Development of micro-calorimeter system to characterize thermal behavior of biofilm

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**NANYANG
TECHNOLOGICAL
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SINGAPORE

**DEVELOPMENT OF MICRO-CALORIMETER SYSTEM TO
CHARACTERIZE THERMAL BEHAVIOR OF BIOFILM**

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**SINGAPORE CENTRE FOR ENVIRONMENTAL LIFE SCIENCES
AND ENGINEERING (SCELSE)**

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CHARACTERIZE THERMAL BEHAVIOR OF BIOFILM**

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Abstract

Environmental biofilms and human microbiomes are complexed microbial communities. Not only the structure, but also the dynamics of the social interactions and the physico-chemical properties of biofilms are highly dependent on local environmental stresses, such as pH, nutrient availability or temperature. Studying of the effect of temperature on microbial biofilms has been mainly limited by the lack of high-precision experimental tools, capable of regenerating complex, well-controlled and reproducible micro-environmental conditions where temperature is the only variable parameter. The objectives of this work was to design, develop, and validate experimental platforms with the potential to (a) reproduce complex but well defined micro-environmental gradients relevant for microbial biofilms (flow velocity, nutrient, temperature), and (b) monitor dynamics of biofilm (development, dispersal, death) using temperature as readout signal. In order to demonstrate the potential for applications of these newly developed experimental set-ups, proof of concept studies were performed. Using *Pseudomonas aeruginosa* and *Pseudomonas putida* as medical and environmental model organisms for biofilms, proof of concept studies included (a) study of early stages of biofilm development at high spatial and temporal resolution under well-defined micro-environmental gradients, (b) assessment of the effect of temperature on biofilm formation using a temperature gradient, and (c) use of biofilm thermal signature to characterise major biofilm behaviour (biofilm formation or dispersal). Three devices were designed, micro-fabricated and validated: a new microfluidic flow through chamber allowing for multidirectional flow conditions as well as spatial and temporal characterisation; a sensitive temperature controlled system to be combined with the flow chamber in order to generate well-defined temperature gradients; a 3D thermopile for high precision temperature sensing. Using these sophisticated devices, we provided preliminary evidences that the initial stage of biofilm formation is a complex phenomenon, highly dependent on the bacterial communities and the local micro-

environmental gradients. *P. aeruginosa* and *P. putida* demonstrated strain specific behaviors within the first 12 hours of biofilm formation (initial attachment, clonal development, first dispersal, and reattachment). These behaviors were also found to be highly dependent on the flow velocity, which indirectly drives nutrient and oxygen availability, and temperature, which impacts on cellular activity and physical characteristics of the local environment. These preliminary results support the need for further research to assess in real time the impact of temperature on social microbial interactions within biofilms, using high precision, complex and well-controlled environmental conditions. Finally, biofilm behaviors (build up, dispersal and eradication) were assessed using temperature as readout parameter using a sensitive thermal sensor combined with the new microfluidic flow chamber. A good correlation between heat signal and biovolume measurements was observed, suggesting the potential of this experimental approach for further applications, such as drug screening.

In conclusion, biofilm development depends on physical and chemical micro-environmental gradients surrounding the local biofilm clusters. Flux measurements are therefore warranted to observe the heterogeneity of biofilms, and the newly developed devices offer users unique experimental conditions to study biofilm behaviors at high spatial and temporal resolutions.

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List of abbreviations

EPS: extracellular polymeric substances

eDNA: extracellular deoxyribonucleic acid

CLSM: confocal laser scanning microscopy

IMC: isothermal micro-calorimeter

FLIM: fluorescent lifetime imaging microscopy

GFP: green fluorescent protein

YFP: yellow fluorescent protein

sRNA: small ribonucleic acid

rhB: Rhodamine B

MEMS: micro-electrical-mechanical system

PID: proportional–integral–derivative

BOE - buffered oxide etching

TMAH: tetramethylammonium hydroxide

PCB: printed circuit board

RTD: resistance temperature detector

PDMS: polydimethylsiloxane

TRIS: Tris (hydroxymethyl) aminomethane

CMOS: Complementary metal–oxide–semiconductor

List of variables

E : total energy of a system

Q : heat

W : work done by a system

V : volume

H : enthalpy

A : area

H : thickness

U : voltage

p : pressure

T : temperature

t : time

S_o : thermoelectric coefficient

ρ : density

$\bar{c}_p$: mean heat capacity

$\dot{Q}$: heat flux

$\dot{W}$: work supplied

C_p : total heat capacity

R : thermal resistance

C : heat lost

r : rate of reaction

r_b, r_n : thermal resistance

R_o : universal gas constant,

E_o : specific activation energy

F : collision factor.

B : regression coefficient

k : thermal conductivity of material

S : heat power sensitivity

s : slope of the regression line

C_{HCl} : concentration of HCl

$Q_{reaction}$: enthalpy of protonation.

Chapter 1- Introduction

1.1 Bacterial biofilms

Biofilms are structured communities of assembled microbial cells. They are enclosed into a highly hydrated and heterogeneous matrix composed of extracellular polymeric substances (EPS) that include polysaccharides, extracellular DNA (eDNA), ions and proteins (Figure 1.1)¹. The composition of this matrix varies depending on the environment and the bacterial communities that it protects. This ultimately affects physical and electrochemical properties of the biofilms, impacts on the cellular communication and, influences the structural and functional integrity of the biofilms²⁻⁵. The proximity of the cells within the matrix facilitates a life-supporting exchange of signalling compounds, metabolites, genetic elements or defensive compounds (Figure 1.2)⁶. The heterogeneous nature of biofilms is a result of micro-environmental gradients within the matrix and a complex bacterial community that is composed of cells with different metabolic capacities and social roles (Figure 1.3)⁶

Figure 1.1 Composition of the biofilm matrix ¹ (taken from McDougald *et al.*, *Nat.Rev.Microbiol*,2012).

Figure 1.2. Extracellular polymeric substance (EPS) matrix influences the establishment of stable physical and chemical gradients that create different localised niches where organisms will stratify according to their growth requirements⁶(taken from Flemming *et al.*, *Nat.Rev.Microbiol*,2016)

Figure 1.3. Social interactions occurring in biofilms involve cooperation or competition between cells. If these interactions are driven by chemical communication, they are also widely influenced by micro-environmental gradients within the matrix⁶ (taken from Flemming *et al.*, *Nat.Rev.Microbiol*, 2016).

Formation of a biofilm starts when bacterial cells make contact with a surface and initiate attachment. When surface conditions such as hydrophobicity and access to nutrients, are favourable, initial attachment becomes irreversible. Cells adhere permanently to the surface and start to divide and form micro-colonies. This biofilm developmental process can be described in 5 stages (Figure 1.4):

Figure 1.4 Stages of the biofilm cycle⁷ (taken from Koo *et al.*, *Nat.Rev.Microbiol.*, 2017).

1-Initial attachment: Free floating planktonic bacterial cells are transported to a conditioned surface by physical forces (sedimentation, the collision caused by the flow), bacterial motility hydrophobicity, pH, substratum, temperature and hydrodynamic forces. Any combination of the above mentioned forces can contribute towards adhesion of bacteria to the surface or initial phase of the attachment.^{3, 8-10}. At this stage, bacterial cells can detach, and are removed by the hydrodynamic or surface repulsive forces.

2-Irreversible attachment: The transition from dynamic and reversible attachment to irreversible binding happens when cells overcome the repulsive factors and maintain their adhesion to the surfaces. Surface-localised bio-adhesins are synthesised to facilitate cell-to-surface and cell-to-cell bindings. For example, *Pseudomonas aeruginosa* utilises type IVa pilus-mediated twitching motility for surface attachment and intercellular adhesion¹¹.

3-Early biofilm formation: Attached cells grow to become clusters and microcolonies where cells are embedded in the EPS matrix. Expression of biofilm formation-related genes leads to the production of polysaccharides which enhance surface colonisation¹². In the case of *P. aeruginosa*, alginate is the most common capsular polysaccharide which is produced for cellular protection while other polysaccharides contribute to maintaining the structure of the biofilm during various developmental stages^{12,13}.

4-Biofilm maturation: Defined three-dimensional structures are characteristic of mature biofilms. The morphology of mature biofilms depends on environmental factors as well as the composition of species within the matrix.^{14,15} Cells in the community share resources to maintain the biofilm architecture. There is a continuous exchange of nutrients and products between the biofilm that subsequently contributes to the formation of various zones of differential nutrient/product gradient.

5- Detachment: Dispersal or detachment can happen when biofilm reaches maximum maturation capacity. Detachment is a critical stage in the biofilm life cycle where some individuals revert to the planktonic state, where they can go on and colonise other surfaces. This allows biofilms from one environment to spread to another one. This is significant in many environments, such as in water distribution systems, the human respiratory tract and other organs whereby biofilms can lead to diseases.^{16–18}

Figure 1.5. Biofilm dispersal can be defined as passive or active dispersal. In passive dispersal, an external force leads to the complete or partial removal of the biofilm. In active dispersal, microbial cells respond to an anti-biofilm stimulus and actively degrade the EPS matrix, thereby releasing free-swimming cells in the environment¹⁹ (taken from Flemming *et al.*, *Microorganisms*, 2017).

Biofilm dispersal can result from a combination of signals, such as nutrient limitation or hydrodynamic forces.^{20,21} It can be defined as active or passive dispersal (Figure 1.5).¹⁹

Active biofilm dispersal is due to hydrodynamic forces, such as shear stress, where a few

cells are continuously removed from the biofilm. Other examples include sloughing, where a part of the biofilm is broken off and removed from the surface. In abrasion, the objects in the fluid collide with the biofilm resulting in the removal of the cells from the biofilm.^{16,21,22} The rate of dispersal is dependent on the flow velocity, nature of the surface and architecture of the biofilms.^{23,24} In passive dispersal, cells can be released from the degraded matrix when the environmental conditions such as nutrient, oxygen, pH are no longer favourable.¹⁹ Passive dispersal occurs by disruption of the EPS matrix which is facilitated by targeting its components or regulatory pathway (Figure 1.6).⁷ Few approaches have been investigated and include treatments targeting the components of the matrix, bacterial adhesion and biofilm initiation. Strategies targeting the EPS matrix include eDNA (DNases), exopolysaccharides (dispersin B and glycoside hydrolases for examples) and proteins (DNABII family antibodies).⁷ PS adhesin-binding antibodies or inhibitors, as well as phage-encoded peptidoglycan hydrolases, have been examined to target cell adhesion and biofilm development⁷. Other biofilm triggers include inhibitors of EPS synthesis and secretion systems, which have demonstrated their potential for disrupting biofilm accumulation. Endogenous pathways controlling biofilm dispersal such as quorum sensing and regulation of cyclic-di-GMP and cyclic-di-AMP levels may also be promising targets for disrupting biofilms.

Dispersal is an important characteristic of biofilms as it leads to further colonisation. Therefore biofilm dispersal is of concern in the management of infection. Since dispersed cells are not protected in the matrix and are more susceptible to antimicrobial agents, control of biofilm dispersal represents a promising approach for the management of chronic and hard to treat infections caused by bacterial biofilms. As biofilms develop and disperse as a result of fast-changing ecological selection, it is important to develop sensitive and precise tools for biofilm research. Also, given the heterogeneous nature of biofilms, it is challenging, yet

essential, to develop accurate experimental procedures to collect high resolution imaging for quantification of the results into the understanding of the evolution of microbial biofilms.

Figure 1.6. Mechanisms of extracellular polymeric substance (EPS) matrix production and secretion represent attractive targets for the development of biofilm-targeting strategies⁷ (taken from Koo *et al.*, *Nat.Rev.Microbiol*,2017).

1.2 Experimental approaches for the study of biofilms under well controlled environmental conditions

Biofilms are often studied using multi-well plates and Petri dishes. While batch cultures with multi-well plates can permit high throughput assessments, the static environment represents a

limiting condition dominated by the absence of nutrient and oxygen supplies and the accumulation of metabolic waste that is deleterious for the microorganisms. Furthermore, conventional multi-well plates do not have a surface material allowing for detailed observations on biofilms using high-resolution microscopy. In the last decades, flow through systems technology has emerged with a three-channel and unidirectional flow cell made from polycarbonate, widely considered as the ‘gold standard’ condition for biofilms studies under flow conditions.²⁵ The apparatus is mounted on a thin glass coverslip, which allows for microscopic examination and the medium is supplied by connecting the tubing between inlets to the medium reservoir (Figure 1.7). This flow cell system enables constant supply of nutrient and oxygen while ensuring the continuous removal of metabolic waste and toxic compounds.

Figure 1.7. Flow cell reactor developed and commercialised by Technical University of Denmark²⁶ (taken from Nielsen *et al.*, *J.Vis.Exp.*, 2011).

However, the design of the chamber does not allow reproduction and control of complex environmental conditions, and the resulting microscopic observations must be carefully interpreted taking into account the undefined conditions existing at the inlet, outlet and central part of the chamber^{27,28}. Other flow systems used to study biofilms include glass or plastic capillary. However, these flow apparatus are limited by a unidirectional flow which

cannot recreate important key environmental gradients, such as gradients of shear forces, chemicals, and/or temperature. These environmental gradients have a critical impact on the structure and dynamics of biofilms at microscale level^{6,29–31}.

To circumvent the limitations of the commercially available flow cell, i.e. unidirectional flow and non-removable substrate, a multi-channel flow cell with a square cavity which support biofilm development was previously designed for forming reproducible and controllable biofilm³². By applying different flow rates and concentrations of nutrients at each channel and manipulating the inlet/outlet functions, one can establish and control key environmental gradients, which enable emerging physical-chemical-biological interactions followed by expression of new properties of biofilms. The flow cell can produce reproducible and stable gradients of shear stress (Figure 1.8).³³

Figure 1.8 Flow cell with hyperbolic profile to generate smooth flow gradient³³ (taken from Nguyen *et al.*, *npj Biofilms Microbiomes*, 2016).

In this microfluidic device, distribution of flow patterns and nutrient concentrations can be well characterised and maintained, which make this system a platform of choice to examine biofilms under known gradients. Furthermore, the coverslip is removable and can be replaced by many other surfaces so that surface-biofilm interactions and compatibilities can be easily investigated. The flow cell is coupled with confocal laser scanning microscopy (CLSM) with automated and controllable stage to allow high precision multi-position image acquisition in

real time. Development of the biofilm under the gradients established can then be monitored and examined in 3D structure with high spatial and temporal resolution. 3D images can be analysed by various powerful software platforms including IMARIS, ImageJ and COMSTAT^{25,3435}. The main limitation of this new flow cell is that it can only recreate well-controlled shear stress gradients. Further development is now needed to develop sensitive tools for other environmental gradients, such as temperature.

