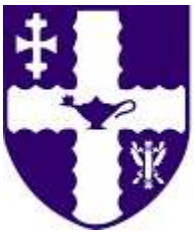

Loughborough University

**Development of a scalable upstream bioprocess for bacteriophage culture**

By Junaid Ali

A doctoral thesis

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Abstract

Since the discovery of antibiotics, there has been a revolution in treating microbial infections. However, with their increasing use, some microbes have developed a form of resistance to many of the available antimicrobial agents. Untreatable infections because of antimicrobial resistance (AMR) have recently become a global threat and it is estimated that by 2050, more than 10 million people will die per annum from resistant bacterial infections. Therefore, novel methods are needed to kill resistant microbes. One such method that may be used to kill resistant bacteria are bacteriophages (phages), viruses that infect microorganisms and cause them to lyse.

Although currently there are a limited number of clinical trials that use phage therapy, bacteriophages are considered as a promising candidate to tackle bacterial infections. Future clinical trials will require far larger volumes of phage than current levels can provide whilst the efficient production of phage must also be investigated. To address these challenges, the optimisation and scale up of two bacteriophages was investigated.

The project examines the bioprocess for T4 and bacteriophage K with the objective of optimising the conditions which contribute to phage propagation and improve upon it where possible using Design of Experiments (DoE). The temperature of infection was shown to play a significant role in the propagation of both, where it was found that 28$^{o}$C resulted in higher titres than 37$^{o}$C. Time, multiplicity of infection (ratio of phage : bacteria) and agitation were also investigated in shake flasks. Moreover, these conditions were then further improved upon through scale up into a stirred tank reactor. The kinetics, including burst size and adsorption were investigated for both phage which gave an insight as to why there may be an increase in titre seen. Titres of >1x10$^{14}$ pfu/ml were shown for T4 phage whilst the highest culture volume published to date (3L working volume) was shown for phage K. The data presented shows a reliable and robust method for both high titre T4 and phage K production in both shake flasks and a 5L bioreactor (3L working volume). Optimisation of bioprocesses allows users to improve yield, efficiency and reduce cost all of which may enable treatments being driven towards the market/clinic. The methods presented in this thesis have resulted in the greatest achievable titres for both phage.

A investigation into a novel method for the characterisation of the bacteriophage infection in both shake flasks and bioreactors is presented by sampling the culture and analysing the DNA/RNA and protein

content. Given the lytic mechanism of phage leading to the release of bacterial endotoxins, DNA/RNA and proteins into the culture, their presence will be enumerated throughout the cultures at various time points.

*Key words: Antimicrobial resistance, bacteriophage, bioprocessing, scale up, manufacturing, stirred tank bioreactor*

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1) 2016 Poster presentation – A scale up model approach to bacteriophage culture. Loughborough University
2) 2016 Poster flash presentation - A scale up model approach to bacteriophage culture. Loughborough University
3) 2017 Podium presentation – A scalable model for the translation of bacteriophage culture to manufacturing scale. Oxford University. Bacteriophages
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*Abbreviations*

| | |
|---|---|
| *Analysis of variance* | *ANOVA* |
| *Antimicrobial resistance* | *AMR* |
| *Brain heart infusion* | *BHI* |
| *Clostridium difficile* | *C. difficile* |
| *Colony forming units* | *CFU* |
| *Critical quality attributes* | *CQA* |
| *Defined medium* | *DM* |
| *Design of Experiments* | *DoE* |
| *Dissolved oxygen* | *DO* |
| *Escherichia Coli* | *E. coli* |
| *Extended spectrum beta lactamase* | *ESBL* |
| *Food and drug administration* | *FDA* |
| *Hospital acquired infection* | *HAI* |
| *Key process input variables* | *KPIV* |
| *Lipopolysaccharide* | *LPS* |
| *Luria broth* | *LB* |
| *Multiplicity of infection* | *MOI* |
| *One factor at a time* | *OFAT* |
| *Optical density (600nm)* | *OD600nm* |
| *Plaque forming units* | *PFU* |
| *Point of infection* | *POI* |
| *Pseudomonas aeruginosa* | *P. aeruginosa* |
| *Research and development* | *R&D* |
| *Response surface methodology* | *RSM* |
| *Revolutions per minute* | *RPM* |
| *Small RNA* | *sRNA* |
| *Staphylococcus aureus* | *S. aureus* |
| *Staphylococcus epidermidis* | *S. epidermidis* |

| | |
|---|---|
| *Stirred tank reactor* | *STR* |
| *Tangential flow filtration* | *TFF* |
| *Target product profile* | *TPP* |
| *Transmission electron microscopy* | *TEM* |
| *Tryptic soy broth* | *TSB* |
| *Urinary tract infection* | *UTI* |
| *Quantitative PCR* | *qPCR* |
| *Quality by Design* | *QbD* |

## Brief background to the PhD

Within recent years, there has been a rise in antibiotic resistance for several different reasons. This is in part attributed to the overuse and misuse of antibiotics. Antibiotics are distributed worldwide and there is evidence that antibiotics are often prescribed incorrectly. Moreover, given that antibiotics are heavily used in animal and plant farming, this increases the rise of bacterial resistance. Within the next three decades, it is estimated that >10 million people will die every year due to contracting infections that are unable to be treated, given that the bacteria will have developed resistance. At this time, resistant infections will be the second greatest killer of humans and therefore shows a real threat.

Bacteriophage i.e. phage, therapy has shown promise in treating resistant organisms, however, their use is currently restricted. Phage lyse bacteria, releasing DNA, RNA and proteins along with endotoxins from the bacterial organism and it would be unwise to allow this to contaminate humans. However, an additional worry is that there is currently no standardised method for phage production and very little evidence to show that their production is being tackled.

This PhD hoped to tackle an area of bacteriophage therapy that is vastly understudied and develop a standardised method for phage propagation. Two bacteriophages (T4 and K) were used against *E. coli* and *S. aureus*. There is evidence in the literature suggesting that there is no standardised method of phage propagation and those who have examined it, generally focus on the conditions which are ideal for the host propagation, rather than the phage propagation. Chapter 3 includes tables with some studies in the literature and shows the wide range of conditions that are used.

Additionally, the PhD also focused on the scale up into a stirred tank bioreactor. Scale up into automated reactors allows a greater level of control and a lower level of variability in the conditions within the culture and may reduce variation in final phage titre. Additionally, producing larger volumes in a single batch is beneficial as it reduces the need for multiple batches to be run which may reduce batch to batch variability and even prevent contaminations.

Finally, the PhD tackled a unique area of phage research, the detection of phage. Currently, the gold standard method is to use the plaque assay. Whilst this is a relatively cheap and simple method, it can take up to 24 hours

to enumerate and determine which phage is present in a sample. It is also further complicated if there is a cocktail of phage in a single sample. The work in chapter 5 shows how phage can be detected throughout the infection cycle based on a nanodrop spectrophotometer reading and a GC-MS method.

## Thesis structure

In chapter 1 there is a literature review which initially examines the development of antibiotic resistance along with current challenges and resistance seen in animals. Whilst antimicrobial resistance is seen in many antibiotics currently in use, there are certain organisms that have developed significant resistance to a wide variety of antibiotics which have been identified as some of the main threats. These are initially examined before a view is given towards clinical issues such as resistance in surgery and how it can effect patients with different conditions such as diabetes as well as those who have infections.

The literature review next examines alternative treatments to resistant bacteria other than antibiotics. These include antimicrobial peptides, bacteriocins and bacteriophages. The advantages and disadvantages is given of bacteriophages and due to the current research in the literature, bacteriophages are chosen as a viable method. Recently, synthetic biology of bacteriophages has been gaining merit. Genetic manipulation of phage has shown promising results in several areas of phage research and a look into this is given. Animal models and clinical trials is also looked at to show the promising results that have been made in vivo.

Finally, the literature review examines the production of phage. There is initially a look at the need for scalable bioprocesses and manufacturing. Bacteriophage bioprocessing is currently in an infantile state and the challenges are given. Manufacturing scientific approaches such as QbD and DoE are shown how they have been previously used and how they may be used in this work i.e. to improve phage production efficiency.

The materials and methods, chapter 2, follows and shows all of the techniques used for host growth and phage propagation. Two bacteriophage – host systems were used throughout this work, T4-*E. coli* and K-*S.aureus*.

Chapter 3 begins with the key process input variables that may be used in order to improve phage titres. A DoE is run in shake flasks using a 20ml volume with each of the factors that may effect the titre examined. Those factors which give the greatest titre are validated over several experiments in order to show reliability and accuracy of the full factorial experiment that was run. Interestingly, a lower temperature of infection showed

that it may significantly improve titres and a full investigation into the temperature of infection was then conducted. The mechanism of infection i.e. adsorption of phage to host, was investigated using a wide variety of conditions which showed that those titres that positively or negatively influence the titre can have a positive or negative effect on the adsorption of phage to its host which may account for the changes in titre. Supplements which may be added to improve the titre are then examined.

The thesis next moves on to the scale of into a stirred tank automated bioreactor. The advantages of automation include reduced variability and less chance of human error. The baseline conditions and those that gave the greatest titre from the shake flask work are initially investigated in the 5L bioreactor. A further DoE investigation was carried out examining pH and DO, as they are less readily controlled in a shake flask system but will influence titre. The adsorption investigation is again carried out in the 5L bioreactor. The extreme levels from the DoE were investigated, similar to the shake flask work which showed the positive and negative influence on titre and adsorption.

The final chapter in this thesis examines a rapid method for examining the bacteriophage culture. Samples were taken throughout the culture to examine the changes in DNA/RNA/Protein concentration. The data showed that the culture could be tracked although no comparison was able to be made between final phage titre or burst size and the quantities of DNA/RNA/Proteins at different time points. The volatile fatty acids were then examined using a GC-MS. Rapid quantification is needed given that the plaque assay requires an overnight culture.

The thesis ends with a conclusion of the main findings and future work that should be complete in order to improve phage titres and further scale up the infection for higher yields to be produced.

# Chapter 1 – Introduction

Chapter overview

This chapter will give an overview into the prevalence of antibiotic resistance and the mechanisms of why bacteria develop resistance. An insight is given into the strategies to combat resistance, with bacteriophage chosen as one potential method. Every clinically used product must be able to be manufactured and scaled up in order to be viable and thus there is an examination of the challenges of manufacturing as well as its need. Moreover, the experimental methods which can be used in manufacturing science as well as to improve a bioprocess are explored.

## 1.1 Evolution of antibiotics and spread of antimicrobial resistance?

### 1.1.1 An introduction to antimicrobial resistance

The discovery of antibiotics is arguably the most important development in medicine in the twentieth century as they are used extensively in the treatment of bacterial, yeast and parasitic infections (Davies & Davies, 2010). They are chemicals used to destroy the cell wall and inhibit DNA, RNA and protein synthesis resulting in microorganism death. Antibiotics have been routinely used for decades to combat bacterial infections in humans and animals. The first 'modern day' antibiotic, penicillin, discovered by Sir Alexander Fleming in 1928, has been widely used throughout the world (Ligon, 2004). In 1940, Florey and Chain took great strides in the development of mass production of penicillin; however, with the Second World War looming, their research was put on hold. Penicillin was extremely effective at treating soldiers of WWII, but it is believed many more lives could have been saved if they had been able to mass produce higher quantities of the drug (Fletcher, 1984). The discovery of penicillin has been complemented by a series of other antibiotics such as the sulphonamides and aminoglycosides, sulphamethoxazole and gentamicin respectively (Huovinen, 2001, Lindgren & Sjöstedt 2016). They are particularly effective in the treatment of urinary tract infections (UTI) and inflammatory diseases (Price *et al*, 2014, Sahu *et al*, 2014). However, with their broad use, some microbes have started to develop resistance. Antimicrobial resistance (AMR) is the broad term that encompasses microbes with the ability to resist all antimicrobial agents, with all classes of microbes able to develop resistance. It is distinguished from antibiotic resistance, the term given to microbes that have the ability only to resist antibiotics (Blair *et al*, 2015). There is a small group of pathogens commonly associated with antibiotic resistant infections, known as the ESKAPE

pathogens. These are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp* (Goren *et al*, 2017). It must be noted that *E. coli* also presents as a common clinical infection.

Antibiotics revolutionised medicine, as before the antibiotic era, more than 80% of patients died if they contracted *Staphylococcal* infections (Lowy 2003). Penicillin dramatically improved the survival rates of those who contracted *Staphylococcus aureus (S. aureus*) infections and mortality in patients decreased from around 36% to 21% after its use began (Van Hal *et al*, 2012). Examples of *S. aureus* infections include pneumonia, toxic shock syndrome and sepsis which can be treated with ease using antibiotics (NHS 2015).

With ever-increasing use of antibiotics, it is believed that AMR may become such a threat that their use may no longer be permitted. All use of antibiotics can lead to the development of AMR, even when necessary, whilst misuse only increases the problem e.g. viral infections if not properly diagnosed. It may be easier to use empirical evidence to decide the best treatment for a patient and prescribe an antibiotic than spend time, effort and money to determine the exact nature of an infection. In addition to misuse, counterfeit antibiotics have also exacerbated the problem and it has been estimated they make up around 5% of total global antibiotics in circulation (Delepierre *et al*, 2012, O'Neill, 2016). In 2011, livestock owners purchased 29.9 million lbs of antibiotics and uses more antibiotics per kg of meat produced than any other country. Whilst use of antibiotics in animals has recently increased, in 1999, Denmark banned all non-therapeutic use of antibiotics in pigs and has consequently seen them become the worlds leading exporter of pork (Levy, 2014). Section 1.1.4 focuses more heavily on antibiotic use in animals.

There have been large investments into AMR research with nearly $2 billion invested between 2003 and 2013 (Simpkin *et al*, 2017). This may be due to there currently being ~$4.7 billion worth of patented antibiotics; if they were to become redundant, the knowledge would need to be in place to immediately replace them. It has also been estimated that by 2050, every year around 10 million people will die due to AMR infections with a resulting $100 trillion dollars total lost, owing to R&D, clinical studies and healthcare costs (Sabiiti 2017). The world health organisation estimates that antibiotic resistance costs more than $1.5 billion every year in healthcare expenses in the European Union which demonstrates AMR as a significant problem (WHO, 2016). Given the challenge in quantifications that can occur, the unpredictability associated with modelling and the

uninformative nature of the current burden these figures has previously been criticised (De Kraker *et al*, 2016). However, despite any criticisms that can be made, it can be stated categorically that AMR is a serious global issue with many implications.

### 1.1.2 Why do bacteria develop resistance?

Resistance develops when an antibiotic targets and destroys susceptible bacteria. If there is a mutant strain, they will be unharmed allowing the bacterial colony to carry the mutation and create a new resistant colony (NHS, 2014). The instance of genetically transferable antibiotic resistance was observed around 1950 in Japan, and since then there has been increasing resistance to antibiotics owing to the evolution of bacteria, greater detection capabilities and greater antibiotic use (Davies & Davies, 2010). More recently, there has been the emergence of super-resistant microbes, or "superbugs", which are more generally classified as multi-drug resistant (MDR) forms of the bacteria.

It is interesting to note that within the same year that streptomycin was given FDA approval for therapeutic use, resistant mycobacterium tuberculosis strains emerged and the effectiveness of gentamicin and cefotaxime was lost within five years of their introduction (Crofton & Mitchison, 1948). Interestingly, resistance to penicillin G and linezolid was reported before their approval for use, but is allowed given the effectiveness against a variety of gram positive and negative bacteria (Bush, 2004).

Antibiotics usually target the cell wall of the bacteria or peptidoglycan biosynthesis, the layer outside the plasma membrane of the bacteria (Beveridge, 1999). There are several classes of antibiotics used to treat a wide variety of microbial infections that work through different mechanisms; the main mechanisms by which resistance develops are outlined in Table 1.1. Resistant bacteria generally prevent drugs inhibiting their growth in different ways. Enzymes, within the bacteria such as β-lactamases inactivate antibiotics, through chemical changes and hydrolysis whilst aminoglycosides inhibit protein synthesis within the bacteria. Secondly, resistant bacteria target the transportation of the drug through the absence of a drug transport protein in the bacteria or intracellular targets of drugs change (Hayes & Wolf 1990, Levy and Marshall 2004).

It should be noted that there are differences between species for resistance against similar antibiotics. Martinez & Baquero (2000) showed that there must be mutations in seven positions in the *gyrA* gene of *Escherichia coli* (*E. coli*) for resistance against quinolones, whereas only two mutations are required on the *gyrA* gene for resistance against quinolones in *Streptococcus pneumonia*.

External factors including salt concentration and temperature have been shown to affect the growth and proliferation of methicillin resistant strains of bacteria (Stapleton & Taylor, 2002). They can be exploited to determine if a strain possesses resistance or not in a laboratory setting and shows how easily resistance can develop and the multitude of factors contributing to resistance applying to all microorganisms (Mohamed *et al*, 2014).

There are several reasons for the development of resistance of microbes within our control. Patients have been given an incorrect dosage or duration of antibiotics and it is estimated that at least 30% of outpatient prescriptions for antibiotics are incorrect (Centre for Disease Control and Prevention, 2020). Moreover, around 80% of antibiotics are used outside of hospitals, in the community care setting, so physicians have reduced control over their use (The Centre for Disease Dynamics, Economics & Policy, 2015). Additionally, users may not always complete the course of antibiotics that are prescribed for them and when bacteria are exposed to antibiotics, they may acquire mutations against that particular antibiotic, forming a resistant colony (McNulty *et al*, 2007). It should also be noted that antibiotics can be bought without a prescription in Latin America which can lead to misuse of antibiotics, highlighted later in this section, allowing the formation of resistant colonies (Llor & Cots, 2009, Mainous, 2009).

AMR is a more significant issue in developing countries with low socio-economic status due to self-medication (Hunt *et al*, 2011, Ramay *et al*, 2015). Because of self-medication of antibiotics and the expense to produce antibiotics, there is the need for cheaper and more controlled distribution of antimicrobial agents.

Table 1.1. A table to show different antibiotic classes, their target and mechanisms by which resistance develops. Adapted from Davies J and Davies D 2010.

| **Antibiotic class** | **Antibiotic example** | **Target** | **Mechanism by which resistance develops** |
|---|---|---|---|
| **β-Lactams** | Penicillin/Methicillin | Peptidoglycan biosynthesis | Hydrolysis of hydroxyl groups |
| **Amioglycosides** | Gentamicin | Translation | Phosphorylation, acetylation of 3 and 2 hydroxyl groups |
| **Glycopeptides** | Vancomycin | Peptidoglycan biosynthesis | Reprogramming peptidoglycan biosynthesis |
| **Macrocyclic** | Fidaxomicin | Protein synthesis | RNA polymerase resistance (Golan & Epstein, 2012) |

### 1.1.3 Challenges in antimicrobial resistance

Global use of antibiotics rose 40% between 2000 and 2010. Moreover, with travel and migration becoming more widespread, this allows for the spread of resistant pathogens (O'Neill 2014). If initially, the first line of defence of drugs fails, there is the need to turn to the second and third line of drugs which can be expensive. Around 700,000 lives per year currently are lost due to AMR infections within the USA and Europe (Simlai *et al*, 2016). Moreover, by 2050, it is estimated that AMR will cause more than 4.1 million and 4.7 million deaths in Africa and Asia respectively per annum (O'Neill, 2014).

An additional challenge of AMR includes hospital acquired infections (HAI) which have been associated with longer stays in hospitals (WHO, 2014) and were estimated to cost around $35 billion per annum in the U.S alone in 2009 (Centre for Disease Control and Prevention, 2009). Increases in prevalence of resistant bacteria

when acquired in a hospital may increase hospital stays, cost and burden. Bacteria are found throughout hospital surfaces and contribute to HAI which can be passed from patient to patient. Consequently, there is now considerable effort in the development of novel antimicrobial therapies for bacterial infections. One report showed that over a 6-month period, patients with any MRSA infection cost around $36,000 in hospital expenses, compared to nearly $20,000 less with a *staphylococcal* susceptible infection (Filice *et al*, 2010).

### 1.1.4 Antimicrobial resistance in animals

In addition to the costs to human health, AMR is an overwhelming concern in modern animal food production which is highly dependent on the vast use of antibiotics for treatment of infections. The antibiotics used are to promote growth and prevent disease in animals which has contributed to the emergence and spread of resistant bacteria in both human and animal populations. Food and drinking sources for animals are supplemented with antibiotics and overuse/misuse may contribute to the development of resistant strains. AMR bacteria in animals may contaminate the carcass and when slaughtered, may colonise humans that eat those animals (Wilhelm *et al*, 2011). Faecal contamination is also a source of cross contamination between animals and humans (Leonard *et al*, 2012). Additionally, trade of animals and food presents the opportunity for global spread of resistant bacteria. Many animals have genetic resistance to antibiotics whilst exposure to the drugs may lead to the diseases becoming ever more fatal (Murray *et al*, 1984, McKinney *et al*, 2010).

There are many infectious microorganisms that originate in animals but have zoonotic properties, crossing the species barriers to infect humans, such as severe acute respiratory syndrome (SARS) which infected 8,400 as of 2003 (Satija & Lal, 2007). Another example is swine flu which originated in pigs but mutated and can infect humans. It was shown to be adamantine resistant, and in 2009 caused almost 30,000 deaths in humans (Mercola, 2009). There are numerous bacteria which have zoonotic properties, and if their resistance were to increase to antibiotics, this may heighten the risk to humans.

## 1.2 Main threats - *C. difficile, E. coli, P. aeruginosa, S. aureus*

There are many bacteria that have developed resistance to antibiotics; however, four that are of major concern to the human population because of the severity of the infections are *Clostridium difficile* (*C. difficile), Pseudomonas aeruginosa (P. aeruginosa), E. coli* and *S. aureus*. *C. difficile*, is a gram-positive bacterium which

causes abdominal and intestinal problems (Cohen *et al*, 2010). It is reported that more than 10% of patients with a seven-day *C. difficile* infection resulted in mortality (Karas *et al*, 2010). In 2011 in the USA, *C. difficile* caused half a million deaths (Centre for Disease Control and Prevention, 2016 a).

*P. aeruginosa*is a gram-negative bacterium which can cause lung infections (Boukerb *et al*, 2014). There is an approximate 60% mortality rate in patients with some resistant *P. aeruginosa* infections (Kim *et al*, 2014).

*S. aureus* is a gram-positive bacterium that can cause skin infections (Kim *et al*, 2014, Tong *et al,* 2015). It has more seriously been shown to be a major cause of health care concern in blood infections (Dantes *et al*, 1978). Around 60% of all *S. aureus* infections are methicillin resistant with an incidence in U.S hospitals of almost 7 per 1,000 admissions, whilst almost 1 in 4 patients with an MRSA infection will die given the unavailability of treatment (Seybold *et al*, 2006). Figure 1.1 shows the percentage of *S. aureus* that is methicillin resistant globally. This shows that those bacteria can cause substantial deaths and if further resistance develops to each, this will result in more invasive treatments, higher rates of mortality, longer hospital stays and consequently increased costs (Segal *et al*, 2016).

Resistant *E. coli* has been shown to be prevalent in several infections including UTI, blood infections as well as HAI. It is of such concern that the UK government has highlighted it as one of the key bacteria causing infections. Previously used co-amoxiclav and ciprofloxacin are nearing a point where their use is no longer suitable. One study showed a 10-fold increase in resistance to ceftriaxone between 2000 and 2010 whilst others have shown statistically significant increases of *E. coli* resistance to antibiotics between 2009 and 2016 for several antibiotics (Sanchez *et al* 2012, Johnston *et al*, 2018).

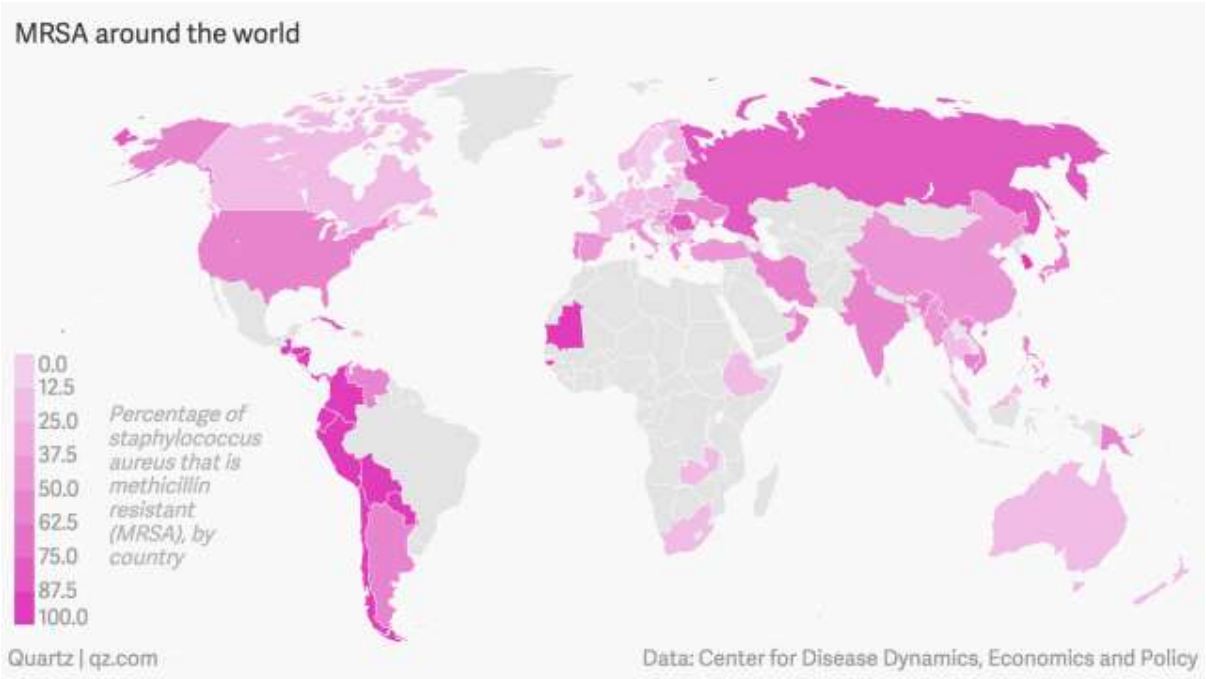



***Figure 1.1*** *– An image to show the percentage of Staphylococcus aureus that is methicillin resistant (Wang 2015)*

**1.3 Resistance and antimicrobial agent production**

**1.3.1 Antimicrobial agent production**

The importance of addressing AMR has been highlighted but a further significant issue is the production of novel technologies to combat resistant organisms. Up until 2014, there was only one new FDA approved antibiotic, probably due to the FDA restrictions, whilst only 5 out of the 506 drugs in R&D development are antibiotics as of 2017 (FDA, 2004, Bassetti *et al*, 2013, Kållberg *et al*, 2018). Pharmacokinetic/pharmacodynamics studies, *in vitro* bioassays, preclinical animal studies and clinical studies must be completed to prove that new drugs have no harm. Additionally, efficacy, safety and immunogenicity studies all need to be done before *in vivo* studies can be undertaken.

If at any point the FDA see a reasonable need to discontinue a drug, they have the authority to immediately cease production and testing (Heinemann *et al*, 2015). This could be difficulty in demonstrating a

drug's efficacy, side effects, being less superior to other drugs or the cost/ability to be produced. Bridging the gap of risk known as the valley of death may help to prevent failed translation of a drug. Risks include funding, regulatory and market issues which must all be avoided for a drug translated to be used clinically.

### 1.4 Clinical issues

Whilst the production of resistance strains has already been highlighted, it is important to note the potential effect that AMR bacteria have in a clinical setting. Given that resistant strains can be difficult to treat, they increase the time that patients can be in the clinic, thereby increasing the cost of treatment.

#### 1.4.1 Surgery

Patients are often administered antibiotics immediately before and during surgery to try to alleviate the possibility of infection or bacteria colonising exposed tissue (Centre for disease control and prevention, 2016). If the threat of AMR continues to grow, routine surgical procedures, which raise the quality of life, may become too risky to carry out in case of a resistant organism. Patients who develop surgical site infections cost nearly four times more than uninfected patients and, in some cases, need an additional two weeks of hospital stays per patient (Whitehouse *et al*, 2002). *S. aureus* is the most common bacteria infecting prosthetic joint replacements and this accounts for half of all infections (Song *et al*, 2013). Around 40% of all prosthetic shoulder joints and around 25% of prosthetic knees are contaminated by *S. aureus* (Tande & Patel, 2014).

#### 1.4.2 Chemotherapy

In USA, around 650,000 people every year undergo chemotherapy treatment for cancer (Centre for Disease Control and Prevention, 2015). Chemotherapy suppresses the immune system and consequently, patients may be more susceptible to infection. Currently, 1 in 14 cancer patients die due to infections from a weakened immune system (Centre for Disease Control and Prevention, 2015). An increased threat from AMR may raise the risk of death in chemotherapy patients.

### 1.4.3 Diabetes

Increasing rates of diabetes in both developed and less developed countries increases the incidence of diabetic foot ulcers. Usually, broken skin can heal easily, but diabetic patients' skin may not heal normally and an ulcer may develop (Brem, 2000). When an ulcer arises, the colonised bacteria spread throughout the limb and amputation may be needed. The bacterial infection can contribute to poor healing of the skin allowing wounds to develop. Additionally, the immune system is weaker due to the higher levels of glucose caused by diabetes, resulting in poorer blood flow to the foot and leg, making wound healing slower (Brem & Tomic-Canic, 2007). Together, ulcers can develop into chronic wounds until amputation is needed (Jeffcoate & Harding, 2003).

In 2015, there were an estimated 135 amputations every week in the UK among diabetic patients, 80% of which may have been avoided if more focused care on the infection were in place (Diabetes UK, 2015). Additionally, amputation leads to a greater mortality rate, up to 80% within 5 years of amputation (Diabetes, 2014). It is estimated that £1 in every £150 that the NHS spends is on diabetic foot ulceration and amputation which may increase if AMR becomes more prevalent (National Diabetes Information Service, 2013). Diabetic foot ulcers are the cause of the longest hospital stays for those with diabetic related illnesses (Thompson *et al*, 1993). Around 15% of diabetic patients develop a non-healing chronic ulcer during their lifetime (Gardner *et al*, 2013). Prolonged treatment for diabetic foot with antibiotics may lead to resistant strains forming. If resistance occurs, and it is unable to be treated, the amputation risk is heightened increasing the cost on the NHS for their treatment.

### 1.4.4 Wound infections (Chronic and acute)

Acute infections are those which are short-term or of recent onset, in contrast to chronic infections, which can develop from acute infections, and are long-term. Both can refer to a bacterial or viral infection. Wounds provide the ideal location for bacteria to colonise given their exposure to the environment. It should be noted that the pH of the skin can be used as a diagnostic technique for bacterial detection given that bacteria will alter the skin pH (Messager *et al*, 2003, Ono *et al*, 2015). In 2012, a study was conducted with 114 burn wound patients. Nearly 70% of patients had an MRSA infection and more than 85% could not be treated with Vancomycin, Clindamaycin, Kanamycin or Erythromycin due to resistance. This may be why burn wound

infections are considered a major risk due to the bacteria colonising the wound and resistance that may have already developed (Alebachew *et al*, 2012).

Although *S. aureus* is a common cause of early burn wound infection, *P. aeruginosa* is the most prevalent bacteria in burn wounds (Church *et al*, 2006). It is known that the primary source of bacterial wound contamination is the patient's own flora i.e their gut, nose, mouth or skin; infections may also arise due to bacteria from the environment (Taneja *et al*, 2013). The wound, possesses a nutrient-rich environment and is an ideal place for bacteria to colonise, proliferate and cause infection. In both chronic and acute wounds, there may be a mixture of bacteria, fungi and viruses which will range dependent on the wound size and depth (Bowler *et al*, 2001).

Wound infections are an enormous challenge for the NHS, with more than 2.2 million wounds managed annually, with approximately 15% unresolved. Managing these wounds includes 29.5 million practice and community nurse visits, 7.7 million GP visits and 3.4 million hospital outpatient visits, at an estimated cost of £4.5-5.1 billion (Guest *et al*, 2015). Rising rates of diabetes, obesity and an ageing population means chronic wound infections are expected to become an ever-increasing challenge.

**1.4.5 Biofilm infections**

A biofilm is a group of cells attached to a surface or interface and enclosed in an extracellular matrix. It is estimated that around 80% of microbial infections are associated with a biofilm infection (Römling & Balsalobre, 2012). Biofilms can be formed by both virulent and opportunistic pathogens (Jacqueline & Caillon, 2014). They can be notoriously difficult to treat as cells within biofilms are 100-1000 times more resistant to antimicrobial agents than planktonic (single and free) bacterial cells. This is because the matrix that encompasses the bacteria is thick and can resist antibiotic penetration (Costerton *et al*, 1999). Biofilms have also been shown to have a level of resistance to cleaning agents such as hydrogen peroxide. During its presence, bacteria release two enzymes, KatA and KatB which neutralise the biocide. However, over periods of exposure, bacterial populations may increase production of these enzymes effectively showing a level of resistance (Macvanin & Hughes, 2010).

Biofilms can form on a variety of surfaces and novel methods are needed for their treatment (Lehman & Donlan, 2016). Biofilms can be polymicrobial and are recalcitrant to treatment compared to mono-microbial infections. The pathogenicity has been shown to increase when two or more species make up a biofilm and eradication of a polymicrobial biofilm is more difficult with harsher treatments needed (Wolcott *et al*, 2013, Coulter *et al*, 2014). Biofilms display resistance to antibiotics through the low metabolic activity of biofilm cells and via horizontal resistance, resistant genes passed between species. Figure 1.2 shows the formation of a biofilm on a surface.

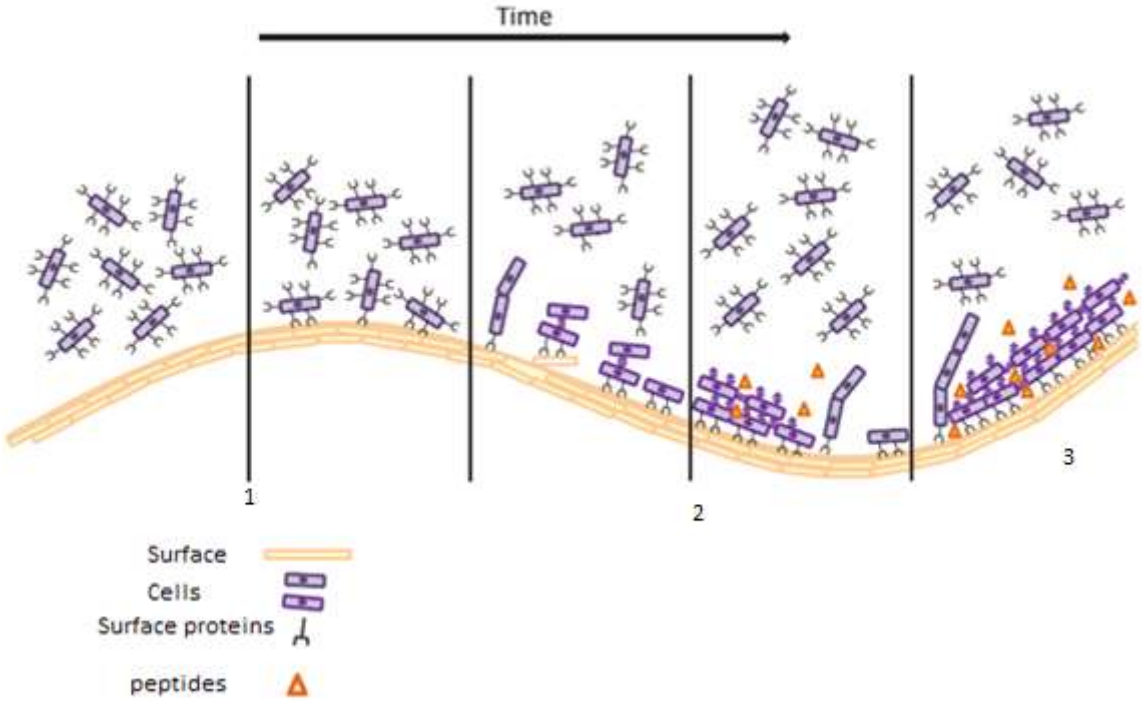



Figure 1.2 - Adapted from Frese et al, 2013. Development of a biofilm attached to a surface. 1) Bacteria beginning to grow on a surface 2) Bacteria continue to grow and a matrix is formed around them 3) Biofilm develops and continues to grow on a surface and will continue to grow unless treated.

The evidence outlined so far has highlighted the urgent need for new antimicrobial agents and potential agents that may be used to tackle the problem will be outlined in the next section.

### 1.5 Developing strategies against antimicrobial resistance (AMR)

#### 1.5.1 Alternatives to treat AMR infections

Dame Sally Davies, the chief medical officer for England, has highlighted AMR as a priority to be tackled with new innovative technologies, showing its threat and the need to develop novel treatments (Gov.UK 2011, Rex, 2014). In 2014, a £10 million prize fund was available to anyone who developed a method to conserve antibiotics for future generations (Longitude prize, 2016). Other prizes for AMR product development include a £2 million prize fund from Innovate UK and the Federal prize offering $20 million (AMR centre, 2016, Gov.uk 2016). Such prizes are produced to incentivise AMR research. A further incentive is the 'new drugs for bad bugs work scheme' (ND4BB) that comprises several projects between industry and academia with almost three quarters of a billion of funding available (Innovative medicines initiative, 2010).

Alternatives to antibiotics will become critical and currently, there are several possible treatments such as antimicrobial peptides and bacteriocins. A promising approach is that of viral bacteriophage (phage) therapy more commonly known as phage therapy and has many potential human applications to treat bacterial infections (Miedzybrodzki *et al*, 2007, Nedialkova *et al*, 2016).

#### 1.5.2 Antimicrobial peptides

Antimicrobial peptides were discovered in 1939 and are effective against both gram positive and gram-negative bacteria. They bind to the lipopolysaccharide (LPS) on the bacteria, leaving the membrane permeable to antibiotics (Malojčić *et al*, 2014). However, they have been shown to potentially be toxic, limiting their clinical use (Bahar & Ren, 2013). Toxicity may be reduced by altering concentrations or reducing the frequency of the doses, although the levels may be insufficient to destroy bacteria to become a viable treatment (Gordon *et al*, 2005). One study used an antimicrobial peptide, EC5, against *E. coli*. An in vitro model was used with polymixin B as a control. At 12.5µg/ml EC5 significantly inhibited *E. coli* growth from $5_{log10}$ colony forming units (cfu)/ml to 0 cfu/ml (Rao *et al*, 2013).

### 1.5.3 Bacteriocins

Bacteriocins are toxins produced by bacteria, inhibiting the growth of bacterial strains that are similar to the bacteria that produce them through intra-species competition (Kjos *et al*, 2016). They are classed (i-iv), based on their size and complexity. Bacteriocins can either be gram negative, less than 60 amino acids, or gram positive, less than 28 amino acids long. Gram positive are further classed I-III depending on their size (Yang *et al*, 2014). An example is a colicin which can form pores on the inner membrane causing leakage of cytoplasmic compounds (Kienker *et al*, 2008). Several bacteriocins have been investigated against MRSA biofilms. It was shown that 10µM nisin A was the most effective against *S. aureus* and *Staphylococcus epidermidis* (*S. epidermidis*) biofilms reducing CFU/ml from $10^8$ to $10^0$ in less than five hours in an in-vitro model using a 96 well plate, $p<0.05$ (Okuda *et al*, 2013). However, nisin A has been shown to become soluble during pH changes and many other bacteriocins have a poor distribution profile, limiting their clinical use (Alvarez-Cisneros, 2011). Any potential therapeutic must have a good distribution profile and must be able to target organisms at the source.

### 1.5.4 Bacteriophages

Phages are the most abundant microorganisms in the biosphere with around $10^{31}$, meaning that they are ten times more abundant than bacteria (Rohwer & Edwards, 2002). Although there is some controversy over who first discovered bacteriophages, it is generally accepted that it arose out of contributions from Twort (1915) and D'herelle (1917) (Duckworth, 1976). They build on work from 1896 when it was noticed that the spread of *Vibrio cholera* was being limited by an unknown substance that was heat liable and could pass through filters (Sulakvelidze *et al*, 2001). Their general structure is shown in figure 1.3.

Given the shear number of phages on the planet it is reasonable to assume that they come in many different shapes and sizes. Phages such as MS2 have only a few kilobases whilst others such as T4, who have one of the largest genomes, have >100,000 kilobases. The most common shape of a phage is detailed below in figure 1.3 and consist of a head, body and tail fibres. However, the smallest phage, such as MS2 are more spherical as they have a less complex structure and it should be noted that the smaller phage have a lower infectivity rate than the larger ones, given their simpler structure. Between different phage that have large genomes, there have been shown to be different rates of infection times and the amount of ATP needed for the packaging

motors showing that even within large phage, differences can occur throughout the entire phage infection process. T4 phage has one of the fastest packaging motors of all bacteriophage which will undoubtably affect the rate of phage infection between species (Lin *et al*, 2017).

However, when phage are propagated using different host strains, there can be differences between the physicochemical properties of phage. Esteban et al recently showed that using three different host organisms, bacteriophage K showed different zeta potentials, of the phage, whilst they also interestingly showed that the diameters of phage were different on average when propagated using different host organisms. Therefore, it is reasonable to believe that different phages would have different physicochemical properties. The authors also showed that at higher concentrations of phage, there was more aggregation between the phage (Esteban *et al*, 2018).

Recently, it was shown that changing the ionic strength of a culture can impact on the aggregation of T4 phage. When at a higher pH, there will be more aggregation compared to a lower pH. However, one author previously showed that phage would separate out and reduce the level of aggregation when maintained at the differing pH levels. At pH 8.6 T4 phage had an average aggregate size of 300nm, pH 7 - 200nm and pH 5.8 - 150nm. However, after 18 hours, the authors showed that at pH 8.6 there was an average aggregate size of 800nm, pH 7 - 250nm and pH 5.8 – 150nm (Szermer-Olearnik et al, 2017).

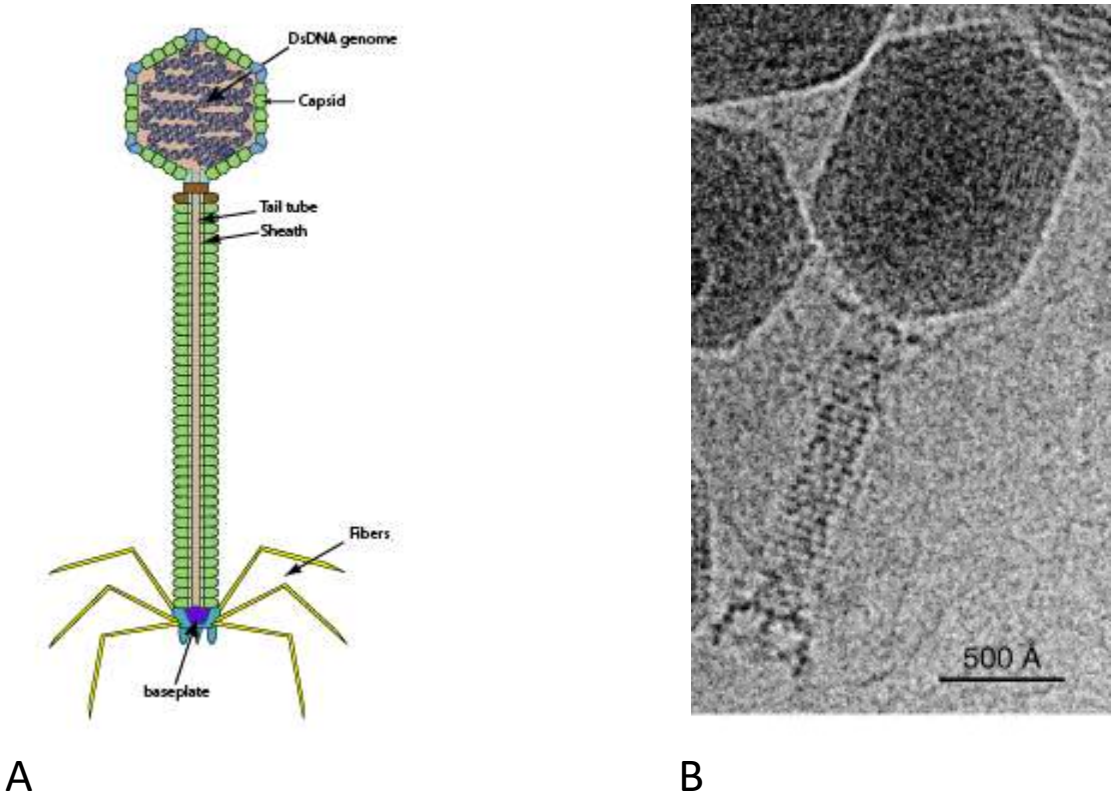



***Figure 1.3*** *– A) A cartoon image to show the structure of a general bacteriophage. Adapted from ViralZone 2016 B) An electron microscopy image of T4 phage taken from Rossmann et al, 2004.*

Phage have previously been used *in vivo* and *in vitro* which will be outlined in greater detail later in this chapter. Conventional phage therapy employs native phage against bacteria that have developed resistance to antibiotics. Treatment with enzymes derived from phages, such as endolysins, can be used to degrade the bacterial cell wall and lyse bacteria that it infects (Mayer *et al*, 2008). It should be noted that lytic phage can result in the release of endotoxins from bacteria which can be harmful to patients if endotoxins are released into the body. Despite this, previous research suggests that there is little responses that will practically occur during phage therapy treatment (Gorski *et al*, 2003). Moreover, phage will not target human cells as they specifically target bacteria.

One study used mice to determine the endotoxin levels from *P. aeruginosa* caused by a modified non-lytic phage (Pf3R). The control lytic phage (PAO1) showed that, 240 minutes after infection, endotoxin levels

were 60-fold higher. However, the non-lytic phage showed endotoxin levels were no more than seven-fold higher (Hagens *et al*, 2004).

Combination therapy uses phage in combination with antibiotics to increase the overall effect. *P. aeruginosa* phage-amikacin combination was shown to eradicate *P. aeruginosa* biofilms in an in vitro 96-well plate model, (p=0.003), compared to the control. The minimum biofilm eradication concentration for phage-amikacin was shown to be $\geqslant 64\ \mu g/ml$. There was also a significant difference of phage-amikacin against planktonic bacteria compared to the control (p=0.001) (Nouraldin *et al*, 2016). A further study showed the effects of antibiotic resistant bacteria when phage therapy and antibiotics were used in combination. Carbenicillin, Gentamicin and Trimethoprim were used in vitro with LKD16 phage against *P. aeruginosa*. The optical density of bacteria was reduced when a combination treatment was used for each antibiotic combination. Carbenicillin-phage treatment was shown to be the most superior between each combination with a threefold reduction in optical density, between expected and observed, compared with a twofold reduction for the other treatments. The minimum inhibitory concentration was examined although there was no significant difference between any of the treatments, p=0.0618, p=0.4907, p=0.6756 for trimethoprim, carbenicillin and gentamicin combination with phage respectively (Torres-Barceló *et al,* 2016).

Bacteriophage therapy currently remains in an infantile state for the treatment of clinically relevant bacteria. This is due to toxicity issues (bacterium DNA/RNA and proteins being released into the patient) but possibly also due to their limited scope of infection. Moreover, the scalable manufacture has not been examined thoroughly and must be for any clinical product. Despite this, there have, however, been clinical trials that have already been completed, begun and others planned. Most notably, of those planned, and currently in the recruitment phase to begin by the end of 2020, researchers in San Diego will administer the first phage therapy intravenously to treat *S. aureus* infections on ventricular assisted devices and is planned for phage 1 and 2 trials. Others include, treatment of a cocktail of 10 phage for *P. aeruginosa* infections in cystic fibrosis patients and one recently completed which assessed the use of phage against *E. coli* UTI infections (clinicaltrials.gov 2020).

**1.5.5 Mechanism of action of phages**

There are two types of bacteriophage (phage) life cycles, lytic and lysogenic figure 1.4. In the lytic life cycle, phages infect bacteria and propagate themselves resulting in an increased number of phage with every infection. In the lysogenic cycle, phages insert their genome into bacteria which can be later activated and revert back to the lytic cycle. The first stage in the phage life cycle is the adsorption onto the bacterial cell wall by binding to specific receptors. The attachment to a cell envelope layer is by the phage tail fibres (Hadas *et al*, 1997, Abedon *et al*, 2011). The phage-host interactions are highly specific, and therefore, phages are only able to lyse certain hosts. It is for this reason that they will not target human cells and can be used to treat humans. It has previously been documented that environmental factors such as pH and temperature in the soil can influence phage binding; however, it is more common that these factors will affect host growth which will prevent the usual propagation of phage (Fister *et al*, 2016). Bacteriophages are known to effectively kill extracellular bacteria to a greater degree than intracellular bacteria, however, one study showed that whilst traditionally an extracellular pathogen, *S. aureus* is able to internalise phage and has some properties of an intracellular bacteria. They added lysostaphin which targeted the extracellular bacteria, leaving only those intracellular bacteria behind. They infected with phage and showed a statistically significantly reduced level of host organism after 3 hours of infection. The authors explained how interactions via immunoglobulin like domains may help phage cross cell membranes (Zhang et al, 2017). Interestingly, an engineered phage was able to successfully target both intracellular and extracellular phage to the same degree against *E. coli* (Moller-Olsen *et al*, 2018).

Once a phage has bound to a bacterium, it must make the lytic-lysogenic decision. Many of the larger phages such as lambda phages, contain host lethal proteins and therefore will adopt the lysogenic cycle. Moreover, this cycle may also be adopted when the multiplicity of infection (MOI) is in favour of the number of phage. The ability of phages to switch between the two cycles is known as the bistable switch and means that two gene expression states exist (Bednarz *et al*, 2014).

Once attached, the phage then injects its genome into the host's cytoplasm where its proteins are produced and expressed. The structural proteins of phage are synthesised and assembled, within the bacteria, which leads to host cell lysis and release of the newly formed phage. The release is due to two proteins, holin and endolysin, which stimulate lysis of the bacterial cell wall allowing release of the phage involved (Klaenhammer & Sturino, 2006). The newly produced phages are able to infect other bacteria and propagate

themselves. This mechanism is adopted by virulent phage; however, other (temperate) phages may adopt a non-lytic cycle, if the bacterial levels are reducing and there is a need to preserve phage (Maurice *et al*, 2013). During the non-lytic cycle, the phage injects its DNA into the host bacteria, and becomes part of the genome of the bacteria. As the new cell has the phage genome, there is the potential to be activated at any time and the cell could lyse at any point. It should be noted that not all phages can use both cycles.

Excretion serves as a route out of the body for the phage. Excretion of phages can be rapid and can occur as soon as there are no more bacteria, or can take some time after all the bacteria have been killed (Newton-Northup *et al,* 2014). Phage DNA may also lie dormant in the daughter cells of any bacteria that the phage has infected if the infection has been through the lysogenic route (Williamson *et al*, 2001).

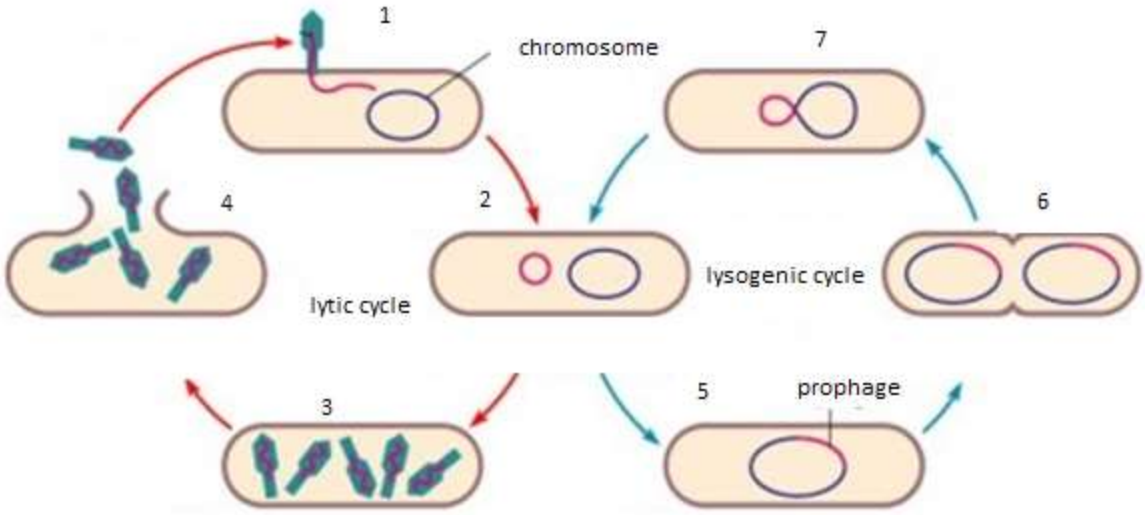



***Figure 1.4*** *- (1-5) Life cycle of a phage. 1) The bacteriophage seeks out the bacterium and the phage injects its genome into the bacterium where in the lytic cycle it replicates independently of the bacterial genome. 2) The phage's genome replicates in the cytoplasm in the lytic cycle 3) The bacteria produce more phage which can go on and infect other bacterium 4) The phages are produced and are able to lyse the bacterium. The phages lyse the bacteria killing it and the newly formed phages are able to repeat the process 5) Phages that infect host cells may enter the lysogenic cycle (temperate phages) rather than the lytic cycle. The cell that has the phage gene*

*incorporated into it can replicate rather than produce new phage. In the lysogenic cycle the phage injects its genome straight into the bacterial genome 6) The cell with the phage genome divides and replicates and the genes that produce phage can be activated at any time 7) With the genome incorporated into the new cell, the phage can be induced to revert back to the lytic cycle Adapted from major differences, 2016.*

### 1.5.6 Synthetic biology for the study of AMR

#### 1.5.6.1 Synthetic biology and AMR research

Synthetic biology combines biological and engineering principles and is interlinked with other areas including biotechnology and systems biology (Weiss & Purnick, 2009). The initial focus for synthetic biology was understanding gene regulation but has now moved towards tuning gene regulation and led to the creation of new methods to tackle AMR (Redden *et al*, 2015). Synthetic biology is an advanced form of genetic engineering, where genetic engineering splices elements from one organism to another for novel functions and enhanced abilities. Moreover, it encompasses synthesising genetic elements or biochemical pathways that do not exist in nature. It has been estimated that the total global market for synthetic biology products is around $7 billion (Erickson *et al*, 2012). Modified phage therapy uses synthetic biology to modify certain aspects of the phages, e.g. use of non-lytic phages preventing bacterial endotoxins release, but cause host cell destruction from within and release of phage progeny (Paul *et al*, 2011, Drulis-Kawa *et al*, 2012, Ando *et al*, 2015).

Targeting genetic sequences of bacteria, to potentiate the power of antibiotics to resistant bacteria has had many positive results against AMR organisms. Resistance gene knockout has shown to be effective in targeting resistance genes with plasmids used to knock out expression of resistance genes. Alternatively, chloramphenicol acetyltransferase was inhibited by designed small RNA (sRNAs) and fully restored chloramphenicol sensitivity in *E. coli* (Eisener-Dorman *et al*, 2009). At 500µg/ml chloramphenicol, >99% of organisms were killed compared to the control. At 250µg/ml, *E. coli* reduction in counts was observed. The new insights from these studies may be used in the future against AMR infections and should be further studied to

establish the effectiveness. However, the techniques required advanced research and can be expensive (Libis *et al,* 2014).

#### 1.5.6.2 Synthetic biology and bacteriophage

Phage therapy has the advantage of acting against specific bacteria but with a corollary drawback that individual phage cannot be used against a range of different bacterial infections. With the emergence of synthetic biology, some phages have been engineered to enable a broader range of applications (Citorik *et al*, 2014). T2 phage was engineered by Yoichi *et al* who swapped the long tail fibre genes from T2 phage with that from a PP01 phage which targets *E. coli 0157:H7*. Their engineered phage was able to successfully infect *E. coli 0157:H7,* the target of the original PP01 phage (Yoichi *et al*, 2005).

Synthetic biology provides a novel method for re-engineering phages for their safe-use. Krom *et al*, (2015) engineered phagemids (a plasmid used in a similar method as a vector) to express toxic proteins and non-lytic antimicrobial peptides. 7-week-old mice were injected with 106 cfu/ml *E. coli* and were left for 1 hour, after which the mice were treated with 100ng/ml phagemids. Death of bacteria was reduced between 2-3 times and was an attractive approach with significant future potential since a key problem with phage therapy is the lysis of bacteria releasing toxins which may harm the patient.

Phages have limited spectrum of infection against bacteria which must be either better understood or modified in a way allowing them to be used effectively. Phages do, however, have the advantage that they will not attack the microbiome or human cells and only target specific bacteria. Scholl *et al*, 2005 designed a phage to infect a range of hosts by degrading barriers to phage adsorption and infection. They found that the K1 capsule of *E. coli* acts as an effective barrier to T7 infection as it is specific to *E. coli BL21*. Thus, they engineered T7 bacteriophage to express an endosialidase enzyme, under a strong capsid promoter, degrading the K1 capsule and showed it could infect *E. coli* K1 strains. Similar to their work, Lu & Collins (2007) demonstrated another example of synthetic biology for AMR. They used the T7 phage and modified it to express dispersin B, an enzyme able to degrade biofilms. Biofilms were grown for 24 hours in 96-well plates before the addition of phage. There was significant removal of the biofilm (99.997%), 4.5 orders of magnitude after around 30 hours of treatment with $10^3$pfu/ml phage, compared to the control, $p<0.05$.

Synthetic biology has the potential to enhance phage therapy and combined with the increase in phage research, this may lead to additional phage therapies emerging into the clinic. Therefore, the need for bioprocessing and manufacturing of phage needs to be developed for their use in a clinical setting. Resistance to antimicrobial agents has been outlined and the issues around organisms that pose significant threat have further been outlined. Although there are several promising approaches to treat AMR infections, this thesis focuses on the specific opportunities with phage therapy and their development.

## 1.6 Bacteriophage therapy

### 1.6.1 Wider applications of phages

Phages have been used in a wider setting than tackling AMR bacteria, for example in hygiene. One study examined hotel staff members' hands who were treated with phage and samples were examined after 4 hours and again after three days against 37 fomites. Samples were taken before and after treatment to measure bacterial counts and determine the route where bacteria were picked up and transferred to. There was a statistically significant reduction in fomites overall, $p=0.04$, whilst there was also a significant reduction in bacteria after treatment on staff members' hands, $p=0.0002$ (Sassi *et al*, 2015). However, the authors gave no indication as to whether or not the bacteria being transferred could be tracked.

Additionally, phage have been used in treatment of food products, in particular meat, to control the levels of pathogens (Sukumaran *et al*, 2015). They may be used to extend the shelf life of products by inhibiting bacterial growth, preventing waste and reducing cost. Approximately 25% of total global food that is produced is lost because of microorganisms (Snyder *et al*, 2018). This is of significant concern for the food industry and may represent a profitable market for phage therapy. Given the value of the food industry, the scale of food wastage and increasing the shelf life of some foods, it may be beneficial to use phage against food spoilage. In 2013, $3.2 \times 10^7$ PFU/4 $cm^2$ of phage FAHEc1 was used to control the levels of *E. coli* O157:H7 in meat. At 5$^{o}$C, there was a four orders of magnitude reduction in pathogenic levels whereas at 37$^{o}$C there was around a three-log reduction when $6.2 \times 10^3$ pfu/piece of phages was used (Hudson *et al*, 2013).

### 1.6.2 Advantages and Disadvantages of Bacteriophage Therapy

Whilst the areas of importance as well as their potential drawbacks have previously been introduced, this section specifically aims to summarise some of the advantages and disadvantages.

#### 1.6.2.1 Disadvantages of bacteriophage therapy

There are several issues that need to be overcome for phage to become a viable alternative to current antibiotic treatments. One issue is the limited spectrum of infectivity. Effort has been directed towards using cocktails of phages in order to expand the host range with synthetic biology highlighted as another potential method to tackle this issue (Lu & Koeris, 2011). However, adding several phages to one culture, to tackle multiple hosts, requires more extensive study to gain approval for clinical use (Clinicaltrials.gov, 2019). Furthermore, as new bacteria evolve, there will be the need for new phages (Nilsson *et al*, 2014).

When a phage infects a bacterium, it causes it to lyse. A concern with any lytic bacterial treatment is the release of endotoxins and super antigens, from the bacteria, which may stimulate an inflammatory response that could result in an adverse health effects such as inflammation (Mani *et al*, 2011). Endotoxins have been shown to reduce inflammatory markers, initiate oxidative stress and more seriously cause organ damage (Alamili *et al*, 2014, Wang & Wang, 2013). The concern regarding the toxicity of phage therapy is twofold: contamination of toxins from the bioprocess in the sample and toxins released during patient therapy. During phage therapy, the patient may experience side effects of contamination and a sample given to a patient may not be sufficiently pure and free of toxins. Currently, there is no efficient purification method to remove all bacterial DNA, RNA, protein and toxin contamination although some have been suggested (Halter & Zahn, 2018). Current methods are time-consuming and costly and methods to efficiently produce phage that are pure enough to be used in clinical trials are rigorous and expensive and may highlight why there have been <50 clinical trials to date. Due to strict government laws, phage must be purified before use in human therapy is allowed. This is ultimately one challenge that must be addressed and the use of non-lytic phages may be an option to consider.

Resistance to phages represents a concern as spontaneous mutations that result in changes or complete loss of phage receptors on the bacteria may occur. Providing the mutation rate of an organism is known, this is then calculable (Drake, 1991). Moreover, selection pressure may occur leaving those bacteria that

have developed resistance to phages able to replicate and pass on the mutations to their progeny (Alekshun & Levy, 2007, Chan *et al*, 2016).

A further challenge for bacteriophage therapy is the confrontation with the natural immune system (Hodyra-stefaniak *et al*, 2015). Phages are foreign objects and induce a strong anti-phage humoral response. Many studies focus on phages for wound infections, as the same humoral response will not be initiated (Lecion *et al*, 2013, Rose *et al*, 2014). Typically, after phage infection, IgM and IgG will be produced to produce antiphage antibodies.

Hodyra-Stefaniak *et al* (2015) tested the effect of phage infection in mice and the levels within the body. The spleen is the major organ for clearance of phage and was examined for phage presence showing $10^7$ pfu/g, 6 hours after infection. Additionally, there was $10^5$pfu/ml of phage in the blood 6 hours after injection with $10^9$pfu/mouse. Shortly after phage infection, IgM levels were 2.52 log lower than control mice which could compromise any humoral function and have lasting negative effects as it is the main antibody in the body. In order to reduce the risk, phages can be engineered to be lysis-deficient. This approach can decrease the level of endotoxins and inflammatory mediators released during treatment. Hagens and Bläsi (2003) engineered phages which were toxic to bacteria and caused death, by delivering toxic proteins, but did not cause cell lysis and thus released "minimal" levels of endotoxin. This may provide an attractive approach for future studies.

#### 1.6.2.2 Advantages of bacteriophage therapy

Phage therapy offers advantages in targeting bacterial infections that have become problematic to address with other antimicrobials. As an example, many antimicrobial agents are ineffective against biofilms due to the thick extracellular matrix and phage may be used against them as they can penetrate the barrier (Mathur *et al*, 2016). Given the increasing amount of research into phage therapy, their potential has been widely explored and they offer a very real potential outlet for AMR.

Re-sensitizing bacteria to antibiotics may be applied to phage resistant bacteria (Podoll *et al*, 2013). Kim *et al*, applied CRISPR-Cas9 to extended-spectrum beta-lactamase (ESBL) producing *E. coli*. Sequences were targeted that were conserved in ESBL mutants restoring sensitivity to multidrug resistant *E. coli*. It was shown that these target sequences can be exploited to re-sensitize multidrug resistant cells where resistance is mediated by genes present on the same plasmid as target genes. There was significant mortality of *E. coli*

compared to the control where over 99% of cells treated with the pESBL plasmid were killed with sequences to restore sensitivity, $p<0.05$ (Kim *et al*, 2016). The authors state that there is huge potential in targeting any resistant bacteria and this should be applied elsewhere, for example against clinically resistant strains.

Another study reprogrammed CRISPR-cas nucleases capable of targeting MRSA using it as a sequence specific antimicrobial. Sequences to target the resistant bacteria were cloned into phagemids to target the bacteria. Exponentially growing cells were infected with phage at a multiplicity of infection of 5 with >99.99% of cells becoming tetracycline sensitive and were therefore killed (Bikard *et al*, 2014).

It is known that antibiotics may have some side effects, particularly in the digestive system, whilst phage show fewer side effects in animal studies and will further only minimally disrupt the normal flora (Loc-Corillo & Abedon, 2011). Moreover, phages replicate at the site of infection and therefore, unlike antibiotics, are immediately available where needed (Drulis-Kawa *et al*, 2012). If phages did not have contamination issues, initially a low dose would be required, given their self-replicating nature, even if the bacterial density were relatively high. Despite the hurdles faced in phage therapy, natural and engineered phages have many beneficial properties that justify continued research and development into their use. Antibiotics can cost up to $1 billion to produce, in R&D, animal models, clinical trials and marketing the drug. In contrast, phages are self-replicating and can propagate themselves meaning that production of new phages is a relatively cheap and quick with many phages being produced within hours (Slama, 2008). It should be noted that although phage therapy will require investment, its potential could suffice the cost. Isolation and characterization of novel phages can be achieved more rapidly and cheaply than antibiotics. Phages can be isolated simply, and are commonly obtained from sewage or rivers, passing them through a 0.22µm filter (Kleiner *et al*, 2015, Azizian *et al*, 2015, Lee & Park, 2015).

One of the specific advantages of using bacteriophage therapy is the initial number of phage needed is low given that they have the ability to propagate themselves, essentially creating more bacteriophage from a single treatment dose. Previously, doses of between ~$1x10^6$ phage and $1x10^{10}$ phage/patient have been used in human treatment showing that large numbers of phage will be needed (Abdulamir et al, 2014, Sarker et al, 2016). There currently exists a huge gap in the knowledge that has previously been highlighted surrounding clinical trials with phage and only through conducting additional trials will regulations increase (Monsur et al, 1970, Cui et al, 2019). Whilst some of these phage doses may seem low, it must be noted that given that phage

will propagate themselves, it may be safer to initially use a low dose rather than a higher dose. Using larger scale bioprocessing may eliminate variability between the phage produced whilst it will also allow single batches to be produced. This will have the specific advantage as when enumerating and carrying out characterisation analysis of the phage produced, there should be fewer differences between the samples. Additionally, given that phage can easily be stored, a single bioreactor culture may be able to be used for multiple treatments (Jończyk *et al*, 2011). The more optimal the phage propagation is, the fewer batches will need to be run in order to produce the phage, reducing overall cost.

As has previously been highlighted, the increasing rate of research into bacteriophage therapy and synthetic biology is also an advantage of bacteriophage therapy and is why it may be in a good position to tackle AMR.

### 1.6.3 Phage therapy against biofilms

High concentrations of disinfectants and biocides have been used to treat biofilms which may be used in the environment or on surfaces. However, such concentrations are undesirable in a clinical setting and phage therapy may alleviate the use of such concentrations of drugs. Biofilms have been found on many different surfaces and medical devices including catheters. Much previous research focuses on biofilms on surfaces, which may not be representative of a biofilm in the body i.e non-human surface (implant material surface) vs body surface (Dalton *et al*, 2011). One study used an *in vitro* model to disrupt an overnight *P. aeruginosa* biofilm on catheters using a two-hour treatment with 10 $\log_{10}$ PFU $ml^{-1}$ of an M4 phage. Catheters initially had a biofilm count of 6.87 $\log_{10}$ cfu $cm^{-2}$ which decreased to 4.03 $\log_{10}$ cfu $cm^{-2}$, $P<0.001$, after phage treatment (Fu *et al*, 2010).

Ryan *et al*, (2012) used a sub-lethal concentration of cefotaxime and T4 phage, and found an increase in clearance zone compared with just the antibiotic. At a combination of 0µg/ml antibiotic and $6x10^9$pfu/ml phage, average plaque diameter was 1.5mm compared to 0.0156µg/ml antibiotic and $5.6x10^{11}$pfu/ml phage gave an average plaque diameter of 3.5mm. The combination of therapies significantly enhanced the eradication of an *E. coli* biofilm in vitro compared to antibiotic treatment alone, $P<0.05$. Additionally, the minimum biofilm eradication concentration was reduced from 256 µg/ml to 32µg/ml when T4 phage ($10^7$pfu/ml) was used in combination with cefotaxime.

**1.6.4 Animal models**

Although there have been a limited number of human clinical trials on bacteriophage therapy, there have been several pre-clinical animal studies. Kishor *et al*, (2016) isolated and characterised phages through the double agar overlay method and used several phages (*SA-BHU1*, *SA-BHU2*, *SA-BHU8*, *SA-BHU15* and *SA-BHU21*, *SA-BHU37*, *SA-BHU47*) in a cocktail mixture against MRSA in a rabbit model of osteomyelitis. Although minimal changes in osteomyelitis were observed with the phage cocktail treatment, oedema (swollen tissue) and erythema (reddening of the skin), both improved. The authors suggested that had a larger dose been used (>$5x10^{12}$ plaque forming units (pfu)/ml) a significant result may have been obtained. The study may also have benefitted from a comparison of results between the large dose ($5x10^{12}$ pfu/ml) and the initial dose ($2x10^{12}$ pfu/ml).

Weiss *et al,* (2009) conducted a pre-clinical study of T4 phage in mice, colonized with ($10^9$ CFU/ml) *E. coli* K-12 in their gut, were used to assess their potential use for clearance but showed no adverse effects. When they compared faecal phage counts to control mice, T4 phage were 300-fold higher than the control when treated with phage and a full recovery was made. Their study confirmed previous animal studies showing phage therapy did not appear to have any adverse effects and paved the way for human phase I trials to begin (Chibani-Chennouf *et al*, 2004). There is currently one for burn wound clinical trial for patients with an *E. coli* infection with several planned, including diabetic foot ulcers and cancer treatments (Clinicaltrials.gov, 2019).

Thermally injured mice infected with *Klebsiella pneumonia* were treated with five *Klebsiella* phages, and showed maximal reduction in bacterial counts 72 hours post phage infection. Mice showed complete recovery of the wound using histopathological examination. On the skin, bacterial counts were found to initially be 7-log initially which decreased to around 4 log, 3 log and 1 log after 24, 48 and 72 hours respectively (Kumari *et al*, 2009).

A further study examined the effect of phages against biofilms *in vivo*. MRSA infections were administered on implants intravenously in 48 rats and left for 15 days. Teicoplanin was administered (20mg/kg/day) after which biofilm formation was shown to be absent when phage was used in combination with the antibiotic whilst cfu/ml was also significantly decreased ($p=0.004$) in the treated group compared to the control group (Yilmaz *et al*, 2013). Although there have been animal models and clinical trials, development

of phage therapy into the clinic is slow because of a multitude of factors including, purification challenges, fear of endotoxin release by bacteria and the limited specificity of phage (Parracho *et al*, 2012).

**1.6.5 Clinical trials**

In 2009, the first clinical trials involving phage was approved by the FDA. To date there have been 42 clinical trials, 15 completed, conducted in human patients for phage therapy, compared to 7108 for stem cell therapy showing the difference between research areas (Parracho *et al*, 2012, Wadman, 2009, Clinicaltrials.gov, 2019).

