Comparison of biomass detachment from two different *Pseudomonas* spp. biofilms under constant shear conditions.

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Abstract

In the context of biofilm development, detachment is of practical importance when placed in a biofilm management perspective. The objective of the present study was to examine biofilm structure and biofilm detachment under controlled conditions for two distinct microorganisms grown under constant shear conditions. Detached biofilm biomass was regularly collected and analyzed over the course of 72h biofilm growth by *Pseudomonas putida* and *Pseudomonas fluorescens* cells, and biofilm structural development assessed using confocal microscopy. The two *Pseudomonas spp.*, which had very similar specific growth rates in planktonic culture, presented remarkably different characteristics in terms of biofilm morphology but surprisingly their detachment behaviour over time were very similar. These findings underline the intrinsic complexity of the detachment phenomenon.

Keywords

Detachment, biofilm, shear, structure, *Pseudomonas*

Introduction

In the context of biofilm development, detachment is a term used to describe the release of cells either individually or in aggregates. Detachment is of practical importance in public health particularly with regard to the dissemination of bacteria associated with device related infection (Fux et al. 2004). The phenomenon also has important implications for the optimum performance of biofilm reactors in wastewater treatment (Telgmann et al. 2004) and in the management of biofouling in water treatment processes (Flemming et al. 2002).

Detachment occurs when local shear forces acting on the biofilm exceed the cohesiveness of the biofilm (Telgmann et al. 2004). However the imposition of elevated shear force is known to alter the biofilm material properties and indeed enhance the supply of nutrients to the biofilm. Stoodley et al. (2002) reported that biofilms grown under low shear conditions were less dense and detached at low fluid shear, while biofilms grown under high shear conditions detached at higher fluid shear. These experiments showed that the onset of detachment occurred at a shear stress of roughly twice that applied during growth. Detachment also plays a role in the development of biofilm structure. A hypothesis postulated by van Loosdrecht et al. (Van Loosdrecht et al. 1995, Van Loosdrecht et al. 1997) and verified by Kwok et al. (Kwok et al. 1998), suggested that biofilm structure is determined by a balance between substrate surface loading (proportional to biomass surface production rate) and detachment force. This evidence is now generally accepted and forms the basis for the assumptions used in spatially structured mathematical models (Kreft et al. 2001, Kwok et al, 1998, Laspidou and Rittmann 2004). The application of this concept has been proposed as a strategy for biofouling control in reverse osmosis systems for water desalination whereby hydrodynamic conditions are selected in a manner that results in a biofilm that has an open or loose structure thereby making them easy to remove (Vrouwenvelder et al. 2011).

Despite the importance of these key inter-relationships between biofilm growth conditions, hydrodynamics and structure, there are few reports in the literature describing systematic studies of biofilm detachment over the development cycle of the biofilm. The objective of the present study was to examine biofilm structure and biofilm detachment under controlled conditions for two distinct microorganisms grown under constant shear stress.

Materials and Methods

Strain cultivation

Bacterial strains used throughout this investigation were Green Fluorescent Protein (GFP) tagged *Pseudomonas putida* PCL1482^{tet} and mCherry-tagged *Pseudomonas fluorescens* PCL1701^{gen} (Lagendijk et al. 2010). These strains were cultivated in King B broth composed of peptone 20g g L^{-1}, potassium phosphate dibasic 1.5 g L^{-1} and magnesium sulphate heptahydrate 1.5 g L^{-1}, supplemented with either 40 µg mL^{-1} tetracycline or 10 µg mL^{-1} gentamicin and incubated at 28^0 C with shaking at 200 RPM, overnight. Culture aliquots (0.5 ml) were stored at -20^0 C.

Prior to biofilm experiments, thawed culture aliquots were used to inoculate 100 mL sterile King B medium, supplemented with appropriate antibiotic, followed by incubation at 28^0C with shaking at 200 RPM overnight. These cultures were then centrifuged at 2760 RCF for 10 minutes (Hettich Universal 320R); the pellet was resuspended in sterile physiological water (0.1M NaCl) and the optical density at 600 nm adjusted to 0.5.

Biofilm growth

Three-channel flow cells devices (DTU Systems Biology, Technical University of Denmark) with individual channel dimensions of 1x4x40 mm were first prepared, sterilized and conditioned as previously described (Crusz et al. 2012).

