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In-vitro approach for improved efficacy of phage therapy against *Pseudomonas aeruginosa*.

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In fulfilment of the requirement of

The degree of Master of Philosophy

In the College of Medical, Molecular and Veterinary Sciences

JAMES COOK UNIVERSITY

April 2024

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All experimental work involving *Pseudomonas aeruginosa* was conducted at James Cook University in Australia. The Australian/New Zealand Standard 2243.3:2010 (Safety in Laboratories) was strictly followed, with live organism work carried out within a BSL2 laboratory to BSL2 standards. All protocols were approved by the Institutional Biosafety Committee, and biological samples were handled in a Class II biosafety cabinet. All disposable materials were placed in heavy-duty Yellow Biohazard Contaminated Waste Bags and then sent for incineration with an authorized facilitator. Human and animal ethics approval was not required for this project.

STATEMENT ON THE CONTRIBUTION OF OTHERS

Nature of Assistance	Contribution	Names, Titles and Affiliations of Co-Contributors
Intellectual support	Proposal writing	Dr. Jennifer Elliman, Dr Jacqueline Picard, and Dr. Richard Squires, conceptual encourage.
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	JCU Veterinary Hospital Cages sewer, and JCU Veterinary post-mortem room.	Dr. Jacqueline Picard, In-field support.

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ABBREVIATIONS

<	less than
>	greater than
°C	degree Celsius
µl	micro litre
µm	micro meter
AIDS	acquired immunodeficiency syndrome
AmpC	an inducible chromosomal enzyme β-lactamase
ANOVA	analysis of variance
APH-3	Aminoglycoside-3'-phosphotransferase
ATB	Antibiotic
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignments Search Tool
BSL2	Biosafety level 2
BV	bacterial vaginosis
$CaCl_2$	Calcium chloride
CFU	Colony-forming unit
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPR-Cas9	CRISPR associated protein 9
CI	confidence interval
CV	crystal violet
DAOPA	Double agar overlay plaque assay
dH_2O	distilled water
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
eDNA	extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EMA	European Medicines Agency
EPS	extracellular polymeric substance
ESBLs	Extended spectrum B-lactamase
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter spp.*
ESKAPEE	ESKAPE + *Escherichia coli*
FDA	Food and Drug Administration
HCl	hydrogen chloride
HIV	The human immunodeficiency viruses
ICNV	International Committee on Virus Nomenclature
ICTV	International Committee on Taxonomy of Viruses
IPB	Israeli phage bank

IQR	interquartile range
JCU	James Cook University
LB	Luria-Bertani
MDR	Multiple drug resistance
MDR-bacterial	multidrug-resistant bacterial infections
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate or magnesium sulphate
m	meter
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MIC	minimal inhibitory concentration
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NIH	National Library of Medicine
OD	optical density
OG1RF	*E. faecalis* strain
OprF	general outer membrane porin of *Pseudomonas aeruginosa*
PAE	*Pseudomonas aeruginosa*
PAO1	reference *Pseudomonas aeruginosa* strain
PBS	Phosphate buffered saline
PFU	plaques forming units
Phage	Bacteriophage
PYO	Pyophage
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
spp.	species
T.E.M.	transmission electron microscopy
TAE	Tris-acetate
TALENs	transcription activator-like effector nucleases
TE	Tris-EDTA
UV	Ultraviolet
VET	Veterinarian
ZFNs	zinc-finger nucleases

ABSTRACT

This study aimed to enhance the effectiveness of bacteriophage therapy for treating *Pseudomonas aeruginosa* (PAE) infection in dogs with external ear canal afflictions by utilising a magistral concoction with a high number of distinct bacteriophages in a cocktail formulation. The project's foundations could be extended to other infections that affect the skin of animals or humans. The project was divided into three phases. The first part included the sample collection of PAE phages from built and natural environments. It took place in water and wastewater reservoirs in the Townsville area. Then, the second part took place in the laboratory, where samples were processed to select the most virulent bacteriophages capable of killing 48 strains of PAE previously isolated from dogs' ears and producing a phage cocktail from them. These selected phages were then used to produce phage cocktails with different numbers in the formulation and tested in a biofilm model. The third phase aimed to identify the most reliable storage method for PAE phages.

In a comparative study, forty bacteriophages were isolated from the Mount Saint John sewage facility and the veterinary hospital drainage at James Cook University, with the latter proving to be a more prolific source for phages efficacious against canine ear PAE strains. Phage cocktails consisting of 10 to 20 phages demonstrated superior efficacy in a biofilm model, whereas exceeding this range led to diminishing returns. All the selected phages were characterised by the PAE strain range and plaque morphology. Twelve distinct phage morphologies were observed via transmission electron microscopy (TEM), and the successful sequencing of seventeen phage genomes was conducted. The most reliable storage method was freezing at -80 degrees Celsius with 50% glycerol as a cryoprotectant. This research represented a significant step forward in applying phage therapy for treating PAE infections affecting the external ear canal, providing valuable insights into phage collection, cocktail creation, and storage.

CHAPTER 1: INTRODUCTION

The discovery of viruses that destroy bacteria, known as bacteriophages or simply phages, started at the beginning of the 20th century (d'Herelle, 1917). The use of phages to treat bacterial infections occurred soon after its discovery. More than a hundred years of research on phage treatment has proven it to be effective (Kutter et al., 2010, Nikolich and Filippov, 2020b). Nevertheless, only countries within the former Soviet Union formally adopted phage therapy. The treatments against bacterial infections are done mainly through antibiotics (Loc-Carrillo and Abedon, 2011). Bacterial resistance to antibiotics is rapidly increasing worldwide, and the creation of new anti-infective therapies is in need (Ventola, 2015). A review on antimicrobial resistance commissioned by the UK government established that at this increasing rate, by the year 2050, there will be few or no antibiotic options to treat "simple bacterial infections" (de Kraker et al., 2016). This fact represents a global health problem. Therefore, phage therapy opens up more possibilities for solving this issue (Kortright et al., 2019) and Western countries are giving serious consideration to the utilisation of phages in the treatment of bacterial infections (Kutateladze and Adamia, 2010).

In most of the therapies where phages are used to treat bacterial infections, lytic phages are preferred since they kill the bacteria soon after they infect them (Monteiro et al., 2019). The first step in the phage life cycle is the recognition and binding to a suitable host. Phages identify and bind to specific receptors on the bacterial surface via their tail fibres. Following attachment, the phage transmits its DNA or RNA into the bacterium, commandeering the host's cellular machinery to produce new phages. Once the bacterium is filled with new phage particles, it bursts, releasing them to infect more bacteria. This process is known as the lytic cycle. Conversely, some phages initiate the lysogenic cycle, where the bacterium is not immediately lysed. Here, the bacterial cell can persist through several cycles, which may be less advantageous when using these phages therapeutically for bacterial infections (Ali et al., 2023).

For now, phage therapy is still experimental in Western countries and approval from the respective Health Ministries is required for compassionate use treatments (Onallah et al., 2024). It is imperative that more clinical studies are conducted to enhance our understanding of phages, including their optimal number in a cocktail, appropriate dosage, and the development of a standardised delivery system. These activities are necessary to ensure that phage-based therapy does not take the form of an experiment but is a standard medical treatment (Zalewska-Piątek, 2023).

PAE is classified as an ESKAPEE pathogen (acronym comprising the scientific names of six highly virulent and antibiotic-resistant bacterial pathogens including: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli*), which is of medical importance since it is a common opportunistic pathogen of people and animals and is intrinsically resistant to commonly used therapeutic antibiotics (Aloke and Achilonu, 2023). This bacterium is known to cause various infections, including otitis externa, an external ear canal inflammation common in dogs, especially those with underlying skin disorders. Otitis externa is a very painful disease for dogs. Sometimes, when therapy fails, for example, if the bacteria affecting the area are resistant to the antibiotics used for treatment, the dog's suffering may lead to a decision to euthanise the dog (Radulescu et al., 2020, Mota-Rojas et al., 2023).

PAE is a bacterium commonly found in soil and water sources contaminated by animals and humans, such as sewage, rivers, and lakes (Diggle and Whiteley, 2020). PAE-phages, which infect this bacterium, are

similarly distributed across these environments. However, determining where the most diverse populations of PAE-phages exist is challenging without employing metagenomic approaches. This project aims to compare the abundance and diversity of phages across different aquatic environments in the Townsville area. Although a metagenomic analysis would provide a more comprehensive understanding of phage diversity. This research focused on isolating phages that target a limited number of PAE strains for the purpose of using these phages. This pragmatic approach provides an initial assessment of phage presence and abundance, albeit with limitations in capturing the full diversity of phages in these environments.

Phage therapy offers a versatile approach to combating bacterial infections, particularly through the use of phage cocktails that combine multiple types of phages (Lin et al., 2017, Regeimbal et al., 2016). This strategy can enhance treatment efficacy by targeting a broader range of bacterial strains and reducing the likelihood of resistance. Instead of removing less effective phages, adding more phages to the cocktail may increase its robustness without compromising effectiveness. This research aims to ensure that increasing the number of phages does not introduce detrimental effects, such as antagonistic interactions, while still improving overall treatment. Although current studies typically focus on phage cocktails containing 2 to 5 phages, there is limited research on the benefits of incorporating more phages into a single formulation. This study addresses this gap by systematically increasing the number of phages in phage cocktails and evaluating whether this enhances treatment efficacy, particularly for an *in-vitro* biofilm model of PAE infections in dogs' ears. The findings could provide valuable insights into how cocktail complexity correlates with its ability to eliminate a wider range of bacterial strains.

The burgeoning field of phage therapy, while promising, is fraught with complexities, particularly concerning the potential for phages to harbour and disseminate antibiotic-resistant genes. Such genes, often sourced from both natural and built environments, pose a significant risk of engendering new strains of antibiotic-resistant bacteria (Modi et al., 2013, Reindel and Fiore, 2017). This fact could lead to the development of new antibiotic-resistant bacterial strains. For this reason, detecting these genes from phages used in therapy is crucial. This project also aims to detect antibiotic-resistant genes in some isolated phages. In addition, for phage therapy to be effective, phages must be stored appropriately to maintain their infectivity. Several diluents can be employed in the concentrated phage solutions, depending on whether they will be stored in the freezer (-80°C), refrigerator (4°C), or lyophilised (Clokie et al., 2011). We aim to test different methods to determine which best suits the JCU laboratory conditions.

To summarise, the aim of this research is to use PAE from cases of canine otitis externa to determine suitable sources of phage against this bacterium. The study also aims to evaluate the efficacy of large phage cocktails *in-vitro* and to develop storage and characterisation protocols that are suitable for the laboratories at James Cook University, Townsville.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Bacteriophages are one of many types of viruses that exist in our ecosystem. However, these particular kinds of viruses exclusively target bacteria. The term bacteriophage comes from the combination of “bacteria” and the Greek word φαγεῖν (phagein), which means "to devour" or “to eat” (d'Herelle, 1917, Abedon, 2022).

This review includes the history of bacteriophages or simply phages, their discovery, and the evolution of their study over the last century focusing on phage therapy. The review will then move on to bacterial skin and soft tissue infections in humans and animals. Finally, the review focuses on ear infections in dogs caused by PAE and phage treatment for this species.

2.2 The history of phage therapy

2.2.1 Initial identification and early trials

The discovery of bacteriophages began in 1915 when Frederick W. Twort, a student at the Brown Animal Research Institute in London, observed a "transmissible lytic principle" while attempting to cultivate the vaccinia virus. Twort noticed that some bacterial colonies had a watery appearance and hypothesised that a bacterial metabolite might be responsible for this lytic effect (Fildes, 1951, Summers, 1991).

Two years later, Felix d'Herelle, working at the Pasteur Institute in France, independently observed the same phenomenon during an outbreak of dysentery among French soldiers. He identified areas of lysis on Shigella culture plates and concluded that an invisible microbe, which he named "bacteriophage," was responsible for destroying the bacteria (Kutter and Sulakvelidze, 2005, d'Herelle, 1917).

In 1919, d'Herelle conducted further studies demonstrating that bacteriophages could provide protection against bacterial infections. Despite the lack of ethical regulations at the time, he tested the safety of phages by inoculating himself and others, ultimately proving their safety and paving the way for the first human trials to treat bacterial dysentery (d'Herelle, 1921, d'Herelle, 1926). This foundational work laid the groundwork for the development of phage therapy, which remains a topic of significant research today.

2.2.2 Rejection of phage therapy in the West and the growth of antibiotic use

Interest in phage therapy grew over time as a promising treatment for infections (Fruciano and Bourne, 2007). However, a 1934 report by the American Medical Association concluded negatively about phage therapy, leading to decreased interest and economic support in the West (Eaton and Bayne-Jones, 1934). Meanwhile, countries in the former Soviet Union, such as Russia, Poland, and Georgia, continued their studies. The introduction and commercialisation of penicillin in 1942 further diminished interest in phage therapy (Maurois, 1959). Despite the discovery of over 40 antimicrobial drugs, bacterial resistance to antibiotics began to emerge by 1959, prompting renewed interest in alternative treatments (Bertoye et al., 1959, Kutateladze and Adamia, 2010).

2.2.3 Increasing evidence of the effectiveness of phage therapy

In the 1980s, extensive human studies at the Bacteriophage Institute in Tbilisi, Georgia, and the Institute of Immunology and Experimental Therapy in Poland showed high success rates in phage therapy, with 518 out of 550 patients having positive outcomes (Weber-Dabrowska et al., 2000). Tailored phage therapy effectively treated drug-resistant bacterial infections (Kutter et al., 2010). In 1988, significant advances in topical phage therapy to treat bacterial infections in animals were made (Soothill et al., 1988), and further studies on bacterial infections in burns in mice were published in 1992 (Soothill, 1992). In 2004, Hagens produced the first genetically manipulated phage, demonstrating its efficacy *in-vitro* and *in-vivo* (Hagens et al., 2004). In 2009, Wright et al. conducted the first controlled clinical trial with phages against PAE in humans, showing high efficacy and safety (Wright et al., 2009). By 2010, studies indicated that phage cocktails were more successful than single phage therapy for various bacterial infections (Jaiswal et al., 2013, Chadha et al., 2016). Figure 2.1 displays a timeline of these highlights in phage therapy history.

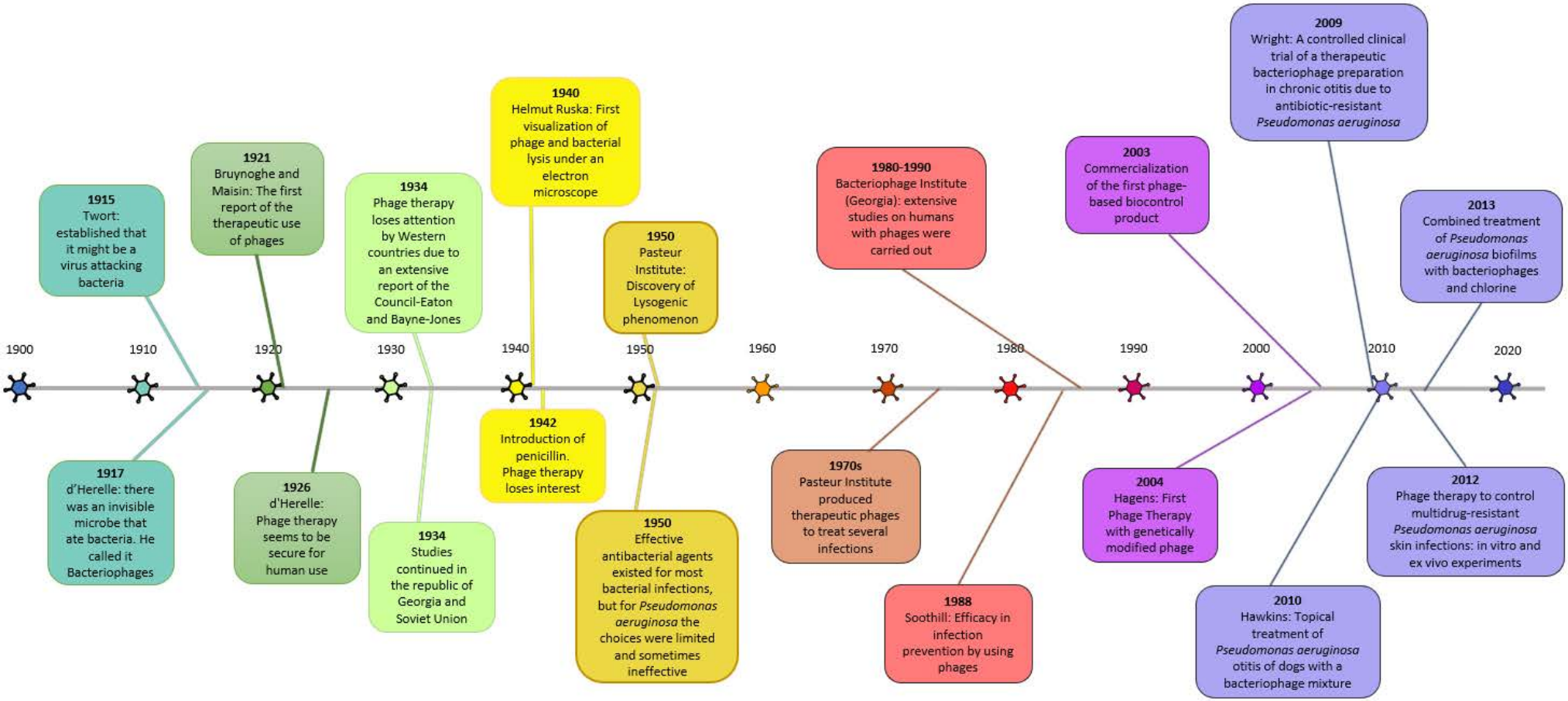

Figure 2.1: Timeline of groundbreaking events in the history of phage therapy.
(Fildes, 1951, d'Herelle, 1917, Bruynoghe and Maisin, 1921, d'Herelle, 1921, d'Herelle, 1926, Eaton and Bayne-Jones, 1934, Ruska, 1940, Maurois, 1959, Peyrieras and Morange, 2002, Bertoye et al., 1959, Vieu, 1979, Kutter and Sulakvelidze, 2005, Soothill et al., 1988, Hagens et al., 2004, Leverentz et al., 2003, Wright et al., 2009, Hawkins et al., 2010, Zyara et al., 2016, Vieira et al., 2012).

2.3 Phage biology

Phages, which are viruses and obligate intracellular microorganisms, attack specific groups of bacteria, often targeting only a subset of species within these groups (Guttman et al., 2004). Recognised as the most abundant and genetically diverse elements of the terrestrial biosphere, phages have colonised every area where bacteria are found, with their numbers exceeding those of bacteria in such environments (Abedon, 2008, Dennehy, 2010, Clokie et al., 2011, Muniesa et al., 2011, Bergh et al., 1989). Their genetic material, protected by a polypeptide coat, is introduced into bacteria to utilize bacterial replication machinery for survival.

2.3.1 Phage habitats

The number of phages has been reported to be between 10^{30} and 10^{32} bacteriophages in the world (Travis, 2003), which surpasses the number of bacteria by approximately one order of magnitude (Suttle, 2005). The majority of viral species identified by the International Committee on Taxonomy of Viruses (ICTV) have been found in viruses isolated in laboratory cultures using specific host organisms. However, these host organisms, particularly when isolated from environmental samples, probably only account for a small fraction, possibly less than 1%, of the total prokaryotic diversity present in those environments (Hugenholtz et al., 1998).

2.3.1.1 Water

Metagenomic studies have revealed that the concentration of phages in aquatic environments can be significantly higher than that of bacteria. For instance, studies conducted on the surface of river water and seawater in northern Europe found that phages are present at concentrations up to ten times greater than bacteria (Bergh et al., 1989, Breitbart et al., 2002, Suttle, 2005). Researchers noted that the concentration of phages varies depending on the year's season (Maranger et al., 1994). When temperature increases, the amount of bacteria increases; therefore, the number of phages does too. This fact shows the active role of phages in aquatic ecosystems mainly regulating bacterial growth (Bergh et al., 1989). Furthermore, specific studies have shown that phages contribute significantly to the turnover of microbial biomass in the oceans, with an estimated 10^{23} infections occurring per second globally (Suttle, 2007). In freshwater ecosystems, phages have also been shown to influence microbial community structure by selectively lysing dominant bacterial species, thereby promoting biodiversity (Fuhrman, 1999).

2.3.1.2 Sediment and soil

Phages are not limited to aquatic environments; they are also abundant in sediments and soils. For instance, studies on continental margin sediments off central Chile revealed mean concentrations of phages as high as 8×10^{12} viruses per square meter (Danovaro et al., 2008, Siem-Jørgensen et al., 2008). This was determined using epifluorescence microscopy with nucleic acid stains, which allowed for accurate viral counts. Direct counts of soil phages using transmission electron microscopy (TEM) have demonstrated concentrations 350 times higher than previous estimates (Ashelford et al., 2003). More recently, phages were identified as a major component of the soil virome, influencing microbial dynamics and nutrient cycling. For instance, studies have reported that phages play a pivotal role in the nitrogen cycle by targeting ammonia-oxidizing bacteria and archaea in agricultural soils (Kimura et al., 2008). Phages have also been found to persist in harsh terrestrial environments, such as permafrost and desert soils, where their ability to remain stable over extended periods contributes to the resilience of microbial ecosystems (Emerson et al., 2018, Williamson et al., 2017)

2.3.1.3 Animals and humans

Phages are not only ubiquitous in aquatic and terrestrial environments but also colonise various niches within animals and humans, including the digestive system, oral cavity, external ear canal, and skin. These environments are particularly rich in microbial life, providing fertile ground for phage proliferation.

In the gastrointestinal tract, for example, the intestinal microbiota comprises a complex community of bacteria, many of which are closely associated with their specific phages. Research has shown that the concentration of phages in the faeces of cattle and sheep can reach levels as high as 10^9 phages per gram (Furuse, 1987). This high concentration underscores the role phages play in regulating the bacterial populations within the gut, which is crucial for maintaining a balanced microbiome and, consequently, the overall health of the host.

An interesting example can be found in the external ear canal of dogs, a niche environment where phages play a role in controlling bacterial populations such as PAE and *Staphylococcus pseudintermedius*, both of which are common pathogens associated with otitis externa in dogs (Pye, 2018). For instance, phages targeting PAE have been isolated and are being explored as potential therapeutic agents to manage chronic and antibiotic-resistant ear infections in dogs (Hawkins et al., 2010). This highlights the potential for phages to influence microbial dynamics in specific animal habitats, with direct implications for animal health.

Despite the extensive presence of phages across these animal and human habitats, our understanding of their ecological roles and interactions with bacterial hosts remains incomplete. The dynamic relationship between phages and bacteria is influenced by numerous factors, including the host's immune system, diet, and the presence of other microbes. Additionally, the specificity of phages to their bacterial hosts means that changes in bacterial populations—due to factors like antibiotic use or disease—can lead to corresponding shifts in phage populations (Abedon, 2008, Wang et al., 2016).

Current research is beginning to unravel the complexities of these interactions, but much remains to be discovered. A more profound understanding of phage biology in these environments could lead to innovative therapeutic approaches, such as phage therapy, and provide insights into the maintenance of microbial balance in health and disease.

2.3.2 Phage taxonomy

Phages used to be classified by their morphology. First, in 1967, David Bradley organised tailed phages into three morphologies by simply using an electron microscope (Bradley, 1967). Then, Ackermann and Eisenstark updated the classification in 1974 (Ackermann and Eisenstark, 1974). Finally, the International Committee on Virus Nomenclature (ICNV) formally endorsed this system. This classification system was used for more than 40 years (Turner et al., 2021) (Table 2.1).

Phage taxonomy is currently changing to a Genome-Based system ((ICTV), 2021). Consequently, numerous viruses have been reclassified from the previous system (Turner et al., 2021). Moreover, phage genomes have unveiled significantly greater genetic diversity than previously recognised, particularly among phages of the order *Caudovirales* (tailed phages), resulting in the establishment of the first subfamilies within the three extant families: *Podoviridae*, *Myoviridae*, and *Siphoviridae* (Turner et al., 2021).

Phage taxonomy system Genome-Based is classified into 1 class, 4 orders, 33 families, 37 subfamilies, and 493 genera. The new class is called *Caudoviricetes*, and the four orders are, *Crassvirales*, *Kirjokansivirales*, *Methanobavirales*, and *Thumleimavirales* ((ICTV), 2021). However, Dr Dann Turner from the University of West England, UK, and an ICTV member, recommends using the previous classification in publications and adding the new description in the annotated sequence records (personal communication).

It is believed that tailed phages are the most abundant phage members in the natural environment, representing about 96% of reported phages in the literature (Ackermann, 2007). We must consider the bias generated when using the plaques technique as a form of isolation. Tailed phages produce more significant plaques than other phages, which are finally used for investigation (Serwer et al., 2007). Furthermore, in electron micrographs of environmental samples, the distinctive forms of tailed phages make them easier to identify than others (Abedon, 2008).

For practical purposes, this literature review will use the previous taxonomic system based on morphology and focus on phages targeting PAE. Most phages reported targeting PAE are included in the order *Caudovirales*.

Table 2.1: Virus Taxonomy - 2020 Release (Previous bacteriophage classification). Classification by International Committee on Taxonomy of Viruses (ICTV) report for Bacteriophages.

Class	Order	Family	Morphology	Nucleic Acid
Caudoviricetes	***Caudovirales***	*Myoviridae*	Icosahedral or elongated head connected to a contractile tail through a neck and a central tube	dsDNA
		Podoviridae	Short, noncontractile tail	dsDNA
		Siphoviridae	Simple, long, noncontractile tail that can have a flexible or rigid tube	dsDNA
		Ackermannviridae	Presence of head, neck, tail. and base plate	dsDNA
		Autographiviridae	Head-tail morphology with contractile tail	large dsDNA
		Chaseviridae	Head-tail morphology with contractile tail	linear dsDNA
		Demerecviridae	Polyhedral head and a long tail	dsDNA
		Drexlerviridae	Simple, long, noncontractile tail that can have a flexible or rigid tube	dsDNA
		Guelinviridae	Icosahedral head with short non-contractile tail	dsDNA
		Herelleviridae	Head-tail morphology with contractile tail, heads generally isometric, and showing capsomers	dsDNA
		Rountreeviridae	Icosahedral capsid, tail is non-contractile.	dsDNA
		Salasmaviridae	Icosahedral capsid, tail is non-contractile.	dsDNA
		Schitoviridae	Short, noncontractile tail	dsDNA
		Zobellviridae	Isometric capsid a short tail	dsDNA

2.3.3 The phage lifecycle

The phage life cycle begins in a given environment, where phages and bacteria coexist. Three types of phage cycles have been defined, the lytic cycle, the lysogenic cycle, and the pseudolysogenic cycle (Abedon, 2008) and an upgrade to this concept has been proposed by Hobbs and Abedon (2016) (Hobbs and Abedon, 2016). Regardless of the mechanism used, bacterial recognition by the phage is the first step in the phage cycle followed by insertion of the genome into the host cell and some level of processing of the DNA.

2.3.3.1 Recognition of a suitable host

Phages with tails have structures that allow them to recognise and bind to their target bacteria. For instance, some phages utilise a single central fibre as a recognition element (Guttman et al., 2004), while others employ fibres located at one end of the tail opposite the head. The binding of phages occurs at specific sites or receptors on the bacterial surface, which vary depending on the phage and the bacterial species. In the case of PAE, phages often target lipopolysaccharides (LPS) on the bacterial cell wall or type IV pili (T4P), facilitating the adsorption and subsequent infection process (Vaitekenas et al., 2021). When a phage approaches a bacterium, its fibres initially adhere reversibly, allowing the phage to recognise specific structures for adhesion. Once the bacterium's specific structure is identified, irreversible binding occurs, positioning the base of the phage plate on the bacterial plasma membrane (Kutter et al., 2005). Recognition structures in gram-negative bacteria include, but are not limited to, LPS, porins, and nutrient transport proteins (Guttman et al., 2004).

2.3.3.2 Insertion of the genome into the host cell

The second stage corresponds to the insertion of the genetic material into the bacterial cell. In order to achieve this, the phages must cross both the bacterial outer membrane and the bacterial inner membrane. This mechanism requires energy, which is obtained through ATP, membrane potential or some enzymatic action (Guttman et al., 2004). As an illustration, phages possessing contractile tails (Myoviridae family) can act like hypodermic syringes. They perforate the outer and inner membranes and introduce the genetic material (Granoff et al., 1999). Another example is by degradation of the bacterial membrane and the formation of a "narrow tunnel". In this way, the phage deposits its DNA. This mechanism is generally used by phages possessing a small tail (Podoviridae family) (Molineux, 2001).

2.3.3.3 Processing of the bacteriophage genome

Once the genetic material has been introduced, phage DNA starts processing. Phages require bacterial replicative and transcriptional machinery to carry out this process. Moreover, some phages encode their own DNA and RNA polymerase or have several replication origin sites in their genome. As a result, phages have an advantage over bacterial genetic material when processing DNA (Kutter and Sulakvelidze, 2005).

The first genes to be transcribed are used to produce elements such as DNA polymerase that help the DNA replication process and other structures associated with the decision of the lytic/ lysogenic pathway. The late genes translate into phagic structures such as head or capsid, tail, and other associated proteins (Kutter and Sulakvelidze, 2005). In the lytic cycle, this leads to the assembly of phage particles and the release of progeny phage into the environment.

2.3.3.4 Phage lifecycle selection

The phage biologist Zack Hobbs and Dr Stephen T. Abedon proposed four possible lifecycle mechanisms (figure 2.2) (Hobbs and Abedon, 2016). First, is it essential to define the phage's "vegetative state," which implies that the virus is in a noninfectious and replicating condition (Doermann, 1953). In addition, bacteriophage genomes that have been integrated into the host genome are known as “prophages” (Saussereau and Debarbieux, 2012).

The first lifestyle is when, after the phage introduces its genetic material into the bacterium, the bacterium enters a vegetative state where the phage DNA begins to replicate and assemble the virion. Then, the bacterial membrane is destroyed without entering a lysogenic state, and virions are released into the environment and ready to infect other bacteria. This scenario represents the strictly lytic cycle. The second lifestyle happens when a phage passes its genetic material to the bacteria, and the virion particles start the process of replication. In this case, phages are released without the destruction of the bacterial membrane. This cycle is known as Chronic, Non-temperate. The third possibility occurs when the viral genome can opt for a pathway in which replication occurs or the phage remains in a latent state included in the bacterial genome (prophage) or in the form of a plasmid with rupture of the bacterial cell membrane later and release new phages. This life cycle is called lytic, temperate and is classically called the lysogenic cycle. There is another comparable state called pseudolysogenic. The main difference resides in the time the phage stays in the prophage form. Pseudolysogeny is a state in which a starving host cell coexists in an unstable association with its viral genome for long periods (Ripp and Miller, 1998). The last scenario to be described is when, as in the previous case, the phage may choose to enter the prophage state or in a state of continuous replication, and then the phage will be released from the bacteria without destroying the bacterial plasma membrane and killing them. This phage life cycle is called chronic, temperate.

2.4 Phage banks

Prior to applying phage therapy or phage-related research, an essential point in that process involves storing as many phages as possible in a given location with easy access. This physical storage is defined as a "phage bank" (Tanir et al., 2021). Phage banks are a good starting point when carrying out studies with many phages since they keep an ample variety of stored phages for a given number of bacteria. What is more, it is unnecessary to look for phages in the environment every time they are needed. Implementing phage banks will shorten the sampling and initial processing times (Bonilla et al., 2016).

Since phages have a narrow range of target bacteria, it is essential to have a wide variety for each bacterial species. For example, this will be important in phage therapy when preparing cocktails containing more than two different phages when high lytic capacity is necessary. In this way, it will be possible to tilt the balance to the therapeutic success (Bull and Gill, 2014). When discussing the number of phages that a bank contains, it refers to each of the phages that were isolated, purified and characterised and are ready to be used in clinical trials or another previously mentioned purpose (Tanir et al., 2021, Yerushalmy et al., 2020).

The initial development of phage banks occurred in Poland and what is now Russia and Georgia (Former Soviet Union countries). In Georgia, for example, the Eliava Institute of Bacteriophages, Microbiology and Virology has developed a phage bank that hosts more than 1000 phages (ELIAVA, 2022). Similarly, the Hirszfeld Institute of Immunology and Experimental Therapy in Poland accounts for more than 850 phages ready to be used (Zaczek et al., 2020). By 2024, many more phage banks have been developed. For instance, the Felix d'Herelle Reference Center for Bacterial Viruses in Canada has more than 400 phages. In the United States, the American Type Culture Collection (ATCC) boasts a collection of over 350 phages, while the Phage Australia biobank has registered 342 phages to date. Countries worldwide are continuously developing new phage banks and expanding the number of phages in those that already exist. This development is possible thanks to government grants and private investors (Lin et al., 2021). Developed countries such as the USA, Japan, Canada, and some from the European Union possess several phage banks; private companies fund the most (Lin et al., 2021). The scenario in countries in Africa, Central America or South America is different, and different approaches should be taken to create phage banks. Since phages can be isolated locally, it was proposed to create national or regional phage banks to tackle this issue (Nagel et al., 2016). In those developing countries, the private sector has not been persuaded to invest in this field. Therefore, developing phage banks in those regions should be stimulated by local governments. Ideally, the objective is to have phage banks worldwide following international standards (Nagel et al., 2016).

Overall, basic phage bank operations include the isolation and characterisation of new phages, maintenance of the phage collection, supply demanded phages, phage training or adaptation, and sharing knowledge that came from exhaustive research (Yerushalmy et al., 2020).

2.5 Phage Therapy

As discussed in Section 2.2, at the beginning of the twentieth century, before the widespread use of antibiotics, the therapeutic potential of phages was met with scepticism, often dismissed as ineffective or even labelled as "snake oil" (Monk et al., 2010, Davies, 2007). Despite early doubts, research continued, and some clinical studies reported encouraging outcomes, contributing to a gradual shift in perception (Monk et al., 2010). However, it is important to acknowledge that, to date, only a limited number of clinical trials have been conducted, and the results have been mixed, with some studies failing to demonstrate clear efficacy.

Phage therapy, defined as the use of bacteriophages to combat bacterial infections, operates through a specific mechanism where phages attach to the bacterial surface, inject their genetic material, and hijack the host cell's enzymatic machinery to replicate. This process culminates in the destruction of the bacterial membrane, releasing new phages ready to infect other bacterial cells. Notably, phages are highly specific and generally do not harm non-target cells (Abedon et al., 2011).

With the rise of antibiotic-resistant bacteria, phage therapy is increasingly being explored as a promising alternative or adjunct to traditional antibiotics, particularly in the fight against multidrug-resistant bacterial strains (Kutateladze and Adamia, 2008). However, the field still faces significant challenges, including the need for more robust clinical evidence to establish phage therapy's efficacy and safety on a broader scale.

2.5.1 Monophagic versus polyphagic therapy

2.5.1.1 Single Phage applications

Experimental intraperitoneal inoculation of mice with a suspension containing a single phage demonstrated the elimination of the target bacteria, *Enterococcus* and *Escherichia coli* respectively, present in the blood (Biswas et al., 2002, Wang et al., 2006). Compared to other phage therapies, monophagic therapy offers several advantages, including simplified treatment and easy purification (Abdul Wahid, 2015).

2.5.1.2 Increasing single phage therapy efficiency

Monophagic treatment also has its limitations. Just as bacteria generate resistance to antibiotics, they also do so against phages (Chadha et al., 2016, Gu et al., 2012). Therefore, to decrease the rate of acquisition of resistance by bacteria to phage therapy, the "phage training" technique has been considered. Phage training consists of repeatedly subjecting a lytic phage to its target bacteria. These repeated interactions allow the bacteria to generate resistance against the phage. In turn, the phage also evolves, generating counter-resistance. It was shown that if the evolved phage faces the first bacterial strain used, bacteria take longer to acquire resistance to the phage. Thus, the virulent effect of the phage is prolonged in time, leading to more efficient phage therapy (Borin et al., 2021).

2.5.1.3 Multiple phage (cocktail) applications

Phage cocktails have also been used against a single bacterium. Phage cocktails include more than one phage in the applied treatment (Chan et al., 2013, Schmerer et al., 2014). Today, it is possible to find many scientific articles that include the use of phage cocktails to treat target bacterial species. For example, the researcher Legesse Kifelew from the College of Science and Engineering, Flinders University in Adelaide, South Australia and his team conducted studies on phage cocktails against multidrug-resistant

Staphylococcus aureus in diabetic mouse wound infections. They employed a cocktail of three phages related to *Staphylococcus* phage K, designated as AB-SA01, and observed a significant reduction in bacterial counts following treatment (Kifelew et al., 2020). However, the study did not explain how these specific phages were selected or whether they evaluated a larger pool. This lack of detail highlights a common gap in phage cocktail studies, where selection criteria are often underreported, limiting insights into cocktail optimisation.

Phage cocktails could also treat polymicrobial infections or as empirical therapy when the cause of the infection has not been determined. In Tiblisim Georgia, the Eliava Institute of Bacteriophage commercially produced generic cocktails, the "Pyophage" (PYO) and the "Intestiphage". As an illustration, the PYO contains phages that attack *Staphylococcus aureus, E. coli, P. aeruginosa, Proteus vulgaris, Proteus mirabilis, and Streptococcus pyogenes*. On the other hand, Intestiphage contains phages that attack about 23 different species present in enteric samples (Nikolich and Filippov, 2020a). Thus, a phage cocktail can contain phages against different species of bacteria. This use may be comparable to broad-spectrum antibiotics.

Researchers agree that phage therapy has greater efficacy when using phage cocktails than monophage (Chadha et al., 2016, Gu et al., 2012). This is because it reduces the possibility of the bacteria generating resistance to all the phages included in the cocktail.

2.5.1.4 The number of different bacteriophages in cocktails

Another critical aspect of multiphage therapy is the number of phages included in each cocktail. The number of phages used in these therapies can vary significantly. *In-vivo* studies have employed phage cocktails containing anywhere from 2 to 12 different phages (Table 2.2). In some cases, researchers have used even more than 12 phages when the goal is to reduce bacterial colonisation or prevent infections (Andreatti Filho et al., 2007).

Table 2.2: Examples of cocktails used against a range of bacterial targets, including the numbers of phage used.

Bacterial Target	Host	Site of infection	Administration	Phages used in cocktail	Comments	Reference
Vibrio cholerae	Rabbit	Enteric	Oral	5		(Jaiswal et al., 2013)
Pseudomonas aeruginosa	Human	Skin	Topical	12		(Jault et al., 2019)
Pseudomonas aeruginosa	Human	Systemic	Intravenous	2		(Jennes et al., 2017)
Acinetobacter baumannii	Human	Systemic	Intracavity, Intravenous	4, 4, 2	Three different phage cocktails were used successively	(Schooley et al., 2017b)
Pseudomonas aeruginosa	Human	Systemic	Intravenous	2		(Duplessis et al., 2018)
Pseudomonas aeruginosa	Dog	Ear	Topical	6		(Hawkins et al., 2010)
Acinetobacter baumannii	Mouse	Skin	Topical, Intravenous	4		(Regeimbal et al., 2016)

The selection of the number of phages in these studies often varies depending on the intended application and experimental design. In some cases, the number of phages was determined empirically, aiming to maximise bacterial lysis through trials with multiple combinations of phages (Schooley et al., 2017b). For example, in the case of Acinetobacter baumannii, three distinct cocktails with 4, 4, and 2 phages were applied successively to combat the infection (Schooley et al., 2017b). In other instances, researchers appear to have included all the phages identified during screening, such as the study targeting PAE in dogs, which utilised six phages (Hawkins et al., 2010). Alternatively, some studies formulated cocktails based on specific bacterial strain coverage, ensuring that the included phages were sufficient to lyse all the targeted strains (Jault et al., 2019). However, in many reports, the rationale behind the number of phages included in the cocktail remains unclear, indicating the need for more standardised approaches in the cocktail formulation.

However, the literature is limited regarding the potential benefits of creating phage cocktails with a larger number of phages and whether this approach could effectively treat a broader range of bacterial strains. While increasing the number of phages in a cocktail might seem to intuitively improve the likelihood of eliminating target bacteria, this assumption oversimplifies the complexity of phage-bacteria and phage-phage interactions. In reality, the efficacy of a phage cocktail is not solely a numbers game; rather, it depends on carefully selecting phages that exhibit strong virulence against the specific bacterial strain in question (Kutter et al., 2010).

Phage therapy is typically customised to achieve therapeutic success against a particular bacterial species. The causative bacteria must first be isolated from the site of infection, after which the most effective phages are selected based on their ability to lyse the target bacterium (Nikolich and Filippov, 2020b). While hypothetically, one might consider increasing the number of phages in a cocktail to 30–40 in an effort to broaden the spectrum of action, this approach could introduce new challenges. The complex interactions among multiple phages and between phages and bacteria might lead to unintended consequences, making it difficult to predict or optimise the therapeutic outcome. Consequently, there is no conclusive evidence or consensus in the literature to support the notion that simply increasing the number of phages in a cocktail will enhance its efficacy. Further research is needed to explore this hypothesis and establish a more nuanced understanding of the dynamics at play.

2.5.2 Combination therapy of phages and antibiotics

The combined use of phages and antibiotics represents a promising treatment model, particularly as phage therapy gains acceptance in Western countries. This does not signify a replacement for antibiotic therapy; instead, it underscores the potential for these two modalities to complement each other effectively. Synergistic effects observed from their joint use have been documented, showing significant therapeutic benefits. For instance, Oechslin et al. highlighted the enhanced efficacy of phage-antibiotic combinations against PAE infections (Oechslin et al., 2017). Similarly, Tkhilaishvili et al. demonstrated that combining phages with antibiotics significantly reduced bacterial counts in biofilms formed by *Staphylococcus aureus* (Tkhilaishvili et al., 2018).

Research indicates that some phages can disrupt biofilms, which are protective structures formed by bacteria. For instance, certain phages can prevent the formation of the bacterial capsule in *Acinetobacter baumannii,* crucial for biofilm integrity, while others may disrupt biofilms formed by *Staphylococcus aureus*

through mechanisms that are not yet fully understood (Gordillo Altamirano et al., 2021, Tkhilaishvili et al., 2018). When phages impair these biofilms, antibiotics can penetrate more effectively, enhancing their bactericidal action. Moreover, the efficacy of combined treatments in eradicating bacteria not encased in biofilms often surpasses that of either treatment modality alone, though this advantage can vary significantly depending on specific environmental and microbial conditions (Easwaran et al., 2020). As an illustration of this synergy is observed when bacterial resistance to specific phages makes previously ineffective antibiotics regain their efficacy. This phenomenon can occur because bacterial adaptations to evade phage attacks - like mutations in or loss of phage receptors - can inadvertently expose new sites that antibiotics can target. Such receptor modifications often involve metabolic functions, compelling bacteria to either form new receptors or express previously silent genes. These alterations can inadvertently create new attachment sites for antibiotics, thereby restoring their effectiveness (Ho et al., 2018, Chan et al., 2016).

2.5.3 Use of phage proteins in therapy

Lytic phages use enzymes encoded by their DNA to destroy the host cell's plasma membrane. This mechanism of destruction is being investigated for clinical use. Lytic proteins are a variant of phage therapy since the physical use of phages is not strictly necessary (Fischetti, 2008). The two most commonly reported are holin and lysin. These are found in most lytic phages and both proteins work together. Lysin molecules accumulate in the bacterial cytoplasm while the number of phages increases. Once the virions reach a certain number, holin synthesis begins. Holins attach to the internal bacterial membrane and eventually form channels. The holin molecules allow lysins to pass through it and destroy the bacterial peptidoglycan (external bacterial membrane), thus generating its destruction (Lin et al., 2017).

From a commercial point of view, laboratories are particularly interested in these proteins since they are chemical structures that can be patented and commercialised (Gerstmans et al., 2020, Todd, 2019). However, unlike antibiotics, which generally have a broad spectrum of bacterial targets, phage products typically only attack the species for which they were created (Fischetti, 2010). Whilst lytic enzymes have shown to be effective against Gram-positive cocci, studies on their effect against Gram negative bacteria are challenging (Viertel et al., 2014, Fischetti, 2010). This is because the Gram-negative bacterial outer membrane protects the peptidoglycan from contact with exogenous endolysin (Viertel et al., 2014).

2.5.4 Use of temperate phages for treatments

Temperate phages, which can undergo lytic and lysogenic cycles, present risks and potential benefits in antibacterial therapy. During the lysogenic cycle, the phage DNA integrates into the bacterial genome without causing immediate lysis, allowing bacteria to persist at the infection site. This integration can confer new properties to the bacteria, such as antibiotic resistance or increased virulence, through gene transfer via transduction (Abedon et al., 2011, Cisek et al., 2017). Moreover, the presence of a prophage can prevent superinfection by similar phages due to immunity, thereby limiting the effectiveness of subsequent lytic phage treatments.

Conversely, some researchers advocate for the strategic use of temperate phages. For example, Regeimbal and colleagues used a cocktail of one temperate and four lytic phages against Acinetobacter baumannii. The temperate phage suppressed capsular formation, enhancing the lytic phages' ability to infect and kill the bacteria in both *in-vitro* and *in-vivo* models (Lin et al., 2017, Regeimbal et al., 2016). Another innovative approach involves equipping temperate phages with a CRISPR-Cas system, allowing them to specifically

target and disrupt antibiotic resistance genes within the bacterial genome upon integration, thereby reviving the susceptibility of bacteria to conventional antibiotics (Yosef et al., 2015).

The potential for inducing lytic cycles in bacteria harbouring lysogenic phages through chemical triggers also merits exploration. A study linked the presence of benzopyrene diol epoxide, a chemical in cigarette smoke, with the induction of the lytic cycle in vaginal lactobacilli, illustrating the complex interactions between environmental factors and phage biology (Pavlova and Tao, 2000). Although this example illustrates a detrimental effect, it suggests that targeted triggers might be harnessed to convert temperate phages within pathogenic bacteria to a lytic state. However, this concept remains largely unexplored, and further investigation is necessary to assess its viability for therapeutic applications, as the use of chemical triggers has not been extensively investigated to date.

2.5.5 Topical administration of bacteriophage

Phage therapy can be administered through various routes, including topical, parenteral, oral, and pulmonary methods, all of which have demonstrated success (Lin et al., 2017). In this review, the focus is on the topical administration of phages. Topical administration involves applying a therapeutic agent directly onto the surface of the skin, which is particularly relevant for the potential treatment of ear infections in dogs (Bohling M, 2021, Kumar and Verma, 2010), as discussed in Chapter 1.

2.5.5.1 Immunological responses to topical phage application

Regardless of the route of administration, phage therapy elicits an immunological response in the mammalian host (Jończyk-Matysiak et al., 2017). In the case of topical administration, phages may penetrate systemically if the epithelial barrier is disrupted. This disruption allows the innate immune system - both cellular and humoral - to recognise and remove phages as foreign bodies (Dąbrowska, 2019, Hodyra-Stefaniak et al., 2015). The innate immune system's non-specific response plays a crucial role in this process.

Adaptive immunity also contributes to phage clearance. Notably, the adaptive response becomes more efficient after the host's first exposure to phages. This efficiency is due to the production of neutralising antibodies that specifically target phage epitopes (Forthal and Moog, 2009). Consequently, the effectiveness of subsequent phage treatments in the same individual may be reduced if similar phages are used (Dąbrowska, 2019).

Although an immune response is triggered by bacteriophage application, Dr. Pincus from the National Institutes of Health in Maryland, USA, reported in 2015 that an appropriate therapeutic dose of phages for treating Methicillin-Resistant Staphylococcus aureus (MRSA) skin infections in mice does not induce an inflammatory response. However, excessively high doses can lead to inflammatory damage to the skin in some cases (Pincus et al., 2015).

2.5.6 Purity of phage preparation for treatment

A crucial aspect of phage therapy is the purity of the phage preparation used for treatment. Historically, interest in phage therapy waned in the early 1900s, partly due to the poor quality and purity of phage preparations available at that time (Frobisher, 1927). Contaminants in phage preparations can include macromolecules such as lipopolysaccharides (endotoxins) from bacteria, culture media components, and cellular debris, among others (Bonilla et al., 2016). If these contaminants are not adequately removed, they can lead to serious adverse effects on the host, such as inflammation, sepsis, and in severe cases, septic shock due to the presence of endotoxins (Düzgüneş et al., 2021, Luong et al., 2020).

Today, several advanced protocols are available to achieve high-purity phage preparations and minimise the risk of such adverse effects. These protocols include methods like dead-end microfiltration, ultrafiltration, density gradient ultracentrifugation, and affinity chromatography (Tanir et al., 2021, Bonilla et al., 2016, Luong et al., 2020) (Table 2.3). Each method has specific characteristics that make it suitable for different types of phage preparations.

