Parameters allowing to study the evolution of the oscillation frequency of streamers at two growth times for growth condition J

	Day 10			Day 35	
	Streamer 1	Streamer 2	Streamer 3	Streamer 1	Streamer 2
Frequency (Hz)	30.3	13.5	13.4	ND	ND
Mean streamer length (mm)	46	29	22	52	47
Mean oscillation amplitude (mm)	21	15	17	3	4
St	0.38	0.18	0.17	ND	ND

4 Discussion

Results showed that, depending on the growth conditions, the observed morphotypes of aggregates were markedly different. Only biofilms (B) and suspended particles (P) associated were observed at high HRT (20h) whereas at low HRT (3h), streamers (S) were also observed. Next paragraphs aim to discuss the influence of growth conditions on biofilm properties and on the competition between those three morphotypes considering transport, detachment and biological processes.

4.1 What is the influence of the apparent SOLR and shear stress on biofilm physical structure?

A main result of our study is the experimental data on the influence of SOLR and shear rates on biofilm thickness and density in a range little studied. The physical structure of biofilms is mainly influenced by the substrate loading on the biofilm surface (SOLR) and the balance between hydrodynamic conditions and the internal cohesion of the biofilm (Stoodley et al., 1998; van Loosdrecht et al., 1995). This cohesion depends on the penetration of soluble substances (oxygen, carbon substrate, etc.) that in turn influence the type of micro-organism and the physiological state. In the present study when full penetration of organic substrate ($\gamma_{S/O_2} \geq 1$) is achieved, L_f increased with the apparent SOLR and decreased with shear stress (Figure 2). This trend is consistent with the majority of studies in the literature (Chang et al., 2020; Kwok et al., 1998; Paul et al., 2012; Wäsche et al., 2002) (Figure SM-4, panel A). However depending on the reactor configuration and on the carbon source, L_f can vary for a given SOLR by an order of magnitude (Figure SM-4).

In addition, an increase of shear stress resulted in an increase of ρ_f (Figure 3). These results are in accordance with other studies where biofilms were developed under turbulent flow and low SOLR (Melo and Vieira, 1999) and under low shear stresses (Wäsche et al., 2002) (Figure SM-4). However, there is no consensus in the literature on the influence of the apparent SOLR on

ρ_f . In the present study, ρ_f increased with the apparent SOLR when grown at 2 Pa. In the same way an increase of ρ_f with SOLR was observed by Trulear and Characklis, (1982) in a rotating annular reactor. On the other hand, a different feature has been observed by Kwok et al. (1998) and Wasche et al. (2002) (Figure SM-4). The former authors observed that the apparent SOLR did not influence ρ_f while the second one related a decrease of ρ_f with increasing the apparent SOLR in tube reactor. However, it must be stressed that Wasche et al. applied shear stresses significantly lower than those used in our work (10 times lower). Moreover, the reactor configuration was different.

When the biomass is growing, an increase of biofilm thickness occurs when the new biomass formed can withstand the shear forces applied on the biofilm, i.e when the cohesion strength is higher than the shear forces applied (van Loosdrecht et al., 1995). At low shear stress, the relation between biofilm thickness and SOLR was almost linear (at 0.5 Pa using Equation 1, $L_f \propto SOLR^{0.98}$). The detachment forces were low resulting in an enhancement of the tendency of microorganisms to grow toward the liquid bulk and to induce sloughing events. To resist to low shear stress, the biofilm did not need to adopt a very cohesive structure and, as a result, micro-organisms favored the creation of new biomass to increase L_f leading to thick biofilms. Thus, by increasing the apparent SOLR, an increase of L_f was detected. As a result, in the upper layers, the substrate availability led to the development of new biomass with low density and could explain the decrease of ρ_f with the apparent SOLR at low shear stress observed in the literature (Kwok et al., 1998; Wäsche et al., 2002). On the contrary, at high shear stress, strong detachment forces were applied on the biofilm resulting in a permanent renewal of cells at the surface of the biofilm and on a thin and homogeneous biofilm. As a consequence, substrates and nutrients were not consumed in totality in the superficial layers, being available for micro-organisms in the basal layers. In this case, the new formed biomass was for one main part