The biofilm experimental setup is illustrated in (Figure 1). Briefly, a flow cell device was connected to a Watson-Marlow 205S peristaltic pump (Watson-Marlow Ltd., Falmouth, England), and to feed tanks each containing 2 L of sterile King B broth, using silicone tubing with a bore of 1 mm and an external diameter of 3 mm (Tube Verisilic, Fisher Scientific).

A 4 mL freshly prepared bacterial suspension was used to inoculate sterile individual flow cell channel, using a sterile syringe. The flow cells were maintained in a horizontal position for 30 min, with no flow, after inoculation to allow initial bacterial adhesion. The inoculated flow cell system was then placed in an incubator (IP250, LTE Scientific) at 28^0 C, before starting the flow at 30 mL h^{-1}. The start of the flow was taken as time zero.

Growth curve

A 100 µL inoculum from an overnight culture, adjusted to an optical density of 0.4 λ_{600nm} was transferred to 100 ml of sterile King B medium, which was incubated at 28^0 C and 200 RPM. A sample of 1ml was taken every hour for 8 hours and its optical density at 600 nm measured. 15 flasks per strain were inoculated 5 were used to measure the growth between 0 and 8 hours, 5 for 8 to 16 hours and 5 for 16 to 24 hours. Results from the 5 replicates were combined and averaged.

Confocal Scanning Laser Microscopy (CSLM)

Horizontal plane images of the biofilms were acquired using a Zeiss LSM 510 confocal microscope (Zeiss, Germany). The excitation wavelength used for detecting GFP was 488 nm, and emitted fluorescence was recorded above 505 nm, whereas mCherry was excited at 543nm and fluorescence emissions recorded above 560 mn. Images were collected through a Meta Plan-Apochromat 63x NA1.4 oil immersion objective with a z-step of 1 µm. The structural quantification of biofilms (total biovolume, substratum coverage and mean thickness) was performed using the PHLIP Matlab program (Xavier 2004). Three-dimensional projections were reconstructed using IMARIS software (Bitplane, Zürich, Switzerland). Images were acquired after 0, 24, 48 and 72 hours growth.

Detachment

During biofilm formation the effluent from individual channels was collected every 8 hours in sterile 250 mL conical flasks that were kept on ice to prevent cell proliferation. From the collected effluent 3x50 mL aliquots were taken in previously weighed 50 mL centrifuge tubes and centrifuged at 2760 relative centrifugal force (RCF) for 10 minutes. The supernatant was carefully discarded and the pellet dried at 60^0 C for 48 hours to determine dry weight.

To establish the baseline effect of the King B Medium on the pellets' dry weight, a flow cell setup was run for 72 h with sterile King B medium with no inoculum, and the effluent was collected and dry weight measured as previously described. Mean baseline values for each time point were used to standardise the dry weight experimental results.

For the 72 h-long experiments 350 ml of sterile King B medium were aseptically replenished after 48 h in each feed tank to ensure that the reservoir did not run dry.

Statistical analysis

Detachment results for *P. putida* and *P. fluorescens* were analysed using one way analysis of variance (ANOVA), to evaluate intra and inter differences in time and experiments with a significance level of 0.05 followed by all pair-wise comparisons with Holm-Sidak method. Comparison of biofilm proprieties were performed using Kruskal-Wallis One Way ANOVA on Ranks followed by all pairwise multiple comparison with Dunn's Method ($p<0.05$). Comparison analyses of acquired data were performed using SigmaPlot© (Systat Software, San Jose, CA)

Results and discussion

Bulk-liquid behaviour

To characterize the behaviour of both *P. putida* and *P. fluorescens* suspended cells, growth curve experiments were performed. An initial latency period was followed by an exponential phase that slowly declined without reaching a plateau in the 24h experimental period. The growth curves reached a stationary phase at around 12h (Figure S1). Calculated growth rates between 6h and 11h (exponential phase) were 0.83 h^{-1} and 0.87 h^{-1} for *P. putida* and *P. fluorescens* respectively. However, while *P. fluorescens* cells entered early-exponential phase at around 8 hours of growth, *P. putida* cells reached the same state after 10 hours.

