

The role of hydrodynamic stress on the phenotypic characteristics of single and binary biofilms of *Pseudomonas fluorescens*

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Abstract This study investigates the phenotype of turbulent ($Re = 5,200$) and laminar ($Re = 2,000$) flow-generated *Pseudomonas fluorescens* biofilms. Three *P. fluorescens* strains, the type strain ATCC 13525 and two strains isolated from an industrial processing plant, D3-348 and D3-350, were used throughout this study. The isolated strains were used to form single and binary biofilms. The biofilm physiology (metabolic activity, cellular density, mass, extracellular polymeric substances, structural characteristics and outer membrane proteins [OMP] expression) was compared. The results indicate that, for every situation, turbulent flow-generated biofilms were more active ($p < 0.05$), had more mass per cm^2 ($p < 0.05$), a higher cellular density ($p < 0.05$), distinct morphology, similar matrix proteins ($p > 0.1$) and identical (isolated strains – single and binary biofilms) and higher (type strain) matrix polysaccharides contents ($p < 0.05$) than laminar flow-generated biofilms. Flow-generated biofilms formed by the type strain revealed a considerably higher cellular density and amount of matrix polysaccharides than single and binary biofilms formed by the isolated strains ($p < 0.05$). Similar OMP expression was detected for the several single strains and for the binary situation, not dependent on the hydrodynamic conditions. Binary biofilms revealed an equal coexistence of the isolated strains with apparent neutral interactions. In summary, the biofilms formed by the type strain represent, apparently, the worst situation in a context of control. The results obtained clearly illustrate the importance of considering strain variation and hydrodynamics in biofilm development, and complement previous studies which have focused on physical aspects of structural and density differences.

Keywords Binary biofilms; biofilm structure and activity; hydrodynamic conditions; phenotypic characterisation; strain variation; turbulent and laminar flows

Introduction

The formation of biofilms is a universal bacterial survival strategy. Important mechanisms involved in biofilm formation and control are complex and only partially understood. A representative biofilm characterisation is essential in order to develop reliable methods to eliminate specific sources of bacterial contamination associated with biofilms. Furthermore, detailed knowledge of the environmental conditions on biofilm characteristics and behaviour is essential in order to create effective control strategies. One of the most important factors affecting biofilm structure and behaviour is the velocity field of the fluid in contact with the microbial layer (Vieira *et al.*, 1993; Stoodley *et al.*, 1999; Pereira *et al.*, 2002). In an engineered sense, the hydrodynamic conditions can be manipulated, as a control parameter. Hydrodynamic conditions will determine the rate of transport of cells and nutrients to the surface, as well as the magnitude of shear forces acting on a developing biofilm. Thus, these conditions significantly influence many of the processes involved in biofilm development (Vieira *et al.*, 1993).

Another important feature encountered in real conditions is the heterogeneity of the microbial community. In fact, significant proportions of all microorganisms are associated in complex multi-species biofilms, performing community level processes (Fux *et al.*,

2005; Møller *et al.*, 1998). According to Fux *et al.* (2005), growth within mixed communities in real world complex environments contrasts with the standardised and idealised conditions in laboratory conditions and it appears that no bacterial strain can truly represent its species. Microbial diversity provides a reservoir of strains with different physiological traits that benefit the performance and stability of the best-adapted strain alone (von Canstein *et al.*, 2002).

The main objective of this study was to evaluate the influence of hydrodynamic conditions and strain variation on biofilm formation and phenotype of *Pseudomonas fluorescens* single and binary biofilms.

Materials and methods

Bacteria

Three different *P. fluorescens* strains were used throughout this work: the type strain (13525) purchased from the American Type Culture Collection used to form single biofilms; two strains isolated from an industrial process (Dogan and Boor, 2003) – D3-348 (extracellular protease negative) and D3-350 (extracellular protease positive) used to form single and binary biofilms.

Bacterial growth was performed in a 0.5 L chemostat (Quickfit, MAF4/41, England) aerated (air flow rate = 0.425 min^{-1}), agitated with a magnetic stirrer (Heidolph Mr 3001), and continuously fed (10 mL/h) with growth media consisting of 5 g/L glucose, 2.5 g/L peptone and 1.25 g/L yeast extract, in phosphate buffer at pH 7 (Simões *et al.*, 2005a). This culture was used to inoculate continuously a 3.5 L reactor also aerated (air flow rate = 0.243 min^{-1}) and agitated. The resulting bacterial suspension was pumped up, passing through the flow cell reactors described elsewhere (Pereira *et al.*, 2001; Simões *et al.*, 2003a, 2005c) and back to the 3.5 L reactor.

