Individual-based modeling for biofilm structure control

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**INDIVIDUAL-BASED MODELING FOR
BIOFILM STRUCTURE CONTROL**

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Interdisciplinary Graduate School

Singapore Centre for Environmental Life Sciences Engineering

2017

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Abstract

Biofilm growth is affected by many factors such as substratum surface properties, fluidic conditions, and medium supply. In this thesis, we investigated biofilm structure development by considering the influence of three key factors: biofilm detachment, oxygen availability, and calcium concentration. This thesis mainly focused on the mathematical modeling of biofilms. Biofilm experiments were also conducted to validate the results of mathematical models.

We first simulated the structure formation of *Pseudomonas aeruginosa* biofilms with three detachment mechanisms: shear detachment (SD), nutrient-limited detachment (NLD), and erosion detachment (ED). The simulation results agreed well with the reported data in terms of the effect of SD on producing smooth biofilms, NLD on hollowing the biofilms, and ED on isolating bacterial clusters. We also discovered that biofilm growth could achieve equilibrium when only SD was enabled. ED was important for biofilm structure formation throughout the simulation period, whereas SD and NLD demonstrated their importance only in late stages. In addition, the effect of SD on biofilm structure was less dependent on the detachment coefficients compared with that of NLD and ED.

We conducted simulations and experiments to evaluate the effect of oxygen availability on the initial biofilm growth of *Pseudomonas putida*. Biofilms were cultured in a square chamber with controllable oxygen gradients. The oxygen distribution in the chamber was obtained from fluid simulation. Confocal images of six-hour biofilms were recorded at different oxygen concentrations, and biofilm surface coverage was higher with a higher oxygen concentration. A regression model was developed to optimize simulation parameters. Individual-

based modeling of biofilms generated similar biofilm structures as experimental observations. In the simulated biofilms, oscillations of biofilm growth rates were characterized by surface coverage and cell number, and the periods of the oscillations were smaller with a higher oxygen concentration.

We also investigated the effect of calcium on the biofilm structure formation of *Shewanella oneidensis*. A biofilm growth chamber, called the microfluidic biofilm reactor in this thesis, was developed for this purpose. Biofilm growth was monitored in the reactor under stable calcium gradients. The biovolume and surface coverage of biofilms were considerably greater in locations where calcium concentrations were higher. Based on the literature, calcium was hypothesized to affect biofilm detachment, EPS production, and bacterial surface motility. The hypotheses on calcium influence were tested by simulations, in which the results were comparable to those of experiments. When larger detachment coefficients and low calcium concentrations were applied, the simulated biofilms showed a slight decrease and large variations in biovolume and surface coverage in late stages, which were suspected to be caused by calcium deficiency as indicated by calcium profiles.

This thesis contributes to the state-of-the-art in biofilm mathematical modeling. Unlike most of the existing biofilm mathematical modeling studies, simulations and experiments were compared in detail. Mathematical models were improved to simulate experimental observations and to test hypothesized mechanisms leading to those observations. The approaches proposed in this thesis demonstrates the potential of using a mathematical modeling approach to prioritizing the physicochemical factors according to their effect on biofilm structures.

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Abbreviations

The following abbreviations are used throughout this PhD thesis:

Abbreviation	Description
EPS	Extracellular polymeric substances
IbM	Individual-based modeling
CA	Cellular automaton
iDynoMiCS	Individual-based Dynamics of Microbial Communities Simulator
2D	Two dimensional
3D	Three dimensional
CLSM	Confocal laser scanning microscopy
WWT	Wastewater treatment
SD	Shear detachment
SDC	Shear detachment coefficient
NLD	Nutrient-limited detachment
NLDC	Nutrient-limited detachment coefficient
ED	Erosion detachment
EDC	Erosion detachment coefficient
DC	Detachment coefficient
DCs	Detachment coefficients

Chapter 1 Introduction

1.1 Importance of Biofilm Research

Bacterial biofilms are complex surface-associated microbial communities encapsulated in a self-generated matrix of extracellular polymeric substance (EPS) (Donlan, 2002). The importance of bacterial biofilms increased since their first scientific description (Zobell and Anderson, 1936) and the first recognition of their ubiquity (Costerton et al., 1978; Geesey et al., 1978).

In the early 21st century, the US National Institutes of Health reported that over 80% of human microbial infections were biofilm-related. Biofilms are involved in many persistent infections (Costerton et al., 1999; Donlan and Costerton, 2002). Furthermore, biofilms are responsible for the failure of various medical devices and implants (Donlan, 2001; Donlan and Costerton, 2002). On the other hand, biofilms have been successfully applied to processes such as wastewater treatment (WWT) (Chen and Chen, 2000; Verma et al., 2006; Wilderer and McSwain, 2004) and contaminated soil and ground-water remediation (Singh et al., 2006; Valls and De Lorenzo, 2002). Biofilms also play a major role in aquatic ecosystems (Battin et al., 2003).

The study of biofilm structure formation could be critical for both constructing robust biofilms and eradicating undesired biofilms. Biofilm structure can influence biofilm growth in various aspects. One important example is that it can change the transportation of nutrient and waste products as well as the mechanical stability of the biofilm (Allison, 2003). Many studies have been conducted to investigate the effects of different factors on biofilm structure formation (Picioreanu et al., 1998b; Stoodley et al., 1999b; Wijeyekoon et al., 2004).

However, because many factors are involved (which are reviewed in Section 2.2.2), the mechanisms behind the formation of complex biofilm structures under different conditions are still not very well elaborated.

In conclusion, biofilm research is important considering that biofilms are highly relevant for the health of both humans and the Earth.

1.2 Motivation

In Section 2.2.2, we review the factors that have a significant effect on biofilm growth. Because of the various factors involved, it is difficult to experimentally confirm which factor contributes the most to the observed biofilm characteristics. Hence, mathematical modeling approaches are particularly important. In contrast to experimental biofilms, mathematical models could evaluate the combined effects of multiple factors as well as the separate effects of each individual factor on biofilm development.

Biofilm mathematical modeling is a mathematical representation of actual biofilm systems. More than four decades have passed since mathematical models were first used to study biofilm processes. Different types of models have been developed, ranging from 1D to 3D and from single species to multispecies (Wanner et al., 2006). All existing biofilm mathematical models have their advantages and disadvantages (which are discussed in Section 2.3). An appropriate model should be selected according to the specific questions and requirements of a study. However, “*models are no more than a simplified representation of reality based on hypotheses and equations used to rationalize observations*” (Picioreanu et al., 2004). Biofilm mathematical modeling is still an

ongoing effort that is improving with the increasing understanding of biofilm development from experimental observations.

Although different models have been developed (which are reviewed in Section 2.3.1), further studies are needed before a complete mathematical modeling system can be developed for various studies. Considering the importance of mathematical modeling in understanding biofilms and need for more comprehensive biofilm models, this thesis focused on developing and improving existing mathematical models to study the effect of different environmental factors on biofilm structure formation.

1.3 Significance and Novelty

This thesis contributes to the state-of-the-art in biofilm mathematical modeling in terms of both model development and applications.

1.3.1 Model Development

Biofilm detachment has been extensively studied in the literature. There are different types of models that describe detachment process (Stewart, 1993). The commonly used quadratic dependence of detachment rates on biofilm thickness is an example. Based the causes of the detachment processes, namely shear rate, nutrient starvation, and weakened bacterial adhesion to the matrix, three detachment hypotheses have been proposed (Chambless and Stewart, 2007). Almost all of the existing studies have focused on the final structures when describing the influence of the detachment process. Thus far, no studies have determined the time frame in which the detachment process would significantly influence biofilm structures and which detachment mechanism is predominant in specific biofilm growth stages. Moreover, when simulating the effects of

detachment on biofilm formation, thickness and biomass or biovolume are most frequently assessed, whereas other parameters such as surface roughness and enlargement are seldom reported. Considering the heterogeneous nature of biofilms, a detailed description of biofilm structures is needed and can be achieved by characterizing them with different parameters.

In this thesis, Chapter 3 focuses on describing the improvements to the existing model in an attempt to study the effect of detachment on biofilm development. The three detachment mechanisms were adopted from the cellular automata (CA) method to the individual-based Dynamics of Microbial Communities Simulator, iDynoMiCS. Biofilms were characterized by five parameters (cell number, thickness, surface coverage, surface enlargement, and surface roughness) with simulation cycles of 400 hours. The relative importance of each of the three detachment mechanisms in different biofilm growth stages (from initial growth to late maturation) is described.

1.3.2 Model Applications

In this thesis, we applied mathematical models to study the biofilm growth of two bacterial species under two chemicals conditions. Specifically, we evaluated the biofilm growth of *Pseudomonas putida* under altered oxygen fluxes and the biofilm growth of *Shewanella oneidensis* under different calcium levels.

P. putida OUS82 is a gram-negative, rod-shaped, aerobic bacterium. In aerobic bacterial biofilms, oxygen availability is crucial as it is an electron acceptor that can affect bacterial growth rates (Alagappan and Cowan, 2004). Nevertheless, owing to difficulties in maintaining a stable oxygen distribution environment in

biofilm growth chambers, limited studies have reported the effect of oxygen on the biofilm structure development of *P. putida*.

In Chapter 4, the effect of oxygen availability on the initial biofilm growth of *P. putida* is described from both experimental and mathematical modeling perspectives. A square biofilm growth chamber was used to conduct the biofilm experiments, in which the oxygen gradient could be controlled by adjusting the input and output configurations. The oxygen distribution in the chamber was obtained from a validated COMSOL model. Images of biofilm growth in the initial six hours were taken by confocal laser scanning microscope (CLSM). Simulation parameters were optimized for the experimental conditions by regression using experimental data. Experimental results showed that a significantly higher surface coverage was obtained with a higher oxygen flux. Mathematical modeling using iDynoMiCS with a developed regression method was conducted, which was successful and showed similar findings as the experimental observations. Moreover, oscillated bacterial growth rates were observed for the simulated biofilms.

Shewanella oneidensis MR-1 is a facultative metal-reducing bacterium that is capable of transferring electrons to metal oxides (Fredrickson et al., 2008; Fredrickson and Zachara, 2008). *S. oneidensis* biofilms are important for various environmental and biological processes (Belchik et al., 2011; Cao et al., 2011; Marshall et al., 2006). Calcium concentration has been identified as an important factor in biofilm formation. Indeed, calcium was found to affect *S. oneidensis* cell–cell adhesion and aggregation (Theunissen et al., 2010). However, the mechanisms by which *S. oneidensis* biofilms are affected by calcium concentration has not been fully elucidated.

In Chapter 5, we focus on the effect of calcium on the biofilm formation of *S. oneidensis*. A microfluidic chamber was developed to culture *S. oneidensis* biofilms under stable calcium gradients. Mathematical simulations of biofilms with different calcium concentrations were conducted using iDynoMiCS. Simulation results were similar to the experimental results with respect to the effect of calcium concentration on overall biofilm growth. Moreover, constant monitoring of the biovolume and surface coverage of simulated biofilms for three days showed that in addition to the initial continuous increasing behavior, a slight decrease and large fluctuations were observed with larger detachment coefficients under low CaCl_2 concentrations. Calcium profiles indicated that biofilm growth caused severe calcium deficiency in the bottom biofilm layers after three days regardless of the supplied CaCl_2 concentrations, which might be one reason for the decrease in biovolume and surface coverage.

1.4 Thesis Outline

Chapter 2 provides an overview of the related work in the field of biofilm experimentation and mathematical modeling.

Chapter 3 discusses the simulation of biofilm structure formation with respect to three types of biofilm detachment processes using iDynoMiCS. This chapter is based on a study (Li C, Yilei Z, Cohen Y. 2015. Individual based modeling of *Pseudomonas aeruginosa* biofilm with three detachment mechanisms. *RSC Adv.* 5:3396–3402).

Chapter 4 discusses the effect of oxygen availability on the biofilm growth of *P. putida* OUS82.

Chapter 5 discusses the effect of calcium concentration on the biofilm growth of *S. oneidensis* MR-1.

Chapter 6 concludes the thesis and gives an overview of future work.

Chapter 2 Literature Review

2.1 An Introduction to Biofilms

Bacterial biofilms are complex surface-associated microbial communities embedded in a self-produced EPS matrix, which is mainly composed of polysaccharides, proteins, nucleic acids, and lipids (Costerton and Lewandowski, 1995; Donlan, 2002; Flemming and Wingender, 2010). Biofilm development progresses through five major steps, including initial reversible adhesion to a surface, EPS production and irreversible binding to the surface, early development and small cluster formation, maturation on the surface, and bacterial dispersion to colonize new locations (Høiby et al., 2011; Stoodley et al., 2002) (Figure 2-1).

Figure 2-1. An illustration of the five stages of biofilm development. (a) Reversible bacterial adhesion to the substratum. (b) EPS production and irreversible attachment. (c) Early development of biofilm clusters. (d) Maturation of biofilm. (e) The dispersion stage. The figure was created by P. Stoodley, K. Sauer, D. G. Davies, and J.W. Costerton (Stoodley et al., 2002) and used with permission from *Annual Review of Microbiology*, Volume 56 ©2002 Annual Reviews (<http://www.annualreviews.org/>).

In nature, most bacteria are found living in biofilms instead of living as individual planktonic cells (O'Toole et al., 2000; Potera, 1996; Watnick and Kolter, 2000). Biofilms constitute a distinct bacterial growth phase that is remarkably different from their planktonic counterparts. It is advantageous for bacteria to live in biofilms since biofilms can grow under limited nutrient conditions, which are challenging for planktonic bacteria (Zobell, 1943). In biofilms, bacteria are presented with microhabitat niches of different growth conditions (Stewart and Franklin, 2008), such as oxygen (De Beer et al., 1994; Ramsing et al., 1993; Xu et al., 1998), carbon dioxide (De Beer et al., 1997; Beyenal et al., 2004), and pH (De Beer et al., 1992; Ramsing et al., 1993). The biofilm EPS matrix brings bacteria cells close to each other, which enhances bacterial cell–cell communication, cooperation, and competition (Bradshaw et al., 1994; Bryant et al., 1967; Christensen et al., 2002; Falsetta et al., 2014). Lastly, biofilms are more resistant to predators or antimicrobial chemicals (Davies, 2003; Hoiby et al., 2010; Mah, T.F. and O'Toole, 2001).

Different theories have been proposed and investigated regarding the increased antibiotic resistance of biofilm-associated cells compared with their planktonic counterparts (Hoiby et al., 2010; Mah, T.F. and O'Toole, 2001; Stewart and Costerton, 2001). There are three commonly recognized mechanisms. One is the limited penetration of antibiotic agents in biofilms, which might be caused by deactivation or sorption of the antibiotic agents in the biofilm surface layers rather than limited diffusion since the biofilms barely function as diffusion barriers for small-sized antibiotic agents (Stewart, 1996; Stewart, 1998). Second, the chemical gradient established in biofilms could alter the antibiotic efficiency directly by altering the antibiotic properties, such as by antagonizing antibiotic

action through decreased pH in deep biofilms, or indirectly by altering the bacterial properties, such as by decreasing bacterial growth rates as a result of nutrient limitation (Wentland et al., 1996; Zhang and Bishop, 1996). Lastly, the increased mutation frequency and horizontal gene transmission of bacteria in biofilms might allow some bacteria to develop increased antibiotic resistant phenotypes (Driffield et al., 2008; Molin and Tolker-Nielsen, 2003).

Biofilm formation is responsible for many chronic persistent infections, such as dental caries and cystic fibrosis, and the failure of indwelling medical devices, such as prosthetic joints and central venous catheters (Costerton et al., 1999; Donlan, 2001). Given the high resistance of biofilms to antibiotic agents, these infections are hard to cure except through surgical removal and replacement (Ong et al., 1999).

On the other hand, biofilms have been successfully applied to WWT plants. In comparison with conventional physicochemical WWT methods, biological treatment methods have the advantage of being more environment-friendly by generating lower levels of secondary pollutants and having a low cost. Of the two biological treatment systems, suspended biofilms and fixed biofilms, fixed biofilm treatment appears to be more applicable as it has many advantages such as higher stability with fewer washout biofilms, higher specific biomass loading rate, and longer cell retention time (Verma et al., 2006). Different types of fixed biofilm WWT reactors have been developed since the end of the 19th century, from the earliest trickling filters to the advanced biofilters that involve the artificial immobilization of biofilms. Conventional fixed biofilm WWT systems, including the trickling filters and the rotating biological contactors, can be applied to treat multicomponent streams, whereas the advanced artificial immobilized

biofilm biofilters can only treat diluted streams or lower toxic levels of pollutants despite being more efficient (Verma et al., 2006).

As a result, biofilm research is becoming increasingly important for both natural and engineered systems. In order to promote the development of beneficial biofilms and inhibit harmful biofilms, the mechanisms of biofilm formation, growth, and detachment should be systematically investigated. Both experimental and mathematical modeling approaches should be applied simultaneously.

2.2 Experimental Biofilms

2.2.1 Lab-cultured biofilms

It is challenging to directly investigate biofilms in nature; thus, biofilm studies are usually conducted using lab-cultured biofilms. Depending on the research purpose, different bioreactors are available nowadays.

Microtiter plates can be used to grow biofilms in static conditions with high throughput efficiencies (Ramage et al., 2001). Petri dishes are the primary choice when biofilms are to be cultured on solid agar (Blair et al., 2008; Das et al., 1998). Dip flow reactors (DFR) are designed for biofilm cultivation under shear force between the air-liquid interfaces (Goeres et al., 2009; Xu et al., 1998). Rotating disk reactors (RDR) are used to culture biofilms under hydrodynamic shear forces, which can simulate natural conditions (Donlan et al., 2004; Zelter et al., 1999). However, due to complex fluid dynamics in the rotating reactors, local hydrodynamic shear stress is uncontrollable. It is difficult to precisely determine the fluidic conditions of the observed biofilms.

On the other hand, biofilm flow cells are more predictable in regard to both flow and chemical conditions (Bakker et al., 2003; Teodósio et al., 2011). Different flow cells have been developed, ranging from silicon tubing to the famous three-channel flow cells (Singh et al., 2000; Sternberg and Tolker-Nielsen, 2005). They have been successfully applied to study biofilm development. However, all of these flow cells have a unidirectional flow, which to some extent limits their application. The flow direction cannot be altered, and substrates always display a decreasing gradient from the inlets to the outlets. Recently, a planar flow cell was designed, which supports the precise control of 2D fluid fields and 2D nutrient gradients as well as real-time imaging of experimental biofilms (Zhang et al., 2011). However, based on the design protocol, the management of this planar flow cell is demanding. Twelve screws are used to seal the perimeter of the flow cell in order to prevent liquid leakage, the pressures of which are hard to balance. In addition, the chamber is made of deformable silicon rubber sheets; it is challenging to ensure that the upper and bottom surfaces are parallel to each other. Therefore, improved biofilm flow cells are in need for high-resolution biofilm experiments.

The whole biofilm flow system is made of various components, including the media bottle, the pump, the tubing, the bubble trap, the flow cell, and the waste bottle (Weiss Nielsen et al., 2011), as illustrated in Figure 2-2. A peristaltic pump or syringe pump is used to pump the media out from the bottle. Because air bubbles could disrupt biofilm development, bubble traps are usually used to prevent air bubbles from entering the flow cells. In order to avoid backflow contamination, the media bottle should be placed at a higher level than the level

of the pump and flow cell, and the waste bottle should be placed on a relatively lower level.

Figure 2-2. An illustration of the biofilm flow cell system. The supplied media is pumped from the media bottle to the bubble trap, where air bubbles would be removed. After bubble removal, the media flows through the flow cell, where biofilms are cultured, to the waste bottle.

2.2.2 Factors important for biofilm development

Biofilm formation and development are influenced by many factors, including substratum surface properties, shear stress imposed by fluid flow, quorum sensing, cyclic di-GMP (c-di-GMP) signaling, and substrate availability.

Bacterial interaction with the substratum solid surface is a complex process that can be affected by solid surface physicochemical properties, bacterial surface properties, and media ionic strengths. The effects of substratum surface properties, such as hydrophobicity, roughness, and surface micropatterns, on bacterial adhesion have been studied. The studies found that bacteria prefer to attach to both hydrophobic surfaces (A.J Mohamed, 2013; Balazs et al., 2003) and hydrophilic surfaces (Baier, 1982). These conflicting results indicate that

different bacterial strains might behave differently with different surfaces. Indeed, a study reported that depending on the strains, bacterial surfaces could be either hydrophilic or hydrophobic (Van Der Mei et al., 1998). Even for the same strain, binding structures with different degrees of hydrophobicity, which are anchored at the bacterial membrane, can result in bacterial surface heterogeneity. In addition, optimal bacterial retention at the interface between the hydrophobic and hydrophilic bands has been identified (Bos et al., 2000). Surface roughness and surface micropatterns have been extensively studied regarding their effects on bacterial adhesion. Consistently, the findings indicate that bacteria prefer to attach to locations that guarantee the largest cell-surface contact area, the least cell deformation, and protection from fluid shear stress (Crawford et al., 2012; Yang et al., 2015). The electrostatic force between the substratum surface and bacterial cells is also important for bacterial adhesion. Changes in the electrostatic force due to fluctuating ionic strength of the liquid media have been found to significantly affect bacterial attachment (Busscher et al., 2008; Li and Logan, 2004).

Quorum sensing is a term used to describe the regulation of bacterial gene expression depending on cell population density (Miller and Bassler, 2001). Quorum sensing is usually accomplished by producing, releasing, detecting, and responding to small diffusible molecules called autoinducers (Bassler, 2002; Nealson et al., 1970). There are three main quorum-sensing systems in bacteria (Bassler, 2002; Bassler and Losick, 2006): the acyl-homoserine lactone (acyl-HSL) system of gram-negative bacteria (Sensing et al., 2001; Whitehead et al., 2001), the peptide-based signaling system of gram-negative bacteria (Dunny and Leonard, 1997; Sturme et al., 2002), and the autoinducer-2 (AI-2) system of both

gram-negative and gram-positive bacteria (Chen et al., 2002; Schauder et al., 2001). Quorum sensing has been shown to influence various aspects of biofilm development, including adhesion (Pratten et al., 2001; Vuong et al., 2000), bacterial motility (Eberl et al., 1996), EPS production (Hammer and Bassler, 2003; Sakuragi and Kolter, 2007), and dispersal (Dow et al., 2003). However, studies on *Pseudomonas aeruginosa* PAO1 found that the effect of quorum sensing on the biofilm structure greatly depends on experimental conditions. The *lasI* mutant, which is a signaling mutant that is deficient in the synthesis of N-(3-oxododecanoyl)-L-homoserine lactone, was revealed to form thin, flat, and densely packed structures. The mutant biofilms could be easily dispersed, which differed from the highly differentiated structures formed by wild-type that was unaffected by sodium dodecyl sulfate (Davies et al., 1998). Stoodley et al. found that the effect of quorum sensing depends on hydrodynamics. In their experimental set-up, quorum sensing did not have a noticeable effect on biofilm structure complexity despite affecting the progression of biofilm colonization on the substratum (Stoodley et al., 1999a). However, in a subsequent study, with different parameters, quorum sensing had a significant effect on the biofilm structure formation of PAO1 (Purevdorj et al., 2002). In another study, *lasI* mutants formed flat homogeneous biofilms that were similar to those of wild-type PAO1 (Heydorn et al., 2002). The conflicting results of different studies might be attributed to the limited methods for characterizing the effects of quorum sensing, as its effects on biofilm functions are considered more important than on biofilm structures (Parsek and Greenberg, 2005).

The synthesis and the breakdown of the intracellular signaling molecule c-di-GMP in cells are regulated by diguanylate cyclases and c-di-GMP-specific

phosphodiesterases, respectively. Many reviews have been published on the mechanism and the function of c-di-GMP signaling in terms of the bacterium itself and the multicellular biofilm community (D'Argenio and Miller, 2004; Hengge, 2009; Hengge et al., 2016; Jenal and Malone, 2006; Liberman and Wedekind, 2011; Mills et al., 2011; Romling et al., 2013; Römling, 2012; Schirmer and Jenal, 2009; Sondermann et al., 2012; Tamayo et al., 2007; Wolfe and Visick, 2008). In bacteria, c-di-GMP signaling has been shown to influence bacterial motility, including flagellum-mediated swimming and swarming and type IV pilus-mediated twitching (Huang et al., 2003; Kuchma et al., 2012; Petersen et al., 2011; Savakis et al., 2012; Wolfe and Visick, 2008). Furthermore, c-di-GMP influences bacterial pathogenesis with enhanced bacterial virulence at low levels (Kuchma et al., 2005; Tischler et al., 2002; Tischler and Camilli, 2005). In biofilm communities, the consensus is that c-di-GMP signaling promotes biofilm formation (Kader et al., 2006; Kirillina et al., 2004; Merritt et al., 2007; Nakhamchik et al., 2008; Simm et al., 2004). Overall, c-di-GMP signaling has two major roles in biofilm formation. One is that c-di-GMP signaling supports bacterial adhesion by influencing bacterial motility when approaching a surface (Romling et al., 2013). The other is that c-di-GMP signaling stimulates the production of many EPS components (Römling, 2012), such as the alginate, Pel, and Psl polysaccharides in *P. aeruginosa* (Hickman et al., 2005; Merighi et al., 2007). However, lower c-di-GMP levels are also thought to contribute to the complexity of 3D biofilm structures (Romling et al., 2013). For example, low c-di-GMP levels could increase extracellular DNA (Ueda and Wood, 2010), which was identified to be important for the heterogeneous structure formation of *P. aeruginosa* biofilms (Barken et al., 2008). In addition, biofilm dispersion could

be regulated by c-di-GMP levels (An et al., 2010; Petrova and Sauer, 2012; Roy et al., 2012).

