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Isolation and characterization of wide host range vibriophages for therapeutic use in aquaculture

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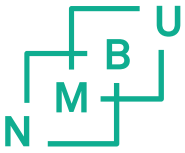

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ACDPHARMA
INNOVATIONS IN AQUACULTURE

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Ingrid Sofie S. Larsen

Abstract

The aquaculture industry is one of the fastest growing food-producing sectors in the world, but the industry is struggling with huge losses due to bacterial infections. Several species of *Vibrio* are ubiquitous to marine environments, and some are responsible for large disease outbreaks in fish and shellfish farms. Antibiotics have been used to fight bacterial infections, but due to emerging resistant bacteria, negative impacts on the natural microflora, as well as the increasing consumer demand for food raised without antibiotics, there is need for alternatives.

Phage therapy represents a promising alternative because of bacteriophages´ ability to infect and kill bacteria with high specificity. Phages have been known for a long time, but their use in therapy have suffered from poorly organised studies and improper diagnostics. One of the main challenges with using phage for therapeutic purposes is the need for very specific diagnoses in order to apply the right phage. Use of broad host range phages can facilitate easier diagnostics, making phage application more efficient. The aim of this study was to isolate and characterize vibriophages suitable for therapeutic use in aquaculture, with particular emphasis on wide host range phages. Mussels and water samples from the coast of Norway were used as search material, and a library of *Vibrio* spp. was established. The library was further used to screen environmental samples for vibriophages. Isolated phages and bacteria were characterized through whole genome sequencing and Sanger sequencing, respectively. Genome analyses and phage susceptibility patterns provided the identification of 10 different *Vibrio* species and 11 different vibriophages. The vibriophages covered all three families within the order *Caudovirales*, and at least one phage showed potential for being wide host ranged. Knowledge acquired during this work will be used to improve the methodology for isolating wide host range vibriophages.

Populærvitenskapelig sammendrag

Akvakulturnæringen er i dag en av de raskest voksende mat-næringene i verden, men næringen sliter med store tap grunnet bakterielle infeksjoner. Antibiotika har lenge vært brukt til å behandle slike infeksjoner, men på grunn av resistensutvikling, negativ innvirkning på den naturlige mikrofloraen og et større fokus blant forbrukere på mat produsert uten antibiotika, har det oppstått et behov for alternativer.

Bakteriofager, virus som spesifikt infiserer og dreper bakterier, representerer et slikt alternativ. Bakteriofager (eller fager) har vært kjent lenge, men deres bruk til behandling av infeksjoner har vært preget av ufullstendig diagnostikk og dårlig organiserte forsøk. En av hovedutfordringene med fagterapien er det smale vertspekteret til fager, noe som krever spesifikk diagnostisering slik at riktige bakteriofager blir valgt i behandlingen. Bruk av fager med et bredere vertsspekter vil stille enklere krav til diagnostiseringen, samt kunne effektivisere behandlingen av koinfeksjoner forårsaket av nært beslektede bakteriearter. Målet med denne studien var å isolere og karakterisere bakteriofager til å bekjempe *Vibrio*-infeksjoner i akvakulturnæringen, med et spesielt fokus på bredspektrede bakteriofager. Blåskjell og vannprøver fra norskekysten ble brukt som prøvemateriale for å lete etter *Vibrio* og vibriofager. Et bibliotek av *Vibrio*-isolater ble opprettet og brukt til å søke etter vibriofager i det samme prøvematerialet. Isolerte bakteriofager og bakterier ble karakterisert via helgenomsekvensering og Sanger sekvensering. Genomanalyser og kartlegging av bakterienes bakteriofagsensitivitet resulterte i identifikasjon av 10 ulike *Vibrio*-arter og 11 ulike vibriofager. Vibriofagene representerte tilsammen alle de tre familiene innen bakteriofagordenen *Caudovirales*, hvorav minst en av fagene viste potensial for å være bredspektret. Kunnskap og erfaringer tilegnet gjennom denne studien vil bli brukt til å forbedre metoder for å isolere bredspektrede vibriofager.

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1. INTRODUCTION

A rising demand for fish and shellfish in the food marked worldwide has led to insufficient supply from wild catches (FAO 2016; Moriarty 1999). As a consequence, the aquaculture industry is now growing faster than any other food-producing sector in the world (Bondad-Reantaso et al. 2005), and it is likely to continue to grow as the world population is estimated to increase, and bypass 9 billions by the year of 2050 (FAO 2016). In 2014, the Food and Agriculture Organization (FAO) of the United Nations estimated aquaculture to produce approximately 74 million tonnes of fish and shellfish, with a net value of USD 160.2 billions. By 2020, production is projected to be more than double and bypass 164 million tonnes (Rao & Lalitha 2015). However, with the expanding aquaculture comes several issues to be solved, like those regarding infectious diseases and health management of farmed species. This is a challenge both when it comes to animal welfare and to economic aspects of the industry (Bondad-Reantaso et al. 2005; Meyer 1991).

1.1 *Vibrio* and vibriosis

According to FAOs report "The state of world fisheries and aquaculture", outbreak of diseases in the last 20 years have led to financial losses worth tens of billion dollars (FAO 2016). Microbial communities in fish farms are huge and complex, containing several potential pathogens. Among these, species belonging to the family of *Vibrioaceae* are common pathogens, causing infections known as vibriosis (Labella et al. 2013; Silva et al. 2014). *Vibrioaceae* is classified into seven genera, of whom the genera of *Vibrio* and *Aliivibrio* are well known in fish farming (Sharma et al. 2017). Several species of *Vibrio* are ubiquitous to marine environments as most of them require salts (halophilic), with some exceptions found in brackish water and freshwater (Poppe 2002). The bacteria are Gram-negative, facultative anaerobic, motile due to flagella and tolerate alkaline environments well (Gomez-Gil et al. 2014). They can exist freely in water, in biofilms or in associations with a host, where some can function as pathogens and others as "probiotics" (Thompson et al. 2005).

Fish and shellfish suffering from vibriosis can show different symptoms, depending on the *Vibrio* species, the animals' immune status and general health. Diffuse symptoms such as

lethargy and reduced appetite can be difficult to spot, while skin ulcers and abnormal swimming patterns can be hard to clarify the causative agent behind (Poppe 2002). Vibriosis usually start with ingestion of the bacteria or by colonization on the host surface, followed by penetration of the skin (Silva et al. 2014). Infections do not necessarily kill the animal, but leave unpleasant wounds and scars, consequently reducing the animal welfare and market price. However, if bacteria are left to spread systemically they can cause acute haemorrhagic septicemia with severe internal bleeding and necrosis. The outcome is usually irreversible damage and high mortality (Poppe 2002).

Being opportunists, *Vibrio* species can reproduce rapidly during favourable conditions, and cause high numbers of infections and rapid death (Suttle 2007). Healthy fish with a normal functioning immune system are usually able to keep pathogens in check and avoid sickness (Moriarty 1999). Problems arise when fish are stressed and their immune status is weakened. This may be when farms are overcrowded, have inadequate food and/or oxygen supply or poor water quality (by accumulation of faeces or inappropriate temperatures), giving opportunistic pathogens a chance to infect (Meyer 1991; Silva et al. 2014). For instance, cold-water vibriosis, caused by *Aliivibrio salmonicida (*earlier *Vibrio salmonicida*), is a well-known disease in aquaculture, but has never been reported in captured fish (Kashulin et al. 2017; Poppe 2002). This implies that conditions in fish farms are stressing the fish, giving opportunistic bacteria a chance to infect (Cabello 2006; Poppe 2002). Also, stress related to handling, sorting and vaccination can make animals more susceptible to infections (Nilsen et al. 2014).

1.1.1 Prevalence of vibriosis

Asia stands for nearly 90% of the global aquaculture production (FAO 2016; Naylor et al. 2000), but the industry is suffering from infections by species such as *V. harveyi*, *V. parahaemolyticus* and *V. anguillarum* (Letchumanan et al. 2016). Similar problems are seen in Norway, the second largest fish exporting country in the world (after China) (FAO 2016). For instance, in 2003 Lillehaug, Lunestad and Grave studied the prescription of antibacterial drugs for use in Norwegian aquaculture from 1991 to 2000. Vibriosis appeared to be one of the main causes behind the prescriptions, affecting a variety of species (Lillehaug et al. 2003). In farming of Atlantic salmon alone, cold-water vibriosis and classic vibriosis were listed among the most

commonly treated diseases. However, due to good vaccination in recent years, this problem is severely reduced in several countries (Poppe 2002). Salmon farms are instead struggling with parasitic infections by sea lice. Cleaner fish, such as wrasse and lumpfish, prey on sea lice and are therefore of high interest to the farmers. However, according to a comprehensive report from 2014 by the Norwegian veterinary institute, the mortality of cleaner fish in some farms were as high as 60% (Nilsen et al. 2014). Bacterial infections were among the causative agents, and several *Vibrio* spp. were detected. In 2016, Institute of marine research confirmed this in their report "Risikovurdering av norsk fiskeoppdrett 2016", listing *V. anguillarum*, *V. splendidus, V. tapetis* and *V. ordalii* as bacteria causing problems for cleaner fish (Svåsand et al. 2016).

1.1.2 Treatment in aquaculture

As the aquaculture industry continues to expand, the need for effective methods to fight infections rises with it. Not surprisingly, the use of antibiotics has increased drastically, both for prevention and for treatment (Cabello 2006; Labella et al. 2013). Developing countries have the highest consumption of antibiotics, frequently using them as prophylactic agents to compensate for poor sanitary conditions in fish farms (Letchumanan et al. 2016). Antibiotics are often distributed into fish feed and are later found in faeces and unconsumed food (Cabello 2006; Poppe 2002). Non-biodegradable antimicrobials can then be dispersed to surrounding niches, affecting the natural microflora. This may exert selective pressure for resistant bacteria, by eliminating the non-resistant ones. In fact, Almeida et al. (2009) claimed that as much as 90% of marine bacteria are resistant to one or more antibiotics (Almeida et al. 2009). Additionally, Cabello (2006) reviewed how prophylactic use of antibiotics affected the aquaculture, and found that several antibiotic resistant determinants detected in the human pathogen *Salmonella Typhimurium,* were firstly discovered in marine species of *Vibrio*. By this, he showed that resistant genes can spread from marine environments to terrestrial animal- and human pathogens. Further, as antibiotic residuals have been retrieved from the tissue of fish and shellfish, there is a potential for affecting the consumers' natural gut microbiota (Cabello 2006; Samulsen et al. 1992).

1.2 Bacteriophages

The rapid spread of antibiotic resistance and consumers demand for food "raised without antibiotics", is pushing the aquaculture industry towards using alternative antimicrobials (Bowman et al. 2016). Bacteriophages, natural viruses that specifically infect and kill bacteria, represents such a promising alternative. Bacteriophages (also referred to as phages, or Φ), typically consist of a RNA or DNA genome, protected by a protein capsid. Of all phages identified today, ~96% of them are tailed bacteriophages, have linear dsDNA and belongs to the order *Caudovirales* (Ackermann 2001). These phages have an icosahedral formed protein cap (or lipoprotein) encapsulating the genome, and a tail structure with tail fibers enabling the phage to attach to the host cell (Fig. 1.1). In this "head-tail" morphology the head serves to protect the genome, while the hollow cylindrical tail is meant for host cell adsorption and transfer of nucleic acids during bacterial infections (Letchumanan et al. 2016; Sharma et al. 2017).

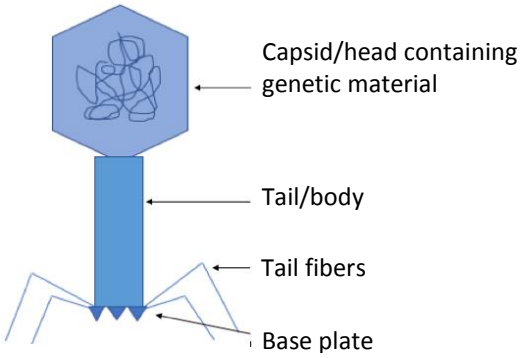


Figure 1.1: "Head-tail" morphology of a tailed bacteriophage, based on the morphology of *Escherichia coli* phage T4 (*Myoviridae*).

According to the International Committee on Taxonomy of Viruses, the order *Caudovirales* consist of three phage families, namely *Siphoviridae*, *Myoviridae* and *Podoviridae*, of which the family of *Siphoviridae* is comprising 61% of the described tailed phages. Families are usually distinguished by their head and tail morphology, as shown in figure 1.2.

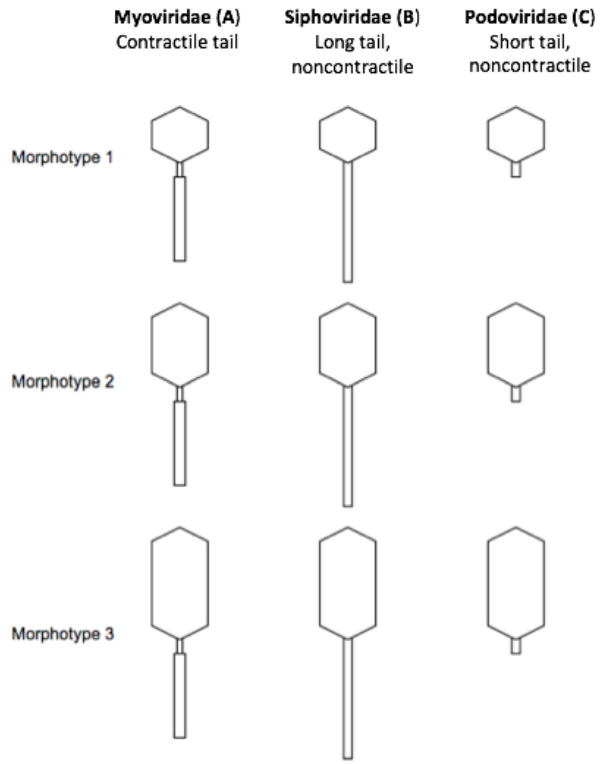


Figure 1.2: The order *Caudovirales* is divided into three families: *Myoviridae*, *Siphoviridae* and *Podoviridae,* based on their tail structure. The families are further subdivided into morphotypes based on their head shape. Modified from Ackermann (2001).