Flow cell reactor

A flow cell described by Pereira *et al.* (2001) was used to form biofilm under different flow conditions on ASI 316 stainless steel slides. It consists of a semi-circular polymethyl methacrylate (PMMA) duct with several apertures on its flat face to fit several coupons where biofilm formation surfaces ($1.75 \times 1.25 \text{ cm}$) were glued. These surfaces were ASI 316 stainless steel slides, and were in contact with the fluid circulating in the system.

Biofilms were formed by recirculating the bacterial suspension, obtained from the 3.5 L reactor at 27°C and pH 7, through two similar flow cell reactors operating in parallel, each one with ten slides for biofilm sampling. One of the flow cells was used to promote laminar flow ($Re = 2000$, $u = 0.204 \text{ m/s}$) and the other turbulent flow ($Re = 5,200$, $u = 0.532 \text{ m/s}$). The biofilms were allowed to grow for 7 days to ensure that steady-state biofilms were used in every experiment (Pereira *et al.*, 2001).

The 3.5 L reactor was continuously fed (1.7 L/h) with sterile minimal nutrient medium containing 50 mg/L glucose, 25 mg/L peptone, 12.5 mg/L yeast extract in phosphate buffer (pH 7) and *P. fluorescens* in the exponential phase of growth, provided by the 0.5 L chemostat, as referred to previously, in order to circulate a diluted bacterial suspension ($6 \times 10^7 \text{ cells/mL}$) in the flow cells. In the case of binary biofilms, two independent 0.5 L chemostats were used (one with D3-348 and the other with D3-350). The flow cell system was inoculated with the two strains and was fed with minimal nutrient medium at a flow rate two times higher (3.4 L/h) than the one used for biofilm formation by a single strain, in order to obtain the adequate dilution rate.

Sampling of biofilms

The biofilm that covered the stainless steel slides was entirely removed using a stainless steel scraper and then resuspended into 10 mL of extraction buffer (2 mM Na_3PO_4 , 2 mM NaH_2PO_4 , 9 mM NaCl and 1 mM KCl, pH 7) and homogenised in a vortex (Heidolph, model Reax top) for 30 sec with 100% power input, according to the methodology described by Simões *et al.* (2005a). Binary biofilms were also streaked onto skim milk agar (Merck, VWR, Portugal) in order to estimate the proportion of protease negative and positive bacteria. Homogenised biofilm suspensions were then used to assess, sequentially, morphological characteristics, respiratory activity, extracellular polymeric substances content, outer membrane proteins (OMP) expression and mass.

Extraction of extracellular polymeric substances

Extraction of the extracellular components of the biofilm was carried out using Dowex resin (50X 8, NA^+ form, 20–50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frølund *et al.* (1996). The biofilms were resuspended in 20 mL of extraction buffer and 50 g of Dowex resin per gram of volatile solids were added to the biofilm suspension and the extraction took place at 400 min^{-1} for 4 hours at 4°C (Simões *et al.*, 2005a). The extracellular components were separated from the cells through centrifugation ($3,777 g$, 5 min).

Respiratory activity assessment

Biofilm respiratory activity assays were performed in a model 53 Yellow Springs Instruments (OH, USA) biological oxygen monitor (BOM) as described previously (Simões *et al.*, 2003a, 2005b). The homogenised biofilm suspensions were placed in a temperature-controlled vessel of the BOM ($T = 27^\circ\text{C} \pm 1^\circ\text{C}$). Each one contains a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation ($[\text{O}_2] = 9.2 \text{ mg/L} - 27^\circ\text{C}$, 1 atm). The vessel was closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, $50 \mu\text{L}$ of a glucose solution (100 mg/L) was introduced into each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to the glucose oxidation and was expressed as $\text{mg O}_2/\text{g}_{\text{biofilm}} \cdot \text{min}$.