Biofilm properties

Qualitative analyses of *P. putida* and *P. fluorescens* biofilms were performed using confocal microscopy to assess the biofilm structural development over time. As shown in Figure 2, structural differences were observed between *P. putida* and *P. fluorescens* biofilms. Following 24h growth, *P. putida* biofilms appear to have covered the entire surface of the substratum with a prominent biomass layer, whereas *P. fluorescens* biofilms seem to be composed of adhered cell clusters spread over the surface. Direct observation of *P. putida* biofilms at 48h and 72h did not reveal differences in morphology when compared to the 24h

samples. Conversely structural changes were observed for *P. fluorescens* biofilms, going from small to larger cell clusters during the experimental timeframe. Interestingly *P. fluorescens* biofilms did not reach complete surface colonization, even after 72h growth. Moreover *P. putida* biofilms were shown to have a carpet-like structure compared to the tower-like structure of *P. fluorescens* biofilms after 72h. These structural observations suggest that *P. putida* is a faster biofilm former compared to *P. fluorescens* under the experimental conditions employed in this study.

Similar *P. fluorescens* biofilm structures were observed in another study using comparable experimental conditions (Korber et al. 1994). Interestingly, Tolker-Nielsen et al. (2000), reported analogous carpet-like *P. putida* biofilm structures, but also tower-like structures, which were not observed in this experiment. It is reasonable to assume that differences in biofilm growth conditions and experimental procedures may lead to various biofilm structural outcomes and make direct comparisons of structure difficult.

For quantitative analysis of biofilms, the structural parameters: total biovolume, substratum coverage and mean thickness were calculated from acquired CLSM data (Figure. 3). Quantitative results confirmed that *P. putida* biofilms were structurally different to *P. fluorescens* biofilms over the course of 72h. Adhesion profiles derived from the 0 h time point showed statistically significant difference ($p<0.05$) in substratum coverage (Figure. 3B, 3E), in which *P. fluorescens* cells covered on average 10% of the total area compared to the 4% of *P. putida* cells. Conversely, after 24h *P. putida* biofilms were shown to have 6 times more total biovolume and substratum coverage compared to *P. fluorescens* biofilms. In addition, *P. putida* biofilms were found to be 1.5 times thicker than *P. fluorescens* biofilms. Based on the conditions set in this study, the results suggest that *P. putida* is faster at forming biofilms compared to *P. fluorescens*. While structural differences were observed between the adhesion profile and early stage biofilms ($p<0.05$) for both *P. putida* and P. *fluorescens*, no significant structural changes were observed after 24 hours of biofilm development. Although these observations are in accord with acquired 3D projections of *P. putida* biofilms (Figure 2), a discrepancy between qualitative and quantitative data was noticed when studying *P. fluorescens* biofilms, which may be ascribed to the intrinsic variability of this biofilm system. Such variability will affect the distribution of the values measured and in turn this will be reflected in a lower power of the statistical tests.

Biomass detachment during biofilm growth under dynamic conditions.

In order to understand the phenomenon of detachment during biofilm growth, the experimental setup was designed to continuously collect and quantify biomass from the effluent. The collected detached biomass of *P. putida* and *P. fluorescens* over the course of 72h biofilm growth are presented in Figure. 4.

The bulk-liquid flow rate utilized during these experiments was sufficient to ensure a dilution rate that greatly exceeded the maximum replication rate of both *Pseudomonas spp.* thus the biomass collected was derived exclusively from the biofilm. An increase in detachment rate was observed for *P. putida* cells in the 8-16 hour period compared to the initial biofilm grown between 0h and 8h. From 16h onwards, the amount of collected biomass plateaued at a value of 0.011g, a ≈2-fold increase compared to early stage biofilm detachment ($p<0.05$). Similar behaviour was observed for *P. fluorescens* biofilms however this was at a later stage. A plateau of detached biomass at a value of 0.013g was observed following 24 hours development, which represents a ≈9-fold increase compared to early stage detachment. Moreover, compared to *P. fluorescens*, the detachment plateau was reached at a faster rate in *P. putida* biofilms. These same experiments were repeated for both organisms at 24h and 48h time points. No significant differences ($p<0.05$) were observed for the acquired data between replicates, hence confirming the reproducibility of the chosen experimental method in this study (Figure S2). These results clearly show that detached biomass gradually reached a steady state over time, and was typically preceded by an initial period of increased biomass release. Substantial detachment events or sloughing events were not observed during biofilm experiments.