1.2.1 Phage life cycle

Phages depend on a living bacterial host for replication and proliferation, since phages lack the necessary metabolic machinery themselves. The phage life cycle starts with phage attachment to specific receptors on the host cell surface. This is achieved by receptor binding proteins (rbp) of the phage tail, recognising receptors on the host cell (Labrie et al. 2010). Potential host receptors are, for example, outer membrane proteins, lipopolysaccharides, flagella, and for Gram-positive bacteria, teichoic acids. Due to this diversity of host receptors, and the specificity of rbps, most phages have a narrow host range (Sharma et al. 2017). After attachment, various mechanisms are used for injecting the genetic material into the host cell, depending on the phage type.

Following genome injection, a phage will continue with a lytic of lysogenic life cycle (Fig. 1.3). Phages following a lytic cycle will take over the host cell and use its machinery for exponential production of new phages (Skurnik & Strauch 2006). This process involves reprogramming of the host, conducted by phage genes. Expression of so-called “early genes” protects viral DNA from enzymatic degradation by host enzymes, while later expressed genes are responsible for

DNA replication, translation and assembly (Kutter & Sulakvelidze 2005). Host cell lysis is usually achieved by production of holins and lysins, enzymes involved in breaking down the cell membrane and peptidoglycan layer, respectively (Doss et al. 2017). As a host cell burst, progeny phages are released to infect neighbouring bacteria, and the cycle is repeated (Sharma et al. 2017).

The alternative lifestyle is a lysogenic cycle. Here, the phage genome is inserted into the host genome, and replicated together with it (Canchaya et al. 2003). In this way, viral genes are passed on in a repressed state to every bacterial clone of the primary host (Skurnik & Strauch 2006). A phage genome residing in a host chromosome or plasmid is called a prophage, and will remain latent in its host until induced to enter the lytic cycle. Induction is often caused by the bacteria being stressed, for instance by antibiotic treatment or DNA damage (Kleppen 2012). Phages able to switch between the lytic and lysogenic cycle are known as temperate phages, whilst virulent phages are restricted to the lytic cycle (Paul 2008; Rao & Lalitha 2015). A key difference between virulent and temperate phages is the regulation of proteins involved in, for instance, degradation of the hosts DNA or cell membrane, disruption of bacterial replication and translation or destruction of essential enzymes. As prophages are depending on a living host, expression of such proteins must be highly controlled by inhibitors (Kutter & Sulakvelidze 2005; Paul 2008).

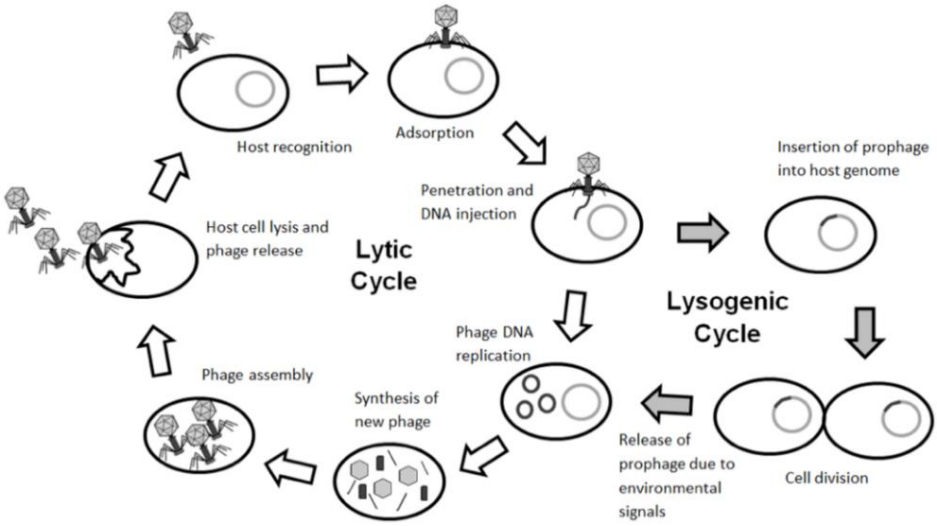


Figure 1.3: Life cycle of phages. Virulent phages are obligated to the lytic cycle, while temperate phages can switch between the lytic and lysogenic cycle. While in the lysogenic phase, prophages protect their host from infections by closely related phages (so-called homo-immunity). Figure adapted from (Doss et al. 2017)

1.3 Phage therapy

The discovery of bacteriophages is credited to Frederick Twort and Felix d´Herrelle who independently observed "parasites with antimicrobial properties" in 1915 and 1917, respectively (Letchumanan et al. 2016; Sharma et al. 2017). D´Herrelle thought the parasites to be viruses and named them bacteriophages ("bacteria eaters"). He quickly understood their potential to treat bacterial infections, introducing the field of phage therapy already in the 1920s (Kutter & Sulakvelidze 2005). In the beginning, physicians and pharmaceutical companies showed huge enthusiasm for this new form of therapy. However, studies and trials were poorly organized and gave varying and inconsistent results, much due to the lack of proper diagnostics. Then, with the discovery of penicillin in combination with outbreak of the second World War, use concerning phages came to a halt (Kutter & Sulakvelidze 2005).

1.3.1. Phage therapy versus antibiotics

Multidrug resistant (MDR) bacteria are becoming a global health problem (WHO 2014), and there is a general perception that frequent use of antibacterial chemicals lead forward resistant bacteria (Poppe 2002; Silva et al. 2014). For instance, when Labella et al. (2013) analysed water samples from the Adriatic coast they found a higher incidence of MDR bacteria near aquaculture centers using antimicrobials, compared to coastal areas. Since phages induce bacterial lysis through other mechanisms than antibiotics, they can lyse MDR bacteria regardless of their drug resistance. Because of this, research concerning phage therapy has reoccurred over the last decades, 100 years after their discovery (Rao & Lalitha 2015). Nevertheless, just as with antibiotics, bacteria can develop resistance towards phages (Skurnik & Strauch 2006). For instance, bacteria may alter their surface receptors, thereby preventing phage attachment. They may also use restriction modification systems to excise injected viral DNA (Doss et al. 2017; Labrie et al. 2010). An important difference is that phages can undergo mutations themselves, resulting in an endless race between new resistance mechanisms in bacteria, and phages adapting to overcome them (Labrie et al. 2010). Additionally, phage adsorbing receptors are often involved in processes important for bacterial fitness. Mutations altering the receptors might then also affect the host, potentially reducing its pathogenicity (Nakai & Park 2002; Skurnik & Strauch 2006). Ideally, therapeutic phages should attach to a

receptor of vital importance for the bacteria, as such receptors are highly conserved (Mobegi et al. 2014). The lower mutation rate of these receptors can leave the phage effective longer.

Considering phages' host specificity they do not conduct selective pressures in the same way as antibiotics (Kutter & Sulakvelidze 2005). Each phage has a limited target range, usually restricted to one or a few bacterial strains (Nakai & Park 2002). Such "obligated bacterial predators" are beneficial as they only affect the targeted host, leaving the unintended bacterial flora unharmed. Phages can therefore be considered more "user friendly" compared to antibiotics, as the narrow host range pose a low risk for allergic side effects to the consumer. Besides, isolation of phages would be relative simple, inexpensive and less time consuming compared to developing new antibiotics (Matsuzaki et al. 2003).

1.3.2 Challenges with phage therapy

Although the field of phage therapy is expanding, there are still several challenges to overcome, as seen in figure 1.4. Early studies of phage therapy often suffered from imprecise identification of the causative bacteria, leading to use of unsuitable phages (Kutter & Sulakvelidze 2005). Moreover, a lack of well characterized phages were a challenge. Phage therapy requires virulent phages, as only obligated lytic phages can guarantee death of the host cell (Almeida et al. 2009). Temperate phages opens for the possibility of transduction, where genetic material is transferred between hosts (Rao & Lalitha 2015). In this way, genes encoding e.g. antibiotic resistance, virulence or exotoxins can be transferred, turning non-pathogenic bacteria into pathogens (Skurnik & Strauch 2006). It is therefore very important that all phages intended for use in therapy are virulent and screened for potentially harmful genes.

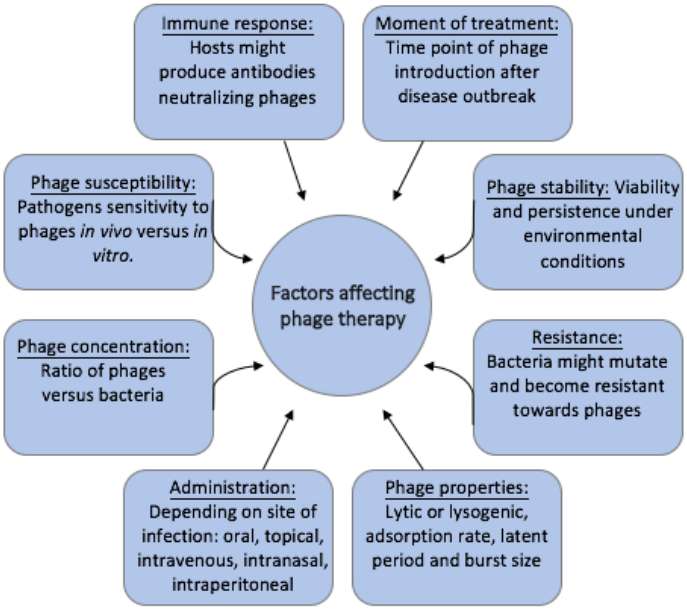


Figure 1.4: Factors affecting the efficacy of phage therapy. Modified from Ly-Chatain (2014).

Considering phages' high abundance and natural diversity they should, in theory, be able to defeat most bacterial infections. However, besides using a suitable phage, phage therapy requires introduction at the correct site of infection, at the right moment and in the right amount (Sharma et al. 2017; Suttle 2007). As phages cannot enter eukaryotic cells, intracellular pathogens can be problematic to treat. Further, since phages are biological agents the *in vivo* behaviour can, unlike antibiotics, differ from one phage to another (Parracho et al. 2012).

1.3.3 Phage therapy in aquaculture

Some fish farms apply probiotic cultures into fish tanks as a protection towards pathogens (Pandiyan et al. 2013). Adding antibiotics to these fish tanks may then eliminate both pathogenic- and beneficial bacteria. In addition, fish and shellfish receiving antibiotics usually require some retention time before slaughtering, due to residues of antibiotics in the meat (Samulsen et al. 1992). Phages, on the other hand, have limited impact on the microflora, as they only affect the target of interest. Moreover, since phages are self-replicating and self-limiting, they will expand with the target host, and also, decrease as the host is eliminated (Doss et al. 2017; Rao & Lalitha 2015). Phages´ stability in the environment prevents them from disappearing completely, but as they stop propagate their concentration is diluted over time. Phage treatment cannot eliminate all target pathogens during a disease outbreak, but

they might reduce the number to a level in which the hosts immune system can cope (Silva et al. 2014). By reducing the infection pressure, phages can protect uninfected individuals.

To prove a phage effective, the concentration of phages should increase as the number of bacteria is reduced (Nakai & Park 2002). Rao and Lalitha (2015) summarized several phage therapy trials in aquaculture were this was the case. For instance, *V. parahaemolyticus*, *V. harveyi* and *V. anguillarum* infections in oysters, shrimps and Atlantic salmon, respectively, were all reduced *in vivo* after phage application. And in 2006, Vinod et al. published a paper claiming that treating *V. harveyi* infected shrimp larvae with bacteriophages gave a survival rate of 86%. In comparison, a 40% survival was observed with antibiotics, due to emergence of resistant bacteria. And for the control group (no treatment) a mean survival of 17% was observed (Vinod et al. 2006). Also, when Karunasagar et al. (2007) compared how antibiotics and phages eradicated biofilms of *V. harveyi* in fish tanks, bacteriophages showed the most promising outcome.

A question to be considered before applying phage therapy in aquaculture is the route of administration. Depending on the site of infection, oral- or bath- administration are the most common methods for treating large numbers of fish simultaneously (Nakai & Park 2002; Rao & Lalitha 2015). However, phage suspensions are rapidly diluted when applied to the water, depending on the volume of the tank and the rate of water exchange. The original concentration of phages, volume of the fish tank and estimated concentration of bacteria are important parameters to consider before utilizing phage treatment. Because of this, phage therapy in open ocean aquaculture systems should be performed by, for instance, oral administration.

Phages outnumber bacteria by a tenfold, with an estimated number of 10^{31} in the biosphere (Labrie et al. 2010; Skurnik & Strauch 2006). They are naturally occurring in all ecosystems, and are constantly protecting animals from fatal infections by pathogens (Doss et al. 2017). In this way, phage therapy can be explained as using the natural nanoflora (viruses) to control the microflora (bacteria). Nevertheless, as shown in figure 1.5, phage propagation lags behind the host in time. In aquaculture, this time gap can result in huge financial losses during disease outbreaks. The idea of phage therapy is to add phages in sufficient amounts to quickly reduce

the bacterial population (Ly-Chatain 2014). Alternatively, they can be used as prophylactic agents, preventing diseases from occurring in the first place (Silva et al. 2014). For instance, fish and shellfish are easily stressed during molting or transport and handling, situations known to make the animals more susceptible to infections (Meyer 1991; Moriarty 1999). By applying phages to fish tanks during transport, the infection pressure of specific pathogens can be reduced, and infections might be prevented. Although, as with all treatments, some prerequisites apply. The phage-host interactions *in vivo* must be thoroughly investigated and understood, and to ensure pure phage preparations, strict regulations must apply during production. If phages are properly characterized, purified and correctly administered, they represent a promising antimicrobial with low environmental impacts (Almeida et al. 2009).

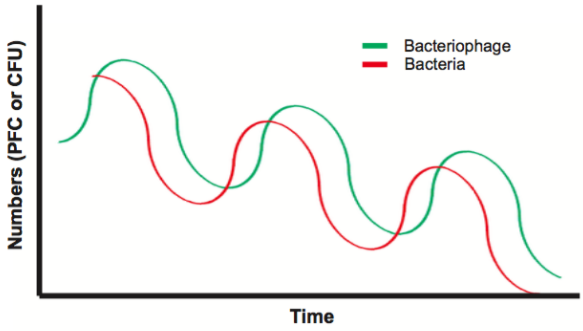


Figure 1.5: Growth dynamic of bacteria and phages. Phages lag behind their host in growth, as they need the bacteria to propagate. High numbers of hosts yield high numbers of phages. However, as phages lyse their hosts, the number of bacteria is reduced. Consequently, the number of phages is reduced as well. Figure adapted from Parracho et al. (2012).