The effect of fluid shear stress on biofilm formation is another interesting biofilm research area. Although shear force can affect the initial adhesion of bacteria (Lecuyer et al., 2011; Roosjen et al., 2005; Wang et al., 2013), its effect on late biofilm growth and detachment is much more noticeable (Kreuter, 1996; Purevdorj et al., 2002). There are two aspects to the effect of shear rate on biofilm structure. One is that shear rate could influence bacterial EPS production and structure properties such as porosity and surface roughness. It was found that a sudden increase in shear rates could increase bacterial EPS production and biofilm density and decrease biofilm porosity for a short period, which would be restored to previous levels after the biofilm adapts to the new shear rate conditions (Qi et al., 2008). The other is that shear rate affects biofilm detachment processes, which occurs only when biofilm external forces are larger than internal adhesion forces. An increase in shear rates has been reported to increase the frequency of biofilm surface erosion or even sloughing events and decrease biofilm thickness and surface roughness (Choi and Morgenroth, 2003; Manz et al., 2005). However, these effects also subside after a short period when the biofilms successfully adapts to the changing conditions (Choi and Morgenroth, 2003; Manz et al., 2005). Therefore, a change in shear rate may only be temporarily effective for biofilm formation, which might be a reason why some studies report that shear rate has no significant effects on biofilm characteristics such as thickness and area mass density (Peyton, 1996).

The effect of oxygen as an electron acceptor on biofilm formation is also important; however, this has not been extensively studied. Experimental

measurements have revealed that oxygen distribution in aerobic biofilms is closely related to the 3D heterogeneous structures of the biofilms (De Beer et al., 1994). Furthermore, dissolved oxygen concentration could affect the growth rates of bacterial biofilms (Jang et al., 2003; Norsker et al., 1995) and oxygen limitation has been found to induce high amounts of biomass detachment in *Shewanella oneidensis* biofilms (Thormann et al., 2005). In a study, *P. aeruginosa* formed normal biofilm structures through microaerobic respiration whereas triple terminal oxidase mutants formed abnormal biofilms with flatter and thinner structures (Alvarez-Ortega and Harwood, 2007). However, in another study, the biofilm formation of *Streptococcus mutans* was significantly repressed when exposed to oxygen, which could influence the expression and maturation of an autolysin called AtlA (Ahn and Burne, 2007). It was also found that the activation of σ^B activity under anaerobic conditions could repress the transcription of the *icaR* gene and thus increase *icaADBC* gene expression and the biofilm formation of *Staphylococcus epidermidis* (Cotter et al., 2009). *Pseudomonas putida* biofilms cultured under turbulent flow conditions have demonstrated that oxygen limitations can lead to increased EPS production rather than carbon limitations, which is suspected to contribute to the relatively lower steady-state shear removal rates of oxygen-limited biofilms and the sloughing events that occur only in oxygen-limited biofilms (Applegate and Bryers, 1991).

The lack of studies in this field may be attributed to the difficulty in maintaining a stable local oxygen supply especially in the range of low oxygen concentrations as well as the difficulty in accurate local monitoring and measurement of oxygen concentration in biofilms. Oxygen sensing has been a critical problem for many years in biological and environmental research. Various oxygen sensing methods

have been developed, among which microelectrodes, nanoparticles, and oxygen planar optodes are the three common options. Oxygen microelectrodes have been successfully applied to study oxygen dynamics in benthic communities decades ago (Rasmussen and Jørgensen, 1992; Reusbech et al., 1980). However, these electrodes have two limitations; they can only measure oxygen profile at single points or in a stepwise manner using microcontrollers, and they are invasive to the measured biofilm structures. Nanoparticles embedded with an oxygen reporter dye can measure the 3D oxygen profile, and this approach has already been applied to measure cellular and intracellular oxygen (Fercher et al., 2011; Kondrashina et al., 2012). However, it is yet to be elucidated whether this method could be adopted for biofilm oxygen sensing as the interactions between nanoparticles and biofilm matrix are complex processes (Fabrega et al., 2009; Lee et al., 2014a; Radzig et al., 2013). On the contrary, planar optodes have been successfully applied to oxygen measurement in biofilm systems (Glud et al., 1998; Köhl et al., 2007; Oguri et al., 2006; Staal et al., 2011). This method can only be used to measure the surface oxygen distribution of the biofilm base as the optodes are coated on the glass slides. However, owing to the highly heterogeneous nature of biofilms, it is usually difficult to extrapolate oxygen data from the biofilm base surface to the whole 3D biofilm domain.

Biomass has a proposed elemental formula of $C_5H_7O_2N$ (Hoover and Porges, 1952) or $C_5H_7O_2NP_{0.074}$ (Droste, 1997), which indicates that at least three additional elements are required for biofilm growth besides hydrogen (H) and oxygen (O), which are carbon (C), nitrogen (N), and phosphorus (P). The C:N:P ratio can influence biomass production in different biological systems (Baird and Middleton, 2004; Joergensen and Scheu, 1999; Misra and Gupta, 2001; Saini et

al., 2004). To sustain bacterial growth, the required C:N:P ratio is 100:5:1 for aerobic WWT and 250:5:1 to 500:5:1 for anaerobic WWT (Ammary, 2014; Henze et al., 1997; Metcalf and Eddy, 1991; USEPA, 1995). In comparison with a lower ratio (384:84), a higher ratio of C:N (384:28) has been reported to promote bacterial attachment and biofilm formation, and the results were not significantly affected by phosphorus concentrations (Thompson et al., 2006). On the other hand, when carbon supply is increased with a constant C:N ratio, denser biofilms with lower porosity could be established, which can hinder nutrient penetration into deep biofilm clusters and result in spatial competition between heterotrophs and nitrifiers as they attempt to grow in biofilm surface layers with higher nutrient fluxes (Wijeyekoon et al., 2004). As a result, nitrification efficiency is suppressed under high nutrient supply conditions (Wijeyekoon et al., 2004). Carbon and nitrogen have also been found to affect the biofilm morphology of *Serratia marcescens*, which displays filamentous structures under higher concentrations and microcolony-type structures under lower concentrations (Rice et al., 2005).

Carbon starvation was reported to induce *P. putida* biofilm dissolution in the late biofilm stages when bacterial clusters reached certain sizes. The detailed mechanism proposed was that carbon starvation causes the decrease in intracellular c-di-GMP level, and combined with the regulation of membrane-associated proteins, which can interact with c-di-GMP, bacteria are released from the EPS matrix and return to the planktonic state (Gjermansen et al., 2005; Gjermansen et al., 2010). A sudden increase in carbon availability was found to induce dispersion in *P. aeruginosa* biofilms, which can be related to increased flagella expression and decreased pilus expression in the dispersed cells (Sauer

et al., 2004). Furthermore, nutrient-limited biofilm detachment and dissolution have been reported; however, the type of nutrient implicated is unknown (Hunt et al., 2004; Tolker-Nielsen et al., 2000). Hollow structures caused by bacterial detachment have been experimentally observed in the biofilm clusters of *P. aeruginosa* (Hunt et al., 2004). The authors proposed that the hollow structures are the result of a combined effect of cell lysis, EPS degradation, and cell dispersion, which can be activated by prolonged nutrient starvation.

Calcium concentration is another important factor in biofilm formation (Geesey et al., 2000). In particular, calcium influences bacterial adhesion forces to the substratum (Cruz et al., 2012). Calcium also affects bacterial binding strength to the EPS matrix (Kierek and Watnick, 2003). EPS production rate has been observed to be calcium-dependent (Patrauchan et al., 2005). Moreover, bacterial motility, which is crucial for biofilm structure formation (Barken et al., 2008; Klausen et al., 2003), can be affected by calcium availability (Cruz et al., 2012). However, quantitative analysis studies of the effect of calcium on biofilm development are still limited.

2.3 Computational Biofilms

Mathematical modeling is a useful tool that can be used to understand biofilm characteristics. Although existing mathematical models are unable to fully describe the actual development of biofilms, they can be applied to study specific biofilm processes with necessary simplifications and assumptions. The five goals of biofilm modeling are understanding the mechanisms of biofilm processes, linking different mechanisms in one computational framework, modifying the

experimental protocols in advance, developing new experimental designs, and improving existing designs (Wanner et al., 2006).

2.3.1 Development of biofilm mathematical models

In a biofilm mathematical model, there are usually four sub-domains, namely the bulk fluid, the mass transfer boundary layer, the solid biofilm including the EPS matrix, and the substratum (Figure 2-3).

Figure 2-3. Computational domain of biofilm mathematical models. The four sub-domains (bulk fluid, boundary layer, biofilm, and substratum) are grouped according to the calculation of the substrate gradients.

For most of the existing model systems, solutes are considered to be fully mixed in the bulk fluid sub-domain, where dilutions of the solutes caused by input and output flows are allowed. In the mass transfer boundary layer, diffusion of the solutes begins, and gradients could be generated. In addition to the diffusion process, the consumption of solutes by the active biomass also affects their distributions when they reach the biofilm sub-domains. On the other hand, the

substratum is often treated as an inert solid surface that is impermeable to both solutes and particulates except on special occasions when the substratum is simulated as a membrane that is permeable to gas or certain chemicals.

$$\frac{\partial X}{\partial t} = -\left(\frac{\partial j_x}{\partial x} + \frac{\partial j_y}{\partial y} + \frac{\partial j_z}{\partial z}\right) + r_X \quad \text{Equation 2-1}$$

where X is the concentration of the solute or biomass of the particulate; t is the time; j_x , j_y , and j_z are the three components of mass flux $\vec{j}$ along the three coordinate axes; and r_X is the net generation rate of X.

Despite the different approaches in biofilm mathematical modeling, some common rules are followed. The first and most important of all is the mass balance of both the solutes and the particulates (Equation 2-1). Fick's law is used when considering the diffusion of solutes (Equation 2-2).

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2}\right) + r_C \quad \text{Equation 2-2}$$

where C is the concentration of the solute; D is the corresponding diffusion coefficient; and r_C is the solute reaction rate (r_C is negative when the solute is consumed and positive when the solute is generated).

Figure 2-4. Graph of the number of papers containing the keyword ‘biofilm mathematical model’ that were published per year as determined with Google Scholar.

For the past four decades, the importance of biofilm mathematical modeling has been increasingly recognized (Figure 2-4). Accordingly, different models have been developed for various research objectives. Depending on the complexities, existing biofilm mathematical models could be classified as analytical models, pseudo-analytical models, 1D models, and multidimensional models (Wanner et al., 2006). Models developed in the 1970s include only the substrate utilization and mass transport process. These simple analytical or pseudo-analytical models were used to study mass flux into biofilms and the concentration distributions of limiting substrates in the biofilms (Harris and Hansford, 1976; Lamotta, 1976; Williamson and McCarty, 1976). Models in the 1980s already have the ability to simulate mixed species biofilms with varied biomass compositions. Although they are 1D models, these second generation models could be applied to study the interactions between different species in biofilms and the overall flux of substrates and metabolic products through the biofilm-liquid interfaces (Kissel et al., 1984; Wanner and Gujer, 1984; Wanner and Gujer, 1986). As more and more studies highlight the heterogeneity of biofilms in terms of both biofilm structure and function (Kinniment and Wimpenny, 1992; Tobergte and Curtis, 1997; Wentland et al., 1996; Xu et al., 1998), there is also an increasing need for mathematical models that could describe heterogeneous biofilms. Complex multidimensional models, using either the individual-based modeling (IbM) or cellular automata (CA) method to locate daughter cells, have been developed since the 1990s in order to study the effects of various physicochemical and biological factors on biofilm morphology (Eberl et al., 2001; Hermanowicz, 1998; Kreft et al., 1998; Kreft et al., 2001; Lapidou and Rittmann, 2004a; Noguera et al., 1999; Picioreanu et al., 1998a; Picioreanu et al., 1998b; Picioreanu et al., 2001;

Picioreanu and van Loosdrecht, 2002; Pizarro et al., 2001; Wimpenny and Colasanti, 1997).

Nevertheless, complex models do not always produce better results. Complex models usually make more assumptions and are more computationally demanding. For example, when simulating the degradation of an organic substrate by a flat and homogeneous biofilm, all of the different types of models were found to generate similar results (Morgenroth et al., 2004). Considering the computational requirements, simple analytical or 1D models would be a better choice. Suitable models should be selected taking into consideration the modeling purpose and capacity.

2.3.2 IbM method vs. CA method

The IbM or individual-based modeling method, as implied by its name, treats bacterial cells as individual particles or small clusters. iDynoMiCS (previously called BacSim), which is an open-source software governed by the CeCILL license under French law and developed by a group of researchers (Kreft et al., 1998; Kreft et al., 2001; Lardon et al., 2011), adopts the IbM method.

Figure 2-5. The shoving method used in iDynoMiCS. (a) Shoving of two bacteria with each other. (b) Biofilm build-up by individual cells in IbM.

In iDynoMiCS, bacteria are simulated as hard spheres (Figure 2-5), and their growth rates are dependent on substrate availability and specific microbial kinetics. The division of a bacterial sphere occurs when the biomass or biovolume criterion is met by generating a new sphere that shares the biomass equally or unequally with the mother sphere. Overlapping between bacterial spheres is avoided by applying a shoving algorithm (Figure 2-5a). In the algorithm, the scale factor S ($S = \frac{R_1^{Shove}}{R_1} = \frac{R_2^{Shove}}{R_2}$) defines the influencing region of the bacterium (dashed circle regions in Figure 2-5a). Once the overlap limit parameter δ ($\delta = R_1^{Shove} + R_2^{Shove} - d_{12}$) becomes larger than the given threshold value, which could be zero, the two bacteria are moved apart by moving one or both bacteria in opposite directions. However, movement of the overlapped bacteria in the software is more complex than that is shown in the figure since more than two bacteria exist in the system (Figure 2-5b). For each bacterium in the system, the displacements caused by all overlapped neighbors are added up and saved, and the movement of all the bacteria are done together after examining the overlap of all the bacteria in order to eliminate bias caused by the movement sequence.

In contrast to the IbM approach, the CA method divides the entire simulation domain into small grids (squares in 2D simulations and cubes in 3D simulations, usually in the micrometer scale) representing bacterial cells. The Unified Multi-Component Cellular Automaton (UMCCA), a model developed in 2004 that is a typical example of CA models, uses the CA algorithm to strategically move divided cells (Laspidou and Rittmann, 2004a; Laspidou and Rittmann, 2004b). According to the UMCCA method, bacteria are simulated as biomass filling the grids, which can also grow depending on the local substrate concentrations and the kinetics of the microorganism. Once the biomass in a grid reaches the defined

threshold, a so-called daughter cell is created by dividing the biomass into two parts. After division, the daughter cell is moved to a new grid that is free of biomass. In Figure 2-6a, the dark black grid is dividing. To locate the daughter cell, the algorithm first identifies all the nearest free grids, which are shown in Figure 2-6b as three white grids outlined by black lines. Then, one of three grids is randomly chosen as the target, and the daughter cell starts to shove neighbors according to the shortest pathway calculated by the algorithm until the chosen free grid is reached. In this case, there are three possible shortest pathways (Figure 2-6c, d, and e). The final shoving path is also randomly chosen.

Figure 2-6. The UMCCA model interpretation of the CA algorithm. (a) The grid that is going to divide. (b) The nearest three equidistant grids to the dividing grid. (c, d, and e) One of the three nearest free grids is randomly chosen (the lower right grid). The daughter cell is placed in a neighboring grid, and the biomass in the neighboring grid is shoved along the shortest path that defined by the algorithm to the chosen free grid (there are three possible shortest pathways).

There are two major differences between IbM and CA models. As discussed previously, the IbM models simulate individual cells, whereas the CA models use

grids to represent cells. Individual cells in the IbM models can move in any 3D direction; however, cells in the CA models can only move in grid-limited directions. In the IbM models, different cells could have different growth parameters, and detachment can be made based on cells, whereas detachment can only be made based on grids in the CA models. These advantages suggest that the IbM models may be a better choice than the CA models. However, the CA method could outcompete the IbM method when biofilm density heterogeneity or biofilm cavity is considered, as the shoving method employed by the IbM method pre-determines the simulated biofilm porosity. Another disadvantage of the IbM models is the larger demands in computational power. When complex biofilms are considered, computational power might also become a limiting factor. Both methods have their strengths and weaknesses. It is difficult to decide which method is superior to the other. The choice of which to use should be made after considering both the objectives of the study and the computational tools (computers) (Laspidou et al., 2010).

2.3.3 Applications of biofilm mathematical models

Mathematical models have been applied to study the effect of various factors on biofilm development, such as detachment (Chambless and Stewart, 2007; Stewart, 1993; Xavier et al., 2005b), substrate availability (Nadell et al., 2010; Xavier et al., 2005a), EPS production (Kreft and Wimpenny, 2001), pH (Picioreanu et al., 2010), etc. Biofilm detachment and substrate availability are two of the most important determinative factors for biofilm structure and function development. Therefore, the applications of mathematical models for studying biofilms under different detachment and substrate availability conditions are reviewed in detail in the following part.

Application 1 – Simulating biofilm structures as influenced by detachment events

Biofilm detachment, which is the process of biomass particle removal from surface-attached biofilms to the liquid phase, is widely believed to have a significant effect on biofilm structures (van Loosdrecht et al., 1995; Stewart, 1993; Xavier et al., 2005b).

Figure 2-7. An illustration of biofilm detachment. (a) The biofilm structure before detachment; the zoomed inset shows the detachment rate (F_{det}), which is in the direction vertical to the surface ($n(x)$ is the unit vector), at a point (x) on the biofilm surface (Γ). (b) The white band is an example of erosion detachment. (c) The sloughing detachment of a large biomass group. The figure was created by Xavier, J. D. B.; Picioreanu, C., and van Loosdrecht, M. C. M. (Xavier et al., 2005b) and used with permission from Biotechnology & Bioengineering, Volume 91, Issue 6, ©2005 John Wiley and Sons (<http://onlinelibrary.wiley.com/>).

Biofilm detachment without human interruption can be divided into two main groups: continuous and sloughing (Figure 2-7) (Stewart, 1993; Xavier et al., 2005b). Continuous detachment refers to the removal of individual or small groups of bacterial cells from the biofilms (Figure 2-7b; white band). Sloughing refers to large amounts of biomass removal, which can severely alter existing

biofilm structures (Figure 2-7c; white biomass cluster). In comparison with continuous detachment events, sloughing detachment events are more random and harder to predict. Therefore, only the continuous detachment process is directly implemented in almost all mathematical models. It is a common practice to implicitly include sloughing in the model frame by allowing large quantities of biomass to detach from the biofilm when the connection between the biomass group and the substratum is broken as a result of continuous detachment.

Various models have been applied to evaluate the effect of detachment on biofilm structure formation. Two approaches with different complexities have been established. One approach is to determine detachment according to the calculated biofilm internal shear stress caused by fluid shear stress (Martin et al., 2013; Picioreanu et al., 2001). Fluid dynamics are solved using Navier-Stokes equations through the lattice Boltzmann method (Chen et al., 1995; Picioreanu et al., 1999a). Then, the shear and normal forces exerted on biofilms by the fluid could be extracted. Zero displacement condition is used at biofilm-substratum interface for biofilm mechanical calculations. By maintaining the mechanical equilibrium of the biofilm, the internal stress at each point in the biofilm structure could be computationally calculated (Picioreanu et al., 2001). Finally, the detachment occurrence is determined according to the maximum-distortion-energy criterion, which is the fourth material failure theory that requires the equivalent stress to be larger than the given ultimate tensile stress for detachment. This approach of implementing detachment in biofilm modeling is more realistic. Results have shown that detachment due to shear stress leads to smoother biofilm structures; on the other hand, sloughing results in rougher biofilm structures, and sloughing events occur more frequently when biofilms are cultured under low liquid shear

conditions combined with high biomass growth rates (Picioreanu et al., 2001). However, many assumptions that are made to calculate biofilm mechanics can compromise the accuracy of the simulation results. Considering the heterogeneity of biofilms, assumptions such as “a biofilm behaves as an elastic material” (Ohashi et al., 1999) and “the biofilm is homogeneous and isotropic” (Picioreanu et al., 2001) would be applicable only to specific conditions. Even if these assumptions could hold, it is difficult to determine the necessary mechanical parameters such as Young's modulus and Poisson's ratio of the specific biofilm. Although both compression and tensile tests have been applied to measure the overall mechanical properties of some biofilms, the biofilms were restricted to cylindrical structures to perform the tests (Körstgens et al., 2001; Ohashi et al., 1999). A study has been conducted to measure the localized elastic modulus of biofilms by monitoring the deformation of biofilm streamers under changing shear stress conditions (Stoodley et al., 1999c). However, this method is only applicable to filamentous biofilms.

The other approach is by applying simplified equations to associate detachment rates or probabilities with different biofilm-relevant parameters such as thickness, biomass density, and nutrient concentrations. Although detachment has been proposed as a combined effect of both biofilm internal processes and fluid flow (Characklis and Bryers, 1981), the implementation of simplified equations in models to represent detachment has been proved to be able to adequately describe experimental observations (Stewart, 1993). Different detachment equations could be found in the literature (Table 2-1). The commonly adopted model of detachment rate is the one in which it is proportional to the square of biofilm thickness (Table 2-1; No. 4). This model of detachment has the advantage of

ensuring a steady state and it could be linked to fluid shear stress (Chambless and Stewart, 2007; Stewart, 1993). It has been confirmed that by increasing the detachment coefficient (DC), smoother and flatter biofilms can be generated (Chambless and Stewart, 2007; Xavier et al., 2005a). Another important detachment model is the one linked with nutrient limitation (Table 2-1; No. 9). This type of detachment could generate hollow structures in large clusters, which have frequently been observed in experimental biofilms (Boles et al., 2005; Kaplan et al., 2003; Purevdorj-Gage et al., 2005; Stapper et al., 2004). The existence of multiple models to describe the detachment process reflects the lack of one general model to cover all possible conditions (Peyton and Characklis, 1993).

Table 2-1. Detachment models

No.	Detachment rate	Description	Sources
1	$\sigma(t)$	t – time	(Reichert, 1994; Wanner and Gujer, 1986)
2	$k_d M^2$	k_d – DC; M – areal biomass density.	(Bryers, 1984)
3	$k_d \rho L_f$	ρ – density; L_f – thickness.	(Chang and Rittmann, 1987; Rittman, 1982)
4	$k_d \rho L_f^2$	ρ – density; L_f – thickness.	(Stewart, 1993)
5	$k_d \rho L_f \tau^{0.58}$	ρ – density; L_f – thickness; τ – shear stress.	(Peyton and Characklis, 1993)
6	$k_d \rho \tau$	ρ – density; τ – shear stress.	(Stewart, 1993)
7	$k_d C_s$	C_s – concentration of the detachment factor.	(Hunt et al., 2003)
8	$k_d (1 - n N_{max}^{-1})$	n – occupied neighbors; N_{max} – all neighbors.	(Chambless and Stewart, 2007)
9	$k_d (1 - C_i C_{i,max}^{-1})$	C_i – local concentration; $C_{i,max}$ – bulk concentration.	(Hunt et al., 2004)

Application 2 – Simulating biofilm structures as influenced by nutrient availability

Apart from nutrient limitation-induced bacterial detachment, nutrient availability has been found to significantly affect both biofilm growth rates and competition between species in biofilms (Allan et al., 2002; Huang et al., 1994; Ohashi et al., 1995; Peyton, 1996). As it is difficult to measure the nutrient distributions of biofilms in real time, mathematical models have been developed accordingly to evaluate the importance of nutrient availability.

The effect of nutrient availability on the development of single-species biofilm structures has been investigated (Picioreanu et al., 1998b; Picioreanu et al., 2000). For simplicity, a parameter that has been defined in the literature is described here (Equation 2-3) (Picioreanu et al., 1998b; Picioreanu et al., 2000). The equation indicates that when G is large, bacterial growth is limited by nutrient transport, and when G is small, bacterial growth is limited by growth rate. Various surface and volume parameters were utilized to characterize the generated biofilm structures. Both 2D and 3D results revealed that when G was large (such as 50), rough biofilms with finger-like colonies separated by water voids were generated. When G was small (such as 5), extremely smooth biofilms with high compactness and density were formed (Picioreanu et al., 1998b). These simulations have been performed by assuming static flow conditions. In subsequent development of the model, dynamic flow was introduced, and the effect of substrate convective transport (detachment was not included) as well as diffusion and bacterial growth on biofilm structure was evaluated (Picioreanu et al., 2000). However, the results revealed that the effect of convection on biofilm structure formation was small compared with that of diffusion. Similar results were obtained with biofilms

simulated under static flow conditions. When G was large (such as 562) rough finger-like or filamentous biofilms structures with low compactness and high porosity were generated, and when G was small (such as 11), smooth biofilms with high compactness were formed (Picioreanu et al., 2000). These findings indicated that substrate availability can significantly influence biofilm structure development. In addition, flow simulations can be neglected when considering substrate availability effects.

$$G = \frac{\text{Maximum biomass growth rate}}{\text{Maximum substrate transport rate}} = l_z^2 \frac{\mu_{max} c_{Xmax}}{D_S c_{S0}} \quad \text{Equation 2-3}$$

where l_z is the biofilm thickness; μ_{max} is the maximum specific growth rate; c_{Xmax} is the maximum biomass density; D_S is the nutrient diffusion coefficient; and c_{S0} is the bulk concentration of the nutrient.

The competition between different species for the same nutrient has also been evaluated, in which two different conditions were considered concerning species metabolism. One condition involved simulating two species with the exact same kinetics to observe the nutrient effects on biofilm structure and cell lineage segregation (Nadell et al., 2010). The results revealed that when nutrient supply was low and could not support the growth of all cells, cell lineage segregation was noticeable, and the generated biofilms had irregular front surface structures with many protrusions. When nutrient supply was slightly increased, both cell lineage segregation and a smooth biofilm front surface could be observed. Finally, when nutrient supply was sufficiently high, a smooth biofilm surface with fully mixed bacterial cell lineages could be observed. The results of this study contribute toward understanding cellular cooperation under different conditions (Nadell et al., 2010). The other simulated condition involved similar species; however, one species produces intracellular storage compounds (H-PHB), and

the other species produces polysaccharides (H-EPS) (Xavier et al., 2005a). Intracellular storage compounds such as polyhydroxybutyrate (PHB) could be consumed by the producing bacteria when the nutrient is limited (Xavier et al., 2005a). The results demonstrated that when nutrient supply was constant with a bulk carbon source of 0.1 g/L, H-EPS bacteria consistently dominated the biofilm, and after 1,500 hours, almost no H-PHB cells could be found. This dominance of H-EPS bacteria may be attributed to the low density of EPS, which can help H-EPS bacteria spread rapidly over the surface and occupy locations with higher nutrient and oxygen availability. However, when nutrient supply was in the feast and famine mode (4 hours of nutrient supply at 0.6 g/L and 20 hours of nutrient absence), H-PHB bacteria became the dominant population in the biofilms. As EPS cannot be consumed and PHB can only be consumed by the H-PHB species, biomass growth rate remained negative for the H-EPS species during nutrient absence because of maintenance, in which active biomass is steadily converted to inert biomass. However, the H-PHB species maintained positive growth rates throughout the entire cycle as they could use PHB instead of nutrients for metabolism. As a result, H-PHB bacteria outcompeted H-EPS bacteria in the biofilm. This study could be applied to optimize WWT systems as the biofilms in these systems are often subjected to variations in the supply of oxygen or other electron acceptors (Xavier et al., 2005a).