Biomass quantification

The dry mass of the biofilm accumulated on the stainless steel slides, after the respiratory activity determination, was assessed by the determination of the total volatile solids of the homogenised biofilm suspensions, according to the *Standard Methods* (1989), method number 2540 A-D. The biofilm mass accumulated was expressed in mg of biofilm per cm^2 of surface area of the slide ($\text{mg}_{\text{biofilm}}/\text{cm}^2$).

Proteins and polysaccharides

The proteins were determined by the Lowry modified method using bovine serum albumin as standard (Sigma, Portugal, Cat. No. P5656) and the polysaccharides by the phenol-sulphuric acid method of Dubois *et al.* (1956) using glucose as standard.

Direct cell counts

After the extraction procedure, the cells separated from the extracellular products were diluted to an adequate concentration, microfiltrated through a Nucleopore[®] (Whatman)

black polycarbonate membrane (pore size 0.22 μm) being thereafter stained with 4',6-diamidino-2-phenylindole – DAPI (Sigma Cat. No. D-9542), a DNA binding stain, as described by Saby *et al.* (1997) and inspected by epifluorescence microscopy (Zeiss AXIOSKOP). The optical filter combination for optimal viewing of stained preparations consisted of a 359 nm excitation filter in combination with a 461 nm emission filter. The micrographs were obtained using a microscope camera (AxioCam HRC, Carl Zeiss). A programme path (AxioVision, Carl Zeiss Vision) was used for image acquisition and processing. Another programme path (Sigma Scan Pro 5) involving object measurement and data output was used to quantify the number of cells.

The mean number of cells was determined from counts of a minimum of 20 microscopic fields, for each membrane.

Scanning electron microscopy observations

Scanning electron microscopy (SEM) inspections were performed according to the procedure described by Simões *et al.* (2003b). The SEM inspections always comprised the observation of at least 15 fields of each biofilm-covered slide. Prior to SEM observations, biofilm samples were gradually dehydrated in an ethanol (Merck) series to 100% (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100% v/v), and dried in a desiccator for 3 days. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10–15 kV. SEM observations were documented through the acquisition of at least 20 representative microphotographs for each experiment.

Statistical analysis

The data were analysed using the statistical programme SPSS version 14.0 (Statistical Package for the Social Sciences). Since low sample numbers contributed to uneven variation, non-parametric Wilcoxon test procedure was used to compare biofilm characteristics. Statistical calculations were based on confidence levels equal to or higher than 95% ($p < 0.05$ was considered statistically significant).

Results and discussion

Turbulent and laminar flow-generated biofilms – metabolic activity, mass and cellular density

Figure 1 presents the respiratory activity, mass and cellular density of turbulent and laminar flow-generated biofilms formed by the several *P. fluorescens* strains as single and binary (by the industrial isolates) biofilms.

Turbulent flow-generated biofilms are markedly distinct in terms of respiratory activity, mass and cellular density from the ones formed under laminar flow (Figure 1). This phenomenon is evident for every strain as single and binary biofilms. Figure 1(a) shows that biofilms formed under turbulent flow were more active than the laminar flow-generated ones ($p < 0.05$). Turbulent flow-generated biofilms formed by the type strain presented the higher respiratory activity values, while D3-348 strain formed biofilms under turbulent flow with the smaller respiratory activity values. Concerning laminar flow-generated biofilms, the higher respiratory activity values were found for the single isolated strains, the type strain biofilms being the ones that presented the smaller respiratory activity. Comparing the respiratory activity of the four distinct biofilms grown under turbulent and laminar flow conditions, they are closely related within the same flow regime ($p > 0.1$).

Biofilm mass results (Figure 1(b)), show that single and binary biofilms formed under turbulent flow had more mass per cm^2 than the ones formed under laminar flow ($p < 0.01$). Similar mass values were found for the turbulent flow-generated biofilms formed by the type strain, D3-348 and the binary strains, the biofilms formed by the

