IMPACT OF HYDRODYNAMIC PERTURBATIONS ON PHYSICAL STRUCTURE AND MICROBIOLOGICAL COMPOSITION OF BIOFILM

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ABSTRACT
In natural environments as well as in industrial processes, microorganisms form biofilms. Hydrodynamic conditions are one of the key parameters influencing the microbiological and physical structures of a biofilm. In the present work, a method has been developed to count and characterize grazing population of a biofilm. We used hydrodynamic conditions to obtain biofilms having different microbiological compositions and superficial structures. We monitored two continuous bubbles columns, inoculated with aerobic activated sludge. One reactor was operated under a stable low hydrodynamic constraint while the second one was exposed to frequent high hydrodynamic perturbations. Microscopic images showed a very dense population of eukaryotic organisms such as rotifers, nematodes and ciliates. We developed an innovative approach to quantify the number of rotifers. The method is based on the analysis of microscopic images. Hydrodynamic perturbations modified both the morphological and microbiological structures: perturbations decreased the quantity of moving organisms, such as rotifers, whereas some streamers appeared.

INTRODUCTION
Biofilms are a widely spread system in biological processes. One key parameter acting on biofilms is the shear stress (Busscher and Van der Mei, 2006). In the literature, this parameter was mainly studied either under constant shear, e.g. comparing two reactors in which biofilms were grown at two different shear rates (Liu and Tay, 2002) or as erosion tests (low shear rate during growth followed by higher shear rate) (Derlon et al., 2008). In this work a growing biofilm is subjected to variable hydrodynamic conditions, because these conditions better represent industrial environments. Two biofilms were grown, one under turbulent and constant hydrodynamic conditions and the other with hourly intense perturbations.
Metazoa populations can be used as bioindicators for the performance of wastewater treatment plants (Duchene and Cotteux, 1993). Many studies focus on eukaryotic microorganisms in activated sludge (Ginoris, a L Amaral, et al., 2007; a. L. Amaral, da Motta, et al., 2004). The originality of the present study is to investigate eukaryotic microorganisms directly into a biofilm. Among the eukaryotes, we focused here on rotifers. The dominant species in our reactor system are up to 100µm long, mobile and graze on bacteria.
The aim of this work was to develop a method to characterize the grazer populations, and especially rotifers. Different hydrodynamic conditions were used to obtain different biofilm structure and test the method that was developed. Both physical and microbiological structures of the biofilm were also investigated.

METHODS

Reactors
The experimental sets-up were bubble column reactors (Figure 1). These 5-litres reactors contained 7 stainless steel rings. Each ring carried 19 polyethylene coupons (133 coupons per reactor). Each coupon has an exposed surface of 5 cm². The reactors were continuously fed with sterilized substrate and unsterilized softened tap water. The feeding solution was as followed: meat extract, yeast extract and peptone 1071mg/L each, NH_4Cl 46mg/L, K_2HPO_4 150mg/L, KH_2PO_4 75mg/L, $MgSO_4 \cdot 7H_2O$ 15mg/L, $Na_2HPO_4 \cdot 2H_2O$ 157mg/L and oligo-elements.

A hydraulic retention time of 65 minutes was maintained to favour biofilm growth against growth of suspended cells. Mixing and oxygen supply were carried out by continuous aeration. Two reactors were operated. A first one was operated under constant hydrodynamic conditions, by maintaining an air flow rate of 1 L/min during the experiment. In the second reactor, hydrodynamic perturbations were generated by an 8-fold increase of the air flow rate for 12 min every 60 min.

Figure 1 : Reactor design and sample treatment. The reactor contains rings and each ring carries removable plastic coupons. Stereomicroscopic images of the biofilm on a coupon are recorded and then analyzed

Sampling and image acquisition
A coupon sampled from the reactor was submerged in particle free reactor solution in a custom made aluminium chamber. Microscopic images of the biofilm were acquired every day or two days with a Leica stereomicroscope M205 FA and a Leica DVC 495 camera.

Samples were observed in transmitted bright field illumination at 22× magnification. The 8-bit acquired images corresponded to of biofilm surface of 6.4×4.8 mm² with a resolution of 3.5 Mega pixels. Images were then treated for three purposes: rotifer counting, movement quantification and morphology characterization.

Image analysis

Rotifer counting was done visually for each image using the Image J multi-point plugin.

