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Isolation and Characterization of Bacteriophages from the Arctic

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Abstract

Bacteriophages are viruses that specifically infect bacteria. These obligate intracellular parasites are Earth's most copious, ubiquitous, and genetically diverse (unexplored) biological entities. Organisms in the Arctic environment constantly experience extreme conditions, therefore life in the Arctic evolved different specialized strategies and molecules to survive which offers huge opportunities to prospect for novel bioactive compounds and potential industrial applications or products for different purposes. In the Arctic, biological resources are little studied, and we know just a little bit of them. This study aims to isolate and characterize various phages from the Arctic Sea and sewage samples collected from waste-water treatment plants (WWTP) in Tromsø, Norway (Arctic). Host bacteria used were isolated from the Arctic Sea and belong to the following genera: *Vibrio* (2 strains), *Flavobacterium, Shewanella, and Maribacter*, while *Escherichia coli* K12 (laboratory strain) was used for isolating phages from sewage samples. Marine bacteria were cultured at 4°C whereas *E. coli* was cultivated at 37°C. Phage isolation was made by preparation of enrichment cultures and plating using the double agar layer (DAL) method. Morphological characterization was performed using negatively stained transmission electron microscopy (TEM). This study found that while marine phages were present in the samples, but they were challenging to grow and further characterize. Based on plaque assay, phages were found in three marine Arctic hosts (none in *Shewanella).* However, five coliphages were successfully isolated and purified from the sewage samples, and their titer was determined. The highest titer was estimated at 8×10^{10} pfu/ml for phage Ec42_SH23 followed by the lowest 2×10^{10} in Ec41_SH23 phage. The TEM analysis revealed that the isolated phages belonged to the caudovirus group, which is known for its long, short, and contractile or non-contractile tails according to the International Committee on Taxonomy of Viruses (ICTV). Moreover, the phage genome was extracted from the isolated coliphages, although it has not been sequenced yet. These findings suggest that the Arctic may be a potential source of novel phages with unique features.

Keywords: Arctic Bacteriophage, Marine Bacteria, Sewage, TEM, Caudovirus

Abbreviations

DAL	Double agar layer
GTA	Gene transfer agents
ICTV	International Committee on Taxonomy of Viruses
ITS	Internal transcribed spacer
LB	Lysogeny broth
PCR	Polymerase chain reaction
PFU	Plaque-forming units
RBP	Receptor-binding proteins
TEM	Transmission electron microscope
VBR	Virus-to-bacteria
VLP	Virus-like particles
WWTP	Waste-water treatment plants

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1 INTRODUCTION

1.1 Background of the Study

The Arctic is a unique ecosystem characterized by extremely cold temperatures, low nutrient availability, and prolonged periods of darkness, making it a challenging environment for life to thrive. Despite these challenges, the Arctic Ocean is home to a diverse array of microorganisms, including bacteria and viruses. All are adapted to this extreme environment. A virus is a biological entity composed of either double or single-stranded DNA or RNA enclosed by proteins and, in some cases, lipids. Viruses can infect all types of living cells, which make them the most copious biological agents in the biosphere. They play an important role in microbial food webs dynamics in a wide range of environments, including many extreme environments. Despite their ubiquity, in the last century, most of the viral-based bioprospection was mainly focused on viruses that cause disease in metazoans. In addition to controlling the climate, half of the earth's oxygen and a significant portion of the protein consumed globally are originated from marine microbes. Microorganisms are the predominant regulators of energy and nutrient cycles in the vast majority of the Earth's oceans and contribute over 90% of the total living biomass in the marine environment. (Suttle, 2007). Due to their extremely small size, viruses only represent about 5% of the global microbial biomass, which is a small proportion of the total prokaryotic biomass (Bar-On & Milo, 2019). The amount of biomass that is killed by viruses each day is estimated to be around 20%-40% (Suttle, 2007). Furthermore, viruses are considered one of the world's largest unexplored reservoirs of genetic diversity (Suttle, 2007). Every millilitre of ocean water contains millions of virus-like particles (VLPs) (Bergh et al., 1989), and viruses are significant agents of mortality in heterotrophic and autotrophic plankton (Proctor & Fuhrman, 1990; Suttle et al., 1990). These findings have prompted a major awareness of the enormous underestimation of the effects of viral infection on microbial species. Microbial communities as well as nutrient and energy cycles are also affected by viruses (Breitbart et al., 2018), which are major players in marine microbial diversity.

Observations using transmission electron microscopy (TEM) have indicated that most viruses present in aquatic environments are bacteriophages, commonly referred to as phages. These phages typically exhibit capsid diameters that are less than 250 nm. Bacteriophages are the most abundant biological entities on Earth and can be found in almost every environment

that contains bacteria, including soil, water, and the human body, which plays a crucial role in the ecology and evolution of bacteria. They can influence nutrient cycling, genetic exchange, and bacterial population dynamics. Arctic phages are of particular interest due to their potential for biotechnological and therapeutic applications. However, compared to phages from other environments, Arctic phages remain largely understudied. Thus, there is a need to isolate and characterize new Arctic phages to better understand their diversity, host range, and functional properties. It may also lead to the discovery of new phage-based therapies and biotechnological applications.

This chapter highlights the abundance and distribution of phages, exploring both their phenotypic and genomic characteristics. It seeks to clarify the diverse life cycles of phages and their potential impacts on their host organisms. Additionally, the chapter provides an overview of research into the prevalence of bacteriophages in the Arctic and molecular investigations into phages found in oceans. Furthermore, this chapter delves into the significance of studies conducted on phages found in sewage and their potential implications. Finally, the chapter concludes by emphasizing the significance of the study and its potential contributions to the broader understanding of phages and their role in various ecosystems.

1.2 History of Bacteriophage Discovery

Bacteriophages, also known as phages, are viruses that specifically infect and replicate within bacteria. Similarly, to other viruses, they are not endowed with any intrinsic metabolic mechanism and all their activities requiring energy depend on their host cells' intracellular machinery. Through mutualistic symbioses, viruses can also establish long-term relationships with their specific hosts, which may increase their fitness or provide competitive advantages to them (Roossinck, 2011). In the case of phages, these symbiotic interactions are common and happen through lysogenic cycles.

The discovery of bacteriophages dates to the early 20th century when researchers were investigating the nature of infectious agents that could cause bacterial lysis. The history of bacteriophage discovery has been the subject of debate, but in 1896, the British bacteriologist Ernest Hankin reported that the waters of the Ganges and Jumna rivers in India had antibacterial action against *Vibrio cholerae* and that ingestion of the water prevented the spread of cholera epidemics. Hankin suggested that an unidentified substance was responsible

for this phenomenon, which was heat-labile and could pass through fine porcelain filters. Russian bacteriologist Gamaleya made a similar observation two years later while working with *Bacillus subtilis* (Deresinski, 2009). However, none of these investigators further explored their findings until Frederick Twort, a British pathologist, and Felix d'Herelle, a French-Canadian bacteriologist at the Pasteur Institute in Paris, independently reported isolating filterable entities that could destroy bacterial cultures and produce small clear areas on bacterial lawns in 1915 and 1917, respectively. D'Herelle coined the term "bacteriophages" to describe these entities. Over the next few decades, D'Hérelle and other researchers continued to study bacteriophages and their properties. They discovered that phages had specific host ranges and could only infect certain strains or species of bacteria. They also found that phages had different morphologies, with some having tails and others having icosahedral or filamentous shapes.

Moreover, the clinical use of phages against bacterial diseases (phage therapy), was developed by D'Herelle and used as a routine in many countries from the 1920s to the 1940s. After World War II, with the widespread use of antibiotics in the latter half of the 20th century, interest in phages declined in Western countries but phage therapy was kept alive in the Soviet Union and Poland (de Freitas Almeida & Sundberg, 2020). In recent years, however, there has been renewed interest in the use of bacteriophages as an alternative therapy to antibiotics due to the rise of antibiotic-resistant bacteria (Gordillo Altamirano & Barr, 2019; Jault et al., 2019; Watts, 2017)

1.3 Abundance and Distribution of the Marine Virus

Phages have been observed in the ocean for more than sixty years (Wommack & Colwell, 2000) but their high abundance has only been recognized recently (Suttle, 2005; Weinbauer, 2004). Even though convincing evidence existed in the late 1970s that viruses abound in the sea (Torrella & Morita, 1979), a decade later quantitative estimates revealed that seawater contains millions of viruses per millilitre (Bergh et al., 1989). The first estimates of virus abundances were based primarily on electron microscopy analysis of concentrated seawater samples. Although these studies found that the particles appeared virus-like and abundant, their estimates were inconsistent and unreliable due to sample preparation artifacts and biases. In addition to this, coupled with the high costs and time required for electron microscopy studies, there have been efforts to develop epifluorescence microscopy (Hara et

al., 1991; Hennes & Suttle, 1995; Noble & Fuhrman, 1998; Suttle et al., 1990) that are more accurate, and high-throughput. Although methodological errors have caused significant underestimates in many cases (Suttle, 2005; Wen et al., 2004), these methods are widely used by scientists and have generally resulted in reproducible abundance estimation. Recent estimates of the deep ocean, for example, differ by an order of magnitude from those taken a few years ago (Ortmann & Suttle, 2005; Parada et al., 2007). As far as the estimates of viral abundance in the water column are concerned, there is a proficient level of agreement and a high degree of confidence when proper procedures are followed. The estimation of the viral abundance in marine sediments remains difficult despite the presence of high numbers of viruses (Danovaro et al., 2001; Helton et al., 2006).

There are around 10-15-fold more viruses than bacteria and archaea in nature (Whitman et al., 1998). A total viral abundance varies along with the abundance and productivity of prokaryotic organisms and the environmental conditions in any given environment. Thus, in the oceans, as the water column goes deeper and further offshore, virus abundance decreases. When specific groups of infectious viruses (Cyanophages) were studied and their titers determined, similar patterns were observed (Suttle & Chan, 1994; Waterbury & Valois, 1993). This trend can be seen from the ratio between viral particles and prokaryotic or bacterial cells that shows the presence of these viruses. In different marine environments, however, there has been a considerable change in the relationship between viral abundance and prokaryotic abundance, as well as in the level of prokaryotic growth. For instance, the average virus to bacteria ratio (VBR) of the surface waters of the Pacific Ocean and the Arctic Ocean is approximately 40 and 10 respectively, while the average VBR of lakes is less than 5 (Clasen et al., 2008). In contrast, the ratio often exceeds 100 in the deep waters of the Atlantic Ocean (Parada et al., 2007). The pattern is large-scale in nature and tends to be influenced by different environmental conditions, but in the end, viral production is influenced by microbial hotspots on the cellular scale and the spatial scale of individual cells. In aquatic environments, the order-of-magnitude variations in viral abundance and VBRs that occur on spatial scales of centimeters in aquatic environments are evidence of this fact (Seymour et al., 2006).

With the help of flow cytometry, a better understanding of the distributions and abundances of viruses can be found in the sea, as it is a high-throughput method of counting viruses. Even though viruses are too small to scatter light predictably, it enables us to count

them by fluorescent staining of nucleic acids. The characteristics of fluorescence and scatter allow the discrimination of subpopulations of viruses and potential hosts. Despite limited data, it has been demonstrated that the most abundant subpopulation of viruses in the Arctic Ocean biome was associated with low fluorescence and high nucleic-acid content heterotrophic prokaryotes (Suttle, 2007). This sub-group of prokaryotes has been argued to be one of the most active members of the prokaryotic community (Lebaron et al., 2001; Longnecker et al., 2006), however, some have questioned this interpretation (Moran, 2007; Sherr, 2006). The phycodnaviruses which infect eukaryotic phytoplankton, exhibit higher fluorescence and scatter than other viruses. Chlorophyll a concentration, which indicates the abundance of photosynthetic cells, is most closely related to viruses with these characteristics (Suttle, 2007). Viral infections, however, may have an impact on microbial diversity, nutrient cycling (Cram et al., 2016; Rodriguez-Brito et al., 2010; Weinbauer & Rassoulzadegan, 2004), and potentially, the efficiency at which carbon is transported from surface waters to deep waters if they preferentially infect cells that are growing more rapidly (Vincent & Vardi, 2023).

1.4 Phenotypic Traits of Marine Phages

Observations of VLP via transmission electron microscopy (TEM) provided the first indication of the global diversity of viruses. A limited number of viral phenotypes have been detected in aquatic samples, primarily comprising tailed or untailed particles bearing capsid heads; this type of particle is characteristic of phages (Sime-Ngando, 2014). Several different types of phages live in aquatic systems, among which are tail phages belonging to the caudovirus group, which are members of the double-stranded DNA virus family and make up 10-40% of the total virus population (Sime-Ngando & Colombet, 2009; Wommack & Colwell, 2000). There are three caudovirus families (**Figure 1**) which dominant quantitatively: Siphoviruses, which have a long, non-contractile tail (e.g., Phage lambda), Podoviruses, with characteristics of the short, non-contractile tail (e.g., Phage T7), and Myoviruses, which have a contractile tail of variable length (e.g., Phage T4) (Sime-Ngando, 2014). Tailed phages are characterized by the presence of protective capsids attached to the tail. Unlike other tailed phages, myoviruses possess a contractile tail sheath. at the distal end of the tail, both myoviruses and siphoviruses possess a baseplate that contains receptor-binding proteins (RBPs), including tail spikes and fibers. However, in podoviruses, the RBPs directly attach to the tail due to the absence of a baseplate. Furthermore, siphoviruses and

podoviruses differ from myoviruses by having a central tail fiber or spike on the distal end of their tails or bases.

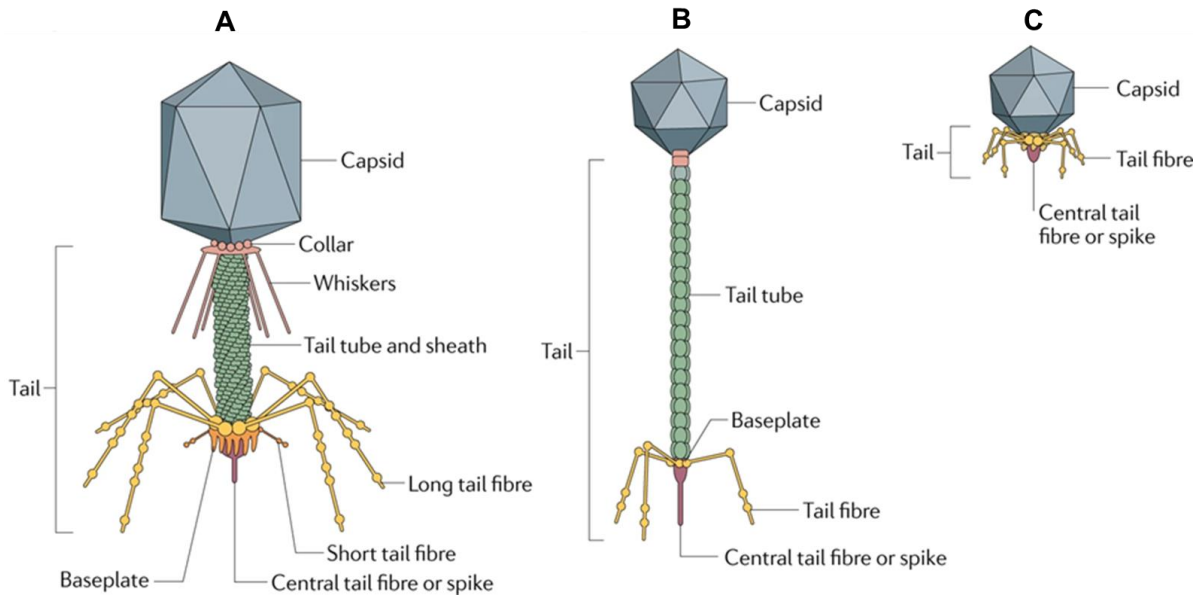

Figure 1: Illustrative frameworks of the tailed phages A) Myovirus; B) Siphovirus and C) Podovirus. The figure is adapted from Nobrega et al. (2018)

Virus abundance is dominated by non-tailed capsids in most studies. The most likely reason for this is the effect of the mechanic shock resulting from the handling, mostly ultracentrifugation (Colombet et al., 2007) because 96% of the 5500 bacteriophages described in this study were tailed particles (Ackermann, 2007). An analysis of the global morphology of marine viruses conducted recently has suggested that non-tailed viruses, which accounted for 50-90% of the viral particles observed, might be the most ecologically significant component of a natural viral community (Brum et al., 2013).