1.3.4 Wide host range phages

The specificity of a phage is both of strengthening and limiting effect for phage therapy. Due to their specificity, the untargeted bacterial flora is unharmed, and narrow host ranged phages have shown promising results in several therapy trials (Karunasagar et al. 2007; Nakai & Park 2002; Silva et al. 2014). However, if different bacterial species are infecting the same host organism, using phages with narrow host range could result in the bloom of another, untargeted host. Successful treatment therefore requires infection and lysis of a broader range of bacteria at the same time. One solution for how to overcome resistant bacteria and/or infections by multiple bacteria, is to use a mixture of different phages, so-called phage cocktails (Mateus et al. 2014). A cocktail consisting of phages with different adsorption

receptors makes resistance development harder for the bacteria (Chan et al. 2013; Doss et al. 2017). However, mixing too many phages in one cocktail could affect non-targeted bacteria, posing similar problems as those seen with antibiotics (Chan et al. 2013). Also, producing polyphage cocktails entails higher development and manufacturing costs (Mateus et al. 2014).

On the other hand, if one single bacteriophage was to be effective against different pathogenic species, or strains, it would be of great advantage when it comes to production, handling and administration. The "ideal" phage for therapy would be an obligated lytic phage with wide host range, long environmental stability, high efficiency (large burst size, and short latent period) and low resistance development in the host (Mirzaei & Nilsson 2015). However, such wide host range phages are rare, making them harder to find (Kelly et al. 2011; Suttle 2007). Also, the term "wide host range" is defined differently among researchers. While some use it for phages infecting different species, others use it to describe a phage infecting different strains of the same species (Ross et al. 2016; Tan et al. 2014; Vinod et al. 2006). Throughout this work, wide host range phages will be used to define phages infecting different species.

2. AIM OF THESIS

Bacteriophages have been known for a long time, but their use in therapy have suffered from varying and inconsistent results. Today, better knowledge about phage-host interactions, in combination with modern technologies in sequencing and diagnostics, have made it possible to identify phages suitable for therapeutic use. The ability to screen phage genomes for known virulent and antibiotic resistance genes, in combination with proper purification and administration, have provided safer and more reliable therapeutic phages. However, one remaining challenge is that most phages have a very narrow host range.

The aim of this study was to isolate and characterize vibriophages as potential therapeutic agents in aquaculture, with emphasis on isolating wide host range phages. The knowledge acquired during this work will be used to improve current methods for isolating and identifying vibriophages intended for therapeutic use.

3. MATERIAL AND METHODS

3.1 General considerations during laboratory work

To minimize risk of contamination, all work concerning bacteria and phages where performed in sanitized laminar air flow (LAF) benches, using gloves disinfected with 70% ethanol and pre-sterilized equipment. Whenever bottles containing growth media were opened, these were opened inside a LAF bench, burned off with a gas-burner, and checked for bacterial growth (opalescent appearance) prior to use. Solutions containing bacteriophages were pipetted with filter tips to reduce the risk of cross-contamination via the pipette.

Growth media and buffers were prepared as recommended by the manufacturer, and sterilized by autoclaving (Appendix A). The exception was medium thiosulfate citrate bile salt sucrose (TCBS; CM0333; Oxoid), which was only heated until boiling. Agar plates were prepared by adding 1.5% of agar powder (VWR Chemicals) to the growth medium, prior to autoclaving. The same applied for preparation of soft agars, but with 0.5% of low melting-temperature agarose (SeaPlaque™ Agarose, Lonza, USA) instead.

Agar plates were stored at 4°C, and liquid media were stored at room temperature. During incubation and storage, plates were kept in airtight boxes or plastic bags to avoid drying out. Incubations were conducted at room temperature unless otherwise specified, and agar plates were inverted to prevent condensation from dripping into the agar surface.

3.2 General methods concerning bacteriophages

Phages require a host for propagation, meaning that phages will grow in basically any medium in which their hosts are growing. Consequently, growth media, pH-values and incubation temperatures were optimized according to the hosts requirements (*Vibrio* spp.). In addition, considerations were made regarding phages sensitivity towards drying, vortexing and vigorous mixing. As most phages tend to require divalent cations for host adsorption, penetration and multiplication (Chhibber et al. 2014; Rountree 1955), 5-10mM of calcium ions were added to all media used for phage propagation.

The best time point for phage introduction is, in general, when host cells are in early- to mid-exponential growth phase. Cells in their stationary phase are growing slow or not growing at all, giving phages little time to proliferate (Sambrook & Russell 2001). Throughout this work, phage infections were carried out at an optical density (OD) of 0.3-0.4. Bacterial cultures used for isolation or enrichment of phages were prepared by inoculating 30μl of glycerol stocks in 5ml growth medium. Cultures were grown over night (ON) at room temperature, before diluted to an OD of 0.1 in fresh growth medium. Once at an OD of 0.3-0.4, cultures were infected with phage solutions. An excess of bacteria versus phages were used to ensure phage-host interactions.

3.2.1 Plaque assay

Plaque assays are used for isolating and/or determining the number of phages present in a solution, giving the concentration in plaque-forming units per ml (PFU/ml). Phages, bacteria, growth medium and agar are mixed together, and phages with a lytic life cycle encountering a receptive host will infect it, replicate, and subsequently release new phage particles upon lysis. As progeny phages encounter nearby bacteria, the cycle is repeated multiple times and visible “holes” (plaques) are formed in the lawn of bacterial growth. The size of a plaque is usually restricted by the host reaching stationary growth phase (Fortier & Moineau 2009). However, other factors can affect plaque formation, such as the size of the phage itself, the phages’ adsorption time and burst size, and the concentration of agar. Phages ability to diffuse in agar is limited, where larger phages have a lower diffusion rate than smaller phages. Small phages therefore tend to form larger plaques.

Plaque assays were conducted as a soft agar overlay method, consisting of two layers of agar. Both layers contained media supporting growth of the host bacterium, with the bottom layer serving as a nutrient supplier and containing 1.5% agar. The upper layer contained indicator bacteria, page solution and 0.5% soft-agar, to improve phage diffusion. In sterile glass tubes, 100μl of lysate, 150μl of exponential growing host culture, and 3ml of soft agar were mixed together and poured on a bottom agar layer. Plates were incubated in airtight boxes, and inspected for plaques the following day. If the concentration of a phage solution (titer) was too high to separate single plaques, serial dilutions were prepared and assays repeated.

3.3 Amplification of nucleic acids

Polymerase chain reaction (PCR) is a common method for amplifying specific DNA fragments, making it a powerful tool in molecular biology. The PCR method is based on binding of short oligonucleotides (primers) complementary to targeted sequences on each side of a region of interest. Primers will recruit DNA polymerase and provide –OH groups on which the polymerase can extend a new DNA fragment (Kleppe et al. 1971). Heat-stable polymerases are required, as the amplification takes place in temperature cycles in a thermal cycler. Initially, high temperatures are used (~90°C) to denaturate dsDNA, before lowering the temperature to allow primer annealing. Then, by increasing the temperature to suit the DNA polymerase, primers are extended 5´-3´direction by incorporating nucleotides to the template. As the cycle is repeated, the number of PCR products increase exponentially. Following PCR, an agarose gel electrophoresis is usually set up to visualize DNA fragments formed by the PCR. By exploiting nucleic acids negative charge, an electric field can be applied to separate DNA fragments by size. Fragments will migrate through a network of agarose polymers towards the gel chambers anode (Sambrook & Russell 2001). Samples are visualized by using DNA binding factors, such as peqGreen, which fluorescence under UV-light. Band sizes are estimated by comparison to a ladder of known band sizes.

3.4 Schematic work flow

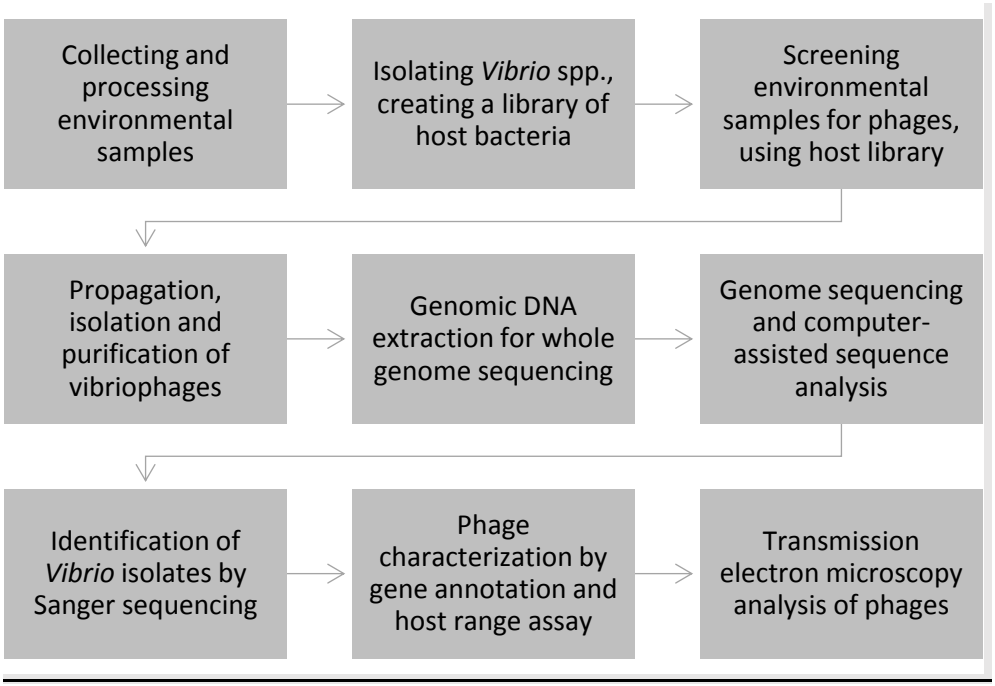


Figure 3.1: Overview of the steps conducted during this thesis, leading to isolation and identification of *Vibrio* spp. and vibriophages.

3.5 Sample material

Ocean water and mussels were used to search for both *Vibrio* spp. and vibriophages. Mussels were chosen as they are filter feeders, and therefore likely to accumulate microorganisms. A fistful of mussels and approx. 1L of water were collected from different locations at the East- and West Coast of Norway. At each sampling point, relevant information about the sampling site was recorded. This included a geographical description, water temperature, biological background and sampling date (see table 4.1). In addition, to indicate if *V. parahaemolyticus* was present in sampling areas, warnings by the Norwegian Food Safety Authorities regarding poisonous mussels was monitored. Samples were kept cold after sampling, and stored at -20°C.

Prior to analysis, mussels and water samples were thawed at 4°C ON. Mussels were crushed and the content centrifuged at 5000 x rpm for 10 min at 4°C to separate liquid from solid parts. Half of the supernatant was filtrated through 0.45µm membrane filters (Sarstedt AG) and stored at -20°C with 15% glycerol, for later use as source material in phage isolation (see

section 3.7). The remaining supernatant was resuspended with the pellet and kept at 4°C for use in isolation of *Vibrio* spp. Ocean water samples were filtered using vacuum-driven Stericup® and Steritop® filters (0.22μm; Millipore). Filtrations were performed under open flame to create a more sterile environment, and the filtered water was stored in pre-sterilized bottles at 4°C until used for phage isolation. Filter membranes were cut out and left rotating in 20ml of saline 6s(T) buffer (Appendix A) at 4°C ON, to dissolve bacteria from the filters.

3.6 Isolation of *Vibrio* spp.

Vibrio spp. were isolated from resuspended mussel pellets and filter membranes containing water sample bacteria. From each sample, 50μl was plated on thiosulfate citrate bile salt sucrose selective agar plates (TCBS, CM0333; Oxoid, Appendix A) and incubated ON. As human pathogenic *Vibrio* spp. often require temperatures around 37°C and most fish pathogens prefer temperatures at 25°C or below, samples were incubated at both temperatures to check for potential human pathogens. Additionally, to avoid excluding *Vibrio* species unable to grow on TCBS, samples were plated on less selective marine agar (MA, 2216; Difco, Appendix A) and incubated ON. To obtain single colonies, each sample was serial diluted and plated using a sterilized Drigalski spatula. Plates were inspected after ON incubation, and colonies obtained from different environmental samples, showing different morphology and growth conditions, were transferred to new MA plates and stored at 4°C for further confirmation of *Vibrio*.

Liver samples obtained from diseased shrimps from Vietnam were provided by Hans Petter Kleppen during this thesis. As samples were thought to contain species of *Vibrio*, it was decided to include them in this work. Samples were cultured on TCBS at 25°C ON, and colonies showing different morphologies were pure cultured 3 times. Pure cultures were grown in 6SYP ON to obtain glycerol stocks, and stored at -20°C.

3.6.1 Identification of *Vibrio* spp. by PCR

Polymerase chain reaction was used to check whether isolated colonies belonged to the genus of *Vibrio* or not. The PCR was performed as described by Dalmasso et al. (2009) using primers targeting a fragment within the *rpoA* gene, a gene encoding the alfa subunit of the RNA polymerase. Primers were claimed to be specific for most species belonging to the genus of

Vibrio, and were assumed to provide a quick method for differentiating *Vibrio* species from other bacteria.

Single colonies were transferred to the bottom of eppendorf tubes, using sterile toothpicks. Cells were opened by microwaving the tubes at 800W for 1 min before resuspended in 500µl ddH_2O. The *rpoA* targeted sequence (242bp) was amplified in a PCR containing 0.625U of One*Taq®* DNA polymerase (BioLabs Inc.), 0.2µM of primer VIB-1 [5'-AAATCAGGCTCGGGCCCT-3'] and VIB-2 [5'-GCAATTTTRTCDACYGG-3'] (Sigma-Aldrich), 200µM dNTPs (BioLabs Inc.), 1x One*Taq* Standard Reaction buffer (BioLabs Inc.), 1µl template and ddH_2O to a final volume of 25µl. All samples, a positive control (*V. anguillarum*) and negative control (ddH_2O) were amplified with MyCycler™ thermal cycler (BIO-RAD) according to conditions described by Dalmasso et al. (2009): 94°C for 3min, and 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. A final extension step was carried out at 72°C for 5 min to ensure completion of any partial amplicons.

