# Design of a biofilm cultivation devices for the analysis of microbial interactions

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**Travail de fin d'études présenté en vue de l'obtention du diplôme de Master Bioingénieur en Chimie et Bio-industries**

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## Abstract

The biofilms are multispecies communities fixed to a surface and producing extra poylmeric substances which encapsulate the cells. The biofilm can be considered as a balance between a planktonic and a biofilm phase.Researches about the interaction and cells communications are important to fully understand all the mechanisms taking place in this system.

For the controlled growth of repeatable biofilms for such researches, cultivation devices as the Drip Flow reactor are needed and have to bemastered. With this master thesis, a methodology of use of Drip Flow reactor hax been developed to obtain more repeatable biofilm growth. Then its adaptation to interactions studies was tested by growing *Bacillus amyloliquefaciens GA1* with supernatant of *Pseudomonas fluorescens 69 (or A214)*. The impacts on growing were investigated by optical density measurement, surface hydrophobicity evaluation and gene expression byReal-Time Quantitative Reverse Transcription PCR (or qRT-PCR). The selected genes were tasA, yuaB (both implicated in the matrix production and properties) and degU (a regulator gene also involved in the cells differentiation).

All results prove the efficacity of the Drip Flow methodology to lead such experiences on biofilms.

# Résumé

Les biofilms sont des communautés de micro-organismes fixés sur une surface et produisant des polymères extracellulaires qui les encapsulent. Suite à sa dynamique, le biofilm peut être considéré comme un équilibre entre une phase planctonique et une phase biofilm (micro-organismes sessiles).

Des recherches sur les interactions et communications des cellules sont importantes pour bien appréhender tous les mécanismes ayant lieu dans ce système. Afin d'effectuer de telles études, une maîtrise des différents systèmes de culture comme le réacteur Drip Flow est nécessaire (afin d'obtenir des résultats répétables particulièrement).

Au cours de ce travail, une méthodologie d'utilisation du réacteur Drip Flow a été mise au point afin d'obtenir une croissance répétable de *Bacillus amyloliquefaciens GA1*.L'adéquation de cette méthodologie aux études des interactions a été testée en étudiant l'impact du surnageant de *Pseudomonas fluorescens 69* (ou *A214*) surla croissance de *Bacillus amyloliquefaciens GA*1. Les impacts ont été étudiés par la mesure de la densité optique, l'évaluation de l'hydrophobicité de surface et l'expression génétique par Real-Time Quantitative Reverse Transcription PCR (ou qRT-PCR).Les gènes sélectionnés étaient tasA, yuaB (tous deux impliqués dans la production de la matrice et de ses propriétés) et degU (un gène régulateur également impliqué dans la différenciation cellulaire). Tous les résultats prouvent l'efficacité de la méthode d'utilisation du réacteur Drip Flow pour mener de telles expériences sur les biofilms.

# Aknowledgements

I would like to thank**Mi**crobial **P**rocesses and **I**nteractions department of Gembloux Agro-Bio Tech and M. Delvigne for such an opportunity of research for my master thesis. Indeed, during my studies, I have been interested more and more by the microorganisms and their applications. This master thesis was such interesting and relevant to what I was looking for that I intend to continue working in the middle of research in microbiology and biotechnology (if possible).

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Finally I would like to thanks all my friends and my family to support me during this important period of my life which ends with this master thesis. Indeed, during this period and with their help,I grew up and shaped the person I am now.

# List of figures

## Summary

# I. Introduction

The biofilms are now considered as the most prolific and predominant life forms of the bacteria. However, a lot of things about these ones are still unknown, misunderstood or need more researches like their real dynamics, exchanges between species, their utiliations of the different substrates,…

To study this bacterial system, some new devices are needed like the Drip Flow reactor allowing to investigate the kinetic of biofilm formation and the biofilm bacterial interactions.

# II. Objectives

Biofilms are complex systems difficult to study because of the difficulties to cultivate them in reactors and their growth variabilities. The Drip Flow reactor allows cultivating them and sampling the biofilm phase but the variabilities stay high.

The main objective of this study is the design of a repeatable cultivation method for the development of biofilms with the Drip Flow reactor and to decrease the variabilities of the growth.With this method, dynamics of the biofilm formation will be evaluated. The dynamics will also be questioned due to the discovery of a possible third fraction of cells in the biofilms, the non adherent sessiles cells (NAS) which could make the link between the adhered and agglomerated sessile cellsor biofilm fraction (from the biofilm phase) and the planktonic cells or planktonic fraction (from the planktonic phase).

This cultivation method will be used for the investigation of the bacterial interactions inside the biofilms due to the possibility of sampling the biofilm phase. The efficiency of this method for this goal will beevaluated by the study of interactions between biofilms of *Bacillus amyloliquefaciens GA1* and supernatant of *Pseudomonas fluorescens 69* (or A214). The study will be based on the growth differentiation and expression of three genes by qRT-PCR.

Some other objectives were added to this study. Indeed, theDrip Flow developed methodologywill also be used to detect and measure the surface hydrophobicity evolution of *Bacillus amyloliquefaciens GA1* biofilms based on the studies about the hydrophobicity of *Bacillus subtilis* strains. The surface hydrophobicity will also be used to investigate the bacterial interactions effects on the expression of hydrophobic metabolites and on the structure of the biofilm matrix.

# III. State of the art

## 1. Biofilm

### A. Introduction and biofilm definition

The predominant life-style of bacteria was often considered as free floating organisms (the planktonic state) but in fact the main state of bacterial existence on Earth is sessile consortia of bacteria adhered to a surface.(Flemming et al., 2016) This system is called biofilm. The definition of a biofilm is then an agglomerate of bacteria sticked to a solid (biotic or abiotic) surface and enclosed into a matrix of extra polymeric substances (EPS) produced by the bacteria themselves. (Azeredo et al., 2016a)(Costerton, 1999)

The definition can also include the fact that the exhibited phenotypes of the biofilm cells have to be different from the free-living ones. (Donlan & Costerton, 2002).

Cell concentration in a biofilm can reach $10^8$ to $10^{11}$ cells by gram of wet weight depending on the strain and the maturation stage. (Flemming et al., 2016) Moreover, the mutations and gene expression differenciation in the biofilms are very high leading to phenotypic differences and high heterogeneity in the biofilm. (Flemming et al., 2016)It is due to gradients (of pH, $O_2$ or nutrients) in the biofilm (caused by the immobilization of the matrix) and social communication between cells (by gene exchanges meditated by the EPS matrix for instance). All these differenciations lead to new emergent properties of interests. Indeed, biofilms can present very interesting applications for industries like water treatment and energy production (biofuels) (Halan et al., 2012).

But they canalsobe useful for fundamental researches like the research of interactions between the species, the subject of this master thesis.

However, through the numerous interests and possible applications of the biofilm, the high capacity of spreading and colonizing surfaces has led to a bad vision of the biofilms. Indeed, the biofilms can cause hygiene problems and food spoilage in the food (dairy) industry. They can also lead to catalyzes of reactions which can cause metal corrosion and then equipment deteriorations but problems of heat exchanges too if they become too thick(Simões et al., 2010a). In the medical field, the biofilms are also the causes of big disorders (especially infections) like the cystis fibrosis studied by M.Holby (Høiby, 2014).

## B. Dynamics of the biofilm formation and dissassembly

The bacterial biofilm formation is a dynamic model divided into several steps: The pre-conditioning of the adhesionsurface (intentionally or not) by macromolecules, the transport of the free-living cellsfrom the liquid phase to the surface, the adsorption of cells on surface (reversibly or irreversibly), the possible desorption of the cells adsorbed reversibly, the irreversibleadsorption of cells on surface, the production of cell-to-cell signal molecules, the substrates transport into the biofilm, the substrate metabolism by the biofilm cells (and the output of the products and coproducts from the biofilm) and the biofilm removal by detachment or sloughing (voluntary for the dispersion of the organism or not).

Cell division, cell growth and EPS production happen also often during the step of substrate metabolism. (Simões et al., 2010b)(Bryers & Ratner, 2004)

In conclusion, the biofilm can be considered as a two phases system. These two phases are the planktonic phase and biofilm phase (or sessile phase). They are in balance because of cellular adhesion from planktonic to sessile phase, biofilm detachment to planktonic phase and cells desorption.

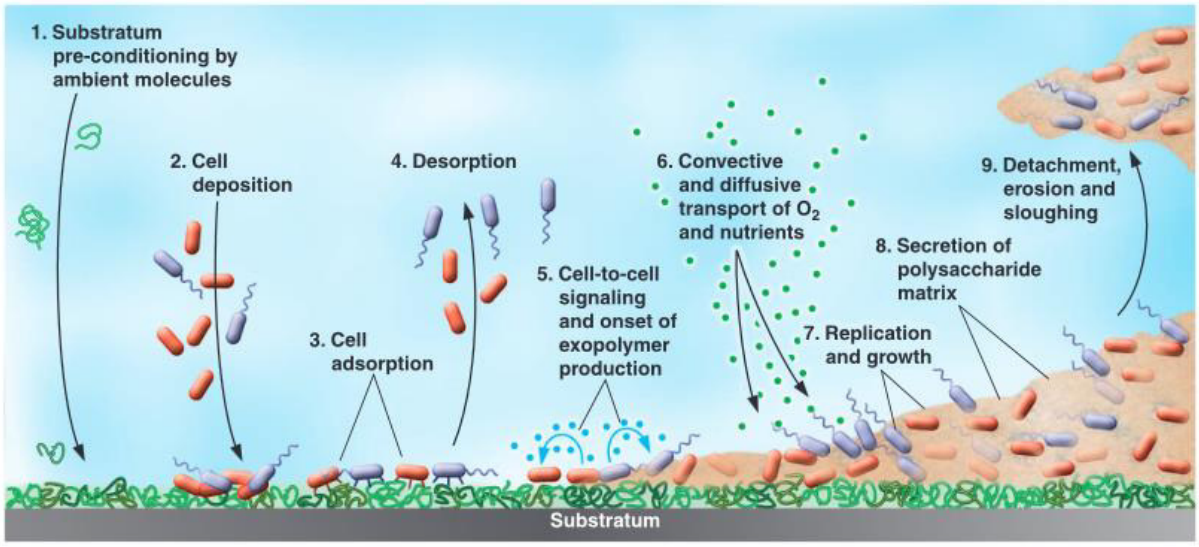


*Figure 1: Schema of the 9 steps of the biofilm formation.* (Bryers & Ratner, 2004).

However these numerous points can be summarized into 3 main stages: The cells attachment, the microcolonies development and the maturation in macrocolonies (with dispersion mechanisms).

The figures2.A and 2.B schematize these 3 main stages.

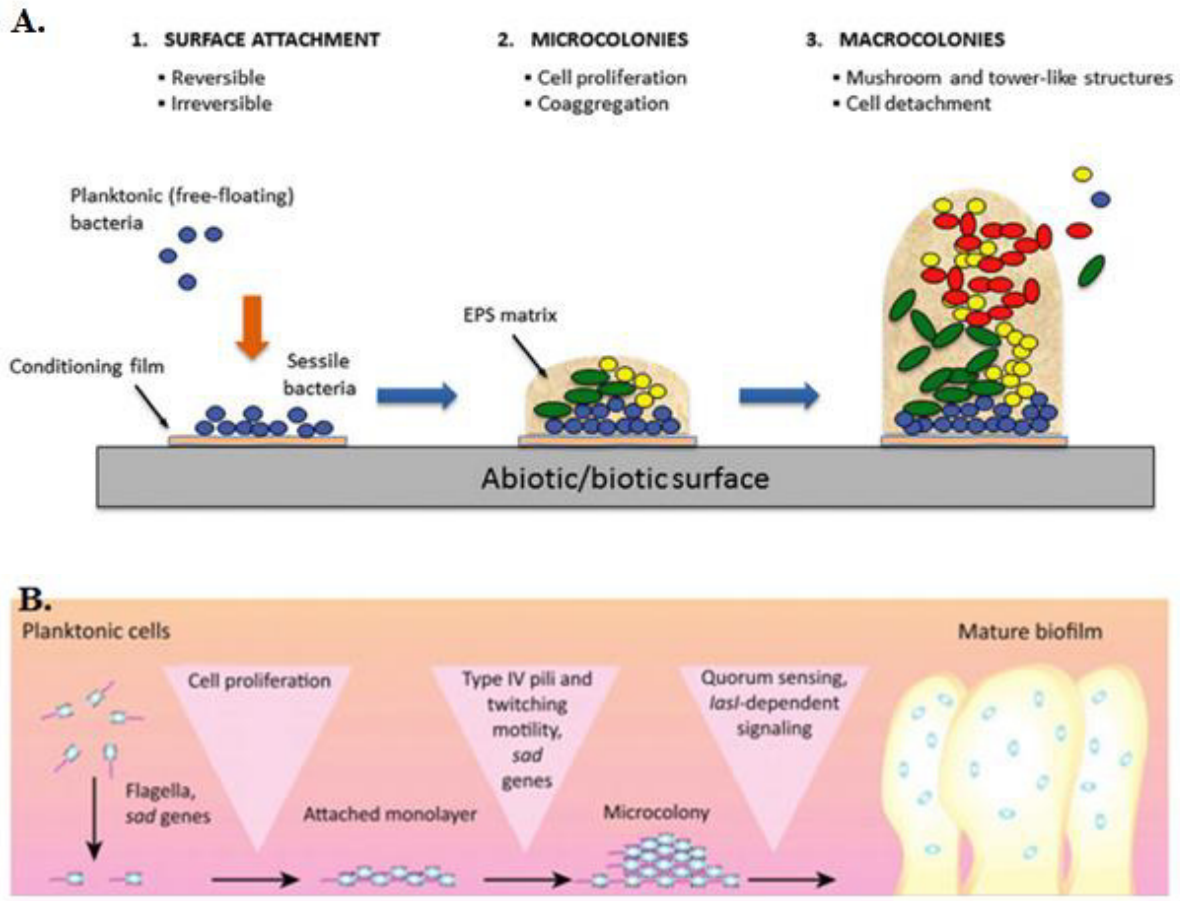


*Figure 2: Two examples of schema showing the 3 main stages of the biofilm formation.*

A: (Dufour et al., 2012).

B: (Costerton, 1999)

During the first step, bacteria adhere to a biotic or abiotic surface. This adhesion can be reversible or irreversible and engaged a lot of physical, chemical and biological parameters. (Dufour et al., 2012)

This step will be more detailed in this state of the art because of its importance for the study of the biofilm formation in a Drip Flow reactor.

Then, bacteria begin to agglomerate and the first adhered cells (called colonizers) multiply themselves to form microcolonies. It's the second step of the biofilm formation. During this one, the cells also begin to produce extra polymeric substances (or EPS).

## C. The Extra Polymeric Substances

The Extra Polymeric Substances (or EPS) are the major fraction of a biofilm. Indeed they form the matrix of the biofilm itself. It's why the EPS represent 90% (against 10% of cells) of the dry weight ratio(Flemming & Wingender, 2010).

The composition of the matrix of EPS is complex but the main components are already known as polysaccharides, proteins, nucleic acids, lipids and humic substances. These extra polymeric substances lead to the adhesion on the surface, the elaboration of the three-dimensional geometry of the film and the immobilization of the cells into the biofilm.So, it is responsible of the both adhesion and cohesion of the biofilm(Flemming & Wingender, 2010).These compoundsare also involved in the communications between bacterial cells because they keep cells at close proximity. However, their roles are more diversified, complex and depending of the compound themselves. Table I below summarizes all the possible function of the EPS compounds into biofilms. All these functions are necessary for the biofilm existence.

Table 1 | **Functions of extracellular polymeric substances in bacterial biofilms**

| Function | Relevance for biofilms | EPS components involved |
|---|---|---|
| Adhesion | Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces | Polysaccharides, proteins, DNA and amphiphilic molecules |
| Aggregation of bacterial cells | Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition | Polysaccharides, proteins and DNA |
| Cohesion of biofilms | Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication | Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and DNA |
| Retention of water | Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of dessication in water-deficient environments | Hydrophilic polysaccharides and, possibly, proteins |
| Protective barrier | Confers resistance to nonspecific and specific host defences during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protoza | Polysaccharides and proteins |
| Sorption of organic compounds | Allows the accumulation of nutrients from the environment and the sorption of xenobiotics (thus contributing to environmental detoxification) | Charged or hydrophobic polysaccharides and proteins |
| Sorption of inorganic ions | Promotes polysaccharide gel formation, ion exchange, mineral formation and the accumulation of toxic metal ions (thus contributing to environmental detoxification) | Charged polysaccharides and proteins, including inorganic substituents such as phosphate and sulphate |
| Enzymatic activity | Enables the digestion of exogenous macromolecules for nutrient acquisition and the degradation of structural EPS, allowing the release of cells from biofilms | Proteins |
| Nutrient source | Provides a source of carbon-, nitrogen- and phosphorus-containing compounds for utilization by the biofilm community | Potentially all EPS components |
| Exchange of genetic information | Faciliates horizontal gene transfer between biofilm cells | DNA |
| Electron donor or acceptor | Permits redox activity in the biofilm matrix | Proteins (for example, those forming pili and nanowires) and, possibly, humic substances |
| Export of cell components | Releases cellular material as a result of metabolic turnover | Membrane vesicles containing nucleic acids, enzymes, lipopolysaccharides and phospholipids |
| Sink for excess energy | Stores excess carbon under unbalanced carbon to nitrogen ratios | Polysaccharides |
| Binding of enzymes | Results in the accumulation, retention and stabilization of enzymes through their interaction with polysaccharides | Polysaccharides and enzymes |

*Table I: Functions of the different EPS components into a biofilm* (Flemming & Wingender, 2010).

# 2. Bacillus s biofilm

## A. Bacillus subtilis

The *Bacillus subtilis* is one of the main species studied to increase the biofilm knowledges. Indeed, it's a good model for the study of biofilm and the mechanisms leading to the different mechanisms ruling the biofilm formation. (Vlamakis et al. - 2013 - Sticking together building a biofilm the Bacillus subtilis way-annotated).

Researches done with these bacteria have proven the dynamic of biofilm formation as explained above but they also proved that cells genetically identical can express different genes which lead to different phenotypes (and subpopulation) with different roles within the biofilm (Vlamakis et al., 2013a).

They are often summarized as motile cells (planktonic phase), EPS-producing cells and spores (biofilm phase) but the diversity is more important as observable in the Figure 3.

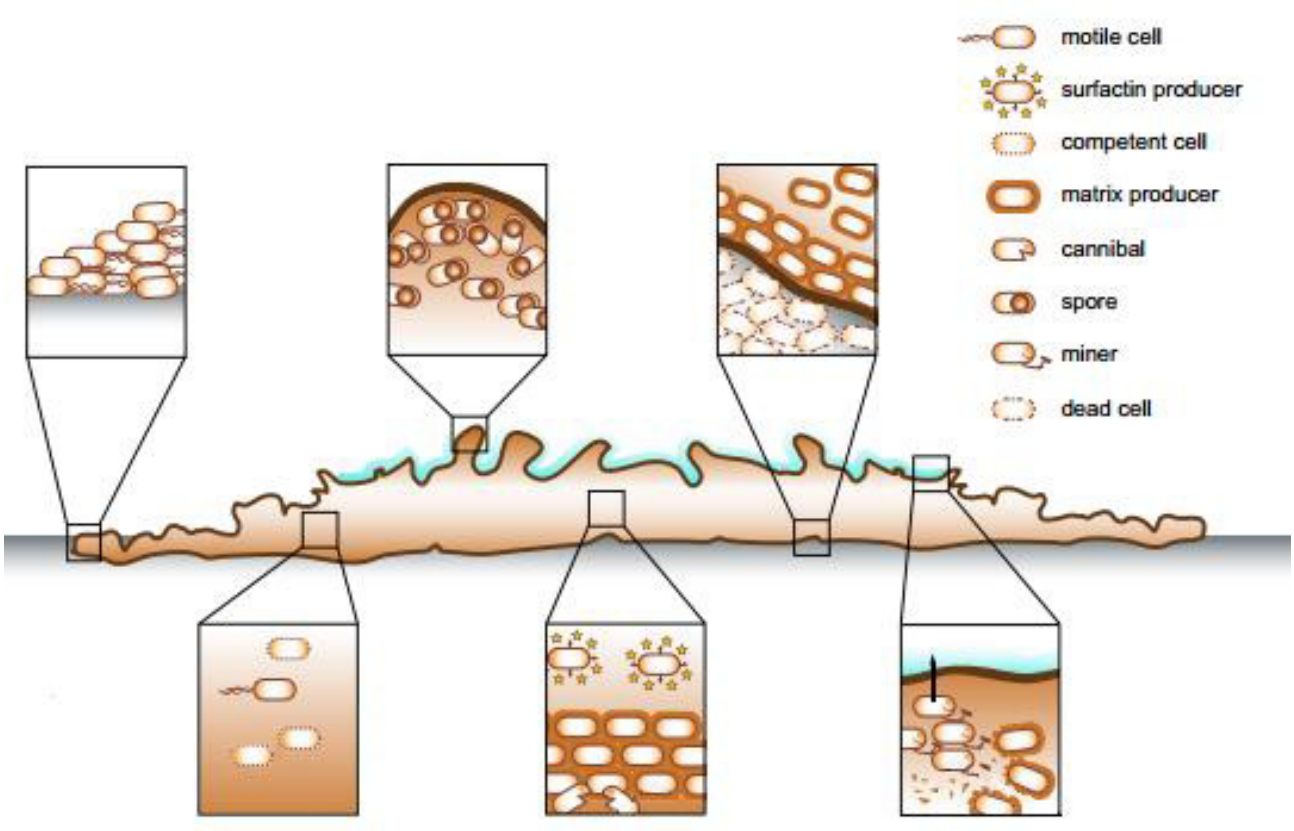


*Figure 3: Cell phenotypes and their role in Bacillus subtilis biofilm* (Mielich-Süss & Lopez, 2015).

The different locations of the subpopulations are dynamic and subject to studies. However, it seems that the motile cells tend to become cells which produce EPS matrix and then spores (Vlamakis et al., 2008).

To generate a biofilm, cells switch from a planktonic to a sessile state by downregulating the expressionof flagellar genes and expressing more genes involved in production of the extracellular matrix. In*B. subtilis*, the switch is doneby nutrient depletion, low oxygen levels or surface adherence(Mielich-Süss & Lopez, 2015).

Concerning the genes involved in the biofilm formation, they are numerous and controlled by some subnetwork as observable in the Figure 4.

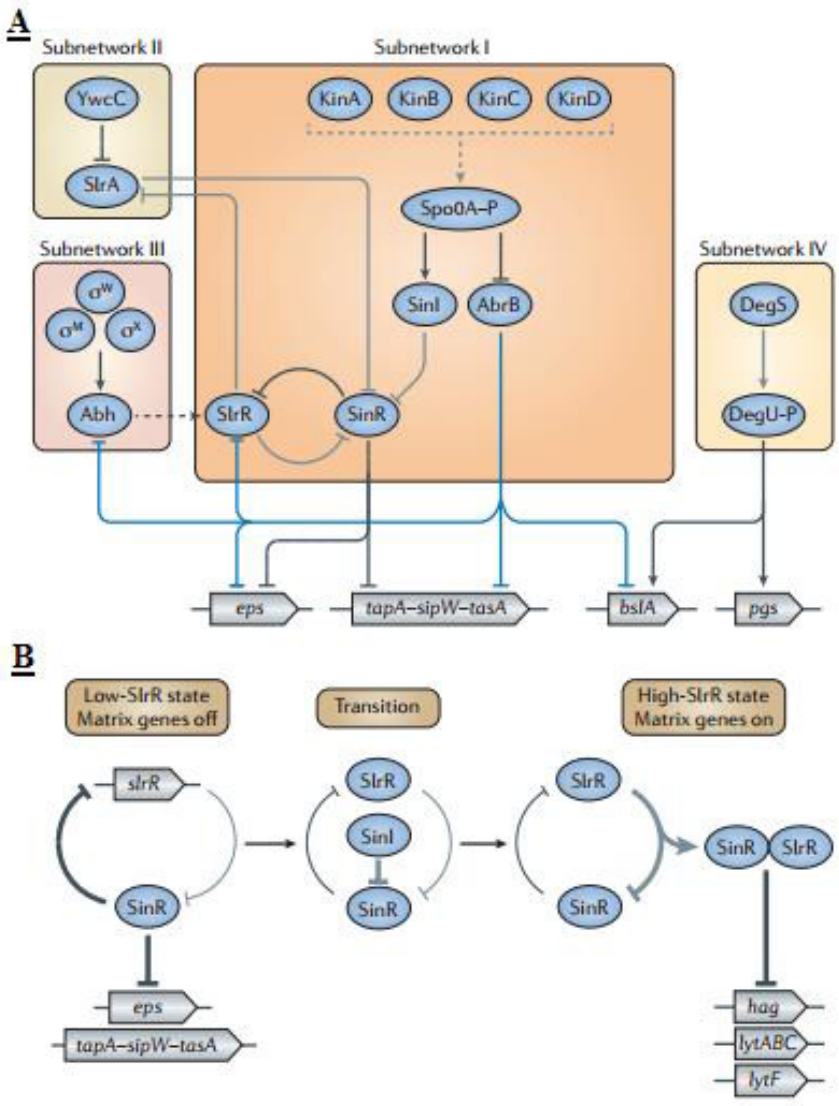


*Figure 4: Genes and regulation pathways involved in the Bacillus subtilis matrix production* (Vlamakis et al., 2013b).

Main genes associated to the biofilm formation (by the production of extra polymeric substances) are the eps operon, the tapA-sipW-tasA operon, the bslA gene and the pgs gene.

The eps operon is a 15 genes operon responsible for the production of the main compounds of the EPS matrix. The pgs operon has the same role, the production of an essential compound of the matrix for the submerged biofilm, γ-poly-dl-glutamic acid (PGA)(Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

The tapA-sipW-tasA operon is mainly responsible of the matrix structural integrity. Indeed, the TasA is an amyloid protein assembled into fibers. These fibers are fixed to the wall of these Gram positive bacterial cells with the help of the protein TapA. TapA is also involved in the formation of the TasA fibers. These TasA proteins are necessary to the biofilm formation, the tasA-defective mutants can't form biofilms as proof. The sipW gene has also a role in the integrity of the matrix. Indeed, it is responsible of the production of a signal peptidase, SipW, which processes TasA and TapA to release them from the membrane (to be fibers fixed to the wall after). But SipW has also another role due to its carboxy-terminal domain, the activation of the eps gene expression for submerged biofilm (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

All these genes are regulated by some regulators like Spo0A, SinR, SinI, SlrR, DegU,….