2.4 Conclusion

In this chapter, we reviewed experiments and simulations that investigated biofilm formation and development. It is clear that many factors can influence biofilm development, and no straightforward approach exists yet for using biofilm mathematical models to study biofilm development. Thus, in this section,

we highlight several questions regarding the application of mathematical modeling to study the effect of various factors on biofilm growth and demonstrate the objectives of this thesis.

2.4.1 Challenges in the control of chemical gradient

As reviewed in Section 2.2.1, the precise control of chemical gradients in the unidirectional flow cells is difficult. In addition, owing to the constraint of unidirectional flow, the biofilm formed near the inlet was significantly different from that formed near the outlet (Lee et al., 2014b). Furthermore, it is difficult to evaluate biofilm development because the growth of the biofilms is affected by various factors such as nutrients, metabolic products, and flow. On the other hand, although the precise control of 2D fluid fields and nutrient gradients is supported in the reported planar flow cell, their management is demanding. A new generation of biofilm flow cell that is capable of chemical gradient control and easy to manage is required to facilitate the investigation of the effect of substrate availability on biofilm growth.

2.4.2 Factors important for biofilm growth

As reviewed in Section 2.2.2, various factors have been revealed to significantly influence biofilm growth. Among the reviewed factors (substratum surface properties, shear stress, quorum sensing, c-di-GMP signaling, and substrate availability), we focus on detachment, oxygen availability, and calcium in this thesis.

The effect of substratum surface properties on biofilm growth is important. However, the effect is most noticeable in the initial stage bacterial adhesion, which is difficult to associate with mature biofilm growth (Bernstein et al., 2014).

Similarly, the main effect of c-di-GMP signaling is on the bacterial transition from the motile to sessile state. It is difficult to model the process of bacterial adhesion because both physical forces (such as van de Waals force and electrostatic forces) (Ong et al., 1999) and biological interactions (through bacterial surface appendages, such as flagella and pili) between the bacterium and the substratum surface are involved (Olsson et al., 2009). The effect of quorum sensing on the biofilm structure depends on the experimental conditions. Considering the conflicting results reported, it has been suggested that quorum sensing is not required for the formation of complex biofilm structures, whereas other factors such as chemical gradient and bacterial species are more important (Nadell et al., 2009).

Therefore, this thesis focuses on three factors: detachment, oxygen flux, and calcium availability. The selected factors showed significant effects on biofilm formation and it is feasible to model their effects.

2.4.3 Challenges in the validation of biofilm mathematical modeling results

As reviewed in Section 2.3, biofilm mathematical models have been frequently used to investigate biofilm development; however, the validation of the model outputs is seldom reported. Many articles only present simulation results without a direct comparison with experimental data (Kreft et al., 2001; Nadell et al., 2010; Picioreanu et al., 1998b; Picioreanu et al., 1999b; Picioreanu et al., 2001). Some articles conduct a qualitative comparison between simulated and experimental biofilm structures by observing similar biofilm structures (Hunt et al., 2004; Picioreanu et al., 2007). In one study, quantitative comparison between simulated and experimental biofilms was performed by calculating different morphological parameters (biovolume, filled space fraction at different biofilm depths, surface

coverage, thickness, and roughness) (Xavier et al., 2004). However, the study did not go further to evaluate the effect of different factors on biofilm growth despite the fact that similar biofilms were generated between the simulations and experiments.

Two reasons can be proposed to explain the lack of reports on the validation of biofilm modeling results. One is that biofilm mathematical modeling is still in the development stage and the available models are not ready for experimental validation. The other is that biofilm formation is a complex process that is influenced by many variables. It is much more challenging to perform variable control when culturing laboratory biofilms than simulating mathematical biofilms. In addition, mathematical modeling usually focuses on one or several conditions; thus, it is difficult to compare the model results with the experimental results quantitatively. Therefore, this thesis reports both the development and application of mathematical models. In addition, simulation results are compared with experimental results quantitatively.

Chapter 3 Effect of Detachment Process on Biofilm Structure Development

3.1 Introduction

Bacterial detachment is widely believed to affect biofilm structure significantly. Biofilm structure is the result of bacterial growth balanced by detachment events (Stewart, 1993; Xavier et al., 2005b). Detachment can significantly influence the formation of biofilm structures. The detachment of multiple bacterial cells could reshape the biofilm and change its spatial heterogeneity (Stewart, 1993). In addition, the reattachment of detached bacteria has been suggested as one reason for the formation of higher-level mushroom structures observed in *Pseudomonas aeruginosa* biofilms (Piciooreanu et al., 2007).

As reviewed in Section 2.3.3, various models have been developed to simplify the simulation of detachment processes. Three detachment models have been proposed to take all detachment causes into consideration (Chambless and Stewart, 2007): shear detachment (SD), which reflects the effect of fluid shearing; nutrient-limited detachment (NLD), which reflects the effect of nutrient limitation or starvation; and erosion detachment (ED), which reflects the effect of cell–cell or cell–EPS matrix adhesion force.

In this chapter, we applied the three detachment models from the cellular automata (CA) method to the individual-based modeling (IbM) software iDynoMiCS (Lardon et al., 2011) to study their effect on the biofilm structure formation of *P. aeruginosa*. Codes with the ability to extract quantitative parameters, including cell number, thickness, coverage, enlargement, and

roughness, from the simulation results were also developed to systematically quantify the effects of the three detachment models.

3.2 Materials and Methods

3.2.1 IbM software – iDynoMiCS

In iDynoMiCS, biofilm structures are developed from the growth and movement of individual bacteria. Neglecting the substratum which is an inert surface in this study, the overall biofilm reactor is divided into three parts in the model: the bulk fluid, in which the medium is considered to be fully mixed and no substrate gradient exists; the boundary layer, in which only diffusion exists and substrate gradients start to be generated; and the solid biofilm, in which both diffusion (with a different diffusivity from that in the boundary layer) and reaction are considered at the same time (Figure 3-1).

Figure 3-1. Computational domain illustration ((i,j,k) is the grid reference for the bacterium).

The whole simulation domain is divided into small grids, and the simulation cycle could be roughly divided into the following three steps: calculate the substrate concentrations in each grid based on the diffusion–reaction equation (Equation

3-1) from bulk fluid to biofilm; perform bacterial biomass growth (Equation 3-2), divide the cells whose diameters reach the threshold diameter, and release the biofilm internal pressure by moving cells apart according to defined algorithm; and process bacterial detachment according to the detachment probabilities calculated by the three models. The cycle is then repeated until the end of the simulation is reached. Care should be taken to ensure that substrate concentrations are considered constant when calculating the biomass growth, and the biofilm is considered static when calculating the substrate concentration due to the different time scales of the processes (Picioreanu et al., 1999b). Triplicates were conducted by using a random number generation algorithm (Lardon et al., 2011). More details regarding the software can be found in published literature (Kreft et al., 1998; Kreft et al., 2001; Lardon et al., 2011).

$$\frac{\partial s}{\partial t} = D \left(\frac{\partial^2 s}{\partial^2 x} + \frac{\partial^2 s}{\partial^2 y} + \frac{\partial^2 s}{\partial^2 z} \right) + r_s \quad \text{Equation 3-1}$$

$$\mu = \mu_{max} \frac{s}{K_s + s} \quad \text{Equation 3-2}$$

where s is the substrate concentration; r_s is the substrate reaction rate; D is the substrate diffusion coefficient; μ is the biomass specific growth rate; μ_{max} is the biomass maximum specific growth rate; and K_s is the substrate half-saturation coefficient. Substrate consumption rate can be calculated by $r_s = \mu/Y_s$ where Y_s is the yield coefficient of biomass production on substrate consumption.

3.2.2 Detachment models

The existing detachment model in iDynaMiCS, which was also called erosion, was only based on biofilm thickness or biomass concentration, which was not comprehensive because other factors, such as nutrient concentration, can also affect detachment. In this study, three different detachment mechanisms adopted

from the CA method (Chambless and Stewart, 2007) were integrated into iDynoMiCS as discussed in the following.

Shear detachment. SD refers to the detachment caused mainly by fluid shear stress. Previous studies have proven that the effect of fluid shear on bacterial detachment can be simplified as a quadratic function of the biofilm local thickness with acceptable accuracy (Chambless and Stewart, 2007; Xavier et al., 2005b). Therefore, instead of applying complex fluid dynamics to the simulation directly, a quadratic function is used as an effective simplification in the current model. The detachment probability of bacteria caused by SD (P_{ds}) is modeled as follows:

$$P_{ds} = K_{ds} \cdot \Delta t \cdot \left(\frac{h_i}{h_{max}}\right)^2 \quad \text{Equation 3-3}$$

where K_{ds} is the detachment coefficient (DC); Δt is the simulation time step; i is a reference of the specific cell; h_i is the distance between the cell i and the substratum surface; and h_{max} is the maximum biofilm thickness at that time point.

Nutrient-limited detachment. When nutrients become limited, cells tend to detach from their original locations (Allison, 2003; Hunt et al., 2004; O'Toole et al., 2000). NLD relates the cell detachment process to local nutrient concentration rather than biofilm thickness. The lower the local nutrient concentration, the higher the probability the bacteria will become detached. The detachment probability of bacteria caused by NLD (P_{dn}) (Chambless and Stewart, 2007; Hunt et al., 2004) is modeled as follows:

$$P_{dn} = K_{dn} \cdot \Delta t \cdot \left(1 - \frac{s_i}{s_{bulk}}\right) \quad \text{Equation 3-4}$$

where K_{dn} is the DC; s_i is the substrate concentration at the location of the bacterium i ; and s_{bulk} is the bulk nutrient concentration.

It should also be noted that more than one nutrient type may exist in experiments, such as oxygen and carbon, but only one was considered to limit the bacterial growth in the current study for simplicity. For multiple nutrients, the equation should be modified and the detachment probability should be obtained by multiplying the effect of each nutrient.

Erosion detachment. In biofilms, bacteria are encapsulated in the matrix and constrained by cell–cell or cell–EPS matrix interactions. Bacteria on the biofilm surface have fewer neighbors, which means they have weaker connections to the biofilm structure and they are more easily detached. The ED model reflects the different detachment probabilities of surface bacteria and bacteria located deep inside biofilms surrounded by neighbors. The detachment probability of bacteria caused by ED (P_{de}) (Chambless and Stewart, 2007) can be modeled as follows:

$$P_{de} = K_{de} \cdot \Delta t \cdot \left(\frac{NB_{free,i}}{NB_{total}} \right) \quad \text{Equation 3-5}$$

where K_{de} is the DC; $NB_{free,i}$ is the number of neighbor grids free of biomass; and NB_{total} is the total number of neighbor grids.

After the calculations, detachment probabilities (P_{ds} , P_{dn} , and P_{de}) were compared with a value between 0 and 1 generated from a uniform distribution function of the random generation algorithm to determine whether the cell should be detached.

3.2.3 Parameters in simulations

Model parameters. All bacterial growth-relevant parameters (Table 3-1) used in the simulation were chosen according to the literature (Chambless and Stewart, 2007; Picioreanu et al., 2007), and are applicable to single species biofilm growth

of *P. aeruginosa* in a flow cell. The detachment coefficients (DCs) were chosen in the range defined by the preliminary simulations (data not shown), which indicated that larger DCs than the maximum values of the ranges led to very few biomass accumulations when all three detachment models were enabled.

Table 3-1. Model parameters for simulating detachment influence on biofilm structures

Parameters	Values	Units
Size of computation domain	200x200x200	μm^3
Size of each grid	4x4x4	μm^3
Bulk concentration of substrate	0.04	$\text{g} \cdot \text{L}^{-1}$
Diffusion coefficient of substrate	4.5×10^{-6}	$\text{m}^2 \cdot \text{day}^{-1}$
Mass transfer boundary layer thickness	20	μm
Initial number of cells	10	
Maximum specific growth rate	0.625	hour^{-1}
Substrate half-saturation coefficient	0.02	$\text{g} \cdot \text{L}^{-1}$
Yield biomass on substrate	0.2	$\text{g} \cdot \text{g}^{-1}$
Average cell radius at division	2	μm
Simulation time step	1	hour
Shear detachment coefficient (SDC) [#]	0.05/0.1/0.15	h^{-1}
Nutrient-limited detachment coefficient (NLDC) [#]	0.001/0.005/0.01	h^{-1}
Erosion detachment coefficient (EDC) [#]	0.025/0.05/0.1	h^{-1}

(#): Three different levels of DCs from slow to fast detachment were used.

Biofilm characterization parameters. Two groups of characterization parameters were applied. One group, including average and maximum biofilm thickness and the cell number inside biofilms, was applied to indicate biofilm growth. The other group, which includes biofilm surface coverage, enlargement, and roughness, was used to evaluate biofilm morphology complexities (Table 3-2).

Table 3-2. Biofilm surface characterization parameters

Characterization parameters	Equations	Descriptions
Surface coverage (SC)	$SC = \frac{A_{cell}}{A_{surface}}$	A_{cell} – the area of the attachment surface that is occupied by bacteria, and $A_{surface}$ – the whole area of the attachment surface.
Surface enlargement (SE)	$SE = \frac{A_s}{A_p}$	A_s – the area of biofilm surface, and A_p – the area of attachment surface which are attached by bacteria.
Surface roughness (SR)	$SR = \frac{\sum_{j,k} h_{j,k} - \bar{h} }{j \cdot k}$	$h_{j,k}$ – biofilm thickness at grid referred to as (j, k) (j is the j^{th} grid in y-direction and k is the k^{th} grid in z-direction), and $\bar{h}$ – the average biofilm thickness.

3.3 Results

Biofilms simulated without considering detachment events were treated as the control. All simulations lasted 400 hours. The results of the simulations were evaluated both qualitatively by 3D structures and quantitatively by characterization parameters.

3.3.1 3D Biofilm structures

Representative biofilm structures at 200 and 400 hours are shown (Figure 3-2 and Figure 3-3, respectively). The two time points were chosen based on the total cell number data (which will be discussed in Section 3.3.2). A similar biofilm structure to that of the control (Figure 3-2a) was observed at 200 hours for all shear detachment coefficients (SDCs) (Figure 3-2b and c). However, at 400 hours, SD showed the effect on thinning the biofilms (Figure 3-3b and c). Similarly, there was no noticeable difference between biofilms under NLD and the control at 200 hours (Figure 3-2d and e), and NLD resulted in the detachment of a large

amount of biomass at 400 hours with the largest NLDC, which might have been sloughing events (Figure 3-3e). No noticeable effect on biofilm structure could be found throughout the simulation period while applying the smallest EDC (Figure 3-2f and Figure 3-3f). However, when the largest EDC was used, fewer colonies than the control presented at 200 and 400 hours (Figure 3-2g and Figure 3-3g). When all the three detachment mechanisms were enabled with the largest DCs, ring-shaped structures were observed (Figure 3-3i; white arrows).

Figure 3-2. Biofilm structures at 200 hours. (a) The control without detachment. (b and c) Biofilms with SDCs of 0.05 and 0.15. (d and e) Biofilms with nutrient-limited detachment coefficients (NLDCs) of 0.001 and 0.01. (f and g) Biofilms with erosion detachment coefficients (EDCs) of 0.025 and 0.1. (h and i) Biofilms with all the three detachment mechanisms with the smallest DCs (SDC = 0.05, NLDC = 0.001, and ED = 0.025) and largest DCs (SDC = 0.15, NLDC = 0.01, and ED = 0.1).

Figure 3-3. Biofilm structures at 400 hours. (a) The control without detachment. (b and c) Biofilms with SDCs of 0.05 and 0.15. (d and e) Biofilms with NLDCs of 0.001 and 0.01. (f and g) Biofilms with EDCs of 0.025 and 0.1. (h and i) Biofilms with all the three detachment mechanisms with the smallest DCs (SDC = 0.05, NLDC = 0.001, and ED = 0.025) and largest DCs (SDC = 0.15, NLDC = 0.01, and ED = 0.1).

It is difficult to directly observe the effects of NLD on biofilm structure formation before sloughing events. Therefore, the cell numbers at different thicknesses of chosen biofilms were plotted (Figure 3-4). Biofilms with SD or ED had the largest cell number in the first layer regardless of the growth stages (Figure 3-4a and c). However, for biofilms with NLD, the layer that was 62 μm away from the substratum surface had the largest cell number at 300 hours (Figure 3-4b), which indicates that deep layers in these biofilms had larger porosities. It is insufficient to draw the conclusion that hollow structure formation has taken place. However, considering the ring-shaped structures found under the All maximum condition

(Figure 3-3i; white arrows), it could be speculated that NLD can lead to the formation of hollow structures in large clusters through promoting the detachment of cells close to the substratum surface (Figure 3-4b).

Figure 3-4. Cell numbers at different heights of biofilm with SDC of 0.15 (a), biofilm with NLDC of 0.01 (b), biofilm with EDC of 0.25, and biofilm with All maximum (SDC = 0.15, NLDC = 0.01, and EDC = 0.25).

3.3.2 Biofilm growth characterization

Different parameters were utilized to evaluate the biofilm growth and development, which are discussed in this section.

Figure 3-5. Total cell number inside biofilms. (a) Cell number in biofilms with different SDCs. (b) Cell number in biofilms with different NLDCs. (c) Cell number in biofilms with different EDCs. (d) Cell number in biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; and All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

Total cell number inside the biofilms was used as an indicator of biomass accumulation on the substratum surfaces. For SD, instead of reaching the constant cell number count, the cell number decreased after reaching the maximum value (Figure 3-5a). NLD resulted in either continuous increasing or constant values of the total cell number before large variations were shown, which might have been sloughing events (Figure 3-5b). Indeed, the sloughing of bacteria with percentages higher than 97% was observed at different time points by plotting the

total cell number of the individual biofilm (Figure 3-6). It could be speculated that if the simulation time could be extended, the bacterial cell number in the biofilms applying NLD will slowly reach constant values followed by sloughing for all the DCs. For ED, the cell number kept increasing with lower rates than those of the control (Figure 3-5c). When all three detachment mechanisms were included at the same time, a similar trend was observed to that including only SD before complex patterns were shown (Figure 3-5d).

Figure 3-6. Total cell number of the individual biofilm with NLDC of 0.01. Different colors indicate the triplicate results. The three time points are when sloughing occurred.

Figure 3-7. Comparison of total cell numbers of biofilms with different detachment models at different time points. (a) Results at 100 hours. (b) Results at 200 hours. (c) Results at 300 hours. (d) Results at 400 hours. All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD. * indicates a significant difference between the respective sample and the control (*t*-test with $p < 0.05$). # indicates a significant difference between the corresponding sample with individual detachment and the sample with all detachment mechanisms (SDC = 0.05 – All minimum, SDC = 0.1 – All middle, SDC = 0.15 – All maximum, NLDC = 0.001 – All minimum, NLDC = 0.005 – All middle, NLDC = 0.01 – All maximum, EDC = 0.025 – All minimum, EDC = 0.05 – All middle, and EDC = 0.1 – All maximum) (*t*-test with $p < 0.05$).

The total cell numbers of the biofilms with different detachment models were compared at four time points (Figure 3-7). At 100 hours, only ED with DCs of

0.05 and 0.1, All middle, and All maximum were significantly influencing the biofilm cell number (Figure 3-7a; columns with *). In addition, no significant difference in cell number was found between biofilms with EDC of 0.05 and All middle or between biofilms with EDC of 0.1 and All maximum (Figure 3-7a). Therefore, ED was the dominant detachment mechanism at this stage, and SD and NLDC were not important. At 200 hours, more biofilms had significant different cell numbers from the control (Figure 3-7b; columns with *). The effect of SD and NLD began to become noticeable. However, no significant difference was found between biofilms with EDC of 0.025 and All minimum or with EDC of 0.1 and All maximum, which reveals that ED still showed a dominant effect. At 300 and 400 hours, almost all biofilms showed significant differences from the control, and the dominant role of ED disappeared (Figure 3-7c and d). Therefore, it can be concluded that SD and NLD are only critical for biofilm growth after 200 hours and ED showed an important effect during the entire simulation period; thus, biofilm structures at 200 hours (Figure 3-2) and 400 hours (Figure 3-3) were selected to represent the biofilm growth at important time points.

Maximum thickness is the distance between the substratum surface and biofilm top surface. The maximum thickness of the control biofilm increased linearly, and for biofilms with SD, a trend of achieving constant values was shown (Figure 3-8a). The maximum biofilm thickness was not influenced by NLD before sloughing occurred (Figure 3-8b). For biofilms with ED, the maximum thickness showed linear increasing similar to that of the control (Figure 3-8c); therefore, the increase rates of maximum thickness can be obtained by linear fitting ($R^2 > 0.95$). The linear fitting results reveal that the increase rates of the biofilm

maximum thickness were significantly lower for biofilms with SD than that for the control (Figure 3-9).

Figure 3-8. Biofilm maximum thickness. (a) Maximum thickness of biofilms with different SDCs. (b) Maximum thickness of biofilms with different NLDCs. (c) Maximum thickness of biofilms with different EDCs. (d) Maximum thickness of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; And all maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

Figure 3-9. Increase rate of biofilm maximum thickness calculated by linear fitting. * indicates a significant difference in increase rate between the biofilm and the control (t -test with $p < 0.05$).

The average thickness of biofilms with only SD showed a similar trend to their maximum thickness; however, a more noticeable equilibrium state was presented, in which average thickness only fluctuated within a small range (Figure 3-10a). The time point when biofilm reached the equilibrium state, which was around 200 hours, was independent of the SDCs. The average thickness of biofilms with only NLD first increased before reaching a short equilibrium state and then increased again until large variations appeared (Figure 3-10b). When ED was enabled alone, continuous increasing of the average thickness was observed without the trend of reaching equilibrium states (Figure 3-10c). When the All maximum condition was applied, the average thickness increased first, reached a turning point, and decreased. For biofilms under the All middle and All minimum

conditions, the average thickness first reached a short-period equilibrium state before increasing again, which was similar to biofilms formed with only NLD (Figure 3-10d).

Figure 3-10. Average biofilm thickness. (a) Average thickness of biofilms with different SDCs. (b) Average thickness of biofilms with different NLDCs. (c) Average thickness of biofilms with different EDCs. (d) Average thickness of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; and All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

3.3.3 Biofilm surface characterization

Surface parameters were calculated and evaluated to compare the biofilm surface properties.

Figure 3-11. Biofilm surface coverage. (a) Surface coverage of biofilms with different SDCs. (b) Surface coverage of biofilms with different NLDCs. (c) Surface coverage of biofilms with different EDCs. (d) Surface coverage of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

No noticeable effect of SD on biofilm surface coverage was shown (Figure 3-11a).

The surface coverage of biofilms including NLD decreased after an initial increase (Figure 3-11b). ED showed a small effect on decreasing surface

coverage during the initial biofilm growth; however, the entire substratum surface was eventually covered (Figure 3-11c). Biofilms with all three detachment mechanisms showed different trends when different DCs were applied (Figure 3-11d). It has been discussed that ED had a dominant effect before 200 hours and SD and NLD were important after 200 hours. For the surface coverage of biofilms including all three detachment mechanisms, it could be expected that SD would show no contribution because it had no effect on surface coverage and ED would play a leading role before 200 hours, after which both ED and NLD would become significant. Indeed, it could be observed that the surface coverage plot showed consistent patterns as expected (Figure 3-11d).

The surface enlargement of biofilms with SD increased very fast at late stages (Figure 3-12a), which corresponded to the observation of the loosely packed surface layers of 3D biofilm structures (Figure 3-3c). When applying only NLD, biofilm surface enlargement increased significantly after 200 hours compared with that of the control (Figure 3-12b), which could be the result of the decreased surface coverage discussed previously (Figure 3-11b). Biofilms with ED also showed increased surface enlargement (Figure 3-12c), which was expected because erosion helped the formation of more isolated bacterial clusters. When all three detachment mechanisms were enabled, the biofilms presented increase and decrease patterns in the surface enlargement plot (Figure 3-12d).

Figure 3-12. Biofilm surface enlargement. (a) Surface enlargement of biofilms with different SDCs. (b) Surface enlargement of biofilms with different NLDCs. (c) Surface enlargement of biofilms with different EDCs. (d) Surface enlargement of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; and All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

Figure 3-13. Biofilm surface roughness. (a) Surface roughness of biofilms with different SDCs. (b) Surface roughness of biofilms with different NLDCs. (c) Surface roughness of biofilms with different EDCs. (d) Surface roughness of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; and All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

Finally, biofilm surface roughness was evaluated. The surface roughness of the control consistently increased before reaching a constant value. Surface roughness of biofilms with SD increased first, reached a short equilibrium state, and increased again (Figure 3-13a). It was tempting to suspect that the shear smooth effect could only be maintained for a short time period, after which the biofilms with SC could be even rougher than the control. This later increase of

surface roughness could also be attributed to the loosely packed biofilm surface layers (Figure 3-3c). NLD did not show a conclusive effect on surface roughness (Figure 3-13b), which is acceptable as NLD had little influence on the biofilm surface properties. The surface roughness of biofilms with only ED kept increasing linearly (Figure 3-13d). Therefore, it could be predicted that these biofilms would become rougher than the control eventually. When all three detachment mechanisms were enabled, one conclusion could be made that the larger the coefficient values, the smoother the biofilms became before large variations occurred (Figure 3-13d).

