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# Macroscale *versus* microscale methods for physiological analysis of biofilms formed in 96-well microtiter plates

L.C. Gomes [a], J.M.R. Moreira [a], J.M. Miranda [b], M. Simões [a], L.F. Melo [a], F.J. Mergulhão [a,*]

[a] LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, Porto, Portugal
[b] CEFT, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Rua Dr. Roberto Frias, Porto, Portugal

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ABSTRACT

Microtiter plates with 96 wells have become one of the preferred platforms for biofilm studies mainly because they enable high-throughput assays. In this work, macroscale and microscale methods were used to study the impact of hydrodynamic conditions on the physiology and location of *Escherichia coli* JM109(DE3) biofilms formed in microtiter plates. Biofilms were formed in shaking and static conditions, and two macroscale parameters were assayed: the total amount of biofilm was measured by the crystal violet assay and the metabolic activity was determined by the resazurin assay. From the macroscale point of view, there were no statistically significant differences between the biofilms formed in static and shaking conditions. However, at a microscale level, the differences between both conditions were revealed using scanning electron microscopy (SEM). It was observed that biofilm morphology and spatial distribution along the wall were different in these conditions. Simulation of the hydrodynamic conditions inside the wells at a microscale was performed by computational fluid dynamics (CFD). These simulations showed that the shear strain rate was unevenly distributed on the walls during shaking conditions and that regions of higher shear strain rate were obtained closer to the air/liquid interface. Additionally, it was shown that wall regions subjected to higher shear strain rates were associated with the formation of biofilms containing cells of smaller size. Conversely, regions with lower shear strain rate were prone to have a more uniform spatial distribution of adhered cells of larger size. The results presented on this work highlight the wealth of information that may be gathered by complementing macroscale approaches with a microscale analysis of the experiments.



## 1. Introduction

Bacteria and other organisms tend to attach to solid surfaces where they grow and produce extracellular polymeric substances (EPS), forming a biofilm comprised either of a single or multiple species (Costerton et al., 1999; Davey and O'Toole, 2000; Stoodley et al., 2002). Biofilms are often unwanted and have severe effects in industrial and biomedical settings. Besides causing additional problems in cleaning and disinfection, biofilms may cause energy losses and blockages in membrane systems and heat exchangers, a phenomenon known as biofouling. Biofouling costs can represent up to 30% of the entire plant operating costs in membrane systems (Melo and Flemming, 2010) and, in specific cases such as power and desalination plants, they may exceed $15 billion per year (Melo and Flemming, 2010). But the biofilms enjoying the worst reputation are undoubtedly those found in the health sector (Bryers, 2008) because they are responsible for more than 60% of all microbial infections (Shunmugaperumal, 2010). Overall, a large percentage of biofilm-related infections is associated with indwelling medical devices (Hancock et al., 2007). For instance, catheter-associated urinary tract infection (UTI) accounts for more than 1 million cases in United States hospitals and nursing homes (Tambyah and Maki, 2000) and the estimated annual cost of caring for patients with these infections is approximately $2 billion (Foxman, 2003).

Intensive studies on the mechanisms of biofilm formation and resistance have prompted the development of multiple *in vitro* platforms where biofilms are formed to simulate "real life" biofilms. When using these systems in the laboratory, it is important to choose the correct operational conditions in order to obtain biofilms that resemble those found in natural environments (Moreira et al., 2013a). Microtiter plates with 96 wells enable a high-throughput, are easy to handle and are cheap to use as only small volumes of reagents are required (Coenye and Nelis, 2010; Duetz, 2007). Additionally, microtiter plates allow "multiplexing", *i.e.*, multiple organisms and/or treatments can be included in a single run (Coenye and Nelis, 2010), and several well established protocols are available for determination of macroscale parameters related to the biofilm (Table 1). Microtiter plates have been extensively used for biofilm studies addressing microbial adhesion (Moreira et al., 2013a; Simões et al., 2010), biofilm inhibition (Cady et al., 2012; Lee et al., 2011) and screening of antimicrobial compounds

* Corresponding author at: Department of Chemical Engineering, Faculty of Engineering University of Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal. Tel.: +351 225081668; fax: +351 5081449.
*E-mail address:* filipem@fe.up.pt (F.J. Mergulhão).

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**Table 1**
Macroscale methodologies used on microtiter plate operation (adapted from Azevedo et al. (2009)).

| Parameter | Method | Reference |
|---|---|---|
| Biofilm biomass | CV assay | Costerton et al. (1999) and Stepanovic et al. (2000) |
| Microbial physiological activity | Fluorescein diacetate assay | Honraet et al. (2005) and Peeters et al. (2008) |
| | Resazurin assay | Peeters et al. (2008) |
| | XTT* assay | Honraet et al. (2005) and Peeters et al. (2008) |
| Microbial cells in the biofilm | Syto9 assay | Honraet et al. (2005) and Peeters et al. (2008) |
| Biofilm matrix | Dimethyl methylene blue assay | Toté et al. (2008) |

* 2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide.

(Pitts et al., 2003; Quave et al., 2008; Shakeri et al., 2007). The lack of detailed knowledge of the hydrodynamics in microtiter plate systems has been, however, the major drawback of this technique in many reported studies (Moreira et al., 2013a).