The first main one is Spo0A with an activity regulated by its phosphorylation pathway (involving Spo0B and Spo0F) caused by 4 kinases (KinA, KinB, KinC and KinD). Phosphorylated Spo0A controls the activity of SinI which is the antirepressor of SinR, the repressor of the eps and tapA-sipW-tasA operons and the slrR gene (which is also a regulator). In fact, SinI will form the complex SinI-SinR and will prevent SinR to bind DNA in only some cells creating, by the way, matrix producer subpopulations. Spo0A will also have an impact on the duration of the gene expression for matrix production. Indeed, the sinI promoter contains a high-affinity activator and low-affinity operators. The activator is first occupied and after the operators with a growing level of phosphorylated Spo0A (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

High concentration of Spo0A-P also leads to sporulation. When this sporulation begins, a mechanism stops the expression of the matrix genes. Indeed, SinR and SinI are dependent of the sinR and sinI genes quantity. If this quantity increases due to the sporulation which allows the presence of two copies of the chromosome longer, gene expression of the matrix production is blocked. Finally, Spo0A also represses the AbrB (which is also a repressor of the eps and tapA-sipW-tasA operons) but also the production of BslA protein (in links with the hydrophobic surface of the biofilm matrix) and even the SlrR and Abh regulatory proteins. The SlrR protein role is to link to SinR to form a complex and preventing SinR to repress the

two operons of matrix production and the slr promoter. There is so a balance between the proteins SinR, SlrR and the gene slrR. At high concentrations of SlrR, the SinR level is low leading to the expression of the genes responsible for the matrix production. But small concentrations of SlrR, the SinR is not inhibited enough and it leads to repression of the slr genes (including slrR) and the matrix production operons. It's SinI (controlled by Spo0A-P) which allows the switch between the high and small SlrR concentrations by bonding SinRThe complex SlrR-SinR also represses the motility (hag) and cell separation genes (lytAB and lytF coding for the autolysins required for the cells chains separation and the cells chains are needed for the biofilm formation). KinA, KinB, KinC, KinD, Spo0A, AbrB, SinI, SinR and SlrR are all the regulators forming the subnetwork I (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

Two other regulators, YwcC and SlrA (from the subnetwork II), also influence the balance between SlrR and SinR. Indeed, SlrA acts as an antirepressor of SinI and YwcC represses the expression of the slrA gene. This system is present in all *B. subtilis* cells (in the opposite of SinI). It's possible that it's a mechanism of response to environmental stresses.

Concerning the Abh regulatory protein (repressed by the Spo0A as explained above), it helps to the transcription of the spr gene and its transcription is controlled by $\sigma^M$, $\sigma^W$ and $\sigma^X$ (extracytoplasmic function RNA polymerase σ-factors).It's all the units composing the subnetwork III (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

Other main regulators, independent from Spo0A and the other regulators are DegS and DegU. These two ones represent the subnetwork IV and are involved in the regulation of the bslA gene and pgs operon expression. Indeed, DegS (a sensor histidine kinase) phosphorylates DegU (the real regulator). This one is also involved in several cellular processes like competence, motility, secretion of degradative enzymes and cell differentiation (with two other key regulators, ComA and Spo0A). The bsla gene is responsible of the secretion of the BslA, a wall amphiphilic protein that works with EPS and TasA amyloid fibers to allow biofilm development. It is also responsible of the formation of a biofilm surface layer which repels water and low surface-tension liquid (methanol, methanol, isopropanol,…) (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

All mechanisms and regulators described above are impacted by some signals. For example, the surfactin (a lipopeptide produced by *Bacillus subtilis,* visible in the Figure 4) works as a signal for the phosphorylation of Spo0A by KinC sensor kinase. The cells producing surfactin

are different from the ones producing matrix (influenced by Spo0A), it is considered as a paracrine signal or unidirectional signal in the opposite with quorum sensing where all cells are impacted by the signal. The results are the same with other compounds like nystatin and valomycin but also chlorine dioxide (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).
Other examples of the impact of signals on regulation of the biofilm development are the link between KinD regulator and the matrix. Indeed, the production of the matrix switches the activity of KinD. At low level of matrix, KinD works as a phosphatase and keep Spo0A at low concentration. But at high level of extracellular matrix, the kinase activity of KinD allows sporulation. Some other compounds of the soil (as tomato root exudates) can have the same impact on KinD activity (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).

The biofilm dispersal also depends of regulation mechanisms but it will not be covered in the case of this master thesis.

## B. Bacillus amyloliquefaciens: genomic comparison with other strains

Comparative analyses between *Bacillus subtilis 168* genome and *Bacillus amyloliquefaciens FZB42* were done by …. to obtain the complete genome of this *Bacillus amyloliquefaciens* strain. Some genes related to the *Bacillus subtilis* biofilm development are then also found in this genome strain accorded to the supplementary tables of this publication as seen in the following table(Chen et al., 2007).

**Supplementary Table 5. Genes probably involved in plant bacterium interactions.**

| *Gene* | *From* | *To* | *Protein* | *Remark* |
|---|---|---|---|---|
| **Root colonization, swarming motility and biofilm formation** | | | | |
| sacB | 3869505 | 3870926 | Levan sucrase | Levan is implicated in the aggregation of wheat root-adhering soil [1] |
| ycdH | 284685 | 285644 | High affinity zinc ABC transporter lipoprotein | Proposed to be involved in surface adhesion[2] |
| RBAM00754 | 769334 | 770713 | Collagen like triple helix with GXT repeats5 | Proposed to be involved in surface adhesion[2] |
| RBAM00751 | 767904 | 769154 | Collagen like triple helix with GXT repeats4 | Proposed to be involved in surface adhesion[2] |
| RBAM00750 | 765780 | 767777 | Collagen like triple helix with GXT repeats3 | Proposed to be involved in surface adhesion[2] |
| yflQ | 842464 | 843552 | Putative surface adhesion protein | Proposed to be involved in surface adhesion[2] |
| sfp | 375046 | 374372 | Phosphopantetheinyl transfease necessary for lipopeptide and polyketide synthesis | Biosurfactant necessary for surface motility and biofilm formation but suppressing biofilm formation in competing microorganisms[4,5] |
| yczE | 375792 | 375145 | Integral membrane spanning protein affecting lipopeptide and polyketide synthesis | Biosurfactant necessary for surface motility and biofilm formation but suppressing biofilm formation in competing microorganisms[4,5] |
| srfABCD | 343826 | 369253 | Surfactin synthetases | Biosurfactant necessary for surface motility and biofilm formation but suppressing biofilm formation in competing microorganisms[4,5] |
| comP | 2993285 | 2995585 | ComX sensor kinase | Regulator of surfactin production[6] |
| swrC | 2413698 | 2413141 | Multidrug efflux pump | Self-resistance against surfactin[6] |
| efp | 2413698 | 2413141 | Similar to elongation factor P | Essential for swarming motility[6] |
| swrB | 1633667 | 1634074 | Swarming protein | Essential for swarming motility[6] |
| swrA | 3354144 | 3353791 | Swarming protein | Essential for swarming motility[6] |
| spo0A | 2393886 | 2393086 | Master regulator of initiation of sporulation | Involved in initial stage of biofilm formation[7] |
| sigH | 117336 | 117992 | Sigma factor H | Involved in initial stage of biofilm formation[7] |
| abrB | 45895 | 45611 | Transition state regulator | Transcription control of biofilm formation[8,9,10] |
| sigW | 199909 | 200472 | ECF sigma factor W | Transcription control of biofilm formation[8,9,10] |
| resE | 2222973 | 2221192 | Sensor histidine kinase controlling aerobic and anaerobic repiration | Transcription control of biofilm formation[8,9,10] |
| sinR | 2427247 | 2427588 | Master regulator of biofilm formation | Transcription control of biofilm formation[8,9,10] |
| sinI | 2427046 | 2427219 | SinR antagonist | Transcription control of biofilm formation[8,9,10] |
| ybdK | 479816 | 480583 | Hypothetical protein | Transcription control of biofilm formation[8,9,10] |
| ycbA | 256238 | 257479 | Sensor histidine kinase | Transcription control of biofilm formation[8,9,10] |
| lytS | 2722139 | 2720358 | Sensor histidine kinase controlling autolysis | Transcription control of biofilm formation[8,9,10] |
| epsA-O | 3285390 | 3274560 | Operon for capsular poly-saccharide biosynthesis | Exopolysaccharide necessary for biofilm formation controlled by sinR[10] |
| yhxB | 936650 | 938392 | Putative phosphohexomutase | Likely involved in exopolysaccharide synthesis[11] |
| yqxM | 2429713 | 2429042 | Hypothetical protein | Member of yqxM-sipW-tasA operon, essential for biofilm formation and controlled by sinR[11] |
| sipW | 2429070 | 2428486 | Type I signal peptidase | Impaired in processing of TasA[11] |
| tasA | 2428421 | 2427636 | Spore coat associated protein | Required for development of complex colony architecture[11] |
| ecsABC | 1006147 | 1008856 | ABC multidrug transport | Protein secretion to extracellular matrix[8] |
| yqeK | 2514458 | 2513898 | Putative HD phosphatase | Predicted role in NAD metabolism[8] |
| ylbF | 1485947 | 1486396 | Positive regulator of ComK | Control of community development[8] |
| ymcA | 1694164 | 1694595 | Hypothetical protein | Control of community development[8] |

*Table II: Bacillus amyloliquefaciens FZ42 genesof interest similar to genes of Bacillus subtilis* (Chen et al., 2007).

## C. Lipopeptides production

One of the interests of the *Bacillus* cultivation is the synthesis of lipopeptide molecules exploitable for diverse applications such as environmental and pharmaceuticalapplications.

These lipopeptides are also produced by *Pseudomonas* species and the different obtained molecules for each species (*Bacillus* and *Pseudomonas)* are in the following Figure5(Raaijmakers et al., 2010; Meena & Kanwar, 2015).

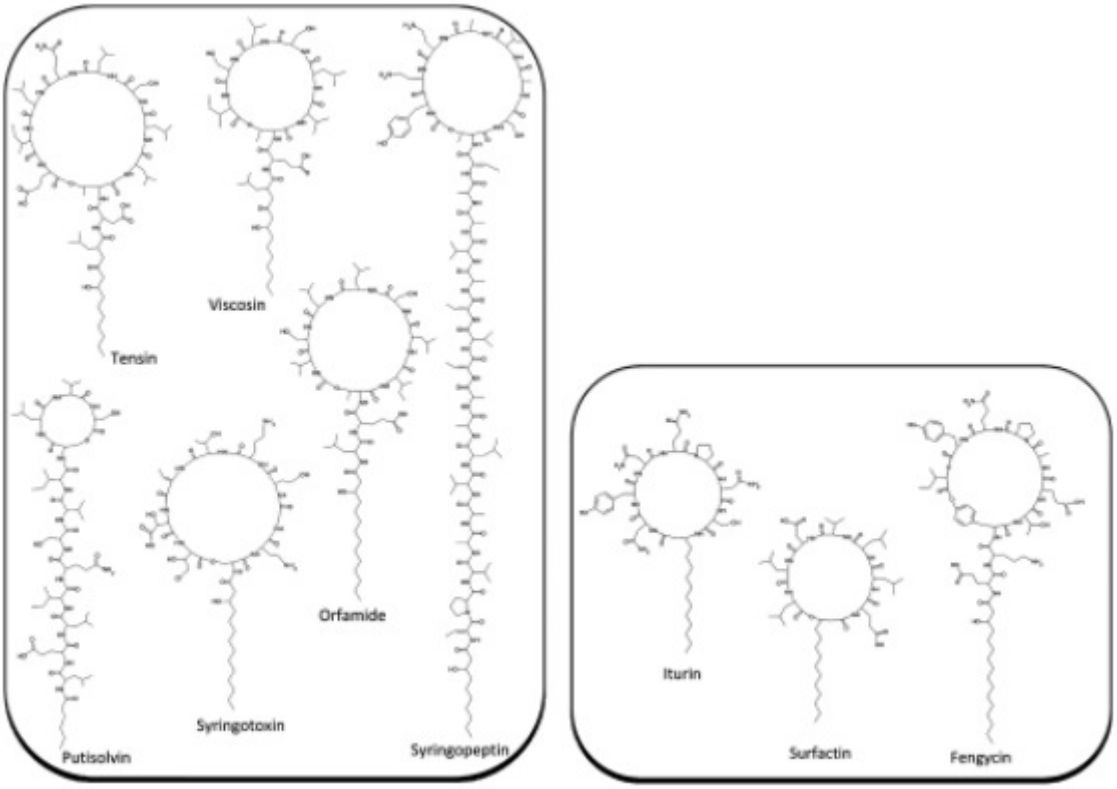


*Figure 5: Exemples of lipopetides produced bt Pseudomonas (left) and Bacillus (right)* (Raaijmakers et al., 2010).

The three main lipopeptides are the ones produced by *Bacillus* species, the iturin, the surfactin and the fengycin. The surfactin is especially used for different applications summarized in the following Figure 6 (Raaijmakers et al., 2010; Meena & Kanwar, 2015).

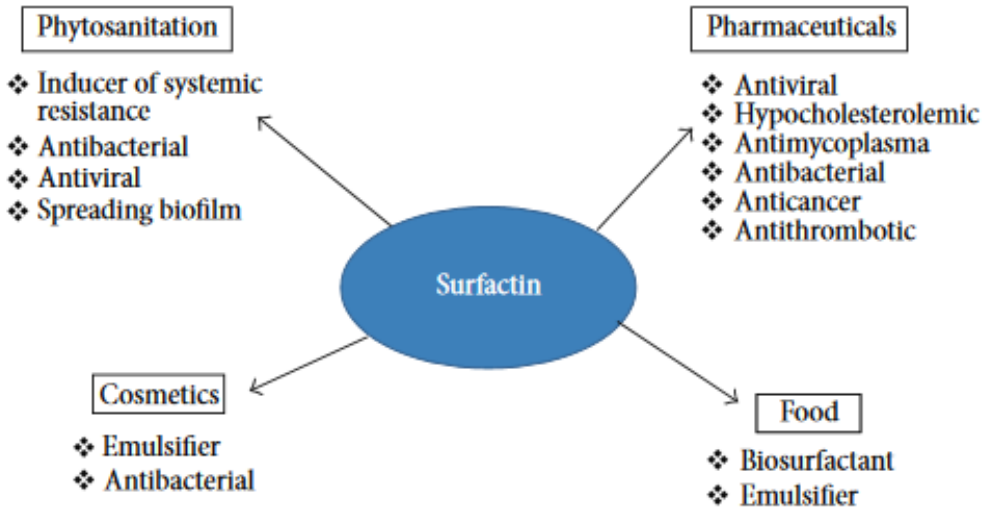


*Figure 6: Application fields of surfactin* (Meena & Kanwar, 2015).

The lipopeptides are important in the researches about biofilm. Indeed, presence of lipopeptides plays an important role on the biofilm formation (but also on the migration of the subpopulations for rhamnolipids of *Pseudomonas aeruginosa* and on the distribution of nutrients depending of the molecules. Indeed, for the surface attachment, these molecules can be oriented in two different configurations:

- The hydrophilic part is exposed to the bacteria and the hydrophobic part to the surface, promoting adhesion to hydrophobic surface.
- The hydrophobic part is fixed to the bacterial cell surface and the hydrophilic part is exposed to the surface, promoting adhesion to hydrophilic surface (Raaijmakers et al., 2010; Meena & Kanwar, 2015).

As a result, depending of the cell surface charge, the substratum surface charge and the lipopeptides charge and hydrophobicity, the impact can be different.
Forexample, the surfactin (of *Bacillus subtilis*) has proven to decrease the biofilm formation on vinyl urethral catheters (Raaijmakers et al., 2010; Meena & Kanwar, 2015).
However the impact can be different. Indeed, as explained on the previous chapter, the surfactin can also promote the matrix formation by helping to the phosphorylation of Spo0A by KinC.

As conclusion, it's important to take into account the lipopeptides. They are important because of their impact on biofilm development and then helpful in the studies about bacterial interactions (especially between *Bacillus* and *Pseudomonas* species). They are also very interesting by the different applications they offer.

# 3. Devices used for the cultivation of biofilms

To make a culture and a research about biofilm, some specific devices are necessary because of the complexity of the bacterial biofilm growth in comparison with the planktonic cells development.

## A. Different kinds of devices

Following the review of Azeredo and al. (Azeredo et al., 2016b), several kinds of devices can be used : The microtiter plates (and their alternatives, the Calgary devices), the Robbins device, the Flow Chamber reactor, the rotary biofilm reactor and the device used in this study, the Drip Flow reactor. Another emergent promising device study is the microfluidic devices. Indeed, they allow the observation of biofilms development, the interactions of the biofilms with their hydrodynamic environment, the factors influencing the biofilms formation. As a result, some mathematical models can be developed(Azeredo et al., 2016b; Janakiraman et al., 2009).However it will not investigated in the case of this master thesis because of its complexity.

To these 5 systems, some others reactors can be added like the moving bed biofilm reactor (MBBR), the integrated fixed film activated sludge (IFAS), membrane-supported biofilm reactors (MBfR), membrane aerated biofilm reactor (MABR) and segmented flow biofilm reactor (SFR). These five other kinds of reactors seem to be mainly used for applications like water treatments, cultivation of catalytic biofilm,… So, they will not be investigated here. For more information some studies are advised like the ones of

The figure VII on the next page illustrates some of the devices used for the study of biofilm.

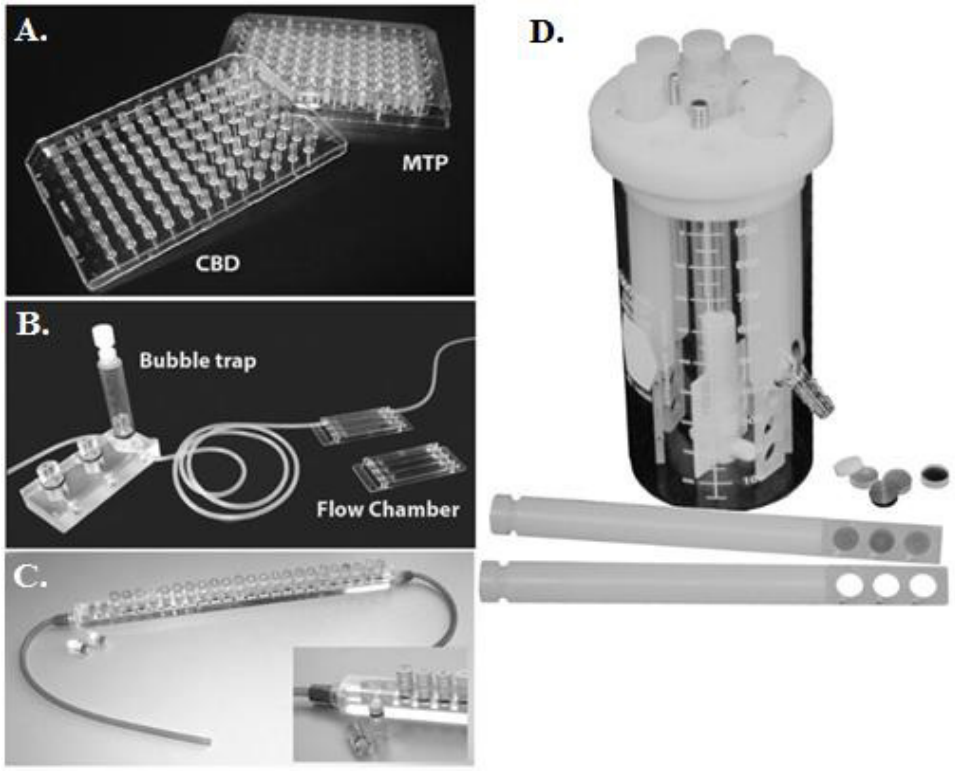


*Figure 7: Illustration of 4 cultivation devices (A: Microtiter plates and Calgary biofilm device, B: Biofilm flow chamber system, C: The modified Robbins device, D: Rotary biofilm reactors)* (Azeredo et al., 2016b).

***The microtiter plates and Calgary devices:*** The most spread and used devices for the study of biofilm are the microtiter plates. Indeed, they are useful because there are numerous polystyrene wellsin one microtiter plate which increase the number of possible experiments and replicates.

The biofilm is then cultivated into the well. At different time of cultivation, one (or more) well(s) can be used to remove the plaktonic cells. After, the biofilm biomass can be stained and quantified with the total biomass adhered to the well(s) surface (Azeredo et al., 2016b; Djordjevic, 2002).

However, some cells, taken into account of the biomass calculation, can come from the sedimentation to the bottom of the well(s) (Azeredo et al., 2016b). To counter this problem, another kind of microplates have been designed, the Calgary biofilm device(Ceri et al., 1999). This device looks like microtiter plates but with plugs inserted into the wells. As a result, the biofilm is formed on plugs which are inserted into the medium with the bacteria (into the wells). The biofilm is then removed from the plegs by sonication for the quantification of the biomass(Müller et al., 2011).

The main advantage of these devices are the number of experiments possible by plate and the possibility to make microscopic analyses and the biofilm ring test (which evaluates the capacity of the biofilm to immobilize microbeams) (Chavant et al., 2007). They are often used for study of the biofilms at the air-liquid interface (with batch cultivation) and for the impact of antibiotics on biofilm (Azeredo et al., 2016b).

***The Robbins device:*** The Robbins device consists of a pipe with numerous holes where coupons can be inserted parallel to the liquid medium flow(Azeredo et al., 2016b; McCoy et al., 1981).

It is principally used for the study of the biofilm formation under controlled conditions (particularly the flow intensity) (Azeredo et al., 2016b; Nickel et al., 1985).

This device exists in another form, the modified Robbins device. This one consists of a square pipe with equally-spaced ports for the insertion of plugs and coupons aligned with the surface and which don't disturbe the flow (in comparison with the original one). Several hydrodynamic conditions can be tested with this device like the laminar and turbulent flow (Azeredo et al., 2016b; Linton et al., 1999)but it is important to be sure that the flow is developed at the coupons place. Another interesting aspect of this device is the possibility to realize long culture experiment (several weeks)(Azeredo et al., 2016b; Teodósio et al., 2011). However, it doesn't allow a direct observation (in comparison with the microfluidics), then the coupons must be retired for observation leading to possible alteration of the biofilms.

***The flow chamber reactor:*** The flow chamber reactor (and its open alternative, the open channel flat plate reactor) is used for the direct inspection of the biofilm development. This inspection can also be done online and continuously in real time (Azeredo et al., 2016b).

The open channel flat plate reactor consists of two connected chambers with liquid medium. The liquid medium goes from a chamber to the other passing along the substratum (located in the connection between the chambers). Fresh medium can be added continuously allowing realizing long cultivation (with big quantities of medium). Moreover, the biofilm growing on the substratum can directly be observable (by the use of lens). However, it can also lead easier to contaminations (Azeredo et al., 2016b).

The closed system (or flow chamber reactor) is close to the open channel flate plate reactor but with aninspection glass or plastic window onto which the biofilm can develop. The biofilm is then encapsulated and the side biofilm of biofilm in contact with the substratum (the window) is visible and images can be obtained by microscopy. The gene expression can

also be investigated with the help of fluorescent intercalants and confocal microscopy (Azeredo et al., 2016b).

A mignature system was designed for several cultivations at the same time with different flow chambers in parallel (Azeredo et al., 2016b; Wolfaardt et al., 1994). It is silicone tubings which provide growth medium in the chambers but bubble traps are needed to prevent small air bubbles in the medium. If bubbles are present in the medium, they can cause biofilm detachment. The main advantage of these devices are theon-line monitoring(Azeredo et al., 2016b).

***The rotary biofilm reactor:*** This reactor exists in three different kinds, the rotary annular reactor, the rotary disk reactor and the concentric cylinder reactor (Azeredo et al., 2016b).

The first one is the rotary annular reactor. It consists of two cylinders, a static outer one and a moving/rotating inner other. The inner cylinder rotation is controlled to create a homogeneous liquid phase. But the main goal of this rotation is the obtainment of a turbulent flow leading to shear stresses(Lawrence et al., 2000). The biofilm development can be done on coupons (of different possible materials) fixed on the outer cylinder. It's with these coupons that the different analyses and observations will be done(Azeredo et al., 2016b).

The second reactor is the rotary disk reactor. In this one, the coupons are fixed on a rotating disk. The rotation is done by the use of a magnetic stirrer and magnet attached to the disk. Due to the rotation, coupons undergo shear stresses but these stresses are the same for all coupons because they are placed at the same radial distance. However this radial distance can also be changed to create different shear stresses on the coupons (Azeredo et al., 2016b).

The third reactor is the concentric cylinder reactor which consists of four (concentric) chambers into which four cylinder sections are rotated at different speeds. Different shear stresses (and then hydrodynamic conditions) but also different strains can be tested at the same time. It's the major advantage of this system. In the opposite, only one kind of surface can be used(Azeredo et al., 2016b; Willcock et al., 2000).

All these reactors are mainly used for the study of the impacts of the shear stresses on the biofilm development. Indeed, the shear stresses can be set up by the cylinders (or disks) rotation frequency independtly of the feed flow rate (and then the dilution rate) leading to specific studies of the both parameters separately. Moreover, for two of these reactors (the rotary annular reactor and the rotary disk reactor), the influence of the surface materials can also be determined by using different materials as steel, PET, PVC or biological surfaces. For

the last reactor (the concentric cylinder reactor), the advantage is the possibility to study four different strains (in the different chambers) at the same time (Azeredo et al., 2016b).

## B. Drip Flow Reactor: advantages and drawbacks

The Drip Flow reactor (Figure VIII), used in this master thesis, is very useful for the biofilm cultivation due to its functioning.

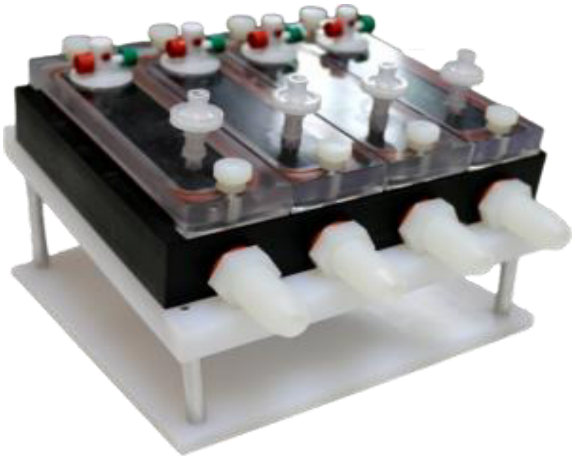

*Figure 8: Illustration of a Drip Flow reactor (Source: http://biofilms.biz/products/biofilm-reactors/)*

Indeed it's a device composed by some chambers into which coupons are inserted. Then the cell suspension (or preculture) and the liquid growth medium can be inserted into the chambers by a needle for a cultivation of 6 hours in batch mode.
The reactor is then tilted of 10° from the horizontal during the experiment allowing the liquid passing along the coupons(Azeredo et al., 2016b; Goeres et al., 2009). The first mode is used for the surface attachment of the cells and the second step allows the biofilm formation on coupons without immersing the cells.

The main advantages of this device are the small needed space, the possibility to study different materials (with coupons) at the same time and also the possibility of sampling the two phases of the biofilm (planktonic and biofilm phases) and study them noninvasively.
However this reactor has also disadvantages and limitations as biofilm heterogeneity on the coupons, low shear stresses (for researches about the impact of the shear stresses on biofilm), low similarity with industrial reactors and also the limited number of chambers (in comparison with microtiter plates for instance) (Azeredo et al., 2016b).

The bacterial adhesion is then an important part of the use of Drip Flow method during the 6 hours of batch mode.

# 4. Bacterial adhesion to surfaces

## A. Theoretical models

The bacterial adhesion can be described with different theoric models (or approaches). The two main ones can be considered as the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory and the thermodynamic approach(Hori & Matsumoto, 2010).

