Biofilm-Derived Planktonic Cell Yield: A Mechanism for Bacterial Proliferation

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Chemical Engineering and Applied Chemistry
University of Toronto

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Abstract

The development of biofilms at solid-liquid interfaces has been investigated extensively, whereas the yield of planktonic cells from biofilms has received comparatively little attention. The detachment of single cells from biofilms has been attributed mainly to the erosive action of flowing liquid or the dispersal of cells from within biofilm microcolonies. The result has been an underestimation of the active role that biofilms can play in microbial proliferation through the production and release of planktonic cells to the environment.

In this study, the cultivation of Pseudomonas sp. strain CT07 biofilms in conventional flowcells, glass tubes and a novel CO$_2$ evolution measurement system was utilized to show that biofilm-derived planktonic cell yield was initiated within 6 hours of initial surface colonization and increased in conjunction with biofilm development. The magnitude of the yield was influenced by the metabolic activity of the biofilm, which was in turn dependent on environmental
conditions, such as carbon availability. The physiologically active region of the biofilm was responsible for the yield of significant numbers of planktonic cells (\( \sim 10^7 \) CFU.cm\(^{-2}\).h\(^{-1}\)), whereas a less active biofilm zone was optimized for survival during unfavourable conditions and shown to be responsible for the subsequent re-establishment of biofilm structure, activity and cell yield. Despite the yield of numerically considerable numbers of planktonic cells (\( \sim 10^{10} \) CFU), a carbon balance revealed that the carbon investment required to maintain this yield was insignificant (\( \sim 1\% \)) compared to the amount of carbon channelled into CO\(_2\) production (\( \sim 54\% \)). Together, these results indicate that biofilm-derived planktonic cell yield represents an efficient proliferation mechanism and support the view that the biofilm lifestyle affords microbes a dual survival-proliferation strategy, where the dominant strategy depends on the prevailing environmental conditions. An alternative model of biofilm development is presented to account for planktonic cell yield during all stages of biofilm development.
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*Pseudomonas aeruginosa* PAO1 was obtained from Prof. P.V. Phibbs at the Pseudomonas Genetic Stock Center, East Carolina University, while the strains containing the plasmids with the mini-Tn7 fluorescent protein constructs and helper plasmid were obtained from Prof. S. Molin, DTU, Denmark.
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CHAPTER 1

Introduction

1.1 Rationale of this thesis

The attachment of microbes to solid surfaces and the elucidation of the subsequent stages of biofilm formation have been studied extensively. In contrast, the continued detachment of single cells from the biofilm has received considerably less attention despite numerous reports of its incidence in single and multispecies biofilms, cultivated under diverse conditions in the laboratory (Karthikeyan et al. 2000; Bester et al. 2005).

The detachment of cells from a biofilm and dispersal into the bulk-liquid has been proposed to be critical for the continued proliferation of microbial species at alternative sites in the environment. In addition, the ability to escape local or global unfavourable conditions (such as limited nutrients or oxygen) through active detachment could enhance the probability of survival (Stoodley et al. 2002) or facilitate biofilm differentiation processes (Stewart et al. 2007).

This study investigated the extent to which biofilms produce and release planktonic cells; whether this yield of cells correlated with biofilm development; how different environmental conditions influenced the yield; which region of the biofilm was responsible for yielding the cells, and ultimately speculated on the significance of biofilm-derived planktonic cell yield.

1.2 Central hypothesis and Research objectives

The central hypothesis of this study is that in contrast to the current view that bacterial cells only detach from biofilms at discrete stages in biofilm development, or in response to environmental
stimuli, biofilms continuously yield a significant number of cells to the planktonic phase. The modulation of the number of cells yielded to the bulk-liquid in response to changing environmental conditions is furthermore hypothesized to be a dual biofilm strategy to promote both the proliferation and survival of the microbes.

1.2.1 Specific research objectives

1.2.1.1 Investigate the magnitude and timing of biofilm-derived planktonic cell yield in relation to biofilm development.

1.2.1.2 Investigate whether the yield of planktonic cells from the biofilm is sustained independently of the environment or modulated in response to the prevailing environmental conditions.

1.2.1.3 Determine whether the metabolically active region of the biofilm is responsible for yielding the planktonic cells and investigate phenotypic differences between the biofilm-derived planktonic cells and the biofilm itself.

1.2.1.4 Develop a theoretical framework to describe the ecological purpose and the implications of the presence of significant numbers of biofilm-derived planktonic cells in environmental, industrial and medical settings.
1.3  Thesis outline and Statement of Authorship

1.3.1  Thesis outline

CHAPTER 1: Introduction and Thesis Rationale

CHAPTER 2: Review of the relevant literature

This chapter contains relevant background information in addition to the material covered in the discussions of chapters 4, 5, 6 and 7.

CHAPTER 3: General experimental procedures

Experimental procedures relevant to chapters 4, 5 and 6 are described, including the bacterial strains used, the green fluorescent protein labelling of one of these strains as well as the configuration, disinfection and inoculation of the different continuous-flow reactors employed for biofilm cultivation.

CHAPTER 4: Planktonic cell yield is linked to biofilm development

This chapter has been published in the Canadian Journal of Microbiology (2009, Volume 55, pages 1195-1206) (excluding Figure 4.2).

Given the previous observations of actively mobile planktonic cells associated with surface-attached growth (Bester et al. 2005) it was hypothesized that biofilms yield these cells through active growth and that this production of cells is correlated to biofilm development, in accordance with the first specific research objective.
CHAPTER 5: Environmental influence on planktonic cell yield

This results contained in this chapter have been published in the *Canadian Journal of Microbiology* (2009, Volume 55, pages 1195-1206) (excluding Figure 5.7).

The results presented in chapter 4 concluded that the biofilm-derived planktonic cell yield is correlated to biofilm development for a number of Pseudomonad species and is initiated within a few hours after initial surface colonization. Since biofilm development is influenced by various chemical and physical environmental factors, the results presented in this chapter evaluate whether biofilm-derived planktonic cell yield was similarly influenced by environmental carbon concentration, bulk-liquid flow velocity and the presence of an oxidizing antimicrobial (specific research objective 2).

CHAPTER 6: Influence of carbon availability on biofilm structure, activity and planktonic cell yield

This chapter has been accepted by the *Journal of Applied Microbiology*.

The results obtained in chapter 5 indicated that environmental conditions, including carbon and likely oxygen availability, influenced not only biofilm development, but also the magnitude of biofilm-derived planktonic cell yield. While the reported response of biofilms exposed to carbon-limited conditions varies widely, the associated effect on biofilm-derived planktonic cell yield has not been investigated. Based on these observations, it was hypothesized that planktonic cell yield by biofilms is an active process, closely correlated to biofilm metabolic activity, which is in turn influenced by carbon availability, amongst other factors.
CHAPTER 7: Metabolic differentiation in the biofilm

This chapter has been published in *Applied and Environmental Microbiology* (2010, Volume 76, pages 1189-1197) (excluding Figures 7.2, 7.8 to 7.12).

The third research objective hypothesizes that one of the primary functions of a metabolically active region in the biofilm would be to promote microbial proliferation through the production and release of planktonic cells to the environment. The cells positioned at the biofilm bulk-liquid interface, provided with optimum access to nutrients and oxygen in the bulk-liquid phase and less constrained by a lack of space due to overlaying cell layers and extracellular polymeric substances (EPS), would be the most likely biofilm region for the yield of planktonic cells. The function of the remaining biofilm regions is hypothesized to be survival and re-establishment of the biofilm, should the metabolically active cells be destroyed by an antimicrobial or removed by physical forces (abrasion, shear or predator grazing). The second part of this objective was to determine whether phenotypic differences existed between the biofilm region responsible for the yield of planktonic cells and the yielded cells.

CHAPTER 8: Significance of biofilm-derived planktonic cell yield.

The last chapter addresses the fourth specific research objective and seeks to define and describe the ecological purpose and the implications of the presence of significant numbers of biofilm-derived planktonic cells in natural, industrial and medical settings.
1.3.2 Statement of Authorship

CHAPTER 4: Planktonic cell yield is linked to biofilm development and

CHAPTER 5: Environmental influence on planktonic cell yield

Elanna Bester, Elizabeth A. Edwards, Gideon M. Wolfaardt

E. Bester designed the experiments outlined in this chapter with suggestions from the co-authors.
E. Bester performed all of the experimental work and data analysis. Writing was completed with input from co-authors.


CHAPTER 6: Influence of carbon availability on biofilm structure, activity and planktonic cell yield

Elanna Bester, Otini Kroukamp, Martina Hausner, Elizabeth Edwards and Gideon M. Wolfaardt

E. Bester designed the experiments for this chapter with advice from O. Kroukamp (CEMS and carbon balance). The experiments were conducted, the data analyzed and interpreted by E. Bester. Writing was completed in consultation with the co-authors.

Accepted for publication: Journal of Applied Microbiology.
CHAPTER 7: Metabolic differentiation in the biofilm

Elanna Bester, Otini Kroukamp, Gideon M. Wolfaardt, Leandro Boonzaaier and Steven N. Liss.

The hypothesis was conceived by E. Bester. The experiments were designed and performed in equal measure by E. Bester and O. Kroukamp. O. Kroukamp developed and described the theoretical model for the quantification of the carbon dioxide evolution rates in consultation with L. Boonzaaier. Data analysis and interpretation were performed by E. Bester and O. Kroukamp. Writing was completed in consultation with the co-authors.


1.4 List of symbols and abbreviations

et al. and others
CEMS carbon dioxide evolution measurement system
CO₂ carbon dioxide
EPS extracellular polymeric substances
in situ in the place
i.e. that is
CHAPTER 2  Review of the relevant literature

2.1  The biofilm life cycle

The formation of a steady-state biofilm at a solid surface has traditionally been considered as a sequential process (Characklis and Marshall 1990) and the currently accepted 5-stage model of biofilm formation, or the ‘biofilm developmental life cycle’ was proposed as a result of direct microscopic analysis and whole-cell protein profiling of the biofilm model organism Pseudomonas aeruginosa (Sauer et al. 2002).

Biofilm development is initiated by the formation of a conditioning layer through the rapid deposition of organic molecules onto a solid substratum, followed by the transport of planktonic cells from the bulk-liquid to the surface by various processes, which may include sedimentation, convective mass transport, diffusion and flagellar-mediated motility (Palmer et al. 2007). The initial attachment of planktonic cells to a solid surface is reversible and believed to be mediated by non-specific van der Waals forces, electrostatic and hydrophobic interactions (Busscher and Van der Mei 2000).

This stage is followed by lasting or irreversible adhesion and takes place presumably once cellular appendages such as pili or fimbriae anchor the cell to the substratum, and (or) extracellular polymeric substances (EPS) are produced. Metabolic processes (i.e., substrate consumption, growth, cell division, and metabolite and EPS production) along with the continual attachment and detachment of cells lead to the formation of microcolonies, where internal cell death and lysis may contribute to the continual cycling of nutrients. During this time, microcolonies may mature and merge to form a confluent biofilm, which continues until a steady-state, often heterogeneous structure is achieved.
Mature biofilm structures often contain void spaces in the form of pores and channels, which provide conduits for the penetration of the bulk-liquid into the biofilm (Lawrence et al. 1991; De Beer et al. 1994). These voids have been shown to aid convective water flow within the biofilm, thereby significantly enhancing the diffusion of oxygen to cells buried in deeper regions of the biofilm. The final stage of development involves the continuous detachment of cells or small portions of matrix-containing cells from the biofilm outer surface, or dispersion of flagellated cells from within microcolonies (Stoodley et al. 2002) (Figure 2.1).

Figure 2.1: The current, widely-accepted model of biofilm development. (1) The initial attachment of free-floating cells to the substratum, (2) irreversible attachment of cells, (3) microcolony formation, (4) maturation of the three-dimensional architecture of the biofilm and (5) dispersion of free-swimming bacteria from within individual biofilm microcolonies to complete the cycle of biofilm development. The bottom section shows photomicrographs of *P. aeruginosa* biofilm development at a glass surface under continuous flow of nutrient medium (Stoodley et al. 2002). (Reproduced with permission of Annual Review of Microbiology from Biofilms as Complex Differentiated Communities, P. Stoodley et al., Volume 56, 2002; permission conveyed through Copyright Clearance Center, Inc.).
2.2 Mechanisms of dispersion or detachment from biofilms

A reduction in the amount of surface-attached biomass may result from various cell-derived or external processes.

2.2.1 External detachment processes

2.2.1.1 Physical removal

Physical removal processes involve those that are described as (i) erosion (the continuous removal of single cells, or small clumps of biomass from the biofilm surface due to shear forces exerted by moving liquid), (ii) abrasion (the removal of small clumps of bacteria from the biofilm due to contact with biofilm carriers or other particulates), (iii) sloughing (the detachment of large fragments of bacteria and matrix material at localized areas of the biofilm), and finally (iv) the removal of the biofilm due to feeding predators (Bryers 1989).

The association of free-floating single cells with biofilms cultivated in a flowing environment has most often been ascribed to the passive erosion of the surface layers of the biofilm due to fluid shear stress (Characklis and Marshall 1990; Trulear and Characklis 1982) despite indications that detachment rates correlate better with biofilm growth rates or substrate utilization rates (Peyton and Characklis 1992; Stewart 1993).
2.2.1.2 Chemical removal

The addition of various chemicals has also been shown to remove biofilms from surfaces. Chen and Stewart (2000) reported on the ability of concentrated salt solutions (NaCl and CaCl$_2$), surfactants (SDS, Triton X-100, and Tween 20), chelating agents (EDTA and Dequest 2006), lysozyme, pH decreases, hypochlorite and monochloramine to remove mixed *P. aeruginosa* and *Klebsiella pneumoniae* biofilms (Chen and Stewart, 2000).

The addition of EDTA, with or without the antibiotic gentamycin, resulted in the killing and (or) detachment of significant portions of *P. aeruginosa* biofilms, likely by chelating divalent cations (i.e. magnesium, iron and calcium), thereby destabilizing the biofilm matrix as well as the lipopolysaccharides in the individual cell membranes (Banin et al. 2006).

2.2.2 Cell-derived detachment processes

Several biological triggers for the detachment or dispersion of single cells from biofilms have been identified, such as changing nutrient conditions, the expression of EPS matrix hydrolyzing enzymes (Boyd and Chakrabarty 1994; Lee et al. 1996; Allison et al. 1998) and cell-to-cell signalling molecules (Dow et al. 2003). Detachment of cells and dispersal into the bulk-liquid is hypothesized to be essential for the continued dissemination of microorganisms at alternative environmental niches. In addition, the ability to escape unfavourable conditions (such as limited nutrients or oxygen) through active detachment could increase the probability of survival (Stoodley et al. 2002).
2.2.2.1 Nutritional status

Significant changes in the adhesion of an Acinetobacter species were observed when cultivated alternately in nutrient rich and nutrient poor media (James et al. 1995), while the addition of excess carbon was found to result in biofilm dispersion (Sauer et al. 2004). Halting the flow of nutrient media initiated a rapid, global dissolution of Pseudomonas putida (Gjermansen et al. 2005) and Shewanella oneidensis biofilms (Thormann et al. 2005). The subsequent omission of the sole carbon source from the growth medium led to a similar dissolution of 4 day-old P. putida biofilms, but not the biofilms formed by S. oneidensis. The latter biofilms were instead found to detach in response to a sudden decrease in oxygen availability and the authors speculated that detachment could be ascribed to the a decline in cell function (such as metabolic activity) due to the starvation conditions, the degradation of a component in the extracellular polymeric matrix material, the accumulation of a signalling molecule, or as part of a biological response process to environmental conditions (Thormann et al. 2005). The regulation of biofilm dispersion has been linked to carbon metabolism via the activity of a carbon storage regulator (csrA) gene in Escherichia coli. Induced expression of csrA led to biofilm dissolution and the dispersion of viable planktonic cells into the environment (Jackson et al. 2002).

2.2.2.2 Hydrolyzing enzymes and signalling molecules

In addition to the response of biofilms to the prevailing nutrient conditions, several other factors have been implicated in biofilm detachment. The secretion of EPS-hydrolyzing enzymes resulted in the dissolution of Pseudomonas fluorescens and P. aeruginosa biofilms (Boyd and Chakrabarty 1994; Allison et al. 1998). The production of an enzyme by Streptococcus mutans led to the detachment of its own biofilm, presumably due to the hydrolysis of adhesive proteins (Lee et al. 1996).
The involvement of cell-to-cell signalling factors in biofilm dispersal was observed for the plant pathogen *Xanthomonas campestris*, where a diffusible signal factor induced the expression of an EPS-hydrolyzing enzyme (Dow et al. 2003). The ability of various oral, non-flagellated bacteria to disperse from young biofilms (9.5 hours after inoculation) has been reported by Kaplan and Fine (2002). The dispersed cells formed new biofilm microcolonies in extended rows radiating away from the initial microcolony, leading to speculation that a temperature gradient resulted in surface movement (Kaplan and Fine 2002). Random transposon mutagenesis of one of these oral isolates, *Actinobacillus actinomycetemcomitans*, indicated that O-polysaccharide as well as a β-N-acetylglucosaminidase enzyme was required for detachment from the biofilm, but not for attachment and biofilm growth (Kaplan et al. 2003a; Kaplan et al. 2003b). Subsequent investigations revealed that the enzyme was active against the EPS matrix of *A. actinomycetemcomitans* as well as *Staphylococcus epidermidis* biofilms and that the enzyme activity led to the rapid dispersion of aggregated biomass and biofilms (Kaplan et al. 2004a; Kaplan et al. 2004b).

In a recent report, Davies and Marques (2009) showed that *P. aeruginosa* biofilms produced the small unsaturated fatty acid, cis-2-decenolic acid. The accumulation of this compound within the biofilm during stagnant conditions resulted in the dispersion of freely-swimming cells from within microcolonies, in a manner similar to that observed for seeding dispersal. The periodic release of cells under flowing conditions was only observed for microcolonies with particular dimensions (>10 µm thick and > 40 µm in diameter), regardless of microcolony age. The addition of the fatty acid to the growth medium of established *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans* biofilms also induced dissolution (Davies and Marques 2009).
2.2.2.3 Seeding dispersion

A spate of recent publications has investigated the dispersal of actively motile planktonic cells from within mature biofilm microcolonies as a new mechanism of cell-driven detachment. This phenomenon has been termed dispersion (Sauer et al. 2002) or seeding dispersal (Purevdorj-Gage et al. 2005) and only takes place at a discrete stage in microcolony development (after 9 days of cultivation on minimal medium with 130 mg.l\(^{-1}\) glutamate and after 3 days of growth on Luria Bertani broth) (Sauer et al. 2002; Purevdorj-Gage et al. 2005). An increased in cell lysis and death in *P. aeruginosa* PAO1 biofilm microcolonies were observed prior to dispersion and has been correlated to the switch of a lysogenic prophage to its infective lytic form in response to the accumulation of reactive oxygen and nitrogen species in the biofilm (Webb et al. 2003; Webb et al. 2004).

The dispersed cells exhibited increased phenotypic differentiation, observed as variations in colony morphology, motility, the rate and extent of subsequent biofilm formation, and the ability to resist antimicrobial treatment; these traits were moreover inherited by subsequent generations (Boles et al. 2004; Mai-Prochnow et al. 2006). These observations have led to speculation that seeding dispersion can generate diversity and enhance survival fitness, although it is as yet not clear whether dispersion is a part of a multicellular-like biofilm developmental program or the result of co-ordinated microbial response to an environmental trigger.

2.2.2.4 Regulation of attachment and detachment by cyclic-di-GMP

In subsequent investigations of *P. putida* and *S. oneidensis* biofilms, beyond what was described under nutritional status (section 2.2.2.1), detachment in response to changing environmental carbon or oxygen concentrations was linked to the expression of proteins containing GGDEF and
EAL domains. Simm et al. (2004) reported that of the 93 prokaryotic genomes sequenced to that date, 691 copies of the GGDEF domain and 503 copies of the EAL domain had been identified. They found that proteins containing these two domains were involved in the synthesis (diguanylate cyclase with a GGDEF domain) or degradation (phosphodiesterase with an EAL domain) of the secondary messenger molecule, cyclic-di(3’→5’)-guanylic acid (c-di-GMP) (Simm et al. 2004). The mechanisms whereby c-di-GMP interact with various proteins and influences transcription within the bacterial cell are under investigation, but it is known to be an allosteric activator of the cellulose synthase complex, responsible for the extracellular cellulose produced by *Gluconacetobacter xylinus* (Ross et al. 1987) and fimbrial production (encoded by the chaperone usher pathway *cupA*) which leads to the autoaggregation of the small colony variant phenotype in *P. aeruginosa* (Meissner et al. 2007).

Thornmann et al. (2006) designated a c-di-GMP regulated gene cluster in *S. oneidensis* as *mxdABCD* (for ‘biofilm matrix deficient’). The *mxdA* gene contained a GGDEF-like domain, while *mxdB* exhibited homology to glycosyl transferases, and some similarity to the AcsAB cellulose synthase from *G. xylinus*. Deletion mutants of both of these genes exhibited limited biofilm formation beyond normal surface attachment under flowing conditions. The *mxdA* mutant phenotype could furthermore be complemented by the expression of a known c-di-GMP-forming diguanylate cyclase.

The induction of a gene encoding for a c-di-GMP-hydrolyzing phosphodiesterase led to the rapid detachment of 20 hour-old biofilms, while the biofilm formed by a strain containing a c-di-GMP-producing diguanylate cyclase continued to increase in thickness. A decrease in oxygen availability, initiated by halting the bulk-liquid flow, led to a significant decrease in the amount of attached biomass for the phosphodiesterase-expressing strain, while the strain with the diguanylate cyclase lost 50% less biomass than the wild-type biofilm under similar circumstances. It was concluded that both attachment to an existing biofilm and detachment from the biofilm is
mediated by c-di-GMP through the *md* gene cluster; likely by the direct allostERIC interaction with the putative glycosyl transferase, *mdB*, thereby modulating the adhesion of cells to the extracellular polymeric substance (EPS) matrix (Thormann et al. 2006).

In a follow-up study by Gjermansen et al. (2006), the overexpression of a gene encoding for a GGDEF domain-containing protein, known to induce the formation of biofilm matrix material in *P. putida*, led to a wrinkled colony morphology on agar plates, thick pellicle formation and increased biofilm establishment in static cultures as well as the formation of large, dense microcolonies in continuous-flow cultures when cultured in minimal and rich growth media. In contrast, the overexpression of an EAL domain-containing gene led to limited attachment of *P. putida* cells during the initial stage of biofilm formation, and the induction of gene expression in a 3 day-old biofilm reduced the amount of attached biomass significantly. The swimming motility of individual cells was also found to be enhanced compared to that of the wild-type. Point mutations in the GGDEF and EAL domains did not result in similar phenotypes as those described above (Gjermansen et al. 2006).

The current model based on these and other studies proposes that the multiple GGDEF and EAL domain-containing proteins integrate the various signals received from the environment and modulate c-di-GMP levels in the cell, which determines whether the cells adhere or disperse.

### 2.2.2.5 Behaviour of single cells at a surface

The behaviour of single cells at a surface has been studied in some detail, mostly during the initial interactions with a surface and subsequent colonization patterns (Lawrence et al. 1992; James et al. 1995; Rice et al. 2000), although usually only for a limited period of time after attachment. In contrast, the association of planktonic cells with mature biofilms has received significantly less
attention, despite previous reports on the presence of notable numbers of single cells ($\sim 10^5 - 10^9$ CFU.ml$^{-1}$) in the effluent from bacterial, yeast and multispecies biofilms, cultivated at dilution rates that greatly exceed the maximum rates of planktonic cell replication (Karthikeyan et al. 2000; Bester et al. 2005; Joubert et al. 2006).

Tolker-Nielsen et al. (2000) reported the presence of large numbers of actively swimming single cells during most stages of Pseudomonas sp. biofilm formation and suggested that surface-associated growth consisted of both a sessile and a planktonic population where the presence of freely swimming cells could afford the biofilm greater flexibility with respect to environmental response (Tolker-Nielsen et al. 2000).

Diverse patterns of initial surface colonization by single cells and subsequent biofilm formation have been reported, and include variations in the amount of time required for irreversible attachment, the orientation of attachment (longitudinal vs. apical) as well as the pattern of microcolony development (Dalton et al. 1996). At least two of the strains studied demonstrated active detachment behaviour; daughter cells of a Vibrio sp. S14 started to detach after only 4 to 5 rounds of attached cell division, and the number of cells shed from the biofilm increased significantly once a monolayer of cells was attached to the surface. In contrast, detachment from a Pseudomonas sp. S9 biofilm was only observed once the biofilm reached a ‘critical mass’, while individual cells moved along the surface between adjacent microcolonies (Dalton et al. 1996).

A similar study conducted by Korber et al. (1989) with P. fluorescens revealed that the detachment of single cells occurred 5 hours after initial surface attachment and correlated with a 1 000-fold increase in the number of cells in the effluent. Subsequent reattachment of detached cells was influenced by bulk-liquid flow velocity, where higher flow velocities reduced recolonization rates, presumably because a greater percentage of cells were unable to overcome the flow rates and remained in the liquid phase (Korber et al. 1989).
Rice et al. (2003) used time-lapse imaging to record the behaviour of single *P. aeruginosa* PAO1 bacteria in young biofilms (3-10 µm thick). It was observed that detaching cells were more actively motile than attached cells and the probability of a cell detaching from a microscope field was estimated at 44%. Due to the nature of the technique used, it was impossible to determine whether these emigrating cells were lost to the bulk-liquid or re-attached to the surface outside the field of view (Rice et al. 2003).

Wilson et al. (2004) quantified the number of detachment events from *P. aeruginosa* PAO1 biofilms, as well as the number of cells represented by each event. The majority of detached biomass was in the form of single (70%) or dividing cells (9%). The detachment of larger clumps, containing more than 100 cells, was observed less frequently (< 0.28% of detachment events), but represented up to 37% of all of the detached cells (Wilson et al. 2004).

These investigations provide valuable insight into the active nature of individual cells during and after surface colonization. In addition to the detachment or dispersion of cells from a colonized surface in response to environmental stimuli (i.e. the addition of chemical agents, or changes in hydrodynamic and nutrient conditions) or when the biofilm reaches a particular stage of development, it is proposed that active cell production and release (herein described as ‘planktonic cell yield’) from biofilms is a continuous, rather than a discrete process, and that the magnitude of this biofilm-to-planktonic cell yield is influenced by environmental conditions.

### 2.3 List of symbols and abbreviations

~ approximately
CFU  colony forming units
mg  milligram
<  less than
l  litre
µm  micrometre
ml  millilitre
>  more than
%  percentage

et al.  and others
mxdABCD  biofilm matrix deficient gene cluster
CaCl₂  calcium chloride
csrA  carbon storage regulator
AcsAB  cellulose synthase gene
cupA  chaperone usher pathway
c-di-GMP  cyclic-di(3’→5’)-guanylic acid
EDTA  ethylenediaminetetraacetic acid
EPS  extracellular polymeric substances
EAL domain  glutamic acid – alanine – leucine sequence
GGDEF domain  glycine – glycine – aspartic acid – glutamic acid – phenylalanine sequence
RNA  ribonucleic acid
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>i.e.</td>
<td>that is</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
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</table>
Experimental procedures relevant to subsequent chapters are described here. This includes the bacterial strains used, the chromosomal insertion of a gene sequence encoding for a green fluorescent protein into one of these strains as well as the configuration, disinfection and inoculation of the different continuous-flow reactors employed for biofilm cultivation. Various methods for the enumeration of biofilm-derived planktonic cells are detailed and the term ‘biofilm-derived planktonic cell yield’ is defined.

3.1 Strains and culture conditions

3.1.1 Strains

An environmental isolate was used as the test organism for all of the experimentation in this study. The Gram negative, rod-shaped and motile bacterium was isolated from water in an industrial cooling system (Bester et al. 2005), and identified by 16S rDNA sequencing as having a 99% similar nucleotide identity to *Pseudomonas* sp. AEBL3 (1489 / 1495 bp) in the National Centre for Biotechnological Information database (NCBI; http://www.ncbi.nlm.nih.gov/) (GenBank accession number DQ 777633) (Wolfaardt et al. 2008) and was designated as *Pseudomonas* sp. strain CT07. Additionally, key observations were compared with a *Pseudomonas putida* strain and the well-characterized *Pseudomonas aeruginosa* PAO1; the latter being one of the recognized model strains in biofilm research. All strains were maintained as glycerol stocks at –80°C. An unidentified microbial community was enriched
from 1 g of soil in 3 g.L\(^{-1}\) tryptic soy broth (TSB) at 22°C ± 2°C with shaking at 250 rpm. The initial culture was subcultured once prior to inoculation into a continuous flow system.

### 3.1.2 Culture conditions

Routine cultivation of the Pseudomonads was carried out in modified AB defined medium (final concentration of 1.51 mM (NH\(_4\))\(_2\)SO\(_4\), 3.37 mM Na\(_2\)HPO\(_4\), 2.20 mM KH\(_2\)PO\(_4\), 179 mM NaCl, 0.1 mM MgCl\(_2\)-6H\(_2\)O, 0.01 mM CaCl\(_2\)-2H\(_2\)O, and 0.001 mM FeCl\(_3\)) (Clark and Maaloe 1967) with 1 mM Na-Citrate-\(\cdot\)2H\(_2\)O as the carbon source, unless stated otherwise. The soil microbial community and a selected set of pure culture biofilms were cultured with TSB. Pre-cultures used as inocula for biofilm studies were incubated at 24°C ± 2°C, with shaking at 250 rpm.

### 3.1.3 Maximum planktonic specific growth rates (\(\mu_{\text{max planktonic}}\))

To establish the critical dilution rate for the continuous-flow systems, the maximum specific planktonic growth rates of the Pseudomonad strains were determined in six 100 ml batch cultures with 1 mM citrate as carbon source (Figure 3.1.1). The cultures were incubated at 24°C ± 2°C with shaking at 250 rpm and samples were taken at 1 hour-intervals for spectrophotometric optical density measurements at 600 nm. The maximum planktonic specific growth rate of *Pseudomonas* sp. strain CT07 with 5 mM citrate at 26°C ± 2°C was determined in a similar manner (Figure 3.1.2).
1: Pure culture Pseudomonads on 1 mM citrate  2: *Pseudomonas* sp. strain CT07 on 5 mM citrate

Figure 3.1: The maximum planktonic specific growth rates of the Pseudomonad species on different concentrations of citrate in the defined growth medium. (1) The µmax planktonic of the various bacteria on 1 mM citrate was calculated as follows: *Pseudomonas* sp. strain CT07 = 0.49 h\(^{-1}\) ± 0.05 h\(^{-1}\), *P. aeruginosa* PAO1 = 0.81 h\(^{-1}\) ± 0.15 h\(^{-1}\) and *P. putida* = 0.86 h\(^{-1}\) ± 0.10 h\(^{-1}\). (2) The µmax planktonic for *Pseudomonas* sp. strain CT07 on 5 mM citrate was determined to be 0.35 h\(^{-1}\) ± 0.10 h\(^{-1}\). Values are given as the average ± the standard deviation of 6 replicate batch cultures.

