Isolation and characterization of bacteriophages from wastewater as potential biocontrol agents for *Escherichia coli*

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Submitted in fulfillment of the academic requirement for the degree of Master of Science in Microbiology

University of KwaZulu-Natal

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Declaration

I, Nontando Percevierance Ntuli, declare that:

1. The work presented in this thesis, except where indicated is my own original research and it has been generated by me.

2. This thesis has not been submitted for any degree or examination at any other University.

3. This thesis does not contain other person's data, pictures, graphs, or other information unless specifically acknowledged.

4. All the sources used or quoted in this thesis have been indicated and acknowledged as complete references.

Signed  …… Date ……21/07.2022

Supervisor ……  ….. Date July 21, 2022

Abstract

Host-specific lytic bacteriophages have regained momentum as an alternative treatment option to control and eliminate pathogenic bacteria. This study aimed to isolate, characterize and evaluate the potential application of *Escherichia coli* phages as a biocontrol agent in wastewater. In this study, four lytic *Escherichia coli* phages were isolated from wastewater for biocontrol purposes, using the double-layer method with *E. coli* (ATCC-25922) as a host. The phage morphology was characterized using transmission electron microscopy, with further parameters such as host range, phage stability at different temperatures, and pH values analyzed additionally. The genome of two selected phages (NPS and NPM) was sequenced, and the capacity of the phage isolate NPM to eliminate *E. coli* from artificial wastewater was evaluated and compared to conventional chlorination. All the four phage isolates showed typical T4 phage appearance with isometric capsids and contractile tails of different sizes, matching the family *Myoviridae* in the order *Caudovirales*. They exhibited a narrow host range limited to *E. coli* isolates, with two exceptions: phage NPS and NPM additionally lysed *Salmonella* Typhimurium (ATCC-14028). The four phage isolates were even able to lyse MDR (multidrug-resistant) *E. coli* isolates, such as the strain FP29. The four phages had burst sizes ranging from 70-115 per host cell and a latency period of 10-20 minutes. All the four bacteriophages were stable at pH 5-9 but completely inactivated at pH 12. Exposure to 60°C for 10 minutes reduced phage titers by 1.5-log, while exposure to 80°C for 10 minutes completely inactivated all four phage isolates. The two genomes (NPS and NPM) were 99% identical and had similar sizes (169 536 bp), but phage NPS differed from phage NPM in view of its host range and plaque morphology. Another difference observed at the genome level was a shift of coding sequences between phage NPS and NPM. Phage isolate NPM achieved a 3.5-log reduction of *E. coli* cells present in artificial wastewater at an MOI of 0.1 in 120 minutes. A 90-minute chlorine treatment achieved a log reduction in the same range, highlighting that phages have the potential as environmentally friendly biocontrol agents in wastewater treatment.

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1. Introduction

Bacteriophages, which are also called simply phages, are viruses that invade and kill bacteria and archaea (Sulakvelidze *et al.,* 2001; Leon and Faherty, 2021). Phages were formally discovered in 1915 by William Twort, a British physician and biomedical scientist. While conducting his study on pox virus, he noticed a voluntary lysis of *Staphylococcus albus* lawn contaminating his plates. He then used a filtrate of this contamination free of bacteria to infect fresh micrococci cultures, and concluded that these agents behaved like parasites, given their need for a living host cell to propagate (Twort, 1915). Two years later, a French-Canadian biomedical researcher by the name of Felix d'Herelle, unconscious of Twort's discovery, observed a similar incidence with *Shigella dysenteriae*. He then published an article in 1917 on his findings regarding a microbe that was antagonistic to bacteria, lysed bacteria in a liquid medium, and on the surface of agar. The clear patches that resulted from this lysis were then named plaques and the microbes responsible for this phenomenon were called bacteriophages (d'Herelle, 1917). However, phage existence can be traced back to the late 1800s, when Hankin reported that there was "something" in the waters of the Ganges River (India), which acted against *Vibrio cholerae,* the causative agent for Cholera and it was tiny enough to pass through the small pore size filters (Hankin,1896).

Most phages have heads with different sizes and shapes (most phages have isometric heads ranging from 20 - 200 nanometers (Sulcius *et al*., 2011)), except for filamentous phages such as M13, f1, and fd with a filament length ranging from 800 nm- 4µm (Hay and Lithgow, 2019). More than 95% of phages possess tails attached to the head that they employ to pass nucleic acid into the bacterial cell during infection (Letkiewics *et al*., 2010, Jonczyk *et al*., 2011). These phages are made up of a nucleic acid core, which contains DNA or RNA (double-stranded or single-stranded), enclosed by a protein-based capsid, protecting the nucleic acid from the environment (Verheust *et al*., 2010; Dion *et al*., 2020).

The capsid has three vital roles; first, it protects the genome (during extracellular life stages); second, it improves phage adsorption to the target cells and third, it aids in the delivery of the phage genome into the cytoplasm of the newly infected bacteria (Gill and Abedon, 2003). A phage uses the host biosynthesis machinery, including

ribosomes, amino acids, and energy-generating systems, and for that reason, can only replicate within a metabolizing bacterial cell (Grabow, 2001; Stone *et al*., 2019). The phages inject their nucleic acid into the bacteria and induce: A) lysis by releasing viral particles called lytic phages or B) they release progeny viruses without lyses the host cell or C) they reside within a bacterial cell as prophage (Verheust *et al.,* 2010). According to the available literature, bacteriophages are the most abundant and diverse entities, estimated at 10^{31} phage particles on the planet (Hendrix *et al*., 1999; Suttle, 2005; Clokie *et al*., 2011; Ji *et al*., 2021). However, Mushegian (2020) reports that there might be even more than 10^{31} viruses on the planet, 10^{31} may be representing tailed phages only. Bacteriophages are present in all the places where bacteria thrive, e.g., soil, oceans, inside plants and animals (Lopes *et al*., 2014, Bond *et al*., 2021), and sewage is where phages are apparently most prevalent (Mattila *et al*., 2015).

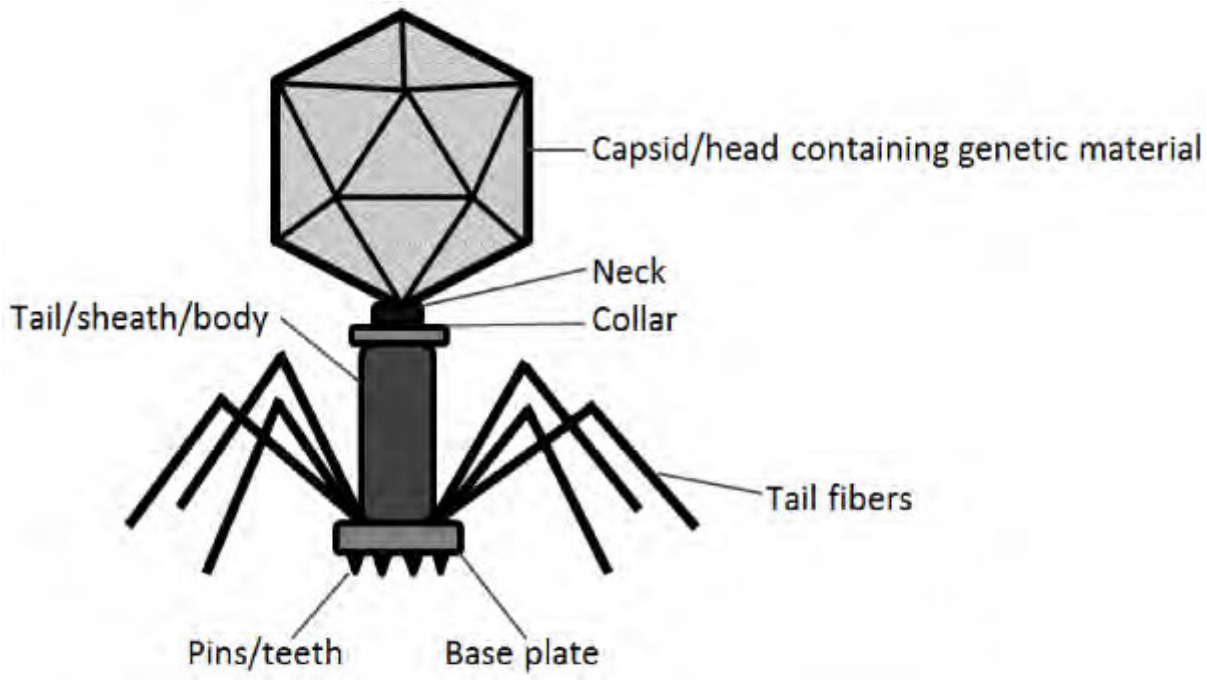

Figure 1.1: Schematic representation of a typical T-type phage particle showing the typical capsid, neck, collar, tail, tail baseplate, tail fibers and pins (Doss *et al*., 2017).

1.1. Bacteriophage classification

Phages were first classified by Bradley in 1967 into six basic morphologies based on structure and nucleic acid (Bradley, 1967), which was eventually approved by the International Committee on Taxonomy of Viruses. The ICTV classified bacteriophages based on structure, capsid size, shape, genetic material, genome size, and resistance to organic solvents, thus grouping them into 3 orders (*Caudovirales*, *Ligamenvirales,* and the Unassigned), 3 families, 16 genera and 30 species (Murphy *et al*., 1995; Sharma *et al*., 2017). The order *Caudovirales* are non-enveloped viruses that include the families *Myoviridae*, *Siphoviridae,* and *Podoviridae* as classified by the ICTV in 1999. However, in 2018 the ICTV issued a report that had re-grouped tailed phages into 5 families, with *Ackermannviridae* and *Herelleviridiae* added to the existing 3 families, 26 subfamilies, 363 genera and 1320 species (Dion *et al*., 2020; Koonin *et al*., 2020). The order *Caudovirales* accounts for 96% of all the known phages. Phages within this order have tails that may be long and contractile or short and non-contractile; they are also known to have double-stranded DNA (Grabow, 2001; Mc Grath and Sideren, 2007; Lopes *et al*., 2014).

The order *Ligamenvirales* is composed of the families *Lipothrixviride* and *Rudiviridae,* which are rod-shaped viruses that may be enveloped or non-enveloped with linear double-stranded DNA. The unassigned order has 13 families, which differ in morphology and nucleic acids (Mc Grath and Sideren, 2007). Transmission electron microscopy (TEM) is one of the most important tools and techniques used in microbiology to classify phage morphology. This tool was introduced in the 1930's - 1940's, and the first phage micrograph was published (Clokie and Kropinski, 2009; Ackermann, 2012). As new phages are being discovered frequently, the ICTV regularly updates the information on phage taxonomy. In 2020, the megataxonomy of viruses was proposed, which provides 15 hierarchical ranks to classify all viruses, now phages are divided into four realms (*Duplodanaviria*, *Monodnaviria, Varidnaviria* and *Riboviria*). These realms are made up of six kingdoms and seven phyla. Moreover, the order *Caudovirales* with the families *Myoviridae*, *Siphoviridae,* and *Podoviridae* is now unified in the class *Caudoviricetes* and the families under it are scheduled to be nullified (Koonin *et al*., 2020; Adriaenssens, 2021).

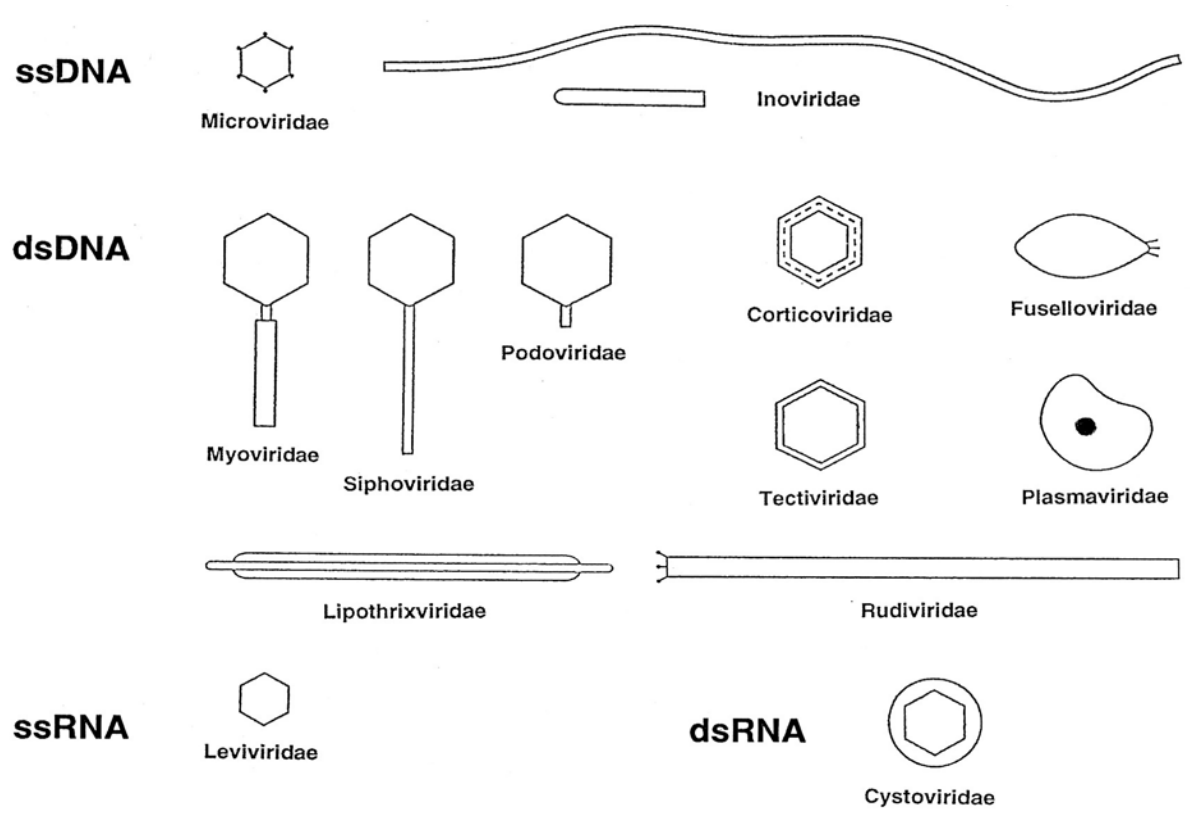

Figure 1.2: Representation of different bacteriophage morphologies (Ackermann, 2007).

1.2. Bacteriophage life cycle and replication

Phages have a remarkable impact in nature; hence it is important to understand their interaction with their host (Clokie *et al*., 2011). Phages are known to undergo two possible life cycles i.e. the lytic and the lysogenic cycle (Ackermann, 2012).

The lytic cycle, which is used by virulent phages, typically lasts for about 30-40 minutes (Jassim and Limoges, 2014). During this cycle, the phage infects and kills the host bacterium and starts to multiply within the host, after which the phage disintegrates the host cell, releasing numerous new phage particles (figure 1.3). The lytic cycle begins with the phage using long tail fibers to attach to a specific receptor on the surface of the bacterial host cell to trigger conformational change on the phage base plate to initiate tail sheath contraction (Goldberg *et al*., 1994; Haq *et al*., 2012).

Once the phage is attached completely, the hollow inner tube pricks the bacterial outer membrane, and the enzyme lysozyme is released to degrade the peptidoglycan layer so that the genetic material of the phage can be inserted into the bacterial host cell (Rakhuba *et al*., 2010).

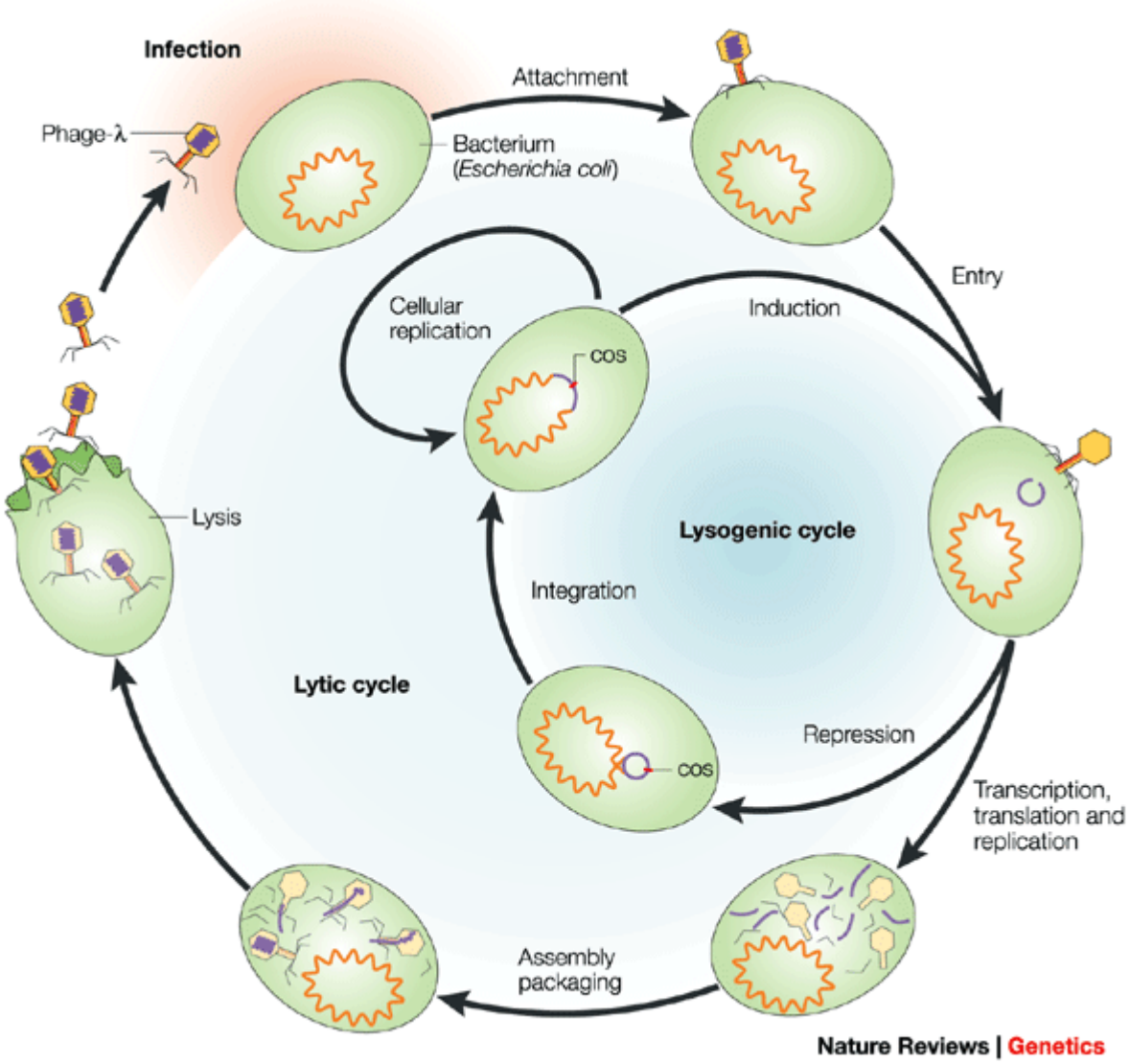

Figure 1.3: The lytic and lysogenic life cycle of the phages (Campbell, 2003).

During penetration, the metabolic machinery of the host bacterium is utilized by the phage to create multiple copies of its own genetic material (Harper and Kutter, 2008). Viruses that contain DNA directly transcribe into messenger RNA molecules that are then used to direct the host cell ribosomes to produce encoded phage proteins. However, RNA viruses have to use reverse transcriptase to transcribe the viral RNA

into DNA, then following the path of DNA viruses for transcription. Later in the translation stage, the proteins are assembled to form a capsid and the tail of the phages that break out of the host cell by typically using holin and endolysins, rupturing its cytoplasmic membrane (Sharma *et al*., 2017). During the final stages of amplification, most tailed phages employ endolysins or lysozyme that hydrolytically break down the bacterial cell wall. This allows new phage particles to leave the host cell and disseminate to the next bacterial host cells until the infection is gone (Gandham, 2015).

The so-called **lysogenic cycle** is specific for temperate or lysogenic phages. In this cycle, the viral genetic material is integrated into the bacterial genome, resulting in a prophage (figure 1.3). In this way, the viral genetic material continues to replicate without killing the infected host, as shown in figure 1.3 (Haq *et al*., 2012). However, the incorporation of the viral genetic material into the host may result in observable changes to the host characteristics (Sharma *et al*., 2017). Bacteria that display a lysogenic infection may have acquired new features, such as toxin production (for example, Shiga toxins) in a non-toxin-forming bacterial strain. This phenomenon is responsible for the pathogenicity of *E. coli* 0157:H7 (Grabow, 2001; Jassim and Limoges, 2014).

1.3. Host range determination

The host range is defined as the type of bacteria that a phage can infect. It is considered as the most important tool in bacteriophage research to determine how efficient the phage is, in terms of how broad or narrow its host range is (de Jonge *et al*., 2019). Hyman and Abedon (2010) described various techniques that can be used to determine host range types, which they referred to as adsorptive, penetrative, bactericidal, productive, plaquing, and lysogenic. They further stated that each host range type depends on the ability of the phage to effectively complete various stages of the infection. To determine the host range of a phage, the spotting technique is widely used. In this method, a small amount of phage lysate, with a high phage titer, is dropped on top of a growing bacterial lawn (Khan Mirzaei and Nilson, 2015). However, this method may sometimes yield false positive results because phages may exist in multiple phage types within a single phage stock and appear to have a broader

host range than is the actual case. To overcome this potential problem, it is crucial to run plaque purification at least three times before determining the host range (Hyman and Abedon, 2010).

Many phages exhibit a narrow host range, this phenomenon can be controlled by factors such as the specificity of phages to host binding proteins, biochemical interactions during an infection, the existence of homologous prophages or distinct plasmids for phages attaching to bacterial pili (Ross *et al*., 2016). The analysis of the phage life cycle is the most reliable mechanism that can be used to elucidate the host range. However, many studies on host range determination focus only on the attachment stage. There are phages that are called monovalent because of their limitation to only bind to one specific receptor on the host surface, and they are most likely to display a narrow host range, while polyvalent phages are capable of binding to numerous unrelated receptors and may therefore show a broad host range (de Jonge *et al*., 2019).

Phage adsorption presents a key step in the infection, and early contact between the bacterium and the virus controls host range specificity. This adsorption step additionally relies on physical-chemical factors such as pH, temperature, and ions present (Silva *et al*., 2016; Rakhuba *et al*., 2010). The distinct relationship linking bacterial receptors and phage-binding domains is occasionally conveyed by enzymatic cleavage. This activates conformational changes in other phage molecules, permitting DNA injection into the host (Silva *et al*., 2016). Receptor sites, which are located on the exterior of the bacteria, can be lipopolysaccharides (LPS), teichoic acids, proteins, flagella, pili, and even capsules (Silva *et al*., 2016; Sharma *et al*., 2017). Phages from the order *Caudovirales* that recognize these receptor sites are called somatic phages (Grabow, 2001). For the infection to happen, the phage has first to recognize a specific receptor located on the surface of the host bacterium (Stone *et al.,* 2019).

Gram-positive bacteria possess teichoic acids in their cell wall, which act as a receptor that a phage will recognize and bind to, while Gram-negative bacteria have an external LPS layer and outer membrane proteins, for example, OmpC, which serve as receptors and enable nutrient diffusion (Sharma *et al*., 2017). A good example for Gram-positive bacteria targeting phages is the *Lactobacillus plantarum* phage B2,

which attaches to the glucose substituent in the teichoic acid. For Gram-negative bacteria targeting phages, a well-established example is the *Escherichia coli* K12 T4 phage, which binds to protein O-8 of the outer membrane protein C (Silva *et al*., 2016). T4 phages generally use OmpC and LPS as their main receptors (Dion *et al*., 2020). The OmpC is the main protein located on *E. coli* cells that T4 phages target. Therefore, T4 phages tend to lose efficiency in infecting *E. coli* if either LPS or OmpC is absent, and the loss of both receptors induces resistance (Rakhuba *et al*., 2010).

1.4. Phage resistance mechanisms

Bacteria can resist bacteriophage infection by using one of the following strategies, namely: voluntary mutations, restriction modifications, abortive infection, and adaptive immunity via CRISPR-cas systems (Oechslin, 2018).

Voluntary mutations: due to the pressure from phage infections, bacteria have evolved mechanisms to co-exist with phages in the same environment. They resist phage attacks via three ways.

1. Blocking phage receptors or changing their shape, this is seen in *Staphylococcus aureus* that produces a cell wall virulence factor called protein A, which binds to the Fc fragment of the immunoglobulin G. When protein A is produced in large quantities, it masks the phage receptor, thus impairing phage adsorption (Labrie *et al*., 2010).

2. Production of an extracellular matrix that becomes a barrier between the phage and its host receptors. *Pseudomonas* spp. and *Azotobacter* spp. produce exopolysaccharides which can induce phage resistance. However, phage 116, which targets *Pseudomonas* spp. produces a lyase that is involved in the dispersion of the EPS matrix as well as reducing the viscosity of the matrix (Labrie *et al*., 2010).

3. Production of competitive inhibitors, which are molecules produced by the bacteria that can bind to phage receptors first, making these receptors unavailable to phages for binding (Labrie *et al*., 2010; Rostøl and Marraffini, 2019). FhuA is an iron transporter and entry point for coliphages, and Microcin J25, which is produced when nutrients are depleted, also uses FhuA as a receptor. Microcin J25 can outcompete coliphages for binding to FhuA.

Restriction modifications: Many bacterial species have restriction-modification systems (R-M systems); the main function of these systems is to shield the cells against foreign DNA, including viruses. For example, when unmethylated phage DNA makes its way into the cell with an R-M system, it will be recognized by restriction enzymes that rapidly degrade it (Labrie *et al*., 2010).

Abortive infection: is a system that renders immunity to bacteria by liberal suicide of an infected bacterium. Abortive infections are very toxic when they are activated as they target different stages of DNA synthesis like inhibiting replication, transcription, and protein synthesis, thus resulting in premature cell lysis. This system has been commonly associated with Gram-positive bacteria such as lactococcal strains. However, some Gram-negative species, such as *Escherichia coli,* have been found to possess this system encoded on plasmids (Fineran *et al*., 2008).

Adaptive immunity: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are DNA sequences derived from phage DNA fragments found in the genome of prokaryotic organisms such as bacteria and archaea that had been previously infected. They are composed of 20-48 base pairs called spacers; when these sequences are incorporated into the phage genomic CRISPR loci, resistance is attained, which will disturb the lytic proliferation of the phage (Castillo *et al*., 2015; Rostøl and Marraffini, 2019).

