Biofilms as multifunctional surface coatings and adaptive systems: a biomimetic approach

By

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Declaration

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ABSTRACT

Biomimicry is an emerging scientific discipline that promotes nature-inspired innovation for sustainable solutions. Several patterns and survival strategies are repeated in Nature and these have been extrapolated into a hierarchical set of biomimetic principles that can be used to investigate the complexity of natural systems. A biomimetic approach was used to review biofilm literature and create a novel framework based on these principles to describe microbial biofilms on a molecular, structural and systems level.

By reinterpreting current biofilm knowledge within a biomimetic framework, this study demonstrates that microorganisms use life-friendly chemistry to integrate biofilm development with growth, giving rise to resource-efficient systems. Furthermore, these structured microbial communities are responsive to their local environment, adapt to changes and, ultimately, evolve to survive.

Subsequently, the application of biomimetic principles to biofilms was investigated using various analytical techniques. Two *gfp*-labelled *Pseudomonas* strains and an environmental multi-species community were selected for this study. Microscopic and spectroscopic techniques were used for biochemical investigations of single-species biofilm composition and structure. The distribution of biomolecules in *Pseudomonas* biofilms was investigated using protein- and glycoconjugate-specific fluorescent stains and confocal laser scanning microscopy (CLSM). CLSM was also used to investigate structural adaptations of *Pseudomonas* biofilms to changes in nutrient availability and hydrodynamic conditions. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy was used to explore biochemical adaptations of single- and multi-species biofilms cultivated in different nutrient media.

ATR-FTIR spectra, visual observations and the quantification of biofilm parameters by digital image analysis of CLSM images support the hypothesis that biofilms are resource-efficient, self-organised systems that are built from the bottom up using life-friendly chemical principles. Both *Pseudomonas* strains adapted to environmental conditions by changing the three-dimensional structure of their biofilms, specifically in terms of biomass, substratum area coverage, average thickness and the surface area of biofilms exposed to the bulk liquid.

In order to study biofilms as a system and investigate the responsiveness of a biofilm community as a whole, a relatively new approach was used to monitor biofilm responses in real time by measuring CO_2 production as an indication of whole-biofilm metabolism. A CO_2 evolution measurement system (CEMS) was combined with metabolic assays and direct plate count methods to monitor biofilm metabolism and biofilm-derived planktonic cell yield in response to environmental changes, i.e. changes in nutrient source and concentration or exposure to antimicrobial compounds (either streptomycin or a solution containing isothiazolone).

The metabolic responses of biofilms, measured as CO_2 production rates, showed that both single- and multi-species biofilms are able to respond rapidly to changes in nutrient availability or exposure to biocides and antibiotics. Multi-species biofilms generally recover faster after environmental changes or antimicrobial exposures, indicating that diversity adds to biofilm resilience and adaptability. Regardless of the conditions, single- and multi-species biofilms are able to maintain some level of metabolic activity, as well as release high numbers of planktonic cells into the effluent. The maintenance of biofilm-derived planktonic cell yield supports the hypothesis that biofilms are active proliferation sites in order to ensure survival – a feature of biofilms that is often overlooked in biofilm research.

This study contributes to the growing field of biomimicry by applying biomimetic principles in biofilm research for the first time. A biomimetic approach can inform novel anti-biofilm strategies, promote biofilm-inspired innovation and explain complex microbial ecological phenomena. Within a biomimetic framework, the increasing degrees of complexity in biofilms are organised in a new way, demonstrating that the biochemical, structural and functional complexity of microbial communities are interconnected and need to be considered together in biofilm studies. To this end, the usefulness of CEMS as a non-destructive technique to study real-time biofilm responses is demonstrated.

OPSOMMING

Biomimiek is ’n ontluikende wetenskaplike dissipline wat natuur-geïnspireerde innovasie vir volhoubare oplossings bevorder. Verskeie patrone en oorlewingstrategieë word in die natuur herhaal. In biomimiek word dié strategieë veralgemeen in ’n hiërargiese stel beginsels wat gebruik kan word om die kompleksiteit van natuurlike stelsels te ondersoek. Biomimiekbeginsels en ’n literatuuroorsig van biofilmnavorsing word hier gebruik om ’n nuwe raamwerk te skep wat mikrobiese biofilms op ’n molekulêre, strukturele en sistemiese vlak beskryf.

Deur huidige biofilmkennis binne ‘n biomimiekraamwerk te herinterpreer, demonstreer hierdie studie hoe mikroorganismes lewensvriendelike chemiese reaksies gebruik om hulpbron-doeltreffende stelsels te skep wat ontwikkeling met die groei van die stelsel integreer. Hierdie gestruktureerde mikrobiese gemeenskappe reageer op hul biotiese en abiotiese omgewing, pas aan by omgewingsveranderinge en evolueer om te oorleef.

Gevolglik is die toepassing van biomimiekbeginsels op biofilms deur ‘n verskeidenheid analitiese tegnieke getoets. Twee *gfp*-gemerkte *Pseudomonas*-stamme asook ’n multi-spesie-omgewingsgemeenskap is gekies vir hierdie studie. Mikroskopiese en spektroskopiese tegnieke is gebruik in biochemiese ondersoeke na die samestelling en struktuur van enkelspesies biofilms. Konfokalelaseraftasmikroskopie (KLAM) en fluoreserende kleurstowwe met proteïen- en glikokonjugaat-spesifisiteit is gebruik om die verspreiding van biomolekule in *Pseudomonas*-biofilms te bestudeer. KLAM is ook gebruik om die strukturele aanpassings van *Pseudomonas*-biofilms wat blootgestel is aan verskillende voedingsmediumkonsentrasies en hidrodinamiese toestande te ondersoek. Die biochemiese samestellings en aanpassings van hierdie biofilms is deur verswakte totale-refleksie Fourier-transformasie-infrarooi (VTR-FTIR) spektroskopie ondersoek.

VTR-FTIR-spektra, visuele waarnemings en die kwantifisering van biofilmparameters deur digitale analises van KLAM-beelde ondersteun die hipotese dat biofilms hulpbron-doeltreffende, self-georganiseerde stelsels is wat van onder af boontoe gebou word deur lewensvriendelike chemiese beginsels. Beide *Pseudomonas*-stamme kon aanpas by omgewingstoestande deur die drie-dimensionele struktuur van hul biofilms te verander, spesifiek ten opsigte van biomassa, oppervlakbedekking, gemiddelde biofilmdikte en die oppervlakarea wat blootgestel word aan die omgewing.

’n Relatief nuwe stelsel, wat biofilms se CO_2-produksie meet as ’n aanduiding van hul algehele metabolisme, is gebruik om biofilms as sisteme te bestudeer. ’n CO_2-evolusie-metingstelsel (CEMS), metaboliese toetse en direkte plaattellingmetodes is gebruik om te monitor hoe veranderinge in omgewingstoestande biofilmmetabolisme en planktoniese sel-opbrengs beïnvloed. Biofilms is blootgestel aan verskillende voedingsbronne en -konsentrasies of aan antimikrobiese middels (streptomisien of ’n oplossing wat isotiasoloon bevat).

Die metaboliese reaksies van biofilms, gemeet as CO_2-produksie tempo’s, wys dat beide enkel- en multi-spesiebiofilms in staat is om vinnig te reageer op veranderinge in voedingsbronbeskikbaarheid of op blootstelling aan antimikrobiese middels. Multi-spesiebiofilms het oor die algemeen vinniger herstel wat daarop dui dat diversiteit bydra tot die veerkragtigheid en aanpasbaarheid van biofilms. Die biofilms is in staat om ’n sekere metaboliese vlak te handhaaf en ’n hoë aantal planktoniese selle vry te stel ongeag die omgewingstoestande. Hierdie resultate het ook bevestig dat biofilms optree as aktiewe selvormingsetels.

Hierdie studie dra by tot die ontwikkelende veld van biomimieknavorsing deur biomimiekbeginsels vir die eerste keer in biofilmnavorsing toe te pas. ’n Biomimiekbenadering kan gebruik word om nuwe anti-biofilmoplossings na te vors, biofilm-geïnspireerde innovasie te bevorder en om komplekse mikro-ekologiese verskynsels te verduidelik. Binne ’n biomimiekraamwerk word die toenemende kompleksiteit van biofilms op ’n nuwe manier gerangskik. Sodoende word die verbintenis tussen die biochemiese, strukturele en funksionele kompleksiteit van mikrobiese gemeenskappe gedemonstreer. Die waarde van CEMS as ’n tegniek vir die nie-destruktiewe bestudering van biofilms word uitgelig.

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For Zoë,

you make me brave.

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LIST OF ABBREVIATIONS

2PLSM	two photon laser scanning microscopy
3D-SIM	three-dimensional structural illumination microscopy
AFM	atomic force microscopy
ATR	attenuated total reflection
AWCD	average well colour development
c-di-GMP	cyclic di-guanosine monophosphate
CEMS	CO_2 evolution measurement system
CFU	colony forming units
CLSM	confocal laser scanning microscopy
CMD	community metabolic diversity
ConA-594	Alexa Fluor® 594 conjugated Concanavalin A
DGC	diguanylyl cyclase
DIA	digital image analysis
eDNA	extracellular DNA
EDS	energy-dispersive X-ray spectroscopy
EDTA	ethylenediaminetetraacetic acid
EM	electron microscopy
EPS	extracellular polymeric substances
ESEM	environmental scanning electron microscopy
FTIR	Fourier transform infrared
gfp	green fluorescent protein
IR	infrared
HGT	horizontal gene transfer
LB	Luria-Bertani
LSM	laser scanning microscopy
MGEs	mobile genetic elements
NMR	nuclear magnetic resonance
OD	optical density
PDE	phosphodiesterase
RCM	Raman confocal microscopy

R-2A	Reasoner's 2A
Re	Reynolds number
SCVs	small colony variants
SEM	scanning electron microscopy
SERS	surface-enhanced Raman scattering
STXM	soft X-ray scanning transmission X-ray microscopy
SYPRO® Ruby	FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain
TEM	transmission electron microscopy
TSB	tryptic soy broth

1. Introduction

Despite decades of research, biofilms remain a source of fascination and frustration for scientists. Most, if not all, microorganisms have the ability to attach to wetted surfaces and embed themselves in a self-produced three-dimensional scaffold of diverse macromolecules (Watnick and Kolter 2000; Neu and Lawrence 2009; Flemming 2011). It is widely accepted that these structured microbial communities, or biofilms, are the dominant mode of life for most microorganisms. The self-produced matrix of extracellular polymeric substances (EPS) that encases a microbial community plays an important role in the biofilm's ability to adapt and survive (Flemming, Neu and Wozniak 2007).

Biofilm research is often driven by the need to prevent biofilm formation (in medical, food, water and several industrial settings) or exploit biofilm function (in wastewater treatment, bioremediation, bio-catalysis processes and microbial fuel cells). Despite continued research efforts to control, eradicate and manipulate biofilms, these microbial communities often evolve new mechanisms that allow them to evade and persist.

To this end, countless studies have set out to uncover the genetic and biochemical secrets of biofilms, mostly focussing on laboratory-cultivated, single-species biofilms. While this is a sensible starting point to elucidate the basic functions of biofilms, we now need to cast our nets wider in order to understand the complexities of naturally occurring, multi-species biofilms. This requires more than novel techniques and experimental studies, i.e. frameworks that combine current biofilm knowledge and explain biofilm phenomena are required. As Sir William Bragg famously said, "The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them." (quoted in Eysenck 1995, p. 1). Ultimately, biofilms function as systems within systems and an approach that considers biofilms at micro and systems levels is required (Stoodley, Sauer, Davies and Costerton 2002).

Biomimicry offers a new lens through which to view biofilms and may offer insights that are particularly relevant to biofilm prevention and utilisation. As an emerging science, biomimicry promotes the study of all life forms in order to understand the strategies living organisms use to survive in the long haul. These strategies, or Life's Principles, represent overarching patterns found amongst

most species and ecosystems, including microorganisms and microbial biofilms (Baumeister, Tocke, Dwyer, Ritter and Benyus 2012). Over the past 15 years, Life's Principles have been distilled into a hierarchical list which can be used as a framework within which nature's ingenuity can be explored as a tool to incorporate natural designs into human innovations.

When Life's Principles are applied to biofilms it becomes evident that these communities function as resource-efficient, multifunctional, adaptive systems. Biofilms can even be regarded as nature's surface coatings that often outperform most man-made coatings in terms of environmental compatibility, functionality and robustness.

Life's Principles of Biofilms

Microorganisms build complex biochemical structures with a small subset of elements, using water as a solvent, giving rise to a hydrated sub-micron layer that serves as a coating scaffold. This layer increases in thickness and complexity as more EPS and microbial cells are added, forming a three-dimensional matrix. By using **life-friendly chemistry**, the matrix can also be broken down into benign constituents that can be reabsorbed by the organisms or the environment.

The development of a mature biofilm is **a resource-efficient process:** environmental conditions, nutrient availability and the metabolic state of microorganisms continuously determine the composition of the EPS matrix and microbial community. The EPS matrix serves many functions: in addition to providing protection against host immune systems and antimicrobial compounds, it also stores nutrients. As the biofilm develops over time, its form fits the functions required by the microbial community.

Biofilm development is a self-organised, ordered process that is directed by cellular responses to environmental conditions. The EPS matrix is built from the bottom up and **integrates development with growth**. Within the EPS matrix, the microbial community fosters cooperative relationships, incorporates feedback loops, uses readily available resources and leverages cyclic processes in order to **be locally attuned and responsive**.

By incorporating diversity, self-renewal, variations and decentralised designs, biofilms are able to **adapt to changing conditions**. Biofilms are also able to integrate unexpected events and reshuffle genetic information. By replicating strategies that work, biofilms are ultimately able **to evolve to survive**.

Given global concerns over environmental degradation, there is mounting pressure on most scientific fields to develop sustainable technologies and environmentally friendly solutions to human design challenges. This includes "green" biofilm eradication alternatives as well as more efficient technologies for harnessing beneficial functions of biofilms. A biomimetic approach to biofilms can therefore be advantageous in many ways. By developing our understanding of biofilms through this multi-level systems approach, we might be able to identify key points of intervention in order to combat biofilm formation. Furthermore, through a biomimetic approach, we might be able to extrapolate the survival strategies of biofilms and interpret these as design principles, which may in turn give rise to sustainable, nature-inspired innovations.

1.1. Central hypothesis and research objectives

This study explored the common principles that underpin the success of biofilms in a wide range of environments. These survival strategies were considered in a hierarchical structure to create a biomimetic framework within which biofilms are viewed as resource-efficient, multifunctional, adaptive systems. This framework was used throughout this study to review our current knowledge of biofilms and to interpret experimental findings on biofilm structure and functions in a novel way. By employing this approach, this study aimed to develop a deeper understanding of these complex systems, which may in turn inform biofilm prevention and utilisation techniques.

Bacterial EPS and biofilms at the solid-liquid interface were the focus of the experimental work of this study as these biofilms are most relevant to industrial anti-fouling applications. Biofilms were evaluated on a basic level as surface coatings and a more complex level as adaptive systems. Established and new experimental techniques were applied to single- and multi-species biofilms to investigate biofilm responses and adaptations.

1.1.1. Specific objectives

1.1.1.1. Create a biomimetic framework to describe biofilms by demonstrating the applicability of Life's Principles to biofilms.

1.1.1.2. Use this biomimetic framework for experimental design to evaluate biofilms as resource-efficient, multifunctional systems by:

1.1.1.2.1. Investigating the effects of environmental conditions on biochemical composition, structure and function of biofilms.

1.1.1.2.2. Comparing the adaptability of single- and multi-species biofilms in response to adverse environmental conditions.

1.1.1.3. Describe biological capabilities of biofilms worth emulating in artificial coatings.

1.2. Thesis outline

CHAPTER ONE: Introduction

This chapter introduces the aims and specific research objectives of this study. It also outlines the thesis structure and contents of following chapters.

CHAPTER TWO: Biofilms as multifunctional coatings and adaptive systems: a biomimetic approach

This literature review uses a biomimetic framework to evaluate current biofilm knowledge. Relevant biomimetic concepts are introduced and then used to describe the principles that underpin the survival of biofilms in a wide range of environments. This hierarchical framework of survival strategies is used throughout this study to review our current understanding of biofilms and interpret experimental findings (specific objective 1.1.1.1).

CHAPTER THREE: Techniques relevant to biofilm studies

The most common biofilm cultivation methods and biofilm analysis techniques are briefly discussed and techniques selected for this study are reviewed in more detail.

CHAPTER FOUR: Techniques and protocol development for the current study

Experimental design, protocol development and techniques used in this study are explained and validated. General experimental procedures used in Chapters 4, 5 and 6 are described, including biofilm cultivation techniques, protocols for spectroscopic and microscopic biofilm studies and the use of a CO_2 evolution measurement system.

CHAPTER FIVE: Resource-efficient coatings: spectroscopic and microscopic investigations of biofilms

The results from this chapter are discussed in terms of the following biomimetic principles: life-friendly chemistry, resource-efficiency and the integration of development with growth. Established techniques were used to demonstrate that microorganisms are able to use a small set of biochemical building blocks to assemble complex biofilm structures. The effect of nutrient concentration and flow rate on biofilm structure was investigated (specific objective 1.1.1.2.1). Results from fluorescent microscope studies showed that biofilm parameters are responsive to environmental conditions.

CHAPTER SIX: Biofilms as adaptable communities that evolve to survive

The results from this chapter are discussed in terms of the following biomimetic principles: responsiveness, adaptability and the ability to evolve to survive. The adaptability of single- and multi-species biofilms was investigated by measuring biofilm CO_2 production rates and biofilm-derived planktonic cell yield as responses to adverse conditions (specific objective 1.1.1.2.2). Biofilms were exposed to nutrient concentration changes, a commercial biocide or high levels of antibiotics. It was hypothesised that multi-species biofilms would recover faster from these negative environmental changes than single-species biofilms.

CHAPTER SEVEN: Learning from nature's multifunctional surface coatings

The last chapter summarises the findings of this study and critically discusses the use of biomimicry as a framework for biofilm research. In conclusion, it addresses the last research objective (1.1.1.3) by describing the strategies of biofilms that allow them to evade anti-biofilm treatments and extrapolating principles from biofilms worth emulating in artificial coatings.

2. Biofilms as multifunctional coatings and adaptive systems: a biomimetic approach

Biofilms are the dominant mode of life for most microorganisms and, despite decades of research, these microbial communities remain a source of fascination and frustration for scientists. Most, if not all, microorganisms have the ability to attach to wetted surfaces and embed themselves in a self-produced three-dimensional matrix of diverse macromolecules (Watnick and Kolter 2000; Neu and Lawrence 2009; Flemming 2011). This surface-associated matrix acts as a scaffold that anchors the microbial community and allows members to take advantage of resources and opportunities in a favourable micro-environment.

Technological advances have allowed researchers to study biofilm structures and speculate about the intricacies of microbial interactions and possible benefits of this preferred communal living. Biofilms fulfil important functions in ecological systems, such as biogeochemical processes (Decho, Visscher and Reid 2005) and carbon cycling (Lyon and Ziegler 2009), and the global food web (Simon, Benfield and Macko 2003), including essential roles in the human microbiome (Huttenhower, Gevers, Knight, Abubucker, Badger et al. 2012). As our understanding of biofilms has increased, we have developed ways of exploiting the functions of these synergistic communities: biofilms are successfully used as purification filters in wastewater treatment and bioremediation systems (Sheng, Yu and Li 2010), as micro-factories in bio-catalysis processes (Wood, Hong and Ma 2011; Halan, Buehler and Schmid 2012) and as energy generators in microbial fuel cells (Du, Li and Gu 2007).

While the adaptive, robust nature of biofilms is beneficial in natural environments and certain industrial processes, biofilms have detrimental effects in other settings. In many engineering sectors biofilms create surface fouling and corrosion that lead to pollution, safety hazards and significant financial losses (Beech and Sunner 2004). Biofilms may also pose a threat to human health within the medical and food sectors, as well as in water treatment and distribution systems (Simões, Simões and Vieira 2010). Despite continued research efforts to control, eradicate and manipulate biofilms, these microbial communities often evolve new mechanisms that allow them to evade antimicrobial treatments and persist in unfavourable conditions.

At the centre of a biofilm's ability to withstand our anti-biofilm strategies is the self-produced matrix of extracellular polymeric substances (EPS) that encases the microbial community. EPS matrixes are more than just glue that holds microbial communities together. These matrixes create sophisticated systems that enable microbial communities to function as self-organised, resource-efficient micro-consortia that are able to adapt to changes within the community and the environment (Flemming et al. 2007).

Numerous reviews have discussed potential biofilm functions as well as the diverse structures and roles of EPS within biofilms. Biofilms have been described as multicellular organisms (Shapiro 1998), microconsortia (Nikolaev and Plakunov 2007), microbial cities (Watnick and Kolter 2000), secret societies (Kolter and Greenberg 2006), landscapes (Battin, Sloan, Kjelleberg, Daims, Head et al. 2007) and living catalysts (Halan et al. 2012) while the EPS matrix has been likened to a sticky framework (Sutherland 2001), the house of biofilm cells (Flemming et al. 2007) and the perfect slime (Flemming 2011).

Although many metaphors have been developed to describe the complexities of biofilms, our understanding thereof is still limited because most biofilm research has focused on single-species biofilms cultivated under controlled laboratory conditions. In order to truly appreciate the multi-functionality and adaptability of biofilms, we need to consider biofilms within the diverse context of their natural environments. Biofilms function as systems-within-systems. Therefore, an approach that considers biofilms at micro levels and systems levels is needed.

Over the past decade, biomimicry has emerged as an acknowledged scientific discipline and offers a new lens through which the complexities of biofilms can be viewed. Biomimicry promotes the study of natural life forms in order to understand the overarching strategies organisms use to survive. The aim is to extrapolate natural principles from these strategies and apply the principles to various human design challenges to innovate sustainable solutions (Baumeister et al. 2012).

This chapter introduces relevant concepts of biomimicry and uses these concepts to explore the common principles that underpin the success of biofilms in a wide range of environments. By organising the survival strategies of biofilms in a hierarchical structure, this study proposes a biomimetic framework within which biofilms are viewed as resource-efficient, multifunctional,

adaptive systems. This framework is used throughout this study to review our current knowledge of biofilms and interpret experimental findings in a novel way. Through this approach, this study aims to develop a deeper understanding of these complex microbial systems, which may in turn inform biofilm prevention and utilisation methods. It is possible that mimicking the design principles of these natural surface coatings may even give rise to new environmentally sound, bio-inspired innovations, as will be discussed in Chapter 7.

GLOSSARY

Alginate: an extracellular polysaccharide consisting of repeat mannuronate and guluronate residues, commonly produced by pulmonary isolates of *Pseudomonas aeruginosa.*

Biofilm: polymicrobial aggregates encased in a matrix of self-produced extracellular polymeric substances (EPS) associated with an interface. Microbial aggregates include bacteria, *Archaea* and eukaryotic microorganisms (i.e. protozoa, algae and fungi). Interface can refer to liquid-liquid, solid-liquid, gas-liquid or solid-gas interface.

Biomimicry: (nature- or bio-inspired innovation) a design discipline and scientific approach that seeks sustainable solutions by emulating the design principles of natural products, processes and systems.

Community: for the purpose of this review 'community' refers to all members of a biofilm, where biomimetic principles are relevant to single- and multi-species biofilms. When discussing single-species biofilms, 'population' is used.

Extracellular polymeric substances (EPS): organic polymers produced and secreted by microorganisms within the biofilm, including polysaccharides, proteins, nucleic acids, lipids, phospholipids, peptidoglycan and other cellular compounds.

Life-friendly chemistry: chemistry that is non-toxic to living tissues and supports life processes.

Life's Principles: a hierarchical list of design principles representing Nature's overarching strategies for sustainability.

Matrix: non-cellular component of a biofilm that may consist of secreted extracellular polymeric substances (EPS), water, absorbed nutrients and metabolites, products from cell lysis and even debris from the environment.

Synergism: the interaction or cooperation of two or more organisms/species in a biofilm to produce a combined effect greater than the sum of their planktonic counterparts.

2.1. Biomimicry: nature-inspired innovation

2.1.1. Key concepts and definitions

The Greek roots of the word "biomimicry" contain its simplest definition: the imitation (*mīmēsis*) of life (*bios*) (Benyus 1997). The natural world has always inspired human innovation (Vincent et al. 2006). Leonardo da Vinci is often credited as one of the first biomimics for his observational sketches of nature, including the workings of the human body (Marshall and Lozeva 2009). Biomimicry first appeared in academic literature in the 1950s and although it was included in the Webster's Dictionary in 1974[1], the concept was only popularised in the 1990s. Figure 2.1 shows the evolution of biomimicry and related fields like bionics and ecological design over the past century.

Biomimicry is formally defined as a novel approach to innovation through "the conscious emulation of life's genius" (Benyus 1997, p. 2). This definition implies specific intent for innovation. By learning from nature's time-tested strategies, it is possible to develop sustainable products, processes or systems. This approach requires a deep understanding of natural patterns and survival strategies. Biomimicry aims to extrapolate and mimic these lessons in human design contexts to create novel solutions, rather than merely creating exact replicas of natural objects.

"Life's genius" refers to natural structures, materials and systems that are well-adapted to the context of the planet. The operating conditions of the planet have significant influences on organisms and ecosystems, including microorganisms and biofilms, and should be taken into account when studying nature's functions and adaptations. Earth's operating conditions are represented by the outer grey circle in Figure 2.2, implying that life is directly or indirectly subjected to these conditions. Figure 2.2 illustrates that, within the Earth's context, certain overarching strategies (Life's Principles) are applied to create self-sustaining systems.

[1] http://www.merriam-webster.com/dictionary/biomimetics, accessed on 25 September 2015.

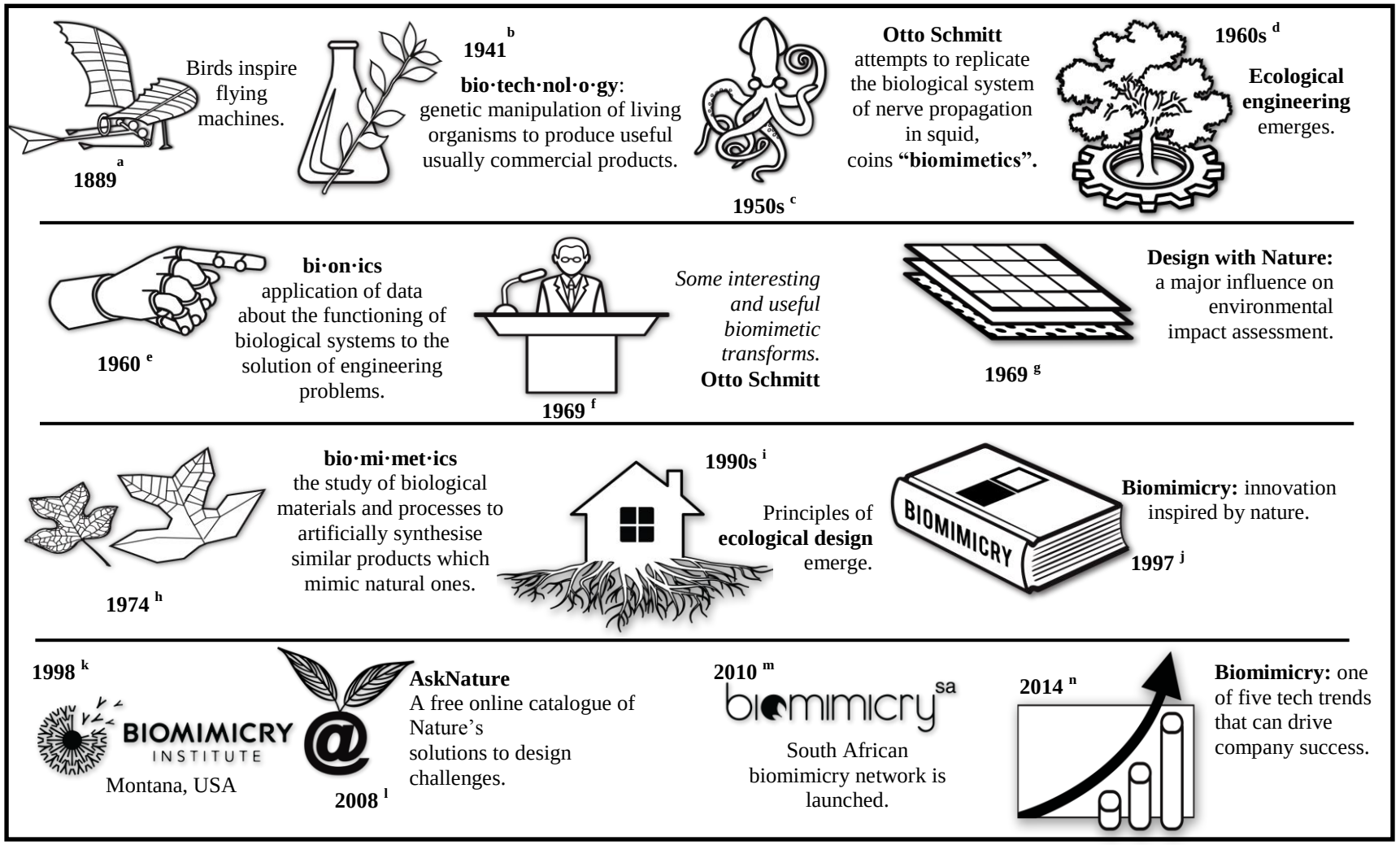

Figure 2.1. The development of biomimicry and related concepts. ([a] Lilienthal 1889, [b,e,h] http://www.merriam-webster.com/dictionary/, [c] Harkness 2001, [d] Odum 1962, [f] Schmitt 1969, [g] McHarg 1969, [i] Todd and Todd 1994, [j] Benyus 1997, [k] http://www.biomimicry.org, [l] http://asknature.org, [m] http://www.biomimicrysa.co.za, [n] http://www.forbes.com/sites/rebeccabagley/2014/04/01/5-tech-trends-you-should-pay-attention-to/ (Websites accessed 25 September 2015)

2.1.1.1. Earth's operating conditions

Of the known factors that influence life, the three main operating conditions that make Earth habitable are sunlight, water and gravity (Baumeister et al. 2012). Electromagnetic radiation from the sun not only warms the planet but is also the primary source of energy for most life forms. Sunlight is linked to various cyclic processes, such as day/night and seasonal changes, which in turn drive ocean currents, climate and water cycles. Water is a crucial component of our blue planet and is the cornerstone of all of nature's chemistry. Earth's unique gravitational properties influence life on macro- and micro-scales: from ocean currents to the transportation of liquids by capillary action.

Life on Earth is subject to change. Some changes are relatively constant while others are dynamic and unpredictable. Cyclic processes, like seasonal changes, hold a measure of predictability and result in unique, context-specific adaptations where organisms take advantage of these cycles. It is often said that change is the only constant on our planet and nature seems to have adapted to this truth. A wide range of forces influence the conditions on Earth and therefore a certain degree of adaptability is always required in order to survive.

Perhaps the most important operating condition acknowledged by biomimetic thinking is the context of a finite planet (Baumeister et al. 2012). There are limits and boundaries to almost all resources on Earth. These limits act at a global scale but certainly affect ecosystems, even at the micro-scale of biofilms. Access to resources and availability of resources constantly change within microbial communities, creating the need for direct feedback loops and diverse metabolic and behavioural responses, as will be discussed in greater detail in this review.

While these operating conditions are universal to the planet, there are always exceptions, such as extremophiles that survive in environments that are never directly or indirectly exposed to sunlight. Nonetheless, Earth's operating conditions work together to create local contexts and niche environments, even on the micro-scale and, therefore, microorganisms are also subject to these conditions for the most part. Earth's operating conditions provide a framework from which to consider the important influence of external factors and environmental conditions when studying biofilms and their functions.

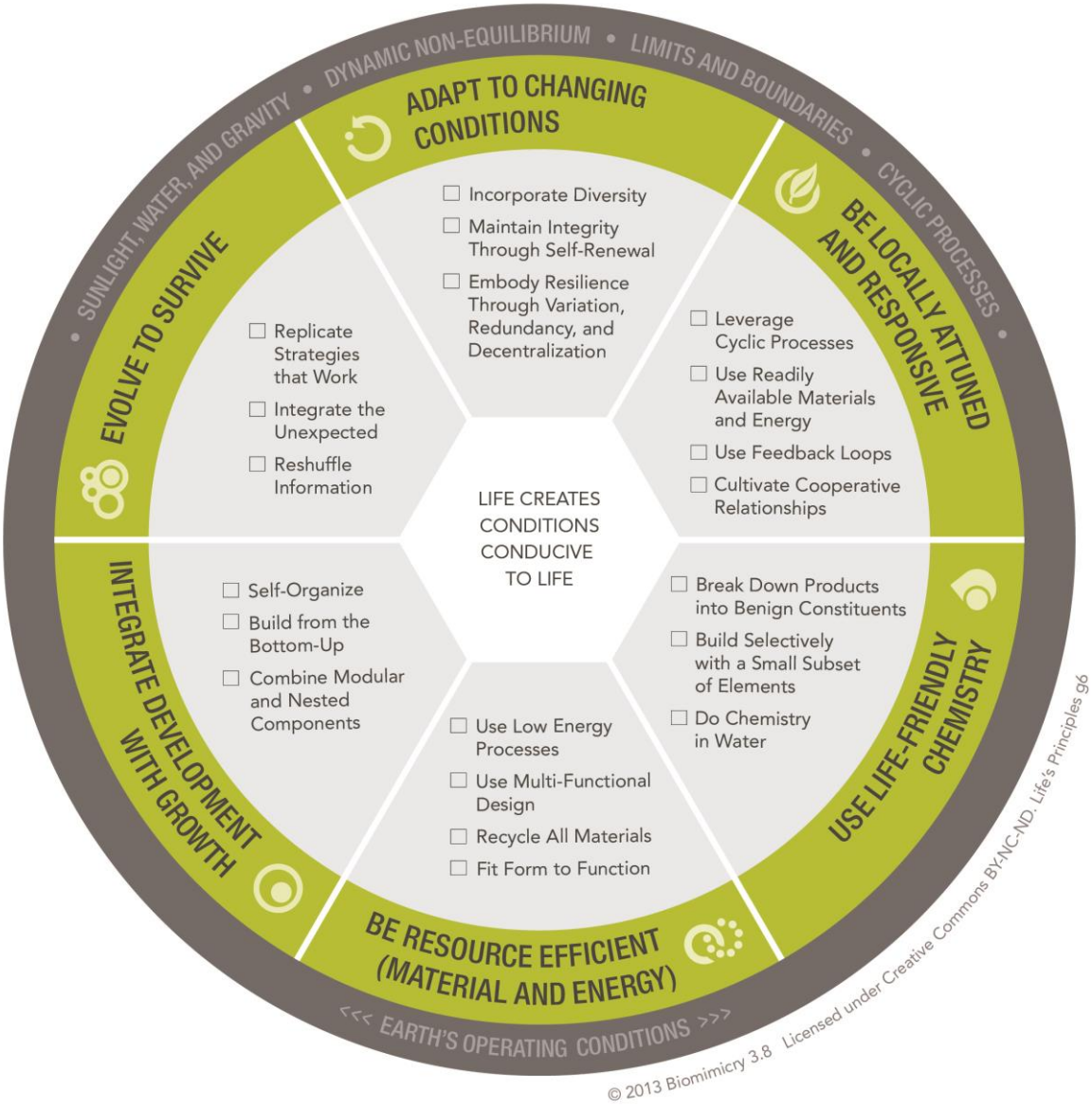

Figure 2.2. Graphic representation of Earth's operating conditions and Life's Principles[2].

[2] http://biomimicry.net/about/biomimicry/biomimicry-designlens/lifes-principles/, accessed on 25 September 2015.

2.1.1.2. Life's Principles

Over the past decade, Biomimicry 3.8[3] has developed the concept of Life's Principles. Their most current version is shown in Figure 2.2. These are a collection of fundamental principles that represent the most common strategies or patterns for survival seen in the natural world. The six main principles are clustered in terms of similarity (Baumeister et al. 2012). While a degree of hierarchy is evident, these principles are interconnected and should be considered together when attempting to understand the complexity of natural systems. The subprinciples will be discussed in more detail in the following section.

Life's Principles are a result of, and subject to, the context of the planet. This relationship is represented by enclosing the principles by the Earth's operating conditions (Figure 2.2). The emergent property of these synergistic principles, "creating conditions conducive to life", is represented by the centre of the diagram.

Within the context of biomimicry, Life's Principles are used as a tool to promote the view of nature as a model, mentor and measure. When looking to the natural world for inspiration, this set of principles begins to explain innovative strategies that emerge from nature and illuminates how natural designs can be emulated in human contexts (*nature as model*). Life's Principles firstly serve as aspirational ideals that reiterate the interconnectedness between human designs and natural systems (*nature as mentor*). Secondly, these principles can be used as sustainable standards that challenge superficial eco-labels that often pass as sustainable designs (*nature as measure*).

The concept of Life's Principles continuously evolves as more patterns emerge and our understanding of natural systems increases. Although the esoteric language is somewhat unconventional in scientific realms, this review aims to show that the current version of Life's Principles can be used as a novel framework which successfully describes the complexity of naturally occurring biofilms (whether containing one or many species), while acknowledging the role the micro- and macro-environment play in creating these adaptive systems.

[3] www.biomimicry.net, accessed 25 September 2015.

2.2. Biofilms and biomimicry

Biofilms are surface-associated polymicrobial aggregates encased in a matrix of self-produced extracellular polymeric substances (EPS) associated with an interface (Neu and Lawrence 2009; Flemming and Wingender 2010). Microbial aggregates may include bacteria, *Archaea* and eukaryotic microorganisms (i.e. protozoa, algae and fungi) and "interface" may refer to a liquid-liquid, solid-liquid, gas-liquid or solid-gas interface. Figure 2.3 shows a brief timeline of the discovery of biofilms and development of the closely related concept of EPS.

EPS comprise a wide range of organic polymers produced and secreted by the biofilm community, including polysaccharides, proteins, nucleic acids, lipids, phospholipids, peptidoglycan and other cellular compounds (Sutherland 2001). Other secondary polymers, not produced by microorganisms, may also form part of the matrix structure (Neu and Lawrence 2009). The EPS matrix may therefore be a complex of secreted polymers, absorbed nutrients and metabolites, products from cell lysis and even humic material and debris from the environment (Sutherland 2001).

EPS are sometimes categorised as either bound or soluble EPS. Bound EPS (sheaths, capsular polymers, loosely bound polymers and attached organic material) are closely bound to cells, while soluble EPS (soluble macromolecules and colloids) are weakly bound to cells or dissolved in the solution. Bound EPS can then be further divided into a two-layer model, where the inner layer consists of tightly bound EPS and an outer layer of loosely bound EPS. Generally speaking, tightly bound EPS account for most bound EPS and are stably bound to the cell surface while loosely bound EPS form a dispersible layer without an obvious edge (Sheng et al. 2010). In this study, "EPS" refers to specific aspects or components and "matrix" to the broader non-cellular structure of the biofilm (Neu and Lawrence 2009).

As our knowledge of biofilms has expanded, it has become evident that the three-dimensional architecture of the matrix, together with the concentration and chemical characteristics of its individual polymeric components, determines many facets of life within a biofilm (Flemming and Wingender 2010).

Biofilms function as complex systems at the molecular, cellular and community level. A biomimetic framework based on Life's Principles can therefore be applied to biofilms in many ways. For example, when studying a single member of a biofilm community, Life's Principles can be used to describe the complexity of a single cell:

- **Life-friendly chemical reactions** happen intracellularly (e.g. metabolism) and externally (e.g. EPS production).

- **Energy requirements** of metabolic reactions are often reduced through enzymatic action.

- Almost all cellular biomolecules consist of **modular and nested components** (i.e. multiple monomers are combined in more complex macromolecules).

- **Feedback loops** are crucial to the metabolic functioning of all organisms, and require a high level of responsiveness to internal and external environments.

- The genetic **adaptability** of microorganisms allows metabolic changes in response to environmental changes and ultimately allows microorganisms to **evolve to survive** in new environments.

In the following section, Life's Principles will be applied to biofilm communities in order to create a novel framework to describe the complexity and synergism that allow biofilms to function as systems-within-systems.

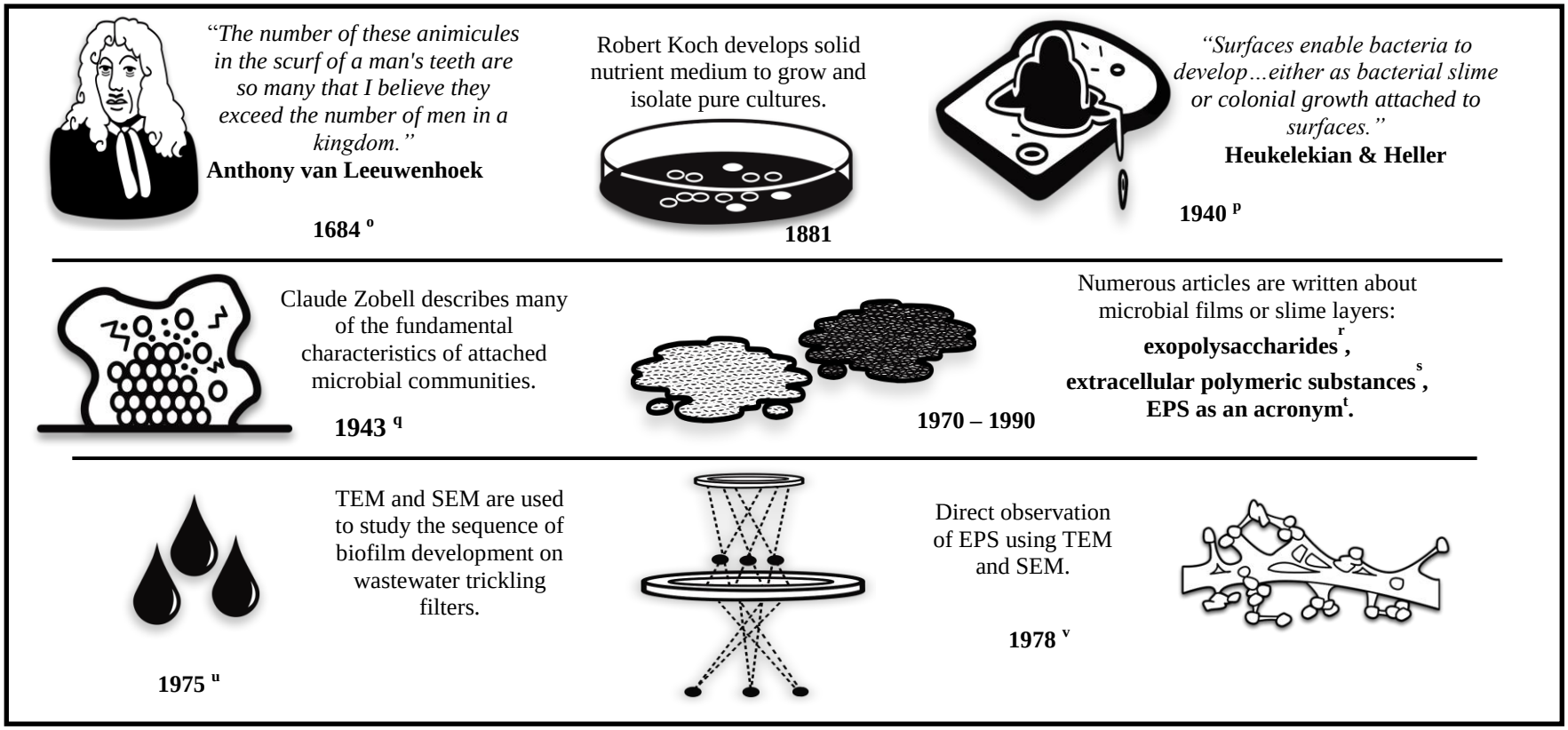

Figure 2.3. A brief history of biofilms and the discovery of EPS. Adapted from Centre for Biofilm Engineering, Montana State University[w] and Neu and Lawrence 2009. ([o] Van Leeuwenhoek 1684, [p] Heukelekian and Heller 1940, [q] Zobell 1943, [r] Sutherland 1977, [s] Geesey 1982, [t] Allison, Sutherland and Neu 2003, [u] Mack, Mack and Ackerson 1975, [v] Costerton, Geesey and Cheng 1978, [w] www.biofilm.montana.edu/brief-history-biofilm.html, accessed online on 5 November 2015.)

2.3. Using life-friendly chemistry: the biochemistry of biofilms

Life-friendly chemistry is the simplest of the six main Life's Principles and refers to the notion that nature's chemistry supports biological processes and is generally safe for living tissue (Baumeister et al. 2012). This is the foundation of biochemistry, as the assembly of elements into monomers and, in turn, into macromolecules is able to perform chemical functions that support life. Biological monomers are elegant combinations of a small subset of elements (mostly C, H, O and N) which give rise to the infinite diversity contained in the four major groups of biomolecules, namely proteins, lipids, carbohydrates and nucleic acids.

Non-toxic chemistry is a prerequisite for life, given that nature's chemistry happens in or near organisms' bodies. Water is a safe, abundant solvent with chemical properties that facilitate biochemical reactions. It stands to reason that if so-called "life-friendly" chemical building blocks are combined in a non-toxic solvent, the products of these reactions can be broken down into benign constituents. This is not to say that nature does not create toxins, as even amongst microorganisms there are known toxin producers. Despite the harmful effects of these compounds, natural toxins are still mostly produced from a small subset of elements using water as a solvent without harming the producer. Most natural toxins are produced locally in small quantities only when needed and normally do not persist in the environment for long periods.

These principles of life-friendly chemistry underpin the formation of biofilms through the production of EPS. On a rudimentary level, the EPS matrix can be viewed as a natural surface coating and it can even be argued that it outperforms many artificial coatings in terms of environmental compatibility, functionality and robustness. By using only a small subset of elements, microorganisms produce and secrete various polymeric substances which create a biodegradable, non-toxic surface coating. Microorganisms manage these elegant chemical assemblies using water as a solvent, giving rise to a highly hydrated, gel-like matrix. Water itself remains a crucial component of the matrix. Since the EPS scaffold is created and maintained by "life-friendly" biochemical processes, the scaffold can be broken down into benign constituents to be reabsorbed by the organisms or the environment.

2.3.1. Small subset of elements: the matrix building blocks

In an aqueous environment, the major components of a biofilm are the microorganisms themselves, the secreted EPS and water (Sutherland 2001). Water can account for up to 90% of wet biofilm mass and in terms of biofilm dry mass, EPS often far outweighs the cellular component depending on strain and culture conditions (Zhang, Bishop and Kupferle 1998; Flemming and Wingender 2010). Although EPS are notoriously heterogeneous, the most common substances are polysaccharides, proteins (including enzymes), extracellular DNA and lipids. As mentioned, these macromolecules are repeat units of monomers containing mostly C, H, O, N and other elements like Ca and Mg.

A rough calculation based on the estimated number of bacterial species (assuming that each strain is able to produce at least one extracellular protein and one extracellular polysaccharide) estimates the potential for more than nine million types of EPS (Staudt, Horn, Hempel and Neu 2003). EPS extraction and characterisation have therefore been the subject of many studies and potential structure-functions of EPS have been reviewed extensively (Wingender, Neu and Flemming 1999; Neu and Lawrence 2009; Sheng et al. 2010; Flemming and Wingender 2010).

2.3.1.1. Polysaccharides

The ubiquitous presence of polysaccharides in EPS matrixes has been demonstrated by various fluorescent microscopic studies and biochemical analyses (see Sutherland 2007 for a comprehensive review of the isolation and characterisation of polysaccharides from a broad range of bacterial biofilms). Although several homopolysaccharides (e.g. sucrose-derived glucans and fructans, cellulose) have been identified within biofilms, heteropolysaccharides consisting of neutral and charged sugar residues are more common.

Polysaccharides are often conjugated to other macromolecules to form structural parts of cell surfaces or integral parts of cellular membranes (e.g. lipopolysaccharides). Membrane glycoconjugates may be cleaved, allowing the polysaccharide component to form a loosely associated layer around the cell, which may in turn be secreted into the extracellular medium. Other glycoconjugates are produced after microbial attachment and help create a niche micro-environment within a biofilm. Glycoconjugate

clusters with no bacterial cells can often be seen in mature biofilms. These clusters may be the result of released EPS or are left behind when microorganisms disperse from the biofilm (Neu and Lawrence 2009).

For most bacteria, polysaccharides are crucial to biofilm formation and although non-producing species may still attach to surfaces and form microcolonies, their ability to develop into mature biofilms is severely compromised (Flemming and Wingender 2010). The presence of polysaccharide producers in a multi-species biofilm may allow non-producers to form part of the biofilm, an example of cooperative relationships in these diverse communities (see section 2.6.4).

2.3.1.2. Proteins

Extracellular proteins, including many enzymes, are another major component of EPS and can even exceed the polysaccharide component (Flemming and Wingender 2010). Most of the non-enzymatic proteins in the matrix have a structural role and two of the best characterised examples are lectins and amyloids. Extracellular lectins are carbohydrate-binding proteins and, together with other cell surface-associated proteins, provide a link between the EPS matrix and surfaces of microorganisms. Amyloids are ubiquitous matrix compounds, forming orderly repeats of protein molecules arranged as fibres that are involved in adhesion to abiotic surfaces and host cells (Neu and Lawrence 2009).

Some structural contributions are made by proteins that are not necessarily secreted as EPS: proteinaceous appendages like pili, fimbriae and flagella can also act as structural elements that stabilise the matrix (Flemming and Wingender 2010).

2.3.1.3. Extracellular enzymes

Many extracellular enzymes have been identified in biofilms and it appears that these enzymes are retained within EPS matrixes by their interactions with polysaccharides (Sutherland 2001). The immobilisation of matrix enzymes in close proximity to the microorganisms creates an external digestive system (Flemming 2011). Extracellular enzymes, mostly hydrolases, can be involved in the

degradation of water-soluble polymers (e.g. many proteins, polysaccharides and nucleic acids), water-insoluble polymers (e.g. cellulose, chitin, lipids) as well as organic matter trapped within the biofilm structure. This efficient use of readily available resources is discussed in section 2.6.1.

