The theoretical and experimental exploration of the use of predatory bacteria to control biofilms

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Submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

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April 2023

Abstract

Membrane based technologies are widely used for treating drinking water in sparsely populated areas, but their effectiveness is significantly diminished by the growth of biofilms and biofouling. Preventing or removing biofilms can increase the life span of membranes and, thus, improve their economic viability. Most cleaning methods involve physical disruption or antimicrobial chemicals and, therefore, require an interruption in the membrane operation. *Bdellovibrio*, a group of predatory bacteria, are a potential alternative to antimicrobials or physical disruption because of its ability to kill a large range of gram-negative bacterial prey and the inability of their prey to develop genetic resistance. However, the use of *Bdellovibrio* in industrial application has not been widespread in part due to the lack of understanding of the dynamics between *Bdellovibrio* and their prey. To compound this, many of the previous investigations into *Bdellovibrio* and biofilm ecology are limited by inaccurate, uninformative, and labour-intensive methods to quantify the population dynamics, which makes it difficult to build comprehensive models to exploit *Bdellovibrio* as a control to biofilms in systems like drinking water membranes.

This thesis aims to develop a set of novel methods and technologies to accurately investigate *Bdellovibrio* and the effect they have on dynamics of their prey; *Pseudomonas sp*, a key gram-negative biofilm forming species.

This research develops the first protocol to use flow cytometry to accurately and rapidly quantify *Bdellovibrio* and *Pseudomonas sp* growth, which makes recording high resolution population dynamics feasible. The protocol was used for the development and experimental validation of mathematical models which aimed to predict *Bdellovibrio* dynamics in batch and chemostat systems. We show the first experimental observation of *Bdellovibrio*-prey oscillations, a key component of predation dynamics and a desired phenomenon for the use of *Bdellovibrio* as a self-sustaining biocontrol. To extend the models for application to systems where biofilms prevail, we demonstrated a new method of deploying flow cytometry and

fluorescent assays to quantify and characterise the effect of nutrients on biofilm growth and predation. The findings suggest that extracellular polymeric substances (EPS) play a vital role in the attachment and persistence of biofilm when under *Bdellovibrio* predation. Thus, in biofilm research, the simple density dependent predator-prey interactions need to be augmented by representing the spatial heterogeneities in biofilm processes and properties such as its detachment, EPS and presence of metabolically damaged cells.

For a more nuanced analysis of predator-prey interactions, at the resolution of individual organisms, this research develops a novel microfluidic device to observe *Bdellovibrio* predation on a 1-D biofilm. This thesis describes both the rationale and novel protocols for combining electron-beam lithography with, the more commonly used, photolithography to create an array of high-resolution channels to constrain biofilms and challenge them with predators. The research demonstrates the opportunities and the technical challenges in using microfluidics. Ultimately, if we are to develop mathematical models that can be parameterised and used effectively in designing strategies for controlling biofilms using predatory bacteria, then observations at the individual scale in microfluidic devices will be invaluable.

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Abbreviations

Abbreviation	Meaning
Chapter 1	
EPS	Extracellular polymeric substance
PFU	Plaque forming unit
OD	Optical density
qPCR	Quantitative polymerase chain reaction
FSC	Forward scatter
FCM	Flow cytometry
WGA	Wheat germ agglutin
Chapter 2	
d-BALO	Delta-Bdellovibrio and like organism
a-BALO	Alpha-Bdellovibrio and like organism
S-layer	Surface layer
LPS	Lipopolysaccharides
DN	Dilute nutrient
FISH	Fluorescent *in situ* hybridisation
PDMS	Polydimethylsiloxane
MIC	Minimum inhibitory concentration
DCNA	Doubly crosslinked nano-adhesive
TPE	Thermoset Polyester
Chapter 3	
IBD	Inflammatory bowel diseases
PI	Propidium iodide
SYBR I	SYBR green I
SSC	Side scatter
ρ	Pearson's correlation coefficient
FITC	Green fluorescence
TAE	Tris acetate-EDTA
HH	High predator: high prey

HL	High predator: low prey
MM	Medium predator: medium prey
LH	Low Predator: high prey
LL	Low Predator: high prey
k-w	Kruskal Wallace
Chapter 4	
X	Prey population size (cells/ml)
Y	Predator population size (cells/ml)
t	Time (h)
k1, k2, k3 and k4	Positive constants
$\frac{dX}{dt}$	Prey population growth
$\frac{dY}{dt}$	Predator population growth
k	Carrying capacity (cells/ml)
μ	Growth rate (h^{-1})
S	Growth limiting substrate concentration (mg/L)
μ_X	Prey maximum specific growth rate(h^{-1})
K_S	Substrate saturation constant (mg/L)
$\frac{dS}{dt}$	Change in substrate concentration
ζ_S	Prey yield (cell/mg substrate)
ζ_X	Predator yield (predator cells/1 prey cell)
μ_Y	Predator maximum specific growth rate(h^{-1})
K_X	Prey saturation constant (cells/ml)
S_0	Initial substrate concentration/substrate concentration in the influent (mg/L)
D	Dilution rate (h^{-1})
X_0	Initial prey concentration (cells/ml)

Y_0	Initial predator concentration (cells/ml)
Chapter 5	
PNAG	Poly-N acetylglucosamine
PBS	Phosphate buffer solution
Chapter 6	
CAD	Computer aid design
UV	Ultraviolet
E-beam	Electron beam
HSQ	Hydrogen silsesquioxane
TMAH	Tetramethyl ammonium hydroxide
IPA	Isopropyl alcohol
JWNC	James Watt nanofabrication centre

Acknowledgments

Firstly, I wish to express my profound gratitude to my PhD supervisors Professor William T. Sloan and Dr Stephanie Connelly, for their great support, kindness and guidance during my PhD. They have not only shown patience and encouragement but provided key insight and shown a genuine interest in my development as a person and researcher. I look forward to working with them further in the future. Additionally, I am grateful for their efforts along with the other principal investigators of the water and environment group to build a pleasant and collaborative work culture.

A special thanks to Dr Rungroch Sungthong, who offered useful advice and training in regard to *Bdellovibrio* research and at the beginning of my PhD, helped make my transition to the lab a success. I am also grateful to Dr Ciara Keating and Dr Marta Vignola for the guidance in microfluidics and flow cytometry respectively.

I would like to thank Robert Harkin and Linda Pollock for their help in training me with nanofabrication in the JWNC as well as the rest of the JWNC staff for carrying out the e-beam exposures and fabrication of the photomask used in this study.

A big thankyou to Julie Russell, Anne McGarrity and the other technicians in the lab. Their dedication and consideration have been vital in maintaining the high standard of lab practice and has facilitated a safe and comfortable environment for me and other lab members. In particular, I would like to thank Julie, Marie Claire Toukam, Lisa Melville and Alysha Hunter for their help with sampling for the chemostat run in this study.

Finally, I want to express my eternal gratitude to my friends and family, for their support and encouragement and especially my parents Idowu and Olayinka Ogundero for their constant love and encouragement. They have gone above and beyond in their emotional and financial support throughout my life and so words alone cannot express my appreciation.

Declaration

With the exception of chapters 1 and 2, which contain introductory material, all work in this thesis was carried out by the author unless otherwise explicitly stated.

1 Introduction

Engineers of the 19th and 20th century transformed the public health and life quality of Western cities by developing infrastructure to deliver energy and clean water into our houses and remove and treat wastewater (Sapkota et al ., 2014). This infrastructure has served us well and we continue to upgrade it to provide ever-safer and more convenient ways of life and a more pristine environment (Beddington et al., 2009). What we have arrived at is a highly centralised paradigm for the technologies and management practices used in the urban water cycle. Here, water is moved through enormous networks and treated in large facilities (Parkinson and Tayler, 2003).

With climate change, population growth, resource scarcity, and rising energy costs there is a growing recognition that these centralised systems, which dissipate water, energy and materials, may not be the most sustainable for all communities, especially those in rural areas (Sapkota *et al* ., 2014). Thus, there is a desire for change and innovation. Indeed the UK water industry has embraced the concept of responsible innovation and have committed to becoming energy-neutral and chemical-free (Beddington et al., 2009). However, given the irreducible energy demands of many existing pumping systems, there is little consensus on how this will be achieved. Part of the problem is that we are locked into existing large-scale energy and water infrastructure; replacing, for example, a huge wastewater treatment plant with a radically new but as yet untried technology carries unacceptable risks for water companies (Beddington et al., 2009).

Decentralised systems are a viable alternative to deliver safe, treated water and reduce water quality failures (Parkinson and Tayler, 2003). Unlike centralised systems, they aim to treat, reuse or dispose of water in relatively close vicinity to the local available sources of water and utilise small to medium scale transportation networks (Sapkota *et al* ., 2014). Decentralised systems therefore have potential to exhibit greater flexibility to accommodate the ever-growing

water needs and challenges of rural communities as well as significantly reducing health and environmental hazards (Sapkota *et al* ., 2014).

Crucially however, the relatively smaller scale of decentralised systems means they do not exhibit the redundancy inherent in extensive grid systems and thus are unable to effectively mitigate the impacts of operation failures (or down time) caused by biofouling (Peter-Varbanets *et al* ., 2009). Biofouling is the nuisance growth of biofilms. Biofilms represent, a mode of bacterial living where bacterial cells stick to each other and to a surfaces with the aid of extracellular structures such as pili and flagella and by excretion of extracellular polymeric substances (EPS) (Núñez *et al* ., 2005). Microorganisms living in biofilms have been shown to survive longer and have greater resistance to environmental stresses such as chlorine, in comparison to free swimming planktonic bacteria (Abberton *et al* ., 2016). Biofilm formation in decentralised systems, as in centralised systems, negatively impacts performance and increases operating cost (Chen *et al* ., 2010). Current strategies to mitigate biofouling include application of damaging chemicals such as chlorine and can produce excess sludge requiring treatment (Monnappa *et al* ., 2013). Thus, there is the need to develop new, effective, and environmentally safe ways to treat water in a decentralised system.

Bdellovibrio bacteriovorus are a species of predatory bacteria that have been investigated as a potential biocontrol for biofilm growth but there is lack of comprehensive understanding of predation dynamics in part due to the lack of reliable multiparametric and fast methods for investigation (Jurkevitch, 2012). This makes it difficult to develop detailed plans for *Bdellovibrio* application in membrane-based drinking water treatment.

1.1 Research aims and objectives

The goal of this thesis is to investigate the dynamics of *Bdellovibrio* predation using novel methods and technologies that will enable a deeper understanding of *Bdellovibrio* and their potential as a biocontrol against biofilms in membrane-based water treatment technologies. Many of the previous studies into *Bdellovibrio*

predation are limited by labour intensive and inaccurate methods such as the plaque forming unit (PFU) method. In the first experimental chapter, we aim to address this by developing a protocol to accurately measure *Bdellovibrio* in a high-throughput manner. Flow cytometry is likely an ideal tool for this with the further benefit of providing multiparametric analysis as has been shown in previous studies (Koch et al., 2014). To confirm this, we aim to demonstrate flow cytometry's ability to accurately measure *Bdellovibrio* samples of varying concentrations and compare the results to the more conventional enumeration methods of optical density (OD), PFU and quantitative polymerase chain reaction (qPCR). We hypothesise that measurements made by flow cytometry will show a high correlation to those made by the aforementioned methods, in particular qPCR which is often regarded as high accuracy tool for microbial enumeration (Iebba et al., 2013).

The forward scatter (FSC) is a key parameter measured by flow cytometry and can be used as a proxy for cell size, because of the nature of *Bdellovibrio's* small cell size in comparison to their gram-negative prey, we hypothesise that the forward scatter can be used to distinguish *Bdellovibrio* and *Pseudomonas*, allowing the measurement of both predator and prey cells at the same time. This will be an improvement on previous studies that typically measure the prey and predator with different methods or methods that are unable to directly measure *Bdellovibrio* such as those that measure the decrease in turbidity as an indication of predation (Im et al., 2014). Addressing this gap in the research enables more robust investigation into the dynamics of *Bdellovibrio* predation.

The success of *Bdellovibrio* in decentralised water treatment systems will rely on being able to predict and engineer the microbial population towards the desired prevention and removal of excessive biofilm growth. In chapter 4, we use the method of flow cytometry established in the previous chapter to measure in a batch system, the growth of *Bdellovibrio* on *Pseudomonas* and the growth of *Pseudomonas* on glucose, a carbon growth limiting substrate. By doing so in a batch system, we aim to reliably quantify key growth parameters such as the

growth rate. These parameters are to be applied to modified Lotka-Volterra models in a batch and chemostat system.

Mathematical models such as the Lotka-Volterra have been demonstrated for microbial predation many times but there have been every few experimentally validated models of *Bdellovibrio* predation and no models have demonstrated the ability to predict predator prey oscillations experimentally (Summers and Kreft, 2019). Predator prey oscillations are a key feature of microbial populations that could be exploited for use in clearing the biofilms of decentralised systems in a self-sustaining manner. However, without the use of mathematical models, it is extremely difficult to predict the necessary environment for this occur. Thus, this study aims to first demonstrate the batch model's ability to predict different outcomes based on the initial prey, predator and glucose concentration. Secondly the study aims to demonstrate the chemostat model's ability to predict the dilution rate and influent glucose concentration that will induce predator prey oscillations.

It is well known that biofilm growth occurs through the interaction of many different processes and can manifest itself its various ways(Gefen and Balaban, 2009). Despite this many biofilm studies, especially those that focus on *Bdellovibrio* predation are limited to only measuring the biomass. This not only limits the understanding of the heterogeneity displayed in biofilm growth and predation but prevents the development of accurate *Bdellovibrio* models. To address this, chapter 5 presents an approach to analyse the growth of biofilm under predation in a high throughput and multiparametric manner.

We present a multi-well plate assay to investigate the growth of biofilm and change in EPS, under predation after growth in different media. Crystal violet is typically used as a means to assess biofilm biomass following exposure to antibiotics, so the study initially compares it to other means of assessing biofilm; flow cytometry, resazurin assay and the wheat germ agglutin assay. We then hypothesise the environment and nutrients available to a biofilm during growth will affect the growth of the total and intact bacteria of the biofilm, the EPS

production and the quantity of planktonic cells in the bulk liquid. We aim to observe whether *Bdellovibrio* predation has any effect on these measurements and demonstrate the necessity to use multiple methods of biofilm assessment in alternative to crystal violet to gain more accurate and in-depth quantification of biofilm growth under predation. This will aid the future development of biofilm predation models.

Though the well plate is based on the end point assessment of static biofilms, there is also the need to monitor biofilm growth and predation in real time, in an open system and at the single cell level. All this can be provided by microfluidics but many of the current investigations that use microfluidics often display methods that are vague, incomplete or inaccessible in language to microbial ecologists who are unfamiliar with the technologies. This makes the devices and the technologies used difficult to replicate and makes it harder for the knowledge gained to be verified and built upon by other researchers. Thus, in chapter 6 we aim to provide a detailed introduction into microfluidic devices and the technologies used to fabricate them for the purpose of single cell analysis. We then provide a comprehensive protocol for the fabrication of a Polydimethylsiloxane (PDMS) microfluidic device with the aim to investigate the effect of biofilm thickness on predation.

Biofilm thickness is an important parameter for biofilm growth and has been demonstrated to reduce the success of antimicrobials (Molobela and Ilunga, 2012). However, it is difficult to control this in an experimental set up, so we aim to build a number of microfluidic devices to restrict *Pseudomonas* growth to a single line in channels of different lengths to model a 1-D intersection of a 'traditional' multilayer biofilm. The length of the channels was to act as a proxy for biofilm thickness and so we hypothesised that at some length the *Pseudomonas* prey was able to outgrow the *Bdellovibrio* predator and avoid complete biofilm removal. As previous, this information would allow the construction of a biofilm predation model where predator prey oscillation could be observed.

Traditional photolithography techniques that are typically used to fabricate microfluidic devices are unable to reliably produce channels of 1μm required to trap *Pseudomonas* cells, so we aim to optimise a protocol for microfluidic fabrication that combines photolithography with electron-beam lithography, a technology that allows for higher resolution structures. Doing so would also increase the throughput of microfluidic design and fabrication, reducing the need for fabrication of new photomasks whenever changes are required to be made to the design.

1.2 Research outline

The following section provides an overview of the chapters and research carried out in this thesis.

1. Chapter 2 will introduce predatory bacteria and *Bdellovibrio* and provide an overview of how they have been investigated as a biocontrol solution to unwanted bacterial growth in different fields such as in clinical human disease, agriculture and water treatment. We will discuss the different factors that may affect predation and review some of the key methods that have been used to investigate microbial predation and biofilm growth in the past including mathematical models, microfluidics, fluorescent staining, microscopy and genetic based techniques.

2. In chapter 3 we describe a novel protocol to measure the number of *Bdellovibrio bacteriovorus* cells of varying density using flow cytometry (FCM) and compare the results with those of other more conventional methods: optical density (OD), plaque forming unit assay (PFU) and quantitative polymerase chain reaction (qPCR). Additionally, we validated the use of flow cytometry by demonstrating its ability to distinguish and count mixed populations of *Bdellovibrio* and the prey *Pseudomonas*.

3. Chapter 4 includes a study, which makes use of mass-action mathematical models and experiments to further study the dynamics between *B.*

bacteriovorus and *Pseudomonas* and a carbon limiting substrate, when present in a batch and chemostat system. Specifically, we conduct batch experiments which grow *Pseudomonas* on different concentrations of glucose and *Bdellovibrio* on different concentrations of prey to obtain parameters for a computer model. We then aim to validate the model by comparing simulations to a series of batch experiments. Using the validated parameters, we develop a chemostat model and simulate the region whereby the dilution rate and influent glucose concentration yields distinct characteristic dynamics such as predator-prey oscillations. Finally, we conduct a chemostat experiment to compare the observed dynamics with those predicted by the model to produce predator-prey oscillations.

4. Chapter 5 presents an microwell plate assay to measure the heterogeneity and complexity of biofilm growth under predation. We study the effect of *Bdellovibrio* predation on *Pseudomonas* biofilms statically grown in different growth media (nutrient rich, nitrogen rich, carbon rich and nutrient poor) in microwell plates. Biofilms are analysed by crystal violet, resazurin, wheat germ agglutin (WGA) assays to assess the biomass, viability and EPS production. We used flow cytometry to further characterise the biofilm and quantify the planktonic cells in the bulk liquid. Additionally, we compare biofilm measurements by flow cytometry to crystal violet, resazurin and WGA assays to validate the method.

5. In chapter 6, we provide an introduction to PDMS microfluidic devices, the techniques and technologies used to develop them and a clear protocol that demonstrates how to design, develop and fabricate a microfluidic device with low aspect ratio growth channels by combining e-beam lithography with traditional photolithography. These growth channels aim to restrict bacterial growth in the microfluidic device using hydrodynamic forces to a single line to model a 2-D intersection of a ‘traditional’ multilayer biofilm and investigate the role of biofilm thickness on predation.

6. The final chapter will act as a summary and outline of the key findings of the thesis and highlight how they will aid potential future fundamental and applied investigations into the use of *Bdellovibrio* as a biocontrol against biofilms in drinking water treatment.

2 Literature review

One novel approach to mitigate biofilm and biofouling in decentralised technologies that is gaining interest is the potential application of biological predators to these biofilm producing bacteria(Feng *et al* ., 2016). Predation of bacteria is an interaction that is widespread and there is evidence that it significantly shapes the ecosystem that it acts within (Jürgens and Matz, 2002) and as such may present the most effective way to prevent and reduce biofilm formation.

Predation as a whole is studied in great depth in larger eukaryotic organisms in the macroscale, whereas its effect on microorganisms such as bacteria are often overlooked in comparison. There have been bacteria predation studies providing some insight into the cell biology, ecology and evolution of bacteria in the environment. They suggest that bacterial predation is a key selective force in a number of factors such as the size and shape of bacteria, distribution, population structure and even possibly in the evolution of pathogenicity (Bohannan and Lenski, 2000).

Predation on bacteria occurs through the action of three key organisms; bacteriophages, protists and predatory bacteria (Johnke et al., 2014). Bacteriophages (also known as phages) are viruses that infect and lyse bacteria. They are highly selective and in some cases one phage will only predate upon a single strain of bacterium (Hanlon, 2007). Phages inject their genome into the bacterial prey's cytoplasm allowing them to replicate inside the bacterial cell to a point where the progeny is then released at the expense of the bacterial cell, in order to infect neighbouring prey cells (Hanlon, 2007). Phages were initially used as a treatment of bacterial infectious diseases in the early 1920's, particularly in eastern Europe, but this was largely abandoned after the practical application of penicillin and other antibiotics in the 1940's (Matsuzaki et al., 2005). This trend has changed in the last few decades, with some recent studies investigating the application of bacteriophages. For example, it has been proposed that the

introduction of strain-specific lytic phages into cholera contaminated environmental reservoirs can reduce the severity of cholera outbreaks and promote the decline of the pathogen (Jensen et al., 2006).

Protists are a diverse group of eukaryotic organisms such as amoeba, protozoa, algae and slime moulds (Finlay, 2004). Many species belonging to the protist group are phagotrophic and so feed by engulfing and ingesting food particles and cells into a phagocytic vacuole (Weisse et al., 2016). Protists have been demonstrated to be important phagocytic predators of bacteria (Sherr and Sherr, 2002) and unlike phages, they are generalist feeders (Bell et al., 2010).

Adiba *et al*, (2010), investigated protozoan grazing on virulent strains of *E. coli* and concluded that bacteria virulence was associated with an increased resistance to protozoan grazing. They proposed that this was an indication as to why so many bacteria virulent to humans exist despite virulence being considered by some as a maladaptation (Kniskern and Rausher, 2001).

Predatory bacteria are defined as specialised bacteria that both 'hunt' and kill bacterial prey in order to obtain nutrients and energy by consuming the prey's macromolecules (Jurkevitch, 2006a). It is important to note that predatory bacteria are distinguished from bacteria that simply secrete growth inhibition metabolites (which is very common) or parasites that form close and potentially non-lethal associations with bacteria (Pasternak et al., 2013). Those bacteria termed as predators must be able to use its motility to find and then kill and feed on their prey (Jurkevitch, 2006a).

In a similar fashion to protists, the term ''predatory bacteria" serves to group many species which are taxonomically diverse. They are distributed over several phyla and exhibit a range of different hunting strategies, which a number of studies have attempted to classify (Martin, 2002). It is particularly difficult to determine a strict classification system because new predatory bacteria are often being discovered and many that already are, have yet to be studied in depth (Pasternak et al., 2013). Moreover, the classification of certain predators may be

biased as the predators are usually only observed in the presence of culturable prey and thus the potential for a single predator to exhibit more than one predatory strategy depending on the prey or environmental conditions is rarely taken into account (Pérez et al., 2016). For example, one species of predator known as *Bdellovibrio exovorus* (discussed below), displays a different strategy from the remaining species of the *Bdellovibrio* genus suggesting that there may be some conditional changes such as prey availability that causes a shift between the two modes of feeding (Chanyi et al., 2013).

Perez *et al* (2016) distinguishes three general types of predation: epibiotic strategies, endobiotic strategies and group attack strategies. In epibiotic strategies (Fig 2.1), the predators do not invade the prey cell but consumes the prey from the outside while attached to the prey cell envelope. The predator divides into two daughter cells which detach to search for more prey, continuing the predation cycle. This group includes the genera *Vampirococcus*, *Micavibrio* and *Bdellovibrio exovorus* (Pasternak et al., 2014).

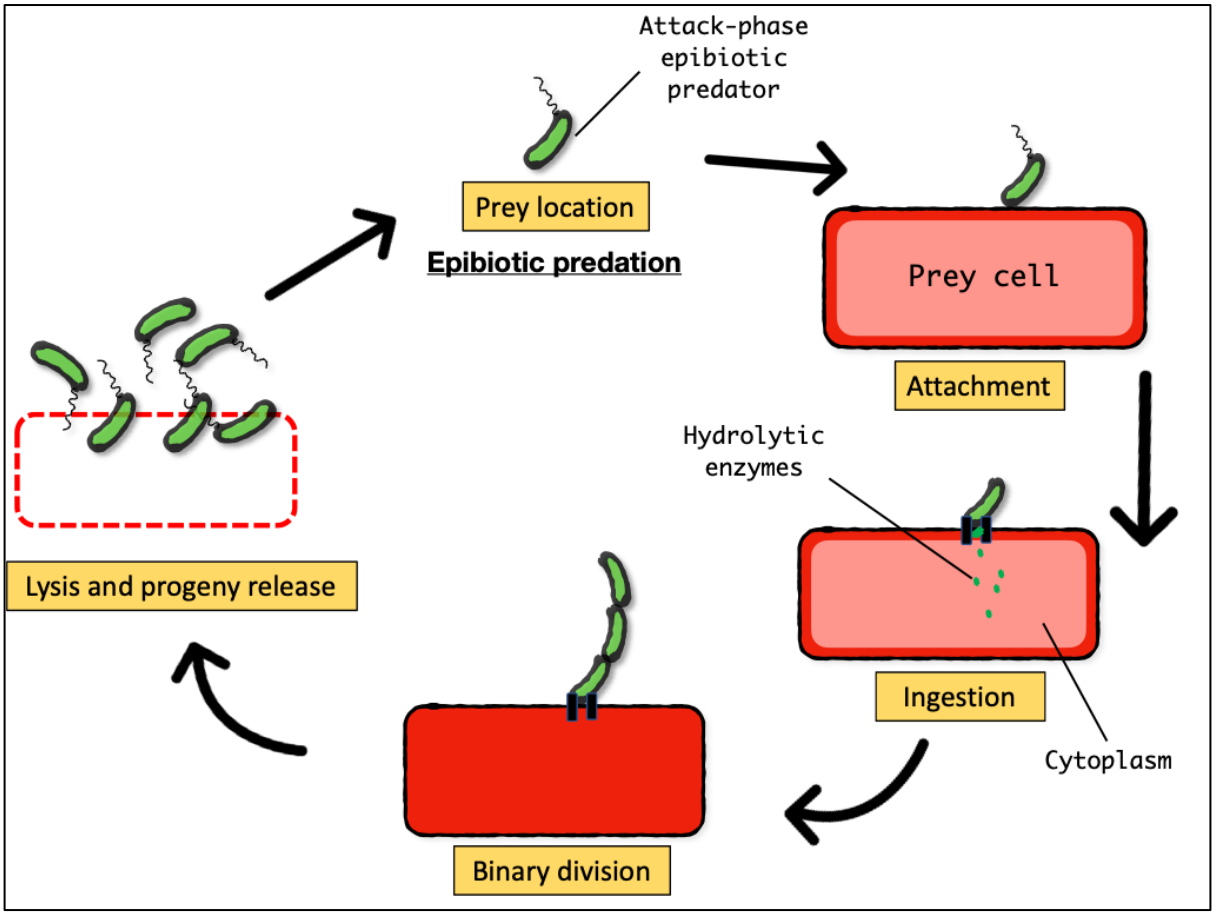

Figure 2.1- Schematic diagram describing the typical life cycle of an epibiotic predatory bacteria.

In endobiotic strategies (Fig 2.2) an individual predatory cell directly penetrates the cell wall, to invade and divide within either the cytoplasm or the periplasmic space. Only one strain in the genus *Daptobacter* is known to invade and replicate in the cytoplasm of the prey, whereas periplasmic invasion is shown in a group of bacteria commonly referred to as delta-*Bdellovibrio* and like organisms (d-BALO), which include *Bacteriovorax* and *Bdellovibrio* species (except *Bdellovibrio exovorus* which are epibiotic predators) (Pasternak et al., 2014). While d-BALOs are deltaproteobacteria, an additional predator genus; *Micavibrio* (mentioned above) belongs to the alphaproteobacterial and hence, is distinguished as an a-BALO (Kandel et al., 2014).

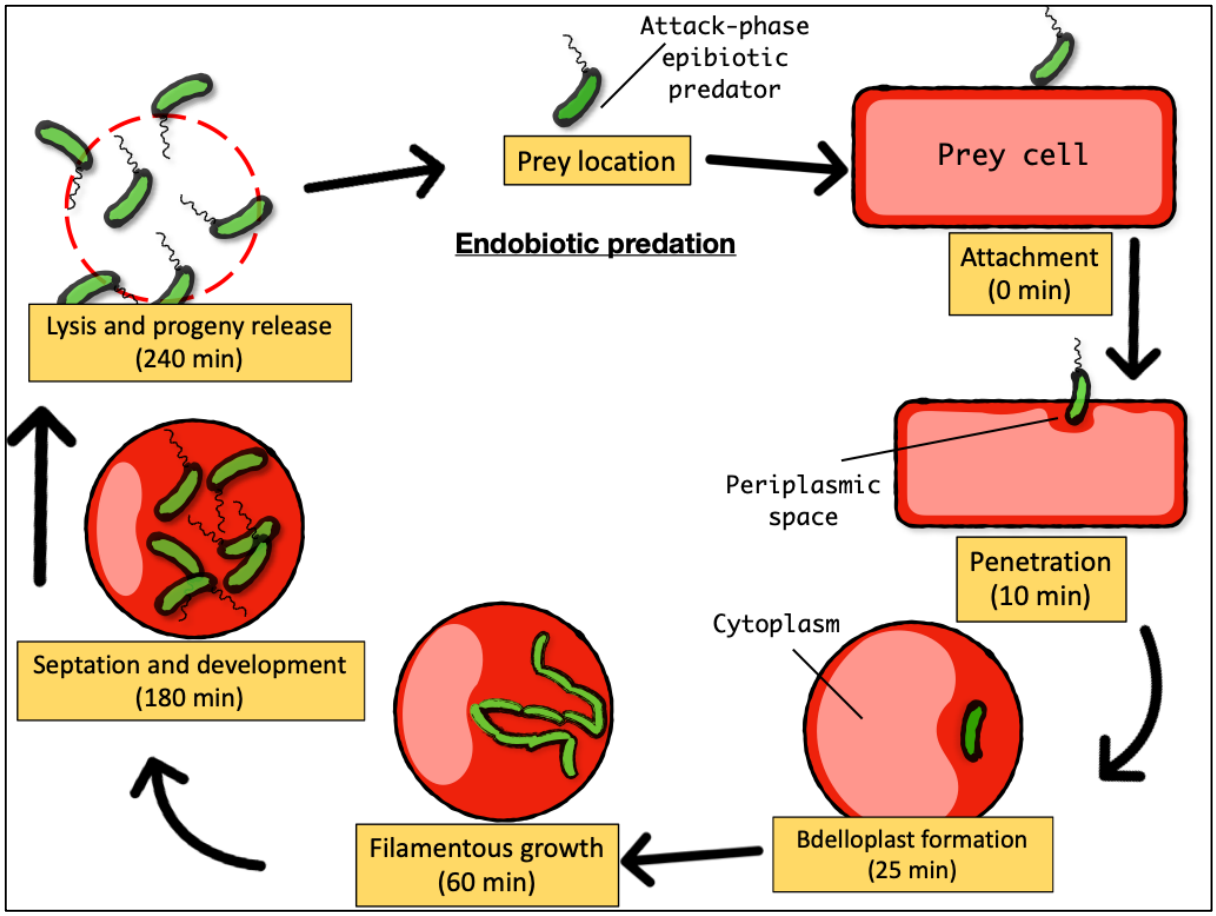

Figure 2.2- Schematic diagram describing the typical life cycle of an endobiotic predatory bacteria.

Group attack (Fig 2.3) is a predation strategy that requires the cooperation of multiple predators to produce and release lytic factors to degrade prey without physical contact (Pérez et al., 2016). This strategy benefits both those predators secreting lytic factors as well as the non-secreting cells as the remains of the lysed prey are readily available as a growth substrate (Mendes-Soares and Velicer, 2013). The mutual benefits of group attack were demonstrated in a study where at high densities, *Myxococcus xanthus* cells cooperated to hydrolyse the growth substrate casein resulting in increased growth rates, whereas at low cell densities, the predators did not grow unless supplemented with already hydrolysed casein (Rosenberg et al., 1977). The *Myxobacteria*-like bacteria such as the *Myxococcus xanthus* are of particular interest because as well as their predation being density dependent, they utilise social gliding motility to search for their prey and an array

of hydrolytic enzymes and secondary metabolites to kill and breakdown the prey for consumption (Pasternak et al., 2014).

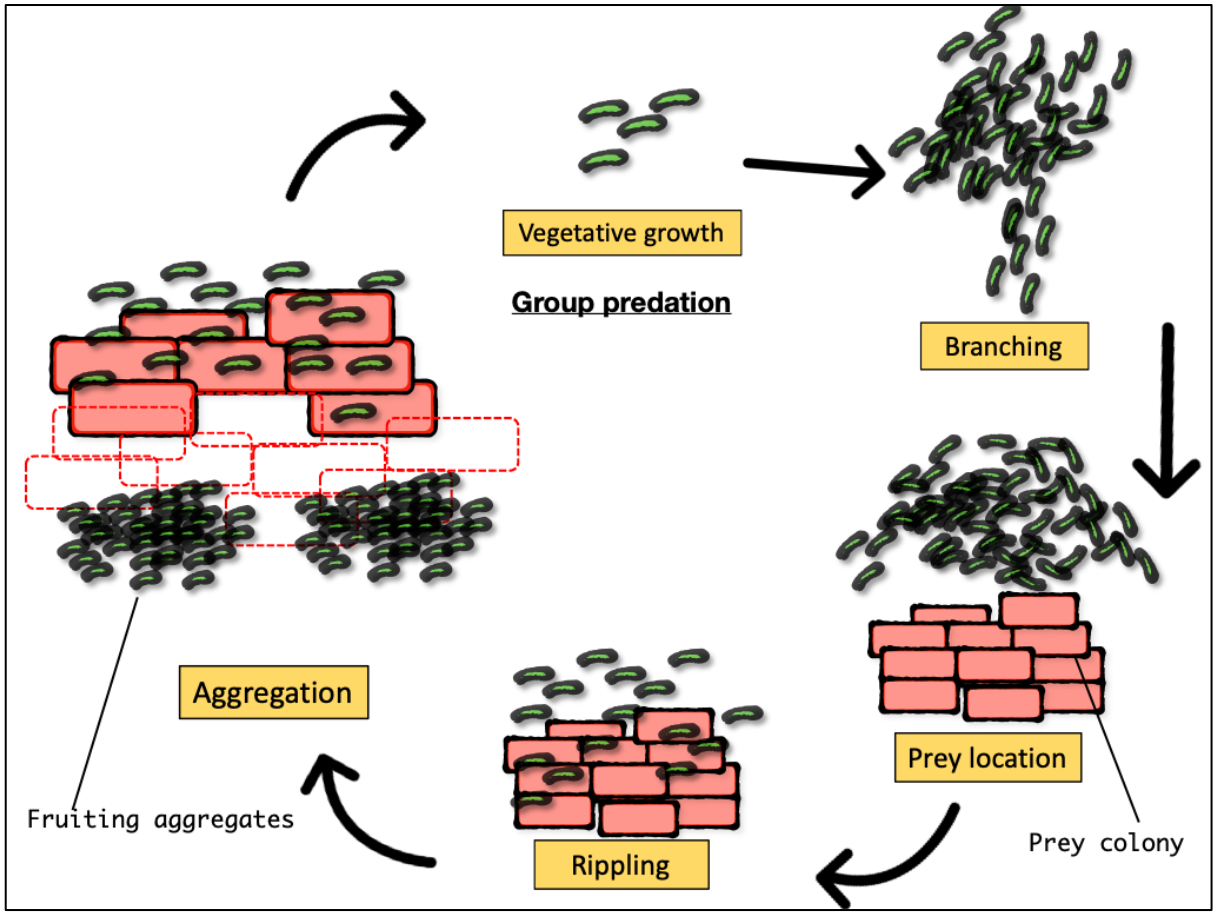

Figure 2.3- Schematic diagram describing the typical life cycle of predatory bacteria that display group attack.

Of all of these varying predatory bacteria, the most extensively studied has been *Bdellovibrio bacteriovorus* which are small (0.25 to 0.5 × 0.75 to 1.25 µm) and highly motile gram-negative bacteria. They are obligate endobiotic predators, and prey exclusively on gram-negative bacteria by entering periplasmic space to grow and reproduce (Jurkevitch, 2012). Similar to protists and phages, the ability to kill and feed upon bacteria has made *Bdellovibrio* and other predatory bacteria an interest for ecological studies and for its potential industrial applications (Chen et al., 2010).

Several traits have been suggested to make *Bdellovibrio* a more attractive biocontrol than other predators. For instance, studies using microcosms of estuarine waters demonstrated that the *Bdellovibrio*, *Halobacteriovorax* (formerly *Bacteriovorax*) responds faster than viruses to the presence of potential prey bacteria, resulting in a significantly higher prey mortality and predator growth (Williams et al., 2016). This may be because unlike viruses, *Bdellovibrio* growth is favoured in environments, where the prey grow in a slow or stationary state (Koval, 2006). In biofilms, the limited nutrient cycling means that most cells grow slowly, explaining why *Bdellovibrio* are well suited to mitigating biofilms (Kadouri and O'Toole, 2005).

The life cycle of *Bdellovibrio* (Fig 2.2) is poorly understood but consists of two spatially and temporally separated phases: the first being a free-swimming attack phase in which the predators find prey by random collision, reaching speeds of 160 μm/s, or over 100 times their length per second (Lambert et al., 2006). Unlike *Myxobacteria*, there is currently no convincing evidence that *Bdellovibrio* use chemotaxis to find suitable prey. Once, the predator has encountered its prey or 'host', the predator will reversibly attach to the prey cell wall, immobilising the prey (Burnham et al., 1968). The predator then uses enzymes to penetrate the prey cell wall and enter the periplasmic space, losing its flagellum in the process. Interestingly, invasion of prey cells by *Bdellovibrio* has been investigated for its potential to explain the mechanism of the mitochondrial endosymbiont theory (Davidov et al., 2006).

The second phase of the life cycle, being the only stage at which the predators can grow and replicate is aptly named the 'growth phase' and is often referred to in literature as the 'intraperiplasmic phase' (Jurkevitch, 2006a). The prey's morphology is altered upon penetration and a round predator-prey hybrid structure known as the 'Bdelloplast' is formed. It has been suggested that this rounding is caused by the activity of the enzyme glycanase which is produced by the predator and solubilises the glycan from the peptidoglycan in the prey bacterial cell wall(Tudor et al., 1990). It is not clear whether there is any benefit to this but penetration of mutant *Bdellovibrio* strains that do not produce glycanase and also

do not cause prey rounding only show a slight delay in their penetration in comparison to the wild type (Tudor et al., 1990).

Once penetrated, the prey is metabolically inactive, as *Bdellovibrio* do not require the host to continue processes such as DNA replication and energy generation, which is not the case with phages (Jurkevitch, 2006a). Upon invasion of the periplasmic space the predator elongates and grows in a filamentous form, degrading the prey's cytoplasm and growing on its source of rich nutrients (Lambert et al., 2007). After adequate multiplication (depending on the size of the prey) inside the 'Bdelloplast', the remains of the ghost prey cells burst, releasing the attack phase progeny into the environment to complete the life cycle (Ruby, 1991).

2.1 Potential applications of predatory bacteria

The focus of this thesis is on developing high throughput methods to quantify the *Bdellovibrio* predation dynamics with the intention to apply these as a suite of rules for the application of *Bdellovibrio* in industrial biotech; in particular in the water industry to control biofilms that cause biofouling. There has been some previous interest in this area (Kim *et al* ., 2013a), but the number of studies is limited. Additionally, the lethal actions of predatory bacteria on human pathogenic bacteria is also being studied for its relevance to basic microbiology and to a lesser extent, its applications in environmental health, medicine, agriculture and other industries (Dwidar et al., 2012b). All of these studies have, to a degree, been inspired by the growing body of literature on biomedical applications of predatory bacteria in a clinical setting to fight infection (Baker et al., 2017). This is an active area of research that is pushing rapidly towards practical solutions. In order to understand the scope of *Bdellovibrio* application; an overview is given below of the challenges and developments in using predatory bacteria as a medical and nonmedical biocontrol agent.

2.1.1 Clinical human disease

One of the most pressing concerns for public and environmental health is the development and spread of antibiotic resistant bacteria (Igbinosa and Odjadjare, 2015). A number of different measures for mitigation are currently being explored by the scientific community, including the use of biological alternatives. *Bdellovibrio* have been proposed as 'living antibiotics' and present a particularly interesting alternative to investigate because their natural life cycle causes death to gram-negative bacteria, which have had no new classes of antibiotics developed to combat them in the last few decades (Parisien et al., 2008).

Moreover, it has been suggested that biological agents such as *Bdellovibrio* are significantly less likely to cause the spread of resistance because they evolve alongside the prey bacteria and attempts to select for resistant prey are rarely successful, as discussed further below (Kadouri and O'Toole, 2005). Other than phenotypic resistance which is temporary (discussed further below) (Shemesh and Jurkevitch, 2003) and biofilm formation which has been shown to be favoured by *Bdellovibrio* below (Kadouri and O'Toole, 2005), there are few other general defence mechanisms to predation shown by prey.

High motility and toxin production has been demonstrated as a bacterial defence against protozoan predation (Matz and Kjelleberg, 2005), however adaptive bacterial defences against *Bdellovibrio* have not been studied in great depth (Pérez et al., 2016). It is known that *Bdellovibrio* cannot attach to and hence predate to bacteria that display paracrystalline protein surface layers (S layers) on their surface (Koval and Hynes, 1991). Similarly, S-layers provide protection to the cyanobacterium *Synechococcus sp.* against heterotrophic nano-flagellate predators, causing the predator to prematurely egest the bacteria shortly after the ingestion (Boenigk et al., 2001).

Bdellovibrio may have many potential uses where antibiotics are currently used, particularly for the health and wellbeing of humans in a clinical setting, where

antibiotic resistance has made it difficult to treat a number of life-threatening diseases (Igbinosa and Odjadjare, 2015).

If *Bdellovibrio* are to be used as a treatment in such a complex system as the human body, in vivo studies are needed to reveal the dynamics of *Bdellovibrio* predation in clinically relevant conditions (Dwidar et al., 2012b). One of the most key in vivo investigations to demonstrate the potential of *Bdellovibrio* as a therapeutic, was that of Nakamura (1972). It used experimental animal models to investigate the alteration of *Shigella* pathogenicity and virulence by the antagonistic interactions of *B. bacteriovorus*. Inoculations of *B. bacteriovorus* suspensions in the eyes of rabbits experimentally infected with *Shigella flexneri* eliminated or substantially reduced severity of keratoconjunctivitis induced by the infection (Nakamura, 1972). The study followed the infection rate of the rabbits for 6 days and indicated that the time of *Bdellovibrio* treatment was important, as the *S. flexneri* infection rate after 6 days was 0% when *Bdellovibrio* treatment was started at 12h following infection, whereas upon simultaneous inoculation of *S. flexneri* and *B. bacteriovorus* at 0 hours the infection rate was higher at 20%.

Moreover, after 12h, as the time of treatment following infection increased further so did the infection rate. Infection rate increased to 25% at 48h and 75% at 72h. This is possibly due to the regrowth of bacteria that survived *Bdellovibrio* predation by displaying temporary phenotypic resistance (Shemesh and Jurkevitch, 2003). Additional experiments showed that simultaneous inoculation of *B. bacteriovorus,* eliminated or reduced the fluid accumulation in the intestinal tract of rabbits associated with shigella infection (Nakamura, 1972).

A more recent study (Baker et al., 2017), investigated the *Bdellovibrio* predation of antibiotic resistant *Klebsiella pneumoniae*, a human pathogen that is responsible for a major proportion of hospital originating infections and associated with high morbidity (Mehrad et al., 2015). Mathematical models of the study provided evidence that suggested *Bdellovibrio* could significantly reduce the K. *pneumoniae* load in serum, though this was followed by a regrowth of prey population. This reduction was shown using two different batches of serum, which

is an important indication that *Bdellovibrio* would not be too patient specific as a potential therapeutic. However, a much wider range of different human serum batches would have to be tested before clinical application. The extent and time of reduction and regrowth also showed to be quite variable among repeats of the same serum batch and even more variation was observed between differing batches. In batch one, significant reduction would begin at varying time points from as early as 24 hours to as late as approximately 48 hours and batch two in contrast, the earliest significant reduction was shown at 60 hours. For all experiments using batch one, they prey had regrown to a population size close to its initial size by 72 hours. The factors (other than initial prey and predator concentration) that determine such varying population dynamics will have to be further understood before *Bdellovibrio* can be applied to a healthcare setting as the results are considerably different to predation experiments in buffer (Shatzkes et al., 2016).

The Baker *et al* (2017) study extrapolates beyond the experimental observations using mathematical models to give further insight to the implications of dynamics observed. In particular it focused on the design of a dosage regime for a *Bdellovibrio* based therapeutic; thus the effect of human serum on predation outcome was considered, which makes an important step from previous papers in the discipline that monitor predation in laboratory media such as dilute nutrient broth (Dashiff et al., 2011).

Although previous studies such as this (Dashiff et al., 2011) were vital in demonstrating the potential use of predatory bacteria as a biocontrol agent for suppressing multidrug-resistant bacteria, laboratory media does not contain factors found in human serum such as antibodies that may display antimicrobial effects on the *Bdellovibrio* and reduce the therapeutic efficacy (Baker et al., 2017). Thus, using media would prove less applicable than serum or even whole blood for further clinical investigations which could answer questions such as the role the human immune system will have on predation (Dashiff et al., 2011). For example, in vivo studies have shown that the zebrafish immune system works alongside *Bdellovibrio* to promote the suppression of the antibiotic resistant *Shigella*

pathogen, resulting in increased zebrafish survival. Subsequently the *Bdellovibrio* cells are cleared by the immune system, with no negative effects on the zebrafish being observed (Willis et al., 2016). Interestingly, the mathematical model of predator-prey dynamics in human serum of Baker *et al.* (2017), suggested that the predators showed reduced attachment to the prey in serum compared to predation in buffer (Baker et al., 2017). Initially it was unclear from the mathematical model whether this was a result of unsuccessful predator-prey attachments not resulting in predation or simply less frequent attachment events occurring. To investigate further, fluorescence microscopy was used to demonstrate that upon initial contact to serum, the predator temporarily changed morphology to a rounded shape, which was correlated to a decrease in predator growth. The attachment delay and associated predator growth delay should be investigated further and the study suggested that it highlights the potential use of pre-conditioning predator cells to serum, thereby increasing their efficiency as a therapeutic agent (Baker et al., 2017).

Notwithstanding the effectiveness of *Bdellovibrio* at killing pathogenic bacteria, the safety of the treatment is of the upmost importance if it to be used medically for humans and even animals. Several studies over the years have suggested that *Bdellovibrio* are likely to be safe for both animals and humans. For example, Shatzkes *et al* (2015), demonstrated that mouse viability was not affected after intranasal or intravenous inoculation of *B. bacteriovorus* and no prolonged inflammatory response was observed (Shatzkes et al., 2015). *Bdellovibrio* have also been shown to be present in the gut of animals and humans (Iebba et al., 2013). One concern regarding the use of *Bdellovibrio* in humans and animals, is whether it could trigger a harmful immune response. As in all gram-negative bacteria, the outer membrane of *Bdellovibrio* contain lipopolysaccharides (LPS), which have a number of functions as well as adding to the structural integrity of the bacteria (Rietschel et al., 1994). The LPS of several bacteria have strong endotoxic activity and induce strong immune responses. Studies in *Bdellovibrio* were carried out to determine whether this was the case and found that the LPS of *B. bacteriovorus* was unique in that it lacked phosphate groups, resulting in a significantly lower

binding affinity for human LPS receptors and reducing the endotoxic activity (Schwudke et al., 2003).

2.1.2 Agriculture

There have been few successful investigations into the potential of *Bdellovibrio* to control plant pathogenic bacteria in agriculture, despite the fact that *Bdellovibrio* are found naturally in soils and can display a rapid response to the presence of large number of prey even in dried soil (Klein and Casida Jr., 1967).

Rice is a staple food for more than half the world's population and vital source of income for millions of households, so as global demands increase with increases in global population, there is the need to enhance productivity of the crop (Muthayya et al., 2014). However, production is greatly reduced by diseases that have been difficult to control (Shrestha et al., 2016).

One such disease is bacterial panicle blight caused by the rice seed borne pathogen *Burkholderia glumae*. Rice fields affected by the disease have often shown have rice production losses as high as 75% (Ham et al., 2011). There are few efficient ways control the disease and currently the most recommended strategies focus on using disease resistant rice strains or controlling the seed of susceptible plants. However, this can provide inconsistent results (Ham et al., 2011). Other methods of control include the use of chemicals, which can be environmentally harmful and are vulnerable to resistance caused by mutations (Shrestha et al., 2016).

Using avirulent bacteria that do not cause disease, to compete with pathogens is one of many biocontrol agents that are disadvantaged by their limited capacity to successfully establish a population to continually antagonise the pathogen. The mechanisms of these control strategies are not well replicated from the laboratory to the field (Jeger et al., 2001). Especially as not enough is known about the conditions required to maximise the effect as well as what conditions favour pathogen growth (Shrestha et al., 2016). Additionally, using recombinant bacteria in widespread control strategies could potentially increase the risks of spread of

genes to unwanted microorganisms, potentially causing harm to human and environmental health (Selvaratnam and Gealt, 1992).

As *Bdellovibrio* have been isolated from the same natural micro-environments as the rice pathogen, they may be closely associated with each other (Jurkevitch et al., 2000), giving the control agent the advantage of exploiting conditions that would otherwise favour the pathogen. Thus, further investigations are needed into the effectiveness of *Bdellovibrio* of biocontrol agents for the rice pathogen, either alone, or in combination with chemical or other biocontrol agents.

One study by Song, (2004), isolated six *B. bacteriovorus* strains and tested their predatory activity against a range of prey cells associated with pathogenicity, antibiosis, or nitrogen fixation in rice paddy fields. The predator strains were found to successfully supress 7 pathogenic prey strain populations whereas no predation was found to occur on the antibiosis-related or nitrogen fixing bacteria (as determined by optical density), which has significant beneficial implications to the field as these processes are vital for plant growth. For example, nitrogen is the greatest nutrient limitation for rice plant growth and any reduction in biological agents that fix nitrogen has to be compensated for by the increased use of nitrogen fertilizers (Ladha and Reddy, 2003) which have adverse environmental effects such as loss of soil fertility (Chung *et al* ., 2018).