1.5. Phage genome

Phages are diverse, and so are their genome sizes. These vary from an approximately 3 300 nucleotide ssRNA coliphage genome to a 500 kilo-basepair (kbp) genome of *Bacillus megaterium* phage G (Hatfull, 2008). Previously phages with genome sizes larger than 200 kbp were referred to as jumbo phages, while those bigger than 500 kbp are called megaphages. As of 2020, the largest complete phage genome was 735 kbp in length (Al-Shayeb *et al*., 2020). The first genome to be sequenced was that of phage φX174 in 1977, which has an ssDNA genome with 5 386 base pairs (bp). Tailed phages with double-stranded DNA currently represent 96% of all sequenced phages (Hatfull, 2008; Fokine and Rossmann, 2014; Hardy *et al*., 2020). Many phages from the family *Siphoviridae* have genomes larger than 20 kbp, while those from the *Myoviridae* have genome sizes typically exceeding 100 kbp (Brüssow and Hendrix, 2002; Hatfull, 2008; Mavrich and Hatfull, 2017).

The size of the phage genome governs how complex the phage morphology can become (Hatfull and Hendrix, 2011), because even the smallest genome of tailed phages encodes for proteins required for DNA packaging, head, tail, tail fibers, DNA replication, transcription regulation, and lysis. As the genome size increases, the virion morphology gets more complicated, and the phage interferes with more cellular activities of its host (Brüssow and Hendrix, 2002). Thus, the effectiveness of a virion depends on how much DNA is packaged inside the capsid; this also interferes with the stability of the virion (Hatfull and Hendrix, 2011). Phages are said to show a genomic mosaic structure. However, not all genes partake in this phenomenon, as some crucial conserved genes like head genes, tail genes, and lysis genes cluster tightly through evolution (Hatfull, 2008). This can be explained by the essential biological function of the proteins they encode for. For example, head genes code for proteins that correspond closely to each other in making up the head structure, and these genes need to co-evolve to perpetuate this close relationship (Hatfull and Hendrix, 2011). Moreover, phages with smaller genomes typically lack tRNA, which can replace host functions under specific conditions of bacterial cell growth (Henry *et al*., 2010). The number of complete sequenced virus genomes is accumulating, to date (May 2022) 4 508 complete phage genomes have been deposited in PATRIC and 4 446 in NCBI Genbank. However, bacterial genomes are still more prevalent (Hatfull, 2008), which poses a challenge to scientists in the field of phage research.

1.6. Phage application

The ability of bacteriophages to kill bacteria, discovered in the late 1800s, led to more studies taking place in several countries to explore the potential application of phages in different fields (Endersen and Coffey, 2020). Increasing numbers of institutions focus on phage research, many companies are producing phage-based products and clinical trials to treat bacterial infections using phage therapy and phages are used additionally as vectors in molecular studies. Below are some of the milestones achieved by phage therapy.

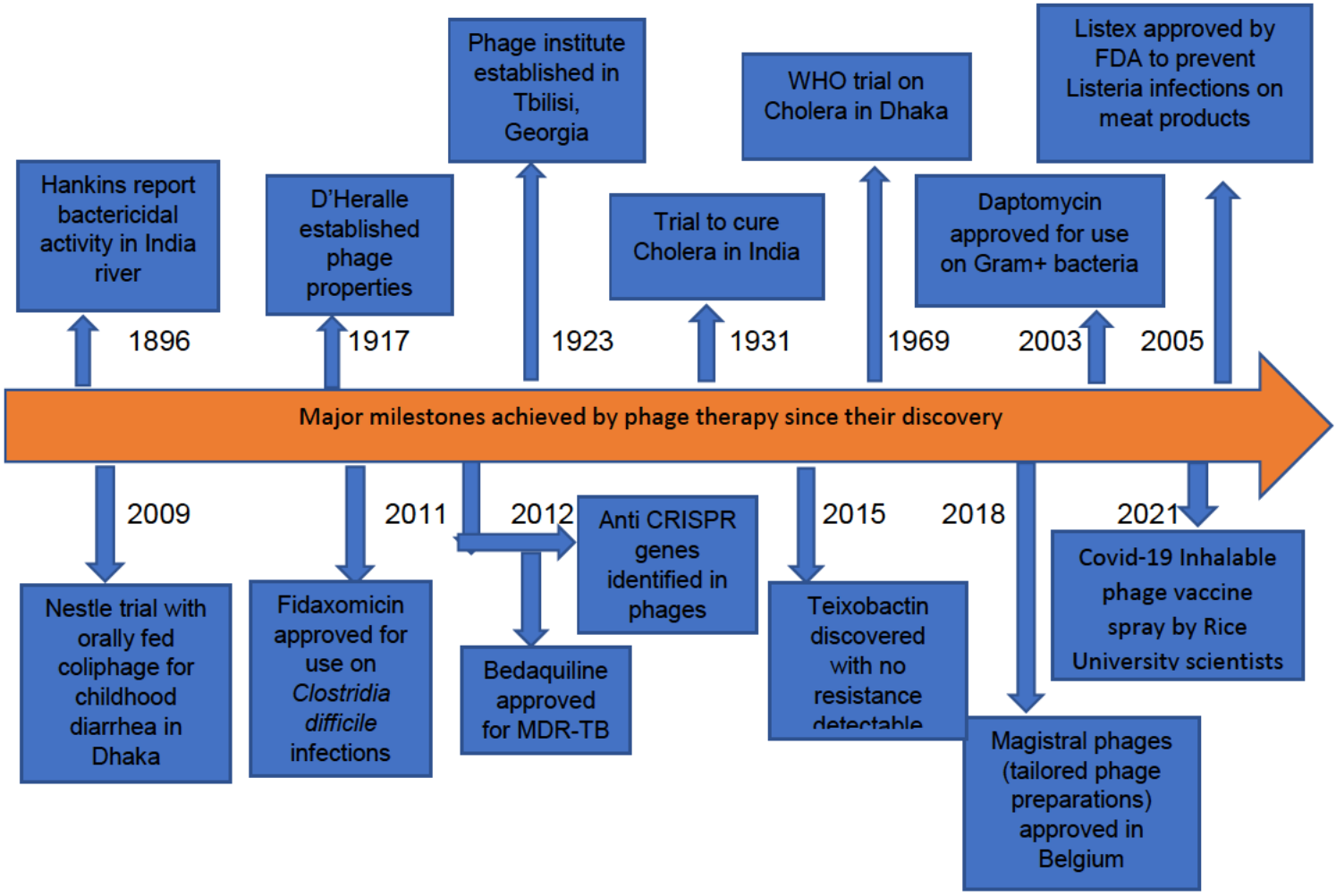

Figure 1.4: Some of the major milestones achieved by phage therapy since the discovery of phages (adopted from Saha and Mukherjee, 2019; National science foundation, 19 July 2021).

For phages to be regarded as efficient to be used for therapy, they need to be lytic, sequenced, and have a high specificity (broad host range) against the target pathogen (Lehman *et al*., 2019).

Phage therapy advantages

- Phage isolation is fast, simple, and cost-effective. Phages are present in soil, water, ocean, plants, sewage, and this makes their isolation easy, fast, and cheap (Badawy *et al*., 2022).
- Bacteriophages are capable of auto dosing, that is they increase in number at the location of the infection (Principi *et al*., 2019). This is because after infection, each virus produces many virions that way, they auto-dose and eradicate the infection faster.
- Phages exhibit low toxicity and are generally regarded as safe (GRAS) (Loc-Carrilo and Abedon, 2011; Endersen and Coffey, 2020; Uyttebroek *et al*., 2022).
- Compared to antibiotics, the development of bacterial resistance to phages is much slower, and phages are effective even against MDR bacteria (Badawy *et al*., 2022).
- Phages remain effective even under relatively harsh environmental conditions and continue to replicate until there is no host bacterium present.
- Phages are specific to their host bacteria; as opposed to broad-spectrum antibiotics, this protects the natural microbiota of the human body (Wienhold *et al*., 2019), thereby avoiding side effects such as an altered gut microbiome (Uyttebroek, 2022)
- Phage therapy is safe and appropriate for humans as they do not infect eukaryotic cells and manifest only minor side effects (Doss *et al*., 2017; Principle *et al*., 2019).

Phage therapy disadvantages

- Most phages exhibit a narrow host range.
- Not all phages are good for phage therapy, as temperate phages may carry undesirable genes and integrate into the host genome.

- Phages are protein-based and can therefore interact with the human immune system and can evolve during use, posing a potential to infect body tissues (allergy).
- Unfamiliarity with phage therapy also serves as a potential limitation (Loc-Carrilo and Abedon, 2011).
- Factors affecting the stability of phage formulations are not yet well understood (Malik *et al*., 2017).
- Phage preparations may present with bacterial debris containing endotoxins that may result in the death of a patient; thus, a costly purification is required (Gandham, 2015).

Even though phage therapy manifests some limitations, most of these limitations can be overcome by isolating new phages and employing well-designed phage cocktails. As they are plenty in nature, with current technology and tools, they can even be engineered and optimized to be fit for a specific purpose. Bacterial infections are on the rise, and with phages showing numerous advantages, phage therapy has a true potential to overcome infections caused by untreatable MDR and XDR bacteria.

Medical application

Bacteriophages were first used for phage therapy at the Hospital des Enfant- maladies in Paris, under the supervision of Professor Victor-Henri Hutinel, who was the chief of the pediatric section in 1919 (Summers, 1999). The phage preparation was ingested by d'Herelle and several hospital interns to confirm its safety before it was administered to a 12-year-old boy suffering from a severe dysentery. The symptoms ceased after one dose of phage therapy, and he recovered after a few days. The confirmation of the efficacy of the phage preparation followed soon after three patients with dysentery recovered within 24 hours of a single dose of treatment (Sulakvelidze *et al*., 2001). However, the first article to report phage therapy was on research conducted by Bruynoghe and Maisin in Belgium in 1921, whereby six patients with boils were injected with phages targeting *Staphylococcus* species, within 48 hours the pain, swelling and fever were lessened (Abedon *et al*., 2011).

A leg ulcer of a 50-year-old woman from Texas successfully responded to phage therapy treatment (figure 1.5(B). Her wound was infected with a Multi-Drug Resistant (MDR) *Pseudomonas* sp. and could not heal after a year of conventional wound care management. After 2 weeks of phage application the wound began to show signs of healing and 2 months later, it was almost closed up (Clark and March, 2006). In 2009, Merabishvili and co-workers used the lytic phage cocktail BFC1 (figure 1.5(A)) to treat patients with burns in a military Hospital in Belgium. The phage cocktail was made up of *Pseudomonas aeruginosa* and *Staphylococcus aureus* targeting lytic phages (Merabishvili *et al*, 2009).

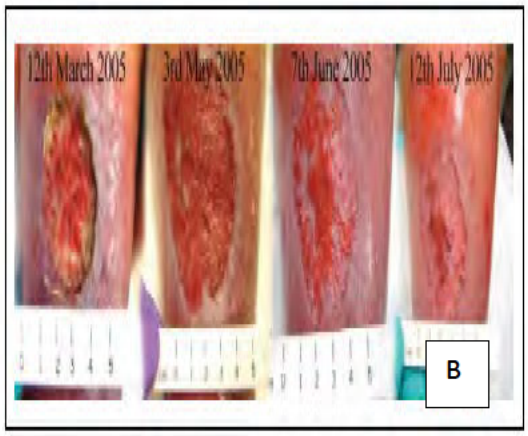

Figure 1.5(A) Phage cocktail BFC 1 that was used to treat wounds infected with *P. aeruginosa* and *S. aureus* in Military hospital patients, Belgium (Merabishvili *et al.*, 2009). (B) Leg ulcer infected with MDR *Pseudomonas* sp. and how the ulcer healed after phage therapy treatment (Clark and March, 2006).

Letkiewics and colleagues reported in 2010 the interaction of phages with bacterial biofilms and showed the safety of phage therapy in treating chronic bacterial prostatitis, which is a serious clinical and social thread affecting men under the age of 50 (Letkiewics *et al.*, 2010). In the Eliava institute in the city of Tbilisi, Georgia, bacteriophages were used for decades to treat eye, ear and nose infections in humans through inhalation (Jassim and Lamoges, 2014).

Sarker *et al*. (2017) reported that the oral application of *Escherichia coli* bacteriophages was safe after they administered a T4-like coliphage cocktail to

healthy Bangladesh children, and no adverse effects were observed clinically and by clinical chemistry. This study further confirmed the results the same authors obtained in a study when investigating the safety of the same phage cocktail in healthy adults and hospitalized diarrhea children (Sarker *et al*., 2017)

Fox (2019) reported that a 15-year-old patient suffering from cystic fibrosis receiving a lung transplant, was infected by an MDR *Mycobacterium abscessus,* which resulted in wounds and swollen nodules across her body. Her doctor collaborated with one of the top phage researchers in the USA, Graham Hatful, who isolated 3 phages that could tackle *M. abscessus*, but 2 out of the 3 were temperate phages. Using a gene editing technique developed in Hatful's lab, the repressor gene was removed to make the phages bactericidal. The cocktail was used on the girl, and in 72 hours, her wounds began to dry, and after 6 weeks of continuous treatment at 12-hour intervals, the infection was gone (Fox, 2019). Phage therapy has also been used in patients with UTI (urinary tract infections), and some of the commonly isolated pathogens were *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and Methicillin-resistant *Staphylococcus aureus*. Patients received intravesical, oral and pelvic installations or combined treatment. In these studies, there were no adverse events reported. However, symptom relief was observed, and there was no recurrent of the UTI reported in 2-48 months of the research (Uyttebroek *et al*., 2022)

Phage therapy gaining momentum has resulted in numerous phage research companies being established, including Biophage Pharma Inc. (Canada), Hexal Genentech (Germany), Technophage (Portugal), Novolytix Ltd (UK), Special Phages Services (Australia), GangaGen (India/USA), Biomed S.A., Biophage Pharma S.A., Proteon Pharmaceuticals in Poland and many more based in the USA (Vandamme and Mortemans, 2018).

To enable long-time storage of medical phage preparations, Zhang and colleagues (2018) investigated the stability of phages in a freeze-dried powder form. In their study, they tested phage formulations with trehalose, sucrose, mannitol, and PEG6000. Trehalose and sucrose showed less phage titer loss after long storage compared to mannitol and PEG. Increased moisture proved to have a positive effect on the viability of the tested phages. These findings highlight the potential of long-term storage of the

phage formulations under ambient temperature, which would be ideal for usage in healthcare facilities (Zhang et al., 2018).

Biotechnology

Phages have been a useful tool in the field of biotechnology, with various studies having been conducted ranging from drug design, assembly of novel proteins, delivery of vaccines, and detection of pathogenic bacteria to the screening of proteins, peptides, and antibodies (Haq *et al*., 2012). Phages have been exploited in vaccine delivery in two modes. Phages can be modified to present an antigen on their exterior or by incorporating a DNA vaccine expression cassette into the phage genome. Unmodified phages have proved to be better for DNA vaccine delivery than standard plasmid vaccines (Clark and March, 2006). The phage capsid coat that covers and protects its DNA from degradation and their ability to display alien molecules on their surface make phages an appropriate candidate for gene therapy. Nanotechnology is advancing, and phages have been used as vectors in nanodevices in the application of nanomedicine (Harada *et al*., 2018).

Host-specific phages have been explored directly for bacterial typing or via antigen-antibody binding for determination of pathogenic bacteria such as *Mycobacterium tuberculosis,* Shiga toxin-producing strains of *Escherichia coli,* and the pathogen *Pseudomonas aeruginosa* (Sharma *et al*., 2017). Furthermore, an *E. coli* M13 phage is extensively used for phage display systems. It is useful in the isolation of peptides that code for specific proteins that may be used to block the receptor-ligand interaction or used as an agonist (Haq *et al*., 2012). In 2005, Dickerson and colleagues conducted a study illustrating an effective approach to treating cocaine addiction in a rodent model. They engineered filamentous phages displaying cocaine-sequestering antibodies on their surface. These phages were administered nasally and then finally, made their way to the nervous system, where the displayed antibody binds to the cocaine molecule and inhibits its action on the brain, thereby blocking behavioral effects (Dickerson *et al*., 2005).

Agriculture and food safety

Not surprisingly, bacteriophages have been used successfully in agriculture. In 1924, Mallman and Hemstreet detected that a filtrate from a decaying cabbage had a bacteriostatic effect against the plant pathogen *Xanthomonas campestris,* which causes black rot in vegetables (Jones *et al*., 2012). A cocktail of Coliphage B44/1 and B44/2 has also been applied on animals infected with enteropathogenic diarrhea-causing *E. coli* strains of serovar O9:K3099. The treatment with phage ceased fluid loss, and all the animals survived (Smith and Huggins, 1983). To combat bacterial contamination of crops, formulations such as Agriphage and Omnilytic have been approved in the USA for the use of crop protection against various bacterial plant pathogens. Moreover, Listex P100 was the first FDA-approved commercial anti-*Listeria* phage formulation, produced by Micreos, the Netherlands that is effective in reducing the presence of *Listeria monocytogenes* on cantaloupes and ready-to-eat meat (Sharma *et al*., 2017). In the United States of America, the Food and Drug Administration (FDA) approved the use of the product ListShield, which is manufactured by Intralytix Ltd for the treatment of food products to inhibit foodborne infections (Meaden and Koskella, 2013). Over the years, Intralytix has innovated more phage-based products, such as Ecoshield, SalmFresh, and Shigashield, which target different foodborne pathogenic bacteria (Goncalves de Melo *et al*., 2018). Bacteriophages are re-gaining momentum worldwide and are now recognized as a viable approach for use in food and safety at various stages of the production of plant and animal-based products (food) and crops (Endersen and Coffey, 2020).

1.7. Wastewater decontamination

A typical wastewater treatment process comprises of technologies such as chemical precipitation, ion exchange, sedimentation, coagulation, and chlorination. These are energy and maintenance-intense, costly techniques that require modern infrastructure to operate (Summers, 2015). As a result, scientists all over the globe are conducting studies assessing how phage-based techniques can be used in wastewater treatment to enhance effluent and sludge quality and enable the removal of pathogens without the need for chemical biocides (Ji *et al*., 2021). Phages have been reported to play a crucial role in wastewater treatment plants where they tackle exopolysaccharides, high molecular weight, and biodegradable polymers, which are produced by bacteria and

aid floc formation by hydrolytic enzymes. Phages also aid in reducing excess biological sludge and excess forming (Johnke *et al*., 2014), and phages have also been used to tackle membrane biofouling (Scarascia *et al*., 2021).

Chlorination

Chlorination is the most common form of disinfection of wastewater worldwide, with small wastewater works typically using calcium hypochlorite tablets or liquid sodium hypochlorite, which contains about 13% of free chlorine (Bekink and Nozaic, 2012). Treating wastewater with chlorine before discharging it to rivers, streams and oceans has saved millions of people from waterborne diseases over the past 10 decades (Bekink and Nozaic, 2012). Chlorine usage has additional benefits, other than removing pathogens, like controlling odor and controlling activated sludge bulking (Övez *et al*., 2006; Freitas *et al*., 2021). The mechanism that chlorine uses to destroy bacteria is still poorly understood. However, chlorine is a halogen, and halogens are strong oxidizing agents. It is believed that chlorine oxidizes the membrane lipids and proteins of bacterial cells, which affects cellular respiration and transport processes resulting in cell death (Maris, 1995). However, chlorination has its shortcomings. When it is used for pre-oxidation in potable water treatment, it can produce toxic trihalomethanes and other chlorinated compounds, which are potentially carcinogenic (Freese, 2008; Ji *et al*., 2021).

Chlorination initially lowers the total number of bacteria, but it may also increase antibiotic resistance and can facilitate the transfer of resistance genes to other pathogenic strains present (Murray *et al*., 1984; Ji *et al*., 2021). Liu and colleagues (2018) demonstrated that chlorine used for disinfection could promote the prevalence of antibiotic-resistance genes, which may lead to the dissemination of these genes in the environment. Although chlorination may kill antibiotic-resistant bacteria, DNA will be released simultaneously into the water, and external antibiotic-resistance genes could still be present. The occurrence of antibiotic-resistance genes in the wastewater may promote antibiotic resistance in bacteria via conjugation and transduction. The extracellular antibiotic resistance genes may persist in water for some time and can later be taken up by competent non-resistant bacteria resulting in widespread antibiotic resistance via transformation (Liu *et al*., 2018).

Phage use as a decontaminating biocontrol agent in wastewater treatment is highly encouraged in developing countries as it is an affordable, sustainable, and eco-friendly approach (Ji *et al*., 2021). Phage performance in any wastewater treatment process is controlled by many factors, and somatic phages, which are reported to be dominant in wastewater, are limited by factors such as host range, pH, temperature, cations, and organic matter concentrations (Jonczyk *et al*., 2011; Sharma *et al*., 2017). Clean, safe water is the most important component of life, as we depend on it for survival. A wastewater treatment plant is the most important method of removing microbial pollutants present in the wastewater for safe disposal back into the environment or reuse. As bacteriophages have been reported to be abundant and to play a crucial role in removing undesirable pathogenic bacteria in wastewater, their application for wastewater treatment is, therefore, of utmost importance (Runa *et al*., 2021).

1.8. Problem statement

Clean, safe water is essential for human life. However, bacteria carrying antibiotic-resistance genes are increasingly excreted to the environment via wastewater treatment plants, thus contaminating rivers with pathogenic bacteria or even multidrug-resistant bacteria (Ji et al., 2021), and Pietermaritzburg, South Africa, is no exception. According to Ngubane et al. (2022) the Darvill wastewater treatment plant serves 51.6% of the 163.993 citizens of the Umsunduzi local municipality; thus it is often operated above capacity and sometimes overflows releasing untreated wastewater back into the environment. This is the main fecal contamination in the Umsunduzi river, which generally decreases water quality in South Africa. In 2019, it was reported that more than half of South Africa's wastewater plants are failing to treat wastewater, which poses a serious danger to the health of the people (Kretzmann *et al*., 2019). *Escherichia coli*, used as a hygiene indicator, is one of the top contaminants of the water bodies receiving sewage (Verlicchi and Grillini, 2020), and pathogenic strains of *E. coli* are amongst the dangerous human bacterial pathogens called ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacte*r spp. and pathogenic strains of *Escherichia coli*) (Skurnik, 2022). *Escherichia coli* is a Gram-negative, facultative aerobic, motile, rod-shaped bacterium residing in human and animal intestines (Lim *et*

al., 2010). For this reason, *E. coli* is found to be prevalent in wastewater. However, this bacterium is regarded as innocuous to its host. The overuse and misuse of antibiotics has led to many bacteria, including pathogenic strains of *E. coli*, becoming resistant, and antibiotic-resistant bacteria are being excessively excreted into the environment and contaminating our water bodies (Ji *et al*., 2021). Since water cleaning techniques are complex, bacteriophages from the environment are widely isolated and characterized as they pose a potential for usage as biocontrol agents (Serwer *et al*., 2004). Water cleaning techniques are expensive and complicated; thus, phages offer a low-cost, sensitive, and eco-friendly alternative to remove undesired bacteria from wastewater.

1.9. Aims of the study: To isolate and characterize *Escherichia coli* specific bacteriophages from wastewater and to further evaluate the potential application of the isolated phage as a biocontrol agent for *Escherichia coli* in wastewater.

1.10. Objectives of the study

1. To isolate *E. coli* bacteriophages as potential biocontrol agents from wastewater using the (double) overlay technique.

2. To characterize bacteriophage isolates using Transmission Electron Microscopy (TEM).

3. To determine the host range of the phage isolates.

4. To determine the multiplicity of infection (MOI), burst size, and latency period of the phage isolates.

5. To test the stability of the phage isolates at different conditions.

6. To extract the DNA of selected phage isolates and to sequence the whole genome.

7. To assess if the selected phages can eliminate *E. coli* from an artificial wastewater sample.

2. Materials and methods

2.1. Culture media

All bacteria were routinely grown using Nutrient broth or Nutrient agar (Biolab) at 37°C and 100 rpm in the dark. Soft agar was prepared using nutrient broth (Biolab) and 0.6% bacteriological agar (Biolab), at pH 7.0. Prior to use, all media were autoclaved at 121°C for 15 minutes.

2.2. Bacteriophage enrichment and isolation

An influent sample was collected from the Darvill wastewater treatment plant (KwaZulu-Natal, Pietermaritzburg, South Africa) using sterile 1L Schott Duran bottles, transported on ice to the laboratory, and stored at 4°C until processing. Phages were enriched following the method described by Clokie and Kropinski (2009) with modifications. Prior to inoculation, debris was removed from 20 mL wastewater samples by centrifugation (Beckman Coulter Avanti-J26 XPI) at 10 000 *g* and 4°C for 10 minutes, followed by filtration using sterile 0.22 µm syringe filters (Acrodisc®, USA). 100 µl of an 18–24-hour culture of *Escherichia coli* ATCC 25922 grown in nutrient broth was added to 10 mL of the filtered wastewater and then incubated at 37°C at 100 rpm. After 24 of incubation, bacterial cells were removed from the culture by centrifugation and filtration, as stated above.

A spot test was performed with *E. coli* ATCC 25922 as a bacterial host to detect the presence of lytic phages in the enrichment culture. 100 µL of an overnight culture of *E. coli* ATCC 25922 was inoculated on Nutrient agar by the spread plate technique, after which 10 µL of the phage lysate was spotted onto the bacterial lawn. The plates were allowed to dry and then incubated at 37°C for 24 hours. The plaques formed on agar plates indicate the presence of phage particles that were selected for isolation.

Phages were isolated from the enrichment culture using a double overlay method. 100 µL of the filtrated enrichment culture was added to 500 µL of *E. coli* ATCC 25922 and allowed to stand for 20 minutes. The mixture was then added to 4.5 mL of 0.6% molten agar and overlaid onto Nutrient agar plates. Single plaques with distinct morphologies were selected and purified.

2.3. Bacteriophage concentration and purification.

Amplification of the phage particles was carried out following the method described by Loessner *et al*. (1993) and Twest and Kropinski (2009) with modifications. A single plaque was picked using a pipette from a soft agar double-layer plate and suspended into 500 µL of 0.85% saline solution, and incubated for 1 hour at 37°C with shaking to remove the phage particles from the soft agar. A 50 µL culture sample of the host bacterium was added to the 500 µL suspension and allowed to stand for 20 minutes to allow the phage particles to attach to the host bacteria. The suspension was then transferred to 8 mL of sterile nutrient broth and incubated at 37°C and 100 rpm for 5 hours. After incubation, the culture was centrifuged at 10 000 *g* at 4°C for 10 minutes, followed by sterile filtration using sterile 0.22 µm membrane filters (Acrodisc, USA). Serial decimal dilutions were prepared from the above filtrate with 0.85% sterile saline and allowed to stand for 20 minutes. 500 µL of the dilutions, together with 100 µL of an overnight culture of *E. coli* ATCC 25922 were then transferred to 4.5 mL molten agar and overlaid on Nutrient agar plates. The plates were left to solidify for 20 minutes; thereafter, they were inverted and incubated at 37°C for 18-24 hours.