Pseudomonas aeruginosa, a model organism for biofilm studies, has been shown to produce a collection of extracellular enzymes (e.g. alkaline proteases, phospholipase C and lipases) that operate alone or synergistically (Tielen, Rosenau, Wilhelm, Jaeger, Flemming et al. 2010). A recent study showed that the extracellular lipase, LipA, is retained near cell surfaces in *P. aeruginosa* biofilms through electrostatic interactions with an extracellular polysaccharide, alginate. This association may protect the enzyme from denaturation and proteolytic degradation while simultaneously exposing the catalytic centre towards the substrate (Tielen, Kuhn, Rosenau, Jaeger, Flemming et al. 2013).

In addition to metabolic functions, extracellular enzymes may act as virulence factors and play a role in cell dispersal. Examples of enzymes that play a role in infectious processes include hydrolytic enzymes excreted by *Candida albicans* (Schaller, Borelli, Korting and Hube 2005), pectic enzymes by *Erwinia chrysanthemi* (Herron, Benen, Scavetta, Visser and Jurnak 2000) and elastase produced by *P. aeruginosa* (Mariencheck, Alcorn, Palmer and Wright 2003).

Cell dispersal and detachment from biofilms rely on enzymatic modification and degradation of structural matrix components, e.g. a soluble β-N-acetylglucosaminidase causes detachment and dispersion of *Actinobacillus actinomycetemcomitans* biofilms (Kaplan, Ragunath, Ramasubbu and Fine 2003). Despite the role of extracellular enzymes in EPS degradation and dispersal, there is no single or simple enzyme mixture that can degrade all polysaccharides in a biofilm (Flemming and Wingender 2010).

2.3.1.4. Extracellular DNA

Although the presence of extracellular DNA (eDNA) was initially attributed to cell lysis within biofilms, various studies have shown that certain strains produce eDNA that is distinctly different from genomic DNA and that it might be actively excreted by living cells (Dominiak, Nielsen and Nielsen 2011). The amount and distribution of eDNA in biofilms vary between species (most likely also between environmental conditions) and eDNA appears to form either grid-like structures or filamentous networks in EPS matrixes (Flemming and Wingender 2010).

Since being identified as an integral component of EPS, it has been suggested that eDNA has a structural role in developing biofilms (Neu and Lawrence 2009). By promoting attractive acid-base interactions between cells and surfaces, eDNA appears to be involved in initial attachment and surface aggregation of microorganisms (Das and Agrawal 2011). In addition to its structural and stabilising roles, eDNA represents an important mechanism for horizontal gene transfer in biofilm communities which facilitates adaptation and evolution by organisms from these communities (Roberts and Kreth 2014).

2.3.1.5. Lipids

While most of the above-mentioned EPS are hydrated hydrophilic molecules, lipids are also common matrix components that add hydrophobic properties to the biofilm micro-environment. Lipopolysaccharides may play a role in the surface adherence of certain species and the interactions between lipids and other components may influence matrix stability. Mutations in lipopolysaccharides have been shown to reduce *P. aeruginosa* attachment to hydrophilic surfaces while attachment to hydrophobic surfaces is increased (Makin and Beveridge 1996). *P. fluorescens* isolates show that lipopolysaccharide-cellulose interactions are important for biofilm development and integrity at liquid-air interfaces (Spiers and Rainey 2005).

Surface-active lipids and biosurfactants have also been identified as EPS in biofilms (Ron and Rosenberg 2001). Surfactants can be surface-bound or excreted and fulfil various roles in swarming

motility, cellular signalling processes and biofilm formation (Sutherland 2001; Van Hamme, Singh and Ward 2006). Rhamnolipid production in *P. aeruginosa* has been linked to biofilm dispersal and the creation of channels in mature EPS matrixes (Davey, Caiazza and O'Toole 2003), while the biosurfactant viscosin appears to increase the surface-spreading ability of *P. fluorescens* biofilms (Alsohim, Taylor, Barrett, Gallie and Zhang 2014). It has also been proposed that biosurfactants like emulsan might enable microorganisms to solubilise and utilise substrates that would be inaccessible otherwise (Flemming and Wingender 2010).

Some biosurfactants have received increased attention because of their antimicrobial activity. Surfactin (from *Bacillus subtilis*) and rhamnolipids (from *P. aeruginosa*) have been used in combination against biofilms of food-borne pathogenic bacteria (Gomes and Nitschke 2012). Lipids may also play a role in the transport of these and other biomolecules into the extracellular space by means of lipid membrane vesicles. Once released into the matrix, these vesicle components can alter the matrix properties and even target competing microorganisms in the biofilm (Flemming and Wingender 2010).

2.3.1.6. Inorganic ions

Inorganic ions are not necessarily produced or secreted by microorganisms but may be present in the environment and play a role in matrix structure and stability. EPS have many sites for adsorption of metals, multivalent cations and organic matter, including aliphatic and aromatic side chains in proteins and hydrophobic regions in polysaccharides (Sheng et al. 2010). Polysaccharides can interact with themselves or heterologous molecules to form gels and multivalent cations often play a significant role in this process (Flemming and Wingender 2010). For example, Ca^{2+} ions are able to form a bridge between polyanionic alginate molecules in *P. aeruginosa* biofilms, creating thick biofilms with increased mechanical stability (Körstgens, Flemming, Wingender and Borchard 2001).

2.3.2. Water as solvent and matrix component

As mentioned, water is a major component of a biofilm and is crucial to all intercellular reactions as well as EPS production and secretion. EPS are also subjected to post-secretion modifications by microorganisms (e.g. enzymatic degradation, excretion of rhamnolipids) and water acts as a solvent for these chemical reactions. In addition to facilitating chemical reactions within and around microorganisms, water is integral to diffusion processes within biofilms and serves as a transport medium for nutrients, cellular communication signals, waste products and microorganisms themselves. The microbial community and EPS retain water within the web-like structure of the matrix by means of electrostatic interactions and hydrogen bonds (Schmitt and Flemming 1999; Sheng et al. 2010). Studies have shown that certain bacteria actively produce polysaccharides in response to desiccation, e.g. increased alginate production in *P. aeruginosa* biofilms prevents evaporative water loss (Chang, Van de Mortel, Nielsen, De Guzman, Li et al. 2007).

The structural role of water and its contribution to the spatial integrity of EPS matrixes is often overlooked. Soft condensed matter physics has recently been used to conceptualise the combined solid-like and liquid-like characteristics of biofilms. Biofilms have subsequently been described as anisotropic colloids that are embedded in a cross-linked polymer gel (Wilking, Angelini, Seminara, Brenner and Weitz 2011). The polymers (as listed in section 2.3.1) contribute to the structural integrity of the matrix by resisting mechanical deformations and also determine biofilm water content. Equilibrium water content can be determined for a given cross-link density and polymer concentration. This can be used to explain how microorganisms adjust the water content of a biofilm by modifying EPS.

2.3.3. EPS break down into benign constituents

Biodegradability of EPS is vital to the success of biofilm communities. The reason for this is that as biofilms develop and mature, microorganisms need to regulate EPS build-up to prevent entrapment in “dead” zones where nutrients become scarce (Flemming 2011). As discussed in section 2.3.1.3, extracellular enzymes are able to break down various EPS as well as organic matter trapped within the

biofilm structure, using water as a medium for these reactions. Since the matrix building blocks are created by life-friendly chemical processes, the metabolic products released by their digestion are non-toxic to the microbial community and can be used as an energy source by the biofilm community and even by organisms in the broader environment. EPS degradation may also be an important survival mechanism during starvation periods, as will be discussed in section 2.4.2.

2.4. Biofilms are resource-efficient systems

Microorganisms face the challenge of being able to perform metabolic functions and behavioural actions while using energy sparingly and minimising waste (in the form of energy or materials). Energy is required to produce energy and therefore energy acquisition and consumption should be carefully regulated. There are countless examples of adaptations that allow organisms to minimise energy consumption – from the use of chemical catalysts to behavioural responses that leverage energy flows advantageously, e.g. using the movement of liquid in the environment for passive transport.

The natural world consists of closed-loop cycles where energy and materials are recycled. The ability to recycle materials greatly reduces energy requirements and resource use within a system – whether a cell, organism or ecosystem. Of course, there is no point in efficient resource usage if the functional needs of an organism are not met in the process. Therefore, biomimicry aims to investigate natural strategies that optimally balance efficiency and effectiveness. This includes studying multifunctional designs in the natural world. EPS matrixes are excellent examples of a single solution that meets multiple needs. Their biochemical building blocks have several functions and the interactions between the building blocks fulfil multiple roles in biofilms, as will be discussed in this section.

Another important sub-principle of the principle of resource-efficiency is the notion of forms fitting their functions, i.e. appropriate three-dimensional structures that facilitate functional requirements and impart properties without unnecessary material usage. This is particularly true on a microscale where chemical bonds and attractive forces between EPS create unique micro-environments for microbial communities. EPS formation is a material- and energy-efficient process that is influenced by environmental conditions and determined by the metabolic state of the microorganisms. As a biofilm

develops over time, its chemical composition and three-dimensional form are altered to fit the functions required by the microbial community. As a multifunctional structure, the EPS matrix anchors the microbial community to a surface, provides mechanical stability and its biochemical properties (e.g. hydrophobicity and electrochemical charges) regulate the micro-environment of the microbial community.

2.4.1. Energy-efficiency: managing energy expenditure within biofilms

In order to reduce energy expenditure, EPS matrixes are generally assembled and modified at ambient temperatures and atmospheric pressures using life-friendly chemical processes. This is mainly achieved through the use of enzymes in synthesis and modification of matrix components (Flemming and Wingender 2010). Enzymes act as selective catalysts that accelerate the rate and specificity of metabolic reactions while allowing these reactions to take place at temperatures and pressures that are safe for organisms. As catalysts, enzymes lower the activation energy required for a chemical reaction and thereby increase the reaction rate significantly.

The immobilisation of enzymes within the EPS matrix creates a biochemically active system and retains the enzymatic activity in close proximity to the microorganisms within the biofilm, thereby optimising the uptake of products (Flemming and Wingender 2010). This "external digestion system" (Flemming 2011) creates continuously changing micro-environments in which the microorganisms appear to self-organise for optimum access to nutrients, ultimately reaching a steady state. The enzymatic degradation of biopolymers within the matrix can provide smaller metabolic products to be used as energy sources by the organisms within the biofilm.

Energy-efficiency also extends to cellular responses, as metabolic pathways are highly regulated processes with many feedback loops, preventing unnecessary energy expenditure. The gene transcription profile of microorganisms in a planktonic state is often significantly different to their biofilm-bound counterparts (Watnick and Kolter 2000). These genetic differences translate into altered rates of respiration, nutrient uptake and cell division as dictated by environmental conditions. During nutrient scarcity, microorganisms can even regulate their metabolic rates as an energy-saving response.

For example, *P. aeruginosa* is influenced by nutritional cues that are integrated as part of a signal transduction pathway that regulates biofilm development via a regulatory protein in its carbon metabolism (O'Toole, Gibbs, Hager, Phibbs and Kolter 2000).

2.4.2. Improving material-efficiency by recycling EPS

Material-efficiency within biofilms is increased by the reuse of EPS and cellular components. The biodegradability of EPS has already been discussed. It is a particularly useful trait during starvation periods. In a multi-species biofilm, EPS can be utilised by the EPS producers or other microorganisms in nutrient-scarce environments (Wolfaardt, Lawrence, Robarts and Caldwell 1995; Zhang and Bishop 2003).

It has been proposed that the EPS matrix also acts as a "recycling yard" where components of lysed cells can be utilised by the remaining biofilm community (Flemming 2011). Cell death and subsequent lysis are normal biofilm events and it appears that regulated cell death might even be important for biofilm development (Bayles 2009). The released genomic DNA may play a role in intercellular adhesion and matrix stability, as discussed in section 2.3.1.4.

Others have investigated the recycling of electrons within biofilms and proposed that microorganisms use electrons from electron-donor substrates to build active biomass (cellular component of biofilm) while producing EPS in the process. EPS are hydrolysed to biomass-associated products, which are then utilised by the active biomass as recycled electron donors (Laspidou and Rittman 2002).

2.4.3. Multifunctional design and the many functions of EPS matrixes

The previous sections demonstrate that biofilms are more than just the sum of their matrix parts. The multi-functionality of the EPS matrix on a biochemical level creates a system with many macro-functions, affording several advantages to biofilm communities. By incorporating multiple functions on a molecular level, the long-term energy and material requirements of a biofilm community are minimised.

On a structural level, biofilms are spatially organised systems that create beneficial micro-environments that can respond to changes in the broader environment and be altered by the microorganisms. In order to truly appreciate the multi-functionality of biofilms, the diverse functions of EPS are briefly summarised in broad categories below (Neu and Lawrence 2009; Flemming and Wingender 2010).

2.4.3.1. Structural EPS

The structural functions of EPS matrixes, including surface adhesion, cell aggregation and cohesion within the biofilm, are attributed mainly to polysaccharides, proteins and eDNA. Initial surface colonisation steps are often facilitated and maintained by surface polysaccharides and proteins that act as adhesives (An and Friedman 1998; Hori and Matsumoto 2010). Specific adhesive polysaccharides can be excreted across the total cell area or at cell poles as a response to surface recognition. This allows microorganisms to switch from a planktonic to adhered state and vice versa.

Interactions between these EPS also contribute to the structural integrity of the matrix. By enabling bridging between cells and temporarily immobilising microorganisms in close proximity, EPS interactions establish high cell densities, cell-cell recognition and communication (Flemming and Wingender 2010).

2.4.3.2. Metabolically active and nutritive EPS

The role of EPS as an external enzymatic digestive system has already been mentioned in section 2.3.1.3. It is thought that EPS can potentially be used as a source of carbon, nitrogen and phosphates through enzymatic action. Additionally, charged groups on polysaccharides and proteins may play important roles in the adsorption of organic and inorganic compounds from the environment. This includes anionic groups (e.g. uronic acids in proteins), cationic groups (e.g. amino sugars in proteins), hydrophobic regions and groups with high hydrogen bonding potential (e.g. polysaccharides). The ability to adsorb and accumulate nutrients from the environment is clearly beneficial to biofilm

communities in nutrient-scarce environments while the adsorption of inorganic ions aids matrix stabilisation and ion exchange.

2.4.3.3. Protective EPS

The sorption characteristics mentioned above might have an additional protective function by adsorbing xenobiotics and/or toxic metal ions. Hydrophilic polysaccharides, and proteins to some degree, interact with water molecules to maintain the highly hydrated state of the matrix which, in turn, protects the microbial community from desiccation. It is believed that the physical barrier formed by polysaccharides and proteins also protects the community from predation, exposure to harmful compounds and host defences during infection (Palmer, Flint and Brooks 2007).

2.4.3.4. EPS for transport and mobility

The biofilm environment is dynamic and there is evidence that shows that the microorganisms remain mobile within regions of the biofilm. Additionally, cell components containing various biomolecules are often transported from the outer membranes of Gram-negative bacteria to the matrix via membrane vesicles (Schooling and Beveridge 2006). Once released into the matrix environment, these biomolecules can alter matrix properties and even act as defence mechanisms against competing microorganisms in the community (e.g. rhamnolipids in *P. aeruginosa*) (Neu and Lawrence 2009).

Information also flows within a biofilm, whether in the form of quorum sensing molecules or DNA. eDNA facilitates horizontal gene transfer between members of the biofilm community and this unique feature will be discussed in more detail in the final section of this review.

2.4.4. The matrix form suits its functions

The three-dimensional architecture of the EPS matrix, together with the chemical characteristics of its polymeric components, facilitates the multiple functions it provides the microbial community (Flemming and Wingender 2010). Various matrix structures and shapes have been reported (Nikolaev and Plakunov 2007), including simple layers, biofilm mats, plaques, bridge-like structures as well as mushroom-shaped structures with channels and pores.

These structures are influenced by environmental conditions and by the microbial community itself. Microorganisms have the ability to modify excreted EPS and thereby alter matrix form to fit the required function. Post-secretion modifications include (Flemming 2011):

- enzymatic degradation of EPS to change matrix structure and stability, possibly to create pores and channels or allow release of microorganisms;

- excretion of rhamnolipids to increase the porosity of the matrix;

- programmed cell death to generate pores and channels and/or provide nutrition for other cells; and/or

- addition of substituents to polysaccharides to influence the charge, structure or hydrophobicity of the polymer (Vuong, Kocianova, Voyich, Yao, Fischer et al. 2004).

While EPS structures have been described for several single-species biofilms, far less is known about the forms and functions of multi-species biofilms in natural environments. It is clear that EPS matrixes respond to environmental conditions and that altered EPS composition and structures create niche micro-environments.

The porous matrix structure allows diffusion of nutrients, oxygen and liquids through the biofilm. This form of passive diffusion contributes to the energy-efficiency of biofilms. As a biofilm grows in

thickness, micro-gradients develop within its structure. For example, actively respiring aerobic organisms may consume oxygen faster than it can diffuse through the biofilm, thereby creating anaerobic zones. Similarly, pH, redox potential, and ionic strength can vary within biofilms (Stewart and Franklin 2008). Beneficial biofilms used in wastewater treatment systems have been studied to some extent and it appears that morphofunctional layers develop within these biofilm mats where anaerobic organisms occupy the deeper oxygen-scarce layers and phototrophs are found in the top layers (Wagner, Loy, Nogueira, Purkhold, Lee et al. 2002).

2.5. Biofilm development integrates development with growth

Following the discussion of matrix components and the multi-functionality achieved by their combination in three-dimensional matrixes, this section examines the formation of these complex structures. As with most natural systems, optimised growth (where a balance is maintained between growth and development) is more important for the stability and longevity of a biofilm than maximised growth (rapid growth without development). While growth is a positive aspect and a necessity for organisms, it is a resource-intensive process and, without regulation, may lead to unsustainable conditions. In this context, development can be regarded as an investment in infrastructure in order to provide functional needs, while creating a stable platform for the following growth phase.

Biofilm development happens from the bottom up. This process allows the components of the system to grow and develop in response to the local environment. Biofilm development is traditionally divided into the following steps:

1) the formation of a conditioning film on a substratum,
2) motility of microorganisms to the substratum,
3) initial reversible or transient attachment of microorganisms through non-specific interactions,
4) so-called “irreversible or “permanent” attachment and
5) the production of EPS to form the matrix structure (Bester, Wolfaardt, Joubert, Garny and Saftic 2005).

Although it is not usually included in models of biofilm formation, cell dispersal is an important function of and factor in the biofilm mode of life and therefore forms part of this discussion. When considering the steps of biofilm formation, it becomes clear that biofilms are the product of simple building blocks that are combined into diverse macromolecule structures. EPS monomers are nested hierarchically into increasingly complex structures that help facilitate efficient growth of the microbial community.

Most natural systems are a result of self-organisation and biofilms are no exception. Although it may start off as a disordered system where primary colonisers attach to a surface at random, the local interactions between microbial members give rise to some form of overall coordination and order in mature biofilms. The switch to sessile state is often triggered by changes in environmental conditions and may be amplified through positive feedback loops. The resulting decentralised system contributes to the adaptability and robustness of biofilms (section 2.7).

2.5.1. Built from the bottom up

Both on a molecular and community level, biofilms start off with simple building blocks that grow into more complex hierarchical structures. Normally, the first structural step of biofilm development is the formation of a conditioning film, described as the accumulation of organic carbon residues onto the wetted surface by means of electrostatic interactions and Van der Waal's forces. Several passive forces, including Brownian motion, sedimentation and convective transport, allow microorganisms to come into contact with and subsequently colonise these primed surfaces. Microorganisms also possess the ability to actively seek out and attach to surfaces by means of propulsion using flagella (Chambers, Stokes, Walsh and Wood 2006). Once attached, microorganisms secrete EPS to anchor themselves and therefore chemically alter the surface.

Although external conditions elicit adhesion responses in multiple microorganisms, it has been hypothesised that microorganisms themselves can stimulate biofilm growth by chemically recruiting and promoting the attachment of other organisms (Chambers et al. 2006). One such recruitment strategy is the chemical alteration of surfaces by microbial footprints. A review published in the early

1990s suggests that many biofilm studies touch on the idea of adhesive surface-priming polymers but that no general definition is used to describe microbial footprints (Neu 1992). For the purpose of this discussion, any polymeric substance left on a surface by a microorganism, whether an excreted molecule or a structural cell component, is considered a microbial footprint.

Very little is known about microbial footprints, but it is assumed that these substances may be involved in initial adsorption and/or desorption processes in early biofilm development. Early studies relied on electron microscopy images and fluorescent staining of these footprints, often after physically removing cells to expose the "former contact side" (Marshall, Stout and Mitchell 1971; Paul and Jeffrey 1985; Levanony and Bashan 1989; Neu and Marshall 1991; Santos, Callow and Botta 1991). It is possible that these substances contain chemical information that influences attachment behaviour and may form part of intra- and interspecies communication signals (Palmer et al. 2007).

Following surface priming, a distinction is made between a transient, initial attachment step and a more permanent, "irreversible" attachment step. Various models have tried to define these different phases (Marshall et al. 1971; Costerton et al. 1978; Beech 2004). It is generally thought that during the initial reversible step, bacteria weakly adhere to the surface by means of Van der Waals and electrostatic forces but still exhibit Brownian motion and can therefore be removed by shear fluid forces. "Irreversible" attachment is considered a time-dependent process and usually involves EPS synthesis. It is assumed that "irreversibly" attached microorganisms no longer exhibit Brownian motion and cannot easily be removed by shear forces.

The term "irreversible" attachment is slightly misleading (Stoodley et al. 2002). Microorganisms may still be mobile within the matrix and may be able to detach from the matrix and disperse by physical or chemical mechanisms. Passive dispersal is mediated by external forces (e.g. fluid shear and abrasion) whereas active dispersal is initiated by the microorganisms themselves via a highly regulated chemical process. Changes in environmental conditions, whether favourable or unfavourable, may lead to biofilm dispersal. The ability to disperse from a biofilm is crucial to the survival of microbial species because it allows the bacterial population to expand (Wood 2009).

Based on the evidence of microbial footprints and microbial mobility within biofilms, this study proposes an alternative structure to describe biofilm formation. During the initial attachment phase, microorganisms may chemically alter the surface before detaching again. Therefore, specific chemical interactions with a surface should not only be limited to that of the more permanent attachment phase. After initial temporary attachment, microbes may still be able to move along the surface and EPS matrix using flagella or type IV pili, allowing the community to self-organise into favourable environments (Stoodley et al. 2002). It is therefore more useful to think of an initial attachment step (which may or may not involve polymer deposits), an immobile phase based on specific interactions with EPS and/or other organisms and a potential mobile phase which may involve movement within, or detachment from, the EPS matrix.

2.5.2. Combining modular and nested components

Biochemically speaking, biofilms are built from the bottom up by fitting multiple units within each other to progress from simple molecular building blocks to complex three-dimensional macro-structures. EPS biosynthesis is genetically regulated and these pathways are the focus of many studies (examples for *P. aeruginosa* are cited in Jain, Franklin, Ertesvåg, Valla and Ohman 2003; Wu, Badran, Arora, Baker and Jin 2004; Jain and Ohman 2005). Most EPS consist of repeated monomers that are assembled intracellularly before being excreted to form the matrix. For example, the biosynthesis of extracellular proteins relies on complex mechanisms for the assembly of amino acids and the folding of protein structures, followed by the translocation of these proteins over cell membranes to the EPS matrix (Sandkvist 2001).

Another example of biochemical modular and nested components is the biosynthesis of polysaccharides. A recent review of extracellular polysaccharide secretion by *P. aeruginosa* describes two general biosynthetic strategies for the production of polysaccharides, specifically alginate, Psl and Pel (Franklin, Nivens, Weadge and Howell 2011). These strategies (a lipid carrier-independent mechanism for alginate and likely for Pel vs. a lipid carrier-dependent mechanism for Psl) describe how modular units are combined into increasing complex structures.

It has been proposed that Pel and alginate are first synthesised as homopolymers and that the lipid carrier-independent mechanism contains a cyclic di-guanosine monophosphate (c-di-GMP) binding protein essential for polymerisation of the polysaccharides. These biosynthesis pathways also appear to involve tetratricopeptide-like repeat proteins as biosynthetic scaffolds to create the more complex structures of Pel and alginate polymers. The isoprenoid lipid carrier-dependent mechanism requires the presence of several proteins as well as the presence of a characteristic repeating oligosaccharide structure. Psl polysaccharides are assembled in association with the isoprenoid lipid (Franklin et al. 2011). (See section 2.7.3 for more on the regulation of the biosynthesis of these polysaccharides.)

The progression from simple to complex structures also applies to the social structures within biofilms. Once at a surface, microorganisms may attach either as single cells or as cell clusters and the primary colonisers may attract other planktonic microorganisms from the environment, giving rise to physiologically and behaviourally integrated microbial communities that have been likened to multicellular organisms (Heydorn, Ersbøll, Hentzer, Parsek, Givskov et al. 2000b). The benefits of this communal living are discussed in section 2.6.4.

2.5.3. Microorganisms self-organise into complex biofilm structures

Often described as a survival or stress response, biofilm formation may be triggered by changes in environmental conditions (favourable or unfavourable), leading to the attachment of microorganisms to a surface and to each other. It has been proposed that cell surface structures and the nature of the encountered surface also play a role in triggering biofilm formation (Blenkinsopp 1991). There are still many unknown aspects regarding the triggers and mechanisms that control the switch from planktonic to sessile state, especially in naturally occurring multi-species biofilms. It is clear, however, that biofilm formation is a complex, ordered process driven by cellular responses and is ultimately regulated on a genetic level (Wood 2009; Ivanov, Boyd, Newel, Schwartz, Turnbull et al. 2012).

One theory regarding the self-assembly of biofilms is based on the genomic and phenotypic diversity of bacterial species (Stoodley et al. 2002). It describes the biosphere as “a continuum of fluids” in which the genomes of numerous bacterial species exist in varying patterns of expression (from dormant to active in either planktonic or sessile expression states). Environmental changes can therefore

resuscitate dormant bacteria and trigger switches from planktonic to sessile state. This universality of bacterial genomes means that the environment becomes a genetic reservoir for microbial communities that self-assemble in response to environmental changes.

It has also been proposed that this coordinated development of complex microbial communities is not solely informed by nutrient availability but may be mediated in part by signal molecules and some type of positioning/motility mechanism (Stoodley et al. 2002). The ability of microorganisms to chemically alter or prime a surface to promote biofilm formation indicates that the biofilm community self-organises from the very start. Communication through chemical signals allows biofilm members to influence and organise microbial distribution patterns within the biofilm. Over time, a level of organisation develops within biofilm communities, giving rise to physiological cooperative systems that are sometimes described as "microconsortia" (Nikolaev and Plakunov 2007).

Single-species biofilms in particular exhibit highly organised patterns with relatively regular cell-cell spacing. This idea of self-organisation extends to established biofilms and more complex community interactions in multi-species biofilms. A recent study showed that a *P. aeruginosa* strain labelled with green fluorescent protein (*gfp*) (PA01 *gfp*) is able to successfully integrate, survive and proliferate within heterogeneous multi-species biofilms (Ghadakpour, Bester, Liss, Gardam, Droppo et al. 2014). PA01 *gfp* is integrated into biofilm structures, regardless of the order of inoculation events.

When multi-species inocula are introduced to flow cells with established PA01 *gfp* biofilms, the new species are able to attach, multiply and cover the existing single-species PA01 biofilms. When PA01 *gfp* is introduced to established multi-species biofilms, the *Pseudomonas* strain is able to incorporate itself in the outer regions of the biofilm near the biofilm-liquid interface. Finally, when the multi-species inocula and PA01 *gfp* are introduced to flow cells simultaneously, more complex biofilm structures develop with the majority of PA01 *gfp* cells clustered closer to the substratum and with varying amounts of PA01 *gfp* cells being released into the effluent over the experimental time period. These co-inoculation experiments illustrate that biofilm communities self-organise into complex three-dimensional social structures and are indeed built from the bottom up.

While biofilms offer many advantages, microorganisms do not participate in this communal form of living altruistically. The need to survive still drives individual behaviour, but ultimately a highly coordinated system with no top-down direction emerges.

2.6. Locally attuned and responsive microbial communities

In order to survive, all organisms need to fit into and integrate with their surrounding environment by adjusting to environmental conditions and local resources. To this end, organisms must be able to detect environmental information and respond appropriately.

The ability to use readily available materials and energy is closely linked to resource-efficiency (section 2.4). In order to make the most of local resources, organisms rely on feedback loops and cyclic processes. Feedback loops provide cyclic information flows that allow organisms to appropriately modify their reactions to the local environment, e.g. response to nutrient availability or scarcity. The shorter and more direct a feedback loop, the faster appropriate changes can be made and the likelihood of negative consequences decreases.

Feedback loops are one example of advantageous cyclic processes in the natural environment, although organisms also develop strategies to benefit from recurrent external phenomena (Baumeister et al. 2012). As discussed in section 2.1.1.1, cyclic processes are part of the operating conditions of the natural world. By taking advantage of predictable changes in the environment, strategies evolve that use these cycles as ways of minimising unnecessary energy or material expenditure.

Biofilms are incredible examples of locally attuned and responsive communities. The composition, structure and physiological activities of biofilms are influenced by and dependent on various internal and external factors (Sutherland 2001; Flemming 2011). Internal factors include the diversity and genetic profile of the microbial community as well as the inherent ability of microorganisms to modify secreted EPS. The physicochemical micro-environment of biofilms is constantly influenced by metabolic processes and diffusion gradients within matrix structures. Biofilm structure and development are influenced by numerous environmental factors, including hydrodynamic conditions,

characteristics of the substratum, temperature, nutrient availability, pH and salinity (Nikolaev and Plakunov 2007).

In addition to making the most of local resources and cyclic processes, microbial communities cultivate cooperative relationships within biofilms, whether a single or multiple species are present. Ecosystems depend on opportunities created by relationships between members, resulting in systems that are greater than the separate members. While mutualism and commensalism are often regarded as cooperative relationships, this study proposes that even competition within a biofilm promotes its ability to survive.

2.6.1. Using readily available materials and energy sources

Regardless of the specific morphology, the effect of the EPS matrix is the same: it immobilises microorganisms and allows the microbial community to function as a microconsortium that creates and regulates its own micro-environment (Flemming and Wingender 2010). While EPS production requires energy expenditure from microorganisms, the adsorptive and nutritive properties of the matrix allow the microbial community to make use of readily available materials and energy sources. EPS are able to sequester dissolved and particulate substances from the environment which can be used as nutrient sources by the microbial community, allowing the biofilm to act as an external digestion system (Flemming et al. 2007; Flemming 2011).

The matrix structure, together with its channels and interstitial voids, allows microorganisms to harness freely available energy through the passive flow of nutrients, enzymes and waste products throughout the biofilm (Sutherland 2001). Micro-chemical gradients often develop as the biofilm community and EPS syntheses increase. The lower substratum-associated layers generally exhibit anaerobic zones while the upper layers function aerobically. Nutrient and pH gradients can also develop in mature biofilm structures (Blenkinsopp 1991). In multi-species biofilms the microbial community is often organised according to micro-environment preferences and symbiotic relationships (Møller, Pedersen, Poulsen, Arvin and Molin 1996; Møller, Sternberg, Pedersen, Christensen, Ramos et al. 1998).

Certain adaptations allow bacteria to maximise access to available nutrients in both nutrient-scarce and nutrient-rich environments. Chitin is a valuable nutrient source for many marine *Vibrio* species. These species express specific structural genes to facilitate binding to chitin (as opposed to other abiotic surfaces). Studies have shown that some species are able to express chitinase and chitin-binding genes selectively in the presence of chitin, thereby maximising their ability to bind to a nutritive surface when it is available. In nutrient-rich environments, the microorgansims are able to attach to any surface but will preferentially attach to a nutritive surface in nutrient-scarce environments (Montgomery and Kirchman 1993; Watnick and Kolter 2000).

2.6.2. Feedback loops influence biofilm processes

Microorganisms use cyclic information flows to appropriately modify internal reactions and responses to their environment, as is evident by the above-mentioned example of *Vibrio* species. While this section focuses on the control of biofilm formation and dispersal via feedback loops, there are numerous examples of other metabolic processes that are regulated in a similar fashion.

Gene transcription influences biofilm formation and development by means of feedback loops, e.g. the regulatory circuitry that controls cell attachment to surfaces and cell detachment from a biofilm. Many bacteria use flagella to propel them in their planktonic state and it appears that the production of flagella and EPS are mutually exclusive.

An example of the genetic feedback control of biofilm formation is the fourfold increase in transcription of *algC* and subsequent production of alginate in certain *P. aeruginosa* strains' biofilms compared to their planktonic counterparts (Garrett, Perlegas and Wozniak 1999). Alginate is produced in copious amounts by pulmonary isolates of *P. aeruginosa* and gives rise to the mucoid character of these biofilms. Interestingly, flagella are absent from these mucoid isolates and studies have shown that sigma factor σ^{22} negatively regulates the synthesis of flagella, while positively regulating alginate synthesis. This feedback system is thought to benefit the biofilm-associated cells in two ways: 1) repressing flagellum synthesis may prevent the destabilisation of the EPS matrix, while 2) alginate production may reinforce the matrix (Watnick and Kolter 2000). A similar system has been described

for decreased flagellin synthesis with increased colanic acid production in *Escherichia coli* (Prigent-Combaret, Vidal, Dorel and Lejeune 1999).

Cell dispersal from biofilms is also initiated through diverse, sophisticated feedback mechanisms in response to environmental cues, such as nutrient and oxygen levels, c-di-GMP and nitric oxide signalling, quorum sensing and other cell-cell signals (Pratt, Tamayo, Tischler and Camilli 2007; Martínez-Antonio, Janga and Thieffry 2008; Barraud, Schleheck, Klebensberger, Webb, Hassett et al. 2009). The regulation and mechanisms of biofilm dispersal have recently been reviewed (McDougald, Rice, Barraud, Steinberg and Kjelleberg 2011).

Cyclic di-GMP (c-di-GMP) is now recognised as a central element in the signal transduction network that regulates the switch from sessile to planktonic state. Cellular concentrations of c-di-GMP are regulated by the opposing feedback activities of multiple phosphodiesterases (PDEs) and diguanylyl cyclases (DGCs). PDE and DGC sensor domains integrate environmental signals that lead to the production or degradation of c-di-GMP. Levels of c-di-GMP influence the microbial mode of life by binding to regulatory molecules that control genetic expression of biofilm or planktonic phenotype. Ultimately, a decrease in c-di-GMP levels leads to cell dispersal (McDougald et al. 2011).

2.6.3. Taking advantage of cyclic processes

Feedback loops that regulate metabolic processes and other responses are examples of intracellular cyclic processes within a biofilm. These regulatory systems allow microorganisms to rapidly respond to cellular needs as well as to changes within the micro-environment. However, biofilms are also attuned to the broader environment and ecosystems they form part of (Battin et al. 2007). As mentioned in section 2.1.1.1, cyclic processes are observed on many levels as part of the operating conditions of the planet. How, then, are biofilms attuned to recurrent phenomena in their environments?

Phototrophic biofilms occur in a wide range of aquatic and terrestrial environments and are often multi-species communities that include aerobic diatoms, green algae and cyanobacteria (Roeselers, Van Loosdrecht and Muyzer 2008). Since these organisms are reliant on photosynthesis for energy

production, diurnal cycles influence these biofilms. The phototrophic community uses light energy to produce organic substrates and oxygen via the reduction of CO_2, which in turn fuels other processes in the broader biofilm community, including the heterotrophic fraction (Roeselers, Van Loosdrecht and Muyzer 2007).

Various studies have shown that diurnal fluctuations impact phototrophic biofilms and that the biological structure of the biofilm mats is affected by the physical and chemical environment. For example, species have been shown to migrate within the mat in response to light and oxygen (Zippel and Neu 2005; Roeselers et al. 2007). The properties and functions of the phototrophic mats can even be manipulated by changes in light conditions and this has many potential biotechnological applications (Roeselers et al. 2008).

There are many other cyclic processes that influence biofilms in natural environments, for example intertidal biofilms that are adapted to the changes in shear forces and nutrient availability related to tidal changes (Magalhães, Bordal and Wiebe 2003). Even in industrial settings nutrient and/or hydrodynamic cycles may emerge that, in turn, influence biofilm development, function and even dispersal.

Cell dispersal and even biofilm sloughing (i.e. loss of biomass) can be vital responses for microbial survival in changing environments. Sloughing may be triggered by unpredicted environmental events (e.g. introduction of a toxic compound) or be part of an adaption to nutrient cycles. For example, decreases in nutrient availability and ultimate starvation have been investigated as biofilm detachment triggers. Sloughing events in *P. aeruginosa* biofilms appear to be linked to nutrient starvation, regardless of stop-flow or continuous flow conditions (Hunt, Werner, Huang, Hamilton and Stewart 2004). Likewise, nutrient conditions have been shown to affect the morphotype of *Serratia marcescens* biofilms. Even mature biofilm morphotypes can be manipulated by nutrient cycles (Rice, Koh, Queck, Labbate, Lam et al. 2005). The active release of planktonic cells from a biofilm is an important function of sessile microbial communities, and is explored further in Chapter 6. These are only a few examples of the responsiveness of microbial communities, but they highlight the importance of understanding the local context of biofilms, especially when developing anti-biofilm strategies.

2.6.4. Cooperative relationships within biofilm communities

The stability of the EPS matrix and the responsiveness of the biofilm community are dependent on synergistic interactions between the various community members (Flemming et al. 2007). Even single-species biofilms offer improved protection from host immune system responses and killing agents like biocides, compared to the planktonic state. Within multi-species biofilms, the additional complexities and opportunities appear to offer even more benefits, such as potential metabolic cooperation, access to a broader communication system and genetic exchange. Interspecies competition is not common in mature, established biofilms. Although one species in a binary biofilm might be seen as the dominant force when comparing growth rates, the second may still remain viable and abundant (Nikolaev and Plakunov 2007).

Many examples of cooperation and synergism have been recorded, where biofilm members have mutual positive influences on each other, e.g. cellulolytic bacteria and methanogenic *Archaea* (Morvan, Bonnemoy, Fonty and Gouet 1996). In other cases, one member may influence the activity of another in a commensal way, e.g. oxygen consumption by aerobic microorganisms facilitates the growth of anaerobic neighbours (Costerton, Lewandowski, De Beer, Caldwell, Korber et al. 1994). As mentioned in sections 2.4.4 and 2.6.3, phototrophic mats normally comprise of multi-layered communities that develop along oxygen gradients where oxygenic phototrophs (e.g. cyanobacteria and diatoms) occupy the top layers, with mixed layers of anoxygenic phototrophs (e.g. green and purple sulphur bacteria) below (Martínez-Alonso, Van Bleijswijk, Gaju and Muyzer 2005). Anaerobes can sometimes be present in the oxic zones of these mats (Cypionka 2000). In addition to oxygen gradients, CO_2 utilisation by the organisms creates pH gradients within phototrophic communities (Roeselers et al. 2007).

Enhanced communication within biofilms enables the cultivation of these cooperative relationships and allows members to be attuned and responsive to their neighbours. Primary colonisers may attract other planktonic microorganisms from the environment and once a biofilm is established, channels and pores within a matrix structure allow other microbes to enter. The EPS matrix may further enhance communication by limiting the diffusion of quorum-sensing molecules as is shown in examples of *P. aeruginosa* and *P. fluorescens* biofilms (Watnick and Kolter 2000).

Comparatively, far less is known about communication in multi-species biofilms although certain quorum-sensing systems, particularly the AI-2 system, have been shown to facilitate interspecies communication (Elias and Banin 2012). Several benefits of intercellular communication have been proposed. This includes possible alteration of species distribution within the matrix, alteration of protein expression in neighbouring cells and/or the introduction of a new genetic trait. Whether one or many species are present, microbes communicate using complex signalling networks to cooperatively restructure the biofilm (Wong and O'Toole 2011).

2.7. Adapting to changing conditions

By being attuned and responsive to its local environment, a biofilm community is able to respond appropriately to dynamic contexts. Hydrodynamic conditions, temperature, nutrient availability and other external factors can have significant effects on biofilm structure and function (Nikolaev and Plakunov 2007). Individual biofilm members, and the community as a whole, constantly need to adjust to these factors in order to survive. Some changes, whether internal or external, may be significant enough to warrant shifts in behaviour, thereby creating longer-term adaptations in survival strategies. This adaptability can be ascribed to functions of self-renewal, diversity and resilience.

Ideally, living systems persist by constantly adding energy and matter to repair and improve the system itself (Benyus 1997). On a molecular and even structural level, systems are continuously disassembled and renewed by actively recycling parts of the system. By preventing parts from breaking down completely (which would compromise the functioning of the entire system), a certain level of system performance is guaranteed. Self-renewal is a proactive maintenance approach that incorporates environmental changes on a continuous basis and, as these changes are built into the parts of the system, the whole system can adapt to changes without complete disruption of functionality (Baumeister et al. 2012).

Diversity adds to the adaptability and stability of systems. By including multiple forms and processes a system is better able to adapt to changes in order to meet its functional needs. This holds true for biofilms: whether within single- or multi-species biofilms, genetic diversity gives rise to variation in biochemical profiles of microorganisms as well as in a range of metabolic and behavioural strategies.

Microorganisms are able to respond to environmental challenges because of these multiple adaptation strategies.

Similar to designed fail-safes in engineering systems, variation, redundancy and decentralisation within a biofilm add to its adaptability and resilience. When one component fails, others can compensate for the loss, either short- or long-term. Resiliency is dependent on functional diversity and the interconnectedness of system components. By incorporating a variety of forms and processes that are not located exclusively together, biofilms are able to maintain function after disturbances. Depending on the nature and the time frame of a disturbance, some members may have a better chance of survival. This is demonstrated in Chapter 6.

2.7.1. Self-renewal helps maintain structural integrity of biofilms

Self-renewal of a biofilm, specifically of its EPS matrix, ensures that a microbial community can persist even when environmental conditions become unfavourable. By constantly adding energy and matter in the form of daughter cells and EPS, microorganisms are able to restore and improve the matrix system.

The creation of daughter cells through cell division is an important part of biofilm development. In spite of spatial constraints within a biofilm, new cells are created and often released into the environment. Several studies, including the results presented in Chapters 4 and 6, demonstrate that biofilms can actively produce and release significant amounts of planktonic cells in the early stages of biofilm development, even as early as 6 hours after biofilm development starts (Bester, Edwards and Wolfaardt 2009). Biofilm formation is therefore not only a survival mechanism for bacteria but also a proliferation mechanism, as released cells can colonise new surfaces or interact with existing biofilms. Very little is known about the interaction between attached and planktonic cells within the reduced-flow zone around the biofilm surface, but it is possible that these cells may be reintegrated into the biofilm (Bester et al. 2009).

As mentioned in section 2.4.4, microorganisms have various means of modifying EPS after secretion and are thereby able to alter and maintain the matrix structure. For example, rhamnolipid surfactants may be actively secreted in *P. aeruginosa* biofilms during the later stages of biofilm development that are characterised by high cell density. Rhamnolipid secretion appears to maintain channels within the matrix (Davey et al. 2003).

In a recent study, *Staphylococcus epidermidis* biofilm isolates from clinical environments were shown to exhibit enhanced biofilm dispersal and self-renewal abilities through the downregulation of a specific quorum-sensing system. Cells released from these biofilms are able to form new biofilms at the same rate as the parent cells, allowing bacteria to establish themselves in niche environments. Thicker microcolonies, increased eDNA release and cell autolysis within the *S. epidermidis* biofilms is reported, suggesting that these biofilms have adapted to their environment by adding additional material to the matrix (Dai, Yan, Parsons, Findlay, Molin et al. 2012). This could suggest that the maintenance of EPS matrixes is an important, yet unrecognised, step in biofilm development.

2.7.2. Incorporating diversity on multiple levels

The biochemical, structural and functional diversity of biofilms has already been discussed in previous sections; therefore, this section focuses on genetic and community level diversification. Genetic diversification is even evident in single-species biofilms cultivated under controlled conditions. Although a single-species biofilm technically develops from a single genetic complement, genetic diversity can develop within the biofilm over time (Boles, Thoendel and Singh 2004). This could be the result of niche micro-environments that develop throughout the biofilm due to nutrient gradients. These micro-environments may cause genetic changes within single-species cell clusters or microcolonies. A niche environment may also allow a mutant to exploit the local environment to its benefit and subsequently establish a microcolony with a genetic profile different from the rest of the biofilm community.

The benefits of synergistic interactions and cooperative relationships of diverse microbial communities have already been mentioned here (section 2.6.4) and elsewhere (Burmølle, Ren, Bjarnsholt and

Sørensen 2014). To understand the potential benefits of species diversity, the analogy of a microbial city is quite useful (Watnick and Kolter 2000). While the city provides access to resources (e.g. nutrients from the environment or in the form of EPS) and opportunities (e.g. protection from environmental factors), there is inherent competition for these benefits in densely populated areas (and, much like us, bacteria cannot always choose their neighbours). For example, some models suggest that polymer production in a biofilm provides a competitive advantage for the producer's lineage by suffocating others, while at the same time pushing its descendants into oxygen-rich environments (Xavier and Foster 2007).

Perhaps our view of competition is restricted by our own negative preconceived notions; therefore, in order to study complex systems and communities like biofilms a less biased perspective is required (Caldwell, Wolfaardt, Korber and Lawrence 1997). Although life in a biofilm is not always "suburban" bliss, the biofilm mode of life generally produces fighting-fit community members that adapt and flourish, or move out.

2.7.3. Resilience through redundancy, decentralisation and variation

On a very basic level, redundancy and decentralisation in a biofilm can be interpreted in terms of its population. In this case, there is strength in numbers, as redundancy means that species may survive despite losing some members as a result of a disturbance (e.g. sloughing event or nutrient starvation). Decentralisation, in terms of population distribution, means that not all members will be subject to the impact of a disturbance. Since the ability to form a biofilm is folded into the genetic "toolkit" of each biofilm member, cells that disperse after a disturbance should theoretically be able to colonise new surfaces and form biofilms.

Redundancies and decentralised design in biofilms can also be interpreted on a genetic level, as microorganisms may possess multiple copies of the genes required for biofilm formation and EPS production. In section 2.6.2, the central role of c-di-GMP in biofilm formation and dispersal was discussed. The genes encoding the enzymes that synthesise and degrade c-di-GMP are redundant and,

although their expression is not well studied in all microorganisms, the effects are the same, i.e. increases in c-di-GMP levels promote biofilm formation (McDougald et al. 2011).

Extracellular polysaccharide production by *P. aeruginosa* is another example of genetic redundancy that leads to increased resilience through variation. As discussed in section 2.5.2, alginate, Pel and Psl have been shown to play a role in biofilm development: alginate is more commonly produced by mucoid strains while non-mucoid strains are able to produce either Pel or Psl as structural EPS. By evaluating a range of clinical and environmental *P. aeruginosa* isolates, a study found significant strain-to-strain variability in terms of the polysaccharides' contribution to EPS matrix structures (Colvin, Irie, Tart, Urbano, Whitney et al. 2012). The results of this study suggest that the strains differ in their expression profiles of these polysaccharides and that Pel and Psl can serve redundant functions as structural matrix components. Further experiments also showed that this genetic redundancy is beneficial to *psl* mutants that are able to upregulate Pel expression and thereby maintain the ability to form biofilms.

Functional gene redundancy is also observed in environmental multi-species biofilms, although it is far more complicated to study and draw conclusions from such complex communities. A comparison of microbial communities isolated from various water streams revealed a diverse range of energy metabolism and nutrient cycling genes that are conserved between the communities from different sites (Dopheide, Lear, He, Zhou and Lewis 2015). This may suggest that environmental biofilm communities develop generally similar functional gene composition and metabolic potential. Although the functional expression of genes was not determined, the results suggest that functional gene redundancy exists in the sampled biofilm communities.

Ultimately, genetic variations give rise to variations on many other levels (see review on the physiological diversity in biofilms by Stewart and Franklin 2008). One variation that has been described for biofilm-forming organisms, specifically for *Pseudomonas* and *streptococci*, is the presence of small colony variants (SCVs). SCVs are slow-growing subpopulations with distinctive phenotypic and pathogenic traits. They appear to be biochemically and morphologically distinct from the parent strain and are less susceptible to antibiotic treatments (Proctor, Von Eiff, Kahl, Becker,

McNamara et al. 2006). SCVs from *P. aeruginosa* biofilms reportedly exhibit accelerated biofilm development rates compared to their wild-type counterparts (Drenkard and Ausubel 2002).

The above-mentioned examples of variations promote resilience and adaptability within the biofilm community, and also make additional survival strategies possible, as is discussed in the following section.

2.8. Biofilms evolve to survive

The elegant chemical assemblies, resourcefulness and adaptive nature of a biofilm culminate in its ability to evolve, thereby ensuring the survival of the microbial community. In fact, the principles discussed in sections 2.3–2.7 enable biofilms to reshuffle genetic information, replicate successful strategies and integrate unexpected events. In order to successfully manage biofilm formation, we need to understand the adaptability and evolution of microorganisms within biofilms.

As mentioned, eDNA has been shown to be a major component of EPS matrixes in single- and multi-species biofilms. Although there are many unanswered questions around the origins and specific mechanisms of eDNA, it appears that microorganisms have the ability to exchange and alter genetic information within a biofilm community, thereby creating new beneficial options for their survival.

There are numerous reports of environmental changes that lead to genetic evolution within biofilms, whether through mutations in single organisms or exchange of DNA between community members. These genetic changes are easily embedded in microbial populations and successful adaptations are replicated in the following generations. The reshuffling of genetic information gives rise to new characteristics and, although these changes might not always be beneficial, they may offer additional strategies and add to the diversity of the system. Likewise, feedback loops and sensitivity to environmental changes allow microorganisms to repeat behaviours and strategies that result in survival.

