CULTURING AIRWAY POLYMICROBIAL COMMUNITIES

**CULTURING AIRWAY POLYMICROBIAL COMMUNITIES
UNDER CONTINUOUS FLOW CONDITIONS**

By AKSHITA PURI, B.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment
of the Requirements for the Degree of Master of Science

McMaster University MASTER OF SCIENCE (2013) Hamilton, Ontario (Biochemistry & Biomedical Sciences)

TITLE: Culturing airway polymicrobial communities under continuous flow conditions

AUTHOR: AKSHITA PURI, B.Sc. (University of Guelph), B.Sc. (Panjab University)

Supervisor: Dr. Michael G. Surette

NUMBER OF PAGES: xiv, 107

ABSTRACT

Microbes are ubiquitous in the biosphere and play important roles in natural ecosystems. They are typically present as diverse, complex communities, and in humans these communities are present on all exposed surfaces and mucosal tissues. The human upper respiratory tract harbors a complex microbiome and the composition includes what are traditionally considered commensal organisms, including a significant proportion of anaerobic bacteria. It is generally assumed that most of the bacteria from any particular environment cannot be readily cultivated, including the human microbiome. Some *in vitro* microfluidic and *in vivo* models are available to study the airway microbial communities, however these methods are expensive, limited and are not practical for experiments manipulating the community. A robust culture-based approach that can propagate these polymicrobial communities has been developed in this study to investigate spatial-temporal changes in bacterial populations *in vitro*. Matrix embedded synthetic bacterial communities, comprised of aerobes and anaerobes, were cultivated in continuous flow cell systems. The structure of communities propagated in these systems was compared to those in static and shaken batch cultures. The data shows that reproducible stable bacterial communities can be propagated with these culture methods, however the community composition varies considerably with the approach used. Only matrix embedded communities, cultured under continuous flow conditions, could successfully retain obligate anaerobes when flow cell systems were operated in an aerobic environment. This optimized method was used for culturing complex and diverse natural communities from clinical samples (sputum). The majority of bacteria present in the

original sample were recovered in flow cell cultures and the methodology was consistent. This study provides an experimental system that can be used for examining microbial community dynamics and community structure-function relationship.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor, Dr. Michael Surette, for allowing me to be a part of his lab as a graduate student. You have been my source of inspiration and I feel extremely fortunate to have had the opportunity to work with you. Thank you for granting me the freedom to think independently and to make my own decisions throughout my graduate training. I appreciate your encouragement, support and your constant faith in me. You have helped me shape my interests and ideas, and it amazes me how much I have learnt in the past two years.

I would also like to thank my committee members, Dr. Nathan Magarvey and Dr. Lori burrows, for their suggestions and advice during my committee meetings. I am extremely grateful for your support, feedback, patience and ideas through this process. Our meetings really helped shape the course of my project and I thank you immensely for that.

My sincerest thanks also go to my wonderful lab mates. Thanks for your continual support, help at the bench and great company. I am very much indebted to you all for creating a stimulating environment and helping me throughout my training.

Finally, I would like to thank my parents for their love and support in all my efforts. Even though I live so far away, you have been a source of strength to me throughout and this distance only motivates me to work harder and make you proud parents one day. I would not have had made it this far without you both.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

URT – upper respiratory tract

LRT – lower respiratory tract

OF – oropharyngeal flora

CF – cystic fibrosis

CFU – colony-forming unit

TVC – total viable count

T-RFLP – terminal restriction fragment length polymorphism

CBA – Columbia blood agar

BHI – brain heart infusion

PCR – polymerase chain reaction

PCoA – principal coordinate analysis

NF-κB – nuclear factor kappa B

LPS – lipopolysaccharides

SEAP – secreted embryonic alkaline phosphatase

AI-2 – autoinducer-2

DECLARATION OF ACADEMIC ACHIEVEMENT

All work in this thesis was completed by me except the following: Tissue culture for host assays was conducted by Julie Kaiser and Jennifer Stearns from the Surette lab.

Processing of Illumina sequencing data was done by Fiona Whelan from the Surette lab.

CHAPTER 1: INTRODUCTION

1.1 Polymicrobial communities

Microorganisms are ubiquitous, thriving in all types of natural environments on earth usually as members of diverse and complex microbial communities[1]. The majority of the phylogenetic diversity on earth is microbial and only about 5000 of noneukaryotic organisms and between 0.1% and 1% of the total bacterial species have been characterized, most of which cannot be readily cultivated[1-3]. The human microbiome (totality of microbial genes and genomes) is comprised of complex communities in various body sites and these habitats are diverse[4-6]. The number of microbes living on and inside the human body is 10 times higher than the total number of human cells, where the genes and metabolic features of both human and microbes together impart characteristics of a 'supraorganism' to humans[4, 5].

Some of the major body sites that have been characterized using both culture-dependent and culture-independent techniques include: gut, vagina, mouth and skin[4]. The polymicrobial communities are dynamic but stable over time, however the proportion of bacterial species that constitute the temporal 'core microbiome' of a body site is low and these communities are inhabited by transient members at different times[7]. The overall diversity in a community can be guided by biological interactions with the invading species, which can determine whether or not the community remains stable and have a corresponding effect on overall ecosystem health[4, 8, 9]. Higher diversity could either

1

increase or decrease the function of the ecosystem[10-12]. Furthermore, microbe-host/environment interactions also play a very important role in the community structure (composition) and stability by keeping a check on the invading organism and defining its potential for pathogenicity[4].

A multitude of microbe-microbe interactions can occur amongst the members of these complex communities where both intra- and interspecies interactions are taking place via the exchange of chemical and physical signals[13]. These interactions can be passive or active, for example, competition for common resources (passive) and the release of secondary metabolites like bacteriocins to directly inhibit neighbouring individual cells/cell populations (active)[13]. Experiments to understand the nature of these interactions reveal some very fascinating properties. Quorum sensing is an example of cooperative behaviour within bacterial populations, where perception of extracellular signals and corresponding response occurs in a concentration dependent manner[13-15]. There are instances when mutants which are adapted to being more fit when present as a minority can arise within actively multiplying populations and consume nutrients from the surrounding environment at a very fast rate[14]. For example, social cheating within cooperative microbial communities arises where these social cheaters exploit common goods without making any contribution themselves[13, 14]. Syntrophic interactions involve two or more microbes that metabolize a common resource in which the metabolic steps of the pathway are carried out by different bacteria[13]. An example where physical interactions have been shown to play a role in influencing the structure of the community

is the oral microbial community. Some members of this community thrive more successfully when isolated as co-aggregated colonies in culture from oral biofilms[13]

1.2 Airway polymicrobial communities

1.2.1 Airway microbiota in health and disease

The airways (upper respiratory tract (URT) and lower respiratory tract (LRT)) harbor diverse microbial communities and its microbial diversity is comparable to that of other body sites like the gastrointestinal tract, skin and oral cavity[16]. Different (nasopharynx, oropharynx, lungs) sites in the airways harbor distinct microbial communities. The nasopharynx is dominated by the members of phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria,* whereas *Bacteroidetes* is the most abundant phylum found in the oropharynx[16-18]. It is noteworthy to mention that obligate anaerobes like *Fusobacterium* spp. and *Prevotella* spp. are prominent and key members of the respiratory tract microbiome[17]. Nasopharynx in healthy individuals is also sometimes colonized by potential pathogens like *Streptococcus pneumonia, Haemophilus influenzae,* and *Moraxella catarrhalis* where colonization usually occurs during childhood. A lot of factors contribute towards the increased risk in carriage of such potential pathogens and have been extensively reviewed elsewhere[19]. Previously, the LRT was regarded as a sterile site and there are still conflicting views regarding its sterility. Charlson *et al.* demonstrated the presence of bacterial sequences in bronchoalveolar lavage samples obtained from healthy subjects where it was observed that the biomass in the lungs was very low as compared to the URT. They concluded that the bacterial

species present in the lungs are either acquired as a consequence of microaspiration or are carryovers from bronchoscopes during sampling procedures, thereby being mostly transient[18]. However, other studies support the existence of a distinct lung microbiome with members of the phyla *Firmicutes, Proteobacteria* and *Bacteroidetes* being most abundant[20-22]. Repeated sampling and monitoring microbial community dynamics over several time points has confirmed the overall temporal stability of airway polymicrobial communities[16]. It can be argued that a community inferred by sequencing does not necessarily imply a viable community. Charlson *et al.* surveyed a population of 62 adults comprising both smokers and non-smokers, and a key outcome of the study was that the airway microbiota in smokers showed more diversity where specific bacterial groups (a marked increase in numbers of Gram positive anaerobes) were significantly altered by smoking[16]. Therefore, environmental factors can also play a pivotal role in microbial community structure, behavior and function. What is lacking is an understanding of the biological function of these microbial communities in both healthy and disease states.

The microbial community composition in airway diseases like asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) is polymicrobial and changes in composition have been implicated as drivers of exacerbations and airway inflammation. Community structure is disturbed in asthma and COPD, and colonization with bacterial groups like *Haemophilus* spp. during early childhood has been linked to an increased risk of development of chronic airways diseases and infections later in life[19]. CF is a classic example of a polymicrobial infection in which the lower airways become more vulnerable and the successful colonization of pathogens more probable, which can

culminate into chronic LRT infections[23, 24]. CF airway microbiome is a very dynamic ecosystem and the community architecture of this system varies with clinical status of the disease[25, 26].

Although principal pathogens like *Pseudomonas aeruginosa, Burkholderia* spp. and *Staphylococcus aureus* have always been implicated in CF airway infections, recent studies have shed more light on the role of oropharyngeal flora (OF) in CF emphasizing the need for adoption of a holistic approach by investigating polymicrobial communities in airway diseases[27]. The detection of anaerobic bacterial groups in CF sputum samples has suggested the important role of these organisms in CF airway microbiology by causing changes in the overall microbial community structure and dynamics[28, 29]. The presence of anaerobes in CF (*Prevotella* spp*., Veilonella* spp*., Fusobacterium* sp.) has made effective treatment even more challenging as a majority of anaerobes are not recovered by standard clinical microbiology protocols[30-32].

1.2.2 Characterizing the airway microbiota

Current knowledge of the microbial community composition of airways in healthy and disease states originates primarily from extensive 16S ribosomal RNA gene sequence analysis[33, 34]. Diagnostic clinical microbiology methods have primarily been designed to select for known pathogens[27, 35, 36]. These methods underestimate the diversity of bacteria present in most samples. Devising new cultivation methods and incorporating changes to existing culture protocols can increase the number of bacterial groups that can be recovered with culturing alone to a great extent. Sibley *et al*. demonstrated that a majority of the CF airway microbiome is culturable[27]. By including a range of different growth

conditions in the culture protocols and applying molecular techniques to reveal the composition of the resultant culture enriched microbial community, it was shown that 84% of the bacterial groups detected in the original sample can be recovered in culture[27]. Based on the aforementioned study, it becomes evident how a change in approach can be effective in capturing the complexity of the airway microbiome with culture-based methods *in vitro*. Our understanding of the biology and behavior of complex communities in airways will improve significantly by conducting studies that can potentially capture the microbial diversity of airways in experiments *in vitro*, and provide an experimental system to study community dynamics and function.

1.3 Methods for cultivating polymicrobial communities

1.3.1 *In vitro* and *in vivo* models utilized in investigation of oral microbiota

Over the past few years researchers have used several *in vitro* and *in vivo* methods in an attempt to better understand the microbial community architecture, spatial organization of species within the community, whole community function, role of environmental factors and community-host interactions. Studies on oral microbial communities have contributed significantly to our understanding of the role of natural microbial communities in health and disease[37, 38]. Batch cultures, continuous cell cultures (chemostats and flow cells) and *in vivo* rat models have been utilized to investigate interspecies interactions operating in complex dental microbial communities which can harbor >500 species[39]. Batch cultures have been used as a method for studying the physiology, metabolism, production of virulence factors, and interactions in bacterial

populations for several years. However, one of the disadvantages associated with using some *in vitro* models (e.g. batch cultures) is that they do not necessarily depict the true patterns of microbial growth and colonization in natural environments[40].

Although we can vary the environmental conditions and study the corresponding effect on the microbial populations in broth cultures, the reality remains that bacteria multiply at a very fast rate in lab cultures[40]. In order to understand the metabolic properties and obtain a better picture of bacterial population dynamics with time, continuous culture chemostat systems (to study planktonic mode of growth) have also been utilized to obtain a steady-state mode of growth in culture[41]. The main principle behind its operation is that by controlling the flow of medium in the vessel, the growth rate can be adjusted and the system can thereby be designed to obtain slower bacterial growth rate by limiting some essential nutrient. The oral cavity harbors heterogeneous bacterial populations and the multispecies interactions in this complex community have been studied in great detail previously. Mixed species oral communities grown in chemostats have been used to investigate the growth behaviour and interspecies interactions[41, 42]. Despite of the advantages, the true ecological niche that exists in natural habitats cannot be generated in chemostat systems as in some cases in nature, microbes grow on surfaces and it can be very hard to mimic the true physical properties essential to support the colonization of a representative community[41, 43]. Additionally, flow cell culture methods (to study biofilm mode of growth) have been used extensively in the past for studying growth and development in mixed-species oral biofilms, are valuable models

that can be used/developed/modified for culturing microbial communities from other body sites in the lab[37, 38].

With the confirmation that *in vitro* methods could propagate reproducible oral communities, detailed studies revealed mechanisms of communication between oral bacteria and also how an organized series of colonization and succession events can guide the formation of plaque biofilms in the oral cavity[44, 45]. Other areas of research included studying oral biofilm community architecture, interspecies interactions which further unraveled the role of specificity in biofilm formation (preference for colonization with certain bacterial groups), the role of physical interactions in biofilm formation, and different mechanisms of metabolic communication[37, 38].

1.3.2 Multispecies interactions in oral microbial communities

Bradshaw *et al* have demonstrated how an obligate anaerobe like *Fusobacterium nucleatum* coaggregates with initial and early colonizers during biofilm formation, and how such physical interactions can have an effect on anaerobe survival[46]. By coaggregating with partner community members like streptococci, *F. nucleatum* permits growth of other obligate anaerobes like members of the genus *Prevotella*. Moreover, the development of oxygen gradients was shown to play a key role in sustaining the existence and growth of obligate anaerobes in the thriving microbial community[46]. Prior to this study Bradshaw *et al* had also determined how mixed community cultures could sustain the growth of obligate anaerobes under aerated conditions in both chemostats and biofilms[47].

Yet another study elucidated the role of other anaerobic residents of these biofilms, *Veillonella*, in establishment of these complex communities where members of this bacterial group were seen to show remarkable specificity towards *Streptococcus oralis*[48]. *Veillonella* utilizes lactic acid produced by *Streptococcus* spp. and fails to grow in its absence in these communities. These specific interactions were further studied in an *in vivo* rat periodontal infection model, where *Veillonella* was unable to cause mono-species infections. On the other hand, infecting a rat with precolonized *Streptococcus mutans* resulted in a mixed infection comprising of *Streptococcus-Veillonella* communities[39]. More mutualistic interactions were uncovered with studies on other community members like *Actinomyces* sp*., Streptococcus gordonii,* and *Streptococcus oralis* where certain multispecies combinations could not survive verifying further how each species has characteristic properties of attachment and colonization in biofilms[49]. The architecture of these heterogeneous populations and the spatial organization has been monitored with time by utilizing confocal laser scanning microscopy[50]. Guggenheim *et al* demonstrated that during the initial colonization events, microbial cells did not form any aggregates and were free floating whereas as time progressed, microcolonies of various sizes and shapes were observed restricting the single cells to the surface of these biofilms[45]. The mechanisms of communication in oral bacteria have been reviewed extensively elsewhere[38]. Intra oral devices like Leeds *in situ* device, have also been utilized for *ex vivo* studies to investigate the structure of these communities in their natural environments[50]. Even though characterizing community interactions in the airways will always be much more challenging as some anatomic sites are not easily

accessible, exploiting culture-based methods can not only help us better understand community dynamics but can potentially provide some valuable insights into whole community function.

1.3.3 Other methods for microbial community cultivation

The different culturing methods that have assisted culture of uncultivable groups from environmental samples like soil have been extensively reviewed elsewhere[51, 52]. Bollmann *et al* developed an *in situ* model for the cultivation of environmental bacteria, which enabled recovery of a very diverse microbial community and some rarely cultivated/uncultured isolates. The inoculum was sandwiched between semipermeable membranes placed in a chamber, and this diffusion chamber was subsequently brought back to the original environment. Post-incubation the growing community was passaged to enrich for rare members of the community[53]. This study brings forward the importance of presence of neighboring bacterial species in a growing microbial community. This is one of the many reasons why some organisms cannot be recovered on synthetic media in a laboratory. A subsequent study by Kim Lewis and coworkers demonstrated how chemical signals and growth factors (siderophores in this case) produced by helper bacteria can be essential to recover rare organisms[54].

Recent advancement in microfluidic based culturing approaches has provided additional opportunities to investigate microbe-microbe, microbe-host and microbe-environment interactions[55, 56]. The dynamic physical and function features of the human intestine were recapitulated in a two-channel microfluidic device lined with a membrane combining an extracellular matrix and human epithelial cell linings. By mimicking the

flow rates and shear rates that exist in a human intestinal environment, researchers were able to maintain a controlled microfluidic environment on this human gut on a chip model[55]. Such an *in vitro* model can be utilized in drug testing and host-microbe interaction studies. Park *et al.* used the microfluidic approach to generate micro-droplets and cultured microbial communities in these to probe symbiotic interactions. By controlling the dilution ratio in the device, such interactions were investigated in various scenarios where at least one of the partners in the symbiont pair was rare. The device was designed in a manner that the distribution of the microbial cells in each droplet could be predicted[56].

Other studies have adopted methods to isolate and cultivate organisms in different ways, and also monitor the phenotypes of the growing populations in order to understand the growth strategies utilized by microbes in natural environments[57-59]. A micro-petridish with a million growth compartments was designed for high throughput culturing and screening of microcolonies for functional applications like detection of enzyme production[59]. Furthermore, application of high throughput culture methods utilizing dilution to extinction approach have resulted in successful culture of novel taxa from seawater[58]. Kishony and coworkers investigated the growth properties of microbes present in a soil microbial community and demonstrated that the presence of neighbouring cells increases the likelihood of formation of visible colonies on growth medium. Thereby, one can boost cultivability by plating intermediate cell densities and increasing incubation times to allow the appearance of more colony types. This can help

overcome the problem of overcrowding of plates at high densities and underestimation of microbial diversity from an environmental sample[57].

1.4 Overall aims of the study

The human upper respiratory tract (URT) harbors complex microbial communities and the composition includes what are traditionally considered commensal organisms including a significant proportion of anaerobic bacteria. However, the upper airways are also colonized by pathogenic organisms, which are carried asymptomatically in most healthy individuals. The balance of asymptomatic carriage and disease is a result of a complex interplay of the microbiome, pathogen, host and environment (**Figure 1.1**). Although bacteria exist predominantly in microbial communities in natural environments, there is a lack of understanding of the complex processes that operate in these communities. The difficulty encountered in culturing these communities in the lab complicates the problem. Some *in vitro* microfluidic and *in vivo* models are available to study the airway microbial communities, however, these methods are expensive and limited and in the context of the human microbiome it is not very feasible to do experiments manipulating the microbial community[60-62]. Work done on culturing oral microbial communities and communities present in other environments like soil and marine habitats provides valuable benchmarking data on microbe-microbe, polymicrobe-host and polymicrobe-environmental interactions[38, 39, 44, 45, 47, 51, 52]. The approaches utilized in these studies can be adopted to conduct similar types of studies on airway polymicrobial communities. Complementation of culture-independent methods that provide information on community composition[63, 64] with culturing can provide invaluable insight into microbial physiology of the airway communities, and will help better understand the role airway microbiome in health and disease.

The central objective of the current study was to develop robust methods to propagate and examine mixed synthetic and natural microbial communities (comprised of aerobes, facultative anaerobes & obligate anaerobes) *in vitro*. This project aimed to develop an experimental system to study the nature and behaviour of the complex bacterial communities that reside in the airways, in terms of the community structure, function and dynamics.

The main goals of the current study were:

a) Develop/optimize different culture methods with synthetic microbial communities *in vitro*

b) Propagate natural microbial communities from clinical samples by utilizing the optimized methods

The current study looked to answer the following questions with the aforementioned goals:

a) Can polymicrobial communities be reproducibly propagated with different culture methods?

b) Can complex polymicrobial communities, comprised of aerobes, facultative anaerobes and obligate anaerobes, be successfully propagated directly from a clinical sample in an aerobic environment?
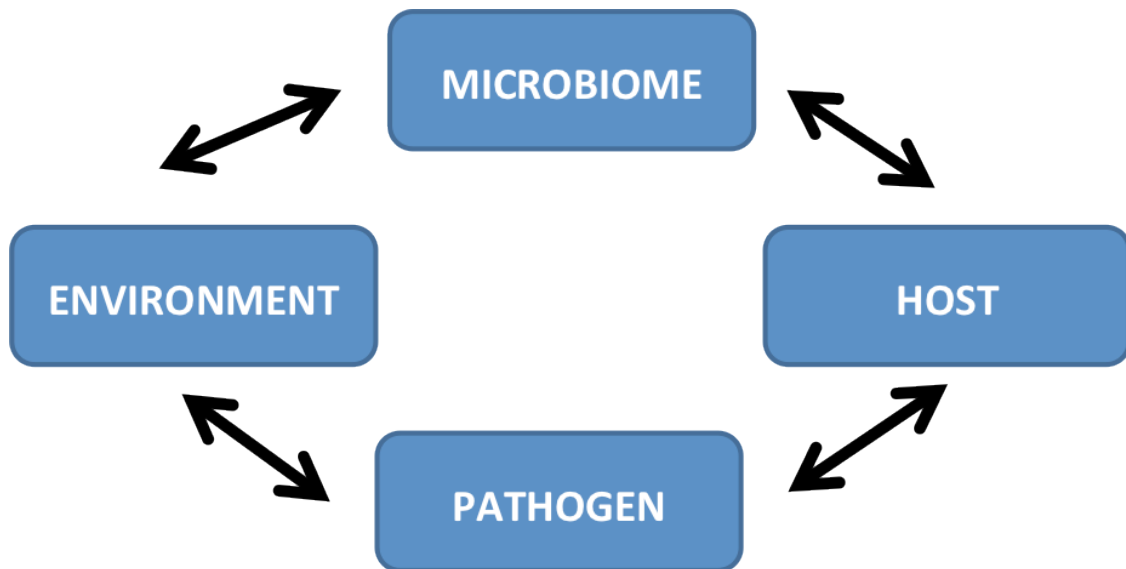
Figure 1.1 - A diagrammatic image depicting the complex interactions in place in the human upper respiratory tract (URT).

CHAPTER 2: MATERIALS AND METHODS

2.1 Culture and molecular methods: synthetic microbial communities

2.1.1 Bacterial strains and growth conditions

All the strains (**Table 2.1**) were recovered from -80°C glycerol stocks and always maintained on Columbia blood agar (CBA) supplemented with 5% sheep blood prior to inoculum preparation. *Achromobacter xylosoxidans* C54, *Staphylococcus lugdunensis* C2101, *Staphylococcus epidermidis* C463 and *Streptococcus salivarius* C412 were incubated for 48 hours at 37°C in air (under 5% CO_2). *Prevotella melaninogenica* C994, *Fusobacterium nucleatum* P6B11 and *Veillonella atypica* C855 were grown in an anaerobic atmosphere (90% N_2, 5% CO_2, and 5% H_2; Bactron, SHEL LAB) for 4 days at 37°C. Obligate anaerobes are those strains that are capable of growing anaerobically but not aerobically. The strains listed in **Table 2.1** were grown on duplicate CBA plates initially and incubated under both 5% CO_2 and anaerobic conditions to confirm conditions for growth.

2.1.2 Batch culture methods (static and shaken)

2.1.2.1 Preparation of inoculum and culture conditions

Aerobic strains (C54, C2101, C463, C412) were incubated overnight in sterile brain heart infusion broth (BHI) (BD) at 37°C under 5% CO_2 conditions. The anaerobes (C994, P6B11, C855) were incubated for 48 hours in BHI broth at 37°C in an anaerobic atmosphere. Inoculum for each microbial community combination (designated as day 0

starting community) was prepared by mixing the bacterial strains in 5ml of BHI, where each bacterial strain was adjusted to be approximately in the final range of 10^5-10^6 colony forming units per milliliter (CFU/ml). Aerobic synthetic communities were incubated under static and shaking conditions at 37°C under 5% CO_2. Mixed synthetic communities were incubated under static conditions in an anaerobic atmosphere for 48 hours, and were subsequently transferred to air and incubated under 5% CO_2 at 37°C. The aerobic communities in batch cultures were subcultured every 24 hours by vortexing the culture for 5 seconds and transferring 50 µl of this culture into sterile 5 ml of BHI broth. Subsequently, composition of the microbial community was characterized at various time points with total viable counts and T-RFLP (**Figure 2.1**).