To purify the phage particles, the above steps were repeated 5 times to obtain a consistent morphology and ascertain purity (Loessner *et al*., 1993).

2.4. Transmission electron microscopy (TEM)

A total volume of 10 µL of pure phage lysate at a phage titer of 10^9 PFU/mL was spotted onto carbon-coated grids (Advanced Laboratory Solutions, JHB, South Africa) and left standing for 5 minutes. The grids were then stained with 2% (w/v) pH 7.2 phosphotungstic acid (PTA) (West Chester, PA 19381, USA) at pH 7.2 for 30 seconds, washed with distilled water, and air-dried for 15 minutes before viewing. The PTA-stained carbon grids were examined using a JOEL JSM-1400 Transmission Electron Microscope at 80 kV. The phage size was determined by measuring the tail and the capsid size.

2.5. Extraction and purification of phage nucleic acids

Extraction of phage isolates nucleic acids was conducted according to the method of Green and Sambrook (2012) with modifications. The crude phage lysate was centrifuged at 10 000 *g,* 4°C for 10 minutes, and filtered through a sterile 0.22 µm filter

(Acrodisc®, USA), and the filtrate was used for extraction of bacteriophage DNA. Ribonuclease A and DNase I (Takara Biotechnology, Darian, China) 1 mg/mL final concentration were added to aliquots of bacteriophage preparations and incubated for 30 minutes at 37°C. Protease K (200 µg) and SDS (0.5% final concentration) were added, and the mixture was incubated at 56°C overnight. Proteins were removed by phenol: chloroform: isoamyl alcohol (25:24:1, Sigma Aldrich, USA) extractions and the nucleic acid was precipitated with alcohol. After washing in 70% ethanol, the pellets were re-suspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and analyzed using 1% agarose gels and Ethidium bromide staining (Sigma Aldrich, USA). The nucleic acid of the isolated bacteriophage was digested with DNase I or RNase A at 37°C according to the manufacturer's instructions. Digested bacteriophage nucleic acids were examined by 1% agarose gel electrophoresis using Transilluminator (Bio-Rad, Hercules, CA).

2.6. Phage nucleic acid sequencing and analysis

The isolated phage DNA was precipitated with 0.1 volume of 3 M Sodium Acetate (pH 5.2) and two volumes of cold ethanol and centrifuged at (15 000 g, 4°C, 30 minutes). The pellets were rinsed twice with 70% ethanol, air-dried, and dissolved in 100 µL sterilized deionized distilled water containing 50 µg/mL RNase A.

The purified phage genomic DNA was sequenced using an Illumina^{R} TruSeq^{R} Nano DNA Library Prep kit (Illumina, CA) and an Illumina HiSeq X-Ten platform to 100 -fold coverage at Guangzhou Gene de-novo Biotechnology Co.Ltd (China). Reads with poor quality were eliminated, and the remaining reads were assembled denovo by SeqMan sequence analysis software (DNASTAR Inc, USA).

Coding sequences were analyzed using the genome annotation tool available in PATRIC (https://www.patricbrc.org/ Davis *et al*., 2020). Homologs of nucleotide sequences and predicted protein sequences were searched using BLASTP & BLASTN tools available at NCBI. MAUVE software was also used to compare phage genomes (https://biotools/mauve). The sequence alignments for selected genes were done using ClustalW or Muscle, and phylogenetic trees were constructed using Mega X (Kumar *et al*., 2018) based on the maximum likelihood and neighbor-joining methods. Additionally, the phage phylogeny was analyzed using Victor software (Meier-Kolthoff and Göker, 2017) TYGS (Type Strain Genome Server) (Meier-Kolthoff

and Göker, 2019), and ANI (Average Nucleotide Identity) for selected phage genomes (Lee *et al*., 2016).

2.7. Establishing plaque-forming units

The phage titers were quantified by counting plaque-forming units using a double-layer method on Nutrient agar with *E. coli* ATCC 25922 as a bacterial host. 100 µL of the phage culture was aliquoted and serially diluted in 0.85% NaCl to achieve desired concentrations. 100 µL of the diluted culture was then mixed with 500 µL of the 24-hour bacterial host culture and allowed to stand for 10 minutes, after which it was then transferred to 4.5 mL of 0.6% molten agar and overlaid on Nutrient agar plates. The plates were incubated at 37°C for 24 hours, and the plaques formed were counted.

2.8. Determination of the latency period and the burst size of the phage isolates

One-step growth curves of the isolated phages were established with *E. coli* ATCC 25922 as bacterial host using a 10-minute interval method as previously described (Bao *et al*., 2011). Host cells (10^8 CFU/mL) and the specific phage were mixed in SM buffer to achieve a multiplicity of infection (MOI) of 10~100. Phage particles were allowed to adsorb for 20 minutes at 37°C, and the mixture was centrifuged at 12 000 *g* for 60 seconds to remove unattached phages. The pellets of infected host cells were re-suspended in 10 mL of pre-warmed Nutrient broth and incubated at 37°C for 2 hours. Samples were collected at 10-minute intervals and immediately centrifuged. Phage titers from the collected samples were then determined using double overlay agar. The latency period and burst size were determined according to the one-step growth curve. Burst sizes were defined as the final titers of phage divided by the initial concentration of host cells.

2.9. Phage stability at different pH and temperature

2.9.1. Thermal stability

The phage stability during thermal exposure was studied at 5 different temperatures (25, 37, 45, 60, and 80°C) following the method described by Verma *et al*. (2009) with modifications. A temperature of 37°C, which is the optimal temperature for phage host growth and replication, was used to compare the effect of temperature on the isolated phages. A high titer phage lysate (10^9 PFU/mL) in Nutrient broth was incubated at the

temperatures stated above for 10 minutes. After incubation, 1 mL of the lysate was withdrawn and serially diluted in 0.85% NaCl. The effect of varying temperature on phage titer was monitored by establishing plaque forming unit using a double-layer plating technique. The plates were incubated at 37°C for 18-24 hours.

2.9.2. The effect of pH

The effect of acidic and alkaline conditions on phage stability was investigated at different pH values following a method described by Jun *et al*. (2013) with modifications. Phage lysate was suspended in 0.85% NaCl at pH values of 3.0, 5.0, 7.0, 9.0, and 11.0 adjusted with hydrochloric acid (HCl) or sodium hydroxide (NaOH) to a final phage titer of 10^9 PFU/mL; the suspension was incubated for 1 hour (volume 1:9) at 37°C. After incubation, 1 mL was serially diluted in 0.85% NaCl, the effect of pH on phage isolates was determined by establishing plaque-forming units using the double layer method as stated in 2.7.

2.10. Host range determination.

In total, 32 selected and representative bacterial strains (Table 2.1) were used to determine the host range of the isolated bacteriophages in replicates. The host range of the isolated phages was determined by spotting 10 µL of the phage lysate (10^9 PFU/mL) of each bacteriophage isolate onto freshly prepared bacterial lawns prepared by spread plating 100 µL of mid-exponential phase cultures of bacterial strains to be tested. Plates were dried for 10 minutes, after 24 hours of incubation at 37°C, the emergence of clear zones on the plates indicated infection of bacterial host by the isolated phage (Majdani, 2016).

Table 2.1 Bacterial strains used in this study for host range determination, their origin, and antibiotic resistance profile.

Name of the bacteria	Origin	strain resistance profile
*Klebsiella pneumoniae K*p124 MDR	Hospital isolate	NOR-AMC-CAZ-ATM-GEN-TOB-CIP-CTX-ERT-MXF-CL
Staphylococcus aureus	ATCC 6538	-

Name of the bacteria	Origin	strain resistance profile
Salmonella Typhimurium	ATCC 14028	-
Enterococcus faecalis	ATCC 29212	-
Micrococcus luteus	ATCC 9341	-
Escherichia coli	ATCC 25922	-
Escherichia coli (Bw1)	Borehole water	-
Escherichia coli (Bw2)	Borehole water	-
Escherichia coli (Bw3)	Borehole water	-
Escherichia coli (Bw4)	Borehole water	-
Escherichia coli (EC 1)	Zebra feces	-
Escherichia coli (EC2)	Zebra feces	-
Escherichia coli (EC3)	Zebra feces	-
Escherichia coli (EC4)	Zebra feces	-
Escherichia coli (EC 9)	Pit latrine sludge	AMP-AMC-ERTGEN-TOB
Escherichia coli (EC10)	Pit latrine sludge	AMP-AMC-TOB
Escherichia coli (EC 15)	Pit latrine sludge	AMP-AMC-TOB
Escherichia coli (EC 20) MDR	Pit latrine sludge	AMC-CAZ-GEN-TOB
Escherichia coli (EC 26)	Pit latrine sludge	GEN-TOB
Escherichia coli (EC 27) MDR	Pit latrine sludge	AMP-AMC-CAZ-ATM-GEN-TOB
		AMP-AMC-CAZ

Name of the bacteria	Origin	strain resistance profile
Escherichia coli (Fp5)	Farm pig	AMP-AMC-CAZ-GEN
Escherichia coli (Fp6)	Farm pig	AMP-AMC-ATM-NOR-CIP
Escherichia coli (Fp29)	Farm pig	AMC
Escherichia coli (Pp 10)	Pet pig feces	-
Escherichia coli (Pp19)	Pet pig feces	AMC-CAZ
Escherichia coli (L20)	Lettuce	-
Escherichia coli (L23)	Lettuce	-
Escherichia coli (L36)	Lettuce	-
Escherichia coli (W26)	Wild beast faeces	AMC-ATM
Escherichia coli (Wd2)	Irrigation water	-

(-) indicates no phenotypic antibiotic resistance known. AMP (ampicillin), AMC (amoxicillin-clavulanic acid), CAZ (ceftazidime – 3rd generation cephalosporin), ATM (aztreonam), CIP (ciprofloxacin), NOR (norfloxacin), GEN (gentamicin), TOB (tobramycin), ERT (ertapenem), CTX (Cefotaxine), MXF (Moxifloxacin) and CL (Cefalexin) resistance.

Bacterial isolates were obtained from the laboratory culture collection. Isolates EC 9, 10, 15, 20, 26 & 27 were reported by Beukes *et al.* (2017), and Fp 5, Fp 6, Fp 29, Pp 10, Pp 19 and W26 were reported by King and Schmidt (2017) and King *et al*. (2020).

The above bacterial strains were grown on either Nutrient agar or in Nutrient broth at pH 7 at 37°C for 18-24 hours. In the latter case, on a shaker at 100 rpm.

2.11. Viability of phage isolates after refrigeration and freezing

The viability of phage isolates was tested after storage in a fridge (4°C) and a freezer (-80°C). 500 mL of the phage lysate was added in 9.5 mL Nutrient broth without the host to a test tube to a final concentration of 10^9 PFU/mL and stored at 4°C for 12 days. Viability was monitored every 48 hours for 12 days by counting the plaque-

forming units using the double-layer agar method with *E. coli* ATCC 25922 as a bacterial host as stated in section 2.7.

The viability of the isolated phages stored at -80°C was monitored for a period of 2 years in replicates. Phage lysate (500 µL) was added in Nutrient broth (9.5 mL) to a final concentration of 10^9 PFU/mL with bacterial host and incubated in a -80°C freezer. Subsamples were collected at 0, 3, 6, 12, 18, and 24 months and filtered 0.22 µm to remove bacterial cells. Viability was monitored by establishing plaque-forming units using the double-layer method, as stated above with *E. coli* ATCC 25922 as a host.

2.12. Phage and chlorine application to treat wastewater

A total volume of 200 mL of Darvill wastewater sample was centrifuged at 10 000 *g* and 4°C for 10 minutes, and then filtered with Whatman No.1 filter paper to remove debris. The pH of the wastewater was adjusted to 7.5 using HCl. 30 mL of the filtered wastewater was transferred to four Erlenmeyer flasks, autoclaved at 121°C for 15 minutes for sterilization. Once the flasks had cooled down, *E. coli* ATCC 25922 was added to a final concentration of 10^8 CFU/mL and incubated at 37°C and 100 rpm (to create an artificial wastewater with only *E. coli* ATCC 25922 present). After 3 hours, one flask was inoculated with phage NPM at a concentration of 1×10^8 PFU/mL, one flask with 5% chlorine solution (sodium hypochlorite) at a concentration of 10 mg/L, one flask with phage NPM (10^8 PFU/mL) and chlorine (10 mg/L) and the last flask was left with only *E. coli* as a control. All four flasks were incubated at 37°C and 100 rpm. A change in bacterial cell number was monitored microscopically (Zeiss primo star) every 30 minutes for 120 minutes by direct cell counts (Helber type counting chamber, Marienfeld, Germany).

2.13. Data analysis

Data were analyzed statistically using Microsoft Excel (2019) and plotted using SigmaPlot version 8.0 where applicable.

Results

3.1. Phage Isolation

3.1.1. Phage enrichment and isolation

Lytic bacteriophages are viruses that infect and destroy bacteria. Therefore, *Escherichia coli* ATCC 25922 was used as a host to investigate the presence of bacteriophages in wastewater samples collected from the Darvill wastewater treatment plant in Pietermaritzburg, KwaZulu-Natal, South Africa. The presence and the lytic activity of bacteriophages present in wastewater samples were detected in the initial enrichment experiments by the decline in *E. coli* biomass over time after the addition of filtered wastewater to *E. coli* ATCC 25922 cultures in Nutrient broth. The data obtained from the initial experiment are presented in figure 3.1.

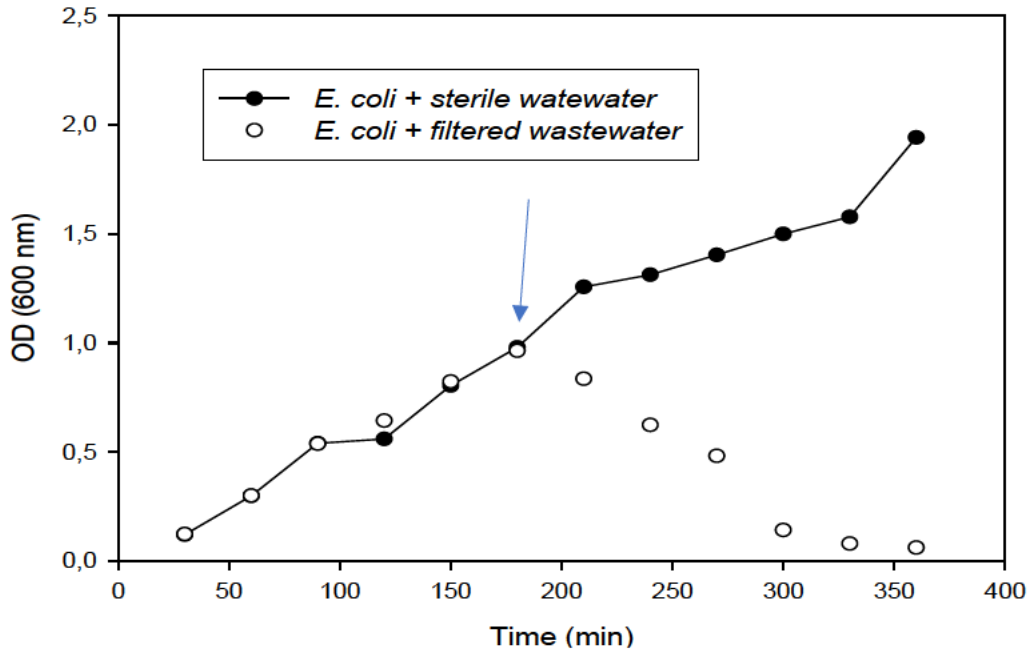

Figure 3.1: The effect of addition of filtered wastewater (10 mL) and sterilized wastewater sample when added to a growing culture of *E. coli* ATCC 25922 at 180 minutes (indicated with an arrow, monitored via OD 600) in Nutrient broth at 37°C, 100 rpm. The values presented are the average of three independent experiments.

Addition of filtered wastewater to a growing culture of *E. coli* ATCC 25922 in Nutrient broth resulted in a decline in bacterial biomass after about 30 minutes, which was not observed when sterile wastewater was added to a parallel culture of *E. coli* in Nutrient broth; thus, indicating the lysis of *E. coli* cells due to the presence of a lytic phage in the wastewater.

In addition to monitoring the decline in bacterial biomass in the presence of bacteriophages in enrichment cultures, the presence of lytic phages was confirmed by assessing the turbidity of an *E. coli* ATCC 25922 culture in Nutrient broth after addition of 10 mL filtered wastewater in test tubes as shown in figure 3.2.

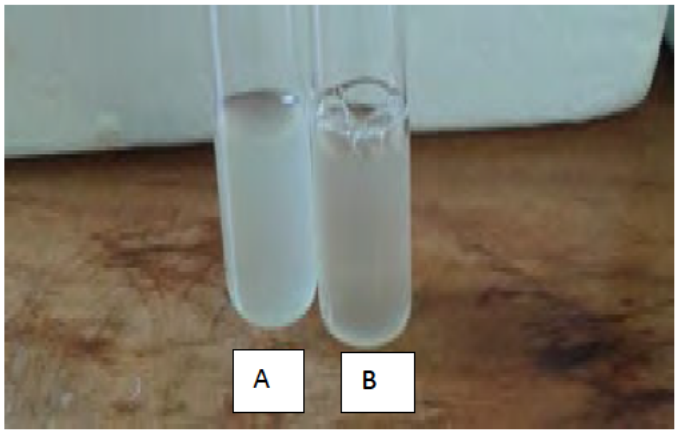

Figure 3.2: Turbidity of *E. coli* ATCC 25922 in Nutrient broth after addition of sterile autoclaved wastewater (A) and filtered wastewater (B) after incubation at 37°C for 18-24 hours.

The presence of lytic activity of bacteriophages in the wastewater was confirmed as the *E. coli* ATCC 25922 culture in test tube B was less turbid upon addition of the 0.22 µm filtered wastewater after incubation, whilst test tube A was more turbid (OD_{600} ≥1.00) due to ongoing growth of *E. coli* cells, indicating that bacteriophages present were inactivated in the wastewater sample during heat treatment.

Lytic phages infecting bacteria cause lysis of the host cells; using solid media with a bacterial lawn present, therefore results in clear patches called plaques if lytic phages are present. Therefore, a spot test on Nutrient agar was carried out to determine the

lytic activity of phages in wastewater samples on24 hour old *E. coli* ATCC 25922 lawn. The plaques formed when performing the spot test are presented in figure 3.3.

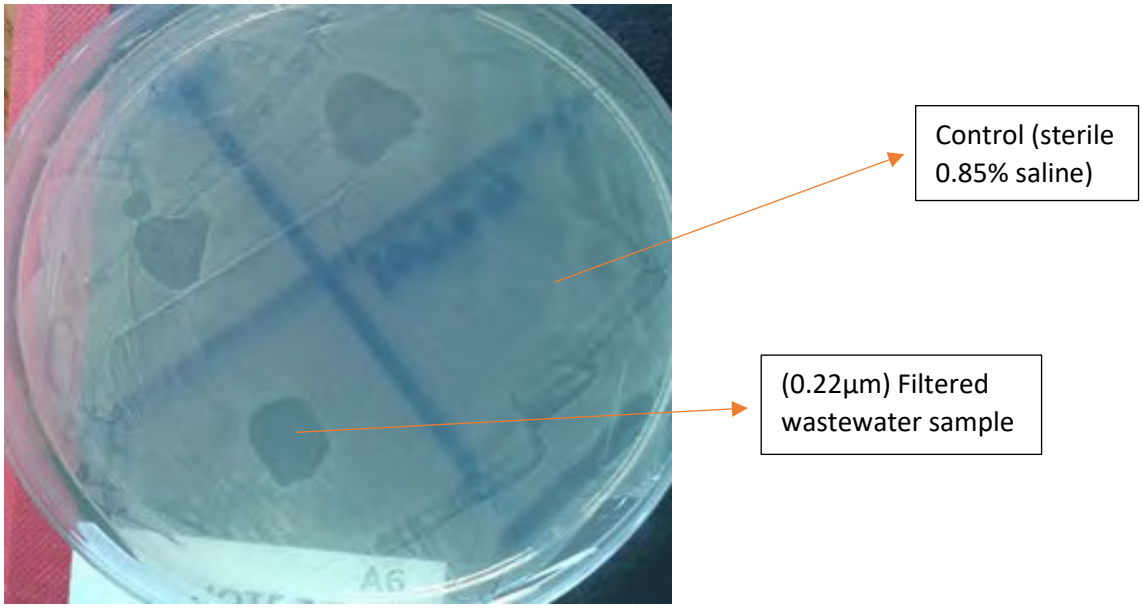

Figure 3.3: Spot test of the filtered wastewater using an *E. coli* ATCC 25922 lawn on nutrient agar to confirm the presence of lytic phages in filtered wastewater. The three quadrants with clear patches were spotted with 10 µL of the filtered wastewater and the fourth quadrant was spotted with 10 µL sterile saline solution as a negative control.

The apparent clear patches in the three quadrants where wastewater filtrate was added onto the host lawn are due to the lysis of *E. coli* ATCC 25922 caused by the lytic bacteriophages present in the filtered wastewater, as shown in figure 3.3. The absence of a clear patch in quadrant four indicates that the visible lysis is due to the presence of lytic phages.

Isolation of individual phage isolates was achieved by employing the double layer method and an enrichment culture with *E. coli* ATCC 25922 as a host, resulting in plaques of different morphology, indicating the presence of multiple lytic phages in the wastewater samples. Consequently, a total of four different bacteriophages infecting *E. coli* ATCC 25922 with different plaque morphologies were selected and used for proliferation and purification.

3.2. Phage characterization

3.2.1. Plaque and phage characterization

After purification using the double-layer method, four lytic phage isolates infecting *E. coli* ATCC 25922 were successfully isolated and characterized based on plaque size and morphology on Nutrient agar plates. In addition, the phage particle morphology was analyzed via Transmission Electron Microscopy.

The first lytic bacteriophage isolate, denoted as *E. coli* phage NPS forms tiny clear plaques in *E. coli* ATCC 25922 lawn on Nutrient agar after incubation at 37°C for 18-24 hours (figure 3.4A).

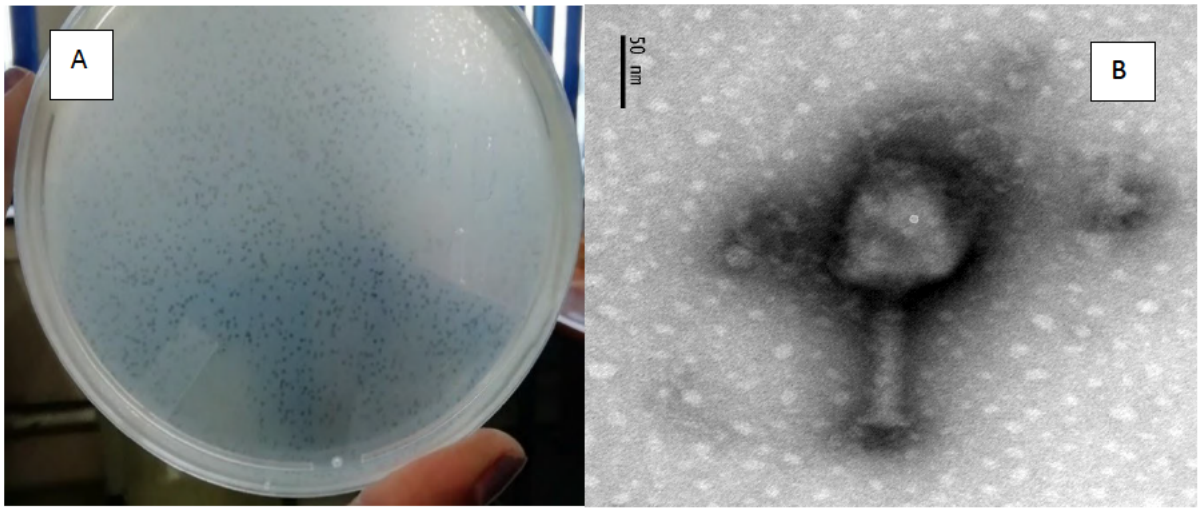

Figure 3.4: Phage isolate NPS (A) plaques formed on Nutrient agar with *E. coli* ATCC 25922 incubated at 37°C for 18-24 hours and (B) TEM micrograph of a phage NPS particle.

Transmission electron microscopy revealed that this phage has an icosahedral head of about 83 nm and a contractile tail of about 74 nm, which are characteristics of members of the family *Myoviridae* within the order *Caudovirale* (figure 3.4B). In addition, the TEM micrograph revealed that this phage has a collar and base plate.

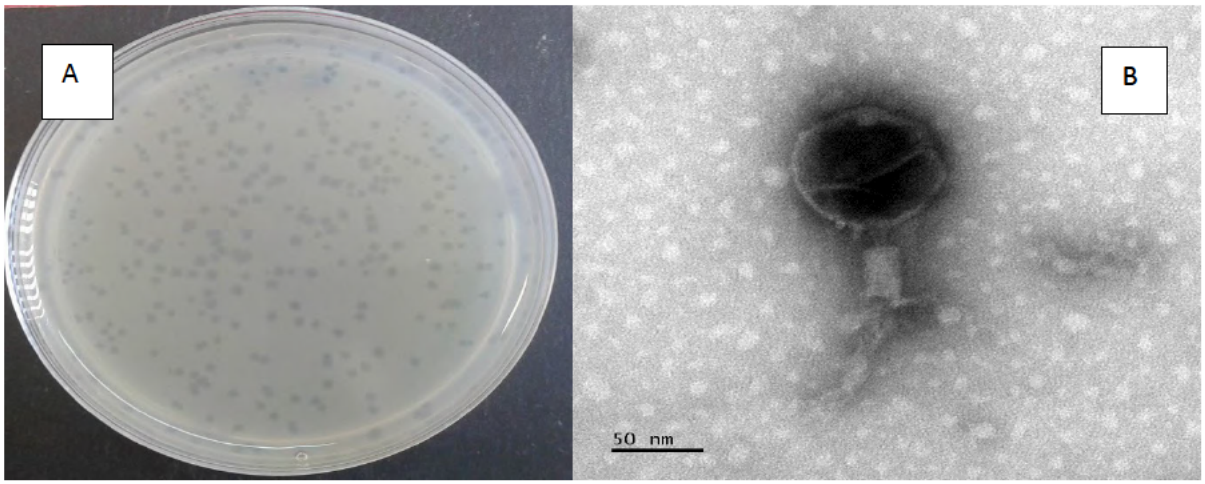

Figure 3.5: Phage isolate YM (A) plaques formed on Nutrient agar with *E. coli* ATCC 25922 incubated at 37°C for 18-24 hours (A) and (B) TEM micrograph of a phage YM particle.