1.3 *Pseudomonas aeruginosa* and *Pseudomonas putida* as model organisms

Biofilm research has been developed using selected bacterial species as model organisms. Amongst these species, *P. aeruginosa* and *P. putida* have been extensively studied for medical and environmental biofilms, respectively.³⁶ (Figure 1.9)

Figure 1.9. Natural or opportunistic niches colonised by *Pseudomonas* spp. Some species can be rhizosphere and soil colonisers (for example, *P. putida*) and others can be found in humans (for example, *P. aeruginosa*). Several *Pseudomonas* spp., including *P. putida* are often found in soils with a history of chemical waste contamination³⁶ (taken from Nikel *et al.*, *Nat. Rev. Microbiol.*, 2014).

P. aeruginosa is an environmental Gram-negative bacterium characterised by its rod-shaped and the production of an odorant and colourful pigment, pyocyanin. *P. aeruginosa* is also an opportunistic human pathogen, classified by the World Health Organization (WHO) as one of the most dangerous bacteria in the world.³⁷ *P. aeruginosa* forms biofilms on both biotic and abiotic surfaces, such as medical catheters, ventilator tube, contact lenses and chronic wounds^{37–39}.

P. putida is an environmental Gram-negative, rod-shaped bacterium, characterised by its broad metabolic versatility, which includes biodegradation of xenobiotic and aromatic pollutants. Because of this, it is mainly found in soil, and especially polluted soil.⁴⁰ *P. putida* is also frequently discovered from plant rhizospheres where they have played an important role to promote plant growth. Like all the members of the genus, *P. putida* forms biofilms on a variety of surfaces.^{41,42} Bacterial cells use flagella to move around and find the surfaces for initial attachment. On both biotic and abiotic materials, the fast transition from reversible to irreversible attachment involves the synthesis of LapA protein^{43,44}. In contrast, in the early stages of biofilm development, the bacterial cells of *P. aeruginosa* seem to move across surfaces using twitching motility and eventually settle in the biofilm clusters.^{45,46} Because of their similarities and differences regarding dynamics of biofilm formation and potential application, *P. aeruginosa* and *P. putida* are ideal candidate model organisms for the development of a new generation of microfluidic systems with the capacity to reproduce complex environmental conditions, including temperature gradients, which will be outlined in this thesis.

1.4 Effect of temperature on development of biofilm

The majority of bacteria relevant to industrial environments, households and the human body are mesophilic and grow optimally at temperatures ranging from 20°C to 40°C. In laboratories, bacteria are typically cultured in incubators set at 25°C, 30°C, 37°C and 42°C. The effect of temperature on bacterial growth has been widely studied and reported in conditions mimicking water pipes⁴⁷ or medical devices⁴⁸.

If changes in the environmental temperature do not seem to affect the biofilm formation of the foodborne pathogen *Listeria monocytogenes*,⁴⁹ temperature does affect the initial adhesion of *Pseudomonas spp.* cells to surfaces. Thus, if lower temperatures tend to increase the initial interactions of cells with surfaces, they also tend to reduce the cells adhesion properties.¹⁵ Temperature also affects gene regulation, including those involved in virulence^{50,51}. Thus, for example in *Vibrio cholera*, a temperature of 15°C results in alterations of c-di-GMP regulation, and hence, in the enhancement of biofilm formation by suppression of surface motility.⁵² In contrast, higher temperatures result in alterations of *Legionella pneumophila* biofilm, with more filamentous cells found at 37°C to 42°C as compared to 25°C where most of the cells are rod shaped⁵³.

Microfluidic apparatus coupled with temperature control systems have recently provided some new but limited insights into the effect of temperature on bacterial biofilms.⁵⁴ Thus, despite being important environmental and medical microorganisms, the effect of temperature on *P. putida* and *P. aeruginosa* is still unknown. In this work, based on the development of the square flow cell, we integrated a temperature gradient generator system to bring another factor of heterogeneity, to examine responses of the opportunistic *P. aeruginosa* and *P. putida* biofilms.

1.5 Micro-calorimetry: concept and applications

1.5.1 Bio-thermodynamics

Heat is probably present in all biochemical processes, where the release or absorption of heat is associated with changes in temperature. The metabolic activity of living organisms releases heat to the environment, and in many higher organisms, this release of energy forms part of their maintenance of constant body temperatures⁵⁵. The relationship between heat and total energy of a system is regulated by the First law of thermodynamics.

For a closed system, the change in internal energy of the system is

$$\Delta E = Q + W \quad (1)$$

where Q is the heat flow into the system and W is the work done by the system.

If we take glucose, which is a common substrate for biological processes, the following reaction can happen under standard conditions,

For an open system, the First law of thermodynamics becomes,

$$dH = \delta Q + \delta W + V dp \quad (5)$$

where H is enthalpy, V is volume and p is pressure. The First law of thermodynamics was applied to microbial reactor systems by Stocker et al.⁵⁶ to yield the formula,

$$\rho V \cdot \bar{c}_P \cdot \frac{dT}{dt} = \dot{Q} + \dot{W} + \sum_e \dot{V}_e \rho \bar{c}_P \cdot (T_e - T) - \sum_j \Delta_{r_j} H \cdot r_j V, \quad (6)$$

where $\bar{c}_P$ is the mean heat capacity of the system, $\dot{Q}$, is the heat flux into the system, $\dot{W}$ is work supplied, the last term is the total molar enthalpy of reactions in the system, the second to last term is the total heat exchange by the flow of material volume into the system. This

model has been successfully applied to quantify heat production of large-scale fermentation reactors by calorimetric measurement. Thermodynamics of microbial growth in large scale and homogenous culture had been characterised by calorimeters for many microbial strains which have proven a close correlation between biomass growths and heat production^{57,58}.

Recent studies have established intracellular temperature to be approximately 0.2-0.9 degrees higher than their environment because cellular activity always produces heat.⁵⁹⁻⁶² (Figure 1.10). By capturing the heat transfer or temperature; we can further understand physiological activities happening inside the biofilm based on their localised temperatures and heat transfers. The heat production rates of some bacterial and eukaryotic cells are listed in Table 1.1.⁶³ Different organisms produce varying amounts of heat depending on their size and metabolic processes under specific conditions. Heat production rates can change significantly when bacterial cells switch their metabolic states from aerobic to anaerobic fermentation. These changes in metabolic states are brought about by the limitations of the electron receptors. Measuring heat production through temperature change can give us details on the actual processes as well as their dynamics.

Figure 1.10 Illustration of heat pathways involved in biochemical processes⁶³ (adapted from Maskow *et al.*, *Methods*, 2015) .

Table 1.1 Metabolic heat production rate of various cells (Adapted from Maskow *et al.*, *Methods*, 2015)

Bacteria	Physiology	Heat power rate (pW/cell)
<i>Escherichia coli</i>	Fermentative	0.05
	Anaerobic respiration	0.2
	Aerobic respiration	0.8
<i>Staphylococcus aureus</i>	Aerobic respiration	2.5
<i>Mycoplasma hominis</i>	Aerobic respiration	11

1.5.2 Heat transfer, thermal sensing and calorimetry

Calorimetry is the study of heat transfer from a system to a well-calibrated environment (heat sink) by measuring the total amount of heat supplied to keep the system at a specific temperature or the temperature change of the system with a calibrated heat capacity.⁶³ As almost all biochemical reactions release or absorb heat (enthalpy), calorimetry is a well-suited method to monitor the process and to detect any changes in the system. Thermal signals from the biological system can be an indicator of their metabolism and their interactions with the environment. Calorimetry offers a non-destructive method for research and industries. It is a method, which requires very minimal sample preparation and works with a variety of samples.

Heat producing bacterial cultures will cause the change in temperature of itself and the surrounding microenvironment. As a temperature gradient between the sample and heat sink develops, a calorimeter is used with various temperature sensors to record the generated temperature changes. Heat produced by bacteria flows towards a temperature gradient as

illustrated in figure 1.11. Heat generated by the bacterial culture with a total heat capacity (c_p) increases the temperature (T) of the culture itself and transfers to the heat sink with a constant temperature (T_0) or environment. R is the heat resistance of the heat conduction pathway and C represents the heat lost by radiation and air convection. In most small calorimeter systems, we can ignore the heat lost by radiation and air convection and hence C can be removed from the calculation.

Figure 1.11 Thermal diagram of a simple calorimeter system including a heat source (sample), a thermal sensing and heat conduction pathway (R , C) and a heat sink.

The equation governing heat transfer of a calorimeter system can be written as,

$$\frac{dQ}{dt} = c_p \frac{dT}{dt} + R(T - T_0) \quad (7)$$

where the rate of heat produced by the source (bacteria culture) is equal to the sum of heat consumed to increase the source temperature and heat lost through the conduction pathway which can be the isolation layers, the calorimeter chamber's wall and the sensor.

Figure 1.12 Illustration of thermoelectric effect with thermocouple and thermopile.

The development of integrated circuits and micro-electrical-mechanical system (MEMS) fabrication technologies led to the invention of micro sensors^{64,65} including chip calorimeter devices in which the heat flow from the sample is detected by a thermopile (thermocouple array) based on a thermoelectric effect^{66,67}. A thermopile is comprised of several thermocouples connected in a series. Each thermocouple is a combination of 2 strips of different materials joint together at one end and open at the other end. When one end of the thermocouple has a higher temperature, an electric voltage is induced with the magnitude depending on the temperature difference (figure1.12).

The working principle of a chip calorimeter is schematically described in figure 1.5⁶⁸. When cells move closely to the sensing junctions, their metabolic activities will release heat that makes the temperature of the sensing junctions higher than the reference junction (cold junction). Due to the thermoelectric effect, the thermopile will generate an electrical voltage that is directly proportional to the temperature difference between the hot and cold junctions. The voltage is measured by a voltmeter. Thermoelectric voltage depends primarily on material properties and temperature differences between hot and cold junctions can be calculated with the Seebeck's effect equation,

$$U=nS\Delta T \quad (8)$$

where V is the thermoelectric voltage, n is the number of thermocouples, S is the thermoelectric coefficient of thermocouple materials, ΔT is the temperature difference between hot and cold junctions.

However, these chip calorimeters have only a limited sensing area, because the hot and cold junctions must remain in the same plane. The sensor can only work for limited flow cell geometry without the capability to access heterogeneity of the development of a biofilm while advances in biofilm understanding require higher temporal and spatial resolutions.

Figure 1.13 Basic principle of chip calorimeter sensor where cells adhered to the hot junction to cause higher temperature and electrical voltage⁶⁸ (taken from Kemp, *Thermochimica Acta*, 2001)

1.5.3 Biological application of calorimeters

Isothermal micro-calorimeter

The isothermal microcalorimeter (IMC) belongs to a group of commercial and conventional calorimeters which are made by various scientific equipment manufacturers including Mettler Toledo, Malvern and TA Instruments^{69,70} (Figure 1.14). The basic building block of IMC is a

glass or metal ampoule which contains the sample with a volume range of 1-20mL⁷¹. There is a thermocouple sensor in contact with the ampoule chamber to measure heat transferred from the samples to the heatsink. The IMC is operated at nearly constant temperature with temperature fluctuations of 10 μ K-100 μ K controlled by a thermostat chamber with oil/water bath to keep the heatsink temperature at a stable range.

Figure 1.14 Isothermal micro-calorimeter apparatus (left) from TA Instruments and its measurement ampoule (right) enclosed by the chamber (centre) (taken from TA Instruments, 2016)

IMC is used to monitor activities of bacterial culture and associate the thermal signature with specific processes. For example, in figure 1.15, *E. coli* was grown in the glass ampoule containing M9 media with glucose and lactose.⁷² The data obtained was split into 3 peaks corresponding to glucose respiration, fermentation and lactose fermentation. Similarly, many other processes such as nitrate reduction and denitrification can be quantified. Further applications of IMC include detection of infections and monitoring drug activities against bacteria.⁷³⁻⁷⁵ However, most of the experiments were carried out in a sealed ampoule with lack of control of the sample and environment making it challenging to link the data with

practical applications. Similarly, isothermal calorimetry of biofilm activities remained limited due to the structure of the ampoule and the sensing chamber not being compatible with the integration of a flow cell for biofilm development.

Figure 1.15 Heat production of *E. coli* in M9 media with various peaks representing metabolic processes of the culture when nutrients were exhausting⁷² (taken from Braissant *et al.*, *FEMS Microbiol.Lett.*, 2010).

Chip calorimeter

Development of the chip calorimeter is motivated by limitations of the commercial isothermal calorimeter.⁷⁶ Advances from microfabrication of thermal sensor and microfluidic channels allow more flexibility in handling and delivery of samples into the system. Miniaturisation of the sensor fabrication allows parallel measurements of multi samples with much smaller volumes. Chip calorimeters are categorised into two sub-groups based on sample handling methods: open chamber and closed chamber chip calorimeter⁷⁷. The basic component of the chip calorimeter is the thermopile sensor, which is based on the thermoelectric effect. The thermopile sensor is placed under a chamber that contains samples separated from the sensor by a thin insulation layer.⁷⁸

Open chamber chip calorimeter

The open chamber chip calorimeter has the sample reaction chamber on top of the sensing area exposed to the environment. Samples are delivered by an inkjet pump or micropipette that is more suitable for the rapid process. The chip calorimeter array was made by Torres et al.^{79,80,81} and consisted of two free-standing thermopiles with one for reference (figure 1.16A). This device was used to measure temperature changes caused by a chemical reaction between 250nl droplets of streptavidin-biotin or RNase A and 2'-CMP mixed electrostatic forces.

Figure 1.16 Open chamber chip calorimeters (A) Dual chamber with one as reference for mixing calorimetry⁸² (taken from Torres et al., PNAS, 2004) (B) Four chambers on free-standing membrane for general purpose calorimetry (taken from Xensor Integration Inc., 2016).

Xensor Integration Inc, Netherlands also manufactured various calorimetric chips^{77,83–85} with an open cavity (figure 1.16 B) separated from a thermopile array by a thin silicon oxide membrane and integrated with heating and temperature control elements. This device is suitable for measurement of thermal properties of materials over time and temperature such as the heat capacity of a polymer or enzyme activity⁷⁷. The company also uses the same technology to manufacture a differential scanning calorimeter, which provides better stability and accuracy by the integration of a reference channel.

The above devices have the advantages of easy usage with simple signal acquisition requirements and provide quick results with sufficient sensitivity. However, exposure of the sample to the environment without a proper control of temperature, humidity and fluidic flow can cause significant signal drift as well as evaporation of liquid sample if the experiment runs at elevated temperature. Therefore, open chamber chip calorimeters work best with samples that require only relatively short reaction time in chemical or material engineering.