A disadvantage of this study and a reason as to why microbiological control is more complex than conventional control strategies, is that it is unable to accurately replicate natural disease relevant environments such as physical habitat structure allowing co-existence of the prey organisms (Stirzaker et al., 1996). This is because unlike liquid medium (which is often used in studies such as this), soil is opaque, making observations and control on the microscale difficult with many conventional technologies (Young and Crawford, 2004). One promising technology is the use of microfluidics, which enables deeper insight into the interactions occurring between different species (Aleklett et al., 2017). This is discussed further below.

2.1.3 Water treatment

The demand of treated water continues to increase and conventional methods for doing so are being deemed to be inadequate for future demands by several studies. Thus, there is the need to implement alternative technologies (Madaeni, 1999). The membrane-based applications have seen a significant recent increase in commercial use in water treatment and other industries (Wallis and Melnick, 1967). However, when microorganisms attach on the membrane surface and to each other by extracellular polymeric substances (EPS), they form a biofilm. Biofilms are a significant problem in a range of fields and a problem in clinical infections because biofilms have an increased resistance to stresses such as antibiotics. Furthermore, the human immune mechanisms such as phagocytosis are often ineffective against biofilms (Meyer et al., 2015) This biofilm growth causes what is known as membrane 'biofouling' in decentralised water systems which reduce permeability and has been cited as one of its biggest disadvantages, preventing large scale use (Kim *et al* ., 2013a).

Controlling biofouling is crucial because the biofilm growth causes a reduction in the performance and lifespan of the membrane. Key strategies for doing so include the use of antibiotics, physical cleaning and quorum quenching; the use of enzymes or whole bacteria to block the quorum sensing of the biofilm forming bacteria (Yeon et al., 2009). As demonstrated by Kim *et al* ., bacteria that had reduced levels of quorum sensing, showed a reduction in the EPS production and developed into a biofilm that was unable to attach effectively on the membrane surface and persist (Kim *et al* ., 2013b). Concerning the widespread use of enzymes in the quorum quenching process, there are doubts over its high costs, catalytic lifetime and large scale efficiency (Lade et al., 2014), despite the advancement of the technology with magnetic enzyme carriers (Yeon et al., 2009).

Bdellovibrio, which have been shown to penetrate deep into large biofilms and replicate, potentially provide continual protection against biofilm formation (Kadouri and O'Toole, 2005), which make them a more likely candidate for biofilm control over protists and bacteriophage predators. Although, there is a surprising

lack of studies into the effectiveness of *Bdellovibrio* in reducing membrane biofouling, one of the key study (Kim *et al* ., 2013a) demonstrated that pre-treating wastewater in low pressure microfiltration membranes with high predator concentrations resulted in significantly larger reductions in *E.coli*, increased water flow rate and ultimately a reduction in biofouling when compared to biofouling in untreated wastewater over 48 hours (Kim *et al* ., 2013a).

Although water treatment systems have a highly complex ecology, physically they are less complex than the human body and as such require investigations into predation dynamics to be approached differently than in studies into *Bdellovibrio* as a clinical application(Feng et al., 2016). For example, in a water treatment system, the reduction of pathogenic bacteria need not be as complete as for clinical and food production settings and reinfection may be less of a concern.

The prey range of *Bdellovibrio* should be considered when used as a biocontrol. For example, many agricultural processes rely on 'beneficial bacteria' such as nitrogen fixers to function and removal of these bacteria by *Bdellovibrio* may hinder production (Dwidar et al., 2012b). Likewise, it may be beneficial and improve membrane performance, to monitor and perhaps target the reduction in bacteria that contribute to biofilm formation and persistence as recent studies have demonstrated that characteristics of biofilms associated with biofouling can be linked to the growth of certain bacterial phenotypes (Yun et al., 2006). It may be easier to monitor and prevent the attack of key processes during biofiltration when using *Bdellovibrio* than protist predators as the bacteria are more selective in their prey (Johnke et al., 2014).

2.2 Predator-prey dynamics

To advance any of the potential applications of predatory bacteria then we will need to bring to bear as much information as possible on the predator-prey dynamics. This means developing a knowledge of the less applied, more fundamental investigations into the population dynamics and interactions between predatory bacteria and their prey (Dwidar et al., 2012b). Many of these studies are

not directly motivated by industrial application, but rather are motivated by a fundamental understanding of the natural history of the organisms.

Improvements in high-throughput sequencing technologies and the ability to sequence whole genomes has led to a progressive increase in focus on molecular biology and genetic studies of *B. bacteriovorus* (Cotter and Thomashow, 1992). A recent study revealed the existence of a predator proteome aptly named the 'predatome', which is significantly different to that of non-predators (Pasternak et al., 2013). This study and others like it, are important in the identification and classification of novel predatory bacteria which has been one of the more extensively focused on areas of predatory bacteria study.

Though these studies may also be useful to help understand predatory bacterial processes and dynamics, the development of *Bdellovibrio* for biocontrol requires more focused studies into the population dynamics displayed between predators and their prey and their influencing factors (Johnke et al., 2014). There is great uncertainty as to what these key factors are, to what extent they combine to play a role in dynamics in different natural or applied settings in comparison to in the laboratory. This is partly because defining predator-prey dynamics has mostly been attempted with physiological and microscopic studies under difficult-to-control conditions (Varon and Shilo, 1969).

The role of factors (predator-prey ratio, biofilm structure, resistance, multiple prey and non-prey etc) that influence *Bdellovibrio*-prey dynamics have been explored in different environments, which makes it complex to apply past observations to the use of *Bdellovibrio* in different and less relevant applications (Dwidar et al., 2012b). The factors that could, putatively, affect predator-prey dynamics are extracted from the more fundamental biological literature. None of these will act in isolation so their effect on any biocontrol strategies that use predatory bacteria may be complex. Nonetheless an attempt is made to set the review in context by speculating on the effects that might arise in real world applications, with emphasis on how this would affect the use of *Bdellovibrio* a biocontrol agent for biofouling. A deep understanding and quantification of the

population growth and structure could give insight into the predator-prey interactions, making it easier to exploit conditions that would optimise the desired predatory bacteria action in a particular application, whether it be complete clearance of a pathogen or removal of a biofilm (Williams and Piñeiro, 2006). To follow on from studies into the action of *Bdellovibrio* on prey bacteria, the combination of antibiotics and other probiotics such as bacteriophages could further enable the successful use of *Bdellovibrio* (Czaplewski *et al* ., 2016).

2.2.1 Resistance

A major disadvantage of antibiotics is that they induce a selection on the bacteria resulting in antibiotic resistance via genetic mutations (Igbinosa and Odjadjare, 2015). Similarly, the development resistance is a key concern for biocontrol agents such as *Bdellovibrio* as it could result in the persistence and regrowth of the targeted prey bacteria which could make *Bdellovibrio* ineffective as a widespread control agent, especially if this resistance gene could spread readily by horizontal transfer (Davies, 1994). Resistance spread of this type has been observed to be enhanced in biofilms by conjugation (Hausner and Wuertz, 1999).

One of the most important papers to have explored resistance to *Bdellovibrio* by prey bacteria was that of Shemesh and Jurkevitch (2003). They demonstrated the presence of a phenotype conferring resistance to a small proportion of the *Erwinia cartona* prey population which was able to survive and grow, leading to an increase in prey cell numbers even in the presence of high *B. bacteriovorus* concentrations ($>10^8$ cells per ml) (Shemesh and Jurkevitch, 2003).

They refuted earlier studies that suggested that the reason a surviving prey population remained was because of the low probability of the predators finding their prey (Varon and Zeigler, 1978). Instead Shemesh and Jurkevitch (2003) proposed that the presence of resistant strains in the prey population explained the co-existence of both the prey and predator. The study further investigated the characteristics of this resistance by mixing concentrated suspensions of susceptible and resistant prey and then exposing them to the predators. While there was

significant decrease in susceptible prey, the resistant prey showed a slight increase in population. Both strains were cultured in identical conditions and the difference in rate of predator-prey encounters that led to irreversible attachment suggested that the susceptible prey were predated on significantly more than resistant prey (Shemesh and Jurkevitch, 2003). It was plausible that in the absence of susceptible prey, the surviving resistant prey face less competition for resources (Hibbing et al., 2010).

In similar fashion to the use of antibiotics, if the resistance displayed in this study was stable across multiple generations, the use of *Bdellovibrio* as a control agent could be less effective (Mohan et al., 2015). Thankfully, this was found not to be the case. The concentrated resistant prey was incubated overnight without the predator, then resuspended in fresh buffer in the presence of the predator. Within an hour, 99.5% of these cells were preyed, demonstrating that this resistance phenotype was only temporary. The study proposed that the appearance and subsequent disappearance of the resistant phenotype could not have been caused by a single reversible mutation as the generation time of the *E. cartonia* strain was approximately 60 mins, which would have only produced 16 generations, which is not enough generations to allow for the population growth of resistant turned sensitive prey cells observed in the predator-free overnight subculture. Therefore, phenotypic plasticity is a more suitable explanation for such an observation (Hibbing et al., 2010).

The study hypothesised that the enzymes released by the predator during unsuccessful prey attachment were responsible for alterations in the prey cell's surface structure which conferred resistance. Although, there was no clear evidence from the study of the altered cell structure of resistant prey, they did show that the resistant prey were larger in size (Shemesh and Jurkevitch, 2003).

Morphology and community structure changes of prey similar to this have previously been demonstrated, following exposure to different stresses such as elevated temperatures and predation by protozoa (Hahn and Höfle, 1998). As the occurrences of stresses in nature are unpredictable, it may be useful to explore

whether there is some specificity of the changes in prey morphology to certain stresses and whether such a change in prey phenotype brought about by one stress such as antibiotics could also increase the tolerance of the prey to *Bdellovibrio* predation (Justice et al., 2008).

Whilst it might be assumed that bacteria that display a resistance phenotype would dominate the population once they appear, however it has shown that this is not the case (Hibbing et al., 2010). Mathematic modelling has provided insight into this by suggesting that in order for the modelled population dynamics to fit the experimental observations, the phenotypic resistant prey would have to display a slightly lower growth rate compared to susceptible non-resistant bacteria in the population (Wilkinson, 2006). In this case, there could be a significant reduction in predator population which would facilitate the slow recovery of the susceptible bacteria because of its growth advantage over the persisters.

Indeed, a similar growth penalty was explored in a study for bacteria persistent to antibiotics, which used microfluidics to predict which bacteria was able to survive exposure to antibiotics based on their slow growth rate (Gefen and Balaban, 2009). This penalty has also been demonstrated by Varon (1979) for *Bdellovibrio* resistant mutants and could need to be factored in for the practical use of *Bdellovibrio*. For instance, a quantification of this resistance phenotype and how it leads to regrowth of sensitive bacteria could give reason to 'cycle' the regulated inoculations or presence of *Bdellovibrio* in order to remove the pressures of *Bdellovibrio* predation that give the slow growing resistant bacteria the advantage over their sensitive counterparts (Gefen and Balaban, 2009).

This resistance phenotype has since been observed in many studies (Baker et al., 2017), whereas mutational resistance emergence of *Bdellovibrio* prey is rarely reported in literature. One such study introduced *Bdellovibrio* to a continuous culture of *Photobacterium leiognathi* prey growing in a chemostat (Varon, 1979). After six days of stable oscillations between the predator and prey populations, the system then changed accompanied by the revelation of a mutant prey resistant

to the predator. Although it should be noted that the basis of this resistance was not extensively examined.

Mutational resistance has been widely reported as a potential limitation for the use of bacteriophage predators as a therapeutic agent (Parisien et al., 2008). Furthermore, Mizoguchi *et al.* (2003), demonstrated a predator-prey arms race in which a bacteriophage responded to the prey's resistance by mutating itself, though it was not established whether this arms race was continuing, or had just been begun. Such an arms race occurring in an application would not be desirable as experimental and theoretical studies have shown that this can alter the characteristics of predator–prey cycles, driving them away from the traditionally observed cycles such as the Lotka-Volterra model in which prey oscillations precede predator oscillations (Cortez and Weitz, 2014). This could make it difficult to predict the predator prey population and optimise the control procedure (Feichtmayer et al., 2017).

There needs to be an increase in studies that investigate the potential resistance mechanisms in *Bdellovibrio* prey making up a biofilm, its ecological implications and how this is relevant to the use of *Bdellovibrio* as a control agent. For example, current investigations into phenotypic resistance such as Shemesh and Jurkevitch have deemed it to be temporary, with the resistant bacteria reverting to the susceptible phenotype. However little attention has been placed on determining if there is in fact also a trigger independent of genetic mutation that causes this or factors influencing the rate at which the switch occurs such as a lack of predator encounter, an adequate interval of time or change in prey density (Hol *et al* ., 2016a).

In addition, there is the need for more studies that observe resistance development and the co-existence of predator and prey over a longer time period. This could help better characterise and predict the effect that prey resistance has on oscillations of predator prey populations such as whether it could cause the two populations to gradually meet equilibrium as is predicted of various mathematical models (Wilkinson, 2006). Unfortunately, the lack of suitable experimental

methods means that in many studies the time incubation of prey with the predator is limited. For example, as discussed above, (Shemesh and Jurkevitch, 2003) the study shows that after contact with the predator, the concentrated resistant prey population begins to decrease significantly after 1 hour but the incubation is then ceased after 3 hours when no further decline in the resistant population size was observed. This time length is possibly too short to observe if there are any long-term penalties or advantages associated with the resistance phenotype when compared to the sensitive bacteria, which could have implications on the widespread use of *Bdellovibrio*.

2.2.2 Predatory-prey ratio

Quantifying and assessing the effect that predator and prey density has on community structure, behaviour and mortality could be crucial for fundamental knowledge as well as optimising the action of *Bdellovibrio* in application and thus, improving system productivity (Strauch et al., 2007).

Many studies that have aimed to elaborate on density mediated predator and prey relationships have focused on either the prey or the predator perspective. This makes it difficult to develop a deeper understanding of how the density-mediated predator and prey behaviour effects population dynamics and vice versa under different conditions (Yair et al., 2003). One study observed the behaviour of luminous photobacterium in the presence of the predator *Bdellovibrio*. Instead of using conventional measurement techniques that require high concentrations such as optical density, using luminous prey allowed them to use low densities more comparable to natural conditions (Varon and Zeigler, 1978).

The study observed predation in different predator: prey ratios by adjusting the prey densities from 10^3 to 10^8 cells per ml. As expected, they found that at low predator: prey ratios (higher prey densities), prey survival increased after 40 minutes. They speculated that this was due to *Bdellovibrio* inability to display chemotaxis and instead encountering prey via random collision. At low predator: prey ratios the probability of a prey being located by random collision is expected

to decrease, leading to the increase in prey survival that was observed. However, measuring only the change in relative light decay of prey in this manner relies on the assumption that there is no significant change in the number or behaviour of predators when incubated at high predator: prey ratios. Without direct observation of the predator, it becomes difficult to make clear conclusions regarding the predator's prey location mechanism and its relationship with prey (Mukherjee et al., 2016).

Studies such as Mukherjee et al. (2016) are important in providing fundamental knowledge that can aid the development of biocontrol applications. For example, if predation rate is more significant at higher predator densities as it is in Mukherjee et al. (2016), the prey population at the surface of the immobile biofilm may favour growth in a planktonic state able to use its motility to avoid predation (Núñez et al., 2005). At which point the vulnerable planktonic bacterial cells can be more effectively targeted by the combination of antibiotics and predatory bacteria (Czaplewski *et al* ., 2016).

Like Varon & Zeigler (1978), Im *et al*. (2014) also used bioluminescent prey to understand population mechanisms from the perspective of the prey (Im et al., 2014). They used a much longer time frame (13 hours) and found that although increased predator: prey ratios, resulted in a more rapid decline of prey numbers (as measured by optical density and bioluminescence decrease), after 13 hours the prey numbers were relatively similar for the different predator: prey ratio treatments. In fact, a predator: prey ratio of 2:1 showed a larger decline in prey numbers than the 4:1 treatment after 13 hours, highlighting the need to further investigate optimal predator: prey ratios in certain conditions for the use of *Bdellovibrio* as a biocontrol.

A contrasting study (Yair et al., 2003), observed the predator prey dynamics by measuring the change in Bdelloplast, attack phase for *Bdellovibrio* and prey cell populations at high (10:1) and low (1:1) predator: prey ratios. They found that predation appeared to be more efficient at the low predator: prey ratio after 350 minutes. At a high predator: prey ratio, there was a rapid loss in prey numbers

within an hour accompanied by an increase in bdelloplasts indicating that they had been penetrated by a predator. The prey population showed a slight regrowth while the majority of predators were in the replicative stage but after two and a half hours there was a slow decline in prey numbers as new progeny attack phase cells were released from the bdelloplasts, ready to search for and predate upon the remaining prey.

The prey population in the low predator: prey ratio remained relatively constant during the first two hours due to the limited number of attack phase cells leaving many prey cells alive to be able to continue replication. After two and a half hours, attack phase progeny from the bdelloplasts was continually released more gradually leading to a second phase of rapid decline. This study ran for only a short time, so it is difficult to draw conclusions as to whether a low predator: prey ratio would be more efficient to use as a biocontrol (Wilkinson, 2006). However, *Bdellovibrio* decrease in size and speed the longer they stay in attack phase starved of prey (Rotem et al., 2015). This could mean using a lower predator: prey ratio could be more efficient as it allowed for a slow release of attack cells to limit the amount of old and less active attack phase cells.

This also highlights the need to better characterise the motility, behaviour and survival of attack phase cells under starvation. As well as the following response to starvation regarding penetration, replication and release of progeny such as if starvation of *Bdellovibrio* attack phase influences the number of progeny released (Rotem et al., 2015).

Microscopy observation showed that many of the prey were predated upon my more than one cell. A similar observation was shown by Im *et al*. (2013), who showed that under high predator densities (>10:1) there was an increase in multiple predator cell attachments on a single prey cell, causing rapid premature lysis. They suggested that in predator: prey ratios as high as 86:1, one in seven prey cells experienced premature lysis.

They also used a model to demonstrate this and found there was a surprisingly higher relative bioluminescence shown in the experimental data compared to the model, especially at higher predator: prey ratios. They proposed that this was because at high predator: prey ratios, premature lysing of cells resulted in a release of nutritious cytoplasmic contents that were utilised by the surviving prey bacteria. This and reduced competition from predated upon cells meant that the remaining prey were more metabolically active and produced a greater bioluminescence.

In membrane filters clogged with biofilms, where cells can easier aggregate to subpopulations of high densities, premature lysis may occur (Chen et al., 2010). This has several additional implications in that the release of cytoplasmic contents could attract more predators or further promote the growth of biofilm (Straley and Conti, 1977). Premature lysis also reduces the number of bdelloplasts which at certain concentrations have been shown to induce growth arrest in a marine strain of *Bdellovibrio* (Varon et al., 1983). It could also be possible that, similar to the predator induced plastic resistance of prey, the prey's premature lysis and subsequent nutrient release is a mechanism to increase the prey cell population and reduce competition between predators under high predator densities. Additionally, it remains to be elucidated what response predators show to premature lysis and whether they cooperate to induce premature lysis and utilise the released nutrients this to increase activity and reduce negative starvation effects.

2.2.3 Multispecies system

The vast majority of predatory bacteria studies have utilised two-species systems, with model organisms such as *E. coli*, *K. pneumonia* and *P. aeruginosa* being favoured as prey because they are well characterised, relatively easy to culture and have relevance to disease and biofilm mitigation in applications (Baker et al., 2017). The addition of a third species in a system has significant effects, so it is clear that current studies are limited in how well they can mimic the field environment and inform on the predator-prey dynamics that would occur

(Wilkinson, 2006). This is especially true for biofilm removal in fields such as water treatment, where biofilms often consist of multiple species which interact closely to provide a number of functions lending to stability and survival where monoculture biofilms would not thrive. Thus, for example, multispecies biofilms have an increased tolerance to antibiotics (Mohan et al., 2015) and protozoan grazing (Yun et al., 2006). In addition, there are many aspects of a multispecies system that can potentially be exploited for the mitigation of biofouling in membrane filters, but this requires further investigation.

For instance, it has been shown by recent meta-analysis studies that an increase in predator species richness is correlated with an increase in prey suppression (Johnke et al., 2014). However, there is the need to investigate the potential mechanisms by which increasing predator diversity can decrease total prey biomass and exploit them to maximise filtration (Johnke et al., 2014).

Similarly, competition between multiple predators targeting the same prey may impact predatory diversity and therefore reduce predation (Feng et al., 2016). Unlike generalist feeders (protists etc) and specialised feeders (phages etc), *Bdellovibrio* exhibit a varied prey range between isolates and various studies have demonstrated that *Bdellovibrio* and their prey rapidly evolve to yield populations that differed in their original predatory and resistance capabilities, respectively (Johnke et al., 2014). This feeding strategy could make the addition of 'alternative prey' a viable strategy in *Bdellovibrio* biocontrol (Wilkinson, 2001). This strategy has been proposed in insect control and involves the addition of 'harmless' alternative prey sources into the environment to support the predator growth when necessary to the level that would lead to the adequate suppression of harmful target prey. In the treatment of drinking water alternative prey could be those that have been heat or UV killed, or non-pathogenic or biofilm forming bacteria (Hespell, 1978).

Multispecies studies such as this could also provide further insight into action of *Bdellovibrio* has on the total bacterial population dynamics including non-prey. This is vital as *Bdellovibrio* display prey preferences and many bacteria are not

susceptible to *Bdellovibrio* predation. For example, bacteria that have paracrystalline surface layers (Koval and Hynes, 1991) could receive a significant selective advantage over susceptible organisms, allowing them to become dominant and accumulate. Alternatively, many species rely on other bacteria to initiate biofilm growth in order to survive in harsh conditions and if these biofilm growing bacteria are removed by *Bdellovibrio* predation, the overall survival of both prey and non-prey bacteria could decrease (Feng et al., 2016). Thus, improving understanding of prey range on biofilm ecology could contribute to successful and safe implementation of predators in water treatment technologies.

It is also possible that multispecies systems could alter our understanding of factors such as density, resistance and host finding, that were previously investigated in two species systems. For example, it is expected that in areas of high prey density, the *Bdellovibrio*'s ability to adhere to prey is not negatively affected. This is based on assumptions from laboratory-based studies and mathematical models that have always used two-cell cultures, including one study which demonstrated that at a low predator to prey ratio (0.1), up to 90% of a *B. bacteriovorus* population was found to attach to *E. coli* (Varon and Shilo, 1968). However, the ratio between prey and non-prey is unpredictable in the field and a high non-prey density could influence the *Bdellovibrio*'s host-finding mechanism (Lambert et al., 2003).

There is evidence that *Bdellovibrio* cells hunt for prey via random collision (Strauch et al., 2007). They possess rapid motility and have been suggested to be weakly attracted towards amino acids and areas of high microbial density in aquatic ecosystems (Chauhan and Williams, 2006). These are all traits that would increase the relative chance of prey encounter; however, they could also increase the chance of encounter with non-prey. Indeed, *Bdellovibrio* appear to collide and attach to many non-prey objects, though these attachments are not irreversible like many encounters with prey(Rotem et al., 2015). This ability for *Bdellovibrio* to readily adhere to surfaces even after they have been rinsed free from medium has been suggested as an adaptation to hunting biofilms adhered to surfaces (Núñez et al., 2005). In biofilms, prey cells would be in close vicinity to each other and

hunting by random collision would prove more efficient rather than hunting planktonic cells (Kadouri and O'Toole, 2005). This could have implications on the length of the life cycle.

It could be, that in areas of high cell (prey and non-prey) densities, the *Bdellovibrio* alters its pattern of motility or hunting strategy to prevent many random non-prey collisions. Bacteria in general, display several methods of movements (Harshey, 2003) and *Bdellovibrio* have been shown to contain several pili gene clusters, as well as genes believed to be related to adventurous gliding (Pasternak et al., 2013) and entry into the periplasmic space (Rendulic et al., 2004).

There is currently a lack of studies that have aimed to characterise the movement of *Bdellovibrio* and whether these patterns are actually 'random' or follow an adaptive pattern. Additionally, the flagellum dependent rapid motility that characterises the predator has shown to not always be necessary depending on the situation. As demonstrated by one study, which observed predation in *Bdellovibrio* mutants that did not possess a functional flagellum and were thus, non-motile. The mutants were unable to grow in liquid cultures but when directly spread onto high-density prey lawns on an agar surface, the mutants showed limited clearing of prey and were able to successfully replicate (Lambert et al., 2006).

Similarly, it has been reported that in prey biofilms, not all of the *Bdellovibrio* population had a flagellum (Núñez et al., 2005), further suggesting that flagellar motility is not absolutely required for Bdellovibrio to enter the periplasm of their prey and motility and adhesion may be adaptive depending on cell concentration or other environmental factors that have yet to elucidated (Núñez et al., 2005).

A switch in motility pattern depending on environmental conditions would require the ability to rapidly adapt. Indeed, *Bdellovibrio* have shown to switch modes of life from a rapid host-dependent lifestyle to being non-motile and host-independent when incubated without the presence of prey (Jurkevitch, 2012) and there are reports that marine biofilm associated *Bdellovibrio* species (Williams,

1988) do not show the random collision predatory behaviour, as they must locate prey to remain near a submerged biofilm community (Williams, 1988). Furthermore their two phase life cycle demonstrates adaptability even in laboratory conditions, where the *Bdellovibrio* switches from the DN (dilute nutrient) broth environment of low nutrients to inside the high nutrient host cell (Jurkevitch, 2012).

If this theory was proven to be correct, it could possibly be exploited to improve the efficiency of *Bdellovibrio* as a biocontrol, for instance a specific non-prey or decoy density could be exposed to the *Bdellovibrio* in a membrane filter to ensure that whichever method of finding prey would be most efficient, would be the method used by the *Bdellovibrio*. However before this can be explored, there is the need for more definitive characterisation and quantification of two culture predatory prey dynamics under varying densities as there is disparity in the literature as to whether a predator is more or less effective in high prey densities (Martin, 2002). Once this can be achieved, the relationship between a predator, its prey and non-prey need to be investigated in multi-species systems, though the methods capable of this are limited (see section below).

2.3 Experimental Methods

Our ability to elucidate the key factors influencing population dynamics is limited by our ability to experimentally observe predators and prey (Chauhan and Williams, 2006). Reliable enumeration of population dynamics requires the combined measurement of predator and prey death and growth rates under different conditions, which many of the current experimental methods are incapable of doing (Feichtmayer et al., 2017). Additionally, most bacteria are not readily culturable, and it may be that the growth and behaviour of predatory bacteria are inadequately characterised because they cannot be co-cultured with their preferred prey (Koval, 2006).

As a result, mathematical models have been relied on to provide predictions about the *Bdellovibrio* and its prey dynamics in nature (Wilkinson, 2006). Mathematical models of predator–prey systems have long been used in the study of ecosystem

dynamics and evolution. They aim to use the observed behaviours of the predator and prey in short term experiments to predict and further understand the longer-term dynamics of the system and provide insight into the parameter values that will yield different population dynamic patterns (Baker et al., 2017). This can help explain many of the features in real predator–prey systems such as the effect of multiple species and prey resistance (Wilkinson, 2001).

However, the majority of models are for highly idealised systems. They make many assumptions, that are not experimentally validated and often neglect processes like, the delay due to replication inside a Bdelloplast, the presence of persistent prey or the spatial structure imposed by biofilm, that might ultimately be important (Wilkinson, 2006). Furthermore, many models have focused on dynamics in a natural environment such as a lake or soil; only a few models are specifically tailored for engineered systems, such as a membrane filter, or medical settings, such as, inside the human body (Baker et al., 2017). Mathematical models and experimental investigations into the *Bdellovibrio*-prey dynamics in an applied setting are crucial for optimising the use of *Bdellovibrio*, specifically the adequate predator: prey ratio, spatial strategy of attack and the timing required for significant prey suppression (Baker et al., 2017).

Protocols of the experimental methods; cell turbidity, viability plating and the double layer agar method (Starr and Stolp, 1976) (Koval, 2006) have been well described previously and so will not be the subject of this review, which will instead discuss the use of these methods in investigations of *Bdellovibrio* predator prey dynamics.

2.3.1 Culture turbidity, Viability plating and plaque forming unit method

In the majority of literature, quantification of predator-prey dynamics has been restricted to conventional techniques. For indirectly evaluating predation, optical density or turbidity in the liquid culture is measured using simple spectrophotometric or coulometric readings to indicate the suppression of the prey population in the presence of the growing *Bdellovibrio* (Yair et al., 2003). As the

prey cell is lysed to release the *Bdellovibrio* progeny, the culture becomes more clear and a reduction in turbidity is seen (Mukherjee et al., 2016). The small size of *Bdellovibrio* cells means that their contribution to turbidity is not significant.

Moreover, the turbidity methods have the advantage over viability counts and double layer agar plating (also known as plaque forming unit method) as it can measure a coculture in its original container without the need to remove a sample which risks contamination. Turbidity measurements can also be used for cocultures in microtiter plates, allowing a much larger number of tests to be carried out and making it easier to monitor predation in real time. Despite this, the use of turbidity as a measurement is limited in that it is not a direct count of prey cells and a decrease in turbidity is shown with prey cell death regardless of cause, as such it provides only an indication of predation.

In contrast, standard dilution plating and viability counts directly measure the change in prey population (Varon and Zeigler, 1978). From this the rate of predation can be revealed and can potentially provide information into predation under different conditions as well as the prey preference of *Bdellovibrio* (Chen et al., 2011).

When using viability counting to measure the quantity of cells in a biofilm, the biofilm must first be detached from its surface. This can be done by adding 30% (volume/volume) acetic acid, which is less invasive but leaves many of the bacterial cells in the biofilm still attached to the surface. Sonication is used to achieve a higher yield of cells, but this approach can lead to false negative results as the procedure can damage the prey cell's ability to replicate. This method is also not as reliable as the bacteria often do not detach as single cells but instead clumps of varying sizes (Pantanella et al., 2013).

Bdellovibrio colonies are not visible, so it is not possible to enumerate their population using general viable count plating methods (Stolp and Starr, 1963). The solution to this is the double agar overlay or plaque forming unit method, which has been adapted from the study of bacteriophages (Stolp and Starr, 1963) and is

used for the direct isolation from environmental samples, enrichment and purification of predator cultures and enumeration of predators (Koval, 2006).

Double agar consists of a base layer of hard agar, and an upper layer of soft agar which contains a large quantity of immobilised prey cells, which replicate to form a dense and turbid lawn covering the top agar (Cormier and Janes, 2014). A sample of *Bdellovibrio* is taken from predator-prey cocultures, diluted to a small quantity of just a few cells and spread over the top agar. Following incubation, a *Bdellovibrio* cell predates upon prey and releases progeny, which continue the cycle to kill more and more prey. The hard agar of the base layer restricts the movement of the *Bdellovibrio* so that it keeps the population of predators within close vicinity of each other and predation only occurs to neighbouring prey cells. When an adequate number of prey cells are lysed, a clear circular area is formed. This is known as a plaque, therefore one plaque forming unit (PFU) represents the quantity of *Bdellovibrio* cells that are capable of lysing a sufficient number of host cells to form one plaque (Koval, 2006).

This measurement is functional and is unable to enumerate the absolute quantity of *Bdellovibrio* cells because those that fail to kill a prey cell because they are dead, non-motile or non-replicative will be unable to produce a plaque and cannot be enumerated, leading to underestimation of the quantity (Chauhan and Williams, 2006). Like viability counting, this method, although well-defined is also limited in that it does not allow real-time estimation of predation and it requires a sample from the coculture, increasing the risk of contamination. Furthermore, these two methods are time consuming and labour intensive (Mukherjee et al., 2016).

Despite their flaws, quantification of Bdellovibrio predator prey dynamics via double-layer method, viability count and turbidity is well defined and still used in a wide range of investigations including cell cycle events and prey preference (Koval, 2006).

2.3.2 Direct microscopic counts and fluorescence

Phase contrast microscopy is often used to monitor growth in culture maintenance by observing the progression of the predation lifecycle, which is not possible with the above-mentioned methods. However, it is rarely used as an accurate measure of quantification and the number of cells is underestimated due to the small size and high mobility of *Bdellovibrio*. It is labour intensive and difficult even with the assistance of a counting chamber and added glycerol to reduce the streaming (Starr and Stolp, 1976).

Fluorescence and bioluminescence is often used as an alternative direct microscopic count method in the study of predation, such as in imaging studies to visualise prey biofilms or in enumeration of the predator (Peeters et al., 2008). Whereas most studies on predator-prey dynamics of *Bdellovibrio* had focused on the evaluation of the prey (Fenton et al., 2010), one recent paper, aimed to monitor predator population change in real time by using *Bdellovibrio* expressing a red dTomato fluorescent reporter protein (Mukherjee et al., 2016). They first validated the fluorescent marker, demonstrating that the predation patterns were similar to those observed in previous studies that used traditional methods (Dashiff et al., 2011) (Wilkinson, 2001). Use of a microplate reader allowed for rapid observation in real time and under different growth conditions including in the presence of different prey (Elkhatib et al., 2014). Specifically, there was a similar pattern to predator growth when quantification was carried out using fluorescence and PFU. As expected, this growth was accompanied by a correlating reduction in prey cell population as measured by the reduction in optical density.

The study was in agreement with previous studies (Wilkinson, 2001), that the addition of non-prey decoy bacterium reduces the predation efficiency of *Bdellovibrio*. They showed that upon the addition of gram-positive *S. epidermidis* to make a 1:1 (volume/volume) ratio culture with *E. coli*, there was a reduced proliferation of the predator compared to that with *E. coli* alone.

Moreover, the study agreed with previous studies investigating the predation efficiency of *Bdellovibrio* with heat killed prey. They found that preheating the prey cells at 65C for significantly reduced the final predator fluorescence after 48 hours when compared non-heat treated cells (Varon and Shilo, 1969).

Flow cytometry has long been a popular tool for the quantification and phenotypic characterization of various microorganisms that have been stained with fluorescence tags (such as sybr green I) and passed through a beam of laser light (Harry et al., 2016). Despite this, flow cytometry has not been used in the simultaneous measurement of *Bdellovibrio* and their prey.

A major benefit of flow cytometry is that is allows for high throughput multiparametric analysis of cell samples which would make it ideal in assessing *Bdellovibrio* predation dynamics in various settings. Several studies have aimed to model *Bdellovibrio* predation but are limited in that they are not experimentally validated or are done so using conventional techniques that are both labour intensive and are not able to measure the predator and prey at the same time (Im et al., 2014).

Another advantage of flow cytometry over conventional techniques such as plate counting is that it acknowledges viable but slow-growing cells that may be non-culturable (Huh et al., 2005). Slow growing bacteria in particular have been shown to be important in the acquiring and spread of antibiotic resistance, however the role of slow-growing predators and prey in predation has yet to be explored (Davies, 1994). This could have consequence for the application of *Bdellovibrio* in water treatment as prey cells in biofilms are more likely to grow slow in comparison to free-living bacteria (Abberton et al., 2016).

2.3.3 Predator prey cultures

In general, the above conventional measurements are used to assess predation dynamics in investigative cocultures such as those in conical flasks containing dilute nutrient broth or buffer (Koval, 2006). The medium is chosen to restrict the

growth of prey cells and limit the chance of contamination, thus, making the measurement of predator growth more accurate. Additionally, it has been cited that *Bdellovibrio* growth is increased in conditions where prey bacteria are slow-growing(Koval, 2006).

Bdellovibrio are aerobic bacteria so as such, require efficient aeration (Jurkevitch, 2012). In conventional cocultures such as flasks or test tubes these conditions are provided by using an appropriate amount of medium to total volume space in a container which is also continually shaken to create a steady flow of oxygen. However, the shaking creates a uniform landscape that is less relevant to natural conditions and prevents the development and analysis of spatially separated subpopulations (Hol *et al* ., 2016b). Another disadvantage of culturing dependent methods is that many prey bacteria are not readily cultured and so investigations into *Bdellovibrio* are limited to well defined and culturable prey strains such as *E. coli* (Pasternak et al., 2013).

2.3.4 Real-time quantitative PCR (qPCR)

Culture independent methods such as Fluorescence in situ hybridisation (FISH) and qPCR have been developed as an alternative to labour intensive culture dependent methods, such as the plaque forming unit method (Bueno, 2014) and are frequently used for the identification of predatory bacteria in natural habitats and in a few cases have been used for the quantification of predator and prey populations (Van Essche et al., 2009).

qPCR is considered the gold-standard for the quantification of nucleic acids in a range of disciplines and can be used in microbiology to measure the abundance of bacteria by targeting the small subunit 16S rRNA gene (Klein, 2002). rRNA genes evolve slowly and are highly conserved so they can be used to distinguish sequences from genera to species (Hirsch et al., 2010).

qPCR uses intercalating fluorescent probes such as (TaqMan) or dyes (SYBR Green) to bind to the target 16S rRNA gene in a sample and as the PCR product increases

so does the fluorescence. This fluorescent is quantified in real time and is used to define the exponential phase of the reaction (Kandel et al., 2014). Plasmids bearing the 16S rRNA gene of the target species are used to construct a standard calibration curve which is then compared to the results from the sample to accurately quantify, in absolute amounts, the initial number of target molecules in the sample (Klein, 2002).

qPCR has the advantage of being much more reliable and faster to perform than conventional techniques as well as being highly sensitive and reproducible (Lee et al., 2006).This has been demonstrated in one study which aimed to explore the dynamics of *Bdellovibrio* populations and their prey in the zero discharge systems of aquaculture (Kandel et al., 2014).They found that the qPCR technique was able to quantify gene expression from small amount of samples, showing a minimal detection limit of 10 16S rRNA copies per 5µl reaction which was equal to two *Bdellovibrio* cells per mL^{-1}. Furthermore, they found that the quantity of predator cells growing on *E. coli* were 2.5 times higher when qPCR was used for enumeration instead of the plaque forming unit method. They suggested that this may be due to decreased predation in soft agar limiting the visual detection of small plaques. Crucially, qPCR simply targets the expression of the 16SrRNA gene and cannot distinguish the physiological state of the bacteria and hence also detects cells that may be dead or non-viable that would not be able to form plaques in the double layer agar method (Hirsch et al., 2010).

This trait may limit the use of qPCR in measuring the predation of prey bacteria but instead could be more useful for measuring the effect that *Bdellovibrio* have on the community of a system as a whole over a longer time period. Indeed, using qPCR the study was able quantify the effect that environmental parameters such as salinity and temperature had on the *Bdellovibrio* community over several months. In similar fashion, one study used qPCR to suggest that patients suffering from irritable bowel disorder and similar diseases showed a reduced amount of *B. bacteriovorus* in their guts in comparison to healthy subjects (Iebba et al., 2013).

Thus, the use of qPCR may be best suited to evaluate the effect that any *Bdellovibrio* control agent has on the bacterial community in a system such as a membrane filter (Kim *et al* ., 2013a). Possibly revealing, if there is a reduction in key pathogenic or biofilm contributing bacteria as well as if the conditions created by the prolonged presence of *Bdellovibrio* favour the growth and possible dominance of a non-prey species (Johnke et al., 2014).

The culture independent and high-throughput nature of qPCR could make the multispecies evaluations much easier to perform than conventional techniques. No post-PCR steps are required, which decreases the risk of cross-contamination due to PCR products.(Klein, 2002). Another advantage is that the technique is highly specific and the 16S rRNA is characterised well enough that with the correct primer design, cultures of non-target do not produce the fluorescent signal indicative of target gene amplification (Van Essche et al., 2009). It has also been demonstrated that even when samples containing target DNA are mixed with non-target DNA from other species, the detection levels of the target DNA are not affected (Van Essche et al., 2009). This could be useful when trying to differentiate between the abundance change of prey and non-prey species in a system. One limitation to evaluating the role of *Bdellovibrio* in multispecies systems is that it may require the use of a large number of reagents which would be costly (Sun and Jiang, 2013).

Although qPCR is often used as a culture independent method for monitoring bacterial populations in a natural environment, when measuring the dynamics of predation under varying experimental conditions, the investigations have usually been carried out using conventional culturing methods.

2.3.5 Microfluidics

Microfluidics is an interdisciplinary technology that aims to study the behaviour of fluids using micrometre-sized (10–100 μm) channels engraved onto a material such as Polydimethylsiloxane (PDMS) making up a microfluidic chip (Salieb-Beugelaar et al., 2010). In cell biology, the microchannels are designed precisely in such a way

that it allows the automated manipulation (directing, mixing, removing etc) of single cell to multicellular organisms (Sia and Whitesides, 2003). Despite microfluidics being a complex and novel technology, microfluidic chips have been reported to be relatively cheap and easy to fabricate (Campbell and Grzybowski, 2004).

Microfluidic devices can be coupled to technologies such as fluorescence microscopy and nucleic acid assays which has made it possible for microfluidics to be used for a range of different microbiology applications including pathogen detection and investigating biofilm formation (Bao et al., 2008). A large proportion of studies using microfluidics have aimed to observe the interaction of bacteria with antibiotics (Mohan *et al* ., 2015). One study (Hol *et al* ., 2016a) fabricated a landscape with two compartments connected by a corridor, one continuously filled with LB medium and one continuously filled with LB medium and the antibiotic kanamycin. This design allowed the population dynamics of non-resistant *E coli* to be observed in the presence of a kanamycin concentration gradient using fluorescence microscopy. They found that surprisingly, a proportion of the bacteria population were able to invade and grow in areas of the antibiotic compartment that contained high concentrations of kanamycin. The persistence and viability of these populations was dependent on motility, with successful invasion of antibiotic compartments only occurring in migrating population that reached a high threshold of density ($>5 \times 10^9$ cells per ml).

A further advantage of microfluidics is that bacteria and the fluids in which they are suspended can be isolated from the device to be rapidly analysed further (Sia and Whitesides, 2003). Thus, the Hol *et al*. (2016a) study isolated a sample of the successfully invaded population to measure the minimum inhibitory concentration (MIC) of kanamycin. They demonstrated that despite the tolerance of the invaded population to lethal concentrations of the antibiotic in chamber, the population showed no change in antibiotic susceptibility compared to the measured MIC before the experiment. This suggests that in a similar fashion to the phenotypic resistance of some species to *Bdellovibrio* (Shemesh and Jurkevitch, 2003), there was an adaptive mechanism independent of genetics conferring temporary

phenotypic resistance to the population allowing them to successfully invade (Corona and Martinez, 2013).

This study is a good example of the benefits of microfluidics. The device creates a diverse mosaic of different microhabitats with physiochemical gradients, allowing the local growth of subpopulations to reach high densities and subsequently migrate to and colonise environments of high antibiotic strategy (Hol *et al* ., 2016a). The spatially structured landscape of the microfluidic chamber additionally allows populations of high densities to continue to grow and show motility. In contrast, when bacteria are observed in unstructured environments such as the shaking flask, there is a mixing effect that creates a uniform environment in which high density populations are saturating and hence, restricted in their movement and growth (Patra and Klumpp, 2014). This prevents the growth and migration of subpopulations and is not resembling of a natural environment (Hol *et al* ., 2016b). Likewise, the spatial structure has been cited as a reason as to why microfluidics has been able to culture multispecies systems not previously possible by conventional methods such as agar plating (Kim *et al* ., 2008).

When studying multispecies interaction, there is the need to establish an adequately relevant environment. This requires mimicking of the specific physical, social and chemical conditions present in local environment that interact to shape bacterial communities (Young *et al* ., 2001). Although there is an incredible amount of diversity in the species found at a macroscale such as a lake or in soil, it is likely that at a microscale the interspecies interactions are much more limited as determined by specific spatial structures (Røder *et al* ., 2016). Thus, unstructured conventional methods such as test tubes, flasks and cuvettes are rarely able to replicate the conditions required for subpopulations of multiple species to continuously grow (Young and Crawford, 2004).

For example, Kim *et al* (2008), failed to co-culture three species: *Azotobacter vinelandii*, *Bacillus licheniformis*, and *Paenibacillus curdlanolyticus* in a test tube containing nutrient rich medium. Depending on the nutrient availability, one species would rapidly grow and without the spatial separation, would out-compete

the others species causing the two ‘losing’ species to rapidly decline (Kim *et al* ., 2008).

However, using a microfluidic device, they were able to synthesise a three-species community by spatially separating each species so that bacteria were unable to migrate and come into direct contact. The device did allow for chemical communication among the species, which was shown to ensure the survival and stability of the community even in a low nutrient environment (Kim *et al* ., 2008). *A. vinelandii’s* function was to supply nitrogen sources by fixing gaseous nitrogen. *B. licheniformis* functioned to ensure the survival of the community under antibiotic pressure by degrading penicillin G with ß-lactamases and *P. curdlanolyticus* used cellulases to provide a carbon energy source, such as glucose (Kim *et al* ., 2008). When the species were attempted to be cultured alone in the device, or when one species was removed, the stability and growth of the community significantly decreased. Thus further demonstrating that microfluidics can be used to construct the spatial complexity of a landscape that governs key syntrophic interactions and allow neighbouring populations of different species to co-exist (Stirzaker *et al* ., 1996). A trait which is essential for investigations to multispecies interactions.

As well as allowing the spatial control of the fluid composition, an adequate design enables the long-term quantification of cell behaviour in a range of tightly controlled parameters such as temperature, media change and cell density, that cannot only better serve to mimic bacteria’s natural environment but also reveal the response of bacteria to varying conditions. Park *et al* (2011) constructed a microfabricated ratchet structure array that was able to arrange populations of both the predatory bacteria *B. bacteriovorus* and its *E. coli* prey at a range of linear, compartmentalised or uniform densities (Park *et al* ., 2011). By measuring fluorescence intensity signals from prey, they were able to characterise the predation rates in nine different experimental conditions, combining two different prey density conditions with three different predator density conditions. Additionally, the concentrator arrays of the device captured and prevented the

escape of motile cells allowing further analysis of the predatory behaviour at single cell levels (Park *et al* ., 2011).

The study agreed with previous investigations that high predator: prey ratios lead to a greater predation rate (Im et al., 2014) Park and colleagues demonstrated that with a higher number of predators, the intensity of fluorescence expressed by prey rapidly decreases after 4 hours and then disappeared completely after 8 hours. This indicated that by this point after the completion of the first predator cycle, almost all the prey cells are infected by predator cells and the life cycle of the predator population is in sync. Whereas at low predator: prey ratios, the inefficiency of the random collision hunting method of *Bdellovibrio* results in many prey cells not colliding with the predator and instead surviving. In this case, the predation cycles are no longer in sync and several cycles occur (Park *et al* ., 2011).

This is depicted by a slower and more gradual decrease in fluorescent intensity in comparison and at a ratio of approximately 0.2 predator to 1 prey, the fluorescent intensities do not approach zero after 8 hours, suggesting there is a small persistent prey population (Park *et al* ., 2011). Although it is not clear from this study whether this is the due to the inefficiency of random collision or the temporary phenotypic resistance discussed earlier (Shemesh and Jurkevitch, 2003). To determine so, may require further studies into the kinetics of predator attachment to prey (Varon and Shilo, 1968).

Sufficient mixing of samples and reagents is necessary when a uniform concentration is required but this has proven difficult when using microfluidics. For the majority of devices, the laminar flow relies on diffusion to mix reactants and cannot provide sufficient rates of mixing which are demonstrated by the turbulent mixing found in macro-scale systems or even with experiments that use shaking flasks. The device used in the Park *et al* (2011) study mentioned above, makes use of microfluidic channels in a Christmas tree structure that act as gradient generators (mixers) at both the top and at the bottom of the device. This meant that microbes could be introduced into the array of concentrator wells uniformly or by varying densities, so that the behaviour of cells in each well could be

observed in a reliable and controllable manner (Park *et al* ., 2011). This Christmas tree structure for the microfluidic channels has been used to create gradients in the study of mammalian and bacterial cells (Jeon *et al* ., 2002) an example of passive mixing; a category of mixing within microfluidics achieved through the fabrication of a specific microchannel structure without the need for external energy or complicated operation (Nguyen and Wu, 2005).

Alternatively, one study fabricated a BAY microbioreactor, as method to increase the mixing rate by active mixing. Active mixers provide external control to the user and include using pressure gradients, electrical voltages, or integrated mechanical or magnetic mixing elements (Ward and Fan, 2015). Using electrodes in digital microfluidics, the BAY microbioreactor can manipulate a droplet of the cell culture in a circular path achieving uniform distribution and mixing rates of up to 10-50 times faster than rates achieved solely by diffusive mixing. The mixing conditions demonstrated by the BAY resulted in growth rates of *E. coli* similar to that of conventional methods at the micro and macroscale, while having the advantage over these methods because they allowed for high-throughput analysis of a long term cell culture (up to five days) even with the use of a small amount of initial culture (~70 µL) (Au *et al* ., 2011).

When fabricating novel microfluidic devices, it is important to consider that the slow laminar rate of many microfluidic devices also reduces the ability of the bacteria to adhere to each other (as measured by shear forces) (Simoes *et al* ., 2007). The small dimensions involved means that many PDMS-based devices cannot deliver the conditions of higher shear forces needed in studies which require denser biofilms (Gomes *et al* ., 2013). This is partly due to the fact that PDMS, a silicon-based polymer which is patterned to create the desired channel structure, is intolerant to high flow rates (like in turbulent mixing), which damage the structure of the microchannels in the device and make it harder to measure or control flow rates (Lu *et al* ., 2004). Though there are some solutions to this such as the inclusion of doubly crosslinked nano-adhesive (DCNA) which strongly seals the microfluidic channels and increasing their tolerance to higher pressures without significantly altering the microchannel structure (You *et al* ., 2013).

Devices made using DCNA have reported a flow rate as high as 10 mL min^{-1} in a 1.5 cm long channel with a 150μm×150μm cross section (You *et al* ., 2015).
Other solutions to allow for high flow rates include the use of alternative polymers to PDMS, such as Thermoset Polyester (TPE) which have been shown to be characteristic of a predictable operation at high pressure (Sollier *et al* ., 2011).

PDMS is widely used because it is relatively simple and cheap to fabricate (Regehr *et al* ., 2009). It also is known for its flexibility and having high optical transparency and gas permeability (Lötters *et al* ., 1997), making it ideal for fabricating varied microenvironments and studying predator and prey bacteria using optical and fluorescence microscopy (Tung *et al* ., 2004).