There are many phage therapies in development throughout Europe and the USA, however, many of these have not yet reached the stage of clinical trials and are in either R&D or the pre-clinical phase (García *et al*, 2008). Although there has been success in a number of animal models, there are relatively few clinical trials that have been conducted in humans (Housby & Mann, 2009). Clinical trials have been successfully implemented against *S. aureus* (Kishor *et al,* 2016) and *C. difficile* in animal models and are ongoing for *staphylococcal* infections (Nale *et al,* 2016). Additionally, animal models with *E. coli* infections show phage therapy may have future applications for clinical treatment of infections (Hagens *et al,* 2006, Schneider *et al*, 2018).

As of 2016, there are six phages approved for use against bacterial organisms on meat (U. S. Food and drug administration 2014). Figure 1.5 shows a graph of the number of clinical trials expected to be complete by their date, which highlights the future increasing need of phage. Additionally, a figure is given based on the number of papers relating to phage and healthcare showing increasing interest in phage healthcare applications over the last decade. The first approved phage product was in 2005 to treat crop diseases by Agriphage, with the first approved trial for meat to control bacteria levels in ready to eat foods in 2006 developed by ListShield (Parracho *et al*, 2012). Recently, there was the first clinically approved phage trial for in-vitro use of AB-SA01 against *S. aureus* infection on ventricular assist device (Drug Development Technology, 2019).

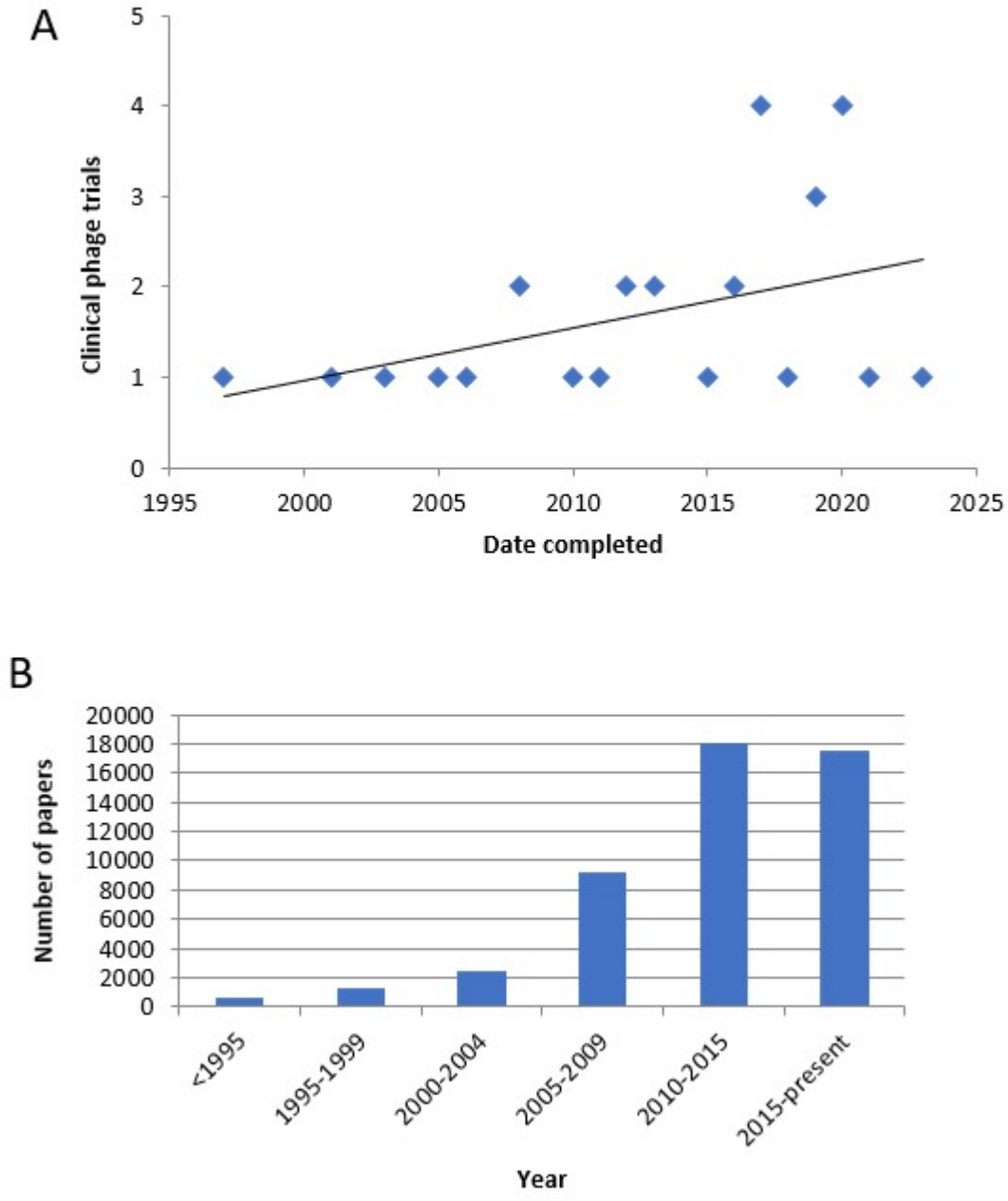



***Figure 1.5*** *– A graph to show the number of A) Phage clinical trials – date of completion. The line represents the increasing trend B) papers relating to phage and healthcare. This data was collected as of December 2019.*

As phages are an emerging therapy, most clinical trials are still in phase I. After passing phase 0, trials where testing is carried out in nonhuman subjects, and the efficacy, pharmacokinetics and safety are tested, drugs are allowed to pass into phase I. This is where 20-100 volunteers are given ascending doses of drugs to test the dose ranging effects in human patients. Once complete, phase II trials can begin where the drug efficacy is tested in 100-300 volunteers before phase III trials where the therapeutic effect is determined in >2000 volunteers (Mahan, 2014). Table 1.2 below shows companies that are carrying out preclinical and clinical trials.

Table 1.2. A table to show different phage clinical trials, the company carrying them out, their location, the target bacteria of the study and the clinical trial phase (García et al, 2008, Clinicaltrials.gov 2019).

| **Company** | **Location** | **Target** | **Stage** |
|---|---|---|---|
| **Ampliphi** | U.S.A | *P. aeruginosa* | Preclinical |
| **Enbiotix** | U.S.A | *P. aeruginosa* | Preclinical |
| **Pherecydes pharma** | U.K | *Staphylococcal* | Preclinical |
| **Pherecydes pharma** | U.K | *E. coli* | Phase I |
| **Technophage** | Portugal | *Pneumonia* | Preclinical |

Sarker *et al*, 2012 used T4 phages or a placebo over 4 days to children in Bangladesh who were hospitalized with acute bacterial diarrhoea caused by *E. coli*. The authors state that there would have been insufficient levels of *E. coli* in the children to propagate the phage needed to see a therapeutic effect. However, no adverse side effects from the treatment were observed. Given the low level of phage used ($3x10^7$ phage), this may have been expected and it would have been interesting to examine a higher level of phage.

One of the other limited phase I clinical trials to have been completed by Ampliphi in 2016, evaluated the safety of AB-SA01 against *S. aureus*. Twelve patients, aged 18-60, were topically administered $1x10^8$ or $1x10^9$ PFU/ml under a bandage of chronic rhinosinusitis for three days. Although the study results have not been posted yet, the efficacy of the treatment is said to be promising (Ampliphi, 2016).

A further project examining the use of bacteriophage is a European research and development project named phagoburn, evaluated the clinical potential of phages for the treatment of burn wounds, specifically against *E. coli* and *P. aeruginosa.* The study used 110 patients with each infection, with 11 clinical partners from France, Belgium and Switzerland. It is the world's first randomised single blind clinical trials performed according to good manufacturing and good clinical practice standards. The trial gave "highly stimulating" and "informative results" with no serious side effects observed (Cordis, 2017). However, the full results are yet to be published as the authors are collecting and analysing all data to be put into an appropriate scientific journal. Interestingly, they highlighted the extensive time needed for production of the phage which remains a key issue.

To date there is only one phase III trial offering evidence of phage therapy being effective against bacteria. In 1963, children between 6 months and 7 years of age were given a treatment against *Shigella* and *E. coli* with phage. The treatment was deemed to be successful although the paper has come under some criticism as it was only a single table in a Russian abstract-type journal (Babalova *et al,* 1968). It remains highly cited given that it is the only reported phase III trials. It is well known that within the Eastern European countries, phage therapy remains widely used, however, the outcomes of clinical studies often go unreported. Table 1.3 below shows some of the clinical trials and the organism they were against.

Table 1.3 – A table to show a variety of current trials that have been completed or withdrawn. In many cases the study organisers refuse to share the reason why the study was withdrawn (Clinicaltrials.gov 2019)

| **Status** | **Organism** | **Intervention** |
|---|---|---|
| **Unknown** | MRSA | Phage cocktail |
| **Unknown** | Primary Immune Deficiency Diseases | Phage OX174 |
| **Completed** | Bacteremia | MicroPhage S. aureus |
| **Completed** | Venous Leg Ulcers | WPP-201 phage |
| **Completed** | Healthy Volunteers | AB-SA01 |
| **Recruiting** | Wound Infection | *E. coli* Phages cocktail |

Phages have been shown to be an emerging therapy with animal models and clinical trials begun. Moreover, their mechanism of action is well known - they have been particularly useful in breaking down biofilms, a major cause of concern. As phage therapy is emerging into the clinic, the bioprocess of phage manufacture must be optimised prior to it entering worldwide clinical trials and treatment for AMR. Whilst phage therapy is not currently used every day and there may be some risks in it as a future treatment, one step towards de-risking its use is its manufacture and showing the optimisation and translation from small to large scale for phage products.

### 1.6.6 Bacterial resistance to phage therapy

Interestingly, there has been some research showing that bacteria have the ability to become resistant to phages. However, phages mutate at a higher rate than bacteria and therefore bacterial resistance to phage therapy is limited (Labrie *et al*, 2010). Whilst this is generally accepted, one study showed that there will be an equal rate of mutation between host and phage, although they used a mathematical model which would need to be experimentally validated (Weitz *et al*, 2005). There are many points throughout the phage's replication cycle that bacterial resistance can occur.

Firstly, there can be interference with the initial adsorption of the phage onto the bacteria. Bacteria can adapt the structure of their cell surface receptors limiting phage interaction and binding. Proteins produced by *S. aureus* have been shown to reduce phage binding, as outer membrane proteins can modify protein A, a receptor that many phage bind to (Khan *et al*, 2002, Labrie *et al*, 2010). Secondly, there can be 'superinfection exclusion', the point where the phage injects its genome into the bacteria to be replicated. The bacteria may initiate a response where proteins interfere with the DNA injection, preventing phage DNA entry (Sturino & Klaenhammer, 2006, Cumby *et al*, 2015).

### 1.6.7 A bridge between AMR, bacteriophage and phage manufacturing

Thus far, the work presented highlights the problem of AMR and some potential solutions. Whilst there has been some debate over the actual numbers of antibiotic resistance infections and deaths highlighted in chapter 1, there is overwhelming evidence that AMR is a huge global problem and novel methods and technologies are needed. One such method is the use of bacteriophage, which have previously been shown to be effective at treating resistant organisms. They offer a potential outlet to the treatment of resistant organisms and must be considered as one treatment of AMR bacteria.

However, despite the current research, there has been very little thought given to bacteriophage bioprocessing and fermentation. Given the state of AMR/phage research, many only produce phage in very small quantities i.e <20ml culture (Maxim *et al*, 2019). Whilst this amount may be suitable for the current needs of some, if phage therapy is to achieve its potential, their production must be examined and evaluated. Without a

viable method of large-scale production, there is a limited chance of phage therapy moving forward with even less chance of it becoming a viable clinical therapy. Therefore, this research will tackle the niche area of bacteriophage fermentation and their bioprocess.

### 1.7 The need for scalable and cost-effective approaches for phage production - an introduction to upstream, bioprocessing

A bioprocess is a method that takes living organisms, such as cells, and obtains desired products from them. It generally begins with an upstream process involving culturing cells using different feeding and processing strategies (Dengl *et al*, 2013). Bioprocessing usually starts at a small scale to optimise the parameters and is then subsequently scaled up (Kensy *et al*, 2009). This is followed by downstream processing to harvest the product and remove contaminants, a necessary regulatory requirement to make products safe. Bioprocesses must maintain identical parameters across batches to ensure that the final products are identical. It is advantageous to use an automated system to minimise variations, improve productivity and reduce changes to the final product (Bareither *et al*, 2013). The difficulties within scale up can be associated with maintaining homogeneity in larger systems, the change in surface to volume ratio and the microenvironment (Baert *et al*, 2016). It should be noted that it took around 15 years for the scalable bioprocess of penicillin to be complete highlighting the need for bacteriophage bioprocessing to be investigated.

#### 1.7.1 The need for manufacturing/scalability within a bioprocess

For any therapeutic product to come to market and meet the supply demands, the bioprocess must be scalable and this is no different for any AMR product. Shake flask cultures have the advantage that they offer high throughput methodologies and therefore several experimental runs can quickly be completed. However, they do not easily allow for the control over several critical conditions: pH and $dO_2$. These key input variables may alter the culture because of changes that occur within the culture i.e waste products given off or the oxygen level decreasing due to cellular usage.

Whilst there is a clear and obvious need for scalability, several translational issues can be derived from a scale up model including variation in the final product, contamination risks and increasing the cost of goods

and mixing. The cost of a scale up model compared to a shake flask model can be considerably increased; however, one must also consider the total yield produced, i.e. how well the cost increase matches the total final product produced (Verma *et al,* 2015).

### 1.7.2 Single use vs multiuse bioreactors

There is some debate in the bio manufacturing industry when deciding whether to use stainless steel or single use bioreactors but the final bioreactor type chosen is generally based on the product and scale of process. Whilst each of the systems has its merits, there are certain advantages and disadvantages of using each. Single use systems are traditionally be used for cell therapeutics given the sterility concerns, as when producing a large batch of product, any contamination risk would need to be prevented to prevent loss of product. Many cell-based therapies are in advanced stages; where there is a need for large scale production for clinical trials, there are many models that are capable of processing large volumes, up to 2000L (Schnitzler *et al*, 2016). However, within the microbiology industry where there can be high numbers of experiments with high turnover, stainless steel bioreactors may be used. There has been limited work directly comparing industrial scale stainless steel and single use reactors but it is important to examine the hydrodynamic effect on the culture and thermofluidics when scaling up a bioprocess into either type of reactor (Delafosse *et al*, 2018).

Within recent years in the bioprocessing industry, a shift has begun towards the use of single use bioreactors which can be particularly important when manufacturing high value products (Shukla & Gottschalk, 2013). In multi-use bioreactors there is the need to ensure there is no carry over between cultures. The time needed to clean a multiuse reactor, or the purchase of further multiuse bioreactors, can add significant downtime and cost to a process. The higher fixed cost of new single use reactors may, however, overtake the upfront cost of a multiuse STR and which is why they remain useful. Scale must be taken into account as larger scale bioreactors will be more expensive. Additionally, multi-use bioreactors are used more within microbial processes where oxygen consumption is higher as there have been questions raised around the oxygen diffusion in single use bioreactor bags (Kusterer *et al*, 2008). Moreover, single-use bioreactors present an environmental concern, aCs although typically they are made of FDA approved plastic, they still must be disposed of after disinfecting the culture (Shukla & Gottschalk, 2013).

**1.8 Bacteriophage Bioprocessing**

Given the advantages presented of using phage, there is a need to establish suitable methods of its manufacture. Typically, the production process begins with the host organism fermentation and then infection of phage into the culture. The next step is to separate the lysed bacteria from the fermentation medium using centrifugation and filtration, followed by a volume reduction step to concentrate the phage (Branston, 2009). At this stage the product, according to regulations, is still classed as being in an impure state and needs to be further purified in order to be used. The phage can then be spotted onto an agar plate and incubated for up to 24 hours to allow the formation of phage plaques, a reliable method for determining phage titre, activity and potency (Sørensen *et al*, 2015). Currently, most of the research with phage is done using shake flasks (<20 ml), however, this may not be an appropriate model as there is limited control over pH and oxygen, which may affect phage production. Moreover, shake flasks also limit the yield, however, their limitations may be limited to R&D rather than manufacturing as for large scale manufacturing experiments, shake flasks would not necessarily be used.

Supplementation with additional carbon sources such as glucose or glycerol may help to increase the phage titre as providing more energy sources for the host cell could improve growth of the cells and therefore increase phage production (Bourdin *et al*, 2014). Bacteria are usually infected in the early log phase as phages need to infect a healthy cell that is continuously growing (Bryan *et al*, 2016).

For any product to be viable it must be scalable to be used as a clinical product; however, it should be noted that if parameters are to be varied, this may be an issue in large scale manufacturing when trying to change the temperature or pH (Soini *et al*, 2008). Much of the work with automated stirred tank bioreactors for phage fermentation is currently done in industry. However, there is still limited commercial work for large-scale manufacture of phages with even less for re-engineered phages (Courchesne *et al*, 2009, Pires *et al*, 2016). Tables 1.4 and 1.5 show currently published stirred tank bioreactor studies for bacteriophage and companies capable of bacteriophage production.

Table 1.4 A table to show different studies in bacteriophage bioprocessing

| Study | Phage | Scale | Type of reactor | Variables |
|---|---|---|---|---|
| **Sauvageau & Cooper 2010** | T4 | 1L | Stirred tank reactor | 37°C, 250rpm, MOI 0.001, 15 hours |
| **Roy et al 2018** | A5II | 100ml | Shake flask | 37°C, 250rpm, MOI 0.001, 8 hours |
| **Nabergoj et al, 2018** | T4 | 25ml | Shake flask | 37°C, 350rpm, MOI 0.1, 8 hours |
| **Lindemann et al, 2002** | Qβ | 20ml | Shake flask | 37°C, 350rpm, MOI 1, 60 hours |

Table 1.5 A table to show different companies who produce bacteriophage from google web search carried out in May 2020

| Company | Phage | Maximum scale available/batch |
|---|---|---|
| **Cellexus** | Phage against *S. aureus* | 50L |
| **Paragon** | Client request | 15/100L |
| **Jafral** | Client request | Capable of 200L, usually 10/50/100L |

Whilst tables 1.4 & 1.5 do not show a complete list of every company that has phage production facilities or phage fermentation studies, they are those who directly give their capabilities. However, there are a limited number of companies who regularly produce bacteriophage, further highlighted by the literature. No

information was able to be provided as to the "client request" either on their website or after direct contact was made with the companies.

It should be noted that there still remains a gap in our knowledge of the effect of external factors on phage propagation. Whilst the work by Greico and this author showed that temperature can significantly improve the overall phage titre obtained there are other variables to consider (Greico *et al*, 2012). Moreover, the influence of temperature has only been investigated in a handful of phage and results may differ for different phage. Bacteriophage have been shown to be stable, in some cases, up to 90$^{o}$C, however, this is atypical and usually phage will loose their propagation and infection ability above 50-60$^{o}$C (Jończyk *et al*, 2011).

Phages again have been shown to be stable at a wide range of temperatures, phage targeting multiple organisms have been shown to maintain their activity between pH 3-11, however, between pH 6-8 phage will usually be at their optimal lytic activity (Feng *et al*, 2003). Outside this range, it is partly due to the host organisms not being able to grow and allow efficient phage propagation. Hydrogen ion concentration has also shown the ability of phage to aggregate, when the pH was less than or equal to the phage isoelectric point, there was a significant increase in phage aggregation which may be useful for downstream purposes (Langlet *et al*, 2007).

Osmotic shock has been shown to reduce viability or phage and also inactivate them in some cases. At 0.5% NaCl, 11 Myoviridae, 7 Siphoviridae and 4 PodoViridae phage were mostly inactivated. Rapid changes in osmotic conditions may cause phage DNA to extrude from the tail or for phage head to break away (Wichels et al, 1998, Jończyk *et al*, 2011).

Tables 1.6 and 1.7 below show the titres reached for T4 phage and phage K respectively within the literature. Although not reported in the studies, the phage per input cell and the phage per input phage was calculated based on the number of host cells at the point of infection and MOI. Currently, using the standard pfu/ml values there is no allowable comparison between different bioprocesses. Given that different authors use different MOIs, process efficiency may vary significantly between one another and by only looking at the final phage titre, this may preclude the efficiency between processes. Moreover, given that different authors also use different OD ranges for the phage infection, this may again mask variations in the bioprocess. However, using these standardised measurements, it allows transparency into the efficiency of a phage bioprocess. The paper published by the author of this thesis, showed for the first time in the literature to our knowledge the first

standardised measurement of bacteriophage infection using phage per input cell and phage per input phage (Ali *et al*, 2018). Tables 1.6 and 1.7 show a wide variation in titres achieved throughout the literature. This was potentially due to the variation in the KPIV used, showing that there is no current standard protocol for phage propagation; this highlights the need for this research.

**Table 1.6.** A table to show the different phage titres achieved in the literature for T4 phage

| Author | Titre reached | Input cell | MOI | Phage per input cell | Phage per input phage |
|---|---|---|---|---|---|
| **Bourdin et al 2014** | 1e11 | 4e8 | 0.1 | 250 | 2500 |
| **Sochocka et al 2015** | 1e12 | 4e9 | 1 | 250 | 250 |
| **Los et al 2007** | 1e6 | 1e9 | 0.1 | 0.001 | 0.01 |
| **Bremmer et al 2016** | 1e11 | 1e8 | 5 | 1000 | 2000 |
| **Sauvageau & Cooper 2010** | 1e9 | 1e9 | 0.001 | 1 | 1000 |
| **Bourdin et al 2013** | 1e9 | 2.4e8 | 10 | 4.2 | 0.42 |

Table 1.6 shows various T4 phage titres achieved from current studies. It is interesting to see the differences in titres achieved throughout the literature, whilst examining the phage harvest per input cell/phage also shows a wide level of variation. Table 1.7 shows the phage K phage per input cell/phage values achieved by different authors. It is interesting to note differences in MOI used between authors, highlighting a lack of standardisation in the MOI to use for phage K infection.

**Table 1.7.** A table to show the different phage titres achieved in the literature for phage K.

| Author | Titre reached | Input cell | MOI | Phage per input cell | Phage per input phage |
|---|---|---|---|---|---|
| **Hsieh et al 2011** | 6e10 | 1e9 | 0.1 | 60 | 600 |
| **O'Flaherty et al 2005** | 1e9 | 1e7 | 1 | 100 | 100 |
| **Rees & Fry 1983** | 1e10 | 2e7 | 0.1 | 500 | 5000 |
| **Narasimhaiah et al 2013** | 1e10 | 6e8 | 0.1 | 16.6 | 166 |
| **Kumar et al 2012** | 5e10 | 1.6e8 | 0.1 | 312 | 3125 |
| **Paliavali et al 2017** | 1e9 | 1e9 | 10 | 1 | 0.1 |

Whilst there is a wide variation in both the input cell and MOI used, the results in the final two columns show that there can be a significant variation in output values. By only reporting the final titre achieved, phage fermentation scientists are unable to get an accurate description of the efficiency of a process. Through our calculation of their stated MOI, input cell and final titre, the efficiency of each process achieved by the different

authors was shown. This demonstrates the need for a unified method for phage production, rather than just phage titre, given that more of the bioprocess is taken into account.

Within microbial experiments, it is common to take a colony from a plate as a starting seeding density for a culture. This ensures that approximately the same number of bacteria will be used as a starting point for any culture. However, the phage literature has shown a wide variation in starting densities of phage. For example, it is common to use an MOI of between 0.1 and 10 for T4 and phage K infections. There are very few processes that would accept a starting concentration to be up to 100 greater in one process compared to another. Moreover, using the 100 times increase in phage seeding density may lead to a wasteful process, as an initial input of phage must be an appropriate level.

## 1.9 Challenges of phage manufacturing

### 1.9.1 Current challenges

Whilst bacteriophages have been shown to potentially play an increasingly important role in both medicine and the biotechnology industry, there is an increasing need to optimise purification strategies. Development into the clinic of phage therapy is slow as phages need to be purified before they can be used because they lyse bacteria, releasing harmful toxins (Paul *et al*, 2011). Additionally, whilst antibiotics are still viable, people may hesitate to use them. However, as phages are used in the food industries there are some commercial sources for large scale phage production (Gutiérrez *et al*, 2016). Studies have shown bioreactor processes for phage manufacturing, however, one challenge is their optimised production (Nabergoj *et al*, 2018). Other challenges lie within engineering phages to give a greater host range. Bioprocess optimisation is critical for developing a scalable and cost-efficient manufacturing process. There has been significant investment into the production of antibodies, vaccines and drug production, given that there is a current clinical need for them. However, given that bacteriophages are not currently used, there is a lack of investment into their bioprocess. Investment is needed in order to show the scalable and optimal process to improve the phage efficiency.

### 1.9.2 Scaling up the phage bioprocess

In the pharmaceutical industry, when a new product is developed, the bioprocess must be scaled up, to increase yield whilst maintaining quality and consistency of the product. A similar approach will need to be taken for the mass production of bacteriophages. Arguably, the greatest challenge currently facing phage scale up is the limited amount of work that has been undertaken and that currently, it is not well understood how to optimally manufacture phage (Garcia *et al*, 2018). Given that conditions will differ significantly when moving from a shake flask culture to a stirred tank reactor, there are numerous studies that need to be undertaken which ultimately have not currently been widely investigated. This includes, agitation rates as the geometry of the culture systems will differ affecting oxygen transfer (Karimi *et al*, 2013). Oxygen level will be one of the key factors that will affect the growth of the host culture and therefore have an effect on phage propagation i.e. too little oxygen available for the host cells will prevent their growth and therefore maybe prevent optimal phage propagation. A further challenge is the rate of shear damage. When moving into bioreactors, mechanical stress, caused by the agitation may destroy or damage the host organisms and phage, which have been shown not to be able to withstand high shear stress even for short periods of time (Malik *et al*, 2017).

The scale up challenge is the final hurdle before large scale manufacture of a product can begin. Heat transfer is also a problem at large scale if there is a desire to change the temperature of a reactor. Mass transfer throughout a fermenter also represents an additional challenge, the equal dispersion throughout a culture leaving gradients. Ensuring the contents are the same throughout each process is important as differences between the above can affect the final product which is highly undesirable. Perhaps the greatest challenge in scale up is the fluid mechanical stress of shear damage caused by agitation or sparging of cultures which may destroy the organisms in the culture (Hewitt & Nienow, 2007). Therefore, optimising the bioprocess will increase yield, improve efficiency, save money and reduce the effect on downstream products.

### 1.9.3 Phage determination

A further challenge of phage therapy is characterisation and release testing. It has previously been highlighted that phages can easily be isolated, but determining which phage they are and what they act against can be difficult. Sequencing phages is the gold standard method to determine which phage has been isolated (Dias-Neto *et al*, 2009). Once a sequence has been complete, they must be quantified. The traditional gold standard method for the quantification of phages uses the plaque overlay technique (Łoś *et al*, 2010). However, if the isolated phage is unknown and its host activity is unknown, it must be tested against a multitude of hosts to determine its lytic activity.

Phage enumeration can be achieved through the traditional plaque assay. Although it is a relatively cheap and simple method and there has been a historical use, it has the drawback that it requires at least 12 hours before results are obtained (Jończyk *et al*, 2011). It must be noted that the plaque assay can result in variable results and whilst is the current gold standard method there are disadvantages which have been previously highlighted (Smither *et al*, 2013, Baer & Kehn-hall, 2014). Whilst it is easy, simple and cheap, it is highly manual and results may differ based on users. Another approach for phage detection is to tag the phage with a fluorescent dye, similar to the process of an antibody, which can be detected by fluorescence microscopy or scanning electron microscopy (SEM) or flow cytometry (Doolittle *et al*, 1996, Naicker & Durbach, 2007, Han *et al*, 2014). One advantage of tagging the phage is that it can then be used in alternative diagnostic assays or detection assays, however, it would not be able to be used for human or animal therapies.

Anderson *et al*, 2011 described an alternative assay based on quantitative real-time polymerase chain reaction (qPCR). The method is based on measuring fluorescence generated during each PCR cycle as the phage-containing specimen is amplified and phage DNA molecules accumulate. The method works by establishing the correlation between the level of DNA and the number of phage particles, validated through the plaque assay. The assay involved the interaction of a single lytic phage particle with a bacterium, which results in the lysis and release of newly formed phage. Their method was shown to be comparable to the traditional plaque assay and took 3 hours compared to 12, giving it a degree of superiority, with less handling requirements. However, their study only examined three bacteriophages and cannot be assumed to produce accurate and reliable results for all phages. Additionally, their methodology required costly equipment and its reproducibility was low.

Critical quality attributes (CQAs) are chemical, physical, biological or microbiological characteristics that should be applied to ensure that the desired product quality is constant (Rathore, 2016). CQAs in bioprocessing may include culture media, reactor volume, culture time and others which must be kept constant in order to keep the product quality constant. They are known to affect product quality and have been described as properties of a product required to remain in a certain range to achieve suitable product performance. Examples of CQA include state, storage stability and size (George, & Ghosh, 2013). Bacteriophage must meet specific CQA's in order to be used such as lytic activity, purification levels, biological, chemical microbiological and physical characteristics.

### 1.9.4 Regulations of phage therapy and purification

Phages have been classified as biological medicinal products and before their use is permitted, they must be purified, a major factor in the limited use of human phage therapy trials. Such products are used to prevent diseases and when a new drug is submitted for clinical trials, it is the FDA or equivalent responsibility to assess a products quality and safety. Bacteriophages are given special attention by EU legislation given their composition. Data from laboratory studies are rigorously examined and after submitting an investigational new drug application, clinical trials may begin. Phage must show an adequate level of purity (<50 Endotoxin units/ml) before their use in humans is permitted. Despite this, clinical trials have been completed examining phage on either wound infections or on patients with immediately life-threatening infections for which there are no alternative treatments (Sarker *et al*, 2012).

Once phages are produced, through infection of their host strain, they need to be characterised through DNA sequencing. Transmission electron microscopy (TEM) has also been used to compare them against a known phage for visual evidence that phage have been produced (Hatfull, 2008, Clokie *et al*, 2011).

A key aspect of good manufacturing practice requires full removal of impurities in a product to regulatory levels (Marathe & McIntyre, 2015). Initially, a sample of phage can contain up to $10^6$ endotoxin units EU/ml (Ceglarek *et al*, 2013). To be used in clinical trials, the British pharmacopeia states that all drugs, including phages, must contain <5 (EU)/kg of body weight before administration to a patient (Malyala & Singh, 2008, British pharmacopeia, 2016). Achieving such levels is often costly and time consuming (Parracho *et al*, 2012).

EndoLISA has been developed to test the levels of endotoxins and can detect from 0.05 to 500 EU/ml (Grallert *et al*, 2011). Other tests include the LAL test developed by Charles Rivers laboratories, to detect endotoxin levels through chromogenic techniques. Current methods that are simple and cheap such as chromatography and caesium chloride precipitation, which can produce purity of between $10^3$-$10^5$EU/ml, work by separation based on density (Monjezi *et al*, 2010, Szermer-Olearnik & Boratynski, 2015). The most significant impurities are typically removed first and the most difficult or expensive separations are left for the final stages of purification. Filtration to remove bacterial debris using a 0.22µm filter is used in small scale phage production after centrifugation (Hudson *et al*, 2013). Once completed, the bacteriophage product is stored in a stable manner with 20%/50% v/v glycerol at -80$^o$C, -20$^o$C or 4$^o$C without the addition of glycerol (Phillips *et al*, 2016). Bacterial DNA, proteins and LPS can differ in viral preparations and therefore effective methods for the removal of bacterial debris remains possibly the greatest challenge (Ceklarek *et al*, 2013).

When larger volumes are produced, spin filters have been shown to be used at large scale production for cell retention (Voisard *et al*, 2013). Also, tangential flow filtration (TFF) has been used as an alternative and for large scale purification which has been shown to remove >99% of RNA contamination (Khan *et al*, 2000, Castro-Mejía *et al*, 2015). Ultra-centrifugation at high speeds, 50,000rpm, may be used for purification of phages for several hours (Yarosh *et al*, 1981). Ultrafiltration has been used with a non-selective absorbent although it is inefficient in the presence of proteins (Bastia *et al*, 2007). Ion exchange, affinity and size exclusion chromatography have also been used for phage purification given that they have shown to be useful in mammalian cell processes (Zakharova et al, 2005, Ceglarek et al, 2013).

There is currently no method of phage purification that offers a complete solution for separation of one phage from another that is of a similar size (Ceglarek *et al*, 2013). Complete isolation of specific phages is difficult but this might not be a particular problem when cocktails of phages are propagated. Cocktails of phages are usually produced independently as different phages will need different times, culture media and hosts to propagate themselves and are mixed prior to use (Chan *et al*, 2013). Phages may also be passaged several times to select for desired attributes known as selective phage development. Drake *et al* (1998) estimated that there would be 1 mutation per genome per replication for lytic phages. During large scale production, this may impact significantly on phage activity.

Although each of the methods produces some level of purity, they still do not offer an effective method to purifying a sample highlighting the importance for developing purification strategies (Oślizło *et al*, 2011, Ceglarek *et al*, 2013). Those methods that can produce a pure enough phage sample can often be expensive and laborious (Merabishvili *et al* 2009).

In the United States and Western Europe, the use of phage therapy is restricted due to safety concerns (Withington, 2001, Brüssow, 2012, Parracho *et al*, 2012, Oliviera *et al*, 2015). However, in some Eastern European countries, phage therapy is available on the market and acceptable to use in medical trials which go unreported (Bhandare, 2015). Clinical trials such as phagoburn within Europe, using phage therapy for burn wound patients, will contribute to optimisation of the regulatory guidelines of phage therapy (Phagoburn.eu, 2013). This development of regulations presents a challenge to developers as guidance on their product is not thorough but ongoing work must product the purest products possible.

The process of purification, manufacturing, quality control and isolation all still need to be addressed and optimized. Before use in human therapy, the bioprocess of phage production and challenges in phage purification must be addressed.

Whilst the purification of phage to acceptable regulatory standards is clearly a major issue for bacteriophage therapy, the scalable manufacture will be crucial for their use to tackle AMR.

## 1.10 Manufacturing Science to improve bioprocesses

### 1.10.1 Quality by Design (QbD)

One cannot ignore the work of Sir Ronald Fisher whose work with experimental design has been hailed as the greatest method to “secure basis of new knowledge” (Kirk, 2015). Whilst currently, many choose to use one factor at a time experiments (OFAT), this has an analytical drawback compared to more advanced experimental designs. One gap in the knowledge that must be bridged is the few studies examining bacteriophage experimental design despite their potential shown in previous sections.

Quality by design (QbD) is a framework for improving processes beginning with pre-defined objectives and emphasis on a systematic approach to develop a process and product based on quality science. Additionally,

there is also an element of risk management that is part of QbD. It can be applied to develop the understanding of key controls to reduce the risk of product failure. In any bioprocess, there are a number of factors that may impact the quality of a product. Changes from raw materials to equipment and even operator variances may impact the quality of a product (Chalk, 2014). During a bioprocess the quality of the final product is of key importance and must be monitored throughout. Whilst all bioprocesses must be scalable, they must also show a level of consistency to achieve certain yields between processes.

The concept of QbD allows manufacturers to define the specifications of a product and design it in a way that meets the needs of its intended use i.e. a phage with an extended host range can target multiple organisms. It also allows the manufacturer to identify any potential source of variability and implement necessary changes to control the bioprocess. QbD is a way to reduce the risk in a bioprocess and reduce uncontrolled variations which are extremely undesirable (Snee, 2014). Before beginning a bioprocess, having information regarding the final product will make it easier to make changes to the bioprocess to achieve the desired product. For example, when engineering a phage or trying to produce a purer phage lysate, the product must have its TPP considered although it may not be fully mapped out.

Specific advantages of QbD include the ability to defining the product target, developing the product to meet the manufacturing requirements, identifying quality attributes and sources of variability. Controlling measures will allow a product to be produced with minimal variations between samples (Yu, 2008). Moreover, QbD can also improve efficiency whilst reduced manufacturing costs of processes, by making a prediction of scale up and validation whilst offering cost savings and improved efficiency of drugs. The commercial viability and potential of a new product is a driver for any bioprocess to improve and maintain the product. Tools including Design of experiments (DoE), risk assessments and analytical technology can be used to make QbD applicable to different fields (Zidan *et al*, 2016). When developing a phage therapy, QbD must be considered in order to be used later in a clinical setting.

Six-sigma is a data driven approach and aims to remove causes of variability in bioprocesses, an example is using on-line analysers to control a process (Luttmann *et al*, 2012). It is a work package that can improve processes to reduce waste and variation, thereby improving efficiency whilst quantitatively described how a process is performing. It is a quality programme that defines, controls, measures, analyses and improves a

process. It aims to reduce output variation so that over time there is no more than 3.4 defects in a process per million opportunities (DelliFraine *et al*, 2014). It has been used in industry such as AstraZeneca who implemented Six Sigma and found several wasteful areas in their processes that were using unnecessary resources. An investment of $100,000 in their process lead to more than $60 million in revenue gains (Haleem *et al*, 2015). Although a QbD approach was not strictly employed within this work, it may be useful in future manufacturing processes.

### 1.10.2 Design of Experiments (DoE)

Design of Experiments is a method used to determine the relationship between input factors and how they affect the outcome of an experiment. The concept is more than 50 years old and was described in detail by Sir Ron Fisher (Fisher, 1971, Mead, 2012). In all experimental procedures, any input factor will be transformed into an output factor, a key part of DoE. Input factors include equipment, cell line used or the protocol and the resulting output of the experiment would be the quality of the desired product and how it performs compared to expectations. DoE changes input factors intentionally to observe changes to the output and enhance the final product. Designing and planning the experiment is the first step of DoE, however, experimental analysis is also critical, including statistical analysis to draw conclusions from the experiment. DoE can be used to gain an insight and understanding into a process as well as determine the best operating conditions of a bioprocess. One of the major benefits to DoE is the influence and interactions between the factors that contribute to the process are considered.

The concept of DoE has been used with great success in some companies such as General Electric and Toyota, to improve work processes and remove wasteful processes but there are still challenges that limit its success (Anthony & Antony 2016). As many people are still unaware of its concept, this ultimately limits its effectiveness and chance to be used to improve a process. Additionally, they do not know about its effectiveness and therefore do not employ it. Moreover, if appropriate parameters are not chosen, this may waste time, effort and resources leading to the final product process being inefficient (Antony, 2014, Baronsky-Probst *et al*, 2016).

One study used a DoE approach to optimise vector expression in to improve HIV-1VLP production in HEK293 cells. The authors used varying concentrations of DNA and PEI given that they were using PEI mediated

transfection for 9 combinations of both. They successfully showed that transfection was dependent on both PEI and DNA concentration rather that cell line and showed that 2.34µg/ml and 5.81µg/ml PEI gave the most efficient transfection and vector uptake (Fuenmayor *et al*, 2018).

DoE has been shown to be a useful concept in several industries, and can be applied to pharmaceutical production of drugs, and has been used minimally for phage production (Yu *et al*, 2014). One study used a three factor, three level experiment for high titre filamentous phage production and incorporated DoE into their study and was the first to employ this methodology to phage production with a second study by González-Menéndez. Greico used 28.5$^{o}$C as a mean temperature and 20$^{o}$C and 37$^{o}$C as the low and high parameters respectively. They also used a mean DO% of 70% with 40% and 100% as the low and high values respectively. Finally, pH was used with a mean value of 6.5 and low and high values of 4.0 and 9.0 respectively. DO concentration had no significant effect for filamentous phage yield but the optimal temperature and pH were found to be 28.1$^{o}$C and 6.9 respectively. Despite this, the study did not consider the time needed for phage propagation which is important for manufacturers whilst they only used a single phage. However, their study did underline the importance of using DoE methodology in order to yield a high phage titre and is commonly used within the bioprocessing industry (Greico *et al*, 2012). Whilst their study used a 700ml working volume in a 3L bioreactor, they were only able to conduct 20 runs given the scale that they were working at. This study will employ techniques and principles of manufacturing science in order to improve the phage bioprocess.

#### 1.10.3 Response surface methodology (RSM)

Response surface methodology (RSM) is a mathematical technique to examine the relationship between input and output factors. Using RSM enables only the most critical runs to be complete and by using the 'mid-levels' of the conditions, operators can make accurate predictions of the outcome based on the mean response from each experiment conducted (Khuri & Mukhopadhyay, 2010). The input variables represent the design space and using RSM the greatest output within the variables can be determined within the design space.

Both the product design space and process design space must be clarified. The product design space is the combination and interaction of input variables and process parameters that are known to produce quality (Rathore, 2009). The process design space is the approach taken to characterise the process i.e. upstream/downstream. Risk assessments to identify parameters for process characterisation can help focus a

study and define the design space. Additionally, they enable researchers to understand risks involved and may reduce costs as high-risk areas are highlighted and may need extra capital.

Liu *et al*, 2018 used an RSM experimental design in order to evaluate supplements and trace elements at varying concentrations for high titre antibody production in hybridoma cells. Serum free media was used along with Ferric Citrate, peptone and trace elements. Experiments were carried out in a static plate, spinner flasks and hollow fibre reactor to examine any differences in volume. Whilst it has been used in other areas, RSM is rarely used in phage fermentation studies but its efficiency has been shown in other areas and may be useful in phage studies.

In the phage bioprocess, DoE and QbD may be adopted in order to produce the best possible product. They ensure better design of products and consequently fewer problems in the manufacturing process, reduced time, cost and effort into developing a product and also waste reduction (Winkle & Rathore, 2009). Using an automated system allows input variables such as pH and temperature to be controlled and prevent deviations from optimal parameters providing consistency between different bioprocesses.

### 1.11 Aims and Objectives

It has been shown that phage-based therapies hold significant promise towards combatting AMR bacteria. However, prior to phage therapy being routinely used in the clinic, there are several challenges that must be overcome. This shows the need for a robust scalable manufacturing process for bacteriophage. One key aspect that must be examined is their production and the bioprocess of bacteriophage and its use may be hindered if it is not addressed and ultimately this work aims to address the following areas. Although the entire manufacturing process requires efficient upstream and downstream processes, this thesis will focus more on the upstream manufacturing of bacteriophage

The aim of this thesis is to optimise the T4 and K bacteriophage manufacturing process in a shake flask model and evaluate their propagation in a litre-scale bioreactor for their upstream manufacturing process. To achieve this aim the following objectives will be undertaken:

1) **Evaluate high phage titre production**
    a. A DoE study will determine the conditions affecting growth and propagation of bacteriophage, specifically T4 phage and phage K. This information will be used to improve titres. These include the MOI, agitation, time of infection and temperature of infection. A DoE methodology gives an analytical advantage over one factor at a time experiments whilst it also reduces the number of experimental runs. This will give a defined set of parameters to be used for the highest titre phage production. Addition of supplements and a defined medium will also be used.

2) **Establish a litre-scale process for bacteriophage production**
    a. Any therapeutic product that may be used will only be viable if its production can be scaled up. Studies will be conducted to identify and establish the scalable production of T4 phage and phage K in stirred-tank bioreactors. Factors such as dissolved oxygen and pH which are readily be controlled in stirred tank bioreactors will be investigated. Moreover, sparging of the culture will be examined to determine its effect on titre. This investigation will show the conditions to

be used in order to achieve similar titres at a shake flask scale compared to a bioreactor scale. The bioreactor scale will allow automation of the process, which may reduce variability and increase titre, given the maintenance of variables i.e. pH/oxygen.

3) **Evaluate the kinetics of bacteriophage infection**

    a. The kinetics of any live organism will give a valuable insight into its nature. Therefore, investigating their kinetics may be useful to back up the results from the shake flask and stirred tank reactor experiments. High and low levels from the DoE will be used to evaluate their effect on the kinetics to provide more information on the effect of multiple conditions on the adsorption and burst size. This will provide an answer as to whether or not any improvement in titre is caused from certain conditions being used i.e. temperature/agitation.

4) **Development of rapid bacteriophage detection methods**

    a. An investigation into the culture around the point of infection, lysis and throughout the remaining culture will be done to determine if analytical methods can be detect the phage of host breakdown. This could be initial work which may take detection times from 24 hours down to a few minutes.

Whilst this thesis primarily focuses on T4 and phage K, the importance of investigating two bacteriophage shows that the techniques presented are transferable. Further investigations of other bacteriophage may follow the methodology presented here. Moreover, showing that the methodology can be used for two phage will exemplify its reliability. The data presented later show variances between the two phage in similar experiments, highlighting the importance of investigating two phage as it can not be assumed that the results from one phage will be similar to another. Comparisons of bacteriophage are not commonly investigated within the literature. Here, the work shows two phage relevant to AMR, shows the ease of applying DoE on two different scale and how to standardise and optimise a phage bioprocess whilst also developing a scalable bioprocess into industrial scale bioreactors.

# Chapter 2 - Materials and Methods

This chapter will focus on the materials and methods used. *E. coli B* was grown using Luria broth (LB) media whilst *S. aureus* was grown in brain heart infusion (BHI) media. A working cell bank was produced for experiments throughout the project to prevent differences in the bacteria used. Unless otherwise stated, all chemicals were purchased from Sigma Aldrich UK.

### *2.1* Host growth

*E. coli B* (University of Reading) was agitated (225rpm) in LB media whilst *S. aureus* (ATCC 19685 (UK supplier LGC standards, Teddington)) was agitated (150rpm) in BHI media (pre-warmed at 37$^{o}$C for at least 30 minutes). A shaking incubator Incu-shake midi (Wolf Labs, UK) was used for all shake flask work. A single colony from a plate was suspended in 20ml media in a 125ml shake flask (Fisher Scientific, Loughborough UK). It was incubated in a Thermo Scientific $CO_2$ incubator (Irvine, UK) at 37$^{o}$C shaking for around 24 hours with a measurement of optical density (OD) 600nm using a Shimadzu UV-1280 UV-Vis spectrophotometer (Japan) taken every two hours in order to produce a growth curve, shown in figure 2.1. Appropriate dilutions were made when necessary where the OD reading >0.7. Additionally, this wavelength was deemed appropriate as it will not cause mutations to the host or phage, it will not be absorbed due to the yellow coloured media and has been used several times for microbiological and in bacteriophage studies (Shibata *et al*, 1954).

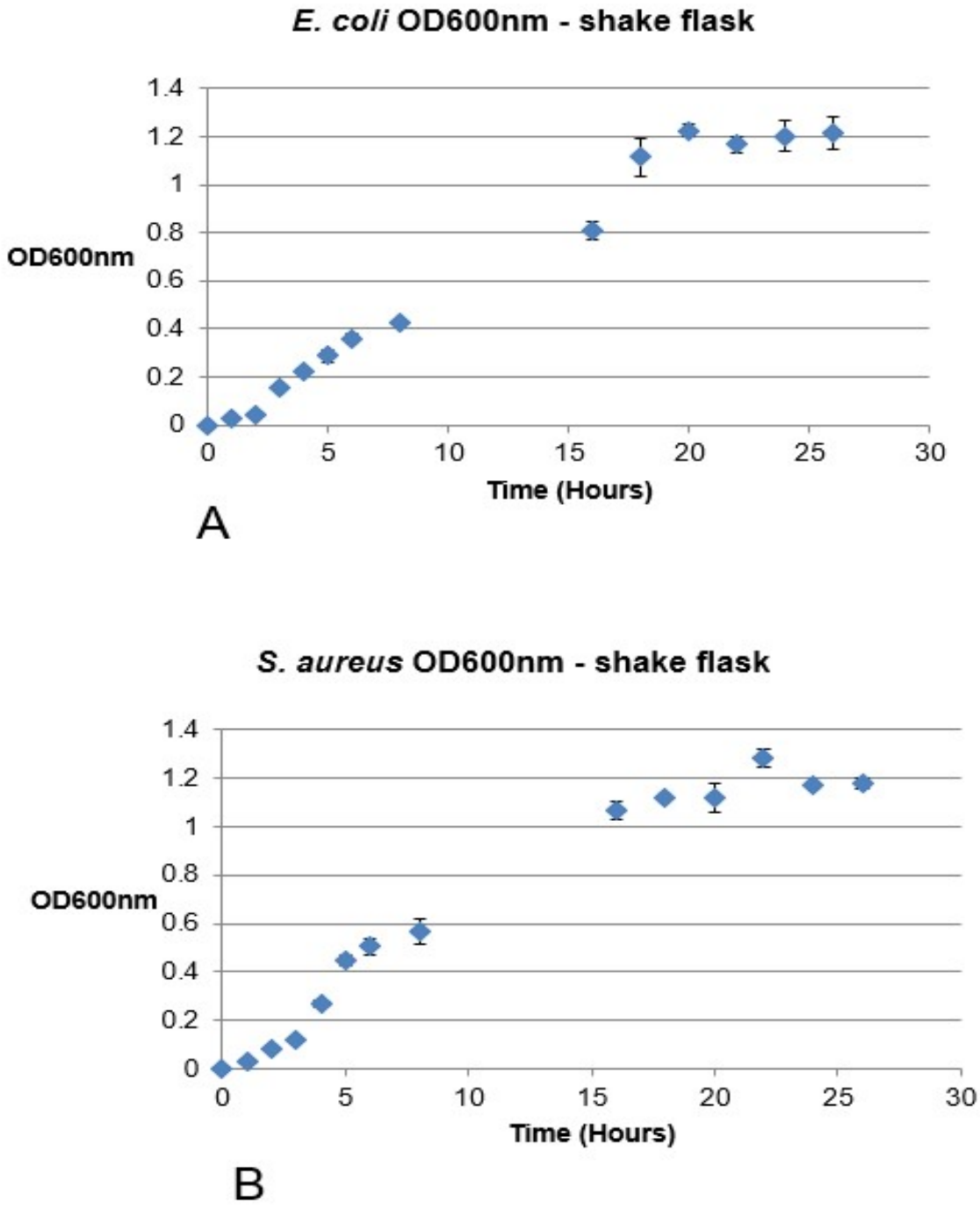



***Figure 2.1.*** *A growth curve of the host organisms in shake flasks over 24 hours. A single colony of bacteria was grown on appropriate agar overnight and picked using a sterile loop before inoculation into 20ml appropriate media. It was incubated at 37°C with appropriate agitation for 16 hours before being diluted to 0.05 OD600nm in 20ml fresh appropriate media. Agitation and incubation was then used and an optical density measurement taken every hour Error bars show 1 standard deviation of a triplicate experiment with triplicate optical density readings.*

### *2.2* Bacterial cell banking

Cells were frozen at -80°C in a 20% glycerol solution (v/v) which will allow them to be kept for longer as it acted as a cryoprotectant (Chan *et al*, 2013). Media was placed into a water bath at 37°C for at least 30 minutes and a single colony from an agar plate was suspended in 20ml appropriate media in a 125ml shake flask. The culture was incubated at 37°C shaking at 225rpm for around 8 hours to mid logarithmic phase. Glycerol was added to the solution to produce a final concentration of 20% and left for around 30 minutes at room temperature (20ml total volume) to allow cells to incorporate the glycerol then aliquoted into 1ml Eppendorf tubes and frozen at -80°C.

### *2.3* Host growth in a 5L bioreactor

Sartorius (Gottinger, Germany) glass bioreactors were seeded with 3 litres media and autoclaved at 121°C for 20 minutes. The vessels were seeded with 1% total volume (v/v) from an overnight culture. This ensured that the culture seeded was ~0.05 OD600nm. Optical density readings were taken when appropriate and the results are shown in figure 2.2. A 10ml sample was taken from the reactor and 1ml of that sample used in the optical density reading.

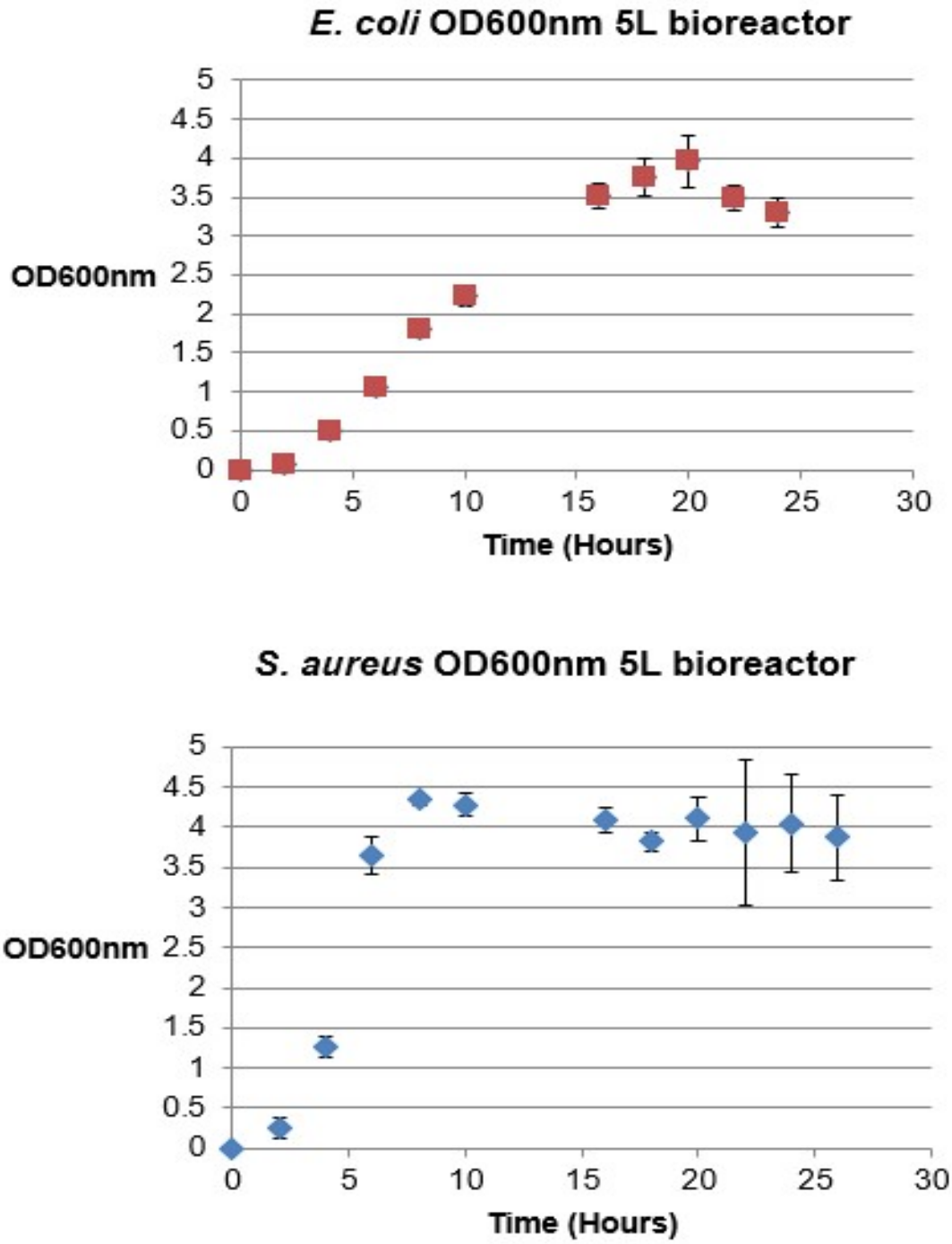
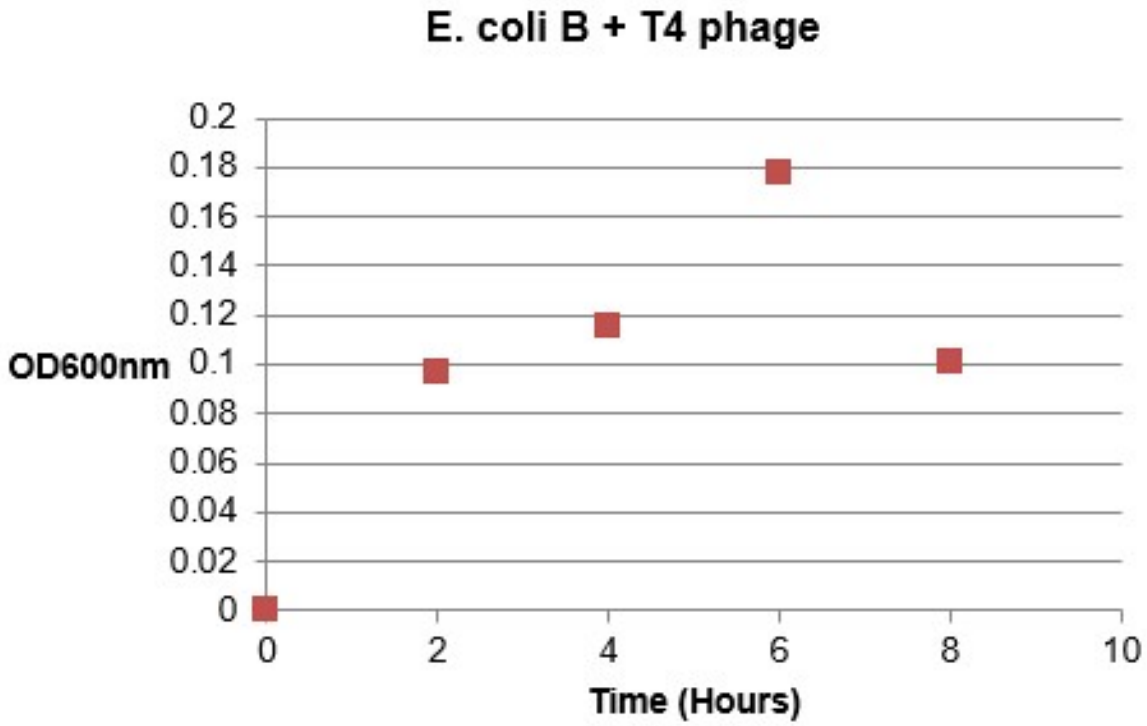



***Figure 2.2.*** *A growth curve of the host organisms in shake flasks over 24 hours in the 5L bioreactor. A single colony of bacteria was grown on appropriate agar overnight and picked using a sterile loop before inoculation into 30ml appropriate media. It was incubated at 37°C with appropriate agitation for 16 hours. The entire culture was inoculated into the bioreactor to maintain a 1% inoculation volume that allowed a starting optical density of roughly 0.05 OD600nm. Error bars show 1 standard deviation of a triplicate experiment with triplicate optical density readings.*

**2.4 Bacteriophage culture in a shake flask**

T4 phage (University of Reading) and phage K (ATCC 19685-B1) were used in all experiments. A single colony of host organism from a plate no more than 1 week old was inoculated in 20ml pre-warmed media and incubated at 37$^{o}$C, with appropriate agitation overnight with in a shake flask. In the morning the culture was diluted and added to fresh media in triplicate to produce an $OD_{600nm}$ 0.05. It was incubated at 37$^{o}$C until it reached $OD_{600nm}$ 0.25 with appropriate shaking. Then, phage was added and incubated at the given MOI, temperature and agitation speed. The optical density readings were taken every 2 hours and the results are shown in figure 2.3. Upon completion of the infection, the culture was centrifuged (Thermo Scientific Waltham, USA) at 4,600 x g for 10 minutes before the culture was filtered through a 0.22µm filter to obtain the phage. Fresh agar was prepared and left to cool.



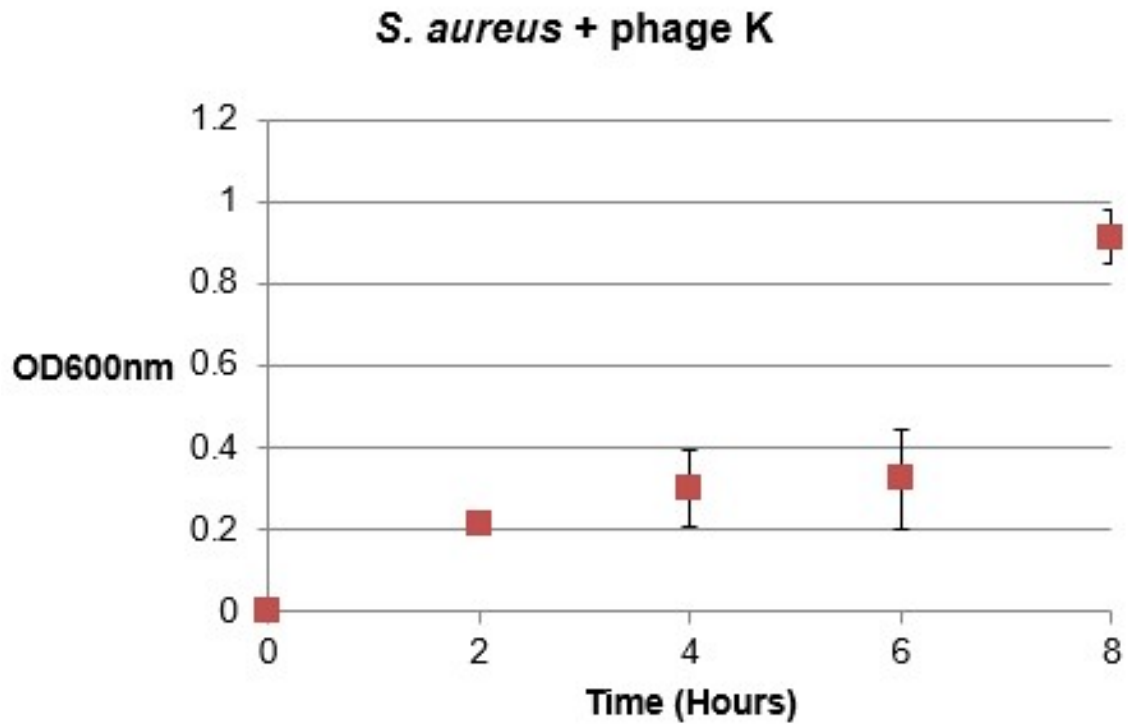




***Figure 2.3.*** *A growth curve of the host organisms with phage in shake flasks over 8 hours. Error bars show 1 standard deviation of a triplicate experiment with triplicate optical density readings.*

**2.5 Bacteriophage culture in a 5L bioreactor**

The bioreactor vessel was seeded with 3L LB media and autoclaved at 121°C for 20 minutes. The vessels were seeded with 1% total volume (v/v) from an overnight culture of host organism and grown at 37°c until it reaches $OD_{600nm}$ 0.25. Then, the temperature was reduced, where appropriate, with the water jacket and the culture infected with phage. Afterwards, a sample was taken and centrifuged at 4,600 x g for 10 minutes. The supernatant was filtered through a 0.22µm filter to obtain the phage. The optical density readings were taken every 2 hours and the results are shown in figure 2.4.

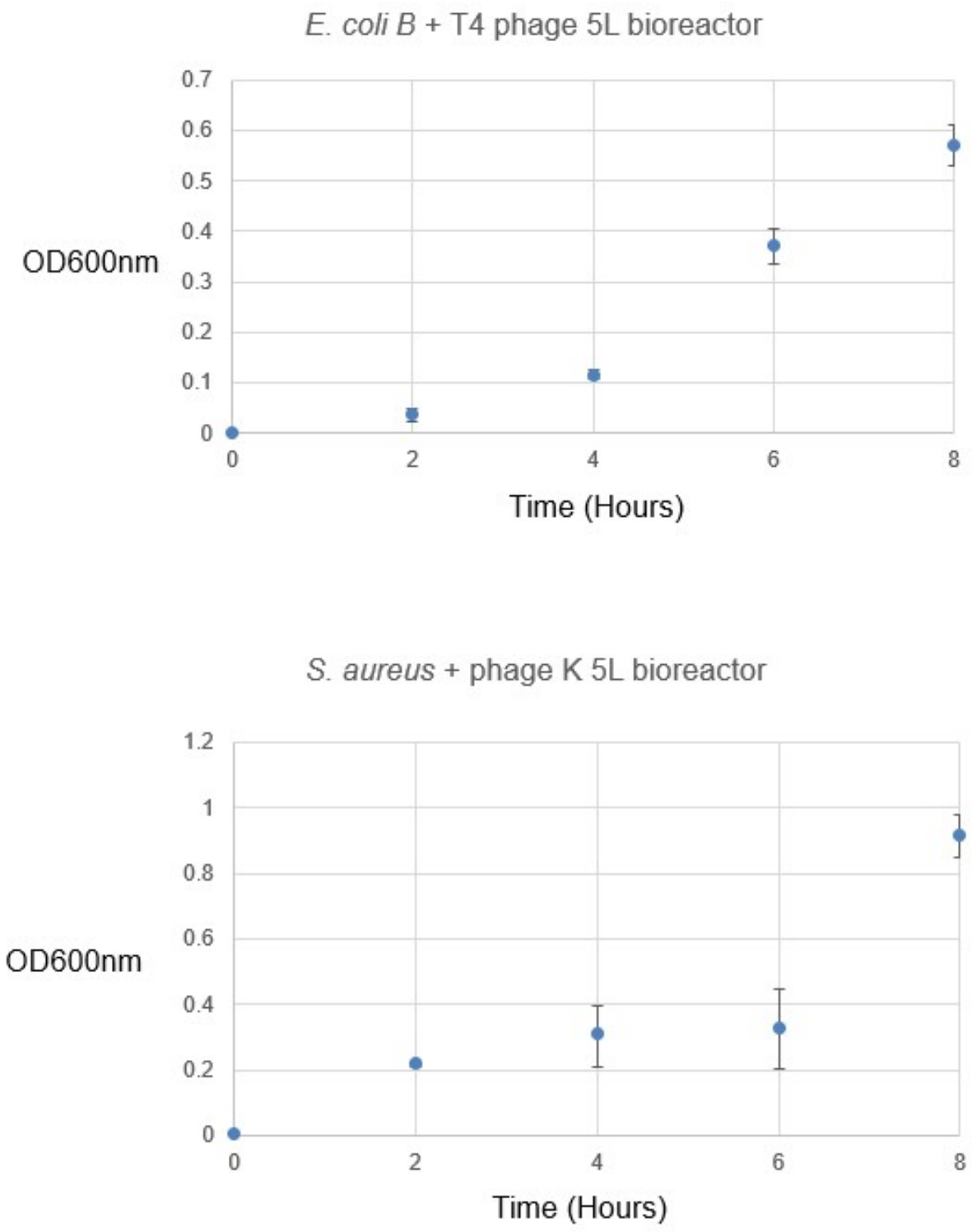



***Figure 2.4.*** *A growth curve of the host organisms with phage over 8 hours. Error bars show 1 standard deviation of a triplicate experiment with triplicate optical density readings.*

**2.6 Phage storage**

It is advised that phages are stored at 4°C; however, for long term storage, phages may be stored at -80°C in a 50% glycerol solution (Jończyk *et al*, 2011). Phages were left for 30 minutes on a bench at room temperature to incorporate the glycerol solution and then put into long term storage. Phage working banks were stored in SM buffer (Esteban *et al*, 2013).

**2.7 Phage concentration**

In order to reduce the total volume and make a concentrated sample PEG-8000, precipitation was done. For a 20ml sample of phage, 5ml of PEG was added to sample and stored at 4°C overnight. The following morning, the sample was centrifuged at 4,600g for 1 hour at room temperature and then the supernatant was decanted with the pellet in appropriate 1ml sterile medium. Phage precipitation occurs due to the addition of the PEG which allows the formation of a gradient whilst the DNA that makes up the phage will be attracted to each other due to the hydrophobic interactions occurring due to the PEG.

**2.8 Host organism and phage storage**

Once stocks of each had been propagated, bacterial agar plates were stored at 4°C for a maximum of one week. Long term storage of bacteria, supplemented with 20% glycerol, were stored at -80°C whilst phage stocks were stored at 4°C. Phage was supplemented with 50% glycerol solution. Glycerol was added to the samples and left for 30 minutes before several inversions to allow full incorporation of the glycerol.

**2.9 Plaque overlay assay**

Once a phage has been filtered through a 0.22µm filter, fresh agar was prepared and left to cool. Top agar (0.6% T4 phage, 0.7% phage K) was prepared and 3ml of an overnight re-suspended culture was added to 5ml of top agar. This mixture was overlaid on the bottom agar and left to cool. Once sufficiently cooled, 10µl of each dilution was spotted onto the plate which was left to incubate at 37°C overnight. The plates were examined for phage plaques. Figure 2.5 shows a schematic of the plaque overlay assay.

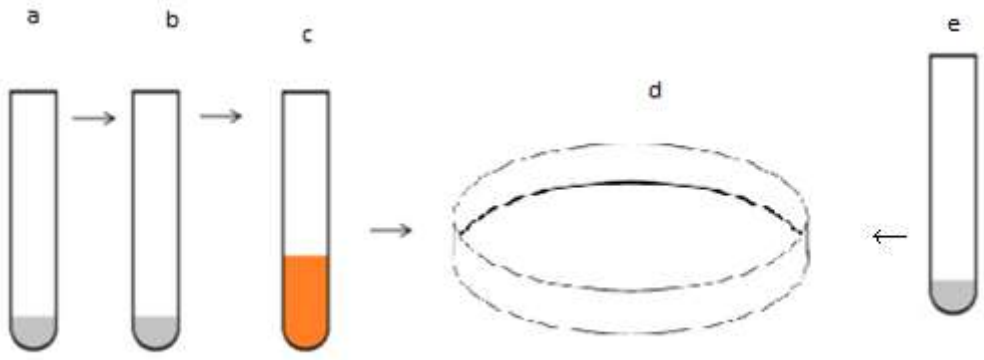



***Figure 2.5.*** *A figure to show the double agar overlay method. A – 3ml of a resuspended overnight culture of bacterial host. B – 5ml of top bacteriological agar. C – 8ml of overnight bacteria and top agar mixture. D – The sample from C is poured onto the bottom agar to make a plate with bottom agar and the top agar mixture. E – Once dried, 10µl of phage dilution is spotted onto the plate*

**2.10 Experimental design**

Minitab16 was used to create all DoE experiments. Full factorial experiments were created with different levels and conditions as seen within the respective organism's literature. The small-scale DoE consisted of a 4 factor 3 level DoE, see chapter 3 for specifics. The 5L DoE consisted of a 2 factor 3 level DoE, see chapter 4 for specifics.

**2.11 Adsorption analysis**

Sacrificial shake flasks were setup to perform the adsorption analysis. Each shake flask that was used was infected with phage and put into an incubator. Once the incubator doors were closed, the time was said to begin. Upon harvest of the cultures, a 1ml sample from the culture was taken and filtered using a 0.22µm filter. The sample was enumerated using the plaque assay.

**2.12 Burst size calculation**

At the point of infection, the flask was swirled by hand 3 times and a 1ml sample was taken and enumerated using the plaque assay. Then the culture was placed into an incubator and left for 30 minutes, after which a 1ml sample of the culture was filtered using a 0.22µm filter. The plaque assay was then performed to determine the increase in titre between the two cultures.

**2.13 Defined medium setup**

The defined medium used was similar to that of Sochocka *et al*, 2015. The medium consisted of 10g/L Hycase amino, 3.5g/L $K_2HPO_4$, 5g/L $Na_2SO_4$ and 1% glucose (v/v). The media was made up using the formulation and filter sterilised using a 0.22µm filter.

**2.14 Nanodrop analysis**

A sample from the shake flask or bioreactor was taken from the culture. This was then filtered using a 0.22µm filter and from that sample a 2µl sample was taken and run on a nanodrop 2000 (Thermo Fisher, Waltham, USA). The DNA/RNA/Protein content was measured through an optical density measurement. The measurement takes place by assessing the quantity of DNA/RNA/Protein in the sample which corresponds to a wavelength. A280 (280nm) was used as the wavelength of absorption given that the DNA and RNA will absorb light at this wavelength without causing mutations or affecting it. It has previously been shown that this wavelength is commonly used when measuring DNA quantity.

