Is biofilm formation a critical step for the valorisation of plastic waste?

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ABSTRACT

English

In the past decades plastic production has increased dramatically and its accumulation leads to great impacts on the environment and human health, which have become issues of global concern. Biodegradation has been suggested as a promising solution in the future in terms of plastic waste management. Some microorganisms are able to generate biodegradable plastics and therefore the use of plastic waste as a carbon source for those microorganisms would achieve the upcycling of the plastic waste and their integration in a circular economy system. Biofilm have been proved to be involved in surfaces biodegradation and may have an important role to play in the context presented.

Bacillus amyloliquefaciens, which is known to be a plastic degrading strain, was studied, and in particular its mutant GA1 was chosen given its particular ability to form biofilms. The dynamics of the biofilm formation and maturation was studied to get a better understanding of the biofilm development over a given surface. The biofilm growth was performed using a drip flow bioreactor and the working conditions were optimized in order to promote the biofilm growth and achieve repeatable results between the different reactor chambers and between different experiments. Also the ability of *Bacillus amyloliquefaciens* GA1 biofilm to degrade poly(ethylene terephthalate) (PET) was tested.

In conclusion, the drip flow bioreactor is a powerful tool for the study of biofilms, thanks to its six different culture chambers and low variability between cultivated biofilms. The dynamic study of *Bacillus amyloliquefaciens* GA1 biofilms shows that the biofilm reaches its maturity after 40 hours of culture which causes significant physiological changes that influence its physical and biochemical properties. Furthermore, the biofilm growth over a PET surface appears to impact the PET surface. Observations using a scattering electron microscope (SEM) indicate that the formation of biofilm on a PET surface has an impact on the integrity of the surface.

Français

Au cours des dernières décennies, la production de plastique a considérablement augmenté et son accumulation conduit à de grands impacts sur l'environnement et la santé humaine, qui sont devenus des problématiques à l'échelle mondial. La biodégradation a été suggérée comme une solution prometteuse en termes de gestion des déchets plastiques. Certains microorganismes sont capables de générer des matières plastiques biodégradables et, par conséquent, l'utilisation de déchets plastiques comme source de carbone pour ces microorganismes permettrait "l'upcycling" des déchets plastiques et leur intégration dans un système d'économie circulaire. Le biofilm s'est avéré être impliqué dans la biodégradation de surfaces et peut avoir un rôle important à jouer dans le contexte présenté.

Bacillus amyloliquefaciens, qui est connue pour être une souche capable de dégrader certains plastiques, a été étudié, et en particulier son mutant GA1 a été choisi en raison de sa capacité à former des biofilms. La dynamique de formation et de maturation du biofilm a été étudiée pour mieux comprendre le développement du biofilm sur une surface donnée. La culture des biofilms a été effectuée à l'aide d'un "drip flow bioreactor" et les conditions de travail ont été optimisées afin de favoriser la croissance du biofilm et d'obtenir des résultats répétables entre les différentes chambres de réacteur et entre différentes expériences. De plus, la capacité du biofilm *Bacillus amyloliquefaciens* GA1 à dégrader le poly(éthylène terephthalate) (PET) a été testée.

En conclusion, le "drip flow bioreactor" constitue un outil puissant pour l'étude des biofilms, grâce à ses six chambres de culture différentes et la faible variabilité entre les biofilms cultivés. L'étude dynamique *Bacillus amyloliquefaciens* GA1 montre que le biofilm atteint sa maturité après 40 heures de culture ce qui entraîne des changements physiologiques significatifs qui influencent ses propriétés physiques et biochimiques. Des observations à l'aide d'un microscope électronique à balayage (SEM) permettent d'affirmer que la formation de biofilm sur une surface de PET a un impact sur l'intégrité de la dite surface.

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Figures

Tables

INTRODUCTION

1. Plastic waste: global overview

1.1. Overview about plastic

Plastics are generally defined as a synthetic carbon polymers derived from the petrochemical industry. Each polymer is composed of 10,000 to 100,000,000 monomers. Different polymers can be mixed to create plastics with the properties of the two polymers (Crawford and Quinn, 2017d). Using 8 basic elements (hydrogen, carbon, nitrogen, oxygen, fluorine, silicon, sulphur and chlorine) it is possible to create thousands of different plastics. Plastic general properties makes it a precious material for human for they are light and quite resistant, easy to shape and produce, not subject to corrosion, electrical insulators, colourful and are produced at low cost. Furthermore, each plastic offers particular properties that may serve in a large range of sectors (Alauddin *et al.*, 1995).

Nowadays, over 30% of plastic are used for packaging mostly due to its physical resistance and its impermeability. Plastic is found in applications ranging from car batteries to irrigation and drainage pipes, from drinking straws to football helmets (Shah *et al.*, 2008).

Depending on their technical and chemical behaviour, plastics are divided in two categories: thermosetting plastics and thermoplastics. Thermosetting plastics become solids after being melted by heating. During the solidification, which is called the curing, the small molecules are chemically linked together, impeding the chains to slip and thus the plastic to flow once heated. Thermosetting plastic doesn't melt after they have been solidified, they undergo a chemical breakdown. (Alauddin *et al.*, 1995). On the opposite, thermoplastics can be melted and solidified an unlimited number of times. A large number of cycles may impact the plastic properties. In solid state, thermoplastic have a glassy behaviour (Alauddin *et al.*, 1995).

1.2. Plastic waste and environmental issues

Plastic production has insanely increased over the last decades despite the oil crisis of 1973 and the financial crisis of 2007, going from 15 million of tons in 1964 to 311 tons in 2014 and it's expected to double in the next 20 years (**Figure 1**).

The biggest plastic producers in 2014 were China, Europe and North America, being responsible for 26%, 20% and 19% of the global production respectively (Crawford and Quinn, 2017b).

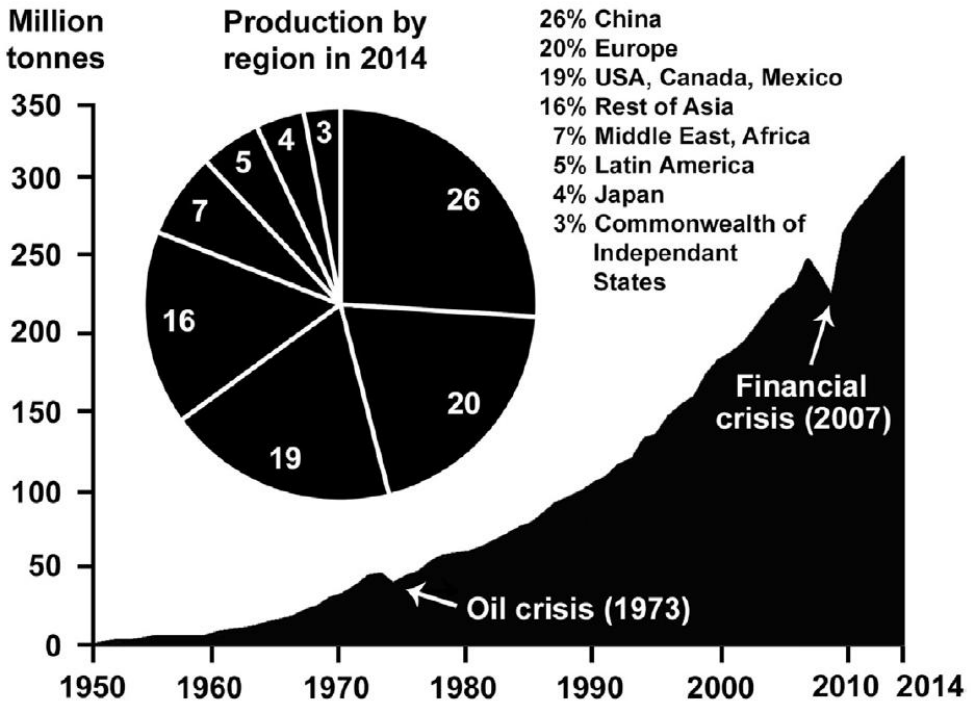

Figure 1: Global production of plastic from 1950 to 2014 (Crawford and Quinn, 2017b)

This huge amount of plastic causes environmental issues, the first of which is the participation to the global warming. Up to 90% of the produced plastic comes from virgin fossil feedstocks which represents 6% of the global oil consumption (Ellen MacArthur Foundation, 2016). The plastic production increases each year and not even the depletion of the fossil feedstock will stop it since we are able now to produce plastic from renewable resources, called "bioplastic". Therefore, it's legitimate to address the impact of plastic production and treatment on the greenhouse gas production.

However, the most concerning environmental issues caused by plastic are focused on the marine environment. In 2016 it was estimated that 8 million tons of plastic per year ended in the ocean and that over 150 million tons were already in. At that rate, in 2050 there'll be as

much more plastic than fish by weight in the oceans (Ellen MacArthur Foundation, 2016). The plastic presence in the oceans is mainly due to open landfills where plastic can be carried by wind or even by water itself in flooding conditions. In addition, many developing countries use open dumps which doesn't prevent plastic from ending in the ocean (Crawford and Quinn, 2017b). Those plastic wastes are classified by their size in two main categories: macroplastics and microplastics. The classification may differ from a source to another. Generally, macroplastics are considered as plastic debris bigger that 5 mm while microplastics are smaller than 5 mm (Arthur, Baker and Bamford (eds), no date; Law, 2017) . Others have more complex definition and classify macroplastics as being bigger than 25 mm and microplastic being between 5 and 1 mm (Crawford and Quinn, 2017b). In this case, the other sizes have their own classification (**Figure 2**).

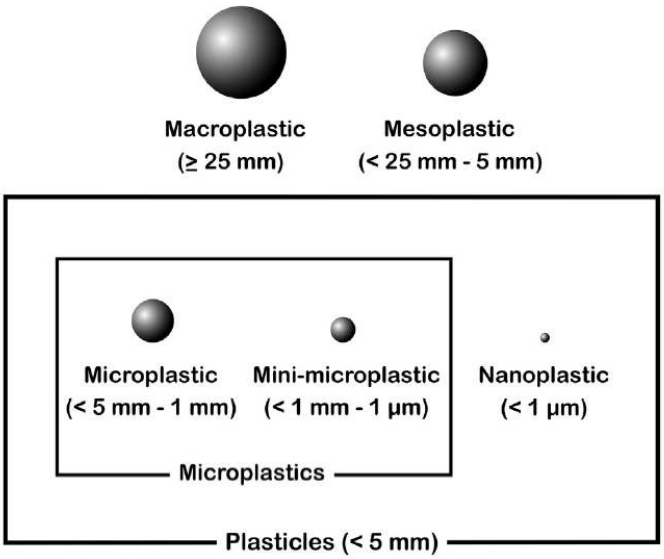

Figure 2: Size classification of plastic debris (Crawford and Quinn, 2017a)

Macroplastics may also be sorted in 3 categories which are floating, beached and submerged plastics depending on where they are found. Floating macroplastics are responsible for the occurrence of gyres in the ocean (Crawford and Quinn, 2017b). Microplastics are classified in primary or secondary microplastics whether they that have been intentionally manufactured or come from macroplastic degradation (Andrady, 2011; Crawford and Quinn, 2017a).

In marine environment, macroplastics and microplactics threatens wildlife. Juvenile animals may become entangled in plastic wastes which then cause injuries and deformations during the animal growth (Webb *et al.*, 2013). Moreover, animals can confuse plastic debris with food. This plastic then remains in the digestive system, leading to physiological perturbation and gastrointestinal blockages (Webb *et al.*, 2013). Moreover, some plastic contains toxic

molecules that can be released into the water as the plastic degrades (Ellen MacArthur Foundation, 2016). But maybe the most disturbing part of plastic pollution is the ability of plastic debris to adsorb toxic substances such a persistent organic pollutants (POP's). The concentration of these substances on the plastic debris can be over 1 million times superior to the concentration in the surrounding water due to weathering which increases the debris surface area, thus creating more reaction sites (Andrady, 2011; Crawford and Quinn, 2017a). Some contaminants can be desorbed from the plastic surface once it reaches a digestive system, due to the pH and temperature change and to physiological activities. Since most of POP's are lipophile, they tend to bioaccumulate in organisms and enter the food web (Outi Setälä, Fleming-Lehtinen and Lehtiniemi, 2013) therefore becoming a threat for humans (**Figure 3**)(Webb *et al.*, 2013; Crawford and Quinn, 2017c).

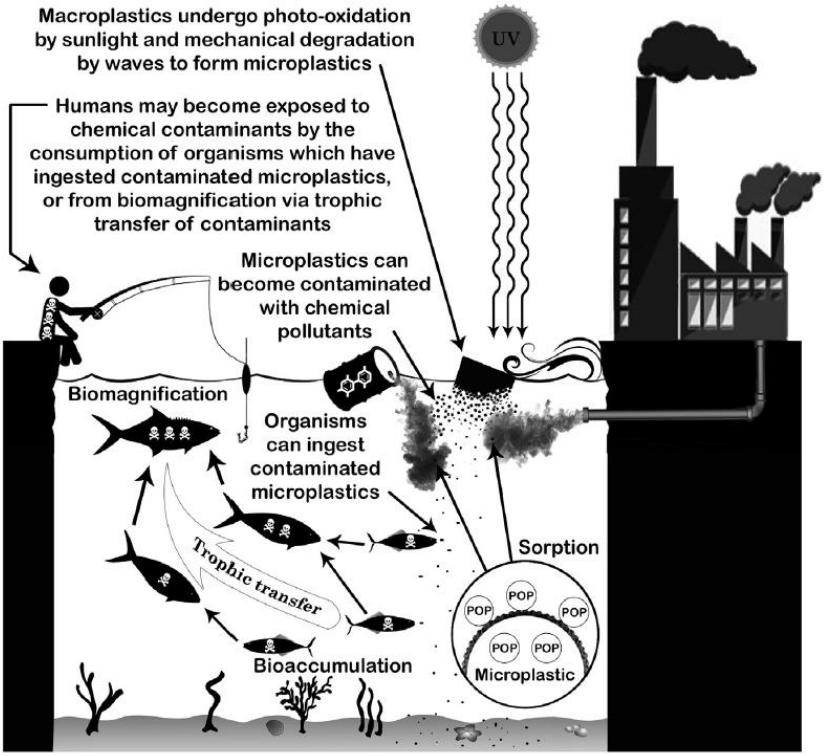

Figure 3: Introduction of pollutants into the food web through contaminated microplastics (Crawford and Quinn, 2017c)

The irregularities caused by weathering also promote the biofilm formation over the plastic, thus allowing microorganisms to travel on long distances and to end in new environments. Beside biofilms, macroscopic fauna like molluscs also travels with plastic debris. Therefore microplastics may become a pathogen vector and increases the risk of introducing invasive

and destructive species into new environments (Webb *et al.*, 2013; Crawford and Quinn, 2017c).

1.3. Plastic waste management nowadays

Plastic waste accumulates over time as a consequence of the lack of solutions at our disposal to get rid of them. Nowadays plastic waste is either incinerated (14% of the global production), recycled (14%) or for the most of them placed in landfills (72%) where they may end up in the marine environment due to the wind and undergo a degradation for several decades (Ellen MacArthur Foundation, 2016).

The mean inconvenient of landfills is the space they occupy and that could serve for more productive activities. Due to the slow degradation of the plastic, occupied area are unavailable for long time periods. The waste stacking creates an anaerobic surrounding which brakes the plastic degradation since it mainly operates by thermooxidative degradation. Moreover, the degradation may lead to the production of toxic compounds, as mentioned before (Webb *et al.*, 2013).

Incineration consists of burning the plastic to use the produced heat as energy. It doesn't suffer from the space inconvenience presented for landfills. However the short percentage of plastic incinerated is explained by the fact that the incineration generates a large amount of harmful compounds such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, heavy, metals, toxic carbon- and oxygen-based free radicals, and greenhouse gases all of which are released into the environment (Webb *et al.*, 2013).

The recycling still at present the most eco-friendly solution to the plastic accumulation for it prevent plastic from ending and degrading in the environment. Recycling is performed either chemically (chemolysis or hydrolysis) or by mechanical processes. Unfortunately, recycling suffers from many drawbacks that retain its development. First of all, only thermoplastic can be recycled, since thermosetting plastics cannot be melted and reshaped. Then, thermoplastics such as PET suffer from an alteration of their properties once they have been melted and solidified, making it more a downcycle than a real recycle. Finally, recycle processes are financially difficult to sustain since the processes are tedious and need specific installations (Webb *et al.*, 2013; Crawford and Quinn, 2017b).

With no intervention, plastics incur a degradation during several decades depending on the plastic. The complete degradation is achieved through 4 main mechanisms: photodegradation,

thermooxidative degradation, hydrolytic degradation (which is insignificant) and biodegradation. Photodegradation operates when UV brings the activation energy required to allow thermooxidation which consist of introducing oxygen into the polymer. As a consequence, the plastic becomes more fragile and eventually breaks into pieces small enough to be biodegraded by plastic degrading microorganisms (Shah *et al.*, 2008; Webb *et al.*, 2013).

2. Plastic waste as a possible new resource in biotech industry using biofilms

2.1. Synthetic plastic biodegradation with special reference to PET

Plastic management remains nowadays quite rudimentary and inefficient. Landfills occupy large areas for long periods, incineration generates highly toxic compounds, recycling is economically inefficient and degradation operates very slowly and can also produce harmful compounds. Therefore biodegradation stands as the most suitable solution to the plastic accumulation in the future from an economical and environmental point of view since biodegradation doesn't lead to toxic co-products. In addition, microorganisms degrading the plastic can be used to our profit by using them in bioindustries.

A complete biodegradation operates in 2 steps: depolymerisation and biomineralisation (when the end products are inorganic products). The process is achieved by enzymatic activity. Two types of enzymes have been proved to be involved in the depolymerisation processes: intracellular and extracellular depolymerases (Gu, 2003; Mohan and Srivastava, 2010). Extracellular depolymerases are responsible for the depolymerisation up to molecules small enough to pass the cell membrane. Intracellular depolymerases achieve the work once oligomers, dimmers or monomers are in the cell. Cells then use these molecules as carbon source and generates inorganic compounds such as CO_2, H_2O o CH_4. It's called biomineralisation (Gu, 2003; Mohan and Srivastava, 2010).

Depolymerisation strongly depends on the polymer characteristics (Shah *et al.*, 2008). Particularly, crystallinity have been proved to have a great impact on the degradation process of plastics since the amorphous parts of the plastics make the polymer chains more accessible for the enzymes while crystalline regions have the opposite effect (**Figure 4**). The molecular weight of the polymers also contributes to its depolymerisation due to the fact that the higher

the molecular weight of polymers is, the less soluble in water they are and thus the less affordable for microbial activity (Webb *et al.*, 2013).

Plastic have been around the world in large quantities for only a few decades, which haven't been enough time for the microorganisms to adapt and develop the ability to degrade them with great efficiency. However, some microorganisms have showed biodegrading activities that may be exploited in the future and among them, Bacillus and Pseudomonas are the most referred genus (Delacuvellerie, 2017).

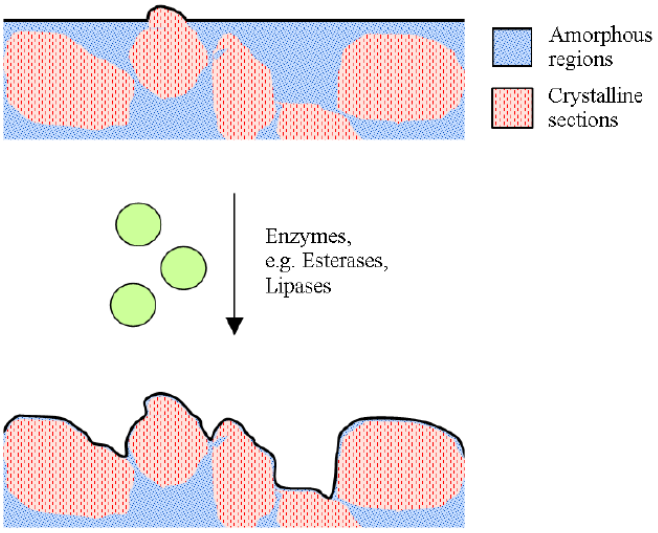

Figure 4: Impact of crystalline regions over enzymatic degradation of plastics (modified from (Webb et al., 2013))

Along with the plastics engineered specially to be biodegradable (Shimao, 2001), the most produced plastics have also been documented as sensible to biodegradation, even if the enzymatic reactions always have a very slow kinetics. Among them can be mentioned the polystyrene (Atiq *et al.*, 2010; Yang *et al.*, 2015), the polyvinyl chloride (Sah *et al.*, 2011), the polyurethane (Howard, 2002) and the polyethylene (Roy *et al.*, 2008; Sah *et al.*, 2011; Kyaw *et al.*, 2012; Yang *et al.*, 2014; Paul and Santosh, 2015; Gajendiran, Krishnamoorthy and Abraham, 2016), more particularly the low-density polyethylene which appear to be the most sensitive to biodegradation. The last four represented in 2016 about 51.9% of the global plastic production (**Figure 5**) (Plastics -The Facts, 2016).

That let us with the poly(ethylene terephthalate) (PET), the polypropylene (PP) and the "others" (**Figure 5**). The last section is far too complex to be studied from the biodegradation point of view and PP isn't degraded by any microorganisms as far as we know, but is a thermoplastic, meaning that it an be recycled. Last standing plastic in the list is PET, which

biodegradation have been focused by researchers for years due to its environmental impact. PET stands for 7% of the global production and its accumulation in the environment has become a global concern (Yoshida *et al.*, 2016). PET contains a high ratio of aromatic groups making it chemically inert and thus resistant to any microbial degradation. In order to compensate this lack of degradation ability, co-polymers were created by associating PET with aliphatic groups aiming to ease the polymer biodegradation but the results weren't totally satisfying. (Kint and Muñoz-Guerra, 1999; Muller, Kleeberg and Deckwer, 2001).

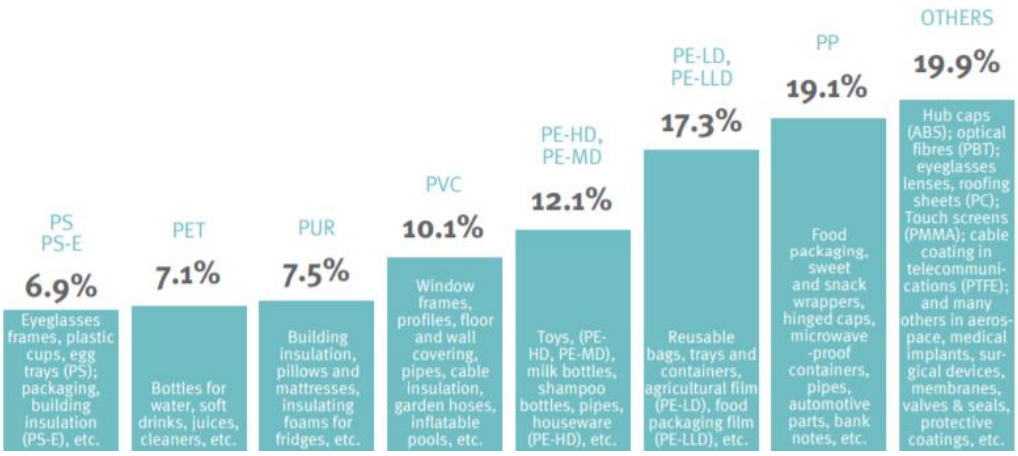

Figure 5: Global production of the different types of plastics in 2016 (modified from Plastics - The Facts, 2016)

However, a new bacteria have been isolated in 2016, *Ideonella sakaiensis* 201-F6, which is able to degrade PET quite efficiently. *Ideonella sakaiensis* 201-F6 is at the present time the only bacteria able to completely degrade PET, from depolymerisation to mineralization. *Ideonella sakaiensis* 201-F6 have been proved to produce a PETase able to degrade the PET into mono(2-hydroxyethyl) (MHET) and terephthalic acid (TPA). The PETase is also able to transform bis(2-hydroxyethyl) (BHET) into MHET. It has also been demonstrated that *Ideonella sakaiensis* 201-F6 is able to metabolise TPA and MHET, achieving thus a complete biodegradation of the PET (**Figure 6**)(Yoshida *et al.*, 2016).