2.1.3 Continuous culture methods

2.1.3.1 Preparation of the matrix embedded microbial community

A 0.4% (w/v) agarose matrix was prepared by dissolving agarose (UltraPure^{TM}) in BHI broth, which was then autoclave sterilized. BHI broth cultures for the bacterial strains of interest were then prepared. Each microbial community combination was prepared by mixing the bacterial cultures with sterile 0.4% (w/v) molten agarose (after cooling it to 45-50°C), where each strain was adjusted to be in the final range of 10^5-10^6 CFU/ml (**Figure 2.2**). 60 µl of this pre-mixed agarose was immediately pipetted into the flow cell channel, ensuring that the matrix solidified only along the length of one wall in the channel to allow clear passage of media through the rest of the flow channel. Therefore, only one surface of the matrix embedded community is directly exposed to the flow of culture medium in the flow cell (**Figure 2.3**). The flow of media was started 30

minutes after matrix deposition. The flow cell slide (with the matrix) and the matrix recovered at the end of a flow cell run are shown in **5.1** (Appendix)**.**

2.1.3.2 Flow cell system design

The components of a flow cell system (**Figure 2.4**) include: a flow cell (ibidi μ-slide I, sterile; ibidi, Germany; catalog#: 80191) with one flow channel (growth chamber) (channel height = 800 μm, channel length = 50 mm, channel width = 5mm), reservoir (250 ml Erlenmeyer flask) with growth medium (BHI medium), pump to draw media (Minipuls 3 peristaltic pump; catalog#: GF-F155001; Mandel Scientific), four channel pump head (Mandel Scientific; catalog#: GF-F117606), eight channel pump head (Mandel Scientific; catalog#: GF-F117608), flow tubing (1 mm ID; Mandel Scientific; catalog#: GF-F1825112), connector tubing (1.52 mm ID; Tygon), PVDF connectors (Mandel Scientific; catalog#: GF-F1179941), male luer lock connectors (ibidi, Germany; catalog#: 10826) and an effluent flask (250 ml Erlenmeyer flask). The thickness of the agarose matrix in the flow channel was 800 μm (based on channel height). For easier access to the matrix in the flow channel, a bottomless one-channel slide (ibidi sticky μ-slide I, sterile; ibidi, Germany; catalog#: 80198) with a self-adhesive underside was used in some cases. After pipetting the matrix into the flow channel, a sterile clear glass microscope slide (Fisherbrand) was firmly placed over adhesive side of the sticky slide and was cured overnight to ensure a tight seal. The tubing, connectors and flasks (with one hole rubber stoppers) were assembled and then sterilized by autoclaving. When the sticky slides were used, glass microscope slides were sterilized with ethanol prior to flow cell assembly.

After preparing the matrix embedded community (see Section **2.1.3.1**), media was drawn through the flow cell continuously with a peristaltic pump at a rate of ~500 µl/hour. For culturing an aerobic community (C54, C2101, C463 and C412) (**Table 2.1**), the flow cell system was operated in air inside a 37°C incubator under 5% CO_2 conditions for a period of 4-7 days. To culture a mixed community (comprised of both aerobes and anaerobes), the flow cell setup was assembled and operated in an anaerobic chamber (90% N_2, 5% CO_2, and 5% H_2; Bactron, SHEL LAB) at room temperature for 48 hours. This step allowed the establishment of anaerobes in the mixed community, as a premature transfer to aerobic conditions could possibly result in the loss of obligate anaerobes. At the 48 hour time point (designated as day 2 (ana); ana: anaerobic environment), the entire setup was transferred into air and operated inside a 37°C incubator under 5% CO_2 conditions for a period of 4-7 days. To prevent back flow of medium from the flow cell into tubing during the transfer step, both upstream and downstream tubing were clamped. 1 ml of effluent culture was collected in 1.5 ml microfuge tubes at several time points for further analysis. The matrix was recovered on the last day and was used for further characterization.

2.1.4 Characterization of microbial community composition

2.1.4.1 Total viable counts

The bacterial composition in batch cultures, flow cell cultures and matrix was determined by serial dilutions of samples at various time points and plating 100 µl of the dilutions on CBA supplemented 5% sheep blood. Plates were incubated at 37°C under 5% CO_2 for 48 hours (aerobic communities) and anaerobic conditions (90% N_2, 5% CO_2,

and 5% H_2; Bactron, SHEL LAB) for 96 hours (mixed communities). Cell numbers for each organism were calculated as CFU/ml. The relative abundance of each species in the community was determined based on the calculated CFUs.

2.1.4.2 Extraction of bacterial DNA

Bacterial genomic DNA was extracted from the broth cultures, flow cell effluent and matrix samples through mechanical lysis with bead beating. The extraction protocol was a modification of a procedure described previously[27]. 300 µl of the sample was added to a 2 ml plastic screw top tube containing 0.2 g of 0.1 mm glass beads (MO BIO laboratories, Inc.). Subsequently, 800 µl of 200 mM $NaPO_4$ (pH 8) and 100 µl of guanidinium thiocyanate-EDTA-sarkosyl (GES) were added to the tube and homogenization was performed for 3 minutes in a benchtop bead-based homogenizer (PowerLyzer^{TM} 24; Medicorp, Inc.). For first enzymatic lysis step, 50 µl of lysozyme (Sigma-Aldrich, 100 mg/ml in ddH$_2$O), 50 µl of mutanolysin (Sigma-aldrich, 10U/µl) and 10 µl of RNase A (Qiagen, 10 mg/ml in ddH$_2$O) were added to the homogenized sample. The sample was mixed by vortexing and incubated at 37°C (waterbath) for 1-1.5 hours. Post-incubation, 25 µl of 25% (w/v) SDS, 25 µl of Proteinase K (Invitrogen, 20 mg/ml) and 100 µl of 5M NaCl were added to each sample. The samples were mixed by vortexing and incubated at 65°C (waterbath) for 0.5-1.5 hours. After incubation the tubes were again spun at maximum speed for 5 minutes on the bench top centrifuge. 900 µl of the supernatant was removed and transferred to a 2 ml tube containing 900 µl of phenol-chloroform-isoamyl alcohol (25:24:1). After vortexing the tubes for 10 seconds (to shear DNA), they were spun at maximum speed on a bench top centrifuge for 10 minutes. After

centrifugation, the top layer was carefully transferred to a 1.5 ml microfuge tube. DNA clean and concentrator-25 kit (Zymo Research) was used for purifying the DNA, where DNA was eluted from the column with 50 µl of water and the samples were diluted to a final concentration of 20 ng/µl. DNA samples were quantified using a Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific).

2.1.4.3 Terminal Restriction Fragment length Polymorphism (T-RFLP) Analysis

For PCR amplification for the T-RFLP analysis, a total of 20 ng of DNA was used as template in a PCR reaction with primers 8f (5'-AGAGTTTGATCCTGGCTCAG-3') labeled with 6FAM (ABI Biosciences), and 926r (5'-CCGTCAATTCCTTTRAFTTT-3') labeled with HEX (ABI Biosciences). Every sample was subjected to three independent polymerase chain reactions and the resulting products were pooled and purified using a DNA Clean and Concentrator-5 column (Zymo Research). 200 ng of each purified PCR product was digested with 20 U of CfoI (Roche) according to the manufacturer's instructions at 37°C for at least six hours. Approximately 5 ng of digested PCR product was injected into ABI 3730 Genetic Analyzer, and fragment analysis was be done using the software package Peak Scanner (ABI Biosystems); LIZ1200 (ABI Biosystems) was used as size standard. The total area under the peak for a given T-RFLP profile was totaled and each T-RF was represented as a percent of the total peak area. T-RFs were assigned by performing the fragment analysis protocol on 16S amplified from purified colonies of each bacterial species (**Table 2.2**).

Table 2.1 - Strains used in this study.

Strain #	Organism ID	Source*	Description
C54	*Achromobacter xylosoxidans*	CF†	Aerobe
C2101	*Staphylococcus lugdunensis*	Sinusitis	Facultative anaerobe
C463	*Staphylococcus epidermidis*	CF†	Facultative anaerobe
C412	*Streptococcus salivarius*	CF†	Facultative anaerobe
C994	*Prevotella melaninogenica*	CF†	Obligate anaerobe
C855	*Veillonella atypica*	CF†	Facultative anaerobe
P6B11	*Fusobacterium nucleatum*	Asthma	Obligate anaerobe

*Clinical sample from which the isolate was recovered
†Cystic fibrosis

Table 2.2 -Terminal restriction fragment (T-RF) sizes for bacterial species used in this study.

Species	8f (5')	926r (3')
Streptococcus salivarius, C412	575	342
Staphylococcus epidermidis, C463	234	225
Achromobacter xylosoxidans, C54	560	63
Staphylococcus lugdunensis, C2101	234	225
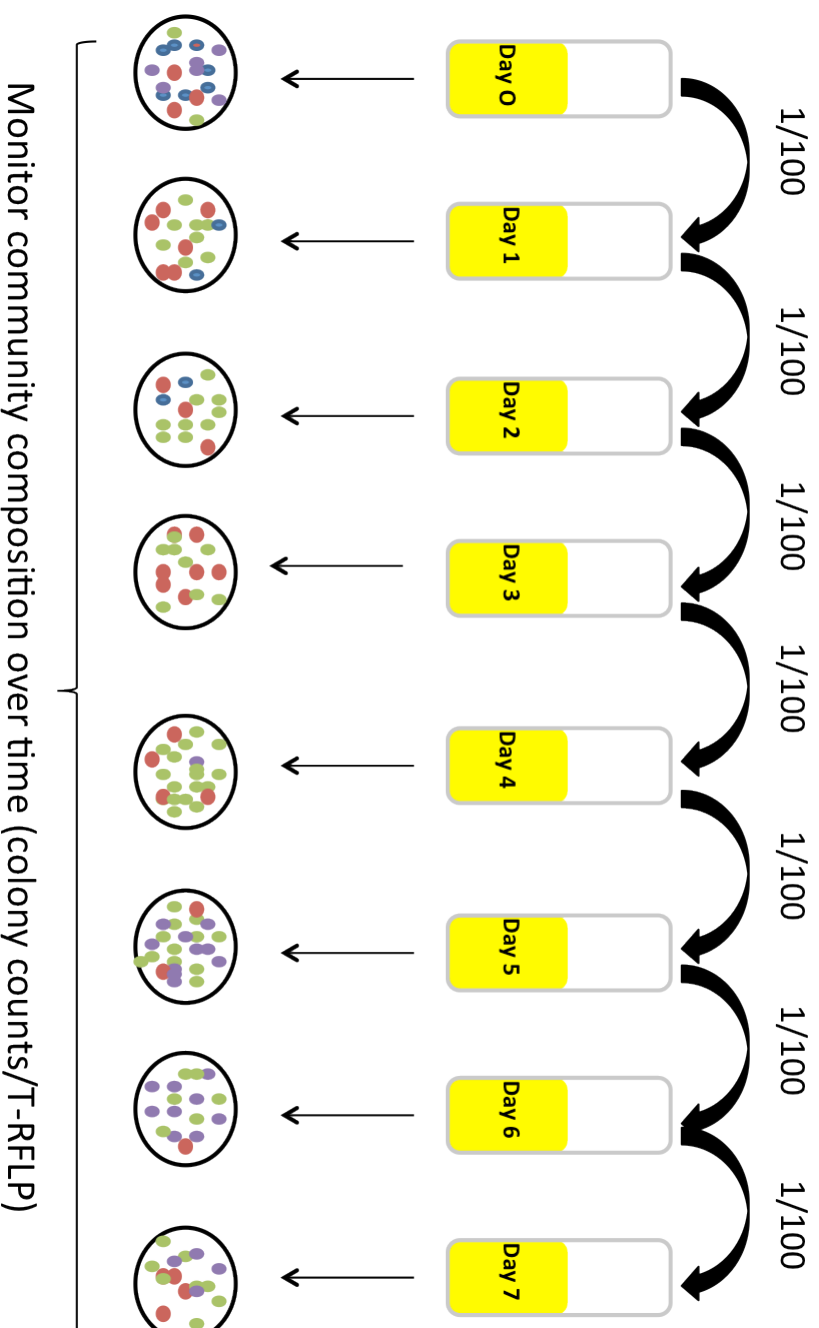
Figure 2.1 - Schematic diagram of the batch cultures.
The cultures were subcultured every 24 hours and the community structure was monitored by total viable counts and terminal restriction fragment length polymorphism.
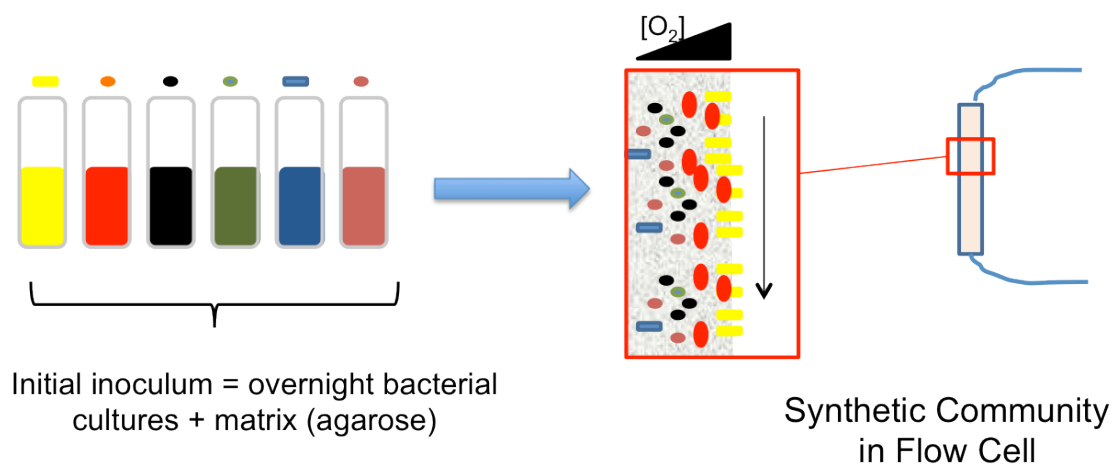
Figure 2.2 - Preparation of the matrix embedded microbial community.
Bacterial cultures were used to prepare the initial inoculum. Inoculum was then embedded in a 0.4 % agarose matrix. This matrix embedded microbial community was cultured in a flow cell under continuous flow of media.
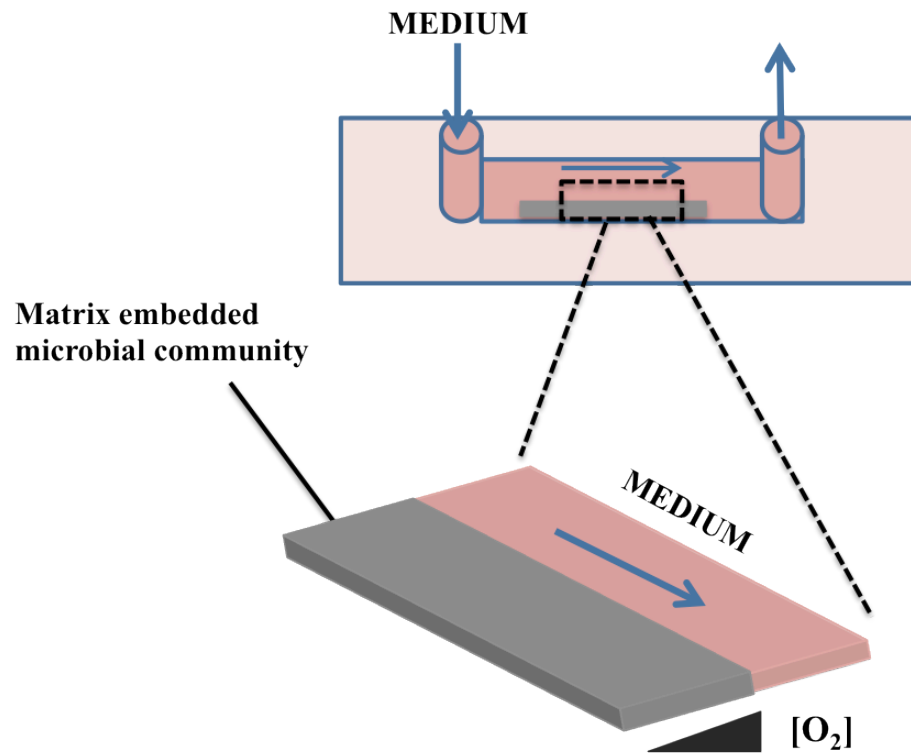
Figure 2.3 - Matrix embedded community in flow cell chamber (ibidi μ-slide).
Only one surface of the matrix embedded community is directly exposed to the flow of culture medium in the flow cell.
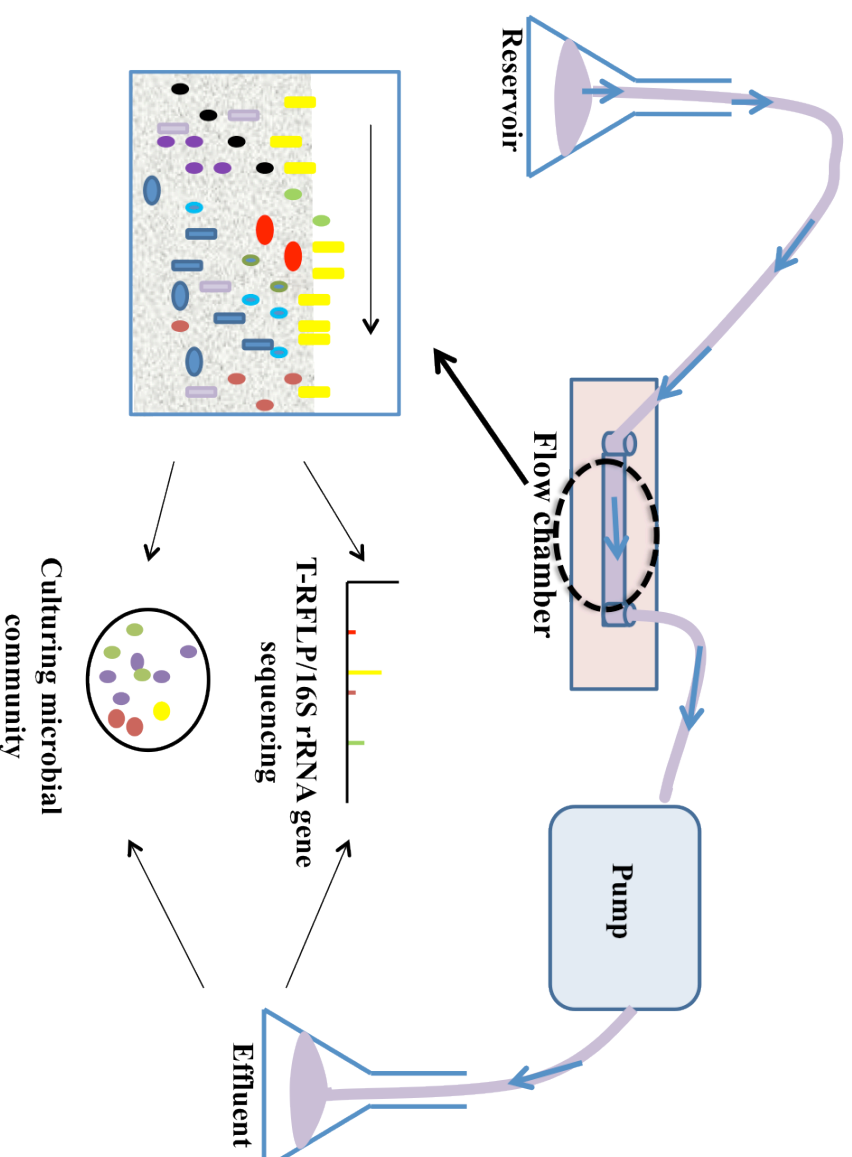
Figure 2.4 - A diagram of the entire flow cell setup.
The matrix embedded community is introduced in the flow chamber, which is connected to the medium reservoir and pump with tubing. The culture media is drawn through the flow chamber at the desired flow rate with a pump. The composition of the growing microbial community can be analyzed with different methods.

2.2 Culture and molecular methods: natural microbial communities

Sputum samples were collected with the informed consent of all the patients. Three sputum samples were obtained on the same day from three asthmatic patients (patient IDs: PN32, PN33 and PN34). These samples were cultured in the flow cell system to propagate natural microbial communities under continuous flow conditions. For comparison, the samples were also cultivated on various media types. Cultivable bacterial communities in the flow cell system were investigated by characterizing the bacterial groups present in the matrix and effluent, followed by an overall comparison to the groups detected in the original clinical sample. Profiling of microbial populations in all the samples was done by sequencing amplicons of the V3 region of the16S rRNA gene on an Illumina MiSeq **(Figure 2.5)**.

2.2.1 Sample preparation and flow cell setup

Upon sputum induction, each sputum sample (PN32, PN33 and PN34) was transferred into a sterile container and was shipped on dry ice. The sputum was then sheared with vigorous passage through a 1cc syringe (without a needle). For propagating communities from the clinical samples in flow cells, the matrix embedded community was prepared as described previously (see Section **2.1.3.1**). Samples PN32, PN33 and PN34 were diluted 1:100 in 0.4% molten agarose. The entire flow cell setup was assembled and operated in the anaerobic chamber (90% N_2, 5% CO_2, and 5% H_2; Bactron, SHEL LAB) at room temperature for 48 hours. At the 48 hour time point (designated as day 2 ana; ana: anaerobic) the setup was transferred into air and operated

inside a 37°C incubator under 5% CO_2 conditions for a period of 5 days. The last time point was designated as day 7 (on day 7, the flow cell system has been operated under an anaerobic and aerobic environment for 2 days and 5 days respectively). 1 ml effluent cultures were collected in 1.5 ml microfuge tubes on day 2 and day 7 for further analysis. The matrix embedded community was also recovered on day 7 and used for further analysis.

2.2.2 Microbiological profiling

The sheared sputum sample was serially diluted in BHI broth (supplemented with L-cysteine hydrochloride hydrate (0.5 g/L)), and 100 µl of the 10^{-3} and 10^{-5} dilutions were cultured on various media types (**Table 2.3**). All the media were prepared as recommended by the manufacturer (BD). The plates used for culturing under anaerobic conditions were pre-incubated in an anaerobic environment for 24 hours. Media were autoclave sterilized, cooled to 55°C and poured into 100 mm Petri dishes. After inoculation, the plates were incubated under aerobic conditions for 48 hours and under anaerobic conditions for 4 days. Post-incubation, the organisms on each media type were pooled (designated as aerobic and anaerobic plate pools), by adding BHI broth (supplemented with 0.5% L-cysteine) onto the surface of agar. DNA was then extracted from the plate pools for further analyses.

2.2.3 Molecular profiling

2.2.3.1 Extraction of bacterial DNA

Bacterial DNA from the sputum, flow cell matrix, and effluent culture samples was extracted as outlined previously (see Section **2.1.4.2**).

2.2.3.2 Illumina PCR amplification of 16S rRNA V3 region and Illumina sequencing

Bacterial community profiling of the 16S rRNA gene was carried out using paired end reads of the V3 region, using bar coded Illumina sequencing as described previously[65]. Bar codes are included in both forward (F) and reverse (R) primers incorporate sequences for flow cell binding and Illumina sequencing.

V3F:(5'aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatctNNNNCCTACGGG AGGCAGCAG-3')

V3R:(5'caagcagaagacggcatacgagatXXXXXXgtgactggagttcagacgtgtgctcttccgatctATTAC CGCGGCTGCTGG-3'),

where XXXXXX denotes the hexameric barcodes described by Bartram *et al.*[65].

Each reaction mixture contained 25 pmol of each primer, a 200 µM concentration of each dNTP, 1.5 mM $MgCl_2$, and I U phusion Taq polymerase (NEB). The PCR conditions involved an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, and ended with an extension step at 72°C for 10 minutes. Amplifications were carried out in triplicate, where the amplified product was purified by separation with agarose gel electrophoresis and gel extraction. The purified product was used for subsequent steps. 250 nt paired-end

sequencing was carried out on a MiSeq Illumina sequencer as per manufacturer's instructions.

2.2.3.3 Sequence processing

PCR products were sequenced using the Illumina Miseq with paired-end reads. Custom Perl scripts were developed in-house to process the sequences. First, Cutadapt[66] was used to trim these sequences to the V3 region, ridding of any sequences surpassing this region. Next, sequences were aligned with their pair using PANDAseq[67]; during this alignment, any mismatches or ambiguous bases were culled. Operational taxonomic units (OTUs) were picked using AbundantOTU and as described previously[68] with a clustering cutoff of 97%. Taxonomy of the resultant OTUs was assigned via comparison of a representative sequence of the unit to the Greengenes reference database[69] using the Ribosomal Database Project (RDP) classifier[70]. Beta-diversity measures and summaries of the relative abundances of taxonomies were calculated using QIIME[71].

2.2.3.4 Clustering and biodiversity of bacterial communities

Bacterial community comparisons were carried out with unweighted UniFrac. Biodiversity of samples (sputum, plate pools, flow cell effluent cultures and matrices) was measured by the Shannon-Weiner diversity index.

2.2.3.5 Statistical analysis

An unpaired Student's *t* test was used to analyze differences in biodiversity between groups.

Table 2.3 – Media used for culture enriched molecular profiling of sputum sample.