**2.15 Fermenter setup**

A 5L Sartorius bioreactor biostat B plus was used for all bioreactor experiments. A working volume of 3L was used for all experiments. Deionised water was used and placed into the bioreactor. Then, the appropriate amount of media powder was dissolved into the water. Agitation was used to help fully dissolve the media powder. Once dissolved, a 2 step pH calibration was performed using pH 7.0 and 9.21 buffer, the bioreactor was autoclaved at 121$^{o}$C for 20 minutes. Upon completion, the bioreactor was sparged with air for a 1 step $DO_2$ calibration. The bioreactor was then set to the required conditions. An image of the bioreactor is shown in figure 2.6.


***Figure 2.6.*** *An image to show the setup of the 5L bioreactor*

**2.16 Fatty acid analysis**

At the point of sample extraction, a 5ml sample was taken from the culture and filtered using a 0.22µmm filter. This was then directly used for GC-MS analysis. 100µl was mixed with an equal volume of MTBE and a 4% v/v of formic acid and 100 ppm of 2-ethylbutyric acid. The sample was shaken for 10 seconds by hand and left. An Agilent technologies (Santa Clara, USA) 17820A system fitted with a G4567A injector and a 5977B MSD quadrupole MS used. The column used was a silica fused stabilwax DA column. The injector was set to 250$^{o}$C. Purified helium was used as a carrier gas with a flow rate of 1.5ml/min used. Agilent technologies masshunter, version B.07.00 was used to analyse the data.

**2.17 Temperature reduction study**

Sacrificial shake flasks were setup in order to conduct the temperature reduction study. All cultures were cultured as set up in the standard way. At the point of infection, 10 individual shake flasks were infected with phage and placed into the appropriate incubator, shaking, static or room temperature. At the appropriate time points, a single shake flask was taken from the incubator, placed into the biological safety cabinet and standard thermometer was used to determine the temperature. For method 1, a digital readout was taken and recorded.

**2.18 Statistical analysis**

All statistical analysis was completed using either Minitab16 (State college PA, USA) or IBM SPSS 23 (Chicago, USA). Paired t-tests and ANOVAs were used to assess statistical significance. The appropriate statistical tests have been highlighted throughout. A P value $<0.05$ was considered to be statistically significant. 1 standard deviation was used throughout the body of work which was deemed more appropriate then standard error of the mean as it estimates how far the average population is from the mean whereas standard deviation shows the degree to which individual samples within the population differ from the mean. Standard deviation was shown using the ± in figures and tables.

# Chapter 3 - Small scale bacteriophage infection

**Chapter overview**

This chapter will examine input variables which may affect the bacteriophage bioprocess and alter the final harvest titre. The chapter aims to determine conditions which give the greatest titre and compare them to current literature titres. They will then be validated in shake flasks to determine reliability. The kinetics will be examined, adsorption and burst size, which may help to explain any improvements in titre seen. Additionally, a supplement and defined medium experiment will be run.

**3.1 Introduction**

Key process input variables (KPIV) and how they affect the key process outputs i.e. phage titre, phage harvest per input cell and phage harvest per input phage will be examined for T4 phage and phage K. Whilst the latter two values are not used currently within the literature, they provide a unified method for all phage biologists/fermentation scientists to show the productivity of a process as phage titre does not take into account the point of infection or the MOI used. The MOI, ratio of phage to bacteria, agitation (revolutions per minute) (RPM), temperature ($^{o}$C) and time of infection (hours) were investigated, as each may contribute to the final phage titre from a culture i.e each is a KPIV. Additionally, a DoE methodology will be followed in order to determine which factors, and levels can be used for high titre bacteriophage production.

Chapter 1 gave advantages of a DoE experimental design over OFAT experiments, but this chapter will focus on small-scale shake flask production with a 20ml culture volume. Experiments were completed for T4 phage targeting *E. coli* and phage K targeting *S. aureus*. T4 phage was chosen as it has been used clinically to treat humans. Although the phage against *S. aureus* was not reported, there have been multiple phage clinical trials targeting *S. aureus* and MRSA (Sarker *et al*, 2012, Clinicaltrials.gov 2019). This highlights the importance of this research as it aims to improve the bioprocess for current usable products.

Currently, there has been a limited amount of research into the phage bioprocess and there is a gap in the knowledge that must be bridged (Wünsche, 1989, Grieco *et al*, 2012). If phage therapy were to ever make it to large scale clinical trials and receive post-market approval, it would be vital that phage propagation and amplification is optimal to reduce wastefulness and improve productivity. Such experiments have been conducted thoroughly for other bioprocesses, such as microbial and stem cell manufacture (Van der Sanden *et*

*al*, 2010, Liu *et al*, 2017). However, there are few studies examining the phage bioprocess but the significant research being undertaken in phage therapy, which may one day replace antibiotics, highlights the importance for this research (Kutateladze *et al*, 2010, Allen *et al*, 2013, Modi *et al*, 2013, Yu *et al*, 2017). Additionally, due to the dearth of solutions that currently exist, phage therapy has been hailed as one of the potential outlets for treatments and therefore their propagation is of vital importance (Wünsche, 1989).

**3.2 Establishing key process input variables**

Initially, a literature review was carried out to examine the KPIV which are commonly used for T4 phage and phage K propagation. Tables 3.1 and 3.2 below shows variables for T4 phage and phage K propagation respectively which may contribute to the infection process and should be addressed. A 4 factor, 3 level full factorial DoE design was used. Using a wider range of conditions allowed a prediction to be made as to where the greatest titres could be achieved. The baseline process was run and the DoE experiment carried out to examine the effects of variables on phage propagation. The baseline conditions were taken from current processes in the literature and acted as a reference point throughout the experimental work. The overall aim of the experiment was to determine the conditions to be used in order to obtain the highest phage titre possible. Tables 3.1 and 3.2 show the variables used in the shake flask experimental design

**Table 3.1.** A table to show the factors and levels used in the small-scale T4 phage DoE. Baseline conditions; 37ºC, MOI 2.5, 225rpm, 3 hours

| Factor | Level |
|---|---|
| **Temperature (ºC)** | 20, 28, **37** |
| **MOI** | 1, **2.5**, 10 |
| **Agitation (RPM)** | 100, **225**, 400 |
| **Time of infection (Hours)** | 1, **3**, 6 |

**Table 3.2**. A table to show the factors and levels used in the small-scale phage K DoE. Baseline conditions; 37ºC, MOI 1, 150rpm, 8 hours

| Factor | Level |
|---|---|
| **Temperature (ºC)** | 20, 28, **37** |
| **MOI** | 0.1, **1**, 10 |
| **Agitation (RPM)** | 100, **150**, 200 |
| **Time of infection (Hours)** | 4, **8**, 16 |

The KPIV were investigated given that there is variation within the levels of each factor used for phage amplification in the literature. T4 phage has previously been propagated over a period of between 1 and 6 hours with agitation of 225rpm, commonly used for *E. coli* and T4 phage growth. Shaking the culture less vigorously i.e 100rpm may cause less shear damage, to the host or phage, and an increased chance of the phage infecting the bacteria as it may be easier for the phage-host attachment to form. However, using 400rpm, may allow for a greater degree of homogeneity within the culture of, pH, media constituents, oxygen and phage/host organisms. Maintenance of oxygen will aid in growth of the host organism for phage propagation. Additionally, the pH must be constant throughout the culture as if in one part it were to be higher/lower, the growth of organisms in that section of the culture flask may differ (Wunschel *et al*, 2005).

An additional factor that was examined was the temperature of infection. Whilst few have considered deviating from the commonly used 37$^{o}$C, it is a variable that may alter the infection and therefore must be considered. The significant research in bioprocessing and scale up in other industries has shown one factor which remains relatively constant throughout a variety of cell types: the temperature at which they grow. Although 37$^{o}$C is widely used for both mammalian cells and microorganisms, Grieco *et al*, 2012 showed in their paper that reducing the temperature may actually benefit the phage infection process and a greater phage titre produced at a reduced temperature. This was an interesting paper to consider and played a role in the development of this experiment.

All bioprocesses must be as efficient as possible especially if phage therapy is to become more prominent. Only a certain number of phage are needed for the initial infection as there is a limit as to the number of phage which can infect a bacteria. If too many phage infect the culture of host cells i.e. high MOI, this may ultimately result in a less efficient process due to the wastage of stock phage. Similarly, if the initial density of phage is too low, the time of the propagation of phage may be increased, i.e. increasing operator time. Therefore, it is important to determine an optimal MOI/time to improve overall efficiency (Kasman *et al*, 2002).

In 2009 Grieco *et al*, used M13, FL, FD and FT filamentous phage and aimed to maximise their yield in a 1L working volume in a 3L bioreactor by examining the pH and dissolved oxygen (DO %). The cultures were left for 10.75 hours, and their data showed that using NZY medium, a pH of 7.4 produced the greatest titres with 100% DO. They built on that work in 2012, where they used a filamentous phage infecting *E. coli* K91. NZY

medium was used with a 700ml working volume in a 3L bioreactor. They examined DO (%), pH and temperature for phage fermentation and found the maximum yield to occur at pH 6.9 at 28.1$^{o}$C whilst DO (%) had no significant effect on phage titre. Their paper highlighted the insignificant amount of investigations into key variables for phage production. Our study aims to initially examine other conditions which were not considered such as the optimisation of the MOI, agitation and time of infection.

Whilst a reduced temperature of infection has been relatively unexplored in bacteriophage bioprocessing, it has been explored in other areas of biologics. A temperature reduction may prevent cells from going through their natural growth cycle at a 'normal' pace, but may in fact be beneficial. Cells have been shown to prolong their life, producing more product at lower temperatures and lower cell death earlier. This may be because the reduced temperature allows the cells to maintain their metabolic activities for longer so that they will have a longer life span (Hoekstra *et al*, 2018). For Chinese hamster ovary cells, this paradox was first thought of over nearly half a century ago (Gerweck, 1977). This effect has also been seen for the production of protein using mammalian cells (Wang *et al*, 2018).

Here, three temperatures were used which were considered in order to clarify the results, that a lower temperature increases phage titre. Additionally, the agitation of infection was investigated. There has been an abundance of research examining factors such as mixing for both microbial and mammalian bioprocesses (Bajpai & Reuss 1982, Micheletti *et al*, 2006, Chang *et al*, 2017). However, this research is lacking for the phage bioprocess specifically. Differences in exact seeding volume and the time of the infection culture all contribute to the bioprocess and quality of the harvest product. Within the experiments, an effort was made to maintain constant factors where possible, such as the infection optical density, to standardise as much of the process as possible.

Within the phage literature it is common to use a point of infection between 0.2-0.4 $OD_{600m}$. However, keeping this constant may be key to improving the phage bioprocess and reducing variability within the study. In each experiment, an OD600nm 0.25 was used as the point of infection. Cultures above 0.28 OD600nm were diluted with media whilst cultures were only infected once they reached a minimum of 0.23 OD600nm.

### 3.3 Aims and objectives

1) Examine key input variables for phage culture and their effect on titre in shake flasks to establish a scale down model of culture
    a. It is important to investigate all of input variables that could affect the phage titre. Narrowing down the factors which contribute to the propagation of phage and growth of the host, will show the where the greatest titres are achieved
2) Validate conditions where greater phage titres can be achieved
    a. Once the greatest titres conditions are established, validation will be required to show reliability of the method for high phage titre production
3) Examine the culture at different temperatures at the point of phage infection
    a. By examining multiple temperatures, we will be able to show the true effect of temperature on phage propagation, given that previous studies have shown its influence on improving titre
4) Examine the adsorption and kinetics at baseline and greatest titre conditions
    a. Examining the kinetics will allow us to gain an insight into the causes of different achievable titres. They will be, the burst size and the rate of adsorption of phage to host cells.

**3.4 Small scale DoE**

Initially, it must be noted that a response surface methodology DoE was set up with the experiment run in full. However, upon completion of the experiment, it was found that the data was unable to fit the response surface model. Upon clarification with statisticians within the university, it was discussed that sometimes, the data produced is unable to fit specific DoE models. The experiment was made again using a full factorial design and run in full with response surface curves produced.

A full factorial DoE was created in minitab to examine the effect of each condition on the phage bioprocess. Given the high number of runs required, each experiment was run in singular with duplicate enumeration plaque assays. A 4 factor 3 level experiment was setup, using DoE this is denoted as $4^k$ or $4^3$ i.e. 4 factors and 3 levels. There are generally three types of experimental designs, comparative objective, screening objective or response surface objective. Examples of each include randomized design, full factorial design or central composite/box-behnken design respectively. Given that the aim of this study was to screen each of the conditions, a full factorial design was chosen.

The contour plots in figure 3.1 below show the design space for the conditions to achieve the greatest T4 phage titre in shake flasks i.e > 5 x $10^{11}$ pfu/ml. Based on all of the data from the DoE experiment, contour plots make predictions where the optimal levels lie for the greatest titre, one advantage of DoE over one factor at a time (OFAT) experiments. Additionally, running the DoE meant that some experiments could be run in parallel, saving time, highlighting the practical advantage of DoE over OFAT experiments.

An MOI 2.5, 225rpm, 3 hours of infection at 28$^o$C gave the greatest titre of T4 phage (2.45 x $10^{13}$ ± 3.5 x $10^{12}$ pfu/ml). Compared to the baseline conditions, MOI 2.5, 225rpm, 3 hours of infection at 37$^o$C, this was a significant improvement when using a paired t-test in phage titre, 4.5 x $10^{10}$ ± 5 x $10^9$ pfu/ml, p=0.0199, and represents around a 500-fold increase in phage titre.

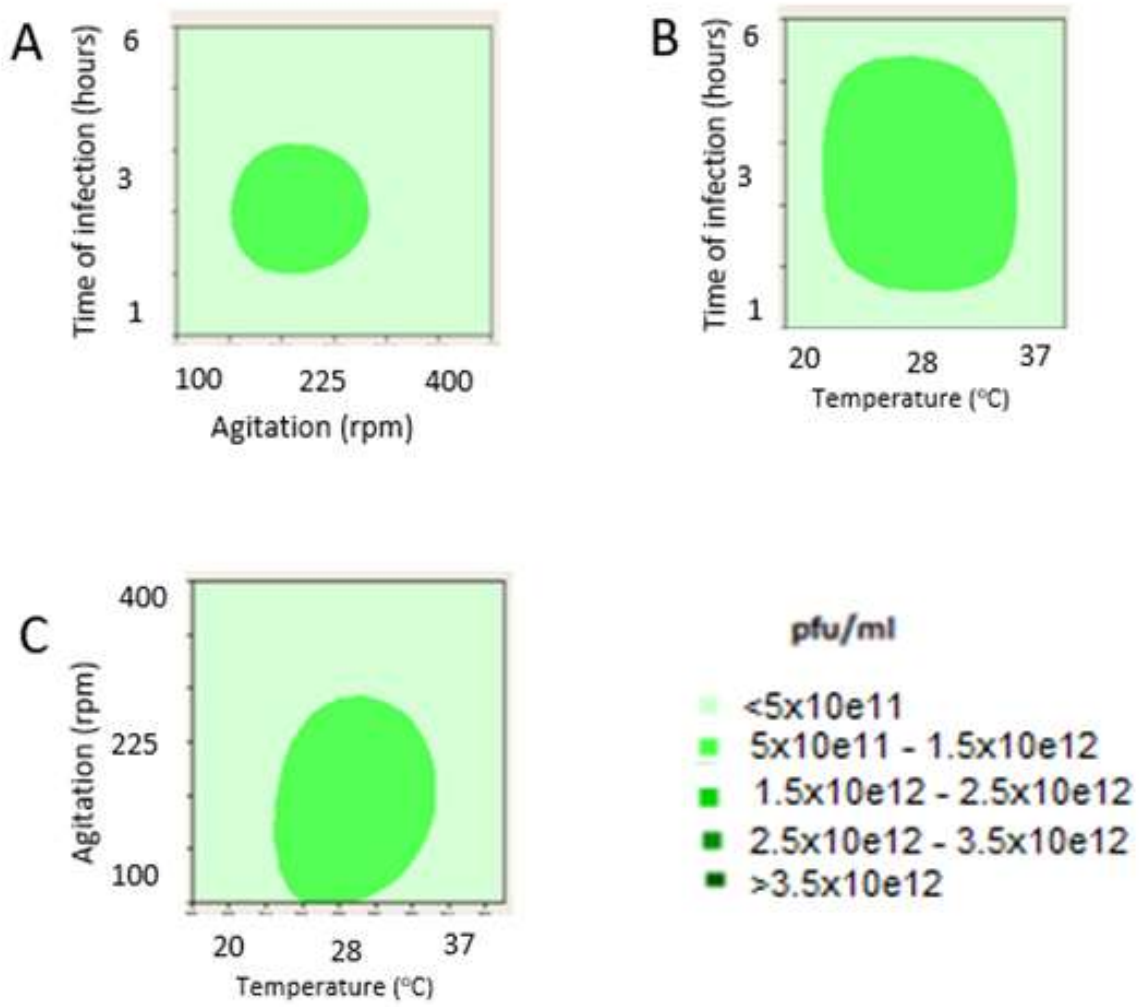



***Figure 3.1.*** *Contour plots which indicates the optimal parameters for the T4 phage infection to produce the greatest titre from the DoE experiment created in Minitab16. Dark green areas represent the level of each conditions where the greatest titre will be produced. The DoE consisted of 81 runs, each were run as singular runs with each experiment enumerated with duplicate plaque assays.* ***a.*** *The conditions where the greatest T4 phage titre can be harvested between infection time (hours) and agitation of infection (rpm)* ***b.*** *The conditions where the greatest T4 phage titre can be harvested between time of infection (hours) and temperature of infection (°C)* ***c.*** *The conditions where the greatest T4 phage titre can be harvested between temperature of infection (°C) and agitation (RPM).*

The contour plots in figure 3.1 predict the levels of each condition used to achieve the greatest T4 phage titres. The darker area indicates the levels where titres achieve > 5 x $10^{11}$ pfu/ml and estimate where similar levels of titres will be achieved. For example, agitation levels of 100rpm, 225rpm and 400rpm were used. Figure 3.1c shows when the infection takes place at 28$^{o}$C, an agitation of 100 to 275rpm may result in a titre of > 5x$10^{11}$ pfu/ml. However, this would need to be validated to confirm the titre achievable. Additionally, it must be noted that these were from a singular experiment, with duplicate plaque assay measurements. Interestingly, figure 3.1a and 3.1c show different levels of agitation that could be used to achieved high T4 phage titres in shake flasks. Figure 3.1a shows that no less than 150rpm can be used whilst 3.1c shows it may be possible to reach the titres at 100rpm. Each of the contour plots must be cross compared, with additional experimental validation runs, before an accurate prediction of conditions to give high titres can be made.

To determine if the same methodology could be applied to another phage, phage K targeting *S. aureus*, was next examined (Nadaf *et al*, 2018). It was important to show that this methodology could be effective for a separate organism to firstly validate the method but also for future studies that may wish to use this methodology. Although specifically phage K hasn't been used in clinical trials, there have been a handful of clinical trials that have used phage therapy to tackle *S. aureus* and MRSA with the name of the phage not given (Clinicaltrials.gov 2019).

Again, contour plots were produced for the small-scale phage K DoE. To keep consistency between experiments, the point of infection was kept at 0.25 OD600nm similar to the T4/*E. coli* B experiments. It was important to maintain consistency within the bioprocess as even minor variations may impact final titre as well as quality, a criticism of current phage fermentation work. The contour plots in figure 3.2 below show the design space that can be used to obtain the greatest titres from the phage K infection.

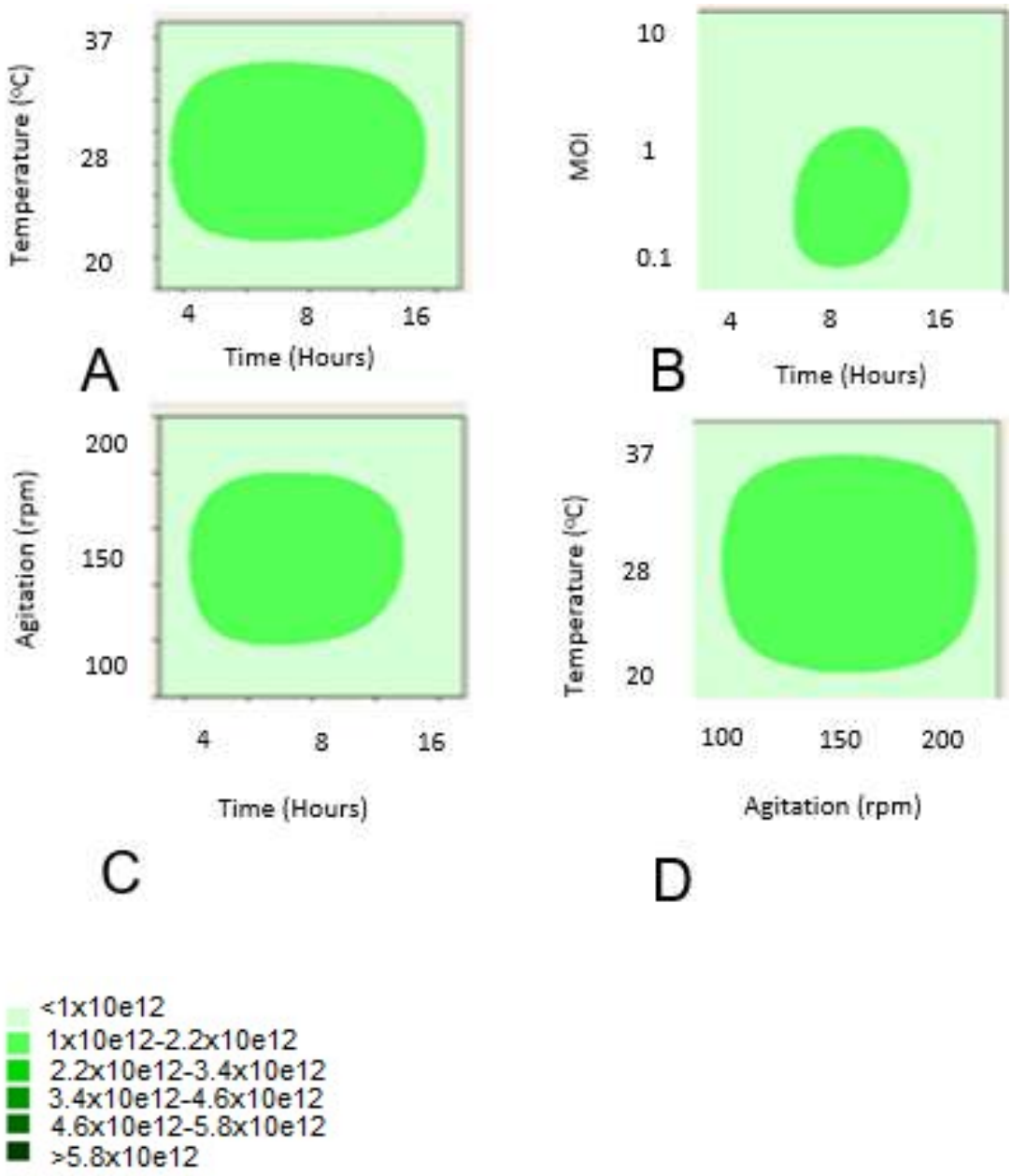



***Figure 3.2.*** *Contour plots which indicates the optimal parameters for the phage K infection to produce the greatest titre from the DoE experiment created in Minitab16. Dark green areas represent the level of each conditions where the greatest titre will be produced. The DoE consisted of 81 runs, each were run as singular runs with each experiment enumerated with duplicate plaque assays.* ***a.*** *The conditions where the greatest phage K titre can be harvested between infection time (hours) and temperature (°C)* ***b.*** *The conditions where the greatest phage K titre can be harvested between time of infection (hours) and MOI* ***c.*** *The conditions where the greatest phage K titre can be harvested between time (hours) and agitation (RPM)* ***d.*** *The conditions where the greatest phage K titre can be harvested between temperature (°C) and agitation (RPM).*

The data showed that the greatest phage K titre was obtained at 28°C. Given that two other temperatures of infection were used, 20°C and 37°C, the plots were able to predict that between 24°C and 32°C, similar titres could be obtained. However, without further validation runs, it would be unwise to assume that these temperatures could produce similar titres. Similar to the T4 phage experiment, the phage K data suggested that infection and propagation is more effective at lower temperatures and adds further weight to the argument that phage propagate to a greater titre at a lower temperature of infection than 37°C. It would be unwise to assume this is the same for all phage as different phage can act differently. Therefore, similar experiments would be needed for different phage, to determine their optimal temperatures of infection for high phage titre.

The contour plot suggested that the greatest phage K titre is achieved at 150rpm. Again, figure 3.2 shows that similar titres could be produced when using agitation speeds of 120-180 rpm. It is known that *S. aureus* is usually agitated at a lower rate than *E. coli*, however, mixing is still a critical factor for the optimal host growth. Due to a lack of significance in titres achieved between each of the MOI values, it was difficult to determine the optimal MOI. A further investigation of the phage K MOI was conducted, presented later in this chapter.

The time of infection was also investigated and the greatest phage K titre was achieved at 8 hours of infection. Longer agitation times were shown to have no improvement on the phage titre, which may be due to the phage entering the lysogenic cycle or attaching onto host debris. This is to preserve themselves or will mean that it can not be detected after the filtration step respectively (Hyman, 2019). In the phage K experiments, 4 and 16 hours of infection were used as the extreme values. Interestingly, figures 3.2b and 3.2c give a different prediction for the optimal time of infection. Figure 3.2b estimated that 8 hours whilst figure 3.2c estimated that 4 hours is the minimum time of infection that could be used to achieve the high titres. This may be interesting to further investigate, as reducing the time of infection from 8 hours to 4 hours would represent a 50% time saving. An 8-hour infection time with the setup and harvest would represent a whole working day; however, saving 50% of that time may be beneficial for the operator and consequently the cost of the process.

One study in 2018 used a similar methodology using a response surface in order to examine the optimal conditions for vB_SauM-phiPLA-RODI phage propagation against *S. xylosus*. Interestingly their results showed that 38°C and 135rpm gave the greatest titre. Whilst the phage used can infect *S. aureus*, their study chose to

use *Staphylococcal xylosus* as the host organism to show the host range (González-Menéndez *et al*, 2018). However, their study gave no indication as to the optimal time of infection. The time is a crucial aspect for improving efficiency of a bioprocess.

Within the T4 phage DoE, the greatest titre was found at 225rpm whilst the greatest titre for phage K was 150rpm in a shake flask. The effect of mixing can have several consequences which may improve or reduce phage titre. A greater agitation rate may cause shear damage to the host cells or phage, which may have a two-fold consequence (Wang *et al*, 2007). Firstly, this may damage or kill host cells available for infection thereby limiting the number of bacteria available for phage propagation. Secondly, the high agitation rate may also damage the phage virus which then can not be detected at harvest in the plaque assay. However, it may also improve the chance of the phage-host interaction. A lower rate of mixing may prevent optimal growth of host-cells causing fewer host cells for phage to infect. This may also cause a degree of non-homogeneity within the culture and lack of oxygen or differences in pH throughout the culture whilst preventing shear damage to both phage and host cells could affect the final titre (Liu *et al*, 2010). The level of oxygen, pH, bacteria and phage must be uniform throughout the culture and a lower level of mixing may prevent this.

Small scale experiments using shake flasks are relatively simple, cheap, easy as well as taking minimal time and effort to setup, allowing more experiments. However, when considering scale up manufacture, one must also consider the time involved to set up a larger stirred tank bioreactor. Setting up a stirred tank bioreactor can take significantly longer than shake flasks with each of the cleaning, sterilisation, calibration steps and down time needed to prepare a bioreactor to begin further experiments as well as seeding, which requires an overnight culture of host cells. By reducing the time of infection, the overall time of batch to batch processes will be reduced. A bioreactor culture model will be examined in further detail in chapter 4.

It has previously been documented that at MOI 4.6, 99% of the host cells will be infected with T4 phage, therefore, adding in additional phage to the infection process may not be beneficial, resulting in a wasteful process (ScienceGateway, 2018). A MOI that was too low initially may prevent enough phage from initially infecting the host cells and therefore the rate of host cell growth could be greater than phage propagation. This may result in an irreversible process, there would be a greater number of bacteria than phage and a culture with an OD600nm outside of the optimal range for phage infection. Furthermore, the bacterial population would be

following their natural growth phase becoming more difficult to infect and less likely to propagate phage (Millard *et al*, 2011). Moreover, a denser population of host organisms would likely prevent optimal infection rates of host cultures given the availability of phage (Kasman *et al*, 2002, Kick *et al*, 2016). Alternatively, an MOI that was too high, would mean that there were free phage within the culture. This will cause stock wastage and increase the cost per phage whilst also reducing phage output per input phage. Although stock wastage currently may not be of great importance, if research continues to increase it will become more important in the future. It is therefore important to use an appropriate MOI to ensure these situations do not occur.

Next, the time of infection was investigated to determine the time of infection that gave the greatest titre. It is known that if phage are left in the culture to infect for too long, they may revert to their lysogenic cycle. When the MOI gets too high after the phage have lysed the host cells, they naturally preserve themselves through the lysogenic cycle. They can also attach onto the bacterial debris or lysed host cells. Binding is still possible as the receptors on the host may be intact, even if the cells are not, reducing the total number of phage able to be harvested (Storms *et al*, 2014). However, by not leaving the infection for an appropriate time, the phage may not be able to propagate to a sufficiently high level. The contour plot in figure 3.1 estimates, that a similar titre of phage could be produced after 2 hours compared to 3 hours for T4 phage whilst figure 3.2C shows phage K achieved titres >1 x $10^{12}$ pfu/ml from 4-15 hours. If the T4 phage process were to be run at 2 hours, compared to 3 hours, this would represent a 33% saving in time, compared to the 3-hour process used in this experiment. This would allow the operator to harvest the product and setup further shake flask experiments, resulting in better equipment utilisation and improved resource management.

A DoE methodology can produce vast amounts of information based on the parameters/levels used. It may be worthwhile others conducting similar experiments for their phage to learn about specific phage bioprocesses. As with the T4 phage infection, the greatest phage K titre was achieved at a temperature of infection of 28$^{o}$C. However, unlike with T4 phage, statistically significant differences were found between the phage K titres based on the temperature of infection, 20$^{o}$C - 28$^{o}$C p=0.004, 20$^{o}$C - 37$^{o}$C p=0.028 and 28$^{o}$C - 37$^{o}$C p=0.014 using a paired t-test.

It may be useful in future to consider the energy needed for the shaking incubator for different agitation rates. Reducing the power and energy used by a shaking incubator may further reduce cost, although this may

only be beneficial if experiments were run over a long period of time. Moreover, for larger stirred tank bioreactors, this may be more of a concern, given the energy cost of power input needed. An insight is given towards the cost per phage is examined later in this thesis based on media cost.

The temperature of infection was an interesting variable to consider, as there are very few studies that have ever examined a lower temperature of infection for phage. However, the results by Grieco (2012) showed that it was an important factor whilst the data presented in figures 3.1 and 3.2 shows a greater titre of phage were produced at 28$^{o}$C compared to 37$^{o}$C for T4 and phage K. It is hoped that this study will add further weight to the current knowledge of phage infection, and that future research will be tailored towards a reduced temperature of infection. Further experimental work presented later in this chapter examines in greater detail the effect of temperature on titre for both phage. Additionally, it may be more beneficial for others to infect different phage at a lower temperature as a greater titre may be produced, allowing phage stocks to last longer in both clinical and academic research.

Grieco was unable to provide a hypothesis as to the reduction in phage temperature, and the resulting improvement in phage titre. Based on the phage mechanism and nature of bacteriophage, slowing the reaction rate with a reduced temperature of infection may allow the DNA to integrate more efficiently therefore producing more phage. This could be tested by sequencing phage DNA either side of two infections, one at 37$^{o}$C and another at 28$^{o}$C. If the temperature was really to have this improved effect and the DNA integration is more efficient at lower temperatures, then there should be a greater similarity in the sequence of the 28$^{o}$C infection temperature compared to the 37$^{o}$C infection temperature. Alternatively, Hadas *et al* 1997 revealed that by preventing the replication of the host organism but allowing it to grow larger, there would be more binding receptors for the phage which would allow more phage to be produced and increase overall phage titre. It has been shown that using *E. coli*, lower temperatures may increase specific glycolysis pathways and other glyconeogenic pathways which may have an effect on the phage propagation (Vasina & Baneyx, 1997, Cruz et al, 2012). Whilst protein production using mammalian cells have shown to increase at lower temperatures, some of the mechanisms may also be applicable in phage bioprocessing. Moreover, thermolabile proteins may also increase metabolism which may be more upregulated at lower temperatures. One study showed that CI857, a thermolabile protein, that is a phage lambda promoter and a regulator of phage propagation in *E. coli*, which may also contribute to T4 propagation (Valdez-cruz et al, 2010).

**3.5 Initial point of infection**

Whilst it is well known that point of infection should be ~0.2-0.4 OD600nm, this work made a conscious effort to maintain, to as great a degree as possible, the point of infection (Bryan *et al*, 2016, González-Menéndez *et al* 2018). Previous studies have indicated that host cells may need to be at a certain density, or metabolic state, for phage propagation to occur optimally (Kasman *et al*, 2002). Dufour *et al* infected at an interestingly low OD600nm, 0.015. Whilst their study aimed to investigate the killing of host cells *E. coli 536*, they wanted to investigate the effect of a low point of infection which saw a reduction in release of endotoxins i.e to use phage early in bacterial treatment (Dufour *et al*, 2017).

The graph in figure 3.3 shows that 0.25 OD600nm gave the greatest phage titre for both T4 and phage K using MOI 2.5, 225rpm, 3 hours, 28$^{o}$C and MOI 0.1, 150rpm, 4 hours and 28$^{o}$C respectively. An infection point of 0.25 OD600nm gave a significantly increased T4 phage and phage K titre compared to 0.3 OD600nm and 0.2OD600nm, $p<0.001$ and $p<0.003$, and $p<0.004$ and $p<0.004$ respectively using a paired t-test. The burst size values are given above each bar. Each of the burst sizes are relative to the final harvest values i.e the greatest burst was seen at 0.25 OD600nm where the greatest harvest titre was seen. The improvement in titre between 0.05 and 1 OD600nm may be due to there being more or less bacteria than required, affecting the phage ability to propagate. At 0.05 OD600nm, there may not be enough phage able to infect the host organisms and therefore the burst will be reduced. At 1 OD600nm, there may be too many host organisms, lowering the number of phage infecting a bacterium, therefore further reducing the number of phage seen in the burst. Additionally, the host cells may not be in the optimal metabolic state for propagation. The cultures were taken from a diluted overnight culture to 0.05 OD600nm and grown to the required optical density then infected with phage.

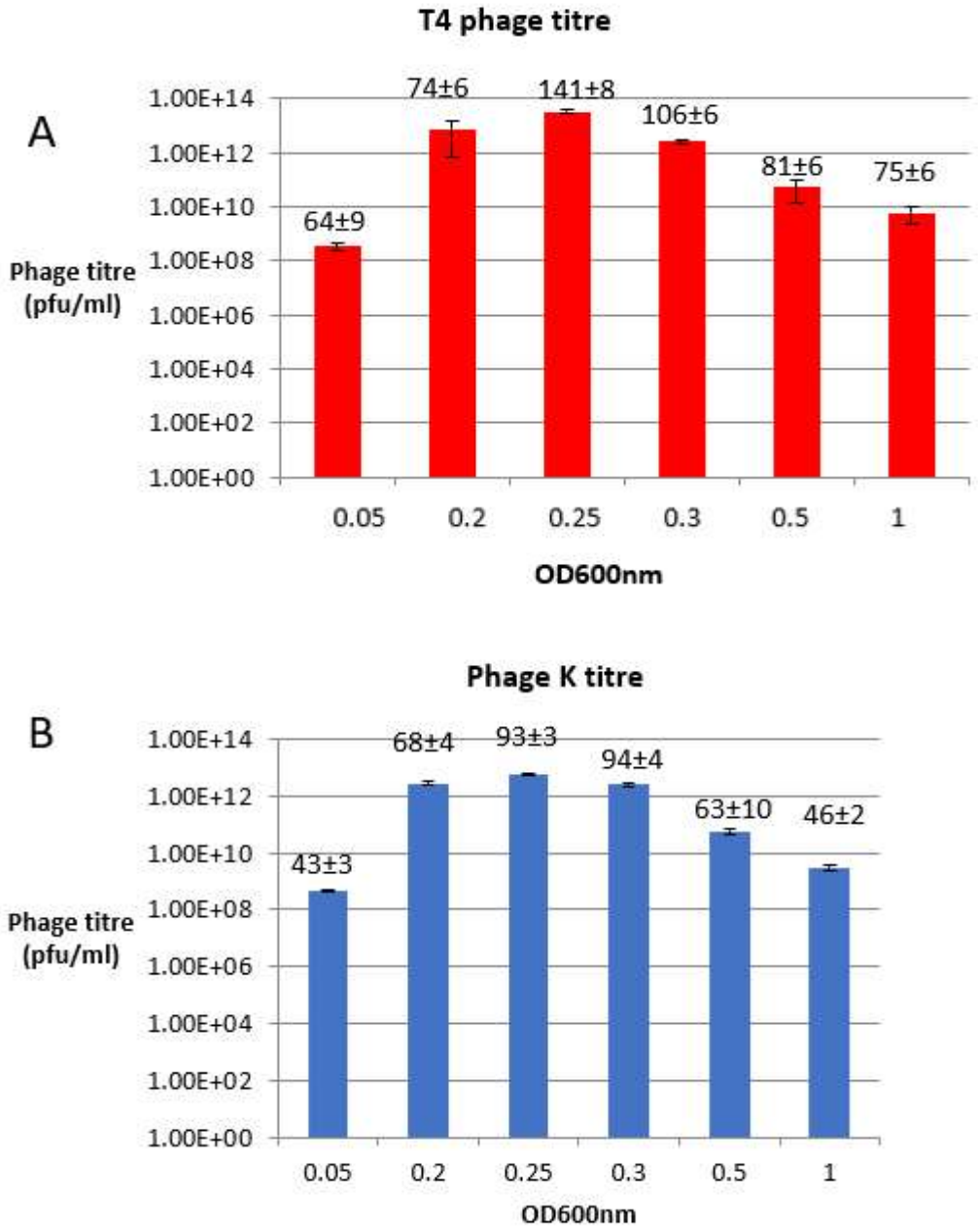


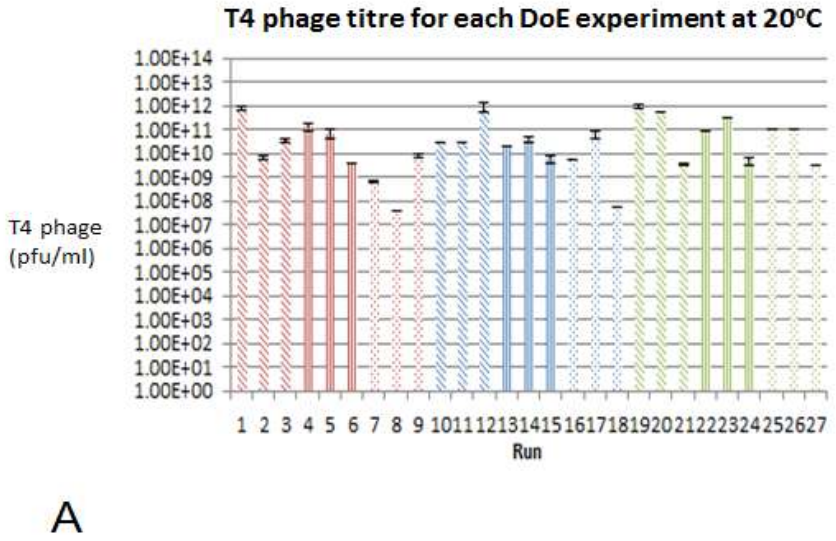

***Figure 3.3.*** *A bar chart to show the phage titres achieved when the point of infection was changed whilst using the greatest titre conditions for A) T4 phage B) Phage K. Error bars show mean ± 1 standard deviation of a duplicate experiment with each experiment enumerated with duplicate plaque assays. Above the bars show the burst sizes achieved with 1 standard deviation of a duplicate experiment with a single burst size measurement. Burst size measurements were calculated based on the initial number of phage that infects the culture, and the number of phage 30 minutes after the infection. 0.25 OD600nm gave the significantly greatest titre compared to each of the other points of infection used whilst it also gave the greatest harvest titre using a paired t-test.*

There has been very little previous research into the point at which bacteriophage infection takes place and even less work examining the effect on titre and burst. However, it is known it should take place when the host cells are in the early log phase. Phillipe *et al* (2018) used an infection point of 0.2-0.3OD600nm with a GC1 phage. Whilst this is still a wide window of infection, it is an improvement in the window of phage infection that many authors use (0.2-0.4 OD600nm) whilst some do not state the point of infection. A second study in 2018 was one of the first to examine the effect on titre of the point of infection. Using a vB_SauM-phiIPLA-RODI phage on *Staphylococcus Xylosus*, they showed that infection at log 7.39 bacteria gave the greatest titre and burst of the phage, significantly higher than at an infection point of log 8.79. This was an important study as they showed the effect that the point of infection can have on titre. However, it may be more beneficial to use bacterial counts as the point of infection, as the optical density can differ based on the host organism. This is because OD is dependent on the ability of light to pass through a culture i.e larger organisms will give a greater OD but have a lower count (González-Menéndez *et al*, 2018). It would be beneficial for future phage bioprocesses to carry out a similar study for other phage to this to show the point of infection where the greatest titres can be produced.

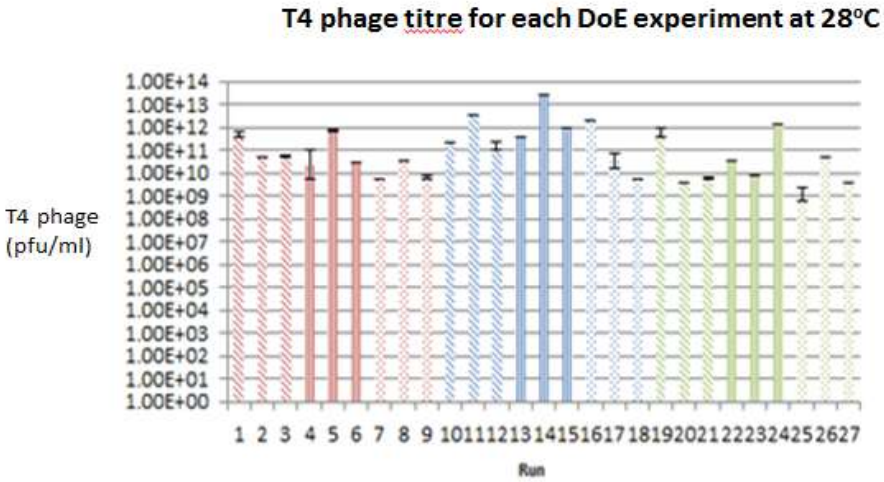
### 3.6 Full factorial DoE study analysis

The bar graphs below show each run for the DoE experiment against the phage titre. Whilst the contour plot is a useful analysis, it can be useful to examine the full data set. Although there was no significance within each of the levels of each KPIV, it is important to note that using certain conditions may give higher titres. Each bar represents one run from the DoE which was enumerated with duplicate plaque assays.

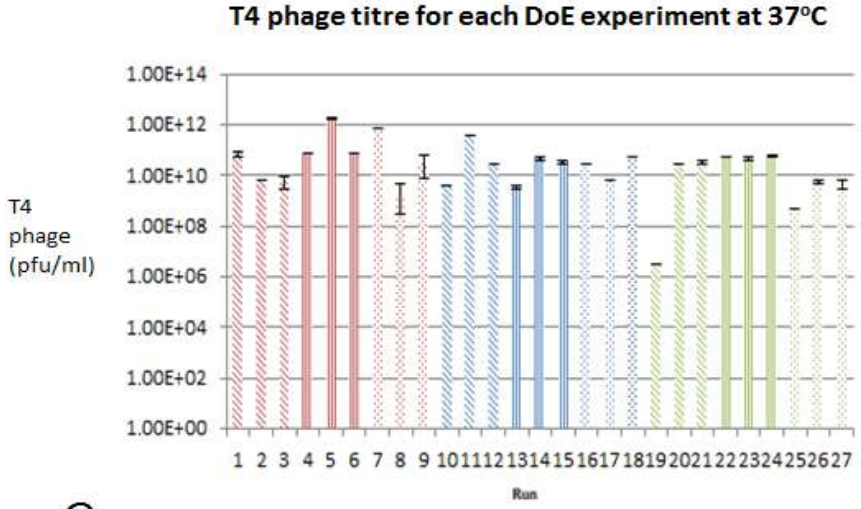
#### 3.6.1 Full factorial DoE analysis for T4 phage infection


T4 phage titre for each DoE experiment at 20°C
T4 phage (pfu/ml)
1.00E+14
1.00E+13
1.00E+12
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Run

A


T4 phage titre for each DoE experiment at 28°C
T4 phage (pfu/ml)
1.00E+14
1.00E+13
1.00E+12
1.00E+11
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Run

B


T4 phage titre for each DoE experiment at 37°C
T4 phage (pfu/ml)
1.00E+14
1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
1.00E+02
1.00E+00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Run

C

1) 1, 100rpm, 1 hour
2) 1, 225rpm, 1 hour
3) 1, 400rpm, 1 hour
4) 1, 100rpm, 3 hours
5) 1, 225rpm, 3 hours
6) 1, 400rpm, 3 hours
7) 1, 100rpm, 6 hours
8) 1, 225rpm, 6 hours
9) 1, 400rpm, 6 hours
10) 2.5, 100rpm, 1 hour
11) 2.5, 225rpm, 1 hour
12) 2.5, 400rpm, 1 hour
13) 2.5, 100rpm, 3 hours
14) 2.5, 225rpm, 3 hours
15) 2.5, 400rpm, 3 hours
16) 2.5, 100rpm, 6 hours
17) 2.5, 225rpm, 6 hours
18) 2.5, 400rpm, 6 hours
19) 10, 100rpm, 1 hour
20) 10, 225rpm, 1 hour
21) 10, 400rpm, 1 hour
22) 10, 100rpm, 3 hours
23) 10, 225rpm, 3 hours
24) 10, 400rpm, 3 hours
25) 10, 100rpm, 6 hours
26) 10, 225rpm, 6 hours
27) 10, 400rpm, 6 hours

**D**

***Figure 3.4****. A bar chart to show T4 phage titre achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where an MOI 1 was used, blue bars show the conditions where an MOI 2.5 was used and green bars show where an MOI 10 was used. The first 3 bars in each set show where a time of 1 hour was used, the second set of 3 bars show where a time of 3 hours was used and the final 3 bars in each set show where a time of 6 hours was used as a time of infection.*

Figure 3.4 shows the titres achieved by the different conditions used in the T4 phage DoE. The graphs show that at 20°C, 8 runs achieved >1e11pfu/ml. However, 11 runs were able to achieve this titre at 28°C and 3 runs at 37°C. Throughout the literature, the most common temperature of infection is 37°C; however this data further suggests that by reducing the temperature, the average T4 phage titre may be increased. They back up the contour plots showing that 28°C is more effective for phage propagation than 37°C.

At MOI 10 (all green bars) only 2 runs achieve >1e12 pfu/ml. Therefore, adding a more phage into the initial infection did not improve the phage titre as within the culture there are a finite number of bacteria that can be infected. As it has been alluded, at MOI >4.6, 99% of host cells will be infected and therefore, there may be free phage within the culture. Interestingly, when MOI 10 was used, the data shows that 14 runs reached a titre of 1e10pfu/ml compared with 19 runs at MOI 2.5 and 17 runs at MOI 1. Brown and Bidle, 2014 explained the negative feedback within cultures. Using a high MOI, phage may be unable to complete their replication cycle, limiting the number of host cells infected whilst prevent host lysis, showing a two-fold mechanism for the reduction in final phage titre. The high MOI may also diminish viral production and cause defective viruses or no viral progeny further limiting the yield of phage. Graphs were based on the temperature and MOI of infection. Similar graphs could be produced in further investigation was needed into the agitation or time of infection.

Next, it was important to determine the number of phage per host cells at the point of infection. As has been seen previously, there are a wide variety of infection conditions at which the infection takes place and the point of infection is somewhat varied within the literature. The most common point of infection for phage is an optical density of 0.2-0.4 OD600nm which represents a range of around $2x10^8$ cfu/ml. It was important in this study to keep a constant point of infection which may lead to a differing phage titre as there would be more or less cells to initially infect. This experiment used an infection point of 0.25 OD600nm given the data in figure 3.3. The graphs in appendix 1 show the T4 phage harvest per input cell and T4 phage harvest per input phage.

The bar charts from the DoE results showed that that using certain conditions, there could be an improvement in phage titre and therefore phage harvest per input cell and phage harvest per input phage. The conditions, which gave the greatest T4 phage titre also gave the greatest phage per input cell and phage harvest per input phage. Temperature seemed to play an interesting role thus far whilst it is the most uncommonnly studied parameter in phage propagation. Therefore, the conditions which gave the greatest titre were used for

the T4 phage infection over multiple temperatures of infection; MOI 2.5, 225rpm at 3 hours infection which will be examined in further detail for the effect of temperature on the bioprocess shown later in this chapter. The data does, however, show a wide variability, and therefore the bioprocess is dependant on a multitude of factors, each must be optimised to obtain the greatest overall titre.

Graphs A1, A2, A3 and A4 shown in appendix 2 show the phage per input cell and phage per input phage for T4 phage and phage K respectively. The graphs in figure 3.7 show the full phage k DoE experiment, similar to the T4 DoE. Each experiment was a single run enumerated with duplicate plaque assays. Error bars show 1 standard deviation.

### 3.6.2 Full factorial DoE analysis for phage K

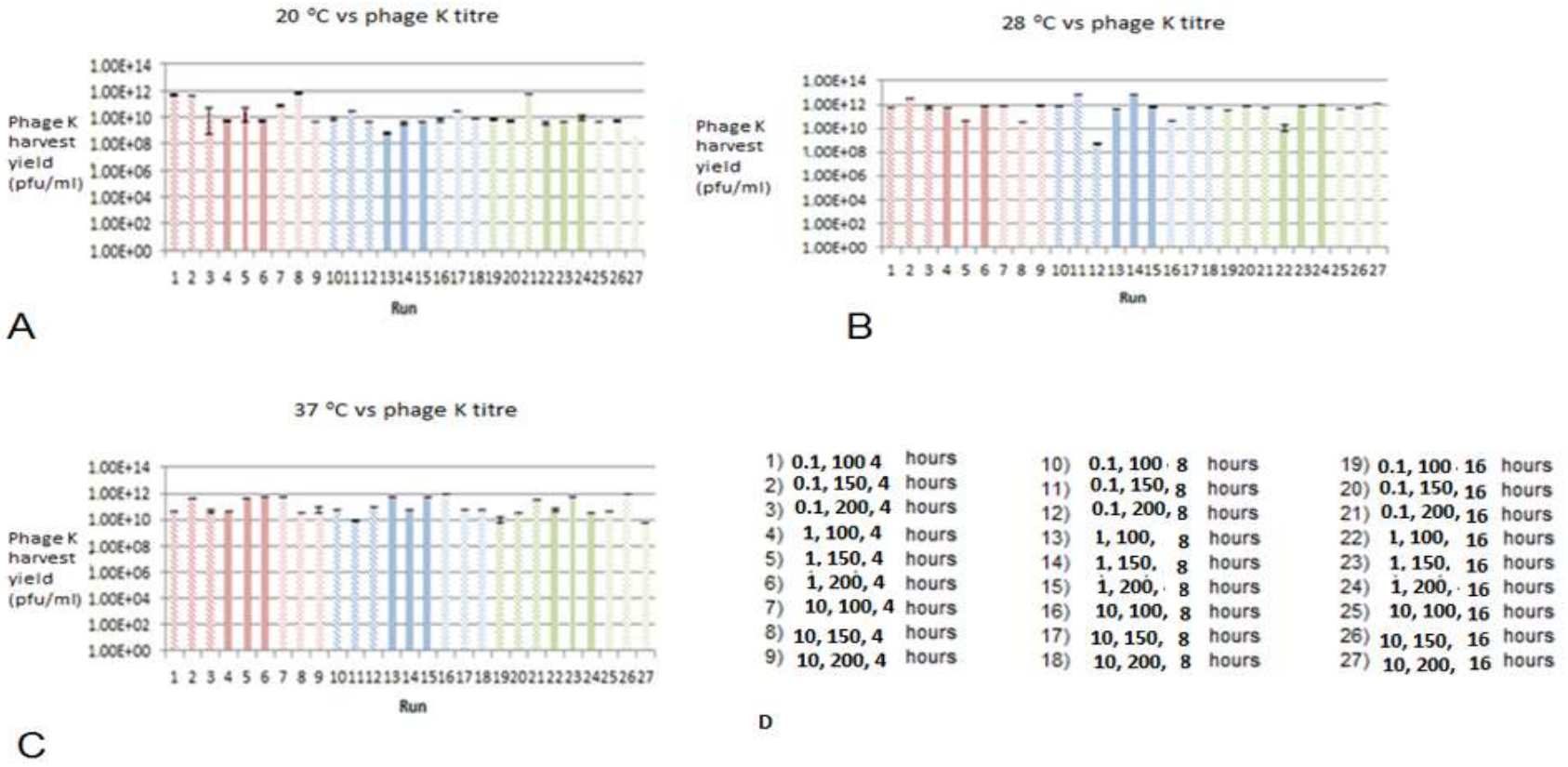



**Figure 3.7.** *A bar chart to show T4 phage titre achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where a time of infection of 4 hours was used, blue bars show the conditions where a time of infection of 8 hours was used and green bars show where a time of 16 hours of infection was used. The first 3 bars in each set show where an MOI 0.1 was used, the second set of 3 bars show where an MOI 1 was used and the final 3 bars in each set show where an MOI 10 was used at the MOI in the experiment.*

The graphs in figure 3.7 show the different phage K titres achieved, based on the temperature at which the infection took place. The graphs show at 20°C and 37°C, titres above 1 x $10^{12}$ pfu/ml were usually unachievable. However, four runs achieved this titre at 28°C. The baseline phage K conditions (MOI 1, 150rpm, 8 hours of infection at 37°C (fig 3.7C run 14)) gave a titre of 7.5 x $10^{9}$ pfu/ml, similar to current literature values (Alves *et al*, 2014). However, the titre was significantly improved in figure 3.7b, run 14, 6 x $10^{12}$ pfu/ml, p=0.027. Additionally, there was a significant improvement in phage titres achieved at all expriments using 28°C compared to those at 37°C, p=0.015, using a paired t-test. Although it has been hypothesised that reducing the temperature may aid in DNA integration, reducing it too far may produce conditions that are unfavourable for host and phage growth.

In addition to the phage titre, it is also important to consider the phage per input cell. Again, as with the T4 phage data, the phage K data was normalised to determine the number of phage per input cell. Figure 3.8 shows the phage K per input cells at the point of infection.

Whilst DoE may be becoming more of an accepted methodology, examining each run individually is more of a common practice in an OFAT experiment. This work will now use the conditions which gave the greatest titre from the DoE, however, future work may wish to investigate any of the other runs that gave similarly high titres or productivities.

**3.7 Interaction analysis**

Figure 3.10 shows the interaction plots from the T4 phage and phage K DoE. Interaction plots examine all interactions of multiple variables within an experiment. They are a useful way of determining relationships between two or more factors as they can show the simultaneous effect of parameters on output titre. Where two lines are not parallel, there is an interaction but when two lines cross there is a statistically significant interaction whilst parallel lines show no interaction. Figure 3.10 shows the mean phage titre values at each condition used. Statistically significant interactions were confirmed using a two-way ANOVA.

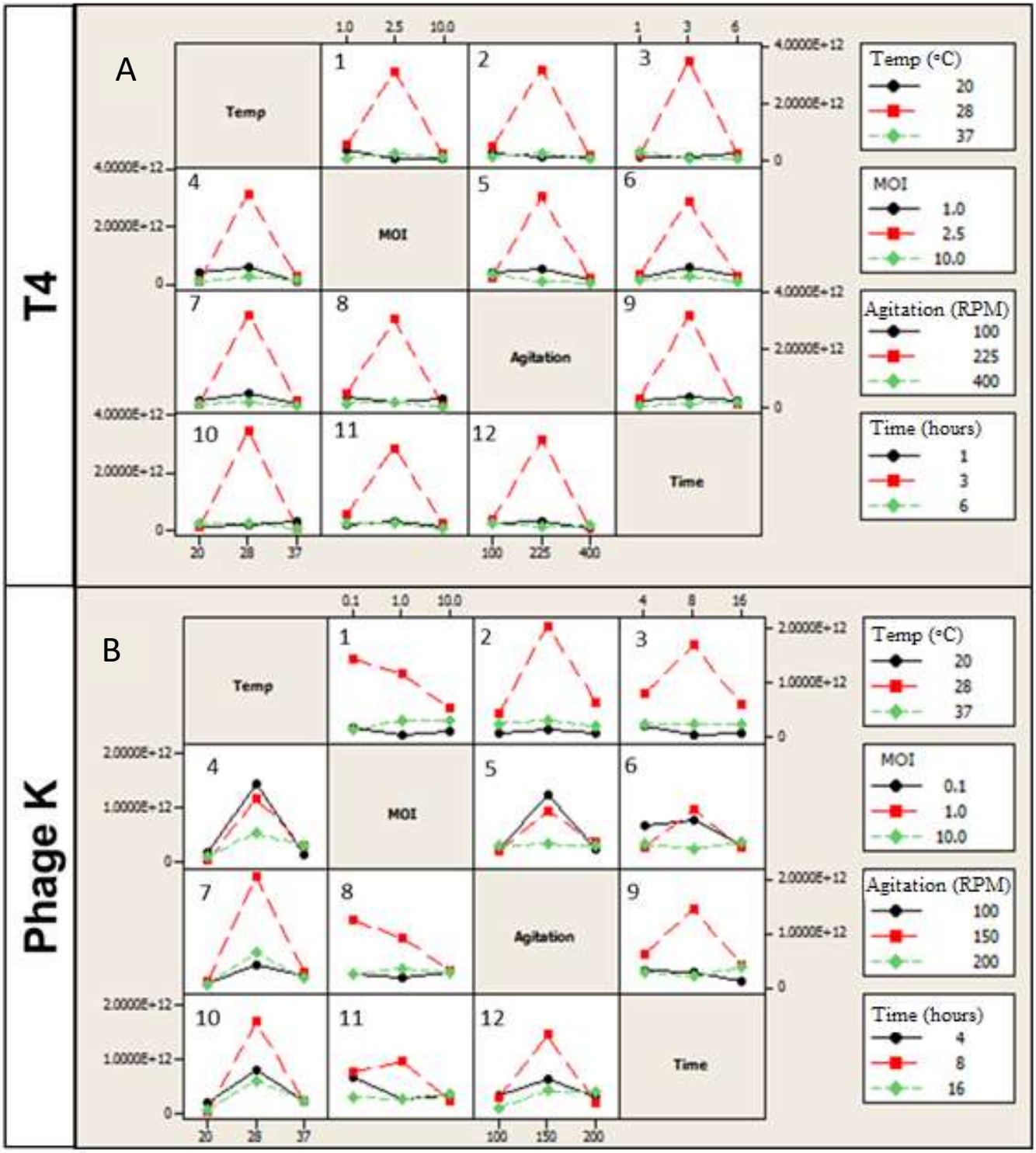



***Figure 3.10.*** *A graph to show the interaction plot analysis of the A) T4 phage B) Phage K small scale DoE.*

*T4 interaction analysis:*

*Temperature:* Figure a shows that the mid-point (28$^{o}$C) gives the greatest titre at all points apart from 1 hour infection. At 1 hour of infection, 37$^{o}$C (plot 3 graph A) gave the greatest titre, this may be because a higher temperature was needed for effective propagation of the host cells to allow phage to propagate themselves in a short infection time.

*MOI:* At 20$^{o}$C, MOI 1 (plot 1 graph A) gave the greatest titre. A low temperature of infection may be more useful to use as the host cells will be unable to propagate as greatly as if 37$^{o}$C were used, however, using the low MOI will mean that the phage will not 'over-run' the host cells and lyse them all.

*Agitation:* Graphs 7, 8, 9 show that 225rpm gave the greatest titre. However, graph 8 also shows that at MOI 10, 100rpm gave the greatest titre. A lower agitation speed may prevent optimal mixing of the culture and therefore, a higher MOI will be needed for the infection.

*Time:* Graphs 10, 11 and 12 show weak interactions between 1 and 6 hours and each MOI used with a strong interaction between 3 hours. Therefore, MOI has a weak effect on titre at 1 and 6 hours. There are several different conditions that give the greatest titre at different times of infections.

*Phage K interaction analysis:*

*Temperature:* Graphs 1-3 plot B show that 28°C gives the greatest titre at all levels used for each condition. There was a statistically significant difference between mean titres for 20/28°C, 20/37°C and 28/37°C p=0.003, p=0.02 and p=0.014 respectively. This analysis is useful for showing the true effect that each temperature has.

*MOI:* The graphs 4, 5 and 6 show that at 100, 150 and 200rpm, there was a different MOI that gave the greatest titre, showing that the MOI may not have a great overall effect on titre given that there is no single MOI at a particular agitation that gives the greatest titre.

*Agitation:* Graphs 7,8 and 9 show that 150rpm gives the greatest titre at each condition used. Therefore, using this plot and the previous MOI analysis, can show that 150rpm can give the greatest titre at each level and condition used.

*Time*: At the mid-point of each condition, 8 hours gave the greatest titre. At 200rpm, 16 hours gave the greatest titre, this may be because the agitation was too high which could have damaged the cells/phage and leave them undetected at harvest

### 3.8 Temperature of infection investigation

The next experiment closely examined the effect of temperature on phage titre, following from the result from the DoE. A reduced temperature had previously been shown, and corroborated here, to improve titre. Each experiment was run in triplicate with triplicate enumerations (n=9). The greatest titre conditions from the small scale DoE were used, changing only the temperature of infection.

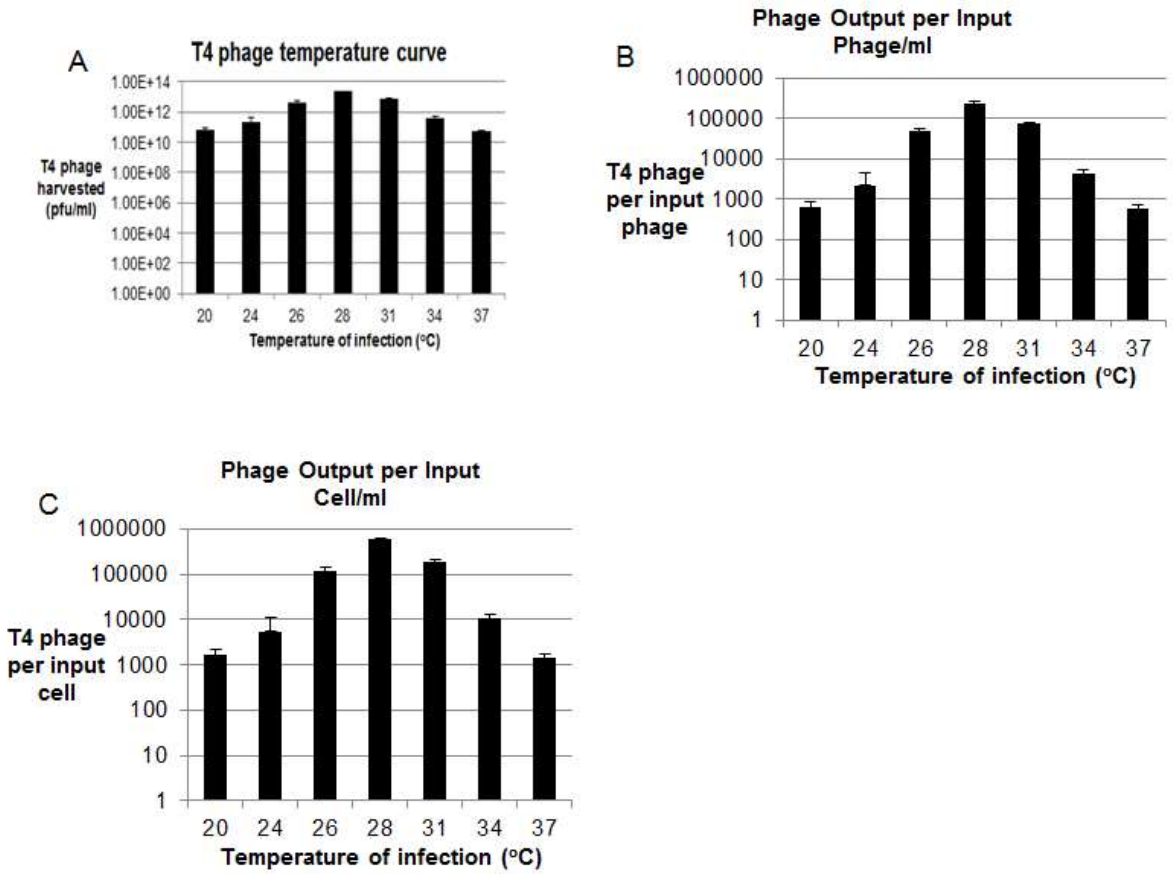



***Figure 3.11.*** *Temperature curve of T4 phage infection A) phage titre B) Phage output per input phage C) Phage output per input cell. Experiments were run in triplicate with each run enumerated by triplicate plaque assays. The conditions used were MOI 2.5, 225rpm and 3 hours. A temperature of infection of 20, 24, 26, 28, 31, 34 and 37°C was used. A peak is seen at 28°C.*

Figure 3.11 shows that by maintaining a constant infection time, MOI and agitation but changing the temperature of infection, there was a change in T4 phage titre. There was a significant reduction in titre between 28$^{o}$C and 20/37$^{o}$C, $p<0.01$ using an ANOVA. Moreover, there was also a significant reduction in titre between 28$^{o}$C and 26/31$^{o}$C, $p<0.05$ using an ANOVA.

Two hypotheses have been presented to account for the titre reduction i) the reduction in temperature is having a negative effect on the growth of host organism thereby preventing cell density to reach over an appropriate range for host infection ii) the reduction in temperature allows phage DNA integration to be improved given that higher temperatures may cause mis-integrations (Baron, 1996, Lee *et al*, 1996). Both of these hypotheses may be in line with the results as further reducing the temperature may prevent host organism growth to a point where there aren't enough bacteria for phage to infect. Increasing it above 28$^{o}$C may be better for host growth and less efficient for DNA integration.

A previous study examined the effect of temperature reduction on titre and showed up to 3 orders of magnitude reduction in titre of *Burkholderia thailandensis* E264 phage when infected at 25$^{o}$C compared to 37$^{o}$C (Shan *et al*, 2014). Additionally, Taj *et al* (2014) showed the effect of T4 phage on *E. coli BL21* and its temperature range. Lysis was found at 15/25/30/37 and 41$^{o}$C, but was shown to be inactivated and have no lysis at 45/55 and 70$^{o}$C. They also showed lytic activity between pH 4 and 10. No study has previously examined the temperature of infection as closely as presented here in figures 3.11/3.12 which shows the significant difference in titres achievable from just a few degrees Celsius change. Of the studies that have examined temperature on phage propagation, there has been no definitive hypothesis given as to why there may be an increase in titre, whilst this is the first study to examine the effect of temperature on phage titre this closely.

Figure 3.12 shows the phage K titre and phage K per input cell/phage at seven different temperatures. The conditions were those that gave the greatest phage output per input phage after 4 hours (MOI 0.1, 150rpm).

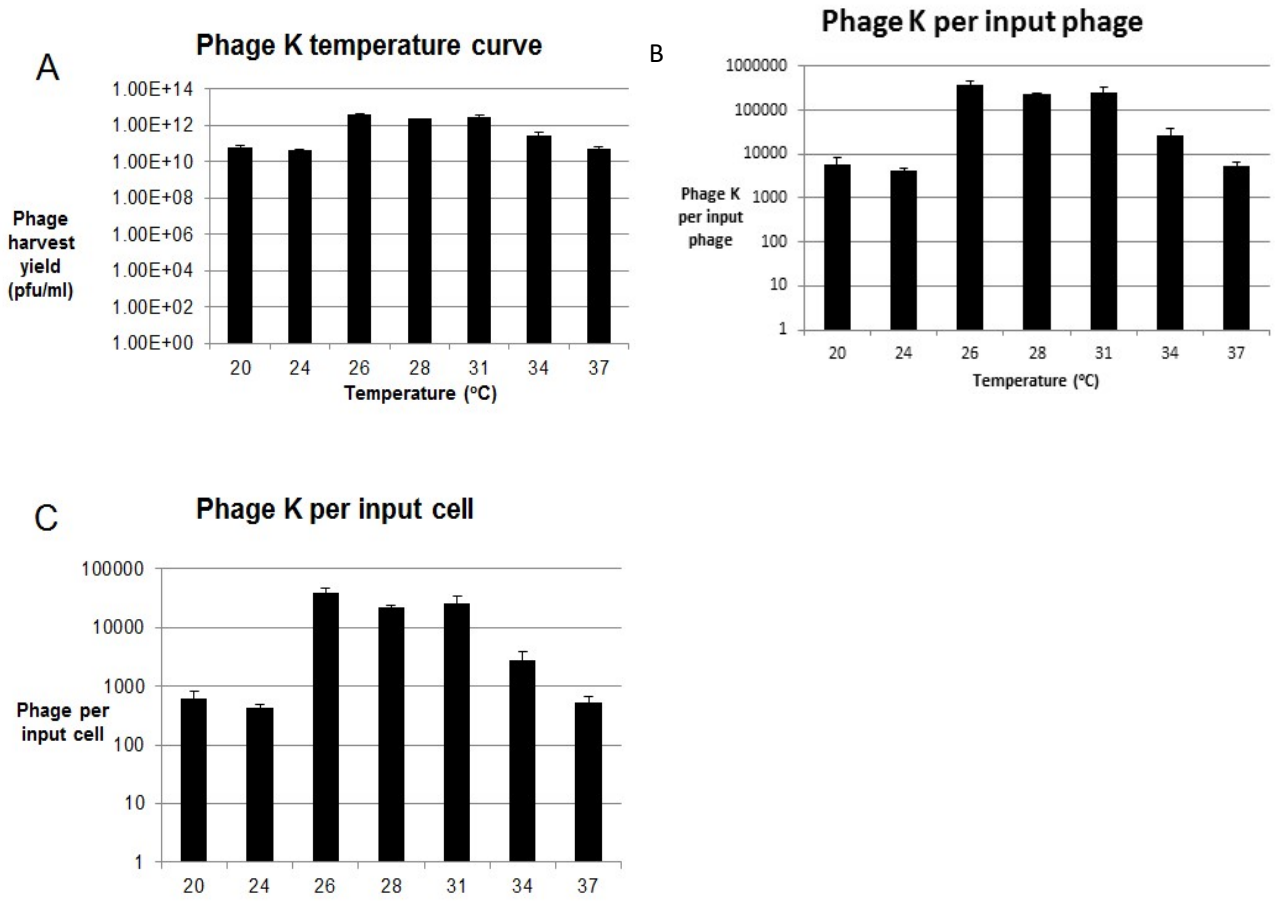



***Figure 3.12.*** *Temperature curve of phage K infection A) phage titre B) Phage per input phage C) phage per input cell. Experiments were run in triplicate with each run enumerated by triplicate plaque assays. The levels for the phage K infection were MOI 0.1, 150rpm and 4 hours. A temperature of infection of 20, 24, 26, 28, 31, 34 and 37°C was used. A peak is seen between 26 and 31°C.*

It is interesting to note that the T4 phage temperature reduction gave a more defined peak for titre, at 28°C, whereas the phage K data suggests that there is a window where the greatest phage titre can be achieved, 26-31°C. This shows one difference between the phage and that they act differently. This experiment may be needed for other phage researchers for their specific phage. Future work may wish to further examine this as less energy will be required to cool down the shaking incubator or stirred tank bioreactor from the pre-culture temperature of 37°C to 31°C compared to 28°C. Where similar titres and phage output per input cell/phage are

produced, 31°C may be used if energy were to become a significant cost and if the result was validated. Importantly, this study has given solid evidence that a reduction in temperature can improve T4 and phage K titre.