3.1.4 Fluorescent labelling of *Pseudomonas* sp. strain CT07

To facilitate routine microscopic and flow cytometric experimentation, a gene sequence encoding for the green fluorescent protein (GFP) was inserted into *Pseudomonas* sp. strain CT07 at a specific, but neutral chromosomal site downstream of the *glmS* gene, using a mini-Tn7 transposon system first described by Bao et al. (1991) and subsequently expanded (Højberg et al. 1999; Koch et al. 2001; Lambertsen et al. 2004).
The pUC19-derived donor plasmids contain the Tn7 ends on either side of the fluorescent protein and antibiotic resistance marker. The transposase genes (*traABCDE*) are located on a second helper plasmid. Neither the donor, nor the helper plasmid is able to replicate in non-enteric bacteria, such as the Pseudomonads. The simultaneous introduction of the donor and helper plasmid into a bacterial cell and subsequent expression of the transposase genes leads to the site-specific insertion of the fluorescent protein into the chromosome and the loss of the plasmids upon cell division.

### 3.1.4.1 Plasmid extraction and purification

The donor (pBK-miniTn7-gfp2, miniTn7(Gm)PA1/04/03-gfp.AAV-a, miniTn7(Gm)PrrnB1-gfp.AAV-a, miniTn7(Gm)PA1/04/03-DSRedExpress-a) (Koch et al. 2001; Klausen et al. 2003; Lambertsen et al. 2004) and helper (pUX-BF13) (Bao et al. 1991) plasmids were amplified and extracted from the respective *Escherichia coli* strains with the Genopure Plasmid Maxi kit (Roche, Catalogue number 03143422001), according to the manufacturer’s instructions.

### 3.1.4.2 Preparation of electrocompetent cells

Electrocompetent cells were prepared as described by Choi et al. (2006). Briefly, a 5 ml pre-culture of *Pseudomonas* sp. strain CT07 was prepared in Luria Bertani broth (LB broth). This culture was aliquoted into four microcentrifuge tubes and centrifuged for 2 minutes at 13,200 rpm. Each cell pellet was resuspended in 1 ml of 300 mM sucrose and centrifuged as before. The wash and centrifugation steps were repeated. After the final centrifugation, each pellet was resuspended in 25 µl of 300 mM sucrose and the contents of all four tubes were combined (Choi et al. 2006).
3.1.4.3  Electroporation

The BioRad Gene Pulser Xcell™ electroporation system was used with the following settings; \( C = 25 \, \mu F, V = 2.5 \, kV, PC = 200 \, \Omega \). Equal volumes of helper and donor plasmid (containing 1.5 – 2 \( \mu g \) of DNA per plasmid) were mixed with 100 \( \mu l \) of electrocompetent cells in a chilled microcentrifuge tube. A maximum of 5 \( \mu l \) of either plasmid was used. The mixture was pulsed in a chilled 0.2 cm electroporation cuvette and transferred to a sterile tube containing 1 ml of LB broth. The cells were incubated at 30°C for 2 hours and plated onto selective (with antibiotic) and non-selective (without antibiotic) LB plates. The remaining cell suspension was concentrated by centrifugation, resuspended in 100 \( \mu l \) LB and plated onto selective LB plates. The plates were incubated for 2-3 days at 30°C.

3.1.4.4  Selecting transformants and verifying the correct insertion

All of the resulting colonies on the selective plates were screened for fluorescence using an epifluorescent microscope. Fluorescent colonies were picked and streaked to single colonies on selective plates and colony PCR was used to verify the site-specific insertion of the fluorescent protein sequence (Figure 3.2) (Lambertsen et al. 2004). Since the donor and helper plasmids are unable to replicate in Pseudomonas sp. strain CT07, the transformants were subcultured for three successive generations in the absence of antibiotic selection, to ensure that any remaining plasmid would be lost from the population. After the subculture routine, the transformants were streaked onto LB plates with antibiotic selection to further verify stable insertion of the mini-Tn7 system.

To ensure that the insertion and expression of the fluorescent protein did not place a detectable metabolic burden on the transformed bacteria, the maximum specific planktonic
growth rate of the transformants was compared to that of wild-type *Pseudomonas* sp. strain CT07 (Figure 3.3.1). The stability and intensity of *gfp* expression was monitored simultaneously with the aid of a luminescence spectrometer (Perkin Elmer LS 50B) with excitation at 475 nm, and emission scan from 490 to 535 nm (Figures 3.3.2).

**Figure 3.2:** PCR-verification of the site-specific insertion of the green fluorescent protein into *Pseudomonas* sp. strain CT07. The forward primer Tn7-GlmS (5′-AAT CTG GCC AAG TCG GTG AC-3′) anneals to nucleotide number 310 from the start site of the *glmS* gene and the reverse primer Tn7-R109 (5′-CAG CAT AAC TGG ACT GAT TTC AG-3′) anneals to nucleotide number 109 of the Tn7R flanking region. An annealing temperature of 56°C was used and the expected size of the PCR product (100-150 bp) was visualized on a 1.2% agarose gel. 1: 100 bp DNA ladder (Fermentas, O’GeneRuler, Catalogue number SM0241); 2: *Pseudomonas* sp. strain CT07 genomic DNA; 3: *Pseudomonas* sp. CT07::*gfp*-2; 4: *Pseudomonas* sp. strain CT07::*gfp*[AAV]; 5: *Pseudomonas* sp. strain CT07::*gfp*[AAV]*P*_rmB1; 6: *Pseudomonas* sp. strain CT07::DSRedExpress; 7: *P. aeruginosa* PAO1 genomic DNA; 8: No template control (sterile dH₂O). Lanes 3 to 6 contain a PCR product of expected size (150 bp), indicating that the fluorescent protein was inserted in the correct chromosomal position.
1: Transformant maximum specific growth rates

2: GFP fluorescence intensity and stability

**Figure 3.3:** The maximum planktonic specific growth rates and the fluorescence properties of the *gfp* transformants as compared to wild-type *Pseudomonas* sp. strain CT07. (1) The maximum planktonic specific growth rates and (2) green fluorescent protein fluorescence intensity and stability of the various *Pseudomonas* sp. strain CT07 transformants on 1 mM citrate medium. The $\mu_{\text{max}}$ planktonic were calculated as follows: *Pseudomonas* sp. strain CT07::*gfp*-2 = 0.47 h$^{-1}$ ± 0.03 h$^{-1}$, *Pseudomonas* sp. strain CT07::*gfp*-2[AAV] = 0.49 h$^{-1}$ ± 0.01 h$^{-1}$, and *Pseudomonas* sp. strain CT07::*gfp*-2[AAV]PrnB1 = 0.46 h$^{-1}$ ± 0.04 h$^{-1}$. (Values given are the average ± standard deviation of triplicate cultures).

The $\mu_{\text{max}}$ planktonic values did not differ significantly from that of the wild-type (0.49 h$^{-1}$ ± 0.05 h$^{-1}$) and suggests that the expression of the fluorescent protein did not adversely affect the metabolism of the organism under these culture conditions. The wild-type *Pseudomonas* sp. strain CT07 exhibited negligible fluorescence, as did the short half-life *gfp*-2[AAV], as expected for the rapid proteolytic degradation of the modified protein (Figure 3.3.2). The expression of the unmodified *gfp*-2 protein from a constitutive promoter correlated well with
the increase in cell density (Figure 3.3.1) and reached maximal intensity at the end of the logarithmic growth phase. Expression of the short half-life gfp-2[AAV] from the growth-inducible PrrnB1 promotor increased during the active logarithmic growth phase, followed by a rapid decline once the cells entered stationary growth. Subsequent comparisons between Pseudomonas sp. strain CT07::gfp-2 and Pseudomonas CT07::gfp-2[AAV]ₚᵣᵣₙₜ₁ biofilm formation in flowcells indicated that the strain with the growth-inducible GFP was impaired in biofilm formation. No significant differences between biofilms formed by the wild-type strain and the Pseudomonas sp. strain CT07::gfp-2 strain were observed.

3.2 Biofilm cultivation and defining biofilm-derived planktonic cell yield

3.2.1 Continuous-flow cultivation of biofilms

Glass tubes and conventional flowcells were selected for the laboratory cultivation of biofilms. The choice of glass tubes allowed microscopic investigation of biofilm formation and the definition of various important system parameters; namely, a homogeneous (glass) attachment surface, total reactor volume, the liquid dilution rate, and the Reynolds number. Since surface irregularities in the glass tubes limited the image quality obtained with confocal laser scanning microscopy (CLSM), conventional flowcells were used whenever images of higher quality was required for subsequent analysis.

3.2.1.1 Glass tubes and flowcells

Square, borosilicate glass tubes with inner dimensions of 2 mm x 2 mm x 152.4 mm (width, depth and length) and a wall thickness of 0.3 mm were obtained from Friedrich & Dimmock,
Inc. (Millville, NJ, 08332 USA). The volume \( V_l \) of the glass tubes was 0.61 ml and cross-sectional area \( A \) was 0.040 cm\(^2\). Multi-channel flowcells were milled from Perspex as previously described (Wolfaardt et al. 1994), with flow chamber dimensions of 5 mm x 6 mm x 33 mm (width, depth and length), with a volume \( V_{ch} \) of 0.99 ml and cross-sectional area \( A \) of 0.30 cm\(^2\), unless stated otherwise. A microscope coverslip (No. 1 thickness) was glued to the top of each flowcell with silicone adhesive.

The flow reactors were connected to the growth medium reservoirs and waste collection flasks with silicone tubing (inner diameter of 1.575 mm) to facilitate aeration, except for a 10 cm piece of tygon tubing immediately upstream of the chambers to discourage back-growth, since tygon tubing is significantly less oxygen permeable than silicone. A continuous flow of growth medium was supplied by a Watson-Marlow 205S peristaltic pump (Figure 3.4).

**Figure 3.4:** A typical laboratory setup for the cultivation of biofilms under continuous-flow conditions with square glass tubes as the flow chambers.
3.2.1.2 Cleaning, disinfection and inoculation

Prior to re-using a glass tube, it was soaked in a 2% (v/v) solution of the liquid detergent Extran MA01 (VWR International, Catalogue number CAEX0993-4) for a day, repeatedly rinsed with distilled water, soaked in 0.5 M Nitric acid for another day, followed by extensive rinsing with dH$_2$O and then drying. The flowcell coverslips were replaced after each experiment.

After assembly, the continuous-flow reactors (Figure 3.4) were disinfected with a dilute commercial bleach solution (final concentration ~0.525% sodium hypochlorite) for a minimum of 2 hours, followed by overnight irrigation with sterile dH$_2$O. Prior to inoculation, the dH$_2$O was displaced by flushing with sterile growth medium. Each flow reactor was inoculated aseptically using a sterile needle and syringe with 0.1 ml of a test strain pre-culture, cultivated in the corresponding growth medium. The inoculated bacteria were allowed to adhere for 0.5 hours (unless specified otherwise) under quiescent conditions, where after a medium flow rate (F) of 15.3 ml.h$^{-1}$ was initiated for the duration of the experiment, resulting in laminar flow (Reynolds number of 2.1 for the glass tube and 0.8 for the flowcell). This corresponds to a dilution rate (D) of $F/V_l = 25.1$ h$^{-1}$ in the glass tubes and 5.5 h$^{-1}$ in the flow chambers, which is 50 and 32 times, respectively, greater than the maximum planktonic specific growth rate of Pseudomonas sp. strain CT07 on 1 mM citrate. It is therefore unlikely that an independently replicating planktonic population of bacteria could persist in the reactor bulk-liquid; cells in the effluent from each reactor were thus assumed to originate from surface-associated growth.

3.2.1.2 Optical Large Area Photometer (OLAPH)

Biofilm development was monitored in real time with the use of an optical large area photometer (OLAPH, German patent number 19947651), which detects and quantifies changes
in visible light scatter owing to the accumulation of biomass in a parallel-plate flowcell (Bester et al. 2005; Saftic et al. 2005). This system measures light scatter over a comparatively large area (± 10 cm²), thereby quantifying macroscopic changes in biofilm development (Figure 3.5).

**Figure 3.5:** A diagram of the various components required to determine biofilm development in a parallel plate flowcell with the optical large area photometer (OLAPH). The optical unit and flowcell were encased in a darkened box to prevent light interference, while an electronic system interfaced with a PC where LabView-based software recorded the light scatter and temperature data at fixed time intervals.

To eliminate the accumulation of biomass due to aggregation and gravitational forces, the rectangular flowcell (48 cm² internal attachment area) was oriented vertically, with inflow of sterile growth medium at the top and outflow at the bottom. The flowcell was connected to the sterile growth media reservoir and Masterflex peristaltic pump (Cole Parmer) with silicone tubing, as described previously. The internal volume of the rectangular flowcell was 12 ml, and
a liquid flow rate of 60 ml.h\(^{-1}\) resulted in a dilution rate (\(F/V_l\)) of 5 h\(^{-1}\) in the system, which is 10- and 14-fold greater than the \(\mu_{max}\) planktonic of *Pseudomonas* sp. strain CT07 on 1 mM and 5 mM citrate, respectively.

The measured changes in light scatter can thus be attributed to the accumulation of attached biomass alone, since any contribution of planktonic cell replication would have been minimal due to the high dilution rate. Disinfection was achieved as described previously and 1 ml of a pre-culture was injected aseptically into the flowcell, with flow stopped for 0.5 hours. Once flow resumed, the scattered light intensity (arbitrary units) was recorded at 5-minute intervals during the incubation period.

### 3.2.2 Definition of biofilm-derived planktonic cell yield

The growth medium dilution rates (D) applied to the various flow chambers exceeded the maximum rate of planktonic cell replication of all of the Pseudomonad strains. It is therefore unlikely that an independently replicating planktonic population of bacteria would persist in the reactors, and the cells collected in the effluent were assumed to originate from surface-associated growth. The tubing was disconnected from the outlet of the flow chambers prior to effluent sampling to minimize the contribution of downstream tubing wall growth to the measured cell numbers. Biofilm formation in the tubing upstream of the reactors was rarely visible, even after extended incubation. Therefore, the only surface that is considered available for biofilm formation is the inner surface area of the flow chamber, which is 12.2 cm\(^2\) in the case of the glass tube. The cell numbers measured in the effluent from the glass tubes
(CFU.ml⁻¹) were normalized with respect to the flow rate (ml.h⁻¹), and the internal surface area of the glass tube (cm²) to determine the biofilm-to-planktonic cell yield (CFU.cm⁻².h⁻¹).

3.2.2 Enumeration of planktonic cell numbers

3.2.3.1 Viable cell counts

Serial dilution in sterile saline (0.9% m/v NaCl) followed by spread plating onto 5 mM citrate modified AB medium with 1.2% (m/v) agar was used for the routine enumeration of suspended cell numbers in flow reactor effluents when the biofilms were cultivated on citrate. Effluent cell counts of biofilms cultivated on TSB medium were plated onto 3 g.l⁻¹ tryptic soy agar plates (TSA). All of the plates were incubated at 24°C ± 2°C for 3 - 4 days.

3.2.3.2 Direct counts

Cell numbers in the effluent from duplicate glass tube-grown *Pseudomonas* sp. strain CT07::gfp-2 biofilms were also enumerated by direct counting with the aid of an epifluorescent microscope (Leica DM 5000 B). The cell suspensions were diluted prior to immobilization on 0.2 µm black, polycarbonate membrane filters with vacuum filtration (VWR International, Whatman, Nucleopore, Catalogue number 28159-252) to achieve approximately 30 cells per microscope field. Between 40 and 50 microscopic fields per filter were captured with a 63X Apoplan oil-immersion lens and a black and white camera (Leica DFC 350 FX). The number of cells per image was counted manually, rather than using automated image analysis, to evaluate the size of each detached particle.
3.2.3.3 Flow activated cell sorting (FACS) or flow cytometry

Serial dilution and spread plating onto agar-solidified growth medium still remains the microbiological golden standard for the enumeration of viable cell numbers in suspension. Another widely-applied technique for the enumeration of microbes in environmental samples is epifluorescent direct counting (Kepner Jr. and Pratt 1994). While these techniques have accepted advantages and disadvantages, both techniques are relatively time consuming in both preparation and execution, as well as the time required to attain the final results (i.e. incubation time, or manual thresholding of each image prior to analysis to achieve accurate results). These considerations make the use of flow activated cell sorting (FACS, or flow cytometry) an attractive alternative for the enumeration of suspended cell numbers.

An accepted technique for flow cytometric enumeration of bacterial cell numbers requires the addition of a known concentration of fluorescent beads to each sample, followed by measurement of the number of events from the size-separated bead and bacterial populations, and extrapolating the bacterial cell count from the number of beads counted in the given sampling volume or time (e.g. Bacteria counting kit for flow cytometry, Invitrogen, Catalogue number B7277). While feasible, this method becomes cost-prohibitive when testing a large number of samples.

The goal was therefore to determine whether a reliable correlation could be obtained between the events per millilitre (measured by the cytometer) and actual cell count per millilitre (as viable cell counts, i.e. CFU.ml⁻¹) for each of the three fixed cytometer flow rates (high, medium and low) of a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, California). Batch cultures of Pseudomonas sp. strain CT07 and Pseudomonas sp. CT07::gfp-2 were diluted to ~10⁵ bacteria per ml in 0.2 μm filtered saline and used to optimize the acquisition parameters of the flow cytometer with a 488 nm (15 mW) argon laser (Figure 3.6).
The best results were achieved with the following instrument settings; the test population was distinguished from debris on a side scatter (Voltage of 452) vs. forward scatter (Voltage E02) dot plot, and the fluorescence properties of the gated events (G1 = R1) displayed on a second dot plot of FL2 (Voltage of 570) vs. FL1 (530/30 nm, Voltage of 750). All of the detection parameters were set to log mode and an amplifier gain of 1. The primary trigger for event acquisition was set on the forward scatter (value of 65), with side scatter as the secondary parameter (value of 270). The combination of primary and secondary trigger parameters eliminated the detection of a significant amount of debris-related events.

To test the robustness of the optimized acquisition parameters, pre-cultures of *Pseudomonas* sp. strain CT07::gfp-2 were serially diluted (10\(^{-3}\) to 10\(^{-6}\)) in 0.2-µm-filtered saline. Fractions of each dilution were used for flow cytometric analysis, serial dilution (if necessary) and spread plating to enumerate the number of cells. The number of CFU.ml\(^{-1}\) in the original pre-culture was also enumerated by spread plating for comparative purposes. The acquisition parameters of green fluorescent events (G1 = R1) was set to 15 seconds or 10 000 000 events. The number of events for each dilution was measured in triplicate for each of the fixed cytometer flow rates and the filtered saline solution was included in the measurements to determine background fluorescent event levels. The entire procedure was performed in triplicate and a minimum of 10 000 events of interest was collected for each sample.

The optimized procedure resulted in a good correlation between the number of fluorescent events measured with the cytometer and the viable cell numbers determined with spread plating for all three flow rates, as well as between the measured and expected viable cell counts for each of the serial dilutions (Figure 3.7). The cell number ratio as determined with flow cytometry versus that of viable counting was calculated to assess whether the flow cytometric protocol was a reliable tool for the quantification of suspended cell numbers (Table 3.1).
1: Wild-type *Pseudomonas* sp. strain CT07

2: GFP-labelled *Pseudomonas* sp. strain CT07

Figure 3.6: The enumeration of planktonic fluorescent cell numbers with flow cytometry. Examples of fluorescence dot plots (left) and total event count histograms (right) as generated by CellQuest software are shown. The FACS instrument settings were optimized to detect (1) wild-type *Pseudomonas* sp. strain CT07 as non-fluorescent (bottom left quadrant on the dot plot and in the first region in the histogram) and successfully detected (2) *Pseudomonas* sp. strain CT07::gfp-2 as fluorescent (right quadrants on the dot plot and second region in the histogram). A low percentage of non-fluorescent events were always detected in labelled samples; it is unknown whether this was due to non-cellular debris, non-fluorescent bacteria or weakly fluorescent bacteria.
Figure 3.7: The correlation between suspended cell numbers as determined by flow cytometry and viable cell counts for serial dilutions of *Pseudomonas* sp. strain CT07::gfp-2. The expected CFU.ml\(^{-1}\) was extrapolated from the number of cells in the original pre-culture and the subsequent dilutions. The following correlations were obtained between the flow cytometric counts and the viable cell counts; High flow rate: \(R^2 = 0.989\), Medium flow rate: \(R^2 = 0.994\), and Low flow rate: \(R^2 = 0.999\) and \(R^2 = 0.999\) between the measured and expected CFU.ml\(^{-1}\).

When the calculated average ratios were applied as multiplication factors to the measured number of events, the best correlation to actual cell number is obtained with the medium flow rate, for a sample with \(~10^5\) bacteria. This multiplication factor underestimates the cell number by 17\%, which is significantly different to preclude the use of this procedure for absolute quantification of cell numbers, but not for gaining qualitative information.
Table 3.1: The ratios of viable counts and cell numbers enumerated with flow cytometry for dilutions of *Pseudomonas* sp. strain CT07::gfp-2. Not all of the ratios are shown, since some of the $10^{-5}$ and all of the $10^{-6}$ dilutions corresponded to event counts below the minimum 10 000 events of interest required.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Flow rate</th>
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<tr>
<td></td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
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<tr>
<td></td>
<td>Ratio ± St dev</td>
<td>Ratio ± St dev</td>
<td>Ratio ± St dev</td>
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<tr>
<td>$10^{-2}$</td>
<td>0.91 ± 0.01</td>
<td>0.94 ± 0.01</td>
<td>0.99 ± 0.01</td>
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<td>$10^{-3}$</td>
<td>0.95 ± 0.03</td>
<td>0.96 ± 0.03</td>
<td>1.01 ± 0.03</td>
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<tr>
<td>$10^{-4}$</td>
<td>0.94 ± 0.03</td>
<td>0.97 ± 0.01</td>
<td>1.02 ± 0.01</td>
<td></td>
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<tr>
<td>$10^{-5}$</td>
<td>0.94 ± 0.04</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Average</td>
<td>0.93 ± 0.01</td>
<td>0.95 ± 0.01</td>
<td>1.01 ± 0.01</td>
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</tbody>
</table>

3.3 List of symbols and abbreviations

bp: base pairs
C: capacitance (µF = microFarad)
CFU: colony forming units
A: cross-sectional area
°C: degrees Celsius
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>D</td>
<td>dilution rate ( (h^{-1}) )</td>
</tr>
<tr>
<td>µF</td>
<td>microFarad</td>
</tr>
<tr>
<td>F</td>
<td>flow rate ( (ml.h^{-1}) )</td>
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<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>µg</td>
<td>micrograms</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>( (m/v) )</td>
<td>mass in volume</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<tr>
<td>µl</td>
<td>microlitre</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>( cm^2 )</td>
<td>square centimetre</td>
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<tr>
<td>mm</td>
<td>millimetre</td>
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<tr>
<td>µm</td>
<td>micrometre</td>
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<td>nm</td>
<td>nanometre</td>
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<tr>
<td>M</td>
<td>molar</td>
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<td>mM</td>
<td>millimolar</td>
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<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>±</td>
<td>plus and (or) minus</td>
</tr>
<tr>
<td>PC</td>
<td>pulse controller (resistance; ( \Omega = \text{ohm} ))</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
</tbody>
</table>
sp. species

V voltage (kV = kiloVolt)

(v/v) volume in volume

$V_1$ volume of liquid

$(\text{NH}_4)_2\text{SO}_4$ ammonium sulfate

$\text{CaCl}_2.2\text{H}_2\text{O}$ calcium chloride dehydrate

$\text{Na}_2\text{HPO}_4$ disodium hydrogen phosphate

dH$_2$O distilled water

FeCl$_3$ ferric chloride

MgCl$_2.6\text{H}_2\text{O}$ magnesium chloride hexahydrate

KH$_2$PO$_4$ potassium dihydrogen phosphate

Na-citrate sodium citrate

NaCl sodium chloride

et al. and others

CLSM confocal laser scanning microscope

DNA deoxyribonucleic acid

FACS flow activated cell sorting

glmS glucosamine synthetase

GFP green fluorescent protein
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td><em>gfp</em></td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>µmax planktonic</td>
<td>maximum planktonic specific growth rate</td>
</tr>
<tr>
<td>No.</td>
<td>number</td>
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<tr>
<td>OLAPH</td>
<td>optical large area photometer</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
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<tr>
<td>i.e.</td>
<td>that is</td>
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<tr>
<td><em>traABCDE</em></td>
<td>transposition genes</td>
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<tr>
<td>TSA</td>
<td>tryptic soy agar</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
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<td>vs.</td>
<td>versus</td>
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CHAPTER 4  Planktonic cell yield is linked to biofilm development*

Based on previous observations of active, motile planktonic cells associated with surface-attached growth (Bester et al. 2005), it was hypothesized that biofilms yield these cells through active growth and that this production of cells is correlated to biofilm development, in accordance with the first specific research objective.

4.1  Experimental procedures

4.1.1  Planktonic cell yield from various pure culture Pseudomonas species biofilms

Pure culture biofilms of Pseudomonas sp. strain CT07::gfp-2, Pseudomonas aeruginosa PAO1 and Pseudomonas putida were cultured in duplicate sets of glass tubes for 144 hours. One mM citrate was supplied as the carbon source and effluent samples were taken at 24 hour-intervals for the enumeration of viable biofilm-derived cell numbers. Additional effluent samples from Pseudomonas sp. strain CT07::gfp-2 biofilms were collected, filtered onto 0.2 µm black, polycarbonate filters and examined with an epifluorescent microscope to assess the size distribution of detached biomass on a qualitative basis.

*Published in Canadian Journal of Microbiology, 2009, Vol.55, p.1195-1206, NRC Research Press Journals (except Figure 4.2).
4.1.2 The interaction between attached and planktonic biomass

A continuous, once-through flow of batch-cultured, planktonic cells was pumped through the OLAPH parallel plate flowcell at a flow rate of 60 ml h\(^{-1}\), resulting in a dilution rate (D) of 5 h\(^{-1}\) in the flowcell, which is 14 times greater than the maximum planktonic growth rate of *Pseudomonas* sp. strain CT07 on 5 mM citrate (\(\mu_{\text{max}}\) planktonic = 0.35 h\(^{-1}\) ± 0.10 h\(^{-1}\)). The batch culture was prepared by sterilizing a 4 litre reservoir containing 3 litres of 5 mM citrate medium and inoculating it with 30 ml of a *Pseudomonas* sp. strain CT07 pre-culture. Continuous mixing and aeration were provided by a magnetic stirrer. The reservoir was connected to the flowcell with tygon tubing to minimize tubing wall growth. The changes in light scatter due to the increase in both suspended biomass flowing into the flowcell and attached biomass on the flowcell surfaces were monitored for 48 hours, while samples of the flowcell effluent and batch culture were taken periodically to assess changes in optical density at 600 nm with a spectrophotometer. After 48 hours, the inflowing cell suspension from the reservoir was replaced with a reservoir containing sterile growth medium only. The washout of unattached planktonic bacteria due to the liquid flow and the biofilm dynamics were monitored for a subsequent 24 hours.

4.1.3 The nature and number of cells yielded during biofilm development

4.1.3.1 Flow cytometry

*Pseudomonas* sp. strain CT07::gfp-2 biofilms were established in two sets of triplicate glass tubes with 1 mM citrate as the carbon source and incubated for 96 hours. To minimize the interference of particulate matter during the subsequent flow cytometric enumeration of
effluent cell numbers, all of the glassware was rinsed twice with 0.2 µm-filtered dH₂O and the rinsing water and nutrient medium were filtered prior to autoclaving.

After inoculation and the initial 0.5 hour stagnant period, the flow of growth medium was initiated and collection of effluent from each tube commenced. Composite effluent samples were collected for 3-hour periods, except for the first 5 displaced reactor volumes (total of 0.2 hours), which were collected separately. Inoculation of one set of tubes was offset by 12 hours to cover full 24-hour sampling during a 15-hour period of each day. Care was taken to inoculate both sets of glass tubes with pre-cultures grown to similar optical densities. Formaldehyde was added to the collection tubes prior to sampling to preserve cell integrity and prevent cell division (final concentration of 3.7% (m/m)). The samples were stored at 4°C until the cell numbers were determined with flow cytometry, which facilitated the enumeration of a large number of samples in a shorter time than direct counts.

To avoid the potential underestimation of cell numbers due to degradation of the green fluorescent protein during storage, 1 ml of each sample was stained with 50 µl of a 6.7 µM SYTO 9 fluorescent stain (Molecular Probes, Invitrogen, Catalogue number L7012) for 0.5 hours in the dark, before dilution in filtered saline and flow cytometric analysis. SYTO 9 is able to diffuse across the cell membrane of *Pseudomonas* sp. strain CT07 and bind to the intracellular nucleic acids. The event rate was kept below 1 500 events.s⁻¹ by selecting an appropriate cytometer flow rate and a minimum of 10 000 of the events of interest was collected for each sample by varying the collection time. The instrument settings used were those determined for *Pseudomonas* sp. strain CT07::gfp-2 as described previously and the validity of these settings was confirmed prior to sample analysis.
4.1.3.2 Direct counts

The effluent cell numbers from duplicate *Pseudomonas* sp. strain CT07::gfp-2 tube-grown biofilms were enumerated simultaneously by direct counting with the aid of fluorescent microscopy, as previously described. The results obtained from digital image analysis of each image in Scion Image for Windows (Scion Corporation, public domain NIH Image program from the U.S. National Institutes of Health, available at http://rsb.info.nih.gov/nih-image/) using digital image filtering steps as described previously (Massana et al. 1997), was compared to the results from manually counting the number of cells per image. Although manual counting was more time consuming, it provided additional qualitative information on the size of the detached particles.

4.1.4 Initial attachment efficiency and dynamics

To complement the previous experimentation, the attachment efficiency and dynamics during the initial stages of biofilm formation were investigated by sampling the effluent with greater frequency during the first 6 hours after inoculation. Three sets of triplicate glass tubes were inoculated as before, but zero-flow incubation periods of 0 and 1 hour were included in addition to the customary 0.5 hours after inoculation. The first 5 displaced reactor volumes were collected individually (0.04 hours per reactor volume), as well as one reactor volume each at 0.4, 1, 3 and 6 hours after the initiation of medium flow and the viable cell numbers enumerated.

To assess the retention time of a non-surface reactive molecule under these hydrodynamic conditions, the conservative tracer fluorescein (Sigma-Aldrich, Catalogue number F6377) was injected into the glass tubes at a final concentration of 1 mg.l⁻¹. The decline of fluorescence owing to the displacement of the fluorescein from the tubes was determined in triplicate with
a luminescence spectrometer (Perkin Elmer LS 50B, excitation at 460 nm and emission at 520 nm) at one minute intervals after the introduction of flow for a total of 12 minutes (0.2 hours).