2.8.1. Microorganisms reshuffle genetic information

Genetic material can be transferred between microbes in both single- and multi-species biofilms (Molin and Tolker-Nielsen 2003). In the case of single-species biofilms, genetic exchange results in the propagation of specific traits, whereas in more complex microbial communities it may lead to novel genetic combinations and the potential emergence of new pathogens through the acquisition of virulence factors, antibiotic resistance genes and other survival strategies (Watnick and Kolter 2000; Madsen, Burmølle, Hansen and Sørensen 2012).

Horizontal gene transfer (HGT) can be seen as a resource-efficient mechanism for the reshuffling of genetic material. It (HGT) lowers the energy and time requirements for reproduction because it is not dependent on locating a compatible mating partner. In biofilms, HGT can occur through direct cell-cell contact (conjugation), DNA uptake by competent microorganisms (transformation) and bacteriophage-mediated transfer (transduction) (Molin and Tolker-Nielsen 2003; Madsen et al. 2012).

Conjugation promotes HGT between donor and recipient cells by physical contact and occurs in liquid cultures as well as in sessile microbial communities. Through conjugation, mobile genetic elements (MGEs), like plasmids and conjugative transposons, may be shared between members of the same or different species. The population density, high cell-cell contact and relatively stable physical environment of a biofilm create an ideal space for HGT. Accelerated rates of conjugation have been reported in biofilms, suggesting that biofilms allow rapid evolution by means of HGT (Watnick and Kolter 2000; Burmølle et al. 2014).

Conjugative plasmids are independent replicons that mediate their own transfer and improve their own survival by promoting microbial interactions and increasing their host's direct fitness. Research points toward interconnectedness between biofilms and plasmids, suggesting that biofilm formation facilitates HGT through plasmids and that, at the same time, plasmids induce biofilm formation (Ghigo 2001; Madsen et al. 2012).

Transduction and transformation require the integration of the external DNA into the host's chromosome or plasmids in order for the DNA to be expressed. Uptake of DNA from the environment occurs at higher rates in biofilms. There is increasing evidence that shows positive feedback exists between transformation and biofilm formation: eDNA is often released when cells enter a competent stage, providing cell-surface and cell-cell adhesion properties, thereby stabilising the matrix (Madsen et al. 2012). It has been proposed that eDNA in turn triggers natural competence in biofilms, which allows for transformation and increases the range of MGEs (Molin and Tolker-Nielsen 2003).

Genetic reshuffling has been described for many microorganisms, especially pathogens. Within biofilms, HGT by *Vibrio cholerae* is induced, suggesting that chemical communication signals within a biofilm may promote DNA exchange and subsequent evolution in *Vibrios* species (Antonova and Hammer 2011). Similar evolutionary strategies have been described for fungal biofilms in terms of genes encoding for adhesins (Verstrepen, Reynolds and Fink 2004). Adhesin proteins play a central role in surface adhesion and subsequent biofilm formation. The structure and sequence homology between adhesin-encoding genes allow domains to be reshuffled and results in the formation of new genes which are a reservoir of cell-surface molecules with novel functions.

A recent review of unisexual reproduction by eukaryotic microbial pathogens highlights that this process allows these pathogens to increase their genetic diversity by reshuffling their genomes while at the same time removing harmful mutations and producing infectious offspring (Feretzaki and Heitman 2013).

2.8.2. Integrating the unexpected

Examples of biofilm responses and adaptations to unexpected environmental events have been mentioned in this review (see section 2.6.1 and 2.7.3). By being locally attuned and responsive, biofilm members can respond to unexpected changes and integrate new strategies, thereby creating new forms and functions. This is probably one of the most useful functions of biofilms for exploitation in biotechnology applications. For example, biofilm reactors are often used as alternatives to biological waste treatment systems because the latter can be disrupted by unprecedented concentrations of

inhibitory organic compounds (so-called "shock loads"). Research has shown that under continuous flow conditions stratified biofilms with uneven biomass distribution develop within these reactors, which limit the biofilms' ability to treat shock loads. Periodic operation of biofilm reactors leads to more evenly distributed biomass in biofilms. Regular variations in substrate concentrations improves biofilm responses to shock loads (Woolard 1997).

MGEs play an important role in the *in situ* transfer and even *de novo* construction of catabolic pathways that allow microbial communities to adapt to unexpected xenobiotic exposure (Top and Springael 2003). Bacteria that can degrade organic xenobiotics seem to rapidly adapt to new xenobiotic compounds by expressing "new" catabolic pathways acquired by means of HGT, mutations and genetic reshuffling. These evolutions have been observed as accelerated biodegradation of pollutants in several habitats.

2.8.3. Biofilms replicate successful strategies

Although biofilms may provide protection in unfavourable environments, this can only be a successful survival strategy if the microorganisms are able to adapt to the changes by replicating strategies that work. Biofilms in natural environments are continuously exposed to changing environments and therefore EPS matrixes provide a space where genetic adaptations can be exchanged. Accelerated adaptations through genetic reshuffling may be important for biofilm formation, which benefits microorganisms and MGEs. Genetic heterogeneity within a biofilm is maintained by HGT and mutations which affect whole genomes, i.e. original DNA and extra chromosomal elements (Rankin, Rocha and Bronw 2011). Biofilm formation is stimulated by MGEs, which, in turn, ensures genetic heterogeneity through modular and mutational adaptations (Madsen et al. 2012).

HGT by plasmids are not always beneficial and microorganisms have developed systems to prevent the "hijacking" of their chromosome by foreign DNA (Madsen et al. 2012). A clustered, regularly interspaced, short palindromic repeats coupled with *cas* genes (CRISPR/cas) system was recently discovered and this system seems similar to an adaptive immune system. In this case, a single microorganism obtains immunity to a specific foreign nucleic acid sequence that allows it to reject

MGEs with this sequence. This "acquired" immunity becomes part of the microbe's DNA and is passed along to its daughter cells (Horvath and Barrangou 2010).

It appears as if biofilm strategies can also be passed along to daughter cells. While biofilm development is often presented as a waiting-out-the-storm survival mechanism, the notion of biofilms as microbial proliferation sites has received far less attention. Although biomass sloughing is recognised as a distinct, though sporadic, event in biofilm cycles, "cell shedding" or planktonic cell yield of active cells has been observed in many studies and is supported by the results of this study.

It has been proposed that the nature of the detachment event may influence the phenotype of the released cells, i.e. cells released during a sloughing event may be more likely to present a biofilm phenotype while actively released daughter cells may present planktonic phenotypes (Donlan 2002). Either way, it seems as if a biofilm is able to prepare and prime the parcel of genetic information it releases in order to ensure its success outside the confines of the EPS matrix.

2.9. Learning from nature's multifunctional coatings

Following the description and demonstration of the biomimetic framework for biofilms, this section focuses on 1) nature-inspired anti-biofilm strategies and 2) the principles of biofilms that are worth emulating in artificial coatings and other applications.

2.9.1. Nature-inspired anti-biofilm strategies

Given their ubiquitous presence and the detrimental effects biofilms can have in a wide range of environments, biofilm prevention has become a research interest for multiple industries. There is an increasing demand for methods that successfully reduce biofilm formation, without increasing microbial resistance or negatively impacting the environment.

Many toxins commonly used in coatings have been discontinued and there are growing concerns regarding the replacement of these toxins with biocides and antimicrobial compounds, such as silver

nanoparticles. Very little information is available on the leaching rates of these alternative compounds from coatings and their subsequent environmental impacts (Chambers et al. 2006).

Biomimicry offers alternative approaches to biofilm prevention. Many organisms have developed physical and chemical antimicrobial strategies, some of which have already been mimicked in environmentally sound biofilm-control applications (Salta, Wharton, Stoodley, Dennington, Goodes et al. 2010). Physical or topographic approaches have inspired biomimetic surfaces that aim to prevent microbial attachment and biofilm formation based on micro- or nanostructures, without the use of antimicrobial compounds.

One of the most well-known examples is a sharkskin-inspired surface. By mimicking the microstructure of sharkskin, a patterned surface with low wettability and antifouling properties is created. Commercially available Sharklet™ adhesive films have been shown to significantly reduce biofilm formation without the use of biocides[4]. This technology has been tested against a range of pathogens, including *P. aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumonia*, *Acinetobacter baumannii*, and *E. coli* (Chung, Schumacher, Sampson, Burne, Antonelli et al. 2007; May, Hoffman, Sogo, Parker, O'Toole et al. 2014).

Other topographic strategies include mimicking mussel adhesive proteins and barnacle cement to grow stable, non-toxic antifouling polymer chains on surfaces (Statz, Finlay, Dalsin, Callow, Callow et al. 2006), ultra-low fouling peptide surfaces derived from natural amino acids (Chen, Cao and Jiang 2009) and nano-engineered surfaces inspired by natural super-hydrophobic surfaces like *Nelumbo* (lotus) leaves (Scardino, Zhang, Cookson, Lamb and De Nys 2009).

Another biomimetic approach to biofilm prevention is to mimic natural antimicrobial compounds and use these compounds as active agents in surface coatings. The mode of action of natural antimicrobials varies: they can disrupt intercellular communication and signalling pathways (e.g. acyl homoserine lactones derivatives) or use small molecules that prevent/disrupt biofilms (e.g. enzymatic degradation of EPS or the use of monoclonal antibodies as anti-biofilm agents) (Blackledge, Worthington and Melander 2013).

[4] http://sharklet.com/our-technology/sharklet-discovery/, accessed 27 April 2016.

Examples that have been investigated include bromoageliferin and oroidin from marine sponges (Richards, Ballard, Huigens and Melander 2008; Stowe, Richards, Tucker, Thompson, Melander et al. 2011), furanones from seaweed (Hentzer, Riedel, Rasmussen, Heydorn, Andersen et al. 2002) and various antimicrobial peptides (Czyzewski and Barron 2008; Batoni, Maisetta, Brancatisano, Esin and Campa 2011; Costa, Carvalho, Montelaro, Gomes and Martins 2011). In addition to their antimicrobial activity, these compounds are generally non-toxic because of life-friendly chemical principles and typically do not lead to antimicrobial resistance.

The most common disadvantage of natural antimicrobial compounds is the relatively short activity of these compounds, which currently limits their use in surface coatings. However, there are countless natural compounds with antimicrobial and/or anti-biofilm properties and we are only beginning to uncover this wealth of resources.

2.9.2. Emulating biofilm design principles

As mentioned in Chapter 1, biofilms can be viewed as "green" surface coatings. The EPS matrix self-assembles using life-friendly chemistry and successfully attaches to almost any wetted surface, often with high percentages of substratum coverage. These living coatings are also multifunctional, as EPS matrixes not only provide mechanical stability to microbial communities but also create beneficial micro-environments.

The most obvious innovations inspired by biofilms relate to the protection of abiotic surfaces. In fact, there are many documented cases where biofilms themselves have been used as protective coatings. It has been shown that aerobic *P. fragi* and the facultative anaerobe *E. coli* DH5a form biofilms on carbon steel and, in doing so, inhibit corrosion of the surfaces. When microorganisms within these biofilms are killed using antibiotics, the protective function is lost and corrosion rates are comparable to those in the sterile medium controls (Jayaraman, Earthman and Wood 1997). Similarly, gramicidin-S-producing *Bacillus brevis* biofilms have been shown to protect mild steel surfaces by inhibiting the attachment of iron-oxidising and sulphate-reducing bacteria (Zuo and Wood 2004). There are also examples of "artificial" biofilms where microorganisms are immobilised in sol-gel materials in order to

impart additional metabolic functions to a surface (Ghach, Etienne, Urbanova, Jorand and Walcarius 2014).

However, simply using biofilms as surface coatings or encapsulating microorganisms in a coating is not biomimicry but rather a form of bio-utilisation. In order to truly mimic biofilms, we first need to acknowledge the deeper principles that allow these structured communities to thrive. Using this biomimetic framework, there are a few biological capabilities of biofilms that are worth highlighting as inspiration for improved coatings.

The labelling of surfaces with macromolecules (inspired by microbial footprints – see section 2.5.1) has been proposed as a bio-inspired approach to corrosion inhibition (Stadler, Fuerbeth, Harneit, Grooters, Woelbrink et al. 2008). Factors like surface charge, hydrophobicity and surface roughness play an important role in microbial attachment and therefore the chemical alteration of a surface may promote or prevent biofilm formation.

Studies have shown that microbial surface interactions can be influenced by labelling surfaces with specific compounds. For example, emulsifying agents and biosurfactants have been used to create anti-adhesive layers on surfaces (Banat, Satpute, Cameotra, Patil and Nyayanit 2014). Conversely, surface deposits of molecules that alter the surface charge (e.g. amino acids) may attract microorganisms and promote attachment (Terada, Yuasa, Kushimoto, Tsuneda, Katakai et al. 2006). Although it is difficult to study microbial footprints, given the microscopic scale and miniscule amounts thereof, understanding the basic glue of biofilms may lead to new antifouling strategies or biomimetic surface coatings.

The environmental compatibility of EPS matrixes has been discussed in Chapter 2. Biofilm decomposition does not result in harmful by-products, compared to many man-made coatings that have been banned based on the environmental toxicity of their constituents (e.g. tributylin, volatile organic compounds, as well as lead, arsenic, mercury and their organic derivatives) (Chambers et al. 2006). The life-friendly chemistry of microorganisms can inspire the production of “green” coatings, as well as countless other eco-friendly materials. Recent examples include biofilm-inspired coatings where surface-active compounds, like enzymes with antimicrobial activity, are cross-linked into bio-

degradable polymer coatings (Kammoun, Haddar, Kallel, Dammak and Sayari 2013; Yuan, Yin, Jiang, Liang, Pehkonen et al. 2013).

Life-friendly chemical processes are energy-efficient and EPS matrixes are generally assembled and modified at ambient temperatures and atmospheric pressures. This is mainly achieved through the use of enzymes in the synthesis and modification of matrix components (Flemming and Wingender 2010). Conversely, the production of most artificial coatings is energy-intensive and requires additional chemical treatments, high temperatures and/or pressures as final curing steps (a "heat-beat-treat" approach) (Baumeister et al. 2012).

Despite the high energy and material inputs, most artificial coatings often only offer a single function, for example killing microorganisms upon contact with the surface. The nature-inspired coatings in the above-mentioned section demonstrate that multi-functionality can be achieved while keeping material use to a minimum (an example of form-fitting function, as discussed in 2.4.4). Super-hydrophobic coatings protect surfaces from corrosion by repelling liquid, prevent biofouling by inhibiting microbial attachment, and may impart additional hydrodynamic properties to a system.

EPS matrixes are excellent examples of multifunctional designs that fit their form to the required functions on a nano- and micro-scale. In addition to covering a surface, the EPS matrix and the microbial community it houses may play an active role in the local environment, for example the cycling of heavy metals by phototrophic biofilms in aquatic systems (Beck, Janssen, Polerecky, Herlory and De Beer 2009). When we consider surface coatings as active, rather than static, components in our industrial systems, future coatings might be able to recycle compounds or purify waste, as well as protect surfaces from corrosion

When we think of biofilms as "active" coatings, there are several other properties worth emulating in synthetic coatings. Firstly, biofilms self-assemble from the bottom up by combining modular and nested components, as discussed in section 2.5. Biomimetic principles of hierarchical and self-organisation have inspired novel supramolecular architectures that have been applied in various biomaterials (Zhang 2003; Sanchez, Arribart and Guille 2005).

Furthermore, biofilms are able to restore and renew themselves through the secretion and modification of EPS, as well as the addition of daughter cells. Bio-inspired autonomic self-healing materials that are responsive to environmental stimuli are being developed, some of which are used as anti-corrosion coatings (Trask, Williams and Bond 2007). By studying biofilm responses to environmental changes, we might be able to identify important triggers for self-renewal, which may inspire novel anti-biofilm strategies or unlock more potential self-healing materials.

By being locally attuned and responsive, biofilm members can respond to unexpected changes and integrate these new strategies, thereby creating new forms and functions. Cell-cell communication is vital for these systems level adaptations and microbial communication mechanisms, like quorum sensing, might offer inspiration for new swarm intelligence optimisation algorithms, which can be used to solve real-world problems (Niu, Wang, Duan and Li 2013).

Examples of biofilm responses and adaptations to unexpected environmental events have been mentioned in this chapter (see sections 2.7 and 2.8). This is probably one of the most useful functions of biofilms for exploitation in biotechnology applications and certainly a design principle worth mimicking in innovations.

2.10. Bionics, ecological engineering, biomimicry: real differences or just semantics?

"*Biomimicry could represent a revolutionary change in our economy by transforming many of the ways we think about designing, producing, transporting, and distributing goods and services.*"

Biomimicry Economic Impact Study, Fermanian Business and Economic Institute, 2010[5]

This review would be incomplete without a critical reflection on the concept of biomimicry. Following the era of computerisation, economists predict that we have entered a new transformational period of innovation, or the 6th innovation wave[6], that will be marked by sustainable technologies that include whole-system design, industrial ecology, green chemistry and nanotechnology, renewable energies and biomimicry. Considering all the sustainability-related buzzwords, the question remains whether there

[5] http://www.sandiegozoo.org/images/uploads/BiomimicryEconomicImpactStudy.pdf, accessed 23 April 2016.
[6] http://www.naturaledgeproject.net/Keynote.aspx, accessed 23 April 2016.

are real differences between related concepts such as bionics, ecological engineering and biomimicry and, more importantly, if these fields will in fact yield sustainable solutions.

There is some overlap between the closely related concepts of bionics, biotechnology, ecological engineering and biomimicry, which is not surprising given their simultaneous development (Figure 2.1). Biomimicry is often distinguished from biotechnology and ecological engineering on the basis of bio-utilisation. While the latter two fields often employ organisms or ecosystems as "workhorses", biomimicry focuses on the extrapolation of natural principles into areas of human design. For example, using spiders to produce silk would be considered bio-utilisation, cloning silk genes into microorganisms to produce silk would be bio-manipulation, but recreating the chemistry of spider silk or mimicking the physical production process would be biomimicry.

Since nature-inspired innovation dates back to the fifteenth century, maybe even further, it could be argued that biomimicry is not a novel approach to innovation (Vincent, Bogatyreva, Bogatyrev, Bowyer and Pahl 2006). Over the past twenty years, the concept has certainly been redefined, developed and trademarked to an unprecedented level. Perhaps the true novelty lies in the context and intention of biomimicry. Inspiration from the natural world may have been an idealistic luxury for past generations, but, given looming global environmental, economic and social crises, there is increasing need for sustainable solutions. Biomimicry promotes technological advances in a "do-more-use-less" manner. By using Life's Principles as design standards, it sets high aspirations for any innovation that claims to mimic nature.

The Da Vinci Index was developed in 2000 by the Fermanian Business and Economic Institute at Point Loma Nazarene University, San Diego, USA. This index was designed to track the progress of biomimicry by considering the number of related academic articles, patents and grants in North America. By 2010, biomimicry was gaining momentum in academic and economic spheres and the Da Vinci Index recorded a 13-fold increase in patents, 5-fold increase in academic papers globally as well as a 3-fold increase in the number of grants for biomimetic research issued in the USA. It was optimistically projected that by 2025 biomimicry could directly represent $300 billion of gross domestic product (USA) and indirectly provide $50 billion by mitigating the negative impacts of

pollution and resource depletion (figures calculated using 2010 dollar values) (Fermanian Business and Economic Institute 2010).

An updated report published in 2013 presented a less optimistic but more balanced picture; a trend doth not an economy make (Fermanian Business and Economic Institute 2013). Although the field of biomimicry continued to expand, the performances of firms applying the technology varied. Uptake by investors has not increased as expected, which could be explained by limited awareness of the concept of biomimicry. This report points out that tangible evidence to support the marketed potential of biomimicry was still lacking (Fermanian Business and Economic Institute 2013).

Other critiques point to the gap between biology and technology: biomimicry needs more standardised, common principles that allow non-biologists to access the so-called genius of nature (Vincent et al. 2006). To this end, the Biomimicry Institute curates AskNature, a comprehensive online library that catalogues natural phenomena and bio-inspired applications[7]. Tools like AskNature will need to be developed in order to promote biomimetic solutions for problems in non-biological fields.

On a philosophical level, biomimicry still needs some development to iron out ambiguities in concepts and language use. Its descriptive and sometimes ad hoc approach to innovation might also contribute to seemingly fragmented results (Mathews 2011). Biomimicry is set apart from fields like bionics by the ethos it promotes (i.e. the concept of nature as mentor, model and measure), but like any movement it risks being misconstrued and used in ways that contradict its own code of conduct. If practiced in its truest form, biomimicry is much more than a bag of technological tricks. It is a revolutionary concept that promotes sustainable progress and technological advancement, while building a scientific and economic case for the conservation of the natural world.

[7] http://www.asknature.org/article/view/why_asknature, accessed 29 April 2016.

2.11. Conclusion

Biofilms are more than the sum of the microbial community, their self-produced glue and the protected spaces they create. Describing the complexity of the microscopic universe within a biofilm is similar to describing the intricacies of the cosmos. Even the smallest unit of a biofilm, a single microorganism, is a universe in itself.

In this review, the complexities of biofilms were organised into the hierarchical framework of biomimetic Life's Principles. These six principles were applied to biofilms on various levels: molecular levels of cellular metabolism and EPS, structural levels of microcolony formation and matrix development as well as levels of function and relationship within the biofilm community and the broader ecosystem.

When Life's Principles are used as a check list, all the boxes are ticked by biofilm functions. This review has demonstrated that:

- The three-dimensional matrix is constructed using **life-friendly chemical principles.** Microorganisms use water and a small subset of elements to construct biological macromolecules and assemble these molecules into a hydrated web-like structure. Because of the biochemical processes used in biofilm formation, the matrix components and even organisms themselves can be broken down into benign constituents when no longer useful.

- Biofilms are **resource-efficient systems** that optimise resource use through low-energy processes and by recycling materials. The EPS matrix can be described as a multifunctional solution to microbial needs by fitting its three-dimensional form to fulfil the functions required.

- Biofilm **development is integrated with its growth**, ultimately forming a system of modular and nested components that are built from the bottom up by a self-organising community.

- By leveraging cyclic processes, feedback loops and cooperative relationships, the biofilm community remains **attuned and responsive** to its local environment, which allows it to make the most of readily available resources.

- Diversity, self-renewal and resilience are evident on various levels (e.g. molecular, structural, community) and ultimately create an **adaptive** community that can **respond to environmental changes**.

- Within a biofilm, microorganisms develop ways of reshuffling genetic information, replicating strategies that work and integrating unexpected events in order to **evolve and survive**.

When viewing biofilms from a biomimetic perspective, these microbial communities can be seen as self-sustaining systems that operate in a way that allows the systems to advance and continue – regardless of which parts of the systems survive. This framework is used to discuss the experimental results presented in this study.

It is not surprising that there are a plethora of techniques available to study these complex microbial communities. The next chapter briefly reviews some of the most common techniques used in biofilm research and highlights the ones used in this study. In Chapter 4 protocols are developed for the relevant techniques. This chapter aims to develop a robust experimental approach by applying a range of techniques to quantify structural features and metabolic rates of biofilms. It is hypothesised that single-species biofilms will produce relatively reproducible results when cultivated under controlled conditions. Multi-species biofilms are then also investigated using these techniques.

In Chapter 5, the application of three biomimetic principles (life-friendly chemistry, resource-efficiency and integrated growth and development) to EPS matrixes are tested through microscopic and spectroscopic investigations. Specifically, this chapter studies the effect of nutrient concentrations and hydrodynamic conditions on biofilm composition and structure in order to identify trends in biofilm adaptations.

Chapter 6 investigates the responsiveness, adaptability and evolution of biofilms when exposed to environmental changes and antimicrobial compounds. It is hypothesised that multi-species biofilms may be less affected and/or recover faster than single-species biofilms. Biofilm responses are measured in terms of whole-biofilm metabolism and planktonic cell yield.

3. Techniques relevant to biofilm studies

This chapter briefly reviews the most common biofilm cultivation methods and techniques used for biofilm analysis. This mini-review is by no means a complete list of technologies available for biofilm research, but rather aims to illustrate the limitations (and risks) of studying biofilms in one or two dimensions only. This last point is a cornerstone of the fundamental questions posed in this study: are laboratory-cultivated biofilms comparable to naturally occurring biofilms and how accurate are our extrapolations of biofilm behaviour from laboratory-scale to natural settings?

These questions, together with the technical overview, serve as motivation for the experimental design used in this study. The selected analytical techniques are reviewed in more detail and protocol development and technique validation are expanded on in Chapter 4.

3.1. Model biofilm organisms and multi-species biofilms

The majority of biofilm research has focused on single-species biofilms cultivated under controlled laboratory conditions. Advances in microscopy, molecular genetics and genome analysis have made it possible to study the structural, molecular and genetic aspects of model biofilm organisms in great detail. Of the most extensively studied organisms include *P. aeruginosa*, *E. coli*, and *V. cholerae* (Davey and O'Toole 2000).

Two well-characterised *Pseudomonas* strains were selected for this study. Pseudomonads are ubiquitous in a wide range of environments and this genus of Gram-negative bacteria has been studied extensively for its role in hospital-acquired infections, plant growth promotion, degradation of certain pollutants and biological pest control. The formation, structure, composition and function of *Pseudomonas* biofilms have been studied using various techniques (Strathmann, Wingender and Flemming 2002; Huang, Ude and Spiers 2007; Ivanov et al. 2012). *P. aeruginosa* is known to form biofilms under controlled laboratory conditions and has become a particularly useful model organism for biofilm studies.

While single-species biofilms are a sensible starting point, it is important to expand our understanding of these complex microbial systems by investigating multi-species biofilms that more closely resemble naturally occurring biofilms. For this purpose, an environmental microbial community consisting of bacteria, yeast and fungi was isolated from a winery wastewater treatment facility.

3.2. Biofilm cultivation methods

Most biofilm research is performed using *in vitro* models that allow control over environmental factors. *In vitro* models can be divided into two broad categories: closed, static batch systems and open, dynamic flow systems (Coenye and Nelis 2010). While batch systems (e.g. Calgary devices, microtiter plates) have played an important role in establishing biofilm research, it is widely accepted that dynamic flow systems are better able to mimic the environments where biofilms naturally occur. The category of open, dynamic systems includes a wide range of reactor vessels with continuous flow: suspended substratum reactors with removable, colonisable material (e.g. CDC biofilm reactors), rotating disk reactors, flow cells, microfluidic devices, modified Robbin's devices and drip flow reactors (McBain 2009).

It is important that the "why" of biofilm research should inform the "how" of biofilm cultivation and "what" of analytical techniques. Biofilm cultivation methods must be compatible with the selected analytical technique and the combination of cultivation/analytical techniques should be appropriate to address the research question. For example, if the structure of the EPS matrix is the focus of the study it is crucial to select a combination of cultivation and analytical techniques that allows non-disruptive investigation of the biofilm. The removal of a biofilm from its aqueous environment (e.g. biofilms cultivated on coupons) for microscopic analysis is unsuitable in this case, since the exposure to air interfaces might disrupt the matrix structure.

In natural environments, biofilms are often exposed to unfavourable conditions such as low nutrient conditions, high shear forces and periods of desiccation. Natural biofilm development can therefore be a slow process (weeks to months). At laboratory level, biofilms are often studied over shorter time periods in more favourable conditions that encourage rapid biofilm development. The selection of a

biofilm cultivation method should therefore balance time and other experimental constraints with the method's ability to mimic natural environments.

It is important to acknowledge the limitations of the selected method when inferring conclusions from laboratory-scale to naturally occurring biofilms. For example, observations of biofilm responses in a shear-free environment (e.g. cultivation in microtiter plates) might not explain biofilm responses in an environment where hydrodynamic forces are at play.

In this study, biofilms were cultivated in continuous, once-through flow systems with bioreactors (either flow cells or silicone tubes). These systems are simple, inexpensive and compatible with a wide range of analytical techniques. Model organisms and established biofilm cultivation protocols were used to compare results with previous biofilm studies and to subsequently validate the methods used here (Chapter 4). Although these systems still create an environment that promotes biofilm development, it is possible to alter environmental factors (e.g. hydrodynamic conditions and nutrient availability – Chapter 5) and introduce disruptions (e.g. antimicrobial compounds – Chapter 6) to investigate biofilm responses.

3.3. EPS extraction and characterisation

Biofilms are complex systems composed of microorganisms, water and EPS (Sutherland 2001). EPS production is a distinct step in the traditional model of biofilm development and therefore many biofilm studies have focused on EPS extraction and characterisation (Bester et al. 2005). EPS include organic polymers produced and secreted by the microbial community (proteins, polysaccharides, extracellular DNA and lipids) as well as other secondary molecules adsorbed from the environment (humic substances, environmental debris etc.). The heterogeneous biochemical compositions and three-dimensional structures of EPS matrixes are influenced by internal factors (e.g. genetic and metabolic profile of microbial community) and environmental conditions (e.g. cultivation method, pH, temperature) (Sheng et al. 2010).

Considering the inherent heterogeneity of the subject matter, the characterisation and quantification of EPS is problematic. While many biofilm studies have made use of extraction methods followed by biochemical assays to investigate EPS, most of these methods require the disruption of the biofilm and therefore do not offer information on the spatial distribution of EPS in relation to microbial cells. The effective separation of EPS from cells, without inducing cell lysis, is another challenge. Nonetheless, EPS extraction and biochemical assays are still widely used and are briefly discussed below. Alternative techniques that allow *in situ* investigations of EPS matrixes are discussed in section 3.4.

3.3.1. EPS extraction methods

In general terms, an ideal extraction technique should 1) effectively release EPS, 2) cause minimal cell lysis and 3) not disrupt or alter EPS components. Extraction efficiency can be expressed as the total amount of EPS extracted from the total EPS pool for a given biofilm sample (Nielsen and Jahn 1999). There is no universal extraction method for the complete quantitative extraction of EPS from a biofilm (Sheng et al. 2010). Although numerous methods have been applied, only a few methods have been thoroughly evaluated to obtain an optimal extraction procedure with high extraction efficiency together with minimal cell lysis and disruption of macromolecules.

It is difficult to determine the extent of cell lysis during EPS extraction. Since proteins and nucleic acids are inherent EPS components, these macromolecules cannot be used as indicators of cell lysis. As an alternative, intracellular markers such as adenosine triphosphate (ATP) and intracellular enzymes (e.g. glucose-6-phospate dehydrogenase) have been used to measure cell lysis during EPS extraction (Frølund, Palmgren, Keiding and Nielsen 1996). Cell lysis has also been evaluated using live/dead cell count, staining methods or cell count coupled with microscopy. These methods are based on cell wall integrity, since cell wall disruption would lead to intracellular content release (Sheng et al. 2010).

Standard EPS extraction procedures generally include sampling and pre-treatment followed by extraction, purification and analysis steps (Nielsen and Jahn 1999). Sampling and pre-treatment steps are normally carried out at low temperatures to prevent the disruption of EPS through enzymatic activity. Homogenisation of biofilm samples is a very common pre-treatment step (Sheng et al. 2010).

One of the first factors to consider when planning EPS extraction is whether EPS need to be separated quantitatively from cell biomass or if only certain EPS components need to be extracted for further analysis. Bound and soluble EPS can normally be separated by centrifugation: bound EPS should be confined to the microbial pellets while soluble EPS should remain in the supernatant (Sheng et al. 2010). Alternatively, soluble EPS can be removed by washing the biofilm sample before extraction. The ionic strength and composition of the washing buffer should be similar to the sample to prevent bound EPS from being washed from the matrix (Nielsen and Jahn 1999).

3.3.1.1. Physical and chemical extraction methods

Extraction methods for bound EPS can be categorised as physical, chemical or a combination thereof. Physical methods apply an external force to extract EPS from cells and dissolve it in solution, e.g. mixing or shaking, sonication, centrifugation or heat treatment (Nielsen and Jahn 1999; Sheng et al. 2010). Chemical extraction methods typically involve the disruption of interactions between EPS and cells. Common methods include the use of alkaline treatment (NaOH/HCHO), ethylenediamine-tetraacetic acid (EDTA), phenol/saline or cation exchange resin (Karadenizli, Kolayli and Ergen 2007; Sheng et al. 2010).

Although the extraction efficiencies of chemical methods are higher than those of physical methods, the use of chemicals may introduce other problems (D'Abzac, Bordas, Van Hullebusch, Lens and Guibaud 2010). Alkaline treatment can cause severe cell lysis and disruption of macromolecules. The EDTA method has high extraction efficiency and causes minimal cell lysis, but the residual EDTA interferes with subsequent protein determination assays. A dialysis step must therefore be included in EDTA extraction methods. The cation exchange resin method has become one of the most widely accepted EPS extraction methods because of its high efficiency and low levels of cell lysis. The resin can be easily removed, eliminating chemical contamination, and therefore subsequent EPS analysis is easier (Jahn and Nielsen 1995).

3.3.1.2. Combined extraction methods

Combinations of chemical and physical extraction methods generally yield more reproducible results. Some examples include:

- Alkaline treatment combined with heat treatment to extract capsular EPS (Breedveld, Zevenhuizen and Zehnder 1990),

- Centrifugation and acetone precipitation, followed by size-exclusion chromatography and nuclear magnetic resonance (NMR) analysis to investigate polysaccharide production (Conti, Flaibani, O'Regan and Sutherland 1994),

- NaCl, formaldehyde and ultrasonication to extract EPS from anaerobic sludge (Jia, Fang and Furumai 1996),

- And scraping, blending and centrifugation followed by anion-exchange chromatography to characterise sugar content from planktonic and biofilm samples (Kives, Orgaz and Sanjosé 2006).

More recently, the efficiency of certain physical (ultrasonication and heating) and chemical (EDTA, H_2SO_4, formaldehyde plus NaOH and glutaraldehyde) extraction methods was compared in terms of EPS yield, cell lysis and EPS disruption (Sun, Li, Mu, Wang, Yu et al. 2012). The results indicate that there is a payoff between EPS yield and cell lysis: longer extraction times increase yield but generally increase cell lysis. The study also reports that some methods give higher EPS yields (formaldehyde plus NaOH and H_2SO_4) while others show lower levels of cell lysis (ultrasonication and H_2SO_4) and EPS disruption (formaldehyde plus NaOH and EDTA).

None of these methods can extract the entire compliment of EPS from biofilms. Therefore a method must be selected and optimised for each experiment, taking the sample characteristics and research objectives into consideration. This makes EPS extraction and subsequent characterisation a tedious process.

3.3.2. Biochemical analysis of extracted EPS

There are numerous assays that have been used to identify and quantify EPS composition. Table 3.1 lists some of the commonly used techniques.

Table 3.1. Assays for biochemical characterisation of EPS.

EPS component		Method	Reference
Polysaccharides	*Total carbohydrate content*	Phenol-sulphuric acid assay	(DuBois, Gilles, Hamilton, Rebers and Smith 1956)
		Anthrone method	(Trevelyan, Forrest and Harrison 1952)
	Acetyl determination	Hydroxylamine in presence of Fe^{3+}	(Hestrin 1949)
	Glucose, fructose and gluconic acid determination	Glucose oxidase method	(Bergmeyer and Bernt 1963)
		Modified glucose oxidase/peroxidase method	(Brivonese 1986)
	Alginate content	Poly(hexamethylenebiguanidinium) chloride assay	(Kennedy and Bradshaw 1984)
	Uronic acid content	Meta-hydroxydiphenyl method	(Blumenkrantz and Asboe-Hansen 1973)
Proteins		Coomassie Brilliant Blue colour reagent	(Bradford 1976)
		Folin–Ciocalteu reagent	(Lowry, Rosebrough, Farr and Randall 1951)
		Bicinchoninic acid (BCA) assays	(Smith, Krohn, Hermanson, Mallia, Gartner et al. 1985)
Extracellular DNA		Diphenylamine assay	(Burton 1956)
		Absorbance at 260 nm	(Manchester 1996)
Lipids		Sulfo-phospho-vanillin reaction	(Frings, Fendley, Dunn and Queen 1972)

3.4. Techniques for whole-biofilm research

Biofilm research has expanded dramatically since the 1970s and technological advances have greatly contributed to our ability to study microbial communities. This section focuses on microscopic and spectroscopic techniques that are routinely used to study biofilms as a whole, i.e. without the physical separation of EPS from microbial cells. The advantages and limitations of most of these techniques are discussed.

Based on the advantages of the individual techniques, as well as the possibility of combined use with others, the following techniques were identified for use in this study:

- **Microscopic**: confocal scanning laser microscopy (section 3.4.1.1) combined with digital image analysis (section 3.4.1.2).

- **Spectroscopic**: attenuated total reflection-Fourier transform-infrared spectroscopy (section 3.4.2.1) and Raman spectroscopy (section 3.4.2.2).

The above-mentioned techniques are valuable tools for biofilm research but often only examine one or two dimensions of biofilms (e.g. biofilm composition or mapping of EPS within a biofilm), thereby generating snapshots of biofilms in a specific time and space. While these techniques provide important building blocks for our understanding of biofilms, as is the case in this study, there is a need to move beyond data collection in order to combine these building blocks into more complex frameworks that attempt to explain naturally occurring biofilms as adaptive and robust systems. To this end, this study proposes the use of a non-destructive, real-time monitoring system that allows for *in situ* investigations of biofilm responses by measuring biofilm CO_2 production. This CO_2 evolution measurement system is reviewed in section 3.4.4.

3.4.1. Microscopic techniques

The most prominent techniques in biofilm research include forms of electron microscopy (EM) and laser scanning microscopy (LSM). Scanning electron microscopy (SEM) has allowed for high resolution visualisation of biofilms (Walker, Verran, Boyd and Percival 2001), and similarly, advances in transmission electron microscopy (TEM) have made near-nanometre resolution of specific biofilm structures possible (Leppard 1992). By coupling TEM and SEM with specialised detectors (e.g. energy-dispersive X-ray spectroscopy or EDS) it is possible to analyse the elemental composition in specific regions of a sample, thereby providing insight into EPS composition and distribution (Lawrence, Swerhone, Leppard, Araki, Zhang et al. 2003; Neu, Manz, Volke, Dynes, Hitchcock et al. 2010).

The major disadvantage of most EM techniques is the destructive pre-treatment of samples. SEM requires fixation, drying and coating of samples with a conductive film, while TEM specimens must be embedded in a resin to stabilise the EPS matrix (Priester, Horst, Van de Werfhorst, Saleta, Mertes et al. 2007). Pre-treatment may alter biofilm morphology, cause polymers in the EPS matrix to collapse and introduce artefacts (Little, Wagner, Ray, Pope and Scheetz 1991).

As a result, environmental SEM (ESEM) has been used as an alternative technique since it requires little to no sample pre-treatment. Fully hydrated samples can be imaged without a conductive coating at a moderate vacuum in a moist atmosphere using ESEM (Donald 2003). While hydrated EPS is often more visible with ESEM than SEM and TEM, there are limitations to the resolution of images generated by ESEM. Various staining and fixation methods have been investigated to overcome these limitations (Priester et al. 2007), but time-resolved and non-destructive biofilm visualisation by ESEM is still not possible (Halan et al. 2012).

Atomic force microscopy (AFM) has been used as a surface analysis technique to study biofilm developmental stages, including the formation of conditioning films on surfaces, cell adhesion and microbial surface footprints (Gómez-Suárez, Pasma, Van der Borden, Wingender, Flemming et al. 2002). Very little is known about EPS interaction with surfaces, especially interactions with ions on

metal surfaces and AFM has therefore become an important tool in microbial-induced corrosion studies (Beech and Sunner 2004).

Although AFM provides information about the morphology of a surface, it provides little information on the chemical composition of an interface. The scanning probe or tip may also damage soft biological samples and it is therefore considered a destructive technique in terms of biofilm structure investigations. It is, however, the only available method that allows for investigation of surface charges and specimen elasticity by measuring minute forces within or between biological molecules (Halan et al. 2012). Other scanning probe techniques used in biofilm research include scanning tunnelling electron microscopy and scanning ion-conductance microscopy.

As the central role of EPS in biofilms became evident, the need for non-destructive techniques that allow *in situ* investigation of hydrated biofilms arose. Microscopic techniques that address this need include confocal laser scanning microscopy (CLSM) and soft X-ray scanning transmission X-ray microscopy (STXM). Since its introduction to biofilm research in the 1990s, CLSM has contributed greatly to our understanding of biofilm structures and the composition of EPS matrixes.

Advances in LSM have led to the development of systems using two-photon excitation called two- or multiphoton laser scanning microscopy (2PLSM). With the emergence of fluorescence lifetime imaging (i.e. measuring the lifetime of an excited fluorochrome) and intensity imaging, in combination with fluorescence staining techniques, it is now possible to collect multiple pieces of information from biofilms using CLSM and 2PSLM (Neu et al. 2010).

Recent advances in super-resolution microscopy have been employed to study intact biofilms, specifically microbial macromolecules, in unparalleled detail. Three-dimensional structural illumination microscopy (3D-SIM) generates high-resolution images by reconstructing multiple cross-section images of a sample. With an approximate 2-fold improvement in resolution in all three dimensions, 3D-SIM images have verified genetic predictions of the role of certain proteins in cell attachment through the use of specific fluorophore-conjugated antibodies (Ivanov et al. 2012). This is a novel approach that allows a deeper understanding of biofilm attachment and EPS composition.

3.4.1.1. Confocal laser scanning microscopy (CLSM)

CLSM was introduced to biofilm research in 1991 and gained popularity because it offered improved images compared to the widely-used fluorescent microscopy (Lawrence, Korber, Hoyle, Costerton and Caldwell 1991). This form of LSM combines traditional epi-fluorescence microscope hardware with a laser light source, specialised scanning equipment and computerised digital imaging (Lawrence and Neu 1999). Laser sources for CLSM use one-photon excitation through continuous visible and ultraviolet or white lasers (super-continuum light sources). When coupled with photomultipliers, these instruments can be used to examine fluorescence emission ranging from 400–750 nm. Together with spectral imaging detection systems, confocal laser scanning microscopes can be used to examine fluorophores with overlapping spectra (Claxton, Fellers and Davidson 2006).

Thicker samples can be imaged with CLSM in order to produce three-dimensional reconstructions and animations of samples (Amos and White 2003). It also allows optical thin sectioning of intact, hydrated samples and its non-destructive nature has made it an invaluable biofilm research tool. As an imaging tool, CLSM allows multiple biofilm features to be visualised and can also be used to quantify biofilm structures (Neu et al. 2010).

CLSM has been particularly important for EPS matrix investigations. Various fluorescent dye-conjugate probes, which bind to specific EPS components, have been used to map the structural and biochemical heterogeneity of the matrix (Halan et al. 2012). Fluorescent probes and CLSM imaging systems have also made it possible to investigate dynamic processes like biofilm metabolism, micro-gradient development within biofilms, as well as biofilm responses to environmental changes and exposure to antimicrobials (Pamp, Sternberg and Tolker-Nielsen 2009).

Although CLSM continues to provide insights into the structural complexity of EPS matrixes and microbial interactions in biofilms, there are limitations to consider. While it is possible to make use of intrinsic properties of samples (e.g. reflection and auto-fluorescence), fluorescent protein labelling of organisms (e.g. chromosomal integration of *gfp*) and reporter gene technology, staining by means of fluorochromes or fluor-conjugated probes may have to be applied (Neu et al. 2010). Stain specificity and efficiency can be problematic. Staining is not a quantitative method, fluorescent probes may

modify biofilm structures and there are limitations to the interpretation of fluorescent probe binding patterns in complex environments such as aqueous biofilms. A second technique is often required to confirm that probes bind specifically to the macromolecules of interest (Lawrence et al. 2003).

There are also limitations with respect to laser penetration (approximately 250 µm in aqueous solutions) and other physical restrictions (e.g. microscope stage dimensions) (Halan et al. 2012). Resolution is dependent on the wavelength of the laser light, numerical aperture of the objective lens and the refractive index of the medium. As a result, the resolution of biological samples showing absorption and scattering is at best in the range of 200–300 nm. Although deconvolution can be applied to sharpen images, this requires high-resolution imaging and is therefore impractical for routine work (Neu et al. 2010).

Despite these limitations, CLSM remains one of the most versatile tools in biofilm analysis and was selected for this study to characterise biofilms as surface coatings, by quantifying certain structural parameters as described in the following section. CLSM, in combination with digital image analysis (DIA), was used to quantify structural biofilm parameters and to investigate the influence of environmental factors on biofilm structure (Chapter 5). The biochemical composition of *Pseudomonas* biofilms was also investigated using CLSM and fluorescent stains (Chapter 5).

3.4.1.2. Digital image analysis (DIA)

Most data from microscopic techniques are now available in digital format, making it possible to quantify various volumetric and structural elements through DIA. Various DIA software packages are available for visualisation and quantification of three-dimensional data sets (e.g. IMAGEJ[8]). Since biofilm quantification has specific challenges, research groups often develop software depending on the research aims (e.g. ISA-3D[9] and ConAn[10] for biofilm structural analysis) (see Neu et al. 2010 for others).

[8] http://imagej.net/Welcome, accessed 24 May 2016.
[9] Beyenal, Donovan, Lewandowski and Harkin 2004.
[10] An Interactive Data Language (IDL)-based programme developed by BioCom, (Uttenreuth, Germany).

3.4.1.2.1. COMSTAT

COMSTAT, a free computer programme written as a script in MATLAB 5.1, was developed for the quantification of biofilm parameters (Heydorn et al. 2000b). More specifically, it is used for digital analysis of CLSM images of biofilms cultivated in flow cells and is therefore an appropriate choice for this study. Quantification of biofilm parameters by COMSTAT is useful for time-based analyses of biofilm development and comparisons of biofilm structures from different microorganisms under steady-state conditions.

In this study, COMSTAT was used to investigate the influence of environmental factors on biofilm structure by quantifying selected biofilm parameters. For the purpose of this study, the following structural parameters were selected for quantification using COMSTAT:

- **Bio-volume**: this represents the total volume of the biofilm and is an estimate of the biomass in the biofilm, calculated by dividing the biomass volume (μm^3) by the substratum area (μm^2). The biomass volume is calculated as "the number of biomass pixels in all images of a stack multiplied by the voxel size [$(\text{pixel size})_x$ x $(\text{pixel size})_y$ x $(\text{pixel size})_z$]" (Heydorn et al. 2000b, p. 2399).

- **Area occupied by bacteria in each layer**: this is defined as "the fraction of the area occupied by biomass in each image of a stack" (Heydorn et al. 2000b, p. 2399). The area occupied by bacteria in the first image of a stack indicates how efficiently the bacteria have colonised the substratum (so-called substratum coverage).

- **Thickness distribution and mean thickness**: thickness is defined as "the maximum thickness over a given location, ignoring pores and voids inside the biofilm" (Heydorn et al. 2000b, p. 2399). This function "locates the highest point (μm) above each (x,y) pixel in the bottom layer containing biomass" (Heydorn et al. 2000b, p. 2399). Mean biofilm thickness is commonly used in biofilm studies as an indication of the spatial dimensions of the biofilm.

- **Surface:volume ratio**: only biofilm surfaces that are exposed to the bulk fluid are included in this calculation. Biofilm surface is defined as "the collection of pixels having at least one

background pixel as neighbour" (Heydorn et al. 2000b, p. 2400). This function indicates what portion of the biofilm is exposed to the bulk fluid and therefore may indicate how the biofilm adapts to environmental changes.

Bio-volume (as indication of biomass) and average thickness are commonly measured biofilm parameters and were useful for comparison of the results of this study with other studies. The percentage of substratum attachment and coverage was of interest in order to describe biofilms as natural surface coatings. Finally, surface:volume ratio indicates the portion of a biofilm that is exposed to the bulk fluid and may explain how a biofilm adapts to environmental changes.

3.4.2. Spectroscopic techniques

Various spectroscopic techniques have been employed in biofilm research, as stand-alone techniques or in combination with microscopic and other techniques. This includes EDS, X-ray photoelectron spectroscopy (XPS) and time-of-flight second ionization mass spectrometry (TOF-SIMS) (Beech 2004). NMR spectroscopy and magnetic resonance imaging (MRI) have been used as a non-invasive techniques to study intact biofilms and have provided valuable insights into biochemical mapping of EPS, transport properties of matrixes and measurements of structure-flow relationships within biofilms (Seymour, Codd, Gjersing and Stewart 2004; Majors, McLean, Pinchuk, Fredrickson, Gorby et al. 2005; Neu et al. 2010).

In situ methods such as infrared (IR) and Raman spectroscopy have become popular in studies of planktonic cultures and biofilm-surface interaction, as well as for the identification of compounds within biofilms (Jiang, Saxena, Song, Ward, Beveridge et al. 2005). Raman and Fourier transform-infrared (FTIR) spectroscopy, specifically attenuated total reflection-FTIR spectroscopy, are reviewed in greater detail in this section. Three-dimensional excitation-emission matrix (EEM) fluorescence spectroscopy, a highly sensitive and non-destructive technique, has been used to study chemical and physical properties of EPS, although these studies typically involve EPS extraction from biofilms prior to analysis (Sheng and Yu 2006).

3.4.2.1. Attenuated total reflection-Fourier transform-infrared (ATR-FTIR) spectroscopy

FTIR spectroscopy is a highly sensitive technique used to detect changes in functional groups of macromolecules. In short, a sample is irradiated with a continuous spectrum of infrared wavelengths and the radiation is recorded simultaneously over all wavelengths by an interferometric modulation. This produces an interferogram that contains all the information of the sample over all wavelengths. The sample-spectrum is calculated from the interferogram by Fourier transform techniques using elaborated mathematical equations (Schmitt and Flemming 1998).