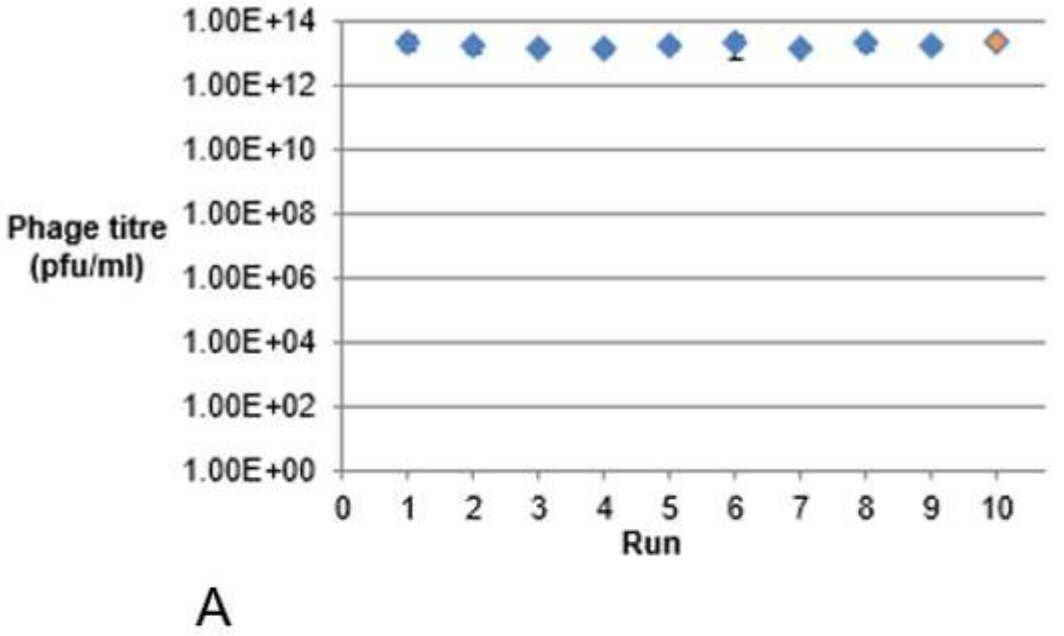
It must be noted that whilst this experiment was conducted in a shake flask, it was relatively easy to reduce the temperature in a short period of time. Section 3.11 will further investigate the mechanism of temperature reduction. However, whilst a temperature reduction in a shake flask may be simple, this may not be the case in a larger scale stirred tank bioreactor. For a temperature reduction in a large-scale bioreactor, this would significantly increase the power required which would increase cost of the process and potentially take away power from the impellor or control unit, increasing variability. This may also cause different profiles in the phage given the different sizes of culture units which may impact the product. It may be worthwhile running a further experiment to grow the host from the initial 0.05OD600nm to 0.25OD600nm at 28°C, however, this has the disadvantage that it would take longer for the host to propagate, increasing overall experimental time and again potentially further increasing cost or phage produced/hour. This experiment may be run for other phage as with the phage K, there is a significant increase in phage titre between 37°C and 34°C. Only reducing the temperature by 3°C may be more viable and if a significant increase in titre were to be seen for other phage, this may be one way to increase titre and yield. It should be noted that cooling mechanisms in bioreactors may significant affect the culture and final phage propagated and therefore using 28°C may not necessarily be the most appropriate temperature, however, for the purpose of this work 28°C was used in the bioreactor to continually try and improve titres achieved.

**3.9 Validation of DoE**

After several experiments using the DoE determined greatest titre conditions, a final experiment was run in triplicate over three days to act as a validation run. This would determine the reliability of the greatest titre conditions, n=9 experiments. Each were enumerated with triplicate plaque assays n=27. Figure 3.13 shows each run and titre achieved,. It takes into account external factors such as temperature of the laboratory,

equipment issues or operator variance. The result from the original DoE experiment is highlighted in orange (run 10).

The graph shows no significant difference was found between any of the runs compared to the DoE run for T4 phage or phage K. The average T4 phage titre from the three-day triplicate experiment was 1.87 x $10^{13}$ pfu/ml, compared to 2.45 x $10^{13}$ pfu/ml from the DoE shown in orange. Given that the experiment was a full factorial design, validation runs were needed to be carried out separately. The average phage K titre from the three-day experiment was 2.4 x $10^{12}$ pfu/ml, compared to 3.5 x $10^{12}$pfu/ml from the DoE. This allowed us to gain confidence in the result and methodology produced for high T4 phage and phage K titres.



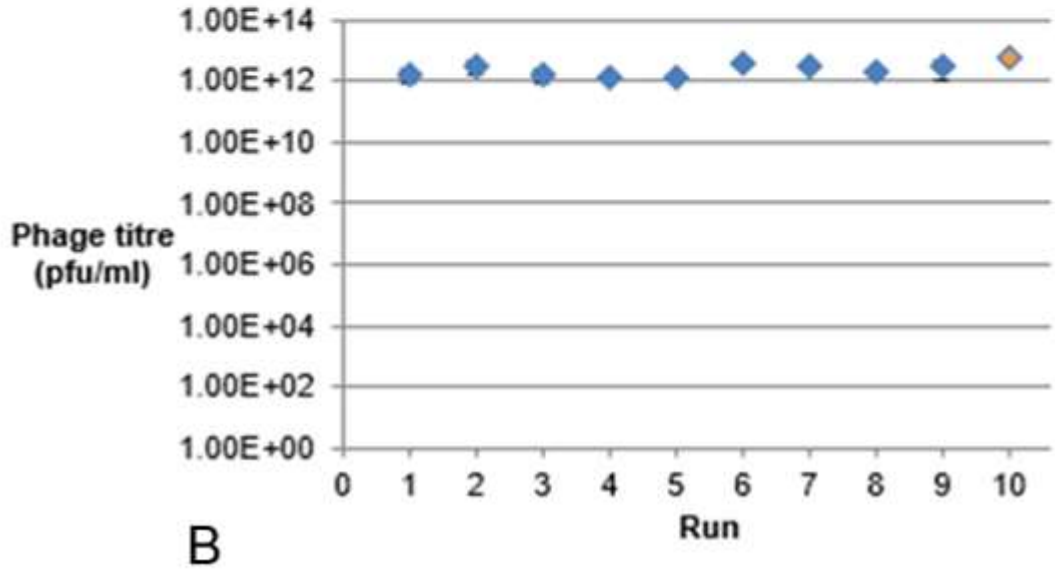




***Figure 3.13***. *A triplicate validation experiment run over 3 days, n=9 runs of the improved conditions for the phage infection. Each point on the graph represents a single run and the error bar is shown for each point. The orange point represents the original DoE titre. A) T4 phage validation B) Phage K validation. The error bars show 1 standard deviation of a triplicate enumeration.*

**3.10 Reduction in conditions of phage K**

The next experiment examined a reduction in MOI and time for phage K to improve efficiency which may make the bioprocess more desirable, shown in figure 3.14. The DoE determined greatest phage K titre was achieved at MOI 1 and 8 hours, but a similar phage harvest per input phage was achieved using an MOI 0.1 and 4 hours, it was important to determine whether a further reduction could give similar titres.

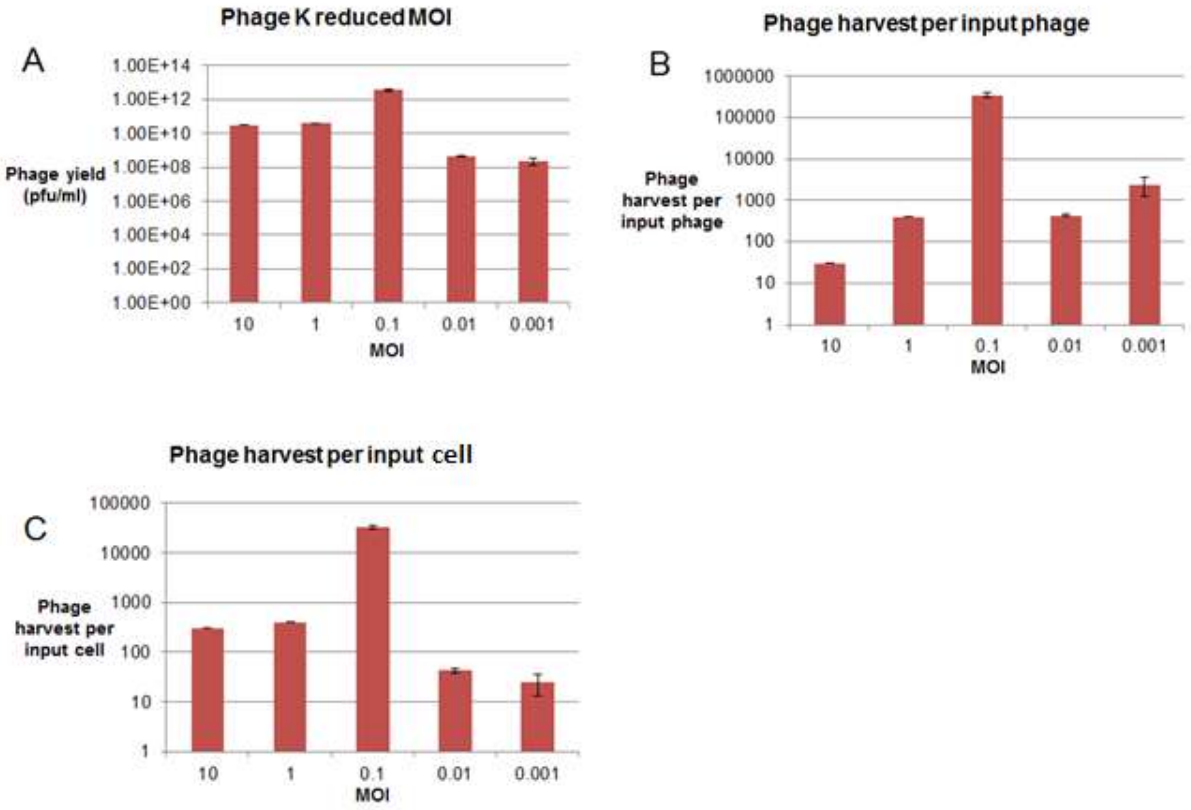



***Figure 3.14.*** *A graph to show the A) phage titre B) phage harvest per input phage C) phage harvest per input cell based on an MOI reduction for phage K. The conditions used were 150rpm, 28°C, 4 hours infection. An MOI, 10, 1, 0.1, 0.01 and 0.001 was used. The graph shows 1 standard deviation of triplicate runs each enumerated by triplicate plaque assays.*

Figure 3.14 MOI 0.1, 150rpm, 4 hours at 28°C, achieved >100,000 phage per input phage and around 1,000 when reduced to 0.01 and 0.001. This was unexpected given that there was such a significant reduction in phage harvest when the MOI was reduced 10 and 100 times to 0.01 and 0.001, p=0.0002 and p=0.002 respectively. Future investigations may wish to focus on examining an MOI close to 0.1 to determine if there is a window where similarly high titres can be achieved. Between MOI 1 and 10, the phage titre significantly decreased compared to MOI 0.1 thereby reducing efficiency of the processes (phage harvest per input phage). It is interesting to note the significant improvement in phage harvest per input phage between MOI 0.01 and 0.001, $p<0.05$ using a paired t-test. Whilst similar titres were achieved, there was 10-fold lower phage used in the infection. When a low viral MOI is used, the infection is more difficult to control and less reliable and a level of negative feedback can be introduced causing variation in titres (Brown & Bidle, 2014). Previous work has shown that reducing the MOI to an undesirable point can have a negative impact on the phage titre achieved which may explain the increase in titre seen (Bryan *et al*, 2016).

Similarly, Agboluaje & Sauvageau 2018 examined the effect of MOI on final phage titre. Using a 1L bioreactor, the study examined T4 phage and showed that using mineral salt medium, at 37°C, 200rpm, a titre of ~$1x10^9$ pfu/ml was achieved at MOI 10. However, as the MOI was reduced to 0.05 and 0.0001, the final harvest titre increased to ~$1x10^{10}$ pfu/ml and ~$1x10^{11}$ pfu/ml respectively. Given that they were using a minimum media, this may have prevented growth of the host organism and a higher titre of phage may rapidly destroy the host organism, preventing phage propagation. However, a lower titre may have allowed a longer infection which may subsequently increase overall titre (Agboluaje & Sauvageau 2018). The next step was to determine if similar phage values could be achieved with a reduction in infection time, shown in figure 3.15.

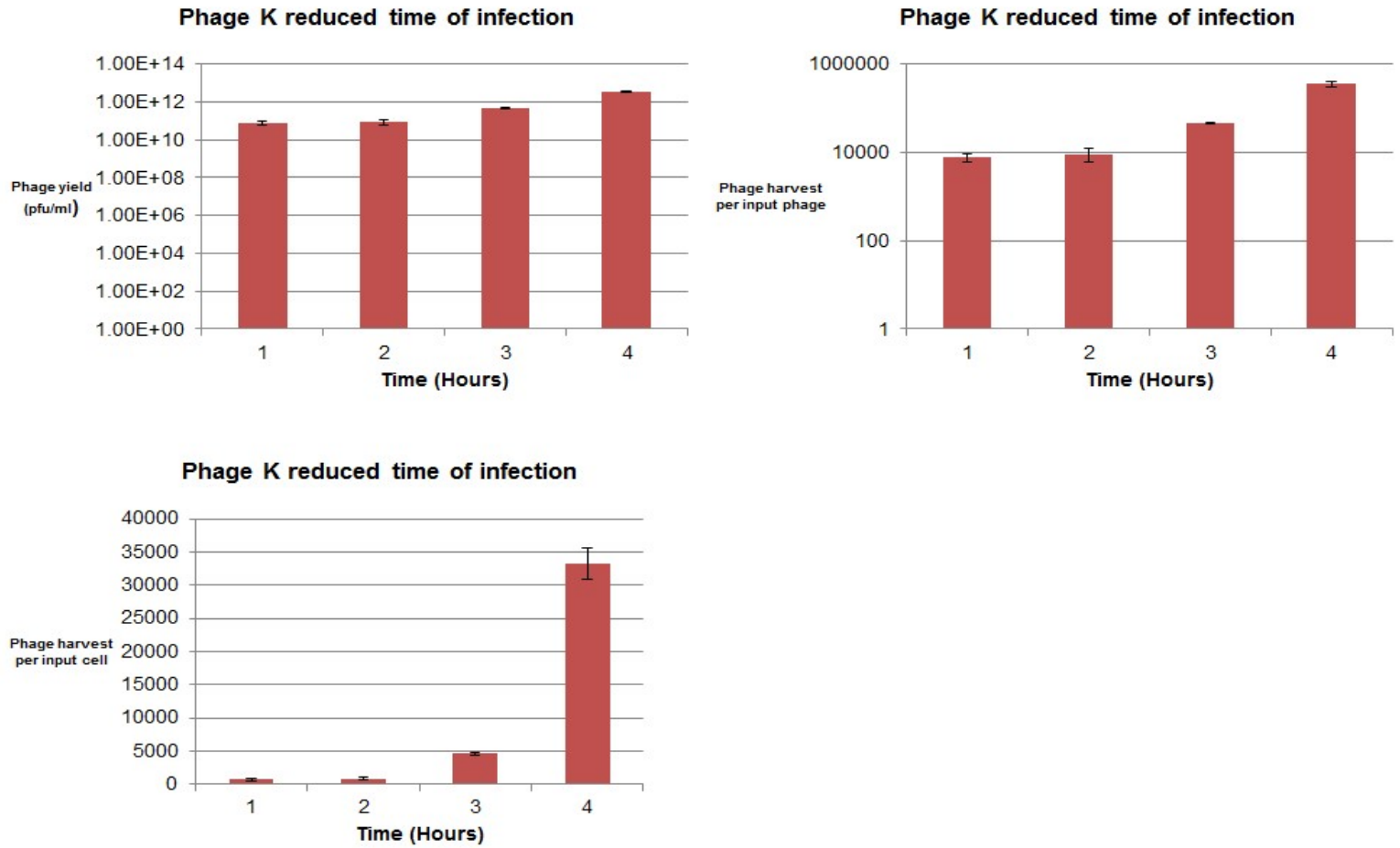



***Figure 3.15.*** *A graph to show the A) phage titre B) phage harvest per input phage C) phage harvest per input cell based on a time of infection reduction for phage K. Infection times of 1, 2, 3 and 4 hours were used. The conditions used were 150rpm, 28°C, MOI 0.1. The graph shows 1 standard deviation of triplicate runs each enumerated by triplicate plaque assays.*

The data showed that after 4 hours infection, >100,000 phage per input phage could be achieved. However, reducing the time to 1, 2 and 3 hours, there was a statistically significantly reduction in the final phage titre compared to 4 hours $p<0.01$ for all respectively using an ANOVA. This was most likely due to the time not being sufficient for the phage K to propagate. Nevertheless, 4 hours infection gave > 1 x $10^{12}$ pfu/ml. Additionally, 4 hours infection would be considered to be a very short time of infection for phage K, with some leaving the culture overnight i.e 16 hours (O'flaherty *et al*, 2005).

Given all of the data collected thus far, figure 3.16 shows the baseline and greatest titre conditions in shake flasks for T4 phage and phage K. The graph shows the statistically significant increase in titre between the conditions for both phage and highlights the importance of this work. Whilst the baseline titres are currently acceptable levels, simple manipulations of the conditions have shown the improvement in titres that can be made.

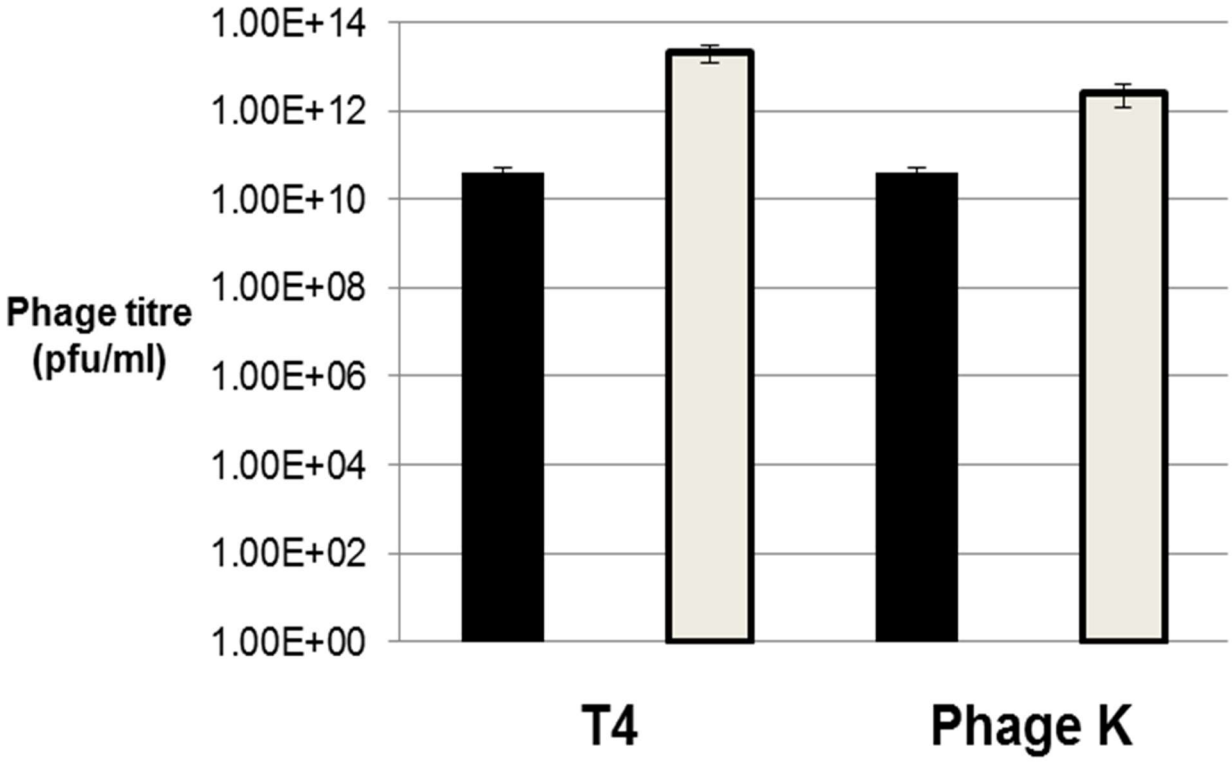



***Figure 3.16.*** *The graph shows a comparison between the baseline phage process parameters (black bars) and the process parameters that provided significantly improved output phage titres (grey bars) from the scaled down model (p≤0.004). Each experiment was performed in triplicate with individual experiments enumerated by triplicate plaque assays (bars represent average output titre, error bars represent 1 standard deviation)*

**3.11 Incubator temperature reduction experiment**

So far, the infection conditions for T4 and phage K have been optimised, validated, and a temperature/time reduction study completed. The data presented thus far has shown that an infection temperature of 28°C can result in an increased phage titre compared to 37°C T4 and phage K. Therefore, the next study examined the culture whilst the incubator was reducing in temperature, from the point of phage infection. Prior to the phage infection beginning, there were three possible methods investigated whilst the incubator reduced from 37°C to 28°C. Each experiment was run in triplicate outlined below:

1) Host culture is infected with phage and culture placed back into shaking incubator which is simultaneously reducing to 28°C
2) Culture placed into biological safety cabinet whilst the shaking incubator reduces to 28°C
3) Culture placed into static incubator, pre-set to 28°C, whilst shaking incubator reduces to 28°C

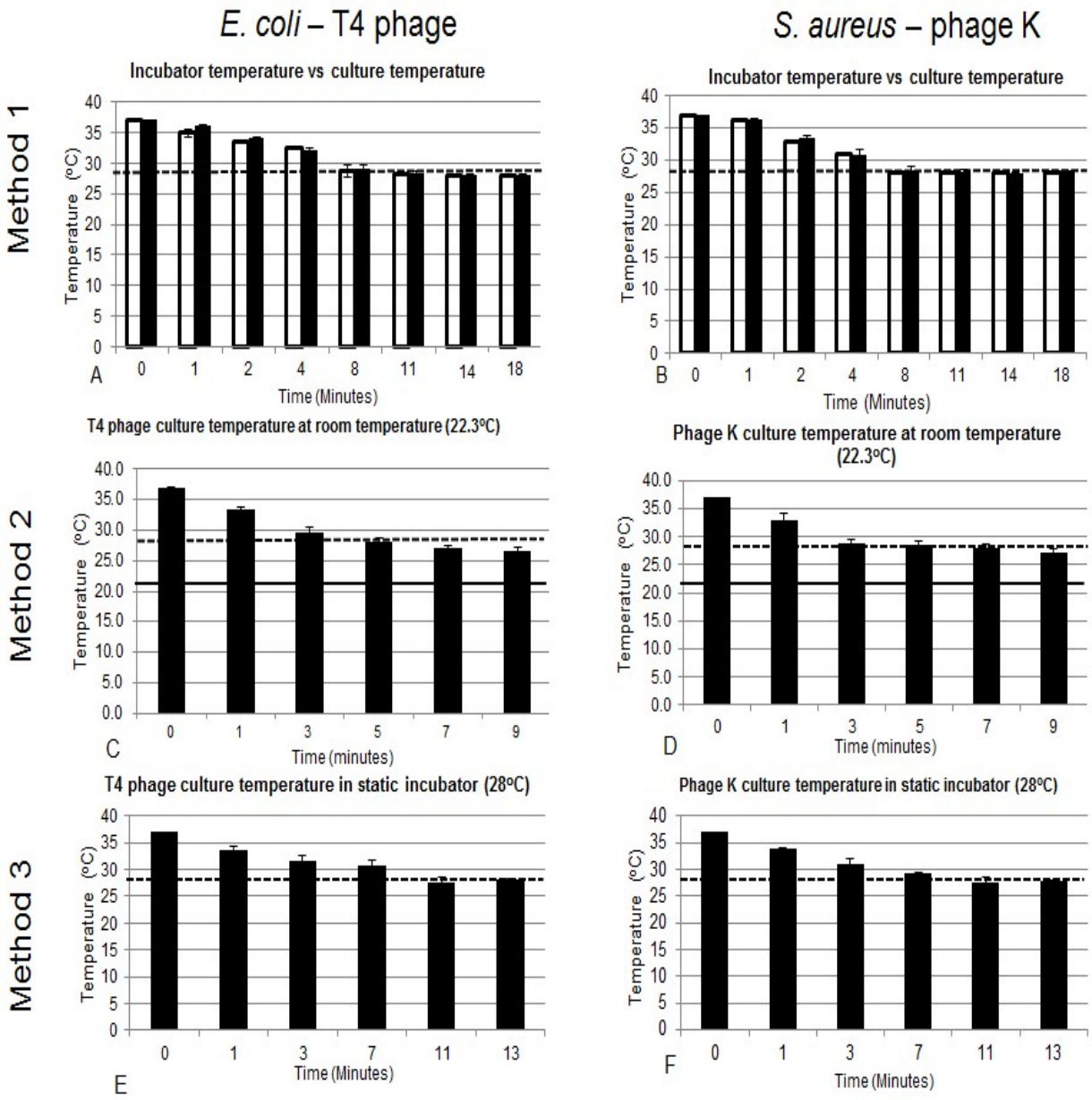



***Figure 3.17.*** *A graph to show the temperature of the incubator and culture which is being reduced from 37°C to 28°C. The error bars show 1 standard deviation of triplicate runs. Method 1 - culture in shaking incubator, method 2 – culture at room temperature, method 3 – culture in static 28°C incubator. Dashed lines show the required temperature, solid lines show the temperature of the culture environment. The incubator temperature in method 1 is shown by the white bars.*

Figure 3.17 shows the incubator and culture temperature when the culture was infected with phage and the shaking incubator was reducing from 37°C to 28°C. The graph shows after around 8 minutes, the shaking

incubator reached 28°C. Additionally, it took around 11 minutes for the incubator to stabilise at 28°C and 14 minutes for the culture. It was important to measure the temperature of the culture to determine how quickly the heat was transferred into the flask.

Method 2 kept the culture at room temperature (in a biological safety cabinet) whilst the shaking incubator temperature was reducing. Figure 3.17 C and D show the temperature of the culture. Here, the culture took around 5 minutes to reach the required 28°C. However, this method had the disadvantage of the culture temperature dropping below 28°C whilst the incubator temperature was reducing to 28°C: after 5 minutes the culture reached 28°C but it took 11 minutes for the shaking incubator to reach 28°C (method 1 A/B). The average temperature of the biological safety cabinet was 22.3°C which reduced the temperature of the culture at a greater rate than in a shaking incubator.

This led to the next experiment which was to keep the culture in a static incubator, method 3, pre-set to 28°C whilst the shaking incubator temperature was reducing. The graph above shows the temperature reduction of the culture took around 11 minutes to reach 28°C and a further 2 minutes to stabilise at 28°C. It may have been interesting to examine a culture placed straight into a shaking pre-set 28°C incubator, however, due to the unavailability of equipment this was not possible.

It must be noted that transferring the culture between a biological safety cabinet, shaking and static incubator will have an effect on the culture and its temperature. However, these experiments have been important as they show how quickly the temperature of the culture reduced. No previous work has examined the culture when the temperature is reduced at the point of infection. Figure 3.18 shows the phage titre reached for method 1 – 3 when the full infection was carried out.

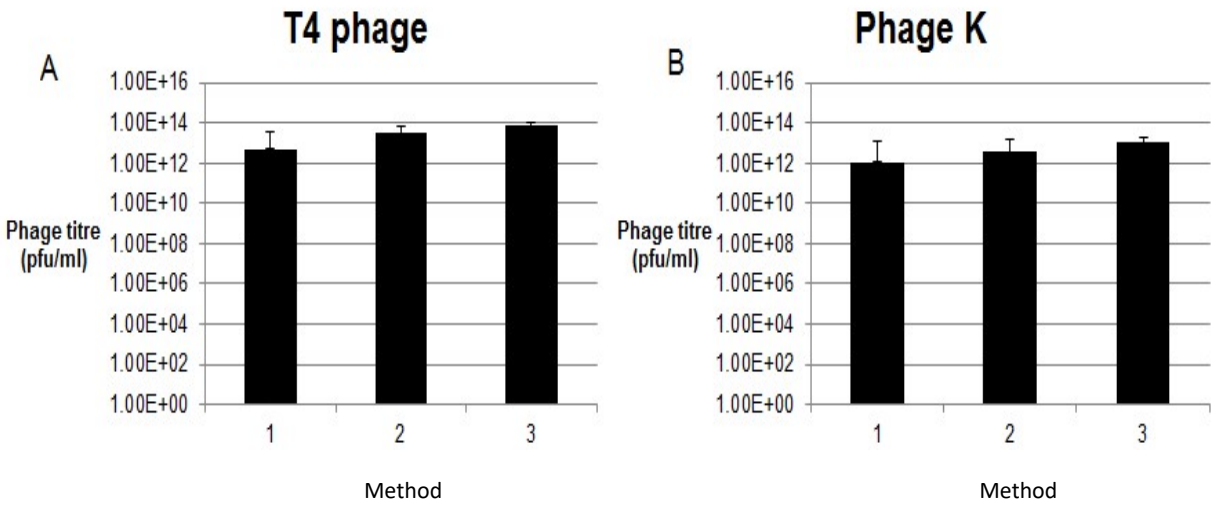



***Figure 3.18****. A graph to show the A) T4 phage B) phage K titre based on each method of temperature reduction. Method 1 - culture in shaking incubator, method 2 – culture at room temperature, method 3 – culture in static 28°C incubator Error bars show 1 standard deviation of a triplicate experiment each enumerated with triplicate plaque assays.*

Figure 3.18 shows that by keeping the culture at 28°C and allowing the shaking incubator to reduce to 28°C, there was an improvement in phage titre compared to the DoE determined greatest titre conditions. Method 1 gave an average T4 phage titre of $5.11 \times 10^{12} \pm 2.1 \times 10^{11}$ pfu/ml compared to $7.89 \times 10^{13} \pm 7.1 \times 10^{12}$ pfu/ml in experiment 3. A statistically significant increase in harvest titres between each method (1-3) for T4 phage and method 2-3 only for phage K, $p<0.05$ using a paired t-test was seen. Method 3 was not taken forward for the 'greatest titre' conditions as it would be important to compare future titres achieved from the DoE. Instead, method 1 was used.

One study showed that at a lower temperature than 37°C, a greater titre of protein could be produced in *E. coli JM109*. Cultures were grown to mid-log phase at 37°C in a shaking incubator and then transferred to shaking water baths for the remainder of the culture. Compared to 37°, statistically significantly increases titres were seen at 15°C and titres were found to continuously increase up to 30°C (Vasina & Baneyx, 1997). Whilst no

studies have been carried out like this for bacteriophage culture, a similar experimental design has been carried out for other microbial processes.

The DoE optimised the conditions for the full infection, however, this experiment optimised the infection process by examining the conditions at the point of infection and how the culture is handled. Although this step may seem minor or unimportant, by optimising this stage, a further improvement in phage titre was seen which further highlights the need for the full optimisation at each stage of the bioprocess.

**3.12 Adsorption analysis**

Whilst it has already been highlighted that there is a limited body of work examining the bacteriophage infection process, those that do sometimes fail to take into account the adsorption of the phage to the host cell, a key part of the kinetics. Examining the kinetics may give an insight into the improvement in the titre in the DoE determined greatest titre conditions compared to the baseline conditions. After the initial infection of phage, the next step is adsorption to the host cells. The adsorption is the key stage as the phage will either be recognised by the host cells and therefore bind to the receptors on the cell wall or not (Rakhuba *et al*, 2010). Although we know that the phage used in this work binds to the host cells used in this work, it is interesting to examine their rate of adsorption and this may play a role in the understanding of why improved titres can be achieved.

Initially, the baseline and greatest titre conditions were used to determine the rate of phage adsorption. Figure 3.19 shows the rate of T4 phage adsorption by examining the free phage. The experiment was run a total of four times, with each point enumerated by duplicate plaque assays. The experiment was run for the first 5 minutes after infection with sacrificial shake flasks set up and sampled every 30 seconds.

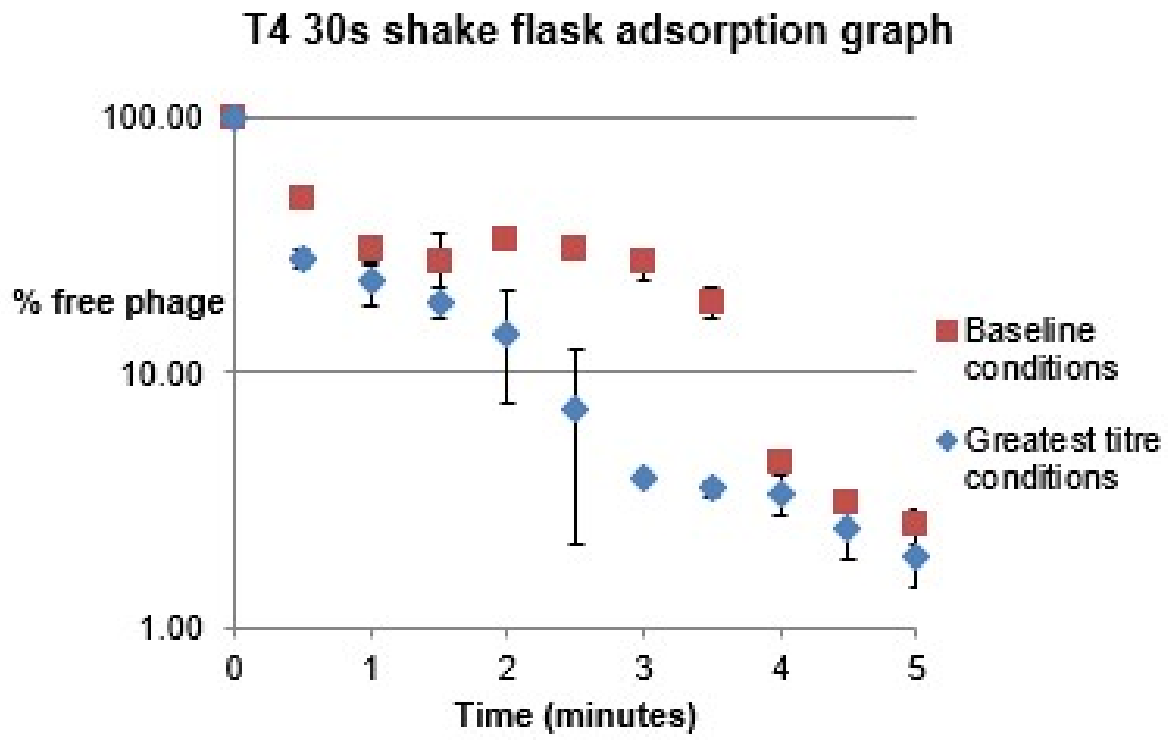



***Figure 3.19.*** *A graph to show the adsorption rate in shake flasks of T4 phage to E. coli based on either the baseline or greatest titre conditions. Error bars show mean ± 1 standard deviation of a quadruplicate experiment each enumerated with duplicate plaque assays.*

At the point of infection, the host was infected by the phage with either the baseline or greatest titre conditions. Sacrificial shake flasks were set up with a sample taken every 30s for the first 5 minutes of the infection. Figure 3.19 shows the adsorption of T4 phage at baseline and greatest titre conditions in shake flasks and shows the greatest titre conditions generally gave the lowest rate of free phage in shake flasks, compared to the baseline conditions. Given that the difference between conditions was the temperature, the significantly lower free phage, must be due to the temperature difference i.e 28°C vs 37°C. A statistically significant difference in free phage was found at all time points between the two conditions, using a paired t-test.

Given that the phage infection is dependent on host cell division and the availability for it to be infected, the higher rate of adsorption of phage to host (seen in the greatest titre conditions) may allow for more phage to adsorb to the host cells for the propagation accounting for the increase in titre between conditions. The next experiments focused on the phage K adsorption to *S. aureus.*

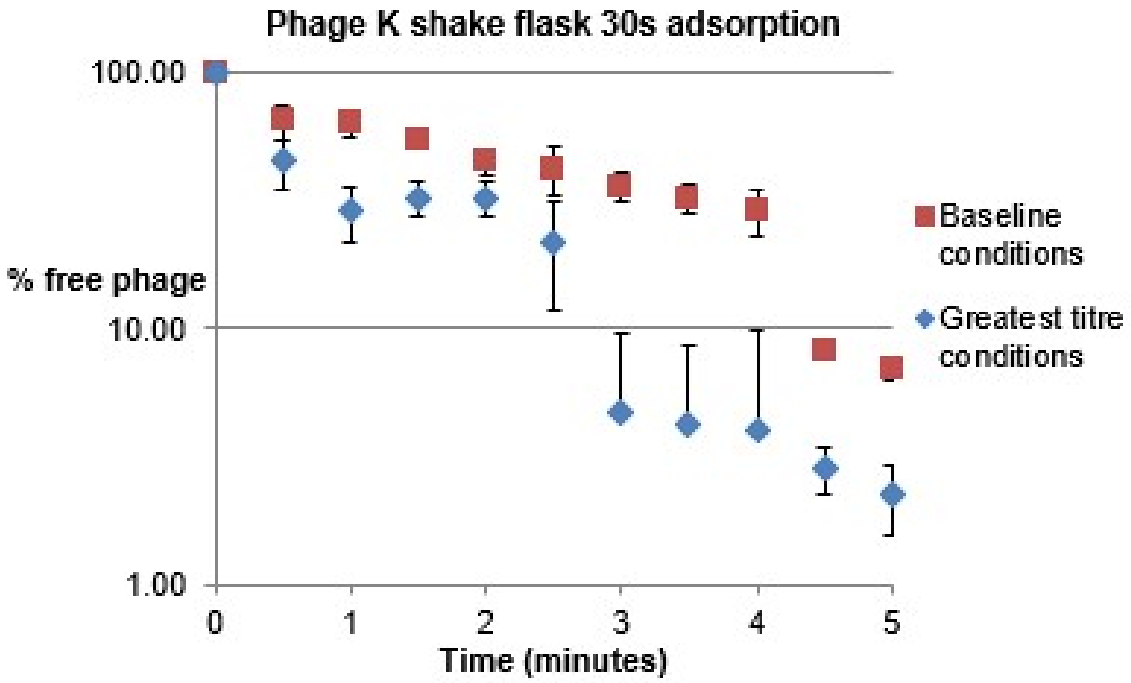



***Figure 3.20.*** *A graph to show the adsorption rate in shake flasks of phage K to S. aureus based on either the baseline or greatest titre conditions. Error bars show mean ± 1 standard deviation of a quadruplicate experiment each enumerated with duplicate plaque assays.*

Figure 3.20 shows the rate of phage K adsorption to its host organism. Similar to the T4 phage infection, the graph shows that the greatest titre conditions gave the lowest rate of free phage. A statistically significant difference in free phage was found at all time points between the two conditions, using a paired t-test.

There can be no single model of adsorption of phage to host organism made given that all phage act in different ways. It was reported that nearly every collision between phage and its host will result in the irreversible binding (Storms & Sauvageau, 2015). However, the results presented here exhibit a linear decline in adsorption and similar results to previous studies (Washizaki et al, 2016).

Table 3.3 shows the burst size and final T4 and phage K titre for the baseline and greatest titre conditions in shake flasks. Statistically significant increases were found between baseline and greatest titre conditions for burst and titre.

***Table 3.3.*** *A table to show the burst sizes achieved and final phage titres achieved, between the shake flask cultures for T4 and phage K for the baseline and greatest titre conditions.*

| **Phage** | **Conditions** | **Burst size average ± 1SD** | **P value** | **Phage titre (pfuml) ± 1SD** | **P value** |
|---|---|---|---|---|---|
| T4 | Baseline | 87.3 ± 4.8 | 0.03 | 4. 8x $10^{10}$ ± 1.3 x $10^{9}$ | 0.0009 |
| T4 | Greatest titre | 113.5 ± 1.5 | | 2.7 x $10^{13}$ ± 1.3 x $10^{13}$ | |
| K | Baseline | 71.5 ± 4.8 | 0.014 | 5 x $10^{9}$ ± 1.4 x $10^{9}$ | <0.0001 |
| K | Greatest titre | 111.8 ± 0.7 | | 5.7 x $10^{12}$ ± 1.7 x $10^{12}$ | |

Alves *et al*, showed that they were able to achieve a burst of 125 for phage K. Whilst this is significantly improved compared to our baseline burst, it is not too dissimilar than our greatest titre burst. However, it is important to note that they measured burst after 1 hour rather than 30 minutes as presented here. Additionally, with a burst of 60, Rees and Fry showed a similar burst to our baseline value whilst they used a burst time of 25 minutes. Rabinovitch *et al*, showed that their process gave a T4 burst size of 60 after 40 minutes of growth.

Therefore, by optimising the process and using the greatest titre conditions there is not only an improvement in titre, but also in burst size. The significant increase in burst between conditions may also explain why there is a greater rate of adsorption and final phage titre seen. Burst size is another measurement, similar to pfu/ml, that has differences in it in the literature and again the time should be standardized across the literature to allow comparisons.

The next experiment examined the adsorption and burst size for T4 and phage K when examining the three conditions at which a shake flask culture was maintained at the point of infection (method 1-3 section 3.11). Given the increase in titre seen between each condition, it would be interesting to note any differences in the kinetics that account for the increase in titre. The results are shown in figure 3.21. Given that there was more of a temperature effect on the culture shake flasks, the experiment was run over the first 10 minutes of the infection, rather than the first 5 minutes.

Statistically significant differences were seen for T4 phage between the culture kept in the shaking incubator and the culture kept in the BSC at 3,6,7,8, 9 and 10 minutes. A significant difference was also seen between the culture kept in the shaking incubator and the culture kept in the 28$^{o}$C static incubator at 3, 7, 8, 9 and 10 minutes $p<0.05$. Additionally, a significant difference was also seen at 3 minutes, $p<0.05$, between the culture kept in the BSC and the culture kept in the 28C static incubator, using a paired t-test.

The graph shows the culture in the shaking incubator generally gave the lowest free phage at each time point i.e highest rate of adsorption (green triangles for T4 phage). This was possibly due to the only culture that was being agitated. Agitation of a culture will ensure that the contents of the shake flask will be evenly spread out i.e all cells/phage, oxygen, pH and media content. Despite this, the culture that was kept in the 28$^{o}$C static incubator gave the greatest titre.

Similar trends were seen in the phage K experiment. Statistically significant differences were seen in the adsorption between the culture kept in the shaking incubator and the culture kept in the BSC at all time points. Moreover, significant differences were seen at all time points between the culture kept in the BSC and the culture kept in the 28$^{o}$C static incubator, $p<0.05$ using a paired t-test. A significant difference was also seen between the culture kept in the shaking incubator and the culture kept in the 28$^{o}$C static incubator at 3, 4, 5 and 6 minutes, $P<0.05$ using a paired t-test.

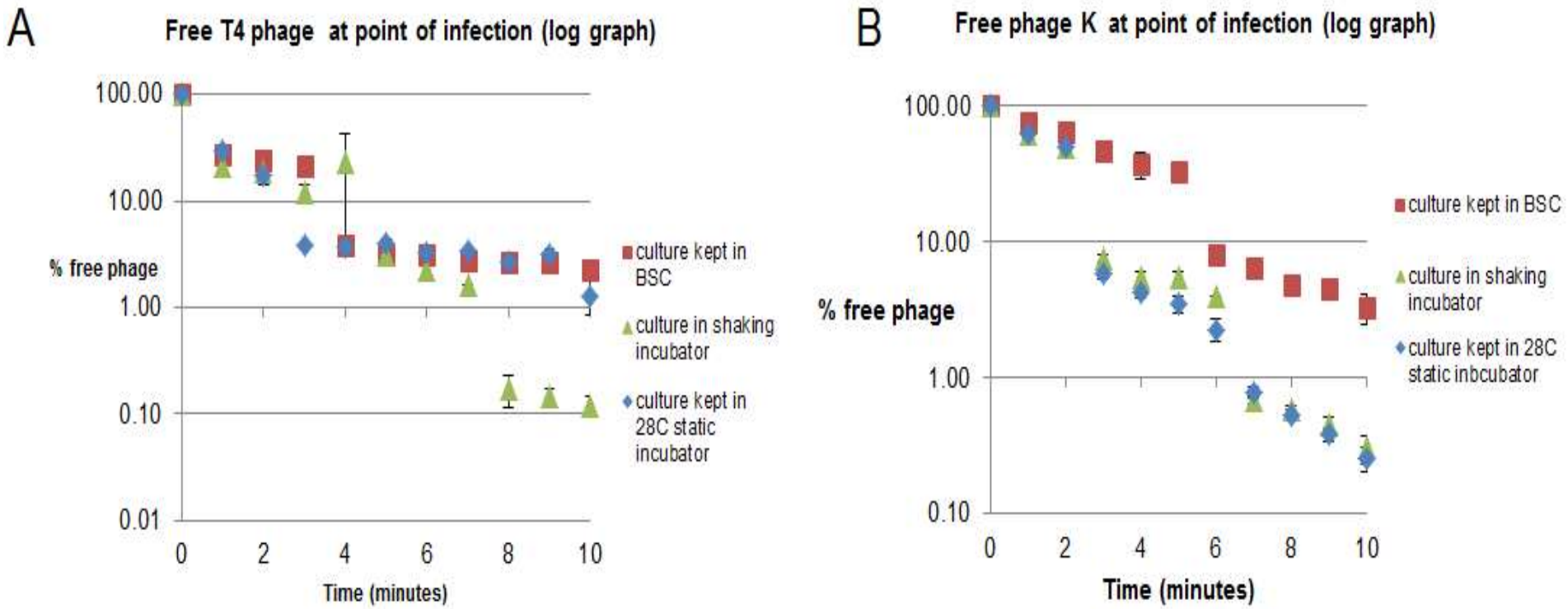



***Figure 3.21.*** *A graph to show the adsorption rate of each method (1-3) of temperature reduction of A) T4 phage B) phage K. The first 10 minutes of the infection was used, sacrificial shake flask cultures were set up and a sample was taken from each shake flask every minute. The graph shows a decline in the number of phage due to the phage adsorbing to the host organism.*

The burst sizes were next calculated for each experiment; T4 Culture in shaking incubator – 80.7 +- 9.3 (method 1), Culture kept in BSC – 65.6 +- 9.5 (method 2), and the culture kept in 28$^{o}$C static incubator – 143.75 +- 2.2 (method 3). The phage K experiment gave burst sizes of; Culture in shaking incubator – 79.5 +- 6.5, culture kept in BSC – 66.6 +- 4.05 and the culture kept in 28$^{o}$C static incubator - 96.9 +- 3.1.

This section has focused on the adsorption and burst size of the bacteriophage infection i.e. the kinetics for phage propagation. They were initially investigated in order to determine whether or not they could give indications as to why statistically significant differences were found between baseline and greatest titre conditions. Therefore, it would be worthwhile investigating these in future experiments for other phage. Figures 3.19 and 3.20 show differences between the rates of adsorption for the baseline and greatest titre conditions where the greatest titre conditions give a higher rate of adsorption. Therefore, if one were to specifically try to improve the rate of adsorption of T4 or phage K, the greatest titre conditions should be used. There may be differences found between the phage after different passages and if one were wanting to have a more homogeneous population of propagated phage i.e. harvest the phage after a single burst then these conditions may help to reduce experimental time. Figure 3.21 shows the rate of adsorption during the changes in incubator and temperature. The graphs generally show that when cultures are kept in the shaking incubator, the greatest rate of adsorption and burst size are seen. The faster and higher phage adsorb to the host organism, potentially more phage will be propagated. Therefore, those conditions that allow the greatest rate of phage propagation and quick adsorption should be used as the burst size has shown that those conditions give the highest final phage titre, highest burst size and highest rate of adsorption which will increase efficiency and reduce overall cost in the experiment.

**3.13 Extremes DoE levels infection**

Thus far, the data given has examined the baseline and greatest titre conditions. It may be interesting to examine the extreme levels used, from the DoE, to determine their effect on the culture and its contribution to the phage infection and propagation. It may also explain why the greatest titre conditions were those that gave the greatest titre. The next experiment aimed to examine the adsorption, burst size and final phage titre at each of the extreme levels for T4 and phage K in shake flasks. Table 3.4 gives the levels used in both culture systems. Previous studies have shown the effect that different media can have on the phage adsorption but limited work has been done on the conditions at which the infection takes place (Storms *et al*, 2010). The study by Storms and colleagues also showed that altering the MOI had very little effect on the adsorption efficiency but the media composition had a greater effect which will be examined later in this chapter.

***Table 3.4.*** *A table to show the levels used in analysing the extreme conditions from the shake flask DoE for T4 and phage K.*

| **Phage** | **Condition** | **Level** |
|---|---|---|
| **T4** | Agitation (RPM) | 100, 225, 400 |
| | Temperature ($^{o}$C) | 20, 28, 37 |
| **K** | Agitation (RPM) | 100, 150, 200 |
| | Temperature ($^{o}$C) | 20, 28, 37 |

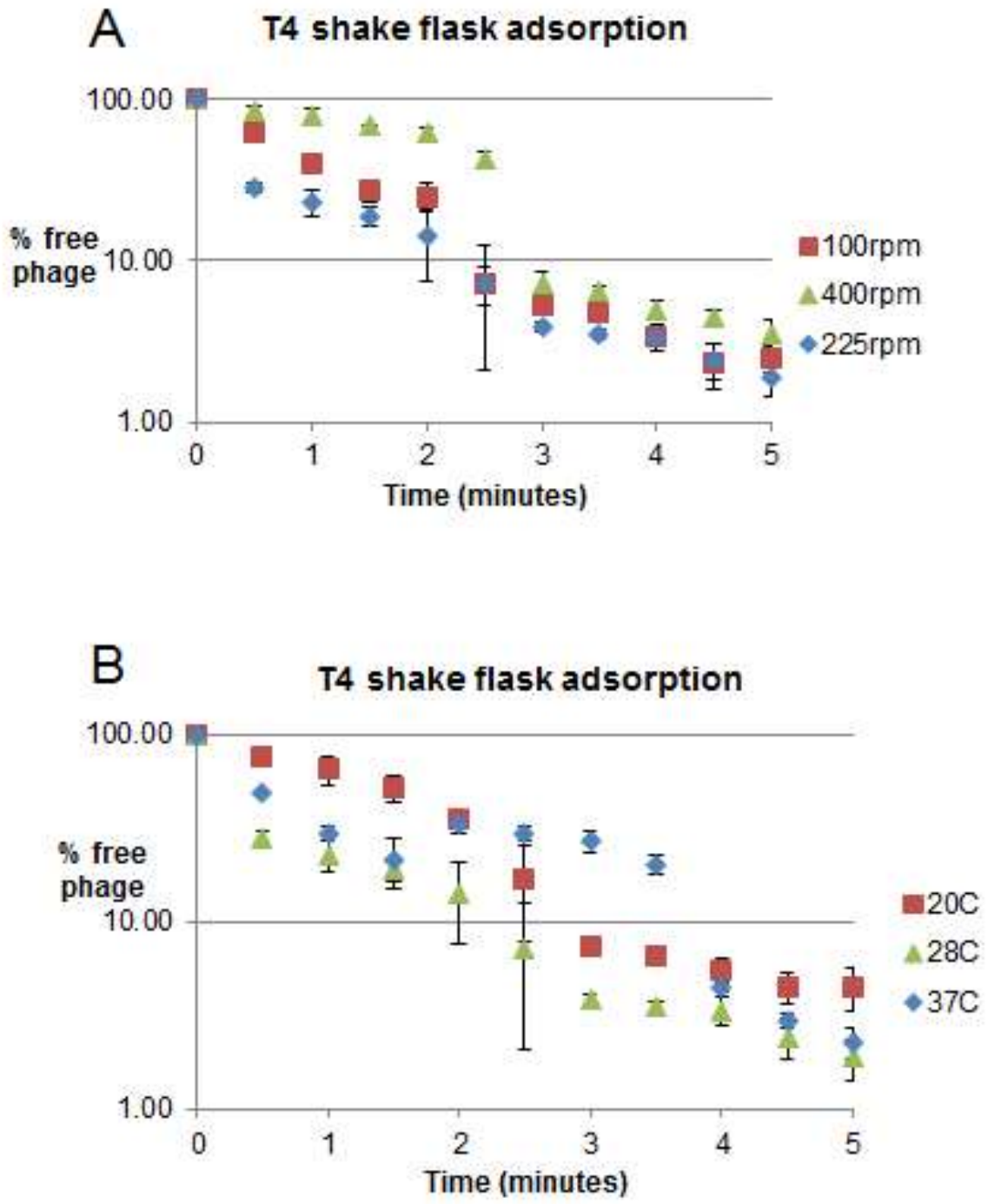



***Figure 3.22.*** *A graph to show the adsorption rate of T4 phage based on the different conditions in shake flasks A) Adsorption of infection B) Temperature of infection. The first 5 minutes of the infection was used, sacrificial shake flask cultures were set up and a sample was taken from each shake flask every minute. The graph shows a decline in the number of phage due to the phage adsorbing to the host organism. Error bars show 1SD of a quadruplicate experiment with each experiment enumerated twice i.e. each point represents n=8 measurements.*

***Table 3.5.*** *A table to show the burst size and final phage titre based on each of the levels in the phage K infection.*

| **Condition** | **Level** | **Burst size ± 1SD** | **Pfu/ml ± 1SD** |
|---|---|---|---|
| Agitation | 100 | 68.5 ± 8.5 | $1.33 \times 10^9 \pm 6.8 \times 10^8$ |
| | 225 | 113.5 ± 1.5 | $2.3 \times 10^{13} \pm 1.3 \times 10^{13}$ |
| | 400 | 57.7 ± 4 | $2.25 \times 10^{11} \pm 5.17 \times 10^{10}$ |
| Temperature **(°C)** | 20 | 68 ± 8 | $3.63 \times 10^{10} \pm 1.39 \times 10^{10}$ |
| | 28 | 113.5 ± 1.5 | $2.3 \times 10^{13} \pm 1.3 \times 10^{13}$ |
| | 37 | 87.3 ± 4.8 | $4.8 \times 10^{10} \pm 1.3 \times 10^9$ |

Figure 3.22 shows the rate of T4 phage adsorption in shake flasks at the extreme levels of the agitation and temperature from the DoE. The graph shows initially that 225rpm gives the statistically highest rate of adsorption whilst 400rpm gives the lowest rate of adsorption in shake flasks. Table 3.5 shows that 100rpm gives a higher burst than 400rpm, however, the final phage titre was significantly higher at 400rpm than 100rpm. The low agitation may be more beneficial to adsorbing phage allowing for a greater burst as more phage will be able to infect the host cells. However, this low agitation throughout the culture may prevent optimal growth of the host and propagation of phage which may explain why the final titre is lower. An agitation of 400rpm may have been too high for the phage/host interaction to occur. Moreover, the high rate of agitation may also cause a breakage of the phage/host interaction during the reversible binding step where the phage is not fully attached onto the host cell.

Figure 3.22 also shows that the greatest rate of adsorption and burst is at 28$^{o}$C. Whilst the lowest rate of adsorption was usually at 20$^{o}$C, the lowest burst and average titre was also seen here. The temperature will affect the ability of the host cells to propagate which may directly influence phage propagation. Additionally, the graph shows that 28$^{o}$C gives the greatest adsorption whilst initially 37$^{o}$C gives a greater rate adsorption than 20$^{o}$C. Interestingly, 37$^{o}$C gave a greater burst size than 20$^{o}$C which may be due to shock to the host cells as they move from the pre-culture temperature of 37$^{o}$C to 20$^{o}$C. However, a greater average titre was seen at 20$^{o}$C as the optimal temperature for host growth, 37$^{o}$C, may cause the host cells to grow to a density that is too high for optimal phage infection.

Both the temperature and agitation could effect the ability of adsorption but also the uniformity of the phage and host system, effecting the adsorption rate. Susceptible bacteria, free phage, bacterium-phage complex, infected cells and distribution throughout the infection process along with conditions will all play a role in phage adsorption. Given that so many external factors, as listed, could affect adsorption, it was critical that as many factors as possible are kept constant i.e. starting media temperature, ambient temperature, temperature of shaking incubator etc. Each of these will play a role in the manufacturing process but will also play a role in growth kinetics.

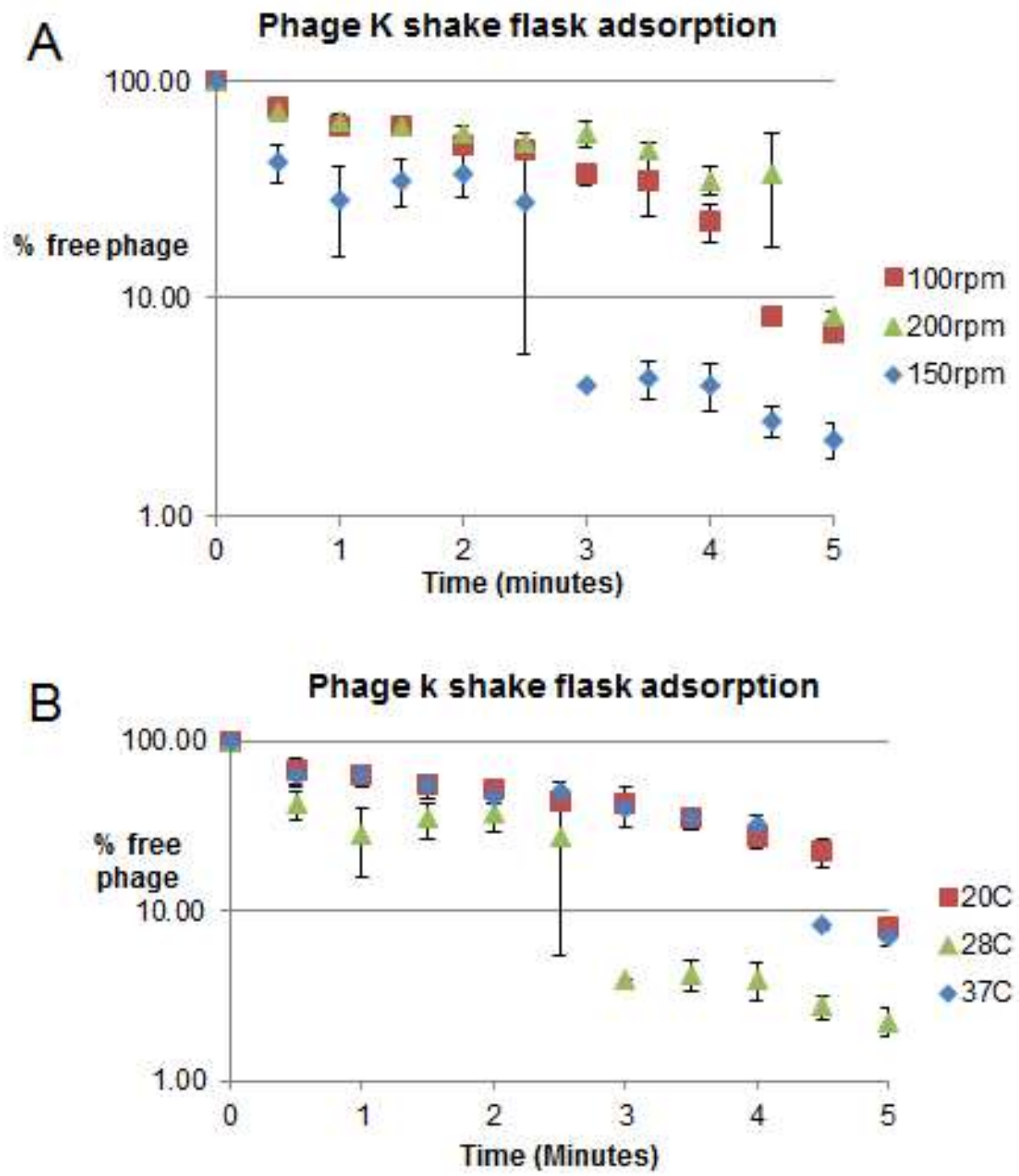



***Figure 3.23.*** *A graph to show the adsorption rate of phage K based on the different conditions in shake flasks A) Adsorption of infection B) Temperature of infection. The first 5 minutes of the infection was used, sacrificial shake flask cultures were set up and a sample was taken from each shake flask every minute. The graph shows a decline in the number of phage due to the phage adsorbing to the host organism. Error bars show 1SD of a quadruplicate experiment with each experiment enumerated twice i.e. each point represents n=8 measurements.*

***Table 3.6.*** *A table to show the burst size and final phage titre based on each of the levels in the phage K infection.*

| **Condition** | **Level** | **Burst size ± 1SD** | **Pfu/ml ± 1SD** |
|---|---|---|---|
| Agitation (RPM) | 100 | 99.1 ± 2.88 | $5.5 \times 10^{11} \pm 8.66 \times 10^{10}$ |
| | 150 | 111.8 ± 0.7 | $5.7 \times 10^{12} \pm 1.7 \times 10^{12}$ |
| | 200 | 92.8 ± 7.1 | $4 \times 10^{11} \pm 8.29 \times 10^{10}$ |
| Temperature (°C) | 20 | 94.75 ± 2.75 | $4.75 \times 10^{11} \pm 8.29 \times 10^{10}$ |
| | 28 | 111.8 ± 0.7 | $5.7 \times 10^{12} \pm 1.7 \times 10^{12}$ |
| | 37 | 71.46 ± 4.8 | $5 \times 10^{9} \pm 1.4 \times 10^{9}$ |

Figure 3.23 shows the phage K adsorption in shake flasks at the extreme levels of the agitation and temperature from the small-scale DoE. The graph shows the greatest rate of adsorption was at 150rpm compared to 100 and 200rpm. Apart from 2 and 2.5 minutes, a statistically significantly lower free phage was

seen at 150rpm compared to 100/200rpm, $p<0.05$ using a paired t-test. Table 3.6 shows the burst size and final titre based on the conditions and shows a statistically significant increase in burst size and final phage titre compared to 100 or 200rpm. Agitation rates that are either too high or too low may prevent optimal growth of the host cells i.e. smaller cell size, which may produce a lower burst (Weinbauer & Höfle, 1998). This would continue throughout the infection, preventing growth and propagation of host and phage, and may be why the greatest final phage titre is seen at 150rpm.

Graph B, figure 3.23, shows the adsorption of phage K at the different temperatures of infection. The graph generally shows 28$^{o}$C gives the lowest free phage. Minor differences were seen between 20$^{o}$C to 37$^{o}$C. Moreover, table 3.6 shows that 28$^{o}$C gave the greatest final phage titre and burst size. A statistically significantly higher titre was seen at 20$^{o}$C compared to 37$^{o}$C, $p<0.05$ using a paired t-test. The significant increase in burst between 28$^{o}$C and 20/37$^{o}$C may help to explain why a greater titre is seen.

Interestingly, Moldovan *et al*, 2007 showed that statistically significant differences were found from 5-40 minutes in the rates of phage λ using *E. coli Ymel* as a host, adsorption at different temperatures of infection. The results showed that 40$^{o}$C gave the greatest rate of adsorption followed by 30, 20 and 4$^{o}$C. However, our results showed that the mid-level (28$^{o}$C) gave the greatest rate of adsorption and at temperatures above or below, there was statistically lower rates of adsorption, possibly due to the phage mechanism of action. Whilst different hosts were used in their work and our work, one can not draw direct comparisons, their study was an early study showing phage adsorption.

Whilst this study showed that there can be statistically significant differences in final titre, burst and adsorption, Cha and colleagues showed using an *Acinetobacter baumannii* phage against its host gave no statistically significant difference in titre throughout 4-55$^{o}$C and pH 4-10. Therefore, a similar study would need to be carried out as this shows their study showed that for a different phage acting against a different organism, there can be vast differences in its mechanism of action (Cha *et al*, 2018).

**3.14 Supplement addition**

The experimental work so far has focused on LB medium for *E. coli* growth and brain heart infusion (BHI) medium for *S. aureus* growth. These two media are commonly used when growing the respected

organisms. Additionally, they both offer cheap solutions if high titres of host organisms are not required. Previous studies have shown that an improvement in phage titre can be found through the addition of supplements to the media and therefore it may be advantageous to determine if our improved bioprocess can further be improved on (Sochocka *et al*, 2015). However, within the studies in the literature that focus on supplementation, there is no examination of the cost of the supplements. It would be unwise to add supplements if there is a minimal improvement in phage titre, with a significant increase in the cost due to the supplements.

One other potential growth medium for these organisms is tryptic soy broth (TSB). With richer constituents that make up the media, it is well known that it can be beneficial to the growth of the organism. Therefore, an experiment was conducted using the greatest titre conditions in TSB media for both T4 phage and phage K in shake flasks.

Carbon sources are vital for the growth and propagation of microorganisms. The nutrients are needed for growth of the microorganisms and supplementation with have previously been shown to improve titre. Lamas-Samanamud & Shipley showed up to a 2 order of magnitude increase in titre, $1x10^{8}$-$1x10^{10}$ pfu/ml, for T7aiiA phage compared to when no glucose was added. They explained that increasing bacterial growth with the carbon source allowed there to be more host organisms to infect whilst also increasing cell size increases likelihood of binding and propagation. However, they also explained the importance of scale up and optimisation of the infection protocol which may firstly improve titre and should be looked at first rather than the addition of supplements, increasing cost of the experiment.

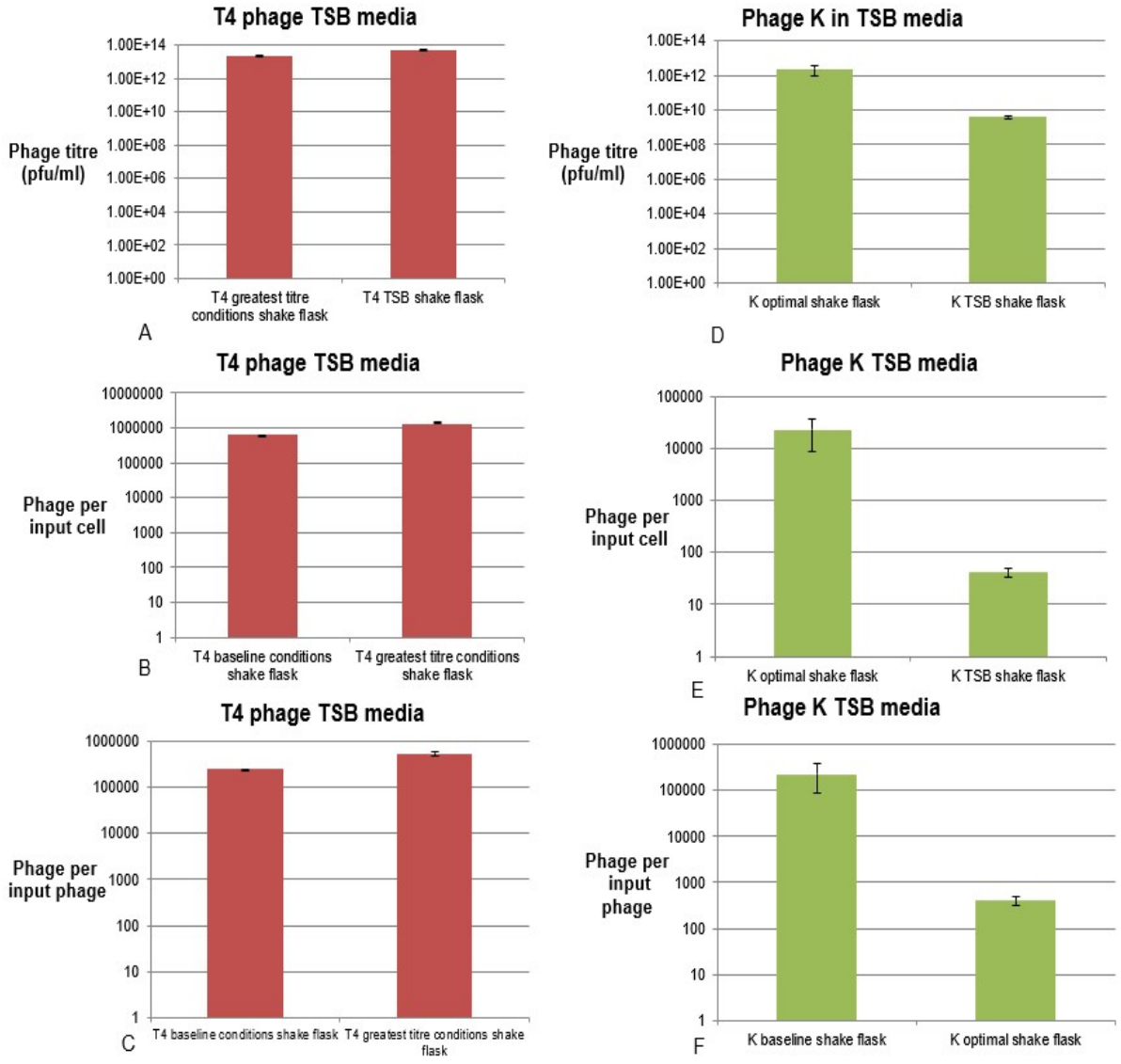



***Figure 3.24.*** *A graph to show the different titres achieved using the greatest titre conditions in LB/BHI or TSB media A) T4 phage titre B) T4 phage per input cell C) T4 phage per input phage D) Phage K titre E) Phage K per input cell F) Phage K per input phage. The experiments were completed in triplicate with each enumerated with triplicate plaque assays with error bars showing 1 standard deviation.*

Figure 3.24 shows the T4 phage and phage K shake flask greatest titre conditions which was conducted in TSB media. The graphs show a statistically significant improvement in phage titre between the greatest titre

conditions in LB media vs the greatest titre conditions in TSB media p=0.007. It should be noted that there was also a statistically significant improvement in baseline conditions in LB media vs greatest titre conditions in TSB media p=0.000024. However, there was a significant decrease in phage K titre between the greatest titre conditions in BHI media and the baseline conditions in BHI media vs the greatest titre conditions in TSB in a shake flask, p=0.00034 and p<0.000001 respectively. Given that the harvest OD of the T4 and phage K culture were >2 OD600nm and >3 OD600nm respectively, the TSB media may have allowed the host organisms to grow to a higher density than optimal for phage infection. Previous authors have shown that *S. aureus* growth can be higher than *E. coli* when using TSB media (Villanueva *et al*, 2018).

The next experiment took the greatest titre process from the DoE, and added supplements into the process. Supplements were either added at 0.05OD600nm and the host cells grown to the point of infection, or added to 0.25 OD600nm and infected with phage. The final phage titres are shown in figure 3.25 and figure 3.26 for the T4 phage and phage K process respectively.