4.2 Results

4.2.1 Planktonic cell yield from various pure culture *Pseudomonas* species biofilms

To establish that the biofilm-derived planktonic cell yield of *Pseudomonas* sp. strain CT07 reported previously (Bester et al. 2005) was not an isolated observation, limited to a particular strain or cultivation condition, the biofilm-to-planktonic cell yield from the well-characterized *P. aeruginosa* PAO1 and a *P. putida* strain was compared to that of the environmental isolate *Pseudomonas* sp. strain CT07::gfp-2 (Figure 4.1).

The various pure culture biofilms yielded ~1 x 10^6 cells per tube attachment area per hour to the effluent as early as 24 hours after inoculation. As the dilution rate exceeded the specific growth rates by at least 30 times for *Pseudomonas* sp. strain CT07::gfp-2, 19 times for *P. aeruginosa* PAO1 and 18 times for *P. putida*, the interpretation of the results is based on the assumption that planktonic growth could not contribute significantly to the effluent cell numbers; these cells were therefore produced, or yielded by surface-associated biomass.
**Figure 4.1:** The biofilm-derived planktonic cell yield from glass tube-cultivated Pseudomonad biofilms. Viable effluent cell numbers were enumerated at 24 hour-intervals and expressed as the log₁₀ of the number of cells yielded from the internal reactor surface available for colonization (cm²), per hour. The yield of cells increased as the biofilms developed and peaked at $2.0 \times 10^7$ CFU.cm⁻².h⁻¹ ± $1.8 \times 10^7$ CFU.cm⁻².h⁻¹ and $2.5 \times 10^7$ CFU.cm⁻².h⁻¹ ± $1.1 \times 10^7$ CFU.cm⁻².h⁻¹ after 96 hours for *P. putida* and *Pseudomonas* sp. strain CT07::gfp-2, respectively and at $1.2 \times 10^7$ CFU.cm⁻².h⁻¹ ± $1.0 \times 10^7$ CFU.cm⁻².h⁻¹ for *P. aeruginosa* after 120 hours of incubation. Values given are the averages of three replicates ± standard deviation.

### 4.2.2 The interaction between attached and planktonic biomass

The assumption that a cell suspension in a fixed volume flow chamber cannot resist removal by continuous flowing liquid when the dilution rates exceed the maximum rate of cell replication was tested at a macroscopic level by simultaneously quantifying suspended and attached growth with the OLAPH (Figure 4.2). A continuous inoculum of planktonic cells was provided from a newly inoculated batch culture. The gradual increase in scattered light intensity, due to suspended biomass growth in the batch culture, as well as biofilm formation on the surface of the flowcell was quantified in real time by the OLAPH.
Following the replacement of the inflowing batch culture with a sterile medium reservoir at 48 hours, the removal of unattached biomass from the flowcell due to the high dilution rate is evident from Figure 4.2. The planktonic cell load flowing into the flowcell prior to the switch consisted of $2 \times 10^8$ CFU.ml$^{-1}$ and contributed 500 arbitrary light scatter units, while the attached biomass alone accounted for 1640 units of the total measured light scatter, indicating that the suspended cells contributed significantly to the measurements under these conditions (23%).

Figure 4.2: Real-time monitoring of the interaction between suspended and attached biomass of *Pseudomonas* sp. strain CT07 with the OLAPH and spectrophotometry. Optical density (OD) measurements of flowcell effluent and batch culture samples were quantified at 600 nm.

However, the maximal yield of planktonic cells from biofilms cultivated in the parallel-plate flowcell under increasing flow velocities in subsequent experimentation did not exceed $2 \times 10^7$ CFU.ml$^{-1}$ (See chapter 5). Considering the direct correlation between the amount of biomass and the measured light scatter, it can be expected that the yielded planktonic cells did not
contribute more than 3% to the light scatter measured during the continuous flow cultivation of biofilms.

### 4.2.3 The nature and number of cells yielded during biofilm development

To investigate whether the yield of cells to the bulk-liquid (Figure 4.1) was a continuous or discrete process, composite effluent samples from triplicate glass tube-cultivated *Pseudomonas* sp. strain CT07::gfp-2 biofilms were collected at 3-hour intervals over a 96-hour period, except for the first five displaced reactor volumes, which was collected as a composite sample after 0.2 hours (Figure 4.3). Each glass tube was inoculated with $1.9 \times 10^7 \pm 0.13 \times 10^7$ cells from a pre-culture. This number was estimated by taking into account the injection volume (0.1 ml) of a cell suspension of known concentration. Assuming that the cells were evenly dispersed within the reactor volume (0.61 ml), the inoculum concentration in the glass tubes was calculated to be $3.3 \times 10^6$ CFU.ml$^{-1}$.

Approximately 35% of the inoculated cells were washed out of the tubes during the time it took to displace 5 reactor volumes (0.2 hours). Twelve hours after inoculation, a gradual increase in the effluent cell numbers was evident over the subsequent 84 hours, with no indication of discrete or transient increases in cell numbers.

The area below the graph in Figure 4.3 was computed separately for each 3-hour fraction, normalized with respect to the flow rate (ml.h$^{-1}$) and collection time (hour), and summed to determine the cumulative number of cells released during 96 hours of growth. Each glass tube biofilm, with a footprint area of 12.19 cm$^2$, produced $4.3 \times 10^9$ cells during the 96-hour incubation time, which translates to an averaged production of $3.5 \times 10^8$ cells per cm$^2$ of attachment surface over the incubation period. The total number of cells produced by the biofilm increased from $2.6 \times 10^8$ after 24 hours, to $5.4 \times 10^8$, $1.0 \times 10^9$ and $2.4 \times 10^9$ after 48, 72 and 96 hours, respectively.
Figure 4.3: The number of fluorescent events, corresponding to SYTO 9-stained *Pseudomonas* sp. strain CT07::gfp-2 single cells, enumerated with flow cytometry from the effluent of glass tube-cultivated biofilms. The values are expressed as the log$_{10}$ of the number of events per ml. Surface attachment was promoted by 0.5 hours of stagnant conditions, where after flow and collection commenced. The first collected fraction (0.2 hours) contained the first 5 displaced reactor volumes; approximately 65% of the inoculated cells remained in each glass tube at this time. The error bars represent the average ± standard deviation from measurements taken of the effluent of triplicate glass tube-grown biofilms.

The nature of the detachment events was determined by microscopic examination of filter-immobilized effluent from glass tube-grown biofilms. A good correlation ($R^2 = 0.87$) was obtained between the direct microscopy counts and the viable cell numbers enumerated by spread plating (Figure 4.4). The protocol for the enumeration of cells with microscopy was in the process of being refined, which may account for the significant difference between the direct and viable counts. Since the two methods showed similar trends, it was decided to use plate counts for all routine sampling, and microscopy for visual examination of the cells.

The majority of the cells immobilized on the filter were either single, or in the process of dividing, rather than clumps of cells contained in matrix material. Interestingly, a significant
amount of the filter-immobilized cells (29% ± 8.9%) were not single cells, but in the process of dividing. These cells were scored as one cell during the manual counting, since the two cells were not yet separated from each other, and would likely give rise to a single colony on an agar plate.

Figure 4.4: Determining the number of planktonic cells yielded from a developing biofilm with direct microscopic counts and viable cell counts. The results are expressed as the log_{10} cells per ml and showed similar trends.

4.2.4 Initial attachment efficiency and dynamics

The displacement of the conservative tracer, fluorescein, from the glass tubes was nearly complete 0.15 hours (3.8 displaced reactor volumes) after the initiation of flow, with 0% ± 0.4% of the initial concentration present in the effluent, compared with 65% of the inoculated cells remaining after 5 displaced reactor volumes (Figure 4.3). These results suggested some degree of association between the inoculated cells and the glass surface that impede the rate of cell wash-out. The subsequent experiment therefore measured biofilm-to-planktonic cell yields more frequently during the early stages of biofilm development and varied the initial
incubation period before flow was introduced to evaluate surface association of inoculated cells (Figure 4.5).

Figure 4.5: The fate of inocula and early biofilm-to-planktonic cell yield as investigated by varying the stagnant incubation time prior to the initiation of flow. The number of bacteria in the first five displaced reactor volumes (0.04 - 0.21 hours), the tenth reactor volume (0.42 hours), as well as 1, 3 and 6 hours after the initiation of continuous flow was determined and plotted as a function of time. The average values of three replicate measurements ± the standard deviation are given.

The data from Figure 4.5 indicate that the number of cells removed by liquid flow declined and stabilized at approximately $1 \times 10^2 \text{cells.mL}^{-1}$ of effluent between 1 and 3 hours after the initiation of flow. An increase in the effluent cell numbers after 6 hours strongly suggests that cell replication and planktonic cell yield by surface-associated cells had commenced. Approximately 80% of the inoculum was washed out of the glass tubes in the first 10 reactor volumes after the 0 hour incubation time, leaving in the order of $1 \times 10^5 \text{cells}$ in the glass tube.
This suggests that 2 cells should on average be attached per microscope field, when observed with a 63X objective (2.14 x 10^{-4} \text{ cm}^2 \text{ per field}), which explains why so few, if any, attached cells can be seen at the surface during the early stages of biofilm formation (< 12 hours).

### 4.3 Discussion

The ability of biofilms to produce and release significant numbers of single cells to the bulk-liquid was confirmed for two Pseudomonas model strains (*P. aeruginosa* PA01 and *P. putida*) in addition to the environmental isolate, *Pseudomonas* sp. strain CT07. The yield of cells to the environment increased as the biofilms developed, before stabilizing at \( \approx 1 \times 10^7 \text{ cells per cm}^2 \text{ of attachment surface per hour} \), approximately five days after surface colonization (strain dependent).

Direct microscopic counts confirmed that most of the released cells were either single or dividing, rather than clumps of cells contained in matrix material, which refuted the possibility that large portions of sloughed-off biofilm accounted for the majority of the observed cell yield, as reported previously for mixed-species biofilms cultivated under turbulent flow (Stoodley et al. 2001).

Continual monitoring of the cell numbers in the effluent from biofilms cultivated for 4 days revealed that the yield of planktonic cells from the surface was a continuous, rather than a discrete process and that it was initiated within 15 hours of initial surface attachment. Subsequent experimentation focussed on the first 6 hours after inoculation and monitored the number of cells in individual reactor volumes, displaced by the initiation of liquid flow. In contrast to the complete washout of the fluorescent tracer within 4 reactor volumes, approximately 80% of the inoculated cells were removed after the displacement of 10 reactor volumes (0.42 hours), when flow was initiated immediately after inoculation. This indicated that some bacteria were able to resist washout by associating with the reactor glass surface.
An increase in the number of cells in the effluent within a few hours after the initiation of flow indicated that replication of the remaining bacteria had commenced. The duration of the stagnant period prior to the initiation of flow did not seem to influence the ability of the inoculum to resist displacement from the reactor by the liquid flow.

Previous simulations carried out by Escher and Characklis (1990) modelled the expected increase in *P. aeruginosa* surface cell density as a function of inoculum cell concentration and a constant shear stress of 0.5 N.m⁻² under laminar flow. According to the model, the transport of cells from the bulk-liquid, attachment to the surface and detachment from the surface all contributed to the initial number of attached cells. Approximately 1.67 hours after the start of the simulation, cell replication and detachment became the major contributing factors to the amount of attached biomass. The rate of biofilm accumulation was found to be dependent on the initial number of attached cells and lower shear rates allowed more cells to attach in the first 1.67 hours of the simulation (Escher and Characklis 1990). The results presented here confirm these earlier simulations and further demonstrate the active role that attached biomass can play in the production and release of daughter cells to the environment.

A spate of recent publications has investigated the ‘seeding dispersal’ of freely swimming cells from within biofilm microcolonies at a discrete time during biofilm development, mostly after the microcolonies had reached steady-state dimensions (Purevdorj-Gage et al. 2005). These observations have been incorporated into the accepted 5-stage model of biofilm development as the final stage where a subset of attached cells detach from the biofilm and return to a free-floating existence, thereby completing the biofilm life cycle. Owing to the nature of the experimentation presented here, it could not be determined whether the mechanism of detachment was passive erosion of cells due to liquid shear forces or the active release of daughter cells to the environment. It is, however, unlikely that active seeding dispersion could be responsible for the effluent cell numbers observed in the first 12 - 24 hours after inoculation, since microcolony formation and maturation had not yet taken place.
Regardless of the mechanism, the requirement placed on the pioneer attached cells to produce daughter cells for surface colonization and biofilm formation, while compensating for the release of cells to the environment, constitutes an additional metabolic burden and (or) required investment of carbon and other resources. If the release of cells from the biofilm is viewed as a proliferation mechanism, then the carbon and resource investment may be offset by the potential benefits.

The fate of the detached cells is unknown; it can be speculated that these cells are free to attach to uncolonized surfaces or interact with existing biofilms elsewhere, provided that cell motility can overcome the prevailing hydrodynamic flow rates outside of the biofilm microenvironment (Korber et al. 1989). Measurements by Lawrence et al. (1987) demonstrated that a bulk-liquid flow velocity of 10 cm.s\(^{-1}\) decreased to 200 µm.s\(^{-1}\) at a distance of 0.2 µm from the solid surface and allowed single \textit{P. fluorescens} bacteria to actively migrate upstream by means of flagellar-driven motility. They also observed cells swimming in a direction perpendicular to the surface (i.e. from slower to faster moving liquid), and proposed that bacteria could utilize this as a rapid, chemotaxis-driven relocation mechanism (Lawrence et al. 1987).

The ability of these biofilm-derived planktonic cells to remain associated with the biofilm by swimming within the reduced-flow microenvironment raises interesting questions about microbial interaction in this zone, given the possible phenotypic differences between the attached and free-floating cells, and ultimately how this impacts our understanding of biofilm ecology in the environment.

Interestingly, a significant amount of the yielded cells (29% ± 8.9%) was in the process of dividing. This raised the question of whether these cells were shed from the biofilm while they were in the process of dividing, which is in disagreement with the proposed biofilm proliferation mechanism where an attached cell elongates and releases a daughter cell to the bulk-liquid upon division. An alternative source of actively dividing, free floating cells could be
located in surface-associated zones with sufficiently reduced flow to allow independent planktonic cell replication, a scenario that could be explored using mathematical modelling.

4.4 Conclusions

4.4.1 Pure culture biofilms can yield significant numbers of planktonic cells to the environment.

4.4.2 Single cells are produced and released as early as 6 hours after initial surface colonization and the number of cells yielded increased in conjunction with biofilm development.

4.4.3 The detachment of single cells described in the early stages of biofilm formation is distinct from the seeding dispersal phenomenon.

4.4.4 An amendment to the existing biofilm life cycle is proposed where detachment is not restricted to the final stage of development, but instead occurs continuously throughout biofilm development.

4.5 List of symbols and abbreviations

~ approximately

°C degrees Celcius

D dilution rate (h⁻¹)

mg milligram

h hour

l litre
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<td>ml</td>
<td>millilitre</td>
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<tr>
<td>gfp</td>
<td>green fluorescent protein</td>
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<td>(m/m)</td>
<td>mass in mass</td>
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<td>OD</td>
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<td>OLAPH</td>
<td>optical large area photometer</td>
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CHAPTER 5  

Environmental influence on planktonic cell yield*

The results presented in chapter 4 indicated that biofilm-derived planktonic cell yield is correlated to biofilm development for a number of Pseudomonad species. Since biofilm development is influenced by various environmental factors, the results outlined here evaluate whether planktonic cell yield was similarly influenced by environmental carbon concentration, bulk-liquid flow velocity and the presence of an oxidizing antimicrobial (second specific research objective). The effect of changes in the concentration of other nutrients, such as nitrogen or phosphate, was not investigated in this study. While it is likely that these nutrients will play a role in sessile-planktonic interactions, the bulk of the literature pertains to the influence of carbon source and concentration on biofilm systems and techniques developed and utilized by our group are furthermore most suited to carbon measurements.

5.1  Experimental procedures

5.1.1  Effects of nutrient type and concentration on the biofilm and biofilm-to-planktonic cell yield

The OLAPH and glass tubes were used to compare biofilm development and the associated

*Published in Canadian Journal of Microbiology, 2009, Vol.55, p.1195-1206, NRC Research Press Journals (except Figure 5.7).
planktonic cell yield by *Pseudomonas* sp. strain CT07 when grown on 1 and 5 mM citrate (n = 2) and by *P. aeruginosa* PAO1 on 1 mM citrate (n = 2). The routine experimentation into biofilm-derived cell yield, carried out with single bacterial isolates, cultivated on a defined growth medium with a single carbon source was complemented with a complex medium (TSB) and an undefined multispecies microbial community, isolated from 1 gram of soil. Planktonic cell yield from triplicate, glass tube-cultivated *Pseudomonas* sp. strain CT07, *P. aeruginosa* PAO1 and the undefined microbial soil community biofilms was measured when cultivated on either 0.30 or 3.0 g.l\(^{-1}\) of TSB at room temperature for 4 days. Viable effluent cell numbers from these biofilms were enumerated at 24-hour intervals by serial dilution and plating on the corresponding growth medium plates.

### 5.1.2 Effect of flow rate on the biofilm and biofilm-to-planktonic cell yield

*Pseudomonas* sp. strain CT07 biofilms were established in eight separate glass tubes at a liquid dilution rate of either 0.15 or 17 h\(^{-1}\). From 72 hours onwards, the medium dilution rate was increased incrementally from 0.15 h\(^{-1}\) to 0.50, 1.7, 4.9, 9.2, 15 and 17 h\(^{-1}\) at 12-hour intervals or decreased from 17 h\(^{-1}\) in the reverse order, to vary the liquid flow rate in the tubes. Before each change in flow velocity, the effluent from three tubes was sampled to enumerate the number of viable cells with serial dilution and spread plating and one glass tube was sacrificed to visualize biofilm formation with the LIVE/DEAD BacLight Bacteria viability kit (Molecular Probes, Invitrogen, Catalogue number L7012) and confocal laser scanning microscopy (CLSM) using a Zeiss LSM 510 microscope. A working solution of BacLight was prepared by mixing 2 µl each of the SYTO 9 dye (3.34 mM) and Propidium iodide (20 mM) in 1 ml Milli-Q water, and storing it at -20°C. Two hundred µl of the working stock was injected into each glass tube with
arrested flow and incubated for 20 minutes in the dark, before CLSM imaging using a 650 nm long pass and a 505 to 530 nm band pass filter in two different channels.

The effect of an increase in flow rate on the biofilm and the associated planktonic cell yield was subsequently investigated with the OLAPH in a separate experiment. To allow for better comparison between glass tube-cultivated biofilms and biofilm quantification with the OLAPH, the Perspex parallel plate flowcell used for previous experimentation was replaced by a flowcell with glass as the attachment surface for this experiment only. The modified flowcell had an internal volume of 12.6 ml and glass attachment area of 42.0 cm². A Pseudomonas sp. strain CT07 biofilm was cultivated for 72 hours on 1 mM citrate at a dilution rate of 0.16 h⁻¹ prior to subsequent 12-hourly increases in the dilution rate to 0.51, 1.9, 18 and 32 h⁻¹. Effluent samples were collected at 24-hour intervals until 72 hours after inoculation, and thereafter before each increase in flow rate to enumerate the cell numbers. To determine how the medium flow rate influenced the availability of the main carbon source in the flowcell, the residual citrate concentration in the effluent was determined with a spectrophotometric citric acid enzymatic assay (Enzytec™ citric acid assay, Scil Diagnostics GmbH, Germany, Catalogue number 1214). Sterile medium and effluent samples were filtered (0.2 µm pore size) to remove the cells and frozen at -20°C until analyses of duplicate samples at 340 nm, using citric acid as standard.

In a follow-up experiment, higher dilution rates were applied to glass-tube cultivated biofilms. Triplicate glass tubes were inoculated and subjected to a dilution rate of 25.1 h⁻¹ for 72 hours, before 12-hourly effluent sampling and incremental increases in the dilution rate to 74.1, 101, 201, 394 and 738 h⁻¹.
5.1.3 Effect of antimicrobial treatment on the biofilm and biofilm-to-planktonic cell yield

The response of both the biofilm and the biofilm-derived cell yield to an adverse environmental condition, in the form of an antimicrobial, was investigated with the OLAPH. A commercial bleach solution, containing sodium hypochlorite as the active ingredient (final concentration of approximately 5.25% NaClO) was selected for the antimicrobial challenge. The applied working concentration was selected after determining the minimum inhibitory concentration (MIC) against planktonic *Pseudomonas* sp. strain CT07. The planktonic MIC was established by inoculating test tubes containing serial dilutions of the bleach stock solution in 5 mM citrate medium and incubating it overnight with shaking. The minimum concentration of bleach at which no growth was observed (in triplicate test tubes) was determined to be a 1 in 10 000 dilution of the original bleach solution (~0.0000525% NaClO).

The flowcell was inoculated with *Pseudomonas* sp. strain CT07 as described previously and supplied with defined growth medium containing 5 mM citrate. The changes in light scatter were logged at 5-minute intervals with the OLAPH and the viable effluent cell numbers enumerated every 24 hours and after each antimicrobial challenge. After 96 hours of incubation, the medium reservoir was replaced with medium containing commercial bleach at a final concentration of 10 times the MIC (0.000525% NaClO) and allowed to flow through the flowcell for 2 hours, before switching the inflow back to sterile medium only. The biofilm was challenged for a second time after 120 hours of incubation; this time with 1 000 times the MIC (0.0525% NaClO) for a 1-hour period, before switching back to sterile medium without antimicrobial for the duration of the experiment.
5.2 Results

5.2.1 Effects of nutrient type and concentration on the biofilm and biofilm-to-planktonic cell yield

The effect of a five-fold increase in citrate concentration in a defined growth medium on Pseudomonas sp. strain CT07 biofilm development (Figures 5.1.1 and 5.1.2) as well as biofilm-derived-planktonic cell yield (Figure 5.1.3) was assessed. The OLAPH measured an initial decrease in scattered light intensity after the initiation of flow, owing to the washout of the unattached cells from the inoculum. This was followed by a lag in the development of the biofilm that varied in duration, namely between 10 - 20 hours for 1 mM citrate, and between 20 - 30 hours for 5 mM citrate as the sole carbon source. The lag phase was followed by an exponential increase in biomass at the attachment surface and finally a quasi steady-state, which typically showed minor fluctuations in the amount of attached biomass, likely due to sloughing events.

The greater yield of cells from 24 hour-old glass tube-cultivated biofilms, supplied with 1 mM citrate, compared with biofilms of similar age cultivated on 5 mM citrate (Figure 5.1.3), is consistent with the shorter lag phase in biofilm development on 1 versus 5 mM citrate (Figures 5.1.1 and 5.1.2). The cell yield from the various biofilms became more consistent with age, presumably due to the attainment of steady-state dimensions.
1: Biofilms on 1 mM citrate

2: Biofilms on 5 mM citrate

3: Biofilm-to-planktonic cell yield on 1 and 5 mM citrate

Figure 5.1: The effect of a five-fold increase in citrate concentration on *Pseudomonas* sp. strain CT07 biofilm development and biofilm-derived planktonic cell yield. The photometric measurement of biofilm development in the parallel-plate flowcell on (1) one mM citrate (n = 2) and (2) five mM citrate (n = 2) show that the individual biofilms develop at different rates, which may account for the larger standard deviations in (3) the biofilm-derived cell yield (n = 2) from pre-steady-state biofilms cultivated in glass tubes.
The relationship between biofilm development and planktonic cell yield was investigated in a similar manner for *P. aeruginosa* PAO1 on 1 mM citrate (Figure 5.2).

1: Biofilms on 1 mM citrate

2: Planktonic cell yield on 1 mM citrate

**Figure 5.2:** *P. aeruginosa* PAO1 biofilm development and planktonic cell yield when supplied with 1 mM citrate medium. (1) Biofilm dynamics as determined with the OLAPH (n = 2) and (2) planktonic cell numbers yielded to the effluent from glass tube cultivated biofilms (n = 2).

Similar heterogeneity in biofilm formation dynamics was evident from the quantification of light scatter data from duplicate biofilms, where steady-state biofilms were established approximately 60 hours after inoculation. Planktonic cell yield from the glass tube-cultivated biofilms increased steadily as the biofilm developed and stabilized after 120 hours.

Pure culture biofilms of *Pseudomonas* sp. strain CT07, *P. aeruginosa* PAO1, as well as a multispecies microbial community, were also cultivated in triplicate glass tubes with different
concentrations of TSB. Similar to the results presented previously (Figures 5.1, 5.3 and 5.5) significant numbers of cells (1 x 10^3 to 1 x 10^6 CFU.cm^{-2}.h^{-1}) were yielded from single or multispecies biofilms as early as 24 hours after inoculation (Figure 5.3). Cell yields typically increased and reached a steady-state within 96 hours. A ten-fold increase in the TSB concentration consistently resulted in a larger yield of planktonic cells from the biofilm to the bulk-liquid during the early stages of biofilm formation. The multispecies biofilm (Figure 5.3.3) not only yielded significantly greater numbers of cells during the early stages of development, but also colonized the glass surfaces of the tubes to a greater extent than the pure cultures did. Significant macroscopic growth was evident after only 24 hours, while pure culture biofilms only became noticeable after 48 to 72 hours of cultivation.

5.2.2 Effect of flow rate on the biofilm and biofilm-to-planktonic cell yield

The influence of changes in growth medium flow rate (expressed in terms of the dilution rate D, h^{-1}) on surface colonization and the associated planktonic cell yield were investigated in glass tubes in three independent experiments (Figures 5.4 and 5.5), and the first of these experiments was repeated in the OLAPH with similar increases in dilution rates (Figure 5.6).

The various growth medium flow rates applied resulted in a range of dilution rates that were lower (0.3 times) and significantly greater (1 480 times) than the maximum planktonic specific growth rate of *Pseudomonas* sp. strain CT07; details on volumetric flow rates, Reynolds number and dilution rates are given in Appendix 1. The parameters were calculated for un-colonized flow chambers and the effects of biofilm formation on these values are therefore unknown.
1: *Pseudomonas* sp. strain CT07

2: *P. aeruginosa* PAO1

3: Mixed community

**Figure 5.3:** The response of biofilm-derived planktonic cell yield to different concentrations of the complex growth medium TSB. The viable effluent cell numbers from triplicate glass tube-cultivated biofilms of (1) *Pseudomonas* sp. strain CT07, (2) *P. aeruginosa* PAO1 and (3) a multispecies soil-derived inoculum were enumerated as described previously (n = 3).

The results from the first two experiments are presented in Figure 5.4.1. Under conditions of very low dilution rates (0.17 to 1.7 h⁻¹) comparatively low cell numbers were enumerated from
the effluent, even though planktonic cell replication could theoretically have contributed to the measurements at dilution rates below 0.50 h\(^{-1}\) (dilution rate \(\leq \mu_{\text{max}}\) planktonic). It is therefore likely that carbon and (or) oxygen limitation resulted in a lower cell yield by restricting biofilm development and metabolic activity. This supposition is supported by notably similar numbers of cells yielded at these low dilution rates, even from biofilms of different ages and cultivation histories (the age of the biofilm was 120 and 168 hours respectively for the “Increase” and “Decrease” in flow rate experiments at the dilution rate of 1.7 h\(^{-1}\) in Figure 5.4.1).

Surface attachment of individual cells with few three-dimensional biofilm structures could be identified during the early stages for the Increase in flow rate experiment (Figure 5.4) and no micrographs are therefore shown. Micrograph A was taken after 132 hours of biofilm growth (dilution rate of 15.3 h\(^{-1}\)). Surface coverage was patchy, while some three-dimensional biofilm formation with a maximum thickness of approximately 35 \(\mu\)m was observed.

Biofilms subjected to a dilution rate of 17.2 h\(^{-1}\) increased slightly in maximal depth (to 39 \(\mu\)m) after 144 hours (micrograph B). Actively motile bacteria were seen swimming among the microcolonies and if the focal plane was adjusted to the planktonic phase of the tube, large numbers of mostly single cells could be seen flowing past the field of view in the same direction as the bulk-liquid flow. In contrast, biofilm development under a gradual decrease in flow rate did not show a similar progression. A 96 hour-old biofilm reached a maximal thickness of 19 \(\mu\)m when cultivated at a dilution rate of 9.2 h\(^{-1}\) (micrograph C of a 96 hour-old biofilm). Surface colonization was sparse, with a limited number of pillar-like structures protruding from the surface. As the liquid flow rate was decreased to dilution rates of 4.9 h\(^{-1}\) (a 108 hour-old biofilm) and 0.15 h\(^{-1}\) (a 144 hour-old biofilm) (micrographs D and E), no significant changes in structural complexity could be discerned.
1: Lower range of dilution rates

Figure 5.4: The relationship between bulk-liquid dilution rate and planktonic cell yield as investigated for glass tube-biofilms cultivated on 1 mM citrate medium. A positive trend was observed at lower dilution rates (1), whereas yields remained relatively stable at dilution rates exceeding 15.3 h\(^{-1}\) (1 and 2). (1) Increase: biofilms were established for 72 hours at a dilution rate (0.2 h\(^{-1}\) < \(\mu_{\text{max}}\) planktonic (0.49 h\(^{-1}\)) to facilitate the presence of an independently replicating planktonic population, followed by 12-hourly increases in dilution rates equaling or exceeding \(\mu_{\text{max}}\). (1) Decrease: biofilms were established at a dilution rate (20.2 h\(^{-1}\) > \(\mu_{\text{max}}\)
planktonic for 72 hours before 12-hourly decreases in dilution rates. Three independent biofilms were established at a dilution rate of 25.1 h$^{-1}$ for 96 hours before more significant increases in the flow rate were applied at 12-hour intervals.

**Incremental flow increase**

A (132 h)  B (144 h)

**Incremental flow decrease**

C (96 h)  D (108 h)  E (144 h)

**Figure 5.5:** CLSM micrographs of *Pseudomonas* sp. strain CT07 biofilms cultivated in the glass tubes at various bulk-liquid flow rates.
Biofilm formation was typically more extensive in the inner corners of the tubes, presumably due to lower shear forces in these areas (Figure 5.5). It is not possible at this time to determine from which part of the tube the planktonic cells were yielded; as a result, it is unknown whether the biofilms attached to the tube corners contributed more to the observed yield under increased flow velocities than the biofilms growing on the tube walls.

The first increase in flow velocity experiment (Figure 5.4) was therefore repeated with the OLAPH (Figure 5.6), where the larger biofilm attachment area of the flowcell would likely reduce the relative contribution of cells yielded from the biofilms growing in the shear-protected corners. In addition, a quantitative measure of biofilm development as well as carbon utilization in the flowcell could be obtained. Similar to the glass tube-cultivated biofilms in Figure 5.4.1, a minimal amount of biomass accumulated at the dilution rate below that of $\mu_{\text{max}}$ ($0.16 \text{ hour}^{-1} < \mu_{\text{max planktonic}}$) applied during the first 72 hours and likely consisted of both an attached and a planktonic cell population (Figure 5.6.1). Carbon utilization peaked at 72 hours, with only $8.2\% \pm 0.1\%$ of the inflowing citrate remaining in the effluent (Figure 5.6.2). Subsequent increases in the flow rate resulted in significantly higher concentrations of excess carbon in the effluent and correlated with more extensive biofilm development.