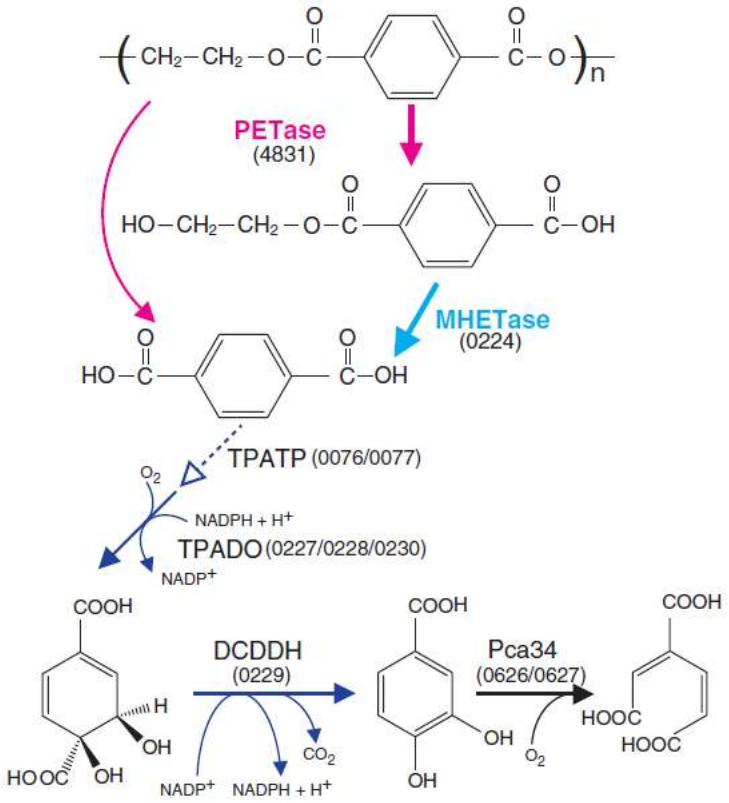

Figure 6: Metabolic path of PET biodegradation by *Ideonella sakaiensis*. The following enzymes are used: PETase, MHETase, TPATP (terephthalic acid transporter, TPADO (terephthalic acid 1,2-dioxygenase) DCDDH (1,2-dihydroxy-3,5-cyclohexadiene-1,4-dicarboxylate deshydrogenase), Pca34 (PCA 3,4-dioxygenase and NADPH co-factors (Yoshida *et al.*, 2016)

2.2. Biofilms as efficient workers for industrial applications

For decades biofilm have been studied for the purpose of getting rid of them. Their resistance to antibiotics and even physical washing made them a threat to human health. Nonetheless, we are now increasingly conscious of the many profitable activities that biofilm is capable of and the industrial applications they can serve, including plastic biodegradation.

2.2.1 What's a biofilm

Generalities

Currently the definition of a biofilm remains a topic of discussion within the scientific community. However, it is generally accepted that a biofilm is an aggregate of microorganisms in which each cell is responsible for the excretion of extracellular polymeric substances (EPS) (H.-C. Flemming *et al.*, 2016), thus creating a complex matrix in which these cells live, develop themselves and eventually which allows the biofilm to adhere to a surface. The EPS composition includes polysaccharides, proteins, lipids and extracellular

DNA (eDNA) (Flemming and Wingender, 2010). However, the exact composition of the EPS is specific to each biofilm, mainly depending of the microorganism composing the biofilm.

The matrix plays multiple roles within the biofilm and its presence is the main reason why biofilms behave differently than free-living bacterial cells. Biofilms are able to produce new metabolites and are far more resistant than the free-living cells. These new abilities offer a significant evolutionary advantage (Stoodley *et al.*, 2002), which justify the very energy-intensive metabolism of EPS (Saville *et al.*, 2011). Thus, one of the most important functions of the matrix is to protect the cells and ensure their survival. This protection operates at different levels and mainly thanks to the matrix sorption properties (H.-C. Flemming *et al.*, 2016). Firstly, the EPS composition and structure act like a gel retaining water and as a consequence the main component of the matrix is water, which can reach up to 97 % of its weight (Flemming and Wingender, 2010), preserving the cells from drought. The presence of water and biopolymers in the matrix gives to the biofilms sorption properties on its surface (Waigh *et al.*, 2015) (absorption concerning the water and adsorption concerning the biopolymers). This implies nutrient access to the cells in a much easier way than for free cells, by allowing the exchanges between the environment and the biofilm in a very efficient way. Moreover, the molecules can be sort of stocked in the matrix after their sorption or their production (H.-C. Flemming *et al.*, 2016), ensuring nutrient access to the cells during a given period. The molecules accumulation leads also to the fact that by producing enzymes and cumulating them in a given area, biofilms are able to degrade biodegradable surfaces and use them as a nutrient source (Mohan and Srivastava, 2010), which couldn't be done as easily by free cells. The occurrence of cumulated enzymes in the matrix gives to it an extracellular digestive role (H.-C. Flemming *et al.*, 2016) which can serve the entire microbial community living in the biofilm. The sorption capacity has no specificity, meaning that in addition to nutrients, all kinds of molecules and ions can possibly be "sorbed". As a consequence biofilms are able to accumulate metal ions present in their environment, some of which are toxic to the cells (Ordax *et al.*, 2010) and thus present antimicrobial activities. However, the matrix properties act as a cell protector against antimicrobials by inducing tolerance. Since the matrix allows the diffusion substances, with no barrier effect, this tolerance is believed to be a particular sort of inhibition called "diffusion-reaction inhibition" (Oubekka *et al.*, 2012) which implies in particular chelation (complex formation with the biopolymers present in the EPS) and enzymatic degradation, giving once again an evolutionary advantage to the biofilm against free-living bacterial cells.

Heterogeneity and communication within the biofilm

The biofilms matrix sorption properties have proved to be very beneficial for the biofilm living cells. Nonetheless the production of matrix also implies constraints for the cellular life, as for instance mobility reduction or accumulation of wastes produced by the cells. In biofilms the slow diffusion of substances coming from the environment leads to the appearance of a gradient of these substances, thus creating a microenvironment specific to each individual cell (**Figure 7**). The most relevant gradients that can be observed are the nutrient gradient, the oxygen gradient and the pH gradient (H.-C. Flemming *et al.*, 2016). All of them have a great influence on the appearance of heterogeneity within the biofilm. In monospecies biofilms, even if the cells have a certain degree of mobility, the gradient appearance force them to adapt to their new environmental conditions by exposing new phenotypes (Kalmbach *et al.*, 1997; Boles, Thoendel and Singh, 2004). In multispecies biofilms, the different microorganisms end up growing in the biofilms parts in which the gradients create the best conditions for their development. Furthermore, multispecies biofilms can show phenotypic differentiations within the strains composing it, just as the monospecies biofilms do (Boles, Thoendel and Singh, 2004).

In addition to the differentiation, the biofilm cells interact with each other. This is valid either for multispecies biofilms, in which the different species may live either in competition or cooperation (Foster and Bell, 2012; Ren *et al.*, 2015), and for monospecies biofilms (Prindle *et al.*, 2016) where the different phenotypes assume different functions in the biofilm.

One of the most mentioned form of communication between cells is the quorum sensing which is defined as a kind of communication between cells based on the concentration of the excreted signalling molecules. This implies that when cells are very close to each other in large quantities, some signalling molecules appear to be in high concentrations, and as a consequence, cells perceive this as a message which lead to the expression of certain genes involved in the formation of the biofilm, by producing EPS for example (Keller and Surette, 2006). It's believed that quorum sensing is strongly involved in the triggering of the biofilm formation (Parsek and Greenberg, 2005; Keller and Surette, 2006; Kim *et al.*, 2016) and can be affected by environmental conditions such as external flow. Quorum sensing continues to be a communication form between the once the biofilm is formed and since it's a phenomenon based on molecule concentrations, its gradient in the biofilm can affect the living cells (H.-C. Flemming *et al.*, 2016).

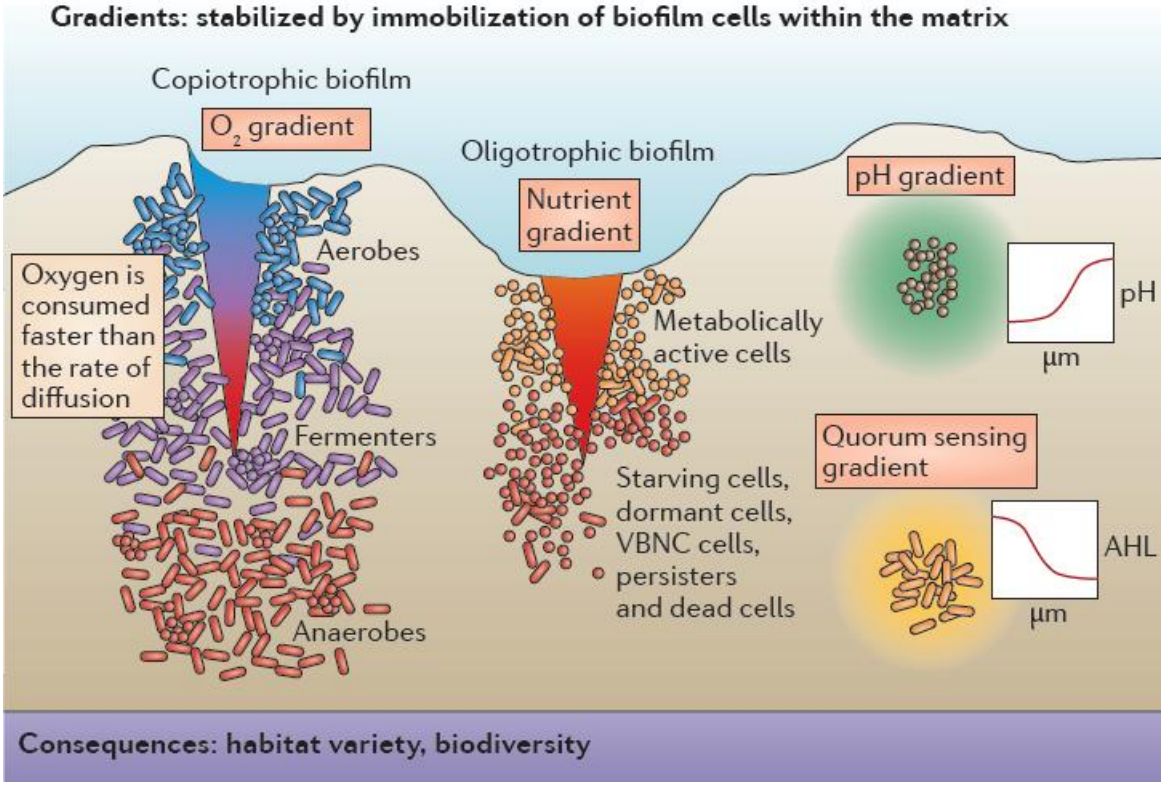

Figure 7: Oxygen, nutrient, pH and quorum sensing gradients contributing to the cells heterogeneity ((H.-C. Flemming *et al.*, 2016)

Next to the quorum sensing, the main form of communication observed in the biofilms is the synergistic interactions. These interactions occur in cases where the different species composing the biofilm live in symbiosis. The activity of the different species ends up providing nutrients for the others, helping to catalyse a given metabolic activity, or protecting other species by consuming or degrading a molecule which would have been toxic for them (Burmølle *et al.*, 2014).

2.2.3 A problem that becomes a solution

Biofilms can be a source of infectious contamination, and their resistance to antimicrobial substances added to their ability to develop on a large range of surfaces has made them a threat, especially in the medical field and in food industries. However, they present properties that are now used in our benefit to optimize chemical or biological processes.

The problematic side of the biofilms

The study of biofilms and their resistance have revealed them as the cause of chronic infections sometimes due to their development on fragile surfaces of their hosts, such as the

lungs, teeth or the inner ear (Satpathy *et al.*, 2016). However biofilm associated infections are very difficult to diagnose (Hall-Stoodley *et al.*, 2012) and to treat since classical antibiotic treatments aren't efficient enough (Malheiro and Simões, 2017)

In the medical field biofilms turns out to be a real problem, since they are able to develop in a large range of surfaces. Biofilm becomes then a threat coming from diverse origins, ranging from washbasin U-bends (Swan *et al.*, 2016) to cardiovascular by-passes, which makes the biofilm a hindrance to surgical science (Walker *et al.*, 2017).

In food industries biofilms are also a topic of concern. For example, concerning *Bacillus* spp., the biofilm formation can lead to the sporulation of the strain. The spores are very resistant, but easy to transfer, becoming a main source of either direct contamination and cross-contamination (Faille *et al.*, 2014). On a larger scale, biofilms are able to develop on stainless steel, which is used in most industries material, and can also be able to resist to ordinary sanitation procedures, thus they can be found at all stages of the industrial process, putting in danger the hygiene conditions of the whole process (Maifreni *et al.*, 2015).

In order to assess the problems caused by the biofilms, different control techniques have been developed. The most know strategy is the cleaning and disinfection of the surfaces by using chemical products as for instance detergents or acids, capable of penetrating and dissolve the matrix which allow antimicrobial substances to gain access to the biofilm cells (Simões, Simões and Vieira, 2010). This is the classical procedure to get rid of any kind of microbial contamination and needs to be adapted to each biofilm. Others control strategies have emerged in the past years, like physical treatments (magnetic fields, electrical fields and sonication), and green technologies such as the use of plant extracts, enzymes, and even phages (Sadekuzzaman *et al.*, 2017)

The biofilms applications nowadays

Besides all the inconvenience they may cause, biofilms can be very useful in many fields. They are nowadays used in diverse application, often related to the decontamination of soil and water or with the industrial production of metabolites. In waste water treatment, biofilm formation appears to be a critical step for the water treatment processes, as they show more efficiency than free-living cells. For this purpose, biofilms can be found in activated sludge for instance (Azari *et al.*, 2017). Specific biofilms are also used for more sophisticated wastewater treatment, as the pharmaceutical products removal (Tang *et al.*, 2017), oil refinery

wastewater treatment, (all two based in the biodegradation of its pollutants (Hodges *et al.*, 2017; Li *et al.*, 2017) and even uranium immobilization (Cologgi *et al.*, 2014). The biofilms sorption abilities also make them a good candidate for the treatment of heavy metals present in waste water (Oyetibo *et al.*, 2017).

In the chemical industrial field, microorganisms have been used as biocatalyst. In that cases, immobilized cells have shown better results than suspended cells. However, the immobilization process implies the use of polymers or organic-inorganic material which reduces the possibilities in terms of microorganism diversity that can be used for that kind of processes. The immobilization also decreases the mass transfer and thus reduces the cells viability and so does the process efficiency (Silveira Martins *et al.*, 2013). Recent researches have tried to find a way to avoid these problems and to that purpose biofilm have been studied as a possible solution due to their ability to immobilize the cells by itself by producing EPS (Rosche *et al.*, 2009). Also biofilms are known to resist to toxic substances, which make them a good candidate in chemical industry applications.

Plastic waste as a new carbon source in bioindustry

Plastic waste has become a problem of major concern during the last years and recycling isn't possible on most plastics and generally struggles economically. Plastic production have a cost and plastic packaging (which is often lost after a short first use) represents alone a loss of between 80 and 120 billion dollars annually since only 5% of the recycled material is retained and used for lower value applications (Ellen MacArthur Foundation, 2016).

To answer the problem, thermochemical depolymerisation of plastic followed by microbial activity to generate higher value biodegradable plastics has recently emerged as an exciting upcycling approach (Ward, Goff and Donner, 2006; Goff, Ward and O'Connor, 2007; Kenny *et al.*, 2008; Guzik *et al.*, 2014). In addition, an increasing number and variety of plastic degrading bacteria are discovered, some of which perform very efficient depolymerisation and may replace the thermochemical depolymerisation. With the right wild strain or performing genetic engineering, both depolymerisation and upcycling of the plastic may be performed by one single strain which will use the plastic waste as a carbon source to produce a high value produce, thus integrating the plastic waste into a circular economic practice (Wierckx *et al.*, 2015).

An even more exciting approach could be the use of biofilms for this purpose. As already mentioned, biofilms have been proved to be more efficient than free living cells in many cases, due to the cell fixation and to the physiological changes incurred by the biofilm. Also, once fixed to a surface, biofilm show most efficient degradation than free-living cells. Biofilms are able to grow on plastic surfaces (Arthur, Baker and Bamford (eds), 2009; Crawford and Quinn, 2017a; Law, 2017) and it has been suggested that biofilm formation promotes the plastic depolymerisation (Gilan, Hadar and Sivan, 2004; Mohan and Srivastava, 2010; Das, 2014; Paul and Santosh, 2015; Sen and Raut, 2015) and that multispecies biofilm benefits from synergistic interaction between the different species. Therefore the use of biofilms using the appropriate consortium legitimately appears to be a good approach regarding the upcycling of the plastic by generating biodegradable plastic.

Bacillus amyloliquefaciens GA1 as a potential strain for plastic biodegradation

Bacillus genus have often been associated with plastic biodegradation and thus retain our attention. *Bacillus* are common and well known microorganisms, easy to manipulate and to integrate into industrial processes. They seem to be quite specific, since they mostly degrade polyethylene, low-density polyethylene (LDPE) and high-density polyethylene (HDPE) (Delacuvellerie, 2017). However, some strains are more remarkable, showing that *Bacillus* may have a larger range of impact concerning plastic degradation. Particularly *Bacillus subtilis*, which is a common strain, produces an enzyme close to the PETase generated by Ideonella sakaiensis (Yoshida *et al.*, 2016). In addition, many *Bacillus* strains are able to form biofilms, which may be a key step for plastic biodegradation. To this effect recent studies have reported *Bacillus amyloliquefaciens* GA1 as a strain able to produce biofilms very efficiently. *Bacillus amyloliquefaciens* GA1 is a non pathogenic strain, and have already been proved to have the ability of degrading LDPE (Paul and Santosh, 2015). Furthermore *Bacillus amyloliquefaciens* GA1 and *Bacillus subtilis* are close strains (Xu and Côté, 2003; Wang and Sun, 2009), which legitimately let us think about *Bacillus amyloliquefaciens* GA1 as a promising candidate for plastic biodegradation.

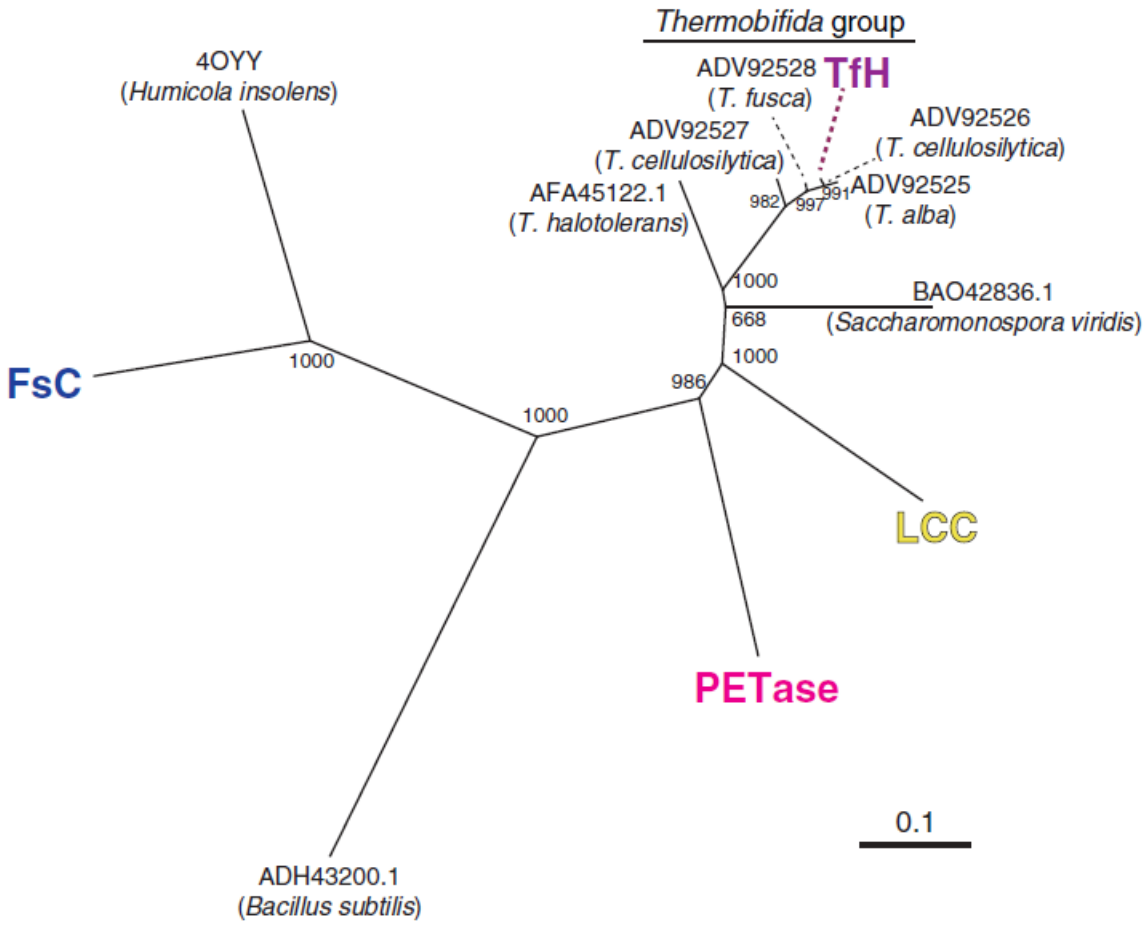

Figure 8: Unrooted phylogenetic tree of known PET hydrolytic enzymes. The GenBank or Protein Data Bank accession numbers (with the organism source of protein in parentheses) are shown at the leaves. Bootstrap values are shown at the branch points. Scale bar, 0.1 amino acid substitutions per single site (Yoshida et al., 2016).

3. Biofilm growth in bioreactors

3.1. Different kinds of bioreactors: particularities and applications

The exploitation of microorganisms in biotechnology applications requires the use of adequate bioreactors. The most common bioreactors are disposable bioreactors using bags as culture ware. They can be divided into mechanically (tipping, stirring, vibrating) and pneumatically driven (airlift, bubbles) (René, 2009). Concerning the production of biodegradable plastics, laboratory stirred tank reactors have been proved to be efficient (Ward, Goff and Donner, 2006; Goff, Ward and O'Connor, 2007).

Although biofilm are about to be considered for biotechnical purposes, no bioreactor have at the present been designed to serve industrial application involving biofilms. They were in fact

considered as a source of problem and researches were mainly focused on finding efficient ways to get rid of them. For now biofilms are studied using laboratory scale bioreactor to fully understand their behaviour. The currently available bioreactors to this aim are summarized in **Table 1** (Azeredo *et al.*, 2017).

Among the different bioreactors presented in **Table 1**, the drip flow bioreactor draw our attention. Drip flow allows to study the biofilm growth over the surface of our choice. Since the biofilm doesn't grow in a liquid phase, its surface can be characterized in addition to other tests. Drip flow bioreactor also allows to quantify the biofilm and to observe its development at different culture times and in several repetitions (Azeredo *et al.*, 2017).

Hopes are that thanks to the understanding of the biofilm dynamics we'll get to take advantage of the biofilms abilities by cultivating them in industrial scale bioreactors to serve biotechnical operations such as the upcycling of the plastic.

Table 1: Biofilm cultivation devices and their respective applications, advantages and limitations (Azeredo *et al.*, 2017)

Device	Application	Advantages	Limitations
Microtiter plate	Screening for biofilm formation capacity Test of anti-biofilm compounds	High-Throughput Inexpensive No need for advanced equipment apart from plate reader Dedicated microscopic-grade microplates allow noninvasive imaging	Loosely attached biofilm may not be measured correctly (can be detached during washing steps) Sensitive to sedimentation End-point measurement Batch mode. Exhaustion of nutrients Direct inspection difficult Usually only short term experiments Possible interference with liquid–air pellicle Sometimes poor reproducibility Results person- or laboratory-dependent Assessment possible only at a sufficiently high cell density Not suitable for investigating early stages of biofilm formation
Calgary device	Screening for biofilm formation capacity Test for biofilm minimal inhibitory concentration (e.g. of antibiotics)	High-Throughput Possibility to change growth conditions Less sensitive to sedimentation No need for advanced equipment apart from plate reader	Each change of medium requires that pegs pass through the liquid–air interface Loosely attached biofilm may not be measured correctly (can be detached during washing steps) Direct inspection difficult End-point measurement Difficulty to collect individual pegs for enumeration Sonication may not remove firmly attached cells Usually only short term experiments Not suitable for investigating early stages of biofilm formation Relatively expensive
The Biofilm Ring Test	Screening for biofilm formation capacity Test of antibiofilm molecules Antibiofilmogram	Rapid high-throughput Easy-handling method (no washing, fixation or staining steps) Well-designed to investigate early stage of biofilm formation Applicable to loosely attached biofilm highly reproducible, not person- or laboratory-dependent	Requires specific magnetic device and scanner Not intended to investigate late stage of biofilm formation Not adapted for biofilms at the air–liquid interface Possible restriction due to the growth medium (e.g. some minimal or salt media spontaneously block the paramagnetic microbeads)
Robbins Device and Modified Robbins Device	Screening of biofilm supporting surfaces Suitable for in-line flow experiments (e.g. utility water systems)	Can run for very long periods without intervention Substratum coupons can be extracted or exchanged during the experiment	Low- to medium throughput Expensive Requires pumps or flow systems Does not allow *in situ* online inspection of biofilms Requires prior knowledge of the flow dynamics inside the device which can limit the operational range
Drip Flow Biofilm Reactor	Visualization and quantification of biofilm formation on coupons at low shear stress	Compatible with coupons of various geometry	Heterogeneity of biofilm development on the coupons
Rotary Biofilm Devices	Evaluation of the effect of material and shear stress on biofilm development	A variety of materials can be compared in similar nutritional and hydrodynamic conditions Constant shear stress field Shear stress and feed flow rate can be set independently Possible to apply high shear stress	Low number of microbial strains can be analyzed The geometry of the coupon is fixed and determined by the reactor design (coupon holder) Expensive
Flow Chamber	Suitable for growing smaller number of biofilms with continuous supply of fresh medium Can be used to study growth physiology, response to stresses (e.g. antibiotics), flow conditions Can in some extent be used to mimic natural flow conditions, e.g. in the body or in other natural environments	Allows direct inspection Allows nondestructive observation of developing biofilms Optimized for on-line *in situ* microscopy Can be designed with glass or other substrata	Low throughput Does not allow direct access to the biofilm cells Requires peristaltic pumps and other special equipment
Microfluidics	Can be designed for special purposes, e.g. mimicking air–liquid interfaces, provide *in situ* mixing of reagents, include customized measuring devices	Can be custom made for specific purposes Versatile Compatible with single cells analysis	Requires special equipment for manufacturing and running systems Can be expensive Operation can be tedious Clogging can occur due to small dimensions

OBJECTIVES OF THIS STUDY

The main purpose of this work was to address the environmental issues caused by the plastic waste in a worldwide scale by exposing the possibility of an environmentally-conscious solution involving biotechnical processes. The most eco-friendly way of treating the plastic waste is by biodegrading it. Nevertheless the simple biodegradation isn't an economically viable solution unless we manage to take advantage of its degradation. A possible way to achieve that is by using the plastic waste as a main carbon source for the microorganisms used in biotechnical applications such as the production of high value biodegradable plastic and therefore performing plastic upcycling. Unfortunately, plastic biodegradation is an extremely long process. In biotechnical processes, biofilms have been proved to be key factors for process yields and acceleration. For those reasons in this study, the research was firstly focused on the biofilm cultivation in bioreactor of a strain able to rapidly create biofilms. The understanding of the biofilm's dynamics is essential in order to be able to exploit its capacities in the future. To do so, the drip flow bioreactor was chosen for the many advantages it presents regarding the biofilm study and its utilization methodology was optimized before carrying any experiment. *Bacillus amyloliquefaciens* was chosen for this study due to its family ties to plastic degrading strains and its ability to form dense biofilms. The biofilm dynamics was studied using the drip flow bioreactor and the eventual degradation impact of the biofilm formation over a PET plastic was tested.