Closed chamber chip calorimeter

Biological applications involving live cells often use a closed chamber chip calorimeter due to its versatility and sophisticated control of samples by integration with microfluidic channels⁷⁶. Using the commercial chip from Xensor Integration, Higuera-Guisset *et al.* constructed a fluidic channel and attached it on top of the cavity to form a closed chamber to study the growth of *E.coli* at different temperatures.⁸⁶ (figure 1.17) Another chip calorimeter with a unidirectional flow cell was used extensively to study biofilm in different aspects.⁸⁷ While most of the other systems use thermopiles made from metal or doped silicon with aluminium, this system employed micro-fabricated thermopiles consisting of 118 BiSb/Sb thin film thermocouple for high-performance sensing.^{78,88,89} The chip was divided into 4 sections under a flow channel insulated by the polymer membrane with 80µm thickness.^{90,91}

(figure 1.18) Responses of *P. aeruginosa* biofilm to the injection of growth medium, lack of nutrient and several antimicrobial agents together with predation were recorded by this chip calorimeter system⁹ as illustrated in figure 1.19.^{2,93,71,94,95}

Figure 1.17 Heat produced by *E.coli* cultures at (A) 37°C, (B) 32°C, (C) 18°C show their dependence on temperature for growth (left) and schematic illustration of the system (right)⁸⁶. (taken from Higuera-Guisset *et al.*, *Thermochimica Acta*, 2005).

Figure 1.18 (A) Thermopile with the flow cell. (B) The chip device with 4 segments on PCB. (C) Cross sectional view (D) device with flow cell⁹⁰. (taken from Lerchner *et al.*, *Thermochimica Acta*, 2006).

Mammalian cells such as cardiomyocyte and fat cells were also studied using a chip calorimeter with stimulated conditions. Johannsen *et al.* developed a ring-shaped thermopile array which can detect a minimum 13nW heat power from 20 cells responding to chemical stimulation.^{96,97} Heat produced from a single brown fat cell was recorded by a pico calorimeter with a free standing resonator where frequency is temperature-dependent.^{98,99} The sensor could detect heat change at 5.2pJ. The same conventional thin film free standing thermopile was integrated with different microfluidic channels to form various chip calorimetric systems for chemical engineering applications.^{100–104} However, due to the in-plane structure of the thermopile, the sensing area is limited to the line along hot junctions which leads to signal loss from other cells far from the hot junction. The configuration and layout of a micro thermocouple also limits the application of the sensing device to other types of flow cells.

Figure 1.19 Response of biofilm in the flow chamber measured by chip calorimeter at (A) different nutrient flow rates and (B) different antibiotic concentrations⁹³ (taken from Buchholz *et al.*, *Antimicrob. Agents Chemother.*, 2010).

The sensitivity and accuracy of calorimetric systems are varied from commercial IMC to chip calorimeter and dependent on the sample volume as well as the stability of the environment

which enclosed the sample and the sensor^{74,75,105}. Most of commercial IMC used closed ampoule without any exchanges of medium. The thermostat controlled by water bath could provide excellent temperature stability of 1mK to 0.01mK thus improve the calorimetric performance^{106,107}. Commercial instruments had sample size of 1mL-4mL with sensitivity of 0.1 μ W-0.2 μ W corresponding to a number 10000-20000 cells in the ampoule⁷⁰. The accuracy of the commercial microcalorimeters was reported at 1%-5% over more than 24hr experiments⁷². The chip calorimeter had sample chamber which contain only volume of 100pL to 100 μ L^{88,93,105,110}. Some device was equipped with a flow system which supplies nutrients to the cultures thus affecting the signal stability. Sensitivity of chip calorimeter is reported from 0.013 μ W to 0.1 μ W for both mammalian and bacterial cultures with the accuracy of 2%-8%^{70,76,103}. The low volume has the advantage to accommodate rare and expensive samples together with smaller time constant (milliseconds to a few seconds compare to tens of seconds to minutes of IMC)⁷⁰. However, the high sensitivity of the chip calorimeter was limited by the extreme low volume of the sample due to the design and layout of the thermal sensor to fit with the chamber dimensions. When sample volumes was taken into account, the sensitivity per unit volume of chip calorimeter is still much lower than the commercial IMC which limited their potential in biochemical application⁷⁶.

Intracellular temperature measurement

Besides the use of a thermopile as an external sensor to measure temperature changes following cell metabolism, there are other approaches to reach intracellular temperatures by bringing thermal sensitive probes inside the cell membrane.¹⁰⁹ This section introduces cellular temperature measured by other technologies mainly micro-thermometers and nanoparticle or molecular probes.

Nano-thermometer

Advanced microfabrication allowed Shirao *et al.*¹⁰⁹ to form metal thermocouple junctions of tungsten-platinum (W-Pt) at the tip of a nano probe with a diameter less than 500nm. The miniaturised thermometer can penetrate the cell membrane and report the actual temperature change of cellular processes. Depending on the concentration, treatment by adding lipopolysaccharide resulted in the temperature increase of up to 0.5°C (figure 1.20). This approach perhaps provides the most accurate temperature readings with a well-calibrated thermocouple and direct contact. However, inserting the tip in the cell membrane could cause damage to the cell structure. The apparatus must be handled and controlled with extreme care because the tip is small and fragile.

Figure 1.20 A) Thermometer nano probe penetrating a cell and (B) intracellular temperature rise measured by the probe when the cell was stimulated.¹⁰⁹ (Taken from Shirao *et al.*, *Nanotechnology*, 2015).

Nanoparticle and molecular probe

The use of fluorescent probes is one of the most convenient applications in biology due to its compatibility with advanced imaging. The cells and their activities can be observed simultaneously by fluorescence from many specific molecular probes. Over the past decades, several fluorescent probes were developed from organic dyes, ligand complexes,

nanoparticles and quantum dots^{61,110}. The temperature can be read out from a single fluorescent intensity, ratiometric intensity or fluorescent lifetime measurement (FLIM). Thermal sensitive fluorescent co-polymer was synthesised by linking thermal sensitive acrylamide units together with fluorescent molecules, water sensitive monomer and a cationic unit^{62,111–113}. This resulted in a molecule that contracts or expands during temperature change. The compression of the polymer chain removes water molecules, which are the fluorescent quencher. Removal of water molecules leads to an increase in the intensity or fluorescent lifetime. This fluorescent probe was used with FLIM to map temperature inside cells and report activity of the mitochondria. Temperature differences up to 0.98°C inside living HeLa cells was detected¹¹³ (figure 1.21). Similar approaches have been used with rhodamine B which is a temperature sensitive fluorescent dye to form ratiometric molecular probes^{114,115} to visualise mitochondrial temperature changes.

Figure 1.21 Mapping cellular temperature of living HeLa cell by fluorescent lifetime imaging¹¹². (Taken from Gota *et al.*, *Analyst*, 2007).

The nano-thermometer based on thermocouple has the sensitivity of 6.97 $\mu\text{V}/^\circ\text{C}$, the accuracy of 0.024°C. The temperature difference of inner cell and the surface was measured at 0.063 $\pm$ 0.057°C¹⁰⁹. Sensitivity and accuracy of fluorescent thermal probes are dependent on the chemistry of the probe and more importantly the detection instrumentation. Ratiometric methods had the temperature resolution of 0.18 °C to 0.5°C with the ratio change 2.2-

2.5%/°C¹¹⁶. FLIM technology which measure the fluorescent relaxation time could bring the resolution below 0.1°C¹¹⁷. Nanotechnology provides thermal sensitive quantum dots encapsulated in polymer¹¹⁸ and nano-diamond sensors which have a resolution of 1.8mK^{119–121}. The major disadvantages of thermometry in micro/nano scale were that fluorescence was often affected by other environmental factor including oxygen and pH which led to less accurate results. Delivery of the probes to the sample required delicate handling system which is micro/nano manipulator with extremely small volume injection system.

Development of fluorescent probe thermometers led to the discovery of the single cell's temperature and the ability to monitor a single cell's metabolism at high spatial resolution. To achieve high measurement and spatial resolution, sophisticated and advanced optical and imaging facilities are essential. The fabrication of these probes is also complicated and expensive which limits the application to only small samples and single cells.

1.6 Objectives of the project

As highlighted in the literature review, there is a need for experimental setups capable of reproducing complex but well defined environmental conditions to further study the behaviour of communal biofilms at high temporal and spatial resolution. One particular physical parameter for which there is a significant gap of knowledge is temperature, which not only influences bacterial growth but also to some extent may characterise specific biological events (Figure 1.22).

Using advanced microfabrication and engineering technologies, this thesis aims at developing a next-generation microfluidic system (Chapter 2), a temperature control system (Chapters 3) and a chip calorimeter (Chapter 4) in order to provide the scientific community with powerful

and sensitive experimental set-ups for applications in both medical and environmental microbiology. As proof of concept, the developed tools were further employed to

- (1) Characterise the dynamics of *P. aeruginosa* and *P. putida* biofilm growth and dispersal under well-defined environmental gradients (Chapter 2),
- (2) Assess the impact of temperature on the behaviour of growing biofilms of *P. aeruginosa* and *P. putida* (Chapter 3) and,
- (3) Study the ‘heat signature’ of *P. aeruginosa* and *P. putida* biofilms (Chapter 4).

Finally, pros and cons of the technologies developed and validated, as well as their potential applications were discussed in Chapter 5. Perspectives of the work initiated in this thesis and possible steps to be taken to improve the developed technologies will also be examined in chapter 5.

Figure 1.22 Overall processes in biofilm development from single cells to dispersal which is affected by the environment and measured by a thermal sensor.

Chapter 2 - A new 2D biofilm growth chamber designed for complex and controlled environmental gradients: From design to the application

2.1 Introduction

Bacteria are social microorganisms! For many years, it was believed that bacteria existed as free-living cells and operated in isolation. It is now widely recognised that most microbial organisms live in organised communities, defined as biofilms. In these complex and well-structured communities, bacteria can play different social roles, characterised by different social phenotypes (cooperation versus competition) as compared to coexisting counterparts. (Figure 2.1)¹²² In response to other organisms and environmental stresses, individuals will engage in complex behaviors that will affect the structure and function of the community.^{123–}

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Biofilm research has traditionally aimed to recreate simple environments where physical conditions and temporal variations are absent. Although this strategy has led to fundamental understandings of biofilm dynamics, it has been significantly limited by the absence of environmental complexities.^{24,31,127,128} The recent introduction and development of microfluidic platforms has provided microbiologists with unique opportunities to manipulate environmental conditions at high temporal and spatial resolutions, relevant to cellular behaviours such as motility or biofilm formation.³³ Strength of these microfluidic devices does not reside in their ability to fully reproduce natural habitats, but rather in their ability to replicate and control specific features of the environments (such as temperature, pH, nutrient availability) allowing for the study of their effects on the community. Multiple microfluidic devices with various designs and features have been developed over the last years^{129–132} providing platforms for the assessment of bacterial responses to specific environmental

signals, chemical gradients and neighbouring cells. These technologies have significantly contributed to filling major knowledge gaps and bringing new insights into bacterial behaviours. However, all the devices developed so far exhibit similar limitations.

Figure 2.1 Social phenotypes in bacterial biofilms. Figures a, c, e and g show simulations/predictions of the known social phenotypes in relation to specific environmental stresses (nutrient availability, toxin secretion for example). Figures b, d, f and h are experimental validations of the simulated/predicted behaviours. Social behaviours and related phenotypes are influenced by spatial distribution (segregation versus mixed) of cell lineages within biofilms, as it increases the frequency of cell interactions¹²² (taken from Nadell et al., Nat. Rev. Microbiol.,2016)

Most devices used in research have unidirectional flows, which significantly reduces the possibility to recreate complex environments. One exception is a platform developed by a group from University of Northwestern^{34,133}. They designed and fabricated a microfluidic device with the capacity to generate multidirectional flow conditions. This device consisted of inlet and one outlet, which are perpendicular to each other with the square chamber cavity (Figure 2.2). However, the device failed to generate and validate complex flow conditions and environmental gradients. Another limitation of most platforms is the non-removable substrate. In other words, the biofilm is growing on a surface that cannot be detached from the system, which does not permit further functional characterisation, for example, genomic and transcriptomic analysis.

Figure 2.2 The two-dimensional flow cell. A) Procedure for fabrication of the planar flow cell. (B) Side-view photograph of the assembled flow cell. (C, D, E) Typical two-dimensional flow patterns: (C) a right-angle (90 degree turn) flow, (D) a 360 degree turn flow, and (E) a symmetric diffusing flow³⁴ (taken from Zhang *et al.*, *Biotechnol. Bioeng.*, 2011)

In an attempt to recreate complex environmental conditions, our group developed a hyperbolic biofilm growth chamber where the effect of flow rates on biofilm development was studied³³. The microfluidic platform designed and micro-fabricated consisted of a hyperbolic profile micro-machined on a polycarbonate sheet with two inlets and one outlet (Figure 1.8). Using *P. putida* as model organisms, we were able to study the initial stages of biofilm development and describe the impact of flow on the bacterial behaviours at high spatial and temporal resolutions.

In order to study the effects of chemical or physical parameters (for example, temperature) on biofilm structure and function, it is imperative to develop, fabricate and validate a new microfluidic platform that allows for multidirectional flow conditions as well as spatial and temporal characterisations. Multidirectional flow conditions will enable us to recreate and study an infinite number of micro-environmental profiles whereas spatial and temporal resolution will enable us to underpin ecological strategies associated with biofilm formation and heterogeneity, especially those related to temperature.

Therefore, using robust engineering and microfabrication techniques, we aimed at developing and validating a precise flow cell apparatus that will help us to gain further insights into previously unexplored aspects of the phenotypic heterogeneity of microbial populations influenced by temperature. In this chapter, we will (1) describe the design and performance of this innovative biofilm growth chamber, (2) show the potential for creating multiple and complex micro-environmental conditions using various flow simulations, (3) demonstrate the precision of the platform using experimental validations and (4) provide biological evidences for reproducible and well controlled biofilm development in this device.

2.2 Materials and methods

2.2.1 Design, fabrication and validation of a next generation biofilm growth chamber

A two dimensional flow chamber with 4 inlets/outlets (Figure 2.3) was developed and patented by the group³². The combination of different inlets and outlets provides with microfluidic conditions for creating physical (flow regime and drag forces) and chemical (for example, nutrients, antibiotics) gradients inside the square cavity.

Figure 2.3 Structure of the square flow cell and its flow channel configuration used throughout this report

The main chamber consists of a squared cavity of 20mmx20mmx1.4mm (Length, width and depth). This design enables the use of standard commercially available glass coverslip (22mmx22mmx0.17mm). Further details of the design and fabrication are available in the relevant patent³². By using different combinations of inlets and outlets, different flow

profiles were simulated using COMSOL Multiphysics simulation (COMSOL Inc., See Appendix 1 for model details).

Particle image velocimetry (PIV) was used to validate the flow field simulated by COMSOL. Fluorescent polystyrene microbeads (diameter=5-8 μ m) were mixed in the standard minimal salts growth medium M9 (See appendix 3 for composition of M9 media) and continuously injected into the flow cells by syringe pumps through 2 opposite inlets at the flow rate of 2mL/h (corresponding simulation Figure 2.5B). After the stabilising time of 30 minutes, the high-resolution camera (AxioCam, Zeiss) recorded the particles movement at the frame rate of 3 frames/second along x-axis, y-axis, y=-2, x=2. Each of the lines (x-axis, y-axis, y=-2, x=-2) have 9 positions with 2mm spacing for the measurement. The acquired images were analysed by OpenPIV software¹³⁴ to obtain the flow velocity of the particles at each position. The mean velocity of three independent experiments was plotted and compared to the simulation. The flow field simulation was considered validated when the measurement from PIV was consistent with the COMSOL model (See appendix 2 for validation results).