Size of amplicons were checked by gel electrophoresis, using 2% UltraPure™ agarose gel (Invitrogen) run in 1x TAE-buffer (Appendix A) at 110V for approximately 1h. Samples were traced by adding 6x loading buffer (Appendix A), and the gel was stained using DNA-binding peqGreen (VWR Peqlab) for visualization under UV-light. 500ng of 100bp DNA ladder (BioLabs Inc.) was used as a reference marker to determine fragment size.

Bacterial isolates showing positive results in the PCR identification were pure cultured three times by repeated streaking on MA, and further grown in 6SYP (Appendix A) over night. Glycerol stocks were made from ON-cultures by freezing down cultures in Cryo-tubes (2ml) at -20°C and -80°C, together with 17% glycerol. Glycerol was added to prevent formation of large ice crystals, which could cause disruption of cells. Stocks at -20°C were used as working stocks, while stocks at -80°C were kept as backups.

3.7 Isolation of vibriophages

Environmental samples were screened for vibriophages using the established collection of *Vibrio* species. Prior to phage isolation and purification, phages were propagated to obtain higher concentrations.

3.7.1 Phage propagation

Filtered mussel- and water samples were challenged with each of the purified *Vibrio* isolates and *V. anguillarum* to propagate vibriophages. Phages in water samples were enriched by mixing 3ml of water with 200µl of exponential growing bacterial culture, and 1ml of 4x YP (final concentration of 1x) (Appendix A). Filtrated mussel samples were enriched equally, but in case the mussel juice contained insufficient amount of salts to support bacterial growth, growth medium 6SYP was used instead of YP. Enrichments were left incubating at room temperature for two days. A control tube without sample material was included for visual comparison of bacterial growth. After incubation, bacterial debris was removed by 0.45µm membrane filtering, and the resulting phage solutions (lysates) were stored in sterile glass tubes at 4°C until used for isolating phages.

3.7.2 Phage isolation and purification

Single phages were isolated from lysates by plaque assays, as described in section 3.2.1. Since the titer of lysates were unknown, phage solutions were serial diluted in TM-buffer (Appendix A). From the diluted phage solutions (10^{-2}, 10^{-4} and 10^{-6}), 100µl was gently mixed with 150µl of log-phase *Vibrio* cultures. Lysates were challenged with the same *Vibrio* isolate they had been enriched with to ensure propagation. Within 5 min after mixing phages and bacteria, 3ml of 6SYP soft agar (~38°C) was added to the tubes, and the mixture was poured on thin 6SYP agar plates. Plates were checked for plaques after ON incubation at room temperature. Throughout this study, *V. anguillarum* and phage KVP40 (Matsuzaki et al. 1992) were included as positive controls.

Single plaques showing different plaque morphology and size were isolated from agar plates using sterile pipette tips, and transferred to eppendorf tubes containing 1ml TM-buffer. Tubes were left at 4°C ON to allow phages to diffuse from the agar. Buffer solutions containing

phages were used in new plaque assays, and the process was repeated three times to increase the likelihood of obtaining single phage isolates. In the final isolation step, two eppendorf tubes containing 1ml of TM-buffer were prepared for each phage and stored at 4°C.

3.8 Extraction of DNA

Prior to whole genome sequencing (WGS) of bacteria and phages, DNA was extracted and quantified. Genomic DNA from *Vibrio* isolates were extracted by use of DNeasy Blood and Tissue Kit (Qiagen, Appendix B), according to the manufactures directions for Gram-negative bacteria. Briefly: ON-cultures were harvested by centrifugation at 11 000 x rpm for 5 min, and the resulting cell pellet was resuspended in lysis buffer ATL. To degrade proteins and disrupt cell membranes, proteinase K (Qiagen) and lysis buffer AL (10% SDS) were added. Tubes were left incubating at 56°C for 1h with occasional vortexing. To ensure DNA binding to the DNeasy Mini Spin Column, EtOH (96%) was added to each sample. Tubes were centrifuged at 8000 x rpm for 1 min to remove unwanted lipids and proteins. Spin columns were transferred to new collection tubes, and washed twice with buffer AW1 and AW2, respectively. Residual ethanol was removed by centrifuging tubes at 14 000 x rpm for 3 min. To elute DNA from the spin columns, 200µl of buffer AE was added to the centre of each column. After 1 min incubation samples were centrifuged at 8000 x rpm for 1 min, and the final elute was stored at -20°C until used for whole genome sequencing.

Before extracting phage DNA, phages were propagated to obtain high-titer lysates. Each phage was enriched with its respective *Vibrio* host by mixing 500µl of purified phage solution with 4.5ml bacterial culture, grown in 6SYP. Enrichments were left incubating for two days, before filtrated into sterile glass tubes using 0.45µm membrane filters. Lysates were used directly in phage DNA extractions.

A protocol for isolating DNA from vibriophages was developed using a combination of DNeasy Blood and Tissue Kit, and methods described by Kot et al. (2014). From each lysate, 150µl was transferred to an eppendorf tube and vortexed together with 120µl of TM-buffer and 10U of DNase I (Sigma-Aldrich). DNase I was added to degrade any residual bacterial DNA passing through the filtration. Samples were incubated at 37°C for 30 min before DNase I was

inactivated with 20µl of 0.5M ethylenediaminetetraacetic acid (EDTA, MERCK). To break down virus capsids, 5.6U of proteinase K (Sigma-Aldrich) and 200µl of lysis buffer AL were added, and samples were incubated at 55°C for 2h with occasional vortexing. Binding of phage DNA to DNeasy Mini Spin Columns was performed following the same procedure as for extracting bacterial DNA. After washing the columns, elution buffer AE was heated to 60°C and DNA was eluted by adding 20µl of buffer AE to the centre of each spin column. Tubes were left incubating for 1 min before centrifuged at 10 000 rpm for 1 min. To obtain maximum DNA yield, the elute was re-loaded into the spin column, left to incubate for 1 min, before repeating the centrifugation step.

3.8.1 Quantification of DNA

Following extraction, DNA concentrations were measured on a Nanodrop 2000 Spectrophotometer (Thermo Scientific), blanked with buffer AE (Qiagen). This instrument exploits nucleic acids ability to absorb ultraviolet light (UV-light) at wavelength 260nm (A_{260}). The purity of viral and bacterial DNA was determined by the ratio of A_{260} to A_{280}, as proteins absorb light at 280nm. Values between 1.8-2.0 were preferred, as lower values indicate contaminations by proteins, and a higher value indicates presence of RNA. However, since both DNA and RNA absorb light at 260nm, the spectrophotometer can yield too high DNA concentrations versus the reality. Therefore, to obtain more accurate values a Qubit® 2.0 fluorometer (Invitrogen™) was used, calibrated with standard solutions provided by the manufacturer. Qubit® dsDNA BR Assay Kit (Invitrogen™) was used for bacterial DNA, and Qubit® dsDNA HS Assay Kit (Invitrogen™) for phage DNA (Appendix B). Qubit® working solution (Qubit® reagent diluted 1:200 in Qubit® buffer) was added to each sample, to a final volume of 200µl. After 2min incubation, shielded from light, the DNA concentration was measured.

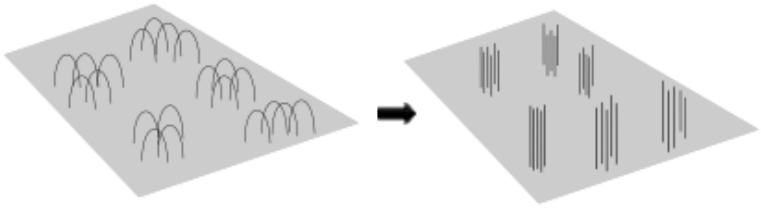
3.9 Whole genome sequencing

To determine the taxonomical class for bacterial isolates and obtain complete genome sequences for the bacteriophages, genomic DNA was sequenced using the Illumina MiSeq platform. In general, the Illumina workflow can be divided into three steps: sample preparation, DNA sequencing and data analysis (Fig. 3.2).

A: Sample preparation

B: DNA sequencing

1) Cluster generation



2) Sequencing by synthesis

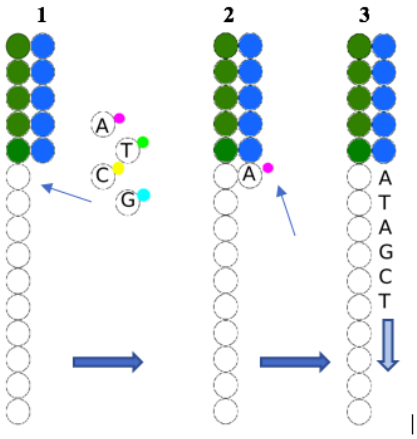


Figure 3.2: Illumina sequencing. **A:** DNA is simultaneously fragmented and tagged with short sequences (tagmentation). The tags (p1) work as primers in a following PCR amplification, during which new primer regions (p2) and index sequences are added. Indexes make it possible to identify the different samples, although being pooled together during the sequencing run. **B**: The DNA sequencing process consists of two main steps. **1)** Cluster generation. Primers added during the indexing- and adapter PCR (p2) are used to bind complementary probes attached to the sequencing flow cell. After binding, bridge amplification generates clusters containing up to 1000 copies of each fragment. **2)** Sequencing by synthesis. Primers bind to the p2 region and DNA polymerase is recruited. Deoxynucleotide triphosphates (dNTPs) are incorporated one at a time, 5' to 3' direction (step 1). After reading the colour of a fluorochrome attached to the dNTP (step 2), the fluorochrome is removed and the next base is incorporated (step 3). Step 1 and 2 is repeated to reveal the sequence.

Prior to every step in the sequencing preparation, reagents were removed from the freezer, thawed at room temperature, visually inspected for precipitation and mixed by inverting. Throughout the whole sequencing preparation, pipette tips were changed between each sample to avoid contamination.

3.9.1 Sample preparation

Samples were prepared for WGS using Nextera XT DNA Sample Preparation Kit (Illumina®, Appendix B) in accordance with the protocol provided by Illumina (Illumina custom protocol, 2016). Briefly: Genomic DNA was fragmented and tagged with universal overhanging tags, mediated by transposons. Indexing- and adapter PCR was set up to attach sample specific short nucleotide sequences (indices) and adapters to each DNA fragment, followed by a bead-mediated fragment length selection. During this selection, fragments <500 and >800 nucleotides were discarded. To minimize over- or underrepresentation of certain samples, tagged and purified DNA libraries were quantified using normalization beads. For optimal cluster generation and MiSeq sequencing, equal volumes of normalized DNA samples were pooled together. Samples were diluted to 8pM in HT1-buffer, before transferred into the sequencing cartridge. A 10% PhiX control library (Illumina, Appendix B) was included in the sequencing run to increase sample diversity.

3.9.2 Genome assembly and gene prediction

Illuminas cloud-based sequencing platform BaseSpace (basespace.illumina.com/home/index) provided raw sequencing data as FASTQ-files. Two FASTQ-files were provided for each sample, representing forward (R1) and reverse (R2) reads. Reads were paired, quality trimmed and *de novo* assembled into longer contigs in Geneious (version 8.1.9). For each sample, draft genome FASTA files were produced and uploaded to the Rapid Annotation using Subsystem Technology (RAST) server (www.rast.nmpdr.org) for gene prediction.

3.10 Identification of *Vibrio* spp. by Sanger sequencing

Vibrio isolates were identified to the level of species by supplementing WGS with an additional Sanger sequencing. Prior to sequencing, a PCR was set up following the same procedure as described in section 3.6.1: *Identification of Vibrio spp. by PCR.* The total volume of each

reaction was set to 50µl, utilizing the extracted DNA from section 3.8 as template. Primers designed by Thompson et al. (2005) were used for amplifying the *rpoA* coding gene, while primers for the 16S rRNA coding gene were designed for this study. The 16S rDNA sequences possible to obtain after Illumina sequencing were aligned with 16S rDNA sequences for *Vibrio* spp. available in the NCBI database. After alignment, conserved regions within the variable region v3 and v6 were discovered. These conserved regions were targeted by forward and revers primers, designed by using the Geneious provided software Primer3. Primer sequences are listed in table 3.1.

Table 3.1: Primer sequences targeting the 16S rRNA and *rpoA* gene.

Primer	Target sequence	Primer sequence	Reference
16S-v3-F	16S rDNA	5'-GACTCCTACGGGAGGCWGCAG-3'	This thesis
16S-v6-R	16S rDNA	5'-ACRACACGAGCTGACGAC-3'	This thesis
rpoA-01-F	*rpoA*	5'-ATGCAGGGTTCTGTDACAG-3'	Thompson et al. (2005)
rpoA-03-R	*rpoA*	5'-GHGGCCARTTTTCHARRCGC-3'	Thompson et al. (2005)

A total of 46 samples (one for each primer pair) and a negative control (ddH_2O), were amplified with MyCycler™ thermal cycler (BIO-RAD). Conditions were followed as recommended by New England BioLabs® Inc. for the One*Taq*® DNA Polymerase: 94°C for 30sec, and 30 cycles of denaturation at 94°C for 20sec, annealing at 54°C or 59°C for 30sec for the *rpoA* and 16S primers, respectively, and extension at 68°C for 1min. A final extension step was carried out at 68°C for 5min to ensure completion of partial amplicons. Amplicons were checked by gel electrophoresis, using 1,5% UltraPure™ agarose gel, as described in section 3.6.1.

Bands of wanted size were cut out from the agarose gel and purified using reagents provided by the Nucleospin® Gel and PCR Clean-Up Kit (Macherey-Nagel) for DNA Purification (Appendix B). Directions were mainly followed as described by the manufacture: Bands were excised from the gel, and 200µl of buffer NTI was added per 100mg gel. Tubes were incubating at 50°C for 5-10min to solubilize the gels. Solutions were then transferred to DNA-binding spin columns and centrifuged at 11 000 rpm for 1min. After discarding the flow through, columns were washed with buffer NT3 and centrifuged at 11 000 rpm for 1min. The washing step was repeated once more, before drying the membranes by centrifugation at 11 000 rpm for 2min.