It is noticed that for parameters such as total cell number, average and maximum thickness, and surface coverage, the variations among replicates were relatively smaller than those of the other parameters, which means that these parameters were more consistent and suitable for characterizing biofilm structure properties. Other parameters, including surface enlargement and roughness, showed large variations. Many factors could affect the variations or error bars in the plots. The first is the difference in the initial conditions. The initial cell number for all simulation was set to 10, but the biomasses of bacteria and their locations were chosen randomly according to the random number generation algorithm by using a Gaussian distribution for biomass and an even distribution for locations on the substratum surface. Second, the DCs applied and the biofilm growth stages also affected the error bars. When NLD was considered, sloughing events might have been the main reason for the sudden increase of error bar values such as those shown in Figure 3-5b. If significant error bars were observed for a long period, such as those shown in Figure 3-11c, this was probably due to the large bacterial detachment that occurred in the early stage. Regarding the control biofilms, the

error bars increased smoothly during development, which could be attributed to the colonial growth effect.

3.3.4 Biofilm detachment characterization

The normalized detachment rate (detached cell number/total cell number) was calculated and plotted (Figure 3-14). It could be observed that the detachment rates changed along with biofilm development. For biofilms with only SD, the normalized detachment rate showed a consistent increase (Figure 3-14a). NLD resulted in constant detachment rates after an initial increase despite the large variations that appeared at the largest NLDC (Figure 3-14b). For biofilms with only ED, the normalized detachment rate maintained small values after initial fluctuations throughout the entire simulation period (Figure 3-14c). According to the definition of ED, the initial attached bacteria had a higher possibility to get detached because they were isolated from each other, which might be the reason of the observed large initial fluctuations. When all three detachment mechanisms were enabled, small normalized detachment rates were presented after large initial fluctuations (Figure 3-14d). However, a slight increase could be observed at late stages.

Figure 3-14. Normalized detachment rate under different detachment conditions. (a) Detachment rate of biofilms with different SDCs. (b) Detachment rate of biofilms with different NLDCs. (c) Detachment rate of biofilms with different EDCs. (d) Detachment rate of biofilms with all three detachment mechanisms: All minimum refers to SDC = 0.05, NLDC = 0.001, and EDC = 0.025; All middle refers to SDC = 0.1, NLDC = 0.005, and EDC = 0.05; All maximum refers to SDC = 0.15, NLDC = 0.01, and EDC = 0.1. Figures were plotted with mean $\pm$ SD.

3.4 Discussion and Conclusion

In this study, we investigated the effect of three detachment models on the structure formation of *P. aeruginosa* biofilms through mathematical modeling. The simulation results agreed well with those of previous reports, including the effect of SD on biofilm smoothening, NLD on biofilm hollowing, and ED on bacterial clusters isolating (Chambless and Stewart, 2007). Compared with the CA method, which was applied in a similar study (Chambless and Stewart, 2007), the IbM method used here is preferred because by using it, different cells can have different growth parameters and detachment can be easily achieved based on the cells

instead of the artificially defined grids in the CA method (Laspidou et al., 2010). As a result, the IbM method provides a more realistic biofilm simulation environment, which could enhance the fundamental understanding of biofilm development.

Experimental work on the shear effects on biofilm development has been reported frequently in the literature (Choi and Morgenroth, 2003; Manz et al., 2005; Peyton, 1996; Qi et al., 2008). Some have proposed that the increasing of shear stress only has a short-term effect and biofilms can adapt to the shear stress change and return to the previously established steady state after a certain period (Choi and Morgenroth, 2003). Others have shown that under higher shearing, elevated detachment occurred; thus, smoother and thinner biofilms could be established (Manz et al., 2005; Qi et al., 2008). The results in this study support the second opinion that higher shear detachment leads to the formation of thinner biofilms. The effect of nutrient limitation on biofilm structure formation is less reported. However, nutrient starvation-induced detachment has been proposed to promote the generation of hollow structures in large biofilm clusters where nutrient concentration is the lowest (Chambless and Stewart, 2007; Hunt et al., 2004), which has been frequently observed in experimental biofilms (Boles et al., 2005; Kaplan et al., 2003; Purevdorj-Gage et al., 2005; Stapper et al., 2004). In the current study, NLD also resulted in the formation of hollow structures in biofilm clusters. Finally, ED alone was hardly evaluated, but one of the findings in this study that ED started to become important for biofilm formation since early biofilm development stages agrees with previous reports (Bester et al., 2005).

SD was applied here to simulate the effect of shear stress on biofilm structures. Any detachment expression that has at least a first-order dependence on biofilm thickness and approaches zero when the thickness of the biofilm approaches zero guarantees a finite steady state of biofilm accumulation (Stewart, 1993). Indeed, the average thickness of biofilms including SD in the current study, which had a second-order dependence on biofilm thickness, showed a steady state after 200 hours. On the other hand, the applied NLD and ED cannot meet the requirements, and no noticeable steady state of biofilm growth was presented.

Initial bacterial adhesion is a complex process. Conflicting results have been reported regarding its effect on biofilm formation. A study conducted by Bernstein et al. revealed that it is difficult to associate initial bacterial adhesion with subsequent biofilm formation (Bernstein et al., 2014). However, there are also studies reporting a significant effect of initial bacterial adhesion on biofilm growth (Arnold and Bailey, 2000; Yang et al., 2012). Although the bacterial adhesion process was not modeled explicitly in the current study, the results here support the opinion that initial bacterial adhesion is important for the formation of subsequent biofilm structures. All of the 3D biofilm structures presented a similar biofilm morphology before the sloughing events, which indicates that the biofilm structures were largely dependent on the initial bacterial seeding.

In addition, the current study showed that different detachment mechanisms played different roles in biofilm structure formation at different biofilm development stages, which has not been previously demonstrated. To be specific, ED was more important at early biofilm stages, whereas SD was more important at late stages and NLD only showed its influence when biofilm clusters became large enough to create a thick nutrient diffusion barrier. Moreover, different

detachment mechanisms had different sensitivities regarding the selection of DCs. A similar conclusion could be drawn from a previous study, although it was not discussed there (Chambless and Stewart, 2007). SD always showed similar effects on biofilm formation regardless of the DCs chosen. For NLD, depending on the coefficient values, sloughing events could happen early or late. For ED, its influence on biofilm formation depended heavily on the coefficient values. Lastly, with detachment enabled, all biofilm parameters, except some conditions discussed before, had smaller error bars than the biofilms formed without detachment, which indicates that detachment could help form more reproducible biofilms when compared with biofilms formed without detachment.

In conclusion, biofilm development with different detachment models was successfully simulated by applying iDynoMiCS. Both 3D observations and structure-characterizing parameters were evaluated, which showed the different effects of these detachment models on the development of biofilm structures. The results of this study were compared with previous experimental and numerical results whenever they were available. Moreover, the effects of different detachment models on achieving the equilibrium states of biofilm growth were also discussed together with the evaluations of the effects of detachment mechanisms at different biofilm growth stages and their dependence on DCs.

Chapter 4 Effect of Oxygen Availability on Initial Biofilm Growth and Colonization

4.1 Introduction

Pseudomonas putida OUS82 is a gram-negative bacterium with a polar flagellum structure. Previous studies have found that initially, *P. putida* biofilms formed separated irregular compact bacterial clusters embedded in the EPS matrix. However, after sufficient culture time and when bacterial clusters reached certain sizes, an abrupt change to loosely packed protruding structures occurred and the structures eventually dissolved (Tolker-Nielsen et al., 2000). It was then proposed that this biofilm structure change was caused by a carbon starvation-induced intracellular cyclic di-GMP level decrease, which finally led to the release of bacteria from the matrix through regulating bacterial membrane-associated proteins (Gjermansen et al., 2005; Gjermansen et al., 2010).

The effect of oxygen as an electron acceptor on biofilm formation is very important. Oxygen distribution could be closely related to the 3D heterogeneous structures of aerobic biofilms (De Beer et al., 1994). In addition, the increase of oxygen availability has resulted in the increased biofilm thickness of aerobic bacteria (Jang et al., 2003; Norsker et al., 1995). Large amounts of biomass detachment were induced in *Shewanella ondidensis* biofilms due to oxygen limitation (Thormann et al., 2005). On the other hand, oxygen has shown a negative effect on the biofilm growth of some bacterial species, such as *Streptococcus mutans* (Ahn and Burne, 2007) and *Staphylococcus epidermidis* (Cotter et al., 2009).

It was reported that planktonic *P. putida* bacteria showed faster growth under a higher oxygen concentration (Alagappan and Cowan, 2004). *P. putida* biofilms cultured under turbulent flow conditions have demonstrated that oxygen limitations rather than carbon limitations can lead to increased EPS production (Applegate and Bryers, 1991). Nevertheless, the reason the effect of oxygen on *P. putida* biofilm formation has not been studied systematically is probably due to the lack of measurement and monitoring techniques. Furthermore, maintaining a stable oxygen gradient is difficult. It would be of great interest to study oxygen limitation-induced biofilm structure evolution with CLSM for biofilm visualization and oxygen planar optode technology for oxygen profile visualization (Glud, 2008; Larsen et al., 2011; Oguri et al., 2006).

In the current study, *P. putida* OUS82 biofilms were cultured at different oxygen fluxes. With the help of a square biofilm growth chamber, it was possible to control and achieve a gradual oxygen gradient inside one chamber with appropriate input velocity and oxygen concentration settings, which made the experiments more feasible and reproducible. Together with the real-time monitoring of biofilm development using CLSM, the effects of oxygen on initial biofilm growth were evaluated. Since only initial biofilm growth was considered, surface coverage was chosen as a parameter to characterize the biofilm growth instead of the commonly used biovolume. The direct measurement of oxygen concentration inside the growth chamber is still challenging. In this work, mathematical modeling was performed to evaluate oxygen effects on biofilm growth. Additionally, three bacterial growth-relevant parameters for the mathematical model were optimized using a regression model. The achievement in this report of simulating the effects of oxygen flux on initial biofilm growth

laid the foundations to study more complex oxygen-related phenomena, such as the biofilm dispersal process of *P. putida*.

4.2 Materials and Methods

4.2.1 Oxygen availability control in a square growth chamber

A square biofilm flow cell was designed and fabricated to control oxygen availability (Yehuda et al., 2016), which consisted of the following four layers: plastic top and bottom layers, a removable glass slide, and a metal supporting layer (Figure 4-1a). These four layers were bonded with four screws, and water leakage was prevented by a rubber ring (Figure 4-1b). With appropriate input configurations, oxygen distributions inside the chamber could be controlled, which were simulated using the commercial software COMSOL Multiphysics (4.3b, COMSOL, Inc.) (Figure 4-1c) and verified by comparing them with the experimental results measured through a surface coating layer of oxygen planar optode (Figure 4-1d) (Yehuda et al., 2016).

In COMSOL, the *Laminar Flow* module was applied to simulating the fluid dynamics inside the chamber assuming that turbulence was negligible, and the *Transport of Diluted Species* module, in which the liquid density and viscosity were not affected by dissolved oxygen and the oxygen transfer was based on Fick's law of diffusion, was applied to simulate the oxygen mixing and diffusion processes. Because it is still challenging at this stage to monitor oxygen distribution using oxygen planar optode during biofilm culture, the same COMSOL model was used to simulate the distribution of oxygen in the chamber after the validation (two input settings were validated and one was shown in Figure 4-1d). The oxygen conditions used in the current study will be discussed

in Section 4.3.1. For the following discussions, the phrase COMSOL O₂ is used to indicate that the oxygen conditions for biofilm culture were simulated using COMSOL.

Figure 4-1. Biofilm square chamber with controllable oxygen availability. (a) Four layers of the square chamber. (b) The assembled square chamber. (c) COMSOL simulated oxygen distribution pattern. (d) Experimental oxygen distribution pattern.

4.2.2 Biofilm growth

Overnight liquid culture of *P. putida* OUS82, which was genetically tagged with green fluorescent protein (GFP) (Tolker-Nielsen et al., 2000), was diluted to OD ≈ 0.01 and inoculated into the square chamber by continuous pumping at a flow rate of 20 ml/h from the left and 60 ml/h from the bottom for 15 minutes. After

this, sterilized M9 minimal medium (48 mM of Na_2HPO_4 , 22 mM of KH_2PO_4 , 9 mM of NaCl , 19 mM of NH_4Cl , 2 mM of MgSO_4 , 0.1 mM of CaCl_2 , and 0.04% w/v glucose) (Lee et al., 2014b) without bacteria inside was supplied as the source media. The oxygen-rich (air-saturated) media was pumped from the left inlet at a flow rate of 20 ml/h, and the oxygen-free (nitrogen-saturated) media was pumped from the bottom inlet at a flow rate of 60 ml/h. Syringe pumps (NE-1000) were used instead of peristaltic pumps to eliminate the flow-rate fluctuations generated by the peristaltic pumping approach. Biofilm fluorescent images were recorded using CLSM (Zeiss LSM 780) for approximately six hours with a time interval of 10 minutes to monitor the biofilm establishment in real time. The excitation wavelength for GFP was 488 nm, and the emission wavelength was in the range of 499 nm to 683 nm. Two oxygen concentrations with three replicates for each (six samples in total) were monitored.

4.2.3 Mathematical modeling of biofilms

Biofilm mathematical modeling was conducted applying iDynoMiCS as it facilitates the addition of new functions (Kreft et al., 1998; Lardon et al., 2011; Merkey et al., 2011). The positions of initial bacterial attachment were extracted from experimental images and inputted into the software as Excel files. This was done to ensure that the starting point of the simulation was the same as that of the experiments because initial attachment has been shown to affect the subsequent biofilm formation significantly (Arnold and Bailey, 2000; Yang et al., 2012). Oxygen was treated as the only chemical that might limit biofilm development because, at the initial biofilm growth stages, hardly any limitations on bacterial growth could be caused by carbon or other chemicals except manually controlled oxygen. The Monod kinetic model of bacterial growth was applied as it is widely

used and can capture the observed effects of substrate concentration on the rate of biomass synthesis (Wanner et al., 2006).

Table 4-1. Model parameters for simulating oxygen effect on *P. putida* biofilm structures

Parameters	Values	Units	Notes
Size of computation domain	212x212x212	μm^3	Same surface area as confocal images
Size of each grid	4x4x4	μm^3	
Simulation time step	10	minutes	Same as experiments
Bulk concentration of oxygen	5.76E-4 (Position H) 8.14E-3 (Position L)	$\text{g} \cdot \text{L}^{-1}$	Same as COMSOL simulation results
Diffusion coefficient of oxygen	2E-4	$\text{m}^2 \cdot \text{day}^{-1}$	(Xavier et al., 2005a)
Mass transfer boundary layer thickness	20	μm	(Picioreanu et al., 2007)
Maximum specific growth rate (μ_{max})	Four levels: 0.2355, 0.471, 0.7065, and 0.942	hour^{-1}	Assumed based on (Xavier et al., 2005a)
Substrate half-saturation coefficient (K_o)	Four levels: 1.75E-4, 3.5E-4, 5.25E-4, and 7E-4	$\text{g} \cdot \text{L}^{-1}$	Assumed based on (Xavier et al., 2005a)
Yield biomass on substrate (Y_o)	Four levels: 0.5, 1, 1.5 and 2	$\text{g} \cdot \text{g}^{-1}$	Assumed base on (Xavier et al., 2005b)
Average cell radius at division	2	μm	
Biomass density	150	$\text{g} \cdot \text{L}^{-1}$	(Picioreanu et al., 2007)

Simulation parameters are listed in Table 4-1. The three bacterial growth-relevant Monod kinetic parameters – the yield of biomass on oxygen consumption (Y_o), the half-saturation coefficient (K_o), and the maximum specific growth rate (μ_{max}) – were treated as variables that need to be optimized. Four levels of these three parameters were assumed at first, covering the range of reported values for the purpose of the following optimization process, which will be discussed in the

next paragraph. Other simulation parameters were chosen according to either the experimental conditions, such as the computational domain and the oxygen bulk concentrations, or the literature reports (Picioreanu et al., 2007; Xavier et al., 2005a), such as the diffusion coefficient of oxygen in liquid and the boundary layer thickness.

To find a suitable set of the three Monod kinetic parameters, full-factorial-design simulations were run with four levels for each parameter (i.e., 64 simulations for one experimental sample and 384 simulations for all six samples). The simulation results of each experimental sample were then built into a regression model using Minitab 17 (State College, PA: Minitab, Inc.), where the surface coverage was treated as the target and the three parameters as well as time were treated as four variables. Second-order regression was considered. The best quadratic regression model was selected with consideration of the goodness of fit and significance of each variable. The obtained regression model was then used to fit the experimental surface coverage data to determine the suitable parameter set (Y_o , K_o , and $\mu_{\max}$). Six sets of such optimized parameters could be respectively extracted from the six experimental samples using the aforementioned method. The three parameters describing Monod kinetics should be constant for treatments with different initial cell densities or substrate concentrations (Galperin and Kaplan, 2008; Simkins and Alexander, 1984; Simkins and Alexander, 1985). Therefore, the six optimized sets of parameters should be the same. Finally, the optimized parameters were implemented in simulations with different oxygen concentrations and the simulation results were compared with the experimental data.

4.2.4 Image processing

The 2D confocal images were exported as TIFF figures and processed using a common-source program called Phlip (Mueller et al., 2006), which was built as a toolbox for MATLAB (MathWorks). Specifically, the exported TIFF images were binarized using the software-defined thresholding method based on the objective threshold selection procedure (OTS) (Xavier et al., 2001). The surface coverage was then calculated as the ratio of occupied pixels to the total image pixels.

4.3 Results and Discussion

4.3.1 Oxygen gradient in the square chamber

The oxygen concentration and shear rate distribution on the glass surface were simulated based on the input flow rates and the oxygen concentrations (Figure 4-2). The COMSOL O₂ figure (Figure 4-2a) indicated a clear transition from high oxygen to low oxygen. The high velocities of both inputs resulted in the narrow transition band, which separated the whole chamber into high and low oxygen regions. The shear rate distribution inside the chamber was quite different according to the COMSOL simulation (Figure 4-2b). High shear rates were only found near the two inlets and two outlets, and the simulated shear rates for all other locations inside the chamber were very small and homogeneous. Images taken at positions H and L were selected for further analysis, as they had different oxygen concentrations but almost identical shear rates according to the COMSOL simulation (Table 4-2).

Figure 4-2. COMSOL flow simulation results. (a) COMSOL O₂. (b) Simulated shear rate distribution.

Table 4-2. Shear rates and oxygen concentrations for the two imaging positions according to COMSOL simulation

	Coordinates (mm) (Origin was the chamber center)	Oxygen concentration (mol.m ⁻³)	Shear rate (s ⁻¹)
Position H	(-7.5, 0)	0.25	1.06
Position L	(5, 5)	0.018	1.07

4.3.2 Biofilm growth in the square chamber

Representative biofilm images of the two positions at six time points were shown (Figure 4-3). Comparing the biofilm development between positions H and L permits the observation that even though the initial attachment patterns were similar (Figure 4-3; images at 0 minutes for positions H and L), they showed different surface colonization after 250 minutes (Figure 4-3; images at 250 and 320 minutes for positions H and L). Biofilm growth under the high COMSOL O₂ was faster than when under the low COMSOL O₂, and larger clusters were found more often for the high COMSOL O₂ than the low COMSOL O₂.

Figure 4-3. Representative biofilm confocal images inside the growth chamber at positions H and L at 0, 60, 120, 180, 250, and 320 minutes (Scale bar: 20 μm). White square: Cluster H1, white oval: Cluster H2, red square: Cluster L1, and red oval: Cluster L2.

Surface coverage plots also showed that the biofilm growth under the two COMSOL O_2 environments had similar initial coverage. However, the surface coverage of the biofilms at position H became significantly larger than those at position L after 250 minutes (Figure 4-4a). Bacterial dispersion became obvious

after 250 minutes upon counting the cluster numbers (Figure 4-4b), particularly for position H. Even though biofilm surface coverage was influenced by bacterial dispersion, the surface coverage plot excluding those dispersed cells (Figure 4-4c) showed consistent results with the overall surface coverage plot (Figure 4-4a). Furthermore, fluctuations could be observed when the data of the three replicates were plotted separately (Figure 4-4d, e, and f). Presumably because of the long sampling interval and few sample points, it was difficult to derive a clear periodic waveform in the increase speed plots (data not shown). Nevertheless, a shorter sampling interval could be easily achieved via simulation, and thus the periodic increase behavior will be discussed in detail in Section 4.3.3.

Figure 4-4. Surface coverage and cluster number of experimental biofilms. (a), (b), and (c) The averaged surface coverage, cluster number, and surface coverage excluding the dispersed cells respectively (error bars: SD). (d), (e), and (f) The surface coverage, cluster number, and surface coverage excluding the dispersed cells of the three replicates separately. * indicates a significant difference between the data under the high COMSOL O₂ and low COMSOL O₂ (t -test with $p < 0.05$).

4.3.3 Simulation of biofilm growth

Biofilm simulation was performed in two steps. The first step was conducted to obtain optimized values for the three Monod kinetic parameters (the yield of biomass on oxygen consumption (Y_o), the half-saturation coefficient (K_o), and the maximum specific growth rate ($\mu_{\max}$)). The second step was applying those optimized parameters to simulate the biofilm growth of the six experimental samples and compare the results from the simulated biofilms with the experimental biofilms to validate the simulation process. Then, more information regarding biofilm development under different COMSOL O_2 was extracted from the simulation results.

Table 4-3. Biofilm simulation reference according to the three parameters

S_(1,1,1)	$Y_o = 0.5$ $K_o = 1.75E-4$ $\mu_{\max} = 0.2355$	S_(1,1,2)	$Y_o = 0.5$ $K_o = 1.75E-4$ $\mu_{\max} = 0.471$	S_(1,1,3)	$Y_o = 0.5$ $K_o = 1.75E-4$ $\mu_{\max} = 0.942$
S_(1,2,1)	$Y_o = 0.5$ $K_o = 3.5E-4$ $\mu_{\max} = 0.2355$	S_(1,2,2)	$Y_o = 0.5$ $K_o = 3.5E-4$ $\mu_{\max} = 0.471$	S_(1,2,3)	$Y_o = 0.5$ $K_o = 3.5E-4$ $\mu_{\max} = 0.942$
S_(1,3,1)	$Y_o = 0.5$ $K_o = 7E-4$ $\mu_{\max} = 0.2355$	S_(1,3,2)	$Y_o = 0.5$ $K_s = 7E-4$ $\mu_{\max} = 0.471$	S_(1,3,3)	$Y_o = 0.5$ $K_o = 7E-4$ $\mu_{\max} = 0.942$
S_(2,1,1)	$Y_o = 1$ $K_o = 1.75E-4$ $\mu_{\max} = 0.2355$	S_(2,1,2)	$Y_o = 1$ $K_o = 1.75E-4$ $\mu_{\max} = 0.471$	S_(2,1,3)	$Y_o = 1$ $K_o = 1.75E-4$ $\mu_{\max} = 0.472$
S_(2,2,1)	$Y_o = 1$ $K_o = 3.5E-4$ $\mu_{\max} = 0.2355$	S_(2,2,2)	$Y_o = 1$ $K_o = 3.5E-4$ $\mu_{\max} = 0.471$	S_(2,2,3)	$Y_o = 1$ $K_o = 3.5E-4$ $\mu_{\max} = 0.942$
S_(2,3,1)	$Y_o = 1$ $K_o = 7E-4$ $\mu_{\max} = 0.2355$	S_(2,3,2)	$Y_o = 1$ $K_s = 7E-4$ $\mu_{\max} = 0.471$	S_(2,3,3)	$Y_o = 1$ $K_o = 7E-4$ $\mu_{\max} = 0.942$
S_(3,1,1)	$Y_o = 2$ $K_o = 1.75E-4$ $\mu_{\max} = 0.2355$	S_(3,1,2)	$Y_o = 2$ $K_o = 1.75E-4$ $\mu_{\max} = 0.471$	S_(3,1,3)	$Y_o = 2$ $K_o = 1.75E-4$ $\mu_{\max} = 0.942$
S_(3,2,1)	$Y_o = 2$ $K_o = 3.5E-4$ $\mu_{\max} = 0.2355$	S_(3,2,2)	$Y_o = 2$ $K_o = 3.5E-4$ $\mu_{\max} = 0.471$	S_(3,2,2)	$Y_o = 2$ $K_s = 3.5E-4$ $\mu_{\max} = 0.942$
S_(3,3,1)	$Y_o = 2$ $K_o = 7E-4$ $\mu_{\max} = 0.2355$	S_(3,3,2)	$Y_o = 2$ $K_o = 7E-4$ $\mu_{\max} = 0.471$	S_(3,3,3)	$Y_o = 2$ $K_o = 7E-4$ $\mu_{\max} = 0.942$

Units of Y_o , K_o , and $\mu_{\max}$ are $g \cdot g^{-1}$, $g \cdot L^{-1}$, and $hour^{-1}$.

Parameter optimization. According to the full-factorial-design method of three variables, each with four levels, there are 64 simulation combinations for each experimental sample in total ($S_{(i,j,k)}$, $i = [1\ 2\ 3\ 4]$, $j = [1\ 2\ 3\ 4]$, and $k = [1\ 2\ 3\ 4]$, which respectively represent the four levels of Y_o , K_o , and $\mu_{\max}$ as shown in Table 4-3).

Biofilm structures affected by the Monod kinetic parameters. The representative simulated biofilm structures of position H replicate 1 were shown (Figure 4-5). Very different biofilm structures were generated, even though the initial conditions (the number and location of attached bacteria) were the same (Figure 4-5i_0). In general, when all other variables were kept the same, biofilms grew faster when Y_o (for example, compare Figure 4-5b with d) or $\mu_{\max}$ (for example, compare Figure 4-5c with d) was increased, and biofilms grew slower when K_o was increased (for example, compare Figure 4-5d and f). Under certain conditions, particularly with small $\mu_{\max}$, no large biofilm clusters were established throughout the simulation period (Figure 4-5a and e). This phenomenon indicated that $\mu_{\max}$ was a very important growth factor under the current modeling conditions.

Figure 4-5. Representative 3D biofilm morphology based on simulation. (a), (b), (c), (d), (e), and (f) respectively refer to $S_{(1,1,1)}$, $S_{(1,1,4)}$, $S_{(4,1,1)}$, $S_{(4,1,4)}$, $S_{(4,4,1)}$, and $S_{(4,4,4)}$, the suffixes 120, 250, and 320 were applied to indicate that the biofilms were simulated for 120, 250, and 320 minutes after attachment, and i_0 refers to the initial adhesion pattern at 0 minutes, which was the same for all simulation runs. Image dimensions: 212 μm wide and 212 μm long.