MATERIAL AND METHODS

1. Laboratory material

- Vortex mixer (Speed from 0 to 2500 min-1, VWR 444-1372, Belgium).
- Double wall autoclave Cisa.
- Screwable HSW SOFT-JECT® syringes of 10 and 20 ml (in Luer Lock version).
- Single use needles (Sterican, Long Bevel 20G x 23/4, 70 mm of length and 0.90 mm of diameter. The reference number is 4665791).
- Falcon tubes of 50 ml and Eppendorf tubes of 2 ml.
- Laboratory glassware (1 L and 5 L flasks).
- Luer Lock connectors (of different sizes) adapted to the silicone tubes.
- Mohr/Hoffman clamps (of different sizes)

2. Microbial strains

Bacillus amyloliquefaciens GA1 was used. The strains were stored at -80°C for conservation.

3. Drip flow bioreactor

3.1. Reactor components

NB: See the complete illustrated list in annex 1

- Reactor base in polysulfone with six channels and six effluent ports (*BioSurface Technologies Corporation, USA, Montana*).
- Reactor covers in polycarbonates (*BioSurface Technologies Corporation, USA, Montana*) with two ports: one for the air filter and one for the medium entry. A needle is fixed in the port dedicated to the medium entry. The covers are screwed to the base using nylon screws.
- Peristaltic pump (Watson Marlow 530S, England).

- Silicone coupons.
- Output silicone tubes (internal/external diameter ratio = 8/12 mm) which has equipped with a Kartell 468 connector allowing a sampling by an Eppendorf tube.
- Silicone tubes of 3/6 mm and 5/9 mm and 6 PharMed BPT tubes of 1.3 mm diameter (reference = SC0743) connected together to bring the medium from the medium flask to the bioreactor chamber. The PharMed tubes are inserted in the Watson Marlow pump.

3.2. Reactor assembly

NB: See the complete illustrated instruction in annex 2

- Insert the coupons into the bioreactor.
- Screw the covers and place the air filters in the ports provided for this purpose.
- Insert the Kartell 468 connectors into the effluent ports of the reactor base and the entry tubes in the needles on the cover port provided to this purpose.
- Pack the reactor in a double layer of aluminum foil.
- Sterilize the reactor in the autoclave at 121°C during 20 minutes in steam conditions.

3.3. Reactor disassembly and cleaning

NB: See the complete illustrated instruction in annex 3

- Place the removed striated coupons in a container filled with water after the sampling.
- Dislodge each pump compartment to remove the entry tube from the peristaltic pump (*Watson Marlow).*
- Remove the entry tubes from the coverts ports.
- Clean up the flask containing the culture medium with water and washing-up liquid.
- Connect the entry tube (the extremity previously placed in the flask) with a connector and circulate water at low debit for a few minutes.

NB: If there are some traces of unknown contamination in the tube, place the end of the same tube in a container of bleach and circulate it to fill the whole of the circuit using a Wilson Marlow 100UR pump. Once the tubes are filled, cut the pump. Leave on for twenty minutes. Then rinse with water.

- Disconnect the output tubes of the reactor base and close the Mohr/Hoffman clamp.
- Fill the output tubes with bleach (two by two) to the Eppendorf tubes for the sampling. Leave on for twenty minutes. Rinse with water by connecting tubes to the faucet (two by two).

NB: Ensure to rinse correctly the output sampler by opening them while rinsing.

- Remove the Sartorius air filters of 0,22 µm and store them.

NB: Check their condition before storage, the filters must stay perfectly white to ensure their performance.

- Remove the covers and wash them using a sponge and washing-up liquid. Rinse with water and ensure to pass water through the needle to check its cleanliness.

NB: Keep attention to the fragility of the coverts and needles.

- Clean the silicone coupons with a sponge and washing-up liquid. Rinse with water.
- Clean the different cells of the reactor in the same way as the covers and the coupons.
- Rinse the effluent ports to ensure that there is no trace of biofilm.

NB: If there are some traces of unknown contamination in the tube, rinse with bleach too.

- All the rinsing water and bleach used for the cleaning of the reactor is collected and autoclaved.
- Place back everything in the reactor corresponding boxes for the next setup and autoclaving.

4. Inoculation and culture conditions

The experiment consists of a formation of bacterial biofilms on inclined silicone coupons. For this formation, two steps are necessary: a batch (or stationary) condition and a continuous condition.

The batch condition consists in inoculating the reactor in a horizontal position with the bacteria (*Bacillus amyloliquefaciens GA1*). This step lasts 6 hours and allows the adhesion of the bacteria and the formation of the biofilm during the following continuous condition.

During the continuous step, the bioreactor is put on a tilted surface of 10°. The peristaltic pump (Watson Marlow, Germany) was set in order to get a 13 $ml.h^{-1}$ flow of culture medium in each channel during as much longer as the experiments needs, which in our case was between 40 and 43 hours. Samplings can be performed during this time, allowing to study the

evolution of the biofilm and it's planktonic phase.

The protocols concerning the setup of the bioreactor, the batch culture condition, the continuous culture condition, the sampling and the reactor clean up are explained in the following points.

4.1. Batch culture condition

- Prepare 200 mL of sterile liquid YPD medium (10 g/L casein peptone, 10 g/L yeast extract and 20 g/L glucose) into a sterile flask (of 1 liter).
- Add in the flask the content of a *Bacillus subtilis* GA1 working seeds to make a preculture.
- Incubate this preculture overnight (approximately 17 hours) at 30°C and 130 RPM.
- Check the Optical Density of the preculture at 600 nm after the night. Adjust the Optical Density to 1by diluting with PBS (Phosphate-buffered saline) if necessary.
- Put the reactor on a tilted surface of 5°. The inclination is done to prevent the inoculating medium of flowing away.
- Inoculate 20 ml of the preculture solution (diluted before to adjust the Optical Density if necessary) into each reactor channel by screwing a 20 ml syringe to the ports used for the medium injection (**Figure 9**).

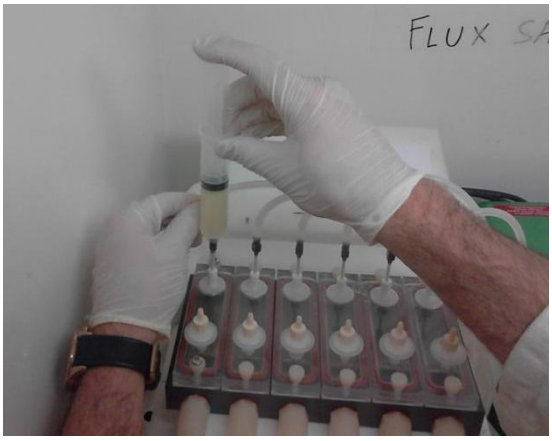

Figure 9: Inoculation of the drip flow chambers with 20 ml of preculture after adjustinfg it OD to 1 with PBS.

- Incubate during 6 hours at temperature of 30°.

4.2. Continuous culture step

- Prepare 3.5 L (or more if needed) of sterilized liquid LB medium (10 g/L casein peptone, 10 g/L yeast extract, 10 g/L NaCl and 2.5 g/L glucose) into 2 sterile flasks of 5 L.
- Insert a silicone tube of 5/9 (internal/external diameter ratio) into the flasks and across a cotton plug to close the flasks.
- Connect the silicone tube from the flasks to the silicone tube for the medium injection (with a 2 ways connector).
- Put the 6 PharMed BPT tubes (from the tubing for the medium injection) into 6 pump cassettes.
- Apply the maximum debit ("MAX" pump button) to bring the medium level at the entry of the reactor channels.
- *NB: This step is often done before the batch culture step to prevent the washing of the fixed cells.*
- Put the reactor on a tilted surface (10°).
- Apply a flow of 13 ml/hour.
- Let this step working during 40 to 43 hours at a temperature of 30° and sample at different times the planktonic and biofilm phases.

4.3. Sampling

4.3.1. Sampling method

- Turn the *468 Kartell* connector of 180° to bring the samplingEppendorff into a 50mlL falcon tube to allow the sampling of the planktonic phase from one of the reactorchannelsl (**Figure 10**).
- Collect at least 10 ml of the planktonic phase (during approximately 50 minutes).
- In order to collect the surface cells (non adherent sessile cells) of the biofilm, gently rinse the coupon (from the same channel than the one for the planktonic phase sampling) with 2mlL of PBS (Phosphate-buffered saline) and collect the solution in a 50mlL Falcon tube.

Figure 10: Planktonic phase collection using Kartell 468 connector.

- After the sampling of the surface cells, put the silicone coupons with the biofilm on it into a 50 ml falcon tube.

NB: Another possibility is to collect the biofilm from another channel (than the one for the planktonic phase) to have the possibility to collect the planktonic phase always from the same channel to see the evolution of the cell concentration in this phase.

- Add 10 ml of sterilized PBS (Phosphate-buffered saline) into the falcon tube.
- Vortex the falcon tube at maximal speed (2500 min^{-1}) until the dissolution of the biofilm into the PBS.
- Turn the coupon upside down into the falcon, and vortex it to be sure that all the biofilm cells are in suspension
- Determine the OD (Optical Density) at 600.0 nm with the spectrophotometer of each sample following the protocol related to the OD measurement.

4.3.2. Sampling plan

Two sampling plans were performed. The first consists in sampling the planktonic phase from one unique chamber of the drip flow and collect the biofilm in the other chambers. During the continuous phase, the samples were collected in duplicate using two drip flows with culture times between 23 et 26 hours. However, this sampling method doesn't allow to eventually correlate the planktonic phase and the biofilm and was therefore abandoned after the two first cultures. The OD was measured without any previous treatment of the samples.

The second sampling plan was used for the rest of the experiments. During the continuous step, Planktonic cells (PC), Biofilm cells (BC) and Non Adherent Sessile cells (NASC) were collected in triplicate (**Figure 11**) from the same chamber. For each sampling time, the optical density at 600 nm was respectively measured.

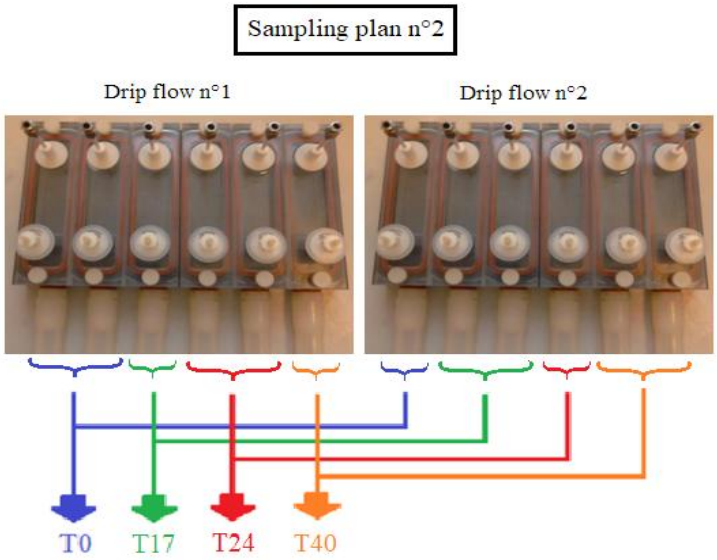

Figure 11: Sampling plan used during the experiments. Sessile, planktonic and non adherent sessile cells were collected from the same reactor chamber in triplicates. Two samples were collected from one bioreactor and the third one from the other bioreactor in an alternative way.

5. Optical density measurement

Before cultures characterization, each sample was treated with ultrasounds using a UW 2070 device (Bandelin Electronic, Germany), in a 9 cycles mode during 40 seconds to 30 KHz (**Figure 3**). The sonication deconstruct the exopolysaccharide matrix in the liquid phase while avoiding the cell damage.

Exactly 10 ml of the samples were then centrifuged at 11325g and 4°C during 15 minutes. The supernatant was removed. Cells were then resuspended in 5 ml of sterile PBS before any analysis (Annexe 4). The cell centrifugation separates the cells from the matrix, allowing for OD measurements of suspended cells alone.

6. Reduction of the variability between the different chambers of the drip flow to an acceptable level

To increase the repeatability of the experiments, the room temperature was fixed at 30°C by warming the room using a stove settled at 100°C and keeping the door closed.

The variation between the different chambers of the bioreactor was reduced by totally immerging the coupons in the inoculating medium. This was achieved by tilting the reactor backward to avoid the inoculating medium from flowing into the waste

Also sample preparation (sonication and centrifugation was performed before measuring the OD (see 6., Material and methods)

7. Study of the link between the biofilm cells and the planktonic cells

7.1 Collection of a potential third type of cells being the bridge between the biofilm and the planktonic phase

See material and methods 4.3.1 and Annexe 4.

7.2. *Bacillus amyloliquefaciens* cultivation in petri dishes

Petri dishes cultivation was performed using LB medium with containing 10g/L of glucose and 15g/L of agar.

7.3 Zeta potential of the cells

The bacterial zeta potential was assessed using the electrophoretic light scattering (ELS) methods. Charged particles are exposed to a scattering light (using a laser). When an electric field is applied to the charged particles, they move in the direction of the electrode with an opposite charge. When the particles are in movement the scattering light incurs a Doppler shift, depending on the velocity of the particle, which determines the electrophoretic movement (EM). The EM depends on the charge of the particle, allowing the determination of the zeta potential. Experimentally, bacteria from working cultures of 24 and 27 hours were harvested by centrifugation (12,000 RPM, 10 min, 4 °C), washed twice with a Milli Q water and the cells were resuspended in a Milli Q water at a final cell density corresponding to an OD_{600nm} of approximately 0.5. Non washed samples were also analyzed to compare the impact of the washing over the zeta potential of the cells. The zeta potential was measured with an automated Delsa Nano C particle size and zeta analyzer, (Beckman Coulter, France). Before injecting any bacterial suspension in the measurement chamber, the latter was

abundantly flushed with deionized water using the Direct-Pure UP, 0.3 mΩ cm 25°C (Rephile, Belgium). The mean of bacterial zeta potential was determined and expressed by (ζ) in mV. Each experiment was performed in triplicate with three independent bacterial cultures (Annexe 5).

7.4. Cytometry

The flow cytometric analysis were performed on the samples beforehand sonicated and filtered with 5 µm filters (VWR, Belgium). The cell suspension concentration is previously adjusted in order to reach an approximate final concentration of 10^3 cells per µL (or diluted to 500 cells per µL if necessary) for the following physiologic parameter analysis.

7.4.1 Sporulation state

Sporulation level is estimated through acridine orange (AO) staining technique (SIGMA-ALDRICH, Belgium) involving flow cytometry for fluorescence measurement. DNA with intercalated AO fluoresces green (525nm); RNA electrostatically bonded to AO fluoresces red (> 630nm). 100 µl of cell suspension is added with 500 µl pH 3 buffered solution A (13.23 mM citric acid, 6.85 mM Na_2HPO_4, 0.1 mM EDTA, 0.2 M anhydrous sucrose) and 500 µl of pH 3.8 buffered solution B (4.95 mM citric acid, 5.5 mM Na_2HPO_4, 0.147 M NaCl) in which AO stock solution is formerly diluted 100 times. After incubation at room temperature for 10 min, samples are centrifuged and resuspended in filtered PBS. Next, the fluorescence profile of the different samples is evaluated thanks to the Accuri C6 flow cytometer (BD Biosciences, NJ USA) on the FL2 channel. Parameters are recorded for 20.000 events with a flow rate of 35 µl.min^{-1} and FSC-H based threshold of 80.000.

7.4.2. Metabolic state

Metabolic state is characterized through Redox Sensor Green (RSG) staining technique (Invitrogen, UK) involving flow cytometry for fluorescence measurement. 1 ml of cell suspension is added with 1 µl RSG. After incubation at room temperature for 25 min, samples are centrifuged and resuspended in filtered PBS pH 7. Next, fluorescence profile of the different samples is evaluated thanks to Accuri C6 flow cytometer (BD Biosciences, NJ USA)on the FL1 channel. Parameters are recorded for 20.000 events with a flow rate of 20 µl.min^{-1} and FSC-H based threshold of 60.000.

8. Maturation of the biofilm

8.1. Contact angle measurement

Contact angle between a milli-Q water drop and the biofilm surface at different culture times was measured using a TRACKER P.N./Tensiomètre/99 (I.T. Concept) tensiometer with automated drop. The device was equipped with a charge coupled device (CCD) camera coupled to a profile video image digitizer board-connected to a computer.

A milli-Q water drop of 2 µl was created by the device and then manually deposed on the biofilm surface. The software was settled in "sessile down" mode and recorded the drop deposition for 300 seconds. The deposit moments were recorded during in "Speed" mode to have the maximum frames per second. Then the rest of the record was set to "slow" mode (minimum frame per seconds). The recording was stopped a few moments after setting the "slow" mode (it is not necessary to record for 300 seconds). The frame of the instant moment when the drop is deposited was stored and used to measure the contact angle automatically using the software. Contact angle was measured manually using printed frames when the irregularity of the biofilm surface led to incorrect contact angle measurements by the software.

9. *Bacillus amyloliquefaciens* GA1 biofilm growth on plastic PET plastic

9.1. PET cristallinity characterization

The cristallinity of the used PET was measured with a Bruker D8 advance X-ray diffraction system, equipped with an Anton Paar TTK 450 sample chamber and a Burker LynxEye detector. Cu$K\alpha$ radiation (λ = 1.54184 Å) was used. X-ray diffraction was measured between 0 and 65° of the 2θ interval. The software was DRIFFACT.EVA (Bruker). The number of measures per degree was settled automatically by the software.

9.2. Biofilm growth on PET in flask

Two cultures were performed in flasks. The first culture was performed over 2 days at 30°C with LB medium with an 60 rpm agitation. The second lasted 3 weeks. The first week LB medium with 2,5g/L glucose was used to accelerate the biofilm formation. The cultures were done with LB medium the two following weeks. The medium was replaced after each week.

Each plastic coupon was cut to obtain 2 pieces colonized by biofilm, one of which was washed using bleach and the other was conserved with the biofilm on it. This allows to observe for the same sample the biofilm and the surface under the biofilm.

9.3 Biofilm growth on PET in drip flow biofilm reactor

Biofilm culture on PET was performed using the same culture conditions used for the cultures on silicone (see material and methods 4.). One chamber was used as control (no inoculation). 4 chambers were inoculated. Two of the four biofilm formed were washed using bleach and the two others were conserved in order to observe the biofilm and the surface on which it has developed.

9.4. Biofilm and PET surface observation using scattering electron microscopy (SEM)

All the samples (washed, non washed, control) went through the same steps. Firstly, the samples were immerged into a 70% ethanol solution for 30 minutes. Then again, they are immerged into a new 70% ethanol solution over night. A last immersion in ethanol 70% is done. The samples are conserved in that solution if not observed the same day. Following that, 2 immersions of 30 minutes are performed on ethanol 90%. Finally an ultimate immersion in absolute ethanol is done for 1h. The consecutive immersions allow the cells to fix to the surface and to dehydrate, which is absolutely necessary for the next steps.

Next step is the drying of the samples. Samples are placed in absolute ethanol and then inserted in a drying chamber "Agar Scientific". CO_2 at its critical point is used to dry the samples.

The final step before the observation is the metallisation of the samples. Here samples were metallised with gold using a JEOL, JFC-1100E ion sputter, fine coat device. Metallisation was performed at 10 mA during 2 minutes.

Finally, observations were done with a SEM JEOL at a 2 kV voltage .

RESULTS AND DISCUSSION

1. Study of the dynamics of the biofilm in the drip flow biofilm reactor

1.1. Study of the biofilm growth on drip flow bioreactor

1.1.1. Optical density

As it has been mentioned before, a drip flow bioreactor presents six individual chambers in which take place the biofilms cultures. Heterogeneity has been pointed out as the major inconvenience of the drip flow bioreactor (**Table 1)**, which brakes the dynamic study of biofilms.

Several explanations to the heterogeneity are suggested. Firstly, optical density measurements are distorted by the fact that the biofilm aggregates and the presence of biofilm matrix had a great influence on the absorbance measured by the spectrometer. Therefore, a sample preparation was suggested, which consist in the sonication of the cells and their centrifugation and suspension in a phosphate buffered saline solution. Also, temperature wasn't stabilized, which could lead to differences between different experiments. Lastly, given that on drip flow conditions the biofilm formation depends on the number of adhering cells after the stationary phase, it is suggested that the inoculation and the stationary conditions have an important role to play in the variability of the results. In that extends, sample preparation was performed and working conditions were optimized to achieve repeatable results between the different reactor chambers (see **6., Material and methods**). Two experiments, A and B, were carried, with (**Figure 12 B**) and without (**Figure 12 A**) the application of the sample preparation and the optimized working condition. For each experiment, four samplings were performed at four different times. At each sampling time, three reactor chambers were sampled and for each chamber the optical density (OD) was measured for biofilm cells (BC), planktonic cells (PC) and non-adherent sessile cells (NASC) (The nature of these different kind of cells is discussed in the part **1.2., Results and discussions**). The impact of the sample preparation and the optimized working condition was evaluated by statistical analysis of the variability of OD measurements relative to each experiment. The statistical analysis was done on the basis of

the coefficient of variation (CV) which allow to compare the variability between different sampling times despite the great differences between the mean values.

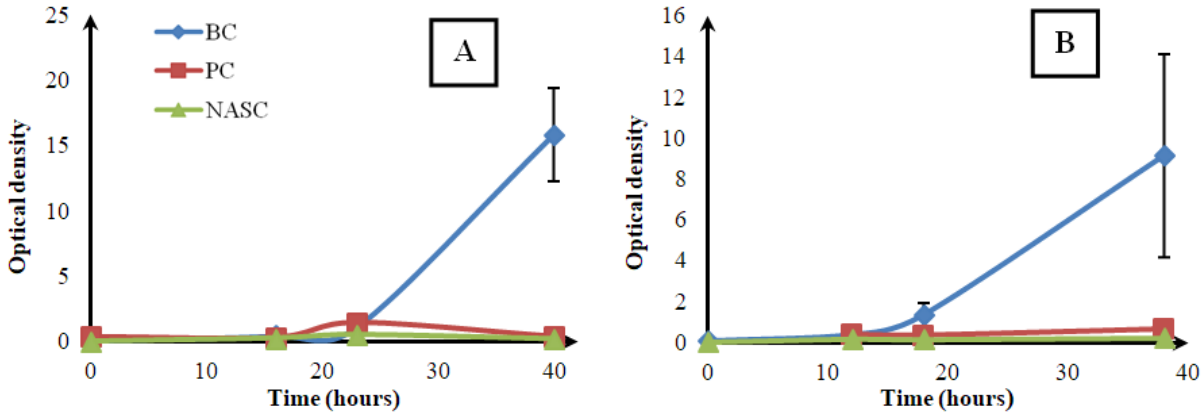

Figure 12: Impact of the sample preparation and optimized working over the optical density measurement of the growth curve of *Bacillus amyloliquefaciens* GA1. No sample preparation nor working condition optimization was performed for experiment A. Both sample preparation and optimized working condition were performed for experiment B.

Table 2: Coefficient of variation values in percentage of the biofilm cells, planktonic cells and non-adherent sessile cells for each sampling time related to a cultivation of *Bacillus amyloliquefaciens* GA1 in drip flow bioreactor without performing sample preparation nor working conditions optimization (experiment A)

Coefficient of variation (%) related to experiment A			
Sampling time (hours)	Biofilm cells	Planktonic cells	Non adherent sessile cells
0	24,8	0,0[1]	29,2
12	6,7	15,4	42,2
18	44,4	45,9	23,6
38	54,4	6,5	34,7
Average CV (%)	32,6	22,6	32,4

[1]No cells were sampled. Time 0 is therefore not used for the average CV calculation for NASC

Table 3: Coefficient of variation values in percentage of the biofilm cells, planktonic cells and non-adherent sessile cells for each sampling time related to a cultivation of *Bacillus amyloliquefaciens* GA1 in drip flow bioreactor after performing sample preparation and working conditions optimization (experiment B)

Coefficient of variation (%) related to experiment B			
Sampling time (hours)	Biofilm cells	Planktonic cells	Non adherent sessile cells
0,0	17,2	5,0	0,0[1]
16,0	10,3	13,8	30,9
23,0	25,2	21,0	21,1
40,0	22,7	17,3	23,7
Average CV (%)	18,9	12,7	25,3

[1]No cells were recovered by the used method. Time 0 is therefore not used for the average CV calculation for NASC

Figure 12 shows the evolution of the OD of experiment A and B and the standard deviation related to each triplicate. Average CV of experiment A stands at 32,6%, 22,6 % and 32,4% for the BC, PC and NASC respectively (**Table 2**) while in experiment B average CV values of 18,9%, 12,7%, 25% are calculated for the BC, PC and NASC respectively (**Table 3**).