DNA was eluted by adding 20µl of pre-heated ddH_2O (70°C) to the columns. After 3min of incubating at 70°C, tubes were centrifuged at 11 000 rpm for 1min, before repeating the elution step by adding another 20µl of ddH_2O.

Finally, 5µl of purified PCR products were mixed with 5µl of the respective primer used in the PCR. One sample was prepared for each of the forward and revers primer. Samples were sent for sequencing by GATC Biotech company, utilizing Sanger sequencing.

3.10.1 Sanger sequencing data analysis

Sequence data was retrieved from GATC Biotech (www.gatc-biotech.com/en/index.html).
ABI chromatogram files were downloaded and processed in Geneious, where each sequence was manually inspected and edited to remove ends of poor base call quality. For each bacterium, the retrieved 16S rDNA sequence was combined with the *rpoA* sequence and used in a nucleotide BLAST (Basic Local Alignment Search Tool) search, looking for sequences of significant similarity. Hits in the NCBI database were evaluated based on query coverage, identity (% similarity) and E-value, and used to identify which species the different bacteria were most similar to. Further, sequences of the 16S rRNA gene were aligned by MUSCLE alignment and a tree was constructed in Geneious, using FastTree 2.1.5 with default settings.

3.11 Characterization of phages

Phages were characterized through whole genome sequencing and host range plaque assays. The morphology of some phages was also studied through transmission electron microscopy.

3.11.1 Genomic analysis

Viral genomes obtained after WGS and assembly were uploaded to the RAST server for gene prediction. Retrieved Genbank files were studied in Geneious, and sequences of structural genes were imported to the search algorithm BLAST in search for similar sequences. Sequences showing high query coverage and identity were used to indicate which family a phage belonged to, given that the matching sequence was derived from a previously characterized phage. Annotated genomes were also used to predict whether phages contained possible toxic genes, lysogeny modules or antibiotic resistance coding genes.

A pan-genome analysis was carried out as described by Frantzen et al. (2017). Such comparative genomics compares genes of all isolates to establish a phylogenetic relationship between them. Using blastp, all protein coding sequences were compared against each other to detect orthologous genes, and these were clustered together into groups using the software GET_HOMOLOGOUS 2.0.10. For each genome in each cluster, a presence/absence matrix was constructed with present genes assigned the value of 1, and absent the value of 0. The 0/1 matrix was processed in R (https://www.r-project.org/) for statistical analysis. In R, the pan-genome matrix was clustered hierarchically using UPGMA with Manhattan distances. The clustered pan-genome was visualized using the heatmap.2 function to produce a heatmap with a dendrogram to represent pan-genomic distances between each bacteriophage sample. Phages which clustered together in the pan-genomic analysis, were studied by whole genome alignment using the Mauve plugin in Geneious.

3.11.2 Host range characterization

Host range plaque assays were performed to describe the phages phenotypes. From undiluted phage lysates, 150µl were distributed to a microtiter plate. Host bacteria were grown in 6SYP ON, before diluted to an OD ~0.1. Once bacteria reached the preferred OD of 0.3-0.4, 150µl of the cell culture was mixed with 3ml of 6SYP soft agar (~38°C), and immediately poured on thin 6SYP agar plates. After plates had solidified, a sterile 48-pin stainless steel replicator (Sigma-Aldrich) was dipped in the microtiter plates containing phages, and carefully spotted on the agar plates containing bacteria. Between each spot assay the replicator was dipped in 96% EtOH, flamed with a gas burner and cooled down in ddH_2O for sterilization. Each phage was tested in triplicates to increase the reliability. Plates were incubated at room temperature, and inspected for plaques the following day.

3.11.3 Transmission electron microscopy

The morphology of an isolated vibriophage was studied by transmission electron microscopy (TEM). Prior to TEM, a high titer lysate was obtained by phage propagation, before the lysate was concentrated by centrifugation.

The indicator host of the selected phage was grown from glycerol stocks ON, and further re-grown from an OD of 0.1 to 0.3 in 5 different glass tubes. Since the titer of the lysate was unknown, different volumes of lysate were added to the different glass tubes: 20µl, 50µl, 100µl, 150µl and 200µl. After ON incubation, the tube showing neither too high, nor too low bacterial growth was filtrated into a 50ml culture of exponential growing host cells (OD 0.3). The enrichment was incubated ON, with rotation, and centrifuged at 7000 rpm for 15min. Centrifugation was done to reduce clogging in the following filtration, where the supernatant was filtered into a 500ml culture of exponential growing hosts, using 0.45µm membrane filters. In order to exchange 6SYP media in the lysate with TM-buffer, a tagential flow filtration (TFF) was performed. Following ACD Pharmaceuticals proprietary methods, the 500ml culture was diafiltrated to a final volume of 50ml.

The phage lysate was concentrated according to descriptions by Deveau et al. (2006): 1.5ml lysate was centrifuged in an Avanti J-26 XP centrifuge at 24 000 x g for 1h in a Beckman JA-18.1 rotor. After centrifugation, the supernatant (~1.4ml) was pipetted of carefully, and the pellet was resuspended in 1ml of 0.1M ammonium acetate, pH 7.5 (Sigma-Aldrich). This step was followed up by another centrifugation at 24 000 xg for 1h, and the washing step was repeated. The remaining ~100µl of concentrated phage solution was stored at 4°C until used in TEM. Bacteriophage KVP40 was included in the TEM analysis as a control. The KVP40 solution was prepared by Eirik Bårdsen according to an ACD Pharmaceuticals proprietary method.

Purified phage solutions were negatively stained with 2% (w/v) uranyl acetate on a carbon formvar membrane grid (FCF400-Cu, Electron Microscopy Sciences) for 30sec. After removing excess of uranyl acetate, phages were visualized in a FEI Morgagni 268 transmission electron microscope (FEI company).

4. RESULTS

4.1 Sample material

Mussels and water samples were collected from the East- and West coast of Norway, in search for *Vibrio* spp. and their phages. From each sampling point, relevant information regarding the sample material and sampling site was gathered. The information is listed in table 4.1.

Table 4.1: Environmental samples used for isolation of *Vibrio* spp. and vibriophages

Location	Material	Water temperature	Environment description	Sampling date
Ulvøya, Oslo Beach area, facing open sea	Mussels Ocean water	20°C ± 1°C	Sandy bottom with low algae vegetation and biological activity[1]	26.07.2016
Malmøya, Oslo Marine harbour	Mussels Ocean water	20°C ± 1°C	Accumulating algae vegetation and some biological activity.	26.07.1016
Hellvik, Rogaland Marine harbour	Mussels Ocean water	18°C ± 1°C	Close to boathouses. High algae vegetation and biological activity.	06.08.2016
Drøbak, Akershus Marine harbour	Mussels Ocean water	20°C ± 1°C	Boat pier with attached algae vegetation	12.08.2016
Sildevika, Østfold Marine harbour	Mussels Ocean water	18°C ± 1°C	Swimming dock with attached algae vegetation	26.08.2016

[1] Biological activity means signs of marine life other than mussels, for instance crabs, starfish, shrimps and sea snails.

No warnings were announced by the Norwegian Food Safety Authorities regarding poisonous mussels at the time of sampling.

4.2 Isolation of *Vibrio* spp.

Culturing of mussel- and water samples on TCBS agar gave lower bacterial growth compared to culturing on MA, as seen in Fig. 4.1, A. Depending on the bacterias' ability to ferment sucrose or not, colonies on TCBS appeared yellow or green, respectively. For incubations conducted at 37°C, few potential human pathogens were revealed, and the ones that grew were discarded seeing they were irrelevant for further work. Cultivation of the same sample, several days apart, showed that storing samples at 4°C was insufficient to suppress bacterial growth (Fig. 4.1, B). Consequently, samples were processed within short time after thawing, in fear of losing sample diversity.

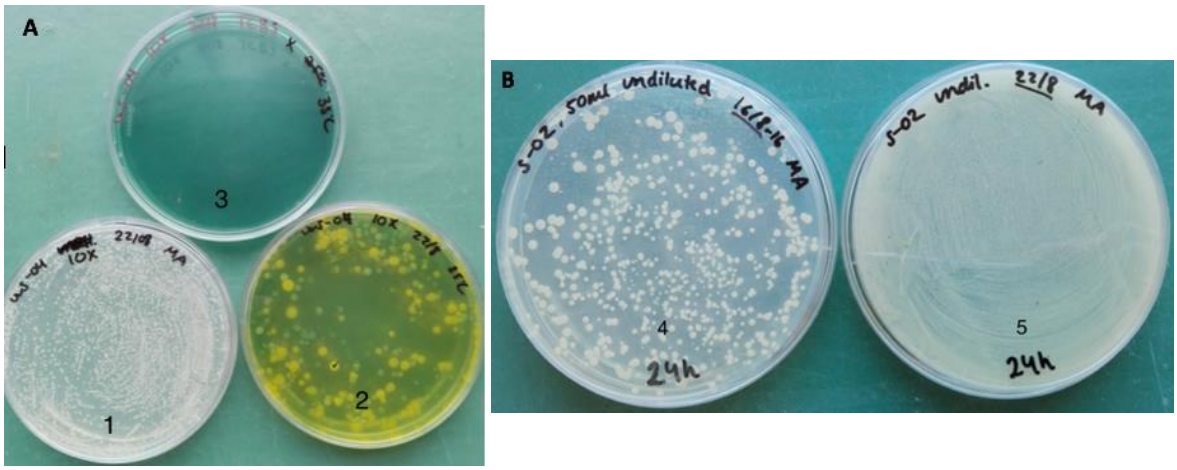


Figure 4.1: Culturing of *Vibrio* spp. on MA and TCBS agar. **A**: Bacterial growth on MA (plate 1) and TCBS (plates 2 and 3) after ON incubation. Plate 1 and 2 were incubated at room temperature, while plate 3 was incubated at 37°C. **B**: Bacterial growth on MA after ON incubation. Both plates were inoculated with 50µl from the same, undiluted sample. Plate 5 was prepared six days after plate 4.

4.3 Identification of *Vibrio* spp. by PCR

Culturing on agar plates resulted in 85 colonies suspected to be *Vibrio* spp. Of these, 40 colonies were isolated from MA and 45 colonies from TCBS. To confirm that isolates belonged to the genus of *Vibrio*, a *Vibrio* specific *rpoA* gene region was targeted by PCR. Amplicons were checked by agarose gel electrophoresis, and an example from 11 colonies is shown in figure 4.2. For the remaining bacterial isolates, results are not shown.

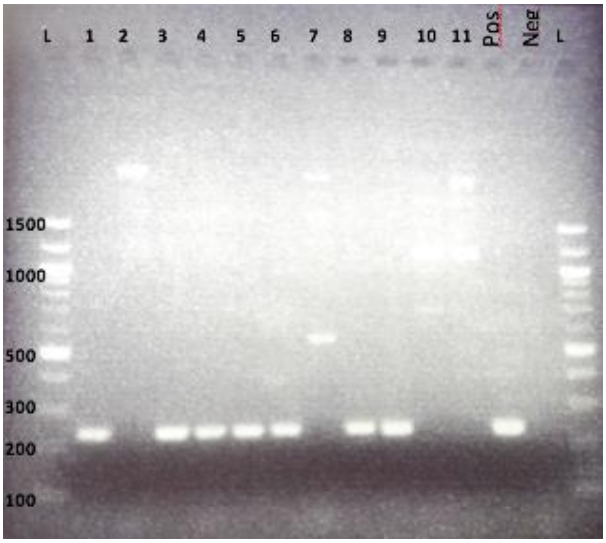


Figure 4.2: Agarose gel of PCR amplicons, revealing isolates positive for a *Vibrio* specific *rpoA* gene region. Positive isolates are indicated by a band size of 242bp**.** Lanes: 1-11; PCR products isolated from bacteria, 12-13; *V. anguillarum* and ddH_2O as a positive and negative control, respectively. Outermost lanes (L); 100bp-ladder with band sizes as stated. The gel was visualized with PeqGreen DNA/RNA loading dye under UV-light.

Of the 85 isolates suspected to be *Vibrio* spp., 43 gave positive results in the PCR analysis. From the 40 colonies isolated on MA, only 12 were positive for the targeted *rpoA* gene sequence (30%). In comparison, 31 out of 45 colonies isolated on TCBS were positive (69%). By combining information about the colony morphology with results from the PCR, 43 isolates were reduced to 23.

4.4 Isolation of vibriophages

A library of 24 purified *Vibrio* isolates, including *V. anguillarum*, was used to screen environmental samples for vibriophages. After 2 days' enrichment for phage propagation, lysates were used in plaque assays. The result from challenging two different *Vibrio* isolates with lysates obtained from the same sample material is shown in figure 4.3. The positive control, consisting of *V. anguillarum* and ΦKVP40, gave small, but clear plaques (Appendix C).

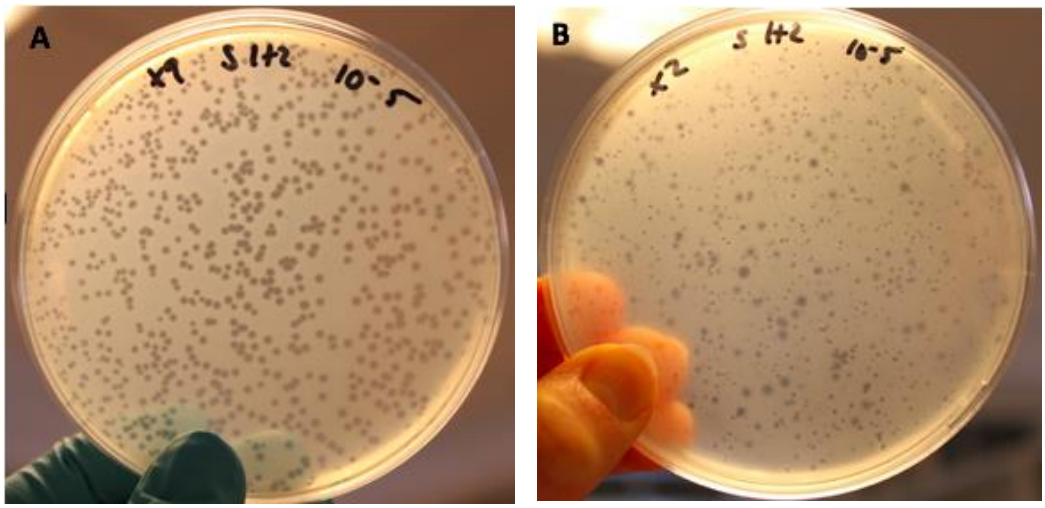


Figure 4.3: Plaque morphology. Two different bacterial isolates were challenged with the same lysate, diluted 10^5x**. A:** Large plaques with clear centers. **B**: Plaques of different size, shape and clarity. All plates were inspected after ON incubation.