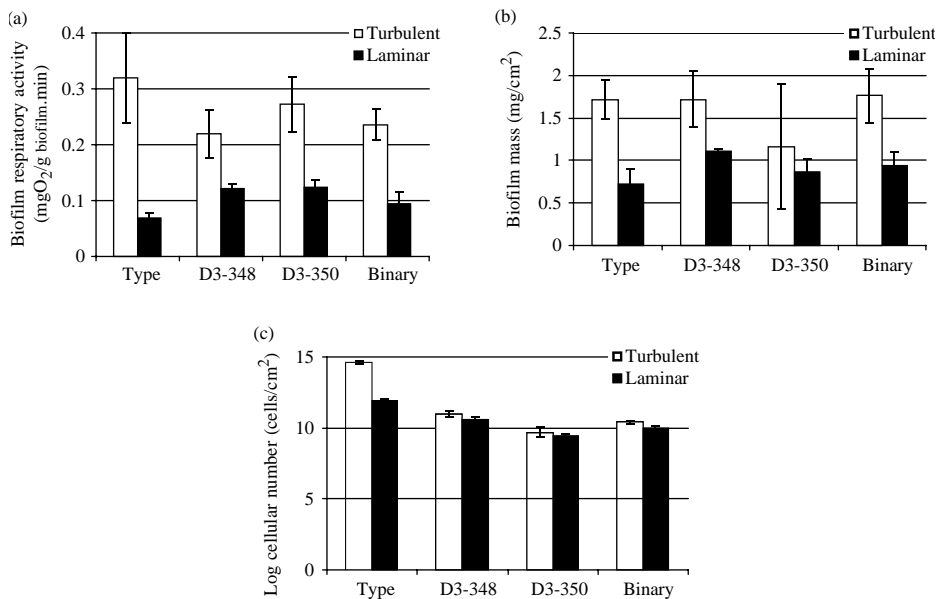


Figure 1 Biofilm respiratory activity (a), mass (b) and cellular density (c) of the several *P. fluorescens* single and binary turbulent and laminar flow-generated biofilms

strain D3-350 being the ones that presented the smaller mass values. However, a statistical analysis of the mass values for the turbulent flow-generated biofilms revealed that they are similar ($p > 0.05$). Although statistical analysis revealed that laminar flow-generated biofilms presented similar mass values ($p > 0.1$), D3-348 strain had the ability to form more mass than the single D3-350 strains and the binary biofilms. The type strain had the lower biofilm mass formation properties under laminar flow conditions.

Biofilms formed under turbulent flow had a higher cellular density per cm² for every situation studied (Figure 1(c)) than the ones formed under laminar flow ($p < 0.05$). The type strain formed both turbulent and laminar flow-generated biofilms with the highest cellular density when compared with the isolated strains as single and binary biofilms ($p < 0.05$). Biofilms formed by the D3-350 strain had the smaller cellular density for the two hydrodynamic conditions tested. Comparing statistically the single and binary biofilms formed by the isolated strains (D3-348 and D3-350) and for the same hydrodynamic conditions, they were similar ($p > 0.05$). Binary biofilm community analysis in terms of relative strain abundance (growth on skim milk medium for detection of protease negative and positive bacteria) revealed that both turbulent and laminar flow-generated biofilms were equally colonised by both strains (50% each strain, $p > 0.1$). Furthermore, the OMP from cells within the several biofilms were isolated and analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), revealing no significant variation within the several strains used, with the biofilm formation under different hydrodynamic conditions and for the binary biofilms (results not shown).

Turbulent and laminar flow-generated biofilms – EPS and structure

Figure 2 presents the matrix proteins and polysaccharides content of turbulent and laminar flow flow-generated biofilms formed by the several *P. fluorescens* strains as single and binary biofilms.

From Figure 2(a) it can be seen that the amount of matrix proteins is similar when comparing turbulent and laminar flow-generated biofilms for the several strains as single and binary biofilms ($p > 0.1$). Matrix polysaccharides contents were different ($p < 0.05$)

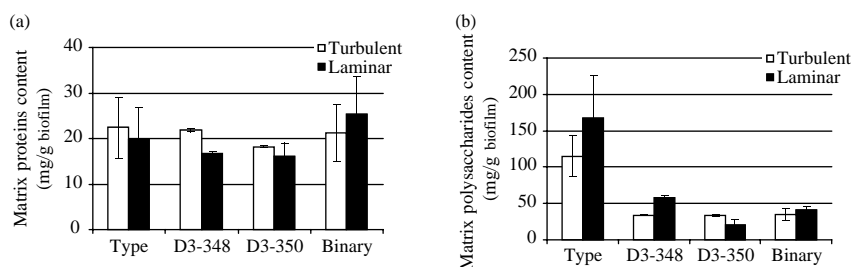


Figure 2 Matrix proteins (a) and polysaccharides (b) content of the several *P. fluorescens* single and binary turbulent and laminar flow-generated biofilms