The second lytic phage isolated and purified was denoted as *E. coli* phage YM, it was distinguished by medium-sized turbid plaques with irregular edges as seen in figure 3.5(A). TEM analysis indicated that phage YM has a roundish head of 83nm and a tail that is about 38 nm long. Interestingly, phage YM has a collar, a narrow region between the head and the tail of about 12 nm in length, as shown in figure 3.5 (B). Therefore, this phage isolate is matching the morphology of the family *Myoviridae* in the order *Caudovirales*.

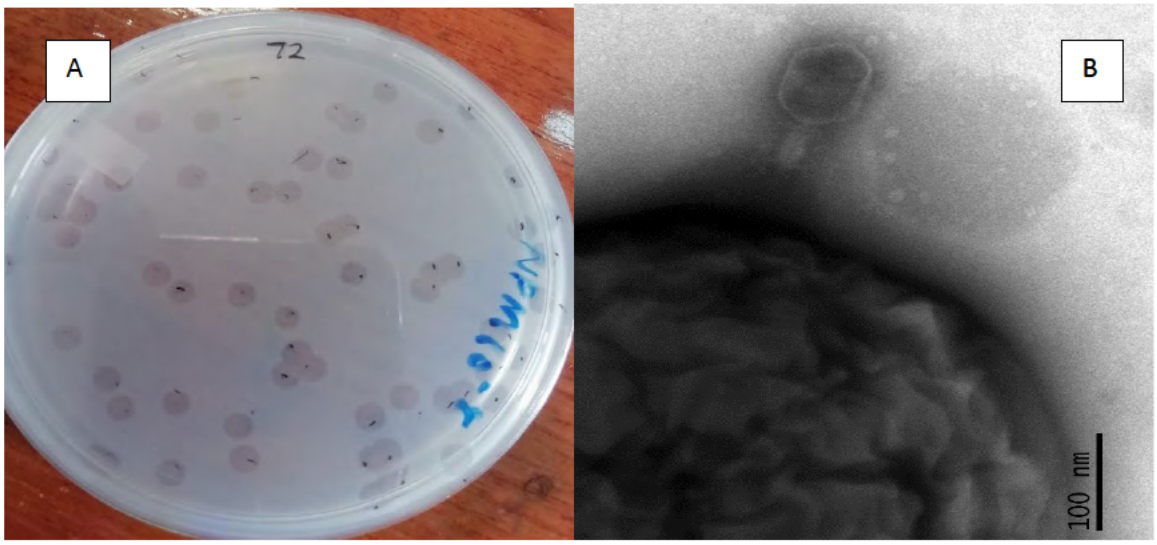

Figure 3.6: Phage isolate NPM (A) phage plaques formed on Nutrient agar with *E. coli* ATCC 25922 as a host incubated at 37°C after 18-24 hours and (B) TEM micrograph of a phage NPM particle.

The third lytic phage isolate, denoted as *E. coli* phage NPM formed large clear plaques using *E. coli* a host as depicted in figure 3.6 (A). The matching TEM analysis of phage NPM revealed an icosahedral head with a length of about 73 nm and a contractile short tail that was about 46 nm long. Additionally, a collar region can be seen between the capsid and the tail. These morphologic characteristics match the members of the family *Myoviridae*.

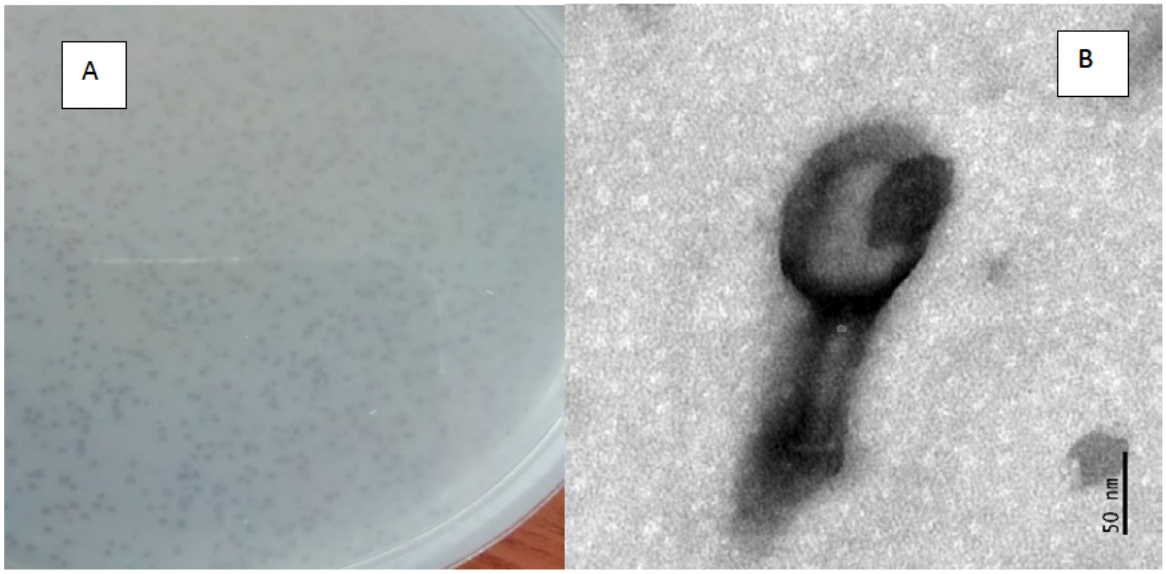

Figure 3.7: Phage isolate YS (A) plaques formed on Nutrient agar with *E. coli* ATCC 25922 as a host after 18-24 hours of incubation at 37°C and (B) TEM micrograph of a phage YS particle.

The fourth lytic phage isolate, *E. coli* phage YS, was characterized by its small turbid plaques that formed in *E. coli* lawns seen in figure 3.7 (A). The TEM micrograph (figure 3.7B) shows that phage YS has a head of about 92 nm and a tail attached to its head that is 100 nm long. Therefore, this isolate matches the characteristics of phages in the order *Caudovirales*.

Table 3.1: Plaque characteristics of phage NPS, NPM, YS and YM with E. coli ATCC 25922 as a host on Nutrient agar after 24 hour incubation at 37°C.

Phage isolate	Plaque morphology	plaque size (cm)*
NPS	small, round, clear, sharp edges plaques	0.12
NPM	big, round, clear, sharp edges plaques	0.46
YS	small, turbid, blunt edges plaques	0.16
YM	medium-big, turbid, blunt edges plaques	0.37

*The values shown are the average of at least 6 plaques measured using a digital caliper.

Table 3.1 shows the characteristics of the plaques formed on *E. coli* ATCC 25922 lawns for each of the lytic *E. coli* phages. These results demonstrate that the four lytic phages can be clearly distinguished based on their plaque morphologies. *E. coli* phage NPS and NPM formed clear sharp edges plaques, while *E. coli* phage YS and YM formed turbid blunt edges plaques

The first step in phage infection is the attachment of the phage onto the LPS or OMP's present in the outer membrane of the Gram-negative bacterial host, subsequently resulting in lysis of the host bacterium. Therefore, to verify the interaction of the phage isolates with the host cells, TEM analysis was performed with a lysate containing *Escherichia coli* ATCC 25922 cells and phage NPM (figure 3.8). Phage NPM was used as a representative phage to demonstrate phage attachment physically onto the outer surface of their host bacteria.

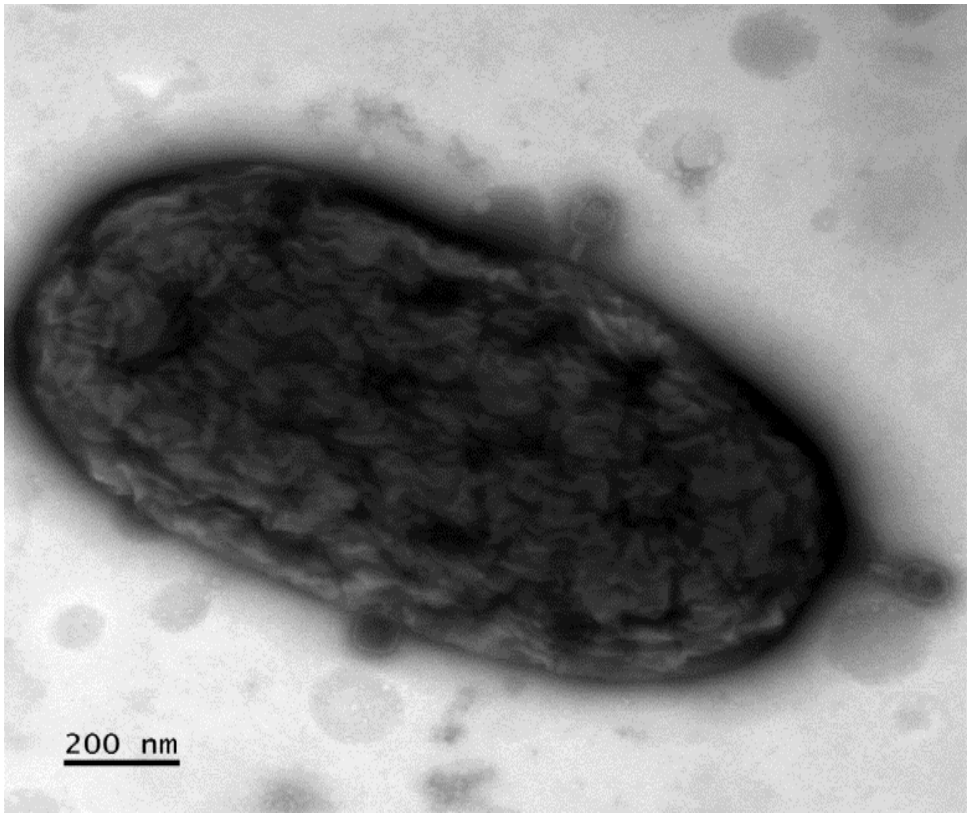

Figure 3.8: TEM micrograph of phage NPM particles attached onto the surface of an individual cell of the bacterial host *E. coli* ATCC 25922.

The TEM analysis demonstrated that multiple *E. coli* phage NPM particles were attached onto a host cell of *E. coli* ATCC 25922 prior to initiating the lytic cycle (Fig 3.8).

3.2.2. Nucleic acid extraction and genomic analysis of the two selected phages

Phage NPS and NPM were selected for further analyses based on their plaque morphology, as they formed clear plaques, which indicates that these two lytic *E. coli* phages are virulent phages. Phage genomes consist of either single or double-stranded DNA or RNA as their nucleic acids. To confirm which nucleic acids *E. coli* phage NPM and NPS genomes are made of, their nucleic acids were extracted and digested with either DNAse 1 or RNAse A, and the digestion was verified via electrophoresis.

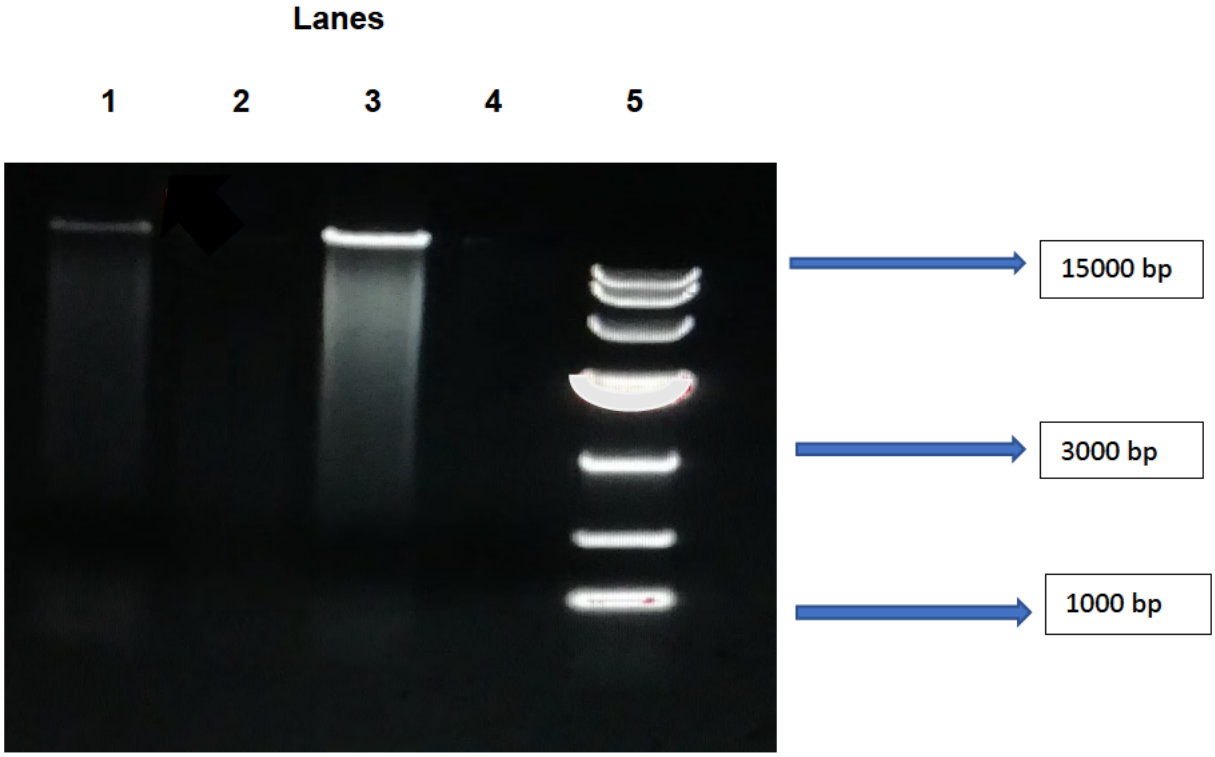

Figure 3.9: Agarose gel showing isolated phage genomic nucleic acids digested with DNAse1 and RNAse A. Lane 1 is *E. coli* phage NPS genomic nucleic acid digested with RNAse A, lane 2 is *E. coli* phage NPS genomic nucleic acid digested with DNAse 1, lane 3 is *E. coli* phage NPM digested with RNAse A and lane 4 is *E. coli* phage NPM digested with DNAse 1. Lane 5 is the DNA size marker used, showing the 15 000 bp expected bands.

As shown in figure 3.9, *E. coli* phage NPS and NPM were selected for genome analysis based on their ability to form clear plaques and, additionally, as they exhibited shorter latent periods. Two enzymes were used to determine whether the genomic nucleic acids of the phages were DNA or RNA. It was apparent that both phages contain DNA as their nucleic acid because digestion took place only in the presence of DNAse I, while no digestion happened with RNAse A (figure 3.9). As expected, given the fact that *Myoviridae* phages are known to have genomes >25 000 bp, the genomic DNA isolated from phage NPS and NPM was larger than 15 000 bp when comparing it to the DNA size marker in lane 5 (figure 3.9).

3.2.3. Genome analysis for the two *E. coli* phages NPS and NPM

Genome annotation and analysis are important in identifying key phage genes such as structural and replication genes and those genes associated with virulence and lysogeny of phages. Moreover, it identifies coding sequences for different genes, their location, size, and enables the prediction of amino acid sequences based on the translation from nucleotide triplet into matching amino acids.

Escherichia coli bacteriophage NPS (PATRIC ID: Bacteriophage APSE-3 Nps1) consists of a circular double-stranded DNA genome that is 169, 536 bp long and has 35.3 % G+C content. Genome annotation using the RAST tool kit available on PATRIC revealed that this genome has 294 coding sequences (CDS) with 254 genes encoding for functional proteins and 40 hypothetical proteins. This genome is composed of genes encoding for essential structural proteins (head: 14, 245, 281-285, 277; neck: 1, 293, 294, tail: 3, 16, 18, 242-244, 264, 197-201, and base plate: 4, 6-9, 13, 23-270) as well as DNA packaging proteins, host lysis proteins such as phage lysozyme and those that are involved in DNA replication and repair, modification and various regulation proteins scattered across the genome. This genome has genes encoding additional proteins like Thioredoxin for redox reactions, acridine resistance and nudix hydrolase, which is characteristic for the lytic T4-like phage. Moreover, this genome encoded 9 tRNA genes, a feature typical for phages of the order *Caudovirales,* family *Myoviridae* and genus *Tequatrovirus*. In addition, genes encoding for proteins involved in lysogeny were not detected in the *Escherichia coli* phage NPS genome, as reflected in figure 3.10 and Table 3.2.

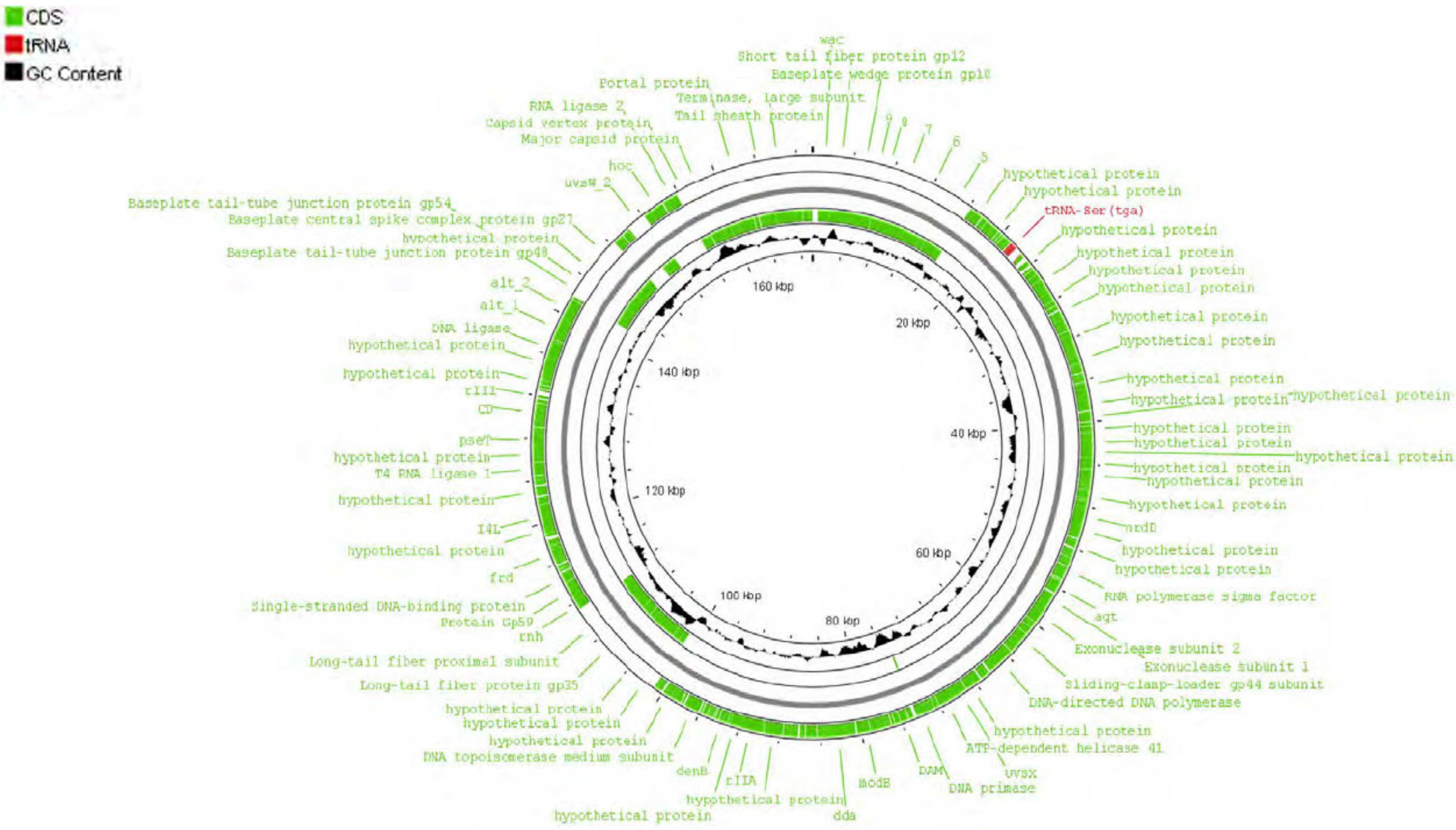

Figure 3.10: Genome map of the isolated and purified lytic *E. coli* phage NPS (169,536 bp)

The following table (table 3.2) summarizes the results obtained when analyzing the genome of *E. coli* phage NPS using the RAST tool kit on PATRIC.

Table 3.2: Coding sequences, location, predicted function and amino acid sequence length based on the genome of phage NPS.

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
1	1	545	-1	Neck protein	181
2	576	2034	-1	Fibritin (wac) protein	485
3	2043	3594	-1	Straight tail fiber	516
4	3590	4250	-1	Baseplate wedge subunit	219
5	4249	6055	-1	Hypothetical protein	601
6	6054	6921	-1	Baseplate wedge tail fiber connector (T4-like gp9)	288
7	6985	7990	-1	Baseplate wedge subunit (T4-like gp8)	334
8	7982	11081	-1	Baseplate wedge initiator (T4-like gp7)	1032
9	11077	13060	-1	Baseplate wedge subunit (T4-like gp6)	660
10	13068	13362	-1	Phospholiphase (ACLAME172)	97
11	13362	13917	-1	Phage protein	184
12	13891	15619	-1	Phage lysozyme	575
13	15602	16693	-1	Baseplate wedge subunit	196
14	16240	16693	1	Head completion protein	150
15	16692	17517	1	DNA end protector protein	274

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
16	17623	18154	1	Tail completion protein	176
17	18203	18929	1	Deoxynucleoside monophosphate kinase	241
18	18928	19159	1	Tail fiber assembly protein	76
19	19155	19614	1	Hypothetical protein	152
20	19687	19945	1	Phage protein	85
21	20021	20255	1	Phage internal core protein	81
22	20313	20499	1	Phage protein	61
23	20498	20897	1	Phage protein	132
24	20899	21187	1	Phage protein	95
25	21994	22660	1	Homing endonuclease	221
26	22757	23108	1	Phage protein	116
27	23176	23311	1	Hypothetical protein	44
28	23316	23469	1	Hypothetical protein	50
29	23495	23964	1	Hypothetical protein	157
30	24076	24247	1	Hypothetical protein	56
31	24210	24474	1	Phage protein	87
32	24530	25049	1	Phage protein	172
33	25092	25686	1	Hypothetical protein	197
34	25727	26336	1	Hypothetical protein	202

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
35	26304	26697	1	Phage protein	130
36	26678	27053	1	Phage protein	124
37	27049	27538	1	Phage protein	162
38	27719	27975	1	Nudix hydrolase	151
39	28565	28506	1	Phage lysozyme	164
40	28565	28982	1	Phage endonuclease	138
41	29015	29117	1	Phage protein	33
42	29123	29668	1	Phage protein	179
43	29664	49994	1	Phage protein	109
44	30000	30363	1	Glycyl radical co-factor	120
45	30362	30584	1	Phage protein	73
46	30576	30843	1	Phage protein	88
47	30842	31121	1	Phage protein	92
48	31180	31642	1	Phage endonuclease	153
49	31649	32195	1	Hypothetical protein	181
50	32187	32529	1	Vilyl-tRNA synthase	113
51	32525	32993	1	T4 like phage protein with macro domain	155
52	32989	33405	1	Phage protein	70
53	33192	33202	1	Phage protein	70

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
54	33571	33575	1	Phage protein	57
55	33571	33757	1	Phage protein	61
56	33766	34348	1	Thymidine kinase	193
57	34390	34603	1	Phage protein	70
58	34615	34909	1	Phage rl lysis inhibition regulator	97
59	34905	35292	1	Phage protein	128
60	35387	35576	1	Phage protein	62
61	35575	35779	1	Phage protein	67
62	35781	35976	1	Phage protein	64
63	35975	36139	1	Phage protein	57
64	36199	36303	1	Phage protein	34
65	36330	36864	1	Phage protein	177
66	36870	37392	1	Phage protein	173
67	37394	37856	1	Phage protein	153
68	37852	38866	1	Thioredoxin, phage-associated	337
69	38862	38961	1	Hypothetical protein	32
70	38980	39949	1	Thioredoxin, phage-associated	322
71	40050	40353	1	Thioredoxin, phage-associated	100
72	40413	40941	1	Thioredoxin, phage-associated	175

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
73	40996	41404	1	Thioredoxin, phage-associated	135
74	41411	42299	1	Thioredoxin, phage-associated	295
75	42307	43327	1	Thioredoxin, phage-associated	339
76	43354	44386	1	Thioredoxin, phage-associated	343
77	44438	45368	1	Thioredoxin, phage-associated	309
78	45364	45679	1	Thioredoxin, phage-associated	104
79	45665	45908	1	Thioredoxin, phage-associated	80
80	45909	46173	1	Glutaredoxin	87
81	46169	46558	1	Phage protein	71
82	46387	46558	1	Hypothetical protein	56
83	46529	46697	1	Phage protein	55
84	46680	47166	1	Pin protease inhibitor	161
85	47202	47379	1	Phage protein	58
86	47421	47895	1	Phage endonuclease, T4-like gp49	157
87	47891	49709	1	Ribonuclease reductase of class III	605
88	49701	50532	1	Phage homing endonuclease	276
89	50528	50999	1	Ribonuclease reductase of class III	156
90	51012	51105	1	Phage protein	30
91	51114	51330	1	Phage protein	71

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
92	51332	51644	1	T4-like RNA viruses enterobacteria phage RB51	103
93	51615	51939	1	Phage protein	107
94	52097	52280	1	Phage protein	60
95	52272	52566	1	Phage protein	97
96	52573	52705	1	Phage protein	43
97	52705	52906	1	Phage protein	66
98	52958	53285	1	Phage protein	108
99	53287	53503	1	Phage protein	71
100	53499	53769	1	Polymerase sigma factor	89
101	53847	54405	1	phage protein	185
102	54388	54607	1	Phage protein	72
103	54608	54926	1	Phage protein	105
104	54894	55098	1	Phage protein	67
105	55161	55275	1	Phage protein	37
106	55341	56544	1	a-gt alpha glycoyl transferase	400
107	56583	56724	1	Phage protein	47
108	56720	57740	1	Phage recombination-related endonuclease gp47	339
109	57736	58000	1	Phage protein	87

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
110	57971	58187	1	Phage protein	71
111	58225	59866	1	Phage recombination-related endonuclease gp46	546
112	59921	60110	1	Phage protein	62
113	60119	60509	1	RNA polymerase binding protein	129
114	60564	61251	1	Sliding camp DNA polymerase accessory protein	228
115	61302	62262	1	Replication factor C small sub-unit	319
116	62263	62827	1	DNA polymerase clamp loader gp62	187
117	62828	63420	1	Phage endoribonuclease translational repressor	122
118	63198	63420	1	phage protein	73
119	63498	66195	1	DNA polymerase T4-like gp43	898
120	66378	66333	-1	Hypothetical protein	34
121	66378	66615	1	Hypothetical protein	78
122	66625	67006	1	Phage immunity	126
123	67013	67265	1	Phage immunity	83
124	67261	67411	1	Phage protein	49
125	67418	68159	1	Hypothetical protein	246
126	68155	68998	1	Glycosyl transferase	280