Enriching environmental samples with different bacteria led to formation of morphologically different plaques. Some were turbid, some clear and some had haloes due to diffusion of degradative viral enzymes. The majority of phages were obtained from mussel samples as these, in general, gave rise to more plaques than water samples.

4.5 Extraction of DNA

Phages and bacteria were characterized through whole genome sequencing. Prior to this, DNA was extracted, a step implying some difficulties regarding phages. The protocol for viral DNA

isolation used by Kot et al. (2014) worked well for the control phage, ΦKVP40, but had to be modified to obtain sufficient DNA concentrations from the remaining phages. Modifications included doubling the input of Proteinase K and increasing the incubation time at 55°C from 30min to 2h. In addition, the elution step was repeated by re-loading the elute to the spin column.

4.6 Identification of *Vibrio* spp. by sequencing

Whole genome sequencing was carried out with a total of 23 bacterial and 25 viral genomes, including ΦKVP40. Various computer-assisted bioinformatic tools were used for analysing the MiSeq data, among them Geneious. Unfortunately, sequencing results for bacterial genomes were insufficient to assemble reads into longer contigs. With a genome size of approx. 5Mb, genomes were larger than expected. This resulted in low coverage. From the few results available, isolates could only be characterized to the genus of *Vibrio,* and not to the level of species. However, this revealed that two of the isolates were not *Vibrio*, but belonged to the genus of *Shewanella* and *Photobacterium*. These bacteria were excluded from further work.

4.6.1 Sanger sequencing

Sanger sequencing targeting the 16S rRNA and *rpoA* gene was performed to identify the 23 bacterial isolates. Data retrieved from GATC Biotech showed that sequences obtained with the *rpoA* targeting primers had a length of approx. 300bp, after trimming ends with low base call quality. For the 16S rDNA forward reads, sequences were approx. 60bp long, suggesting that the primers did not work as intended during sequencing. This might be due to differences in chemical and physical conditions between the PCR and sequencing reactions. However, 16S rDNA reverse reads returned with a length of approx. 600bp and were used to identify bacteria in a NCBI BLAST analysis. As a result, 10 different *Vibrio* species were identified (Appendix D). Combining 16S rDNA- and *rpoA* gene sequences gave no additional information to the identifications. The phylogenetic tree constructed in Geneious presented the species relationship (Fig. 4.4), and results from the host range assay (see table 4.2) were included to reveal different phage sensitivity profiles.

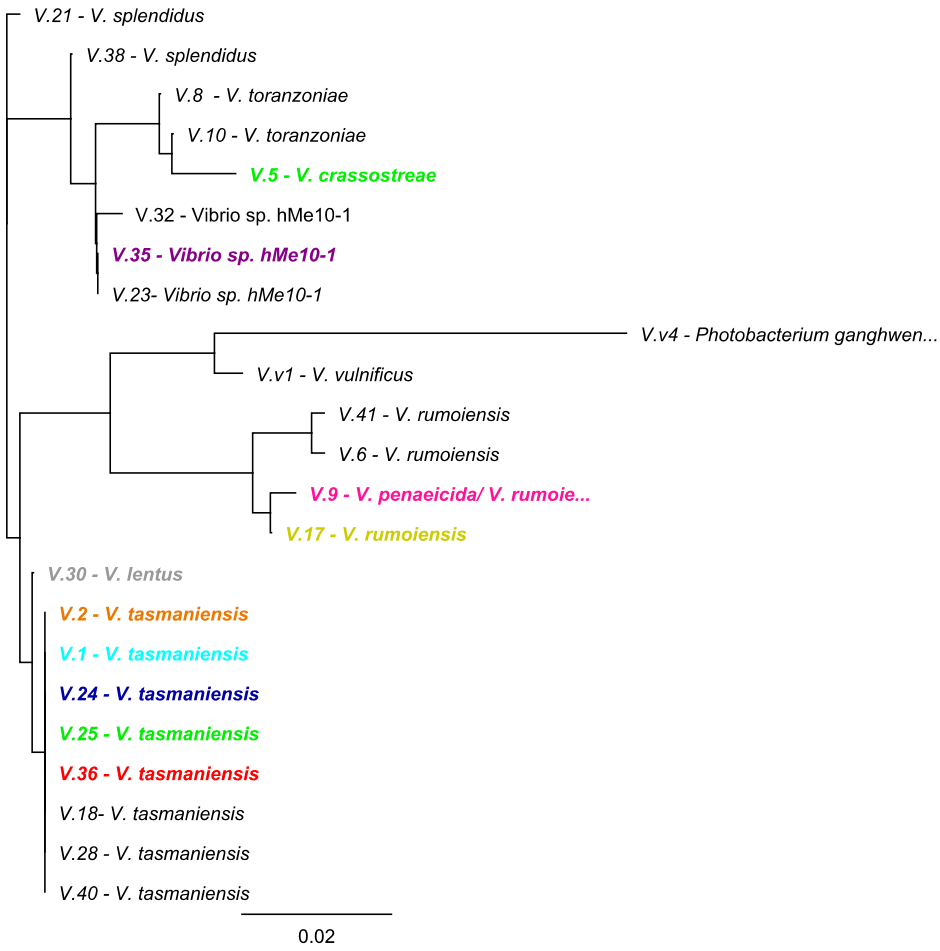


Figure 4.4: Phylogenetic tree based on the v3-v6 region of 16S rDNA sequences. Bacteria were isolated from Norwegian mussels and ocean water, with 2 isolates obtained from Vietnamese shrimp liver (V.v1, V.v4). The tree was designed using Geneious (version 8.1.9) with default settings for FastTree 2.1.5, after conducting a MUSCLE alignment of partial 16S rDNA sequences. FastTree calculates horizontal branch lengths according to Jukes-Cantor distance model. Colours show different phage sensitivity profiles, with black indicating no sensitivity discovered (no suitable phage found).

Bacterium V.9 was identified as *V. penaeicida* or *V. rumoiensis* in the BLAST analysis, with a sequence similarity of 99.8% and 99.9%, respectively. A following sequence alignment between the query sequence of V.9 and an isolate identified as *V. rumoiensis*, revealed 3 possible single nucleotide polymorphisms (SNPs). These were all too ambiguous to give a final identification (Appendix E). Isolate V.23, V.32 and V.35 got a match in the NCBI database with *Vibrio* sp. hMe10-1 (accession: KX453229), with a 100% identity score. However, clustering in the phylogenetic tree implied bacterium V.32 to have evolved slightly from the others. This suspicion was strengthened by the fact that V.32 gave yellow colonies on TCBS, while V.35 and V.23 both appeared green (Appendix D). Bacterium V.v4 was identified as a *Photobacterium*, and was excluded from further work.

Several bacteria identified as the same species showed different sensitivity towards phages. For example, of the eight bacteria identified as *V. tasmaniensis,* five of them were infected by different phages (Fig. 4.4). On the other hand, bacteria V.5 –*V. crassostreae* and V.25 –*V. tasmaniensis* shared the same phage sensitivity profile, although they were identified as different species. The same was observed for V.24 –*V. tasmaniensis* and V.35 –*Vibrio* sp. hMe10-1, where both were sensitive to the same phage.

4.7 Characterization of vibriophages

Unlike the bacterial genomes, viral genomes were small and assembled well after WGS. Each genome was annotated in RAST for gene prediction, and predicted structural genes were blasted in BLASTP in search for matching sequences. Sequences of previously described genes, showing significant similarity and query coverage, were used to characterize phages into families (table 4.3). Annotated genomes were checked for known toxic-, integrase- and antibiotic resistance coding genes, but none were detected.

The pan-genome analysis clustered phages into six groups based on genome similarities, meaning the presence or absence of protein coding genes (Fig. 4.5). Phages within each group had approximately the same genome size, with genome sizes ranging from 33kb to 111kb between the groups. Whole genome alignments were conducted for phages clustered together to identify genetic differences within each group. However, as most genes were identified as “hypothetical proteins”, this gave limited information.

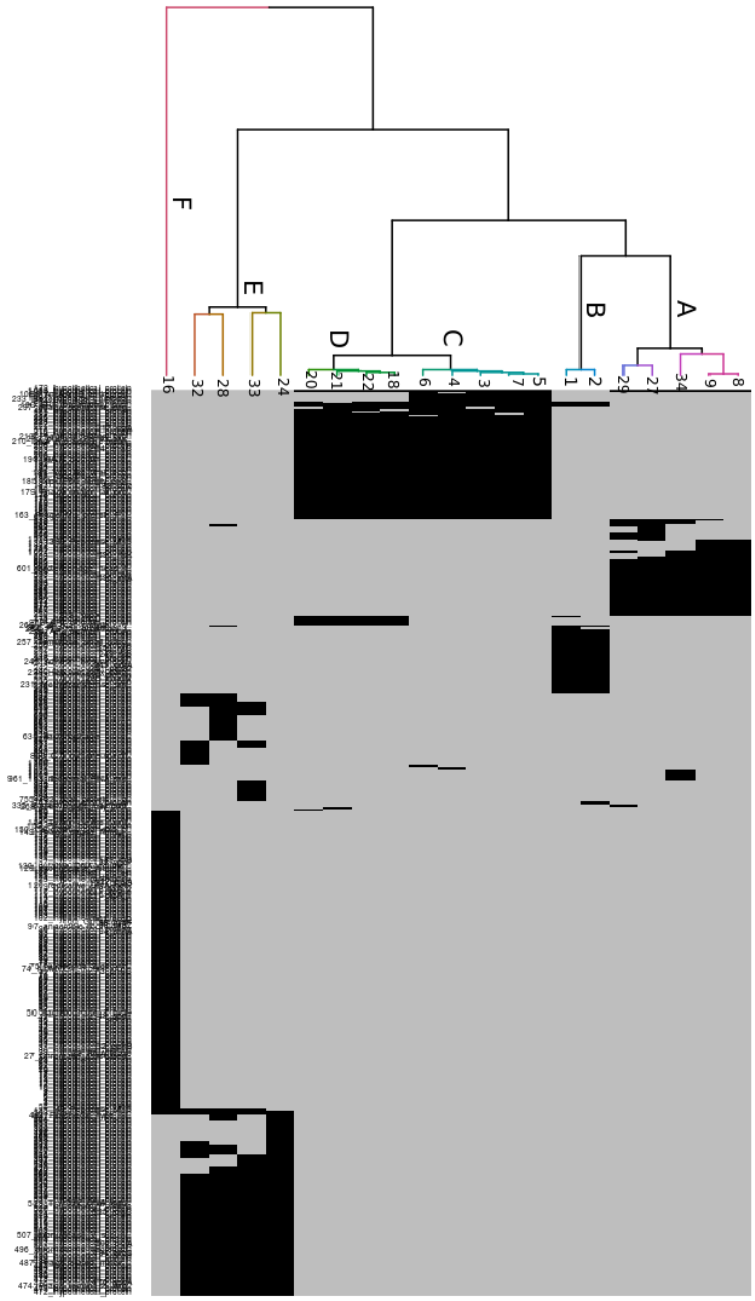


Figure 4.5: Pan-genome heatmap of viral genomes. Phages were clustered based on their protein coding genes, with black and grey areas indicating the presence and absence of genes, respectively. Numbers within each group (A to F) represents individual phages. Each gene (shown to the left) is arranged according to the phage grouping, and not according to the position in the genome.

4.7.1 Host range characterization

Plaque assays were conducted to test the vibriophages infection range. Simultaneously, this revealed the bacterias´ sensitivity towards phages (phage profile). Phage KVP40 and *V.anguillarum* were included as a control and for comparison. Results are shown in table 4.2.

Table 4.2: Host range assay of 25 phages (rows) against 11 bacteria (columns) [1,2].

	Bacterium										
Phage	**V.1**	**V.2**	**V.5**	**V.9**	**V.17**	**V.24**	**V.25**	**V.30**	**V.35**	**V.36**	**V.a**
Φ1	0	0	0	1	0	0	0	0	0	0	0
Φ2	0	0	0	1	0	0	0	0	0	0	0
Φ3	0	0	0	0	0	1	0	0	0	0	0
Φ4	0	0	0	0	0	1	0	0	0	0	0
Φ5	0	0	0	0	0	1	0	0	0	0	0
Φ6	0	0	0	0	0	1	0	0	0	0	0
Φ7	0	0	0	0	0	1	0	0	0	0	0
Φ8	0	0	0	0	0	1	0	0	0	0	0
Φ9	0	0	0	0	0	1	0	0	0	0	0
Φ16	0	0	1	0	0	0	1	0	0	0	0
Φ18	0	0	0	0	0	1	0	0	0	0	0
Φ19	0	0	0	0	0	1	0	0	0	0	0
Φ20	0	0	0	0	0	1	0	0	0	0	0
Φ21	0	0	0	0	0	1	0	0	0	0	0
Φ22	0	0	0	0	0	1	0	0	0	0	0
Φ24	1	0	0	0	0	0	0	0	0	0	0
Φ25	0	1	0	0	0	0	0	0	0	0	0
Φ27	0	1	0	0	0	0	0	0	0	0	0
Φ28	0	1	0	0	0	0	0	0	0	0	0
Φ29	0	1	0	0	0	0	0	0	0	0	0
Φ31	0	0	0	0	0	0	0	1	0	0	0
Φ32	0	0	0	0	0	1	0	0	1	0	0
Φ33	0	0	0	0	0	0	0	0	1	0	0
Φ34	0	0	0	0	0	0	0	0	0	1	0
ΦKVP40	0	0	0	0	1	0	0	1	0	1	1

[1] Black boxes: clear plaques; grey boxes: turbid plaques; white boxes: no plaques detected.
[2] Numbers indicate the results credibility. 1: plaques detected in two or three out of three possible. 0: plaques detected in one or none out of three possible.