The acceptable level of endotoxins in preparations intended for intravenous administration, including those containing phages, is generally considered to be less than 5 endotoxin units (EU) per kilogram of body weight per hour, based on guidelines from regulatory bodies like the Food and Drug Administration (FDA) and the European Pharmacopoeia ((FDA), 2012, Spoladore et al., 2021). This guideline is not specific to phages but applies to any biological or pharmaceutical product intended for intravenous use to prevent adverse reactions such as fever, inflammation, or septic shock. The recommendation for limiting endotoxin exposure in topical applications, particularly over large surface areas or mucosal surfaces where systemic absorption may occur, is based on established guidelines from regulatory authorities such as the FDA and the European Pharmacopoeia and is typically limited to 100 endotoxin units (EU) per square meter. These guidelines are designed to mitigate the risk of adverse reactions, including inflammation and other systemic effects, by setting stringent limits on allowable endotoxin levels in pharmaceutical products.

In the case of topical treatments for ear infections in dogs, where the infection site already contains a high level of endotoxins due to the bacterial presence, the introduction of a relatively small number of endotoxins from the phage preparation is not expected to have a significant impact on the patient (Miedzybrodzki et al., 2012, Steele et al., 2020). In addition, some studies have demonstrated that the treatment with certain β-lactam antibiotics can even produce more endotoxins than phage applications (Dufour et al., 2017). Therefore, endotoxin removal will not be a priority in the purification process described in subsequent chapters.

Table 2.3: Example of bacteriophage purification techniques. Table adapted from Luong et. al. (2020).

Method	Characteristics
Dead-end Microfiltration	Used to retain both bacterial cells and particulates
Ultrafiltration	Any material smaller than the cross-flow membrane pore of 100 kDa passes through the membrane, whereas larger suspended phage particles remain in the retentate stream
Density gradient ultracentrifugation	A technique used to isolate and purify a wide range of phages purely on the basis of their density
Affinity chromatography	The most reliable method used to remove endotoxins

2.5.7 Efficacy of topical application

Some studies based on topical treatment with phages for their specific bacteria have shown a significant reduction in the number of bacteria at the site of infection. This decrease was achieved between 2 to 24 hours after applying the phage treatment. Moreover, no adverse effects were reported after the application of these treatments (Vieira et al., 2012, Goode et al., 2003).

Clinical trials conducted on burn patients who became infected with PAE also demonstrate the effectiveness of topical phage treatment. However, the phage cocktail used was not stable over time due to the decrease concentration of phages in the cocktail. This led to the early termination of the study since it was believed that a lower number of phages would not be effective for the treatment (Jault et al., 2019). This fact contradicts studies that indicate that phages can remain stable over extended periods of time in the environment where they are located (Varga et al., 2015, Shkoporov et al., 2021, Serwer et al., 2019).

2.6 Otitis externa produced by *Pseudomonas aeruginosa* & treatment issues

2.6.1 *Pseudomonas aeruginosa*, taxonomy and general characteristics

PAE is a heterotrophic, motile, Gram-negative rod-shaped bacterium, approximately 1–5 µm long and 0.5–1.0 µm wide. It is a facultative aerobe capable of growth via aerobic and anaerobic respiration. PAE thrives at 37°C but can survive in temperatures ranging from 4°C to 42°C. Commonly found in soil, it is also present in animal and human-polluted water sources, including sewage and hospital environments (Diggle and Whiteley, 2020).

PAE colonies are typically identified by the production of pyocyanin, a greenish-blue pigment that induces oxidative stress in the host by affecting the mitochondrial electron transport chain (Lau et al., 2004). Pyocyanin also promotes apoptosis in neutrophils and limits macrophage phagocytosis of apoptotic cells *in-vitro* (Bianchi et al., 2008). In addition to pyocyanin, PAE produces a range of other virulence factors, including exotoxins such as Exotoxin A, which inhibits protein synthesis in host cells, contributing to tissue damage and immune evasion. Endotoxins, present in the outer membrane of PAE, are also significant contributors to the inflammatory response and can exacerbate tissue damage during infections. Other factors, like elastases and alginate, further enhance the bacterium's ability to cause and maintain infections like otitis externa.

2.6.2 Infection with *Pseudomonas aeruginosa*

PAE is capable of colonising and causing disease in various hosts, including plants, nematodes, insects, and mammals (Gellatly and Hancock, 2013). It is considered an opportunistic pathogen because it primarily infects immunocompromised individuals (Mekic et al., 2011). Many body locations can act as an origin of infection for PAE. These sites include subcutaneous tissue, skin, heart valves, eyes, urinary tract, lungs, and ears; moreover, the location varies depending on the vulnerability of the patient and the entry point (Holloway et al., 2013).

2.6.2.1 Antibiotic Resistance in *Pseudomonas aeruginosa*

PAE infections are particularly challenging to treat due to the bacterium's inherent capacity to withstand numerous antibiotic classes and its ability to acquire resistance through intrinsic, acquired, and adaptive mechanisms (Gellatly and Hancock, 2013, Boucher et al., 2009) (Table 2.4). The World Health Organization has categorised PAE as a critical priority species, highlighting its inclusion on the list of bacterial pathogens that most urgently require the development of new antibiotics (Tacconelli et al., 2018).

2.6.2.2 Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*

PAE exhibits intrinsic resistance encoded in its bacterial chromosome. For example, the low permeability of PAE's outer membrane restricts the access of small hydrophilic molecules, such as β-lactam antibiotics, limiting these molecules from fully accessing the porin channels located within the outer membrane (Delcour, 2009). Additionally, several efflux pump families have been identified in PAE, which actively eject antibiotics from the bacterial cell (Livermore, 2002). For instance, the up-regulation of the MexAB-OprM efflux pump system compromises the effectiveness of fluoroquinolones, penicillins, cephalosporins, and certain carbapenems, thus increasing resistance to a variety of antipseudomonal medications (Livermore, 2002). Another intrinsic resistance mechanism involves the spontaneous production of an inducible

chromosomal enzyme, β-lactamase (AmpC). PAE secretes this enzyme into its periplasmatic space (the space between the plasma membrane and the outer membrane), where it hydrolyses antibiotics such as penicillins and cephalosporins (Jacoby, 2009).

In addition to its intrinsic mechanisms, PAE can acquire resistance through various strategies. These include but are not limited to, acquiring new genetic mutations that aid in survival or obtaining resistance-conferring DNA from other bacteria. For example, the mcr-1 gene, which provides resistance to colistin, can be transferred to PAE through horizontal gene transfer (HGT), a process involving the movement of genetic material between bacteria via DNA elements such as plasmids. The three main means of HGT are transduction (transfer of DNA via phage), transformation (transfer of naked DNA from lysed cells from the environment through the cell wall), and conjugation (transfer of DNA from one cell to another by direct contact via a bridge).

PAE also exhibits adaptive resistance, which occurs when it is subjected to stressors, such as exposure to subinhibitory concentrations of antibiotics. Under these adverse environmental conditions, altered gene expression leads to the overexpression of resistance-conferring genes. Additionally, PAE can form biofilms under these conditions, providing further protection against antibiotics (Breidenstein et al., 2011).

Table 2.4: Intrinsic, acquired and adaptative resistance mechanisms

Resistance Class	Resistance mechanism	Example(s)
Intrinsic	Efflux pumps	MexAB-OprM, MexEF-OprN (cephalosporins, quinolones, carbapenems)
	Outer membrane impermeability	Oprd, OprB, OprF (aminoglycosides, quinolones, carbapenems)
	B-lactamases	AmpC (penicilins)
Acquired	Mutations	DNA topoisomerase, DNA gyrase (quinolones) MexZ (quinolones, cefepimes, aminoglycosides)
	Horizontal transfer	Extended spectrum B-lactamase (ESBLs), Metallo-B-lactamases (penicillins, cephalosporins, carbapenems), mcr-1 (colistin)
Adaptative	Membrane changes	AmpC upregulation (penicillins)

2.6.2.3 Biofilm Formation

A crucial factor to highlight is that antibiotics have been found to be less effective against bacteria that form biofilms (Høiby et al., 2010). A biofilm is a structured community of bacteria enclosed within a self-produced matrix of extracellular polymeric substance (EPS), which allows the bacterial population to remain together in a specific location and offers protection against environmental stressors, including antibiotics (Karatan and Watnick, 2009, Rasamiravaka et al., 2015). Biofilm formation is a continuous process where bacterial populations organise into these communities, replicate, and potentially slough off components to produce new biofilms (Costerton et al., 1999).

In PAE, the EPS accounts for 85% of the total biofilm biomass. It comprises biomolecules, exopolysaccharides, extracellular DNA (eDNA), and polypeptides, which together form a highly hydrated polar mixture that contributes to the biofilm's structural scaffold and architecture (Rasamiravaka et al., 2015) (Table 2.5). Consequently, different biofilm morphologies can emerge depending on PAE strains and/or nutritional circumstances (Flemming and Wingender, 2010). Furthermore, the exceptional capacity of PAE to establish biofilms in a variety of conditions renders antibiotic treatments ineffective, promoting chronic infectious illnesses (Høiby et al., 2010).

Table 2.5: Biofilm development stages

Stage	Process
I	Reversible attachment of planktonic bacteria to a growth-friendly surface.
II	Bacteria establish microcolonies in the extracellular polymeric material matrix after irreversible adhesion.
III	The confluence of bacterial microcolonies results in a more organised phenotype with non-colonized space.
IV	Bacteria expand to surrounding areas, eventually covering the entire surface.
V	Bacteria scatter from the sessile structure and return to a planktonic state, where they can disseminate and colonise other surfaces.

2.7 Otitis externa in dogs

2.7.1 Canine ear structure

The pinna is the external ear's most protruding feature. It features a concave inner surface and a convex outer surface. The concave surface of the standing ear generates a conchal chamber. The apex of the pinna is the distal tip, while the helix is the lateral and medial free borders of the pinna. The tragus is the rostrolateral boundary of the distal section of the ear canal. The annular cartilage forms the ear canal proximally (near the skull), while the auricular cartilage, which fans out to form the pinna, forms the ear canal distally (away from the skull). The concha cavity takes the shape of a funnel, thickening proximally to produce the conchal tube. The conchal tube forms the vertical ear canal and the horizontal ear canal, which limits by the tympanic membrane (Nuttall, 2014). An air-filled tympanic cavity forms the dog's middle ear. The tympanic membrane is detached from the external ear and the inner ear by the vestibular and cochlear systems (Nuttall, 2014) (Figure 2.2).

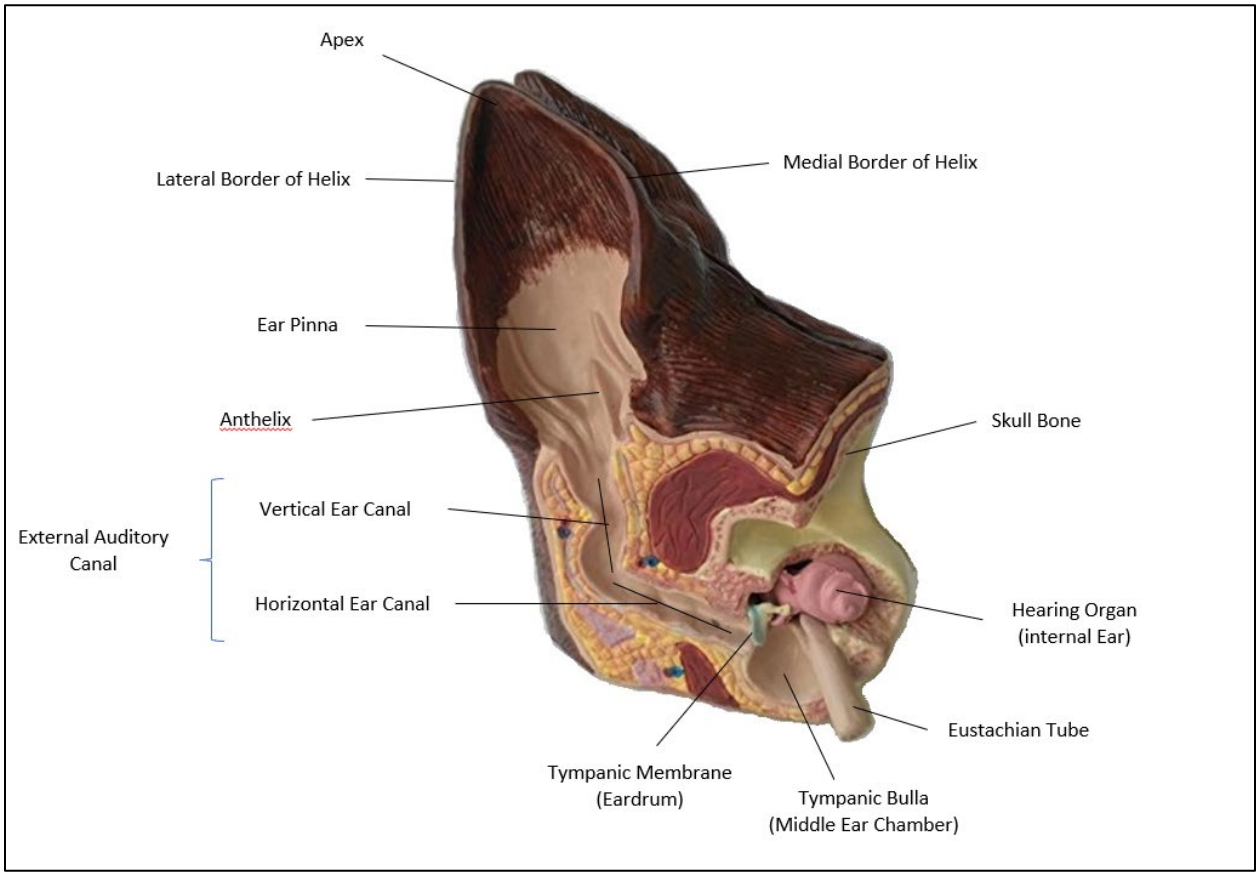

Figure 2.2: Normal ear model and Internal anatomy (Image by HeineScientific).

Understanding the detailed structure of the canine ear is crucial for several reasons. The complex anatomy, including the vertical and horizontal orientation of the ear canal, predisposes dogs to the accumulation of debris, moisture, and pathogens, which are common contributors to otitis externa (Cole et al., 1998, Nuttall, 2014). The ear's funnel shape and the presence of cartilaginous structures also create an environment that can retain moisture and reduce air circulation, further encouraging bacterial and fungal growth (Harvey et al., 2001). Another important consideration is the challenge posed by this anatomy in

effectively delivering ear treatments, as medications must navigate the elongated and curved ear canal to reach the site of infection (Paterson, 2016). Therefore, a thorough understanding of the ear structure is essential for diagnosing, managing, and treating otitis externa in dogs effectively.

2.7.2 Definition and causes of otitis externa

The normal flora in the ear canal is mostly Gram-positive, with a predominance of bacterial species in the external auditory canal. This flora contains commensal and sometimes pathogenic bacteria (Holloway et al., 2013). Otitis externa is defined as the inflammation of the external ear canal, which includes the pinna, vertical and horizontal canals, and extends up to the tympanic membrane. This inflammation can result from various causes, leading to symptoms such as redness, swelling, and discomfort (Pye, 2018).

The development of otitis externa in dogs is multifactorial, involving predisposing, primary, secondary, and perpetuating factors (Scott DW, 2001). Predisposing factors increase the risk of developing otitis externa but do not directly cause the condition. They include anatomical features such as pendulous pinnae, narrow ear canals, and excessive hair growth within the ear canal. Environmental factors like high humidity and frequent swimming can also predispose dogs to ear infections by creating a moist environment conducive to microbial growth (Rosser, 2004). The primary factors are the direct causes that initiate inflammation in the ear canal. Common primary factors include allergic skin diseases such as atopic dermatitis and food allergies, ectoparasites like ear mites (Otodectes cynotis), foreign bodies, and endocrine disorders such as hypothyroidism (Saridomichelakis et al., 2007). The secondary factors contribute to or exacerbate existing inflammation, often following primary causes. Bacterial infections, particularly with organisms like PAE, and fungal infections such as *Malassezia pachydermatis* are typical secondary factors (Barnard and Foster, 2018). Perpetuating factors sustain or worsen ongoing otitis externa, making treatment and recovery more challenging. They include chronic changes like hyperplasia of the ear canal lining, stenosis (narrowing) of the ear canal, and biofilm formation by bacteria, which can protect pathogens from treatments (Scott DW, 2001).

Table 2.6: Factors that might affect the external ear integrity

Predisposing factors	Ear structure/conformation Blockage Significant hair growth Increased wetness Inappropriate treatment
Primary factors	Ectoparasites Foreign bodies Allergic skin disease Endocrinopathies Immune diseases Immunosuppression Ear canal tumours
Secondary factors	Yeast Bacteria
Supportive/perpetuating factors	Ulceration Otitis media Epidermal and glandular hyperplasia

2.7.3 *Pseudomonas aeruginosa* as a causal agent of otitis externa

PAE is the most common Gram-negative isolate in cases of infectious otitis externa (Scott DW, 2001). PAE is a significant pathogen in these infections due to its numerous virulence factors, including the production of exotoxins, enzymes, and its ability to form biofilms. These virulence factors enable PAE to adhere to the ear canal's epithelial cells, evade the host immune response, and resist antibiotic treatment (Livermore, 2002, Rasamiravaka et al., 2015). Additionally, the biofilm formation by PAE provides a protective environment that enhances its survival and persistence within the ear canal, making infections chronic and difficult to treat (Høiby et al., 2010).

Otitis externa caused by this bacteria can present one or more of the following clinical signs: head shaking, aural pruritus, ear odour, erythema, alopecia, signs of self-trauma to the pinnae and pre-auricular region, shyness around the head, ear canal discharge, aural hematoma, and external ear canal ulceration (Scott et al., 2001, Barnard and Foster, 2018).

2.7.4 Antibiotic therapy in otitis externa

Most cases of otitis externa are primarily surface infections, and since the ear canal has poor blood supply compared to many other organs, topical treatment is the preferred treatment method (Barnard and Foster, 2018, Mactaggart, 2008). However, the removal of exudate is critical to the success of this therapy. Treatment is likely to fail if the medication does not penetrate the entire length of the ear canal. The selection of the proper medication for the treatment of otitis externa should be made based on cytological examination of ear canal exudates and otoscopic examination of inflamed ear canals (Holloway et al., 2013).

An overgrowth of PAE as the sole agent or together with other bacteria or yeast is linked and found in most chronic cases of bacterial otitis externa in dogs. Therefore, topical antibiotic treatment targeting PAE is preferred for these cases (Barnard and Foster, 2018). Table 2.7 describes the antimicrobial agents frequently used to treat infections related to PAE. Moreover, up to 40% of PAE isolates produce biofilms lining the ear canal that raise the antimicrobial minimal inhibitory concentration (MIC) required to treat the infection (Pye et al., 2013, Patrick Hensel, 2021). Despite careful treatment with antibiotics, a large number of cases may not resolve or may relapse (Pye, 2018). Thus, alternative or adjunct therapies are required.

Table 2.7: Antimicrobial agents frequently used to treat *Pseudomonas aeruginosa* infections

Antimicrobial agent	Drug(s) Examples	Used in topical treatment	Observation(s)
Aminoglycosides	- Gentamicin - Amikacin - Netilmicin - Tobramycin	Yes, with an anti-inflammatory in dogs' ears = Momentomax; Otomax.	-Gentamicin, for animal use
Carbapenems	- Imipenem - Meropenem - Doripenem	No topical preparation	
Cephalosporins	- Ceftazidime - Cefepime	No topical preparations	
Fluoroquinolones	- Ciprofloxacin - Levofloxacin	Topical formulations available	-Enrofloxacin, norfloxacin, marbofloxacin and pradofloxacin for animal use -Ciprofloxacin used as second-line antibiotic in animals
Penicillins with β-lactamase inhibitors	- Ticarcillin/Clavulanic acid -Piperacillin/Tazobactam	Ticarcillin/Clavulanic acid - Topical formulations available	-Ticarcillin/Clavulanic acid (Timentin®)- for animal use
Monobactams	- Aztreonam	No topical preparations	
Phosphonic acids	- Fosfomycin	No topical preparations	
Polymyxins	- Colistin - Polymyxin B	Polymyxin B - Topical formulations available	

2.7.5 Phage therapy in otitis externa

As previously discussed in this review, phage therapy has shown promising results against several infections (Jaiswal et al., 2013, Jennes et al., 2017, Schooley et al., 2017a, Duplessis et al., 2018). In addition, a study conducted by Catherine Hawkins and her team in 2010 investigated the efficacy of phage therapy against PAE infections in dogs' ears, yielding encouraging outcomes. The study involved ten dogs, each of which received a single dose of a topical preparation containing six different bacteriophage isolates active against PAE. The specific dose administered was $1x10^5$ plaque-forming units (PFU) per millilitre, with a single 0.2 mL dose instilled directly into the external auditory canal of one ear per dog (Hawkins et al., 2010).

Following bacteriophage treatment, a rise in bacteriophage counts was observed in all dogs. These counts were measured by collecting ear swabs at various time points post-treatment and performing plaque assays to quantify the bacteriophages present (Hawkins et al., 2010). This increase in bacteriophage presence coincided with clinical improvement, as identified by a reduction in symptoms such as aural pruritus, erythema, and discharge, along with a decrease in bacterial counts as confirmed by culture results (Hawkins et al., 2010).

These findings suggest the potential utility of this bacteriophage mixture in treating PAE-induced otitis externa. However, Hawkins et al. emphasised the necessity for further studies to validate these results, particularly due to the small sample size and the need to assess long-term outcomes and potential resistance development (Hawkins et al., 2010). Their success serves as a foundation for expanding research to develop and test phage cocktails against PAE from otitis externa cases in different regions, such as Townsville, QLD.

2.8 Conclusion

Bacteriophages, found in diverse environments wherever their bacterial hosts are present, play a crucial role in bacterial ecology and pathogenesis. Phage therapy offers a promising strategy to counter the rising challenge of bacterial resistance to antibiotics, an issue of global concern. This project aims to explore and enhance the efficacy of phage therapy specifically targeting PAE infections, with a focus on treating otitis externa in dogs.

The research will be conducted in three phases, each addressing specific hypotheses central to advancing phage therapy, particularly in the James Cook University context. The first phase will test the hypothesis that certain environments would yield a higher abundance of PAE-specific phages, providing a better source for therapeutic phage collection. The second phase will focus on the hypothesis that increasing the number of phages in a cocktail would significantly improve the efficacy and spectrum of treatment in an *in-vitro* model. The final phase will test the hypothesis that phage stability during storage would vary depending on the storage conditions, influencing the long-term viability and effectiveness of the phages.

Overall, the outcomes of this project will underscore the potential of phage therapy as a viable and effective treatment option for antibiotic-resistant infections, particularly in veterinary medicine. If phage therapy continues to advance and becomes integrated into routine therapeutic practices, either as a standalone treatment or in combination with other therapies, the treatment of PAE-induced otitis externa in dogs could become a routine, effective alternative to conventional antibiotic therapy.

CHAPTER 3: GENERAL MATERIALS AND METHODS

3.1 Preparation of *Pseudomonas aeruginosa*

3.1.1 *Pseudomonas aeruginosa* recovery

In this study, 48 *Pseudomonas aeruginosa* (PAE) isolates were utilised. Forty-seven isolates were collected from dogs diagnosed with otitis externa, primarily from the North Queensland region, with most samples originating from Townsville. Additionally, the commercially available reference strain PAE ATCC 27853 was included to facilitate reproducibility and comparison of results across different laboratories. The samples were collected at the JCU VET General Practice and Referral Hospital in Townsville, Queensland, and stored in the Veterinary Pathology and Biomedical Sciences Laboratory at James Cook University (JCU) from 2006 to 2023. All PAE strains underwent antibiotic sensitivity tests, which are detailed in Appendix 2. PAE strains were removed from storage (-80°C), streaked onto Cetrimide agar (Appendix 1) for recovery, and incubated at 37°C for 24 hours in the JCU Veterinary Diagnostic Facility. Once recovered, they were stored individually in microbial storage with beads (MICROBANK™ Pro-Lab Diagnostics) at -80°C until required.

3.1.2 Working Bacterial Solution

To initiate the experimental procedures, beads containing PAE were taken from microbial storage and inoculated into 10 ml of Luria-Bertani/Lysogeny broth (LB broth) (Appendix 1) for bacterial growth at 37°C overnight. This step was necessary to revive and proliferate the bacterial cells to create a working stock solution suitable for subsequent experimental use.

The bacterial solution (stock solution) was stored at 4°C and renewed every two weeks to ensure the viability and consistency of the bacterial culture throughout the study. Renewing the stock involved re-inoculating fresh LB broth with beads from the original microbial storage, followed by the same overnight incubation process. Aliquots from this refreshed stock were used as needed to cultivate fresh cultures.

After incubation, the bacterial culture in LB broth was then diluted in SM buffer (Appendix 1) and adjusted to McFarland's scale 0.5 turbidity standard using a spectrophotometer (SPECTROstar® Nano microplate reader) at 570 nm. This adjustment was crucial for standardising the bacterial concentration across experiments, with the final working suspension corresponding to approximately 1.5 x 10^8 colony-forming units per millilitre (CFU/ml), equating to an optical density (OD) between 0.26 and 0.27 (Table 2.3.1 - Appendix 2).

3.2 Preparation of *Pseudomonas aeruginosa* phages

3.2.1 Phage isolation and purification using double agar overlay plaque assay

The Double Agar Overlay Plaque Assay (DAOPA) was employed to isolate PAE phages directly from environmental samples without prior enrichment. This method was used to detect plaques, clear zones on a bacterial lawn, which indicate phage activity as they lyse bacterial cells.

For the phage screening, 10 PAE strains were selected based on their antibiotic resistance profiles, which are detailed in Appendix 2. The selected strains represent a diverse range of resistance levels to key antibiotics commonly used to treat ear infections in dogs, such as Amikacin, Gentamicin, and Ticarcillin. This selection strategy was critical to ensure that the phage therapy's potential efficacy could be assessed across different clinical scenarios. Additionally, strains from different years were included to examine possible changes in phage susceptibility over time. Due to time and resource constraints, only 10 PAE strains were chosen, but these strains are representative of the broader resistance patterns observed in the full set of 48 PAE strains.

Environmental samples were collected and filtered to remove debris, resulting in a phage-containing filtrate. Briefly, 1 mL of this filtrate was mixed with 1 mL of LB broth supplemented with 1 M $CaCl_2$ and 1 M $MgCl_2$ to reach a final concentration of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Appendix 1) to stabilise the phages (Bonilla et al., 2016). This LB broth also contained an overnight culture of PAE at a concentration of approximately 10^8 CFU/mL. The phage-bacteria mixture was then combined with 3 mL of melted LB top agar (Appendix 1), a semi-solid medium prepared by adding agar to LB broth at a lower concentration than that used in LB agar (Appendix 1). This mixture was quickly poured onto a pre-solidified layer of LB agar (25 mL), creating an overlay agar layer capable of producing a confluent lawn of bacteria within it (bacterial lawn). The plates were incubated overnight at 37°C, allowing any phages present to infect and lyse the bacteria, resulting in the formation of visible plaques (Bonilla et al., 2016).

Individual plaques, representing single phage particles, were carefully selected using a sterilised pipette. The pipette was inserted at a 90° angle over the plaque, rotated gently to loosen the agar, and then slightly angled to remove the plaque-containing agar. The excised agar, containing the phage, was transferred into a tube with 1 mL of SM buffer and left undisturbed for 30 minutes to allow the phages to diffuse into the solution. After this, the mixture was centrifuged at 8000 RPM for 5 minutes. The supernatant, referred to as the phage extract, was carefully collected without disturbing the pellet and transferred into a fresh container. Phage extracts were subjected to three rounds of purification by re-plating them on fresh propagating overlay lawns to ensure that each resulting phage population was derived from a single, stable phage (purified phage), and stored at 4°C for further analysis.

3.2.2 Phage propagation via liquid lysate

Following isolation, purified phages were propagated to obtain high-titer phage stocks for subsequent experiments. This method was adapted from Bonilla et al. (2016), with modifications to accommodate the specifics of this study.

To propagate the phages, 250 µL of an overnight PAE host culture was added to 5 mL of LB broth, which was supplemented with 1 M $CaCl_2$ and 1 M $MgCl_2$ to reach a final concentration of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Appendix 1). This was incubated with agitation for 1 h at 37°C (Bioline 8000 Series). After the initial hour, 1 mL of purified phage was added to the bacterial culture. The mixture was then incubated at 37°C with agitation for 6 hours or until the bacterial lysate became clear. After this time, the phage-bacterial mixture was centrifuged at 8000 RPM for 10 minutes to remove bacterial debris. The supernatant, containing the phages, was carefully collected and filtered through a 0.45 µm filter (Millipore Millex HV 0.45 µm) to ensure purity.

To confirm the success of the propagation and assess the concentration of the phage solution, spot assays were performed. When the phage concentration was lower than $1x10^9$ plaque forming units per millilitre (PFU/mL), additional rounds of propagation were conducted until the desired concentration was achieved. The target concentration was set at $1x10^9$ PFU/mL, as this high-titer phage stock ensures experimental reliability and effective bacteriophage activity in therapeutic or research applications. The final phage lysates were stored in sealed containers, protected from light, at 4°C until further use.

3.2.3 Spot Assay

The double agar overlay spot assay was adopted to determine phage killing capacity and to estimate the concentration of phages in solutions. This method is based on standard bacteriophage techniques that have been developed from D'Herelle's plaque assay (1917), with modifications by Armon and Kott to fit the specifics of this study (Armon and Kott, 1993). Briefly, 2 mL of an overnight bacterial culture, diluted in SM buffer (Appendix 1) to reach an optical density (OD570 - to measure bacterial growth at a wavelength of 570 nm) between 0.26 and 0.27, was mixed with 3 mL of melted LB top agar (Appendix 1). The solution was quickly poured onto the bottom layer containing LB agar (Appendix 1), resulting in a bacterial lawn.

After the agar solidified, 10 µL of the solution containing the phage was spotted onto the top agar in a pattern, as shown in Figure 3.1. The spots were left to dry in a Class II biosafety cabinet for 25 minutes. This was followed by incubation at 37°C overnight. The next day, the plates were inspected for clear zones (plaques) where spots had been placed, which indicated successful phage infection and bacterial lysis. The diameters of the plaques were compared to assess any variations, and the number of plaques was used to estimate the concentration of phages in the solution.

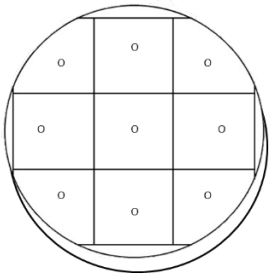

Figure 3.1: Distribution of spots for killing profile in 90mm Petri dish. Circles denote the position of spots in the agar plate.

3.3 Concentration Method

3.3.1 Ultracentrifugation

For environmental samples that produced a low number of plaques, where phage propagation was not possible due to the nature of the phages (e.g., phages with lysogenic life cycle or temperate phages, which tend to produce cloudy or opaque plaques (Abedon, 2021)), ultracentrifugation was performed to concentrate the samples. This process was used to attempt the isolation of lytic phages that may have been present in very low concentrations, preventing plaque formation in the DAOPA assay.

Briefly, 50 mL or 100 mL of the phage-containing solution was ultracentrifuged at 200,000g for four hours (Optima L-90K ultracentrifuge, TY 50.2 Ti rotor; Beckman Coulter). The supernatant was discarded, and the pellet was gently resuspended in 500 µL of SM buffer using a transfer pipette. The suspensions were then filtered through a 0.45 µm filter and stored at 4°C, protected from light with aluminium foil until use (Elliman, 2006).

3.4 Analysis of bacteriophages' lytic activity against host bacteria to determine their killing profile.

Forty-eight strains of PAE were used in this study, comprising 47 clinical isolates provided by the JCU VET diagnostic facility's culture collection and one reference strain, *Pseudomonas aeruginosa* ATCC 27853™ (Section 3.1.1). The clinical isolates were collected from dogs in the North Queensland region and exhibited varying antibiotic resistance profiles, as detailed in Appendix 2. These PAE isolates were screened against bacteriophages, which were isolated and purified as described in Sections 3.2.1 and 3.2.2.

The double agar overlay spot assay (Section 3.3) was used but with volumes adjusted for 150 mm agar plates. Briefly, 6 mL of an overnight bacterial culture was diluted in SM buffer to reach an OD570 between 0.26 - 0.27 ($1.5x10^8$ CFU/mL) and was mixed with 9 mL of melted LB top agar. The solution was quickly poured onto the bottom layer containing LB agar, resulting in a bacterial lawn. After the top agar solidified, 10 µL of the phage solution (or cocktail) was spotted with a micropipette onto the top agar in a pre-determined position, as shown in Figure 3.2. The spots were left to dry in a Class II biosafety cabinet for 25 minutes, followed by overnight incubation at 37°C. The next day, the plates were inspected for zones of clearance (plaques), indicating successful phage infection and bacterial lysis.

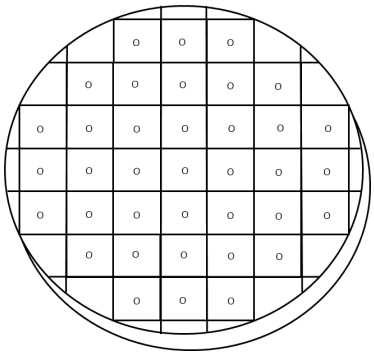

Figure 3.2: Distribution of spots for Killing Profile in 140mm x 15mm glass Petri dish. Circles denote the position of spots in a large agar plate.

CHAPTER 4: PSEUDOMONAS AERUGINOSA PHAGES FROM DIVERSE ENVIRONMENTAL WATER SOURCES AND BUILT LOCATIONS

4.1 Introduction

Bacteriophages have colonised every possible area where their host, bacteria, are found (Muniesa et al., 2011). To find PAE phages, samples were collected where *Pseudomonas aeruginosa* (PAE), their target, might be found. PAE is an opportunistic pathogen that is ubiquitous, typically found in moist environments such as water, including effluent water (Crone et al., 2020). Recommendations on potential sites to search for PAE phages are available in the literature (Aghaee et al., 2021), but the best locations for phage isolation against PAE remain unknown in some regions (Emencheta et al., 2021). This study was conducted in the city of Townsville, North-East Queensland, where no previous data has been recorded regarding preferred phage collection sites for PAE. The primary aim of this section is to identify optimal locations for collecting phages against PAE.

The city of Townsville is located at latitude -19.256944 and longitude 146.849444, on the northeastern coast of Queensland, Australia. Due to its geographical position, Townsville experiences a tropical savanna climate. The Ross River flows through the inner city and its suburbs, extending 19 km from the Ross River Dam to Cleveland Bay in the Coral Sea. The Ross River Dam is the primary potable water source for Townsville. Other water sources include several rivers and streams, which contribute to the moist environments ideal for PAE.

Townsville also houses several wastewater treatment facilities, such as the Mount St. John Wastewater Treatment Plant, Cleveland Bay Purification Plant, and Condon Water Purification Plant, which process urban sewage and stormwater. Additionally, environments where dogs are commonly found, such as veterinary clinics, kennels, and pounds, were considered for sample collection. Notably, the James Cook University (JCU) Veterinary Emergency Centre and Hospital provides emergency services and has its own water waste management systems, while the JCU necropsy facility processes deceased animals for research and teaching purposes.

Identifying the best sampling sites for PAE phage is a complex task with multiple variables influencing the process, including the specific isolates used as hosts for phage isolation. Ideally, repeated sampling over time at different locations would provide a more comprehensive understanding, but due to time and resource constraints, this study was limited to a single-season collection.

The phages identified were isolated and propagated to obtain high concentrations of phage stocks for further analysis. This analysis included eliminating repeated isolates by comparing the phages' killing ability against 48 PAE strains isolated from dogs with otitis externa. Additionally, a comparison of the killing range of phages between the sample sites was performed.

4.2 Materials and Methods

4.2.1 Sampling methodology

Samples were collected from four different locations in the Townsville region, North Queensland, Australia, in 2022. A single round of sampling was conducted due to time and resource constraints, meaning that repeated sampling over time was not possible for this study. The materials used for the collection of samples are detailed in Table 4.1.

Table 4.1: Contents of Sample Kit

• Sterile plastic bottles with screw tops (1 litre)	• Plastic water pump
• Disposable gloves	• Swabs (Amies transport)
• Disposable apron	• Polystyrene box
• Disposable head cover	• Ice gel pack
• Disposable face mask	• Disposable plastic boots cover

For the collection of aqueous samples, personal protective equipment, including gloves and overalls, was worn. Water was collected into two 1-litre sterile screw-capped bottles. For the sample taken from the wastewater treatment plant, a funnel was used to pour a portion of the contents of the water tank into the bottle. For both the JCU veterinary morgue wastewater sample and the Ross River sample, the bottle was submerged approximately one centimetre below the surface to collect the water. Samples were labelled, stored cold in a polystyrene box with an ice gel pack, and transported to the laboratory within 1 hour. For sampling from the veterinary hospital, commercial swabs with transport medium (SARSTEDT™ Amies Agar Gel Transport Swabs) were used, as described by J. Shen et. al. (Shen and Hartmann, 2021). Briefly, four swabs in their transport medium were used. They were pressed against the wet walls of the drainage chamber in a rotary motion. The swabs were put back in their transport medium container tube. The samples were labelled, stored cold in a polystyrene box with an ice gel pack, and taken to the laboratory within 1 hour.

4.2.2 Sample Location selection criteria

Four locations for sample collection, natural and built environments, were selected considering the chances of finding PAE's phages, easy access, and diversity of origin. For finding phage, locations that are likely to have exposure to PAE (target bacteria), the presence of animals (including dogs), humid spaces and places with microbial-contaminated waters were considered. Natural and built environments were selected from those we could most easily access. Samples obtained from built environments included Mount St John Wastewater Treatment Plan, Veterinary Pathology and Biomedical Sciences JCU necropsy room, and the JCU VET General Practice and Referral Hospital. The natural location selected was the Pontoon in Ross River.

4.2.2.1 Pontoon in Ross River

Samples coming from the natural environment were collected in “The Pontoon”, Douglas QLD 4814, an area where the Ross River has stagnant water sites, and microbial biota might be present. Geolocation: Latitude: -19.316781178120873, Longitude: 146.75141461444593 (https://gps-coordinates.org/my-location.php?lat=-19.316781178120873&lng = 146.75141461444593)

4.2.2.2 Mount St John Wastewater Treatment Plan

Mount St John Wastewater Treatment Plan is located at Mount St John Rd, Mount St John, QLD 4818, with coordinates of Latitude: -19.277778, Longitude: 146.716667. This wastewater treatment plant serves the northern suburbs and industrial areas of Townsville. It has a capacity of 25,000kL per day and uses an activated sludge process to remove organic matter and nutrients from the wastewater. Once sewage arrives at the wastewater treatment plant, it undergoes three cleaning stages. The first stage is the primary treatment, where all unwanted (solid) items present in the wastewater are removed. The secondary treatment stage is where the dip cleaning of wastewater takes place. Next, bioreactors (beneficial bacteria) are used to break down the organic solids and reduce the nutrient levels in the sewage and clarify the water. The tertiary treatment is called disinfection, where clear water is treated with chemical products or/and ultraviolet light to eliminate microorganisms.

The sample was taken from a sample collector located at the end of the primary treatment process before the wastewater entered the bioreactor tank. This raw material is collected in a twenty-four-hour container that automatically receives samples every hour and is kept refrigerated at 4°C.

4.2.2.3 The Veterinary Pathology and Biomedical Sciences at James Cook University

The Veterinary Pathology and Biomedical Sciences JCU necropsy room is located at 1 James Cook Dr, Townsville, QLD 4811, with coordinates of Latitude: -19.329167, Longitude: 146.762222. The necropsy room is equipped with stainless steel tables and machinery required for corpse processing, including scissors, axes for breaking bones, and saws of various sizes. The room features an impermeable floor with two long drainage lines for the removal of blood and other liquid waste.

The first collection site for this study was inside this necropsy room. A sample was collected from the entrance pipe of one of the longitudinal drainage lines. The sample appeared reddish in colour. A second sample was taken outside the building, where liquids from the necropsy room drain. To collect this sample, we lifted a concrete cover to access the wastewater chamber and used a one-time pump to draw the sample into a 1-litre container. This sample was also reddish but paler than the first, potentially due to dilution. Both samples were transported to the laboratory for processing.

During the collection process, it was observed that the reddish colour of the samples resembled a cleaning product commonly used in the facility—"MultiClean" (Cyndan Chemicals)—which is applied daily for cleaning. To provide clarity, the cleaning and disinfectant chemicals used in the necropsy room are listed in Table 4.2.

Table 4.2: Chemicals used at JCU Veterinary Necropsy Room.

Chemical name	Characteristics
CHEMEX BLEACH	Used on hard surfaces. Disinfectant, stain removal.
Antec Virkon	Multi-purpose disinfectant
Trigene II	To control bacteria, fungi, viruses, and bacterial spores in veterinary hospitals
MultiClean	Detergent/Solvent Cleaner
Sparkle	Heavy duty detergent
Ethanol 70%	Used as antiseptic
MICROSHIELD* 4 Chlorhexidine	Antiseptic skin cleanser for external use, hand and body washing

4.2.2.4 JCU VET General Practice and Referral Hospital

The JCU VET General Practice and Referral Hospital is located at 1 Solander Rd, Douglas QLD 4814, with coordinates of Latitude: -19.325556, Longitude: 146.769444.

Vet Hospital is a location site with much movement of animals, mainly companion animals, including dogs. The first collection point was the theatre room, where a recently deceased dog was at first sight at one of the operating tables. It was noticeable that were traces of blood on the operating table and the floor. The sample was collected from the drain, which, as in the necropsy room, is located on the floor. We proceeded to lift the lid and collect a swab sample from there. Not a single bacteriophage was obtained from this sample, which may be due to the high level of chemicals used for this place too.
A second sample was collected from the external courtyard's sewer of the vet hospital, which houses cages for medium-large dogs waiting to be collected. The area is covered by a sheet metal roof that shields it from rain but remains open to dust, wind, and some rainwater. The sample was taken from a chamber on the floor covered with a steel lid accessible only with specific tools. The lid was pried open, and the walls of the chamber were found to be damp and blackened with earth, sand, hair, and other residues of dirt and contamination. Two swabs were used to collect samples from the walls as there was no liquid for bottle collection. The swabs were wholly blackened and dirty and were introduced like this in the transport medium. The samples were then taken to the laboratory for analysis.

4.2.3 Bacteriophage Transport and Isolation

4.2.3.1 Transport

Plastic bottles with 500ml capacity and swabs in their transport media were placed in a polystyrene box with an ice gel pack and taken from the collection site to the laboratory. In the lab, wastewater samples contained in bottles were kept in the fridge at 4°C until use. Swabs were placed in 10 ml SM buffer and then kept in the fridge at 4°C until use. The processing time since the sample arrived at the lab was less than 2 hours.

4.2.3.2 Isolation

Samples were centrifuged at 8000 RPM for 10 minutes at 4°C (Sorvall RC6 PLUS TM Centrifuge, Thermo Fisher Scientific), after which the supernatant was separated from the pellet and filtered using 0.45 µm filters (Millex® 33mm Durapore PVDF 0.45 µm Sterile) to remove debris and bacteria.

Phage presence was determined by exposing aliquots from each filtrate to 10 PAE strains, as described in Section 3.2.1. The bacteria were prepared on agar plates as per Section 3.1, and the double agar overlay plaque assay (DAOPA), detailed in Section 3.2, was used to detect plaques on the bacterial lawns. Total plaques were counted and recorded.

4.2.4 Extraction of plaques from DAOPA

The resulting individual distinct plaques were differentiated based on their morphology, specifically the size and clearness of the zones of clearance. Plaques ranged in size from approximately 1 mm to 5 mm in diameter. Plaques with diameters greater than 3 mm and with clear centres (indicating strong lytic activity) were selected for extraction. For assays where plaques larger than 3 mm were not present, smaller plaques (less than 3 mm) were used. It should be noted that plaque size can be influenced by several factors, including the concentration of agar used in the overlay, which affects phage diffusion. In this study, we standardised both the agar concentration and the volume (as described in Section 3.2) to ensure consistency across all experiments.

The selected plaques were extracted using a sterile 1 mL capacity tip attached to a propipette and placed in 1 mL SM buffer in a microfuge tube. The extracts were left for 20 minutes at room temperature to allow the release of phages from the agar. The extracts were then centrifuged at 8000 RPM (Sorvall RC6 PLUS TM Centrifuge, Thermo Fisher Scientific), and the resulting supernatant was decanted into a new microfuge tube for further analysis.

4.2.5 Purification of extracted phages

Each supernatant was subjected to ten fold serial dilution in SM buffer in a 96-well plate. Dilutions were spotted using the double agar overlay spot essay described by Pallavali et al, with modifications (Pallavali et al., 2017), (as described in Section 3.4). Once the top agar containing PAE had solidified, 10ul of phage dilution using a 10-fold titration range, from $1x10^{1}$ to $1x10^{12}$ PFU/ml, were spotted in previously determined sites on the soft agar. The spots were left to dry out prior to incubation at 37°C overnight. The plates were then analysed for lytic plaque formation.

Dilutions that presented non-convergent plaques were used to conduct a DAOPA (as per section 3.3) to check homogeneous plaque formation (this was considered as purified phages). The process of extracting plaques following spot assay (section 3.4) and DAOPA was repeated until homogenous plaques appeared on the plates considering this outcome as a purified phage.

Purified phages were then propagated to reach high concentrations (approximately $1x10^{9}$ PFU/ml) as explained in section 3.4 and stored in a sealed container protected from light at 4°C until use.

4.2.6 Elimination of replicate isolates

Purified phages isolated from diverse wastewater sources were tested against 10 distinct PAE strains previously isolated from dogs with otitis externa (see Chapter 3, Section 3.7; Appendix 3). These 10 strains were selected from the initial 48 strains based on their antibiotic resistance profiles (see Section 3.2.1). The interactions between each purified phage and the 10 PAE strains were assessed, creating a unique phage-killing profile for each phage.

Phages exhibiting identical killing profiles against the 10 PAE strains were considered as replicates, and only one phage from each profile group was retained for further testing. However, it is acknowledged that phages with similar lytic profiles may still behave differently in combination, particularly when targeting complex bacterial populations. This limitation means that relying solely on the killing profile to determine uniqueness may not fully account for the complexity of phage-bacteria interactions.

To address this, the retained phages were further tested against a broader set of 38 PAE isolates (see Section 3.1.1), bringing the total to 48 distinct PAE strains. Only one representative phage for each killing profile against these 48 strains was retained for continued testing, ensuring the uniqueness of the phages with a higher degree of certainty. Nevertheless, it is important to note that this approach primarily serves as a preliminary screening method and does not provide a definitive measure of phage purity or uniqueness.

For the killing profile tests, the purified phages stored at high concentrations (see Chapter 3) were diluted to a working concentration of 1.10^6 PFU/mL. This concentration was selected based on previous studies using phage cocktails for treating PAE infections, particularly otitis externa in dogs (Hawkins et al., 2010). Despite the robust design of these tests, the possibility of slight variations in phage activity or latent interactions that are not detected during initial screening must be considered when interpreting the results.

4.2.7 Definition of killing capacity and killing range

Killing capacity refers to the ability of a phage to lyse bacterial cells, typically measured by a reduction in bacterial counts. While the formation of clear zones (plaques) in a bacterial lawn is a common way to visualise this interaction, it is not the sole measure of a phage's killing efficiency. Various studies have reported differing degrees of plaque clarity and size, but there is no standardised nomenclature to describe this characteristic (Jurczak-Kurek et al., 2016, Pallavali et al., 2017, Mattila et al., 2015, Alves et al., 2016, Issa et al., 2019).

The killing range of a phage refers to the diversity of bacterial strains that a single phage can infect and lyse. Assessing the killing range is crucial for evaluating a phage's potential efficacy in phage therapy, particularly when the therapy is intended for empirical use against a broad spectrum of bacterial strains (Hyman and Abedon, 2010).

4.2.7.1 Analysis of plaques formed by concentrated isolates

The interaction between the purified phage dilutions ($1x10^6$ PFU/mL) and the bacterial lawns ($1.5x10^8$ CFU/mL) on agar plates resulted in four possible types of bacterial lawn lysis. These lytic effects were classified based on the degree of bacterial clearance and were numbered depending on the phage's ability to lyse the bacteria (Jurczak-Kurek et al., 2016), as shown in Figure 4.1.

[0]: No lysis, indicating the bacteria are resistant to the phage.

[1]: Very turbid lysis, with significant bacterial growth remaining inside the cleared area.

[2]: Turbid lysis, showing a scant layer of bacterial growth within the cleared zone.

[3]: Clear lysis, with no bacterial growth inside the cleared zone.