Surface coverage regression based on full-factorial-design simulations. As an example, the experimental biofilm surface coverage of position H replicate 1 was plotted together with the data from the 64 simulations (Figure 4-6).

Figure 4-6. Experimental and simulated biofilm surface coverage of position H replicate 1.

Most of the surface coverage from the simulated biofilms differed from that of the experimental biofilms, which indicated that the best-optimized values for the

three parameters (the yield of biomass on oxygen consumption (Y_o), the half-saturation coefficient (K_o), and the maximum specific growth rate ($\mu_{\max}$)) might lie in between the four chosen levels of parameters. Therefore, regression models that could cover the surface coverage for the whole 3D space of the three parameters were built based on the simulation data (surface coverage = $f(Y_o, K_o, \mu_{\max}, Time)$; Y_o – yield of biomass on oxygen consumption, K_o – half saturation coefficient, and $\mu_{\max}$ – maximum specific growth rate).

Figure 4-7. The main effects plots for simulated biofilm surface coverage before (a) and after (b) logarithm transformations.

All 64 simulation results were utilized for each of the six samples to obtain the best quadratic regression model in Minitab. Because surface coverage showed an exponential relationship with time and linear relationships with the other three variables (Y_o , K_o , and $\mu_{\max}$) in the main effects plot (Figure 4-7a), logarithmic

transformations were performed so that the exponential relationship became linear while the linear relationships were maintained (Figure 4-7b). This step was performed to ensure the good quality of the developed regression model for the following analysis. The obtained model (Equation 4-1 to Equation 4-3 for position H replicates 1, 2, and 3, and Equation 4-4 to Equation 4-6 for position L replicates 1, 2, and 3) fit all of the simulation data with a goodness of more than 97%, which was sufficiently high to ensure the accuracy of the regression models in the 3D space of the three parameters (Y_o , K_o , and $\mu_{\max}$).

$$\begin{aligned} \log_2(\text{SC1}) = & -6.2510 + 0.7033 * \log_2(Y_o) + 0.1876 * \log_2(\mu_{\max}) \\ & + 17.967 * \text{Time}/1440 + 0.4749 * \log_2(Y_o) \\ & * \log_2(\mu_{\max}) + 7.435 * \log_2(Y_o) * \text{Time}/1440 \\ & - 0.4148 * \log_2(K_o) * \text{Time}/1440 + 7.428 \\ & * \log_2(\mu_{\max}) * \text{Time}/1440 \end{aligned} \quad \text{Equation 4-1}$$

$$\begin{aligned} \log_2(\text{SC2}) = & -6.0623 + 0.7230 * \log_2(Y_o) + 0.2209 * \log_2(\mu_{\max}) \\ & + 18.325 * \text{Time}/1440 + 0.4617 * \log_2(Y_o) \\ & * \log_2(\mu_{\max}) + 7.197 * \log_2(Y_o) * \text{Time}/1440 \\ & - 0.3852 * \log_2(K_o) * \text{Time}/1440 + 7.204 \\ & * \log_2(\mu_{\max}) * \text{Time}/1440 \end{aligned} \quad \text{Equation 4-2}$$

$$\begin{aligned} \log_2(\text{SC3}) = & -5.7293 + 0.7051 * \log_2(Y_o) + 0.2348 * \log_2(\mu_{\max}) \\ & + 17.822 * \text{Time}/1440 + 0.4324 * \log_2(Y_o) \\ & * \log_2(\mu_{\max}) + 6.836 * \log_2(Y_o) * \text{Time}/1440 \\ & - 0.3625 * \log_2(K_o) * \text{Time}/1440 + 6.842 \\ & * \log_2(\mu_{\max}) * \text{Time}/1440 \end{aligned} \quad \text{Equation 4-3}$$

$$\begin{aligned} \log_2(\text{SC4}) = & -8.400 - 1.2835 * \log_2(Y_o) - 0.1823 * \log_2(K_o) \\ & - 1.6168 * \log_2(\mu_{\max}) - 9.9000 * \text{Time}/1440 \\ & - 0.15434 * \log_2(Y_o) * \log_2(K_o) + 0.38424 \\ & * \log_2(Y_o) * \log_2(\mu_{\max}) + 5.9109 * \log_2(Y_o) \\ & * \text{Time}/1440 - 0.14734 * \log_2(K_o) * \log_2(\mu_{\max}) \\ & - 2.3309 * \log_2(K_o) * \text{Time}/1440 + 5.8149 \\ & * \log_2(\mu_{\max}) * \text{Time}/1440 \end{aligned} \quad \text{Equation 4-4}$$

$$\begin{aligned} \log_2(\text{SC5}) = & -8.692 - 1.3138 * \log_2(Y_o) - 0.1871 * \log_2(K_o) \\ & - 1.7080 * \log_2(\mu_{\max}) - 10.200 * \text{Time}/1440 \\ & - 0.15710 * \log_2(Y_o) * \log_2(K_o) + 0.39063 \\ & * \log_2(Y_o) * \log_2(\mu_{\max}) + 5.9759 * \log_2(Y_o) \\ & * \text{Time}/1440 - 0.15447 * \log_2(K_o) * \log_2(\mu_{\max}) \\ & - 2.3572 * \log_2(K_o) * \text{Time}/1440 + 5.8566 \\ & * \log_2(\mu_{\max}) * \text{Time}/1440 \end{aligned} \quad \text{Equation 4-5}$$

$$\begin{aligned}
\log_2(\text{SC6}) = & -8.372 - 1.2282 * \log_2(Y_o) - 0.1827 * \log_2(K_o) \\
& - 1.6082 * \log_2(\mu_{\max}) - 9.5040 * \text{Time}/1440 \\
& - 0.14916 * \log_2(Y_o) * \log_2(K_o) + 0.38081 \\
& * \log_2(Y_o) * \log_2(\mu_{\max}) + 5.8877 * \log_2(Y_o) \\
& * \text{Time}/1440 - 0.14636 * \log_2(K_o) * \log_2(\mu_{\max}) \\
& - 2.2899 * \log_2(K_o) * \text{Time}/1440 + 5.7847 \\
& * \log_2(\mu_{\max}) * \text{Time}/1440
\end{aligned}$$

Equation 4-6

where $\log_2(\text{SC})$ refers to the log transformation of the surface coverage.

Figure 4-8. Surface coverage obtained through the simulation and the regression model for position H replicate 1 (where $R_{(x,x,x)}$ has the same definition as $S_{(x,x,x)}$, except that S refers to simulation while R refers to the regression).

The corresponding surface coverage could be calculated for each experimental sample using the regression equations (Equation 4-1 to Equation 4-6). For example, surface coverage of position H replicate 1 was plotted. Overall, the surface coverage obtained from the regression model matched that obtained from the mathematical simulations (Figure 4-8). Exceptions happened when the simulated surface coverage reached and stayed at 1, which meant the substratum surfaces were fully occupied, whereas regression obtained a surface coverage that continued increasing to become larger than 1. This odd phenomenon could be easily understood and removed by limiting the surface coverage to 1. However, because the experimental values of surface coverage were far from 1 (Figure 4-4), the regression models (Equation 4-1 to Equation 4-6) could be applied with satisfactory accuracy under the current experimental conditions.

The optimized parameter sets of Y_o (the yield of biomass on oxygen consumption), K_o (the half-saturation coefficient) and $\mu_{\max}$ (the maximum specific growth rate) could be obtained from the regression model using experimental data. In particular, regression equations were applied to fit the experimental data (for example, Equation 4-1 for position H replicate 1) using a MATLAB curve fitting toolbox within the simulated parameter ranges (Y_o in [0, 2], K_o in [1.75E-4, 7E-4], and $\mu_{\max}$ in [0.2355, 0.942]). The best fitting results with the largest R^2 value were chosen. The same procedure was done for all six samples (Equation 4-1 to Equation 4-6). Previous studies suggested that even though differences might exist in the Monod kinetic parameters for treatments with different initial cell densities or substrate concentrations, one single value for each of the parameter should be able to describe the bacterial growth for a wide range of substrate concentrations (Galperin and Kaplan, 2008; Simkins and

Alexander, 1984; Simkins and Alexander, 1985). The results here show that in spite of the small differences between the mean values, no significance was found by *t*-test ($p > 0.05$) between the values of the respective parameters (Y_o , K_o or $\mu_{\max}$) obtained from the high COMSOL O₂ condition at position H and the low COMSOL O₂ condition at position L (Figure 4-9), which indicates that the three parameters should be constant for different COMSOL O₂ conditions. The average values of Y_o , K_o and $\mu_{\max}$ from position H ($Y_o = 0.976 \text{ g.g}^{-1}$, $K_o = 0.000201 \text{ g.L}^{-1}$, and $\mu_{\max} = 0.674 \text{ hour}^{-1}$) were determined as the best-optimized results for further simulations.

Figure 4-9. Optimized parameters for position H (high COMSOL O₂) and position L (low COMSOL O₂). Bars are plotted as mean $\pm$ SD, calculated from the three replicates.

Biofilm structures simulated with different COMSOL O₂ using optimized Monod kinetic parameters. The three optimized parameters ($Y_o = 0.976 \text{ g.g}^{-1}$, $K_o = 0.000201 \text{ g.L}^{-1}$, and $\mu_{\max} = 0.674 \text{ hour}^{-1}$) were applied to simulate

biofilm development for all six experimental samples. The simulated biofilm morphologies (Figure 4-10) were compared with experiments (Figure 4-3).

Figure 4-10. Representative simulated biofilm structures at positions H and L at 0, 60, 120, 180, 250, and 320 minutes (Scale bar: 20 μm). White square: Cluster H1, white oval: Cluster H2, red square: Cluster L1, and red oval: Cluster L2.

At the initial stages, only small biofilm clusters were established and bacterial dispersal on the surface was not obvious in experiments for either the high (Figure 4-3; position H, 0–120 minutes) or the low (Figure 4-3; position L, 0–120 minutes)

COMSOL O₂ conditions. Thus, the simulated biofilm structures (Figure 4-10; both positions, 0–120 minutes) were very similar to the experimental structures. Bacterial dispersal became noticeable at 250 and 320 minutes, which could be the result of oxygen limitation inside the biofilm clusters. Bacterial colonization at new locations was observed (Figure 4-3; both positions, 250 and 320 minutes), especially for the high COMSOL O₂, where larger clusters were formed. The differences between experimental and simulated biofilm structures could be found. Unlike *Pseudomonas aeruginosa* biofilms, whose initial clusters are usually formed by aggregation, initial biofilm colonies of *P. putida* have been found being formed by bacterial colonial growth (Tolker-Nielsen et al., 2000). From this perspective, bacterial motility was ignored in the current model for simplicity when only the initial biofilm formation was concerned. The results in this study should lead to the conclusion that bacterial motility should be treated carefully if late stage biofilms are studied in the future.

Quantification of biofilms simulated with different COMSOL O₂ using optimized Monod kinetic parameters. Similar surface coverage plots were obtained for positions H and L from simulations (Figure 4-11a and b) compared with experiments (Figure 4-4). Two-way ANOVA showed that time had a significant effect on biofilm growth ($p < 0.0001$ for both positions), whereas no significant difference was shown between the simulation and experimental results ($p = 0.95$ for the high COMSOL O₂ and $p = 0.24$ for the low). Therefore, it could be concluded that the chosen parameters ($Y_o = 0.976 \text{ g.g}^{-1}$, $K_o = 0.000201 \text{ g.L}^{-1}$, and $\mu_{\max} = 0.674 \text{ hour}^{-1}$) could be applied to simulate biofilms with sufficient accuracy for different COMSOL O₂ levels. Moreover, it could be observed that the simulations could catch the experimental biofilm growth

process regardless of the initial bacteria numbers and locations. The average surface coverage increase speeds before and after 250 minutes, when the surface coverage increased rapidly, showed significant differences for both experimental and simulated biofilms (Figure 4-12).

Figure 4-11. Surface coverage plots. (a) The mean surface coverage of simulated biofilms. (b) The individual results of the three replicates based on simulation data. (c) The mean surface coverage calculated by the regressed models. (d) The individual results of the three replicates based on regression data.

Figure 4-12. The average surface coverage increase speeds before and after 250 minutes for both the experimental and simulation biofilms. * indicates the significant differences between the corresponding average speeds before and after 250 minutes by *t*-test with $p < 0.05$.

$$SC = 2^{-a+b*Time} \quad \text{Equation 4-7}$$

$$SC1 = 2^{-6.37316+0.012892*Time} \quad \text{Equation 4-8}$$

$$SC2 = 2^{-6.20440+0.012983*Time} \quad \text{Equation 4-9}$$

$$SC3 = 2^{-5.87929+0.012592*Time} \quad \text{Equation 4-10}$$

$$SC4 = 2^{-6.28484+0.010557*Time} \quad \text{Equation 4-11}$$

$$SC5 = 2^{-6.51581+0.010555*Time} \quad \text{Equation 4-12}$$

$$SC6 = 2^{-6.24974+0.010495*Time} \quad \text{Equation 4-13}$$

where SC1–SC6 refer to surface coverage of the six samples (position H replicates 1, 2, and 3 and position L replicates 1, 2, and 3).

The relationship between the surface coverage and time could be obtained based on the regression model and the optimized values for Y_o , K_o , and $\mu_{\max}$, which could be summarized as a general formula (Equation 4-7). The detailed relationships between the surface coverage and time at positions H and L could be obtained (Equation 4-8 to Equation 4-10 for experiments at position H, and Equation 4-11 to Equation 4-13 for those at position L), which gave surface coverage data (Figure 4-11c and d) very close to both the simulated (Figure 4-11a and b) and experimental results (Figure 4-4).

Figure 4-13. Constants of the equations relating biofilm surface coverage to time. (a) The results of constant a . (b) The results of constant b . Figures were plotted as mean $\pm$ SD. * indicates the presence of a significant difference between the two groups by t -test with $p < 0.05$.

In the general formula (Equation 4-7), the constant a was determined by the initial attachment pattern, which was not directly related to the COMSOL O_2 ($a_{\text{Position H}}/a_{\text{Position L}} = 0.97 \pm 0.04$). The constant b could be linked to COMSOL O_2 , and showed a significant increase when the COMSOL O_2 increased ($b_{\text{Position H}}/b_{\text{Position L}} = 1.22 \pm 0.02$) (Figure 4-13). A similar

exponential relationship between initial surface coverage and time has been previously reported (Gottenbos et al., 2000; Haruff et al., 2003).

As mentioned previously, fluctuating surface coverage was observed in the experimental data even though it was not very obvious. Another set of simulations were performed with a one-minute time step to study the oscillations in detail. The three characteristics of simulated biofilms: surface coverage, average thickness, and total cell number, were then plotted (Figure 4-14a, c, and e). The oscillations of total cell number for both positions H and L were directly observable from the cell number plot (Figure 4-14e), whereas both the surface coverage and average thickness only showed blur oscillations with small magnitudes, which were difficult to distinguish (Figure 4-14a and c). Therefore, the increase speeds of the three characteristic parameters were also plotted (Figure 4-14b, d, and f).

From the three increase speed plots (Figure 4-14b, d, and f), it was clear that the increase rates of all the three characteristic parameters, especially those of the surface coverage and total cell number, showed periodic waveforms. The time interval between oscillation peaks was 68.0 ± 1.4 minutes for position H and 87.8 ± 1.3 minutes for position L. It was suspected that the periodic waveform of the increase speeds was caused by oxygen-limited bacterial growth inside the bacterial clusters. Very similar oscillating patterns of bacterial growth rates inside biofilms was observed in the literature, which was concluded to be collective bacterial behaviors that resulted from metabolic co-dependence between the interior and peripheral cells during nitrogen limitation (Liu et al., 2015).

Figure 4-14. Biofilm characteristics, and their increase speeds, with simulation step of 1 minute. (a), (c), and (e) The biofilm surface coverage, average thickness, and total cell number. (b), (d), and (f) Increase speeds of the three characteristics respectively. Solid lines are the averaged values calculated from the three replicates of position H and position L. Dashed lines indicate the error bars, calculated as SD.

Oxygen distributions on the bottom surfaces of biofilms simulated with different COMSOL O₂ using optimized Monod kinetic parameters. To relate

the observed biofilm growth behaviors to oxygen availability, oxygen profiles on the glass surface were plotted based on biofilm mathematical modeling results (Figure 4-15).

Figure 4-15. Oxygen distributions on the glass surfaces based on biofilm mathematical modeling results. (a) to (f) Oxygen contour plots (Image dimensions: 212 μm x 212 μm). The colors from red to blue indicate the decrease in oxygen concentration. (a), (b), and (c) Respective results of position H replicate 1 at 120, 250, and 320 minutes. (d), (e), and (f) Respective results of position L replicate 1 at 120, 250, and 320 minutes. (g) and (h) Maximum and minimum oxygen concentrations on the biofilm bottom surfaces, respectively.

The contours in the figures (Figure 4-15a–f) represented the same oxygen concentration based on biofilm mathematical modeling, and the colors from red to blue indicate the decrease of oxygen concentration. It is clear from the oxygen profile contour figures that the oxygen concentration on the surface decreased in accordance with the increase in biofilm clusters, especially at the center of the biofilm clusters (Figure 4-15a–f). The maximum oxygen on the bottom surface of simulated biofilms showed a smooth decrease with biofilm growth (Figure 4-15g). However, the minimum oxygen concentration showed clear fluctuations for both positions H and L (Figure 4-15h).

Figure 4-16. Comparison between cell number increase speeds and minimum oxygen concentration on the bottom surface of simulated biofilms for the whole 350 minutes (a) and for the period from 100 to 150 minutes (b).

Upon comparing the periodic waveform of cell number increase speeds with the minimum oxygen data of the simulated biofilms, it was notable that the minimum oxygen fluctuations could be closely linked with the peaks or valleys of the cell number increase figure (such as the circles in Figure 4-16a). Upon detailed inspection, it could be determined that complex relationships existed between the minimum oxygen and bacterial increase speeds (Figure 4-16b). The four arrows

in Figure 4-16b show four such relationship scenarios. Arrow 1 shows a minimum oxygen concentration valley at a bacterial growth speed peak, which indicates that a fast bacterial growth speed leads to greater oxygen consumption. Arrow 2 shows a minimum oxygen concentration peak at a bacterial growth speed valley, which is the opposite of the arrow 1 scenario and reflects that more oxygen could be left in the biofilm as a result of slower bacterial growth. Arrow 3 shows a bacterial growth speed peak at a minimum oxygen concentration peak, which indicates a faster bacterial growth speed at higher oxygen concentrations. Finally, arrow 4 shows a bacterial growth speed valley at a minimum oxygen concentration valley, which reflects that the minimum oxygen availability limits the bacterial growth. These four seemingly conflicting observations prove that the oxygen supply and bacterial growth in biofilms are more complicated than simple cause and effect relationships. While more or less oxygen availability from modeling results can respectively support or limit bacterial growth, fast or slow bacterial growth can in turn respectively lead to oxygen depletion or accumulation in the model.

The area of the four chosen clusters (circled in Figure 4-3 and Figure 4-10), two from the high COMSOL O₂ and two from the low, were plotted (Figure 4-18) to clarify the relationship between the oxygen concentration and cluster growth. Note that the four clusters were chosen such that the initial size of the two clusters from the high COMSOL O₂ (Cluster 1: 28.07 μm^2 and Cluster 2: 6.03 μm^2 from the experimental results) were comparable of the two from the low (Cluster 1: 27.72 μm^2 and Cluster 2: 8.96 μm^2 from the experimental results). Overall, the simulated clusters showed similar increase patterns to the experimental clusters (Figure 4-17). The cluster profile at both positions (Figure 4-18) could directly

demonstrate that the increase in cluster size led to the decrease of oxygen concentration inside the clusters. Besides, the minimum oxygen concentration inside the clusters showed obvious fluctuations for both the large and small clusters, and for both positions H and L.

Figure 4-17. Comparison of the areas of the chosen clusters between the experimental and simulation results.

Figure 4-18. Area of and minimum oxygen concentration in the four chosen clusters of the simulated biofilms at position H (a) and position L (b). H1, H2, L1, and L2 are the four clusters circled in Figure 4-3 and Figure 4-10.

The decrease rate of oxygen concentration and the increase rate of cluster area were plotted and the peaks of the rates were marked to quantify the fluctuations in the minimum oxygen concentration and cluster area plots (Figure 4-19 and Figure 4-20). The first peaks for the oxygen decrease rate were not counted for further analysis, as they were caused by the manual bacterial seeding process. Two phenomena could be observed: There are more peaks for the profiles at position H, and there are more peaks for the increase rate of the cluster area profiles than for the decrease rate of oxygen concentration profiles (Figure 4-19 and Figure 4-20).

Figure 4-19. The decrease rate of the minimum oxygen concentrations in and the increase rate of cluster area for the two clusters in position H.

Figure 4-20. The decrease rate of the minimum oxygen concentrations in and the increase rate of cluster area for the two clusters in position L.

The data also show that the peaks of the decrease rate of the minimum oxygen concentration in the chosen clusters usually occurred at the same time as or just one step (one minute) after the peaks of the increase rate of the cluster area (Figure 4-21; with a probability larger than 60% for clusters H1 and H2, and 90% for clusters L1 and L2). The percentage plot also reveals that the minimum oxygen profile for clusters at position H (cluster H1 and cluster H2 in Figure 4-21) was less related to the cluster area growth compared to that at position L (clusters L1 and L2 in Figure 4-21) by giving lower percentages.

Figure 4-21. The percentage of the oxygen decrease rate peaks that occurred at the same time or one minute after the cluster area increase rate peaks.

Combining these observations, it could be concluded that oxygen availability can significantly affect the initial biofilm development of *P. putida* in terms of both the whole biofilm structures and individual bacterial clusters.

4.4 Conclusion

We studied the effect of oxygen availability on the initial biofilm growth of *P. putida* OUS82 in this work. Compared with conventional biofilm flow cells, such as the popular three-channel one, the square growth chamber applied in the current study is preferred, as both the shear rate and the chemical gradient could be precisely controlled. However, as the real-time monitoring of oxygen during biofilm culture remains a challenge, the oxygen conditions were simulated for biofilm experiments using a COMSOL model, which has been validated using an oxygen planar optode sensing method. Biofilm experimental results showed that

the oxygen flux had an important impact on the initial surface biofilm colonization. Bacteria spread faster on the surface under high-oxygen conditions and slower under low-oxygen conditions.

A new approach that combines regression method with biofilm mathematical modeling was proposed to simulate experimental biofilm characteristics accurately. Consistent results with experimental biofilms were obtained from biofilm computational simulations when comparing the biofilm structures directly and biofilm surface coverage. More information was extracted from the simulation results that could not be achieved from the current experiment protocols. Oxygen profiles on the biofilm bottom surface based on biofilm mathematical modeling results indicated that bacterial consumption resulted in decreased oxygen concentration inside the biofilm clusters, which reflected that the different bacterial colonization speeds on the surface were related to the altered oxygen availability.

Periodic oscillations of bacterial growth rates, in terms of both biofilm surface coverage, average thickness, and cell number, were observed. Although the exact mechanism was unclear, these oscillations may be at least partially related to the oxygen availability, as there were corresponding fluctuations in oxygen concentration profiles. Moreover, the periods of the oscillations were shorter under high-oxygen conditions and longer under low-oxygen conditions .

In the current study, the oxygen profiles were either simulated using COMSOL or extrapolated from the biofilm mathematical modeling results. Therefore, the measurement of oxygen distribution in the biofilm system during biofilm culture

is needed and included in future plans to give a comprehensive conclusion regarding the effect of oxygen on biofilm growth.

Chapter 5 Effect of Calcium Availability on Biofilm Formation

5.1 Introduction

Biofilms are surface-associated microbial communities embedded in self-produced EPS matrix and are the most widely distributed life models on Earth (Stoodley et al., 2002). The microorganisms that live in biofilms are exposed to the immediate microenvironments created by the EPS matrix. The heterogeneous microenvironments generated by the EPS matrix make the biofilm a robust and stable system and tolerant of harsh external conditions and antimicrobial agents through limited diffusion, covalent or noncovalent binding, or biochemical reactions (Flemming et al., 2016; Flemming and Wingender, 2010; Stewart and Costerton, 2001).

Calcium has been identified as an important factor in biofilm formation (Geesey et al., 2000). In detail, calcium enhances the bacterial adhesion forces to the substratum (Cruz et al., 2012), and promotes bacterial binding strength to the EPS matrix (Kierek and Watnick, 2003). The EPS production rate was also observed to be calcium dependent, with faster production rates at higher calcium concentrations (Patrauchan et al., 2005). Bacterial motility, which is very important for biofilm structure formation (Barken et al., 2008; Klausen et al., 2003), was discovered to be decreased by higher calcium availability (Cruz et al., 2012). Therefore, the quantitative study of biofilm formation under controlled calcium gradients is required for a better understanding of calcium's influence on biofilm activities.

Shewanella oneidensis MR-1 is a facultative metal-reducing bacterium that is capable of transferring electrons to metal oxides (Fredrickson et al., 2008;

Fredrickson and Zachara, 2008). *S. oneidensis* biofilms are important for many biotechnological and environmental processes (Belchik et al., 2011; Cao et al., 2011; Marshall et al., 2006). In this study, a novel microfluidic biofilm reactor was developed by integrating a biofilm growth chamber with a gradient generator, with which *S. oneidensis* MR-1 biofilm growth was studied under various calcium gradients. Biofilm mathematical modeling by iDynoMiCS was conducted to provide insights into the possible reasons for the observed biofilm behaviors.

5.2 Materials and Methods

5.2.1 Fabrication of the microfluidic biofilm reactor

The microfluidic biofilm reactor consists of three layers: the gradient generator, the biofilm growth chamber, and the glass substratum (Figure 5-1a). The gradient generator and biofilm growth chamber were made of polydimethylsiloxane (PDMS) with a base-to-reagent ratio of 10 to 1. The gradient generator was fabricated using silicon wafer with complementary features patterned via standard photolithography, and the biofilm growth chamber was fabricated using an aluminum mold fabricated by micromachining. The three layers were bonded together using a plasma bonding method. The bonded biofilm reactor was sterilized with UV radiation for one hour before each biofilm culture experiment.