In general, most phages could only lyse the host used for their first isolation. Together they infected 10 of the 23 bacterial isolates, yielding nine different host range profiles. No phage was active against bacterium V.6, V.8, V.10, V.18, V.21, V.23, V.28, V.32, V.38, V.40, V.41, V.v1 or V.v4. These bacteria were therefore excluded from table 4.2. A total of thirteen phages showed lytic activity towards bacterium V.24 (see appendix F). However, twelve of these were also isolated with the same bacterium. Vibriophage Φ31 was the only phage able to lyse bacterium V.30 –*V. lentus*, but due to poor genome assembly after whole genome sequencing it was not included in the pan-genome analysis (Fig. 4.5).

Worth noting is Φ32, which lysed bacterium V.24, but was originally isolated with bacterium V.35 (table 4.3). The two bacteria were identified as different *Vibrio* species, and were obtained from different locations in Norway (Appendix D). Similar observations were made for phage Φ16, which was isolated with bacterium V.5, but able to lyse both V.5 and V.25.

4.7.2 Transmission electron microscopy

With a genome size of 111kb, bacteriophage Φ16 was believed to be a *Myoviridae*. However, during BLASTP analysis some annotated genes were matched with genes previously isolated from a *Siphoviridae*. As this phage could lyse two different *Vibrio* species (table 4.2), it was decided to study its morphology by transmission electron microscopy (TEM). Results are shown in figure 4.6.

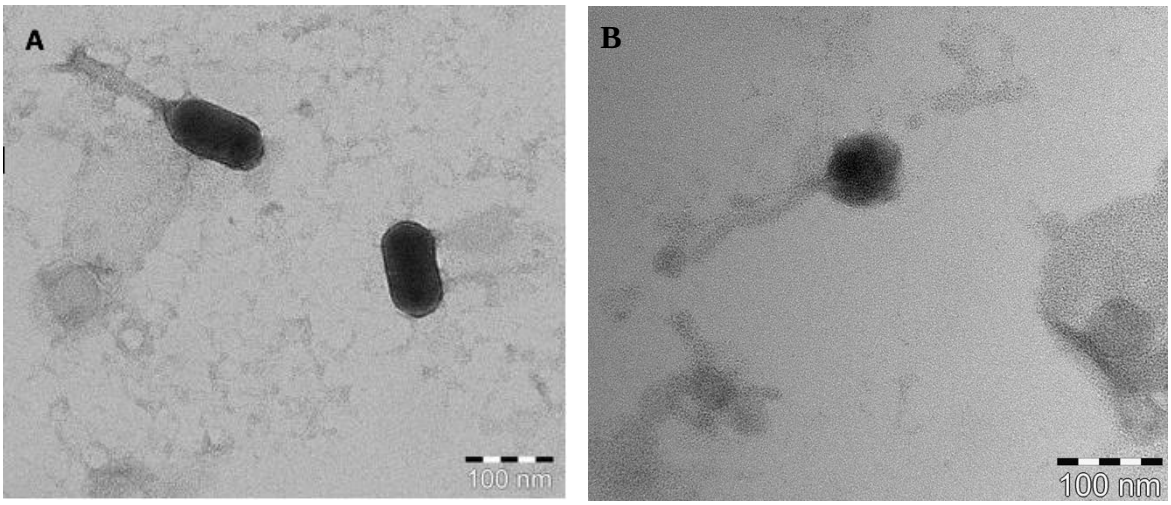


Figure 4.6: TEM analysis of the Φ16 lysate. **A**: Phages with morphology resembling a *Myoviridae*, with receptor binding proteins visible at the end of the contractile tail. **B**: A phage with similar morphology to a *Siphoviridae*. Phages were stained with 2% uranyl acetate.

The TEM analysis revealed that the lysate of Φ16 could contain two different phages. According to morphology description of the order *Caudovirales* (Fig. 1.2), the majority of phages resembled myoviruses, morphotype 2 (Fig. 4.6, A). These phages had a ~150nm long, contractile tail. Additionally, one of the detected phages was suspected to be a *Siphoviridae*, based on its ~200nm long, noncontractile tail (Fig. 4.6, B). This phage had a head shape resembling morphotype 1. However, only one such phage was detected within the same grid.

Bacteriophage KVP40 was also studied by TEM analysis, both as a control and for visual comparison. This phage has previously been characterized as a *Myoviridae*, morphotype 2 (Matsuzaki et al. 1992; Miller et al. 2003), which agreed with observations in this study (Fig. 4.7). One phage had its tail contracted, demonstrating the process of injecting DNA into a host cell (Fig. 4.7, A). The encircled phage in figure 4.7, B stood out from the rest with a head shape resembling morphotype 1. This phage could be a contaminating phage, but due to its contractile tail it was still believed to be a *Myoviridae.*

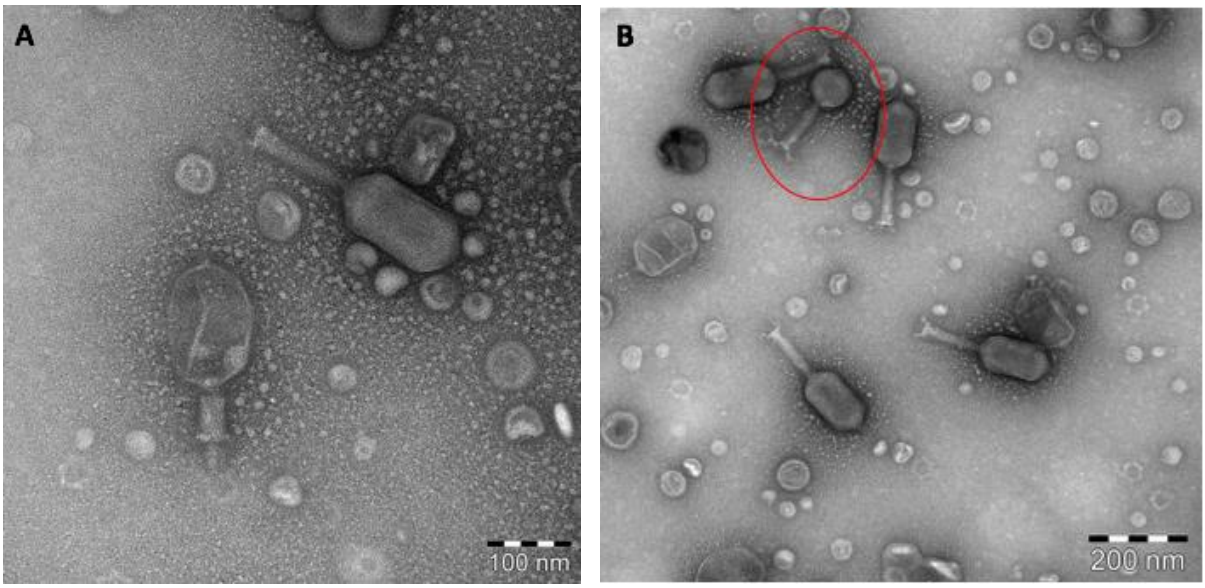


Figure 4.7: TEM analysis of phage KVP40**. A**: A phage with its tail stretched out, and one phage with its tail contracted. **B**: With the majority of phages resembling *Myovirdiaes*, morphotype 2, the encircled phage stood out with its head shape resembling morphotype 1. Phages were stained with 2% uranyl acetate.

Results from whole genome sequencing, pan-genome analysis, host range assays and TEM analysis were combined to characterize vibriophages isolated in this study (table 4.3). A total of 24 phages were isolated, including Φ31 (table 4.2). These were divided into 6 groups based on the pan-genome analysis, but according to the phages host range and their indicator bacteria, 11 different subgroups were identified.

Table 4.3: Overview of vibriophages isolated in this study. Information derived from WGS, pan-genome analysis, host range plaque assays and TEM analysis. All phage isolates were obtained from Norwegian mussel- and ocean water samples.

Phage group	Phage subgroup	Phage	Genome size	Virus family[1]	Source[2]	Indicator host	Host range	Possible host species[3]
A	A1	Φ8	33kb	*Podoviridae*	Mussels, Drøbak	V.24	V.24	*V.tasmaniensis*
		Φ9	33kb	*Podoviridae*	Mussels, Drøbak	V.24	V.24	*V.tasmaniensis*
	A2	Φ34	33kb	*Podoviridae*	Mussels, Drøbak	V.36	V.36	*V.tasmaniensis*
	A3	Φ25[4]	33kb	*Podoviridae*	Mussels, Ulvøya	V.2	V.2	*V.tasmaniensis*
		Φ27	33kb	*Podoviridae*	Mussels, Ulvøya	V.2	V.2	*V.tasmaniensis*
		Φ29	33kb	*Podoviridae*	Mussels, Drøbak	V.2	V.2	*V.tasmaniensis*
B	B1	Φ2	37kb	*Podoviridae*	Mussels, Ulvøya	V.9	V.9	*V.penaeicida/ V.rumoniensis*
		Φ1	37kb	*Podoviridae*	Water, Ulvøya	V.9	V.9	*V.penaeicida/ V.rumoniensis*
C	C1	Φ5	43kb	*Siphoviridae*	Mussels, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ7	43kb	*Siphoviridae*	Mussels, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ3	43kb	*Siphoviridae*	Mussels, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ4	43kb	*Siphoviridae*	Mussels, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ6	43kb	*Siphoviridae*	Mussels, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ19[4]	43kb	*Siphoviridae*	Water, Hellvik	V.24	V.24	*V.tasmaniensis*
D	D1	Φ18	45kb	*Siphoviridae*	Water, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ22	45kb	*Siphoviridae*	Water, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ21	45kb	*Siphoviridae*	Water, Hellvik	V.24	V.24	*V.tasmaniensis*
		Φ20	45kb	*Siphoviridae*	Water, Hellvik	V.24	V.24	*V.tasmaniensis*
E	E1	Φ24	86kb	*Siphoviridae*	Mussels, Sildevika	V.1	V.1	*V.tasmaniensis*
	E2	Φ33	86kb	*Siphoviridae*	Mussels, Drøbak	V.35	V.35	*Vibrio sp. hMe10-1*
	E3	Φ32	86kb	*Siphoviridae*	Mussels, Hellvik	V.35	V.24, V.35	*V.tasmaniensis, Vibrio sp. hMe10-1*
	E4	Φ28	86kb	*Siphoviridae*	Mussels, Hellvik	V.2	V.2	*V.tasmaniensis*
F	F1	Φ16	111kb	*Myoviridae/ (Siphoviridae)*	Mussels, Sildevika	V.5	V.5, V.25	*V.crassostreae, V.tasmaniensis*

[1] According to NCBI BLASTP analysis of structural genes.

[2] Source material for phage isolation

[3] Based on most related sequence in the NCBI database for the v3-v6 region of the 16S rDNA sequence (see appendix D).

[4] Φ19 and Φ25 did not cluster into groups during the pan-genome analysis, but were categorized into group C and A, respectively, based on their genome size, structural genes and host range.

5. DISCUSSION

5.1 Isolation and identification of *Vibrio* spp.

In this study, environmental samples from Norwegian mussels and ocean water, and a few from Vietnamese shrimp, were cultivated on MA and TCBS in search for *Vibrio* spp. Of the bacteria isolated on TCBS, approximately 70% belonged to the genus of *Vibrio*, compared to only 30% on MA. This selectivity of TCBS can be explained by the mediums high pH (8.5-9.5) and content of bile salts, inhibiting growth of most *Enterobacteriaceae* and Gram-positive bacteria, respectively. However, some strains cultivated on MA were unable to grow on TCBS. Further, whole genome sequencing and Sanger sequencing revealed that some of the bacteria cultivated on TCBS belonged to the genus of *Photobacterium* and *Shewanella*. As *Photobacterium* is a genus within the *Vibrionaceae* family, and *Shewanella* was previously included in this family, both are related to *Vibrio* (Balwos et al. 1992). This similarity can explain their ability to grown on TCBS. Overall, cultivation on TCBS was found to be an efficient way of screening environmental samples for *Vibrio* spp., but cultivation on MA should be included to avoid excluding some species.

5.1.1 Whole genome sequencing

Working with unidentified environmental isolates meant not knowing their genome size, and the number of genomes added to the sequencing run was based on previous experience with other bacterial species. Unfortunately, the bacterial genomes turned out to be larger than expected, resulting in low coverage. To produce a good assembly, a 50x coverage is recommended (Pightling et al. 2014). The MiSeq platform can yield around 25 million reads of approximately 300bp each in one sequencing run. As the bacteria had a genome size of around 5Mb, the required amount of reads per genome would be 5Mb/300bp x50 = ~830 000 reads. Turning this around, the maximum number of genomes added in one sequencing run would be (25 000 000 reads x 300bp/read) / (5 000 000bp x 50 coverage) = 30 genomes. Considering the fact that a viral genome is only ~1% the size of a bacterial genome (Hatfull 2008), loading of 23 bacterial and 25 viral genomes in one sequencing run should, in theory, work. However, since a portion of the sequencing reads are filtered out due to low quality scores, the total number of reads will be lower. Nevertheless, viral genomes assembled well because of their small size.

5.1.2 Sanger sequencing

Whole genome sequencing of bacterial isolates was supplemented with a Sanger sequencing targeting the 16S rRNA and *rpoA* genes. The *rpoA* gene was included as several species of *Vibrio* show high similarity in the 16S rDNA sequence, making it difficult to separate species based on this marker alone (Park et al. 2010; Thompson et al. 2005). According to Thompson et al. (2005), strains within the same species of *Vibrio* share ~98% sequence similarity in the *rpoA* gene. It was therefore expected that sequences obtained with the *rpoA* targeting primers would contribute in the separation of *Vibrio* species. However, combining the *rpoA* and 16S rDNA sequences gave no improved match in the NCBI database, compared to blasting the 16S rDNA sequence alone. A likely explanation is that the *rpoA* gene sequences obtained after Sanger sequencing had a length of approximately 300bp each, while the targeted gene should have been 951bp. As the *rpoA* gene is a strictly conserved housekeeping gene, the length of only 300bp is unlikely to add any additional information to that obtained with the 16S rDNA sequence.