Hadas *et al*, 1997 first used each of the supplements in their T4 phage infection process and infected at OD450nm 0.4, MOI 0.5. They explained the need for the cells to use the supplements to increase their size. Given the work presented thus far which shows titres are significantly reduced >OD600nm 0.25, this work used the standard infection point of 0.25 OD600nm. Supplements were added at either 0.05 OD600nm and grown to the point of infection, or added at the point of infection. The results by Hadas *et al* showed that these supplements may have an improvement in adsorption and titre, compared to the control, although no work was done to examine burst size or improving conditions. Table 3.7 shows the burst size achieved from our experiments. Moreover, for ease, they normalised their final phage titres but gave no clear indication as to how they were derived and therefore we were unable to compare their data set to ours.

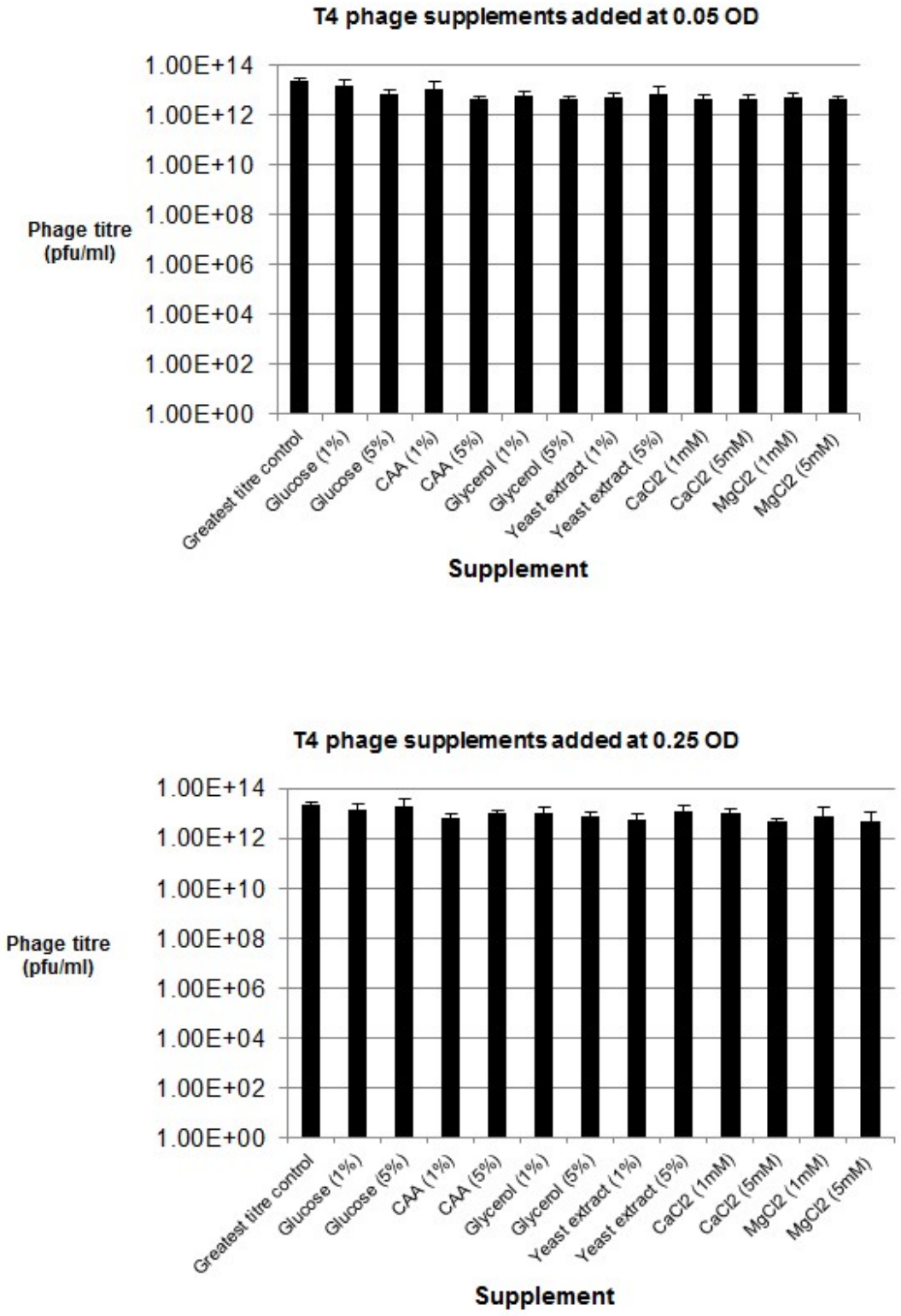
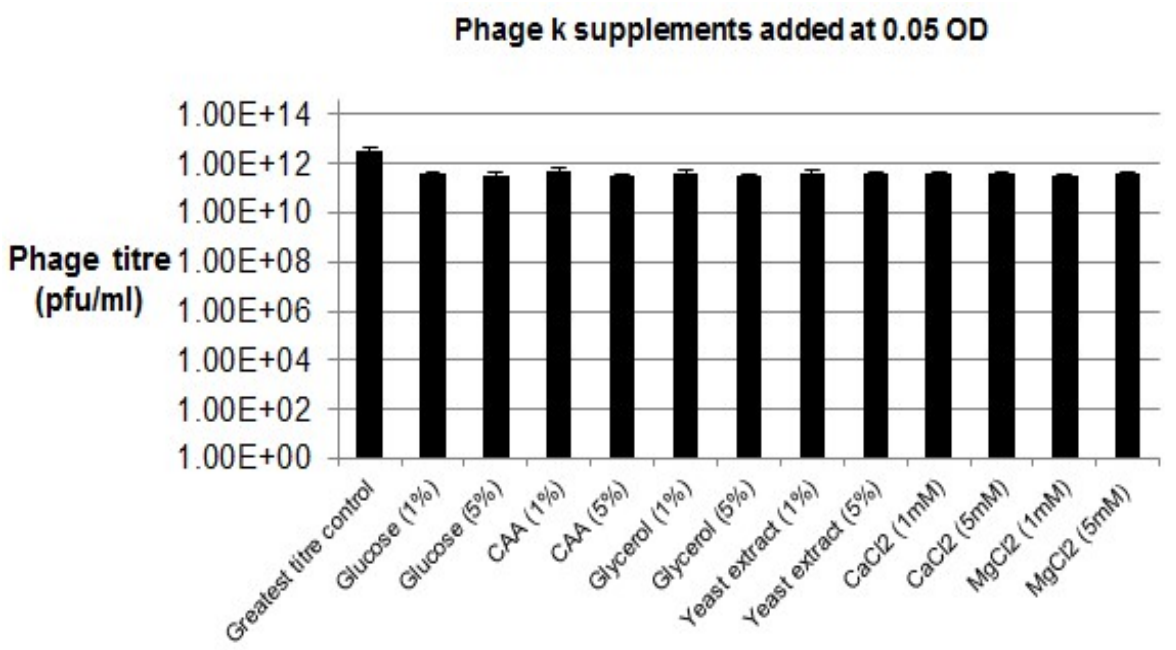



***Figure 3.25.*** *A graph to show the different T4 phage titres achieved based on the point of infection for supplement addition A) 0.05 OD600nm. Only this data was completed by PDP student Callum Pearce. B) 0.25 OD600nm. Error bars show 1 standard deviation of a triplicate experiment, each with triplicate enumerations*

The graphs above in figure 3.25 show the T4 phage titre achieved when using the greatest titre conditions. The data shows that the control experiment gave the greatest titre and addition of supplements made no improvement. Moreover, there was a significantly lower titre between the control experiment and each supplement, $p<0.05$, using a paired t-test, apart from 5% glucose at an infection of 0.25 OD600nm. Additionally, there was a significantly lower titre between the control and each supplement experiment when added at 0.05 OD600nm. Whilst it has been hypothesised that the addition of supplements can increase cell size, and therefore there is more chance for phage infection and phage propagation, it was not seen here (Golec *et al*, 2014). This may be because supplements increase cell size and increase the optical density outside the optimal range for phage infection. Also, there may be a theoretical ceiling of the titre that can be achieved which may already have been hit through the optimisation of the conditions. Whilst it is known that the optimal optical density for phage infection is 0.2-0.4 OD600nm, none of the harvest OD were within this range for the T4 phage supplement addition.

The graph below in figure 3.26 shows the data for the phage K experiments when supplements were added at either 0.05 OD600nm or 0.25 OD600nm. The graph shows that all addition of supplements significantly reduced final titre compared to the control experiment. This may be because the additional nutrients provided in the growth medium allowed additional growth of the host organism resulting in a lower phage propagation.



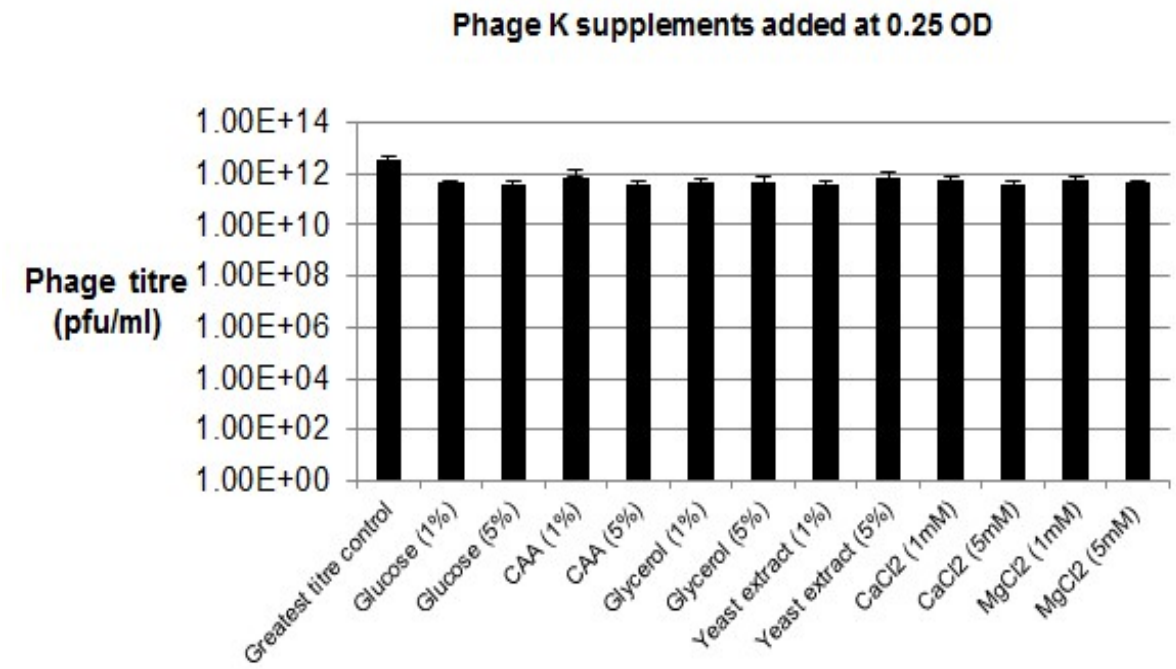




***Figure 3.26.*** *A graph to show the different phage K titres achieved based on the point of infection for supplement addition A) 0.05OD600nm B) 0.25OD600nm. Error bars show 1 standard deviation of a triplicate experiment, each with triplicate enumerations*

Hadas *et al* also showed a significant improvement on titre between their control and glucose supplemented T4 phage infection, with the supplemented infection giving a higher titre. Moreover, there was an improvement in burst size and adsorption rate when glucose was added to the infection. Whilst table 3.7 shows that there was no significant improvement in the control and glucose supplemented burst size, it was significantly increased from the baseline burst (T4, 87.3±4.8 and K 71.46±4.79). However, by first optimising the conditions, there was a significant increase over the baseline in burst which had no additional cost.

A similar result was found for the phage K experiment to the T4 phage experiment. No improvement in titre was achieved when supplements were added to the culture at either starting OD600nm, figure 3.26. Again, the harvest optical density for each supplement was above the optimal 0.2-0.4 range. The next experiment examined the burst size achievable when the supplements were added at 0.05 OD600nm, shown in table 3.7 below.

***Table 3.7.*** *A table to show the burst size achieved for the control experiment and when supplements were added for T4 phage and phage K*

| Phage | Supplement | Burst size (±1SD) |
|---|---|---|
| T4 | Control | 113.5 +- 1.5 |
| | Glucose (1%) | 111.65±1.65 |
| | CAA (1%) | 155±5 |
| | Glycerol (1%) | 136.65±13.35 |
| | Yeast extract (1%) | 133.3±3.3 |
| | CaCl2 (1mM) | 156.65±16.65 |
| | MgCl2 (1mM) | 124.95±11.65 |
| K | Control | 111.8 +- 0.7 |
| | Glucose (1%) | 144.8±31.8 |
| | CAA (1%) | 98.3±1.7 |
| | Glycerol (1%) | 96.6±0 |
| | Yeast extract (1%) | 87.75±15.25 |
| | CaCl2 (1mM) | 67.9±5.4 |
| | MgCl2 (1mM) | 73.4±3.3 |

To determine the effects of supplement addition on burst size, an overnight culture of the host bacterium was diluted to 0.05 OD600nm and the supplements added, grown at standard conditions to 0.25 OD600nm and infected with phage. T4 phage showed a significant increase in burst size for T4 phage in all of

the experiments, apart from glucose, compared to the control, whilst phage K showed a significant increase in burst for glucose but a significantly lower burst for the rest, tables 3.8 and 3.9. Given that burst size is dependent on either shock to the host cells or the cells not being in early logarithmic phase, it must be either of these two factors having an effect on the burst sizes seen. Xie & Yang 2016 showed that $Ca^{2+}$ and $Mg^{2+}$ can reduce the survival and growth of *S. aureus* by up to 20% of a culture whereas there was no effect and improved growth of the *E. coli*. The ions can destabalise the membrane leading to a reduced growth. Harper *et al*, 2018 showed Casamino acids and yeast extract can alter *S. aureus* pathogenicity/virulence and reduce its growth. Hess *et al*, 2015 showed that glycerol can reduce the development of *S. aureus* growth by interfering with lipid formation. Together, this information may help to explain why supplement addition may have a negative effect on host and burst size.

The next experiment examined the effect of using a defined medium. Defined media can be used rather than more complex media i.e undefined media for several reasons: cost, standard medium no matter where it is bought from and keeping/knowing all constituents of the medium used. The baseline and greatest titre conditions for T4 and phage K were used in shake flasks as shown in figure 3.27 below. The medium recipe was taken from Sochocka *et al*, 2015 and is as follows:

- 10g/L HyCase amino
- 3.5g/L DiPotassium Phosphate
- 5g/L Sodium Sulfate
- 1% Glucose

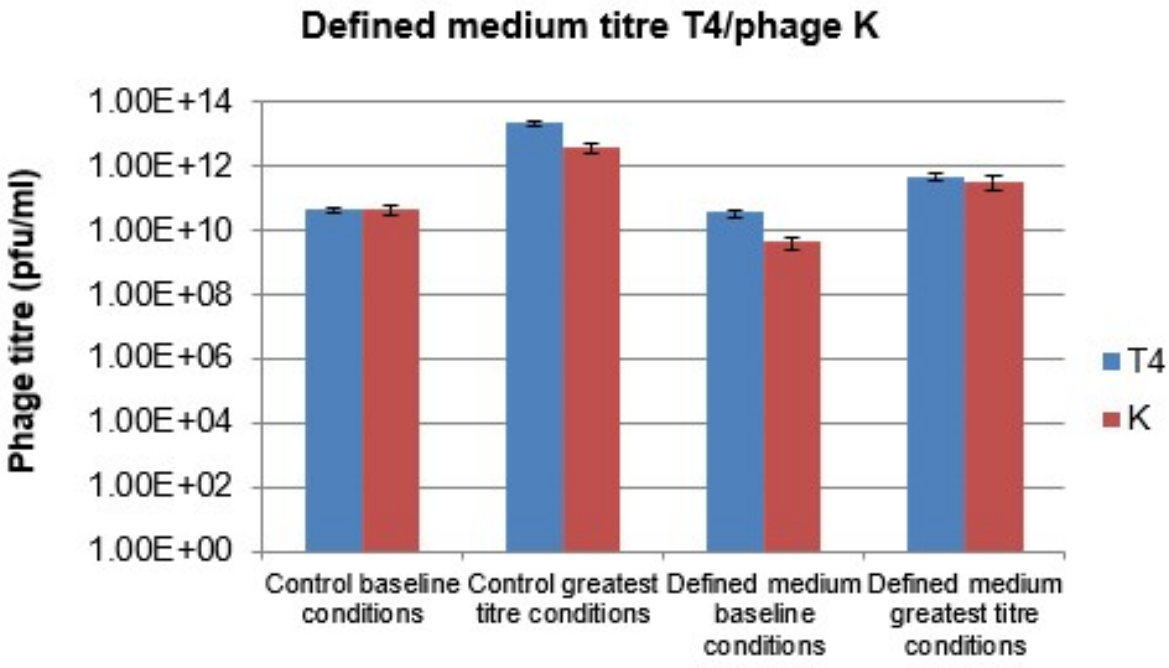



***Figure 3.27.*** *A graph to show the T4 and phage K titre reached using the baseline conditions and greatest titre conditions in shake flasks using the standard media or the defined media. Error bars show 1 standard deviation of a triplicate experiment with a triplicate plaque assay enumeration.*

The graph shows that there was no significant difference between T4 baseline (control) and defined medium baseline titre. However, there was a statistically significant reduction in titre between control greatest titre and defined medium for T4 and phage K, $p<0.05$ using a paired t-test. There was also a significant decrease between baseline/greatest titre conditions and defined medium for phage K. Given the limited nutrients that were used and that made up the medium, it may not have been able to support the propagation of the phage or growth of host.

Despite our results, Sochocka *et al*, 2015 showed significantly greater titres when using their process compared to ours using T4 phage. Using their defined medium, a titre of $1.2 \times 10^{16}$ pfu/ml was achieved. However, the conditions used in the infection were only partially stated, pH 7.0, poor aeration and poor mixing were used. If the conditions that had been used were stated, it would have been interesting to compare them to our greatest titre conditions seen here to determine if this work could have achieved a similarly high titre.

Figure 3.28 and table 3.8 show the adsorption and burst sizes each media used (LB/BHI, defined medium and TSB) for the baseline and greatest titre conditions.

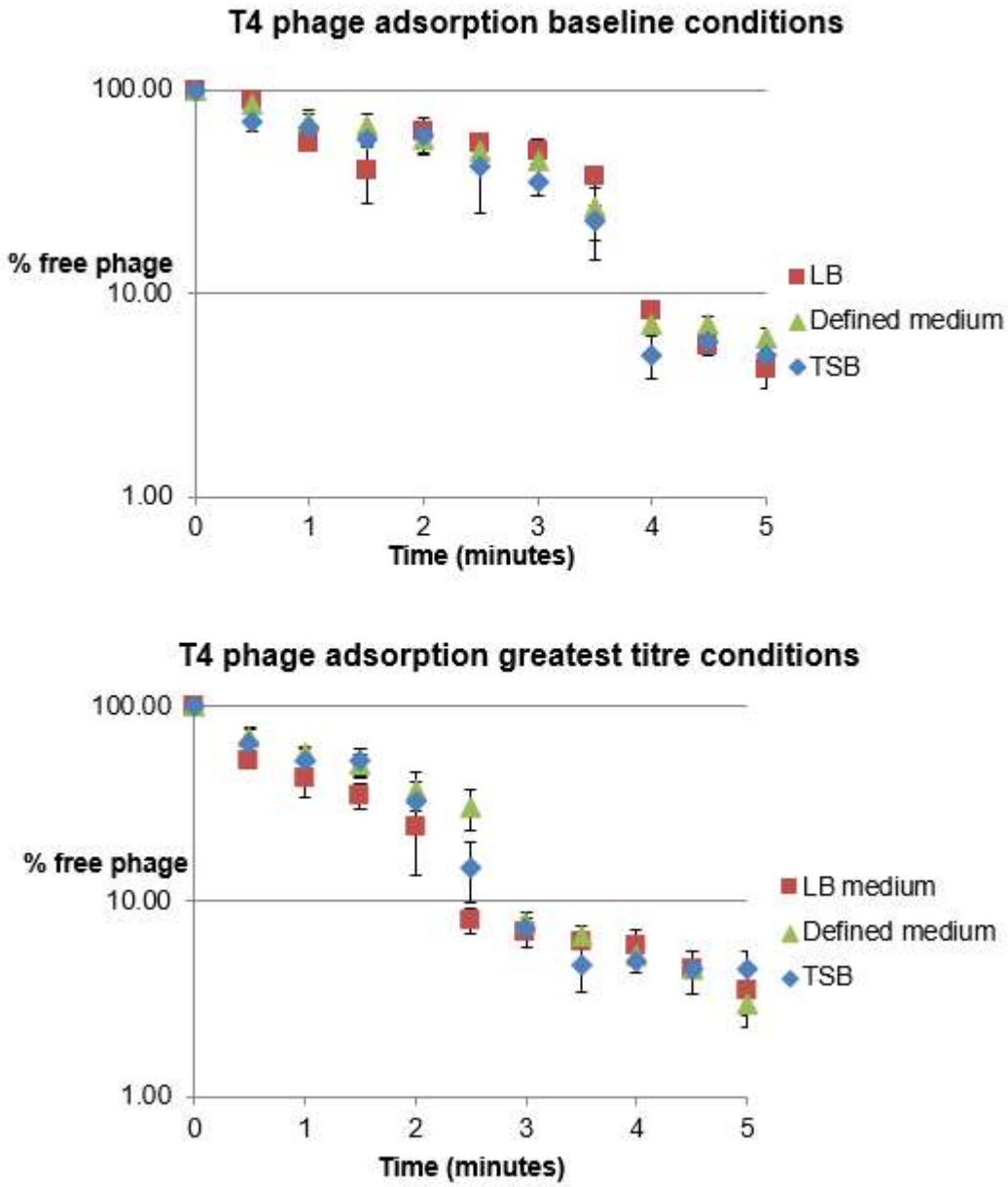



***Figure 3.28.*** *A graph to show the rate of adsorption of T4 phage to its host cell using LB media, defined media or TSB media using A) Baseline conditions B) Greatest titre conditions. The error bars show 1 standard deviation of a duplicate experiment each with duplicate plaque assay enumerations. The graph shows the infection rate for the first 5 minutes of the infection. Error bars show 1SD of a quadruplicate experiment with each experiment enumerated twice i.e. each point represents n=8 measurements.*

***Table 3.8.*** *A table to show the burst size achieved when using LB, defined and TSB media using the baseline and greatest titre conditions for T4 phage*

| Conditions | Media | Burst size (±1SD) |
|---|---|---|
| Baseline | LB | 80.7±9.3 |
| | DM | 75.5±4.2 |
| | TSB | 48.45±3.15 |
| Greatest titre | LB | 113.5 +- 1.5 |
| | DM | 104.4±6.9 |
| | TSB | 86.6±10 |

There was a minor observable difference in the adsorption rate but no significant difference between LB/DM burst size at baseline conditions. Also, there was a significantly reduced burst between LB/DM and TSB at greatest titre conditions and LB/TSB at baseline conditions, $p<0.05$ using a paired t-test. Despite this, the graph shows no difference in the rate of adsorption between each media, suggesting that the conditions play a significant role in the burst size given the increase in burst between them and that there was no difference in adsorption. It is also interesting to note the differences between the baseline and greatest titre adsorption between the media used as its in line with previous results presented here i.e. the greatest titre gives a higher adsorption than the baseline conditions for LB/BHI media. Figure 3.24 shows a significant increase in titre between LB and TSB titre, but a significant decrease in burst as seen in table 3.7 and 3.8. The media may have increased cell size given its richness and caused a reduction in burst (Parada *et al*, 2006). Figure 3.29 shows the experiments for phage K.

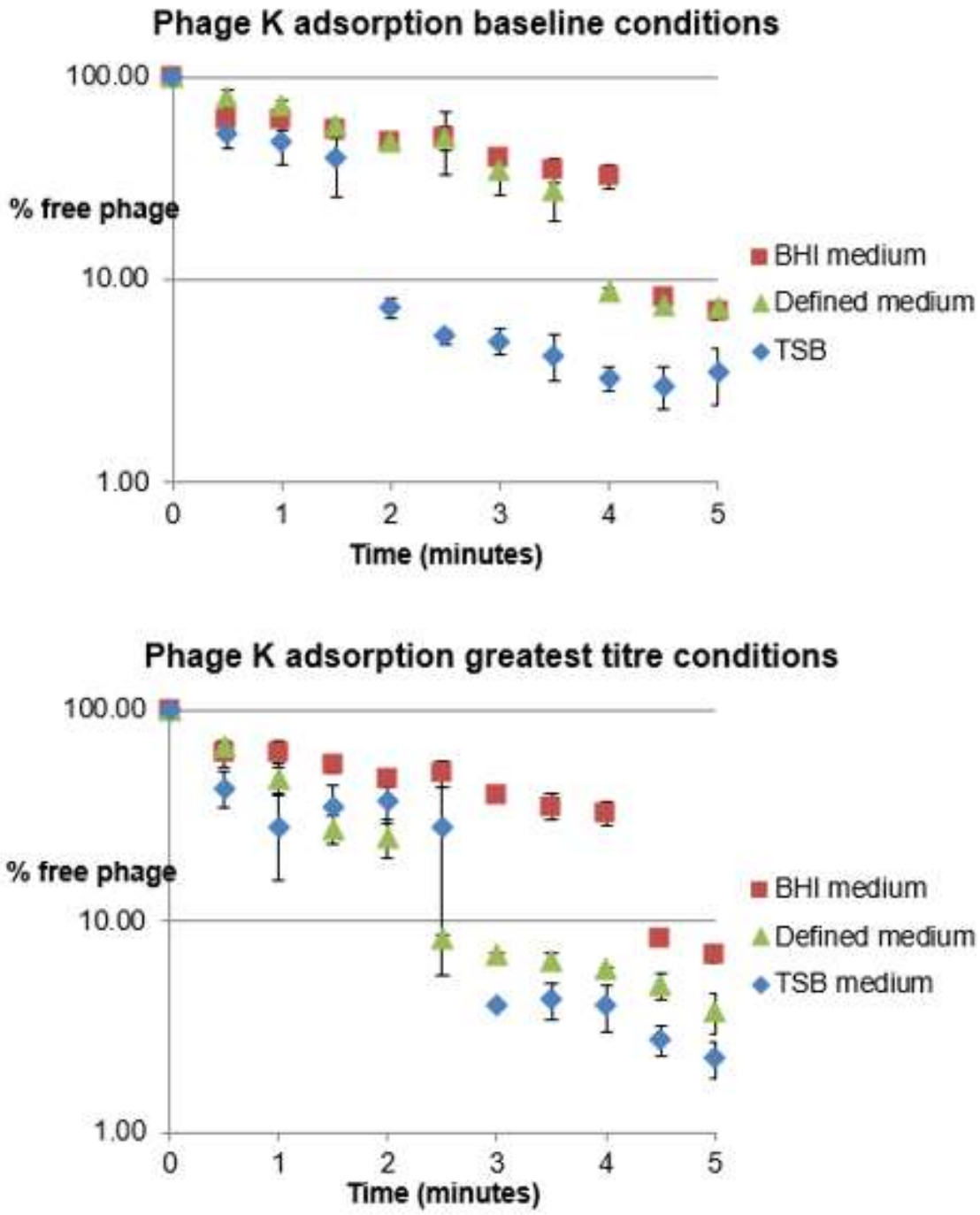



***Figure 3.29.*** *A graph to show the rate of adsorption of phage K to its host cell using BHI media, defined media or TSB media using A) Baseline conditions B) Greatest titre conditions. The error bars show 1 standard deviation of a duplicate experiment each with duplicate plaque assays. The graph shows the infection rate for the first 5 minutes of the infection. Error bars show 1SD of a quadruplicate experiment with each experiment enumerated twice i.e. each point represents n=8 measurements.*

***Table 3.9.*** *A table to show the burst size achieved when using LB, defined and TSB media using the baseline and greatest titre conditions for phage K*

| Conditions | Media | Burst size (±1SD) |
|---|---|---|
| Baseline | BHI | 79.5±6.5 |
| | DM | 62.2±3.1 |
| | TSB | 66.65±3.35 |
| Greatest titre | BHI | 111.8 +- 0.7 |
| | DM | 63.3±8.1 |
| | TSB | 79.95±3.35 |

Significant differences are seen in the burst size at baseline conditions between DM/TSB and BHI as shown in table 3.9. However, TSB showed the greatest rate of adsorption even though there was a significantly lower burst between TSB and BHI, $p<0.05$ using a paired t-test. Despite the greatest burst being in the BHI media, it gave a significantly lower rate of adsorption from 3 minutes onwards compared to DM and TSB for greatest titre conditions. Also, the greatest titre conditions showed a significantly higher burst in TSB than defined medium whilst from 3 minutes onwards there was a significantly higher rate of adsorption. BHI gave the greatest significant burst but it had the lowest adsorption. Abedon *et al*, 2001 showed that there can be an inverse relationship between host cell density and the adsorption for some phage if the density is too great, using a simulated model i.e. a richer media (TSB) may increase cell density and decrease burst. However, despite their model being based on a large number of repeats, their data was not based on experimental runs which would be needed for validation.

One study in 2010, examined the effect of media on adsorption of T4 phage to *E. coli B*. Their study used mineral salt medium, nutrient broth and TSB media to examine the effect of host growth on adsorption. Their results showed that TSB gave the greatest rate of adsorption compared to the other media used. Given that TSB was the richest media of each, this may have allowed the cells to grow larger, resulting in the increased adsorption efficiency. After 5 minutes of infection, around 99.9% of phage had adsorbed to the host cell.

However, they used a far lower MOI, 0.1, compared to our 2.5. This meant that they only achieved a titre of $1x10^{11}$ pfu/ml (Storms *et al*, 2010).

**3.15 Conclusion**

This chapter has focused on T4 phage and phage K propagation with the aim to improve their titres at small scale through simple manipulations of the culture parameters. T4 phage has previously been used in human trials and therefore, was an ideal candidate to improve its bioprocess. If future larger scale clinical trials were to be conducted, one of the primary concerns would be optimising production in single batch cultures as running multiple batch experiments disadvantages including differences between cultures, different titres or characteristics or worse, risk of contamination. Additionally, small scale experiments are often high throughput, cheap, easy, cost effective, less time consuming and as presented here, conditions can be optimised. Therefore, these small-scale experiments are needed in order to move forward to produce larger volumes.

The initial aim of this experiment was to examine the KPIV to examine their effect on titre and validate the greatest titre conditions to show a reliable and robust process for shake flask scale phage propagation which was completed. A full factorial DoE was chosen to initially give an overview to a process and give an insight into where optimal parameters lie for production. It was useful as there were a number of factors that contributed to the phage bioprocess whilst within each factor, different levels. Each were taken from the literature and due to the wide variety, DoE was also useful as contour plots were produced. Figures 3.1 and 3.2 estimated the levels within each factor, where the greatest titres of both phages were achieved. Once the DoE was complete, the conditions which gave the greatest phage titre was validated in triplicate over three days to provide confidence and reliability in the results. OFAT experiments were used to examine other conditions such as further reducing the MOI and time to try and further improve the bioprocess.

The temperature at which the infection took place, a factor that is hugely understudied in an already understudied area of bacteriophage research, had a significant influence on titre. The temperature curve graphs showed that a significantly improved phage titre could be achieved by using 28$^{o}$C, compared to the most commonly used 37$^{o}$C. Two previous studies by Grieco (2009 & 2012) showed a temperature reduction may improve the phage titre, but despite the first a decade old, 37$^{o}$C still seems to be the temperature of

bacteriophage infection of choice for authors. This highlights the importance of this study as it has added further clarity to the T4 and phage K infection conditions. This work also built on the studies by Grieco as it examined the time of infection for both T4 and phage K, a key area of bioprocessing optimisation as reducing it improves process time/efficiency. This factor has rarely been examined and therefore adds novelty to the field. It is hoped that this study has laid a foundation for future bioprocessing bacteriophage work that will examine how to further improve the bacteriophage bioprocess efficiency. This work was one of the initial aims of the chapter and was successfully completed.

Within the phage literature, the gold standard method of enumeration is the phage titre measured by plaque forming units (pfu/ml). However, a further look into the process to determine the phage output per input cell at the point of infection, and phage output per input phage showed that, the phage output per input phage is more representative than phage titre. These two measures are previously unreported in phage literature, but examining how many phage are produced from each phage that infects the culture, gives a far clearer understanding of how efficient the bioprocess is. For example, although MOI 0.1 and 1 at 150rpm, 4 hours, 28$^{o}$C gave similar phage K titres, by using the phage harvested per input phage, it was a better measure as 10x less phage were used in the process that used an MOI 0.1. Plaque forming units gives no standardised measurement of unit whereas these values do.

In the T4 phage bioprocess, the data showed that the lowest levels used, in the DoE, for the MOI and time gave the lowest phage harvest per input phage. However, an experiment was run to determine if a further MOI and time reduction for phage K could give similar values. Although significantly reduced titres were seen when there was a further time and MOI reduction, the study was important to show that there was a cut-off point at which the highest phage harvest per input phage could be achieved. The differences in MOI seen may be accounted for my cytokines or interleukins which may supress propagation at higher or lower MOI for different phage (Weber-Dabrowska *et al*, 2000, Van Belleghem et al, 2017).

It is interesting to see that there was a lack of a unified method for the phage fermentation. Tables 3.3 and 3.4 show the multiple levels of each variables currently used within the literature and therefore there was a need to determine the factors which gave the greatest titres. All phage biologists may benefit from using these

conditions to obtain the greatest possible titres of phage. Additionally, a similar methodology could be carried out for other phage which are of interest to others.

One advantage of completing the experiment with different phage was to show that there were differences between the two organisms and their fermentation must be carried out at different levels for optimal propagation. Therefore, the greatest titre conditions shown for T4 and K are not standard for all phage and it cannot be assumed that by using the conditions presented here, will be the optimal conditions for other phage. One difference was the MOI needed to obtain the greatest titres and the agitation used. Additionally, the temperature study showed a range of temperatures could produce the greatest phage K titre whereas there was a more defined peak in titres for T4 phage. Additionally, the TSB media study showed significant differences between the organisms, T4 showed an improvement in the titre whereas phage K showed a significantly reduced titre. The differences can be accounted for by ultimately being different organisms and having different host organisms, but it is interesting to note these differences and shows the importance of repeating this experimental work for all phage of interest to others, especially those with clinical importance.

Overall, the study showed that there was a lack of significance between many of the levels used for both phage in their DoE experiments. Despite this, the study was able to provide a useful basis for the further experimentation, such as the conditions that should be taken forward for further investigation. Table 3.10 summarises the greatest titre conditions. Additionally, looking at the phage harvest per input cell and phage harvest per input phage was also important and it is hoped that these values will be given more importance in bacteriophage bioprocessing in the future.

The aim of this chapter was to examine conditions which may improve the bacteriophage titre achievable. Compared to current literature values, statistically significant increases in titre have been achieved and are some of the current greatest achievable titres for T4 and phage K. A model has been established that is reliable and robust for the small-scale manufacture of the two phage. The final aim of this chapter was to examine the kinetics to gain insights into the causes of differences seen. The kinetic investigation showed that there can be significant improvements in the greatest titre conditions compared to the baseline which may explain why there is an increase in titre.

***Table 3.10.*** *A table to show the greatest titre conditions for T4 and phage K to be taken forward when LB and BHI media was used for T4 and phage K respectively*

| | **MOI** | **Agitation (RPM)** | **Time (Hours)** | **Temperature (°C)** |
|---|---|---|---|---|
| **T4 phage** | 2.5 | 225 | 3 | 28 |
| **Phage K** | 0.1 | 150 | 4 | 28 |

**3.16 Chapter bridge**

The work provided here has examined the shake flask phage infection bioprocess. By examining the conditions at which the infection takes place, it allows a determination of those conditions to be used to produce the greatest titres of T4 and phage K. It was important to run the experiment for multiple phage to show that the methodology could be used and may be appropriate for future investigations. The kinetics were also examined and gave a possible insight into the reasons why greatest titres can be achieved using certain conditions. Whilst the shake flask experiments are useful and offer a high throughput methodology for phage fermentation, the next chapter will examine the scale up of the bioprocess which is a challenge that must be overcome for large scale phage manufacture, and important for the increasing demand of phage therapy.

Penicillin is the most notable antibiotic that in its early development was hindered due to the production issues and faster development could have increased the number of lives saved (Ligon, 2004). If a new drug candidate is identified then it is important that production scale up is not a limiting factor. Therefore, this work next focuses on scale-up of phage manufacturing

# Chapter 4 -

# Scale up of bacteriophage infection

Chapter overview

This chapter aims to take the baseline and greatest titre conditions from the small-scale shake flask bacteriophage bioprocess and translate them into larger stirred tank bioreactors for both T4 phage and phage

K to improve yield. The work presented here will focus on batch bioprocessing since there is very little previous work and it is important to scale up the bioprocess into a bioreactor to achieve greater yields. The chapter will also explore the scale up process of the T4 and phage K bioprocess examining it in a 5L stirred tank bioreactor (STR) whilst taking into account conditions such as pH and dissolved oxygen (DO%) which can more readily be controlled in the 5L bioreactor. It is less common for the pH and DO (%) to be controlled in shake flask systems and these parameters will be examined to determine if they have any impact on the phage infection. The greater degree of control in the 5L, gives a specific advantage of scale up. Additionally, lowering the window of variation by improving control may allow the titre to be less variable. Their contribution to the phage bioprocess will also be examined by investigating their effect on the kinetics of infection; burst and adsorption.

**4.1 Introduction**

Bacteriophage manufacture tends to be run in batch or fed-batch bioprocesses in multi-use reactors and the research is still in early stage of development. Once these processes are better understood, then there is likely to be greater use of single use vessels and continuous bioprocesses (Chon & Zarvis-Papastoitsis, 2011). For small scale stirred tank bioreactor non-clinical production work, stainless steel glass bioreactors seem to therefore be an obvious choice given the early stage of the research. Moreover, due to the low oxygen transfer that occurs arising from less efficient mixing and the open question of whether single use bioreactors are less appropriate for scale up than multiuse bioreactors, glass autoclavable STR's were used in these experiments (Al-mashhadani *et al*, 2015). Therefore, where high oxygen demands are needed i.e. in microbial cultures, glass autoclavable reactors should be used.

The nature of stirred tank bioreactors has several operating boundaries, which are summarised in figure 4.1. The figure shows that as aeration in the culture increases, foaming and cost start to affect the culture and cost-efficiency of the product. An increase in agitation will lead to more cells being damaged and in contrast low agitation may also lead to pockets of $CO_2$ within the culture which lower the pH at the pocket site. Combinations of agitation and aeration will have an impact on the cost, titre and cell health within the bioprocess. Each of the factors which in combination may contribute to a lower yield although bioprocess engineering approaches can be used to overcome these challenges within larger stirred tank bioreactors (Shuler *et al*, 2017).

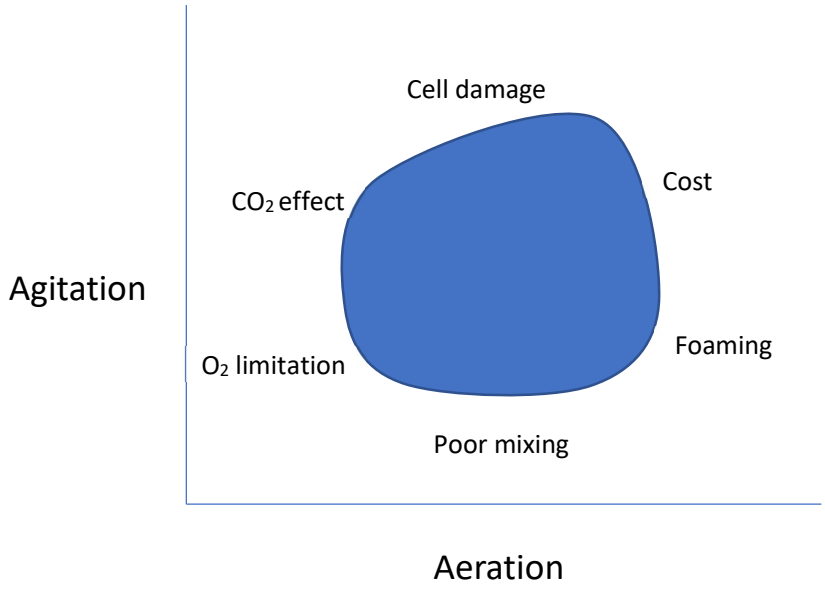



***Figure 4.1.*** *Boundaries to scale up on agitation and aeration {adapted from bioprocess engineering basic concepts (Shuler et al, 2017)*

Before beginning any treatment with bacteriophage, the phage will need to be manufactured. Whilst the small-scale experiments were useful and showed that by altering the conditions, statistically significant increases in titre could be made, scale up into stirred tank reactors will also be useful. They provide the potential to reduce variability between the cultures and also allow an increase in yield. By producing more phage in a single batch i.e. in a bioreactor, this will allow more phage to be created and reduce the overall cost, rather than running multiple shake flask cultures. In experiments presented in this chapter a 3L working volume was used, 150 times more than the 20ml culture in the shake flasks. It is clearly more viable to run a single bioreactor culture than 150 shake flasks. Within the shake flask model, variability will be found within the working volume, initial bacterial concentration, phage infected into the culture and potentially even during the culture i.e. cold spots within an incubator or differences in mixing. Each of these parameters will alter the final phage produced and it is therefore clear why running a single culture will be more appropriate.

One further advantage of running a bioreactor culture compared to multiple shake flask cultures is that one batch will be produced which could be used for a single treatment group. Previously it has been shown that $1x10^{14}$ phage was administered per animal (Samoylova et al, 2017). However, if 10 animals were to be treated,

this would be outside of the range of phage produced in our shake flask model and therefore larger cultures would be needed. Whilst governmentally regulated current clinical trials often fail to mention the amount of phage used in a culture, studies have used ~ $1x10^{12}$ phage for treatment of humans per kg of body weight (Morris et al, 2012). Therefore, if each adult weight roughly 70kg and multiple participants are needed, it is clear to see how the number of phage can rapidly increase showing why more phage will be needed to be produced.

Whilst there have been both batch, fed-batch and continuous phage models studied in bioreactors, this work only examined batch culture whilst it may be a platform for further work to increase the titres of phage produced using the later models (Mancuso *et al*, 2018, Roy *et al*, 2018, Mutti & Corsini 2019).

It is important to improve the bioprocess at a small scale to understand the basics of the process. However, the scale up process may be even more important to increase the yield. Chapter three improved the small-scale production of T4 and phage K, and these are relevant to treatment of antibiotic resistant microorganisms. For phage therapy to be used commercially and clinically, their large-scale production must be explored. The manufacture of phage remains highly understudied to this day. Korsmeyer, 2016 highlighted some of the key challenges for manufacture of nanoparticles, which can be extended to any emerging therapeutics. The key challenges included the scale-up, optimal production and improving manufacturing efficiency, if these are not addressed, then production may be hinder. It is therefore important to look at the challenges of scale up for phage manufacture. As previously highlighted, moving from a shake flask to an STR, can drastically alter the product output if suitable changes in scale up are not made. Therefore, scale-up must be thoroughly explored when translating any process from a shake flask to stirred tank bioreactor.

**4.2 Aims and objectives**

1) To successfully scale up the shake flask baseline and greatest titre conditions into a 5L bioreactor (3L working volume) and establish a scale-up model
    a. To achieve statistically improved or equal titres in the 5L bioreactor as to the achieved titres in the shake flask. This will show the establishment of a scale-up model providing it is reliable

2) To design and run a 5L DoE experiment for T4 phage and phage K in the 5L bioreactor.
    a. A DoE to determine the conditions of agitation and pH to show if they contribute to the phage infection and how they improve the titre
3) To examine the effect of increasing the rate of sparging on the phage bioprocess
    a. To select the rate of sparging which gives the greatest titre of bacteriophage and show its effect on the kinetics
4) To examine the kinetics of the infection in a 5L bioreactor
    a. To determine whether the kinetics contribute to the increase in titre

**4.3 Initial scale up**

Scale up of biological processes from shake flask scale to an impeller based stirred tank reactor can be based on several things; mixing time, tip speed or power per unit volume (energy dissipation rate) (Johnson *et al*, 2014). However, achieving similar titres in the first instance between culture systems is key. If similar titres are unable to be achieved, it may be necessary to run experiments examining each of the above factors in order to optimise the process. Given the differences in geometry between the bioreactor and shake flask there will be differences that affect the mixing and therefore the culture. The work presented here did not take into account the energy dissipation rate or tip speed but assumed that given the length of time of the experiment, complete mixing would be achieved in both culture systems. However, future work must examine the geometry much closer in order to create a more reliable and robust bioprocess which could result in greater titres that those achieved here (Rodriguez *et al*, 2014).

Difficulty of scale up is a key point that is often failed to be fully taken into account and this needs to be overcome for a product to become clinically viable. Within the microbiology and cell culture industry, large scale bioprocessing has been well researched and for many decades (Varley, 1999, Enfors, 2001). High throughput processes and large-scale manufacture allow the biopharmaceutical industry to create large batches of therapeutic products. Manufacturing larger batches of a product in a single reactor may be more beneficial than multiple reactors since it may prevent risk of contamination, differences in final product characteristics or yield.

The challenge of scale up is that, generally, most processes are non-linear. It is unwise to assume that the conditions from one bioprocess can be scaled up into larger reactors as there are several factors that must be taken into account. These include, amongst others, the reaction kinetics, chemical equilibrium, fluid and thermodynamics. Incrementally increasing the scale, e.g from 5L to 10L to 20L to 50L, can prove to be valuable (Madsen *et al*, 2017). This is, however, often not viable in terms of cost and time when quickly trying to scale up a process. It is, however, important that full experimental domain of the process has been fully understood as part of the scale up process. Further experiments would be needed in the future, for larger stirred tank bioreactors to improve the yield.

The experiments here used a 5L Sartorius biostat B plus with a 3L working volume for all experiments. Six Rushton impellors were used, which is fairly typical in microbial bioprocesses. Mammalian cell culture would typically use marine blade impellors for axial flow which gives gentler mixing to prevent cell damage. However, oxygen transfer is less of a requirement in mammalian cell work given that they require less oxygen as lower cell numbers are achieved compared to microbial work (Hewitt & Nienow, 2007). Additionally, microbial cells are generally more robust and less susceptible to sheer damage whilst mammalian cells have no protective cell wall (Betts & Baganz, 2006). For microbial bioprocesses, a 3L working volume may seem to be a small volume, although current bacteriophage research does not typically extend beyond shake flask cultures (Ferrara *et al*, 2015).

If a concentration of $1x10^{12}$/kg phage were needed for treatment of a patient, it is conceivable that the amount needed could be achieved in a single shake flask for 1 individual. However, using bioreactor models will allow less batch to batch variability between cultures and more phage produced for future use.

As phage rely on their host cells for their propagation, the translation of the growth of the host organism will be an important factor for the successful propagation in a stirred tank system. As the small-scale experiments had shown an improvement in phage titre by using certain factors, the work already presented thus far has shown a degree of optimisation of the phage bioprocess. However, the scale up into stirred tank bioreactors was yet to be tackled. The baseline and greatest titre conditions were examined in a 5L bioreactor. Figure 4.2 shows the T4 phage scale up process.

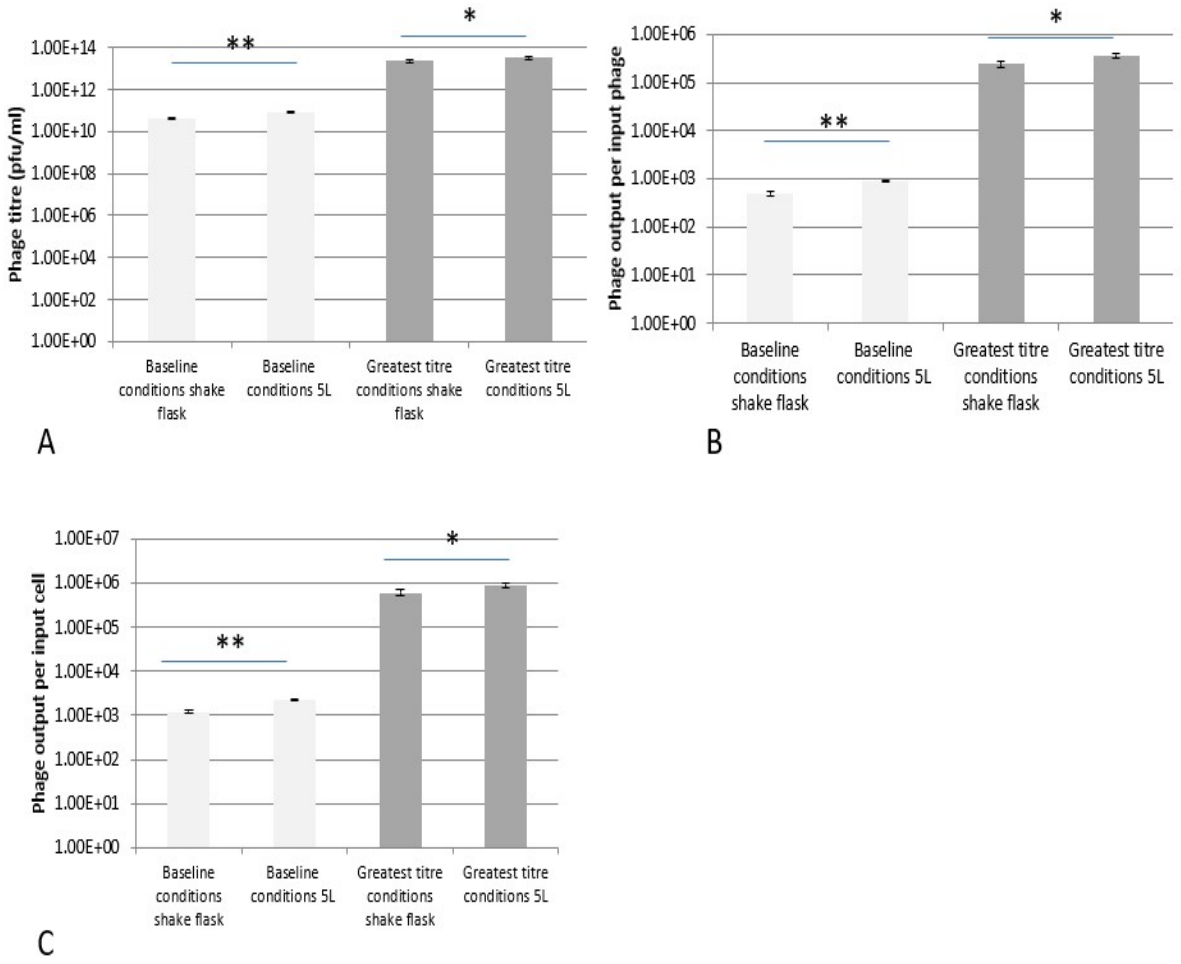



***Figure 4.2.*** *Baseline and greatest titre conditions run in the shake flask and 5L for the T4 phage infection process. A) Phage titre (pfu/ml) B) Phage output per input cell C) Phage output per input phage. The pH was maintained at 7.0 for both cultures. Experiments were run in triplicate (n=3), with enumeration plaque assays run in duplicate. Error bars show 1 standard deviation. * p=0.05, ** p<0.001.*

Figure 4.2 shows bar charts for the T4 phage bioprocess using the baseline and small scale DoE determined greatest titre conditions in shake flasks and the 5L bioreactor. The graph shows the phage titre (pfu/ml) and normalised to phage output per input cell and phage output per input phage. The data showed a statistically significant increase between the baseline and greatest titre T4 phage titre and between the shake flask and the 5L bioreactor, $p<0.001$ and $p=0.05$ respectively. When compared to a shake flask, there are a number of advantages when using an STR; a larger volume of product produced i.e a greater yield, a more throughly controlled environment for homogenity and less handling which may prevent contamination. Achieving >1 x $10^{13}$ pfu/ml in a STR system, shown in figure 4.2, is some of the current greatest titres achieved

and shows the advantage of optimisation and scale up (Ali *et al*, 2018). However, the reliablity of the experiment could have been improved, as only a single 20ml sample was taken, and concentrated to a final 1ml volume. Taking multiple samples from the culture and enumerating each may have been more appropriate.

Bourdin *et al*, 2014 used a 16L fermenter for their T4 phage infection. Agitation at 400rpm and sparging with 1L/min air was used in culture of the phage. Whilst only a 10-fold decrease in titre was achieved, between their process and our baseline experiment, it helped shape our experiment and so 400rpm was used as the upper level and 1L/min air was used to sparge the culture.

The graphs in figure 4.2 show that there was a significant increase in phage output per input cell and phage output per input phage between baseline and greatest titre conditions in shake flasks in both culture systems. This was because the normalised values were calculated from the phage titre, $p<0.001$ and $p=0.05$ respectively.

Our 5L experiments maintained 0.25 OD600nm as a point of infection similar to the shake flask experiments and an effort was made to maintain an infection within a 0.03 OD600nm range. The small range of the point of infection may contribute to the small error bars seen in the bioreactor cultures. Upon completion of the T4 phage 5L scale up, experiments were completed for the phage K bioprocess. Similar to the T4 phage scale up, translating the phage K process into a 5L bioreactor was important to show that this methodology can be used for multiple phage which may be applied and used by others for their specific bacteriophage bioprocess. Figure 4.3 shows the scale up from the shake flask to the 5L bioreactor for baseline and greatest titre conditions for phage K.

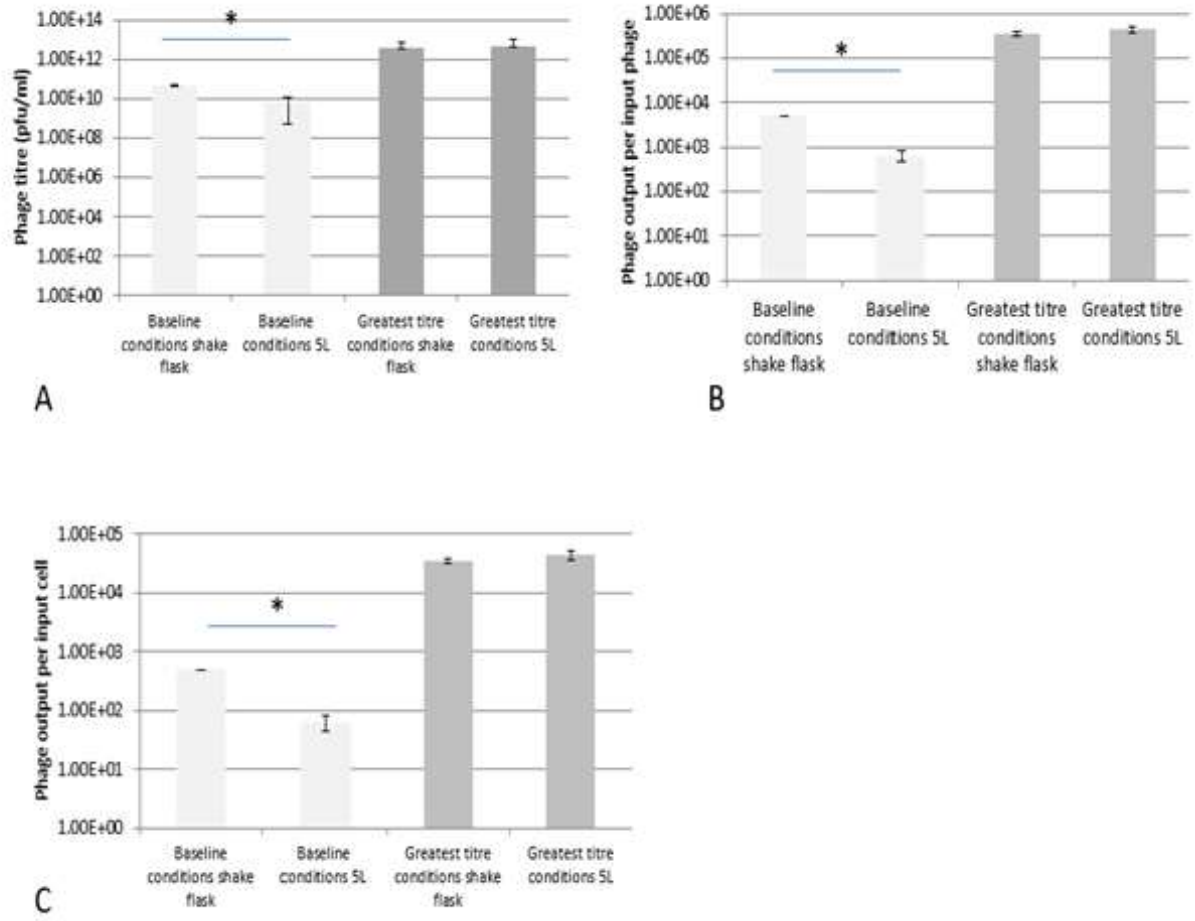



***Figure 4.3**. Baseline and greatest titre conditions run in the shake flask and 5L for the phage K infection process. A) Phage titre (pfu/ml) B) Phage output per input phage C) Phage output per input cell. Experiments were run in triplicate (n=3), with enumeration plaque assays run in duplicate. Error bars show 1 standard deviation. *p<0.05*

The phage K scale up plots show no statistically significant differences in the culture systems between the greatest titre conditions. However, a statistically significantly reduced phage titre in baseline conditions in the 5L compared to the shake flask work was found, $p<0.05$ using a paired t-test. Given that there was no statistically significant difference between the greatest titre conditions, in the shake flask or 5L bioreactor, no further experiments in the scale up were completed. This result showed that the greatest titre conditions were scalable. It is also interesting to note the higher variation seen in the phage k baseline conditions, something that is observed in further experiments.

The scale up challenges of product manufacture has been well documented already. However, in practice, achieving similar titres can often be considered a successful process, especially if translated into a stirred tank bioreactor. A 5L STR can be considered a gateway to further larger STR cultures, given the differences in mixing and sheer damage caused by impellors compared to shake flask culture. Whilst there are differences between different volumes of STR, there is a much greater difference in mixing that occurs between impellors, in a stirred tank bioreactor and a shaking incubator agitating a culture. Our process has shown that using both the small-scale DoE determined greatest titre conditions, similar titres were achieved in a 5L compared to a shake flask. The small-scale experiments, used a 20ml volume whilst the scale up process used a 3000ml volume. Therefore, the process had been scaled up 150x with the total product yield and productivity (phage output per input phage) 150 times more in the STR than in the 20ml shake flask cultures. Whilst triplicate experiments were run, further experiments may be useful in order to improve statistical significance found.

Greater titres were been achieved in the 5L bioreactor when using the greatest titre conditions, over the shake flask possibly due to the greater control over the culture i.e. pH and $O_2$. There can be fewer differences in conditions which may affect the product output. This will improve cell growth, productivity as well as potentially product quality (Xu *et al*, 2017).

One of the main aims was to successfully scale up the process into a 5L stirred tank bioreactor which figures 4.2 and 4.3 show. Given the initial successful scale up into a stirred tank bioreactor, this paves the way for further experiments at the 5L scale and potentially future larger scale experiments. One challenge is the initial scale up given the differences in system but by making appropriate increases in bioreactor scale, similar titres can be achieved. As the scale increases, the conditions will need to be reviewed in order to continuously produce the greatest titres possible.

One study in 2011 used T4 phage infecting *E. coli DSM 613*, in 3L working volume in a biostat B, similar to our study. However, their study aimed to examine the purification of phage using chromatography. After a 5-hour culture at 37$^{o}$C, infecting with $2x10^{10}$ pfu/ml, around a $1.4x10^{10}$pfu/ml titre was reached. The authors acknowledged that a similar titre had been achieved as to the infected concentration, however, there was no control over pH, which dropped from 6.5 to 5.7 and a high titre was not the objective of the study. However,

they were able to optimise the point at which T4 cultures needed to be purified via HPLC based on the DNA in the culture from the lysed host cells (Smrekar *et al,* 2011).

A second study using *S. aureus* phage 44AHJD examined scale up. Initially, cultures were grown in a 250ml erlenmeyer flask, 50ml working volume. Using MOI 0.1, 200rpm, 37$^{o}$C and 16 hours, a titre of 1.5x10$^{7}$pfu/ml was achieved using LB broth, but when repeated in terrific broth 1x10$^{9}$pfu/ml was seen. Maintaining the MOI, but using 30$^{o}$C, 500rpm, 10 hours, pH 7 in a 1.5L working volume, 5x10$^{9}$pfu/ml was achieved in the fermenter. However, their study gave no indication or justification for the change in conditions between the shake flask and fermenter work. Despite this, it was one of the first studies examining a *S. aureus* phage in a fermenter (Kumar *et al*, 2012).

In 2015, Ferrara, showed their process for the production of phage antibodies. Through scaling up their process, they were able to produced more than 4 times the titre of their shake flask experiment in a 5L working volume in their fermenter. Whilst their process showed an increase in titre, they explained it was possibly due to the minimal variations between batch processes and a greater control over the process. However, obtaining equal titres between systems allows for additional experiments.

**4.4 5L DoE**

After the initial scale up was complete, it was important to investigate more readily controlled factors in the 5L bioreactor than in shake flasks (pH and DO%). With so few studies examining large scale culture of bacteriophage infection, this study will be hugely important for future T4 phage and phage K culture.

The agitation was an important factor to investigate as the change in scale, culture volume, mixing patterns and shear damage, will all be affected. During the shake flask infection process, ammonium, and other waste products release, may cause changes in pH consequently affecting the growth of the host organism or phage which may affect the final phage titre. Whilst the pH change was not measured in the shake flask infection, it can be assumed that there would have been some waste given off. However, controlling this in a bioreactor will prevent changes and may therefore reduce variation within the culture. This may be why a further improvement in titre was seen in this study (Alford, 2006). It should be noted that although controlling the pH and agitation in the bioreactor were important, it may have caused some variation in the final titres between

culture systems, as the pH was not controlled in the shake flask experiments and the agitation would have been different.

A two factor, general factorial design was created to examine the effects of pH and agitation on phage titre. Table 4.1 below shows the levels used. The conditions which gave the greatest titre conditions in the shake flask were used in the 5L; MOI 2.5, 3 hours, 28$^{o}$C for T4 phage and MOI 0.1, 4 hours, 28$^{o}$C for phage K. The levels used were to maintain a wide variation, which could later be narrowed down if necessary, given the difference in scale. Additionally, it was shown that the scale up had been successful for both organisms whilst it was important to use a wide variation of the agitation and pH range given the limited literature available, which was however used to determine factors in the DoE (Greico *et al*, 2009, Ferrara *et al*, 2015). Studies tend to focus more on *E. coli*/T4 phage which determined the *S. aureus*/phage K factors.

*Table 4.1. A table to show the factors and levels used in the 5L DoE*

| **Factor** | **Levels** |
|---|---|
| **pH** | 6.4, 6.9,7.4, 7.9 |
| **Agitation (RPM)** | 50, 225, 400 |

Firstly, it must be noted that the agitation is not scalable. Given that 225 and 400rpm were used in the shake flask model, this cannot be assumed to be the same as in the bioreactor. This is because the dimensions and mixing rates will vary. Additionally, if larger stirred tank systems were to be used, these agitations would then be changed again. The DoE consisted of 12 runs, and due to the time needed for a single run in the 5L bioreactor, each was run as a single experiment and enumerated with duplicate plaque assays. Given the time constraints

of the experimental side of the PhD, and the time needed for a single bioreactor run, single runs were deemed appropriate. However, the greatest titre runs were validated with additional runs later, figures 4.7/4.8. These were deemed to be the most important runs to be validated, providing that there was no statistically significant difference, those conditions which gave the greatest titre could be confirmed. The results from the experiment are shown below in the bar graphs, figure 4.4 and 4.5. The phage titre was again used to calculate the phage output per input cell and phage output per input phage similar to the shake flask experiments.

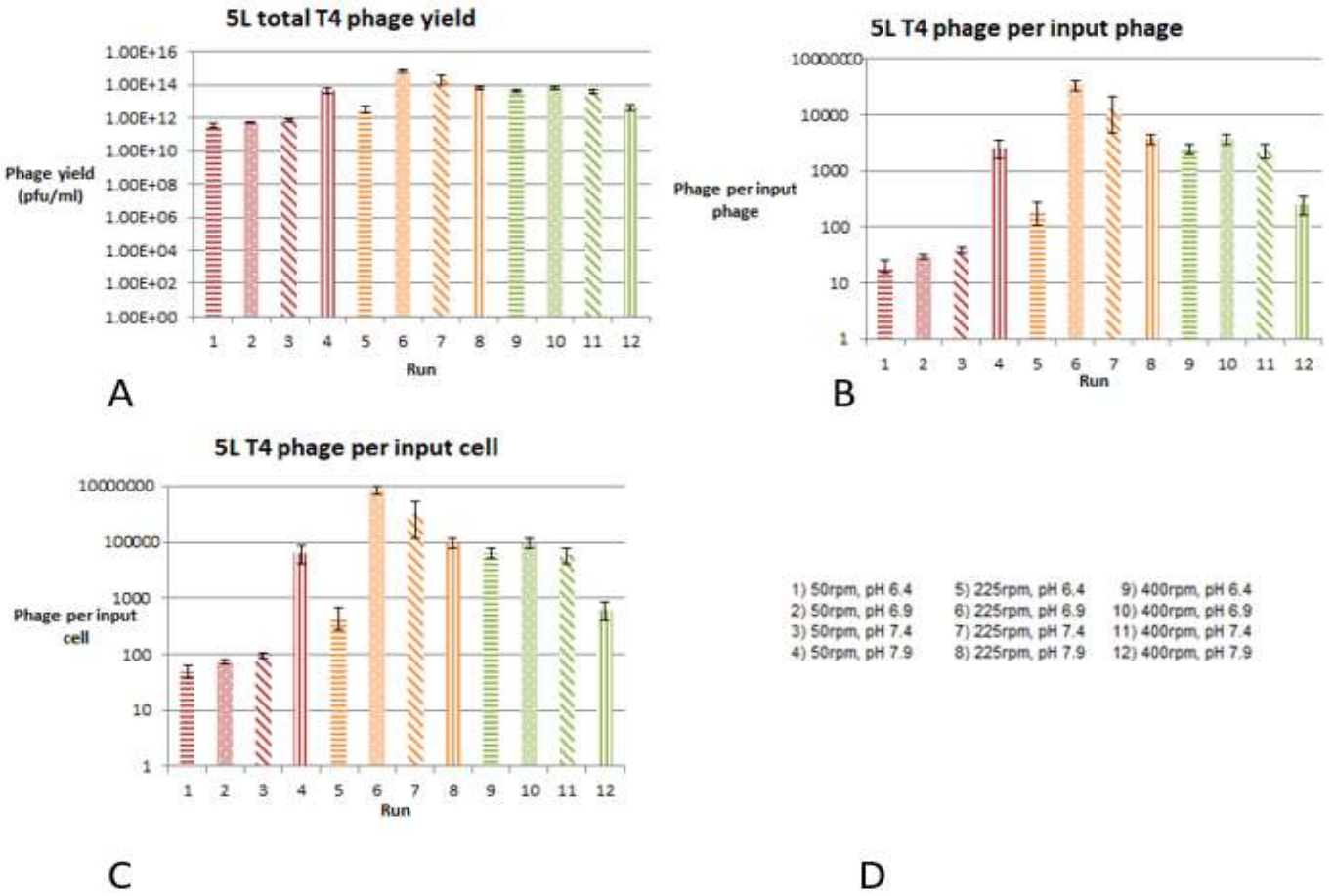



***Figure 4.4.*** *A graph to show the T4 phage titres achieved from each run in the 5L DoE A) Phage titre B) phage output per input phage C) Phage output per input cell D) Experimental conditions. Error bars represent 1SD of a single run with duplicate plaque assays. No statistical significance was found between any set of conditions. All red bars show the conditions where 50rpm was used, orange bars show the conditions where 225rpm was used and all green bars show the conditions where 400rpm was used.*

Figure 4.4 shows the T4 phage titre, phage output per input cell/ml and phage per input phage/ml. The graph shows 225rpm, pH 6.9 gave the greatest phage titre, run 6. No statistically significant difference was observed between each level of the two different variables (pH or agitation) used for the phage titre using a paired t-test when an average of the titres was used.

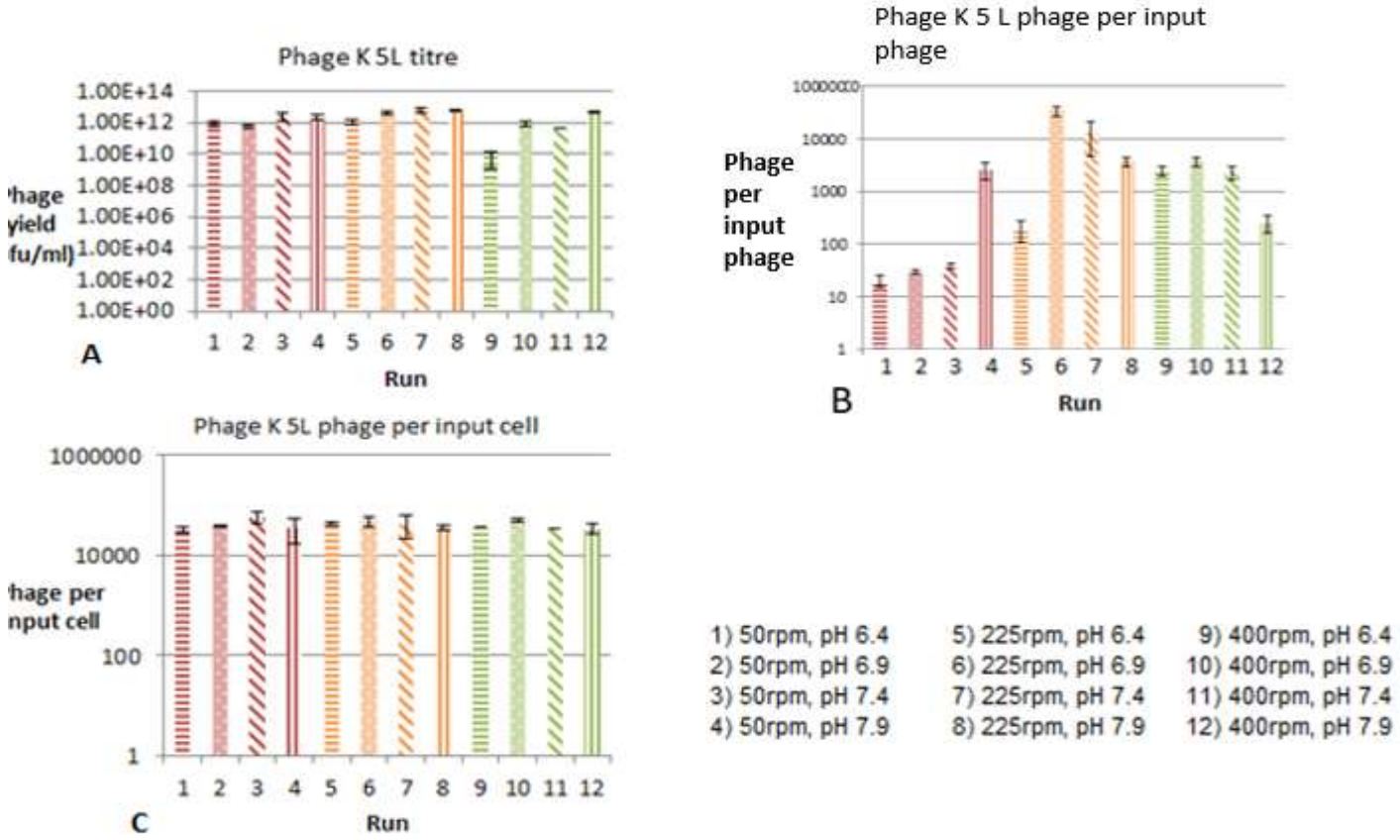



***Figure 4.5.*** *A graph to show the phage K titres achieved from each run in the 5L DoE A) Phage titre B) phage output per input phage C) Phage output per input cell D) Experimental conditions. No statistical significance was found between any set of conditions. Error bars represent 1SD of a single run with duplicate plaque assays. No statistical significance was found between any set of conditions. All red bars show the conditions where 50rpm was used, orange bars show the conditions where 225rpm was used and all green bars show the conditions where 400rpm was used.*

Figure 4.5 shows the phage K 5L DoE runs with the phage titre, phage output per s and phage output per input phage. Whilst no statistically significantly differences were seen between any of the conditions or levels used, $p<0.05$ using a paired t-test, the graphs show that 400rpm gave the greatest variation between each run, 9-12, compared to those at 50rpm or 225rpm. The graphs also show that run 7, pH 7.4, 225rpm gave the greatest titre ($6.67x10^{12} \pm 2x10^{12}$ pfu/ml). This was significantly higher than the baseline in the shake flask but similar to that of the greatest titre conditions ($6 \times 10^{12}$ pfu/ml). However, the overall yield was increased given the increase in scale. The surface and contour plot below in figure 4.6 were used to estimate where similar titres could be achieved based on predictions made from each level used from the T4 phage and phage K 5L DoE. Additionally, a main effects plot was created in order to determine the conditions which gave the greatest average mean titre as shown below. The result from figure 4.6 agrees with the full DoE bar chart analysis that pH 7.4/225rpm gave the greatest phage K titre and pH 6.9/225rpm gave the greatest T4 phage titre. It must be noted that each experiment was run as single runs.