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
127	69075	70257	1	Phage recombination protein	393
128	70249	70594	1	Phage capsid and scaffold	114
129	70603	72031	1	DNA helicase/ primase	475
130	72089	72272	1	Discriminator of mRNA degradation	60
131	72273	72531	1	Phage protein	85
132	72591	72885	1	Hypothetical protein	97
133	72897	73254	1	Phage protein	118
134	73255	73420	1	Hypothetical protein	54
135	73423	74452	1	DNA primase	342
136	74448	74649	-1	Phage protein	66
137	74640	74730	1	Hypothetical protein	29
138	74748	75237	1	Hypothetical protein	162
139	75307	76087	1	DNA adenine methyltransferase	359
140	76147	76666	1	Hypothetical protein	172
141	76734	76980	1	Head decoration protein	81
142	76972	77098	1	Hypothetical protein	41
143	77078	77285	1	Phage protein	68
144	77634	77326	1	Phage protein	113
145	77634	78120	1	Phage protein	161

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
146	78142	78298	1	Phage transcription modulator	51
147	78294	78459	1	Molybdenum ABC transporter	54
148	78451	78922	1	Phage protein	156
149	78930	79113	1	Molybdenum ABC transporter	60
150	79180	79804	1	Hypothetical protein	207
151	79800	80403	1	Phage modA or modB ribosyltransferase	200
152	80528	81275	1	Phage putative anti-sigma factor	248
153	81584	82904	1	Phage protein	103
154	81584	82904	1	DNA helicase	439
155	82910	83171	1	Phage protein	86
156	83157	83403	1	Phage exonuclease	81
157	83395	83638	1	Phage exonuclease	80
158	83637	84321	1	Phage exonuclease	227
159	84384	84885	1	Transcriptional regulator	166
160	84887	85436	1	Transcriptional regulator	182
161	85509	86001	1	Transcriptional regulator	163
162	86066	86156	-1	Hypothetical protein	29
163	86173	86389	1	Cef modifier of suppressor tRNA	71
164	86388	86802	1	Phage protein	137

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
165	86804	86981	1	Phage protein	58
166	86983	87752	1	Phage protein	123
167	87347	87752	-1	Hypothetical protein	134
168	87690	89766	1	DNA topoisomerase large sub-unit	605
169	89562	89766	1	Phage rIIA lysis inhibitor	67
170	89776	91954	1	Phage lysis inhibitor	725
171	91965	92904	1	Hypothetical protein	312
172	92932	93127	1	Phage endonuclease	64
173	93165	93495	1	Phage protein	109
174	93427	93985	1	Phage endonuclease	185
175	94065	94329	1	Phage protein	78
176	94408	94507	1	Phage protein	32
177	94574	94686	1	Phage protein	37
178	94693	94891	1	Phage protein	65
179	94890	94998	1	Phage protein	35
180	95006	95222	1	Phage protein	71
181	95280	95739	1	Phage protein	152
182	95826	95985	1	Acridine resistance	152
183	95977	96118	1	Phage protein	186

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
184	96122	97451	1	DNA topoisomarase	442
185	97447	97597	1	Transcriptional regulator	49
186	97731	97951	1	phage protein	75
187	98064	98700	1	Transcriptional regulator	211
188	98710	99040	1	Phage protein	109
189	99036	99192	1	Phage protein	51
190	99188	99656	1	Hypothetical protein	155
191	99655	99952	1	Hypothetical protein	98
192	100022	100154	1	Hypothetical protein	43
193	100237	100516	1	Phage anti-restriction nuclease	92
194	100512	100665	1	Hypothetical protein	50
195	100677	100950	1	Phage anti-sigma factor	90
196	100950	101607	-1	Phage holin	218
197	101637	102420	-1	Receptor recognizing protein	260
198	102456	105402	-1	Phage tail fiber	981
199	105410	106061	-1	Phage tail connector	216
200	106123	107239	-1	Phage long tail fiber	236
201	107247	111117	-1	Phage long tail fiber	1289
202	111221	112939	1	Phage ribonuclease	305

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
203	112147	112417	1	Phage double stranded DNA binding protein	89
204	112394	112733	1	Transcriptional regulator	112
205	112744	113383	1	DNA helicase loader	212
206	113384	114017	1	Phage homing endonuclease	210
207	114076	114982	1	Single stranded DNA binding protein	301
208	115002	115113	1	Hypothetical protein	36
209	115126	115354	1	Phage protein	75
210	115399	115786	1	Phage protein	128
211	115924	116167	1	Phage protein	80
212	116177	116510	1	Phage protein	110
213	116521	116767	1	Phage protein	81
214	116766	117348	1	Dihydrofolate reductase	193
215	117367	117715	1	Phage protein	115
216	117760	118312	1	Thymidylate synthase	183
217	118312	118411	1	Hypothetical protein	33
218	118429	118531	-1	Hypothetical protein	32
219	118537	118636	-1	Hypothetical protein	32
220	118626	118941	1	Thymidylate synthase	104
221	118964	119228	1	Phage protein	87

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
222	119181	119508	1	Phage protein	108
223	119498	121763	1	Ribonucleotide reductase of class Ia (aerobic)	754
224	121814	122492	1	Ribonucleotide reductase of class Ia (aerobic)	225
225	122635	123445	1	Phage associated homing endonuclease	269
226	123405	123501	1	Hypothetical protein	31
227	123613	124084	1	Ribonucleotide reductase of class Ia (aerobic)	156
228	124111	124522	1	Phage endonuclease	136
229	124574	125699	1	RNA ligase	374
230	125760	126267	1	Phage alc transcription terminator	168
231	126257	126611	1	Hypothetical protein	117
232	126607	126907	1	Phage outer membrane lipoprotein	99
233	126903	127134	1	Phage protein	976
234	127130	127433	1	Phage protein	100
235	127429	128335	1	3’-Phosphotase, 5’-polynucleotide kinase	301
236	128334	128532	1	Phage protein	65
237	128524	128725	1	Phage protein	66

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
238	128727	129003	1	Phage protein	91
239	129065	129593	1	Phage protein	175
240	129586	129823	1	Phage protein	78
241	129819	130158	-1	Phage protein	112
242	130523	130734	1	Phage tail fiber protein	193
243	130735	130972	1	Phage tail fiber protein	78
244	130972	131281	1	Phage tail fiber protein	102
245	131337	131673	1	Phage head assembly chaperon protein	111
246	131337	132069	1	Hypothetical protein	82
247	132137	132320	1	Hypothetical protein	60
248	132425	132641	1	Hypothetical protein	36
249	132425	132641	1	Phage protein	72
250	132754	133087	1	Phage protein	110
251	133154	133529	1	Phage protein	124
252	133569	133857	1	Phage protein	95
253	133856	134054	1	Phage protein	65
254	13450	134257	1	Phage protein	68
255	134249	134708	1	Hypothetical protein	152
256	134704	135559	1	Phage protein	284

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
257	135558	135828	1	Phage protein	89
258	135824	137288	1	Phage protein	487
259	137284	137437	1	DNA ligase, phage associated	62
260	137525	139574	1	Phage protein	682
261	139577	141635	1	Putative RNA polymerase ADP-ribosyltransferase	685
262	141695	141986	1	Hypothetical protein	96
263	142014	142980	1	Phage protein	321
264	142979	144074	1	Phage tail assembly protein	364
265	144082	145855	1	Phage baseplate tail tube cap gp 48	590
266	145851	146385	1	Phage baseplate hub	177
267	146332	147505	1	Phage baseplate hub	390
268	147501	148254	1	Phage baseplate hub subunit	250
269	148304	148931	1	Phage baseplate hub subunit gp26	208
270	148930	149329	1	Phage baseplate wedge subunit	132
271	149395	149809	1	single stranded DNA binding protein	137
272	149808	150033	1	Phage protein	74
273	150061	150229	1	Phage protein	55
274	150284	150515	-1	Phage DNA helicase	76
275	150540	152052	-1	Phage DNA helicase	503

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
276	152102	152783	1	Hypothetical protein	226
277	152792	154211	1	Capsid and scaffold protein	472
278	154306	154522	1	Phage protein	71
279	154508	154787	1	Phage protein	92
280	154796	155801	-1	RNA ligase	334
281	155830	157114	-1	Capsid vertex protein	427
282	157197	158763	-1	Major capsid protein	521
283	158781	159591	-1	Phage pro-head assembly (scaffolding) protein	269
284	159621	160260	-1	Phage pro-head assembly (scaffolding) protein	212
285	160259	160685	-1	Phage capsid and scaffold	141
286	160684	160924	-1	Phage pro-head core protein	79
287	160923	162498	-1	Phage portal (connector) protein gp20	254
288	162581	163073	-1	Phage tail tube protein	163
289	163189	165169	-1	Phage tail sheath	659
290	165200	167033	-1	phage terminase, large subunit	610
291	167016	167511	-1	Phage terminase, small subunit	164
292	167519	168338	-1	Tail sheath stabilizer and completion protein	272

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
293	168379	169150	-1	Phage neck protein	256
294	169151	169493	-1	Phage neck protein	113
tRNA1	21284	21357	1	tRNA-Gln-TTG	73
tRNA2	21538	21442	1	tRNA-Leu-TAA	84
tRNA3	21450	21521	1	tRNA-Gly-TCC	71
tRNA4	21534	21608	1	tRNA-Pro-TGG	74
tRNA5	21610	21697	1	tRNA-Ser-TGA	87
tRNA6	21699	21772	1	tRNA-Thr-TGT	73
tRNA7	21776	21850	1	tRNA-Met-CAT	74
tRNA8	21861	21945	1	tRNA-Pseudo-GTA	84
tRNA9	22663	22736	1	tRNA-Arg-TCT	73

Escherichia coli bacteriophage NPM (PATRIC ID: *Escherichia coli* NPM) has a circular double-stranded DNA genome that is 169,536 base-pairs long with a G+C content of 35.3 %. The RAST tool predicted 268 coding sequences, of which 24 encode for hypothetical proteins and 9 for tRNA genes, highlighting that the *E. coli* phage NPM matches the family *Myoviridae*. This genome is made up of the essential phage genes encoded for structural proteins, e.g., capsid proteins at CDS 6,134-140,160, 130, tail and tail fibers proteins at 58, 59, 61, 62, 98, 99, 141, 149, and 162. In addition, this genome has genes encoded for DNA packaging proteins (CDS 143 & 143), large and small terminase subunit, lysis proteins at CDS 180, and replication and modification proteins are present in the genome of phage NPM. Again, genes encoding for proteins

involved in lysogeny or in bacterial virulence were not detected in the genome on *E. coli* phage NPM as shown in figure 3.11 and table 3.3.

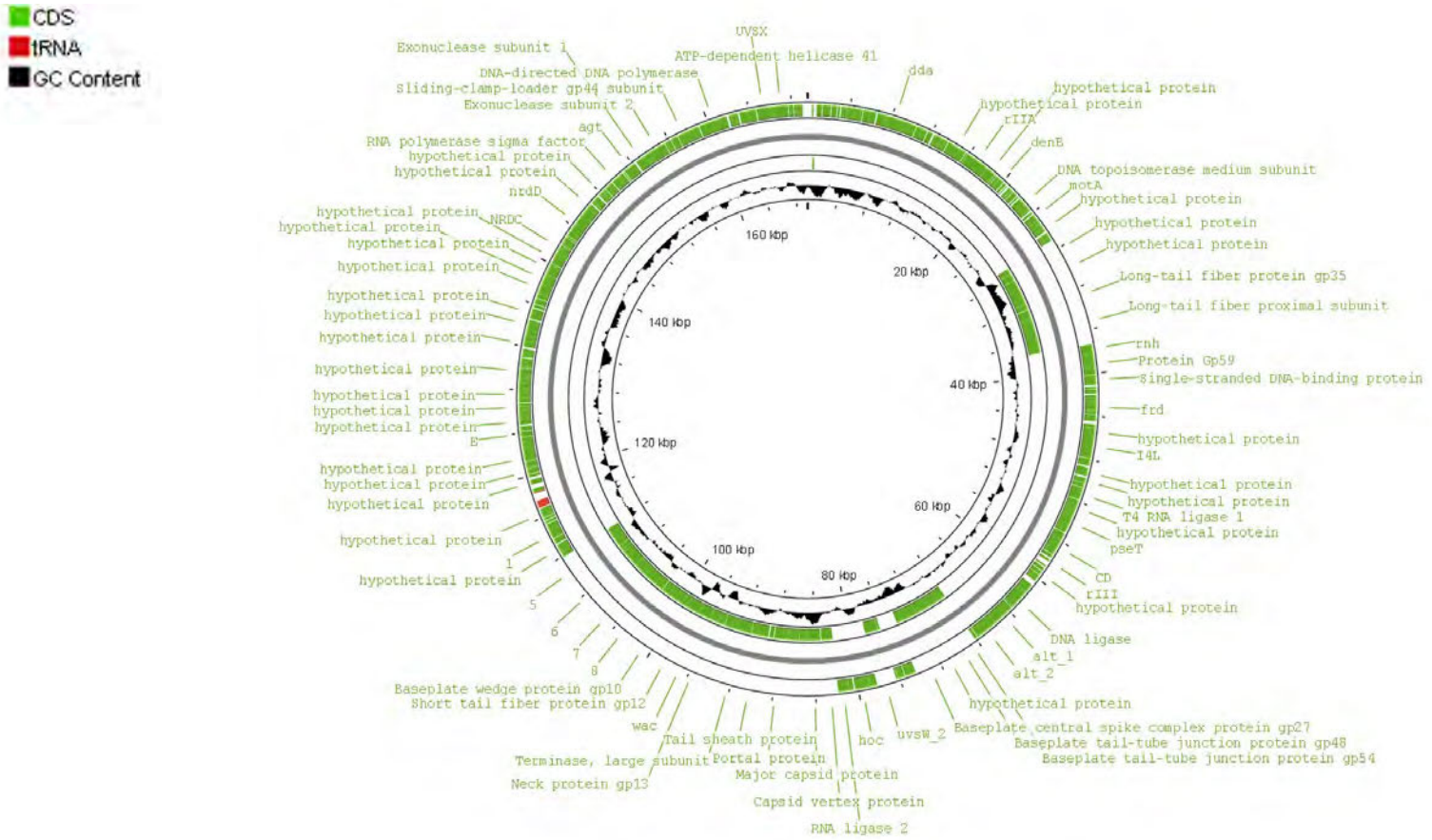

Figure 3.11: Genome map of the isolated and purified lytic *E. coli* phage NPM (169,536 bp).

Table 3.3: Coding sequences, location, predicted function, and amino acid sequence length based on the genome of the lytic *E. coli* phage NPM.

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
1	2	557	1	DNA primase	184
2	553	754	-1	Phage protein	66
3	853	1342	1	Hypothetical protein	162
4	1412	2192	1	DNA adenine methyltransferase	259
5	2252	2771	1	Hypothetical protein	172
6	2839	3085	1	Phage capsid and scaffold	81
7	3183	3390	1	Phage protein	68
8	3389	3731	1	Phage protein	113
9	3739	4225	1	Phage protein	161
10	4399	4563	1	Molybdenum ABC transporter	54
11	4556	5627	1	Phage protein	156
12	5035	5218	1	Molybdeunum ABC transporter	60
13	5285	5909	1	Hypothetical protein	207
14	5905	6508	1	Phage modA or modB ribosyltransferase	200
15	6633	7380	1	Putative anti-sigma factor	248
16	7381	7693	1	Phage protein	103
17	7689	9009	1	DNA helicase	439
18	9015	9276	1	Phage protein	86

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
19	9262	9508	1	Phage exonuclease	81
20	9500	9743	1	Phage exonuclease	80
21	9732	10426	1	Phage exonuclease	227
22	10489	10990	1	Transcriptional regulator	166
23	10992	11541	1	Transcriptional regulator	182
24	11614	12106	1	Transcriptional regulator	163
25	12278	12494	1	cef modifier of suppressor tRNAs	71
26	12493	12907	1	Phage protein	137
27	12909	13086	1	Phage protein	58
28	13088	13460	1	Phage protein	123
29	13456	13726	1	Phage protein	89
30	13795	15613	1	Phage DNA topoisomerase large subunit	605
31	15667	15871	1	Phage rIIA lysis inhibitor	67
32	15881	18059	1	Phage rIIA lysis inhibitor	725
33	18070	19009	1	Hypothetical protein	312
34	19037	19232	1	Phage endonuclease	64
35	19270	19600	1	Phage protein	109
36	19613	20090	1	Phage endonuclease	158
37	20170	20434	1	Phage protein	87

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
38	20480	20612	1	Phage protein	43
39	20677	20791	1	Phage protein	37
40	20798	20996	1	Phage protein	65
41	20992	21103	1	Phage protein	36
42	21111	21327	1	Phage protein	71
43	21331	21844	1	Phage nucleoid disruption protein Ndd	170
44	21931	22090	1	Acridine resistance	52
45	22227	23556	1	DNA topoisomerase	442
46	23552	23702	1	Transcriptional regulator	49
47	23836	24064	1	Phage protein	75
48	24169	24805	1	Transcriptional regulator	211
49	25815	25145	1	Phage protein	109
50	25141	25297	1	Phage protein	51
51	25293	25761	1	Hypothetical protein	155
52	25760	26057	1	Hypothetical protein	98
53	26127	26259	1	Hypothetical protein	43
54	26342	26621	1	Phage anti-sigma nuclease	92
55	26617	26770	1	Hypothetical protein	50
56	26782	27055	1	Phage anti-sigma nuclease	90

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
57	27055	27712	-1	Phage holin	218
58	27472	28525	-1	Receptor recognizing protein	260
59	28561	31507	-1	Phage tail fiber	981
60	31515	32166	-1	Phage tail connector tube	215
61	32228	33344	-1	Phage long tail fiber	371
62	33352	37222	-1	Phage long tail fiber	1289
63	37326	38244	1	Phage ribonuclease	305
64	38252	38522	1	Phage double-stranded DNA binging protein	89
65	38499	38838	1	Transcriptional regulator	112
66	38834	39488	1	Phage DNA helicase loader	217
67	39489	40122	1	Phage-associated homing endonuclease	210
68	40181	41087	1	Single-stranded DNA binding protein	301
69	41231	41459	1	Phage protein	75
70	41504	41891	1	Phage protein	128
71	42029	42272	1	Phage protein	80
72	42282	42615	1	Phage protein	110
73	42626	42872	1	Phage protein	81
74	42871	43453	1	Dihydrofolate reductase	193

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
75	43472	43820	1	Phage protein	115
76	43865	44417	1	Thymidylate synthase	183
77	44731	45046	1	Thymidylate synthase	104
78	45069	45333	1	Phage protein	87
79	45663	47868	1	Ribonucleotide reductase of class Ia, alpha	754
80	47919	48597	1	Ribonucleotide reductase of class Ia, beta	225
81	48740	49550	1	Phage homing endonuclease	269
82	49718	50189	1	Ribonucleotide reductase of class Ia, beta	156
83	50216	50627	1	Phage endonuclease	136
84	50679	50804	1	RNA ligase	374
85	51865	52372	1	Phage alc Transcriptional terminator	167
86	52362	52716	1	Hypothetical protein	117
87	52712	53012	1	Phage outer membrane lipoprotein RZ1	99
88	53008	53239	1	Phage protein	76
89	53235	53538	1	Phage protein	100
90	53534	54440	1	3’-phosphotase, 5’-polynucleotide kinase	301

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
91	54439	54637	1	Phage protein	65
92	54629	54830	1	Phage protein	66
93	54832	55108	1	Phage protein	91
94	55170	55698	1	Phage protein	175
95	55691	55928	1	Phage protein	78
96	55924	56263	1	Phage protein	112
97	56259	56841	1	Dcmp deaminase	193
98	56840	57077	1	Phage tail fibers	78
99	57077	57386	1	Phage tail fibers	102
100	57443	57778	1	Phage head assembly chaperone protein	111
101	57925	58174	1	Hypothetical protein	82
102	58530	58749	1	1,4-alpha-glucan(glycogen) branching enzyme	72
103	58859	59192	1	Phage protein	110
104	59259	59634	1	Phage protein	124
105	59674	59962	1	Phage protein	95
106	59961	60159	1	Phage protein	65
107	60155	60362	1	Phage protein	68
108	60432	60813	1	Hypothetical protein	126

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
109	60809	61664	1	Phage protein	284
110	61663	61931	1	Phage protein	89
111	61929	63393	1	DNA ligase	487
112	63389	63578	1	Phage protein	62
113	63630	65679	1	Putative RNA polymerase-ADP-ribosyltransferase	682
114	65682	67740	1	Hypothetical protein	685
115	67800	68091	1	Phage protein	96
116	68119	69085	-1	Tail assembly protein	321
117	69084	70179	-1	Baseplate tail tube cap T4-like gp48	364
118	70187	71960	-1	Baseplate hub	590
119	71956	72415	-1	Baseplate hub	152
120	72437	73610	-1	Baseplate hub subunit	390
121	73606	74359	-1	Phage baseplate	250
122	74409	75036	1	Baseplate hub subunit T4-like gp26	208
123	75035	75434	1	Baseplate wedge subunit T4-like gp25	132
124	75500	75914	1	Single-stranded DNA –binding protein	137
125	75913	76138	1	Phage protein	74
126	76166	76334	1	Phage protein	55
127	76389	76620	-1	Phage DNA helicase	76

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
128	76645	78157	-1	Phage DNA helicase	503
129	78207	78888	1	Hypothetical protein	226
130	78897	80316	1	Capsid and scaffold protein	472
131	80423	80627	1	Phage protein	67
132	80613	80892	1	Phage protein	92
133	80901	81906	1	RNA ligase	334
134	81935	83219	-1	Capsid vertex protein	427
135	83302	85696	-1	Major capsid protein	521
136	84886	83219	-1	Phage pro-head assembly (scaffolding) protein	269
137	85726	86365	-1	Phage pro-head assembly (scaffolding) protein	212
138	86364	86790	-1	Phage capsid and scaffold	141
139	86789	887029	-1	Phage pro-head assembly	79
140	87028	88603	-1	Phage portal vertex of the head	534
141	88686	89178	-1	Phage tail fibers	263
142	89294	91274	-1	Phage tail sheath	659
143	91305	93138	-1	Phage terminase, small subunit	610
144	93121	93616	-1	Phage terminase, large subunit	164

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
145	93624	94443	-1	Tail sheath stabilizer and completion protein	272
146	94484	95255	-1	Phage neck protein	256
147	95256	96186	-1	Phage neck protein	309
148	96217	97675	-1	Phage fribritin (wac) protein	485
149	97684	99235	-1	Phage straight tail fiber (short)	516
150	99231	99891	-1	Baseplate wedge subunit and tail pin	219
151	99890	101696	-1	Hypothetical protein	601
152	101695	102562	-1	Baseplate wedge tail fiber connector T4-like gp9	288
153	102626	103631	-1	Baseplate wedge subunit T4-like gp8	334
154	103623	106722	-1	Baseplate wedge initiator T4-like gp7	1032
155	106718	108701	-1	Baseplate wedge subunit T4-like gp6	660
156	108709	109003	-1	Phage-encoded phospholipase (ACLAME 172)	97
157	109003	109498	-1	Phage protein (ACLAME 782)	164
158	109532	111260	-1	Baseplate hub structural protein/lysozyme	575
159	111243	111834	-1	Baseplate wedge subunit	196
160	111881	112334	1	Phage head completion protein	150

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
161	112333	113158	1	DNA end protector protein	274
162	113264	113795	1	Phage tail completion Protein	176
163	113844	114570	1	Deoxynucleoside monophosphate kinase	241
164	114569	114800	1	Tail fiber assembly protein	76
165	114796	115255	1	Hypothetical protein	156
166	115328	115586	1	Phage protein	85
167	115662	115896	1	Phage internal (core) protein	77
168	115954	116140	1	Phage protein	61
169	116139	116538	1	Phage protein	132
170	116540	116828	1	Phage protein	95
171	118398	118749	1	Phage protein	116
172	119136	119610	1	Hypothetical protein	157
173	119851	120115	1	Phage protein	87
174	120171	120690	1	Hypothetical protein	172
175	120733	121327	1	Phage host specificity protein	197
176	121368	121977	1	Hypothetical protein	202
177	122319	122694	1	Phage protein	124
178	122690	123179	1	Phage protein	162
179	123175	123616	1	Nudix hydrolase	146

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
180	123652	124147	1	Phage lysozyme	164
181	124206	124623	1	Phage endonuclease	138
182	124769	125309	1	Phage protein	179
183	125305	125635	1	Phage protein	109
184	125641	126004	1	Autonomous glycyl radical cofactor	120
185	126003	126225	1	Phage protein	73
186	126217	127283	1	Phage protein	88
187	126483	126762	1	Phage protein	92
188	126821	127283	1	Phage endonuclease	153
189	127290	127836	1	Hypothetical protein	181
190	127828	128170	1	Valyl-tRNA synthase	113
191	128166	128634	1	T4-like phage protein with macro domain	155
192	128630	128843	1	Phage protein	70
193	128839	129046	1	Phage protein	68
194	129042	129216	1	Phage protein	57
195	129212	129398	1	Phage protein	61
196	129407	129989	1	Thymidine kinase	193
197	130031	130244	1	Phage protein	70
198	130256	130550	1	Phage rI lysis inhibition regulation	97

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
199	130546	130933	1	Phage protein	128
200	131028	131217	1	Phage protein	62
201	131216	131420	1	Phage protein	67
202	131422	131617	1	Phage protein	64
203	131606	131780	1	Phage protein	57
204	131971	132505	1	Phage protein	177
205	132511	133033	1	Phage protein	173
206	133035	133497	1	Phage protein	153
207	133496	134507	1	Thioredoxin, phage associated	336
208	134621	135590	1	Thioredoxin, phage associated	322
209	135691	135994	1	Thioredoxin, phage associated	100
210	136054	136582	1	Thioredoxin, Phage associated	175
211	136637	137045	1	Thioredoxin, phage associated	135
212	137052	137940	1	Thioredoxin, phage associated	295
213	137948	138968	1	Thioredoxin, phage associated	339
214	138995	140027	1	Thioredoxin, phage associated	343
215	140079	141009	1	Thioredoxin, phage associated	309
216	141005	141320	1	Thioredoxin, phage associated	104
217	141306	141549	1	Thioredoxin, phage associated	80