In conclusion, *Bdellovibrio* predatory bacteria represent a key biocontrol against the action of biofilms in many different applications, the success of which is often limited by the same issues; lack of reliable methods that enable high-throughput and thorough investigation into the dynamics of predation. However, advancements in microbiology and the development of methods such as flow cytometry and microfluidics will increase the chance of understanding the important factors of predation such as predator and prey density and the diversity of microbial populations and biofilm growth. Ultimately this knowledge is key in the development of systematic action plans for deploying *Bdellovibrio* to prevent biofilm growth and biofouling in decentralised water treatment.

3 Validating flow cytometry as a method for quantifying *Bdellovibrio* predatory bacteria and its prey for microbial ecology

This chapter is the basis for the peer reviewed journal publication:

Ogundero, A., Vignola, M., Connelly, S., Sloan, W.T., 2022. Validating Flow Cytometry as a Method for Quantifying *Bdellovibrio* Predatory Bacteria and Its Prey for Microbial Ecology. Microbiol. Spectr. 10, e01033-21. https://doi.org/10.1128/spectrum.01033-21

Ayo Ogundero led the research by contributing to the study design, experimental work, data analysis and manuscript writing.

3.1 Introduction

Host dependent strains of *Bdellovibrio* are a group of obligate predatory bacteria that kill and consume other bacteria to survive and reproduce (Jurkevitch, 2012). It is this ability to lyse cells as a function of reproduction that has led to increasing interest of *Bdellovibrio* as biocontrol agents, in particular for their mitigation of biofilms (Chen et al., 2010). For example, the human gut and intestinal microbiota population is negatively affected by the excessive biofilm growth of gram-negative bacteria but *Bdellovibrio* has been investigated as a potential probiotic to restore balance to the ecosystem and help treat conditions such as Inflammatory bowel diseases (IBD)(Iebba et al., 2013).

Biofilms are described as the most common natural state of bacteria, where free swimming planktonic bacterial cells group together and are embedded in a self-

produced extracellular polysaccharide matrix which helps to further anchor the cells to a substrate and facilitate continuous growth in this sessile state (Hall and Mah, 2017). This state increases bacteria's tolerance to stresses such as antibiotics and other antimicrobial agents. The mechanisms providing this defence are not well characterised and vary depending on many factors such that biofilm-based infections are both persistent and difficult to control (Hall and Mah, 2017).

Effective use of predatory bacteria as control agent for biofilms(Chen et al., 2010) requires a deeper understanding of how they interact with each other and the ecological dynamics with other microorganisms (Dwidar et al., 2012b). Presently, our ability to explain the key factors that may influence *Bdellovibrio* predator-prey dynamics, such as predator: prey density, resistance, and space is limited by how we enumerate both the predators and prey.

Many of the current experimental methods are cumbersome and arguably incapable of doing this accurately (Mukherjee et al., 2016). Flow cytometry has recently been demonstrated as an inexpensive and versatile method to accurately count total and intact microorganisms, with the ability to distinguish populations based on cellular features such as size and nucleic acid content(Brown et al., 2015) (Wang et al., 2009). This has been applied to mixed cultures from many different environments including drinking water(Hammes et al., 2008), soil and sediment(Porter et al., 1997) and activated sludge(Brown et al., 2019) (Brown et al., 2015). *Bdellovibrio* has been investigated in relevance to these environments (Richardson, 1990) (Fry and Staples, 1976) (Feng et al., 2017) (Iebba et al., 2013) but the use of flow cytometry to quantify the predatory bacteria has yet to be compared to other conventional methods.

Measuring optical density (OD) or cell turbidity with a spectrophotometer is a common method to study bacterial growth(Monod, 1949). When light passes through a microbial culture it is scattered. This scattering is recorded by a spectrophotometer and can be used as an indication of the biomass present (Sutton, 2011). OD is widely used, largely because of the speed and ease of

measurement. This has also made the method a common preference for culture inoculation and harvest (Myers et al., 2013).

The small size of *Bdellovibrio* makes it difficult to directly measure the bacteria (Lambert and Sockett, 2008) so optical density is instead often used to indicate the suppression of a prey population in the presence of growing *Bdellovibrio* (Varon and Shilo, 1969). A limitation here is that OD provides only an indication of predation rather than a direct measure as a decrease in optical density is shown with prey cell death irrespective of cause(Mukherjee et al., 2016). Additionally, optical density is not as sensitive or accurate as other methods, making its use limited when trying to get a deeper understanding of predator dynamics (Mukherjee et al., 2016).

The plaque forming unit (PFU) assay, also known as the double agar layer assay, is another of the most common laboratory techniques associated with *Bdellovibrio*(Varon and Shilo, 1968). Here dilutions of predatory bacteria prey are spread upon a cloudy lawn of prey bacteria on an agar plate to form clear zones in the agar. These clearings are known as plaques and are assumed to be formed by the initial growth of a single predator and are thus termed a plaque forming unit (Cormier and Janes, 2014).

Like colony forming unit plating techniques, the PFU assay is a popular and standard technique for obtaining a viable count because it is relative accurate and requires less specialist equipment and reagents to perform than newer techniques such as quantitative polymerase chain reaction (qPCR) (Koval, 2006). However, there is now an emphasis on the importance of high-throughput research in microbiology which has called for new approaches as the PFU assay is time consuming, slow and vulnerable to human error (Koval, 2006).

In recent years genetic techniques have been widely deployed to accurately detect and quantify microbial populations (Fey et al., 2004) (Baldwin et al., 2003). qPCR is one method that has been increasingly used and seen as a high standard measure due to its speed, high sensitivity and reproducibility (Lee et al., 2006).

qPCR offers accurate detection using several different approaches (Ponchel et al., 2003). To quantify *Bdellovibrio* populations in aquaculture zero discharge systems, one study made use of a *Taq hydrolysis* probe, a short fluorescent DNA sequence that is designed to complement and bind to a highly conserved region of the 16S rRNA specific to *Bdellovibrio* aquaculture(Kandel et al., 2014). Using PCR, the targeted region is replicated in turn increasing the fluorescence produced. This fluorescence is recorded in real time and used to accurately quantify, in absolute amounts, the initial number of target molecules (16S rRNA) and thus the organisms that carry them in the sample (Klein, 2002).

qPCR is mostly used to quantify either the total bacteria or a singular species present in environmental samples because it requires a large number of expensive reagents when compared to flow cytometry, which make it less suited in regular experimental work and in measuring growth of samples with multiple species (Sun and Jiang, 2013).

Flow cytometry (FCM) is a useful tool for quickly and reliably counting total microorganisms that have been stained with a fluorescence tag (usually SYBR green I) and enumerates cells via passage of the sample through a beam of laser light (Troussellier et al., 1993) (Hammes et al., 2008) and, more advanced machines are able to sort and collect these cells (Ibrahim and van den Engh, 2007).

Flow cytometry is also able to assess metabolic activity(Nir et al., 1990) and viability (Diaper et al., 1992) (Berney et al., 2007) by use of a gating system to allow the user to distinguish and analyse cell populations of different properties (Troussellier et al., 1993). The FCM gating system has been used previously to distinguish bacteria populations in freshwater with low and high nucleic acid cell content based on their green fluorescence and side scatter measurements.

Recently, advanced applications of FCM have further characterised the structure and phenotypic properties of sub-groups within microbial communities in order to generate a unique fingerprint (Günther et al., 2012). As an alternative or in

combination with molecular analysis, it can establish the dynamics and biodiversity that contribute to the stability of microbial communities in natural and engineered systems (Props et al., 2016) (Koch et al., 2013) (Prest et al., 2014).

As well as total cell count, flow cytometry can be used to estimate the quantity of non-viable cells by staining a population with propidium iodide (PI) and SYBR green I(SYBR I)(Barbesti et al., 2000). PI can only enter bacteria with damaged cytoplasmic membranes, causing a reduction in the SYBR I stain fluorescence which allows those cells with intact membranes to be counted. This value is subtracted from the total count of the same sample to estimate the number of membrane damaged cells (Berney et al., 2007). The ability of flow cytometry to distinguish between intact and membrane-damaged cells is important as *Bdellovibrio* have been shown to be able to predate and reproduce in heat-killed cells with damaged membranes, which would alter any model of predator prey dynamics (Hespell, 1978).

Surprisingly, there are few studies investigating *Bdellovibrio* predation using flow cytometry, despite the fact that the size difference between the predator and their prey is reflected in the difference of forward-scattered (FSC) light signals in correlation with distinctive side-scattered (SSC) light signals, making it possible to distinguish *Bdellovibrio* from prey cells.

Direct cell count measurement using microscopy can also be considered a conventional method for measuring *Bdellovibrio* but is not included in this study. We believe PFU and qPCR in particular are sufficient as reliable and accurate measures of cell quantification to validate FCM. Plaque forming units are considered the standard method for determining *Bdellovibrio* viable count (Lambert and Sockett, 2008) and qPCR has also been shown to be the gold standard in measuring bacterial total count especially when considering mixed cultures found in environmental samples (Venieri et al., 2012), (Kirakodu et al., 2008). Previous data has shown that direct cell counts and measurements by PFU are comparable for quantification of *Bdellovibrio* (Williams et al., 2019) but unlike PFU and qPCR, direct cell count measurements require a higher concentration per

sample to measure accurately without the use of sophisticated hardware and software that may not available in many labs (Broadaway et al., 2003). This makes the method less desirable and labour intensive in many microbial ecology studies such as growth time lapses.

This study aimed to determine whether flow cytometry could serve as a method for quantifying *Bdellovibrio* count as an alternative to the methods of optical density, the plaque forming unit assay and qPCR. Filtered *Bdellovibrio* cell samples in a range of concentrations were prepared on different days and quantified by the four methods. The cell counts for flow cytometry were then compared to the other methods using a linear regression model and the Pearson's correlation coefficient (ρ). Additionally, the study aimed to show that flow cytometry could accurately count and distinguish mixed populations of *Bdellovibrio* and their prey *Pseudomonas*.

3.2 Methods

3.2.1 Bacterial strains and growth conditions

The type strain *Bdellovibrio bacteriovorus* strain HD100 (DSM no. 50701) was used throughout this study and was grown by predation on *Pseudomonas sp*. (DSM no. 50906) using standard culturing methods (Herencias et al., 2017; Martínez et al., 2016). *Pseudomonas* cells were grown in LB broth at 30°C with shaking (150rpm) for 16 hours and resuspended in supplemented Ca/Mg-HEPES buffer (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, pH 7.6) to an optical density (600nm) value of 10. This prey suspension was stored at 4°C for later use. 50µl of *B. bacteriovorus* from a glycerol stock stored at -80°C was added to 1 ml of the prey suspension in 10ml dilute nutrient broth at 30°C with shaking (200rpm) for 24 hours and then sub-cultured twice at 24-hour intervals by transferring 200µl of the culture and 1ml of the prey suspension to 10ml Ca/Mg-HEPES buffer (predator: prey ratio 1:10).

In total seven replicate cultures were prepared for use in the quantification of *B. bacteriovorus* in pure culture. For the validation of the FCM gating system, an eighth culture was also grown as described. Additionally, a pure *Pseudomonas* culture was grown in LB for 16 hours.

3.2.2 Preparation of predator filtrate samples for enumeration and comparison

Seven replicate cultures were grown as described then prepared for enumeration. To remove the prey and harvest the predator alone each culture was filtered twice through a 0.45µm pore size syringe filter (Fisherbrand™). An aliquot of each filtrate was then diluted by 10^{-3} times to produce two *Bdellovibrio* samples from each culture; the original at a 'high' concentration (~10^7 to 10^9 cells/ml) and the dilution of 'low' concentration (~10^4 to 10^6 cells/ml)· Through this, the concentration range of the prepared samples was estimated to be from 10^4 to 10^9 cells/ml. The average cell counts of predator filtrate samples were then obtained from triplicate measurements using flow cytometry and compared with three quantification methods: Optical density (OD), qPCR and the plaque forming unit (PFU) assay. An aliquot of each filtrate is plated on solid LB agar plate to confirm the removal of prey.

3.2.3 Flow cytometry

Total cell count measurements of each of the high and low concentration predator filtrate samples were performed using a Bd Accuri C6 plus flow cytometer. Samples were initially fixed 1:1 v/v with glutaraldehyde (1% in DI water) stored in the dark at 4°C and analysed within 1 hour. Prior to staining, where necessary, samples were diluted to achieve an events per second reading of less than 600 on the flow cytometer. Dilutions were made in filtered (0.22 µm Sartorius™ Minisart™ Plus Syringe Filters, Fisher scientific) DI water. The samples were each stained with 10 µL/ml of SYBR Green I (10,000 x in DMSO, Thermofisher) previously diluted 1:100 in Ethylenediaminetetraacetic acid (1 mM, Sigma-Aldrich) and incubated in the dark at 37°C for 13 min before measurement(Hammes et al., 2008).

Gating was used to distinguish selected signals (*B. bacteriovorus* and *Pseudomonas* cells) from each other and from the background (inorganic and organic particles) using a dot plot of forward scatter (FSC-A) vs green fluorescence (FITC-A). This was achieved with the aid of negative controls consisting of the DI water used for dilutions, HEPES buffer used for growth, and a sample of the predator/prey co-culture further filtered (0.22 µm) to remove any bacterial cells. We found that the limit of detection for this flow cytometry protocol to be 10^4 cells/ml due to the background and instrument noise. However, this could be improved to 10^3 cells/ml by increasing the volume of sample measured.

3.2.4 Optical density

To measure the OD of each of the high and low concentration predator filtrate samples, 1ml of each sample was aliquoted in polystyrene semi micro cuvettes (Fisherbrand™) for measurement and each sample was measured in triplicate by optical density (600nm) using the Hach DR 2800 Portable Spectrophotometer. We found the limit of detection for optical density to be 10^7 cells/ml for measuring *Bdellovibrio*. Due to the lack of sensitivity with the spectrophotometer, optical density was only used to measure samples from 10^7 to 10^9 cells/ml as quantified by flow cytometry.

3.2.5 Plaque forming unit assay

The plates used for the PFU assay comprised two layers. The bottom layers (dilute nutrient broth, 1.5% (weight/volume) agar, supplemented with 2 mM calcium chloride and 3mM magnesium chloride) of the double layer agar plates were prepared in advance. To plate, an aliquot of each of the high and low concentration predator filtrate samples was prepared in a range of ten-fold dilutions. Triplicates of each dilution were then mixed carefully with 500µl of a *Pseudomonas* suspension (OD=10) and dilute nutrient broth, 0.7% (weight/volume) agar supplemented with 2 mM calcium chloride and 3mM magnesium chloride. This mixture was then poured over the base of the double layer agar to form the top

layer. Once dry, the plates were incubated at 30°C for 3-4 days and the number of formed plaques then counted. The PFU/ml is read from a plate with 30 to 300 plaques and the original sample concentration calculated from the volume plated and the dilution used as shown in the equation below.

PFU/ml = number of plaques/ (dilution factor * volume (ml) plated) (3.1)

The typical limit of detection for the plauque forming unit assay was 10^3 cells/ml.

3.2.6 Standard curve preparation for qPCR

qPCR was performed for each of the high and low concentration predator filtrate samples of *B. bacteriovorus* to enumerate the total copy number of the 16S rRNA gene (492 bp conserved locus specific to the *Bdellovibrionaceae* family) based on a previously developed protocol (Van Essche et al., 2009).

The total 16S rRNA gene copy number of a sample was determined by comparison with a standard curve of known concentrations of a plasmid containing the 16S rRNA gene. The number of cells was inferred from the value of total copy number based on reports of *Bdellovibrio* having an approximate copy number of 2 16S rRNA genes per cell (Ruby, 1992).

To amplify a fragment of 492 bp of the *B. bacteriovorus* HD100 16S rRNA gene to use as a standard for qPCR, PCR was performed on a pure *Bdellovibrio* culture using a protocol previously developed (Van Essche et al., 2009). The PCR reaction contained 12.5 µL of PCR mastermix (Lambda Biotech), 1 µL of each primer ((10 µm); BbSF216: 5′-TTTCGCTCTAAGATGAGTCCGCGT-3′ and BbSF707: 5′-TTCGCCTCCGGTATTCCTGTTGAT-3′) previously designed (Davidov et al., 2006), 2 µL of DNA and 8.5 µL of PCR grade water. Positive (*Bdellovibrio* DNA) and negative (no DNA) controls were included.

The PCR was performed with a GeneTouch thermal cycler (Bioer) using a thermal profile of: denaturation and enzyme activation at 95 °C, 2 min, followed by 36

cycles of 95 °C for 30 s, annealing at 51 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. PCR products were verified by gel electrophoresis (1% agarose gel in 0.5% Tris acetate-EDTA (TAE) buffer stained with 10 µL of SYBR safe DNA gel stain (Thermofisher) ; 110 V for 1 hour) and the gel was digitized with a Gel Doc™ XR+ imager (Bio-rad).

The standard fragment was then cloned with the pGEM®-T easy plasmid vector system (Promega). The DNA was quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermofisher) and a 10-fold dilution series for the standard curve was prepared from 9 to 9 × 10^6 plasmid copies per reaction using a calculation previously described (Van Essche et al., 2009). qPCR for the standard curve was always performed in combination with the qPCR assays for the *Bdellovibrio* samples.

3.2.7 qPCR

After preparation of the high and low concentration predator filtrate samples aliquots of each were first frozen at -80°C to ensure complete lysis of the cells. As in Van Essche *et al*, the qPCR assay used primers Bd347F (5' - GGAGGCAGCAGTAGGGAATA- 3') and Bd549R (5'- GCTAGGATCCCTCGTCTTACC- 3') and a TaqMan nucleic acid staining probe, Bd396P (5'- TTCATCACTCACGCGGCGTC- 3'), which is labelled with 5' FAM (6-carboxyfluorescein) and 3'TAMRA (6-carboxytetramethylrhodamine) (Van Essche et al., 2009). The thermal profile was also as stated previously with the data collected during the annealing and extension steps (Van Essche et al., 2009). Reactions were run in a C1000™ thermal cycler (Bio-rad) and included 12.5 µL of Jumpstart Taq Readymix for High Throughput Quantitative PCR (Sigma-Aldrich), 2 µL of each primer (10 µM), 1.25 µL of the TaqMan probe (1 µM), 5 µL of DNA and 2.25 µL PCR grade water as previously described (Kandel et al., 2014). The reaction efficiency was calculated using the slope of the standard curve to be on average 104.34% with an R^2 value of 0.99. We found the limit of detection to be approximately 10^3 cells/ml.

3.2.8 Validation of FCM gating system

To confirm that the FCM gating system developed was accurate in distinguishing between *Bdellovibrio* and their larger prey, *Pseudomonas,* a sample each of pure *Bdellovibrio* filtrate and *Pseudomonas* were prepared separately to ~10^9 cells/ml as above. Aliquots of each sample were prepared and then diluted from high (10^8 - 10^9 cells/ml) to medium (10^6 -10^7 cells/ml) and to low (10^4 -10^5 cells/ml) concentrations and measured in triplicate using FCM as described above. Following this, aliquots of the prey and predator with known concentrations were mixed together in different ratios and again measured in triplicate using FCM. The preparations were as follows: high predator: high prey (HH), high predator: low prey (HL), medium predator: medium prey (MM), low predator: high prey (LH) and low predator: low prey (LL).

3.2.9 Statistical analysis

The statistical analysis was performed using R software. Pearson's correlation coefficient (ρ) was calculated to find the relationship between cell quantification by FCM and the other methods. Linear regression models were used to find the significant differences in measurement between FCM and the other methods tested. Natural log transformations of FCM, PFU and qPCR values were used while the OD values were transformed using the BoxCox method (appendix Table 8.1). This was to allow the data to satisfy assumptions of normality and homoscedasticity. Note that when the gradient of the relationship between log-transformed variables is one it reflects a perfectly linear relationship between the raw, untransformed variables.

To validate the gating system, Kruskal Wallis (k-w) and Bonferroni tests were used to find the significant difference between sample preparations (mixed vs pure) of the same concentration.

3.3 Results and Discussion

To validate flow cytometry as a suitable alternative to other methods of cell quantification, specifically optical density, the PFU assay and qPCR, isolated samples of *Bdellovibrio* within a range of different concentrations (10^4 to 10^9 cells/ml) were quantified using each method. Linear regression and Pearson's correlation analysis between the FCM results and the results obtained from each of the conventional methods was determined.

3.3.1 Optical density

A linear relationship was observed between log transformed measurements of cell count using FCM and the Box-Cox transformed measurements of optical density (Fig 3.1A, correlation coefficient (ρ): 0.784. P-value<0.05). However, this translates to a non-linear relationship between the untransformed values. The low R^2 value (0.456) suggests that enumeration provided by FCM cannot repeatedly and reliably predict the values provided by OD. This is expected and highlights why optical density is not typically used to measure *Bdellovibrio* concentration.

The moderate correlation between the two methods could arise from differences in the material quantified and the means of detection of each measurement method. OD is not a direct measure of cell numbers, rather it is an indication of the total biomass concentration in a sample including intact cells, damaged cells and debris. Such debris however can contribute to the overall turbidity read by a spectrophotometer (Griffiths et al., 2011) thus resulting in an overestimation of cell count. By contrast, damaged cells and debris may be omitted from quantification using FCM by use of targeted gating and selective staining as applied here.

Further, OD value only shows a linear correlation with biomass concentration at lower concentrations, the cut off for which will differ depending on the spectrophotometer used and the path length of the cuvette used (Sutton, 2011). The small size of *Bdellovibrio* also contributes to how difficult it is to accurately

measure cell concentration (Lambert and Sockett, 2008). Whilst so, quantification using OD provides near-instant estimation of cell numbers with minimal sample preparation when measuring high concentrations which is advantageous over the other methods applied here. One observation from this study is that the speed of OD as a measurement method and its moderate correlation between cell number as determined by FCM, made optical density useful for estimating the required dilution necessary for a sample to be quantified by FCM and PFU assays.

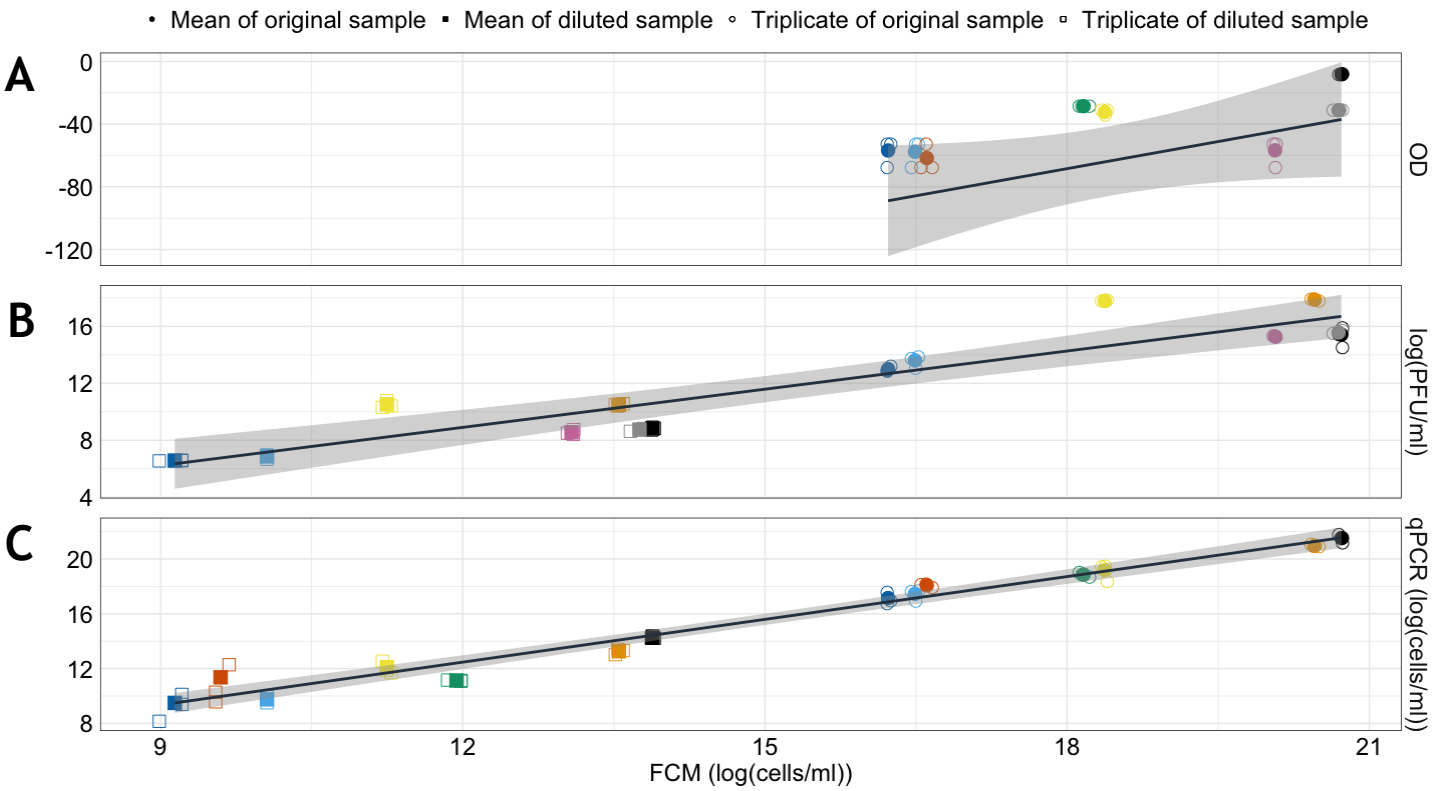

Figure 3.1- Liner regression plot with confidence intervals (grey) to compare quantification when using flow cytometry (natural log (cells/ml) with other methods. A) Optical density (Box-Cox transformation, lambda=-0.6). Each bold point represents the mean of three observations. Pearson's correlation coefficient: 0.784. P-value<0.05. Box-Cox transformed OD=-192.967 + natural log (FCM)8.339. R2= 0.456. **B)** Plaque forming unit method (natural log (PFU/ml)). Each bold point represents the mean of three observations. Pearson's correlation coefficient: 0.923. P-value<0.0001. Natural log (PFU)= -1.821 + natural log (FCM)0.894. R2= 0.84. **C)** qPCR (natural log(cells/ml)). Each bold point represents the mean of three observations. Pearson's correlation coefficient: 0.987. P-value<0.0001. Natural log(qPCR)=-0.023 + natural log (FCM)1.041. R2=0.972.

3.3.2 Plaque forming unit assay

A highly linear relationship was determined between the log transformed relative cell numbers quantified by FCM and the PFU assay (Fig 3.1B, correlation coefficient (ρ): 0.923. P-value<0.0001), and the high R^2 value (0.84) suggests that results provided by FCM are repeatable and reliable as an alternative to PFU for the

assessment of relative cell numbers and that the relationship can be used to convert between measurements. Any increase in the cell numbers determined by FCM corresponds to smaller increase in cells given by PFU method (as determined by the slope: 0.894). Differences in the count between the two methods are expected as the FCM protocol employed enumerates the total cells whereas the PFU method can only quantify the viable cells (Chauhan and Williams, 2006).

Cells present in a sample that are dead or are unable to replicate for whatever reason, will not form plaques, and thus will not be enumerated using the PFU method, by contrast, they will be enumerated using FCM. Non-viable cells may still be important to quantify when investigating *Bdellovibrio* as they could affect the living cell's ability to find prey when at high densities, which may play some role in maintaining predator and prey growth cycles. Additionally, were the method to be applied to quantification of prey cells to map predator/prey dynamics, it has been shown that non-viable but intact *E.coli* cells can still act suitable hosts for *Bdellovibrio* growth (Hespell, 1978).

Further discrepancy could occur because the cell count obtained from plating techniques often result in an underestimation because plaques can be formed by multiple cells originating close to each other, despite efforts to reduce this by sufficient diluting and spreading of the sample (Chauhan and Williams, 2006). Furthermore, whilst FCM may register false positives, cells counted using PFU are less likely to be false positives as plaques can only be achieved by the initial presence and sufficient replication of a lytic predator cell. By contrast, an observed count in flow cytometry is not necessarily specific to a *Bdellovibrio* predator cell and can possibly be achieved in a number of different outcomes such as bacterial cells of similar size, or cell debris from prey cultures that may pass the filter during predator-prey separation. This study measured pure cultures of *Bdellovibrio* that were filtered from *Pseudomonas* that are larger in size, therefore, in this instance, flow cytometry is likely to show a high specificity. This also remain true for future studies involving predatory bacteria and a gram-negative host species such as *E. coli*. Thus, FCM could allow the accurate enumeration of *Bdellovibrio* cells after long term incubation with prey cells

without the need of staining the cells prior to co-culturing which would limit the time of study (Garcia et al., 2018).

Were enumeration of living cells a priority for FCM, as opposed to total cell numbers as enumerated here, this could be achieved by using a live cell count assay which makes use of both the SYBR I and PI dyes instead of a total cell count assay for FCM (Vignola et al., 2018). It is anticipated that this could provide a better comparison to the PFU assay as this method is able to distinguish live and intact cells from those that have damaged membranes and would not be able to replicate and subsequently produce a plaque in the PFU method (Evans et al., 2012). Even so, it is important to note that even FCM estimates of live cell abundances are likely to be higher than the counts given by the PFU method, as PFU methodology is more vulnerable to several biases including human error, plaques originating from multiple cells that are close together or the influence of culture environment for the double layer agar plates which could result in the insufficient cultivation of bacteria (Chauhan and Williams, 2006).

3.3.3 qPCR

qPCR was used to measure the copy number of the *Bdellovibrio* 16SrRNA gene in a sample. This value was used to calculate the total cell count in a sample, based on reports of the copy number of the 16sRNA gene being two per single cell (Van Essche et al., 2009).

There was a significant correlation between the relative cell numbers quantified by FCM and qPCR (Fig 3.1C) (correlation coefficient (ρ): 0.987. P-value<0.0001, R^2 0.972), with qPCR proving to be a better suited comparison to FCM than PFU. This is expected because unlike the PFU assay, qPCR and are FCM are both measures of the total cell number, so it is expected to be a better suited comparison (Lee et al., 2006). The absolute cell numbers estimated by FCM have an almost perfect linear relationship with those given by qPCR method (as determined by the slope: 1.041), however the difference was negligible. As such, the data suggests FCM is an excellent method for quantification, enabling accurate and rapid quantification.

3.3.4 Validation of FCM gating system

Whether measured alone or mixed with different concentrations of *Bdellovibrio*, the flow cytometry gating system (Fig 3.2) was able to accurately quantify the *Pseudomonas* population.

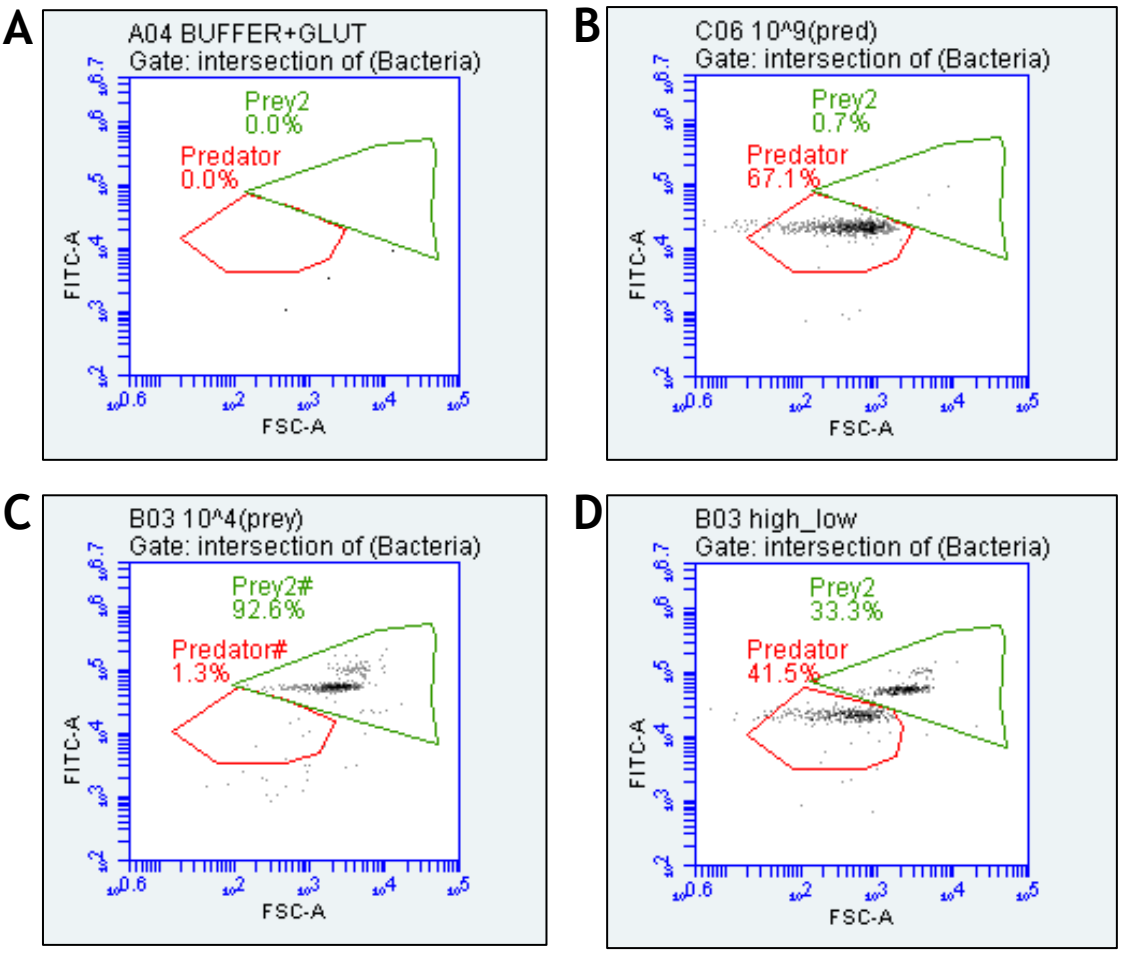

Figure 3.2- Dot plot of forward scatter (FSC-A) vs green fluorescence (FITC-A) used for flow cytometry gating. A) Growth buffer and glutaraldehyde **B)** *Bdellovibrio predator* alone **C)** *Pseudomonas* prey alone **D)** *Pseudomonas* prey and *B. bacteriovorus* predator species mixed.

As determined with the Kruskal Wallis test, no significant difference was found between FCM measurements of *Pseudomonas* samples of different mix ratios (Fig 3.3, k-w test: p-value>0.05). Similar results were found with the *Bdellovibrio* samples, in that the FCM gating was able to accurately distinguish and count *Bdellovibrio* in lone or mixed samples. One discrepancy observed was that there

was found to be a statistical difference between the predator samples of high concentration (k-w test: p-value: 0.048). The Bonferroni test was applied to reveal that the difference in *Bdellovibrio* populations was specifically between the "high: high" vs the "high: low" predator: prey populations (p-value: 0.048) whereas there was no significant difference in these samples when measured against the lone high predator sample (Bonferroni test, respective p-values: 1 and 0.295).

Although statistically different, both *Bdellovibrio* populations were measured to be the same order of magnitude (10^9 cells/ml) and in practicality the difference between the two is considered to be low, with the population in the "high: high" sample being a 20% increase from the "high: low" sample. Additionally, the choice of threshold value for significance, ($P < 0.05$) is largely subjective and a value of 0.01 could have also been chosen to make the test of significance more strict (Dahiru, 2008). Thus, with the other results, there is still confidence in FCM as a useful and accurate tool for quantifying species in a mixed culture using gating to identify each species, provided that the species are of distinct size.

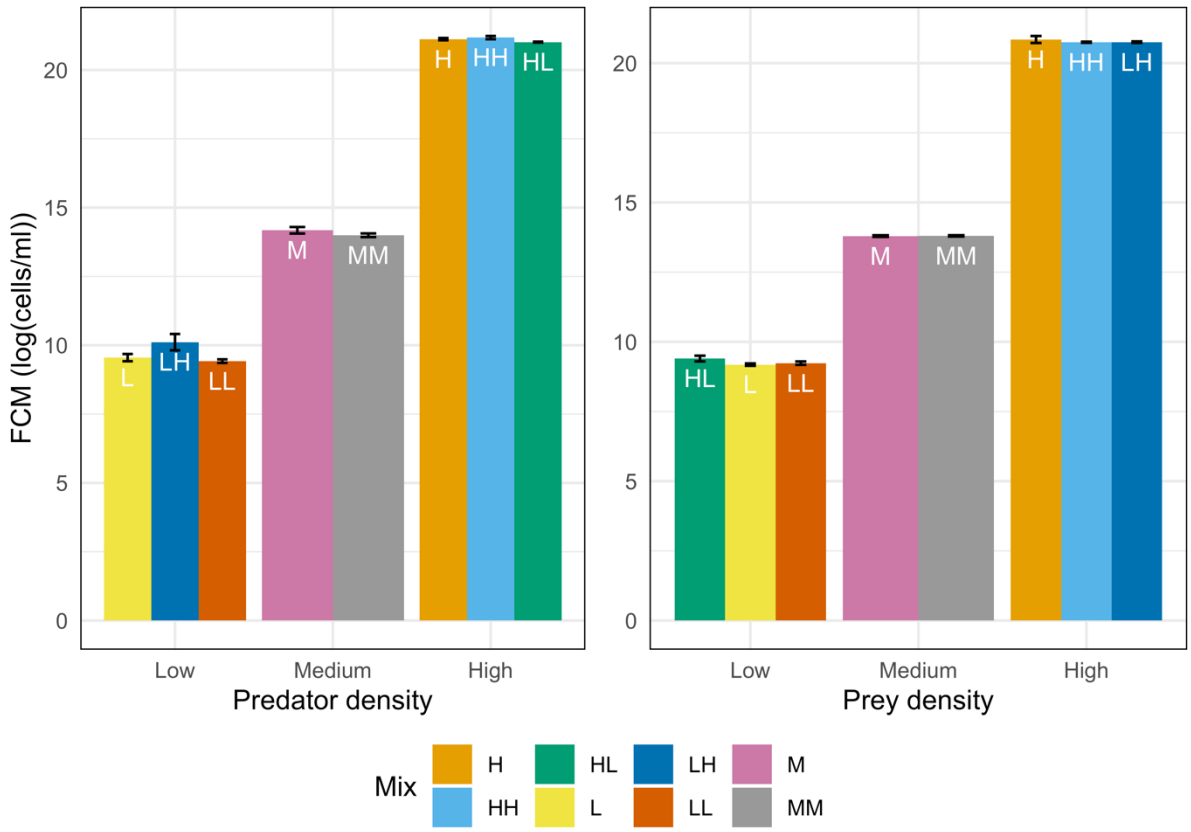

Figure 3.3- FCM count (log(cells/ml) of the A) *Bdellovibrio* predator and B) the *Pseudomonas* prey at different densities (low, medium and high) and different mix conditions. (L) monoculture of low density, (M) monoculture of medium density, (H) monoculture of high density, (LL) low predator: low prey densities, (LH) low predator: high prey densities, (HL) high predator: low prey densities, (HH) high predator: high prey densities and (MM) medium predator: medium prey densities.

3.4 General discussion

FCM was demonstrated as an effective and rapid tool for enumerating both pure and predator-prey co-cultures. Thus, it offers significant benefit over the conventional methods against which it was compared. Neither PFU nor qPCR can enumerate mixed cultures in a single assay. Whilst OD can crudely enumerate total cell count in a mixed sample, OD measures can inaccurately enumerate cell concentration in a sample containing cells of varied size because larger cells are able to absorb and scatter more light (Griffiths et al., 2011). Thus, application of optical density as a means of cell quantification is limited to mono-culture samples

as it cannot distinguish between different cells. Inaccurate results are produced when monitoring growth of cultures in which multiple cell morphologies are exhibited such as in a typical *Bdellovibrio*-prey co-culture (Vojinović et al., 2006). Not only would the small size of *Bdellovibrio* contribute little to the differing light scattering properties of a sample that also contained much larger prey cells such as *Pseudomonas*, but the included presence of the swollen bdelloplasts would also increase the inaccuracy of the reading (Koval, 2006).

The direct application of FCM to the co-culture of interest here was underpinned by the difference in the mean size of the two species in interest (*B. bacteriovorus*: 1.2 x 0.4 µm (Iebba et al., 2014), *Pseudomonas*: 1-3 x 0.5 – 0.7 µm (Rhodes, 1959)), which in turn enabled a simple gating system to accurately quantify each species in a mixed sample. Gating systems such as this cannot always be used alone, even when investigating two populations of different size as the signal detected is a complex function of a number of different parameters such as particle orientation and cellular content (Wittrup et al., 1988). Thankfully bacterial populations even in pure cultures display different levels of heterogeneity (Müller and Nebe-von-Caron, 2010) and with advancement in flow cytometry there are a number of options to detect this (Comas and Vives-Rego, 1998).

Flow cytometry has been shown to distinguish predatory bacteria and their prey by using fluorescent protein expressing cells as an alternative to dyes for quantification was demonstrated by a previous study which incubated tdTomato-expressing *B. bacterivorous* with different mutants of GFP-expressing V. *cholerae* to investigate predator attachment (Duncan et al., 2018). The study used FCM 30 minutes and 1 hour after starting the predator/prey co-culture to find that there was more attachment (displayed by green and red double positive events) in the coculture of prey mutants that could not produce the O-antigen, suggesting that they were more likely to be attacked.

Distinguishing and quantifying the predator and prey in this manner could prove more specific as the gating strategy can easier pick out several differing

fluorescents than when it is determined largely by the forward scatter as is done in the current study. However, staining the cells after incubation in this manner would prevent the influence of long-term incubation on the photostability of the fluorescent protein which could reduce the accuracy of quantification (Mamontova et al., 2015).

Another alternative would be to quantify bacteria using a 16SrRNA targeting fluorescent probe to combine flow cytometry with fluorescence in situ hybridization (FISH)(Clarke and Pinder, 1998; Zoetendal et al., 2002). Though there are no current studies that have used flow-FISH to quantify *Bdellovibrio*, despite the fact that the technique has long been used to quantify a number of different bacteria in mixed populations (Wallner et al., 1993). Additionally, FISH has previously been successful in the identification of *Bdellovibrio* cultured on *E. coli* and in environmental samples following enrichment (Mahmoud et al., 2007). In flow-FISH, the probe used to stain the samples is similar to that used in qPCR, making it a more specific and sensitive method of quantification when using environmental samples that contain a number of different bacteria and typically have lower concentrations of *Bdellovibrio* (Koval, 2006).

In the case reported here however, FCM's ability to distinguish and count mixed predator: prey populations at the same time makes it ideal to study the effect of predator: prey density on predation. This represents a key factor for the application of *Bdellovibrio* as a biocontrol and is one that is yet to be investigated using flow cytometry. Several past studies have aimed to characterise the effect of predator: prey density, however they have all used conventional techniques that may be limited (Im et al., 2014). In addition, there still remains debate over whether predation is favoured in high or low predator: prey densities (Yair et al., 2003). Investigating this using new online flow cytometry technology which uses automation could be more accurate and easier to perform high-throughput which would allow monitoring of growth continuously and for a longer duration (days to weeks) and in more detail so that a more complex model of predator-prey dynamics could be built (Hol et al., 2016b) (Besmer et al., 2014).

In conclusion, the tests performed show that flow cytometry represents a rapid and accurate alternative method for distinguishing and measuring *Bdellovibrio* and *Pseudomonas* cell counts as compared to the methods of optical density, the plaque forming unit assay and qPCR. When compared to optical density, a fast but largely inaccurate method, flow cytometry shows a non-linear relationship. When compared to plaque forming unit, which is the most common method for *Bdellovibrio* enumeration, flow cytometry shows a non-linear relationship with the raw variables but a strong linear relationship when both variables are log transformed. In this case, the cell number measured using FCM overestimate those in plaque forming unit, which is to be expected as PFU/ml is a viable cell count only. When compared to qPCR, which is seen as the gold standard for measuring 16srRNA as a proxy for cell count, FCM shows a very strong linear relationship. The population measured using FCM slightly underestimate those from qPCR which was expected as free DNA was not present in cells that can be detected by qPCR but not by the FCM gating system used. As it has been validated as a suitable method, FCM can be used in further studies to measure *Bdellovibrio*, specifically in investigating the effect of density on predation and prey survival. Through this, further predator/prey population models can be constructed to aid their application in biofilm control.

4 Bdellovibrio predator - prey modelling

Biofilms cause biofouling in membrane filters which can reduce their permeability. This often means that transmembrane pressures have to be increased to maintain the desired flow rate through the membrane, which increases the energy demand and can, ultimately, render the technologies too costly in comparison to others (Chen et al., 2010). *Bdellovibrio* represent a biological alternative to chemical and antibiotic removal of biofilms in different fields (Dwidar et al., 2012a; Klein and Casida Jr., 1967). Application of *Bdellovibrio* in such a manner requires a deep understanding of its predator dynamics, which is vital for predictions of the different possible outcomes from any system designed to combat biofouling.

The complexity of the interactions between different species and with their environment in real biological systems makes it difficult to predict the population dynamics with certainty. However, it is often possible to distil the most important factors into a mathematical model that can capture phenomena. This is important in validating our conceptual models of the system (Li et al., 2007). It is also important in engineering design because in the absence of model predictions, it would be necessary to explore the range of possible system behaviours entirely through experimentation on a trial-and-error basis, which would be costly and fraught with practical difficulties. A mathematical model allows the design to progress through a rational exploration of the systems behaviour under a broad range of operating conditions, quickly and with little cost. Thus, optimal strategies and operating conditions can be explored in silico. The simplifications that are implicit in any mathematical representation of the biological process mean that there will be a degree of uncertainty in the model predictions and, therefore, it may well be necessary to validate the predictions empirically. However, the modelling serves as a useful guide to significantly constrain the amount of experimentation required.

Models are also particularly useful tools for understanding complex systems as an alternative to experiments because they can allow for precise control of a number

of parameters that would otherwise be difficult and expensive to conduct experimentally.

Lotka (1920) and then Volterra (1925) derived the same set of equations to describe predator-prey interactions in two different systems: plant-herbivore and fish predation respectively. Despite the fact that organisms are discrete and so strictly, population number ought to be an integer variable, they assumed that populations size could be considered a real variable and they used two coupled ordinary differential equations to describe the rate of change of predator and prey densities. Their model necessarily employed a number of simplifying assumptions:

- The prey population finds ample food at all times.
- The food supply of the predator population depends entirely on the size of the prey population.
- The rate of change of population is proportional to its size.
- During the process, the environment does not change in favour of one species, and genetic adaptation is inconsequential.
- Predators have limitless appetite.

Whilst these assumptions are clearly an oversimplification of the biological reality, the solution of Lotka Volterra equations has been shown to reproduce phenomena that are observed in times series of predator and prey populations growth (Varon and Zeigler, 1978). In particular, the oscillatory behaviour where the prey species grows, causing an increase in the predators which in turn kill the prey causing the prey population to decrease. With their food source low, the predators starve, and their population decreases which allows the prey species to regrow and continue the cycle. Whilst the phenomena are one of the most interesting results from the equations, oscillatory predator prey dynamics have rarely been observed in the real world.

The classical Lotka Volterra equations are given by,

$$\frac{dX}{dt} = k_1 X - k_2 XY \ , \qquad (4.1)$$

$$\frac{dY}{dt} = k_3 YX - k_4 Y \ , \qquad (4.2)$$

where X is the prey population size (cells/ml), Y is the predator population size (cells/ml), t is time (h) and k_1, k_2, k_3 and k_4 are postive constants.

Equation 4.1 represents the rate of change of the prey population. The first term describes the growth of the prey with k1 describing the prey maximum growth rate (h^{-1}). In the absence of any other terms $\frac{dX}{dt} = k_1 X$ has a solution $X = X_0 e^{k_1 t}$ (Begot et al., 1996) and so it represents exponential growth. Exponential growth is also referred to as the Malthusian growth model, first constructed by Malthus in 1798 to describe population growth as exponential under optimal conditions (Malthus, 2007). The intrinsic growth of the population is not constrained in any way, for example, by lack of food and so if there were no other terms in equation 4.1 then the population would rapidly blow-up to enormous numbers. Most naturally occurring bacteria live in oligotrophic environments or are subject to feast and famine cycles of substates and so this assumption is severely limiting for real microbial communities.

The second term in equation 4.1 describes the rate by which the prey population declines as a result of predation. The assumption here is that the rate of decline is proportional to the number of predators and the number of prey. k_2 is the death rate of prey for each predator attack. Inherent in this relationship is that a predator is equally likely to find and consume a prey no matter what the prey density is.

Equation 4.2 represents the rate of change of the predator population. The first term in equation 4.2, describes the growth rate of the predator. k_3 represents the reproduction rate of predators per 1 prey and, similar to the rate of predation $k_2 XY$, it is determined by the abundance of interactions between the prey X and predator Y. k_4 represents the intrinsic death rate of the predator (h^{-1}).

The application of the model was further developed by Lotka and Volterra with the addition of the assumption that the growth (or death) of the populations would be proportional to the product of their biomass densities as according the mass equation principle (Cohen J. E. et al., 1990). Though this does not yield a change in the equations, it allows for scientists to use measurements of cell biomass instead of cell numbers using methods such as measuring the wet or dry weight. Optical density measurements are also often obtained as they can be correlated well to either the biomass or the number of cells per volume (Sutton, 2011). This was especially vital at the time as these were the most common and reliable methods available.

One of the earliest *Bdellovibrio* predator models used the Lotka-Volterra equation to describe the interaction between *Bdellovibrio* and different density populations of photobacterium prey (Varon and Zeigler, 1978). The study measured the light intensity decrease of the prey as a result of predation by different predator densities and used it to determine the predation coefficient k2 and from the Lotka- Volterra model estimated equilibrium populations at which the rates of change were equal to zero.