The first theory was used for the description of the interaction between a colloidal particle and a surface. This kind of interaction is then the sum of the Van Der Waals and Coulomb interactions. Indeed, if the Van Der Waals attractive force is dominant, the particle is not able to detach from the surface by Brownian motions. The particle is then fixed irreversibly to the surface. On the opposite, if the particle is too far from the surface, the Coulomb repulsive interactions take the lead because the Van Der Waals interactions decrease with the distance (Hori & Matsumoto, 2010; Marshall et al., 1971).

For the bacterial adhesion, the ionic strength has a big importance. Indeed, the surface and the bacteria are often charged negatively in solution. As a result, they repulsed each other. However, a higher ionic strength can decrease this repulsion energy (and the energy barrier for the cells adhesion) because counter ions come in the electrical double layers and partly hide the negative charge. On the contrary, a lower ionic strength will increase the energy barrier. The Figure 9illustrates these energy barrier changes.

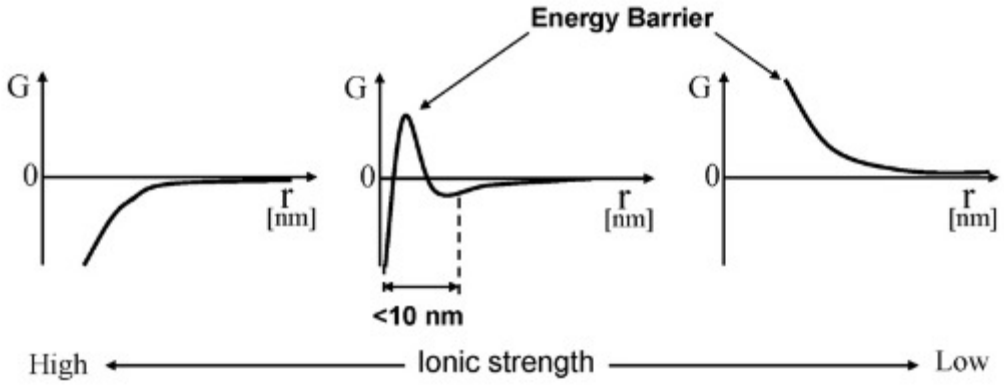


*Figure 9: Bacterial adhesion energy barriers changes depending on the ionic strength* (Hori & Matsumoto, 2010).

With the DLVO theory, the bacterial adhesion can be summarized as a two-phase process: The physical reversible adhesion and the irreversible molecular (and cellular) adhesion. During the first stage, the bacterium approaches by swimming or Brownian motion and adheres reversibly to the surface. During the next step, the bacterial pili, flagella and EPS help to the irreversible adhesion between the bacterium and the surface. Indeed, they can overcome the energy barrier because of their small radii. However, if the energy barrier becomes higher because of a lower ionic strength or if the cell is farther from the substratum, these structures (or compounds for the EPS) have difficulties to reach the surface. The bacterial adhesion is then compromised. On the opposite, the bacterial cell adhesion is promoted at high ionic strength because the energy barrier disappears leading to a fast irreversible adhesion (Figure IX)(Hori & Matsumoto, 2010).

The other alternative of model is the thermodynamic model. This approach is based on the surface free energies calculations following this equation:

$$\Delta G_{adh} = \gamma_{sm} - \gamma_{sl} - \gamma_{ml}$$

With $\Delta G_{adh}$ = Total free energy.

$\gamma_{sm}$ = Free energy of the interface between the surface and the micro-organism.

$\gamma_{sl}$ =Free energy of the interface between the surface and the liquid.

$\gamma_{ml}$ =Free energy of the interface between the micro-organism and the liquid.

The adhesion is then reached if the total free energy ($\Delta G_{adh}$) is negative (Hori & Matsumoto, 2010; Absolom et al., 1983; Busscher et al., 1984).

This model presents a problem: it doesn't include the distance dependence of the adhesion. However, it allows explaining one observation, the fact that the bacteria with a hydrophobic surface adhere preferentially to hydrophobic surfaces and it's the opposite for the hydrophilic bacteria. The hydrophobicity is, indeed, an important factor of the bacterial adhesion and aggregation. This factor is mainly due to the hydrogen bonds which can be seen as simple Lewis acid-base interactions (electron-donor and electron-accepter interactions) (Hori & Matsumoto, 2010; An & Friedman, 1998).

The thermodynamic model and its explanations lead to the creation of an extended DLVO theory by Van Oss (van Oss et al., 1987). With this one, the hydrophobic/hydrophilic interactions but also the osmotic interactions are taken into account. However, the osmotic part is so small that it can often be neglected. The extended DLVO equation is then:

$$\Delta G_{adh} = \Delta G_{VDW} + \Delta G_{dl} + \Delta G_{AB}$$

With $\Delta G_{adh}$ = The total adhesion energy.

$\Delta G_{VDW}$ = The energy term related to the Van Der Waals interactions.

$\Delta G_{dl}$ = The energy term related to the electric double layer interactions.

$\Delta G_{AB}$= The energy term related to the acid-base interactions which includes the hydrophobic (attractive effect) and hydrophilic (repulsive effect) interactions.

In fact, it has to be noted that the real bacterial adhesion is much more complex and presents a lot of deviation to these models. Indeed, a lot of parameters like the presence of organic or inorganic matter on the surface, the physicochemical properties of the surface, the composition of the cell membrane or the pili and flagella lengths influence the fixation of the bacteria to a surface (Hori & Matsumoto, 2010).

## B. Factors influencing adhesion

The factors influencing the adhesion are numerous and depending on the bacterial strain and the surface. The following parameters are bacteria-dependent cell surface parameters:

***Presence of polysaccharides:***

These compounds can be divided into two categories concerning the bacterial adhesion, the lipopolysaccharides and the extra polymeric substances (Hori & Matsumoto, 2010).

Concerning the first one, they concern principally the Gram-negative bacteria because they are the major compounds of the outer membrane of these bacteria. These lipopolysaccharides are composed of lipids (lipid A), polysaccharides core and other polysaccharides composing the O antigen units at the extremity (Figure 10) (Caroff & Karibian, 2003).

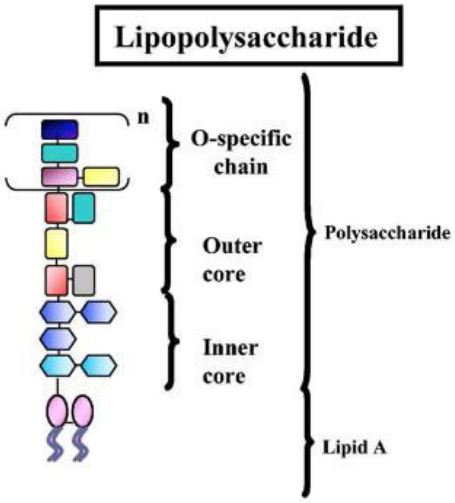


*Figure 10: The schematic structure of the bacterial lipopolysaccharides*(Caroff & Karibian, 2003).

It's in these antigen units that we can find specific structures are able to make hydrogen bonds with mineral surfaces. Precisely, it's the B-band units (or the serotype-specific antigen), composed of two to five saccharides, which bind to the surfaces with an energy of $2.5kT$ J (where $k$ is the Boltzmann constant and $T$ is the temperature) (Hori & Matsumoto, 2010; Jucker et al., 1997).

The bacterial lipopolysaccharides are strongly depending on the strain, especially the O-chains which are like a fingerprint for the bacteria(Caroff & Karibian, 2003).

*Extra polymeric substance:*

As explained in the point 1.E, the different compounds of the EPS have different functions (Flemming & Wingender, 2010). The adhesion function is mainly due to polysaccharides, proteins, DNA and amphiphilic molecules. The interactions which they form are noncovalent bonds, such as electrostatic attraction and hydrogen bonds (The role of intermolecular interactions: studies on model systemsfor bacterial biofilms). The force of these interactions is very low in comparison with a covalent bond but they are numerous. Indeed, the compounds responsible of the adhesion have a large number of binding sites. As a result, thetotal binding force exceeds the force of the covalent bonds between carbons (Hori & Matsumoto, 2010; H-C. Flemming., J, 2001; Flemming & Wingender, 2001).

***The presence of bacterial nanofibers:***

These cell appendages have already showed an influence on the bacterial adhesion in the DLVO theory. However, their real roles will be investigated in the following lines.

The first nanofiber of interest is the pilus (or fimbria). It consists in a hair-like complex of protein subunits with a bigger one (in the form of a helix) called major pilin. At the extremity of the protein subunits, an adhesin can be found. It's the extremity of this nanofiber which is responsible for the adhesion to surface and host (like mammalian cells). The major responsible is then the adhesin. However the protein subunits can also influence this phenomenom(Hori & Matsumoto, 2010; Soto & Hultgren, 1999).

It has to be noted that the specific composition of a pilus differs in function of bacterial strains. The most studied pilus in links with the bacterial adhesion of the biofilm is the type IV pilus found in a lot of Gram-negative bacteria (like *Pseudomonas aeruginosa*) and which helps to the movements of the cells by gliding or twitching. This pilus is involved in nonspecific adhesion to abotic surface (and so biofilm formation) but also specific in specific bond with some molecules (Hori & Matsumoto, 2010; Mattick, 2002; Wall & Kaiser, 1999).

Some other pili like type 1 pili and P pili are also known but will not be aborded here. Concerning the Gram-positive bacteria, less pili are known and also limited to some species (like *Streptococci* and *Corynebacteria*). However some information already exist like the fact that these pili are thinner and that they consist of some copies of pilin unit with a little number of supplementary proteins. These pili seem to be also involved in the adhesion and propagation of the Gram-positive bacteria (Hori & Matsumoto, 2010; Scott & Zähner, 2006; Bonds et al., 2007).

Another kind of nanofibers seems to play also a role in the bacterial adhesion like the autotransporter adhesins (ATADs), an adhesin from *Caulobacter crescentus* or the peritrichate nanofiber from cells of the archae (or SM1 euryarchaeon)(Hori & Matsumoto, 2010). However, these nanofibers are produced by Gram negative bacteria or are rare so they will not be investigated in the case of this master thesis.

Concerning the parameters in links with the surface, they include the energy parameters described in the theoretical models. Moreover they are difficult to identify because bacteria can morphologically change in contact with the surface(Renner & Weibel, 2011). However some parameters have been identified such as:

*Surface roughness and topography:*It's on the nanometer scale that the roughness of the surface can increase the adhesion of cells as reported in a study with titanium surfaces (Renner & Weibel, 2011; Truong et al., 2009).

In this study, they also conclude that the surface roughness represents the most important parameter of the cells adhesion. Indeed this parameter can also influence other physicochemical properties of the surface (as surface energy for example). But it isn't all, the roughness can also prevent the cells of shear stresses(Renner & Weibel, 2011)

Concerning the topography, the ways it influence the cells adhesion are still not known but a study proved that the adhered bacteria patterns are influenced by the topography of the surface (on the nanometer scale) (Renner & Weibel, 2011; Hochbaum & Aizenberg, 2010).

*Chemical properties:* It's mainly with these properties that the regulations of biofilm formation are done (to prevent biofilm formation) by covalent changes, non-covalent changes, released of samll molecules and degradation of polymeric substances(Renner & Weibel, 2011). The chemical composition is also important because it also impacts the cells adhesion. For example, the surface energy can be changed by chemical composition (and

temperature).Very useful materials to study biofilm are self-assembled monolayers (or SAMs) because with these ones, the functional groups presented to the cells are controlled(Renner & Weibel, 2011; Ulman, 1996). It allows the study of the impacts of the chemical composition on the biofilm formation. For instance, SAMs with hydrophobic and hydrophilic groups are good substrates for the cells adhesion. In the opposite, SAMs with monosachharides groupes are bad ones (Renner & Weibel, 2011).

Finally, the use of antimicrobial and bactericidal coatings is also a form of control of the biofilm formation.

# 5. Multispecies biofilm and interactions

As mentioned in the introduction, biofilms are often multispecies communities. So, the interactions inside are important and numerous. Indeed, the cellular aggregation is a common strategy used to increase local cell density and cells interactions. As a result, the EPS represent the medium of becterial interactions.

However, the presence of several bacterial species in the biofilm also leads to competition for the nutrients (Moons P, Michiels C, 2009; Banks & Bryers, 1991). Competitive bacteria can even produce some metabolites that inhibit or inactivate other populations to take the advantage (or also compounds which decrease the cellular adhesion like lipopolysaccharides as explained in previous lines) (Moons P, Michiels C, 2009). With this mechanism, the gain of nutrients is transformed into metabolites production. But a research(Tait & Sutherland, 2002) proved that, in dual-species biofilm, long-term coexistence is possible even with toxins production. In fact, the two species form different separated microcolonies preventing the contact between sensible populations and toxins. As a result, the formed biofilms were thinner compared tosingle-species biofilm. This phenomenom was also observed by M.Rao in 2005 (Rao et al., 2005). When the two species produce a toxin, the advantage is given to the species producing the toxin with the smaller secretion time and the bigger relative toxicity(Rao et al., 2005). The competition is even more complicated with more species involved (Moons P, Michiels C, 2009).

The productions of such metabolites are regulated by some signals. These signals can be quorum sensing (QS) signals (the needed cell concentrations are easily reached in biofilm), peptides signals (from other strain with the example of *Streptococcus salivarius* producing a lantibiotic, the salivaricin A, which promotes production of other lantibiotic in close species

as studied by M.Upton in 2001) or cell-surface proteins (with the example of CdiA and CdiB of *E.coli*) (Moons P, Michiels C, 2009; Upton et al., 2001; Aoki et al., 2005).
The production of such metabolites is not the only antagonism mechanism. For instance, some bacteria are able to reduce the cell adhesion of other bacteria (like the effect of *Staphylococcus sciuri* on *Listeria monocytogenes*) (Leriche & Carpentier, 2000). Another mechanism is to change the environment by acidifing this one (a mechanism adopted by *Lactococcus lactis*)(Leriche et al., 1999).
The last and most extreme way of antagonism is the feeding of the competitors. Indeed, some bacteria as *Bdellovibrios* fix to other bacteria, break the membrane of their victim and kill them (Núñez et al., 2005).

All these mechanisms were competition and antagonism ones but bacteria can also cooperate in a biofilm. The species are then all close together if they have interests in this cooperation. These interest are often by production of nutrients useful by the others species or degradation of nefast metabolites by other species (Moons P, Michiels C, 2009). The biofilm shows then a more important development.The best example of such cooperation is the nitrifying biofilm formed by *Nitrosomonas* and *Nitrobacter* species (Schramm et al., 1996). An interesting application of interspecies cooperation inside a biofilm is the degradation of complex molecules as benzyl alcohol (by *Pseudomonas putida R1* and *Acinetobacter C6*) or the herbicide linuron (by *Variovorax WDL1* and *Comamonas testosteroni WDL7*) (Moons P, Michiels C, 2009; Breugelmans et al., 2008; Christensen et al., 2002)

Interspecies communications allowing cooperation (or antagonism) are based on signal molecules. These ones influence logically the biofilm development (Davies et al., 1998).
The bacteria can possess receptors to molecules they produce themselves or receptor of molecules produced by other bacteria (like the receptor of AHL of *E.Coli*(Van Houdt et al., 2006). The effects of the different signal molecules can be various like the increase of antibiotics tolerances, biofilm mass increase, gene expression increase for the production of molecules (like amylase). The communication can also be done by horizontal genes transfers (with conjugation and conjugative plasmids) that create new interesting phenotypes for the biofilm (Ghigo, 2001).

As already mentioned above with the separation in microcolonies (preventing contact between toxin and sensible bacteria), the interactions between the species have an impact on the spatial organization of the biofilm (and not just its formation). Indeed, according to the works of M.Liu (Liu et al., 2016), the spatial distribution of the bacteria can represent the kinds of interactions between the species involved as seen in the following Figure XI.

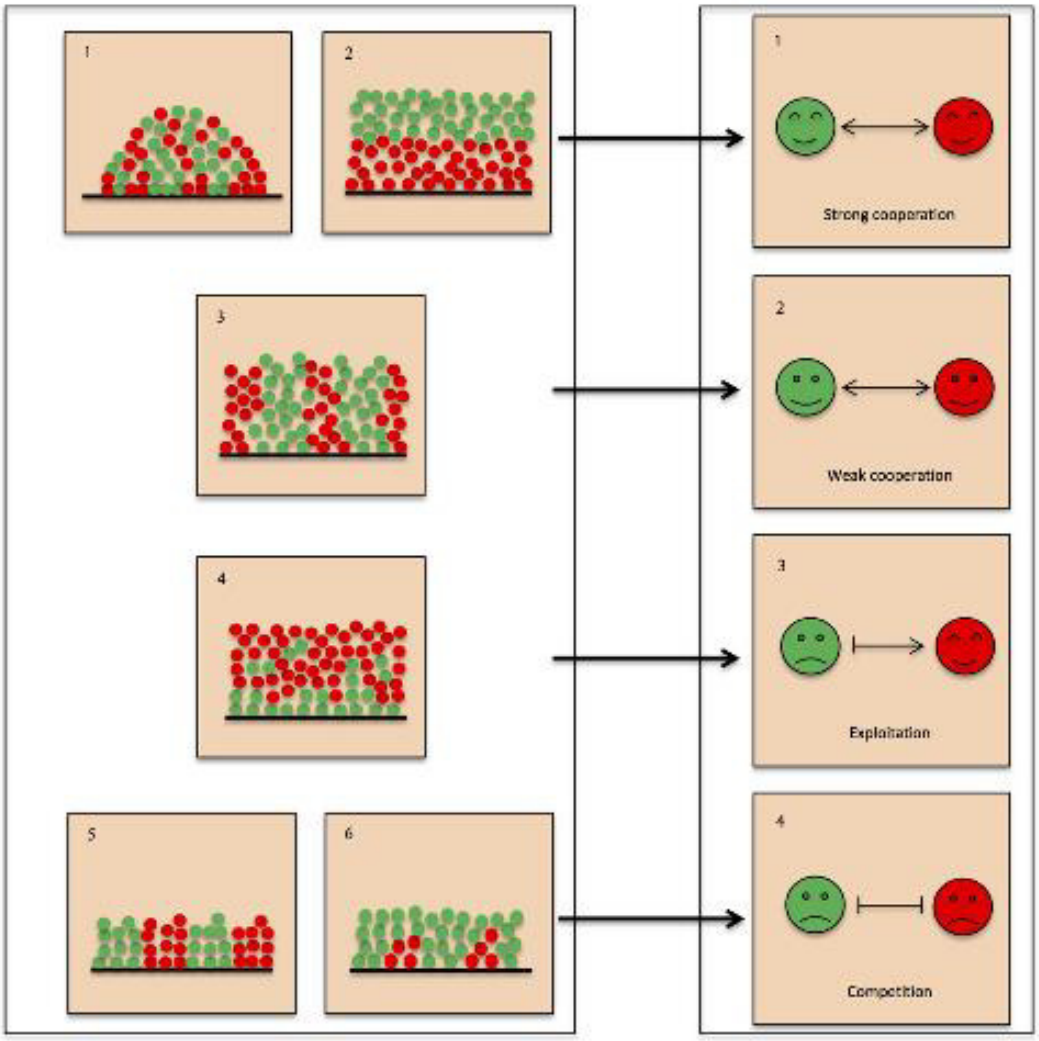


*Figure 11: Representation of the spatial organization of two species in a biofilm depending of their interactions* (Liu et al., 2016).

As a conclusion, interactions between the species in a biofilm are complex because involving physico-chemical processes, gene transfer and expression and cells locations. More researches and investigations are needed to fully understand the mechanisms ruling the communication and interactions between the bacterial species. To this end, the devices used for biofilm growth have to provide repeatable results and the possibility to study these mechanisms. It's the main goal of this study with the Drip Flow reactor.

# IV. Materials and methods

## 1. Drip Flow Reactor

### A. Materials

- Reactor base in polysulfone with six chambers and six effluent ports (*BioSurface Technologies Corporation, USA, Montana*). An image is available in the Appendices (Figure 2).
- Reactor covers in polycarbonates with two holes (for nylon screws to fix the covers to the reactor base) and two ports: one emplacement for the air filter attachment and one emplacement for the medium entry across the needles (*Delvo*, length of 50 mm and diameter of 2 mm). A Teflon base adjusts the injection level of the needles. An image is available in the Appendices (Figure 3).
- Bacterial air vents (0.22 µm of pore size, *Sartorius*).
- Peristaltic pump (*Watson Marlow 530S*). An image is available is the Appendices (Figure 4).

  *NB: Another peristaltic pump (Watson Marlow 100UR) may be necessary for the reactor cleaning.*

- Nylon (or steel) screws.
- Silicone coupons (coupons with silicone edges and coupons with streaks). An image is available in the Appendices (Figure 5)
- Output silicone tubes (internal/external diameter ratio = 8/12 mm) which has an extremity with a *Kartell 468* connector allowing a sampling by an Eppendorf tube. An image of the *Kartel 468*connector is available in the Appendices (Figure 6).
- Entry silicone tubes of different internal/external diameter ratios (3/6 mm and 5/9 mm).The different tubes of 5/9 mm are linked with T form connectors to the tubes of 3/6 mm to allow the medium entry in the 6 channels of the reactor.The entry tubes are also composed with 6 *PharMed BPT* tubes of 1.3 mm diameter (reference = SC0743) for the insertion into the peristaltic pump. An image of the *Pharmed BPT* tubes is available in the Appendices (Figure 7).
- *Luer Lock* connectors (of different sizes) adapted to the silicone tubes diameters.

- Adjustable micropipettes of maximum 100 and 1000 µL.
- Hoffman compressor clamps.
- Aluminium foil.
- Autoclave.
- Laboratory glassware.
- Liquid sterilized YPD medium (10 g/L ofcasein peptone, 10 g/L of yeast extract and 20 g/L of glucose).
- Liquid sterilized LB medium (10 g/L of casein peptone, 10 /L of yeast extract, 10 g/L of NaCl and 2.5 g/L of glucose).
- Sterilized phosphate-buffered saline (PBS) solution (8.0 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of $Na_2HPO_4$ and 0.24 g/L of $KH_2PO_4$).

## B. Operations in DFR

These methods were the ones used after the different steps of optimization. All these steps and changes in the methodology will be explained in the results part (point optimization).

***Reactor set up and sterilization:***

- Insert the silicon coupons adaptedto the experiments into the 6 reactor chambers (in the case of the coupons with streaks, put them on the coupons with edges to prevent biofilm formation under the coupons).
- Put the covers on the different reactor channels and fix them with the nylon (or steel screws).
- Put the clean Sartorius bacterial air vents in the covers ports provided for this purpose. An image of the reactor after this step is available in the Appendices (Figure 8).
- Insert the Kartell 468 connectors (linked to the output silicone tubes and the sampling eppendorf) into the effluent ports of the reactor base.
- Insert the entry tubes (the part of the tubes with a ration external/internal diameter of 3/6 mm) into the covers ports for the medium injection.
- Pack the reactor into a double layer of aluminum foil.
- Sterilize the reactor in the autoclave at 121°C during 20 minutes in steam conditions.

***Inoculation and batch culte step:***

*NB: All these steps are done under laminar flow hood.*

- Prepare 200 mL of sterile liquid YPD medium into a sterile flask with baffles (of 1 liter).
- Add in the flask the content of a *Bacillus subtilis GA1* working seeds or a colony from a petri dish to make a preculture.
- Incubate this preculture overnight (approximatively 17 hours) at 30°C and 130 RPM.
- Check the Optical Density of the precultureat 600 nm after the night. Adjust the Optical Density to 1by dilution with PBS (Phosphate-buffered saline).
- Put the reactor on a straight surface and tilt the reactor back of 5° to prevent the inoculum of leaving the reactor chambers. An image of the reactor assembled with the different tubes on the tilted surface is available in the Appendices (Figure 9).
- Inoculate 20 mL of the preculture solution (diluted before to adjust the Optical Density if necessary) into each reactor channels by screwing a 20 mL syringe to the ports for the medium injection. An image of the inoculation step is available in the Appendices (Figure 10).
- Incubate during 6 hours at 30°C.

***Continuous culture step:***

*NB: All these steps are done under laminar flow hood.*

- Prepare 4 liters of liquid LB medium into flasks of 5 liters.
- Insert silicone tubes of 5/9 (internal/external diameter ratio) into the flasks and across cotton plugs to close the flasks. Sterilize the flasks.
- Connect the silicone tubes from the flasks to the silicone tubes for the medium injection (with a *Luer Lock* connector).
- Put the 6 PharMed BPT tubes (from the tubing for the medium injection) into 6 pump cassettes.
- Apply the maximum flow (“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 by an important flow.*

- Put the reactor on an inclined surface (10°).
- Apply a debit of 9.75 mL/hour (or 2.4 turns per minute).

- Let the system workingtill 40 hours (or more)and sample at different times the planktonic and biofilm phases.

***Reactor disassembly and cleaning:***

*NB: This protocol is valid only for wild-type but no GMO or pathogenic microorganisms.*

- Place the removed slides 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. Keep the lights open to facilitate the next manipulations.
- 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 sametube in a container of bleach and circulate it to fill the whole of thecircuit 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 Hoffman compressor clamps.
- 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 from the reactor covers and store them.
  *NB: Check their condition before storage, the filters must stay perfectly white to ensure their performances.*
- Unscrew the screws (anti-horloger sense) 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 covers 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 bleachtoo.*

# 2. Sampling preparation

## A. Materials

- The drip flow reactor assembled and working (following the previous steps).
- Peristaltic pump (*Watson Marlow 530S*).
- Sterilized iron clamp.
- Vortex mixer (*VWR International, n°444-1372*).
- Ultrasonic homogenizers (*Bandelin sonopuls HD 2070*).
- High speed centrifuge (*Beckman Avanti®J-25*).
- Falcon conical centrifuge tubes of 15 and 50 mL.
- Sterilized phosphate-buffered saline (PBS) solution (8.0 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of $Na_2HPO_4$ and 0.24 g/L of $KH_2PO_4$).

## B. Methods

***Sampling of the planktonic phase:***

- Turn the *468 Kartell*connector of 180° to bring the sampling Eppendorf tube into a Falcon conical centrifuge tube of 50 mL to sample the planktonic phase from one of the reactor channel.
- Collect the planktonic phase from one reactor chamber during one hour (or until having more than 11 mL of solution).
- If the sample is not directly used for the analysis, keep it at 4°C.

***Sampling of the biofilm phase:***

- Open a reactor chamber by removing the screws and the cover.
- Use the sterilized iron clamp to sample the silicon coupon from the chamber. *NB: Don't deteriorate or remove the biofilm on the coupon by touching it with the clamp. To prevent this, grab the coupon by the silicone edges.*

- Rinse gently the biofilm surface with 2 mL of PBS solution, sample the removed cells in an Eppendorf tube to obtain the NAS cells.
- Put the coupon into a Falcon conical centrifuge tube of 50 mL. Add 10 mL (of 11 mLif qRT-PCR analysis is planned) of PBS (Phosphate-bufferedsaline) solution into the tube.