In marine viruses, the phenotypic characteristics and viral morphology reflect the selective pressures that these communities face, and they give insight into the host range, the replication of viruses, and their function (Suttle, 2005; Vincent & Vardi, 2023). A myovirus, for example, has a large host range and is generally lytic, which is a competitive advantage that can be transferred to species that are r-strategist in nature and thrive with elevated production rates in fluctuating environments (Breitbart et al., 2018). A *podovirus*, on the other hand, is highly specific to its host, while a *siphovirus* is intermediate between the two. Furthermore, several siphoviruses can insert their genome into their hosts for several generations (i.e., lysogeny), which is similar to a stable environment characteristic of K-

strategist species (Breitbart et al., 2018; Suttle, 2007). As a result of the ability of viruses to potentially cope with almost any type of environmental situation and interface (Hurst, 2011), it is not surprising that they can develop along the continuum of r-k selection. This, in turn, may explain their ubiquity, thus resulting in the concept of a virosphere, which stems from the typical r (e.g., prokaryotes) to k (e.g., vertebrates) strategy (Suttle, 2007).

1.5 Diversity of Marine Viral Genome

According to the ICTV, most of the viral species reported, have only been isolated from laboratory cultures representing not even 1% of the total viruses in environmental samples (Hugenholtz et al., 1998). Consequently, there is a high degree of diversity among environmental viruses, though most of them are unknown (Rohwer & Edwards, 2002). It is, therefore, crucial to employ molecular approaches to access the largest unexplored reservoir of biodiversity on the earth (Hambly & Suttle, 2005), which are not only characterized by whole genome sequencing of phages (Allen et al., 2011) but also assembled using metagenomics datasets (Rosario et al., 2009). Since no gene is universally conserved among viruses, polymerase chain reaction (PCR)-based methods are limited to a limited number of viral groups; however, PCR primers are based on previously identified sequences described in public databases, which means a major portion of the existing diversity of these viral groups is not covered. It has been reported that viral metagenomics offers a comprehensive overview of viral diversity in uncultured samples (Breitbart et al., 2002), and thus has revealed a richness and diversity of viral communities that was not previously known (Edwards & Rohwer, 2005). The following methods can be applied to describe marine viral diversity.

1.5.1 Whole Genome Comparison

The lack of reference bacterial phage genomes in reference databases constitutes one of the fundamental barriers to the integration of viruses into microbial ecology studies. Currently, reference databases only contain sequenced phages from 8 out of the 29 bacterial phyla with cultured isolates, which is a very small percentage (0.001%) of the total genome sequence databases (Bibby, 2014). They include *Proteobacteria, Firmicutes, Bacteriodetes, Actinobacteria, Cyanobacteria, Chloramydiae, Tentericutes,* and *Deinococcus-Thermus*. Comparative genomics has revealed that there are a significant amount of genomic diversity and novelties that can be found within the genomes of these few phages, as well as hypotheses about how phage genomes adapt to aquatic environments. In a comparison study

of 26 T4-like genomes of *myoviruses* that infect diverse marine *Cyanobacteria* (*Prochlorococcus*, *Synechococcus*), the genetic structure of the viruses was revealed to be highly syntenic, with genes for DNA replication being observed in all genomes, followed by genes for virion structure, previously described genes, and several hypothetical genes (Sime-Ngando, 2014). Additionally, to the cyanophage-encoded photosynthetic and phosphate stress genes that have already been described, genes that appear to serve numerous putative functions (e.g., phytanoylCoA dioxygenase, 2-oxoglutarate) have been described along with genes that may be responsible for niche diversification and niche expansion (Sullivan et al., 2010). According to the first genome sequence of a deep-photic marine cyanobacterial siphovirus that was recently revealed, the lysogenic lifestyle of this virus was prevalent, as well as significant differences in size and divergence from previously sequenced siphoviruses, and the virus did not contain photosynthetic genes, which are consistently found in other marine cyanophages (Sullivan et al., 2009). Also, a genomic and functional analysis of a new marine siphovirus isolated from marine *Vibrio* revealed the genome of this new siphovirus to be significantly larger (80,598 bp) than the genomes of other *siphoviruses*, as well as a novel shell symmetry, which allows it to hold together against a variety of chemical, physical, and environmental stressors with remarkable stability (Baudoux et al., 2012). It is expected that whole genome sequencing and reconstruction of unrepresented phages will provide significant insight into viral diversity, ecology, and evolution and provide molecular tools to study groups of viruses, as well as provide deep insight into phages that are currently absent from the database.

It has indeed been shown through comparisons of several whole genomes that within certain viral taxonomic groups, certain genes are conserved across all of them. The conservation of these genes in cultured and environmental viruses can be studied using PCR amplification and sequencing to study both groups of viruses. These types of genes include structural proteins such as gp20, which codes to produce capsids in T4 phage-like viruses, DNA polymerases for podovirus similar to T7, or RNA-dependent RNA polymerase fragments (Culley et al., 2007), which have been used in the identification of new groups of marine picornaviruses. According to the data from the conserved gene studies, viral diversity is abundant in the environment and its characterization is largely unknown (Breitbart & Rohwer, 2005).

1.5.2 Metagenomic Analysis

A revolution in microbiology has taken place because of the advent of the concept of metagenomics, which enables the assessment of microbial communities in complex environments without the dependency on culture (Simon & Daniel, 2011). Metagenomic analysis of viral diversity or an analysis of viromes has found that the diversity of viral populations in the environment is very high and largely uncharacterized (Angly et al., 2006; Roux et al., 2012). There has been metagenomic analysis conducted on 184 viral assemblages collected from 68 sites around the world over the span of a decade in four major oceanic regions (Angly et al., 2006) that shows that most of the viral DNA and protein sequences do not match the ones in the current databases. There are likely several hundred thousand species in the world, and the richness of each region varies along a latitude gradient that runs from north to south. However, it was found that most viral species are widespread in environments, which supports the idea that marine viruses are also widely dispersed and that local environmental conditions produce viral strains that are enriched by selective pressure. Despite the vast geographical distances between sample locations within each of these biomes, a study on comparative viral metagenomics demonstrated that freshwater, marine, and hypersaline environments were distinct from each other based on comparative viral metagenomics. This suggests that the viral communities from related environments are genetically similar (Roux et al., 2012).

As an unexpected result of metagenomics of marine RNA viruses, it has been discovered that picorna-like viruses dominate the RNA virome, indicating their position as representatives of the superfamily of eukaryotic viruses (Kristensen et al., 2010). According to Lang et al. (2009), the majority of marine RNA viruses infect eukaryotes, primarily protists, which are the predominant hosts of most of these viruses (Culley et al., 2007). The abundance of RNA viruses in coastal seawater was found to be equal to or greater than that of DNA viruses in a recent quantitative study on total RNA and DNA mass in viral fractions (Steward et al., 2013). Similarly, Roux et al. (2012) reported similar results in freshwater systems. It seems likely that protists contribute more to the dynamics of marine viruses than one might expect based on the relatively low abundance of picorna-like viruses due to their small genomes, which means they are below the detection limit of fluorescence-based counting methods, suggesting that marine viruses will be made up of many more protists than one might expect. Additionally, 129 genetically novel and distinct viruses have been

identified depending on whole genome assemblages from temperate and subtropical seawater in a recent metagenomic study (Labonté & Suttle, 2013). Most of these viruses are single-stranded DNA viruses, which are economically significant to plants and animals. Aquatic viruses have been primarily assumed to contain double-stranded DNA and infect bacteria, but the discovery of RNA and ssDNA viruses changes this view fundamentally. The lack of a significant fraction of viruses in aquatic ecosystems likely contributes to the apparent discrepancy between the diversity of viruses derived from genomes and those derived from metagenomes (Ignacio-Espinoza et al., 2013).

Overall, the metagenomic study of viruses is increasingly emerging as an important tool for understanding the evolution of viruses, changing the idea of what constitutes viruses, and revealing novel groups of viruses and virus-like agents (Kristensen et al., 2010). A considerable difference exists in the gene composition of marine DNA viromes compared to known phages. There is a large number of unidentified genes in the virome, many of which could be contained in VLPs called gene transfer agents (GTAs), which are DNA carriers that carry DNA from one host to another (Lang et al., 2012). There has been considerable evidence that marine metagenomes are composed of bacterial genes, but several sequences have been found to be homologous to conserved genes of large DNA viruses of eukaryotes, leading to the discovery of a wide range of previously understudied viruses as well as suggesting that new classes of virus-like agents might exist.

1.6 Phage Life Cycle

A better understanding of the life choices bacteriophages makes and how environmental factors affect them is necessary before determining the potential impacts phages may have on polar ecosystems. The phages can adopt one of three major lifestyle choices presented in **Figure 2**. Many factors might influence these choices, including the genetics and physiological state of the phage and the host. A productive, lysogenic, or pseudolysogenic lifestyle (Miller, 2006) is commonly used to describe them. The Arctic and Antarctic have not yet been studied for pseudolysogeny, but each of these choices will be discussed in detail in this section. Although the causes of these differences are not known, it is believed that the loss rates of virus particles in freshwater environments might be higher, which may result in a greater abundance of virus particles, while the high VBR in the deep

ocean may be attributed to the presence of viral accumulation zones (Hara et al., 1991; Parada et al., 2007; Proctor & Fuhrman, 1990).

1.6.1 Choosing a Life of Productivity

Bacteriophages produce progeny virions through their productive life cycle as implied by their name (Saye, 1989). There comes a time in the life cycle of every phage when it undergoes a productive cycle. Viral progenies are assembled from phage nucleic acid and capsid proteins after the host is infected. According to Miller and Day (2008), productive life cycles can be distinguished as either lytic or chronic.

1.6.1.1 Lytic productive cycle

After lysing the host cell, progeny phage particles are released into the environment during the end of lytic cycles. **Figure 2.A** illustrates the lytic productive cycles of double-stranded DNA bacteriophages that infect *E. coli* (Saye, 1989). There are several parameters that are dependent on the environment during the lytic period of each phage species. Virions are released into the environment during the latency period, which is the interim between infection and the release of the progeny virions by the infected cells. The period of phage growth is usually longer in oligotrophic environments than it would be in a laboratory setting, in which the host has access to nutrients to supply energy and macromolecular building blocks for phage growth (amino acids and nucleotides) (Kokjohn & Sayler, 1991). When nutrients are scarce, progeny phage burst size, which measures virions production on average, is smaller than when nutrients are abundant (Kokjohn & Sayler, 1991). Most phage-like particles are found in the environment resulting from this lifestyle.

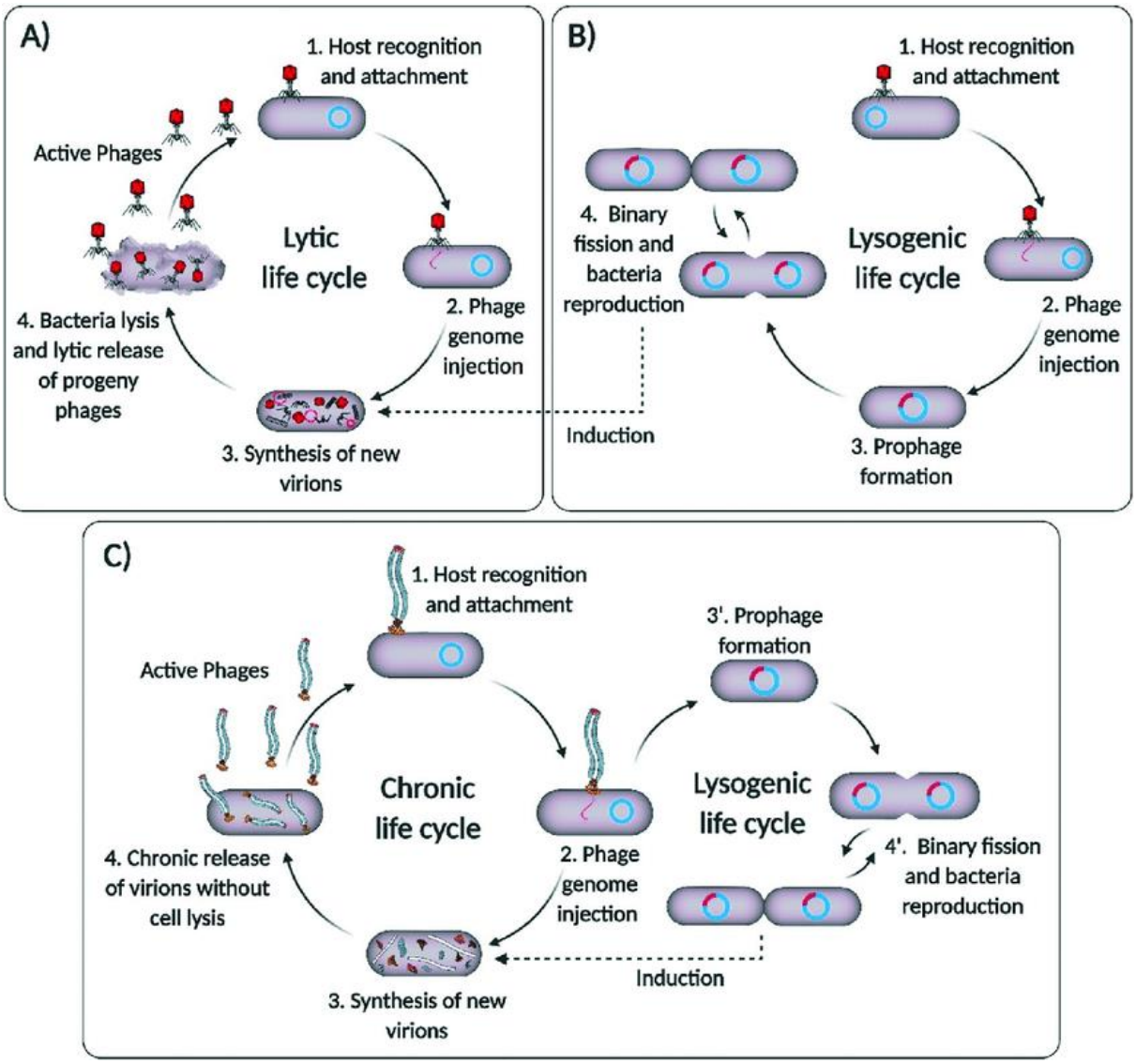

Figure 2: Several types of phage life cycles (A) The lytic cycle (B) Lysogenic cycle (C) The phage chronic lifestyle. The figure is adapted from Bayat et al. (2021)

1.6.1.2 Chronic life cycle

Alternatively, many filamentous phages are characterized by a chronic life cycle (**Figure 2.C**) that has a longer period of productive life in comparison to other phage types. Unlike the lytic productive cycle that ends with the death of the host cell, the chronic productive cycle continues long after infection. In fact, the infected cells continue to release virions through the cellular membrane of the host throughout this process (Miller & Day, 2008). The phage particles are slowly produced until the cellular membrane of the host is no longer feasible. Chronic infections are difficult to identify in nature and are little studied, despite being potentially important to natural microbial ecosystems.

1.6.2 Effect of Lytic Conversion

As previously reported, certain phage genomes often contain host-like genes that alter the phenotype of the host during the latent phase more frequently than was originally believed (Miller & Day, 2008). The presence of diphtheria toxin is a classic example of this phenomenon. This virulence factor is encapsulated within the genome of corynebacteriophage rather than the genome of the bacterium itself (Barksdale & Arden, 1974; Holmes & Barksdale, 1970; Hyman & Abedon, 2008; Miller & Day, 2008). In addition, cholera toxins (Hyman & Abedon, 2008) and *Shigella* toxins in *E. coli* O157:H7 (Canchaya et al., 2003) are among the other examples. The bacterium can not produce toxins in either of these cases, so it is not pathogenic unless it is infected or lysogenized by a bacteriophage that can code for the toxins (Miller & Day, 2008). Several pathogenic bacteria produce toxins, but some cyanophages also convert toxins into lytic proteins. There are several genes present (*psbA and psbD*) in these phage genomes that encode a polypeptide that forms a part of the core of the reaction center of their hosts' photosystem II (Angly et al., 2006; Bailey et al., 2004; Lindell et al., 2005; Mann et al., 2003). There is a rapid turnover of these proteins in the cell due to their labile nature. During the lytic growth of phages, it appears that the genes were expressed for maintaining photo-centers for the development of young virions (Bailey et al., 2004; Clokie et al., 2006; Lindell et al., 2005; Mann et al., 2003).