The chemical gradients and the fluid flow fields inside the reactor were simulated using COMSOL Multiphysics 4.3b (COMSOL, Inc.) (Figure 5-1b). *Laminar Flow* and *Transport of Diluted Species* modules were applied. The effluents of the nine outlets (Figure 5-1a and b) were collected to evaluate the calcium gradient generation, which was determined by mixing and diffusion in the

upstream gradient generator channels and the rectangular growth chamber. The calcium concentration was quantified using the Ion Chromatography process with defined concentrations of CaCl_2 as the standard (Rieman and Walton, 2013).

5.2.2 *Shewanella oneidensis* MR-1 biofilm growth

S. oneidensis MR-1 tagged with a green fluorescent protein (MR-1-gfp) was used for biofilm culture (Zhang et al., 2014). Chemically defined MM1 media (Ding et al., 2014) (30 mM of HEPES, 7.5 mM of NaOH, 28.04 mM of NH_4Cl , 1.34 mM of KCl, and 4.35 mM of NaH_2PO_4), either with or without 0.68 mM CaCl_2 , were supplied as the growth media for MR-1-gfp. Biofilms of MR-1-gfp were cultivated in the fabricated microfluidic biofilm reactor. The medium was supplied for bacterial growth via syringe pumps (NE-1000) at a 3 mL/h flow rate for both inlets. MM1 containing either 0.68 mM CaCl_2 or 0 mM CaCl_2 was delivered via the two inlets. Calcium gradients were generated by the gradient-generating channels upstream of the center chamber. MR-1-gfp suspension diluted from overnight cultures ($\text{OD}_{600} \approx 0.1$) was inoculated into the central chamber through the cell inoculation inlets located at each side of the growth chamber (Figure 5-1b). Media flow was stopped for two hours to facilitate bacterial adhesion.

Biofilm growth and development was monitored by imaging biofilm structures using CLSM (ZEISS LSM780). The excitation wavelength used was 488 nm, and the emission wavelength was in the range 508–535 nm. Images were further processed by the MATLAB toolbox, Phlip (Mueller et al., 2006).

5.2.3 Biofilm mathematical modeling

Biofilm mathematical modeling software package, iDynoMiCS, was adopted (Kreft et al., 1998; Lardon et al., 2011; Merkey et al., 2011). The source code was modified accordingly to give the capacity of relating EPS production and bacterial surface motility to local calcium concentration. The developed detachment mechanisms in Chapter 3 were also applied to reflect the effect of calcium on the biofilm matrix cohesiveness (Li et al., 2015). The simulation parameters were listed in Table 5-1. Parameters not listed in the table include detachment coefficients (DCs), EPS production rates, and bacterial motility speeds, which were varied in simulations to check their influence on the simulated biofilm structures.

Table 5-1. Model parameters for simulating the effect of calcium on the biofilm growth of *S. oneidensis*

Parameters	Values	Units
Size of the computation domain	212x212x212	μm^3
Size of each grid	4x4x4	μm^3
Mass transfer boundary layer thickness	20	μm
Simulation time step	1	hour
Bulk concentration of lactate	1.78	$\text{g} \cdot \text{L}^{-1}$
Lactate diffusion coefficient	9.1584E^{-4}	$\text{m}^2 \cdot \text{day}^{-1}$
Bulk concentration of oxygen	0.00816	$\text{g} \cdot \text{L}^{-1}$
Oxygen diffusion coefficient	1.728E^{-4}	$\text{m}^2 \cdot \text{day}^{-1}$
Maximum specific growth rate	0.47	hour^{-1}
Lactate half-saturation coefficient	0.0011748	$\text{g} \cdot \text{L}^{-1}$
Oxygen half-saturation coefficient	0.000192	$\text{g} \cdot \text{L}^{-1}$
Bacterial density	150	$\text{g} \cdot \text{g}^{-1}$
EPS density	75	$\text{g} \cdot \text{g}^{-1}$
Biomass yield on lactate	0.215	$\text{g} \cdot \text{g}^{-1}$
Biomass yield on oxygen	0.072	$\text{g} \cdot \text{g}^{-1}$
Biomass yield on calcium	0.43	$\text{g} \cdot \text{g}^{-1}$

5.3 Results and Discussion

5.3.1 Calcium gradient in the microfluidic biofilm reactor

As described, the calcium gradient inside the fabricated microfluidic biofilm reactor was simulated using COMSOL (Figure 5-1b). A clear calcium gradient was generated inside the chamber perpendicular to the flow direction. Moreover, the slow diffusion process meant that the calcium concentrations did not change significantly in accordance with the flow direction inside the growth chamber (data not shown).

Figure 5-1. Microfluidic biofilm reactor. (a) The structure of the reactor. The reactor consists of three layers, including the gradient generator layer, the 2 mm biofilm growth chamber layer, and the glass substratum. (b) COMSOL simulation of the calcium gradients in the chamber. (c) Validation of the calcium gradient in the chamber.

The simulation results were validated by measuring the calcium concentrations of the nine effluents with the same parameters used for the simulations by the Ion Chromatography method. As shown in the results (Figure 5-1c), the experimentally measured calcium concentrations of the nine effluents matched well with the simulation-obtained results, with 0.92 goodness of fit. Therefore, the microfluidic biofilm reactor was confirmed as being able to generate a stable calcium gradient for the biofilm development of *S. oneidensis*.

The gradient generator was first developed by Jeon et al. and was demonstrated by generating hydrofluoric acid gradients to etch glass coverslips (Jeon et al., 2000; Jeon et al., 2007). After this, gradient generators have been applied to various studies such as the dosing effect of chemicals to attached cells (Lin et al., 2004; Ruan et al., 2009) and the chemotaxis of cells under chemical gradients (Li Jeon et al., 2002; Walker et al., 2005). Compared with the existing gradient generator design, the microfluidic biofilm reactor developed in this study has two advantages. First, an additional layer (Figure 5-1; growth chamber layer) that is separate from the gradient generator layer was fabricated. This additional layer has four large channels, two of which can be used as cell inoculation channels to prevent the micro-channels clogging, and the other two can be used as outputs to lower the pressure in the chamber. Second, the nine output channels (Figure 5-1; gradient generator layer) can be used as sampling points during experiments, as long as the pressure can be balanced.

5.3.2 Biofilm development under calcium gradients

S. oneidensis MR-1-gfp biofilms were cultured under the defined CaCl_2 gradient. Only the results of one of the two conducted experiments are reported here. Although the other experiment also showed increased biofilm formation at higher

calcium concentrations, it was difficult to analyze them together, as the biofilm CLSM images were taken at different time points. Representative day 7 biofilm images were shown for three CaCl_2 concentrations, 0.024 mM, 0.34 mM, and 0.66 mM (Figure 5-2a, b, and c). The biofilm images show that the biofilm was much thicker and denser at 0.66 mM CaCl_2 with a biovolume of $4.33 \times 10^6 \mu\text{m}^3$ per image. However, less biofilm was formed at 0.34 mM CaCl_2 ($2.19 \times 10^5 \mu\text{m}^3$ per image) than that at 0.024 mM CaCl_2 ($6.99 \times 10^5 \mu\text{m}^3$ per image). This unexpected phenomenon was suspected to be caused by the chamber wall effects. The biovolume and surface coverage of the generated biofilm were also calculated (Figure 5-2d). The results showed that the two parameters had similar exponential relationships with the CaCl_2 concentration. When the CaCl_2 concentration changed from almost zero (0.0084 mM) to nearly the supply concentration (0.67 mM), the biovolume of the biofilms first showed a small decrease (0.0084 mM at $6.35 \times 10^5 \mu\text{m}^3$ per image to 0.34 mM at $2.19 \times 10^5 \mu\text{m}^3$ per image). After that, the biovolume increased rapidly (0.34 mM at $2.19 \times 10^5 \mu\text{m}^3$ per image to 0.67 mM at $4.33 \times 10^5 \mu\text{m}^3$ per image), and the same development pattern was shown for biofilm surface coverage. Exponential fitting could be applied to relate biovolume and surface coverage to CaCl_2 concentration with an R^2 larger than 0.7.

Figure 5-2. Biofilm development in the microfluidic biofilm reactor with CaCl_2 gradients. (a), (b), and (c) Representative biofilm structures at Day 7, taken at locations near the chamber inlets with CaCl_2 concentrations of 0.024 mM, 0.34 mM, and 0.66 mM. (d) The biovolume per image of $424.68 \mu\text{m} \times 424.68 \mu\text{m}$, and the surface coverage together with the exponential fitting curves of the two parameters of the biofilms growing under different CaCl_2 concentrations.

5.3.3 Mathematical simulations of biofilm development with different calcium concentrations

Models of the CaCl_2 concentration effect on biofilm development. The biofilm responses to altered CaCl_2 concentrations were simulated; three hypotheses were made based on the literature reports. The first was that the CaCl_2 concentration could affect detachment events, the second was that the CaCl_2

concentration could influence the bacterial EPS production rates, and the last was that the CaCl₂ concentration could impact the bacterial surface motility.

The influence of the CaCl₂ concentration on biofilm detachment was simulated as a simple inhibition model (Equation 5-1), and the detachment methods described in Chapter 3 were applied here. The equation permits the anticipation that more detachment events could happen for decreased CaCl₂ concentrations.

$$P_{d,i} = \left(\frac{S_{Ca,bulk}}{S_{Ca,bulk} + S_{Ca,i}} \right) \cdot (K_{ds} \cdot \Delta t \cdot \left(\frac{h_i}{h_{max}} \right)^2 + K_{dn} \cdot \Delta t \cdot \left(1 - \frac{S_{lac,bulk}}{S_{lac,i}} \right) + K_{de} \cdot \Delta t \cdot \left(\frac{NB_{free,i}}{NB_{total}} \right)) \quad \text{Equation 5-1}$$

where $P_{d,i}$ is the detachment probability of the biomass inside the grid; $S_{Ca,bulk}$ and $S_{lac,bulk}$ refer to the bulk concentrations of CaCl₂ and lactate; $S_{Ca,i}$ and $S_{lac,i}$ refer to the local concentrations of CaCl₂ and lactate; h_i refers to the local biofilm thickness and h_{max} refers to maximum thickness; $NB_{free,i}$ refers to the free neighbors and NB_{total} refers to all possible neighbors; Δt is the simulation time step; K_{ds} , K_{dn} , and K_{de} are the three DCs (here $K_{ds} = K_{dn} = K_{de} = 0.01$ and $K_{ds} = K_{dn} = K_{de} = 0.1$ were used).

Even though exponential fittings were applied to relate the biovolume and surface coverage data with CaCl₂ concentrations, the influence of CaCl₂ concentrations on EPS production rates and bacterial surface motility were simulated using three different models (Figure 5-3). The three models were applied for three reasons. First, the three models were very simple to implement. Second, the models could guarantee the same bacterial behaviors at 0 mM CaCl₂ and 0.68 mM CaCl₂. Most importantly, the first derivatives of the three models had different relationships with the altered CaCl₂ concentrations. Increasing the CaCl₂ concentrations meant that the first derivatives of the Monod model, linear model, and quadratic model were respectively decreasing, constant, and increasing. The first derivatives

represent the parameter's increasing speed. Therefore, the three models could represent three different scenarios.

Figure 5-3. Models of the CaCl_2 concentration influence. (a) The three models for EPS production rates. (b) The same three models for bacterial surface motility speeds.

Simulated biofilm structures. The simulated biofilm structures at day 3 for the different models of CaCl_2 concentration influence at different CaCl_2 concentrations with the two chosen DCs were shown (Figure 5-4). Obvious differences can be observed between the biofilms formed with higher CaCl_2 concentrations and those formed with lower CaCl_2 concentrations. Taking the linear influence model with DCs of 0.1 as an example, only small bacterial clusters were generated at 0.17 mM CaCl_2 , whereas much larger biofilm clusters were formed at 0.68 mM CaCl_2 . The two DCs applied in simulations made it clear that less biomass could accumulate on the surface with the larger DCs, which was true for all the considered CaCl_2 concentrations from 0 mM to 0.68 mM. However, it was difficult to determine from the biofilm structures whether there were different effects in the three calcium influence models, as similar structures were generated by applying the three models with the same CaCl_2 concentrations.

Figure 5-4. Simulated biofilm structures at day 3 with different calcium influence models and calcium concentrations.

Characterization of simulated biofilms. The simulated biofilm structures were also characterized using different parameters. Besides the biovolume and surface coverage parameters, which were used to characterize the experimental biofilms, two more parameters were considered, namely the cell number count and thickness.

Figure 5-5. The biovolume and surface coverage of the simulated biofilms. (a) and (b) The biovolume of biofilms simulated with DCs of 0.1 and 0.01. (c) and (d) The surface coverage of biofilms simulated with DCs of 0.1 and 0.01. (e) The biovolumes of the simulated day 3 biofilms for five CaCl_2 concentrations. (f) The surface coverage of the simulated day 3 biofilms for five CaCl_2 concentrations.

The biovolume and surface coverage showed very similar growth patterns (Figure 5-5). The time series data showed observable differences between the three

calcium influence models, particularly for the larger DCs (Figure 5-5a and c). When the DCs were 0.1 and the CaCl_2 was 0.34 mM, biofilms simulated using the quadratic model showed very slow biovolume and surface coverage increases before 56 hours and showed fluctuations after 56 hours. Biofilms simulated with the linear model also showed biovolume and surface coverage fluctuations at 0.34 mM CaCl_2 after 66 hours. No fluctuations were observed for simulations with 0.68 mM CaCl_2 or simulations that applied the Monod model throughout the simulation period. The biovolume and surface coverage increased smoothly and rapidly over time when the smaller DCs was used (Figure 5-5b and d). Plotting the two parameters of day 3 biofilms at different CaCl_2 concentrations showed that the biovolume increased as the CaCl_2 concentrations increased for all the three applied calcium influence models (Figure 5-5e), which was consistent with the experimental results. However, different phenomena were observed in the surface coverage plot (Figure 5-5f). Only two of the six plotted conditions had their largest surface coverage at 0.68 mM CaCl_2 , and the other four had their largest surface coverage at 0.51 mM CaCl_2 instead. The cell number count, both the total cell number including the EPS and the bacterial cell number, showed very similar results as biovolume and surface coverage (Figure 5-6a, c, and e).

Figure 5-6. The cell number and average thickness of the simulated biofilms. (a) and (b) The total cell number counts of biofilms, including both bacterial cell and EPS, with DCs of 0.1 and 0.01. (c) and (d) The bacterial cell number count of biofilms with DCs of 0.1 and 0.01. (e) and (f) The average thickness of simulated biofilms with DCs of 0.1 and 0.01.

The average thickness plot shows that biofilms with no CaCl_2 were initially thicker than those with CaCl_2 supply, especially for the smaller DCs (Figure 5-6e and f). Considering that the total cell counts of the initial biofilms with or without

CaCl₂ were similar (Figure 5-6a and b), the decrease in the average thickness for biofilms with CaCl₂ supply indicated that the bacteria were able to move on the surface and colonize new locations. However, when there was no CaCl₂ present, bacteria could only grow where they attached, leading to the formation of thicker biofilm clusters connected by empty channels. Indeed, it has been confirmed that lower surface coverage was obtained in the absence of CaCl₂ (Figure 5-5c and d). The fluctuations observed in biovolume and surface coverage figures are more obvious in the average thickness plot. biofilms simulated with all the three calcium influence models showed vibrations at late stages at 0.34 mM CaCl₂ with DCs of 0.1. However, there were still no fluctuations observed with DCs of 0.01 or at 0.68 mM CaCl₂. Therefore, it could be speculated that both the detachment and CaCl₂ supply were responsible for the observed fluctuations. Biofilm-oscillated accumulation has been reported as being caused by regrowth after detachment (Lewandowski et al., 2007). In this study, the fluctuations in biofilm characteristics could also be caused by regrowth after detachment, as fluctuations were only observed at the high detachment rate.

Bacterial density was also evaluated (Figure 5-7). Similar to the literature reports (Sarkisova et al., 2005), the calcium concentrations here had no significant influence on the bacterial cell density for all simulated conditions, even though a larger biovolume was generated under higher calcium concentration conditions.

Figure 5-7. Bacterial density of the simulated biofilms with DCs of 0.1 (a) and 0.01 (b).

Calcium distributions on the bottom surfaces of the simulated biofilms.

Calcium distributions on the bottom biofilm surfaces were plotted to evaluate the effect of calcium on the simulated biofilm structures (Figure 5-8). The contour figures clearly show that along with the biofilm growth, calcium was consumed and heterogeneous calcium distributions were established. Zero or close to zero CaCl_2 concentration was found in the centers of the bacterial clusters at day 3 for both 0.34 mM CaCl_2 and 0.68 mM CaCl_2 (blue color in Figure 5-8). Meanwhile, high concentrations of CaCl_2 still existed on the substratum surfaces in the biofilm-free locations (red color in Figure 5-8).

Figure 5-8. Calcium distributions on the substratum surfaces. Contours in the images refer to the same CaCl_2 concentrations. Colors from red to blue indicate decreasing CaCl_2 concentrations. Scale bar: 50 μm .

The maximum and minimum CaCl_2 concentrations on the substratum surfaces were also plotted for quantitative evaluation (Figure 5-9). It could be observed that even though the overall decreasing trends for CaCl_2 concentrations were similar for the three calcium influence models, obvious differences existed between them upon detailed inspection. At 0.34 mM CaCl_2 , the smallest CaCl_2 concentrations were found when applying the linear model for both the maximum and minimum CaCl_2 concentrations, while the other two models showed no significant differences between one another (Figure 5-9a and b). However, at 0.68 mM CaCl_2 , the largest CaCl_2 concentrations were found when applying the quadratic model for the maximum CaCl_2 concentration plot (Figure 5-9c). No obvious differences could be observed for the minimum CaCl_2 concentration plot (Figure 5-9d). Therefore, it could be summarized that there were differences between the three models with regard to their effect on the calcium distributions on the substratum surfaces. However, it was difficult to draw a conclusion based

on the existing data with regard to how the calcium distribution was influenced by the three models.

Figure 5-9. CaCl₂ concentrations on the substratum surfaces. (a) and (b) The results of maximum and minimum CaCl₂ concentrations on the surface for 0.34 mM CaCl₂ supply. (c) and (d) The results of maximum and minimum CaCl₂ concentrations on the surface for 0.68 mM CaCl₂ supply.

5.4 Conclusion

In this study, the effect of calcium on the biofilm formation of *S. oneidensis* was investigated. A microfluidic biofilm reactor, which could generate a continuous and stable chemical gradient in the chamber, was developed and used to culture biofilms under a defined calcium gradient. Experimental results showed significant effects of calcium concentrations on *S. oneidensis* biofilm growth. Higher calcium concentrations resulted in larger biovolume and surface coverage.

Biofilm mathematical modeling using iDynoMiCS, was applied to simulate the effect of calcium on biofilm formation. To investigate the association between calcium concentration and biofilms growth, calcium concentration was hypothesized to influence biofilm detachment, EPS production, and bacterial surface motility. The hypothesis on biofilm detachment was simulated using an inhibition model, whereas three models (the Monod model, linear model, and quadratic model) were tested for the hypotheses on EPS production and bacterial surface motility.

Simulation results were similar to experimental results with respect to the effect of calcium concentration on overall biofilm growth, which demonstrated the possibility of further applications of the mathematical models to test other mechanisms which might be causing the observed calcium effects. The calculated biovolume and surface coverage results demonstrated that in addition to a continuous increasing behavior, a slight decrease and large fluctuations existed with larger DCs at low CaCl_2 concentrations. Calcium profiles from the simulation results indicated that biofilm growth caused severe calcium deficiency in the bottom biofilm layers after three days regardless of the supplied CaCl_2 concentrations, which might be one reason for the observed decrease in biovolume and surface coverage.

The results presented in this chapter are currently still in the preliminary stage. It could be noted that the simulated biofilms were only qualitatively compared with the experimental results; there are two reasons for this practice. One is that the experiments were only conducted twice, and only one experiment gave analyzable results. Therefore, although it might be true that calcium can significantly affect the biofilm growth of *S. oneidensis*, more experiments should

be conducted to give statistically significant results. The other is that the effect of calcium on the biofilm growth of *S. oneidensis* in the current study was assumed; the three hypothetical models have not been validated. Hence, quantitative comparison between the simulation and experiment was not performed, but will be conducted in future studies.

Chapter 6 Conclusion

In this thesis, we have discussed applying experimental and mathematical modeling approaches to evaluate the effects of three factors, biofilm detachment, oxygen availability, and calcium concentration, on biofilm structure formation. The next section outlines the key contributions of the thesis and describes the further development of biofilm mathematical models.

6.1 Key Contributions of the Thesis

In this thesis, new detachment models were implemented in an existing IBM software package (iDynoMiCS) to study the influence of three different detachment mechanisms, SD, NLD, and ED, on biofilm structure formation. The models were validated according to their abilities to generate similar results as reports in which the detachment mechanisms were investigated in a CA model (Chambless and Stewart, 2007). In addition, the proposed models were used to study the relative importance of different detachment mechanisms in different biofilm developmental stages. By characterizing the biofilms using various parameters, including cell number, thickness, and surface coverage, SD and NLD were only effective in late biofilm growth stages, whereas ED was important from the beginning. Moreover, in comparison with NLD and ED, SD was less dependent on the coefficient values.

The effect of oxygen availability on the initial biofilm growth of *P. putida* OUS82 was studied from both experimental and mathematical modeling perspectives. A square biofilm growth chamber, which is similar to the reported planar flow cell (Zhang et al., 2011) but is easier to manage, was used to conduct biofilm experiments, in which the oxygen gradient could be controlled by adjusting the

input and output configurations. Biofilm images were taken by CLSM. Simulation parameters were specifically optimized for the experimental conditions. A new approach combining regression with biofilm mathematical modeling was proposed to simulate experimental biofilm behaviors with high accuracy. Using regression method to optimize simulation parameters has been used in a previous study and proven to be an effective approach (Xavier et al., 2004). The results showed that the models could generate biofilm structures that were similar to experimental images. Periodic oscillations of bacterial growth rates, in terms of both biofilm surface coverage and cell number, were observed. Although the exact mechanism was unclear, these oscillations may be at least partially related to oxygen availability, as there were similar fluctuations in the corresponding oxygen concentration profiles. Moreover, the periods of the oscillations were shorter under high-oxygen conditions and longer under low-oxygen conditions. Therefore, oxygen availability was demonstrated to not only influence the biofilm development of *P. putida* but also cause oscillations in biofilm growth speeds. Oscillated biofilm growth has been observed previously due to metabolic co-dependence (Liu et al., 2015). However, the exact causes of the oscillations observed in this study need to be further evaluated. The proposed method could be readily applied to study biofilm formation with various chemical gradients.

Finally, the effect of calcium on the biofilm formation of *S. oneidensis* MR-1 was investigated. A microfluidic biofilm reactor, which could generate a continuous and stable chemical gradient in the chamber, was developed with improvements to existing gradient generators (Jeon et al., 2000; Jeon et al., 2007; Lin et al., 2004) and used to culture biofilms under a defined calcium gradient. Again,

iDynoMiCS was applied to simulate the effect of calcium on biofilm formation. To investigate the association between calcium concentration and biofilm growth, calcium concentration was hypothesized to influence biofilm detachment, EPS production, and bacterial surface motility as suggested by literature (Cruz et al., 2012; Patrauchan et al., 2005). The hypothesis on biofilm detachment was simulated using an inhibition model, whereas three models (the Monod model, linear model, and quadratic model) were tested for the hypotheses on EPS production and bacterial surface motility. Simulation results were similar to experimental results with respect to the effect of calcium concentrations on overall biofilm growth. The calculated biovolume and surface coverage results demonstrated that in addition to a continuous increasing behavior, a slight decrease and large fluctuations were observed with larger DCs under low CaCl_2 concentrations. The observed fluctuations were suspected to be caused by regrowth after detachment as observed in previous reports (Lewandowski et al., 2007). Calcium profiles obtained from the simulation results indicated that biofilm growth caused severe calcium deficiency in the bottom layers after three days regardless of the supplied CaCl_2 concentrations, which might be the reason for the decrease in biovolume and surface coverage.

6.2 Improvements for the Current Studies

There are several possible improvements for the studies in this thesis.

As both fluid shear stress and nutrient transport are influenced by fluid flow, it might be important to simulate flow directly. However, the influence of fluid flow was neglected in this thesis and most studies because it is difficult to accurately simulate the interactions between fluid flow and biofilms. In addition, as biofilms are highly heterogeneous, biofilm deformation under stress is extremely complex.

Assumptions such as “a biofilm behaves as an elastic material” (Ohashi et al., 1999) and “the biofilm is homogeneous and isotropic” (Picioreanu et al., 2001) are acceptable only under specific conditions. In addition, there is also a study demonstrated that the convection transport of substrate by flow was not important for the biofilm formation (Picioreanu et al., 2000). Therefore, it is to be evaluated whether the inclusion of fluid simulation to the current biofilm mathematical model can result in better biofilm modeling results.

Another possible improvement for the current studies is to measure the oxygen distribution on the biofilm substratum surface, which could serve to validate the mathematical modeling regarding the effect of oxygen on biofilm growth. Oxygen planar optodes have been developed for biofilm measurement (Glud, 2008; Larsen et al., 2011; Oguri et al., 2006). However, oxygen planar optode sensing method that can be used to monitor oxygen in real time during biofilm development with high accuracy is still in development. Moreover, biofilm growth under different oxygen availabilities (more than two) should be evaluated.

The effect of oxygen availability on biofilm growth has been demonstrated for various strains. Oxygen distribution in aerobic biofilms showed close relationships to the 3D heterogeneous structures of the biofilms (De Beer et al., 1994). Dissolved oxygen concentration could affect the growth rates of bacterial biofilms (Jang et al., 2003; Norsker et al., 1995). Oxygen limitation could induce the dispersal of *S. oneidensis* biofilms (Thormann et al., 2005). Moreover, different bacterial strains respond differently when exposed to oxygen. In contrast to *S. oneidensis*, the biofilm formation of *S. mutans* was significantly repressed under high oxygen supply (Ahn and Burne, 2007). For *P. putida* biofilms cultured under turbulent flow conditions, oxygen limitations led to more EPS production

than carbon limitations (Applegate and Bryers, 1991). Therefore, the effect of oxygen availability on biofilm formation is a more complex process than simply inhibiting or promoting biofilm growth. The current study only treated the oxygen as a bacterial growth-limiting factor for simplicity. Further improvement of the model will be conducted in the near future to investigate the various roles oxygen may play in biofilm development. In addition, bacterial detachment and motility processes were neglected in the current study, which should also be taken care of in the future studies.