This was the first DoE study examining the conditions that can be controlled in a 5L bioreactor for T4 phage and phage K which have shown a significant increase in titre compared to the shake flask conditions. Whilst DoE was previously a niche area, and few used it as an experimental design, in the wider bioprocessing industry it has now become more accepted. However, it is still not used widely in phage studies.

The graph in figure 4.6 shows output analysis of the DoE. Graphs A/B and E/F show the response surface curve and the levels where the greatest titres are achieved. They show the whole design space (levels used) and those levels which gave the greatest titre. Use of DoE at a 5L scale has never previously been applied focusing on the conditions shown here. However, it has been particularly useful to show the shape of the curves and the levels where the greatest titres were achieved are. Moreover, graphs C and D show a main effects plot and where the mean average titre achieved at each level. Although graph D shows the greatest average mean titre was achieved at 7.9, this did not take into account the agitation.

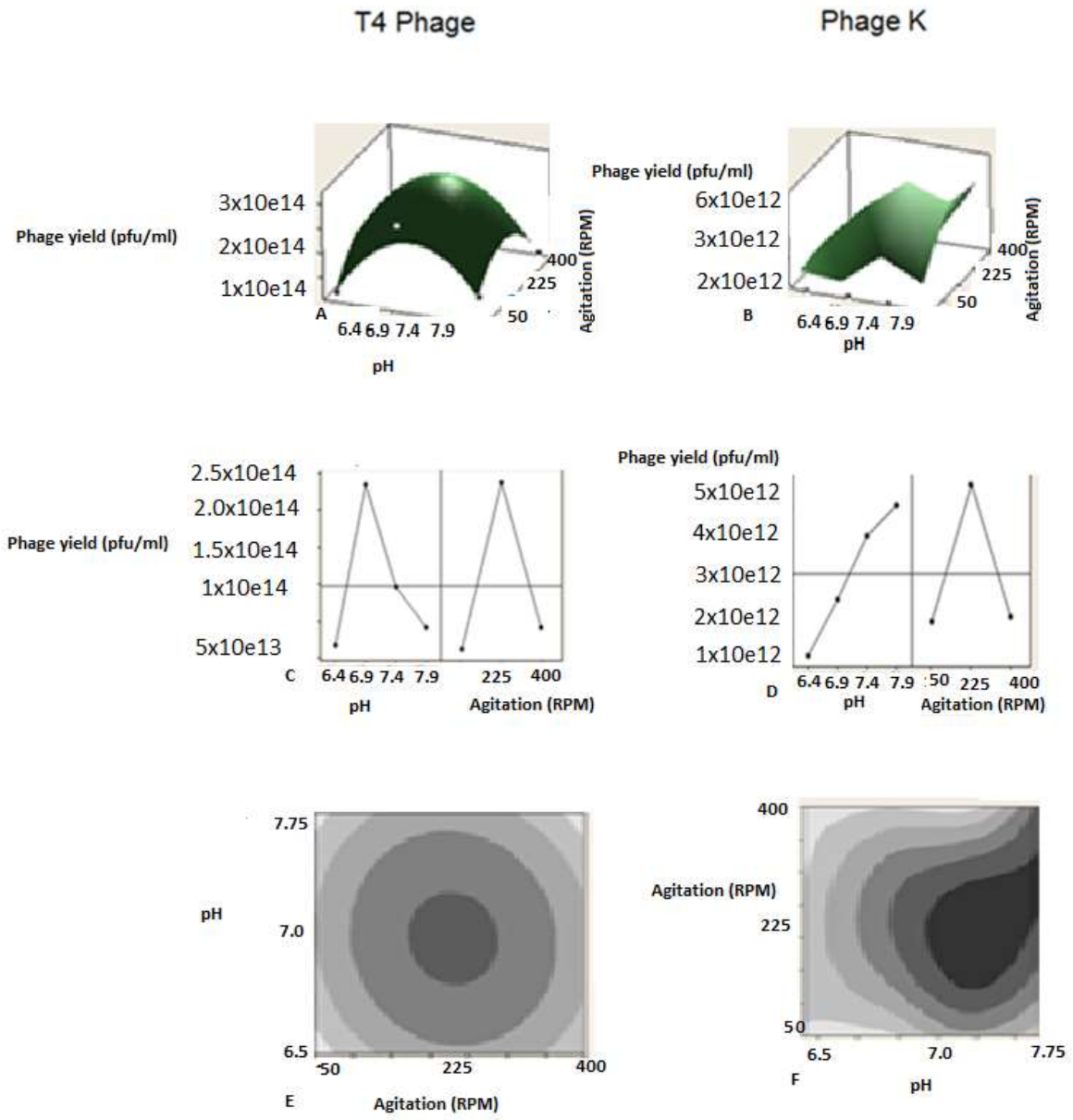



***Figure 4.6.*** *A figure showing the conditions where the greatest T4 phage and phage K titres are achieved in the 5L bioreactor, 3L working volume for pH and agitation (**A**) A surface plot showing the agitation and pH where the greatest T4 phage titre is produced (**B**) A surface plot showing the agitation and pH where the greatest phage K titre is produced (**C**) A main effects plot showing the level of pH and agitation where the greatest T4 phage titre is produced (**D**) A main effects plot showing the level of pH and agitation where the greatest phage K titre is produced (**E**) A contour plot showing the agitation and pH where the greatest T4 phage titre is produced (**F**) A contour plot showing the agitation and pH where the greatest phage K titre is produced.*

Figure 4.6 gives a surface, contour and a main effects plot for the 5L T4 phage and phage K 5L bioreactor DoE. The main effects plots show a line connecting the titre at each level is not parallel to the line along the x axis and therefore there is a main effect present i.e the factors (pH and agitation) have an effect on titre. Each plot takes into account all of the data from the DoE and shows the mean average result from the DoE at each level/condition. Each of the plots show that 225rpm and pH 6.9 give the greatest mean T4 phage titre. The greatest mean T4 titre achieved in the 5L bioreactor was $>1x10^{14}$pfu/ml. Given that the DoE sparged the culture with 1L/min air, there was a further statistically significant improvement (from figure 4.2 greatest titre 5L conditions) in the phage titre, possibly due to the availability of oxygen in the culture, $p<0.05$ using a paired t-test. Sparging of the culture will be examined in greater detail later in this chapter.

The phage K greatest titre from the DoE was $>1x10^{12}$ pfu/ml. pH 7.9 gave the greatest average titre as shown by the main effects plot in figure 4.6 D. However, there is some disagreement between the main effects and contour plot, the latter shows the relationship between pH and agitation and when both are taken into account, the greatest average phage K titre was at pH 7.4. To determine if a pH >7.9 could give an even greater titre, the conditions for phage K which gave the greatest titre in 5L were run at pH 8.4. However, there was a statistically significantly reduction in titre when the higher pH was used ($5x10^{11} \pm 1.6x10^{11}$ pfu/ml) $p<0.05$ using a paired t-test. This data is not shown as a figure. González-Menéndez *et al*, 2018 B showed that phage K may be more suited to higher pH levels which may explain why there is a greater titre seen at higher pH levels than T4 phage.

This study improved on the experiments in the previous chapter which examined a combination of factors to give the greatest phage titre in shake flasks. This experiment took forward those conditions into a 5L bioreactor, and further improved on the bioprocess by optimising the pH and agitation, further improving the titre. However, given the time needed for each bioreactor run, along with the time needed to clean the bioreactor and set it up for additional experiments and the experimental time available, only single runs were used for each condition. It would be important to repeat the DoE at least once again to ensure reliability of the results.

It was important to validate the conditions which gave the greatest titre as without validation, it would be unwise to base the conclusions made from a single run in a 5L bioreactor. Therefore, three further validation

runs were completed which took the DoE conditions that gave the greatest titre to validate and show variation between the runs. Each run was enumerated with triplicate plaque assays and error bars show 1 standard deviation. This was further important as it showed the reliability of a number of experimental runs.

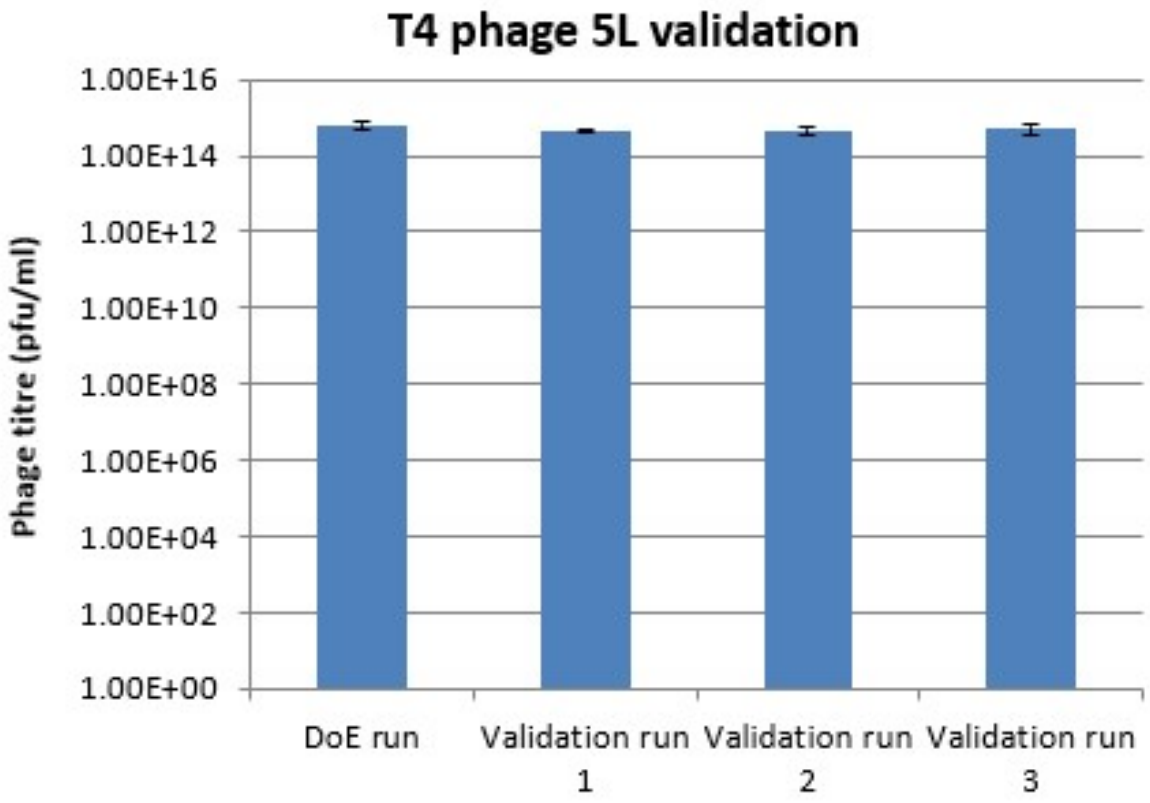



***Figure 4.7.*** *A graph to show the T4 phage validation runs which gave the greatest titre from the 5L DoE A) T4 phage titre (pfu/ml). Error bars show 1 standard deviation of a single experiment with triplicate plaque assay enumerations. No statistical significant difference was found between the initial DoE run and sny of the validation runs.*

Figure 4.7 shows that there was no statistically significant difference in each validation run compared to the initial T4 phage 5L DoE run which gave the greatest titre, pH 6.9, 225rpm and therefore it was assumed to be a reliable result.

The conditions which gave the greatest phage K titre (pH 7.4. 225rpm) were next validated in the 5L as shown in figure 4.8. Due to variation in titre between the first three validation runs; two further validation runs were completed.

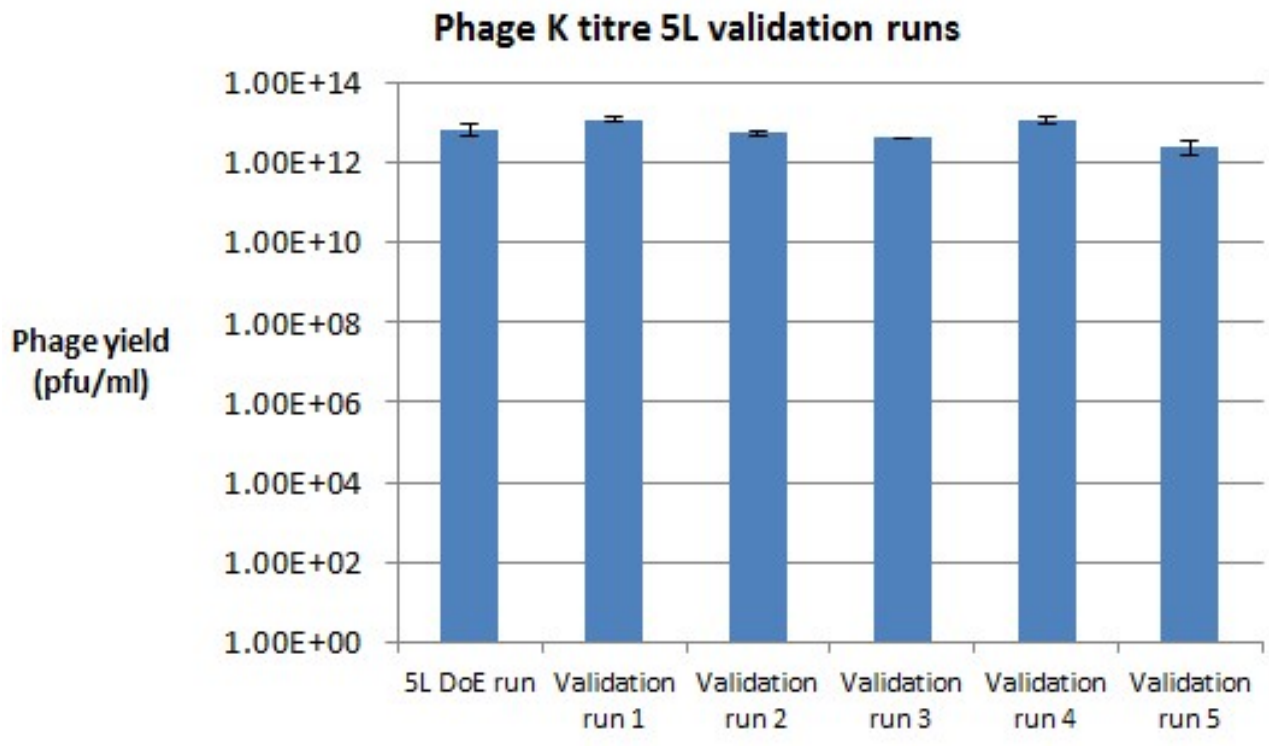



***Figure 4.8.*** *A graph to show the phage K validation runs which gave the greatest titre from the 5L DoE A) phage K titre (pfu/ml). Error bars show 1 standard deviation of a single experiment with triplicate plaque assay enumerations.*

Validation runs 1/2/3 (Figure 4.8) show a degree of variation between the runs, with around a 30% increase in titre between run 3 and run 1, $4x10^{12}$ and $1.13x10^{13} \pm 1.69x10^{12}$ pfu/ml respectively. Although there was no statistically significant difference (given the high variation in the 5L DoE run) between the initial 5L DoE titre achieved, and each of the validation runs, there was a high level of variation between the 5L DoE run and the 5 validation runs, $6.67x10^{12} \pm 2.05x10^{12}$ pfu/ml, $6.77x10^{12} \pm 3.82x10^{12}$ pfu/ml, respectively. However, run 8 (225rpm, pH 7.9) from the 5L phage K DoE, showed a similar phage titre but a far lower variation, $5.67x10^{12} \pm$

4.7x10$^{11}$, and was therefore validated in triplicate. Run 8 used pH 7.9 whereas run 7 used pH 7.4. The higher pH may reduce the growth of host organism and prevent the variability in its growth, thereby improving the overall phage titre seen (Jin & Kirk, 2018)

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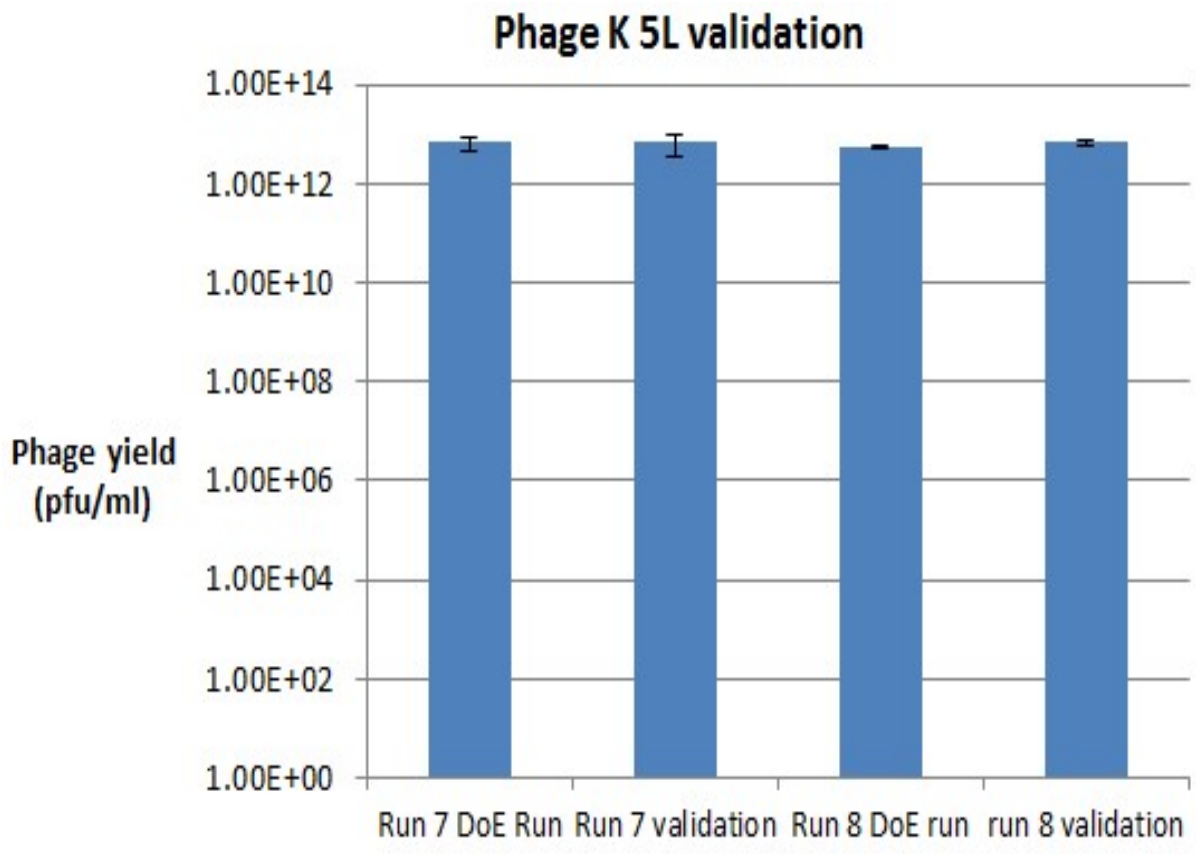



***Figure 4.9.*** *A graph to show the validation runs and the titre achieved from run 7 and run 8 from the phage K 5L DoE, given the variation seen in run 7. Error bars show 1 standard deviation of a single experiment with triplicate plaque assay enumerations*

Figure 4.9 shows the DoE and validation for runs 7 and 8 from the phage K 5L experiment. A wider degree of variation was found for run 7 than run 8 in both the DoE and validation experiments. In the DoE, a lower titre was found in run 8 compared to run 7, 5.7x10$^{12}$ ± 0.5x10$^{12}$ pfu/ml and 7x10$^{12}$ ± 2x10$^{12}$ pfu/ml

respectively. The run 8 validation gave a titre of $7x10^{12} \pm 1x10^{12}$ pfu/ml which was higher than the DoE indicating that similar titres can be achieved using either run 7 or run 8 conditions. It is important for manufacturing processes to use conditions that give lower variation so that titres can be achieved consistently. The validation between runs 7 and 8, however, showed no statistically significant difference in phage titre between run 7 (five replicates) and run 8 (three replicates).

The variation between runs 7 and 8 in the validation experiments may be accounted for by the harvest OD600nm readings, which shows the density of the culture made up of all intact cells, phage and debris. The validation for run 7 had a harvest OD600nm average reading of 0.5±0.1 compared to 0.49±0.01 for run 8. The lower variation seen for run 8 in the harvest optical density reading may account for the reduced variation in final titre. If there are more cells present in the culture within run 7, the rate of phage adsorption and propagation may be different and account for a difference in phage titre. Optical density may not be the most accurate measure and a colony forming unit assay may have been more appropriate. It should, however, be noted that optical density provides a quick, simple and easy measure.

No additional validation runs were needed for T4 phage. Throughout this work, we have shown that phage K can give had greater variations in titre achieved and some different results to T4 phage. One study examined the genome of phage K given variances seen in previous literature examining phage K. The paper showed that there were several uncharacterised open reading frames with their function unknown which may help to explain variation in the phage K results (Ajuebor *et al*, 2018).

The 5L DoE showed an improvement in T4 phage titres achieved between shake flask and 5L STR, $2.45x10^{13}$ and $2.43x10^{14}$ pfu/ml, respectively. This is possibly due to the greater control in the stirred tank system. Additionally, the 5L DoE experiment showed that further optimisation of the process leads to a further improvement in titre. Additionally, a water jacket fitted to the bioreactor allows constant temperature control of the culture, something that in a shaking incubator may not occur due to slight changes in the environment or cold spots in an incubator which may alter the infection process.

It is widely accepted that whilst scale up can be an important and difficult challenge, the benefits can be countless. Bioprocesses that are conducted at the manufacturing scale, benefit from those processes with

less variation. When investing money into a process, achieving certain specifications and titres is more important that minimal increases in titres, provided that they are consistent.

Whilst this study has examined a batch model of production, there have been advances in other industries examining fed-batch and continuous processing. Growth rate tends to be higher given that nutrients are continuously being added to the culture whilst the efficiency is improved as the fermenter will continuously be operating (Fisher *et al*, 2019). One study examined a three-stage chemostat model of T3 phage production. They used 3 bioreactors, one to continuously culture fresh host bacteria which was continuously drawn into the second reactor, where the propagation took place. The final reactor had phage and host cells in which would allow them to continuously draw off samples of the culture. As long as the initial feed tank had media in, the first reactor would never run out and meant that the culture could be run continuously. This had a specific advantage as there was a volumetric increase in product, however, only a titre of ~$1x10^{11}$pfu/ml was produced. There is less of an ability to alter the conditions in continuous systems. Moreover, any potential contaminations could have meant severe losses to harvest product (Mancuso *et al*, 2018).

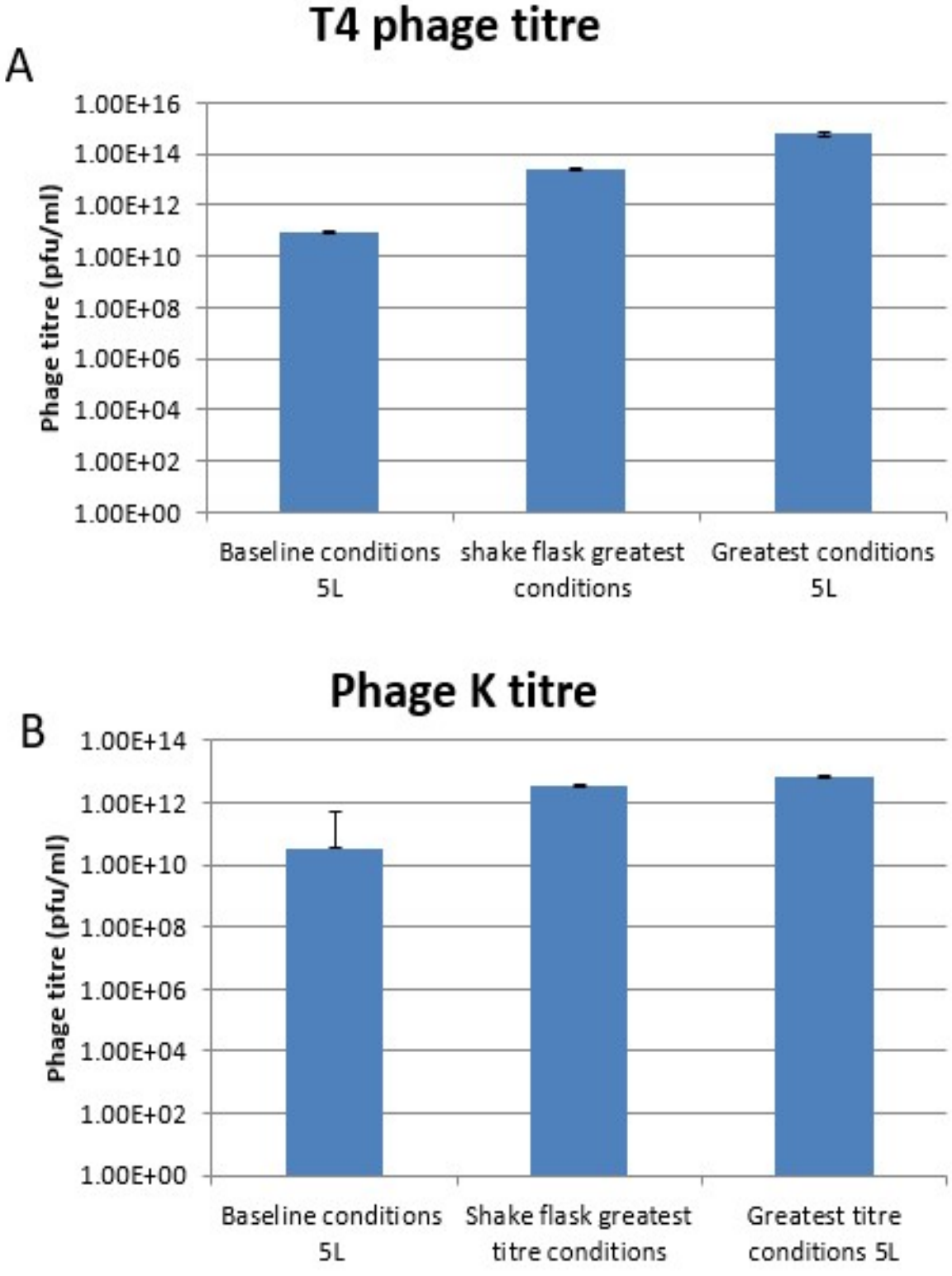



***Figure 4.10.*** *A graph to show the baseline conditions from the 5L, and the improvement in titre shown in the shake flask greatest titre conditions and the greatest titre conditions in the 5L bioreactor A) T4 phage titre B) Phage K titre*

Figure 4.10 above shows how important effective scale up can be. An order of magnitude improvement in titre was observed between the greatest titre conditions in shake flasks and in the 5L STR for T4 phage, p=0.02 using a paired t-test. This was likely due to the control over the culture that was available in the 5L bioreactor, compared to the shake flask. Subtle changes in the environment or power (electricity) to the system may cause deviations in a shaking incubator which could prevent the constant culture environment maintenance, and therefore product yield. Additionally, the total product yield had been increased by up to 150 times.

As the conditions in the 5L had been optimised and validated, those conditions were next examined whilst sparging with air. An increase in sparging the culture with air may be beneficial as it will increase the oxygen available in the culture medium which may increase host cell growth rate and therefore phage propagation. The media was conditioned to 100% DO prior to seeding the bioreactor. Figure 4.11 shows the phage titre when sparging was increased from 1L/min at the greatest conditions, to 2/3 L/min.

The next experiment examined the effect of increasing the rate of sparging on the phage K titre. Sparging a culture with air is common in batch microbial cultures as it can prevent the limitation of oxygen diffusion throughout the culture (Saba *et al*, 2017). Whilst shaking and stirring has been investigated, there is currently a limited body of work investigating stirred tank culture production of phage. This chapter will later present the influence of stirring and sparging on titre and how it can influence the phage bioprocess.

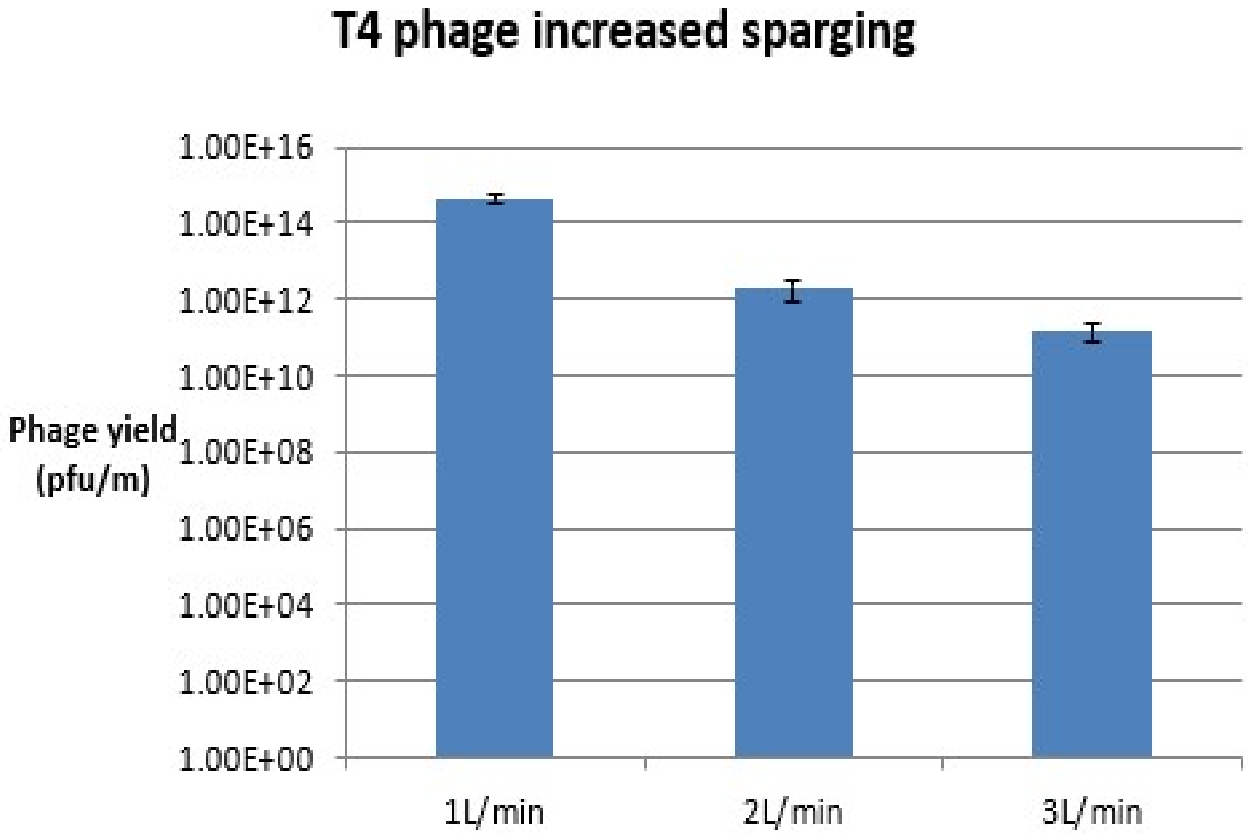



***Figure 4.11.*** *A graph to show the phage titre achieved when increasing the sparging rate of air from 1L/min air to 2/3 L/min. The greatest titre conditions were used MOI 2.5, 225rpm, 3 hours, 28°C, pH 6.9. A) T4 phage titre. The error bars show 1 standard deviation of a duplicate experiment for 2/3 L/min sparging with a single experiment run as a control for 1L/min, each enumerated with triplicate plaque assays.*

The conditions which gave the greatest titre was used and the culture was sparged at 2 and 3 L/min air. Statistically significantly reduced T4 phage titres were achieved when the sparging rate increased, 1-2 L/min p=0.0016 1-3 L/min p=0.00016 using a paired t-test and ANOVA 2-3L/min p=0.003 using an ANOVA. The increase in sparging rate may reduce the ability of the phage to bind onto the host cells, cause shear damage and bubbles bursting, whilst there may also be improved conditions for host growth, all consequently reducing phage titre. This may also be accounted for by the high rate of sparging, causing shear damage to host cells or phage or the host cells utilising the available oxygen to improve their growth as seen in table 4.2 (Weuster-Botz, *et al*, 2002). The kinetics are examined in figure 4.13 in this section.

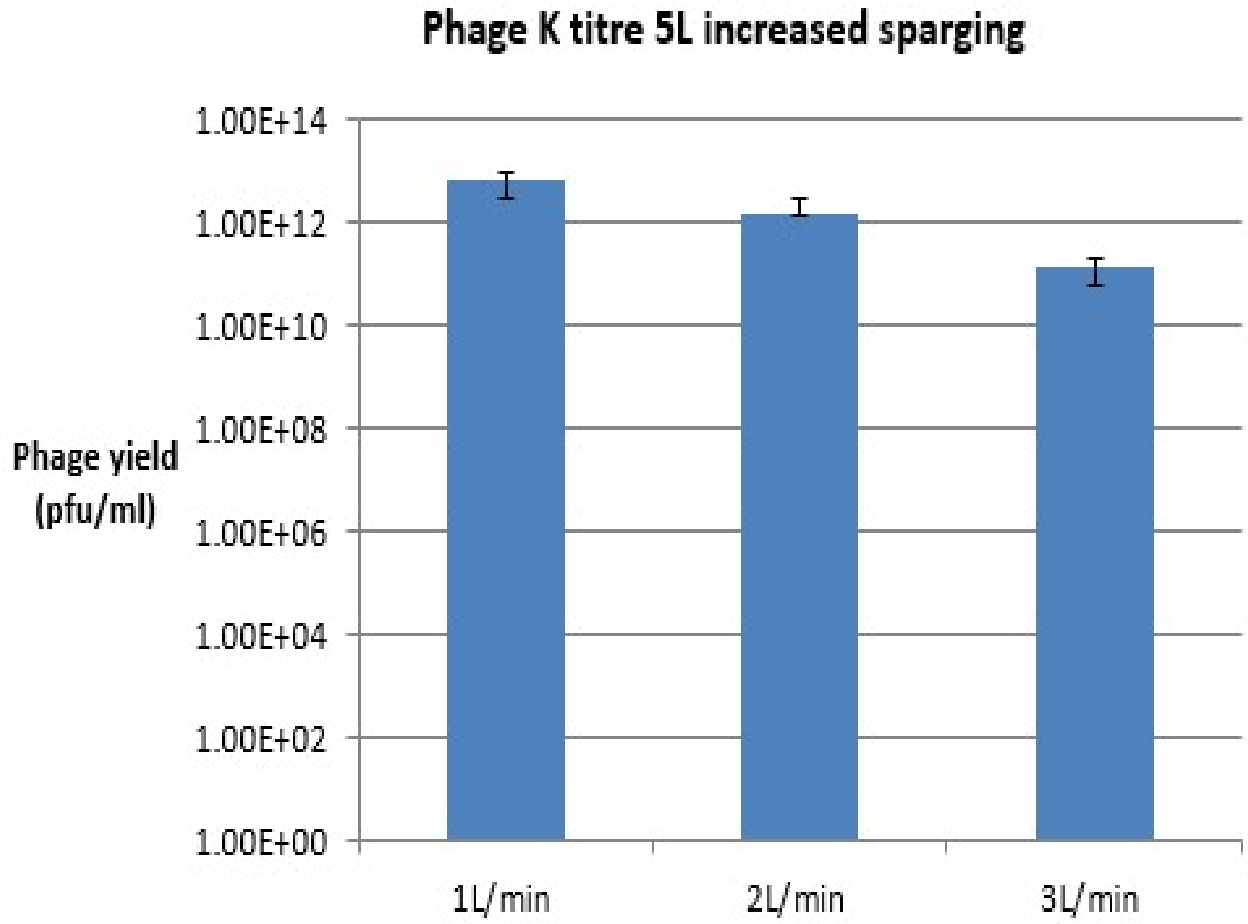



***Figure 4.12.*** *A graph to show the phage titre achieved when increasing the sparging rate of air from 1L/min air to 2/3 L/min. A) Phage K titre. The greatest titre conditions were used; MOI 0.1 225rpm, 4 hours, 28°C, pH 7.4. The error bars show 1 standard deviation of a duplicate experiment for 2/3 L/min sparging with a single experiment run as a control for 1L/min, each enumerated with triplicate plaque assays.*

Figure 4.12 shows the phage K titre, when 2 and 3 L/min air was sparged into the culture. The graph shows a statistically significant reduction in phage titre between 1-2 L/min and 1-3 L/min air, $p<0.05$ using a one-way ANOVA. Additionally, there was a significant decrease in phage titre between 2-3 L/min air $p=0.003$ using a paired t-test.

No previous literature has examined the effect of sparging with air at different rates and the effect on titre. However, there has been some work showing that as the sparging rate of air increases, the titre of different of different viruses when produced decreases (Kioukia *et al*, 1996, Maranga *et al*, 2004). The majority of the literature examining bacteriophage cultures in a stirred tank system is done through mathematical models (Tang et al, 2002, Weitz *et al*, 2005, Smith & Thieme 2012, Shu *et al*, 2017). Bioreactor models can be expensive and require specific knowledge and practical experience to run the reactors. Moreover, fermentation studies can be time intensive and require a lot of setup. However, by combining mathematical research and wet experiments, the research may progress and studies may increase in the coming years (Krysiak-Baltyn *et al*, 2018, Nabergoj *et al*, 2018).

One study examined a *Salmonella* lytic phage, PVP-SE1. The study experimentally determined the conditions to use in a 250ml shake flask of titre, adsorption, burst and latent period and was then used as a basis for the model with around $1x10^{11}$pfu/ml was achieved. Given each of the interactions, population dynamics and adsorption models run, there was low variation seen in the first 3 hours of infection but given the unpredictability of the population dynamics and the self-replicating nature of phage, there were some statistically significant differences between the shake flask and bioreactor model. However, there was good agreement between the experimental and model data for the host organism (Santos *et al*, 2014).

To determine why the increase in sparging rate may have an effect on the titre, an experiment was run to determine the adsorption rate in the 5L bioreactor when the sparging rate was increased from 1L/min to 2 and 3 L/min shown in figure 4.13. Whilst a hypothesis was made that an increase in cfu may lead to a lower titre, it was important to examine the effect on adsorption and burst size. The effect of sparging and its effect on final cfu/ml is seen later in this section in table 4.2. The greatest titre conditions from the 5L DoE were used with each experiment run in duplicate and each enumerated with duplicate plaque assays.

Firstly, a statistically significant decrease was seen for the adsorption between all time-points at 1 to 2/3 L/min for T4 phage, $p=0.005$ and $p=0.004$ respectively. Statistically significant differences were seen at all time points for 1-2/3 L/min $p<0.05$ whilst from 5 minutes onwards there was a significant difference in free T4 phage between 2 and 3L/min. Additionally, a significant decrease was also seen for phage K at 1-2/3 L/min at all time points, $p=0.03$ and $p=0.017$ respectively. Moreover, a significant decrease was seen between 1-2L/min at

3 and 4 minutes, 1-3L/min at 2 and 3 minutes and 2-3L/min at 2, 4, 5, 8 and 10 minutes, $p<0.05$ for phage K. Statistically significant differences were confirmed by paired t-tests between all time points at all sparging rates.

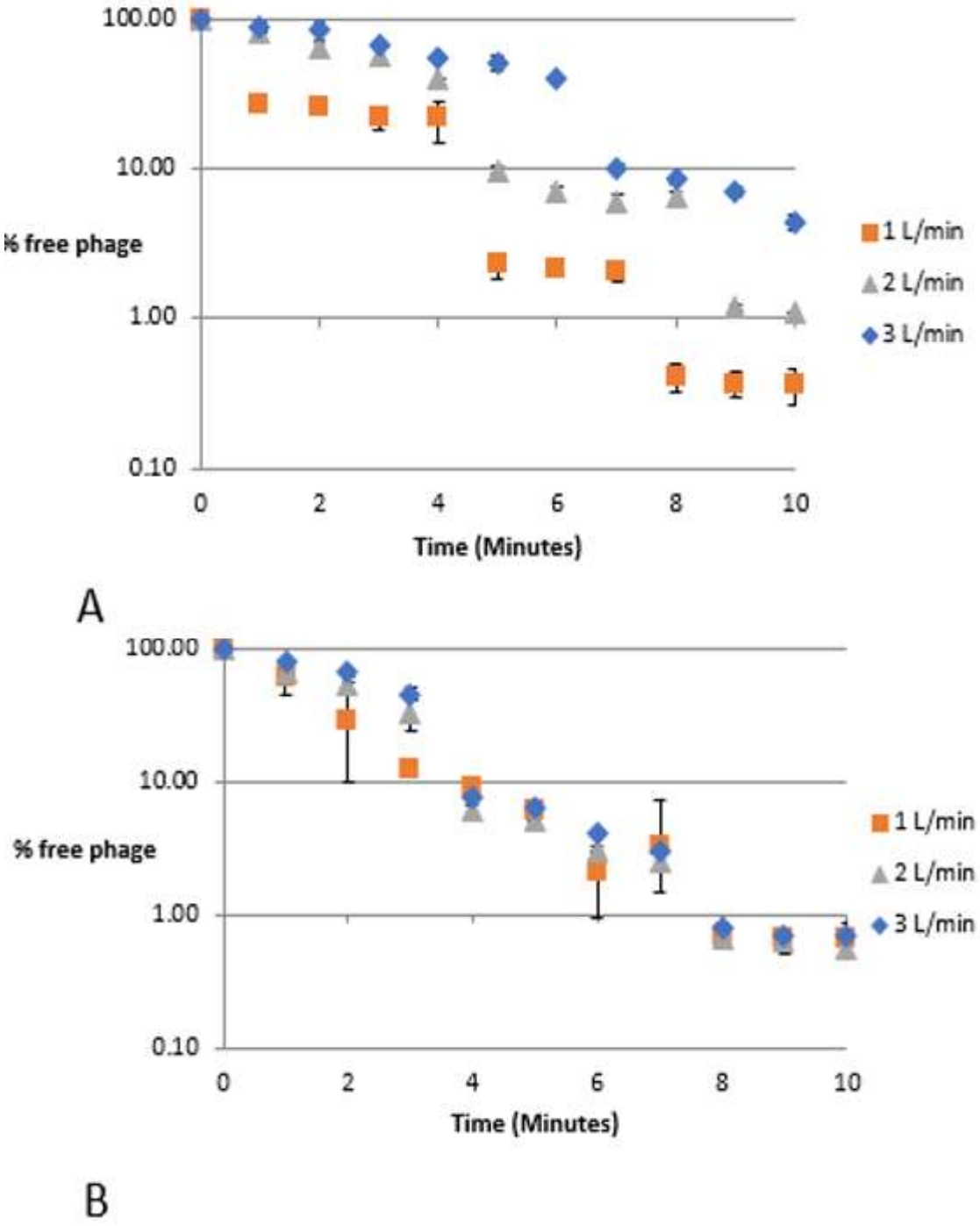



***Figure 4.13.*** *A graph to show the rate of T4 and phage K adsorption in the 5L bioreactor at 1/2/3 L/min sparging of air A) T4 phage B) phage K. The first 10 minutes of the infection were used in the bioreactor, given the time it took for sampling. Orange Squares represent 1L/min, green triangles represent 2L/min and blue diamonds represent 3 L/min. Each condition was run n=2 times with each point showing 1 standard deviation of n=2plaque assays.*

It is interesting to note the three distinct rates of adsorption (% free phage) that are seen for the T4 phage data whereas the phage K data shows less of a distinct pattern as there were fewer statistically significant differences. Whilst previous data showed the effect of sparging on phage titre, the adsorption data shows it has less of an effect on the free phage K. One possible explanation for this is the length of the tail of both phage. It has been reported that phage K has a tail length nearly 3 times the length of T4 phage (Rees & Fry, 1981, Yap & Rossmann, 2014). This increase in tail size may allow phage K to adsorb more easily to its host cell when the sparging rate is increased. If there is a longer tail, there is an improved chance of the phage being able to bind or interact with its host organism and adsorb to its host (Rees & Fry 1981, Bartual *et al*, 2010). Phage K may also be more robust or more easily able to bind to its host cell compared to T4 phage. It is important to note no antifoam was added within the first 10 minutes of the culture which could have an effect on the culture and therefore the phage adsorption. Previously, it has been shown for viral culture that when antifoam is added given that it is a surfactant and can damage the cell membrane in both microbial and mammalian cultures (Velugula-Yellela *et al*, 2017, Dill *et al*, 2019)

Next, the burst sizes were measured in duplicate to determine the effect sparging has on them. It was interesting to note the significant differences seen between the burst sizes, when T4 distinctly had three adsorption rates when the sparging rate was increased. The T4 phage burst size was as follows: 1L/min - 118.9 ± 1.55, 2L/min – 77.2 ± 2.8, 3L/min – 63.3 ± 3.3. However, there were also statistical significances between sparging rates in the phage K burst sizes, despite there being no difference in the rate of adsorption: 1L/min – 134.4 ± 9.4, 2L/min – 79.8 ± 4.8, 3L/min – 59.4 ± 3.2. Literature surrounding phage culture and propagation in stirred tank systems is scarce, however, examining their behaviour at different conditions is important. The significant differences seen between the final harvest titres were also seen for T4 phage in the burst size and adsorption. However, significant differences were found in between the burst size and final titre for phage K but less so for the adsorption which may be due to the tail length. If the phage tail can bind onto the host more easily, it would be expected that there would be less of an effect on adsorption, but the sparging would have an effect on the host cells and phage throughout the culture i.e a lower burst and titre.

One study examined the burst rate of T4 infecting *E. coli K12* in a 25ml working volume in a glass bioreactor vial. Using 2L/min air a burst size of 89 was achieved. Whilst this was lower than our baseline value,

the difference in host organism would have an effect on its kinetics accounting for the lower value (Nabergoj *et al*, 2018).

At the point of harvest, an OD measurement was taken as well as a 1ml sample of the culture. This was diluted to an appropriate level and 100 µl plated onto appropriate agar to determine the cfu/ml. The table below shows the final OD measurement and cfu/ml. This experiment could have been improved as only a 1ml sample was taken from a 3L working volume which may not be representative of the culture.

*Table 4.2. The table below shows the average harvest optical density and final colony forming unit count of the 5L stirred tank reactor culture. The table shows the outcome of a duplicate technical repeats for 2 and 3L/min and a single experiment for 1L/min air.*

| **Phage** | **Sparging rate of air (L/Min)** | **Harvest OD600nm ± 1SD** | **cfu/ml ± 1SD** |
|---|---|---|---|
| **T4** | 1 | 0.204 | $3.6x10^8 \pm 3.3x10^7$ |
| | 2 | 1.05 ± 0.03 | $1.79x10^9 \pm 3.28x10^8$ |
| | 3 | 2.09 ± 0.03 | $2.8x10^8 \pm 4x10^8$ |
| **K** | 1 | 0.523 | $4.83x10^8 \pm 1.38x10^8$ |
| | 2 | 2.07 ± 0.07 | $1.95x10^9 \pm 3.69x10^8$ |
| | 3 | 3.91 ± 0.01 | $3.2x10^9 \pm 3.11x10^8$ |

During the infection, the cultures were sparged with the various rates of air throughout the infection, the media was conditioned for at least 1 hour to allow the DO% to reach 100%. Table 4.2 shows that as the sparging rate increases, the final number of cells also increases. The increase in cells may have had a two-fold consequence on phage yield. Firstly, it would have been outside the optimal range for phage infection as the host cells would have used the additionally available oxygen to grow at an increased rate. As the cell density increases >0.4 OD600nm, host cells become less prone to phage infection. Secondly, the increase in sparging may have had an effect on phage adsorption due to bursting of bubbles and shear damage to phage, preventing the phage-host interaction. Increasing the oxygen available may improve cell health during infection or provide more cells for infection for an increased yield, however, this work showed that by increasing the oxygen there was a resultant decrease in titre (Swift, 2014). It should be noted that at 3L/min air, there was a large variation in the cfu data for the T4/*E. coli* experiment. Whilst previous authors have shown it to be a highly variable assay, it may have been more appropriate to use dry cell weight as a measure. However, cfu assays are a generally accepted as they can show the number of colonies formed and identify different microorganisms (Lumley *et al*, 1997, Cassoli *et al*, 2016). The experiment may have benefitted from dry cell weight measurements or additional samples and measurements from the bioreactor culture given that it was a 3L working volume.

A statistically significant decrease in phage titre was seen when the sparging rate of air was increased above 1L/min. Also, the final optical density and colony forming unit measurements, given in table 4.2 above, was increased as the sparging rate increased and there was an increase in final host cell titre. The increase in cell titre and harvest OD meant it may have been outside the optimal range for phage to infect their bacteria. Once the host cells reach a density outside this optimal range, 0.2-0.4OD600nm, they become more difficult to infect and less likely to produce phage, resulting in lower titres. The increase in availability of oxygen will allow for an improvement in the growth profile of the host organism and if conditions are more suited to the host, it will dampen the ability of phage propagation (Turner *et al*, 2017). It may also be interesting to see the effect of 0.5L/min sparging on the titre.

**4.5 Rate of adsorption**

The adsorption experiment was completed in the 5L bioreactor for the baseline and greatest titre conditions. It must be noted that in the 5L bioreactor, there is a need to discard some of the sample culture, which may be in the sampling tube, and re-sample. Therefore, the sampling method was changed to sample every minute rather than every 30s as in the shake flask and whilst it was completed every 30s for the shake flask model it took longer to sample the bioreactor as in the shake flask model, sacrificial flasks were used. The data shows that the baseline conditions gave a lower rate of adsorption compared to the greatest titre conditions. Figure 4.14 shows a statistically significant decrease in free phage at between 4-5 minutes at the greatest titre conditions. It is worthwhile noting that there is a significant decrease in the free phage at baseline conditions between 5-6 minutes and the data shows that there is a 1-minute delay in this significant reduction in free phage $p<0.0001$.

There was no statistically significant difference between the baseline and greatest titre conditions throughout the infection between conditions, $p=0.66$. However, significant differences were found between conditions at 1, 3, 5, 6, 8 and 10 minutes between the baseline and greatest titre conditions for T4 phage adsorption in the 5L STR, $p<0.05$ using a paired t-test. The data suggests that when the infection takes place at a lower temperature of infection, the adsorption rate is greater i.e lower free phage at the greatest titre conditions than baseline conditions. This may help to explain why there is a greater titre between the conditions. Additionally, there was a significant difference in burst size between baseline and greatest titre conditions for T4 phage in the 5L bioreactor 102.4 ±2.4 and 118.9 ±1.6 respectively.

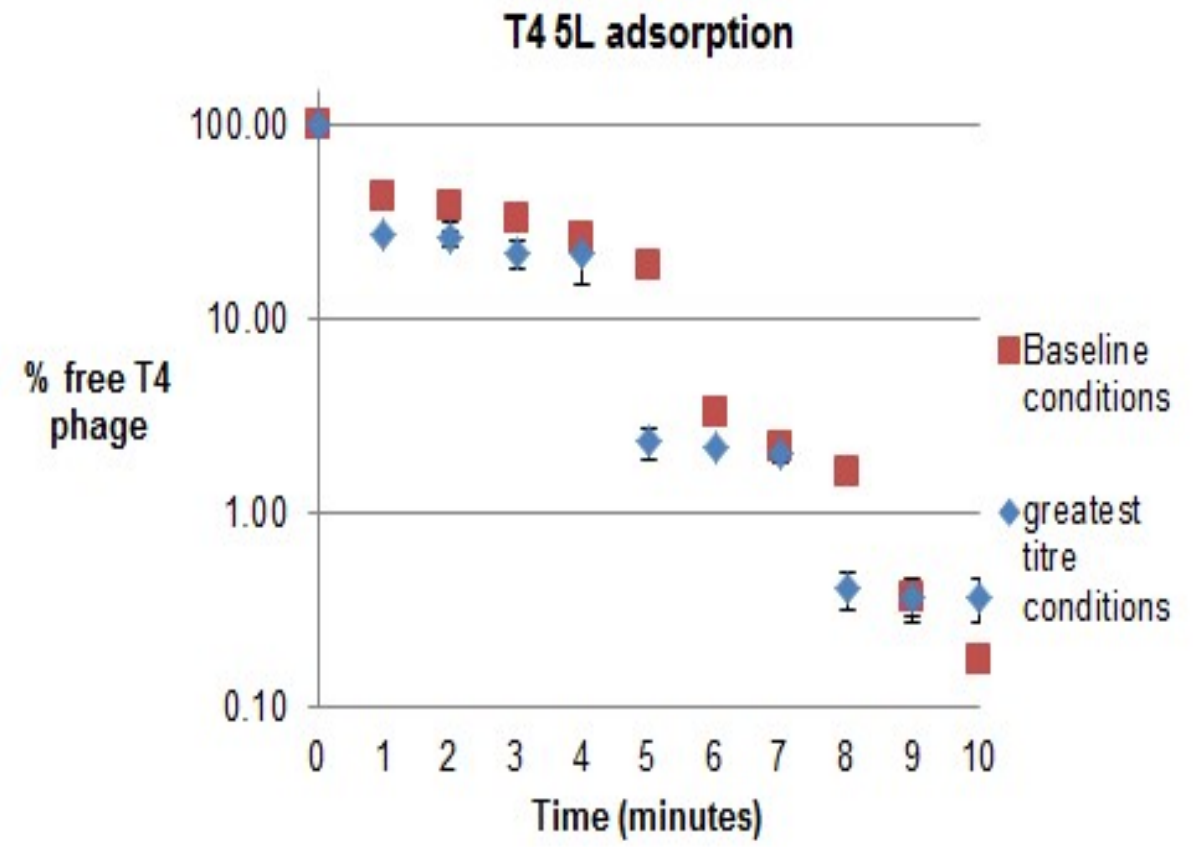



***Figure 4.14.*** *A graph to show the rate of T4 phage adsorption to the host cells when using the baseline and greatest titre conditions in the 5L bioreactor. Error bars show 1SD of n=4 experimental runs with duplicate plaque assay enumerations for each run.*

Whilst some previous work has investigated how the MOI effects adsorption, there has been limited work examining the effect of temperature of infection or agitation on adsorption rate of T4 phage or phage K. Tokman *et al* (2016) showed that at 30°C, phage LP-048 can adsorb to its host more efficiently than at 37°C. They hypothesised that as the phage required a rhamnose gene for adsorption to the host (*Listeria Monocytogenes*) upregulation of the GlcNAc gene, responsible for expression of WTA that is rhamnoslyated, will lead to more efficient binding although this was phage specific. The additional rhamnosylation that took place to a greater degree at 30°C compared to 37°C will ensure that more of the binding/adsorption can take place. Whilst T4 phage commonly binds to the LPS of its host, the genes of which are down regulated with a reduced temperature, there may be other genes that are thought to be less important in binding, upregulated leading to more efficient binding (Tokman *et al*, 2016).

The adsorption experiment was repeated in the 5L bioreactor for phage K. Again, a sample was taken every minute to determine the rate of phage adsorption based on the free phage left in the sample, given the sampling method required in the bioreactor.

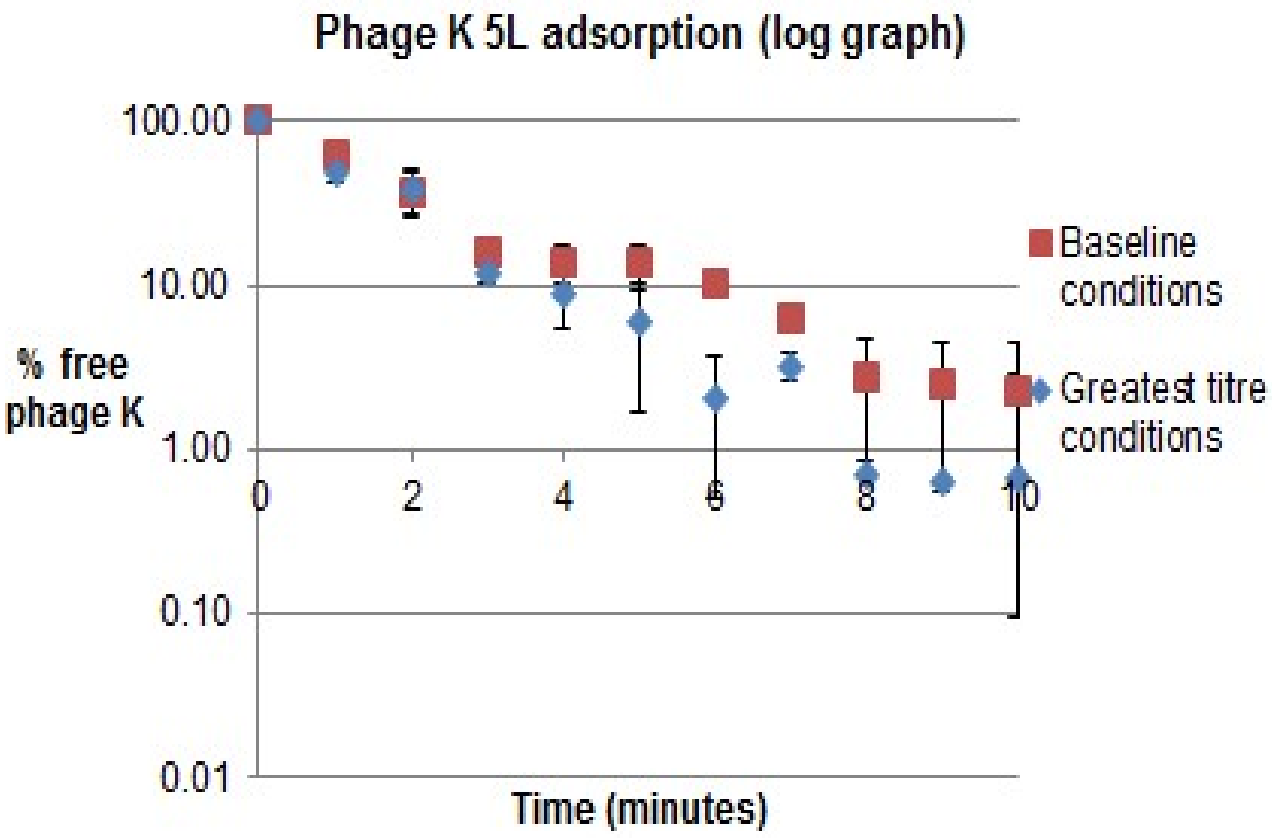



***Figure 4.15.*** *A graph to show the rate of phage K adsorption to the host cells when using the baseline and greatest titre conditions in the 5L bioreactor. Error bars show 1SD of n=4 experimental runs with duplicate plaque assay enumerations for each run.*

Figure 4.15 shows that there was no statistically significant difference between the conditions at which the adsorption takes place. However, there was a statistically significant difference at 6 and 7 minutes between

the baseline and greatest titre conditions, $p<0.05$ using a paired t-test. The results from this study show that by using the greatest titre conditions, the phage may adsorb to their host cell quicker. Therefore, if the phage is beginning its replication process quicker, it is likely to be able to infect more hosts quicker. Although this was seen more clearly for T4 phage, there was a statistically significant difference in the burst size for baseline and greatest titre phage K conditions, 80.6 ±0.6 and 134.4 ± 9.4. This showed that although there was little difference in the adsorption, the burst was significantly affected and may explain why a greater titre was seen between the two conditions.

The next experiments focused on the difference between the shake flask and 5L adsorption at both baseline and greatest titre conditions. Each experiment was repeated in duplicate to ensure reliability of the results. Examining these conditions may explain why greater titres can be achieved in the greatest titre conditions compared to the baseline whilst optimising these conditions may further help to improve the titre.

Given the sampling method of the 5L STR, a sample was taken every minute for the first 10 minutes, compared to every 30s in sacrificial shake flasks for the first 5 minutes after infection, there may be some differences between the adsorption data. Figure 4.16 shows the phage K adsorption at baseline and greatest titre conditions between shake flasks and the 5L bioreactor.

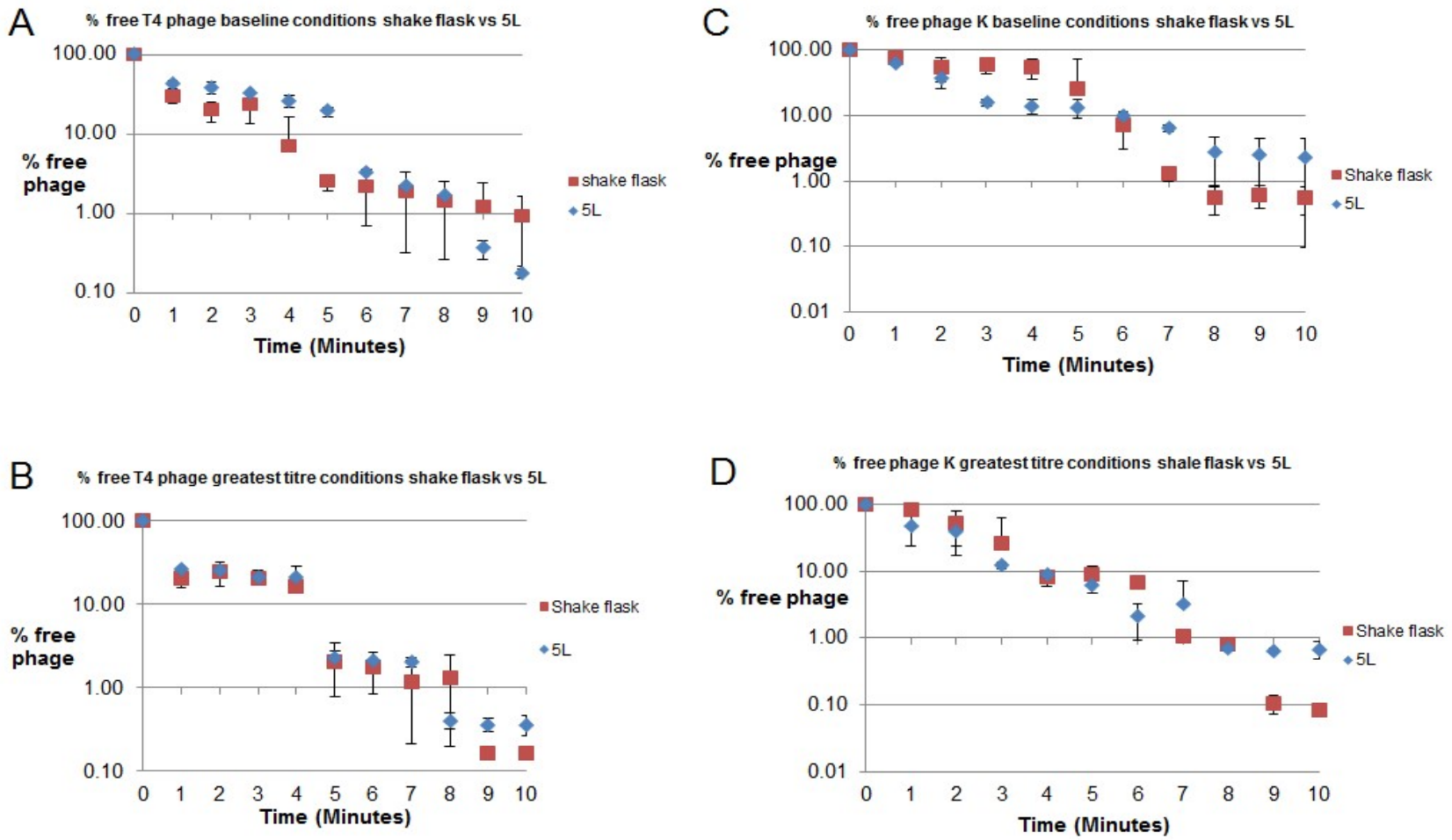



***Figure 4.16.*** *A graph to show the rate of adsorption to the host cells between the shake flask and the 5L DoE A) T4 baseline conditions B) T4 greatest titre conditions C) Phage K baseline conditions D) Phage K greatest titre conditions. Error bars show 1 standard deviation of a duplicate experiment with duplicate plaque assay enumerations.*

Figure 4.16 shows that there are few statistically significant differences between culture systems and the rate of free phage after the initial infection. At the greatest titre conditions, the shake flask gives a significantly greater rate of T4 phage adsorption compared to the 5L at 9 and 10 minutes which was also seen for phage K (graphs B and D). Given that the 5L bioreactor gave greater titres than the shake flasks at both conditions it may be assumed that it was due to the greater control from the bioreactor. Previous authors have shown that due to viruses' mechanisms, adsorption can become highly variable when more than 90% have adsorbed (Bull *et al*, 2014, Denes *et al*, 2015). Also, the shake flask culture will not control pH or oxygen which could play some role in the variation seen.

The difference between the mixing patterns in both culture systems may cause the variation seen in the results. Given that the geometry between the two culture systems was not closely examined this could have had an effect and may explain the variation. It was previously shown that there are certain genes that are linked with phage adsorption and that mutations within these genes between phage in a culture can play a role in the variation of the phage adsorption to the host organism (Denes *et al*, 2015). This could even occur between experiments that use the same sample of phage but on separate occasions due to mutations in the phage. Additionally, variation and mutations between host organism may see different numbers of receptors on the hosts cell surface which cause additional variation (Bull *et al*, 2014). Therefore, between batches of phage used between experiments this may have accounted for the variation seen. Whilst the same batch of phage was not used for each of the experiments presented in figure 4.16, a sample of phage stored at either appropriate temperatures could have mutations occur and therefore experiments between culture systems should use the same batch and be run as closely together as possible.

**4.6 An investigation of the DoE extreme levels in an STR**

The next experiment examined the rate of adsorption in the STR when the extreme levels of the 5L DoE were used. Each experiment was run as an OFAT. The greatest titre conditions were used whilst only 1 of the other variables were changed e.g T4 phage, MOI 2.5, 225rpm, 3 hours, 28$^{o}$C with an extreme 7.9 pH used. This was then changed to pH 6.9 and 6.4 to complete graph C figure 4.17. The experiments were repeated for agitation and temperature. Each experiment was run in duplicate with each experiment enumerated with duplicate plaque assays. Table 4.3 shows the levels used whilst figure 4.17 shows the rate of adsorption for the extreme and mid-levels of agitation, pH and temperature used in the 5L DoE.

*Table 4.3. A table to show the extreme levels of the conditions used for the agitation, temperature and pH in the 5L bioreactor DoE*

| Phage | Culture system | Condition | Level |
|---|---|---|---|
| T4 | 5L | Agitation (rpm) | 50, 400 |
| | | Temperature ($^{o}$C) | 20, 37 |
| | | pH | 6.4, 7.9 |
| K | 5L | Agitation (rpm) | 50, 400 |
| | | Temperature ($^{o}$C) | 20, 37 |
| | | pH | 6.4, 7.9 |

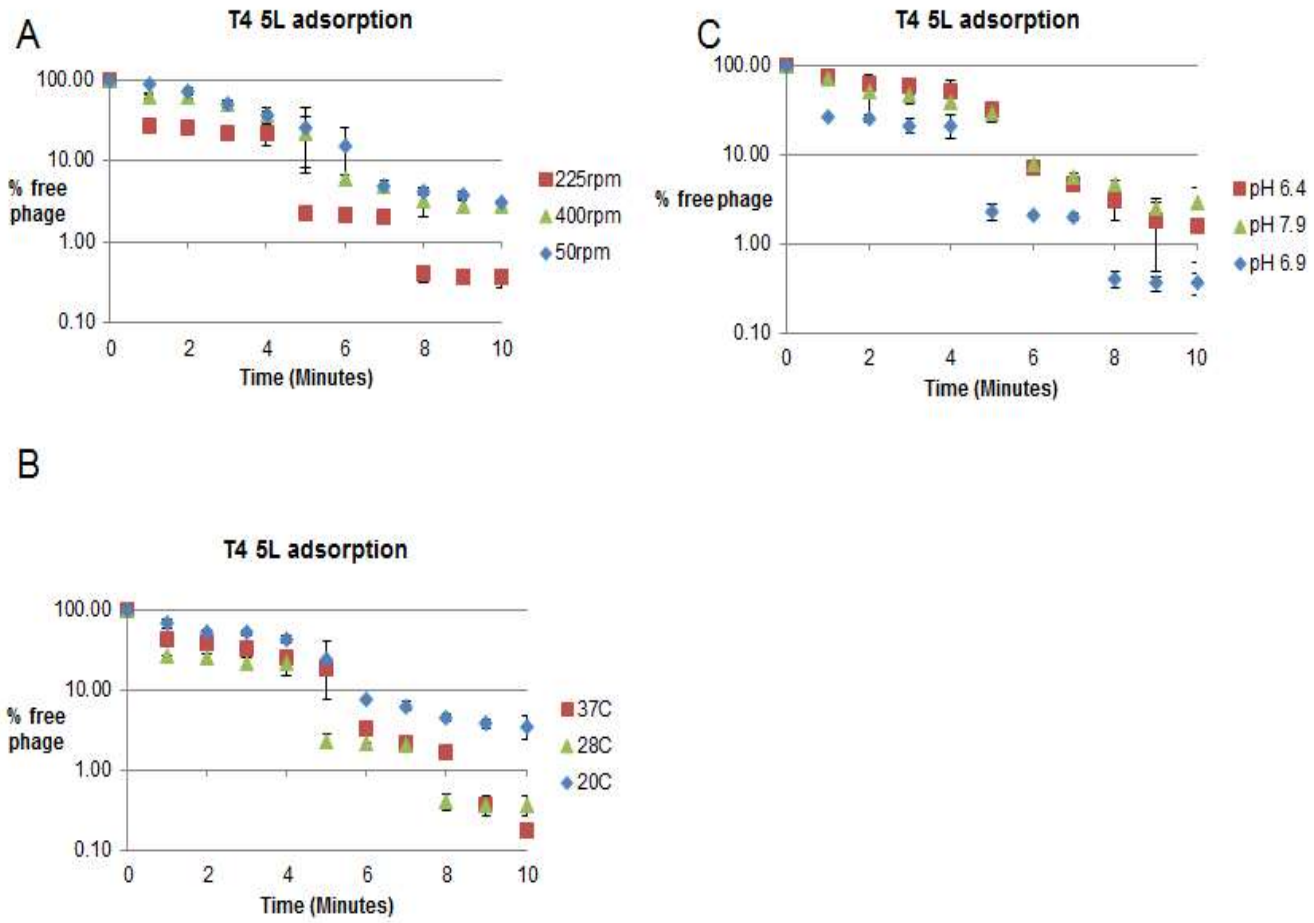



***Figure 4.17.*** *A graph to show the adsorption of T4 phage to its host cell in the 5L bioreactor A) Agitation (rpm) B) Temperature (°C) C) pH. The phage was seeded using MOI 2.5 with the other conditions altered one factor at a time.*

Figure 4.17 graph A shows that at 225rpm there was a statistically lower free phage from 5 minutes onwards compared to 50 and 400rpm at all time-points. Moreover, pH 6.9 also gave a statistically significantly lower free phage than pH 6.4 and 7.9 from 3 minutes onwards apart from at 9 minutes. This may explain why there is a greater titre when the mid-range levels were used. Additionally, table 4.4 below shows the burst size and final titre achieved and the final phage titre when the experiments were run to completion using the indicated levels of each conditions. Previous authors have shown the effect of MOI on the adsorption (Storms *et al*, 2015)

*Table 4.4. A table to show the burst size and final phage titre for T4 in the 5L bioreactor and the extreme and centre point levels*

| Condition | Level | Burst size ± 1SD | Pfu/ml ± 1SD |
|---|---|---|---|
| Agitation (rpm) | 50 | 55.9 ± 1.3 | $5.75x10^{11} \pm 2.17x10^{11}$ |
| | 225 | 114.9 ± 1.5 | $2.2x10^{14} \pm 1.86x10^{14}$ |
| | 400 | 62.5 ± 1.7 | $1.2x10^{13} \pm 4.74x10^{12}$ |
| Temperature (°C) | 20 | 58 ± 8 | $4.15x10^{10} \pm 3.51x10^{10}$ |
| | 28 | 118.9 ± 1.5 | $2.2x10^{14} \pm 1.86x10^{14}$ |
| | 37 | 102.4 ± 2.4 | $4.3x10^{10} \pm 1.5x10^{10}$ |
| pH | 6.4 | 70.4 ± 6.3 | $3.5x10^{12} \pm 1.12x10^{12}$ |
| | 6.9 | 111.9 ± 1.5 | $2.2x10^{14} \pm 1.86x10^{14}$ |
| | 7.9 | 63.8 ± 6.3 | $6.25x10^{13} \pm 8.29x10^{12}$ |

Together, figure 4.17 and table 4.4 show that the mid-levels generally gives the greatest rate of adsorption and burst size/phage titre. However, temperature seemed to have less of an effect on the adsorption in the 5L bioreactor although 28°C did give a significantly higher final titre and burst compared to 20 or 37°C. The burst and adsorption may all contribute to the final titre achieved being the greatest at the mid-level.