Several studies have demonstrated that lytic conversion in cyanophages facilitates lateral gene transfer mediated by transduction, which could lead to the evolution of these genes among cyanobacteria (Miller, 2004; Replicon et al., 1995; Saye, 1989; Saye et al., 1987). Lindell et al. (2004) suggested that lateral gene transfer among cyanophages may be an essential component of maintaining this organism's fitness in polar environments. According to Zeidner et al. (2005), uncultured environmental cyanophages and prophages of *Synechococcus* show evidence of transduction between these two genera (*Synechococcus* and *Prochlorococcus*). In addition to protecting cells against photobleaching, these gene products also play a vital role in facilitating photosynthetic processes. The phages have most likely contributed substantially to the enrichment of the bacterial gene pool by transferring these genes through the infection of their hosts (Replicon et al., 1995) and assisting in host evolution (Miller, 2004; Zeidner et al., 2005).

1.6.3 Lysogeny

Besides productive life cycles, many phages have the capability of initiating a reductive infection, where the phage genome is retained in a quiescent state inside the host's cells and no viral progeny particles are generated during this process. This reductive lifestyle can be classified into lysogeny or pseudolysogeny (Miller, 2004; Miller & Day, 2008). During lysogeny, the phage genome (also referred to as prophage) forms a prolonged relationship with the host genome by integrating itself within the host cell (**Figure 2.B**). Prophages can be embedded into the host's genome or maintained as plasmids in the cytoplasm, depending on the viral species. Subsequent activation of the prophage may cause it to enter a productive cycle, resulting in the production of progeny virions and the subsequent death of the host. There are several reasons why this activation could occur, including either genetic expression of the phage genome (Roux & Brum, 2018) or in response to the host's exposure to a variety of stressors in the environment. UV radiation, mitomycin C, or desiccation are a few of the common stressors which affect DNA integrity and can lead to DNA damage (Miller & Day, 2008). A repressor molecule encoded in the genomes of most prophages prevents their lytic functions from being expressed by repressing the release of their lytic products (Miller & Day, 2008; Ptashne, 2004). This repressor has both positive and negative autoregulation in the cytoplasm. In response to the stressors referred to above, the degradation of protein results in the induction of prophages that initiate lytic growth and contribute to the induction of phage degradation (Ptashne, 2004).

1.6.3.1 Effect of lysogenic conversion

A lysogenic cell, which contains an integrated prophage, may undergo both phenotypic and genotypic changes as a result of its presence (Miller & Day, 2008). Superinfection immunity is one of the most common forms of lysogenic conversion (Ptashne, 2004). A repressor of lytic expression is responsible for initiating this process by repressing the expression of lytic proteins. Upon lysogenization of a bacterium, it provides the bacterium immunity to infection by viruses belonging to similar and closely related species of the lysogen (Miller & Day, 2008). The lytic expression of lytic genes is repressed in the cytoplasm of lysogenized bacteria because of the presence of a soluble repressor. In the phage/prophage genome, these molecules interact with the operator which inhibits their expression (Ptashne, 2004). There is a balance between the concentration of bound repressor molecules as well as those which are free in the cytoplasm of the bacterium that maintains the

binding of repressor to the prophage promoter or operator. Superinfecting phage genomes enter the cytoplasm and are immediately bound to free repressor proteins which prevent them from expressing their lytic functions, thus rendering the host immune to the infection which is caused by these phage genomes.

The host's phenotype may be altered by the expression of additional genes in many phages during reductive growth. The components of the cell envelope can be altered as a result of these changes. Depending on the host and the changes that have been made, certain bacteriophages have been shown to be resistant to infection, while others are sensitized (Saye, 1989). In addition, new metabolic systems have also been acquired (Cavenagh & Miller, 1986; Ptashne, 2004). There is evidence that many cyanobacterium phages carry a portion of the photosynthetic complex, which is capable of being expressed during both lysogeny and productive growth (Lindell et al., 2005; Lindell et al., 2004; Mann et al., 2003). It is also possible to associate the acquisition of bacteriocin production with prophage presence in the host cell (Ivánovics et al., 1976), as well as the acquisition of new restriction-modification systems (Rocha et al., 2001) and pili production (Karaolis et al., 1999), along with the synthesis of antibiotics (Martinez-Molina & Olivares, 1979). A combination of lytic conversion and lysogenic conversion has been shown to contribute to the expression of phage-encoded toxins (Mahenthiralingam, 2004), such as diphtheria toxin (Barksdale and Ardon, 1974) and cholera toxin (Hyman & Abedon, 2008).

There is a strong correlation between lysogenic conversion and fitness increase. Frye et al. (2005) suggested that phenotypes are more likely to succeed in occupying niches when they have greater flexibility and mobility. Introducing alternative phenotypes can influence the composition of bacterial communities, resulting in the dominance of lysogens in some cases (Edlin et al., 1975). The utilization of new metabolites or the expression of new virulence factors may open new ecological niches for lysogeny (Lindell et al., 2005; Mahenthiralingam, 2004; Miller & Day, 2008). Hence, lysogeny can be described as the positive symbiotic relationship between bacterium and phage (Miller & Day, 2008). In turn, this allows the phage to be protected against the environment that could otherwise cause virion decay, while also providing the host with the opportunity to establish additional niches and increase its fitness levels. Natural selection favors lysogeny due to its benefits, so it is not surprising that it appears to be widespread. The proportion of environmental bacteria that

harbor prophages varies between 21 and 60%, according to Ackerman and DuBow (1987). The number may even be underestimated. A study conducted by Ortmann et al. (2002) in a fjord in British Columbia found that 80% of heterotrophic bacteria were lysogens while approximately 0.6% were found to contain bacteriophage genomes that could be induced by mitomycin C. A similar result was reported by Miller et al. (1992) in a study conducted in Tennessee (Fort Loudon Lake) where 80% of the *Pseudomonas aeruginosa* sequence that found to hybridize with *Pseudomonas* phage genomes. Most bacteria have prophages or vestiges of prophages in their genomes, as determined by the sequencing of their whole genomes. Multiple prophages are found in many of them. For example, at least 18 prophages are identified in the *E. coli* O157:H7 strain Sakai. Approximately 16% of the genome is taken up by this portion, which includes a gene for the Shigella toxin, which is a crucial factor in making O157:H7 play such an important role in food contamination (Canchaya et al., 2003; Miller & Day, 2008).

1.6.4 Pseudolysogeny

In 1971, Baess introduced the concept of pseudolysogeny. After reviewing the early literature on the topic, Baess defined pseudolysogeny as a phage-host interaction in which phages elicit an unproductive, unstable response from their hosts (Miller & Ripp, 1998; Miller & Day, 2008). During this stage of development, there is no evidence of lysogeny or productive growth. Instead, the viral genome resides in limbo for prolonged times as a pre-prophage within the host cell (Miller & Ripp, 2002). Miller and Day (2008) compare pre-prophagy to true lysogeny which has a linear inheritance pattern instead of an exponential one. A pseudolysogenic pre-prophage was proposed by (Miller & Ripp, 2002) in the context of severely starved hosts in which the phage was unable to acquire sufficient energy and substrates to allow the phage to function as a productive or lysogenic agent. Cellular starvation is described as a common phenomenon in environmental ecosystems (Koch, 1979; Morita, 1997). The polar regions, in particular Antarctica, exhibit this characteristic. The availability of nutrients varies with the time in every ecosystem (Miller & Day, 2008). A pre-prophage can be converted into a true prophage during these periods, or phage growth can begin in some cases (Miller & Ripp, 1998; Miller & Day, 2008; Miller & Ripp, 2002; Ripp & Miller, 1997, 1998). No reports of pseudolysogeny were found in the Arctic during the literature review of this thesis.

1.7 Phages in the Arctic

There have been decades of fascination among microbiologists and other scientists regarding the ecology and molecular biology of bacteriophages. Our understanding of how the cell works and the basis of molecular biology was founded on studies of *E. coli* and its phages T4 and λ. Phages have been the subject of many studies concerning their molecular biology and ecology (Abedon, 2008). However, the polar regions have received little attention. Despite some studies on Arctic and Antarctic phages that identified and characterized specific phages, most studies focused on identifying VLPs or only provided metagenomic data. Even so, these studies provide valuable information regarding the ecology of these viruses as well as their importance for the functioning of polar ecosystems. An analysis of the evolutionary relationships among bacteriophages around the world was conducted by Hendrix et al. (2002). Their results suggest that dsDNA-tailed phages share a common ancestry, indicating genetic exchange among bacteriophages throughout evolutionary time (Hendrix et al., 2002). They propose that these complex phage genomes are mosaics associated with a common genetic pool through lateral gene transfer. The adaptation to these unique environments may have led to the development of unique characteristics of phages from polar regions, as many of their characteristics are similar to those of their more temperate relatives. Exploring the molecular genetics and biology of these viruses will provide a fascinating insight into how the most fundamental of organisms have evolved to cope with the stresses of extreme cold and seasonal variations found in the Arctic environment.

There have been few studies conducted on bacteriophages and their hosts in the Canadian Arctic and North Atlantic seaboard. Wells and Deming (2006a) investigated the impact of viral predation on bacterial growth in the bottom waters of Franklin Bay in the Canadian Arctic. In subzero-temperature bottom waters during February and March 2004, they used epifluorescence microscopy to estimate the relative abundances of VLPs and bacteria. The concentrations of VLPs in seawater were generally one order of magnitude higher than bacterial densities ($1x10^6$ to $4x10^6$/ml versus $1x10^5$ to $3x10^5$/ml, respectively). Bacteriophage infection plays a significant role in regulating bacterial populations in these oligotrophic waters as it results in substantial viral-induced death of bacteria comparable to or exceeding intrinsic bacterial growth rates. There was a similar concentration of VLPs in these bottom waters to that observed in the summer months (Steward et al., 1996), but the

concentrations were higher than those observed in Arctic surface waters (Bergh et al., 1989). Steward et al. (1996) found that VLP concentrations ranged from 10^6 to 10^7 /ml in Bering and Chukchi seawater during the summer months (August and September 1992), while bacterial cell concentrations ranged from 5×10^5 /ml. Generally, there was around a five-to-one ratio between the VLPs and bacteria in most of the cases. In their study, the researchers found that the viral lysis may have contributed to 3 to 25% of the bacteria mortality (depending on the depth and station of the study). The size of the bacterial population was determined by the amount of bacteriophage lysis, which was equal to or even more important than the amount of flagellates grazing the bacteria. Because viruses are species-specific (Ackerman & DuBow, 1987; Miller, 1998), Steward et al. (1996) caution that viruses may destroy specific bacterial communities in these polar seas. A high diversity of species could be regulated by conditions like these, which could result in the succession or the extinction of species (Fuhrman, 1992). According to Payet and Suttle (2008), there are spatial and seasonal correlations between phage and bacterial counts in the Beaufort Sea and Amundsen Gulf of the Canadian Arctic, as well as biotic and abiotic variables. It was found that in the spring and summer months, the abundance of VLPs was 1.5 to 2 times greater than in the winter months, ranging from 10^5 to 10^6 /ml. In addition, there was a four- to six-fold increase in bacteria and phytoplankton during these periods. A total of 72% of the VLPs described in the study were identified as bacteriophages, while the remainder were identified as phytoplankton-infecting agents. Those results suggest that bacteriophage and algal phage populations in Arctic waters can be controlled by bacteriophages and algal phages.

During June 2001 and April 2002, Winter et al. (2004) carried out an interesting study in the North Sea where they looked at how viral infection and production cycled throughout the day during the period of the study. They reported that the rate of phage infection (55 to 64%) was highest at night, while several times more bacterial activity was recorded during the day by incorporation of leucine [14C]. The highest levels of lysis and phage production were found during the peak period of bacterial activity between noon and early afternoon. According to these data, induction of productive growth occurs when the host cells are at their most metabolically active level so that they are able to produce the maximum number of virions and induce productive growth.

There have been several papers that have investigated viral activity in the communities of microbes in polar ice. In an Arctic glacier, Säwström et al. (2002) investigated cryoconite holes. From these holes, they found that bacterial concentrations ranged from $1x10^4$ to $4.5x10^4$ /ml of water. Compared to the top of the hole, the level of bottom materials was slightly higher. The VBR was found to range from 0.2 to 8.1 in this study. The heterotrophic nanoflagellates in the ecosystem have been found to be directly dependent on VLPs as well as bacteria for energy. Arctic winter sea ice brines contain higher levels of viral and bacterial concentrations than seawater, thereby increasing the likelihood of host-pathogen interaction (Wells & Deming, 2006b). In the coldest ice horizons (–31 to –24°C), the highest levels of VLPs were obtained, with levels as high as 8×10^7 /ml of brine. There were bacteria counts of 1×10^7 CFU/ml of brine, but most commonly between $2x10^6$ to $4x10^6$ CFU/ml. These data demonstrate that despite temperatures as low as -12°C in sea-ice brines, viral production and bacterial growth can continue unabated. Researchers have examined a variety of freshwater ecosystems including glaciers and lakes in the Arctic and alpine environments (Hofer & Sommaruga, 2001; Maurice et al., 2010; Säwström et al., 2007). Several freshwater Arctic habitats were found to have 10^4-10^6 heterotrophic bacteria and 10^4-10^8 VLPs per ml of water (Säwström et al., 2007). Despite using mitomycin C to induce phage particle production as the criterion for determining the presence of lysogenic bacteria in these populations, the authors could not detect lysogenic bacteria.

Maurice et al. (2010) found that three northern temperate lakes contained lysogens in their bacterial populations. These lake waters contained 10-fold greater amounts of VLPs (10^7 /ml) than bacteria (10^6 /ml), as detected by seasonal variation in the populations. There was a decrease in numbers in the winter months as compared to the summer months. Twenty-three of the thirty samples analyzed showed that a considerable proportion of bacteria were lysogenized by temperate phages. The percentage of bacteria infected with a virus was approximately 2% in all three lakes. Lysogenized bacteria were found in 5 to 73% of these populations, with little seasonal variation. During the winter, the most eutrophic lake showed higher levels of lysogeny than during the spring. In winter under the ice, lysogen numbers were lower in two other lakes that were more oligotrophic. Spring marked a dramatic increase in their frequency. According to these findings, reductive infections are more likely to occur at high metabolic levels. Hofer and Sommaruga (2001) studied viruses in an alpine lake to determine their seasonal dynamics. In the winter, VLPs were most abundant beneath the ice

covering the lake (5×10^6 /ml). The VBR was typically between 2 and 6, with bacterial concentrations slightly lower than VLP concentrations. Most of the bacterial mortality was caused by virus-mediated lysis, accounting for 5 to 28% of the mortality rate. A decrease in VLPs was observed following the ice breakup. Upon removal of the ice cover, these microbes are more likely to encounter more lethal solar UV radiation, according to the authors. In this study, filamentous phages were found to be associated with filamentous bacteria. Freshwater aquatic systems rarely harbor these microorganisms. As part of their study, Middelboe et al. (2002) explored the northern waters between Canada and Greenland. During the experiment, bacteria were grown, and phages lysed in subzero water. Throughout the sampling stations, the bacteria count was approximately 10^6 CFU/ml, whereas the concentration of VLP was two- to five-fold higher. Approximately 6 to 28% of bacteria died because of viral infection. A *Pseudoalteromonas* species was also isolated and characterized along with its associated bacteriophage. In their sampling range, they observed phage-host interactions throughout and determined that phage production was substantial even at temperatures as low as 0°C.

Polar Sea ice collected northwest of Svalbard, Arctic, Norway, and was characterized by three phage-host systems (Borriss et al., 2003). The study was conducted on phages that infect *Flavobacterium hibernum*, *Shewanella frigidimarina*, and *Colwellia psychrerythraea.* Bacteria and phages have both evolved to survive in freezing conditions. There is no restriction for the hosts to grow at 0°C. While there was a maximum growth rate for the phages below 14°C and well-formed plaques were produced at 0°C. In electron microscopy, the phages were identified as belonging to the siphovirus and myovirus families of tailed, dsDNA phages. Each of them infected only the species from which it was isolated. Borriss et al. (2007) have isolated a bacteriophage from the Arctic Sea ice infecting a psychrophilic *Flavobacterium* species. It has a 36 kbp genome with a 30.6% GC content. There is no known phage genome with a lower GC content, which may be related to the virus's isolation from a very cold environment. According to the analysis of the sequences and proteins of this bacteriophage, it is a mesophilic nonmarine siphovirus closely related to the bacteriophage SPP1 that infects *B. subtilis* (Alonso et al., 1997). As viruses evolved, they exchanged genetic material with each other when they infected hosts with quite distinctive characteristics.