  *NB: For the measurement of the surface hydrophobicity, the biofilm on the coupon must be intact. So the coupon is putted into a Petri dish.*
- Vortex the Falcon tube (and its content) with the vortex mixer (*VWR International, n°444-1372*)at 2500 $min^{-1}$ during one minute. Then, return the coupon into the tube and vortex it again at 2500 $min^{-1}$ during one minute. This step allows the removing of the biofilm from the coupon and the dissolutioninto the PBS solution.
- If the sample is not directly used for the analysis, keep it at 4°C.

***Preparation of the samples for the different analyses:***

- For the measurement of the surface hydrophobicity (point 3 of the Materials and methods), only the coupon into a Petri dish is necessary. The biofilm on the coupon must stay intact.
- For the qRT-PCR analyses, 1 mL of the different liquid samples (the planktonic cells and the biofilm cells diluted in PBS solution) must be centrifuged into an Eppendorf tube with the centrifuger at 15000 RPM during 3 minutes. The supernatant is removed and the eppendorf with the pellet is stored at -80°C till the analyses.
- For the rest of the analyses (optical density and cytometry), the liquid sample must be homogenized with the ultrasonic homogenizerswith an intensity of 30% during 40 seconds and 9 cycles. This step disolves the matrix into the solution.

  Then, 5 (or 10 mL if no cytometry analysis is planned) of the sample is transferred into falcon conical centrifuge tubes of 15 mL and centrifuged with the high speed centrifuger at 8000 RPM during 15 minutes. The supernatant is removed (or conserved for a possible analysis of the produced metabolites). The pellet is then diluted into 5 mL of PBS solution for optical density analyses (Point 4 of the Materials and methods).

  The rest of the homogenized samples are kept for the possible cytometric analyses.

  A draft which summarizes all the sampling preparation steps is available in the Appendices (Figure 11)

# 3. Hydrophobicity analysis

## A. Materials

- Tracker (*IT Concept, France*) with a straight capillary fitted to a syringe of 2.5 mL.
- CCD camera coupled to a profile video image digitizer board-connected to a computer.
- MilliQ water.
- Biofilm(s) on coupon(s).

## B. Methods

***The parameters of the saved image:***

- Fill the syringe with milliQ water and fix it to the tracker.
- Create a drop by bringing down the piston with the help of the button "*Descente*".
- Open the software TRACKER.
- Open the focus adjustement on the software. Adjust the focus by placing the horizontal white line at the edge of the image of the drop (the half of the straight in the drop and the other half out the drop).

  Click on "Go" to notice the contrast difference. Change the offset ad the gain (to adjust the white and black coloration) to have a contrast difference of 200.
- Open the histogram adjustement. Trace a rectangular form which includes the center of the drop. The histogram is correct if the two achieved peaks are almost similar.

*NB: The verticality of the capillary on screen can be modified but it is not necessary for the analysis.*

- Move the drop in the capillary with the button "*Montée*"
- Open the tab "Volumetric calibration". Put the colored superior limit at the border of the capillary and click on GO. Repeat the step until the volume is constant.

***The parameters of the measurement:***

- Open the tab "Set up measurement" to choose the parameters.

  The parameters are the following: Deposit contact angle, sessile down, drop of 2 µL, time of 300 seconds, save the data and images, the precision must be L/R High Precise and the sampling must be 1 sample by second.

***The measurement:***

- Put the coupon on the surface support of the Tracker and adjust the position (vertical and horizontal position and the inclination) of the surface with the different screws to have an approximate straight biofilm surface on screen (to place the drop).
- Click on "Run measurement". The drop of 2 µL is formed.
- Click on "Drop deposit" and open the "Speed" option. Then click on "*Speed*". *NB: The logo of the speed option is a car.*
- Bring down the capillary with the help of a screw to place the drop on the biofilm surface.
- When the drop is placed, bring up the capillary.
- Click on "Slow", close the tab of the "Speed option" and click on "Stop" to bring the measurement to an end.

***The analyses of the results:***

- On the result screen, click on "Calculations" and "Movie".
- Click on play and find the first stable image corresponding to the placed drop (after the drop deposit) by clicking on the icons  .
- Click on "One image analysis".
- Place the blue continuous lineabove the edge between the drop and the surface. Create and place the blue discontinuous line on the edge between the drop and the surface by pushing the bouton « CTRL » while adjusting the blue line.

  *NB: If the blue line is not present; click on the icon*  *and if there is an orange line, click on the icon*  *to remove it.*
- Click on "Go" to make the contact angle measurement.

  *NB: If an error message appears, try to change a bit the position of the continuous and discontinuous lines.*

# 4. Optical density analysis

## A. Materials

- *Genesys 10S UV Vis* spectrophotometer (*Thermo Scienific, USA*).
- Spectrophotometer cuvettes in polystyrene.

- Sterilized hoshphate-buferred saline (PBS) solution (8.0 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L of $Na_2HPO_4$ and 0.24 g/L of $KH_2PO_4$).
- Samples pellets diluted in 5 mL of the PBS solution following the point 2 (*preparation of the samples for the different analysis).*

### B. Methods

- Open the spectrophotometer and use the mode "ATC Base".
- Adjust the absorption at 600 nm.
- Fill a cuvette with the PBS solution and place it in the "B" emplacement of the spectrophotometer.
- Wait that the given optical density is stable and push the button "Définir comme blanc" to define the blank.
- Fill the cuvettes with the liquid samples and place them at the different emplacements of the spectrophotometer (excepted the "B" emplacement).
- Push and keep pushing the button corresponding to the emplacement where is the sample you want the optical density until the optical density is given.
- Wait a stable optical density. If this one exceeds 0.8, dilute the sample with the PBS solution.

  *NB: The acceptable range is between 0.1 and 0.8.*

## 5. qRT-PCR analysis

### A. Materials

- Mini-kit for RNA extraction and isolation, NucleoSpin® RNA (*Macherey-Nagel, Ref :740955.250*).
- IC Green One Step Mix (Nippon Genetics, n°LS4303HR).
- RTase solution (20x FastGene, Nippon Genetics, Lot: 606-011).
- Forward and reverse primers for the gene YuaB, DegU, TasA and the gene of the gyrase.
- 96 wells PCR plate.
- Eppendorf® PCR Cooler
- *NanoDrop™ 2000* spectrophotometer.
- The samples pellets at -80°C.

## B. Methods

***RNA isolation:***

The mini-kit protocol to follow is the "RNA preparation from up to $10^9$ bacterial cells" and after this protocol, proceeds with the step 5 of the RNA standard protocol of the mini-kit.
The entire protocol is available in the Appendices (Part 1).

***RNA quantification:***

- Open the nanodrop program and choose quantification of acid nucleic and the RNA quantification precisely.
- Rinse the surface and the arm of the nanodrop spectrophotometer with RNase-free water.
- Put a drop of 2 µL of RNase-free water on the support and close the arm. Use this RNase-free water as blank for the next analysis (click on the blank option in the nanodrop program).
- Remove the drop from the surface and the arm with a soft tissue.
- Put 2µL of the sample on the support and close the arm. Click on "Read" to get the quantification of the RNA in the samples.
  *NB: The ratios 260/280 and 260/230 must be as close as possible from 2 to have a good sample.*
- Remove the drop with a soft tissue and rinse with RNase-free water before each sample analysis.

***Reverse transcription quantitative PCR analysis :***

- Dilute all the samples to approximatively the same concentrations (between 10 and 50 ng/mL) with Rnase-free water.
- Prepare a mix for each pair of primers with this composition: 10µL of ICR Green Mix solution, 1µL of RTase solution, 0.8 µL of the forward primer, 0.8 µL of the reverse primer and 2.4 µL of RNase-free water.
  *NB: This composition is for the analysis of one sample. The quantities have to be adjusted to the number of samples.*
- Place the 96 wells PCR plate into the PCR cooler.

- Add 15µL of the mix and 5 µL of a diluted sample into a well. Repeat the step for each sample and each primers mix.
  *NB: Manipulate precisely to avoid air bubble into the wells. A little centrifugation(less than one minute, the RPM is not relevant) may be necessary to burst the air bubbles and bring the solution at the bottoms of the wells.*
- Insert the plate into the PCR system and set a new experiment with these parameters.

*Experiments properties:*

Define the name of your experiment.The instrument is the "StepOnePlus™ Instrument (96 wells)" and the type of experiment is "Quantitation".

*Methods and materials:*

The quantification method is the "Comparative $C_T$ ($\Delta\Delta C_T$), the reagent to detect the target sequences is the "SYBR® Green Reagent", the ramp speed is the "Standard (~ 2 hours to complete a run)" and the type of template is the "RNA".

*Targets:*

Identify your target genes. The reporter is SYBR for all the genes and there is no quencher.

*Samples:*

Name the different samples. The distribution on the plates will be done in a following step.

*Relative quantitation settings:*

Choose the reference sample (the sample corresponding to the time 0 for the analysis of the RNA expression evolution in function of cultivation time) and define the gyrase as the endogenous control.

*Run method:*

The reaction volume per well is 20 µL and the method is the following:

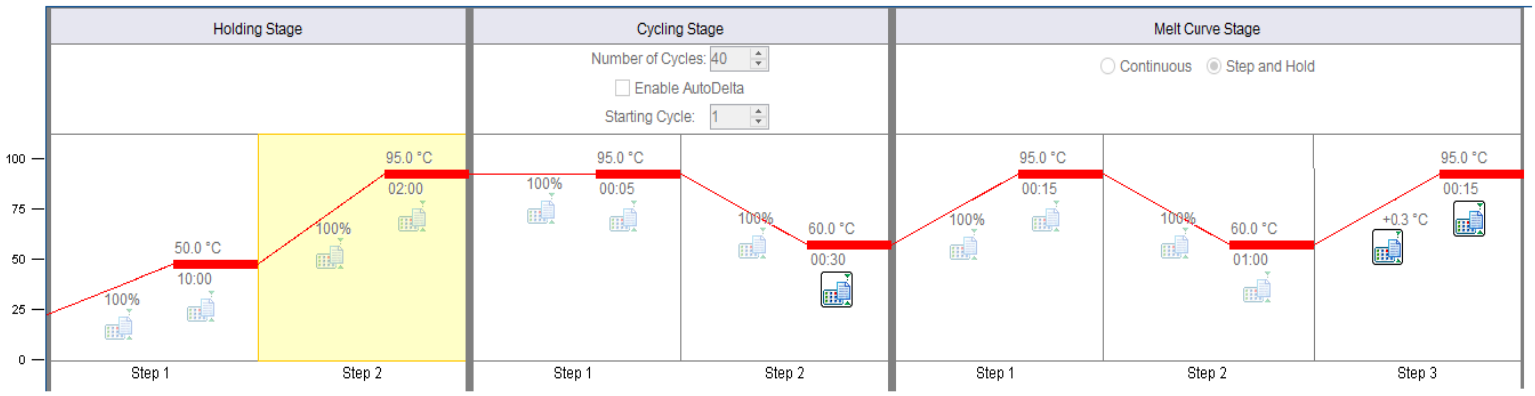


*Figure 12: Run method of the quantitative PCR analysis*

- Change nothing in the *Reaction setup* and *Materials list*.
- Click on "Finish designing experiment" and on "Edite Plate Layout"
- Click on "Assign Targets and samples" and distribute all the targets (the genes) and the samples to the wells as the distribution done during the preparation of the plate.
- After verification of the parameters, click on "Start Run".

# V. Results and discussions

## 1. Optimization of the biofilm cultivation

As mentioned in the state of the art, the major problem of this reactor was its important biofilm growth variabilities between the reactor chambers.At the beginning of the experimentations, the standard deviations between the replicates were so important that no result could lead to interesting conclusions (Figure 13). Indeed, aninteresting result could be caused by a change in the cultivation parameters or by the growth variabilities between the chambers.

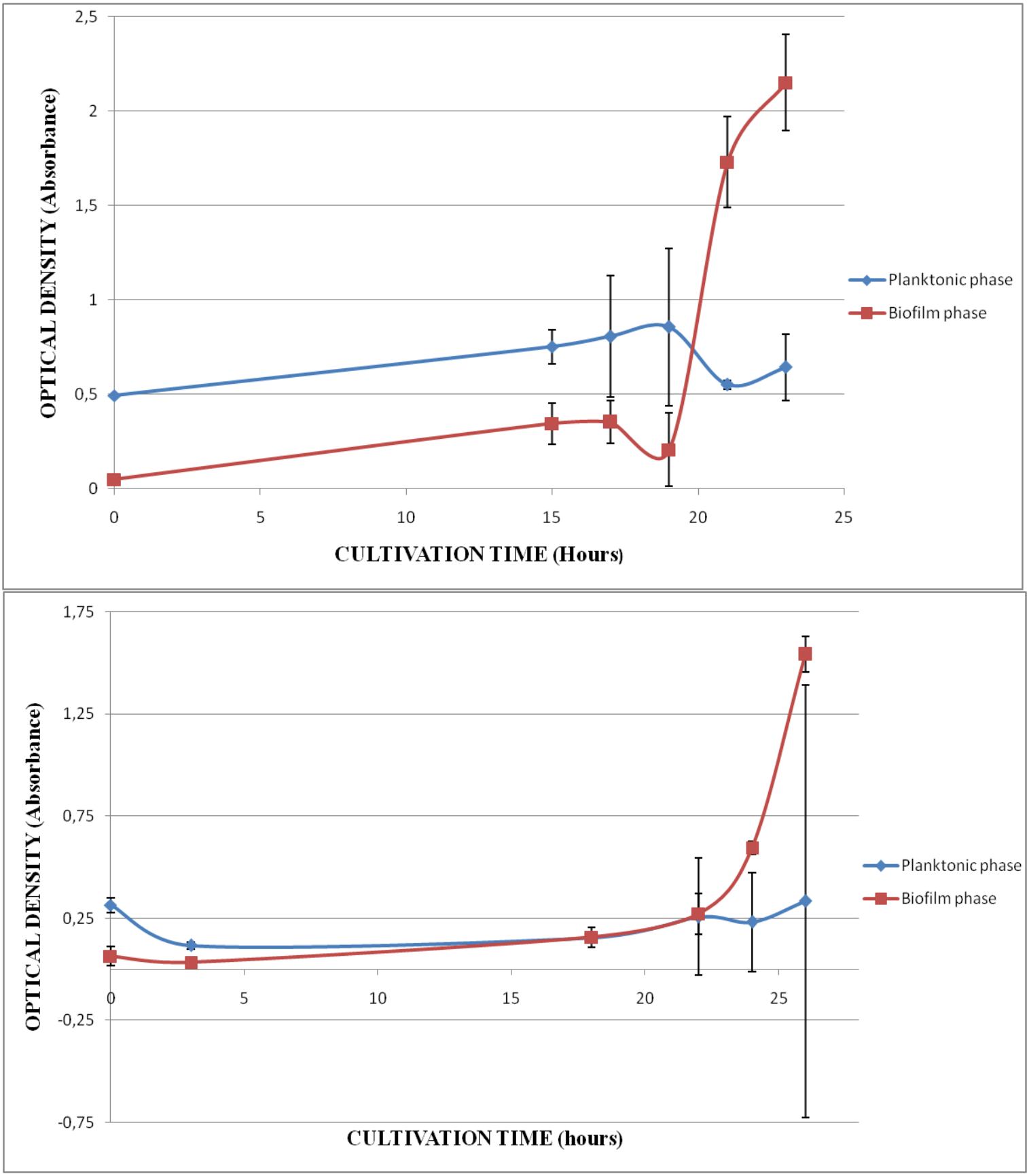


*Figure 13: Two examples of the evolution of the optical density of the Bacillus amyloliquefaciens GA1 biofilm and planktonic phases in function of the cultivation time without sample preparation, temperature control and new inoculation method (and with standard deviations).*

Then, the first step of this master thesis was to decrease these variabilities and obtain repeatable results to develop the profile of the biofilm formation dynamic by measuring the optical density. So, some modifications in the methodology of the use of the Drip Flow reactor were done to create a new method of use.The done modifications can be classified in three points: The sampling (and the preparation of the samples), the cultivation temperature and the inoculation method (for the batch culture step involved in the adhesion of the cells to the surface). The results of the development of this new method of Drip Flow reactor use are in the Figure 15.

***The sampling and the preparation of the samples:***

The sampling method is important to make the link between the planktonic phase evolution and the biofilm phase. For the different other studies using the Drip Flow reactor, the sampling way of the planktonic phase was done by taking samples at different times but from the same chamber. It looked not logical if a link between the evolution of the biofilm and the planktonic phase has to be done.The methodology has been adapted by sampling, at each time, the planktonic phase first and then the biofilm phase from the same chamber.

Some steps of homogenization and centrifugation were also added to the protocol.These modifications allownormalizing the procedure but also to keep only the cells for the analyses. Indeed, the homogenizations by ultrasonication allow removing and dissolving the matrix. Indeed, by keeping the biofilm matrix, the optical density analyses were not optimal because of the matrix interfering with the cells light absorption. As seen in the Figure13, the evolution of the planktonic and biofilm phases can be very different between the experiments due to the matrix. In comparison, the two experiments, observable in the Figure 15 (part 2 of the Results), were obtained with homogenizations by ultrasound. The profiles between the experiments stay the same and represent really the cell concentrations evolution, in the opposite of the experiments from the Figure 13which represent the biofilm and planktonic phase evolution due to the presence of the biofilm matrix. However, the measurement of the optical density doesn't seem the best way to quantify the evolution of the biofilm and planktonic phases, a measurement of the dry matter seems a better alternative.

The difference of the optical densities between the Figure 13 and the Figure 15 are caused by the centrifugation step which concentrates the samples but is necessary to keep only the cells and not the different compounds of the matrix.

***The cultivation temperature:***

The cultivation temperature was not controlled and dependent of the room temperature at the beginning of the manipulatios. As a result, the growth of the biofilms could be inhibited by the fluctuations of temperatures during the days and the nights. Some systems were imagined to bring continuously the LB medium at 30°C (the cultivation temperature of the *Bacillus amyloliquefaciens GA1* strain) but were inappropriate. It's only with the control of the temperature of the room that a cultivation temperature of approximately 30°C was obtained. The impact can be seen by comparing two optical density evolutions at different weeks with and without the control of the temperature (Figures 15.A and 15.B). At the same time of cultivation, the optical density (which represents the cell concentration) is higher for the Figure 15.B (with control of the temperature) than the Figure 15.A (without control of the temperature).

It confirms again the importance of the temperature parameter for the bacterial cultures even for biofilm cultures.

***The inoculation method:***

The first method of inoculation (for the batch culture) was very different. At the beginning, the inoculations were done by adding 10 mL of diluted preculture into each reactor chambers. Moreover the reactor wasn't inclined during this step. However, this kind of inoculation was not efficient. Indeed, the volume wasn't enough and the diluted precultures leave each coupon in a different way (because of preferential liquid paths). To prevent this kind of problems leading to growth variabilities between the 6 chambers, the coupons must be immerged (in 20 mL of diluted preculture) and the reactor must be tilted to prevent the diluted preculture to go out the chambers. By this method, the entire coupon surface has the same probability of bacterial adhesion (excepted in the case of spoiled coupons). It's one of the most critical point to obtain repeatable results as illustrated by the difference of variabilities between the experiments of the Figure 15. The standard deviation after 43 hours of continuous cultivation is 1.499 for the Figure 15.B (with immersion of the coupons). In comparison, the standard deviation of the biofilm sample of the 36 hours time of cultivation is 4,961 for the Figure 15.A (without immersion of the coupons). As the standard deviation increases with the time of cultivation, the comparison between these two values shows the importance of the immersion of the coupons into the diluted preculture to prevent important variabilities between the reactor chambers results.

All these changes in the methodology of use of the Drip Flow reactor lead to the possibility to obtain a dynamic of the biofilm formation and evolution in cells concentration by the measures of the optical density. This dynamic will be explained in the next part of the results.

## 2. Dynamics of the biofilm formation and third fraction of cells

Before investigating the dynamic of biofilm formation, some explanations will be given about the hypothesis of the existence of a third biofilm fraction which consists of the **N**on-Adherent **S**essile **C**ells present on the biofilm surface. They are sessile cells encapsulated into the matrix but non adherent to the rest of the biofilm. Indeed, some observations during biofilms cultivations led to this hypothesis due to the fact that some parts of the cultivated biofilms seem less adhered to the surface and were removed just with the gravity by tilting the coupons. These observations were often done during the sampling of the biofilms after an important cultivation time (between 24 and 40 hours) and a high biofilm growth.

Planktonic fraction of cells (forming the planktonic phase) is motile cells which lead to the formation of a biofilm by sticking to a surface and create subpopulations with other different phenotypes(Mielich-Süss & Lopez, 2015). In the opposite, the cells in the biofilm phase are adhered and agglomerated cells resulting from the cellular adhesion and the production and encapsulation into an extracellular matrix. But as explained in the lastparagraph, some parts of the biofilms were less bonded to the rest of the biofilm. It leads to the hypothesis of the third fraction of cells which is part of the biofilm phase. Then, following this hypothesis, the biofilm is composed of two phases and three cellular fractions.The planktonic cells fraction forms the planktonic phase (without extracellular matrix) and the biofilm phase (with extracellular matrix) is formed by the adherent sessile cells fraction (or ASC) and the non-adherent sessile cells (or NACS) called respectively biofilm and third fraction in the Figure 15.A and the rest of the Figure involved with these two fractions.

Non-adherent sessile cellscould allow studying the biofilm dynamics and the correlation between the main phases of a biofilm, the planktonic phase and the biofilm phase. The Figure 14 illustrates two ways to represent the biofilm dynamics and the correlation between the two phases. With more study about the non-adherent sessile cells (microscopic observations, phenotypic and genomic analyses), the exact situation of the biofilm dynamic between these two hypotheses could be investigated.

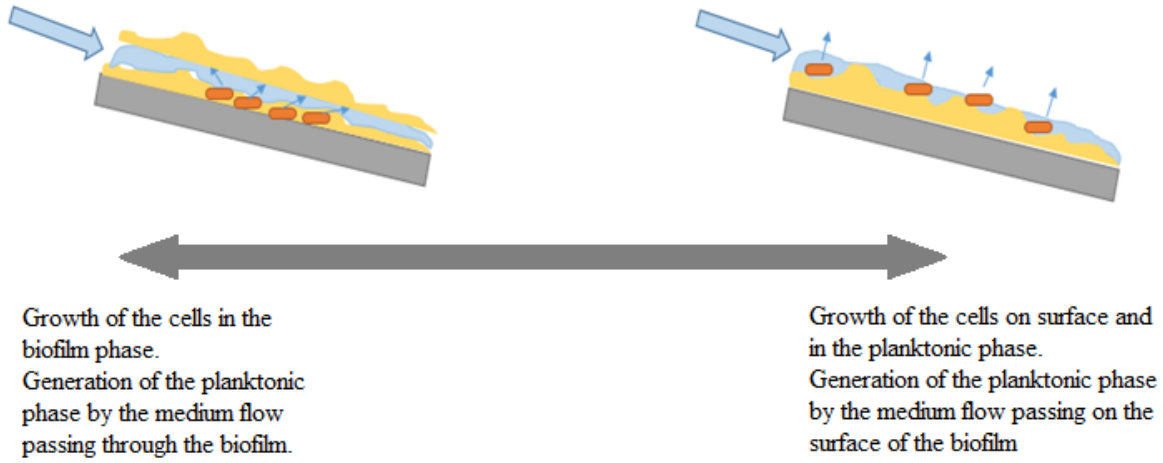


*Figure 14: Hypothetical dynamics of the biofilm and planktonic phase in Drip Flow reactor.*

The best way to sample these non-adherent sessile cells showed to be a gentle rinse of the biofilm surface with PBS. Sampling with a sterile swab was not efficient and not repeatable because depending of the operator and the manipulation of the swab. This isolation makes this kind of cells open to study and analyses and also confirms the hypothesis of the presence of non-adherent cells.

The dynamic of biofilm formation (in term of cellular concentrations) is observable in the Figure 15.Afor the three cells fractions. As seen in the Figure 15.B, the two fractions of cells from the biofilm phase were combined (due to other analyses). Another figure useful for the discussion about the dynamic of biofilm formation is the Figure 20 (part 4 of the Results) which represents the optical densities evolution of the three fractions for the experimentdedicated to the analysis of the RNA expression of three genes. This experiment was done with coupons immersion, temperature control and preparation of the samples.
It has to be noted that the variabilities are important for the biofilm fraction in the Figure 20. These variabilities are due to the fact than a stuck of biofilm was lost during the sampling of one reactor chamber. So, it doesn't reverse the conclusion obtained about the importance of the coupons immersion for the batch culture step and the cells adhesion to the surface.

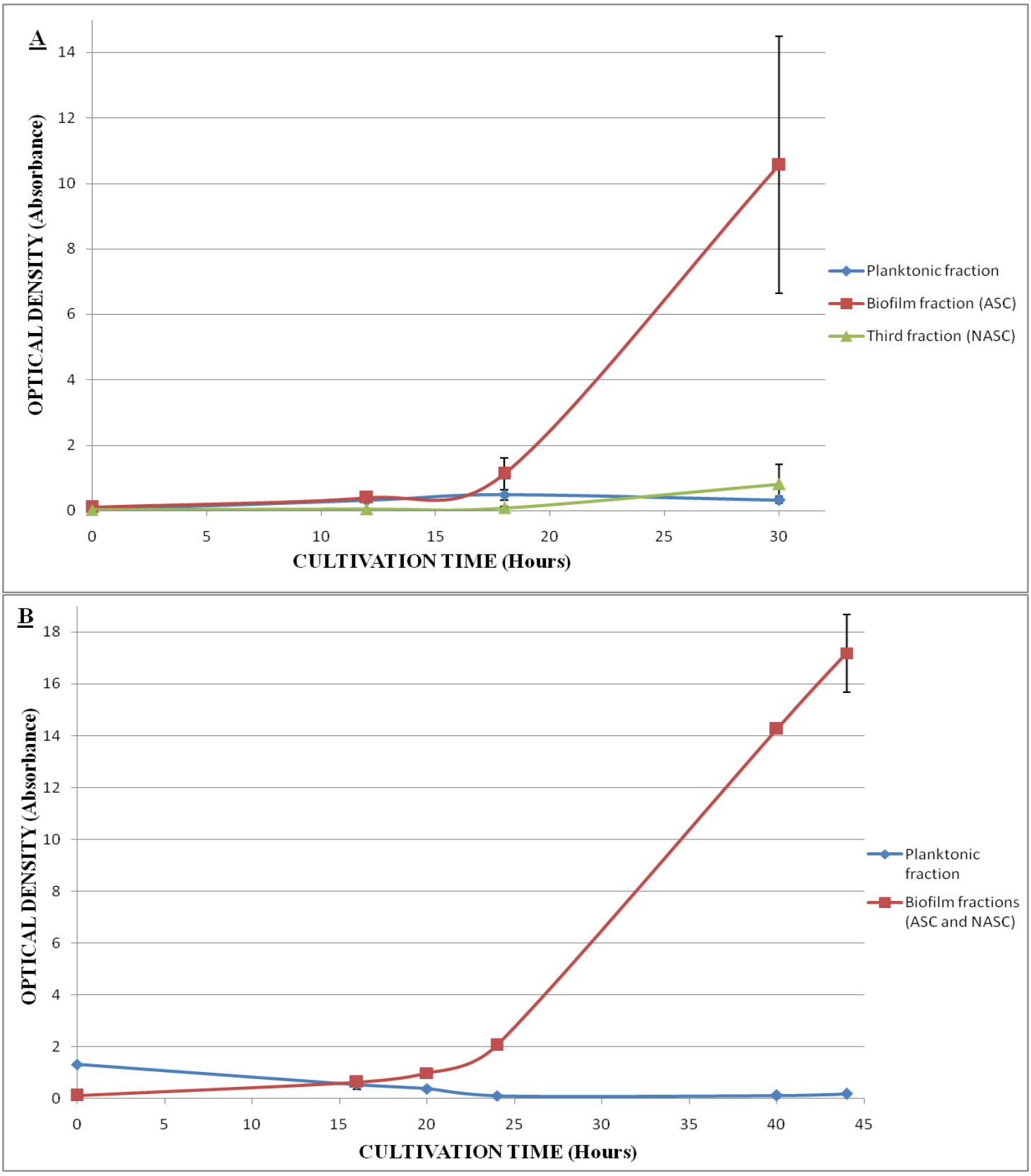


*Figure 15: Two examples of the dynamic of biofilm formation (A: Evolution of the optical densities of the three fractions: planktonic fraction, biofilm fraction (or **A**dherent **S**essile **C**ells) and the third fraction (or **N**on-**A**dherent **S**essile **C**ells) with sample preparation.*

*B: Evolution of the optical densities of the two phases (without matrix): planktonic phase (or planktonic fraction) and biofilm phase (consisting of two fractions, the **A**dherent **S**essile **C**ell and the **N**on-**A**dherent **S**essile **C**ells) with samples preparation, temperature control and inoculation by immersion).*

The evolution of the optical densities (and, so, cellular concentrations) can be divided into two parts, a latent part and an important growth part. These two parts are the same as the two first steps of a normal representation of a microorganism growth, the latent phase and the exponential growth phase.