Rotifer movement was quantified using a custom-made Image J script (Figure 2). Two images of the same biofilm location were acquired at a time interval of 2 seconds. By digitally subtracting these two images, differences between them, mainly due to organism moving, can be detected. The resulting image was then binarized using the Otsu thresholding method. One opening step is finally done to remove the isolated pixels. The coverage of this final image was then used to quantify the differences between the two images

$$Coverage = 100 \; x \; \frac{Number \; of \; black \; pixels}{Total \; number \; of \; pixels} \quad (1)$$

The "Analyze Particles" plugin was used to extract the number and the size of particles from the image. A filtering treatment has also been done using this tool. Particles with an area lower than 750 µm² can be excluded from the calculations.
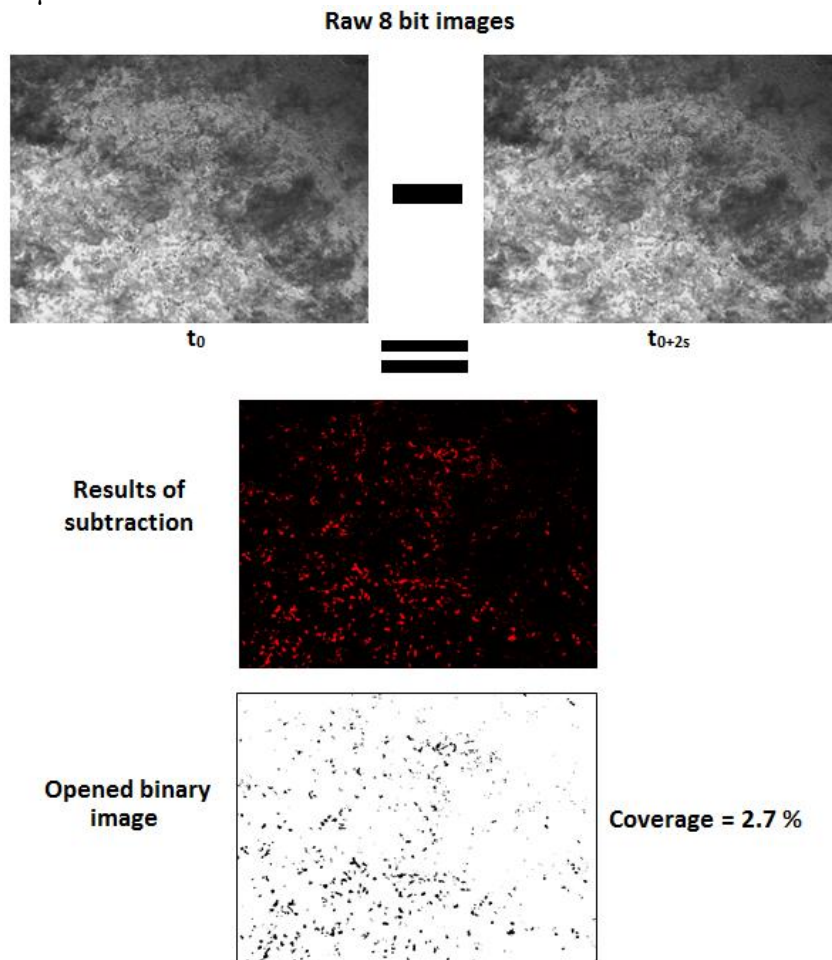

Figure 2 : Image processing from two raw images to the final subtraction image. Raw images are subtracted and the resulting image is binarized and opened. The numerical result, the coverage, quantifies differences between raw images