directed to fill the biofilm porosity and the remaining part to increase L_f . An increase of the apparent SOLR at such high shear stress thus led firstly to an increase of L_f , the extent of which strongly decreased with the applied shear stress ($L_f \propto SOLR^{0.65}$ at 2 Pa and $L_f \propto SOLR^{0.42}$ at 9 Pa) and secondly to an increase of ρ_f ($\rho_f \propto SOLR^{0.3}$ at 2 Pa). To explain these trends various hypothesis can be drawn. The effect of shear stress on biofilm physical properties may be explained partly by a modification of the microbial community. Some authors observed that increasing shear stress decreases the bacterial diversity suggesting that high shear stresses slow down biofilm maturation (Catão et al., 2019; Rickard et al., 2004; Rochex et al., 2008). These results are in accordance with ours since for biofilms developed at high shear stress, less substrates gradients should occur due to the significant lower thickness leading in turn to less diverse microenvironment and thus to more homogeneous physiological states in the biofilms. In addition, physiological adaptation mechanisms associated to EPS production can also partly explain the influence of shear stress: (i) a physiological change in the type of EPS produced by the microorganisms leading to stickier EPS through no change on the yields of production (Menniti et al., 2009), (ii) an increase in the EPS yields of secretion with the shear stress applied (Fish et al., 2017; Ohashi and Harada, 1994; Polst et al., 2018; Ramasamy and Zhang, 2005). Lastly, a physical process can partly explain the observations. By increasing the shear stress, the fluid creates pressures and vibrations that can cause the biofilm to physically consolidate by modifying the physical arrangement of individual polymers and cells (Coufort et al., 2007; Laspidou and Rittmann, 2004; Paul et al., 2012; Stoodley et al., 2002). These different responses lead to an increase in the biofilm cohesiveness which may explain the increase of ρ_f with shear stress.

4.2 What is the influence of HRT on the competition between morphotypes?

Another main finding of our study is that HRT significantly influence the competition between suspended biomass, biofilms and streamers. To date, in biofilm research, competition between

morphotypes has referred to competition between attached biomass and suspended biomass (Filali et al., 2012; Tijhuis et al., 1994). In the present study, in addition to these two morphotypes, a third one has been quantified: long streamers. Streamers with tails ranging from 0.5 cm to 5 cm were observed and could reach 30% of the total biomass in the reactor suggesting a real contribution in the biodegradation processes.

The substrate flux available for the attached morphotypes depends on the concentration of substrates in the bulk which is governed by the dilution rate and by the relative difference in the substrate consumption rate between the three types of biomass. Streamers were found only at low HRT (3h) whatever the carbon source and the apparent SOLR. In the present study, a decrease in HRT mainly led to a decrease in the carbon substrate concentration in the bulk. This goes against the trends for a chemostat without attached biomass where an increase in HRT lead to a carbon substrate concentration decrease. As in the present reactor the sludge residence time is dissociated to the HRT, the residual carbon substrate concentration depends on different parameters such as the sludge age, the decay rate and the apparent substrate affinity constant K_S . For an HRT of 20 h, the residual carbon substrate concentration is higher than the one for an HRT of 3h which is in accordance with the results obtained by di Biase et al., 2021. In our system, this could be partly explained by the presence of streamers at low HRT that could lead to a decrease of the apparent K_S at low HRT and as a result to a decrease of the residual carbon substrate concentration. Due to the flapping movement of such structure in the bulk, the external mass transport rate at the streamer-bulk interface is increased compared to the one at the biofilm-bulk interface leading to a decrease of the apparent K_S (Scheidweiler et al., 2019; Taherzadeh et al., 2012). As a result, micro-organisms of streamers are less substrate limited than those of biofilm. Moreover, micro-organisms of streamers benefit larger retention times compared to those of suspended biomass but conversely are more susceptible to shear stresses than those of biofilms (Taherzadeh et al., 2010; Xia et al., 2021).