1.8 Molecular Studies of Marine Bacteriophages

Filée et al. (2005) evaluated the distribution of bacteriophages that are similar to T4-type globally. A DNA primer designed to amplify the T4 g23 gene was assessed on samples collected from British Columbia fjords, the Gulf of Mexico, and the Arctic Ocean. An analysis of the phylogenetic relationships between the T4-like bacteriophages in many environments, including the Arctic samples, led to the discovery of five previously uncharacterized subgroups of T4-like bacteriophages. The results of their study demonstrate the ubiquitous presence and diversity of T4-like viruses throughout the world as well as their ability to infect a significantly broader range of hosts than previously believed.

Short and Suttle (2005) and Chenard and Suttle (2008) examined the phylogenetic diversity of cyanophages. In addition to isolates from the Arctic Ocean, Gulf of Mexico, and northeast Pacific Oceans, Chenard and Suttle examined samples from Lake Constance, Germany, and a few freshwater lakes in northern Ontario, Canada. The presence of the psbA gene was examined in phages that infect *Synechococcus* and *Prochlorococcus* species. Despite cyanophage phylogenies differing between freshwater and marine cyanophages, the gene is widespread among cyanophages. Phages that cause infection of *Synechococcus* species have an evolutionary history different from those that cause infection of *Prochlorococcus* species.

A cyanophage structural gene g20 was examined by Short and Suttle (2005) to determine its diversity. This gene was found to be widespread across a wide range of samples taken from the Arctic, Southern Ocean, Northeastern and Southeast Pacific Oceans, a catfish production pond, lakes in Canada and Germany, and deep in the Chukchi Sea. All these sites were found to contain identical sequences of this gene. Closely related hosts and viruses occur worldwide, and wide-ranging habitats have likely allowed lateral gene transfer between phage communities. An analysis of metagenomes of phages from four ocean regions revealed there are many differences between the viral sequences currently in databases and those found in the Arctic Ocean, the Pacific off British Columbia, the Gulf of Mexico, and the Sargasso Sea (Angly et al., 2006). Many species were found worldwide, representing an exceedingly high diversity. In the different regions, there was a predominance of different phage assemblages. The Sargasso Sea is home to many single-stranded DNA phages, which are classified as cyanophages, and a previously unknown group of prophage-like sequences that suggest a high

degree of lysogeny in the Arctic. All the sampling regions were observed to have the most viral species present. They differed depending on the type of virus that predominated in the area. The presence of any specific group of phages was not excluded from any of the samples (Angly et al., 2006).

Wichels et al. (1998) studied the diversity of bacteriophages in the North Sea and found elevated levels of VLPs (10^4 to 10^7 /ml) in the water. They collected eighty-five different viruses in the vicinity of Helgoland, Germany, and investigated 22 different phage-host systems. As a result of morphological studies, 11 of the phages were classified as myovirus and 7 as siphovirus; four of the phages were podoviruses. In a DNA-DNA hybridization, 13 species of phages were identified with no sequences shared among them. The infected cocci belong to the genus *Pseudoalteromonas* and are gram-negative, facultatively anaerobic, and motile.

1.9 Phages from Sewage

A process called activated sludge involves converting solids and pollutants into a biological form, and it is an established method for the treatment of sewage and industrial wastewater globally (Jenkins et al., 2003; Martins et al., 2004). A mixture of micro and macro-organisms, containing viruses, bacteria, protozoa, and metazoa, forms activated sludge, which converts organic materials into a liquid mixture low in suspended solids and organic compounds (Jenkins et al., 2003; Martins et al., 2004; Wagner et al., 2002). Wastewater treatment is one of the most significant biotechnological processes currently available. Water treated this way does not pose an ecological threat to rivers and lakes because organic and inorganic compounds are reduced by a selected bacterial community (Ju & Zhang, 2015). Flocs, which are aggregates of biomass, are distinctively organized. In summary, this process initiates with the introduction of influent into aeration basins where aeration is used to increase the oxygen supply to the wastewater, which promotes the growth of microbial communities and the formation of aggregates. Afterward, the biomass flocs settle rapidly in secondary clarifiers, where the liquid phase is removed, along with the increased biomass flocs. The primary aspect of the procedure is the recycling of much of the settled biomass back to the plant's head. Aside from being fast and occupying a small footprint, activated sludge has the advantage that it contains the most suitable populations for managing the

incoming wastewater. The process is primarily controlled by maintaining sludge age, in which some of the biomass is discarded to maintain a predetermined biomass concentration.

A considerable variation in VLPs values in wastewater entering WWTPs is likely due to the variability between sites (Gulino et al., 2020) or within sites (Brown et al., 2019) and the less efficient methods used in VLP detection (Wu & Liu, 2009). It is predicted in the literature that activated sludge contains 10^8 to 10^9 VLPs per ml of mixed liquor, representing a highly diverse and abundant population of phages (Otawa et al., 2007). The values reported for faeces (Hoyles et al., 2014) are similar to those reported for sewage, considering that each defecates 150 to 200 grams of waste, contributing an average of 200 liters of water to society's sewage systems. A metagenomic investigation that compared it to the soil, plant-associated, and other artificial systems revealed 36% more viral DNA there, supporting the high abundance of phages, which primarily consist of siphovirus family members (Wang et al., 2018). There had been relatively little research on phage communities prior to 2011, despite an understanding of activated sludge and viruses involved (Shapiro & Kushmaro, 2011). This status has improved with the introduction of metagenomics investigations and next-generation DNA sequencing (NGS) of isolated phages (Brown et al., 2019; Dyson et al., 2016; Dyson et al., 2015; Liu et al., 2015; Petrovski et al., 2012; Taylor et al., 2019). Several bacterial genomes present in activated sludge systems are now known to contain regions of the CRISPR-Cas system, indicating that they have previously been infected by phages, as the presence of these regions is suggestive of previous infections. Thus, the presence of these phages complicates the determination of host/phage relationships, and consequently phage recovery (Liu et al., 2017).

A variety of gut bacteria strains have been reported to be infected by bacterial phages found in raw sewage such as *E. coli* (Grami et al., 2023; Jebri et al., 2017), *Shigella* (Montso et al., 2019; Muniesa et al., 2004), *Enterobacter* (Wangkahad et al., 2015), *Klebsiella* (Muniesa et al., 2003; Wangkahad et al., 2015), *Salmonella* (Carey-Smith et al., 2006), *Pseudomonas* (Essoh et al., 2015), *Staphylococcus* (Synnott et al., 2009), *Enterococcus* (Bonilla et al., 2010; Vijayavel et al., 2014), *Aeromonas* (Wangkahad et al., 2015), and *Bacteroides* (Jebri et al., 2017). However, the number of studies that quantify infectious phages is relatively low, except for those that infect *E. coli* usually recognized as coliphages.

A second group of cultivable phages are those that infect the most abundant phylum of bacteria in our guts: the Bacteroidetes.

Electron microscopy has been used to study non-cultivable phages in sewage, and the results of these studies highlighted a huge diversity of these microbes. There are a variety of morphological forms that can be seen in electron microscopy images, with myovirus, podovirus, siphovirus, and microvirus being the most common (Brown et al., 2019; Wu & Liu, 2009). The metagenomics studies have found that raw sewage samples have a very variable metagenome composition, with a high proportion of sequences that have not yet been characterized and a predominance of DNA phages (Cantalupo et al., 2011; Gulino et al., 2020; Tamaki et al., 2012). By combining existing metagenomic data regarding sewage viromes and performing further analyses, sewage virome profiles will be able to be analyzed more comprehensively. Although most of the phages in raw sewage are derived from the gut of humans and therefore could probably be found in the peripheral microbiota, it is possible to assume that most of the phage populations in raw sewage can be found in the gut of humans (García‐Aljaro et al., 2019). As part of a study conducted in New York, crAssphage, the most abundant phage found in human feces, was found in the wastewater of all 14 WWTPs tested (Gulino et al., 2020). The authors also used real-time qPCR as a method for detecting phages in municipal wastewater in 2021, reporting 10^6 and 10^7 copies per milliliter, which is 10,000 times higher than the maximum number of phages discovered too far by the culture of Bacteroides host strains (Payan et al., 2005). Further, in another study that examined WWTPs in New York, only a low percentage of samples were found to be infected by phages that were capable of infecting bacteria whose function is to cycle carbon and sulfur, and these bacteria could grow in biofilms formed in the sewer systems (Gulino et al., 2020). In a study conducted by Aghaee et al. (2021) reported the most reliable sources of *Pseudomonas* phage isolation are sewage and sewage-contaminated ambience. Furthermore, the phages found in hospital sewage lysed the bacteria most efficiently for the determination of the host range.

Phages might provide a specific method for interfering with the growth of problematic bacteria in activated sludge if it were clarified what impact they might have on them (Thomas et al., 2002). Several bacterial species may be capable of reproducing rapidly, which leads to the serious problems associated with bulking and foaming, caused primarily by filamentous bacteria, which is difficult to treat because of the limited treatment options (Petrovski et al.,

2011; Tandoi et al., 2017; Wanner, 2014). The primary intention is to reduce the population levels of these organisms below the threshold necessary to sustain bulking or foaming. Phage therapy has demonstrated great interest in controlling bouts of bulking (Jassim et al., 2016). Several lytic phages have been isolated, and their genomes sequenced for most of these bacteria responsible for foaming. However, much remains to be learned before these phages can be used on an industrial scale to control foaming despite being highly effective in controlling foaming under laboratory conditions. For instance, Liu et al. (2015) isolated four *Gordonia* phages from a WWTP and showed 10 times more effective in controlling *Gordonia* host than in non-phage treatment.

This brief overview highlights the abundance and diversity of phages in raw sewage and wastewater treatment plants. Although there is still a lot to be learned, scientists are seeking answers to many questions, including whether this density of phages has any effect on human health and the potential for application.

1.10 Scope and Significance of the Study

The Arctic environment is quite different from others. Organisms inhabiting Arctic environments are consistently exposed to severe conditions, such as frigid temperatures, elevated salinity levels, and prolonged periods of either constant illumination or darkness. Because of this extreme condition, life in the Arctic evolved different specialized strategies and molecules to survive which offer huge opportunities to prospect for novel bioactive compounds and potential industrial applications or products for different purposes. Despite the importance of phages in Arctic ecosystems, little is known about their diversity, abundance, and function. Besides, several studies focus on molecular methods only and not on virus isolation. In particular, there is a lack of information on the genetic and morphological diversity of phages in the Arctic, as well as their interactions with Arctic bacterial communities. This knowledge gap is partly due to the difficulty of isolating and characterizing phages from Arctic environments, which are characterized by low bacterial biomass and high viral diversity. This rich biodiversity and extreme environment are the catalyst to work in this project and I believe the Arctic would be the next frontier in viral-based bioprospection.

There is still a lot to be learned from Arctic viruses, especially phages. In this study, I aim to use a collection of Arctic bacteria as hosts for finding Arctic phages. The bacteria used are part of the UiT Marine Bioprospecting group collection, whereas the samples used for virus isolation were collected during a teaching cruise (BIO 3612-2022 course).

This study will contribute to our understanding of the diversity and function of phages in Arctic ecosystems. The findings will provide valuable insights into the role of phages in regulating the diversity and abundance of Arctic bacterial communities, which is important for understanding the biogeochemical cycles and carbon fluxes in the Arctic. The genetic and morphological characterization of Arctic phages will also provide a basis for future studies on their potential biotechnological applications, such as phage therapy and biocontrol. This is the first project aimed at systematically isolating marine phages in Tromsø. Additionally, this study will provide a methodological framework for isolating and characterizing phages from low-biomass environments, which may apply to other extreme environments beyond the Arctic.

2 AIM

The major objective of the present study is to isolate and characterize phages from the Arctic Sea and sewage samples. To fulfill this goal, the following aims are also defined.

- Evaluate the survival of the Arctic marine bacteria during the preparation of double-agar plates.
- Isolate bacteriophages from the Arctic.
- Grow and prepare stocks of the phages found.
- Image the phages found by electron microscopy.
- Phage genome isolation and sequencing analysis for further characterization.

3 BACKGROUND AND PRINCIPLES OF METHODS

Phage isolation and characterization are essential methods for studying the biology and ecology of bacteriophages in various environments, including the Arctic Sea and sewage. The following **Figure 3** is an overview of the methods and principles used for phage isolation and characterization from these environments.

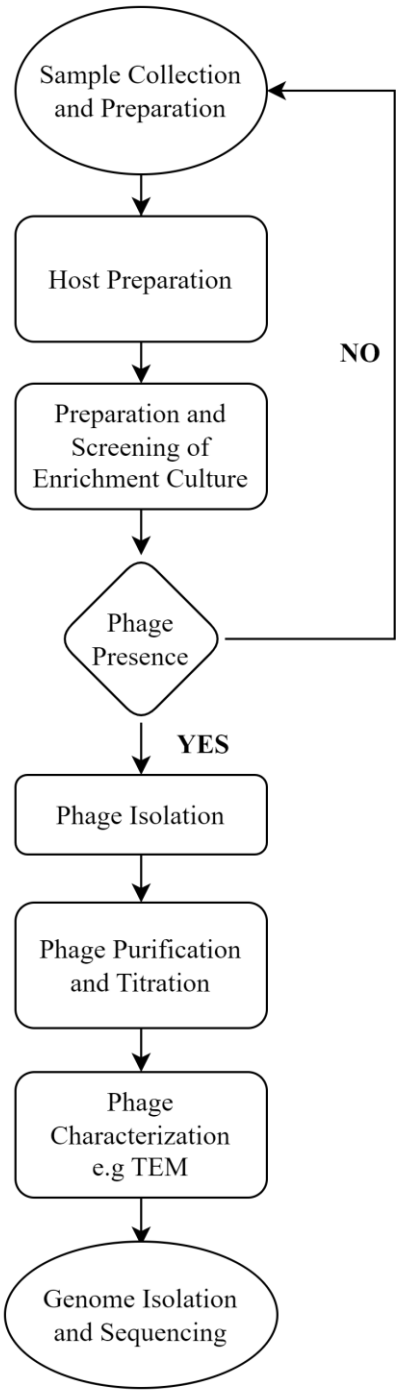

Figure 3: The flowchart provides an overview of the methodology for phage isolation and characterization.

3.1 Phage Isolation

The first step in phage isolation involves the collection of environmental samples, such as seawater or sewage. These samples are then filtered in 0.22 or 0.45 µm filters to remove bacteria and other large particles present in the sample. The resulting filtrate, which contains the phages, is mixed with a suitable host bacterium in the so-called enrichment culture. The mixture is then incubated, and the resulting plaques (clear zones) on the bacterial lawn indicate the presence of phages.

Once plaques are observed, the next step is to isolate individual phage particles from the mixture. This is typically done by picking a single plaque with a sterile pipette tip and eluting the phages from the plaque using a buffer solution. This eluate is then purified and concentrated using techniques such as ultracentrifugation or polyethylene glycol (PEG) precipitation if a concentrated stock is needed. For conventional phage work, the filtered lysate is enough to be used as a working solution.

3.2 Phage Characterization

Characterizing phages involves determining their morphology, genome size, host range, and other biological properties. The following are some common techniques used for phage characterization; Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) can be used to visualize the morphology of phages. TEM can reveal details such as the shape, size, and structure of the phage particle.

3.3 Genomic DNA Isolation and Sequencing

The genomes and DNA genes of phages can be sequenced using next-generation sequencing technologies, which can reveal information about the phage's genetic makeup, including its gene content, organization, and phylogeny.

The principles of phage isolation are based on the fact that phages are highly specific to their host bacteria. By enriching a particular host bacterium, researchers can isolate phages that specifically target that bacterium. Additionally, the formation of plaques on a solid growth medium allows for the isolation of individual phages, which can then be further characterized and studied.

Overall, phage isolation and characterization methods involve a combination of microbiology, virology, and molecular biology techniques. These methods are crucial for studying the diversity, ecology, and potential applications of phages in various environments.