The main classes of biomolecules can be identified by means of vibrational spectroscopy, due to their absorbance in different frequency regions in the medium infrared part of the spectrum (wavelength between 2.5 and 20 µm) (Pistorius, DeGrip and Egorova-Zachernyuk 2009). The position of absorption is expressed in wavenumbers (cm^{-1}), which is reciprocal of the wavelength. Functional groups of molecules absorb in a range between 4000–1500 cm^{-1}; double and triple bonds display higher wavenumbers than single bonds. The range below 1500 cm^{-1} is significant for deformation-, bending- and ring-vibrations and is commonly referred to as the "fingerprint" region of a spectrum. Band intensities, changes in band widths and shifts in peak positions provide structural and functional information of the sample in question (Karadenizli et al. 2007).

IR spectroscopy has been applied to microbiology for the general identification of microorganisms and to identify specific members within biofilm communities. Although most structural and functional groups of different bacteria are the same and therefore produce similar spectra, the quantity and distribution of functional groups vary among microbial strains. Modern FTIR spectrometers, together with advanced software, can provide fingerprint spectra for microbial isolates and allow the detection of differences in order to distinguish different bacterial strains (Schmitt and Flemming 1998; Pistorius et al. 2009).

In the transition mode, microbial samples are removed from the substratum or aqueous solution, transferred to IR transparent material and subsequently dried on the window material (in the case of a biofilm sample this would alter the EPS matrix structure). Solid samples in powder form can also be

mixed with an IR transparent material such as KBr or KCl and moulded into pellets under high pressure. Sample homogeneity, particle size and thickness determine the quality of the spectrum.

ATR-FTIR spectroscopy is a non-destructive online technique that can be used to record the appearance of chemical groups on the surface of an internal reflection element exposed to the aqueous phase. This makes it possible to monitor biofilm development and metabolic activity *in situ* and in real time, directly at the substratum-liquid interface (Blenkinsopp 1991; Jiang et al. 2004). ATR-FTIR spectroscopy has been used to study biofilm development, EPS composition, as well as bio-corrosion processes facilitated by biofilms (Beech and Sunner 2004; Karadenizli et al. 2007).

It is difficult to avoid spectral interference by buffers and media. This limits the use of ATR-FTIR spectroscopy for biofilm studies in aqueous environments (Schmitt and Flemming 1998). If relying on external standards to identify molecules, it is important to use small-chain organic molecules of known structure and functional groups to assist the interpretation of spectra. Furthermore, the selection of appropriate standards can be complex when little information is known about the sample in question.

In this study, ATR-FTIR spectroscopy was used to investigate the biochemical composition of *Pseudomonas* and multi-species biofilms cultivated in different nutrient media (Chapters 4 and 5). The FTIR equipment used in this study required dehydrated biofilm samples and was therefore considered a destructive technique.

3.4.2.2. Raman spectroscopy

A typical Raman spectroscopy system consists of a non-ionising laser, monochromator, sample holder and detector. In brief, the laser is used to irradiate the sample and incident photons can be absorbed, scattered and/or pass through the material without interference. Light scattering takes place as the incident photons distort electron clouds; if the vibrational state of a molecule is altered and energy transfer from the photon to the molecule or vice versa occurs, the light scattering process becomes inelastic and is called Raman scattering (Tu and Chang 2012).

Various lasers can be used as excitation sources, but those with longer wavelength excitations produce less background fluorescence and are better suited for biological samples. Raman scattered photons are

collected by a detector and used to generate highly specific spectra that provide vibrational information specific to chemical bonds and symmetry of molecules in the sample. Recent advances have addressed problems such as low sensitivity and fluorescence rejection, thereby allowing for well-resolved spectra, often with sharper bands than IR spectra (Das and Agrawal 2011).

Within the field of microbiology, Raman spectroscopy has been used for the identification of microorganisms and, more recently, for biochemical investigations of biofilms (Jarvis and Goodacre 2004; Du, Wan, Lu, Rasco and Wang 2012). Spectra of microbial samples consist of complex biochemical information as the spectra contain contributions of all biomolecules present in the sample. Surface-enhanced Raman spectroscopy (SERS) and resonance Raman (RR) spectroscopy have been used to avoid the complexity of general Raman spectra by enhancing the contribution of specific molecules. SERS increases the signal level by exploiting the interaction between a nanoscale metal surface (e.g. silver nanoparticles) and the sample molecules (Ivleva, Wagner, Horn, Niessner and Haisch 2008). While the addition of signal-enhancing materials may improve spectra resolution, this is often only useful if a limited number of biomolecules are being investigated.

The lack of comprehensive databases limits the use of Raman spectroscopy for the analysis of complex samples. Some attempts have been made to generate spectra databases of biomolecules to address this limitation. Experimental conditions influence spectra and this should be taken into account when drawing comparisons between sample and reference spectra. One example is a database of Raman spectra of biomolecules that serve as building blocks in cells; this is a useful reference for the interpretation of Raman spectra of biological samples (De Gelder, De Gussem, Vandenabeele and Moens 2007). The reference spectra include all 5 nucleic acid bases as well as certain amino acids, fatty acids, saccharides and primary metabolites. The spectra of these groups of biomolecules can clearly be distinguished from each other:

- **DNA and RNA bases:** the spectrum of each base contains an intense band in the 600–800 cm^{-1} region assigned to ring breathing vibrations. It is possible to distinguish between all 5 bases.

- **Amino acids:** The presence of proteins is identified by amide I and amide III bands around 1300 and 1655 cm^{-1}.

- **Fatty acids and fats:** Spectra of four unbranched saturated fatty acids show three characteristic bands between 1050 cm^{-1} and 1150 cm^{-1}, a band at 1296 cm^{-1} and group of bands in the 1400–1500 cm^{-1} region.

- **Saccharides:** shifts in peak positions can be seen between the spectra of monosaccharides and their corresponding dimers.

Since the FTIR equipment used in this study required dehydrated biofilm samples, Raman spectroscopy was considered as a non-destructive alternative for biochemical profiling of hydrated biofilms (Chapter 4). The addition of nanoparticles for improved signal strength was also investigated.

3.4.3. Combining microscopic and spectroscopic techniques

All of the above-mentioned techniques have limitations and therefore are often used in combination with others to provide a more comprehensive understanding of biofilms. For example, TEM, CLSM and STXM have been used together to map EPS in fresh-water biofilms to illustrate the value of multi-microscopic analyses for an improved biochemical understanding of biofilm organisation (Lawrence et al. 2003). Microscopic and spectroscopic approaches have also been combined successfully. A recent study combined SEM, CLSM, ATR-FTIR and Raman spectrocsopcy to characterise and analyse *Salmonella* biofilm formation in food processing environments (Wang, Ding, Wang, Xu and Zhou 2013).

Raman confocal microscopy (RCM) is gaining popularity in biofilm research. The integration of Raman spectroscopy with a confocal microscope makes it possible to record the three-dimensional distribution of compounds with high spatial resolution. This fast, non-destructive technique is ideal for the analysis of biological samples, as only small sample volumes with little to no sample preparation are required. Since water causes little interference in Raman spectra, RCM makes it possible to study the biochemical composition and structural development of intact, hydrated biofilms (Pätzold, Keuntje

and Anders-von Ahlften 2006). More specifically, it has been used to correlate structural appearances within biofilms (i.e. data obtained from fluorescent stains and CLSM) with biochemical composition of these structures (as determined from Raman spectra) over time (Ivleva, Wagner, Horn, Niessner and Haisch 2009). Although most studies have focused on single-species biofilms, RCM has the potential to allow insights into more complex, multi-species communities.

3.4.4. CO_2 evolution measurement system (CEMS)

The CO_2 evolution measurement system (CEMS) allows for *in situ* investigations of biofilm responses (Kroukamp and Wolfaardt 2009). Gaseous CO_2 production by biofilms is measured as an indication of biofilm metabolism; the measured CO_2 production rates represent the metabolic activity of active cell mass in biofilms and can therefore be used to determine whole-biofilm metabolic response in real time. Although most studies using this system have focused on single-species biofilms, it has been used to investigate multi-species biofilms (Kroukamp, Dumitrache and Wolfaardt 2010).

In brief, biofilms are cultivated in a continuous flow system (as show in Figure 3.1). The CEMS is essentially a silicone tube biofilm reactor encased in a sealed Tygon® tube with the annular space being connected to a CO_2 analyser. Given the relatively high permeability of silicone tubing to both O_2 and CO_2 (compared to that of Tygon® tubing), as well as the higher gaseous CO_2 concentration in the lumen of the silicone tubing due to biofilm metabolic activity, it is assumed that a fraction of CO_2 produced by the microorganisms will diffuse across the silicone tube wall to the annular space. A CO_2-free sweeper gas is used to transport CO_2 in the annular space to an absolute, non-dispersive, infrared CO_2 gas analyser.

The mathematical relationship that describes the fraction of CO_2 that will cross the silicone tube wall has been described in detail (Kroukamp and Wolfaardt 2009). Using these calculations it is possible to relate measured CO_2 in the annular space to the CO_2 concentration in the lumen of the silicone tube. During the development of this technique, CO_2 transfer across the silicone tube wall to the gas phase was tested by measuring the gaseous CO_2 concentrations for various concentrations of dissolved CO_2 (0% to 4% (v/v) of a dissolved CO_2 solution at room temperature). These calibration measurements

provide a reliable indication of the dissolved CO_2 (R^2 = 0.999) and measured CO_2 concentrations produced by biofilms in subsequent experiments were moreover found to be within this range (Kroukamp and Wolfaardt 2009).

Microorganisms produce CO_2, which is excreted in a dissolved form into the bulk-liquid phase, where it can be transported to the gas phase or converted into bicarbonate ions and eventually carbonate ions (Jones and Greenfield 1982). The conversion of CO_2 from the gas phase is dependent on the ion concentration, pH and temperature of the liquid (Frahm, Blank, Cornard, Oelßner, Guth et al. 2002). Temperature fluctuations are minimised by submerging the CEMS in a temperature-controlled water bath. Liquid and gas flow rates also influence mass transfer rates from the gas-to-liquid phase via membranes and it is therefore necessary to ensure constant gas flow rates during an experiment using gasflow regulators.

The CEMS has been used to perform biofilm carbon mass balances by measuring carbon channelling in biofilms, thereby providing insight into carbon allocation in biofilm formation and maintenance. The first published study using the CEMS determined whole-biofilm CO_2 production rates of *Pseudomonas* sp. strain CT07 biofilms in real time and showed that less than 5% of inflowing carbon was retained in the biofilm, suggesting that biofilms serve as catalytic entities that are able to transform carbon from the surrounding environment (Kroukamp and Wolfaardt 2009).

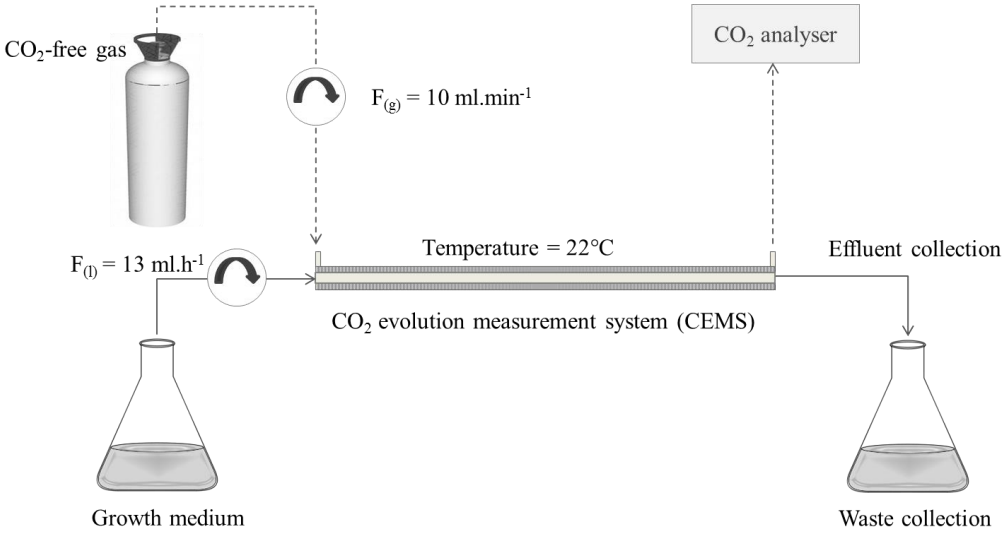

Figure 3.1. Diagram of a CO_2 evolution measurement system. Adapted from Bester, Kroukamp, Wolfaardt, Boonzaaier and Liss 2010.

In a subsequent study, the CEMS was used to measure whole-biofilm CO_2 production rates of single- and multi-species biofilms as an indication of biofilm development and metabolic activity. The growth curves (measured as CO_2 production in $\mu mol.h^{-1}$) make it possible to compare *Pseudomonas* and multi-species biofilm development in terms of acclimation time, maximum growth rates and steady-state metabolic activity (Kroukamp et al. 2010). The nature of the inoculum has been shown to also have a significant effect on acclimation time and biofilm development.

Since the CEMS allows real-time, non-destructive monitoring of biofilms it can also be used to investigate metabolic responses of steady-state biofilms to changes in environmental conditions. Studies using CEMSs have shown that changes in temperature, carbon availability and nutrient type elicit rapid metabolic responses (Kroukamp and Wolfaardt 2009; Bester et al. 2010; Kroukamp et al. 2010). Additionally, the CEMS can be used to study dynamic biofilm behaviour, such as sloughing events and recovery after chemical or physical disruption. More recently, the metabolic responses of *Pseudomonas* biofilms to, and recovery from, exposure to antimicrobial compounds were investigated (Jackson, Kroukamp and Wolfaardt 2015).

Ultimately, the CEMS should allow investigations of biofilm structure-function relationships, biofilm development and metabolic responses. On its own, the CEMS does not provide information on the structural development or biochemical composition of a biofilm. In order to address this limitation, the CEMS has been combined with microscopic analysis and other biofilm assays. Given the simplistic design of the system, it is easy to collect effluent containing biofilm-derived planktonic cells for further analysis. Flow cells and other bioreactors can be included in series with CEMSs for microscopic or spectroscopic studies of biomass accumulation and/or biofilm composition. In a study that combined a CEMS, CLSM and cell count techniques, it was shown that carbon availability affects biofilm architecture, planktonic cell yield and biofilm metabolic activity (Bester et al. 2010).

The benefits of non-destructive, real-time biofilm monitoring make the CEMS a valuable experimental tool. It was selected for this study because it allows biofilms to be studied as systems. Whether composed of a single strain or a heterogeneous community, studying the overall response of a biofilm to environmental changes provides more insight into the true synergistic functions of these complex microconsortia.

3.5. Conclusion

- Two well-characterised *Pseudomonas* strains were selected as model biofilm-forming organisms for this study. In order to test biomimetic principles against biofilms that more closely resemble the complexity of naturally occurring biofilms, a multi-species environmental culture was used in this study.

- A continuous, once-through flow system and a range of bioreactors were selected as biofilm cultivation systems for microscopic and spectroscopic studies.

- CLSM and DIA (using COMSTAT) were selected as a non-destructive approach to validate biofilm cultivation techniques (Chapter 4) and to investigate the influence of environmental conditions on biofilm parameters (Chapter 5).

- CLSM was used to investigate the biochemical composition of *Pseudomonas* biofilms using selective fluorescent stains (Chapter 5). ATR-FTIR and Raman spectroscopy were used to investigate the biochemical composition of single- and multi-species biofilms (Chapter 5).

- A CEMS was selected as a non-destructive, real-time investigations of whole-biofilm metabolic responses to environmental changes (Chapter 6).

4. Protocol development for the current study

The previous chapter reviewed some of the most common techniques used for biofilm studies and provided motivation for the techniques selected for this study. This chapter expands on the specific experimental systems and methods used in this study. The first section of this chapter contains general descriptions of culture conditions, biofilm cultivation techniques and experimental systems (CLSM, ATR-FTIR and Raman spectroscopy, and a CEMS) used in this study.

In the second part, protocol development, experimental design and statistical analysis of data are explained. This chapter aims to standardise and validate the aforementioned techniques by collecting data from single-species biofilms. These optimised protocols are applied to multi-species biofilms in following chapters. Lastly, results generated during protocol development are used to evaluate the appropriateness of the selected techniques, followed by a discussion of experimental limitations. The conclusions from this chapter inform the experimental design for the following sections of the study.

4.1. General experimental methods

4.1.1. Strains and culture conditions

4.1.1.1. *Pseudomonas* strains

Two well-characterised *Pseudomonas* strains (wild type strains, as well as strains containing *gfp* genes) were kindly donated by the Biofilm Ecology Group (Ryerson University, Canada). This included an environmental strain that was previously isolated and subsequently identified as closely related to a known *Pseudomonas* species by 16S rDNA sequencing (Bester et al. 2005). This isolate was designated as *Pseudomonas* sp. strain CT07 (GenBank Accession No. DQ 777633).

A *gfp* gene was inserted into the chromosome of *Pseudomonas* sp. strain CT07 and *Pseudomonas aeruginosa* PA01 as previously described (Bester et al. 2009), and are hereafter referred to as PCT07 *gfp* and PA01 *gfp*, respectively.

4.1.1.2. Multi-species culture

Microbial biofilms were isolated from a winery wastewater-treatment cooling tower. Samples were homogenised by vigorous mixing and serial dilutions were made in test tubes containing 3 $g.L^{-1}$ tryptic soy broth (TSB) medium. These dilutions were incubated overnight on a rotating wheel at 26 ± 2 °C, after which the contents were combined and used to make freezer stocks (40% glycerol, final concentration). A new freezer stock was used for each experiment requiring a multi-species inoculum. All freezer stocks were kept at -80 °C.

Repeated DNA sequencing of random biofilm samples from this specific wastewater treatment system confirmed the presence of a broad spectrum of microorganisms (De Beer 2016). At genus level, the following groups of bacteria were regularly present in these biofilms: *Acetobacterium*, *Actinomyces*, *Aeromicrobium*, *Amaricoccus*, *Arcobacter*, *Bdellovibrio*, *Bosea*, *Devosia*, *Dysgonomonas*, *Fluviicola*, *Kaistia*, *Lactobacillus*, *Leadbetterella*, *Mezorhizobium*, *Myroides*, *Paracocci*, *Patulibacter*, *Pseudoxanthomonas* and *Trichococcus*. In terms of the fungal community, *Candida* was the most abundant among the identified genera, followed by *Trichosporon* and *Fusarium*. It is important to remember that many environmental species are difficult to culture under laboratory conditions and that the species listed here may not be the dominant organisms present in biofilms in this study.

Serial dilutions of two random freezer stocks were plated out on 3 $g.L^{-1}$ TSB and Reasoner's 2A (R-2A) agar-solidified nutrient medium to compare the microbial community distribution between freezer stocks. The average culturable number of colony forming units (CFU) for both freezer stocks was approximately 5 x 10^{8} $CFU.ml^{-1}$.

4.1.1.3. Culture conditions

Routine cultivation for biofilm studies was carried out in either TSB medium or modified defined AB medium (Table 4.1) as described by others (Bester et al. 2009). Pre-cultures for biofilm studies were incubated for 18 hours on a rotating wheel at 26 ± 2 °C in a nutrient medium corresponding to the nutrient medium used in the experiment.

Modified defined AB medium was prepared as follows: stock solutions were prepared and sterilised at 121 °C for 15 minutes (see Table 4.2 for stock solution concentrations). For 1 L of modified defined AB medium, 100 ml solution A was added to approximately 800 ml deionised water and sterilised at 121 °C for 15 minutes. Subsequently, 1 ml sterile solution B and 1 ml sterile solution C were added aseptically. Varying amounts of the sterile Na-citrate stock solution was added, depending on the concentration required for the experiment. The final volume was adjusted to 1 L by adding sterile deionised water. All chemical compounds were purchased from Sigma Aldrich, South Africa.

4.1.1.4. Planktonic growth rates

In order to introduce comparable amounts of planktonic cells into bioreactors, planktonic growth rates of PA01 *gfp* and PCT07 *gfp* were determined. The cultures were incubated for 18 hours on a rotating wheel at 26 ± 2 °C in modified defined AB medium with either 1 mM or 10 mM citrate as sole carbon source.

Spectrophotometric optical density (OD) measurements and corresponding cell counts (calculated as $CFU.ml^{-1}$ by direct plate counting method) were recorded for serial triplicate dilutions of these cultures. An OD corresponding to approximately 10^7 $CFU.ml^{-1}$ was calculated for PA01 *gfp* and PCT07 *gfp* in modified defined AB nutrient medium with either 1 mM or 10 mM citrate. For subsequent experiments, the OD of overnight cultures was measured and the cultures were diluted with sterile medium to an OD corresponding to approximately 10^7 $CFU.ml^{-1}$. Diluted cultures were then used as inocula for experiments (unless described otherwise).

Table 4.1. Modified defined AB medium (Clark and Maaloe 1967).

Component	Final concentration (mM)
$(NH_4)_2SO_4$	1.51
Na_2HPO_4 (anhydrous)	3.37
KH_2PO_4	2.20
NaCl	179.0
$MgCl_2 \cdot 6H_2O$	0.1
$CaCl_2 \cdot 2H_2O$	0.01
$FeCl_3$	0.001
Na-citrate$\cdot 2H_2O$	1 or 10

Table 4.2. Stock solutions for modified defined AB medium.

Stock solution	Compound	Concentration (mM)
A	$(NH_4)_2SO_4$	15.1
	Na_2HPO_4 (anhydrous)	33.7
	KH_2PO_4	22.0
	NaCl	1790.0
B	$MgCl_2 \cdot 6H_2O$	100.0
	$CaCl_2 \cdot 2H_2O$	10.0
C	$FeCl_3$	1.0
Carbon source	Na-citrate	1000.0

4.1.2. Continuous, once-through flow systems for biofilm cultivation

Figure 4.1 illustrates the general experimental system used in this study, consisting of a medium reservoir, a peristaltic pump, a bioreactor and waste bottle. Depending on the experiment, flow cells or silicone tubing were used as bioreactors. Flow cells were made from poly(methyl methacrylate) (PMMA, commercially known as Perspex) or quartz.

The bioreactor was connected to the medium reservoir by silicone tubing (inner diameter 1.5 mm x 3.175 mm, outer diameter 0.082 mm) via a peristaltic pump. The bioreactor was connected to a waste bottle by Tygon® tubing (inner diameter 1.5 mm x 3.175 mm, outer diameter 0.082 mm). Tubing was connected using straight or T-barbed plastic connectors (inner diameter 1.58 mm, Cole Palmer, IL,

USA). The medium reservoir did not require a sterile air inlet because the silicone tubing is permeable to gas and allows for O_2/CO_2 exchange.

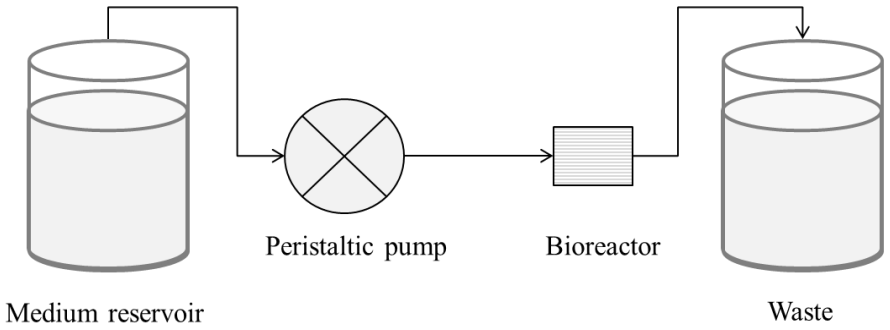

Figure 4.1. Diagram of a continuous, once-through flow system.

4.1.2.1. Flow rates and Reynold numbers

Reynolds number (Re) is a dimensionless value used to predict similar flow patterns in different fluid flow situations. In order to compare flow conditions between bioreactors (Perspex and quartz flow cells), the dimensions of the bioreactors were used to calculate flow rates that corresponded to Re of 0.80 or 1.85 (Table 4.3).

Table 4.3. Flow rates and Reynolds numbers calculated for flow cells.

Re	Flow rate ($ml.h^{-1}$)	
	Perspex flow cell	*Quartz flow cell*
0.80	13.0	14.5
1.85	30.0	33.5

4.1.2.2. Cleaning and disinfection of bioreactors

Thorough cleaning steps were taken to prevent the carryover of organic matter from previous biofilm experiments. Immediately after the termination of an experiment, bioreactors were disinfected with a commercial bleach solution (final concentration 7.0% m/v sodium hypochlorite) to oxidise organic matter.

After disinfection, the microscope slides (on Perspex flow cells) and tubing were removed from bioreactors and any residual silicone rubber was scraped off with a scalpel. Flow cells were vigorously scrubbed with a small brush using hot water and soap to physically remove any remaining organic matter.

When preparing a bioreactor for an experiment, the bioreactor was disinfected with a commercial bleach solution (final concentration 0.525% m/v sodium hypochlorite) at a flow rate of approximately 15 $ml.h^{-1}$ (Re = 0.8) for at least 2 hours. The inlet tubing was then aseptically connected to sterile deionised H_2O and the bioreactor was washed overnight at a low flow rate.

4.1.2.3. General bioreactor inoculation procedure

Following the overnight wash step, the inlet tubing of the bioreactor was aseptically connected to a reservoir containing sterile nutrient medium. Bioreactors (duplicate or triplicate) were connected to a single medium reservoir to eliminate variability in nutrient concentration and were flushed with nutrient medium for 1 hour at a flow rate of approximately 15 $ml.h^{-1}$ (Re = 0.8). Thereafter the flow was stopped and the inlet tubing was clamped to prevent air bubbles from entering the bioreactors.
For single-species biofilm experiments, overnight *Pseudomonas* pre-cultures were diluted with sterile nutrient medium to a suspension of approximately 10^7 $CFU.ml^{-1}$ (section 4.1.1.4). This diluted culture was injected directly into each bioreactor through the inlet tubing, using a sterile 1 ml syringe with a 0.5 x 16 mm sterile hypodermic needle. The needle was removed gently and the puncture was sealed with silicone rubber. For multi-species biofilm experiments, overnight cultures were injected directly without dilution. The inoculum was allowed to adhere to the silicon tubing for 1 hour under stagnant conditions before nutrient flow was resumed (flow rate was dependent on specific experiment). Biofilms were cultivated at room temperature (20 ± 2 °C).

4.1.3. CLSM

4.1.3.1. Flow systems with flow cells

For microscopic studies, biofilms were cultivated in continuous, once-through flow systems (section 4.1.2) with multi-channel flow cells milled from Perspex bases (prepared by the Biofilm Ecology Group, Ryerson University, Canada). Perspex flow cells had the following channel dimensions: 40 mm (length) x 5 mm (width) x 4 mm (depth). Channel inlets and outlets were created by boring holes (3 mm diameter) in the sides of the flow cell.

Closed channels were formed by sealing the open end with microscopy glass coverslips (48 x 60 mm, No 1, Ted Pella Inc.), cemented to the flow cell using 100% silicone rubber that contains no antimicrobial compounds. Silicone tubing was then cemented into the inlet and outlet holes. These flow cells were used for microscopic studies of biofilms.

4.1.3.2. Image acquisition for DIA

Microscopic observations and image acquisition were performed by a Carl Zeiss LSM 780 confocal microscope with an Elyra S.1 super-resolution platform. A "Plan-Neofluor" 40x/0.6 Corr M27 objective was used to obtain images. Images were acquired at 2.0 µm intervals up through the biofilm using an inverted microscope (the first image taken was at or below the glass substratum). The number of images per stack varied according to the thickness of the biofilm. It is recommended that a minimum area of 10^5 µm^2 should be investigated to obtain representative data of *Pseudomonas* biofilms (Korber, Lawrence, Hendry and Caldwell 1993); therefore a scan area of 1.2 x 10^5 µm^2 was select for each image stack (353.9 µm x 353.9 µm).

The 488 nm laser line from an Argon laser, together with a GaAsP detector (493–598 nm) were used in image scanning. ZEN 2011 software package, running on a Z800 workstation, was used to generate simulated fluorescence projections (Z-stacks) and sections through the biofilms.

Additional settings are listed below:

- Frame size: 1024 x 1024
- Pixel dwell: 6.30 µsec
- Bit depth: 8 bit
- Pixel size (x and y): 0.35 µm
- Beam splitters: MBS-488/561/633

4.1.3.3. Digital image analysis using COMSTAT

ZEN 2011 software package was used to export biofilm images (Z-stacks) in tagged image file format and these images were converted to greyscale using IrfanView[11], a free graphic viewer programme. Greyscale images were subsequently imported to MATLAB 5.1 for analysis using COMSTAT (Heydorn et al. 2000b).

4.1.3.4. Experimental design and statistical analysis

Two independent experimental rounds were performed to quantify specific biofilm parameters and investigate experimental reproducibility (Heydorn, Ersbøll, Hentzer, Parsek, Givskov et al. 2000a). In each round, biofilms were cultivated in three separate flow cell channels. Images were acquired from random positions in the middle of each flow cell channel (at least 10 mm from the inlet or outlet). Seven image stacks were acquired from each channel, i.e. 21 images stacks were analysed per round and 42 image stacks were analysed per biofilm parameter (Figure 4.2).

Variation between duplicate rounds of experiments and variance between flow cell channels containing the same strain was minimised on various levels:

- **Experimentally**: Standardised disinfection and inoculation procedures were followed. For example, all three flow cell channels were inoculated from a single overnight culture. The main

[11] http://www.irfanview.com/, accessed 6 May 2016.

factors that influence biofilm development were kept constant as far as possible (i.e. temperature, nutrient concentration, flow rate and the history of the cultures used for inoculation).

- **Image acquisition**: Images were acquired in the middle of flow cell channels to minimise the effects of biomass accumulation and structural irregularities of the flow cell walls (Heydorn et al. 2000a).

- **Digital image processing**: Care was taken to ensure reproducibility of image threshold setting. Threshold values (a value that determines which pixels represent biomass and which are ignored as background) were assigned manually for each image stack. All image acquisitions and analyses were performed by one operator to avoid individual influences and the same computer monitor was used to ensure constant brightness and contrast settings (Heydorn et al. 2000b).

Statistical analysis of data was performed in GraphPad Prism®. Shapiro-Wilk test was used as a normality test, followed by single-factor ANOVA (normal distribution) or Kruskal-Wallis one-way analysis of variance (non-normal distribution). In the event of a rejected null hypothesis, either Tukey's test for the comparison of means (significant difference determined by single-factor ANOVA) or Dunn's multiple comparison test (significant difference determined by Kruskal-Wallis one-way analysis of variance) was applied to determine which sample pairs were significantly different from each other.

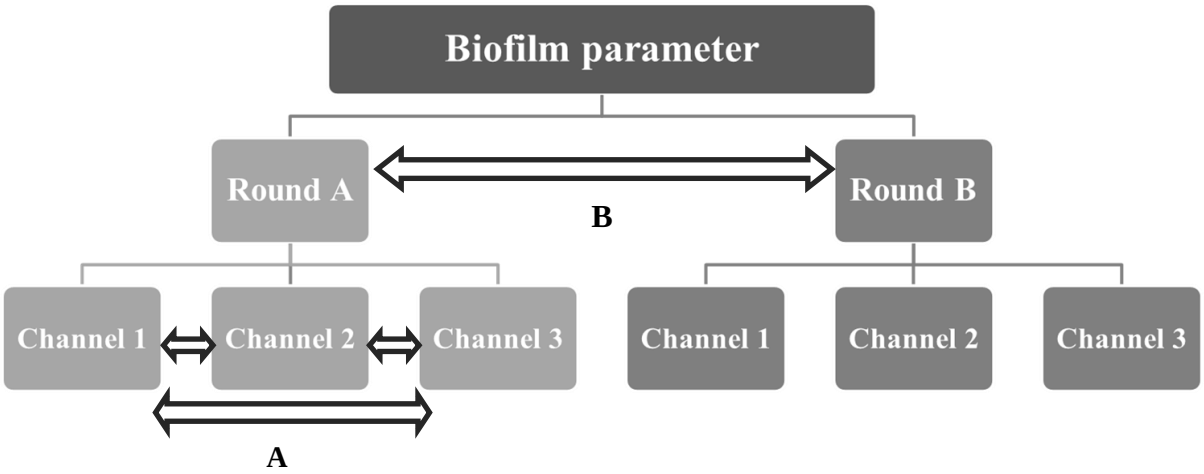

Figure 4.2. Schematic representation of CLSM experiments and the statistical analysis of data. Variance between biofilm parameters was determined between biofilms per round (A) and experimental rounds (B).

4.1.4. ATR-FTIR spectroscopy

4.1.4.1. Flow systems with silicone tube bioreactors

Biofilms were cultivated in duplicate in continuous, once-through flow systems using silicone tubes as bioreactors (inner diameter of 4 mm x 7 mm, length 270 mm) (see section 4.1.2). General disinfection and inoculation procedures were followed.

4.1.4.2. Sample preparation for ATR-FTIR spectroscopy

Nutrient medium flow was suspended and outlet tubing was disconnected from bioreactors. Biofilms were harvested by applying force to the bioreactor tubing using a glass bottle as a rolling pin. Biofilms and residual nutrient medium were collected from the silicon tubing in sterile tubes (approximately 35 ml per sample). Samples were dialysed for 18 hours at 4°C against 40 L RO-H_2O in dialysis tubing with a 14 000 molecular weight cut-off. After dialysis, samples were lyophilised for 48–72 hours.

4.1.4.3. ATR-FTIR measurements

Infrared spectra were obtained at ambient temperature, using a NEXUS 630 FTIR instrument (Thermo-Fischer, Cape Town, South Africa) containing a Smart Golden Gate Diamond ATR accessory with a type IIa diamond crystal. Spectra were recorded with a Ge-on-KBr beamsplitter and DTGS/Csl detector.

Dried biofilm samples were finely ground and deposited on the internal reflection crystal. The spectra were recorded from 4000 cm^{-1} to 400 cm^{-1} with a spectral resolution of 4 cm^{-1} and were the sum of 32 individual scans. OMNIC (version 7) software was used for data acquisition. GraphPad Prism® 5 was used to process data and spectra are presented as normalised absorbance.

4.1.5. Raman spectroscopy

4.1.5.1. Flow system with quartz flow cells

Biofilms were cultivated in a continuous, once-through flow system using quartz flow cells as bioreactors (section 4.1.2). Quartz tubes (Friedrich & Dimmock, NJ, USA) were prepared with the following dimensions: 5 mm width x 5 mm depth x 60 mm length; 0.9 mm wall thickness. Silicone tubing was cemented in the quartz tube openings using 100% silicone rubber that contains no antimicrobial compounds.

4.1.5.2. Silver colloid preparation for SERS

Silver colloids were prepared by the reduction of silver nitrate with hydroxylamine as previously described (Leopold and Lendl 2003). These solutions were used immediately after preparation for SERS. Between 100 and 200 µl of the silver colloid solution was injected directly into the quartz flow cell containing a biofilm before measurements were taken.

4.1.5.3. Raman spectroscopic measurements

The system consisted of an Argon ion laser (operating at 514.5 nm with average power of 200 mW), a sample chamber and a SPEX double monochromator fitted with a Synapse iCCD camera (Horiba Scientific, NJ, USA) as detector. Because of the high resolution of the system, only a small portion of the spectrum is imaged onto the camera. In order to obtain the complete spectrum, the gratings were rotated in unison in order to scan across the complete spectrum. The different spectral regions recorded in this fashion were stitched together using custom-written software. Toluene and silicon samples were used for wavelength calibration (data not shown).

4.1.6. CEMS

4.1.6.1. Flow systems with CEMSs

Each CEMS consisted of an inner silicon tube (inner diameter 1.6 mm, outer diameter 2.4 mm, length 1500 mm) encased by an outer Tygon® tube (inner diameter 4.8 mm, outer diameter 7.9 mm). Two CEMSs were used as bioreactors in a continuous, once-through flow system and biofilms were cultivated in the inner silicon tube. CEMSs were immersed in a heating/cooling water bath, set at 20 ± 2°C.

The annular space between the inner and outer tube was connected to a compressed CO_2-free air supply (Air IG Zero, pure oxygen 20%, pure nitrogen 80%, Instrument Grade supplied by Afrox, South Africa). This sweeper gas carried CO_2 produced by the biofilm to a dispersive, infrared LI-820 CO_2 gas analyser (LI-COR Biosciences, NE, USA). The sweeper gas flow rate was controlled by a GFC mass flow controller (Aalborg, NY, USA) at a rate of 10 $ml.min^{-1}$.

4.1.6.2. Disinfection, inoculation and culture conditions

Two CEMSs were connected to the growth medium reservoir and waste collection bottle with silicone tubing (inner diameter 1.575 mm) and straight plastic connectors (inner diameter 1.58 mm, Cole Palmer, IL, USA). After the system was assembled, it was disinfected with a commercial bleach solution and washed overnight with sterile deionized H_2O (as described in section 4.1.2.2). Prior to inoculation the system was connected to a medium reservoir and flushed with sterile nutrient medium.

Each CEMS was inoculated aseptically with 950 µl of an 18-hour pre-culture using a sterile needle and syringe. The inoculum was allowed to adhere to the silicon tubing for 1 hour under stagnant conditions before nutrient flow was resumed (15 $ml.h^{-1}$). Pre-cultures and nutrient medium were dependent on the specific experiment.

4.1.6.3. Biofilm-derived planktonic cell yield determination

Effluent from each CEMS was collected at the outlet. Serial dilutions of the collected effluent were made in sterile saline solution (0.9% w/v NaCl) and were incubated on agar-solidified nutrient medium. Effluent from *Pseudomonas* biofilms was incubated on agar-solidified nutrient medium containing the same nutrient source and concentration as supplied to the biofilm (typically TSB or modified defined medium with Na-citrate as carbon source). Effluent from multi-species biofilms were plated on duplicates of three types of agar-solidified nutrient medium: agar with TSB, R-2A agar and agar with Luria-Bertani (LB) broth and Congo Red. Agar plates were incubated at 26 ± 2 °C and effluent cell numbers ($CFU.ml^{-1}$) were enumerated after 5–7 days.

4.1.6.3.1. Agar-solidified nutrient medium for multi-species cultures

Three types of agar-solidified nutrient medium were used for the enumeration of multi-species cultures and contained the following (all chemicals supplied by Sigma Aldrich, South Africa):

- **TSB agar**: either 3.0 $g.L^{-1}$ or 0.3 $g.L^{-1}$ TSB with 12 $g.L^{-1}$ agar.

- **R-2A agar:** 15 $g.L^{-1}$ agar, 0.5 $g.L^{-1}$ casein acid hydrolysate, 0.5 $g.L^{-1}$ dextrose, 0.5 $g.L^{-1}$ proteose peptone, 0.3 $g.L^{-1}$ dipotassium phosphate, 0.024 $g.L^{-1}$ magnesium sulfate, 0.3 $g.L^{-1}$ sodium pyruvate, 0.5 $g.L^{-1}$ soluble starch, 0.5 $g.L^{-1}$ yeast extract.

- **1% LB agar with 2% Congo Red:** 10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ sodium chloride, 10 $g.L^{-1}$ agar and 2% Congo Red stock solution.

Congo Red stock solution was prepared as follows: 0.1 g Congo Red and 0.05 g Coomassie Brilliant Blue were dissolved in 35 ml 70% ethanol and 15 ml deionised H_2O (total volume = 50 ml).

4.2. Protocol development and method validation

4.2.1. Characterisation of biofilm structures using microscopic techniques

4.2.1.1. Biofilm cultivation for CLSM

Pseudomonas biofilms (PA01 *gfp* and PCT07 *gfp)* were cultivated in a continuous, once-through flow system using Perspex flow cells as bioreactors (section 4.1.2). General disinfection and inoculation procedures were used. Overnight pre-cultures were cultivated in modified AB defined medium with 10 mM Na-citrate on a rotating wheel at 26 ± 2 °C (section 4.1.1.3). Pre-cultures were diluted with sterile nutrient medium to a suspension of approximately 10^7 $CFU.ml^{-1}$ (section 4.1.1.4) and 100 µl of the diluted culture was injected directly into each channel under stagnant conditions.

Nutrient flow was resumed after 1 hour and biofilms were cultivated in modified AB defined medium with 10 mM Na-citrate at room temperature (20 ± 2°C) and a flow rate of 13 $ml.h^{-1}$ (Re = 0.8). After 96 hours, nutrient flow was suspended. Inlet and outlet tubing of bioreactors were clamped and

bioreactors were disconnected from the system. Bioreactors were transported to the microscope unit and were kept at room temperature (20 ± 2 °C) during CLSM analysis.

4.2.1.2. Quantification of biofilm parameters

To investigate the reproducibility of the biofilm cultivation techniques used in this study, certain biofilm parameters of PA01 *gfp* and PCT07 *gfp* biofilms were quantified using CLSM and DIA by COMSTAT (section 4.1.3.3). Three biofilm parameters were selected for this part of the study: percentage surface area coverage, bio-volume and average biofilm thickness.

Three biofilms were cultivated as described above in two independent experimental rounds (Round A and B) for both *Pseudomonas* strains (PA01 *gfp* and PCT07 *gfp).* In each experimental round, three flow cell channels (Channel 1–3) were inoculated from a single overnight culture (either PA01 *gfp* or PCT07 *gfp* in modified AB defined medium with 10 mM citrate). Seven image stacks were acquired from each biofilm, i.e. 21 image stacks were analysed per experimental round and 42 image stacks were analysed per biofilm parameter.

COMSTAT offers an additional connected-volume filtration process through which background signals can be reduced by removing pixels which are not connected to the substratum in a vertical line by other pixels (Heydorn et al. 2000b). After fixed threshold values were set, CLSM images were analysed with and without the connected-volume filtration process (data not shown). When the three-dimensional structures of the biofilms in this study were considered, it appeared that the connected-volume filtration process excluded biomass protrusions from the biofilms and therefore underestimated certain biofilm parameters. Although absolute values for biofilm parameters were not vital for this study, it was decided that values generated without the connected-volume filtration process were more accurate and these are reported in section 4.3.1.

4.2.1.3. Statistical analysis of biofilm parameters

Variance between biofilms per round (A in Figure 4.2) and between experimental rounds (B in Figure. 4.2) was determined by statistical analyses (section 4.1.3.4). Normality tests were performed, followed by one-way analysis of variance (single-factor or Kruskal-Wallis). In the case of a rejected null hypothesis, a comparison test was applied to determine which sample pairs were significantly different from each other (Table 4.4). Scatterplots were used to represent the 7 values measured per biofilm for each parameter and the variance between experimental rounds was represented by box-and-whiskers plots.

Table 4.4. Summary of statistical tests for CLSM data (p = 0.05).

Data sets compared	Normal distribution	Non-normal distribution
Channel 1 vs. Channel 2 vs. Channel 3 (7 values per channel)	Single-factor ANOVA	Kruskal-Wallis one-way analysis of variance
Round 1 vs. Round 2 (21 values per round)	Unpaired t test	Mann-Whitney U test

4.2.2. Biochemical characterisation using spectroscopic techniques

4.2.2.1. Biofilm cultivation for ATR-FTIR spectroscopy

PA01 *gfp* and PCT07 *gfp* biofilms were cultivated in duplicate in a continuous, once-through flow system using silicone tubing as bioreactors (section 4.1.2). General disinfection and inoculation procedures were followed. Bioreactors were inoculated with 1 ml of an overnight culture under stagnant conditions. Overnight pre-cultures were cultivated in either a complex nutrient medium (3 $g.L^{-1}$ TSB) or defined nutrient medium (modified defined AB medium with 10 mM citrate as carbon source) on a rotating wheel at 26 ± 2 °C (section 4.1.1.3).

After 1 hour, nutrient flow was resumed (either 3 $g.L^{-1}$ TSB or modified defined AB medium with 10 mM citrate) at a flow rate of 13 $ml.h^{-1}$ and biofilms were cultivated at room temperature (20 ± 2 °C). After 96 hours, biofilms were harvested from bioreactors, dialysed overnight against RO-H_2O and

lyophilised for 48 hours (section 4.1.4.2). The spectra of dried biofilm samples were recorded as described in section 4.1.4.3.

4.2.2.2. Biofilm cultivation for Raman spectroscopy

The fluorescent properties of the *gfp*-labelled *Pseudomonas* strains created interference with Raman light scattering and therefore the wild type strains (PA01 and PCT07) were used. PA01 and PCT07 biofilms were cultivated in duplicate in a continuous, once-through flow system using quartz flow cells as bioreactors (section 4.1.2). General disinfection and inoculation procedures were followed.

Overnight pre-cultures were cultivated in modified defined AB medium with 10 mM citrate as carbon source on a rotating wheel at 26 ± 2 °C (section 4.1.1.3). Bioreactors were inoculated with 300 µl of an overnight culture under stagnant conditions. After 1 hour, nutrient flow (modified defined AB medium with 10 mM citrate as carbon source) was resumed at a flow rate of 14.5 $ml.h^{-1}$ (Re = 0.8) and biofilms were cultivated at room temperature (20 ± 2 °C).

After 96 hours, nutrient flow was suspended. Inlet and outlet tubing of bioreactors were clamped and bioreactors were disconnected from the system. Bioreactors were kept at room temperature (20 ± 2 °C) during analysis using Raman spectroscopy (section 4.1.5). Spectra were recorded, after which 100–200 µl of a silver colloid solution was injected directly into the bioreactor and spectra were recorded again.

4.2.3. Investigation of biofilm growth rates

4.2.3.1. Biofilm cultivation in CEMSs

The initial CO_2 production rates of PA01 and multi-species biofilms were compared. Biofilms were cultivated in duplicate over three experimental rounds in continuous, once-through flow systems with CEMSs as bioreactors (section 4.1.6). General disinfection and inoculation procedures were followed

(section 4.1.6.2). Experimental conditions, as well as nutrient type, concentration (3 $g.L^{-1}$ TSB) and flow rate (15 $ml.h^{-1}$) were the same for PA01 and multi-species biofilms.

Overnight pre-cultures were cultivated in 3 $g.L^{-1}$ TSB at 26 ± 2 °C on a rotating wheel (section 4.1.1.3). CEMSs were inoculated with 950 µl of an 18-hour pre-culture under stagnant conditions. After 1 hour, nutrient flow was resumed at a flow rate of 15 $ml.h^{-1}$ and biofilms were cultivated for 48 hours at 20 ± 2 °C.

4.2.3.2. Biofilm-derived planktonic cell yield

Direct plate count methods were used to determine biofilm-derived planktonic cell yields (section 4.1.6.3). The growth medium flow rate in this CEMS setup would typically exceed the maximum specific growth rate of *Pseudomonas* and it is therefore likely that cells collected from the biofilm effluent would originate from biofilms rather than independently replicating planktonic microbial populations (Bester et al. 2010).

After 48 hours, effluent from the CEMS was collected and serial dilutions were made in sterile saline solution (0.9% w/v NaCl). Serial dilutions of PA01 biofilm effluent were plated in duplicate on agar-solidified medium (3 $g.L^{-1}$ TSB). Serial dilutions of multi-species biofilm effluent were plated in duplicate on three types of agar-solidified medium (3 $g.L^{-1}$ TSB, R-2A and 1% LB with 2% Congo Red) (section 4.1.6.3.1). Planktonic cell yield was subsequently determined by direct colony counts after incubation for 5–7 days.

4.3. Results

4.3.1. Quantification of *Pseudomonas* biofilm parameters

The two *Pseudomonas* strains formed distinct biofilm structures under the selected experimental conditions (Figures 4.3 and 4.4). PA01 *gfp* biofilms formed dense mats, similar to reports by other

studies where citrate is used as carbon source (Heydorn, Ersbøll, Kato, Hentzer, Parsek et al. 2002; Klausen, Heydorn, Ragas, Lambertsen, Aaes-Jørgensen et al. 2003). In the areas where significant amounts of biomass accumulated, the biofilm was a uniform mat containing dense microcolonies that vary in size (Figure 4.3). Microcolonies have defined edges and were often thicker than the surrounding mat-like layer. Voids were visible in the mat-like parts of the biofilm (indicated by the arrow in Figure 4.3.A). Highly variable growth patterns were visible throughout the length of the flow cell channel (images not shown). Areas with dense growth and microcolonies were interspersed with areas where only individual cells are visible on the surface. This explains the variance calculated for the biofilm parameters using COMSTAT (Table 4.5).

Quite the opposite was observed for PCT07 *gfp* biofilms (Figure 4.4). Mushroom-shaped microcolonies with small substratum-bound bases were located close to each other and were often connected by bridge-like structures containing cells (indicated in Figure 4.4.C). These "bridges" between microcolonies were not always connected to the substratum and appeared to float between colonies, protruding into the lumen of the channel. Overall, PCT07 *gfp* biofilms contained more biomass (2.8 ± 1.1 $\mu m^3.\mu m^{-2}$ bio-volume) than PA01 *gfp* biofilms (1.1 ± 1.0 $\mu m^3.\mu m^{-2}$ bio-volume).