The influence of HRT on the carbon residual concentration and on the relative proportion of the three morphotypes can be described as follows. At low HRT, when the dilution rate is high, the residual substrate concentration and the suspended biomass concentration are low. In the biofilm reactor used, the solid retention time of suspended biomass is equal or lower to the HRT. Indeed the suspended biomass is associated both to detachment of attached biomass and to growth of suspended micro-organisms in the flocs (Aqeel et al., 2019; Tijhuis et al., 1994). At this low HRT, the growth rate of micro-organisms is lower than the dilution rate. As a result, the main mechanism responsible of the presence of suspended biomass is detachment of attached biomass and the concentration of suspended biomass are low. As a consequence, biofilms and streamers are the predominating morphotypes. Streamers benefit from their flapping movement to increase their external mass transfer compared to biofilm but are more susceptible to physical disturbance (Taherzadeh et al., 2012; Xia et al., 2021). On the other hand elevated hydrodynamic resistance of biofilms confers in a certain way stability to the streamers because it allows streamers to form *de novo* from the biofilm after sloughing events (Scheidweiler et al., 2019).

In contrast at high HRT, the attached morphotypes (biofilms and streamers) do not benefit significantly from their ability to be attached and to benefit higher residence time than micro-organisms in suspended biomass. When the HRT is in the same order of magnitude as the generation time of micro-organisms in the suspended biomass, the concentration of suspended biomass is associated not only to biofilm detachment but also to biomass growth. This leads in turn to higher consumption rates of suspended biomass. The absence of the streamer morphotype in such conditions can be explained by both mass transfer and detachment considerations. For a given apparent SOLR, the substrate concentration is higher in the bulk and the advantage of flapping movement for streamers can become less useful. Moreover the presence of high concentration of suspended biomass can lead to strong abrasion phenomena

in addition to the high shear stress. Due to their structure, streamers are more susceptible to shear stress variations (Xia et al., 2021) and to abrasion which could also explain the absence of the streamer morphotypes at high HRT.

Community composition in the different morphotypes was not analyzed in the present study. However recently Scheidweiler et al., 2019 observed a similar community composition in biofilms and streamers grown during 10 d in porous media using a complex inoculum (hundreds of OTUs). Their results suggest that biofilms and streamers could benefit to each other. Streamers allow biofilms to expand space exploitation whereas biofilms with elevated resistance to flow allows streamers to form *de novo* after sloughing events. The similar community composition in biofilms and streamers suggests that micro-organisms in biofilms and streamers could produce EPS with different properties or in different quantities. This aspect should be further studied in future works.

4.3 Dynamics of streamers formation: Importance of mass transport, reaction and detachment processes

The third main result of our study is the new knowledge bring on the evolution of physical and biophysical parameters of streamers during their formation and maturation. In the literature, streamer formation has been attributed independently to: (i) unstable or turbulent hydrodynamic conditions (Rusconi et al., 2011, 2010; Stoodley et al., 1998), (ii) low nutrient availability conditions (Garny et al., 2009; Scheidweiler et al., 2019; Stoodley et al., 1998). The present study is the first experimental evidence of bacterial streamers development during more than one month under well-controlled growth conditions in terms of substrate availability and hydrodynamics. During the first days of growth under carbon substrate limitations (low HRT), the biofilm reactor was dominated by many long streamers composed by filamentous bacteria attached to the substratum. Micro-organisms are known to adapt their morphology structure and become elongated when strong substrate limitations occurs and under turbulent flows such as those encountered in the present study (Dias et al., 1968; Rice et al., 2005; Stoodley et al.,

1998). As long as the substrate is available, the microorganisms use this substrate to divide, grow and secrete EPS. These biological processes require space. Due to the low residual substrate concentration, growth in the form of biofilm then decreases the availability of substrate as the diffusion distance increases with the aggregate size. Thus a favourable direction to propagate is in the flow direction in the streamers morphotype (Taherzadeh et al., 2012). Indeed, such morphology is beneficial from a mass transfer as well as from a detachment point of view as already discussed in section 4.2.