During the first part (the latent one), the cellular concentration (represented by the optical density)of the biofilm fraction and the third fraction (respectively ASC and NASC) increase slowly. The planktonic fraction has a different profile between the Figures15.A and 15.B, its cellular concentration increases slowly as the two other fractions for the Figure 15.A but it decreases slowly for the Figure 15.B. The real profile seems to be a decrease according to the Figure 20 and the fact that the experiment of the Figure 15.Awas done without temperature control and immersion of the coupons for the inoculation. However, on the Figure 20, a little increase of cells concentration of the planktonic fraction is visible around 20 hours and can be due to little stuck of biofilms leaving out the coupons. It can be confirmed by the decrease of the cells concentration of the third fraction (NASC) after 20 hours. The NAS cells have been sampled with the planktonic fraction may be because of a flow rate change.

During the second part, the cellular concentration of the biofilm fraction (ASC) greatly and rapidly improves (after 23 hours of continuous cultivation according to the Figures 15.B and 20 and after 18 hours following the experiment of the Figure 15.A. However, the 23 hours time will be kept because of the not controlled temperature and the non immersion of the coupon for the experiment of the Figure 15.A). In the opposite, the cellular concentration of the planktonic fraction rapidly decreases in function of the cultivation time which is probably due to an increase of hydrophobicity (explained in the next part of the Results) preventing the medium flow to take cells from the biofilm.

The evolution of the third fraction of cells (NASC) is quite low (Figure 15.A). Indeed, the optical density (and the cells concentration) stays approximatively the same for this new discovered fraction of cells (optical density of 0.1469 after 12 hours of cultivation and optical density of 0.1927 after 36 hours of cultivation) in comparison with the evolution of the planktonic fractionbut especially when compared with the biofilm fraction (ASC). It could be explained by the surface limitation, these cells seem to be cells in surface of the biofilm and the biofilm surface is limited by the coupon surface on the opposite of the adherent sessile cells (biofilm fraction). The cells concentration of this fraction can even decrease as visible on the Figure 20 but it is explained above.

As a conclusion, most important evolution is the one of the biofilm fraction (ASC) and the two phases of these evolutions can be linked to two stages of the biofilm formation, the microcolonies development and the maturation in macrocolonies. Then the first stage of the biofilm formation, the surface attachment takes place during the batch culture step. The second stage of this formation, the microcolonies development takes place till approximatively 23 hours of continuous cultivation and is visible by a slow increase of the optical densities of the biofilm and third fraction and by a slow decrease of the optical density of the planktonic fraction. Then, after approximatively 23 hours of cultivation, the third stage is reached, the macrocolonies development. So, the increase of the biofilm and third fraction increases more rapidly, especially the biofilm fraction. In the opposite, the planktonic fraction optical density decreases faster. If the experiment worked longer, it's possible that an increase of the planktonic fraction would be observable due to the dispersion mechanisms.

Another method has been useful for the detection of this stage of the biofilm formation linked to the matrix production and its properties. It's the surface hydrophobicity measurement with interesting results which will be explained in the next chapter.

## 3. Evolution of the surface hydrophobicity as a proxy for evaluating biofilm formation

Observations on the *Bacillus amyloliquefaciens GA1*biofilm surface during the non-adherent cells isolations led to a hypothesis, the surface hydrophobicity increases for this strain biofilm. After 40 hours of continuous cultivation, the biofilms on coupons arewrinkled(and hydrophobic) as observable with the Figure 16.

*Figure16: Aspect of a biofilm after 40 hours of continuous cultivation with Drip Flow reactor.*

To confirm this observation of the surface hydrophobicity, experiments have been done in Petri dishes with agar LB medium. Drops of water with methylene blue dye were deposited on surfaces of colonies of *Bacillus amyloliquefaciens GA1* at different times of cultivation.

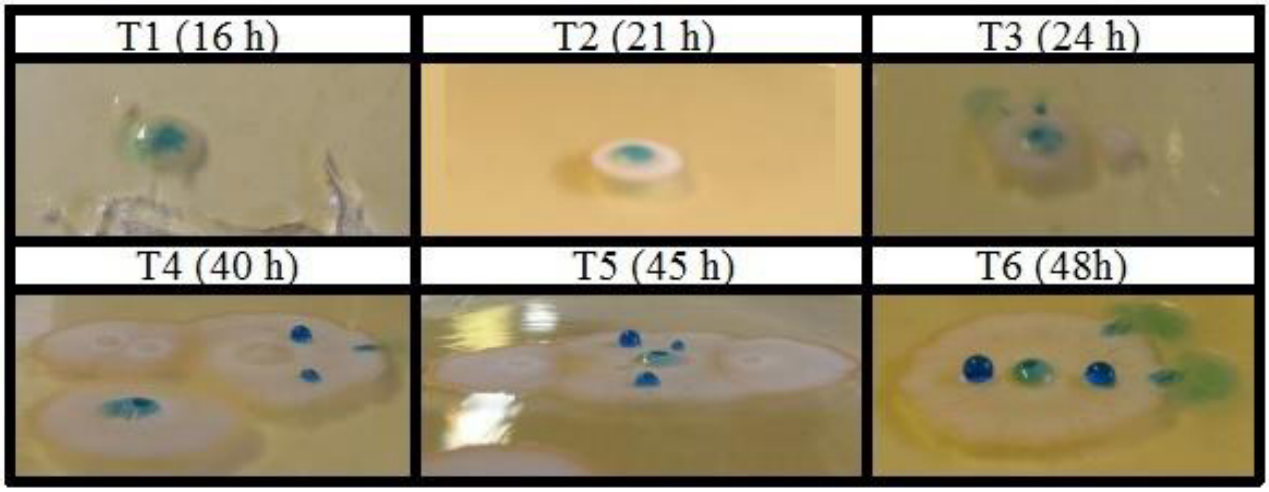


*Figure 17: Blue drops deposits on Bacillus amyloliquefaciens GA1 colonies at differentcultivation times.*

This method allows making some observations but also confirms the hypothesis of an increase of the hydrophobicityfor *Bacillus amyloliquefaciens GA1* in the same way than *Bacillus subtilis*strains(Kobayashi & Iwano, 2012).

The hydrophobicity of *B. subtilis* strains is due to a production of BslA. The bslA gene is also found in the genome of *Bacillus amyloliquefaciens FZB42*(but called yuaB)as mentioned in the state of the art. The hypothesis of the production of BslA and the increase of the surface hydrophobicity for the *Bacillus amyloliquefaciens GA 1* strain can be confirmed by the blue drops deposits experiment. Indeed, the hydrophobicity of colonies of *Bacillus amyloliquefaciensGA1* increases greatly between 24 and 40 hours. It's only after 40 hours that real drops are formed, before 24 hours of cultivation, the drops spread rapidly.Then, the contact angles are more important after 40, 45 and 48 hours than the ones after 16, 21 and 24 hours resulting from an increase of the hydrophobicity. The strain produces hydrophobic BslA along its culture and particularly after 24 hours of cultivation.

Another important observation is the surface heterogeneity of the colonies; the drops are different at different places of the same colony. Examples of colonies after 45 and 48 hours are the better to show the heterogeneity. The drops on the centrum of the colonies are more spread than the ones on the peripheral part (and on the white rings visible in the colonies).However, in the most peripheral part (at the edges of the colonies), the drops spread very quickly (and end in the agar LB medium as seen in the image corresponding to 48 hours of cultivation in the Figure17). This heterogeneity will be also detected with the Tracker method to invest the surface hydrophobicity and it will be discussed after the results of the Tracker method.

The method hassome other defects. First, it is not a quantitative method but a qualitative one, the differences of surface hydrophobicities can just be observed and not measured. Secondly, the method is adapted to Petri dish cultivation and not with the Drip Flow reactor coupons because the blue drop can penetrate the coupon and alter the next biofilm formation on the coupon. Moreover, the observations would be more difficult on coupons due to the silicon edges and the aspect of the biofilm surface (Figure 16). All these reasons lead to the use of another method, the Tracker method which allows measuringprecisely the contact angles between a drop and the surface and then quantify the surface hydrophobicity. The drops can even be directly deposed on the biofilm which has grown on the silicon coupon which is useful for culture with Drip Flow reactor.

An experiment to quantify the evolution of this surface hydrophobicity was done on a 44 hours cultivation of *Bacillus amyloliquefaciens GA1* strain. Samplings were done after 0

hours (directly after the batch step), 16 hours, 20hours, 24 hours, 40 hours and 44 hours. The results were the followings (Figure 18).

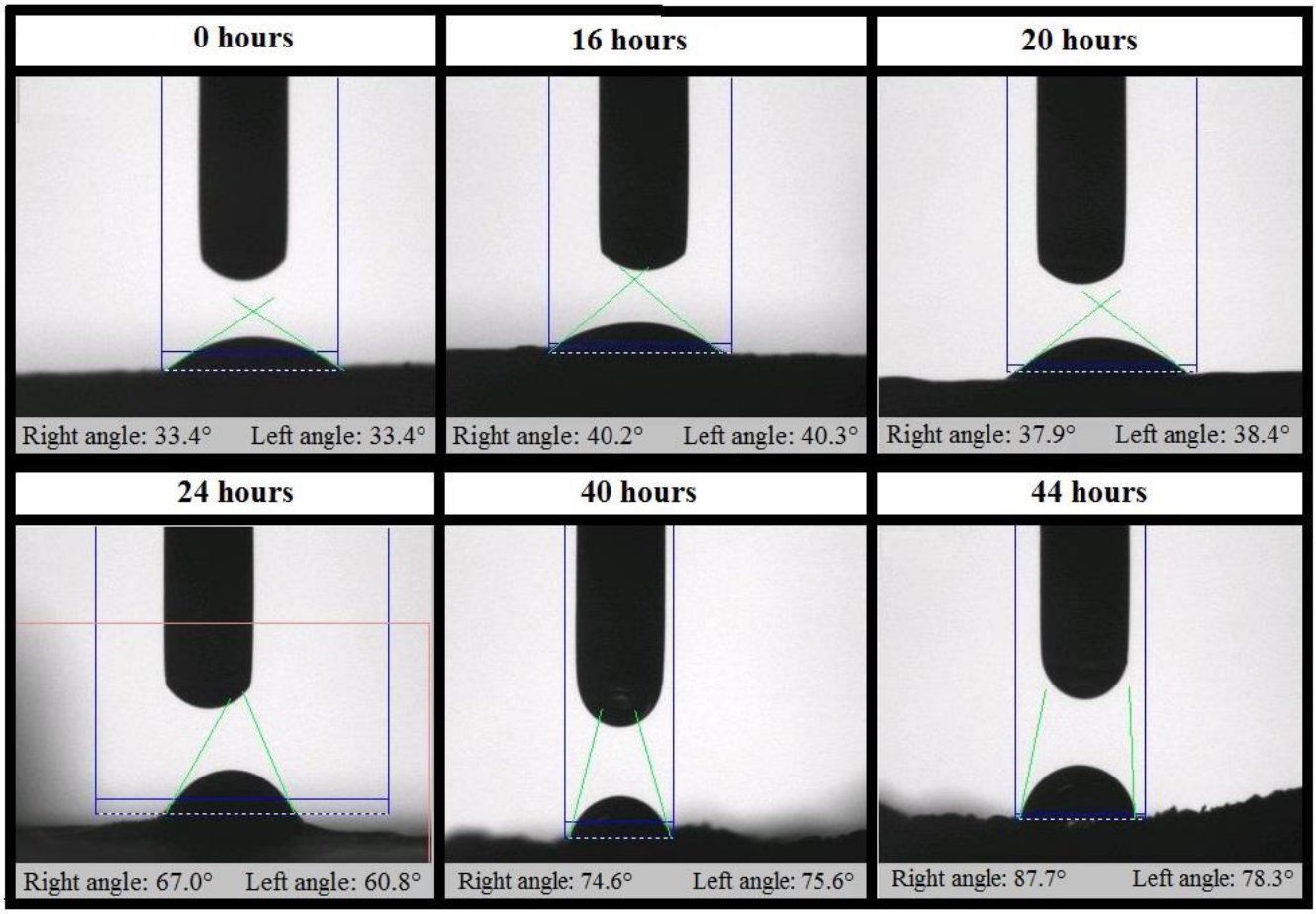


*Figure 18: Evolution of the surface hydrophobicity (between 0 and 44 hours of continous cutivation) measured by the Tracker method.*

An increase of the surface hydrophobicity is clearly observable and the results are close from the results obtained with the blue drops, the surface hydrophobicity increases greatly between 20 hours and 24 hours of continuous cultivation. It can be compared to the blue drops deposits method if the time of the batch cultivation step (6 hours) is added. Then the increase of hydrophobicity is between 26 and 30 hours of cultivation. It can be associated with the observations of the blue drops methods which lead to the conclusion of an important hydrophobicity increase between 24 and 40 hours (due to a probable production of BslA). But with this new method, the evolution is quantitative due to the measures of the contact angles. Then the surface hydrophobicity evolution is the following for *Bacillus amyloliquefaciens GA1* strain:

- Between 0 and 20 hours, there is a slight increase of the surface hydrophobicity (from approximatively 33° to approximatively 38°). The result for the time of 16 hours seems abnormal, it's more important that after 20 hours. However it can be due to the surface heterogeneity. This point will be developed below.
- Between 20 and 24 hours, the increase of the contact angles are more important as mentioned above (from 37.9°to 67.0°).
- Between 24 and 44 hours, the increase stays marked but less than between 20 and 24 hours (from 60.8° to 87.7°).

However, due to the heterogeneity (because of wrinkles) of the surface, the measurement of the angles can be complicated. The presence of disparities (like mounds and wrinkles) can alter the form of the drops or hide the drop from the CCD camera. As a result, in function of the place of the drop deposits on the biofilm, the obtained angles can be different.For example, some measures done after 44 hours of continuous cultivation revealed contact angles of 90.7° to 103.3°. They can be due to the biofilm wrinkles. If not, the evolution of the surface hydophobicities between 24 hours and 44 hours can be considered as important as the evolution of the surface hydophobicities between 20 hours and 24 hours. However the production of BslA stays more important between 20 and 24 hours because the evolution of the hydrophobicity is fast (4 hours) in comparison with the evolution between 24 and 44 hours which is slow (20 hours).

Due to the heterogeneity of the biofilm surface, some places of the biofilm canalso rapidly absorb the drop. Then, no measure is possible.It was observed for the biofilm sample cultivated during 24 hours (of continuous cultivation). The Figure 19 (on the next page) represents this phenomen. The fraction of time between the images 45 and 46 from this figure is less than one second proving the rapid absorption of the drop. This problem is probably caused by some less mature biofilm parts or by parts needing more medium and become hydrophilic to absorb the liquid medium more easily. More investigations will be needed about the biofilm heterogeneity and its link with the different cells phenotypes present in the biofilm.

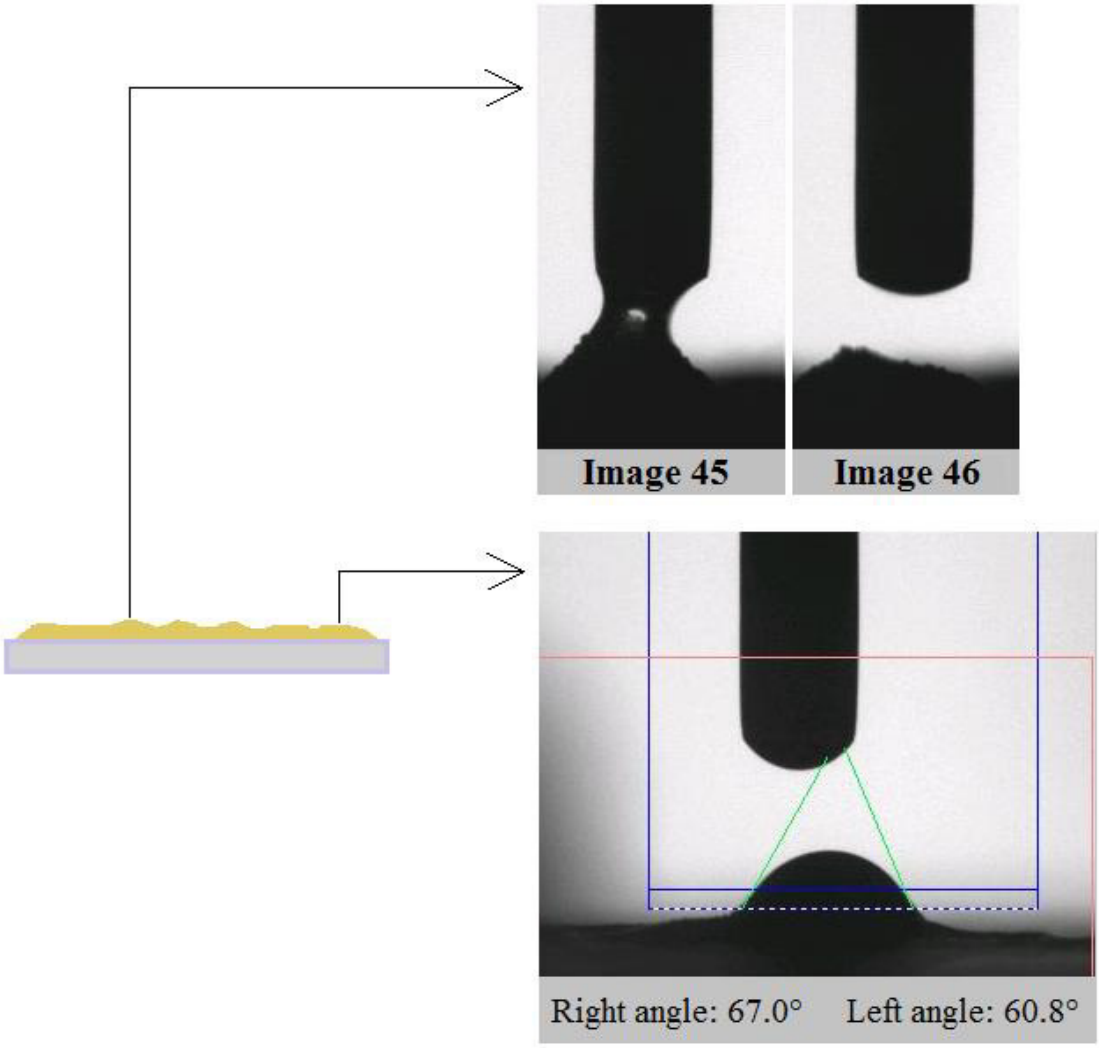


*Figure 19: Example of the biofilm surface heterogeneity leading to rapid drop absorption after 24 hours of continuous cultivation.*

Excepted these problems of heterogeneity, the measurement of the contact angles reveals itself as a good method to study the biofilm formation dynamics (as the measurement of the cellular concentration by optical density explained in the second part of the Results). Indeed, the small increase of the hydrophobicity between 0 and 20 hours can represent the second stage of the biofilm formation, the biofilm microcolonies development (Figure 2 of the State of the art). After 20 hours, the bigger increase of the hydrophobicity can represent the third phase, the biofilm maturation in macrocolonies. Then the first stage (or the surface attachment) is obtained with the batch culture step.

To verify the production of BslA (or YuaB), the RNA expression of the gene yuaB was done by qRT-PCR.

# 4. Transcriptomic analyses of biofilm maturation in Drip Flow conditions

Selected genes for these analyses were the tasA, degU and yuaB genes. The first one, tasA, is a gene related with the production of an amyloid protein associated to the structural integrity of the biofilm matrix(Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015).The degU gene is a key gene of the matrix production and cell differentiation inside the biofilm with the ComA and Spo0A genes(Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015). The last selected gene, yuaB (or bslA currently) is a gene associated with the production of the BslA protein responsible for the hydrophobic layer on *Bacillus subtilis* biofilm (Kobayashi & Iwano, 2012). These three genes were selected to study the evolution of the matrix (for tasA and yuaB) and the cell differentiation (with the regulation of the matrix production (for degU) and to notice the expression changes in the study of the bacterial interactions with the Drip Flow reactor. They are all associated to *Bacillus subtilis* in studies but they are also present in the genome of *Bacillus amyloliquefaciens FZB42* close to the GA1 strain (Chen et al., 2007).
The selected primers for the three genes are the following:

- tasA forward primer = GAACCCGACAGCACGGATTT
  tasA reverse primer = TCGTCAAATGCCGGGAACAG

- degU forward primer = ATGTCTCGGTAGCGCATCAG
  degU reverse primer = TTGCGCTGAATCACGGTTTG

- yuaB forward primer = GACCGCTTTCCGTATCTTCA
  yuaB reverse primer = GTGAAAAAGATTGCCCTGGA

An analysis of the RNA expression of the 3 kinds of cells (biofilm agglomerated and adhered, planktonic and non-adherent sessile cells) was done with a Drip Flow reactor culture of 40 hours (OD of the inoculation was 0.941 A). The evolution of the optical densities of the three fractions is in the Figure 20 and the results of qRT-PCR for each cell fraction are represented by the Figure 21.

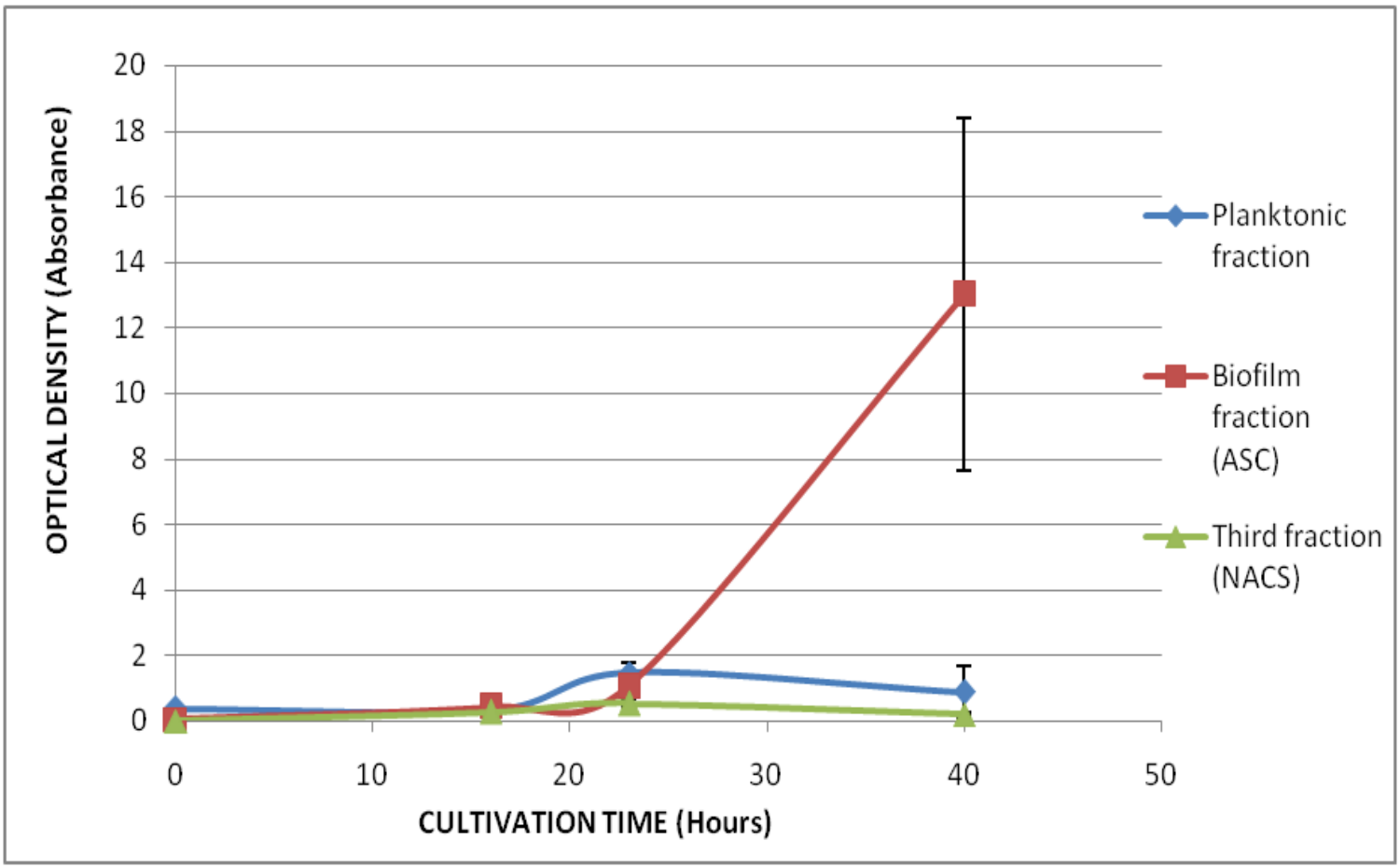


*Figure 20: Optical densities evolution in function of time for the cultivation dedicated to the qRT-PCR analysis.*

It has to be noted that the analyses of the third replicate of the 40 hours cultivation time samples wasdone 1 month after the analyses of the other replicates and with a different ramp speed (the fast one). It could explain the differences of expression in comparison with the two other replicates of this cultivation time.

No trend can be highlighted for the non-adherent sessile cells (third fraction). In the opposite, the evolutions of the three genes for the adhered and agglomerated biofilm cells are important after 40 hours of cultivation (Figure 21). Although the big variabilities between the replicates, the trend clearly proves an increasement of the expression of thesethree genesespecially the yuaB and the degU gene.