Despite the recent development of techniques for the macroscale characterization of biofilms, microscopy is still used to analyze morphological and structural details of biofilms *in vitro*, especially in flow cells and on coupons retrieved for microtiter plates (Hannig et al., 2010). The complexity and structural heterogeneity of biofilms have been unraveled by techniques such as scanning electron microscopy and confocal laser scanning microscopy (CSLM). SEM can be used to produce clear images of microorganisms on opaque substrata across a wide range of magnifications (Norton et al., 1998). At higher magnifications (up to 100,000×), individual cells are readily distinguished (Serra et al., 2013; Stewart et al., 1995). For these reasons, it is recognized that SEM gives a detailed insight into the ultrastructure of bacteria and their environment (Alhede et al., 2012; Hannig et al., 2010), although it does not allow observation of the biofilm interior (Stewart et al., 1995). The main difficulty of SEM is that sample preparation might induce shrinkage and other structural changes due to dehydration (Hannig et al., 2010). Conversely, CLSM allows *in situ* and real-time examination of biofilms, but lower magnification and resolution are available (Fedel et al., 2007; Stewart et al., 1995). The use of the CLSM is also useful to obtain clear photomicrographs of densely packed microorganisms (Palmer and Sternberg, 1999). Mainly because of its excellent resolution properties, SEM will, in spite of its limitations, continue to be an important tool for biofilm research.

This study compares two well established macroscale techniques (crystal violet and resazurin assay), that provide bulk data from a biofilm, with SEM visualization for the analysis of biofilms formed in 96-well microtiter plates. Analysis of the hydrodynamics at a microscale using computational fluid dynamics (CFD) provided additional information that can explain the results obtained with SEM. Biofilm formation in microtiter plates was performed in shaking and static conditions because it is widely known that hydrodynamics influence biofilm development (Liu and Tay, 2002; Stoodley et al., 2001; Wäsche et al., 2002), not only in terms of nutrient and oxygen supply (Moreira et al., 2013a, 2013b), but also by the shear forces, which can modulate cell adhesion to a surface (Busscher and van der Mei, 2006; Simões et al., 2007; Teodósio et al., 2013; van Loosdrecht et al., 1995). CFD is a numerical simulation technique that enables the simulation of fluid flow and/or heat/mass transfer problems, providing fast and detailed information of general flow phenomena and other parameters which are difficult to obtain experimentally (Xia and Sun, 2002). CFD has the advantage of allowing a quick assessment of different alternatives and changes, including geometrical and flow parameters, without spending much time and resources (Hilgenstock and Ernst, 1996). Prior to CFD simulation, a computational model must be generated and a meshing operation is performed. Meshing corresponds to the division of the region under study into numerous elements (also known as cells) where the equations from the computational model are solved (Xia and Sun, 2002). CFD is a microscale fluid analysis technique that can be performed at different precision levels much like microscopy where the use of different magnifications can provide different levels of detail. In fact, in microscopy, the use of higher magnifications increases the detail, but this is obtained at a higher operational cost as the number of images that are required to analyze the same region of interest increases. Likewise, in CFD, the use of a more refined mesh (with a higher number of cells of smaller volume) increases numerical accuracy but also with an increased computational cost as the simulations will take longer to run. The main objective of this work was to compare macroscale and microscale methods that can be used for biofilm analysis to see if the information that can be extracted from them is redundant or if they provide complementary data. This information was used to assess the effect of hydrodynamics on cell physiology and biofilm location.

## 2. Materials and methods

### 2.1. Bacterial growth

*Escherichia coli* JM109(DE3) from Promega (USA) was used to produce biofilms because this strain had already demonstrated a good biofilm formation capacity at 30 °C in the same culture medium used in this work (Teodósio et al., 2011). An overnight culture of *E. coli* was obtained by inoculation of 500 μl of a glycerol stock (kept at −80 °C) to a total volume of 0.2 l of inoculation media previously described by Teodósio et al. (2011). This consisted of 5.5 g $l^{-1}$ glucose, 2.5 g $l^{-1}$ peptone, and 1.25 g $l^{-1}$ yeast extract in phosphate buffer (1.88 g $l^{-1}$ $KH_2PO_4$ and 2.60 g $l^{-1}$ $Na_2HPO_4$), pH 7.0. The culture was grown on a 1 l shake-flask, incubated overnight at 30 °C with orbital agitation (120 rpm). Cells were harvested by centrifugation (for 10 min at 3202 *g*) and appropriate dilutions in sterile saline (NaCl 0.85%) were performed to obtain an inoculum containing approximately $10^8$ cells $ml^{-1}$.

### 2.2. Biofilm formation in 96-well microtiter plates

Biofilms were produced by pipetting 20 μl of inoculum into six wells of sterile 96-well polystyrene, flat-bottomed microtiter plates (maximum well volume = 0.39 ml, Orange Scientific, USA, catalogue number 4430100) filled with 180 μl of nutrient media. The media was previously described by Teodósio et al. (2011) and consisted of 0.5 g $l^{-1}$ glucose, 0.25 g $l^{-1}$ peptone, 0.125 g $l^{-1}$ yeast extract and phosphate buffer (0.188 g $l^{-1}$ $KH_2PO_4$ and 0.26 g $l^{-1}$ $Na_2HPO_4$), pH 7.0 (a 10× dilution of the inoculation medium described in Section 2.1). Microtiter plates were placed for 24 h at 30 °C in an orbital shaking incubator (50 mm of orbital diameter) at 150 rpm (CERTOMAT® BS-1, Sartorius AG, Germany) and without shaking.

### 2.3. Biofilm analysis

#### 2.3.1. Macroscale assays

The total amount of biofilm formed was measured by the crystal violet (CV) assay and the metabolic activity was determined by the resazurin assay.

The CV assay is frequently used for quantification of biofilm biomass (Pitts et al., 2003) because crystal violet binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al., 2003). The procedure used in this work was fully described by Moreira et al. (2013a) and it has been shown that the results obtained by CV staining can be correlated with those obtained by plate counts (Alnnasouri et al., 2011). After the 24 h incubation, the