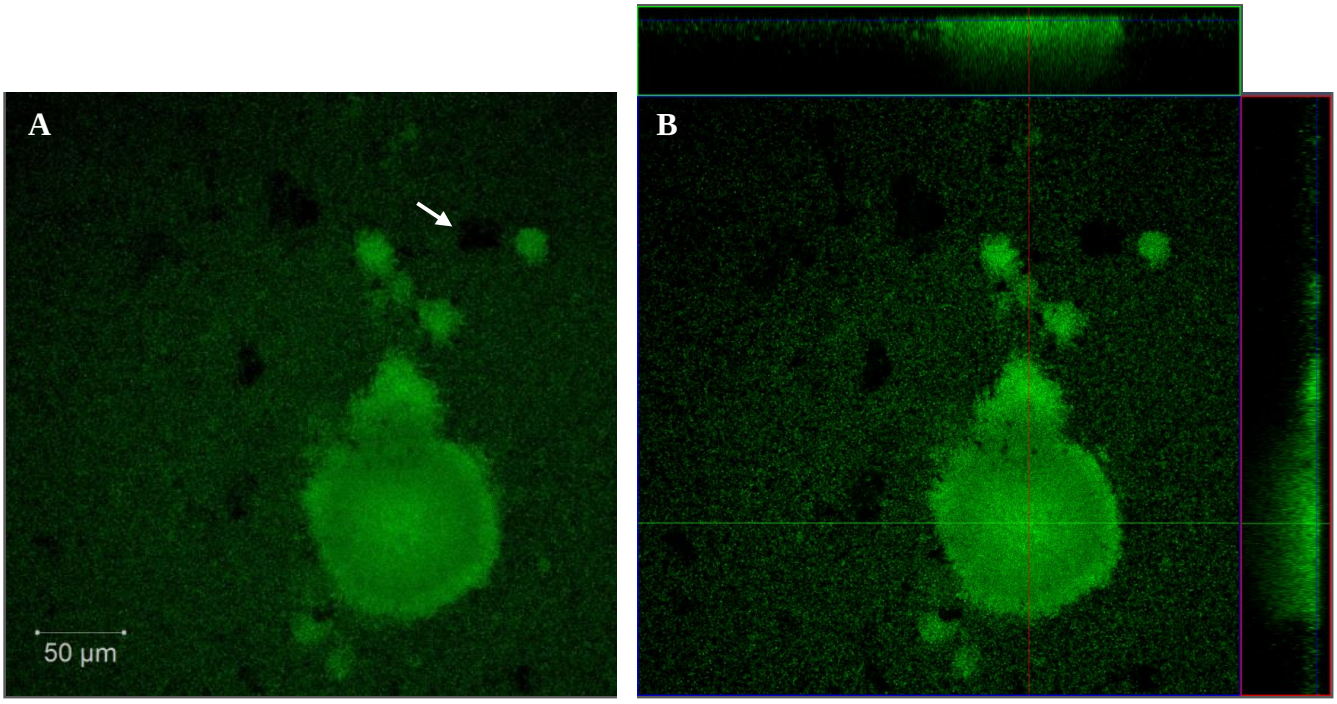

Figure 4.3. CLSM images displaying characteristic structures of PA01 *gfp* biofilms cultivated at high nutrient concentrations and low flow rates. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$. **A**. Image captured using a 40x objective. Arrow indicates void in biofilm. **B**. Ortho-view at the same z-position as A (approximately 8 µm from substratum).

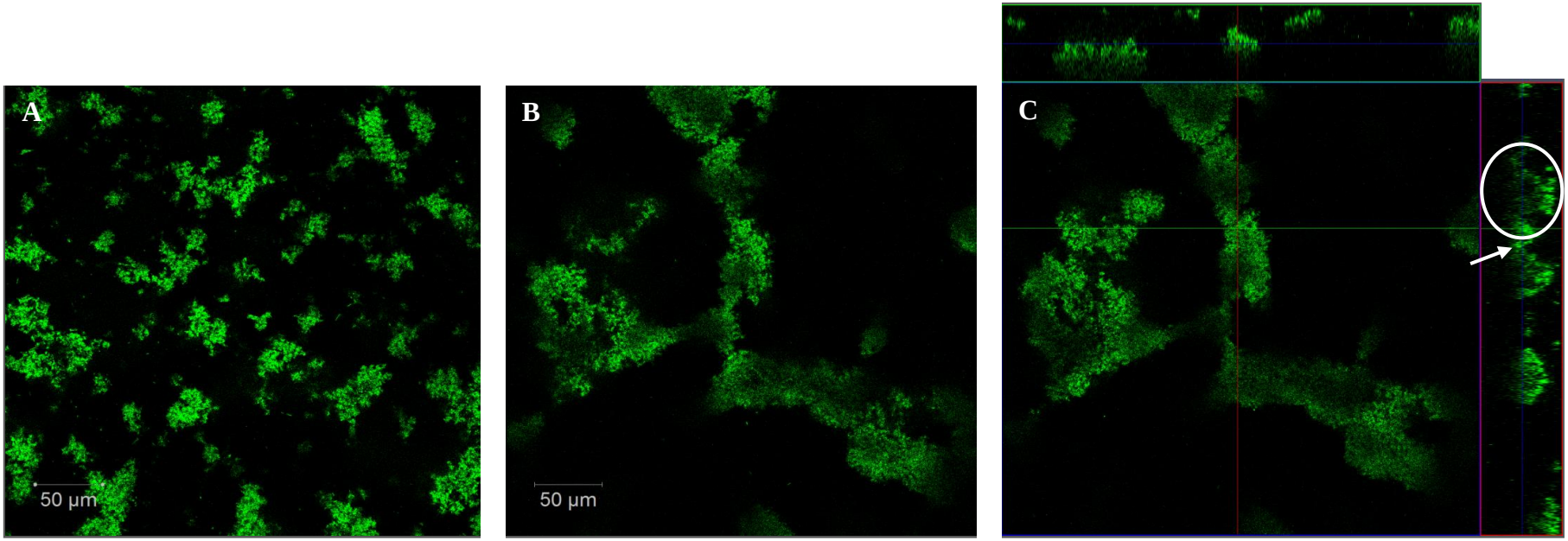

Figure 4.4. CLSM images displaying characteristic structures of PCT07 *gfp* biofilms cultivated at high nutrient concentrations and low flow rates. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$. **A**. Image captured using a 40x objective (approximately 8 µm from substratum). **B**. Image captured using a 40x objective (approximately 28 µm from substratum). **C**. Ortho-view at the same z-position as B (approximately 28 µm from substratum). Circle indicates mushroom-shaped colony. Arrow indicates bridge-like structure connecting two mircocolonies.

4.3.2. Statistical analysis of biofilm parameters

The average thickness of PA01 *gfp* biofilms is used as an example to discuss the variance between biofilms per round (A in Figure 4.2) and the variance between experimental rounds (B in Figure 4.2), as determined by statistical tests. Scatterplots (Figure 4.5.A) and box-and-whiskers plots (Figure 4.5.B) show the variance between biofilms per round and between experimental rounds, respectively. A summary of significant differences calculated within the experimental rounds (A and B) and between the experimental rounds (A vs. B) is presented in Table 4.6.

When considering the average thickness of PA01 *gfp* biofilms, no significant differences were observed between biofilms per round or between experimental rounds. Similarly, at these specific growth conditions, the biomass production by PA01 *gfp* biofilms was reproducible (i.e. no significant differences in bio-volume within or between rounds). Although no significant difference was observed for surface area coverage by PA01 *gfp* per round, a significant difference was observed between the two experimental rounds. PCT07 *gfp* biofilms were reproducible in terms of bio-volume, but significant differences were observed for average thickness and surface area coverage.

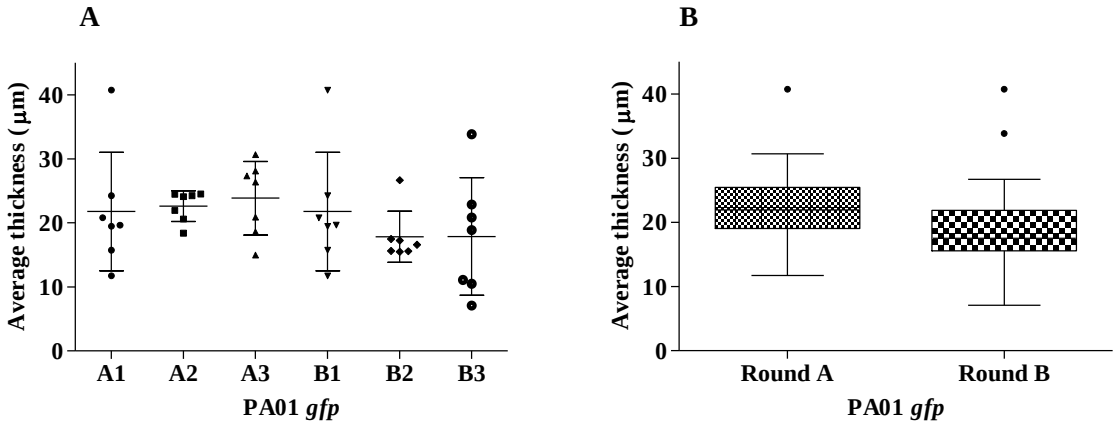

Figure 4.5. **Average thickness of PA01 *gfp* biofilms cultivated at high nutrient concentrations and low flow rates.** CLSM simulated fluorescence projections were analysed using COMSTAT. In experimental rounds A and B, triplicate biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 13 ml.h^{-1}. **A.** Scatterplots of 7 values (mean value with standard deviation) per biofilm for both experimental rounds. **B.** Box-and-whiskers plot of 21 values per experimental round, showing outliers, calculated using Tukey's test.

Table 4.5. Biofilm parameters of *Pseudomonas* strains cultivated at high nutrient concentrations and low flow rates. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$. Average values with standard deviations were calculated from 21 measurements over duplicate experimental rounds.

	Surface area coverage (%)	**Bio-volume ($\mu m^3.\mu m^{-2}$)**	**Average thickness (µm)**
PA01 *gfp*	13.9 ± 11.3	1.1 ± 1.0	1.8 ± 1.8
PCT07 *gfp*	19.1 ± 4.6	2.8 ± 1.1	5.7 ± 2.7

Table 4.6. Significant differences recorded for PA01 *gfp* and PCT07 *gfp* biofilm parameters. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$ in two experimental rounds (A and B). Significant differences within an experimental round (A or B) or between experimental rounds (A vs. B) are indicated by * (p = 0.05).

	Surface area coverage			**Bio-volume**			**Average thickness**		
	A	B	A vs. B	A	B	A vs. B	A	B	A vs. B
PA01 *gfp*	-	-	*	-	-	-	-	-	-
PCT07 *gfp*	-	*	*	-	-	-	*	*	*

4.3.3. Biochemical composition of biofilms

Figure 4.6 shows reproducible ATR-FTIR spectra for PA01 *gfp* and PCT07 *gfp* biofilms. When cultivated in TSB medium, the peak positions on the *Pseudomonas* biofilm spectra were almost identical. When comparing the ATR-FTIR spectra of the *Pseudomonas* biofilms cultivated in defined modified AB medium with citrate as carbon source, peak position shifted and it was possible to distinguish between the spectra of PA01 *gfp* and PCT07 *gfp* biofilms. The spectra of PA01 *gfp* biofilms are comparable with other ATR-FTIR spectroscopic studies of *P. aeruginosa* biofilms (Suci, Vrany and Mittelman 1998). Band assignments and the biochemical compositions of *Pseudomonas* biofilms are discussed in greater detail in sections 4.4 and 5.4.1.

Figure 4.7.A shows the Raman spectra of PA01 and PCT07 biofilms. PA01 biofilms generated very weak Raman signals and therefore the spectra were difficult to interpret. While both strains formed visible biofilms in the quartz flow cells, PCT07 biofilms formed clumps of biomass and it was possible that the operator was able to better focus the laser on areas containing dense biomass and therefore generated more resolved spectra. Figure 4.7.B shows the 1000–1800 cm^{-1} region of PCT07 biofilm spectra.

SERS was used in an attempt to better resolve biofilm spectra. Solutions containing silver colloids were added to solutions of various external standards (e.g. proteins and polysaccharides). Figure 4.8 shows the improvement in peak resolution when an active silver colloid solution was added to a solution of dextran, a branched polysaccharide. Despite repeated attempts, no well-resolved spectra for biofilms using this technique were obtained and therefore it was decided to revert to ATR-FTIR spectroscopy for further analysis (see Chapter 5).

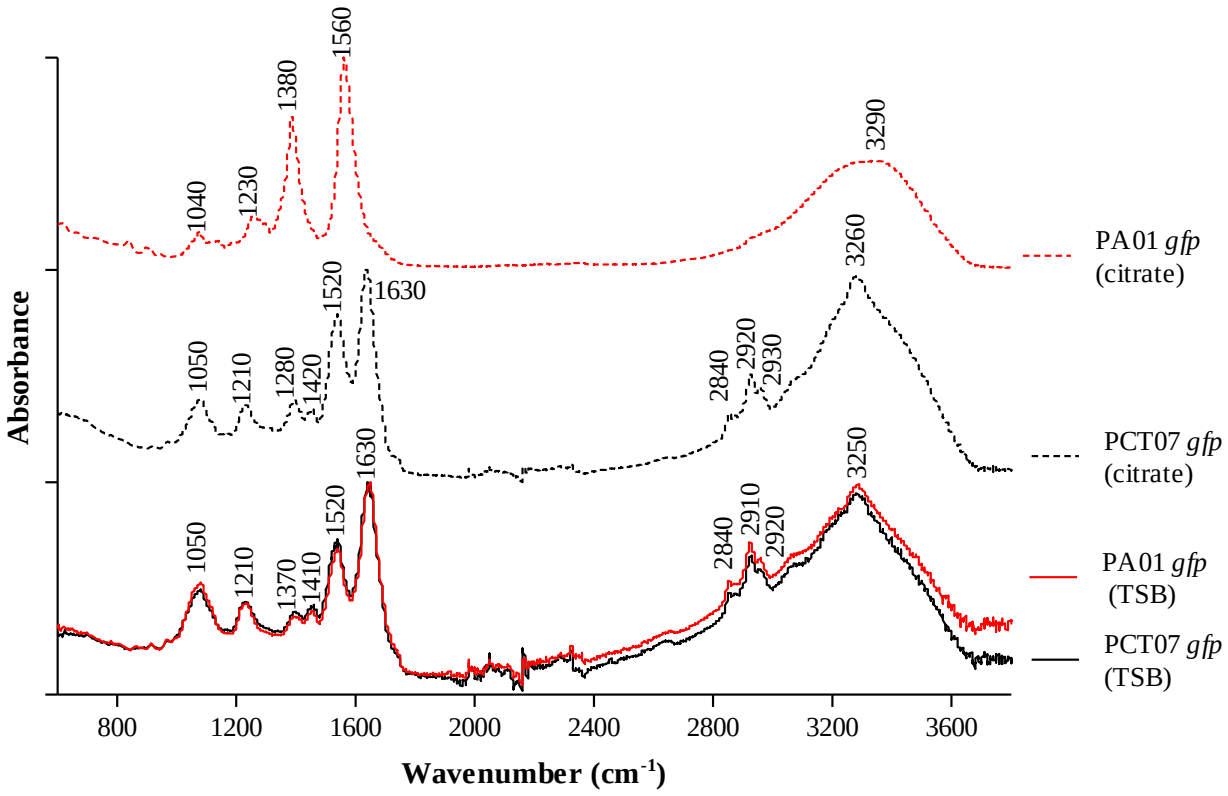

Figure 4.6. **Normalised ATR-FTIR spectra of lyophilised PA01 *gfp* and PCT07 *gfp* biofilms cultivated in complex or defined nutrient medium.** Biofilms were cultivated in continuous, once-through flow systems with silicone tube bioreactors for 96 hours in either 3 $g.L^{-1}$ TSB or modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$. Absorbance is presented in arbitrary units.

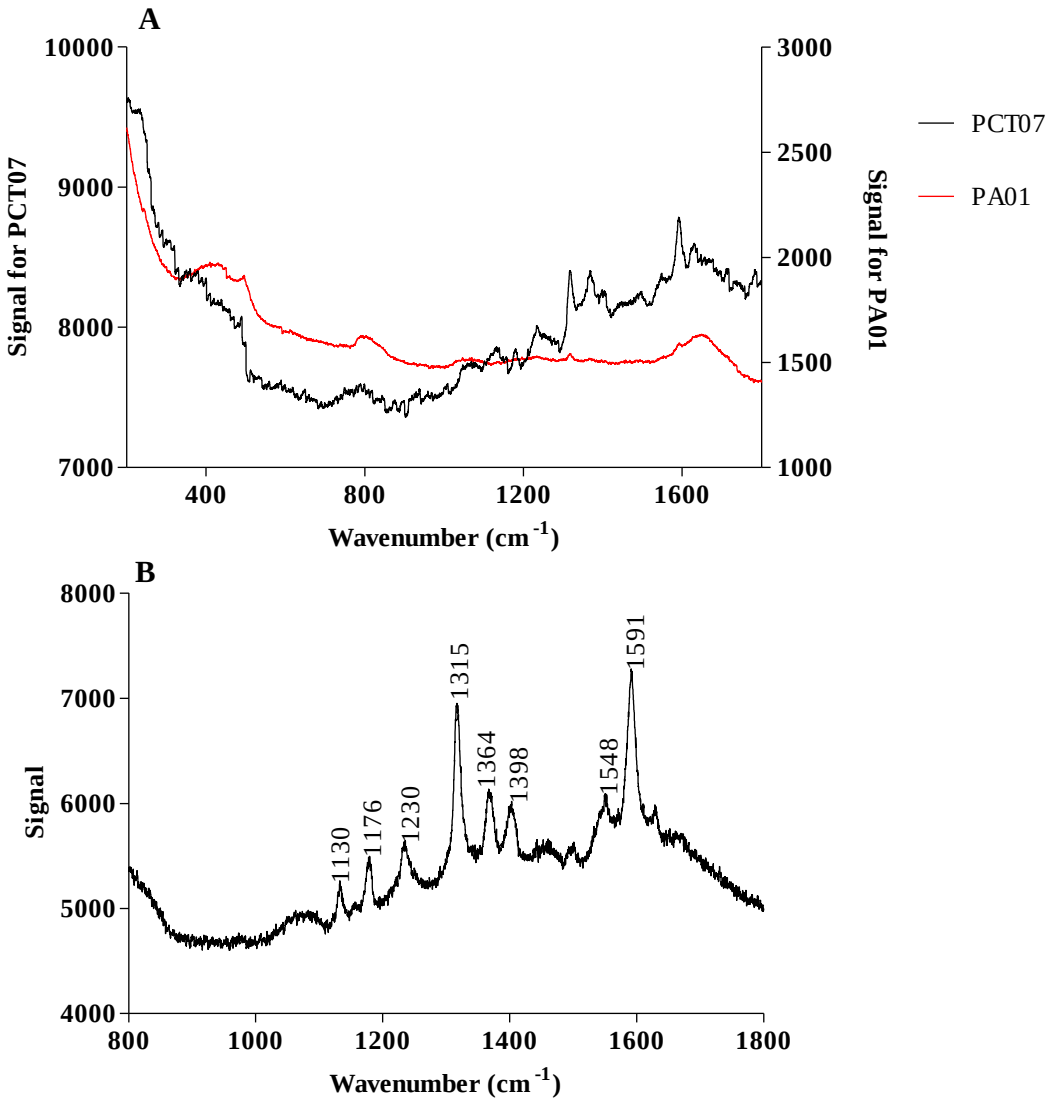

Figure 4.7. Raman spectra of PA01 and PCT07 biofilms cultivated in defined nutrient medium. Signal is presented in arbitrary units. **A.** Biofilms were cultivated in continuous, once-through flow systems with quartz flow cells for 96 hours in modified defined AB medium with 10 mM citrate at a flow rate of 14.5 $ml.h^{-1}$. **B**. Spectra of a PCT07 biofilm with peak resolution.

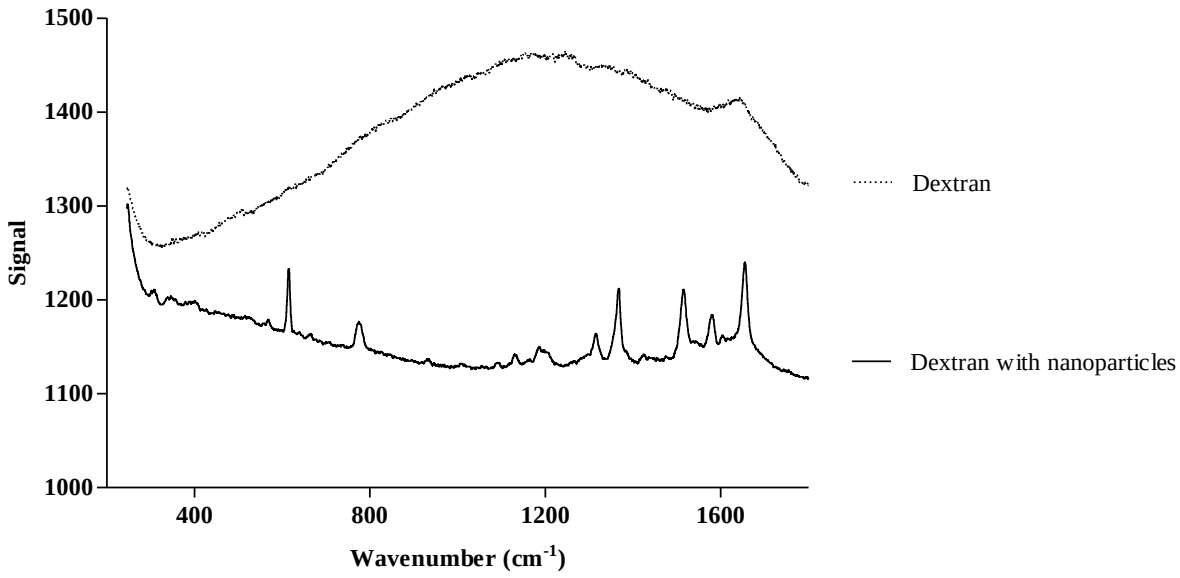

Figure 4.8. Raman spectra of a dextran solution, with and without active silver colloids. Signal is presented in arbitrary units.

4.3.4. Biofilm CO_2 production rates and planktonic cell yield

In three independent experiments, initial CO_2 production rates of PA01 biofilms reached a steady state of 80–95 µmol.h^{-1} within 36 hours (Figure 4.9.A). Despite the variance in the initial rates of multi-species biofilms (Figure 4.9.B), CO_2 production reached a steady state of 80–95 µmol.h^{-1} within 48 hours. Similar CO_2 production rates for PA01 and multi-species biofilms have been observed elsewhere (Kroukamp et al. 2010). Both the single- and multi-species biofilms reached stable CO_2 production rates within 48 hours of inoculation, with multi-species biofilms showing accelerated CO_2 production rates within the first 24 hours.

The planktonic cell yield of PA01 and multi-species biofilms was determined 48 hours after inoculation. For PA01 biofilms, each CEMS was inoculated with 900 µl of an overnight culture containing approximately 7.0 x 10^8 CFU.ml^{-1} and after 48 hours the biofilm effluent contained an average of 5.7 x 10^8 CFU.ml^{-1} planktonic cells (Figure 4.9.C). For multi-species biofilms, each CEMS was inoculated with 900 µl of an overnight culture containing approximately 1.1 x 10^9 CFU.ml^{-1} planktonic cells and after 48 hours the biofilm effluent contained an average of 1.2 x 10^9 CFU.ml^{-1}. No significant differences were observed between planktonic cell counts of overnight cultures and biofilm effluent at 48 hours for PA01 or multi-species biofilms.

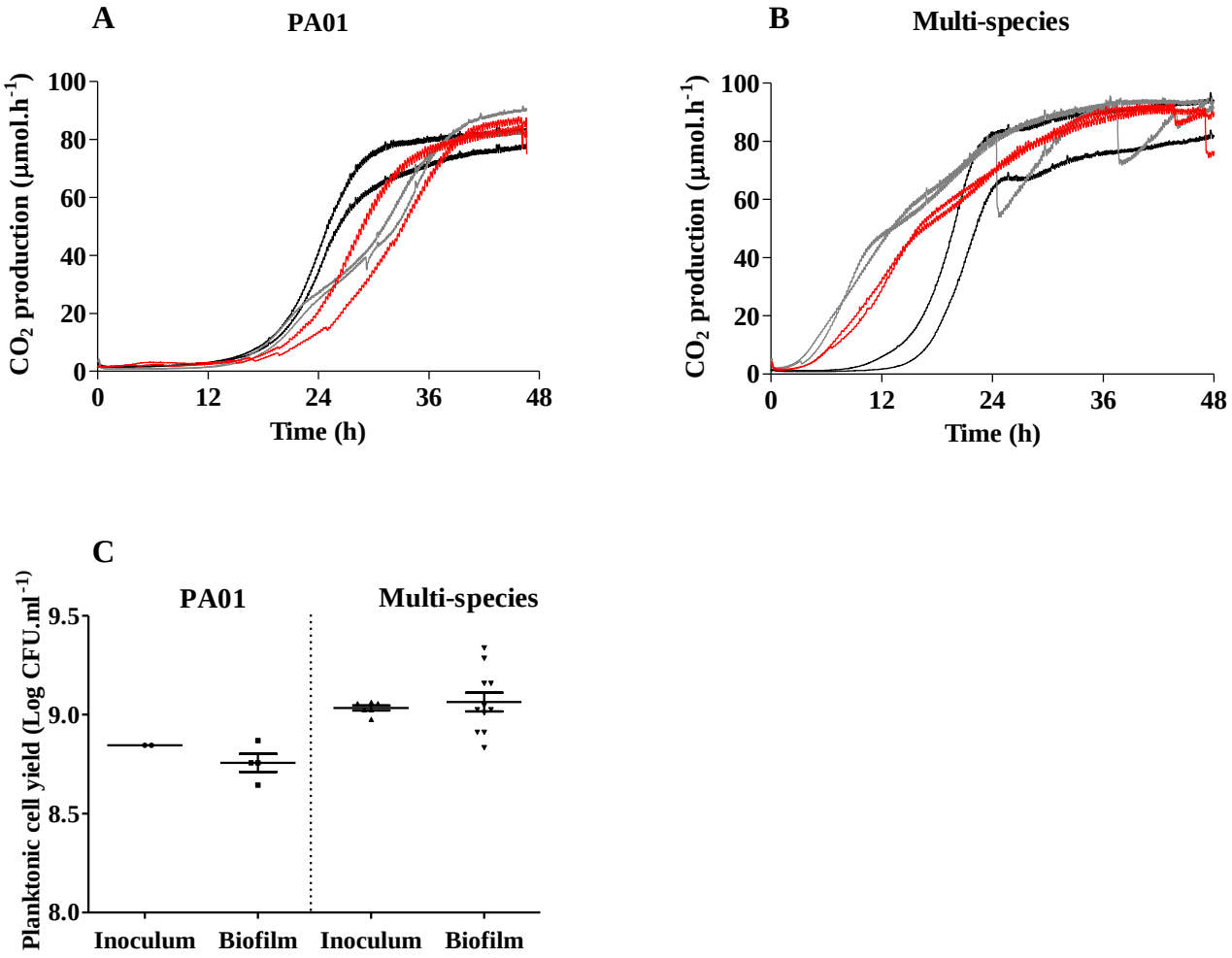

Figure 4.9. **CO_2 production rates and biofilm-derived planktonic cell yield of PA01 and multi-species biofilms.** Biofilms were cultivated in continuous, once-through flow systems with CEMSs as bioreactors for 96 hours in 3g.L^{-1} TSB medium at a flow rate of 15 ml.h^{-1}. Two biofilms were cultivated in three independent experimental rounds (rounds indicated in red, black and grey). **A**. CO_2 production rates of PA01 biofilms. **B.** CO_2 production rates of multi-species biofilms. **C.** Planktonic cell yield of biofilms calculated by direct plate count methods.

4.4. Discussion

The above-mentioned investigations were performed to evaluate the appropriateness of the selected techniques for addressing the central hypotheses in this study. To this end, the reproducibility of results and the notion of "reproducible" biofilms are discussed in this section. Biofilm development is a complex, stochastic process and no two biofilms are identical structural copies. Despite this inherent heterogeneity, studies report that it is possible to cultivate "reproducible" biofilms that exhibit similar traits, e.g. biofilm thickness, three-dimensional structures and viable cell counts (Heydorn et al. 2000a). For the purpose of this study, biofilms were considered reproducible when they exhibit similar time-based structural development and biomass accumulation (Jackson, Beyenal, Reese and Lewandowski 2001).

Well-characterised *Pseudomonas* strains, together with established biofilm cultivation techniques and CLSM protocols, were used to investigate whether reproducible biofilms can be cultivated under controlled environmental conditions. Certain "normal" biofilm structures and attributes were observed for PA01 *gfp* and PCT07 *gfp* biofilms using CLSM and statistical analysis of measured biofilm parameters indicated that the two strains generally produce biofilms that have reproducible traits. Less variance was recorded for PA01 *gfp* biofilm parameters, which may be due to the uniform, mat-like structures of these biofilms, compared to the irregular structures of the mushroom-shaped microcolonies of PCT07 *gfp* biofilms. The results in Table 4.6 demonstrate that the selected experimental system and conditions produced "reproducible" PA01 *gfp* biofilms, as well as PCT07 *gfp* biofilms with some variation. The same statistical model was applied to analyse biofilm parameters of both *Pseudomonas* strains cultivated under different nutrient concentrations and flow rates.

Although PA01 *gfp* biofilms appeared to cover more of the flow cell surfaces than PCT07 *gfp* biofilms, the percentage surface area coverage, as calculated by COMSTAT, is lower for PA01 *gfp* than PCT07 *gfp* biofilms. This could be due to the positioning of image collection within the flow cells. Figure 4.3 is an example of a typical PA01 *gfp* biofilm cultivated under these conditions. Areas exhibiting this kind of growth were present in all PA01 *gfp* biofilms in both experimental rounds. However, under these conditions, biofilm growth patterns varied throughout the flow cell channels. Images were collected in random positions in the middle section of the flow cell channel to minimise so-called wall

hydrodynamic effects, as recommended by several studies. If images were only collected from areas where dense biofilm mats are visible, higher values may be reported for surface area coverage.

As with most experiments, time, costs and appropriate sample size needed to be balanced. It is time-consuming and costly to capture biofilm images with sufficient resolution and therefore the sample size per biofilm was limited to seven image stacks. Each stack captures 1.2 x 10^5 µm^2 of the flow cell surface area, that means 8.4 x 10^5 µm^2 (or 0.84 mm^2) is captured out of a total flow cell area of 200 mm^2. Ideally, more samples per biofilm should be included to better represent biofilm growth throughout the flow cell channel.

The laser intensity was kept constant for all CLSM observations to allow comparisons between strains. The laser was set to a low intensity to prevent photobleaching of thicker biofilm samples. In this case, PA01 *gfp* cells generated weaker fluorescent signals than PCT07 *gfp* cells. Manual threshold setting in COMSTAT was used to compensate for the difference in signal strength, but this may have led to an underestimation of certain parameter values. Additionally, a correction feature ("smacking") was used to compensate for images captured on non-flat surfaces. This may influence the calculation of average thickness This study was not concerned with absolute parameter values calculated by DIA, but rather focused on the identification of trends in biofilm responses (both structurally and metabolically) to changes in environmental conditions.

Reproducible ATR-FTIR spectra were generated for PA01 *gfp* and PCT07 *gfp* biofilms. Generally, the double peak formations between 1500 and 1700 cm^{-1} are indicative of proteins (amide I at 1648–1658 cm^{-1} and amide II at 1548–1550 cm^{-1}), while the peaks between 1000 and 1200 cm^{-1} most likely indicate the presence of RNA or DNA bases. The presence of proteins in *Pseudomonas* biofilms was further supported by the peaks at amide positions observed in the Raman spectra of PCT07 biofilms (Figure 4.7.B). ATR-FTIR spectra are interpreted in greater detail in Chapter 5.

The FTIR equipment used in this study required dehydrated biofilm samples and Raman spectroscopy was therefore considered as a non-destructive alternative for biochemical profiling. Although Raman spectroscopy has been used for biochemical investigations of biofilms elsewhere, the system available for use in this study did not produce resolved spectra for *Pseudomonas* biofilms. ATR-FTIR

spectroscopy is therefore used to investigate the biochemical composition of multi-species biofilms and the spectra are compared to the *Pseudomonas* biofilm spectra in Chapter 5.

The reproducible ATR-FTIR spectra and data gathered from DIA of CLSM images demonstrated that the general cultivation methods using bioreactors (whether silicone tubing or Perspex flow cells) in continuous, once-through flow systems are appropriate for the cultivation of relatively reproducible *Pseudomonas* biofilms. Although these systems still create "ideal" environments, it is possible to alter environmental conditions and monitor changes in biofilm structures and metabolic responses, as is shown in Chapter 5.

While numerous studies have demonstrated the use of CLSM, ATR-FTIR and Raman spectroscopy for non-destructive biofilm response analysis, the available equipment and other logistical considerations limited the use of these techniques in this study. For example, nutrient flow needed to be discontinued and flow cells removed from the flow systems to transport biofilms to the CLSM unit. For this reason, no time-lapse experiments were performed and the study of biofilms using CLSM is limited to a specific moment in time.

The CEMS provided a viable alternative that allows real-time monitoring of single- and multi-species biofilm growth rates without disrupting nutrient flows (Chapter 6). Initial experiments generated reproducible CO_2 production rates for PA01 and multi-species biofilms over a 48-hour time period. Biofilm-derived planktonic cell yield was similar to planktonic cell counts of overnight cultures for both the single- and multi-species inocula. Based on the advantages discussed in section 3.4.4, the CEMS, in combination with metabolic assays, was selected for further use in this study to investigate metabolic responses of single- and multi-species biofilms to environmental changes.

4.5. Conclusion

The aforementioned results confirmed that:

- Continuous, once-through flow systems with selected bioreactors can be used to cultivate single-species biofilms with reproducible traits,

- ATR-FTIR spectroscopy generates reproducible spectra of two *Pseudomonas* biofilms and biochemical differences are visible between the two strains,

- CLSM and DIA can be used to identify structural traits and quantify certain parameters of PA01 *gfp* and PCT07 *gfp* biofilms,

- ATR-FTIR spectroscopy and CLSM provide useful biochemical and structural information about biofilms, but due to certain limitations these techniques do not allow real-time investigations of biofilm responses,

- The CEMS is a non-destructive alternative for investigations into the metabolic responses of single- and multi-species biofilms in order to describe the adaptive nature of these microbial communities.

5. EPS matrixes as resource-efficient coatings: biochemical investigations of composition and structure

The EPS matrix is an integral part of a biofilm. It influences the physicochemical properties of a biofilm, as well as the microbial activity within a biofilm. This chapter investigates the biochemical compositions and three-dimensional structures of biofilms using spectroscopic and microscopic techniques.

Three of the biomimetic principles that form part of the proposed framework for describing microbial biofilms are examined here (Figure 5.1). Firstly, the concept of life-friendly chemistry in biofilms is explored. The biochemical compositions of biofilms from two *Pseudomonas* strains and a multi-species environmental culture are compared using ATR-FTIR spectroscopy. To test whether microorganisms are in fact able make use of only a small subset of elements to produce and secrete complex EPS, *Pseudomonas* biofilms were cultivated in a modified defined nutrient medium (which contained only C, H, O, N, Ca, Mg and other trace elements) and the EPS distribution was investigated using fluorescent stains and CLSM.

In order to investigate resource efficiency within microbial communities and the integrated growth and development of biofilms, CLSM is used to study the structures of *gfp*-labelled *Pseudomonas* biofilms cultivated in different nutrient concentrations and hydrodynamic conditions. From a biomimicry point of view, resource efficiency is understood within the context of energy and material use. Energy and material expenditure is minimised when physical structures are designed to fit their function. It was hypothesised that biofilm morphology of *Pseudomonas* biofilms would be influenced by environmental conditions in order to provide the microbial community benefits like optimal access to nutrients. To this end, CLSM and DIA are used to quantify certain biofilm parameters and identify trends in structural adaptations of biofilms.

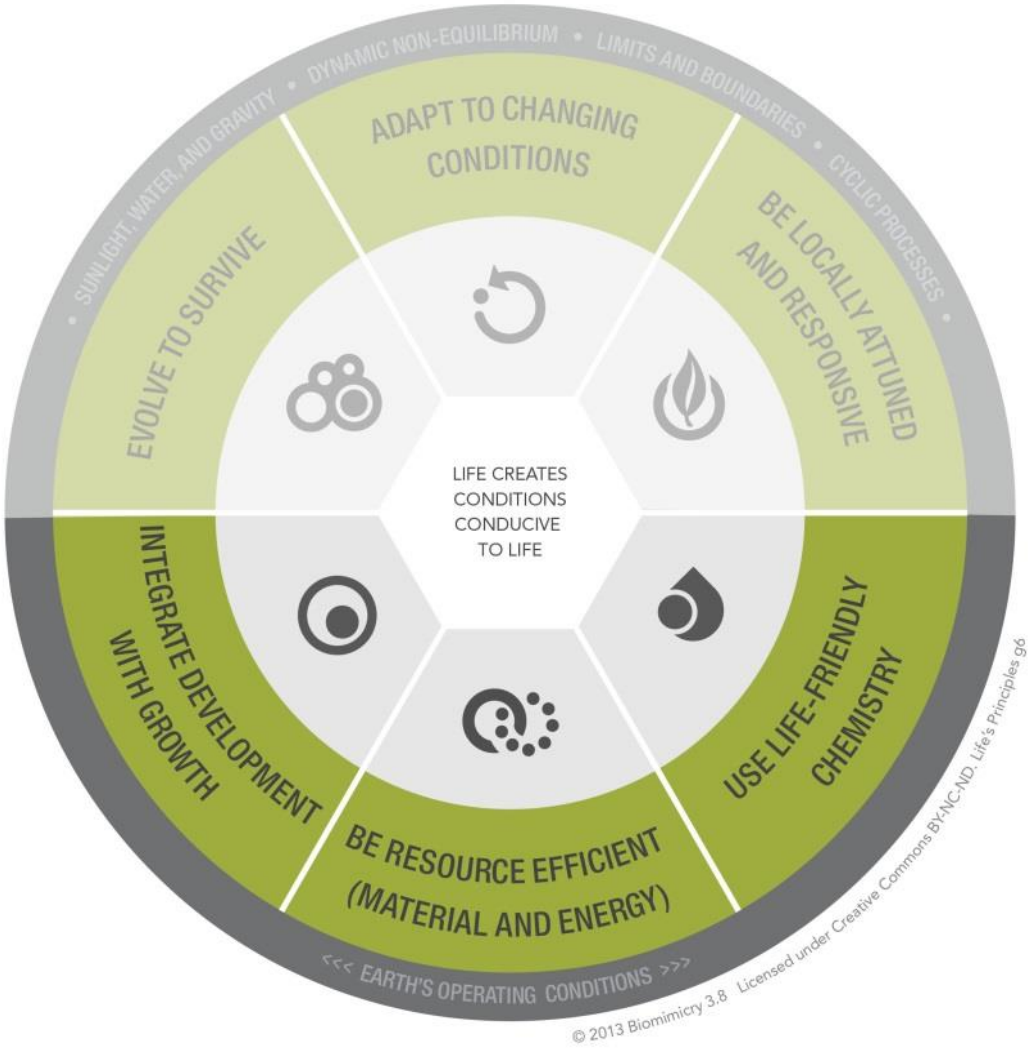

Figure 5.1. Graphic representation highlighting three Life's Principles: use life-friendly chemistry, be resource-efficient and integrate development with growth. Adapted from original[12].

[12] http://biomimicry.net/about/biomimicry/biomimicry-designlens/lifes-principles, accessed on 25 September 2015.

5.1. Biochemical investigations of biofilms

5.1.1. Biofilm cultivation for ATR-FTIR spectroscopy

The following experiments were performed in duplicate. Duplicate biofilms were cultivated in a continuous, once-through flow system using silicone tubing as bioreactors (section 4.1.4). General disinfection and inoculation procedures were followed (section 4.1.2). Bioreactors were inoculated with 1 ml of an overnight *Pseudomonas* pre-culture (PA01 *gfp* or PCT07 *gfp)* or a multi-species environmental pre-culture (section 4.1.1). Overnight pre-cultures were cultivated in 3 $g.L^{-1}$ TSB medium on a rotating wheel at 26 ± 2 °C (section 4.1.1.3). Biofilms were cultivated in 3 $g.L^{-1}$ TSB medium at room temperature (20 ± 2 °C) and at a flow rate of 13 $ml.h^{-1}$ for 96 hours, before being harvested from the bioreactors (section 4.1.4.2).

After harvesting biofilms, a centrifugation step (10 000 g for 30 minutes) was included in an attempt to separate EPS (expected in the supernatant fraction) from the cellular component (pellet fraction) (Tielen et al. 2010). The pellet fraction was re-suspended in RO-H_2O. Intact biofilm samples, as well as supernatant and re-suspended pellet fractions, were dialysed for 18 hours at 4 °C against 40 L RO-H_2O in dialysis tubing with a 14 000 molecular weight cut-off. After dialysis, samples were lyophilised for 48–72 hours (section 4.1.4.2).

Using the above-mentioned protocol, PA01 *gfp* and PCT07 *gfp* biofilms were also cultivated in modified defined AB medium with 10 mM citrate at a flow rate of 13 $ml.h^{-1}$ for 96 hours, before being harvested from the bioreactors. Biofilms were dialysed and lyophilised. The spectra of all the lyophilised biofilm samples were recorded as described in section 4.1.4.3.

5.1.2. Biofilm cultivation and staining for CLSM

Biofilms were cultivated in triplicate in a continuous, once-through system using Perspex flow cells as bioreactors (section 4.1.3.1). General disinfection and inoculation procedures were followed (section 4.1.2). Flow cells were inoculated with 100 µl of a diluted overnight *Pseudomonas* pre-culture (PA01 *gfp* or PCT07 *gfp)* under stagnant conditions. Pre-cultures were diluted with sterile nutrient medium to a suspension of approximately 10^7 CFU.ml^{-1} (section 4.1.1.4). Biofilms were cultivated in modified defined AB medium with 1 mM citrate at room temperature (20 ± 2 °C) and at a flow rate of 13 ml.h^{-1} for 96 hours.

5.1.2.1. Staining procedure

Biofilms were incubated with either FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain (SYPRO® Ruby) or Alexa Fluor® 594 conjugated Concanavalin A (ConA-594) before being imaged using CLSM. Invitrogen™ stains were purchased from Thermo Fischer Scientific Inc., MA, USA.

Stock solutions of ConA-594 were prepared by dissolving 5 mg lyophilised ConA-594 in 0.1 M sodium bicarbonate (final concentration 1.25 mg.ml^{-1}, approximate pH 8.2). Dissolved ConA-594 stain solution was divided into 200 µl aliquots and stored at -20 °C. For each experiment, one aliquot was thawed and diluted with 10 mM phosphate buffer (final concentration 0.25 mg.ml^{-1}, approximate pH 8.2). The diluted stain was centrifuged for 30 seconds and no aggregates or pellets were visible. Of this diluted stain solution, 800 µl was injected into each flow cell channel (flow cell internal volume = 800 µl). SYPRO® Ruby was not diluted before use. For these experiments, 800 µl SYPRO® Ruby was injected directly into flow cell channels. Control experiments showed that there was no overlap between green (*gfp*-labelled organisms) and red (SYPRO® Ruby or ConA-594) signals (data not shown). Control experiments included capturing images of flow cell channels containing medium and stain (without biofilms) to ensure that the stains did not bind to the flow cell surface or have interaction with the nutrient medium.

Biofilms were cultivated for 96 hours, at which point the stain was introduced. Flow was suspended and the inlet tubing of the flow cell was clamped. The fluorescent stain was gently injected into the flow cell channel. Biofilms were incubated with the stain for 30 minutes in the dark. Flow was resumed for 5 minutes at a flow rate of 13 $ml.h^{-1}$ to remove unbound stain. Flow was suspended and inlet and outlet tubing were clamped. Bioreactors were disconnected from the system and transported to the microscope unit.

5.2. Influence of nutrient concentration and flow rate on biofilm development

5.2.1. Biofilm cultivation for CLSM

Biofilms were cultivated in triplicate in a continuous, once-through system using Perspex flow cells as bioreactors (section 4.1.3.1). General disinfection and inoculation procedures were followed (section 4.1.2). Flow cells were inoculated with 100 µl of a diluted overnight *Pseudomonas* pre-culture (PA01 *gfp* or PCT07 *gfp)* under stagnant conditions. Pre-cultures were diluted with sterile nutrient medium to a suspension of approximately 10^7 $CFU.ml^{-1}$ (section 4.1.1.4). Overnight pre-cultures were cultivated in modified AB defined medium (concentration of Na-citrate corresponding to the nutrient medium supplied in the experiment) (section 4.1.1.3).

Biofilms were cultivated at room temperature (20 ± 2 °C) in different nutrient media and hydrodynamic conditions. The four sets of environmental conditions are summarised in Table 5.1. After 96 hours, nutrient flow was suspended. Inlet and outlet tubing of bioreactors were clamped and bioreactors were disconnected from the system. Bioreactors were transported to the microscope unit and were kept at room temperature (20 ± 2 °C) during CLSM analysis.

5.2.2. Experimental design and statistical analysis

Two experimental rounds were performed for each environmental condition set out in Table 5.1 (section 4.1.3.4). In each experimental round, three flow cell channels were inoculated from a single diluted overnight culture (either PA01 *gfp* or PCT07 *gfp* in modified AB defined medium with a final concentration of either 1 or 10 mM citrate).

The growth and development of *Pseudomonas* biofilms were compared in terms of surface area coverage, bio-volume, average thickness and surface:volume ratios for the four environmental conditions investigated. Seven image stacks were acquired from each channel, i.e. 21 image stacks were analysed per round and 42 image stacks were analysed for each environmental condition investigated.

The variance between these biofilm parameters within an experimental round (A in Figure 5.2), between duplicate rounds (B in Figure 5.2) and between environmental conditions (C in Figure 5.2) was analysed using GraphPad Prism®. Shapiro-Wilk test was used as a normality test and subsequent tests were performed as summarised in Table 5.2. In the event of a rejected null hypothesis, either Tukey's test for the comparison of means (significant difference determined by single-factor ANOVA) or Dunn's multiple comparison test (significant difference determined by Kruskal-Wallis one-way analysis of variance) was applied to determine which sample pairs were significantly different from each other.

Table 5.1. Nutrient concentrations and flow rates for the cultivation of *Pseudomonas* biofilms.

Combinations of nutrient concentration [C] and flow rates F
Low [C] Low F
Low [C] High F
High [C] Low F
High [C] High F
[C] *(mM citrate* in modified defined AB medium*)*
Low [C] = 1
High [C] = 10
F *(ml.h^{-1})*
Low F = 13
High F = 30

Table 5.2. A summary of statistical tests for CLSM data (p = 0.05).

Data sets compared	**Normal distribution**	**Non-normal distribution**
Channel 1 vs. Channel 2 vs. Channel 3 (7 values per channel)	Single-factor ANOVA	Kruskal-Wallis one-way analysis of variance
Round 1 vs. Round 2 (21 values per round)	Unpaired t test	Mann-Whitney U test
Condition 1 vs. Condition 2 vs. Condition 3 vs. Condition 4 (42 values per condition)	Single-factor ANOVA	Kruskal-Wallis one-way analysis of variance

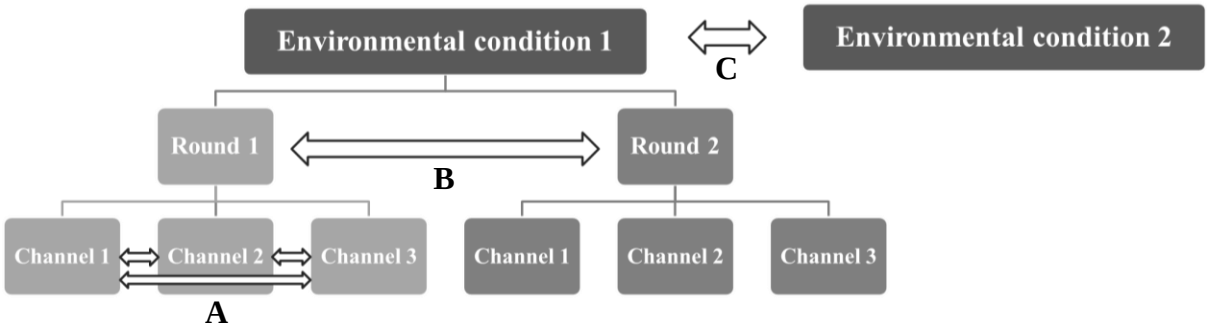

Figure 5.2. Schematic representation of CLSM experiments and the statistical analysis of data. Variance between biofilm parameters was determined between biofilms per round (A), between experimental rounds (B) and between environmental conditions (C)**.**

5.3. Results

5.3.1. Biochemistry of *Pseudomonas* and multi-species biofilms

ATR-FTIR spectra of PCT07 *gfp*, PA01 *gfp* and multi-species biofilms cultivated in TSB medium are presented in Figures 5.3–5.5 (spectra for intact biofilm samples as well as pellet and supernatant fractions are shown separately). Spectra of PCT07 *gfp* and PA01 *gfp* biofilms cultivated in TSB medium or defined nutrient medium with citrate are compared in Figure 5.6. Band assignments of ATR-FTIR spectra are summarised in Table 5.3.

When comparing the spectra of the intact *Pseudomonas* and multi-species biofilms cultivated in TSB medium, peaks occurred at similar wavenumbers. The only noticeable differences were variations in peak intensity. The common peaks may indicate the presence of alcohols (1070, 1220, 1380 and 3260 cm^{-1}), amides (1220, 1530, 1640 and 3190 cm^{-1}), carboxylic acids (1400 and 1420 cm^{-1}) and aldehydes (2620 cm^{-1}). The broad peaks between 2800 and 3400 cm^{-1} are typically associated with C-H stretching found in aliphatic compounds and intermolecular bonds involving O-H stretching (Figure 5.7).

The spectra of intact biofilms and pellet fractions for both *Pseudomonas* biofilms and the multi-species biofilms were almost identical. The spectra of intact biofilms and pellet fractions showed similar peak positions, shapes and absorbance intensities. However, the spectra of the supernatant fractions showed shifts in certain peak positions. For example, a weak peak was recorded at 1220 cm^{-1} for the PA01 *gfp* and PCT07 *gfp* supernatant fractions (compared to medium peaks at the same position on the spectra of the intact biofilms and pellet fractions). A strong peak was detected at 1400 cm^{-1} in the supernatant spectra, while two weak peaks occurred between 1370 and 1440 cm^{-1} in the spectra of the intact biofilms and pellet fractions.

Changes in nutrient conditions affected the biochemical composition of *Pseudomonas* biofilms (Figure 5.6). The spectra of PCT07 *gfp* biofilms cultivated in TSB medium and modified defined AB medium with citrate were almost identical, but there were noticeable changes in the spectra of PA01 *gfp* biofilms cultivated in the two types of media. The spectrum of a PA01 *gfp* biofilm cultivated

in defined nutrient medium showed a decrease in peak intensity at 1050 cm^{-1}, an increase in peak intensity at 1380 cm^{-1}, a shift at position 1560 cm^{-1} and no peak at 1630 cm^{-1}. The defined peaks between 2800 and 3400 cm^{-1} merged into a single, broad peak at 3290 cm^{-1}, possibly indicating less of a carbon backbone in this biofilm (compared to its counterpart cultivated in TSB medium).