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
218	141550	141814	1	Thioredoxin, phage associated	87
219	141810	142026	1	Phage protein	71
220	142028	142199	1	Hypothetical protein	56
221	142321	142807	1	Pin protease protein	161
222	142843	143020	1	Phage protein	58
223	143062	143536	1	Phage endonuclease T4-like gp49	157
224	143532	145350	1	Ribonucleotide reductase of class III	605
225	145342	146173	1	Phage associated homing endonuclease	276
226	146169	146640	1	Ribonucleotide reductase of class III	156
227	146632	146764	1	Phage protein	37
228	146755	146971	1	Phage protein	71
229	146973	147280	1	Phage protein	103
230	147256	147580	1	Glutaredoxin	107
231	147738	147921	1	Phage protein	60
232	147913	148207	1	Phage protein	97
233	148214	148346	1	Phage protein	43
234	148346	148547	1	Phage protein	66
235	148599	148926	1	Phage protein	108
236	148928	149144	1	Phage protein	71

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
237	149140	149410	1	Phage protein	89
238	149488	150046	1	T4-like phage RNA polymerase sigma factor	185
239	150059	150248	1	Phage protein	62
240	150249	150567	1	Phage protein	105
241	150742	150916	1	Phage protein	57
242	150928	152185	1	a-gt alpha glycosyl transferase	400
243	152361	153381	1	Phage recombination-related endonuclease gp47	339
244	153377	153641	1	Phage protein	87
245	153621	153828	1	Phage protein	68
246	153824	155507	1	Phage recombination-related endonuclease gp46	560
247	155562	155751	1	Phage protein	62
248	155760	156150	1	RNA polymerase binding protein	129
249	156205	156892	1	Sliding clamp DNA polymerase accessory protein	228
250	156943	157903	1	Replication factor C small subunit	319
251	157904	158468	1	DNA polymerase clamp loader gp62	187
252	158469	158838	1	Endoribonuclease translational repressor	122

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
253	158839	159061	1	Phage protein	73
254	159139	161836	1	DNA polymerase	898
255	161962	162256	1	Hypothetical protein	97
256	162266	162647	1	Phage immunity	126
257	162654	162906	1	Phage immunity	83
258	163059	163800	1	Hypothetical protein	246
259	163796	164639	1	Glycosyl transferase	280
260	164716	165898	1	Phage recombination protein	393
261	165890	166235	1	Phage capsid and scaffold	114
262	166244	167672	1	Phage DNA primase/ helicase	475
263	167730	167913	1	Discriminator of mRNA degradation	60
264	167914	168172	1	Phage protein	85
265	168232	168526	1	Hypothetical protein	97
266	168538	168895	1	Phage protein	118
267	168896	169061	1	Hypothetical protein	54
268	169064	169535	1	DNA primase/ DNA helicase	157
tRNA1	116925	116998	1	tRNA-Gln-TTG	73
tRNA2	116999	117086	1	tRNA-Leu-TAA	87
tRNA3	117091	117165	1	tRNA-Gly-TCC	74

CDS	Start	Stop	Strand	Predicted function	Amino acid sequence length
tRNA4	117175	117249	1	tRNA-Pro-TGG	74
tRNA5	117251	117338	1	tRNA-Ser-TGA	87
tRNA6	117304	117494	1	tRNA-Thr-TGT	76
tRNA7	117340	117494	1	tRNA-Met-CAT	77
tRNA8	117502	117589	1	tRNA-Pseudo-GTA	87
tRNA9	118304	118380	1	tRNA-Arg-TCT	76

As for the phage isolate NPS, the genome of the phage isolate NPM revealed that it is also a member of the family *Myoviridae* because it has all the genes typically present in the genome f T4-like phages. In addition, the presence of tRNA genes in both *E. coli* phage NPS and NPM clusters them to the genus *Tequatrovirus*. Phage NPS and NPM are clearly distinguishable phenotypically, as phage NPS forms small clear plaques and phage NPM forms big clear plaques in lawns of the host bacterium *E. coli* ATCC 25922. These two lytic *E. coli* phages have similar genes that are arranged differently within the genome as depicted in figure 3.15. However, *E. coli* phage NPS has genes encoded for long tail fibers on CDS 200 with 237 amino acid sequence length while *E. coli* phage NPM has this gene on CDS 61 with 371 amino acid sequence length. In addition, it was noted that phage NPM genome is encoded for dCMP deaminase an enzyme essential for DNA biosynthesis while it was absent on phage NPS genome. The two genomes comprise of four main encoded protein groups:

1. DNA packaging proteins = terminase enzymes (small and large subunits) and endonuclease
2. Structural proteins= tail proteins, tail fibers, major capsid proteins, scaffold proteins, portal proteins.

3. Host lysis proteins= endolysins (lysozyme) and holins
4. DNA replication, modification, and regulation proteins= thymidine kinase, DNA binding proteins, nucleotide-binding proteins, thymidylate synthase, DNA polymerase.

In addition, both genomes encode for tRNA genes. However, out of the 294 (NPS) and 268 (NPM) predicted protein-coding genes, 20% were phage proteins, which were hypothetical proteins (majority) and transcription modulator, transporters, mRNA metabolism modulator, and membrane proteins. Phage NPS and NPM also had no lysogeny genes detected; both phages have no bacterial virulence or AMR genes present

3.2.4. Phylogenetic and genome nucleotide identity analysis for *E. coli* phage NPS and NPM.

The phylogenetic analysis aids in understanding the evolutionary relationship among organisms. The phylogeny of phages NPS and NPM was analyzed based on their genome (figure 3.12). Both phage NPS and NPM were closely related to other *E. coli* phages such as VB_EcoM-JB75, vB_EcoM-fFHoEco02, vB_EcoM-fFiEco0, and vB_EcoM-G3G7. The genome-based comparison of phage NPS, NPM, and closely related phages revealed that these two phages (NPS and NPM) belong to the family *Myoviridae* and closely cluster with phages vB_EcoM-JB75, vB_EcoM-fHoEco02 and vB_EcoM-fFiEco06 (figure 3.12).

A selected phage protein (major capsid protein) was also compared with those of closely related phages. *E. coli* phage NPS and NPM are 99% identical, they share similar genes as seen in figure 3.13 and they fall into the same cluster and are related to a *Shigella* phage JK23

Furthermore, genome comparisons were done for *E. coli* phage isolate NPS, NPM and two closely related phages (vB_EcoM-fFHoEco02, and vB_EcoM-fFiEco06), originally isolated in a Finland wastewater treatment plant. This analysis revealed that Phage vB_EcoM-fFiEco06 (accession number MG781190) and vB_EcoM-hHoEco02 (accession number MG781191) have 98.6 % sequence similarity to phage NPM and 98.4% sequence similarity to phage NPS. It was evident that phages NPS and NPM

are larger in genome size than the two phages that were isolated in Finland. Even though phage NPS and NPM have identical genomes based on the genome size, they differ in the arrangement of their genome and some encoded proteins, as depicted in figure 3.15.

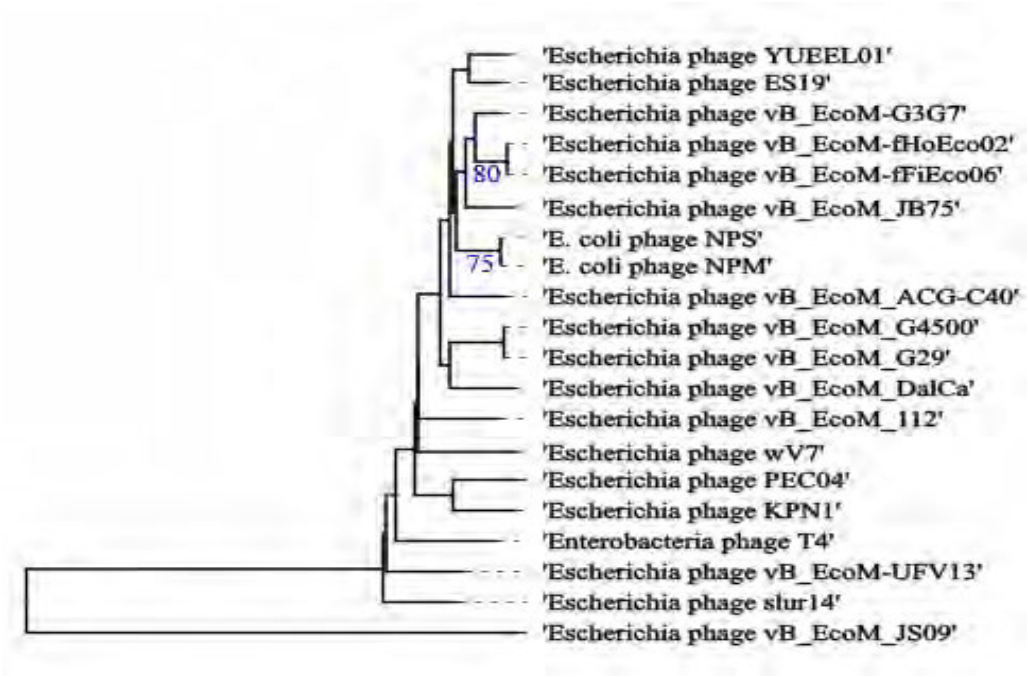

Figure 3.12: Genome comparison result for the *Escherichia coli* phages NPS, NPM and closely related phages using a whole genome data set. The tree was inferred with FastME 2.1.456 and GBDP distances were calculated from genome sequences. Branch lengths are scaled in terms of the GBDP distance formula d5; numbers above branches are GBDP pseudo-bootstrap support values from 100 replications.

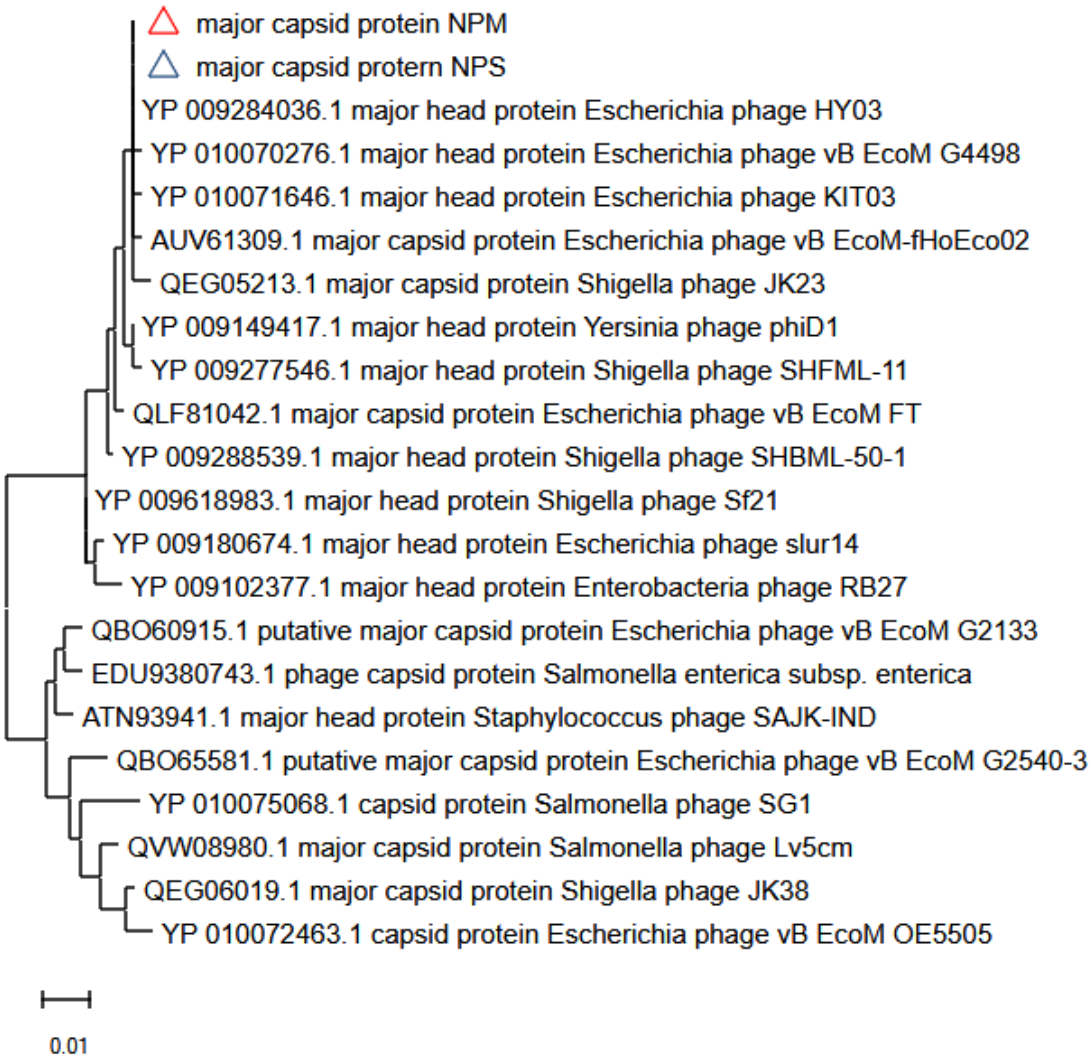

Figure 3.13: Phylogenetic analysis of the *E. coli* phages NPS (blue triangle) and NPM (red triangle) based on the predicted amino acid sequence for the major capsid protein. The sequence alignment was performed using MUSCLE and MEGAX and the maximum likelihood method with 1000 bootstrap replicates. The scale bar indicates the percentage of genetic variation.

The comparison of genomes of *E. coli* phage NPS and NPM in view of average nucleotide identity confirms that phage NPS and NPM are members of the order *Caudovirales*, family *Myoviridae,* and resemble T4-like phages in the *Tequatrovirus* genus as they showed the highest similarity to two closely related phage genomes.

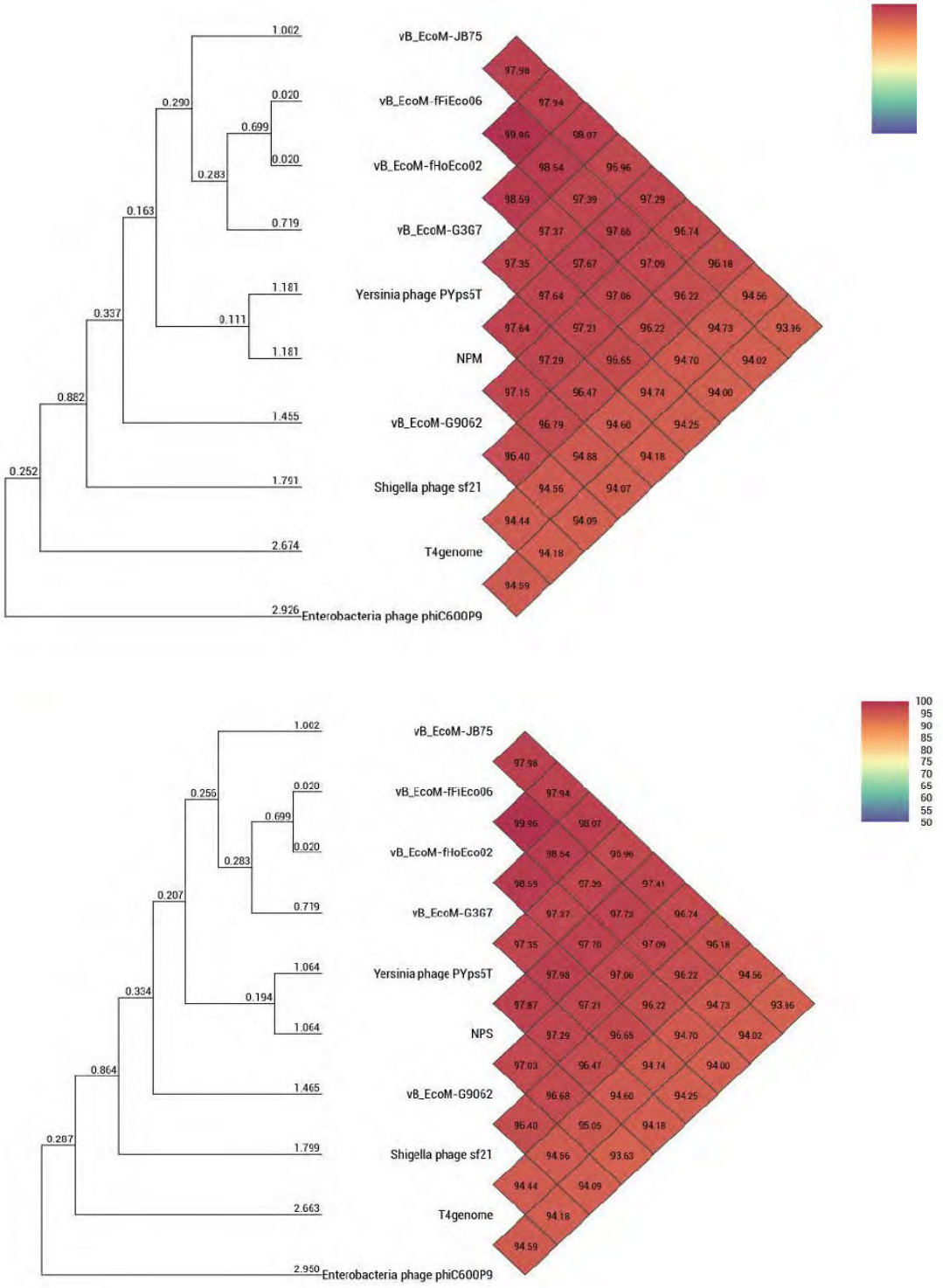

Figure 3.14: Average nucleotide identity (ANI) analysis for the genomes of *E. coli* phage NPM (top), NPS (bottom) and closely related phages.

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A

B

C

D

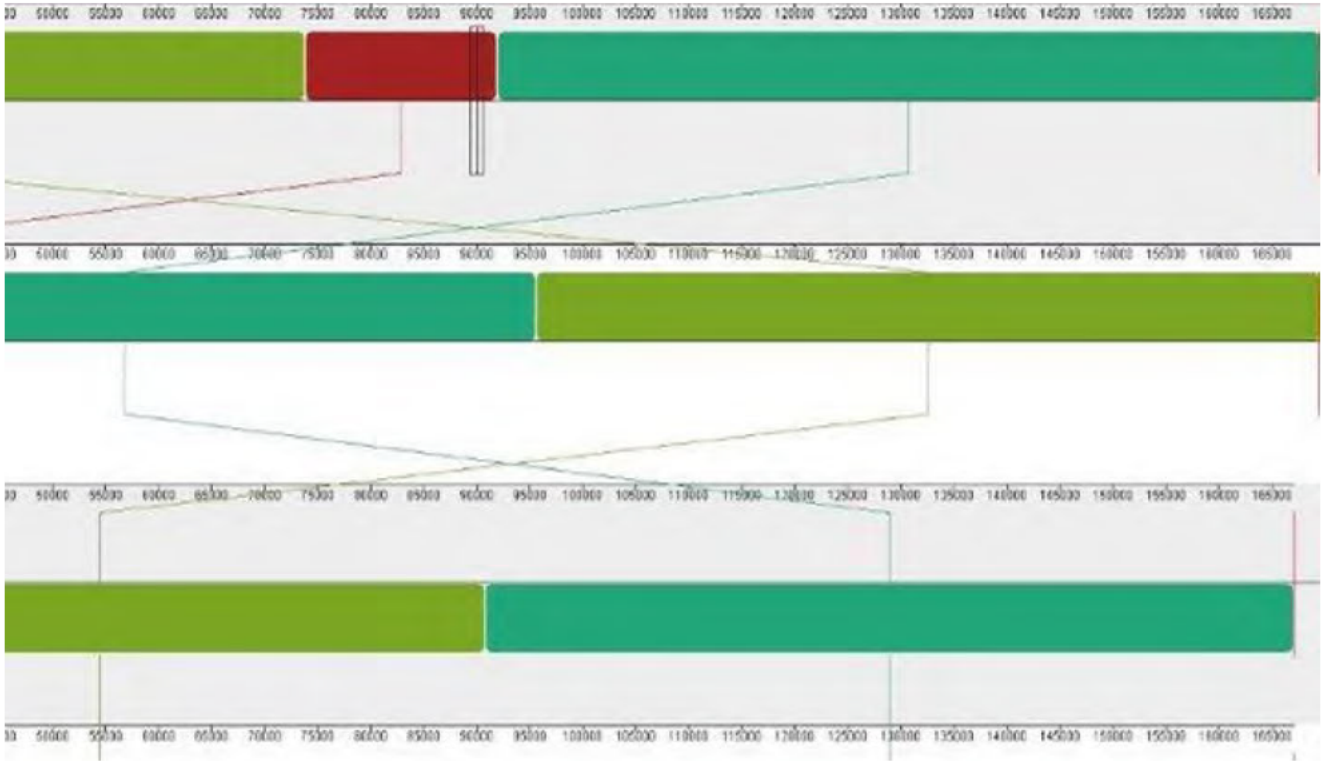

Figure 3.15: Genome based comparison of lytic *E. coli* phage NPS (A), NPM (B), with vB_EcoM-fFiEco06 (C) and vB_EcoM-fHoEco02 (D) at DNA level.

Finally, the genomes of the two lytic *E. coli* phages NPS and NPM, were compared to the genomes of the two closely related *E. coli* phages that were originally isolated in Finland (vB_EcoM-fFiEco06 and vB_EcoM-fHoEco02). The genomic arrangement of genes encoding for similar proteins is highlighted in Figure 3.15 via color coding. It demonstrates that the genomes of the four phages are composed of three similar gene clusters. However, it shows that the arrangement of these clusters differs between the two phages NPS and NPM as well as between the two phages NPS and NPM and the two phages from Finland.

3.2.5. One-step growth curves of the four lytic *E. coli* phages

The growth of the isolated phages was analyzed to determine the efficiency of phages to infect bacterial host cells by establishing latent period and burst size, which suggest the progeny production rate of the phages. Phages with shorter latent periods and higher burst sizes are regarded as more suitable for phage therapy as they can eliminate target bacteria more efficiently. Therefore, one-step growth curves of the four isolated bacteriophages were established with *E. coli* ATCC 25922 as the bacterial host.

The growth of the four phage isolates NPS, NPM, YS and YM mostly shows the typical pattern for lytic bacteriophages, i.e. the latent period, followed by the rise period, and then finally reaching the plateau phase as shown in figure 3.16. *E. coli* Phage NPM (B) had essentially no latent period and YS (C) had a short latent period of about 10 minutes, while phages YM (D) and NPS (A) had a latent period of about 20 minutes. The rise period was about 60 minutes for phage NPS, 80 minutes for NPM and YS, and, finally, 70 minutes for phage YM. The estimated burst sizes of the four phages were 107 (YS), 75 (YM), 87 (NPS), and 102 (NPM) phage particles per infected cell, respectively.

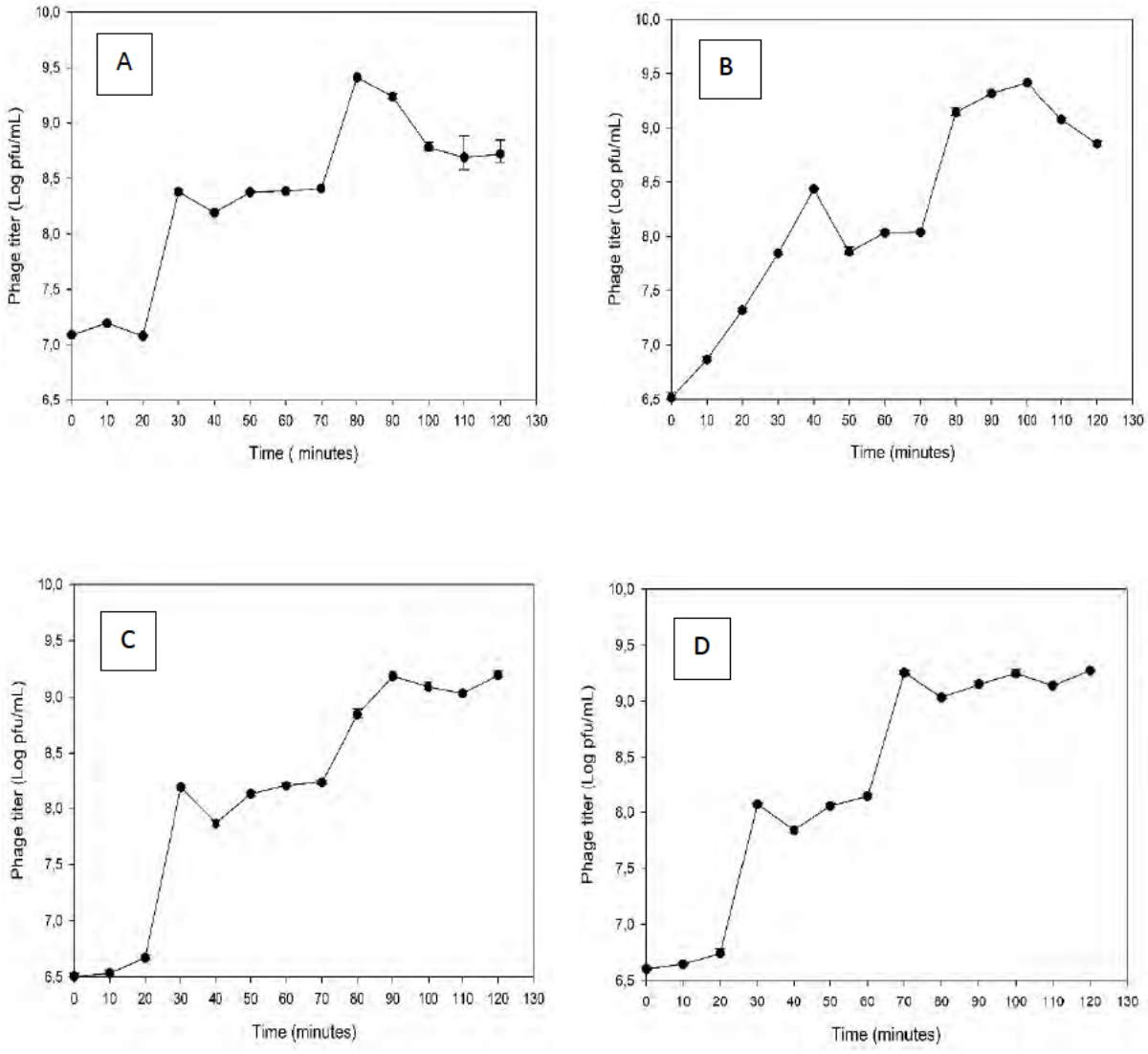

Figure 3.16: One-step growth curves of phage isolate NPS (A), NPM (B), YS (C) and YM (D) using their host bacterium *E. coli* ATCC 25922 at an incubation temperature of 37°C. All the data points shown are the means of 3 independent experiments and the error bars show the standard deviation of the mean.