Interestingly, the yield of planktonic cells to the effluent (Figure 5.6.1) seemed to reach a plateau between 84 and 96 hours ($1.98 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1} \pm 0.43 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1}$ to $1.78 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1} \pm 0.35 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1}$) whereas the attached biomass increased exponentially (Figure 5.6.1). When the biofilm reached a steady-state thickness, the planktonic cell yield increased to $7.63 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1} \pm 0.77 \times 10^6 \text{ CFU.cm}^{-2} \cdot \text{h}^{-1}$. It is likely that the detection limit of the photometer was reached at this point with subsequent underestimation of further biofilm accumulation. This suggests that prior to reaching a steady-state, the biofilm channelled relatively more resources into attached cell and EPS production, while still
allocating some carbon to maintain a base yield of $1 \times 10^6$ CFU.cm$^{-2}$.h$^{-1}$ to the environment. Once the biofilm established its optimal dimensions with respect to the prevailing environmental conditions, it responded to the increased availability of carbon with greater planktonic cell yield to the bulk-liquid. However, in the absence of more frequent sampling of the yielded cell numbers, this hypothesis remains to be tested.

The potential contribution of the yielded planktonic cells to the scattered light measured by the OLAPH was taken into account and found to be minimal as detailed previously in chapter 4. A positive correlation ($R^2 = 0.86$) was obtained between the yield ($\log_{10}$ CFU.cm$^{-2}$.h$^{-1}$) from the glass tubes (Figure 5.5) and that of the OLAPH flowcell (Figure 5.6) for the following dilution rates; 0.15, 0.50 (0.49 for flowcell), 1.7 (1.8 for flowcell) and 17 h$^{-1}$. This indicates that the biofilm grown in the significantly larger OLAPH flowcell responded in a similar manner as glass tube-cultivated biofilms to changes in flow rate, even though the hydrodynamic properties (e.g. wall shear rate) certainly differed, suggesting that increases in shear rate at these relatively low values may not have a significant effect on cell yield from the biofilm. This is contrary to the prevailing assumption that detachment or erosion of single cells from the biofilm is due to the shear forces exerted by liquid flowing over the biofilm surface.

The results presented here indicate that carbon, and likely oxygen availability played a more prominent role in not only biofilm development, but also planktonic cell production by the biofilm, likely by governing the metabolic activity of the attached cells.
1: Biofilm biomass and planktonic cell yield

![Graph showing biofilm biomass and planktonic cell yield](image)

2: Effect of dilution rate on substrate utilization

![Graph showing effect of dilution rate on substrate utilization](image)

**Figure 5.6:** The relationship between biofilm development, biofilm-to-planktonic cell yield, dilution rate and carbon utilization. **(1)** The biofilm was cultivated for 72 hours at a low dilution rate (0.15 h\(^{-1}\)) prior to the initiation of 12-hourly increases in the flow rate, with concurrent enumeration of biofilm-derived planktonic cell numbers. **(2)** Once the dilution rate exceeded \(\sim 4\) times the \(\mu_{\text{max}}\) planktonic (dilution rate of 1.9 h\(^{-1}\)) the availability of carbon (represented by the unutilized citrate in the effluent) increased significantly. Beyond this
point, where nutrient and likely oxygen limitation were alleviated, biofilm development showed an immediate response and released more cells to the bulk-liquid.

5.2.3 **Effect of an antimicrobial agent on the biofilm and biofilm-to-planktonic cell yield**

The hypothesis that the yield of planktonic cells from the biofilm is closely linked to the physiological status of the biofilm was tested by challenging a mature biofilm with an oxidizing antimicrobial and monitoring the effect on both the attached biomass and the biofilm-derived planktonic cell numbers. Similar to observations made in other experiments, biofilm-derived cell yield to the bulk-liquid stabilized once the biofilm biomass reached a *quasi* steady-state (Figure 5.7).

The first antimicrobial addition (10x MIC) at 96 hours did not result in the removal of the biofilm or a reduction in the number of planktonic cells yielded to the effluent. The second addition of an increased concentration of the antimicrobial (1000x the MIC) at 120 hours not only succeeded in removing a portion of the biofilm, but also halted the production and release of cells to the bulk-liquid. Subsequent biofilm recovery was accompanied by the re-establishment of planktonic cell production. The extent of biofilm accumulation after the second antimicrobial challenge was significantly greater than the previous steady-state, while the suspended cell yield returned to similar values as before. While it is only possible to speculate about the nature and composition of the biofilm after the antimicrobial challenge, the biofilm may have responded by increasing EPS production as a protective measure against future challenges.
Figure 5.7: The response of a *Pseudomonas* sp. strain CT07 biofilm to a challenge with an oxidizing antimicrobial. Changes in biofilm structure were quantified by measuring the intensity of scattered light with the OLAPH, while the biofilm-to-planktonic cell yield was enumerated from the effluent with spread plates. The biofilm reached a semi steady-state approximately 72 hours after inoculation. The first bleach challenge took place after 96 hours of incubation, with a final concentration of 10x the MIC for a period of 2 hours. Neither the biofilm, nor the cell yield (at 98 hours) was significantly affected by this challenge. A second challenge was applied at 120 hours, this time with 1 000x the MIC for a duration of 1 hour. This challenge removed a significant portion of the attached biomass and reduced the planktonic cell yield at 121 hours to zero (this data point was therefore not included).

5.3 Discussion

In the previous chapter it was shown that biofilm-to-planktonic cell yield is correlated to biofilm development. Since it is known that biofilm development, structure and activity are influenced by various environmental factors, a secondary hypothesis was formulated; namely
that the biofilm-derived planktonic cell yield will likewise be dependent, likely indirectly, on environmental conditions.

The relationship between biofilm development and planktonic cell yield, in addition to the effect of an increase in carbon concentration on both, can be seen from the data presented in Figure 5.1.3. The number of cells released from biofilms supplied with 1 and 5 mM citrate continued to increase up to 96 hours after inoculation before it levelled off, which is a significant time after the respective biofilms (Figures 5.1.1 and 5.1.2) had reached a semi steady-state with respect to the amount of biomass. It is also interesting to note that the planktonic cell yield from biofilms, supplied with different concentrations of carbon, stabilized at similar levels (3.0 x 10⁷ CFU.cm⁻².h⁻¹), whereas the average amount of steady-state biomass of the 1 mM citrate biofilms exceeded that of the 5 mM biofilms (1600 vs. 1300 arbitrary units of scattered light). The difference in the amount of biofilm biomass may be indicative that the availability of another essential nutrient, likely oxygen, was limited at the higher carbon concentration. The observation that biofilms composed of different amounts of biomass yielded a similar number of cells has implications for a common simplification employed in mathematical models of fluid shear-mediated erosion of single cells from the biofilm. Chambless et al. (2007) and several other modelling studies assumed that the rate of cell detachment is proportional to the square of the thickness of the biofilm, to achieve a steady-state where biofilm growth is balanced by detachment. The results presented here dispute the validity of this assumption and indicate that factors other than the amount of biomass at the surface or the thickness of the biofilm are more important determinants of planktonic cell yield. This clearly warrants further investigation.

Another important environmental factor that influences biofilm dynamics is the characteristics of the bulk-liquid flow. Fluorescent particle tracking with CLSM has provided valuable insight into liquid flow within and above biofilms. Stoodley et al. (1994) observed the flow of particles
over and around biofilm microcolonies and even against the direction of the laminar bulk-liquid flow (Stoodley et al. 1994). The average flow velocity in the biofilm was only half of what was measured for uncolonized surfaces and was moreover found to be dependent on the bulk-liquid velocity (De Beer et al. 1994). The precise effect of bulk-liquid hydrodynamics on biofilm development and structure is, however, difficult to define because biofilm formation at a solid surface will in turn impact on the flow hydrodynamic characteristics.

It is well known that hydrodynamic and mass boundary layers exist above the biofilm surface in a flowing environment (Lewandowski and Beyenal 2007). The hydrodynamic boundary layer is composed of a gradient of liquid flow velocities, with maximal flow rates in the bulk-liquid and slower flow rates near the biofilm surface. A mass (or concentration, or diffusion) boundary layer is contained within the hydrodynamic boundary layer. The mass boundary layer is the result of reduced flow velocities near the biofilm surface as well as biofilm activity, such that mass transport becomes diffusion dominated, in contrast to convective transport in the bulk-liquid. Greater bulk velocities will decrease the thickness of the hydrodynamic boundary layer, thereby reducing the distance across which nutrients must diffuse to reach the biofilm surface, but simultaneously enhancing the shear and deformation forces acting on the biofilm and increasing the probability of biofilm detachment.

In recent investigations, detachment from biofilms grown in an annular reactor was found to increase rapidly in response to a step-increase in shear stress, both in the number of detached particles and particle volumes, followed by a return to pre-disturbance steady-state detachment rates, despite continued exposure to the higher shear stress (Choi and Morgenroth 2003). The authors concluded that low shear forces resulted in the formation of erosion-dominated biofilms with reduced density, which rendered the biofilm more susceptible to sloughing under higher shear forces. The authors did not, however, comment on the similar rates of detachment observed for lower and higher shear forces (1.1 and 3.1
N.m$^{-2}$) even though detachment from the biofilm was explicitly ascribed to fluid velocity-mediated erosion.

Similarly, the empirical analysis of drinking water biofilms cultivated in another rotating annular reactor was conducted in combination with predictive modelling of growth and mass balance equations (Tsai 2005). Biofilms were cultivated under three different shear forces (0.070, 0.17 and 0.29 N.m$^{-2}$) while monitoring the number of cells in the biofilm as well as the liquid phase. It was found that the higher shear forces reduced the amount of attached biomass ($10^3 - 10^4$ CFU.cm$^{-2}$ at 0.29 N.m$^{-2}$, versus $10^5 - 10^7$ CFU.cm$^{-2}$ at 0.07 - 0.17 N.m$^{-2}$), while the number of free floating cells in the bulk-liquid increased slightly. Since the dilution rate in the system was insufficient to support planktonic cell replication in the bulk-liquid, it can be concluded that these cells were produced by the biofilm. The modelling results indicated that an increased shear rate resulted in an increase in the net specific biofilm growth rate and a similar increase in the specific rate of detachment from the biofilm.

Fluctuations in the rate at which the bulk-liquid flows over the biofilm certainly occur in natural environments and will therefore have an impact on the structure and activity of a newly developing or established biofilm through physical forces, as well as nutrient availability. The dependence of microenvironment substrate availability on bulk-liquid flow velocities was demonstrated previously for Pseudomonas fluorescens biofilms (Caldwell and Lawrence 1986) that became growth limited after rapid substrate depletion of 100 mg.l$^{-1}$ glucose in the absence of liquid flow, whereas a higher glucose concentration (1 g.l$^{-1}$) was sufficient to sustain biofilm growth even in the absence of flow.

In the experiments described here, the biofilms cultivated initially under lower flow velocities were thicker and more extensive than those cultivated at higher velocities. This has been observed previously for microbial biofilms cultivated in streamside flumes, which simulated
biofilm development in natural streams under slow and fast flows (Battin et al. 2003). They reported an enlarged biofilm surface area under slow flow and attributed this to an adaptive mechanism that facilitates greater mass transport of nutrients from the bulk-liquid to the biofilm.

No significant correlation was observed between the comparatively low fluid shear stresses applied in this study and the number of cells yielded by the biofilm. Biofilm formation was typically more extensive in the inner corners of the glass tubes, presumably because of lower shear forces in these areas. It was not possible to determine from which part of the tube the planktonic cells were yielded; therefore, we cannot comment on whether the biofilms attached to the tube corners contributed more to the observed yield under increased flow velocities than the biofilms growing on the tube walls. The first increase in flow velocity experiment (Figure 5.5) was therefore repeated with the OLAPH (Figure 5.7), where the larger biofilm attachment area of the flowcell would likely reduce the relative contribution of yielded cells from biofilms growing in the shear-protected corners; however, the results were similar when compared as cell yield per overall biofilm footprint area. This suggests that factors other than fluid shear, such as substrate loading rates of carbon and oxygen, and thus indirectly biofilm growth rate, played a more important role in the number of cells yielded by the biofilm.

As early as 1990, Bakke et al. (1990) stated that in environments where substrate availability is dependent on flow velocity, the reported correlation between flow velocity (i.e., shear) and erosion from the biofilm may obscure the influence of substrate loading and active biofilm growth on detachment. The analysis of empirical data and detachment models have also suggested that detachment rates are more dependent on biofilm growth rates or substrate utilization rates than shear-related removal forces (Peyton and Characklis 1992; Stewart 1993). The relative importance of flow velocity or substrate loading on biofilm structure and activity has indeed been debated, apparently without reaching a consensus (Van Loosdrecht et al.
1997; Wimpenny and Colasanti 1997a; Wimpenny and Colasanti 1997b). The impact of carbon availability on biofilm activity, structure and planktonic cell yield was subsequently investigated as detailed in chapter 6.

The hypothesis that biofilm-derived cell yield is indirectly influenced by the prevailing environmental conditions is further supported by evidence that an antimicrobial agent not only removed a portion of the biofilm, but also reduced the yield from the biofilm. The subsequent increase in planktonic cell yield was directly correlated to the recovery of the biofilm in the absence of the antimicrobial challenge. It is speculated that a particular region of the biofilm produces most of the biofilm-derived planktonic cells; this region is hypothesized to be metabolically active and located at the biofilm bulk-liquid interface. This location would allow for optimal access to nutrients and oxygen, the removal of metabolic waste products and release of daughter cells, but also be the first to be affected by the presence of an antimicrobial agent. To this end, the metabolic activity of various regions of the biofilm and the correlation of activity with planktonic cell yield was investigated in chapter 7.

5.4 Conclusions

5.4.1 The number of planktonic cells yielded increased in conjunction with biofilm development and levelled off after the biofilm reached a steady-state.

5.4.2 The dynamics of biofilm development and biofilm structure, as well as the extent of biofilm-to-planktonic cell yield are influenced by environmental parameters.

5.4.3 Greater carbon availability generally allows larger cell yield from developing biofilms. Upon achievement of a steady-state biofilm, the excess carbon no longer provides an advantage.
5.4.4 The bulk-liquid flow velocities applied here influenced the biofilm and biofilm-derived cell yield not through erosive action due to liquid shear, but rather by limiting mass transport of nutrients to the biofilm surface.

5.4.5 Partial biofilm killing and removal, due to the action of an oxidizing antimicrobial agent, significantly reduced the yield of cells to the environment. A resumption of planktonic cell yield was dependent on biofilm recovery.

5.5 List of symbols and abbreviations

- approximately
- CFU colony forming units
- °C degrees Celsius
- D dilution rate
- g grams
- h hour
- < less than
- ≤ less than or equal to
- l litre
- ml millilitre
- µl microlitre
- m² square metre
- cm² square centimetre
µm micrometre
nm nanometre
mM millimolar
N Newton
n number
% percentage
± plus and (or) minus
x times

NaClO sodium hypochlorite
et al. and others
CLSM confocal laser scanning microscopy
µmax planktonic maximum planktonic specific growth rate
MIC minimal inhibitory concentration
OLAPH optical large area photometer
sp. species
TSB tryptic soy broth
i.e. that is
CHAPTER 6

Influence of carbon availability on biofilm structure, activity and planktonic cell yield*

The results outlined in chapter 5 indicated that environmental conditions, including carbon and likely oxygen availability, influenced not only biofilm development, but also the magnitude of biofilm-derived planktonic cell yield. While the reported response of biofilms exposed to carbon-limited conditions varies widely, the associated effect on biofilm-derived planktonic cell yield has not been investigated. Based on these observations, it was hypothesized that planktonic cell yield by biofilms is an active process, closely correlated to biofilm metabolic activity, which is in turn influenced by carbon availability, amongst other factors (second specific research objective). To test this hypothesis, changes in biofilm activity, architecture and planktonic cell yield were monitored with conventional methods (i.e., continuous flowcells for confocal scanning laser microscopy and the enumeration of viable effluent cell numbers) in combination with a novel CO$_2$ evolution measurement system (CEMS), recently developed to monitor in situ biofilm respiration rates as an indicator of metabolic activity (Kroukamp and Wolfaardt, 2009). The CEMS has the added advantage of lending itself towards performing carbon mass balances, which can provide an indication of how inflowing carbon is allocated towards establishing and maintaining a biofilm, in addition to yielding planktonic cells to the effluent.

*The results presented in this chapter have been accepted for publication by the Journal of Applied Microbiology.
6.1 Experimental procedures

Biofilms were cultivated in two types of continuous flow systems in a once-through mode, namely (i) flowcells for microscopy and (ii) a CO₂ evolution measurement system (CEMS) as developed by Kroukamp and Wolfaardt (2009). The growth medium flow rate applied for both systems was 15.3 ml.h⁻¹.

6.1.1 Conventional flowcells

Biofilms were cultured in multiple flow chambers of conventional Plexiglas flowcells with modified dimensions of 5.0 mm x 6.0 mm x 33 mm (width, depth and length) and volume of 1.0 ml. The flowcells were prepared and disinfected as described previously (Chapter 3).

6.1.2 The carbon dioxide evolution measurement system (CEMS)

Each CEMS consisted of an inner silicone tube (ID = 1.6 mm, OD = 2.4 mm, Length = 1500 mm, volume = 3 ml) as the biofilm attachment surface, completely encased by and outer Tygon tube (ID = 4.8 mm, OD = 7.9 mm). Biofilms were cultivated in the silicone tube with a continuous, once-through flow of nutrient medium supplied by a peristaltic pump. The silicone tubing is significantly more permeable to O₂ and CO₂ than the Tygon tubing (the permeability coefficients of the silicone tubing for the two gases are 50 and 200 times, respectively, greater than that of the Tygon tubing) and thereby facilitates the diffusion of accumulated dissolved CO₂ in the bulk-liquid of the silicone tube (due to biofilm metabolic activity) across the silicone tube wall and into the annular space between the two tubes. The annular space was connected to a compressed CO₂-free air supply (TOC grade, CO₂ < 0.5 ppm, CO < 0.5 ppm, O₂ =
20 – 22% and THC < 0.1 ppm, Linde, Canada), which provided the sweeper gas to carry the CO\textsubscript{2} to a non-dispersive, infrared LI-820 CO\textsubscript{2} gas analyzer (LI-COR Biosciences, Nebraska) with the gas flow rate controlled by a peristaltic pump (F\textsubscript{g} = 1400 ml.h\textsuperscript{-1}). The CEMS tubes were immersed in a heating/cooling water bath set at a constant temperature of 23°C. A diagram of the setup is given in Figure 6.1.

Kroukamp and Wolfaardt (2009) measured the gaseous CO\textsubscript{2} concentrations for various known concentrations of dissolved carbon dioxide (0% to 4% (v/v) of a dissolved CO\textsubscript{2} solution at room temperature) flowing through the CEMS and showed that measurements in the gaseous phase provided a reliable indication of the dissolved CO\textsubscript{2} concentration (R\textsuperscript{2} = 0.999). The measured concentrations during the subsequent biofilm experimentation were moreover found to be within this range (Kroukamp and Wolfaardt 2009). The CO\textsubscript{2} produced by microbes is excreted in a dissolved form (CO\textsubscript{2}\text{aq}) into the bulk-liquid phase (Jones and Greenfield 1982) where it can be transported to the gas phase CO\textsubscript{2}\text{(g)} or converted into bicarbonate ions (HCO\textsubscript{3}\textsuperscript{-}) and eventually carbonate ions, depending on the ion concentration, pH and temperature of the liquid (Frahm et al. 2002).

### 6.1.3 Disinfection, inoculation and culture conditions

The flow reactors were connected to the growth medium reservoirs and waste collection flasks with silicone tubing (ID = 1.575 mm). After assembly, the systems were disinfected with a commercial bleach solution (final concentration 0.525% sodium hypochlorite) for 2 hours, followed by overnight flushing with sterile dH\textsubscript{2}O. Prior to inoculation, the dH\textsubscript{2}O was replaced by sterile medium.
**Figure 6.1:** A diagram of the two continuous-flow systems used for biofilm cultivation; namely conventional flowcells and the CEMS (Kroukamp and Wolfaardt 2009).

Each CEMS and flowcell chamber was inoculated aseptically with 1.0 and 0.1ml, respectively, of a *Pseudomonas* sp. strain CT07::gfp-2 pre-culture using a sterile needle and syringe. The inoculum was allowed to adhere for 0.5 - 1 hour under stagnant conditions, before initiating the flow of the defined growth medium containing 1 mM citrate ($F_l = 15.3$ ml.h$^{-1}$).

The biofilms were allowed to develop for 5 days ($t = 0 - 120$ hours) before the inflowing medium was switched to modified AB medium without added citrate for a period of 8 days ($t = 120 - 312$ hours). One mM citrate medium was re-introduced and supplied for an additional 4 days ($t = 312 - 408$ hours).
6.1.4 Viable and direct counts of biofilm-derived planktonic cells

The growth medium flow rate resulted in a dilution rate of 5.0 h\(^{-1}\) for the CEMS and 15 h\(^{-1}\) for the flowcell chamber, which significantly exceeded the maximum specific growth rate of *Pseudomonas* sp. strain CT07::gfp-2 (\(\mu_{\text{max planktonic}} = 0.47 \text{ h}^{-1} \pm 0.03 \text{ h}^{-1}\)). It is therefore unlikely that an independently replicating planktonic population of bacteria would persist in the reactors and the cells collected in the effluent were assumed to originate from surface-associated growth. Effluent from each flow chamber and CEMS was collected at the outlet after disconnecting the downstream tubing prior to sampling, followed by serial dilution and spread plating onto 5 mM citrate agar plates. Effluent cell numbers (CFU.ml\(^{-1}\)) were enumerated after 3 - 4 days and normalized with respect to the flow rate (ml.h\(^{-1}\)) and the internal reactor surface area (cm\(^2\)) to determine the biofilm-to-planktonic cell yield (CFU.cm\(^{-2}\).h\(^{-1}\)).

Additional effluent was collected directly from each CEMS outlet (n = 2) and preserved for total microscopic cell counts by the addition of formaldehyde (2.0% (m/m) final concentration; Hobbie et al. 1977) and storage at 4°C. Each sample was diluted in saline to achieve a minimum of 20 cells per microscope field after incubation with the fluorescent nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI, final concentration of 5 µg.ml\(^{-1}\)). Five millilitres of each sample was vacuum filtered onto a 0.2 µm black, polycarbonate filter (25 mm diameter, Nucleopore, Whatman) before mounting the filter onto a glass slide and fixing a coverslip into place with a drop of CITIFLUOR antifadent mounting medium AF2 (Electron Microscopy Sciences, Catalogue number 17971-25,). Multiple microscope fields (40 – 50) were captured at random from each filter for digital image analysis with a 63x oil-immersion lens (Plan-Apochromat 63 x/1.4) of a Zeiss Axiovert 200M Inverted Epifluorescent microscope. The average cell dimensions (length and width) and the number of cells in each microscope field were determined with image analysis with Scion Image for Windows (Scion Corporation, public

6.1.5 CLSM and COMSTAT image analysis

The extent of biofilm development was examined daily (except at 24, 288 and 384 hours) with a 20X Plan-Apochromat objective and Zeiss LSM 510 CLSM microscope, with excitation at 488 nm and detection of GFP fluorescence emission with a long pass 505 nm filter. Ten to twelve microscope fields along a central transect of each flow chamber, from the in- to the outlet, were chosen at random and a stack of images in the Z-direction captured with the Zeiss LSM 510 software (version 3.2 SP2) at 1.39 µm intervals and stored for subsequent analysis with COMSTAT.

Each Z-image stack was exported from the Zeiss LSM Image Browser (version 4.2.0.121) as a raw series of single images, converted to greyscale tagged image file format (TIF) files with the freeware IrfanView (Version 4.10) for analysis with COMSTAT (http://www.im.dtu.dk/comstat), which runs as a script in MatLab (The MathWorks, Massachusetts, USA) using the Image Processing Toolbox (Heydorn et al. 2000).

COMSTAT allows the user to select an appropriate threshold for each image stack, which distinguishes the biomass from the background and moreover facilitates noise reduction through a connected-volume filtration process, which removes biomass pixels that are not directly, or indirectly connected (via biomass in the previous slice) to the substratum. The smacking function was used when required to correct for a non-flat substratum by producing a composite image of a user-defined number of image slices prior to analysis. A selected number of the COMSTAT functions were used for biofilm analysis: the biomass of each image
stack, expressed as the volume of biomass per substratum area (µm³/µm²), the maximum thickness of the biofilm (µm), the biofilm surface area-to-volume ratio (µm²/µm³), and the dimensionless roughness coefficient, which provides an indication of the variation in biofilm thickness and structural heterogeneity (Heydorn et al. 2000).

On the final day of carbon limitation, a separate biofilm was counterstained with the red fluorescent, lipophilic membrane stain FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethyl-amino)phenyl)hexatrienyl)pyridinium dibromide, Molecular Probes, Invitrogen, Catalogue number T3166) by carefully injecting 0.2 ml of a 5 µg.ml⁻¹ working solution into the flow chamber and incubating for 20 minutes, before simultaneous CLSM visualization of green fluorescence in the first channel (excitation at 488 nm, emission detection with a band pass 505/530 nm filter) and red fluorescence in the second channel (emission detection with a long pass 650 nm filter). This step was included to confirm GFP expression during carbon-limitation and the absence of non-fluorescent contaminants.

6.1.6 Determining the origin of the planktonic cells released during carbon limitation

In a separate experiment, six flowcell chambers were prepared, disinfected, inoculated with *Pseudomonas* sp. strain CT07 (wild type) and cultivated for 5 days with 1 mM citrate as before. The purpose of this experiment was to determine whether the cells released from biofilms were formed before or during carbon-limitation; i.e., whether biofilms have the ability to sustain cell growth and yield by utilizing nutrient reserves stored in the biofilm matrix. The staining efficiency of a vital fluorescent stain, 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE) (Molecular Probes, Invitrogen, Catalogue number C1157) was tested in four of the flow chambers; 1.0 ml of a 50 µM working solution of the stain was injected directly into two chambers and incubated with arrested flow for 0.5 hours
before restarting the flow of nutrient medium, while the stain was added to the nutrient medium reservoir (final concentration of 50 µM) and allowed to continuously irrigate the remaining two flow chambers for 1 hour, before replacing the inflowing fluid with growth medium only. The four flow chamber biofilms were counterstained with the red fluorescent membrane stain FM4-64, as described previously, prior to the collection of image stacks in the Z-direction at three random microscope fields for each flowcell channel with CLSM for COMSTAT image analysis and the quantification of biofilm biomass (µm³.µm⁻²) and maximum biofilm thickness (µm).

The remaining two 120 hour-old biofilms were stained with the vital stain (5(6)-CFDA, SE; CFSE) using the continuous flow-through method, and two to three Z-image stacks were collected per flowcell channel for image analysis before replacing the inflowing growth medium with the defined medium only (i.e., without added carbon) for a 8-day period. After the carbon-limited period, the inflowing medium was switched back to defined medium containing 1 mM citrate for 6 days. At the end of the experiment, the biofilms were counterstained with FM4-64 to evaluate the extent of biofilm recovery once carbon was re-introduced. Flowcell effluent was collected periodically throughout the experiment and preserved with formaldehyde for the enumeration of green, vital stained cell numbers using direct microscopic counts, while a portion of each sample was stained with DAPI to determine the total cell numbers, as detailed previously. It was assumed that replicating cells would lose their vital stain fluorescence, thus facilitating the determination of the degree of proliferation (growth and cell division) during carbon limitation.
6.1.7 Carbon balance

To complete a carbon balance for the biofilms cultivated in the CEMS, various samples were collected to account for carbon inflow into the CEMS, carbon accumulation within the CEMS and carbon outflow from the CEMS, namely; (i) carbon content in the inflowing growth medium (with or without added citrate), (ii) gaseous CO₂ production (biofilm respiration due to the active metabolism of citrate), (iii) carbon content in the aggregate traps (likely the loss of attached biomass due to sloughing events), (iv) carbon content in the biofilm effluent (dissolved CO₂, unutilized citrate, single cells, small cell aggregates and soluble microbial products, such as EPS) and (v) carbon content in the attached biomass retained within the CEMS (Kroukamp and Wolfaardt 2009).

The citrate content of sterile growth medium, with and without added citrate, was determined with an Enzytec™ enzymatic citric acid analysis kit (Scil Diagnostics, GmbH, Germany, Catalogue number 1214). The amount of citrate not utilized by the attached biomass was determined by collecting effluent from the CEMS silicone tubes. The samples were filtered to remove any cells (0.2-µm pore size syringe filter) and stored at -20°C prior to analysis.

Off-gas CO₂ measurements of the biofilm growing in the CEMS were taken at 24-hour intervals, and more frequently after the changes in growth medium composition at 120 and 312 hours. The aggregate-free effluent from the CEMS was collected in a vessel containing sodium azide to inhibit microbial activity (final azide concentration ≥ 1 mg.ml⁻¹). The vessel content was agitated continuously by a magnetic stirrer bar. The carbon content of the azide-treated effluent samples was determined with a catalytic combustion, non-dispersive infrared total carbon analyzer (TOC-V<sub>CSH/CSN</sub>, Shimadzu, Kyoto, Japan) at 24-hr intervals. These discrete concentration measurements, together with the gas and liquid flow rates, were used to calculate the cumulative amount of carbon flowing into and out of the CEMS.
A removable aggregate trap, consisting of a 1.5 ml micro-centrifuge tube, was inserted into the effluent tubing, downstream of the CEMS, to collect biofilm aggregates that were sloughed off at discrete, but random periods during biofilm cultivation. The trap inlet port was positioned below the outlet port to allow aggregates, with greater density than the liquid, to sink to the bottom of the tube, while the aggregate-free effluent flowed into the vessel containing sodium azide. The contents of the aggregate traps were collected at 24-hour intervals and preserved for analysis and the traps were disinfected prior to re-insertion into the system. The amount of carbon collected in the aggregate traps was determined with the total carbon analyser after homogenization with equal volumes of 1 M NaOH. At the end of the experiment (408 hours), the attached biomass retained in the silicone tubes was removed by rolling a heavy, round medium bottle over the tubes and collecting the displaced material. The carbon content was determined in a manner similar to that of the aggregate traps.

Additional effluent from the CEMS was collected at discrete time points and preserved for microscopic direct counting of biofilm-derived planktonic cell numbers with DAPI. The dimensions of individual biofilm-derived planktonic cells (length and width) were also determined using image analysis with the Scion Image for Windows software. The following formula was used to calculate the volume of the cell from a two-dimensional image (Massana et al. 1997):

\[ V = \frac{\pi}{4} W^2 \left( L - \frac{W}{3} \right) \] (1)

where \( V \) is the volume of the cell (\( \mu m^3 \)), \( W \) is the width of the cell (\( \mu m \)) and \( L \) is the length of the cell (\( \mu m \)).