The study measured prey suspensions of 'low' density (10^3-10^5 cells/ml) in an effort to model prey and predator populations seen in nature, which was in contrast to higher density cultures that were favoured previously by similar experimental studies (Varon and Zeigler, 1978). The use of high-density cultures at the time was favoured because optical density measurements are not sensitive enough at low concentrations. It was, however, widely preferred due to its speed and lower technical requirements than methods such as flow cytometry (Begot et al., 1996). Regardless higher concentrations are not always characteristic of natural habitats and importantly not characteristic of the concentration (10^4 cells/ml) often found after ultrafiltration of surface water in drinking water treatment (Buysschaert et al., 2018).

They used prey that lost its ability to fluoresce upon penetration by the predator and demonstrated that the rate of light decay of the prey increased with the predator/prey ratio. Additionally, they showed that under the measured conditions (10^3cells-10^5cells/ml) , the rate of light decay is increased with increasing predator density or decreasing prey density (Varon and Zeigler, 1978). From the data (using Lineweaver burke solution) they estimated that the predation coefficient to be 3.0 x 10^{10} per min. They concluded that if the *Bdellovibrio* predators display no chemotaxis towards prey cells and successful encounters occurred by chance, then the predation coefficient would be equal to the rate of encounter multiplied by the probability of penetration for each predator/prey encounter. Thus, the probability of penetration for each encounter was estimated (3%) using the predation coefficient and the rate of encounter (1.0 x10^8 per min) which was taken from a previous study (Hespell et al., 1974).

The Varon and Zeigler study represents an example of investigating predator/prey dynamics by turning original and previous experimental data into a set of useful parameters to insert into a simple mathematical model, as well as demonstrating how the model can be improved on using new information (Varon and Zeigler, 1978). Despite this, there are several ways in which it can be further improved upon. For instance, the Lotka-Volterra model assumes exponential growth of prey bacteria with unlimited access to nutrients and that any decrease to the growth result is only a result of predation. Though this may be true for a time, long term growth of bacterial populations in batch cultures and in nature will be restricted by competition for resources. Typically growth in batch culture is characterised by four phases: (1) lag phase, (2) logarithmic or exponential growth phase, (3) stationary phase, and (4) death phase (Liu, 2017).

An alternative for modelling prey growth is to use logistic growth (also shown in the Verhulst model) (Verhulst, 1838), which is able to model growth from the exponential to the stationary phase, where the population of the prey grows high enough that competition ensues and resources in the system begin to deplete and waste products build up resulting in the eventual deceleration and then cessation of growth as seen in the stationary phase (Vadasz et al., 2001).

Logistic growth of the population is thus given by,

$$\frac{dX}{dt} = k_1 \frac{(k-X)}{k} X \quad . \qquad (4.3)$$

The maximum population that a particular environment can sustain is expressed as the carrying capacity k (cells/ml), a finite parameter to quantify the upper limit of bacterial growth imposed by the limited resources. The prey intrinsic growth rate value k_1 (h^{-1}) is also used here and is important because a higher growth rate will result in the carrying capacity being reached faster. Furthermore, when the population is far below the carrying capacity, the density dependent effect becomes almost negligible and the population change is most affected by the intrinsic growth rate (Wilkinson, 2006).

In ecology, logistic growth models have been to model population growth in food, fisheries and agriculture and provide a guide for culling to prevent disease or over-grazing and allow for successful regrowth. One study used a modified Lotka-Volterra predator prey model using logistic growth to investigate the effect of predator: prey ratio on population growth (Im et al., 2014). Like Varon and Ziegler (Varon and Zeigler, 1978), they showed that higher initial predator: prey ratios resulted in faster decline in prey population soon after inoculation (>1 hour). They also used the model to validate experiments that suggested high predator: prey ratios resulted in premature lysis of the prey. However, growth was only monitored for 1 hour, so it is unclear whether this trend continues for multiple life cycles of the *Bdellovibrio* predation.

Additionally, logistic growth models only describe population growth and not substrate consumption. Like the Lotka-Volterra model it assumes that there is an unlimited and optimum nutrient availability to the prey whereas in nature and in membrane systems, this is not always the case and nutrients such as carbon and nitrogen can be present in concentrations limiting to growth (Kampen, 2014). When all other nutrients are saturated and non-limiting, but one key nutrient is too low, the growth rate is proportional to the concentration of the limited nutrient. At optimal concentrations the growth rate will be a maximum value with

exponential growth displayed as it is in the Malthusian model(Bren et al., 2013). However, when the nutrient concentrations increase to too high a value it can inhibit growth causing the growth rate to fall (Johnson and Stokes, 1965).

This relationship between the growth rate of the bacteria and one limiting substrate has been often described by the Monod model (Monod, 1949),

$$\mu = \mu_X \frac{S}{K_S+S} \ , \tag{4.4}$$

where μ (h^{-1}) is the growth rate of a prey species (previously k_1), S is the growth limiting substrate concentration (mg/L), μ_X (h^{-1}) is the maximum specific growth rate of the prey and the K_S (mg/L) is the substrate saturation constant. The saturation constant is the concentration of growth rate-limiting nutrient that supports half the maximum specific growth rate. The Monod model is well suited for microbes in batch and in chemostats. Instead of using exponential or logistic kinetics, we can model the prey bacteria growth on a rate limiting substrate with Monod kinetics. Thus, in the absence of predators, equation 4.3 can be altered:

$$\frac{dS}{dt} = -\left(\frac{\mu_X S}{K_S+S}\right)\frac{X}{\zeta_S}, \tag{4.5}$$

$$\frac{dX}{dt} = \left(\frac{\mu_X S}{K_S+S}\right) X \ , \tag{4.6}$$

where ζ_S is the prey yield, which is the number of new prey cells produced per mass of substrate consumed and is assumed to be constant. Equation 4.5 represents the rate of substrate degradation. In a batch experiment, after the initial substrate has been added, it is assumed that additional substrate is neither added to the system nor produced and it is, therefore, only removed by prey bacteria activity, hence the minus sign. Mass balance principles are maintained so the growth rate is proportional to the prey concentration. The rate of change of the prey bacteria is represented in equation 4.6. It is expected that when the substrate concentration is high enough and saturating, the maximal growth rate is achieved and the growth rate of the prey is constant, as in logistic growth(Owens and Legan, 1987).

Monod kinetics are often preferred over logistic growth to model bacterial growth because the saturation constant (K_S) and the maximum specific growth rate of the prey (μ_X) values are linked more directly to experimental data. Whereas the carrying capacity (K) value in logistic growth, is more based in mathematics (Kargi, 2009).

Monod kinetics is perhaps also widespread because of its similarities to the Michaelis–Menten model, though there are some discrepancies, and these two models can be wrongly equated. The saturation constant in the Monod model is empirical, based on experimental data, and is an approximation of all of the processes that are responsible for bacterial growth on a substrate (Merkel et al., 1996). Whereas the saturation constant in the Michaelis–Menten model is only able to describe the direct relationship between a substrate and one specific reactions with a single enzyme species involved in the substrate utilisation (Robinson and Tiedje, 1983). Bacterial growth via even just a singular substrate is a complex process involving many different enzymatic reactions (Liu, 2017). So, it is less suited to bacterial growth models.

Another limit of the initial Lotka-Volterra equation 4.1, is in its ability to predict predator growth. Similarly to the k1 (prey birth rate) value, the predator grows exponentially where it should instead be subject to some kind of saturation constant that accounts for the time taken for predators to process the prey, over-and-above the time it takes to find the prey (Wilkinson, 2006). Of course, it is well known that once a predator invades the periplasmic space of the prey, the prey bacterium becomes non-viable but the predator enters the intracellular replicative portion of its life cycle (about 3-4 hours), during which time the predator cannot interact with other bacteria and progeny bacteria are waiting to be released (Sockett, 2009). This can be described by the Holling type II model, which uses Monod kinetics(Liu and Chen, 2003) and has been shown to be better suited to protist (DeLong and Vasseur, 2012) and *Bdellovibrio* modelling when used with Monod prey kinetics (Summers and Kreft, 2019).

The saturation constant used in Monod kinetics is versatile and has the ability to simplify processes that may involve more than one enzyme, which may make it suitable to describe the kinetics of *Bdellovibrio* obligate growth using the prey bacteria as the 'substrate'. Thus, when the prey concentration is saturated, the predator will grow at a maximum rate. Applying Monod kinetics results in the set of equations:

$$\frac{dS}{dt} = -\left(\frac{\mu_X S}{K_S+S}\right)\frac{x}{\zeta_S}, \tag{4.7}$$

$$\frac{dX}{dt} = \left(\frac{\mu_X S}{K_S+S}\right)X - \left(\frac{\mu_Y X}{K_X+X}\right)\frac{Y}{\zeta_X}, \tag{4.8}$$

$$\frac{dY}{dt} = \left(\frac{\mu_Y X}{K_X+X}\right)Y. \tag{4.9}$$

Here, equation 4.7 describes for limiting substrate utilisation and remains the same as in equation 4.5 but the rate of change of prey (equation 4.8) is now a function of predation. This action is determined by the predator growth rate under Monod kinetics and the predator yield (ζ_X) ,which is the number of new predator cells produced per prey consumed. The rate of change of the predator is included with Monod kinetics by taking into account the prey concentration (X) which in this case is the growth-limiting 'substrate', the maximum specific growth rate of the predator (μ_Y) and the prey saturation constant (K_X). The prey saturation constant is the concentration of prey that supports half the maximum specific growth rate of the predator. It is assumed that *Bdellovibrio* does not grow or directly interact with the substrate and, as above, the predator does not grow in the absence of prey (Lambert and Sockett, 2008). Of course, this model does not take into account the potential development of host-independence, which is rare occurrence under very particular conditions (Dashiff and Kadouri, 2009).

A predator utilises prey differently than prey utilises an organic substrate; at low prey concentrations, it can be expected that the predator's chance of encountering prey decreases. This phenomenon is better captured by the Holling type III functional response. The type III functional response is similar to type II in that at increasing levels of prey density, the rate of the predation increases until

saturation occurs. But at low enough levels of prey density, there is a lag that takes into account that the predator is less efficient in catching the prey.

Type III functional responses may arise from a variety of mechanisms and are typical of predators which show some kind of learning behaviour such as developing a more specialised way of killing prey. It is also used to describe predators responding to increased chemical stimuli from prey or in cases where a larger amount of energy is expended in finding the prey than is returned. (Real,1977).

This model has primary been used to describe vertebrate predators but there are examples of the model fitting microbial predation well. In chemostat, the Monod model is not sufficient for describing *Tetrahymena pyriformis* protozoa predation on *E. coli* (Jost et al., 1973). The Monod model predicted that when glucose substrate was kept at a saturating concentration then the population densities of the prey and predator would grow in typical sustained oscillations. However experimental data showed that the populations displayed damp oscillations with the potential to develop into steady states. This was better predicted by the Holling type III response because the rate of predation decreased when the prey population decreased significantly. In further studies, shifting the functional response increases the species richness and stability of more complex food (Rall et al., 2008).

The Holling type III functional response has not previously been shown in *Bdellovibrio*. However, many studies have described the random collision-based hunting method of *Bdellovibrio* (Jurkvitch, 2006. Wilkinson 2001). The lack of strong chemotaxis or specific searching for prey suggests that the encounter rate, the chance of a *Bdellovibrio* cell successfully colliding with a prey cell would be a major determinant in the overall predator attack rate and would shift the functional response from Holling type II to type III. Here, the searching efficiency and the chance of encountering a prey would increase with prey density. This could explain why *Bdellovibrio* is strongly associated with high bacteria environments such as biofilms and downstream sewage treatment plants (Staples

and Fry, 1973) (Summers and Kreft, 2019). This relationship can be displayed by modifying equations 4.7,4.8 and 4.9 to the following:

$$\frac{dS}{dt} = -\left(\frac{\mu_X S}{K_S+S}\right)\frac{X}{\zeta_S}, \tag{4.10}$$

$$\frac{dX}{dt} = \left(\frac{\mu_X S}{K_S+S}\right)X - \left(\frac{\mu_Y X^2}{K_X+X^2}\right)\frac{Y}{\zeta_X}, \tag{4.11}$$

$$\frac{dY}{dt} = \left(\frac{\mu_Y X^2}{K_X+X^2}\right)Y. \tag{4.12}$$

This equation involves squaring the value of X to take into account the incremental amount of prey needed to increase the efficiency of predation.

The main difficulty in determining the Monod and Holling parameters for prey microbial growth in batch study has been to accurately quantify the prey bacteria and the growth limiting substrate at the low concentrations where we expect to see the Monod kinetics displayed. Past research has often used indirect methods; estimating the substrate concentration based on start and end values (Bren et al., 2013). Additionally, the substrate concentration measured in these batch cultures are often higher than required for saturation which can still provide accurate values of the maximum growth of the bacteria but has led to inconsistent results for the saturation constant (Kovárová-Kovar and Egli, 1998).

The use of low substrate concentrations is preferred but requires more sensitive measurement and measurements over a shorter time period time to prevent dramatic nutrient change (Button, 1985). Use of low bacteria concentrations in cultures can help minimise nutrient flux and allows time for the glucose to be filtered from the bacteria and measured accurately to directly represent the substrate concentration present in the culture (Owens and Legan, 1987). Moreover, this brings about the problem of needing a sensitive method for quantifying prey growth.

The previous growth history of cells used is also important as it can determine the lag time in adaptation to the new environment with changes in nutrient concentration and the yield when growing on a substrate (Liu, 2017) (Graham and

Canale, 1982). A previous study (Law and Button, 1977) explored the relationship between a marine bacterium and glucose. They reported that when the bacteria were grown in minimal medium and glucose, which was additionally supplemented with amino acids, the saturation constant decreased, and the maximum growth rate increased compared to being grown without amino acids.

The saturation constant can be determined by monitoring bacterial growth under different growth rates. As expected, the complex nature of bacteria growth means that different saturation constant values for a substrate can be observed with the same bacteria species when they are exposed to different abiotic conditions. So, any value estimated for these parameters applies strictly within the observed experimental conditions. For example, changes in saturation constant and growth rate are observed with changes in media composition ,temperature and pH (Kovárová-Kovar and Egli, 1998) (Kovárová et al., 1996). Thus, it is ideal that as many of these conditions are kept constant as possible.

This can be achieved in a chemostat, a continuous culture that allows the control of fluid (bacteria or substrate) input into the system as determined by the concentration present in a reservoir system and the precise control of dilution rate (Senn et al., 1994). Furthermore, the saturation constant is more accurately determined in a chemostat because unlike bacteria growing in batch, oxygen and nutrients can be continuously provided and the resulting stable environment means that the cells are well adapted and exist in a steady state.

Steady state exists when bacteria have the same specific growth rate as the dilution rate, so that the bacteria concentration does not change. The bacteria in this state, are also more physiologically similar to each other, which lends to a higher experimental reproducibility (Höfle, 1983).

Bacterial growth in chemostats is commonly investigated in engineering, through the use of models or as an experimental system. In a chemostat the predator/prey/substrate system in equations 4.10,4.11 and 4.12 can now be described as:

$$\frac{dS}{dt} = \mathrm{D}(S_0 - \mathrm{S}) - \frac{1}{\zeta_S}\left(\frac{\mu_X S}{K_S+S}\right)X, \tag{4.13}$$

$$\frac{dX}{dt} = -\mathrm{DX} + \left(\frac{\mu_X S}{K_S+S}\right)X - \frac{1}{\zeta_X}\left(\frac{\mu_Y X^2}{K_X+X^2}\right)Y, \tag{4.14}$$

$$\frac{dY}{dt} = -\mathrm{DY} + \left(\frac{\mu_Y X^2}{K_X+X^2}\right)Y, \tag{4.15}$$

where S_0 represents the substrate concentration (mg/L) in the inflow and D the dilution rate (h^{-1}).

Very similar models have been presented previously to describe the relationship between a single predator, its prey and nutrient consumed by the prey in a chemostat (Li and Kuang, 2000) (Canale et al., 1973), although the use of the Holling type III response function for prey is unique to this research. Alternative variants of the model have been proposed. For example, where the prey yield (the number of new cells per g substrate) is no longer constant but a linear function of the substrate. This was in response to the models with constant yield failing to replicate the oscillatory behaviour observed experimentally in a chemostat (Pilyugin and Waltman, 2003) (Rana et al., 2015). Other theoretical studies have shown that including a function for yield , resulted in multiple limit cycles characteristic of an increased chance of co-existence between multiple species and as well as hopf bifurcation (Jia and Zhang, 2012; Pilyugin and Waltman, 2003).

Unfortunately, there is lack of experimental validation for the effect of variable yield for both the prey and *Bdellovibrio* predator. This change in prey concentration produced as a result of variable substrate concentration and prey yield is also accompanied by changes in cell size which could further vary the predator yield (Sockett, 2009) and reduce the reliability of the parameter estimation especially when cell numbers are used in alternative to biomass (Graham and Canale, 1982) (Shehata and Marr, 1971).

One study used Monod kinetics with constant yield to describe the dynamics between glucose (as the carbon growth limiting substrate), *Aerobacter aerogenes*, the bacterium prey and, *Tetrahymena pyriformis*, the protozoan predator (Canale et al., 1973). The study routinely measured in both batch and continuous cultures bacterial-limiting nutrient concentration, bacterial density (OD), and protozoan biovolume (coulter counter). The maximum growth rates were calculated by measuring the maximum slope of the bacterium and protozoan concentration changes with time on semi-log paper. Saturation constants (K_s and K_x) were calculated by measuring the growth rates of both prey and predator against the soluble carbon and prey optical density respectively (Canale et al., 1973).

Obtaining the saturation constant initially from batch culture is useful because it effects the steady state concentration of the substrate and can be used to control chemostat conditions (Canale, 1969).Observations in the batch experimental data led them to include equations to express the interactions between the bacterium prey, the useable substrate and the refractory substrate which is unaffected by bacterial action. Additionally, the study adds a term to express the death rate as a result of starvation. This modified Monod model was reported to fit well with experimental data from batch (Canale et al., 1973).

In chemostat we can remove *Bdellovibrio* predators before they become unviable as a result of starvation so we can expect the death rate of the predator to be negligible. However, in batch cultures, starved or unviable bacteria remain in the system and may have an effect on the dynamics as is has previously been reported that *B. bacteriovorus* cultured in the absence of prey had a reduced viability of 50% after 10 hours (Hespell et al., 1974) which occurs parallel to decrease in ATP, though there is some debate as to whether this pattern is common in nature (Wilkinson, 2006). Previous studies have demonstrated that ignoring starvation in predation models, led to more regular oscillations (Wilkinson, 2006).

In the Canale study (Canale et al., 1973), different constant flow rates of the chemostat were used (with the same initial substrate concentration at a saturating level) to observe different responses including 1) predator washout. 2) damped

oscillations 3) steady state (Canale et al., 1973). The model was not always able to duplicate these results at different dilutions, even with the alteration of the model parameters. They proposed that this could be solved with the addition of time delays to represent the lag or handling time that it takes for the predator to process its prey (Canale et al., 1973).

Various Lotka-Volterra based models exist for predation that take into account either exponential, logistic or Monod-type growth kinetics. For *Bdellovibrio*, models have been further developed by acknowledging the effect of Bdelloplast growth, heterogeneity (Hol et al., 2016b), the addition of the *Bdellovibrio* bacteriophage predator (Crowley et al., 1980), and the presence of a decoy prey (Hobley et al., 2006). Beyond this, there are many factors of a predator/prey systems that have not been sufficiently considered in regards to *Bdellovibrio* but have been explored further in other predator prey models such as chaos and periodic changes in temperature or nutrient supply (Liu and Chen, 2003) (Hui and Chen, 2006). Such models could be applied to further investigate the dynamics of *Bdellovibrio* predation in natural or engineered systems.

Most problematic biofouling occurs via biofilms, where the spatial structure of the microbial community clearly plays a role in the community's robustness to perturbations and its ability to resist predation, the basic tenets of the predator-prey models that have been described should hold for any system where predation occurs. Thus, to test whether this is the case, in this thesis, predator-prey dynamics are explored in the simple batch and chemostat systems that have been described above. The rationale used here is initially to derive parameters for the models from careful experimentation in highly simplified scenarios. Then to use the parameters to simulate more complex dynamics of predatory prey interactions.

The aim then is to validate the results of the simulations using further careful experimentation. Thus, the modelling is being used to guide experimentation. In an engineering application it is desirable to then design a system where there is no requirement to inoculate with the predator, instead they remain in the system while continuing to keep the, potentially biofouling, prey at bay. Thus,

maintaining predator-prey oscillations or cycles is of particular interest. The modelling should elucidate the ranges in the key parameters that will deliver predator prey cycling in the laboratory experiment.

The research is organised into a logical progression of tasks:

- Develop a growth media for the prey, a *Pseudomonas sp.,* that allows the effect of a single limiting resource to be quantified using standard Monod kinetics. In particular, optimise the inocula media such that the growth-rate of the prey is a function of different concentrations of glucose alone.

- Obtain the critical biological model parameters: growth rates, yields and saturation constants for both prey and predator. This is achieved in a series of batch experiments with the prey growing on different glucose concentrations and with the predator growing on different concentrations on prey.

- Develop computer models based on the mathematical models: equations 4.10,4.11,4.12, for batch culture and equations 4.13, 4.14 and 4.15 for a chemostat.

- Apply the batch computer model, with the empirically derived parameters, to simulate the dynamics of a predator feeding on prey which is, in turn, growing on glucose, for a variety of initial conditions.

- Conduct a series of laboratory batch experiment to implement the scenarios simulated by the computer model and compare the experimental and simulated population dynamics. Then, if necessary, adjust the model parameters to improve the match.

- Use parameters in the chemostat model to simulate the predator-prey population dynamics for a fixed set of initial conditions but a wide range of values for two variables: dilution rates and influent substrate concentrations.

Then, for these two variables map out regions of the parameter space where distinct characteristic dynamical regimes are predicted to occur: stable co-existence; oscillatory dynamic (limit cycle) co-existence; simultaneous prey and predator extinction; predator only extinction).

- Based on the map of different predicted behaviours, select a set of experimental conditions that are predicted to deliver the oscillatory predator-prey dynamics, the most interesting for control in engineering. Then implement an experimental chemostat for these conditions and compare the observed dynamics with those predicted.

- Based on the comparison of simulated and experimental chemostat dynamics, augment the experimental results to explain any discrepancies.

4.1 Methods

4.1.1 The predator and the prey

The wild-type *Bdellovibrio bacteriovorus* strain HD100 (DSM no. 50701) was used throughout this study and was grown by predation on *Pseudomonas sp*. (DSM no. 50906) using standard culturing methods (Herencias et al., 2017). *Pseudomonas* cells were grown in LB broth at 30°C for 16hours and resuspended in Ca/Mg-HEPES buffer (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, pH 7.6) to an optical density (600nm) value of 10. *B. bacteriovorus* was then grown on this prey suspension in DN broth at 30°C for 24 hours then sub-cultured twice at 24-hour intervals in in Ca/Mg-HEPES buffer. To remove the prey and harvest the predator alone for growth experiments each culture was filtered through a 0.45µm pore size syringe filter (Fisherbrand™, product code: 15216869).

4.1.2 Inocula History

It is known that bacteria can alter their kinetic properties in response to environmental changes such as the substrate available for growth (Ahmad and Holland, 1994). Previous studies investigating the kinetic relationship between bacteria and a growth limiting substrate initially grow bacteria in a rich media such as LB before inoculation into a minimal media (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, 0.05% casein hydrolysate, pH 7.6) with varying concentration of the growth limiting carbon substrate, glucose. Changing the environment directly from a high to lower nutrient environment could potentially influence the reported kinetic values. Additionally, the different growth methods used between researchers lead to inconsistent reporting of kinetic parameters.

To investigate the effect of inocula history on Monod kinetics (Fig 4.1), *Pseudomonas* was grown overnight at 30°C and 200rpm in LB media and then sub-cultured overnight in different media; minimal media (Fig 4.1A),minimal media further supplemented with varying concentrations of glucose (Fig 4.1B), which was the carbon growth-limiting substrate (0, 0.5, 2.5, 20mg/L) and LB broth (Fig 4.1C). *Pseudomonas* from each culture is then resuspended to 10^5 cells/ml (as determined by flow cytometry) in microwell plates with minimal media supplemented with varying concentrations of glucose (0, 0.5, 2.5, 20mg/L). Growth was then observed at 30°C for 18 hours by OD using microwell plate reader.

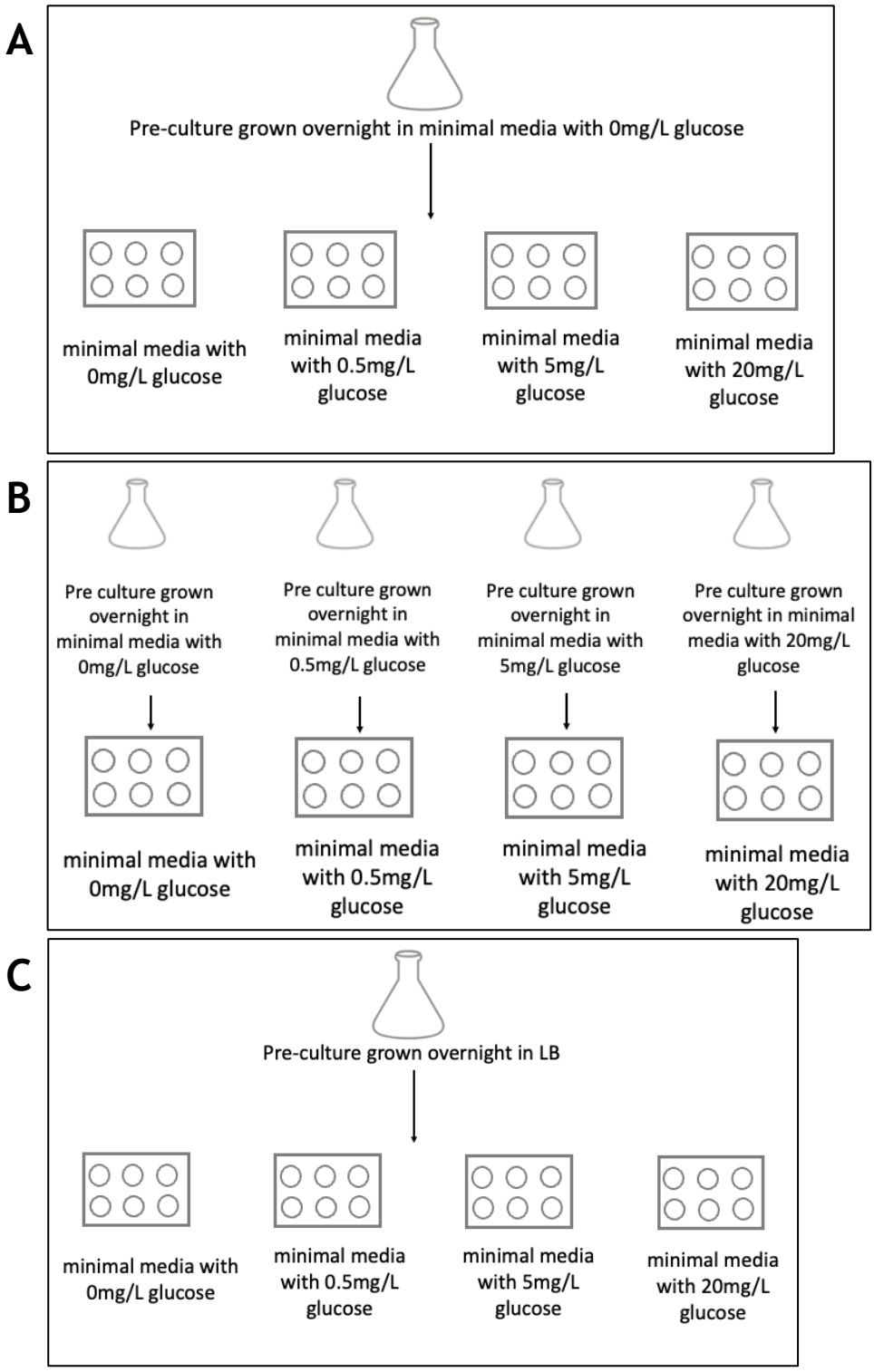

Figure 4.1- Schematic representation of inocula history experiment. Bacteria is initially grown in LB then to gain different inocula, the bacteria are grown overnight again in different media: **A)** HEPES minimal media, **B)** minimal media supplemented with glucose and **C)** LB broth before being grown in microplates with different concentration of glucose supplemented minimal media.

LB was chosen as the *Pseudomonas* inocula media for future kinetic experiments as it produced the highest concentration of initial bacteria, and when the inocula was grown on glucose showed the typical Monod relationship.

4.1.3 Identifying the Monod model parameters

To obtain the Monod parameters that describe relationship in batch between the substrates (glucose) and the prey bacteria, *Pseudomonas*, a culture was grown overnight in LB medium, and 10^5 cells/ml was suspended into six 500ml flaks with 100ml minimal media, with varying levels of glucose (0, 0.5,1, 2.5, 5, 20mg/L).

The cells were grown for 16 hours at 30°C and 200rpm shaking as it is the optimum conditions at which we wish to view the predator dynamics of *Bdellovibrio*. To observe the prey growth, aliquots were taken from each culture and resuspended 1:1 v/v in glutaraldehyde then stored at 4°C and measured by flow cytometry within 3 days. To observe the substrate concentration depletion in the culture, aliquots were taken every 2 hours and immediately filtered, then stored at -20°C and measured using the sigma glucose assay kit according to instructions. Absorbance was recorded at 570nm using a multiplate reader.

For the prey limiting assay, *B. bacteriovorus* filtrate was prepared as above to 10^5cells/ml and used to inoculate *Pseudomonas* cultures of varying concentration (10^5-10^9 cells/ml) in 250ml conical flasks with 50ml of Ca/Mg-HEPES buffer. The culture was then incubated for 72 hours at 30°C. To observe the prey and predator growth, aliquots were taken at regular time periods from each culture and resuspended 1:1 v/v in glutaraldehyde then stored at 4°C and measured by flow cytometry within 3 days.

4.1.4 Computer models

The numerical solutions of equations 4.10-4.12 and 4.13-4.15 were implemented in two separate MATLAB computer codes. The codes used the robust, ordinary differential equation package ODE45. They are included in appendix 8.2.

4.1.5 Batch predator prey simulations

The computational model was used to simulate the dynamics in five flasks (Fig 4.2). These five dynamics were randomly chosen based on them differing in the initial conditions as well as showing difference between the observed behaviour, through the speed and degree of change of the prey and predator population.

4.1.6 Batch predator prey experiments.

The conditions simulated in the batch computational model were mirrored in a set of 250ml conical flasks in the laboratory. The flasks were filled with 50ml of the minimal media supplemented with different concentrations of glucose, *Pseudomonas* and *B. bacteriovorus*. Following inoculation of prey and predator, substrate depletion and prey and predator growth were measured routinely for 72 hours.

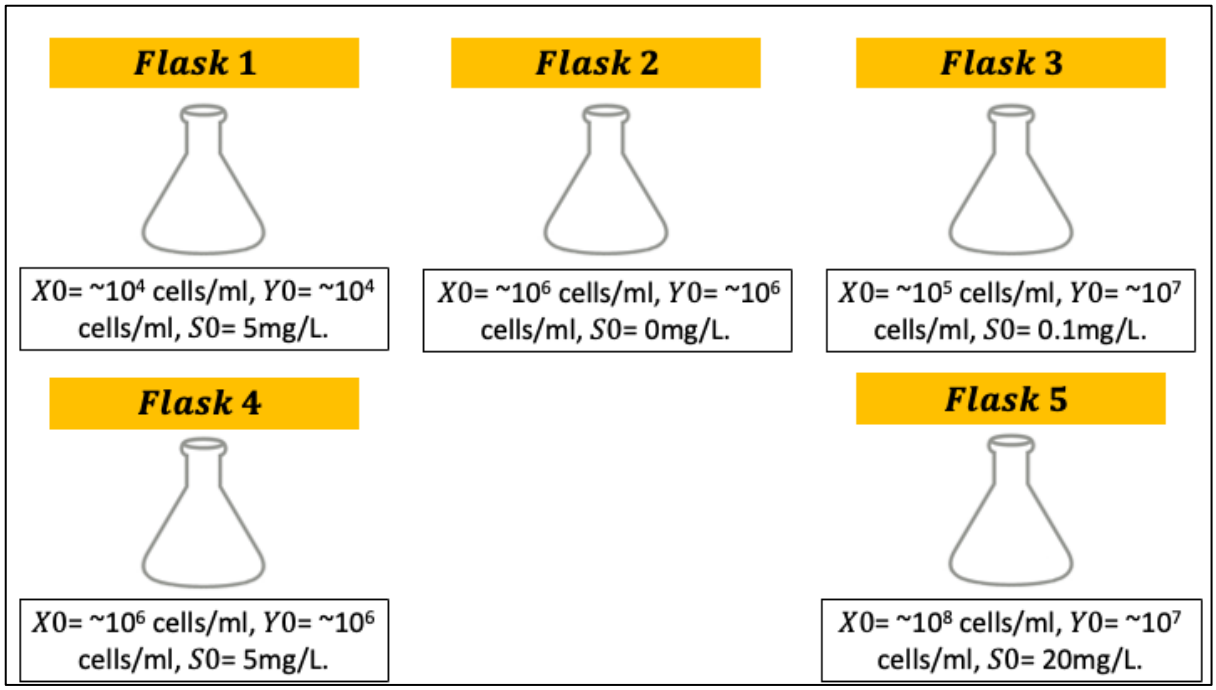

Figure 4.2- Schematic diagram represents the flasks for the batch experiments. Initial conditions with different initial prey (X_0), predator (Y_0), and glucose substrate (S_0), concentrations that were randomly selected are shown.

4.1.7 Chemostat Simulations

The chemostat model was run for every combination of S_0, influent glucose concentration, and D, dilution rate selected from vectors with 100 equal increments in the ranged (0.5,25) mg/l and (0.01,1) hr^{-1}. The dynamics of predator and prey abundances were for each combination were simulated for 40 hydraulic retention times, 40/D hours. If the simulated abundances of predator and prey at the end of the simulation were zero, then the dynamics were classified as 'washout'. If the prey abundance remined constant over the last 20 retention times and the predator abundance dropped to zero, this was classified as predator washout. If both abundances were constant, this was classified as stable co-existence. If the abundances of predator and prey over the last 20 retention times crossed their mean values more than 4 times then the solution was deemed to be oscillating and, thus, exhibiting a 'limit cycle'.

4.1.8 Chemostat Monod parameter experiment run 1

To observe the relationship in chemostat between the substrates (glucose), the prey bacteria *Pseudomonas* and the predatory bacteria *Bdellovibrio*. *Pseudomonas* and *Bdellovibrio* cultures are used to inoculate a bioreactor chamber filled with minimal media (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, 0.05% casein hydrolysate, pH 7.6) further supplemented with 25mg/L glucose. Following this, the bioreactor is connected to an inlet and outlet bottle, to remove volume from the reactor while also providing the reactor with minimal media further supplemented with 25mg/L glucose. Using peristatic pumps, the medium is supplied at a constant dilution rate for 168 hours. Samples of the substrate, prey and predator are obtained from the effluent routinely across the experiment run of 168 hours. Concentration of the samples were determined as in the batch study.

4.1.9 Chemostat Monod parameter experiment run 2

To determine the growth of biofilm within the bioreactor and explain the discrepancies observed in the first chemostat run, we repeated the chemostat run as above but instead for 72 hours with the addition of glass microscope slides placed in the reactor to collect biofilm. Samples of prey and predator were obtained from the effluent routinely and at the end of the run the glass slides were removed and the biofilm was reconstituted into PBS by sonication. The concentration of the sample was determined by flow cytometry as above and then used to calculate the total volume of bacteria growing as a biofilm in the reactor.

4.1.10 Flow cytometry

Samples were prepared by fixation with glutaraldehyde (1% in DI water) and where necessary diluted to achieve an events per second reading of less than 600 on the flow cytometer. Dilutions were made in filtered (0.22 µm Sartorius™ Minisart™ Plus Syringe Filters, Fisher scientific, product code: 10730792) DI water. Samples were stained for the total cell count.

For total count: the samples were each stained with 10 µL/ml of SYBR Green I (SGI) and incubated in the dark for 13 min before measurement. Stock solutions of SGI (10,000 x in DMSO, Thermofisher, product code: S7653) were diluted 1:100 in EDTA (1 mM).

Gating was used to distinguish selected signals (*B. bacteriovorus* and *Pseudomonas*) from each other and from the background (inorganic and organic particles) with the aid of negative controls consisting of the DI water used for dilutions, HEPES buffer used for growth, and a sample of the predator/prey co-culture further filtered (0.22 µm) to remove any bacterial cells.

4.1.11 Glucose measurement

To measure the amount of glucose in a sample, a glucose coulometric assay kit (Sigma-Aldrich, product code:MAK263-1KT) was used. Samples were first prepared by immediate filtering and then freezing at -20°C. Samples were then further prepared and measured at 570nm in a microwell plate according to instructions.

4.2 Results

4.2.1 Inocula History

We initially grew six cultures of *Pseudomonas*. One grown on LB broth (Fig 4.1C), one grown in minimal HEPES medium (Fig 4.1A) and the others grown in minimal HEPES medium supplemented with four different amounts of glucose (0, 0.5, 2.5 and 20mg/L) (Fig 4.1B), the growth rate-limiting nutrient. We then measured the growth curve of each of the six different *Pseudomonas* inocula grown on HEPES medium supplemented with casein hydrolysate and four different amounts of glucose (0, 0.5, 2.5 and 20mg/L).

The growth rates of *Pseudomonas* cultures fed different glucose concentration is plotted when inoculated from: HEPES with 0mg/L (Fig 4.3A); HEPES supplemented

with the same glucose concentration (Fig 4.3B); and LB broth (Fig 4.3C). Whatever the initial growth media of the inocula, the growth curve resembled that described by the Monod model; *Pseudomonas* shows limited growth without glucose and grows rapidly as the glucose concentration increases until saturation is reached and no further increase in growth rate is observed. When grown in HEPES supplemented with the same amount of glucose concentration as in the inocula, there was less variation between repeats in the specific growth rate.

Using the solver in excel we calculated the maximum growth rate and saturation constants for each inocula condition. When previously grown in HEPES with 0mg/L glucose, the maximum growth rate and the saturation constant were the lowest (0.121 h^{-1} and 0.06mg/L). The highest maximum growth rate (0.141 h^{-1}) was observed when grown in the same amount of glucose as in the inocula, though the saturation constant was only 0.07mg/L. When grown previously in LB the maximum growth rate was 0.138 h^{-1} and the highest saturation constant of 0.236 mg/L was displayed. In experiment, the *Pseudomonas* also shows a constant growth rate even at 0mg/L glucose concentration which is in contrast to what was expected and what the model predicted, where no growth is shown at 0mg/L glucose.

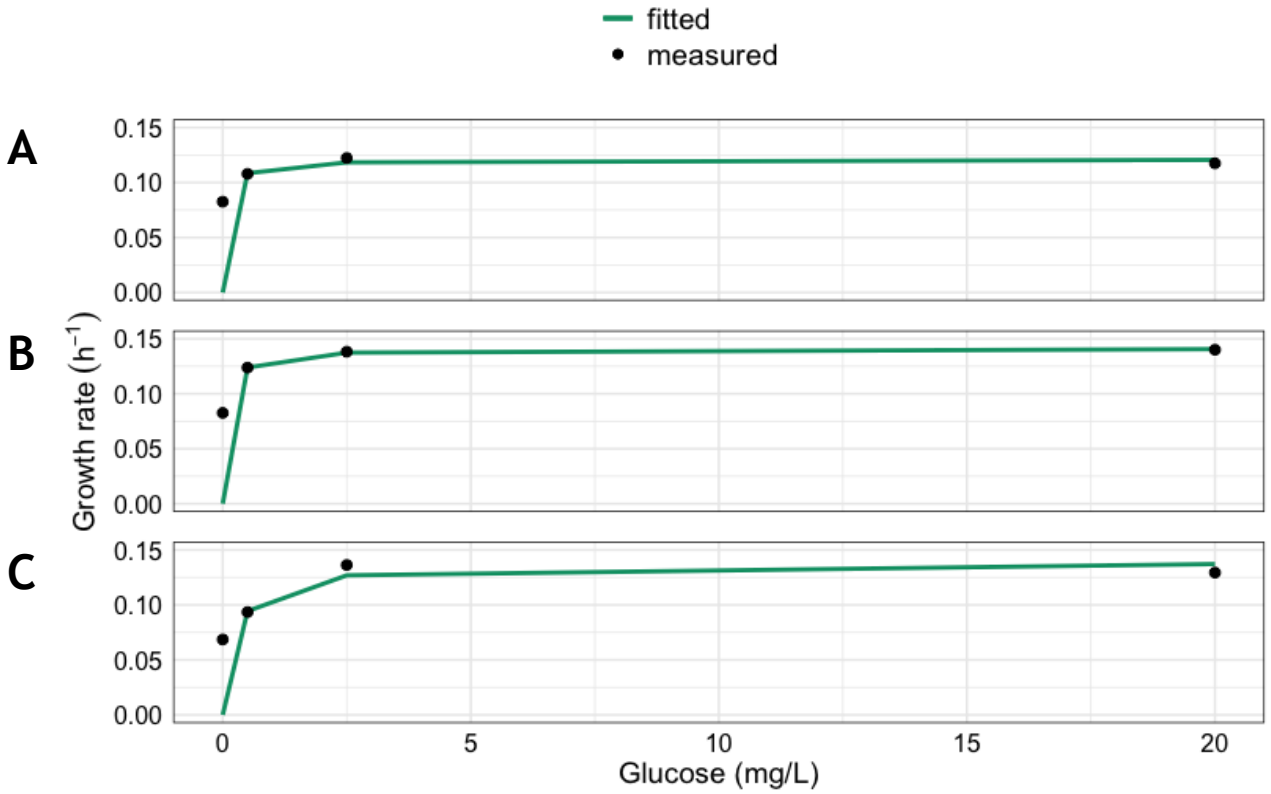

Figure 4.3- Specific growth rate (h^{-1}) of *Pseudomonas* vs the initial concentration of glucose (mg/L). The saturation constant and maximum growth rates are calculated to model the relationship using the Monod equation. The bacteria were previously grown on **A)** supplemented HEPES buffer with zero glucose **B)** supplemented HEPES buffer with the same concentration of glucose as measured (0, 0.5,2.5,20 mg/L) and **C)** LB buffer.

4.2.2 Batch predator prey system

To measure the growth of prey more accurately and reliably capture the growth parameters that are critical to the batch model, we use flow cytometry to measure the batch growth of *Pseudomonas* on minimal media supplemented with a wider range of glucose concentrations ranging from 0 to 20mg/L. *Pseudomonas* growth on glucose fits Monod theory well (Fig 4.4B). We plotted the observed growth rate as a function of the initial glucose substrate. As the initial glucose concentration increases, *Pseudomonas* growth rate increases exponentially up to approximately 2.5 mg/L where the growth rate levels off.

We used the Monod model (equation 4.10) to characterise the relationship between bacterial growth rate and the concentration of glucose. We plot a Lineweaver-Burk plot which is a linearised forms of the Monod equation (Fig 4.4A). With the plot, we use the intercept and gradient to determine the value of the maximum growth rate (1.0 h^{-1}) and the saturation constant (0.18mg/L). We use the Lineweaver-Burk plot rather than the excel solver in this instance because of the increased precision and the ability to quantitatively estimate the error.

As shown previously, the model is estimated to show no growth (Fig 4.4B) in the absence of glucose, which is unlike the growth observed in experimentation, where *Pseudomonas* grows at a higher rate of 0.51 h^{-1}. For all other concentrations of glucose, the fitted model is similar to the observed measurements.

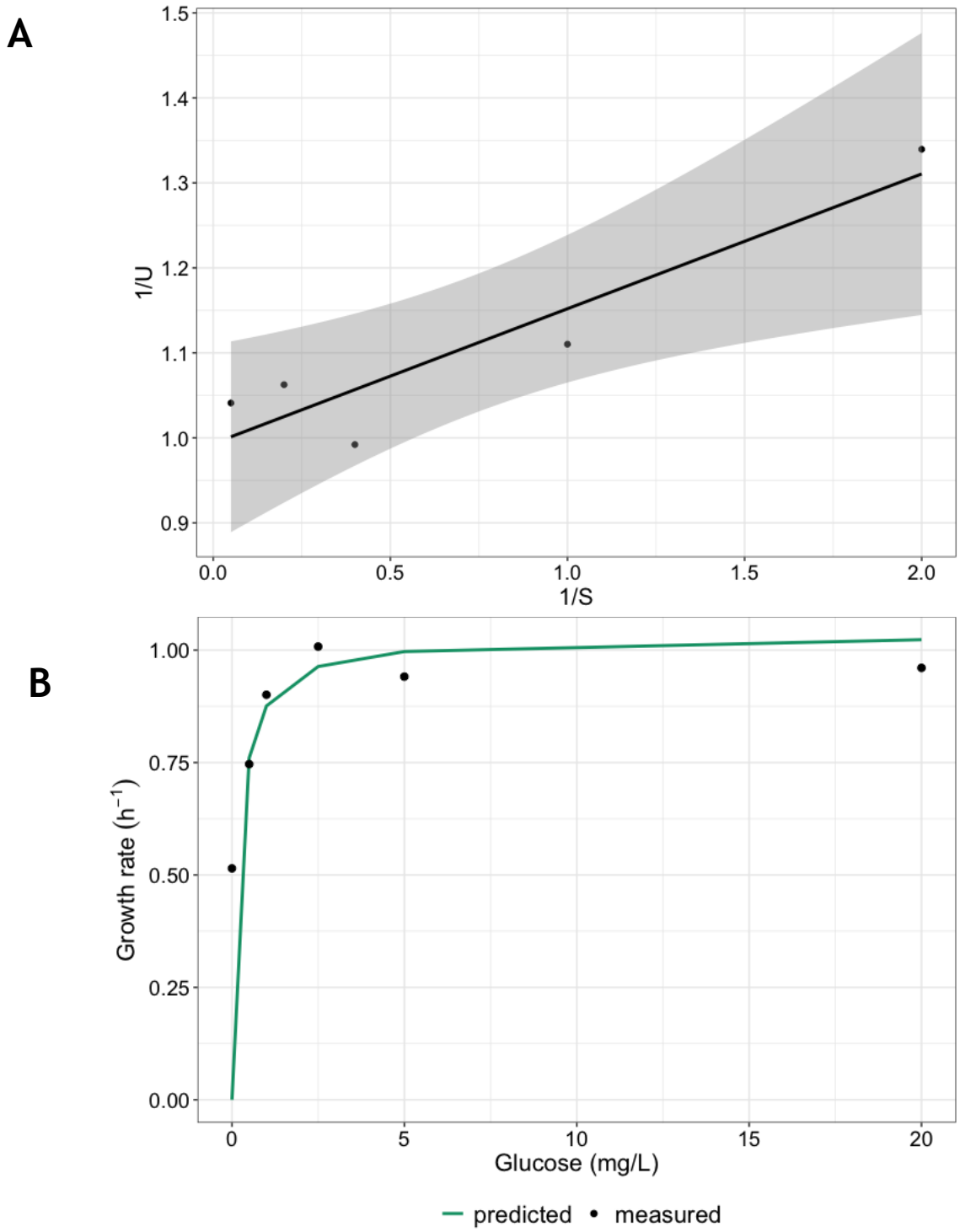

Figure 4.4- Relationship of *Pseudomonas* growth with glucose. A) Lineweaver Burk plot with confidence intervals (grey) of the relationship between specific growth rate (h^{-1}) of *Pseudomonas* vs the initial concentration of glucose (mg/L). **B)** The saturation constant (0.18 mg/L) and maximum growth rates (1.0 h^{-1}) are calculated to model the relationship using the Monod equation.

We measured the growth curve of *Bdellovibrio* grown on Ca/Mg-HEPES buffer inoculated with different concentrations of *Pseudomonas* ranging from a severely limiting level (0 cells/ml), to saturating level (10^9 cells/ml).

Figure 4.5 shows a typical growth in a batch culture of *Bdellovibrio* and *Pseudomonas*. For the 72 hours there is little to no change in the concentrations of prey and the predator when the initial *Pseudomonas* concentration is 10^7 cells/ml or lower. However, when the prey concentration is increased to 10^8 cells/ml the predator population increases slowly for the first 24 hours before rapidly increasing and then levelling off at around 44 hours. Simultaneously the prey population shows little change at high initial prey concentrations for the first 24 hours before rapidly decreasing as a result of predation. The prey population then levels off after 48 hours.