4 MATERIALS AND METHODS

4.1 Sample Collection and Preparation

For phage isolation, environmental samples were collected in 50 ml sterile falcon tubes during the BIO-3612 Cruise on September 08, 2022, from Stakkevollveien 1, Tromsø, Norway. Samples were collected approximately 800 m away from the seashore by the boat RV Hyas; Depth: 19-24 m (**Figure 4**). Samples were stored in an ice box before and during transfer and then stored at 4°C at the lab. The samples were filtered using 50 ml syringe filters (Henke-Jet, Germany), and transferred from the original tube to a sterile one. The samples were then stored in a cold room at 4°C until further use. Details of the samples are shown in **Table 1**. Sewage samples used were part of the Marine Bioprospecting research group sample collection, previously collected from the Breivika wastewater treatment facility and prepared as described above for the marine samples.

Figure 4: Sign indicating the sampling site for samples 53-58

Table 1: Source of collected samples for phage isolation.

Sample No	Date	Source
41	20.04.2022	Inlet pipes (crude sewage)
42	20.04.2022	Inlet pipes (crude sewage)
43	20.04.2022	Inlet pipes (crude sewage)
44	20.04.2022	Filtered sewage
45	20.04.2022	Filtered sewage
53	08.09.2022	Sea surface water
54	08.09.2022	Sea surface water
55	08.09.2022	Sea water from collected sediments (bucket 1)
56	08.09.2022	Sea water from collected sediments (bucket 2)
57	08.09.2022	Sea water from collected animal tray (long incubation)
58	08.09.2022	Sea water from collected sediments (bucket 2)

4.2 Bacterial Strains and Culture Preparations (Host)

Six pure bacterial strains were provided by the Marine Bioprospecting research group, at the UiT The Arctic University of Norway. The marine bacterial strains except *E. coli* and *Vibrio* were isolated from a Bioprospecting Cruise in 2019 in the Arctic Ocean Norway and characterized as part of the Ph.D. candidate Ataur Rahman's work. Additionally, the ampicillin-resistant *E. coli* K12 strain was used to isolate phages from sewage samples. The bacterial strains were grown according to Moebus and Nattkemper (1983). From stock, the marine bacterial strains were cultured on FMAP agar plates (appendix 4) while *E. coli* on LB (Lysogeny Broth) agar (Millipore, Spain) containing 20 ml media and incubated at 4°C and 37°C, respectively to grow. From the marine bacterial culture plate, a single colony was picked and transferred to liquid FMAP media (appendix 3) to grow under agitation in the cold room at 4°C. The bacterial species used are presented in the following Table 2 along with their source and characteristic features.

Table 2:Host bacteria and their characteristics features for the isolation of phages.

ID code	Isolation Source	Genus/Species	Cell Wall	Characteristics of the Genus
MBP 016.7	Sediment (158 m)	***Flavobacterium***	G (-)	Cold water disease on salmonids and Rainbow Fry disease on rainbow trouts
KC1RTL 01	Infected Sea Urchin	***Vibrio***	G (-)	Seafood spoilage, foodborne infection
SUK GRAA1 BAC GENOME	Sea Urchin	***Vibrio***	G (-)	Seafood spoilage, foodborne infection
MBP011.13.1	Sediment (158 m)	***Shewanella***	G (-)	Spoilage of Seafood, foodborne infection
MBP019.6	Zooplankton	***Maribacter***	G (-)	Cold water disease on salmonids and Rainbow Fry disease on rainbow trouts
E.coli K12	Laboratory strain used for cloning	***E.coli***	G (-)	Food poisoning, urinary tract infections

4.3 Bacterial Behaviour Test in Double-Layer Agar Plates

A double-layer agar technique described by Adams (1959), was used to check whether the host bacteria would survive during the heat shock of double-layer agar plate preparation (**Figure 5**) and bacterial lawn formation. This test was made considering that the Arctic marine bacteria thrive in cold environment. The soft agar (0.7% agar, appendix 5) bottle was heated, and 3 ml of media was transferred to a glass tube. The tubes were kept inside a water bath at 55°C. Right before making the plates one tube from the water bath was removed, waited for it to cool down, and 300 µl of fresh target bacterial cultures were added. The mixture was briefly vortexed and added to the plate. The plates were incubated at 4°C until the bacterial lawn formed.

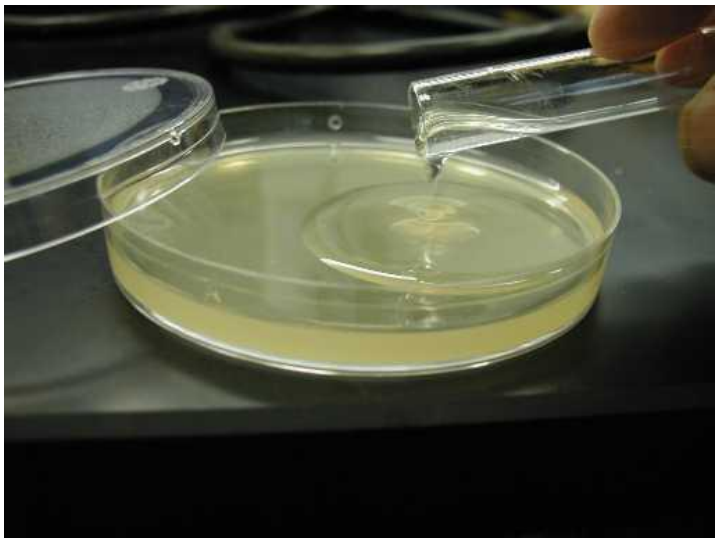

Figure 5: Double agar-layer (DAL) preparation. The picture shows the addition of the top-agar (0.7%) containing the host bacteria to an agar plate.

4.4 Phage Isolation

To obtain phages for isolation, various samples were examined for the presence of phages. These samples included surface seawater, sediment seawater, seawater that had undergone prolonged incubation with various marine organisms, as well as wastewater collected from both the Arctic Sea and a wastewater treatment plant. Phages were isolated by following the enrichment method (Sambrook et al., 1989; Spencer, 1955) with some modifications. Phage isolation is made by mixing a sample that could contain phages with a growing population of host cells (enrichment cultures). Over time, phages may establish specific interactions with their host cells, leading to an increase in the phage population, and they can be detected through screening of the enrichment cultures.

4.4.1 Preparation of Enrichment Culture

For isolating phages, an enrichment culture was prepared for each sample according to the following **Table 3**. Enrichment cultures were incubated under gentle agitation at cold temperature and sampled after 2-5 days to detect marine phage presence. While enrichment culture for coliphages were incubated in a shaking incubator at 37°C and sampled after 24 h.

Table 3: Components used for enrichment culture preparation.

Components	Volume
Sample	3.5 ml
5x liquid media	1 ml
Overnight bacterial culture	0.5 ml
Final volume	5 ml

4.4.2 *Screening the Enrichment Cultures for Phages*

A small fraction of each enrichment culture was transferred to clean Eppendorf tubes, and double-agar plates were prepared using specific bacteria. Plates were divided into a 3x3 grid. The Eppendorf tubes were briefly centrifuged at 5000 × g to pellet the cells. On the other hand, 10% chloroform was added to the samples instead of centrifuging. These two strategies were chosen for distinct reasons: chloroform treatment is efficient in killing cells and thus avoids contamination from phage-resistant bacteria. Pelleting the cells allows the crude separation of cells and the phage-containing supernatant, but sometimes it is not enough to avoid contamination. Regardless of the treatment, 10 µl of each sample was dropped in the designated space in the plates and incubated at 4℃ for marine phages and 37℃ for coliphages until phage plaques appeared.

4.4.3 *Plaque Purification*

The soft agar overlay method (Adams, 1959; Sambrook et al., 1989) was utilized to purify bacteriophages from individual plaque isolates. After detecting phage, it is useful to make several rounds of plaque purification to adapt the phage to the host strain and avoids problems of having mixed phages in the same stock. Plaque purification was done in three passages. Starting from the right sample a 10-fold serial dilution was performed (0 to -3 for marine) and (0 to -9 for coliphages). For passage 1 the plaque from the enrichment sampling was used as a starting material. In each dilution, 200 µl of the right bacteria was added and double agar plates (one plate for each dilution) were prepared. The plates were incubated until plaques appeared. The size, edge, and boundaries of the plaque's morphology were observed and categorized as either small (less than or equal to 1 mm), medium (1-2 mm), or large (greater than 2 mm) and classified as either clear or diffused type plaques. Using a sterile pipette tip, a clear plaque was picked up and resuspended in 100 µl of liquid media in

Eppendorf tubes. The tubes were then briefly centrifuged by using a bench centrifuge and incubated for 10 min at 4°C for marine phages and 37°C for coliphages to adapt the phages. The above process was repeated three times for each phage isolate until a uniform appearance of plaques was achieved.

4.4.4 Preparation of Phage Stocks

This step is used to prepare crude phage stocks that can be stored at -80°C (long-term preservation) and 4°C (working solutions). Highly purified phages can be obtained by ultracentrifugation, but often it is not needed for building a collection and performing phage characterization experiments. The method for preparing phage stocks was conducted using the soft agar overlay method, which had been described previously.

The isolated phages are named FL53-58_SH22, VHU53-58_SH22, VIU53-58_SH22, MR53-58_SH22, and Ec41-45_SH23 for *Flavobacterium*, *Vibrio*, *Maribacter*, and *E.coli* respectively. The beginning two characters represent the starting letters of the bacteria hosting the phages, after which comes the sample number. While "SH" follows, it stands for my name initials and is followed by the year of phage isolation. However, "HU" and "IU" denotes the source of *Vibrio* that was isolated from a healthy or infected sea urchin, respectively. After the isolation of the single phage strain, the plate with high confluency (plaques) was chosen. The top agar layer was harvested using a sterile glass spreader and transferred to a 50 ml falcon tube followed by the addition of 4 ml of culture media and gently vortexed the mixture. The tubes were centrifuged at 4500 × g for 10 minutes at 4°C. Without disturbing the pellet, the supernatant was collected and filtered it using sterile syringe filters (VWR, 0.45 µm PES). After filtration, aliquots were frozen at the -80°C freezer (2x200 µl aliquots in cryotubes, no glycerol added) as stocks. The rest of the sterile lysate was kept in the fridge and used as a working solution.

4.4.5 Phage Titration

The phage solution was 10-fold diluted from 0 to -9 to determine the phage titer. Double agar plates were prepared using the appropriate host. The dilutions were plated in 10 µl drops. The plates were incubated at 4°C for marine phages and 37°C for coliphages until plaques appear and counted the plaques in the appropriate dilution. Phage titer was calculated in the form of plaque-forming units (PFU/ml) by the following equation.

Pfu/ml = pfu counted/dilution × volume of phage dilution plate (Nasser et al., 2019)

4.5 Phage Genomic DNA Isolation

The present study employed a commercially available Norgen Biotek phage DNA extraction kit (Thorold, ON, Canada) to extract phage DNA, adhering to the manufacturer's recommended protocol. The clarified phage supernatant obtained during the preparation of phage stock was utilized as the starting material for this process. In a 15 ml tube, 1 ml of the phage lysate was transferred. To avoid host genomic DNA contamination in the phage DNA elution, 10 µl RNase-free DNase I (Thermo Fisher Scientific Baltics UAB, Lithuania) was added followed by DNase I inactivation at 75°C for 5 minutes. Also, 10 µl of Protease-free RNase A (Thermo Fisher Scientific Baltics UAB, Lithuania) was added. After adding 500 µl of Lysis Buffer B, the mixture was vortexed vigorously for 10 seconds. To increase the DNA yields, 4 µl of Proteinase K was added and incubated at 55°C for 30 minutes. The lysate was mixed occasionally 3 times during incubation by inverting the tube. After incubation, 320 µl of isopropanol was added and vortexed. A spin column was assembled with one of the provided collection tubes followed by applying up to 650 µl of the lysate to the column and centrifuged for 1 minute at 6000 x g. The flowthrough was discarded and reassembled the column and the collection tube. This process was repeated until the entire lysate had passed through the column. To wash the column, 400 µl of Wash Solution A mixed with absolute ethanol was applied to the column and centrifuged for 1 minute at 6000 x g and discarded the flowthrough and reassembled the spin column with its collection tube. The washing process described above was repeated twice and finally, the column was spined for 2 minutes to thoroughly dry the resin at 14000 x g. The column was then placed into a fresh 1.7 ml elution tube and 75 µl of Elution Buffer B was added to the column followed by centrifugation for 1 minute at 6000 x g. The purified DNA samples were stored at -80 °C for long-term storage.

4.6 Phage DNA Quantification and Restriction Digestion

Phage DNA was quantified according to the Qubit™ broad range (BR) kit (Invitrogen, USA) manufacturer instructions. The experiment involved setting up a specific number of Qubit™ tubes to analyze standards and samples. The Qubit™ working solution was prepared by diluting the Qubit™ dsDNA BR Reagent 1:200 in Qubit™ dsDNA BR Buffer. For the standards, 190 µl of Qubit™ working solution was added to each tube, followed by 10 µl of each Qubit™ standard, and mixed by vortexing for 2-3 seconds. Similarly, for each sample

assay tube, 198 µl of Qubit™ working solution was added, followed by 2 µl of the sample, and mixed by vortexing for 3-5 seconds. All tubes were then incubated at room temperature for 2 minutes. The samples and standards were measured using the Qubit™ 4 Fluorometer (Thermo Scientific, USA).

For restriction analysis, approximately 100 ng of each phage genome was treated with fast digest BamHI restriction endonuclease (Thermo Scientific, USA), and the resulting DNA fragments were separated using a 0.7% agarose gel stained with GelRed (Bio-Red, USA). The reaction mixture (**Table 4**) was pipetted on ice and incubated at 37°C for 5-30 minutes. Subsequently, the samples were loaded onto a 0.7% agarose gel and run side-by-side. The marker used was 1 Kb Plus DNA Ladder (Invitrogen, USA). The profiles of the separated DNA fragments were visualized after exposure to UV light, and a picture was taken.

Table 4: Chemical components and the volumes used in phage genome digestion.

Components	Ec41_SH23	Ec42_SH23	Ec43_SH23	Ec44_SH23	Ec45_SH23
DNA	20	17	17	15	20
Enzyme	2	2	2	2	2
10x green buffer	3	3	3	3	3
Water	5	8	8	10	5
The final volume (µl)	30	30	30	30	30

4.7 Transmission Electron Microscopy

Transmission electron microscopy was conducted on three phage isolates (Ec41_SH23, Ec44_SH23, and FL54_SH22), and phage morphotypes were determined using negative staining as described previously (Brenner & Horne, 1959). TEM analysis was conducted on fresh phage lysates. A clear spot or plaque less than 24 hours old was repeatedly washed using LB medium and phage lysate was collected carefully without disturbing the soft agar. The sample is briefly vortexed and brought to the TEM lab. To prepare the image, a clean surface covered with parafilm was used, onto which 5 µl of the specimen was deposited. To prepare for electron microscopy, a 400-mesh copper acid grid was coated with carbon and placed on drops for 5 minutes. Afterward, the grid was washed four times with distilled water drops. Staining was then performed with 1% uranyl acetate (Sigma-Aldrich, Darmstadt,

Germany) for 20 seconds, followed by blotting to remove excess uranyl acetate and allowing the grid to dry for a few minutes. The resulting sample was observed using a Zeiss transmission electron microscope (Carl Zeiss LEO EM 906 E, Germany) with an accelerating voltage of 100 kV in the microscope lab at the Faculty of Health Science, UiT The Arctic University of Norway. The phage morphology was noted and compared to that of known phages.

5 RESULTS

The present study employed municipal sewage water as well as various saltwater samples collected in the vicinity of Tromsø City as the primary sources for the identification, isolation, and study of bacteriophages.

5.1 Cultures of the Arctic Marine Host Bacteria

The marine hosts cultured on FMAP plates after being successfully retrieved from the glycerol stock in the freezer (-80°C) are shown in **Figure 6**. Despite being cold-adapted bacteria, the heat shock during DAL preparation did not impair the formation of a bacterial lawn (important for phage screening) in any of the hosts used. However, it took 2-3 days for *Flavobacterium, Vibrio,* and *Shewanella* to form a confluent bacterial lawn whereas *Maribacter* took 5 days to appear. On the other hand, *E. coli* lawns fully developed within 24 hours.