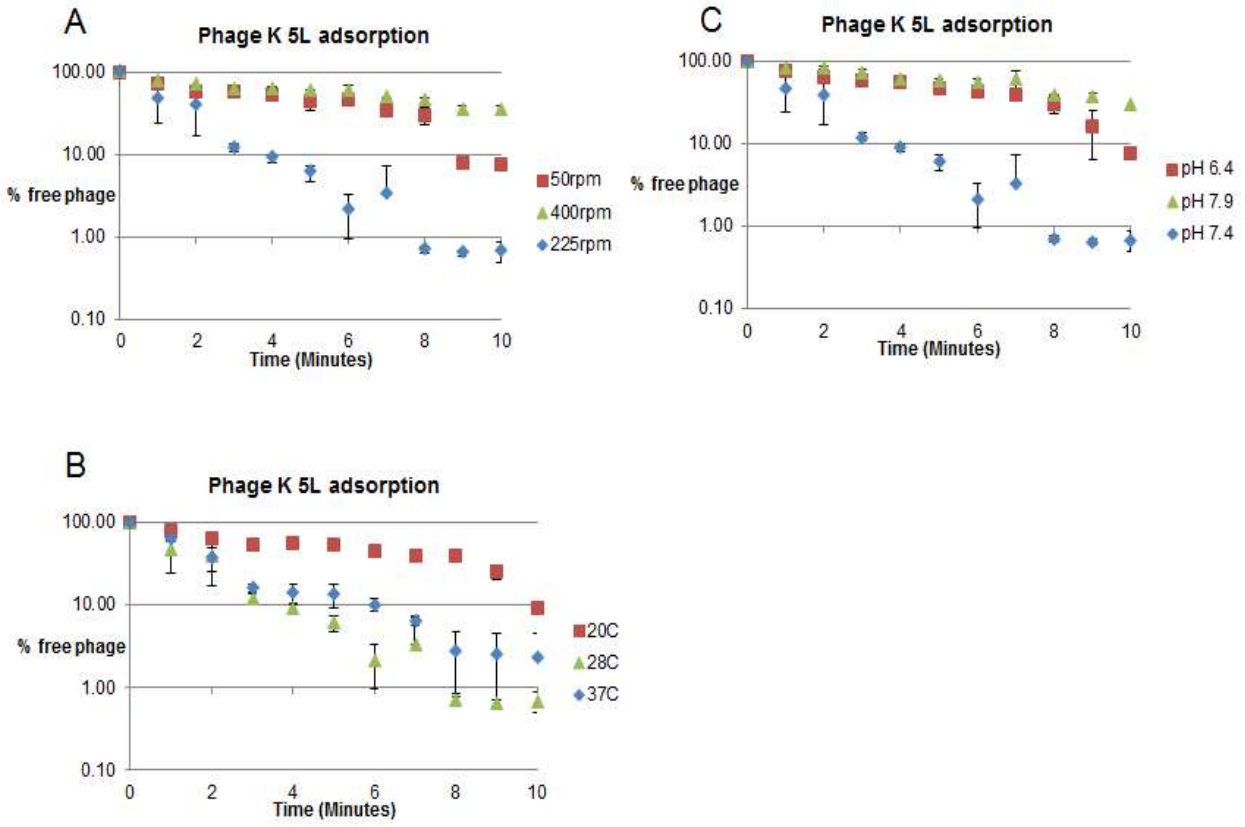



***Figure 4.18.*** *A graph to show the adsorption of phage K to its host cell in the 5L bioreactor A) Agitation (rpm) B) Temperature (°C) C) pH. The phage was seeded using MOI 0.1 with the other conditions altered one factor at a time.*

Figure 4.18 shows that from 3 minutes onwards, 225rpm gives a statistically significantly lower free phage K compared to 50 or 400rpm. Additionally, pH 7.4 gave the statistically lowest rate of free phage compared to pH 6.4 or pH 7.9 from 3 minutes onwards. Similar to the T4 phage experiments, there seemed to be less statistically significance in free phage at 28°C compared to 20/37°C. Table 4.5 below shows the burst size and final titre achieved and the final phage K titre when the experiments were run to completion using the indicated levels of each conditions.

*Table 4.5. A table to show the burst size and final phage titre for phage K in the 5L bioreactor and the extreme and centre point levels*

| Condition | Level | Burst size ± 1SD | Pfu/ml ± 1SD |
|---|---|---|---|
| Agitation (rpm) | 50 | 106.4 ± 3.6 | $4.5x10^{12} \pm 1.1x10^{12}$ |
| | 225 | 134.4 ± 9.4 | $4.2x10^{12} \pm 6.9x10^{11}$ |
| | 400 | 111.05 ± 2.5 | $4.5x10^{11} \pm 1.1x10^{11}$ |
| Temperature (°C) | 20 | 88.2 ± 2 | $4.5x10^{12} \pm 1.1x10^{12}$ |
| | 28 | 131.4 ± 9 | $4.2x10^{12} \pm 6.9x10^{11}$ |
| | 37 | 80.6 ± 0.6 | $4.2x10^{9} \pm 1.7x10^{9}$ |
| pH | 6.4 | 60 ± 5 | $3.8x10^{10} \pm .3.1x10^{9}$ |
| | 7.4 | 132.4 ± 4 | $4.2x10^{12} \pm 6.9x10^{11}$ |
| | 7.9 | 83 ± 3 | $5.3x10^{12} \pm 4.3x10^{11}$ |

Table 4.5 shows that the mid-point levels of each variable used gave the greatest burst size and final titre with the exception of 50 and 225rpm. However, a statistically significantly lower burst and rate of adsorption was seen 50 and 225rpm and 50 and 400rpm from 5-10 minutes respectively, $p<0.05$ using a paired t-test. An agitation of 50/400rpm may be less appropriate for the burst and adsorption accounting for the lower burst/adsorption rate compared to 225rpm. However, this low rate of agitation may be more appropriate for

the long-term infection for an increased phage K titre. Additionally, figure 4.6 graph F shows that 50rpm may be used to obtain similar titres to 225rpm when the full infection is carried out.

Graphs 4.17/4.18 and tables 4.4/4.5 show clearly the improvement in burst and adsorption when the mid-levels of each of the 5L DoE were used. Whilst the DoE showed that it was these levels that gave the greatest titres, the kinetics experiments may explain why. Where there is a greater burst, it may increase the likelihood of a greater final titre, and where the adsorption takes place at a more rapid rate, more phage will infect the host, increasing titre.

One study examined the effect of pH and temperature of iLp84 phage on *Lactobacillus paracasei*. The study showed no statistically significant difference in the rate of the phage between pH 5 and 8. However, there was an order of magnitude reduction in adsorption when pH was increased to 9 whilst a 3-log reduction was seen in the rate of adsorption at pH 4. Additionally, they also saw a 25% increase in burst when the experiment was carried out at 30$^{o}$C compared to 37$^{o}$C (Mercanti et al, 2015). The results presented in their study show a similar trend, despite it being with a different phage where the burst was increased at a lower temperature. However, Figure 4.17 and 4.18 C show statistically significant differences in the rates of adsorption at a 0.5 change in pH, most likely due to the phage. The stability in adsorption may have been because lactobacillus is stable between pH 5 and 8 whilst minor changes in pH can have a significant effect on *E. coli* and *S. aureus* growth (Cleghorn & Bowden, 1989, Wallin-Carlquist *et al,* 2010, Wang *et al*, 2014).

The experiments presented above for T4 and phage K examined the extreme DoE conditions and their effect on adsorption, burst size and final phage titre. They showed the importance of each factor and its effect on the phage infection kinetics. It is interesting to note the species-specific differences such as the temperature, where 20$^{o}$C gave a higher burst than 37$^{o}$C for phage K but the opposite for T4 phage. This may be true for each individual phage and therefore others may need to carry out similar experiments for their own specific phage. Table 6 below show the burst size and phage titre that were achieved between the baseline and greatest titre conditions for T4 and phage K for ease.

*Table 4.6. A table to show the burst size and final phage titre between the baseline and greatest titre conditions and the significance for T4 phage and phage K in the 5L bioreactor*

| Phage/Scale | Conditions | Burst size average ± 1SD | P value | Phage titre (pfu/ml) ± 1SD | P value |
|---|---|---|---|---|---|
| T4/5L | Baseline | 102.4 ± 2.4 | 0.055 | $4.3x10^{10}$ ± $1.5x10^{10}$ | 0.02 |
| T4/5L | Greatest titre | 118.9 ± 1.6 | | $2.2x10^{14}$ ± $1.9x10^{14}$ | |
| K/5L | Baseline | 80.6 ± 0.6 | 0.029 | $4.2x10^{9}$ ± $1.7x10^{9}$ | P<0.000001 |
| k/5L | Greatest titre | 134.4 ± 9.4 | | $4.2x10^{12}$ ± $6.9x10^{11}$ | |

Sections 4.5 and 4.6 have examined the influence of the kinetics on the T4 and phage K infection in a stirred tank bioreactor. Notably, figure 4.16 showed the difference in adsorption between a shake flask and a 5L bioreactor for both conditions. It was interesting to note that usually, after 10 minutes, the shake flask model gave a greater level of adsorbed phage, compared to the shake flask. However, the greatest burst and final titre was seen in the stirred tank bioreactor. This was probably due to the greater level of control within the process.

Figures 4.17 and 4.18 show the rate of adsorption whilst tables 4.4 and 4.5 show the burst size and final phage titre when the temperature, pH and agitation level was changed. The conditions which gave the greatest burst and final titre also saw the greatest rate of adsorption of both phage to the host organism. Therefore, the extreme conditions i.e. the highest and lowest level used should not be used when trying to improve rate of

adsorption, burst or final titre. Therefore, the mid-level conditions should be used to improve the process. Using these conditions may prevent the significant development and propagation of the host culture and allow more optimal phage propagation.

## 4.7 STR sparging DoE

Next, a separate DoE was run in order to examine the effect of sparging and agitation with the greatest titre conditions used. The agitation in the STR may affect the rate at which the air can be dissolved into the culture and given that the sparging rate was altered, a DoE was created in order to examine the effect on titre. Figure 4.19 shows the titre achieved based on each run from the DoE. Error bars are shown which indicate 1 standard deviation of each titre achieved from a single bioreactor run with duplicate plaque assays enumerations. This would be the first DoE study to examine the effect of sparging on a culture and the effect on temperature.

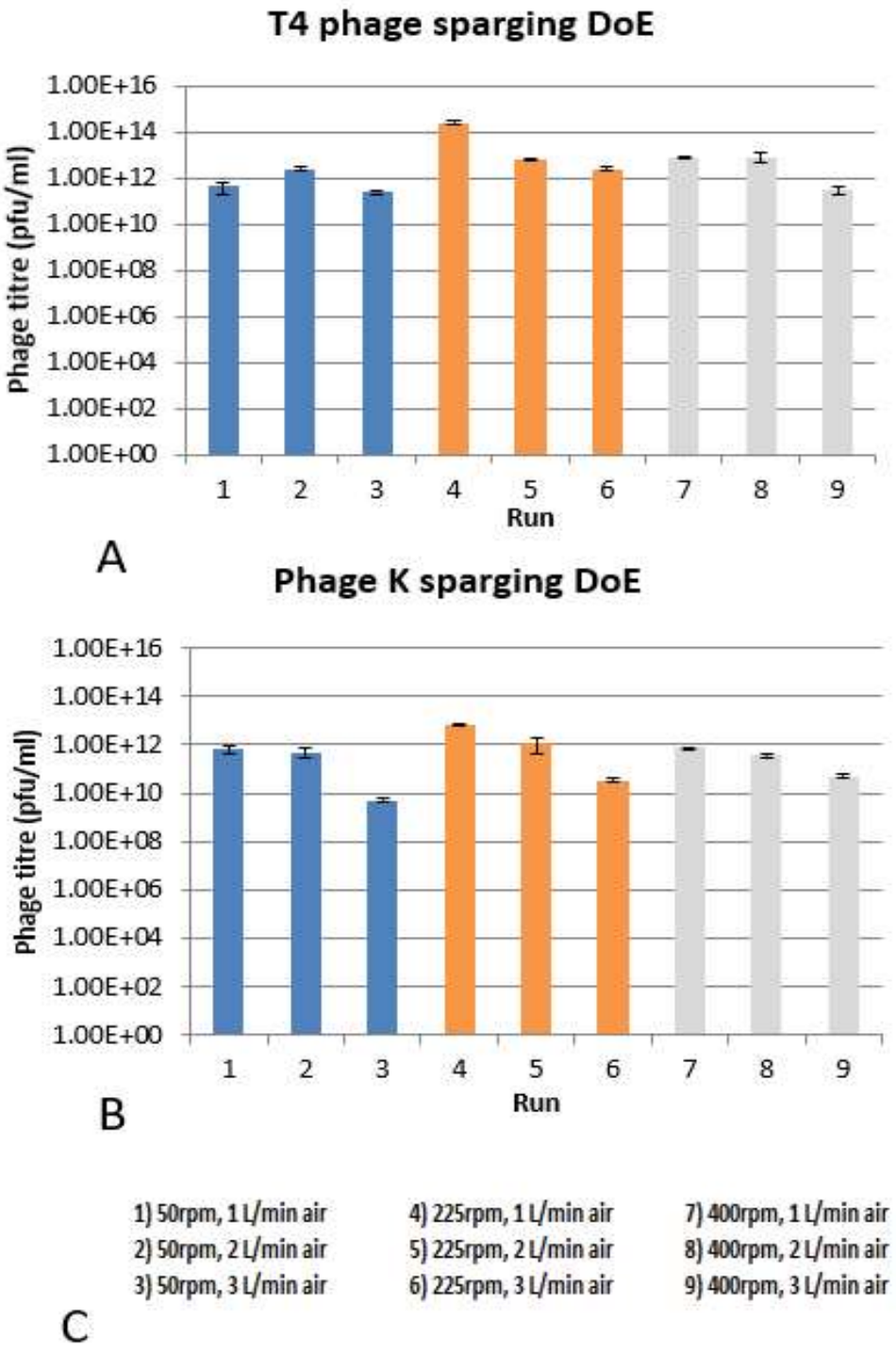



***Figure 4.19.*** *A graph to show the titres achieved in the A) T4 phage B) phage K sparging DoE C) The conditions used for each experiment. The greatest titre conditions were used for both phage. Error bars show 1 standard deviation of each titre achieved from a single stirred tank run with duplicate plaque assays enumerations. All blue bars show the conditions where 50rpm was used, orange bars show where 225rpm was used and grey bars show where 400rpm was used. Each bar shows n=1 5L bioreactor experimental run.*

Figure 4.19 shows the titre achieved from the T4 and phage K sparging DoE. Both graphs indicate that run 4, 225rpm, 1L/min air gave the greatest titre. Given that the initial 5L DoE showed that 225rpm gave the greatest titre and the experiments in figures 4.11 and 4.12 (1L/min air) this result may have been expected.

Generally, the processes show that as the sparging rate increases for each agitation level, the titre reduces. Figure 4.19, graph A shows that 2L/min, 50rpm gave a statistically significantly greater titre than at 1L/min, 50rpm, $p<0.05$ using a paired t-test. The low agitation rate may prevent additional air dissolving into the culture medium whilst a higher rate of air (2L/min) may be better for oxygen availability for the culture. No statistically significant difference was found for these parameters for phage K. There was a statistically significantly lower titre seen for run 4-5 and 8-9 for phage K. This could have been due to 2 and 3L/min sparging of air damaging the cells and phage or preventing binding when the bubbles burst which may have accounted for the reduced final titre.

Scaling the process into a stirred tank reactor meant that this experiment was needed, given that the impellor speed may have an impact on the way that the bubbles can dissolve in the culture which would in turn have an effect on the adsorption, burst size and final phage titre.

**4.8 Medium investigation in an STR**

Given that chapter 3 examined the effect of defined medium on the shake flask bioprocess, the defined medium study was scaled up into a 5L bioreactor. The experiment was only completed for T4 phage due to the time available. Figure 4.20 shows the phage titre achieved using baseline and greatest titre conditions. The graph shows that there was a statistically significant decrease in titres achieved for both baseline and greatest titre conditions between LB media and the defined media, $p<0.05$ using a paired t-test. No air was used to sparge the cultures as given that there was a reduction in titre, and a reduced host growth, sparging may have affected the kinetics in a poor choice of media and the titre may have been even further reduced. This was a similar result to the shake flask model where no improvement in titre was found and is therefore perhaps unsurprising. It may be that the defined medium is unable to support the growth of host and phage and reduced titres were seen. Given that future bioprocesses may need to move towards defined medium, it was important to show that the

process could at least be scaled into a 5L bioreactor. Future investigations could carry out more extensive studies on components to make up a defined medium to improve titres. However, they would need to be as cost effective as undefined medium to be viable. Future studies may also wish to examine the burst size of the defined medium in the 5L bioreactor.

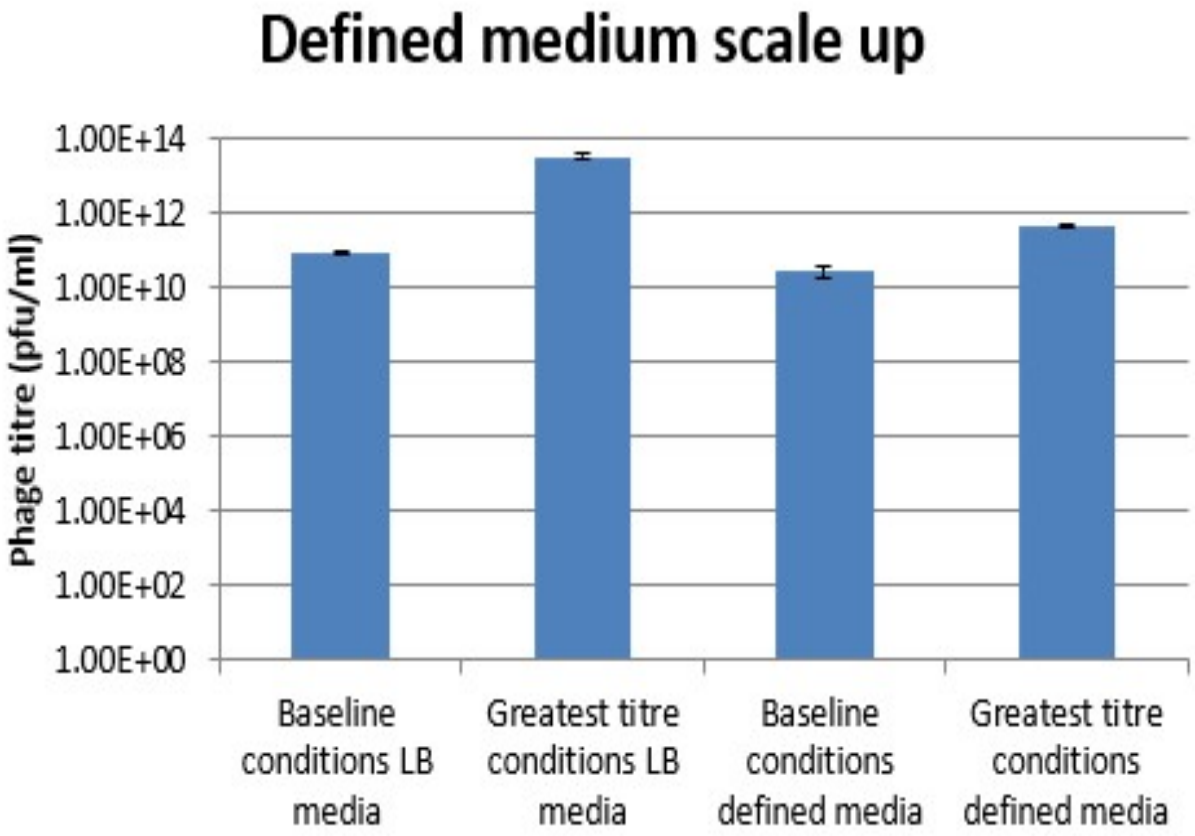



***Figure 4.20.*** *A graph to show the T4 phage titre achieved using the baseline and greatest titre conditions in the 5L bioreactor with the defined medium from Sochocka et al, 2015. Error bars show 1 standard deviation of a duplicate experiment with duplicate plaque assay enumerations. Each bar shows n=2 experimental runs.*

Given that the results thus far have shown the creation of a robust model for batch production of phage, as stated earlier, the work my Mancuso and colleagues highlighted the need to move towards continuous culture. Another study examined the effect of continuous culture of T4 phage and its effect of single stranded

DNA production. 8 parallel bioreactors were run, each with a 10ml volume and showed that only a 20% reduction in DNA was found when phage infection took place compared to the control. Whilst phage titre was not measured, the study adds value to the field showing that phage have been used in bioreactors elsewhere as phage contamination of microbial processes can be devastating when taking place on an industrial scale i.e. if phage is not properly contained in one lab and contaminated a large scale microbial production, there may be titre loss (Kick *et al,* 2017).

Sochocka *et al*, used completed their defined medium study in an 8L working volume in a fermenter for T4 phage. Whilst our experiments focused on shake flask and a 3L volume, there has previously been larger scale studies run for phage bioprocessing specifically. It will be important that future experiments focus on larger fermenter studies to continuously improve the manufacturing of bacteriophage. Other industries have seen the use of 200L volumes for plant and animal cell production (Ramirez *et al*, 2018, Zhu *et al*, 2018). Additionally, wave bag reactors have also been used in the microbial cell fermentation (Kurt *et al*, 2018). Given their lower cost, enhanced configuration and handling due to its shape, some studies tend to focus towards the use of wave bags rather than bioreactors (Scholz & Suppmann, 2016). It may be interesting for future studies to compare the results presented here to a wave bag system.

**4.9 Conclusion**

This chapter has explored the scale up of the T4 phage and phage K bioprocess from a shake flask culture system to a stirred tank 5L bioreactor system with a 3L working volume. The scale up challenges were initially eluded to but using the small-scale DoE determined greatest titre conditions they were successfully translated into a 5L culture system. Optimising a process and reducing variation enables fewer variable processes, and results, and allows more control over a process thereby improving the scalability. This was a hugely critical part of the process as without achieving a similar titre in the 5L, the process could not have been taken forward whilst enthusiasm for future larger scale experiments would have been dampened with more research needed.

One of the initial aims of this chapter was to successfully scale up the shake flask baseline and greatest titre conditions into a 5L bioreactor which was completed. Whilst this study shows that similar titres were achieved between culture systems, there can be a lot of work to achieve a similar titre, for all cell types, in a

stirred tank system compared to a shake flask system. There is a huge shortage of knowledge that exists within the bacteriophage bioprocessing field, and the research presented thus far has added an important study to examine the small scale production of T4 and phage K.

Upon successful completion of the scale up, the second aim of the chapter was to design and run a DoE experiment examining the pH of the culture and the agitation. In the shake flask experiments, 225rpm and 150rpm gave the greatest titre for T4 phage and phage K respectively. A wide variation in levels was used in the 5L DoE (50-400rpm) to allow an estimation to be made as to the level that gave the greatest titre. Whilst this is a big range for shake flasks, it is far less significant in a stirred tank bioreactor given the 3L working volume of culture. Future studies may wish to examine agitation more closely or sheer damage caused to host cells or phage. Additionally, a closer examination of the pH at which the bioprocess took place may be beneficial. pH changes in the shake flask system were not controlled in our shake flask experiments, and whilst it can be controlled in a shake flask, there are few who choose to do so for all bioprocesses. The changes in the pH that occur would prevent homogeneity throughout the culture, whilst it may also affect the rate of host growth and phage propagation due to waste products given off.

Upon completion of the 5L DoE, the conditions which gave the greatest titres were validated to determine the reliability of the bioprocess. The validated processes were then sparged at either 2 or 3 L/min air, the third aim of the chapter. This would increase the overall air within the culture which although did not improve phage titre was interesting to see how it affected the culture. The final part of this chapter aimed to examine the kinetics of the infection in the 5L to provide insight into the increases in titre produces and successfully showed significant differences in burst, adsorption and final titre between baseline and greatest titre conditions. This chapter has developed a robust process for batch phage bioprocessing and future studies may use this work as a starting point for future fed-batch or continuous process studies.

It should also be taken into account that given that a reduced temperature of infection saw an improvement in titre in the 5L bioreactor, this may bring a significant energy saving. If further large-scale experiments were to be run, additional money would be saved which would improve the environmental concerns and increase cost effectiveness and efficiency of a process.

Whilst our study has shown that between the two scales used the methodology can be used, between a 5L scale and a larger stirred tank reactor, there may be differences in the experiments carried out. It is hoped that this study will pave the way for future larger-scale bacteriophage manufacturing whilst the methodology used here has been shown to two separate phage, it could also be applied to other phage bioprocesses.

Chapter bridge

This chapter has examined the scale up of the bacteriophage bioprocess for T4 phage and phage K. Whilst this is a key aspect of bioprocessing, future work must examine the further scale up of the bioprocess to make a clinically relevant bioprocess. However, there is one area that may be useful to examine, alternative characterisation methods. Throughout this body of work, it has been highlighted that the plaque assay is the gold standard method of enumeration. However, this method whilst simple and cheap can take up to 24 hours to complete. Therefore, it may not be the most efficient method to examine the final phage titre achieved. Chapter 5 examines a novel method for characterising the burst size and phage titre.

# Chapter 5 –

# Alternative characterisation methods

**Chapter overview**

This chapter aims to explore a novel method for detection of the bacteriophage bioprocess by using a nanodrop2000 to measure the DNA, RNA and protein content from the culture. It offers a simple and cheap method to determine the amounts for each of DNA, RNA and proteins at various time points. It is hoped that this method, if validated, could be an indicator for titre or burst that is comparable to the conventional plaque assay and have the advantage of being a more rapid method since the plaque assay can take up to 24 hours to obtain results. Two methods will be used, firstly a method based on the nanodrop2000 which requires only a 2µl volume and secondly a method based on GCMS.

**5.1 Introduction**

Upon detection of a new bacteriophage, there is a standard protocol that most researchers follow. They screen a variety of host organisms to determine their infectivity range or hosts, complete a number of microscopy analysis for visualisation of the phage, extract the DNA to determine its sequence, often running it through the NCBI database and carry out infection assays to determine its stability at different temperatures or pH levels (Ciacci *et al*, Moodley *et al*, 2019, Newase *et al*, 2019). However, the one factor that is constant is the need for its titre determination through the plaque assay. It has so far been argued that on the horizon may be a new era of bacteriophage therapy and therefore novel and more rapid methods of detection will be needed. Modern analytical research has allowed us to improve detection times of a measurement, reduced times needed in the analytics, improved accuracy of results as the greater detection capabilities has removed background noise and improved reliability/accuracy and the enhanced capabilities has allowed us to be able to detect sensitive elements and trace elements (Ehsan & Yang, 2018, Guerra *et al*, 2019, Lin *et al*, 2019, May, 2019).

In addition to new methods being needed for the rapid detection, the manufacturing industry highlights the need for rapid methods of detection and characterisation (Marshall *et al*, 2018, Mutalik *et al*, 2019, Yasin *et al*, 2019). Currently, when manufacturing large batches of phage i.e in the phagoburn trial, manufactures would need to run a plaque assay at the end of their bioreactor run to determine the titre of phage produced. However, this may not be sufficient as there is a need to know rapidly how many phage have been produced. Therefore,

this chapter aims to explore a potential method for the rapid detection and characterisation of the culture and determine how many phage have been produced, rather than having to go through the laborious plaque assay. Moreover, when running experiments such as the DoE from chapter 3, or any of the adsorption assays, there will be multiple samples that need to be enumerated. This can often end up with multiple samples that are waiting to be analysed given that an overnight culture of host bacterium is needed for the plaque assay. If more experiments were to be run on an industrial scale, rapid characterisations would be needed. Whilst it was previously highlighted that the production of penicillin was held up due to the production, characterisation is equally as important as production. More rapid characterisation means that patients will be able to be treated in a more time efficient manner which will allow them better chances of recovery (Chen et al, 2019, Cornut *et al*, 2019). Additionally, new capabilities will allow research to advance which may lead to better testing and treatments.

Both the baseline and greatest titre conditions from the DoE were run in shake flasks and 5L bioreactor with samples takes at the point of infection (POI), 10/20 minutes after the point of infection and at burst i.e 30 minutes. Samples were also taken at 1 and 1.5 hours after the point of infection and at harvest. Use of the nanodrop device allows the operator to take a filtered 2µl sample from the culture and to instantly give a measure of the total DNA, RNA or protein content (Li *et al*, 2019). The nanodrop method has two significant advantages. Firstly, it requires an extremely small volume. There is no need for a lengthy purification and volume reduction step as is needed in the plaque assay method whilst smaller volumes are usually easier to control. Secondly, a reading will be taken within seconds and a measurement made giving it a further advantage over the plaque assay which requires an overnight incubation period. Moreover, it also prevents the need for cuvettes when taking measurements. Additional measurements will be able to be made as setting up multiple plaque assays whilst is relatively easy will not be as quick as putting a sample of the culture onto a nanodrop spectrophotometer and analysing the sample. Unlike other instruments which measure DNA content, it has the ability to scan all wavelengths, 220-750nm. Nanodrop has previously been used to quantify extracted phage DNA only (Duyvejonck *et al*, 2019).

Given the mechanism of phage infection, the phage infects the bacteria and lyse them releasing the host bacterial DNA, RNA and proteins into the culture. Use of the nanodrop could provide a rapid method for detection of burst or allow a direct comparison to final phage titre.

Recently, there has been an interest in using gas chromatography-mass spectrometry (GCMS) for rapid analytical technology. It has the ability to detect substances within a sample. By heating the sample, chemical differences within the sample can be observed by the mass spectrometer. The sample is heated, and the ions travel down a column in the gas chromatogram before being detected by the mass spectrometer. The ions will travel at different speeds based on their density which will be detected in the mass spectrometer (Beale *et al*, 2018). It is for this reason that both pieces of equipment are needed.

Here, this study applies GCMS to volatile fatty acids. Different volatile fatty acids (VFA's) will be examined, all which have different numbers of carbons in their structure. Volatile fatty acids have between 2-8 carbons which will determine their size and the time taken to be detected within the mass spectrometer. They are formed from larger molecules such as fats, carbohydrates or proteins and can also be byproducts from digestion. However, they are also the main source of energy for microorganisms (Ghidotti *et al*, 2018). They can, however, be harmful to the environment given their pungent odor and increase mobility of heavy metals (Banel & Zygmunt, 2011). Whilst they have a presence within the culture medium, given that they are a byproduct, their total amount may increase which may be detectable. This may offer an additional solution to detecting differences within a phage culture.

The overall aim of this chapter is to improve process analytical technology for bacteriophage analysis. As it has been previously eluded to, the current gold standard method for bacteriophage analysis is the plaque assay. Whilst this is cheap and simple, it only allows the user to determine which phage is present and how much of it there is in a sample. Process analytical technology is used and given merit to by the FDA to analyse and control manufacturing processes through measurement of critical parameters. Currently, there are few additional methods used to analyse phage cultures and we present two alternative methods here.

**5.2 Aims and objectives**

1) To determine whether there is a correlation between the level of DNA/RNA/Protein at different time points throughout the infection and the different conditions/culture systems
    a. The baseline and greatest titre conditions were run in both the shake flask and 5L bioreactor and samples taken at different time points throughout the infection to show levels of DNA/RNA and proteins and track their levels throughout the infection process
2) To determine if there is any correlation between levels of DNA/RNA and proteins and final phage titre/burst
    a. Manufacturing processes will benefit from a rapid method to determine if there can be a correlation at any point of the infection of the measured values and final phage titre/burst
3) To examine volatile fatty acid concentration, based on phage-host system and determine if there are differences between the cultures.
    a. Using VFAs may allow an alternative method for examining the bacterial-phage culture and show any differences that can allow us to distinguish between phage.

**5.3 T4 phage nanodrop analysis**

Figures 5.1 to 5.6 show the DNA, RNA and protein content from the baseline and greatest phage titre experiments in shake flasks and the 5L bioreactor for T4 and phage K. They were measured by examining the filtered supernatant from the culture. The experiment was completed in quadruplicate with duplicate nanodrop measurements giving a total of n=8 measurements for each culture using each condition.

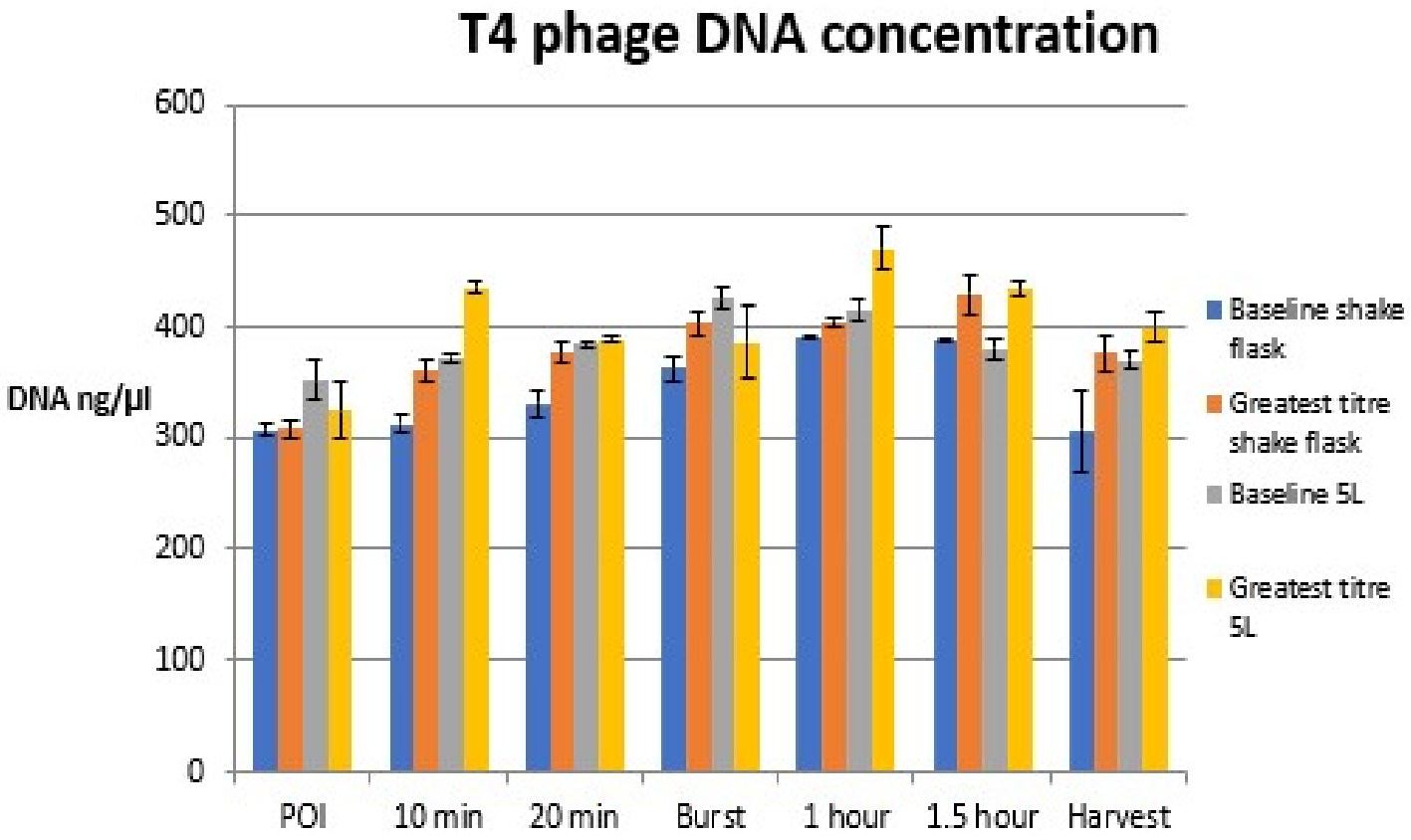



*Figure 5.1. T4 phage DNA concentrations throughout the infection using baseline and greatest phage titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

It was interesting to note that the bioreactor greatest titre conditions gave the highest harvest phage titre from each of the experiments using the data from chapters 3 and 4. It is possible that the higher harvest titres would result in higher amount of bursts of host organism and higher levels of DNA. Figure 5.1 shows the DNA concentration in the T4 phage at both conditions in both culture systems. The figure shows that the greatest titre conditions in the 5L bioreactor gave the highest DNA content at most time points. From Figure 5.1 it can be seen that the amount of DNA in the filtered supernatant is significantly higher for the greatest titre conditions for both culture systems (shake flask and STR) after 10 minutes of infection, $p<0.05$ using a paired t-test. It has previously been shown that DNA content can be related to antibody content. Whilst our experiment aimed to show DNA content based on time, the previous study by Hsu *et al* showed that they could estimate final titres upto a 98.1% accuracy (Hsu et al, 2001). More recently, Virok and collegues showed that herpes simplex virus could be quantified based on a PCR method of DNA (Virok *et al*, 2015).

From Figure 5.1 it can also be seen that the DNA content seems to increase upto 1 hour and subsequently there seems to be some decrease in DNA content which continues to the point of harvest. It might be assumed that as the phage breaks down the host organism, the DNA content will continue to increase throughout the infection. It has previously been reported that bacteriophage have DNase enzymes on their surface (Kim *et al*, 2000, Broudy *et al*, 2002, Rato, 2011). When the enzymes come into contact with the free DNA in the culture, there may be some breakdown of the DNA which could lead to a reduction of DNA content. It follows that when there is sufficient phage with DNase enzymes to break down the DNA in the culture then the DNA content of the culture will reduce and in Figure 5.1 this seems to take place after 1 hour. It also shows that between 1.5 hours and harvest, there was a statistically significantly reduced DNA concentration for each culture, $p<0.05$ using a paired t-test. Given that the T4 phage infection took 3 hours and the DNA concentration is increasing in the first 90 minute of culture, then it seems that the DNA was being broken down by the DNase on the phage surface in the remaining 90 minute period of the culture and this may explain the reduced DNA detected at harvest.

The data in Figure 5.1 also shows that there is a statistically significant increase in DNA concentration from the POI to 10 minutes after POI for the greatest titre conditions in ths shake flask, $p<0.001$ for both. It is known that typically bacteriophage replication can take around 15 minutes when inside the host organism. The data in Figure 5.1 shows that the DNA content increases between the POI and 10 minutes in the 5L bioreactor

and the greatest titre shakeflask and therefore the host organism may have broken down earlier than is typically the case.

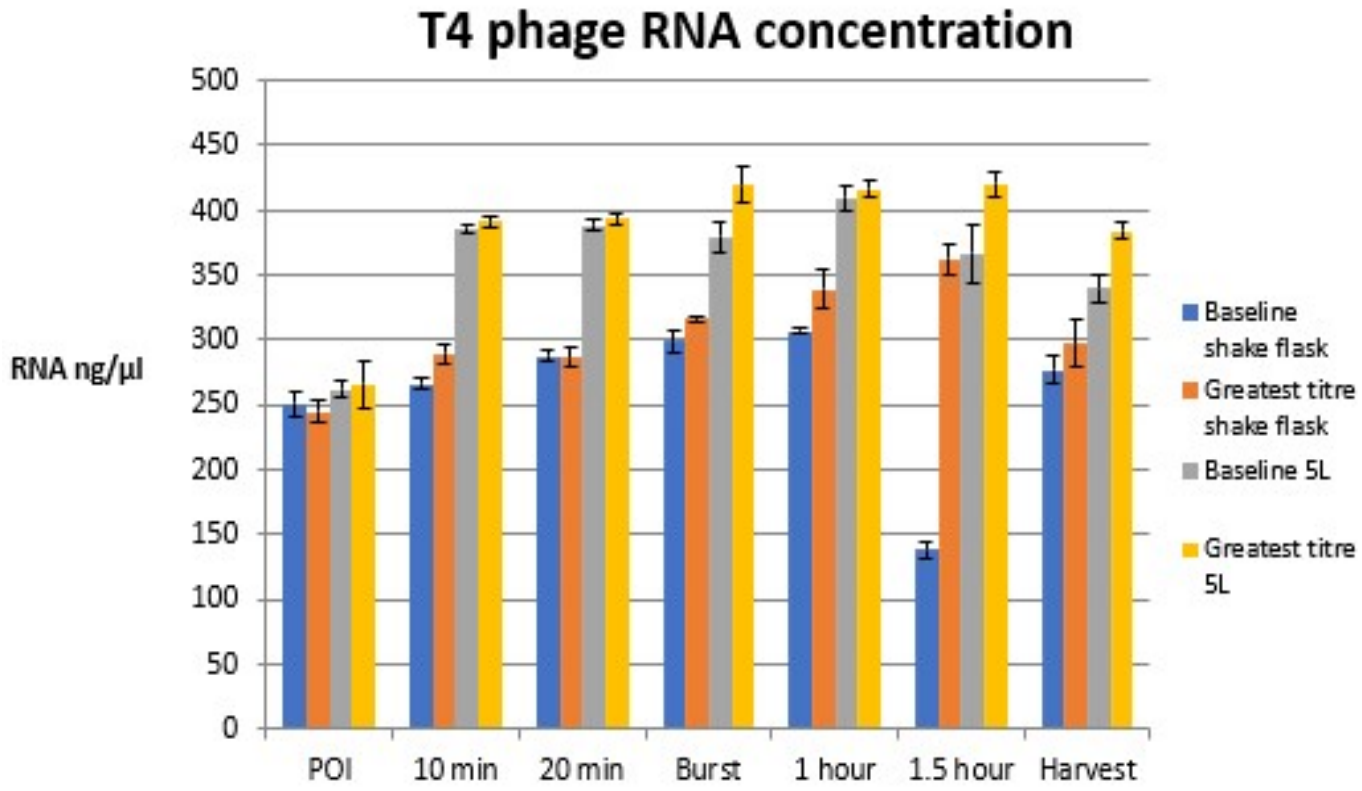



*Figure 5.2. T4 phage RNA concentration throughout the infection using baseline and greatest titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

In chapters 3 and 4, data was given showing that the burst size and harvest phage titre are greater in the 5L bioreactor compared to the shake flask. It could be anticipated that if there are more bursts then there should be more RNA in the culture. Figure 5.2 gives the RNA concentration in the culture for T4 phage at both conditions and culture systems and shows that there is generally a far greater RNA concentration in the 5L bioreactor compared to the shake flasks at all time points. It also shows that between the POI and 1.5 hours there is an increase in RNA content whereafter there seems to be a decrease. In addition to there being DNase on the phage surface, there is also RNase which will cause the breakdown of the RNA in the culture. Figure 5.2 also shows that there was a statistically significant decrease in RNA between 1.5 hours and harvest when the greatest titre conditions were used in both culture systems $p<0.001$. This could be attributed to an increase in the phage with higher amount of RNase present on the surface which leads to a reduction in RNA in the culture. There may be a discrepancy in the baseline shake flask data at 1.5 hours.

Previously it has been examined for non-microbial cultures that RNA can be used in quantification from cell lysates, however, RNA is unstable and samples must either be stored appropriately, stored in buffer or analysed within an appropriate time frame (Svec *et al*, 2013). For all experiments presented here, samples were analysed immediately. Previous authors have also used northern blot to analyse rna and quantify cells (Streit *et al*, 2009).

Figure 5.2 shows that after 10 minutes, there was a statistically significant increase in RNA in the baseline and greatest titre cultures in the 5L bioreactor compared to POI, $p<0.001$. The studies within chapters three and four assumed a standard burst time of 30 minutes. However, the data presented in Figures 5.1 and 5.2 show an increase in DNA and RNA content from POI to between 10 and 20 minutes suggests that there may be some burst that takes place before 30 minutes.

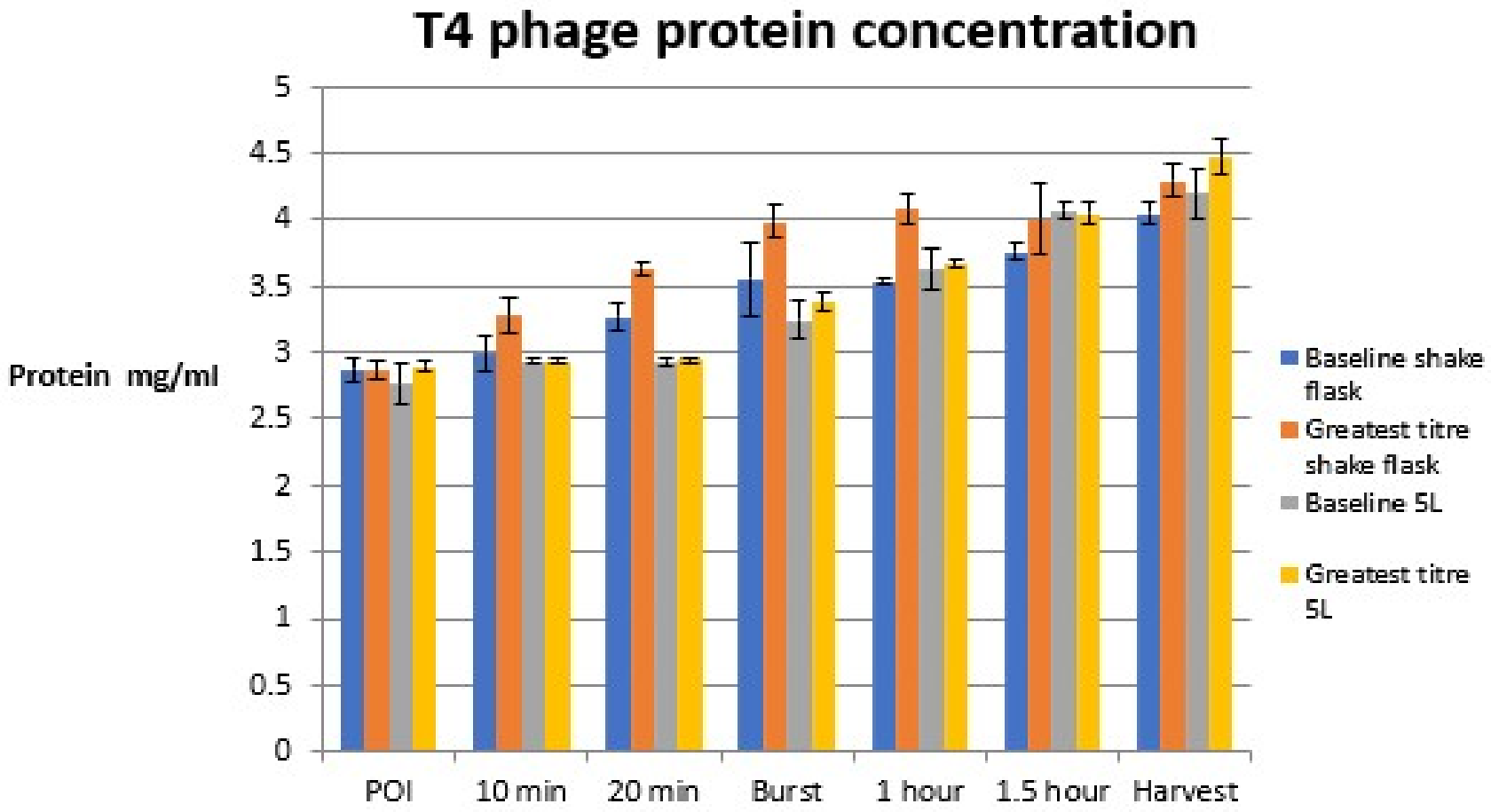



*Figure 5.3. T4 phage protein concentration throughout the infection using baseline and greatest titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

Figure 5.3 shows that the protein content increases continually throughout the whole infection period for both conditions and culture systems, in contrast to the results for the DNA and RNA content. Whilst phage have DNase and RNase on their surface to respectively allow the breakdown of free DNA and RNA within the culture, phage have no mechanism for the breakdown of proteins. It follows then that any protein released from the host organisms will not be broken down by the phage whilst in the culture. The error bars seen for the protein content are slightly larger than those of the DNA and RNA data and consequently repeating the numbers of experiments may reduce the size of the error bars and increase accuracy. It can, however, be seen that there is a clear increase in the protein content throughout the whole of the infection for each culture system and each condition.

Figure 5.1 and 5.2 showed that there were some cases where there was a statistically significant increase in DNA and RNA respectively, after 10 minutes for multiple conditions and culture systems. However, the only significant increase in protein content was using the greatest titre conditions in shake flasks between POI and 10/20minutes and burst. Given that the data showed statistically significant increases in DNA, RNA and protein content after 10 minutes, there may have been some level of burst prior to the standard 30 minutes used in the experiments in chapters 3 and 4. However, a future experiment using microscopy may be needed to visually examine this burst or running a burst size plaque assay experiment to confirm that, the burst could have taken place earlier than expected.

No correlation was able to be found between T4 phage DNA/RNA/Protein concentration and final phage titre or burst size. Given that only a 2µl sample was used, there may have been issues around the small volume which could be open to variation. Whilst small sample sizes are easier to handle, store and maintain and can often be cheaper, a larger volume would give a more accurate measurement of the sample. Additionally, given that there is limited information around the phage biology and the bioprocess, there could have been other underlying issue that contributed to the lack of correlation. Assays must both be sensitive in order to detect changes but also be specific. They can not measure within a very wide range or small range as this will limit assay sensitivity and therefore by examining either all of the contents a more accurate measurement could be made. However, this was the first stage in a new assay development and can be built on in future (Saah 1997).

Figures 5.1-5.3 show that there was a reduction in DNA and RNA content between 1.5 hours of the infection and harvest. Given that phage have DNase and RNase on their surface, it was expected that there would be some breakdown of the DNA and RNA in the culture; this work has shown the level and rate of breakdown.

**5.4 Phage K nanodrop analysis**

Figures 5.4-5.6 show the experiment for phage K using both culture conditions and both culture systems. It was again important to show that the experiment could be repeated for multiple phage-host systems.

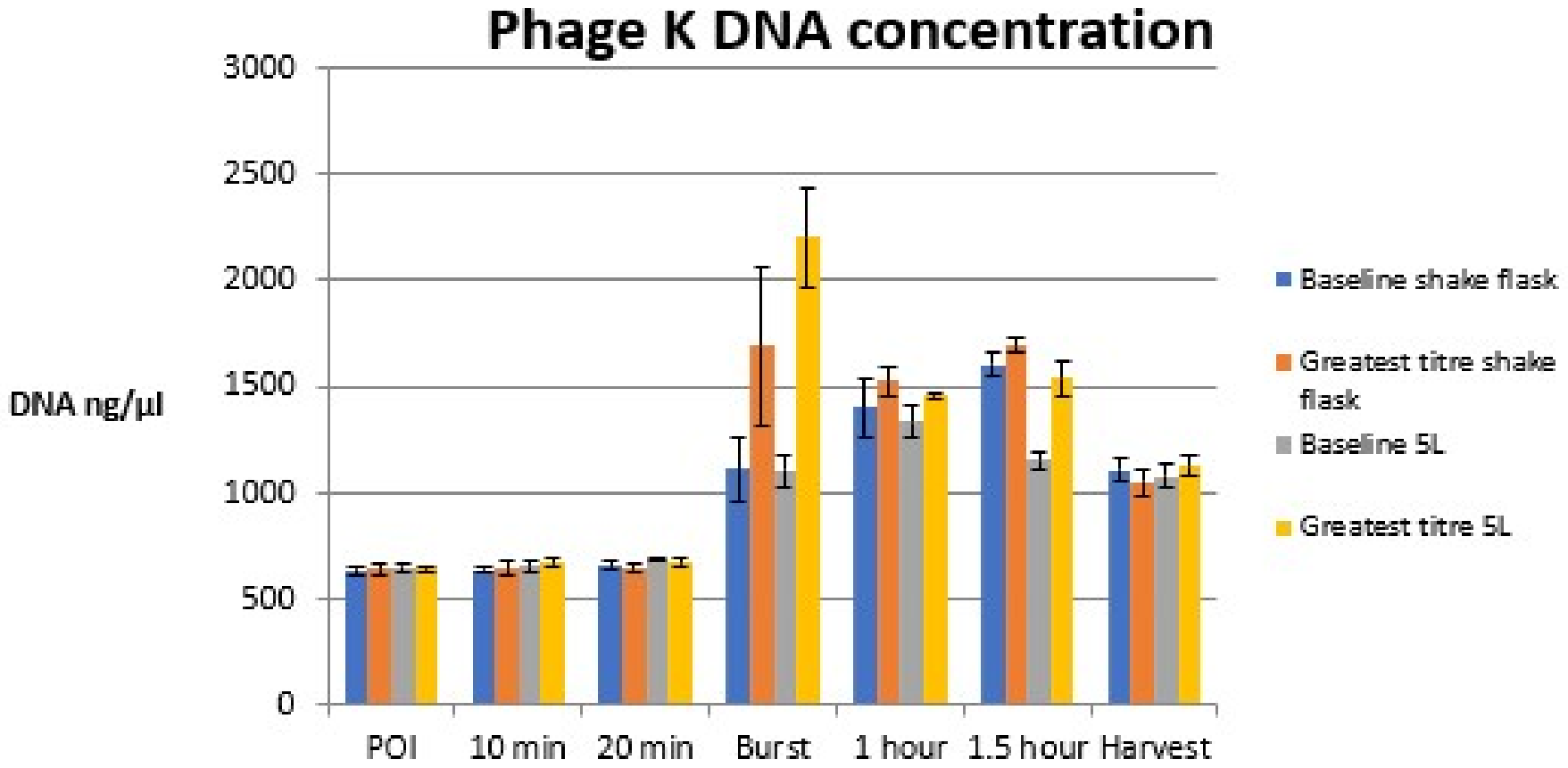



*Figure 5.4. Phage K DNA concentration throughout the infection using baseline and greatest titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

Figure 5.4 shows the concentration of all DNA in the culture when the phage K experiments were run at both conditions in both culture systems. Whilst there was an increase in DNA in the T4 phage experiments prior to burst at30 minutes, the data showed no statistically significant increase in DNA for the phage K experiments until burst. Whilst there is some evidence that the burst can take less than 30 minutes for different phage, figure 5.4 suggested that phage K takes >20 minutes for any burst given that there was no increase in DNA content. Only after 30 minutes burst was there a statistically significant increase seen in DNA content for each experiment, highlighting a major difference between the two phage. This further gives the importance of repeating the experiment for different phage.

When using the standard plaque assay, if any bacteria were toremain in the culture, phage activity may be overestimated. One study showed that there may be a statistically significant overestimation in phage activity that is shown through plating compared to a qPCR technique (Weld *et al*, 2004, Liu *et al*, 2014). This was due to cell-surface attached phage during the incubation period of plating and host cells still being metabolically active during the plaque assay. However, this work has shown free DNA/RNA and protein concentration where the culture has been purified from bacteria through centrifugation and filtration. Additionally, due to there being no need for plating, there was no chance of further propagation of phage. To determine the point at which burst occurred, this study had to take a sample of phage at 10/20/30 minutes after infection and carry out the overnight plaque assay. However, the result from the nanodrop shows free DNA that must have been released from the burst due to its increase and there was no need for plaque assay i.e. no need for a long incubation period and no chance of overestimation of phage activity.

Whilst phage K also has DNase on its surface, a reduction in DNA content was seen between 1.5 hours and harvest. There was a statistically significant reduction in DNA concentration for all cultures except the baseline conditions in the 5L bioreactor between 1.5 hours and harvest, $p<0.0001$. Although no statistically significant reduction was seen for the baseline conditions in the 5L bioreactor, the study may have benefitted from further repeats.

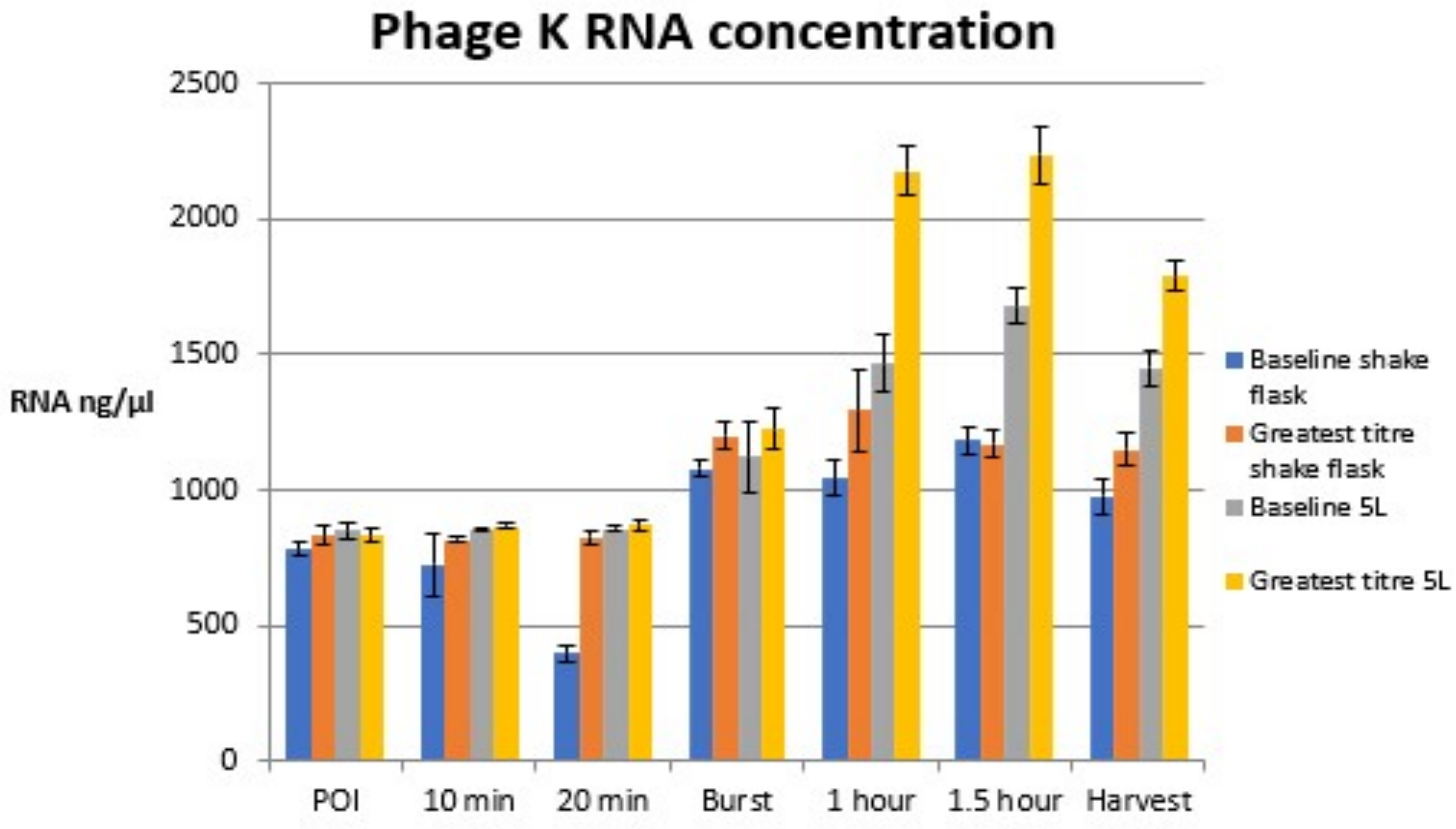



*Figure 5.5. Phage K RNA concentration throughout the infection using baseline and greatest titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

Figure 5.5 shows the greatest average RNA concentration present in the culture and is seen in the 5L bioreactor when the greatest titre conditions were used. Similar to the DNA study for phage K, the data shows no statistically significant difference between the RNA at POI and 10/20 minutes. However, there was a significant increase in RNA between POI and burst for all cultures, $P<0.001$. The data for the 5L bioreactor experiments suggest that there is a breakdown in the RNA between 1.5 hours and harvest i.e 4 hours, although it was only statistically significant for the baseline shake flask experiment and both 5L bioreactor experiments, $p<0.001$ using a paired t-test. Given that phage K has RNase on its surface, this may be attributed to the reduction in RNA content.

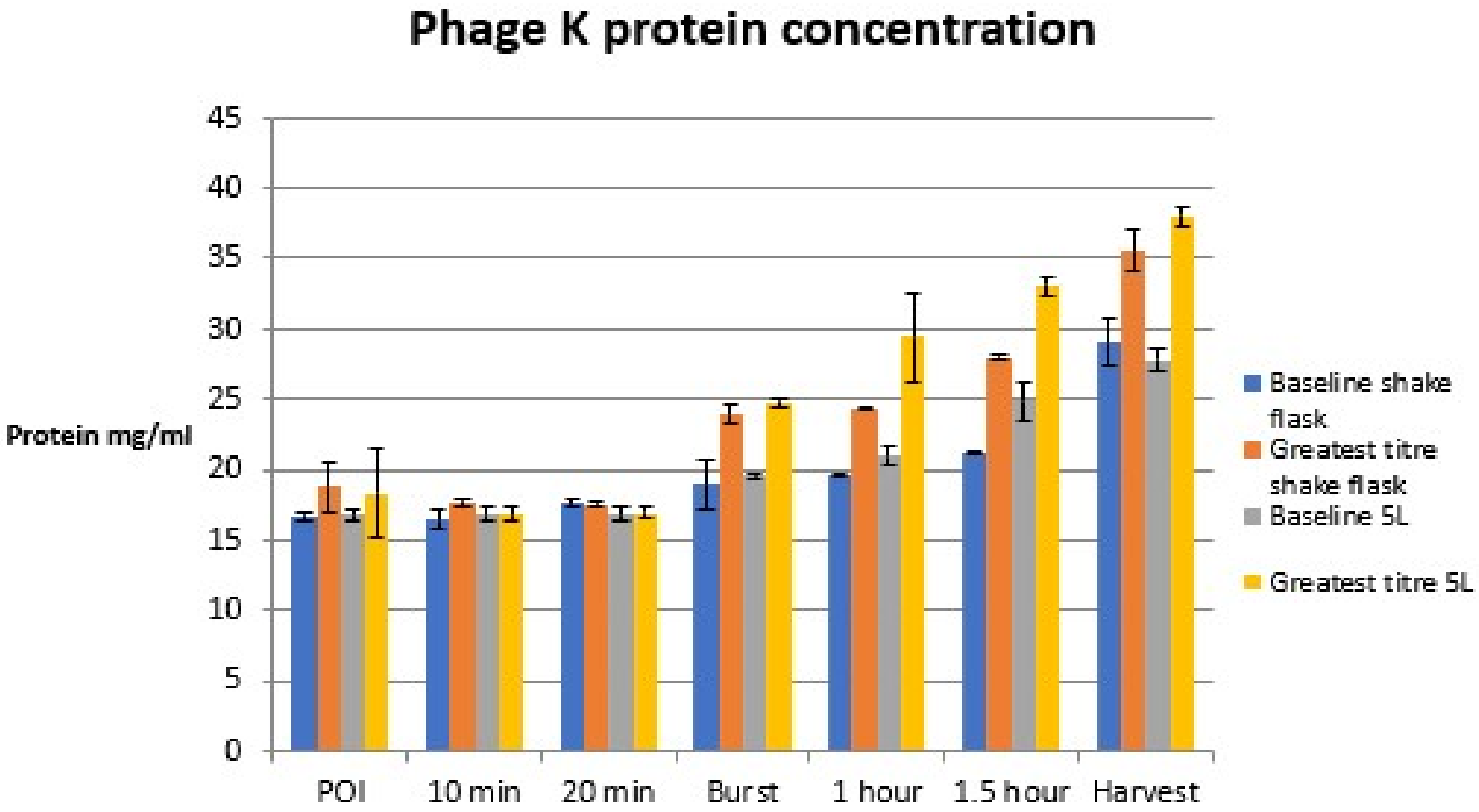



*Figure 5.6. Phage K protein concentration throughout the infection using baseline and greatest titre conditions in shake flasks and 5L bioreactor. All blue bars show the baseline shake flask infection, orange bars show the geratest titre conditions in a shake flask infection, grey bars show the baseline 5L infection and yellow bars show the greatest titre in a 5L infection. A total of n=4 measurements were analysed for each time point of each experiment.*

Figure 5.6 shows the protein content for all experiments at both conditions using both culture systems for phage K. The graph shows a continuous increase in protein throughout the infection process. Initially, the data shows that there was no statistically significant increase in protein between POI and burst, similar to the DNA and RNA results for phage K. However, between POI and burst, there was a significant increase in protein concentration for each culture $p<0.01$. Given that phage K has no mechanism for protein breakdown, it was expected that there would be a continuous increase in protein.

Figures 5.4-5.6 shows that it takes at least 30 minutes for phage K burst to occur, given that the data for DNA, RNA and protein showed no statistically significant increase until 30 minutes i.e burst. This was unlike the T4 phage data which suggested there may be a degree of burst at either 10 or 20 minutes when using certain conditions or culture systems.

Previous studies have shown that proteins can be used for quantification of product through fluorescence and spectrophotomic assays and have a use in downstream purification (Kennedy & Jones, 1994, Hawkins *et al*, 2009, Brestrich et al, 2018, Whongsiri *et al*, 2018). Although crude protein concentration has never previously been used for bacteriophage quantification, quantitative PCR measurements have previously been used and have been shown to provide a useful method for direct quantification, specifically for phage (Peng *et al*, 2018). Although previous studies used have examined spectrophotometry methods for linking quantification of titres to DNA/RNA/Protein concentration in a crude lysate have been used and the method here was based on a spectrophotometry method, other spectrophotometry assays may have been more useful to try (Pellicer *et al*, 2018). The advantage of the nanodrop method was that it allowed the user to use a small amount, but using a larger sample of the whole crude lysate may have been more beneficial. If this work were to be repeated, the 1ml sampling method that was used for optical density analysis of the final culture using a Shimadzu UV-1280 UV-Vis spectrophotometer could be used in this case. The sample could be purified from host organism through centrifugation and filtration and then be analysed using a larger volume. This may provide an alternative method that could allow a direct comparison between DNA/RNA/Protein concentration in the sample to burst size or final phage titre. However, there would be several impurities in the sample that still remain although may offer an alternative solution.

The data was further analysed to determine if there was a correlation between burst or harvest and DNA/RNA/Protein concentration, but no correlation could be made. This was also the case for the harvest concentrations. Whilst the method presented above may not be a fully functional method for detecting the phage infection, the data has shown that the burst can be detected by the concentration of DNA/RNA and protein. To be a future useful method, there would need to be significant development of the method, however, given that the data was a duplicate sample of a duplicate experiment i.e n=4 measurements, the experiment would need to be repeated over multiple samples, phage/host systems and experiments to determine its reliability despite the results shown. An additional experiment could have been run for DNA/RNA/Protein concentration for each culture system and time point, with a sample taken every minute to show the increase in their respective concentrations and the exact point at which burst occurs.

Whilst there may not be value in taking specific method forward for T4/K enumeration, given the time it takes to run the experiment it would be worthwhile running a similar experiment for other phage. If a method were developed where final phage titre or burst size could be estimated through a nanodrop method, which takes minutes compared to overnight in the current plaque assay, it would be extremely valuable. Additional work may wish to examine carbohydrates, sugars, amino acids, proteins or fats present in the culture at different time points which may be able to be used to relate to burst size or final phage titre. They have been used in both aerobic and anaerobic cultures as methods for quantification which may have merit here. Improving times for quantification and detection is of high importance and these additional factors could be investigated to improve times on the current plaque assay (Mauerhofer *et al*, 2019).

Process analytical technology has advanced in recent years and has reduced variability by using real time, rapid monitoring and on-line measurements. This has also allowed operations to see greater control over processes (Dai et al, 2016). The work presented here has shown a rapid method for phage culture monitoring, if a DNA/RNA/Protein measuring system were to be implemented this could be incorporated into a bioreactor which would prevent the need for sampling, reducing sterility concerns and allowing real time detection. Additionally, knowing the latent period may become more critical in future for phage infection as reducing this may reduce process time. There may also be value in examining whether or not there is any correlation between DNA/RNA/Protein content and which bacterial/phage system was used which could be used to establish a model

of detection of phage. Previously, flow cytometry has been used to differentiate between viruses (Khalil *et al*, 2016).

The graphs in figures 5.1 - 5.6 have shown a novel method for examining bacteriophage culture. They show that there can be a significant increase in DNA/RNA/Protein content in the culture which can be detected. Whilst the experiment was not able to make a comparison between DNA/RNA/Protein and titre or burst size, the data did allow us to examine the differing concentrations based on culture system/conditions and time. Given that impurity is a huge challenge that phage therapy faces, this may provide the basis for further experiments to help control the level of impurity in the cultures. The graphs show DNA and RNA reduce from 1.5 hours-harvest, further experiments could be run examining the addition of phage to the culture which may then further reduce the levels of impurities as proteins are easier to purify. It should be noted that the data for the control experiment, where no infection took place was not fully run and only 30 minutes after the infection took place i.e. burst, was the DNA/RNA and protein content examined and is shown in table 5.1 below. Table B1 in appendix 2 shows the burst size and final pfu/ml when the experiments were repeated for chapter 5 to ensure there was no difference between within the experiments throughout this thesis.