Medium	Supplements	Growth conditions (5%CO_2, anaerobic* or both)
Columbia blood agar (CBA)	5% sheep blood	Both
Columbia colistin naladixic acid agar (CNA)	5% sheep blood	Both
Brain heart infusion agar (BHI)		5% CO_2
Trypticase soy agar	0.5% yeast extract	5% CO_2
Brain heart infusion agar (BHI)	L-cysteine hydrochloride hydrate (0.5 g/L), hemin (10 mg/ml), Vitamin K (1mg/ml)	Anaerobic
Trypticase soy agar	0.5% yeast extract, L-cysteine hydrochloride hydrate (0.5 g/L), hemin (10 mg/ml), Vitamin K (1mg/ml)	Anaerobic
McKay agar (MK)		Both
Mannitol-salt agar (MSA)		5% CO_2
MacConkey agar (MAC)		Both
Fastidious anaerobe agar (FAA)		Anaerobic

*Anaerobic conditions = 90% N_2, 5% H_2 and 5% CO_2
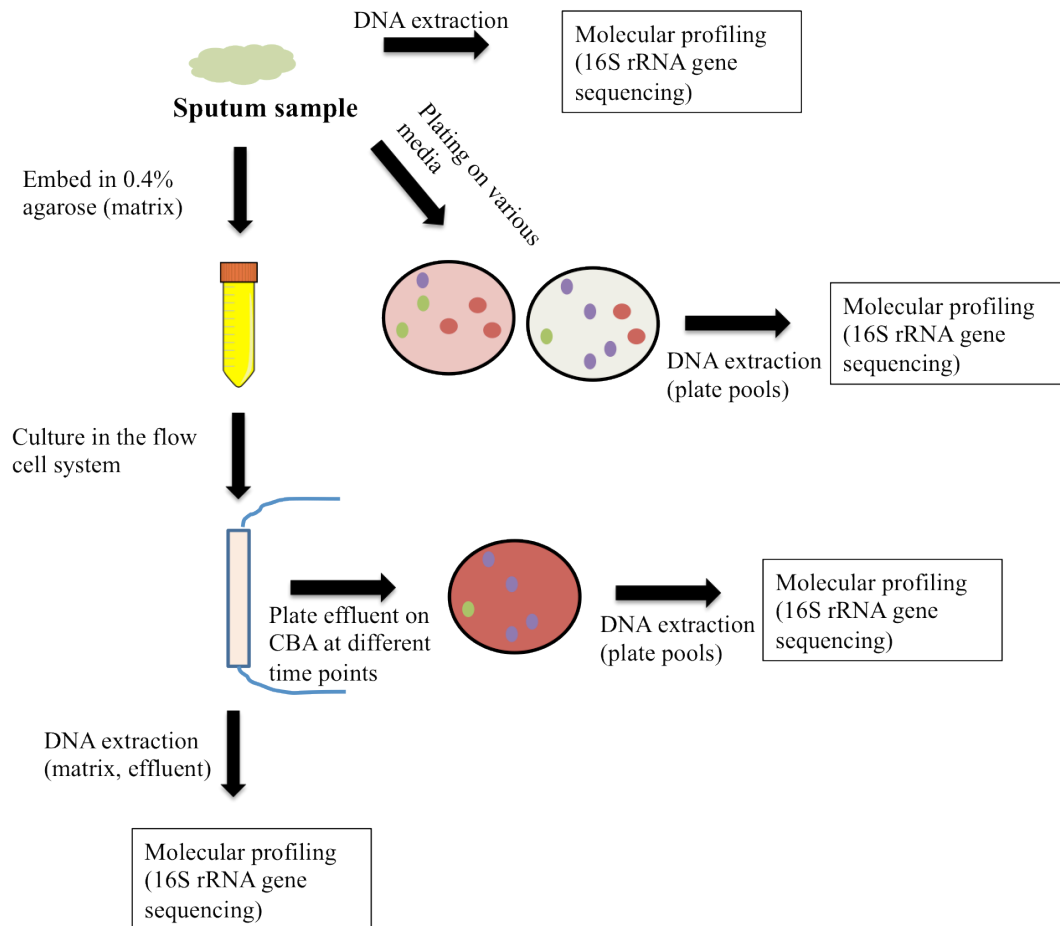
Figure 2.5 - Schematic diagram of the different methodologies utilized for characterizing bacterial populations in the sputum sample.
The bacterial communities in the sputum sample were characterized by direct molecular profiling of the original sample, culturing the sample on different media types and propagating it in the continuous flow cell system. After cultivation, the bacterial populations in the flow cell effluent cultures, matrix, and plate pools were analyzed with direct molecular profiling.

CHAPTER 3: EXPERIMENTAL INVESTIGATIONS

3.1 Develop/optimize methods to construct and propagate synthetic microbial communities *in vitro*

3.1.1 Introduction

The principal goal of this study was to culture synthetic microbial communities under continuous flow conditions. Batch cultures have been used as a method of studying the physiology, metabolism, production of virulence factors, and interactions in bacterial populations for several years (discussed previously, see Section **1.3.1**). However, one of the disadvantages associated with this system is that it does not necessarily depict the patterns of microbial growth and colonization in many natural environments including the naso- and oro-pharynx. Flow cells were used, first, to be able to capture the development of airway microbial communities in a physical environment that approximates the natural context, and second, to do this *in vitro,* in a cost-effective and reproducible manner.

The first aim was to study and compare the structure and diversity of synthetic microbial communities temporally with different culture methods. An evaluation and comparison of these methodologies was important to inform the design of experiments for culturing both synthetic and natural microbial communities. To construct synthetic communities, bacterial isolates cultured from clinical samples in previous studies were acquired from the Surette laboratory strain collection (**Table 2.1**). The strain list represents bacterial species that are commonly isolated from the upper respiratory tract[27,]

[30, 31]. The growth and abundance of these organisms was monitored under different culture conditions over various time points. The second aim of this study was to culture matrix embedded mixed (both aerobes and anaerobes) microbial communities under continuous flow conditions. One can expect that culturing communities by embedding them in a matrix will provide the community some spatial structure, and with time as microorganisms start growing within the matrix, oxygen, pH and nutrient gradients will get established in the community (**Figure 3.1A**). The structural localization of aerobes and anaerobes in the matrix was characterized by dissecting the community, where bacterial numbers were determined with total viable counts (**Figure 3.1B**).

3.1.2 Results

3.1.2.1 Growth of multispecies bacterial communities in static, shaken and flow cell effluent cultures

The relative abundances in a simple four-species aerobic synthetic community, when grown with the three culture methods, are shown in **Figure 3.2**. The communities were propagated under aerobic (5% CO_2) conditions and incubated at 37°C. For comparative purposes, all the cultures were started on the same day. The bacterial numbers were determined with total viable counts. It is evident that the overall community composition varies with the culture method used, where the most diverse communities are propagated with static and flow cell cultures. All four bacterial species in this simple community were able to persist or grow when propagated with static and flow cell cultures. However, in shaken cultures, there was a significant decrease (*t* test, *P* < 0.001) in the $\log_{10}$ viable counts of *Achromobacter* and *Streptococcus* on day 7 as compared with the initial inoculum. *Staphylococcus* spp. were the most abundant organisms cultured in shaken batch cultures. These communities were dynamic and exhibited changes in composition over time. Some differences in overall community composition between replicates were also seen.

3.1.2.2 Total viable counts are reliable for characterizing simplified synthetic communities

The composition in the bacterial communities, over different time points, was characterized with both total viable counts and T-RFLP. The relative abundances in a simple four-species aerobic synthetic community, when grown with three culture

methods, where the structure was determined with both total viable counts and T-RFLP, are shown in **Figure 3.3**. The communities were propagated under aerobic (5% CO_2) conditions and incubated at 37°C. For comparative purposes, all the cultures were started on the same day. The overall community composition (for all three culture methods) determined by total viable counts largely mirrored the one determined by T-RFLP.

3.1.2.3 Matrix embedded communities are diverse

It is important to characterize the community composition within the matrix as some organisms can be missed in flow cell effluent cultures (**Figure 3.4**). The communities were propagated under aerobic (5% CO_2) conditions and incubated at 37°C. The relative abundances in a simple four-species aerobic synthetic community, in flow cell effluent cultures and matrices on day 7, are shown. The overall community composition is similar between replicates. The compositions of flow cell effluent cultures, at different time points, and matrix embedded communities are shown in detail in **Table 3.1**. All bacterial species grow within the matrix embedded communities, and some organisms are missed in the effluent cultures.

3.1.2.4 Mixed synthetic communities retain obligate anaerobes when cultured under aerobic conditions in flow cell cultures

Viable numbers of obligate anaerobes (*Prevotella melaninogenica*, *Fusobacterium nucleatum*) were present in the flow cell effluent cultures and matrix, when the communities were cultured under aerobic conditions. The compositions of flow cell effluent cultures (at different time points) and matrix embedded communities are shown in detail in **Table 3.2**. The relative abundances in a simple six-species mixed synthetic

community, in flow cell effluent cultures and matrix, are shown in **Figure 3.5**. The communities were grown in an anaerobic environment for 2 days (the 48 hour time point is designated as day 2 (ana); ana: anaerobic environment), and were incubated at 37°C. The flow cell setup was transferred to an aerobic environment (5%CO_2, 37°C) at the 48 hour time point. The changes in composition under aerobic conditions were followed thereafter (flow cell system was kept aerobic for 6 days). Although not as abundant as other anaerobes in the community, like *Veilonella atypica*, *Prevotella* and *Fusbacterium* were able to persist and grow under aerobic conditions (**Figure 3.6**). There was a significant increase (*t* test, $P < 0.05$) in the $\log_{10}$ viable counts of *Prevotella* in the day 6 aerobic effluent cultures as compared to day 2 anaerobic cultures (**Table 3.2**).

3.1.2.5 Growth in matrix embedded mixed synthetic communities

Prevotella and *Fusobacterium* were more abundant in the matrix in comparison with flow cell effluent cultures, in a simple six-species community cultured under aerobic conditions (**Figure 3.7**). The relative abundances in flow cell effluent cultures and matrices on day 7 are shown. The matrix embedded communities showed more diversity, where all the organisms present in the starting community were able to persist and grow in the matrix. To evaluate the growth and changes in community composition within the matrices over different time points, four flow cells were started in parallel from the same starter culture (six-species community). One flow cell was sacrificed at each time point and the composition was subsequently determined with total viable counts. The compositions in the matrix embedded communities, at different time points, are shown in

detail in (**Table 3.3**). The relative abundances in these communities are shown in **Figure 3.8**.

3.1.2.6 No spatial patterns within the matrix embedded mixed synthetic community

The community structure in the matrix was spatially characterized to measure patterns of bacterial species along the length of the community (**Figure 3.1B**). Spatial distributions in a simple six-species community cultured under aerobic conditions, is shown in **Figure 3.9**. The matrix was recovered on day 7 and dissected into parts, where the bacterial numbers were determined with total viable counts. Flow cell was dominated by the same community of organisms, throughout the whole vertical profile. *Veillonella atypica* was most abundant in part 4, but the differences were not significant. Total proportions of *Prevotella* and *Fusobacterium* also did not change significantly. Conclusively, no spatial heterogeneity was observed and the communities can be described as being spatially stable within the matrix.

3.1.3 Discussion

In this study, my aim was to optimize culture methods for growing bacterial communities in the lab, and investigate whether a polymicrobial bacterial community is maintained and reproduced in separate methods. An *in vitro* flow cell model was optimized to propagate mixed communities, comprised of aerobes and anaerobes. Such a model would be beneficial for studying microbe-microbe and polymicrobe-host interactions in near future studies. As a first step, simple aerobic communities were propagated with batch and flow cell culture methods, and the growth behavior of organisms in these cultures was studied. The key findings were that the overall community structure depends on the culture method used, where most diverse communities can be propagated with static batch cultures and the flow cell system. Aeration in shaken batch cultures results in spatial homogeneity. One or two organisms take over this culture and flourish, whereas other members of community either die or merely persist. On the other hand one can expect spatial heterogeneity in static batch cultures where the presence of multiple niches can result in the successful growth of multispecies communities in these cultures.

Composition of aerobic synthetic communities was characterized with both TVCs and T-RFLP to: 1) evaluate the effectiveness of TVCs as a method for community profiling and, 2) determine (with T-RFLP) whether any major bacterial groups are being missed in culture. The data shows high similarity in the community compositions deduced with both methods. Although some organisms can be missed in culture and a better approach is to use both culture-dependent and culture-independent methods in

tandem[27], for studies on simple synthetic communities (up to 10 organisms in culture), TVC was proven to be reliable for characterizing community composition as observed in this study and others[47]. Furthermore, TVCs give information about the living members of the community. Data from TVCs for the flow cell cultures established that multispecies communities develop robustly with the flow cell system. Thereafter, it was important to determine whether communities grown in the matrix can proliferate over time. The data shows that matrix embedded communities are diverse, stable and reproducible, where significant increase in growth was observed within the matrix over time. The communities that developed in the flow cell systems were reproducible both within a flow cell run and between independent flow cell runs. Differences in the community composition between the effluent cultures and matrix were also recorded. Most diverse communities were recovered in the matrix and this finding provided further incentive for culturing mixed species (aerobes and anaerobes) communities in flow cell systems. Although effluent cultures can serve as predictors of the community dynamics within the matrix over time, it is essential to analyze the community structure within the matrix. The main limitation of my flow cell model is the inaccessibility of the matrix over the course of the experiment.

We hypothesized that the development of oxygen gradients will permit the establishment of obligate anaerobes in a mixed community of aerobes and anaerobes, under aerobic growth conditions. My data indicates that this is the case and possibly steep oxygen gradients develop in the entire flow chamber and downstream tubing as obligate anaerobes could also be recovered efficiently in the effluents cultures. In order to

determine the effect of aeration on the recovery of anaerobes, communities were also cultured under higher flow rate conditions. Communities cultured under relatively higher flow rates of medium (1.5 ml/hour) did not show any significant changes in the overall community structure. All community members of a six-species mixed community (with obligate anaerobes) were recovered in flow cell matrices under these conditions (in an aerobic environment), and an increase in bacterial numbers over time was also recorded (**5.2**, Appendix).

The community composition of mixed synthetic communities within flow cell matrices was also studied. An increase in bacterial numbers (including the obligate anaerobes) was recorded when the flow cell system was transferred to aerobic conditions. As discussed above, diverse aerobic synthetic communities can be propagated in static batch cultures. I also wanted to investigate whether mixed communities of aerobes and anaerobes can be successfully propagated in batch cultures. One wouldn't need to utilize a relatively technically challenging method like flow cell culturing if mixed communities can be conveniently propagated in batch cultures. Loss of obligate anaerobes was recorded in batch cultures when a six-member mixed community was propagated in BHI growth medium (**5.3**, Appendix). Steep oxygen gradients that can protect obligate anaerobic organisms from the toxic effects of oxygen do not establish in these static batch cultures. The static batch cultures did not undergo subculturing, and one culture was analyzed for each time point in the experiment. The rationale behind this approach was that if oxygen gradients develop in batch cultures, they could get disrupted during subcultures. Apart from the lack of oxygen gradients, pH and nutrient gradients, which

are also crucial, can develop and it is possible that nutritional requirements of some bacterial species couldn't be realized. The accumulation of toxic products could also be responsible for declining numbers of some community members in batch cultures. Multispecies communities can exist in chemostat cultures under aeration, however the survival of anaerobes is dependent on the initial inoculum, community members and the amount of oxygen in the culture[47].

I previously hypothesized that the members of a mixed community in the flow cell will demonstrate spatial patterns where the anaerobes are located away from the flow of media and aerobes are directly exposed to the flow. Oxygen gradients would be expected to establish along the length of the community in this model. Spatial characterization of the matrix embedded mixed community did not reveal any striking differences in the spatial patterns, where obligate anaerobes were located in all sections of the community. This model airway bacterial community did not include any obligate aerobes, which can serve as better indicators of community spatial patterns. Conclusively, I have optimized the flow cell system to propagate mixed species communities in the lab. These communities are diverse and reproducible and in all cases the obligate anaerobes could be recovered efficiently when the flow cell setup is operated in aerobic conditions. The optimized system can be utilized for propagating much more complex communities directly from airway samples.

Table 3.1 - Composition of flow cell effluent cultures and matrix embedded communities (aerobic synthetic community).

Species	Day 0† (Effluent)	Day 1(Effluent)	Day 3(Effluent)	Day 5(Effluent)	Day 7(Effluent)	Day 7(Matrix)
Achromobacter xylosoxidans	5.47 ± 0	5.49 ± 0.83	8.10 ± 0.70	8.59 ± 0.12	8.5 ± 0.18	5.03 ± 0.61
Staphylococcus lugdunensis	5.98 ± 0	8.54 ± 0.21	8.34 ± 0.61	8.85 ± 0.26	8.61 ± 0.16	8.77 ± 0
Staphylococus epidermidis	5.90 ± 0	8.45 ± 0.34	8.30 ± 0.42	7.85 ± 0.49	8.07 ± 0.6	8.55 ± 0.06
Streptococcus salivarius	5.20 ± 0	8.30 ± 0	8.65 ± 0.06	8.77 ± 0.24	8.66 ± 0.02	7.40 ± 0.70

Results are shown as $\log_{10}$ CFU/ml ± standard deviations for three independent experiments (atleast two technical replicates were included in each experiment).

†Day 0 refers to the initial inoculum (starting microbial community).

Table 3.2 - Compositions of flow cell effluent cultures and matrix embedded communities (mixed synthetic community).

Species	Day 0 (ana)†	Day 2(ana) Effluent	Day 2(ana)+Day 2(aer) Effluent	Day 2(ana)+ Day 6(aer) Effluent	Day 2(ana)+Day 6(aer) Matrix
Veilonella atypica	4.53 ± 0.0.3	4.89 ± 3.59	8.38 ± 0.58	8.21 ± 0.34	8.06 ± 0.63
Prevotella melaninogenica	4.49 ± 0.6	0	6.91 ± 0.12	6.32 ± 0.57	7.25 ± 0.63
Fusobacterium nucleatum	5.4 ± 0.5	5.69 ± 0.39	3.68 ± 3.12	6.25 ± 0.59	7.23 ± 0.42
Staphylococcus lugdunensis	5.2 ± 0.15	7.60 ± 0.64	8.25 ± 0.50	8.03 ± 0.37	8.41 ± 1.15
Staphylococcus epidermidis	5.3 ± 0.2	4.81 ± 3.92	2.648 ± 1.5	4.92 ± 3.5	7.25 ± 0.59
Streptococcus salivarius	5.2 ± 0.2	7.81 ± 0.58	7.95 ± 0.83	7.85 ± 0.34	6.96 ± 0.52

Results are shown as $\log_{10}$ CFU/ml ± standard deviations for three independent experiments (atleast two technical replicates were included in each experiment); ana: anaerobic, aer: aerobic.
†Day 0 refers to the initial inoculum (starting microbial community).

Table 3.3 - Composition within flow cell matrices over different time points.

Species	Day 0(ana)†	Day 2(ana)	Day 2(ana)+Day 2(aer)	Day 2(ana)+Day 6(aer)
Veilonella atypica	5.41 ± 1.92	4.73 ± 6.7	8.90 ± 0.13	8.06 ± 1.5
Prevotella melaninogenica	5.58 ± 2.16	5.46 ± 3.12	7.42 ± 0.06	7.23 ± 1.5
Fusobacterium nucleatum	6.32 ± 1.16	5.30 ± 1.52	7 ± 0	7.29 ± 1.2
Staphylococcus lugdunensis	6.90 ± 2.3	8.69 ± 0.72	9.47 ± 0.10	8.12 ± 1.9
Staphylococcus epidermidis	6.97 ± 2.12	4.45 ± 6.29	4 ± 5.65	7.49 ± 1.99
Streptococcus salivarius	6.92 ± 1.22	9.12 ± 0.18	8.42 ± 0.60	6.94 ± 1.22

Results are shown as $\log_{10}$ CFU/ml ± standard deviations for two independent experiments (atleast two technical replicates were included in each experiment); ana: anaerobic, aer: aerobic.
†Day 0 refers to the initial inoculum (starting microbial community).

A

[O$_2$]

Matrix embedded bacterial community in the flow chamber

Flow (microaerophilic/anaerobic)