By comparing the absolute value of detached biomass from *P. putida* and *P. fluorescens* biofilms, an apparent distinction emerges, in which *P. putida* biofilms, having a significantly higher total biovolume, are more prone to biomass detachment over a given time period. Given that *P. putida* and *P. fluorescens* biofilms have different structural proprieties, it is likely that these structures might respond differently to shear stresses, hence different levels of collected biomass. This was shown in a recent *in silico* model (Laspidou and Rittmann 2004), where prominent structural features of a biofilm were more prone to surface detachment, compared to flat homogenous structures. Moreover, in highly structured biofilms, as observed for *P. fluorescens*, the presence of voids within biofilms have been shown to harbour free planktonic cells which are systematically released in the bulk of the

liquid as part of the planktonic yield theory (Bester et al. 2005-2013). This could also explain the observed variations of collected biomass between *P. putida* and *P. fluorescens* biofilms over the course of the experiments. Furthermore, when investigating *Pseudomonas sp.* biofilms under dynamic conditions, Bester et al. (2009) reported an increase in bulk-liquid collected colony forming units over time that levelled at around 96h of growth. The findings presented in this paper are in accord with the above mentioned studies in which similar detachment profiles were obtained for two structurally different biofilms by two different organisms.

Conclusion

This study investigated the monoculture biofilm development of *Pseudomonas putida* and *Pseudomonas fluorescens* strains under dynamic conditions, and assessed biomass detachment. The level of collected biomass was found to be similar for both organisms, regardless of their differences in biofilms structure and proprieties.

Biomass detachment is an intrinsic phenomena of the biofilm development process , partially dictated by the environment under which they develop. A better understanding of these factors will facilitate the control or management of biofilms with strategically selected conditions that promote or limit biomass detachment. Nevertheless, the investigation about detachment is still in its early stage and deeper knowledge is required, particularly on mixed-species biofilms, to help shed light on the significance of bacterial interactions amidst dynamic environments.

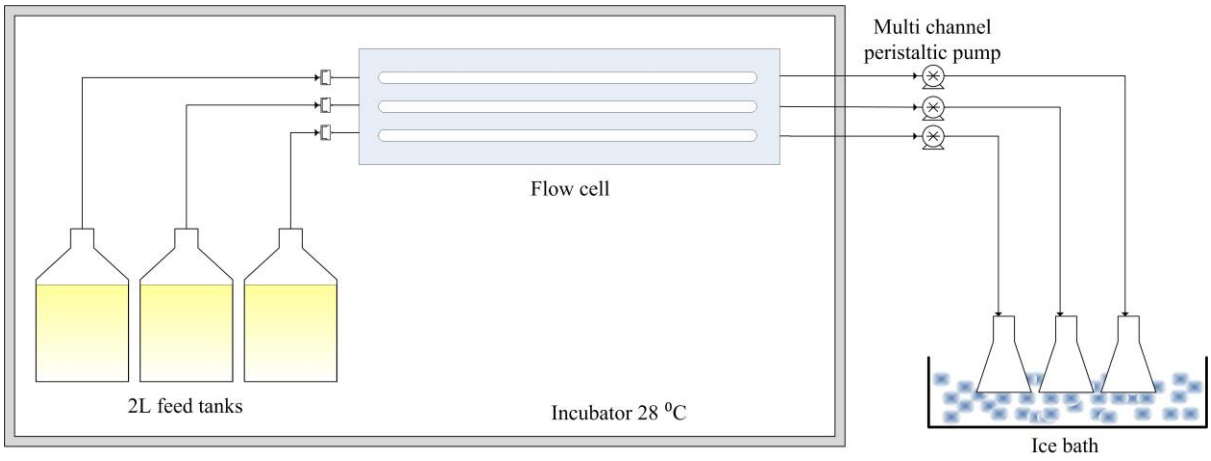

Figure 1. Schematic diagram of the system employed for biofilm growth and imaging. Sterile medium introduced into individual flow cell channel, by means of a peristaltic pump, from the feed tanks through the flow cell and collected in sterile flasks kept on ice to prevent rapid cell proliferation. Luer-lock junctions were used to inoculate the system with the bacterial suspension.