*Table 5.1 – A table to show the difference in DNA/RNA and protein between POI and burst when no phage infection was carried out*

| Experiment | DNA at POI (ng/µl) | RNA at POI (ng/µl) | Protein at POI (mg/ml) | DNA at burst (ng/µl) | RNA at burst (ng/µl) | Protein at burst (mg/ml) |
|---|---|---|---|---|---|---|
| T4 baseline shake flask | 301 ± 6 | 234 ± 8 | 2.8 ± 0.1 | 309 ± 11 | 241 ± 3 | 2.9 ± 0.3 |
| T4 baseline 5L | 307 ± 5 | 249 ± 7 | 2.7 ± 0.1 | 312 ± 13 | 247 ± 8 | 3.1 ± 0.4 |
| T4 greatest titre shake flask | 330 ± 11 | 256 ± 3 | 2.9 ± 0.2 | 308 ± 5 | 261 ± 5 | 3.2 ± 0.2 |
| T4 greatest titre 5L | 315 ± 7 | 263 ± 7 | 2.8 ± 0.1 | 309 ± 8 | 258 ± 13 | 3.3 ± 0.1 |
| K baseline shake flask | 645 ± 18 | 714 ± 11 | 16.6 ± 3.2 | 644 ± 16 | 728 ± 4 | 17.1 ± 2.9 |
| K baseline 5L | 656 ± 11 | 727 ± 16 | 17.2 ± 2.8 | 642 ± 16 | 729 ± 11 | 17.3 ± 2.2 |
| K greatest titre shake flask | 642 ± 11 | 744 ± 11 | 17.8 ± 2.2 | 648 ± 17 | 728 ± 16 | 16.2 ± 2.4 |
| K greatest titre 5L | 652 ± 8 | 721 ± 19 | 17.9 ± 2.8 | 651 ± 11 | 749 ± 13 | 18.1 ± 3.3 |

The data shows that there is no significant difference between the DNA/RNA or protein concentration within either of the conditions between the point of infection and the burst size. Whilst it would have been valuable to look at the DNA/RNA and protein at all points that were taken for the full experiment, it was important to show that there was no difference between the POI and burst. Therefore, any differences seen within the experiment must be due to the phage infection.

Whilst the nanodrop was unable to show a correlation between DNA, RNA and Protein concentration to burst or final pfu/ml value, the experiments were repeated and the final burst size and phage titres are given in appendix 2 to show that the experiment was run to the same degree as all of the other experiments that have been presented in this work.

**5.5 Extraction and quantification of volatile fatty acids**

The previous experiments showed an alternative method of analysis for the bacteriophage infection during culture, however, it was unable to be related to burst size or final harvest titre. Significant method and assay development would need to be carried out in order to address the assay sensitivity and take steps in order to create a more robust and reliable method for culture examination to relate it to burst and final titre. However, the data did prove valuable as it showed the respective levels of DNA/RNA and proteins throughout the culture. Rapid quantification has been given merit and has a role to play in process analytical technology. Therefore, whilst the results thus far in chapter 5 have not been able to provide a method for estimating phage burst or titre based on the nanodrop method, there was value in further investigating the issue. VFA's were chosen to be examined within the culture next as their presence will change due to the metabolism of the host organisms (Zhou et al, 2016). Therefore, by examining them in the phage infection, there should be a notable change in their quantities. Moreover, the technology was available during the research but this is not to mean that there aren't other viable components that could be analysed in the future.

An experiment was run using the samples collected from the nanodrop experiment and analysed using gas chromatography, specifically VFA's were examined. It is known that they will be released through the natural biological metabolism (Wang *et al*, 2020) but the following experiments aim to specifically examine whether or not they can be detected and to what extent. Figure 5.7 shows the fatty acids chosen and the relative abundances compared to the starting amount at various time points throughout the infection cycle from the small-scale greatest titre conditions. Given that VFAs are produced and metabolised by many microbial communities, their levels can be detected through simple gas chromatography analysis. During the host organism burst, VFAs will be released into the culture which can then be monitored. Previous studies have shown that VFA has been detected in wastewater and human urine (Yarimtepe *et al*, 2017, Zhao *et al*, 2017). However, GCMS has never been used for the analysis of bacterial/phage propagation in cultures to specifically analyse VFA's. At the point of infection, a sample was taken with further samples collected after 5, 10, 30, 60 and 90 minutes after infection of the phage. Several fatty acids were chosen and analysed by gas chromatography and mass spectrometry analysis for their abundance in the culture. Figure 5.7 A shows there was a far greater differences in the phage K cultures compared to the T4 cultures which can be accounted for in the media used as BHI media has more fatty acids present naturally than LB media. Moreover, the change in VFA concentration after t=0 can only be explained by it being a biological trend and possibly due to the burst of the host organism and the phage. It is estimated that around 75% of total energy metabolised is from VFAs in humans and they can also be metabolised by bacteria and can be substrates of amino acids used for energy (Penner *et al*, 2009). Therefore, the additional VFAs seen in the culture may be used as an energy source for either the remaining bacteria in the culture or possibly even the phage (Lee *et al*, 2014).

The fatty acids shown in figure 5.7 shows only a significant increase in response for acetic acid for T4 phage culture. This is most likely due to the media contents as BHI broth naturally has more fatty acids present that LB broth given that the latter is largely composed of yeast and tryptone. Additionally, previous authors have shown differences in VFAs between cultures of *E. coli* and *S. aureus* and explain it could be due to their specific metabolism, growth patterns or fatty acid composition (Patrignani *et al*, 2008, Wang *et al*, 2018). It may also be expected that if this experiment were to be run with other organisms/phage, differences in VFAs would be seen as one author saw the differences in VFAs between *E. coli* and *Salmonella enteridis and Listeria* (Siroli *et al*, 2015).

Graph 5.8 below shows the VFA concentration over time in the bioreactor. However, given that few responses were seen for the T4 phage culture, the phage K culture was taken forward into the bioreactor only. An examination of the VFAs from bacteriophage culture has never previously been shown and it may warrant additional investigation as although again, a direct comparison was unable to be made between VFA concentration and final phage titre or burst size, the experiment did add value as it showed the culture can be tracked and that the differences in the culture are attributed to the phage burst and microbial metabolism. Moreover, given that the experiments here used a burst time of 30 minutes, figures 5.7-5.8 show increases in the concentrations of VFA's due to the metabolism and their release. The fatty acids were chosen as they were previously identified in the culture in house. Additionally, the large range of acids were chosen as their number of carbons ranges to give a greater range of data. Due to a freezer breaking down a large number of samples were lost and unable to be recovered and therefore there was a lack of data to produce error bars i.e. each graph represents a single run in figure 5.7.

It is interesting to note that figure 5.7 shows an increase in VFA for phage K for acetic acid around 1 hour whilst there seems to be an increase in propanoic acid and isobutyric acid around the point of burst. Whilst it should be noted that these were single runs, it has previously been shown that acetic acid is a byproduct of catabolism of lactic acid. Microorganisms will typically use lactic acid as one of the final substances for energy through propionic fermentation. Moreover, acetic acid is also one of the end stage VFA's produced which may explain why there is a later spike in the levels of acetic acid (McSweeny *et al*, 2004). Propanoic acid and isobutyric acid are also some of the most abundant VFA's which may also explain why they are seen sooner i.e. if more are produced, they may be detected earlier (Dijkstra *et al*, 2013).

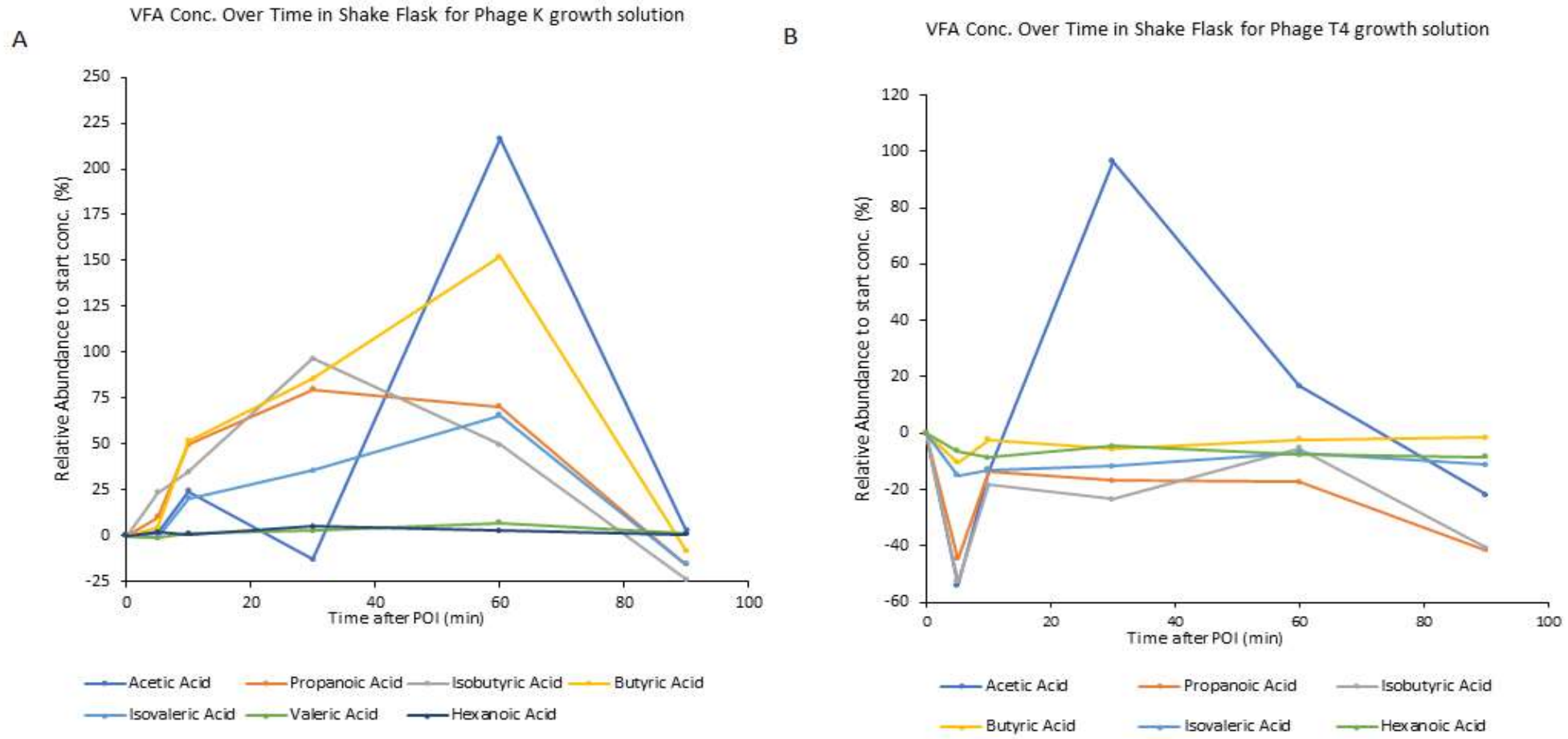
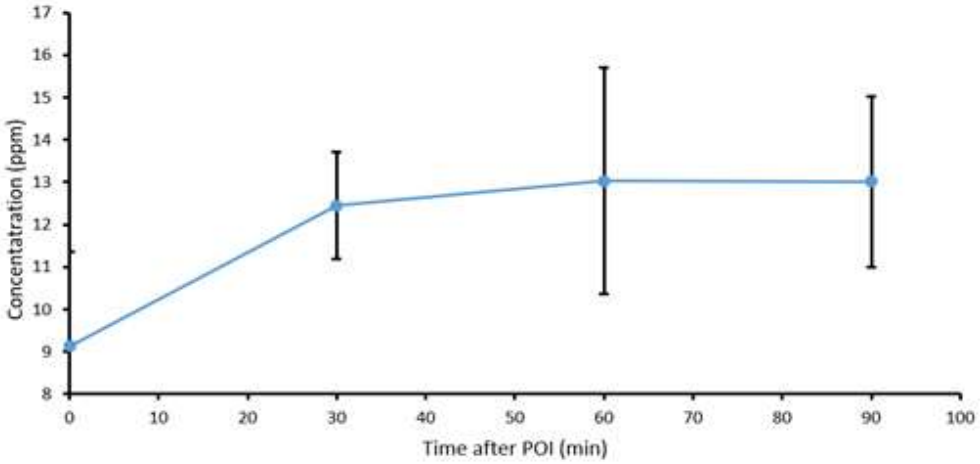
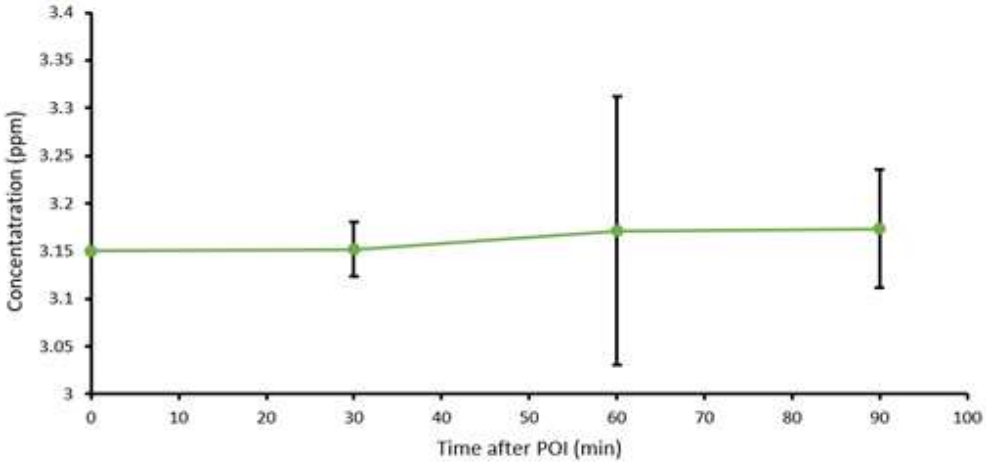



***Fig. 5.7*** *Graphs comparing average percentage relative abundance to start concentration of volatile fatty acids; acetic acid, propanoic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid and hexanoic acid, against time A) Phage K-S. aureus B) T4-E. coli in a shake flask. A mixture of baseline and greatest titre conditions were used in the sample to determine a specific difference between the two cultures. The full 20ml sample was taken from the shake flask and used for analysis.*

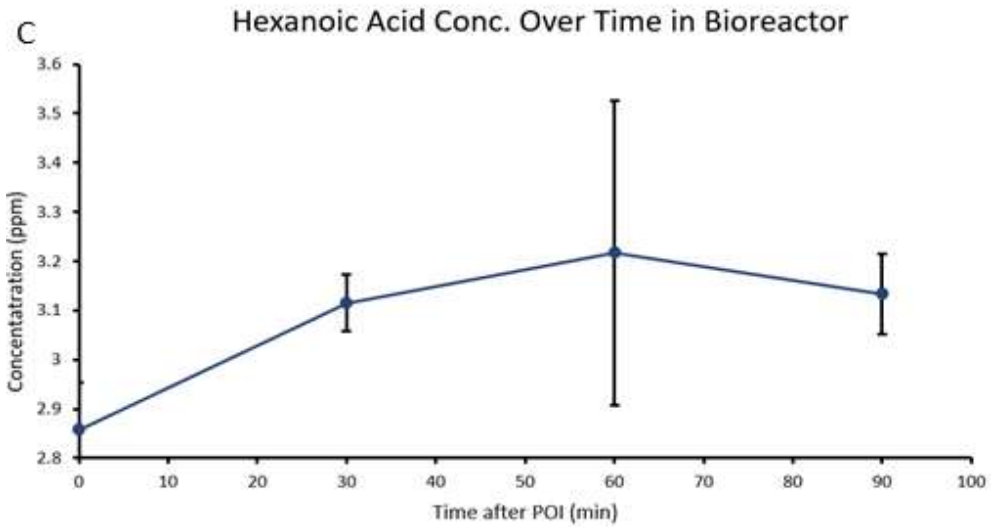
A


Isovaleric Conc. Over Time in Bioreactor
Concentatration (ppm)
Time after POI (min)

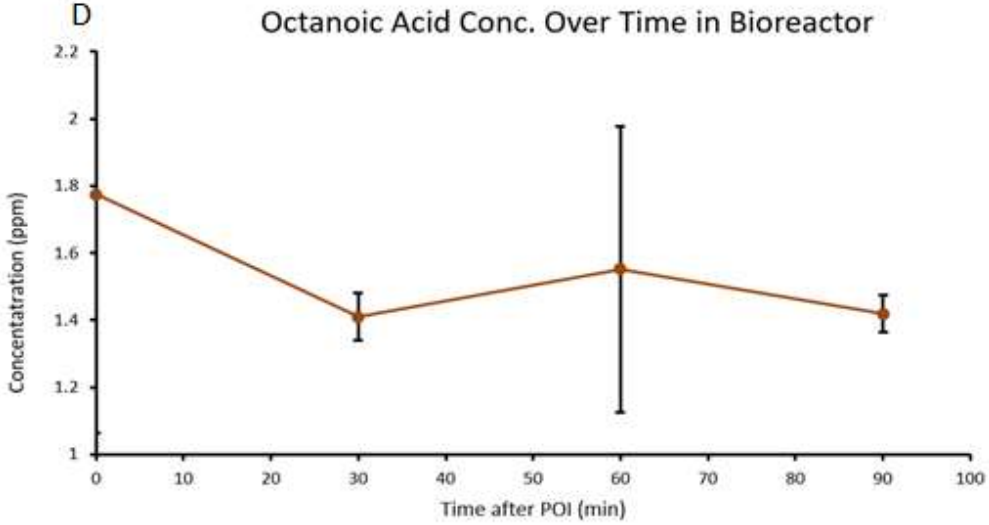
B


Valeric Acid Conc. Over Time in Bioreactor
Concentatration (ppm)
Time after POI (min)

C


Hexanoic Acid Conc. Over Time in Bioreactor
Concentatration (ppm)
Time after POI (min)

D


Octanoic Acid Conc. Over Time in Bioreactor
Concentatration (ppm)
Time after POI (min)

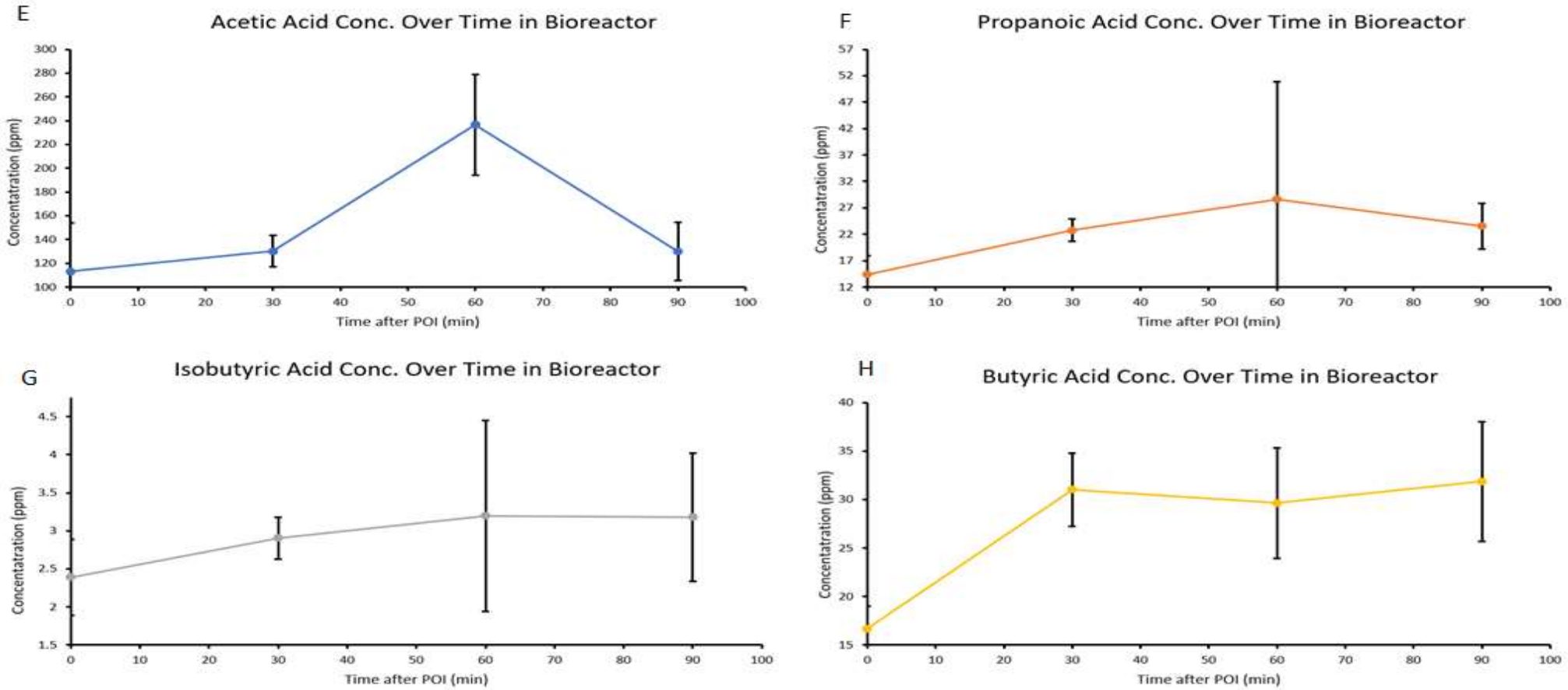



*Figure 5.8 - A graph to show the concentrations of eight different volatile acids and their concentrations for phage K in the bioreactor culture. A) isovaleric concentration, B) Valeric acid concentration, C) Hexanoic acid concentration, D) Octanoic acid concentration, E) Acetic acid concentration, F) Propanoic acid concentration, G) Isobutyric acid concentration, H) Butyric acid concentration. A single 10ml sample was taken from the bioreactor and used for analysis. Samples were mixed together to show the increase in VFA concentration at each of the indicated time points in triplicate.*

Figure 5.8 examined the phage K cultures in the bioreactor given that there was little change in the T4 VFA profile. Statistically significant increases in all VFAs were seen apart from valeric acid, similar to the shake flask culture with a statistically significant decrease in octanoic acid concentration between POI and burst (30 minutes). This was expected as when bacterial cells burst they release VFAs whilst Octanoic acid may have been absorbed by the environment or been metabolised. It is worthwhile noting that after 60 minutes, all VFAs were increased by 100% except butyric acid and acetic acid. The high average variation in VFAs may be accounted for due to differences in frequency in burst or size or subtle differences in bacterial size. Statistically significant differences were calculated between POI and 30 minutes for acetic acid, propanoic acid, isobutyric acid, butyric acid, isovaleric acid and hexanoic acid in the phage K sample $p<0.05$. The increase in VFA over time could be accounted for as when the media is metabolised, they may be produced by either of the host organisms. However, given that they are volatile, they will break down or be utilised as an energy source. Despite the significant increases, there was no correlation made between burst size and final phage titre. Figures 5.1-5.6 examined the DNA/RNA and protein where no correlation could be made either. However, here there was also a lack of correlation found. One reason for being more specific in what to examine i.e. specific vfas over total dna is to reduce the range of what can be examined. However, using VFAs did not allow a more direct comparison between their levels at any point in the culture and final phage titre or burst.

One of the key differences between mammalian and microbial cultures is that microbial can utilise such energy sources that would not be utilised by mammalian cells (Wenzel *et al,* 2018). If the VFAs were left without a lid on, within several hours due to their volatility, they would break down even without the presence of organisms to utilise them as an energy source (Strazzera *et al*, 2018).

A criticism of this experiment may be that for the bioreactor culture, only a single 10ml sample was taken from the culture. Compared to the 3L volume, this may not be representative of the culture, despite it being continuously mixed throughout the culture and mixed when sampling occurred. If this experiment were to be repeated, it would be important that multiple samples were taken from the culture, at the point of sampling and each analysed individually to give a more representative overview of the culture.

Samples of 6 different acids were analysed to determine how many parts per million there were between species samples. Figure 5.9 below shows the comparison between species.

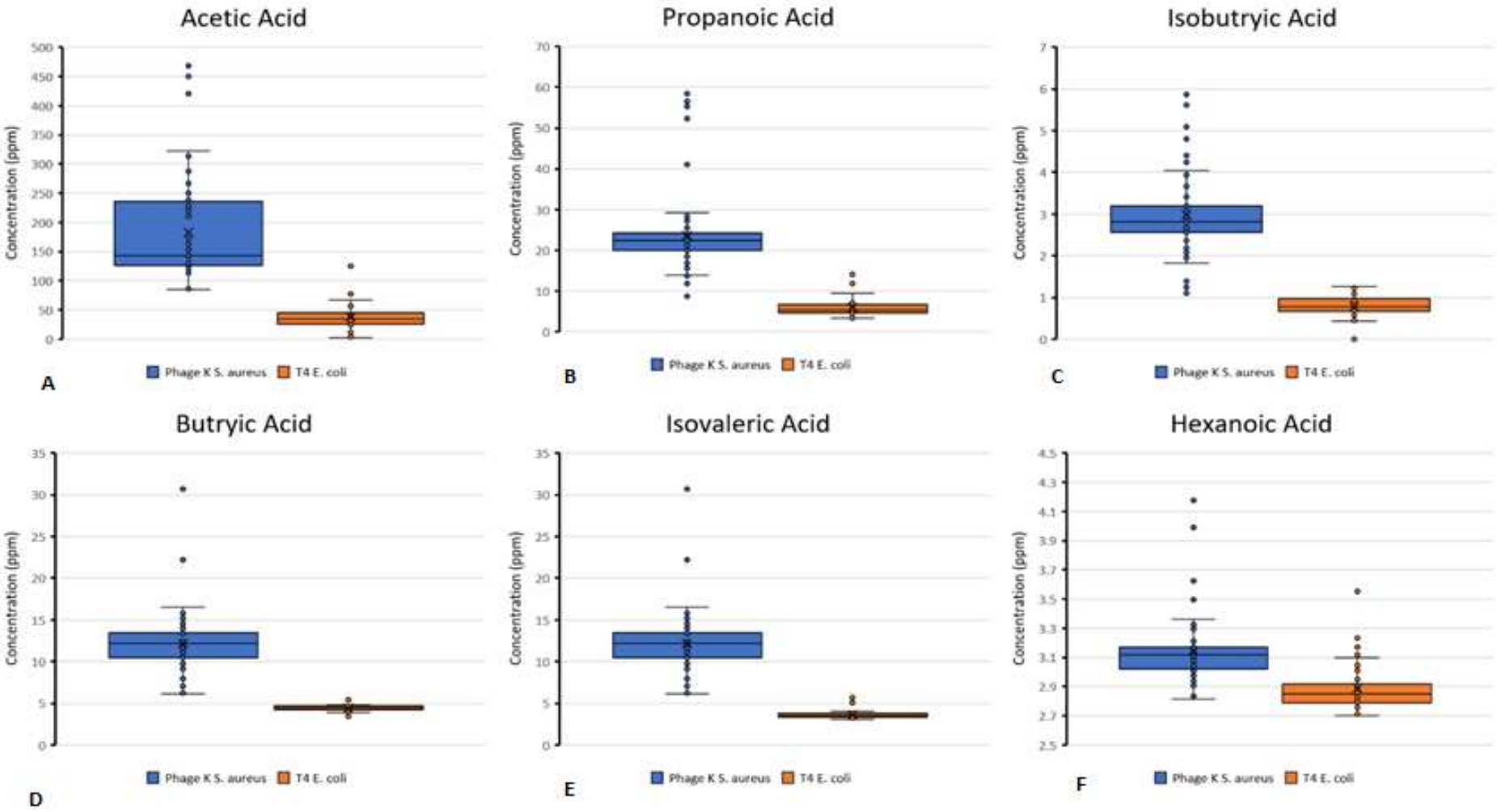



*Figure 5.9 – A graph to show the concentrations of A) Acetic acid B) Propanoic acid C) Isobutyric acid D) Butyric acid E) Isovaleric acid F) Hexanoic acid. The graph shows the parts per million (ppm) of each of the different acids based on the different organisms used, when samples from the different species were taken with phage infection. Samples were taken from the harvest sample to be analysed*

For each acid analysed, there was a statistically significant increase in the PPM for phage K compared to T4, where phage K was higher, apart from hexanoic acid. Whilst figure 5.7 showed that there was a lack of variation in the hexanoic acid concentrations, there was no statistically significant difference between the T4 phage data or the phage K data for hexanoic acid. However, between all other experiments in figure 5.9, there was a higher concentration for the phage K compared to T4, $p<0.1$. Additional repeats of the data may have allowed a significant result to be obtained.

These assays have been run as whilst previously, there was less of a rapid need for phage enumeration and examination, given the concern of antimicrobial resistance and the potential outlet that phage offer, more rapid methods will be needed in future. Additionally, they both may offer greater reliability than the plaque assay which whilst is quick, cheap and simple is open to operator interpretation and variability (Blanchard-channell & Stott, 1989, Shurtleff *et al*, 2016). The differences between the cultures seen may be accounted for by either the host organisms metabolism or the difference in culture media. It is important to note that for the T4 phage media, valeric acid is not present, and may be why there was no differences seen. This is because, BHI media naturally contains more fatty acids, where as LB media contains yeast and tryptone which have naturally less fatty acids. It has previously been shown that numerous different factors play a role in VFA concentration including pH, broth and that phage can have more of an effect on VFA concentration seen than abiotic factors (Jie *et al*, 2014, Zhang *et al*, 2017, Heyer *et al*, 2019). This work has also been useful as it may be taken forward in the future and use it as method for differentiating between different phage.

The bacteriophage/host organism work was carried out by the author of this thesis. However, once samples had been taken and purified, they were passed to collegues within the university. I would like to thank Dr Jim Reynolds, Dr Liam Heaney, Joshua Bain and Rebecca Donahue who ran the GCMS analysis of the bacteriophage samples collected presented in this section. In particular Joshua who ran the analysis and provided figures 5.7 - 5.9. Their experimental work and analysis was critical to figures 5.7-5.9 and they were extremely useful in aiding the authors understanding of the work they carried out.

### 5.6 Other potential methods of phage characterisation

Although this study was unable to relate either the DNA/RNA/Protein concentration to either the burst size or final phage titre for a rapid method of enumeration, we did show that we could track the infection process. Other studies have previously taken a similar approach with different experimental techniques, they aim to find a method for more rapid enumeration. Table 5.3 below shows some other studies and their advantages/disadvantages.

*Table 5.3 – A table to show other studies examining rapid phage enumeration methods.*

| Author | Characterisation method | Advantages | Disadvantages |
|---|---|---|---|
| Tjhung *et al*, 2014 | Phage were transduced with fluorescence genes which could be detected through fluorescence scanning | Took 90 minutes to obtain a reading | Could only detect up to $10^6$ pfu/ml |
| Liu *et al*, 2014 | qPCR with use of membrane dye for enumeration | Can examine intact bacterial cells | Significant differences between their method and standard methods |
| Peng *et al*, 2018 | qPCR method for fluorescence-based enumeration | Results obtained in 2-4 hours | Only tested for T4 phage and requires molecular biology techniques |

Whilst many of the current methods for phage detection involve qPCR or other specialist molecular biology techniques, the advantage of our method is that it is a quick, simple and cheap method to use. It requires no specialist techniques and the results are obtained instantly whilst also being an affordable method. However, our results were unable to be correlated to either final phage titre or burst size. Despite this, no previous work examined a similar method for detection or tracking of the infection process. The results presented here have shown that there may be either some leakage of DNA/RNA/Protein prior to the 30 minutes, of what was previously thought to be burst, given the significant increase in each seen. Additionally, the results also show the difference between T4 and phage K i.e there is no DNA/RNA/Protein detected for phage K before 30 minutes. Bacteriophage have also been used to detect and enumerate bacteria, recent advances in their use have been described by Farooq *et al*, 2018.

Whilst this study has not definitively correlated phage growth and VFA concentration, it highlights a further potential avenue for future investigations similar to the nanodrop experiments. The increase in VFA concentration has been attributed to the burst of bacteria due to the phage, whilst the research highlighted one key difference in that there were greater differences seen in the phage k experiments compared to the T4 experiments. This research could be used to identify phage or bacteria through a library-based approach or comparison. It has also highlighted that phage infection does cause changes in concentration.

### 5.7 Conclusion

The data shows a rapid method for analysis of the cultures within the cultures. Further development of this method or other methods to analyse the cultures would be needed in the future if high throughput experiments continued. Whilst the current gold standard measurement of phage enumeration is through the plaque assay, it can take up to 24 hours to complete. This is undesirable for manufacturers who may require rapid methods to determine titres. However, this work may be an initial step towards developing a more rapid method for phage enumeration.

Interestingly, the T4 phage work showed that there may be some burst within the first 10-20 minutes after infection, given the significant increases seen. However, a burst size experiment would need to be run after this time point in order to confirm that burst had taken place and phage had been produced in this more rapid manner. Whilst many report that phage takes on average 30 minutes to burst, there may be some level of burst within the first 10 minutes i.e the phage enzymes may breakdown the bacterial wall causing DNA/RNA/Proteins to spill out into the culture, causing the increase seen. It was interesting to note that no significant increase was seen for the phage K experiments and that an increase was only seen after 30 minutes. The second section focused on a GCMS method to detect bacterial burst and volatile fatty acids that are seen within the phage propagation cycle. The results show that there were far greater differences detected within the phage K culture compared to the T4 phage culture. Whilst this was most likely due to media, it would be interesting to grow the cultures using the same media to reduce differences and standardise the process to a greater extend. Additionally, maintaining the same conditions such as agitation would also allow the study to be improved. Whilst this may not allow the organisms to propagate to the greatest extend, there would be some value in showing the cultures when using the same media. The method did show that the culture could be tracked and it may be worthwhile examining other fatty acids to determine if there is any correlation between fatty acids and phage titre or burst. It must be noted that if these methods were to be used in future, they should be used to complement the plaque assay rather than replace it. The work initially set out to find a more rapid method to find an indication of what burst or final titre could be. However, it would always be imperative that the gold standard method be used in order to obtain the most amount of data possible.

Developing a method for rapid enumeration or even a method to show a correlation between burst size or final phage titre is important. If a method can be developed, is reliable and accurate it may prevent the need for the plaque assay. It has been highlighted that the plaque assay is the gold standard measurement, despite many showing its variability, and that there is no need for other enumeration assays (Anand *et al,* 2010). However, in the future, if phage are more relied on, there will be a greater need for their rapid detection and this work has shown some evidence that the bacteriophage infection can be tracked. However, there will need to be further investigations into the phage enumeration techniques, such as examining other sugars, amino acids or VFAs that may be present in the culture.

It is not only rapid enumeration and analytical technology that needs to be investigated. Information regarding the infection process from the phage and host cells are important. The work here has shown that the levels of DNA/RNA and protein can be traced throughout the entire culture. The nanodrop work showed that the levels of DNA/RNA can be reduced, possibly due to DNase or RNase on the phage that will break them down. This may be a useful took to investigate purification of the culture. Moreover, the technique could be expanded on to detect different phage/host organisms or further identify information about the culture, phage or host bacteria.

In 2018, a study using droplet digital PCR was used for the detection and enumeration of phage. The method used a digital PCR technique, using primers of the organism for quantification of DNA or RNA targets. A droplet reader measures the amount of amplified target after the PCR reaction and software can calculate the number of copies made, linking it back to the original sample and therefore detecting the initial phage titre. Whilst there was good agreement between the sample placed on the reader and the plaque assay result, it required specialised equipment and a partial genome sequence was needed (Morella *et al*, 2018). Their study did, however, show that a rapid method could be used to detect phage titre within three hours, a fraction of the time compared to the plaque assay. Despite this, eventually there would need to be an instant method for phage enumeration. Machines such as the ViroCyte from Sartorius do offer an automated and rapid viral enumeration, however, their use is limited possibly due to there being a lack of data on its efficacy.

It could be argued that bioanalytics is the greatest area of research that needs to be tackled. Downstream processing can often be forgotten as harvesting the product is important but detecting and purifying the product is just as vital. It may be useful for future research to create and design a method for online analysis of the DNA/RNA/Protein content in the culture. Perhaps a sensor such as the oxygen or pH sensor used in our 5L experiment could be created which detected increases in DNA/RNA/Protein and show the user when burst had occurred. However, with new methods there will undoubtedly be new problems to tackle such as its validation but it may lead to new potentials in phage downstream analytics.

The initial aim of this experiment was to determine whether there was a correlation between the levels of DNA/RNA/Protein throughout the infection and to the final phage titre and burst. Moreover, volatile fatty acids cultures were also to be examined with both used as a rapid method for bacteriophage detection and

relate it to final phage titre or burst. However, there was no correlation made between each of the experiments used at each of the time points and burst or final phage titre for either T4 or phage K. However, this work did show that the infection could be tracked based on the DNA/RNA/Protein content. Additionally, there was also an indication that where greater titres were achieved i.e greatest titre conditions or cultures in the 5L bioreactor, there was a greater amount of DNA/RNA/Protein in the culture. The data also showed that there are significant changed in VFA concentrations at different time points in the culture and therefore these methods may warrant further investigation in the future.

# Chapter 6 – Conclusion and future work

**6.1 Overall conclusion**

Antimicrobial resistance, as shown in chapter 1, is currently a significant concern with no immediate solution to fully alleviate its concern. However, bacteriophage therapy has been shown to provide a potential outlet to AMR but as of yet, there is a lack of research surrounding their production. This thesis has shown a simple and effective method for the optimisation of the fermentation of two phage and a method for scaling up into a 5L bioreactor, to increase volume of phage produced. Future work could follow similar methods as to those presented here for clinically relevant phage or other phage where specific interests lie.

Although research into phage therapy to target AMR is slow, there has been a rise in the number of studies published, whilst clinical trials are also beginning to increase. They offer a solution to tackling AMR whilst multiple studies have shown their effectiveness against resistant organisms, biofilms, animal models and treatment of meat (Callaway *et al*, 2011). Given that phage typically have higher mutation rates than bacteria, it is less likely that bacteria will become resistant to phage than antibiotics. Despite this, there is a lack of research examining their efficient production. Given that penicillin's use was limited due to its production, there is the need for early research into the optimal production and scale up of bacteriophage.

The aim of this work was to determine the conditions that give the greatest phage titres by examining different conditions and a variety of levels that may contribute to phage titre. Two bacteriophage-host systems were chosen; T4-*E. coli* and K-*S. aureus*. They were chosen as they have been used to treat humans and improving the bioprocess for clinically relevant bacteriophage was important and may be used in the treatment of antimicrobial resistant organisms. Moreover, using two phage-host systems was important as it would show validity of the method and the results may be misleading if they have not been backed up with additional studies. However, given the differences seen between the phage, these experiments would be needed to be repeated for other phage if required, to improve their titres, given the bespoke nature of phage. Out of the two phage that were chosen to be used in this study, phage K is the less studied phage, possibly because there is more general research into *E. coli* over *S. aureus*. Additionally, there is more media attention given to *E. coli* over *S. aureus*, one additional reason for the greater depth of research into it and as shown by the research presented here with the T4-*E.coli* system is less variable to work with.

A Design of Experiments (DoE) was employed as it has significant benefits over OFAT experiments, reducing the number of experiments needed, improving experimental efficiency and allows response surfaces to be created for improved analysis (Beg *et al*, 2019). There will be fewer runs required in a DoE and the entire experiment will be streamlined. Its efficient nature of experimental design was once slow to be used within the biologics industry, however, there are many examples of its use to date. Despite this, it is still an underused tool for phage studies. Given the hypothesis of changes in input factors causing changes in output factors, estimations of the output factors can be made and can be useful when examining factors leading to process improvements. Some concerns remain surrounding its validity and reliability, but these have been alleviated by users running additional validation runs (Lee & Gilmore, 2006). Usually, after a DoE has been complete, the centre point runs i.e. the experimental conditions with the mid-points of each level of each factor will be repeated and compared to the original output variable from the DoE. Additionally, the highest/lowest levels can also be run i.e. to show reliability (Toms et al, 2017). Response surfaces can then be produced in order to visualise the levels which give certain responses i.e. highest yields or titres.

The data showed that by using certain conditions for both phage (T4: MOI 2.5, 225rpm, 3 hours and 28$^{o}$C, K: MOI 0.1, 150rpm, 4 hours and 28$^{o}$C) there could be a statistically significant improvement in titre. These conditions were then taken forward into a 5L stirred tank bioreactor (3L working volume) and the process was shown to be scalable i.e similar titres produced between the shake flask and the STR. Shake flask processes are most cost effective, have lower risk, higher throughput system and easy to use. The improvements were shown to be translatable during the initial scaling where other processes can take significant time before achieving similar titres between two scales (Wu *et al*, 2018). The bioprocess was then further optimised as the pH which could be more readily controlled in the STR system was investigated. Additionally, given the differences in stirring, the agitation was also investigated. T4 phage gave the greatest titre at 225rpm and pH 6.9 whilst phage K gave the greatest titre at pH 7.4 and 225rpm in the STR system.

Given that the temperature of infection was shown to give greater titres when reduced, an investigation as to how to handle the culture during the shaking incubators temperature reduction study was run. The experiment showed different temperature reduction profiles and provided a method that further improved final phage titre showing that temperature and its reduction was important. The data showed that significantly improved titres were seen compared to the previous 'greatest titre conditions' when the culture was placed into

a static 28°C incubator, immediately after infection, whilst the shaking incubator was reducing in temperature. T4 phage showed that 28°C gave the greatest titre in a defined peak, whereas phage K showed that similar titres were achieved between 26°C and 31°C. This data showed that the most influential factor on phage titre was temperature given that during the shake flask DoE there were few statistically significant differences between the other conditions used.

Whilst there was little previous work examining temperature as an input factor this study showed its significant effect on titre. Future work may benefit from a temperature reduction for greater titres for other phage given the results shown here that two phage gave statistically significantly greatest titres at lower temperatures. Moreover, statistically significant increases were observed compared to the baseline in the greatest titre conditions. Additionally, the current literature standard greatest titres achievable have been further improved on in this work through mainly simple manipulations of culture conditions. The manipulation of conditions to give the greatest titre may have also reduced the variability of the experimental runs and showed why there were consistently high titres with low variability and was translated into a scalable culture. Given that the small-scale data showed that different conditions gave the greatest titres for the two different phage, similar studies may need to be carried out for all bacteriophage.

Additionally, the kinetics of the adsorption were investigated. In both the shake flask cultures and the bioreactor experiments adsorption and burst size were shown to be statistically significantly increased for greatest titre conditions compared to baseline conditions. If phage can adsorb to host organisms faster and give more phage in the initial burst, this may explain why a greater final titre was achieved. Two hypotheses were given for the improvement in titre; the reduction in temperature allowed the optical density to be in a more optimal range for phage infection whilst reducing the temperature allowed better integration of phage DNA. Given that the lowest and greatest levels of the conditions were used and showed statistically significant reductions in adsorption, burst and final titre compared to the mid-levels used, this may explain why the greatest and lowest levels gave lower titres.

An additional experiment was carried out in chapter 5 which investigated the DNA/RNA and protein concentration of both cultures at different times throughout the bacteriophage infection. Given that the plaque assay can take up to 24 hours in order to enumerate the sample, there is a need for a more rapid method. The

data showed that it may be a useful indicator for determining when burst occurred, and showed the breakdown of DNA and RNA in the culture within minutes when a plaque assay would normally be required. Further development of the method may be required if this were to be used as a rapid enumeration assay. Given the results in chapter 5, the natural progression of the assay would be a more in-depth study into other rapid methods of characterisation. Producing an assay which could reliably give titre whether that be based on optical density of a PCR method would be beneficial as long as it maintains accuracy, is cheap and simple. Whilst the DNA/RNA and proteins showed no correlation to titre or burst, spectrometry analysis has proven valuable in other areas of research (Redhair *et al*, 2019, Ryan *et al*, 2019). Additionally, examination of endotoxin levels may also be useful. This work further took mass spectrometry analysis of VFA's and examined their levels between the two phage organisms used. A future experiment could examine adding in additional phage at the point that the DNA/RNA concentration starts to decrease to further decrease their levels for purification purposes. Given that the work shown in chapter 5 has showed their levels increase and subsequently decrease in the culture, this could prove valuable to remove further DNA and RNA.

Whilst this work had advanced the literature in several ways, the most novel areas of the work are as follows:

- A standardised process for T4 and phage K have been produced in both shake flasks and a 5L bioreactor
- A method for scaling up the process into a litre scale bioreactor shown with statistically significant improvements in T4 and phage K titres between current literature titres
- A standardised method has also been created for the culture of the two phage in a 5L bioreactor (3L working volume)
- Insights have been given into the mechanism and kinetics of the two phage which may have provided information as to why a greater titre is achieved.

**6.2 Future work**

It would be extremely important that the 5L DoE was repeated. However, this was difficult given the time, money and resources available. Additionally, it would be important to further increase scale to increase the yield.

Although the DoE determined greatest titre conditions were used throughout the work, a further study around other conditions that achieved similarly high titres or productivities may warrant further investigation such as oxygen transfer or phase of host cell growth. It may also be useful to examine whether a reduction in time for the T4 phage infection could give a similar titre or productivity. Additionally, other phage bioprocesses would need to be run for other phage-host systems.

Future bacteriophage bioprocessing studies must take into account the need for increasing the yield achievable. Given that one of the early drawbacks to penicillin research was the yield needed were not producible, early research into the bacteriophage bioprocess will be critical. Another aspect that may be critical is manufacturing phage in continuous processes. Whilst batch and fed-batch are an easier method of production, there has been countless research in other therapeutic production processes showing that greater titres can be achieved by switching to continuous processing (Zydney, 2016). Given that batch processes are cheaper as they require less media and consumables they are the most commonly used. They also reduce risk of contamination and offer more flexibility (Jameel *et al*, 2015). The general bioprocessing field is certainly heading towards continuous processing and whilst this may currently not be the case for phage, it seems logical that the phage bioprocessing field will adopt continuous studies as other bioprocessing research areas have eventually continuous studies may be needed for greater yields. Given that currently, the field focuses on batch studies, this work has provided us with a solid base for further fed-batch and continuous studies which may be an area for further study. Additionally, a further examination into defined media may be important.

LB media powder comes at a cost of ~£2.50/L and BHI media powder comes at a cost of ~£3.50/L, any cheap defined media that could give similar high titres to the greatest titre conditions could be produced, would bring additional benefits given that bioprocesses have a need to be cost effective and reducing final cost per phage produced.

Most notably, there was an increase in titre when the temperature of infection was reduced and must be investigated when developing phage bioprocesses. Whilst our work examined control of DO% and pH, there would be a need to assess additional control parameters, however, future work may include examination of toxin levels in the culture, substrate and metabolite levels. Online analytics will become more important, chapter 5 showed the concentrations of DNA/RNA/Proteins in the culture given the host organism breakdown and a novel tool may be developed which could be incorporated into a bioreactor to show the time points that the host organism is breaking down. Although spectrophotometry and PCR have previously been used for the quantification of viruses, and specifically phage, these methods can be complicated whereas the plaque assay is cheap and simple (Refardt, 2012). A more rapid method will need to be developed in order to improve the time taken to quantify samples of phage. Given the work showed in chapter 5, that DNA and RNA levels can be reduced after a certain point during the infection, it may be interesting to look at whether or not adding in additional phage could further reduce the contaminant levels, providing this did npt have a negative impact on titre of phage output per input phage.

The initial aim of this work was to try and improve the conditions for T4 phage and phage K to achieve high titres which was successful. Through the studies presented here, it may improve the chances of phage therapy being used in future. We have shown statistically significant improvements in titre, with around four orders of magnitude increase in titre from baseline T4 phage to final 5L phage titre, with an additional increase in scale from 20ml to 3L, with statistically significant improvements in phage K titre and scale up.

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# Appendix 1 –

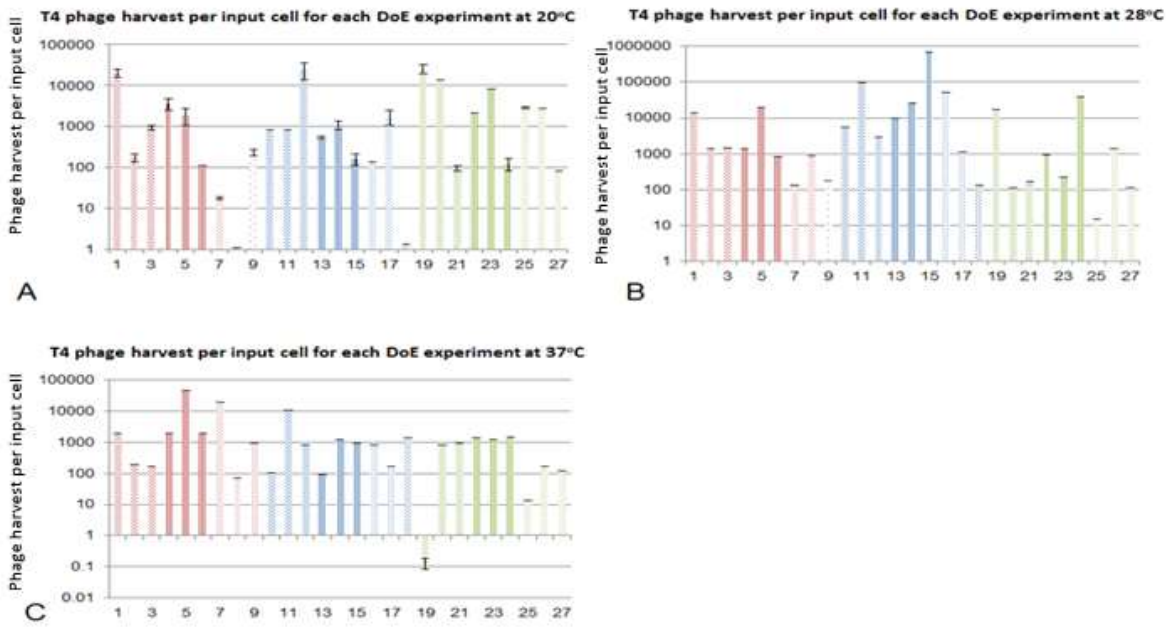


***Figure A1.*** *A bar chart to show T4 phage harvest per input cell achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where an MOI 1 was used, blue bars show the conditions where an MOI 2.5 was used and green bars show where an MOI 10 was used. The first 3 bars in each set show where a time of 1 hour was used, the second set of 3 bars show where a time of 3 hours was used and the final 3 bars in each set show where a time of 6 hours was used as a time of infection.*

Using MOI 10, the data often resulted in a pfu value of 1 x $10^{10}$ pfu/ml. This would initially seem to be a good titre, however, when a minimal phage per input cell is produced, it is an undesirable process to use. This has a 2-fold consequence; stock wastage of phage resulting in an inefficient bioprocess and cost implications. Using a higher MOI requires more phage stock solution which will increase cost and is undesirable in bioprocessing. Although very little stock can be used to propagate phage, future needs may be more demanding, and therefore wastage will become a more important issue. Developing a sustainable bioprocess where there is minimal wastage and maximal product will become imperative if large scale clinical trials are to be held for phage. Using a larger amount of phage when scaling into larger bioreactor processes will cost more time, effort and money to produce the phage required to infect a culture. Also, the media needed to run a stirred tank bioreactor will be far greater than a 20ml process in a shake flask and whilst the total yield may be improved, efficiency may not. Obtaining minimal product from certain runs due to the MOI being too high is undesirable and using a lower MOI may reduce the cost per phage and improve titre. However, conducting the DoE, multiple conditions and levels were examined together and the data shows conditions where a greater phage titre and therefore phage per input cell were produced.

The data shows that MOI 10 generally gives the lowest phage per host cells suggesting that increasing the MOI may not improve the titre or phage per input cell. Several authors have used MOI 10 in their infection process showing the importance of the study as we may be able to improve efficiency (Bourdin *et al*, 2013, Bryan *et al*, 2016).

Despite the infections which used MOI 10, giving a low phage titre, several runs gave >1,000 phage per input cell when the infection took place at 28°C. The greatest phage titre and phage harvest per input cell was at MOI 2.5, 225rpm, 3 hours of infection 28°C, figure 3.5b, run 15. At 20°C of infection, 4 runs produced >10,000 phage per input cell, whereas at 37°C, only 3 runs gave 10,000 phage per input cell. At 28°C, 8 runs also gave >10,000 phage per input cell. DNA integration and perhaps more mis-integrations which in turn produce less viable phage that may not infect the host may occur at high temperatures, whilst low temperatures may prevent host growth reducing the chance for infection and further phage propagation.

In addition to phage titre and phage harvest per input cell, one must also consider the phage harvest per input phage. This will determine how efficient the process is and gives a further insight into the bioprocess.

Although one process may give a reasonable titre, if more phage stock is needed initially to achieve this greater titre, it may not be as good as the final titre suggests. The graphs in figure 3.6 show the phage harvest per input phage based on the temperature of infection.

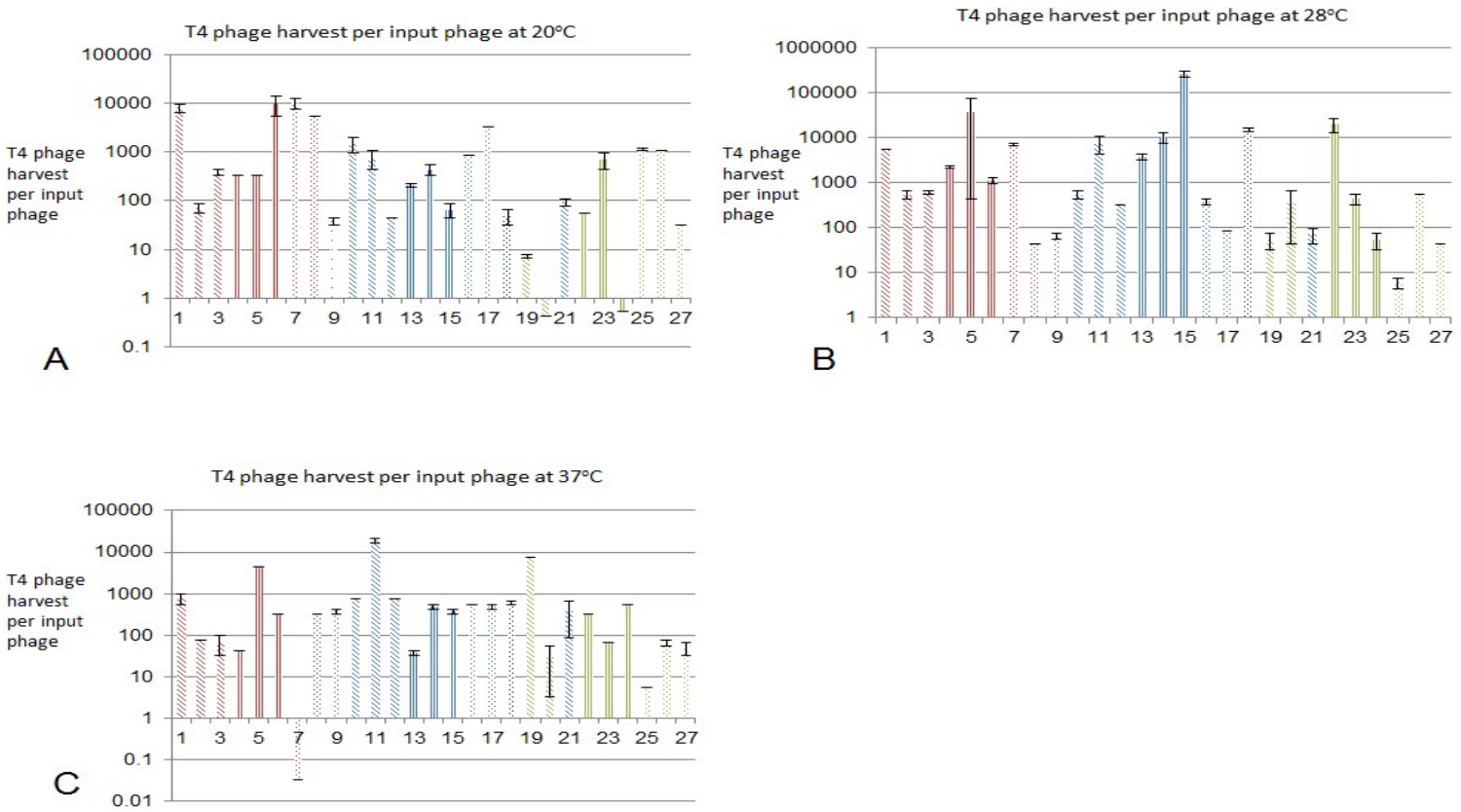


**Figure A2.** *A bar chart to show T4 phage harvest per input phage achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where an MOI 1 was used, blue bars show the conditions where an MOI 2.5 was used and green bars show where an MOI 10 was used. The first 3 bars in each set show where a time of 1 hour was used, the second set of 3 bars show where a time of 3 hours was used and the final 3 bars in each set show where a time of 6 hours was used as a time of infection.*

The greatest phage harvest per input phage occurred at MOI 2.5 at 28°C figure 3.6b run 15. Although the pfu value is the gold standard measure of phage titre, the phage harvest per input phage is far more representative of the bioprocess. This may be more useful in a bioprocess and gives a specific advantage over just using phage titre, for all phage biologists and fermentation scientists. It allows a standard measurement of phage between studies as the MOI is taken into account.

There was a significant difference in phage output per phage input between MOI 1 and 10, p=0.036 with MOI 1 giving a greater average phage output than MOI 10, using a paired t-test. It must be noted that within the bacteriophage literature, the only reportable quantitative measurement is phage titre and this study has shown that the titre may not be as important as many believe it to be. It is hoped this will encourage others to show their phage harvest per input phage in the future as it is a better representation of the bioprocess compared to the phage per input cell and phage titre (pfu/ml).

Although there was a lack of significance between the levels used for the MOI, time and agitation, there was one variable that stood out, the temperature of infection. At 28°C, 11 runs showed >1000 phage output per input phage could be produced, compared to 9 runs and 3 runs at 20°C and 37°C respectively.

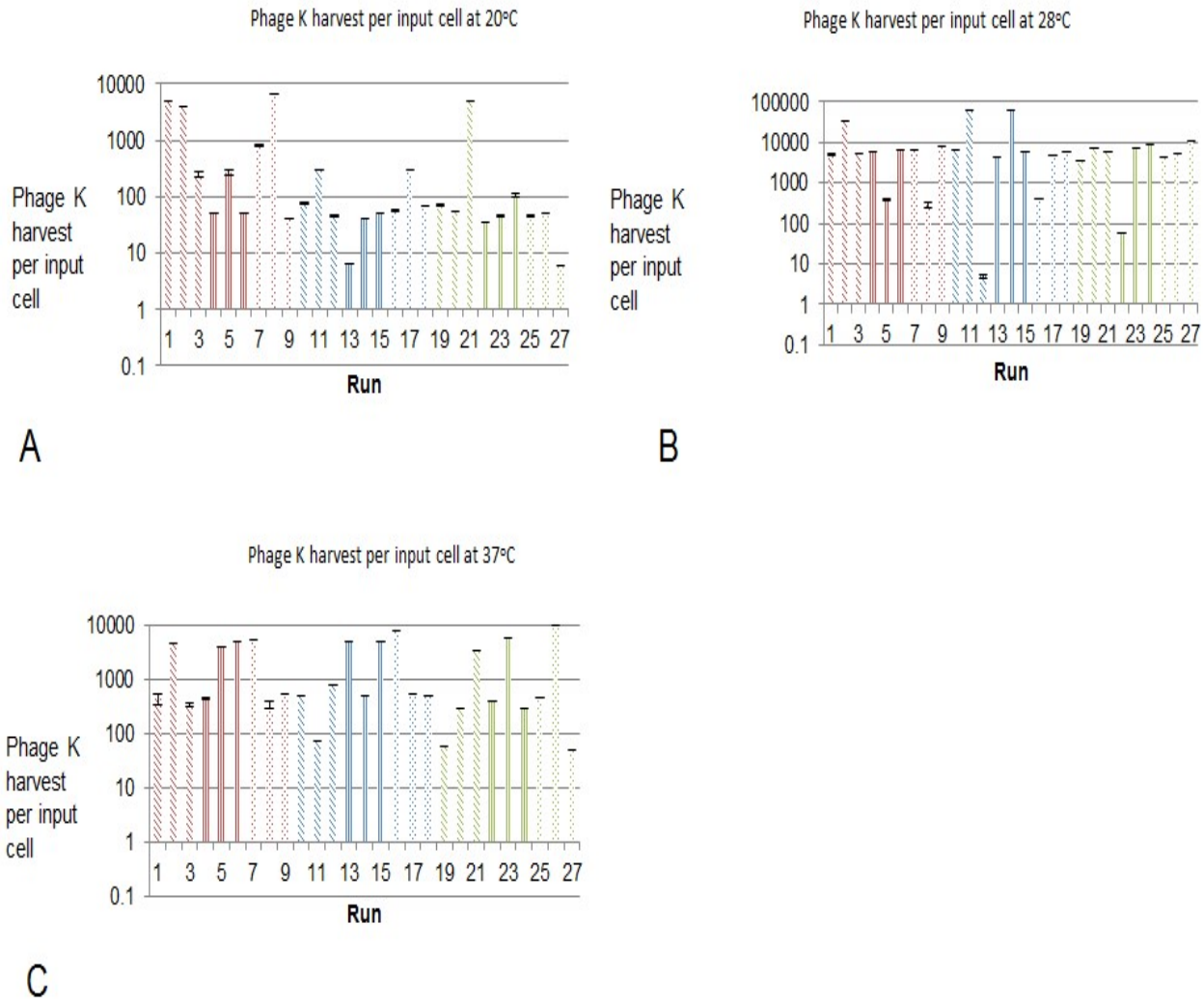


**Figure A3.** *A bar chart to show phage k harvest per input cell achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where a time of infection of 4 hours was used, blue bars show the conditions where a time of infection of 8 hours was used and green bars show where a time of 16 hours of infection was used. The first 3 bars in each set show where an MOI 0.1 was used, the second set of 3 bars show where an MOI 1 was used and the final 3 bars in each set show where an MOI 10 was used at the MOI in the experiment.*

Figure 3.8 shows the normalised data for the phage K per input cell from the DoE. It was important to do this as normalised data is more representative of a bioprocess. At 28$^{o}$C, the greatest phage titre and consequently phage per input cell was produced. Three runs produced over 10,000 phage per input cell, each at an agitation rate of 150rpm, two at 8 hours and 1 at 4 hours, two at MOI 0.1 and 1 at MOI 1. Statistically significant differences found between 20/28$^{o}$C p=0.004, 20/37$^{o}$C p=0.028 and 28/37$^{o}$C p=0.014. Interestingly, at 20$^{o}$C, 10 runs gave >100 phage per input cell whilst 2 runs gave <100 phage per input cell at 28$^{o}$C. At 28$^{o}$C, 5 runs gave <1000 phage per input cell but at 37$^{o}$C, 17 runs gave <1000 phage per input cell, figure 3.8b. The data was next normalised to phage harvest per input phage, figure 3.9

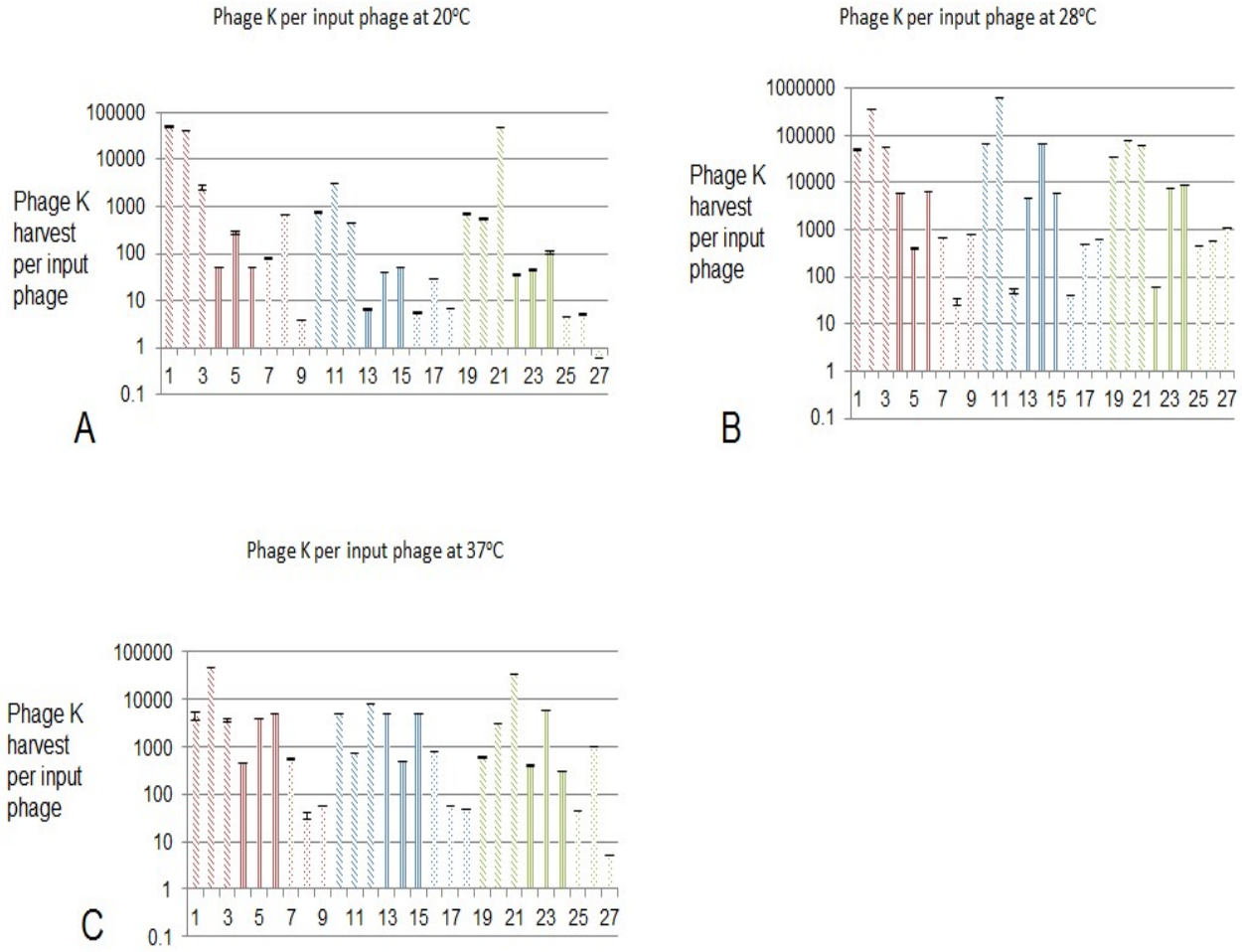


**Figure A4.** *A bar chart to show phage k harvest per input phage achieved from each of the shake flask DoE experiments based on the temperature of infection A) 20°C B) 28°C C) 37°C D) The conditions at which the experiments were run. Error bars represent 1 standard deviation of a singular experiment each enumerated with duplicate plaque assays. All red bars show the conditions where a time of infection of 4 hours was used, blue bars show the conditions where a time of infection of 8 hours was used and green bars show where a time of 16 hours of infection was used. The first 3 bars in each set show where an MOI 0.1 was used, the second set of 3 bars show where an MOI 1 was used and the final 3 bars in each set show where an MOI 10 was used at the MOI in the experiment.*

At 20°C, 5 runs gave >1,000 phage harvest per input phage, however, this was increased to 16 runs at 28°C. Moreover, at 28°C, 11 runs gave <1,000 phage harvest per input phage whilst at 37°C this was increased to 14. This data shows the improvement in the bioprocess that can be seen by simply examining the temperature and the normalised data. Statistically significant differences were found between 20/28°C p=0.04, 20/37°C p=0.03 and 28/37°C p=0.014 using a paired t-test.

The data shows that using MOI 0.1, 4 hours (run 1-3) gives less variation between the titres achieved compared to 8 hours (run 10-12) using 4 hours over 8, gave more consistency within the harvest titre and therefore, the process at 4 hours were used as the 'greatest titre conditions' given that there is better agreement, 50% time saving and a lower MOI in run 2 compared to 11 at 28°C. Therefore, the greatest titre to be used for phage K is MOI 0.1, 4 hours, 150rpm, 28°C.

# APPENDIX 2

**Assay validation**

A new assay must be validated in order to show it is accurate, specific and reproducible and that the correct target will be analysed. Additionally, validation will provide a degree of reliability to the assay to be used in future studies. Moreover, the experiments must be run in accordance with standard protocols and experiments analysed using current and appropriate techniques in order to clarify that the experiments were run accurately. Therefore, each of the experiments were run to harvest and plaque assay and burst size measurements taken for each experiment. This would ensure that the infection that took place as usual. Table 5.2 below shows the burst size and final phage titre from each of the experiments. Each quadruplicate experiment was enumerated separately with a duplicate plaque assay enumeration i.e. n=8 measurements in total.

*Table B1 – A table to show the burst size and final phage titre at baseline and greatest titre conditions in a shake flask and 5L bioreactor. Error shows 1 standard deviation of quadruplicate experiment with duplicate enumeration assays.*

| Phage/condition/scale | Burst size | Final phage titre (pfu/ml) |
|---|---|---|
| **T4 baseline shake flask** | 82.2 ± 3.1 | $3.67 \times 10^{10} \pm 1.1 \times 10^{10}$ |
| **T4 greatest titre shake flask** | 119 ± 7.4 | $2.17 \times 10^{11} \pm 6.9 \times 10^{12}$ |
| **T4 baseline 5L** | 114 ± 15.8 | $8.17 \times 10^{10} \pm 6.9 \times 10^{9}$ |
| **T4 greatest titre 5L** | 120 ± 6.6 | $2.83 \times 10^{14} \pm 6.9 \times 10^{13}$ |
| **K baseline shake flask** | 72.2 ± 1.6 | $4.67 \times 10^{9} \pm 9.4 \times 10^{8}$ |
| **K greatest titre shake flask** | 113.3 ± 1.2 | $4.5 \times 10^{12} \pm 7.6 \times 10^{11}$ |
| **K baseline 5L** | 81.1 ± 1.6 | $8 \times 10^{9} \pm 8.2 \times 10^{8}$ |
| **K greatest titre 5L** | 131 ± 4.15 | $5 \times 10^{12} \pm 1.2 \times 10^{12}$ |

It was important to run the burst size and harvest titre measurements for each experiment. A statistically significant difference was found between the phage K 5L baseline and the T4 phage 5L baseline experiments for the final phage titre, there were no other significant differences for either the burst size or final phage titre compared to the standard measurements from chapter 3 and 4. The significant increased titres here may have been anomalies in the results. Also, there may have been some uncontrollable environmental or equipment factors that caused these changes.

Whilst the experiment had been completed 4 times, with a single sample taken and its DNA/RNA/Protein profile measured twice using the nanodrop, it would be important for a future work to validate our results and run the experiment multiple times with additional profile measurements using the nanodrop. Additionally, it would have been useful to serially dilute the sample in order to determine the limit of detection from our sample. Additionally, it may be useful to back up the results of DNA/RNA/Protein quantification using alternative methods in order to compare the readings to the nanodrop. Whilst other techniques to carry out measurements exist such as spectrophotometry, it would have been impractical to use them, given that there may have been some phage in the sample which could have given false optical density readings, as (the sample was only filtered from the culture (Haque *et al*, 2003). The sample would have needed to be purified of phage before such a measurement could be taken.