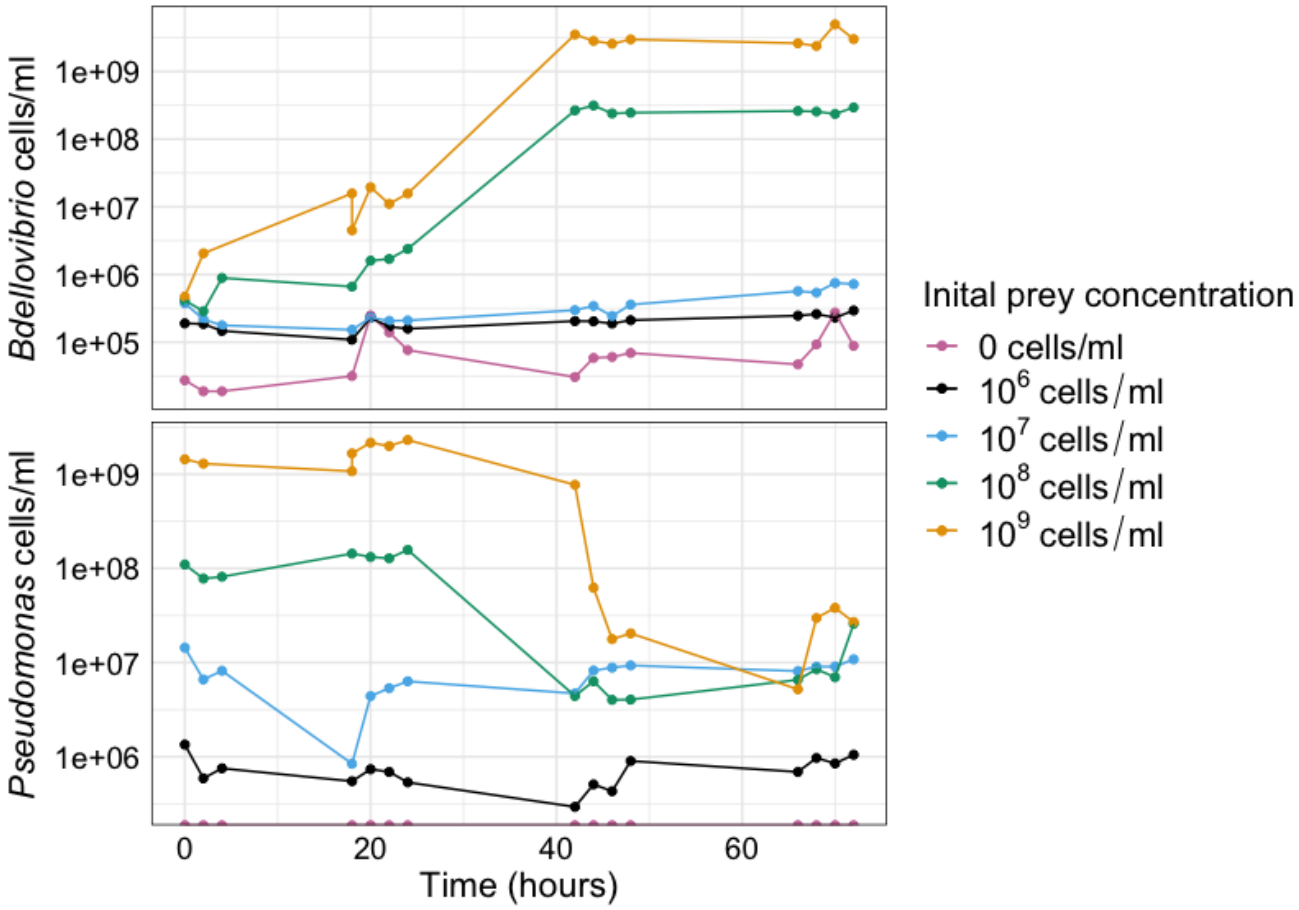

Figure 4.5- Growth curve of *Bdellovibrio* and *Pseudomonas* (cells/ml) population during batch culture with different initial prey concentrations.

Figure 4.6 shows the *Bdellovibrio* specific growth rate as a function of *Pseudomonas* prey cell density. This concurs with the observations of figure 4.5; the predator growth rate shows no increase at low concentrations of prey but when the prey concentration increases the predator growth rate rapidly increases. We determined the maximum growth rate and saturation constant when using both Monod ($0.266h^{-1}$ and 5.59×10^7 prey cells/ml respectively) and the Holling type III functional response ($0.244h^{-1}$ and 2.74×10^{15} $cells^2/ml^2$ respectively).

The observed pattern of growth better resembles the Holling type III equation rather than the Monod equation, which is unable to overestimate the increase in growth rate when increasing prey concentration from 10^6 cells/ml to 10^7 cells/ml. Neither the Monod nor Holling models were able to predict the decrease in predator population observed in the absence of prey.

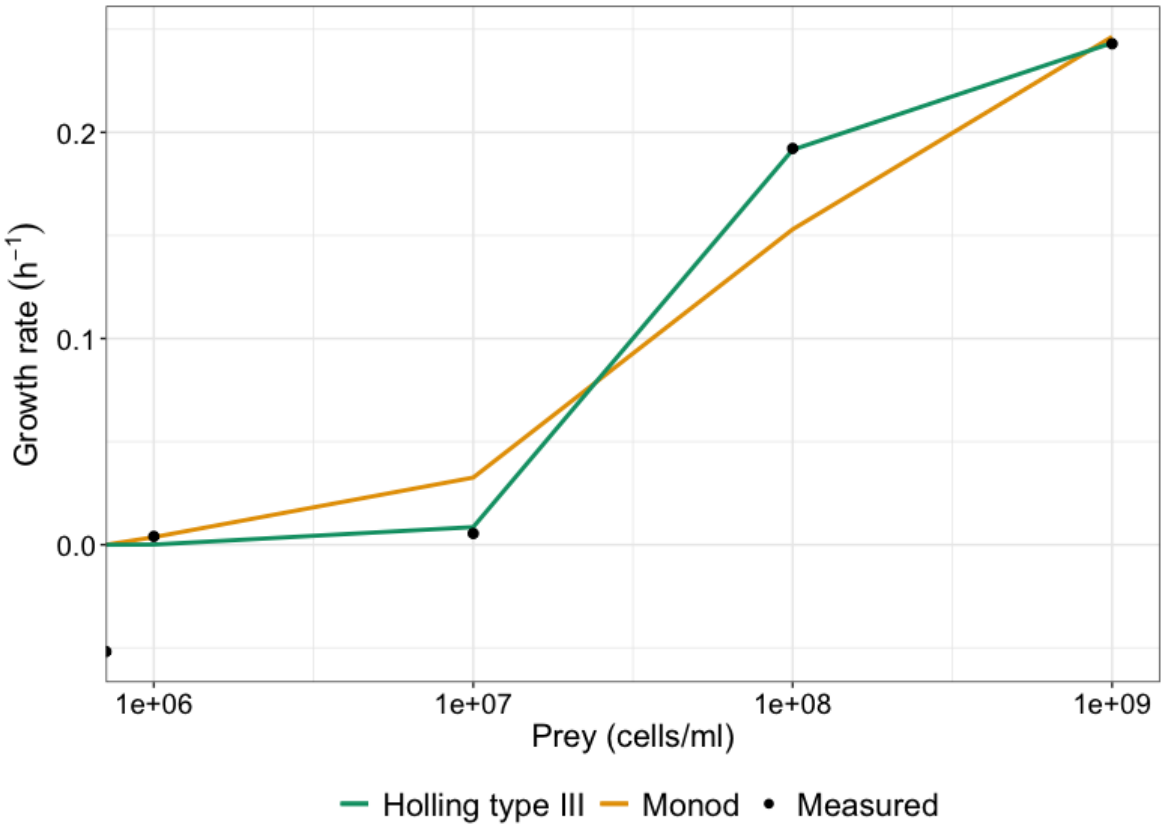

Figure 4.6– Specific growth rate (h^{-1}) of *Bdellovibrio* vs the initial concentration of prey (cells/ml) in batch culture. The saturation constant and maximum growth rates are calculated to model the relationship using the Monod equation (0.266^{h-1} and 5.59×10^{7} prey cells/ml) and Holling type III functional response ($0.244h^{-1}$ and 2.74×10^{15} $cells^2/ml^2$).

The batch experiments above allowed us to estimate the parameter values for the predator prey growth kinetics (maximum growth rates and saturation constants); these are summarised and presented in table 4.1.

Table 4.1- kinetic parameters used in model.

Kinetic parameter	Value
Maximum prey growth rate	1.0 h^{-1}
Maximum predator growth rate	0.24 h^{-1}
Predator yield	1.95 *Bdellovibrio* cells/*Pseudomonas* cell
Prey yield	3.1 x 10^7 cells/mg of glucose
Glucose saturation constant	0.18mg/L
Pseudomonas prey saturation constant	2.74 x10^{15} $cells^2/ml^2$

4.2.3 Batch validation/fitting

Using these parameters in a model created with equations 4.10, 4.11 and 4.12 in MATLAB. With these parameters we predicted the outcome of a batch system with a range of initial conditions for glucose concentration, predator and prey population. A summary of the conditions and the predicted and observed system responses is presented in figures 4.7-4.11.

Using the computer model for the simultaneous batch growth of predator and prey (equations 4.10-4.12) we simulated a range of the possible predator prey dynamics. This was achieved by the appropriate selection of initial conditions. Fig 4.7 shows a simulation where the initial conditions lead to the rapid depletion of glucose and a large increase in both the predator and prey concentrations from ~10^5 cells/ml to ~10^8 cells/ml. Both populations plateau but the prey population subsequently decreases from ~10^8 cells/ml to ~10^6 cells/ml. Fig 4.8 shows a simulation where the initial conditions lead to no significant change in the concentration of prey or predator. In Fig 4.9 the initial conditions were predicted by the simulation to show fast depletion of glucose and an increase in the prey from ~10^5 cells/ml to ~10^6 cells/ml but this was not enough to cause any significant change in the predator. Fig 4.10 shows a simulation where the initial conditions cause rapid depletion in glucose and increases in the prey (~10^6 cells/ml to ~10^8 cells/ml) and predator (~10^6 cells/ml to ~10^8 cells/ml), followed by no change in the concentration of the predator but a significant decrease of the prey from ~10^8

cells/ml to a lower concentration than the initial. Similarly, Fig 4.11 presents the simulation where there is rapid depletion of glucose, a large increase in the predator (~10^7 cells/ml to ~10^9 cells/ml) followed by a plateau whereas the prey concentration increases shortly (~10^8 cells/ml to ~10^9 cells/ml in under six hours) before significantly declining from ~10^9 cells/ml to ~10^5 cells/ml. For Fig 4.7-4.11, we have superimposed the concentrations of predator and prey measured in the set of batch experiments that attempt to recreate the simulated results.

When the initial glucose concentration was zero or low (below the saturation constant) the model was not able to accurately predict the growth of the prey and the predator (Fig 4.8 and 4.9). Due to the lack of sensitivity of the glucose assay we were unable to accurately capture the behaviour of glucose in these systems and compare it to the modelled behaviour. Regardless, the model predicted that at these glucose concentrations there would not be any significant growth in the prey population. With the prey population remaining at such a low concentration well below the prey saturation constant, it is predicted by the model that the predator would also not show significant growth. However, in experiment, the *Pseudomonas* prey was still able to grow even in the absence of glucose. As expected, this results in an increase in the predator population. Interestingly, in these cases the prey population predicted by the model is better fitting to the measured population after a number of hours, by which point the predator appears to have grown to a saturating amount.

Notably, when the initial glucose concentration is higher (5mg/L and above), the model is able to predict the growth of the prey and the predator (Fig 4.7, 4.10 and 4.11). As expected, the prey bacteria grow on the glucose present and subsequently the predator population also grows which in turn results in the decrease of the prey population. Additionally, the largest population of predator (1 x10^9 cells/ml) is shown when the prey also grows to the largest population (1x10^9 cells/ml) (Fig 4.11) and for all batch experiments if the prey population did not reach 1 x 10^7 in the first six hours, the predator population would take longer (over 24 hours) to reach a saturating level.

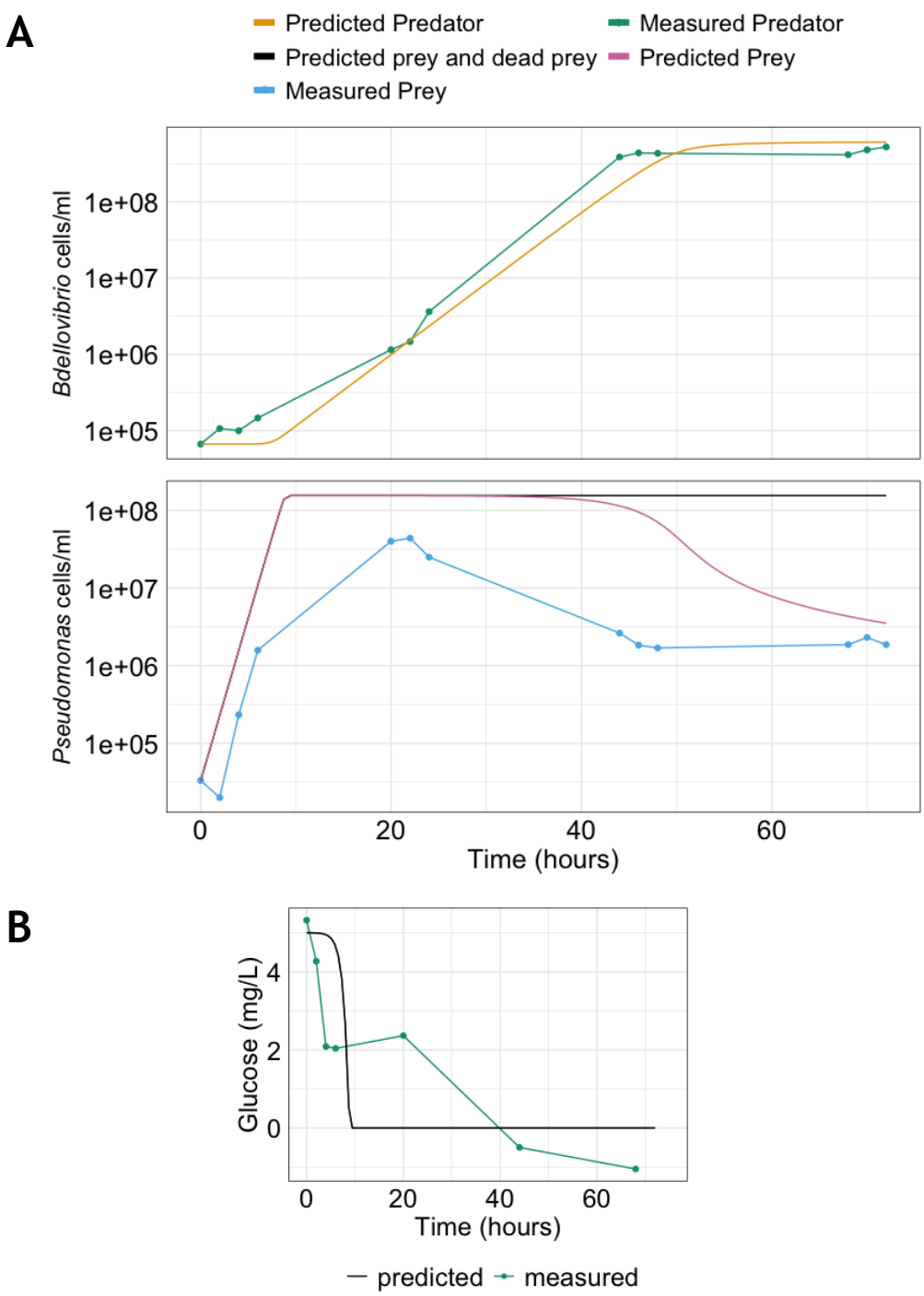

Figure 4.7- Batch flask 1. Transient curve of **A)** *Bdellovibrio* and *Pseudomonas* (cells/ml) population and **B)** glucose (mg/L) during batch culture with different initial prey ($\boldsymbol{X_0}$ = ~10^4 cells/ml), predator ($\boldsymbol{Y_0}$= ~10^4 cells/ml) and glucose substrate ($\boldsymbol{S_0}$= 5mg/L) concentrations. For which the model predicts a large increase in both the predator and prey concentrations followed by a plateau and then a decrease of the prey population.

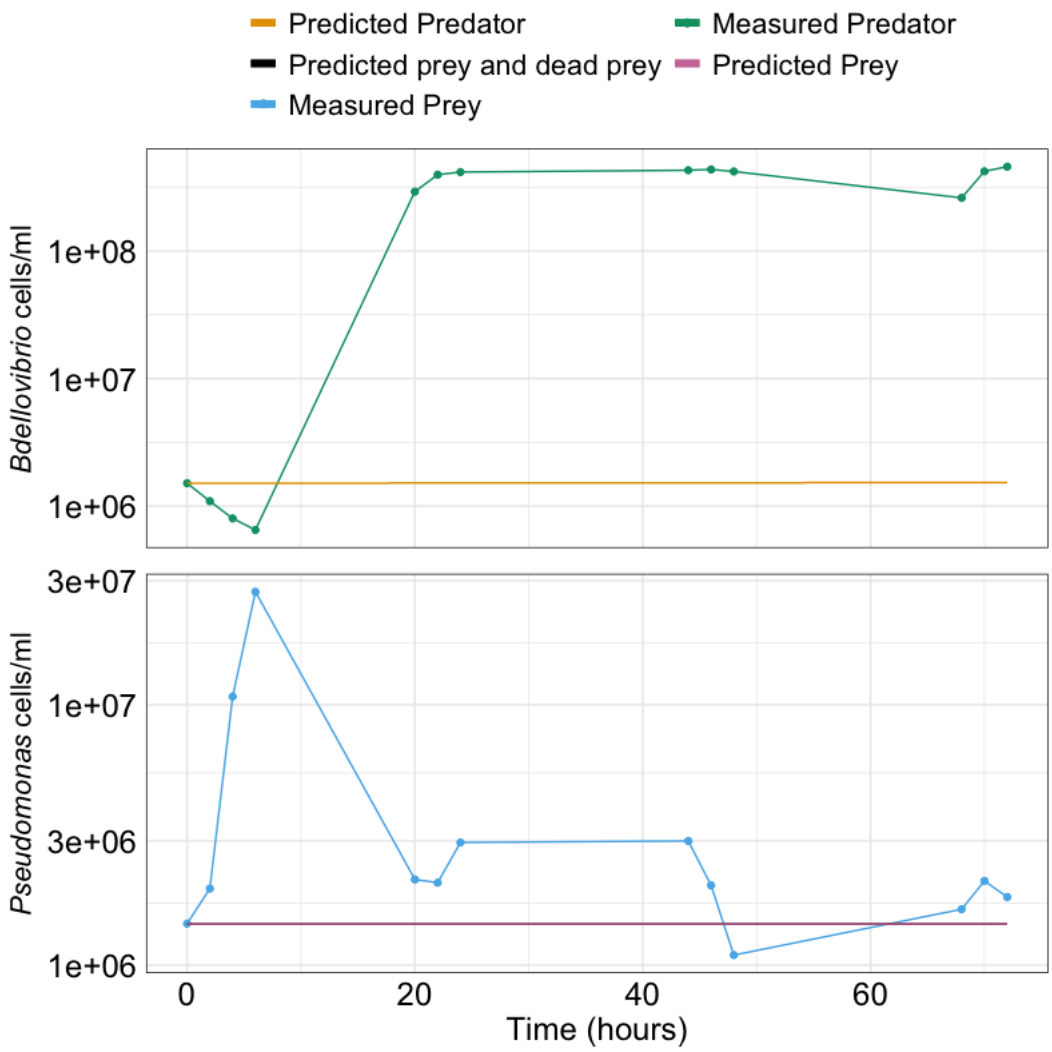

Figure 4.8- Batch flask 2. Growth curve of *Bdellovibrio* and *Pseudomonas* (cells/ml) population during batch culture with different initial prey ($\boldsymbol{X_0}$ = ~10^6 cells/ml), predator ($\boldsymbol{Y_0}$= ~10^6 cells/ml) and glucose substrate ($\boldsymbol{S_0}$= 0mg/L) concentrations. For which the model predicts no change in the predator or prey population.

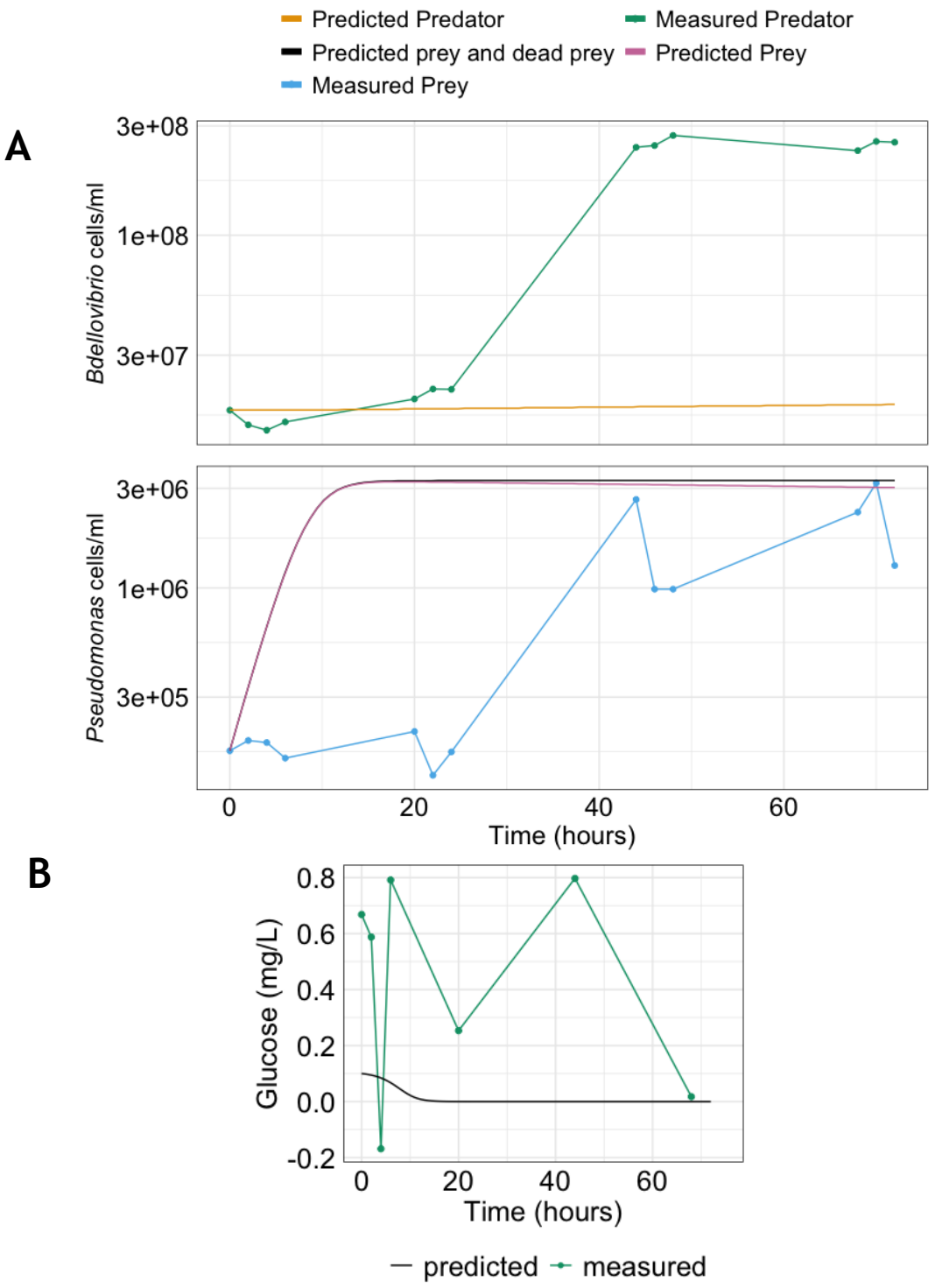

Figure 4.9- Batch flask 3. Transient curve of **A)** *Bdellovibrio* and *Pseudomonas* (cells/ml) population and **B)** glucose (mg/L) during batch culture with different initial prey ($\boldsymbol{X_0}$ = ~10^5 cells/ml), predator ($\boldsymbol{Y_0}$= ~10^7 cells/ml) and glucose substrate ($\boldsymbol{S_0}$= 0.1mg/L) concentrations. For which the model predicts an increase in the prey but not enough to support an increase in the predators.

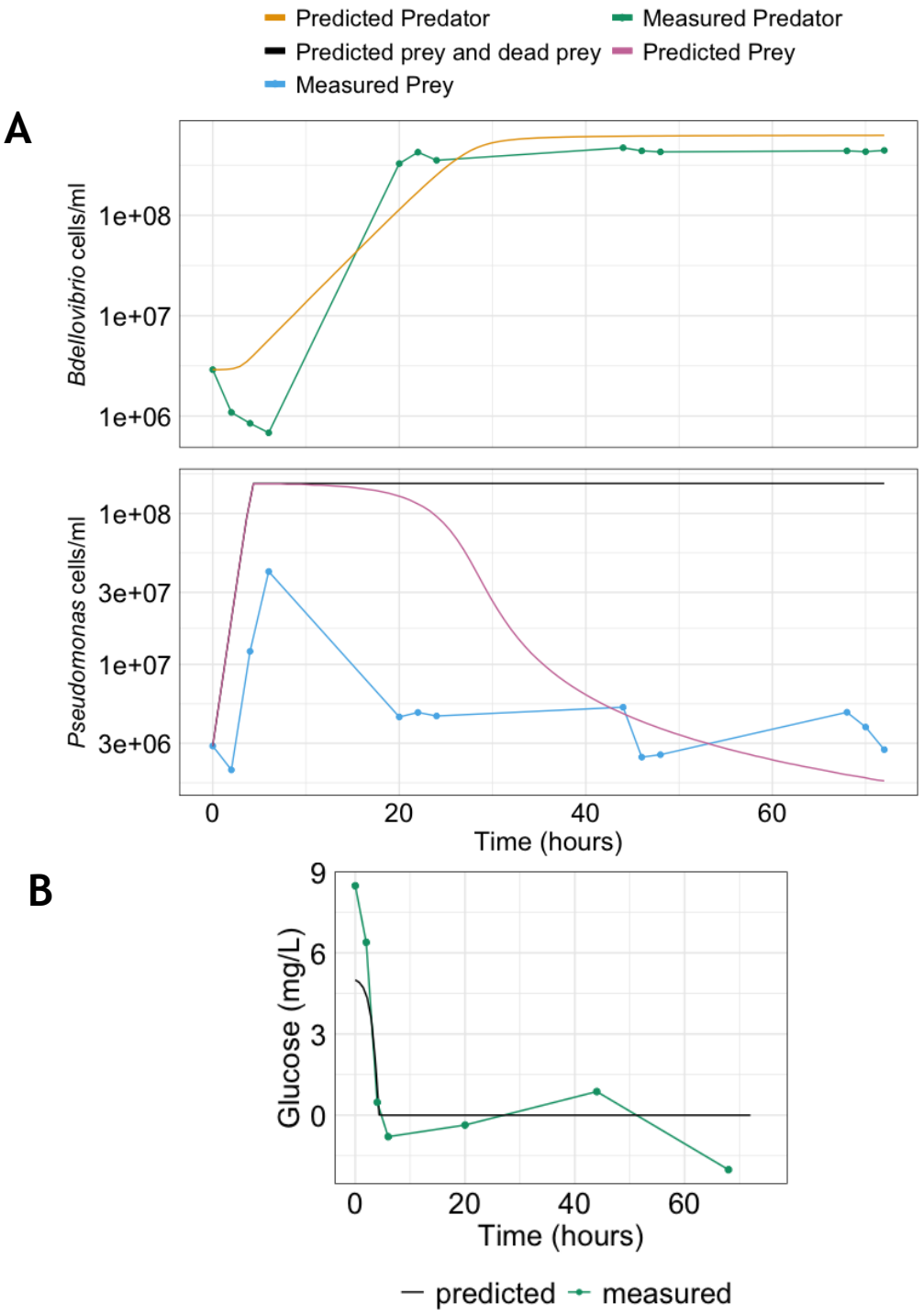

Figure 4.10- Batch flask 4. Transient curve of **A)** *Bdellovibrio* and *Pseudomonas* (cells/ml) population and **B)** glucose (mg/L) during batch culture with different initial prey ($\boldsymbol{X_0}$ = ~10^6 cells/ml), predator ($\boldsymbol{Y_0}$= ~10^6 cells/ml) and glucose substrate ($\boldsymbol{S_0}$= 5mg/L) concentrations. For which the model predicts an increase in the predator and prey populations followed by a large decrease in the prey.

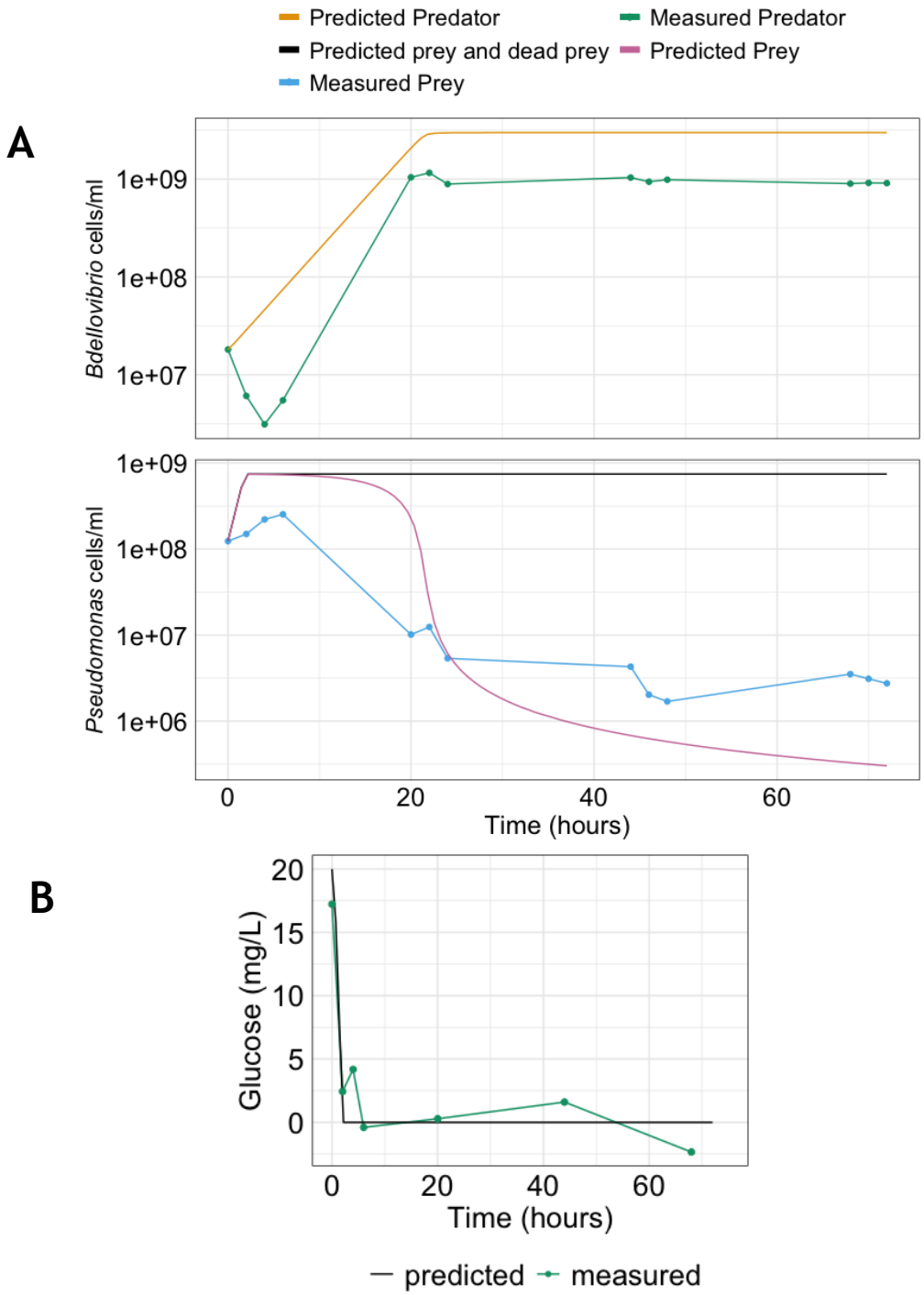

Figure 4.11- Batch flask 5. Transient curve of **A)** *Bdellovibrio* and *Pseudomonas* (cells/ml) population and **B)** glucose (mg/L) during batch culture with different initial prey ($\boldsymbol{X_0}$ = ~10^8 cells/ml), predator ($\boldsymbol{Y_0}$= ~10^7 cells/ml) and glucose substrate ($\boldsymbol{S_0}$= 20mg/L) concentrations. For which the model predicts a large increase in the predator population and a large decrease in the prey population.

4.2.4 Simulations to explore different classes of population dynamics in a chemostat.

The growth kinetic parameters that were now validated, at least for high concentrations of glucose in batch system, were used to simulate predator prey dynamics using the chemostat mathematical model (solving equations 4.13,4.14 and 4.15). We systematically simulated the dynamics for 100x100 plausible combinations of dilution rate and the influent glucose concentration and classified the dynamics using the heuristic rules outlined in the methods above as bring either: prey and predator washout, predator washout, stable co-existence and limit cycle oscillations (Fig 4.12).

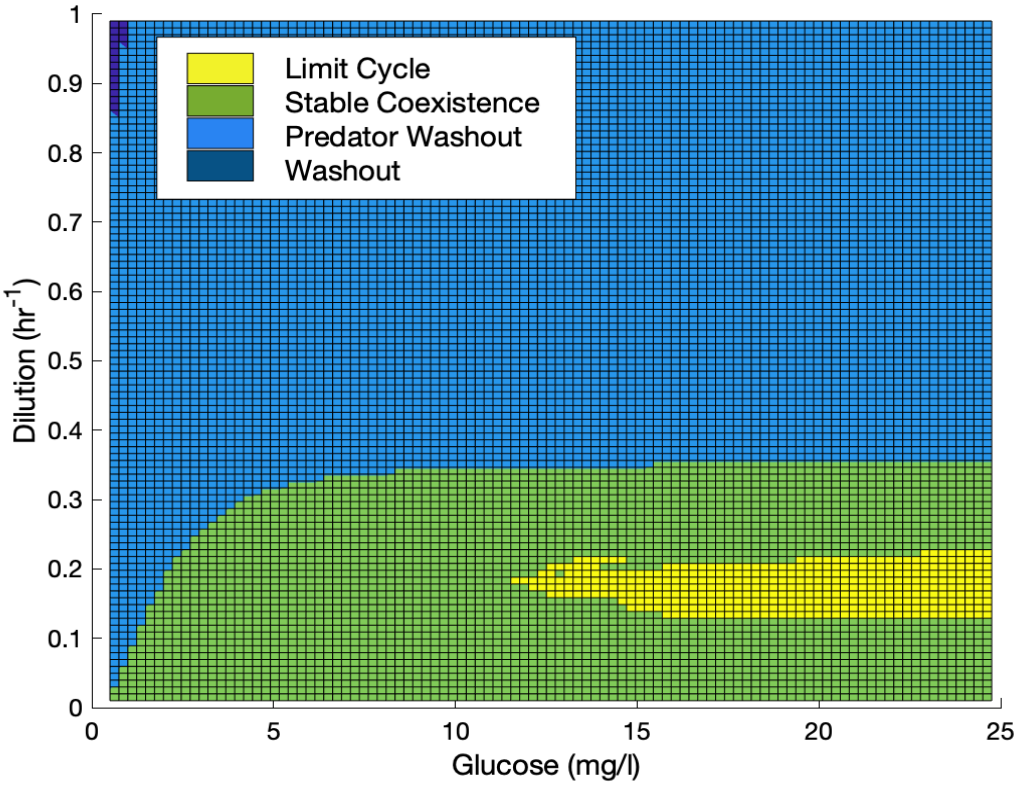

Figure 4.12- Model predicted surface plot. Displays the different behaviours of *Bdellovibrio* and *Pseudomonas* in chemostat culture depending on the glucose in the influent (mg/L) and dilution rate (h^{-1}).

The outcome of the predator and prey populations is largely determined by the dilution rate of the chemostat, and at the dilution rates higher than 0.4h^{-1}, the predator is washed out regardless of the glucose concentration in the influent. There is a small region in the parameter space, when the dilution rate is really high (approximately above 0.82 h^{-1}), and the glucose concentration is low (approximately below 1mg/L), the prey is also washed out along with the predator.

At a low enough dilution rate and a high enough influent substrate concentration the prey and predator growth are more stable and there is a small parameter space in which there is a limit cycle, regular oscillations in the substrate, predator and prey populations.

Figure 4.13 shows the predicted growth of the predator and prey populations under the conditions required to induce oscillations ($X0$= ~10^7 cells/ml, $Y0$= ~10^7 cells/ml, $S0$= 25mg/L. D=0.17 h^{-1}). As expected, *Pseudomonas* prey species grows in response to spikes in glucose, this increase in the prey species provides enough of a food source to the *Bdellovibrio* predator allowing its population to grow and decrease the prey species, this is in turn results in a recovery of the glucose concentration in the reactor. We recreated these conditions in the chemostat and recorded the abundance of predator and prey through time. It is apparent that the experimental results (Fig 4.14) are able to predict some oscillation and the oscillation of predator and prey are, reassuringly out of phase. However, the amplitude is an order of magnitude lower than in the simulations the period is twice as long.

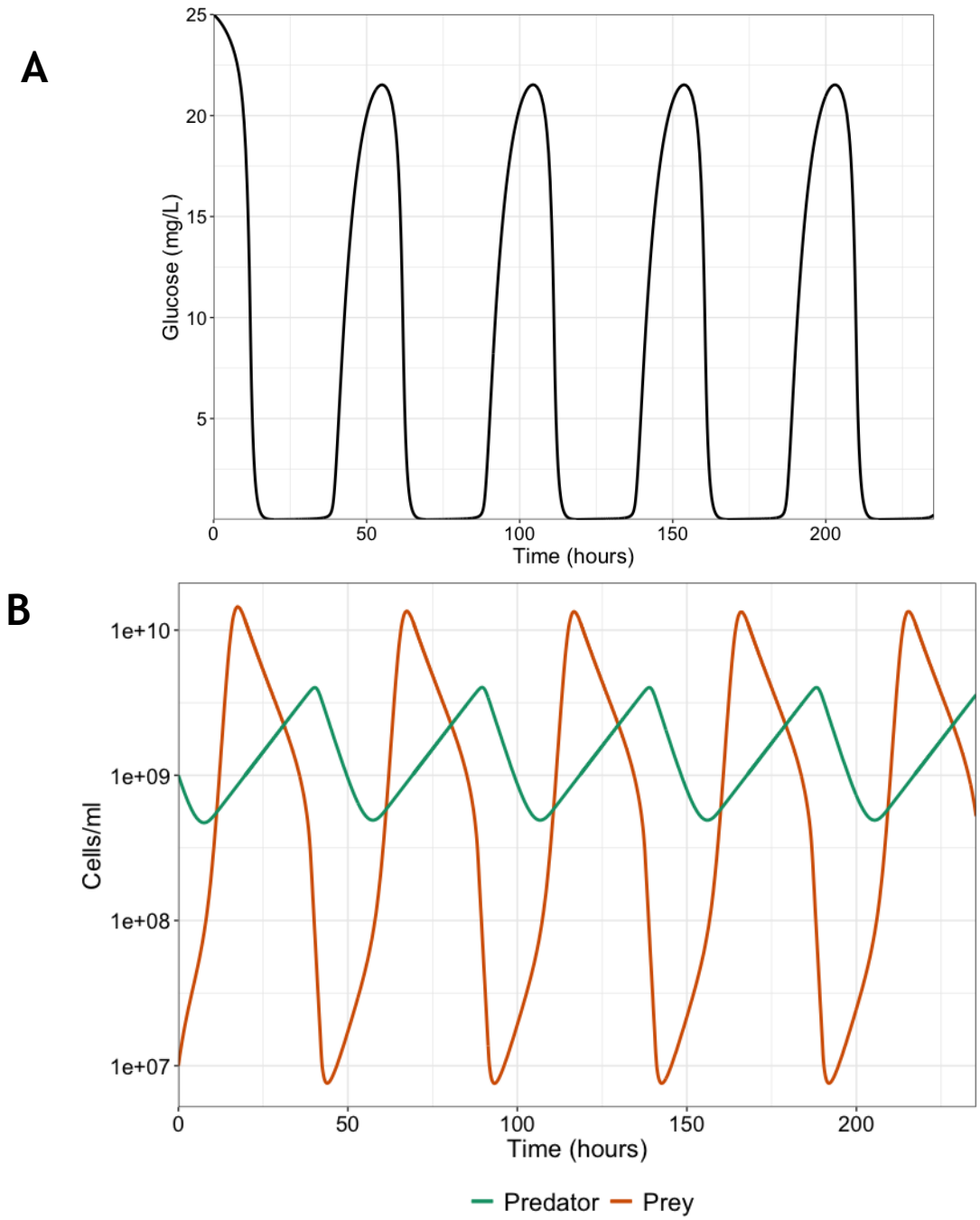

Figure 4.13- Model predicted transient response during chemostat culture. A) for glucose and **B)** for *Bdellovibrio* and *Pseudomonas* (cells/ml). Initial prey ($\boldsymbol{X0}$): 3.5 x 10^7 cells/ml, initial predator ($\boldsymbol{Y0}$): 6.55 x 10^7 cells/ml, and initial glucose substrate ($\boldsymbol{S0}$): 25mg/L., dilution rate (D): 0.17 h^{-1}.

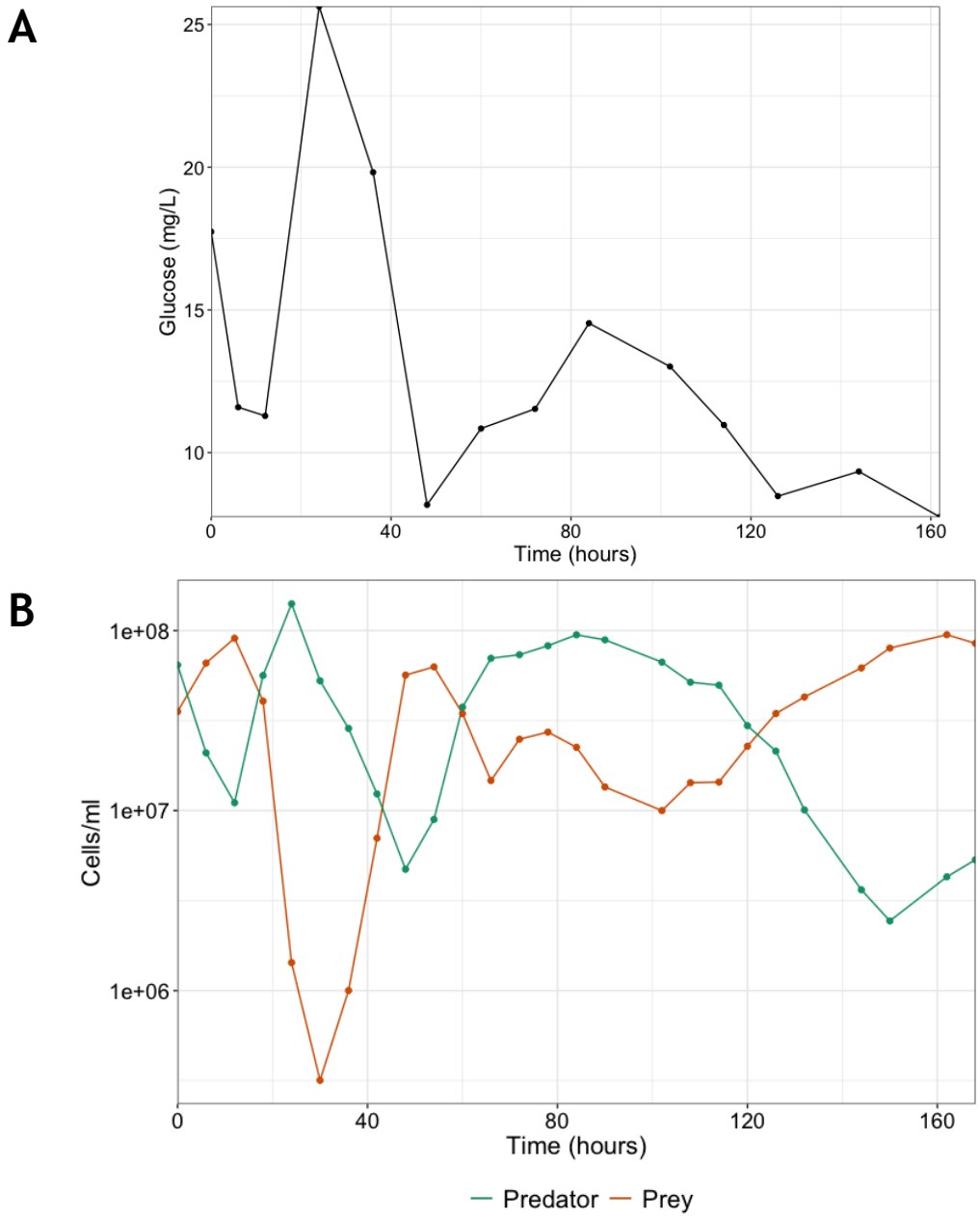

Figure 4.14- Experimentally observed transient response during chemostat culture. A) for glucose and **B)** for *Bdellovibrio* and *Pseudomonas* (cells/ml). Initial prey ($\boldsymbol{X0}$): 3.5 x 10^7 cells/ml, initial predator ($\boldsymbol{Y0}$): 6.55 x 10^7 cells/ml, and initial glucose substrate ($\boldsymbol{S0}$): 25mg/L., dilution rate (D): 0.17 h^{-1}.

There are a number of reasons why the experimental chemostat results might deviate from the simulation. It might be that our experimental determination of the model parameters was not completely accurate. To qualitatively assess the likelihood of this we subjectively adjusted parameters to see if we could create

simulated dynamics that better fit the experimental data. We were able to reduce the simulated cell abundances to values more in line with those in the experiment, the most significant value being the prey maximum growth rate. Reducing this value from 1.0 h^{-1} to 0.6 h^{-1} significantly reduced the maximum values of the populations observed in the model and made them more comparable to the experimental data (Fig 4.15).

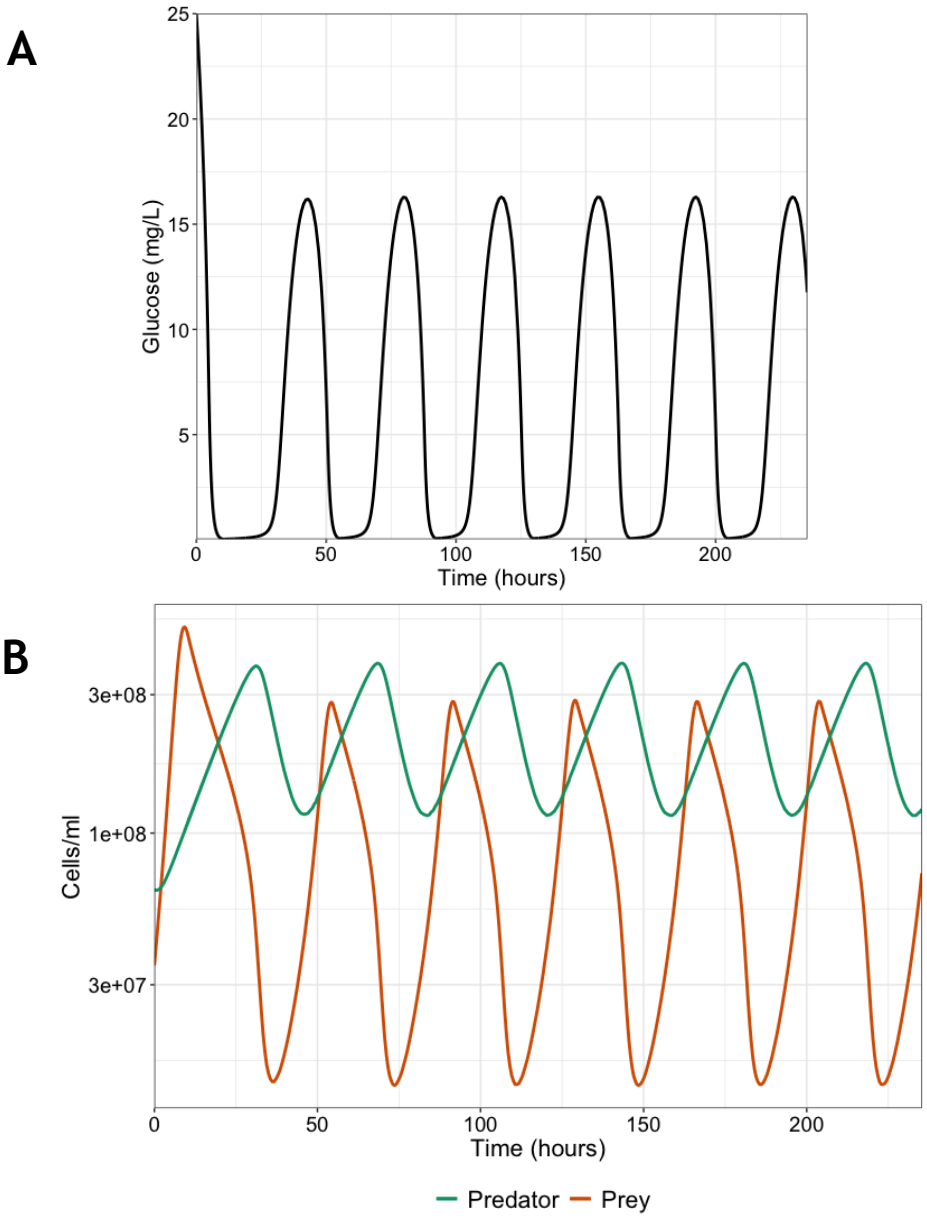

Figure 4.15- Altered model predicted transient response during chemostat culture. A) for glucose and **B)** for *Bdellovibrio* and *Pseudomonas* (cells/ml). Initial prey ($\boldsymbol{X0}$): 3.5 x 10^7 cells/ml, initial predator ($\boldsymbol{Y0}$): 6.55 x 10^7 cells/ml, and initial glucose substrate ($\boldsymbol{S0}$): 25mg/L., dilution rate (D): 0.17 h^{-1}. Growth parameters used were the same as previous models excluding the maximum prey growth rate: 0.5 $h^{-1,}$ Prey yield: 3.1 x 10^7 cells/mg of glucose and *Pseudomonas* prey saturation constant: 2.74 x10^{15} $cells^2/ml^2$.

As discussed, the model is not in fact capturing some of the important phenomena that are occurring in the experiment. It may be the assumption of a mixed

planktonic community is a gross oversimplification and that the bacteria are forming biofilms on the walls of the chemostat. Biofilm cells typically exhibit slower growth rates than their planktonic counterparts thus, this could explain why decreasing the maximum prey growth in the model yielded more comparable results to the experimental data.

To determine the effect of biofilm growth on the chemostat growth, the experiment was repeated with the similar conditions but instead the biofilm growth on glass slide was obtained and reported below. As before, the model is able to predict the oscillations, but it is otherwise inaccurate. Though there was biofilm found to be growing in the reactor, the total count was similar to the total planktonic bacteria growing in the bulk liquid at 72 hours (Table 4.2) and the total number of bacteria growing in the reactor still did not account for the overestimation shown in the model.

Table 4.2- Measurement of the free living planktonic bacterial cells in the bulk liquid and the bacterial cells present in biofilm attached to the reactor surface.

Species	State of cells	Number of cells
Bdellovibrio	Planktonic	1.23E+09
Pseudomonas		5.57E+10
Bdellovibrio	Biofilm	1.19E+09
Pseudomonas		4.03E+10
Bdellovibrio	Planktonic + Biofilm	2.42E+09
Pseudomonas		9.60E+10

4.3 Discussion

The objective of this study was to gain a quantitative understanding of the relationship between a simple predator-prey system including a single growth limiting organic nutrient.