RESULTS AND DISCUSSION

The goal of this work was to develop a method to identify eukaryotic population distribution within a biofilm, especially the grazer population. In the present work, hydrodynamic perturbations were used to develop two different structures of biofilms. The morphologies of these two biofilms obtained are presented on the Figure 3. The perturbed reactor was characterized by the presence of filamentous and fluffy structures, so called streamers. This result is consistent with recent studies attributing the presence of streamers to unstable hydrodynamic conditions (Rusconi et al., 2011). The physical structure of biofilms grown in the stable reactor is more homogeneous and streamers were not present.
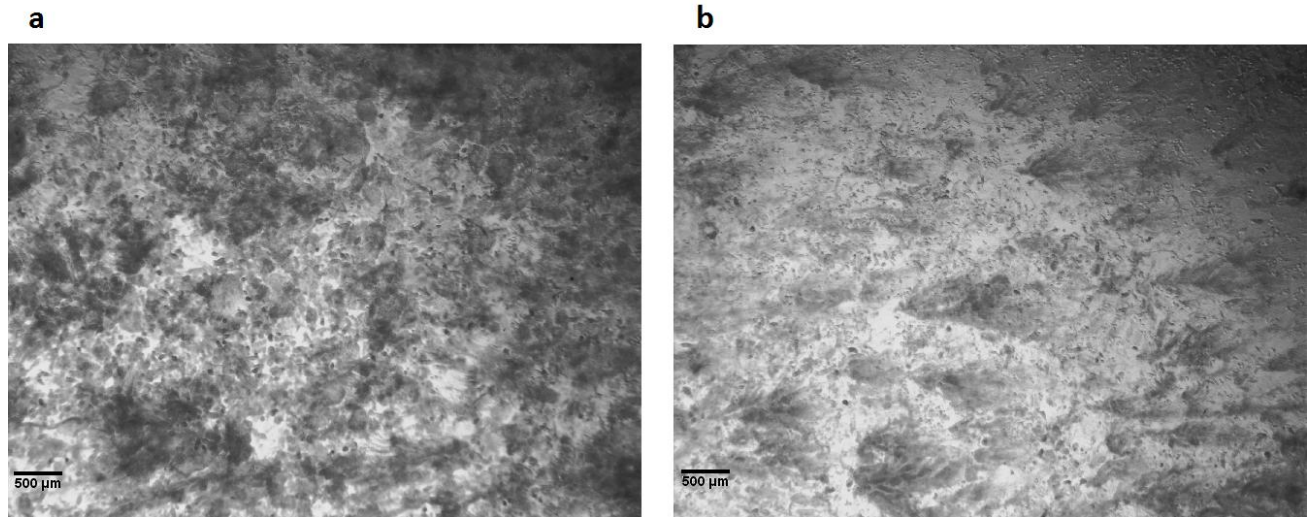

Figure 3: Microscopic images of two biofilms (Bright field illumination), (a) grown under constant hydrodynamic conditions and (b) grown in a hydrodynamically perturbed reactor (b). Note the different texture of the two biofilms.

An image analysis approach based on coverage surface was developed to characterize the presence of grazing eukaryotes. By digitally subtracting two images taken at the same location with a time delay of 2 seconds, movements and abundance of moving organisms can be estimated. Grazing organisms such as rotifers, nematodes or some ciliates keep moving to find their substrate. The coverage is directly linked to movements of grazers which can be detected and quantified. Figure 4 represents the mean coverage after 29 days for each reactor. The mean value was calculated from 14 images taken in different locations of a given coupon. The hourly perturbed reactor presents approximately two times lower coverage than the reactor under constant shear. Based on visual microscopic observations, the number of rotifers was lower in the perturbed reactor, whereas sessile ciliates were more abundant. Biofilms grown in the two reactors are different in terms of physical structure (presence of streamers) and population density of grazing microorganisms as suggested by the coverage magnitude. The 8-fold increase of the air flow rate is likely to (i) induce biofilm detachment and so deprive grazers of their feeding source, (ii) hinder their colonization and favor sessile ciliates and (iii) mechanically wash them out of the biofilm because of an increase in shear force.
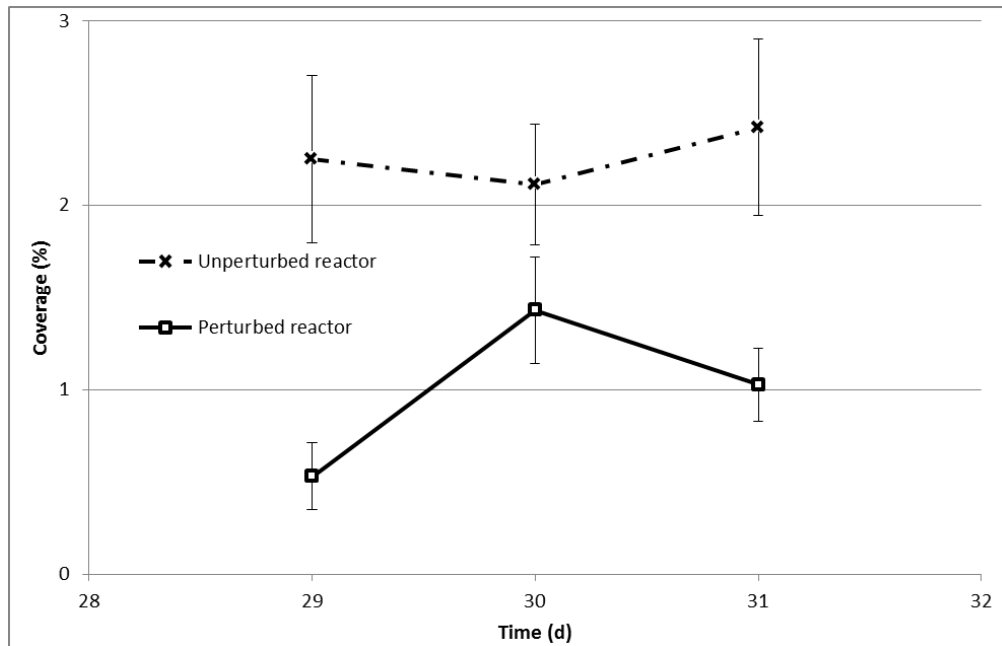
Figure 4: Impact of the hydrodynamic conditions on the detected movement. Each point represents the mean coverage calculated from 14 images pairs. All images pairs per point originate from the same coupon. The 14 resulting coverages are then averaged. It appears that under constant hydrodynamic conditions the coverage is higher which means that more movements are detected. This emphasizes the denser population of moving and grazing organisms in biofilms grown under steady conditions.