The carbon content of each cell was estimated using an allometric conversion formula, as described by Posch et al. (2001), where the amount of carbon in a cell is determined from the
average cell volume. For a cell stained with the fluorescent DAPI stain and visualized using epifluorescent microscopy, the conversion factor is as follows (Posch et al. 2001):

\[
Cell \text{ carbon} = 218 \times V^{0.86}
\]

(2)

where the amount of carbon in the cell is measured in femtograms. The average \( \pm \) standard deviation of the length and width of 500 cells were determined to be 1.9 \( \mu m \) \( \pm \) 0.7 \( \mu m \) and 0.6 \( \mu m \) \( \pm \) 0.1 \( \mu m \), respectively.

6.1.8 Statistical analysis

Statistical analysis of replicate measurements was performed using one-way analysis of variance (ANOVA) and Tukey’s test for the comparisons of means (P value = 0.05).

6.2 Results

6.2.1 Biofilm architecture

\textit{Pseudomonas} sp. strain CT07::gfp-2 biofilm development at the glass surface of replicate flowcell chambers (n = 3) was investigated with CLSM and COMSTAT image analysis. Biofilm biomass (biomass per surface area) and maximum thickness increased as the biofilm developed under carbon-replete conditions (0 – 120 hours) (Figure 6.2.1). The omission of citrate from the growth medium after 120 hours had an initial ambiguous effect on the measured biofilm parameters as seen by the variation in the amount of attached biomass
(volume and thickness) until these parameters appeared to stabilize at a significantly reduced steady level \((P < 0.05)\) at 264 hours (after 6 days of carbon limitation). A similar response pattern was seen for the biofilm surface area-to-volume ratio and roughness (Figure 6.2.2) where a significant increase in both parameters at 264 hours \((P < 0.05)\) indicated that the biofilm architecture had become more heterogeneous with a probable increase in specific biofilm surface area. The re-introduction of carbon after 8 days (after CLSM examination at 312 hours) led to a rapid and significant increase in the biofilm biomass, as well as a reduction in roughness and biofilm surface area-to-volume ratio \((P < 0.05)\).

A separate biofilm was counterstained with the red fluorescent membrane stain FM4-64 to confirm GFP expression during carbon limitation. No significant difference in the distribution of biomass or biofilm thickness could be detected between the red and green fluorescence and it was concluded that the images captured for the duration of carbon limitation were representative of the true extent of the biofilms under these conditions.

A series of CLSM projections, representative of *Pseudomonas* sp. strain CT07 biofilms on various days are shown in Figure 6.3 to corroborate the biofilm parameters quantified with image analysis (Figure 6.2). Biofilm development under carbon-replete conditions (48 - 120 hours) was evident from an increase in the amount of biomass at the surface, in addition to an increase in the size and structural heterogeneity of dense cell clusters extending from the surface into the bulk-liquid. The removal of citrate from the growth medium after 120 hours resulted in a gradual reduction in the number and size of dense cell clusters from 192 hours and onwards. The re-introduction of citrate into the growth medium at 312 hours (after 8 days of carbon limitation) initiated a rapid increase in the number of dense microcolonies at the surface.
1: Biofilm biomass and maximum thickness

Figure 6.2: Various biofilm parameters were quantified by CLSM and COMSTAT image analysis. Four parameters; (1) biofilm biomass (bio-volume per surface area; \( \mu m^3.\mu m^{-2} \)), maximum thickness (\( \mu m \)), (2) biofilm surface area-to-volume ratio (\( \mu m^2.\mu m^{-3} \)) and the roughness coefficient were quantified at 24-hour intervals during cultivation on modified AB defined
growth medium with 1 mM citrate (t = 0 - 120 hours), modified AB medium without added citrate (t = 120 - 312 hours; shaded area) and finally AB medium with 1 mM citrate (t = 312 - 408 hours).

6.2.2 Biofilm-derived planktonic cell production

Since the bulk-liquid dilution rates in both continuous-flow systems greatly exceeded the maximum planktonic growth rate of *Pseudomonas* sp. strain CT07::gfp, it is assumed that cells in the effluent from these systems were produced and released by the surface-attached biofilms. In contrast to the biofilm architecture, the rate of planktonic cell production responded rapidly to changes in carbon availability; the yield of viable cells from the CEMS-grown biofilms decreased by 40%, 88%, 94% and 99% after 1, 3, 5 and 24 hours, respectively, of carbon limitation when compared to the yield before the limitation (Figure 6.4). After 312 hours of carbon limitation (8 days), the yield from the CEMS-grown biofilms was reduced to 4.9 x 10^5 cells.cm\(^{-2}\).h\(^{-1}\) ± 3.9 x 10^5 cells.cm\(^{-2}\).h\(^{-1}\), which is 2 orders of magnitude less than the cell production rate at 120 hours (5 day-old biofilm), prior to the initiation of carbon limitation (3.6 x 10^7 cells.cm\(^{-2}\).h\(^{-1}\) ± 4.4 x 10^7 cells.cm\(^{-2}\).h\(^{-1}\)).

A one order of magnitude reduction in the viable cell numbers was also enumerated for the flowcell-grown biofilms for the same period (1.4 x 10^7 cells.cm\(^{-2}\).h\(^{-1}\) ± 0.2 x 10^7 to 3.4 x 10^6 ± 2.9 x 10^6 cells.cm\(^{-2}\).h\(^{-1}\)). The re-introduction of carbon after 312 hours resulted in an increase in yield from the CEMS-grown biofilms within one hour to 1.2 x 10^7 cells.cm\(^{-2}\).h\(^{-1}\) ± 1.6 x 10^7 cells.cm\(^{-2}\).h\(^{-1}\) and stabilized after 24 hours at a similar order of magnitude to that observed prior to the carbon limitation. The cumulative number of viable planktonic cells yielded to the bulk-liquid phase from the CEMS-grown biofilms during the entire cultivation period was calculated at 7.2 x 10^10 CFU ± 9.6 ± 10^8 CFU.
Figure 6.3: A time series of CLSM micrographs representative of the GFP-labelled *Pseudomonas* sp. strain CT07 biofilms. The biofilms were cultivated under carbon-replete (t = 48 - 120 hours and 336 - 408 hours) and carbon-limited conditions (t = 144 - 312 hours).
Figure 6.4: Biofilm-derived planktonic cell production during alternating periods of excess carbon and limited carbon (shaded area), as enumerated from the effluent of conventional flowcells (n = 3) and the CEMS (n = 2). The number of viable cell numbers per effluent volume was normalized with respect to the medium flow rate (ml.h$^{-1}$), and the internal reactor area available for biofilm colonization (cm$^2$) and expressed as a the log$_{10}$ of that value to enumerate the active yield of planktonic cells per reactor area per hour.

6.2.3 Carbon dioxide production and carbon utilization rates

Kroukamp and Wolfaardt (2009) detailed a linear increase in the rate of CO$_2$ production by *Pseudomonas* sp. strain CT07 biofilms when supplied with increasing citrate concentrations exceeding 0.5 mM; the CO$_2$ detected by the CEMS can thus be concluded to be the result of active biofilm metabolism of citrate. The amount of CO$_2$ detected in the off-gas increased with biofilm development in the CEMS and reached a plateau after 72 hours; with a similar pattern observed for citrate utilization (Figure 6.5).
The omission of citrate after 120 hours of cultivation resulted in a 91% decrease in whole-biofilm CO₂ production rate within 1 hour of the switch, with a subsequent 6% decrease over the next 24 hours (relative to the steady-state value at 120 hours), where after the CO₂ production rate stabilized for the duration of the carbon limitation period. The re-introduction of carbon after 312 hours led to a rapid increase in the rate of CO₂ production to 63%, 78%, 99% and 115% of the 120-hour steady-state production rates after 1, 3, 5 and 24 hours, respectively. No statistically significant difference in CO₂ production rates was observed between the values before carbon limitation and those observed after re-establishment of steady-state production following the re-introduction of carbon, indicating that the biofilm respiration rates had recovered to pre-carbon limitation levels (P = 0.05).

Figure 6.5: Carbon dioxide production rates (µmol of C.h⁻¹) of CEMS-grown biofilms, as measured in the gaseous phase, were monitored along with the rate of citrate utilization (µmol of C.h⁻¹) for biofilms exposed to alternating periods of carbon excess and limitation (shaded area). Both parameters are expressed in terms of the carbon content produced or utilized per hour.
6.2.4 Determining the origin of the planktonic cells released during carbon limitation

According to information provided by Invitrogen, the non-fluorescent vital stain (5(6)-CFDA, SE; CFSE) used to stain the wild-type *Pseudomonas* sp. strain CT07 biofilms for this experiment diffuses into cells where esterases cleave off the diacetate groups to generate a fluorescent product that binds readily to intracellular proteins. The cell remains fluorescent until it divides, leading to the equal distribution of fluorescence between the two daughter cells, thereby halving the original fluorescence intensity. Planktonic bacteria were labelled with the CFDA/SE isomer without unwanted effects on viability or adhesion (Fuller et al. 2000) and Ueckert et al. (1997) showed reliable detection of *Lactobacillus plantarum* cells, labelled with the CFSE isomer, through a maximum of eight generations with flow cytometry.

To evaluate the staining efficiency, duplicate *Pseudomonas* sp. strain CT07 flowcell-biofilms were incubated with the stain under stagnant conditions for 0.5 hours or continuous flow-through conditions for 1.0 hours. After counterstaining with the red membrane stain, FM4-64, all four biofilms were investigated with CLSM and COMSTAT image analysis. The average percentage of biomass stained by the vital stain was 71% ± 13% for the flow-through conditions and 70% ± 15% for the stagnant incubation conditions. The averaged maximum vital-stained biofilm thickness was 95% ± 12% and 98% ± 4.2% of the total maximum biofilm thickness for the flow-through and stagnant conditions, respectively, indicating that the stain was able to diffuse through multiple cell layers and reach the attachment surface. Despite the expected lower metabolic activity of cells in the basal zone of the biofilm, sufficient esterase activity was retained to hydrolyze the vital stain.

Duplicate 120 hour-old biofilms were subsequently stained with the vital stain using the flow-through method, prior to omitting the carbon source from the inflowing growth medium for 8 days. Flowcell effluent was collected periodically and preserved for the enumeration of vital-
stained and total cell numbers (DAPI) using direct microscopy, while the biofilms were subjected to CLSM to evaluate vital stain retention by the biofilm during carbon limitation. The amount of green, vital-stained biofilm biomass declined during carbon limitation (Figure 6.6.1) in a manner similar to what was observed for the *gfp*-labelled *Pseudomonas* sp. CT07 biofilms (Figure 6.2.1). The re-introduction of carbon into the flowcell influent after 312 hours resulted in an increase in attached cell division and growth, thereby leading to the displacement of the vital stain from the biofilm. This increase in biofilm biomass was demonstrated by counterstaining the biofilms with FM4-64 at 432 hours (5 days after the re-introduction of carbon). Simultaneous visualization of green and red fluorescence confirmed that only 5% ± 3% of the biofilm biomass still retained the vital stain, while the total amount of biomass had recovered to levels observed prior to the carbon limitation period.

The number of vital-stained cells released from the biofilms during carbon limitation (168 – 312 hours) declined by one order of magnitude (results not shown), while the average percentage of vital-stained effluent cells collected at five sampling points during carbon limitation, as enumerated with direct fluorescent counting, was found to be 48% ± 5.8% (Figure 6.6.2).

Re-introduction of carbon (after 312 hours) led to a greater percentage of vital-stained cells being yielded from the biofilm for the ensuing 24 hours, most likely due to the rapid increase in growth and cell division of the remaining vital-stained attached biomass. Thereafter, the number of vital-stained cells yielded by the biofilms declined significantly, which correlated to the low percentage of vital-stained biomass remaining in the biofilm; suggesting near-complete turnover in biofilm biomass.
1: Vital-stained biofilm biomass

![Image of vital-stained biofilm biomass](image)

2: Percentage of vital-stained cell yield of total biofilm-to-planktonic cell yield

![Image of percentage yield](image)

Figure 6.6: *Pseudomonas* sp. strain CT07 wild-type biofilms were stained at 120 hours with a vital green fluorescent stain prior to the omission of the carbon source from the influent.
During the 8-day carbon limitation period (t = 122 - 312 hours; shaded area), the amount of green fluorescent biofilm biomass (µm$^3$.µm$^{-2}$) was monitored using CLSM and COMSTAT image analysis (1) in addition to the number of vital-stained fluorescent cells yielded to the bulk-liquid (2), expressed as a percentage of the total number of yielded cells as determined with direct microscopic counts. (1) The insets show CLSM micrographs representative of the biofilm biomass; the micrographs taken at 312 and 360 hours show the green vital-stained fluorescence only, while the 432-hour micrograph provides a visual indication of the distribution of the vital-stained biomass (green-yellow fluorescence) within the total biofilm biomass (red fluorescence). The white scale bars represent 100 µm.

6.2.5 Carbon balance

The carbon balance determined for the CEMS-cultivated biofilms is given in Figure 6.7 and Table 6.1. On average 27% ± 4.1% of the inflowing carbon was metabolized by the biofilm and detected as CO$_2$ in the off-gas phase of the reactor, while 39% ± 3.6% of the carbon exited the reactor in the liquid phase as a combination of dissolved CO$_2$ and microbial products (such as soluble EPS) and 28% ± 4.8% as unutilized citrate. Kroukamp and Wolfaardt (2009) found that the amount of dissolved CO$_2$ exiting the CEMS was at least equal to that of the gaseous CO$_2$ measured in the off-gas. Duplicate biofilms, removed from the reactors at the end of the experiment, retained 6.5% ± 2.8% of the inflowing carbon, while dense biomass aggregates (i.e., sloughing events) and biofilm-derived single planktonic cell yield in the effluent accounted for 3.4% ± 1.5% and 1.0% ± 0.2 % of the inflowing carbon, respectively.
A carbon balance was completed to determine the fate of the carbon flowing into the CEMS for both periods of carbon excess and carbon limitation. The cumulative carbon content of the various fractions are expressed as a percentage of the cumulative inflowing carbon and were as follows: the carbon fraction in the gas phase (detected as CO₂), the aggregates of dense sloughed biomass flowing out of the reactor, the carbon retained as biofilm biomass after 408 hours (this value is thus not cumulative), the carbon in the biofilm-derived planktonic cell yield, the unutilized citrate and the non-cellular fraction of carbon leaving the reactor in the liquid phase, which consisted of dissolved CO₂ and likely soluble microbial products, such as EPS. The values represent the average of duplicate biofilms, where the final balance closed within 3% and 9% over the entire cultivation period (408 hours).

Calculations show that an average of 1 580 ± 0.98 µmol of carbon was retained as biofilm biomass during the first 120 hours of biofilm cultivation (with carbon supply). The biofilm converted carbon into planktonic cell production at an average rate of 0.66 µmol of C.h⁻¹ ± 0.62 µmol of C.h⁻¹ during this period. This measured rate of carbon conversion into planktonic cells decreased to 0.02 µmol of C.h⁻¹ ± 0.03 µmol of C.h⁻¹ during the carbon-limited period, which
correlates with the reduction in the number of viable cells enumerated from the effluent during the same period (Figure 6.3).

**Table 6.1:** The results from the carbon balance determined for the CEMS-cultivated biofilms, expressed as the cumulative amount of carbon flowing into the CEMS (as citrate and dissolved CO₂), the measured amount of carbon accumulated in the CEMS as attached biomass after 408 hours and the cumulative amount of carbon flowing out of the CEMS (as gaseous CO₂, dense biomass aggregates, biofilm-derived planktonic cell yield, citrate not utilized by the biofilm, dissolved CO₂ and non-cellular carbonaceous microbial products). All values are expressed in mmol of carbon as the average to two replicates ± the standard deviation.

<table>
<thead>
<tr>
<th>Total Carbon IN</th>
<th>Carbon accumulated</th>
<th>Total Carbon OUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate and CO₂</td>
<td>Biofilm biomass</td>
<td>Gaseous CO₂</td>
</tr>
<tr>
<td>mmol</td>
<td>mmol</td>
<td>mmol</td>
</tr>
<tr>
<td>Carbon in = 19.7</td>
<td>1.28 ± 0.55</td>
<td>5.37 ± 0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.67 ± 0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.62 ± 0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.74 ± 0.71</td>
</tr>
</tbody>
</table>

**SUM of accumulated carbon and carbon out = 20.9 ± 0.87**
The periodic release of dense aggregates from the biofilm continued during the carbon-limited period, although less carbon was collected from the aggregate traps compared to pre-carbon limitation values. It is thus likely that a combination of single cell release and the sloughing-off of dense biomass aggregates from the biofilm resulted in the reduction of the attached biomass volume per attachment area as observed by COMSTAT image analysis (Figure 6.2). The re-introduction of carbon led to an increase in the rate of aggregate sloughing from the biofilm.

6.4 Discussion

The ability of microorganisms to respond and rapidly adapt to fluctuations in environmental conditions, such as carbon availability, enable optimal substrate utilization and enhance survival fitness. Since the low concentration of available carbon in natural water environments limits the growth of most heterotrophic microbes (Brown et al. 1977, Morita 1993) the reported affinity of bacteria for solid surfaces in these environments is hypothesized to be an adaptive response to facilitate the scavenging of surface-associated organic material (Kjelleberg et al. 1982; Mueller 1996). The ability of biofilm-associated cells to sense prevailing nutrient conditions and modulate their structural organization and species composition accordingly has been documented (Wolfaardt et al. 1994; James et al. 1995; Nielsen et al. 2000; Karthikeyan et al. 2001) and undoubtedly aids in microbial survival and propagation in the environment.

The response of recently adhered bacteria and mature biofilms to carbon-limited conditions has been varied and evidence for both increased adhesion to and detachment from solid substrata in response to carbon limitation has been reported. The attachment of a marine *Pseudomonas* sp. was found to increase after 10 hours of carbon limitation (Wrangstadh et al.
1986), while the adhesion of starved *Vibrio* cells increased within 5 hours, along with a marked decreased in cell volume and oxygen consumption rates, and the production of an unidentified polymer (Brown and Lester 1982). A river-water microbial community exhibiting increased attachment under glucose-limited conditions, but little attachment during nitrogen limitation despite the production of copious amounts of EPS has also been described (Brown et al. 1977).

Significant changes in the surface behaviour of an *Acinetobacter* species were observed when cultivated alternately in nutrient rich and poor media (James et al. 1995). Under nutrient limiting conditions, division of attached bacteria resulted in densely packed, sessile microcolonies. In contrast, excess nutrients prompted an increase in cell size and dissemination across the surface, likely to facilitate colonization elsewhere. This alternating behaviour suggests a bacterial survival response, where the continued existence of the species became the primary objective during starvation periods rather than proliferation.

Halting the flow of nutrient medium resulted in the rapid dissolution of *Pseudomonas putida* (Gjermansen et al. 2005) and *Shewanella oneidensis* biofilms (Thormann et al. 2005). The *S. oneidensis* biofilms were found to detach in response to a sudden decrease in oxygen availability rather than carbon limitation (Thormann et al. 2005). Subsequent studies indicated that both attachment to and detachment from the biofilm was mediated by the signalling molecule, 3′,5′-cyclic diguanylic acid (c-di-GMP) through the *mxdABCD* gene cluster, likely by the direct allosteric interaction with the putative glycosyl transferase, *mxdB*, thereby modulating the adhesion of cells to the matrix (Thormann et al. 2006). Gjermansen et al. (2006) came to a similar conclusion soon thereafter, by showing that proteins containing GGDEF and EAL-domains were responsible for inducing cell attachment or detachment, likely through the production or degradation of c-di-GMP (Gjermansen et al. 2006). The current model based on these and other studies propose that the multiple GGDEF and EAL domain-containing proteins integrate the various signals received from the environment and modulate
c-di-GMP levels in the cell, which determines whether the cells adhere or disperse into the bulk-liquid.

Contrary to these observations, the addition of excess carbon (succinate, glutamate or glucose) resulted in the dispersion of 4 day-old *P. aeruginosa* PAO1 biofilms, with the dispersed cells exhibiting marked increases in the expression of the *fliC* gene (encoding for a flagellum protein) and genes involved in ATP synthesis and energy metabolism (Sauer et al. 2004). The authors suggested that active swimming by means of flagella not only aided in the release of single cells from the biofilm and associated matrix material, but may also constitute a chemotactic response to greater nutrient availability. The regulation of biofilm dispersion has also been linked to carbon metabolism via the activity of the *csrA* gene (encoding for a carbon storage regulator protein) in *Escherichia coli*. Induction of *csrA* expression led to biofilm dissolution and the dispersion of viable planktonic cells into the environment (Jackson et al. 2002).

The development of the *Pseudomonas* sp. strain CT07 biofilms was evident not only from the accumulation of biomass at the glass surface, as visualized by conventional microscopy (CLSM), the increase in whole-biofilm CO₂ production, and the yield of viable planktonic cells to the bulk-liquid, the latter of which was shown to be related to biofilm development in chapter 5. The biofilm CO₂ production rate was furthermore correlated to the rate of biofilm carbon utilization (i.e., citrate consumption) where an absence of added carbon reduced the biofilm respiration rate to the background levels seen prior to inoculation (due to dissolved CO₂ from atmospheric origin).

Despite a significant reduction in biofilm biomass during the carbon-limited period, the biofilm respiration rates recovered to 99% of the pre-limitation CO₂ production rate within 5 hours of the re-introduction of carbon. Kjelleberg et al. (1982) reported a similar rapid increase in
oxygen consumption of planktonic, starved *Vibrio* cells upon the addition of nutrients and furthermore noted that this response was not preceded by protein synthesis for cells starved for either 5 hours or 5 days (Kjelleberg et al. 1982). The swift response of the remaining biofilm cells to the re-introduction of carbon, first evident in the increase in CO₂ production and planktonic cell yield and the subsequent re-growth of biofilm biomass, demonstrates the powerful metabolic capacity of attached microbes when access to nutrients become unrestricted. The increase in biofilm roughness and surface area-to-volume ratio during the carbon-limited period suggests that the reduction in the amount of attached biomass may provide biofilms with a strategic advantage, since an increase in the specific surface area of the biofilm could enhance nutrient capture from the bulk-liquid, as previously suggested (Battin et al. 2003). This change in biofilm architecture could not only facilitate more efficient capture and utilization of available nutrients during starvation conditions, but also position the biofilm to derive maximal benefit from an increase in nutrient concentration through a rapid response, which was evident in this study.

Numerous reports of spatial differentiation in biofilm metabolic activity exist, with the biofilm cell layers exposed to the bulk-liquid exhibiting greater activity than cells in the biofilm centre and at an inert, non-nutritive attachment surface (Wentland et al. 1996; Werner et al. 2004; Rani et al. 2007; Bester et al. 2010). A previous investigation into the rate of *Pseudomonas* sp. strain CT07 accumulation at a surface during the initial stages of biofilm formation (12 - 18.5 hours after inoculation) indicated that it was greater than the maximum specific rate of growth in a planktonic batch culture (1.2 vs. 0.62 h⁻¹) (Bester et al. 2005). The fast growth rate during this initial stage may be attributed to unrestricted access to nutrients and colonization space. It is unlikely that this rate could be maintained once the cells become embedded in an EPS matrix and overlaying layers of cells - with the notable exception of the cell layer at the biofilm bulk-liquid interface. An enlarged biofilm surface area-to-volume ratio would increase the
relative number of cells in this layer with the potential to maintain a high growth rate under favourable environmental conditions or respond rapidly to nutrient availability after a period of limitation; it is therefore likely that the biofilm cells at the bulk-liquid interface are responsible for the production and release of single cells to the bulk-liquid.

The continuous release of viable single cells to the effluent during carbon limitation, coupled to the reduction in biofilm biomass may be due to either the continued yield of single cells from the biofilm, sloughing events, or dispersion of the biofilm in response to carbon (in this case) or oxygen limitation, as reported previously (Delaquis et al., 1989; Gjermansen et al., 2005; Thormann et al., 2005; Delille et al., 2007). Delille et al. (2007) indicated that carbon limitation resulted in the gradual detachment of cells from a Pseudomonas fluorescens biofilm over a 20-hour period, before the amount of attached biomass stabilized at a reduced level (Delille et al., 2007). In the present study, a gradual change in biofilm biomass was also observed, but the reduction in planktonic cell release was almost immediate (40% reduction after 1 hour and 99% after 24 hours in the CEMS). These results warrant a critical evaluation of the widely held assumption that fluid-mediated shear (i.e., erosion) is the major contributing factor to detachment from biofilms; if erosion alone was responsible for the removal of cells from the carbon-limited biofilm, one would expect that the fraction of green, vital-stained fluorescent cells released into the bulk-liquid would reflect the same ratio of attached cells that were originally stained with the vital stain (i.e., 71% ± 13%). Instead, the results suggest that a combination of attached cell division and erosion contributed to the release of green fluorescent cells (48% ± 6% of the total number of planktonic cells) to the bulk-liquid, since only active cell division can reduce the fluorescence intensity of cells.

Based on the carbon mass balance, it was estimated that an average of 1 580 µmol ± 0.98 µmol of carbon had been accumulated as biofilm biomass during the first 120 hours of cultivation (with added carbon), with concurrent carbon investment into biofilm-derived planktonic cells
at an average rate of 0.66 µmol of C.h⁻¹. This carbon investment decreased to 0.02 µmol of C.h⁻¹ during the carbon limitation period, indicating that a minute fraction of the accumulated biofilm biomass would be required to sustain this yield of cells over periods much longer than the 8 days of carbon limitation applied here. The CO₂ production rate during this period of reduced cell production could furthermore be expected to be in the same order as that seen in Figure 4 (i.e. background levels, likely below the detection limit). The evidence presented for continued attached cell division, together with a reduced rate of planktonic cell release from the biofilm for the duration of the carbon-limited period, may be indicative of the utilization of an alternate carbon reserve, such as the biofilm matrix or citrate reserves stored within the matrix (Wolfaardt et al. 1995) and lends support to models suggesting that detachment rates correlate better with biofilm physiology (i.e., biofilm growth rates) than shear-related removal forces (Stewart 1993).

While the cumulative number of viable planktonic cells yielded to the bulk-liquid phase from the CEMS-grown biofilms was numerically significant (7.2 x 10¹⁰ CFU ± 9.6 x 10⁸ CFU), the carbon investment required to produce the cells was comparatively small (1.0% ± 0.2% of the total carbon input). The small amount of carbon required to maintain the yield of planktonic cells may explain how the pioneer, surface-attached cells are able to form a multi-layered biofilm through growth and cell division, while simultaneously yielding cells to the environment as early as 6 hours after introduction to new surfaces (Bester et al. 2009) as well as the continued production and release of cells during carbon limitation (with the latter presumably due to the metabolism of carbon reserves stored in the biofilm). While the fate of biofilm-derived planktonic cells was not investigated here, it is likely that these cells may maintain a free-floating existence which could allow for the colonization of new surfaces, or the interaction with existing biofilms.
The comparatively small amount of carbon retained in the biofilm (6.5% ± 2.8% of the total inflowing carbon) was similar to that reported previously and affirms the suggestion that while biofilm formation represents a small carbon investment, it functions as an efficient catalyst for the transformation of carbon in the environment (Kroukamp and Wolfaardt, 2009). The release of biofilm-derived planktonic cells and loss of dense biomass aggregates to the environment both represent small carbon investments and together with the demonstrated ability of biofilms to maintain a reduced rate of planktonic cell yield during starvation periods, these results demonstrate the remarkable efficiency of biofilm-associated microbes to utilize environmental resources to promote microbial proliferation. This re-enforces the suggestion that the biofilm lifestyle facilitates not only microbial survival but also functions to promote proliferation.

We used a pure culture in this study to develop an approach for measuring carbon channelling and to demonstrate the role of biofilms in contributing to the free-floating population. In a recent study of early biofilm development, Kroukamp et al. (2010) showed that this approach is equally applicable to mixed-species biofilm communities and should therefore provide a useful experimental model to investigate the role of biofilm function in environmental processes.

6.5 Conclusions

6.5.1 Whole-biofilm respiration rates can be measured as an indicator of biofilm metabolic activity.

6.5.2 Carbon deficiency led to changes in biofilm structure; most notably a gradual decrease in the amount of attached biomass and an increase in the biofilm surface area-to-
volume ratio and roughness. The altered architecture could enhance nutrient scavenging by the biofilm during carbon-deficient conditions and enable the biofilm to respond rapidly once carbon availability increases.

6.5.3 Carbon deficiency also reduced biofilm metabolic activity and planktonic cell yield to a significant extent. Despite this reduction in activity, evidence for continued attached cell division and liberation from the biofilm was presented.

6.5.4 The carbon-related cost to the biofilm to produce significant numbers of planktonic cells for release into the environment was found to be comparatively low, which sheds light on the ability of surface-attached microbes to utilize environmental resources to promote microbial proliferation.

6.5.5 The ability of biofilms to not only persist during adverse conditions, but also promote microbial proliferation to the environment was demonstrated and re-enforces the suggestion that the biofilm lifestyle facilitates not only microbial survival but also functions to promote proliferation.

6.6 List of symbols and abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming units</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celcius</td>
</tr>
<tr>
<td>ID</td>
<td>inner diameter</td>
</tr>
</tbody>
</table>
**OD**  
**outer diameter**

**F<sub>t</sub>**  
**flow rate** \((\text{ml.h}^{-1})\)

**µg**  
**micrograms**

**h**  
**hour**

**L**  
**length**

**ml**  
**millilitre**

**(m/m)**  
**mass in mass**

**cm<sup>2</sup>**  
**square centimetre**

**mm**  
**millimetre**

**µm**  
**micrometre**

**µm<sup>2</sup>**  
**square micromtres**

**µm<sup>3</sup>**  
**cubed micromtres**

**nm**  
**nanometre**

**M**  
**molar**

**mM**  
**millimolar**

**µmol**  
**micromoles**

**µM**  
**micrometre**

**ppm**  
**parts per million**

**%**  
**percentage**

**±**  
**plus and (or) minus**

**t**  
**time**
(v/v) volume in volume
V volume
W width

HCO$_3^-$ bicarbonate ion
CO$_2$ carbon dioxide
CO$_2$ (aq) dissolved carbon dioxide
CO$_2$ (g) gaseous carbon dioxide
CO carbon monoxide
dH$_2$O distilled water
O$_2$ oxygen
NaOH sodium hydroxide
THC total hydrocarbon
TOC total organic carbon

ATP adenosine triphosphate
ANOVA analysis of variance
et al. and others

mxdABCD biofilm matrix deficient gene cluster
CEMS carbon dioxide evolution measurement system
csrA carbon storage regulator
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>CFDA/SE</td>
<td>carboxyfluorescein diacetate succinimidyld ester</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyld ester</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>c-di-GMP</td>
<td>cyclic-di(3’→5’)-guanylic acid</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
</tr>
<tr>
<td>EAL domain</td>
<td>glutamic acid – alanine – leucine sequence</td>
</tr>
<tr>
<td>GGDEF domain sequence</td>
<td>glycine – glycine – aspartic acid – glutamic acid – phenylalanine</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>gfp</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>µmax planktonic sp.</td>
<td>maximum planktonic specific growth rate</td>
</tr>
<tr>
<td>i.e.</td>
<td>that is</td>
</tr>
<tr>
<td>vs.</td>
<td>versus</td>
</tr>
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</table>
CHAPTER 7  

Metabolic differentiation in the biofilm*†

7.1 Introduction

It has been shown by various means that the biofilm layers in closest proximity to nutrients and (or) oxygen are more metabolically active than the cells located in the centre of the biofilm, or at a non-nutritive solid surface (Huang et al. 1995; Wentland et al. 1996; Werner et al. 2004; Rani et al. 2007). The goal was therefore to investigate whether the proposed metabolically active region of the biofilm can be linked to the yield of biofilm-derived planktonic cells to the effluent, in addition to possible differences in the expression of selected genes between the biofilm layers and the yielded cells. To conduct this investigation, a practical technique was developed to structurally fractionate the biofilm into two regions. Initially, a significant increase in fluid shear was briefly applied to remove the shear-susceptible layer of the biofilm. This method was subsequently expanded into the shear-mediated removal of the susceptible biofilm region by a moving air-liquid interface (i.e. air bubble). This enabled the comparison of metabolic activity, protein content and gene expression profiles between the yielded planktonic cells, the shear-susceptible biofilm layer and the persistent base layer of the biofilm.