The important increase of the yuaB expressionconfirms the contact angle measurment, the surface hydrophobicity is much more important in mature biofilm due to the BslA production which leads to an hydrophobic layer, the same comportment than *Bacillus subtilis* biofilm. For the degU gene, the evolution seems logical. The cells differentiate into subpopulations with the same genome but with different phenotypes. More the biofilm is developped and important, more the number of subpopulations (and different phenotypes) are necessary to the viability of the biofilm. It can be compared to the different tissues and organs of an organism

(complex lifestyle).Moreover, the degU expression increases parallel to yuaB which is logical due to its role of regulation of the yuaB expression. It could be interesting to lead a new experiment to a bigger time of cultivation to notice the evolution of the degU expression.

Concerning the planktonic cells, the result of the yuaB expression for the second replicate of the 0 hour of continous cultivation time samples looks abnormal in comparison with the other results (Figure 21).Without this abnormal result, the trend would show an increase of the yuaB expression as the evolution of the biofilm fraction (ASC) yuaB expression.

Another interesting information is the evolution of the degU and tasA expressions, the degU expression is more important at the beginning of the continous step of cultivation than after 40 hours of cultivation. It seems but finally looks logical according to the reviews (Vlamakis et al., 2013a; Mielich-Süss & Lopez, 2015)which explain that the master regulators like degU are more expressed when the cells are sessile. Then at time 0, the planktonic phase may have been formed by sessile cells driven by the medium flow. These cells, which were dividing into subpopulations, expressed more the degU regulator.

These three figures provide interesting information but it could also be very interesting to compare the expression of each gene between the three kinds of cells. These comparisons are possible with the Figure 22 on the following page.

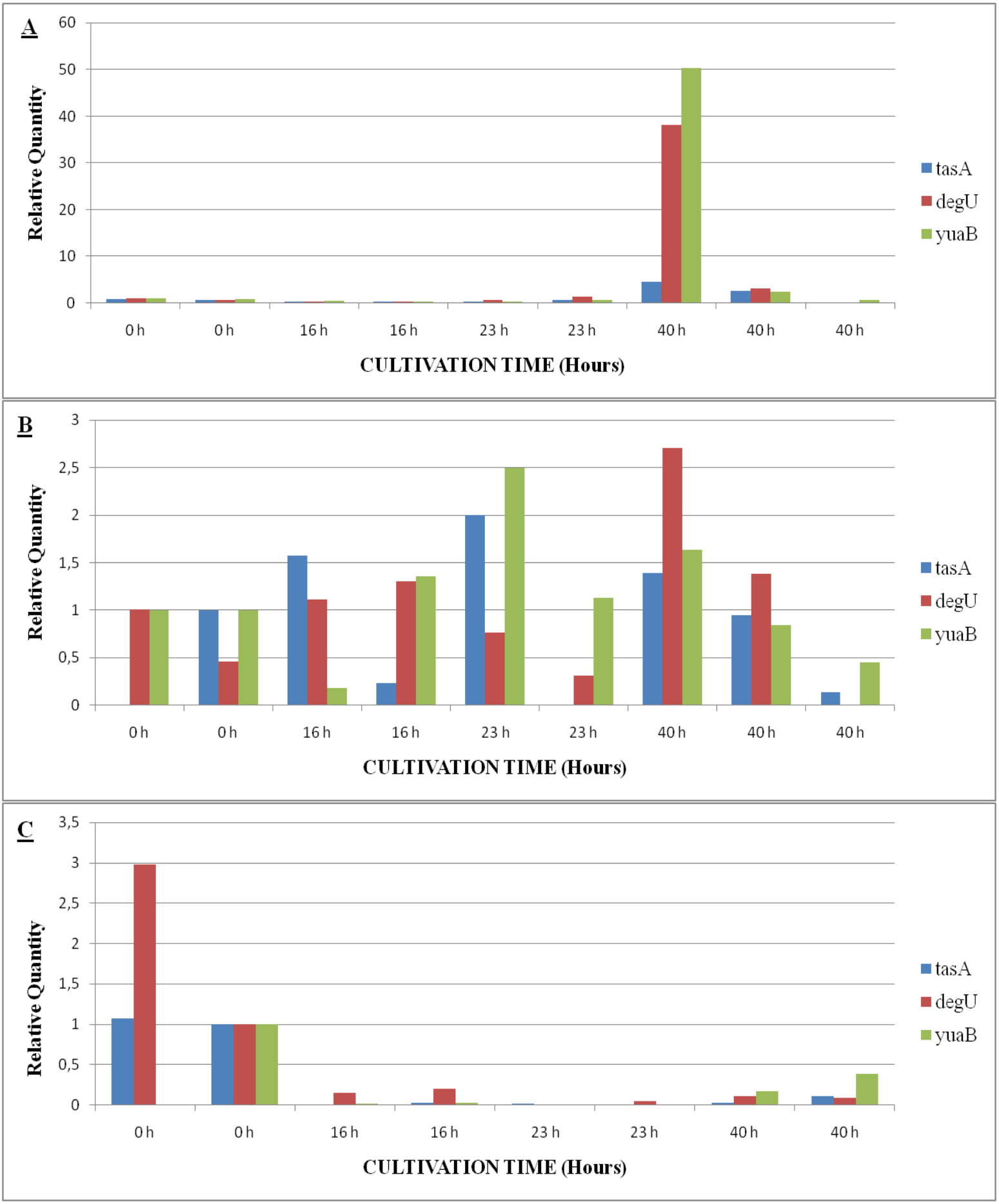


*Figure 21 : Quantification of the RNA expression of three genes from the different fractions of a biofilm* (A: Biofilm fraction (ASC), B: Third fraction (NASC), C: Planktonic fraction).

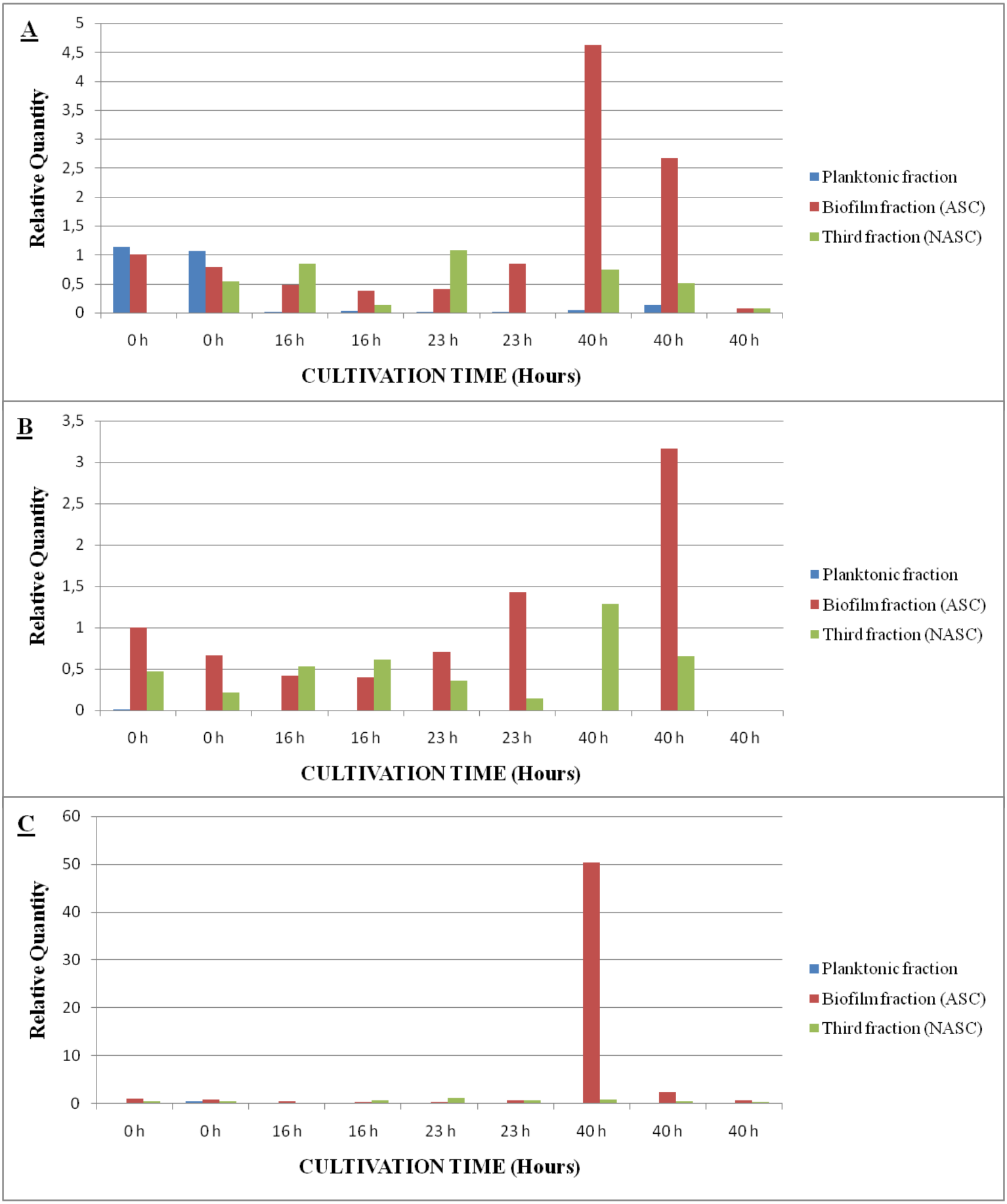


*Figure 22:Quantification of the RNA expression of three genes from the different fractions of a biofilm* (A: tasA, B: degU, C: yuaB)*with time 0 as reference for the relative quantification.*

For each gene, the RNA expression is more important for the adhered and agglomerated biofilm cells. It looks logical because the tasA and yuaB genes are essential for the biofilm matrix which is much more important and developped for this kind of cells. Concerning the degU, following the explainations about its higher expression for the sessile cells. It is normal that this kind of cells which are sessile, encapsuled into the matrix and sessile shows the better expression of this gene.

This quantification method and the obtained results are necessary to the study of the biofilm dynamics and the method will also be useful for the study of the interactions inside the biofilm (point 6 of the Results).

## 5. Global discussion about biofilm formation investigations in DFR

As seen in the 3 previous covered points, the biofilm formation can be investigated in the Drip Flow reactor by different methods such as measure of the optical density and surface hydrophobicity.

For the cultivation of *Bacillus amyoliquefaciens GA1*, the Figure Y shows the parallel between the evolution of the optical density (and then cells concentration) and the surface hydrophobicity. Both parameters evolutions allow the distinction between the microcolonies stages and the macrocolonies stages of the biofilm formation. For this strain, the switch happens after 20 hours of continuous cultivation. Indeed, the surface hydrophobicity and the cells concentration of the biofilm fractions both increase suddenly after this time

It proves the adequation of these two methods to study the biofilm formation. The other method presented in this master thesis (the qRT-PCR) can also be useful to link the result of the hydrophobicity with the expression of yuaB gene for instance. But no figure was added to show the parallel of these two kinds of results because of the difficulties to interpret the qRT-PCR results and the variabilities of these results.

However these differents methods were all used to study the interactions of bacterial species in biofilm cultivated with Drip Flow reactor. The results will allow noticing the efficiency of this reactor (and the developed method to use it) for such interactions studies.

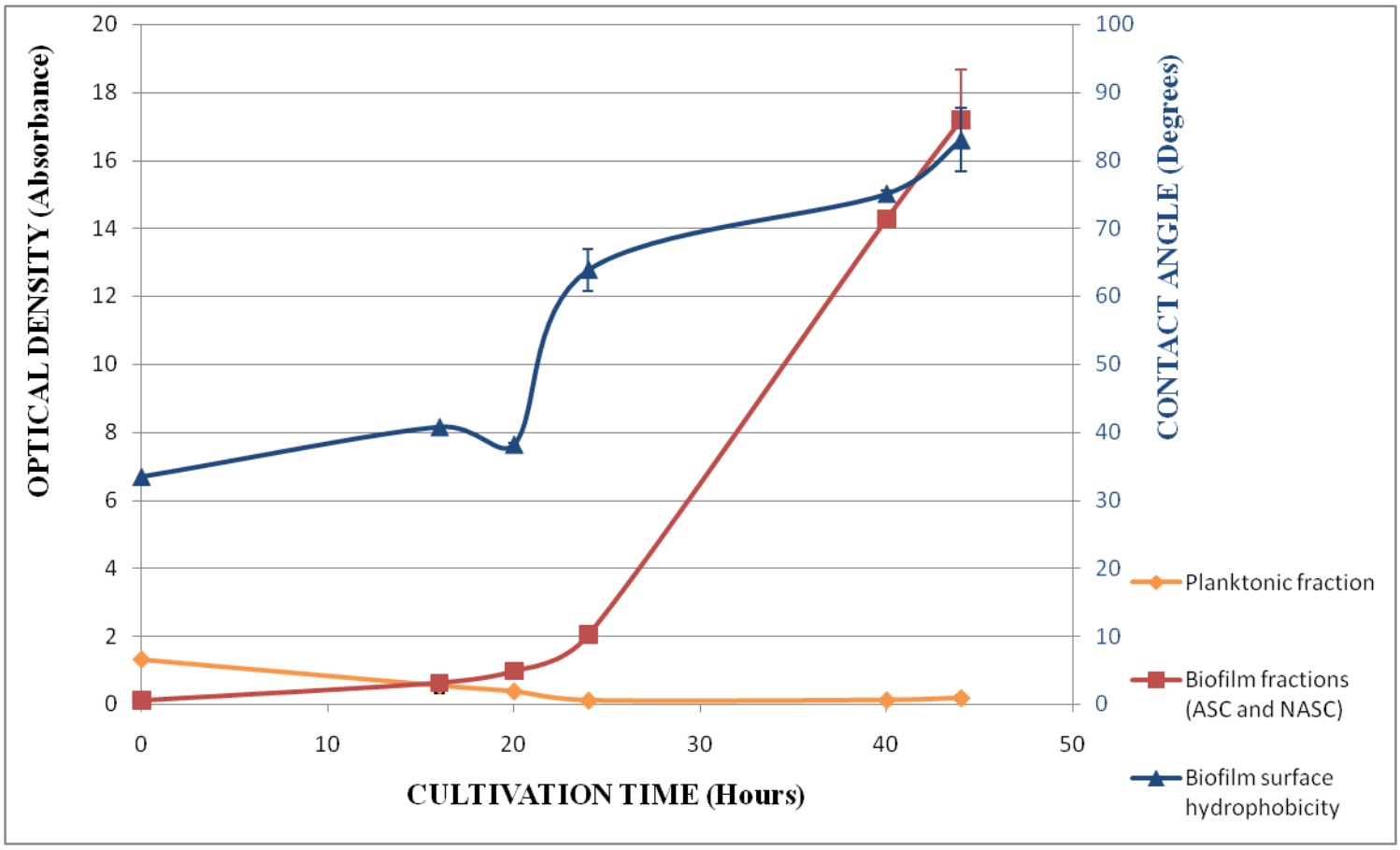


*Figure 23: Parallel between the evolution of the surface hydrophobicity and the optical density evolution.*

# 6. Adaptability of the Drip Flow reactor method to study the bacterial interactions in biofilms

The interaction between *Bacillus amyloliquefaciens GA1* and *Pseudomonas fluorescens 69*(or *A214*) was investigated with the Drip Flow reactor to notice the efficacity of this device.

The experiment was done by adding supernatant of *Pseudomonas fluorescens 69*prepared after 24 hours of cultivation at 28°C and 150 RPM. The profile of the supernatant metabolites can be obtained by UPLC analysis (*ACQUITY UPLC H-Class,* the column is*ACQUITY UPLC BEH C18 Column*of 130Å, 1.7 µm, 2.1 mm X 50 mm). The obtained profile is the Appendices(Figure 12)but needs more investigation.

The supernatant was added in the liquid LB medium (1/25 of the volume) for three chambers. The others chambers were provided with normal LB medium. The cultivation was done during 40 hours to study the impacts of the supernatant on the biofilm development and the surface hydrophobicity.

The result was directly observable on the coupons (Figure 24).

*Figure 24: Difference of biofilms between chambers with Pseudomonas fluorescens 69 supernatant (the three on the right) and without supernatant (the three on the left).*

The biofilms aspects were very different between the biofilms provided with and without supernatant. Without supernatant, the biofilm growth seemed to be less important.

It's different from results obtained with another method of cultivation such as microtiter plates (Figure 25).

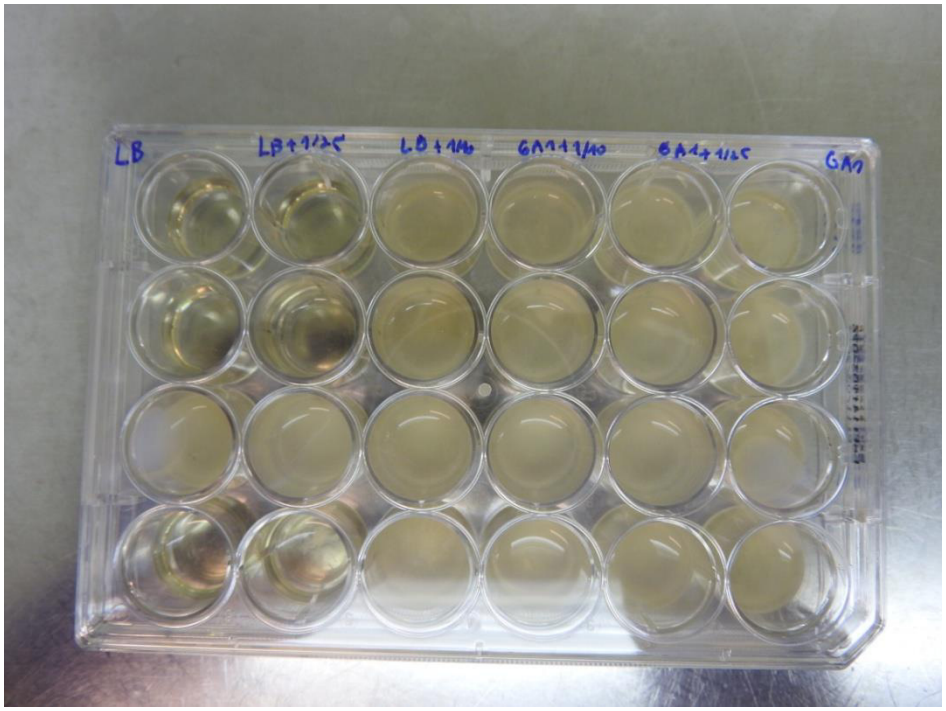


*Figure 25: Interactions experiment in microtiter plates (the three columns on the left represent LB medium with Pseudomonas supernatant and the three columns on the right represent the biofilm formation of Bacillus amyloliquefaciens with 1/10, 1/25 and without supernatant of Pseudomonas fluorescens 69 (or A214).*

Indeed, simply by observations, no trend can be highlighted with the Figure 25. Moreover, the planktonicand the biofilms fractions can't be sampled separately with this method of biofilm cultivation.

The optical densities from the Figure 26 tend to confirm the results highlighted in the Figure 24.

According to the means and standard deviations, the optical densities between biofilms cultivated with and without supernatant are significantly different. The biofilms grow better without supernatant of *Pseudomonas fluorescens 69.* It is possible that a metabolite of this supernatant altered the growth of the *Bacillus amyloliquefaciens GA1*.

The results for the planktonic phase are different as seen in the Figure27.

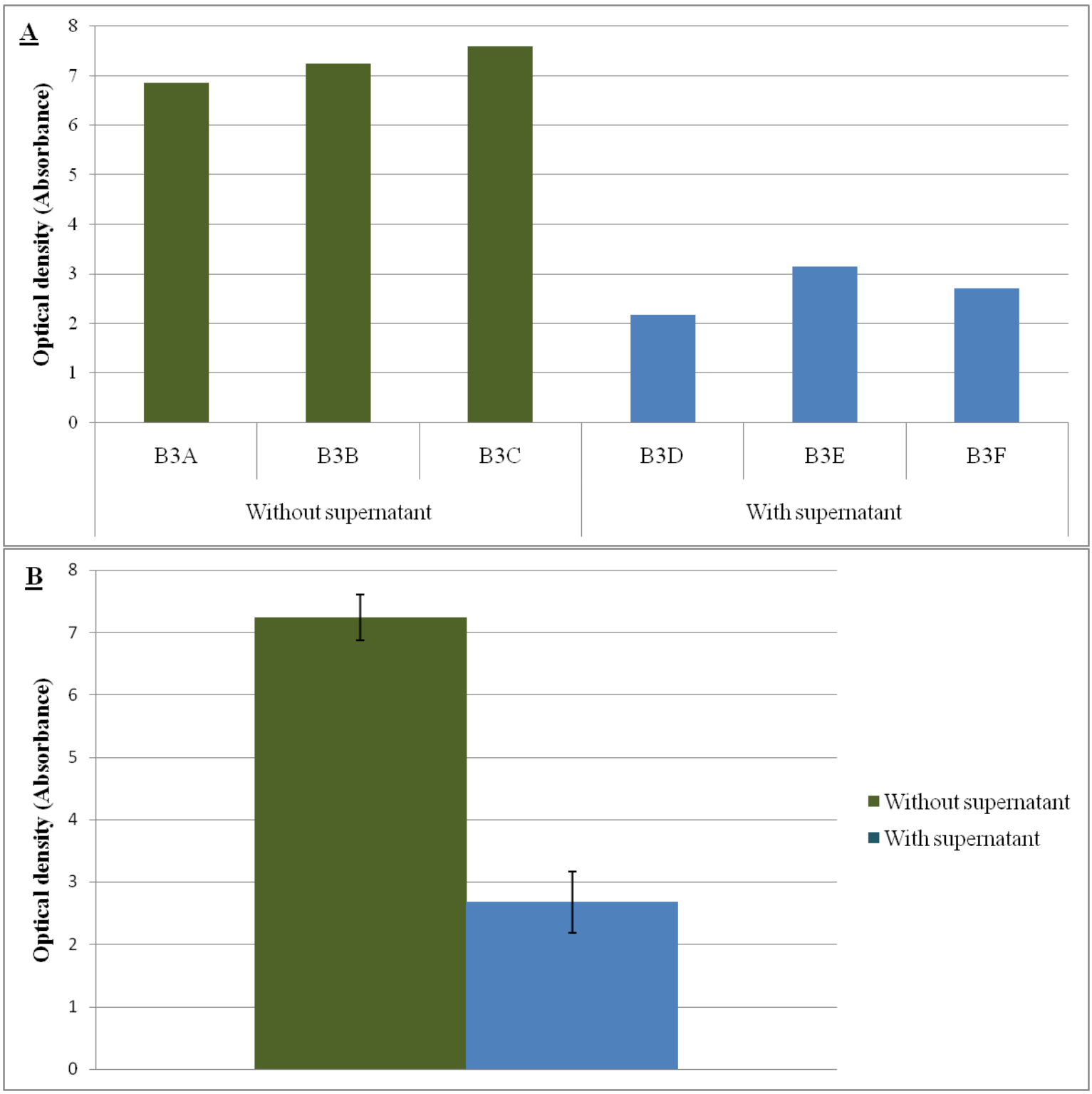


*Figure26:A: Difference of optical density between the biofilm fractions samples provided with and without supernatant of Pseudomonas fluorescens 69, B: Means and standard deviations of the optical densities of the biofilm fractions samples.*

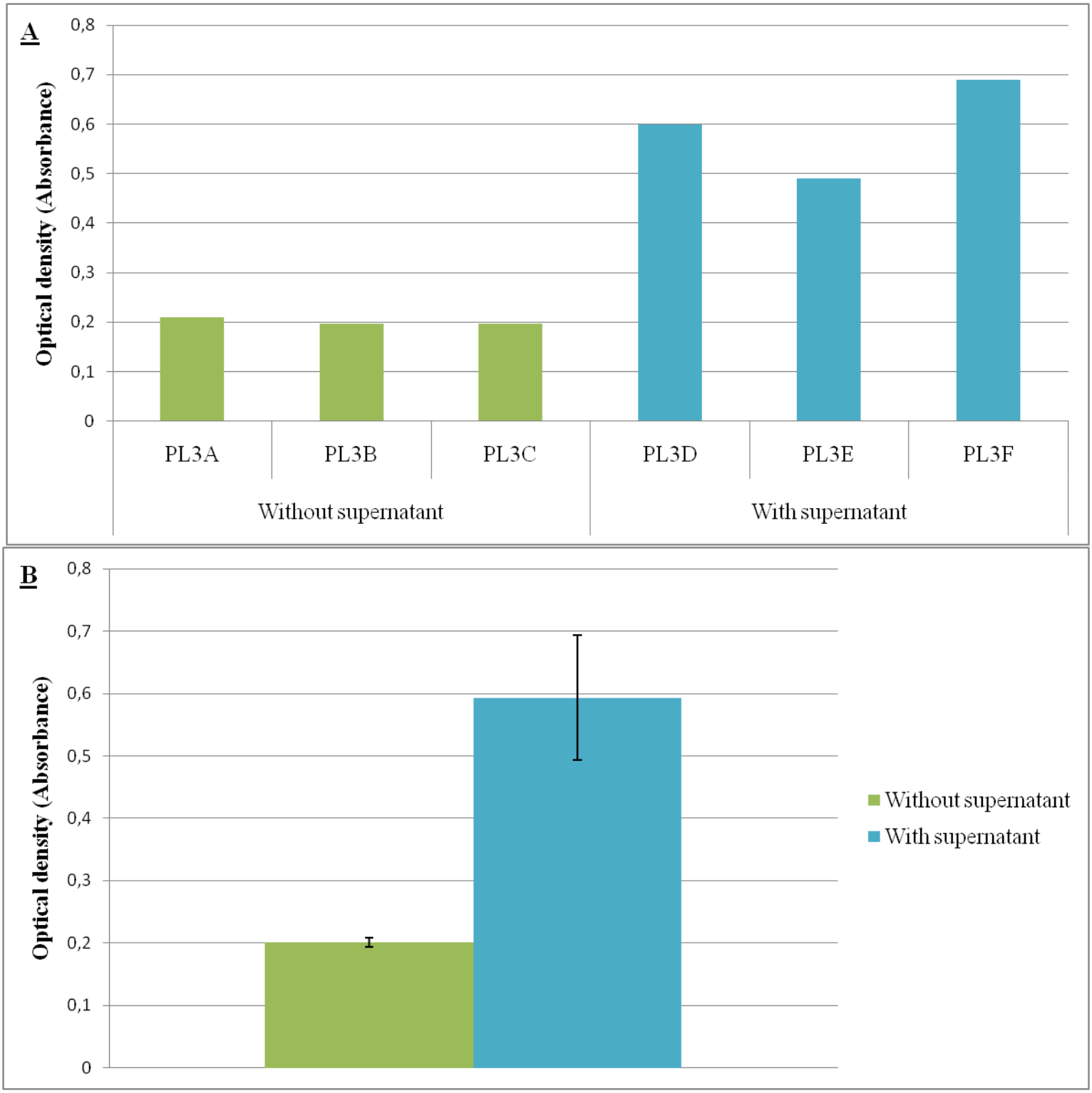


*Figure 27: A: Difference of optical density between the planktonic fraction samples provided with and without supernatant of Pseudomonas fluorescens 69, B: Means and standard deviations of the optical densities of the planktonic fraction samples.*

These results show the opposite, the optical densities of the planktonic samples cultivated with supernatant are significantly higher than the ones without.