2.2.2 Study of early stages of biofilm development flow patterns

Flow cell set up was prepared under sterile conditions in a biosafety cabinet. A solution of 95% ethanol was pumped in for 30 minutes under UV exposure, followed by sterile water for 60 minutes and ultimately, minimal salts growth media (See appendix 3 for composition of M9 media) supplemented with 0.04% casamino acids and 0.04% glucose. Careful inspection of the flow cell was then performed to ensure that there was no bubble entrapped in the cavity. M9 media was injected continuously for 30 minutes by a dual syringe pump (KDS Inc.) at the flow rate of 2mL/h for each syringe to form a stable flow field before inoculation of bacterial suspension.

Strains used in this chapter included *P. aeruginosa* GFP (PAO1) and *P. putida* YFP (OUS82). The strains were initially grown onto Lysogeny (LB, Sigma-Aldrich) agar plates to obtain single colonies after 24h incubation at 37°C. A single colony of the strain was inoculated in 4mL of LB medium and incubated overnight at 30°C in a shaking incubator (100rpm). The resulting bacterial inoculum/suspension was diluted in fresh M9 medium to obtain an optical density of 0.008-0.009 at 600nm. This diluted suspension was used to inoculate the flow cell using the inoculation inlet at a flow rate of 10mL/h for 5 minutes. The inoculation inlet was then blocked until the end of the experiment and the flow cell was left for 30 minutes without any flow to allow bacteria to efficiently attach to the glass coverslip surface. The flow rate was set at 2mL/h for the 2 inlets during the entire course of the experiment, which was performed at 25°C. Three independent experiments were performed using *P. putida* and two experiments using *P. aeruginosa* to ensure reproducibility. The experiments were performed one after the other using different flow cells.

Figure 2.4 Flow design and map of 17 positions across the flow chamber selected for confocal image acquisition. Positions were chosen based on the flow field simulation by COMSOL.

The microscopic imaging was carried out in real time with the time interval of 20 minutes using confocal laser scanning microscopy (CLSM, in the Zeiss LSM780) and a x40 objective on 17 defined positions (figure 2.4). The geometric centre of the flow cell was defined as the reference point (0,0) for the coordinates to locate positions within the flow cell in 2D. Z-stacks images of 21 slices for each position were acquired by excitation laser wavelength of 514nm and an emission range of 524-600nm for strains with yellow fluorescent protein (YFP); 488nm excitation and 500-580nm emission range for strains with green fluorescent protein (GFP). The images were then quantified in batch processing using Imaris software (Bitplane, Zurich) and the surface segmentation function was used to obtain biovolume measurements. Using the above configuration, each image frame had dimensions of 212 μ m $\times$ 212 μ m. The biovolume measurement at each position was reported as the mean biovolume per area of 100 μ m $\times$ 100 μ m, which was calculated by dividing the average biovolume measurement from independent experiments (3 for *P. putida* and 2 for *P. aeruginosa*) at each frame (212 μ m $\times$ 212 μ m) to the factor 4.49.

2.3 Results

Simulation/Validation of the flow velocity:

As per COMSOL simulation, the configuration of 1 inlet/1 outlet (Figure 2.5A) results in a homogenous flow velocity pattern, while a higher number of inlets leads to the formation of flow velocity gradient in which the vertical central axis is the symmetrical axis (Figure 2.5B and C). The flow velocity also increases significantly in areas close to the inlets/outlets due to a significant change in cross-section of the flow channel.

Figure 2.5 Visualization of the flow field in flow cell cavity at total flow rate 4mL/h by COMSOL simulation under laminar flow. A. One inlet, one outlet. B. Two inlets one outlet. C. Three inlets one outlet.

The flow patterns of all the above flow cell configurations were simulated by COMSOL software to visualise the flow velocity distribution within the chamber cavity (Figure 2.5). In order to generate reproducible flow gradients and separate nutrient and inoculated bacteria inoculation, combination of 2 inlets and 1 outlet (figure 2.5B) was used in biofilm experiments. Two opposite channels were used as inlets for nutrition while a third channel was used as outlet. As shown in Figure 2.6, the flow appears to be very disruptive and turbulent at the regions 3mm closer to the inlet/outlet openings. Beyond the disruptive areas, in the vertical direction (y), flow velocity increases from 0 to maximal value while in the horizontal direction (x), the velocity is stable. The numerical value of flow velocity was extracted and plotted in figure 2.6 to define the biofilm growth area with stable flow velocity gradient. With reference point (0,0) at the centre of the chamber cavity, the stable area starts from $y=-7$ to $y=9$, $x=-7$ to $x=7$. For convenience and symmetry, we defined the area of 14mmx14mm square in the centre of the flow cell to observe biofilm development. Along the y-axis, the flow velocity increases linearly from upstream (close to the inlets) to downstream (closer to the outlet).

Figure 2.6 Variations in the simulated flow velocity along (A) vertical dimension and (B) horizontal dimension of the chamber; each line represents velocity magnitude across the horizontal or vertical axis of the chamber with 2mm spacing. Centre of the flow cell is the origin (0,0).

2.3.1 Study of *P. putida* biofilm development under well controlled flow conditions

We examined the distribution of biofilm in the square flow cell with respect to time and spatial localisation across the chamber. The first set of positions consisted of positions (0,7.0),(0,3.5),(0,0),(0,-3.5) and (0,-7.0) which reside along the vertical y symmetry axis, where the flow velocity increased linearly from the upstream to the downstream. The biovolume measurements at respective positions in this set were plotted for the first 5h of biofilm development and are shown in Figure 2.7. Interestingly, during the selected time period from 200 minutes to 300 minutes, a major disruptive dispersal occurred very quickly bringing down the biovolume measurements to almost the initial values. Dispersal was

recorded to be initiated at the upstream position (0,7.0) and to propagate towards the downstream position close to the single outlet, with the last dispersion event observed at position (0,-7.0). Other dispersal time points related to positions included: 220 minutes for position (0,7.0), 240 minutes for position (0,3.5), 260 minutes for position (0,0) and (0,-3.5) and 280 minutes for (0,-7.0). After 5h of growth, the biofilm has totally dispersed at the centre positions.

Figure 2.7 Mean biovolume measurements during 5 hours of *P. putida* biofilm development along the central y-axis

Illustrations of data are provided as microscopic images at selected time and positions in Figure 2.8. At 220-240 minutes, biofilm dispersal is visible at position (0,7.0) with many small aggregates and single cells spread around the main clusters. Dispersion event propagated from the upstream to the downstream within an hour and the majority of biomass was removed from the surface along the central vertical axis. The confocal images also illustrate that the last dispersion took place at downstream position (0,-7.0). Biofilm dispersion started downstream, i.e. close to the outlet as previously observed.³³

Figure 2.8 Projected confocal laser scanning microscopy images at selected time points of biofilm along y-axis. Scale bar is 50 μ m.

The second set consisted of positions (-7.0,0),(-3.5,0),(0,0),(3.5,0) and (7.0,0) which reside along the vertical x axis. As the flow field simulation demonstrated in figure 2.6, at a given y-position, flow velocity does not change significantly along the x-axis with lower velocity at more central positions. Figure 2.9 depicts the biovolume measurements of biofilm taken

along the horizontal x-axis. Dispersion started at position (0,0) at the centre and propagated to the two sides of the flow cell near the inlets. Consistent with the symmetry of the flow configuration, positions (-3.5,0) and (3.5,0) exhibited the same behavior with similar biovolume and dispersal event at 340-360 minutes. Positions (-7.0,0) and (7.0,0) also showed the same growth patterns and dispersal behavior at 440-460 minutes. As compared to position (0,0) for which dispersal occurred at 260 minutes, positions located 3.5mm away from the centre experienced dispersal with 100 minutes delay.

Figure 2.10 shows the confocal microscopic images of biofilm development and dispersal along the x-axis with selected time points. The biomass, structure and dispersal events were symmetrical over the centre of y-axis. Further from the centre (0,0), biofilm can achieve much higher biovolume with bigger cluster before dispersions. Although the flow velocity is similar, the differences of biofilms in terms of structures and biovolume are significantly different when the biofilm was 3.5mm from the centre in the horizontal direction as compared the same distance from the vertical direction.

Figure 2.9 Mean biovolume measurements during 10h of *P. putida* biofilm development along the x-axis of the square camber.

Figure 2.10 Projected confocal laser scanning microscopy images at selected time points showing *P. putida* biofilm development along x-axis. Scale bar is 50 μ m

2.3.2 Study of *P. aeruginosa* biofilm development under well controlled flow conditions

The biofilm development of *P. aeruginosa* was investigated using the same methods that were employed to study the development of *P. putida* biofilm. . Biovolume measurements illustrate the development and dispersion of *P. aeruginosa* biofilm from the upstream (0,7.0), center (0,0) to downstream (0,-7.0) along the y-axis (Figure 2.11). The onset of dispersion took place after 440 minutes of growth at the furthest position from the outlet. Within 60 minutes, the dispersion propagated downwards up to position (0,-7.0) next to the outlet. As compared to *P. putida*, biofilm dispersal occurred 200 minutes later in *P. aeruginosa*, however the duration of complete dispersal was almost identical in both species.

Figure 2.11 Mean biovolume measurements of *P. aeruginosa* biofilm during 10h growth along the y-axis.

Figure 2.12 Projected confocal laser scanning microscopy images at selected time points of *P. aeruginosa* biofilm along y-axis. Scale bar is 50 μ m

Figure 2.12 shows confocal images of events at selected time points (440 minutes to 520 minutes) and positions (0,7.0), (0,0) and (0, -7.0).

Biovolume measurements along the x-axis shown in Figure 2.13 show a similar trend as observed with *P. putida* (Figure 2.9). The difference resides in the maximum biovolume and latest dispersal that took place more than 200 minutes later as compared to *P. putida*.

Figure 2.13 Mean biovolume measurement of *P. aeruginosa* during 10h of biofilm development along the x-axis.

Figure 2.14 shows confocal microscopy images of events that occurred (taken along the x-axis) within the 2h window of dispersal propagation that started at the centre position. In *P. aeruginosa*, the biofilm completely dispersed within 80-100 minutes, which is consistent with results achieved in *P. putida*.

Figure 2.14 Projected confocal laser scanning microscopy images at selected time points of *P. putida* biofilm development along x-axis. Scale bar is 50 μ m.

Figure 2.15 Distinct behaviors of *P. aeruginosa* and *P. putida* during the first 3h of biofilm development. The distinct surface motility behavior of both strains impact on their ecological strategy. The spatial distribution of *P. aeruginosa* on the surface continuously changes while *P. putida* remains attached to the surface at the same position. Scale bar is 50 μ m.

P. putida and *P. aeruginosa* exhibit major differences in the first hours of biofilm formation. Figure 2.15 shows the first 3h of biofilm development for both bacteria. While *P. putida* attaches and forms aggregates, which grow and remain at the same positions, *P. aeruginosa* cells did not form aggregates at such early stages, but instead, continued to move across the substratum, most likely leaving substances (such as rhamnolipids) on the surface. As a consequence of that, *P. aeruginosa* grew in the flat and wide spread biofilm covering the surface, while *P. putida* developed in a clustered, patchy biofilm.

During the dispersal, *P. putida* cells might have been immediately released in the environment whereas cells of *P. aeruginosa* might have first rearranged at the surface of substratum, resulting in a flat and homogeneous layer prior to being dispersed in the environment (Figure 2.16).

Figure **2.16** Differences in morphological transformation of *P. aeruginosa* and *P. putida* during dispersal. *P. aeruginosa* first develops into an even and thin biofilm layer before major dispersal while *P. putida* dispersed fast as compared to *P. aeruginosa*. At time t_1 the biofilm reached maximal biovolume, followed by dispersal. t_2 :20 minutes after t_1 . t_3 :20 minutes after t_2 Scale bar is 50 μ m.

2.4 Discussion

Biofilm development is a complex biological phenomenon that varies with time and space. Sophisticated experimental tools that can recreate complex well defined environmental conditions and allow quantifiable observations at microscale levels are required to study biofilm behavior and social interactions as function of specific physical or chemical parameter. In this chapter, we have developed and validated a next generation flow cell apparatus consisting of 4 inlets/outlets that allow for generation of stable and controlled flow gradients. The gradients in the flow cell can be further translated into complex, but defined, physical and chemical microenvironments. The microfabrication by micro-milling and laser bonding allowed precise control of multi-channel dimensions and eliminated the formation of

toxic by-products which can affect biofilm development. Simulation and validation of the flow profile demonstrated that the flow appears to be chaotic and unstable, at regions 3mm close to the inlet/outlet openings. This problem will be overcome by extending the channel length and funnel profile at the openings to avoid drastic changes of the cross-section area. However, beyond these regions, the flow velocity remained controlled, stable and consistent with the simulation. Considering that experiments were run at constant and stable flow rate, extrapolation of the flow pattern provides some insights into the nutrient and oxygen availability at the different regions across the chamber.

Figure 2.17. Zones of biofilm development in the square flow cell

Using the square chamber together with precise set of flow regimes and real-time imaging, the development of *Pseudomonas spp.* biofilm was investigated at high spatial and temporal resolution for over 10 hours. Observations could be classified into 2 categories: biomass build up and biofilm dispersal as a function of time and space. Considering the biomass build up, four zones were defined across the chamber (Figure 2.17). Zone A consisted of the most upstream positions, showing minimal biomass development. Considering that the flow

velocity observed in the region was minimal, we speculated that there was minimal supply of nutrients and oxygen. Zone B consisted of the regions along the inlet sides. Positions in this region exhibited similar and homogeneous biomass build up. This correlated to a symmetrical supply of nutrients and oxygen. It is important to note that flow rate is directly proportional to the dimensions of the region. The central region (zone C) where the symmetrical flows merged, demonstrated a heterogeneous, yet reproducible, biomass build up. This result was most likely due to variability in the nutrient and oxygen supply together with accumulation of metabolic waste along the flow towards the outlet. Nutrient and oxygen limitation and accumulation of toxic metabolites can partially explain the onset and propagation of dispersal within the flow cell (from upstream zone A through upstream zone B and downstream zone C, Figure 2.17) Thus, in Zone A, the area where flow is minimal, biofilm dispersal started the earliest as compared to Zone C and propagated along the y axis, in the same direction as the flow. This is not consistent with previously published observations, where biofilm dispersal propagates from lower to higher flow velocity in opposite direction of flow. However, the flow cells employed in the publications were designed to assess and quantify the impact of shear forces on biofilm behavior indicating that dispersal was most likely a result of shear forces coupled with nutrient and oxygen limitations, and metabolites accumulation³³. In our present experiments, there was no significant shear force to influence biofilm development. The last dispersal occurred close to the outlet suggesting that nutrient/oxygen limitations and metabolic waste accumulation are not the only parameters that could influence biofilm dispersal in this environment. Since dispersal is initiated upstream, signalling molecules secreted prior and during dispersal may potentially trigger biofilm dispersal in the downstream regions. This would require further investigations.