when comparing turbulent and laminar flow-generated biofilms between the several studied single and binary strains biofilms. Biofilms formed by the type strain had the higher amount of matrix polysaccharides for both turbulent and laminar flow-generated biofilms than single and binary turbulent and laminar flow-generated biofilms formed by the isolated strains (Figure 2(b)). Matrix polysaccharides content of the isolated strains as single and binary biofilms is similar ($p > 0.1$) when comparing hydrodynamic similar biofilms. The matrix polysaccharides content comparison between the isolated strains' biofilms and the type strain revealed a significant statistical difference ($p < 0.05$) for both turbulent and laminar conditions. In a biofilm control process, the barrier protection provided by the EPS matrix increased the resistance of biofilm-resident bacteria to environmental stress conditions (Simões *et al.*, 2005a). When antimicrobial agents are applied in the control of biofilms, besides the physical hindrance of antimicrobial diffusion, the protective property of the EPS matrix could also be due to others factors such as absorption or catalytic destruction of the aggressive agent on the biofilm surface (Simões *et al.*, 2003b).

The physiological parameters analysed (Figures 1 and 2) play a significant role in the context of biofilm control using chemical agents (Simões, 2005). Comparing the effect of turbulent flow conditions on the studied physiology of the several different strain biofilms (Figures 1 and 2), statistical similarity was detected for the single and binary biofilms when comparing the metabolic activity, mass and matrix proteins. Turbulent and laminar flow-generated biofilms formed by the type strain revealed a considerably higher cellular density and amount of matrix polysaccharides than the biofilms formed by the isolated strains (single and binary); consequently, in the context of biofilm control by chemical agents, biofilms formed by the type strain represent, apparently, the worst situation.

Concerning the distinct properties of turbulent and laminar flow-generated biofilms, according to several authors (Stoodley *et al.*, 1999; Vieira *et al.*, 1993), they are mainly related to the cellular responses to the hydrodynamic stress and due to the passive transport of cells and nutrients by the flow through the biofilm. A cascade of parameters such as mass transport, substrate concentrations, diffusion gradients, detachment–attachment mechanisms, relationships between different microorganisms and flow rates arguably have significant influence on biofilm structure and behaviour.

Figure 3 displays SEM microphotographs representative of the several fields observed in each biofilm-covered metal surface.

As observed in Figure 3, biofilms grown under turbulent flow look very different from the ones grown under laminar flow. The existence of a higher number of cells in the biofilms formed under turbulent flow is evident as well as an almost nonexistent biofilm matrix when compared with the laminar flow-generated biofilms. Therefore, the hydrodynamic conditions play an important role in the biofilm architecture, being responsible for the differences obtained (Pereira *et al.*, 2002). Additionally, some morphological differences are encountered when comparing the different strains and association (single

or binary) for the same hydrodynamic condition. These differences can be, in part, associated with the different biofilm characteristics emphasised by Figures 1 and 2.

Although the morphology of the biofilm may have been altered by the dehydration process, SEM results provide good comparative information and demonstrate the existence of heterogeneity within the biofilms (Figure 3). This heterogeneity could be behind the reduced susceptibility of sessile microorganisms to antimicrobial agents, which could