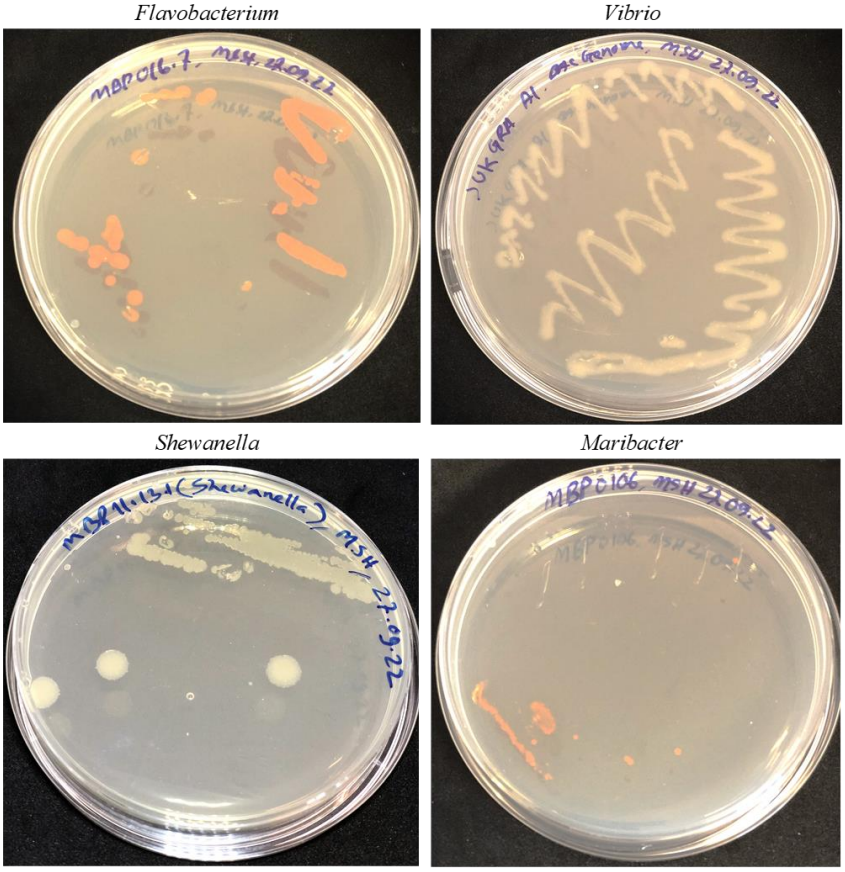

Figure 6: Marine bacterial cultures on FMAP media plates.

5.2 Isolated Phages and Screened Enriched Cultures

Following the preparation of the enrichment cultures, an initial screening was done to confirm the presence of bacteriophages in the samples at set time points. A clear zone over the bacterial lawn is supposed to be observed due to the lytic activity of the phages when a phage is present. Initially, we assessed two alternatives for treating the collected samples from the enrichment cultures: centrifugation and chloroform treatment. Both have the same objective: avoid the appearance of bacterial colonies in the area in which drops are added to the double-agar plates. Phage lysis was easier to see in samples treated with chloroform. When non-chloroform centrifuged samples were tested, often we saw growth of bacteria inside the drop zones, making it impossible to verify phage presence. Therefore, chloroform treatment was chosen for the rest of the plaque isolation process to remove the bacterial growth in the drops.

Screening of the enrichment cultures confirmed the presence of phages (**Figure 7**) in all samples of the following hosts: *Flavobacterium*, *Vibrio* (from healthy sea urchin), *Maribacter*, and *E. coli*. Also, phage presence was detected in only one sample in the *Vibrio* isolated from the infected sea urchin. On the other hand, there was no clear zone of inhibition observed for the *Shewanella* host, hence no presence of phage against it in the tested samples. Appendix 7 shows the consolidated data from marine sample analysis.

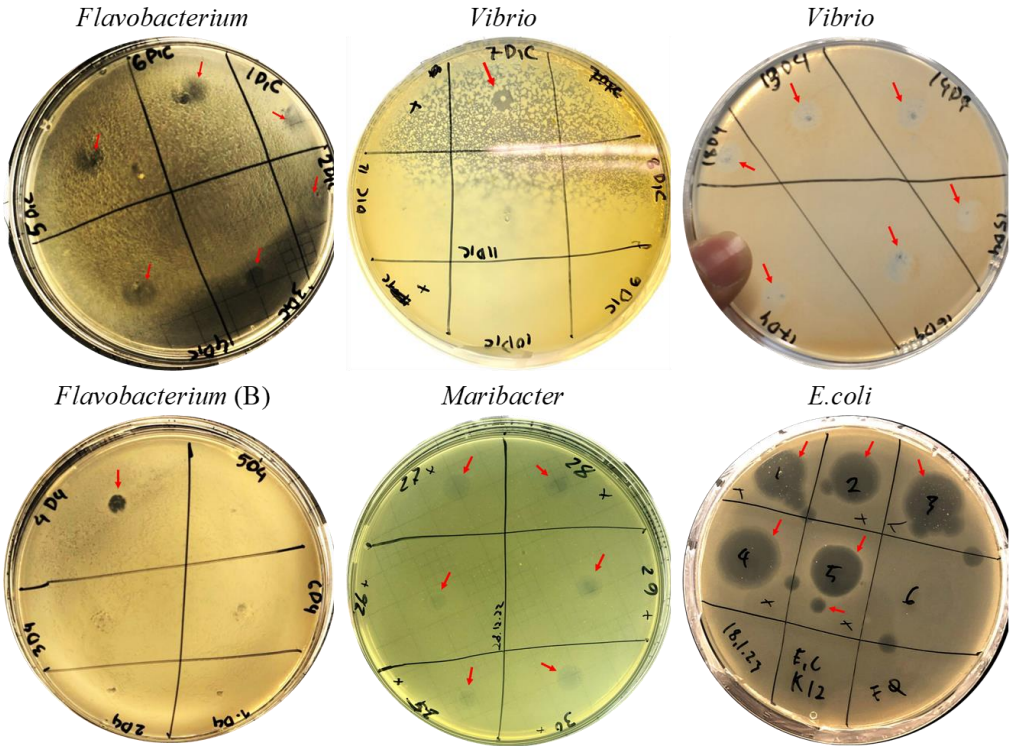

Figure 7: Presence of phages from the screening of enrichment culture. The arrow indicates bacterial lysis due to phages. Flavobacterium (B) represents centrifuged treated samples while others are chloroform treated.

5.3 Phage Growth and Plaque Purification

For subsequent characterization, phage isolation was performed by repeated plaque purification. To collect the pure phages, plaque purification was done for all the positive samples individually. Samples used in this step were collected from the lysis zone that appeared during the screening of the enrichment culture. During the purification of the marine phages, no plaques appeared when we tried to grow them on a whole plate. However, plaque formation was seen when placed as drops in the titration step which were later collected and stored for further analysis.

However, plaques appeared all over the plates in all the purification stages (Passage 1, passage 2, and Passage 3) of the coliphages. To isolate a single phage strain one of the plates carrying the best number of plaques was chosen and from it, one single plaque was collected. This material was diluted in a 10-fold dilution series, used for plating and this process was repeated three times. The following phages Ec41_SH23, Ec43_SH23, and Ec45_SH23 showed uniform plaques of morphology (**Figure 8, Figure 9**, and **Figure 10**); round-shaped clear regions ranging from 1-4 mm in size throughout the purification steps. In contrast, 3

distinct types of plaque appeared in the first purification stage for Ec42_SH23 (**Figure 11**). However, in the later stage, they appeared as homogenized plaques (**Figure 12**).

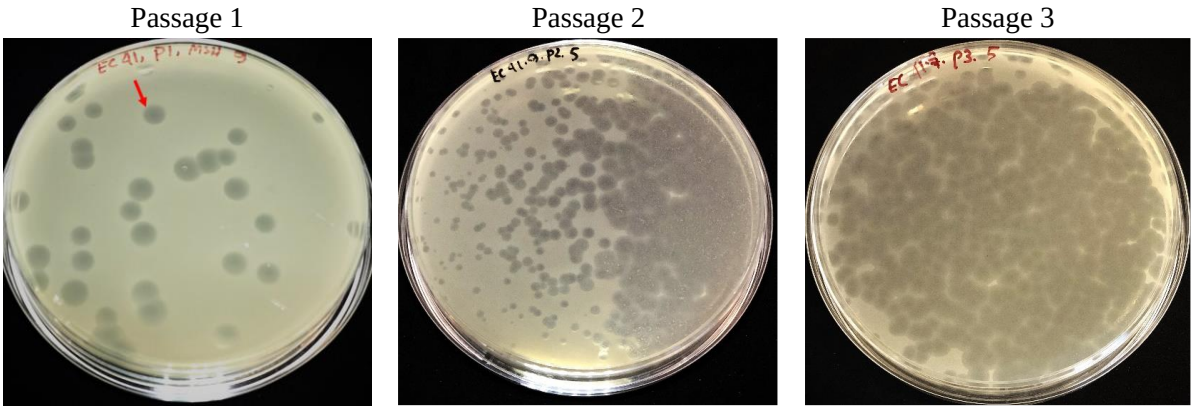

Figure 8: Phage purification outcome of Ec41_SH23, the process shown at three different passage stages. Arrow indicates the plaque formation on Amp-resistant E. coli bacterial lawn.

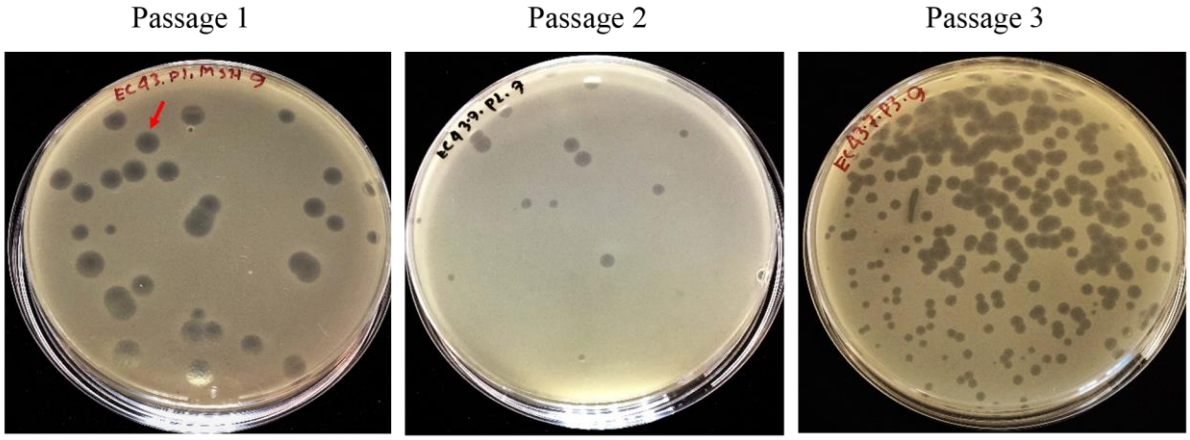

Figure 9: Phage purification outcome of Ec43_SH23, the process shown at three different passage stages. Arrow indicates the plaque formation on Amp-resistant E. coli bacterial lawn.

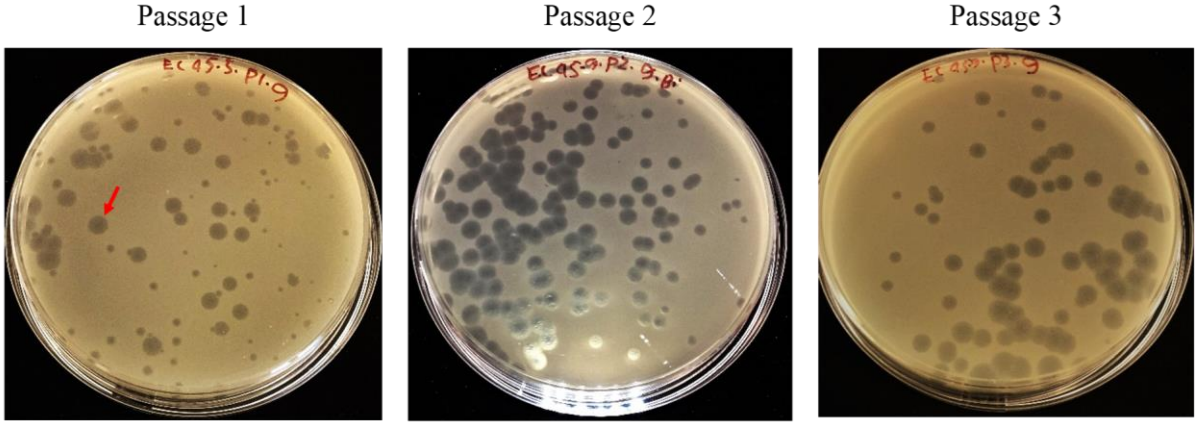

Figure 10: Phage purification outcome of Ec45_SH23, the process shown at three different passage stages. Arrow indicates the plaque formation on Amp-resistant E. coli bacterial lawn.

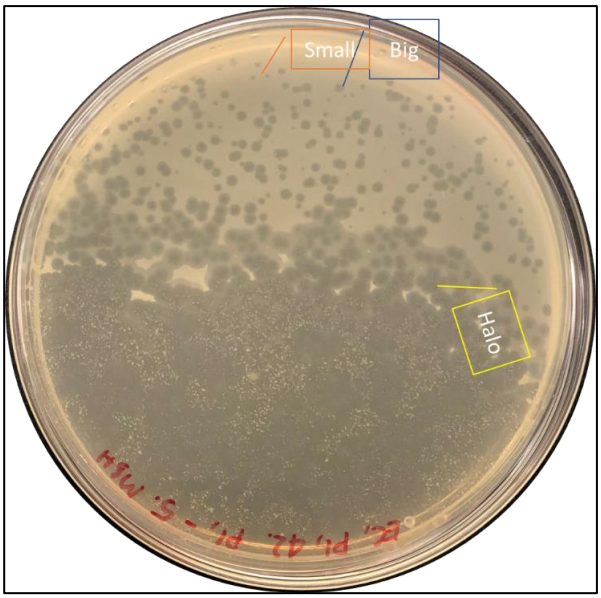

Figure 11: Three different plaque morphotypes are shown in the plate containing Ec42_SH23 phages in the passage 1 purification process. The plaques are formed on Amp-resistant E. coli bacterial lawns.

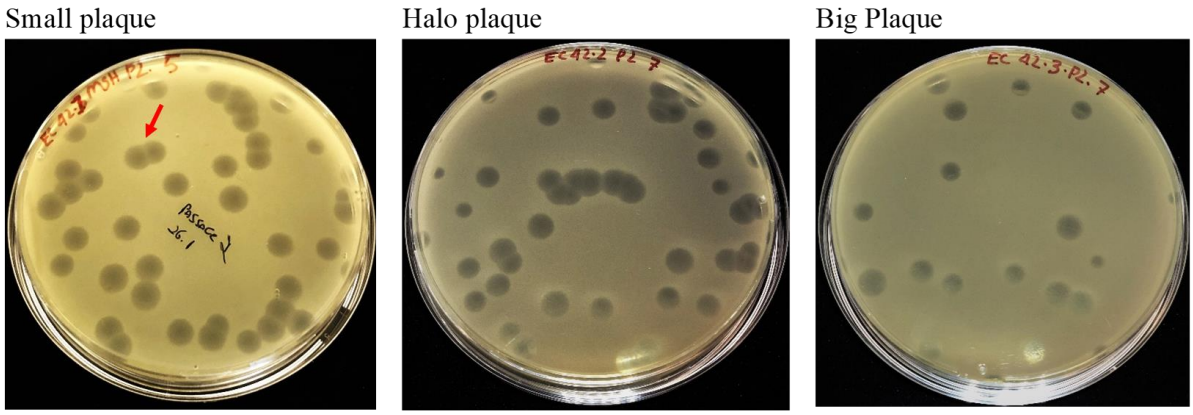

Figure 12: Uniform plaque morphotype of Ec42_SH23 phage in passage 2 purification process. Arrow indicates plaques formation on Amp-resistant E. coli bacterial lawns.

Figure 13: Uniform plaque morphotype of Ec42_SH23 phage in passage 3 purification process. The plaques are formed on Amp-resistant E. coli bacterial lawns.