When comparing the behaviors associated with the biofilm development of *P. putida* and *P. aeruginosa*, significant similarities as well as clear differences were observed. In both the

strains, biovolume loads were found to be higher under higher flow allowing both biofilms to sustain at higher rate of nutrient supply. In addition, the dispersal of biofilm in both the strains initiated close to the single outlet of the square chamber (zone where metabolites produced upstream are accumulated). In contrast to the flat and homogenous morphology of *P. aeruginosa* biofilm, the *P. putida* biofilm was highly heterogeneous, comprising separated aggregates that reached a maximal size before biofilm dispersal was observed. As compared to *P. putida*, the reduction of biovolume measurements was not as abruptive in *P. aeruginosa*. A clear transition was observed with *P. aeruginosa*, with biovolumes slightly decreasing before a significant drop in biofilm biomass, illustrating a massive removal of cells from the surface.

This variation in the biofilm architecture may be attributed to the differences in surface motility between the two species. In literature, swarming motility has been shown to significantly influence biofilm formation and architecture. By using *P. aeruginosa* as a model organism, Shrout *et al.* described ‘a model for nutritional contribution in early biofilm development’¹³⁵. According to this model, nutrient availability controls bacterial communication and surface motility following the attachment of cells to the substratum to influence the initial coverage of the substratum. Thus, bacterial cells with low motility will tend to form aggregates, resulting in structured/patchy biofilms. In contrast, bacterial cells with high surface motility will tend to move across the surface, resulting in a homogeneous and flat biofilm (Figure 2.18).

If nutrient starvation and oxygen limitations were the only factors to cause biofilm disruption, the onset of dispersal should start downstream and then propagate upstream. However, since the pattern observed differs from this scheme, other biofilm dispersal triggers might have contributed to the early dispersal event observed. Signalling molecules released from dispersal occurring upstream may potentially trigger biofilm dispersal downstream. Further

experiments are now warranted to better understand the dynamics of biofilm dispersal in this complex environment.

Figure 2.18. Model for nutritional contribution in early biofilm development¹³⁵ (Taken from Shrout *et al.*, *Mol. Microbiol.*, 2006).

2.5 Conclusion

We have successfully developed a next generation flow through biofilm growth chamber with the unique capacity to generate multiple, complex, well defined and reproducible environmental gradients. The strengths of this flow cell reside mainly in its unique design which allows for (1) multidirectional flow regime and therefore multiple gradients (physical and chemical) that can be defined and controlled, and (2) removal and substitution of the glass coverslip substratum for further phenotypic and genetic assessments. This latter feature enables = the substitution of the glass coverslip with, a commercially available PEN membrane (Leica Microsystem). This will allow for laser microdissection of biofilm areas of specific interest. Isolated samples of $1 \mu\text{m}^2$ could be then further analysed at DNA and RNA levels, adding another dimension to the understanding of biofilm structure, function and dynamics. . However, this system also exhibits few limitations. Firstly, the design requires specific microfabrication technologies, which may render the production quite challenging. The flow system cannot be autoclaved and it is difficult to follow cleaning and decontamination procedures. Therefore, it is recommended to dispose the

flow cell after use., In addition, the environmental gradients generated by the multidirectional flow can be divided into 3 zones where data has to be analysed carefully (Figure 2.17).

As a proof of concept, we demonstrated the biological application of this next generation flow through chamber by investigating the dynamic patterns of biofilm development and dispersal of two phylogenetically related bacterial strains *P. aeruginosa* and *P. putida*. The unique capacity of *P. aeruginosa* to glide onto surfaces using twitching motility and leaving behind a distinct trail of polymeric matrix, resulted in the build-up of a very distinct biofilm structures. Multiple scientific questions could now be further addressed using this new system that is able to generate both homogeneous and complex environmental conditions. Because homogeneous distribution of nutrients and oxygen is present in the 2 symmetrical zones along the inlets, it is now possible to study the impact of a single physical or chemical parameter in the same biological culture. For example, there is no system that enables the assessment, quantification, evaluation of the effect of temperature in a single biological sample to date. Temperature effect on biofilm is evaluated by splitting the same bacterial communities into multiple tubes, wells, flow cells and culturing them in different incubators with different temperatures. These samples, although considered as biological replicates are not identical as they grow in different conditions where the environment is not well controlled. This particular flow cell can be adapted to generate a temperature gradient where the only parameter that will vary is temperature, which is demonstrated in chapter three.

Chapter 3 - Bacterial biofilm development over a thermal gradient

3.1 Introduction

Temperature plays a major role in the kinetics of many biochemical processes. The rate of reaction (how fast a reaction can progress) is always a product of the reaction rate coefficient $k(T)$, which is temperature dependent. The relationship between the reaction rate coefficients with temperature was expressed by Arrhenius's Law

$$r = F e^{-E_o/RT} \quad (9)$$

Where T is the absolute temperature (K), R_o is universal gas constant, E_o is specific activation energy of the process and F is the collision factor. Arrhenius's Law has been applied extensively in biochemical engineering to describe temperature dependence of processes.

In microbiology, temperature control is a critical to obtain optimal growth of cultures in an incubator or large-scale bioreactors. Most bacterial species grow faster at certain high temperatures from 30°C to 37°C while the growth rate declines rapidly and cells become inactive in low temperature environments such as refrigerators. Based on Arrhenius's Law, Ratkowsky et al.³⁵ proposed a modified equation

$$\sqrt{r} = B(T - T_0) \quad (10)$$

where B is a regression coefficient and T_0 is the optimal growth temperature of bacteria which is the intrinsic property of that particular strain or species. This equation was experimentally verified by several batch cultures³⁵.

Almost all mammalian cell lines and bacterial species function effectively within a small temperature range and low or elevated temperatures can lead to decreased functional

efficiency and ultimately cell death. Growth patterns of many organisms vary depending on temperatures^{136,137}. Microorganisms are classified into 3 large groups with respect to their temperature preference. Mesophilic organisms, which include most of human pathogens, grow well in temperature ranging from 20 to 45°C.¹³⁸ Organisms growing optimally at lower temperatures (even below 4°C) are classified as psychrophiles.¹³⁹ Thermophile organisms grow optimally at temperatures over 45°C.¹⁴⁰ In practice, control of temperature is therefore a critical parameter that enables the control of bacterial growth and death. In research laboratories, optimal temperature is required to ensure optimal growth in experimental conditions and in industrial environments, temperature is used as a treatment to eliminate or avoid microbial contaminations.¹⁴¹

Molecular mechanisms by which bacteria sense temperature and adapt to different ranges of temperatures have been a subject of many studies using a variety of axenic cultures. Biological thermosensors that have been identified and characterised include proteins, nucleic acids or membranes (figure 3.1)¹⁴². Structure of specific proteins can change at certain temperatures, which in turn alters the protein functionality. If the thermosensitive protein has a key role in gene regulation, the effect of temperature may be significant. Furthermore, activity of other thermosensors such as sRNA is highly temperature dependent. In *Listeria monocytogenes*, for example, small RNA *pfrA* is destabilised at 37°C, temperature indicative of host invasion¹⁴³. This results in the expression of several virulence-related genes, such as those encoding for adhesins, or immune modulating factors. Lastly, changes in temperatures can result in alterations of the membrane structure which in some cases results in the activation of a two-component signal transduction pathway.¹⁴⁴

Figure 3.1. Illustration of biological thermosensors. (A) DNA thermosensor: At low temperature, DNA is folded, allowing binding of repressor molecules and inhibition of transcription. At higher temperature, melting of the bend promotes transcription. (B) RNA thermosensor. At low temperatures, the structure prevents translation as opposed to higher temperatures where translation initiation occurs. (C) Protein thermosensor. At low but not high temperatures, dimerisation of a repressor protein promotes binding to DNA and inhibition of transcription. (D) Lipid-protein thermosensor. Alteration of the membrane fluidity and thickness can occur at different temperatures affecting thus transmembrane protein-lipid interactions and regulates protein activity as a function of temperature (Taken from Sengupda *et al.*, *Curr.Biol.*, 2013).¹⁴⁵

Our present knowledge of effect of temperature on bacterial growth, behavior and function is based on classic molecular biology studies. These studies mostly used axenic homogeneous cultures of model bacterial strains grown in culture conditions where temperature is not the only variable parameter. In *P. aeruginosa*, the *pel* operon is thermoregulated and since it regulates the production of matrix exopolysaccharides and redox cycling in biofilms, both the processes are indirectly thermoregulated as well.^{146,147} There is a significant knowledge gap in understanding the impact of temperature on biofilm development and behavior mostly due to the non-availability of an experimental set up that allows accurate and high content assessment of temperature in biofilms. In this chapter, we aimed at developing a sensitive

temperature controlled system, which combined with the technology developed in the previous chapter, will enable us to study behavioral and functional patterns of model bacterial species that are temperature regulated.

3.2 Material and methods

3.2.1 Experimental apparatus

The experimental system designed and further developed in this chapter is based on a cooling and heating apparatus placed at the two opposite inlets of the flow through chamber developed in chapter 2. (Figure 3.2) Cooling and heating apparatus consists of a hollow aluminium block, which allows exchange of heat with the media (in the tubing) before it enters into the flow chamber.

Figure 3.2 Building blocks of the temperature gradient flow cell

The hot block is heated by a Kapton film heater (Omega Engineering Inc.), where temperature is monitored by a 4-wire thermistor (Omega Engineering Inc.), providing temperature feedback to an auto tunable proportional–integral–derivative (PID) controller with solid state relay output to control the DC power supply to the heater. The cold block at the other hand is cooled down by a thermoelectric (Peltier's) unit which has one surface

attached to the aluminum unit. The other surface can be effectively cooled down by a heatsink continuously blown by a miniature DC exhaust fan unit. The temperature of the cold side is also monitored and controlled by a second PID controller. The system can be then mounted on the motorised microscope stage enclosed in an on-stage incubator as shown in figure 3.3.

Figure 3.3 Assembled flow cell with temperature gradient control system positioned on microscope stage of the Confocal Laser Scanning Microscopy. (1),(2): Inlets; (3): Outlets. (4): hot block heat exchanger. (5) cold block heat exchanger.

Validation of temperature simulation

Rhodamine B (rhB, Sigma-Aldrich) is one of the most popular fluorescent dyes used to visualise temperature as its fluorescent intensity changes significantly and reversibly as a function of temperature. The fluorescent intensity of rhodamine B 10 μ M solution in a multi-well plate at different temperatures was read by spectrophotometer (Tecan Infinite 200Pro, Excitation at 545nm, Emission at 565nm) to assess the dependency of fluorescence on temperature. For calibration, the flow cell was filled with a solution of Rhodamine B (10 μ M) and mounted on the microscope under temperature controlled conditions (30°C). The temperature was raised up to 42°C in 2°C increments. At each step, the temperature was left

constant for 2h to allow the system to reach a steady state prior to scanning. Fluorescent intensity of the rhB was assessed at 64 locations (frames) across the flow cell using confocal scanning mode (Leica SP8 Laser Confocal Microscopy system). The positions of 64 locations in the flow cell are illustrated in figure 3.4. Laser excitation was set at 543nm and 10% power. Fluorescent emission was collected by a hybrid detector from 554nm to 600nm. With zoom factor of 7.00 and 1000Hz speed, each frame contains $16 \times 16 = 256$ pixels and measured at $83\mu\text{m} \times 83\mu\text{m}$. Each pixel has an intensity value from 0 to 256. By using confocal scanning mode, the out of focus signal was eliminated by the pin hole of 1 airy unit giving the optical section of $7.2\mu\text{m}$. The brightest image of each location/ frame was acquired and extracted at the mean fluorescent intensity at each temperature point. The mean of signal intensity was plotted against temperature for calibration.

Figure 3.4 Map of locations of image acquisition to quantify temperature in the flow cell.

In order to visualise the temperature distribution of the system to be used for biofilm growth, we used the same image acquisition parameters as calibration to acquire fluorescent intensity of rhodamine B ($10\mu\text{M}$) across 64 locations. The temperature of the on-stage incubator was

set at 35°C, the hot block at 42°C, and the cold block was kept at 30°C. The entire flow cell was then filled with the prepared rhB solution and incubated for 4h to stabilise the system before performing any experiment. To prevent formation of any air bubbles at elevated temperatures, the liquid supplying the hot end was pre-heated to 50°C and de-gassed in an ultrasonic bath before being withdrawn into the gas tight syringe (KDS, 20mL glass syringe). The stable state temperature distribution inside the flow chamber was simulated by COMSOL Multiphysics 5.1 (COMSOL Inc.) software at the flow rate of 1 and 2mL/h (See Appendix 4 for the simulation model).

3.2.2 Biofilm development under temperature gradient

Strains of *P. putida* YFP (OUS82) and *P. aeruginosa* GFP (PAO1) were used to investigate the effect of temperature on biofilm behaviors. Following the preparation of the flow cell as described above, bacterial inoculum was prepared and injected inside the chamber, as described in chapter 2 while media was continuously supplied by syringe pump placed inside the temperature controlled chamber. M9 supplemented with glucose and casamino acid was used as a nutrient medium. To generate a temperature gradient across the flow cell, the two inlets were kept at 30°C and 42°C, respectively. The flow cell system was mounted on the stage of a confocal microscope (Zeiss LSM 780), inside a temperature controlled chamber keeping the system at a constant temperature of 35°C. The experiment was performed for 10h and biofilm development was monitored in real time at multiple positions. The same acquisition parameters as mentioned in chapter 2 (with the exception of the time interval lowered down to 10 minutes) were used in this study. Images acquired were processed and analysed using Imaris software, as described in chapter 2. Experiments were performed in duplicates for both *P. aeruginosa* and *P. putida* to ensure reproducibility of the observations.

3.3 Results

Simulation and validation of the temperature gradient

Temperature distribution in the flow set up of 2-inlets/1-outlet, a flow regime of 1 and 2 ml/h and temperature ranging from 30 to 42°C as shown in figure 3.5 with the gradient established across the x-axis were simulated. According to the simulation the spectrum of gradient was dependent on the flow rate as a broader temperature gradient (at the region of interests in the flow cell) was obtained with a flow rate of 2mL/h (Figure 3.5B) as compared to 1mL/h (Figure 3.5A).

Figure 3.5 Simulations of temperature distribution across the biofilm growth chamber. Cold and hot sides set at 30°C and 42°C, respectively, (A) flow rate of 1mL/h. (B) flow rate of 2mL/h.

To ensure that rhB was a good chemical candidate to monitor temperature, we assessed the fluorescent intensity of 10 μ M rhB solution using a spectrophotometer. Results showed a good correlation between rhB fluorescence and temperature suggesting that it could be used for further validation of the temperature gradients (figure 3.6). A linear relationship of fluorescent intensity with temperature was observed when the mean intensity of rhB was plotted from the calibration experiment ranging from 30°C to 42°C (figure 3.7). Based on the slope on the linear fitting curve of calibration data, we converted the acquired signal intensity

into temperature from the gradient validation experiment where fluorescent intensity of rhB was acquired.