The comparison between the average CV values of experiments A and B values show that the variability of the OD measurements decreases considerably, proving the efficiency of the established procedures to achieve a better repeatability between the reactor chambers.

The sonication of the samples was meant to break the cell aggregates and the centrifugation permitted the separation between the cells and the matrix. This procedure allowed to measure the optical density of the BC, PC and NASC suspended alone and thus have results closer to the reality. The stabilization of the temperature avoided temperature fluctuation which could have an impact on the cell development. The inoculation method optimization permitted the immersion of the coupons which allowed the cells to adhere to the coupons in the same conditions from a chamber to another and as a consequence the amount of adhered cells in each chamber became very similar.

1.2. Link between the biofilm and the planktonic phase: presence of a third type of cells?

Many models have been suggested in the literature for the biofilm formation. In particular, *Bacillus subtilis* (which is phylogenetically close to *Bacillus amyloliquefaciens* as discussed in part 2.2.3, Introduction) biofilm formation model (**Figure 13**) have been described as following: firstly, suspended cells (defined as planktonic cells) population grows in a agitated nutritive medium. The suspended cells meet a surface of an immerged or partially immerged material and eventually stick to it, which results in cell differentiation. The cells starts then producing matrix which allows to create a chain of aggregated cells that becomes the biofilm. Once the biofilm reaches its mature state, cells sporulate and eventually detach from the biofilm (Vlamakis *et al.*, 2013). This model implies that the formed biofilm is surrounded by planktonic cells and that the eventual detached cells end up in the planktonic phase.

In drip flow bioreactor (DFB) conditions, the biofilm formation model differs from the presented afore. In DFB conditions, each chamber contains a coupon of a given material and

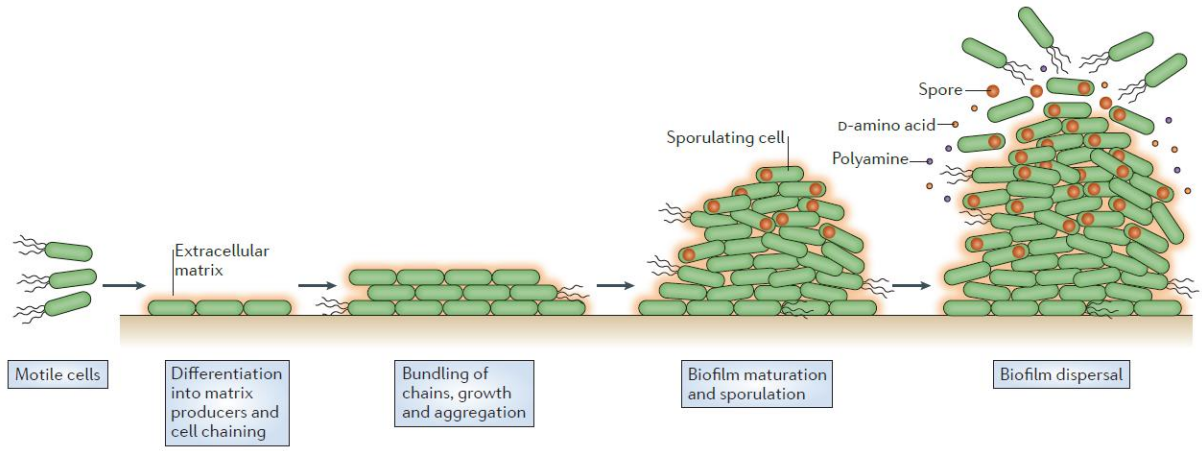

Figure 13: ***Bacillus subtilis*** **biofilm formation occurs following different stages. Cells first adhere to a surface and incurs differentiation. EPS are produced and biofilm is build until biofilm reaches its mature state, after which sporulation and cell detachment occurs (Vlamakis *et al.*, 2013).**

is inoculated with a preculture planktonic phase, in which the coupon is completely immerged. The reactor is kept immobile during 6 hours, which is called the stationary state. During this time all the cells in contact with the coupon have better chance to adhere to the surface thanks to the absence of fluidic movement. After 6 hours, the inoculating phase is entirely removed, theoretically leaving the surface with only the adhered cells. Then fresh nutritive medium is brought to the coupon with a low flow rate. This is called the continuous state. Adhered cells then follows the formation model presented afore and the fresh medium continuously goes through the biofilm and is collected in a waste. The analysis of the medium after its flow over the biofilm shows that suspended cells are present, making it a planktonic phase. However since the inoculating phase is removed to uniquely retain the adhered cells on the coupon, where does this new planktonic phase come from?

Two main hypotheses are suggested to understand the presence of a planktonic phase (**Figure 14**). The first one states that the planktonic cells grow in a thin liquid layer slowly flowing over the biofilm surface. The second hypothesis comes from the observation of less adherent cells on the biofilm surface. This observation led to the hypothesis of a third "type" of cells (in addition to the planktonic cells and the biofilm cells), presenting characteristics between those of the biofilm cells and those of the planktonic cells and eventually being the intermediate state between the two.

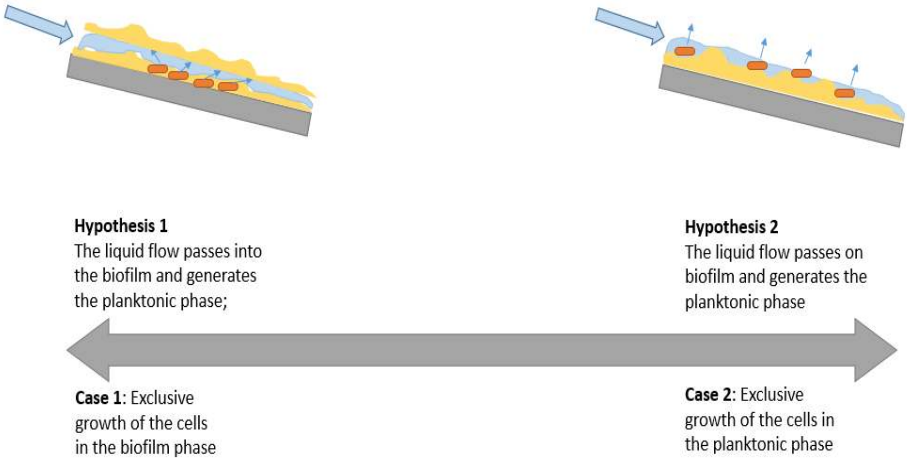

Figure 14: Hypothesis formulated to understand the presence of a planktonic phase in drip flow bioreactor conditions. Planktonic cells could come either from the biofilm (hypothesis 1) or from a thin liquid phase over the biofilm in which they grow (hypothesis 2).

The third "type" of cell would be defined as biofilm cells laying on the surface of the biofilm, in a sessile but non adherent state therefore being non adherent sessile cells (NASC) (**Figure 15**).

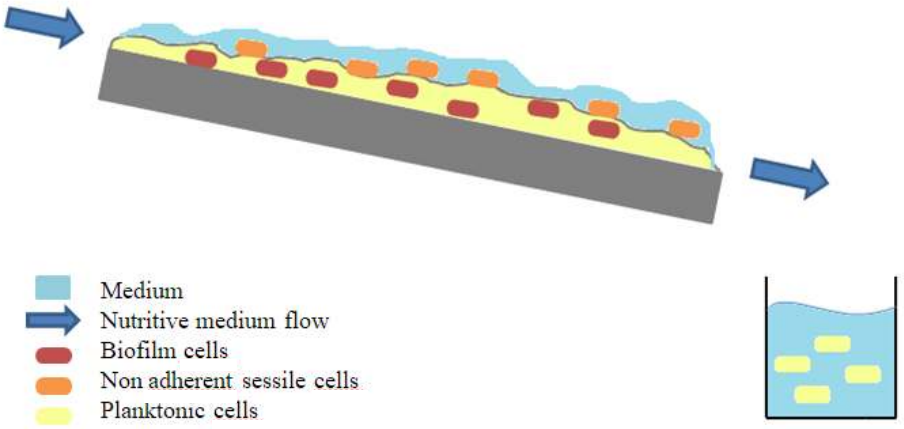

Figure 15: Detailed hypothesis 2 stating that the planktonic cells comes from the biofilm. The biofilm would generate non adherent sessile cells on its surface which would have intermediate characteristics between the biofilm cells and the planktonic cells. The non-adherent sessile cells would be washed by the medium flow to end in a suspended state, becoming the planktonic phase after incurring a differentiation due to their new environment.

These cells would detach themselves from the biofilm, creating thus the planktonic phase. It's commonly accepted that biofilm releases cells once it has reached its maturity, but the release of cells during the whole duration of the biofilm formation has never been studied, even if it has been suggested (Boles, Thoendel and Singh, 2004). Therefore, those NASC were

collected (see **4.3., Material and methods**) and studied at different levels to see if they actually are involved in the occurrence of the planktonic phase by comparing them to the biofilm cells and the planktonic cells.

1.2.1. Polymorphism observation

The cultivation of *Bacillus amyloliquefaciens* GA1 on petri dishes have proved that different morphotypes are active at the same time. As mentioned before, those morphotypes are the result of the cell differentiation which is necessary for the biofilm formation. The three different kinds of cells were though collected and cultivated individually in petri dishes in order to eventually observe isolated morphotypes specifics to each kind of cell.

Two main morphotypes are observed (**Figure 16**), one creating big colonies, the other creating small colonies. The big colonies show a swarming phenomenon, possibly a consequence of matrix production

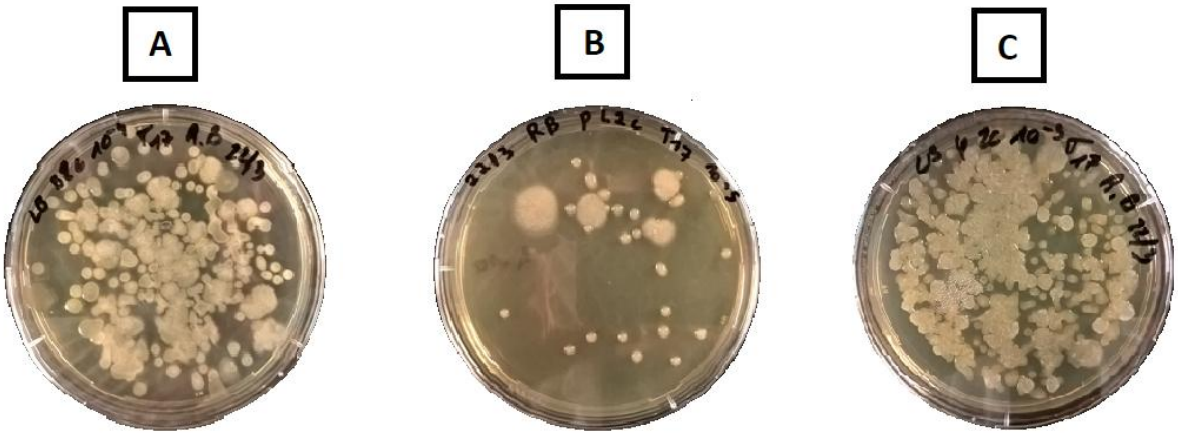

Figure 16: Petri dishes cultures of *Bacillus amyloliquefaciens* GA1 after isolating the Biofilm cells (A), planktonic cells (B) and non adherent sessile cells (C).

However, no isolation of the morphotypes was achieved by this method despite the fact that the different cells were correctly isolated. Consequently, no conclusion concerning the differences between those types of cells could be done on the morphotype basis and the link between the non adherent sessile calls (NASC) and planktonic cells (PC) remains theoretical. However the fact that each petri dish presents the same diversity of morphotypes clearly implies that both biofilm and suspended cells are able to differentiate and adapt to a new environment.

1.2.2. Zeta potential of the biofilm, planktonic and non adherent sessile cells

The first step for a biofilm formation is the cell adhesion to a surface. It is admitted that the adhesion can depend on the surface properties of the material on which the biofilm is spreading (Parkar *et al.*, 2001; Simões, Simões and Vieira, 2010). Also, biofilm cells need to adhere to each other (Del Re *et al.*, 2000) and the matrix composition and quantity may not be the only responsible. Given this, it seems legitimate to expect from the different kind of cells to have different physico-chemicals properties affecting especially the adhesion capacity of the cells (Hori and Matsumoto, 2010; Hong *et al.*, 2012). The most evident physico-chemical property is the zeta potential (Hori and Matsumoto, 2010; Hong *et al.*, 2012). The physico-chemical properties may be affected by physical treatments such as high speed centrifugation and washing/resuspension. On Gram + bacteria, centrifugation have very little impact on the cells while the washing and resuspension can't be neglected (Pembrey, Marshall and Schneider, 1999). The effect of these treatments also strongly depends on the strain.

To determine the effect of the washing and resuspension in milli-Q water, the zeta potential of biofilm, planktonic and non adherent sessile cells was measured after 24 h of culture (**Figure 17 and 18**). Zeta potential of washed cells after 27 hours of culture was measured to determine the culture time contribution to the zeta potential (**Figure 19**).

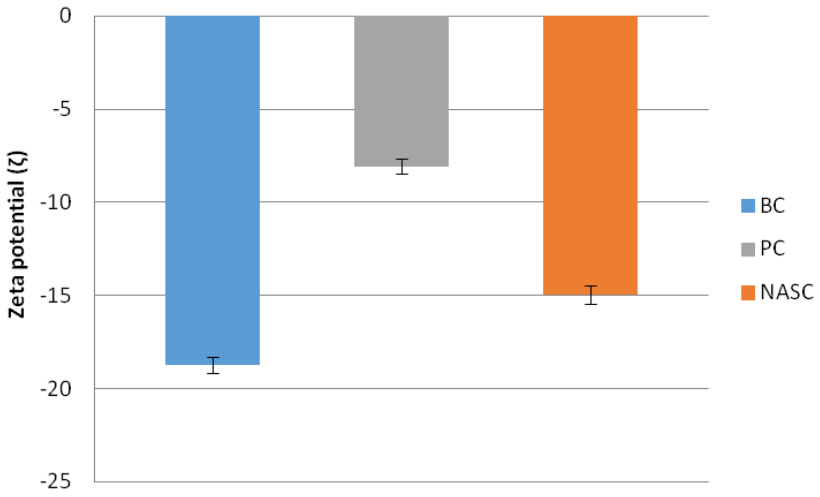

Figure 17: Comparison of zeta potential values of non washed nor resuspended biofilm cells (BC), planktonic cells (PC) and non adherent sessile cells (NASC) after a 24 hours of culture on dripflow reactor.

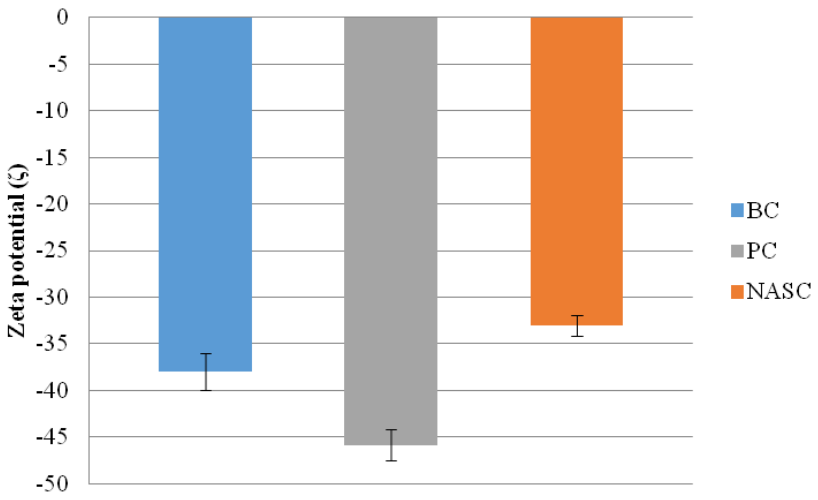

Figure 18: Comparison of zeta potential values of washed and resuspended biofilm cells (BC), planktonic cells (PC) and non adherent sessile cells (NASC) after a 24 hours of culture on dripflow reactor. Cells were washed and resuspended with milli-Q water

Figure 17 and **Figure 18** clearly show that washing and resuspension have a great impact on the zeta potential values. The change of pH and the absence of charged particles in milli-Q water may be the most likely reasons for this phenomenon. Biofilm cells and non a adherent sessile cells were less affected by the treatment. The presence of matrix around those cells may contribute to the impact of the treatment.

Figure 19 compared to **Figure 28** shows that the hydrophobicity of the biofilm can dramatically increase in a very short period of time, meaning that biofilm cells physiology have changed and that the biofilm is reaching its mature state. Therefore, biofilm and planktonic cells' zeta potential is logically impacted.

On the opposite, non adherent sessile cells zeta potential doesn't significantly changes meaning that they have a different behaviour than the two other types of cells.

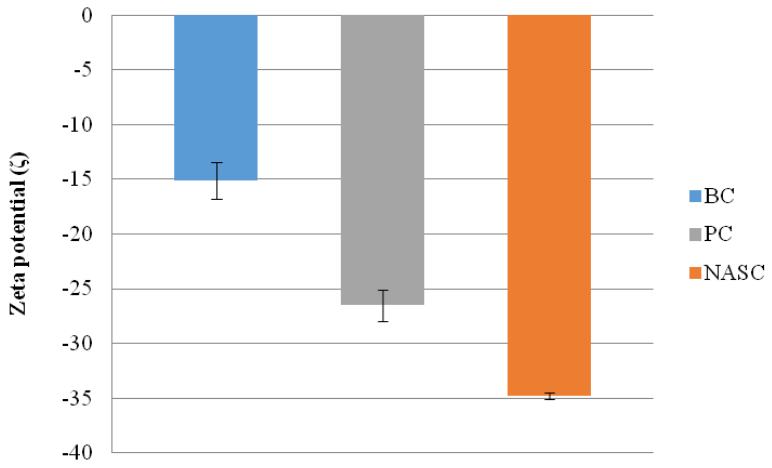

Figure 19: Comparison of zeta potential values of washed and resuspended biofilm cells (BC), planktonic cells (PC) and non adherent sessile cells (NASC) after a 27 hours of culture on dripflow reactor. Cells were washed and resuspended with milli-Q water.

All the samples showed negative zeta potential values. It has been stated that silicone (Gu and Dongqing, 1998), and multiple common surfaces like minerals (Hong *et al.*, 2012) or PET (Campagne, Perwuelz and Leroux, 2009; Güney *et al.*, 2015) have also negative zeta potential. Therefore, it becomes logical that biofilm cells present a lower zeta potential in terms of absolute value than planktonic cells since they need to adhere to these surfaces. Also, they need to aggregate to form a biofilm and therefore the lower their external electric charge is, the easiest for them to approach each other. Moreover, low zeta potential and EPS production have been proved to be correlated. EPS production appears to be promoted when the zeta potential is close to 0 (Nguyen *et al.*, 2016).

After 27 hours of culture, the NASC show a high zeta potential in comparison to the biofilm cells. That might be explained by their position and function in the biofilm. Biofilms are meant to grow in a bidirectional way to colonize surfaces. A gain of width would bring negative consequences to biofilm such as starvation. If the biofilm is too thick, the nutrients coming either from the upper or the lower surface will not be able to reach every cell as they are consumed in the first layers of the biofilm. Also biofilms cells replicate and the new cells have to replace old ones in the same way as in our skin new cells are created while old ones are permanently rejected. Considering this and admitting that the biofilm must have a maximum width it becomes likely to have non adherent sessile cells at its surface with great zeta potential allowing them to leave the biofilm. Those cells presumably end in the medium and contribute to the occurrence of a planktonic phase in the drip flow.

1.2.3. Flux cytometry

Flow cytometry is commonly used to study phenotypic heterogeneity between microbial populations. Biofilm are known to show such heterogeneity and therefore the phenotype analysis of the three kinds of cells using flow cytometry can lead to the establishment of phenotypes divergences or convergences regarding the different kind of cells, which could help to determine if the NASC are actually involved in the occurrence of the planktonic phase. To that extend FSC and SSC profiles were firstly analysed to compare the size and granularity distributions of the different types of cells. Two colorants were also used to study the cell physiology: acridine orange and redox sensor green (RSG).

Acridine orange was used to visualize the sporulation rate of the different types of cells, which is related to the viability of the populations and can give information about the environmental conditions perceived by the cells (McFeters *et al.*, 1991). RSG was used to compare the respiratory activities of the different kind of cells, which allow to compare the metabolic activity of each type of cell (Stewart and Franklin, 2008). Differences in the metabolic activities may mean different functions in the biofilm and therefore a physiological difference between the cells

Sampling was performed for each type of cells after 21 and 40 hours of culture.

FSC profile didn't show significant differences between the three types of cells. No differences are observed between the two sampling times either. At this point the three types of cells can't be considered as different by their size (**Figure 20**).

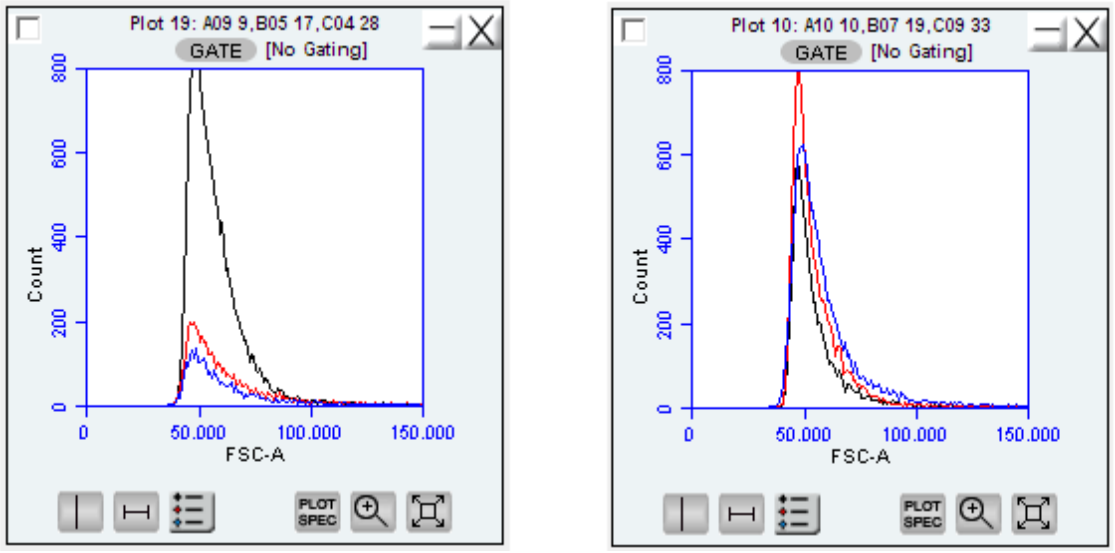

Figure 20: FSC profile of biofilm cells (black), non adherent sessile cells (red) and planktonic cells (blue) after 21 hours of culture (left) and 40 hours of culture (right).

SSC profile (**Figure 21**) shows that planktonic cells and non adherent sessile cells (NASC) have close granularities after 21 hours of culture. After 40 hours of culture the three profiles evolve due to the biofilm maturation which engender cells physiology alteration.

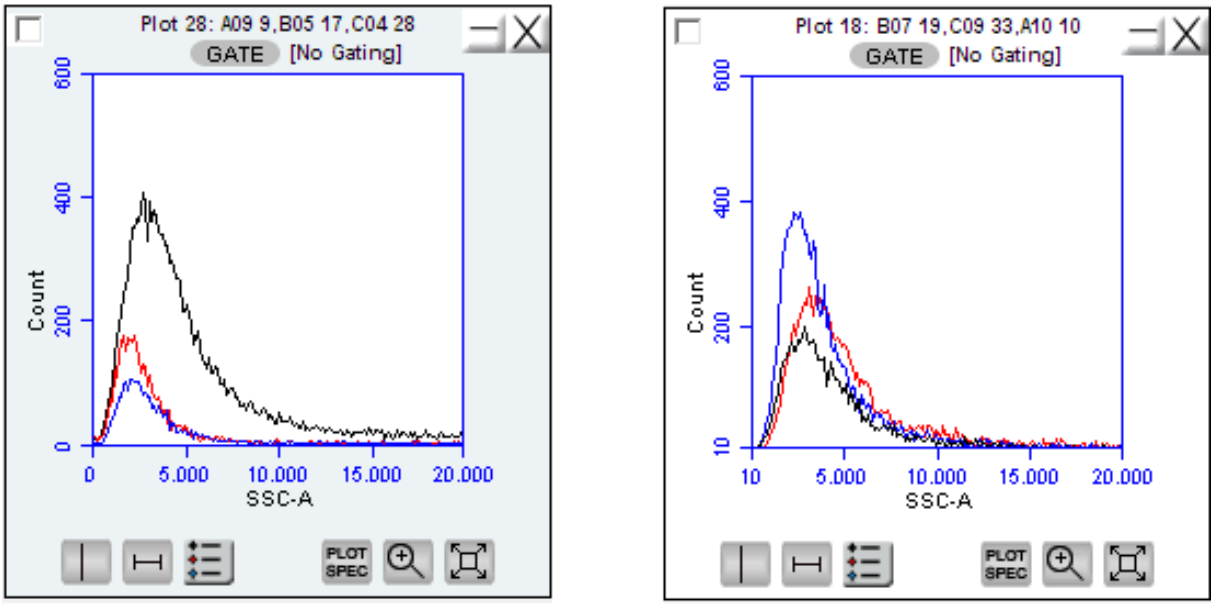

Figure 21: SSC profile of biofilm cells (black), non adherent sessile cells (red) and planktonic cells (blue) after 21 hours of culture (left) and 40 hours of culture (right).