Estimating the growth kinetics is difficult and there are inconsistencies in the reported values for growth kinetics. It has also been reported that microorganisms

can alter their kinetic properties because of the conditions of their environment such as temperature, pH, substrate used for growth, agitation speed (Ahmad and Holland, 1994). One of these conditions is the media used to grow the bacteria culture prior to it being used as an inoculum. It has previously been demonstrated that the growth media used to grow the inoculum can affect the bacterial growth and gene expression (Khan et al., 2013). Studies on the growth dependence on growth substrates such as glucose often grow the inoculum in LB media or some other high nutrient medium in order to maximise the growth of the inoculum (Bren et al., 2013). However, if switching environments in this way has a significant effect on the bacteria's ability to consume glucose it could result in discrepancies in the measured growth kinetics and reduce the reliability of the model. This study found that *Pseudomonas* relationship with glucose could be well described the Monod curve, despite the difference in inocula media.

As expected, the inoculum grown previously in the minimal media (0mg/L glucose) showed the lowest saturation constant and maximum growth rate. This is likely a result of the lack of nutrient reserves within the inoculum bacteria and indicate that the physiological state of the inoculum bacteria plays a role in glucose consumption.

Inoculum grown from nutrient rich LB broth during the pre-inoculation stage had slightly lower saturation constant and maximum growth rate values compared to the inoculum previously grown in the same minimal media composition pre and post inoculation. When switching from a complex media to minimal media post inoculation, it is likely that the inoculum bacteria require considerable adaptation before they can efficiently use the glucose carbon source to reach maximum growth rate. Whereas the inoculum grown previously in minimal media may require less adaptation time and can quickly make use of the glucose in the system. Nonetheless, with both inoculum conditions showing Monod behaviour and the differences in kinetics being subtle, LB was chosen as the inoculum growth media for *Pseudomonas* in the following experiments because it showed the maximum concentration reached after incubation and allowed for a more direct comparison to many previous studies on microbial growth kinetics.

Past literature into growth dynamics of *Bdellovibrio* feeding on prey bacteria often highlights that the analyses are limited in the methods available to measure the bacterial abundances. Here we move away from techniques such as measuring optical density or dry weight and instead use the flow cytometry as a more direct measurements of cell numbers (Ogundero et al., 2022). Using flow cytometry, we measured the growth of *Pseudomonas,* our prey population, in response to increasing concentrations of a single caron source, glucose. This relationship between growth of *Pseudomonas* and glucose concentration adhered to a Monod model (Fig 4.4), with maximum growth rate of 1.0 h^{-1} and a saturation constant of 0.18 mg/l for glucose. This is similar to values reported in previous literature. It should also be noted that the growth rate observed here is significantly higher than the maximum growth rate of *Pseudomonas* observed in the inocula study. It is likely that the low growth rate was caused because sealed microplate well in which the culture was placed, do not provide significant mixing and oxygen exchange compared to shaking conical flasks (Chaturvedi et al., 2014).

The experimental data shows that the *Pseudomonas* was able to grow in the absence of glucose, which cannot be replicated by a Monod growth model, which assumes a single limiting resource and thus zero resource mean zero growth. Thus, it suggests that, despite our best efforts, glucose was not the only growth limiting substrate present. We used casein hydrolysate as a nitrogen source in the media, which may have been utilised for growth.

Many studies select yield and saturation constant values from previous literature rather than obtaining them experimentally, which is difficult to do because it requires measurements of glucose at the microgram per litre range (Nielson et al. 2003). Here, assay kits that allow specific substrates to be quantified by absorbance reading, enabled us to measure the degradation of the glucose substrate when provided to *Pseudomonas*. The cell yield coefficient is defined as the unit amount of cell mass produced per unit amount of substrate consumed. By measuring both the direct cell count and the glucose concentration during the batch culture run we observed an average yield of 3.1 x 10^7 *Pseudomonas* cells/mg of glucose. This value is important in predicting the growth of bacteria as the

higher the value, the more efficiently a substrate is determined to be degraded and the higher the maximum population is reached. Yield coefficients dependent on the nature of substrate being degraded and the intrinsic physiological mechanisms of the bacteria.

A bottleneck in many previous studies has been an inability to accurately measure both *Bdellovibrio* and their prey cell numbers simultaneously. Typically, with *Bdellovibrio*, predation is solely measured through the depletion of the prey (measured in turn by optical density or plate count) or the predator numbers are determined through the use of plaque forming assays which is tedious and prone to error (Ogundero et al., 2022). Using flow cytometry, the predator and prey populations can be directly quantified.

The growth of *Bdellovibrio* on increasing concentrations of prey is displayed as a sigmoid curve which is best described by the Holling type III function. The *Bdellovibrio* are unable to show significant population growth at low prey concentrations and only show an increase in growth rate at prey populations of 10^8 cells/ml. Indeed, there is a lot of evidence *Bdellovibrio* found predominantly in environments of high prey density such as biofilms and downstream sewage and water treatment systems (Staples & Fry 1973).

This could be the result of a number of traits of *Bdellovibrio* predation. Firstly, the predator is thought to show no specific searching or chemotaxis mechanism in hunting their prey. Instead, the predator relies on speed and high frequency ‘random’ collisions with the prey cell wall to engage in attachment and subsequent invasion of the periplasmic space (Straley and Conti, 1977).Thus, it is likely that at low enough prey densities the chance of a significant successful collisions in the population is extremely low and the trade-off between energy used to find prey and the energy received from predation for the production of new progeny is no longer favourable. As *Bdellovibrio* cells age without being able to invade a prey cell, the cell becomes slower which further decreases the chance of successful collisions.

Additionally, at high predator to prey ratios, multiple predators can attach to a single prey cell causing the prey cell to burst prematurely. It is possible that if enough of these events happen at low prey density, it prevents the predator cells from invading and replicating inside of the prey cell, so as a result the population doesn't significantly increase. But at high prey densities, there is less premature prey lysis and the single predator cells are able to easily find a single prey cell to replicate within. This could also help explain why at the lower prey concentrations the prey shows a large drop in population faster than in the higher concentrations where the significant decrease is delayed (Fig 4.5).

Using the simple model for predator and prey dynamics we simulated the population dynamics in four different scenarios of batch growth, with different initial conditions of bacteria numbers and substrate concentrations. We then experimentally reproduce the conditions that had been simulated and measure the numbers of bacteria and the substrate concentration through time. There was a good match between the experimental measurements and the model predictions in scenarios where the glucose concentration was more than 5mg/l. For scenarios where the glucose concentration was lower than 5mg/l there was a poor correlation. This poor correlation is a result of the inability of the Monod model to capture the observed growth at very low substrate concentrations due to the casein hydrolysate (nitrogen source) added to the medium, which has a knock-on effect on prey and subsequently predator populations in the simulations.

Using the calculated parameters in a model created with equations 4.13, 4.14 and 4.15 in MATLAB, we mapped the combinations of initial conditions for glucose concentration in the influent and, predator and prey population in a chemostat that would lead to a range of different dynamics: prey and predator washout, predator washout, stable co-existence and limit cycle oscillations. As expected, the potential for predator washout is the largest of the outcomes as it slow growing in comparison to the prey so even moderate dilution rates remove all the predator before it can significantly grow its population in the reactor. In contrast, the prey has a much faster growth rate and is able to grow at high dilution rates. If using *Bdellovibrio* in an applied manner, for example, to control the bacteria

numbers in a biofiltration system then it would be important to characterise the potential for predator washout and thus choose dilution rates that ensure the predator presence in the system.

Predator washout would require re-inoculation of the system but being able to keep the predators growing in a limit cycle would keep the system more self-sustainable and efficient. The potential for predator prey oscillations is comparably smaller in the chemostat we describe, which is to be expected as the prey's growth is reliant on one substrate and the action of one predator. Whereas in a natural system with multiple sources of organic substrates and potentially multiple prey and predator species, limit cycles are more commonly observed (Wilkinson, 2006).

To test the fit of the model we ran a chemostat for 168 hours with initial conditions that would lead to limit cycle oscillations: Initial prey ($X0$): 3.5 x 10^7 cells/ml, initial predator ($Y0$): 6.55 x 10^7 cells/ml, and influent glucose substrate ($S0$): 25mg/L., dilution rate (D): 0.17 h^{-1}. As expected, the predator and prey populations exhibit regular oscillations. Though previous studies have theoretically demonstrated this (Summers and Kreft, 2019), we demonstrate the first experimentally observed oscillations in *Bdellovibrio* and their prey. The observation and understanding of this phenomenon is necessary to unleash the true potential of *Bdellovibrio* as a self-sustainable solution to mitigate biofilms in decentralised systems. Unlike the addition of chemicals such as chlorine, repeated dosage would not be as necessary or as frequent, as the predators would continue to grow on the remaining prey. Additionally, both predicting and inducing oscillations could aid in the identification of key 'low' points in the prey population where chlorine dosing could be considered more efficient, maximising the potential to mitigate biofouling while minimising the harmful effects of frequent chlorine usage such as resistance development.

Using the model in application with the ability to control the initial parameters ($X0$, $Y0, S0$ and D) required, would allow for the induction of predator prey oscillations in a decentralised membrane system, with relative ease and without

the need for extensive modelling knowledge. The model also removes the need for trial-and-error experimentation to search for this precise parameter space. Thus, the model increases the speed and efficiency as well as reducing the time and cost associated with all the extra experimentation.

The extent to the predator growth and the action of prey removal over time can also be predicted with models such as the one in this study in order to optimise and maintain the process. Unfortunately, the chemostat model could not accurately replicate the regularity of the oscillations as well as the maximum and minimum populations reached.

One potential reason for this is that the *Pseudomonas* prey additionally grows as a biofilm on the glass surface of the reactor. *Pseudomonas* species has been shown to grow significant biofilm in chemostats in as little as 12 hours (Jass et al., 1995). Compared to the planktonic bacteria, bacteria growing in biofilm can consume significant nutrients from the bulk liquid while typically displaying a slower growth rate and is not as easily removed from the reactor by washout. This biofilm cannot be quantified by sampling from the effluent and can reduce the measured total count of the bacteria in the reactor. Additionally, the predator *Bdellovibrio* does not form biofilm but has been shown to be able to move and predate within biofilms. This is less of a concern in batch studies where there is not a constant supply of nutrients to allow for significant biofilm development and planktonic bacteria remain in the system and are not removed by dilution.

To investigate the effect of biofilm growth on the validity of the model, we repeated the chemostat experiment for 72 hours, and at the end of this period also measured the growth of biofilm on a glass slide which was then used to approximate the total bacterial growth in the reactor.

We demonstrate that the prey species does grow as biofilm attached to the glass surface and that *Bdellovibrio* can be found within these biofilms. The bacterial total count in the bulk liquid is similar to the total count attached to the reactor

surface for both the predator and the prey, though it is not confirmed whether the biofilm to planktonic bacteria ratio changes over time.

The presence of the *Pseudomonas* biofilms on the chemostat wall means that our chemostat model, where all bacteria are assumed to be planktonic and the population is continually being depleted by bacteria leaving in the effluent, is flawed. In order to have a chance of capturing the behaviour in the chemostat then the equations would have to be augmented with ones that explicitly model the biofilm population and action of the predator on the biofilm. The rate at which bacteria attach and detach would be required and it could not be assumed that the growth kinetics of the predator and prey in biofilms would be the same as for planktonic populations. It is known that planktonic bacteria and biofilms exhibit phenotypic differences and respond to environmental conditions differently.

Moreover, as the chemostat observes oscillations in nutrient concentration, it is expected that the biofilm will respond in changes to their physical and chemical structure as well as the total biomass present within the biofilm. So, whilst it is tempting to construct a mathematical model that has a representation of the biofilm to see if the observed dynamics could be reproduced, it would comprise a large number of parameters that we have not derived experimentally. Merely calibrating parameters would not allow us to determine whether the model was really capturing the processes at play. The premise of this chapter was that if we measure key parameters in advance, we could predict dynamics. Thus, it is suffice to say that our chemostat model is missing representations of critical phenomena and we have strong circumstantial evidence that it requires explicit representation of the biofilm.

Thus, moving forward, in future research, we need to characterise the kinetics of our predator and prey in biofilms. Indeed, a biofilm model will ultimately have more practical application than the chemostat model, our rationale was that the chemostat was a simpler system to begin to observe predator prey dynamics. Indeed, the complexities of biofilms are such that a much more intensive experimental programme would be required. For example, it has been reported

that at higher nutrient concentrations, oxygen and waste can accumulate creating unfavourable conditions that can lead to the detachment of *Pseudomonas putida* biofilms. Whereas lower nutrient concentrations didn't allow for an over accumulation of density and resulted in more compact structures (Salgar-Chaparro et al., 2020). The structure of biofilms is key when considering how they develop in response to growth limiting nutrients as they are heterogenous and contain pores and channels that allow for diffusion. In thick or compact biofilms there is lower diffusion and less nutrients delivered to bacteria deeper within the biofilms and likewise there is less removal of waste products. These bacteria are slower growing and less metabolically active than both planktonic bacteria and bacteria on the outer surface of the biofilm. All of these behaviours might have an effect on the dynamics.

Similarly, progeny predatory bacteria that are developing within bdelloplasts from biofilm forming bacteria may be less likely to be removed from washout than their counterparts in the bulk liquid. Retaining these predators within the system can then lead to a further unexpected decrease of the prey bacteria within the biofilm and the bulk liquid. There is little information on how *Bdellovibrio* predation is affected by biofilm structure but there have been many studies that have demonstrated that thicker, less porous biofilms prevented the diffusion of antibiotics and limits the predation of bacteriophages to the upper regions of biofilms (Kim et al., 2009).

The pursuit of a biofilm predator-prey model with experimentally quantifiable parameters is an avenue of research that warrants the substantial experimental effort required. It has the potential to inform the use of *Bdellovibrio* in membrane filter treatment systems, and predict conditions that would induce oscillations such that biofilms are kept at bay by predation without the need for repeated dose with fresh predator populations (Feichtmayer et al., 2017).

In conclusion, the study utilized a model of nonlinear ordinary differential equations to express the interaction between *Bdellovibrio* predators, *Pseudomonas* prey and glucose. Using experimentally obtained growth parameters, the model

was able to predict the dynamics of *Bdellovibrio* predation in batch. The prey bacteria growth on a substrate adheres to Monod kinetics and the *Bdellovibrio* predator growth on prey adheres to the Holling type III functional response, which could explain why *Bdellovibrio* is predominantly found in high prey density environments such as biofilms. Using the parameters in a chemostat model we were able to predict the parameters needed to cause different system behaviours including predator prey oscillations. Oscillations were induced in the continuous system, but it was unable to accurately duplicate the predicted concentrations. Biofilm growth which was found to be significant within the continuous system is likely to have a profound effect on the system. Capturing the dynamics in a verifiable, validated model would require a substantive experimental programme. Nonetheless, the ability to induce predator prey oscillations is vital in the application of *Bdellovibrio* as a self-sustainable biocontrol.

5 Development of a multi-parametric well plate assay to measure biofilm growth in response to *Bdellovibrio* predation

Current membrane technologies represent a more environmentally and economically sustainable method for cleaning drinking water (Guo et al., 2012). However, they are yet to be used on a larger scale because it is susceptible to unwanted biofilm growth and deposition that reduces the operational performance (Chen et al., 2010). Biofilms are a problem in many water distribution systems and can result in undesired odour or taste, deterioration and clogging of pipes and system materials (Guo et al., 2012).

Bdellovibrio and like organisms are small and motile gram negative bacteria that invade the periplasmic space of other gram negative bacteria to consume the host's macromolecules for growth (Jurkevitch, 2006b). Many studies have investigated their ability to kill bacteria and demonstrated both its ability to clear biofilms and to kill pathogenic bacteria that make it a potential biological control against biofilm growth in membrane-based water treatments (Kim et al., 2013). Most of these investigations have focused on fundamental aspects of understanding *Bdellovibrio* predator/prey dynamics and further insight into this will be enable more effective explorations to the use of *Bdellovibrio* in water treatment (Feichtmayer et al., 2017).

The previous chapter demonstrated the importance of mathematical models in capturing the dynamics that determine prey cell growth and interaction with *Bdellovibrio* predators. That chapter focused on testing models for planktonic communities as a precursor for the more complicated case of biofilm communities, where the distribution in space of predators, prey and resources becomes important.

A wide range of models have been developed in the past to describe biofilm growth (Wang and Zhang, 2010). Many of these models evaluate biofilm growth based on the rate of utilization of the growth-limiting nutrient. However, there are many examples of the biofilm activity being modelled based on the rate of other substances such as antibiotics and organic compounds of interest. One commonly used biofilm model is the one-dimensional Wanner–Gujer model (Wanner and Gujer, 1986). It uses mass conversion principles, and the biofilm growth is determined by substrate consumption, diffusion of molecular substrates and biomass detachment. The various approaches to modelling biofilms are reviewed by McBain (McBain, 2009). To understand and quantify biofilm growth under *Bdellovibrio* predation, it is first necessary to understand the key factors affecting biofilm growth. Therefore, this chapter begins by reviewing critical properties and processes in biofilms development such as, biofilm density and thickness, biofilm attachment and extracellular polymeric substances (EPS).

Like the Monod model, key aspects of biofilm models include the yield coefficient and maximum growth rate. Models such as Monod that describe the growth of planktonic bacteria on a single substrate in bulk liquid often do not take into account the diffusion of the substrate as it is assumed that the environment is well mixed and bacteria have equal access to nutrients (Wang et al., 2015).

Diffusion is more important when modelling the growth of biofilms as biofilm growth is separate from growth in the bulk liquid. Nutrients and other substrates must travel from the bulk liquid to the surface of the biofilm as described by film theory and then diffuse through the biofilm in a linear fashion as described by Fick's second law (Wang et al., 2015). Likewise, diffusion is important in removal of waste products from the biofilm. This diffusion coefficient is largely affected by the viscosity of the liquid, biofilm density and thickness, and the extracellular polysaccharide (EPS).

Simple and ideal biofilm models view the biofilm as a continuum which disregards the discrete nature at the scale of individual cells and considers the biofilm to be uniformly distributed in the matrix and in the case of 1D models, uniform in its

thickness. These can help simplify the process of biofilm growth such as diffusion into a number of quantifiable parameters. However, in reality, the heterogenous nature of biofilms, means that nutrients and oxygen can reach deeper parts of the biofilm faster where pores in the biofilm are present. Cells on the outer parts of these pores are likely to show accelerated growth in comparison to other cells deeper in the biofilm (Dufour et al., 2010). This is more difficult to model, however.

Diffusion within biofilms could have relevance in regard to predation. *Bdellovibrio* show very little signs of displaying chemotaxis and their method of prey hunting is driven by random collision which is influenced by the flow in an environment (Strauch et al., 2007). The flow of nutrients and removal of waste in a biofilm creates pores that allow deeper access to the biofilm for *Bdellovibrio*. This could give rise to a feedback loop whereby the flow of nutrients and *Bdellovibrio* affects the structure of the biofilm which then in turn affects the flow of *Bdellovibrio*.

Resistance to antimicrobial agents has been the most extensively investigated feature of biofilms (Stewart and William Costerton, 2001) and has been attributed to factors such as the exopolysaccharide matrix providing an effective barrier that restricts penetration by limiting the diffusion rate of the antimicrobial agents (Drenkard, 2003). In some antibiotic treatments, a reduced diffusion rate may only delay the clearing of the biofilm. However, this is not observed in biofilms such as those formed by *Pseudomonas aeruginosa*, a key human pathogen in water treatment biofouling. The bacteria is well known for its ability to form biofilms with large quantities of EPS, a trait that limits the diffusion rate sufficiently to reduce the concentration of incoming antibiotics (Ibrahim et al., 2001). Furthermore, at lower antibiotic concentrations enzymes such as ß-lactamases can successfully degrade the antibiotics, ensuring survival (Giwercman et al., 1991).

Unlike with antibiotics, there is little evidence that gram negative bacterial species develop resistance to *Bdellovibrio*, but even without such resistance or means to kill the predatory bacteria, EPS could cause a delay in the delivery of *Bdellovibrio* and other predators through the biofilm by limiting the penetrability,

which could allow time for regrowth of bacteria and could have confounding impacts on the operational performance of the water treatment system (Chen et al., 2010).

EPS is also shown to be key in the initial attachment of planktonic cells to surfaces as well as influencing many functions associated with biofilm growth (Drews et al., 2006). The effect of predation on early biofilm formation has yet to be thoroughly investigated and the presence of *Bdellovibrio* could promote EPS and biofilm formation as it would provide a stable source of prey as well as refuge from stresses.

In general, EPS can be quantified using fluorescent lectins; proteins that binds to specific polysaccharides. One of the most common is the commercially available wheat germ agglutin-Alexa Fluor 488 fluorescent conjugate (WGA) which binds to poly-N acetylglucosamine (PNAG) residues(Skogman et al., 2012). PNAG residues are produced by many bacteria including *E. coli* and *Pseudomonas* and have been reported to be a crucial component of bacterial attachment and biofilm persistence as well as increasing protection against certain antibiotics (Colvin et al., 2011).

Idealised biofilm models also do not take into account the growth of the planktonic cells in the bulk liquid (Wang and Zhang, 2010). This is an important factor when modelling biofilm growth in engineered systems as the planktonic and biofilm bacteria are not likely to react in the same way but do interact and influence the growth of one another. For instance the planktonic bacteria suspended in the bulk are likely to have a higher and more constant rate of substrate removal when compared to the bacteria present in the inner layers of the biofilm (Revilla et al., 2016).

Additionally, the growth of the biofilm, which is a large determinant of spread, is increased by the attachment of planktonic bacteria to pristine surfaces to start a new biofilm as well as in its attachment to already existing biofilm. The rate at which this occurs is vital for the overall modelling of biofilm growth in an active

bioreactor as well as the potential early biofilm formation inside a clean bioreactor. Adhesion has been shown to be influenced by nutrient availability, surface conditions and shear stress (Lu et al., 2004).

Attachment and growth of a biofilm is counteracted by biofilm detachment, as shown in biofouling, and can occur due to liquid shear stress or sloughing. This can be very difficult to observe and quantify so there is a lack of knowledge on the process, and it is also often omitted from biofilm models. Detachment in biofilms can also be caused by predator grazing but the effect of predation by *Bdellovibrio* on biofilm attachment has not been adequately investigated (Derlon et al., 2012).

Crystal violet is a common stain for assessing antimicrobial activity on biofilm growth. However, this method only stains adherent cells, planktonic cells are often ignored in measurements despite the fact they can recolonise the surface. In regards to predation, bacteria show a level of temporary phenotypic resistance, this development may result in a temporary disadvantage to bacteria such as reducing its ability to adhere to surfaces or to other cells in biofilm (Shemesh and Jurkevitch, 2003). Flow cytometry has previously been shown to be a reliable and rapid tool to measure predatory and their bacteria in culture (Ogundero et al., 2022) and so could be an alternative screening tool to measure biofilm and planktonic bacteria cultured with *Bdellovibrio*. This method is more informative than crystal violet staining and could additionally provide an estimation of cell size based on the forward scatter reading, and the ability to measure intact cells, whereas crystal violet can only provide one value for the whole well and does not distinguish between the live, dead cells and matrix within the biofilm.

Given the lack of understanding as to how biofilm heterogeneity can affect *Bdellovibrio* predation, the emphasis of this chapter is placed on the investigation and discussion of novel and high-throughput experimentation that can be used to study the effect of *Bdellovibrio* predation on key biofilm parameters typically used in modelling. Though, it is not intended as a description or validation of a biofilm model it can be used to aid the development of such in the future.

To do so we develop a static microplate assay to evaluate the capability and dynamics of *B. bacteriovorus* predation of *Pseudomonas* biofilms when the biofilm is previously grown in a nutrient rich (LB), nitrogen rich (HEPES buffer supplemented with casein hydrolysate), carbon rich (HEPES buffer supplemented with glucose) or a nutrient poor (HEPES buffer) media. Staining of the biofilm (by crystal violet and resazurin) and the biofilm matrix (by WGA), and direct enumeration of the biofilm and planktonic cells by flow cytometry were used to assess the effect of nutrient availability on biofilm formation and predation.

We aim to demonstrate the assays' ability to be more informative than the typical crystal violet assays alone in regard to assessing the anti-biofilm capability of *B. bacteriovorus*. Such an assay could also be used as a high throughput method to investigate predatory capability in different environments and with different prey species, in similar fashion to assays typically used in pharmaceutical settings (Skogman et al., 2016).

We hypothesise that the methods of flow cytometry and the resazurin assay will show a high correlation with crystal violet for quantifying biofilm growth. We also hypothesise that the *Bdellovibrio* predation will have different effects on the growth of the total and intact bacteria of the biofilm, the EPS production and the quantity of planktonic cells in the bulk liquid depending on the nutrients available to the biofilm during initial growth.

5.1 Methods

The wild-type *Bdellovibrio bacteriovorus* strain HD100 (DSM no. 50701) was used throughout this study and was grown by predation on *Pseudomonas sp.* (DSM no. 50906) using standard culturing methods (Herencias et al., 2017). *Pseudomonas* cells were grown in LB broth at 30°C for 16 hours and resuspended in supplemented Ca/Mg-HEPES buffer (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, pH 7.6) to an optical density (600nm) value of 10. *B. bacteriovorus* was then grown on this suspension in DN broth for 24 hours then sub-cultured twice at 24-hour intervals in in Ca/Mg-HEPES buffer. To remove the prey and harvest the

predator alone for growth experiments each culture was filtered through a 0.45µm pore size syringe filter (Fisherbrand™, product code: 15216869).

5.1.1 Biofilm microplate study

Pseudomonas was grown as described above and then diluted to 10^6 cell/ml. This suspension was added to a microplate well diluted in 180ul of different media; LB, Ca/Mg-HEPES buffer supplemented with casein hydrolysate (0.05%), Ca/Mg-HEPES buffer supplemented with glucose (20mg/L) and Ca/Mg-HEPES buffer alone. The suspensions were grown at 30°C for 24 hours. In replicate plates, the media and planktonic cells were then removed, and the biofilms were grown for a further 24 hours in Ca/Mg-HEPES buffer alone or with 10^5 cells/ml of *Bdellovibrio* suspended in Ca/Mg-HEPES buffer. The biofilm was analysed at 24 hours and 48 hours using crystal violet, resazurin, WGA and flow cytometry intact and total cell count.

5.1.1.1 Crystal violet and resazurin assay

Crystal violet and resazurin and staining was used as an indirect method for the total and viable number of cells within the biofilm. To begin, the planktonic cells were removed from the biofilm by pipetting 300µl of the media out. The biofilms were then washed once with phosphate buffer solution (PBS) and stained with 50µl of 20mM resazurin for 20 mins at room temperature and in the dark and at 200rpm. Fluorescence was then measured at 560nm.

To reduce waste and make the process more efficient, the same biofilm was directly measured with crystal violet as a counterstain. Such methods have been used previously (Skogman et al., 2012). The resazurin stain was removed and crystal violet was added to stain for 5 minutes at room temperature and in the dark. The stained biofilm was then washed once with PBS and then solubilized with 300µl of 33% acetic acid. Absorbance was then measured at 595nm.

5.1.1.2 WGA assay

Using a replicate plate, the biofilms were grown under the same conditions. Planktonic cells were removed, and the biofilm was washed once with PBS. Then 200 µl of 10mg/ml WGA (WGA-Alexa Fluor 488 fluorescent conjugate, Molecular Probes Inc., Eugene, OR, USA) in PBS was added to the biofilm and stained for 2 hours at 4C in the darkness. The stained wells were then washed once with PBS, air-dried for 15 minutes and then solubilized with 300µl of 33% acetic acid. Plates were then covered with a seal, sonicated for 30 seconds and then incubated at 37°C. The plate was then sonicated again for 30 seconds and measured for fluorescence at 495/520nm.

5.1.1.3 Flow cytometry

A third replicate plate was also used to measure planktonic and biofilm growth using flow cytometry. The planktonic bacteria in the media were removed from each well and then prepared for flow cytometry total and intact count as described below. To remove the biofilm attached to the well surface, the well was washed once with PBS and then sonicated for 1 min. The PBS/Biofilm mixture was then removed and prepared for flow cytometry total and intact count as described below.

To determine the optimal regime for biofilm removal, samples of biofilm were grown in a well plate and then sonicated for different length of time (1-10 minutes). The concentration was calculated by agar plate count. The biofilm removal procedure that delivered the highest cell count (CFU/ml) was chosen in this study (data not shown).

Samples for flow cytometry were prepared by fixation with glutaraldehyde (1% in DI water) and where necessary diluted to achieve an events per second reading of less than 600 on the flow cytometer. Dilutions were made in filtered (0.22 µm Sartorius™ Minisart™ Plus Syringe Filters, Fisher scientific, product code: 10730792) DI water. Samples were stained for both the total and the intact cell count.

For total cell count: the samples were each stained with 10 µL/ml of SYBR Green I (SGI) and incubated in the dark for 13 minutes before measurement. Stock solutions of SGI (10,000 x in DMSO, Thermofisher, product code: S7653) were diluted 1:100 in EDTA (1 mM).

For intact cell count: the samples were stained with 10 µL/ml of SYBR Green I/Propidium iodide (SGI/PI) and incubated in the dark for 13 minutes before measurement. Stock solutions of SGI (10,000 x in DMSO) was mixed with PI (1.6mM) and diluted 1:100 in EDTA (1 mM) for a final concentration of 0.6mM PI and 100x for SGI.

Gating was used to distinguish selected signals (*B. bacteriovorus* and *Pseudomonas*) from each other and from the background (inorganic and organic particles) with the aid of negative controls consisting of the DI water used for dilutions, HEPES buffer used for growth, and a sample of the predator/prey co-culture further filtered (0.22 µm) to remove any bacterial cells.

5.1.2 Statistical analysis

The statistical analysis was performed using R software. Pearson's correlation coefficient (ρ) was calculated to find the relationship between cell quantification by crystal violet assay and the other methods of resazurin, wheat germ agglutin (WGA) assay, and total and intact cell count by flow cytometry. Linear regression models were used to find the significant differences in measurement between crystal violet and the other methods tested.

The Kruskal-Wallace test was used to find the significant difference between biofilms grown for 24 hours in different media.

The Wilcoxon rank test was also used to find the significant difference between the growth of biofilms pre grown in different media and exposed to *Bdellovibrio* or HEPES media as a control. The Wilcoxon rank test was also used to find the

significant difference between the percentage of cells growing in the well as biofilm (as measured by flow cytometry) when exposed to *Bdellovibrio* or HEPES media as a control.

Measurements were based on one biological repat with four repeats for crystal violet, resazurin and WGA and three repeats for the total and intact cell counts by flow cytometry.

5.2 Results and discussion

5.2.1 Static well plate assay

The growth of biofilms has previously been shown to be affected by the environmental conditions such as the nutrients available (Straub et al., 2020). There is little information on how the growth of biofilm in different conditions influences *Bdellovibrio* predation. We investigated this through the use of 96 well plate staining methods, which are useful end point measurements.

Crystal violet is commonly used as an indirect method for measuring biofilms in well plates. A greater understanding of the heterogeneity of biofilms, requires alternative methods such as flow cytometry and fluorescent lectin assays that are more specific and can offer more accurate quantification and characterisation. Doing so could also give insight into the methods by which *Bdellovibrio* can inhibit biofilm growth and help guide the development of prey-predator models in engineered systems.

We assessed the correlation of crystal violet with total and intact cell counts by flow cytometry and the spectrofluorometric assays of WGA and resazurin (Fig 5.1). The correlation analysis showed a significant positive relationship between crystal violet and resazurin (correlation coefficient (ρ): 0.984, P-value<0.001, R^2 0.966), the flow cytometry total cell count (correlation coefficient (ρ): 0.923, P-value<0.001, R^2 0.837), and the flow cytometry intact cell count (correlation coefficient (ρ): 0.925, P-value<0.001, R^2 0.841). This demonstrates the validity of

these measurements for assessing the cell density within biofilms as an alternative to crystal violet. The is an important clarification as flow cytometry has the benefit of being a direct cell count that is necessary when modelling the growth of biofilms. Additionally, flow cytometry can be used to measure other aspects of biofilm such as the average forward scatter, which is a proxy to cell size, the ability to measure planktonic cells in the well and the ability to sort cells within the biofilm for further observation.

The resazurin assay showed a slightly higher correlation to crystal violet than the flow cytometry counts but this is as expected as the resazurin and crystal violet assays were performed on the same biofilms. Resazurin is non-toxic to bacterial cells so there is the potential to use this assay as an alternative to crystal violet which is destructive, allowing for further analysis and increasing the throughput of anti-biofilm assays (Skogman et al., 2016).

In contrast, there was no correlation between the crystal violet assay and the WGA assay (correlation coefficient (ρ): -0.189, P-value:0.5, R^2= -0.039). This is expected as the EPS is a small part of the biofilm in comparison to the bacterial cells. Especially when the biofilm was grown in HEPES with casein hydrolysate, there was a higher number of EPS compared to the cell count than when the biofilm is grown in LB. This highlights that the WGA assay cannot be used specifically to solely assess the quantity of a biofilm but instead acts as a useful tool to characterise and model the biofilm in addition to other assays that measure the cell quantity.

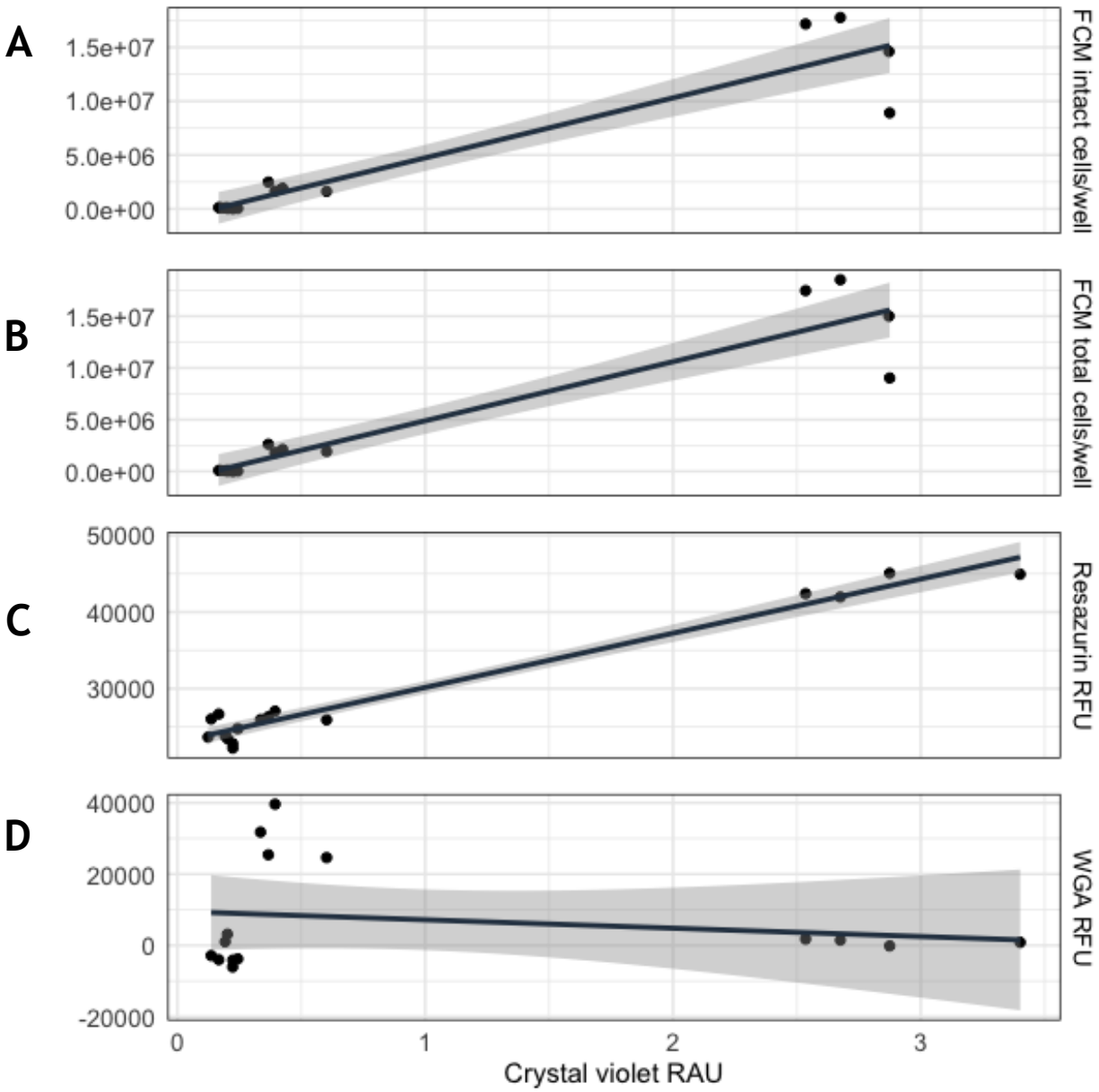

Figure 5.1- Correlation between the endpoint signal generated by crystal violet assay and other biofilm quantification methods. A) Intact cell count by flow cytometry (Pearson's correlation coefficient: 0.925. P-value<0.0001. R^2= 0.844), **B)** total cell count by flow cytometry (Pearson's correlation coefficient: 0.922. P-value<0.0001. R^2= 0.837), **C)** resazurin (Pearson's correlation coefficient: 0.984. P-value<0.0001. R^2= 0.966.) and **D)** WGA (Pearson's correlation coefficient: -0.189. P-value=0.5. R^2= -0.039). RFU stands for relative fluorescence units and RAU stands for relative absorbance units. Each point represents an observation from a biofilm grown in one well.

The study showed that the growth of *Pseudomonas* biofilm after 24 hours differed depending on the media it was grown in (Fig 5.2). Biofilms grown in nutrient rich LB showed a significantly higher value than biofilms grown in HEPES and HEPES with glucose (p-value<0.05) when measured by crystal violet and a significantly higher value than biofilms grown in HEPES when measured by resazurin and cell count by flow cytometry(p-value<0.05). This is expected as LB medium is developed for optimum growth of many bacterial species including *Pseudomonas*.

The lowest value across these methods was measured for the biofilm grown in HEPES buffer which contains no nutrients for growth. Interestingly, similarly low values were shown in the biofilm grown in the HEPES buffer with glucose, but this was deemed not be statistically significant despite the difference in concentration being two orders of magnitude. There was higher biofilm growth when grown in HEPES buffer with casein hydrolysate, suggesting that *Pseudomonas* biofilm growth is more dependent on a nitrogen source than carbohydrate source, though the difference between biofilms grown in the two media was also not deemed to be statistically significant when measured by crystal violet, resazurin, and flow cytometry.

The WGA assay showed that the most EPS was produced in biofilms grown in HEPES buffer with casein hydrolysate, even more so than in LB grown biofilms and significantly higher than biofilms grown in HEPES (p-value<0.05). This differs from previous reports of *Pseudomonas* biofilm grown under high nutrient conditions shown an increase in biofilm thickness, biomass and EPS content. The WGA assay method used in this study could underrepresent the total quantity of EPS in biofilms as it quantifies and binds specifically to PNAS, an adhesive protein that has been shown to be vital in the early attachment of *Pseudomonas* biofilms. Though this is thought to make up a large portion of biofilms, there have been many studies that have demonstrated the composition of EPS can change depending on the growth conditions (Molobela and Ilunga, 2012). *Pseudomonas* biofilms grown at higher nutrient concentration can display increased oxygen limitation and waste accumulation as the bacteria reach high concentrations, resulting in an increased amount of detachment from the biofilm (Rochex and

Lebeault, 2007). Thus, it could be that as the biofilm sheds in a nutrient rich LB, there is less need to synthesis PNAS to attach to surfaces.

When growing in HEPES with casein hydrolysate, the conditions are less optimum for outright growth and so attachment to the surface in early biofilm colonisation is more important. EPS also can serve as sources of nutrients for biofilm growth allowing bacteria to display growth even in hostile environments (Ansari et al., 2012). The increased amount of EPS in the biofilm grown in HEPES with casein hydrolysate could explain why there was more growth here than when grown in HEPES with glucose and HEPES (p-value<0.05) as determined by the other biofilm quantification methods. It also demonstrates the importance of quantifying and characterising the composition of EPS to better understand the relationship between biofilm structure and function and in turn for the development of biocontrol strategies to disrupt biofilm and prevent biofouling (Ansari et al., 2012).

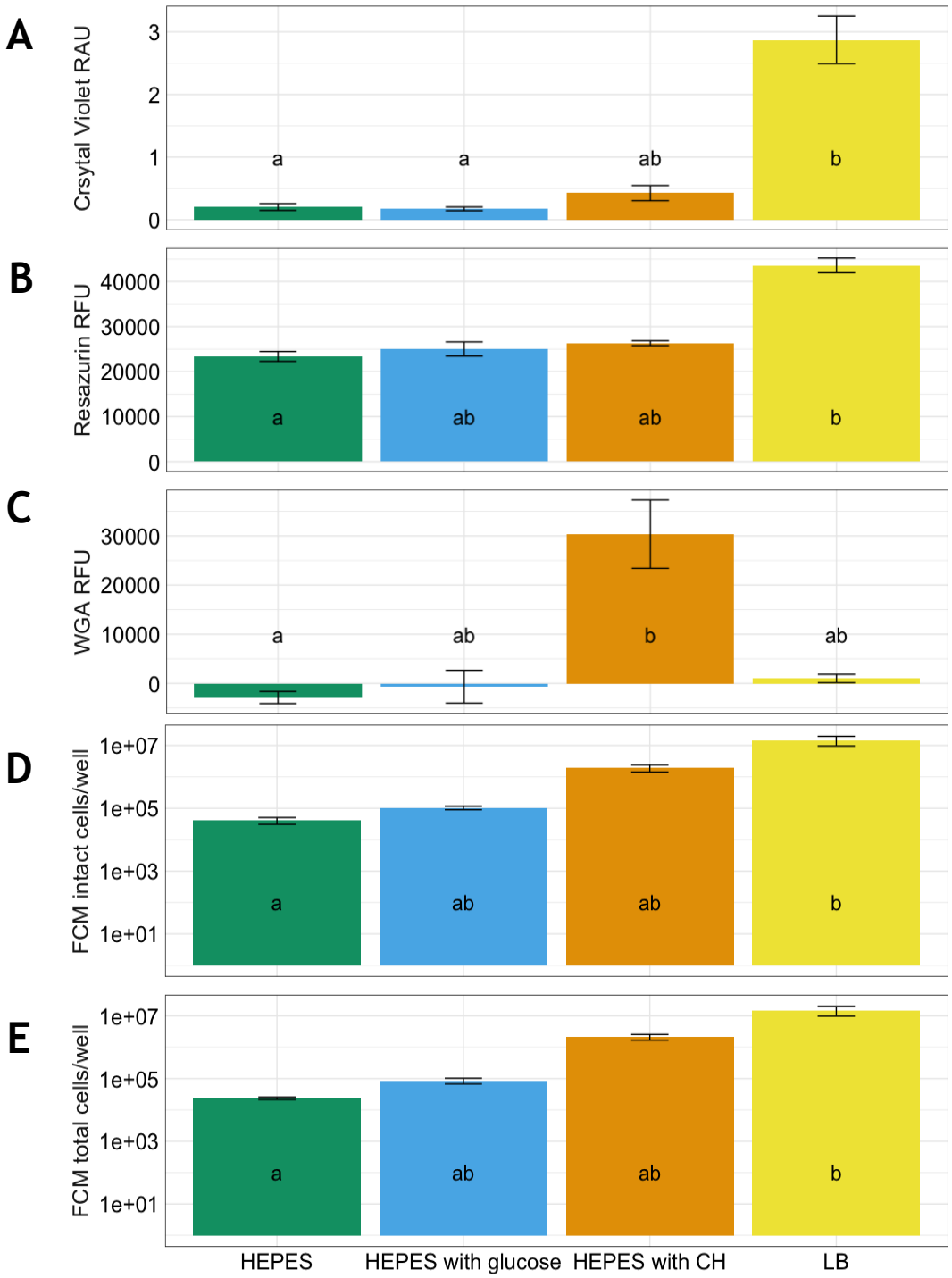

Figure 5.2- The growth of biofilms after 24 hours in different media is measured by different methods. A) biomass is measured by crystal violet, **B)** viability is measured by resazurin, **C)** EPS is measured by WGA and **D)** the intact cells/well and **E)** total cells/well is measured by flow cytometry. Standard deviation bars are shown. Variables labelled with the same letter are not statistically significant ($p>0.05$)

Having established the ability of the resazurin, WGA and flow cytometry methods to give a more complete characterisation of biofilms than just crystal violet, we apply this to characterise biofilms with or without *Bdellovibrio* predation. This study demonstrates the ability of *Bdellovibrio* to inhibit further biofilm growth, as assessed by crystal violet, WGA, flow cytometry and resazurin assays (Fig 5.3). The biofilms that were grown in HEPES and HEPES with glucose show no significant decrease in crystal violet, resazurin and the flow cytometry counts that are closely related to biomass quantity. The low level of anti-biofilm activity in this instance is likely because of the lack of nutrients that these media provide; *Pseudomonas* is unable to reach a large enough concentration to initiate significant growth in the predator to then cause the subsequent decline in *Pseudomonas*. As stated in the previous chapter, *Bdellovibrio* predation relies on random collision that is less successful at lower concentrations of prey, as such the predator has a high saturation constant (2.74×10^{15} cells^2/ml^2) necessary to induce a significant increase in growth rate. Additionally, many gram-negative bacteria display a temporary resistance to *Bdellovibrio* which allows them to persist at low concentrations (Shemesh and Jurkevitch, 2003).

In contrast, there is a significant reduction in the crystal violet and both flow cytometry counts for biofilms grown in HEPES with casein hydrolysate and a significant reduction in crystal violet, resazurin and intact cell count for biofilms grown in LB. With these two media, more nutrients are supplied to *Pseudomonas*, so they are able to grow to higher concentrations (>10^6 cells/well) increasing the chance of significant *Bdellovibrio* predation. This concurs with similar observations in the past, that biofilms were susceptible to attack by *Bdellovibrio* and could cause significant reduction in biofilm over 24 hours (Kadouri and O'Toole, 2005).

Biofilms grown in HEPES with casein hydrolysate did not show a significant decrease in resazurin which is a viability stain that binds to oxidative molecules found in metabolically active cells. This is initially surprising as there was a significant decrease in the flow cytometry intact cell count, though it is likely that the resazurin assay is less reliable at low biofilm concentrations (Sandberg et al., 2009).

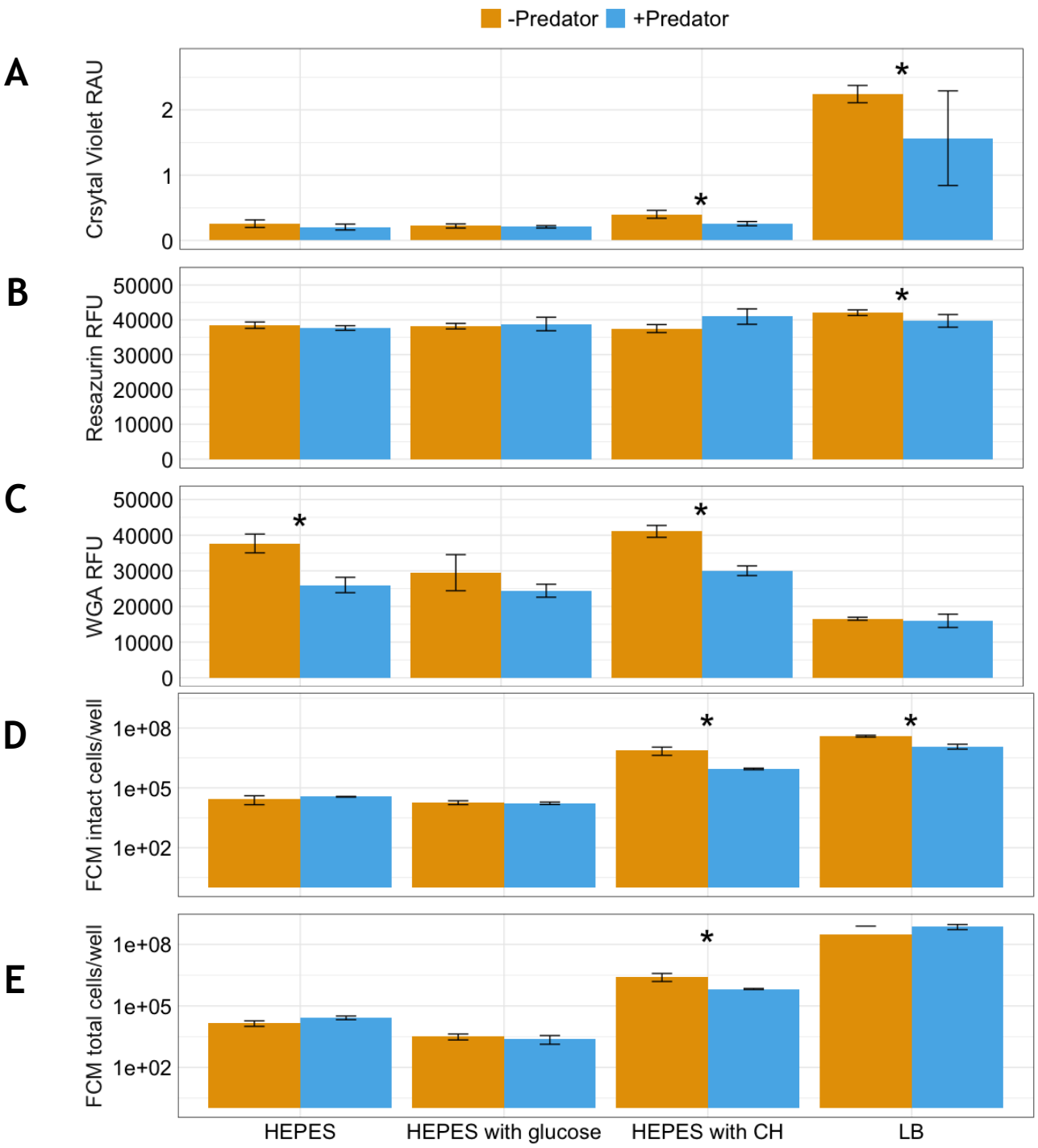

Figure 5**.3- The effect of *Bdellovibrio* predation on *Pseudomonas* biofilms pre-grown in different media** and then exposed to *Bdellovibrio* (orange) or the HEPES buffer (no predation) control (blue). **A)** biomass is measured by crystal violet, **B)** viability is measured by resazurin, **C)** EPS is measured by WGA and **D)** the intact cells/well and **E)** total cells/well is measured by flow cytometry**.** Standard deviation bars are shown. * is used to indicate statistical difference ($p<0.05$) to biofilms grown without predation.