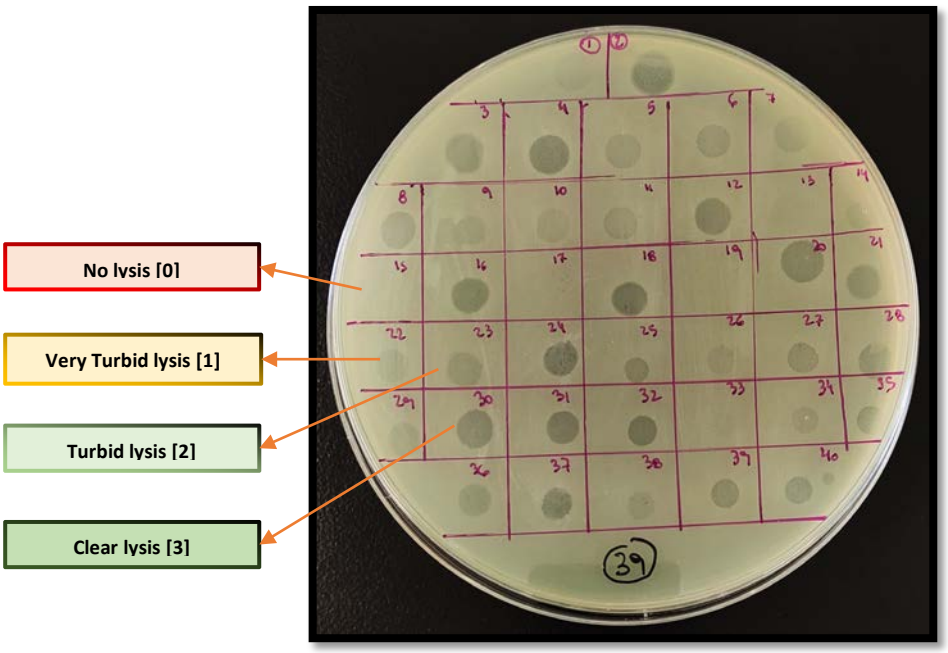

Figure 4.1: Classification of Bacterial Lawn Lysis by Phage Isolates.
This image depicts the results of a Double Agar Overlay Spot Assay, illustrating phage-induced lysis on a *Pseudomonas aeruginosa* lawn (PAE39). Phage interactions produce four types of bacterial lysis: [0]: No lysis, indicating bacterial resistance to the phage. [1]: Very turbid lysis, with visible bacterial regrowth in the cleared area. [2]: Turbid lysis, showing partial bacterial clearance. [3]: Clear lysis, indicating complete bacterial clearance.

For practical purposes in this project, phages were divided into two categories based on their killing capacity. The first category includes phages classified as [0] and [1], which are ineffective at killing the PAE strains tested, indicating minimal or no impact on bacterial growth. The second category includes phages classified as [2] and [3], which display effective killing capacity, significantly reducing or preventing bacterial growth.

4.3 Results

4.3.1 Number of bacteriophages in four sample collection sites.

The number and killing spectrum of bacteriophages after the screening against ten clinical isolates of PAE, from ear infections in dogs, varied depending on the site of origin.

4.3.1.1 Ross River

At The Pontoon (Ross River, Townsville, QLD), samples were collected from specific locations with stagnant water. Animal faeces were observed in the area of sample collection, along with the presence of ducks, other birds, and small fish and insects in close proximity. Vegetation was found both inside and outside the water, in varying states of decay. The water near the shore was murky but became clearer as the distance from the riverbank increased. The sample was taken from an area that contained debris such as dirt, sand, small sticks, and other impurities. After collection, the sample was transported to the laboratory for processing.

A total of 15 bacteriophages were counted (Table 4.3). Only 5 from 10 selected PAEs produced plaques on initial growth, Section 4.2.3, and a small number of plaques were counted in each case. All plaques were large (>5mm), and presented moderate bacterial growth inside the plaque, score 1 (Figure 4.2). The isolation of phages was not possible for this sample. Although phages were detected on the initial screening plates, they were lost during the subsequent isolation steps. The same bacterial strains were used for both phage screening and phage isolation, so the phages should have been able to grow on them. This procedure was repeated twice for each phage extracted from the original agar plate, but the absence of plaques was consistent.

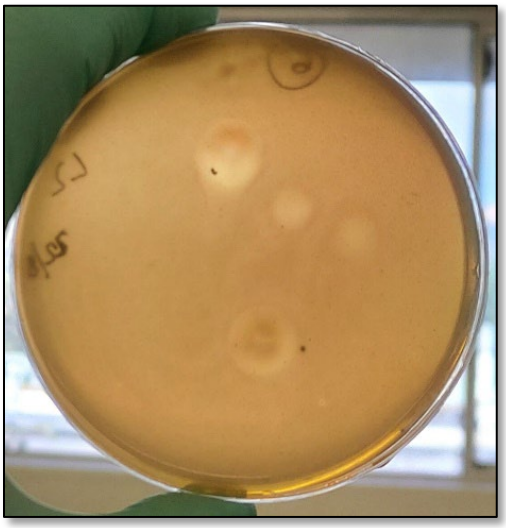

Figure 4.2: Example of plaques observed from Ross River sample. Four plaques on a lawn of PAE formed from environmental sample taken at Ross River.

After unsuccessful attempts to propagate the bacteriophages, a concentration method, ultracentrifugation, was used prior to planting to increase the number of phages that could be found (Section 3.5.1). In spite of this, the same outcome was obtained. For this reason, it was decided to carry on with the sample collection of the next location.

4.3.1.2 Mount St John Wastewater Treatment Plant.

At the second location, Mount St John Wastewater Treatment Plant, a total of 1,196 bacteriophage plaques were counted during the first screening (see Table 4.3). Most PAE strains, except for "PAE9", produced more than ten bacteriophage plaques. For "PAE2", 415 plaques were counted on the bacterial lawn, representing the highest number of plaques observed across all tested sites. PAE4, PAE5, and PAE6 each produced over 100 plaques. The PAE9 strain, however, exhibited resistance to all phages present in the sample, as no plaques were formed on its bacterial lawn.

The morphology of the bacteriophage plaques varied in terms of size, shape, and opacity (Figure 4.4). At this stage of the experiment, it could not be confirmed whether the bacteriophages were replicates or if they belonged to a single species or strain.

4.3.1.3 JCU Veterinary Necropsy Room

Two wastewater samples were collected from different locations at the JCU necropsy room: one from inside the room's drainage system and another from the external wastewater chamber. Both samples were reddish in colour, similar to a cleaning product, "MultiClean," which is used daily to clean the facility.

Screening these samples against the 10 different strains of PAE did not result in the detection of bacteriophages. Despite repeated efforts and testing, no plaques were observed during the screening process, indicating the absence of phages in these samples.

4.3.1.4 The JCU VET General Practice and Referral Hospital

The last location selected for sampling was the JCU VET General Practice and Referral Hospital. A total of 421 bacteriophages were counted (Table 4.3). Of note, the sample type used here was a swab, which was technically a smaller volume than water samples. The majority of plaques were counted on the layer of PAE5 which gave a total number of 292 phage plaques. In regard to PAE1, PAE2, PAE8 and PAE9, no phage plaques were present. Again, at this stage of the experiment, we could not confirm there are not phages belonging to the same strain or species.

Table 4.3: Total number of plaques per location

Pseudomonas aeruginosa strain	Ross River	Mt St John Wastewater Treatment Plant	JCU Veterinary Hospital	JCU Vet Necropsy room
Pae1	7	14	0	0
Pae2	4	415	0	0
Pae3	2	83	37	0
Pae4	0	130	3	0
Pae5	0	128	295	0
Pae6	1	268	27	0
Pae7	0	65	51	0
Pae8	0	28	0	0
Pae9	1	0	0	0
Pae10	0	65	8	0
Total of plaques	**15**	**1196**	**421**	**0**

4.3.1.5 Mount St John Wastewater Treatment Plan – Second sample

In order to increase the number of isolated phages and check for variability, a second sample from Mount St John Wastewater Treatment Plan was taken two months after the first collection. The same 10 PAEs were use for screening (Table 4.4). A total number of 1001 plaques were counted this time. This collection was not compared with other sites since the objective was to discover more phages that could kill those PAE that had few or no phage options for treatment.

Table 4.4: Results from the second sample taken from Mount St John Wastewater Treatment Plan.

	Mt. St. John Wastewater Treatment Plant - 2nd collection -
PAE	**Plaque count**
Pae1	49
Pae2	16
Pae3	124
Pae4	136
Pae5	28
Pae6	416
Pae7	140
Pae8	3
Pae9	0
Pae10	89
TOTAL	1001

4.3.2 Comparison of phage plaque numbers regarding the site of collection

The statistical analysis to compare the means of phage plaques counted at each site of collection was the Kruskal-Wallis statistical test, a nonparametric test that compares three or more unmatched groups. Statistically significant results were those with a p-value of ≤ 0.05. Analytical statistics were performed using GraphPad Prism, version 10 (GraphPad Software).

4.3.2.1 Comparison of phage plaque numbers from Ross River and Mt. St. John

The number of plaques counted from the sample collected at Ross River was significantly lower than the number of phages counted from the sample collected at Mt. St. John (P value = 0.0021) (Figure 4.3).

4.3.2.2 Comparison of phage plaque numbers collected from Ross River and Vet Hospital

The number of plaques counted from the sample collected at Ross River was not statistically significant compared to the number of phages counted from the sample collected at the JCU Vet Hospital (P value= 0.1583) (Figure 4.3)

4.3.2.3 Comparison of phage plaque numbers collected from Mt. St. John and Vet Hospital

The number of phage plaques counted from the sample collected at Mt. St. John was bigger than the number counted at the JCU Vet Hospital. Even though there were notable differences in the counting, the statistical analysis provided a P value of 0.5261, meaning the counting was not statistically significant (Figure 4.3).

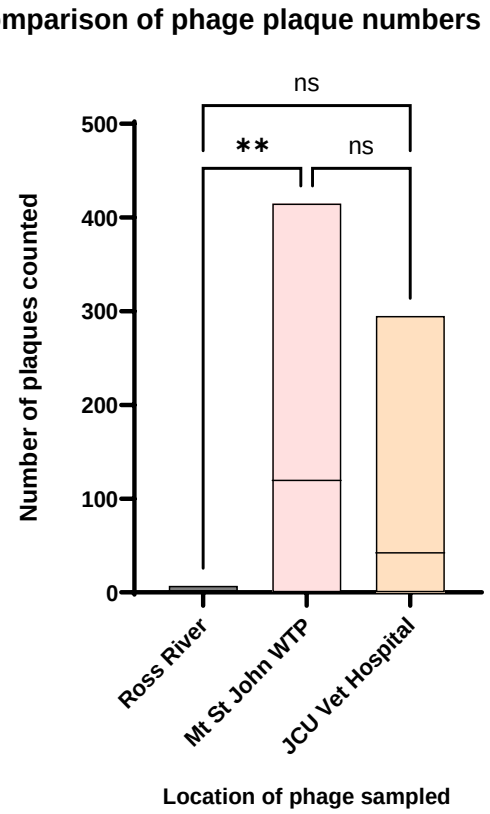

Figure 4.3: Comparison of the means of the phage plaques counted from samples collected from Ross River, Mt. St. John, and JCU Vet Hospital.
Ross River mean=3.0, Mt. St. John mean=132.9, and JCU Vet Hospital mean=70.17. Mean with 95% CI is displayed. P-values: Ross River vs. Mt St John WTP= 0.0021, Ross River vs. JCU Vet Hospital= 0.1583, and Mt St John WTP vs. JCU Vet Hospital= 0.5261.

4.3.3 Bacteriophage isolation

The distinct individual plaques identified in section 4.3.1 were differentiated based on their size and clearness (see Figure 4.4). From each plate containing a PAE strain, five plaques were selected and extracted for further analysis. The selection process prioritised plaques with larger and clearer centres, as these were more likely to represent phages with strong lytic activity.

To clarify, this extraction process was performed for each of the 10 PAE strains tested. For every strain, plates were screened for bacteriophage activity, and five representative plaques were extracted from each positive plate. This allowed for a consistent selection of phages across all samples.

Figure 4.4: Different phage plaque morphotypes on *Pseudomonas aeruginosa* lawns.
Note darker plaques indicating better clearance and paler plaques indicating less clearance. Also, note the size variation in clear plaques, indicating different bacteriophages.

4.3.4 Purification of extracted phage

The process of extracting plaques following spot assay (section 3.4) and DAOPA was repeated until homogenous plaques appeared on the plates (Figure 4.5).

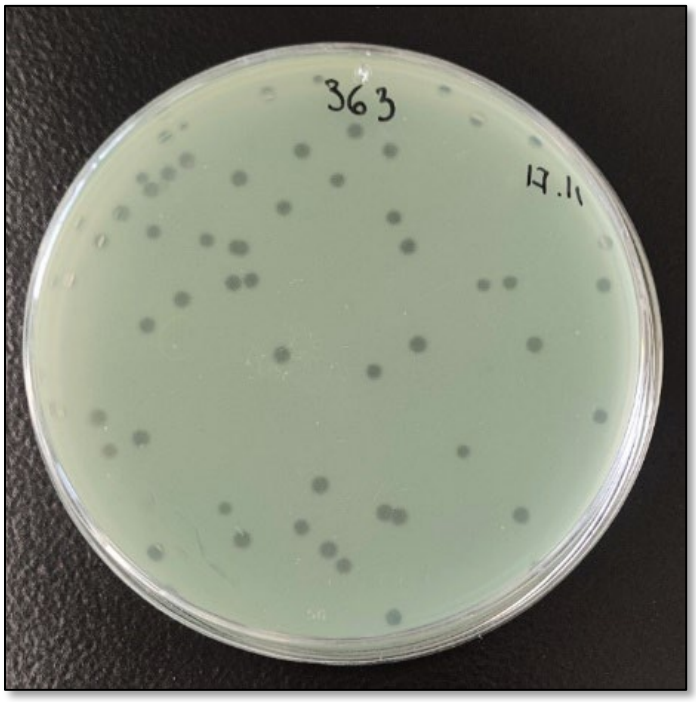

Figure 4.5: Homogeneous plaques on lawn of PAE in LB soft agar. Example used is PAE 7 with phage 24 (Ph24).

4.3.4.1 Elimination of repeated isolates

The initial phage screening was conducted using 10 PAE strains (Table 4.3), creating a killing profile based on these isolates. After this initial screening, the killing profile was expanded to 48 PAE strains. By identifying repeated phage isolates early on with the first 10 PAE, the likelihood of encountering repeated killing profiles during the extended screening with 48 PAE was significantly reduced. This process helped ensure that unique phage isolates with distinct killing profiles were selected for further analysis.

4.3.4.2 Purified phages obtained from collection sites.

Following extraction, purification, and propagation, the repeated phage isolates from distinct collection sites were identified and eliminated. Phages were then labelled with unique identifiers to track their source and ensure no repetition of isolates (Table 4.5).

Table 4.5: Phage labelling and repeated isolates by collection site.

Collection Sites								
Mt St John Wastewater Treatment Plant			JCU Veterinary Hospital			Mt. St. John Wastewater Treatment Plant 2nd collection		
N	Phage label	Repetitions	N	Phage label	Repetitions	N	Phage label	Repetitions
1	Ph1	0	1	Ph8	0	1	Ph11	0
2	Ph2	1	2	Ph9	0	2	Ph25	0
3	Ph3	3	3	Ph10	1			
4	Ph4	0	4	Ph16	0			
5	Ph5	0	5	Ph17	1			
6	Ph6	0	6	Ph18	0			
7	Ph7	0	7	Ph22	2			
8	Ph12	4	8	Ph23	0			
9	Ph13	0	9	Ph24	0			
10	Ph14	0	10	Ph27	0			
11	Ph15	0	11	Ph28	2			
12	Ph19	0	12	Ph29	0			
13	Ph20	1	13	Ph35	0			
14	Ph21	0						
15	Ph26	0						
16	Ph30	3						
17	Ph31	0						
18	Ph32	0						
19	Ph33	0						
20	Ph34	1						

Note: N: The number of isolated phages from each site; Phage label: The unique identifier assigned to each isolated phage; Repetitions: The number of times a phage was repeatedly isolated across different sites.

4.3.5 Killing range of phages isolated from collection sites.

4.3.5.1 Killing range of phages isolated from Mt. St. John Wastewater Treatment Plant.

After the extraction and purification process described in sections 4.2.4 and 4.2.5, a total of 20 phages were successfully purified from this location. These phages were tested against 48 PAE strains, and the

results are displayed in Table 4.6. The killing performance of phages was evaluated based on the formation of plaques, and a scoring system (0 to 3) was used to indicate the degree of bacterial lysis (section 4.2.6.1).

Table 4.6: Killing performance of **bacteriophages isolated from Mt. St. John Wastewater Treatment Plant.**

PAE Strain Tested	Phage Isolates																			
	Ph4	Ph2	Ph12	Ph3	Ph30	Ph21	Ph32	Ph6	Ph31	Ph5	Ph26	Ph7	Ph34	Ph14	Ph19	Ph15	Ph13	Ph20	Ph33	Ph1
PAE 19	3	3	2	3	3	3	3	3	3	2	3	3	3	3	3	3	1	3	2	1
PAE 43	3	3	2	3	3	3	3	3	3	2	2	2	3	3	3	2	2	2	1	1
PAE 33	3	3	2	3	3	3	3	2	3	3	3	1	2	3	3	3	2	2	1	1
PAE 48	3	3	2	3	3	2	3	2	3	2	1	2	2	3	3	2	2	2	2	1
PAE 6	3	3	2	3	3	3	3	2	3	2	3	2	2	3	2	3	1	1	2	0
PAE 40	3	3	2	3	3	2	3	2	3	2	1	2	2	3	3	2	2	2	1	0
PAE 17	2	2	3	2	3	3	3	1	3	1	3	2	2	3	2	3	2	1	0	0
PAE 10	3	3	1	3	3	3	3	3	3	0	3	2	3	0	0	3	0	0	3	1
PAE 16	3	3	1	3	3	3	3	0	2	3	3	0	3	3	3	3	1	0	0	0
PAE 34	3	3	3	2	2	3	3	2	2	2	0	2	1	3	1	2	2	2	0	2
PAE 3	3	2	0	2	3	3	3	3	3	3	3	3	2	0	0	3	0	0	3	0
PAE 29	3	3	0	3	3	3	3	3	3	0	3	3	3	0	0	3	0	0	3	0
PAE 5	0	3	1	3	3	3	3	0	3	3	3	0	2	3	0	3	0	0	0	0
PAE 25	2	2	3	2	0	2	0	3	0	3	3	3	0	2	2	1	2	0	0	0
PAE 7	0	0	3	0	2	1	1	3	2	3	2	3	0	3	0	0	1	0	1	0
PAE 41	2	3	2	3	0	1	0	3	0	3	1	3	2	0	1	0	2	2	0	0
PAE 39	3	3	2	2	2	2	2	2	2	2	0	2	0	1	0	0	0	2	0	1
PAE 32	3	3	2	3	0	2	0	0	0	0	0	0	2	0	2	0	1	2	0	1
PAE 36	3	3	2	3	0	0	0	0	0	1	0	0	2	0	3	0	1	0	0	1
PAE 45	3	3	2	2	0	0	0	1	0	1	0	1	0	0	0	0	2	3	0	1
PAE 1	3	3	1	3	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	3
PAE 8	3	2	1	2	0	0	0	0	0	0	0	0	1	3	3	0	0	0	0	0
PAE 9	1	0	1	0	3	1	2	0	3	0	2	0	0	0	0	1	0	0	0	0
PAE 28	3	3	0	3	0	0	0	0	0	0	0	0	2	0	3	0	0	0	0	0
PAE 31	3	3	0	3	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2
PAE 42	2	2	3	2	0	0	0	0	0	0	0	0	1	0	0	0	1	2	0	0
PAE 23	0	0	2	0	3	2	2	0	3	0	1	0	0	0	0	0	0	0	0	0
PAE 20	3	2	3	2	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
PAE 27	0	0	0	0	0	0	0	3	0	3	0	3	0	3	0	0	0	0	0	0
PAE 21	2	2	1	2	0	0	0	1	0	1	0	3	0	0	0	0	0	0	0	0
PAE 4	0	0	3	0	3	0	0	0	0	0	2	0	0	0	0	0	3	0	0	0
PAE 26	0	0	1	0	0	0	0	2	0	2	0	2	0	1	0	0	1	0	2	0
PAE 24	2	1	3	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	1	1
PAE 38	0	1	3	1	0	0	0	1	0	1	0	1	0	0	0	0	2	0	0	0
PAE 13	3	0	2	0	0	0	0	0	0	0	0	0	1	0	0	0	1	2	0	0
PAE 46	0	1	0	0	1	2	2	0	0	0	2	0	0	0	0	0	0	0	0	1
PAE 12	3	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
PAE 35	0	1	0	0	0	2	0	1	0	0	2	0	0	0	0	0	0	0	0	1
PAE 18	3	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
PAE 11	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
PAE 47	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
PAE 37	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
PAE 22	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total Sensitive Strains [2] - [3] %	**62 %**	**54 %**	**52 %**	**54 %**	**38 %**	**40 %**	**35 %**	**33 %**	**35 %**	**33 %**	**33 %**	**35 %**	**35 %**	**29 %**	**29 %**	**27 %**	**23 %**	**27 %**	**15 %**	**6 %**

Note: In the left column are the PAE strains that have been sorted based on the highest cumulative counts. The degree of lysis is indicated in colours, which are clear or cloudy spots on the bacterial lawn where the phages have killed the bacteria. The colours are: [0 - red]: No plaque formation, [1 - yellow]: Very turbid lysis, [2 - light green]: Turbid lysis, [3 - dark green]: Clear lysis.

4.3.5.1.1 Killing range of phages isolated from Mt. St. John Wastewater Treatment Plant – Second Sample.

A second sample was collected from Mt. St. John wastewater treatment plant at a different period of time. The decision was made to isolate phages that only affect PAE strains that lack phages capable of effectively destroying them. As a result, two phages were successfully purified, as shown in Table 4.7.

Table 4.7: Killing performance of **bacteriophages isolated from Mt. St. John Wastewater Treatment Plant.**

PAE Strain Tested	Phage Isolates	
	Ph25	Ph11
PAE 3	3	3
PAE 41	3	3
PAE 26	3	3
PAE 27	3	3
PAE 19	2	3
PAE 48	3	2
PAE 40	3	2
PAE 34	2	2
PAE 33	2	2
PAE 6	2	2
PAE 7	2	2
PAE 25	2	2
PAE 21	2	2
PAE 43	1	2
PAE 17	1	2
PAE 39	1	2
PAE 37	2	1
PAE 36	1	1
PAE 1	1	1
PAE 45	1	1
PAE 31	1	1
PAE 38	1	1
PAE 32	1	0
PAE 35	1	0
PAE 24	1	0
PAE 46	1	0
PAE 12	1	0
PAE 16	0	0
PAE 29	0	0
PAE 5	0	0
PAE 12	0	0
PAE 13	0	0
PAE 28	0	0
PAE 4	0	0
PAE 18	0	0
PAE 20	0	0
PAE 10	0	0
PAE 11	0	0
PAE 8	0	0
PAE 9	0	0
PAE 42	0	0
PAE 23	0	0
PAE 14	0	0
PAE 15	0	0
PAE 2	0	0
PAE 22	0	0
PAE 30	0	0
PAE 47	0	0
PAE 44	0	0
Total Sensitive Strains [2] - [3] %	30 %	33 %

Note: The table above illustrates the killing range that phages have on different strains of *Pseudomonas aeruginosa* by plaque formation. The degree of lysis is indicated in colours, which are clear or cloudy spots on the bacterial lawn where the phages have killed the bacteria. The colours are: [0 - red]: No lysis, [1 - yellow]: Very turbid lysis, [2 - light green]: Turbid lysis, [3 - dark green]: Clear lysis.

4.3.5.2 Killing range of phages isolated from JCU VET General Practice and Referral Hospital

After the extraction and purification process previously described (4.2.4 and 4.2.5) on selected phages for this location, a total number of 13 phages were effectively purified. The same 48 PAE were used against these 13 phages. The results of these interactions can be seen on Table 4.8. Numbers denoted killing ability of phages against PAE strains, Section 4.2.6.1. In addition, there are only three PAE strains, PAE30, PAE44, and PAE47 (words in red), that are not affected by any bacteriophage isolated from this location (Table 4.8).

Table 4.8: Killing performance of **bacteriophages isolated from** JCU Vet Hospital.

PAE Strain Tested	Phage Isolates												
	Ph18	Ph16	Ph17	Ph28	Ph8	Ph27	Ph9	Ph10	Ph24	Ph29	Ph23	Ph22	Ph35
PAE 19	3	3	3	3	3	3	3	3	3	1	3	3	2
PAE 48	3	3	3	3	1	3	3	2	3	2	3	2	0
PAE 43	3	3	3	3	2	3	2	2	3	3	3	1	0
PAE 34	3	3	3	1	2	3	2	2	3	2	3	3	0
PAE 33	3	3	3	3	1	3	2	2	3	1	1	2	2
PAE 40	3	3	3	3	2	3	2	2	3	1	2	1	1
PAE 6	3	3	3	3	1	3	2	2	3	1	1	2	2
PAE 3	3	3	3	1	2	3	3	3	3	1	3	0	1
PAE 25	3	3	3	2	0	3	2	3	3	2	1	3	1
PAE 10	3	2	3	3	2	2	2	2	2	3	1	2	1
PAE 17	3	3	3	1	2	3	3	1	3	1	3	0	0
PAE 16	3	3	3	1	0	3	2	3	3	1	0	2	2
PAE 29	3	3	2	1	2	0	3	3	0	1	1	2	3
PAE 5	3	3	3	1	0	3	3	0	3	1	3	0	0
PAE 7	3	2	1	2	2	2	1	2	2	1	3	1	0
PAE 32	1	2	3	1	3	2	0	3	0	1	3	0	3
PAE 36	0	3	3	0	3	3	0	3	0	0	3	0	3
PAE 24	3	3	3	0	2	3	2	3	0	0	0	0	0
PAE 1	3	3	3	1	0	2	0	0	3	0	1	0	0
PAE 21	3	2	0	3	0	1	1	0	0	2	0	1	2
PAE 31	3	2	3	1	2	0	2	1	0	1	0	0	0
PAE 12	2	1	0	2	2	1	1	1	1	1	1	0	2
PAE 13	2	3	3	2	0	0	1	0	0	3	0	0	1
PAE 28	1	2	3	1	0	3	0	0	3	1	0	0	0
PAE 4	2	2	2	2	0	2	0	0	2	0	2	0	0
PAE 18	1	1	1	1	2	0	2	3	0	3	0	0	0
PAE 20	2	2	1	2	1	0	2	0	1	1	1	1	0
PAE 41	2	2	0	2	2	0	1	0	0	2	0	2	0
PAE 11	3	3	1	3	0	0	0	0	0	3	0	0	0
PAE 45	2	2	0	2	2	0	1	0	0	2	0	0	0
PAE 8	3	2	0	2	0	0	0	0	0	2	0	0	0
PAE 9	3	1	0	2	2	0	0	0	0	1	0	0	0
PAE 26	2	2	0	2	0	0	0	0	0	2	0	0	0
PAE 27	2	1	0	1	2	0	0	1	0	1	0	0	0
PAE 39	1	1	3	1	0	0	0	0	0	2	0	0	0
PAE 42	2	1	0	2	0	0	0	0	0	2	0	0	0
PAE 23	0	1	2	1	0	0	0	0	0	2	1	0	0
PAE 14	0	1	0	0	2	0	2	1	0	0	0	0	0
PAE 15	2	1	0	2	0	0	0	0	0	1	0	0	0
PAE 38	0	0	2	0	1	0	0	1	0	0	0	0	0
PAE 2	0	0	1	0	1	0	0	0	0	1	0	0	0
PAE 35	0	0	1	0	0	0	1	0	0	0	0	0	0
PAE 37	0	0	0	0	0	0	0	0	0	1	1	0	0
PAE 46	0	0	0	0	0	0	1	1	0	0	0	0	0
PAE 22	0	0	0	1	0	0	0	0	0	1	0	0	0
PAE 30	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 47	0	0	0	0	0	0	0	0	0	0	0	0	0
PAE 44	0	0	0	0	0	0	0	0	0	0	0	0	0
Total Sensitive Strains [2] - [3] %	67 %	62 %	52 %	46 %	42 %	42 %	40 %	35 %	35 %	33 %	25 %	21 %	19 %

The table above illustrates the killing range that phages have on different strains of *Pseudomonas aeruginosa* by plaque formation. The degree of lysis is indicated in colours, which are clear or cloudy spots on the bacterial lawn where the phages have killed the bacteria. The colours are: [0 - red]: No lysis (PAE unaffected by phage), [1 - yellow]: Very turbid lysis, [2 - light green]: Turbid lysis, [3 - dark green]: Clear lysis.

4.3.6 Comparison of phage killing range based on their source location

The sample from Mt. St. John had more phages that were ineffective against PAEs compared to the JCU Vet Hospital sample. Specifically, we observed five PAEs (PAE 15, PAE 2, PAE 30, PAE 14, and PAE 44) in the Mt. St. John sample that were completely resistant to all phages tested (Table 4.6). In contrast, the JCU Vet Hospital sample showed only three PAEs (PAE 30, PAE 47, and PAE 44) that exhibited complete resistance to the phages tested (Table 4.8). Notably, PAE 30 was resistant across both sites, while PAE 40 was resistant only at the Mt. St. John site.

The JCU Vet Hospital produced two phages with a killing range exceeding 60%, meaning these phages were able to lyse more than 60% of the PAE strains tested. Additionally, one phage from this site had a killing range of over 50% based on their killing capacity [2] and [3]. In contrast, Mt. St. John had only one phage with a killing range above 60%, and three phages with ranges over 50% of the PAE strains tested. The majority of the phages isolated from Mt. St. John exhibited a killing capacity below 40%, as shown in Figure 4.6.

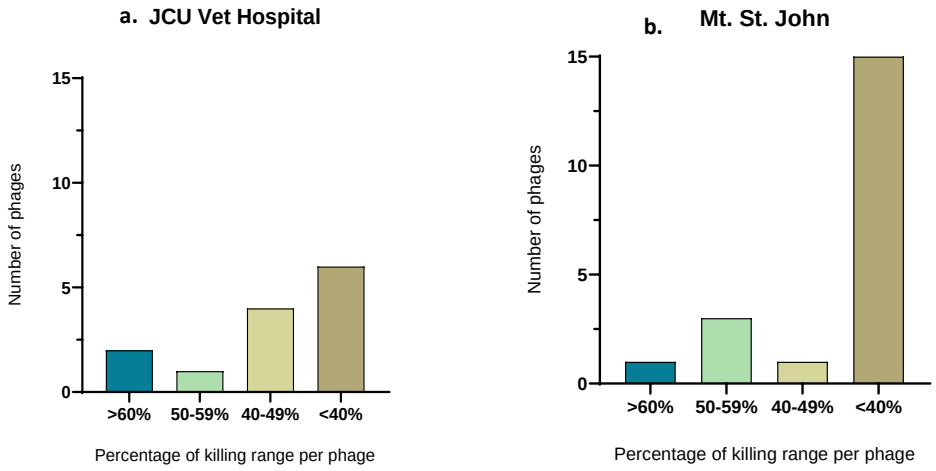

Figure 4.6: The number of phages in each location, categorised by their percentage of killing capacity. a. JCU Vet Hospital, b. Mt. St. John. Note: The data shown in Figure 4.6 were normalised against the different numbers of phages isolated from each site.

4.4 Discussion

4.4.1 Sample collection

Four locations for collecting samples were selected based on their potential to harbour phages that target PAE, which may affect dogs (Diggle and Whiteley, 2020). The selection criteria included factors such as exposure to PAE, presence of animals (including dogs), humidity, and contaminated water sources as stated in the literature (Khan Mirzaei and Nilsson, 2015, Aghaee et al., 2021). Each site was chosen with the aim of isolating lytic phages against specific PAE strains relevant to otitis externa in dogs, considering the critical role of host specificity in phage recovery (Hyman, 2019). Water sources such as the Ross River were preferred due to their high microbial and organic content, which is conducive to phage proliferation (Khan Mirzaei and Nilsson, 2015). However, factors such as agar concentration, multiplicity of infection (MOI), and phage kinetics can significantly influence the number of plaques observed, as evidenced by the variability in plaque counts across different sites (Weinbauer, 2004). Although enrichment procedures were not utilised, the standardised conditions were maintained to minimise variability. It is also crucial to note that the plaques observed may represent a mixture of different phages, necessitating further purification steps to isolate individual phage types accurately.

4.4.1.1 Ross River Sample

The Ross River location was chosen with the intention of finding phages against PAE in water reservoirs of natural environments. Previous studies have shown that rivers in certain parts of the world, such as Germany (Danube River), India (Ganga River, Ganges River), and Siberia (Inya River), contain a large number of phages targeting PAE, especially in highly polluted areas (Rathor et al., 2022, Raman Mishra and Nath, 2020, Knezevic et al., 2009). Some of these environments were highly polluted.

The Pontoon also had evidence of faecal contamination (Section 4.3.1.1), and the minimal phage isolated was therefore unexpected. In addition, the phages from Ross River samples seemed to present a lysogenic-like behaviour. It was evident the presence of bacterial growth inside the plaques and the inconsistent propagation results that has been previously reported (Altamirano and Barr, 2021, Jurczak-Kurek et al., 2016). However, this is not a conclusive indicator. Additional tests such as integrase gene detection or plate replication assay are required to verify lysogenic phages. For the purpose of this study, phages that presented these characteristics were considered potential lysogenic phages. This means that these phages were not suitable as a therapy. Lytic phages to treat PAE infections were required for this project. This location was disregarded from further analysis, given the low number of phages and likely lysogenic behaviour. Although PAE is an environmental bacterium, found in natural waters (Crone et al., 2020), this location in Townsville may not be a suitable location to source PAE phages. The limit of this study was that only one sample was selected from this location during one seasonal period. In addition, further sampling could be done to confirm these characteristics over other seasons and waterflow levels.

On the other hand, the PAE search was not performed on this site either. This is a typical place where these environmental bacteria should be found. Had it been done, it could have been confirmed that its presence is not a limiting factor for finding phages for this bacterium in this place.

4.4.1.2 JCU Veterinary Pathology and Biomedical Sciences

Samples from the JCU Veterinary Pathology and Biomedical Science were collected at two sites, inside (necropsy room) and outside (wastewater chamber) the building.

Upon analysing the samples collected from the two locations in and around the necropsy room, no phages were counted. The frequent use of chemicals, which include microbicidal products, may have prevented the presence of phages in the samples (Table 4.2). The literature refers to the conjunct use of disinfectant and phages as a therapeutic option. But to use the combination to this end the concentration of chemicals must be in lower ranges (Agún et al., 2018, Stachler et al., 2021). Interestingly, the reddish colour of the collected samples resembles the "MultiClean" product, a specific cleaning product used as a detergent in combination with other chemicals to clean the area. The bactericidal properties of some chemicals would destroy the host, preventing the virus from replicating within it. On the other hand, products such as "Trigene II", also used in this room, have virucidal properties. Therefore, the virus could be destroyed by the direct action of these chemicals (Lin et al., 2020, Fernandez et al., 2022). To confirm this hypothesis, further research would be needed to test the effect of these chemicals on viable phages and PAE. The impact of such environmental factors underscores the importance of considering the local conditions and their potential effects on phage viability when selecting sampling sites.

4.4.1.3 Mt St John Sample

The literature suggests that wastewater samples contain a wide range of phages (Mattila et al., 2015, Loc-Carrillo and Abedon, 2011, Aghaee et al., 2021). Therefore, a strategic decision was made to collect samples from the Mount St John Wastewater Treatment Plan. From this site, 1196 plaques were counted using 10 PAE strains for screening as mentioned in Section 4.2.3. Most of the bacterial strains tested were susceptible to at least one of the bacteriophages in the sample, except for "PAE9", which did not show any plaque formation after the DAOPA assay. Since the sampling protocol of this site includes a sample change every 24 hours, explained in 4.3.1, taking a daily aliquot might eventually provide phages capable of attacking all PAE tested. The number of diverse phages isolated from the wastewater treatment plant, in this case, Mt. St. John Wastewater Treatment Plant, is a suitable source of phages against PAE isolated from otitis externa infections in dogs. While primarily sourced from human waste, animal waste can also enter the plant through drainage systems, further contributing to phage diversity.

4.4.1.4 Second Sample from Mt. St. John

A second sample was taken from Mt St John to increase the number of bacteriophages in the stock for PAE strains with limited or no isolated phages, such as PAE1, PAE8, and PAE9. Only plaques from phage-deficient PAE isolates were extracted this time, following the procedure in Section 4.2.3. This sampling allowed a comparative analysis of plaque counts between the two collection times, though this sample was not included in the kill range analysis. Due to time and resource limitations, only two bacteriophages were effectively purified.

Interestingly, PAE9 was the only strain among the ten tested that exhibited an intermediate level of sensitivity to Doxycycline, whereas all other strains (PAE1 to PAE10, excluding PAE9) were resistant to this antibiotic (Appendix 2, Table 2.2). This unique antibiotic profile suggests that PAE9 may possess distinct resistance mechanisms or environmental adaptations, which could also influence its susceptibility to phages. Despite the additional sampling, no phages capable of lysing PAE9 were isolated, indicating that its unique characteristics might contribute to its resilience against both antibiotics and phages. This finding highlights the importance of considering not only the host's environment but also its antibiotic resistance

profile when selecting and interpreting phage isolation results. Further research is required to target environments closely related to dogs to isolate phages that are effective against this particular strain.

4.4.1.5 Veterinary Hospital Sample

Only samples from the JCU Vet Hospital were collected using swabs in transport media, in contrast to the samples collected from Ross River, Mt St John Wastewater Treatment Plant, and the JCU Vet Morgue. This site yielded less material to be processed according to the protocol described in Section 4.2.3, compared to other samples. The total number of plaques counted in this case was 421 PFU/ml for the PAEs used as screening. The samples were diluted tenfold during processing.

It is essential to consider this dilution factor when selecting the collection site and the method for that location, as the sampling method used directly impacts the phage yield and interpretation of results. We compared the actual numbers obtained from the different sampling types to assess their efficacy. In the end, 13 phages were effectively purified from the JCU Vet Hospital samples using the recovery protocol outlined in Section 4.2.3.

4.4.2 Site comparison of phage numbers

The sites that provided more significant number of phages were obtained using two different collection methods (bottle as recipient and swabs with transport media). This allowed Mt St John (bottle as recipient) to provide more phages counted again the same ten PAEs used for screening. In terms of sample potential to obtain phages, and since the results from the Vet Hospital sample were diluted ten times, this represented the most significant sample to be considered for collection. However, in real terms, a more comprehensive range of phages was isolated from the Mt. St. John wastewater treatment plant. In the end, both sites showed to be excellent sources of bacteriophages, and both can be considered for future collections.

Information in the literature was not found specifying the exact number of bacteriophages present at a single site for PAE even though is stated that these locations are a great source of PAE phages (Aghaee et al., 2021). Therefore, this research can serve as a reference point for future studies on this line of research.

On the other hand, when looking for phages that can be used as antipseudomonal agents, it is not advisable to collect samples from natural environments such as Ross River or built environments like the JCU Vet Necropsy room where chemicals are often used for disinfection.

4.4.3 Sites comparison over killing range.

The killing ranges of isolated phages were compared between the two main collection sites: Mt St John Wastewater Treatment Plant and the JCU Veterinary Hospital. Phages isolated from the Veterinary Hospital demonstrated a higher overall killing range against the 48 PAE strains tested, with seven out of thirteen phages exhibiting a range above 60%. In contrast, the majority of phages isolated from Mt St John showed lower killing ranges, with sixteen out of twenty phages presenting less than 40% effectiveness. This suggests that the Veterinary Hospital, due to its direct association with canine hosts, yielded phages better suited for treating otitis externa in dogs.

This discrepancy in phage quality may be attributed to the Veterinary Hospital's unique environment, which directly correlates with the clinical context of PAE infections in dogs. Consequently, phages isolated from such environments may possess a more targeted efficacy. Future studies could benefit from screening

these phages against a broader range of PAE isolates, including those from human infections, to determine if the relative killing ranges change.

The limited phage diversity from the Mt St John samples, despite the high overall count, suggests that while wastewater treatment plants are prolific sources of phages, they may not always yield the most clinically relevant isolates for veterinary applications. As such, collection sites must be carefully chosen based not only on the bacteria of interest but also on the infection context and the habitual environment of the affected animal (Hyman, 2019). Moreover, the analysis of PAEs isolated from the samples collected in this study would provide further insights into the dynamics of phage-host interactions, enhancing our understanding of the host-specificity of isolated phages. Selective enrichment and characterisation of these PAE isolates could be a valuable addition to future studies.

Overall, samples obtained from Mt St John and JCU Vet Hospital are reliable sources of lytic phages that can be used for phage treatments. Based on the collection method, the nature of the patient involved, and the permissions required for sampling, the Vet Hospital is best suited to obtaining bacteriophages that target PAEs causing otitis externa in dogs.

CHAPTER 5: FORMULATION AND OPTIMISATION OF PHAGE COCKTAILS

5.1 Introduction

Phage cocktails are mixtures of different bacteriophages that can target and kill specific bacteria. They are used as an alternative or complementary treatment for bacterial infections, especially those resistant to antibiotics (Oechslin et al., 2017). Phage cocktails have several advantages over single phage therapy, such as broader host range, higher efficacy, and lower risk of bacterial resistance (Ryan et al., 2011). They have been applied in various fields, including medicine, veterinary applications, agriculture, and food safety (Molina et al., 2021, Witzany, 2020).

The rationale for using phages in cocktails stems from the need to enhance the efficacy of phage therapy against *Pseudomonas aeruginosa* (PAE). By combining multiple phages with different bacterial targets, the overall spectrum of bacterial strains that can be lysed is broadened. This reduces the likelihood of bacterial resistance emerging during treatment, as the simultaneous presence of multiple phages makes it more difficult for bacteria to develop resistance to all components of the cocktail (Chan et al., 2013, Kortright et al., 2019). The phages selected for this study are primarily lytic phages, as lytic phages are known for their ability to kill bacteria directly by lysing the host cells. Lysogenic phages, which integrate their genome into the bacterial DNA, were not prioritised in this study due to the potential risk of transferring genes that could enhance bacterial resistance or virulence (Pires et al., 2016). Therefore, only phages with clear lytic activity were selected based on their killing profiles, as described in Chapter 4.

An important consideration when using phage cocktails is the lack of standardisation in the concentration of individual phages used in formulations. This refers to the fact that, across different studies, the concentration of phages within cocktails varies significantly. Some researchers use lower concentrations (Hawkins et al., 2010), while others suggest that higher concentrations can enhance efficacy (Ryan et al., 2011). For instance, phage concentrations in cocktails can range from $1x10^5$ to $1x10^9$ PFU/ml. However, there is no universally accepted concentration that guarantees optimal efficacy. In this study, a concentration of $1x10^6$ PFU/ml was chosen based on previous successful applications and adjusted according to the specific needs of treating PAE.

This work aims to explore the potential of using a large number of lytic phages, previously isolated (Chapter 4), in a cocktail formulation against PAE causing otitis externa in dogs, as well as a control strain of PAE (ATCC 27853). To our knowledge, this is the first study investigating the use of more than 20 phages in a single cocktail to treat a bacterial infection. It is hypothesised that increasing the number of phages in a cocktail can enhance its spectrum and bactericidal activity.

5.2 Materials and Methods

5.2.1 Bacterial strains used for the study

For this study, 48 PAE strains were sourced from the James Cook University (JCU) diagnostic facility culture collection (Appendix 2). Of these, 47 strains were isolated from ear infections in different dogs over a period of 19 years (up to 2024), while one strain, PAE29 - ATCC 27853, was included as a standard control strain. These strains had undergone prior testing for antibiotic resistance and phage susceptibility as detailed in Chapter 4.

To evaluate the phage killing ability of the cocktails, all 48 PAE strains were tested using the spot assay technique described in Section 4.2.7.1. Following this initial screening, three PAE strains were selected based on their phage susceptibility profiles. The selected strains included: A control strain (PAE29 - ATCC 27853), which is a well-characterised strain known for its biofilm-forming capacity and is frequently used in antimicrobial studies (Cao et al., 2017). The use of this strain may allow other researchers to replicate this work. A resistant strain (PAE22), which showed resistance to most phages in the preliminary spot assays (Chapter 4). A sensitive strain (PAE10), which demonstrated a high degree of susceptibility to multiple phages. An intermediate strain (PAE11), which exhibited partial susceptibility to phages, showing a moderate level of lysis in the spot assays.

These selected strains were used in subsequent biofilm assays to assess the efficacy of the phage cocktails in reducing biofilm formation and bacterial activity. Detailed characteristics of each isolate, including their resistance profiles and killing range, are summarised in Table 5.2 (Section 5.2.4).

5.2.2 Cocktail Formation

5.2.2.1 Phage selection for cocktail formulation

Phage selection for cocktail formulation was based on three main criteria: the killing capacity of individual phages, the killing range of each phage, and the potential for combining phages to broaden the spectrum of bacteria targeted.

The killing capacity of individual phages refers to their ability to lyse specific strains of PAE. This was measured using spot assays (Section 4.2.7.1) and phages that produced either clear lysis (with no bacterial growth inside the cleared zone) or turbid lysis (where a scant layer of bacterial growth is observed within the cleared zone), indicating strong and moderate killing capacity, respectively, were prioritised for inclusion in the cocktail.

The killing range refers to the number of bacterial strains a phage can effectively lyse. The killing range was assessed by determining how many PAE strains were lysed by each phage. This is akin to the "host range" described by Hyman and Abedon, where the phage's ability to infect and kill different bacterial strains is influenced by factors such as adsorption resistance, phage-genome uptake blocks, and bacterial resistance mechanisms (Hyman and Abedon, 2010).

The third criterion for phage selection involved combining phages that, together, could maximise bacterial coverage by lysing strains that were resistant to other phages. This approach was intended to ensure that the cocktail had a broader bacterial killing spectrum.

Phages were ultimately selected from the results of the killing performance tests described in Section 4.3.5. The phages were categorised based on their killing efficiency and their ability to target strains not lysed by others.

5.2.2.2 Selection of concentration and number of phages to form the cocktails

The concentration of phages used in this study was set to $1x10^6$ PFU/ml, drawing on findings from two previous studies. Hawkins et al. demonstrated that phage concentrations of $1x10^5$ PFU/ml in cocktails effectively reduced bacterial load (Hawkins et al., 2010), which was initially considered for this study. However, experiments by Ryan et al. showed that higher phage concentrations ($1x10^8$ – $1x10^9$ PFU/ml) resulted in the complete survival of mice treated with phage therapy, even for phages that were less effective at lower concentrations (Ryan et al., 2011). Based on these findings and considering the potential effects of the host immune system, the concentration used in this study was adjusted to $1x10^6$ PFU/ml, a balance between efficacy and safety in a veterinary context.

Multiplicity of Infection (MOI), which refers to the ratio of phage particles to host cells (Abedon, 2016), is an important factor in determining the efficacy of phage therapy. By increasing the phage concentration, the likelihood of more effective bacterial killing increases, particularly for phages with higher MOIs.

Thirty phage solutions, stored at concentrations of $1x10^9$ PFU/ml or higher (Section 4.2.5), were diluted while preparing the cocktails to ensure a final individual concentration of $1x10^6$ PFU/ml per phage in each cocktail. Six phage cocktails were developed by serially adding five phages at a time to create formulations containing 5, 10, 15, 20, 25, and 30 phages. Initially, five phages were added to six 250 ml containers, with one container set aside as the 5-phage cocktail. For the remaining five containers, the volumes of the individual phage solutions were adjusted accordingly, and the volume of SM buffer was reduced to maintain the correct concentration. This process was repeated, progressively adding five more phages to each of the remaining containers until all cocktails were complete. The resulting phage cocktails were stored at 4˚C until use (Table 5.1).

Table 5.1: Phage cocktails composition

Number of phages in the Cocktails	Phage Code (Ph)	Stock Concentration (PFU/ml)	Dilution required to reach $1x10^6$ (PFU/ml)	Phage stock dilutions	Aimed Concentration (PFU/ml) in 50ml	Volume of each phage stock solution added to cocktails (ul)	Total Phage Solutions added (ul)	SM buffer added to reach 100 ml (ml)
5	3	$1x10^{12}$	6	10ul/1ml, 10ul/100ml	$1x10^6$	10	1130	98.87
	2	$1x10^{13}$	7	10ul/10ml, 10ul/100ml	$1x10^6$	10		
	4	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	8	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		
10	10	$1x10^8$	2	1000ul/100ml	$1x10^6$	1000		98.73
	12	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10	140	
	17	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		
15	28	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		98.68
	29	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	30	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		
20	5	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10	50	
	6	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		98.54
	13	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		
25	16	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		97.32
	18	$1x10^{12}$	6	10ul/1ml, 10ul/100ml	$1x10^6$	10		
30	19	$1x10^{12}$	6	10ul/1ml, 10ul/100ml	$1x10^6$	10	140	97.18
	23	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		
	27	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		
	31	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	32	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		
	34	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10	1220	
	9	$1x10^8$	2	1000ul/100ml	$1x10^6$	1000		
	7	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	11	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	14	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		
	15	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10	140	
	21	$1x10^{11}$	5	100ul/1ml, 10ul/100ml	$1x10^6$	10		
	22	$1x10^9$	3	100ul/100ml	$1x10^6$	100		
	25	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		
	26	$1x10^{10}$	4	10ul/100ml	$1x10^6$	10		

5.2.3 Phage cocktail screening on PAE strains

Phage cocktail's host ranges were tested against 48 stored PAE strains by using the spot assay technique described in section 4.2.7.1.