The FL-2 channel was used to observe the cytograms of acridine orange stained cells (**Figure 22**). After 21 hours of culture biofilm cells and NASC react to the acridine orange nearly

identically. On the opposite, planktonic cells present a totally different profile. After 40 hours differences can be observed between NASC and biofilm cells, but NASC cytogram still closer to biofilm cells cytogram than to the planktonic cells cytogram.

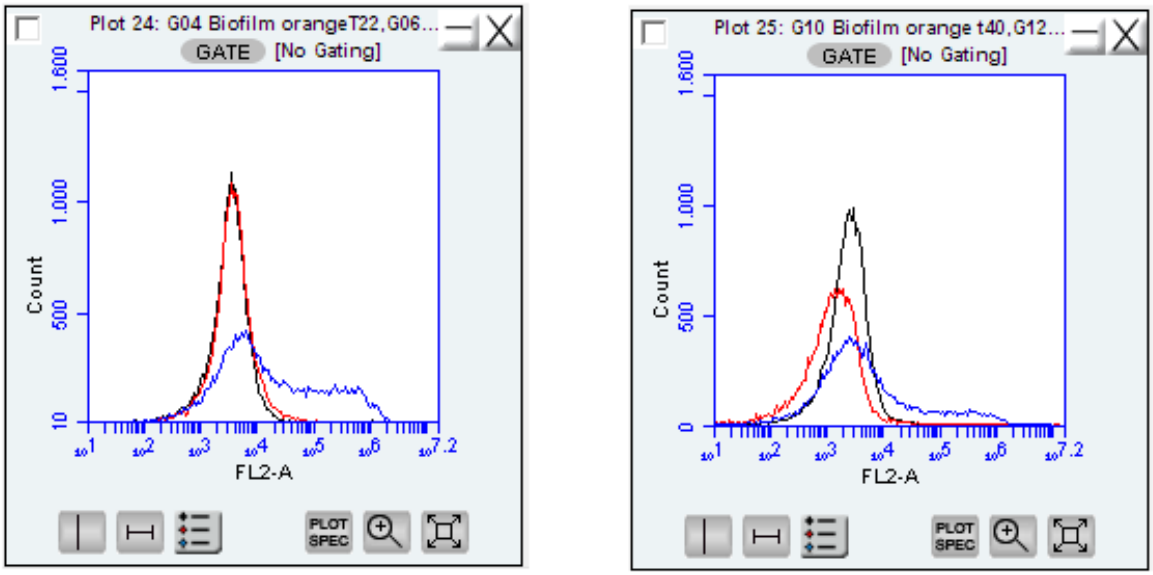

Figure 22: FL-2 profile of acridine orange stained biofilm cells (black), non adherent sessile cells (red) and planktonic cells (blue) after 21 hours of culture (left) and 40 hours of culture (right).

Planktonic cells cytogram expands to the right, particularly after 21 hours, meaning that there are two subpopulations reacting differently to the dye, as a consequence of their physiological differences. The planktonic subpopulation showed in the right part of the FL2-A axis shows that a percentage of the planktonic cells are sporulating meaning that they are less viable that the biofilm cells and the non-adherent sessile cells. This can be explained by the fact that the nutrient contained in the fresh media are all consumed by the biofilm cells and the non adherent sessile cells. Therefore the planktonic cells are suspended in a poor solution, maybe are in starvation state. After 40 hours the sporulation level of the planktonic cells is lower than for 21 hours.

The fact that after 40 hours NASC and biofilm cells present different profiles also means that once the maturation of the biofilm is achieved big physiological changes incur to the cells.

The metabolic state of the three kinds of cells was determined using RSG colorant (**Figure 23**). The FL-1 channel was used. Also, preculture cells in exponential phase were stained with RSG and a cytogram of the FL-1 over time was done (**Figure 24**) in order to determine the time necessary for the RSG to make an effect on the cells. That also gave us an idea of the response given by cells in full metabolic activity. After 21 hours of culture, biofilm cells and

planktonic cells have strongly different profiles. NASC have a profile intermediate between the two. After 40 hours of culture the three cytogram evolve in comparison to the 21 hours cytogram. The three types of cells have different metabolic states. NASC present at time 40 hours a profile close to the biofilm cells profile at time 21 hours.

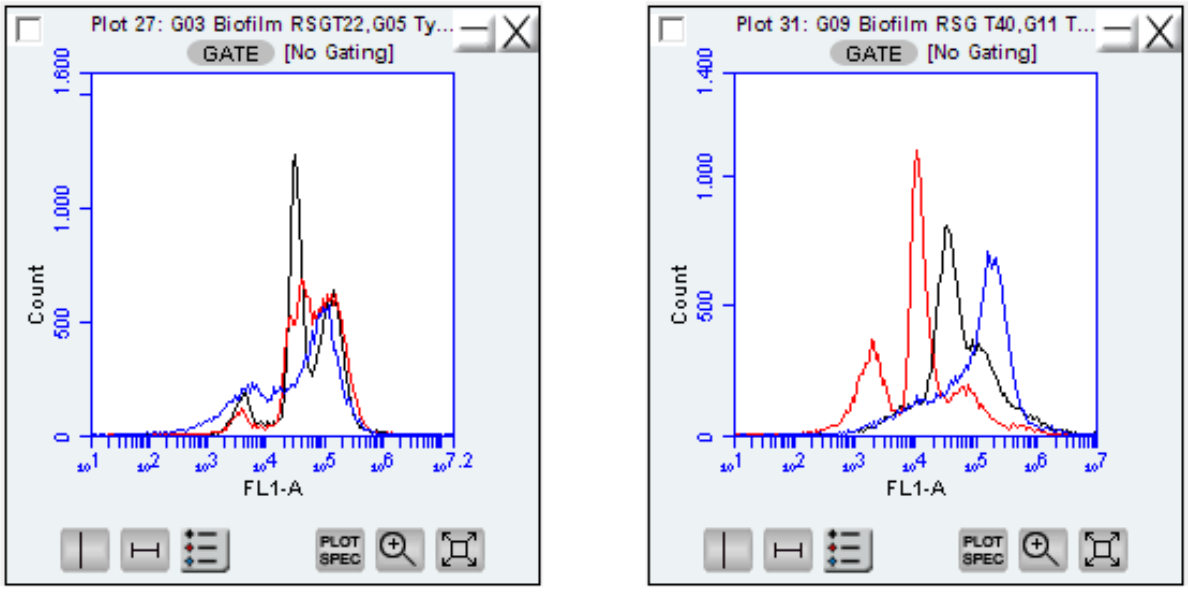

Figure 23: FL-1 profile of RSG stained biofilm cells (black), non adherent sessile cells (red) and planktonic cells (blue) after 21 hours of culture (left) and 40 hours of culture (right).

RSG cytograms show that there are different subpopulations for each type of cells, in particular for the biofilm cells and the NASC. This is caused by the differentiation occurring during the biofilm formation.

Using the preculture cells as a reference (**Figure 24**), it can surprisingly be observed that the planktonic cells have the most active metabolism compared to the other types of cells, which increase from 21 hours to 40 hours the same way that their sporulation rate decrease during the same time period.

While biofilm cells' metabolic state hardly evolve, the NASC metabolic activity dramatically decrease from time 21h to 40h. In drip flow conditions, it has been observed that after 40 hours, the biofilm is fully mature and produces hydrophobic molecules at its surface to protect it. Therefore surface cells have a protective function and hardly have access to nutrients, explaining their lower metabolic activity. On the opposite, biofilm assures its nutritive income by growing around the area where the medium drop falls (**Figure 25**), assuring absorption of the media despite the hydrophobicity of the biofilm surface. This explains why biofilm cells

don't tend to sporulate and continue to have a strong metabolic activity despite the hydrophobicity.

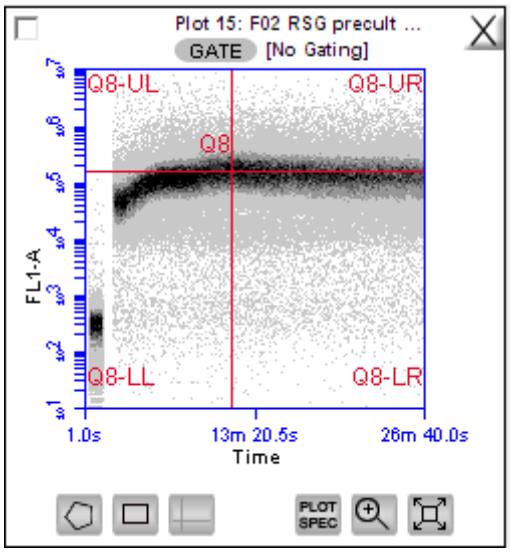

Figure 24: FL-1 profile over time of preculture cells of *Bacillus amyloliquefaciens* GA1 in exponential phase stained with RSG. The dynamic analysis shows that FL-1 response of RSG stained cells is maximal 13 minutes after the staining and stay constant for at least 15 minutes.

Figure 25: *Bacillus amyloliquefaciens* GA1 biofilm developed over a smooth silicone surface. The biofilm develops around the area where the nutritive medium arrives

From a general point of view, the results show that NASC closer to the biofilm cells than to the planktonic cells, which seem logical since they are collected on the biofilm surface. However, after 40 hours of culture, the NASC can't be considered a biofilm cells since their cytogram present substantial differences in comparison with the biofilm cytogram. After 21 hours of culture the metabolic state of the NASC seems to be between the biofilm and the planktonic cells metabolic state. As a conclusion, it's legitimate to think NASC may be cells developed in the biofilm which could lead to the occurrence of the planktonic phase.

1.3. Maturation of the biofilm over time

1.3.1. Hydrophobicity of the biofilm: contact angle measurement

In nature the hydrophobicity of the biofilm is an essential behaviour for the biofilm survival (Kobayashi and Iwano, 2012). They are often founded in surfaces bigger than themselves and usually get their nutrients by the lower layer (Vlamakis *et al.*, 2013), given that the hydrophobic layer becomes a protective tool for the biofilm impeding liquid flows like heavy rains to wash them away. From a more general point of view, this external layer protects the

biofilm from several environmental threats. However, in drip flow conditions, the biofilm didn't show a hydrophobic behaviour during the early stages of the biofilm formation. It was suggested that the production of hydrophobic compounds depended on the maturation state of the biofilm. Therefore the evolution of the biofilm's surface hydrophobicity would become an interesting approach for the study of the dynamics of the biofilm in terms of maturity.

Firstly, the ability of the biofilm to produce hydrophobic compounds was tested by cultivating single colonies in petri dishes and observing its eventual hydrophobic behaviour. Then a dynamic study of the evolution of the hydrophobicity of the biofilm's surface was performed.

Hydrophobicity in petri dish cultures

Single colonies cultivation in petri dishes shows a hydrophobic behaviour of the biofilm. As the culture time increased so did the hydrophobicity of the colony. This kind of behaviour has already been studied for *Bacillus subtilis* and BslA and TasA have been proved to be responsible for this phenomenon (Kobayashi and Iwano, 2012)

For the a given colony, its hydrophobicity depended on the place where the measure was taken. Visually, the centre of the colony and the borders showed hydrophilic behaviours while the area between the two showed more hydrophobic behaviours (**Figure 26**).

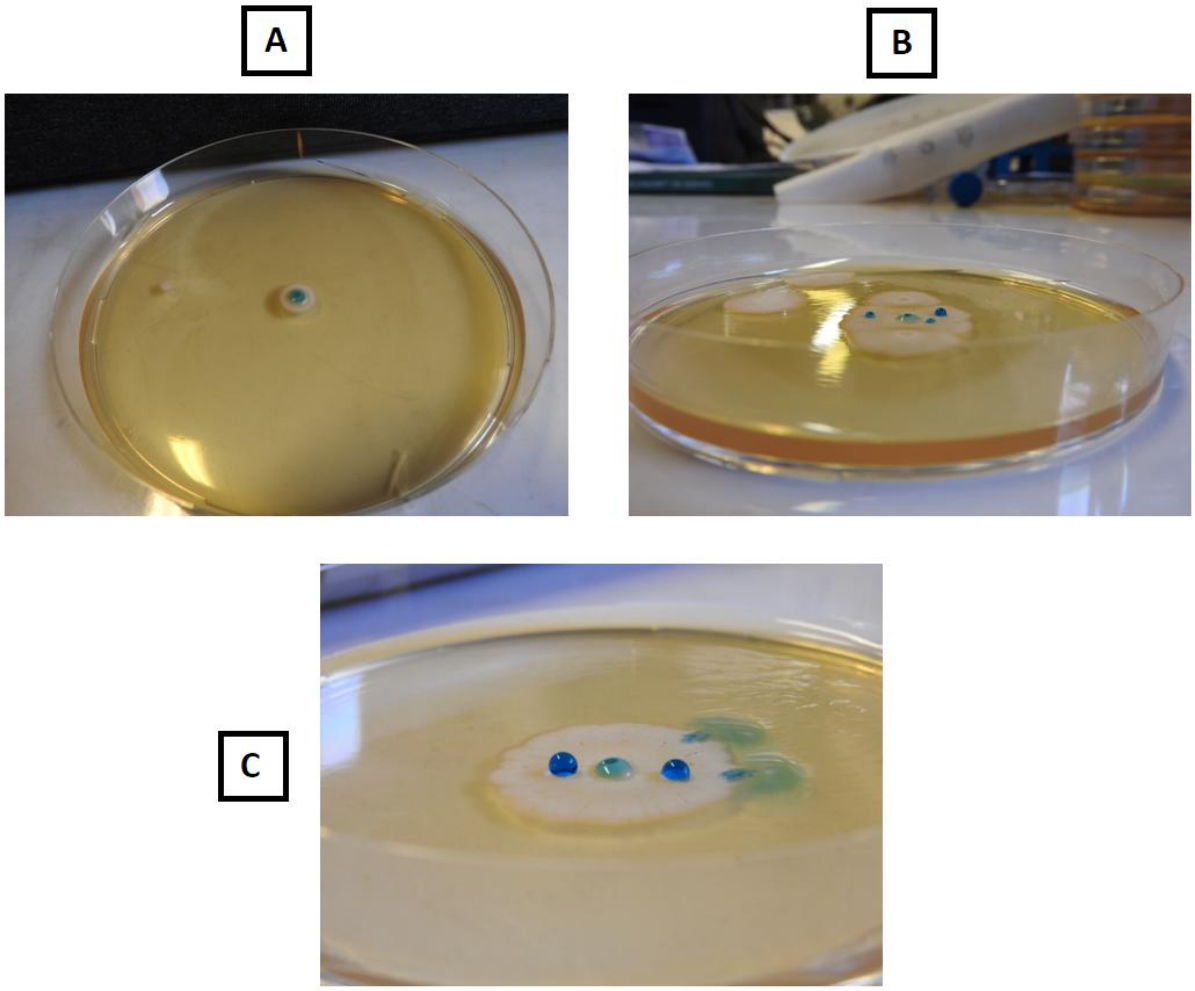

Figure 26: Colored water drops on *Bacillus amyloliquefaciens* GA1 after 16 h (A) and 40 h (B,C) of culture at 30 °C.

Hydrophobicity in drip flow bioreactor cultures

The contact angle between the biofilm surface and milli-Q water (which surface tension is very close to the nutritive medium) was measured using the TRACKER (see **8.1., Material and methods**). Pictures from the tracker (**Figure 27**) clearly show the rise of the contact angle during the culture. The values calculated by the tracker were corrected manually since the irregularity of the biofilm surface causes false results.

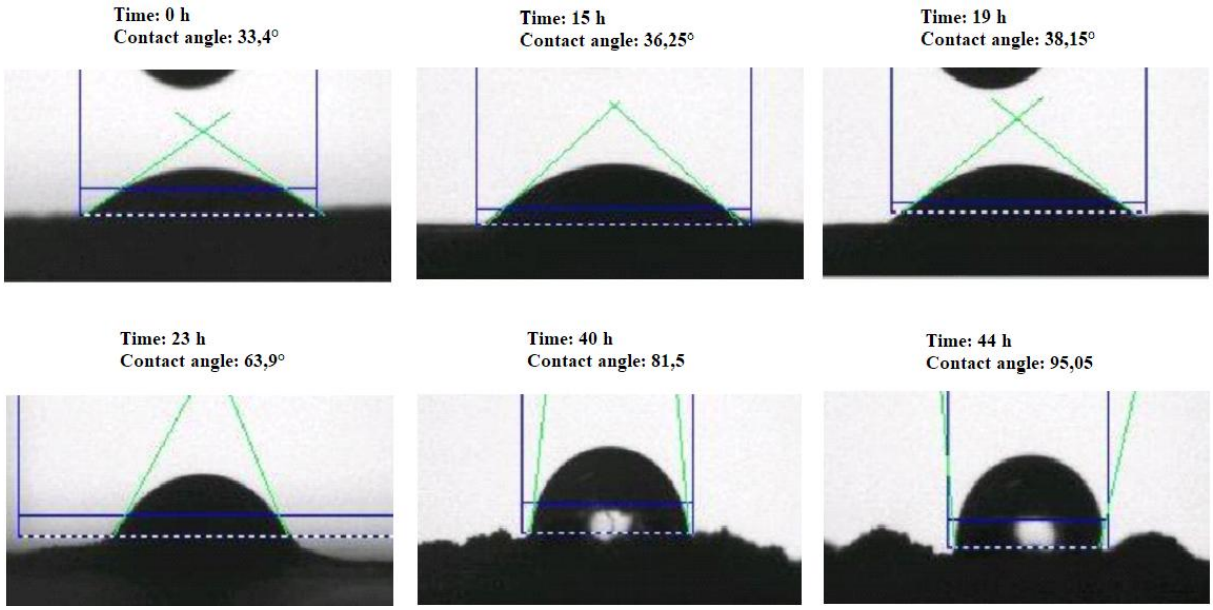

Figure 27: Pictures from the tracker showing the contact angle between *Bacillus amyloliquefaciens* GA1 biofilm and milli-Q water for different sample times.

Figure 28 shows the evolution of the contact angle during the experiment in parallel with the OD measurements of the same samples. It can be observed that the hydrophobicity dramatically rises between the 19th and the 23rd hour of culture. This perfectly matches with the moment where the biofilm starts to grow rapidly after a phase of latency during which biofilm nearly didn't grow. Therefore, it can be concluded that there is a physiology turnover bringing the biofilm to its maturation.

Concerning *Bacillus subtilis*, it have been proved that bslA, which is an amphiphilic protein, and TasA amyloid fibres induce the hydrophobicity of the biofilm. Particularly bslA amphiphilic proteins appears to form a hydrophobic layer on the surface of mature biofilms (Kobayashi and Iwano, 2012). Given the resemblances exposed between *Bacillus subtilis* and *Bacillus amyloliquefaciens* it is legitimate to expect the same impact of bslA and TasA on the two strains. Therefore bslA and TasA expression of the very same samples presented afore were quantified by rtqPCR (Bouchat, 2017) for the biofilm cells and for the non adherent sessile cells. Results showed a clear increase of the bslA and TasA expression after 40 hours of culture , which correspond with the maximal contact angle measured. Also, after 40 hours of culture the upper layer structure of the biofilm surface completely changes to become

irregular (**Figure 30**) which is likely to be caused by the bslA layer created observed on mature biofilms.

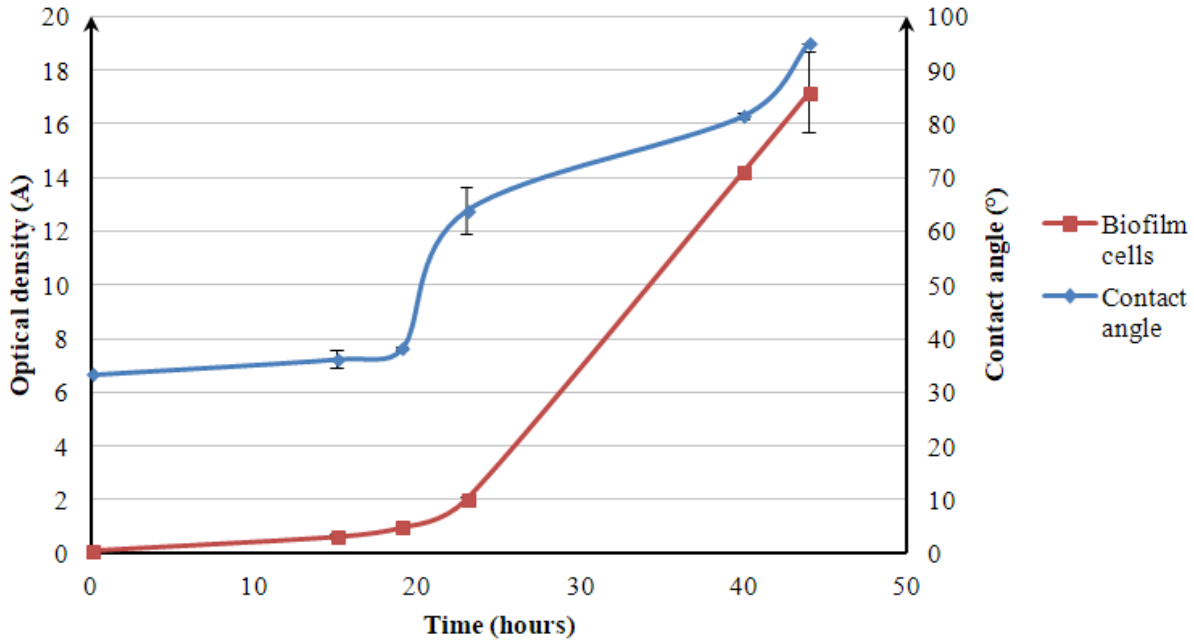

Figure 28: Evolution of the contact angle of the biofilm showed in Figure 27 against the evolution of the optical density relative to the same biofilms.

The dynamic analysis of the contact angle supported by the quantification of TasA and BslA leads to the conclusion that the biofilm reaches its maturity after 40 hours of drip flow bioreactor culture in our specific conditions.

The hydrophobicity of the biofilm is a determinant parameter that needs to be taken into account when studying the biofilm growth. In drip flow condition the hydrophobicity establish one of the limits of the device since the nutritive medium arrives drop by drop falling from the needle to the biofilm. Even if it has been stated that biofilms grows around the drop deposit spot, there is no guarantee that it will always be the case. Therefore, a drop of nutritive medium landing on the biofilm surface wouldn't penetrate the biofilm surface and reach the biofilm cells, which could therefore suffer from nutrients lacks.

Experiments longer than 40 hours will though risk to suffer from this nutrient lack if the culture is carried in drip flow like conditions. From a plastic degradation point of view, this may constitute a problem since degradation operates over months. Indeed as mentioned before plastic molecules are quite inert and the processes involved in its degradation aren't yet totally understood.

2. Biofilm culture on PET surfaces

It's commonly admitted that biofilm is a necessary step for plastic degradation. Biofilm development and adhesion to the surface are determinant and depends on the properties of the biofilm and the material surface. In particular the cristallinity of the PET impacts its degradation and was therefore measured. Cultures in flasks and drip flow were tested and compared. Eventual degradation was observed using scattering electron microscopy (SEM).

2.1. Cristallinity of the PET

Plastic coupons were autoclaved before their use either in flasks or drip flow. Thermal treatments are known to impact the crystallinity degree of polymers, which have a great impact on their degradation (Kint and Muñoz-Guerra, 1999; Gu, 2003; Mohan and Srivastava, 2010). Therefore the crystalline state our PET was measured after 1 and 2 autoclaves to test its stability regarding high temperatures. **Figure 29** shows the results of the RX measurements No impact of the temperature on the plastic cristallinity was observed.

The observable differences in the counts axis are due to a different placement of the plastic coupon leading to the measurement of a thinner part of the plastic.

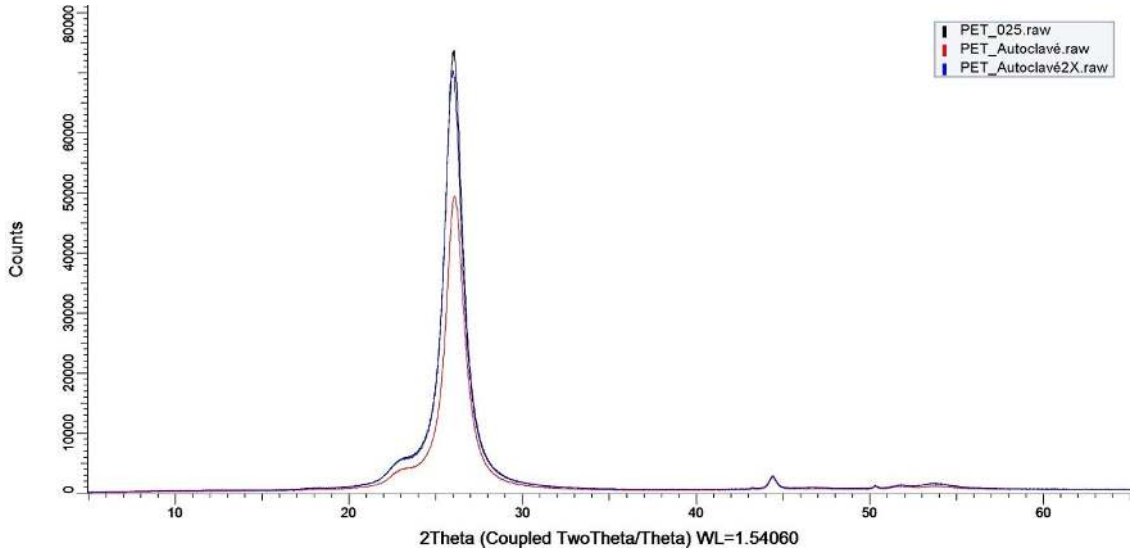

Figure 29: PET cristallinity before autoclave treatment (black) and after 1 (red) and 2 (blue) autoclaves.