After predation, biofilms pre-grown in HEPES with casein hydrolysate show a significant decrease in EPS as indicated by the WGA assay, which could suggest that the action of *Bdellovibrio* predation reduces the attachment ability of biofilms through some action. This was supported by the evidence that when *Pseudomonas* was grown in HEPES with casein hydrolysate, the percentage of total cells that existed as biofilm (as opposed to planktonic cells in the bulk liquid) significantly decreased after predation (Fig 5.4). The WGA assay showed a decrease in EPS after predation with the biofilms pre grown in HEPES but there was no significant decrease in the other biofilm assays, signifying that the decrease in WGA is not the result of a decrease of bacteria alone.

Interestingly, biofilms grown in LB did not show a significant decrease in the flow cytometry total count but did show a significant decrease in the intact cell count and resazurin, suggesting that *Pseudomonas* pre-grown in LB had a large portion of damaged and potentially metabolically inactive cells still present within the biofilm even after predation. This was not reflected in the crystal violet assay as this method non-specifically stains both bacteria and the surrounding biofilm matrix and there was shown to be no significant decrease in the EPS measured by the WGA assay. The ability for these biofilms to maintain EPS level could also aid in the attachment and persistence of damaged and non-intact cells within the biofilm as it was also shown that the percentage of total cells within the well that existed as biofilm did not significantly change after predation (Fig 5.4). These cells are likely to be present deeper within the biofilm, shielded from predators. Likewise, biofilms grown in HEPES with glucose showed no significant decrease in WGA after predation and a significant increase in the percentage of cells that existed as biofilm (p-value<0.05).

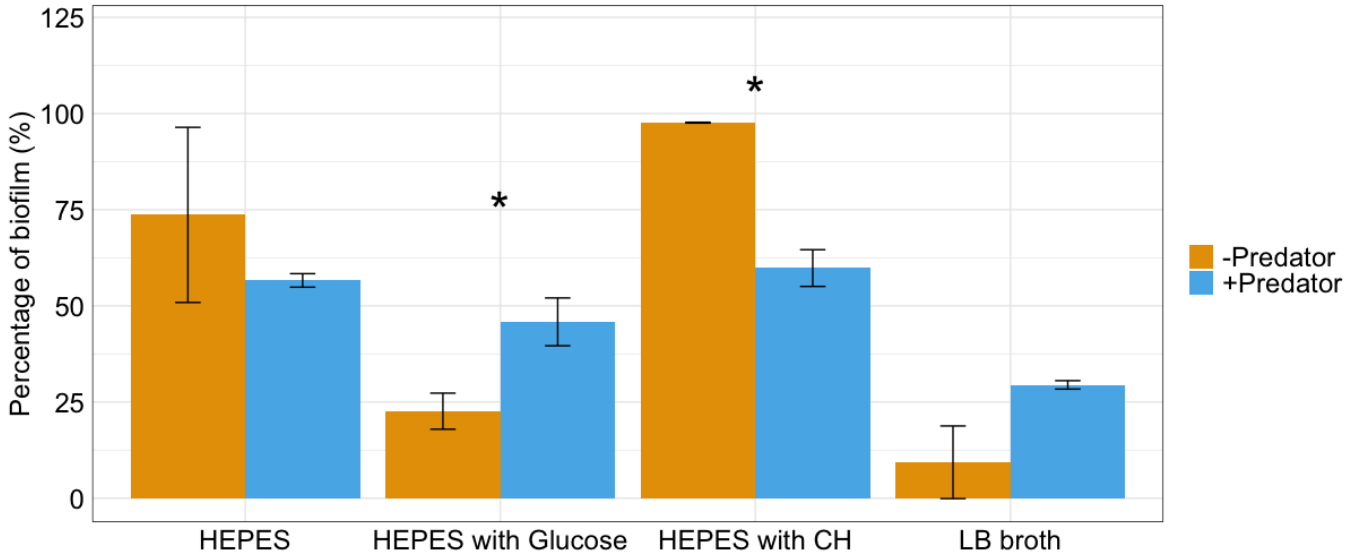

Figure 5.4- The effect of *Bdellovibrio* predation on the percentage of *Pseudomonas* in the well that exist as biofilm. Biofilms were grown in different media and then exposed to *Bdellovibrio* (orange) or the HEPES buffer (no predation) control (blue). Biofilm and planktonic bacteria are then measured by the total count flow cytometry method. Standard deviation bars are shown. * is used to indicate statistical difference ($p<0.05$) to planktonic cells grown without predation.

Flow cytometry in this instance has the advantage over crystal violet and other traditional biofilm assays as it is generally more specific, accurate and can also measure the concentration of *Bdellovibrio* cells, which could interfere with the crystal violet reading. This also demonstrates that *Bdellovibrio* are able to predate and grow within biofilms though we observed that *Bdellovibrio* predominantly are found within the bulk liquid with the *Pseudomonas* planktonic bacteria regardless of nutrient availability, with less than 20% of the total *Bdellovibrio* cells in the well found in the biofilm (data not shown).

5.3 Conclusion

We demonstrate that flow cytometry and fluorescent assays can be used to investigate the dynamics of static biofilms. The static biofilm assay allowed for the rapid screening of the effect of different media on the persistence of biofilms

exposed to *Bdellovibrio* predation. Through this we observed, as has been previously, that a small population of biofilm is able to persist after predation. Our results also suggest that fully understanding the anti-biofilm effect of *Bdellovibrio* under different environments requires an understanding of its effect on the EPS matrix, as its persistence may contribute to the biofilm survival. Similarly, the reduction in EPS synthesis, the detachment of biofilm and the increase in planktonic bacteria could be a direct response to predation. The addition of the WGA assay to measure EPS and flow cytometry to measure the total and intact cells of both the biofilm and planktonic bacteria in the bulk liquid are overlooked in conventional biofilm assays like crystal violet but are critical in rapidly assessing the predation dynamics of biofilms. For example, a similar experiment could be carried out by instead measuring the growth of biofilms formed on different concentrations of a growth limiting substrate in order to calibrate a mathematical model of the biofilm and derive growth characteristics.

Further assessment of *Bdellovibrio* predation of biofilms should include tests on the dose-dependence of biofilm inhibition by adding different initial concentrations of *Bdellovibrio*. This would also allow for measurement of the yield. Furthermore, the biofilm was grown under static conditions which limits the growth of the biofilm in comparison to applying shaking or under flow conditions such as a chemostat, microfluidic device or a membrane-based filter (Buchholz et al., 2012). Applying flow would not only aid biofilm growth but also *Bdellovibrio* growth which shows optimum predation at 200rpm.

Microfluidics devices in combination with fluorescence microscopy would allow us to assess the biofilm adhesion, formation, cell size and EPS formation in the presence of *Bdellovibrio* predation in a continuous system whereby fresh medium is supplied. These variables can be addressed using 96 well plates in static assays but microfluidics and microscopy with the addition of selective fluorescent staining could allow biofilm formation and predation in real time and for longer, while still maintaining the high-throughput nature (Merritt et al., 2005). This is important in measuring dynamics and growth parameters throughout a typical biofilm life cycle

and key to constructing mathematical models such as attachment and detachment rate, lag phase, growth rate, yield and saturation constant.

6 Development and optimisation of a protocol to combine HSQ planarization and soft lithography for fabricating a PDMS microfluidic device

In the past, the study of *Bdellovibrio* dynamics relied on conventional methods such as flasks or plate cultures. Glass microscope coverslips are used in batch studies to provide the surface for bacteria to grow and can be analysed by microscopy or gravimetric investigations (Bakke and Olsson, 1986).

Biofilm investigations are difficult to reproduce and hence comparing data concerning biofilm growth under predation is also difficult. Reproducibility can be increased through the use of 96 well plates which can be used for high-throughput spectrometric analysis. 96 well plate and channel based devices can also be used to study early biofilm development in real time non-destructive analysis when combined with microscopy (Dashiff and Kadouri, 2011). The static nature of these assays makes it difficult to study mid and late-stage biofilm development which is accompanied by the maturation and detachment of biofilms.

Currently, there has been a growing interest in alternative methods such as microfluidics. Microfluidic devices contain small channels typically in the micrometre scale that allow the control and manipulation of fluids (Sia and Whitesides, 2003). In turn, this makes it possible to study predator/prey interactions in real time and at a single cell level as well as in multi-species communities with high reproducibility (Yuan et al., 2017).

This can provide more accurate measurement on the predator/prey interactions under varying conditions more applicable to understanding biofilm mitigation in water treatment systems, where biofilm growth and biofouling is not determined

by one factor or one microorganism species alone but instead is a manifestation of the many different spatial and temporal factors (such as nutrient availability, temperature, metabolic products) acting upon certain sub-populations within the engineered system (Bachmann and Edyvean, 2005).

Like EPS, biofilm thickness has been cited as an important parameter in the transport rate of certain antimicrobial agents (Bakke and Olsson, 1986) and when biofilm thickness is increased, the diffusion distance also increases (Stewart, 2003). This has been demonstrated in investigations into resistance of thin (average cell density ~3.5 log colony-forming units (cfu) cm^{-2}) and thick (average cell density ~7.6 log cfu cm^{-2}) *P. aeruginosa* biofilms to hydrogen peroxide (Mah and O'Toole, 2001). It was shown that in thin biofilms, survival was increased when compared to planktonic cells but overall, hydrogen peroxide was still able to penetrate the biofilm. In contrast, thicker biofilms were able to restrict penetration of hydrogen peroxide. They concluded that the restricting EPS works in synergy with the catalase-mediated destruction of this hydrogen peroxide to protect the *P. aeruginosa* population (Mah and O'Toole, 2001).

It is important to characterise the extent to which *Bdellovibrio* can penetrate prey biofilms because thicker biofilms may be able to delay penetration by *Bdellovibrio* as the biofilms do with many antibiotics (Mah and O'Toole, 2001). This may result in the regrowth of prey bacteria at a rate greater than the rate of predation, which would reduce the effectiveness of bacteria as a biocontrol agent (Wilkinson, 2006). Furthermore, the large quantity of energy expended by the attack phase *Bdellovibrio* when penetrating the EPS may reduce their motility and in the absence of prey, the survival of the attack phase *Bdellovibrio* will also be reduced (Lambert et al., 2006). However, this has yet to be shown and there are very few studies into the effect of biofilm thickness on predation by protists (Huws et al., 2005) and bacteriophages (Ibrahim et al., 2001) and currently no extensive studies have been done with predatory bacteria.

To date the single study that has briefly considered the effect that thicker biofilms may have on predation is that by Kadouri and O'Toole (2005) who observed that

Bdellovibrio were able to predate upon *E. coli* and *P. fluorescens* in 'thin' biofilms, inducing a 4-log reduction after 24 hours. Using flow cells to control biofilm thickness, the study then utilised 'thicker' biofilms of 30 micrometres in depth (Kadouri and O'Toole, 2005) which when exposed to a *Bdellovibrio* population exhibited a 5-log reduction after 48 hours (Kadouri and O'Toole, 2005).

This study was very important in being one of the first to demonstrate that *Bdellovibrio* were able to successfully predate upon biofilms however there remains the need for more thorough investigations into the role that the thickness of biofilms has on predation. For example, the 'thick' biofilm used is relatively thin compared to biofilms observed to grow in biofilters (Huws et al., 2005). Furthermore, the study did not state the thickness of the initial 'thinner' biofilms.

These thin biofilms were also developed on agar plates and not using the flow cell like the thicker biofilm; therefore, it does not make for a reliable comparison as to what effect biofilm thickness has on persistence and predation. The thick-thin biofilm experiment was instead used by the authors to demonstrate that thicker biofilms could be penetrated by *Bdellovibrio* (Kadouri and O'Toole, 2005), which would make them a useful biocontrol against biofilms in water treatment (Chen et al., 2010). Additionally, biofouling in water treatment systems is increased with biofilm thickness (Bakke and Olsson, 1986) so it is important that *Bdellovibrio* are able to penetrate and clear thick biofilms.

Protists such as *Colopdi maupasi* have been investigated for its potential as a biocontrol (Huws et al., 2005). It is thought that the relatively limited penetration of biofilms observed in *C. maupasi* is explained by its characteristic to graze on prey inhabiting exposed surfaces of the biofilm (Huws et al., 2005), rather than becoming integrally with the extracellular matrix as predatory bacteria might (Núñez et al., 2005). Predatory bacteria biocontrol may also be advantaged over the use of bacteriophages because they consume even dead cells (Hespell, 1978), which have been modelled to accumulate in the interior of thicker biofilms with slow nutrient transfer (Roberts and Stewart, 2004).

Microfluidics could be a vital tool to further analyse the dynamics found from these experiments in addition to the influence of biofilm thickness and flow rate. Through extensive research we aim to navigate the plethora of different manufacturing techniques to try to optimise the design and manufacture by soft lithography of a bespoke microfluidic device. It is hoped that with such a device we can observe predator prey dynamics in different conditions at a single cell level and create a predator-prey model for *Bdellovibrio* that will later inform future investigations into the application of *Bdellovibrio* in drinking water membrane technologies and a range of industrial fields (Oyedara et al., 2016).

6.1 Microfluidics

Microfluidic devices are made using micro- or nano-fabrication, which describes a collection of technologies and processes used to make structures with microscale and smaller features (Altissimo, 2010). Within this field, are soft lithography techniques that aim to fabricate structures in elastomeric molds using pattern transfer (P. Kim et al., 2008).

Its popularity is also partly caused by its multidisciplinary nature, which facilitates the fabrication of devices and provides deeper investigations into scientific phenomena. However, there can be difficulties when trying to understand how and when to apply the different nanofabrication techniques for those not from that background. This is exacerbated by, amongst other things: the growth in micro technologies, the inconsistent and complex use of terminology and the many different techniques and process that have been developed, which can initially cause confusion for inexperienced users when deciding which processes will be required for their needs. Thus, prior to developing our own device we review the different methods involved in the fabrication of microfluidic devices, addressing the advantages and pitfalls of the different methods.

Though many devices can be made relatively simply without high technology facilities, the lack of standard and informative methods sections from previous studies make it more difficult to replicate previous devices and studies. This is a

big problem as scientific advances rely on the understanding and repeatability of recent discoveries. Through extensive research we aim to navigate the plethora of different manufacturing techniques to try to optimise the design and manufacture by soft lithography of a bespoke microfluidic device.

6.1.1 Soft lithography

Polymethyldisiloxane (PDMS) is a polymer commonly used as a device material for biological studies because it is optically transparent, biocompatible and gas permeable making it ideal for studying predator and prey bacteria growth using optical and fluorescence microscopy (Tung *et al* ., 2004). From a fabrication stand point, it has become widely used because it is inexpensive, flexible and can be easily cast over a mold to replicate the inverse pattern (P. Kim et al., 2008).

Patterning PDMS is the final stage of soft lithography, with the first and most critical stage being the fabrication of the mould (master) on which PDMS is imprinted (Jenkins, 2013). PDMS can be imprinted by a master with features as small as 10nm, so it is the master that determines the resolution of the final device. Any process that produces rigid relief structures will suffice for PDMS patterning, though the most commonly used is photolithography (Wolfe et al., 2010).

6.1.2 Photolithography

In contact-photolithography, an optical image projection system and UV light source are used to pass UV rays are through a photomask to transfer the 2-D pattern onto a photosensitive elastomer or ‘photoresist’, that is uniformly coated onto a silicon wafer.

The photomask (also known as mask) pattern is first designed by a computer aid design (CAD) system and transferred to a surface using e-beam lithography (see below) and etching (Jenkins, 2013). The mask acts as a ‘stencil’ as the parts of the mask that are optically transparent expose the photoresist in this pattern and

changing the structure of the exposed photoresist (Gonzalez-Macia et al., 2010). Washing the resist with the appropriate developing solution will leave a raised pattern that depends on the type of resist material used. For positive resists the area exposed to the e-beam is made weaker and a layer is removed by the developed solutions. Whereas for negative resists the exposed area is made resistant to the developer solution and the weaker unexposed areas are removed (Gonzalez-Macia et al., 2010).

Another category of photolithography is known as image-projection photolithography, which also utilizes ultraviolet (UV) light source but unlike contact-photolithography, this method does not have the photomask in close contact with the light source. This projects a de-magnified pattern onto the photoresist rather than a 1:1 image. Because of this the method can achieve higher resolutions of line width (approximately 20nm). This has been used in applications involving semiconductors (Wolfe et al., 2010). A great disadvantage of projection photolithography is that is very costly and not readily available to average users.

Contact photolithography systems are relatively inexpensive, and the UV patterning can be carried out over a large area with a high throughput, which has made it the dominant method of pattern replication.

6.1.3 Resists

Choosing the correct photoresist is an important step in soft-lithography, successful application depends on the properties of the resist, including adhesion to substrate and film thickness.

Photoresists come in two main categories: positive photoresists and negative photoresists. Upon exposure to UV, positive photoresists are made vulnerable to removal by a developer solvent and able to maintain their pattern. In contrast, a negative resist that has been exposed to UV is able to maintain its pattern structure when the developer solvent is used (Jenkins, 2013).

To coat the silicon wafer with photoresist, a small volume is poured onto the wafer and spun off. The speed at which the resist is spun determined the final thickness of the photoresist film, but its uniformity and overall quality is determined by the temperature, humidity, volume of resist dispensed and the spreading time. Additionally, improper cleaning or handling of the wafer can affect the uniformity of the photoresist.

Following the spinning procedure, the resist must be soft baked in order to remove any excess resist and make the coated resist more stable and uniform in preparation for further processes such as coating more resists, etching and UV exposure.

The soft bake also has an effect on the solvent development of the resist. For example, in negative resists, an insufficient time or temperature will leave residual solvent from the spinning procedure which will prevent the full exposure of UV to the photoresist. So, when developer is applied, the exposed resist is unintentionally removed, which lead to too small or partly eroded structures. When the soft bake is applied on too high a temperature or for too long, the developing time can be drastically increased, and the resists can show ‘cracks’. Thus a compromise must be achieved (Cui, 2016). Typically, photoresists have a soft bake temperature of 80-100°C .

Ovens are widely used for soft baking of resists, but they are not as reliable because of the temperature differences that can be displayed in various locations. For processes where the temperature is more critical, vacuum hot plates are ideal as they can heat the wafer with greater accuracy and reproducibility. Heating the wafer from the back of the substrate surface results in a more uniform evaporation of the residual solvent preventing the formation of a surface skin which can be often seen when soft baking resists in an oven. The speed at which hotplates heat up the substrate also reduces the chance of contamination (Mack et al., 1994).

Negative resists have the advantage over positive resists in that they are often cheaper, and a shorter processing time. SU-8 is a negative tone photoresist which

is commonly used for pattern transfer in PDMS. SU-8 is made of epoxy groups that are crosslinked to form a strong network when exposed to UV. The unexposed areas are not crosslinked making them weaker and soluble to a developer (Jenkins, 2013). SU8 is flexible and various formulations are easy to spin over a silicon wafer, creating features of a few microns high up to several millimeters. Additionally the thickness of the same SU8 formulation can be changed by altering the spin speed (Yuan et al., 2017).

6.1.4 Multi-layer devices

The majority of PDMS microfluidic devices fabricated by soft lithography are 2-D structures. These structures are channel-based geometries that enable a large number of functions including diffusion, mixing and droplet generation. By controlling the fluid in the device, it is possible to study, for example, the behaviours and interactions of biological cells with other cells, environmental signals and antibiotics (Yuan et al., 2017).

One challenge with SU8 and photolithography is in making multilayer (3D) devices. The thickness of the device is determined by the thickness of the resist and the photomask can only control the width and length of features (Li, 2016). Multilayer devices can be made with multiple cycles of SU8 photolithography by spinning and developing the resist layers of different thickness in order from the smallest to the largest features (Meyer et al., 2015). This is necessary to create active devices or moving parts such as integrated valves and pumps (Meyer et al., 2015).

The primary driver of microfluidic devices that can be applicable to a range of uses is the ability to make masters with high resolution and accuracy in a reliable and high-throughput fashion. Unfortunately, the resolution limit of a photomask (1μm) restricts its use for single-cell trapping. For the proposed device of the project, the bacterial prey cells are trapped in channel sizes (0.45 μm) that are too narrow to be formed using SU8 so an alternative must be found (Wheeler et al., 2003).

Between them, single cells in the same environment exhibit physiological and biochemical differences. In relation to biofilm formation, cellular heterogeneity likely has a close relationship with antimicrobial resistance, stress tolerance and potentially even predation (Park et al., 2011).

However, conventional studies of measuring bacterial growth rely on bulk-based studies where bacteria are measured as a whole population, ignoring the interactions and ecology among individual cells. Single cell analysis has the potential to investigate cell heterogeneity further and provides further understanding to the bacterial ecology (Balaban et al., 2004).

The small size of bacteria makes it difficult to analyse with traditional biological tools. Additionally, the intracellular and extracellular components that are involved in single cell-cell interactions are present in very small concentrations. Therefore, highly sensitive detection methods are necessary when trying to perform high throughput analysis (Yuan et al., 2017).

Flow cytometry has previously been demonstrated as a reliable tool for characterising fluorescently labelled bacteria and can be used for single cell analysis. However, they are not capable of monitoring cells continuously in real time and require large sample volumes and even larger volumes of reagents. This is in contrast to microfluidics which is able to control microlitres of fluid and can be combined with other technologies such as microscopy and fluorescent labelling to observe cell dynamics in real time (Yuan et al., 2017).

To analyze single cells, they must first be successfully isolated and trapped in a desired location. Different strategies based on microfluidic technology have been developed for manipulating single cells in different ways for varying analysis. These include hydrodynamic, electrical, optical, acoustic and magnetic methods (Chen et al., 2015).

The Hydronamic method uses the interactions between fluid and the structure of the microfluidic device to manipulate single cells. This is the most commonly used

method for single cell trapping because it is simple to design and can be integrated with other analysis method. Cells can be fixed to desired positions by designing structures of similar size to the cell and various shapes can be used like holes, channels and dams. Common hydrodynamic methods include micro trapping and droplet generation and generally cause less damage than other cell trapping methods, which is vital for monitoring the further growth of the cell (Chen et al., 2015).

One study reported the design and optimization of a two-layered microfluidic platform with microstructures to allow for single cell capture and subsequent culture of stem cells (Chen et al., 2015). The first layer enabled the seeding of thousands of cells across an array of channels. Underneath this layer was an array of micro-wells that contained a U-shaped trap. This U-shaped hydrodynamic trap allows for the spread and growth of the trapped single cells for further and long-term analysis. An advantage of this method is than the microwells geometry, size could be adjusted to increase the capture efficiency with a range of cellular organisms (Chen et al., 2015).

Such devices that can monitor the growth of single individual cells are important in measuring the interaction of predation, cell phenotype and growth in the absence of space. And is in contrast to methods used in previous chapters and other literature, which monitor growth in batch experiments where the whole population of bacteria cells are well mixed and measured together.

One method for fabricating a multilayer device with channels on the nanometer scale includes the use of several etch processes (Ou et al., 2011). This process works by first spinning on a positive resist layer onto a silicon wafer and then patterning it to reveal the relief of the channels. The width of the resist and hence the channels can then be reduced by oxygen plasma etching or thinning while maintaining its shape. The patterned substrate is then processed by a dry etch step which bombards it with ions that reduce the depth of the substrate at a selective rate depending on the substrate's properties and thickness (Ou et al., 2011). The resist remaining after development is typically etched at a much slower rate and

acts as a mask, shielding the silicon below from etching. In contrast, the parts of the substrate that no longer have resist after being developed are etched, revealing 3-D features in the substrate. This is a useful step in achieving channels that are deeper than the spun resist (Long et al., 2013).

In this process, etching could potentially by replaced by electron beam (e-beam) lithography. It works in very similar fashion to photolithography but utilizes e-beams to pattern resists rather than UV. E-beam is the typical approach to creating nano-arrays as it is known for its very high resolution which is typically 20nm to 1 microns as determined by the thickness of the e-beam resist used (Altissimo, 2010). Although it can be quite expensive, it offers reasonable job speed and higher reliability in the quality of nano-arrays, when compared to using multiple etch processes (Vieu et al., 2000).

Hydrogen silsesquioxane (HSQ) is a negative e-beam resist that is commonly used as an etch mask but also has gained interest for patterning high resolution lines with minimum edge roughness (Haffner et al., 2007). HSQ contains silicon hydrogen bonds which are converted to silanol when exposed to electron beams. Silanol is stronger than the silicon hydrogen bonds so when the resist is washed by a developer such as tetramethyl ammonium hydroxide (TMAH), the exposed parts of the resist remain whereas the silicon hydrogen bonds are broken, washing the unexposed areas of resist away (Min et al., 2010). Unlike most resists it has good stability when viewed under a scanning electron microscope (SEM) which is useful for accurate measurements of depth and circumvents the need for metallisation (Min et al., 2010).

With the high resolution and high-throughput advantages of using HSQ in soft-lithography, we propose a different method for creating a multilayer device with nanometre width channels; one that removes the need for dry etch and plasma thinning which can cause channel collapse, especially with high aspect features (Min et al., 2010). Instead, e-beam lithography is used to make high resolution HSQ resist lines of 1.1 micron that narrow to 350nm to limit the growth of bacterial prey cells to a single cell line (Long et al., 2013). Photolithography will then be

used to pattern SU-8 lines of 20-micron depth over the e-beam lines to template the channels that will use hydrodynamic forces to load and deliver cells and media to the narrow growth channels.

6.2 Methods

6.2.1 Microfluidic device design and fabrication

We aimed to develop a protocol to fabricate a silicon master as a negative mould for a PDMS microfluidic device by combining e-beam lithography with photolithography using the facilities at the James Watt Nanofabrication centre (JWNC), a clean room at the University of Glasgow. Because of the complexity of the methods (in particular the fabrication of multilayer HSQ resist channels by planarization), alternative methods were conducted at different fabrication stages to assess the most optimal for fabricating the master with the desired dimensions and quality (Fig 6.1). Importance was also placed on the speed and waste produced in these steps.

For all of the e-beam lithography steps (HSQ planarasation, e-beam patterning and development) the optimal procedure was assessed using a dose test whereby sets of 9 resist lines with 1µm depth and width, are patterned on a silicon substrate. Each set of resist lines were to be exposed to different e-beam doses ranging from 500 to 4500 µC/cm^2 in intervals if 1000 µC/cm^2. The e-beam exposure for patterning is carried out using the VB6 electron-beam tool by staff at JWNC.

The effect of the different e-beam methods was assessed after the resist development stage using a combination the Bruker Dektak XT height profiler to measure depth of the patterned channels and microscopy to measure the width and length of the patterned channels and observe the presence of substrate damage or residual resist.

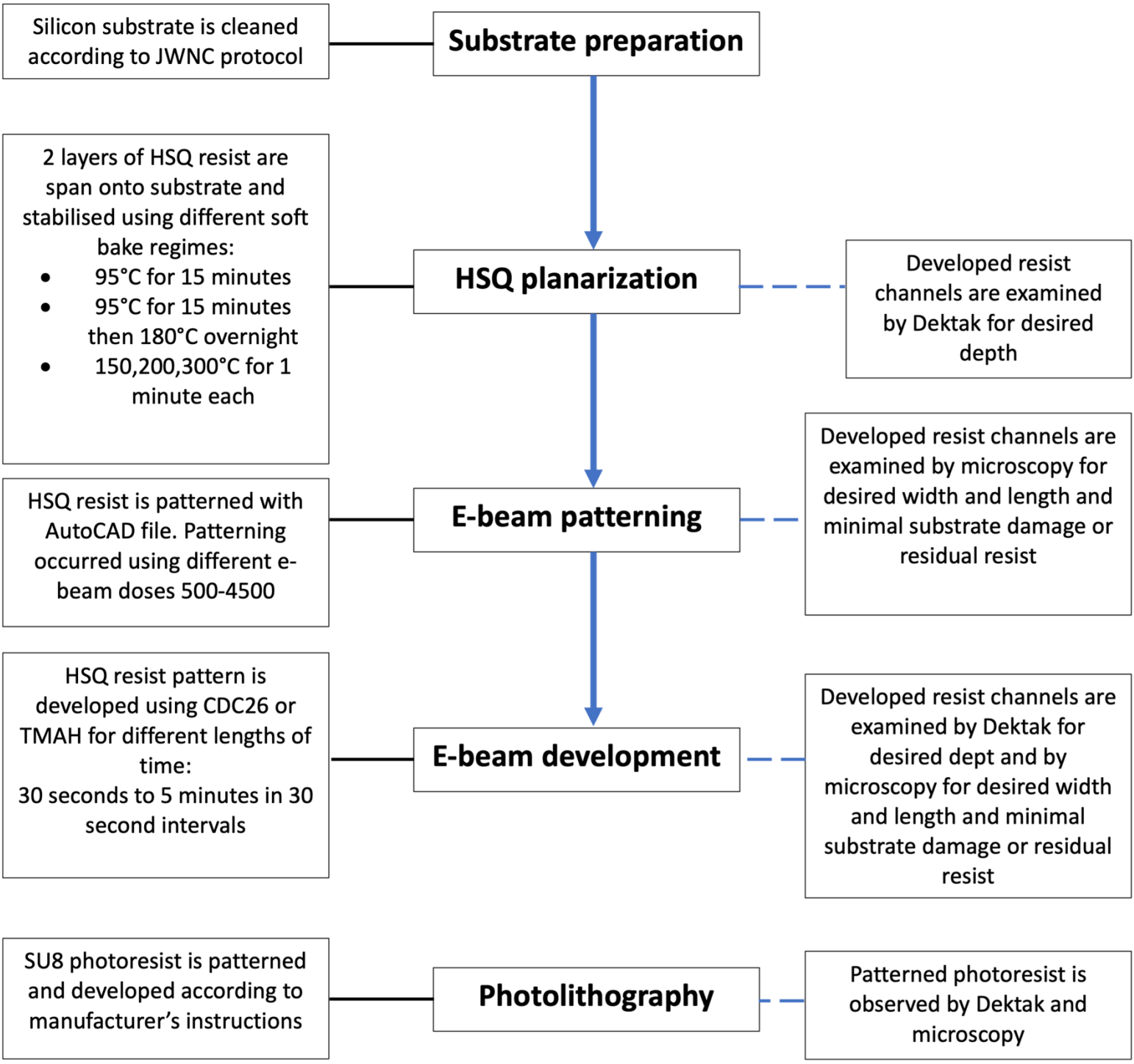

Figure 6.1- Flow chart summarising the development of the workflow of e-beam lithography and photolithography to pattern negative channels on silicon substrate. Boxes on the left summarise the alternative methods attempted at each fabrication stage in an effort to find the most optimal for the desired device dimensions. Boxes on the right summarise the methods used to confirm this.

The fabricated silicon master was coated with a silane agent in the JWNC and then transferred to a clean laboratory to carry out the PDMS moulding and curing and the bonding of the PDMS to a glass slide. As with the e-beam lithography steps, alternative methods were used in each stage to determine the most optimal for fabricating the PDMS device with the desired dimensions and quality (Fig 6.2). This was assessed using microscopy and by loading the device with a cell culture as described below to observe if there was any leaking and if the device could trap the cells in a single line as intended.

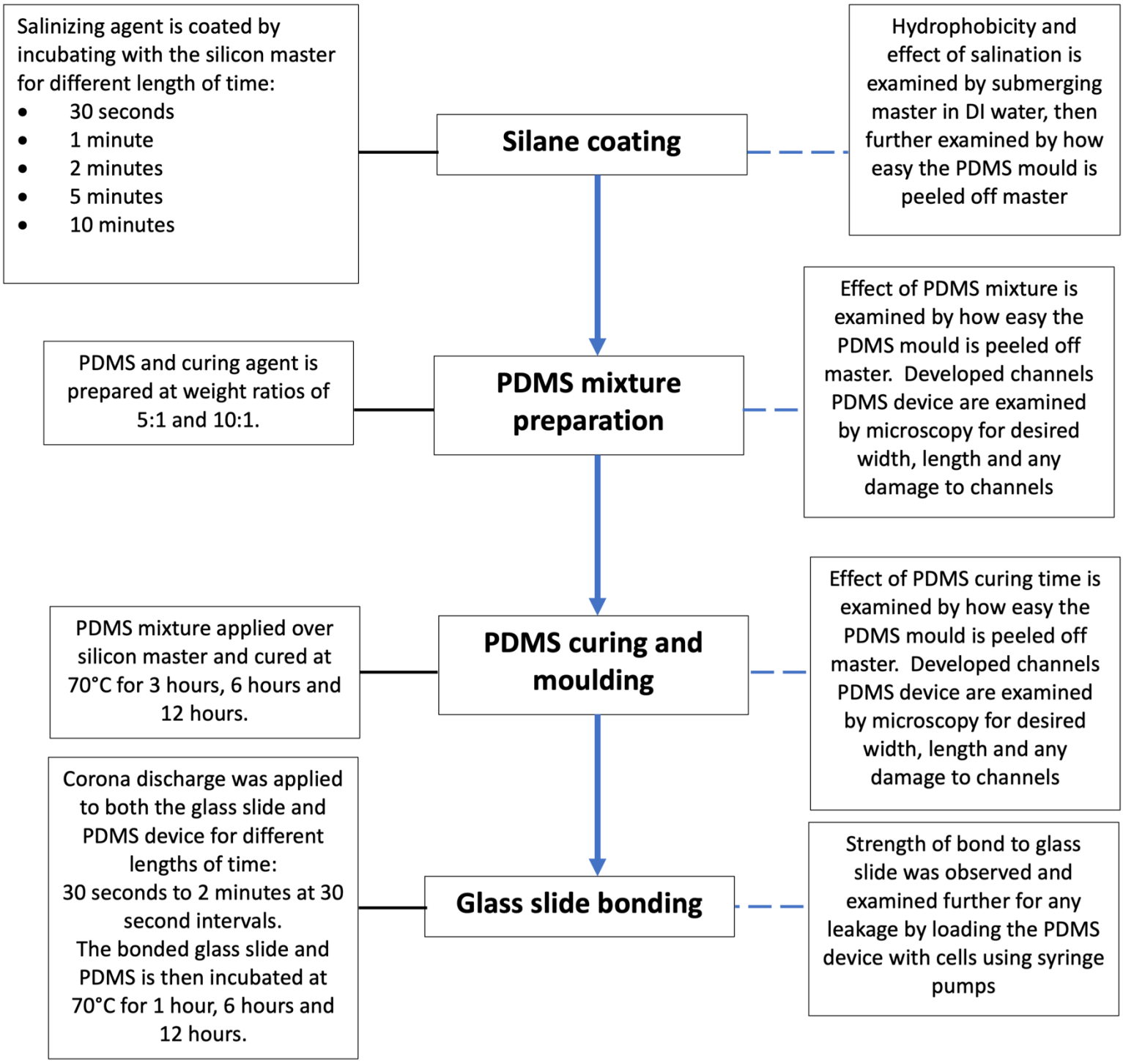

Figure 6.2- Flow chart summarising the development of the workflow of PDMS device fabrication. Boxes on the left summarise the alternative methods attempted at each fabrication stage in an effort to find the most optimal for the desired device dimensions and success. Boxes on the right summarise the methods used to confirm this.

6.2.2 Microfluidic device bacteria culture

Pseudomonas sp. (DSM no. 50906) using standard culturing methods (Herencias et al., 2017). *Pseudomonas* cells were grown in LB broth at 30°C for 16 hours and resuspended in supplemented Ca/Mg-HEPES buffer (25 mM HEPES, 2 mM calcium chloride, 3mM magnesium chloride, pH 7.6) to an optical density (600nm) value of 10.

Two syringe pumps were used to drive the solutions into the parallel feeding channels of the PDMS device. In order to clean the PDMS surface, the channels were filled with isopropyl alcohol (IPA) solution at 10μL/min and left to rest for at least one hour. Following the sterilisation step, the channels were filled with DI water and then filled with LB medium. Lastly, 10^4 cells/ml *Pseudomonas* was injected into the top inlet at 5 μL/min to flow into the growth channels and LB media into the bottle inlet at 0.4 μL/min. This difference in flow rate between the two parallel feeding channels leads to a strong flow that forces the bacteria cells further into the shallow growth channels. Thereafter the top inlet syringe was changed to LB and the flow rate was changed to 0.4 μL/min and the bottom inlet was reduced to 0.2μL/min to start the PDMS device cell culture. The PDMS device was mounted onto a temperature-controlled chamber and mounted onto a microscope stage. Images were taken with the evos fl auto 2, an epi-fluorescence inverted microscope (Olympus Inc., UK).

6.3 Results and discussion

The ability of microfluidic technology to influence the spatial distribution of bacteria is vital not only to replicate the structures present in environment or engineered systems but also in the investigation of physical structure to prevent bacterial growth. The method proposed allows for different channel size and geometries to be patterned by e-beam lithography to contain bacteria for observation, while the larger channels are patterned by photolithography with a single reusable photomask. This can aid the high-throughput production and

optimisation of high-resolution microfluidic devices in research as it prevents the need to fabricate new photomasks every time a design change is needed.

There is need to draw together a disparate set of methods to successfully fabricate the high-resolution device to capture bacterial cells in the manner that we propose. This is especially critical given the importance of the geometric features for flow control in this device, the lack of well reported PDMS devices that can capture single cell dynamics and the uniqueness of applying e-beam lithography directly for soft lithography. Each successful combination of methods was hard won and required many hours of trial and error. Therefore, the processes are laid out as detailed and precise lists of instructions and their rationale. Many microfluidics publications only give broad indications of the methods, which makes reproducing the results and arduous task. The aim here is to give sufficient detail that any researcher could reproduce the device, given the appropriate instruments.

6.3.1 Substrate preparation

Prior to lithography, new substrates must be adequately cleaned to ensure the removal of any dust or material. This is important as any contaminant will prevent the spinning of a uniform layer of resist and reduce the accuracy of patterning (Altissimo, 2010). Any artefact or surface irregularity in the resist pattern will be transferred to the PDMS mould as PDMS casts over a master with great accuracy (Patel et al., 2013). Thankfully there are numerous procedures that can be used to clean substrates prior to processing but care needs to be taken to ensure the cleaning procedure does not damage the surface of the substrate. We used the following suite of procedures (table 6.1), which was previously developed and standard practice in the JWNC facility.

Table 6.1- Table detailing the protocol for substrate (e.g., silicon wafer) cleaning and preparation.

Substrate preparation
• Measure the thickness of silicon wafer • Cut into a 1 inch x 1 inch square. • Put the substrate in a beaker of methanol and ultrasonicate for five minutes. • Without letting the substrate dry, (to avoid white drying marks) transfer to a beaker of acetone and ultrasonicate for 5 minutes • Without letting the substrate dry, transfer to beaker of IPA for 1 minute (no ultrasonication needed) • Blow dry the substrate carefully with a nitrogen gun • Dispose of the solvents in unchlorinated waste

6.3.2 E-beam lithography

To prepare a layer of resist suitable for patterning, the resist is poured onto a clean wafer which is spun at a particular speed to create a uniform layer of resist of desired thickness. The thickest single HSQ layer that can be formed is only 600nm so a bilayer must be spun in order to achieve the desired 1μm thickness for the growth channels of the microfluidic device in this study. Following the spinning procedure, the resist layer is patterned by e-beam lithography and then developed to reveal the lines that will make up the growth channels. (Fig 6.3).

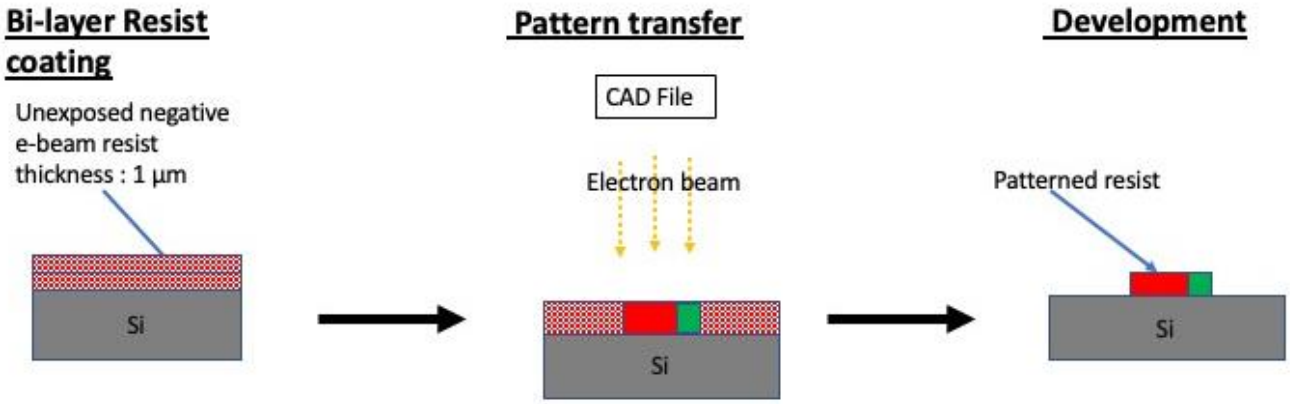

Figure 6.3- E-beam lithography stage of the process. Schematic overview: HSQ e-beam resist is spun twice to a clean silicon wafer for a thickness of 1µm then exposed to electron beams and developed to reveal the pattern relief. The green block represents where channel narrows.

Through literature searches and trial and error, the soft bake regime was identified, over the e-beam dosage and solvent development, as the crucial component in generating multiple layers of HSQ, as it is the process that increases the initial stability of the resist. HSQ planarization is a process whereby multiple layers of resist are alternately spun onto the wafer and soft baked to stabilize.

Common HSQ planarization methods utilized for the fabrication of electronic-photonic integrated circuits often use extremely high temperatures (>400°C) in combination with nitrogen exposure (Büyükköse et al., 2011). However, the process has not previously been used in the fabrication of PDMS devices and hence no standard protocols exist. We conducted three methods of HSQ planarization to find the most reliable way of fabricating the lines of resist for the PDMS growth channels. The spinning steps are the same for each method but the soft bake step changes.

The different soft baking regimes that were tested here were similar to those previously reported (Holzwarth et al., 2007). However, they were altered with the intention of reducing the temperature and thus the expertise required for these methods and to ensure that the structure of multilayer resists would not be changed by the subsequent soft lithography process. The success of each method

was to be assessed after patterning and development and ranked according to the quality and height using optical microscopy and Dektak measurement. We found that both soft bake methods 2 and 3 were successful and produced similar results whereas method 1 without a significant soft bake step was unable to produce resist layers higher than a single 500nm layer. Method 2 was chosen going forward as the preferred method due to its speed though method 3 was used when higher temperature vacuum hotplates were not available.

Table 6.2- Table detailing the three methods used to fabricate two layers of HSQ with an estimated total thickness of 1µm.

<table>
<tr><td colspan="3">HSQ planarization</td></tr>
<tr><td colspan="3">HSQ layer 1 spin

• Pipette 200µl of HSQ resist to cover the wafer then quickly spin 1800 rpm for 60 seconds (for 500nm).
• Once finished check the backside of wafer and clean any residual resist with acetone and a cotton bud.</td></tr>
<tr><td>Soft Bake method 1

• Place the wafer on a vacuum hot plate at 95˚C for 15 minutes.</td><td>Soft Bake method 2

• Place the wafer on a vacuum hot plate at 95˚C for 15 minutes.
• Place wafer in an oven at 180˚C overnight.</td><td>Soft Bake method 3

• Place the wafer on a vacuum hot plate at 150˚C,200˚C and 300˚C for 1 minute each.</td></tr>
<tr><td colspan="3">HSQ layer 2 spin

• Pipette 200µl of HSQ resist to cover the wafer then quickly spin at 1800 rpm for 60 seconds (for 500nm).
• Once finished check backside of wafer and clean any residual resist with acetone and a cotton bud</td></tr>
<tr><td>Soft Bake method 1

• Place the wafer on a vacuum hot plate at 95˚C for 15 minutes.</td><td>Soft Bake method 2

• Place the wafer on a vacuum hot plate at 95˚C for 15 minutes.
• Place wafer in an oven at 180˚C overnight.</td><td>Soft Bake method 3

• Place wafer on a vacuum hot plate at 150˚C,200˚C and 300˚C for 1 minute each.</td></tr>
</table>

6.3.3 Patterning

To transfer a pattern onto a resist it must first be designed by an AutoCAD software (Altissimo, 2010). The software gives the ability to separate features of the pattern into different layers, so that you can chose which layers are transferred. This tool is important when making devices of multiple layers as different features can be patterned at different stages of the fabrication process and aligned to each other using patterned markers. For example, in this device plan, the growth channels, cell loading channels and alignment markers were separated into three different layers. The growth channels and alignment markers were patterned onto the substrate as one e-beam job and then the cell loading channels with the same alignment markers were used to make the photomask so that the cell loading channels could be aligned and patterned to the e-beam patterned substrate using photolithography.

6.3.4 Development

To remove the unexposed HSQ resist and reveal the desired pattern, the substrate must be washed in a developer, usually TMAH or the less potent CD26 (for channels of <600nm depth) (table 6.3). The effectiveness of this is determined by different factors. The e-beam exposure dose, the temperature and the development time are considered to be the most important and care must be taken to determine the optimal conditions to remove enough residual resist and leave clear well defined features without degrading or altering the properties of the pattern (Haffner et al., 2007). For example, we found that when the HSQ resist lines were exposed to TMAH for longer than 30 seconds or the e-beam dose was lower than 2500 $\mu C/cm^2$, the lines significantly degraded. However, when the dose was higher than 3500$\mu C/cm^2$ or if CD26 was used as the developer, the channels were ill defined and thicker than the desired width. The lowest development time and lowest dose possible was chosen to be optimal, providing that the desired channel dimensions and the minimal presence of damage to or residual resist on the master was achieved. This was to reduce the cost and time associated with the method.

To ensure a reliable protocol for development and test the suitability of the three different HSQ planarization methods, dose tests were carried out before fabricating the channels for the microfluidic device. This was carried out by patterning lines of 1µm depth and width and altering parameters such as the exposure dose (Fig 6.4). Typically dose tests should be carried out with every new pattern design, with any change in the developer or resist and at regular intervals because the quality of the e-beam machine and the clean room environment may change over time. Following development, features should be examined to confirm quality and height by optical microscopy followed by Dektak measurements

Table 6.3- Table detailing the chemical development of HSQ.

HSQ resist development
• Place the substrate in beaker of TMAH warmed up to 21°C for 30 seconds. Gently swirl the beaker around. • Without letting the substrate get dry transfer it to a beaker of RO water. • Blow dry the substrate carefully with a Nitrogen gun • Dispose of the solvents in unchlorinated waste

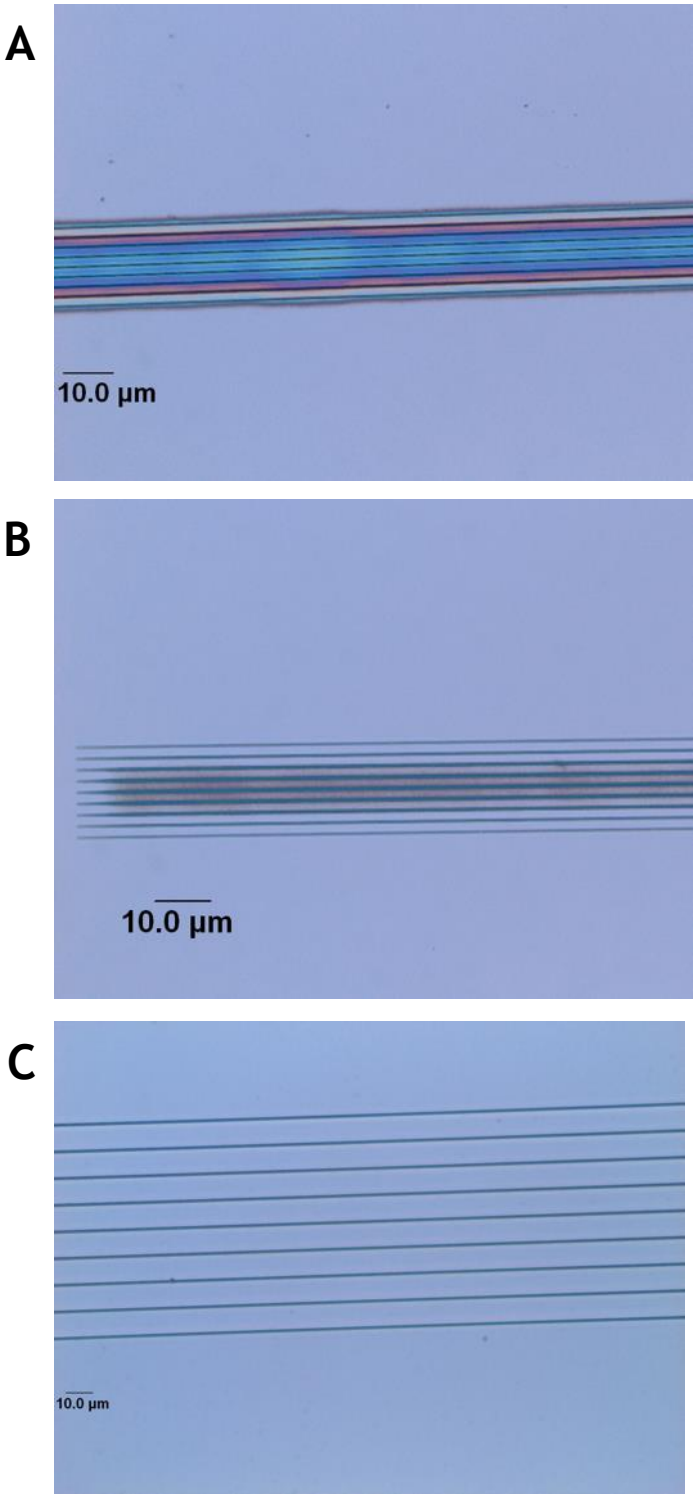

Figure 6.4– Microscope view of channels after development. E-beam dose of **A)** 4500 **B)** 3500 and **C)** 2500 µC/cm^2.