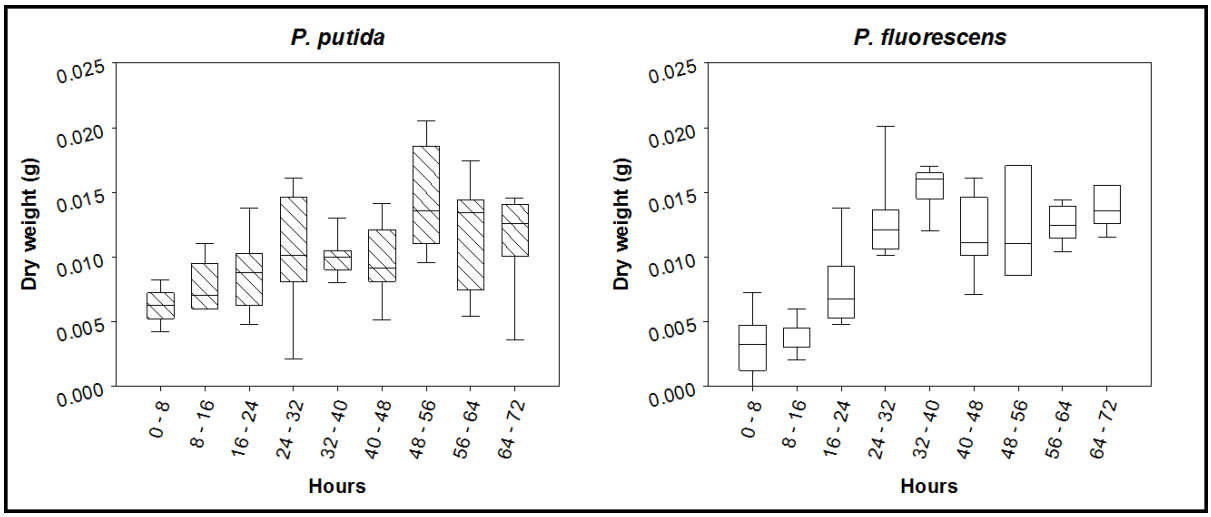

Figure. 2. Representative shadow projections of GFP-tagged *P. putida* and mCherry-tagged *P. fluorescens* biofilms grown for 0, 24, 48 and 72 hours. White scale bar represent 10µm.