Obligate anaerobes

Facultative anaerobes

Obligate aerobes

B

Part 1

Part 2

Part 3

Part 4
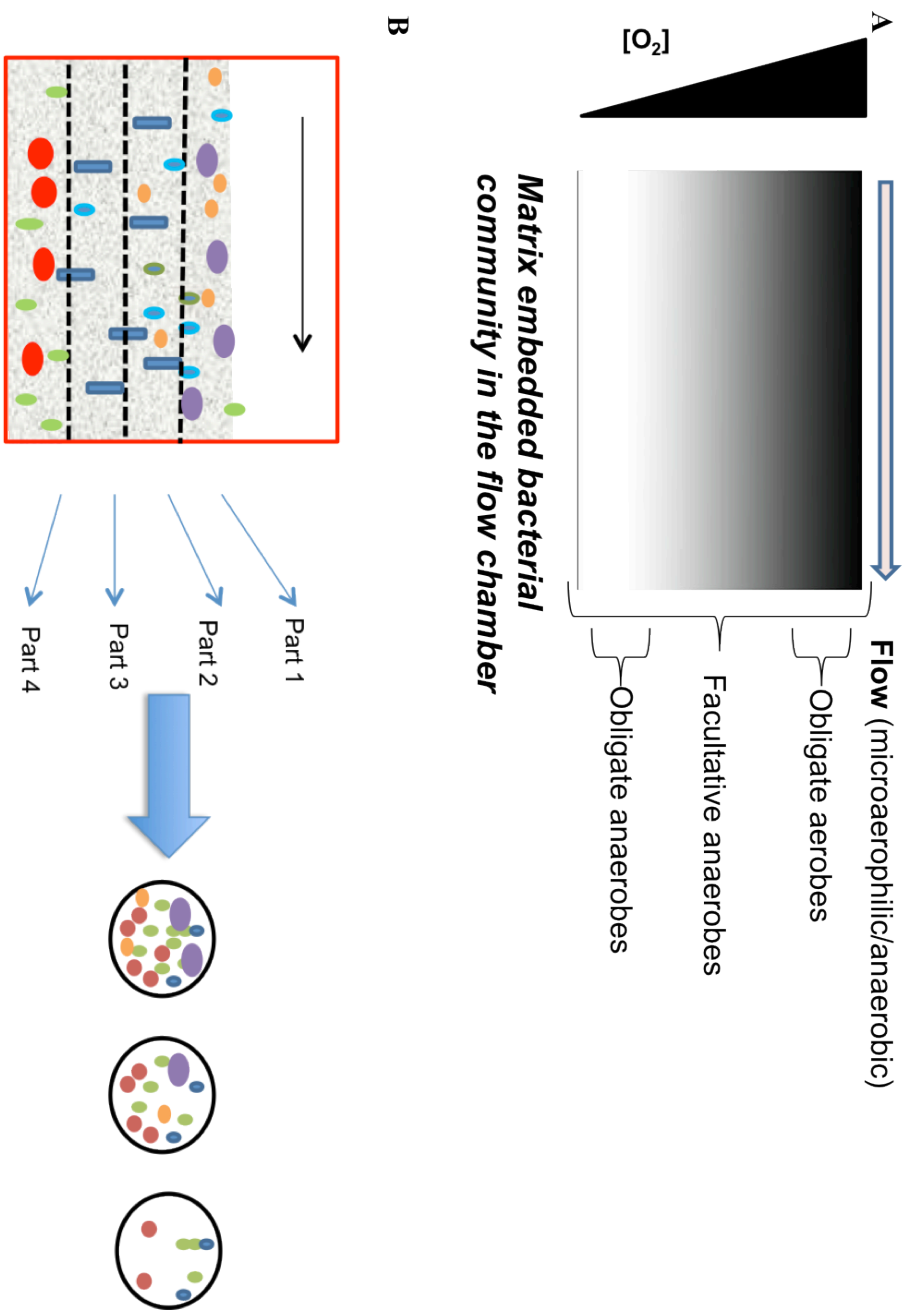
Figure 3.1 - Proposed model for spatial distribution of microbes in the matrix embedded community. (A) Proposed model for spatial localization of microbes along the length of the matrix embedded community in the flow cell channel. (B) Spatial structure within the community can be characterized by dissecting the community. Total viable counts on different sections of the community can be done for analyzing the spatial composition.
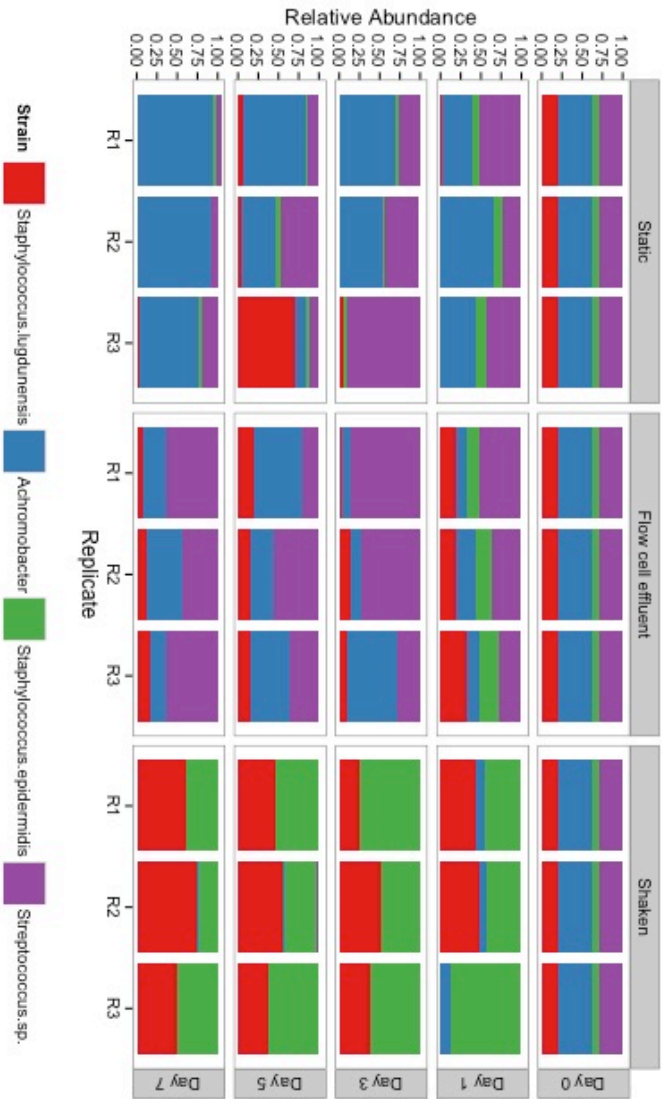
Figure 3.2 – Comparison of compositions, in an aerobic synthetic bacterial community, between static, shaken and flow cell cultures.
Relative abundances of bacterial species in a four-species community are shown. The community structure at different time points, when propagated with static, shaken and flow cell cultures under 5% CO_2 (incubated at 37°C), is shown. Day 0 refers to the initial inoculum (starting community). The data for only flow cell effluent cultures is represented on the plot. The results for one experiment (with three technical replicates) are shown here. Relative abundance for each species was determined by total viable counts.
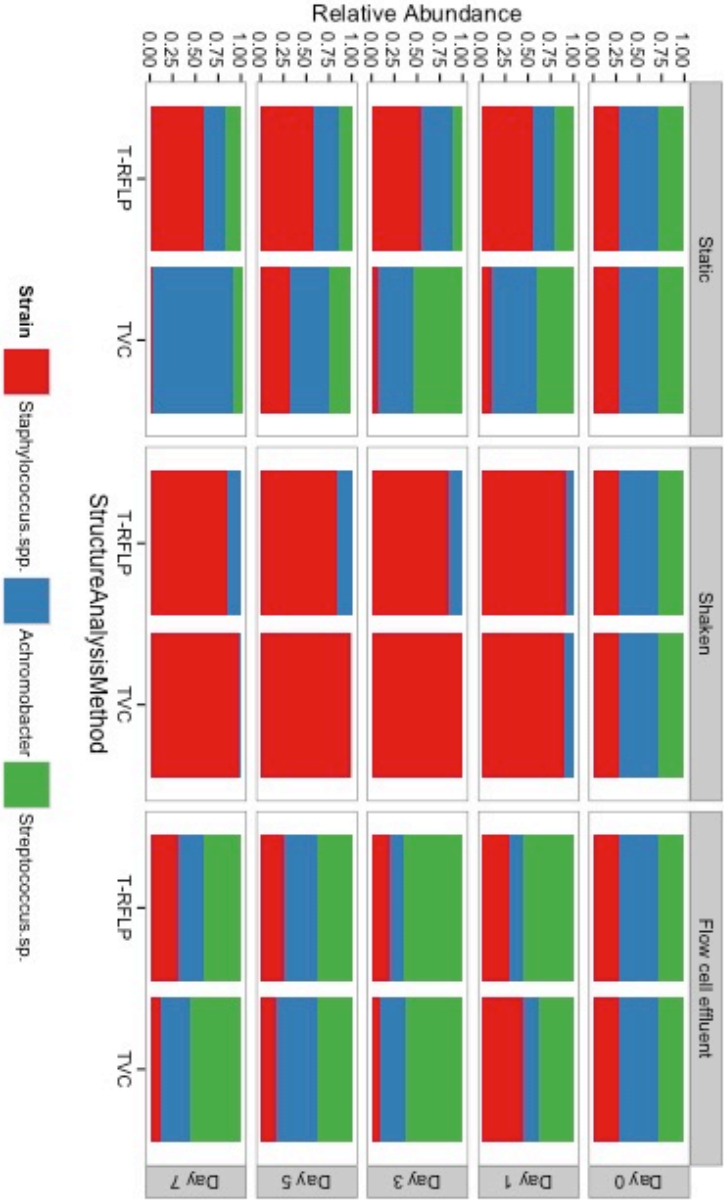
Figure 3.3 - Comparison of community compositions, in an aerobic synthetic community, with total viable counts and T-RFLP (grown with three culture methods).
The structure of a simple four-species bacterial synthetic community at different time points when propagated with static, shaken and flow cell cultures, under 5% CO_2 (incubated at 37°C) is shown. The data for only flow cell effluent cultures is represented on the plot. Day 0 refers to the initial inoculum (starting community). The results for one experiment are shown (the average of three technical replicates for each method, is represented on the plot)

Figure 3.4 - Comparison of compositions, in an aerobic synthetic community, between flow cell effluent cultures and matrix.
The structure of a simple four-species bacterial synthetic community in the flow cell (FC) effluent cultures and matrices on day 7 is shown. The results for one experiment are shown (three technical replicates: R1, R2, R3). Starting community refers to the initial inoculum on day 0. Relative abundance for each species was determined by total viable counts.
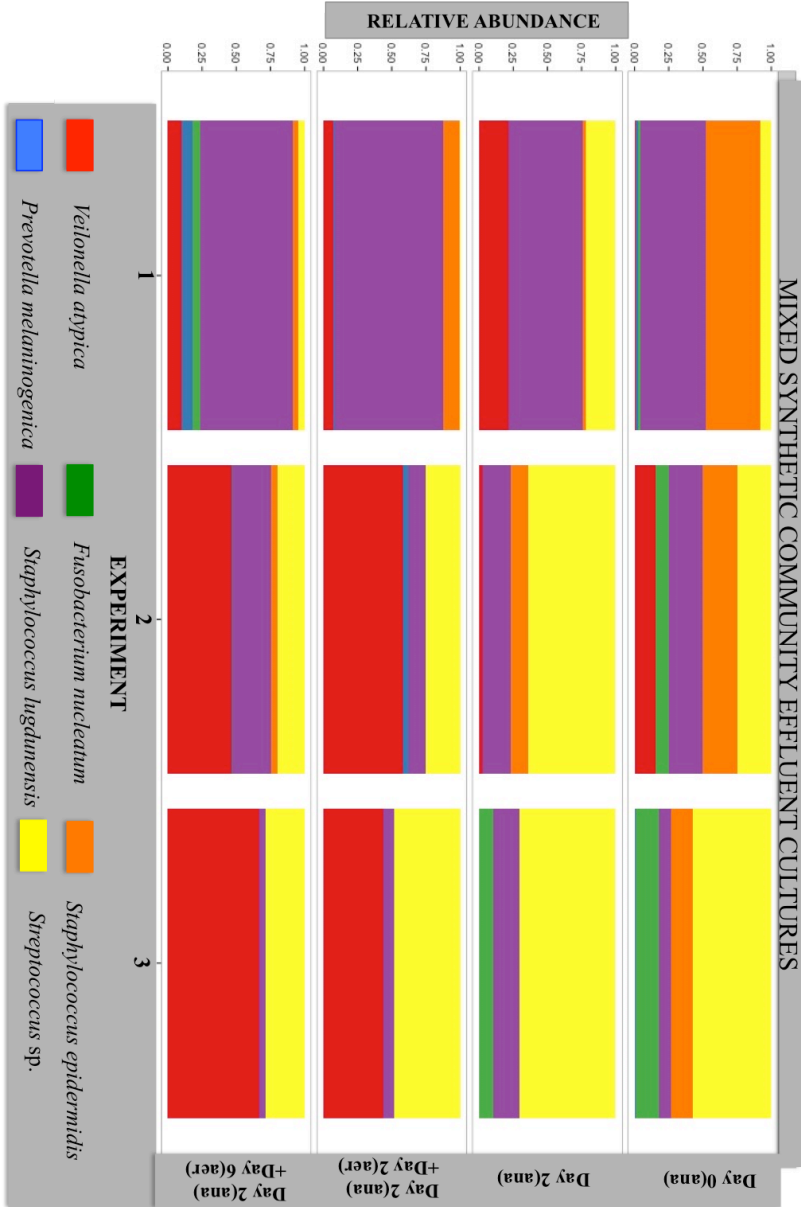
Figure 3.5 - Composition of a mixed synthetic community in flow cell effluent cultures at different time points. The structure of a simple six-species bacterial synthetic community, in the flow cell effluent cultures, is shown. The results for three independent experiments (the average of three technical replicates for each experiment is represented on the plot) are shown. Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs (the community was grown anaerobically for first 48 hours); ana: anaerobic, aer: aerobic. Relative abundance for each species was determined with total viable counts.
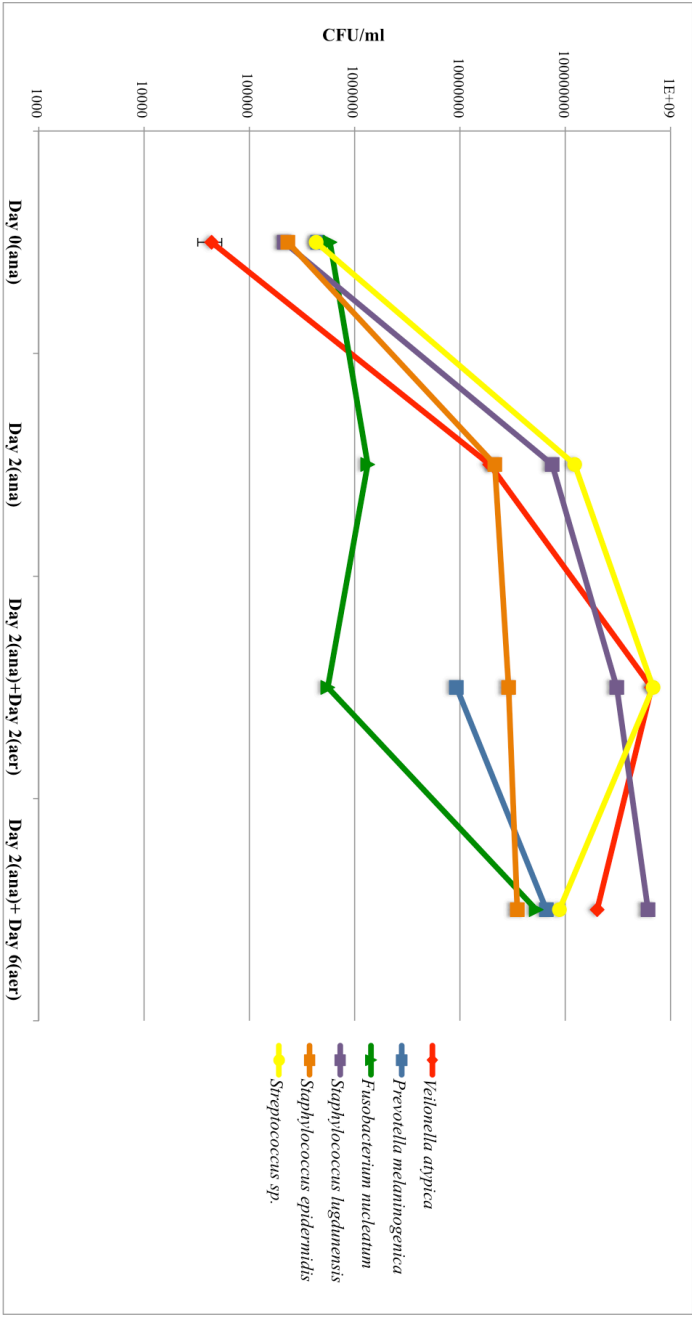
Figure 3.6 – Bacterial growth dynamics of a mixed synthetic community.
Bacterial levels are in Colony Forming Units/ml. The average CFU/ml for each bacterial species in a mixed community are present on the plot. The results for three independent experiments are shown (with two technical replicates per experiment). Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs (the community was grown anaerobically for 48 hours); ana: anaerobic, aer: aerobic. The data for only flow cell effluent cultures is represented on the plot
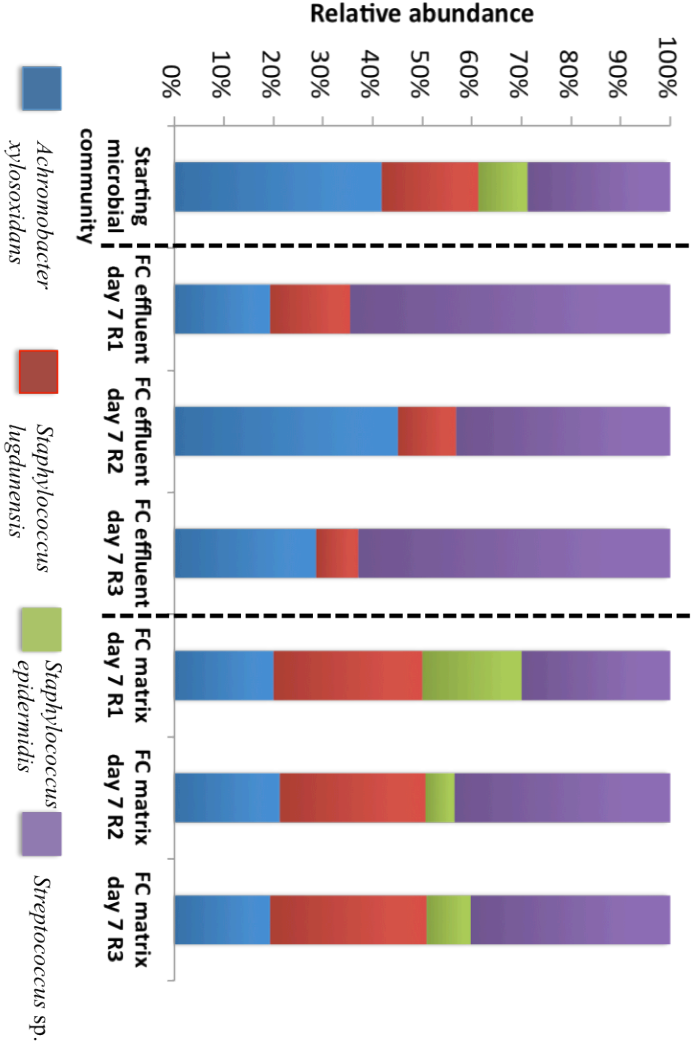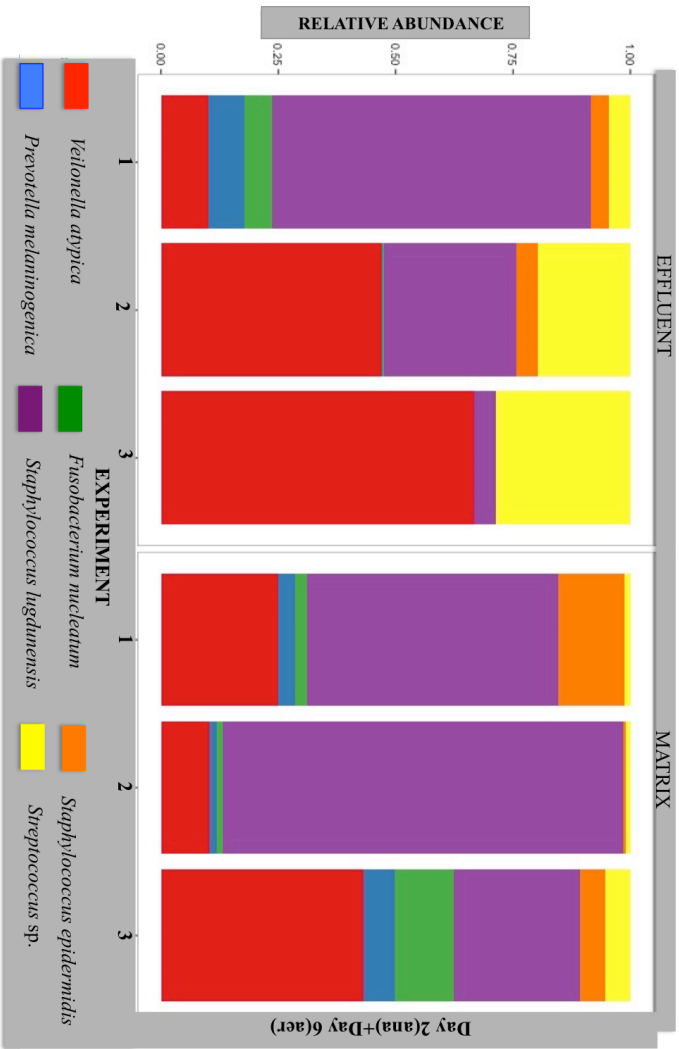
Figure 3.7 - Comparison of compositions, in a mixed synthetic community, between flow cell effluent cultures and matrices.
The structure of a six-species bacterial synthetic community in the flow cell effluent cultures and matrices, on day 6 of incubation under aerobic conditions, is shown. The results for three independent experiments (the average of three technical replicates for each experiment is represented on the plot) are shown. Day 0(ana) refers to the starting community (initial inoculum) and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs; ana: anaerobic, aer: aerobic. Relative abundance for each species was determined by total viable counts.
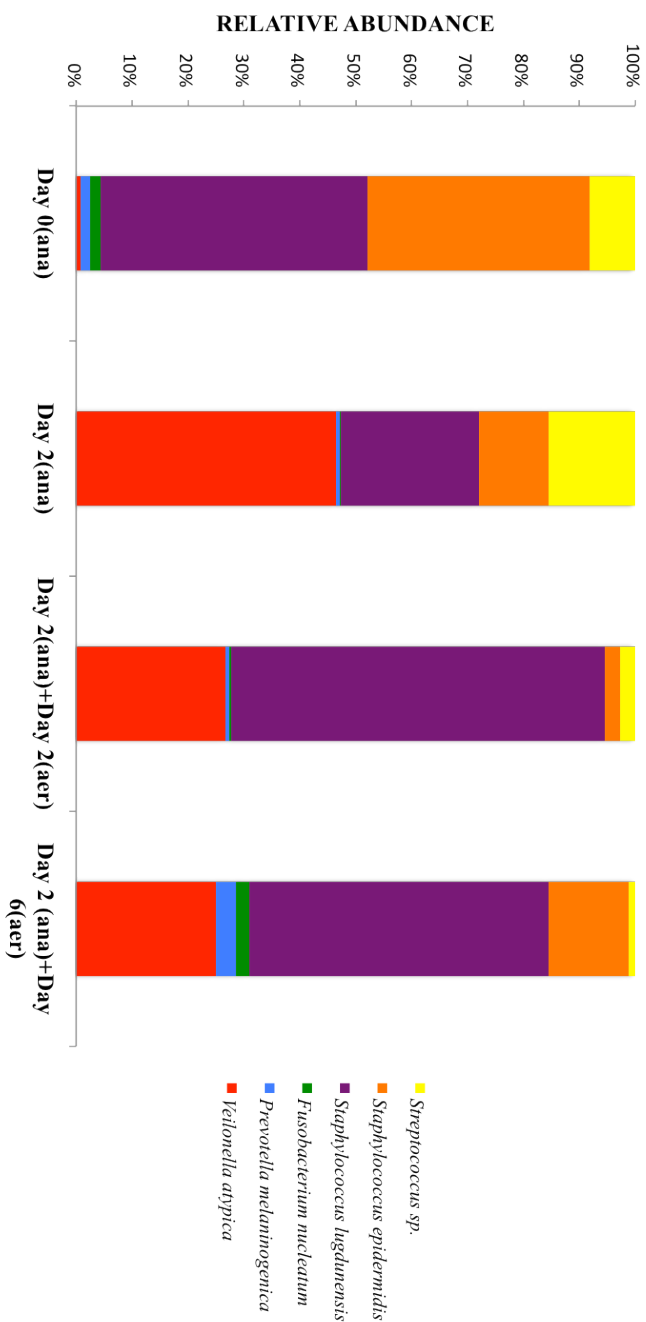
Figure 3.8 - Comparison of the compositions, in a mixed synthetic community, between matrices at different time points.

The structure of a simple six-species bacterial synthetic community, within the flow cell matrices, is shown. The results for two independent experiments are shown (the average relative abundance is represented on the plot). Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs; ana: anaerobic, aer: aerobic. Relative abundance for each species was determined by total viable counts. The matrix from one flow cell was sacrificed at each time point to deduce community dynamics within the matrix over time.
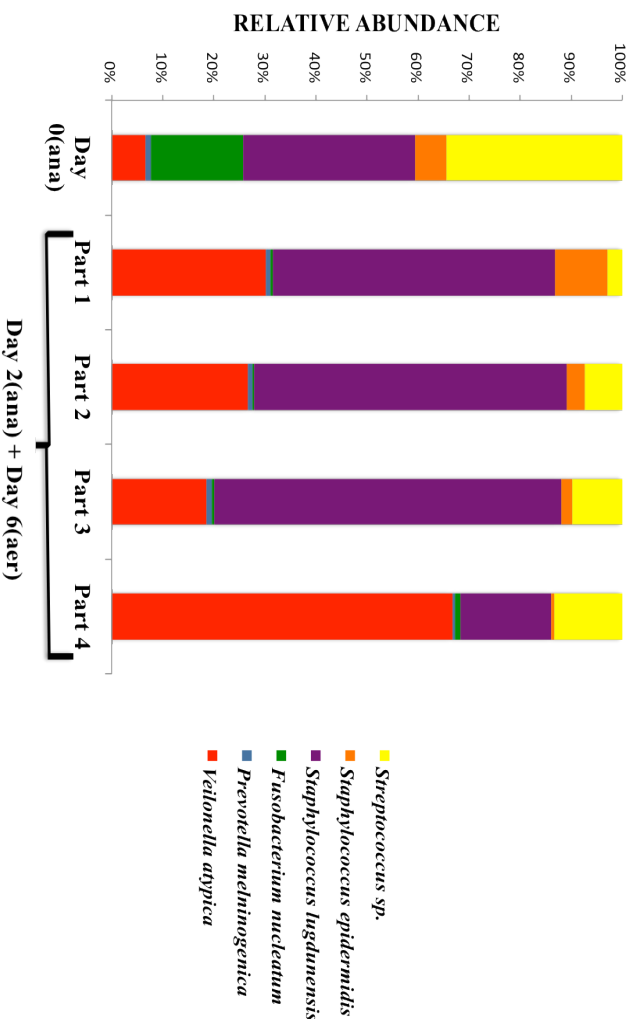
Figure 3.9 - Spatial characterization of matrix in a mixed synthetic community. The structure of a simple six-species bacterial synthetic community in the flow cell matrix is shown. The results for two independent experiments (average relative abundances are represented on the plot). Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs. Relative abundance for each species was determined by total viable counts. The matrix was recovered on day 7, and was dissected into four parts to measure patterns of bacterial species within the matrix.

3.2 Propagate natural communities from clinical samples under continuous flow conditions

3.2.1 Introduction

A robust culture-based method (a flow cell system operating under continuous flow conditions), that can support growth of polymicrobial communities recovered from natural environments has been developed in this study to: 1) culture complex communities from airway samples; 2) investigate bacterial population dynamics over time within these communities. The data from synthetic microbial communities demonstrates that reproducible bacterial communities can be cultured with the flow cell system. Using the methodology developed with the synthetic communities, I sought to characterize the structure of polymicrobial communities cultured from sputum samples in the flow cell system. First and foremost, it was necessary to compare the composition of communities propagated in the flow cell and the original sputum sample to evaluate the utility of the flow cell method. By determining the organisms recovered by cultivation in the flow cells, one can assess the cultivable diversity. Community compositions in the original sputum samples, flow cell effluent cultures and matrices were analyzed by 16S rRNA gene sequencing.

Culture enriched molecular profiling of the sputum sample was also conducted in parallel for additional comparison with flow cell communities. Culture enriched molecular profiling involves plating the sample on different media types (selective and non-selective) for recovering diverse bacterial populations. The use of selective media

inhibits the growth of fast growing organisms and selects for rare/less abundant members of the community. The organisms are then harvested off the surface of plates and molecular profiling is conducted on these enrichment pools to analyze the community composition in the original sample. The purpose behind aforementioned comparisons was to evaluate the consistency of this method, for incorporation in future studies relating to airway polymicrobial communities.