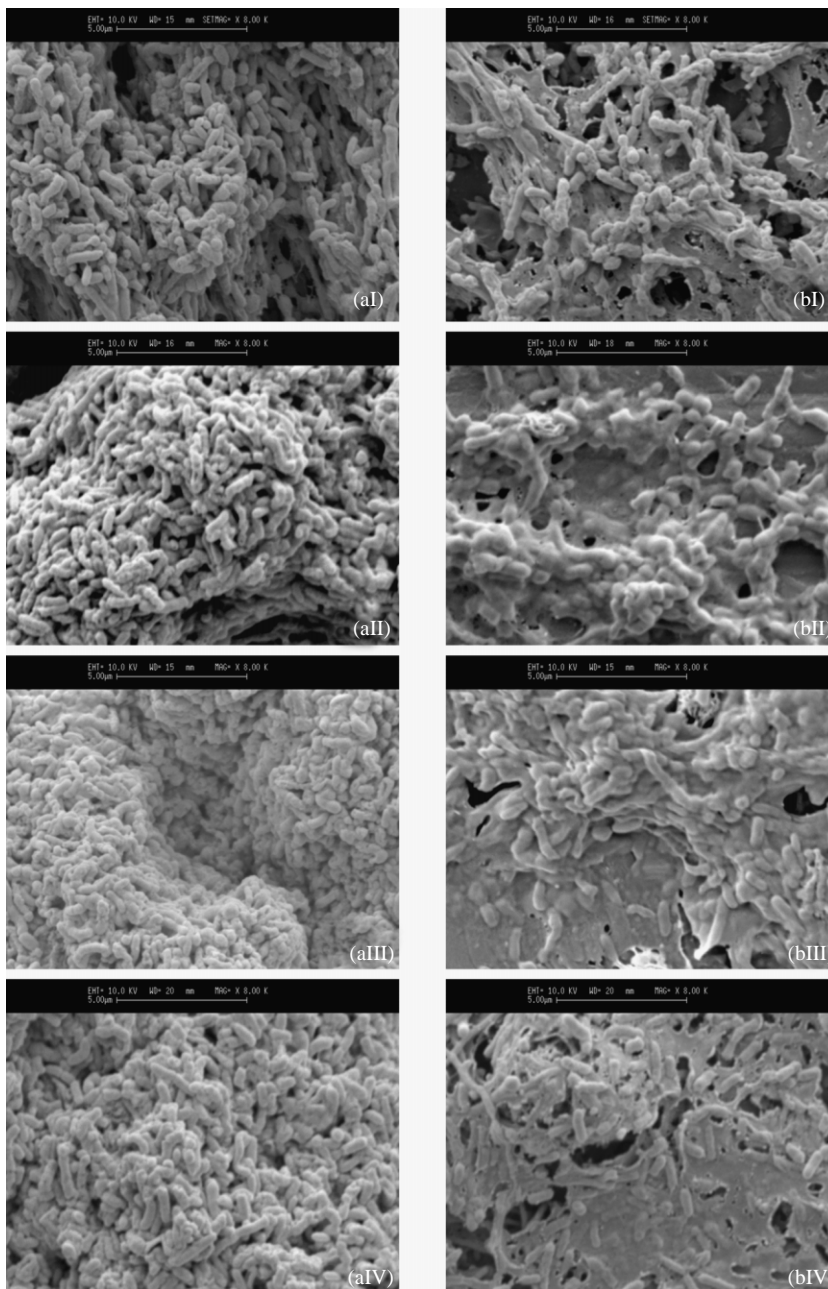


Figure 3 SEM photomicrographs of 7-day old biofilms formed under turbulent (a) and laminar (b) flow by *P. fluorescens* type strain (I), D3-348 (II), D3-350 (III) and by the binary isolated strains (IV). 8000X magnification, bar = 5 µm

lead to the existence of persistent microorganisms that are recalcitrant to further treatments (Simões *et al.*, 2005a). Figure 3 also reinforces that both turbulent and laminar flow-generated binary biofilms did not show evidence of remarkable differentiation as a consequence of the interactions promoted by the two isolated strains. Previous studies using *Pseudomonas* spp. in binary species biofilms (Cowan *et al.*, 2000) demonstrated the potential for commensal interactions in the presence of organic compounds. Other apparent protective effects found within microorganisms have been mentioned (Møller *et al.*, 1998; Whiteley *et al.*, 2001). The binary biofilms studied present characteristics (Figures 1, 2 and 3) that seem to be an average of the values of each single biofilm, evidencing neutral interactions between the two isolated bacteria.

Conclusions

The data provided in this study emphasise that *P. fluorescens* biofilm formation under different hydrodynamic conditions triggers significant quantitative metabolic, morphological and biochemical changes, illustrating the importance of considering hydrodynamics in biofilm development, and complements previous studies which have focused on physical aspects of structural and density differences. The results found for the type strain were comparable with the ones obtained with the isolated strains as single and binary biofilms, except for the cellular density and matrix polysaccharides content, highlighting the phenomenon of strain variation. Biofilms formed by the type strain represent, apparently, the worst situation in the context of control. Binary biofilms reveal an apparent coexistence of the isolated strains with neutral interactions.

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