†The first two authors contributed in equal measure to the work presented in this chapter (except Figures 7.2 and 7.8 - 7.12).
The effect of air bubbles on biofilm stability has mostly been studied in a dental context, where biofilm removal is the goal. Gomez-Suarez et al. (2001) utilized a single bubble to investigate the strength of bacterial cell adhesion to various surfaces. According to the authors, the probability of cell detachment due to the movement of an air bubble over an attached cell is determined by several factors, namely, collision efficiency, bubble-bacteria attachment efficiency, and the stability of the bubble-bacteria aggregate. For a bubble spanning the entire width of a flow chamber, the collision efficiency is expected to be equal to 1, although the velocity of the bubble may also influence the detachment efficiency since a rapidly moving bubble will result in a thicker liquid film surrounding the bubble, which in turn decreases the collision efficiency. Bacterium-substratum adhesion forces of $\sim 10^{-9}$ N were estimated, which is significantly smaller than the detachment force of a bubble moving over an attached cell (up to $10^{-7}$ N) (Gómez-Suárez et al. 2001).

Liquid flow in most environments – in nature, industry, and clinical or dental settings – typically shows much variation. It can be expected that microbial biofilms have evolved to manage this variability and even to utilize the resulting differences in flow to optimize activity (i.e., the prevention of excessive biomass accumulation for the maintenance of optimum gradients of nutrients and gases) or to relocate to more favourable environments.

Furthermore, increased shear is a recognized strategy to remove unwanted microbial growth from surfaces; therefore, methods to measure the effect of shear on biofilms, including biofilm recovery after partial shear-induced removal should contribute to our overall understanding of this important form of microbial existence. A technique was developed to measure real-time CO$_2$ production as an indicator of biofilm activity and this method was combined with confocal laser scanning microscopy (CLSM) and cell yield measurements to study structure-activity relationships in biofilms. This approach is an extension of the carbon dioxide evolution measurement system (CEMS), first described by Kroukamp and Wolfaardt (2009) and applied
in chapter 6 and allowed the determination of differences in the metabolic activity of the whole biofilm versus that of the shear-susceptible biofilm region, and to compare biofilm-derived planktonic cells with those growing in batch culture. The modified carbon dioxide production measurement reactor (CMR) system is described in detail below.

Much of the focus in biofilm research in recent years has been on the changes in gene expression that take place when planktonic cells attach to a surface and subsequently form a biofilm. Various genes involved in biofilm formation have been identified (O'Toole and Kolter 1998a; O'Toole and Kolter 1998b), some of which were found to encode for flagella, pili, surface adhesins and cell-to-cell signalling factors, amongst others (Davies et al. 1998; Pratt and Kolter 1998).

Prigent-Combaret et al. (1999) reported a 38% differential gene expression between biofilm and planktonic populations of E. coli K12 (Prigent-Combaret et al. 1999). Profound differences in whole-cell protein profiles were also observed when planktonic populations of Pseudomonas aeruginosa were compared to their attached counterparts, and when the various stages of biofilm development were compared to each other (Sauer et al. 2002). The protein profiles of dispersed cells were, interestingly, more similar to that of planktonic cells than those in the biofilm maturation stage, which led to speculation that detached or dispersed planktonic cells are in transition from an attached to a planktonic phenotype.

A recent investigation using whole-cell protein analysis and DNA microarrays confirmed that biofilm-detached cells exhibited a gene expression pattern different from that of attached cells (Sauer et al. 2004). This work did not, however, attempt to establish whether the bacteria retain the biofilm-derived phenotype when restricted to a planktonic existence, or whether a population of cells exhibiting a different phenotype may play a significant role in an enclosed system.
7.2 Experimental procedures

7.2.1 Removing the region of the biofilm responsible for the observed yield

To investigate if the removal of the shear-susceptible biofilm region would affect planktonic cell yield from the biofilm, *Pseudomonas* sp. strain CT07 biofilms were established in replicate glass tubes as described previously. Effluent was collected from each tube immediately after initiating flow (t = 0), 8 and 16 minutes after and thereafter at 24 hour-intervals to determine the number of viable biofilm-derived planktonic cells. After 144 hours of incubation at a growth medium flow rate of 15.3 ml.h\(^{-1}\), five consecutive pulses of increased flow rate (498 ml.h\(^{-1}\)) were applied to the biofilms. The duration of each pulse (5 s) was sufficient to displace one reactor volume, which was collected separately for each of the 5 pulses to enumerate the viable cell numbers. One reactor volume from each tube was collected twelve minutes (5 reactor volumes displaced), 24 and 48 hours subsequent to the last pulse to determine the yield from the biofilm.

7.2.2 Flowcell cultivation of biofilms

Biofilms were cultured in multi-channel flowcells with modified chamber dimensions (7.22 mm x 3.00 mm x 60.0 mm; width, depth and length) as previously described. Watson-Marlow 205S peristaltic pumps supplied 5 mM citrate defined growth medium to the biofilms at a constant flow rate (F\(_i\)) of 37.5 ml.h\(^{-1}\) (Reynolds number of 2.00). The medium dilution rate in the flow chambers (D = 28.5 h\(^{-1}\)) significantly exceeded the maximum specific planktonic growth rate of *Pseudomonas* sp. strain CT07::gfp-2 in 5 mM citrate medium (\(\mu_{max}\) planktonic of 0.35 h\(^{-1}\) ± 0.05 h\(^{-1}\)). The system was disinfected as described previously and each chamber was
inoculated with 0.2 ml of a *Pseudomonas* sp. strain CT07::gfp-2 pre-culture and incubated for 0.5 hours, before the initiation of medium flow.

### 7.2.3 Experimental conditions and perturbation

Biofilms were cultured in multiple flow chambers (n = 9) for 8 days. Various parameters were investigated at 24-hour intervals, including whole-biofilm CO₂ production, total direct and viable biofilm-derived cell numbers in the effluent (n = 6). The extent of biofilm formation on the glass coverslip was monitored in three additional flow chambers with CLSM and image analysis.

After 5 days of incubation, a single air bubble was introduced into all of the flow chambers to remove the shear-susceptible fraction of the biofilm. Each bubble was generated by disconnecting the silicone tube from the growth medium reservoir upstream of the peristaltic pump. An air bubble, large enough to fill the entire flow chamber, was introduced at a volumetric flow rate of 37.5 ml.h⁻¹ (as controlled by the pump head) and allowed to move through the chamber. The flowcell was tilted vertically with the inlet at the top to ensure that the air bubble spanned the entire chamber cross-section and contacted all sides as it travelled through the chamber. The displaced biofilm material (containing the shear-susceptible biofilm region as well as the bulk-liquid) was clearly visible as a slimy, milky-white layer ahead of the bubble, which increased in size as the bubble sheared off the attached biomass as it moved through the chamber, before the biomass was collected at the outlet for subsequent analyses. After collection, the up- and downstream tubing were reconnected, and six of the flow chambers were refilled with medium to measure recovery over 2 to 3 days (three flow chambers for CO₂ production and effluent cell number enumeration and 3 flow chambers for CLSM). Cells from the non-shear-susceptible biofilm (i.e. the biomass that resisted shear
removal) in the three remaining flow chambers were removed by sonication and the cell numbers determined using direct microscopy, as described below.

### 7.2.4 Viable and total cell counts

Approximately 1.5 ml of effluent was collected directly from each chamber outlet \( (n = 6) \) and preserved for subsequent microscopic enumeration of total cell numbers by the addition of formaldehyde. Sample dilution, DAPI-staining, image acquisition and digital image analysis with Scion Image for Windows were performed as detailed previously.

Additional effluent from two of the six chambers was collected from the chamber outlet for serial dilution and spread plating onto 5 mM citrate agar plates to confirm cellular viability in the effluent.

### 7.2.5 Dispersion of shear-susceptible and base biofilm layers

The bubble-displaced content of each flow chamber was collected as described and the volume determined (approximately 1.3 ml). This fraction contained liquid medium, single (i.e. non-aggregated) biofilm-derived planktonic cells, and the shear-susceptible portion of the biofilm biomass. To enumerate the number of cells in these samples it was necessary to disperse the cells from the biofilm matrix. Methanol was added to each sample to a final concentration of 1% (v/v), followed by sonication in an ultrasonic cleaner (Elma Ultrasonic LC20/H) at 35°C at 35 kHz for 15 minutes (Lunau et al. 2005). The samples were preserved by the addition of formaldehyde and stored prior to enumeration by direct microscopic counts.
The number of cells in the non-shear-susceptible base biofilm layer that remained in the flow chambers after the bubble perturbation was also enumerated by direct counting. Three of the emptied flow chambers were filled with 1% methanol, sealed and sonicated as described previously. After sonication, the content of each chamber was collected in a microcentrifuge tube with repeated up and down pipetting to aid in the removal of biomass from the surfaces. Each chamber was filled with methanol for a second time and sonicated and the contents were collected to determine the removal efficiency of the first sonication step; analysis of the results indicated that the second sonication step removed two orders of magnitude fewer cells than the first, indicating that the first removal step was sufficient. These samples were preserved in the same manner as the shear-susceptible samples prior to direct microscopic counting.

7.2.6 CLSM and COMSTAT image analysis

The extent of biofilm formation in three independent flow chambers was examined daily with CLSM, using a 20x Plan-Achromat objective (excitation at 488 nm and emission detection of the GFP with a long pass 505 nm filter). Ten microscope fields, each with an area of 0.002 cm², were chosen at random along a central transect ranging from the chamber inlet to outlet, and a stack of images was captured in the Z-direction for subsequent image analysis with COMSTAT, as described previously. Only a selected number of the COMSTAT functions were used for the analysis of the biofilm: the biovolume of each image stack (µm³.µm⁻²), the mean thickness of the biofilm (µm), the maximum thickness of the biofilm (µm), the biofilm surface area-to-volume ratio (µm².µm⁻³), and the dimensionless roughness coefficient.
7.2.7 Carbon dioxide production measurement reactor (CMR) setup

The CMR was situated downstream of the flow chambers (Figure 7.1) and allowed the real-time, non-destructive measurement of the CO$_2$ produced by the whole biofilm as well as the contributions of the shear-susceptible biofilm region, the remaining base biofilm layer after the removal of the shear-susceptible layer, and the biofilm-derived planktonic cells leaving the flowcell in the effluent.

Figure 7.1: A schematic diagram of the experimental setup showing the flowcell and CO$_2$ production measurement reactor (CMR).

The CO$_2$ production rates of the different biofilm regions were measured in the gas phase using two different measuring regimes; namely, an open- and closed-loop configuration. The CMR consisted of a 20 ml serum vial, sealed with a butyl rubber stopper containing four ports (one
port each for the inflowing liquid, outflowing liquid, inflowing gas, and outflowing gas), as well as a magnetic stirrer bar. All of the tubing connecting the flow chambers, CMR, and CO₂ gas analyzer consisted of Tygon, which has very low gas permeability. Sterile growth medium with or without dissolved CO₂ (for \( k_a \) determination) or flowcell effluent (to measure steady-state biofilm CO₂ production rate) was delivered to the CMR at a constant flow rate (\( F_l = 37.5 \text{ ml.h}^{-1} \)) provided by a Watson Marlow peristaltic pump.

Air without CO₂ (grade TOC < 0.5 ppm CO₂, Linde Canada) was bubbled through the liquid in the CMR at a constant gas flow rate (\( F_g = 1530 \text{ ml.h}^{-1} \)) provided by a second peristaltic pump. The gas flow rate was determined by volumetric displacement. Off-gas CO₂ concentrations were measured with an absolute, non-dispersive, infrared LI-820 CO₂ gas analyzer (LI-COR Biosciences, Nebraska).

### 7.2.7.1 Theory for the open loop system configuration

Measurement of the total amount of CO₂ produced by an intact (\textit{in situ}) biofilm growing in a flowcell was accomplished by using the CMR in an open loop configuration (clamp 3 was closed, while clamps 1, 2 and 4 remained open, referring to Figure 7.1).

A CO₂ mole balance over the flowcell at steady-state (with no accumulation) can be written as follows:

\[
0 = \text{mol CO}_2 \text{ into flowcell} - \text{mol CO}_2 \text{ out of flowcell} + \text{mol CO}_2 \text{ generated within flowcell}
\]

\[
= c_{FC,in} \cdot F_{l, FC,in} - c_{FC,out} \cdot F_{l, FC,out} + CER_{FC}
\]

\[
= c_{FC,in} \cdot F_{l, FC,in} - c_{A,CMR,in} \cdot F_{l, FC,out} + CER_{FC}
\] (1)
Where $F_{l,FC,in} = F_{l,FC,out}$ is the liquid flow rate into and out of the flowcell respectively (litre.h$^{-1}$); $c_{FC,in}$ is the CO$_2$ concentration in the sterile liquid medium entering the flowcell ($\mu$mol CO$_2$.litre$^{-1}$ of liquid medium); $c_{FC,out}$ is the CO$_2$ concentration exiting the flowcell and serves as the feed to the CMR (this assumes that all of the CO$_2$ produced in the flowcell reached the CMR since the connecting Tygon tubing is highly impermeable to CO$_2$, thus recognizing that $c_{FC,out} = c_{A,CMR,in}$) and CER$_{FC}$ is the CO$_2$ production rate ($\mu$mol of CO$_2$.h$^{-1}$) of all microbial cells within the flowcell (sum of CO$_2$ contributions from all three in situ biofilm fractions, namely, the biofilm-derived planktonic cells and the shear-susceptible and base biofilm layers).

For a gas that does not interact with the liquid phase, the gas mole balance across the CMR can be written as:

$$V_l \frac{\partial c_{l,CMR}}{\partial t} = F_{l,CMR,in} \cdot c_{l,CMR,in} - F_{l,CMR,out} \cdot c_{l,CMR} + k_l a (c^* \cdot c_{l,CMR}) V_l + CER_{CMR}$$

(2)

Where $V_l$ is the liquid volume in the CMR (litre); $c_{l,CMR}$ is the dissolved gas concentration in the liquid phase in the CMR ($\mu$mol gas.litre$^{-1}$ of liquid medium); $F_{l,CMR,in}$ is the liquid flow rate into the CMR (litre liquid medium.h$^{-1}$); $F_{l,CMR,out}$ is the liquid flow rate out of the CMR (litre liquid medium.h$^{-1}$) where $F_{l,CMR,in} = F_{l,CMR,out}$; $k_l a$ is the volumetric transfer coefficient (h$^{-1}$); $c^*$ is the concentration of gas in the liquid phase that is in equilibrium with the gas phase ($\mu$mol gas.litre$^{-1}$ liquid medium) and CER$_{CMR}$ is the gas produced in the system ($\mu$mol of CO$_2$.h$^{-1}$).

Dissolved CO$_2$ interacts with water, however, and can convert to bicarbonate, carbonate and carbonic acid, depending on environmental conditions such as the pH, temperature, and ionic strength of the solution (Schumpe et al. 1982). To accommodate the conversion of dissolved CO$_2$ to the bicarbonate ion under the experimental pH conditions, Bonarius et al. (1995) introduced $c_A$ as a term to include both of the forms of CO$_2$ in the liquid phase, i.e., the sum of the dissolved CO$_2$ ($c_{CO_2}$) and the bicarbonate ion ($c_{HCO_3}$) or $c_A = c_{CO_2} + c_{HCO_3}$ (Bonarius et al.
The equilibrium constant, $K_1$, which relates the equilibrium of dissolved CO$_2$ and the bicarbonate ion

$$K_1 = \frac{c_{HCO_3^-} - c_H}{c_{CO_2}}$$

can then be used to find a relationship between $c_A$ and $c_{CO_2}$

$$c_{CO_2} = \frac{c_A}{(1 + 10^{pH-10-pK_1})}$$

(3)

It should be noted that only the dissolved form of CO$_2$ can move across the gas-liquid interface and be transferred to the gas phase (i.e., bicarbonate ions cannot). Therefore, at steady-state and CER$_{CMR}$ of 0 (CO$_2$ produced by the biofilm-derived planktonic cells in the CMR from the effluent is negligible; see below), substituting equation 3 into equation 2 (taking the “transfer” of dissolved CO$_2$ to bicarbonate ions due to the pH range of the experiment into account) yields:

$$0 = F_{l,CMR,in} \cdot c_{A,CMR,in} - F_{l,CMR,out} \cdot c_{A,CMR} + k_1 a_{CO_2} \left( \frac{c_A}{(1 + 10^{pH-10-pK_1})} - \frac{c_{A,CMR}}{(1 + 10^{pH-10-pK_1})} \right) V_l$$

(4)

A gas phase CO$_2$ balance around the CMR yields:

$$\frac{p}{RT} V_g \frac{\partial x_{g,CMR}}{\partial t} = \frac{p}{RT} F_{g,CMR,in} x_{g,CMR,in} - \frac{p}{RT} F_{g,CMR,out} x_{g,CMR} - k_1 a_{CO_2} V_l \left( \frac{c_A}{(1 + 10^{pH-10-pK_1})} - \frac{c_{A,CMR}}{(1 + 10^{pH-10-pK_1})} \right)$$

(5)

where $V_g$ is the headspace volume (litre); $F_{g,CMR,in}$ is the total gas volumetric flow rate into the CMR (litres of gas.h$^{-1}$); $F_{g,CMR,out}$ is the total gas volumetric flow rate out of the CMR ($F_{g,CMR,in} = F_{g,CMR,out}$); $x_{g,CMR,in}$ is the CO$_2$ concentration of gas flowing into the CMR (ppm, µl of CO$_2$.litre$^{-1}$ of total gas); $x_{g,CMR}$ is the CO$_2$ concentration in the gas phase in the CMR (ppm, µl of CO$_2$.litre$^{-1}$ of
total gas); P is the pressure in the CMR (kPa); R is the universal gas constant (litre.kPa.K⁻¹.mol⁻¹) and T is the temperature of the gas in the CMR (in K).

At steady-state and with $x_{g, \text{CMR}, \text{in}} = 0$ (CO₂-free air was used for gas flow) equation 5 becomes:

$$0 = -\frac{P}{RT} F_{g, \text{CMR}, \text{out}} \cdot x_{g, \text{CMR}} - k_{l}a_{CO_2} \cdot V_{l} \left( \frac{c_{g}^{*}}{1+10^{pH_{10^{-pK_{1}}}}} \right) \left( \frac{c_{A, \text{CMR}}}{1+10^{pH_{10^{-pK_{1}}}}} \right)$$  (6)

Substituting equation 6 into equation 4 yields:

$$0 = F_{l, \text{CMR}, \text{in}} \cdot c_{A, \text{CMR}, \text{in}} - F_{l, \text{CMR}, \text{out}} \cdot c_{A, \text{CMR}} - \frac{P}{RT} F_{g, \text{CMR}, \text{out}} \cdot x_{g, \text{CMR}}$$  (7)

Considering the Henry’s law relationship of CO₂ concentration in the gas phase in equilibrium with the liquid phase and equation 3 yields:

$$c_{g}^{*} = \frac{P}{RT} x_{g, \text{CMR}} = \frac{c_{A}}{H} \left( \frac{P}{RT} x_{g, \text{CMR}} \right)$$  (8)

where H is the dimensionless Henry’s law coefficient (litre of liquid.litre⁻¹ of gas). Equation 6 and equation 8 yield:

$$c_{A, \text{CMR}} = \left( 1 + 10^{pH_{10^{-pK_{1}}}} \right) \left( \frac{P}{RT} x_{g, \text{CMR}} \right) \left( \frac{F_{g, \text{CMR}, \text{out}}}{k_{l}a_{CO_2}V_{l}} + \frac{1}{H} \right)$$  (9)

Substituting equation 9 into equation 7 yields:

$$c_{A, \text{CMR}, \text{in}} = \left( 1 + 10^{pH_{10^{-pK_{1}}}} \right) \left( \frac{P}{RT} x_{g, \text{CMR}} \right) \left( \frac{F_{g, \text{CMR}, \text{out}}}{k_{l}a_{CO_2}V_{l}} + \frac{1}{H} \right) + \frac{P}{RT} F_{g, \text{CMR}, \text{out}} x_{g, \text{CMR}}$$  (10)

By substituting equation 10 into equation 1, it is possible to determine CERFC (CO₂ contribution from all of the biomass fractions in the flowcell). The CO₂ contribution of the base biofilm
region was determined by solving equation 1 after the bubble had removed the shear-susceptible biofilm layer, and the CO$_2$ contribution of the shear-susceptible fraction was calculated as the difference between the total biofilm CO$_2$ production and that of the base layer.

To determine the CO$_2$ contribution of the shear-susceptible biofilm fraction and the planktonic cells (i.e., biofilm-derived planktonic cells as well as planktonic cells harvested during exponential growth in batch culture), the CMR was used in a closed loop configuration (clamps 1 and 2 were closed after a known volume of effluent was pumped into the CMR, followed by closure of clamp 4; clamp 3 remained open with no flow from the compressed CO$_2$-free air tank) (Figure 7.1).

### 7.2.7.2 Theory for closed loop system configuration

In the closed loop (batch) system, the total amount of CO$_2$ at a specific time was considered a combination of the CO$_2$ in the liquid and gas phases (Chai et al. 2008):

$$m_t = c_{A,CMR} V_l + x_{g,CMR} \frac{p}{RT} V_g$$  \hfill (11)

where $m_t$ is the number of the moles of CO$_2$ in the system at time $t$. In the closed loop it is assumed that the CO$_2$ in the liquid phase is in equilibrium with the gas phase, similar to equation 8 (Dřímal et al. 2006).

$$m_t = (1 + 10^{pH - pK_1}) \frac{p}{RT} \cdot \frac{x_{g,CMR}}{H} V_l + x_{g,CMR} \frac{p}{RT} V_g$$

$$= x_{g,CMR} \frac{p}{RT} \left( (1 + 10^{pH - pK_1}) \frac{V_l}{H} + V_g \right)$$  \hfill (12)
It can therefore be seen that the total amount of CO\textsubscript{2} in the closed vessel can be determined by measuring the CO\textsubscript{2} concentration only in the gas phase.

By measuring the total amount of CO\textsubscript{2} in the system at different time intervals, it is thus possible to determine the rate of CO\textsubscript{2} production (\(\mu\text{mol of CO}_2\cdot\text{h}^{-1}\)) by microbes in the CMR. During short time intervals (< 10 minutes), the increase in the total amount of CO\textsubscript{2} in the system can be attributed primarily to respiration and not to a significant increase in cell numbers.

### 7.2.7.3 Defining the essential parameters for the CMR

#### 7.2.7.3.1 Determining the volumetric transfer coefficient (\(k_\text{la}, \text{h}^{-1}\))

A common, but likely less precise means to estimate the volumetric transfer coefficient of CO\textsubscript{2} for a system, is to assume a linear relationship with the oxygen transfer coefficient, since it is simpler to measure the latter experimentally (Bloemen et al. 2003). In this study, the \(k_\text{la}\) was determined using the static method, as described previously (Blanch and Clark, 1996), which involves solving both the gas and liquid mass balances for the CMR. Steady-state off-gas measurements were obtained for known dissolved CO\textsubscript{2} concentrations (\(c_{A,\text{CMR,in}}\)) in the open loop system configuration, and the \(k_\text{la}\) was calculated by rearranging equation 10.

Known concentrations of CO\textsubscript{2} were generated by agitating solid state CO\textsubscript{2} pellets (dry ice) in the 5 mM citrate growth medium until saturation. This concentrated dissolved CO\textsubscript{2} solution was diluted in sterile medium to concentrations that resulted in off-gas readings similar to those obtained from the flowcell effluent. The concentration of dissolved CO\textsubscript{2} was determined
by measuring the inorganic carbon content with a catalytic combustion, non-dispersive infrared total carbon analyzer (TOC-VCSH/CSN; Shimadzu, Kyoto, Japan). Growth medium, without the addition of the dissolved CO$_2$, was used as a blank to determine the off-gas reading for growth medium equilibrated with atmospheric CO$_2$.

### 7.2.7.3.2 Determining the headspace volume ($V_g$, L)

Since the CMR system was assembled in the laboratory, it was necessary to determine the headspace ($V_g$, in litres) experimentally. A liquid-free CMR was prepared and flushed with CO$_2$-free air until no CO$_2$ could be detected in the off-gas. Precise volumes of growth medium with known dissolved CO$_2$ concentrations, prepared as described above, were injected into the CMR, and the CO$_2$ in the off-gas was measured in the closed loop system configuration. Known concentrations of dissolved CO$_2$ allowed the determination of $V_g$ according to equation 12.

### 7.2.7.3.3 Calculation of the dimensionless Henry’s coefficient (H)

The dimensionless Henry’s coefficient ($c_{gas}/c_{liq}$; litres of gas/litres of liquid) for CO$_2$ was calculated to be 1.12 at 22°C with data from Schumpe et al. (1982) and corrected for the ionic strength of the growth medium (Schumpe et al. 1982).
7.2.7.4 Open loop calculations to determine steady-state whole-biofilm CO₂ production

Whole-biofilm CO₂ production rates were measured at 24-hour intervals with the CMR in an open loop configuration, i.e., continuous, once-through flow of both the liquid and gas phases. Effluent exiting the flowcell entered the CMR at a known flow rate \( F_l = 37.5 \text{ ml.h}^{-1} \) while being sparged with CO₂-free air as before \( F_g = 1530 \text{ ml.h}^{-1} \). Equation 1 was used in conjunction with equation 10 to determine the CO₂ production of the \textit{in situ} whole biofilm. The values for the various parameters were those applied during the \( k_a \) determination, except that in this case the \( c_{A,CMR,in} \) was calculated from the experimental steady-state gas phase CO₂ values captured during measurements by the off-gas analyzer.

7.2.7.5 Closed loop calculations to determine CO₂ production of the shear-susceptible biofilm region and planktonic cells

The closed loop system configuration was selected as a means to distinguish the CO₂ produced by the whole biofilm (originating in the flowcell upstream of the CMR) and that produced by the biofilm-derived planktonic cells swept along by the exiting liquid flow, as well as the shear-susceptible biofilm fraction. The closed loop method measures the increase in CO₂ in the CMR only as a result of respiring suspended cells collected in a known volume of flowcell effluent or bubble-displaced content. In contrast, an open loop system measures dissolved CO₂ in the continuous-flowing liquid, thus also measuring CO₂ that originated from biofilm cells upstream in the flowcell.

In the closed loop configuration the liquid flow into and out of the CMR was halted, while continuous stirring of the liquid phase in the CMR was maintained by the magnetic stirrer. The
gas was continuously circulated through the system via a peristaltic pump at a flow rate of 1530 ml.h\(^{-1}\). Prior to the closed loop measurement, the flowcell effluent entered the CMR under open loop conditions, with the liquid flowing through the CMR at a constant rate and CO\(_2\)-free air being sparged into the liquid. After a steady-state off-gas CO\(_2\) reading was obtained, the liquid flow was halted while the liquid continued to be flushed with the CO\(_2\)-free air for 3 to 5 minutes to drive off the residual dissolved CO\(_2\) that originated from the flowcell biofilm. The liquid in the CMR was subsequently sparged with ambient air until steady-state CO\(_2\) off-gas readings were reached, after which the gas flow was closed, resulting in a continuous recirculation of gas in the headspace through the system. A linear increase in CO\(_2\) measurements was recorded for at least 10 minutes or until the measurement exceeded the upper limit of the CO\(_2\) analyzer (as in the case of the shear-susceptible layer responses, where the detection limit was exceeded after approximately 4.5 minutes).

The CO\(_2\) produced in the CMR was calculated with equation 12 with parameters and variables as before and with \(V_g\) as the headspace volume (in litres). The calculation involved the arbitrary choice of two CO\(_2\) measurements \((x_{g0}\) and \(x_{g1}\)) at specific time points \((t_0\) and \(t_1)\).

It was shown that CO\(_2\) production by the biofilm-derived effluent cells, when not concentrated, was below the closed loop detection limit. This confirmed that the effluent cells produced a negligible amount of CO\(_2\) relative to the biofilm, and so there was no need to take this fraction into account for steady-state open loop measurements.

To measure the CO\(_2\) contribution of planktonic cells exiting the flowcell, effluent from three 48 and 72 hour-old biofilms were collected on ice for 1.5 hours (approximately 160 ml) and the biofilm-derived effluent cells were concentrated by centrifugation at a relative centrifugal force (RCF) of 3000 for 20 minutes at 4°C. After the removal of the supernatant, the remaining cell pellets were resuspended in 3.3 ml of sterile growth medium, of which 3 ml was used to
determine CO\textsubscript{2} production in the closed loop configuration, and the remainder to determine the viable cell numbers after spread plating.

Batch cultures of planktonic cells were incubated until exponential phase of growth (as monitored by optical density measurements at 600 nm), before harvesting the cells by centrifugation and resuspension as described for the biofilm-derived effluent cells. The viable cell numbers and CO\textsubscript{2} production rate for a fixed volume of this suspension were determined in addition to values for an original batch sample and a one in 10 dilution of the latter. The centrifugation and resuspension steps taken in preparation of the batch culture samples were found not to influence the CO\textsubscript{2} production rate per cell number for the various batch culture samples (correlation of $R^2 = 0.998$ between the amount of CO\textsubscript{2} produced per cell number for the various dilutions of exponential phase cells).

### 7.2.8 Real-time PCR reference and target gene selection

A number of genes were selected for analysis with Real-time PCR, which have been shown or are hypothesized to be expressed differentially in the biofilm and planktonic lifestyles. These include the gene for flagellin production, which polymerize to form flagella ($fliC$) (Prigent-Combaret et al. 1999; Sauer and Camper 2001; Sauer et al. 2004), a molecular chaperonin ($groEL$) and a Family 2 glycosyltransferase.