2.2. Comparison between the drip flow and the flasks culture efficiency

Flasks tests showed a huge variability between flasks that were in the same conditions. The variations come from the random factor arising from the turbulent flow of the medium due to flask agitation and the slight differences in the plastic placement in the flasks.

The biofilm formation was either non observable or inconsistent as a consequence of the medium agitation which tends to extract cells from the plastic to bring them back into the culture medium.

Biofilm was always present on the medium air interface between the medium and the air or in the bottom of the plastic coupon. This is explained by the fact that the surface presents more oxygenation and the bottom benefits from less turbulence than the rest of the flask.

Drip flow tests proved that *Bacillus amyloliquefaciens* GA1 is able to grow on PET surfaces as easily than on the silicone (**Figure 30**).

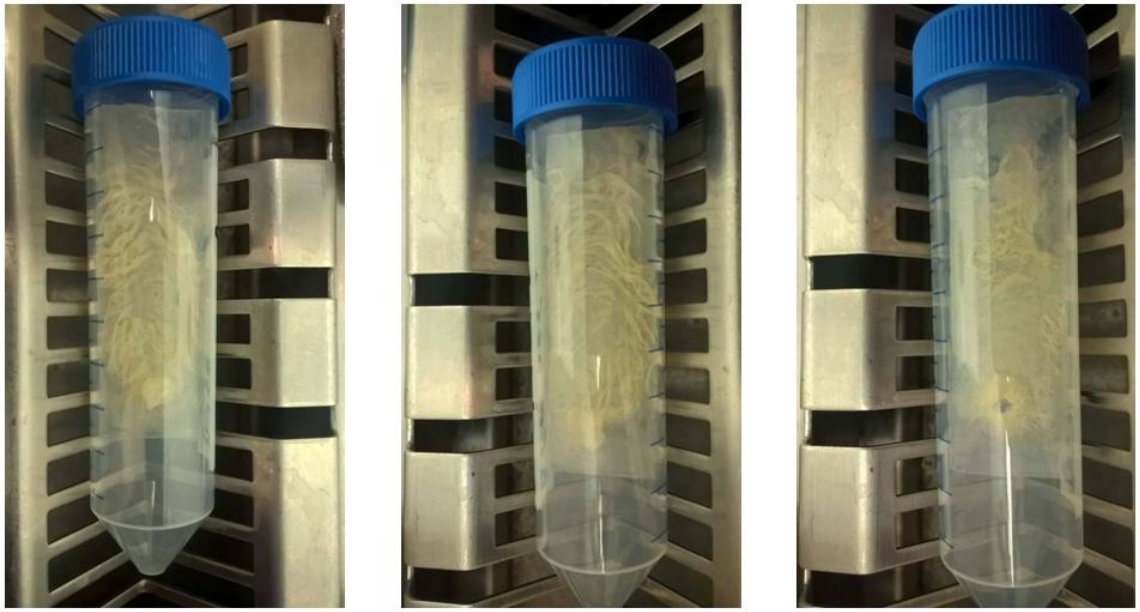

Figure 30: ***Bacillus amyloliquefaciens*** **biofilm on PET surface after a 54 h cultivation in drip flow bioreactor.**

However biofilm showed a very little adhesion to the PET surface while in the same conditions they were strongly attached to the silicone.

Repeatability between chambers stills acceptable considering the large number of random parameters (**Figure 31**). Biofilm growth on silicone and PET showed close trends and

visually biofilms were similar (**Figure 25 and 30**). Given that it is legitimate to consider that the biofilm dynamics is comparable in the two growth conditions (plastic and PET).

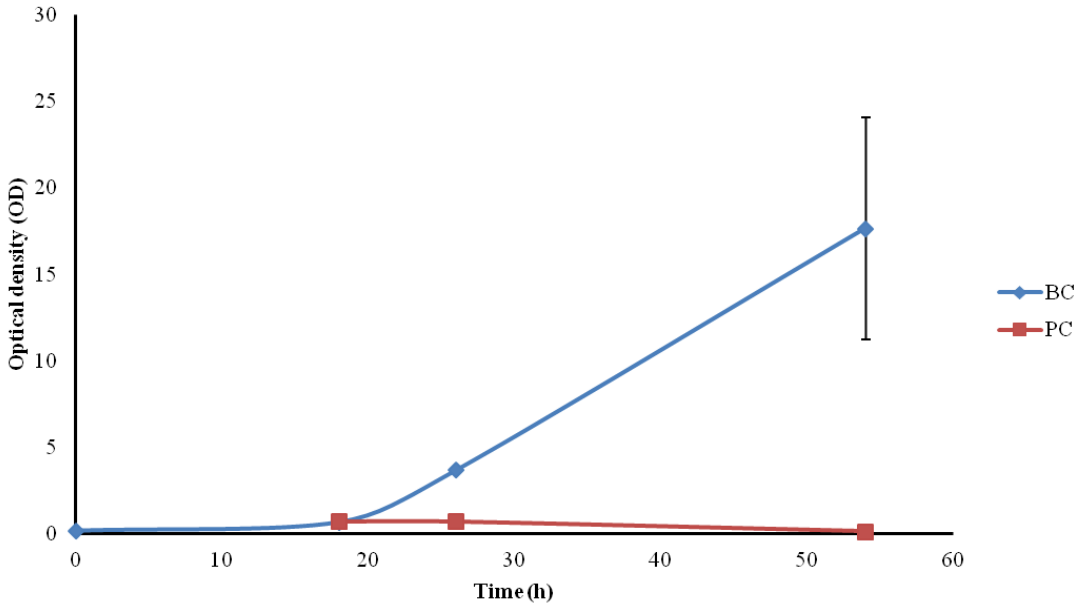

Figure 31: Evolution of the optical density of planktonic cells (PC) and biofilm cells (BC) from a 54 hours culture of *Bacillus amyloliquefaciens* GA1 on PET after performing sample preparation (sonication and cells washing) and working conditions optimization.

2.3. Bacterial adhesion to the PET and surface

Biofilm adhesion to PET surface was much lower than to silicone surface. Since it has been suggested that the biofilm development on PET and on silicone are comparable, the adhesion differences should find an explanation away from the biofilm matrix composition. Therefore physico-chemical parameters were studied.

Firstly, the PET and silicone hydrophobicity was measured using the same technique used to measure the biofilm surface hydrophobicity. Results show that PET surface is much more hydrophobic than silicone surface (**Figure 32**), which makes the PET more difficult to stick on. If the biofilm is wet due to the nutritive medium it will rip away from the PET surface more easily than from the silicone surface.

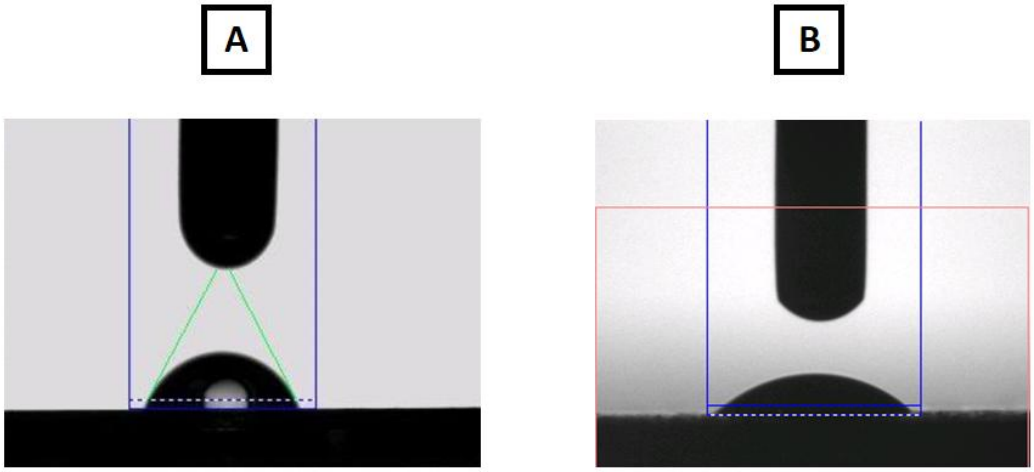

Figure 32: Comparison between the contact angle formed by a drop of milli-Q water on a PET surface (A) and a smooth silicone surface (B)

PET surfaces were also observed by SEM. It follows that plastic surface is remarkably smooth, with nearly no natural irregularities (**Figure 35**). On the opposite, observation of the silicon surface carried in our lab with a WHX digital microscope show that silicone surface is very rough, with cavities going up to 40 µm (**Figure 33**)

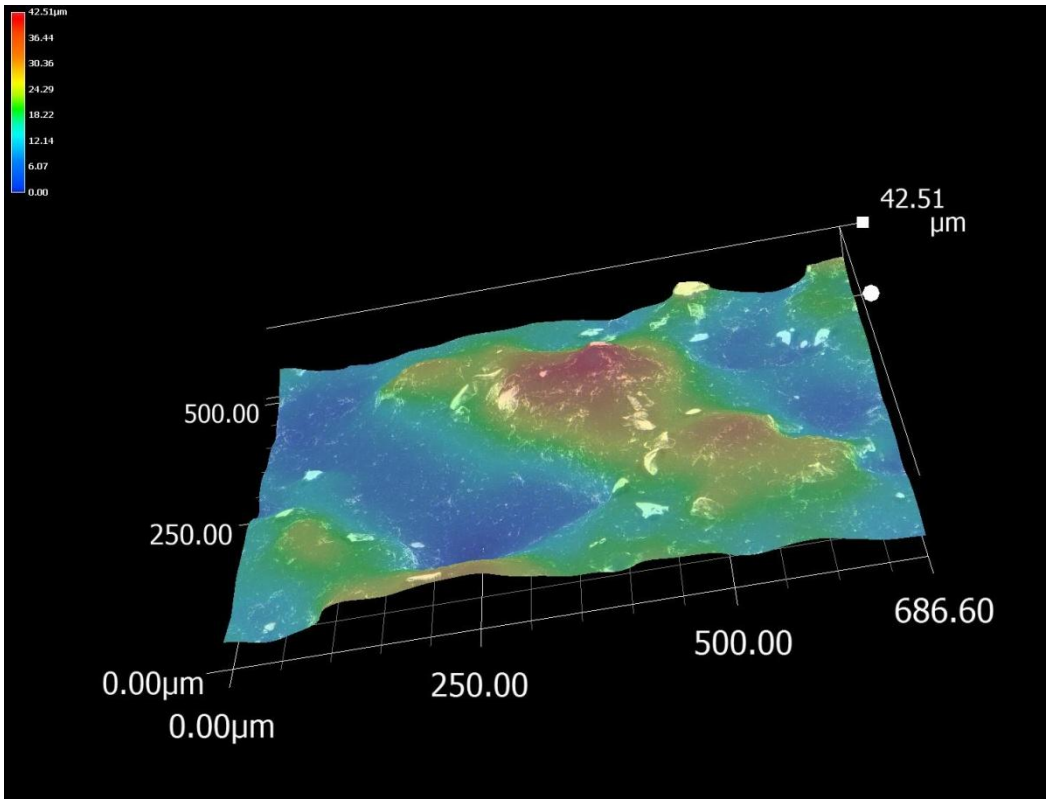

Figure 33: Smooth silicone surface observation with a VHX digital microscope.

The surface roughness is a well known factor influencing the biofilm adhesion (Hong *et al.*, 2012). The rougher the surface is, the better is the adhesion. Furthermore, SEM observations showed that biofilm often tended to develop over the PET surface irregularities (**Figure 38, 39, 40**).

2.4. Observations of eventual plastic degradation due to biofilms activities using scattering electron microscopy (SEM) technique.

Three experiments were performed: a flask culture over 2 days, a flask culture over 3 weeks and a 40 hours culture in drip flow bioreactor.

As expected the flasks experiments were less compelling than the drip flow experiences. Drip flow offered matured biofilms (**Figure 34**) while flasks experiments barely achieved to have visible biofilm formation on the plastic surface.

PET samples from the flasks tests seems to have suffer some physical alteration (**Figure 36 and 37**) when compared to the controls (**Figure 35**) (the controls all had the exact same appearance). However the observations aren't enough significant to conclude any real impact of the microorganisms over the plastic. On the opposite, biofilm tests showed more compelling results. Some areas appear to be damaged in very particular ways (**Figure 38 and 39**). Moreover, in some cases rests of biofilm matrix can be observed perfectly fitting the borders of some unusual cavities (**Figure 40**).

Figure 34: Biofilm after 40 hours of culture in drip flow bioreactor on a PET surface. Biofilm have been fixed to the surface by immerging it in consecutive ethanol solutions of increasing purity.

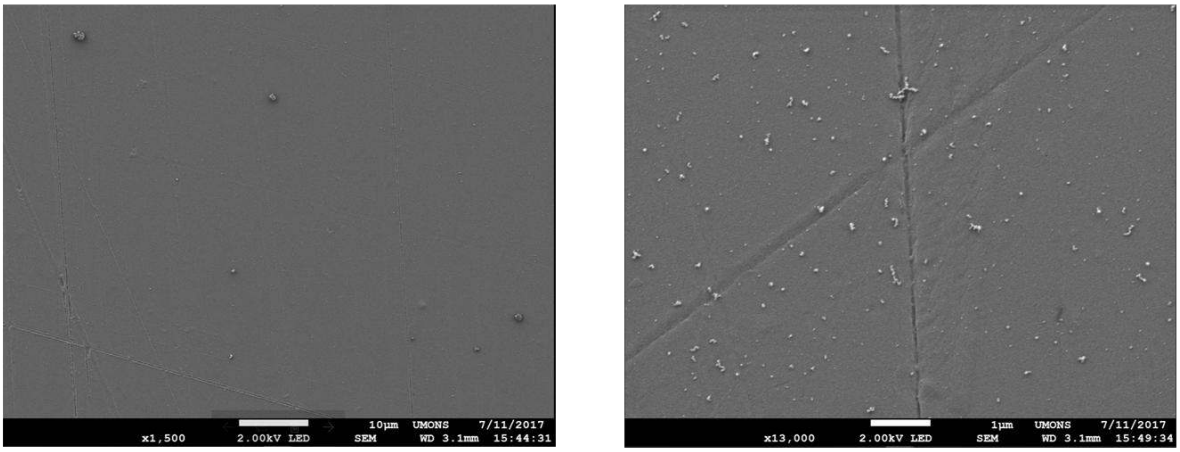

Figure 35: Control PET surface. Surface have been washed with bleach.

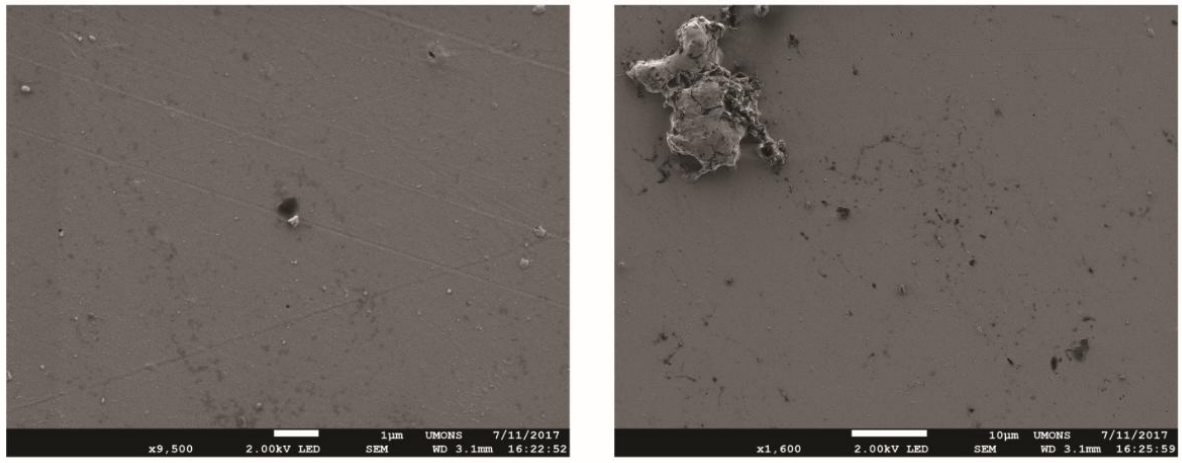

Figure 36: PET surface washed with bleach after a flasks cultures.

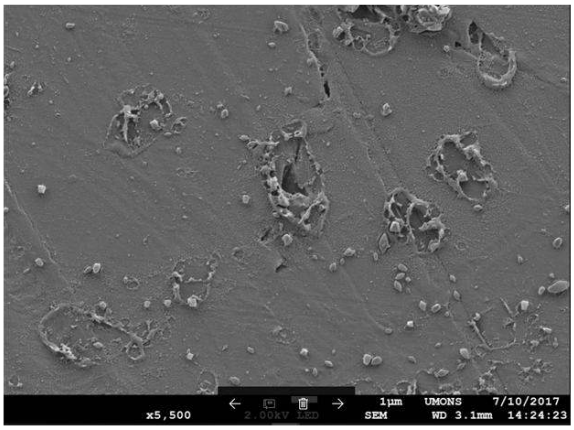

Figure 37: PET surface washed with bleach after a flasks cultures.

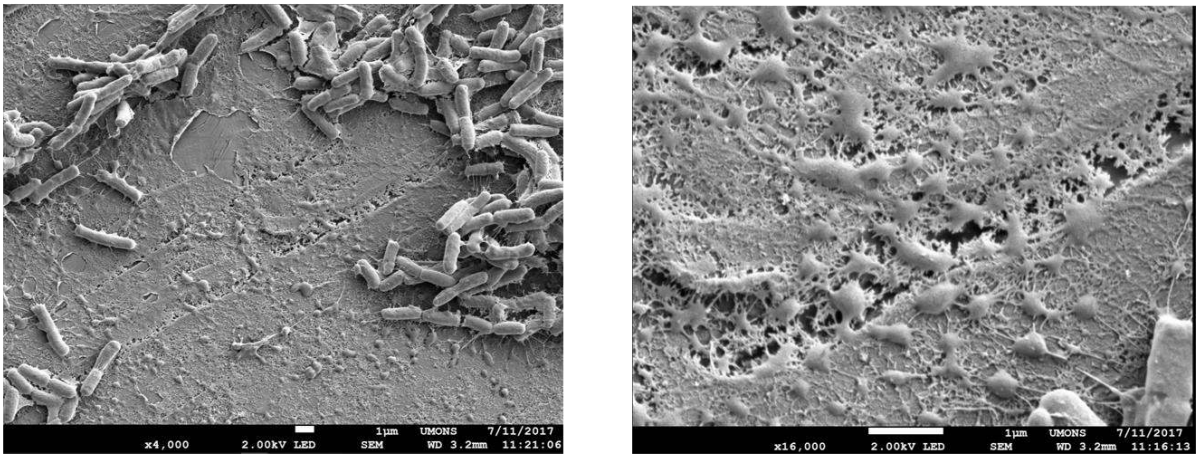

Figure 38: Biofilm formation after a 40 hours culture in drip flow bioreactor over PET surface cavities covered with matrix

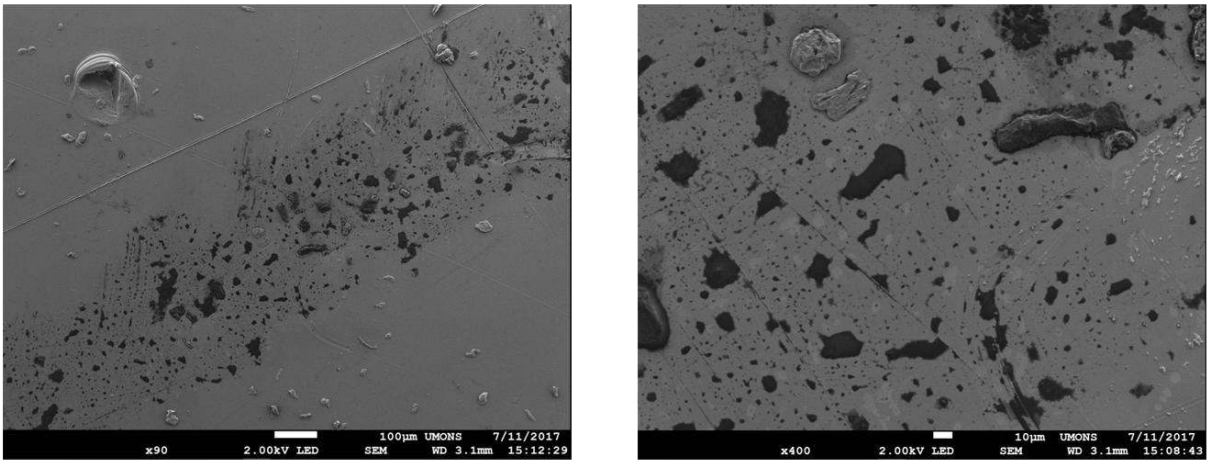

Figure 39: PET surface washed with bleach after a 40 hours drip flow culture

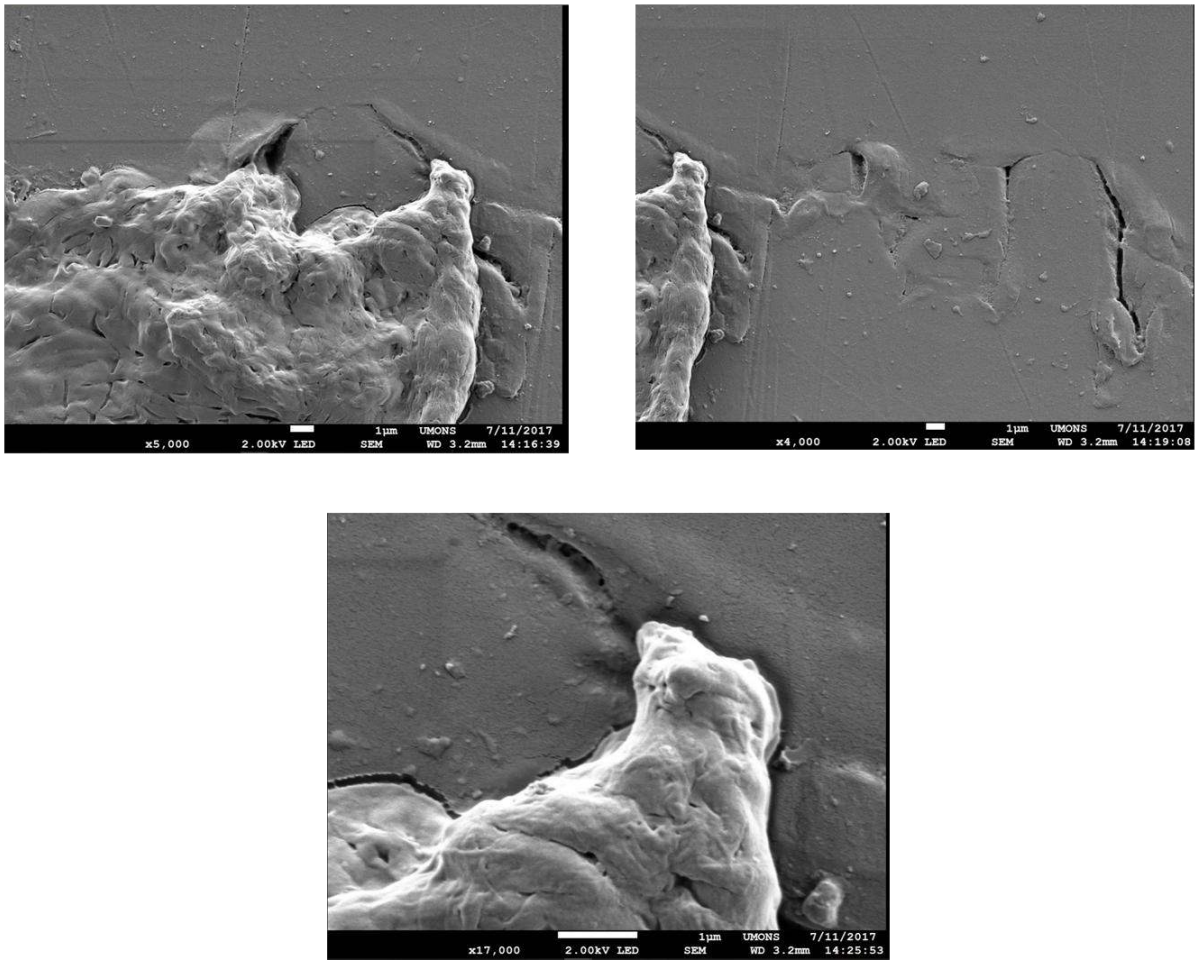

Figure 40: PET surface after biofilm have been detached without using bleach. Biofilm were cultivated during 40 hours in drip flow bioreactor.