For the final experiment, which involves investigating the effect of calcium on the biofilm growth of *S. oneidensis*, it would be better to monitor biofilm growth at different time points instead of imaging only on day 7. This approach would make it possible to compare the simulations with experimental observations quantitatively. Moreover, because the effect of calcium on bacterial cell–substratum and cell–EPS matrix interactions is frequently reported (Cruz et al., 2012; Kierek and Watnick, 2003), it should be included in the future studies.

6.3 Further Development of Biofilm Mathematical Modeling

The output of a biofilm mathematical model significantly depends on the input parameters (Xavier et al., 2004). Recently, the initial biofilm development of *P. putida* OUS82 has been monitored at high temporal (10 minutes) and spatial resolutions (1 μm) (Lim et al., 2016). Proper image processing could allow using the biofilm results to build the next generation of biofilm mathematical models.

These next generation of biofilm mathematical models are expected to interact actively with the user, which means that the simulation results can be checked occasionally and the simulation processes (such as detachment or not) could be externally controlled by experimental results at different time points. In this

manner, the models could be validated in a step-by-step manner, and the output of the biofilm mathematical modeling can thus be more instructive. These next generation models are also expected to the potential to prioritize the physicochemical factors according to their effect on biofilm structures.

6.4 List of Publications

Journal Papers –

Published:

1. Li C, Yilei Z, Cohen Y. 2015. Individual based modeling of *Pseudomonas aeruginosa* biofilm with three detachment mechanisms. *RSC Adv.* **5**:3396–3402.
2. Xu Q*, Li C*, Kang Y, Zhang Y. Long term effects of substrate stiffness on the development of hMSC mechanical properties. *RSC Adv.* **5**:105651-105660.

In preparation:

1. Li C*, Ding, Y*, Kuddannaya, S, Zhang, Y, Yang, L. Influence of surface chemical modifications on bacterial adhesion. In preparation.
2. Li C, Roizman-Sade D, Zhang, Y, Cohen, Y. Effects of oxygen availability on initial growth and surface colonization of *Pseudomonas putida* biofilms. In preparation.
3. Li C, Zhang YD, Cao B, Zhang, Y, Cohen, Y. Influence of calcium on *Shewanella oneidensis* MR-1 biofilm formation and development. In preparation.

Conference Papers –

1. Badjo W*, Li C*, Xu Q, Kuddannaya S, Zhang Y, and Kang Y. Cellular stiffness measurement for 3D biological printing. 1st International

Conference on Progress in Additive Manufacturing, 26-28 May 2014,
Singapore.

*: As co-first authors.

Bibliography

- A.J Mohamed XZ and XL. 2013. Effect of surface topography on hydrophobicity and bacterial adhesion of polystyrene. *Int. Conf. Manip. Manuf. Meas. Nanoscale*:228–233.
- Ahn SJ, Burne RA. 2007. Effects of oxygen on biofilm formation and the AtlA autolysin of *Streptococcus mutans*. *J. Bacteriol.* **189**:6293–6302.
- Alagappan G, Cowan RM. 2004. Effect of temperature and dissolved oxygen on the growth kinetics of *Pseudomonas putida* F1 growing on benzene and toluene. *Chemosphere* **54**:1255–1265.
- Allan VJM, Callow ME, Macaskie LE, Paterson-Beedle M. 2002. Effect of nutrient limitation on biofilm formation and phosphatase activity of a *Citrobacter* sp. *Microbiology* **148**:277–288.
- Allison DG. 2003. The biofilm matrix. *Biofouling* **19**:139–150.
- Alvarez-Ortega C, Harwood CS. 2007. Responses of *Pseudomonas aeruginosa* to low oxygen indicate that growth in the cystic fibrosis lung is by aerobic respiration. *Mol. Microbiol.* **65**:153–165.
- Ammary BY. 2014. Nutrients requirements in biological industrial wastewater treatment. *African J. Biotechnol.* **3**:236–238.
- An S, Wu J, Zhang LH. 2010. Modulation of *Pseudomonas aeruginosa* biofilm dispersal by a cyclic-di-gmp phosphodiesterase with a putative hypoxia-sensing domain. *Appl. Environ. Microbiol.* **76**:8160–8173.
- Applegate DH, Bryers JD. 1991. Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes. *Biotechnol. Bioeng.* **37**:17–25.
- Arnold JW, Bailey GW. 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. *Poult. Sci.*:1839–1845.
- Baier RE. 1982. Conditioning surfaces to suit the biomedical environment: recent

- progress. *J.Biomech.Eng* **104**:257–271.
- Baird ME, Middleton JH. 2004. On relating physical limits to the carbon : nitrogen ratio of unicellular algae and benthic plants. *J. Mar. Syst.* **49**:169–175.
- Bakker DP, Van der Plaats A, Verkerke GJ, Busscher HJ, Van der Mei HC. 2003. Comparison of velocity profiles for different flow chamber designs used in studies of microbial adhesion to surfaces. *Appl. Environ. Microbiol.* **69**:6280–6287.
- Balazs DJ, Triandafillu K, Chevolot Y, Aronsson BO, Harms H, Descouts P, Mathieu HJ. 2003. Surface modification of PVC endotracheal tubes by oxygen glow discharge to reduce bacterial adhesion. *Surf. Interface Anal.* **35**:301–309.
- Barken KB, Pamp SJ, Yang L, Gjermansen M, Bertrand JJ, Klausen M, Givskov M, Whitchurch CB, Engel JN, Tolker-Nielsen T. 2008. Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environ. Microbiol.* **10**:2331–2343.
- Bassler BL. 2002. Small talk: Cell-to-cell communication in bacteria. *Cell* **109**:421–424.
- Bassler BL, Losick R. 2006. Bacterially Speaking. *Cell* **125**:237–246.
- Battin TJ, Kaplan LA, Denis Newbold J, Hansen CME. 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* **426**:439–442.
- De Beer D, Huisman JW, Van den Heuvel JC, Ottengraf SPP. 1992. The effect of pH profiles in methanogenic aggregates on the kinetics of acetate conversion. *Water Res.* **26**:1329–1336.
- De Beer D, Glud A, Epping EHG, Kühl M. 1997. A fast-responding CO₂ microelectrode for profiling sediments, microbial mats, and biofilms. *Limnol. Oceanogr.* **42**:1590–1600.

- De Beer D, Stoodley P, Roe F, Lewandowski Z. 1994. Effects of biofilm structures on oxygen distribution and mass transport. *Biotechnol. Bioeng.* **43**:1131–1138.
- Belchik SM, Kennedy DW, Dohnalkova AC, Wang Y, Sevinc PC, Wu H, Lin Y, Lu HP, Fredrickson JK, Shi L. 2011. Extracellular reduction of hexavalent chromium by cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **77**:4035–4041.
- Bernstein R, Freger V, Lee J-HJ, Kim Y-G, Lee J-HJ, Herzberg M. 2014. “Should I stay or should I go?” Bacterial attachment vs biofilm formation on surface-modified membranes. *Biofouling* **30**:367–76.
- Bester E, Wolfaardt G, Joubert L, Garry K, Saftic S. 2005. Planktonic-cell yield of a pseudomonad biofilm. *Appl. Environ. Microbiol.* **71**:7792–7798.
- Beyenal H, Davis CC, Lewandowski Z. 2004. An improved Severinghaus-type carbon dioxide microelectrode for use in biofilms. *Sensors Actuators, B Chem.* **97**:202–210.
- Blair KM, Turner L, Winkelman JT, Berg HC, Kearns DB. 2008. A molecular clutch disables flagella in the *Bacillus subtilis* biofilm. *Science* (80-.). **320**:1636–1638.
- Boles BR, Thoendel M, Singh PK. 2005. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Mol. Microbiol.* **57**:1210–1223.
- Bos R, Van Der Mei HC, Gold J, Busscher HJ. 2000. Retention of bacteria on a substratum surface with micro-patterned hydrophobicity. *FEMS Microbiol. Lett.* **189**:311–315.
- Bradshaw DJ, Homer KA, Marsh PD, Beighton D. 1994. Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology* **140**:3407–3412.
- Bryant MP, Wolin EA, Wolin MJ, Wolfe RS. 1967. *Methanobacillus omelianskii*, a symbiotic association of two species of bacteria. *Arch. Mikrobiol.* **59**:20–31.

- Bryers JD. 1984. Biofilm formation and chemostat dynamics: Pure and mixed culture considerations. *Biotechnol. Bioeng.* **26**:948–58.
- Busscher HJ, Van De Belt-Gritter B, Dijkstra RJB, Norde W, Van Der Mei HC. 2008. *Streptococcus mutans* and *Streptococcus intermedius* adhesion to fibronectin films are oppositely influenced by ionic strength. *Langmuir* **24**:10968–10970.
- Cao B, Ahmed B, Kennedy DW, Wang Z, Shi L, Marshall MJ, Fredrickson JK, Isern NG, Majors PD, Beyenal H. 2011. Contribution of extracellular polymeric substances from *Shewanella* sp. HRCR-1 biofilms to U(VI) immobilization. *Environ. Sci. Technol.* **45**:5483–5490.
- Chambless JD, Stewart PS. 2007. A three-dimensional computer model analysis of three hypothetical biofilm detachment mechanisms. *Biotechnol. Bioeng.* **97**:1573–1584.
- Chang HT, Rittmann BE. 1987. Mathematical modeling of biofilm on activated carbon. *Environ. Sci. Technol.* **21**:273–80.
- Characklis WG, Bryers JD. 1981. Fouling biofilm development: a process analysis. *Biotechnol. Bioeng.* **23**:1923–1960.
- Chen CY, Chen SD. 2000. Biofilm characteristics in biological denitrification biofilm reactors. *Water Sci. Technol.* **41**:147–154.
- Chen S, Dawson SP, Doolen GD, Janecky DR, Lawniczak A. 1995. Lattice methods and their applications to reacting systems. *Comput. Chem. Eng.* **19**:617–646.
- Chen X, Schauder S, Potier N, Van Dorsselaer A, Pelczar I, Bassler BL, Hughson FM. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. *Nature* **415**:545–549.
- Choi YC, Morgenroth E. 2003. Monitoring biofilm detachment under dynamic changes in shear stress using laser-based particle size analysis and mass fractionation. *Water Sci. Technol.* **47**:69–76.
- Christensen BB, Haagensen JAJ, Heydorn A, Molin S. 2002. Metabolic

- commensalism and competition in a two-species microbial consortium. *Appl. Environ. Microbiol.* **68**:2495–2502.
- Costerton JW, Geesey GG, Cheng KJ. 1978. How bacteria stick. *Sci. Am.* **238**:86–95.
- Costerton J, Lewandowski Z. 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49**:711–45.
- Costerton J, Stewart PS, Greenberg E. 1999. Bacterial biofilms: a common cause of persistent infections. *Science (80-.).* **284**:1318–1322.
- Cotter JJ, O’Gara JP, Mack D, Casey E. 2009. Oxygen-mediated regulation of biofilm development is controlled by the alternative sigma factor σ_B in *Staphylococcus epidermidis*. *Appl. Environ. Microbiol.* **75**:261–264.
- Crawford RJ, Webb HK, Truong VK, Hasan J, Ivanova EP. 2012. Surface topographical factors influencing bacterial attachment. *Adv. Colloid Interface Sci.* **179–182**:142–9.
- Cruz LF, Cobine PA, De La Fuente L. 2012. Calcium increases *Xylella fastidiosa* surface attachment, biofilm formation, and twitching motility. *Appl. Environ. Microbiol.* **78**:1321–1331.
- D’Argenio DA, Miller SI. 2004. Cyclic di-GMP as a bacterial second messenger. *Microbiology* **150**:2497–2502.
- Das JR, Bhakoo M, Jones M V, Gilbert P. 1998. Changes in the biocide susceptibility of *Staphylococcus epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. *J. Appl. Microbiol.* **84**:852–858.
- Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science (80-.).* **280**:295–298.
- Davies D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug Discov.* **2**:114–22.

- Ding Y, Peng N, Du Y, Ji L, Cao B. 2014. Disruption of putrescine biosynthesis in *Shewanella oneidensis* enhances biofilm cohesiveness and performance in Cr(VI) immobilization. *Appl. Environ. Microbiol.* **80**:1498–1506.
- Donlan RM, Costerton JW. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15**:167–19.
- Donlan RM, Priede J a, Heyes CD, Sanii L, Murga R, Edmonds P. 2004. Model system for growing and quantifying *Streptococcus pneumoniae* biofilms in situ and in real time. *Appl. Environ. Microbiol.* **70**:4980–4988.
- Donlan RM. 2001. Biofilms and device-associated infections. *Emerg. Infect. Dis.* **7**:277–281.
- Donlan RM. 2002. Biofilms: microbial life on surfaces. *Emerg. Infect. Dis.* **8**:881–890.
- Dow JM, Crossman L, Findlay K, He Y-Q, Feng J-X, Tang J-L. 2003. Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc. Natl. Acad. Sci. U. S. A.* **100**:10995–11000.
- Driffield K, Miller K, Bostock JM, O’neill AJ, Chopra I. 2008. Increased mutability of *Pseudomonas aeruginosa* in biofilms. *J. Antimicrob. Chemother.* **61**:1053–1056.
- Droste RL. 1997. Theory and practice of water and wastewater treatment. Canada: John Wiley and Sons.
- Dunny GM, Leonard BAB. 1997. Cell-cell communication in gram-positive bacteria. *Annu. Rev. Microbiol.* **51**:527–564.
- Eberl HJ, Parker DF, Van Loosdrecht MCM. 2001. A new deterministic spatio-temporal continuum model for biofilm development. *J. Theor. Med.* **3**:161–175.
- Eberl L, Winson MK, Sternberg C, Stewart GSAB, Christiansen G, Chhabra SR, Bycroft B, Williams P, Molin S, Givskov M. 1996. Involvement of N-acyl-L-homoserine lactone autoinducers in controlling the multicellular

- behaviour of *Serratia liquefaciens*. *Mol. Microbiol.* **20**:127–136.
- Fabrega J, Renshaw JC, Lead JR. 2009. Interactions of silver nanoparticles with *Pseudomonas putida* biofilms. *Environ. Sci. Technol.* **43**:9004–9009.
- Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai CH, Gonzalez-Begne M, Watson G, Krysan DJ, Bowen WH, Koo H. 2014. Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms in vivo. *Infect. Immun.* **82**:1968–1981.
- Fercher A, Borisov SM, Zhdanov A V., Klimant I, Papkovsky DB. 2011. Intracellular O₂ sensing probe based on cell-penetrating phosphorescent nanoparticles. *ACS Nano* **5**:5499–5508.
- Flemming H-C, Wingender J. 2010. The biofilm matrix. *Nat. Rev. Microbiol.* **8**:623–33.
- Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.* **14**:563–575.
- Fredrickson JK, Zachara JM. 2008. Electron transfer at the microbe-mineral interface: A grand challenge in biogeochemistry. *Geobiology* **6**:245–253.
- Fredrickson JK, Romine MF, Beliaev AS, Auchtung JM, Driscoll ME, Gardner TS, Nealson KH, Osterman AL, Pinchuk G, Reed JL, Rodionov D a, Rodrigues JLM, Saffarini D a, Serres MH, Spormann AM, Zhulin IB, Tiedje JM. 2008. Towards environmental systems biology of *Shewanella*. *Nat. Rev. Microbiol.* **6**:592–603.
- Galperin Y, Kaplan IR. 2008. Zero-order kinetics model for the Christensen-Larsen method for fugitive fuel age estimates. *Gr. Water Monit. Remediat.* **28**:94–97.
- Geesey GG, Mutch R, Costerton JW, Green RB. 1978. Sessile bacteria: An important component of the microbial population in small mountain streams. *Limnol. Oceanogr.* **23**:1214–1223.

- Geesey GG, Wigglesworth-Cooksey B, Cooksey KE. 2000. Influence of calcium and other cations on surface adhesion of bacteria and diatoms: A review. *Biofouling* **15**:195–205.
- Gjermansen M, Nilsson M, Yang L, Tolker-Nielsen T. 2010. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms: Genetic elements and molecular mechanisms. *Mol. Microbiol.* **75**:815–826.
- Gjermansen M, Ragas P, Sternberg C, Molin S, Tolker-Nielsen T. 2005. Characterization of starvation-induced dispersion in *Pseudomonas putida* biofilms. *Environ. Microbiol.* **7**:894–904.
- Glud RN, Santegoeds CM, De Beer D, Kohls O, Ramsing NB. 1998. Oxygen dynamics at the base of a biofilm studied with planar optodes. *Aquat. Microb. Ecol.* **14**:223–233.
- Glud RN. 2008. Oxygen dynamics of marine sediments. *Mar. Biol. Res.* **4**:243–289.
- Goeres DM, Hamilton M a, Beck N a, Buckingham-Meyer K, Hilyard JD, Loetterle LR, Lorenz L a, Walker DK, Stewart PS. 2009. A method for growing a biofilm under low shear at the air-liquid interface using the drip flow biofilm reactor. *Nat. Protoc.* **4**:783–788.
- Gottenbos B, Van Der Mei HC, Busscher HJ. 2000. Initial adhesion and surface growth of *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* on biomedical polymers. *J. Biomed. Mater. Res.* **50**:208–214.
- Hammer BK, Bassler BL. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol.* **50**:101–114.
- Harris NP, Hansford GS. 1976. A study of substrate removal in a microbial film reactor. *Water Res.* **10**:935–943.
- Haruff HM, Munakata-Marr J, Marr DWM. 2003. Directed bacterial surface attachment via optical trapping. *Colloids Surfaces B Biointerfaces* **27**:189–195.
- Hengge R. 2009. Principles of c-di-GMP signalling in bacteria. *Nat. Rev.*

- Microbiol.* **7**:263–273.
- Hengge R, Gründling A, Jenal U, Ryan R, Yildiz F. 2016. Bacterial signal transduction by cyclic di-GMP and other nucleotide second messengers. *J. Bacteriol.* **198**:15–26.
- Henze M, Harremoës P, Jansen J, Arvin E. 1997. Wastewater treatment Second edit. Springer.
- Hermanowicz SW. 1998. A model of two-dimensional biofilm morphology. *Water Sci. Technol.* **37**:219–222.
- Heydorn A, Ersbøll B, Kato J, Hentzer M, Parsek MR, Tolker-nielsen T, Givskov M, Molin S. 2002. Statistical analysis of *Pseudomonas aeruginosa* biofilm development: impact of mutations in genes involved in twitching stationary-phase sigma factor expression. *Appl. Environ. Microbiol.* **68**:2008–2017.
- Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. *Proc. Natl. Acad. Sci. U. S. A.* **102**:14422–7.
- Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. 2010. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* **35**:322–332.
- Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen PØ, Molin S, Givskov M, Tolker-Nielsen T, Bjarnsholt T. 2011. The clinical impact of bacterial biofilms. *Int. J. Oral Sci.* **3**:55–65.
- Hoover SR, Porges N. 1952. Assimilation of dairy wastes by activated sludge-II. The equations of synthesis and rate of oxygen utilisation. *Sewage Ind. Waste.*:306–312.
- Huang B, Whitchurch CB, Mattick JS. 2003. FimX, a multidomain protein connecting environmental signals to twitching motility in *Pseudomonas aeruginosa*. *J. Bacteriol.* **185**:7068–7076.
- Huang CT, Peretti SW, Bryers JD. 1994. Effects of medium carbon-to-nitrogen ratio on biofilm formation and plasmid stability. *Biotechnol. Bioeng.* **44**:329–336.

- Hunt SM, Hamilton MA, Sears JT, Harkin G, Reno J. 2003. A computer investigation of chemically mediated detachment in bacterial biofilms. *Microbiology* **149**:1155–1163.
- Hunt SM, Werner EM, Huang B, Hamilton A, Stewart PS, Hamilton MA. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. *Appl. Environ. Microbiol.* **70**:7418–7425.
- Jang a, Bishop PL, Okabe S, Lee SG, Kim IS. 2003. Effect of dissolved oxygen concentration on the biofilm and in situ analysis by fluorescence in situ hybridization (FISH) and microelectrodes. *Water Sci. Technol.* **47**:49–57.
- Jenal U, Malone J. 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. *Annu. Rev. Genet.* **40**:385–407.
- Jeon NL, Dertinger SKW, Chiu DT, Choi IS, Stroock AD, Whitesides G. 2000. Generation of solution and surface gradients using microfluidic systems. *Langmuir* **16**:8311–8316.
- Jeon NL, Dertinger SKW, Chiu DT, Choi IS, Stroock AD, Whitesides GM, Jeon NL, Whitesides GM, Campbell K, Groisman A. 2007. Generation of gradients having complex shapes using microfluidic networks. *Lab Chip* **7**:8311–8316.
- Joergensen RG, Scheu S. 1999. Response of soil microorganisms to the addition of carbon, nitrogen and phosphorus in a forest Rendzina. *Soil Biol. Biochem.* **31**:859–866.
- Kader A, Simm R, Gerstel U, Morr M, Römling U. 2006. Hierarchical involvement of various GGDEF domain proteins in rdar morphotype development of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **60**:602–616.
- Kaplan JB, Meyenhofer MF, Fine DH. 2003. Biofilm growth and detachment of *Actinobacillus actinomycetemcomitans*. *J. Bacteriol.* **185**:1399–404.
- Kierck K, Watnick PI. 2003. The *Vibrio cholerae* O139 O-antigen polysaccharide is essential for Ca²⁺-dependent biofilm development in sea water. *Proc. Natl.*

Acad. Sci. U. S. A. **100**:14357–62.

Kinniment SL, Wimpenny JWT. 1992. Measurements of the distribution of adenylate concentrations and adenylate energy charge across *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* **58**:1629–1635.

Kirillina O, Fetherston JD, Bobrov AG, Abney J, Perry RD. 2004. HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. *Mol. Microbiol.* **54**:75–88.

Kissel BJC, McCarty PL, Asce, M.Street RL. 1984. Numerical simulation of mixed-culture biofilm. *J. Environ. Eng.* **110**:393–411.

Klausen M, Aaes-Jørgensen A, Molin S, Tolker-Nielsen T. 2003. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Mol. Microbiol.* **50**:61–68.

Kondrashina A V., Dmitriev RI, Borisov SM, Klimant I, O'Brien I, Nolan YM, Zhdanov A V., Papkovsky DB. 2012. A phosphorescent nanoparticle-based probe for sensing and imaging of (intra)cellular oxygen in multiple detection modalities. *Adv. Funct. Mater.* **22**:4931–4939.

Körstgens V, Flemming HC, Wingender J, Borchard W. 2001. Influence of calcium ions on the mechanical properties of a model biofilm of mucoid *Pseudomonas aeruginosa*. *Water Sci. Technol.* **43**:49–57.

Kreft JU, Booth G, Wimpenny JW. 1998. BacSim, a simulator for individual-based modelling of bacterial colony growth. *Microbiology* **144**:3275–3287.

Kreft JU, Picioreanu C, Wimpenny JW, van Loosdrecht MC. 2001. Individual-based modelling of biofilms. *Microbiology* **147**:2897–2912.

Kreft JU, Wimpenny JW. 2001. Effect of EPS on biofilm structure and function as revealed by an individual-based model of biofilm growth. *Water Sci. Technol.* **43**:135–41.

Kreuter J. 1996. Nanoparticles and microparticles for drug and vaccine delivery. *J. Anat.* **189** (Pt 3):503–505.