Figure 3.6 Fluorescence intensity of rhodamine B at different temperatures measured by spectrophotometer.

Figure 3.7 Calibration curve of rhodamine B fluorescent temperature reporter

Although the flow cell cavity has dimensions of 20mm×20mm, we were only interested in the area of 14mm×14mm at the centre where the flow is not disturbed by the turbulence at the inlets/outlets. Based on the calibration and the validation data, the estimated temperature gradient in the central area of 14mm×14mm are represented in the below heat map (figure 3.8). As the gradient primarily built across the inlet-to-inlet direction (x-axis), the estimated and measured temperature profiles along the x direction were plotted and compared (figure 3.9). The heat map showed that we can obtain approximately 1.0°C difference over a 2mm

distance in the flow cell. Consecutive measurement at 3h interval demonstrated a constant temperature gradient.

Figure 3.8 Heat map of the estimated temperature across the central area of 14mm×14mm.

Figure 3.9 Temperature gradient based on A) COMSOL simulation and B) Measurement of rhodamine B fluorescence intensity. X-axis is the position along the horizontal direction.

Figure 3.10 Overtime changes in biovolume measurements along the central y-axis in the region where temperature is approx. $35 \pm 0.36^\circ\text{C}$.

Impact of temperature on *P. putida* biofilm behavior at early stages of development

The first few hours of biofilm formation of *P. putida* OUS82 were monitored along the y-axis in a zone where temperature is constant at approximately $35 \pm 0.36^\circ\text{C}$. The first dispersion event was found to initiate at the uppermost and centred area (Zone A, position 0,7.0) and propagated downstream with the last dispersal event observed at the position near the outlet channels. This was already identified as the area, which has the least nutrient supply rate in the chapter 2. The dispersion sequence initiated at 250 minutes (as compared to 240 minutes in chapter 2).

Figure 3.11 Confocal Laser Scanning Microscopy images of *P. putida* biofilm along y-axis at the important time points of dispersal. Scale bar is 50 μ m

Similar analysis was applied for biofilms developed along the x-axis that experienced a temperature gradient ranging from 32.0-39.0°C. Biofilms observed at higher temperatures dispersed earlier (within 30 minutes) than their equivalent positions in the lower temperature range (Figure 3.12 and 3.13). We observed that at temperatures below 35.5°C biofilms tend to have increased biovolume as compared to those at positions at higher temperatures. This was even more evident for positions (7.0,7.0), (0,7.0) and (7.0,-7.0) near the inlets. Positions located in the temperature of approximately 32.0°C had the highest biovolume measurement and the most delayed dispersal initiation time point (around 500 minutes).

Figure 3.12 Quantification of *P. putida* biofilm development along x-axis under temperature gradient

Figure 3.13 Confocal Laser Scanning Microscopy images of *P. putida* biofilm along y-axis at selected time points. Scale bar is 50µm

The combined effect of temperature and flow configuration differences were shown in figure 3.13 during the examination of 4 distinct positions [(3.5,3.5), (3.5,-3.5), (-3.5,-3.5) and (-3.5,3.5)] which have the unique conditions for comparison (low temperature-low flow, low temperature-high flow, high temperature-high flow, high temperature-low flow respectively). Position (3.5,3.5)- (low flow-low temperature) has higher biovolume compared to positions (-3.5,3.5) and (-3.5,-3.5) at the high temperature end but dispersed earlier than position (3.5,-3.5) due to lower flow (figure 3.14). For all positions, the dispersal events took place in the same order as in chapter 2, with respect to the flow velocity gradient. Biovolume was found higher in position with lower temperature in the flow cell.

Figure 3.14 Quantification of *P. putida* biofilm at 4 positions with different fluidic and thermal conditions.

Impact of temperature on *P. aeruginosa* biofilm behavior at early stages of development

Exposure of *P. aeruginosa* to temperature gradient resulted in different biofilm morphology and behavior as compared to *P. putida*. Figure 3.15 depicts the mean biovolume measurements along the x-axis with a temperature gradient ranging from 32°C to 39°C. The first dispersion event took place at highest temperature and near the inlet with very small biovolume built up. Positions (-3.5,0) and (0,0) dispersed at 460 minutes followed by the last two at lower temperature (figure 3.15). As seen in chapter 2, *P. aeruginosa* grown under room temperature (*ie*, 25°C) dispersed after 640 minutes at position (7.0,0) with the maximum biovolume load of 22000 μm^3 . Increase of temperature up to 32°C resulted to increased biovolume (33000 μm^3) but earlier dispersal (520 minutes). This distinct growth behavior at 32°C observed with *P. putida* was not observed with *P. aeruginosa*.

Figure 3.15 Quantification of *P. aeruginosa* biofilm development along x-axis under temperature gradient.

Figure 3.16 Confocal laser scanning microscopy images of *P. aeruginosa* biofilm along y-axis at the important time points of dispersal. Scale bar is 50μm

3.4 Discussion

By combining this unique biofilm growth chamber with the temperature controlled system it is now possible to generate experimental conditions where temperature would be the only environmental parameter to vary. In chapter 1, we demonstrated that areas located in Zone B, which consist of the 2 symmetrical areas along the inlets, have similar environments (nutrient, oxygen, flow, etc...) and this translates into similar biological phenotypes. Thus, manipulation of the flow rate or the temperatures at the 2 inlets, this homogeneous zone can be enlarged or reduced. Thus, for the first time, we assessed and quantified the impact of temperature on the same biological sample, in the same bacterial community.

Temperature difference across the flow cell formed an environment for biofilm to express different morphologies along a continuous gradient in a single chamber. This allowed us to mimic natural and human body environments where temperature gradient play a key role for the selection and development of bacterial biofilm^{51,148}. Loose structures with less biomass were found in biofilms developed at higher temperature (37-39°C) as compared to the same condition at lower temperature (32-35°C). More importantly in this experiment, high temperature also induced early dispersal of biofilm structure which was dispersed much later in the flow cell at lower temperature. While nutrient supply was equivalent at both sides, the dispersal at high temperature could involve other molecular mechanisms in addition to nutrient starvation and quorum sensing-induced dispersal. Faster image acquisition sequence every 10 minutes, allowed us to closely follow dispersal events. With the introduction of a temperature gradient along the x-axis, the symmetrical behavior of biofilm dispersal along the x-axis showed in the previous chapter was no longer observed (figure 3.17). For *P. putida*, optimal temperatures for biofilm development ranged between 25°C to 35°C. At higher temperatures, lower biovolumes and early biofilm dispersal (3h) were observed. For *P.*

aeruginosa, optimal temperatures for biofilm development ranged between 32°C to 37°C. At higher temperatures, lower biovolumes and early biofilm dispersal (4h) were observed.

Figure 3.17 Illustration of biofilm development and dispersal patterns under temperature gradient in a flow set up of 2 inlet/1 outlet and a flow rate of 2 ml/h.

The behavior of biofilm development and dispersal subjected to well-controlled temperature gradient under highly reproducible environment selection was studied for the first time (to the best of our knowledge) on two phylogenetically similar bacterial strains of *P. aeruginosa* and *P. putida*. In both strains, maximal biofilm biovolume measurements were generated at temperatures ranging from 35 to 37°C, and were lower when biofilm was exposed to temperatures greater than 37°C. Yet, biofilm produced by *P. aeruginosa* is significantly more resilient to exposure to temperatures ranging from 37 to 39°C. Indeed, if biofilm dispersal was found to be delayed as compared to *P. putida*, the remaining biofilm after this first dispersal event was also found to be significantly higher.

3.5 Conclusion

Temperature is an important and universal physical parameter that is known to regulate multiple biological processes. Our present knowledge of the effect of temperature on the growth, behavior and function of bacterial biofilms is fairly poor; partially due to the lack of experimental set ups with the capacity to generate accurate and stable temperature gradients. In this chapter, we successfully developed a sensitive temperature controlled system, which combined with the next generation flow through chamber developed in the previous chapter, has enabled us to study behavioral patterns of *P. aeruginosa* and *P. putida* at relevant temperatures. The experimental system described here could now be further employed as a model for the behavior of more diverse microbial communities of either collection of well-defined model strains or clinical isolates. This would lead to addressing questions on the behavior patterns in complex biofilms affected by temperature responses using medically relevant communities such as those in the human airways^{149–151}, skin^{152,153}, vagina¹⁵⁴ and mouth¹⁵⁵.

Finally, as shown in this chapter, temperature affects major biological processes that are relevant to biofilms. These processes involved cellular modifications that are likely to generate heat. Biofilm relevant events such as maturation or dispersal could potentially be characterised through monitoring of temperature. In the next chapter, we aimed at developing an innovative 3D Thermopile or Chip Calorimeter with the objective to describe heat signature profiles of biofilm dispersal.

Chapter 4 - Concept and fabrication of a 3D thermopile for chip calorimeter applications

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4.1 Introduction

Temperature or heat is a central and fundamental physical parameter affecting, but also characterising, many vital biological processes^{63,156,157}. Almost all enzymatic reactions occurring in a living cell, release small amounts of heat, which can be monitored using sensitive thermal sensors^{84,101,158,159}. Science that relates to the monitoring of chemical and biological processes in a small volume at constant temperature using heat flow is called Isothermal Micro-Calorimetry (IMC). IMC has numerous advantages which include minimal and non-destructive sample preparations, fast results, and continuous real time observations of fluctuating heat production, allowing for the monitoring of bacterial metabolic activity (Figure from chapter 1) or cell replication⁷². Most of the classical IMC systems have also significant limitations, which include a 1h equilibration phase, during which the samples cannot be monitored; samples are placed in closed environments where nutrient and oxygen are rapidly changing, and metabolic waste accumulates. Finally, the heat signals that are generated by the samples that are captured by the system are non-specific, as it corresponds to the sum of all chemical and physical processes occurring in the samples. The latter limitation is of particular concern, and highlights the importance of carefully designed IMC experiments with appropriate controls that should be performed in parallel.

To circumvent some of these limitations, thermal sensors based thermopiles have been designed and fabricated at the micrometre scale^{85,160–162}, where some of them have an opened chamber for the samples^{77,79,82}. Conventional thermopile sensors were fabricated on silicon wafer to form free standing structures where the silicon under the hot junction was etched completely to enhance thermal insulation and sensitivity^{77,78}. Usually those thermocouples with a few micrometres in width are laid on a silicon wafer surface by consecutive thin film deposition and etching. Bulk silicon was selectively removed by alkaline back etching to generate free-standing membranes as hot junctions (figure 4.1). However, those free-standing thermopiles were fabricated as in-plane structure, which limits the sensing area to one dimension and, have limited efficiency and flexibility, due to the fact that hot and cold junctions share the same plane.

Figure 4.1 Conventional micro-fabrication technologies to form thermopile on free standing membrane (Taken from Herwaarden *et al.*, *Thermochimica Acta*, 2005)⁷⁷ (a) Aluminium- n type silicon (b) Doped polysilicon (p-n) thermopile (c) Combination of a and b processes.

Three dimensional thermopiles with vertical thermocouples have been recently reported^{163–165}. (Figure 4.2) In the 3D design, which has the potential to almost double the packing

density of thermocouples, the hot junctions are now located at the top plane, while cold junctions are placed at the bottom plane. Further, the device became less fragile due to the absence of the free-standing membrane. The sensing area at the top of the surface has significantly increased, thus improving the efficiency and flexibility of the device, as compared to an in-plane 2D thermopile layout.

Figure 4.2 Illustration of advanced 3D thermopile structures. (A) Bridge structure (reproduced from Huang *et al.*, *J. Micro/Nanolithography. MEMS. MOEMS*, 2013)¹⁶³. (B) Stacked p/n junctions (taken from Allison *et al.*, *Sensors & Actuators A*, 2003)¹⁶⁵.

As discussed in Chapter 1, IMC has been widely used in the medical field, for the study of antimicrobial, bactericidal or bacteriostatic effects, the detection of bacterial or viral contaminations in human samples; and in environmental microbiology, for the assessment and monitoring of bacterial activities in various samples, including soil.^{73,166}

Micro-calorimetry has been shown to be a powerful strategy to study biological processes generating heat, including in biofilm settings, we speculated that the dynamic nature of bacterial biofilms observed in the previously chapters could be further characterised and

studied using heat flow fluctuations. Ultimately, each biofilm, each biological and ecological event driven by enzymatic metabolic reactions could be monitored and associated to a specific “heat signature”. In this chapter, we developed a new generation of chip calorimeter which employs a thermocouple array in a 3D architecture, allowing for a higher sensing element density and a multichannel device for calorimetric mapping. The innovative aspects of this new device reside in its 3D architecture which is based on the crystalline structure of the silicon substrate. In this design, the fragile free standing membrane is not required and the sensing area is extended to fit any growth surface. As a proof of concept, we used this newly developed chip calorimeter to monitor biofilm development of *P. aeruginosa* and *P. putida*, and elucidate the heat signature of biofilm dispersal.

4.2 Material and methods

4.2.1 Concept and design of a novel chip calorimeter for biofilm process applications

Figure 4.3D illustrates the structure of the newly designed 3D micro-fabricated thermopile, which is laid on the template of a 3D etched substrate. Use of a monocrystalline silicon (100) wafer with anisotropic orientation in an alkaline etching solution resulted in a sloped wall structure with a slope angle of 54.5°. This allowed thin film deposition of thermoelectric metals on the etched structure. The top of the 3D structure was the hot junction, while the bottom of the etched area was the cold junction. This design allowed the biofilm to be developed on a thin substrate, which was directly on top of the hot junction, so that heat can be transferred through the thermopile structure thereby generating an electrical signal.

Figure 4.3 Thermopile on silicon substrate with 2D and 3D microstructures (Adapted from Huang *et al.*, *J. Micro/Nanolithography. MEMS. MOEMS*, 2013)

Figure 4.4 Schematic structure of the proposed chip calorimeter with a cell layer that is growing and producing heat transferred through the isolation layer above hot junction of the sensing structure.

Figure 4.5 Thermal circuit of the proposed chip calorimeter.

Figure 4.4 illustrates the structure and components of a chip calorimeter which employed a 3D thermopile as a sensing element. Thermal resistance R in equation 7 is composed of the resistance from the isolation layer (R_1) and the sensor that is contributed by many sensing islands (r_i). Figure 4.5 represents the thermal circuit of the isolation layer and thermal sensors from figure 4.4.