5.4 Phage Titer

Since the marine phages did not grow in the purification stages, only the coliphage stocks were made and their titer was determined. The phage titer was ascertained by 10-fold serial dilutions followed by the plating of dilution drops. Plaques were counted after overnight incubation at 37°C and the titer of the isolated phage was determined to be 2×10^{10}, 8×10^{10}, 3×10^{10}, 5×10^{10}, and 4×10^{10} pfu/ml for phage Ec41_SH23, Ec42_SH23, Ec43_SH23, Ec44_SH23, and Ec45_SH23, respectively. Phage Ec44_SH23 was isolated by Frøde, and the rest of the analysis was done by me. **Figure 14** shows the plaques of the bacteriophages after titration.

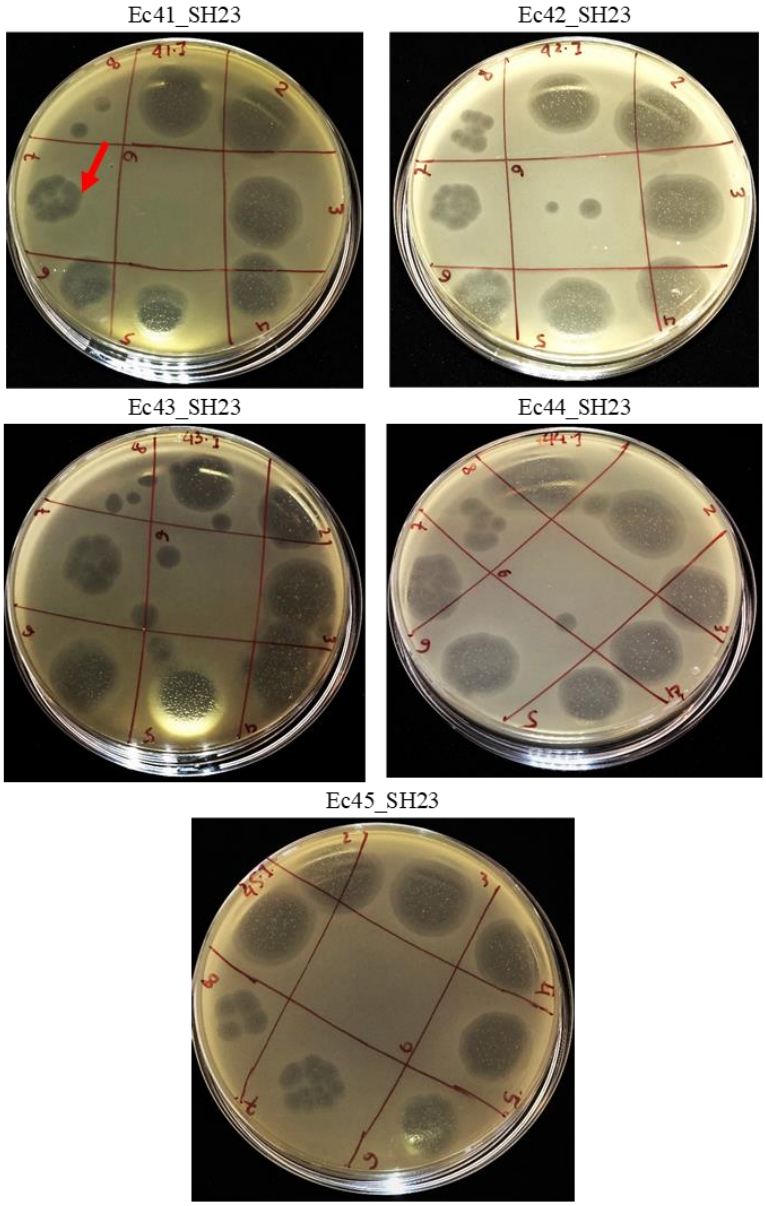

Figure 14: Visualizing phage stock titration: correlating plaque formation with phage concentration at different dilutions. Arrow indicates the plaque formation on Amp-resistant E. coli bacterial lawn.

5.5 Phage Genomic DNA and Restriction Profiles

The amount of nucleic acid extracted from phages using Qubit™ 4 Fluorometer (Thermo Scientific, USA) is presented in **Table 5**. Based on the measurements, the amount of nucleic acid extracted from the phages ranged from 4.63 ng/µl to 6.83 ng/µl. The results revealed that phage Ec44_SH23 had the highest value of 6.83 ng/µl, whereas phage Ec41_SH23 had the lowest value of 4.63 ng/µl.

Table 5: Qubit fluorophore measurements of extracted phage DNA.

Phage	DNA Concentration (ng/µl)
Ec41_SH23	4.63
Ec42_SH23	5.65
Ec43_SH23	5.62
Ec44_SH23	6.83
Ec45_SH23	4.64

The restriction enzyme BamHI was used to distinguish the isolated phages by a restriction digestion profile. The pattern of the digestion is shown in **Figure 15**. For phage Ec41_SH23, BamHI digestion yielded several bands, compared to only three bands for the other phages.

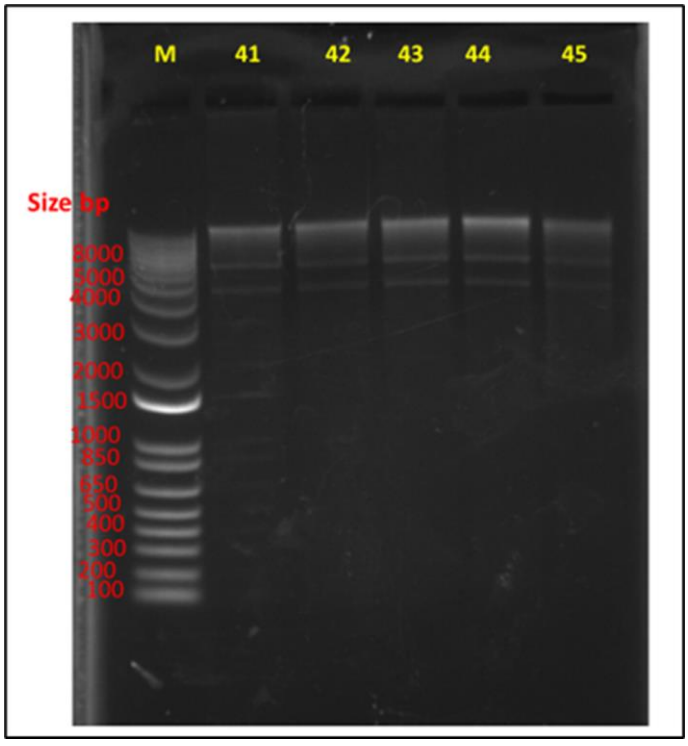

Figure 15: Restriction profiles of BamHI digested coliphage nucleic acid; separated by electrophoresis in an agarose gel. Lanes: M- 1Kb marker, 41-45 represents the phage number Ec41_SH23 to Ec45_SH23.

5.6 Morphological Characteristics

Morphological analysis of samples Ec41_SH23, Ec44_SH23, and FL54_SH23 was made under a TEM. The TEM micrographs obtained from high-titer lysates showed that the virion morphology of the phages Ec41_SH23, and Ec44_SH23 was similar to caudovirus which are tailed bacteriophages with an icosahedral head that contains the viral genome and is attached to the tail by a connector protein (Turner et al., 2021). Ec41_SH23 phage exhibited both podovirus (**Figure 16.A**) and myovirus (**Figure 16.B**) type morphologies. However, only the podovirus morphotype was found on the Ec44_SH23 phage (**Figure 17.A**). While *Flavobacterium* phage showed unusual characteristics like a round head with a short tail (**Figure 17.B**).

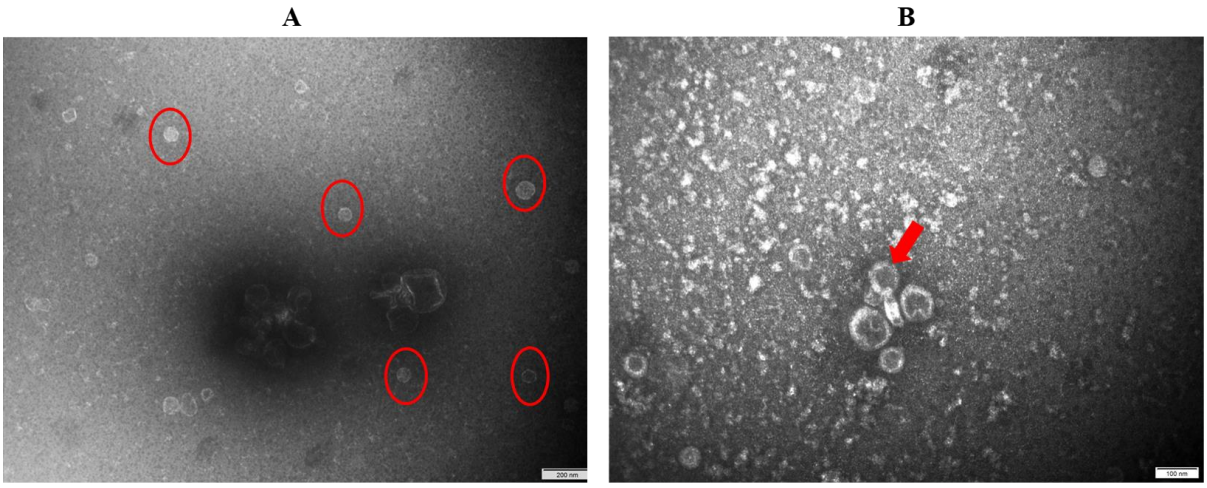

Figure 16: TEM of phage Ec41_SH23 with negatively stained 1% uranyl acetate. In A, the Red circle indicates podovirus-like morphotypes; scale bar is 200 nm. In B, Arrow indicates myovirus-like morphotypes; scale bar 100 nm.

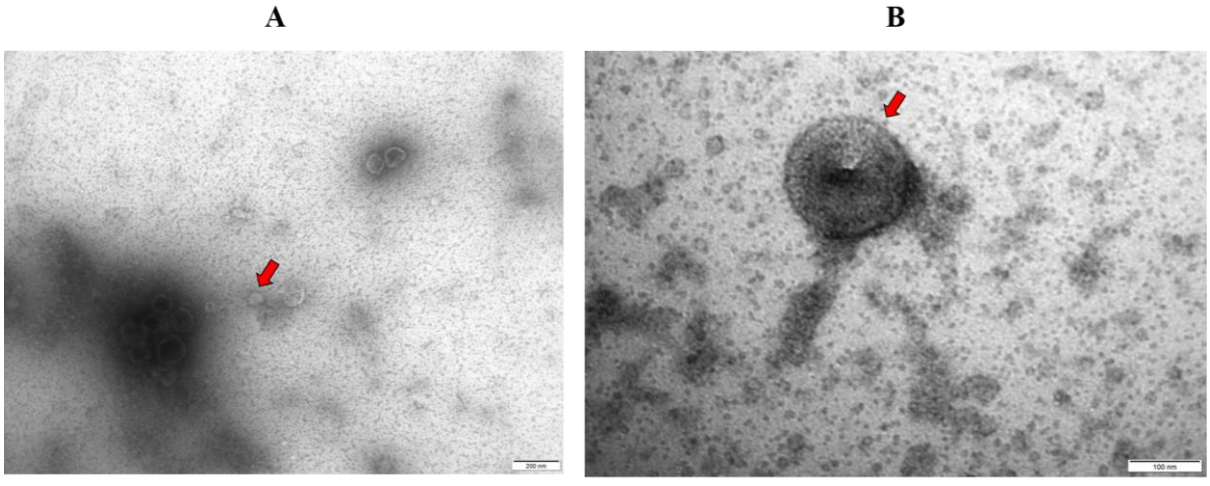

Figure 17: TEM image of phage A) Ec44_SH23 and B) FL54_SH22 with negatively stained 1% uranyl acetate. In A, the arrow indicates podovirus-like morphotypes; scale bar 200 nm. In the B, arrow indicates a round head with short contractile tail morphotypes; scale bar 100 nm.

6 DISCUSSION

The isolation of phages is possible from a variety of sources including seawater, sewage water, soil, and other waste products. This study aimed to isolate and characterize phages from the Arctic environmental sources, including sewage and marine water. In this study, bacteriophages infecting *Flavobacterium, Vibrio, Shewanella*, and *Maribacter*, as well as an antibiotic-resistant *E. coli* that is pathogenic in humans were found. This study was designed to isolate bacteriophages from Arctic seawater and sediment as well as sewage, following a method described by (Spencer, 1955).

The use of bacteriophages for treating different bacterial diseases has been developed in the 1920s and is being used since then in different countries. Considering that phages largely feed on specific bacteria, they have been found to possess bactericidal properties. The use of phage therapy was kept alive in Poland and Georgia. As a consequence of the multidrug-resistant strains of bacteria that have emerged in the past decade, more and more people all over the globe are dying every year because of infections caused by these resistant organisms (Murray et al., 2022). The evolution of drug resistance is a good example of the fact that microbes can evolve with each generation. Phages are being preferred as an alternative to broad-spectrum antibiotics because, unlike antibiotics, they are highly specific for a particular strain of bacteria, which prevents the development of resistance against general strains (Brüssow et al., 2005). Phages are considered non-living entities outside of their host cells, and in order to replicate and perform other metabolic functions they must be in contact with their host cells (Carlton, 1999). The lethal nature of bacteriophages has been known since they were discovered.

6.1 Isolation and Purification of Arctic Marine Phages

Phages found in this project were likely lytic based on the development of clear zones of lysis against different host bacteria. Out of the five marine bacteria used as hosts in this study, only *Shewanella* had no hints of phage presence. However, it was not possible to purify and follow up with any of these marine phages because they did not grow in the purification process. Explanations for this are likely the lack of specific micronutrients in the growth media that were present in the original marine samples, or stability in the temperatures used in the laboratory cultures. According to Borriss et al. (2003), arctic marine phages are sensitive

to temperature, and their plaque formation is optimal at 0°C. The complex nature of the special marine environmental conditions under ordinary laboratory conditions may have hindered the isolation systems. One possible explanation could be the fact that phages and bacteria from the Arctic Sea and seafloor are highly sensitive to temperature. The temperature sensitivity of these phages may also have implications for their survival and persistence in the Arctic marine environment, which is characterized by extreme and variable temperature regimes. Nevertheless, the inability to purify these phages may also be due to several factors, including low phage concentration in the sample, the presence of phage inhibitors, or the presence of other microorganisms that interfere with phage isolation. Further studies are needed to investigate the potential reasons for the inability to purify the phages from the arctic marine samples.

A variety of environments, including the Dead Sea, ice-covered mountains, and Antarctic lakes, have been reported as possible phage-host habitats (Hofer & Sommaruga, 2001; Kepner Jr et al., 1998; Oren et al., 1997). Bacteriophages are believed to be a ubiquitous part of the marine environment with a constant temperature, which includes an environment with sea ice. Sea ice is known to contain high levels of viruses, according to Maranger et al. (1994) However, no viruses were isolated from this cold environment by this group. As viruses play a dynamic role in microbial communities at sea, their abundance changes over the season. Especially during the long winter period in the northern hemisphere, the dynamics of the phage–host relationship are less known. Nevertheless, both the abundance and diversity of the microbial community are subject to dramatic fluctuations (Niu et al., 2014). There is evidence that the viral community can also play a significant role in shaping the microbial community during the frosty winter months by being highly diverse and active. We know little about the physiological adaptations and peculiarities of psychrophilic phages, as well as their host specificity, phenotypic and genotypic diversity. Using the permanently cold environments of the Arctic Sea we describe the finding of cold-adapted phages using samples collected in Tromsø.

The transmission of viruses also play a significant role in the evolution of ecosystems and the diversity of their hosts (Suttle, 2007). Even though several studies have been conducted to understand the diversity of microbial populations in different environments, little information is available regarding the isolation and cultivation of bacteriophages living in the Arctic.