5.2.4 Selection of PAE for use in the Biofilm Assay

Based on the results of the previous screening (Section 5.2.3), three PAE were selected to perform the biofilm assays study: one resistant, one sensitive, and one intermediate to phage cocktails (Table 5.6). One extra PAE, PAE29 (ATCC 27853) was selected as a biofilm control since it is a well-known strain with biofilm-forming capacity (Appendix 2) (Table 5.2).

Table 5.2: *Pseudomonas aeruginosa* selection for biofilm assay.

Pseudomonas aeruginosa	Level of resistance to cocktails	Observations
PAE 10	Sensitive to 6 cocktails	Sensitivity level: high
PAE 11	Intermediate to 1 cocktail (Cocktail 5). Sensitive to 5 cocktails.	Sensitivity level: intermediate
PAE 22	Resistant to 6 cocktails.	Sensitivity level: resistant
PAE 29 (Control: ATCC 27853)	Sensitive to 6 cocktails.	Sensitivity level: high

5.2.5 Development of Biofilm Assay

5.2.5.1 Selection of staining assays

Two assays were performed in combination to assess the impact of phage cocktails on PAE biofilms. These assays included crystal violet staining and the MTT reduction test, as described by Kwiatek (Kwiatek et al., 2017). The observed effects were later compared to those of the positive control, which consisted of biofilms without phage treatment, ensuring that biofilm growth under normal conditions was accurately assessed.

The crystal violet staining, in a biofilm assay study, measures the total biofilm biomass by staining both the extracellular matrix and the bacterial cells attached to the bottom of the wells (Syal, 2017).

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is used to determine metabolic activity of bacterial cells. Briefly, bacteria with an active electron transport system reduce the tetrazolium salt to a water soluble purple formazan product, which can be read by the microplate reader (Ghasemi et al., 2021). The combination of these two assays allows the user to determine both whether biofilm is being lost or inhibited as well as whether bacteria still present are active.

5.2.5.2 Biofilm Formation

The biofilm assay used in this project was modified from three methodologies (Alves et al., 2016, Kwiatek et al., 2017, Walencka et al., 2006). Briefly, four PAE strains were selected based on their varied sensitivity to phage cocktails, as outlined in Section 5.2.4. These strains included PAE10 (high sensitivity), PAE11 (intermediate sensitivity), PAE22 (resistant), and PAE29 (control, ATCC 27853). These strains were chosen to represent a range of responses to phage treatment and to provide a comprehensive analysis of biofilm formation and inhibition. After incubation, the bacterial broths were diluted in LB broth to a final optical density between 0.25 - 0.27 using a spectrophotometer (SPECTROstar® Nano microplate reader) at 570nm, (bacterial concentration $1.5x10^8$ CFU/ml) (Appendix 2).

Biofilms were grown in 96-well plates by adding 200µl of diluted bacterial culture in LB broth for the crystal violet staining and 100µl for the MTT reduction test. In the 96 well plate, the chosen distribution was as follows; Six columns were selected for the later introduction of cocktails, three columns for the positive control (no phage addition), two columns were selected for the negative control (only broth – no bacteria or phage addition), and one empty column for the blank (Figure 5.1). Biofilms were incubated statically for 24 hours at 37°C in a moist environment by covering the wells with a paper towel roll moistened with sterile water and the plate cover.

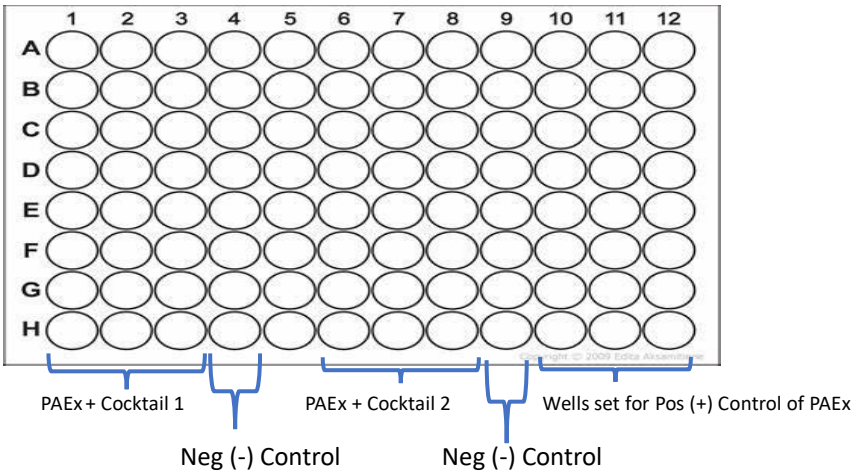

Figure 5.1: 96-well plate layout for biofilm assays. PAEx refers to the PAE strain being tested.

5.2.5.3 Biofilm washing and introduction of phage cocktails

After 24 hours, planktonic cells present in the liquid were removed by pipetting followed by washing twice with 200 µl of PBS (Appendix 1) for the CV protocol and 100 µl for the MTT protocol. Each wash step also had PBS removed by pipetting. These washing and phage cocktail introduction protocols were adapted from Alves et al. and Walencka et al. (Alves et al., 2016, Walencka et al., 2006).

After this point, stored phage cocktails in LB broth were added to their respective columns: 200 µl for the crystal violet staining and 100 µl for the MTT assay. Only supplemented LB broth was added to the negative and positive control. Biofilms were then incubated for 24 hours at 37°C in a moist environment by covering the wells with a moistened paper towel roll and the plate cover. The washing step was repeated prior to starting the staining protocols.

5.2.5.4 Biofilm Crystal Violet Staining

200 µl of methanol (Sigma, Australia) was added to each well and left statically for 15 min. The methanol was then removed by pipetting, and the plates were left to dry at room temperature for 20 min. Next, 200 µl of 0.1% crystal violet (CV) was added to each well and left for 15 min. After staining, the CV was removed from the wells by plate inversion over the sink, and the wells were washed by submersion in three successive containers of tap water. The plates were left to dry at room temperature for 20 min. To extract the biofilm-bound dye, 200 µl of 70% (v/v) ethanol (Sigma, Australia) was added with a multichannel pipette and incubated at room temperature for 10 min. The absorbance was read at 570 nm using a microplate reader. 70% ethanol was used as a blank.

5.2.5.5 Biofilm MTT Reduction Test.

100 µl of MTT solution (0.5 mg/ml in LB medium) (Appendix 1) was added to each well. The microwells were incubated in the dark for 4 hours at 37°C. The MTT solution was then replaced with 100 µl of lysis solution (10% w/v sodium dodecyl sulphate (SDS) in 50% v/v dimethylformamide (DMF)) (Appendix 1) to stop the reaction. The plates were incubated for 30 min in the dark. The absorbance was read at 570 nm in the microplate reader.

5.2.5.6 Confirmation of biofilm production

All four selected PAE strains were used to test both biofilm formation and biofilm measurement. To ensure the biofilms were reproducibly and effectively produced, each PAE biofilm assay was performed three times under the same conditions to confirm the consistency and replicability of biofilm production across experiments.

5.2.6 Statistical analysis for the biofilm assay study.

For the statistical analysis, and considering the nature of the sample, a one-way ANOVA statistical test was chosen to compare the means for each cocktail among each other and against the growth control. Analytical statistics were performed using GraphPad Prism, version 10 (GraphPad Software). A p-value less than or equal to 0.05 was considered significant (Table 5.3).

Table 5.3: Key to values presented by using GraphPad Prism, version 10 (GraphPad Software).

P value	Summary
< 0.0001	****
0.0001 to 0.001	***
0.001 to 0.01	**
0.01 to 0.05	*
≥ 0.05	ns

To evaluate the overall pattern of cocktail efficacy on biofilm biomass and activity, a nested one-way ANOVA was performed using all individual replicate data points obtained from each cocktail tested against each of the four PAE strains. For example, all replicate measurements from cocktail (5) against PAE10, PAE11, PAE22, and PAE29 were included in the analysis to assess trends across the entire dataset.

5.3 Results

5.3.1 Selection of bacteriophages for cocktail formulation

The 48 PAE strains represented one hundred percent of the population tested. The killing range percentage (KR%) indicates the proportion of bacteria a single phage can kill among the PAE population (Table 5.4) and is based on the data in Tables 4.6, 4.7, and 4.8. Bacteriophages were selected according to their lytic ability (scoring 2 or 3) and killing range among these 48 PAE strains, prioritising phages that killed strains not affected by others.

Table 5.4: Phage Selection.

N	Selected Phage	KR%	N	Selected Phage	KR%	N	Selected Phage	KR%
1	Ph3	47%	**11**	Ph5	27%	**21**	Ph34	30%
2	Ph2	47%	**12**	Ph6	27%	**22**	Ph9	40%
3	Ph4	56%	**13**	Ph13	10%	**23**	Ph7	33%
4	Ph8	37%	**14**	Ph16	60%	**24**	Ph11	33%
5	Ph10	43%	**15**	Ph18	53%	**25**	Ph14	26%
6	Ph12	40%	**16**	Ph19	23%	**26**	Ph15	27%
7	Ph17	57%	**17**	Ph23	23%	**27**	Ph21	40%
8	Ph28	30%	**18**	Ph27	40%	**28**	Ph22	21%
9	Ph29	44%	**19**	Ph31	34%	**29**	Ph25	30%
10	Ph30	36%	**20**	Ph32	30%	**30**	Ph26	33%

N: Order number; Phage: Phage name; % in killing range: Number of PAE strains capable of being effectively destroyed by individual phage.

5.3.2 Interaction between phage cocktails and *Pseudomonas aeruginosa* strains.

5.3.2.1 Expected killing range of phage cocktails.

The effectiveness of individual phages was evaluated using spot assays (Section 4.2.5), where phages classified as [2] or [3] demonstrated significant lytic activity. A classification of [2] indicated moderate bacterial lysis, while [3] represented clear lysis, suggesting a higher capacity to kill or inhibit bacterial growth (Section 4.2.7.1). These classifications were used to select phages for the cocktail formulation based on their lytic ability and coverage across the 48 PAE strains tested.

In this section, Table 5.5 presents the expected killing range of the phage cocktails, which estimates the combined effect of multiple phages working together. Unlike the individual lytic profiles shown in Chapter 4, the data here reflects the expected impact of the cocktail combinations, aiming to achieve broader bacterial coverage by exploiting the additive effects of different phages within the formulation.

Table 5.5: Expected killing range after phage selection for cocktails.

PAE Strains Tested	Phage Cocktails					
	Cocktail (5)	Cocktail (10)	Cocktail (15)	Cocktail (20)	Cocktail (25)	Cocktail (30)
PAE 29 (ATCC 27853)						
PAE 48						
PAE 43						
PAE 33						
PAE 40						
PAE 6						
PAE 3						
PAE 25						
PAE 10						
PAE 16						
PAE 19						
PAE 20						
PAE 32						
PAE 36						
PAE 24						
PAE 1						
PAE 21						
PAE 31						
PAE 12						
PAE 13						
PAE 28						
PAE 4						
PAE 18						
PAE 17						
PAE 7						
PAE 41						
PAE 34						
PAE 11						
PAE 5						
PAE 45						
PAE 8						
PAE 9						
PAE 26						
PAE 27						
PAE 39						
PAE 42						
PAE 23						
PAE 14						
PAE 15						
PAE 38						
PAE 2						
PAE 35						
PAE 37						
PAE 46						
PAE 22						
PAE 30						
PAE 47						
PAE 44						

The table above illustrates the potential killing range that phages cocktails would have on different strains of *Pseudomonas aeruginosa* by plaque formation. The degree of lysis is indicated in colours, which are clear or cloudy spots on the bacterial lawn where the phages have killed the bacteria. The colours are: [0 - red]: No plaque formation, [1 - yellow]: Very turbid lysis, [2 - light green]: Turbid lysis, [3 - dark green]: Clear lysis.

5.3.2.2 Actual killing range of cocktails.

The spot assays of each cocktail against each PAE strain are shown in Table 5.6. The cocktail with 5 phages in the formulation was able to effectively destroy 63% (30/48) of the PAE strains tested, the cocktail with 10 phages eliminated 73% (35/48) of PAEs tested, the cocktail with 15 phages eliminated 71% (34/48) of

PAE tested, the cocktail with 20 phages, 77% (37/48), the cocktail with 25 phages also reached a killing range of 77% (37/48), and the cocktail with 30 phages decreased to 64% (31/48) of killing range. There was variation in the actual results from the expected results, primarily with more of the PAE strains being less sensitive to the cocktails than expected. Of note, see PAE strains 7, 11, 27, and 38 as examples of this.

Table 5.6: Interaction between phage cocktails and *Pseudomonas aeruginosa* strains.

PAE Strain Tested	Phage Cocktails					
	Cocktail (5)	Cocktail (10)	Cocktail (15)	Cocktail (20)	Cocktail (25)	Cocktail (30)
PAE 29 (ATCC 27853)						
PAE 48						
PAE 43						
PAE 33						
PAE 40						
PAE 6						
PAE 3						
PAE 25						
PAE 10						
PAE 16						
PAE 19						
PAE 20						
PAE 32						
PAE 36						
PAE 24						
PAE 1						
PAE 21						
PAE 31						
PAE 12						
PAE 13						
PAE 28						
PAE 4						
PAE 18						
PAE 17						
PAE 7						
PAE 41						
PAE 34						
PAE 11						
PAE 5						
PAE 45						
PAE 8						
PAE 9						
PAE 26						
PAE 27						
PAE 39						
PAE 42						
PAE 23						
PAE 14						
PAE 15						
PAE 38						
PAE 2						
PAE 35						
PAE 37						
PAE 46						
PAE 22						
PAE 30						
PAE 47						
PAE 44						
Total Sensitive Strains ([2] - [3])	**63% (30)**	**73% (35)**	**71% (34)**	**77% (37)**	**77% (37)**	**64% (31)**
Total Resistant Strains ([0] - [1])	**37% (18)**	**27% (13)**	**29% (14)**	**23% (11)**	**23% (11)**	**36% (17)**

The table above illustrates the killing range that phage cocktails have on different strains of *Pseudomonas aeruginosa* by plaque formation. The degree of lysis is indicated in colours, which are clear or cloudy spots on the bacterial lawn where the phages have killed the bacteria. The colours are: [0 - red]: No plaque formation, [1 - yellow]: Very turbid lysis, [2 - light green]: Turbid lysis, [3 - dark green]: Clear lysis. PAEs that appear in bold letters were selected for the biofilm assay.

5.3.3 Selection and confirmation of biofilm formation for selected *Pseudomonas aeruginosa* strains.

Based on the results showed in Table 5.6, the following PAE strains were selected for biofilm formation: PAE10, PAE11, PAE29, and PAE22. These strains were chosen because they produced biofilms that were suitable for testing with both the crystal violet staining and the MTT reduction assay, as previously noted in Section 5.2.4. PAE29 served as the control strain due to its well-established biofilm-forming capacity (Figure 5.2).

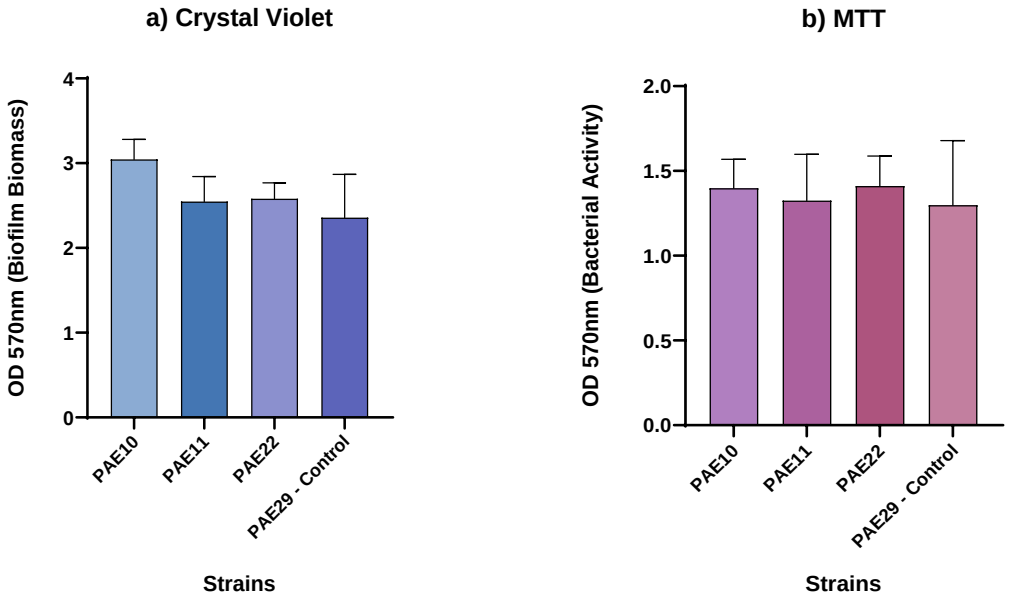

Figure 5.2: Level of biofilm biomass and activity produced by four strains of PAE over a 24-hour period, as measured by OD at 570 nm using both a) crystal violet staining and b) MTT assay (Section 5.2.4). PAE29 was used as the control strain to benchmark biofilm formation.

5.3.4 Effect of phage cocktails on *Pseudomonas aeruginosa* growth in biofilm assays.

To examine the effects of different phage combinations on biofilms, phage cocktails consisting of 5, 10, 15, 20, 25, and 30 phages were tested against the selected biofilm-producing PAE strains (Section 5.2.4). Crystal violet and MTT assays were used to determine biomass reduction and bacterial activity reduction, respectively. The growth control, representing 100% biofilm production, was used as a reference to compare the reduction in biofilm biomass and activity after the application of phage cocktails.

The following sections highlight the interaction of phage cocktails with four specific PAE strains, each selected for their distinct biofilm production characteristics: PAE10 (high sensitivity to phage cocktails), PAE11 (moderate sensitivity), PAE29 (intermediate sensitivity and control strain), and PAE22 (resistant). This approach allows for a detailed analysis of the different effects the phage cocktails had on biofilm biomass and activity for each strain, demonstrating the variability in responses.

5.3.4.1 Effect of cocktails against PAE 10

PAE10 was selected due to its high sensitivity to phage cocktails based on the results obtained in section 5.3.2.2, making it an ideal strain for assessing the efficacy of different phage combinations on biofilm reduction. After 24 hours of phage cocktail application, cocktail (5) vs the growth control showed no statistically significant difference, indicating that this cocktail did not improve treatment efficacy in reducing biofilm biomass in the *in-vitro* model.

However, cocktails (10) to (30) exhibited significant reductions in biofilm biomass compared to the control (Figure 5.3A). The activity analysis revealed a similar effect when comparing the cocktails to the biofilm activity control (Figure 5.3C). All cocktails significantly reduced biofilm activity in comparison to the control.

When comparing the cocktails among each other, cocktail (5) vs cocktails (10) to (25) were statistically significant, suggesting that increasing the number of phages in the cocktail formulation significantly enhanced the efficacy of the treatment in the *in-vitro* model. Cocktails (10) to (25) did not differ significantly from one another, but cocktail (30) showed a significantly higher biomass than cocktail (25), indicating it was less effective (Figure 5.3B). In terms of activity, cocktail (30) did not differ significantly from cocktails (10) to (25), indicating that although biomass was not reduced, bacterial activity was still inhibited (Figure 5.3D).

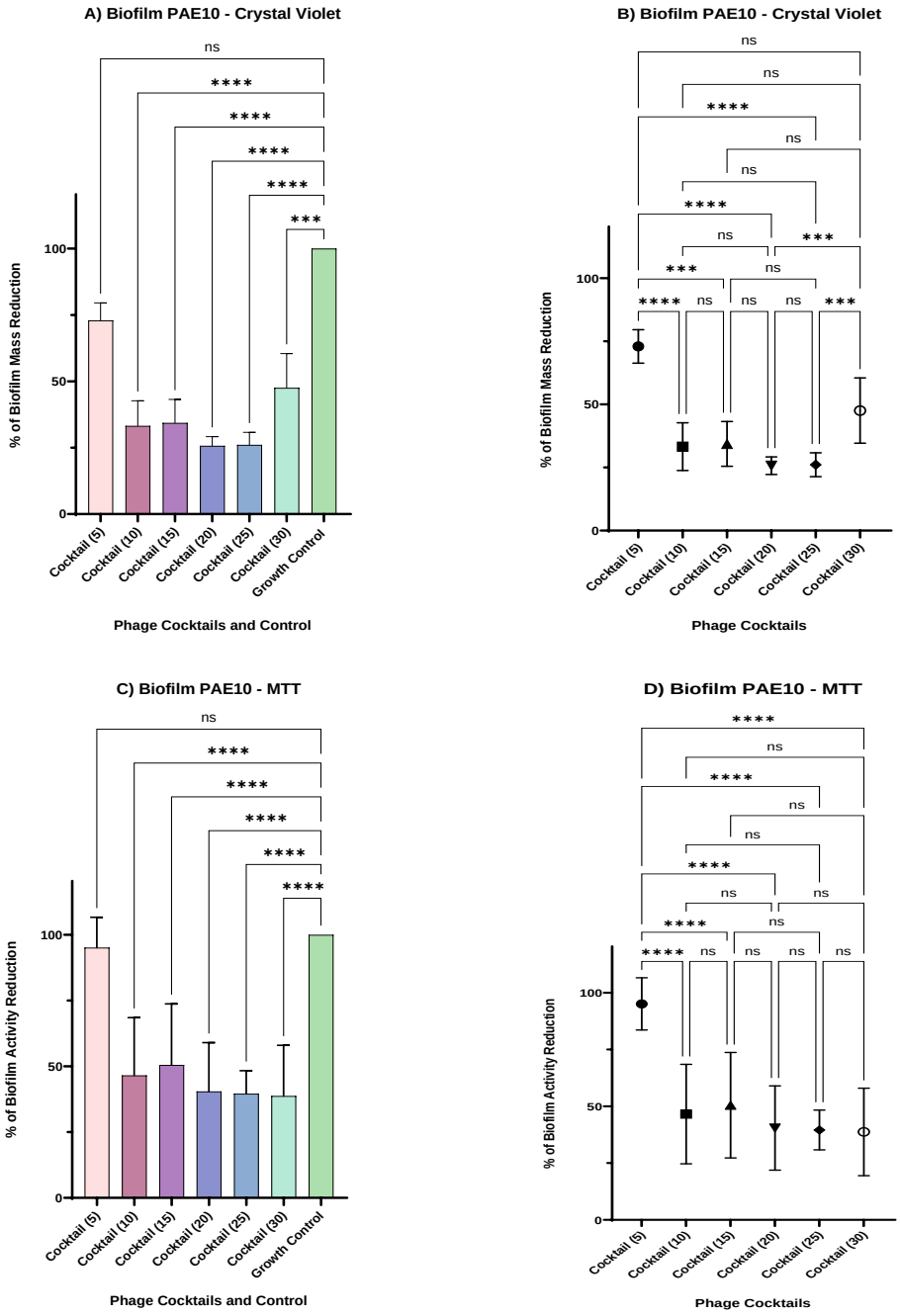

Figure 5.3: A) Comparison between cocktails and growth control (100%) in percentage of biofilm biomass for PAE10, B) Comparison between cocktails in percentage of biofilm biomass for PAE10, C) Comparison between cocktails and growth control in percentage of biofilm activity for PAE10, D) Comparison between cocktails in percentage of biofilm activity for PAE10.

5.3.4.2 Biofilm biomass and activity reduction for PAE 11

PAE11, like PAE10, was considered sensitive to all cocktails (Section 5.2.4). Compared to PAE10, the biofilm biomass reduction for PAE11 was also significant for cocktails (5) to (25) in comparison to the control (Figure 5.4A). However, unlike with PAE10, cocktail (30) did not produce a significant reduction in biomass compared to the control.

The activity reduction for all cocktails significantly reduced biofilm activity compared to the control (Figure 5.4C). When comparing the cocktails among each other, cocktail (5) was not significantly different from cocktail (10) in terms of biomass reduction for PAE11. There was a steady decrease in biomass reduction up to cocktail (30), which had a significantly higher biomass than all other cocktails, indicating its reduced efficacy.

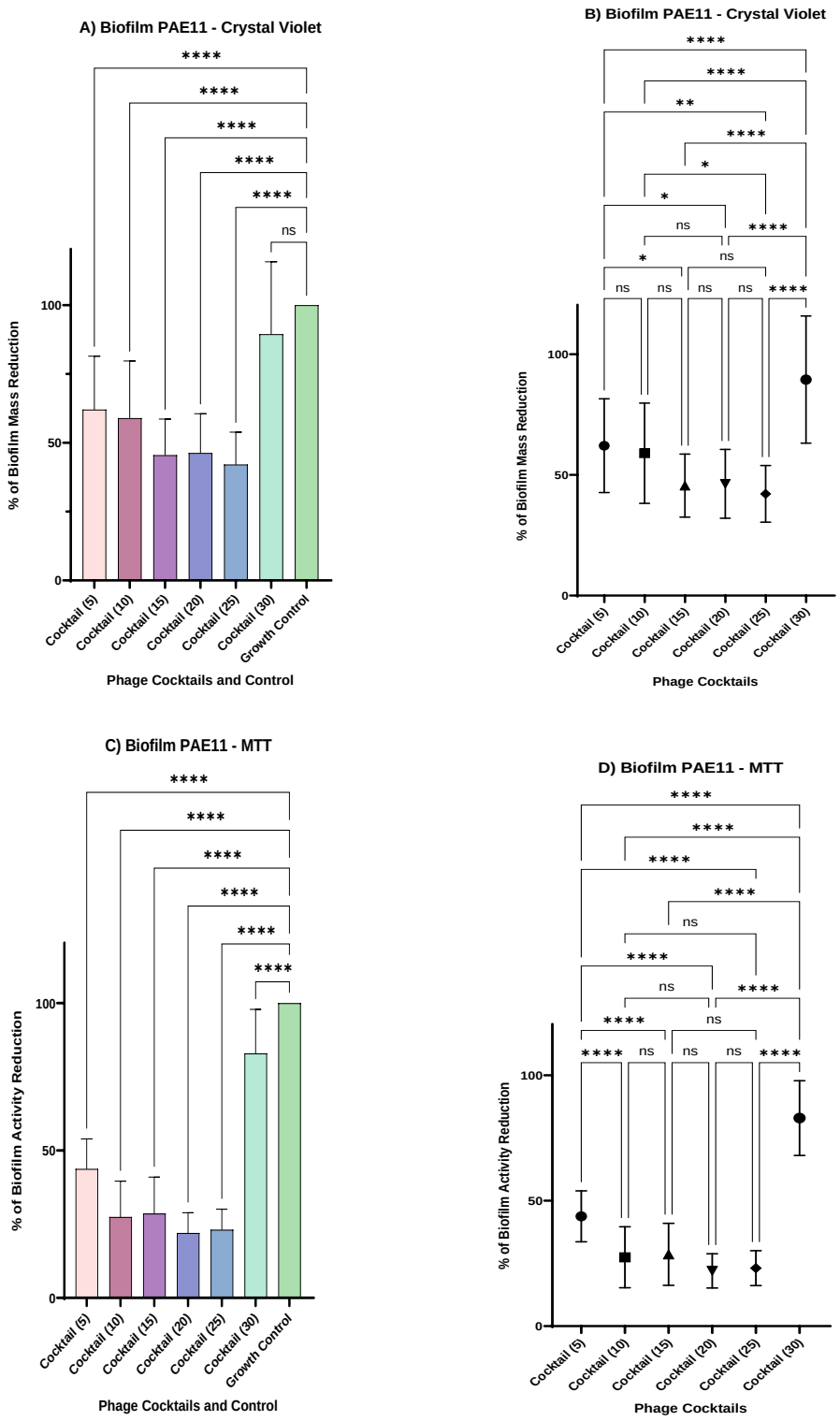

Figure 5.4: A) Comparison between cocktails and growth control (100%) in percentage of biofilm biomass for PAE11, B) Comparison between cocktails in percentage of biofilm biomass for PAE11, C) Comparison between cocktails and growth control in percentage of biofilm activity for PAE11, D) Comparison between cocktails in percentage of biofilm activity for PAE11.

5.3.4.3 Biofilm biomass and activity reduction for PAE 29

PAE29, considered to have an intermediate level of resistance to the phages tested, showed a significant reduction in biofilm biomass when compared to the growth control, following a similar pattern to PAE10 (Figure 5.5A). However, the numerical percentage reduction was lower than that observed for PAE10.

The activity analysis for PAE29 mirrored the trend of biomass reduction, where cocktail (10) and cocktail (25) produced similar reductions in biofilm biomass, with the percentage of activity reduction being higher than that of biomass (Figure 5.5C). A statistically significant difference between cocktails (25) and (30) was observed for biomass reduction, while the reduction in biomass for cocktail (30) was not statistically significant compared to most other cocktails (Figure 5.5B).

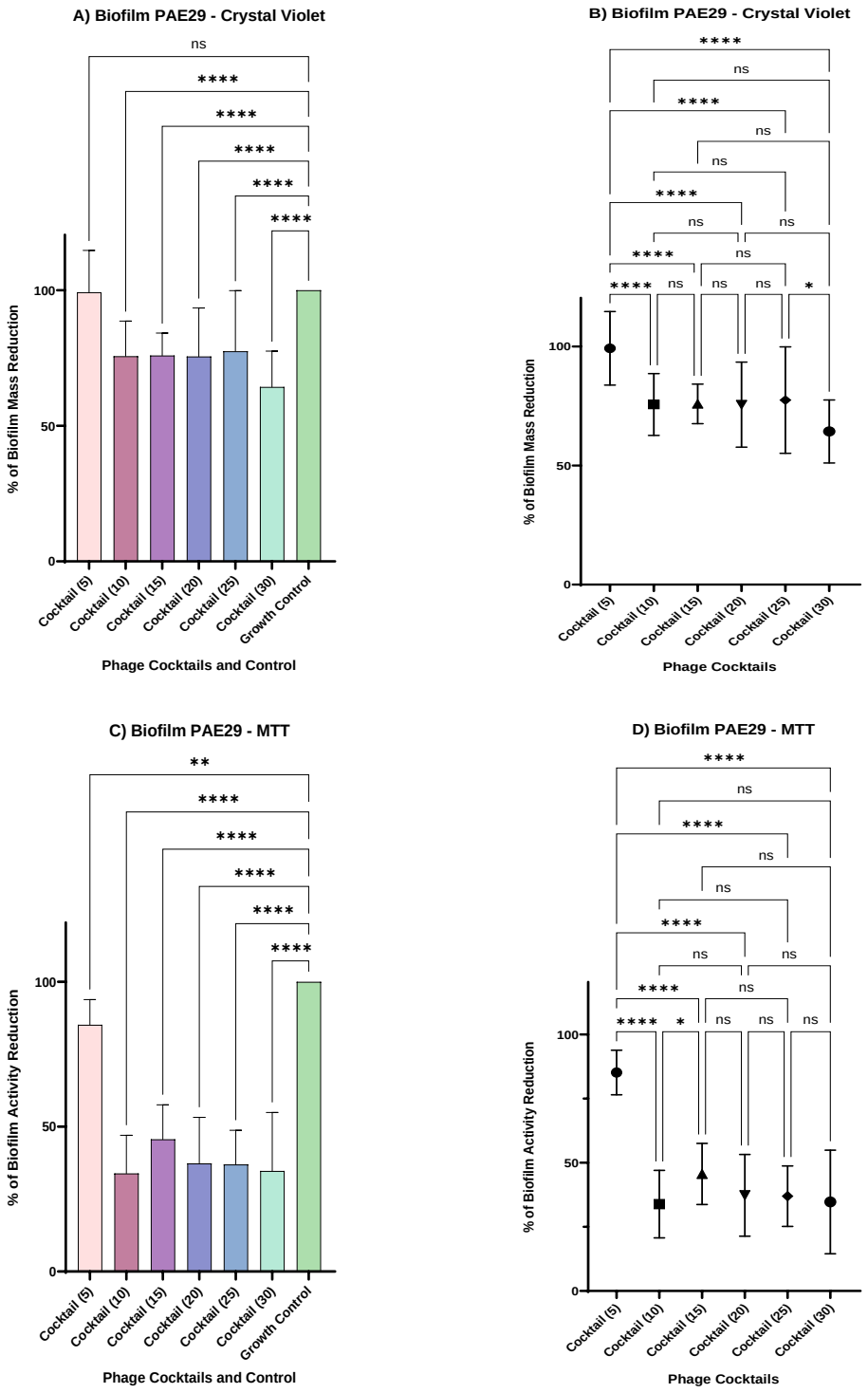

Figure 5.5: A) Comparison between cocktails and growth control (100%) in percentage of biofilm biomass for PAE29, B) Comparison between cocktails in percentage of biofilm biomass for PAE29, C) Comparison between cocktails and growth control in percentage of biofilm activity for PAE29, D) Comparison between cocktails in percentage of biofilm activity for PAE29.

5.3.4.4 Biofilm biomass and activity variation for PAE 22

PAE22, which was resistant to all phage cocktails in the spot assay, exhibited a unique response in the *in-vitro* biofilm assay. A small but significant reduction in biomass was observed with cocktails (10) and (15). However, cocktails (20) and (25) resulted in a significantly larger biofilm biomass formation compared to the positive control (Figure 5.6A). This effect was unique to PAE22 and was not observed in any of the other PAE strains.

The activity reduction showed a similar trend to previous strains, although the percentage reduction in biofilm activity for PAE22 was much lower (Figure 5.6C). Comparisons among cocktails revealed a sharp increase in biofilm biomass production with cocktails (20) and (25), in contrast to cocktail (15). A similar pattern was observed for biofilm activity.

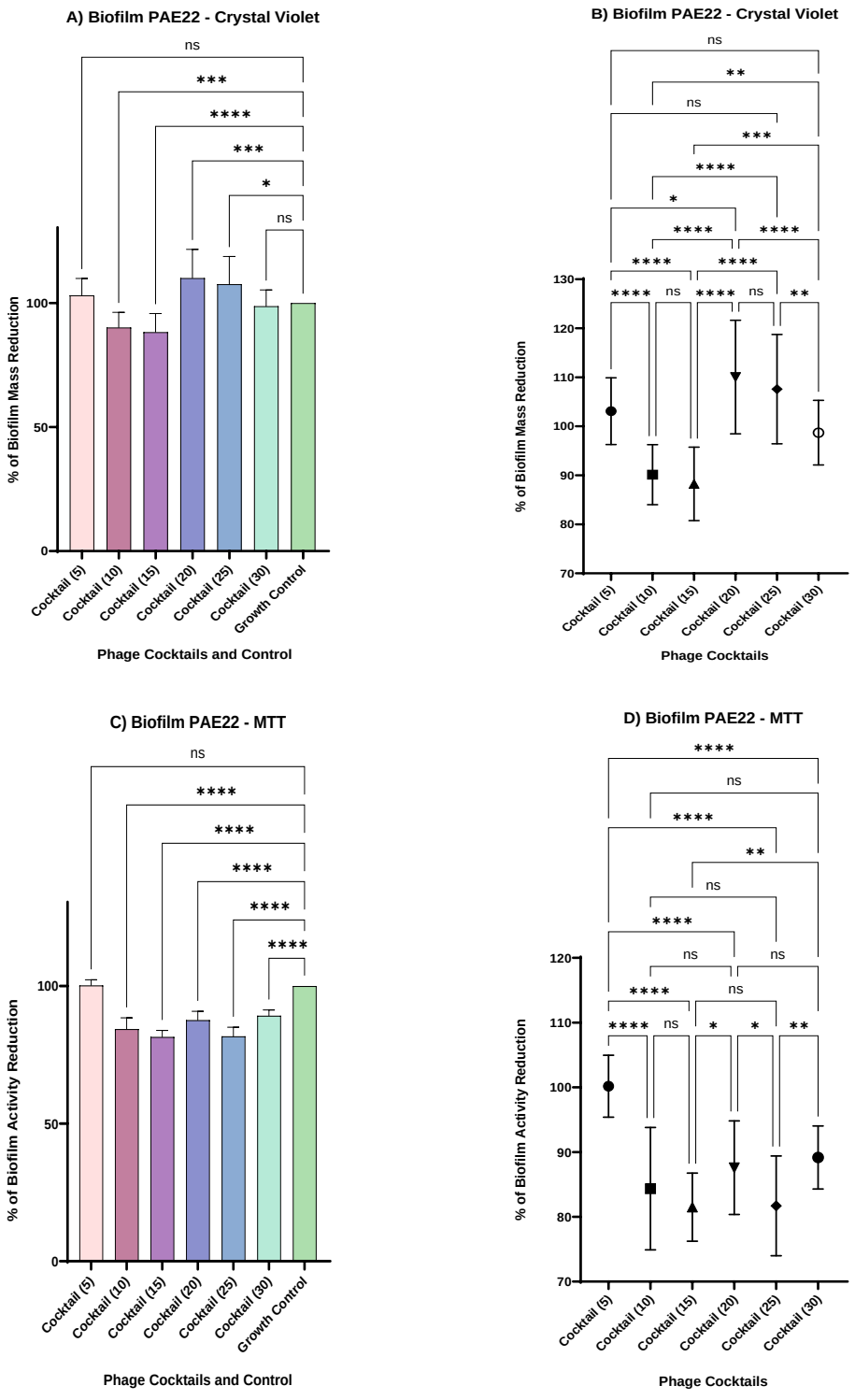

Figure 5.6: A) Comparison between cocktails and growth control (100%) in percentage of biofilm biomass for PAE22, B) Comparison between cocktails in percentage of biofilm biomass for PAE22, C) Comparison between cocktails and growth control in percentage of biofilm activity for PAE22, D) Comparison between cocktails in percentage of biofilm activity for PAE22.

5.3.4.5 Overall effect of cocktails on biofilms

The overall trend observed across individual PAE strains was that cocktails, starting with cocktail (5), had some effect on biofilm biomass and activity, with an increased effect seen at cocktail (10), followed by a reduction that remained stable within a defined range until cocktail (30), (Figure 5.7-A). To determine whether this general trend was statistically significant, a nested one-way ANOVA was performed using all individual data points obtained from each cocktail against each of the four PAE strains tested. Although each strain was tested independently, the statistical analysis was used to identify patterns in biofilm reduction across the four strains tested.

Regarding biofilm mass reduction, the comparison between cocktails and the growth control yielded statistically significant results (Figure 5.7-B). This indicates that the use of phage cocktails consistently reduced biofilm biomass in the tested strains.

There was a significant reduction in biofilm biomass and activity when comparing the cocktail (5) vs cocktails (10) to (30). There was no statistically significant difference between cocktails (10) to (25), but cocktails (10), (15), (20), and (25) showed a statistically significant reduction compared to cocktail (30) and the growth control (Figure 5.7).

Three critical segments were identified: 1) A minimum number of phages required to initiate biofilm reduction in biomass and activity, 2) A sufficient number of phages that achieved the maximum biofilm reduction or plateau, and 3) An excessive number of phages that can cause rebound effect on biofilm growth.

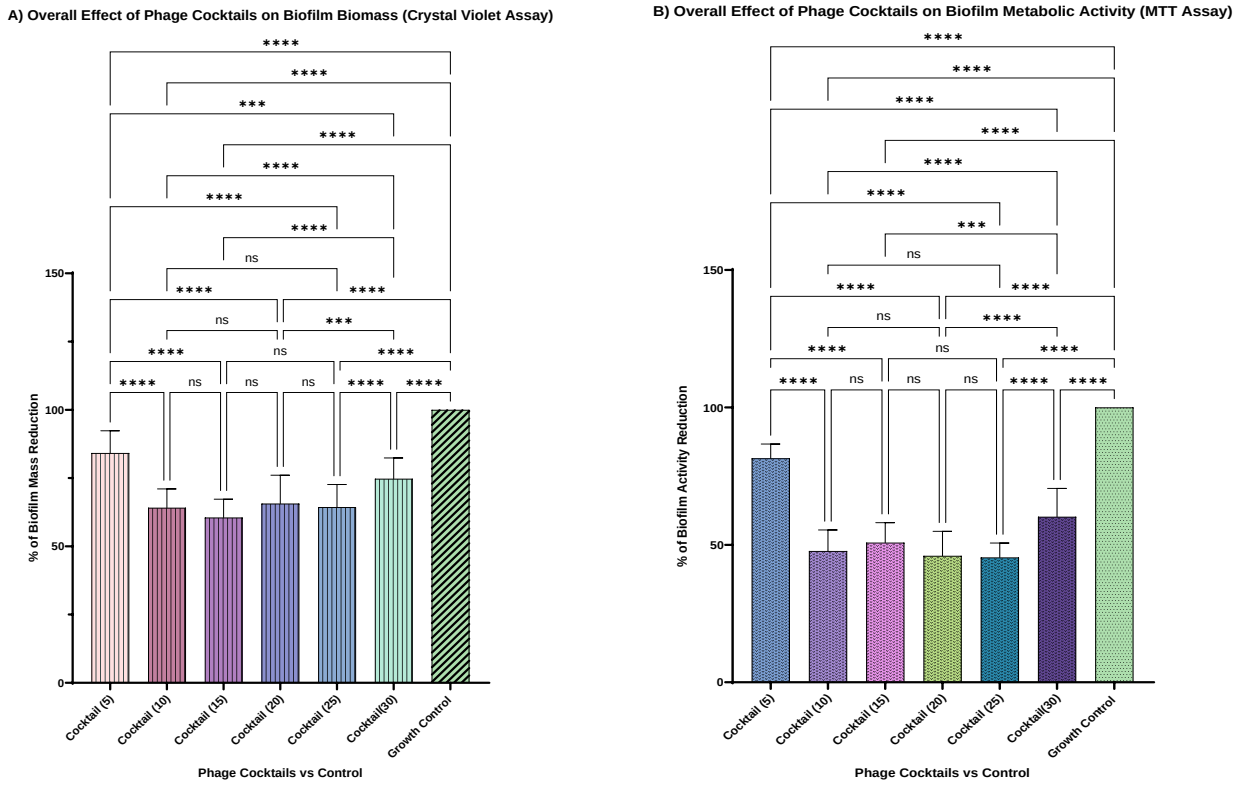

Figure 5.7: A) Comparison in the percentage of biofilm biomass formation between the average values of cocktails and growth control, B) Comparison in the percentage of biofilm activity between the average values of cocktails and growth control.

5.4 Discussion

Bacteriophage selection for cocktail preparation

The phages purified in Chapter 4 were used to create cocktails and proceed with the biofilm assay. A selection of the most effective phages was carried out based on their characteristics. Phages were grouped into cocktails with an increasing number of phages in each formulation.

To determine which phages to include in the cocktails, several factors were considered: phage killing capacity (the phages' ability to kill individual strains), range of killing (how many PAE strains they killed), and the potential combination of different phages that could broaden the spectrum of bacteria targeted and killed when used together. However, the ability of combined phages to broaden the bacterial killing spectrum could not be determined a priori and was therefore confirmed experimentally as part of this study.

Phages were classified into four categories based on their killing capacity. Phages with classifications of [2] and [3] were considered to have effective killing capacity, meaning they could prevent or reduce the bacteria's development. Since the ultimate goal of phage selection was to create cocktails for potential *in-vivo* use, phages with classifications [2] and [3] were prioritised, as they showed higher potential for clinical efficacy.

Regarding the range of killing, Ph16 had a killing range of 60%, Ph17 at 57%, and Ph4 at 56%. Conversely, less effective phages such as Ph13 and Ph22 had killing ranges of only 10% and 21%, respectively. These findings align with some literature reports, where investigators select the most potent phages for experimentation, showing killing ranges starting from 40% (Li et al., 2010).

The third criterion for the cocktail formulation was to select phages that could broaden the bacterial spectrum targeted and killed. Some phages had a narrow killing range, such as Ph13, Ph19, Ph22, and Ph23. Certain bacterial strains, such as PAE44, PAE37, and PAE30, were resistant to all phages used, and combining phages did not improve the cocktail's efficacy against these strains. Further sampling and screening for new phages using the double agar assay could potentially address this issue.

Formation of cocktails considering the number in the formulation

Once the working concentration and phages to be used were defined, cocktails were created. Most studies involving phage cocktails typically use 2 to 5 phages (Jaiswal et al., 2013, Jennes et al., 2017, Duplessis et al., 2018, Regeimbal et al., 2016). We began with a cocktail containing 5 phages, then incrementally added 5 different phages to each new formulation until reaching a final cocktail containing 30 phages. Due to project limitations, 30 phages were the upper limit. Literature indicates that 12 phages have been used in a cocktail to treat burn wound infections in humans (Jault et al., 2019).

Cocktails against 48 PAE strains on plates

The interaction between the phage cocktails and 48 stored PAE strains was analysed. Forty-seven of the strains were clinical samples, and one strain, ATCC 27853, was used as a bacterial control. ATCC 27853 is a well-known strain used globally to test antibiotic efficacy, biofilm development, and metabolic activities of *Pseudomonas* spp. It was originally obtained from a human blood sample in 1971 (Cao et al., 2017). ATCC 27853 can serve as a control organism for future phage cocktail testing or as a benchmark in experiments

that examine changes in antibiotic sensitivity following serial phage application (Gordillo Altamirano et al., 2021).

When comparing the actual clearance of bacteria (Table 5.6) in the spot assay to the expected clearance (Table 5.5), the results showed that some phage combinations were less effective than individual phages. For instance, some individual phages that produced a level [3] clearance when tested alone only produced level [2] clearance when used in a cocktail. This highlights the need to consider more factors when creating phage cocktails, as an excessive number of phages could potentially reduce the overall effectiveness.

Conversely, synergistic effects were observed with certain combinations. For example, phages that individually had no effect on PAE11, PAE23, and PAE15 showed some level of clearance when combined in cocktail (5). Similar synergistic interactions were observed in previous studies, where one phage created optimal conditions for another to increase bacterial killing efficiency (Schmerer et al., 2014, Gu et al., 2012).

It was also observed that the optimal number of phages for bacterial clearance in the spot assay was 15. Increasing the number to 30 phages decreased bacterial clearance, suggesting that phage cocktails have a saturation point beyond which additional phages interfere with bacterial killing (Molina et al., 2022).

Cocktails against four PAE strains in biofilms

After the spot assay results, three clinical PAE strains with varying levels of resistance were randomly selected, along with ATCC 27853, for biofilm assays (Section 5.2.4). The effects of phage cocktails on biofilm biomass and activity were analysed.

For PAE10, PAE11, and PAE29, an initial reduction in both biomass and bacterial activity was observed with cocktail (5). However, the addition of more phages in cocktails (10) to (25) significantly improved the reduction in biofilm biomass, with cocktail (10) producing the greatest reduction in PAE10. There was no statistical difference between cocktails (10) to (25), indicating that increasing phage numbers beyond 10 did not enhance the efficacy in the *in-vitro* model. This could be due to the saturation of bacterial receptors by phages, as reported by Knezevic and Petrovic (Knezevic and Petrovic, 2008).

The addition of 30 phages, however, resulted in less biofilm reduction, which might be due to competition between phages for bacterial receptors, causing a rebound effect (Worley-Morse et al., 2014). This suggests that cocktails with more than 15 phages may not provide additional benefits and could even reduce efficacy. One possible explanation for this outcome is that at higher concentrations, phages exhibit lysis inhibition, which causes a delay in the release of phages (Young, 1992). We hypothesise the rebound effect in our cocktail may be related to a competition between different phages at increased numbers of different phages, particularly if they are competing for the same receptors. This outcome, along with the stable biofilm inhibition seen from 10 to 25 phages, provides a basis for optimising a broad-spectrum cocktail. Phages cannot be added beyond a certain point and may need to be substituted.

Analysis of results PAE 22

When examining the effect of phage cocktails on PAE22, a strain seen to be resistant to all cocktails in the spot assay, a different scenario was found. Only cocktails with 10 and 15 phages produced a reduction in biofilm biomass compared to the growth control. Surprisingly, cocktails with 20 and 25 phages led to an

increase in biofilm production. This has been observed in other studies, where prolonged exposure to phages can cause resistant bacterial strains to increase biofilm production over time (Pires et al., 2011).

One possible explanation for this phenomenon is that the low metabolic activity of the bacterial cells within the biofilm could interfere with the phages' lytic cycle, leading to an increase in biofilm mass in resistant cells (Cerca et al., 2007). Additionally, bacteria might sense the presence of phages and respond by producing more biofilm to protect themselves. Although there is no direct evidence of this in the literature, it has been hypothesised that phages may trigger receptors located in the bacterial membrane, and then through a cascade of intracellular signals, silent genes are switched on (Davidson, 2017). In this line, they produce more bioproducts, and those bioproducts help to build a denser biofilm, but again, there is no evidence in the literature supporting this thought.

In terms of biofilm activity, although the reduction for PAE22 was smaller compared to other strains, it was still statistically significant. The difference between biofilm biomass and activity could suggest that while the phages did inhibit bacterial activity, the surviving bacteria produced more extracellular matrix, leading to increased biofilm mass. The MTT reduction assay would reflect the number of live bacterial cells, whereas the crystal violet staining would measure both bacterial cells and the extracellular bioproducts forming the biofilm.