The $groEL$ protein is classified in the first subgroup of molecular chaperonins or heat shock proteins (Hsp 60) and performs essential functions in the cell, which include assisting in protein folding and translocating secreted proteins under both normal and stress conditions (Feltham and Gierasch 2000; Hartl and Hayer-Hartl 2002). Forced down-regulation of $groEL$ in *Streptococcus mutans* led to impaired biofilm formation (Lemos et al. 2007), while proteomic
analysis of *Campylobacter jejuni* indicated that biofilms exhibited increased levels of GroEL and its cofactor GroES, compared to planktonic cells of the same organism (Kalmokoff et al. 2006).

Glycosyltransferases catalyze the glycosylation of proteins, which is the transfer of various glycans to specific amino acid residues within the protein sequence, and leads to the classification of as either N- or O-linked glycosylated proteins. Both of these linkage types have been identified in bacteria, as reviewed by Szymanski and Wren (2005); examples include the O-linked glycosylation of flagellin and N-linked glycosylation of various polymers, which may include flagella, pili and lipopolysaccharides (Szymanski and Wren 2005). The fimbriae of *Streptococcus parasanguinis* are composed of multiple subunits of the Fap1 protein, which has been found to be extensively glycosylated with N-acetylglucosamine and glucose residues (Wu et al. 2007). *S. parasanguinis* mutants lacking the Fap1 protein were shown to adhere poorly to surfaces, and were consequently deficient in biofilm formation (Froeliger and Fives-Taylor 2001). While the inactivation of one of the glycosyltransferases responsible for Fap1 glycosylation did not alter initial surface attachment, the resulting biofilm consisted of only 30% of the corresponding wild-type biofilm biomass (Wu et al. 2007). Thormann et al. (2006) identified a putative family GT-2 glycosyltransferase in *Shewanella oneidensis* and found that a knock-out mutant was similarly severely impaired in biofilm formation subsequent to normal adhesion (84% - 94% less biomass than the wild-type). While not confirmed, the authors speculated that the putatively identified glycosyltransferase formed part of a gene cluster responsible for exopolysaccharide matrix production (Thormann et al. 2006).

The expression levels of the target genes (*fliC*, *groEL* and a glycosyltransferase) were compared to that of two reference genes, namely *16S rDNA* and an alternative sigma factor ($\sigma^v$) encoded by the gene *rpoS*, also known as the stationary phase sigma factor.
Multiple investigations in different prokaryotes have shown that the levels of \textit{\textit{rpoS}} increase during the transition from late exponential to stationary growth phases (Latifi et al. 1996) and as reviewed (Loewen and Hengge-Aronis 1994; Loewen et al. 1998). The regulation of \textit{\textit{rpoS}} and \textit{\textit{RpoS}} levels in \textit{E. coli} has been studied extensively, revealing a complex and as yet not completely understood system involving control at the transcriptional, translational, post-translational and protein stabilization levels (Loewen et al. 1998). Unfortunately the regulation of \textit{\textit{RpoS}} in Pseudomonads has not received as much attention, except in recent reports of transcriptional regulation (Potvin et al. 2008). The use of \textit{\textit{rpoS}} as a reference gene for real-time PCR would ideally require either constitutive expression, or regulation at a translational or post-translational level. Since the mode of regulation in \textit{\textit{Pseudomonas}} sp. strain CT07 is still unknown, the expression levels of \textit{\textit{rpoS}} were determined for each planktonic growth phase.

All of the molecular biology methods (i.e. plasmid isolation, restriction enzyme digests, gel electrophoresis, ligations and cloning) were performed according to standard protocols (Sambrook et al. 1989; Ausubel et al. 1993) or by following the instructions provided by the manufacturer.

\subsection*{7.2.9 Primer design for Real-time PCR}

Since the genome sequence of \textit{\textit{Pseudomonas}} sp. strain CT07 is unknown, degenerate primers were initially designed by analysis of the amino acid sequences of the selected genes from \textit{P. aeruginosa} PA01 (Stover et al. 2000), \textit{P. putida} KT2440 (Nelson et al. 2002) and \textit{Pseudomonas fluorescens} Pf-5 (Paulsen et al. 2005) obtained from the Pseudomonas Genome Database (Winsor et al. 2005). Degenerate primers were designed to conserved regions of the gene sequences for endpoint PCR. The resulting PCR products were cloned into the pGEM-T vector (Promega, Catalogue number A3600) and sent for sequencing at a commercial facility.
Upon comparison of these sequences using the BLAST function (tblastx) of the NCBI database (National Centre for Biotechnological Information database; http://www.ncbi.nlm.nih.gov/), it became evident that most of the nucleotide sequences displayed significant amino acid sequence similarity to genes from either \textit{P. fluorescens} Pf0-1 or \textit{P. fluorescens} Pf-5. The amino acid sequences from these organisms were thus used for the design of subsequent primers. This approach proved to be successful and the nucleotide sequences of the putative genes were sequenced in both directions and used for the design of primers for Real-time PCR (Table 7.1).

7.2.10 Differential gene expression and protein content in biofilms and planktonic cells

\textit{Pseudomonas} sp. strain CT07 biofilms were cultured in glass tubes (\( n = 4 \)) on 1 mM citrate growth medium at a flow rate of 15.3 ml.h\(^{-1}\) as described previously. Batch culture flasks (\( n = 3 \)) containing 100 ml of 1 mM citrate growth medium were inoculated with \textit{Pseudomonas} sp. strain CT07 and incubated at room temperature, with shaking at 300 rpm. Samples were extracted in the lag, early exponential, exponential and stationary phase, as monitored by optical density measurements (600 nm).

7.2.10.1 TRI Reagent for simultaneous RNA and protein extraction

TRI Reagent (Sigma Aldrich, Catalogue number T9424-100 ml), a solution consisting of phenol and guanidine thiocyanate, was used to simultaneously extract RNA and total protein from biofilms and planktonic cells of \textit{Pseudomonas} sp. strain CT07. This commercially available solution was modified from the one-step total RNA isolation reagent developed and described previously (Chomczynski and Sacchi 1987; Chomczynski 1993).
### Table 7.1

The sequences, annealing temperatures and amplicon lengths of the Real-time PCR primer pairs used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplicon length (bp)</th>
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Planktonic cells from batch cultures or biofilm-derived planktonic cells were harvested by centrifugation and resuspension in 0.5 to 1.0 ml of TRI Reagent. The bulk-liquid inside each
biofilm-colonized glass tube (~0.6 ml) was drained by inversion of the tube and collection of the contents in a separate microcentrifuge tube containing 1 ml of TRI Reagent. The inversion removed the biofilm-derived planktonic cells within the bulk-liquid, as well as the shear-susceptible fraction of the biofilm.

The remainder of the attached biomass in the non-shear-susceptible base biofilm region was removed by injecting TRI Reagent into the tubes, followed by repeated washing of the solution over the inner surface with a pipette. Complete removal of biomass from the inner surface was confirmed with epifluorescent microscopy. The TRI Reagent samples were stored at -70°C until further analysis.

7.2.10.2 RNA extraction and cDNA conversion

RNA was extracted from the TRI Reagent samples according to the manufacturer’s protocol. All filter pipette tips and microcentrifuge tubes were double autoclaved, while pipettors and work spaces were treated with Rnase Away (Molecular BioProducts, Catalogue number 7000) to minimize RNA degradation. The isolated RNA was stored in 75% ethanol at -20°C.

The RNA was subsequently solubilized in diethylpyrocarbonate (DEPC) -treated dH₂O and all residual DNA was removed with the Ambion TURBO DNA-free™ kit (Ambion, Catalogue number AM1907). The efficient removal of DNA was verified by Real-Time PCR with 10 to 130 ng of RNA for all of the samples using the 16S rRNA primer set, according to the protocol described below. The DNA-free RNA (4 µl) was converted to cDNA using Roche’s Transcriptor First Strand cDNA synthesis kit with random hexanucleotide primers (Roche, Catalogue number 04 379 012 001).
7.2.10.3 Internal control for RNA extraction efficiency and cDNA conversion

A fragment of the *Arabidopsis* chlorophyll synthetase gene was included in each sample as an internal control for RNA extraction and cDNA conversion efficiency. Since the gene originates from a plant, it is unlikely to bear any sequence similarity to the genome of *Pseudomonas* sp. strain CT07. This was confirmed by end point PCR of *Pseudomonas* sp. strain CT07 genomic DNA using primers for the *Arabidopsis* insert. The plasmid with the *Arabidopsis* gene insert was obtained from the UHN Microarray Centre (Toronto), and transformed into *E. coli* DH5α for amplification. The plasmid was subsequently extracted, linearized with *SacI*, excised from a 1.8% agarose gel, and purified. *In vitro* T7 transcription of the plasmid, using the Promega Riboprobe® in vitro transcription system (Promega, Catalogue number P1440) was performed and resulted in a bright 1 kb RNA fragment, as well as several unexpected smaller fragments. The *Arabidopsis* RNA was added to each TRI Reagent sample at a known concentration prior to extraction and cDNA conversion.

7.2.10.4 Real-time PCR, external standard curves and relative quantification of gene expression

Real-time PCR allows the determination of the number of transcripts of a specific target gene at a particular point in time. The Roche LightCycler software monitors the increase in fluorescence intensity, which corresponds to double stranded DNA product formation once during each PCR cycle and in doing so can detect target concentrations spanning 10 orders of magnitude.

For relative quantification of gene expression with Real-time PCR, the copy number of the target gene in a given sample is expressed relative to the number of copies of a reference or
housekeeping gene in the same sample. Ideally, a reference gene with known constitutive expression under all conditions should be selected for relative quantification; unfortunately no such gene has been identified in this test organism. In order to overcome this limitation, it was decided to use both the 16S rRNA and the rpoS genes as reference genes. The use of 16S rDNA as a reference gene has been evaluated previously and was found to be the most constant in expression level among different environmental conditions (Tasara and Stephan 2007).

Real-Time PCR was performed with the Roche LightCycler® 2.0, using 2.0 µl of the 4x FastStart DNA Master PLUS SYBR Green I Master Mix (Roche, Catalogue number 3515869001), 1 µl of an appropriate dilution of cDNA and 0.75 µl of each 5 µM primer stock solution per 10 µl reaction. All reactions started with a 10 minute activation step at 95°C, followed by 30 to 40 cycles of quantification (consisting of 10 seconds of denaturation at 95°C, 4 to 5 seconds of annealing at either 59 or 60°C and 9 or 14 seconds of amplification at 72°C).

External standard curves, with defined concentrations of the target and reference genes, were generated to quantify the starting copy number of each gene for each sample, on the condition that the PCR amplification efficiencies are similar in both the sample and standard curve reactions. Stock solutions of the target and reference genes were prepared using endpoint PCR with the Real-time PCR primer pairs (Table 7.1). The amplified products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, Catalogue number A9281) prior to determining the concentration of double stranded DNA (OD 260 nm), and calculating the number of gene copies.

Standard curves of these serially diluted PCR products were generated for each target and reference gene by amplifying five of the different dilutions, each in triplicate, with the LightCycler using the settings described above. Once an acceptable PCR efficiency was obtained (E ≥ 1.75), the standard curve was saved and used for the subsequent quantification
of copy number in the experimental samples. Two of the standard curve dilutions with known copy numbers were included as positive controls for each set of Real-time PCR reactions. The $\log_{10}$ of the number of target gene copies per µl of sample, as determined with real-time PCR, was normalized against the $\log_{10}$ of the number of reference gene copies per µl of sample.

### 7.2.10.5 Statistical analysis

Significant differences between the average copy number ratio's of the samples, as well as the CO₂ production rates, direct cell counts, and biofilm parameters were determined with Single-factor analysis of variance (ANOVA, $P = 0.05$). In the event of a rejected null hypothesis, Tukey’s test for the comparison of means was applied to determine which sample pairs were significantly different from each other.

### 7.2.10.6 Protein extraction and concentration determination

Proteins were isolated from the TRI Reagent samples in accordance with a modified protocol (Molecular Research Centre Inc.) which uses acetone for precipitation and a combination of glycerol, guanidine hydrochloride and ethanol for protein purification. The extracted protein was solubilized in a 1% SDS, 50 mM Tris HCl (pH 8), 62.5 mM N-Lauroylsarcosine buffer with incubation at 60°C according to (Banerjee et al. 2003). Any insoluble material was removed by centrifugation and the supernatant stored at 4°C.

The protein concentration in each sample was determined with the BCA™ protein assay kit (Pierce, Catalogue number 23225). A concentration range of bovine serum albumin (BSA, 5 - 250 µg.ml⁻¹) in the same solubilization buffer was used for calibration at 562 nm. A portion of
the protein samples were diluted in the solubilisation buffer and assayed according to the enhanced protocol at 60°C. Since the BCA assay does not reach a fixed end point, all of the duplicate standards and samples were processed within 10 minutes of one another.

### 7.2.10.7 1D Tris-Tricine Polyacrylamide Gel Electrophoresis (1D-PAGE)

The total protein content isolated from the various samples as described above was subjected to size separation on a 9% acrylamide gel. One microgram of protein from each sample was boiled in a Tricine loading buffer for 5 minutes, prior to loading it onto the gel. The gel consisted of both a stacking and resolving gel. The samples were electrophoresed at 30 V for 30 minutes, followed by 125 V until complete migration had been achieved.

### 7.3 Results

#### 7.3.1 Removing the region of the biofilm responsible for the observed yield

The hypothesis that the planktonic cells yielded to the bulk-liquid originated from a particular region of the biofilm was tested by removing the shear-susceptible region of the biofilm through the application of brief pulses of increased fluid shear, while monitoring the effluent cell numbers (Figure 7.2).

The increased flow velocity during the 5 pulses significantly increased the shear forces acting on the biofilms. Biofilm-derived planktonic cell yield declined by 2 orders of magnitude (from $1.6 \times 10^8$ CFU.cm$^{-2}$.h$^{-1} \pm 2.1 \times 10^8$ CFU.cm$^{-2}$.h$^{-1}$ to $2.3 \times 10^6$ CFU.cm$^{-2}$.h$^{-1} \pm 0.7 \times 10^6$ CFU.cm$^{-2}$.h$^{-1}$).
as a result of the pulses, when comparing the yield from 144 hour-old biofilms to that at 0.2 hours subsequent to the last pulse (equal to the time required to replace five reactor volumes). The biofilm-to-planktonic cell yield recovered to \(7.5 \times 10^6\) CFU.cm\(^{-2}\).h\(^{-1}\) ± \(7.2 \times 10^6\) CFU.cm\(^{-2}\).h\(^{-1}\) and \(3.5 \times 10^7\) CFU.cm\(^{-2}\).h\(^{-1}\) ± \(2.6 \times 10^7\) CFU.cm\(^{-2}\).h\(^{-1}\) within 24 and 48 hours, respectively of the increased shear application. The large standard deviations are indicative of the heterogeneous response of replicate biofilms to the treatment. Observation of the glass tubes during each pulse revealed that macroscopic aggregates of biomass were removed by the increased shear and the amount of attached biomass was significantly reduced by the series of pulses. It should be noted, however, that these aggregates were not efficiently dispersed by vortexing alone. It is thus likely that the cell numbers enumerated from each sample collected during the pulses significantly underestimate the amount of biomass removed from the surface by the increased fluid shear.

### 7.3.2 Validation of the CMR experimental system and defining system parameters

The steady-state CO\(_2\) concentrations (\(x_g; \mu l\) of CO\(_2\).litre\(^{-1}\) of air) measured in the off-gas for the corresponding dissolved CO\(_2\) concentrations was highly linear (\(R^2 = 0.997\)) and was moreover within the range of the off-gas measurements taken during the subsequent experimentation.

A \(k_a\) value of 21 h\(^{-1}\) was calculated for the experimental setup described here. The influence of the gas flow rate on \(k_a\) measurements was also investigated, and lower flow rates were found to decrease the \(k_a\) value, which is in agreement with Blanch and Clark (1996). Our results indicate that the \(k_a\) was not constant with varying dissolved CO\(_2\) concentrations and that care has therefore to be taken when determining \(k_a\) for the particular experimental conditions.
Figure 7.2: The application of increased liquid shear to remove the biofilm region responsible for the yield of planktonic cells. (1) The biofilm-derived cell yield in the effluent was determined with spread plating and expressed as the log$_{10}$ of the cell number yielded per attachment area per hour. The washout of unattached inoculum cells is evident up to 2 hours after the initiation of liquid flow at a velocity of 15.3 ml.h$^{-1}$. After 144 hours of incubation, five pulses of increased liquid flow were applied ($F(l) = 498$ ml.h$^{-1}$) with simultaneous collection of the effluent. The recovery of biofilm-derived cell yield was monitored for a subsequent 48 hours. (2) Each pulse resulted in the displacement of approximately one reactor volume, which was collected separately for the enumeration of viable cell numbers.

For known dissolved CO$_2$ concentrations that had off-gas readings in a similar range to what was measured for the whole biofilm, the open and closed loop configurations yielded results that were within 5% of each other. Both pH and $k_a$ may have noticeable influences on the determination of dissolved CO$_2$ concentration from off-gas measurements using equation 10.
In the current experimental system, the effluent pH (6.92) did not differ significantly from that of the sterile growth medium (pH 6.97).

7.3.3 Steady-state whole-biofilm CO$_2$ production

Whole-biofilm CO$_2$ production rates were measured at 24 hour-intervals for the first 120 hours, at 2 and 4 hours after the bubble perturbation, and thereafter again at 24-hour intervals for the remainder of the experiment (Figure 7.3) using the open loop system configuration.

The removal of the shear-susceptible biofilm region led to a significant reduction (P < 0.05) in the rate of CO$_2$ production in the flowcell; only 28% ± 29% of the pre-disturbance CO$_2$ production rate was maintained by the remaining non-shear-susceptible base biofilm region 2 hours after the perturbation (total 122 hours of incubation). CO$_2$ production in the base biofilm region recovered to 51% ± 41% of the previous steady-state production rate within 4 hours after the removal of the shear-susceptible biofilm fraction and to 104% ± 42% after an additional 24 hours. Interestingly, the post-disturbance rate of CO$_2$ evolution stabilized at a higher level than what was observed previously (136% ± 64% at 48 hours after the bubble, although this increase was not statistically significant at a P value of 0.05).
Figure 7.3: The average whole-biofilm CO\textsubscript{2} production rate, as measured for six biofilms (24 to 120 hours) and three biofilms (122 hour and 124 - 192 hours), using the open loop system configuration. After the acquisition of the steady-state measurements of the respiration rate at 120 hours, an air bubble was introduced into each flowcell to remove the shear-susceptible biofilm region. Three of the biofilms were sacrificed at this stage to determine the cell numbers in the base biofilm region, while open loop measurements continued on the three remaining flowcells at 2 and 4 hours after the bubble perturbation, and thereafter at 24-hour intervals for a total of 192 hours.

7.3.4 Biofilm-to-planktonic cell yield

In addition to the respirometry, the number of free-floating cells produced and released into the effluent by the biofilms was determined with viable counts as well as direct fluorescent counting and image analysis (Figure 7.4).
Microscopic direct counts facilitated the comparison of the cell numbers in the shear-susceptible and base biofilm regions, which was not possible with viable counting methods since these fractions required dispersion using sonication and preservation with formaldehyde.

**Figure 7.4:** The number of planktonic cells produced by the biofilms and released to the bulk-liquid was enumerated from the flowcell effluent with direct fluorescent counts and viable counts at 24-hour intervals. The average planktonic cell yield from six biofilms was enumerated from 24 to 120 hours and three biofilms from 144 to 192 hours.

The yield of cells from the biofilms initially decreased less than one order of magnitude (a significant decrease for the viable cell counts, but not the direct counts at a P value of 0.05) after the introduction of the bubble despite the fact that a significant fraction of the attached biomass was removed, as suggested by the reduction in the rate of CO₂ production after the bubble (Figure 7.3) and shown by a series of CLSM micrographs of the biofilm taken over the course of the experiment (Figure 7.5). Planktonic cell yield from the biofilm recovered to pre-
disturbance levels after 168 to 192 hours of incubation (48 and 72 hours after the bubble disturbance; statistically there was no significant difference compared to 120 hours, with a P value of 0.05).

7.3.5 Biofilm structure

The bubble perturbation removed a large portion of the biofilm biomass at the glass surface; mostly single cells and a few smaller cell aggregates were observed attached to isolated regions of the glass along the central transect of the flow chamber (Figure 7.5 After bubble, glass), while more extensive biomass survived the perturbation along the edge of the chamber; likely as a result of reduced shear forces in these areas (Figure 7.5 After bubble, edge). Image analysis was not performed at this point due to insufficient amounts of biomass at the area normally viewed (i.e., central transect) of the flow chambers.

COMSTAT image analysis of the CLSM-acquired images provided information on biofilm architecture in terms of the amount of biomass and the average thickness (Figure 7.6.1), the roughness coefficient and the surface-to-biovolume ratio (Figure 7.6.2) and the maximum thickness (Figure 7.6.3). At the magnification applied, CSLM only allowed the observation of the biofilms growing on the glass coverslips (35% of total surface area available for attachment). However, the observed changes in biomass volume and the average biofilm thickness showed a similar trend as the whole-biofilm CO₂ production and did not reach a steady-state in the first 120 hours of cultivation.

Biofilm architecture at the glass surface was also significantly altered after the perturbation; the bubble-mediated removal of the shear-susceptible biofilm region resulted in significant changes (P < 0.05) in all of the measured biofilm parameters at 144 hours (24 hours after the
perturbation). Analysis of these parameters indicated that the biofilms were rougher, had a larger surface area-to-volume ratio, consisted of less biomass and exhibited a reduced thickness compared to the pre-disturbance biofilm (Figure 7.6).

Figure 7.5: CLSM micrographs taken at 24-hour intervals at random locations of the *gfp*-labelled *Pseudomonas* sp. strain CT07 biofilms cultivated in conventional flowcells under continuous-flow conditions. (A) 24 hour, (B) 48 hour, (C) 72 hour, (D) 96 hour and (E) 120 hour. (After bubble, glass) The single cells at the glass surface, and (After bubble, edge) the biomass along the edge of the flowcell where the glass coverslip meets the Plexiglas 1 hour after the perturbation, (F) 24 hours after the perturbation (144 hour) and (G) 48 hours after the perturbation (168 hour). Micrographs collected 1 hour after the bubble perturbation and along the edges of the flow chambers were not included in the image analysis with COMSTAT.
Biofilm biomass and the surface area-to-volume ratio at 144 and 168 hours (24 and 48 hour after the disturbance) recovered to the levels measured for the 72 and 24 hour-old biofilms, respectively, while the mean biofilm thickness recovered to the values observed for a 96 hour-old biofilm (P = 0.05).

These observations, together with the increased steady-state whole-biofilm CO₂ production and recovery of biofilm-to-planktonic cell yield, indicated that biofilms remained metabolically active during the first 48 hours after the disturbance and that rebuilding of biofilm structure occurred soon after the disturbance.

### 7.3.6 Comparison of CO₂ production rates.

The average rate of CO₂ production per cell was compared between planktonic cells in the exponential phase of growth (cultured in batch), concentrated biofilm-derived planktonic cells, the shear-susceptible biofilm layer (both *in situ* and in the CMR after removal from the flowcell) and the base biofilm layer (Figure 7.7). The average cell number of the non-shear-susceptible base biofilm layer at 120 hours was \(4.8 \times 10^8 \pm 1.9 \times 10^8\) while that of the shear-susceptible fractions was \(6.9 \times 10^8 \pm 2.5 \times 10^8\). Thus, approximately half of the bacterial cells were removed from the flowcell by the bubble perturbation while only \(28\% \pm 29\%\) of the pre-disturbance CO₂ rate of production was maintained by the remaining base biofilm after the bubble removal event. Using the average cell numbers in the respective biofilm layers, it was possible to calculate the steady-state CO₂ production rates per cell, as indicated in Figure 7.7.
Figure 7.6: COMSTAT image analysis of five biofilm parameters was averaged for three biofilms cultivated under the same conditions as described previously. (1) The average amount of biofilm biomass at the surface ($\mu m^3/\mu m^2$) and the average biofilm thickness ($\mu m$), (2) the roughness coefficient and surface area-to-volume ratio ($\mu m^2/\mu m^3$) and (3) the maximum thickness. The absence of sufficient biofilm biomass at the glass surface after the bubble
perturbation at 121 hours (Figure 7.5) did not allow the capture of images suitable for analysis, and hence this data point was not included.

Figure 7.7: The average rate of CO$_2$ production per cell was compared between (exp phase) planktonic cells in the exponential phase of growth; (yield) concentrated biofilm-derived effluent cells; (shear) the shear-susceptible biofilm and (base 1 hour) the non-shear-susceptible base biofilm layer 1 hour after the bubble perturbation. Activity of the shear-susceptible biofilm layer was determined in situ, as part of the whole biofilm (shear in situ) and as suspended cells after removal from the biofilm (shear).

7.3.7 Differential gene expression and protein content in four phases of planktonic growth

To develop the technique and verify whether the expression of the reference genes (16S rDNA and rpoS) remained unchanged, the copy number of the reference and target genes was quantified in four phases of growth in batch culture (Figure 7.8.1). In addition to determining
the number of gene copies, the viable cell number and total protein content were also determined for each sample (Figure 7.8.2).

The ratio of rpoS expression versus that of the 16S rDNA gene remained consistent during all phases of planktonic cell growth, and no statistically significant differences were detected. This validated their selection as references genes and use in subsequent real-time PCR experiments. Statistical analysis of the results indicated that the expression of fliC and groEL declined in the stationary phase, when compared to the other three phases (P < 0.05) (Figure 7.8.1). The amount of protein extracted from each phase of growth correlated well with the viable cell numbers, as determined by serial dilution and spread plating (R² = 0.86), thereby confirming that the total protein concentration could be used as an indicator of total biomass.

7.3.8 Differential gene expression in the biofilm

To evaluate whether the target genes were expressed differentially in surface-associated environments, four glass tubes were inoculated with Pseudomonas sp. strain CT07 as previously described. The shear-susceptible and persistent base biofilm regions and associated planktonic cell yield were harvested by sacrificing two tubes each after 72 and 96 hours of growth (Figure 7.9). Between 6 and 8 ml of effluent was collected from each tube to ensure that a sufficient amount of RNA could be extracted from the biofilm-derived planktonic cells.

The log₁₀ of the different ratios of target to reference gene copies per µl was determined with Real-time PCR, and the results for 72 and 96 hours were averaged prior to statistical analysis. It was decided to exclude the shear-susceptible biofilm fraction from the ANOVA analysis, since biofilm-derived planktonic cells were present in the bulk-liquid phase in each glass tube, which
was collected as the air bubble removed the shear-susceptible biofilm region. It is therefore not possible to differentiate between RNA extracted from these two sources.

Statistical analysis revealed a significant difference between the expression of the *fliC* and *groEL* genes when comparing the persistent base biofilm region and the newly yielded planktonic cells after 72 and 96 hours of growth \((P < 0.05)\). The expression levels of both genes were higher in the yielded planktonic cells. The expression of the *rpoS* gene did not change relative to that of the 16S rDNA gene for these samples, as confirmed previously for the batch cultures.

As mentioned previously, an internal control (*Arabidopsis* RNA) was added to each sample to monitor RNA extraction efficiency and its conversion to cDNA. Significant differences in the copy numbers of the control gene were, however, detected between both the batch culture and the biofilm samples. This could be ascribed to the cumulative effects of RNA degradation during storage, as well as incomplete extraction and conversion processes. In addition, the presence of several unexpected RNA fragments after *in vitro* transcription of the *Arabidopsis* DNA could have contributed to an overestimation and variation in the amount of control RNA added to each sample. These results were therefore not taken into account.
1: Real-time PCR relative quantification of gene expression

![Graph showing gene expression ratios for different phases of planktonic cell growth.]

2: Total protein content and viable cell count

![Graph showing total protein content and viable cell count for different phases of planktonic cell growth.]

**Figure 7.8:** The expression of selected genes, the total protein content and the viable cell numbers were determined for four phases of planktonic cell growth in batch culture. (1) The relative quantification of the expression of selected genes using Real-time PCR, and (2) the simultaneous determination of total protein content and planktonic cell numbers from four different phases of growth in batch cultures of *Pseudomonas* sp. strain CT07. Samples were...
taken in the lag, early exponential, exponential and stationary phase, as monitored by optical density measurements (600 nm). The average ± standard deviation of the various parameters sampled from triplicate batch cultures is shown.

![Figure 7.9](image_url)

**Figure 7.9:** The relative quantification of the three target genes (*fliC*, *glyc* and *groEL*) versus the reference genes (*rpoS* and *16Sr DNA*) in the non-shear susceptible base biofilm region, the shear-susceptible biofilm fraction and the biofilm-derived planktonic cells yielded to the bulk-liquid using real-time PCR. The average s± standard deviation of replicate glass tubes containing 72- and 96-hr old biofilms (total n = 4 biofilms) are given.

### 7.3.9 Differential total protein content in the biofilm

*Pseudomonas* sp. strain CT07 biofilms were established in 10 square glass tubes as previously described. Biofilm effluent as well as the two biofilm fractions (shear-susceptible and the persistent base biofilm) were harvested from two tubes each at 12, 24, 48, 72 and 96 hours to
extract total protein content. The viable effluent cell numbers were also quantified by spread plating after serial dilution. This experiment was replicated.

A positive correlation ($R^2 = 0.84$) between the yield of biofilm-derived planktonic cells (CFU.cm$^{-2}$.h$^{-1}$) and the amount of protein isolated from these samples (µg protein.cm$^{-2}$.h$^{-1}$) was observed (Figure 7.10), similar to results presented previously for suspended cells from batch cultures (Figure 7.8.1).

**Figure 7.10:** A comparison between the yield of biofilm-derived planktonic cells and the amount of protein extracted from the effluent. The different methods of enumeration correlated reasonably well ($R^2 = 0.84$). The average ± standard deviation of replicate experiments are given; the effluent from two tubes per sample time, per experiment were sampled for viable cell and total protein content determinations.
The total protein content isolated from the shear-susceptible and non-shear-susceptible base biofilm regions (Figure 7.11) likely originated from cellular as well as non-cellular fractions, i.e. extracellular proteins excreted and trapped in the EPS as well lysed cell content. Draining the liquid content from the glass tubes removed a visible amount of biomass from the surface, consisting of a creamy, slime-like mass, which increased as the biofilm aged. The non-shear-susceptible fraction of the biofilm that remained in the tube was often substantially less visible, but still accounted for at least half of the total protein isolated from the entire biofilm.