The SEM observations give legitimacy to the hypothesis that biofilm have a great impact over surface degradation. Even though *Bacillus amyloliquefaciens* isn't known to produce PETases, its implication in low-density polyethylene degradation has already been discussed and there is a possibility that our strain has the ability to degrade PET or at least to produce similar enzymes to PETases since it is phylogenetically close to *Bacillus subtilis*, which is close to *Idonella sakaiensis* (see 2.2.3)

CONCLUSION AND PERSPECTIVES

The cultivation methodology in drip flow bioreactor (DFB) was optimized. We managed to obtain repeatable results from the different chambers of the drip flow which allowed us to study *Bacillus amyloliquefaciens* biofilm dynamics.

Biofilm cells (BC), planktonic cells (PC), and non adherent sessile cells (NASC) laying on the biofilm cells were studied. No link could be made between the PC and the NASC. However, the three types of cells can be considered as different population, each of one having their subpopulations. NASC exposed a different zeta potential than the two other types of cells, and during the biofilm maturation their respective metabolic activities evolved in different ways, supporting the fact that they are three different populations of cells.

Experiments showed that the biofilm incur significant physiological changes due to cell differentiation after approximately 20 hours of culture. The evolution of the biofilm surface hydrophobicity and the BslA and TasA quantification indicates that the maturation of the biofilm was achieved after 40 hours of culture. This conclusion was also supported by the evolution of the metabolic activity of the different types of cells observed on flow cytometry with RSG.

Poly(ethylene terephtalate) (PET) biodegradation by *Bacillus amyloliquefaciens* was tested on flasks and on DFB. The results were observed with a scattering electron microscope (SEM). DFB experiments were much more efficient in terms of biofilm formation and SEM observations showed that the biofilm formation over PET coupons in DFB led to a physical alteration of the surface.

From a general point of view, the DFB shows itself as a powerful tool for the biofilm study, allowing the study of several strains at once and even the interactions of these strains together. It also allows the study of the biofilm development over time and on different surfaces, which is essential for the study of the biofilm dynamics.

In the future, further experiment should be performed, using different types of plastics (in particular LDPE which have been proved to be sensitive to *Bacillus amyliquefaciens* activity),

different pre-treatments of these plastics and using different mutants with special biofilm formation abilities. The most exciting perspective would be the designing of a completely artificial microorganism able to massively produce PETases and form biofilms efficiently.

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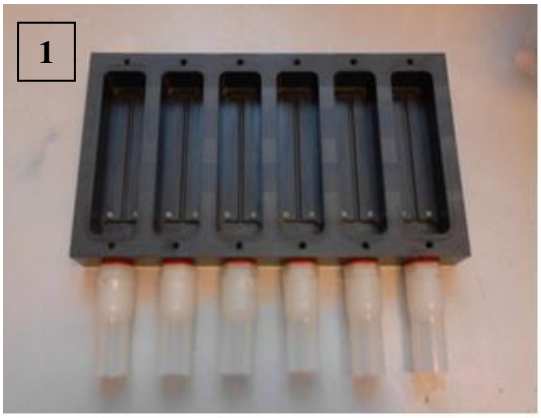
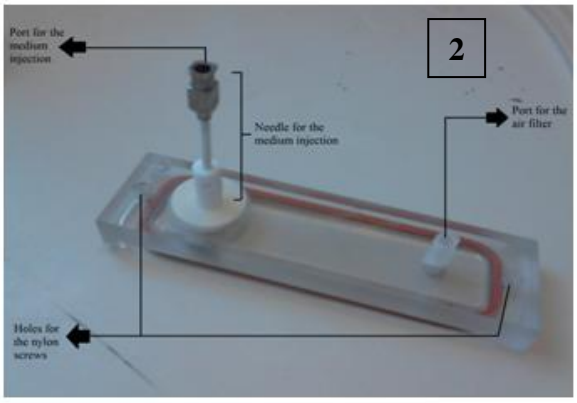
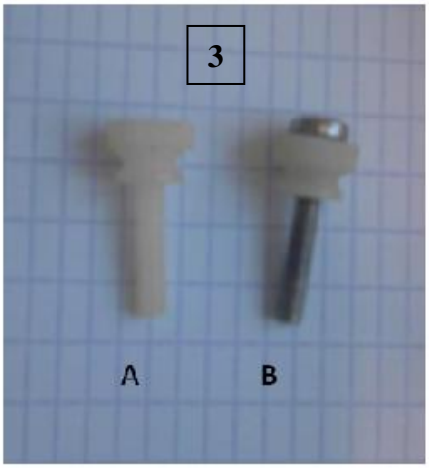
ANNEXES

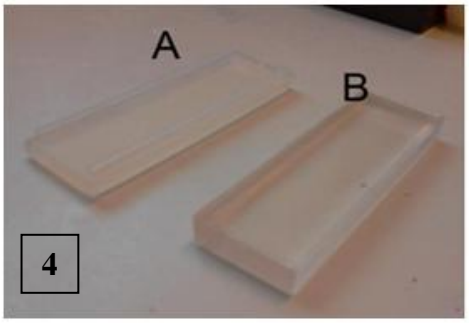
ANNEXE 1 : Drip flow bioreactor components

- Reactor base in polysulfone (1) with six channels and six effluent ports (*BioSurface Technologies Corporation, USA, Montana*).
- Reactor covers in polycarbonates (2) (*BioSurface Technologies Corporation, USA, Montana*) with two holes (for nylon screws (3) to fix the covers to the reactor base) and two ports: one emplacement for the air filter attachment and one emplacement for the media entry across the needles (length of 50 mm and diameter of 2 mm , *Delvo*). These needles have also a Teflon base to adjust the injection level. The covers are closed using nylon screws (or steel screws for the room at the left side of the reactor n°1).
- Bacterial air vents (0.22 µm of pore size, Sartorius).
- Waste rectangular silicone coupons (4A) and rectangular silicone coupons with silicone streaks (4B)(waste silicone coupons may serve as culture coupon).

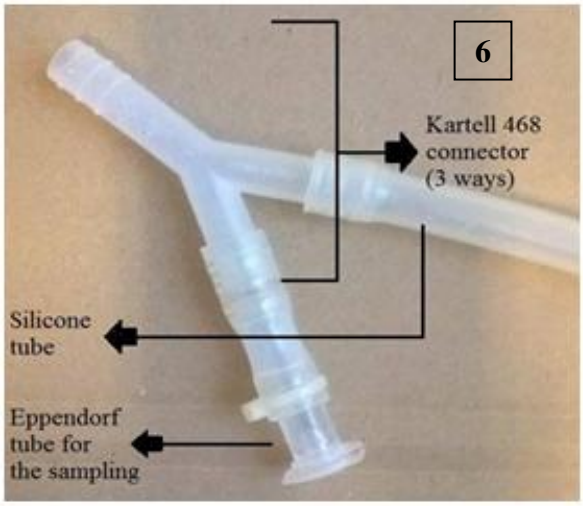


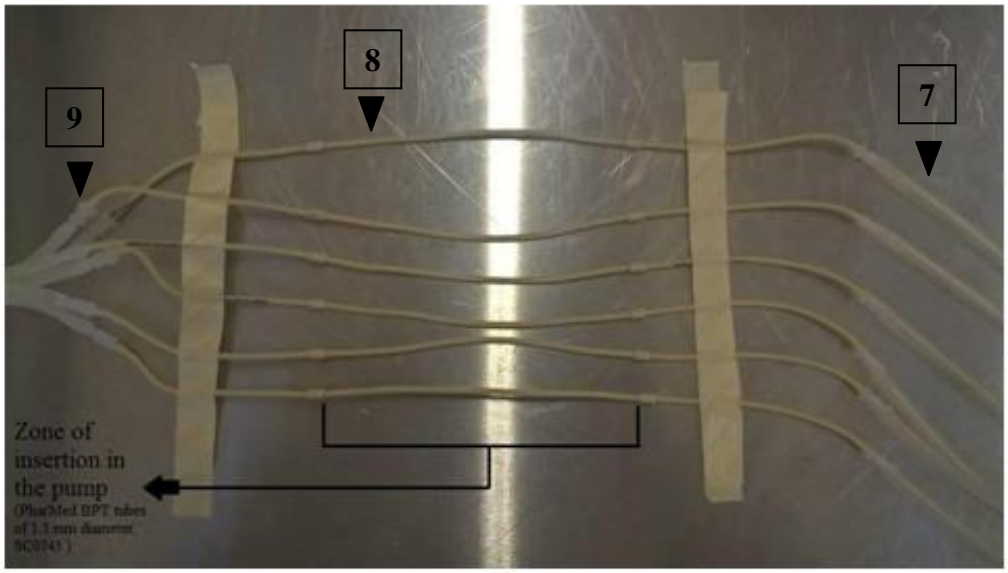






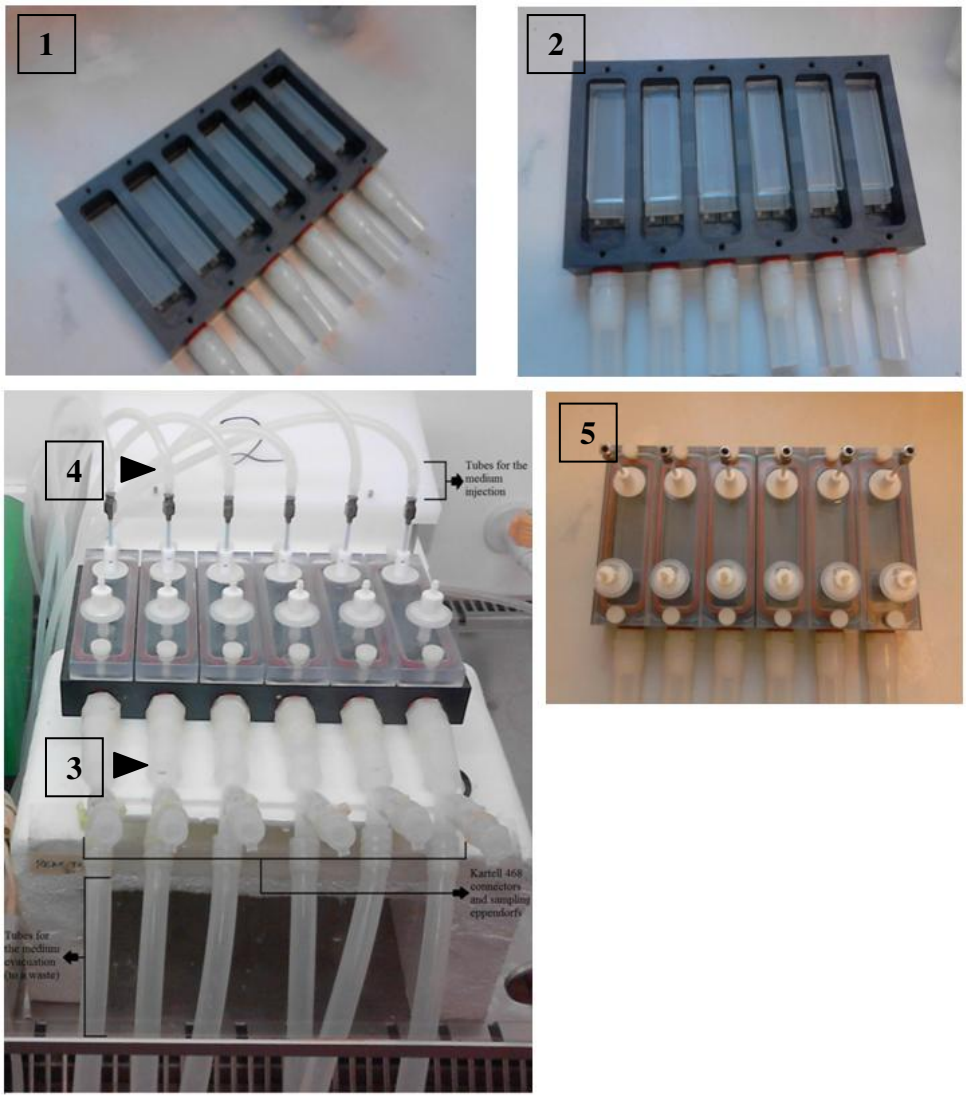
- Peristaltic pump (5) (Watson Marlow 530S, England).
- Output (6) siliconee tubes (internal/external diameter ratio = 8/12 mm) equipped with a Kartell 468 connector allowing a sampling by an Eppendorf tube.
- Entry siliconee tubes of different internal/external diameter ratios (3/6 mm and 5/9 mm). The different tubes of 5/9 mm are linked to the tubes of 3/6 mm (7) to allow the medium entry in the 6 channels of the reactor. This link is made with connectors in T form, allowing 3 ways. The entry tubes are also composed of 6 PharMed BPT tubes (8) of 1.3 mm diameter (reference = SC0743) for the insertion into the peristaltic pump (Watson Marlow 530S, England). The link between these tubes and the tubes with another diameter (3/6 mm) is made by Luer Lock connectors (9).





ANNEXE 2: Reactor assembly

- Insert the waste silicone coupons (1) against the screws into the 6 channels of the reactor base. Put the striated/smooth silicone coupons (2) over the waste silicone coupons and against the top of the reactor base.
- Put the coverts on the different reactor channels and fix them with the nylon (or steel) screws. Add the clean Sartorius air filters (3) in the covers ports provided for this purpose.
- Insert the Kartell 468 connectors (4) (linked to the output silicone tubes and the sampling eppendorf) into the effluent ports of the reactor base. Insert the entry tubes (5) (the part of the tubes with a ration external/internal diameter of 3/6 mm) into the covert ports for the medium injection.
- Pack the reactor in a double layer of aluminum foil.
- Sterilize the reactor in the autoclave at 121°C during 20 minutes in steam conditions.



ANNEXE 3: Reactor disassembly and cleaning

- Place the removed striated slides are placed in a container filled with water after the sampling.

NB: Distilled water is not necessary.

- Raise the window of the laminar flow hood to facilitate access to the device. The hood is then turned off, as well as the pump.

The peristaltic pump

- Dislodge each pump compartment to remove the entry tube from the peristaltic pump (*Watson Marlow).*

NB: Place all the pump compartments back into the pump to avoid any loss of materials.

Vials and tubes

- Remove the entry tubes from the coverts ports.
- Clean up the flask containing the culture medium with water and washing-up liquid.

NB: If there is presence of a contamination into the flask, add 10 mL of bleach and rinse with water after 15 minutes.

- Connect the entry tube (the extremity previously placed in the flask) with a connector and circulate water at low debit for a few minutes.

NB: If there are some traces of unknown contamination in the tube, place the end of the same tube in a container of bleach and circulate it to fill the whole of the circuit using a Wilson Marlow 100UR pump. Once the tubes are filled, cut the pump. Leave on for twenty minutes. Then rinse with water.

- Disconnect the output tubes of the reactor base and close the Mohr/Hoffman clamp.
- Fill the output tubes with bleach (two by two) to the eppendorf tubes for the sampling. Leave on for twenty minutes. Rinse with water by connecting tubes to the faucet (two by two).

NB: Ensure to rinse correctly the output sampler by opening them while rinsing.

Silicone coupons, covers and reactor base

- Remove the Sartorius air filters of 0,22 µm and store them.

NB: Check their condition before storage, the filters must stay perfectly white to ensure their

performance.

- Unscrew the screws of the covers and store them.

NB: Keep attention to the fragility of these screws.

- Remove the covers and wash them using a sponge and washing-up liquid. Rinse with water and ensure to pass water through the needle to check its cleanliness.

NB: Keep attention to the fragility of the coverts and needles.

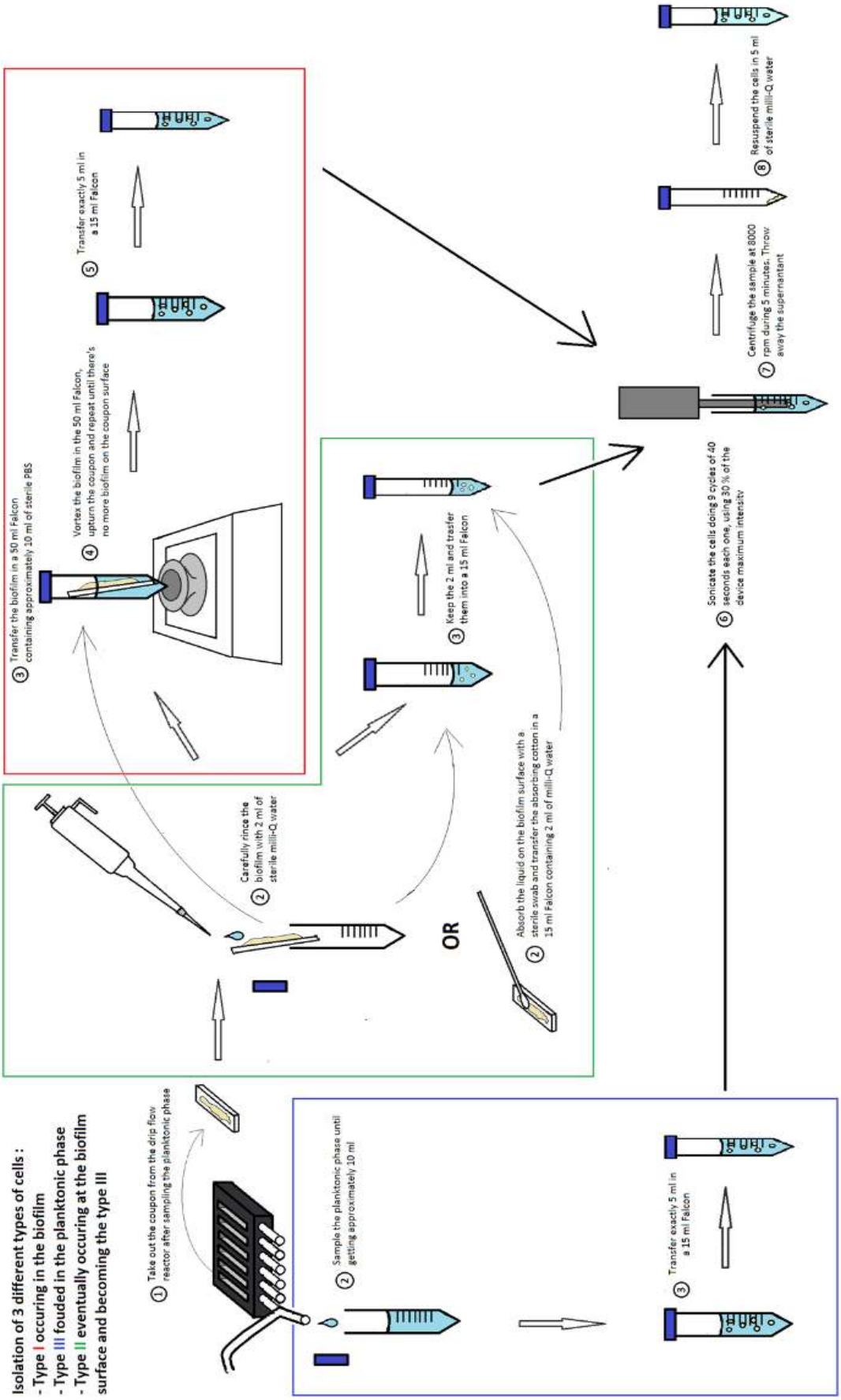
- Clean the siliconee coupons with a sponge and washing-up liquid. Rinse with water.
- Clean the different cells of the reactor in the same way as the covers and the coupons.
- Rinse the effluent ports to ensure that there is no trace of biofilm.

NB: If there are some traces of unknown contamination in the tube, rinse with bleach too.

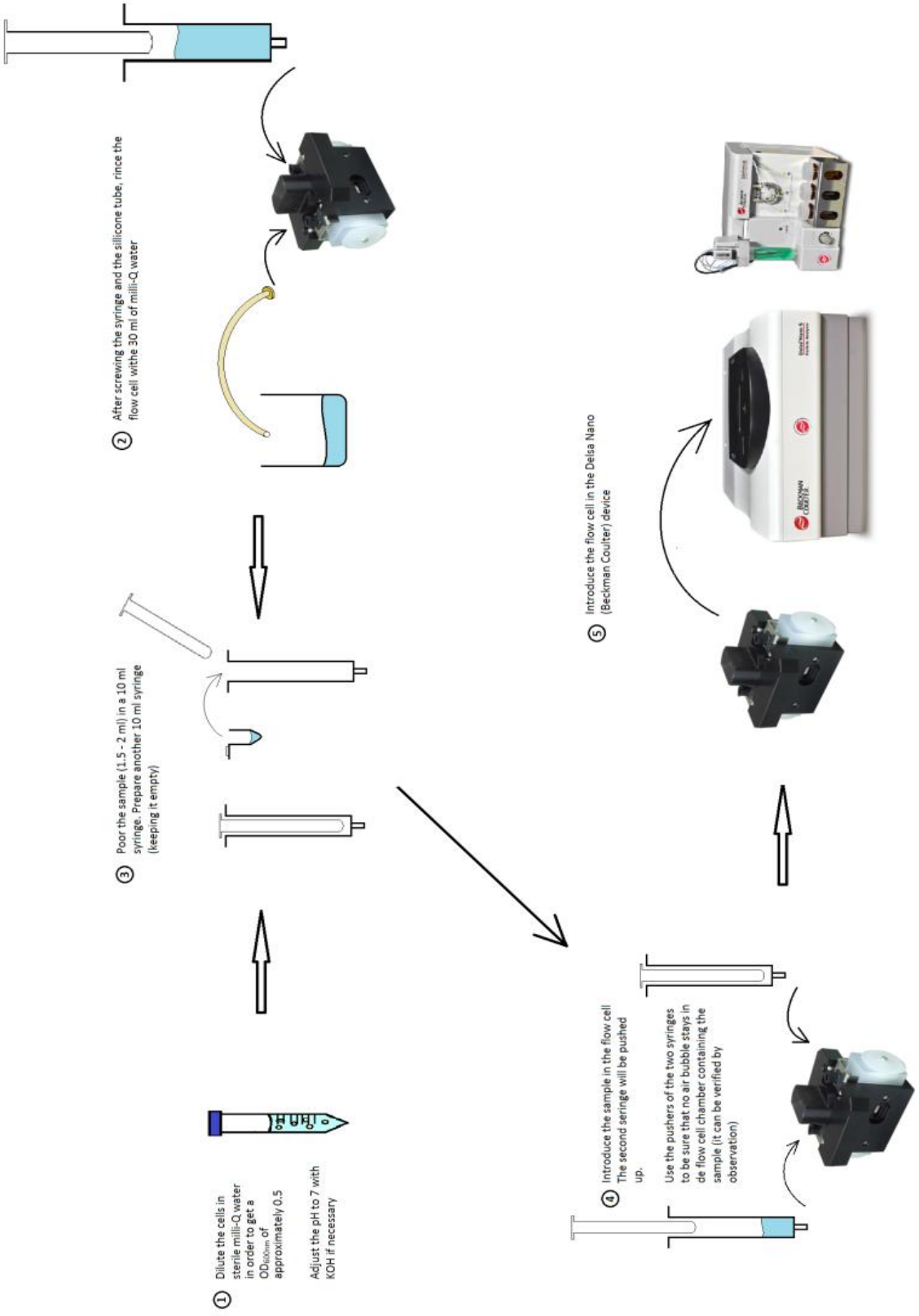
Storage

- Place back everything in the reactor corresponding boxes for the next setup and autoclaving.