It would be interesting to explore the antibiotic sensitivity of this PAE strain when combined with these cocktails, to check if this small decrease may still help to improve antibiotic therapy. This could be done by comparing the minimal inhibitory concentration of the antibiotic after treated together with the cocktail in a similar biofilm assay test. Similarly, further research could investigate whether specific phages in cocktail 20 triggered the increased biofilm production, potentially by testing those phages individually against PAE22.

Conclusions and further research

Overall, this study has demonstrated that a phage cocktail containing 10 to 20 phages can significantly reduce biofilm biomass and activity in an *in-vitro* model. However, it also revealed limitations, such as the observed rebound effect with the cocktail (30) and the difficulty in treating highly resistant strains like PAE22. This highlights the need for further research into the interactions between phages and bacterial biofilms, especially when increasing phage numbers in cocktails. Future studies should focus on investigating the exact limit at which increasing phage numbers no longer improves efficacy and may even hinder bacterial clearance, exploring the use of additional assays to quantify biofilm biomass and bacterial activity more accurately, potentially using methods such as fluorescent staining or flow cytometry to complement crystal violet and MTT assays, as these techniques can distinguish viable from non-viable cells within biofilms (Berney et al., 2007). Furthermore, testing the effectiveness of phage cocktails in combination with antibiotics could help determine whether phage-mediated biofilm reduction enhances antibiotic susceptibility.

The current phage cocktail shows commercial potential, but the risk of antibiotic resistance genes within the phages and the challenges of long-term storage must be addressed. Developing a universal phage cocktail for treating otitis externa infections in dogs would require ongoing research, both to optimise cocktail formulations and to ensure the safety and efficacy of phage-based therapies.

CHAPTER 6: PHAGE CHARACTERISATION

6.1 Introduction

Research institutes in Australia and around the world are actively isolating new phages, particularly targeting bacteria that are difficult to treat with antibiotics, such as *Pseudomonas aeruginosa* (PAE) (Yerushalmy et al., 2020). For clinical applications, a phage cocktail must undergo thorough characterisation to ensure its safety and efficacy (Modi et al., 2013, Reindel and Fiore, 2017). When phages inject their genome into bacteria, there is a potential risk of transferring genes responsible for antibiotic resistance or virulence factors to the bacterial pathogens. Lysogenic phages may integrate their genetic material into the bacterial chromosome, contributing to antibiotic resistance or altering bacterial virulence (Carrias et al., 2011). Therefore, genomic analysis is essential to avoid the use of phages carrying these undesirable genes.

The genome analysis of phages enables the exclusion of phages that possess genes associated with lysogeny, virulence, or antibiotic resistance. This ensures that only lytic phages, which are safe for therapeutic use, are selected (Salmond and Fineran, 2015). As sequencing technologies become more accessible, even for smaller laboratory groups, the number of sequenced phage genomes is rapidly growing (Salisbury and Tsourkas, 2019). However, trained researchers and specialised software are required to efficiently and accurately analyse the sequencing data, with various tools and platforms available for this purpose (Ho et al., 2023).

In this study, sequencing was carried out after cocktail development as funding for molecular work was not available prior to the development of cocktails. Once funding was sourced, two approaches were used to characterise the phage cocktail produced in Chapter 5. First, transmission electron microscopy (T.E.M.) was performed to confirm the purity of the phages and reveal their morphological features for taxonomic classification. Second, genome sequencing was conducted to ensure the phages did not carry any antibiotic resistance, lysogeny, or virulence genes and to further confirm their taxonomic classification.

6.2 Materials and Methods

6.2.1 Morphological observation of bacteriophages through transmission electron microscope (TEM)

6.2.1.1 Sample preparation for TEM and sequencing

To ensure the purity and family identification of 15 selected phages, transmission electron microscopy (TEM) was conducted. These phages represented the initial 15 used in the biofilm assays and were chosen based on their lytic efficiency, diversity in host range, and their inclusion in previously successful phage cocktails (Chapter 5). The selection was also influenced by the stability of phage solutions and the high yield obtained during propagation, ensuring enough concentration for TEM analysis.

From the 15 phages selected for TEM, 12 were subsequently chosen for DNA sequencing. The final selection was based primarily on the DNA concentration obtained after propagation and the laboratory capacity to sequence only 12 phages. Specifically, those with the highest titres (~$1x10^{13}$ PFU/ml) after propagation were prioritised for sequencing (Cao et al., 2011, Quick, 2018).

Phages were propagated as previously described (Section 4.2.5), and the effectiveness of propagation was confirmed using the spot assay technique (Section 3.2.2). Ultracentrifugation was performed on 50 ml of the propagated phage solution as outlined in Section 3.3.1. After ultracentrifugation, 250 µl of SM buffer was added to the tube, and the solution was allowed to sit overnight at 4°C to concentrate the phage pellets. The concentrated phage solution was flushed using a transfer pipette and transferred to microfuge tubes for further processing.

For TEM analysis, 50 µl of each phage concentrate was dispatched on a gel ice pack to the University of Queensland Department of Microscopy and Microanalysis. In parallel, 200 µl of the ultraconcentrated phage solution was used for the DNA extraction protocol.

6.2.1.2 Measuring phages and family definition from TEM images.

The phages were measured based on parameters such as head diameter (width perpendicular to the tail) and tail length (Figure 6.1). These measurements were taken from five images per phage sample, and for each image, a minimum of three phages were counted to ensure accuracy. This resulted in an average measurement for each phage morphology and allowed for the classification of the phages into their respective families.

Viruses with a tail length in the range of 3-40 nm were classified as *Podoviridae*, viruses with a sheath and tail length in the range of 80-485 nm were classified as *Myoviridae* and viral particles with no sheath and a tail length of 79-539 nm were classified as *Siphoviridae*. These classifications followed the methodology of Ackermann (Ackermann, 1998), and the measurements are reported with standard deviations to account for the observed variance (Table 6.1).

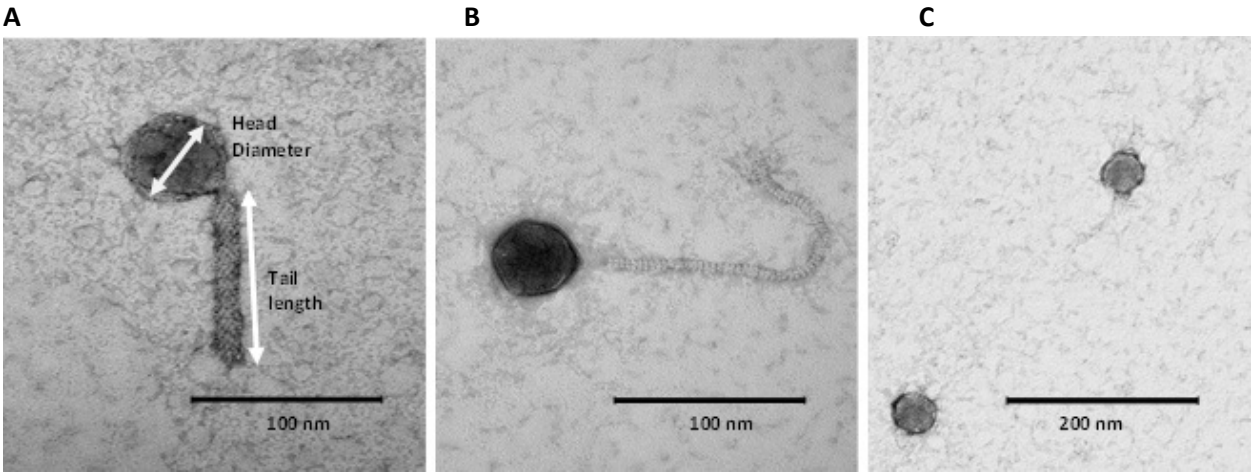

Figure 6.1: Examples of diversity in phage morphology from the isolated phage collection in this study. (A – *Myoviridae* (Ph5), B – *Siphoviridae* (Ph8), C – *Podoviridae* (Ph16)). Measurement parameters are shown in A. Phages were identified and counted from five TEM images per sample, with an average of three phages per image.

6.2.2 Phage Sequencing

6.2.2.1 Estimation of PAE phage genome size.

Since the exact genome sizes of the PAE phages were initially unknown, estimating these sizes was essential to determine the DNA concentration required for sequencing. Based on known genome sizes of PAE phages within the *Caudovirales* order, typically ranging from 43 kb to 89 kb (Campbell et al., 2021), a default size of 50 kb was assumed for calculations. Using this estimate, we aimed to achieve a concentration of 100–200 fmol in 48 µl for Nanopore sequencing, translating to at least 2.08–4.16 fmol/µl. According to the NEBioCalculator (NEB), approximately 100 ng of 50 kb genomes, equivalent to 3.25 fmol or 1.955 x 10^9 copies, was necessary to meet the sequencing requirements.

To achieve these concentrations, phage solutions were amplified to titres of 1x10^9 PFU/ml in a 50 ml volume. Following ultracentrifugation and DNA extraction as described in Section 6.2.1.1, a total volume of 250 µl was collected for subsequent DNA extraction.

6.2.2.2 DNA extraction and ethanol precipitation

The DNA extraction and ethanol precipitation were performed as per the method of Dr. Jennifer Elliman (Elliman, 2006). Briefly, a dry block heater (Ratek Laboratory Equipment, model DBH30) was preheated to 56°C. In a microfuge tube (Sarstedt; Germany), 16 µl of 500 mM Tris-EDTA (TE) (Appendix 1), 20 µl of 10% sodium dodecyl sulphate (SDS) (Appendix 1), and 4 µl of 20 mg/ml proteinase K (Sigma) to 200 µl of phage suspension were added. Then, 160 µl of water was added to make the final volume 400 µl. The tube was incubated in the block heater at 56°C for 1.5 hours to lyse the phage and release the DNA. To degrade any RNA present, 4 µl of a 1:10 diluted 10 mg/ml solution of Ribonuclease A (RNAse A) was added to the tube and incubated at 56°C for an additional hour. An aliquot of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma) was taken to 56°C and then added in equal volume (400 µl) to the tube. The tube was vortexed and incubated in the block heater for 30 minutes to separate the DNA from proteins and other contaminants. The tube was centrifugated at 5000 g for 10 minutes. As a result, the DNA was in the upper

aqueous phase, while the proteins and other contaminants were in the lower organic phase or at the interface. The upper aqueous phase was transferred to a new microfuge tube, and an equal volume (400 µl) of chloroform was added. This was then mixed and centrifuged to remove any residual phenol from the DNA solution, and the aqueous phase was collected in a new microfuge tube.

The DNA was precipitated by adding 0.1 volume (40 µl) of 3 M sodium acetate (pH 5.2) (Appendix 1) and 2 volumes (800 µl) of ethanol (Sigma, Australia) to the tube. Then, the tube was mixed and stored at -20°C for one hour. The tube was centrifuged at 5000 g for 15 minutes to pellet the DNA. The supernatant was discarded, and the pellet was washed with 70% ethanol. The pellet was air-dried and resuspended in distilled water.

6.2.2.3 Determination of DNA concentration and purity

The concentration of DNA was measured using a spectrophotometer (Implen NanoPhotometer™) by analysing the absorbance of light at 260 nm (O.D.260nm). The NanoPhotometer determines the purity of DNA samples by measuring the absorbance at 260 nm and 280 nm and calculating the 260/280 ratio (Appendix 4). A 260/280 ratio within the range of 1.8 to 2.0 is typically considered indicative of pure DNA. Ratios lower than 1.8 suggest potential protein contamination, while higher values may indicate RNA contamination (García-Alegría et al., 2020). Additionally, the 260/230 ratio was used to assess the level of salt contamination, where values below 1.5 can signal salt presence that may inhibit enzymatic reactions during nanopore processing.

To further evaluate the DNA integrity, agarose gel electrophoresis was performed (Appendix 4). This method allowed for the assessment of DNA fragment size and the presence of any degradation or shearing, ensuring that the samples were suitable for subsequent sequencing procedures.

6.2.2.4 Assessment of DNA integrity

The integrity and quantity of DNA samples were assessed by agarose gel electrophoresis. The gel was composed of 1% agarose (Progen molecular biology grade agarose) (W/V) in 1x Tris-acetate (TAE) buffer (Appendix 1) with 5 µl/100 ml gel red and run at 90V. A mixture of 3 µl of DNA solution and 1 µl of orange G front run (Sigma Chemicals, Australia) was loaded into the agarose wells.

DNA integrity was evaluated by visualising clear, high-molecular-weight bands above the top marker band (more than 12 kb in size; Hyper Ladder™ 1Kb Plus, Meridian Bioscience®, Australia). A lack of smearing indicated that the DNA was intact and not sheared. Gels were analysed using a GelDoc 1000 (Biorad, Australia).

6.2.2.5 Library Preparation and Sequencing for MinION Whole Genome Sequencing

To analyse the phage genomes, 12 DNA samples were selected based on their high titres and suitability for sequencing after quality control assessments, including concentration and integrity verification (Sections 6.2.2.3 and 6.2.2.4). The selected samples included Ph2, Ph3, Ph5, Ph8, Ph9, Ph10, Ph12, Ph16, Ph17, Ph18, Ph28, And Ph29, chosen for their strong lytic activity and representation within the phage cocktail formulations discussed in Chapter 5.

The Native Barcoding Kit EXP-NBD104 from Oxford Nanopore Technologies plc was utilized according to the manufacturer's instructions (see Appendix 4). The twelve selected DNA samples were multiplexed for adaptor addition using the Nanopore ligation kit SQK-LSK109, followed by the sequencing process.

6.2.2.6 Whole genome sequencing, assembly and analysis

Phage whole-genome sequencing was performed on a MinION sequencer using MinKNOW software v.23.04.03 (Oxford Nanopore Technologies plc). All reads were analysed and base-called using the software, and each genome was automatically separated by MinKNOW using the unique barcodes attached to the sequences. This data was subsequently sent to Dr. Bhavya Papudeshi at Flinders University, South Australia, for detailed bioinformatic processing and analysis.

The fastq sequences were processed using Sphae v1.4.5 (https://github.com/linsalrob/sphae). Initially, the reads were passed through fastp v0.23.4 (Chen et al., 2018) to remove any adaptor regions or low-quality sequences. Once cleaned, the reads were assembled using Flye v2.9 (Kolmogorov et al., 2020), an assembler optimised for long-read data, to construct contigs. These assembled contigs were then assessed for completeness using CheckV v1.0.1 (Nayfach et al., 2021).

To verify the viral origin of the contigs, ViralVerify (Antipov et al., 2020) was employed, ensuring that only complete viral genomes were retained for subsequent analysis. Following this verification, the contigs were annotated with Pharokka, a rapid standardised tool for bacteriophage genome annotation (Bouras et al., 2022). Pharokka conducts sequence similarity searches within a dedicated viral gene database containing over 17,000 complete viral genomes, enabling comprehensive annotation. Pharokka was also used to screen for antimicrobial resistance (AMR) genes, as it incorporates this functionality within its annotation pipeline.

For genes initially labelled as hypothetical, further analysis was conducted using Phynteny (Grigson et al., 2020), which leverages synteny information, and Phold (Bouras et al., 2024), which incorporates structural data to enhance gene assignment accuracy. Finally, taxonomic classification was performed using Pharokka's alignment to closely related genomes in the INPHARED database (Cook et al., 2021), utilising Mash (Ondov et al., 2016) for efficient similarity searches.

6.3 Results

6.3.1 Characterisation of Phages Through Transmission Electron Microscope (TEM)

6.3.1.1 Morphological Observation Through Transmission Electron Microscopy (TEM)

Out of the 35 distinct phage concentrates ("purified phage solutions") (Section 4.2.6), 15 phage concentrates stored in LB broth were selected for purity checks and morphological characterisation (Ph2, Ph3, Ph4, Ph5, Ph8, Ph9, Ph10, Ph12, Ph16, Ph17, Ph18, Ph28, Ph29, Ph30, Ph32). These 15 phage concentrates were chosen based on their lytic activity and inclusion in previously effective phage cocktails (Chapter 5). The rationale behind selecting these specific phages was to represent a diverse range of host interactions and maximise the effectiveness of potential treatments.

From the 15 phage concentrates, the morphology of 12 was successfully characterised (Figure 6.2). However, it was not possible to define three phage concentrates (Ph4, Ph29, Ph32) due to time and resource constraints that prevented further TEM analysis. The selected phages for morphological characterisation are part of the "Cocktail (15)" formulation (Section 5.2.2.2). Additionally, two distinct morphotypes were observed in three phage concentrates (Ph5, Ph28 and Ph18) (Figure 6.2, [m]) in spite of these isolates being purified by three cycles of plaque selection and growth on lawns (Section 3.2.1).

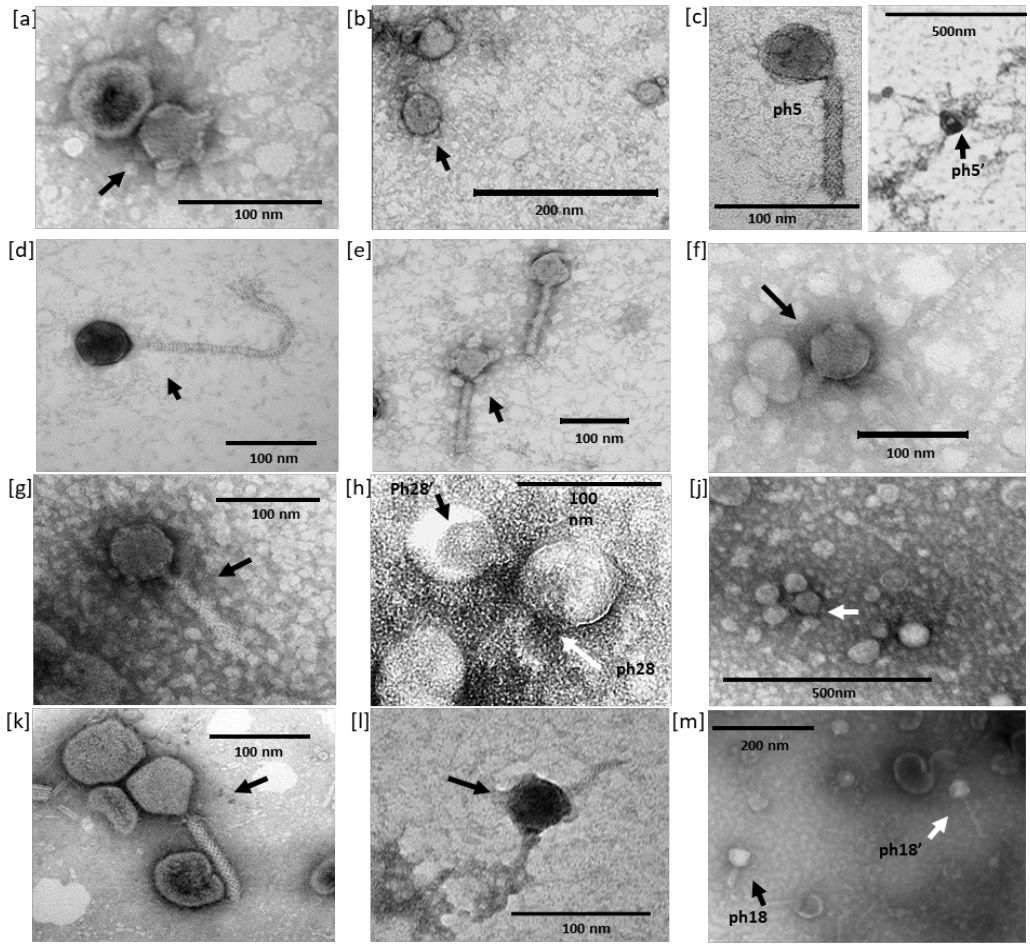

Figure 6.2: Transmission electron microscope view of twelve phage concentrates in this study. [a] - Ph2; [b] - Ph3; [c] - Ph5; [d] - Ph8; [e] – Ph10; [f] - Ph12; [g] - Ph17; [h] - Ph28; [j] - Ph30; [k] - Ph9; [l] - Ph16 and [m] - Ph18. Black and white arrows indicate the phages' position.

The TEM images revealed that all observed bacteriophages are non-enveloped viruses indicating that they lack a lipid membrane surrounding their protein capsid. The phage concentrates that presented two different phage morphotypes were named as, for Ph5: Ph5 and Ph5'; for Ph28: Ph28 and Ph28', and for Ph18: Ph18 and Ph18'. This means that fifteen phages were morphologically characterised.

All phages belong to the order Caudovirales. Two out of fifteen phages (Ph8, Ph18') were long-tailed, non-contractile with icosahedral heads, and were classified within the *Siphoviridae* family. Six out of fifteen phages (Ph5, Ph10, Ph17, Ph28, Ph9, and Ph18) were long-tailed, contractile with icosahedral heads, placing them in the *Myoviridae* family. The remaining seven phages (Ph2, Ph3, Ph5', Ph12, Ph28', Ph30, and Ph16) were short-tailed with icosahedral heads classifying them within the *Podoviridae* family (Figure 6.2). The measured sizes of these phages, with variances, are documented in Table 6.1.

Table 6.1: Morphological descriptions of characterised phages and their respective families based on TEM observations.

TEM Code	Phage Code	Structure	Size (nm)	Family
A	Ph2	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 43±1 b. Tail length: 13±1	*Podoviridae*
B	Ph3	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 46±3 b. Tail length: 10±3	*Podoviridae*
C	Ph5	a. Head: Icosahedral b. Tail: Long, contractile and thick	a. Head diametre: 56±5 b. Tail length: 95±15	*Myoviridae*
	Ph5'	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 43±3 b. Tail length:18±3	*Podoviridae*
D	Ph8	a. Head: Icosahedral b. Tail: Long, non-contractile and thin	a. Head diametre: 59±5 b. Tail length: 241±2	*Siphoviridae*
E	Ph10	a. Head: Icosahedral b. Tail: Long, contractile and thick	a. Head diametre: 66±2 b. Tail length: 123±5	*Myoviridae*
F	Ph12	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 47±1 b. Tail length: 11±1	*Podoviridae*
G	Ph17	a. Head: Icosahedral b. Tail: Long, contractile and thick	a. Head diametre: 70±5 b. Tail length: 130±5	*Myoviridae*
H	Ph28	a. Head: Icosahedral b. Tail: Short, contractile and thick	a. Head diametre: 60±3 b. Tail length: 52±3	*Myoviridae*
	Ph28'	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 45±2 b. Tail length: 10±2	*Podoviridae*
J	Ph30	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 70±3 b. Tail length: 18±2	*Podoviridae*
K	Ph9	a. Head: Icosahedral b. Tail: Long, contractile and thick	a. Head diametre:56±2 b. Tail length: 112±1	*Myoviridae*
L	Ph16	a. Head: Icosahedral b. Tail: Short	a. Head diametre: 48±1 b. Tail length: 26±1	*Podoviridae*
M	Ph18	a. Head: Icosahedral b. Tail: Short, contractile and thick	a. Head diametre: 55±5 b. Tail length: 60±5	*Myoviridae*
	Ph18'	a. Head: Icosahedral b. Tail: Long, non-contractile and thin	a. Head diametre: 60±5 b. Tail length: 119±1	*Siphoviridae*

6.3.2 Whole Genome Sequencing analysis

6.3.2.1 Long-reads polished, assembled, and annotated.

A total of twelve phage genome solutions were sequenced and processed. Due to challenges in isolating individual phages, some genome solutions contained sequences from multiple distinct phages, as seen in phage solutions such as Ph5, Ph10, Ph17, and Ph28, each of which revealed genomes from two or more phage types.

The genome lengths ranged from approximately 42 kb to 94 kb, with GC content percentages varying from 49% to 62%. The sequenced genomes were all linear. Taxonomic classification at the lowest level identified the phages primarily within the genera *Phikmvvirus*, *Bruynoghevirus*, *Samuniavirus*, *Detrevirus*, and *Pakpunavirus*, with some unclassified genomes. The classical classification primarily placed these phages within the families *Podoviridae* and *Myoviridae*.

The coding sequence (CDS) counts varied significantly among genomes, reflecting the diversity of the phage collection. Key details, including genome type, length, CDS counts, hypothetical protein numbers, and GC content, are summarised in Table 6.2.

Table 6.2: General features of the PAE-Phage Genomes

Phage Code	Unique Phage Label	Clasical Clasification	Lowest Taxa Classification	Total Read Length (bp)	Genome Circular/Linear	CDS Count	Hypothetical Proteins	GC Content (%)
ph2	ph2	*Podoviridae*	*Phikmvvirus*	43,363	Linear	64	32	62%
ph3	ph3	*Podoviridae*	*Phikmvvirus*	43,357	Linear	64	32	62%
ph5	ph5-1	*Podoviridae*	*Bruynoghevirus*	48,419	Linear	144	91	53%
ph5	ph5-2	*Podoviridae*	*Phikmvvirus*	47,582	Linear	112	61	62%
ph8	ph8	*	*Samunavirus*	94,268	Linear	171	113	55%
ph9	ph9	*	*Detrevirus*	51,120	Linear	103	51	59%
ph10	ph10-1	*Podoviridae*	*Phikmvvirus*	47,298	Linear	133	81	62%
ph10	ph10-2	*Podoviridae*	*Bruynoghevirus*	45,074	Linear	104	64	52%
ph12	ph12	*Podoviridae*	*Bruynoghevirus*	53,228	Linear	134	95	54%
ph16	ph16	*Podoviridae*	*Bruynoghevirus*	45,266	Linear	86	54	52%
ph17	ph17-1	*	*Unclassified*	50,920	Linear	186	160	57%
ph17	ph17-2	*	*Detrevirus*	48,787	Linear	145	96	59%
ph17	ph17-3	*Myoviridae*	*Pakpunavirus*	70,833	Linear	160	110	49%
ph18	ph18	*Podoviridae*	*Bruynoghevirus*	45,354	Linear	86	54	52%
ph28	ph28-1	*Podoviridae*	*Bruynoghevirus*	45,060	Linear	81	49	52%
ph28	ph28-2	*Myoviridae*	*Pakpunavirus*	92,765	Linear	195	131	49%
ph30	ph30	*Podoviridae*	*Bruynoghevirus*	45,320	Linear	87	55	52%

***** Data not provided from the analysis

6.3.2.2 Genomic Features Analysis.

The sequenced phage genomes were analysed to assess the presence of specific genetic elements, including integrases, recombinases, transposases, antimicrobial resistance (AMR) genes, virulence factor genes, anti-CRISPR spacers, and defence genes.

No AMR genes, virulence factor genes, anti-CRISPR spacers, or defence genes were identified across the sequenced genomes, supporting the therapeutic potential of these phages. Only one phage genome (Ph5) contained a recombinase, while no transposases were detected in any of the genomes. These findings are summarised in Table 6.3, which details the presence or absence of each targeted genetic element in the analysed genomes.

Table 6.3: Summary of Genetic Elements in Phage Genomes

Phage Code	Unique Phage Label	Integrases	Recombinases	Transposases	AMR genes	Virulence factor genes	anti-CRISPR spacers	Defense genes
Ph2	Ph2	No	No	No	No	No	No	No
Ph3	Ph3	No	No	No	No	No	No	No
Ph5	Ph5-1	No	No	No	No	No	No	No
	Ph5-2	No	No	No	No	No	No	No
Ph8	Ph8	No	Yes	Yes	No	No	No	No
Ph9	Ph9	Yes	No	No	No	No	No	No
Ph10	Ph10-1	No	No	No	No	No	No	No
	Ph10-2	No	No	No	No	No	No	No
Ph12	Ph12	No	No	No	No	No	No	No
Ph16	Ph16	No	No	No	No	No	No	No
Ph17	Ph17-1	Yes	No	No	No	No	No	No
	Ph17-2	Yes	No	No	No	No	No	No
	Ph17-3	No	No	No	No	No	No	No
Ph18	Ph18	No	No	No	No	No	No	No
Ph28	Ph28-1	No	No	No	No	No	No	No
	Ph28-2	No	No	No	No	No	No	No
Ph30	Ph30	No	No	No	No	No	No	No

6.3.2.3 Visualisation of Phage Genomes.

All phage genomes from the characterised samples were confirmed to be linear. To maintain consistency in data presentation, a circular visualisation format was used for all phage genomes. The visualised genomes included Ph2, Ph3, Ph5 (Ph5-1, Ph5-2), Ph8, Ph9, Ph10 (Ph10-1, Ph10-2), Ph12, Ph16, Ph17 (Ph17-1, Ph17-2, Ph17-3), Ph18, Ph28 (Ph28-1, Ph28-2), and Ph30, as shown in Figures 6.3 to 6.19. Notably, in these visualisations, certain genes with identical descriptors appearing consecutively may need further validation. This additional analysis will help determine if they represent two distinct genes or if misalignment or base-calling errors artificially split a single gene. Such validation is essential to ensure accurate gene representation prior to final publication.

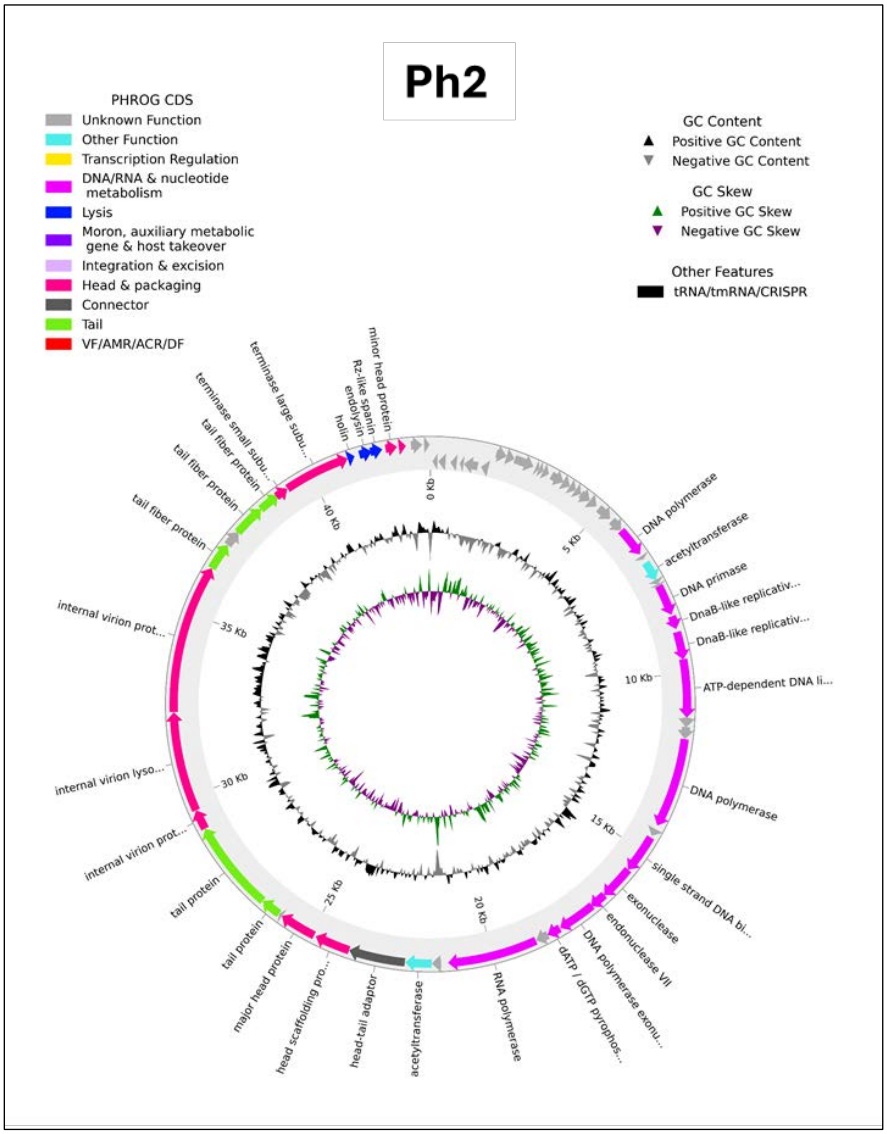

Figure 6.3. Genome map of phage Ph2, a *Pseudomonas aeruginosa*-infecting *Podoviridae* classified within the genus *Phikmvvirus*. The map was generated with Pharokka (Bouras et al., 2022) using annotation from the INPHARED database (Cook et al., 2021). Coding sequences (CDS) are shown as coloured arrows, with functional genes in blue, pink, purple, green, and dark-grey. Hypothetical proteins in grey. Inner rings represent GC content (black) and GC skew (green/purple). Although Ph2 has a linear genome, it is shown here in circular format for visual consistency.

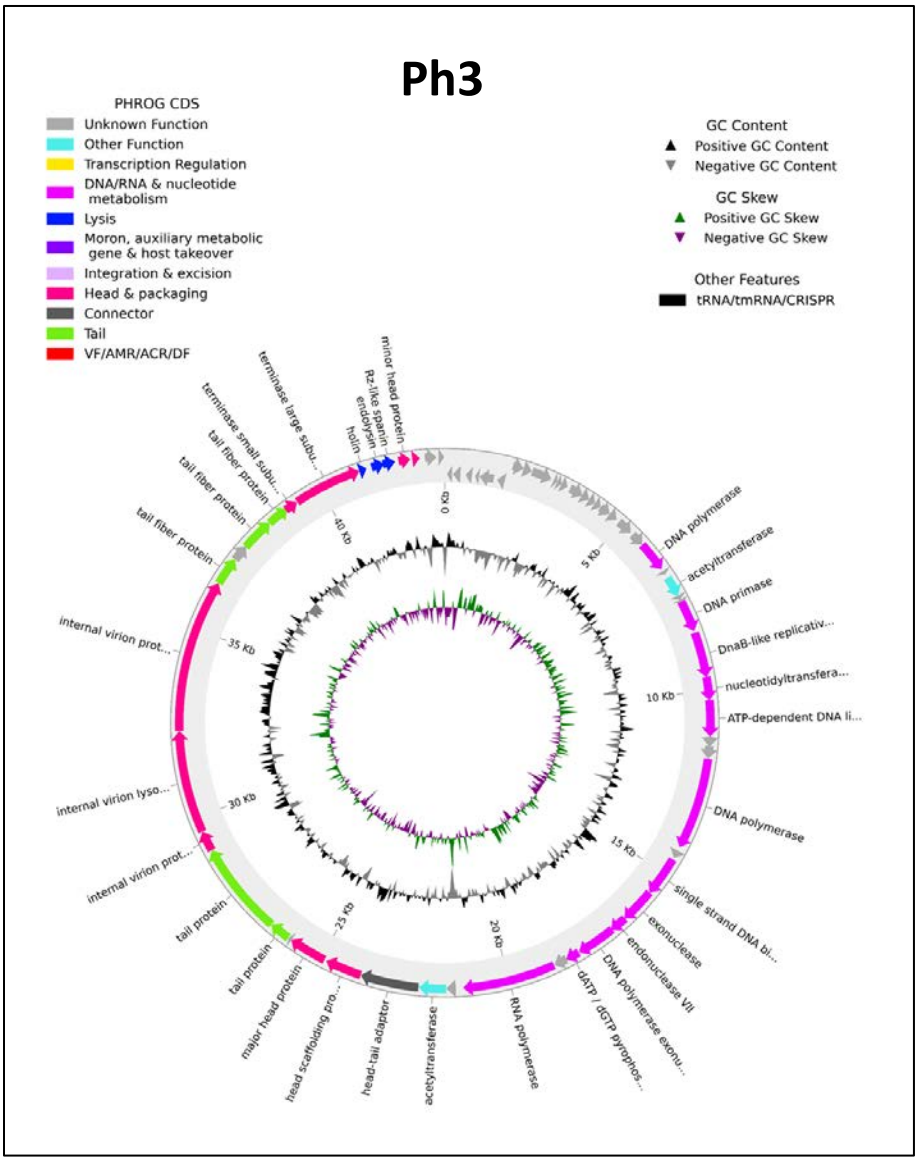

Figure 6.4. Genome map of phage Ph3 (*Podoviridae, Phikmvvirus*), isolated against PAE. Visualisation was performed in Pharokka with annotation against INPHARED. CDS are displayed as arrows (with functional genes in blue, pink, purple, green, and dark-grey. Hypothetical proteins in grey). Inner rings show GC content and skew. Genome is linear but displayed in circular format.

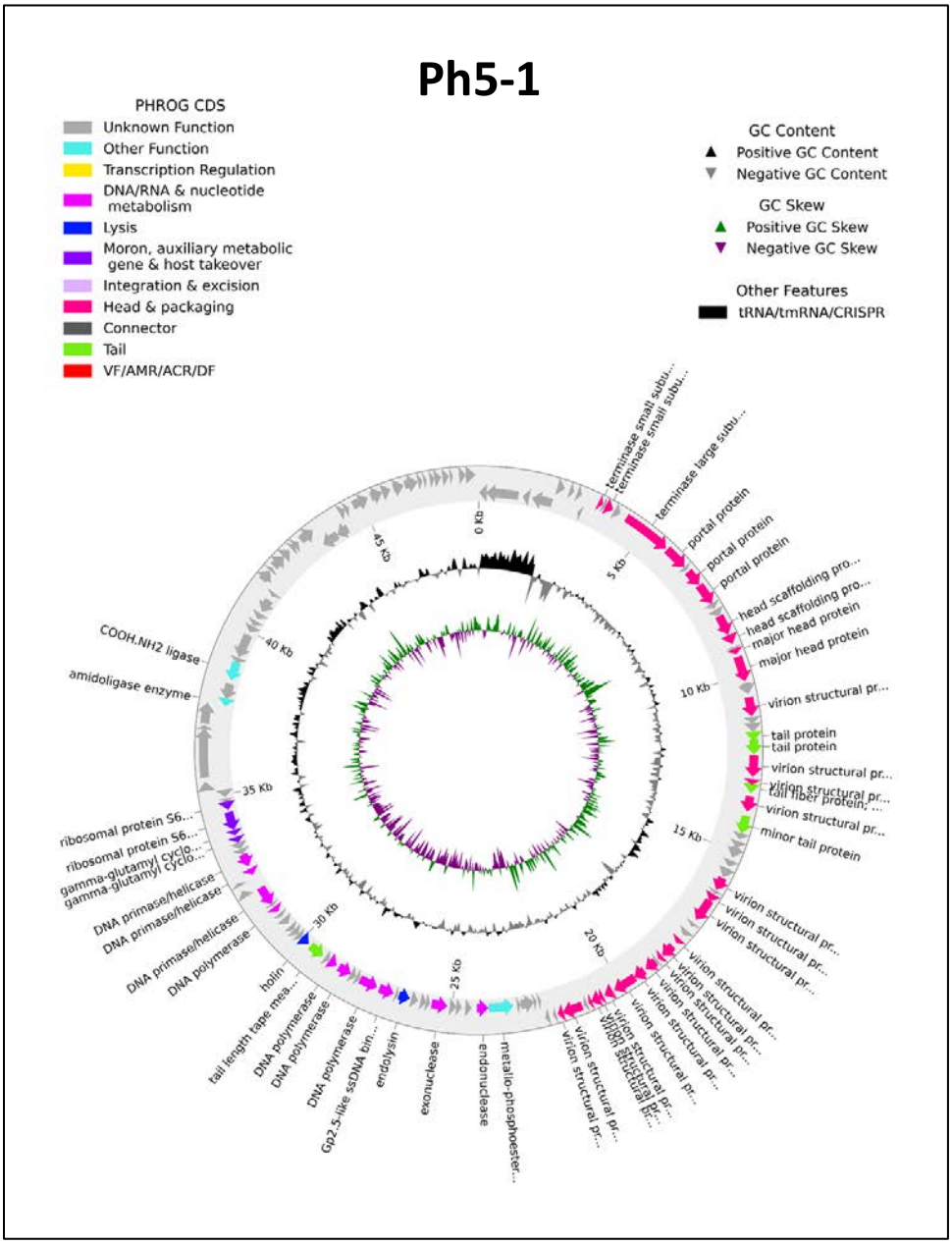

Figure 6.5. Genome map of phage Ph5-1 (*Podoviridae, Bruynoghevirus*), infecting PAE. Generated with Pharokka and INPHARED. CDS are shown as arrows (with functional genes in blue, pink, purple, green, and dark-grey. Hypothetical proteins in grey). GC content and GC skew are shown in inner rings. Genome displayed circularly for consistency.

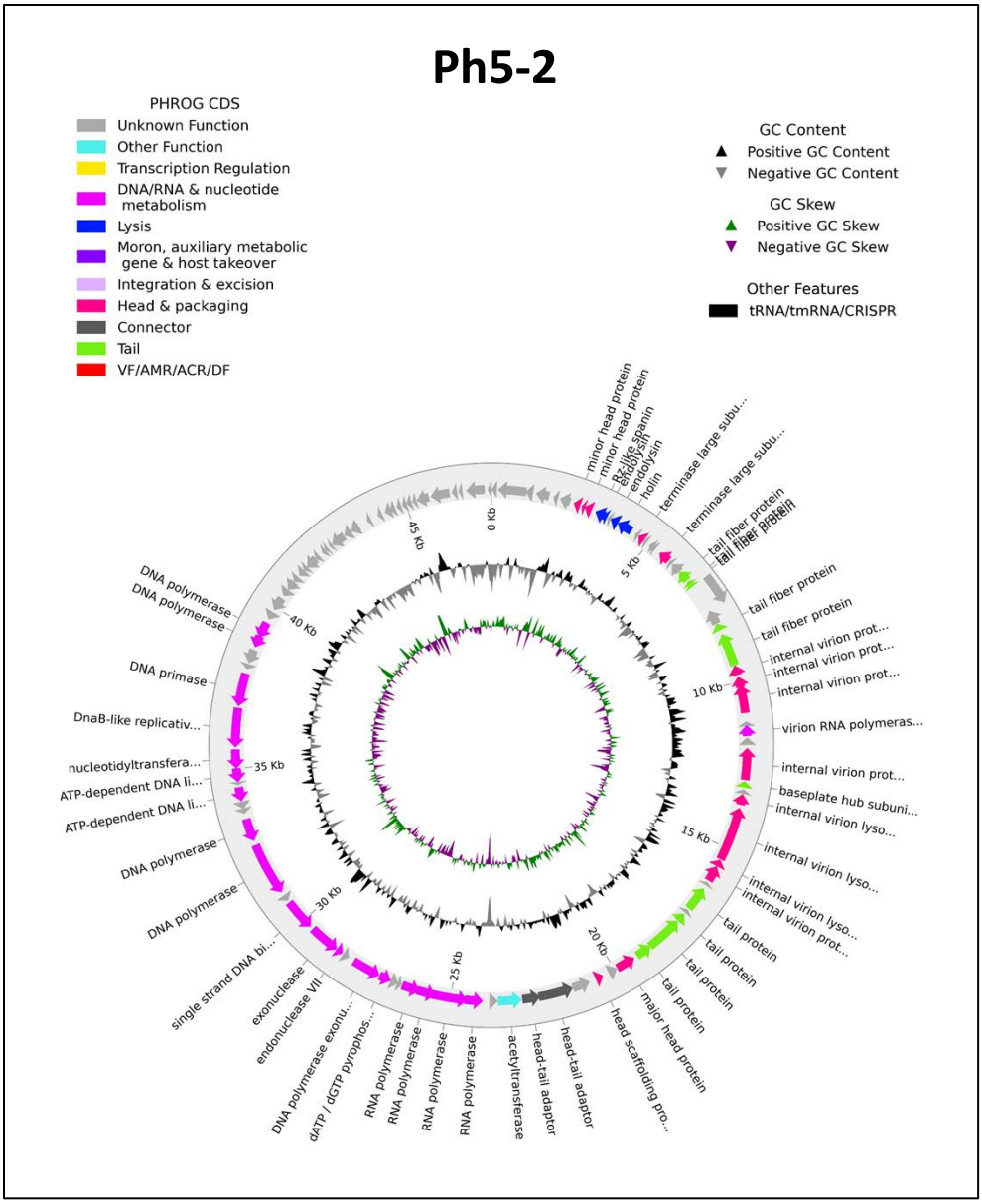

Figure 6.6. Genome map of phage Ph5-2 (*Podoviridae, Phikmvvirus*), a PAE-infecting phage. Annotation and visualisation were performed in Pharokka with INPHARED database support. Arrows represent CDS (with functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings show GC content and skew. Genome is linear but presented in circular view.

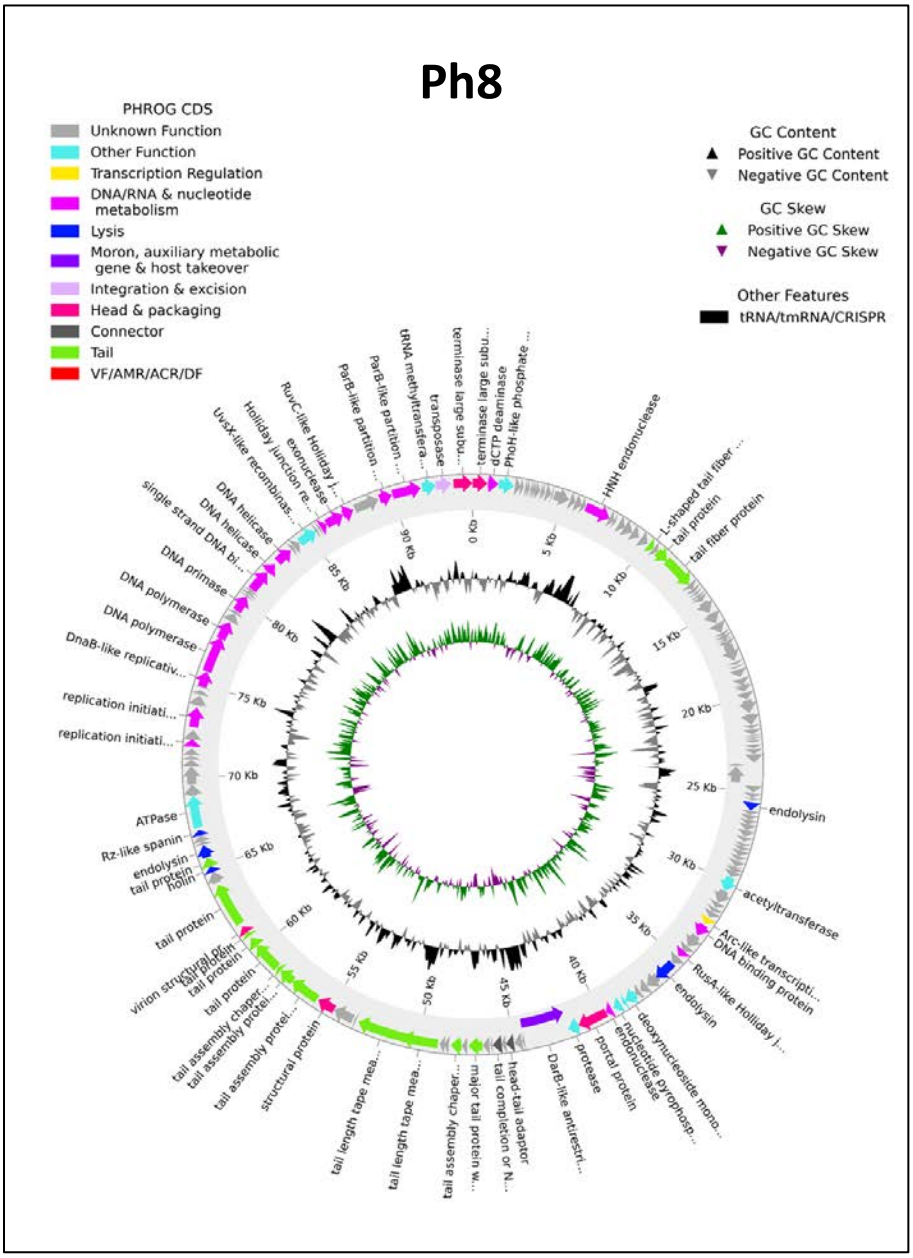

Figure 6.7. Genome map of phage Ph8 (*Siphoviridae, Samunavirus*), isolated against PAE. Visualisation and annotation performed in Pharokka. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey.), with GC content (black) and GC skew (green/purple) displayed as inner rings. Genome is linear but represented circularly.