3.2.2 Results

3.2.2.1 Compositional differences in bacterial communities characterized with different culture methods

For characterizing the structure of bacterial communities cultured in the flow cell system, I examined the flow cell effluent and matrix embedded communities for all three clinical samples in depth with direct molecular profiling. Three sputum samples were obtained on the same day from three asthmatic patients (patient IDs: PN32, PN33 and PN34). These samples were cultured in the flow cell system, where the system was kept in an anaerobic environment for 2 days, followed by transfer to aerobic conditions. The community composition, on day 2 (designated as day 2 anaerobic) and day 7 (designated as day 7 aerobic), in the flow cell effluent cultures and matrices was analyzed. For added comparison, the samples were also cultivated on various media types. Comparison with the communities cultured by plating the sputum sample on selective and non-selective media (14 growth conditions in total, see **Table 2.3**) further assists in assessing the effectiveness of the flow cell system in cultivating polymicrobial communities. Community composition of these samples was compared to the original sputum sample (starting community). Technical flow cell replicates of sample PN33 were also included in the analysis to evaluate the consistency of this method. Community composition in two replicates (replicate1 and replicate 2) of sample PN33 was examined in parallel. A third flow cell replicate of this sample was used for determining the community structure within the matrix on day 2, to infer the composition of the matrix embedded community prior to transfer into an aerobic environment. Differences in bacterial community

composition between flow cells effluent cultures, matrix and original sputum sample are described below.

Sequencing data from all the samples generated a total of 1,263,433 sequences, where an average of 54,931 sequences per sample was recorded (**Table 3.4**). The sequencing revealed a community dominated, on average, by the bacterial phyla Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria and Proteobacteria. At the genus level, I identified a total of 25 distinct genera in the data that were present at an abundance of 1% or higher (in at least one sample). The most abundant genera were: *Streptococcus, Veillonella, Rothia, Prevotella* and *Haemophilus*. The percent relative abundances in bacterial communities cultured from samples PN32, PN33 and PN34, in flow cell effluent and matrix, are shown in **Figure 3.10, Figure 3.11, Figure 3.12, Figure 3.13** and **Figure 3.14**. For samples PN32 and PN33, effluent cultures were collected under anaerobic conditions on day 2 and the community composition in these was also characterized and is shown on the plots.

The most abundant groups detected in matrix on day 7 were *Streptococcus*, *Veillonella* and members of the family Lachnospiraceae. Overall, the community composition in the flow cell effluents on day 7 was very similar to the composition in the matrix on day 7. *Streptococcus* and *Veillonella* were the most abundant genera detected in the flow cell effluent cultures and matrices on day 2 (**Figure 3.10, Figure 3.11, Figure 3.12 and Figure 3.13**). Certain anaerobic groups like *Prevotella* were not as abundant in culture (plate pools, effluents and matrices) as compared to the original sample. *Rothia,*

which was abundant (~10%) in the original sample, was not very efficiently recovered with the flow cell culture method.

Interesting differences in the compositions of bacterial communities that developed in technical replicates of PN33 were also observed. Three common dominant genera in the matrix embedded communities of replicates 1 and 2 were *Streptococcus, Veillonella* and *Campylobacter* (**Figure 3.11, Figure 3.12**). However, Lachnospiraceae, (also dominant in matrix embedded communities of PN32 and PN34 samples; see **Figure 3.10, Figure 3.11** and **Figures 3.14**), was not very abundant in replicate 2, where *Fusobacterium* became the most abundant OTU and constituted 50% of the total bacterial population (**Figure 3.12**). It is noteworthy that *Fusobacterium* was not detected in the plate pool communities for this sample. Conclusively, complex and dynamic microbial communities can be cultivated with the flow cell system, and major population shifts were observed when the flow cells were transferred to an aerobic environment on day 2.

3.2.2.2 Diverse natural communities can be propagated under continuous flow conditions

To determine the community membership, relative abundances of bacterial genera in the sputum sample (genera detected in >0.0001 are shown, which is a percent relative abundance of >0.01%) are plotted in order of decreasing abundance. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell samples (both matrix and effluent) are indicated on this same plot to illustrate the cultivable members of the flow cell communities. The abundance rank orders, for the three sputum samples (including the technical flow cell replicates and day 2 matrix

embedded community), are shown in **Figure 3.15**, **Figure 3.16**, **Figure 3.17**, **Figure 3.18** and **Figure 3.19**.

The average number of genera ascertained via direct molecular detection from sputum was 21.5 (+/-1). The average number of genera detected in the matrix was 17.25 (+/-2.75), and in the effluent (day 7) was 18 (+/-1.25). Around 86% of the genera present in the original sample could be recovered in the flow cell communities on day 7. Analysis of the microbial composition in the matrix embedded community on day 2, revealed the recovery of 17 genera as compared to an average of 11.33 (+/-1.52) genera detected in the effluent cultures on day 2 (**Figure 3.18**). Conclusively, the majority of genera detected in the sputum were recovered with flow cell culturing.

A comparison of the community compositions of bacterial communities developing in the technical flow cell replicates of PN33 (**Figure 3.16**, **Figure 3.17**), revealed a high similarity in the overall community structures for these samples. There were also instances when recovery of some groups was seemingly difficult under certain growth conditions. For example, some aerobic organisms, like *Rothia* and *Neisseria*, that were detectable on day 2 in the anaerobic effluent of all samples (and in the matrix of sample PN33 on day 2, see **Figure 3.18**), couldn't be recovered in the matrix/effluent on day 7 (**Figure 3.16**, **Figure 3.17** and **Figure 3.19**).

3.2.2.3 Flow cell culturing is a consistent method

To investigate the relationship between the sputum and flow cell bacterial communities, unweighted UniFrac was used as a measure of phylogenetic beta diversity in the communities. As compared to weighted UniFrac, which reveals community

differences that incorporate relative abundances, unweighted UniFrac is a qualitative measure that shows differences based on the community membership[72]. Comparison of the sputum, flow cell effluent and matrix samples revealed that primary clustering in this data set is by day (**Figure 3.20**), where the communities existing in the flow cell effluents and matrix showed highest similarity. Based on separation of flow cell communities by day (the original sputum samples also cluster separately), it can be concluded that the communities cultured with flow cell method are reproducible.

3.2.2.4 Biodiversity of the microbial communities in flow cell effluents and matrix

The biodiversity of the microbial communities propagated in the flow cells, plate pools and original sputum sample was evaluated with Shannon diversity index (**Figure 3.21**), which reflects both the species richness and evenness in the sample[73]. Significant differences were observed between the biodiversity of bacterial communities in the original sample and the communities cultivated from the original samples by different methods (plating on different media types and flow cell culturing). However, no significant difference was observed between the biodiversity of the communities recovered in the matrix and effluent cultures on day 7.

3.2.3 Discussion

The data on culturing polymicrobial communities from sputum samples in flow cell systems, shows that a majority of organisms present in the original sample can be recovered in flow cell cultures (both effluent and matrix). I have successfully developed a culture-dependent method that can be utilized for propagating complex (aerobes and anaerobes) polymicrobial communities *in vitro*. Obligate anaerobic species were able to persist in the community when the system was operated under aerobic (5% CO_2) conditions. With slight variations in the protocol, this methodology can be utilized in future studies for culturing more diverse complex communities, conveniently under aerobic conditions, from airway samples.

In all cases *Streptococcus* and *Veillonella* were the most abundant organisms (at genus level) observed in the flow cell cultures (both effluent and matrix). This is not surprising, as these organisms are prominent members of the URT[16, 32]. During the first 48 hours (anaerobic incubation), these organisms flourish and become dominant members of the community. A 1/100 dilution of the original sample was used for inoculation of flow cells, and this might have resulted in overcrowding in the developing community. The rationale behind this dilution for inoculation was to prevent the loss of less abundant/rare members of the community and disruption of important community interactions. However, the main caveat of using low dilutions is that only a few cell divisions will occur before reaching saturation, which might obscure the underlying diversity in the community. Using higher dilutions can decrease the competition and permit the growth of less abundant organisms[74]. A 1/1000 dilution of a sputum sample

from asthmatic patients has been shown to recover a simplified diverse community of the airway microbiome, when the samples are plated on different media types and cultured under varied growth conditions (Surette lab, unpublished data). Based on the data from this study, one can predict that embedding the inoculum in a matrix and providing a physical structure to the community can possibly retain important community interactions. Therefore, for future experiments, a 1/1000 dilution of the original sample should be utilized for inoculation of flow cells.

Despite of the high abundances of some organisms, ~50% of the genera present in the original sample were still detected in the flow cell effluent cultures and matrix on day 2. The overall viability of the bacterial populations in the flow cells was determined by plating effluent cultures on CBA (supplemented with 5% sheep blood). The comparison of the plate pool communities, recovered from effluents, with the original effluent sample (**5.4**, Appendix) confirmed that the communities propagated with flow cell culturing are viable.

Although the community composition in all the samples characterized in this study was determined by molecular analysis, the population shifts observed in the communities between day 2 and day 7 provide evidence that some, if not all, organisms are growing in the community. A shift in composition was observed when the flow cells were transferred from an anaerobic environment to aerobic conditions on day 2. This implies that communities in flow cells are dynamic, where the increase or decrease in relative abundance of an organism in a community may possibly be due to changes in environmental conditions. Obligate anaerobes were detected in lower proportions in most

cases (this observation was mirrored in anaerobic plate pool communities). The protocol followed for sputum sample collection can affect the efficiency of recovery of strict anaerobes[27]. The sputum samples obtained in this study were not transported anaerobically and the samples were received few hours after sample collection. This can possibly explain the low recovery of anaerobes in overall community.

It is noteworthy to mention that bacterial community present in the flow cell technical replicate of sample PN33 behaved differently, where high abundance of the obligate anaerobe *Fusobacterium* was recorded. This result was not observed in other technical and biological flow cell replicates; and anaerobic plate pool communities. Further analysis revealed that the same species was behaving differently in the technical replicates, where in replicate 2, ~20,000 sequences of the same OTU were detected as compared to 1 sequence detected in replicate 1 (**5.5**, Appendix). One can speculate that this observed shift in community composition of one bacterial community could be an outcome of underlying complex microbe-microbe/microbe-environment interactions or even both.

As discussed previously (Section **1.3.2**), studies have implicated the role of coaggregation in survival of obligate anaerobes like *Prevotella* spp. in mixed bacterial cultures[46, 47]. Anaerobic (including strict anaerobes) organisms can be cultured in flow cell system, and one possible mechanism guiding the development of this complex microbial community can be coaggregation. To analyze this further, I sought to determine whether coaggregates are formed in flow cell communities with fluorescence microscopy. Coaggregate formation was recorded where, very large coaggregates were observed on

day 7 (**5.6,** Appendix). Therefore, coaggregate formation could play an important role in formation of complex communities in the flow cells. The development of steep oxygen gradients within a community can sustain the growth/presence of obligate anaerobic organisms. It is already known that steep oxygen gradients develop in biofilms, and a depth of 8-22 um is sufficient for the establishment of anoxic conditions[47]. Thus, the anaerobic members of the community are protected from toxic effects of oxygen[47]. Some organisms in a bacterial community can also play a major role in oxygen consumption, thereby providing growth conditions favorable for anaerobes. A previous study demonstrated how *Neisseria* is a major consumer of oxygen in a ten-member mixed bacterial community propagated in chemostat cultures, thereby allowing growth of anaerobic organisms in the community[47].

The genera rank abundance plots were generated to reflect the cultivable diversity in flow cell communities. Relative abundances of taxa in a bacterial community are important to deduce the overall community structure, however the dominant organisms can obscure underlying information about the variety of taxa present[74]. To evaluate the utility of using flow cell method for culturing complex polymicrobial communities, it is necessary to determine the community membership in the flow cell samples (both effluent and matrix). Even though some organisms were present in relatively low proportions, it does not necessarily mean that those organisms are absent in the community. Therefore, I compared the organisms detected in flow cell communities with the community composition in the sputum. The data shows that a majority of the organisms present in the initial inoculum can be recovered in the matrix embedded community on day 7.

Conclusively, the method I have developed is effective in propagating diverse communities directly from the clinical sample. However, some aerobic organisms like *Rothia* and *Neisseria,* couldn't be recovered from the flow cells efficiently and this could possibly be due to the prolonged anaerobic incubation step in the initial stages of community development. Although these aerobes persist in the culture, they are not reproducing in the community. Therefore, shortening the first step of anaerobic incubation should be evaluated in future studies. It will be interesting to see whether decreasing the anaerobic incubation can have any effect on the community structure and efficiency of recovery of anaerobes.

PCoA revealed that community composition in the effluent cultures and matrix was most similar. Collecting effluents is the only way to sample the community over different time points, as our flow cell model is a closed system. The results demonstrate high similarities between the community composition in the flow cell effluents and matrix, substantiating that effluent cultures can be considered good representatives of the matrix embedded community dynamics and composition. Therefore, the growing community in flow cells can be surveyed at various intervals to study overall community structure and function. Furthermore, the separation of samples based on day validates the consistency of this method. The community members contribute to the separation of the samples, where sputum samples group separately suggesting that the community composition in the flow cell cultures is different from the starting community. It will be interesting to see whether changes in protocol and further optimization in future can propagate flow cell communities that are more similar to the initial inoculum.

Shannon diversity indices of samples showed a significant difference in the diversity of the original clinical sample and the flow cell communities. Shannon diversity is an indicator of species richness and evenness. Therefore, different growth rates can have an impact on the diversity index as evenness in the growing communities can sometimes substantially differ from the initial inoculum. Another example of this is plate pool communities, where culture enrichment selects for some organisms. More abundant organisms in the regrown communities can have impact on the calculated diversity index and does not necessarily mean that there are major variations in the variety of taxa present. The Shannon diversity of the effluent and matrix communities on day 7, were not significantly different which also demonstrates that a high level of similarity exists between these two communities.

Conclusively, flow cell method is a consistent method that can be utilized effectively for propagating diverse bacterial communities from airway samples. Further optimization can potentially improve the efficiency in recovering some organisms. Some technical challenges were encountered during the cultivation of complex microbial communities in the flow cell system and future experiments should be designed keeping these in mind. Due to the heavy growth of the bacterial communities in the matrix and effluent cultures, there were instances when back growth of biofilms into the inlet tubing was observed. Such samples can't be used for further analysis, as the media flowing into the flow cell is not sterile anymore. Formation of bubbles in the tubing is another common occurrence, where pre-incubation of the tubing and sterile medium at 37°C (prior to flow cell setup) can circumvent this.

Table 3.4 - List of samples and number of sequences/sample (Illumina sequencing)

Sample	Sequences/sample
PN32 sputum	41099
PN32 PP* aerobic	159865
PN32 PP anaerobic	49654
PN32 effluent anaerobic day 2	34592
PN32 effluent aerobic day 7	35565
PN32 matrix day 7	128742
PN32 effluent anaerobic day 2 PP	28248
PN32 effluent aerobic day 7 PP	26345
PN33 sputum	52731
PN33 PP aerobic	89090
PN33 PP anaerobic	41102
PN33 effluent anaerobic day 2 (replicate 1)	23284
PN33 effluent aerobic day 7 (replicate 1)	14331
PN33 matrix day 7 (replicate 1)	60887
PN33 effluent anaerobic day 2 (replicate 2)	43585
PN33 effluent aerobic day 7 (replicate 2)	137872
PN33 matrix day 7 (replicate 2)	37601
PN33 matrix day 2 (replicate 3)	88443
PN34 sputum	15066
PN34 PP aerobic	63397
PN34 PP anaerobic	34164
PN34 effluent aerobic day 7	31697
PN34 day 7 matrix	26073

*PP denotes plate pools

Figure 3.10 - Percent relative abundance of organisms (at the genus level) in bacterial communities cultured from sputum sample PN32.
Bars are coloured with genus level assignment for each group. Percent relative abundances of bacterial genera (for all representative sequences >0.1%) can be seen on the bar plot. The composition in the original sputum sample (PN32), which is the expected composition in regrown communities, is also shown. Data for plate pool (PP), effluent culture and matrix communities are shown.