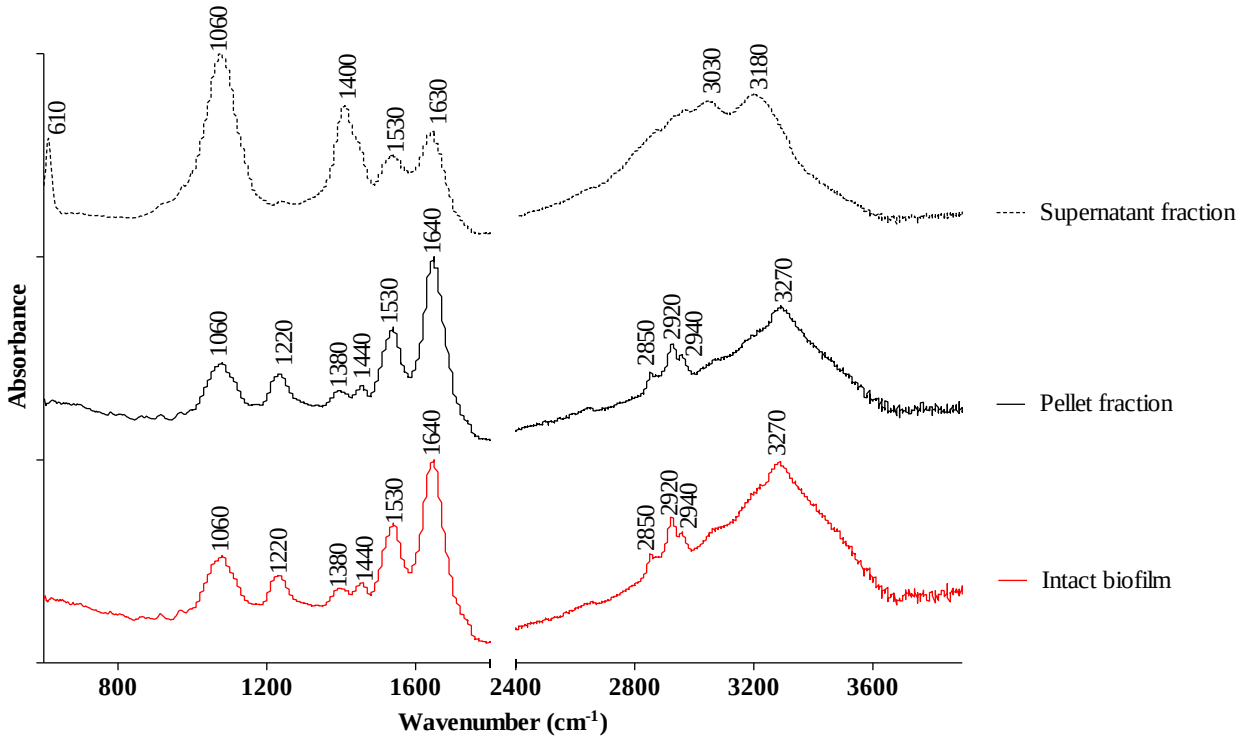

Figure 5.3. Normalised ATR-FTIR spectra of lyophilised PA01 *gfp* biofilm samples cultivated in a complex nutrient medium. Biofilms were cultivated in continuous, once-through flow systems with silicone tube bioreactors for 96 hours in 3 $g.L^{-1}$ TSB.

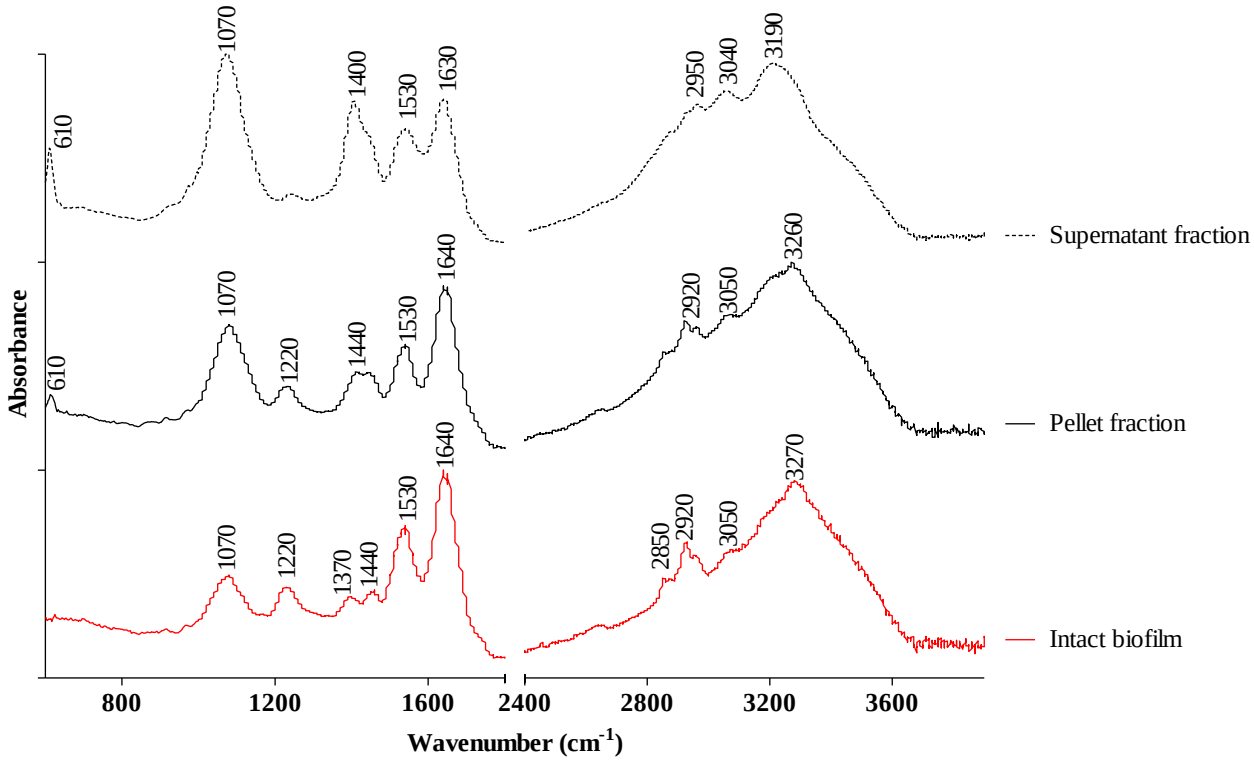

Figure 5.4. Normalised ATR-FTIR spectra of lyophilised PCT07 *gfp* biofilm samples cultivated in a complex nutrient medium. Biofilms were cultivated in continuous, once-through flow systems with silicone tube bioreactors for 96 hours in 3 g.L^{-1} TSB.

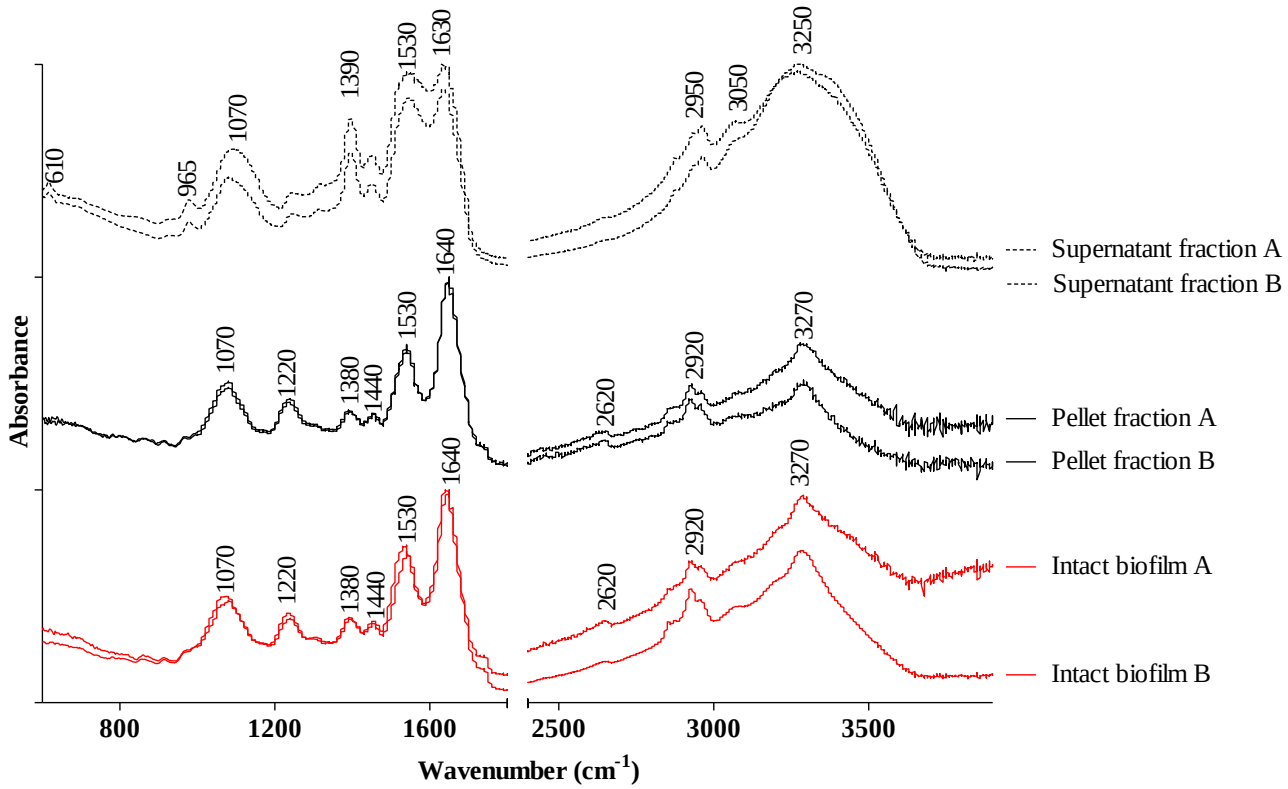

Figure 5.5. Normalised ATR-FTIR spectra of lyophilised multi-species biofilm samples cultivated in duplicate in a complex nutrient medium. Biofilms were cultivated in continuous, once-through flow systems with silicone tube bioreactors for 96 hours in 3 g.L^{-1} TSB.

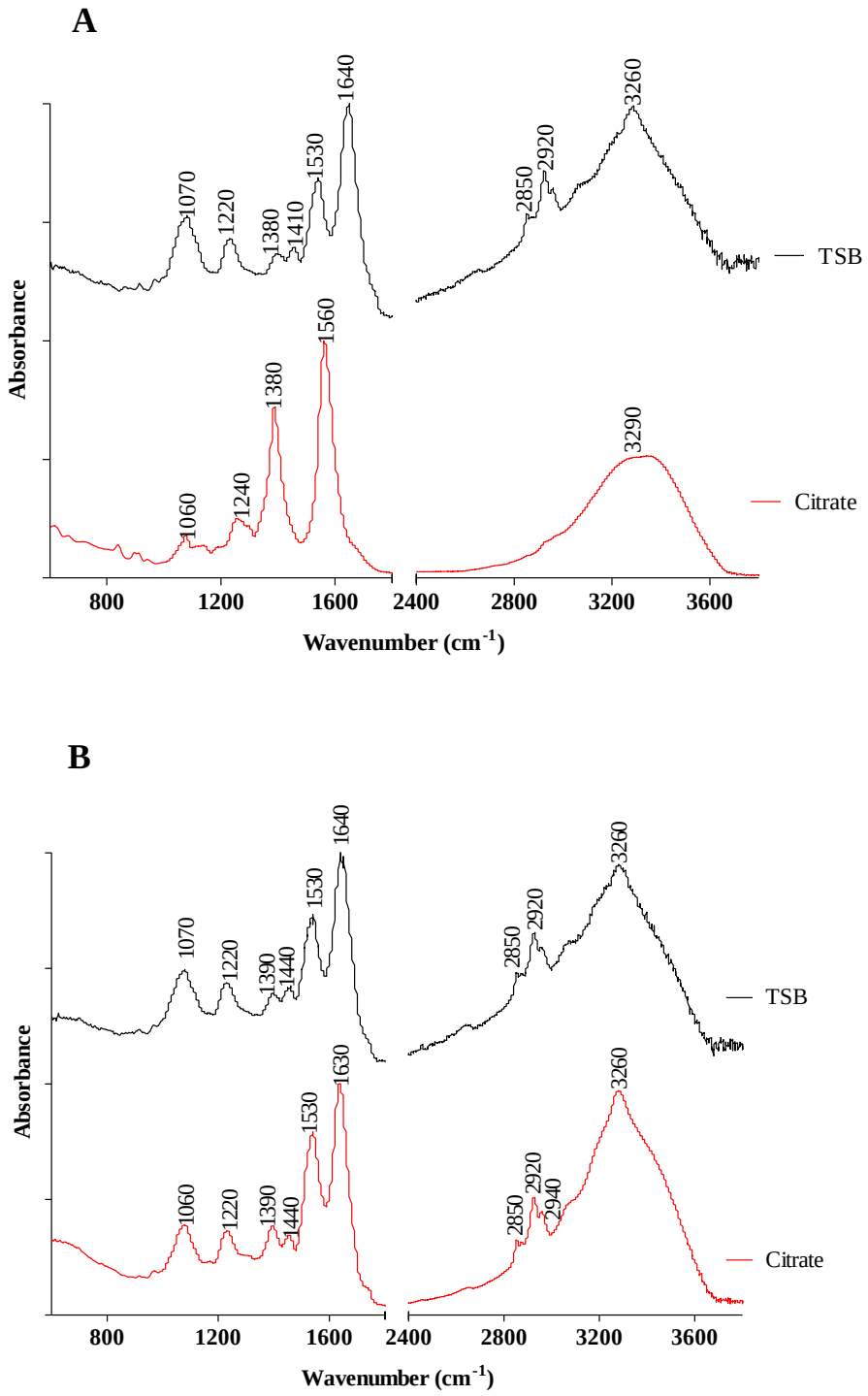

Figure 5.6. Normalised ATR-FTIR spectra of lyophilised *Pseudomonas* biofilms cultivated in complex or defined nutrient medium. Biofilms were cultivated in continuous, once-through flow systems with silicone tube bioreactors for 96 hours in either 3g.L^{-1} TSB medium or modified defined AB medium with 10 mM citrate. **A.** PA01 *gfp* biofilms. **B.** PCT07 *gfp* biofilms.

Table 5.3. IR absorption bands of *Pseudomonas* and multi-species biofilms relating to functional groups. Range and assignments compiled from Coates 2000; Ojeda, Romero-Gonzalez, Pouran and Banwart 2008).

Wavenumber (cm^{-1})	Range (cm^{-1}) and intensity [a]	Assignment	Group and class
1070	1015–1200 (vs)	C-O stretch	C-OH in alcohols
	1025–1060 (vs)	C-O stretch	CH_2-OH in primary alcohols
	1080–1120 (s)	C-O stretch	C-OH in secondary or tertiary alcohols
1220	1100–1230 (s)	C-C-N bending	C-C-N in amides
	1180–1280 (s)	C-N stretch	C-N in aromatic amides
	1250–1350	P=O stretch	Organic phosphates
	1260–1350	O-H in-plane bend	Primary or secondary alcohol
1380	1310–1410	O-H bend	Phenol or tertiary alcohol
1400, 1420	1400–1440 (m)	O-H in-plane bend	OH in carboxylic acids
1530	1500–1650	N-H bend, C-N stretch	Amide II
1640	1580–1640 (s)	NH_3 deformation	NH_3^+ in amino acids, Amide I
2620	2650–2750 (w-m)	C-H bend	-CHO in aldehydes
2850	2700–2850 (m)	C-H stretch	$-CH_3$ attached to O or N
2920, 2940	2850–2990 (m-s)	C-H antisym and sym stretching	$-CH_3$ and $-CH_2$ in aliphatic compounds
3190	3180–3200 (s)	NH_2 sym stretch	$-NH_2$ in primary amides
3260	3250–3420 (s)	OH stretch	-OH in alcohols and phenols

[a] v = very, s = strong, m = medium, w = weak

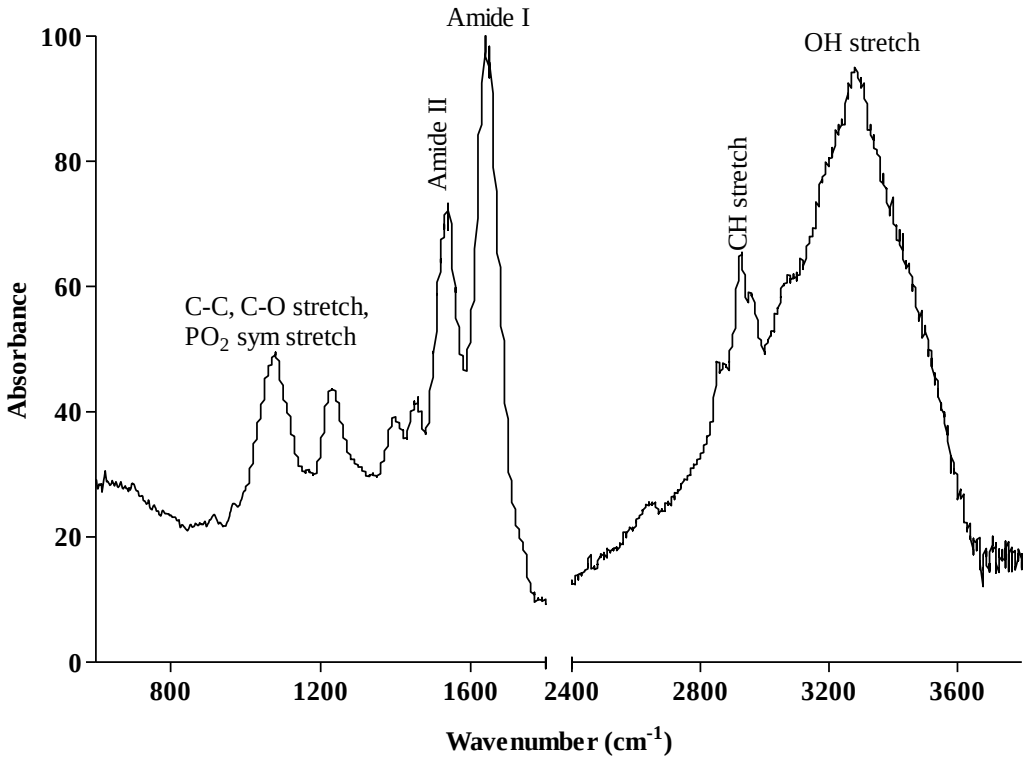

Figure 5.7. General band assignment of FTIR spectra. Adapted from Delille, Quilès and Humbert 2007.

5.3.2. Presence and distribution of proteins and glycoconjugates in *Pseudomonas* biofilms

The spatial distribution of extracellular proteins and glycoconjugates within *Pseudomonas* biofilms was investigated using two fluorescent stains. SYPRO® Ruby stain is a permanent stain that interacts non-covalently with most classes of proteins and has been used to label glycoproteins, lipoproteins and phosphoproteins in bacterial biofilms (Berggren, Chernokalskaya, Steinberg, Kemper, Lopez et al. 2000). ConA-594 is a fluoro-conjugated lectin that selectively binds to α-mannopyranosyl and α-glucopyranosyl residues and it is used for the detection of glycoconjugates[13].

Figure 5.8 shows protein-specific staining of PCT07 *gfp* and PA01 *gfp* biofilms with SYPRO® Ruby stain. The stain was located within microcolonies of both *Pseudomonas* biofilms. The bound stain was localised to cell surfaces in PCT07 *gfp* biofilms, while more diffuse, cloud-like staining patterns surrounded microcolonies in PA01 *gfp* biofilms.

No clear images were obtained for PCT07 *gfp* biofilms stained with ConA-594 stain. This could indicate low levels or the absence of glycoconjugates in these biofilms. In PA01 *gfp* biofilms, ConA-594 was mainly bound to the outer layers of the microcolonies (Figure 5.9). At a higher magnification, it is evident that the stain was localised between bacteria in microcolonies and not necessarily bound to cell surfaces.

[13] https://www.thermofisher.com/order/catalog/product/C11253, accessed 19 April 2016.

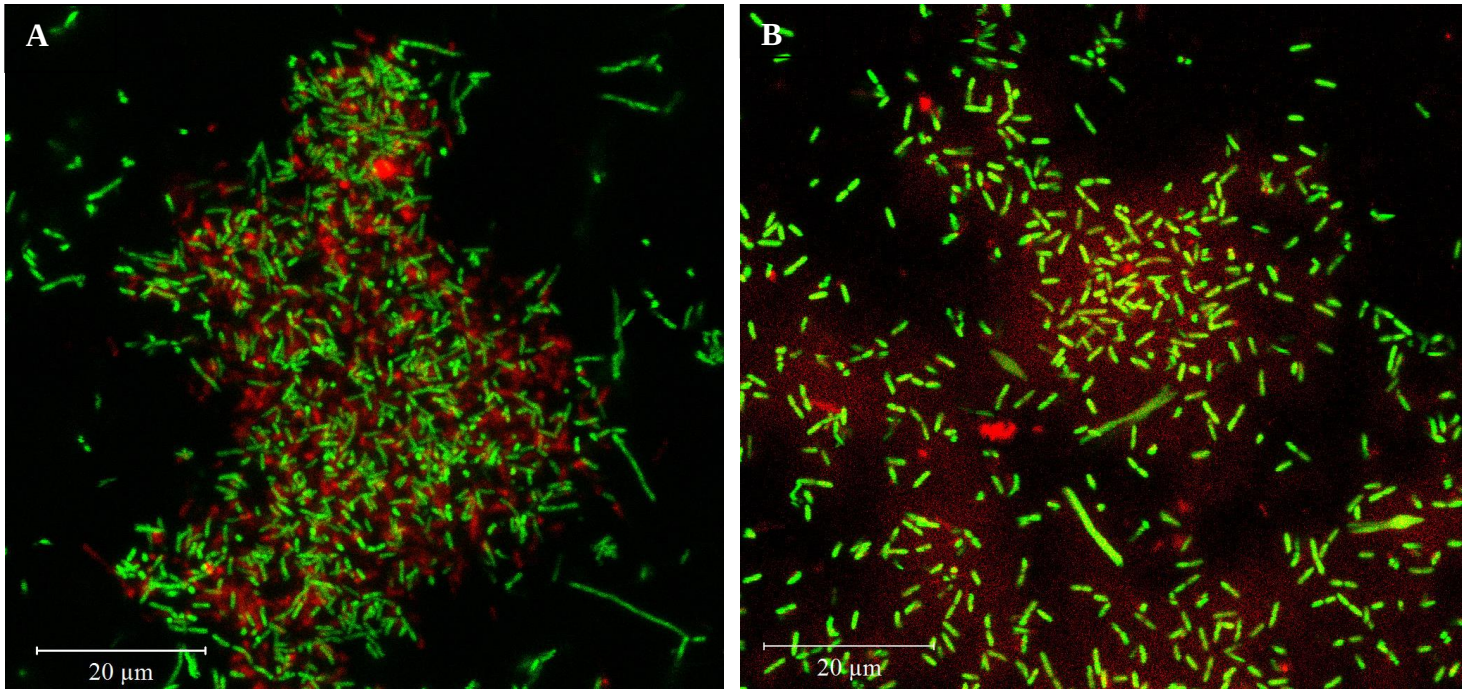

Figure 5.8. Protein-specific staining of *gfp*-labelled *Pseudomonas* biofilms. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 1 mM citrate at a flow rate of 13 $ml.h^{-1}$. CLSM images of *Pseudomonas* (green) with SYPRO® Ruby (red) captured using a 100x objective and are presented as maximum intensity projections. **A.** PCT07 *gfp*. **B.** PA01 *gfp*.

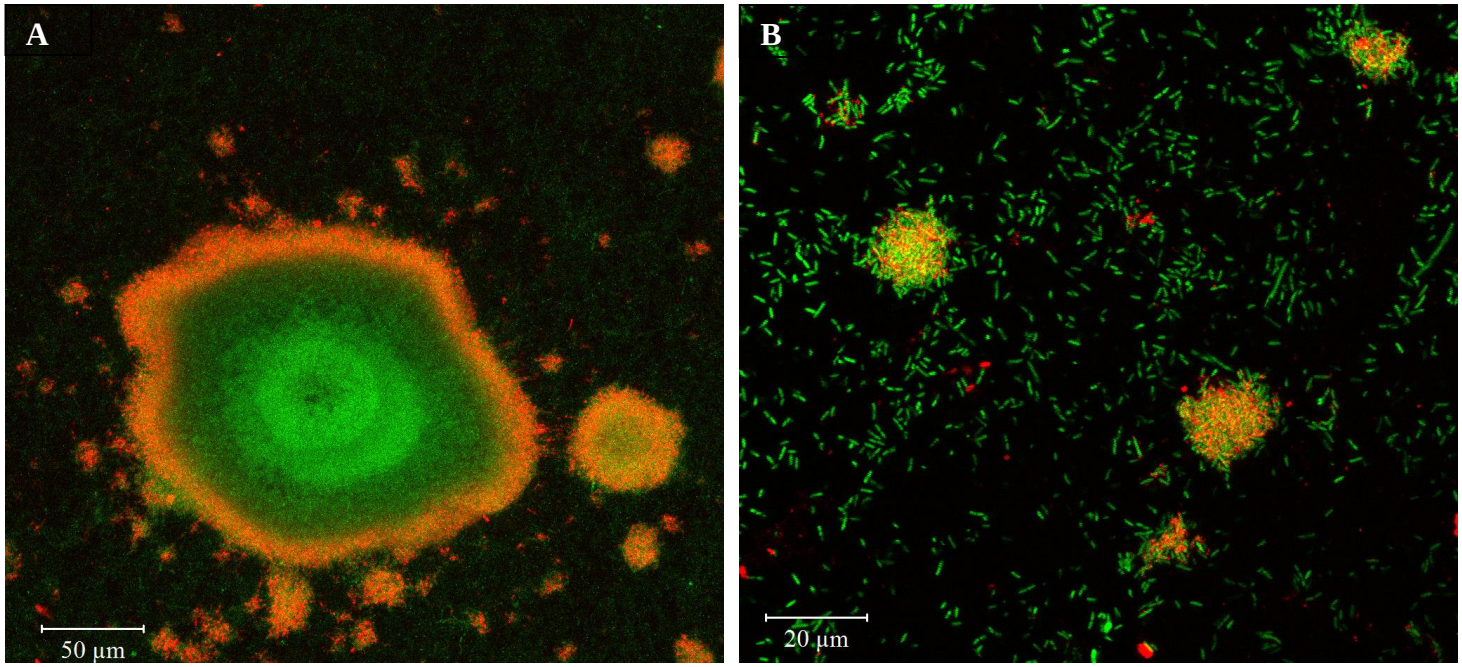

Figure 5.9. Glycoconjugate-specific staining of a PA01 *gfp* biofilm. Biofilms were cultivated in continuous, once-through flow systems with flow cell bioreactors for 96 hours in modified defined AB medium with 1 mM citrate at a flow rate of 13 $ml.h^{-1}$. CLSM images of PA01 *gfp* (green) with ConA-594 (red) are presented as maximum intensity projections. **A.** Captured using a 40x objective. **B.** Captured using a 100x objective.

5.3.3. Influence of environmental conditions on *Pseudomonas* biofilm structure

The effect of nutrient concentration and flow rate on biofilm development was investigated by monitoring differences in PCT07 *gfp* and PA01 *gfp* biofilms cultivated in four environmental conditions (Table 5.1). Distinct biofilm features for PCT07 *gfp* and PA01 *gfp* biofilms are visible in Figures 5.10 and 5.11 and the typical growth patterns observed for these biofilms are described in Tables 5.3 and 5.4. Four biofilm parameters were quantified by means of DIA of CLSM images. The calculated parameter values for PCT07 *gfp* and PA01 *gfp* biofilms are presented as scatterplots in Figures 5.12 and 5.13, respectively. The average values with standard deviation are summarised in Table 5.6. Variance between triplicate biofilms per round and between the two experimental rounds is summarised in Table 5.7.

In certain instances the typical growth patterns seemed to contradict the measured biofilm parameter values. For example, through visual observation the biofilms in Figures 5.11.C and 5.11.D appeared to cover more of the substratum than those in Figures 5.11.A and 5.11.B, although the calculated surface area coverage indicated the opposite. Of these contradictions can be explained by the variation in biofilm development throughout flow cell channels. The CLSM images were captured at random points and did not always include areas of "typical" growth. The scatterplots (Figures 5.12 and 5.13) and statistical analysis (Table 5.7) offered insight into these variations and help explain the apparent inconsistencies between visual observations and calculated values.

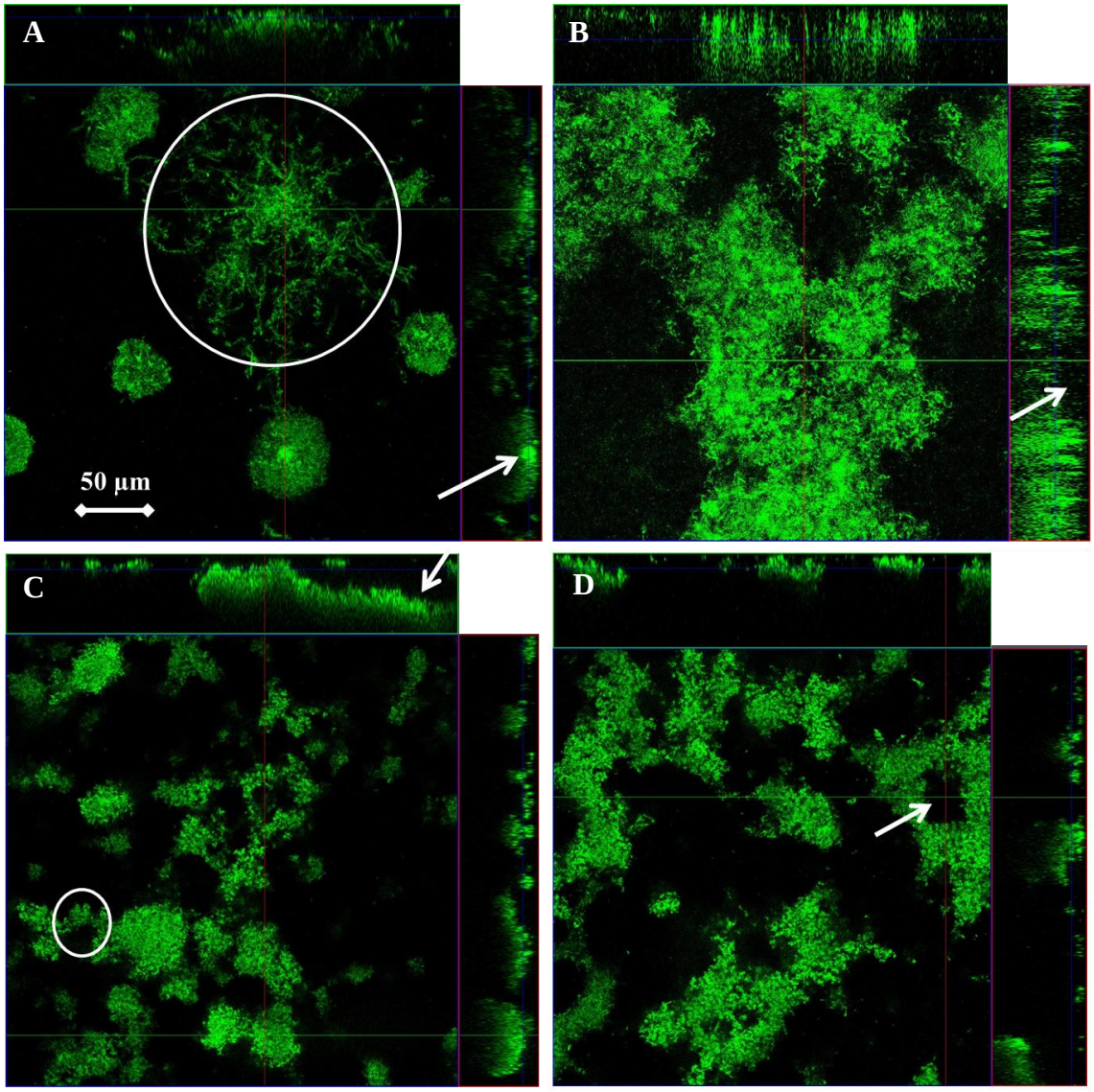

Figure 5.10. PCT07 *gfp* biofilms cultivated at different nutrient concentrations and flow rates. Biofilms were cultivated for 96 hours in modified defined AB medium with 1 mM citrate (**A.** flow rate 13 ml.h^{-1}, **B.** flow rate 30 ml.h^{-1}) or 10 mM citrate (**C.** flow rate 13 ml.h^{-1}, **D.** flow rate 30 ml.h^{-1}). Three-dimensional CLSM images in ortho-view were acquired using a 40x objective.

Table 5.4. Typical growth patterns and common characteristics observed for PCT07 *gfp* biofilms using CLSM. Biofilms were cultivated in combinations of high/low nutrient concentrations and high/low flow rates.

	Low flow rate (13 ml.h^{-1})	**High flow rate (30 ml.h^{-1})**
Low nutrient concentration (1 mM)	***Figure 5.10.A*** ▪ Small, isolated microcolonies and individual cells visible on substratum. ▪ Higher cell densities in the centre of microcolonies (indicated by arrow). ▪ Few visible connections between colonies, but bridge-like connections appear between some colonies; these connections are not always attached to the substratum. ▪ Areas with more undefined, filamentous growth appear (indicated by circle).	***Figure 5.10.B*** ▪ Small, isolated microcolonies with more individual cells visible on substratum (compared to Figure 5.10.A). ▪ Large, asymmetrical microcolonies with patchy connections between some of the larger colonies. ▪ Large patches of filamentous growth visible. ▪ Large bulges of biomass with hollow spots also occur (indicated by arrow).
High nutrient concentration (10 mM)	***Figure 5.10.C*** ▪ Bulky, "fluffy" biofilms with mushroom-shaped microcolonies. ▪ Microcolonies vary in size, shape and cell density. ▪ Small microcolonies on substratum located close to each other, often connected by bridge-like structures made up of cells (indicated in circle). ▪ "Bridges" between microcolonies not always connected to the substratum (indicated by arrow).	***Figure 5.10.D*** ▪ Similar pattern of microcolony distribution on substratum with more uniform microcolonies (compared to Figure 5.10.C). ▪ Mostly dense microcolonies with some patchy colonies visible at times. ▪ Many bridge-like connections between microcolonies which create channels within the biofilm. ▪ Voids in the bigger biomass structures (indicated by arrow).

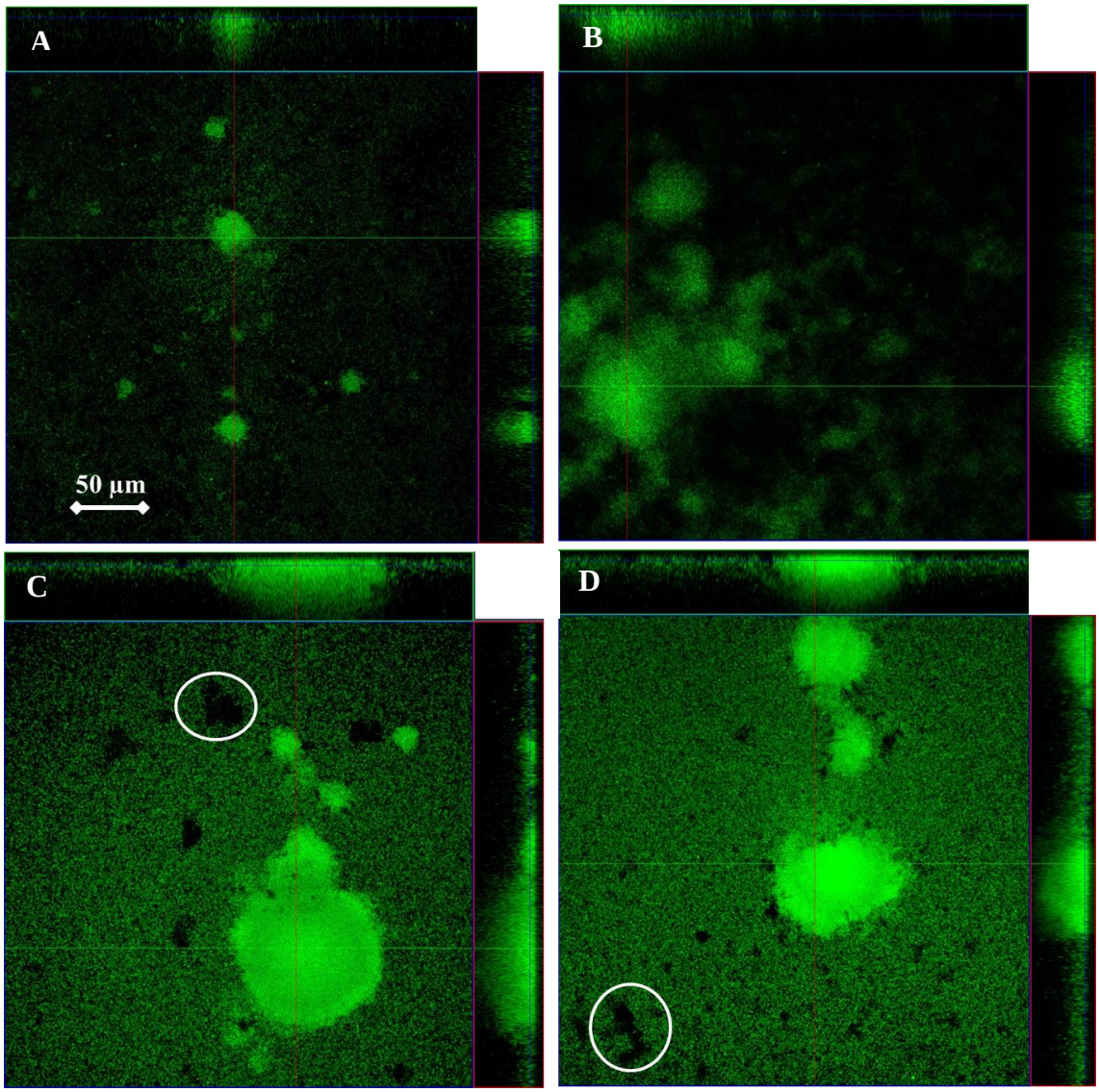

Figure 5.11. PA01 *gfp* biofilms cultivated at different nutrient concentrations and flow rates. Biofilms were cultivated for 96 hours in modified defined AB medium with 1 mM citrate (**A.** flow rate 13 ml.h^{-1}, **B.** flow rate 30 ml.h^{-1}) or 10 mM citrate (**C.** flow rate 13 ml.h^{-1}, **D.** flow rate 30 ml.h^{-1}). Three-dimensional CLSM images in ortho-view were acquired using a 40x objective.

Table 5.5. Typical growth patterns and common characteristics observed for PA01 *gfp* biofilms using CLSM. Biofilms were cultivated in combinations of high/low nutrient concentrations and high/low flow rates.

	Low flow rate (13 ml.h^{-1})	**High flow rate (30 ml.h^{-1})**
Low nutrient concentration (1 mM)	***Figure 5.11.A*** ▪ Areas of mat-like growth with undefined edges, marked with patches of denser growth without defined microcolonies. ▪ Small microcolonies spread out throughout channel. ▪ Voids visible in mat and stretch from substratum throughout biofilm (not visible in this example).	***Figure 5.11.B*** ▪ Defined microcolony structures visible, often grouped close together. ▪ Microcolonies not necessarily uniform in shape but have clearly defined edges. ▪ Microcolonies surrounded by mat-like growth. ▪ Mat-like growth haphazardly throughout channels, very thin surface coverage at times.
High nutrient concentration (10 mM)	***Figure 5.11.C*** ▪ Highly variable growth throughout channels: small patches of microcolonies interspersed with areas of single cells attached to surface. ▪ Areas with mat-like growth containing microcolonies appear denser than surrounding mat. ▪ Voids visible in mat-like growth, often at substratum level, but covered by cells further from substratum (indicated by circle).	***Figure 5.11.D*** ▪ Single microcolonies within mat-like growth; microcolonies sometimes surrounded by clear zone. ▪ Defined microcolony structures significantly thicker that mat-like growth (similar to Figure 5.11.C). ▪ Sparse mat-like growth; spaces visible between cells. ▪ Voids appeared in mat-like growth, similar to Figure 5.11.C, often covered by cells further from substratum (indicated by circle). ▪ Edges of mat-like areas were patchy with less defined microcolonies.

5.3.3.1. Effects of nutrient concentration on biofilm structure

Changes in nutrient concentration produced noticeable differences in the three-dimensional biofilm structures of both *Pseudomonas* strains. Nutrient concentration affected microcolony morphology, patterns of surface colonisation and the frequency of physical connections between microcolonies.

PCT07 *gfp* biofilms

At low nutrient concentrations (1 mM citrate) and low flow rates (13 $ml.h^{-1}$) defined and separate microcolonies, interspersed with filamentous growth, were visible at low nutrient concentrations (Figure 5.10.A). These growth patterns were noticeably different to the irregular-shaped microcolonies with many bridge-like connections at higher nutrient concentrations (Figure 5.10.C). PCT07 *gfp* biofilms cultivated at low nutrient concentrations and low flow rates were slightly thinner (3.2 ± 1.2 µm), contained less biomass and covered less of the substratum (10.8 ± 3.4%) than PCT07 *gfp* biofilms cultivated at the same flow rate and high nutrient concentrations (Figure 5.10.C, Table 5.6).

There were more pronounced differences in biofilm structures between high and low nutrient concentrations at higher flow rates (30 $ml.h^{-1}$). At low nutrient concentrations (1 mM citrate), PCT07 *gfp* biofilms were significantly thicker (16.5 ± 13.1 µm) with less compact structures (Figure 5.10.B) than their counterparts cultivated in 10 mM citrate (Figure 5.10.D).

Figure 5.12 shows that parameters measured at low nutrient concentrations and high flow rates varied more than those measured at high nutrient concentrations and high flow rates, although this variance was only statistically significant for the calculated surface:volume ratios (Table 5.7).

PA01 *gfp* biofilms

At low flow rates (13 ml.h^{-1}), PA01 *gfp* biofilms exhibited the reverse trend seen for PCT07 *gfp* biofilms at the same flow rate. PA01 *gfp* biofilms cultivated at low nutrient concentrations (Figure 5.11.A) produced thicker biofilms (20.9 ± 7.1 µm), contained more biomass and covered a larger surface area (59.5 ± 17.9%) than their counterparts cultivated at high nutrient concentrations (Figure 5.11.C, Table 5.6).

The same was observed for PA01 *gfp* biofilms cultivated at higher flow rates (30 ml.h^{-1}). At this flow rate, PA01 *gfp* biofilms cultivated in low nutrient concentrations (Figure 5.11.B) were thicker (8.6 ± 4.6 µm) and covered more of the substratum surface (36.2 ± 16.8%) than their counterparts cultivated at higher nutrient concentrations (Figure 5.11.D). Again, more variation was recorded for measurements at low nutrient concentrations and, at high flow rates, there was significant variation in surface area coverage and average thickness between biofilms per round (Table 5.7).

5.3.3.2. Effects of flow rate on biofilm structure

Flow rate appeared to have a greater effect on biofilm parameters of both *Pseudomonas* strains at low nutrient concentrations than at higher nutrient concentrations.

PCT07 *gfp* biofilms

Changes in PCT07 *gfp* biofilm structures under different flow rate conditions were clearly visible (Figures 5.10.A and 5.10.B, Table 5.4). At low nutrient concentrations, PCT07 *gfp* biofilms cultivated at low flow rates contained significantly less biomass and covered less of the substratum than their counterparts cultivated at high flow rates. More variability was recorded for measured parameters at low nutrient concentrations, especially at higher flow rates (Figure 5.12).

Conversely, at high nutrient concentrations only small changes in PCT07 *gfp* biofilm structures were observed. Biofilms cultivated in higher flow rates were more compact with more channels visible within the biomass, compared to the mushroom-shaped microcolonies with floating bridge-like

connections at lower flow rates (Figures 5.10.C and 5.10.D). While average biofilm thickness and bio-volume did not change significantly between low and high nutrient flow rate conditions at 10 mM citrate, there was a slight increase in surface area coverage under higher flow rates at high nutrient concentrations.

PA01 *gfp* biofilms

Similar to PCT07 *gfp* biofilms, significant changes were detected between biofilms cultivated in low and high flow rates at low nutrient conditions. At low flow rates (Figure 5.11.A), PA01 *gfp* produced thicker biofilms (20.9 ± 7.1 µm) with higher surface area coverage than its counterparts cultivated at higher flow rates (Figure 5.11.B). More variability in measurements was recorded for parameters at low nutrient concentrations (Figure 5.13), although the variation was only significant for average thickness and surface area coverage (Table 5.7).

Very few changes in biofilm growth patterns were visible between flow rate conditions at high nutrient concentrations and no significant differences were recorded for any of the parameters when comparing PA01 *gfp* biofilms cultivated at low and high flow rates in 10 mM citrate.

Table 5.6. *Pseudomonas* biofilm parameters quantified by means of DIA of CLSM images. Biofilms were cultivated in combinations of high/low nutrient concentrations ([C]) and high/low flow rates (F). Average values with standard deviation were calculated from 42 measurements.

Parameter	Biofilm	Low [C] Low F	Low [C] High F	High [C] Low F	High [C] High F
Surface area coverage (%)	PCT07 *gfp*	10.8 ± 3.4	17.3 ± 9.6	19.1 ± 4.6	24.4 ± 6.9
	PA01 *gfp*	59.5 ± 17.9	36.2 ± 16.8	13.9 ± 11.3	23.0 ± 12.5
Bio-volume ($\mu m^3.\mu m^{-2}$)	PCT07 *gfp*	1.2 ± 0.3	5.4 ± 5.0	2.8 ± 1.1	2.9 ± 0.9
	PA01 *gfp*	7.9 ± 4.1	3.6 ± 2.1	1.0 ± 1.2	1.9 ± 1.5
Av. thickness (µm)	PCT07 *gfp*	3.2 ± 1.2	16.5 ± 13.1	5.7 ± 2.7	5.0 ± 1.9
	PA01 *gfp*	20.9 ± 7.1	8.6 ± 4.6	1.8 ± 2.4	3.0 ± 2.4
Surface:volume ($\mu m^2.\mu m^{-3}$)	PCT07 *gfp*	10.8 ± 3.4	6.7 ± 1.6	4.0 ± 1.3	4.2 ± 0.7
	PA01 *gfp*	7.9 ± 1.2	7.8 ± 1.7	5.1 ± 1.2	5.1 ± 1.3

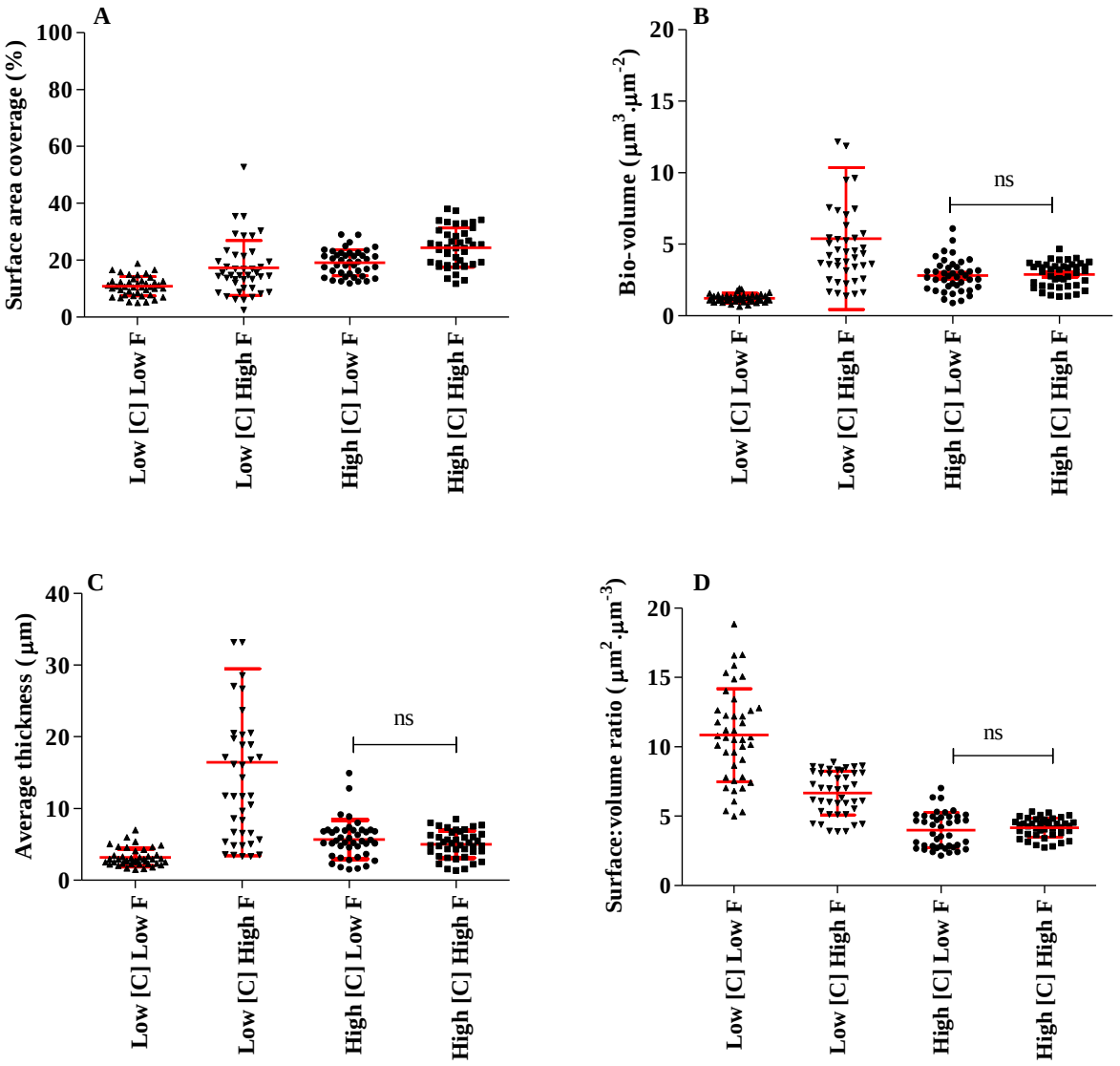

Figure 5.12. Biofilm parameters measured for PCT07 *gfp* biofilms cultivated in four environmental conditions. A. Surface area coverage. **B.** Bio-volume. **C.** Average thickness. **D.** Surface:volume ratio. Biofilms were cultivated in combinations of high/low nutrient concentrations (indicated as [C]) and high/low flow rates (indicated as F). Values represent 7 measurements of triplicate biofilms in two independent experimental rounds. Mean with standard deviation indicated (ns = difference not significant, determined by Tukey's multiple comparison test).