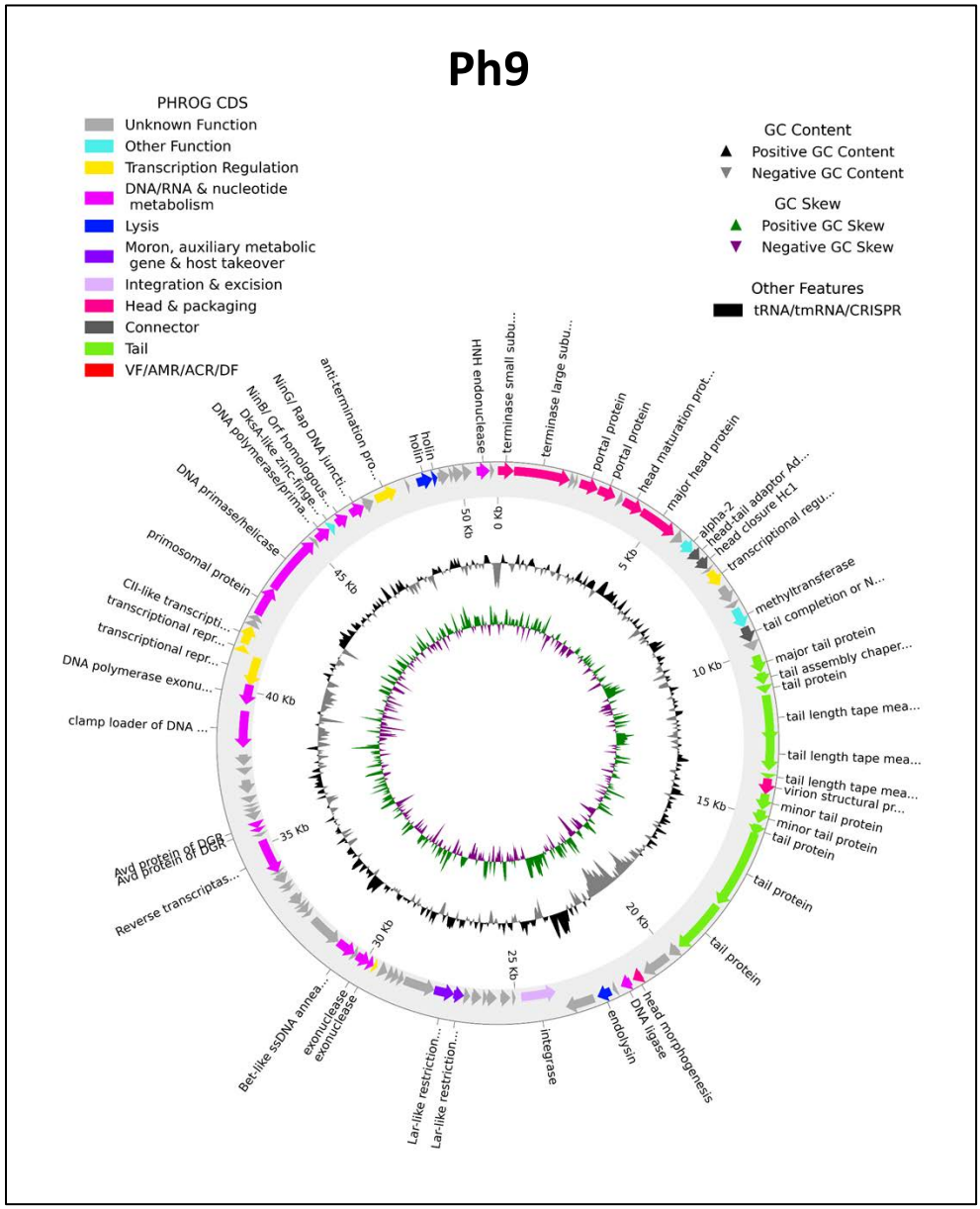

Figure 6.8. Genome map of phage Ph9 (*Detrevirus*, unclassified family within *Caudovirales*), infecting PAE. Pharokka with INPHARED was used for annotation. Arrows show CDS (functional genes in blue, pink, purple, green, yellow and dark grey. Hypothetical proteins in greyvv). Inner rings display GC content and skew. Genome is linear, visualised circularly.

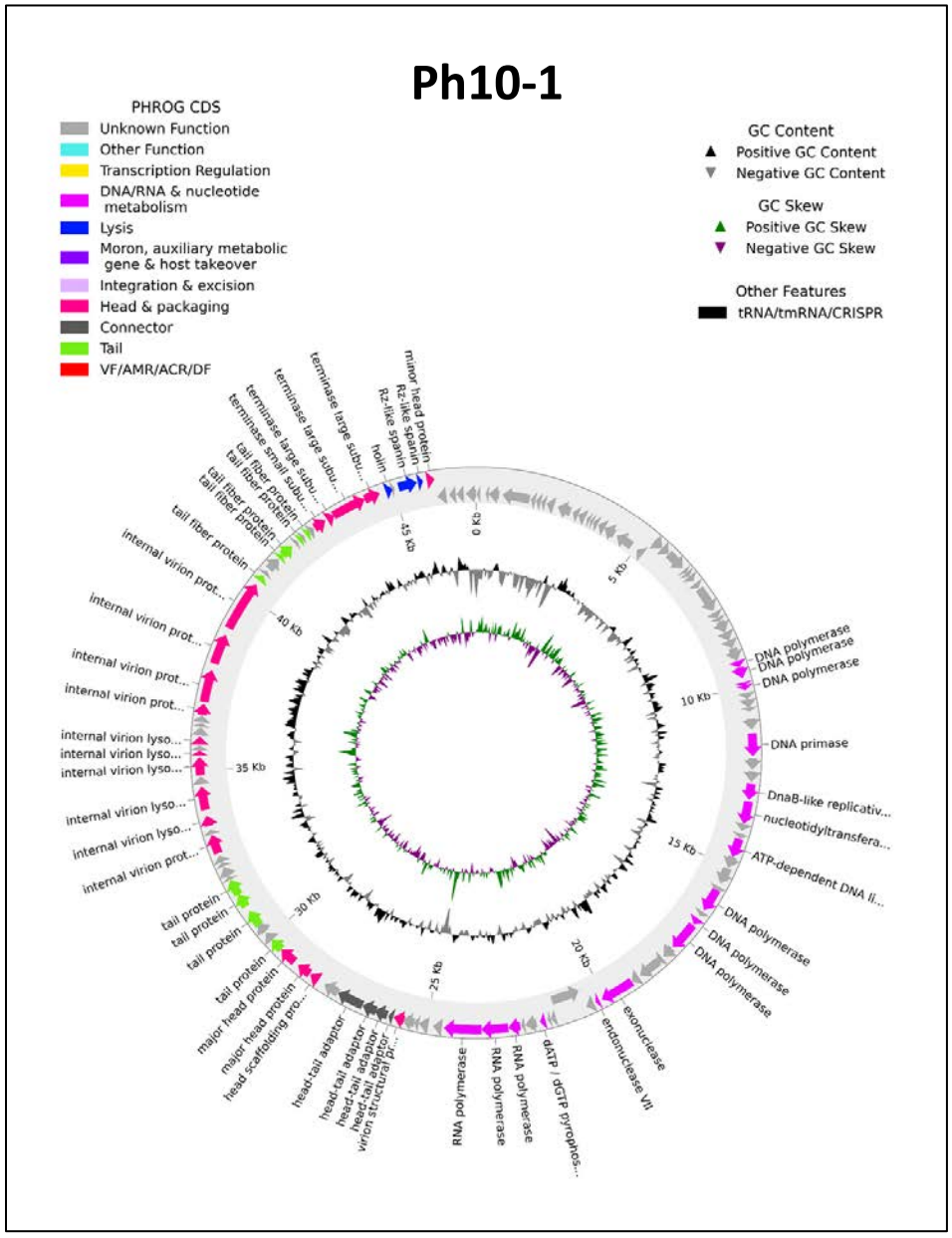

Figure 6.9. Genome map of phage Ph10-1 (*Podoviridae, Phikmvvirus*), targeting PAE. Map generated in Pharokka with annotation via INPHARED. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings display GC content and skew. Genome is linear but displayed circularly.

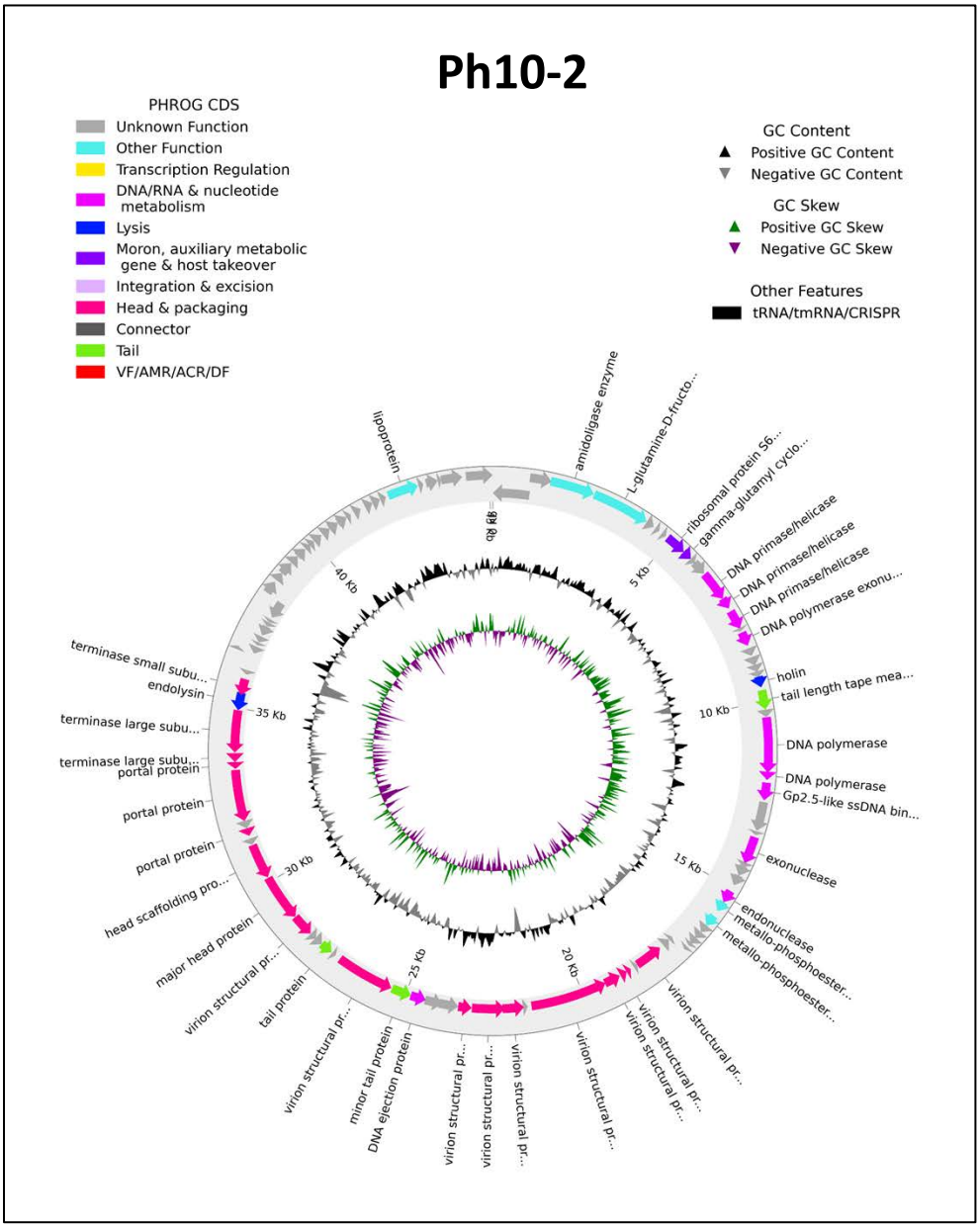

Figure 6.10. Genome map of phage Ph10-2 (*Podoviridae, Bruynoghevirus*), isolated against PAE. Visualisation and annotation were performed using Pharokka and INPHARED. CDS represented as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). GC content/skew are shown in inner rings. Genome displayed in circular format.

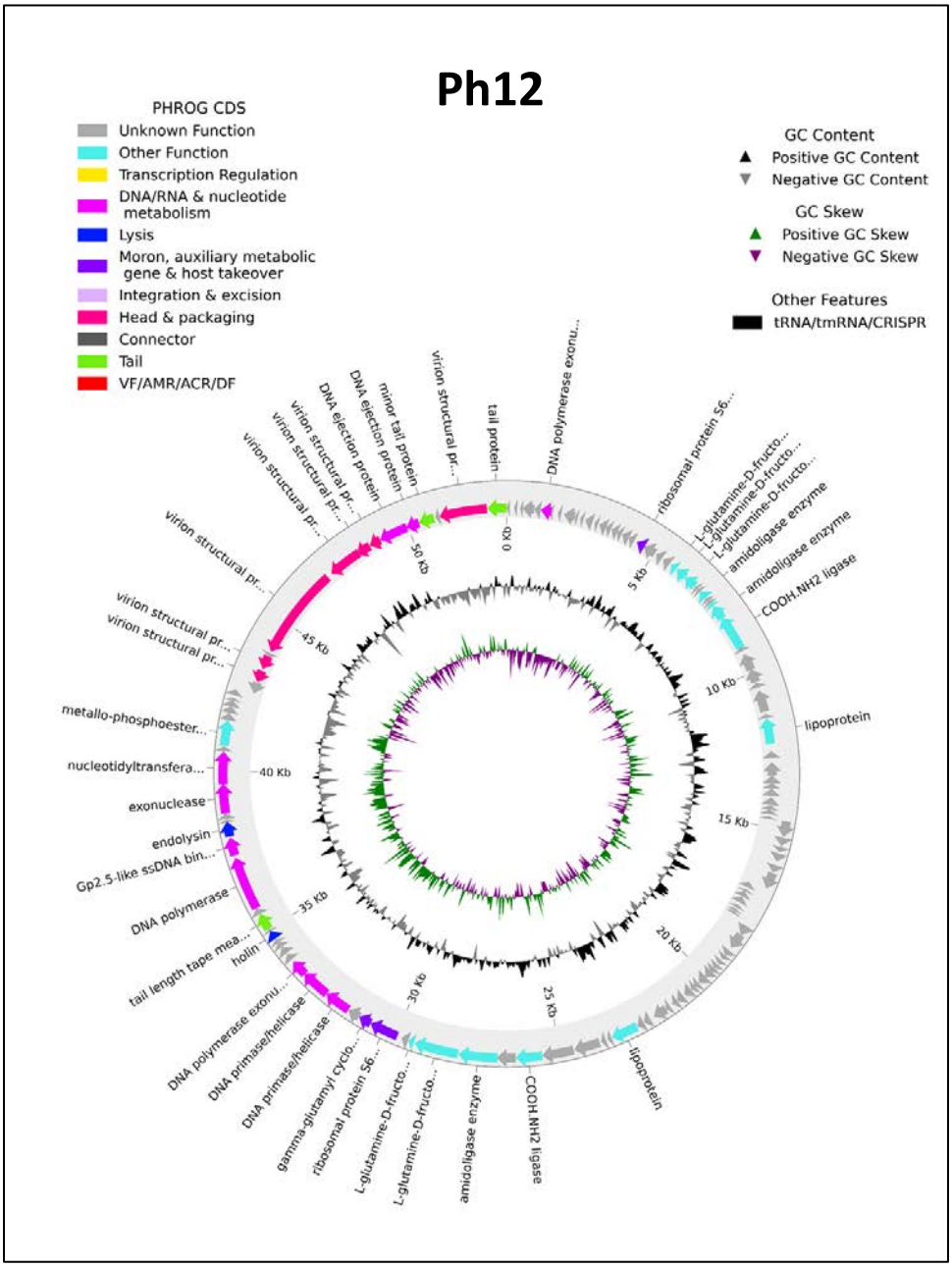

Figure 6.11. Genome map of phage Ph12 (*Podoviridae, Bruynoghevirus*), a PAE-infecting phage. Generated with Pharokka and annotated against INPHARED. CDS are represented by arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey), with GC content (black) and skew (green/purple) shown in inner rings. Genome is linear but circularly visualised.

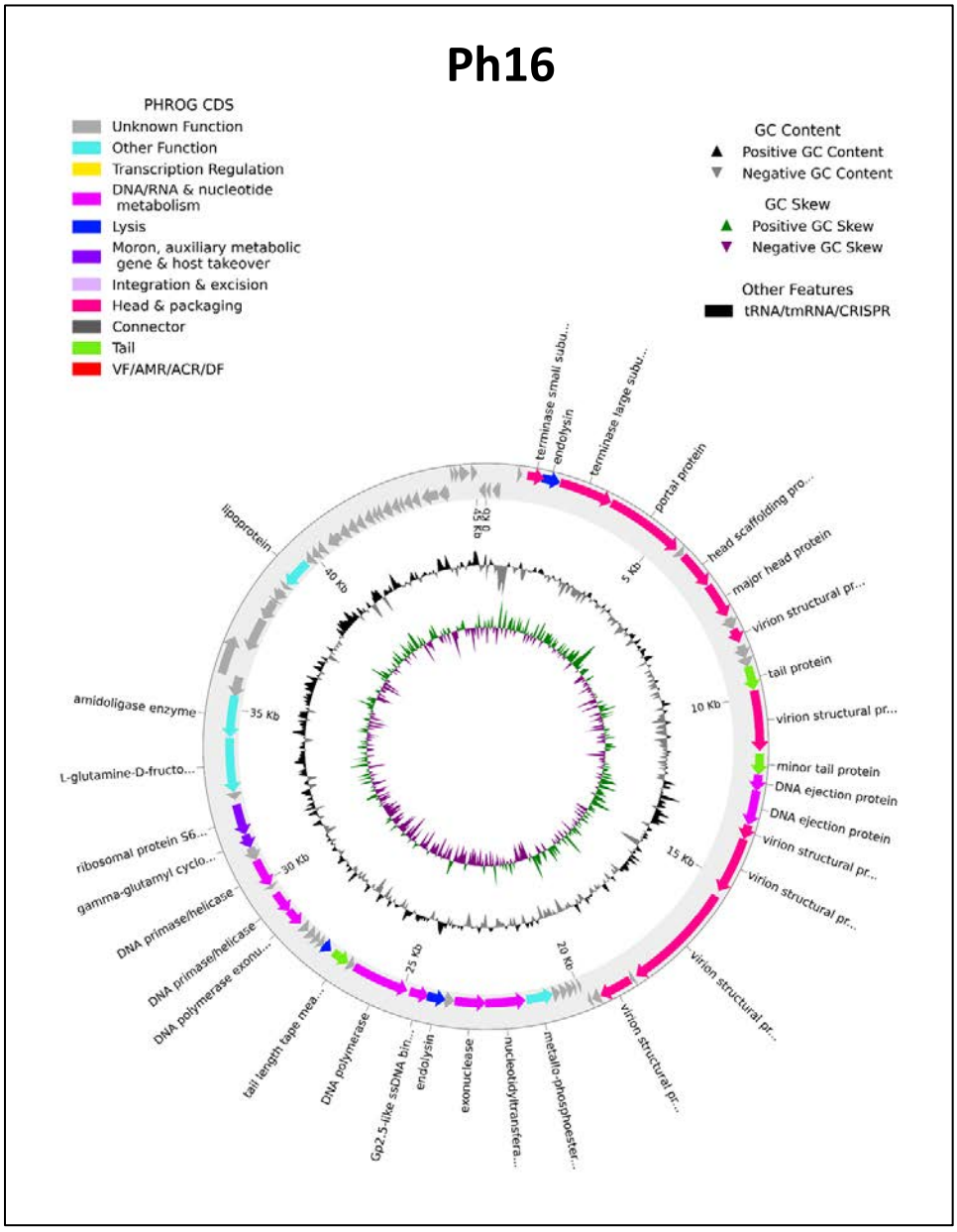

Figure 6.12. Genome map of phage Ph16 (*Podoviridae, Bruynoghevirus*), isolated against PAE. Visualisation performed in Pharokka with annotation via INPHARED. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). GC content and skew shown in inner rings. Genome is linear but represented circularly.

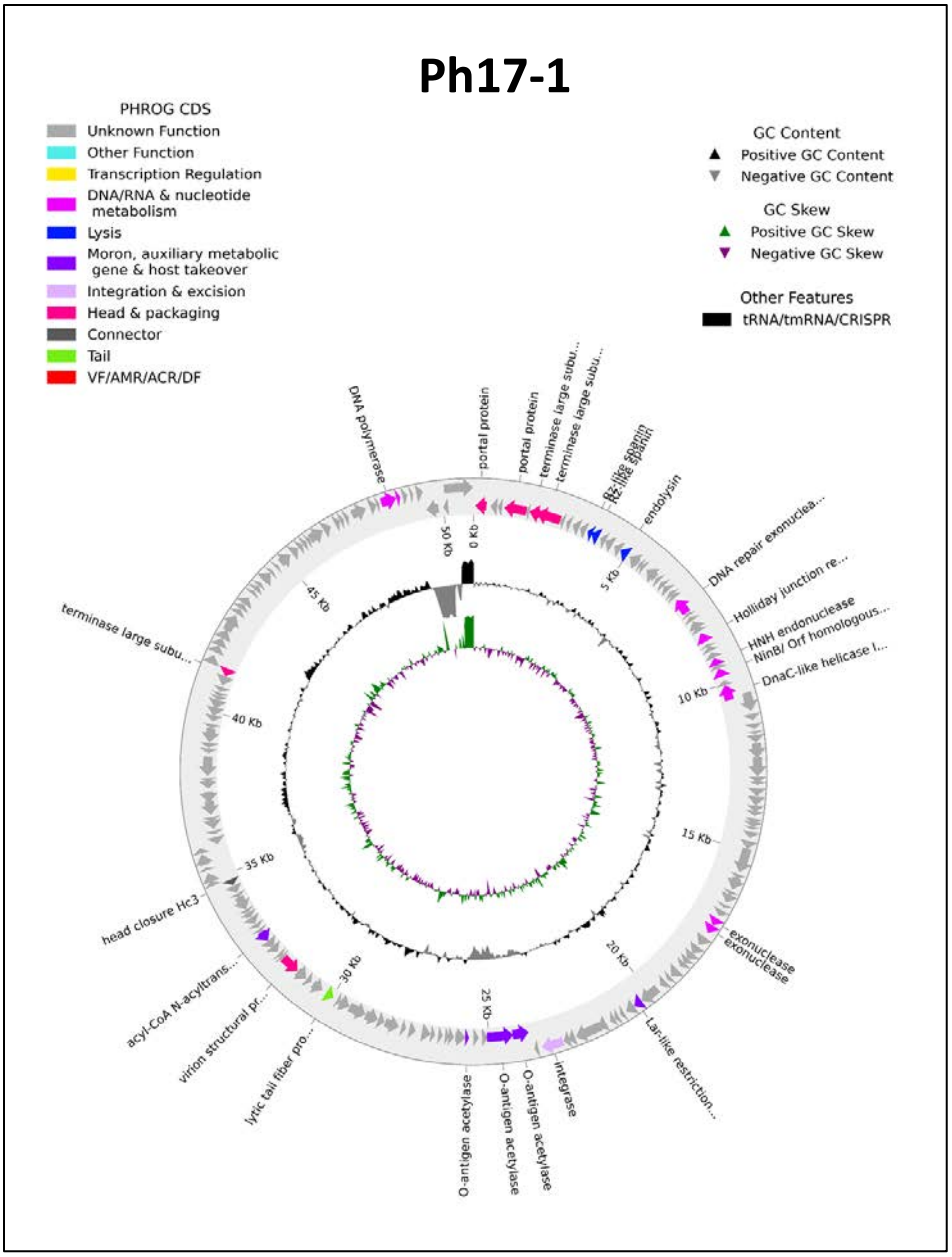

Figure 6.13. Genome map of phage Ph17-1 (unclassified *Caudovirales* phage), infecting PAE. Generated in Pharokka with INPHARED annotation. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). GC content and skew displayed in inner rings. Genome is linear but displayed circularly.

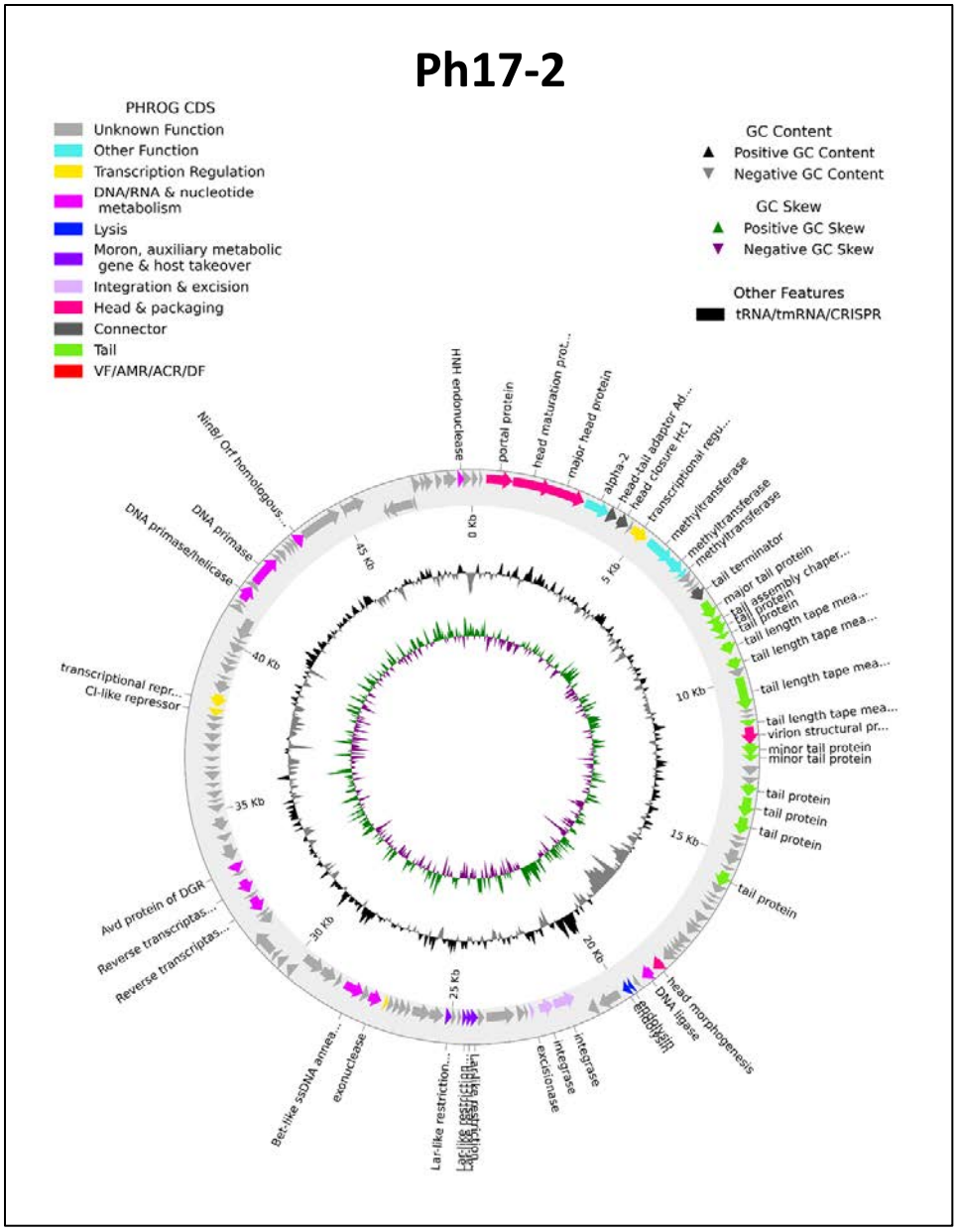

Figure 6.14. Genome map of phage Ph17-2 (*Detrevirus*), isolated against PAE. Pharokka with INPHARED was used for annotation and visualisation. CDS represented as arrows (functional genes in blue, pink, purple, green, yellow, and dark grey. Hypothetical proteins in grey). Inner rings show GC content and skew. Genome displayed circularly.

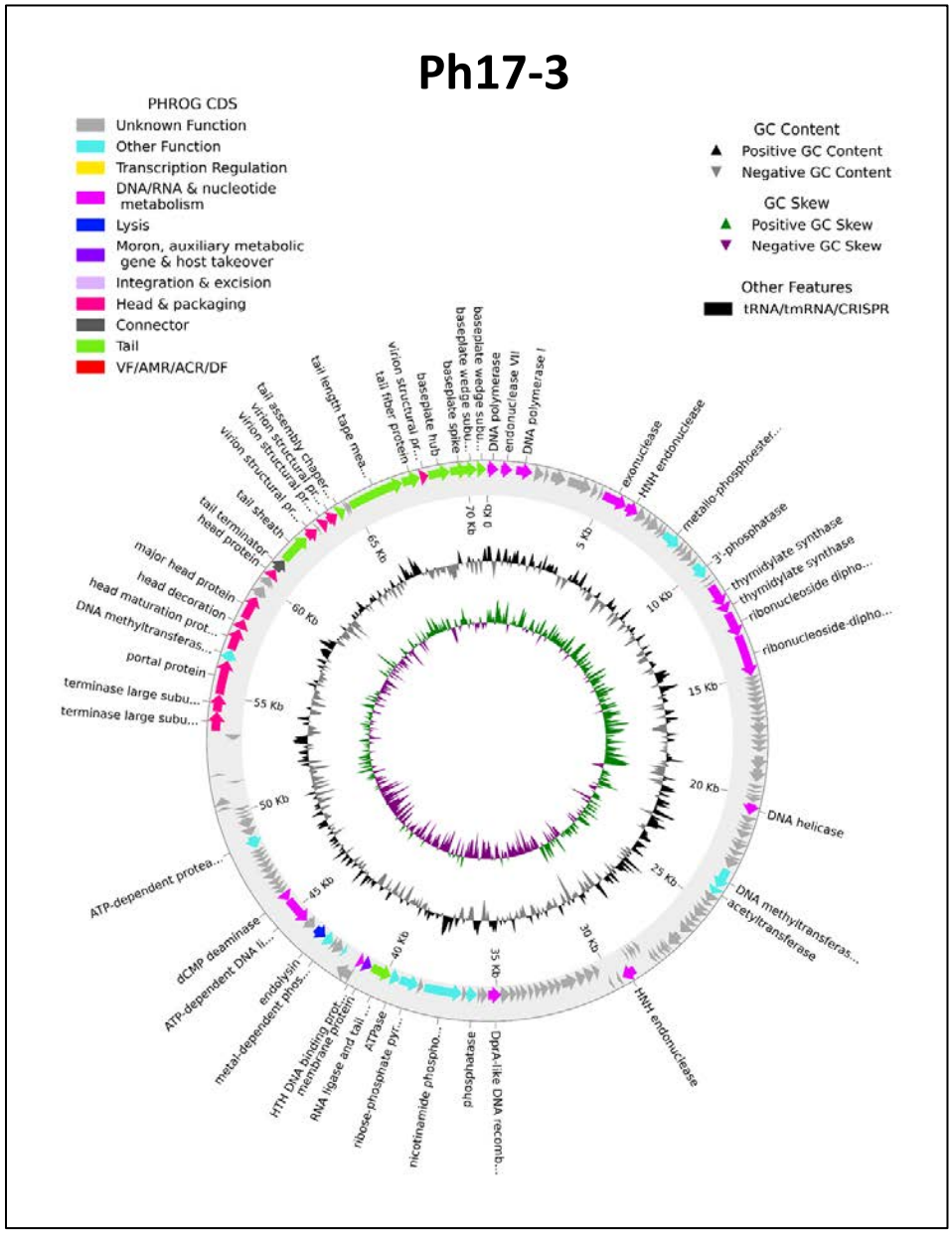

Figure 6.15. Genome map of phage Ph17-3 (*Myoviridae, Pakpunavirus*), infecting PAE. Generated with Pharokka and annotated against INPHARED. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). GC content (black) and skew (green/purple) in inner rings. Genome linear, shown circularly.

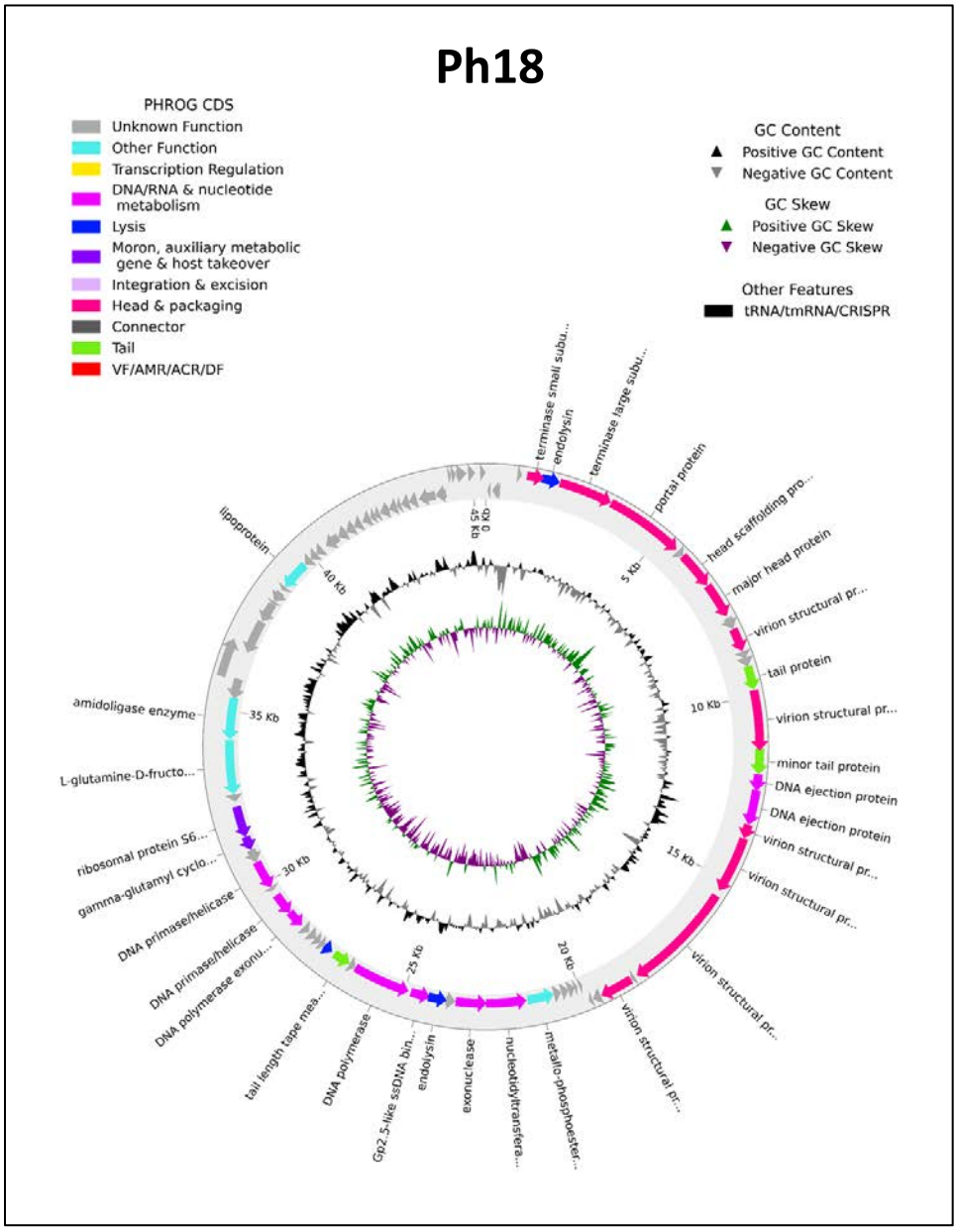

Figure 6.16. Genome map of phage Ph18 (*Podoviridae, Bruynoghevirus*), a PAE-infecting phage. Map generated with Pharokka, annotation via INPHARED. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings represent GC content and skew. Genome is linear but circularly presented.

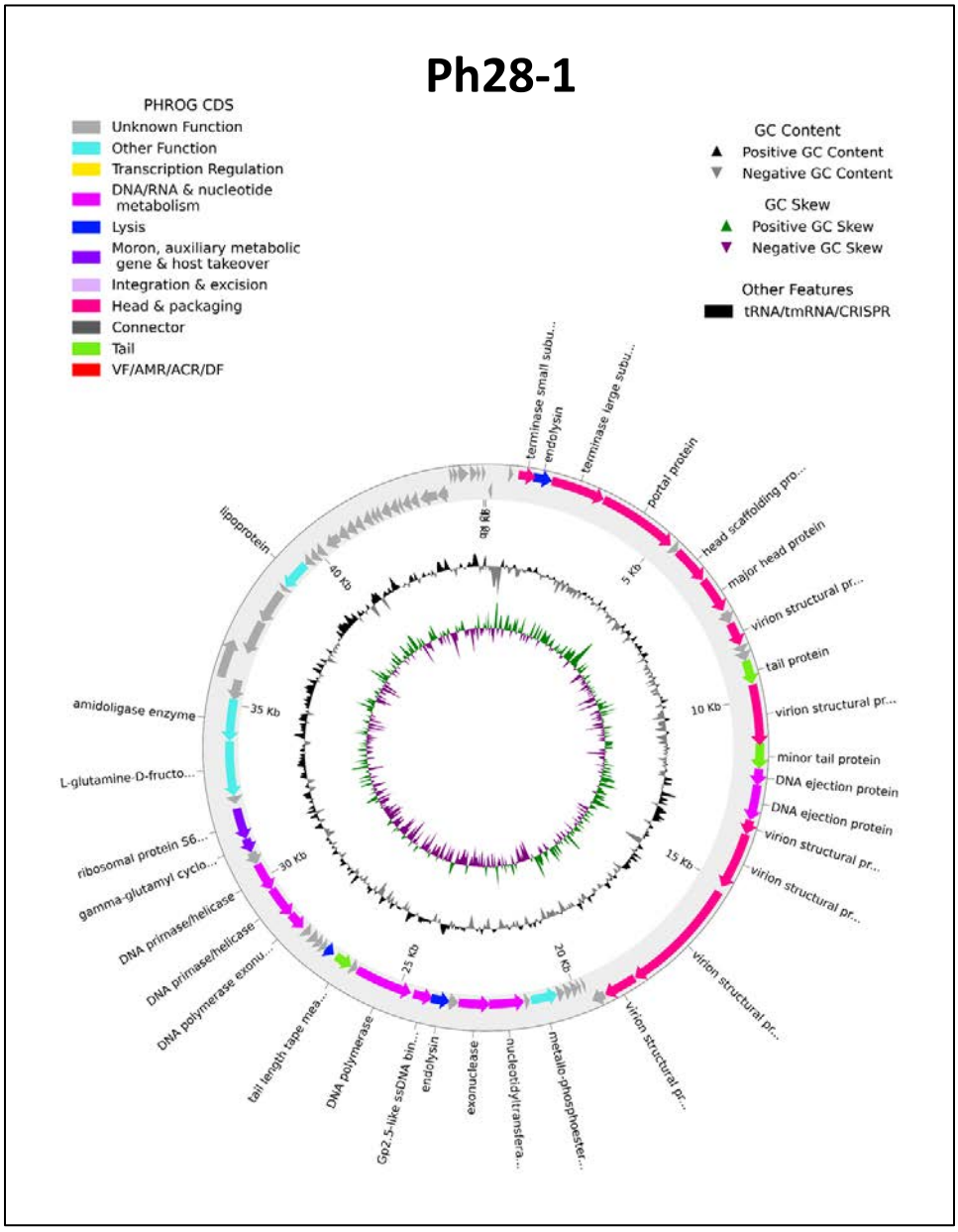

Figure 6.17. Genome map of phage Ph28-1 (*Podoviridae, Bruynoghevirus*), targeting PAE. Generated using Pharokka with INPHARED annotation. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings show GC content and skew. Genome linear, displayed circularly.

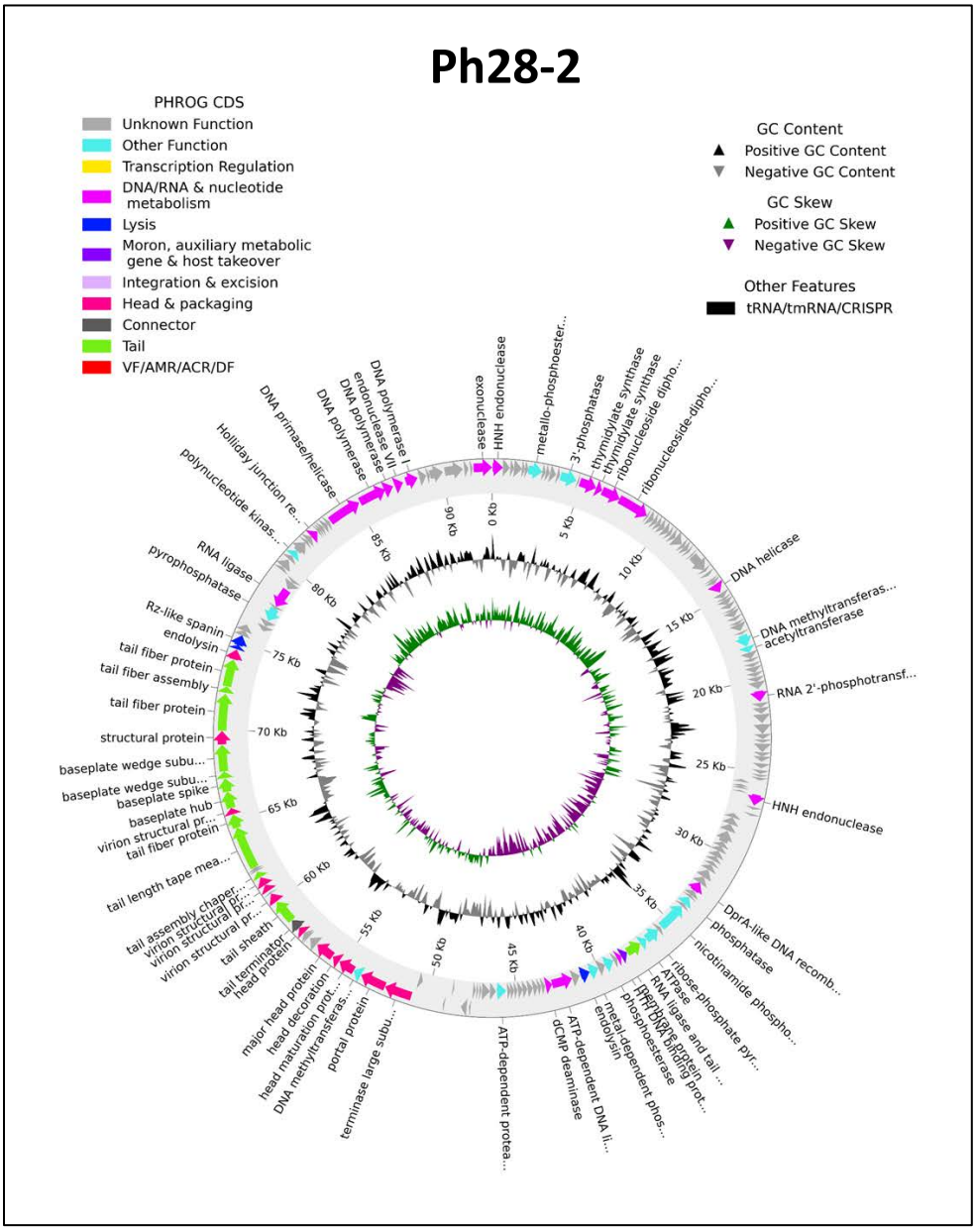

Figure 6.18. Genome map of phage Ph28-2 (*Myoviridae, Pakpunavirus*), infecting PAE. Visualisation and annotation performed with Pharokka and INPHARED. CDS shown as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings display GC content and skew. Genome is linear but circularly shown.

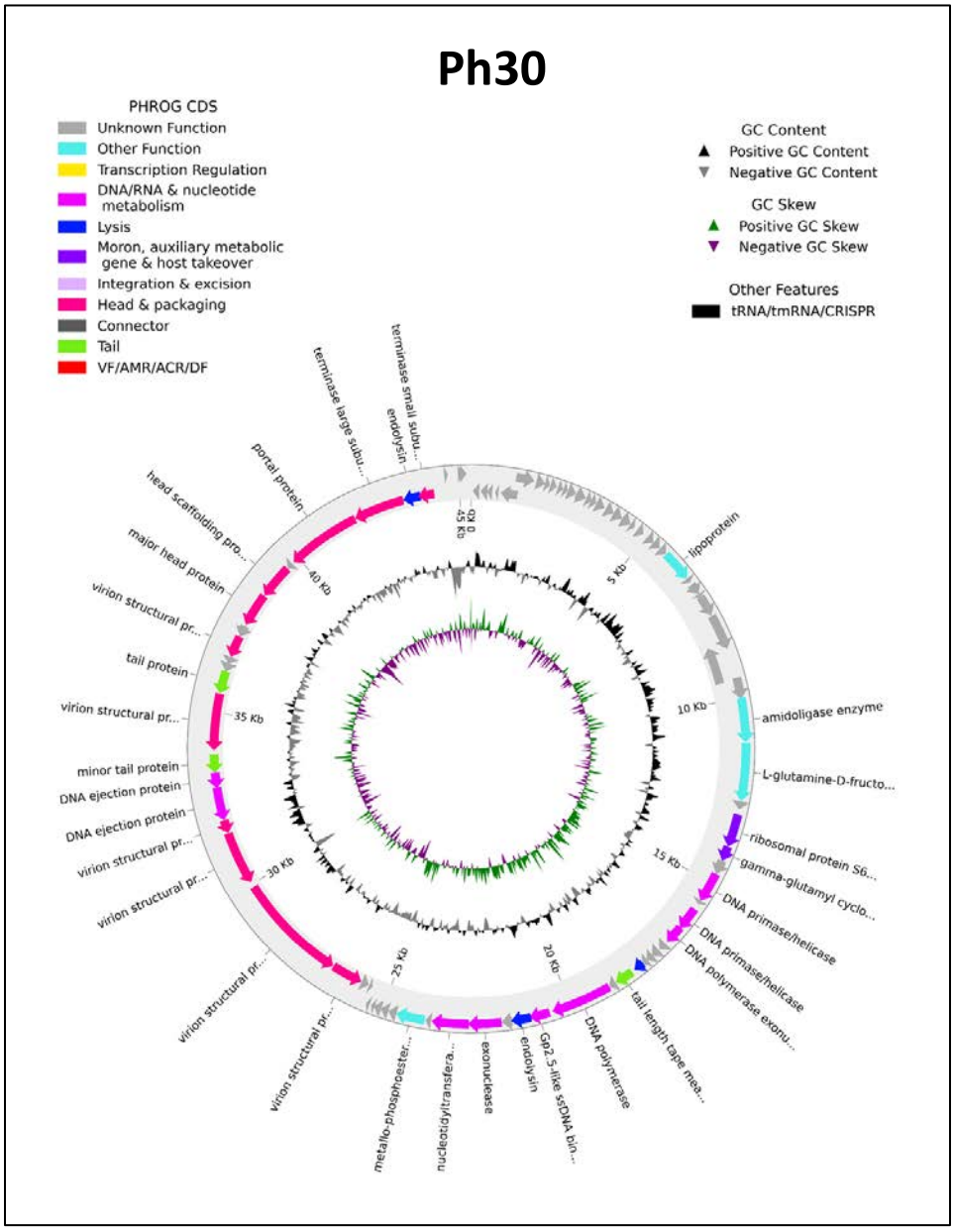

Figure 6.19. Genome map of phage Ph30 (*Podoviridae, Bruynoghevirus*), a PAE-infecting phage. Generated with Pharokka and annotated against INPHARED. CDS represented as arrows (functional genes in blue, pink, purple, green, and dark grey. Hypothetical proteins in grey). Inner rings show GC content and skew. Genome linear, visualised circularly.

6.4 Discussion

This chapter provides an in-depth characterisation of the phages used in this study, focusing on morphological classification through TEM, genomic sequencing, and subsequent gene analysis. The findings are significant for understanding the safety and applicability of phages for therapeutic use, particularly against PAE infections. Each method applied in this study contributes to assessing the phages' lytic potential, genetic safety, and classification accuracy, guiding the selection of suitable candidates for phage therapy.

Morphological Characterisation and TEM Insights

Transmission electron microscopy (TEM) confirmed that all observed phages belonged to the order *Caudovirales*, consistent with literature reporting that most isolated phages fall within this order (Ackermann, 1998, Sada and Tessema, 2024). However, unlike the literature indicating that the majority of phages isolated from wastewater tend to belong to the *Myoviridae* family (Gunathilaka et al., 2017, Hassan et al., 2021), the present study found a predominance of *Podoviridae*. This discrepancy could be attributed to specific isolation conditions or the host range specificity of the PAE strains targeted in this study. Such findings underscore the value of morphological characterisation combined with molecular methods to verify classification and enhance our understanding of phage diversity within different environments.

Interestingly, some phage concentrates (Ph5, Ph10, Ph17 and Ph28) presented multiple morphotypes, potentially due to overlapping plaque morphologies during isolation in spite of serial plaque isolation (Section 3.2.1). Such challenges underscore the need for refined isolation techniques and demonstrate the value of combining molecular analysis with morphology-based methods to verify phage identity and minimise the risk of misclassification.

Genomic Features Analysis

The sequencing of twelve phage genomes revealed insights into their genetic structure, potential virulence, and safety profiles. This analysis not only helps in characterising the phage but also addresses the critical concern of minimising the transfer of Minimise the risk of misclassification antibiotic resistance and virulence factors when applying phages for therapeutic use (Reindel and Fiore, 2017). The detection of certain lysogeny-related genes, such as integrases and recombinases, in phages like Ph8 and Ph17-1 suggests the presence of lysogenic elements, which could be a safety consideration for phage therapy due to the potential for horizontal gene transfer (HGT).