Total thermal resistance is calculated by,

$$R = R_1 + R_2 \quad (11)$$

$$\frac{1}{R_2} = \sum_{i=1}^n \frac{1}{r_i} = \sum_{i=1}^n \frac{A_i k_2}{H_2} = \frac{k_2}{H_2} \sum_{i=1}^n A_i \quad (12)$$

Where R_2 is the equivalent thermal resistance of the thermopile, k_2 is the thermal conductivity of material (silicon), A_i is the average cross-section area of the hot junction and cold junction perpendicular to the direction of heat flow. As the sum of the average cross section area is equal to half the total area of the isolation layer ($A = 2A_i$), we can write equation (10) as below,

$$\frac{1}{R_2} = \frac{Ak_2}{2H_2} \quad (13)$$

Total thermal resistance is,

$$R = \frac{H_1}{Ak_1} + 2 \frac{H_2}{Ak_2} \quad (14)$$

As the thermal resistances (R_1, R_2) are in series, the heat flux $\dot{Q} = \dot{Q}_1 = \dot{Q}_2$. According to Newton's law of cooling, we have

$$\dot{Q}_1 = \Delta T_1 / R_1 = (T_2 - T_1) \frac{Ak_1}{H_1} \quad (15)$$

$$\dot{Q}_2 = \Delta T_2 / R_2 = (T_1 - T_0) \frac{Ak_2}{2H_2} \quad (16)$$

$$(T_2 - T_1) \frac{Ak_1}{H_1} = (T_1 - T_0) \frac{Ak_2}{2H_2} \quad (17)$$

$$\frac{T_2 - T_1}{T_1 - T_0} = \frac{H_1 k_2}{2H_2 k_1} \quad (18)$$

Equation (16) calculates the ratio between temperature differences across the isolation layer and the sensing layers which is dependent on the thickness (H_1) as well as thermal conductivity of the isolation material (k_1). This is because the materials and dimensions of the sensing structure are chosen for a given fabrication process. A thinner isolation layer (H_1) will help to improve the temperature difference which leads to a higher sensitivity. The same principle is applied to thermal conductivity when isolation material can be changed from polymer ($k_1 < 0.8 \text{ W/m.K}$) to glass ($k_1 = 1.38 \text{ W/m.K}$) then to metal or silicon membrane ($k_1 = 100\text{-}130 \text{ W/m.K}$). Optimisation of the heat transfer pathways also contributes significantly to the sensitivity of the device.

4.2.2 Fabrication process

The proposed thermopile is composed of several Ni/Au thermocouples connected in series on an alkaline etched Si (100) substrate. There are 4489 thermocouples distributed over the chip area of $20\text{mm} \times 20\text{mm}$. Each thermal leg is $180\mu\text{m}$ long, $50\mu\text{m}$ wide and $30\mu\text{m}$, each overlapping with the other to form hot and cold junctions. Due to crystallographic properties of silicon (100), alkaline etching will form a cavity with sloped side walls at an angle of 54.7° . The top and bottom surfaces of the cavity are where the thermocouple forms the hot and cold junctions respectively (figure 4.6). The sloped side wall enables conventional photolithography to pattern the thermal leg more easily than the vertical side wall. This template creates a 3D structure thermopile where the hot and cold junctions are no longer on the same plane but two parallel planes. Unlike the conventional micro-fabricated thermopile,

when both hot and cold junctions are in the same plane, the sensing area is limited to only a small portion of the entire chip area; the proposed 3D thermopile utilises the whole upper plane as a hot junction so that the whole chip area can now function as the sensing area. This new advantage offers more experimental choice, allowing the experimental chamber to be flexible in size. The spatial resolution can also be investigated as the thermopile can be divided into smaller sensing areas.

Figure 4.6 Fabrication process of silicon based thermopile

The fabrication process started with wet oxidation of a 4-inch silicon wafer (100) to grow 500nm oxide as an etching mask (figure 4.6a). Shipley S1813 photoresist and its developer were used in all photolithography steps of the fabrication process. The thermal oxide film was patterned by photolithography using Karl-Suss MA6 mask aligner and buffered oxide etching (BOE, $\text{HF}:\text{NH}_4^+=5:1$) solution to form etching windows (figure 4.6b). The silicon wafer was etched in tetramethylammonium hydroxide (TMAH) 25% at 80°C to form cavities with 7μm depth (figure 4.6c). The square cavity had the dimensions of 200μm with 100μm spacing. Due to silicon crystallography, the cavity side wall was sloped at 54.7° which

enables the deposition and coverage of the thermoelectric materials in the later steps. The thermal oxide film was then removed by dipping in BOE solution for 15 minutes (figure 4.6d). After extensive wafer cleaning and drying, Ni thermocouple legs with 400 nm thickness were deposited by electron beam evaporation and patterned by a lift-off process (figure 4.6e). Similarly, 400nm Au thermocouple legs and wire bonding pads were formed by sputtering and lift-off process (figure 4.6f). The width of the thermocouple was 50 μ m with the spacing of 250 μ m. The density of the thermocouple per area was 25mm⁻² in the prototype which can be easily increased with smaller feature size. After wafer fabrication, 6 chips were obtained from each wafer using a dicing saw machine. The fabricated chip was then glued on a printed circuit board (PCB) and gold wire bonding was carried out to form the connection between the chip bond pads and the PCB metal pads. All bonding pads on the silicon chip and PCB was plated with gold by either sputtering or electroplating to improve conductivity and eliminate minor thermoelectric effects at the wire joints caused by different metal junctions. The bonding pads and gold wire were then covered by crosslinked SU-8 3000 epoxy photoresist (MicroChem) with thickness of 180 μ m for mechanical and chemical protection which leave only the sensing area exposed to the environment. The fabricated devices were kept in desiccator with reduced humidity to minimise oxidation and delamination of materials caused by air humidity

4.2.3 Characterisation of the device

After wire bonding and epoxy packaging, a reliability test was carried out by measuring electrical resistance to assess possible material failures at many metal junctions. Due to the high number of metal junctions that were connected by thin film deposition and lift-off, low resistance reflected the junction array integrity with strong layer-to-layer adhesion without any missing junctions.

Morphology of thermocouple elements on the chip were also investigated using scanning electron microscopy (SEM, Hitachi 3500N, Tokyo, Japan) to confirm the 3D structure of the standing thermopile with the hot and cold junctions. The devices were mounted on the rotating stage by carbon and aluminium tape to improve system electrical conductivity. The stage was then placed in high vacuum chamber to avoid electron beam scattering by air molecules. Electron beam was generated by a potential of 5kV to minimize the electron bombardment effect on the thin film surfaces.

Temperature sensitivity

Performance of the device was evaluated by a Joule heating experiment (figure 4.7). A foil heater powered by a DC source was attached on top of the device which was covered by a glass cover slip with 170 μm thickness. The temperature of the heater was monitored by a resistance temperature detector (RTD) thermometer (SA1-RTD-4W, accuracy $\pm 0.15^\circ\text{C}$, Omega Engineering). The whole system was placed inside an environment chamber with a pre-set temperature of 25°C to minimise the temperature fluctuation of the surrounding environment.

Figure 4.7 Illustration of the joule heating experiment based on a DC foil heater, which is controlled and monitored by a RTD temperature sensor on top of the sensing device. The bottom of the device is bonded on a printed circuit board, which is placed on an aluminium block acting as a heat sink.

The temperature of the heater was first raised up to 49.6°C and then cooled down step by

step to achieve the output signal at different temperatures. The sensor output was acquired by a nano-voltmeter (Model 34420A, Agilent) and recorded by computer using the LabView program (National Instruments) at the frequency of 1Hz throughout the entire course of experiment. Once targeted temperatures were achieved, the system was maintained for more than 45 minutes to allow stabilisation. Two independent experiments, each including 3 cycles, were performed to ensure reproducibility of the results.

Heat power sensitivity

The heat power sensitivity was assessed using flow reaction experiments. The flow rate was started at 1 $\mu\text{L}/\text{min}$ and increased up to 3, 5, 7 and 9 $\mu\text{L}/\text{min}$. A specific flow chamber with a width of 4 mm, height of 1.5 mm and an effective length on the chip of 20 mm (Figure 4.8), made in polydimethylsiloxane (PDMS, Sigma-Aldrich) was fabricated for this experiment. The bottom of the flow channel was covered by a thin glass coverslip with a thickness of 170 μm and attached to the thermopile device. Two inlets were designed to allow the mixing of Tris (hydroxymethyl) aminomethane (TRIS, 0.1M) and hydrochloric acid (HCl, 0.01M) solutions following their entry into the channel (figure 4.8). The TRIS concentration was 10 times higher than HCl to ensure that the reaction was almost complete. The whole set up was placed in an environmental chamber to minimise temperature disturbance. The injections were controlled by a computer. The bottom of the PCB was attached onto an aluminium block acting as a heat sink with a water bath underneath it to remove the heat regenerated. The output voltage was read by a nanovoltmeter and recorded by a computer using the LabView program. The experiment was performed using 3 independent devices and for each device, the mixing was repeated 3 times.

Figure 4.8 (a) Illustration of the flow reaction experiment in which HCl and TRIS were injected from different syringe and mixed in the chamber. (b) The actual flow chamber on top of the chip calorimeter with 2 inlets and 1 outlet.

4.2.4 Thermostat chamber to enhance background stability

To increase sensitivity of a biological micro-calorimeter, an environment with minimal temperature fluctuations is required. Therefore, the chip calorimeter and the flow cell previously designed (Chapter 2) were both enclosed in a customised double-wall thermostat chamber, which employed several PID controllers and high precision thermal sensors to stabilise the system at the ambient temperature of 25°C (figure 4.9). The flow cell was integrated in the thermostat chamber on top of a thermoelectric unit, which has a similar thermoelectric power as our micro-fabricated sensor. The flow cell was run with only sterile water for more than 10h to evaluate the stability of the system. The output voltage was read by a nano-voltmeter and recorded by a computer using the LabView program.

Figure 4.9 Assembly of the chip calorimeter and flow cell inside the thermostat chamber.

4.2.5. Monitoring of biofilm development using a chip calorimetry

P. putida YFP (OUS82) and *P. aeruginosa* PAO1 GFP described earlier in this work were used in this chapter as model organisms. The chip calorimeter was mounted onto the flow cell and the glass coverslip placed onto the sensing surface of the thermopile sensor. The chip calorimeter and flow cell were then placed in the thermostat chamber set at 25°C. Inoculation of the flow cell with either of the strains was performed following the protocol described in chapter 2. For *P. aeruginosa*, biofilm development of PAO1 GFP was monitored during 10h using the chip calorimeter under different glucose concentrations (0.01%, 0.04%, 0.10%). The calorimetric signal (average $\pm$ STDV) was plotted over time for each glucose concentration. For *P. putida* YFP (OUS82), biofilm was grown for 8h prior exposure to kanamycin (Sigma-Aldrich, 60 or 120 μ g/mL). The effect of kanamycin on biofilms was assessed for 11h using heat signal monitored by the chip calorimeter. The heat signal (average $\pm$ STDV) was plotted for each experiment over time.

4.3 Results

4.3.1 Characterisation and performance of micro-fabricated chip device

The 3D thermopile was fabricated by a CMOS compatible approach and packaged by SU-8 epoxy passivation. A material failure was measured by electrical resistance assay at the metal junctions. Low resistance of $15.24 \pm 3.35 \Omega$ was obtained for 11 out of 14 fabricated silicon chips. The 3 other devices had a resistance greater than $0.5 \text{ k}\Omega$ which indicated possible failures in thermoelectric junctions or wire bond pads. Devices with low resistance were then characterised by a combined approach of microscopy, change of temperature and reaction calorimetry to evaluate their performance. The SEM image confirmed the micro-structure of the fabricated thermocouple array. As illustrated by the images in Figure 4.10b, the micro-structure of the fabricated thermocouple array consisted on a thin metal film covering the sloped sidewalls without any debris or crack found on edge. The two metals overlap on the top of the ridges and at the bottom of the valleys to form hot junctions and cold junctions, respectively.

Figure 4.10 (a) The thermopile chip was diced from the silicon wafer and bonded on a PCB with gold wire bonding. (b) SEM image of the hot and cold junctions of the fabricated thermopiles.

The temperature sensitivity was then assessed using a joule heating experimental set up. The output signals from the chip calorimeter were plotted at temperature differences generated by the foil heater. Results of 3 independent experiments are shown in figure 4.11. When the

temperature was decreased by reducing the voltage supplied to the heater, the output signal dropped accordingly (figure 4.11a).

Figure 4.11 (a) Response of the chip calorimeter to Joule heating. (b) Output voltage vs. temperature plot with linear regression.

Figure 4.12 (a) Response of the chip calorimeter at different flow rates in TRIS/HCl mixing reaction. (b) Output signal vs. flow rate plot with linear regression.

The average output signal at different temperatures was plotted against the temperature in figure 4.11b that shows the linear response of the output signal to the variation of temperature. The temperature sensitivity of the device estimated by the slope of the regression line was $78 \pm 4 \mu\text{V/K}$, which is comparable to other devices in the literature.^{101,167}

Different flow rates of acid and base solutions in the protonation mixing flow cell led to different amounts of heat generated by changing the kinetics of the reaction. The thermopile output signal at different flow rates was plotted in figure 4.12a. The baseline fluctuation when the flow rate is zero was estimated by the standard deviation at 50 nV. The average signal outputs at different flow rates were plotted against the flow rate (figure 4.12b). The slope of the regression line was estimated as $s = 0.5 \mu\text{V} \cdot \text{min} \cdot \mu\text{L}^{-1}$. The enthalpy (heat) of protonation of TRIS is -47.4 kJ/mol ¹⁶⁸. The heat power sensitivity S was calculated using the equation below:

$$S = \frac{s}{C_{\text{HCl}} \times Q_{\text{reaction}}} = \frac{0.5 \times 60}{0.01 \times 47400} = 63.2 \text{ mV/W} \quad (19)$$

where s is the slope of the regression line, C_{HCl} is the concentration of HCl, Q_{reaction} is the enthalpy of protonation. The heat power sensity of 63.2mV/W represents the output when one unit of thermal power transferred through the sysem.

4.3.2 Evaluation of the system stability under flow conditions

Figure 4.13A shows the thermoelectric voltage representing the temperature fluctuations of the water in the flow cell. Results suggest that at least 2h are required prior to any experiment for the system to stabilise. Figures 4.13 B and C show temperature fluctuations at the outer wall and the centre of the thermostat, respectively. The temperature deviation inside the thermostat chamber is lowered down to $10.7 \mu\text{K}$, suggesting that the system could achieve comparable thermal stability with the literture¹⁶⁸ to provide a low noise environment for the sensor.

Figure 4.13 Performance of the thermostat chamber throughout a 12h experiment. (A) Stabilisation of the thermopile sensor inside the chamber, (B) Temperature of the outer chamber for coarse control (T1a1), (C) Temperature of the inner chamber for fine control (T2a2)

4.3.3 Use of micro-calorimetric to assess *P. putida* biofilm development and susceptibility to antimicrobial agents.

Calorimetric measurements acquired during *P. putida* YFP (OUS82) biofilm growth are shown in figure 4.14. Overall, within the time period of 10h, the maximal signal acquired from the biofilm was 11 μV , which is equivalent to a heat production rate of 0.17mW. Three steps were observed based on the output signal observed during the experiments. In the first step, from 0 to 7h, the signal output increases gradually from 0 to 7.42 uV. At 7h and for about 30 min, the heat signal drastically dropped from 7.32 to 5.15uV. Last, from 7.5h to 10h, the heat signal increases on a stepwise manner as compared to the step 1.