3.2.6. pH and thermal stability of the four lytic *E. coli* phage isolates

Phage stability is a crucial requirement for phage therapy, applications, and survival in the environment. Various external factors, including acidity and temperature, determine the stability, viability, and infectivity of bacteriophages. It is therefore important to determine the stability of bacteriophages at potentially adverse environmental conditions to select for highly effective phages. Therefore, the stability

of the four isolated bacteriophages was evaluated at pH values ranging from 3-11. The three phages (NPS, NPM, YS) were stable at pH values between 3 and 9 with maximum stability at pH 7, while phage YM was most stable at pH values between 5-9 with a maximum stability at pH 7- 9. All four phage isolates were highly sensitive to an alkaline pH of 11 due to protein denaturation, with more than 3 log reductions of phage titers compared to the optimal pH 7. However, acidic pH values <7 resulted in about 1 log reduction of phage titers for phage NPS, NPM, and YS, whereas phage YM was highly sensitive to an acidic pH of 3 with more than 2 log titers reductions from the titers at the optimal pH (7-9).

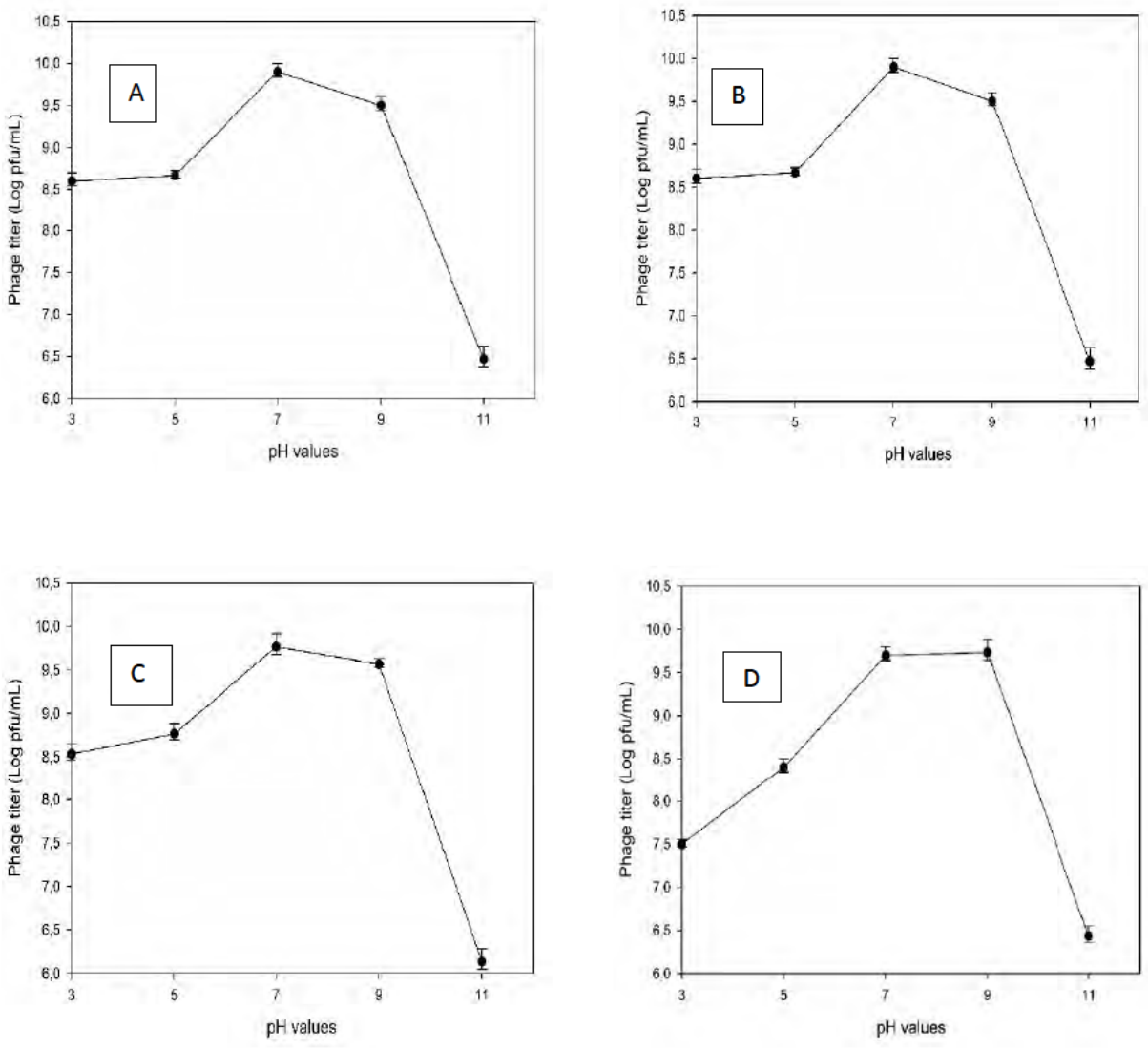

Figure 3.17: Stability of the four *E. coli* phage isolates NPS (A), NPM (B), YS (C) and YM (D) after 1 hour exposure to different pH values at 25°C. All the data points shown are the means of 3 independent experiments and the error bars show the standard deviation of the mean.

As temperature affects the survival and infectivity of bacteriophages for their bacterial host, the isolated phages were exposed to different temperatures. The four lytic *E. coli* phage isolates were all stable between 25-60°C. However, an optimal thermal stability was observed at 37°C for the three phage isolates NPS, YS and YM, but phage NPM exhibited an optimal thermal stability at 45°C. While exposure to 80°C completely inactivated the phage particles, an increase in temperature from 25 to 37°C caused more than 1 log increase in phage titer for all four phage isolates enabling optimum growth and replication, decreasing by more than 1 log of the phage titer when exposed to 60°C.

The titers of phages NPS, NPM, YS and YM were 1- 1.3-log lower at 25°C when compared to 37°C, the optimum temperature. At 45°C there was a 0.2-log reduction for phages NPS and YM, about 0.1- log increase for phage NPM indicating a potentially higher temperature optimum, and no decrease or increase was observed for phage YS. A 60°C heat treatment resulted in a 1.5-log reduction for phage NPS, a 0.4-log reduction for phage NPM, a 0.5- log reduction for phage YS and a 1.2-log reduction for phage YM.

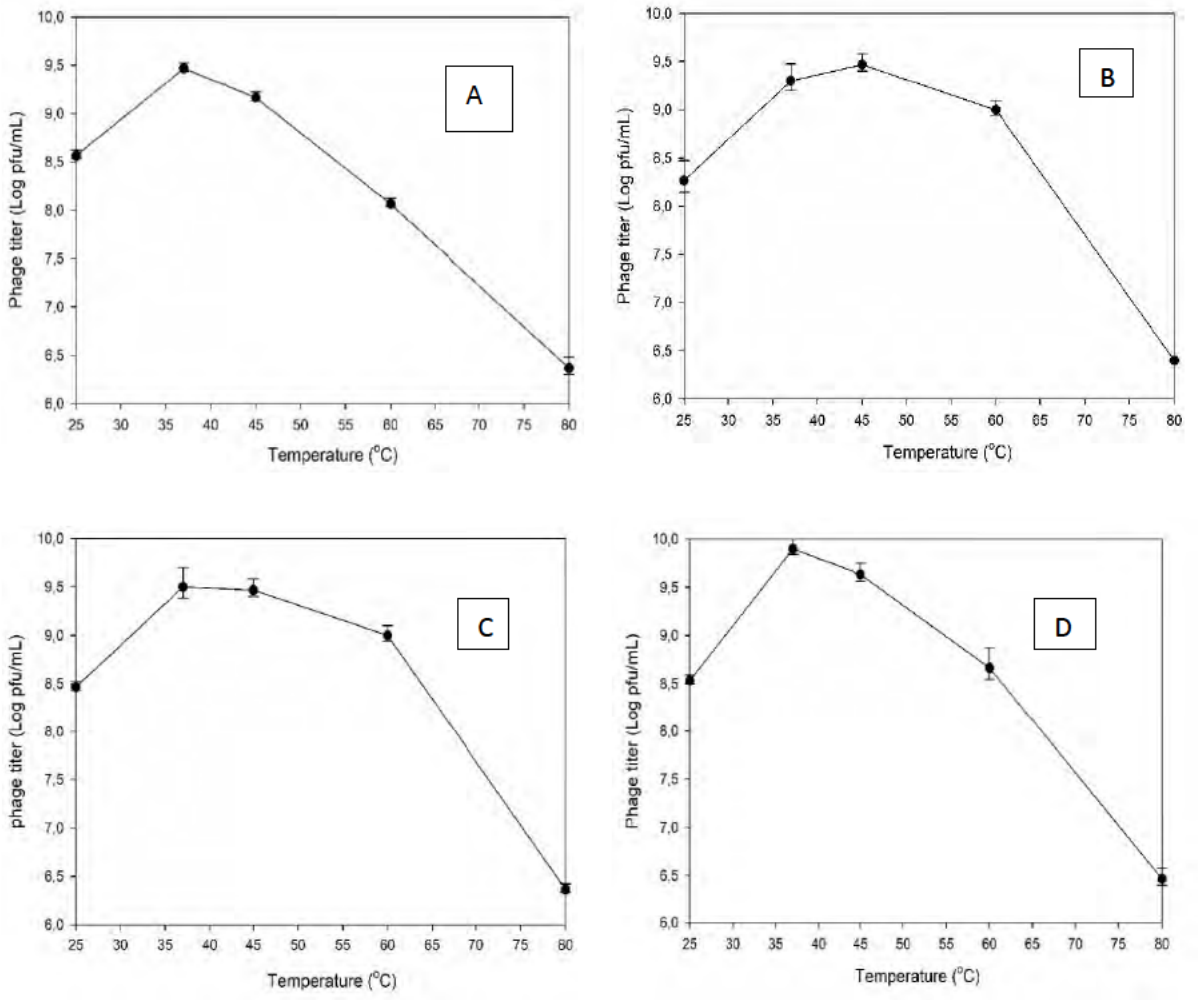

Figure 3.18: Thermal stability of the four phage isolates NPS (A), NPM (B), YS (C) and YM (D) after exposure to different temperatures for 10 minutes. All data points shown are the means of 3 independent experiments and the error bars show the standard deviation of the mean.

3.2.7. Host range of the four lytic *E. coli* phage isolates

A bacteriophage's host range, which is defined as the span of bacterial hosts that a phage is capable of infecting, can be used to predict the therapeutic potential of phages. As lytic phages are generally highly host-specific, they differ fundamentally from other antimicrobial agents, such as broad-range antibiotics. Therefore, the host range of the four phage isolates was evaluated using various representative bacteria (table 3.4).

Table 3.4: Host range of *E. coli* phage isolates NPS, NPM, YS, and YM for various bacterial hosts.

Bacterial strains	Origin	Resistance profile	Phage isolates			
			NPS	NPM	YS	YM
Klebsiella pneumoniae KP 124 (MDR)	Greys Hospital effluent	NOR-AMC-CAZ-ATM-GEN-TOB-CIP-CTX-MXF-CL-ERT	-	-	-	-
Staphylococcus aureus	ATCC 6538	NA	-	-	-	-
Salmonella Typhimurium	ATCC 14028	NA	+	+	-	-
Enterococcus faecalis	ATCC 29212	NA	-	-	-	-
Micrococcus luteus	ATCC 9341	NA	-	-	-	-
Escherichia coli	ATCC 25922	NA	+	+	+	+
(*E. coli*) Bw1	Borehole	NA	+	+	-	-
(*E. coli*) Bw2	Borehole	NA	-	-	-	-
(*E. coli*) Bw3	Borehole	NA	-	-	-	-
(*E. coli*) Bw4	Borehole	NA	-	-	+	+
(*E. coli*) Ec1	Zebra feces	NA	+	+	-	-

Bacterial strains	Origin	Resistance profile	Phage isolates			
			NPS	NPM	YS	YM
(*E. coli*) Ec2	Zebra feces	NA	+	+	-	-
(*E. coli*) Ec3	Zebra feces	NA	+	+	-	-
(*E. coli*) Ec4	Zebra feces	NA	+	+	+	-
(*E. coli*) Ec9 (MDR)	Sludge	AMP-AMC-ATM-GEN-TOB	-	-	-	+
(*E. coli*) Ec10 (MDR)	Sludge	AMP-AMC-ERT-GEN-TOB	-	-	-	-
(*E. coli*) Ec15	Sludge	AMP-AMC-TOB	-	+	-	-
(*E. coli*) Ec20 (MDR)	Sludge	AMC-CAZ-GEN-TOB	-	-	-	-
(*E. coli*) Ec26	Sludge	GEN-TOB	-	-	-	-
(*E. coli*) Ec27 (MDR)	Sludge	AMP-AMC-CAZ-ATM-GEN-TOB	-	-	-	-
(*E. coli*) Fp5	Farm pig faeces	AMP-AMC-CAZ	-	-	-	-
(*E. coli*) Fp6 (MDR)	Farm pig feces	AMP-AMC-CAZ-GEN	-	-	+	-
(*E. coli*) Fp29 (MDR)	Farm pig feces	AMP-AMC-ATM-NOR-CIP	+	+	+	+
(*E. coli*) Pp10	Pet pig feces	AMC	+	+	+	+
(*E. coli*) Pp19	Pet pig feces	AMC-CAZ	+	+	-	-

Bacterial strains	Origin	Resistance profile	Phage isolates			
			NPS	NPM	YS	YM
(*E. coli*) L20	Lettuce	NA	-	-	-	-
(*E. coli*) L23	Lettuce	NA	-	-	-	-
(*E. coli*) L36	Lettuce	NA	+	+	+	+
(*E. coli*) W26	Wild beast feces	AMC-ATM	+	+	-	-
(*E. coli*) Wd2	Irrigation water	NA	+	+	+	+

(+) indicates lysis and (-) indicates no lysis after (24-48 hours at 37°C) when applying the spot test on bacterial lawn in Nutrient agar. ATCC= American Type Culture Collection. NA= no phenotypic resistance known. AMP (ampicillin), AMC (amoxicillin-clavulanic acid), CAZ (ceftazidime – 3rd generation cephalosporin), ATM (aztreonam), CIP (ciprofloxacin), NOR (norfloxacin), GEN (gentamicin), TOB (tobramycin), ERT (ertapenem), CTX (Cefotaxine), MXF (Moxifloxacin) and CL (Cefalexin) resistance.

The above table depicts the host range of the four isolated phages against various *E. coli* isolates, representative Gram-positive bacteria, and two Gram-negative pathogens from the family *Enterobacteriaceae*. The four isolated phages lysed even multi-drug resistant strains of *E. coli*. While phage YS and YM were specific to *E. coli* strains; with YS infecting 8 *E. coli* strains and YM infecting 7 *E. coli* strains out of 25 *E. coli* strains tested, phages NPS and NPM had a broader host range, lysing 12 and 13 out of 25 tested *E. coli* strains respectively. In addition, phages NPs and NPM lysed *Salmonella* Typhimurium ATCC 14028. Interestingly, all four phages were able to kill an MDR *E. coli* strain (FP 29), which is resistant to 5 classes of antibiotics. Phage NPM could additionally lyse EC 15, which is resistant to 3 classes of antibiotics. However, none of the four phage isolates was able to lyse the MDR *Klebsiella pneumoniae* and the Gram-positive bacteria.

3.2.8. Viability of the four lytic *E. coli* phage isolates under storage conditions

A proper method of storage should be used to ensure the safe keeping of bacteriophages for extended use. Low-temperature preservation methods have been widely used to maintain phage stocks during long-term periods. Therefore, the storage of phage suspensions in Nutrient broth in the fridge (4°C) and a freezer with host bacteria present (-80°C) was evaluated. Freezing caused a maximum of 0.5-log reduction of the phage titers over the storage period of 24 months, while the viability of phages was already decreased by ≤ 1-log after only 2 days of storage and was lost almost completely (≤ 5 -log) after 10 days storage in the fridge (4°C).

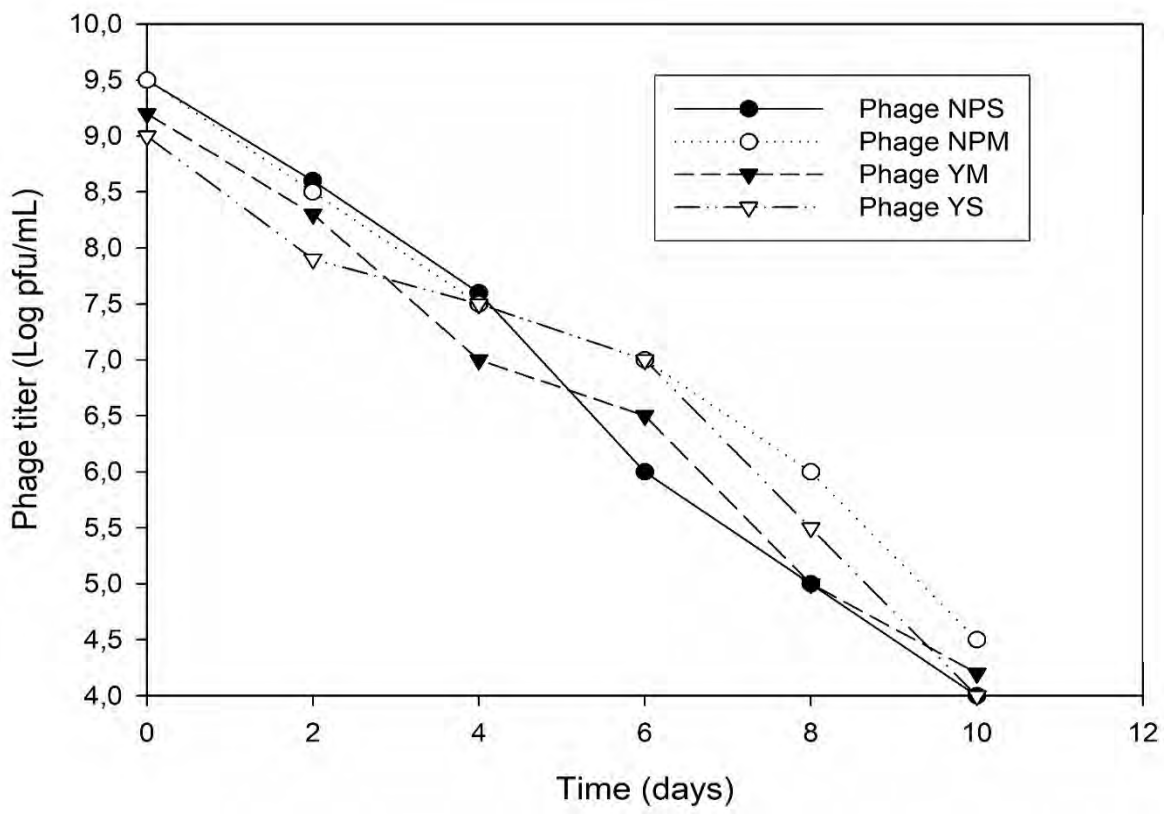

Figure 3.19: Viability of the lytic *E. coli* phage isolates NPS, NPM, YS and YM at 4°C in nutrient broth for 10 days.

It was remarkable that the titers of the four phage isolates decreased substantially when stored in nutrient broth at 4°C, a temperature commonly used in the laboratory to store solutions (or similar) without the host cells present. After only two days of storage in the fridge at 4°C an average of 1 log reduction in phage titer was detected. Thereafter, the reduction was rapid, with a total average of about 5- log reduction after 10 days of storage. These findings suggest that phages should not be stored without host cells in liquid media at 4-8°C for longer than 4 days if high titers are desired.

However, the four phage isolates-maintained viability when frozen with the host bacteria at -80°C over a 24-month period. The two white phages NPS and NPM showed a 0.5-log reduction, the two phages YM and YS had a 0.7-log reduction over 24 months of storage at -80°C in the presence of host cells in a complex medium. These results indicate that freezing phage particles with their host at -80°C is more suitable for phage storage over extended periods of time.

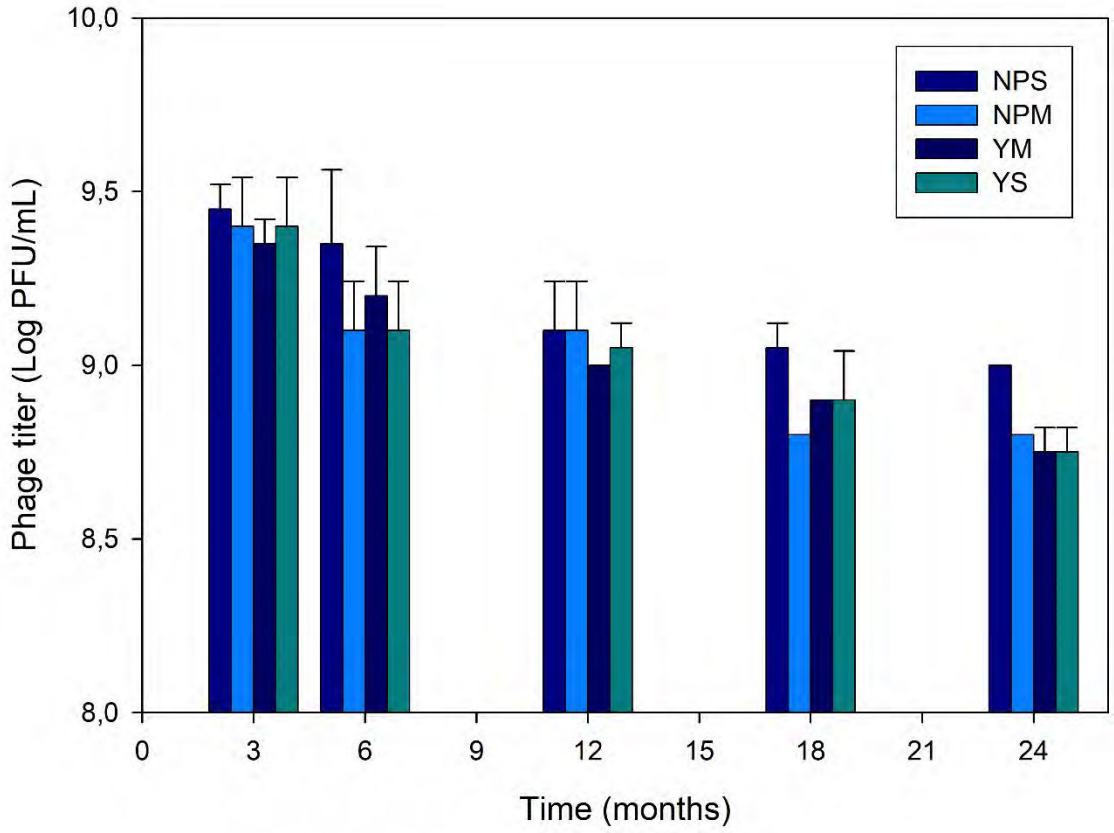

Figure 3.20: Viability of the four phage isolates after freezing (-80°C) with the host (*E. coli* ATCC 25922) over a 24 month's period. All data points shown are the means of three independent experiments and the error bars shown are the standard deviation of the mean.

3.3. Application of the lytic *E. coli* phage NPM in comparison to chlorine in the treatment of wastewater

Chlorine is used globally to treat wastewater and is regarded as the most effective antimicrobial agent, even at relatively low concentrations. While lytic bacteriophages are known to infect and kill bacteria and are regarded as important regulators of bacteria in aquatic environments, their use in wastewater as a bio-control agent is only poorly established in South Africa. Lytic *E. coli* phage NPM was selective for this application experiment based on its latency period (no latent period) and high burst size (102 phage particles per infected cell).

When employing chlorine treatment at a concentration of 10 mg/mL to eliminate spiked *E. coli* cells present in sterile wastewater, *E. coli* counts started to decrease after 30 minutes as expected on microbiological grounds. A 3.5-log reduction was observed after 90 minutes. However, the lytic bacteriophage NPM was effective for the removal of *E. coli*, as a viable count decline of 3.5-log was observed after 120 minutes. Interestingly, when chlorine and phage NPM were combined, a 3.5- log reduction was reached in only 60 minutes, suggesting that a combined treatment could be more efficient in eliminating undesired bacteria from wastewater. In addition, it indicates that the phage NPM can tolerate the biocide chlorine at a concentration toxic to *E. coli*.

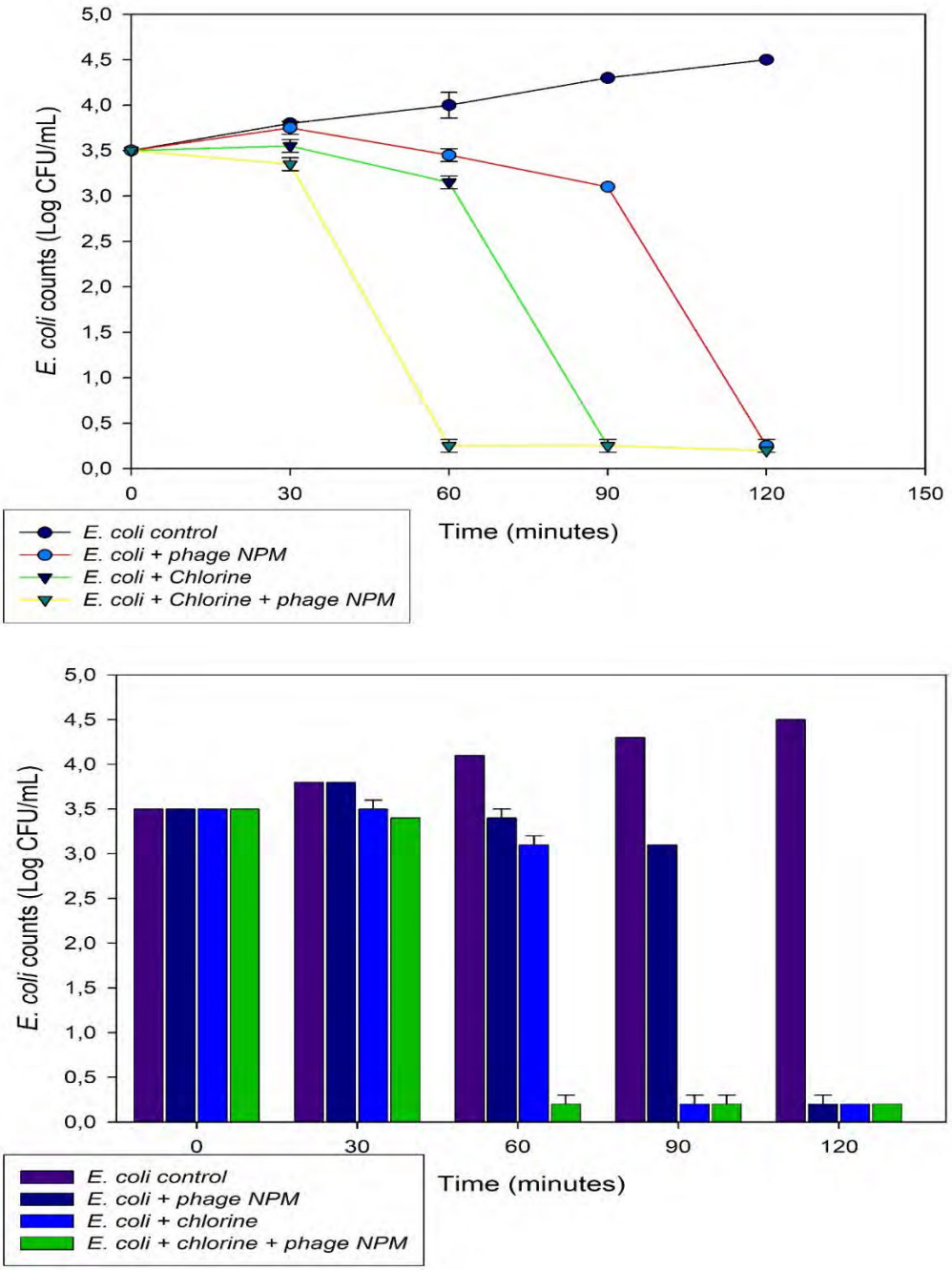

Figure 3.21: Application of chlorine versus phage NPM for the elimination of *E. coli* in wastewater treatment over the period of 120 minutes. All data shown are the average of three replicates and the error bars shown are the standard deviation of the mean.