As an alternative to excluding phages with such genes, future studies could employ genome-editing techniques to remove or inactivate these elements. Techniques such as recombineering-assisted SpCas9 modification, which uses CRISPR-Cas9 with negative selection to eliminate parental phage genomes (Isaev et al., 2022), could enable precise genome modification. Other potential tools include CRISPR–Cas9-based bacteriophage genome editing, synthetic biology methods like CRISPY-BRED, CRISPY-BRIP, and targeted nucleases such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) (Mahler et al., 2023),(Usman et al., 2023). However, these processes, while promising, are beyond the scope of this current study but could be a future direction for ensuring therapeutic phage safety.

Circular vs Linear Genomes and Visualisation Challenges

In this study, linear genomes were mapped using a circular visualisation approach for consistency in data presentation. However, this approach may introduce ambiguity, particularly for linear genomes, as it does not account for phage packaging mechanisms, such as direct repeat regions, that can pose challenges in genome assembly. Studies have shown that Nanopore sequencing may not reliably assemble these repeat regions, potentially misrepresenting circular genomes as linear (Elek et al., 2023).

Following genome annotation, sequencing outputs were reviewed by a bioinformatician (Section 6.2.2.6) and some genomes initially interpreted as circular were subsequently confirmed to be linear after careful reanalysis. This issue has been previously reported in the literature, where phage genomes with complex termini or packaging strategies were initially misclassified due to limitations in assembly algorithms (Turner et al., 2021). In certain cases, adjacent genes with identical descriptors were identified, suggesting potential misalignments or sequencing artefacts.

In certain cases, adjacent genes with identical descriptors were identified, suggesting potential misalignments or sequencing artefacts. Further validation is necessary to confirm whether these occurrences represent distinct genes or artefacts due to base-calling errors. These findings underscore the need for additional polishing steps or alternative sequencing technologies to improve the precision of genome mapping, especially for genomes with direct repeat regions.

Future Applications and *in-vivo* Potential

These findings provide a foundation for further testing these phages *in-vivo* settings, particularly for treating ear infections in dogs, given the topical nature of PAE-related infections. Beyond otic applications, other topical infections caused by PAE could also be considered for treatment trials using these characterised phages. By testing these phages in a controlled *in-vivo* model, their efficacy, stability, and safety can be better evaluated, paving the way for their therapeutic application.

Limitations and Future Directions

This study, while comprehensive, has certain limitations. Due to time and resource constraints, seventeen phages were sequenced in detail, but some isolates could not be purified to exclude multi-phage presence. The observed discrepancies between TEM classifications and genetic data highlight the complexity of classifying phages accurately, especially those with similar morphologies or mixed-genome occurrences. Addressing these limitations in future work will be essential for advancing therapeutic phage development.

Refining isolation methods to enhance the purity of individual phage samples, possibly through improved plaque-purification techniques, would be beneficial. Incorporating alternative sequencing technologies or high-resolution polishing tools could also yield higher fidelity in genome representation, particularly for linear genomes. Moreover, employing genetic editing techniques, as previously discussed, could remove lysogenic or other undesired elements, ensuring therapeutic phage safety.

In conclusion, this section of the study provides a robust characterisation of PAE-targeting phages, setting a foundation for their safe therapeutic use. The absence of AMR and virulence genes, coupled with the genetic diversity of the characterised phages, offers promising candidates for treating PAE infections, especially in veterinary contexts. With continued refinement in phage isolation, genome validation, and possible genetic modifications, these phages hold substantial potential for future clinical applications.

CHAPTER 7: STORAGE STABILITY OF PSEUDOMONAS AERUGINOSA PHAGES

7.1 Introduction

Maintaining the functional and structural integrity of phages over time is essential for developing a robust phage therapy protocol (Fortier and Moineau, 2009). Effective preservation methods allow phage collections to be stored long-term without compromising their viability or infectivity, which is critical for both research and therapeutic applications (Pfeifer et al., 2021).

Various preservation techniques are available for long-term phage storage, including freeze-drying (lyophilisation), storage at -80°C, and refrigeration at 4°C (Gonzalez-Menendez et al., 2018, Ackermann et al., 2004). However, the efficacy of these methods is debated in the literature. For instance, while lyophilisation is often recommended for long-term storage, Ackermann and colleagues reported significant losses in phage titres due to ampule vacuum failures (Ackermann et al., 2004). In contrast, a more recent study by Zhenhe Xu (Xu et al., 2023b), supports lyophilisation as a viable method when properly managed, showing stability across a range of bacteriophages.

Phages stored at -80°C and 4°C have shown no significant differences in stability, suggesting both methods as feasible options for maintaining phage viability as also shown by Fortier and Moineau (Fortier and Moineau, 2009). This study aims to determine the most suitable preservation technique for *Pseudomonas aeruginosa* (PAE) phages at the James Cook University (JCU) facility, specifically for potential future therapeutic applications. Given the study's time constraints, three methods, lyophilisation, storage at -80°C, and refrigeration at 4°C were tested.

For this investigation, three phages (Ph2, Ph26, and Ph32) were selected from those isolated in Chapter 4, representing a diverse range of infectivity profiles and stability characteristics. This selection was based on their high lytic activity and suitability as potential candidates for therapeutic use. The preservation efficacy of each method was assessed over a ten-month period to identify the optimal conditions for long-term phage storage at JCU.

7.2 Materials and Methods

7.2.1 Storage Methods and Initial Phage Concentration.

Out of the 35 isolated phages described in Chapter 4, three phages (Ph2, Ph26, and Ph32) were selected for testing. These phages were chosen based on their high lytic activity, a crucial factor for therapeutic applications, and their representation of diverse infectivity profiles. This selection ensures the evaluation of storage methods across a spectrum of phage characteristics rather than limiting the study to a narrow subset.

The selected phages were individually propagated in LB broth (Section 3.2.3) to produce a master stock solution with a high infectivity titre (> 1×10^9 PFU/ml), as recommended for stability experiments (Gould, 1999). These titres ensure sufficient phage viability for downstream storage testing and comparative analysis. The propagated phages were centrifuged at 8,000 RPM for 10 minutes and then filtered through a 0.45 µm filter (Millipore Millex HV 0.45 µm, Section 3.2.3) for purification.

Aliquots were prepared by dividing the master stock solution into thirty 0.5 ml aliquots and sixty 0.25 ml aliquots, which were dispensed into 1.2 ml cryogenic vials (Corning®). The 0.5 ml aliquots were allocated for the fridge storage method, while the 0.25 ml aliquots were divided equally between the freezer and lyophilisation methods. This division allowed three replicates per phage for each storage condition to be tested monthly over a ten-month period. The aliquots were processed further according to the specific requirements of each storage method.

Quantification of surviving phage was conducted to ensure reproducibility across the methods. The phage titre for each aliquot was determined using the double agar assay method (Section 3.2.1). This method provides an estimation of plaque-forming units (PFU) in the samples. The dilution required for accurate plaque counting is directly indicative of the concentration of viable phages in the sample. For example, phage Ph2 required a maximal dilution of 10^{-16}, meaning its initial concentration was approximately $1x10^{16}$ PFU/ml. Similarly, Ph26 and Ph32 required maximal dilutions of 10^{-13} and 10^{-12}, corresponding to approximate initial concentrations of $1x10^{13}$ and $1x10^{12}$ PFU/ml, respectively. These measurements ensured that the starting titres for each phage were known, enabling meaningful comparisons across the different storage methods.

Although the three phages had different starting concentrations, these were precisely measured, allowing for comparisons relative to their respective initial titres. This approach facilitated the assessment of phage stability and viability across various storage conditions, accounting for the inherent differences in initial phage concentrations.

7.2.1.1 Fridge Storage (2-4˚C)

Storing phages at 2 - 4°C has long been recognised as a standard method for preserving phage viability over extended periods. For this study, the fridge storage protocol was adapted with modifications based on Bonilla et al. (Bonilla et al., 2016). Briefly, bacteriophages suspended in LB broth were aliquoted into 0.5 ml cryogenic vials, labelled with the phage code and processing date, and stored in a light-protected container at 4°C until use.

7.2.1.2 Freezer-80 ˚C

The storage in the freezer at -80˚C was done as per the freezer method of Bonilla (Bonilla et al., 2016). Glycerol to a final concentration of 50 % [v/v] was added as a cryoprotectant to 30 of the 0.25 ml phage solutions. These aliquots were then stored in a light-protected container labelled with the phage code and date of elaboration and placed in a freezer at -80˚C.

To prepare the samples for testing, the frozen solutions were thawed at room temperature for 20 min and gently mixed by pipetting.

7.2.1.3 Lyophilisation and Freezer-80˚C

The storage method by using lyophilised bacteriophage solutions was done as per the method of Ackermann (Ackermann et al., 2004). Glycerol to a final concentration of 50% [v/v] (Appendix 1) was added as a cryoprotectant to 30 of the 0.25 ml phage solutions, resulting in a 0.5 ml phage-glycerol mix. These aliquots were then stored at -80˚C for 24 hours and then freeze-dried (Scanvac, LabGear, Australia) over a period of 6 hours. After that, aliquots were stored in a light-protected container labelled with the phage code and date of elaboration and placed in a freezer at -80˚C until use.

To prepare the samples for testing, the lyophilised solutions were resuspended in SM buffer until the volume reached 0.5 ml to recover the original volume.

7.2.2 Statistical Analysis

To determine any significant differences in phage titres among storage conditions and time, the Log_{10} values of bacteriophage titres were subjected to statistical analysis using the GraphPad Prism 10 software (GraphPad Prism version 10.0.3 for Windows, Boston, Massachusetts, USA).

All assays were performed in triplicate, but the initial reading was done in duplicates. The lyophilisation method yielded poor results due to the lack of stability presented by this method. Therefore, this method was discarded from the statistical analysis. The phage titres obtained from the fridge and freezer methods were compared and expressed as the mean ± standard deviation (SD). For statistical significance determination, Student's t-tests were used, with a significance level of $*p \leq 0.05$.

7.3 Results

The three selected phages (Ph2, Ph26, and Ph32) were counted in triplicate each month for each storage method, and the mean counts are expressed in $\log_{10}$ (PFU/ml) values in Figure 7.1, where the standard deviation is also shown.

After the reconstitution of the vials for the lyophilisation method, which included the addition of SM buffer, titres were extremely low or no viable phages were found on the counting plates for all methods. Due to poor results for the lyophilisation method and the lack of data to be able to be compared with other methods, this process was only repeated for the first four months and then excluded from the statistical analysis. The results obtained for this method were as follows: For phage Ph2, no plaques were counted for the second, third, and fourth months. In the case of Ph26, readings in the order of four Log_{10} for the second month tested and five Log_{10} for the third month were detected, representing a drop in concentration/viability of ten $logs_{10}$ from the initial reading. A similar situation was found for Ph32 (Figure 7.1).

When analysing the data for the fridge and freezer methods, some of the replicate vials did not yield plaques in the double agar essay at the working concentration required for phage therapy. This was considered a failed storage; however, there may have been phage at a lower concentration remaining. This limits accurate statistical analysis as these “no phage” vials must be reported as 0 PFU/ml for the statistical analysis, which results in very large standard deviations and changes the mean (Figure 7.1).

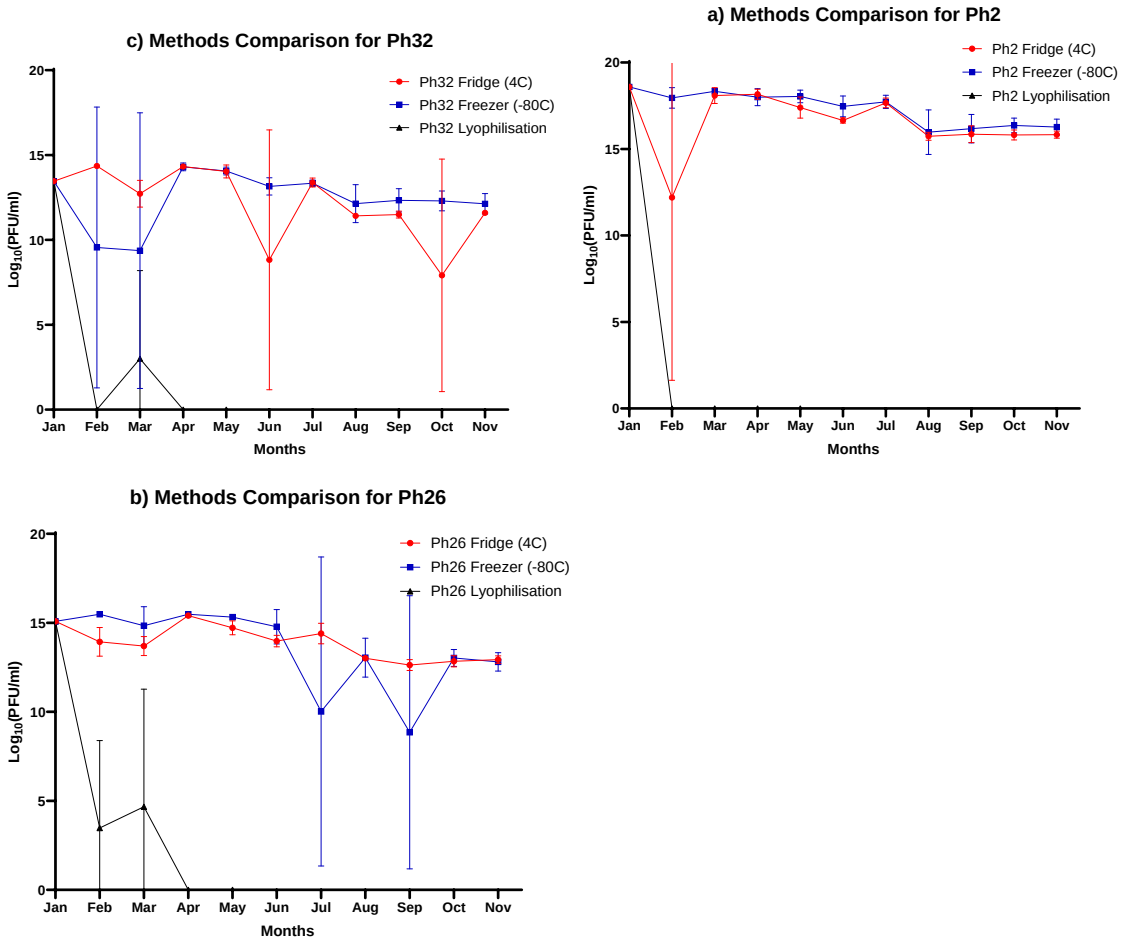

Figure 7.1: Stability in ten months period of three bacteriophage solutions using three different methodologies. [a] bacteriophage Ph2; [b] bacteriophage Ph26; and [c] bacteriophage Ph32. Vertical bars represent standard deviation of the means. Failed vial data is included and recorded as 0.

The number of occurrences of the extreme values (0 PFU/ml) obtained from the fridge and freezer methods in the ten-month period were also analysed. Ph2 was found to be the most stable among the three phages tested since only one out of thirty reads of 0 PFU/ml was obtained for the fridge method, this indicates its better performance. However, phages Ph26 and Ph32 had two out of thirty (0 PFU/ml) values obtained for the freezer method, and Ph32 had two out of thirty (0 PFU/ml) values obtained for the fridge method. This suggests that the freezer method was more unstable during these ten months for Ph26 and Ph32, (Figure 7.2).

If these 0 PFU/ml vials are excluded from analysis, the freezer (-80°C) method and fridge method were equally effective, with all phages remaining relatively stable until seven months, after which there was a 2 $\log_{10}$ drop in concentration followed by a lower level of stability. All data that was recorded as 0 is listed in Table 7.1. Of note, Ph2 had only one early failed vial in the fridge method, while Ph26 only had 2 failed vials late in the process in the freezer method. Ph32 had failed vials in both methods throughout the process for a total of 4 failed vials.

Table 7.1: Inconsistent readings for the fridge and the freezer methods.
All failed vials relative to the total number of vials tested are recorded as failed vials/total vials, e.g. 1/3.

Month/Test	Fridge (2-4°C)	Freezer (-80°C)
January		
February	1/3 vial was counted as 0 PFU/ml for Ph2	1/3 vial was counted as 0 PFU/ml for Ph32
March		1/3 vial was counted as 0 PFU/ml for Ph32
April		
May		
June	1/3 vial was counted as 0 PFU/ml for Ph32	
July		1/3 vial was counted as 0 PFU/ml for Ph26
August		
September		1/3 vial was counted as 0 PFU/ml for Ph26
October	1/3 vial was counted as 0 PFU/ml for Ph32	
November		

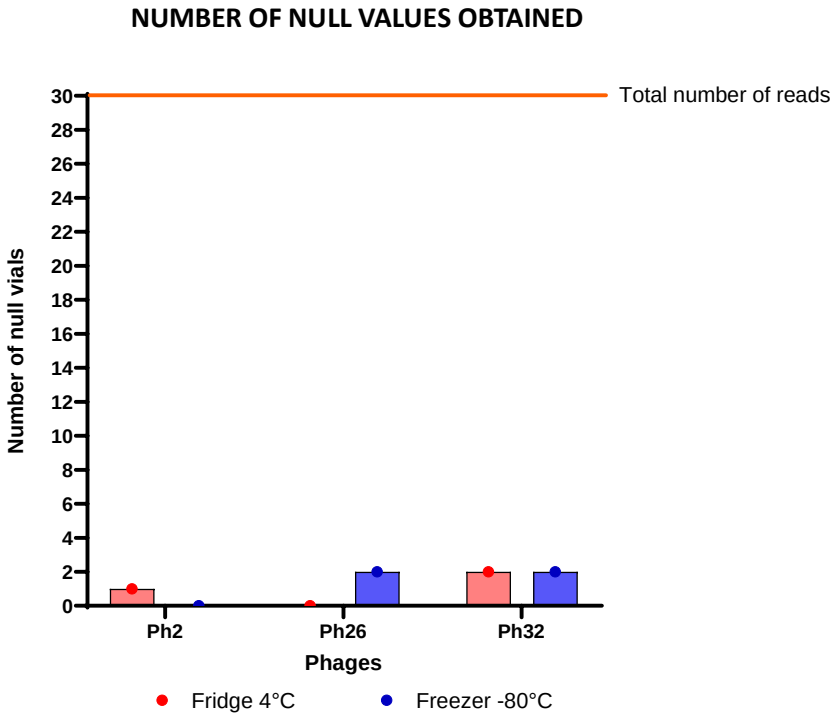

Figure 7.2: Number of occurrences of the extreme values (0 PFU/ml) obtained from the fridge and freezer methods in a ten-month period. The orange horizontal bar represents the total number of reads (30) in a ten-month period.

To better understand the trends in storage stability, the data were also analysed separately, excluding the 0 PFU/ml values observed for the fridge and freezer methods in February for Ph2, July and September for Ph26, and February, March, June, and October for Ph32 (Figure 7.3). While excluding these outliers allows for a clearer interpretation of general trends, it is acknowledged that this approach is not statistically rigorous and reflects the need for additional replicates to achieve greater reliability in future analyses.

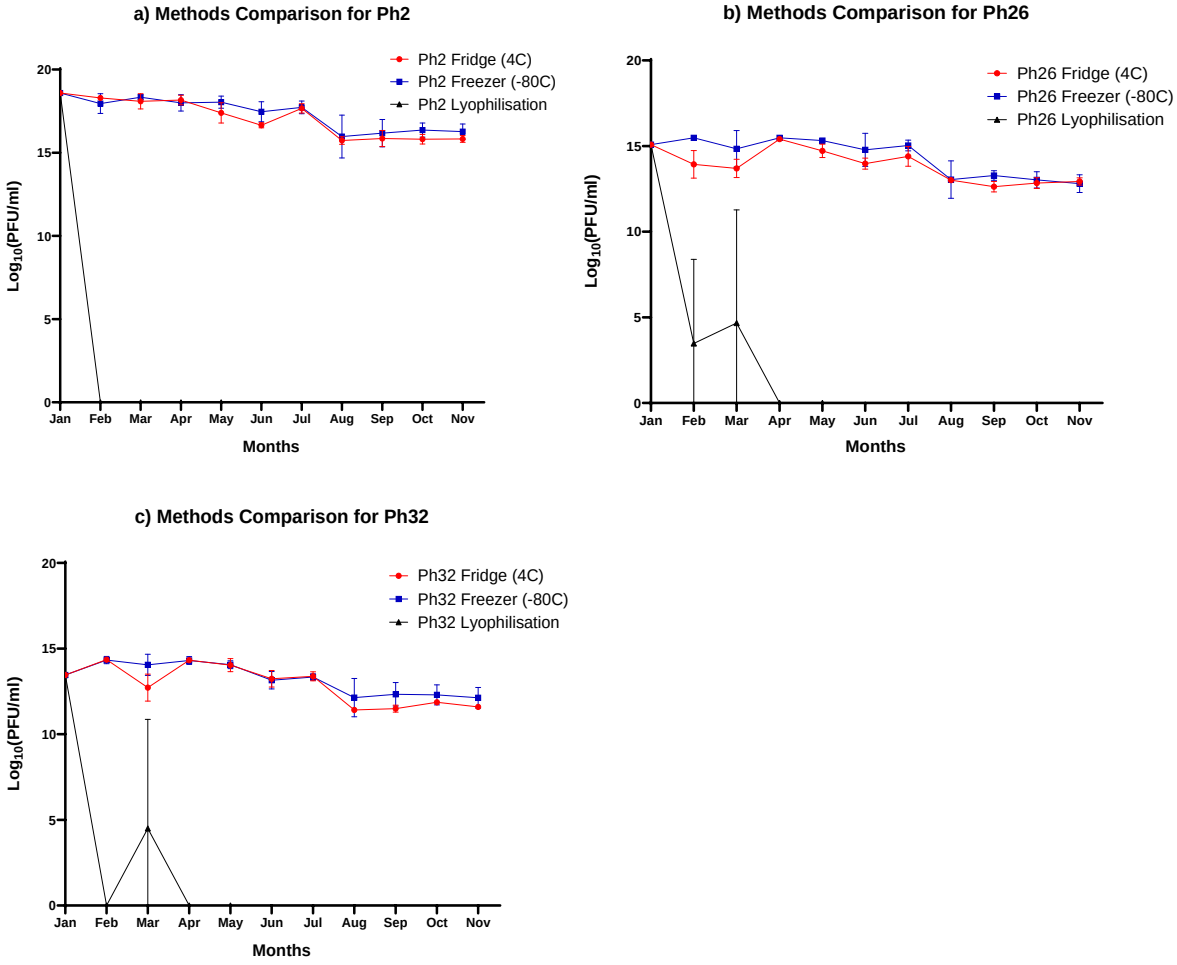

Figure 7.3: Stability of three bacteriophage solutions using three different methodologies with failed vial data removed. [a] bacteriophage Ph2; [b] bacteriophage Ph26; and [c] bacteriophage Ph32. Vertical bars represent the standard deviation of the means.

As the method using lyophilisation was rejected before analysis, the fridge and freezer (-80°C) data is presented in Figure 7.4. The scale has been altered to observe variation. For each phage, there were no significant differences between the two methods (a) phage Ph2; p=0.1385, b) phage Ph26; p= 0.2618, c) phage Ph32; p= 0.4484).

However, there was approximately one log_{10} decrease in titre for Phages Ph2 and Ph32, and two log_{10} decrease in titre for phage Ph2 after the sixth month of the tests. From the sixth month until month ten, the titres remained relatively stable for each phage.

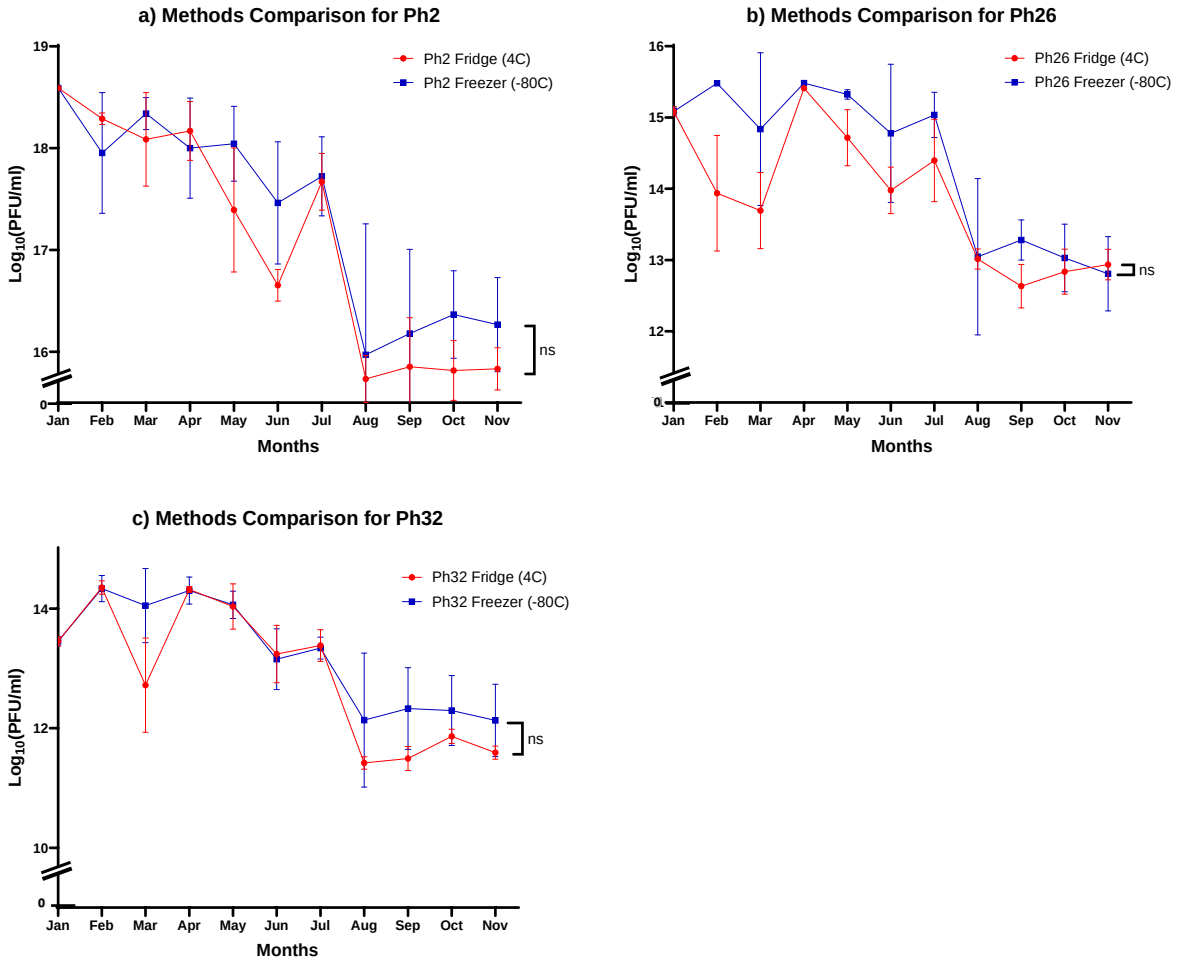

Figure 7.4: Stability in a ten-month period of three bacteriophage solutions using two different methodologies fridge (2-4°C) in red colour and freezer (-80°C) represented in blue colour; [a] bacteriophage Ph2; [b] bacteriophage Ph26; and [c] bacteriophage Ph32. Vertical bars represent the standard deviation of the means. Failed vial data is not included.

7.4 Discussion

The stability of three storage methods that included, lyophilisation (with storage at -80°C), fridge (4°C), and freezer (-80°C), were compared over a ten-month period for three selected phages (Ph2, Ph26, and Ph32). In order to obtain more accurate results high titre phages were developed as suggested by E. Gould (Gould, 1999). From the phage stock solutions, triplicate samples were prepared for each month and were kept at their respective storage temperatures until the samples were needed for a spot assay. Hence, each stored vial/cryotube underwent one spot assay under identical conditions to ensure consistency. Even though those precautions were taken into account for this work, some measurements failed since they were below the working phage concentration of $1x10^6$ PFU/ml (Section 5.2.2.2), considering this outcome as 0 PFU/ml. The reasons behind the failure in some readings, particularly observed with the fridge and freezer storage methods, may be attributed to the inherent instability of temperate phages, which are known to exhibit lower lytic efficiency and reduced detectability under certain conditions (Lillehaug, 1997). This is consistent with previous findings that highlight the challenges of propagating and preserving such phages, including their structural fragility, genetic instability, and heightened sensitivity to environmental stresses (Tovkach et al., 2012).

Regardless of their host, taxonomic position, or morphology, tailed phages are often considered stable, and those with large capsids measuring 100 nm in diameter exhibit high stability (Ackermann et al., 2004, Puapermpoonsiri et al., 2010). Among experimental models, phages belonging to the T series, the λ group, and ϕ29 and its relatives have demonstrated remarkable resilience, maintaining viability for a decade or more. The T4 and T7 phage groups have also been observed to be particularly resistant compared to other phage types (Burnet, 1933). In our study, the phages characterised via transmission electron microscopy (TEM) were classified within the order *Caudovirales*. From the three phage morphotypes selected for this experiment, only Ph2 underwent characterisation via TEM and by sequencing due to time and budget constraints. Ph2 belongs to the *Podoviridae* family (short-tailed) (Section 6.3.3.1). Future research endeavours will aim to determine the familial classification of Ph26 and Ph32.

It has been demonstrated that low-temperature preservation, including temperatures ranging from 2-4°C, is a reliable method for conserving phages in a long-term period (Ackermann et al., 2004, Gonzalez-Menendez et al., 2018). This work matches the description of previous reports, considering the fridge method suitable for future use under JCU laboratory conditions. The difference with previously mentioned works is that they used SM buffer as a preservative, and this work used LB broth. The choice of preservative is critical as it impacts the osmotic balance and nutrient availability, potentially influencing phage viability. Further comparative studies are recommended to determine whether LB broth offers distinct advantages over SM buffer in specific applications.

For the freezer (-80°C) method and using glycerol 50% v/v as a cryoprotectant, Ackermann reported to obtained disappointing results, which may be due to the inactivation of phages after faster freezing or due to rapid thawing, compared to the fridge method (4°C) (Ackermann et al., 2004). In contrast to Ackermann's results, our experiments with the -80°C method and using the same preservative demonstrated similar performance to the fridge method. Similar results to ours were obtained by Fortier, who used glycerol 15% v/v as a cryoprotectant and suggested long-term viability without specifying exact durations, and Xu, who demonstrated effective phage stability over a 12-month period using 10% dimethyl sulfoxide (DMSO) and 30% glycerol as cryoprotectants (Fortier and Moineau, 2009, Xu et al., 2023a).

A study on phage storage conditions using sugars as cryoprotectants, such as sucrose or trehalose, demonstrated enhanced stability over 56 weeks at three temperatures (4°C, -20°C, and -80°C) (Mazumder, 2022). In contrast, our study used glycerol (50% v/v) as the cryoprotectant for the freezer and lyophilisation methods (-80°C) and LB broth for the fridge method (4°C), rather than the sugars employed in Mazumder's work. Although different cryoprotectants were used (glycerol in this study versus sucrose or trehalose in

Mazumder's work), the underlying principle remains that an effective cryoprotectant, whether a sugar or polyol, is essential for protecting phages during low-temperature storage by minimising ice crystal formation, osmotic stress, and desiccation damage.

The lyophilisation method is considered an important technique mostly for pharmaceutical companies (Bhosale et al., 2021), this method presents several limitations when used for conserving phages. An essential factor in the stability of the lyophilisation method is using a preservative to protect the sample from the harsh conditions this method entails. Although there are a vast number of preservatives available for use, only glycerol 50% v/v was used as per the method of Ackerman (Section 7.2.1.2) due to time limitations. Lyophilising presents a time-consuming process that increases handling and processing times, which may limit its scalability for routine laboratory or industrial applications (Jameel et al., 2021). The equipment used for lyophilisation is not only complex and expensive but also requires careful calibration and maintenance to ensure reproducibility and effectiveness (Bhosale et al., 2021). Stability issues have been reported, particularly when phages are exposed to harsh desiccation conditions, leading to the loss of structural integrity or infectivity (Ackermann et al., 2004, Bhosale et al., 2021). These factors limit the method's practicality for routine or large-scale use, particularly in resource-constrained laboratories. Inadequate calibration or inappropriate conditions during lyophilisation can result in substantial loss of phage viability, compromising downstream applications such as therapeutic development or long-term archiving.

From the lyophilisation method, very poor results were obtained. This may be due to the cryoprotectant used in this case or may be the phages used may not resist the freeze-dry protocol. This protocol includes leaving the vials open inside the dry-freeze chamber. Some works suggested the use of an encapsulation technique, using trehalose or whey protein, to protect and improve phage stability in the harsh conditions of the freeze dryer (Petsong et al., 2021). When we finalised the lyophilisation procedure, it was noticed that the vials were oily around the surface, and the volume of the vials was reduced unequally after the set time. This inconsistency in volume meant that when reconstituting the vials to 0.5 ml with SM buffer, varying amounts of buffer had to be added to each vial. This led to variability in the final phage concentration among the vials, introducing a higher range of error during titre measurements. In line with our findings, Ackermann classifies this method of conservation as "a failure" since they obtained a quick decrease in the concentration of phages during the first year of storage and had difficulties when handling the materials used (Ackermann et al., 2004). On the other hand, Puapermpoonsiri demonstrated in his study that phages suspended in SM buffer were stable to lyophilisation in the presence of 0.5 M of sucrose (Puapermpoonsiri et al., 2010). In this line of research, other investigators agreed that this is probably the most satisfactory method of preserving viruses for very long periods, but this will depend on the variations in the technical procedures, along with the specific design of the freeze-drying equipment and the cryoprotectant selected (Gould, 1999). If this work were to be repeated, lyophilisation using whey protein, trehalose or sucrose as a cryoprotectant, such as used by Puapermpoonsiri rather than glycerol, will be put in place, as will a control lyophilisation with no preservatives.

The comparable performance of the fridge and freezer storage methods suggests that both are viable options for maintaining phage stability under laboratory conditions. Despite a reduction of approximately 2 $\log_{10}$ in phage titres over ten months, these methods preserved phages at concentrations sufficient for practical applications. The absence of statistically significant differences between the two methods highlights their similar efficacy in preserving phage viability. However, given the observed titre reduction after ten months, further studies are necessary to explore the long-term stability of these methods, particularly beyond the ten-month timeframe. Such research could provide critical insights into optimising storage protocols for extended durations, especially for clinical or therapeutic applications.

CHAPTER 8: GENERAL DISCUSSION

Introduction

The discovery and use of antibiotics have tremendously helped human and animal health. However, antibiotic resistance represents a considerable challenge to physicians and veterinarians when solving "simple" bacterial infections. Consequently, new treatment strategies are needed to overcome this issue. Phage therapy emerges as a promising alternative in this context (Harper et al., 2011, Abdul Wahid, 2015). The use of phages, or viruses that infect bacteria as a treatment option is not a new concept. In fact, it started in the early 20th century with D'Herelle and Twort (d'Herelle, 1917, Abdul Wahid, 2015). Many studies have been conducted since then (Altamirano and Barr, 2019), but now, under the increasing pressure for new therapies against antibiotic-resistant bacteria, phage therapy offers hope for the treatment of these harmful bacteria.

Pseudomonas aeruginosa (PAE) is a pathogen that represents a serious threat to both human and animal health. This bacterium has endogenous genes that confer natural resistance to some antibiotic groups, such as aminoglycosides, fluoroquinolones, B-lactams, and tetracyclines (Pang et al., 2019). Moreover, it has the ability to acquire additional genetic material, which enhances its antibiotic resistance. This bacterium also has the ability to produce biofilms, which increases its chances of surviving in the host environment. Bacteria in biofilms are difficult to treat since most antibiotics cannot penetrate this biological structure. Some phages are able to penetrate and destroy biofilms that these bacteria produce (Harper et al., 2014). This is one of the reasons why lytic phages can work synergistically with antibiotics (Gordillo Altamirano et al., 2021). PAE is the most common bacterial cause of chronic otitis externa in dogs (Bourély et al., 2019). This study used 48 strains of PAE isolated from infections in the external ear of dogs and stored at the JCU diagnostic facility.

Comparison with phages isolated from different locations.

Bacteriophages can be found in the same environment as the bacteria they target are located. Since PAE can be found in contaminated soil and water reservoirs, the sample collection plan was designed to look for PAE phages in these locations and confirm these as suitable locations.

Phages were isolated from two of the built-contaminated environments: the Mount St John wastewater treatment plant and the vet hospital cage drainage. In contrast, isolation of phages was not feasible in other built environments, such as the JCU Vet Necropsy room, which uses a high number of disinfectants and other cleaning chemicals. Similarly, no phages were isolated from the natural environment of the Ross River during this study. While this suggests that highly sanitised environments like the JCU Vet Necropsy room may not be ideal for phage collection, it is premature to conclude that natural environments such as rivers and soils are unsuitable for phage isolation. Many studies have demonstrated the successful isolation of phages from natural environments, including soils and water. Future studies testing a broader range of natural environments and varying sampling conditions may provide a clearer understanding of their potential as sources of antipseudomonal phages.

Nine out of ten bacterial strains used as screening for the wastewater treatment plant were susceptible to at least one of the bacteriophages in the sample. The sampling protocol of this site includes a sample change every 24 hours. The literature indicates phage populations change rapidly at wastewater sites

(Gulino et al., 2020). It is likely that taking a daily aliquot might eventually provide phages capable of attacking all PAE tested. This project only involved samples being taken on two occasions, with the second sampling adding two phages to the phage collection (only those PAEs that did not supply phages were utilised for the screening process). A total of 22 unique phage were collected from the wastewater treatment plant. While the Mt. St. John treatment plant is primarily sourced from human waste, animal waste can also be present through drainage systems, and this may assist in isolation of phage that are effective against animal isolates of PAE. On the other hand, six out of ten PAE used as screening provided phages for the sample collected in the vet hospital cage-drainage. This led to a total of 13 phages in the library from this source. Of note, this was based on swab samples rather than water samples, so direct comparison of total phage numbers is limited.

The diversity measured in host ranges was compared among the two location sites where phages were isolated. Most phages with the widest host range were obtained from the JCU Veterinary Hospital. The PAEs used for screening were obtained from ear infections in dogs, which could partially explain the higher affinity of the phages isolated from the vet hospital to these strains compared to those obtained from Mt. St. John. Although the number of phages isolated from the vet hospital was lower than that from Mt. St. John, the quality of the veterinary hospital phages was seen to be better. However, this observation might also depend on the epidemiology of PAE at each location, as differences in local bacterial populations could influence the specificity and effectiveness of the isolated phages. This indicates that when looking for phages to treat a particular infection, in the case of otitis externa in dogs, going to a veterinary hospital will be the best option if collection from only one site is possible. In the end, both sites were proven to be excellent sources of bacteriophages, and both can be considered for future collections, both against PAE and other potential target bacteria. These findings align with the project's objectives of identifying optimal sampling sites for phage isolation and establishing a robust library of phages for therapeutic applications. This achievement underscores the importance of targeted sampling strategies in maximising phage yield and diversity.

Phage Cocktail Formulation

After the selection of isolated phages for cocktail formulation, *in-vitro* studies were conducted. First, the double agar assay was used to compare the killing range of individual phages versus the killing range of cocktails. Secondly, the biofilm tests were performed, and four PAEs with different sensitivities to phages were exposed to different cocktails. The number of phages included in the formulation ranged from 5 to 30. The rationale for using higher numbers of phages in cocktails was the limited literature available on large-scale phage cocktails (Jault et al., 2019), and no specific practical upper limit was found. However, increasing the number of phages in a cocktail introduces several challenges, including antagonistic interactions or lack of synergy between phages, difficulties in preparation, and stability issues since greater diversity increases the likelihood of interactions between phages during treatment. These factors should be carefully addressed in future studies to optimise cocktail design. Verification assays, such as kill curves, which measure the rate and extent of bacterial lysis over time, can help assess the dynamics and potency of phage activity (Storms et al., 2020). Similarly, efficiency of plating (EOP), which evaluates the relative ability of a phage to form plaques on different bacterial strains compared to its original host, can be used to quantify host range and detect potential cross-infectivity or resistance patterns (Kropinski et al., 2009). These tools provide valuable insights into phage-host interactions and can enhance cocktail development strategies.

After examining bacterial clearance using the spot assay technique, it was found that the expected clearance was not always achieved when using phage preparations. In some cases, combining different phage preparations resulted in antagonistic effects, reducing the bacterial load less effectively than individual phages. Antagonistic interactions may arise from competition for the same bacterial receptor, inhibition of phage replication by other phages, or interference in adsorption kinetics (Bull and Gill, 2014, Scanlan et al., 2015). Conversely, specific interactions between cocktails and bacterial strains had a synergistic effect, likely due to enhanced bacterial lysis by phages targeting different receptors or pathways (Chan and Abedon, 2012). These observations suggest that factors such as receptor overlap, phage-host specificity, and replication dynamics need to be considered when preparing cocktails with a high number of phages to control PAE infections. Understanding phage-host specificity is particularly critical for designing targeted therapies, as phages with complementary host receptor specificities are more likely to act synergistically, improving therapeutic outcomes (Oechslin et al., 2017). For instance, leveraging phages with diverse receptor targets could help overcome bacterial resistance mechanisms, enhancing the efficacy of phage cocktails in clinical settings. Additionally, after testing the cocktail with 20 phages in the formulation, no improvement in effect was observed. A decrease in cocktail effectiveness was noted with the cocktail containing 30 phages compared to cocktails containing 10, 15, 20, and 25 phages in the formulation. These findings indicate the importance of optimising phage diversity and number in cocktail design to maximise efficacy and avoid potential antagonistic effects.

In the biofilm test, and in contrast to the double agar assay, the optimal number of phages in the cocktail formulation was found to be 10. A plateau effect was observed until cocktail 25, after which adding more than 25 phages did not enhance bacterial clearance and, in some cases, even caused a rebound effect. Although this study hypothesised that the saturation point may result from phages sharing the same receptor sites, further investigations are necessary to confirm this mechanism. For instance, the use of mutant libraries could help identify bacterial receptor variants associated with phage susceptibility. Additionally, whole-genome sequencing (WGS) of both PAE strains and phages could reveal the receptors targeted by specific phages. Such studies would provide critical insights into the molecular basis of phage-host interactions, enabling more rational design of phage cocktails in future research.

The biofilm assay results suggested three critical phases. First, a minimum number of phages is required to initiate a reduction in the biomass and activity of the biofilm. Second, a sufficient number of phages can achieve the maximum reduction or plateau of the biofilm. Finally, an excessive number of phages can lead to a rebound effect on biofilm growth. The exact number of phages required to reach these phases is likely strain-dependent, and further research to identify the cellular and molecular differences between PAE strains could help elucidate the mechanisms involved.

A key factor influencing phage efficacy against biofilms is their ability to penetrate the extracellular matrix (ECM) of the biofilm. The ECM poses a significant barrier to phages, as its dense and heterogeneous structure limits phage mobility and accessibility to bacterial cells (Sutherland et al., 2004; Harper et al., 2014). This suggests that future work should focus on determining how specific phages interact with and degrade the ECM. For example, enzymatic degradation of biofilm components by phage-encoded depolymerase could enhance penetration and improve efficacy (Cornelissen et al., 2011). Further examination of whole-genome sequencing could identify phage genes encoding such enzymes, and targeted experiments could assess their activity and contribution to biofilm reduction. Additionally,

advanced imaging techniques like confocal laser scanning microscopy (CLSM) could be used to visualise phage penetration and biofilm disruption in real-time (Gutiérrez et al., 2017). By integrating these approaches, future studies could better understand the interplay between phage characteristics, ECM composition, and biofilm dynamics, enabling more effective phage therapy strategies against PAE biofilms.

Phage Characterisation

To characterise the phages used in the cocktails, both TEM and genome sequencing were conducted. TEM provided morphological insights and confirmed the purity of the phage samples, while genome sequencing assessed the presence of antimicrobial resistance (AMR) genes and other genetic elements. Developing a phage library requires a comprehensive understanding of the isolated phages to ensure their safety and therapeutic efficacy (Modi et al., 2013). This study successfully sequenced twelve phage genome solutions, revealing significant diversity. However, challenges in isolating individual phages led to the identification of multiple distinct phage genomes within some solutions, such as Ph5, Ph10, Ph17, and Ph28. The sequenced genomes ranged from 42 kb to 94 kb in length, with GC content varying between 49% and 62%. Most phages were classified within the families *Podoviridae* and *Myoviridae*, encompassing genera such as *Phikmvvirus*, *Bruynoghevirus*, *Samuniavirus*, *Detrevirus*, and *Pakpunavirus*.

Genomic analysis revealed no AMR genes, virulence factor genes, anti-CRISPR spacers, or defense genes across the sequenced genomes, affirming their therapeutic potential. Only one phage genome (Ph5) contained a recombinase, while no transposases were detected. These findings emphasise the safety of the phages tested in this study for therapeutic applications.

Despite the absence of AMR genes in this dataset, vigilance is required as phages have been reported to carry genes such as ‘bla’, ‘tet’, and ‘erm’ in other studies (Colavecchio et al., 2017, Enault et al., 2017). Advanced genomic screening and targeted bioinformatic approaches will continue to play a critical role in ensuring the safe application of phages in therapy. Moving forward, the library can be expanded, and genetic engineering techniques, such as CRISPR-Cas9-based editing (Isaev et al., 2022, Mahler et al., 2023), can be used to address any potentially problematic genes identified in future studies.

Storage Stability of Phages

All phage libraries require storage under conditions that ensure the survival of the phages for future use. Various preservation methods have been explored in previous studies (e.g., Fortier and Moineau, 2009; Golec et al., 2011), which evaluated the effects of cryoprotectants and storage temperatures on phage viability. This project utilised three storage methods that were feasible within the available laboratory resources, of which one (lyophilisation in glycerol) was found to be ineffective and was subsequently abandoned. For the remaining two methods, refrigeration at 4°C (fridge) and freezing at -80°C (freezer), three different phages were tested to evaluate their stability over a 10-month period. The analysis demonstrated that these two methods were suitable for laboratory conditions despite occasional intermittent sample failures. Excluding these failed samples, phage titres exhibited only a 1–2 log reduction in concentration after 10 months. Of note, two out of three phages showed better stability at -80°C compared to refrigeration, while one phage remained stable under both conditions.

These findings align with broader observations in the field, as reviewed by Ackermann, who discussed how phage stability can be influenced by factors such as capsid composition, tail structure, and host origin (Ackermann et al., 2004). Specific studies, such as that of Puapermpoonsiri, have demonstrated

differences in resilience among phage families under certain preservation conditions (Puapermpoonsiri et al., 2010). Building on these insights, studies have shown that stability can vary significantly among different phage families, with tailed phages such as *Myoviridae* and *Siphoviridae* often displaying higher resilience under specific storage conditions than *Podoviridae* (Mazumder, 2022, Golec et al., 2011). However, even within families, individual phages may exhibit variability due to unique genetic or structural features. Therefore, storage protocols should ideally be optimised for each phage isolate or family to ensure maximum viability and functionality.

Future work could involve testing phages from additional families and expanding storage duration beyond 10 months to better understand long-term stability trends. These studies should include a comparative analysis of storage capacity across different phage families and hosts, ultimately informing tailored storage strategies for diverse phage collections.

In conclusion, this project identified that the veterinary hospital drains in Townsville were the best location for collecting phages against PAE sourced from dogs. However, human sewage processing sites were also effective. A library of 35 phages was isolated and stored from these two locations. The use of cocktails with a larger than typical number of phages identified a minimum, sufficient and excessive number of phage patterns, which varied with different PAE strains. The cocktail containing 15 phages has been effectively characterised via sequencing and TEM. Lastly, the best method for storage in the JCU laboratories was determined from a selection of three.

Completing this project has addressed many of my questions, but many more have arisen. In a future approach, I would like to take samples from various natural environments during different seasons to determine if there are any variations in the number of phages counted according to changes in temperature. In addition, by collecting multiple sewage samples on different occasions, screening with the most resistant PAEs could determine if the continuous replacement of samples from the water treatment plant increases the chances of finding phages that can destroy these bacteria.

Following this line of research, I would like to expand the library from 35 to 300 phages. From this, the most broad-spectrum phages (these phages will be more likely to be used for clinical purposes) would be characterised through sequencing and TEM. An extended library will not only provide more chances to find phages for treatments but also will allow us to see if the phages contained in the library would affect other *Pseudomonas* species, such as *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas stutzeri.* This larger bank of phages would be useful in developing new cocktails and testing them in clinical cases in dogs with otitis externa. Additionally, I would like to expand the treatment to other infections related to PAE such as mastitis in cows.

Lastly, it would be interesting to investigate what is actually happening at the molecular level when a large number of diverse phages interact with a single bacterial strain, thus, explaining the reason behind the plateau and loss of efficacy that we detected with the phage cocktails containing a large number of diverse phages in the formulation.