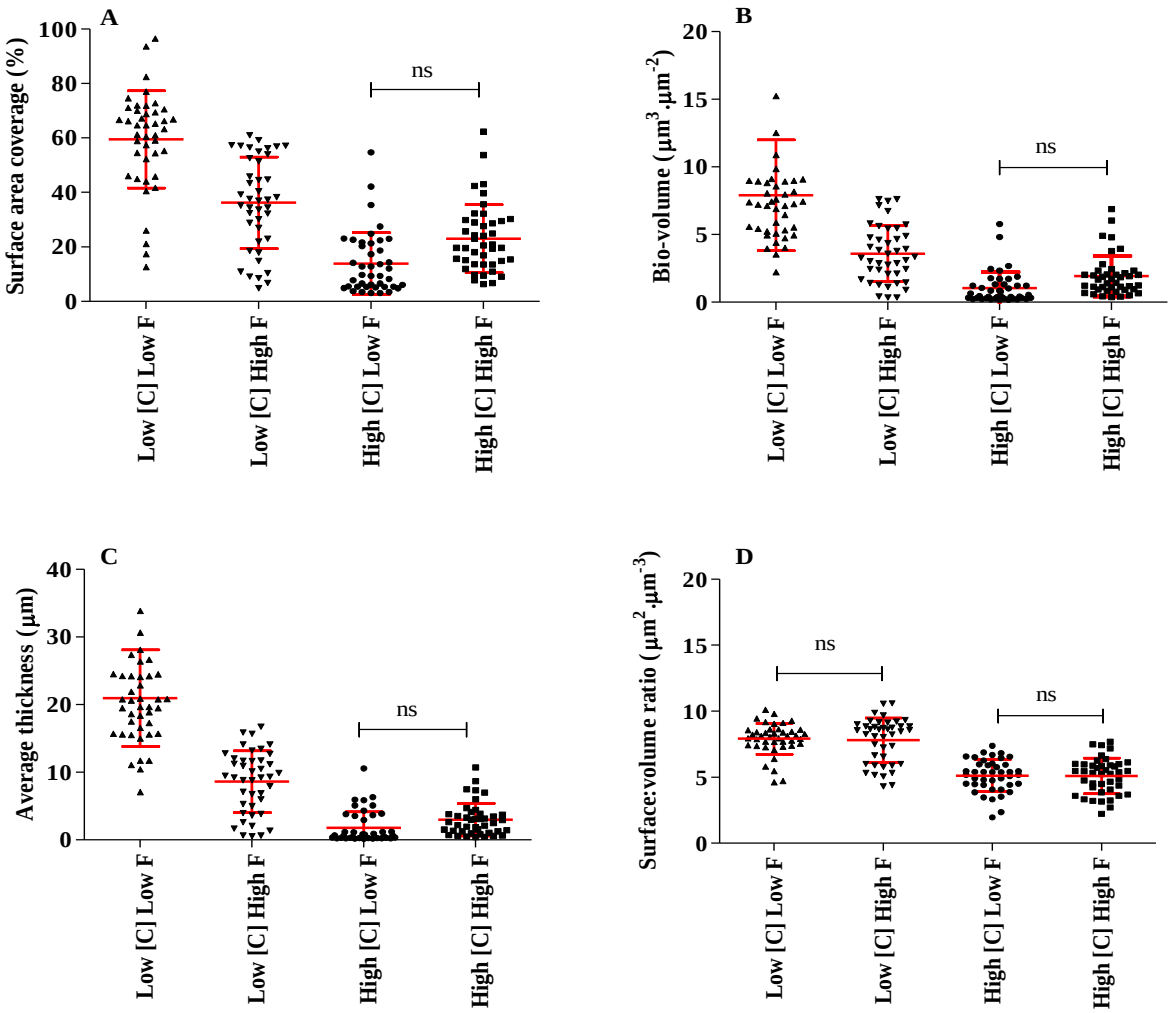

Figure 5.13. Biofilm parameters measured for PA01 *gfp* biofilms cultivated in four environmental conditions. A. Surface area coverage. **B.** Bio-volume. **C.** Average thickness. **D.** Surface:volume ratio. Biofilms were cultivated in combinations of high/low nutrient concentrations (indicated as [C]) and high/low flow rates (indicated as F). Values represent 7 measurements of triplicate biofilms in two independent experimental rounds. Mean with standard deviation indicated (ns = difference not significant, determined by Tukey's multiple comparison test).

Table 5.7. Significant differences recorded for parameters of *Pseudomonas* biofilms cultivated in four different environmental conditions. Biofilms were cultivated in combinations of high/low nutrient concentrations (C) and high/low flow rates (F). A and B denote experimental rounds. Significant differences are indicated by * (p = 0.05).

		Surface area coverage			Bio-volume			Average thickness			Surface:volume ratio		
Strain	**Condition**	**A**	**B**	**A vs. B**	**A**	**B**	**A vs. B**	**A**	**B**	**A vs. B**	**A**	**B**	**A vs. B**
PCT07 *gfp*	Low [C] low F	*	*	*							*	*	*
	Low [C] high F	*									*	*	*
	High [C] low F		*	*	*	*	*					*	*
	High [C] high F		*	*				*	*	*			*
PA01 *gfp*	Low [C] low F		*										
	Low [C] high F	*	*		*			*	*	*			*
	High [C] low F			*									*
	High [C] high F												*

5.4. Discussion

5.4.1. Life-friendly chemistry

It is widely accepted that, within a biofilm, microorganisms are able to produce a wide range of complex biomolecules in the form of EPS (Staudt et al. 2003). From a biomimetic perspective, this is achieved by combining a small subset of elements (mostly C, H, O, N) in an aqueous environment to give rise to hydrated three-dimensional biochemical scaffolds. Based on this life-friendly chemistry, biofilms can be viewed as biodegradable surface coatings.

Using ATR-FTIR spectroscopy, functional chemical groups were identified within biofilms, indicating the presence of a diverse range of macromolecules within the biofilms (Figure 5.7). The presence of polysaccharides was supported by peaks between 1000–1300 cm^{-1} and 3200–3400 cm^{-1}, which corresponded with O-H stretching and C-H stretching. Peaks between 1000 and 1300 cm^{-1} could indicate the presence of nucleic acids and phospholipids. Amide peaks (1500–1700 cm^{-1}) in the IR spectra most likely indicated the presence of proteins in the biofilms. Colorimetric assays (Pierce™ BCA Protein assay kit) confirmed the presence of proteins in lyophilised intact biofilm samples cultivated in TSB medium (data not shown).

The spectra of intact *Pseudomonas* biofilms were comparable to spectra of *P. putida* biofilms (Ojeda et al. 2008) and *P. fluorescens* biofilms (Quilès, Humbert and Delille 2010), despite differences in biofilm cultivation and sample preparation techniques. These two studies both used online FTIR methods to investigate biofilm development in real time and showed that changes in biochemical profiles can be seen between planktonic cultures, early stages of biofilm development and mature biofilms. Within the limitations of the experimental system used in this study, the ATR-FTIR spectra offered simplistic biochemical snapshots of the biofilms examined. With more advanced equipment, FTIR and Raman spectroscopy could be used to further investigate the adaptability of biofilms on a biochemical level.

It is important to point out that spectra presented here were of complex samples containing whole organisms and therefore intracellular biomolecules and structural cellular compounds contributed to the spectra. Although differences were observed between the spectra of intact biofilm samples and supernatant fractions (which presumably contain loosely bound EPS and less cellular material), additional assays would be necessary to distinguish EPS from cellular components. The separation of cellular components from EPS is problematic and perhaps even impossible, considering that cell death and subsequent lysis are normal events in biofilm life cycles. EPS extraction and purification fall outside the scope of this study. For the purpose of this study, it was sufficient to note that there are differences between the spectra of intact biofilms and centrifuged samples.

Fluorescent stains and CLSM confirmed the presence of EPS in *gfp*-labelled *Pseudomonas* biofilms. A protein-specific stain successfully bound to microcolonies of both PCT07 *gfp* and PA01 *gfp* biofilms (Figure 5.8). In PCT07 *gfp* biofilms cell-associated binding patterns were observed. It is possible that the stain bound to cell surface proteins and not necessarily proteins secreted as part of an EPS matrix. In PA01 *gfp* biofilms, the binding pattern of the protein-specific stain appeared more cloud-like, surrounding the microcolonies, and could therefore indicate the presence of extracellular proteins in the biofilm.

P. aeruginosa is known to produce a range of extracellular polysaccharides including alginate, an anionic polysaccharide composed of non-repetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids (Franklin et al. 2011). Fluorescently labelled ConA-594, a glycoconjugate-specific lectin, has been used to detect alginate and other polysaccharides produced by *P. aeruginosa* (Strathmann et al. 2002). As expected, glycoconjugates were detected in PA01 *gfp* biofilms using ConA-594 (Figure 5.9). Glycoconjugates appeared to be concentrated within microcolonies and very little stain was detected in areas where biofilms form thin, mat-like structures. More specifically, glycoconjugates appeared to be localised on the outskirts of large microcolonies, similar to other reports (Bagge, Schuster, Hentzer, Ciofu, Givskov et al. 2004). Although the cellular density of the microcolony may have limited the diffusion of the stain, it is highly unlikely that the ConA-594 stain would have been completely prevented from reaching the interior of microcolonies, given the hydrated, porous nature of the EPS matrix. It is therefore likely that glycoconjugates accumulated in the outer layers of PA01 *gfp* microcolonies.

Glycoconjugate production by PA01 *gfp* biofilms cultivated in modified defined medium (with citrate as sole carbon source) highlights the life-friendly chemistry of biofilms. In this case, the microorganisms only had access to C, H, O, N, Ca, Mg, Cl and trace amounts of Fe. These biochemical building blocks were subsequently assembled within an aqueous environment to produce complex macromolecules. The ability of *Pseudomonas* strains to form biofilms within different nutrient environments also points to the adaptability of biochemical processes within biofilms. Both strains were able to produce biofilms when provided with a complex, nutrient-rich medium (TSB), as well as when provided with a defined medium with a single carbon source (Figure 5.6).

5.4.2. Resource efficiency

On a structural level, biofilms are spatially organised systems that create beneficial micro-environments, which can be altered by the microorganisms in response to environmental changes. By comparing structures and measured parameters of two *Pseudomonas* strains cultivated in four different environmental conditions, it is clear that the three-dimensional form of a biofilm is able to fit its function in response to nutrient availability and hydrodynamic conditions.

Both *Pseudomonas* strains developed more variable biofilm structures at low nutrient conditions and, at these nutrient conditions, significant changes were observed between low and high flow rates for the four measured parameters (surface area coverage, bio-volume, average thickness and surface:volume ratio). At high nutrient concentrations (10 mM citrate), changes in flow rate had no significant effects on parameters measured for PCT07 *gfp* or PA01 *gfp* biofilms (Figures 5.12 and 5.13).

Although the “high” flow rate (30 $ml.h^{-1}$, Re = 1.85) was double the “low” flow rate (13 $ml.h^{-1}$, Re = 0.8), both flow rates were still considered to be laminar flow conditions. Biofilms were generally between 3 and 20 µm thick and it is therefore possible that the biofilms did not experience much shear force at either flow rate. Other studies have reported significant changes in biofilm structures when comparing laminar and turbulent (Re > ± 3000) flow conditions (Stoodley, Dodds, Boyle and Lappin-Scott 1998). Hydrodynamic conditions affect mass transfer and drag, and have been shown to influence biofilm development. Microbial communities might benefit from higher flow rates through the prevention of metabolic waste accumulation within biofilm structures.

Of the measured parameters, surface:volume ratios provide unique insights into biofilm responses to environmental conditions. These ratios indicate how much of a biofilm is exposed to the bulk liquid. For the most part, changes in flow rates did not seem to affect this ratio, but significant differences in surface:volume ratios were recorded between low and high nutrient concentrations (when the flow rate was kept constant). For both *Pseudomonas* strains, the ratios were higher at low nutrient concentrations than at high nutrient concentrations, that is, when less carbon was available, biofilm structures adapted to increase surface area exposure to the bulk liquid, thereby increasing access to nutrients from the environment.

More variability was recorded for parameters measured at low nutrient concentrations, compared to biofilms cultivated in high nutrient concentrations. This highlights the responsiveness and adaptability of microorganisms: the microbial community made use of a variety of growth patterns and three-dimensional architectures in order to optimise growth under lower nutrient concentrations. The structural heterogeneity observed here demonstrates the embodiment of resilience through variation (a subprinciple of Life's Principles discussed in Chapter 6).

Based on the data presented here, it cannot be concluded that nutrient concentration has a greater effect on *Pseudomonas* biofilms than flow rate. However, it was evident that, under the four conditions investigated, microbial communities were able to respond to environmental conditions in energy- and material efficient ways that promoted their survival. The three-dimensional matrix spatially organised the microbial community and allowed them to make the most of available resources. This emphasises the hypothesis that biofilms are multifunctional solutions for survival in diverse conditions.

5.4.3. Integrate development with growth

The traditional model of biofilm development supports the biomimetic description of biofilms as self-organised communities that are built from the bottom up, with increasingly complex three-dimensional structures and social networks. While experimental constraints prevented time studies of biofilm development in this study, the development of *Pseudomonas* biofilms under similar growth conditions has been well documented and results from studies also support the notion of biofilms being built from the bottom up using modular units (Bester et al. 2010; Bester, Wolfaardt, Aznaveh and Greener 2013).

The CLSM "snapshots" of PCT07 *gfp* and PA01 *gfp* biofilms at 96 hours revealed interesting structural adaptations to environmental conditions. Firstly, PCT07 *gfp* and PA01 *gfp* developed distinct biofilm features and it was possible to differentiate between the strains cultivated in any of the four environmental conditions investigated. Voids were visible in mature biofilm structures at high nutrient concentrations, both in the mushroom-shaped colonies of PCT07 *gfp* biofilms as well as in the mat-like areas of PA01 *gfp* biofilms. It is possible that these voids developed as the biofilm was built up or that channels were created at a later stage through mechanisms such as programmed cell death (Flemming 2011) or rhamnolipid production (specifically in PA01 *gfp* biofilms) (Davey et al. 2003). Regardless of the mechanism, these voids promote nutrient accessibility and the removal of waste products within mature biofilms.

Under high nutrient concentrations (10 mM citrate), both *Pseudomonas* strains formed more compact, structurally ordered biofilms than their counterparts at low nutrient concentrations (1 mM citrate). Building on the metaphor of biofilms as microbial cities (section 2.7.2), the biofilm structures at high nutrient concentrations can be likened to apartment complexes, accommodating large populations in high densities when there were ample (nutritional) opportunities. Conversely, at low nutrient concentrations, large sprawling structures protruded into the lumen of the flow cells, possibly in an attempt to pool resources in order to maximise access to nutrients.

During these microscopic investigations, biofilm images were captured in the middle part of flow cells (away from flow cell walls) to limit the effect of additional hydrodynamic variations (Heydorn et al. 2000b). When looking at biofilm development throughout the flow cell channels, it is clear that biofilm growth is very different in corners and along channel walls, compared to the centre of the flow cell. By capturing images in the middle of the flow cell, variations are limited, but it also means that important spatial adaptations in biofilm architecture may have been ignored. We should study biofilms in corners and on harder-to-access surfaces, especially when investigating biofilm eradication strategies.

5.5. Conclusions

In support of the proposed biomimetic framework for biofilms, the aforementioned results showed that:

- Microorganisms made use of readily available biochemical building blocks within an aqueous environment to create complex three-dimensional structures containing cells and secreted macromolecules.

- Biofilms responded to environmental conditions through spatial and structural adaptations.

- The strains used in this study were able to form biofilms and persist in a range of environmental conditions.

- Biofilms are self-organised systems that were built from the bottom up, using simple building blocks (cells and EPS) in increasingly complex structures (microcolonies).

- Even though the data did not necessarily explain biofilm responses in natural environments, ATR-FTIR spectra and DIA of CLSM images provided detailed snapshots of biofilms at a specific time of development.

6. Biofilms as adaptive systems that evolve to survive

This chapter further develops the proposed biomimetic framework for biofilms, by demonstrating that biofilms adhere to the remaining three Life's Principles (Figure 6.1). As discussed in Chapters 4 and 5, certain restrictions limited the use of CLSM, ATR-FTIR and Raman spectroscopy in this study. In order to overcome these limitations, a CEMS is used to investigate biofilms on a more comprehensive systems level.

A CEMS measures CO_2 production by biofilms as an indication of whole-biofilm metabolism (Kroukamp and Wolfaardt 2009). This system monitors biofilm metabolic responses to environmental changes in real time. Using a CEMS, biofilms can therefore be studied on a systems level, rather than focusing on individual members or subgroups of the populations. Measuring whole-biofilm metabolism in the form of CO_2 production is an effective way to monitor how the microbial community's metabolism is affected by nutrient conditions (Kroukamp et al. 2010). By measuring CO_2 production and biofilm-derived planktonic cell yield, this study demonstrates that single- and multi-species biofilms are locally attuned and responsive communities that are able to use readily available resources and cultivate cooperative relationships.

A CEMS has also been used to investigate biofilm recovery following disruptions by antibiotic treatments (Jackson et al. 2015). This study compares the responses of single- and multi-species biofilms to repeated exposures of an industrial biocide containing isothiazolone. Isothiazolone biocides act as electrophilic agents, which are able to induce cell death by disrupting growth and metabolism through interactions with critical enzymes (Cóllier, Ramsey, Waigh, Douglas, Austin et al. 1990).

The responsiveness and adaptive nature of multi-species biofilms are also investigated by exposing these biofilms to antibiotics. The effects of streptomycin on PA01 biofilms have been investigated using a CEMS approach (Jackson et al. 2015). Streptomycin is an aminoglycoside that targets protein synthesis and can disrupt the integrity of bacterial outer membranes (Taber, Mueller, Miller and Arrow 1987). Using an established protocol (Jackson et al. 2015), multi-species biofilms are exposed to increasing concentrations of streptomycin in order to investigate 1) whether multi-species biofilms are able to adapt to changing conditions and 2) whether these diverse communities are able to evolve to

survive under stress conditions. Biofilm responses were measured in terms of CO_2 production, biofilm-derived planktonic cell yield, colony morphology diversity and community metabolic diversity.

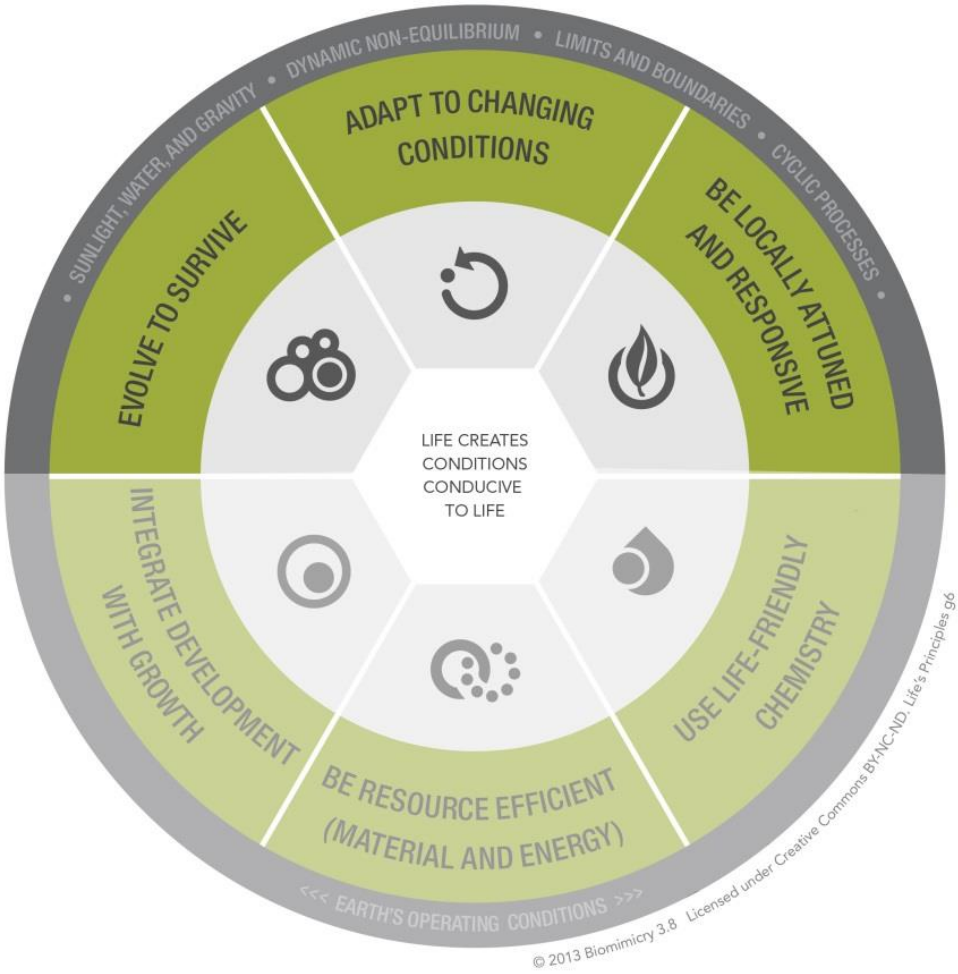

Figure 6.1. Graphic representation highlighting three Life's Principles: be locally attuned and responsive, adapt to changing conditions and evolve to survive. Adapted from the original[14].

[14] http://biomimicry.net/about/biomimicry/biomimicry-designlens/lifes-principles/, accessed on 25 September 2015.

6.1. Single- and multi-species biofilm responses to environmental changes

6.1.1. Biofilm cultivation in CEMS

In each of the experiments listed below, biofilms were cultivated in duplicate in continuous, once-through flow systems with replicate CEMSs as bioreactors (section 4.1.6.1). General disinfection and inoculation procedures were followed (section 4.1.6.2). Pre-cultures and nutrient medium were dependent on the experiment in question, as described in sections 6.1.5–6.1.7. CEMSs were inoculated with 950 µl of an 18-hour pre-culture under stagnant conditions. After one hour, nutrient flow was resumed at a flow rate of 15 $ml.h^{-1}$ and biofilms were cultivated at 20 ± 2 °C. CO_2 production was measured over time.

6.1.2. Biofilm-derived planktonic cell yield

In order to determine biofilm-derived planktonic cell yields, effluent was collected from CEMS outlets. Serial dilutions of the effluent were made and diluted samples were plated on agar-solidified nutrient medium (section 4.1.6.3). Samples were then incubated at 26 ± 2 °C for 5–7 days, after which effluent cell numbers were calculated as $CFU.ml^{-1}$.

Statistical analysis of planktonic cell yield data was performed in GraphPad Prism®. In the event of a rejected null hypothesis (significant difference determined by single-factor ANOVA), Tukey's test for the comparison of means was applied to determine which sample pairs were significantly different from each other.

6.1.3. Community analysis of multi-species biofilms

Ecoplates™ (Biolog Inc., CA, USA) were used for community analysis of planktonic cell-yield of multi-species biofilms. These plates consist of three replicate sets of 31 structurally-diverse carbon source substrates and blank wells on a transparent 96-well plate. Each well contains a tetrazolium salt that produces a colour product when it is reduced. The rate of colour development was measured spectrophotometrically as an indication of community level physiological activities (Baho, Peter and Tranvik 2012).

6.1.3.1. Sample preparation

Multi-species biofilms were cultivated in continuous, once-through flow systems with replicate CEMSs and 1.5 ml of the effluent was collected at various time points. Biofilm effluent was centrifuged at 10 000 rpm for 30 minutes. The supernatant was discarded and the pellet fraction was re-suspended in 1.5 ml sterile saline solution (0.9% w/v NaCl). The dissolved pellet fraction was again centrifuged at 10 000 rpm for 30 minutes. The supernatant was discarded and the pellet was re-suspended in 1.5 ml sterile saline solution. Serial dilutions were prepared from this sample. Each Ecoplate™ well was inoculated with 100 µl of the 1000 times dilution. Ecoplates™ were incubated in the dark at 26 ± 2 °C and colour development was measured at a wavelength of 590 nm at 24-hour intervals for 168 hours using an automated plate reader (PowerWave Microplate HT spectrophotometer, BioTek, VT, USA). The data was collected using Gen5 software and processed using GraphPad Prism®.

6.1.3.2. Average well colour development (AWCD)

The colour development in Ecoplates™ wells was calculated at 24-hour intervals (0–168 h) and expressed as the average net absorbance of each well at 590 nm (Garland and Mills 1991):

$$\text{AWCD} = \sum_{i-1}^{31} (C_i - r)/n$$

C_i = absorbance of the i^{th} substrate measured as OD at 590 nm at various time points

r = the comparable absorbance of the blank well

n = number of substrates (i.e. 31)

Negative ($C_i - r$) values were set to zero (Chen, Li, Chen, Huang, Hua et al. 2013). AWCD was plotted against time and these graphs were used as an indication of the reliability of the experiment (see Figure 6.2 as an example, other AWCD graphs are not shown).

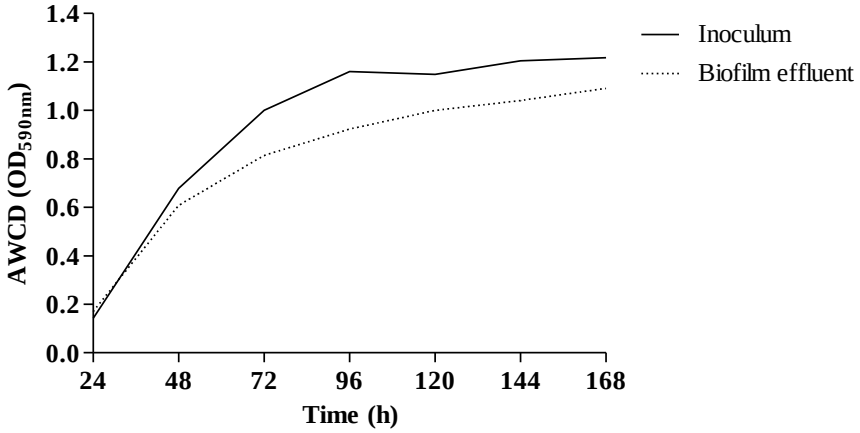

Figure 6.2. Rate of average well colour development (AWCD) of a multi-species inoculum and multi-species biofilm effluent. Measured as the average net absorbance of each Ecoplate™ well at 590 nm.

6.1.3.3. Community metabolic diversity (CMD)

CMD was calculated by summing the number of wells with positive responses (average of three absorbance values per substrate). A well was considered to have a positive response if the absorbance value was greater than 0.25 after all corrections were made (i.e. subtraction of blank value and control absorbance at t = 0) (Zak, Willig, Moorhead and Wildman 1994). Negative values were set to zero. CMD was expressed as the number of carbon substrates utilised by biofilm-derived planktonic cells.

6.1.4. Diversity index for multi-species biofilms

Effluent collected from multi-species biofilms was plated in duplicate on 1% LB agar-solidified nutrient medium with 2% Congo Red. After 5–7 days, different colony morphologies could easily be identified. Colony morphologies were described in terms of colony form, elevation and margin. Colonies were counted and grouped together according to the most common morphology types.

The diversity index is the inverse ratio of the average total colony count per plate to the number of distinct colony morphologies, i.e. a decrease in diversity measured on the index indicates a decrease in colony morphology diversity, which may indicate a decrease in community diversity:

$$\text{Diversity index} = 1 \div \frac{\text{average total colony count per plate}}{\text{number of distinct colony morphologies}}$$

6.1.5. Changes in nutrient conditions

6.1.5.1. Single-species biofilms

Two CEMSs were inoculated with 950 µl of PA01 pre-cultures in 3 $g.L^{-1}$ TSB and were supplied with 3 $g.L^{-1}$ TSB at a flow rate of 15 $ml.h^{-1}$. CO_2 production was allowed to reach a steady state before biofilm effluent was collected. Effluent was collected 72 hours after inoculation, after which the nutrient supply was changed to modified defined AB medium with 10 mM citrate as sole carbon source. CO_2 production reached a steady state within 48 hours of the nutrient medium change, at which point effluent was collected and the nutrient supply was changed to modified defined AB medium with 1 mM citrate as sole carbon source. After approximately 24 hours, effluent was collected and nutrient supply was changed to 3 $g.L^{-1}$ TSB.

Serial dilutions of the collected effluent were plated in triplicate on agar-solidified medium (nutrient type and concentration corresponded to the nutrient medium supplied to the CEMS at the time of collection). Biofilm-derived planktonic cell yield ($CFU.ml^{-1}$) was determined by the direct plate count method. Average values with standard deviation were calculated for each nutrient condition.

6.1.5.2. Multi-species biofilms

A freezer stock containing a sample of an environmental biofilm was incubated in 3 $g.L^{-1}$ TSB for 18 hours on a rotating wheel at 26 ± 2 °C (section 4.1.1.2). Two CEMSs were inoculated with 950 µl of the pre-culture and supplied with 3 $g.L^{-1}$ TSB at a flow rate of 15 $ml.h^{-1}$. CO_2 production was allowed to reach a steady state before biofilm effluent was collected. Effluent from each CEMS was collected 48 hours after inoculation, after which the nutrient supply was changed to 0.3 $g.L^{-1}$ TSB. After 48 hours, effluent was collected and the nutrient supply was changed to 3 $g.L^{-1}$ TSB. Final effluent samples were collected 144 hours after inoculation.

Serial dilutions of the collected effluent were plated in duplicate on three types of agar-solidified medium (3 $g.L^{-1}$ TSB, R-2A and 1% LB with 2% Congo Red) (section 4.1.6.3.1). Biofilm-derived

planktonic cell yield (CFU.ml^{-1}) was determined by the direct plate count method. Average values with standard deviation were calculated for each nutrient condition. The community diversity of biofilm effluent (collected 48, 96 and 144 hours after inoculation) was described by means of a diversity index and Ecoplate™ assays.

6.1.6. Exposure to a biocide

6.1.6.1. Isothiazolone solution

Busan® 1064A, a commercial biocide commonly used to combat biofilm formation in industrial cooling towers, was supplied by Buckman Laboratories, South Africa. This biocide solution contains 1–3% active mixture of 5-chloro-2-methyl-1,2-thiazol-3-one and 2-methyl-1,2-thiazol-3-one in a 3:1 ratio.

Various concentrations of Busan® 1064A (diluted in sterile 3 g.L^{-1} TSB) were tested on PA01 biofilms cultivated in 3 g.L^{-1} TSB in CEMS. CO_2 production was not significantly affected by 90 minute exposures to 1:5000 or 1:1000 dilutions of the biocide (data not shown). Sixty minute exposures to a 1:100 dilution of the biocide showed rapid responses in biofilm CO_2 production rates and this dilution ratio was used in subsequent experiments.

6.1.6.2. Single-species biofilms

The following experiment was performed twice: two CEMSs were inoculated with 950 µl of a PA01 pre-culture in 3 g.L^{-1} TSB and supplied with 3 g.L^{-1} TSB at a flow rate of 15 ml.h^{-1}. CO_2 production was allowed to reach a steady state before biofilm effluent was collected. Effluent from each CEMS was collected 48 hours after inoculation, after which the nutrient supply was changed to a 1:100 dilution of the biocide (diluted in 3 g.L^{-1} TSB) for 1 hour at a flow rate of 15 ml.h^{-1}. After the biocide exposure, the nutrient supply was changed to biocide-free medium (3 g.L^{-1} TSB) at a flow rate of 15 ml.h^{-1}. Biocide exposure was repeated once CO_2 production had stabilised (at 96 hours for the first experimental round and at 120 hours for the second experimental round).

Effluent was collected from each CEMS before each biocide exposure. Serial dilutions of the collected effluent were plated in triplicate on agar-solidified medium (3 $g.L^{-1}$ TSB). Biofilm-derived planktonic cell yield ($CFU.ml^{-1}$) was determined by the direct plate count method. Average values with standard deviation were calculated.

6.1.6.3. Multi-species biofilms

The following experiment was performed twice: a freezer stock containing a sample of an environmental biofilm was incubated in 3 $g.L^{-1}$ TSB for 18 hours on a rotating wheel at 26 ± 2 °C. Two CEMSs were inoculated with 950 µl of the pre-culture and supplied with 3 $g.L^{-1}$ TSB at a flow rate of 15 $ml.h^{-1}$.

Effluent from each CEMS was collected 48 hours after inoculation, after which the nutrient supply was changed to 1:100 dilution of the biocide (diluted in 3 $g.L^{-1}$ TSB) for 1 hour at a flow rate of 15 $ml.h^{-1}$. After the biocide exposure, the nutrient supply was changed to biocide-free medium (3 $g.L^{-1}$ TSB) at a flow rate of 15 $ml.h^{-1}$. Biocide exposure was repeated once CO_2 production had stabilised (at 120 hours).

Effluent was collected from each CEMS before each biocide exposure. Serial dilutions of the collected effluent were plated in duplicate on three types of agar-solidified medium (3 $g.L^{-1}$ TSB, R-2A and 1% LB with 2% Congo Red). Biofilm-derived planktonic cell yield ($CFU.ml^{-1}$) was determined by the direct plate count method. Average values with standard deviation were calculated. The community diversity of biofilm effluent (collected 48, 120 and 192 hours after inoculation) was described by means of a diversity index and Ecoplate™ assays.

6.1.7. Exposure to an antibiotic

6.1.7.1. Streptomycin solutions

Streptomycin sulfate (Melford Laboratories Ltd., Suffolk, UK) was dissolved in sterile TSB nutrient medium (final concentration: 3 $g.L^{-1}$ TSB). Biofilms were exposed to solutions containing 4000, 8000 or 12 000 $mg.L^{-1}$ streptomycin (Jackson et al. 2015).

6.1.7.2. Multi-species biofilms

The following experiment was performed twice: a freezer stock containing a sample of an environmental biofilm was incubated in 3 $g.L^{-1}$ TSB for 18 hours on a rotating wheel at 26 ± 2 °C. Two CEMSs were inoculated with 950 µl of the pre-culture and supplied with 3 $g.L^{-1}$ TSB at a flow rate of 15 $ml.h^{-1}$.

Effluent from each CEMS was collected 48 hours after inoculation, after which the nutrient supply was changed to nutrient medium (3 $g.L^{-1}$ TSB) containing 4000 $mg.L^{-1}$ streptomycin for 4 hours at a flow rate of 15 $ml.h^{-1}$. Biofilms were subsequently exposed to increasing concentrations of streptomycin at 96 hours (8000 $mg.L^{-1}$ streptomycin) and at 144 hours (12 000 $mg.L^{-1}$ streptomycin).

Effluent was collected before each exposure and 2 hours after each exposure. Serial dilutions of the collected effluent were plated in duplicate on three types of agar-solidified medium (3 $g.L^{-1}$ TSB, R-2A and 1% LB with 2% Congo Red). Biofilm-derived planktonic cell yield ($CFU.ml^{-1}$) was determined by the direct plate count method. Average values with standard deviation were calculated. The community diversity of biofilm effluent (collected 48, 120 and 192 hours after inoculation) was described by means of a diversity index and Ecoplate™ assays.

6.2. Results

Single- and multi-species biofilms cultivated in CEMSs were exposed to either changes in nutrient conditions or to a biocide solution containing isothiazolone. CO_2 production and biofilm-derived planktonic cell yield were measured as indicators of biofilm metabolic responses to these changes. Additionally, the responses of multi-species biofilms to the antibiotic streptomycin were also investigated.

6.2.1. Biofilm responses to nutrient availability

6.2.1.1. Single-species biofilms

The metabolic responses of PA01 biofilms to changes in nutrient conditions were measured in terms of CO_2 production and biofilm-derived planktonic cell yield (Figures 6.3 and 6.4). When PA01 biofilms were exposed to a complex nutrient medium (TSB), CO_2 production stabilised approximately 60 hours after inoculation at 80–90 $\mu mol.h^{-1}$, at which point the biofilm-derived planktonic cell yield was approximately 7.8 x 10^8 $CFU.ml^{-1}$. An initial decrease in CO_2 production was measured after the nutrient medium was changed to defined nutrient medium (modified defined AB medium), but CO_2 production returned to 80–90 $\mu mol.h^{-1}$ within 48 hours (Figure 6.3). Planktonic cell yield was significantly lower at this point (120 hours after inoculation), although biofilms still released large numbers of planktonic cells into the effluent (3.5 x 10^7 $CFU.ml^{-1}$) (Figure 6.4).

When the biofilms were exposed to a defined nutrient medium with a low carbon concentration (modified defined AB medium with 1 mM citrate), CO_2 production rapidly decreased and stabilised at a lower level (20–25 $\mu mol.h^{-1}$) (Figure 6.3). There was no significant change in planktonic cell yield when biofilms were cultivated in modified defined AB medium with 10 mM or 1 mM citrate (Figure 6.4). A rapid increase in CO_2 production was observed when the nutrient medium was changed back to the complex nutrient medium (TSB), with CO_2 production reaching 80–90 $\mu mol.h^{-1}$ within 24 hours (Figure 6.3).

6.2.1.2. Multi-species biofilms

The metabolic responses of multi-species biofilms to changes in nutrient concentration were measured in terms of CO_2 production (Figure 6.5), biofilm-derived planktonic cell yield (Figure 6.6), carbon source utilisation by biofilm-derived planktonic cells (Figure 6.7) and a diversity index (Figure 6.8). When multi-species biofilms were exposed to a complex nutrient medium (TSB), CO_2 production stabilised approximately 35 hours after inoculation at 90–105 $\mu mol.h^{-1}$, at which point the biofilm-derived planktonic cell yield was approximately 8.7 x 10^8 $CFU.ml^{-1}$. When the nutrient concentration was decreased from 3 $g.L^{-1}$ to 0.3 $g.L^{-1}$, a rapid decrease in CO_2 production and significant decrease in planktonic cell yield were observed. CO_2 production stabilised at 15–20 $\mu mol.h^{-1}$ within 10 hours of the nutrient medium change. When nutrient medium was changed to 3 $g.L^{-1}$ TSB, a rapid increase in CO_2 production and significant increase in planktonic cell yield was measured. A sloughing event was recorded at 120 hours (Figure 6.5): the sharp decline in CO_2 production corresponded with visible biomass in the biofilm effluent.

Ecoplates™ were used to investigate the effect of environmental changes on the metabolic diversity of multi-species biofilms. More specifically, these assays were used to estimate the functional potential of a microbial community to utilise different carbon substrates before and after perturbations (in this case in the form of nutrient changes or the addition of antimicrobial compounds). Shifts in metabolic diversity patterns, measured as changes in absorbance of Ecoplates™, relate to shifts in community composition (Chen et al. 2013). CMD represents the total number of carbon substrates on an Ecoplate™ that a microbial community can effectively metabolise and therefore it serves as an indication of diversity in carbon source utilisation.

When comparing the carbon substrate utilisation by biofilm-derived planktonic cells of multi-species biofilms exposed to changes in nutrient concentration, the only significant change was observed in the effluent collected from biofilm A1 at 48 hours (27 substrates utilised) and 144 hours (23 substrates utilised) (Figure 6.7). Greater morphological diversity was observed in colonies cultivated from the multi-species inoculum compared to those cultivated from biofilm effluent. Diversity in colony morphology of biofilm-derived planktonic cells decreased over the course of the experiment (Figure 6.8).

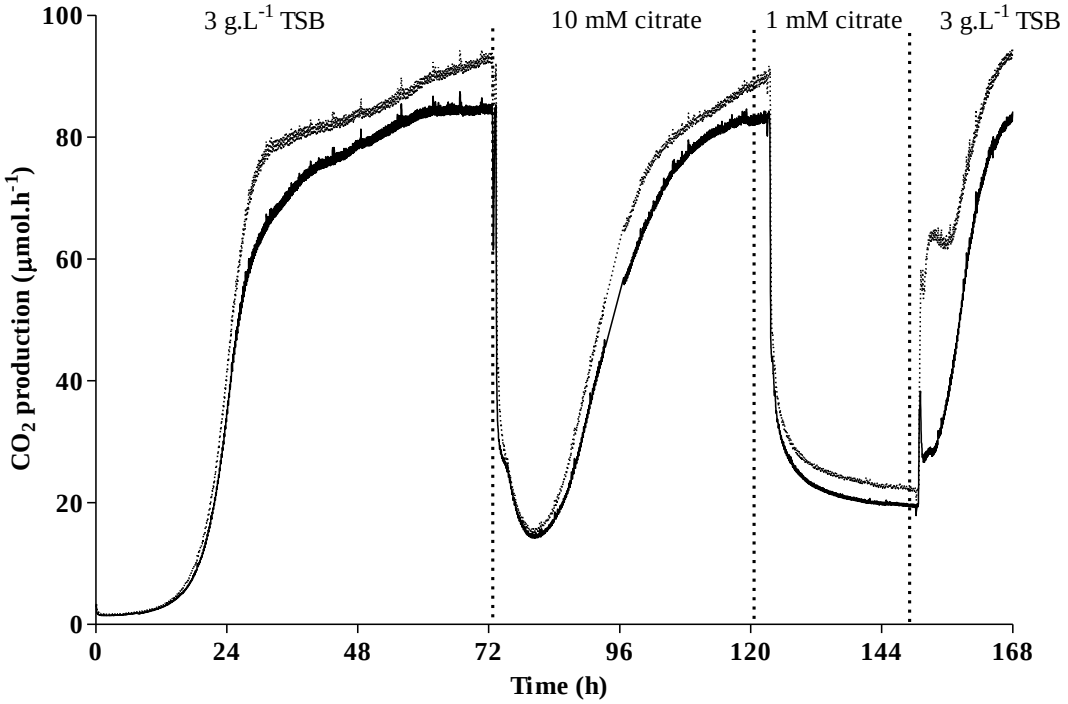

Figure 6.3. CO_2 production of PA01 biofilms in response to changes in nutrient conditions. Duplicate biofilms were cultivated in continuous, once-through flow systems with replicate CEMSs as bioreactors at a flow rate of 15 $ml.h^{-1}$. Biofilm effluent was collected from each CEMS before nutrient medium was changed, as indicated on graph.

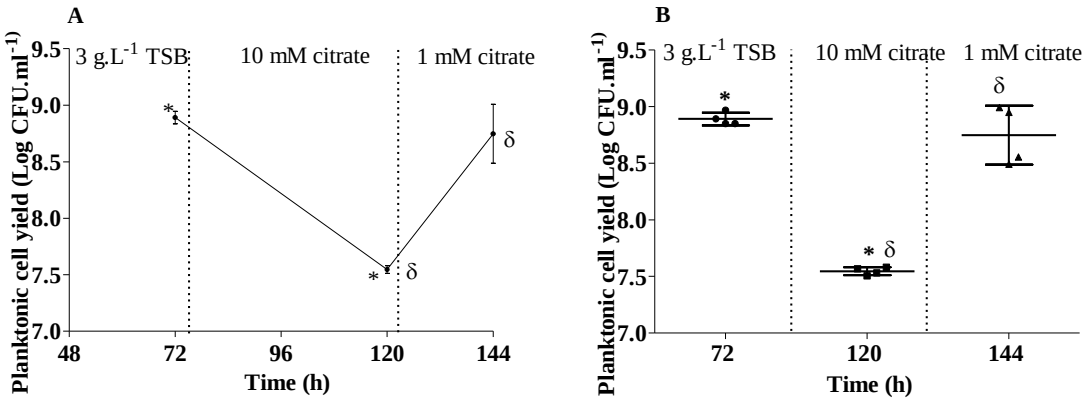

Figure 6.4. Biofilm-derived planktonic cell yield of PA01 biofilms in response to changes in nutrient conditions. Serial dilutions of biofilm effluent were used to determine cell yield by direct plate count method. **A.** Average values with standard deviation are reported and significant differences are indicated with * and δ ($p < 0.005$). **B.** All data points shown.

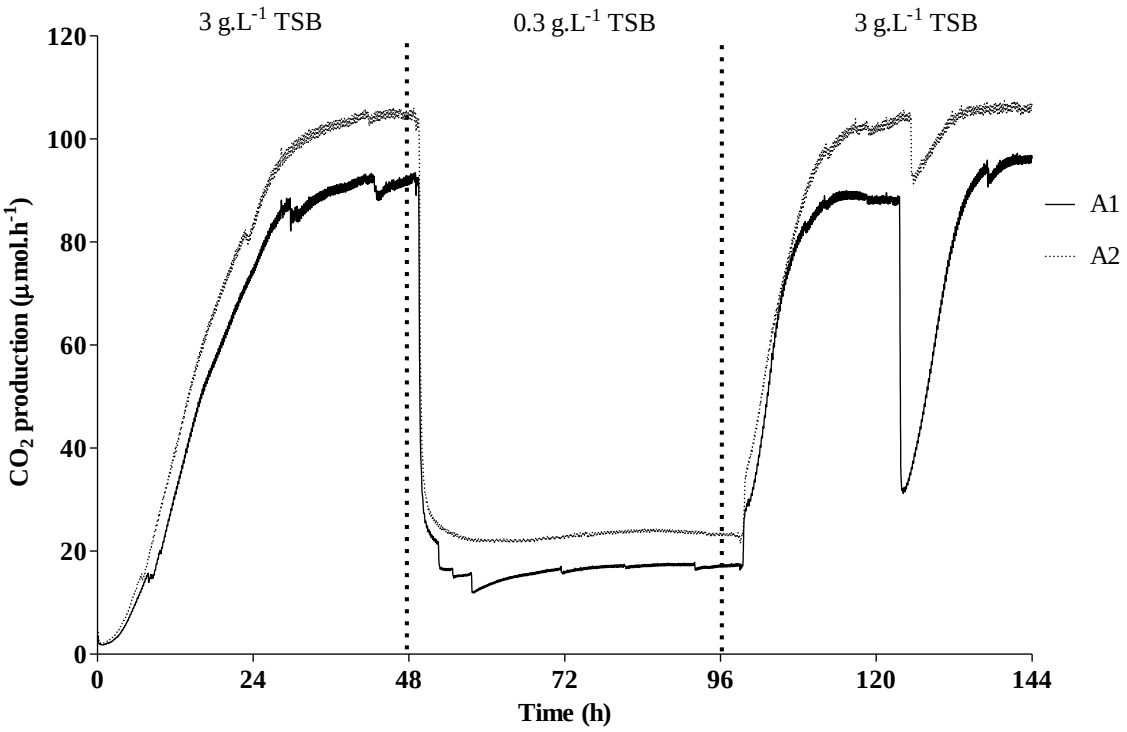

Figure 6.5. CO_2 production of multi-species biofilms in response to changes in nutrient conditions. Duplicate biofilms (A1 and A2) were cultivated in continuous, once-through flow systems with replicate CEMSs as bioreactors in 3 g.L^{-1} TSB at a flow rate of 15 ml.h^{-1}. Biofilm effluent was collected from each CEMS before nutrient medium was changed, as indicated on graph.

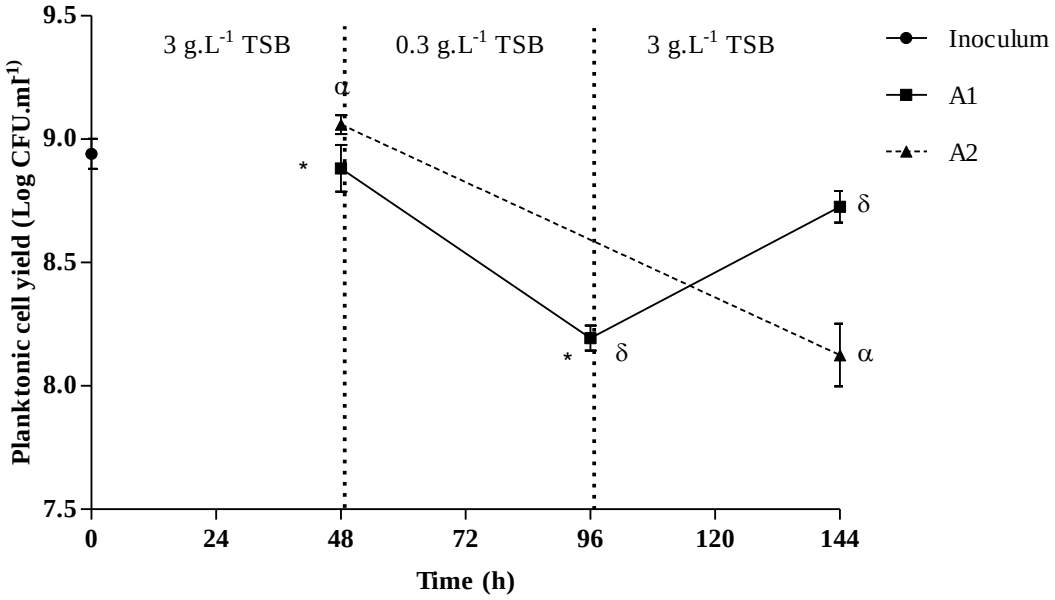

Figure 6.6. Biofilm-derived planktonic cell yield of multi-species biofilms exposed to changes in nutrient conditions. Serial dilutions of biofilm effluent were used to determine cell yield by direct plate count method. Effluent dilutions were plated in duplicate on three types of agar-solidified nutrient medium (3 g.L^{-1} TSB, R-2A and 1% LB with 2% Congo Red). Average values with standard deviation are reported for duplicate biofilms A1 and A2. Significant differences are indicated with *, δ and α ($p < 0.005$).