ANNEXE 4: Sample collection and preparation before analysis



ANNEXE 5: Sample preparation for zeta potential measurement

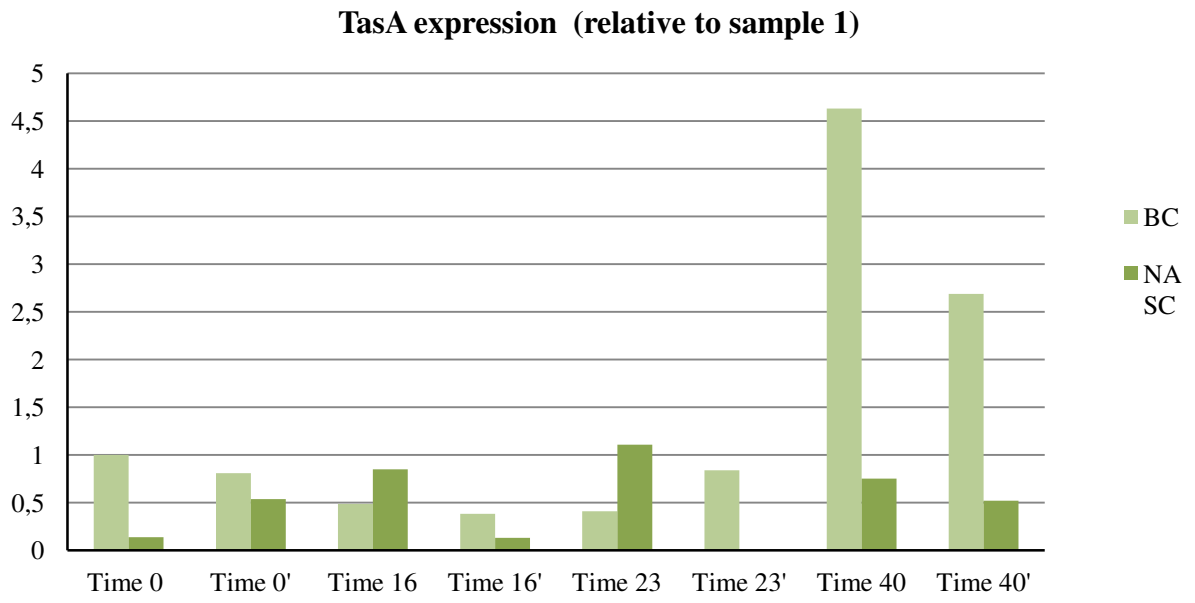


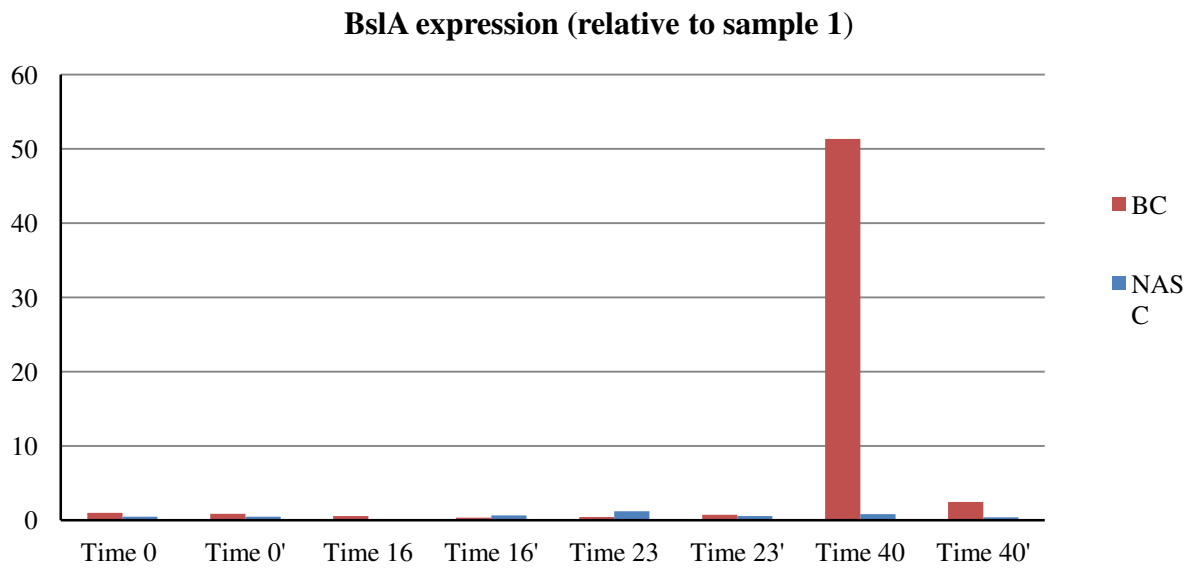
ANNEXE 6: Mean values and standard deviations of experiments carried with and without sample preparation and working condition optimisation.

Mean OD values standard deviation of experiment A			
Sampling time (hours)	Biofilm cells	Planktonic cells	Non adherent sessile cells
0	0,19 +/- 0,05	0	0,04 +/- 0,01
12	1,74 +/- 0,12	1,90 +/- 0,29	0,73 +/- 0,31
18	6,60 +/- 2,94	1,64 +/- 0,75	0,64 +/- 0,15
38	45,63 +/- 24,80	3,31 +/-0,22	0,96 +/- 0,33

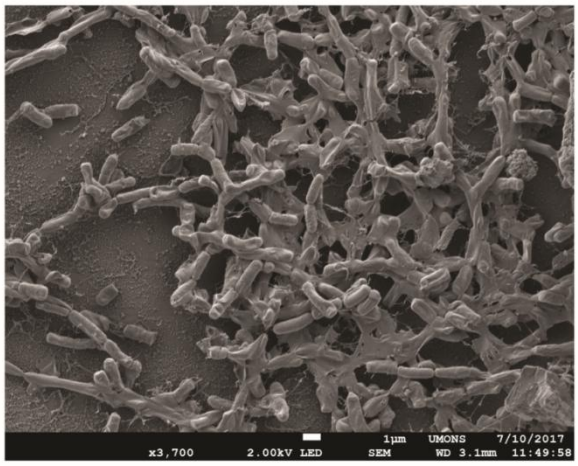
Mean OD values standard deviation of experiment B			
Sampling time (hours)	Biofilm cells	Planktonic cells	Non adherent sessile cells
0	0,06 +/- 0,01	0,38 +/- 0,02	0
12	0,42 +/- 0,04	0,33 +/- 0,05	0,26 +/- 0,08
18	1,05 +/- 0,26	1,46 +/- 0,31	0,53 +/- 0,11
38	15,76 +/- 3,58	0,42 +/- 0,19	0,19 +/- 0,05

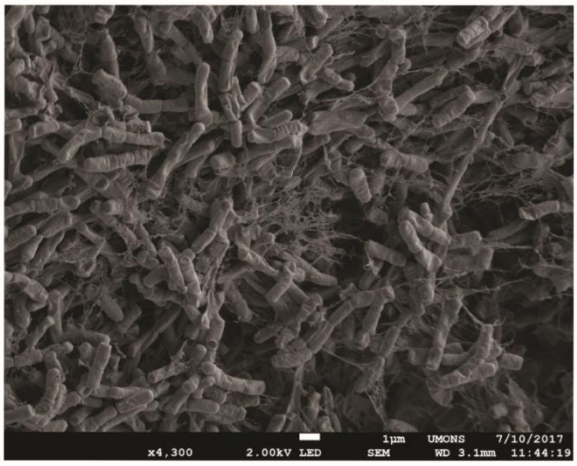
ANNEXE 7: TasA and bslA charts (modified from Bouchat R. 2017)



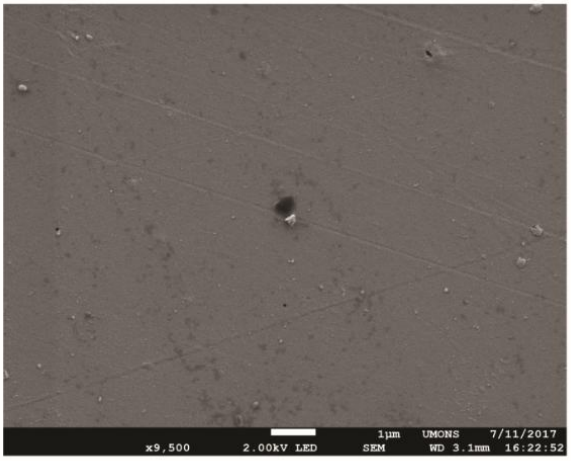


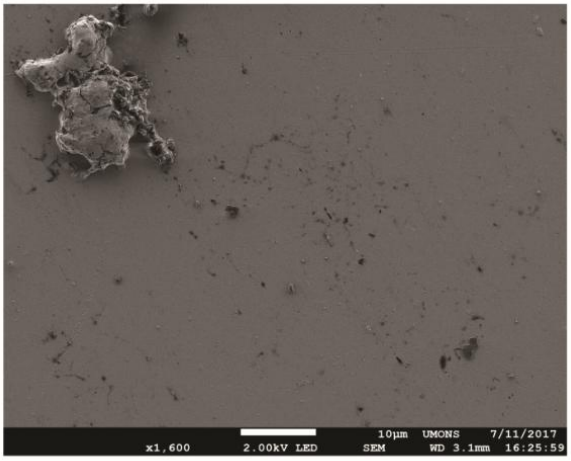
ANNEXE 8: SEM observations

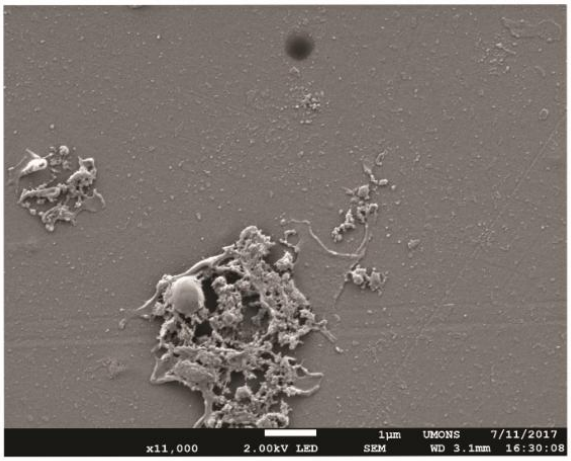




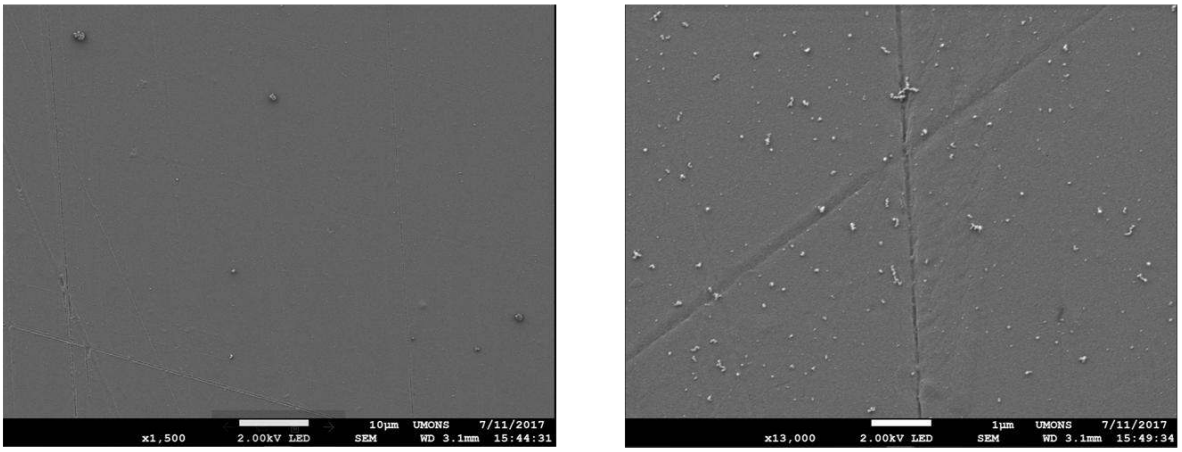
Biofilm formed on PET surface after a 2 days culture in flask with LB medium at 30°C with a 60rpm agitation.



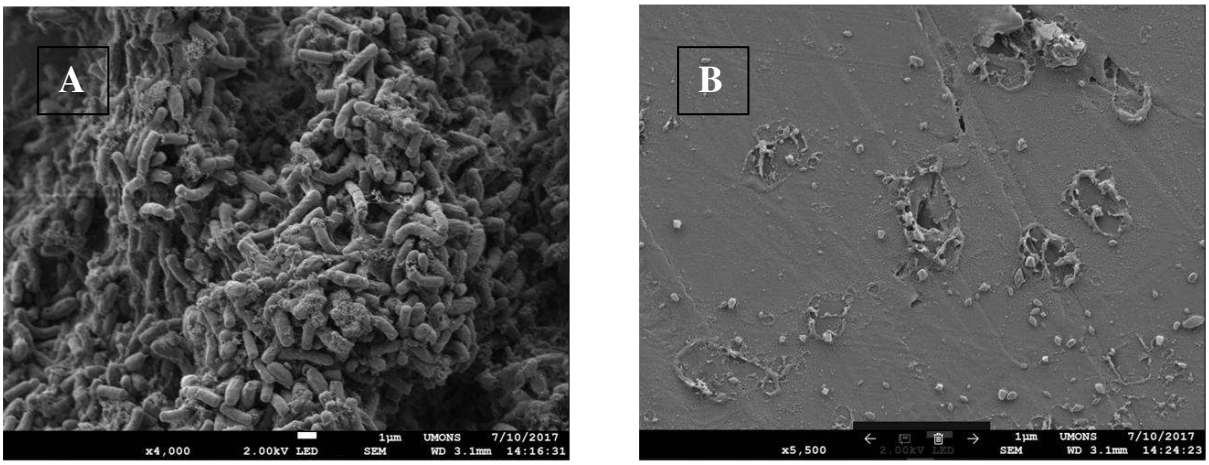




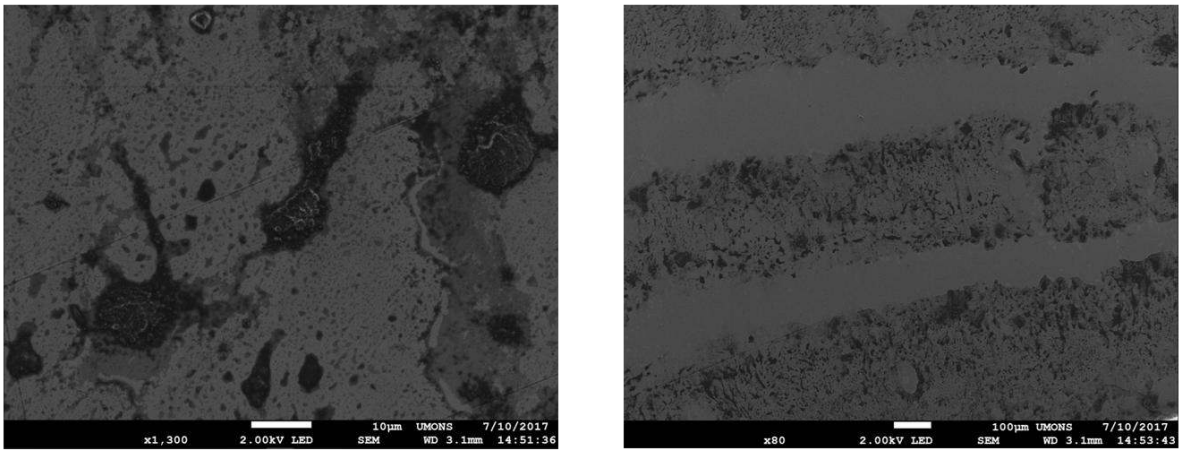
PET surface washed with bleach after a 2 days culture in flask.



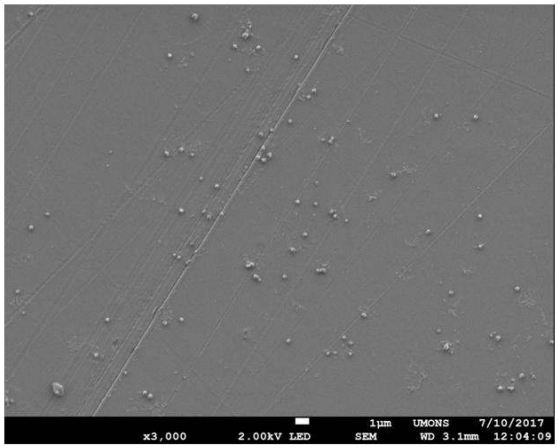
Control PET surface after 2 days in flask. Surface have been washed with bleach.

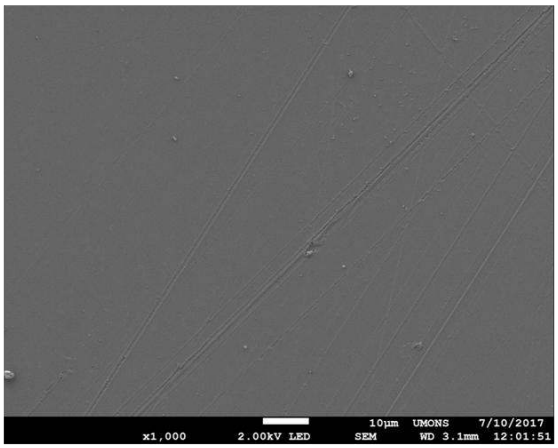


Biofilm formed on PET surface after a 3 weeks culture in flask (A). Rests of biofilm over cavities of the PET surface (B).

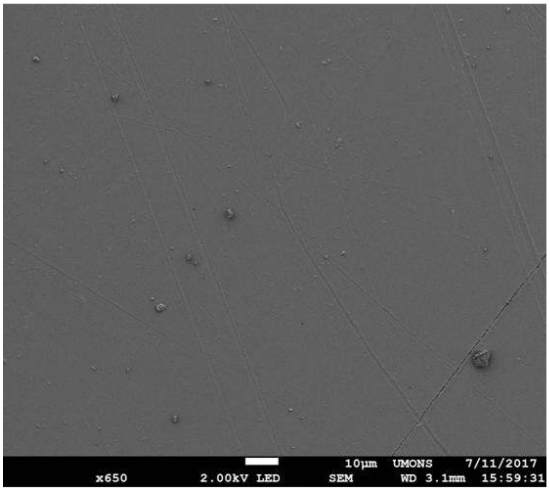


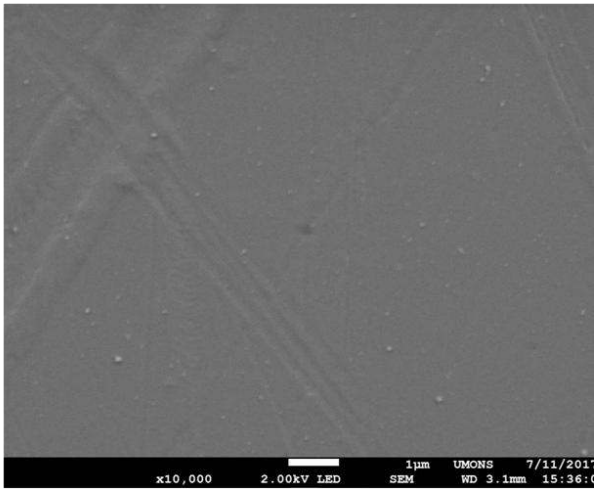
PET surface washed with bleach after a 3 weeks culture in flask.



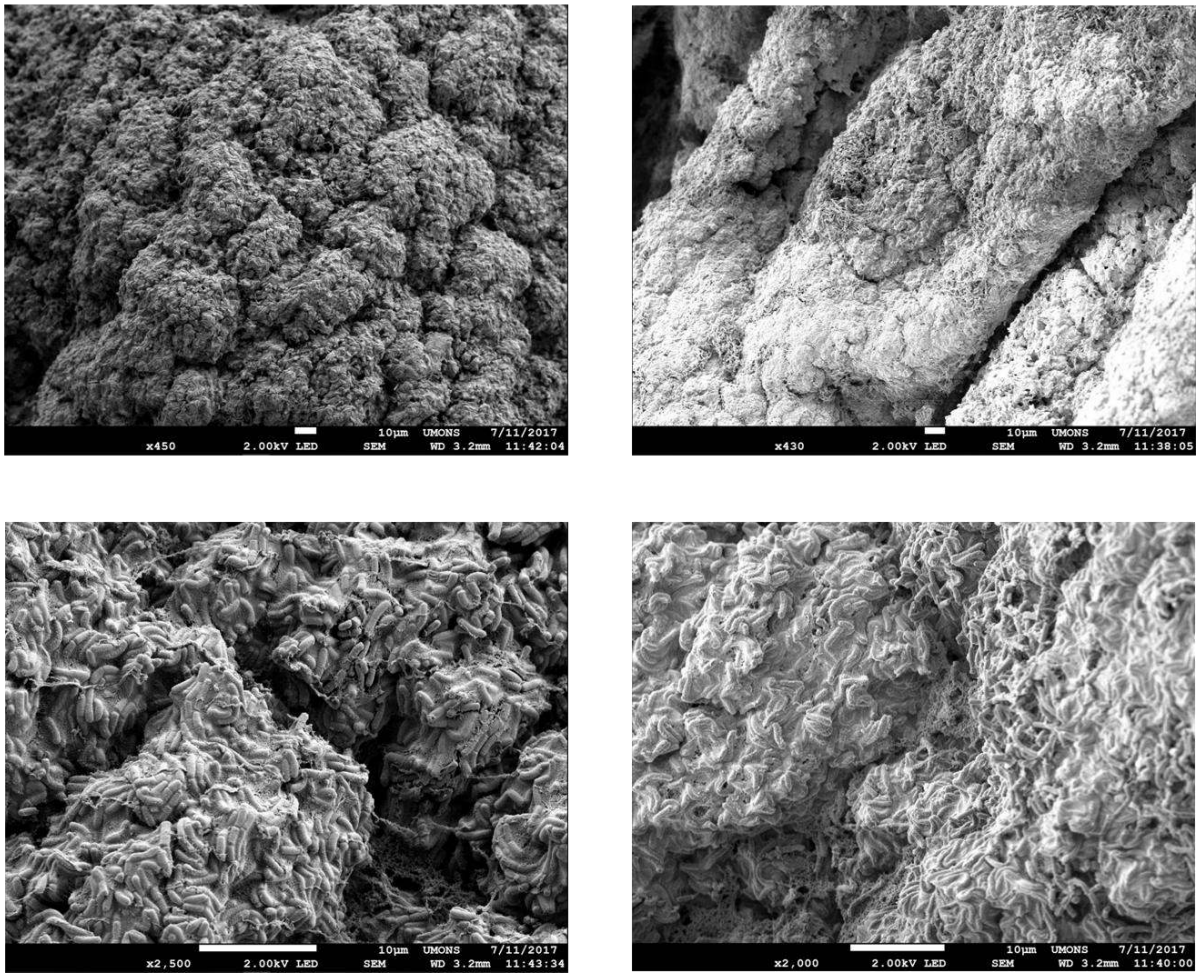


Control PET surface after 3 weeks in flask. Surface have been washed with bleach.

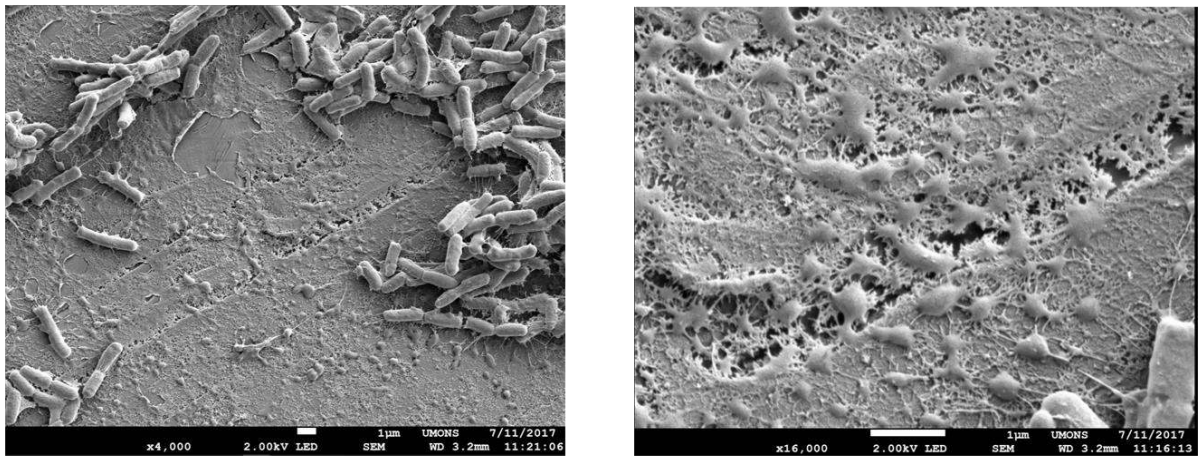




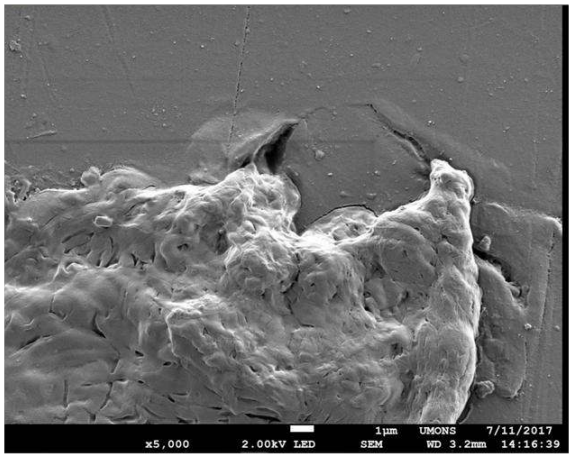
Control PET surface after 40 hours in culture.

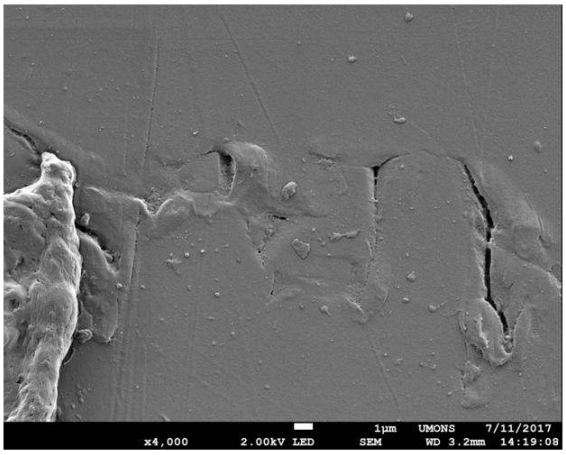


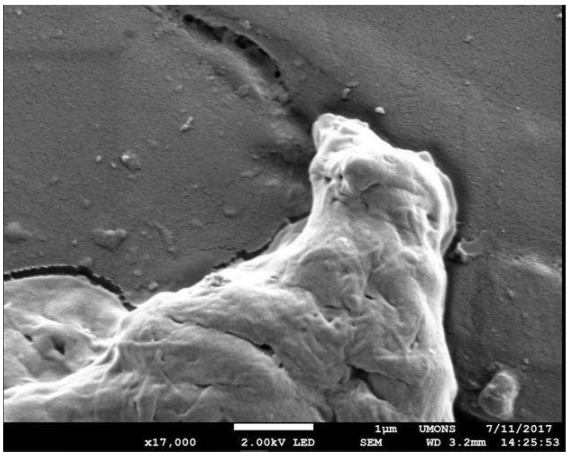
Biofilm formed on PET surface after a 40 hours culture in drip flow bioreactor.



Biofilm formation after a 40 hours culture in drip flow bioreactor over PET surface cavities covered with matrix







PET surface after biofilm have been detached without using bleach. Biofilm were cultivated during 40 hours in a drip flow bioreactor.