Our experimental results thus suggest that during the first days of growth, filamentous bacteria ensured the structure of streamers. Due to the low density of the structure, oscillation frequency and oscillation amplitude were high, thus enhancing the mass transfer but in the same time increasing the drag force and thus the detachment risk. Strouhal numbers ($0.4 > St > 0.2$) were in the range of that observed in the industry for propulsive engines or in the nature for aquatic animals (Taylor et al. 2003). Nevertheless, the L_S/D_S ratio was relatively far from the optimal Fineness ratio of 0.45 found by Stoodley et al. (1998) which provides the minimum drag for the maximum volume.

After several days (7 to 16 days), sloughing events occurred decreasing the number of streamers attached to the substratum. After this period, a densification of the streamers was observed. Such phenomena can be explained both by physical and biological processes. The flapping movement of the filamentous streamers in the bulk could favor the “filtration” of bacterial cells from the bulk liquid (P and detached biofilm particles). The filamentous bacteria form a cohesive backbone for the non-filamentous bacteria and act as a substratum for a subsequent growth as it has been already observed in flocs (Cenens et al., 2000; Sezgin et al., 1978). As a result, denser streamers with a more sophisticated microbial ecology are formed and oscillation amplitudes decrease significantly reducing the mass transfer and the drag forces. In addition, the L_S/D_S ratio decreases leading to a streamlined shape which also reduces the drag coefficient

(Taherzadeh et al. 2010), possibly explaining the lower number of sloughing events observed on streamers.

4.4 Practical implications

By analyzing the impact of several growth conditions on different biological morphotypes presences, our results show their broad potential for adaptation to fluctuating environmental conditions. In addition they provide quantitative results on a wide range of shear stress and apparent SOLR which are generally encountered in industrial pipes and biofilm reactors. Thus our results and analysis could help to design biofilm reactor conditions or to choose appropriate contact times for anti-biofilm actions.

From an engineering point of view, where a stable operation of biofilm reactors is expected (wastewater treatment for example), control of biofilm mass, thickness, density and surface shape are important aspects. While desirable for conventional filtration processes, the sloughing is an unwanted phenomenon for most of the other biofilm processes. Fluffy and thick biofilms which lead to instabilities (in terms of treatment efficiencies and on separation processes) due to frequent sloughing events have in general to be avoided. Thus compact and stable biofilms are desirable. Then, for “designing” biofilm structure, the results presented in the present chapter highlighted that hydrodynamic shear force, apparent SOLR and HRT seem to be effective tools to control biofilm physical and morphological properties and thus nutrient removal quality.

In a context of “negative” biofilms, the results obtained could help to predict the optimal contact time required for an antimicrobial agent to act on biofilm structures encountered in industrial pipes. Indeed, biofilm thickness and density directly impact on the characteristic time of transport of the antimicrobial agent in the biofilm and thus on the time required for the agent to act on the basal layers of the biofilm (Pechaud et al., 2012; Stewart, 2003).

5 Conclusion

The main conclusion of this work are summarized as follows:

- (i) In the absence of streamers, an increase of shear stress promote the formation of thinner and denser biofilms.
- (ii) The influence of SOLR on biofilm density and thickness depends on the hydrodynamic conditions applied.
- (iii) Low HRT (3h) (i.e high dilution rate) favor the development of attached morphotypes (streamers and biofilms) and as a result decreases the concentration in suspended biomass. In contrast high HRT promotes the formation of biofilms and suspended biomass (no streamers observed).
- (iv) The presence of streamer has been attributed to the residual concentration in the bulk and to the hydrodynamic conditions of growth.
- (v) Further studies are needed to analyze more precisely this architectural plasticity especially by analyzing dynamically the chemical and physical properties of EPS and the microbial population involved in each morphotype.

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