A deeper analysis of the particle distribution on the images shows the direct link between the coverage, the number of rotifers and the number of particles (Figure 5 and Figure 6). Rotifers were counted on each image and the result was then correlated to either the coverage (Figure 5a) or to the number of particles (Figure 5b) on the image after processing.

Figure 5a shows the correlation between coverage and the number of manually counted rotifers on each raw microscopic image. The y-axis is the coverage of the corresponding binary image. A linear correlation with R^2 value of 0.82 fits the experimental results. This correlation confirms the previous hypothesis: the number of moving and grazing organisms (i.e., rotifers), can be estimated by the image analysis method proposed in the study. This method, much faster than manual counting, is thus able to quantify rotifers.
Within the biofilms grown under constant hydrodynamic conditions, we counted between 200 and 500 rotifers whereas the maximum value for the reactor with variable hydrodynamic conditions is 124 rotifers. Hence, it appears that hydrodynamic conditions can play a role in the population density.

Figure 5b presents on the y-axis the number of particles detected on the binary images. The total number of particles per image does not fit very well the number of rotifers. It is likely that an important number of particles are counted but do not correspond to movements of rotifers. As a consequence, a procedure was implemented by applying a filter based on the size of the particle. Particles, characterized by a surface higher than 750μm², were only taken into account for the particles counting as well as for the coverage. Based on this filtering method, pixels clusters, that more likely represent a moving rotifer, can be selected by minimizing the noise.
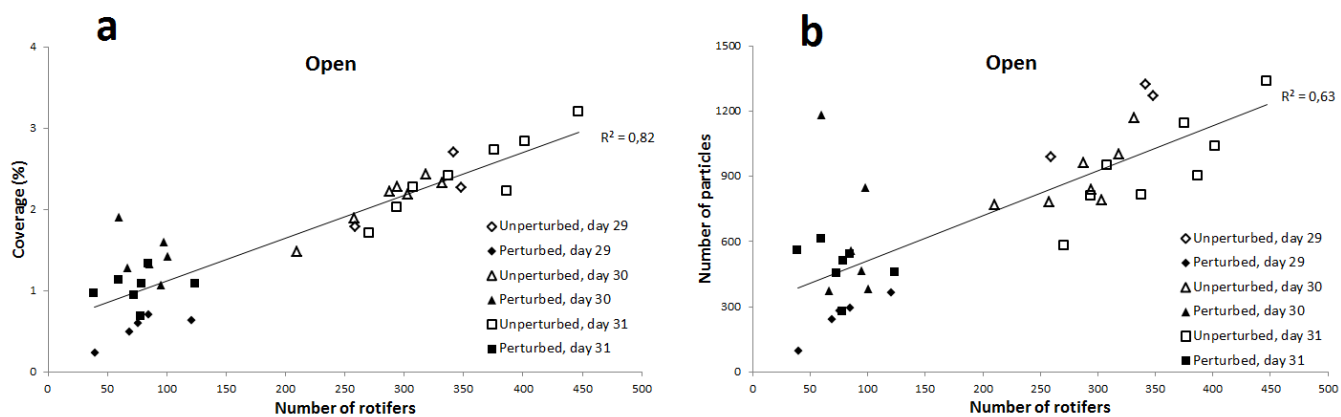
Figure 5: Correlation between the number of rotifers per image (x axis) and either the coverage (a) or the number of particles (b). Calculations have been done on opened binary images.