After having established the optimal procedure for e-beam lithography, the HSQ channels for the PDMS device were fabricated and features observed by microscopy and the Dektak (Fig 6.5).

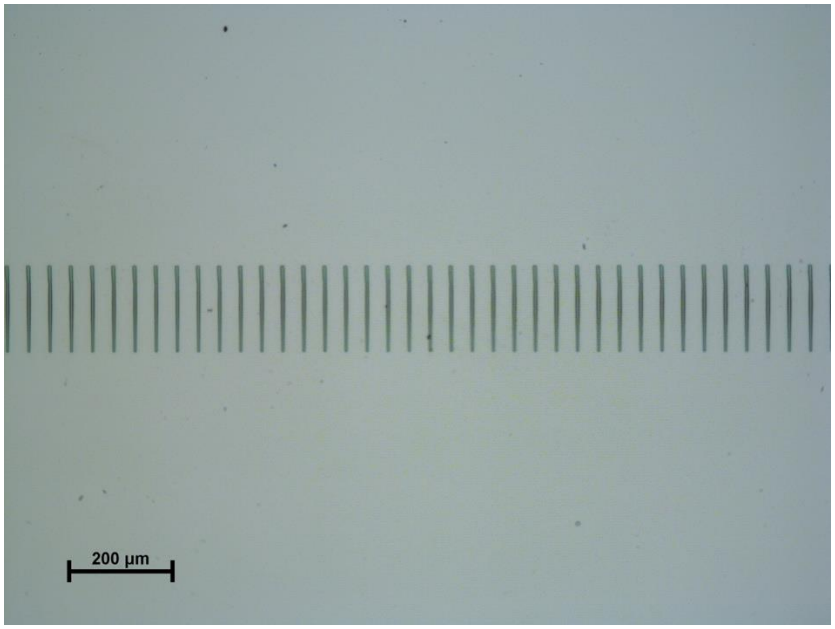

Figure 6.5- Microscope view of 1µm width e-beam generated HSQ channels after development.

6.3.5 Photolithography

While the higher resolution growth channels were pattered with e-beam lithography, such a technique is inefficient for making the larger channels used for transporting media and bacteria. Photolithography using SU-8 photoresist is the traditional process for making microfluidic channels of 20 µm depth. The steps involved are similar to that of e-beam lithography but crucially, patterning the resist is done with UV rays instead of e-beam and instead of using AutoCAD directly, the pattern is derived from irradiating UV through a photomask (table 6.4).

SU8 photoresist is spun over the HSQ patterned substrate and then using a MJB4 or MA6/8 mask aligner machine, is aligned to the photomask so that the large SU8 media channels connect to the HSQ growth channels (Fig 6.6). The markers

patterned in e-beam lithography and in the photomask are critical to this and any misalignment of the channels will disable the function of the device. Following this, development is achieved by using the commercially available EC solvent. As with the e-beam process, the success of this procedure is determined by several factors including the resist spin speed, baking times, UV dose and development time. However, as the dimensions of SU8 channels were not as precise, we fabricated them based on manufacturer's instructions, which we observed gave the desired channel dimensions.

Table 6.4- Table detailing photolithography stage for SU8 resist layers of 20 µm depth

SU8 Photolithography
Spinning and soft bake • Spin SU8-3025 on the substrate at a rate of 4000rpm for 30 seconds • Soft bake the substrate on a vacuum hot plate at 95°C for 15 minutes • Place on watch glass to cool
Patterning • Align the substrate to the photomask using MJB4 or MA6/8 • Expose to UV (Hard contact for 10 seconds and exposure for 30 seconds)
Hard bake • Bake on a vacuum hot plate for 1 min at 65°C then 5 minutes at 95°C.
Development • Place the substrate in beaker of EC solvent for 5 minutes. Gently swirl the beaker around. • Without letting the substrate get dry transfer it to a beaker of IPA for 1 minute. • Blow dry the substrate carefully with a Nitrogen gun • Dispose of solvents in unchlorinated waste

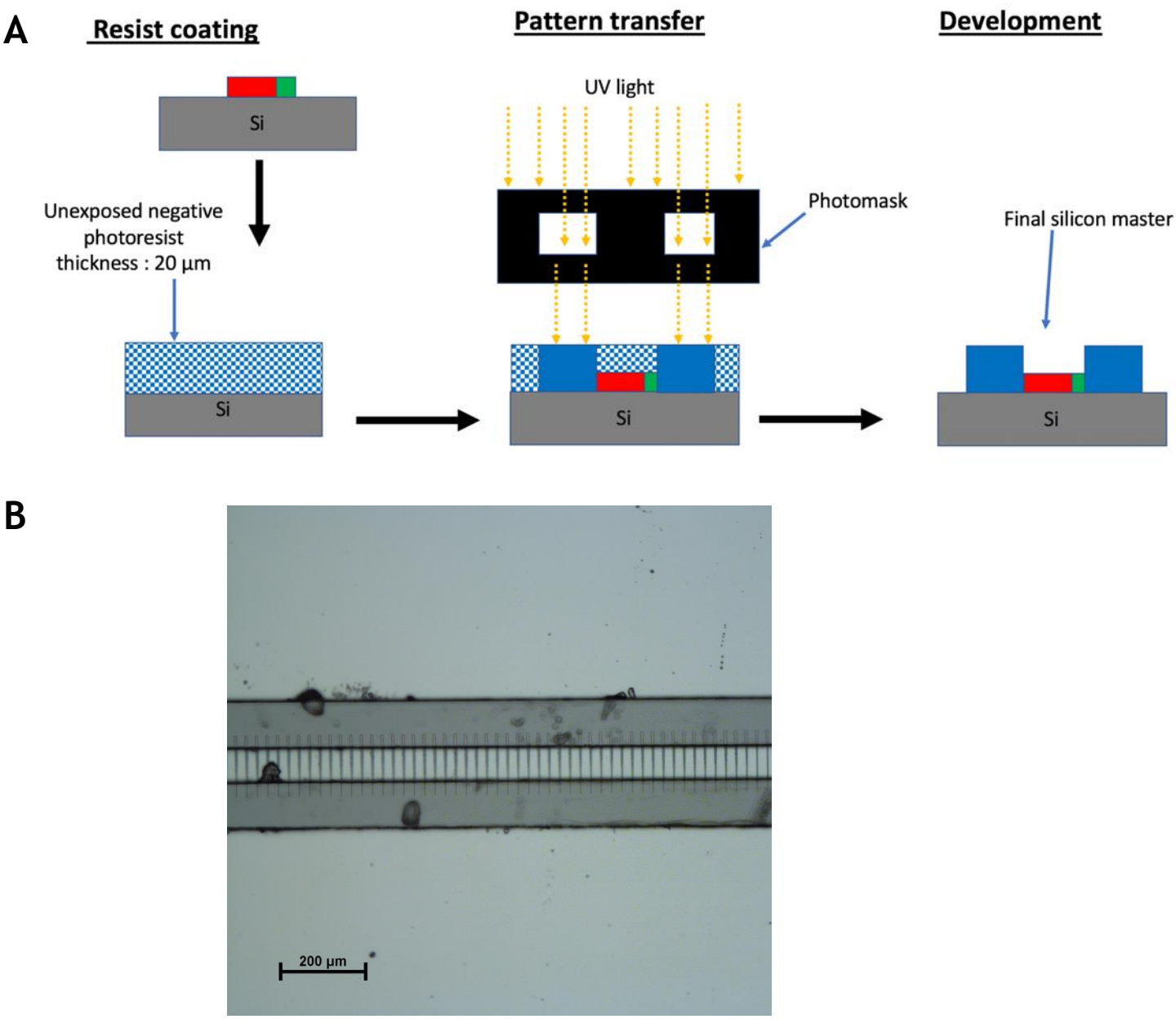

Figure 6.6- Photolithography stage of the process. A) Schematic overview: SU8 photoresist is spun on to the patterned master clean for a thickness of 20 µm then exposed to UV through the photomask and developed to reveal the pattern relief. **B)** Microscope view of photoresist channels over the e-beam channels after development.

6.3.6 PDMS device fabrication

To prevent PDMS from adhering to the silicon master and make it easier to peel off when it has been moulded, the master has to be coated with a salinizing agent such as Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Patel et al., 2013). We found that while there appeared to be no significant effect on increasing the time of silane coating exposure past 1 minute, doing so for more than 1 minute left a strong residue over the silicon master. To avoid this, 1 minute was chosen as the time for silane coating.

Following conformation of the achieved dimensions by microscopy and Dektak measurements, the HSQ/SU8 patterned silicon master was ready to imprint the pattern into PDMS for the fabrication of the microfluidic device (table 6.5). The process to prepare PDMS for patterning, is relatively unchanged in many microfluidic devices; with the PDMS mixture being mixed with a curing agent at a weight ratio of 10 to 1 and cured at 70ºC in order to harden the material. After moulding, the PDMS is punched with holes in the inlet and outlet using a biopsy punch slightly smaller than the diameter of the tubing intended to be used. This is then plasma or corona treated to help bond the PDMS to a glass slide (table 6.6) so that the channels can be viewed under microscope (Fig 6.7). The treatment also converts PDMS to a hydrophilic form allowing solution to flow along the channels easily without bubbles forming (Jenkins, 2013).

As with the other processes in microfluidic fabrication, the preparation of PDMS and the bonding technique used is critical in the success of the device and efforts should be made to optimise the process where necessary. For example, we found that the addition of more curing agent (a 5:1 PDMS: curing agent weight ratio) and longer curing times, increased the strength of the PDMS mould but also made peeling off the silicon substrate more difficult and left artefacts that may have negatively impacted the bonding process.

Additionally, we find that applying the corona discharge to the glass slide and PDMS device for longer than 30 seconds decreased the strength of the bond so that it could be easily broken.

Table 6.5- Table detailing the protocol for PDMS device fabrication. Includes silane coating of the resist coated substrate, PDMS curing and moulding.

PDMS device fabrication
Silane-coating • Clean the sample with a nitrogen gun • Plasma ash the sample at 150 watt for 3-5 minutes • In fume hood, add 40 µl of the salinizing agent to the side of the petri dish next to the sample • Leave the petri dish covered with watch glass for 1 minute • Place the covered petri dish on hot plate at 150°C for 15 minutes • Place the sample on watch glass to cool for 1 minute • Wash the wafer for 1 minute in water • If the process was successful, the water should not stick to the master
PDMS curing and moulding • Mix the PDMS mixture and curing agent at a weight ratio of 10 to 1. • Degas the mixture in a vacuum for 25 minutes or until all bubbles are gone. • Place wafer in a Petri dish of similar size and pour PDMS mixture on top. Pop any bubbles that may form • Degas for another 20 minutes. • Place the dish containing the device and PDMS mixture into the oven at 70°C for 3 hours. • After the PDMS mixture has hardened, move the dish to a sterile hood and use a sharp tool to cut around the master carefully to remove the PDMS.

Table 6.6- Table detailing the protocol for bonding PDMS device to a glass slide

Bonding to glass slide
• Clean the PDMS and the glass slide with IPA and then distilled water • Dry carefully with a nitrogen gun • Treat the glass slide and then the PDMS with the corona discharge for 30 seconds each by moving the discharge gun approximately 2cm above the surface, being sure not to burn it. • Quickly press the PDMS chip evenly to the glass slide for 30 seconds • Heat in an oven for 1 hour at 70°C Allow to cool and treat with UV for 15 minutes.

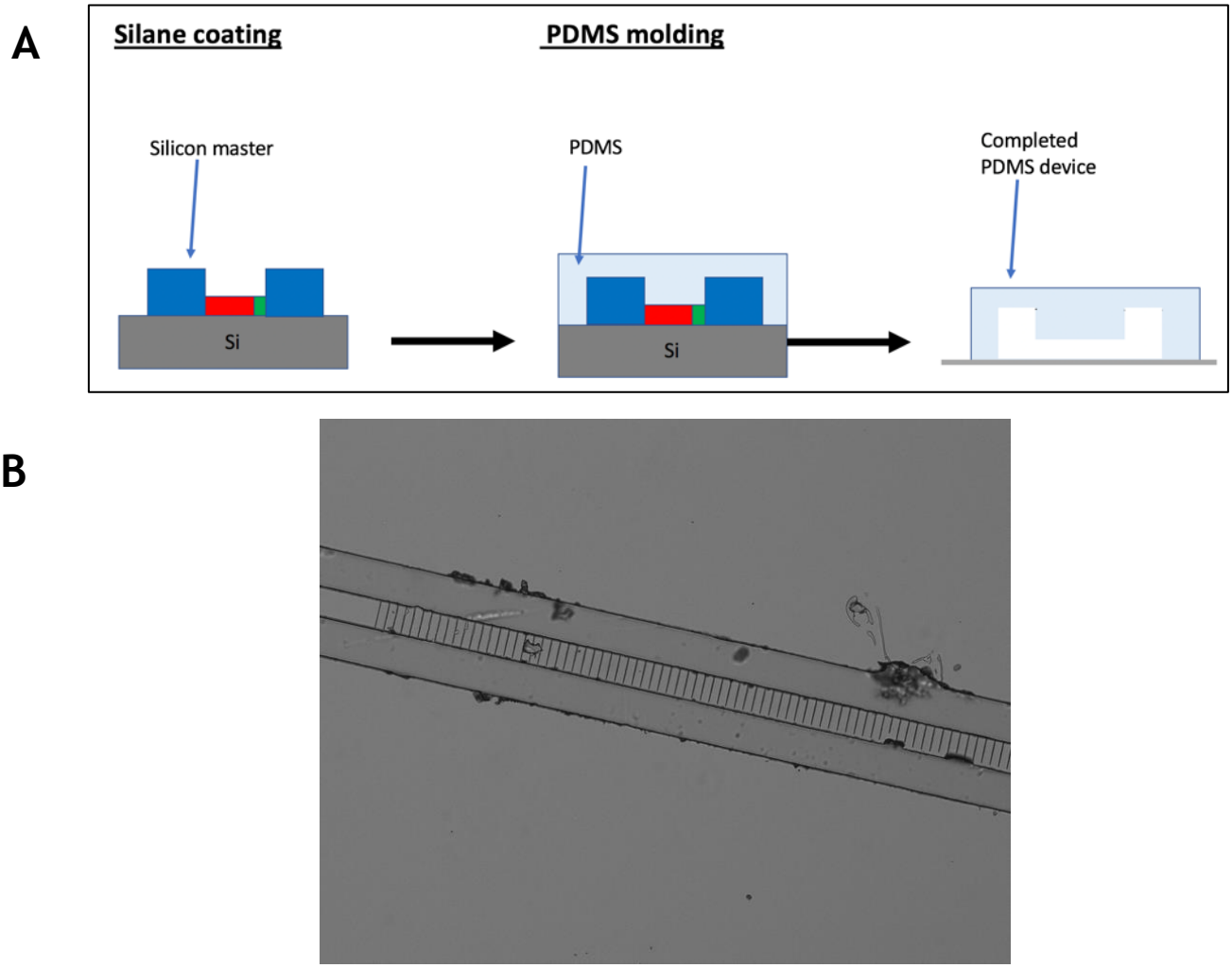

Figure 6.7– PDMS device fabrication. A) Schematic overview: A patterned silicon master is coated with a silane agent to aid the peeling off of PDMS. PDMS is cast to the master, removed and then bonded to glass slide using corona treatment. **B)** Microscope view of PDMS device after fabrication.

Using the optimal procedures we determined for e-beam lithography and HSQ planarization combined with traditional soft lithography, we fabricated 100 narrow micro channels on a PDMS device that aims to use hydrodynamic focusing to capture bacterial cells before exposure to *Bdellovibrio* (Fig 6.8). These growth channels are approximately 1 µm in height and 0.9-1.2 µm wide. The growth channels narrow to approximately 0.45µm wide for the last 10µm of length at the opposite side of the growth chamber connecting to the larger feeding channel. This is to prevent the *Pseudomonas* bacteria cells (approximate size: 0.5 µm in width and 2.0-2.5 µm long) from flowing out of the growth channels and ensures a

monolayer of cells without trapping the smaller *Bdellovibrio* cells (approximate size: 0.4 μm in width and 1.2 μm long). The overall length of the growth channel could be chosen from 5μm to 75μm in order to investigate the growth of different cell monolayer lengths. The growth channels were connected to two 50 mm wide and approximately 20 mm deep feeding channels. The media flow in the feeding channels continuously delivers fresh nutrients/*Bdellovibrio* to the trapped bacterial cells inside the growth channels and removes metabolic waste.

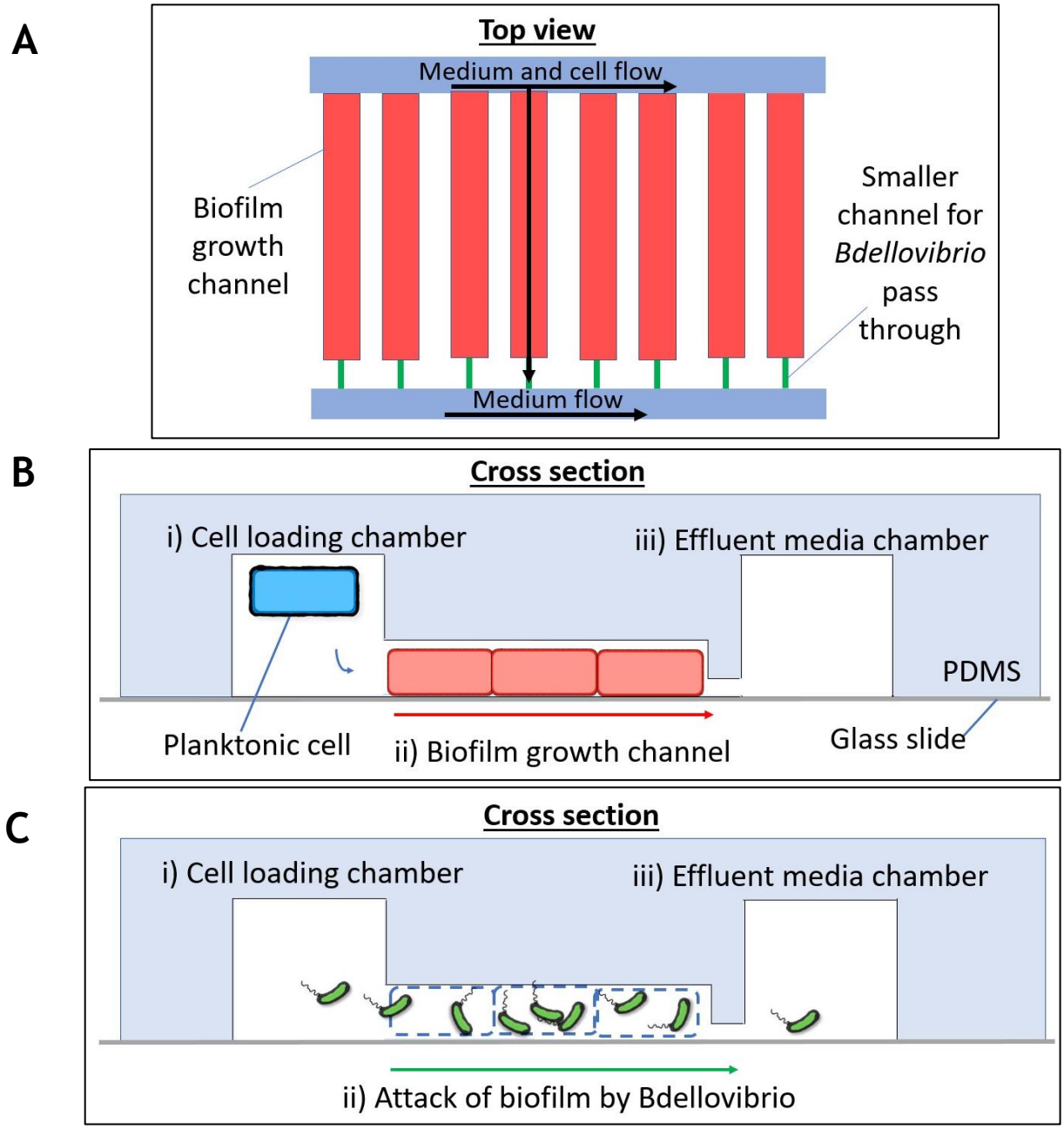

Figure 6.8- Design of microfluidic device to investigate the effect of biofilm thickness on predation. Cells (blue) are loaded through the medium chambers where they enter the biofilm growth channel and attach to the surface to grow as biofilm (red). The length of this channel can vary and will determine the thickness of the growing biofilm as at the end of the channel there is a smaller microchannel blocking the prey cells from passing through to the opposite chamber. This is not the case with predators (green) which sufficiently lyse the prey bacteria in the biofilm to reach the opposite media chamber where it may be sensed or collected as effluent for further analysis.

With these dimensions we were able to load bacterial cells on these devices and confine their positioning in growth channels within minutes (Fig 6.9). This is in contrast to close ended mother machine devices, where cell loading can take up to a few hours. This is vital in investigating the metabolic and growth dynamics of bacteria as prolonged time before observation could influence the results of such experiments.

The open-ended nature of this design relied on the narrow end on the growth channel and the difference in the pressure between the two feeding channels to trap the bacterial cells and prevent them from flowing out of the growth channel. However, the flow regime used for the top and bottom feeding channels caused too much pressure in the central growth channels causing the bacteria to deform the narrower end of the growth channels and pass through. In combination with the leaking at the feeding channel at the top (Fig 6.9), this allowed bacteria to accumulate in the feeding channel at the bottom.

This has consequences for the observation of growth dynamics as these cells could influence the flow of nutrients and waste in the growth channels. Thus, it is important to experiment further with the flow regime and thickness of the narrow end in order to prevent this from happening. We also fabricated growth channels of lower width (<0.8 μm), though these required higher flow rates that resulted in subsequent leaking and growth channel collapse.

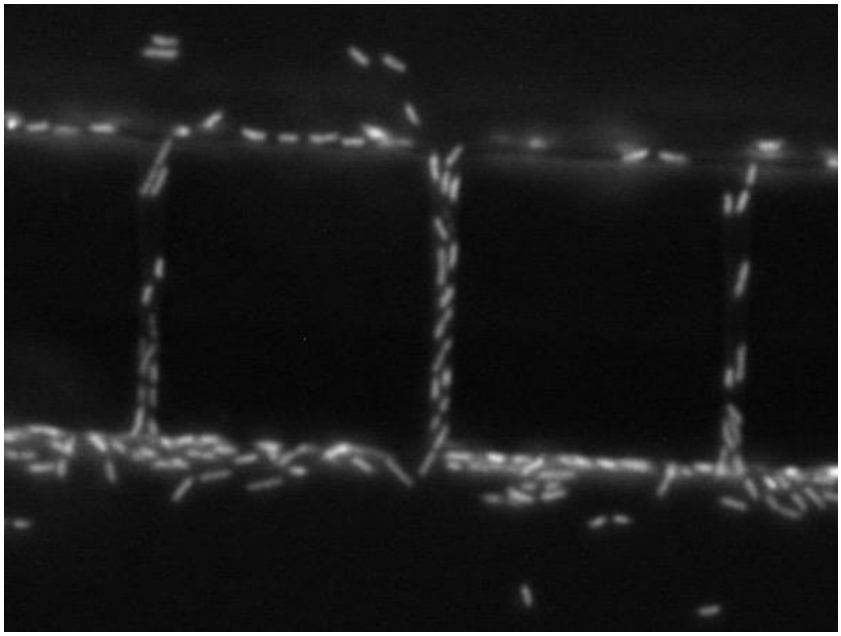

Figure 6.9- Microscope view of *Pseudomonas* cells trapped in growth channel of PDMS device. The feeding channel for loading cells is at the top and feeding channel for media is at the bottom. Media and cells are loaded through these channels at the inlets from the left and removed from the device along with any waste via the outlets to the right.

Unfortunately, further experimentation on this and the growth dynamics was not able to be carried out as the device was prone to significant leaking from the inlet and the edge of the device bonded to the glass slide. This is not likely to be a consequence of the channel fabrication of the device but instead the flow rates that were used and importantly, the cleaning and bonding technique for the glass slide and PDMS device. Despite this setback, we were still able to demonstrate the ability of e-beam lithography to be combined with photolithography in a device and we were easily able to visualise bacterial cells which would enable cell to cell investigation.

Leaking is a common issue with microfluidic devices (Borók et al., 2021), they can prevent the device from operating as intended, can interfere with imaging and results in unsafe biological contamination of the microscope. Despite this there are a lack of studies that investigate mechanisms to increase bonding strength of PDMS devices.

For bonding we used a handheld corona discharge system because of its low cost and ease of use. The success of this method was shown to vary, and we found that greater reliability in bonding when treating both surfaces for 30 seconds rather than longer times (>1minute) that are often reported. We also found that after 1 hour, increasing the time of incubation at 70°C for the glass bonded PDMS device seemingly gave no significant increase in bond strength. Though it is important to note that the process of corona discharge is influenced by many other factors such as the distance used for treatment and the condition of the surfaces used which make it difficult to assess, though there are methods to quantify bond strength of PDMS devices (Borók et al., 2021).Using Oxygen plasma treatment may be a better method for bonding this PDMS to a glass slide and would allow for more uniform testing. Solving the bonding problem is likely to involve a significant research effort that could not be achieved in this time-limited PhD.

With the significant leaking of the device, we were unable to achieve our ultimate goal of challenging the model biofilms with *Bdellovibrio* predators, to investigate the effect of biofilm thickness on predation and establishing the length whereby predation cannot outcompete prey growth. There is still the need for further studies that both investigate *Bdellovibrio* predation in continuous flow and single cell settings such as in microfluidics as well as investigations that aim to quantify the effect *Bdellovibrio* predation has on different biofilm characteristics.

6.4 Conclusion

Modelling *Bdellovibrio* predation is vital in developing action plans to mitigate biofouling in decentralised drinking water treatment. However, there are a lack of models that capture the dynamics between the predator and the prey when growing as biofilm. This is likely due to the lack of high-throughput techniques that are able to reliably measure the heterogeneity and complexity of biofilm growth. We developed a protocol for fabricating a microfluidic device to investigate the predation dynamics on an array of simplified, model biofilms.

Microfluidics presents a promising technology to further these investigations into predation of biofilms by *Bdellovibrio*, especially at a single-cell level. And the success of fluorescent probes such as WGA in the static assay of chapter 5 suggests that fluorescent probes would be useful to assess and quantify biofilm growth by microscopy. Here we demonstrate a method to fabricate nanosized features using traditional photolithography combined with e-beam lithography. This particular device was intended to allow predation to be viewed against a single cell line of prey cells, that would act as a model 1-D biofilm. However, the process as a whole can also be rapidly and accurately applied for other device designs, that would house more complex biofilm structures. Importantly, the device would also allow the high-throughput and real-time observation of bacteria in different growth conditions and revealing a range of different parameters such as growth rate, attachment rate, EPS production that conventional biofilm assays ignore.

Currently a protocol to evaluate and increase the bonding strength of the PDMS device to the glass slide is needed to mitigate the leaking found in the device but with this and further development the methods proposed in this study would also lend to the linking of microorganism population function to population quantity which is vital in the effective application of *Bdellovibrio* for biofilm mitigation. Unfortunately, preventing leaking and maximising bond strength are significant issues and difficult to troubleshoot in a high throughput manner and so despite our extensive efforts we were unable to achieve a solution.

7 Conclusion

Bdellovibrio predatory bacteria have been identified as a potential mitigation to biofilm growth in many applications including dentistry, agriculture and drinking water treatment. There is a need to collect more reliable data on the predator-prey dynamics in order to advance any future uses of predatory bacteria. This entails carrying out more basic studies of population dynamics and interactions between predatory bacteria and their prey (Dwidar et al ., 2012), which is difficult as the methodology and research techniques typically used in microbial ecology are lacking where *Bdellovibrio* is concerned.

Therefore, a key aim of this thesis was to explore and develop novel methodologies, such as flow cytometry and microfluidics, to measure and observe *Bdellovibrio* and the growth of gram-negative bacteria such as *Pseudomonas* species, a key biofilm forming species in drinking water systems.

With these methods we aimed to conduct fundamental investigations into the dynamics of *Bdellovibrio* predation with the aim of developing mathematical models that can begin to inform strategies for the application of predatory bacteria in industrial practice like the removal of biofilms in membrane-based drinking water treatment. We focused on capturing the fundamentals of predatory prey interactions in simplified abstractions of potential industrial applications.

Many of the basic ecological investigations into dynamics of predatory bacteria and their prey are limited by the lack of precise, dependable and fast methods for counting and characterising cells. For the first time, we demonstrated in a published article, that flow cytometry is a reliable tool for simultaneous *Bdellovibrio* and *Pseudomonas* quantification, highlighting its potential to expand on the current knowledge in the field. We established a procedure for distinguishing and counting *Bdellovibrio* in lone and mixed populations based on their size and fluorescence. The validity of this method was demonstrated by comparing the method to the more traditional enumeration approaches in optical

density (OD), plaque forming unit assay (PFU), and quantitative polymerase chain reaction (qPCR).

While there has been at least one demonstration of using flow cytometry in the enumeration of *Bdellovibrio* (Duncan et al., 2018), this study is the first description of a protocol to distinguish *Bdellovibrio* and their gram-negative prey based on size rather than measuring the fluorescence of proteins expressed by the organisms, so the protocol could be used with other gram-negative bacteria which are larger than the predator such as *E. coli*. Additionally, this is the first study to validate flow cytometry for *Bdellovibrio* enumeration by comparison to other quantification methods. This represents a significant advancement in *Bdellovibrio* ecology research, where the majority of literature makes use of plaque forming unit assay that is both inaccurate and takes days before results can be obtained. This will serve not only the capture of experimental data but also the regular maintenance and observation of *Bdellovibrio* cultures.

One limitation of this study is the lack of comprehensive comparison between the flow cytometry results and an absolute count by microscopy. The accuracy of flow cytometry is not in question as it has been compared to qPCR which is seen as a gold standard for microbial enumeration, but microscopy does provide the advantage of being able to distinguish bdelloplasts from uninfected prey cells. As of yet, flow cytometry has not been shown to be able to do this, but with the advancement of fluorescent tags and the identification of different genes characteristic of the *Bdellovibrio* predation cycle, there is the potential to do so. Likewise developing the flow cytometry protocol beyond the use of SYBR green, which is a non-specific DNA stain, is necessary to be able to distinguish bacteria in multi-species communities.

With the development of a reliable method for measuring *Bdellovibrio* and the gram-negative prey *Pseudomonas*, we were able to then go on to use this as means to investigate the population dynamics of the two species. While quantification methods such as flow cytometry can be used to monitor growth and potential contamination in membrane-based drinking water systems, monitoring is only one

part of the process involved in microbial engineering. Successful application will rely on the ability to predict microbial ecology and engineer them towards particular goals such as biofilm removal by *Bdellovibrio* predation.

Mathematical models, in particular, are an important step in the design and troubleshooting of biocontrol and water treatment procedures, and their use is widely studied for microbial predators but less so with *Bdellovibrio*. Mathematical models depend upon of a set of parameters that can represent the growth dynamics of populations. A thorough understanding and quantification of these parameters can give insight into predator-prey interactions, making it easier to exploit conditions that would optimise the desired predatory bacteria action in a specific application, such as complete pathogen clearance or biofilm removal (Williams and Pieiro, 2006).

We employed mass-action mathematical models based on the Lotka-Volterra set of equations to investigate the dynamics of *B. bacteriovorus* and *Pseudomonas* in the presence of a carbon limiting substrate (glucose) in a batch and chemostat setting. Specifically, we used flow cytometry and a coulometric glucose assay to quantify both bacteria populations and the glucose substrate concentration in batch cultures over time. From this we gained parameters for the model: maximum growth rate, yield and saturation constants to predict the outcome of batch cultures with different initial values of the prey, predator and substrate.

By validating the model for bacterial growth in batch experiment, we add to one of the few studies that has aimed to do so and demonstrate that although the nature of *Bdellovibrio* predation is complex it can in part be simplified by mathematical models. It is also the first time that *Bdellovibrio* predation can be described by the Holling type III function, which we propose is the result of the low specificity, random collision method of locating prey displayed by *Bdellovibrio* and could explain why *Bdellovibrio* as so strongly associated with high-density bacteria environments such as biofilms.

Using these same parameters in a chemostat model we examined what circumstances related to glucose concentration in the influent and dilution rate are necessary for the many possible solutions of the prey and predator culture: prey and predator washout, predator washout, predator-prey stability and predator-prey oscillations.

To test the ability of the model to predict for predator-prey oscillations, we conducted a chemostat experiment to compare the outcome. To our knowledge, we demonstrated the first example of *Bdellovibrio* predator prey oscillations in a chemostat and expands upon previous studies that have only theoretically demonstrated this (Summers and Kreft, 2019). Predator-prey oscillations are of great interest in microbial ecology and are of significance in regard to mitigating biofouling in membrane filtration because it allows the prey to regrow up until a point that is still manageable but also allows the continuous periodic growth of *Bdellovibrio* so that the system is effectively self-sustainable and repeated inoculation of the predator is not required. This is especially important in decentralised systems where regular maintenance or care may not be possible.

It should be noted that there were discrepancies in the actual concentrations achieved; the oscillations in experiment were generally of lower amplitude. By significantly decreasing the maximum growth rate of the prey we found that the model was a better fit. Thus, we proposed that the discrepancies were the result of prey biofilm growth on the chemostat chamber wall. This altered the dynamics of the predator-prey system as the biofilm are generally slow-growing and more resistant to washout in comparison to planktonic species in the bulk liquid.

We only measure biofilm growth in the chemostat at one time point, which is not enough information to accurately model the biofilm growth, or even to describe the relationship with the biofilm and planktonic bacteria in the bulk liquid in relation to the process of attachment and biofouling as well as any influence on this from predation. It was not possible to measure biofilm periodically with the bioreactor setup used in this study but there are a range of bioreactors such as the modified Robbins device that allow for such. Microfluidics also presents an

interesting alternative for measuring predator-biofilm dynamics as the rate of attachment and detachment of prey bacteria to the surface can be observed.

Regardless, the ability of the model to capture some of the oscillations displayed is significant as it demonstrates the power of models to guide experimental planning. Trying to find the parameter space required for oscillations entirely through trial-and-error experimentation would have been difficult and wasteful. Further experimental validation of the model's ability to predict other outcomes in chemostat such as predator washout would increase the confidence in the model.

In order to expand on the dynamics that we have captured for *Bdellovibrio* predation in chemostat, there is a strong need for investigating the effect of predation specifically on biofilms in different environments. The formation of biofilm and its removal by predation is not determined by the cell concentration alone, but a combination of chemical and biological processes as well as the exchange between planktonic bacteria within the bulk liquid. It is well known that mature biofilms require a combination of an associated surface, the microorganisms and the extracellular polymeric substances (EPS) in the biofilm matrix to develop (Elkhatib et al., 2014). Additionally, the heterogeneity of a biofilm and its environment has previously been shown to effect biofilm development, maturity and its tolerance to stresses. Heterogeneity can be displayed in a number of ways in the biofilm: distribution and composition of microbial species, biofilm thickness and EPS production. All of which is affected by the nutrients available in the environment.

We investigated the effect of predation on the growth and EPS production of static biofilms grown previously in a nutrient rich (LB), nitrogen rich (HEPES buffer supplemented with casein hydrolysate), carbon rich (HEPES buffer supplemented with glucose) and nutrient poor (HEPES buffer) environment by measuring end-point changes in biomass (crystal violet) and viability (resazurin) using microplate staining assays that have been traditionally used to measure susceptibility of biofilms to antimicrobials. With the addition of a wheat germ agglutin (WGA) assay to quantify poly-N acetylglucosamine (PNAG) residues in biofilm EPS and flow

cytometry to quantify the total and intact cells in the biofilm and in the bulk liquid as planktonic cells.

The addition of these methods addresses the limitations of previous studies investigating *Bdellovibrio* predation of biofilms, first by improving the accuracy of cell quantification through the use of a direct enumeration by flow cytometry. Secondly, by providing more informative analysis of the heterogeneity of biofilm growth rather than only taking into the account the biomass of the biofilm.

The role of biofilm EPS on *Bdellovibrio* predation has rarely been considered despite the fact that EPS has been shown to increase biofilm survival after exposure to some antimicrobials. We show that retention of EPS may be key in the adhesion of biofilms to surfaces and in the persistence of metabolically damaged cells existing in the biofilm when exposed to *Bdellovibrio* predation. In contrast, the reduction of EPS in biofilms during predation may be linked to a change in the proportion of biofilm cells to planktonic cells in the system.

We demonstrate for the first time the power of flow cytometry to measure *Pseudomonas* biofilm cells and *Bdellovibrio* cells, which has the benefit over traditional biofilm quantification such as crystal violet or turbidity measurements as it allows direct enumeration of cells and can distinguish between the prey and predator species. This is key for the development of mathematical models that can be used to predict and quantify biofilm growth under predation and necessary for the development of biofilm mitigation plans in engineered systems.

Additionally, with flow cytometry we can gain information on the cell size and concentration of the planktonic bacteria which can aid the quantification of other parameters that are key in biofilm growth such as predator yield and biofilm attachment. And with the development of commercially available fluorescent stains there is the potential to target and quantify other characteristics of biofilm growth with flow cytometry such as the PNAG and the terminal mannosyl residues of biofilm EPS, proteins and nucleic acids. This is necessary to assess the

antibacterial and antifouling effect of *Bdellovibrio* against different biofilm components.

When using end-point measurements of static well plates to investigate biofilm growth such as here, there is a limit to how much can be understood with regards to how *Bdellovibrio* can affect the whole process of biofilm attachment removal and development. Microfluidics combined with time-lapse microscopy represents a useful tool to aid this as it would allow continuous application and monitoring of microbial aggregate and biofilm growth and can be combined with the quantification of EPS with non-invasive stains such as WGA.

Though many microbial ecologists recognize the benefits and applications of microfluidics, many can struggle to understand the processes needed to design a reliable protocol and fabricate the desired devices due to lack of an engineering background. Thus, we introduced microfluidic devices, the techniques and technologies used to develop them and a clear protocol that demonstrates how to design, develop and fabricate microfluidic devices.

Biofilm thickness is another growth parameter that could potentially be investigated with the use of microfluidics and is anticipated to have a significant impact on predator-prey dynamics due to the combined effect of prey bacteria deeper in biofilms being shielded from predation in thicker biofilms.

Biofilm thickness is difficult to manage ordinarily, which is a key reason as to why biofilm thickness has not previously been considered in regard to *Bdellovibrio* predation dynamics. Thus, we aimed to limit bacteria growth in the microfluidic device to a single 1-D line to represent a 1-D intersection of a 'typical' layered biofilm. Each prey bacterium is attached to the channel surface as well as another bacterial cell on either side of it, generating a single cell line of biofilm, similar to growth found in a mother machine (Long et al., 2013). This structure was to force predators to attack and penetrate single prey cells at a time. The length of the biofilm growth channels could vary by design and served to represent the biofilm thickness.

The resolution required to fabricate such a device is not possible with traditional photolithography techniques, so we presented a new, simple and reliable method of doing so that combined e-beam lithography and Hydrogen silsesquioxane (HSQ) resist planarization with photolithography. We were able to construct a PDMS device with the required channel geometry needed to trap *Pseudomonas* cells, demonstrating for the first time, the suitability for HSQ planarization to rapidly create high resolution structures in PDMS and also a demonstration of combining e-beam lithography with photolithography for PDMS structures.

Unfortunately, we were unable to test the effect of biofilm thickness with this device as we had significant issues with leaking and maintaining the integrity of the PDMS channels which we perceive to be the result of the cleaning and bonding of PDMS to the glass slide and the optimising the flow speed that can be used for the device.

A successful device of this kind with growth channels that restrict the depth of bacterial growth would be very useful to gain insight into microbial predator and prey dynamics at a single cell level. We could apply this approach in a similar manner to the batch and chemostat modelling study where we measure bacterial growth over time to gain kinetic parameters. In this case we can monitor biofilm growth to capture key parameters and apply it to the current predator prey models developed in this study. Combined, this knowledge would be key in predicting and optimising the use of *Bdellovibrio* as a biofilm control agent in membrane-based technologies.

7.1 Future perspectives

Overall, the future of decentralised drinking water systems that exploit *Bdellovibrio*, requires more progress into the fundamental understanding of their dynamics with prey species. This thesis has contributed to the development of this approach in particular through the addition of an accurate and high-throughput flow cytometry protocol to quantify *Bdellovibrio*. We have demonstrated the usefulness of this protocol with the development of mathematical models which

we used to predict and further investigate the dynamics between *B. bacteriovorus* and its prey *Pseudomonas*. These models can be progressively built on by including the presence of bdelloplasts, multiple different organic substrate and different prey and non-prey species to better mimic the environment in nature and engineered sytems.

Similarly, there is the need to investigate more thoroughly the effect of *Bdellovibrio* predation on bacterial growth in its entirety, including the complete phases of biofilm growth in combination with the planktonic bacteria. We have addressed this in part with the microplate assay in chapter 5, but progress can be made by using the assay to investigate the effect of predation on biofilm growth under different concentrations of a growth limiting substrate to gain model parameters (such as the yield, attachment and detachment rate) and develop a model similar to those in chapter 4. This can be utilised to better gain an understanding of biofilm growth under predation and get closer to building a model that can be used to predict the growth of biofilm and biofouling under predation in membrane filtration systems.

Importantly, we observe how *B. bacteriovorus* struggles to cause significant further decrease to *Pseudomonas* populations of 10^6 cells/ml or less. This indicates that it is unable to ensure the complete removal of prey to the same extent as chemicals such as chlorine or antibiotics. However, with an understanding of *Bdellovibrio* predation dynamics, the predator could be combined with other antibacterial agents such as chlorine or even other biological predators such as bacteriophages. These antibacterial agents can potentially be used to target prey cells that are able to evade *Bdellovibrio* such as gram-positive bacteria. Thus, more extensive studies need to be conducted to investigate the action of *Bdellovibrio* in the presence of antibacterial chemical or other biological predators, especially in regard to the temporary phenotypic resistance that is displayed by prey species under *Bdellovibrio* predation (Shemesh and Jurkevitch, 2003).

Complete removal of the prey may not be necessary in membrane technologies; the addition of *Bdellovibrio* could be sufficient in reducing the overall effect of

biofouling and increase the lifespan of membrane filters which are currently vulnerable to biofilm growth and biofouling, reducing their effectiveness and making them dysfunctional after some time. It is this lack of durability after consistent biofouling and the financial and energy cost associated with membrane drinking water filtration systems that has limited their application on a larger scale (Kim *et al* ., 2013a). Therefore, it is also necessary to characterise the effect of *Bdellovibrio* predation on the long-term performance and durability of membrane filtration systems. Though these kinds of large-scale investigations can be costly and time-consuming to operate, fundamental investigations and the development of mathematical models such as those in this thesis are vital in improving their efficiency and likelihood of success.

8 Appendix

8.1 Chapter 3

Table 8.1- The mean and standard deviation for optical density (600nm) values from three observations and the Box-Cox transformations (lambda=-0.6).

OD(600nm)	Standard deviation	Box-Cox transformed OD
0.00233333	0.00057735	-61.637923
0.00266667	0.00057735	-57.607402
0.00266667	0.00057735	-56.801539
0.00266667	0.00057735	-56.713789
0.00666667	0.00057735	-32.227299
0.007	0	-31.051573
0.008	0	-28.532486
0.052	0.002	-8.156392
0.11	0.01	-4.599662

8.2 Chapter 4

8.2.1 MATLAB code for batch predator prey model

```
%name of function
function simple_batch
%choose time range of model output. Timerange= linspace (start point (h), end point (h), time interval)
timerange = linspace(0,72,100);  % time in hrs

%legend
% s0 is the initial substrate concentration (mg/L)
% x0 is the initial prey concentration (cells/ml)
% y0 is the initial predator concentration (cells/ml)
```

```
% z0 is the initial 'dead' prey concentration (cells/ml)
% eta_x is the yield of predator consuming prey (predator cells per 1 prey cell)
% eta_S  is the yield of prey feeding on substrate (prey cells/ml per mg substrate)
% mu_prey = maximum growth rate of prey feeding on substrate (h^-1)
% ksat_S is half saturation constant for prey feeding on substrate (mg/L)
% mu_pred = max growth rate of predator (h^-1)
% ksat_pred = half saturation constant of predator feeding on prey (cells^2/ml^2)
% State variable 1 (a(:,1)) is the prey (cells/ml)
% State variable 2 (a(:,2))  is the predator (cells/ml)
% State variable 3 (a(:,3))  is the number of dead prey (cells/ml)
% State variable 4 (a(:,4)) is the substrate (mg/L)

% parameters
mu_prey = 1.0 ;
mu_pred = 0.24;
eta_x = 1.95;
eta_S = 3.1*10^7;
k_S = 0.18;
k2 = 2.74 *10^15;

%intial conditions
x0 = 1 *10^6;
y0 = 1 *10^6;
S0 = 5;
z0 = 0;

%Define state variables
a0 = [x0 y0 z0 S0]';

%Define function
[t,a] = ode45(@(t,a) differntials(t,a,mu_prey, mu_pred, k2, eta_x, eta_S,
k_S),timerange,a0);
```

```
%transfer data to csv
xlswrite('batchmodelTEST.xlsx',[t(:),a(:,1),a(:,2),a(:,3)+ a(:,1),a(:,4)]);

hold on

%Viewing the result
figure(1)
tiledlayout(4,1)
nexttile
plot(t,a(:,1))
hold on
plot(t,a(:,2))
plot(t, a(:,3)+ a(:,1))
xlabel('Hours')
ylabel('Number')
legend('Prey', 'Predator', 'Prey + Dead Prey')
nexttile
plot(t,a(:,2))
xlabel('Hours')
ylabel('Number')
legend('Predator')
nexttile
plot(t,a(:,1))
xlabel('Hours')
ylabel('Number')
legend('Prey')
nexttile

plot(t,a(:,4))
xlabel('Hours')
ylabel('mg/l')
legend( 'substrate')
hold off
```

```
function dadt = differntials(t,a,mu_prey, mu_pred, k2, eta_x, eta_S, k_S)

%Equations
dadt = zeros(size(a));
m_prey = mu_prey*a(4)/(k_S + a(4));
m_pred = mu_pred*a(1)^2/(k2 + a(1)^2);

dadt(1) = m_prey*a(1) - m_pred*a(2)/eta_x ;
dadt(2) = + m_pred*a(2);
dadt(3) = m_pred*a(2)/eta_x;
dadt(4) = -m_prey*a(1)/eta_S;
```

8.2.2 MATLAB code for chemostat predator prey model

```
function chemostat_predator

%legend
% s0 is the influent substrate concentration (mg/L)
% x0 is the initial prey concentration (cells/ml)
% y0 is the initial predator concentration (cells/ml)
% eta_x is the yield of predator consuming prey (predator cells per 1 prey cell)
% eta_S  is the yield of prey feeding on substrate (prey cells per mg substrate)
% mu_prey = maximum growth rate of prey feeding on substrate (h^-1)
% ksat_S is half saturation constant for prey feeding on substrate (mg/L)
% mu_pred = max growth rate of predator (h^-1)
% ksat_pred = half saturation constant of predator feeding on prey (cells^2/ml^2)
% D is the diluation rate (h^-1)
% State variable 1 (a(:,1)) is the prey (cells/ml)
% State variable 2 (a(:,2))  is the predator (cells/ml)
% State variable 4 (a(:,4)) is the substrate (mg/L)

%model parameters - these values could be estimated in batch experiments.
```

```
S0 = 25;
eta_S = 3.1*10^7;
eta_x = 1.95;
D = 0.17
mu_prey = 1.0;
mu_pred = 0.24;
ksat_S = 0.18;
ksat_pred = 2.74*10^15;

% State variable 1 (a(:,1)) is the prey (cells/ml)
% State variable 2 (a(:,2))  is the predator (cells/ml)
% State variable 3 (a(:,3)) is the substrate (mg/L)



n = 40  %run the model for approx n retention times
tau = [0 n/D]; %

%Initial conditions
Start_S = S0
Start_prey =  3.5*10^7
Start_pred = 6.55*10^7

%Define state variables
a0 = [  Start_prey Start_pred Start_S ]

%Define function
[t,a] = ode45(@(t,a) differntials(t,a,D,S0,mu_prey, mu_pred, ksat_pred, eta_x,
eta_S, ksat_S),tau,a0);

%transfer data to csv
xlswrite('chemostatmodelcsvtable.xlsx',[t(:),a(:,1),a(:,2),a(:,3)])

%Viewing the result
```

```
figure(1)

plot(t,a(:,1))
hold on
plot(t,a(:,2))

plot(t,a(:,3))

legend('Prey', 'Predator','Substrate' )
hold off

%Then tau - the real time
tt = t;
S = a(:,3);
x = a(:,1);
y = a(:,2);

figure(2)
plot(tt,S);
title('Substrate unscaled')

figure(3)
plot(tt,x);
title('Prey unscaled')
figure(4)
plot(tt,y);
title('Predator unscaled')

figure(5)
plot(x,y)

%Equations
```

```
function dadt = differntials(t,a,D,S0,mu_prey, mu_pred, k2, eta_x, eta_S, k_S)

dadt = zeros(size(a));
m_prey = (mu_prey)*a(3)/(k_S + a(3));
m_pred = mu_pred*a(1)^2/(k2 + a(1)^2);

dadt(1) = -D*a(1) + m_prey*a(1) - m_pred*a(2)/eta_x ;
dadt(2) =  -D*a(2)+ m_pred*a(2);
dadt(3) = D*(S0-a(3))-(m_prey)*a(1)/eta_S ;



if (a(1) + dadt(1)) < 0
    dadt(1) = -a(1);
    a(1) = 0.0;
end

if (a(2) + dadt(2)) < 0
    dadt(2) = -a(2);
    a(2) = 0.0;
end
if (a(3) + dadt(3)) < 0
    dadt(3) = -a(3);
    a(3) = 0.0;
end
```

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