It can be explained with the Figure 23. At high cultivation time, more the surface of the biofilm is hydrophobic, less the optical density of the planktonic phase is important. At high

surface hydrophobicity, the cells are more isolated from the medium flow. Then the medium can't penetrate and drive the cells as efficiently as after lesser cultivation time. This phenomenom is the same with the comparison between the biofilms cutivated with and without supernatant. According, to the Figure 24, the biofilms cultivated with supernatant seem to have a lesser hydrophobic surface which leads to a more important planktonic phase than the ones cultivated without supernatant.

The measurement of the contact angles with the Tracker method was done to confirm this observation and this hypothesis. The results are in the Figure 28.

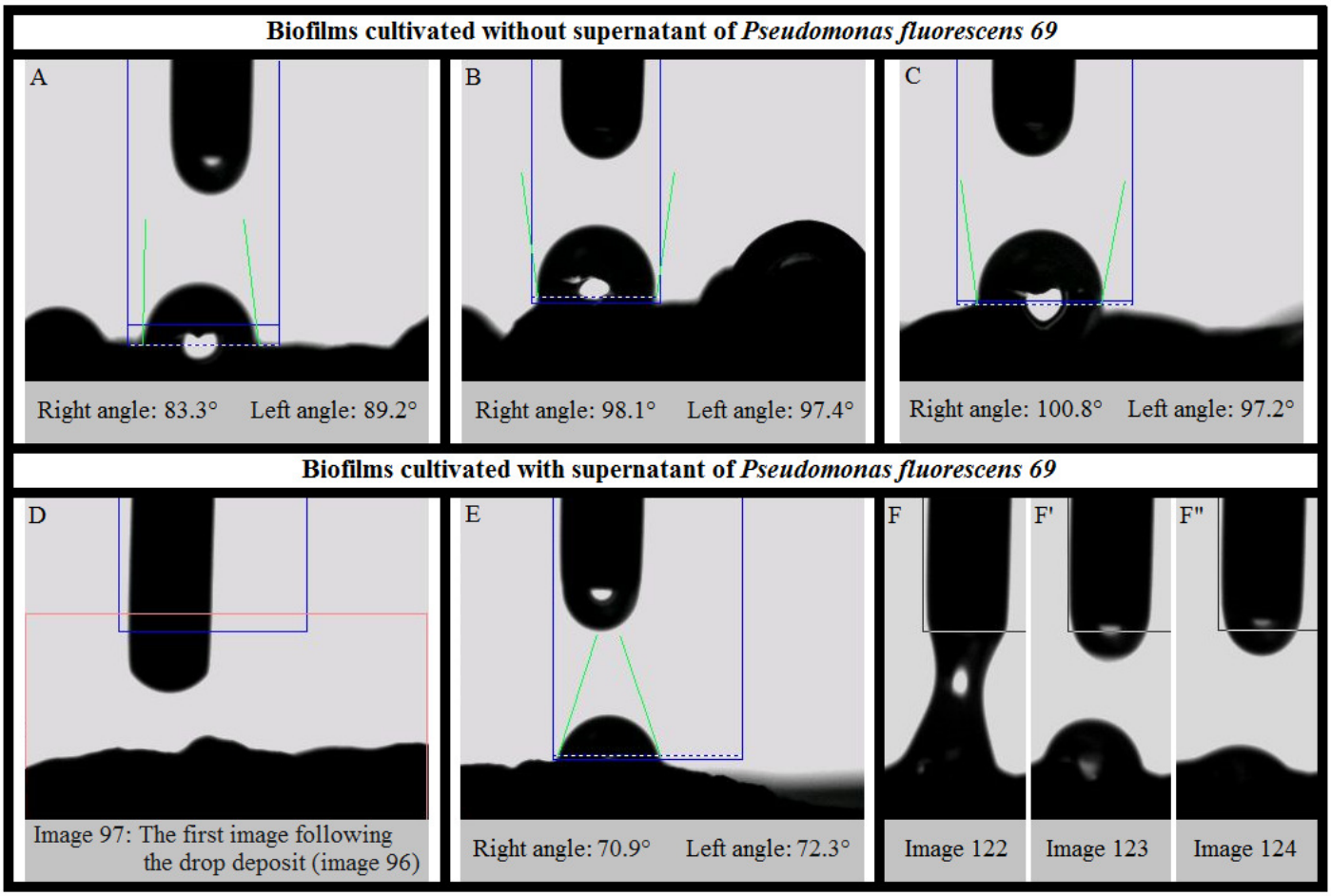


*Figure 28: Comparison of the surface hydrophobicity between biofilms cultivated with and without supernatant of Pseudomonas fluorescens 69.*

These results confirm the hypothesis. The contact angles (and the surface hydrophobicity) are higher for the biofilms cultivated without supernatant of *Pseudomonas fluorescens 69* (between 83.3° and 100.8°) than the ones cultivated with supernantant (where the drops are

directly absorbed by the biofilm for two replicates and the contact angle was only between 70.9° and 72.3° for the third replicate).

To explain this difference of hydrophobicity, a qRT-PCR analysis was done for the gene yuaB (but also tasA and degU).
The results of this analysis are in the Figure 29.

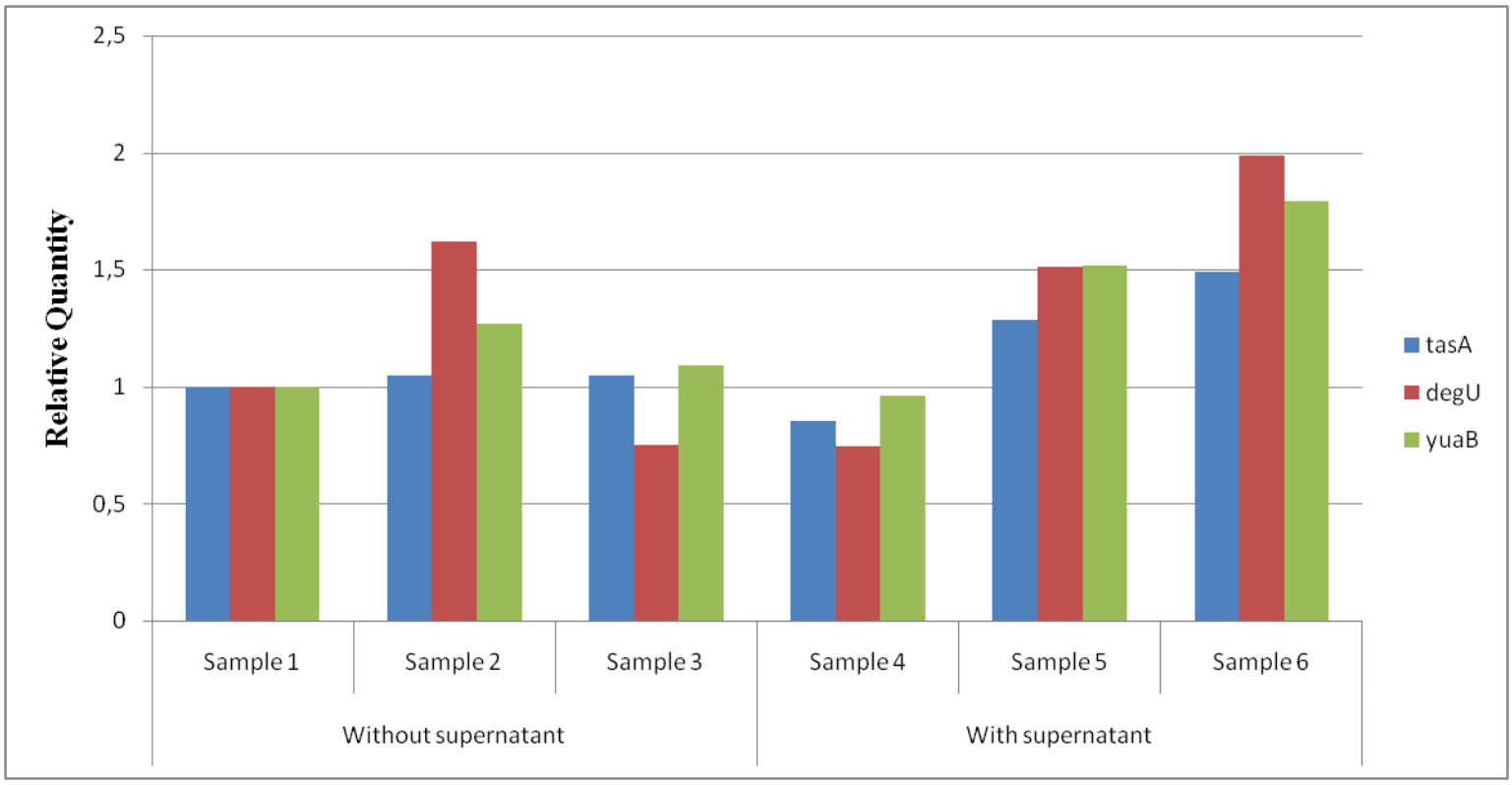


*Figure 29:Comparison of the tasA, degU and yuaB expression between biofilms cultivated with and without supernatantof Pseudomonas fluorescens 69.*

The first thing to notice is that results are not exactly the same for the replicates due to the complexity of living systems. However, in the opposite of the previous qRT-PCR (Figures21 and 22), the replicates results are closer.
Concerning the genes involved in the matrix integrity (tasA) and the hydrophobic protein production (yuaB), the expected result was a higher expression for the biofilm cultivated without supernatant due to the biofilms aspects and the measurement of the contact angles. However the results were the opposite. For two replicates (samples 5 and 6), the expression is more important. It could be explained by two hypotheses.
The first one is that the cells provided with supernatant had just begun to produce TasA and BslA proteins because they have firstly to adapt to their new environment. They were

thenproducing more matrix proteins than the cells cultivated without supernatant which have already a stable and hydrophobic matrix.
The second hypothesis is that the expressions are so relatively close that the differences can be considered as due to the living systems complexity.If the expressions are considered as the same, the cause of the aspect and the lower surface hydrophobicity is a metabolite present in the supernatant and not a change in the genetic expression.
Concerning the degU gene expression, it is also more important (two times more important for a replicate) for the cultivation with supernatant. It can be explained by the hypothesis that the biofilm cells have to differentiate into more subpopulations for the biofilm adaptation to an environment with *Pseudomonas fluorescens 69* supernatant. But it can slso confirms the fact that the production of matrix is beginning du to the DegU role as regulator of the matrix formation.

As a conclusion to all these results, it's probable that the surfactant of *Pseudomonas fluorescens 69* (or A214) possesses a metabolite harmful for *Bacillus amyloliquefaciens GA1*. Then, the two species formed microcolonies to prevent contact between this metabolite and the *Bacillus* strain. As a result to this disposition, a thinner biofilm was obtained (as proven by the optical densities and hydrophobicity results) (Tait & Sutherland, 2002).
The impact of this metabolite also influences a bit the gene expression of the matrix production, probably by shifting the time of EPS production.

If more studies are necessary about the interactions between *Bacillus amyloliquefaciens GA1* and *Pseudomonas fluorescens 69*(or A214) to understand the effects of the supernatant on the biofilm formation and to provide information about bacterial interactions inside a biofilm, the Drip Flow reactor shows itself as a very interesting device adapted to this kind of studies. Indeed, the samplings of the planktonic phase and the biofilm phase, the possibility to study the biofilm surface (hydrophobicity measurement) and the possibility to study the gene expressions in the different phases allowed by the Drip Flow reactor offer a lot of interesting applications.

# VI. Perspectives

A lot of perspectives follow this study. A promising continuity to this one would be the study of the metabolites of the *Pseudomonas fluorescens 69 (*or *A214)* supernatant which influence the biofilm formation and the growthof *Bacillus amyloliquefaciens GA1* strain. Some UPLC analyses of the different phases obtained with the Drip Flow devices and the comparison with the metabolites profile of the supernatant would be a good beginning.

Another interesting continuity would be the coculture of *Bacillus amyloliquefaciens GA1* and *Pseudomonasfluorescens 69* in Drip Flow reactor. It would be necessary to the direct study of the bacterial interactions. The study of the spatial organization of the two strains byFISH techniques following the works of M. Wenzheng Liu (Liu et al., 2016)would be integrated to the study.

Finally, the use of the Drip Flow reactor would be also interesting study the effect of multispecies communities on the production of interesting metabolites like the lipopeptides. These studies can lead to new industrial concepts of production using multispecies biofilm. The perspectives of the degradation of waste like plastics by biofilm could be also be investigated by the use of the Drip Flow device according to the works of M.Ongenae Adrien which can also have environmental and industrial consequences.

# VII. Conclusions

The Drip Flow reactor represents one of the best devices to make studies of the biofilms due to the sampling possibilities. However an important precaution has to be taken about the inoculation method and the cultivation condition as the temperature to increase the biofilm growth but decrease the variability between the reactor chambers as proven in this master thesis.

With this device and an attention to the parameters mentioned above, studies about the surface of the biofilm are currently possible. But the DFR offers also the possibilities to make classical analysis like measurement of the optical densities or gene expression. With this study for instance, the study of the evolution of three genes (tasA, degU and yuaB) is now possible.

The only disadvantage that highlights also this master thesis is the problem in links with the flow cytometry analysis. But it can be replaced by scanning method for the phenotypes observations. Another problem is the impossibility to control the $O_2$ and $CO_2$ exchanges with this device.

In conclusion, this study has clearly showed the advantages and the importance of the use of the Drip Fow reactor device to study the biofilm and the compatibility of the device with the future bacterial interactions studies. The Drip Flow reactor will also probably be one of the most used devices to study biofilms dynamics as shown in this master thesis with the discovery of a new important fraction of cells in the biofilm.

# VIII. Bibliography


Absolom D.R. et al., 1983. Surface thermodynamics of bacterial adhesion . Surface Thermodynamics of Bacterial Adhesion. *Applied and Environmental Microbiology*, **46**(1), 90–97. Available at: http://aem.asm.org/cgi/content/long/46/1/90.

An Y.H. & Friedman R.J., 1998. Concise review of mechanisms of bacterial adhesion to biomaterial surfaces. *Journal of biomedical materials research*, **43**(3), 338–348.

Aoki S.K. et al., 2005. Contact-dependent inhibition of growth in Escherichia coli. *Science (New York, N.Y.)*, **309**(5738), 1245–1248.

Azeredo J. et al., 2016b. Critical review on biofilm methods. *Critical Reviews in Microbiology*, **0**(0), 1–39. Available at: https://www.tandfonline.com/doi/full/10.1080/1040841X.2016.1208146.

Azeredo J. et al., 2016a. Critical review on biofilm methods. *Critical Reviews in Microbiology*, **7828**(January 2017), 1–39. Available at: https://www.tandfonline.com/doi/full/10.1080/1040841X.2016.1208146.

Banks M.K. & Bryers J.D., 1991. Bacterial species domiance within a binary culture biofilm. *Applied and Environmental Microbiology*, **57**(7), 1974–1979.

Bonds S.I. et al., 2007. Revealed in Gram-Positive. , **318**(December), 1625–1629.

Breugelmans P. et al., 2008. Architecture and spatial organization in a triple-species bacterial biofilm synergistically degrading the phenylurea herbicide linuron. *FEMS Microbiology Ecology*, **64**(2), 271–282.

Bryers J.D. & Ratner B.D., 2004. Bioinspired Implant Materials Befuddle Bacteria. *ASM News*, **70**(5), 232–237.

Busscher H.J. et al., 1984. Measurement of the Surface Free Energy of Bacterial Cell Surfaces and Its Relevance for Adhesion. , **48**(5), 980–983.

Caroff M. & Karibian D., 2003. Structure of bacterial lipopolysaccharides. *Carbohydrate Research*, **338**(23), 2431–2447.

Ceri H. et al., 1999. The Calgary Biofilm Device : New Technology for Rapid Determination

of Antibiotic Susceptibilities of Bacterial Biofilms The Calgary Biofilm Device : New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *Journal of Clinical Microbiology*, **37**(6), 1771.

Chavant P. et al., 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. *Journal of Microbiological Methods*, **68**(3), 605–612. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0167701206003381 [Accessed August 16, 2017].

Chen X.H. et al., 2007. Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium Bacillus amyloliquefaciens FZB42.

Christensen B.B. et al., 2002. Metabolic commensalism and competition in a two-species microbial consortium. *Applied and Environmental Microbiology*, **68**(5), 2495–2502.

Costerton J.W., 1999. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science*, **284**(5418), 1318–1322. Available at: http://www.sciencemag.org/cgi/doi/10.1126/science.284.5418.1318.

Davies D.G. et al., 1998. The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm. *Science*, **280**(5361), 295–298. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9535661%5Cnhttp://www.sciencemag.org/cgi/doi/10.1126/science.280.5361.295.

Djordjevic D., 2002. Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. *Applied and Environmental Microbiology*, **68**(6), 2950–2958. Available at: http://aem.asm.org/content/68/6/2950.short.

Donlan R.M. & Costerton J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin.Microbiol. Rev.*, **15**(2), 167–19.

Dufour D., Leung V. & Lévesque C.M., 2012. Bacterial biofilm: structure, function, and antimicrobial resistance. *Endodontic Topics*, **22**(1), 2–16. Available at: http://usyd.summon.serialssolutions.com/link/0/eLvHCXMwY2BQMDNKNjdONTdITjQ2NjdOM0xNTU2zSAV2DBItUwyS0oxRhrGRSnM3UQYbN9cQZw9dcDMxvgBy3kI8UjsV2H2IB1b6ZvHAessoHtSnMo-viHcNCQAyDMUYeBNBy8LzSsDbx1L4DnQG5qYecM-e7Dttque7q2cA85wtFw.

Flemming H.-C. et al., 2016. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, **14**(9), 563–575. Available at: http://www.nature.com/doifinder/10.1038/nrmicro.2016.94.

Flemming H.-C. & Wingender J., 2010. The biofilm matrix. *Nature Publishing Group*, **8**.

Flemming H.-C.C. & Wingender J., 2001. Relevance of microbial extracellular polymeric

substances (EPSs)--Part II: Technical aspects. *Water science and technology : a journal of the International Association on Water Pollution Research*, **43**(6), 9–16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11381977%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/11381954.

Ghigo J.M., 2001. Natural conjugative plasmids induce bacterial biofilm development. *Nature*, **412**(6845), 442–445.

Goeres D.M. et al., 2009. A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nature protocols*, **4**(5), 783–788.

H-C. Flemming., J W., 2001. Relevance of microbial extracellular polymeric substances (EPSs) - Part I:Structural and ecological aspects. , 1. Available at: http://www.iwaponline.com/wst/04306/wst043060001.htm.

Halan B., Buehler K. & Schmid A., 2012. Biofilms as living catalysts in continuous chemical syntheses. *Trends in Biotechnology*, **30**(9), 453–465. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0167779912000741 [Accessed August 14, 2017].

Hochbaum A.I. & Aizenberg J., 2010. Bacteria pattern spontaneously on periodic nanostructure arrays. *Nano Letters*, **10**(9), 3717–3721.

Høiby N., 2014. A personal history of research on microbial biofilms and biofilm infections. *Pathogens and Disease*.

Hori K. & Matsumoto S., 2010. Bacterial adhesion: From mechanism to control. *Biochemical Engineering Journal*.

Van Houdt R. et al., 2006. N-acyl-L-homoserine lactone signal interception by Escherichia coli. *FEMS Microbiology Letters*, **256**(1), 83–89.

Janakiraman V. et al., 2009. Modeling growth and quorum sensing in biofilms grown in microfluidic chambers. *Annals of Biomedical Engineering*, **37**(6), 1206–1216.

Jucker B.A. et al., 1997. Adsorption of bacterial surface polysaccharides on mineral oxides is mediated by hydrogen bonds. *Colloids and Surfaces B: Biointerfaces*, **9**(6), 331–343. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0927776597000386 [Accessed August 16, 2017].

Kobayashi K. & Iwano M., 2012. BslA(YuaB) forms a hydrophobic layer on the surface of Bacillus subtilis biofilms. *Molecular Microbiology*.

Lawrence J.R., Swerhone G.D.W. & Neu T.R., 2000. A simple rotating annular reactor for replicated biofilm studies. *Journal of Microbiological Methods*, **42**(3), 215–224. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0167701200001950 [Accessed

August 16, 2017].

Leriche V. & Carpentier B., 2000. Limitation of adhesion and growth of Listeria monocytogenes on stainless steel surfaces by Staphylococcus sciuri biofilms. *Journal of Applied Microbiology*, **88**(4), 594–605.

Leriche V., Chassaing D. & Carpentier B., 1999. Behaviour of L. monocytogenes in an artificially made biofilm of a nisin-producing strain of Lactococcus lactis. *International Journal of Food Microbiology*, **51**(2–3), 169–182.

Linton C.J., Sherriff A. & Millar M.R., 1999. Use of a modified robbins device to directly compare the adhesion of Staphylococcus epidermidis RP62A to surfaces. *Journal of Applied Microbiology*, **86**(2), 194–202.

Liu W. et al., 2016. Interspecific bacterial interactions are reflected in multispecies biofilm spatial organization. *Frontiers in Microbiology*.

Marshall K.C., Stout R. & Mitchell R., 1971. Mechanism of the Initial Events in the Sorption of Marine Bacteria to Surfaces. *Journal of General Microbiology*, **68**(3), 337–348.

Mattick J.S., 2002. Type IV pili and twitching motility. *Annual review of microbiology*, **56**(February 2002), 289–314. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12142488%5Cnhttp://www.annualreviews.org/doi/abs/10.1146/annurev.micro.56.012302.160938.

McCoy W.F. et al., 1981. Observations of fouling biofilm formation. *Canadian Journal of Microbiology*, **27**(9), 910–917. Available at: http://www.nrcresearchpress.com/doi/abs/10.1139/m81-143%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/7306879%5Cnhttp://www.nrcresearchpress.com/doi/abs/10.1139/m81-143.

Meena K.R. & Kanwar S.S., 2015. Lipopeptides as the antifungal and antibacterial agents: Applications in food safety and therapeutics. *BioMed Research International*, **2015**.

Mielich-Süss B. & Lopez D., 2015. Molecular mechanisms involved in Bacillus subtilis biofilm formation. *Environmental Microbiology*, **17**(3), 555-.

Moons P, Michiels C A.A., 2009. Bacterial interactions in biofilms. *Critical Reviews in Microbiology*, **35**(3), 157–168.

Müller P. et al., 2011. Potential of shock waves to remove calculus and biofilm. *Clinical Oral Investigations*, **15**(6), 959–965.

Nickel J.C. et al., 1985. Tobramycin resistance of cells of Pseudomonas aeruginosa growing as biofilm on urinary catheter material. *Antimicrob Agents Chemother*, **27**(4), 619–624. Available at: http://dx.doi.org/10.1128/AAC.27.4.619.

Núñez M.E. et al., 2005. Predation, death, and survival in a biofilm: Bdellovibrio investigated by atomic force microscopy. *Colloids and Surfaces B: Biointerfaces*, **42**(3–4), 263–271. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0927776505000974 [Accessed August 16, 2017].

van Oss C.J., Chaudhury M.K. & Good R.J., 1987. Monopolar surfaces. *Advances in Colloid and Interface Science*, **28**(C), 35–64.

Raaijmakers J.M. et al., 2010. Natural functions of lipopeptides from Bacillus and Pseudomonas: More than surfactants and antibiotics. *FEMS Microbiology Reviews*, **34**(6), 1037–1062.

Rao D., Webb J.S. & Kjelleberg S., 2005. Competitive interactions in mixed-species biofilms containing the marine bacterium Pseudoalteromonas tunicata. *Applied and Environmental Microbiology*, **71**(4), 1729–1736.

Renner L.D. & Weibel D.B., 2011. Physicochemical regulation of biofilm formation. *MRS bulletin / Materials Research Society*, **36**(5), 347–355.

Schramm A. et al., 1996. Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes . Structure and Function of a Nitrifying Biofilm as Determined by In Situ Hybridization and the Use of Microelectrodes. *Applied and Environmental Microbiology*, **62**(12), 4641–4647. Available at: http://dx.doi.org.ezproxy.lib.uh.edu/10.1016/S0273-1223(97)00333-8.

Scott J.R. & Zähner D., 2006. Pili with strong attachments: Gram-positive bacteria do it differently. *Molecular Microbiology*, **62**(2), 320–330.

Simões M., Simões L.C. & Vieira M.J., 2010a. A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*, **43**(4), 573–583. Available at: http://linkinghub.elsevier.com/retrieve/pii/S0023643809003430 [Accessed August 14, 2017].

Simões M., Simões L.C. & Vieira M.J., 2010b. A review of current and emergent biofilm control strategies. *LWT - Food Science and Technology*, **43**(4), 573–583.

Soto G.E. & Hultgren S.J., 1999. Bacterial Adhesins : Common Themes and Variations in Architecture and Assembly MINIREVIEW Bacterial Adhesins : Common Themes and Variations in Architecture and Assembly. , **181**(4), 1059–1071.

Tait K. & Sutherland I.W., 2002. Antagonistic interactions amongst bacteriocin-producing enteric bacteria in dual species biofilms. *Journal of Applied Microbiology*, **93**(2), 345–352.

Teodósio J.S. et al., 2011. Flow cell hydrodynamics and their effects on E. coli biofilm

formation under different nutrient conditions and turbulent flow. *Biofouling*, **27**(1), 1–11.

Truong V.K. et al., 2009. Effect of ultrafine-grained titanium surfaces on adhesion of bacteria. *Applied Microbiology and Biotechnology*, **83**(5), 925–937.

Ulman a, 1996. Formation and Structure of Self-Assembled Monolayers. *Chemical Reviews*, **96**(4), 1533–1554.

Upton M. et al., 2001. Intra- and interspecies signaling between Streptococcus salivarius and Streptococcus pyogenes mediated by SalA and SalA1 lantibiotic peptides. *Journal of Bacteriology*, **183**(13), 3931–3938.

Vlamakis H. et al., 2008. Control of cell fate by the formation of an architecturally complex bacterial community. *Genes and Development*, **22**(7), 945–953.

Vlamakis H. et al., 2013b. Sticking together: building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology*, **11**.

Vlamakis H. et al., 2013a. Sticking together: building a biofilm the Bacillus subtilis way. *Nature Reviews Microbiology*, **11**(3), 157–168. Available at: http://www.nature.com/doifinder/10.1038/nrmicro2960.

Wall D. & Kaiser D., 1999. Type IV pili and cell motility. *Mol Microbiol*, **32**(1), 1–10. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10216854.

Willcock L. et al., 2000. A new technique for the performance evaluation of clean-in-place disinfection of biofilms. *J. Ind. Microbiol. Biotechnol.*, **25**, 235–241.

Wolfaardt G.M. et al., 1994. Multicellular organization in a degradative biofilm community. *Applied and Environmental Microbiology*, **60**(2), 434–446.