- Kuchma SL, Griffin EF, O'Toole GA. 2012. Minor pilins of the type IV pilus system participate in the negative regulation of swarming motility. *J. Bacteriol.* **194**:5388–5403.
- Kuchma SL, Kuchma SL, Connolly JP, Connolly JP. 2005. A three-component regulatory system regulates biofilm maturation and type III secretion in *Pseudomonas aeruginosa*. *Society* **187**:1441–1454.
- Kühl M, Rickelt LF, Thar R. 2007. Combined imaging of bacteria and oxygen in biofilms. *Appl. Environ. Microbiol.* **73**:6289–6295.
- Lamotta EJ. 1976. Internal diffusion and reaction in biological films. *Environ. Sci. Technol.* **10**:765–9.
- Lardon L a, Merkey B V, Martins S, Dötsch A, Picioreanu C, Kreft J-U, Smets BF. 2011. iDynoMiCS: next-generation individual-based modelling of biofilms. *Environ. Microbiol.* **13**:2416–2434.
- Larsen M, Borisov SM, Grunwald B, Klimant I, Glud RN. 2011. A simple and inexpensive high resolution color ratiometric planar optode imaging approach: application to oxygen and pH sensing. *Limnol. Oceanogr. Methods* **9**:348–360.
- Laspidou CS, Kungolos A, Samaras P. 2010. Cellular-automata and individual-based approaches for the modeling of biofilm structures: Pros and cons. *Desalination* **250**:390–394.
- Laspidou CS, Rittmann BE. 2004a. Modeling the development of biofilm density including active bacteria, inert biomass, and extracellular polymeric substances. *Water Res.* **38**:3349–3361.
- Laspidou CS, Rittmann BE. 2004b. Evaluating trends in biofilm density using the UMCCA model. *Water Res.* **38**:3362–3372.
- Lecuyer S, Rusconi R, Shen Y, Forsyth A, Vlamakis H, Kolter R, Stone HA. 2011. Shear stress increases the residence time of adhesion of *Pseudomonas aeruginosa*. *Biophys. J.* **100**:341–350.
- Lee J-H, Kim Y-G, Cho MH, Lee J. 2014a. ZnO nanoparticles inhibit

- Pseudomonas aeruginosa* biofilm formation and virulence factor production. *Microbiol. Res.* **169**:888–96.
- Lee KWK, Periasamy S, Mukherjee M, Xie C, Kjelleberg S, Rice SA. 2014b. Biofilm development and enhanced stress resistance of a model, mixed-species community biofilm. *ISME J.* **8**:894–907.
- Lewandowski Z, Beyenal H, Myers J, Stookey D. 2007. The effect of detachment on biofilm structure and activity: The oscillating pattern of biofilm accumulation. *Water Sci. Technol.* **55**:429–436.
- Li B, Logan BE. 2004. Bacterial adhesion to glass and metal-oxide surfaces. *Colloids Surf. B. Biointerfaces* **36**:81–90.
- Li C, Yilei Z, Cohen Y. 2015. Individual based modeling of *Pseudomonas aeruginosa* biofilm with three detachment mechanisms. *RSC Adv.* **5**:3396–3402.
- Li Jeon N, Baskaran H, Dertinger SKW, Whitesides GM, Van de Water L, Toner M. 2002. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat. Biotechnol.* **20**:826–30.
- Lieberman JA, Wedekind JE. 2011. Base ionization and ligand binding: How small ribozymes and riboswitches gain a foothold in a protein world. *Curr. Opin. Struct. Biol.* **21**:327–334.
- Lim CP, Nguyen Quoc Mai P, Roizman Sade D, Lam YC, Cohen Y. 2016. Biofilm development of an opportunistic model bacterium analysed at high spatiotemporal resolution in the framework of a precise flow cell. *npj Biofilms Microbiomes* **2**.
- Lin F, Saadi W, Rhee SW, Wang S-J, Mittal S, Jeon NL. 2004. Generation of dynamic temporal and spatial concentration gradients using microfluidic devices. *Lab Chip* **4**:164–167.
- Liu J, Prindle A, Humphries J, Gabalda-sagarra M, Asally M, Lee DD. 2015. Metabolic co-dependence gives rise to collective oscillations within

- biofilms. *Nature* **523**:550–554.
- van Loosdrecht MCM, Eikelboom D, Gjaltema A, Mulder A, Tjihuis L, Heijnen JJ. 1995. Biofilm structures. *Water Sci. Technol.* **32**:35–43.
- Mah, T.F. and O'Toole GA. 2001. Mechanisms of biofilm resistance to antimicrobial agents. *TRENDS Microbiol.* **9**:34–39.
- Manz B, Volke F, Goll D, Horn H. 2005. Investigation of biofilm structure, flow patterns and detachment with magnetic resonance imaging. *Water Sci. Technol.* **52**:1–6.
- Marshall MJ, Beliaev AS, Dohnalkova AC, Kennedy DW, Shi L, Wang Z, Boyanov MI, Lai B, Kemner KM, McLean JS, Reed SB, Culley DE, Bailey VL, Simonson CJ, Saffarini DA, Romine MF, Zachara JM, Fredrickson JK. 2006. c-type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*. *PLoS Biol.* **4**:1324–1333.
- Martin KJ, Picioreanu C, Nerenberg R. 2013. Multidimensional modeling of biofilm development and fluid dynamics in a hydrogen-based, membrane biofilm reactor (MBfR). *Water Res.* **47**:4739–51.
- Van Der Mei HC, Bos R, Busscher HJ. 1998. A reference guide to microbial cell surface hydrophobicity based on contact angles. *Colloids Surfaces B Biointerfaces* **11**:213–221.
- Merighi M, Lee VT, Hyodo M, Hayakawa Y, Lory S. 2007. The second messenger bis-(3'-5')-cyclic-GMP and its PilZ domain-containing receptor Alg44 are required for alginate biosynthesis in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **65**:876–895.
- Merkey B V., Lardon L a., Seoane JM, Kreft J-U, Smets BF. 2011. Growth dependence of conjugation explains limited plasmid invasion in biofilms: an individual-based modelling study. *Environ. Microbiol.* **13**:2435–2452.
- Merritt JH, Brothers KM, Kuchma SL, O'Toole GA. 2007. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. *J. Bacteriol.*

189:8154–8164.

Metcalf, Eddy. 1991. Wastewater engineering, treatment, disposal, and reuse
Third edit. McGraw-Hill, Inc., New York.

Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. *Annu. Rev. Microbiol.*
55:165–99.

Mills E, Pultz IS, Kulasekara HD, Miller SI. 2011. The bacterial second
messenger c-di-GMP: Mechanisms of signalling. *Cell. Microbiol.* **13**:1122–
1129.

Misra C, Gupta SK. 2001. Hybrid reactor for priority pollutant-trichloroethylene
removal. *Water Res.* **35**:160–166.

Molin S, Tolker-Nielsen T. 2003. Gene transfer occurs with enhanced efficiency
in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr.*
Opin. Biotechnol. **14**:255–261.

Morgenroth E, Eberl HJ, van Loosdrecht MCM, Noguera DR, Pizarro GE,
Picioreanu C, Rittman BE, Schwarz AO, Wanner O. 2004. Comparing
biofilm models for a single species biofilm system. *Water Sci. Technol.*
49:145–154.

Mueller LN, de Brouwer JFC, Almeida JS, Stal LJ, Xavier JB. 2006. Analysis of
a marine phototrophic biofilm by confocal laser scanning microscopy using
the new image quantification software PHLIP. *BMC Ecol.* **6**:1.

Nadell CD, Xavier JB, Foster KR. 2009. The sociobiology of biofilms. *FEMS*
Microbiol. Rev. **33**:206–224.

Nadell CD, Foster KR, Xavier JB. 2010. Emergence of spatial structure in cell
groups and the evolution of cooperation. *PLoS Comput. Biol.* **6**:e1000716.

Nakhamchik A, Wilde C, Rowe-Magnus DA. 2008. Cyclic-di-GMP regulates
extracellular polysaccharide production, biofilm formation, and rugose
colony development by *Vibrio vulnificus*. *Appl. Environ. Microbiol.*
74:4199–4209.

- Nealson KH, Platt T, Hastings JW. 1970. Cellular control of the synthesis and activity of the bacterial luminescent system. *J. Bacteriol.* **104**:313–322.
- Noguera DR, Pizarro G, Stahl D a., Rittman BE. 1999. Simulation of multispecies biofilm development in three dimensions. *Water Res. Sci. Technol.*
- Norsker N, Nielsen P, Hvitved-Jacobsen T. 1995. Influence of oxygen on biofilm growth and potential sulfate reduction in gravity sewer biofilm. *Water Sci. Technol.* **31**:159–167.
- O'Toole G, Kaplan HB, Kolter R. 2000. Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**:49–79.
- Oguri K, Kitazato H, Glud RN. 2006. Platinum octaethylporphyrin based planar optodes combined with an UV-LED excitation light source: An ideal tool for high-resolution O₂ imaging in O₂ depleted environments. *Mar. Chem.* **100**:95–107.
- Ohashi A, De Silva DG V, Mobarry B, Manem JA, Stahl DA, Rittmann BE. 1995. Influence of substrate C/N ratio on the structure of multi-species biofilms consisting of nitrifiers and heterotrophs. *Water Sci. Technol.*
- Ohashi A, Koyama T, Syutsubo K, Harada H. 1999. A novel method for evaluation of biofilm tensile strength resisting erosion. *Water Sci. Technol.*
- Olsson ALJ, Mei HC Van Der, Busscher HJ, Sharma PK. 2009. Influence of cell surface appendages on the bacterium-substratum interface measured real-time using QCM-D. *Langmuir* **25**:1627–1632.
- Ong YL, Razatos A, Georgiou G, Sharma MM. 1999. Adhesion forces between *E. coli* bacteria and biomaterial surfaces. *Langmuir* **15**:2719–2725.
- Parsek MR, Greenberg EP. 2005. Sociomicrobiology: The connections between quorum sensing and biofilms. *Trends Microbiol.* **13**:27–33.
- Patrauchan MA, Sarkisova S, Sauer K, Franklin MJ. 2005. Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. *Microbiology* **151**:2885–2897.

- Petersen E, Chaudhuri P, Gourley C, Harms J, Splitter G. 2011. *Brucella melitensis* cyclic di-GMP phosphodiesterase BpdA controls expression of flagellar genes. *J. Bacteriol.* **193**:5683–5691.
- Petrova OE, Sauer K. 2012. Dispersion by *Pseudomonas aeruginosa* requires an unusual posttranslational modification of BdlA. *Proc. Natl. Acad. Sci.* **109**:16690–16695.
- Peyton BM, Characklis WG. 1993. A statistical analysis of the effect of substrate utilization and shear stress on the kinetics of biofilm detachment. *Biotechnol. Bioeng.* **41**:728–35.
- Peyton BM. 1996. Effects of shear stress and substrate loading rate on *Pseudomonas aeruginosa* biofilm thickness and density. *Water Res.* **30**:29–36.
- Picioreanu C, Kreft J-U, Klausen M, Haagensen J a. J, Tolker-Nielsen T, Molin S. 2007. Microbial motility involvement in biofilm structure formation – a 3D modelling study. *Water Sci. Technol.* **55**:337–343.
- Picioreanu C, Van Loosdrecht MCM, Heijnen JJ. 1999a. Discrete-differential modelling of biofilm structure. *Water Sci. Technol.*
- Picioreanu C, Xavier JB, van Loosdrecht MCM. 2004. Advances in mathematical modeling of biofilm structure. *Biofilms* **1**:337–349.
- Picioreanu C, van Loosdrecht MCM, Heijnen JJ. 1998a. A new combined differential discrete cellular automaton approach for biofilm modelling: Application for growth in gel beads. *Biotechnol. Bioeng.* **58**:101–116.
- Picioreanu C, Loosdrecht MCM Van, Heijnen JJ. 1999b. Multidimensional modelling of biofilm structure. *Microb. Biosyst. new Front.*
- Picioreanu C, van Loosdrecht MC, Heijnen JJ. 1998b. Mathematical modeling of biofilm structure with a hybrid differential-discrete cellular automaton approach. *Biotechnol. Bioeng.* **58**:101–116.
- Picioreanu C, van Loosdrecht MCM. 2002. A mathematical model for initiation of microbiologically influenced corrosion by differential aeration. *J.*

Electrochem. Soc. **149**:B211.

- Picioreanu C, van Loosdrecht MCM, Curtis TP, Scott K. 2010. Model based evaluation of the effect of pH and electrode geometry on microbial fuel cell performance. *Bioelectrochemistry* **78**:8–24.
- Picioreanu C, Loosdrecht MCM Van, Heijnen JJ. 2000. Effect of diffusive and convective substrate transport on biofilm structure formation: A two-dimensional modeling study. *Biotechnol. Bioeng.* **69**:504–515.
- Picioreanu C, Loosdrecht MCM Van, Heijnen JJ. 2001. Two-dimensional model of biofilm detachment caused by internal stress from liquid flow. *Biotechnol. Bioeng.* **72**:205–218.
- Pizarro G, Griffeath D, Noguera DR. 2001. Quantitative cellular automaton model for biofilms. *J. Environ. Eng.* **127**:782–789.
- Potera C. 1996. Biofilms invade microbiology. *Science* **273**:1795–1797.
- Pratten J, Foster SJ, Chan PF, Wilson M, Nair SP. 2001. *Staphylococcus aureus* accessory regulators: Expression within biofilms and effect on adhesion. *Microbes Infect.* **3**:633–637.
- Purevdorj-Gage B, Costerton WJ, Stoodley P. 2005. Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology* **151**:1569–1576.
- Purevdorj B, Costerton JW, Stoodley P. 2002. Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* **68**:4457–4464.
- Qi P-S, Wang W-B, Qi Z. 2008. Effect of shear stress on biofilm morphological characteristics and the secretion of extracellular polymeric substances. 2008 2nd Int. Conf. Bioinforma. Biomed. Eng.:3438–3441.
- Radzig MA, Nadtochenko VA, Koksharova OA, Kiwi J, Lipasova VA, Khmel IA. 2013. Antibacterial effects of silver nanoparticles on gram-negative bacteria: Influence on the growth and biofilms formation, mechanisms of action. *Colloids Surfaces B Biointerfaces* **102**:300–306.

- Ramage G, VandeWalle K, Wickes BL, López-Ribot JL. 2001. Standardized method for in vitro antifungal susceptibility testing of *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **45**:2475–2479.
- Ramsing NB, Kuhl M, Jorgensen BB. 1993. Distribution of sulfate-reducing bacteria, O₂, and H₂S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. *Appl. Environ. Microbiol.* **59**:3840–3849.
- Rasmussen H, Jørgensen BB. 1992. Microelectrode of seasonal oxygen uptake in a coastal sediment: Role of molecular diffusion. *Mar. Ecol. Prog. Ser.* **81**:289–303.
- Reichert P. 1994. Aquasim - A tool for simulation and data analysis of aquatic systems. *Water Sci. Technol.*
- Reusbech NP, Sørensen J, Blackburn TH, Lomnolt JP. 1980. Distribution of oxygen in marine sediments measured with microelectrodes. *Limnol. Oceanogr.* **25**:403–411.
- Rice SA, Koh KS, Queck SY, Labbate M, Lam KW, Kjelleberg S, Al RET. 2005. Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J. Bacteriol.* **187**:3477–3485.
- Rieman W, Walton HF. 2013. Ion exchange in analytical chemistry: International series of monographs in analytical chemistry. Elsevier.
- Rittman BE. 1982. The effect of shear stress on biofilm loss rate. *Biotechnol. Bioeng.* **24**:501–506.
- Romling U, Galperin MY, Gomelsky M. 2013. Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. *Microbiol. Mol. Biol. Rev.* **77**:1–52.
- Römmling U. 2012. Cyclic di-GMP, an established secondary messenger still speeding up. *Environ. Microbiol.* **14**:1817–1829.
- Roosjen A, Boks NP, van der Mei HC, Busscher HJ, Norde W. 2005. Influence of shear on microbial adhesion to PEO-brushes and glass by convective-

- diffusion and sedimentation in a parallel plate flow chamber. *Colloids Surf. B. Biointerfaces* **46**:1–6.
- Roy AB, Petrova OE, Sauer K. 2012. The phosphodiesterase DipA (PA5017) is essential for *Pseudomonas aeruginosa* biofilm dispersion. *J. Bacteriol.* **194**:2904–2915.
- Ruan J, Wang L, Xu M, Cui D, Zhou X, Liu D. 2009. Fabrication of a microfluidic chip containing dam, weirs and gradient generator for studying cellular response to chemical modulation. *Mater. Sci. Eng. C* **29**:674–679.
- Saini VK, Bhandari SC, Tarafdar JC. 2004. Comparison of crop yield, soil microbial C, N and P, N-fixation, nodulation and mycorrhizal infection in inoculated and non-inoculated sorghum and chickpea crops. *F. Crop. Res.* **89**:39–47.
- Sakuragi Y, Kolter R. 2007. Quorum-sensing regulation of the biofilm matrix genes (*pel*) of *Pseudomonas aeruginosa*. *J. Bacteriol.* **189**:5383–5386.
- Sarkisova S, Patrauchan MA, Berglund D, Nivens DE, Franklin MJ. 2005. Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J. Bacteriol.* **187**:4327.
- Sauer K, Cullen MC, Rickard a H, Zeef L a H, Gilbert P, Davies DG. 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J. Bacteriol.* **186**:7312–7326.
- Savakis P, De Causmaecker S, Angerer V, Ruppert U, Anders K, Essen LO, Wilde A. 2012. Light-induced alteration of c-di-GMP level controls motility of *Synechocystis* sp. PCC 6803. *Mol. Microbiol.* **85**:239–251.
- Schauder S, Shokat K, Surette MG, Bassler BL. 2001. The LuxS family of bacterial autoinducers: Biosynthesis of a novel quorum-sensing signal molecule. *Mol. Microbiol.* **41**:463–476.
- Schirmer T, Jenal U. 2009. Structural and mechanistic determinants of c-di-GMP signalling. *Nat. Rev. Microbiol.* **7**:724–735.
- Sensing ALQ, Fuqua C, Parsek MR, Greenberg EP. 2001. Regulation of gene

- expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu. Rev. Genet.* **35**:439–68.
- Simkins S, Alexander M. 1984. Models for mineralization kinetics with the variables of substrate concentration and population density. *Appl. Environ. Microbiol.* **47**:1299–1306.
- Simkins S, Alexander M. 1985. Nonlinear estimation of the parameters of Monod kinetics that best describe mineralization of several substrate concentrations by dissimilar bacterial densities. *Appl. Environ. Microbiol.* **50**:816–824.
- Simm R, Morr M, Kader A, Nimtz M, Römling U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* **53**:1123–1134.
- Singh P, Schaefer A, Parsek M, Moninger T, Welsh M, Greenberg E. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature* **407**:762–4.
- Singh R, Paul D, Jain RK. 2006. Biofilms: implications in bioremediation. *Trends Microbiol.* **14**:389–397.
- Sondermann H, Shikuma NJ, Yildiz FH. 2012. You’ve come a long way: C-di-GMP signaling. *Curr. Opin. Microbiol.* **15**:140–146.
- Staal M, Prest EI, Vrouwenvelder JS, Rickelt LF, Kuhl M. 2011. A simple optode based method for imaging O₂ distribution and dynamics in tap water biofilms. *Water Res.* **45**:5027–5037.
- Stapper AP, Narasimhan G, Ohman DE, Barakat J, Hentzer M, Molin S, Kharazmi A, Høiby N, Mathee K. 2004. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture, but is not essential for biofilm formation. *J. Med. Microbiol.* **53**:679–690.
- Sternberg C, Tolker-Nielsen T. 2005. Growing and analyzing biofilms in flow cells. *Curr. Protoc. Microbiol.* **1B**:1–15.
- Stewart PS. 1993. A model of biofilm detachment. *Biotechnol. Bioeng.* **41**:111–117.

- Stewart PS. 1996. Theoretical aspects of antibiotic diffusion into microbial biofilms. *Antimicrob. Agents Chemother.* **40**:2517–2522.
- Stewart PS. 1998. A review of experimental measurements of effective diffusive permeabilities and effective diffusion coefficients in biofilms. *Biotechnol. Bioeng.* **59**:261–272.
- Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**:135–38.
- Stewart PS, Franklin MJ. 2008. Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* **6**:199–210.
- Stoodley P, Jørgensen F, Williams P, Lappin-Scott HM. 1999a. The role of hydrodynamics and AHL signalling molecules as determinants of the structure of *Pseudomonas aeruginosa* biofilms. *Biofilms good, bad, ugly*:323–330.
- Stoodley P, Sauer K, Davies DG, Costerton JW. 2002. Biofilms as complex differentiated communities. *Annu. Rev. Microbiol.* **56**:187–209.
- Stoodley P, Boyle JD, DeBeer D, Lappin-Scott HM. 1999b. Evolving perspectives of biofilm structure. *Biofouling* **14**:75–90.
- Stoodley P, Lewandowski Z, Boyle JD, Lappin-Scott HM. 1999c. Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: An in situ investigation of biofilm rheology. *Biotechnol. Bioeng.* **65**:83–92.
- Sturme MHJ, Kleerebezem M, Nakayama J, Akkermans a DL, Vaughan EE, de Vos WM. 2002. Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol.* **81**:233–243.
- Tamayo R, Pratt JT, Camilli A. 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu. Rev. Microbiol.* **61**:131–48.
- Teodósio JS, Simões M, Melo LF, Mergulhão FJ. 2011. Flow cell hydrodynamics and their effects on *E. coli* biofilm formation under different nutrient

- conditions and turbulent flow. *Biofouling* **27**:1–11.
- Theunissen S, De Smet L, Dansercoer A, Motte B, Coenye T, Van Beeumen JJ, Devreese B, Savvides SN, Vergauwen B. 2010. The 285 kDa Bap/RTX hybrid cell surface protein (SO4317) of *Shewanella oneidensis* MR-1 is a key mediator of biofilm formation. *Res. Microbiol.* **161**:144–152.
- Thompson LJ, Gray V, Lindsay D, Holy A Von. 2006. Carbon : nitrogen : phosphorus ratios influence biofilm formation by *Enterobacter cloacae* and *Citrobacter freundii*. *J. Appl. Microbiol.* **101**:1105–1113.
- Thormann KM, Saville RM, Shukla S, Spormann AM. 2005. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. *J. Bacteriol.* **187**:1014–1021.
- Tischler AD, Camilli A. 2005. Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. *Infect. Immun.* **73**:5873–5882.
- Tischler AD, Lee SH, Camilli A. 2002. *Vibrio cholerae* vieSAB locus encodes a pathway contributing to cholera toxin production. *Society* **184**:4104–4113.
- Tobergte DR, Curtis S. 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *FEMS Microbiol. Ecol.* **24**:11–25.
- Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bacteriol.* **182**:6482–6489.
- Ueda A, Wood TK. 2010. Tyrosine phosphatase TpbA of *Pseudomonas aeruginosa* controls extracellular DNA via cyclic diguanylic acid concentrations. *Environ. Microbiol.* **2**:449–455.
- USEPA. 1995. Industrial waste treatment, a field study training program, volume 2 Second edi. California State University, Sacramento and California Water Pollution Control Association for the USEPA.
- Valls M, De Lorenzo V. 2002. Exploiting the genetic and biochemical capacities of bacteria for the remediation of heavy metal pollution. *FEMS Microbiol.*

- Rev.* **26**:327–338.
- Verma M, Brar SK, Blais JF, Tyagi RD, Surampalli RY. 2006. Aerobic biofiltration processes - Advances in wastewater treatment. *Pract. Period. Hazardous, Toxic, Radioact. Waste Manag.* **10**:264–276.
- Vuong C, Saenz HL, Götz F, Otto M. 2000. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* **182**:1688–1693.
- Walker GM, Sai J, Richmond A, Stremler M, Chung CY, Wikswo JP. 2005. Effects of flow and diffusion on chemotaxis studies in a microfabricated gradient generator. *Lab Chip* **5**:611–8.
- Wang H, Sodagari M, Ju L-K, Zhang Newby B. 2013. Effects of shear on initial bacterial attachment in slow flowing systems. *Colloids Surf. B. Biointerfaces* **109**:32–9.
- Wanner O, Gujer W. 1984. Competition in biofilms. *Water Sci. Technol.* **17**:27–44.
- Wanner O, Gujer W. 1986. A multispecies biofilm model. *Biotechnol. Bioeng.* **28**:314–328.
- Wanner O, J. Eberl H, Morgenroth E, R. Noguera D, Picioreanu C, E. Rittmann B, van Loosdrecht MCM. 2006. Mathematical modeling of biofilms. *Adv. Dent. Res.* 2006 IWA Publishing. Vol. 11.
- Watnick P, Kolter R. 2000. Biofilm, city of microbes. *J. Bacteriol.* **182**:2675–2679.
- Weiss Nielsen M, Sternberg C, Molin S, Regenberg B. 2011. *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* biofilm in flow cells. *J. Vis. Exp.*:1–5.
- Wentland EJ, Stewart PS, Huang CT, McFeters G a. 1996. Spatial variations in growth rate within *Klebsiella pneumoniae* colonies and biofilm. *Biotechnol. Prog.* **12**:316–321.

- Whitehead NA, Barnard AML, Slater H, Simpson NJL, Salmond GPC. 2001. Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol. Rev.* **25**:365–404.
- Wijeyekoon S, Mino T, Satoh H, Matsuo T. 2004. Effects of substrate loading rate on biofilm structure. *Water Res.* **38**:2479–2488.
- Wilderer PA, McSwain BS. 2004. The SBR and its biofilm application potentials. *Water Sci. Technol.* **50**:1–10.
- Williamson K, McCarty PL. 1976. A model of substrate utilization by bacterial films. *J. (Water Pollut. Control Fed.* **48**:9–24.
- Wimpenny JWT, Colasanti R. 1997. A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. *FEMS Microbiol. Ecol.* **22**:1–16.
- Wolfe AJ, Visick KL. 2008. Get the message out: Cyclic-Di-GMP regulates multiple levels of flagellum-based motility. *J. Bacteriol.* **190**:463–475.
- Xavier JB, Picioreanu C, van Loosdrecht MCM. 2004. Assessment of three-dimensional biofilm models through direct comparison with confocal microscopy imaging. *Water Sci. Technol.* **49**:177–85.
- Xavier JB, Schnell A, Wuertz S, Palmer R, White DC, Almeida JS. 2001. Objective threshold selection procedure (OTS) for segmentation of scanning laser confocal microscope images. *J. Microbiol. Methods* **47**:169–80.
- Xavier JB, Picioreanu C, van Loosdrecht MCM. 2005a. A framework for multidimensional modelling of activity and structure of multispecies biofilms. *Environ. Microbiol.* **7**:1085–103.
- Xavier JDB, Picioreanu C, van Loosdrecht MCM. 2005b. A general description of detachment for multidimensional modelling of biofilms. *Biotechnol. Bioeng.* **91**:651–669.
- Xu KD, Stewart PS, Xia F, Huang CT, Mcfeters GA, Huang CT. 1998. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* **64**:4035–4039.

- Yang L, Liu Y, Wu H, Song Z, Høiby N, Molin S, Givskov M. 2012. Combating biofilms. *FEMS Immunol. Med. Microbiol.* **65**:146–157.
- Yang M, Ding Y, Ge X, Leng Y. 2015. Control of bacterial adhesion and growth on honeycomb-like patterned surfaces. *Colloids Surfaces B Biointerfaces* **135**:549–555.
- Yehuda C, Christopher S, Vishal P. 2016. US2016010045 Method of manufacturing a device for supporting biological material growth and device therefrom. United States US 2016/0010045 A1.
- Zelver N, Hamilton M, Pitts B, Goeres D, Walker D, Sturman P, Heersink J. 1999. Measuring antimicrobial effects on biofilm bacteria: From laboratory to field. *Biofilms* **310**:608–628.
- Zhang TC, Bishop PL. 1996. Evaluation of substrate and pH effects in a nitrifying biofilm. *Water Environ. Res.* **68**:1107–1115.
- Zhang W, Sileika TS, Chen C, Liu Y, Lee J, Packman AI. 2011. A novel planar flow cell for studies of biofilm heterogeneity and flow-biofilm interactions. *Biotechnol. Bioeng.* **108**:2571–2582.
- Zhang Y, Ng CK, Cohen Y, Cao B. 2014. Cell growth and protein expression of *Shewanella oneidensis* in biofilms and hydrogel-entrapped cultures. *Mol. Biosyst.* **10**:1035–42.
- Zobell CE. 1943. The effect of solid surface upon bacterial activity. *J. Bacteriol.* **46**:39–56.
- Zobell CE, Anderson DQ. 1936. Observations on the multiplication of bacteria in different volumes of stored seawater and the influence of oxygen tension and solid surfaces. *Biol. Bull.* **71**:324–342.