Figure 4.14 (A) Calorimetric signal acquired from *P. putida* biofilm growth in 2 independent experiments and their mean value. (B) Correlation of biovolume and calorimetry of *P. putida* biofilm development.

As the experimental conditions used here were similar to the ones performed in Chapter 2, we compared the calorimetric signal with the average biovolume measurements obtained in similar conditions. Of interest, we observed a correlation between the pattern of the heat signal (Figure 4.14 A) and the pattern of the biovolume measurements estimated from the confocal images acquired in chapter 2 (Figure 4.14 B).

Response of *P. putida* biofilm to antibiotic exposure was assessed using kanamycin at 60 and 120 ug/ml (Figure 4.16). Prior to drug exposure the biofilm build up resulted in an overall heat signal of 8.37uV. Following administration of kanamycin, at either concentration, the signal slowly dropped. However, for kanamycin at 60 ug/ml, the heat signal remained stable at a value of 5.18uV, whereas for 120 ug/ml, the signal further dropped to reach the baseline level. This suggests that 60 ug/ml of kanamycin was only able to kill a percentage of the biofilm as compared to 120 ug/ml where most likely all the biofilm-embedded cells were eradicated.

Figure 4.15 Response of *P. putida* biofilms to kanamycin treatment. Biofilms of *P. putida* were grown for 8h prior exposure to 60 ug/ml (red) or 120 ug/ml (blue) kanamycin for 11h. Heat production was monitored over the 10h of experiments.

4.3.4 Use of micro-calorimetric to assess the impact of glucose concentrations on the early stages of *P. aeruginosa* biofilm formation

Figure 4.17 shows the calorimetric signals of *P. aeruginosa* PAO1 growing as biofilm under varying concentrations of glucose. Results indicate that in the presence of 0.01% glucose (0.1g/L), minimal heat was produced by the biofilm (maximal output signal of 2.17uV at

7h). At the 7h time point, the signal dropped to 0uV suggesting biofilm dispersal. At higher concentrations of glucose (0.04% to 0.10%), significantly higher signals were recorded (up to 6.85uV) up to 11h. Heat signal started to drop down and the drop was more significant for 0,04% glucose as compared to 0.1% glucose.

Figure 4.16 Calorimetry of *P. aeruginosa* biofilm in M9 with various glucose concentrations

4.4 Discussion

In this chapter, we aimed at using temperature or heat as a signal output to characterise biological events occurring in biofilms such as biofilm development, biofilm dispersal, or bacterial death. We developed a new 3D thermopile with a monocrystalline silicon (100) wafer with an improve design and structure. The estimated temperature sensitivity and heat power sensitivity of this device was found to be lower than the previously reported chip calorimeters.^{96,169,170} The apparent decreased sensitivity is in fact due to the larger volume of the flow cell that was used in this set-up.^{83,108} Almost all the chip calorimeters described in

the literature can only work with chamber containing a few nLs to hundreds of μ Ls.¹⁰³ In contrast, our system has been designed to accommodate much larger flow cells with volumes in the range of 1mL. The apparent decreased sensitivity was, however, compensated by using high performance materials, such as silicon (above 100 μ V/K). Other materials that can also perform as well as silicon (100) include bismuth, antimony, telluride alloys.¹⁶⁸ The major inconvenience of such materials is that they are not compatible with CMOS process, and therefore, special equipment and procedures need to be established to accommodate thin film deposition and patterning of materials. Furthermore, in the current experimental design, a standard glass cover slip was used to isolate the chip calorimeter from the reaction chamber. Glass coverslip is commonly used in biological applications, for example, biofilm culture to allow for microscopic assessments. The sensitivity and time response of the current chip calorimeter could be further improved by replacing the glass cover slip with thinner membranes to improve thermal isolation and heat transfer between the fluid in the reaction chamber and the thermopile device.

The 3D thermopile combined with the new flow cell was employed to study biofilm development and behaviors using *P. aeruginosa* and *P. putida*. The objective was to evaluate the potential of the 3D thermopile as an experimental approach in the field. First, the heat production or heat signal was monitored while *P. putida* was cultured in conditions similar to the ones described in chapter 2. Heat signal and estimated biovolume correlated until 7h, which was previously identified as the onset of biofilm dispersal. Following dispersal, biovolume and heat signal correlated inversely suggesting that other biological and physical components of the system also generated heat. These components most likely include dispersed cells, which have now switched to planktonic mode. If dispersed cells do not contribute to the estimated biovolume, they are metabolically active, which can also influence the thermal signal.¹⁷¹ Other factors include reattachment of dispersed cells that may also

generate heat. These preliminary results should, however, be carefully interpreted, as the experimental approach has several limitations. First, the total biovolume of the biofilm cannot be experimentally quantified using confocal images, and as a consequence the biovolume measurements used here are only a rough estimation. Then, the current design of the 3D thermopile can only monitor the heat generated by the entire sample, independent of the locations within the chamber. However, we have shown in chapter 2 that biofilm dispersal does not occur homogeneously within the chamber but rather sequentially, depending on the locations within the chamber. The correlation between biofilm dispersal and heat signal can only be speculated and more experiments are required to confirm the phenomenon..

The potential use of the 3D thermopile for screening of antimicrobial agents was evaluated using *P. putida* biofilms exposed to kanamycin at increasing concentrations. Here, we observed a progressive and significant concentration dependent decrease in the heat produced by the biofilm over the course of the treatment. With the highest concentration of kanamycin (mention the concentration) the signal dropped to baseline suggesting a complete eradication of the biofilm. In contrast, after 8h, the heat production observed with the lowest kanamycin concentration remained stable at 5.18uV, suggesting that kanamycin reached its maximum killing, leaving tolerant or resistant biofilm alive. These results are consistent with the literature where response of *P. putida* biofilms to kanamycin, tetracycline and ciprofloxacin exposures were reported using calorimetric signals as readouts^{94,172}. Kanamycin was found to be the quickest (rapid eradication time) and most effective biofilm eradicator, as compared to tetracycline and ciprofloxacin.. Additional flow cells experiments aiming at quantifying the killing effect of kanamycin under these experimental conditions are now warranted to validate the interpretation and the usefulness of these systems for screening of anti-biofilm drugs.

Finally, the impact of nutrient availability on biofilm development was further studied using *P. aeruginosa* as a model organism. Nutrient starvation, especially glucose, is a well understood phenomenon and it is considered as a major cause of *P. aeruginosa* dispersal.^{20,173} Our preliminary results supported the previously described hypothesis that the early dispersal occurring at the upstream regions in the flow cell was caused by nutrient limitation due to minimal exchange of media volume at low flow rate. Thus, increased concentrations of glucose in the medium resulted in a significant increased biovolume and a drastic delay in the onset of dispersal. However, since the heat signal observed represented the sum of the heat produced in the entire chamber, and not at specific locations, it remains difficult to fully understand which are the factors that trigger biofilm dispersal in this complex microenvironment. Further improvements are needed to achieve a high spatial resolution with the 3D thermopile.

4.4 Conclusion

A thermopile with a new 3D architecture was designed, fabricated and combined to a biofilm growth chamber in order to monitor the dynamics of biofilm development and dispersal using calorimetric signal. The device was validated by Joule heating and sensitivity of the apparatus was characterised using protonation mixing. The 3D structure thermoelectric sensor allowed high density and surface packed sensor arrays to couple with the flow cell glass coverslip bottom. The calorimetric data confirmed and supported our observations on biofilm early dispersal, showing the correlation between biomass and temperature changes. Early dispersal of the biofilm due to nutrient (glucose) limitation was detected by the chip calorimeter; however, additional experiments are warranted to fully validate the observations. The device also demonstrated its potential usefulness for the screening of antimicrobial drugs, study of flow and dispersal patterns in various environmental conditions providing users with a fast, simple and non-destructive experimental approach.

Chapter 5 Conclusion and future directions

Combining precision engineering micro-fabrication procedures and thermal sensing methodologies, we developed the next generation of experimental tools that allows reproducing complex and well defined micro-environmental gradients which are highly relevant for biofilms studies. These experimental tools include a high precision microfluidic platform consisting of a flow through chamber with 4 inlets/outlets, a temperature gradient system and a 3D thermopile for chip calorimeter applications. These tools have the potential to bridge significant knowledge gap in understanding the role and impact of temperature on complex communities. Thus, as examples of the possible applications, these tools and procedures were used to examine biofilm development of two phylogenetically-related bacteria: *P. aeruginosa* and *P. putida*, and assess the effect of temperatures on biofilm behaviors.

The unique design of the biofilm growth chamber presented in chapter 2 allows for multiple and well controlled flow set ups and therefore environmental gradients, such as temperature gradients. For the first time to the best of our knowledge, biological conditions within the chamber can be controlled and/or defined by the users through manipulations of the flow set up (number of inlets/outlets) and/regime (flow velocity). Its removable and interchangeable surface also enables the users to easily access the samples for further studies. The size and form of the substrate is also universal, allowing the users to replace the glass coverslip with other substratum of interest, such as polyethylene naphthalate (PEN) membranes, which are widely used for laser microdissection, or substratum covered with mammalian cells for study of host bacteria interactions. The developed platform has some limitations. First, the design of the channel results in a chaotic flow at proximity of the inlets openings. This issue has been recently addressed by extending the channel length and funnel profile, and a new

technology disclosure entitled ‘Design, validation and application of a biofilm growth chamber for highly reproducible biofilms for mono specie to poly-species communities exposed to defined flow regimes and chemical gradients’ is in the process of being submitted to NTUitive’. Due to the dimensions of the chamber the control of the flow velocity and ultimately, the micro-environments, is difficult. Future improvements in the flow cells aim at addressing this point by significantly reducing the dimensions of the cavity. Finally, like many other commercialised flow cell apparatus, this biofilm growth chamber cannot be sterilised via autoclaving. Therefore, further improvement of the technology will aim at addressing this important feature.

There are numerous potential applications for such a unique experimental platform. Models used here as proof of concept were relevant for both environmental and medical microbiology fields. Using *P. putida* and *P. aeruginosa*, we examined the first few hours of biofilm development at high spatial and temporal resolution. We observed reproducible differences in the behavior of these two species in the stage preceding the irreversible attachment. A manuscript entitled: “The first 10 hours in the evolution of biofilm: Similarities and differences in the behavior of *P. aeruginosa* and *P. putida*” is currently under preparation.

In order to study the role and impact of temperature on biofilms, we designed and validated an experimental temperature gradient system that can be combined with the flow cell apparatus developed in chapter 1. This technology is described in a second technical disclosure “Apparatus to support bacterial biofilm development over temperature gradient” that is in the process of submitted to NTUitive. This combined system uses user defined experimental conditions where temperature is the unique variable parameter. This is a unique and exceptional strength of the tool, which has the potential to extend our understanding of temperature effect on biological and cellular processes. The main limitations of the

temperature gradient system are 1) the dependency of the gradient on the flow set up of 2-inlet/1-outlet which limit the flexibility of the flow cell, and 2) the lack of real-time temperature sensing and the long stabilisation time before we can inject the bacterial cells. To improve the flexibility of having more combination of inlets and outlets, cooling and heating blocks with smaller dimensions can be designed and coupled with micro heat exchangers to improve the stabilisation time. Thermal sensing fluorescent probes can be integrated as a thin film coated on glass coverslip to report real-time temperature. As proof of concept and preliminary study, we examined the impact of temperature on *P. aeruginosa* and *P. putida* biofilm behaviors in their early stages of development (first 10h). Interestingly, distinct responses to varying temperatures were observed for these two species that grew and dispersed differently at 32°C and 39°C. These results are coherent considering the natural habitats and potential for pathogenicity of these species.

Finally, to overcome the limitations of microscopic imaging to assess biofilm behavior at high spatial and temporal resolution, we developed a 3D thermopile for chip calorimetry applications. This highly sensitive apparatus enables detection of heat signal and this signal can be correlated to biological processes and events. This technology does not require imaging systems or fluorescent tagged microbial organisms. This experimental approach becomes of particular interest for the study of complex microbial communities, such as those found in the environment or in the human body. The major shortfall of the system is that heat signal captured is non-specific and represents the sum of all biochemical and physical processes occurring in the samples. This shortfall can be easily overcome by running control experiments in parallel. Using this approach, we aimed at defining the heat signature of biofilm build up and dispersal. The results demonstrated the capabilities of chip calorimeter in monitoring the biofilm growth and dispersal which can be correlated with results from biovolumes quantified from microscopic experiments.

The first step of biofilm development (initial attachment) appears to be a complex phenomenon with species-dependent behavioral patterns that result from ecological selection. Our preliminary observations support the significant role of temperature and chemical gradients in this ecological selection. The tools and procedures developed in this thesis provide users with optimal experimental conditions to further examine the impact of temperature on biofilms structure and dynamics, microbial ecology and host-microbe interactions.

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Appendix 1 COMSOL Simulation model of the flow cell

Figure i) Flow cell model for laminar flow field simulation

The flow field was simulated under laminar flow mode of COMSOL 5.1 for water with the following properties,

Density: 1000kg/m^3

Dynamic viscosity: 10^{-3}Pa.s

Compressibility: Incompressible flow

Reference pressure: 1atm

Initial value: velocity=0, pressure=0

Appendix 2 Experimental validation results of the flow set up

Figure ii) Flow velocity measured by PIV (dotted lines) along horizontal direction at the center and 2mm downstream from the center. Solid lines are simulation results

Figure iii) Flow velocity measured by PIV (dotted lines) along vertical direction at the center and 2mm to the right from the center. Solid lines are simulation results.

Appendix 3 Components of the standard minimal M9 media used to grow *Pseudomonas* spp. biofilm

No.	Chemical name	Formula	Supplier	Concentration
1	Sodium phosphate dibasic	Na ₂ HPO ₄	Sigma-Aldrich	48.0mM
2	Potassium dihydrogen phosphate	KH ₂ PO ₄	Merck	22.0mM
3	Ammonium chloride	NH ₄ Cl	Sigma-Aldrich	19.0mM
4	Sodium chloride	NaCl	Sigma- Aldrich	9.0mM
5	Magnesium sulfate heptahydrate	MgSO ₄ .7H ₂ O	Merck	2.0mM
6	Calcium chloride dihydrate	CaCl ₂ .2H ₂ O	Sigma-Aldrich	0.1mM
7	Glucose	D(+)-Glucose	BDH	0.04% w/v
8	Casamino acids		DB Bacto	0.2% w/v

Appendix 4 COMSOL Simulation model of the temperature distribution across the flow cell when a temperature gradient system is applied.

Figure iv) The model for temperature gradient simulation

The flow cell model for heat transfer and flow simulation included the flow cell consisting of multilayers which are assumed to be in perfect adhesion. The flow condition was the same as the flow field simulation under laminar flow condition in figure i. The temperature of the media was maintained by the heat exchangers (hot and cold sides) which also help to maintain the differences of temperature in the solid parts of the flow cell at 2 sides.