Articles describing viruses isolated from the polar regions and their biological characteristics are quite a few. A study by Olsen (1967) reported that psychrophilic bacteriophages had been isolated from seawater and sediment samples of a marine environment. A psychrotrophic bacterial species called *Brochothrix thermospacta* is lysed by cold-adapted phages which have been isolated from non-marine samples, such as refrigerated products (Greer, 1983). There is substantial evidence to suggest that viruses are widespread and abundant in Antarctic aquatic environments and that they are capable of changing their lifestyle in accordance with their microbial hosts, ranging from pseudo-lysogenic to lytic in response to climatic conditions that favor microbial growth (Anesio & Bellas, 2011; Säwström et al., 2008). There are numerous DNA viruses adapted to extreme conditions in Antarctica and the Arctic, including bacteriophages, circular ssDNA viruses, and algae phycodnaviruses. A study reported previously by Luhtanen et al. (2018) identifies a sensitive bacterial host that is capable of supporting the replication of a phage in the laboratory that was found in Antarctic water samples and sea ice samples. There have been only three virus-host systems isolated to date from the Arctic, while seven are from the Baltic Sea ice (Borriss et al., 2003; Luhtanen et al., 2014; Yu et al., 2015).

There has been considerable research devoted to understanding the diversity of microbes on the sea, but little is known about the isolation and cultivation of bacteriophages from the Arctic. Based on the findings of this study, it is shown that psychrophilic phages can be found in the Arctic Sea ice and their presence is heavily dependent on low-temperature conditions to persist.

6.2 Isolation and Purification of Coliphages from Sewage

Coliphages were successfully isolated from Arctic sewage samples using an *E. coli* host bacterium and plaque assay. Five out of six sewage samples were tested positive for the presence of coliphages, with varying titers ranging from 2×10^{10} to 8×10^{5} pfu/ml.

In general, the fecal contamination of wastewater causes a large diversity of coliforms to be present in sewage. As a result, sewage water contains enteric pathogens as it is a reservoir for them. As a result of biogeographic studies on bacteriophages, it has been proposed that bacteriophages are not equally abundant everywhere and that many factors affect phage numbers in the starting material of extraction (Abedon et al., 2017; Tan et al.,

2020), including temperature, the source of choice, disinfectant level in sewage, sewage flow rate, and exposure to sunlight or radiation. A wide variety of bacteriophages have been found to live in sewage water (Ballesté et al., 2022). The wastewater from hospitals can be considered a highly selective environment for the development of resistant bacteria (Mattila et al., 2015). In previous studies, it has been reported that phages specific to a wide range of bacteria were identified from wastewater samples, including *E. coli; P. aeruginosa; Acinetobacter sp., Klebsiella sp*., and *Enterococcus faecalis* (Aghaee et al., 2021; Del Rio et al., 2021; Grami et al., 2023; Kumari et al., 2010; Manohar et al., 2019; Martins et al., 2022). In our present study, it was found that ampicillin-resistant *E. coli-specific* phages were isolated successfully from sewage water collected from various sides of the WWTP in Brevika, Tromsø. In our wastewater, we are likely to find phages due to the presence of bacteria from the hospital setting. This overpopulation of bacteria leads to the availability of a huge range of hosts for all types of phages (Rasool et al., 2016). Samarahan (2020) conducted a study in Malaysia that demonstrated the ease with which phages can be isolated from sewage. In contrast, Mattila et al. (2015) found that it was challenging to isolate resistant phages from sewage drains in Finland. It is associated with host density as well as surrounding environmental conditions that bacteriophages can recover from and kill precise pathogenic bacteria (Echeverría-Vega et al., 2019).

This study isolated lytic bacteriophages against *E. coli* from sewage water and determined their titers. Following the presence of clear lysis zones against the host bacteria, all the phage isolates obtained with a slightly modified DAL method were identified as lytic phages shown in **Figure 8 - Figure 13**. During the phage purification process, all the phages except Ec42_SH23 exhibited similar types of plaque morphology e.g. round, clear, size; 1-4 mm. Grami et al. (2023) observed that *Escherichia* phage fBC-Eco01 isolated from a wastewater sample collected from Oued Borj Ecedria produced plaques with a diameter of 1.8 ± 0.2 mm. A uniform plaque of approximately 1 mm in diameter is formed by both vB_Eco4M-7 and ECML-117 when applied to the O157:H7 (ST2–8624) strain of *E. coli* (Necel et al., 2020). In another study, Vahedi et al. (2018) isolated specific enteropathogenic *E. coli* phage from hospital sewage which displayed about 2-3 mm size of plaque on BHI agar. This larger plaque size in our study could be due to the extreme environment in the Arctic where organisms may evolve more efficiently to adapt to the conditions. Several factors determine how large the plaque will be, including how soon the bacteria in the agar

will reach the stationary phase and stop reproducing, and its ultimate size, because phages can only reproduce in actively growing cells. Once this point has been reached, the plaques will cease to spread. Also, Abedon and Yin (2009) found that plaque size is associated with phage diffusivity. An increase in phage diffusion velocity leads to an increase in plaque size. The diffusion rate of phages is influenced by certain aspects of their properties, such as their diameter and whether they aggregate or not. Agar concentration in the overlay layer is also a factor. Serwer et al. (2007) reported that the initial isolation of *Bacillus thuringiensis* phage resulted in the production of small plaques (<1 mm) when using a 0.4% agarose overlay. However, the size of the plaques increased as the concentration of agarose gel decreased to 0.2%. Plaques were generated by the coliphage T4 across all agarose concentrations examined. Nevertheless, the size of the plaques decreased with increasing agarose concentration but eventually stabilized around 0.2% to 0.6% agarose (Serwer et al., 2007). This variation in plaque size could also be due to the antagonistic effect of the phages among them. Dominant phages inhibit the growth of others. This effect was highlighted in higher diluted plates where the small plaques (≤1 mm) appeared big, approximately 3-4 mm. However, round homogenized plaque formation appeared later.

A plaque assay demonstrated that the phage titers of the stocks obtained in this study were 2×10^{10} to 8×10^{10} pfu/ml with clear plaques. Similar results were reported by several previous studies (Maal et al., 2015; Montso et al., 2019; Niu et al., 2014; Perera et al., 2015; Qamar et al., 2019; Zhang et al., 2018). Since phages with high titers are considered ideal from a phage therapy perspective, the phages isolated in this study have the potential to be ideal for therapeutic application.

6.3 Characterization Using TEM

Phage identification is best accomplished through negative staining of purified viruses and electron microscopy as there are no universally conserved genes like the 16S rRNA gene in bacteria or the internal transcribed spacer (ITS) region in fungi that can serve as universal markers (Tan et al., 2020). Phages can be classified according to their morphological characteristics. Our TEM results revealed different morphotypes for the selected phage isolates of this study. The structure of isolated coliphages is characterized by the presence of icosahedral heads, long tails that are noncontractile, and extremely short tails. The phage isolates were categorized into the myovirus and podovirus morphology, based on their

features (Ackermann, 2007). A study conducted by Zhang et al. (2021) also observed that phages infecting *E. Coli* O103, which produces Shiga toxin had similar morphological characteristics. On the other hand, Montso et al. (2019) reported myovirus characteristics of eight *E. coli* O177-specific phages. In addition, the traits mentioned resembled those exhibited by bacteriophages of *E. coli* that are similar to T1-7 (Harada et al., 2018; Truncaite et al., 2012). While significant progress has been made in studying coliphages, there has been a lack of specific focus on the isolation and characterization of arctic phages. However, the phage being investigated in this thesis is particularly noteworthy in this regard.

The electron micrograph of the FL54_SH22 phage (**Figure 14**.**B**) exhibited a unique round head with a short contractile tail-like morphology, which is distinct from previously described phages. This observation raises the possibility of a completely new phage with unique phenotypic characteristics. It is worth noting that the host bacterium used in this study was also novel, which could contribute to the distinct morphology of the phage. Holmfeldt et al. (2007) reported a similar phenotype for the phage φ4:1, identified as a myovirus family member by TEM analysis using 2% sodium phosphotungstate staining. However, Borriss et al. (2003) isolated and characterized flavophages using *Flavobacterium hibernum* as a host from the Arctic sea ice and reported a siphovirus morphology from TEM analysis. Previous studies on flavophages from both marine and freshwater environments have reported diverse morphotypes, including siphovirus, myovirus, and podovirus (Bartlau et al., 2022; Holmfeldt et al., 2007; Jiang et al., 1998; Laanto et al., 2011). Nevertheless, further investigation is required to categorize the FL54_SH22 phage isolated in this study.

7 FUTURE PERSPECTIVE

In the future, the isolation and characterization of bacteriophages from arctic samples can be further explored to uncover new insights and potential applications in various fields. Here are some future perspectives that could be pursued:

- Biological characteristics of the phages: The isolated phages will be further characterized based on their host range, growth curves, and sensitivity to different parameters such as temperature, pH, etc.
- Full genome sequencing and genomic analysis: Through genomic analysis, the isolated phages can be further characterized, providing insights into their genetic diversity, evolution, and potential functional applications. This could lead to the discovery of new phage-encoded enzymes, toxins, or other biologically active compounds that could have potential biotechnological or therapeutic applications.
- Exploration of the Arctic Virome: While this study focused on the isolation and characterization of bacteriophages, future research can expand to explore the entire virome of the Arctic environment, including viruses that infect eukaryotic organisms. This could reveal new virus-host interactions and provide insights into the ecology and evolution of viruses in extreme environments.
- Applications in aquaculture and wastewater treatment: The isolated bacteriophages can be potentially applied in aquaculture, including their use as biocontrol agents in food and agricultural industries. Additionally, they could be used in WWTP to combat bacterial contamination in the environment. Future studies can investigate the efficacy of arctic phages in these applications and optimize their use.
- Phage therapy: The emergence of antibiotic-resistant bacteria has renewed interest in the use of bacteriophages as an alternative therapeutic option. Future studies can investigate the potential of arctic phages as candidates for phage therapy, including their safety, efficacy, and the development of phage cocktails to target multiple bacterial strains.

Overall, the isolation and characterization of bacteriophages from arctic samples is a promising avenue for future research, with potential applications in biotechnology, bioremediation, and phage therapy. Continued exploration of the arctic virome and the genetic

and functional diversity of arctic phages can lead to discoveries and opportunities for innovative solutions to global challenges.

8 CONCLUSIONS

A noteworthy feature of this research is that it is the first study in Norway to describe the isolation and characterization of phages from the Arctic Sea and sewage that are capable of effectively fight foodborne bacteria and Amp-resistant *E. coli*. This study focused on the isolation and characterization of bacteriophages from arctic sewage and sea samples. The main findings are that this study verified that the arctic environment harbors a diverse range of bacteriophages that can infect a variety of bacterial hosts. During the experiment, isolating and purifying marine phages was the most challenging part. While we were able to obtain some hints of their presence, the phages did not grow into full plaques on agar plates. Despite repeated attempts using different conditions and techniques, including different media formulations, we were unable to obtain pure phage cultures. Although this limits the conclusions that can be drawn from the study, the findings suggest that marine phages may have unique growth requirements that differ from those of previously studied phages. Further research is needed to explore these growth requirements and to develop methods for isolating and characterizing marine phages more effectively.

However, coliphages were easily isolated and subjected to TEM analysis. The results revealed the presence of caudovirus characteristics in the phages. The caudovirus family of phages is known for its characteristic morphology, which includes an icosahedral head, a contractile tail, and tail fibers. The identification of caudovirus characteristics in the isolated coliphages suggests that they belong to this family. This information provides valuable insights into the morphological properties of the isolated phages and can guide future research on their genome analysis.

Further, nucleic acid of coliphages was successfully extracted; but, due to time constraints, sequencing has not yet been performed. However, they will be subjected to sequencing analysis to gain insights into their genetic makeup and potential applications. The sequencing results can provide information on the genome size, gene content, and phylogenetic relationships of the isolated coliphages. Future research can further explore the genetic and functional diversity of arctic phages and their interactions with bacterial hosts, as well as their potential as alternatives to antibiotics in the face of antibiotic resistance.

Overall, this study contributes to our understanding of the diversity and ecology of bacteriophages in extreme environments and provides insights into their potential applications in biotechnology and healthcare. Two different but important groups of phages were studied from samples obtained in the Tromsø region: the challenging-to-grow and environmentally relevant marine phages, and the conventional and clinically important sewage phages.

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10 APPENDIX

Table 6: Reagents used in the preparation of FMAP media.

Reagents	ID	Distributor
Marine Broth	11753513	BD Difco™, USA
Marine Agar	11718183	BD Difco™, USA
Peptone from casein (Tryptone)	107213	Merck, Germany

Appendix 1: Composition of Marine Broth (BD Difco™ 2216, USA)

Peptone 5 g/l, Yeast extract 1 g/l, Ferric Citrate 0.1 g/l, Sodium Chloride 19.45 g/l, Magnesium Chloride 5.9 g/l, Magnesium Sulfate 3.24 g/l, Calcium Chloride 1.8 g/l, Potassium Chloride 0.55 g/l, Sodium Bicarbonate 0.16 g/l, Potassium Bromide 0.08 g/l, Strontium.

Appendix 2: Composition of Marine Agar (BD Difco™ 2216, USA)

Peptone 5 g/l, Yeast extract 1 g/l, Ferric Citrate 0.1 g/l, Sodium Chloride 19.45 g/l, Magnesium Chloride 8.8 g/l, Sodium Sulfate 3.24 g/l, Calcium Chloride 1.8 g/l, Potassium Chloride 0.55 g/l, Sodium Bicarbonate 0.16 g/l, Potassium Bromide 0.08 g/l, Strontium Chloride 34m g/l, Boric Acid 22m g/l, Sodium Silicate 4m g/l, Sodium Fluoride 2.4m g/l, Ammonium Nitrate 1.6m g/l, Disodium Phosphate 8m g/l, Agar 15 g/l.

Appendix 3: Preparation of liquid FMAP medium

Liquid FMAP medium is prepared with 15 g/l of marine broth (Difco2216), 5 g/l of peptone from caseine (tryptone). Then 300 ml of seawater is added and completed to 1 litre with MiliQ water. The mixture is divided into 200 ml aliquots and autoclaved at 121°C for 120 minutes.

Appendix 4: Preparation of FMAP medium plates.

Liquid FMAP medium is prepared with 15 g/l marine broth (Difco2216), 5 g/l of peptone from caseine (tryptone), 15 g/l of agar. Then 300 ml of seawater is added and completed to 1

litre with MiliQ water. The mixture is autoclaved at 121°C for 120 minutes. After sterilization Plates are prepared by adding 20 ml of media.

Appendix 5: Preparation of soft FMAP medium

Soft FMAP medium is prepared with 15 g/l marine broth (Difco2216), 5 g/l of peptone from caseine (tryptone), 0.7% (7 g/l) of agar. Then 300 ml of seawater is added and completed to 1 liter with MiliQ water. The mixture is divided into 200 ml aliquots and autoclaved at 121°C for 120 minutes.

Appendix 6: Preparation of 5x concentrated FMAP medium

The 5x concentrated media is made with 15 g/l marine broth (Difco2216), 5 g/l of peptone from caseine (tryptone), 60 ml of seawater added, and completed to 200 ml with MiliQ water followed by autoclaving at 121°C for 120 minutes.

Appendix 7: Summary of results from the marine sample analysis

Culture No	Sample Used	Bacterial Species	Results from screening the enrichment cultures	Results from titration
1	53	*Flavobacterium*	Positive	Positive
2	54	*Flavobacterium*	Positive	Positive
3	55	*Flavobacterium*	Positive	Positive
4	56	*Flavobacterium*	Positive	Positive
5	57	*Flavobacterium*	Positive	Positive
6	58	*Flavobacterium*	Positive	Positive
7	53	*Vibrio*	Positive	Positive
8	54	*Vibrio*	Positive	Positive
9	55	*Vibrio*	Negative	
10	56	*Vibrio*	Negative	
11	57	*Vibrio*	Negative	
12	58	*Vibrio*	Negative	
13	53	*Vibrio*	Positive	Positive
14	54	*Vibrio*	Positive	Positive
15	55	*Vibrio*	Positive	Positive
16	56	*Vibrio*	Positive	Positive
17	57	*Vibrio*	Positive	Positive
18	58	*Vibrio*	Positive	Positive
19	53	*Shewanella*	Negative	
20	54	*Shewanella*	Negative	
21	55	*Shewanella*	Negative	
22	56	*Shewanella*	Negative	
23	57	*Shewanella*	Negative	
24	58	*Shewanella*	Negative	
25	53	*Maribacter*	Positive	
26	54	*Maribacter*	Positive	
27	55	*Maribacter*	Positive	
28	56	*Maribacter*	Positive	
29	57	*Maribacter*	Positive	
30	58	*Maribacter*	Positive	