Achievement of Objectives and Future Directions

This project successfully achieved its objectives, including the establishment of a 35-phage library, the identification of optimal sampling sites, the formulation of effective phage cocktails, and the evaluation of storage methods. The first of these future directions is to expand sampling efforts to include diverse environments and seasons, which could uncover novel phages with broader host ranges. Collecting samples across these variables and expanding the library to include up to 300 phages would significantly enhance the diversity and therapeutic potential of the collection. Another key area for further research involves investigating the molecular interactions between phages and biofilms, including receptor-binding dynamics and extracellular matrix (ECM) penetration, to optimise phage cocktail formulations. In addition, *in-vivo* testing of the efficacy of these phage cocktails in treating canine otitis externa and other PAE-related infections in animal models represents a critical step in validating their therapeutic potential. Whole-genome sequencing of the phage library, accompanied by thorough functional annotation, and the use of advanced techniques such as CRISPR-based genome editing to eliminate undesirable genes, could further enhance the safety and therapeutic potential of the phages. Last but certainly not least, a broader focus on advanced characterisation methods would ensure the development of robust and safe therapeutic agents for future clinical applications.

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APPENDIX 1: Agars, culture media and general reagents

1.1 $CaCl_2.2H_2O$ (1M)

Product	Brand	Weight (g)	Notes
$CaCl_2.2H_2O$	Sigma-Aldrich, CO., USA	1.47	Molecular Weight (Mwt)= 147.02 g/L Desired stock volume = 10 ml 1000 ml ---------- 147.02 g $CaCl_2.2H_2O$ 10 ml ---------- x = **1.47 g** $CaCl_2.2H_2O$ Dissolve $CaCl_2.2H_2O$ in 10ml doubly distilled water. Autoclave at 121°C for 15 minutes.

1.2 $MgSO_4 \cdot 7H_2O$ (1 M)

Product	Brand	Weight (g)	Notes
$MgSO_4.7H_2O$	AnalaR, BDH Chemicals, Australia	2.03	Mwt = 203.3 g/L Desired stock volume = 10 ml 1000 ml ---------- 203.3 g $MgSO_4 \cdot 7H2O$ 10 ml ---------- x = **2.03 g** $MgSO_4 \cdot 7H2O$ Dissolve $MgSO_4.7H_2O$ in 10ml doubly distilled water. Autoclave at 121°C for 15 minutes

1.3 Phosphate buffered saline (PBS) (pH 7.2 - 7.5)

Product	Brand	Weight (g)	Notes
PBS	Amresco, USA.		Thermo Scientific Pierce 20X Phosphate Buffered Saline Dilution needed: 1/20 Volume done: 500 ml 1/20 = 25 ml 20X PBS Stock + 450 ml doubly distilled water. Autoclave at 121°C for 15 minutes

1.4 SM buffer

Product	Brand	Weight (g)	Volume (ml)	Notes
NaCl	Univar, Ajax Finechem, Australia	5.8		Dissolve 5.8g NaCl in 1000ml SM solution.
$MgSO_4$	AnalaR, BDH Chemicals, Australia	0.9632		$MgSO_4$ (anhydrous) Molecular Weight (Mwt)= 120.4 g/L Desired stock volume = 1000 ml 1 M ---------- 120.4 g/L 0.008 M ----- X= 0.9632 g/L Dissolve 0.9632g $MgSO_4$ in 1000ml SM solution.
1M tris-HCl (pH 7.5)			50ml	See 1.6
2% gelatin			5 ml	See 1.5
				Autoclave SM buffer preparation at 121°C for 15 minutes

1.5 2% gelatin

Product	Brand	Weight (g)	Notes
Gelatin	Sigma-Aldrich, CO., USA	0.2	Dissolve 0.2g gelatin in 10ml double distilled water. Use a lab hotplate with heating and Magnetic Stirrer. Autoclave at 121°C for 15 minutes

1.6 tris-HCl (1M) (pH 7.5)

Product	Brand	Weight (g)	Notes
tris	Sigma-Aldrich, CO., USA	12.14	Dissolve tris in 80ml double distilled water and adjust pH to 7.5. Make up to 100ml with double distilled water. Autoclave at 121°C for 15 minutes.

1.7 Luria-Bertani (LB) medium (pH 7.0 - 7.2)

Product	Brand	Weight (g)	Notes
Tryptone	Amresco, USA.	10	
NaCl	Chem-Supply, Pty. Ltd., Australia	10	
Yeast extract	Astral Scientific, Australia	5	
			Make up to 1000ml with double distilled water, adjust pH to 7.0 - 7.2. Autoclave at 121°C for 15 minutes.

1.8 LB agar (for base - normal)

Product	Brand	Weight (g)	Notes
Luria-Bertani (LB) medium			See 1.7
Agar-agar	Sigma-Aldrich, CO., USA	15	Make up to 1000ml with double distilled water, adjust pH to 7.0 - 7.2. Autoclave at 121°C for 15 minutes.

1.9 LB agar (for top - soft)

Product	Brand	Weight (g)	Notes
Luria-Bertani (LB) medium			See 1.7
Agar-agar	Sigma-Aldrich, CO., USA	4.5	Make up to 1000ml with double distilled water, adjust pH to 7.0 - 7.2. Autoclave at 121°C for 15 minutes.

1.10 Cetrimide agar

Product	Brand	Weight (g)	Notes
Pseudomonas Cetrimide Agar (USP, EP)	Oxoid, England	22.65	Dissolve 22.65g cetrimide in 500ml double distilled water. Bring to the boil to dissolve completely. Autoclave at 121°C for 15 minutes

1.11 Crystal violet staining

Product	Brand	Weight (g)	Notes
Crystal violet	Gurr Diagnostics, England.	0.1	For biofilm staining protocol better performance by using 0.1% CV instead of 1% v/v (Normal preparation). 0.1g CV + 10ml Ethanol (once it is dissolved) take it to 100ml with double distilled water (90ml). For 500ml: 0.5g CV + 50ml Ethol + 450ml dH2O. Autoclave at 121°C for 15 minutes

1.12 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)

Product	Brand	Weight (g)	Notes
MTT	ThermoFisher Scientific	0.05	Desired stock volume = 100 ml LB Dissolve MTT in LB medium to 0.5mg/ml. For 100ml LB medium add 50mg of MTT. Filter-sterilize the MTT solution by using a 0.22uM filter into a sterile, light protected container. Store the MTT solution, protected from light at 4°C for frequent use or -20°C for long term storage.

1.12 Lysis solution

Product	Brand	Weight (g)	Notes
Lysis solution			Desire solution volume: 500ml lysis solution: (10% w/v sodium dodecyl sulphate (SDS) in 50% v/v dimethylformamide (DMF)) Prepare 250 ml of DMF 50% and 250ml SDS 10%. Adjust pH to 4.7
SDS			See 1.13
DMF			See 1.14

1.13 10% sodium dodecyl sulphate (SDS)

Product	Brand	Weight (g)	Notes
SDS	OmniPur, Calbiochem, Japan.	10	Desired stock volume: 100ml. Dissolve 10g SDS in 80ml doubly distilled water. Make up to 100ml with doubly distilled water. Store 10% SDS stock solution at room temperature (15°C to 25°C). Do not store in the fridge, as the SDS will re-precipitate.

1.14 50% [v/v] dimethylformamide (DMF)

Product	Brand	Volume (ml)	Notes
DMF	Sigma-Aldrich, CO., USA	50	Desired stock volume: 100ml. Dissolve 50ml DMF in 50ml with double distilled water. Result, 100ml DMF at 50% v/v.

1.15 50% [v/v] Glycerol

Product	Brand	Volume (ml)	Notes
Glycerol	Merck, KGaA, Germany	0.5	Desired stock volume: 1ml. Glycerol to a final concentration of 50% [v/v] Dissolve 500ul glycerol in 500ul LB media.

1.16 tris-EDTA (TE) buffer

Product	Brand	Volume (ml)	Notes
TE buffer			Combine 10ml 1M tris-HCl with 20 ml 0.5M EDTA and make up to 800ml with double distilled water. Adjust pH to 8.0. Make up to 1000ml with double distilled water. Autoclave at 121°C for 15 minutes.
1M tris-HCl		10	See 1.6
0.5M EDTA		20	See 1.17
Doubly distilled water		970	

1.17 EDTA (0.5M) (pH8.0)

Product	Brand	Weight (g)	Notes
EDTA	Univar, Ajax Finechem, Australia	186.12	Dissolve EDTA in 800ml with double distilled water. Adjust pH to 8.0. Make up to 1000ml with double distilled water when EDTA has dissolved. Autoclave at 121°C for 15 minutes.

1.18 10% Sodium acetate (pH 5.2)

Product	Brand	Weight (g)	Notes
Sodium acetate	Sigma-Aldrich, CO., USA	10	Dissolve sodium acetate in 50ml doubly distilled water. Adjust pH to 5.2 with acetic acid. Make up to 100ml with doubly distilled water.

1.19 50x Tris-acetate (TAE)

Product	Brand	Weight (g)	Volume (ml)	Notes
TAE				Combine ingredients and make up to 1000ml with double distilled water. Autoclave at 121°C for 15 minutes.
tris		242		See 1.6
Glacial acetic acid	Univar, Ajax Finechem, Australia)		57.1	
0.5M EDTA (pH8.0)			100	See 1.17
Double distilled water			842.9	

2) CALCULATION OF VOLUME OF CYLINDER FOR ADDITION OF TOP AGAR

Diameter (d): 140mm

Radius (r): 70mm

Pi (π): 3.1415

Height (h): 1mm

$V= \pi.r^2.h = 3.1415 \times (70mm)^2 \times 1mm = 15{,}400mm^3$

mm^3 to ml = 15.4ml ≈ 15ml (I used 15ml because is the biggest tube I get to pour in the petri dish)

Concentration in % of top agar, bacteria, and phage solution for 100mm x 15mm petri dish

- 5ml Total solution ---------- 100%
- 3ml Top Agar ----------x= **60%**
- 1ml Bacteria/Phage solution ------x= **20%**

Concentration in % of top agar, bacteria, and phage solution for 140mm x 15mm petri dish

- 100% -------- 15ml Total solution required
- 60% ---------- **9ml** (Top Agar)
- 20% ---------- **3ml** (Bacteria/Buffer)
-

Result:

-9ml Top agar

-3 ml Bacteria (500ul bacteria in logarithmic growth at 0.5 McFarland + 2.5ml Broth for 2hs at 37C)

-3ml SM buffer

TOTAL: 15ml

APPENDIX 2: *Pseudomonas aeruginosa* strains

Estimating the viable count of bacteria was done through the serial dilution method (Dr. Jacqueline Picard, Senior Lecturer, Veterinary Sciences at James Cook University). Briefly, five colonies of *Pseudomonas aeruginosa* were suspended in a microcentrifuge tube containing 1 ml of LB broth. The first well in a 96-well plate was filled with 200ul of this suspension, while the subsequent wells (columns 2 to 12) were filled with 100ul of LB broth each. The concentrated suspension in the first well was then serially diluted by taking 100ul of the suspension and adding it to the second well, and this process was repeated until the 24th well. The absorbance of the suspension was then measured at 570nm using a SPECTROstar® Nano microplate reader. Immediately after the measurements, 100ul of the suspension in the 24th well (was poured onto the centre of three LB agar plates and spread out using an L-shaped glass spreader. After incubation for 24 hours at 37°C, the number of bacterial colonies were counted and averaged. This count was then used to determine the counts of the dilutions where absorbance was noted and determine the absorbance of a working concentration of PAE. As a result, the final working suspension was estimated to have around 1.5x10^{8} colony-forming units (CFU/ml), which equates to an OD between 0.26 and 0.28 (Table 2.1).

Table A2.1: *Pseudomonas aeruginosa* concentration (CFU/ml) vs Absorbance (OD)

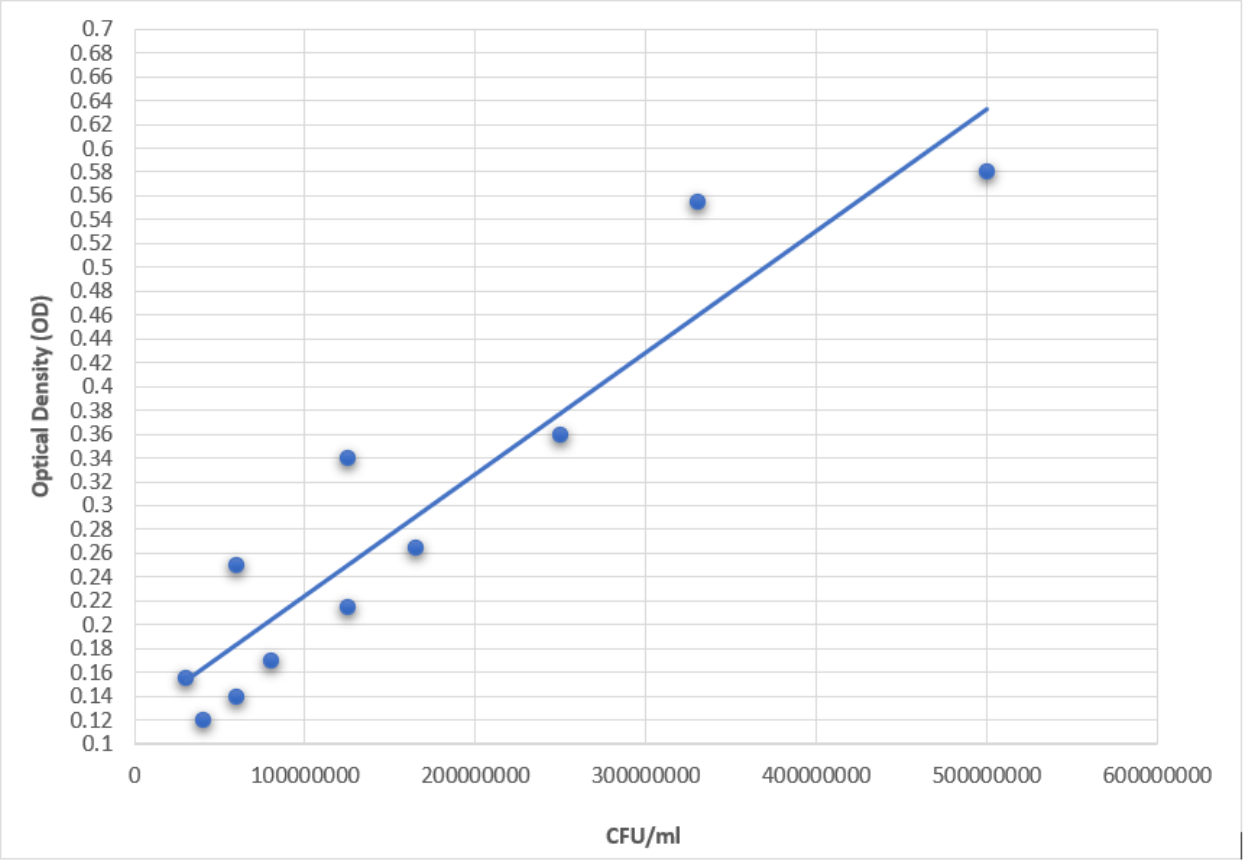


Table A2.2: Bacterial strains used.

PAE	Code	Year isolated	Amikacin	Gentamicin	Carbenicillin	Ticarcillin	Polymyxin B	Enrofloxacin	Pradofloxacin	Ceftazidime	Imipenem	Doxycycline	Aztreonam	Tobramycin	Piperacillin & Tazobactam
1	18-423-3	2018	S	S	S	S	S	I	S	S	S	R	NT	S	NT
2	18-556	2018	I	R	R	I	R	I	I	I	I	R	NT	NT	NT
3	18-441-1	2018	S	R	I	I	S	I	I	S	I	R	NT	NT	NT
4	18-926-1	2018	I	R	I	R	S	R	R	S	NT	R	NT	NT	NT
5	18-1272	2018	S	S	S	S	S	R	S	S	S	R	S	S	NT
6	18-1293	2018	S	I	I	I	S	I	I	S	NT	R	NT	NT	NT
7	19-1144	2019	S	S	NT	R	S	R	R	S	NT	R	NT	NT	NT
8	19-1323-2	2019	S	I	S	I	S	I	I	S	S	R	S	NT	NT
9	20-399	2020	S	I	I	I	S	I	I	NT	NT	I	NT	NT	NT
10	20-811-1	2020	S	I	NT	S	S	I	I	S	S	R	NT	S	NT
11	19-289	2019	S	R	NT	I	S	R	R	S	S	R	S	NT	NT
12	18-1315-4	2018	S	S	NT	S	S	S	R	S	I	R	I	NT	NT
13	18-1312	2018	S	S	S	S	S	R	R	S	I	R	I	NT	NT
14	19-23	2019	S	R	NT	S	S	S	S	S	S	R	S	NT	NT
15	19-571	2019	S	S	NT	S	S	R	S	S	S	R	I	NT	NT
16	20-623	2020	S	S	NT	I	S	I	S	S	S	S	S	NT	NT
17	18-1389	2018	S	S	NT	I	S	R	R	S	S	R	S	NT	NT
18	18-1816	2018	S	S	NT	S	S	S	R	S	I	R	I	NT	NT
19	08-380	2008	S	S	NT	I	NT	R	I	S	S	R	S	NT	S
20	19-323-2	2019	S	S	NT	S	S	R	S	S	S	S	S	NT	NT
21	21-1542	2021	S	R	NT	S	S	I	S	S	R	I	S	NT	NT
22	19-442-1	2019	S	S	I	I	S	S	S	S	S	R	I	NT	NT
23	19-876-2	2019	S	S	NA	R	S	R	R	S	S	R	R	NT	NT
24	19-931	2019	S	S	NT	I	S	I	I	S	S	R	I	NT	NT
25	20-675	2020	S	S	R	R	S	I	S	S	S	R	I	NT	S
26	20-712	2020	S	I	NT	I	S	I	S	S	S	R	I	NT	NT
27	18-550	2018	S	S	I	I	S	S	S	S	S	R	I	NT	NT
28	19-328	2019	S	S	NT	I	S	I	S	S	S	R	I	NT	NT
29	ATCC 27853		S	S	NT	I	S	R	I	S	S	R	S	NT	NT
30	19-1597	2019	S	I	R	R	S	R	R	S	S	R	R	NT	NT
31	22-749	2022	S	S	NT	S	S	S	S	S	S	R	S	NT	S
32	06-109	2006	S	S	NT	I	NT	I	I	S	S	S	I	NT	S
33	18-199	2018	S	S	NT	I	S	R	I	S	S	R	I	NT	S

Table A2.2: Bacterial strains used (Cont.).

34	18-297	2018	S	S	S	R	S	S	R	S	S	R	R	NT	S
35	22-2088	2022	S	S	NT	R	R	R	R	S	S	R	R	NT	R
36	18-437	2018	S	S	NT	S	NT	I	S	S	S	R	S	NT	S
37	18-577	2018	S	S	NT	S	S	R	I	S	S	R	I	NT	S
38	05-725	2005	S	S	NT	I	NT	R	R	S	S	R	I	NT	S
39	19-876	2019	S	S	NT	I	NT	I	I	S	S	R	R	NT	R
40	18-343	2018	S	S	S	S	S	I	S	S	S	I	S	NT	S
41	19-418	2019	S	S	NT	I	NT	R	S	S	S	R	I	NT	S
42	19-339-1	2019	S	S	NT	I	NT	S	I	S	S	R	S	NT	S
43	19-283	2019	S	S	NT	I	NT	R	I	S	S	R	I	NT	S
44	17-1051	2017	S	S	NT	S	S	R	R	S	S	S	R	NT	I
45	18-557	2018	S	S	S	I	S	S	S	S	S	I	S	NT	S
46	20-1278	2020	S	S	NT	R	NT	I	I	S	S	R	I	NT	S
47	21-259	2021	S	R	NA	S	S	R	I	S	S	R	S	NT	S
48	18-197-3	2018	S	S	NT	I	S	I	S	S	S	R	S	NT	S
49	23-1454	2023	R	R	NT	S	S	R	R	S	S	R	S	S	S

*NT: Non-Tested.

APPENDIX 3: Raw data

Table A3.1- Number of isolate phage-replicates obtained per location. All isolates are stored in the JCU microbiology phage collection place.

Collection Sites								
Mt St John Wastewater Treatment Plant			**JCU Veterinary Hospital**			**Mt. St. John Wastewater Treatment Plant 2nd collection**		
Number of phages	Phage label	Number of Replicates	Number of phages	Phage label	Number of Replicates	Number of phages	Phage label	Number of Replicates
1	Ph1	0	**1**	Ph8	0	**1**	Ph11	0
2	Ph2	1	**2**	Ph9	0	**2**	Ph25	0
3	Ph3	3	**3**	Ph10	1			
4	Ph4	0	**4**	Ph16	0			
5	Ph5	0	**5**	Ph17	1			
6	Ph6	0	**6**	Ph18	0			
7	Ph7	0	**7**	Ph22	2			
8	Ph12	4	**8**	Ph23	0			
9	Ph13	0	**9**	Ph24	0			
10	Ph14	0	**10**	Ph27	0			
11	Ph15	0	**11**	Ph28	2			
12	Ph19	0	**12**	Ph29	0			
13	Ph20	1	**13**	Ph35	0			
14	Ph21	0						
15	Ph26	0						
16	Ph30	2						
17	Ph31	0						
18	Ph32	0						
19	Ph33	0						
20	Ph34	1						

Table A3.2.1 - Raw data on absorbance reading for crystal violet measure. Effect of phage cocktails on PAE10 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data PAE10						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(5) and (30)	(10) and (15)	(20) and (25)
2.301	0.877	0.95	1.11	0.486	2.397	3.275	2.856	3.11
2.495	0.691	1.398	0.783	0.875	1.868	3.275	3.023	3.071
2.449	1.236	0.742	0.803	0.67	1.469	2.3	3.02	2.736
2.251	1.514	1.108	0.747	0.685	1.911	3.275	2.926	2.739
2.141	0.962	1.04	1.386	1.214	1.561	3.275	3.16	2.701
2.202	1.168	1.52	0.756	0.588	1.45	3.275	2.789	3.152
2.355	1.296	0.798	0.785	0.781	2.056	3.275	3.305	3.011
2.529	1.622	1.206	0.879	0.693	2.102	3.275	3.417	3.389
2.181	1.548	1.144	0.676	0.737	1.541	2.607	2.986	3.344
2.565	0.877	1.077	0.652	1.365	1.615	3.275	2.87	2.444
2.495	0.66	0.944	0.943	0.887	1.701	3.275	3.253	2.629
2.029	0.853	1.292	0.591	0.926	1.058	3.275	3.071	2.987
2.234	0.823	1.292	0.69	1.019	1.792	3.275	2.584	2.479
2.434	0.707	1.514	0.626	0.511	2.454	3.275	2.542	2.605
2.669	0.906	0.88	0.721	0.781	1.147	3.275	3.141	2.416
2.886	0.95	0.982	0.736	0.834	1.766	3.275	3.416	2.617
2.139	0.908	1.114	0.71	0.604	1.119	3.275	3.165	3.128
2.4	0.92	0.603	0.686	0.799	1.007	3.275	2.991	2.513
2.487	0.685	0.634	0.52	0.692	1.54	3.275	3.115	2.906
1.649	0.774	0.977	0.651	0.796	1.061	3.275	3.243	2.91
2.238	1.108	1.389	1.253	0.588	1.171	3.275	2.922	2.753
2.736	1.439	0.59	0.809	0.733	1.28	3.275	2.871	2.818
2.232	0.821	0.947	0.833	0.805	1.187	3.275	3.01	2.695
2.657	0.972	0.968	0.727	0.795	1.134	3.275	3.417	2.663

Table A3.2.2 - Raw data on absorbance reading for MTT measure. Effect of phage cocktails on PAE10 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(5) and (30)	(10) and (15)	(20) and (25)
1.356	1.068	0.472	0.565	0.581	1.253	1.744	1.451	1.582
1.578	0.273	0.313	0.837	0.696	0.365	2.329	1.452	2.075
1.487	0.408	0.367	1.254	0.678	0.521	1.65	1.321	1.378
1.683	0.405	0.854	0.879	0.598	0.39	2.009	1.248	1.403
1.908	0.477	0.352	1.562	0.627	1.04	1.59	1.288	1.39
1.974	0.538	1.405	0.405	0.764	0.728	2.14	1.333	1.375
1.262	1.152	0.689	0.147	0.597	0.627	1.468	1.365	2.094
1.795	1.142	1.278	0.501	0.225	0.316	3.409	1.47	1.538
1.499	1.163	0.246	0.399	0.976	0.589	1.778	1.375	1.468
1.766	0.91	0.475	0.51	0.619	0.379	1.407	1.351	1.646
1.685	0.795	0.892	0.337	0.922	0.409	1.84	1.311	2.455
1.545	0.704	0.956	0.254	0.856	0.535	1.847	1.182	1.378
1.534	0.288	0.7	0.611	0.484	1.179	1.699	1.251	1.35
1.75	0.74	0.854	0.658	0.498	1.244	1.559	1.361	2.182
1.642	0.398	0.338	0.895	0.623	0.352	1.675	1.271	1.57
2.016	0.65	0.904	0.959	0.424	0.498	1.9	1.375	1.465
1.56	0.462	1.114	1.089	0.63	1.149	1.636	1.44	1.578
1.505	0.285	0.632	0.595	0.17	0.541	1.553	1.931	1.848
1.456	0.4	0.517	1.197	0.625	0.546	1.693	1.818	1.551
1.689	0.372	0.313	0.648	0.743	0.349	1.554	1.323	1.45
1.794	0.292	0.89	0.68	0.45	0.673	1.573	1.364	3.038
1.739	0.797	0.65	1.377	0.733	0.458	1.788	1.557	1.573
1.306	0.65	0.82	0.885	0.528	1.265	1.631	1.283	1.332
1.514	0.828	0.458	0.305	0.676	0.514	1.942	1.447	1.955

Table A3.2.3 - Raw data on absorbance reading for crystal violet measure. Effect of phage cocktails on PAE11 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls			
Cocktail Formulation						Cocktail Formulation			
(5)	(10)	(15)	(20)	(25)	(30)	(5)	(10) and (15)	(20) and (25)	(30)
2.401	1.459	1.398	1.792	1.452	2.719	3.103	2.37	2.507	2.389
2.209	1.368	1.304	1.603	0.841	2.147	3.142	1.96	2.456	2.165
2.658	0.945	1.028	1.038	0.926	2.546	2.981	2.432	2.522	2.232
1.336	1.127	0.895	1.193	1.52	1.916	3.079	2.137	2.304	2.161
2.113	0.666	1.126	1.484	1.339	2.852	3.253	2.005	2.268	2.236
1.566	0.852	1.078	1.516	1.035	2.809	3.21	2.637	2.478	2.309
1.684	2.174	1.076	1.764	1.001	2.516	3.26	2.537	2.567	1.818
2.736	3.029	1.421	2.208	1.494	2.148	3.26	2.647	2.559	2.457
2.609	1.34	1.245	0.909	1.217	1.048	3.053	1.6	2.607	1.894
2.176	1.702	1.668	0.746	1.601	0.868	3.194	1.923	2.651	2.032
2.889	1.237	0.996	0.905	0.884	2.336	3.092	1.69	2.372	1.823
1.392	1.447	1.294	1.035	0.875	1.153	3.138	1.227	2.6	1.705
1.254	0.799	0.788	1.463	0.704	2.364	3.256	2.064	2.718	1.82
1.22	2.135	1.405	1.342	0.628	1.473	3.204	2.227	2.58	1.771
2.076	0.673	0.873	1.269	1.568	1.633	3.212	1.773	2.88	1.797
1.55	1.052	1.043	1.572	1.274	1.935	3.26	2.502	3.083	1.896
2.28	1.454	0.866	1.026	1.165	1.688	3.223	3.143	2.861	2.766
1.873	1.165	1.436	1.105	0.969	1.757	3.152	2.807	2.02	2.513
3.066	1.779	0.6	0.644	0.636	1.806	3.199	2.48	1.988	2.648
2.502	1.002	0.456	0.906	0.704	1.383	3.223	2.631	2.342	2.377
0.958	1.233	0.837	0.621	0.949	1.784	3.26	2.63	2.31	2.545
2.509	1.642	0.745	0.62	0.97	1.911	3.212	2.769	2.324	2.783
1.277	1.858	0.645	1.522	0.964	1.873	3.26	2.893	2.986	2.594
1.218	2.462	1.229	1.099	0.992	3.072	3.26	2.865	3.134	2.496

Table A3.2.4 - Raw data on absorbance reading for MTT measure. Effect of phage cocktails on PAE11 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						
	Cocktail (5)	Cocktail (10)	Cocktail (15)	(20) REP	(25) REP	Cocktail (30)
	1.01	0.435	0.778		0.464	1.163
	1.508	0.326	0.54	0.328	0.332	1.296
	0.826	0.448	0.31	0.411	0.312	0.88
	1.22	0.196	0.26	0.302	0.284	1.463
	0.926	0.587	0.328	0.451	0.289	1.433
	0.954	0.424	0.229	0.339	0.161	1.729
	1.152	0.617	0.227	0.265	0.495	0.917
		0.261	0.372	0.388		1.371
	1.3	0.394	0.701	0.397	0.451	1.448
	1.021	0.239	0.248	0.198	0.232	1.283
	0.91	0.518	0.591	0.153	0.435	1.309
	0.745	0.429	0.497	0.227	0.314	1.239
	0.793	0.325	0.517	0.389	0.257	1.205
	0.739	0.294	0.331	0.339	0.215	1.734
	1.218	0.243	0.188	0.243	0.47	
	1.244	0.231	0.269	0.357	0.496	1.122
	0.901	0.516	0.56	0.411	0.469	1.394
	0.826	0.213	0.406	0.32	0.337	1.068
	0.756	0.686	0.316	0.171	0.415	0.977
	0.705	0.164	0.159	0.538	0.271	1.267
	0.793	0.736	0.611	0.345	0.403	1.669
	0.692	0.189	0.224	0.182	0.207	1.378
	0.762	0.224	0.384	0.407	0.251	
	0.984	0.32	0.341	0.458	0.439	1.473

Blank corrected based on Raw Data (570)						Positive controls			
Cocktail Formulation						Cocktail Formulation			
(5)	(10)	(15)	(20)	(25)	(30)	(5)	(10) and (15)	(20) and (25)	(30)
1.01	0.435	0.778	0.631	0.464	1.163	1.692	1.491	0.844	1.476
1.508	0.326	0.54	0.328	0.332	1.296	2.317	1.34	1.404	1.589
0.826	0.448	0.31	0.411	0.312	0.88	2.211	1.285	1.291	1.261
1.22	0.196	0.26	0.302	0.284	1.463	1.486	1.396	1.025	1.247
0.926	0.587	0.328	0.451	0.289	1.433	2.48	1.584	1.563	1.312
0.954	0.424	0.229	0.339	0.161	1.729	2.471	1.34	1.319	1.256
1.152	0.617	0.227	0.265	0.495	0.917	2.025	1.429	1.459	1.492
0.912	0.261	0.372	0.388	0.752	1.371	2.047	1.225	1.579	1.603
1.3	0.394	0.701	0.397	0.451	1.448	2.314	1.647	1.568	1.579

1.021	0.239	0.248	0.198	0.232	1.283	2.222	1.336	1.866	1.679
0.91	0.518	0.591	0.153	0.435	1.309	1.996	1.323	1.326	1.559
0.745	0.429	0.497	0.227	0.314	1.239	1.846	1.239	1.151	1.453
0.793	0.325	0.517	0.389	0.257	1.205	1.962	1.713	1.589	1.501
0.739	0.294	0.331	0.339	0.215	1.734	2.112	1.05	1.472	1.597
1.218	0.243	0.188	0.243	0.47	0.6	1.806	1.237	1.891	1.991
1.244	0.231	0.269	0.357	0.496	1.122	2.28	1.553	1.843	1.934
0.901	0.516	0.56	0.411	0.469	1.394	2.496	1.451	1.831	1.752
0.826	0.213	0.406	0.32	0.337	1.068	2.458	1.387	1.492	1.671
0.756	0.686	0.316	0.171	0.415	0.977	2.215	1.384	1.493	1.733
0.705	0.164	0.159	0.538	0.271	1.267	2.362	1.324	1.278	1.261
0.793	0.736	0.611	0.345	0.403	1.669	2.251	1.006	1.872	1.713
0.692	0.189	0.224	0.182	0.207	1.378	2.417	1.336	1.322	1.648
0.762	0.224	0.384	0.407	0.251	0.401	2.235	1.431	1.741	2.105
0.984	0.32	0.341	0.458	0.439	1.473	2.258	0.293	1.784	2.049

Table A3.2.5 - Raw data on absorbance reading for crystal violet measure. Effect of phage cocktails on PAE22 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(5) and (30)	(10) and (15)	(20) and (25)
1.809	2.236	2.571	2.797	2.842	1.792	2.143	2.663	1.846
1.945	2.292	2.646	2.621	2.513	1.684	2.146	2.417	2.469
1.726	2.546	2.32	2.708	2.826	2.037	1.932	2.574	2.47
1.661	2.209	2.153	2.642	2.709	1.986	1.764	2.495	2.281
1.738	2.379	2.461	2.917	2.486	1.635	2.226	2.62	2.443
1.775	2.38	2.305	2.827	2.7	2.116	2.387	2.599	2.432
1.87	2.418	2.412	2.558	2.814	1.942	2.356	2.438	1.986
1.849	2.477	2.214	2.707	2.443	1.934	2.439	2.691	2.526
1.915	2.571	2.503	1.722	2.839	1.56	1.642	2.645	2.673
1.884	2.63	2.201	1.899	2.572	1.81	2.117	2.795	1.983
2.074	2.257	2.362	2.146	2.484	1.812	1.794	2.365	2.44
1.904	2.074	2.063	2.37	2.195	1.752	1.427	2.918	2.056
1.971	2.291	2.265	2.478	2.352	1.721	1.769	2.468	2.204
1.728	2.332	2.167	2.602	2.248	1.854	1.646	2.72	1.979
2.079	2.114	2.305	2.21	2.16	1.971	1.884	2.461	2.039
1.798	2.206	2.283	2.423	2.249	1.818	1.697	2.462	2.483
1.935	2.389	2.074	2.495	2.186	1.633	1.476	2.677	1.77
2.125	3.046	1.94	2.518	2.371	1.778	1.544	2.341	1.914
1.968	2.004	2.289	2.518	2.198	1.857	1.576	3.078	2.767
1.914	2.153	1.841	2.505	2.231	1.844	1.408	2.443	2.392
1.74	2.39	2.488	1.949	2.266	1.702	1.592	2.615	2.632
1.871	2.293	2.21	2.418	2.137	1.791	1.313	2.593	2.006
2.024	2.271	2.065	2.195	2.159	1.804	1.626	2.228	2.156
1.967	2.199	2.128	2.471	2.111	1.83	1.99	2.611	2.097

Table A3.2.6 - Raw data on absorbance reading for MTT measure. Effect of phage cocktails on PAE22 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(5) and (30)	(10) and (15)	(20) and (25)
1.688	1.321	1.45	1.614	1.377	1.56	2.136	1.608	1.518
2.484	1.197	1.229	1.301	1.316	1.658	2.158	1.432	1.388
1.707	1.156	1.171	1.419	1.098	1.668	1.507	1.379	1.356
1.703	1.153	1.156	1.213	1.135	1.519	1.377	1.262	1.223
1.708	1.295	1.134	1.342	1.093	1.639	1.631	1.241	1.373
1.629	1.14	1.106	1.208	1.172	1.454	1.946	1.345	1.392
1.678	1.159	1.173	1.561	1.235	1.76	1.591	1.415	1.458
1.758	1.235	1.157	1.501	1.186	1.484	1.608	1.562	1.413
1.765	1.486	1.246	1.219	1.282	1.511	1.697	1.522	1.505
1.707	1.24	1.236	1.255	1.198	1.642	2.081	1.347	1.241
1.816	1.139	1.04	1.144	1.176	1.493	1.602	1.244	1.098
1.688	1.258	1.099	1.166	1.143	1.53	1.788	1.181	0.82
1.885	1.172	1.206	1.226	1.156	1.393	1.452	1.256	1.309
1.737	1.101	1.213	1.132	1.261	1.528	1.705	1.275	1.229
1.825	1.245	1.084	1.347	1.348	1.412	1.449	1.246	1.404
1.631	1.063	1.113	1.344	1.175	1.443	1.639	1.343	1.401
1.637	1.44	1.422	1.288	1.485	1.541	1.731	1.941	1.637
1.57	1.456	1.152	1.276	1.071	1.563	2.066	1.552	1.529
1.568	1.153	1.157	1.154	1.027	1.454	2.099	1.521	1.435
1.651	0.973	0.964	1.1	0.958	1.511	1.525	1.37	1.337
1.685	0.976	1.032	1.172	1.04	1.475	1.354	1.324	1.412
1.814	1.001	1.062	1.13	1.023	1.464	1.7	1.293	1.469
1.832	1.069	1.143	1.195	1.131	1.489	1.732	1.512	1.533
1.68	1.03	1.07	1.214	1.095	1.673	1.487	1.694	1.599

Table A3.2.7 - Raw data on absorbance reading for crystal violet measure. Effect of phage cocktails on PAE29 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(5) and (30)	(10) and (15)	(20) and (25)
2.176	1.555	2.584	2.52	2.734	1.265	1.814	3.063	3.112
2.755	1.746	1.816	1.199	1.97	1.884	2.143	1.964	2.369
2.651	1.616	2.152	1.847	1.947	1.906	2.263	1.704	2.143
1.345	2.167	1.97	1.627	1.061	1.046	2.304	2.203	2.186
2.736	1.528	2.053	1.515	1.189	1.945	2.682	2.103	2.14
2.381	2.077	1.819	1.755	2.102	1.134	2.804	2.039	2.869
2.918	1.472	2.053	2.141	2.625	1.501	2.859	3.418	2.129
2.64	1.578	2.036	1.337	1.93	1.247	2.4	2.734	2.988
1.778	1.983	1.692	1.771	2.7	1.754	2.114	3.283	3.032
2.836	1.709	1.795	2.182	1.481	1.838	2.655	3.204	1.453
2.532	1.949	1.802	2.168	2.228	1.185	1.907	1.964	1.702
2.603	2.02	1.921	2.181	2.303	1.929	2.642	2.165	3.173
1.776	1.902	1.766	2.478	2.639	1.205	2.284	2.155	2.63
2.596	1.79	1.712	2.122	2.538	1.089	1.891	1.797	2.047
2.55	1.547	1.446	1.558	2.597	1.32	2.487	1.84	1.992
2.575	1.072	1.622	0.826	1.784	1.54	2.029	2.506	2.273
2.125	2.275	1.923	1.687	2.094	1.339	1.974	3.073	3.203
2.688	2.219	1.713	1.816	1.188	1.838	2.572	1.949	3.379
2.241	2.797	1.446	2.113	1.352	1.789	2.923	2	2.87
2.376	1.577	1.239	2.635	1.531	1.57	2.321	1.977	2.664
2.267	2.275	1.466	1.743	0.933	1.493	2.118	2.313	1.865
2.004	1.819	1.784	1.785	1.583	1.373	2.4	2.419	2.603
2.397	1.755	1.639	2.454	1.987	1.915	2.598	2.163	2.113
2.13	1.446	1.806	1.552	1.583	1.19	2.267	2.569	2.471

Table A3.2.8 - Raw data on absorbance reading for MTT measure. Effect of phage cocktails on PAE29 growth in biofilm essay. Data include outliers highlighted in red. Calculations had been done without outliers.

Blank corrected based on Raw Data (570)						Positive controls		
Cocktail Formulation						Cocktail Formulation		
(5)	(10)	(15)	(20)	(25)	(30)	(10) and (15)	(20) and (25)	(5) and (30)
1.552	0.518	0.467	0.614	0.264	0.577	1.312	1.075	3.335
1.905	0.326	0.44	0.262	0.701	1.086	1	1.049	2.252
1.687	0.346	0.705	0.708	0.3	1.209	1.139	1.014	1.856
1.53	0.593	0.569	0.389	0.573	0.339	0.989	1.371	1.718
1.478	0.282	0.72	0.328	0.358	1.547	0.995	2.245	1.88
1.496	0.411	0.659	0.677	0.401	0.67	1.185	1.143	2.487
1.532	0.559	0.521	0.288	0.567	0.222	1.132	1.156	1.65
1.635	0.131	0.383	0.156	0.119	0.288	1.221	1.16	1.954
1.388	0.411	0.471	0.287	0.294	0.957	1.071	1.405	1.739
1.452	0.404	0.423	0.599	0.56	0.408	1.144	1.057	2.313
1.8	0.255	0.814	0.474	0.654	1.013	1.356	1.025	1.623
1.605	0.473	0.634	0.449	0.401	0.941	1.189	0.819	1.802
1.694	0.191	0.532	0.38	0.322	1.18	1.193	1.012	2.683
1.489	0.498	0.578	0.801	0.362	0.519	0.988	0.982	1.605
1.461	0.51	0.593	0.215	0.896	0.405	1.547	1.121	1.545
1.463	0.28	0.758	0.447	0.498	0.308	1.293	1.168	1.512
1.829	0.667	0.326	0.531	0.44	0.454	1.115	1.203	1.538
1.661	0.738	0.514	0.721	0.426	0.264	1.347	1.494	2.235
1.765	0.461	0.58	0.437	0.373	0.82	1.251	1.142	1.68
1.762	0.272	0.537	0.488	0.404	0.453	1.236	1.353	1.624
1.689	0.399	0.245	0.52	0.386	0.351	1.977	1.327	1.572
1.793	0.229	0.515	0.512	0.61	0.924	1.363	1.359	1.492
1.565	0.227	0.421	0.131	0.463	0.627	2.788	1.369	1.453
1.201	0.561	0.755	0.176	0.584	0.072	1.315	1.346	3.077

Outlier's calculations, based on the 1.5 IQR rule.

Example:

Cocktail (5). Blank corrected based on Raw Data PAE10 for crystal violet measure.

Cocktail (5)
2.301
2.495
2.449
2.251
2.141
2.202
2.355
2.529
2.181
2.565
2.495
2.029
2.234
2.434
2.669
2.886
2.139
2.4
2.487
1.649
2.238
2.736
2.232
2.657

Data set arranged in order from smallest to largest.

Cocktail (5)	
1.649	
2.029	
2.139	
2.141	
2.181	
2.202	
2.232	Q1
2.234	
2.238	
2.251	
2.301	
2.355	Q2
2.4	
2.434	
2.449	
2.487	
2.495	Q3
2.495	
2.529	
2.565	
2.657	
2.669	
2.736	
2.886	

Find Quartile 1 (Q1).

L= (25/100) x N

L= (0.25) x (24) = 6

Find Quartile 3 (Q3).

L= (75/100) x N

L= (0.75) x 24 = 18

Find the interquartile range (IQR).

IQR= Q3 - Q1 = 18 - 6 = 12

Find the upper boundary.

Upper boundary = Q3 + 1.5xIQR = 2.495 + 1.5x0.263= 2.8895

Find the lower boundary.

Lower boundary = Q1 - 1.5xIQR = 2.232 - 1.5x0.263= 1.8375

Results:

Quartile 1: 2.232

Quartile 3: 2.495

Interquartile (IQR): 0.263

Upper Bound: 2.8895

Lower Bound: 1.8375

Outlier: Measure 20: 1.649

Table A3.3 Bacteriophage titre determination, represented as the number of plaque-forming units (PFU/ml) present in the samples over an eleven-month period. Counts listed are multiplied by the factor in brackets in each column for actual PFU/ml.

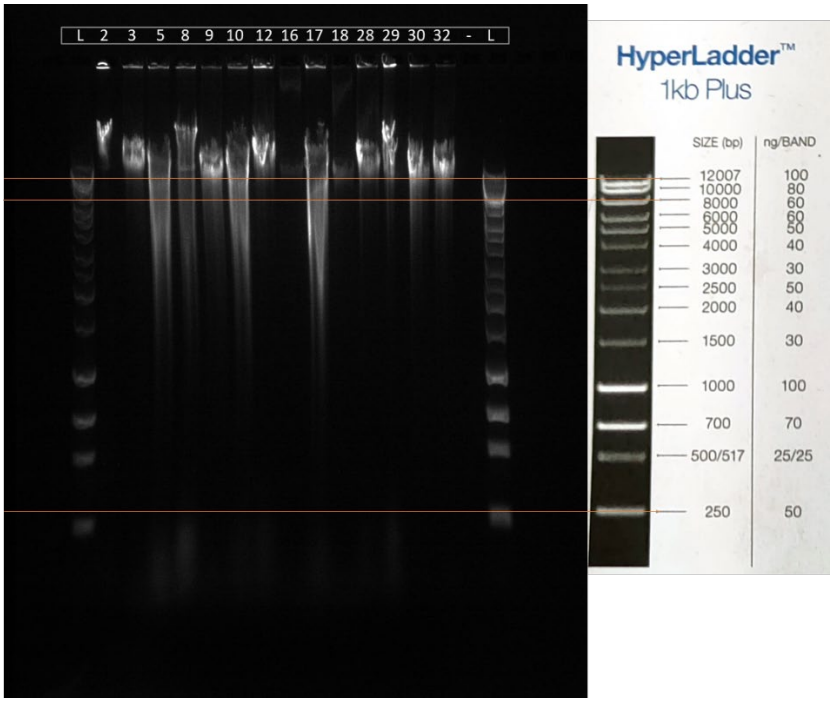
2023	Fridge 4C			Freezer -80C			Lyophilization		
	1.1.4 (10^{16})	**1.7.1 (10^{13})**	**1.10.1 (10^{12})**	**1.1.4 (10^{16})**	**1.7.1 (10^{13})**	**1.10.1 (10^{12})**	**1.1.4 (10^{16})**	**1.7.1 (10^{13})**	**1.10.1 (10^{12})**
January	392	135	32	392	135	32	392	135	32
(Initial count)	388	110	25	388	110	25	388	110	25
February	176	24	204	252	301	150	-	$9x10^{6}$	-
	-	27	303	152	290	-	-	-	-
	215	1	188	19	324	309	-	-	-
March	344	20	36	302	4	-	-	-	-
	42	2	1	148	270	41	-	-	$1x10^{9}$
	126	3	4	236	293	311	-	$22x10^{8}$	-
April	288	258	224	286	316	116	-	-	-
	76	237	224	30	301	320	-	-	-
	149	270	184	116	289	220	-	-	-
May	25	139	238	45	241	210			
	100	45	127	242	301	98			
	6	23	43	125	179	76			
June	3	4	-	112	113	40			
	5	15	38	7	380	4			
	6	14	8	31	5	18			
July	56	101	25	19	-	15			
	23	7	13	77	180	35			
	79	22	44	100	65	20			
August	**(10^{15})**	**(10^{12})**	**(10^{11})**	**(10^{15})**	**(10^{12})**	**(10^{11})**			
Dilution decreased in 1 log from this date	4	13	3	1	1	258			
	4	7	3	3	9	2			
	10	12	2	272	209	5			
September	15	8	2	20	30	17			
	12	5	3	2	-	115			
	2	2	5	87	12	5			
October	14	12	-	22	35	92			
	4	3	6	64	4	7			
	5	9	9	9	9	12			
November	8	7	3	50	22	62			
	10	15	5	6	6	10			
	4	6	4	21	2	4			

APPENDIX 4: Phage sequencing

1.1 Determination of DNA quality

Barcodes	Phage codes	ng/ul	fmol/ul	ul 200fmol	Water to add to 48	Concentration (ng/ul)	260/280	260/230
Barcode1	**Ph2**	680	20.4	9.8	38.2	284	1.86	1.5
Barcode2	**Ph3**	1880	56.4	3.5	44.5	254	1.91	1.59
Barcode3	**Ph5**	5471	164.13	1.2	46.8	144	1.94	1.47
Barcode4	**Ph8**	5065	151.95	1.3	46.7	73.5	2.53	1.47
Barcode5	**Ph9**	2495	74.85	2.7	45.3	46	2.14	1.95
Barcode6	**Ph10**	4375	131.25	1.5	46.5	38	2.23	1.58
Barcode7	**Ph12**	2785	83.55	2.4	45.6	37	2.24	2.05
Barcode8	**Ph16**	94.5	2.835	48	0.0	56	1.89	1.07
Barcode9	**Ph17**	7020	210.6	0.9	47.1	107	1.82	1.18
Barcode10	**Ph18**	102.5	3.075	48	0.0	79.5	1.89	0.99
Barcode11	**Ph28**	1955	58.65	3.4	44.6	242	1.86	1.49
Barcode12	**Ph29**	6265	187.95	1.1	46.9	24	2.66	2.18

1.2 Gel electrophoresis of phages DNA.



1.3 Refer the link for the complete genome sequencing protocol, Native Barcoding Kit 24 V14, SQK-NBD114.24, by Oxford Nanopore Technologies plc (Manufacturer's instructions). The protocol is permanently stored at JCU Data storage.

Appendix 4 - links

3) Fasta-files (with the complete phage genome) This is permanently store at the JCU Data storage and temporary store for examination linked OneDrive.

This is the link that contains the file that will be submitted to NCBI ones the file is fully analysed prior to publishing:

Appendix 4 - links