# IX. Appendices

## 1. Protocol of the RNA extraction

- Resuspend the bacterial pellet in 100 µL of TE buffer (10 mM of Tris-HCl and 1 mM of EDTA, pH 8) containing 1 mg/mL of lysozyme by vortexing.
- Incubate at 37°C for 10 minutes.
- Add 350 µL of Buffer RA1 and 3.5 µL of β-mercaptoethanol and vortex the solution.
- Place a NucleoSpin® filter (the filters with violet rings) in a collection tube of 2 mL. Add the solution in the filters and centrifuge at 11000 x g for 1 minute.
- Transfer the lysate into an Eppendorf tube, add 350 µL of ethanol 70% and vortex the solution.
- Place a NucleoSpin® RNA column (with blue ring) in a collection tube of 2 mL. Load the lysate to the column. Centrifuge at 11000 x g for 30 seconds.
  *NB: The maximal volume of this step is 750 µL. Repeat the step if the maximal volume of lysate exceeds this capacity.*
- Place the column into a new collection tube. Add 350 µL of Membrane Desalting Buffer (MDB) to the column. Centrifuge at 11000 x g for 1 minute.
- Prepare a DNase reaction mixture by mixing 10 µL of reconstituted rDNase and 90 µL of reaction buffer for rDNase in a sterile 1.5 mL eppendorf tube.
- Add 95 µL of the Dnase reaction mixture in the column (on the center of the silica membrane). Incubate at room temperature for 15 minutes.
- Add 200 µL of the buffer RAW2 in the column. Centrifuge at 11000 x g for 30 seconds. Empty the collection tube and place back the column.
- Add 600 µL of the buffer RA3 in the column. Centrifuge at 11000 x g for 30 seconds. Empty the collection tube and place back the column.
- Add 250 µL of the RA3 in the column. Centrifuge at 11000 x g for 2 minutes to dry the membrane, repeat this step if the membrane is not dry enough. Place the column into a sterile nuclease-free collection tube of 1.5 mL.
  Add 60 µL of RNase-free water and centrifuge at 11000 x g for 1 minute to elute the RNA.
- Keep the RNA on ice (or at -20°C) till the next steps.

## 2. Choice of the primers

They were selectioned with NCBI Primer-Blast with another pair for each gene. Then the efficacy of each primer pair was tested (Figure 1) to finally selection the tasA, degU and yuaB genes with the best efficacies. The efficacies were obtained by realizing a qRT-PCR with the diluted (10, 100 and 1000 folds) genome of *Bacillus amyloliquefaciens GA1* extracted with a DNeasy Blood & Tissue Kit from Qiagen.

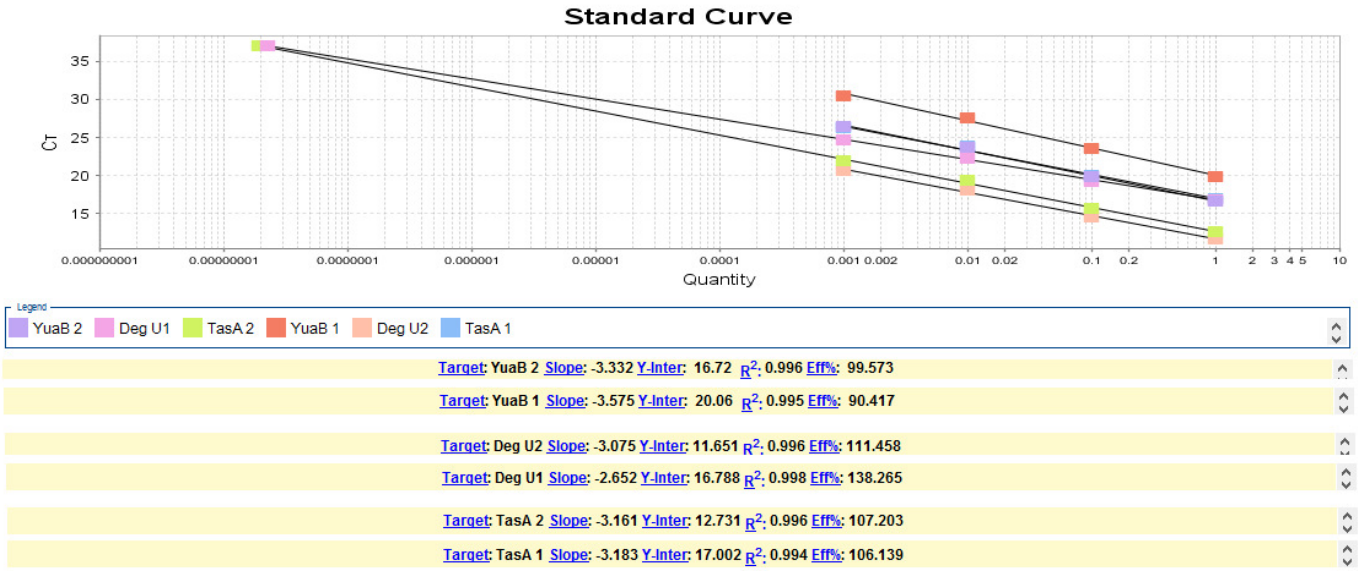


*Figure 1: Efficacies of the primers pairs*

# 3. Additional figures

*Figure 2: Reactor base*

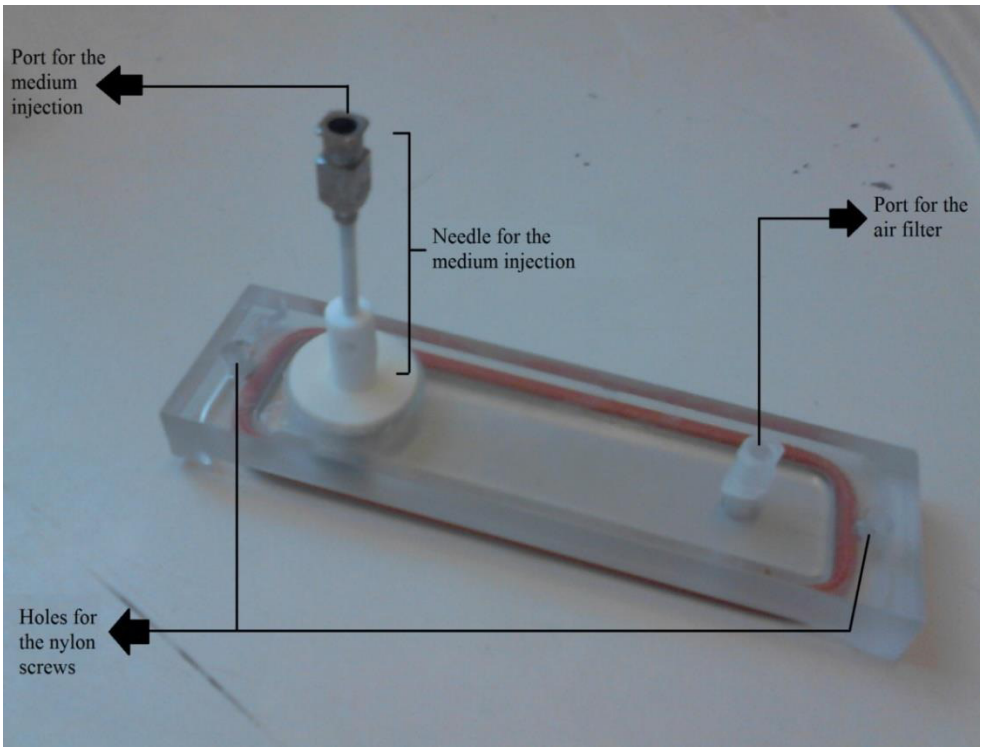


*Figure 3: Reactor covers*


*Figure 4: Watson Marlow 530 S peristaltic pump.*

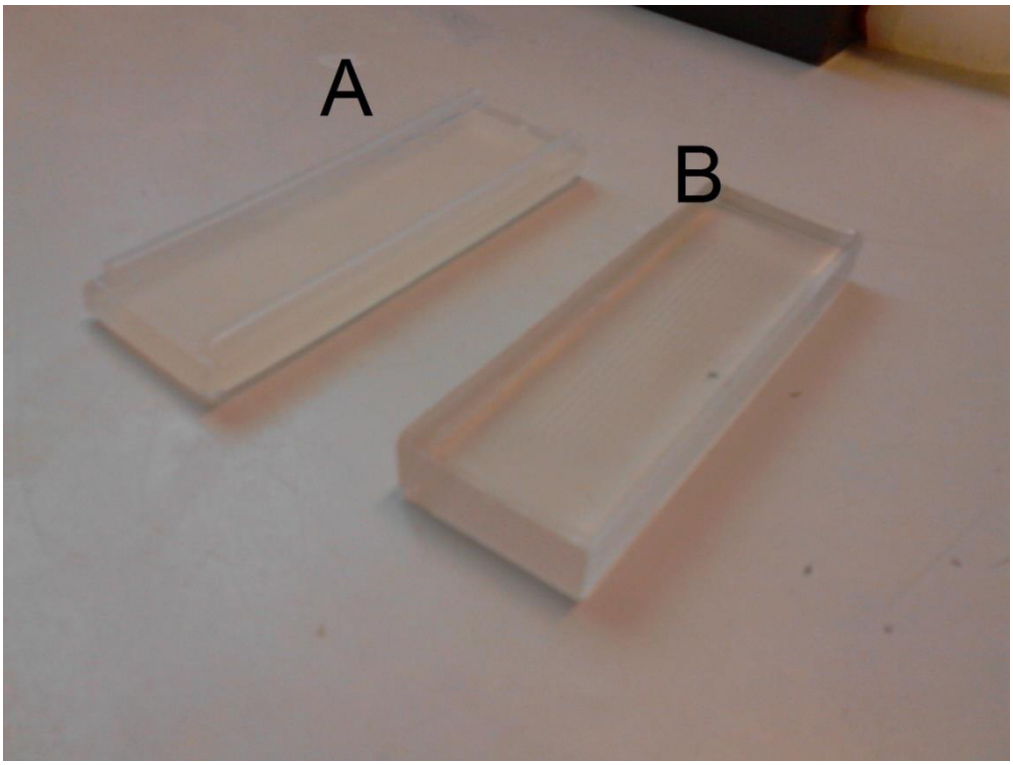


*Figure 5: Silicone coupons (A: With silicone edges, B: Striated)*

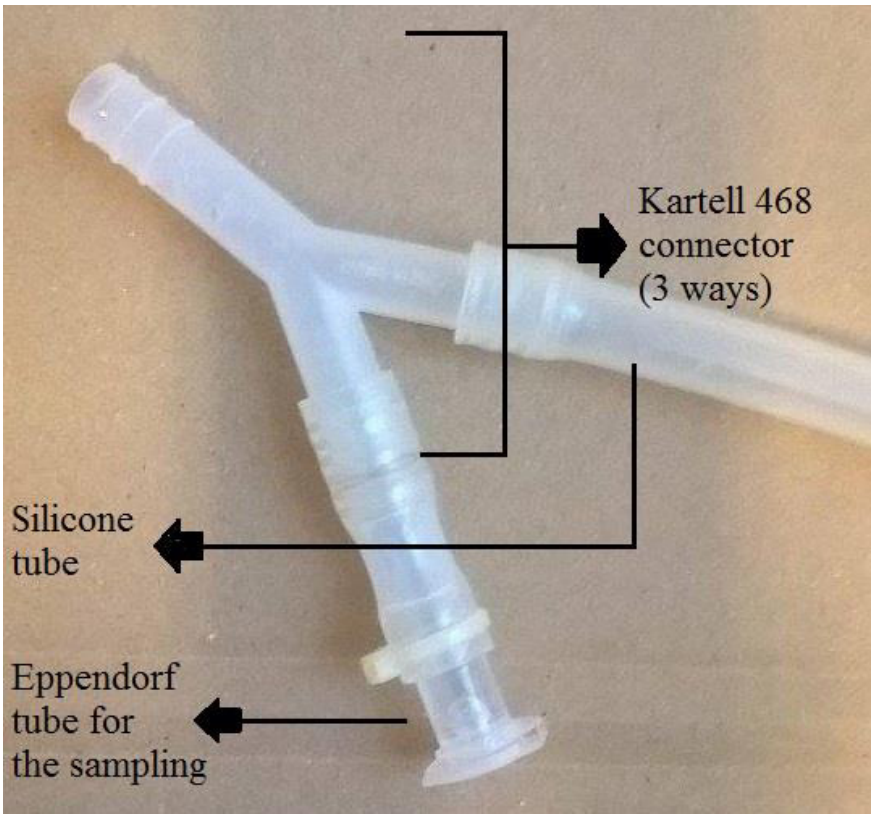


*Figure 6: Kartell 468 connector for the medium output and the sampling.*

*Figure 7: BPT Pharmed tubes to insert into the peristaltic pump.*

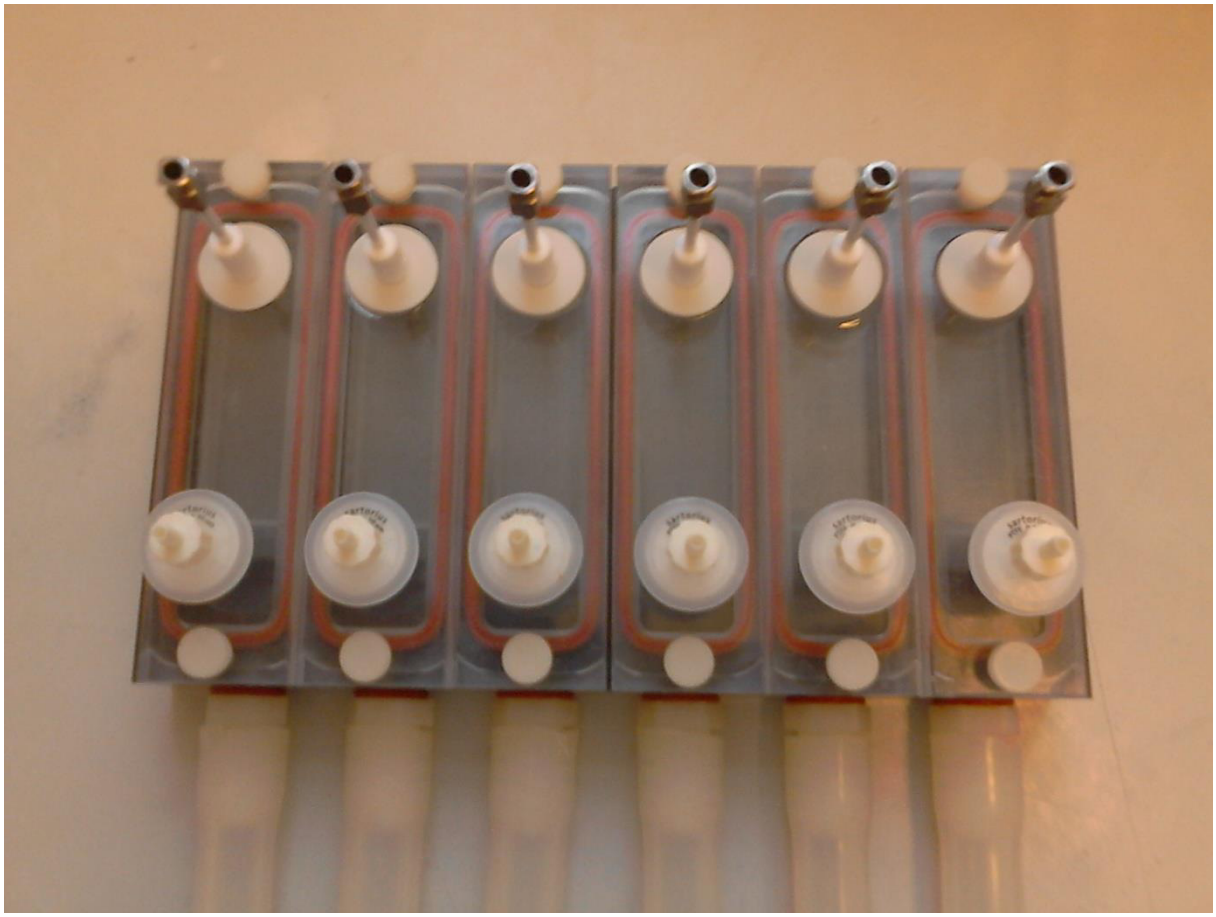

*Figure 8: Assembled Drip Flow reactor (without the tubes).*

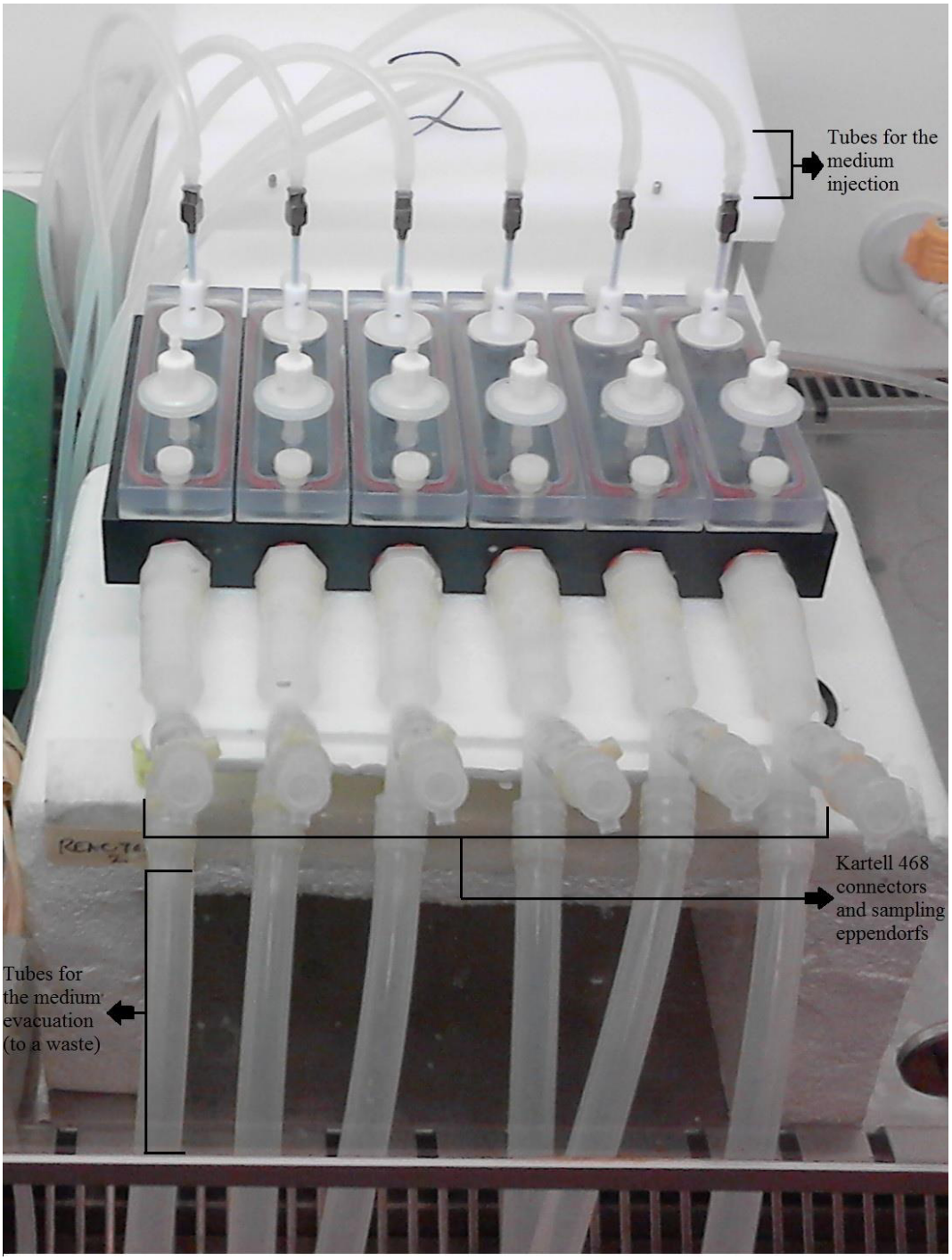


*Figure 9: Configuration of the different tubes and reactor on tilted surface.*

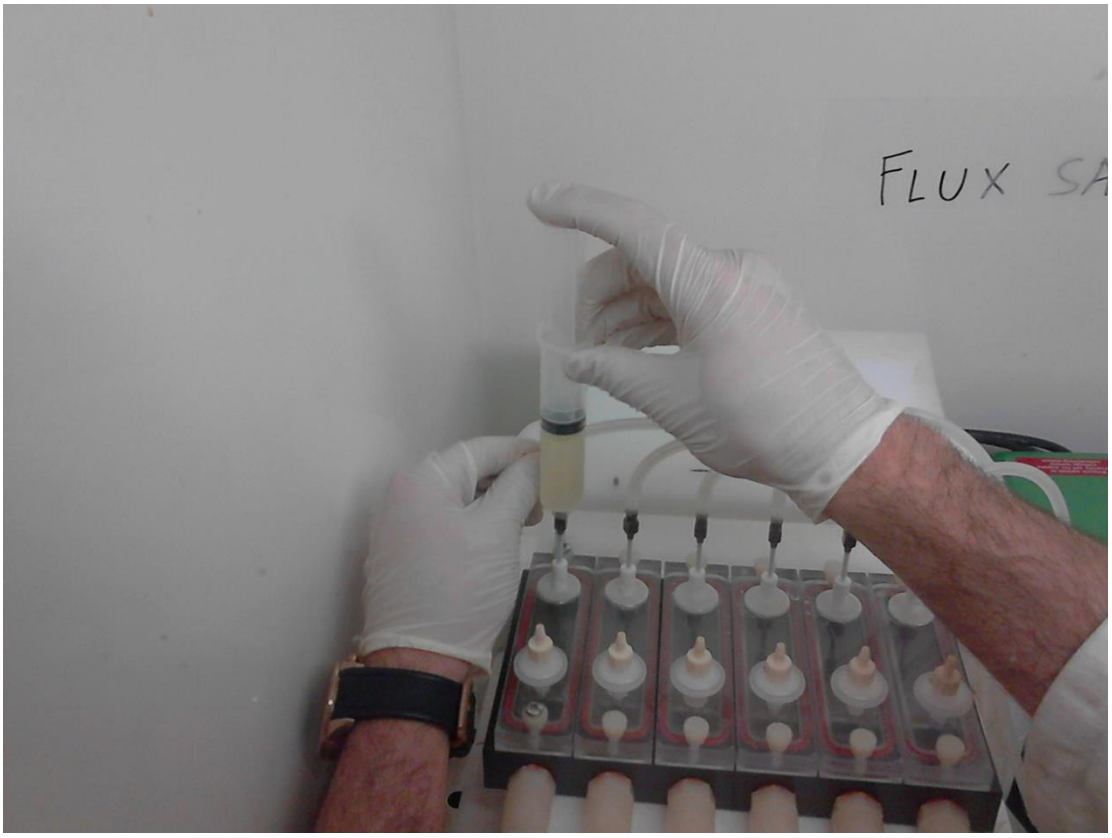


*Figure 10: Inoculation step.*

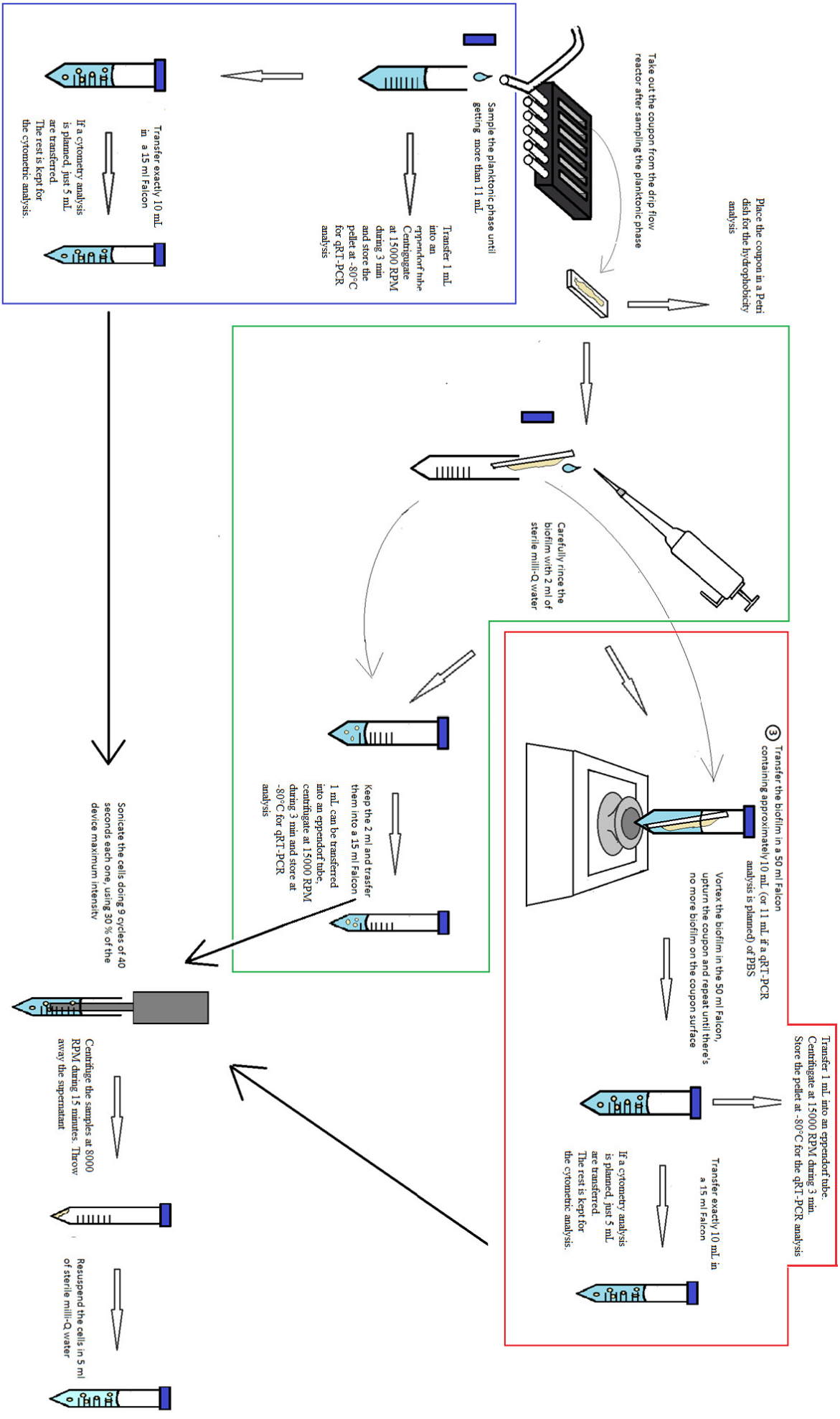


*Figure 11: Draft of the different samples preparation steps.*

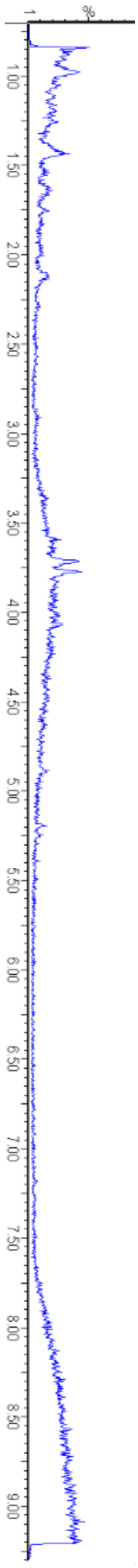


*Figure 11: Metabolites profile of the supernatant of Pseudomonas fluorescens 69 (or A214).*