Figure 6a represents the relation between the number of rotifers and the coverage after this filtering operation. Figure 6b shows the relationship between rotifer population size and the number of particles. By comparing Figure 6a and Figure 6a, it is possible to observe that the linear correlation between the coverage and the number of rotifers remained almost unchanged with a R^2 value from 0.82 to 0.81.
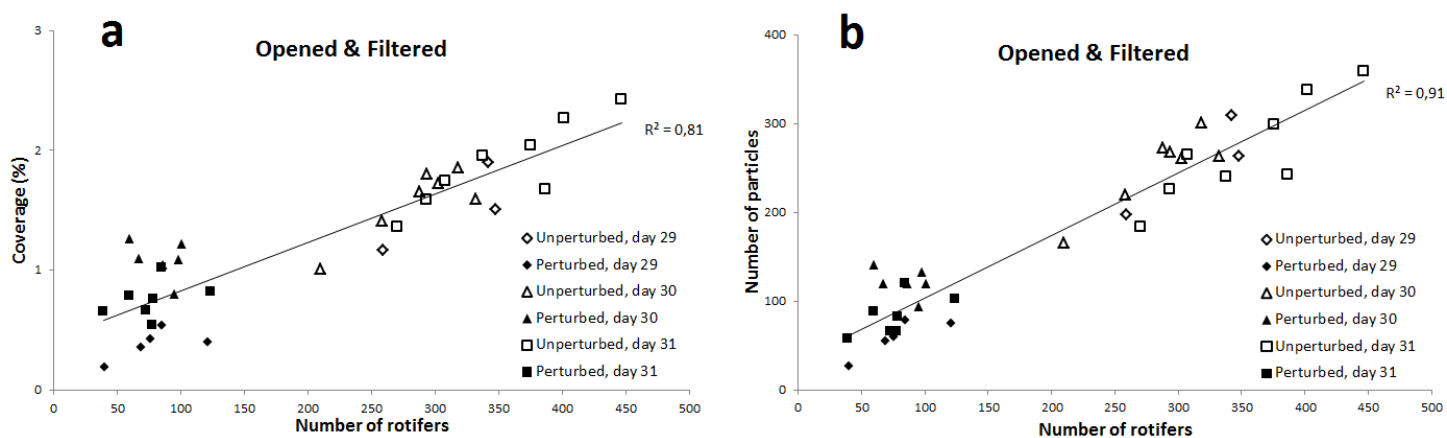

Figure 6: Correlation between the number of rotifers per image (x axis) and either the coverage (a) or the number of particles (b). Only particles with an area higher than 750μm² are considered for the calculations

However, this filtering treatment has a positive impact on the correlation with a R^2 value increasing from 0.63 to 0.91 (Figure 6b). This indicates the low contribution of the removed particles on the coverage despite their number. Indeed, the average coverage undergoes a less 1.5-fold decrease whereas the average number of particles decreases from 721 to 174 - more than 4-fold decrease. So an important number of particles were counted but they are not significant as the coverage suggests it.

CONCLUSION

The high correlations between the number of rotifers and either the coverage or the number of particles on processed images indicate that rotifer numbers can be extracted from biofilm images using the proposed method. During the analysis process, it is necessary to exclude areas smaller than 750µm² because these do not represent rotifer movements. This filtering method allows a better estimation of the number of rotifers, especially considering the number of particles.

Biofilms grown under different hydrodynamic conditions do not have the same morphological structure as well as microbiological structure. This innovative subtracting method of one location at two different times seems to be efficient for both biofilms. It helps to estimate the numbers of rotifers and thus clearly distinguish the two biofilms.

As the literature indicates, hydrodynamic conditions can influence the biofilm structure by many ways such as substratum access, shear stress, nutrients transfer... Our work suggests that predation could be one more aspect that hydrodynamics have to structure the biofilm.

Further experiments are in progress to confirm these results. In addition, the frequency and the strength of hydrodynamic perturbations can also have a strong impact on both the physical and the microbiological structures of biofilms

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