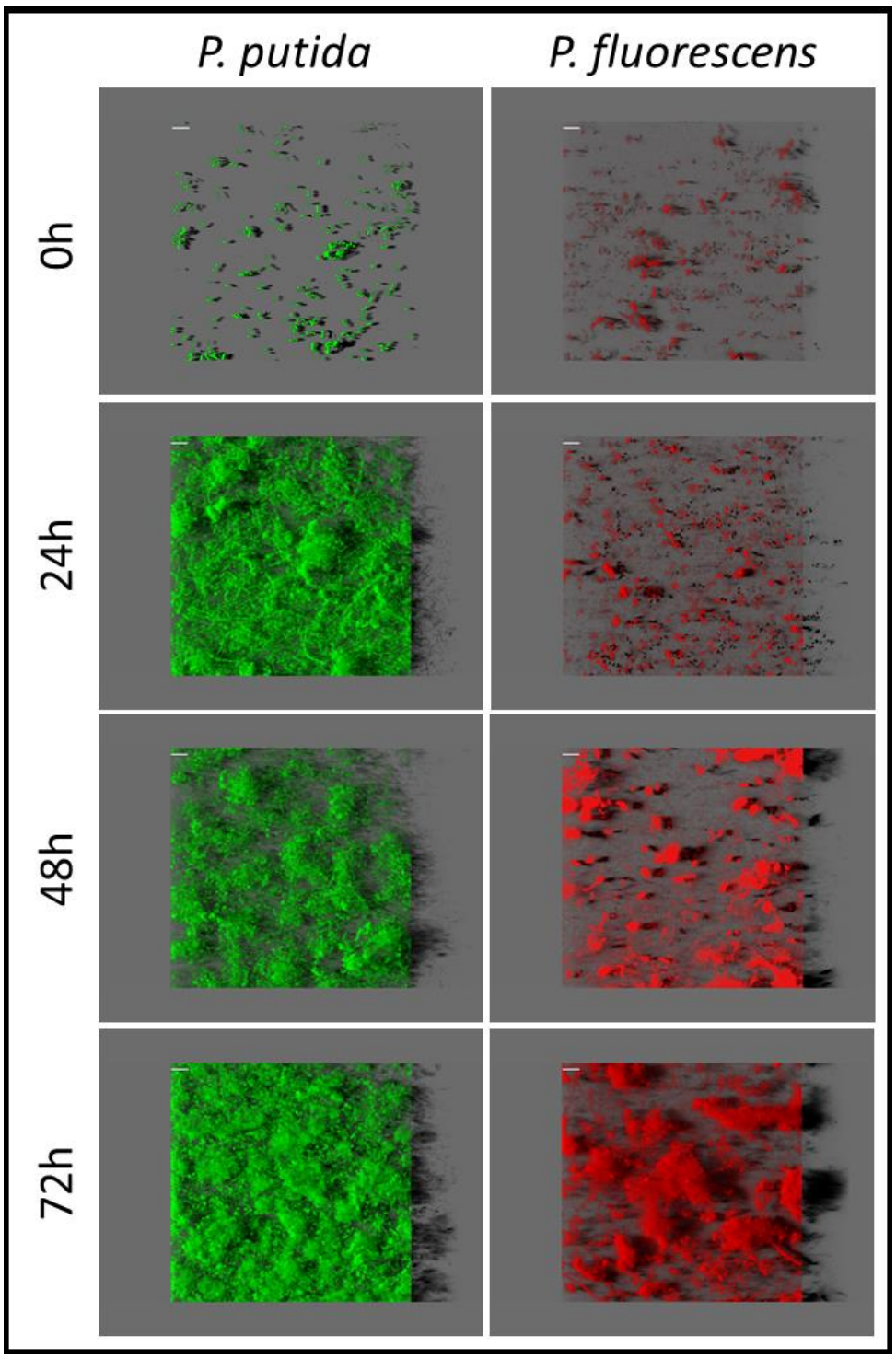

Figure. 3. Biofilm proprieties analysed by PHLIP of *P. putida* (A, B, C) and *P. fluorescens* (D, E, F) following dynamic growth at 0, 24, 48 and 72 hours. Each box represents at least 12 z-stacks from 3 independent biofilms.

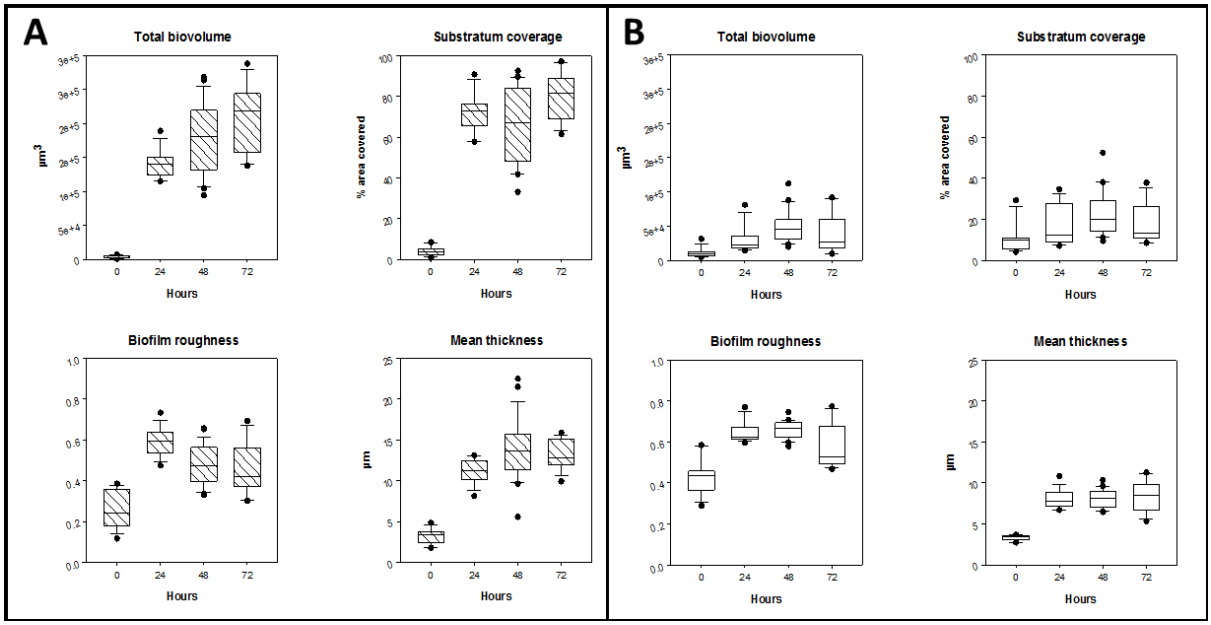

Figure. 4. Dry weight measurements from collected detached biomass during the 72 hours runs. Samples were taken every 8 hours in triplicates for three independent flow cell channels.

Figure. S1. Experimental growth curves of *P. putida* and *P. fluorescens* cells grown at 28^{0}C and 200 RPM. Each points is the mean value of at least 5 absorbance (λ600) measurements from five independent cultures. Error bar represent standard deviation of the mean.

Figure. S2. Dry weight measurements from collected detached biomass during the 24 and 48 hours runs. Samples were taken every 8 hours in triplicates for three independent flow cell channels.

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