Figure 3.11 - Percent relative abundance of organisms (at the genus level) in bacterial communities cultured from sputum sample PN33 (technical replicate 1). Bars are coloured with genus level assignment for each group. Relative abundances of bacterial genera (for all representative sequences >0.1%) can be seen on the bar plot. The composition in the original sputum sample (PN33), which is the expected composition in regrown communities, is also shown. Data for plate pool (PP), effluent culture and matrix communities are shown
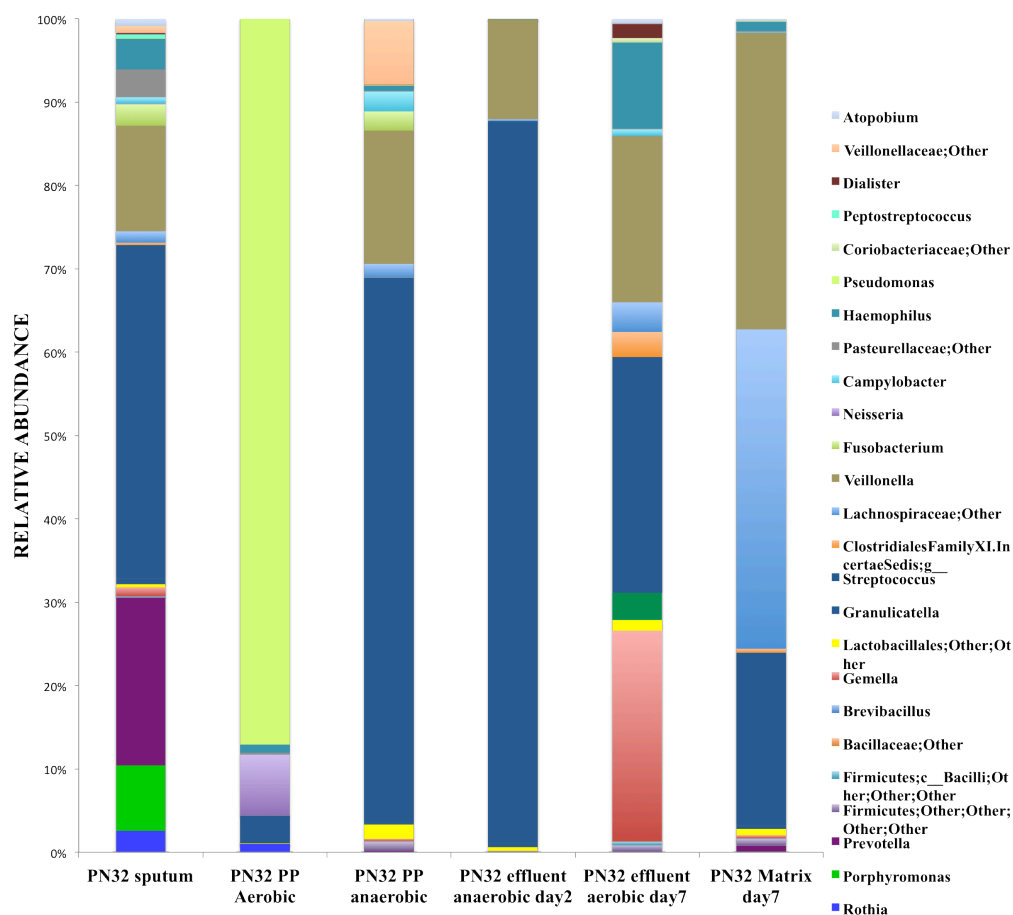
Figure 3.12 - Percent relative abundance of organisms (at the genus level) in bacterial communities cultured from sputum sample PN33 (technical replicate 2). Bars are coloured with genus level assignment for each group. Relative abundances of bacterial genera (for all representative sequences >0.1%) can be seen on the bar plot. The composition in the original sputum sample (PN33), which is the expected composition in regrown communities, is also shown. Data for plate pool (PP), effluent culture and matrix communities are shown.
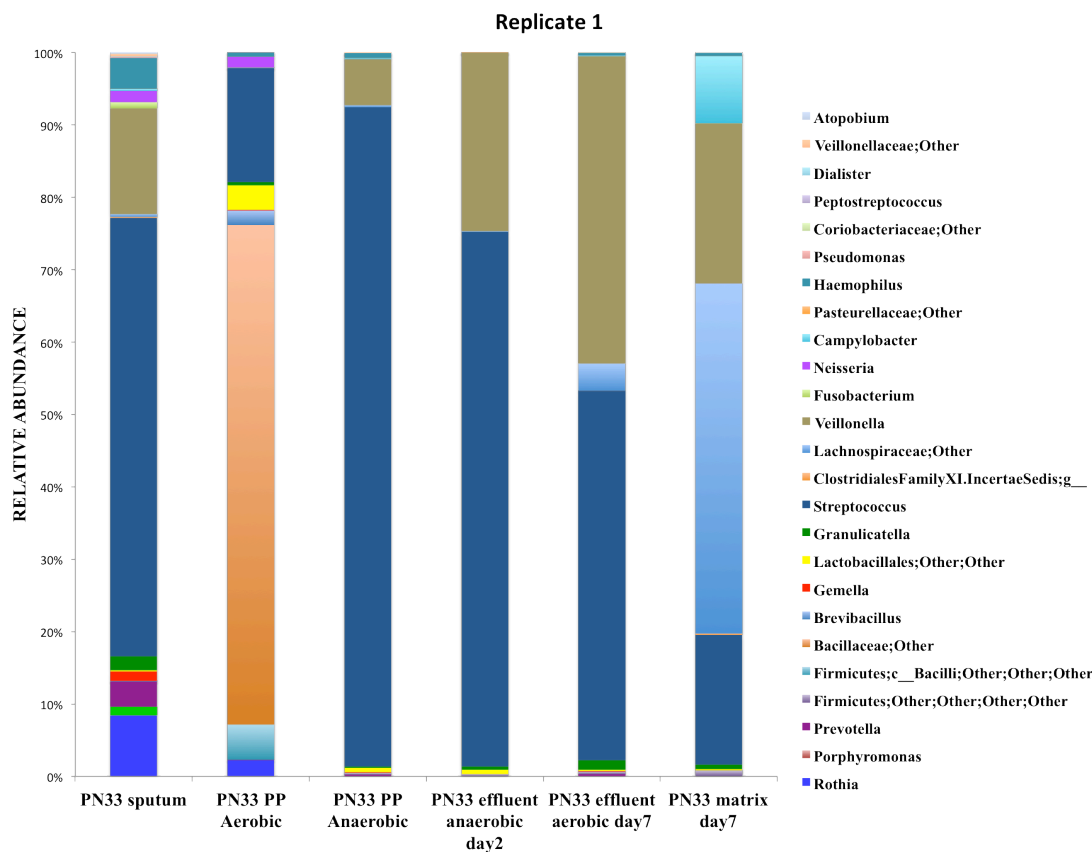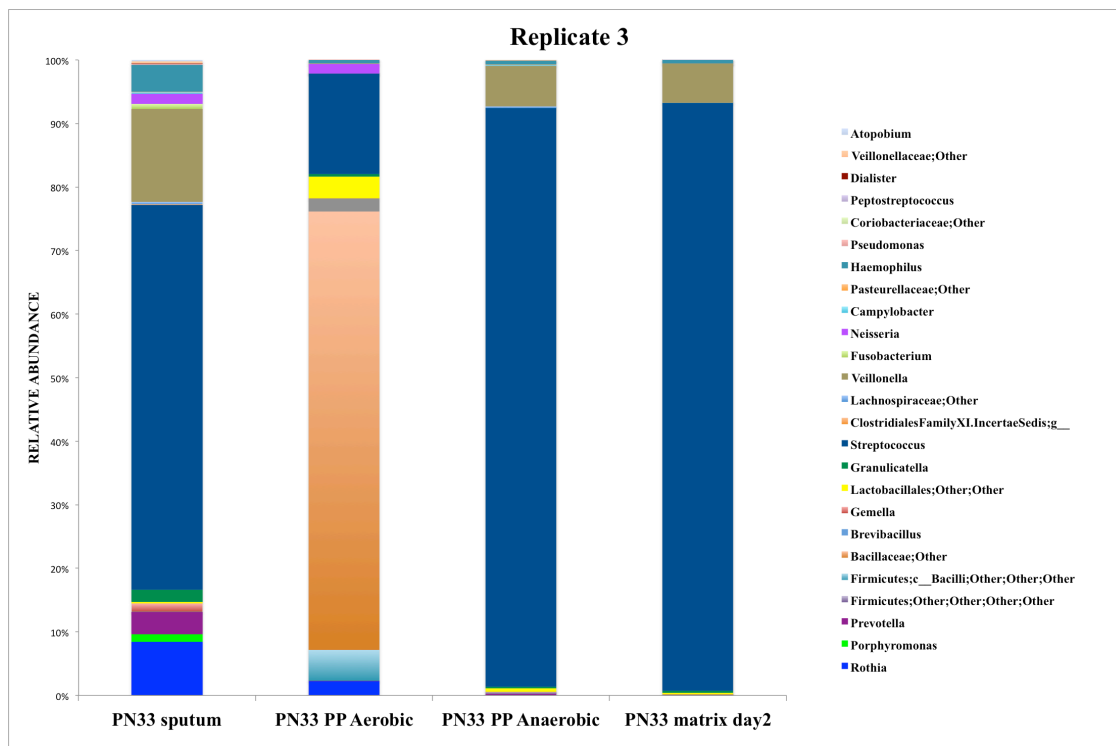
Figure 3.13 - Percent relative abundance of organisms (at the genus level) in bacterial communities cultured from sputum sample PN33 (technical replicate 3). Bars are coloured with genus level assignment for each group. Relative abundances of bacterial genera (for all representative sequences >0.1%) can be seen on the bar plot. The composition in the original sputum sample (PN33), which is the expected composition in regrown communities, is also shown. Data for plate pool (PP) and matrix embedded community on day 2 are shown.
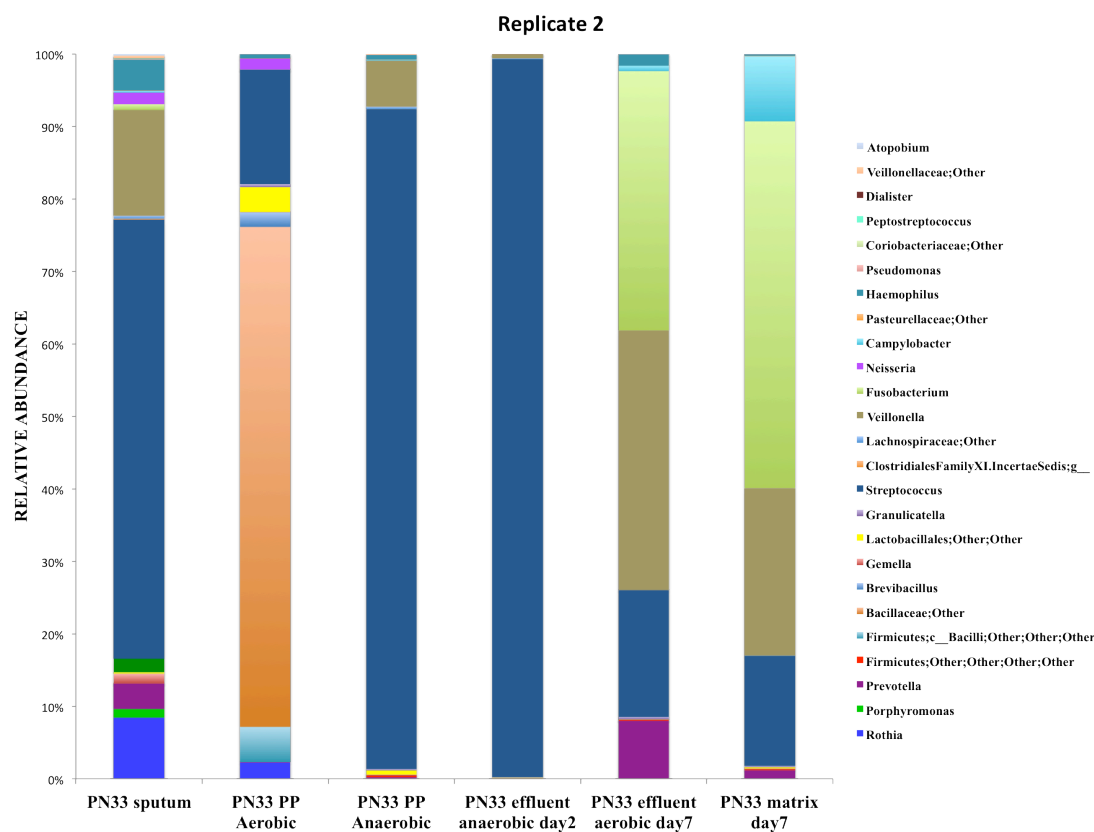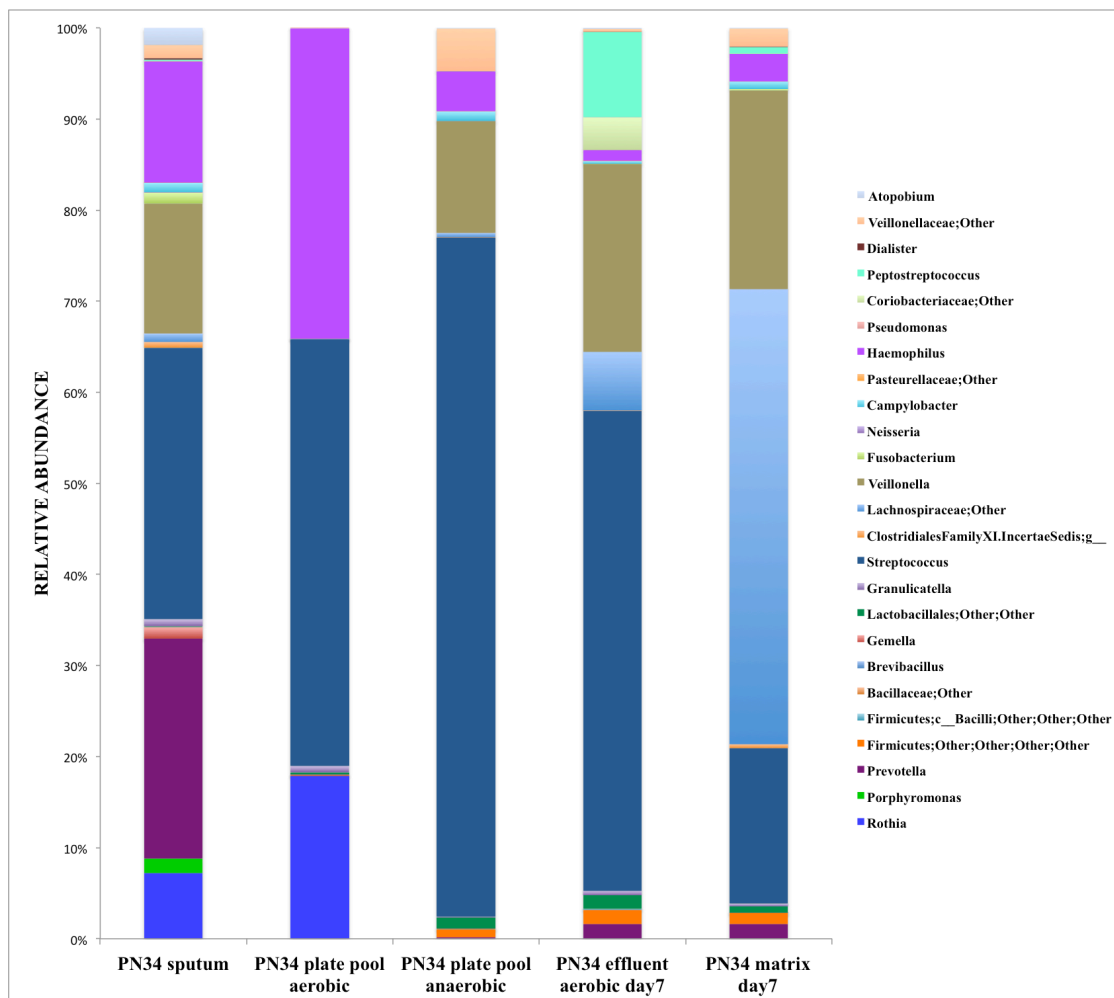
Figure 3.14 - Percent relative abundance of organisms (at the genus level) in bacterial communities cultured from sputum sample PN34.
Bars are coloured with genus level assignment for each group. Relative abundances of bacterial genera (for all representative sequences >0.1%) can be seen on the bar plot. The composition in the original sputum sample (PN34), which is the expected composition in regrown communities, is also shown. Data for plate pool (PP), effluent culture (aerobic, day 7) and matrix communities are shown.
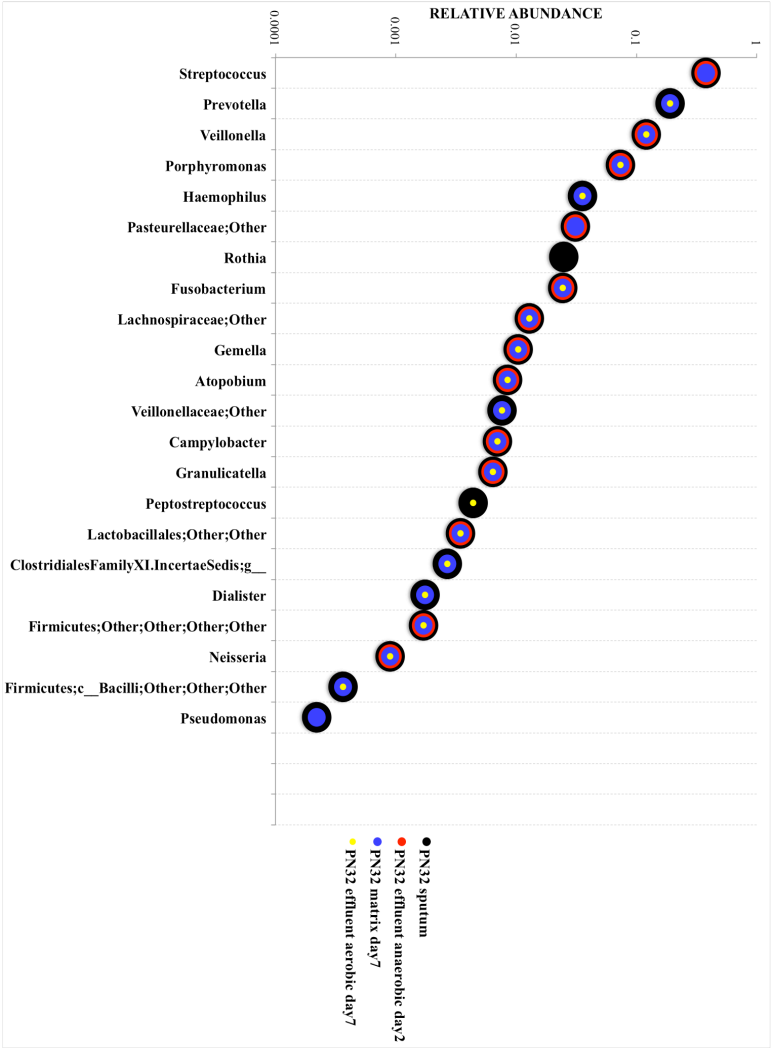
Figure 3.15 - Determining the community membership in flow cell communities grown from sputum sample PN32. Relative abundances of bacterial genera in the sputum sample (for all representative sequences >0.01%) are plotted in order of decreasing abundance for determining the community membership. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell samples (both matrix and effluent) are indicated on this same plot, to illustrate the cultivable members of the flow cell communities. Black, red, yellow and blue illustrate the sputum, anaerobic effluent culture on day 2, aerobic effluent culture on day 7 and matrix embedded community respectively.

Figure 3.16 – Determining the community membership in flow cell communities grown from sputum sample PN33 (technical replicate 1).
Relative abundances of bacterial genera in the sputum sample (for all representative sequences >0.01%) are plotted in order of decreasing abundance for determining the community membership. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell samples (both matrix and effluent) are indicated on this same plot, to illustrate the cultivable members of the flow cell communities. Black, red, yellow and blue circles illustrate the sputum, anaerobic effluent culture on day 2, aerobic effluent culture on day 7 and matrix embedded community respectively.

Figure 3.17 - Determining the community membership in flow cell communities grown from sputum sample PN33 (technical replicate 2).
Relative abundances of bacterial genera in the sputum sample (for all representative sequences >0.01%)) are plotted in order of decreasing abundance for determining the community membership. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell samples (both matrix and effluent) are indicated on this same plot to illustrate the cultivable members of the flow cell communities. Black, red, yellow and blue circles illustrate the sputum, anaerobic effluent culture on day 2, aerobic effluent culture on day 7 and matrix embedded community respectively.
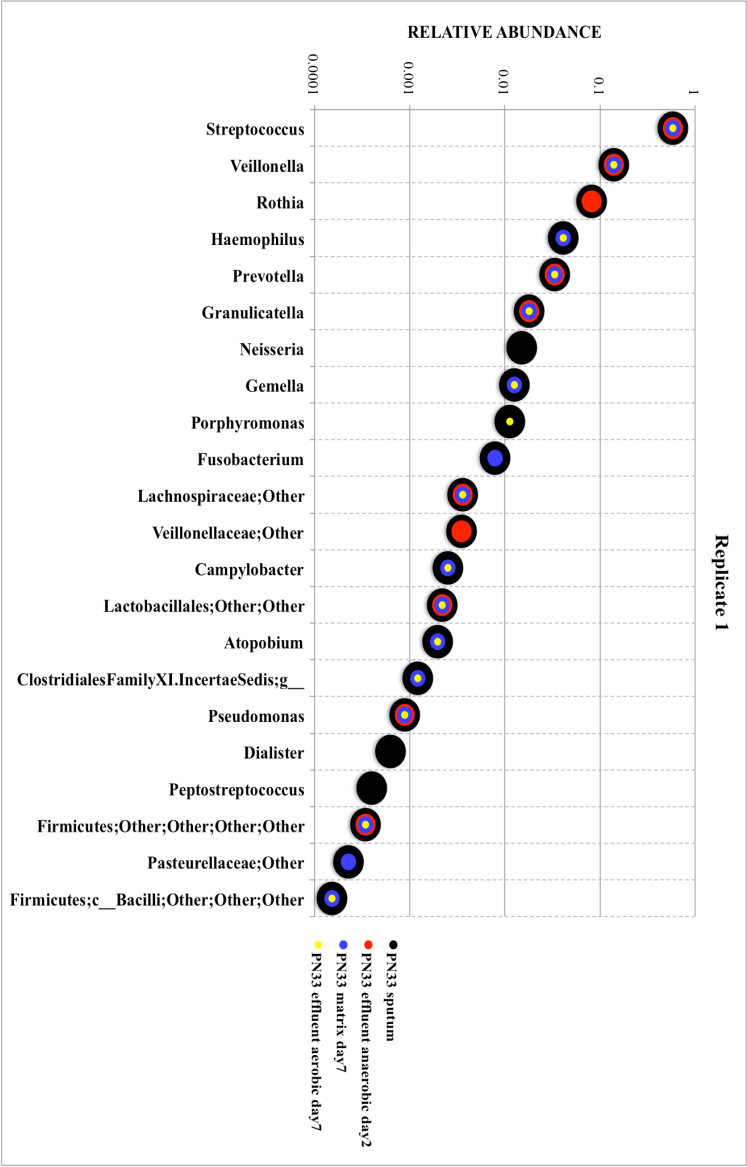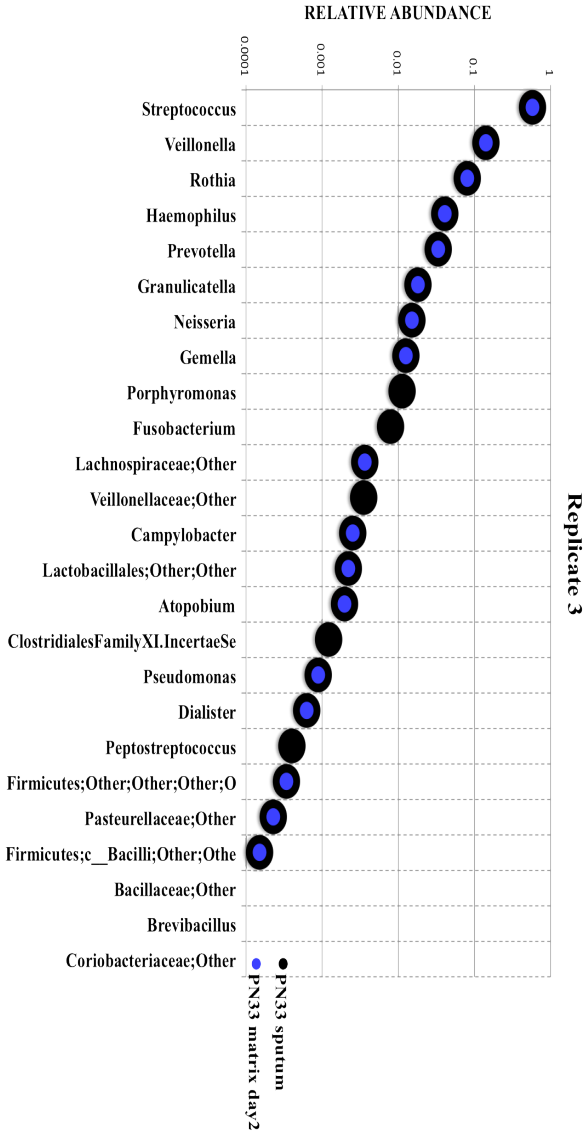
Figure 3.18 - Determining the community membership in a flow cell matrix embedded community grown from sputum sample PN33 (technical replicate 3). Relative abundances of bacterial genera in the sputum sample (for all representative sequences >0.01%) are plotted in order of decreasing abundance for determining the community membership. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell matrix on day 2 when community is anaerobic, are indicated on this same plot, to illustrate the cultivable members of the flow cell communities. Black and blue circles illustrate the sputum and matrix embedded community respectively.
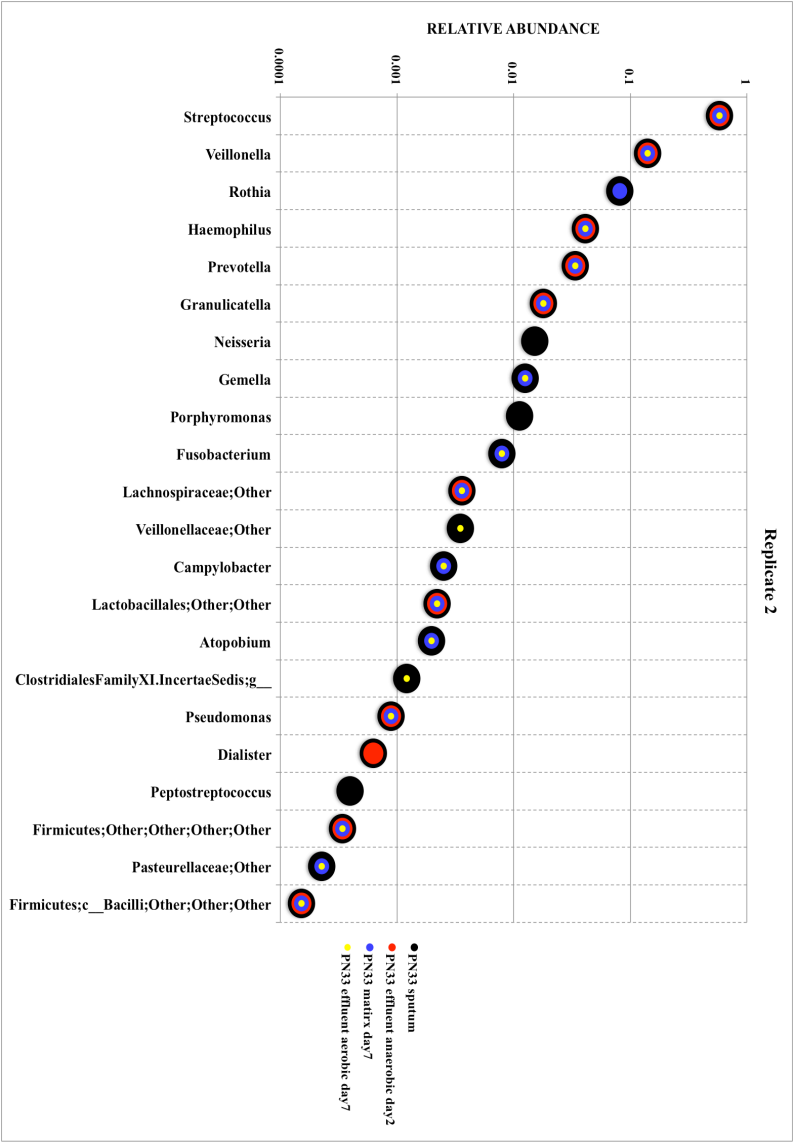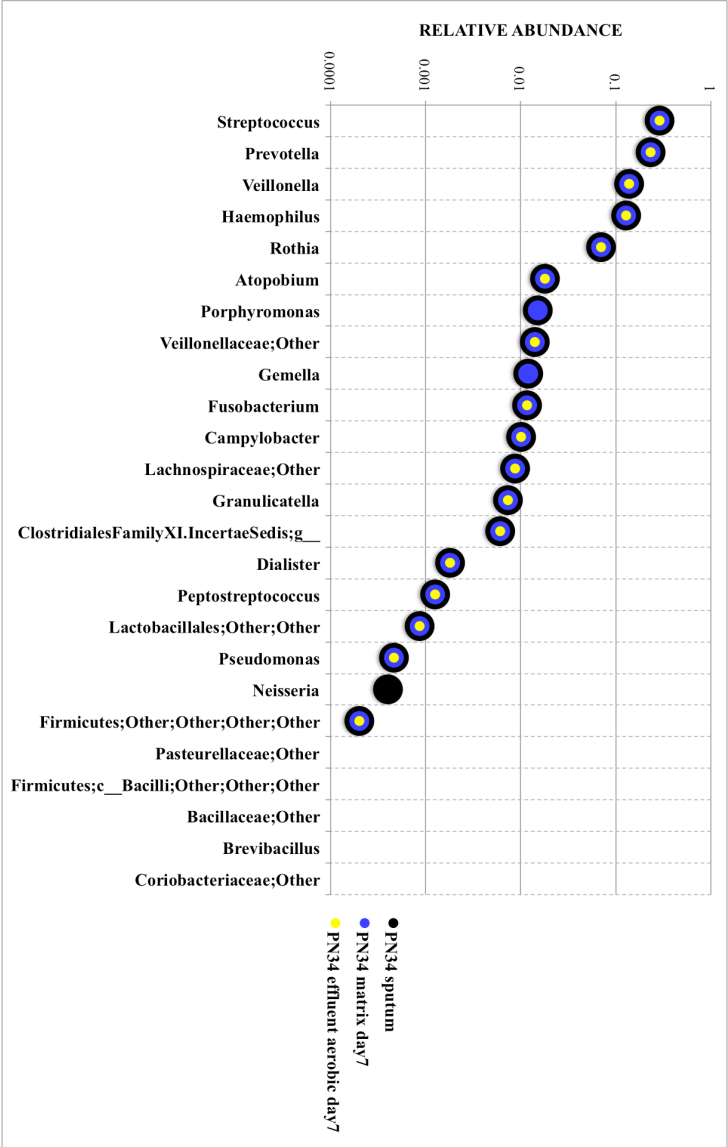
Figure 3.19 - Determining the community membership in flow cell communities grown from sputum sample PN34. Relative abundances of bacterial genera in the sputum sample (for all representative sequences >0.01%) are plotted in order of decreasing abundance for determining the community membership. This represents the predicted community composition in the original sample. The organisms recovered in the flow cell samples (both matrix and effluent) are indicated on this same plot, to illustrate the cultivable members of the flow cell communities. Results for anaerobic effluent culture on day 2 are not shown here. Black, yellow and blue circles illustrate the sputum, aerobic effluent culture on day 7 and matrix embedded community respectively.
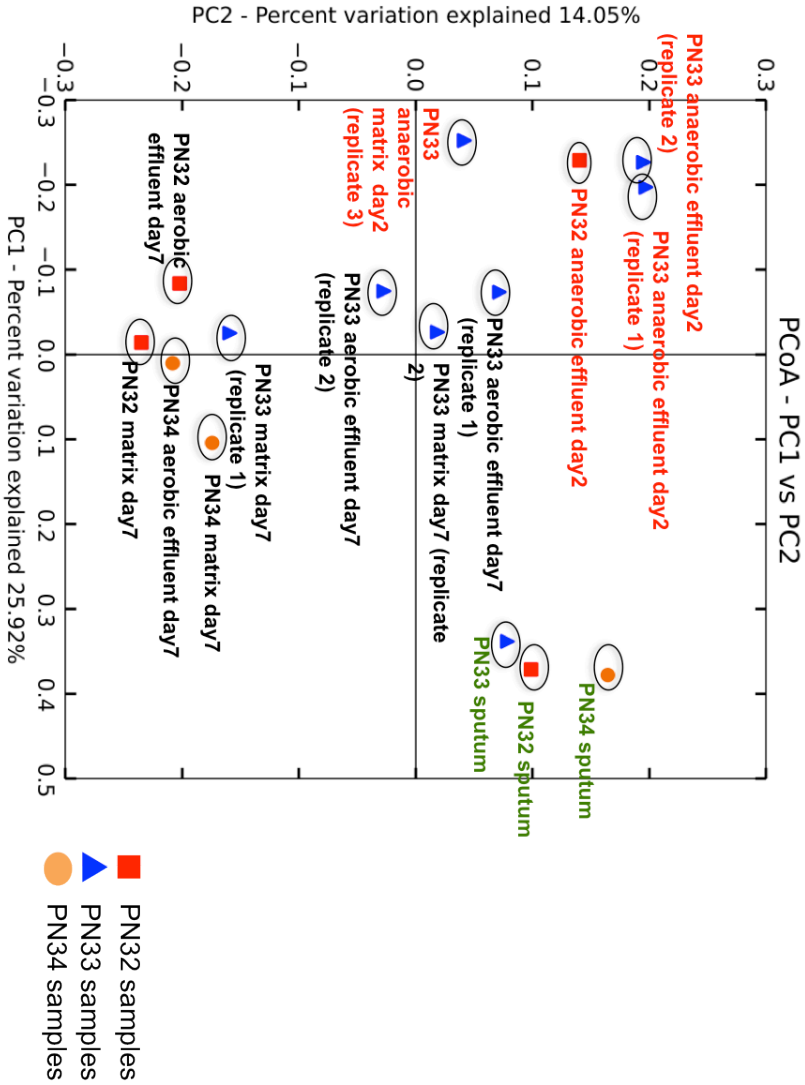
Figure 3.20 - Principle coordinate analysis (PCoA) plot showing variations between flow cell samples (effluents and matrices) and original sputum sample.
The plot demonstrates that primary clustering is by day, and high similarity is seen between the flow cell effluent and matrix embedded communities. Unweighted UniFrac metrics were used to generate the plot.
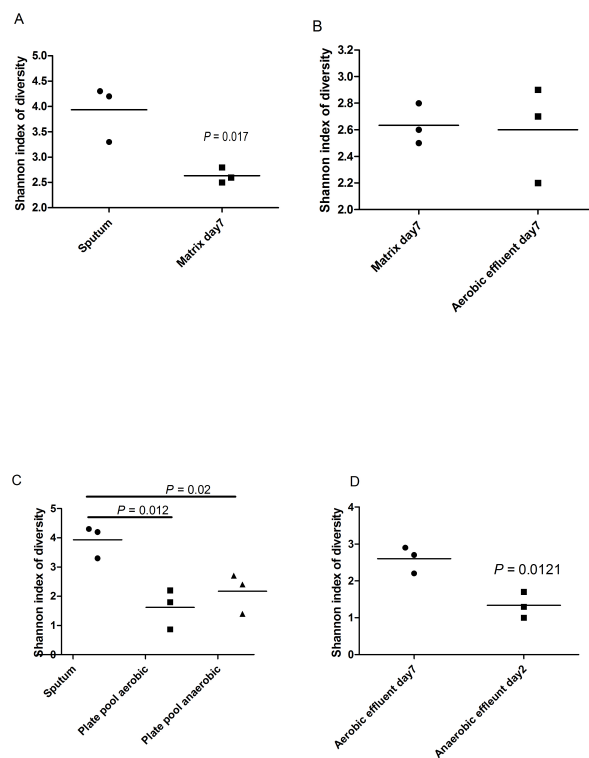
Figure 3.21 - The Shannon diversity index in flow cell, plate pool and sputum samples.
A) Original sputum samples and matrix embedded communities on day 7. (B) Flow cell effluent culture and matrix embedded communities on day 7. (C) Original sputum samples and plate pool communities. (D) Flow cell effluent community on day 7 and day 2.