Figure 7.11: The total protein content of the shear-susceptible and non-shear-susceptible biofilm regions, as well as the biofilm-derived cell yield was determined over a 4-day period. The protein content of the biofilm-derived planktonic cell yield (µg.cm\(^{-3}\)) was normalized to the internal volume of each tube, while that of the biofilm regions were normalized with respect to the surface area available for attachment (µg.cm\(^{-2}\)).
One microgram of the total protein content from the various samples taken at 72 and 96 hours were separated on 1-dimensional tris-tricine polyacrylamide gels to compare and detect any distinct banding patterns, which could be indicative of different phenotypes (Figure 7.12). No observable difference between the biofilm-derived planktonic cells or the biofilm fractions could be observed with 1-D PAGE. The results presented here cannot differentiate between cellular or extracellular proteins, since the extraction technique was not optimized for the isolation of either fraction of proteins.

**Figure 7.12:** The separation of total protein content isolated from the different biofilm regions and biofilm-derived planktonic cells on 1D tris-tricine PAGE and stained with silver. The proteins were extracted after (A) 72 and (B) 96 hours of incubation. The samples are flanked on either side by a protein standard (M), and the samples from duplicate tubes are ordered as follows: (1 and 4) Biofilm-derived planktonic cells, (2 and 5) shear-susceptible biofilm region and (3 and 6) non-shear removable biofilm.
7.4 Discussion

Spatial differences in biofilm cohesivity have been observed after the application of increased shear forces. Coufort et al. (2007) subjected both aerobic and anaerobic biofilms, cultivated on ethanol or waste water, to increased shear stress and found that the biofilm layer at the bulk-liquid interface was removed by slight increases in shear rates (0.2 Pa), whereas the middle and base biofilm layers were able to resist removal when exposed up to 2 Pa and 13 Pa, respectively (Coufort et al. 2007). Total organic carbon analyses indicated that the sensitive top layer of the biofilm contained approximately 60% of the total biofilm biomass, while the remaining two layers each represented approximately 20%. In a follow-up study, biofilms grown under similar conditions exhibited comparable degrees of heterogeneity in the susceptibility of the various biofilm layers to shear and abrasion (Derlon et al. 2008). It was also indicated that the basal biofilm layer contained active microorganisms, as characterized by oxygen uptake rates, but no details were provided on the methodology or time lapse after the removal of the less-cohesive upper biofilm layers.

The goal of this investigation was not to measure the shear forces generated by the moving air bubble or the extent to which the *Pseudomonas* sp. strain CT07 biofilms can withstand difference shear forces, but, rather, to selectively remove a region of the biofilm to evaluate biofilm metabolic response in terms of CO₂ production and biofilm-derived planktonic cell yield, as well as structural adaptation of the biofilm, by utilizing microscopy and image analysis. Other environmental perturbations, such as increased fluid shear, particle abrasion, or an antimicrobial challenge could also be utilized to achieve a similar outcome. The choice of an air bubble proved to simplify execution since it facilitated the quick and efficient removal of the shear-susceptible biofilm region for immediate downstream analysis of changes in CO₂ production rates, which was an important consideration for the timely capture of the expected rapid response in microbial metabolism. The use of an air bubble furthermore yielded
reproducible results, as can be seen from the notably similar numbers of cells enumerated from the different shear-susceptible regions after dispersion (no statistically significant difference; P value of 0.05).

The application of increased fluid flow rates, although effective, proved to be less precise; better standardization of the duration of the pulses, through automated rather than manual control, could potentially improve the outcome. The use of an air bubble enabled the expansion of the initial hypothesis of metabolic differentiation in the biofilm to include speculation on a possible structure-function relationship. It is proposed that the various biofilm regions perform functions related to either survival or proliferation.

The results presented here indicate that the shear-vulnerable layer, which in this case contained half of the biofilm cells, produced 72% of the CO$_2$ in the system. This is consistent with previous indications of spatial heterogeneity in biofilm metabolic activity. In the past, most experimental strategies used for the determination of biofilm activity utilized microscopy, in combination with fluorescent reporter genes or probes that target various indicators of physiological activity in the cell. Several fluorescent stains have been applied, such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Huang et al. 1995), acridine orange (Wentland et al. 1996) as well as the commercially available BacLight viability kit (Korber et al. 1997). The use of alkaline phosphatase as a reporter gene indicated that protein synthesis occurred at the bacterial colony-air interface and at the biofilm-liquid interface for biofilms cultivated under continuous flow conditions (Werner et al. 2004). Rani et al. (2007) also reported on stratified DNA replication, protein synthesis, and respiratory activity in staphylococcal colonies. Moreover, these activities were found to be co-located at the biofilm-air (upper 31- to 38-µm layers) and the biofilm-nutritive substratum interface (14- to 16-µm layers) of 153- to 172-µm thick colonies. The authors identified four cell physiologies, namely; active aerobic growth, active anaerobic growth, non-active or dormant but viable cells at the
interior of colonies, and dead cells (approximately 10% of the total), and suggested that the dormant cells may regain activity if exposed to oxygen and (or) nutrients (Rani et al. 2007).

Although the reporter genes and fluorescent probes have proven to be useful, the techniques usually suffer from inherent disadvantages (Stewart and Franklin 2008); such as incomplete penetration of fluorescent stains and the production of artefacts and perhaps most significantly generally only allow end point analysis due to cellular toxicity. Reporter gene technologies may circumvent this problem but require prior genetic manipulation and it is unknown what, if any, changes in cell physiology may occur as a result of reporter gene expression. The need for genetic manipulation further constrains analysis to pure culture studies.

The results show that biofilm-derived single cells as well as the cells in the shear-susceptible biofilm layer (in situ) are metabolically less active – as indicated by a lower average CO$_2$ production rate on a per cell basis – than exponentially growing planktonic cells but more active than cells in the base biofilm layer one hour after exposure to the bulk-liquid (i.e., after the removal of the shear-susceptible layer) (Figure 7.7). The rapid increase in the CO$_2$ production rate by the base biofilm layer provides empirical support for numerous suggestions that these cells are less active (i.e., dormant, but not dead) for reasons such as diffusion limitation. The basis for spatial heterogeneity in biofilm physiological activity is widely accepted (as reviewed by Spormann 2008; Stewart and Franklin 2008). Limited diffusion of nutrients and oxygen into the biofilm from the bulk-liquid and waste products out of a multi-layered biofilms, is one of the simplest explanations since the absence of complete exchange with the environment, in concert with microbial activity, leads to the formation of chemical gradients in the biofilm. The bacteria in the biofilm respond to the gradients, likely by altering gene expression patterns as determined by global regulators. The remarkable resistance of
biofilms towards the actions of many antimicrobials may in part be due to the insensitivity of dormant cells in the regions of the biofilm where limited diffusion reduces metabolic activity.

In addition to the rapid response of the cells in the base biofilm layer to oxygen and nutrient availability, the average per cell CO₂ production rate of the shear-susceptible layer, after removal from the flowcell, was found to be significantly higher than the in situ rate. These results demonstrate that biofilms possess a remarkable ability to not only respond rapidly to environmental changes, but also retain a powerful metabolic capacity when access to nutrients and oxygen becomes unrestricted. Unlike persister cells that are apparently programmed primarily for survival, the primary function of cells from this shear-susceptible region is probably to optimize proliferation through the production and release of the significant numbers of single cells observed in the bulk-liquid.

It should be pointed out that the term ‘persisters’ usually refer to cells that can survive high antibiotic concentrations; while the disturbance in the present study was shear. In contrast to the small percentage of persisters (less than 1% of the total biofilm population in E. coli, according to Harrison et al. 2005), we found that approximately 50% of the biofilm cells could withstand the applied shear in this case. An increase in shear would likely result in the removal of a greater percentage of biofilm biomass, but this was outside the scope of this investigation. The increase in CO₂ production after the base layer was exposed (Figure 7.3) indicates that, similar to the cells in the shear-susceptible region that rapidly increase their activity when transferred to a planktonic state, these previously dormant (or less active) cells can speedily respond to re-establish overall biofilm activity at the surface. The findings reported here are thus in agreement with previous indications of activity in the basal biofilm layer (Derlon et al. 2008) and may be classed as inactive or dormant, but viable microorganisms (Rani et al. 2007).
The observation that biofilm-derived planktonic cells exhibit respiration rates significantly lower than that of planktonic cells in the exponential phase of growth was somewhat unexpected. Previous results had indicated that the biofilm-derived planktonic cells were more susceptible to killing by an antimicrobial than chemostat-cultured planktonic cells and it was speculated that the greater sensitivity was due to a higher metabolic activity, or differential gene expression (Bester et al. 2005). Per cell CO$_2$ production rates on par with that of the shear-susceptible biofilm layer (the most likely source of the cells observed in the effluent) suggest rather that these cells may be in a lag phase due to recent cell division and separation from the biofilm. The duration of this lag phase and subsequent biofilm-forming ability is of interest to our group and was investigated in a subsequent study (Kroukamp et al. 2010).

Numerous investigations into biofilm-specific gene expression patterns have been, and are being conducted in the hope of identifying genes, of known and unknown function, involved in biofilm formation. The prevailing rationale behind most of these studies is to identify genes or regulatory pathways that can be targeted to prevent or eliminate unwanted biofilm formation in medicinal or industrial environments.

The purpose of this investigation into the expression of a selected number of genes was not to identify a unique gene that is expressed only when a bacterium becomes associated with a surface, but rather as a means to distinguish biofilm-derived planktonic cells from the cells in the biofilm. The presence of single cells in the bulk-liquid of continuous-flow systems is most often ascribed to passive erosive forces with little consideration being given to the role of microbial activity in producing these cells and modulating the yield of cells in response to environmental changes.
The expression of $fliC$ was found to be downregulated in *E. coli* K12 biofilms (Prigent-Combaret et al. 1999), as well as *P. putida* biofilms, where biofilms as young as 6 hours exhibited differential gene expression profiles compared to that of planktonic bacteria (Sauer and Camper 2001). The expression of $fliC$ was upregulated in 3 day-old biofilms, which was attributed to detachment or dispersion of cells from mature biofilms. In contrast to mature biofilms, flagella have also been shown to be involved in the initial attachment of cells to the substratum, since flagella-negative mutants exhibited poor attachment to surfaces as diverse as polyvinylchloride plastic, soil, seeds and potato roots (De Weger et al. 1987; DeFlaun et al. 1994; O'Toole and Kolter 1998a).

The results presented here show that the expression of two putative genes ($fliC$ and $groEL$) in non-stationary phase planktonic cells as well as biofilm-derived planktonic cells was significantly higher than the expression in the non-shear-susceptible base biofilm region and planktonic cells in the stationary phase of growth. The finding that the copy number of the putatively identified $groEL$ gene is higher in planktonic cells than the biofilm, is contradictory to that of Kalmakoff et al. (2006) for *Campylobacter jejuni* biofilms, but not entirely unexpected for metabolically active planktonic cells, since the main functions of $groEL$ in the microbial cell is thought to be related to protein folding and transport, which is associated with active protein synthesis in the cell. The increased expression of $fliC$ in biofilm-derived planktonic cells, relative to that of the cells attached to the glass surface in the persistent base biofilm region, suggests that these cells are producing or have already acquired flagella. The average per cell CO$_2$ production rate measured for the biofilm-derived cells was also higher than that for the base biofilm layer. Taken together, these results suggest that the biofilm-derived cells are not only being yielded from the biofilm with the necessary means to respond to chemotaxis signals in a planktonic environment, but also with some metabolic activity.
The interpretation of gene expression results, acquired from the whole biofilm should however, be approached with caution. The acknowledged heterogeneity of biofilms does not only apply to architecture or composition, but also to the physiology of the cells positioned in various regions of the biofilm (Stewart and Franklin 2008). Whole biofilm transcriptome or proteome analyses can only yield information on the global trends, averaged for the entire biofilm, and moreover only for a single moment in time (Beloin and Ghigo 2005). A recent advance in determining the expression levels of target genes in localized areas in the biofilm utilized cryo-embedding and dissection of the biofilm, followed by the isolation of areas of interest (500 to 48 000 µm²) with laser capture microdissection microscopy in tandem with multiplex, quantitative real-time reverse transcriptase PCR (Lenz et al. 2008). Results showed that the expression of selected genes (involved in fatty acid synthesis, pyocyanin synthesis and an alkaline protease) varied significantly depending on the depth in the biofilm (as the distance from the substratum) while no statistically significant difference in 16S rRNA expression was observed.

Visual inspection during the removal of the shear-susceptible biofilm and bulk-liquid from the glass tubes by inverting and draining the contents into a microcentrifuge tube, indicated that a macroscopic amount of biomass with a creamy-white colour and viscous consistency was removed from the surface, while comparative little biomass remained in the tube. In light of these observations, the finding that the shear-susceptible and base biofilm regions accounted for almost equal amounts of protein throughout the 96-hour cultivation period was unexpected. It can be speculated that the non-shear-susceptible fraction of the biofilm likely contained less EPS and more densely-packed cells with reduced metabolic activity, than that of the shear-susceptible fraction. The dispersion of the cells from the two regions of flowcell-cultivated biofilms, and the quantification of cell numbers with epifluorescent direct counts,
also indicated that these regions of the biofilm contained a similar number of cells (in the same order of magnitude).

Further applications of this approach may be useful in the study of biofilm form-function relationships. The rapid recovery of metabolic activity after the bubble perturbation suggests that biofilms may utilize shear-related removal forces to maintain an optimized architecture which facilitates the maximal utilization of resources; the increase in the non-shear-susceptible base biofilm roughness once exposed to the bulk-liquid (i.e. after the removal of the shear-susceptible region) implies greater rates of nutrient and oxygen transfer to the biofilm, which is confirmed by the higher subsequent metabolic activity. It is not known whether the cell distribution or EPS composition in the biofilm was altered in response to the shear-mediated removal of the susceptible region, as has been observed for biofilms cultivated under higher flow rates (Pereira et al. 2002), and so we cannot speculate on whether the resulting biofilm could withstand subsequent changes in shear to a greater degree. Considering that relatively few real-world environments are bubble and turbulence free, such adaptation can be expected, even if it is not a requirement for survival.

Finally, the measurement of CO$_2$ as an indicator of biofilm metabolic activity described here overcomes a number of the limitations associated with the other available methods, as described by Stewart and Franklin (2008). A notable improvement is that this approach is not dependent on fluorescent stains that are susceptible to incomplete penetration, or the cellular toxicity characteristic of many of these stains. The approach also overcomes the requirement of working with pure cultures that are amenable to genetic manipulation, typical of reporter gene technologies.
7.5 Conclusions

7.5.1 Spatial differentiation in biofilm metabolic activity was observed with respect to changes in the CO$_2$ production rate and biofilm-derived planktonic cell yield.

7.5.2 The shear-susceptible biofilm region produced more than two-thirds of the CO$_2$ measured for the whole biofilm, while consisting of approximately half of the total number of cells and total protein content.

7.5.3 The removal of the shear-susceptible region of the biofilm resulted in a significant and extended reduction in the number of planktonic cells yielded to the environment. An indication of the beginning of a recovery in cell yield was observed three days after the removal of the shear-susceptible biofilm region.

7.5.4 The remaining non-shear-susceptible base biofilm region contributed significantly less to the CO$_2$ production rate measured for the intact biofilm, confirming previous observations of viable, but less active cells in the areas of the biofilm that are not in direct contact with the bulk-liquid environment.

7.5.5 The persistent base biofilm region did, however, respond rapidly to the availability of nutrients and oxygen once the overlaying cell and matrix material had been removed, thereby initiating the recovery of biofilm structure and biofilm-derived planktonic cell yield.

7.5.6 The average per cell CO$_2$ production rate of the shear-susceptible biofilm region was found to increase significantly after the removal from the flow chamber and a brief incubation in a suspended environment. This indicates that while the shear-susceptible biofilm region is also partially nutrient limited while in the biofilm, the cells retain a powerful metabolic capacity to respond when nutrient access becomes unrestricted.
7.5.7 Based on the metabolic activity of the shear-susceptible biofilm region, as well as the reduction in planktonic cell yield after its removal from the whole biofilm, it is most likely this region that is responsible for the production of the majority of cells observed in the bulk-liquid.

7.5.8 The observed metabolic differentiation in the biofilm provides the underlying evidence for speculation on the division of function / labour in the biofilm. The location and resulting metabolic activity of the shear-susceptible biofilm region allowed for the yield of significant numbers of cells to the environment, thereby actively promoting the proliferation of microbes. While the persistent base layer contributes significantly less to the activity of the whole biofilm, it was able to resist removal from the surface. Its persistence or survival at the surface initiated the subsequent recovery of biofilm activity, biomass and likely planktonic cell yield to the environment.

7.5.9 Preliminary evidence indicates that biofilm-derived planktonic cells exhibit a different pattern of gene expression than cells in the persistent, non-shear-susceptible biofilm region.

7.5.10 The increased expression of putatively identified genes involved in protein folding and translocation as well as flagellar synthesis, indicate that the yielded cells are metabolically active and likely equipped for a planktonic lifestyle. The ability of free-floating, motile cells to respond to chemotactic signals and colonize alternative sites can promote microbial proliferation in the environment.
7.6  List of symbols and abbreviations

~  approximately
bp  base pairs
kb  kilobases
\( \text{CER}_{\text{FC}} \)  carbon dioxide evolution rate in the flowcell
°C  degrees Celcius
CFU  colony forming units
c  concentration
CTC  5-cyano-2,3-ditolyl tetrazolium chloride
D  dilution rate
\( F_g \)  gas flow rate
\( F_l \)  liquid flow rate
\( \mu g \)  micrograms
H  Henry’s coefficient
\( kH \)  kiloHertz
h  hour
K  Kelvin
<  less than
l  litre
ml  millilitre
\(\mu l\) microlitre

\(cm^3\) square centimetre

\(cm^3\) cubed centimetre

\(mm\) millimetre

\(\mu m\) micrometre

\(\mu m^2\) square micrometres

\(\mu m^3\) cubed micrometres

\(nm\) nanometre

\(mM\) millimolar

\(mol\) moles

\(\mu mol\) micromoles

\(m_t\) number of moles at time \(t\)

\(\geq\) more than or equal

\(N\) Newtons

\(n\) number

\(Pa\) Pascals

\(kPa\) kiloPascals

\(ppm\) parts per million

\(\%\) percentage

\(\pm\) plus and (or) minus

\(P\) pressure
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>universal gas constant</td>
</tr>
<tr>
<td>RCF</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>seconds</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>(v/v)</td>
<td>volume in volume</td>
</tr>
<tr>
<td>$V_g$</td>
<td>headspace volume</td>
</tr>
<tr>
<td>$V_l$</td>
<td>liquid volume</td>
</tr>
<tr>
<td>$k_{ja}$</td>
<td>volumetric transfer coefficient</td>
</tr>
<tr>
<td>$x_g$</td>
<td>gas concentration</td>
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
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</table>

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma_s$</td>
<td>alternative sigma factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>et al.</td>
<td>and others</td>
</tr>
<tr>
<td>CEMS</td>
<td>Carbon dioxide evolution measurement system</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CMR</td>
<td>Carbon dioxide measurement reactor</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
</tr>
<tr>
<td>fliC</td>
<td>flagellin gene</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>gfp</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>Hsp60</td>
<td>heat shock protein 60</td>
</tr>
<tr>
<td>µmax planktonic</td>
<td>maximum planktonic specific growth rate</td>
</tr>
<tr>
<td>groEL</td>
<td>molecular chaperonin</td>
</tr>
<tr>
<td>GroEL</td>
<td>molecular chaperonin</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>rpoS</td>
<td>stationary phase sigma factor</td>
</tr>
<tr>
<td>RpoS</td>
<td>stationary phase sigma factor</td>
</tr>
<tr>
<td>i.e.</td>
<td>that is</td>
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</table>
The rationale behind biofilm-related research has three broad goals; to eliminate the formation of deleterious biofilms responsible for persistent human infections or biofouling, to promote biofilm development for bioprocesses, and lastly to use biofilms as model systems to delineate the fundamental aspects of microbial ecology.

The results from various experiments presented here indicate that pure culture and multispecies biofilms are capable of producing and releasing significant numbers of planktonic cells to the bulk-liquid under varied environmental conditions. This yield of biofilm-derived planktonic cells is a continuous process, correlated to the development of the biofilm at a solid surface, and is initiated within 6 hours of initial surface colonization. The release of cells from these young, developing biofilms (≤ 3 days) is therefore distinct from the widely accepted seeding dispersal phenomenon, where only a subset of mature biofilm microcolonies breaks open to liberate large numbers of free-swimming planktonic cells to the environment. Based on these observations, it is proposed that the current model of biofilm development be amended to include planktonic cell yield from the biofilm throughout all phases of development, rather than limiting the release of planktonic cells from mature microcolonies only (Figure 8.1).

The proposed model proceeds through similar stages as the current model, namely; (i) initial contact of cells with the surface (planktonic cells from the bulk-liquid landing on the surface or cell division of an existing attached cell with the daughter cell moving away from the parent along the surface to colonize open areas), (ii) firm attachment, (iii) attached cell growth and cell division, where some daughter cells remain associated with the surface and others detach;
the cells that detach from the surface may re-attach elsewhere to an existing biofilm or an uncolonized surface or become entrained in the bulk-liquid flow and is thereby transported out of the immediate environment, (iv) these processes continue, with an increase in the number of planktonic cells yielded to the bulk-liquid as the amount of attached biomass increases until the biofilm attains a steady-state as determined by the prevailing environmental conditions (i.e. nutrient availability and bulk-liquid flow velocity amongst other factors).

The intricate relationship between bulk-liquid flow rate, substrate loading rate, biofilm metabolic activity, and the ultimate effect on the yield of planktonic cells to the environment, has often been overlooked in the analysis of experimental results. This may lead to an underestimation of the role that attached biomass can play in the proliferation of microbes in the environment, the potential significance for biofouling control programs and medicinal therapeutics, as well as the interaction between attached and free-floating cells in these environments.

Contrary to the frequent assumption that fluid-mediated erosion (i.e. increases in bulk-liquid velocity) is responsible for the physical removal of single cells from the biofilm, the availability of carbon and the associated effect on biofilm metabolism were found to exert a greater influence on the number of planktonic cells released from the biofilm; hence the term ‘planktonic cell yield’ is proposed in place of ‘erosion’ to describe this phenomenon. These findings may not apply to biofilms cultivated under turbulent flow, or subjected to high abrasion rates, but are nevertheless representative of numerous biofilms growing in the environment, e.g. trickling filters for water purification, ground water flow, potable water flow distribution, industrial systems, and even dental water lines.
Figure 8.1: The proposed model of biofilm development. A flow diagram outlining the various steps is given in the top panel, with a graphical representation of biofilm formation in the middle panel, while the bottom panel shows CLSM micrographs of 24, 72 and 96-hour old GFP-labelled *Pseudomonas* sp. strain CT07 biofilms (Chapter 6). In the middle panel, the dark grey cells have reduced metabolic activity, likely due to restricted diffusion, while the biofilm-derived planktonic cells and the cells at the bulk-liquid interface are physiologically more active. The white scale bar represents 50 µm.
Results indicate that the biofilm itself retained a comparatively small amount of carbon (6.5% of the total inflowing carbon) (Chapter 6 and Kroukamp and Wolfaardt 2009), while it was responsible for converting approximately 54% of the inflowing carbon into CO₂ and 16% into microbial products (dissolved extracellular polymeric substances, biofilm-derived cell yield and dense biomass aggregates). This suggests that biofilms function as efficient catalytic units for the utilization and transformation of carbon in the environment and in concert with the metabolic capacity of biofilm cells, observed as the rapid physiological response of these cells to nutrient and oxygen availability, shed light on the documented ability of surface-associated microbial communities to catalyze the removal of chemical oxygen demand (COD) from waste water.

The carbon investment required to maintain the production and release of numerically significant numbers of planktonic cells to the environment (~10¹⁰ CFU) was found to be comparatively low (~1% of the inflowing carbon), which may explain how the pioneer attached cells are able to develop a multi-layered, three-dimensional biofilm structure through growth and cell division, while simultaneously yielding cells to the environment. The release of biofilm-derived planktonic cells and loss of dense biomass aggregates to the environment both represent small carbon investments and together with the demonstrated ability of biofilms to maintain a reduced rate of planktonic cell yield during starvation periods, these results demonstrate the ability of biofilm-associated microbes to utilize environmental resources to promote microbial proliferation. While the fate of the yielded cells was not investigated here, it is likely that these cells may maintain a free-floating existence, colonize new surfaces, or interact with existing biofilms.

The latter scenario may provide for an interesting area for future investigations since little is known concerning the interaction of free-floating cells with existing biofilms. The potential for pathogen integration, survival, multiplication and subsequent release is of great concern in
drinking water distribution systems, in intensive care units in medical facilities and for recreational activities on lake shores. The continual surface colonization and the concurrent release of large numbers of planktonic bacteria in industrial facilities will not only challenge biofouling control programs, but also add to operational costs. The potential for these biofilm-derived planktonic cells to remain associated with the biofilm by swimming within the reduced-flow microenvironment furthermore raises interesting questions about microbial interaction in this zone, given the phenotypic differences between the attached and free-floating cells and ultimately how this impacts our understanding of biofilm ecology in the environment.

The observed spatial differentiation in biofilm metabolic activity lays the foundation for speculation on the division of labour or function within the biofilm and the fate and (or) purpose of biofilm-derived cell yield in the environment, as Stewart and Franklin (2008) put it; “Elucidating the physiologies of biofilm-associated bacteria is necessary for our understanding of infection, ecological processes and bioreactor design, as well as other processes that are mediated by microorganisms”.

It is most likely that the metabolically active, shear-susceptible biofilm region is responsible for the yield of planktonic cells to the environment. The biofilm cell layers that are oriented towards the bulk-liquid benefit from frontline access to nutrients and oxygen, which sustains the physiological activity and active proliferation of microbes through the associated planktonic cell yield, but this region will likely also bear the brunt of an antimicrobial challenge, shear-removal event (such as an air bubble) or grazing predators. The inhibition or removal of this region exposes the persistent base biofilm region, located at the attachment surface, to oxygen and nutrients. While this region contributes significantly less to the activity of the whole biofilm, its persistence and survival at the surface initiated the subsequent recovery of biofilm activity, biomass and planktonic cell yield to the environment.
While the practical implications of the knowledge gained here is relevant to the aforementioned industrial and medical environments, it is also of fundamental significance to efforts aimed at understanding the role of surface-attached populations in the proliferation of microbes in the environment. According to the $r-K$ strategy of population selection, a microbial population strives to either optimize the ability to reproduce or conserve resources, but not both (Andrews and Harris 1986; Atlas and Bartha 1998). The presence of a metabolically active biofilm region responsible for the proliferation of microbes in the environment supported by a less active, but more persistent foundation of cells contrasts with this proposition.

Costerton et al. (1995) suggested that the planktonic form of existence can allow microbes to persist as inactive ultramicrobacteria during oligotrophic conditions, in addition to serving as a propagation mechanism, whereas biofilm formation permits growth and multiplication of microbial species (Costerton et al. 1995). Although this statement may be valid, the well-known ability of biofilms to persist despite unfavourable environmental conditions, such as desiccation, starvation, and antimicrobial onslaughts (Kjelleberg et al. 1982; Kjelleberg and Hermansson 1984; Mueller 1996; Costerton et al. 1999; Decho 2000), could also be viewed as a survival mechanism. The production and release of large numbers of planktonic cells to the environment by surface-associated microbial populations as reported here, can aid in the dissemination of microbes in the environment. When all of these considerations are taken into account, surface-associated microbial growth could be described as a simultaneous survival and proliferation strategy, where the dominant strategy will likely depend on the prevailing environmental conditions.
References


Appendix I

Table A1: Important hydrodynamic properties of the continuous-flow devices (glass tubes, OLAPH flowcell and conventional flowcells) employed for biofilm cultivation in this study. See the footnotes for sample calculations for each parameter.

<table>
<thead>
<tr>
<th>Volumetric flow rate (m³.s⁻¹)</th>
<th>Reynolds number (Re)</th>
<th>Dilution rate (h⁻¹)</th>
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</thead>
<tbody>
<tr>
<td><strong>Glass tubes [Dimensions: 2 mm (W) x 2 mm (H) x 152 mm (L)]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 x 10⁻¹¹</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td>8.4 x 10⁻¹¹</td>
<td>0.04</td>
<td>0.50</td>
</tr>
<tr>
<td>2.9 x 10⁻¹⁰</td>
<td>0.15</td>
<td>1.7</td>
</tr>
<tr>
<td>8.3 x 10⁻¹⁰</td>
<td>0.41</td>
<td>4.9</td>
</tr>
<tr>
<td>1.6 x 10⁻⁹</td>
<td>0.77</td>
<td>9.2</td>
</tr>
<tr>
<td>2.6 x 10⁻⁹</td>
<td>1.3</td>
<td>15</td>
</tr>
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<td>2.9 x 10⁻⁹</td>
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</tr>
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</tr>
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<td>6.3</td>
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<td>3.4 x 10⁻⁸</td>
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<td>200</td>
</tr>
<tr>
<td>6.7 x 10⁻⁸</td>
<td>33</td>
<td>390</td>
</tr>
<tr>
<td>1.3 x 10⁻⁷</td>
<td>62</td>
<td>740</td>
</tr>
</tbody>
</table>
\[ Re = \frac{v_s \cdot 4 \cdot Rh}{\nu} \]

where \( v_s \) = average flow velocity (m.s\(^{-1}\)), \( Rh \) = hydraulic radius (m) = surface area perpendicular to flow (m\(^2\)) / wetted perimeter (m), and

\( \nu \) = kinematic viscosity (m\(^2\).s\(^{-1}\)) taken as \( 1.004 \times 10^{-6} \text{ m}^2\text{s}^{-1} \) for water at 20°C.

<table>
<thead>
<tr>
<th>Volumetric flow rate (m(^3)s(^{-1}))</th>
<th>Reynolds number (Re) (^\text{§})</th>
<th>Dilution rate (h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLAPH Flowcell [Dimensions: 30 mm (W) x 6.0 mm (H) x 60 mm (L)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.2 x 10(^{-10})</td>
<td>0.03</td>
<td>0.16</td>
</tr>
<tr>
<td>1.7 x 10(^{-9})</td>
<td>0.09</td>
<td>0.51</td>
</tr>
<tr>
<td>6.4 x 10(^{-9})</td>
<td>0.35</td>
<td>1.9</td>
</tr>
<tr>
<td>OLAPH Flowcell [Dimensions: 30 mm (W) x 6.0 mm (H) x 60 mm (L)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 x 10(^{-8})</td>
<td>3.3</td>
<td>18</td>
</tr>
<tr>
<td>1.1 x 10(^{-7})</td>
<td>5.9</td>
<td>32</td>
</tr>
<tr>
<td>Flowcell flow chamber [Dimensions: 5.0 mm (W) x 6.0 mm (H) x 33 mm (L)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3 x 10(^{-9})</td>
<td>0.77</td>
<td>15</td>
</tr>
<tr>
<td>Flowcell flow chamber (Chapter 7) [Dimensions: 7.2 mm (W) x 3.0 mm (H) x 60 mm (L)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.05 x 10(^{-8})</td>
<td>2.0</td>
<td>29</td>
</tr>
</tbody>
</table>

\(^\text{§}\) Reynolds number (Re) (Lewandowski and Beyenal 2007):