Discussion

Isolation and characterization

Bacteriophages are ubiquitous and diverse in the biosphere and can be isolated using simple and straightforward methods from all the environments inhabited by bacteria and archaea (Sadekuzzaman *et al*., 2017; Ji *et al*., 2021). Bacteriophages are now recognized to play a critical role in controlling bacterial pathogenesis in humans, animals, and plants, as lytic phages can be employed as so-called “living” antibiotics. Additionally, bacteriophages are now regarded as safe, efficacious, and innovative alternatives to the use of antibiotic therapy (Loc-Carrilo and Abedon, 2011; Endersen and Coffey, 2020). Studies have revealed that phages are more prevalent in sewage than in other environmental sources (Matilla *et al*., 2015; Kiljunen *et al*., 2018; Topka *et al*., 2019; Badawy *et al*., 2022). Therefore, the presence of lytic phages that are infecting *E. coli* ATCC 25922 in wastewater samples from the Darvill wastewater treatment plant (WWTP) is not surprising. The four isolated lytic phages were *Escherichia coli* specific and belonged to the tailed phages (Ackermann, 1998); their phenotype represents a typical T4 bacteriophage as defined by Ackermann (2004).

Bacteriophages present a great morphologic variability and their primary classification in the past was based on six groups established by Bradley in 1967 (Bradley, 1967). They are initially differentiated on the bases of their morphology, and thus Transmission Electron Microscopy was employed. Transmission Electron Microscopy (TEM) analysis revealed that the four lytic phage isolates analyzed in this study have typical morphological characteristics of phages within the family *Myoviridae* in the order *Caudovirales*; phages in this family are characterized by icosahedral capsids and rigid contractile tails of different sizes (Sharma *et al*., 2017; Dion *et al*., 2020). The four lytic *E. coli* phages isolated in this study have icosahedral capsids, a collar between the capsid and the tail (*E. coli* phage YM and NPM), and rigid contractile tails with a baseplate at the end of the tail (*E. coli* NPS and YS) as shown in figures 3.4-3.7. They all resemble, therefore, morphological characteristics described by Zinke *et al*. (2021) for T4-like *Myoviridae* phages. However, with recent developments in the virus taxonomy, the ICTV has formally approved the possibility of classifying viruses known only from genomic sequences (Koonin *et al*., 2020), and tailed phages are now grouped into families based on their genomes. The Myoviruses have been officially

rectified as *Ackermannviridae*, *Chaseviridae,* and *Herelleviridae* (Turner *et al*., 2021), which are characterized by a long, rigid, and contractile tail with a sheath around a central tube (Zinke *et al*., 2021).

Characterization of bacteriophages is also based on the nucleic acids they possess in their capsid. In the present study, two phage isolates (*E. coli* phage NPS and NPM) were selected for genome analysis based on their ability to produce clear plaques and their short latency period and high burst size, indicating their potential for effectively eliminating their host cells.

Genome classification

Bacteriophage T4 contains DNA inside its capsid, which preserves the genome under environmental conditions outside the bacterial host (Verheust *et al*., 2010). The phage capsid also carries host recognition sensors; once the phage is attached, it moves the genome into its host (Leiman *et al*., 2003). In this study, the genomes of lytic *E. coli* phage NPS and NPM were sequenced. It was discovered that these two phage genomes are 99% identical and have the same genome size of 169 536 bp. They contain 9 tRNA genes, which is a common feature for the phage genomes in the genus *Tequatrovirus*, in the family *Myoviridae* (Nikapitiya *et al*., 2022), and they carry genes that encode for acridine (antimicrobial agent discovered before penicillin) resistance, but not for a medically relevant antibiotic. The phage NPS genome has 294 coding sequences, of which 40 encode for hypothetical proteins and the NPM genome has 268 coding sequences, of which 24 encode for hypothetical proteins (table 3.2 and 3.3). It was noted that phage NPS has more coding sequences, some of which were characterized as hypothetical “phage proteins”. However, when compared to the sequences available in Genbank (NCBI), the majority of these predicted “phage proteins” were confirmed as hypothetical proteins with yet unknown functions. Fremin *et al*. (2022) recently conducted a large-scale comparative genomics analyses specifically for small genes < 50 amino acids, as they are often overlooked. They reported that more than 40 000 small gene families were predicted in phages and 5000 of these small gene families were predicted to encode for anti-CRISPR proteins, more than 9000 were predicted to encode for secreted or transmembrane proteins, and novel core phage proteins such as baseplate and phage tail proteins. *Myoviridae* T4-like phages infecting *E. coli* are complex and tailed, with double-stranded genomic

DNA that is typically over 125 000 bp, and are among the most studied viruses with genomes typically containing about 274 open reading frames and about 40 encoding for structural proteins (Kutter and Stidham, 1994; Ackerman, 2008; Yap and Rossmann, 2014). These findings confirm that these two phages resemble a typical T4 phage as Cerritelli *et al*. (1997), Kostyuchenko *et al*. (1999), and Zinke *et al*. (2021) reported that a mature T4 phage is made up of double-stranded DNA covered with a capsid, tail and tail fibers attached to the base plate. The head of a T4 phage consists of shell major capsid proteins, capsid vertex, and scaffolding proteins, which are located on gene product=gp 20-40 (Leiman *et al*., 2003). Mutations of capsid proteins result in different capsid shapes, such as icosahedral and prolate heads (Black *et al*., 2015). *E coli* phage NPS has capsid proteins encoded on coding sequences (CDS) 251-261, while *E. coli* NPM has capsid proteins encoded on CDS 130-140 (table 3.2 and 3.3). The phage endonuclease cleaves dsDNA concatemers into suitably sized DNA fragments for packaging (Franklin *et al*., 1998), and in both phage NPS and NPM genomes, genes encoding phage endonucleases were present.

The phage T4 uses 25 000 bp of its genome for encoding proteins for the contractile tail morphogenesis and assembly (King and Laemmli, 1971; Matsui *et al*., 1997; Burda and Miller, 1999). The phage T4 is made up of two protein cylinders called the base plate and the fibers, the inner cylinder is known as the tail tube, and it is used to pass DNA from the head into the infected cell, and the outer cylinder is the tail sheath that covers the tail tube (Leiman *et al*., 2003; Dion *et al*., 2020). All these essential and characteristic features were encoded in the genomes of *E. coli* phage NPS and NPM, further validating them to be members of the *Myoviridae* family, potentially belonging to the genus *Tequatrovirus*. Gene products (gp) gp5, gp6, gp7, gp8, and gp9 are important in the assembly of the base plate located under the tail (Yap and Rossmann, 2014). These proteins were also encoded by the genomes of *E. coli* phage NPS and NPM (table 3.2 and 3.3).

During the infection, the T4 phage uses gp9 to form an attachment site for the long tail fibers on the baseplate. This gene product is necessary to translate the message from the long tail fibers to initiate conformational change in the base plate from a hexagon to a star-like shape, which then activates tail contraction (Kostyuchenko *et al*., 1999; Leiman et al., 2003). This protein was encoded on the genomes of both *E. coli* phage NPS and NPM. During the infection process, the phage recognizes an *E. coli* cell by

long tail fibers that protrude from the base plate and bind to the host receptors. The phage then presents the base plate to the receptors using short tail fibers which are initially present under the base plate. The gene gp5 encodes phage lysozyme, which is an essential enzyme that lyses the bacteria at the end of phage replication. Each phage of the two *E. coli* phages, NPS and NPM had a lysozyme gene present in their genome. Phage holins and endolysins are responsible for attacking the cytoplasmic membrane and peptidoglycan of Gram-negative hosts, thus, resulting in lysis. The presence of these genes could explain the efficiency of the two phages NPS and NPM, to rapidly kill *E. coli,* as all the above-mentioned enzymes were encoded in the genome of both phages (table 3.2 and 3.3).

Virulent bacteriophages are characterized by forming clear plaques and the absence of integrases, transposase, and repressor genes in their genome (Chang *et al*., 2005). Integrases are proteins encoded by tyrosine recombinase genes if expressed, resulting in integrated prophage, and they are the only phage-encoded proteins that facilitate a lysogenic life cycle and prevent superinfection (Fogg *et al*., 2011). *E. coli* phage NPS and NPM, as expected on microbiological grounds, have DNA polymerase encoded in their genome. This enzyme is a multifunctional protein located on gp43 that has high activity for DNA synthesis and is involved in phage DNA replication (Karam and Konigberg, 2000). This explains why the two lytic phages efficiently replicate and rapidly kill their host bacterium, *E. coli*.

When comparing the genomes of *E. coli* phages NPS and NPM with two closely related phages isolated from a wastewater treatment plant in Finland (vB_EcoM-fFiEco06 and vB_EcoM-fHoEco02), it was evident that *E. coli* phages NPS and NPM are similar in size and contain similar gene clusters. However, they differ in genome organization; this could possibly explain the slight difference observed in the host range with phage NPM lysing *E. coli* Ec15, an *E. coli* isolate that was isolated in a pit latrine sludge with resistance to ampicillin, amoxicillin, and tobramycin, while phage NPS could not (table 3.4). It was also notable that the genome of the *E. coli* phage NPM carries a gene encoding for the enzyme dCMP deaminase, which is used to catalyze the formation of dUMP ($dCMP+H_2O=dUMP+NH_3$). This enzyme is essential in DNA biosynthesis and is known from several enterobacterial phages, including T4-like phages (McGaughey *et al*.,1996). However, dCMP deaminase is not encoded in the genome of *E. coli* phage NPS. In addition, results obtained from the phylogenetic

analysis confirmed the provisional assignment of the two lytic *E. coli* phages NPS and NPM to the genus *Tequatrovirus*.

Stability and performance of the phages

Bacteriophages with shorter latent periods and high burst size are ideal candidates for phage therapy as they adsorb fast and produce numerous new virions. Phages from the family *Myoviridae* have been reported to have burst sizes typically ranging from 50 - 100 PFU/cell (Chang *et al*., 2005; Park *et al*., 2012). The four lytic phages reported in this study are within the same range, with burst sizes of 107, 102, 87, and 75 PFU/cell established for *E. coli* phage NPS, NPM, YS, and YM, respectively. Some *E. coli* phages attach to the host bacteria within the first 10 minutes and have therefore a short latent period (Zhou *et al*., 2015). Similar results were found by Necel *et al*. (2020) for the two *E. coli* bacteriophages vB_Eco4M-7 and ECML-117, which had 10 minutes latent period and a bust size of 100 PFU/cell (Necel *et al*. 2020). This was a similar case for *E. coli* phage YS, as it had a latent period of 10 minutes. However, some phages may exhibit even shorter latent periods, like *E. coli* phage vB-EcoS-95, with a reported latent period of only 4 minutes after adsorption (Topka *et al*., 2019). Interestingly, *E. coli* phage NPM had no apparent latent period. *E. coli* phage NPS and YM reported in this study had latent periods of 20 minutes, which is similar to a polyvalent broad-spectrum *Tequatrovirus* EP01 coliphage reported by Zhou *et al*. (2022).

Tailed bacteriophages can strive even under extreme environmental conditions (Ackermann *et al*., 2004). The stability of bacteriophages is one of the critical parameters to study in phage research because these factors determine the occurrence, viability, applicability, and storage of bacteriophages. Inappropriate conditions can inactivate a phage through damage to its structural elements (Ackermann *et al*., 2004). The pH-induced and thermal inactivation are the most studied parameters in phage characterization (Rakhuba *et al*., 2010), and it is reported that T4-like *Myoviridae* phages are unstable at pH< 5 (Ly-Chatain, 2014). In this study, the stability of phages was evaluated in the pH range of 3-11 with 1 hour of exposure. Three out of the four lytic *E. coli* phages (NPS, NPM, and YS,) were stable at pH 3-9 with an overall titer reduction of < 1.5 log PFU/mL between pH 3- 5 and a minimum of < 0.5 log reduction at pH 9. *E. coli* phage YM was stable at pH 5-9, and maximum

stability was achieved at pH 7 and 9 for phage YM, with 1.2-log phage titer reduction at pH 5 and 2.1-log reduction at pH 3. There was a significant titer reduction at pH 11 for all the phages with 3-log PFU/mL. However, at pH 12, no viable phages were detected. Similar results were presented by Niu *et al*. (2009) when evaluating the stability of phages under acidic conditions. These authors found that the titer of a lytic *E. coli* O157:H7 phage was reduced by 1.9- log PFU/mL at pH 3. The significant decline in phage titers observed at pH 11 in the present study could be caused by the denaturation of the capsid proteins due to the high concentration of hydroxyl ions present at pH 11 (Niu *et al*., 2009). The broad-spectrum *Escherichia coli* phage EP01 was also stable at pH 4-10, and phage titers decreased dramatically when exposed to strong acidic or alkaline conditions. This phage, EP01, was inactivated at pH 3 and 12 (Zhou *et al*., 2022). The pH in WWTP effluent ranges typically between 6.5-8.4 (Jeong *et al*., 2016). Therefore, it is important that the four lytic *E. coli* phages reported in this study are stable at pH 5-9, which are the pH values typically encountered in WWTPs. These results confirm that these four lytic *E. coli* phages could be employed to eliminate *E. coli* present in the WWTP effluents.

Temperature is among the most crucial factors in phage survivability; it plays a crucial role in attachment, penetration, and multiplication and affects the latent period (Jonczyk *et al*., 2011). Several studies have demonstrated that exposure of phages to high temperatures inactivates them due to their nucleic acids and essential proteins being denatured (Wang *et al*., 2001; Bao *et al*., 2011; Yamaki *et al*., 2014). Yamaki and colleagues noted that *Myoviridae* phages considerably lost activity with 3.5-log PFU/mL after 60 minutes of exposure to 60°C. However, the recently reported broad-spectrum *E. coli* phage EP01 exhibited remarkably high thermal stability, exposure to 60°C for 1 hour resulted only in minimal phage titer loss, and even exposure to 80°C did not completely inactivate all the phage particles as phage titers were still detectable (Zhou *et al.,* 2022). *E. coli* phage NPM and YS showed minimal titer reduction (≤0.5-log) at exposure to 60°C for 10 minutes, while phage NPS and YM titers decreased by 1.2-1.5-log PFU/mL at 10 minutes of exposure to 60°C. These results confirm that phages respond differently when subjected to heat treatment (Wilkowske *et al*.,1954) and highlight the need for testing their heat stability. There were no viable phage particles detected after exposure of *E. coli* phages NPS, NPM, YS, and YM to temperatures above 80°C, suggesting that exposure to 80°C and above will inactivate

these four *E. coli* phages completely. However, such extreme temperatures are not usually encountered in South African WWTP effluents; temperatures usually range from 14-22 °C (Makuwa *et al*, 2022), highlighting again the potential of the four lytic *E. coli* phage isolates as biocontrol agents under ambient temperatures. Bao *et al*. (2011) found similar results for the two *Salmonella* phages they investigated.

Host range

Host range determination is a crucial factor in bacteriophage characterization and is governed by the phage recognition of and attachment to its host. This initial step determines the specificity of the phage as phages use specific receptors to attach to the host. It is reported that most phages exhibit a narrow host range (de Jonge et al., 2019). However, there are lytic phages that represent a broader host range such as phage SP116, which lysed 72% of the tested *Salmonella* isolates representing various serovars (Bao *et al*., 2019). Studies suggest that phages with a broader host range (able to target various strains from the same or even other species) are suitable for phage therapy as they can target a wider range of target bacteria. The phages YM and YS isolated in this study exhibit a narrow host range as they could only infect *E. coli*. However, additional lysis was observed in *Salmonella* Typhimurium ATCC 14028 for the lytic *E. coli* phage NPS and NPM. Yildirim *et al*. (2018) found similar results for their Eco-phage 13 isolated from sewage, which was effective against one *Salmonella* Typhimurium strain, highlighting that cross-species infectivity is possible for bacteriophages even though they may exhibit a narrow host range. Niu *et al*. (2020) also found that their T5 vB_EcoS-AKFV33 phage isolate was capable of lysing STEC (Shiga-toxin-producing *Escherichia coli*) and *Salmonella* stains. Zhou *et al*. (2022) recently demonstrated similar results for their EP01 phage, which could infect 31 out of 59 *E. coli* and 1 out of 4 *Salmonella* strains tested. This phage also showed a weak lytic capacity for *Salmonella* spp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Proteus mirabilis*. Out of the 24 environmental *E. coli* isolates tested, including isolates with resistance to various antibiotics (excluding the original isolation host: *E. coli* ATCC 25922), *E. coli* phage NPS could infect 45% and NPM 50% of the tested *E. coli* isolates, while phage YS could infect 29% and phage YM only 25% of the *E. coli* isolates tested. When the four phages were tested against antibiotic-resistant and

multidrug-resistant *E. coli* strains, all four phages successfully infected and lysed *E. coli* strain FP29, which was isolated from farm pig feces and is resistant to 5 classes of antibiotics (King and Schmidt, 2017). It was also noted that phage NPM and NPS were able to lyse *E. coli* strains with resistance to Amoxicillin clavulanic acid and Ampicillin but not those resistant to Gentamycin and Tobramycin. In 2018 Peng and Yuan conducted a study to demonstrate that phages can kill MDR *E. coli* strains (Peng and Yuan, 2018). Such results are intriguing as bacteria are becoming more resistant to antibiotics; thus, phages could be an alternative to treat bacterial infections. In addition, hospitals and WWTPs are frequently reported as releasing antibiotic-resistant strains of *E. coli* (Mukherjee *et al*.,2021); results obtained from this study highlight the potential of lytic phages to treat such effluents.

The host range of bacteriophages depends on the recognition and adsorption step, the first part of the phage life cycle. It has been reported in previous studies that bacteriophages bind to specific receptors present on the surface of bacteria before they can initiate the infection (Washizaki, *et al*., 2016). *Escherichia coli* receptors typically recognized by phages are OmpC proteins and lipopolysaccharide (LPS), which the phage attaches to using the long tail fibers. *E. coli* phage NPS notably differs in the long tail fibers nucleotide sequence from phage NPM, which may account for the difference observed in the host range. The T4-like phages use the long tail fibers to reversible bind onto the LPS of *E. coli*, while the short fibers interact with the heptose moiety of the host's LPS for irreversibly binding (Silver *et al*., 2016). Silver *et al*. (2016) also reported that coliphages had no preference for proteinaceous or polysaccharide receptors. They investigated 26 coliphages and found that 10 phages required cell wall proteins for adsorption, 8 required sugar moieties, and the other 8 required both structures for adsorption. If these receptors are not accessible to the phage or have been altered by mutations, that could induce phage resistance. Indeed, studies showed that the alteration of such bacterial structures is the main cause of phage resistance (Mangalea and Duerkop, 2020).

Viability

Ackermann (2004) recorded that bacteriophages belonging to the order *Caudovirales* are resistant to freezing temperatures and can remain viable for as long as 10-12 years. The viability of the four lytic *E. coli* phages NPS, NPM, YS, and YM was

determined at refrigeration and freezing temperatures for storage purposes. All the four lytic *E. coli* phages reported in this study remained viable at 4°C, -20°C, and -80°C. However, at 4°C, viability was only retained for the period of 10 days with an about 3-3.5-log reduction in phage titers over time in the absence of host cells. Interestingly, other studies found opposing results. *Siphoviridae E. coli* phage P-6 and P-7 remained viable in phosphate-buffered saline at 4°C for up to 90 days (Litt and Jaroni, 2017), and *E. coli* phage APTC-EC-2A also remained viable at 4°C for four months in Luria-Bertani broth (Hon *et al.*, 2022). In addition, at -80°C phage NPS, NPM, YS, and YM particles remained viable for 24 months with only minor titer loss (< 1-log reduction) recorded, which is longer than what Hon *et al*. (2022) reported for their *Myoviridae E. coli* phage APTC-EC-2A, this phage remained viable at -80 for four months with a minor reduction in phage titer. Manohar and Ramesh (2019) evaluated the viability of three lytic bacteriophages: *Escherichia* phage ECP311, *Klebsiella* phage KPP235, and *Enterobacter* phage ELP140 after lyophilization (freeze-drying). These phages were freeze-dried using six distinct additives: glucose, sucrose, gelatin, mannitol, polyethylene glycol, and sorbitol. After freeze drying, the phages were stored at temperatures 4°C and 37°C and rehydrated using saline to test their viability at five months intervals of up to 20 months. The use of sucrose, gelatin, and their combination proved to be beneficial in maintaining the viability of phages after lyophilization. When the lyophilized phages were stored at 4°C, their viability was maintained for up to 20 months. However, 37°C storage resulted in reduced activity of the phages after 10 months. These results suggest an alternative to storing bacteriophages for extended periods using additives.

Phage NPM application for biocontrol of *E. coli* vs chlorine

This study aimed to isolate *E. coli* phages for potential bio-control applications in wastewater treatment processes. To evaluate the biocontrol potential of the isolated phages, one isolate NPM, was selected based on having no detectable latency period and the ability to produce clear sharp plaques, using artificial wastewater. The results show that phage NPM is able to remove *E. coli* completely from a spiked artificial wastewater in 120 minutes at an MOI of 0.1. Chlorine, which is a common chemical used in wastewater treatment plants, when employed at a 10 mg/mL concentration as used in the local WWTP, could kill all *E. coli* cells in 90 minutes. The use of both chlorine and phage NPM resulted in the elimination of *E. coli* in just 60 minutes.

However, studies showed that chlorine treatment might have a negative impact on bacteriophages. Briè *et al*. (2018) reported that 10 minutes of exposure of phage MS2 to 100-200 mg/L of free chlorine resulted in decreased infectivity of the phage but did not alter the binding capacity of phage MS2 to *E. coli* K12. This could explain the efficiency of phage NPM to lyse *E. coli* even in the presence of 10 mg/mL of chlorine. However, after 120 minutes of combined treatment (e.g., phage NPM and chlorine), the lytic phage NPM titers could not be detected using the double overlay technique. This could indicate that chlorine may have damaged the phage particles due to the long exposure of 120 minutes, which may be a disadvantage when using this type of combined treatment. Zhang and Hu (2012) attempted to remove biofilms of *Pseudomonas aeruginosa* using wastewater phages and chlorine treatment. Their results highlighted that a combination of phage and chlorine treatment resulted in biofilm growth reduction of 95-97% and removed pre-existing biofilm. Their findings also suggest that a combination of chlorine and phage treatment is a promising method to control and remove bacterial biofilms from various surfaces. Scarascia *et al*. (2021) evaluated the feasibility of using bacteriophages and UV-C irradiation as a combined treatment for anaerobic membrane bioreactors for wastewater treatment. Their results show that the combined treatment resulted in better log cell removal of *Acinetobacter* species tested. Their findings suggest that other combination treatments, such as phage plus UV -radiation, might be even better than phages and chlorine.

Study limitations

A limitation of the current study was that the stability of phage isolates was not tested in the presence of pollutants such as phenol and heavy metals, which could be present in wastewater treated in WWTP systems and might affect the ability of the phages to eliminate target bacteria.

Conclusion and future research direction

As expected, bacteriophages targeting *E. coli* were present and successfully isolated from wastewater samples collected at the Darvill wastewater treatment plant in Pietermaritzburg, South Africa. Wastewater treatment plants receiving fecal matter, which naturally contains diverse phages, are a well-established source of bacteriophages due to the presence of their host bacteria. This study demonstrated

that bacteriophages could be isolated from such samples, demonstrating that WWTPs are an excellent source for phage isolation. The results obtained in this study concur with other studies reporting that the majority of phage isolates belong to the order *Caudovirales* based on their morphology. The formation of clear plaques on agar plates indicates the ability of phages for the lysis of bacteria, thus potential candidates for biocontrol purposes as they do not integrate into the host genome. Whole genome analysis revealed that the two selected *E. coli* phages in this study are highly similar, yet they are different in their morphology and genome organization. It was also noted that these two phages isolated in this study were similar to phages isolated far away in Finland, which proves that phages of similar characteristics are ubiquitous and are related to each other, and evolve similarly when adapting to a specific host bacterium.

As demonstrated in this study, the phage isolates analyzed exhibited a narrow host range, which agrees with previous studies highlighting that *E. coli* phages are mostly specific to their host. Moreover, lytic phages may be used as an environmentally friendlier way than chlorine to combat antibiotic resistance, as they can kill antibiotic-resistant bacteria. This research also validated the stability of the four analyzed phages after storage in the fridge and freezer and further confirmed that freezing phage particles with their host at -80°C is more suitable for phage storage over extended periods of time. Finally, an application study conducted in this research project suggests that the bacteriophages isolated were able to eliminate *E. coli* from the spiked wastewater just as well as the established biocide chlorine. However, they may require more time. It is worth mentioning that the phages isolated in this study were able to attack even an MDR *E. coli* strain and that the two sequenced *E. coli* phage isolates (NPS and NPM) showed no lysogeny, no bacterial virulence, and no genes encoding resistance against clinically relevant antibiotics.

There is surprisingly limited information on phage application in wastewater treatment plants in Southern African countries. This study gives a first insight into the potential of *E. coli* bacteriophages in environmentally friendly wastewater plant applications. Further research should be done on pathogenic bacteria associated with wastewater, including hospital effluents, such as *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterococcus faecalis,* and *Klebsiella pneumoniae,* including antibiotic-resistant strains, as they are dangerous bacterial pathogens for humans. The ability of bacteriophages to reduce foaming bacteria, biomass bulking in wastewater, and

understanding phage interaction with chlorine and using UV + phage as a combination treatment are areas that still need more research. Finally, more phage stability (Chloroform, metal) and biofilm elimination capacity testing are outstanding for *E. coli* phage NPS, NPM, YS, and YM.

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