CHAPTER 4: OVERALL DISCUSSION AND FUTURE DIRECTIONS

Microbes exist predominately in communities in natural environments and there is lack of understanding of the complex processes that operate in these communities. The goals of this study were: 1) to develop methods to propagate and examine mixed synthetic and natural communities *in vitro*, 2) and have a system that can be utilized in studying the community structure and dynamics over time. A flow cell system operated under continuous flow conditions was optimized. We adopted an approach where matrix embedded communities were cultured under continuous flow conditions with the aim of capturing the development of microbial communities in the natural context. Simple synthetic bacterial communities were propagated in the system for initial optimization, and analyses of bacterial populations furthered my understanding of community dynamics and growth behaviour of bacterial species under different culture conditions (batch vs. continuous). The development of natural oxygen gradients in the microbial communities plays a pivotal role in retaining the obligate anaerobic members in the growing community[47]. Moreover, some interspecies interactions within the community can also have a major impact on microbial growth and survival[45, 75]. This kind of complexity was also perceived in the microbial communities propagated in my model as obligate anaerobic organisms could successfully thrive under aerobic conditions, when present as members of mixed bacterial communities (aerobes and anaerobes) propagated under flow conditions. The ultimate goal of propagating complex bacterial communities directly

from airway samples was also achieved with the flow cell model. The microbial diversity of the sputum sample could be captured to a great extent in the flow cell communities.

The flow cell method is inexpensive and the desired growth environment can be attained in the system by controlling the flow of media or changing growth conditions (e.g. temperature). The flow cell can also be utilized for microscopy and the growth of microbes can be visually assessed over the course of time[37, 46]. However, this system comes with its own disadvantages, where most importantly extra care must be taken to ensure sterility in the entire flow cell setup. Formation of bubbles, leakage, and back growth of biofilms into inlet tubing are other technical difficulties encountered while using this system. Nevertheless, this method can serve as a powerful tool for discerning the complexity of polymicrobial communities.

By growing communities under conditions that more closely resemble natural environments, one can study the relationship between changes in community composition and function over time. In future studies, communities propagated in this system can be exposed to antimicrobials, assayed for enzyme activities and chemical products. The community behaviour in response to environmental stimuli can be investigated. Furthermore, host immune response to these communities can also be characterized. The results from some preliminary experiments conducted to study microbial community function are described below.

Autoinducer-2 (AI-2) is an inter-species signaling molecule, and can provide an idea about the cell density and overall fitness of the microbial community as the AI-2 levels are linked to cell numbers[13]. A previous study from our lab demonstrated that AI-2

signaling could direct the outcome of microbe-microbe interactions, where it played an important role in the regulation of certain *P. aeruginosa* virulence factor genes[76]. AI-2 has also been found to play a role in biofilm formation, where in some cases it promotes biofilm formation, whereas in others (eg. *Staphylococcus epidermidis*) it inhibits biofilms[77]. Therefore, AI-2 can be used to monitor overall community function. In some preliminary experiments, AI-2 activity in cell-free supernatants from bacterial cultures, and flow cell effluent and matrix cultures was recorded (**5.7**, **5.8**, **5.9**, Appendix). AI-2 was detected in all the samples.

Host responses in the form of cell viability and NF-κB activity were measured in some preliminary experiments. A human lung epithelial cell line (BEAS-2B) that closely resembles primary human airway epithelial cells was used[78]. To compare the cytotoxicity of the factors produced by communities cultured in flow cells, the airway epithelial cell line BEAS-2B was treated with the culture supernatants from the bacterial communities growing in the flow cells (**5.7**, **5.11** Appendix). The effect of supernatants from mono-species cultures on the viability of epithelial cells was also investigated in parallel to see whether changes in the microbial community correspond to any negative interactions with the host. Cell-free supernatants from bacterial cultures, flow cell effluent cultures and matrix failed to kill human airway epithelial cell lines.

NF-κB, is a transcription factor that can cause induction of many inflammatory cytokines[79]. A previous study showed how a mixture of two bacterial species could be more potent in inducing NF-κB activation as compared with the individual bacterial species. The study demonstrated the role of synergistic interactions in inducing bone loss,

in a mouse periodontitis model[79]. A reporter monocyte cell line, THP-1-blue, was used to investigate whether underlying community interactions in mixed synthetic communities could induce NF-κB activation. The cells release secreted embryonic alkaline phosphatase (SEAP) in the detection culture medium on NF-κB activation. Cell-free supernatants from bacterial cultures were assayed for NF-κB activation in preliminary experiments (**5.7**, **5.10**, **5.12**, Appendix). High background levels of alkaline phosphatase in *Prevotella* supernatants were recorded, which makes this reporter cell line ineffective for future work. However, the assay was effective in detecting NF-κB activity in cell-free supernatants from other organisms (e.g. *Streptococcus* sp.). Therefore, alternatives for this reporter cell line should be considered in future work such as luciferase based reporter cell lines.

In this thesis, I have presented my studies on developing simple *in vitro* systems for propagating polymicrobial communities. Initial studies with synthetic communities showed that least diverse communities were propagated in shaken batch cultures (studies on aerobic communities). Therefore, shaken batch cultures were not chosen for culturing mixed (aerobes & anaerobes) communities, and all the comparisons were conducted between static batch cultures and flow cell cultures. Continuous flow cell cultures supported growth of anaerobes in mixed synthetic communities, whereas static cultures lost community diversity and did not support robust growth of anaerobes as expected. The flow cell system, when applied to clinical samples from the lower respiratory tract, was able to capture most of the microbes present, and supported robust growth of anaerobes. Future experiments should explore different incubation times and culture

media. A limitation of the matrix embedded growth is that it cannot be continuously sampled over time, however effluents also contained a significant diversity of microbes (including the anaerobes). Since the effluent can be continuously sampled, it may be suitable for time course studies and to examine the effect of changing the growth parameters on community composition. The methodology I have developed is simple and reproducible and should allow for further studies on polymicrobial communities recovered from natural environments.

CHAPTER 5: APPENDICES
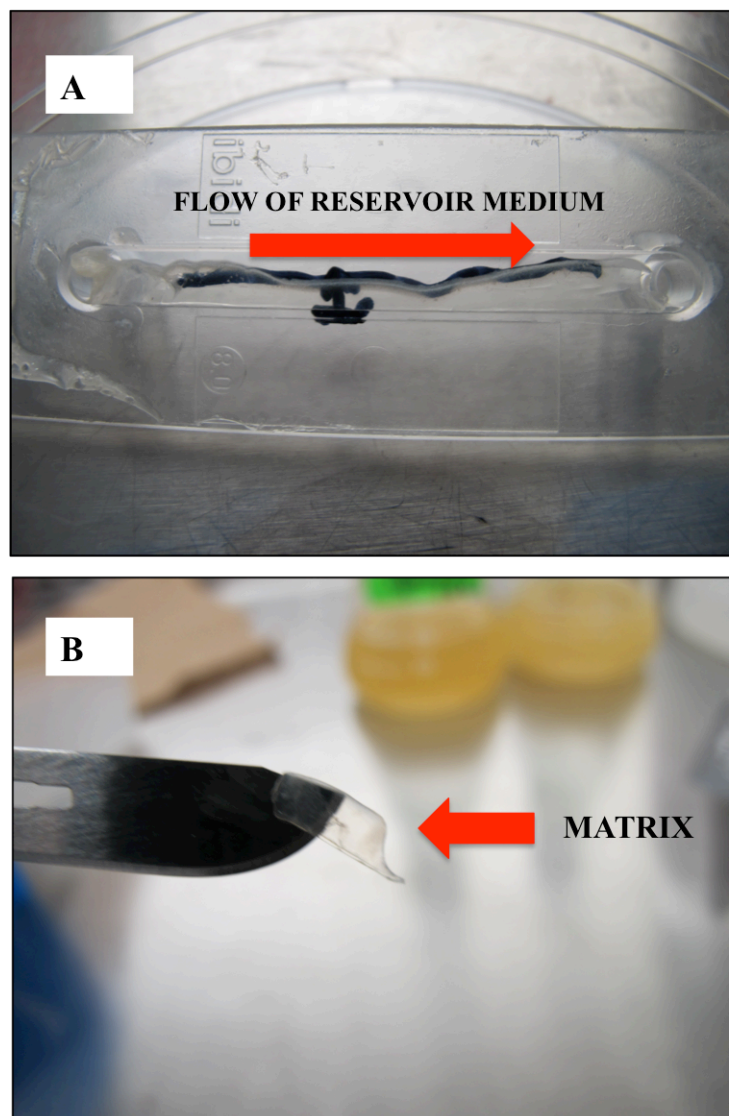
5.1 - **Images of the flow cell and agarose matrix in the flow channel.**
A) The flow cell (ibidi) used for culturing communities under continuous flow conditions is shown. The matrix is deposited along the length of one wall, allowing the passage of medium reservoir through the rest of the channel. B) The matrix recovered on day 7 from the flow channel is shown.

	Day 0(ana)	Day 2(ana)	Day 2(ana) + Day 2(aer)
■ Streptococcus salivarius	2.20E+05	1.00E+09	4.00E+09
■ Staphylococcus epidermidis	7.40E+05	5.00E+07	6.00E+08
■ Staphylococcus lugdunensis	1.90E+05	7.20E+08	3.00E+09
■ Fusobacterium nucleatum	2.16E+05	8.80E+04	1.06E+08
■ Prevotella melaninogenica	1.13E+07	8.80E+06	3.00E+09
■ Veilonella atypica	1.26E+06	3.20E+06	4.00E+09
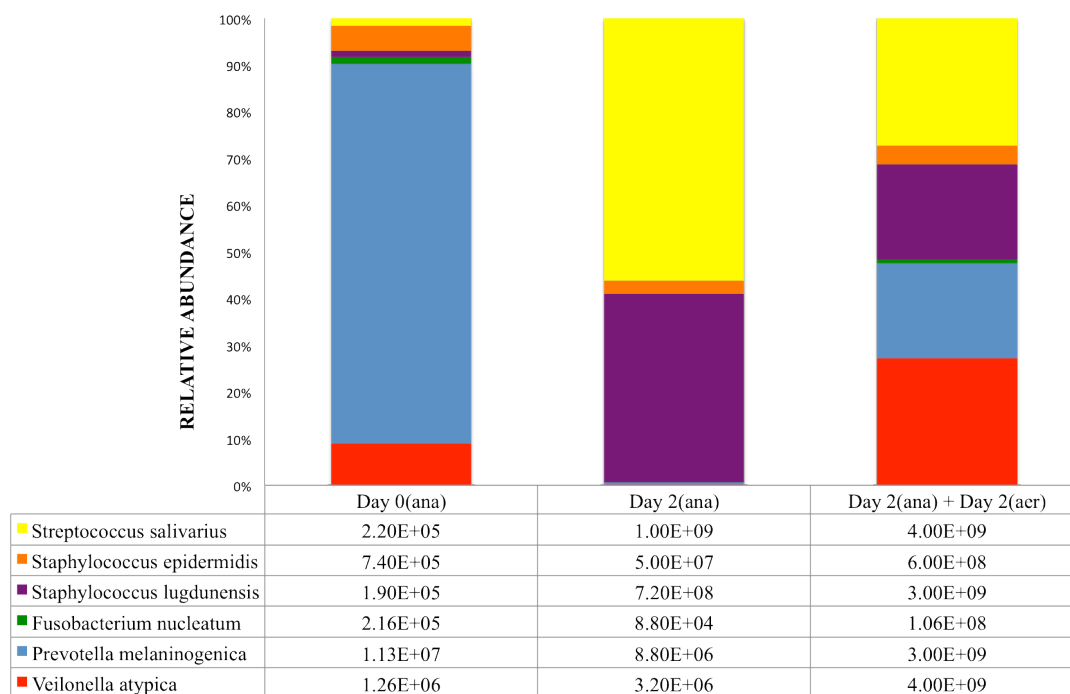
5.2 - **The effect of flow rates on the composition of a mixed species synthetic community.**

A flow cell community was cultured under high flow rates (1.5 ml/hour) and the composition within the matrix embedded community is shown. Higher flow rates did not affect the proportions of obligate anaerobes (CFU/ml values are shown below the bar plot). Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs. The matrix from a flow cell was sacrificed on day 2 (ana) and day 2 (ana) + day 2(aer); ana: anaerobic, aer: aerobic. Relative abundance for each species was determined by total viable counts. The results for one experiment are shown.

	Starting community	Static day0 anaerobic	Static day 2 aerobic	Static day 4 aerobic	Static day 6 aerobic
Streptococcus sp.	62000	2.00E+05		8.80E+03	
Staphylococcus epidermidis	300000		2.00E+08	4.00E+05	3.80E+08
Staphylococcus lugdunensis	360000	2.50E+07	8.00E+07	4.20E+07	2.90E+07
Fusobacterium nucleatum	1400000	1.00E+04			
Prevotella melaninogenica	1320000	7.00E+03			
Veilonella atypica	60000	6.80E+07	4.00E+07	2.40E+06	1.04E+07

5.3 – Composition of a mixed species synthetic community in static batch cultures.
The structure of a six-species bacterial synthetic community propagated in static batch cultures is shown. Three batch cultures were inoculated on the same day from a starter culture. One culture was sacrificed at each time point (the culture was vortexed before plating). The results for one experiment are shown. Day 0(ana) refers to the starting community (initial inoculum), and Day 2(ana) refers to the time point when transfer to aerobic conditions occurs; ana: anaerobic, aer: aerobic. Obligate anaerobes could not be recovered under aerobic conditions. Relative abundance for each species was determined by total viable counts.
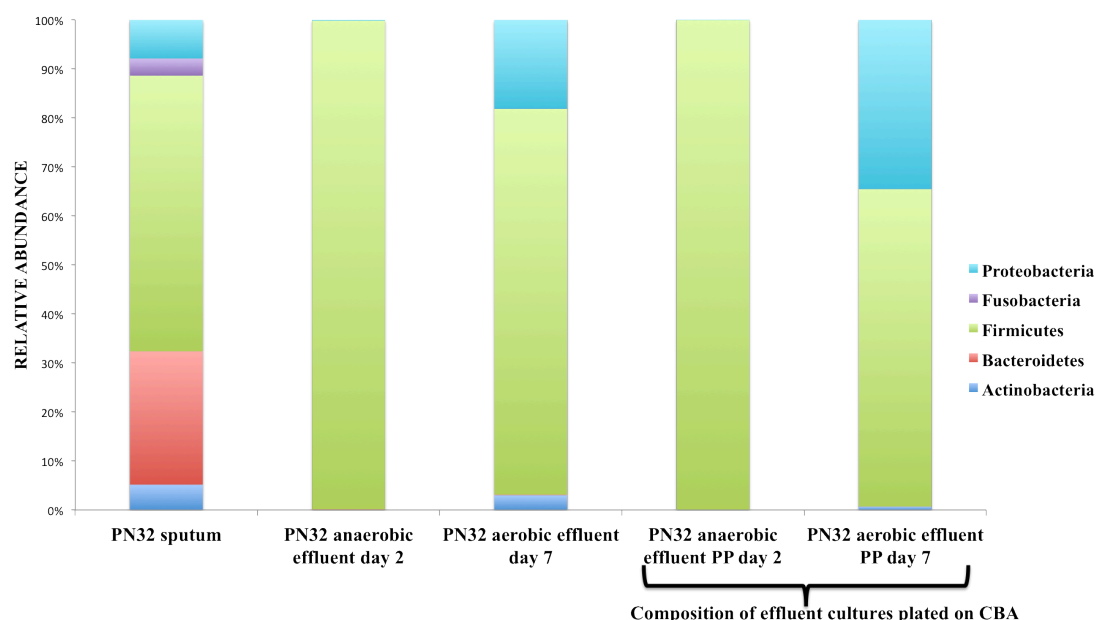
5.4 - Comparison between the compositions of communities in flow cell effluent cultures and communities cultured from effluents by plating (PN32).

To characterize the structure of viable bacterial populations within flow cell effluent cultures, the effluents were plated on Columbia Blood Agar (supplemented with 5% sheep blood) on day 2 and day 7. The communities within the plate pools (PP) were compared with original effluent sample. The plate pool communities are similar in structure to the effluent communities. Relative abundance of organisms (at the phylum level) in bacterial communities cultured from sputum sample PN32 are shown. Bars are coloured with phylum level assignment for each group. Relative abundances of bacterial phyla (for all representative sequences >1%) can be seen on the bar plot. The composition in the original sputum sample (PN32), which is the expected composition in regrown communities, is also shown. Data for effluent cultures (day 2 and day7) and plate pool communities of effluent cultures (day 2 and day 7) are shown.
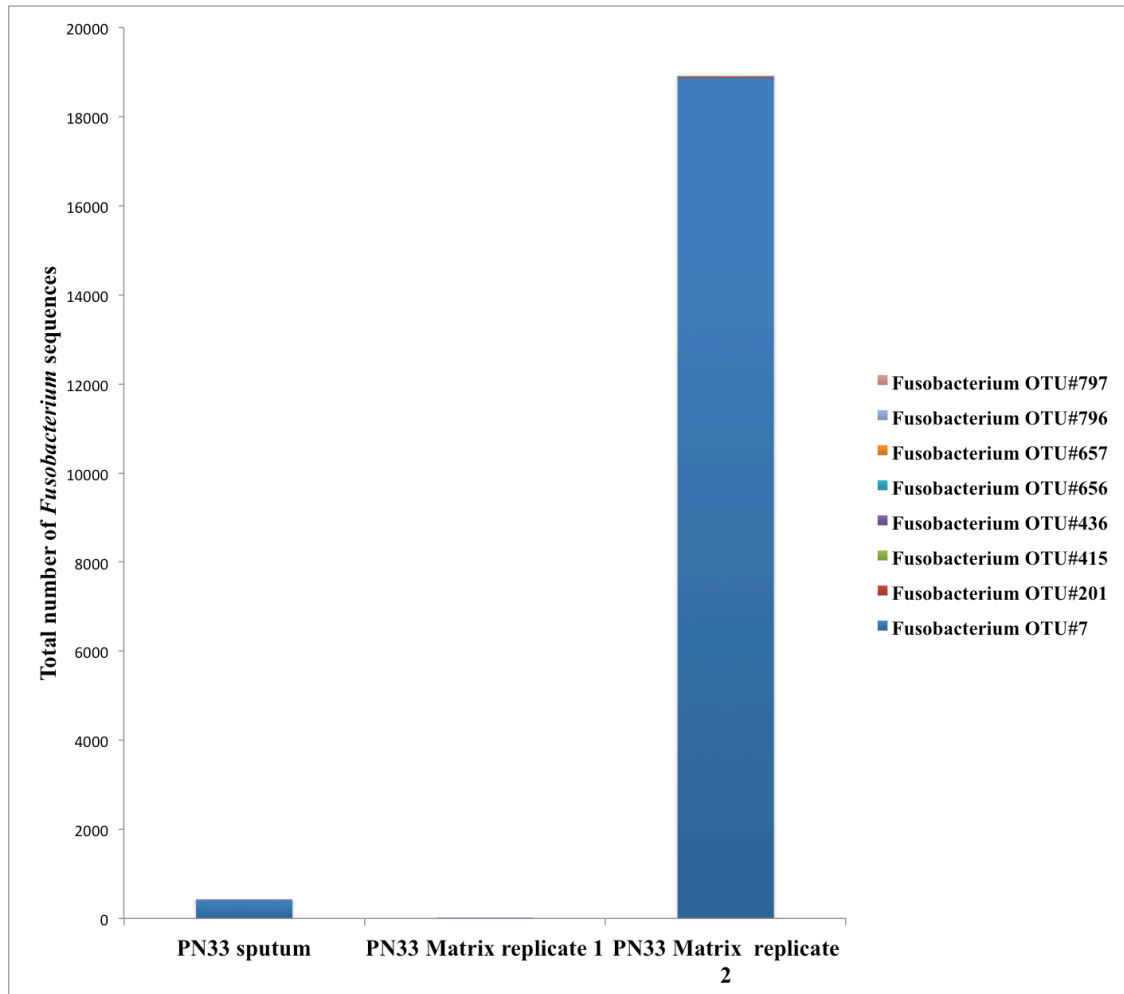
5.5 - **Comparison of the community structure, within the genus *Fusobacterium*, between the original sputum sample (PN33) and matrix embedded communities.**
OTUs (down to species level) were compared in *Fusobacterium* to investigate whether the same OTU is behaving differently in technical replicates. The number of sequences of each unique OTU in the total number of *Fusobacterium* sequences is represented on the plot. OTU#7 is more abundant in the matrix of technical replicate 2 as compared to the original samples and technical replicate 1.
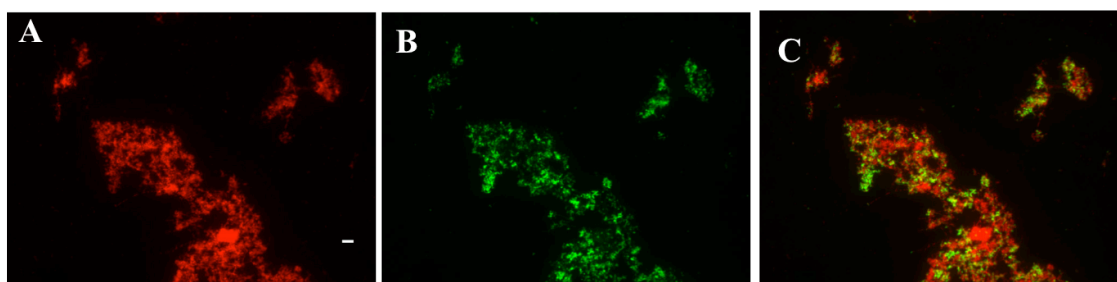
5.6 **- Coaggregates are detected in flow cell effluent cultures.**
Coaggregates were detected in the flow cell effluent communities on day 7 (coaggregates in the effluent sample of PN33 (technical replicate 2) are shown). Bacterial communities in effluent culture were stained with the nucleic acid stain, DAPI (A), and bacterial cell wall peptidoglycan stain, WGA-488 conjugate (Alexa-Fluor®) (B). (C), is an overlay image of (A) and (B). Microscopic observations and image acquisition was performed on Olympus BX-51 with a 20X objective. Bar, 5 μm.

5.7 - Preparation of cell-free supernatants

Cell-free culture fluids (supernatants) were obtained from BHI broth cultures of the aerobic and anaerobic test strains (see **Table 2.1**). After incubation, cultures were spun down at 13000 rpm at maximum speed on a bench top centrifuge for 5 minutes. The resulting supernatant was filter sterilized with a 0.2 µm pore size filter (Millipore). Supernatants from the flow cell effluent cultures and matrix were also collected similarly, where the matrix was diluted 1:10 into sterile BHI medium (due to its small volume (60 µl)) before centrifugation, and the supernatants were collected thereafter. Aliquots of the supernatants were made in 1.5 ml microfuge tubes and were stored at -20°C.