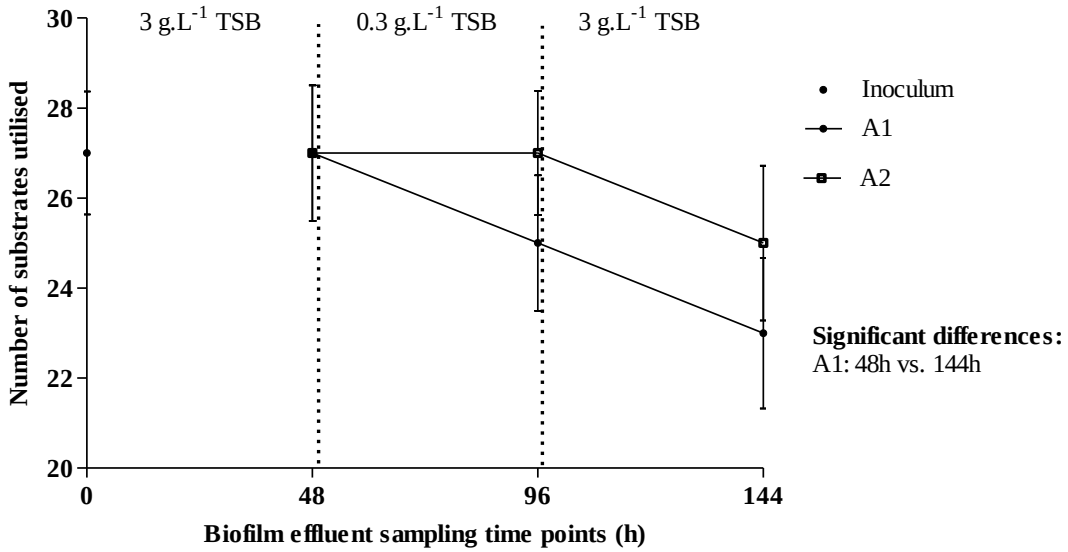

Figure 6.7. Carbon substrate utilisation by planktonic cells from multi-species biofilms exposed to changes in nutrient conditions as indication of community metabolic diversity (CMD). Effluent from duplicate biofilms (A1 and A2) was collected and treated by a series of centrifugation and wash steps before serial dilutions were made. Diluted samples were incubated in Biolog Ecoplates™ and absorbance at 590 nm was recorded every 24 hours. Each data point is the average number of positive reactions calculated for a sample after 144 and 168 hours of incubation (with standard deviation) ($p < 0.005$).

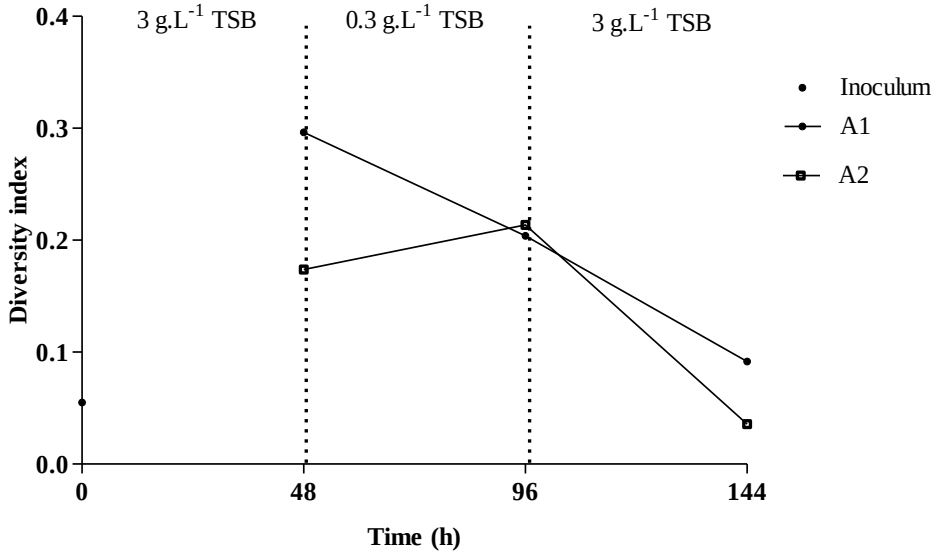

Figure 6.8. Colony morphology diversity of planktonic cells from multi-species biofilms exposed to changes in nutrient conditions. Effluent was collected from duplicate biofilms (A1 and A2) and serial dilutions thereof were plated on agar-solidified nutrient medium (1% LB with 2% Congo Red). The diversity index is the inverse of total colony plate count: total distinct morphologies ratio.

6.2.2. Biofilm responses to biocide exposure

6.2.2.1. Single-species biofilms

The metabolic responses of PA01 biofilms to biocide exposure were measured in terms of CO_2 production (Figure 6.9) and biofilm-derived planktonic cell yield (data not shown). CO_2 production reached 80–90 µmol.h^{-1} 48 hours after inoculation. A sharp decrease in CO_2 production was observed when biofilms were exposed to a 1:100 dilution of a commercial biocide containing isothiazolone. After the biocide exposure, biofilms were supplied with biocide-free nutrient medium until CO_2 production stabilised. Within 12 hours of the first biocide exposure, CO_2 production increased to 80–90 µmol.h^{-1} within 48 (Round A) to 72 hours (Round B). After the second 1-hour biocide exposure, there was a similar decrease in CO_2 production in both experimental rounds. CO_2 production increased within 12 hours of the exposure (Figure 6.9).

Planktonic cell yield was determined before each biocide exposure and 72 hours after the second exposure. No significant changes in biofilm-derived planktonic cell yield were observed before and after biocide exposures. Biofilms released approximately 10^8 CFU.ml^{-1} at each measured interval.

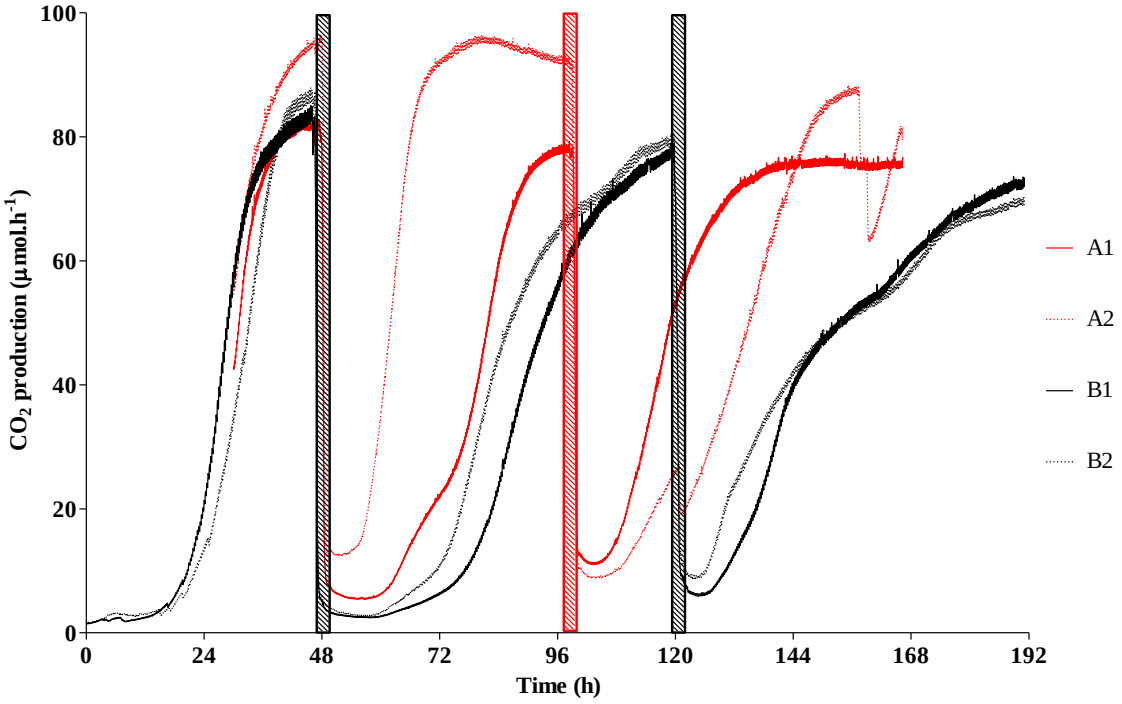

Figure 6.9. CO_2 production of PA01 biofilms in response to biocide exposure. Duplicate biofilms were cultivated in continuous, once-through flow systems with CEMS as bioreactors in two independent experimental rounds, A and B. Biofilms were cultivated at 20±2 °C in 3 $g.L^{-1}$ TSB at a flow rate of 15 $ml.h^{-1}$. Biofilms were exposed to 1:100 dilution of a commercial biocide containing isothialozone for 1-hour intervals (indicated by highlighted areas on graph). Biofilm effluent was collected from CEMS before and after biocide exposure.

6.2.2.2. Multi-species biofilms

The metabolic responses of multi-species biofilms to biocide exposure were measured in terms of CO_2 production (Figure 6.10), biofilm-derived planktonic cell yield (Figure 6.11), carbon source utilisation by biofilm-derived planktonic cells (Figure 6.12) and a diversity index (Figure 6.13).

CO_2 production stabilised at 85–95 µmol.h^{-1}, approximately 30 hours after inoculation. A sharp decrease in CO_2 production was observed after biofilms were exposed to a 1:100 dilution of a commercial biocide containing isothiazolone. CO_2 production rates rapidly increased within the 12 hours after initial exposure and stabilised at 85–95 µmol.h^{-1} within 72 hours after the initial biocide exposure. No significant changes in planktonic cell yield were recorded after the first exposure in round A, while there was a slight decrease in planktonic cell yield in round B as biofilms recovered after the first biocide exposure (Figure 6.10). Overall, planktonic cell yield was significantly lower after the second exposure (192 hours) when compared to the initial yield at 48 hours (Figure 6.11).

No significant change in carbon substrate utilisation by biofilm-derived planktonic cells was observed for round A, while a significant decrease was recorded in round B between cells collected at 48 and 192 hours (Figure 6.12). Colony morphology diversity decreased after the first biocide treatment and then remained relatively stable between the first and second exposure (Figure 6.13).

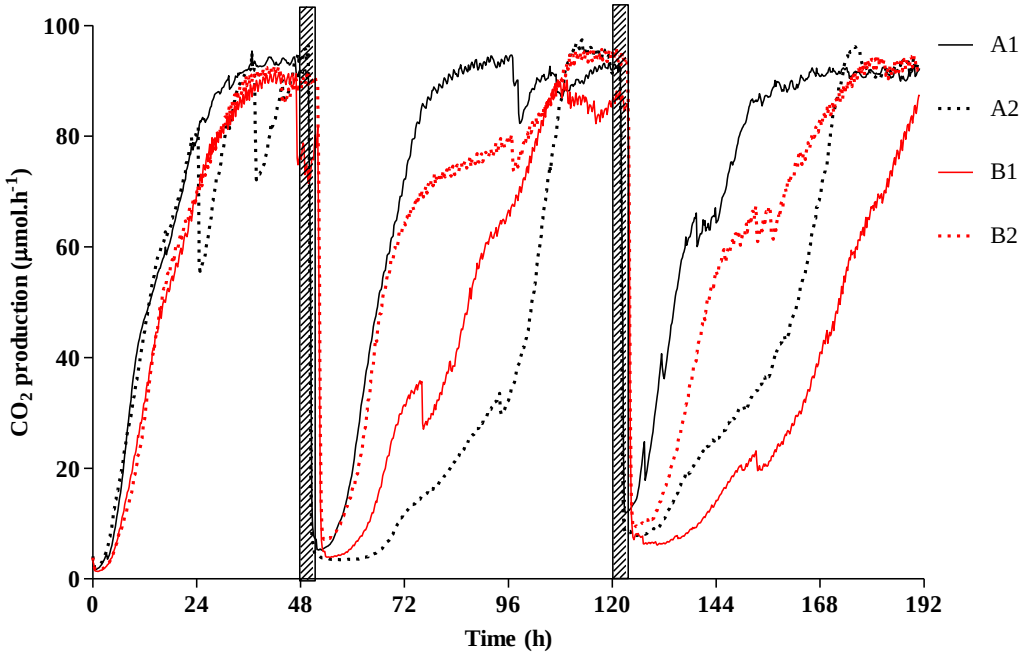

Figure 6.10. CO_2 production by multi-species biofilms in response to biocide exposure. Duplicate biofilms were cultivated in continuous, once-through flow systems with replicate CEMSs as bioreactors in two independent experimental rounds, A and B. Biofilms were cultivated at 20 ± 2 °C in 3 g.L^{-1} TSB at a flow rate of 15 ml.h^{-1}. Biofilms were exposed to 1:100 dilution of a commercial biocide containing isothialozone for 1-hour intervals (indicated by highlighted areas on graph). Biofilm effluent was collected from each CEMS before biocide exposure.

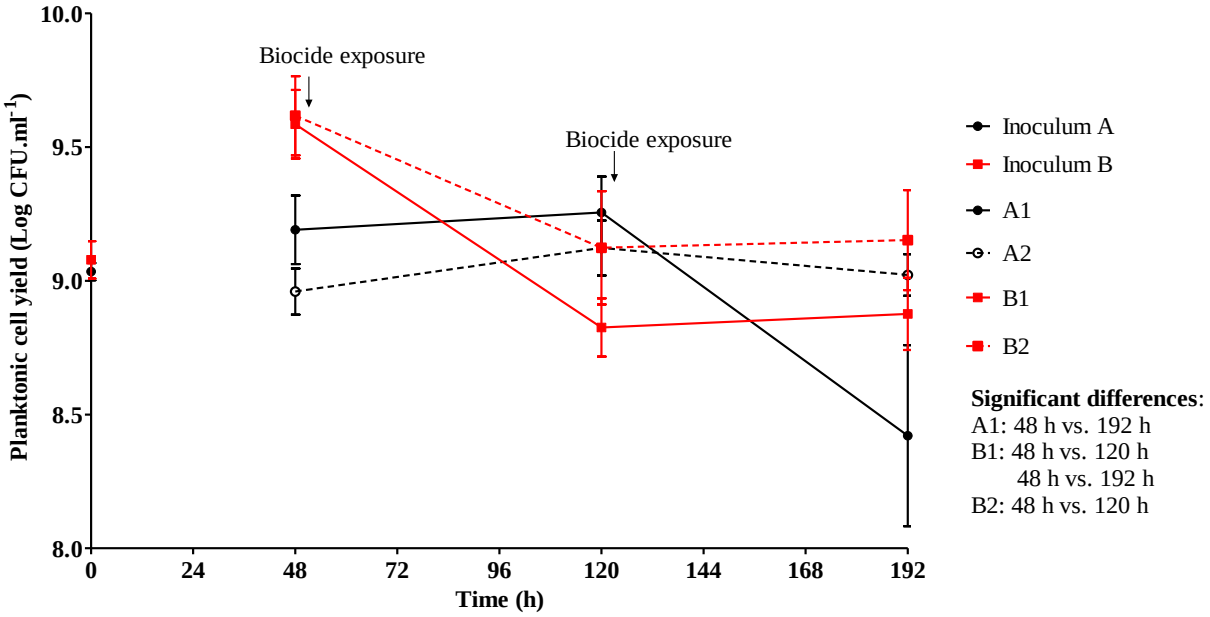

Figure 6.11. Biofilm-derived planktonic cell yield of multi-species biofilms exposed to biocide. Serial dilutions of biofilm effluent were used to determine cell yield by direct plate count method. Effluent dilutions were plated in duplicate on three types of agar-solidified nutrient medium (3 g.L^{-1} TSB, R-2A and 1% LB with 2% Congo Red). Average values, with standard deviation, are reported for duplicate biofilms in experimental rounds A and B ($p < 0.005$).

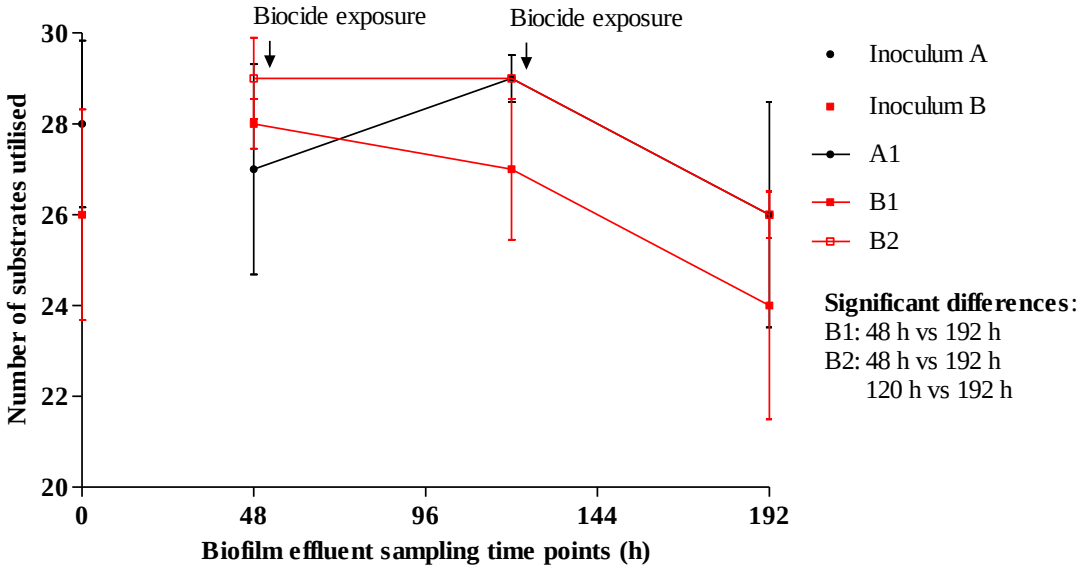

Figure 6.12. Carbon substrate utilisation by planktonic cells from multi-species biofilms exposed to biocide as indication of community metabolic diversity (CMD). Effluent from duplicate biofilms in experimental rounds A and B was collected prior to biocide exposures. Effluent samples were treated by a series of centrifugation and wash steps before serial dilutions were made. Diluted samples were incubated in Biolog Ecoplates™ and absorbance at 590 nm was recorded every 24 hours. Each data point is the average number of positive reactions calculated for a sample after 144 and 168 hours of incubation (with standard deviation) ($p < 0.005$).

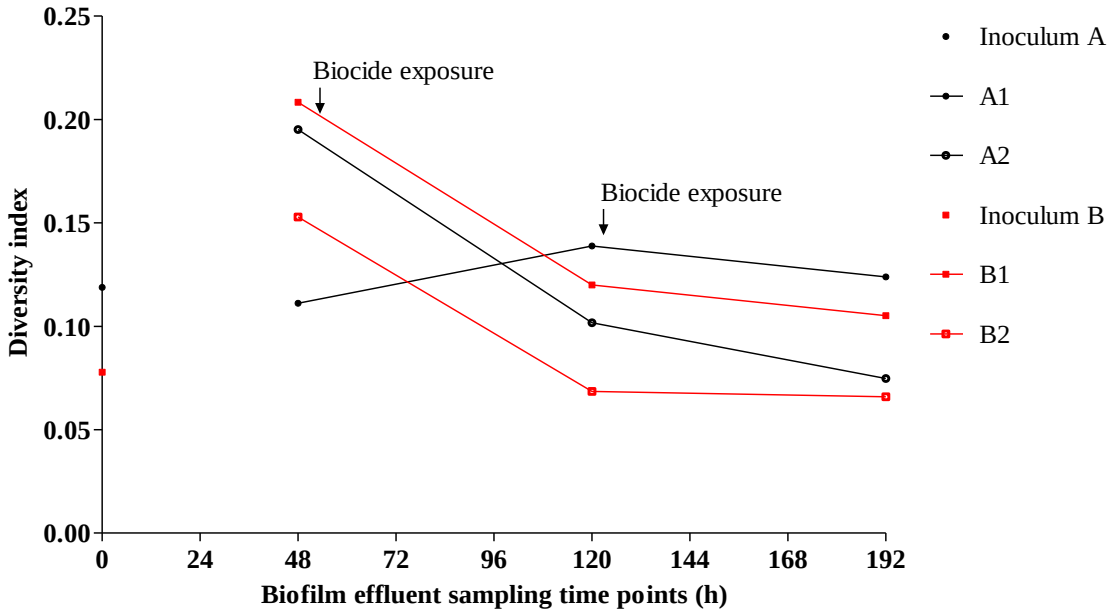

Figure 6.13. Colony morphology diversity of planktonic cells from multi-species biofilms exposed to biocide. Effluent from duplicate biofilms in experimental rounds A and B was collected prior to biocide exposures. Serial dilutions of biofilm effluent were plated on agar-solidified nutrient medium (1% LB with 2% Congo Red). The diversity index is the inverse of total colony plate count: total distinct morphologies ratio.

6.2.3. Biofilm responses to antibiotic exposure

6.2.3.1. Multi-species biofilms

The metabolic responses of multi-species biofilms to increasing concentrations of streptomycin were measured in terms of CO_2 production (Figure 6.14), biofilm-derived planktonic cell yield (Figure 6.15), carbon source utilisation by biofilm-derived planktonic cells (Figure 6.16) and a diversity index (Figure 6.17).

Sloughing events were recorded in both experimental rounds within the first 48 hours: sharp decreases in CO_2 production corresponded with visible biomass in the effluent. After exposure to 4000 $mg.L^{-1}$ streptomycin, there was a rapid decrease in CO_2 production and significant decreases in planktonic cell yield (measured 2 hours after exposure, Round B, Figure 6.15). CO_2 production steadily increased within 48 hours to levels comparable to pre-exposure production. Planktonic cell yield recovered within 48 hours of the exposure, although it remained slightly lower than the yield before the first exposure to antibiotics (Figure 6.15).

When biofilms were exposed to 8000 $mg.L^{-1}$ streptomycin, CO_2 production decreased at a slower rate and recovered faster than after the initial exposure. Planktonic cell yield decreased significantly after the second exposure and then, within 48 hours, increased to levels comparable to cell yield prior to the second exposure (at 96 hours). Despite the higher dose of streptomycin administered at 144 hours (12 000 $mg.L^{-1}$), CO_2 production only decreased slightly and recovered rapidly (Figure 6.14). A decrease in planktonic cell yield was observed 2 hours after the exposure, but the yield reached levels comparable to those recorded prior to the third exposure within 48 hours (Figure 6.15).

Very few changes in carbon substrate utilisation by biofilm-derived planktonic cells were recorded throughout the experiment (Figure 6.16). Diversity appeared to decrease after the first exposure, with a further decrease after the second exposure (round A) and then stabilised (Figure 6.17).

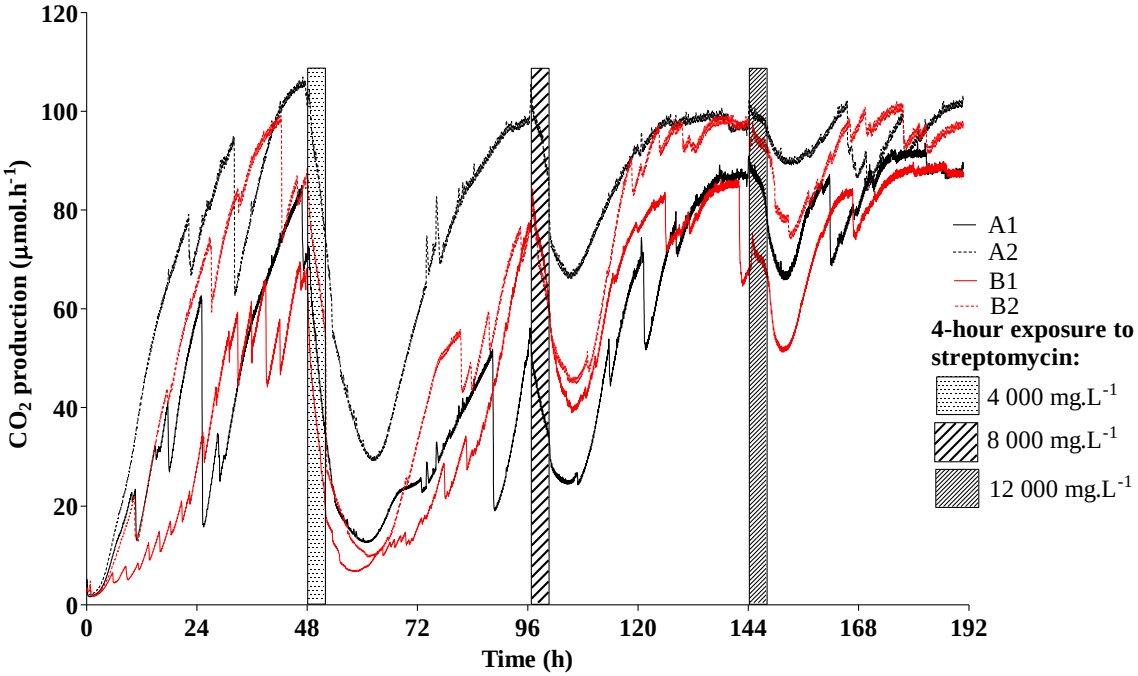

Figure 6.14. CO_2 production by multi-species biofilms in response to antibiotic exposure. Duplicate biofilms were cultivated in continuous, once-through flow systems with replicate CEMSs as bioreactors in two independent experimental rounds, A and B. Biofilms were cultivated at 20 ± 2 °C in 3 g.L^{-1} TSB at a flow rate of 15 ml.h^{-1}. Biofilms were exposed to increasing concentrations of streptomycin (dissolved in 3 g.L^{-1} TSB), indicated by highlighted areas on graph. Biofilm effluent was collected from each CEMS before and 2 hours after exposures.

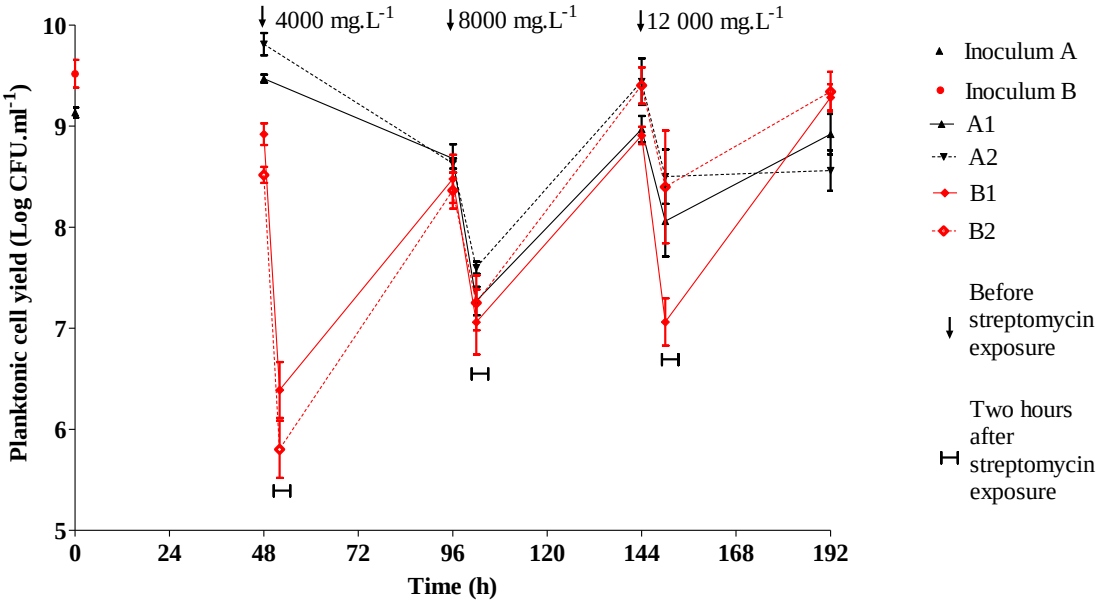

Figure 6.15. Biofilm-derived planktonic cell yield of multi-species biofilms exposed to antibiotics. Serial dilutions of biofilm effluent were used to determine cell yield by direct plate count method. Effluent dilutions were plated in duplicate on three types of agar-solidified nutrient medium (3 g.L^{-1} TSB, R-2A and 1% LB with 2% Congo Red). Average values with standard deviation are reported ($p < 0.005$).

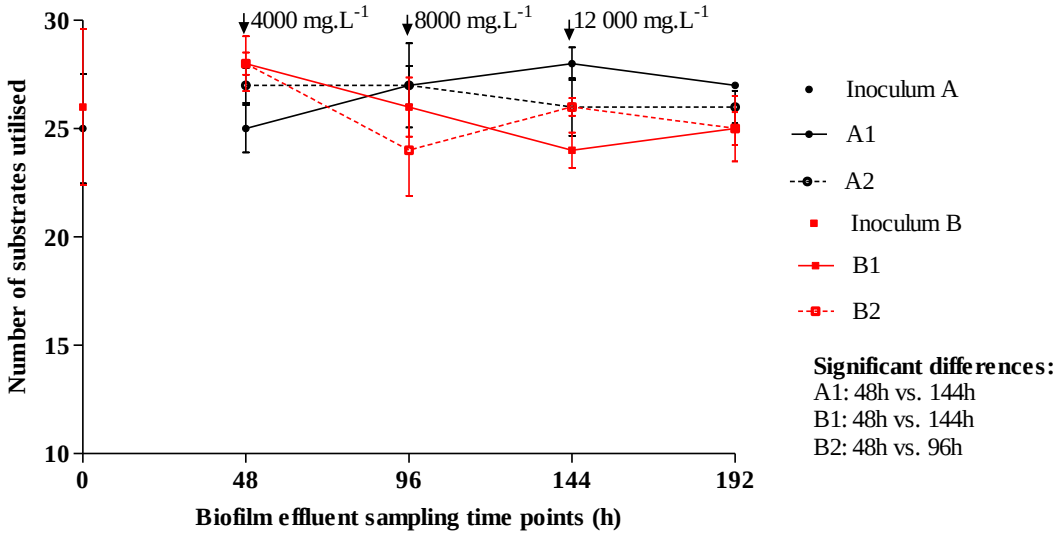

Figure 6.16. Carbon substrate utilisation by planktonic cells from multi-species biofilms exposed to antibiotics as indication of community metabolic diversity (CMD). Effluent from duplicate biofilms in experimental rounds A and B was collected prior to antibiotic exposures. Effluent samples were treated by a series of centrifugation and wash steps before serial dilutions were made. Diluted samples were incubated in Biolog Ecoplates™ and absorbance at 590 nm was recorded every 24 hours. Each data point is the average number of positive reactions calculated for a sample after 144 and 168 hours of incubation (with standard deviation) ($p < 0.005$).

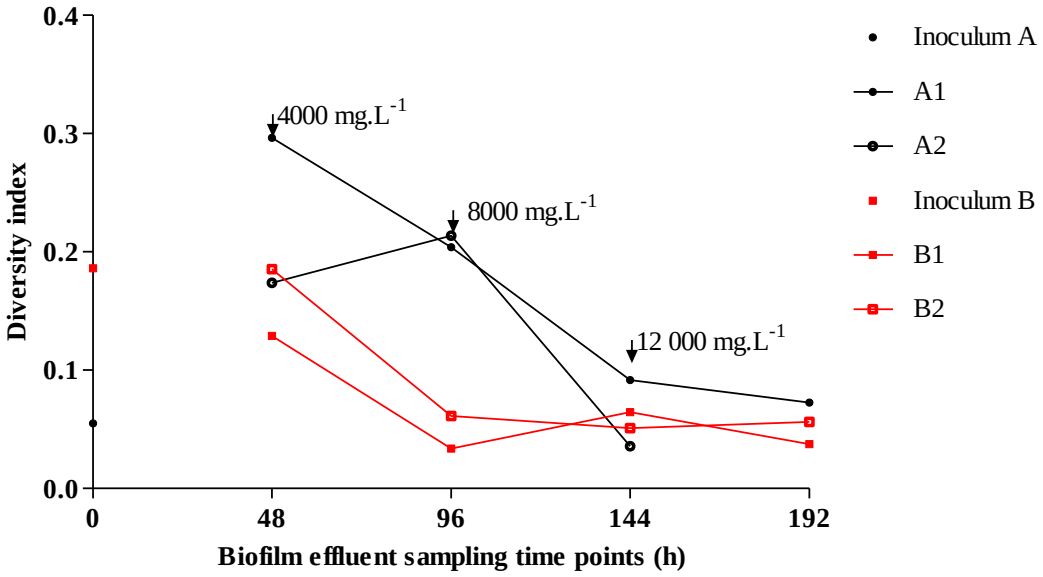

Figure 6.17. Colony morphology diversity of planktonic cells from multi-species biofilms exposed to antibiotics. Effluent from duplicate biofilms in experimental rounds A and B was collected prior to antibiotic exposures. Serial dilutions of biofilm effluent were plated on agar-solidified nutrient medium (1% LB with 2% Congo Red). The diversity index is the inverse of total colony plate count: total distinct morphologies ratio.

6.3. Discussion

6.3.1. Locally attuned and responsive communities

The results in this chapter showed that both single- and multi-species biofilms were attuned to their local environments and responded rapidly to changes in nutrient availability or the presence of antimicrobial compounds.

The metabolic profiles of biofilms exposed to changes in nutrient conditions demonstrate the ability of microbial communities to utilise readily available materials and energy sources from the environment (Figures 6.3 and 6.5). PA01 biofilms responded rapidly to changes in carbon source and concentration. When switched from a complex, nutrient-rich medium to a defined nutrient medium, CO_2 production rates recovered within 24 hours (Figure 6.3). Even at lower carbon concentrations, the biofilms were able to maintain high planktonic cell yields (in the order of 10^7 CFU.ml^{-1}) (Figure 6.4). At low carbon concentrations, CO_2 production rates were lower but stable (±20 µmol.h^{-1}) and responded almost instantaneously when a more complex nutrient medium became available.

When environmental carbon levels decreased, a sudden decrease in CO_2 production rates (from 90–105 µmol.h^{-1} to 15–20 µmol.h^{-1}) and lower planktonic cell yields (9.3 x 10^8 CFU.ml^{-1} vs. 1.6 x 10^8 CFU.ml^{-1}) was recorded for multi-species biofilms (Figure 6.5 and 6.6). These changes were reversed within a couple of hours when higher nutrient concentrations were introduced. These results indicate that single- and multi-species biofilms were able to respond to and recover from changes in nutrient availability, while maintaining a high level of metabolic activity and planktonic cell yield.

When comparing the responses to biocide exposure, rapid decreases in CO_2 production were recorded for both single- and multi-species biofilms. The decrease in metabolic activity (Figures 6.9 and 6.10) could indicate biocide-induced cell death or that the microbial community was able to adapt its metabolism in order to respond to the negative environmental stimulus. Biofilm-derived planktonic cell yield of PA01 biofilms was not affected by the biocide exposure and, although there was a significant decrease when comparing cell yield at 48 and 192 hours, multi-species biofilms maintained the ability

to release high numbers of cells into the effluent even after repeated biocide exposure (Figure 6.11). Other investigations showed that PA01 biofilms are less susceptible to lower biocide concentrations than planktonic cultures (data not shown). Cooperative relationships within a biofilm support the community's ability to respond swiftly and appropriately to changes in the environment. To gain further insight into biofilm responsiveness on a systems level, microscopic techniques could be combined with a CEMS to study structural changes in biofilm communities under changing environmental conditions.

As discussed in Chapter 2, feedback loops allow microorganisms to regulate internal reactions to their environment and respond as a system. To this point, it would be interesting to further investigate the regulation of biofilm-derived planktonic cell yield. From the results above, it appears that the number of cells released by the biofilms ($CFU.ml^{-1}$) did not exceed the number of planktonic cells in the inoculum. It is possible that there is a feedback mechanism regulating optimal cell release as a function of the biofilm mode of life.

It should be taken into account that planktonic cell yield of multi-species biofilms was calculated using agar-solid media that promotes bacterial growth. The yeast and fungi component of the planktonic community may have been overlooked and therefore planktonic cell yield underestimated in these calculations. Future studies should incorporate additional yeast- and fungi-specific culture techniques.

6.3.2. Adapting to changing conditions

Being attuned to environmental changes allows microbial communities to adapt appropriately to dynamic contexts. The results presented in this chapter show that biofilms responded to changes in nutrient availability and to exposure to antimicrobial compounds by adapting their metabolic rates, planktonic cell yield and, in the case of multi-species biofilms, level of diversity. From a biomimetic perspective, adaptation is achieved through self-renewal, the incorporation of diversity and resilience through variation, redundancy and decentralisation.

Biofilms are able to persist because microbial communities can maintain structural and functional integrity by adding energy and matter in the form of EPS and daughter cells. These processes heal and

improve the biofilm system. The high planktonic cell yields from single- and multi-species biofilms before and after the disruptions introduced in these experiments support this notion, but would need further investigation by combining microscopic studies with a CEMS.

Planktonic cell yield is a vital function of biofilms. Metabolic processes and genetic information, including genetic adaptations, are duplicated in daughter cells. The release of "redundant" cells into the micro- and macro-environment creates a decentralised system. Considering the planktonic cell yields measured in all the experiments presented in this chapter, it appears that even in adverse conditions, such as lower carbon availability or the presence of antimicrobial compounds, the production and release of cells were prioritised by the microbial community as an investment policy for survival (Figures 6.4, 6.6 and 6.11).

Metabolic assays and a rudimental diversity index were used to track broad changes in community diversity of multi-species biofilms. When nutrient medium was changed from high to low carbon concentrations, no significant changes were observed in the carbon substrate utilisation by planktonic cells derived from multi-species biofilms. The only exception was a decrease from 27 to 23 substrates that was detected for biofilm A1 after the biofilms were changed back to high carbon concentrations (Figure 6.7). An overall decrease in colony morphology diversity was recorded for multi-species biofilms exposed to changing nutrient concentrations (Figure 6.8).

A similar trend was observed when multi-species biofilms were exposed to biocide solutions. In round B, the carbon substrate utilisation by biofilm-derived planktonic cells decreased by a total of 3 substrates (Figure 6.12), coupled with a noticeable decrease in colony morphology diversity after the first biocide exposure (Figure 6.13).

Although some fluctuations were recorded for carbon substrate utilisation by planktonic cells released from multi-species biofilms exposed to streptomycin, there were no significant changes when comparing substrate utilisation before and after repeated exposures (192 h) (Figure 6.16). Colony morphology diversity decreased noticeably after the first antibiotic treatment and continued to decrease after repeated exposures (Figure 6.17).

Changes in carbon substrate utilisation could indicate metabolic adaptations by biofilm members or shifts in the community composition. Despite the decrease in colony morphology diversity in these experiments, various morphologies were detected during the course of the experiments. Given the simplicity of the methods it was not possible to say whether the morphologies present belong to different species. These results suggest that multiple species were able to survive within these biofilms despite environmental changes or exposure to antimicrobial compounds, but that some species were less affected and survived in greater numbers.

Since this study was not concerned with the presence of specific species within the biofilms but rather the functioning of the community as a system, these methods provided fast, cost-effective insights into general community composition. It should be taken into account that the agar-solidified LB nutrient media supports growth of most bacterial species but it is not an ideal culture medium for yeast and fungi. Therefore the diversity index does not necessarily capture shifts in yeast and fungi populations. Additional culture techniques should be included in future studies. Ecoplate assays can however be used to analysis metabolic diversity of multi-species communities, including yeast and fungi. Future work could include more accurate analysis of species diversity during such experiments.

When comparing the CO_2 production rates of PA01 and multi-species biofilms exposed to biocides, the multi-species biofilms recovered faster and were able to reach pre-exposure CO_2 production rates even after repeated exposures (Figure 6.10), while PA01 recovery times were longer after the second exposure (Figure 6.9). It is possible that the biocide is not equally effective against all members in the multi-species biofilms and that the species that survived the first exposure were present in greater numbers thereafter. It is also plausible that there is greater diversity in the multi-species biofilms (in terms of EPS composition, metabolic responses and genes) and that diversity allowed these biofilms more opportunities for adaptation and survival.

6.3.3. Evolving to survive

The responsiveness and adaptability of biofilms underpin the ability of these communities to evolve and survive in a wide range of conditions. The results presented here show that both single- and multi-species biofilms were able to integrate unexpected events, such as sudden changes in carbon source and

availability or the addition of antimicrobial compounds. Biofilms responded by adapting their metabolism at a community level while still maintaining the ability to release planktonic cells into the environment.

Environmental conditions are easily manipulated in a CEMS without sacrificing the biofilms, allowing biofilm metabolic responses to be monitored on a systems level in real time. A CEMS can be used to further investigate biofilms' ability to replicate successful survival strategies. Previous studies have shown that the history of the inoculum influences biofilm growth rates and other metabolic responses (Jackson et al. 2015). Planktonic cells released from *P. fluorescens* biofilms are able to establish biofilms and high CO_2 production rates within 20 hours, while *P. fluorescens* biofilms from overnight pre-cultures required 40 hours to reach the same levels (Kroukamp et al. 2010). Future studies should use biofilm-derived planktonic cells as inoculum for repeat experiments. This approach could be used to compare responses of biofilms cultured from cells previously exposed to biocides and antibiotic compounds.

Microorganisms in a biofilm are more likely to develop resistance to antimicrobial compounds, through the reshuffling and sharing of genetic information (Molin and Tolker-Nielsen 2003). To understand biofilm behaviour on a systems level, other techniques can be combined with a CEMS to provide more insight into resistance mechanisms and survival strategies. Genomics could be used to study the transfer of genetic information between community members. Microscopic and spectroscopic techniques can be installed in series with a CEMS, allowing real-time investigations into these complex systems.

6.4. Conclusions

In support of the proposed biomimetic framework for biofilms, the aforementioned results showed that:

- Single- and multi-species biofilms are attuned to changes in their environment, as they responded rapidly to changes in carbon sources and nutrient concentration, as well as exposure to biocides (single- and multi-species biofilms) and antibiotics (multi-species biofilms).

- Biofilm adaptions to changing nutrient conditions and exposure to antimicrobial compounds included changes in CO_2 production rates, biofilm-derived planktonic cell yield and community diversity.

- Even when nutrient availability decreased or antimicrobial compounds were introduced, single- and multi-species biofilms still released high numbers of planktonic cells into the effluent, embodying resilience through redundancy and decentralisation.

- Biofilms successfully incorporated unexpected environmental events and were able to survive in a wide range of adverse conditions, such as low nutrient concentrations and exposure to antimicrobial compounds.

- A CEMS, in combination with direct plate count methods and metabolic assays, allowed whole-biofilm responses to environmental changes to be measured in real time.

7. Conclusions

There are three broad goals that drive biofilm research: 1) the prevention of detrimental biofilm formation that leads to biofouling or persistent human infections, 2) the control and manipulation of beneficial biofilms in biotechnological processes and 3) the investigation of fundamental aspects of microbial ecology using biofilms as model systems (Bester 2010).

To date, most research has focused on single-species biofilms cultivated under controlled conditions, while more complex heterogeneous microbial communities in natural environments have received less attention. A large body of information has been gathered on biofilm features and functions, but there is a need to knit the vast array of data together into comprehensive frameworks that acknowledge the complexity and adaptability of biofilms.

To this end, a novel biomimetic framework was proposed as a systems-based approach to biofilm research. Using biomimetic principles in a hierarchical structure, a review of biofilm literature demonstrated that biofilms adhere to all of these principles. The biomimetic framework proposes that, within the context of its environment, microorganisms apply these principles and, in doing so, create dynamic systems that ensure their survival. Subsequently, these principles form the hypotheses investigated experimentally in this study.

In order to develop a robust experimental approach, common methods and analytical techniques used in biofilm research were reviewed and, based on their advantages and limitations, three techniques were selected for this study. ATR-FTIR spectroscopy and CLSM were considered destructive techniques, while a relatively new technique, the measurement of whole-biofilm metabolism using a CEMS, was selected for real-time, *in situ* investigations into biofilm responses. Single-species biofilms were used to standardise and validate the experimental techniques, yielding reproducible results. Optimised protocols were applied to investigate more complex multi-species biofilms.

Considering that randomness is part and parcel of biofilm development, reproducibility seems an elusive goal in this field of research. Of course there is a place for statistical analysis and reproducibility studies in biofilm research. By demonstrating that a cultivation technique yields

reproducible biofilms, conclusions about biofilm behaviour can be drawn with more certainty. Furthermore, given the numerous types of biofilm cultivation systems used worldwide, demonstrating reproducibility is important for comparative studies in order to unify biofilm knowledge.

Nonetheless, when biofilm research is driven by expectations of reproducible results, the risk arises that the true adaptive nature of biofilms might be overlooked. When considering the stochastic nature of biofilm development and the microbial community's inherent abilities to adapt and evolve, it is possible that many insights into these secret societies are masked by standard deviation bars and p values.

A biomimetic approach proposes that variations and heterogeneity encountered in biofilms are vital characteristics of these communities. In the case of this study, high variability was recorded for PA01 *gfp* biofilm parameters measured in low nutrient concentrations (Table 5.7). At first glance, it appears that the cultivation technique does not deliver reproducible biofilms. However, when the scatterplot representations of data at low and high nutrient concentrations are considered together (Figure 5.13), variation in biofilm structures at low concentrations appears to be an adaptation to the environment, rather than an experimental error.

This highlights the importance of studying biofilms using frameworks that acknowledge the complex and adaptive nature of biofilms. To this end, it is important to study trends in biofilm responses rather than focusing on absolute values. It is also necessary to combine various biofilm analysis techniques in order to better interpret variance in biofilm parameters. As demonstrated in this study, microscopic and spectroscopic techniques provide valuable data about biofilms on biochemical and structural levels. To interpret these results on a functional level, methods that measure real-time biofilm responses *in situ* are needed.

The results presented here show that CEMS is a valuable tool for tracking the activity of biofilm communities. Furthermore, it has the potential to be combined with other analytical techniques, e.g. flow cells for microscopic or spectroscopic studies can be installed in series with CEMS bioreactors. This would allow insight into complex responses of biofilms in order to expand the biomimetic framework proposed here. For example, to better understand resource-efficiency in biofilm

communities, the relationship between biofilm morphology and nutrient utilisation could be investigated by combining CLSM, CEMS and biochemical assays in order to study actual resource use, growth rates, structural development and planktonic cell yield. Specifically relating to work presented in this study, future studies would include repeat experiments of environmental changes and antimicrobial exposures using biofilm-derived planktonic cells as inoculum to investigate biofilms' resistance mechanisms and survival strategies.

The biomimetic framework presented here, together with the experimental findings, point to the most important function of biofilms: survival. The success of a biofilm is not measured by the survival of single organisms but by the ability of a microbial community to respond and adapt to ensure the survival of DNA. In this study, CLSM and DIA data showed that both *Pseudomonas* strains were able to multiply significantly in relatively short periods of time and subsequently develop biofilms in all four of the environmental conditions investigated. It is easy to consider the thickest biofilm containing the most biomass a thriving population, and therefore assume the environmental condition is favourable.

From a biomimetic perspective, it is rather irrelevant to speculate which of the four environmental conditions is the "most favourable" because it is evident that biofilm composition and structure are products of a microbial communities' functional needs within a particular environment. Simply put: the microbial community is doing what needs to be done in order to ensure its survival (by replicating DNA and releasing it into the environment). For example, a low nutrient/high flow environment might be considered the least favourable condition, since access to nutrients might be most limited in this case. However, under these conditions, both *Pseudomonas* strains produce thicker biofilms with a greater surface area exposure than at high nutrient concentrations, possibly indicating a more effective nutrient delivery rate per surface area at low nutrient concentrations.

Similarly, when viewing the whole-biofilm metabolic responses presented in Chapter 6, it should not be concluded that a microbial community is faring "better" when its CO_2 production is higher. As the results from this study showed, regardless of the environmental condition or challenge, single- and multi-species biofilms maintained a level of metabolic activity and invested in their survival through the release of high numbers of cells. While a sloughing event might seem like a structural failure, it

might in fact be an example of a biofilm's ability to self-regulate in order to ensure optimum functioning.

One of the most important features of this framework is that it highlights the intrinsic links between the biochemical, structural and functional complexities of biofilms. This holistic approach may, in turn, give rise to novel anti-biofilm strategies. Where most conventional strategies treat the "symptoms" (i.e. use chemicals to eradicate biofilms), the biomimetic framework can be used to find new points of intervention. For example:

- Which environmental conditions enable microbial survival in the system and how can these conditions be altered to inhibit biofilm formation?
- Which cyclic processes are the microorganisms dependent on?
- How can biofilm formation be prevented, considering the structure is built from the bottom up?
- Are there ways to disrupt self-renewal processes within a biofilm?
- How can cooperative relationships in a biofilm be disrupted?

The heterogeneity and adaptability of biofilms make them an inexhaustible source of research potential. The proposed framework can be expanded on by including more case studies and a broader review of current biofilm knowledge to elaborate on each Life's Principle. This study not only contributes a new perspective to biofilm research but furthers the field of biomimicry through academic research. As we come to understand even more strategies of the natural world, biomimicry and its related concepts will evolve, which, in turn, will require development and expansion of this framework.

While no model will ever encompass the complexity of all microbial communities, this biomimetic framework is a workable model that can be used to position future research questions, inform experimental designs and interpret findings in novel ways. The biomimetic framework developed here to describe biofilms can add value in the main fields of biofilm research, namely biofilm prevention, biofilm manipulation and microbial ecology.

"Although human genius through various inventions makes instruments corresponding to the same ends, it will never discover an invention more beautiful, nor more ready nor more economical than does nature, because in her inventions nothing is lacking, and nothing is superfluous."

Leonardo da Vinci, ca 1500[15]

[15]Quoted in Thompson 1999 p.23.

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