5.8 - Autoinducer-2 expression assays

AI-2 production in flow cell effluent cultures and matrix embedded communities were measured using a *Vibrio harveyi* bioassay[80]. MM32 *Vibrio harveyi* reporter strain was used for this assay. *Salmonella* sp. cell-free supernatants were used as positive controls. For conducting the assay, *V. harveyi* MM32 strain was inoculated into 5 ml Autoinducer Bioassay (AB) medium. The culture was incubated overnight (16 hours) with aeration at 30°C, where the overnight culture was then diluted 1:5000 in fresh AB medium and the cell-free supernatants were added to the diluted *V. harveyi* culture at a 10% (v/v) final concentration (each supernatant-dilute culture mixture was setup in triplicate). A negative medium control (10% AB medium) and bacterial growth medium control (10% BHI) was also included in the assay. The assay was conducted in a 96-well microtiter plate (Costar, clear bottom black plate), where the plate was UV irradiated for 30 minutes prior to use. Light production was measured using a luminescence plate reader (SpectraMax M3, Molecular Devices).

A

Fold Activation

- ■ *Veilonella atypica*
- ■ *Prevotella melaninogenica*
- ■ *Fusobacterium nucleatum*
- ■ *Staphylococcus lugdunensis*
- ■ *Staphylococcus epidermidis*
- ■ *Streptococcus sp.*

B

Fold Activation

- ■ FC effluent day 2(ana)
- ■ FC effluent day 2(ana) + day 2(aer)
- ■ FC effluent day 2(ana) + day 6(aer)
- ■ FC matrix day 2(ana) + day 6(aer)
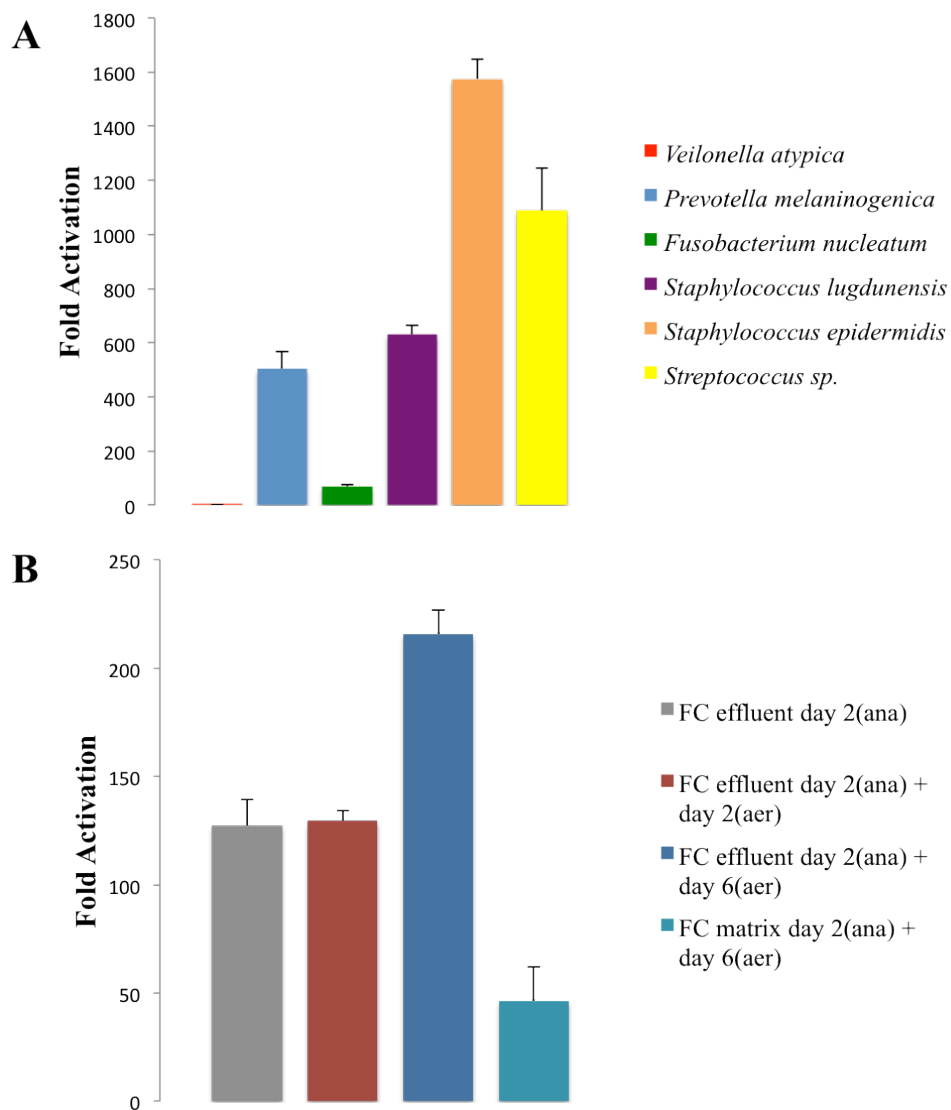
5.9 - Detection of autoinducer-2 (AI-2) in bacterial cultures and a mixed species synthetic community.
AI-2 activity is represented as fold induction of luminescence over medium control, using the *Vibrio harveyi* reporter system. BHI medium was used for growing bacterial cultures and was also used as the medium reservoir for flow cell culturing. Culture supernatants were added at 10% to the assays. The AI-2 activity in the bacterial cultures (A), and flow cell (FC) communities (both effluent and matrix) (B) is shown. Day 2(ana) refers to the time point when transfer to aerobic conditions occurs; ana: anaerobic, aer: aerobic. The matrix was diluted 1:10 into sterile BHI medium. Error bars indicate standard deviations.

5.10 NF-κB activation (SEAP reporter assay)

THP1-Blue cell line (InvivoGen) is a reporter cell line derived from the human THP-1 monocytic cell line that expresses a secreted embryonic alkaline phosphatase (SEAP) gene under the control of NF-κB and AP-1 inducible promoter. Cells were cultured in RPMI 1640 containing 10% heat inactivated fetal bovine serum (FBS), penicillin, streptomycin and normocin. SEAP is secreted by the cells upon NF-κB activation and can be detected in HEK-Blue^{TM} Detection medium (InvivoGen). Cells were seeded at a density of 3 x 10^4 cells/well in a 96-well microtiter plate (BD Biosciences). After 3 days, the media was removed and replaced with HEK-Blue^{TM} Detection media after washing the wells 2X with phosphate buffered saline (PBS). Cell-free supernatants obtained from test strains (see **Table 2.1**), flow cell effluent cultures and matrix embedded communities were added to the experimental wells at 1% (v/v), 2% (v/v), 5% (v/v) and 10% (v/v) final concentrations. THP-1 blue cells were also challenged with a *Salmonella enterica* LPS (100 ng/ml) (Sigma-Aldrich; catalog #: 1770) as a positive agonist. Additionally, bacterial growth medium control (10% BHI) was also included in the assay. Cells were then incubated for 16 hours at 37°C under 5% CO_2 conditions. Absorbance at 620 nm was read with a plate reader (SpectraMax M3, Molecular Devices).

5.11 Cell viability (Cytotoxicity assay)

BEAS-2B cells were cultured in complete Dulbecco' Modified Eagle Medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum, 50 U/ml penicillin and 500 μg/ml streptomycin. At 80% confluency the cells were seeded at a density of 10^4 cells/well in a 96-well microtiter plate (BD Biosciences). After 48 hours media was removed, the wells were washed gently once with PBS and fresh DMEM was added to the culture wells. Cell-free supernatants obtained from test strains (see **Table 2.1**), flow cell effluent cultures and matrix embedded communities were added to the experimental wells at 1% (v/v), 2% (v/v), 5% (v/v) and 10% (v/v) final concentrations. The control wells included a non-treated negative control, a completely lysed positive control and a bacterial growth medium control (10% BHI). Cells were then incubated for 24 hrs at 37°C under 5% CO_2 conditions. Post-incubation, supernatants were collected by centrifugation and used with the lactate dehydrogenase (LDH) cytotoxicity kit (Promega) to measure cell cytotoxicity. Absorbance at 490 nm was read at 24 hours with a plate reader (SpectraMax M3, Molecular Devices).

% Cytotoxicity= (Experimental-untreated/lysis control-untreated) x 100
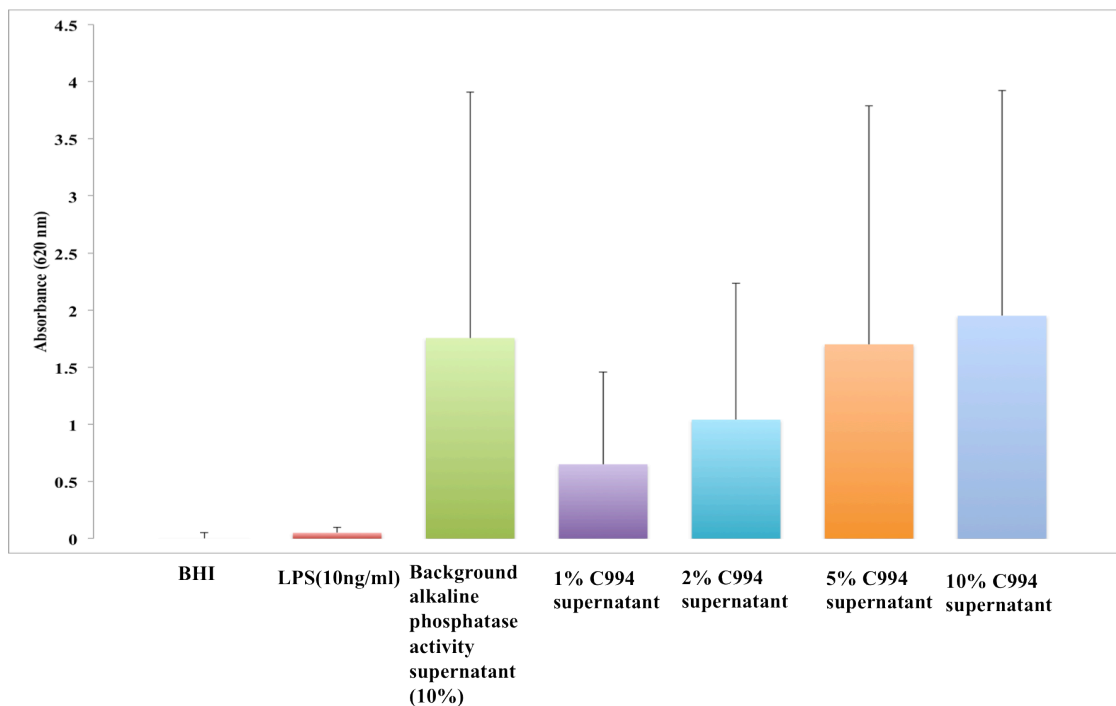
5.12 **Analysis of NF-κB activation in *Prevotella melaninogenica*.**
THP-1-Blue cells were stimulated in triplicate with each concentration (1%, 2%, 5% and 10%) of *P. melaninogenica* (C994) supernatant. *E. coli* LPS (10 ng/ml) was used as a positive control. Background alkaline phosphatase activity was detected in the C994 supernatants (10% supernatant was added to detection culture medium (minus THP-1 blue cells)). SEAP reporter assay cannot be used for detecting NF-κB activation in bacterial communities with *P. melaninogenica*. Error bars indicate standard deviations.

REFERENCES

1. Colwell, R.R. Microbial diversity: the importance of exploration and conservation. *J Ind Microbiol Biotechnol* 18, 302-7 (1997).

2. Alain, K. & Querellou, J. Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* 13, 583-594 (2009).

3. Pace, N.R. A Molecular View of Microbial Diversity and the Biosphere. *Science* 276, 734-740 (1997).

4. Gonzalez, A. et al. Our microbial selves: what ecology can teach us. *EMBO Rep* 12, 775-784 (2011).

5. Turnbaugh, P.J. et al. The Human Microbiome Project. *Nature* 449, 804-810 (2007).

6. Kuczynski, J. et al. Direct sequencing of the human microbiome readily reveals community differences. *Genome Biol* 11, 210 (2010).

7. Caporaso, J.G. et al. Moving pictures of the human microbiome. *Genome Biol* 12, R50 (2011).

8. Kolar, C.S. & Lodge, D.M. Progress in invasion biology: predicting invaders. *Trends in Ecology & Evolution* 16, 199-204 (2001).

9. van Bruggen, A.H.C. & Semenov, A.M. In search of biological indicators for soil health and disease suppression. *Applied Soil Ecology* 15, 13-24 (2000).

10. Cardinale, B.J., Palmer, M.A. & Collins, S.L. Species diversity enhances ecosystem functioning through interspecific facilitation. *Nature* 415, 426-429 (2002).

11. McCann, K.S. The diversity-stability debate. *Nature* 405, 228-233 (2000).

12. Ives, A.R. & Carpenter, S.R. Stability and Diversity of Ecosystems. *Science* 317, 58-62 (2007).

13. Duan, K., Sibley, C.D., Davidson, C.J. & Surette, M.G. Chemical interactions between organisms in microbial communities. *Contrib Microbiol* 16, 1-17 (2009).

14. Hibbing, M.E., Fuqua, C., Parsek, M.R. & Peterson, S.B. Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Micro* 8, 15-25 (2010).

15. Bonnie L, B. How bacteria talk to each other: regulation of gene expression by quorum sensing. *Current Opinion in Microbiology* 2, 582-587 (1999).

16. Charlson, E.S. et al. Disordered Microbial Communities in the Upper Respiratory Tract of Cigarette Smokers. *PLoS ONE* 5, e15216 (2010).

17. Hilty, M. et al. Disordered Microbial Communities in Asthmatic Airways. *PLoS ONE* 5, e8578 (2010).

18. Charlson, E.S. et al. Topographical Continuity of Bacterial Populations in the Healthy Human Respiratory Tract. *American Journal of Respiratory and Critical Care Medicine* 184, 957-963 (2011).

19. García-Rodríguez, J.Á. & Fresnadillo Martínez, M.J. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *Journal of Antimicrobial Chemotherapy* 50, 59-74 (2002).

20. Borewicz, K. et al. Longitudinal analysis of the lung microbiome in lung transplantation. *FEMS Microbiology Letters* 339, 57-65 (2013).

21. Erb-Downward, J.R. et al. Analysis of the Lung Microbiome in the "Healthy" Smoker and in COPD. *PLoS ONE* 6, e16384 (2011).

22. Sze, M.A. et al. The Lung Tissue Microbiome in Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine* 185, 1073-1080 (2012).

23. Fahy, J.V. & Dickey, B.F. Airway Mucus Function and Dysfunction. *New England Journal of Medicine* 363, 2233-2247 (2010).

24. O'Sullivan, B.P. & Freedman, S.D. Cystic fibrosis. *Lancet* 373, 1891-904 (2009).

25. Yang, L., Jelsbak, L. & Molin, S. Microbial ecology and adaptation in cystic fibrosis airways. *Environ Microbiol* 13, 1682-1689 (2011).

26. Sibley, C.D. & Surette, M.G. The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. *Can J Microbiol* 57, 69-77 (2011).

27. Sibley, C.D. et al. Culture Enriched Molecular Profiling of the Cystic Fibrosis Airway Microbiome. *PLoS ONE* 6, e22702 (2011).

28. Worlitzsch, D. et al. Antibiotic-resistant obligate anaerobes during exacerbations of cystic fibrosis patients. *Clinical Microbiology and Infection* 15, 454-460 (2009).

29. Tunney, M.M. et al. Detection of Anaerobic Bacteria in High Numbers in Sputum from Patients with Cystic Fibrosis. *American Journal of Respiratory and Critical Care Medicine* 177, 995-1001 (2008).

30. Field, T.R., Sibley, C.D., Parkins, M.D., Rabin, H.R. & Surette, M.G. The genus Prevotella in cystic fibrosis airways. *Anaerobe* 16, 337-44 (2010).

31. Jewes, L.A. & Spencer, R.C. The incidence of anaerobes in the sputum of patients with cystic fibrosis. *J Med Microbiol* 31, 271-4 (1990).

32. Itzhak, B. Anaerobic bacteria in upper respiratory tract and head and neck infections: Microbiology and treatment. *Anaerobe*.

33. Peters, B.M., Jabra-Rizk, M.A., O'May, G.A., Costerton, J.W. & Shirtliff, M.E. Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* 25, 193-213 (2012).

34. Bakaletz, L.O. Developing animal models for polymicrobial diseases. *Nat Rev Micro* 2, 552-568 (2004).

35. Gibson, R.L., Burns, J.L. & Ramsey, B.W. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 168, 918-51 (2003).

36. Smith, A.L., Fiel, S.B., Mayer-Hamblett, N., Ramsey, B. & Burns, J.L. Susceptibility testing of Pseudomonas aeruginosa isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. *Chest* 123, 1495-502 (2003).

37. Kolenbrander, P.E., Andersen, R.N., Kazmerzak, K., Wu, R. & Palmer, R.J., Jr. Spatial organization of oral bacteria in biofilms. *Methods Enzymol* 310, 322-32 (1999).

38. Kolenbrander, P.E. et al. Communication among oral bacteria. *Microbiol Mol Biol Rev* 66, 486-505, table of contents (2002).

39. McBride, B.C. & Van der Hoeven, J.S. Role of interbacterial adherence in colonization of the oral cavities of gnotobiotic rats infected with Streptococcus mutans and Veillonella alcalescens. *Infection and Immunity* 33, 467-472 (1981).

40. Keevil, C.W. Continuous culture models to study pathogens in biofilms. *Methods Enzymol* 337, 104-22 (2001).

41. Marsh, P.D. et al. The influence of growth rate and nutrient limitation on the microbial composition and biochemical properties of a mixed culture of oral bacteria grown in a chemostat. *J Gen Microbiol* 129, 755-70 (1983).

42. Schmidt, J.K., Konig, B. & Reichl, U. Characterization of a three bacteria mixed culture in a chemostat: Evaluation and application of a quantitative terminal-restriction fragment length polymorphism (T-RFLP) analysis for absolute and species specific cell enumeration. *Biotechnology and Bioengineering* 96, 738-756 (2007).

43. Bradshaw, D.J. & Marsh, P.D. in Methods Enzymol (ed. Ron, J.D.) 279-296 (Academic Press, 1999).

44. Grenier, D. & Mayrand, D. Nutritional relationships between oral bacteria. *Infection and Immunity* 53, 616-620 (1986).

45. Guggenheim, M., Shapiro, S., Gmür, R. & Guggenheim, B. Spatial Arrangements and Associative Behavior of Species in an In Vitro Oral Biofilm Model. *Applied and Environmental Microbiology* 67, 1343-1350 (2001).

46. Bradshaw, D.J., Marsh, P.D., Watson, G.K. & Allison, C. Role of Fusobacterium nucleatum and Coaggregation in Anaerobe Survival in Planktonic and Biofilm Oral Microbial Communities during Aeration. *Infection and Immunity* 66, 4729-4732 (1998).

47. Bradshaw, D.J., Marsh, P.D., Allison, C. & Schilling, K.M. Effect of Oxygen, Inoculum Composition and Flow Rate on Development of Mixed-Culture Oral Biofilms. *Microbiology* 142, 623-629 (1996).

48. Periasamy, S. & Kolenbrander, P.E. Central Role of the Early Colonizer Veillonella sp. in Establishing Multispecies Biofilm Communities with Initial, Middle, and Late Colonizers of Enamel. *Journal of Bacteriology* 192, 2965-2972 (2010).

49. Palmer, R.J., Kazmerzak, K., Hansen, M.C. & Kolenbrander, P.E. Mutualism versus Independence: Strategies of Mixed-Species Oral Biofilms In Vitro Using Saliva as the Sole Nutrient Source. *Infection and Immunity* 69, 5794-5804 (2001).

50. Wood, S.R. et al. Architecture of Intact Natural Human Plaque Biofilms Studied by Confocal Laser Scanning Microscopy. *Journal of Dental Research* 79, 21-27 (2000).

51. Pham, V.H. & Kim, J. Cultivation of unculturable soil bacteria. *Trends Biotechnol* 30, 475-84 (2012).

52. Stewart, E.J. Growing Unculturable Bacteria. *Journal of Bacteriology* 194, 4151-4160 (2012).

53. Bollmann, A., Lewis, K. & Epstein, S.S. Incubation of Environmental Samples in a Diffusion Chamber Increases the Diversity of Recovered Isolates. *Applied and Environmental Microbiology* 73, 6386-6390 (2007).

54. D'Onofrio, A. et al. Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria. *Chemistry & biology* 17, 254-264 (2010).

55. Kim, H.J., Huh, D., Hamilton, G. & Ingber, D.E. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab on a Chip* 12, 2165-2174 (2012).

56. Park, J., Kerner, A., Burns, M.A. & Lin, X.N. Microdroplet-Enabled Highly Parallel Co-Cultivation of Microbial Communities. *PLoS ONE* 6, e17019 (2011).

57. Ernebjerg, M. & Kishony, R. Distinct Growth Strategies of Soil Bacteria as Revealed by Large-Scale Colony Tracking. *Applied and Environmental Microbiology* 78, 1345-1352 (2012).

58. Cho, J.-C. & Giovannoni, S.J. Cultivation and Growth Characteristics of a Diverse Group of Oligotrophic Marine Gammaproteobacteria. *Applied and Environmental Microbiology* 70, 432-440 (2004).

59. Ingham, C.J. et al. The micro-Petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. *Proceedings of the National Academy of Sciences* 104, 18217-18222 (2007).

60. Cash, H.A., Woods, D.E., McCullough, B., Johanson, W.G., Jr. & Bass, J.A. A rat model of chronic respiratory infection with Pseudomonas aeruginosa. *Am Rev Respir Dis* 119, 453-9 (1979).

61. Huh, D. et al. A Human Disease Model of Drug Toxicity–Induced Pulmonary Edema in a Lung-on-a-Chip Microdevice. *Science Translational Medicine* 4, 159ra147 (2012).

62. Sibley, C.D. et al. Discerning the complexity of community interactions using a Drosophila model of polymicrobial infections. *PLoS Pathog* 4, e1000184 (2008).

63. Rogers, G.B. et al. characterization of bacterial community diversity in cystic fibrosis lung infections by use of 16s ribosomal DNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 42, 5176-83 (2004).

64. Wooley, J.C., Godzik, A. & Friedberg, I. A primer on metagenomics. *PLoS Comput Biol* 6, e1000667 (2010).

65. Bartram, A.K., Lynch, M.D.J., Stearns, J.C., Moreno-Hagelsieb, G. & Neufeld, J.D. Generation of Multimillion-Sequence 16S rRNA Gene Libraries from Complex Microbial Communities by Assembling Paired-End Illumina Reads. *Applied and Environmental Microbiology* 77, 3846-3852 (2011).

66. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads (2011).

67. Masella, A., Bartram, A., Truszkowski, J., Brown, D. & Neufeld, J. PANDAseq: paired-end assembler for illumina sequences. *BMC Bioinformatics* 13, 31 (2012).

68. Ye, Y. Identification and Quantification of Abundant Species from Pyrosequences of 16S rRNA by Consensus Alignment. *Proceedings. IEEE International Conference on Bioinformatics and Biomedicine* 2010, 153-157 (2011).

69. DeSantis, T.Z. et al. Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Applied and Environmental Microbiology* 72, 5069-5072 (2006).

70. Wang, Q., Garrity, G.M., Tiedje, J.M. & Cole, J.R. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Applied and Environmental Microbiology* 73, 5261-5267 (2007).

71. Caporaso, J.G. et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7, 335-6 (2010).

72. Lozupone, C.A., Hamady, M., Kelley, S.T. & Knight, R. Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. *Applied and Environmental Microbiology* 73, 1576-1585 (2007).

73. Haegeman, B. et al. Robust estimation of microbial diversity in theory and in practice. *ISME J* 7, 1092-101 (2013).

74. Franklin, R.B., Garland, J.L., Bolster, C.H. & Mills, A.L. Impact of dilution on microbial community structure and functional potential: comparison of numerical simulations and batch culture experiments. *Appl Environ Microbiol* 67, 702-12 (2001).

75. Bradshaw, D.J., Marsh, P.D., Watson, G.K. & Allison, C. Role of Fusobacterium nucleatum and coaggregation in anaerobe survival in planktonic and biofilm oral microbial communities during aeration. *Infect Immun* 66, 4729-32 (1998).

76. Duan, K., Dammel, C., Stein, J., Rabin, H. & Surette, M.G. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. *Mol Microbiol* 50, 1477-91 (2003).

77. Xu, L. et al. Role of the luxS quorum-sensing system in biofilm formation and virulence of Staphylococcus epidermidis. *Infect Immun* 74, 488-96 (2006).

78. Reddel, R.R. et al. Transformation of Human Bronchial Epithelial Cells by Infection with SV40 or Adenovirus-12 SV40 Hybrid Virus, or Transfection via Strontium Phosphate Coprecipitation with a Plasmid Containing SV40 Early Region Genes. *Cancer Research* 48, 1904-1909 (1988).

79. Settem, R.P., El-Hassan, A.T., Honma, K., Stafford, G.P. & Sharma, A. Fusobacterium nucleatum and Tannerella forsythia Induce Synergistic Alveolar Bone Loss in a Mouse Periodontitis Model. *Infection and Immunity* 80, 2436-2443 (2012).

80. Field, T.R., Sibley, C.D., Parkins, M.D., Rabin, H.R. & Surette, M.G. The genus Prevotella in cystic fibrosis airways. *Anaerobe* 16, 337-344 (2010).