As a result of the high similarity for the 16S rDNA sequences and the short *rpoA* sequences, bacterial isolates could only be identified to the level of species (Fig. 4.4). However, as previously shown by Tan et al. (2014), isolates of close genetic relationship often show a high diversity in phenotype. For instance, of the eight isolates identified as *V. tasmaniensis*, five different phage sensitivity profiles were observed. Also, from the three isolates having a 100% sequence similarity to Vibrio sp. hMe10-1, only one of them was susceptible to a phage. According to this, phage typing can serve as a useful tool in the differentiation of bacterial strains, making it easier to map the diversity.

5.2 Isolation and characterization of vibriophages

The 24 vibriophages isolated from Norwegian mussels and ocean water samples covered all three phage families of the order *Caudovirales*. Together, phages lysed 10 of the 23 bacterial strains, and were divided into 11 different phage groups based on their genotype, host range and indicator strain (table 4.3).

To obtain sufficient phage DNA for WGS, the input of Proteinase K was doubled and the incubation time was quadrupled, compared to the control phage (ΦKVP40). One possible

explanation for the need for these modifications is that the lysate was contaminated by proteins from the host bacterium. If so, contaminating proteins might have prevented efficient Proteinase K treatment of the phage capsids, thereby preventing DNA release. Moreover, it is known that phages having terminal proteins (TP) covalently bound to their genomes, require prolonged proteinase K digestion to achieve successful DNA isolation (H.P. Kleppen, pers. comm.). However, phages with terminal proteins bound to the DNA have, so far, only been detected in podoviruses (Kleppen 2012).

5.2.1 Virulent and temperate phages

Successful phage therapy requires efficient lysis of the host, making it essential to know whether a phage is virulent or temperate (Doss et al. 2017). For plaque assays, there is a general appreciation of virulent phages yielding clear plaques, as they lyse most of the hosts they infect. Temperate phages typically give turbid plaques due to prophages residing within the host genome, providing resistant lysogenic clones (Kutter & Sulakvelidze 2005). However, the interaction between a phage and its host can be complex and difficult to determine based on plaque morphology alone (Gallet et al. 2011). For example, obligate lytic phages can cause turbid plaques if the bacteria develop resistance through mutations, and temperate phages can cause clear plaques if all, or most, phages choose a lytic pathway. Consequently, to check whether the phages were virulent or not, phages and their corresponding indicator host were sequenced by whole genome sequencing. The plan was to identify identities between the isolated phages and prophage sequences carried by the host. However, as WGS of bacteria did not work out as planned, an alternative approach was used. Viral genomes were checked for integrase coding genes, enzymes expressed by temperate phages to catalyze the integration of viral DNA into the host genome (Groth & Calos 2004). Yet, as with the resistance- and toxic- coding genes, no such genes were detected. Another possible approach would be to study lysis curves in liquid cultures. If the introduced phage is a temperate phage with specificity for the bacteria of interest, the growth curve should drop and stagnate a bit until prophages make the bacteria resistant. Once resistant, bacteria will continue to grow.

5.2.2 Phage diversity

Phage-host interactions can be difficult to predict. The pan-genome analysis divided phages into six different groups (Fig. 4.5), and since phages within each group shared the majority of protein coding genes, they were expected to share the same host range. However, during host range analysis, nine different infection profiles were detected (table 4.2). Bacteriophage Φ24, Φ33, Φ28 and Φ32, for example, were clustered together in the pan-genome analysis (group E), but infected different hosts. Moreover, when combining genomic data (genome size and virus family) with phages lytic ability, 11 different vibriophages were discovered (table 4.3). For instance, Φ25, Φ27, Φ28 and Φ29 were isolated with indicator V.2 and had the same host range. Consequently, they were suspected to be the same phage. However, genome sequencing revealed that Φ28 was a siphovirus, while Φ25, Φ27 and Φ29 were identified as podoviruses. Another interesting point was that they all infected the same host, even though Φ28 had been isolated from a different location than the rest. The same was observed for Φ8 and Φ9. Like all phages clustered into group C and D (table 4.3), Φ8 and Φ9 were isolated with bacteria V.24. Nevertheless, phages in group C and D were isolated from material obtained at the West coast of Norway, and were identified as siphoviruses. Φ8 and Φ9, on the other hand, were isolated from the East coast and were found to be podoviruses. This proves that the infection range is not necessarily restricted to the local host community, and that a large diversity of phages can be found within limited sample material.

As mentioned, phages which clustered together in the pan-genome were expected to have similar host ranges due to their genomic similarities. Seeing this was not always the case, genome alignments were performed within each group to detect if the different phenotypes could be explained by differences in the genotype. The idea was that phages' host range would be reflected by differences in receptor binding proteins (rbp). If sequence variations were to be detected within the same rbp, this could explain why otherwise similar phages infected different hosts. However, few genes encoding known rbps were detected, and most of the observed sequence variation was found in genes encoding "hypothetical proteins". Information derived from sequence alignments was therefore insufficient to identify which genes were responsible for the different host specificities.

5.2.3 Wide host range phages

A limiting factor in phage therapy is the narrow host range of phages, which means that one would need a large collection of phages to cover the bacterial diversity (Mateus et al. 2014). However, if a phage could infect a broader range of hosts, successful treatment could be achieved with just a few different phages, making production and treatment both easier and cheaper (Ross et al. 2016).

Regarding host range, vibriophage KVP40 infected four different species and outcompeted all phages isolated in this study (table 4.2). It has previously been suggested that a wide host range is related to the size of the phage genome (Letchumanan et al. 2016; Tan et al. 2014), due to the possibility of more genes encoding receptor binding proteins for host adsorption. For instance, ΦKVP40 has a genome size of 244kb, and its flexibility in host adsorption is assumed caused by multiple genes encoding tail associated proteins (Letchumanan et al. 2016; Matsuzaki et al. 1992). Although sequence alignments were insufficient to detect a possible correlation between the vibriophages genetic content and host range, there might be a connection between their genome size and host range. Φ16 and Φ32 were the only two phages able to infect another species than their indicator host. Interestingly, these phages were also among the largest with a genome size of 111kb and 86kb, respectively (table 4.3).

Compared to ΦKVP40, the host range of Φ16 and Φ32 was limited. Furthermore, the infection range of Φ16 could be questioned. When challenged with bacterium V.25, turbid plaques were observed (table 4.2). Hence, Φ16 could be a temperate phage, protecting the bacteria from lysis. However, since infections of the indicator host (V.5) produced clear plaques, this is rather unlikely. Other explanations could be that bacterium V.25 is resistant to the phage due to some resistance mechanism, or that lysis was caused by other factors than the phage itself. According to Mirzaei and Nilsson (2015), growth inhibition during spot assays might be a result of “lysis from without”, meaning that bacteriosins produced by the indicator bacteria, or lytic enzymes produced by the phage, are causing the lysis (Ross et al. 2016). Mirzaei and Nilsson (2015) argument that during spot assays, the large drop of undiluted phage solution can cause false positives. In comparison, during plaque assays one usually test different concentrations of the lysate, which are dispersed into the agar together with the host. In this way, growth inhibition is observed as plaques form over an entire plate, instead of in one spot. This narrows

down the risk of misinterpret lysis caused by bacteriosins or enzymes, particles that do not reproduce, for being the work of a phage. Nevertheless, if “lysis from without” was the case for the Φ16 lysate, growth inhibition would probably be observed for several bacteria in the host range assay, and not just for V.5 and V.25.

The credibility of Φ16s’ host range was questioned further after genome sequencing and TEM analysis. According to the genome size of 111kb, the phage was suspected to be a *Myoviridae.* However, Φ16 had protein coding genes similar to genes previously isolated from both a *Myoviridae* and a *Siphoviridae* phage. TEM analysis revealed phages with morphological similarities to the myovirus ΦKVP40 (Fig. 4.6, A and 4.7), but a phage resembling a *Siphoviridae* was also detected (Fig. 4.6, B). The *Siphoviridae* could represent a contaminating phage, as only one such phage was detected within the same grid. Nevertheless, whether or not the lysate contained several siphoviruses cannot be said for certain. The host flexibility of Φ16 should therefore be asserted with caution. If the lysate contains two different phages, the observed host range may be a result of a phage cocktail, rather than the work of a single wide host range phage.

5.2.4 Phage-host co-existence

According to Doss et al. (2017) and Labrie et al. (2010), phages are found wherever there are bacteria. Enriching environmental samples with bacteria isolated from the same sample should then guarantee isolation of phages against the exact same bacteria. However, during this study it was discovered that some samples harboring *Vibrio* spp. did not harbor a corresponding phage, implying that phage and host might not always co-exist. A more likely explanation though, would be that phages were present in low concentration to begin with and further lost during filtration and handling. This is not uncommon, and has been reported before (Tan et al. 2014).

5.3 Finding wide host range phages

In 1926, d’Herelle identified the first phage against *Vibrio*, and multiple vibriophages have been discovered since (Rowe et al. 1992). During this study, little doubt was given towards finding vibriophages. However, the challenge was finding lytic phages infecting a wider range

of *Vibrio* species, due to the lack of descriptive and concrete protocols for doing so. Phages are usually isolated from the same environment as their host, but phages isolated with a co-occurring host will often have a narrow host range, specific for the same strain they were isolated with (Mirzaei & Nilsson 2015; Ross et al. 2016; Vinod et al. 2006). Consequently, in an attempt to isolate wide host range phages, environmental samples were enriched with different *Vibrio* isolates, obtained from different locations. The idea was that enriching samples with both co-occurring and non-co-occurring bacteria would increase the chance of finding phages with a wider host range. Since two of the isolated phages showed lytic potential towards two *Vibrio* species, this worked to some extent. However, phages with even broader host range might have been obtained if the isolated bacteria had not been derived from the same type of environment. Despite samples being obtained from different locations along the coast of Norway, samples were collected from the same climate. It is possible that using indicator bacteria isolated from e.g. tropical areas to screen water samples from Norway, will increase the likelihood of isolating phages with a broader host range. For instance, if phages in Norwegian water samples can be isolated with bacteria from Vietnam, these phages might have receptor binding proteins interacting with a host receptor conserved among several bacterial species.

Results from this study have led to the understanding that finding wide host range phages can be demanding. Consequently, the alternative of "developing" them should be considered. In 2016, Mapes et al. presented a host range expansion (HRE) method to obtain phages able to lyse several strains of the bacterium *Pseudomonas aeruginosa*. Their method was based on adding different bacterial strains to each row in a microtiter plate, before serial diluting a mixture of four phages in each row. Lysates were pooled from wells having the lowest concentration of phages, but still complete lysis of the host bacteria. The pooled sample was used in a new round of the HRE method. After 30 cycles, they were left with a cocktail of phages with expanded host range. Although this method was conducted using different strains of the same bacterial species, it should in theory work when using different species. The expanded host range of the final phage cocktail was suggested to be caused by mutations and/or recombination among the phages (Mapes et al. 2016). Hence, instead of searching for a "perfect" phage in nature, one might be able to breed forward the desired phage.

Enriched mussel samples gave rise to 17 of the 24 vibriophages, with the remaining ones isolated from ocean water. This was expected since the high concentration of bacteria in filter feeders is thought to accumulate corresponding phages (Karunasagar et al. 2007). However, as shown in this study, phages isolated from samples with a high concentration of host bacteria are likely to have a narrow host range. According to Duffy et al. (2006), host expansion can affect phages ability to infect the original host. Due to this cost in fitness, it is natural that most phages have a narrow host range. Nevertheless, Duffy et al. (2006) also suggested that there are a broad range of ecological conditions favoring phages with an expanded host range. The lower abundance of relevant hosts in the ocean might be such a condition, giving phages with a wider host range an advantage. Based on these assumptions and the fact that the ΦKVP40 was isolated from ocean water (Matsuzaki et al. 1992), chances of finding wide host range phages is believed to be higher in water samples than in mussels.

As most *Vibrio* spp. are opportunists, they respond to favorable conditions by rapid growth (Suttle 2007). Colleting water during the high season of *Vibrio* (usually summer months) can resemble the process of looking for phages in mussels, in which both are likely to yield opportunistic phages. Such phages (known as r-strategists) typically have small genomes, replicate fast and are highly lytic with large burst size (Mirzaei & Nilsson 2015; Suttle 2007). As the host expands, the number of viral infections will increase correspondingly, consequently reducing the level of bacteria back to “normal”. This “boom-and-burst” cycle is likely to select for efficient, but narrow host ranged, phages. Consequently, screening water samples during winter months might seem to be a better strategy for finding wide host range phages. However, according to a mini-review by Paul, J. (2008) it was found that “the greatest occurrence of lysogeny was during winter months, when bacteria and primary production was lowest”. Starving host cells seems to promote a selection for temperate phages due to the phages ability to switch to a lysogenic cycle. During starvation, the low activity of the host is thought to promote expression of viral genes encoding lysis repressors, making the prophage remain latent within the host genome (Kutter & Sulakvelidze 2005). This considered, one is faced with the problem where searching for vibriophages during high season of *Vibrio* is likely to yield narrow ranged, but lytic, phages. Alternatively, looking for phages when there is a low abundancy of *Vibrio* is more likely to favour wide host ranged, but lysogenic, phages (Suttle

2007). Consequently, the best time for sampling water might be during spring or late autumn, when the concentration of *Vibrio* is neither at its highest, nor at its lowest.

Even though samples included in this study were collected during summer months, two of the isolated phages appeared to infect two different species of *Vibrio*. This contradicts with the hypothesis of narrow ranged phages dominating during the bloom of *Vibrio*. A possible explanation to this is that no warnings were announced by the Norwegian Food Safety Authorities regarding poisonous mussels at the time of sampling. The concentration of *Vibrio* might not have reached a dominating level, giving less specific phages a chance to infect and propagate.

5.4 Phage therapy *in vitro* versus *in vivo*

It is important to keep in mind that phage therapy is a form of active treatment, making establishment of physical contact between the phage and its host a critical factor for success. However, achieving this *in vivo* can deviate greatly from *in vitro* experiments (Skurnik & Strauch 2006). A phage showing broad host range and high efficiency of infection in lab, might not do the same in real life. Assumptions and conclusions derived from this study were all based on *in vitro* observations, where factors such as growth media, concentrations, pH and temperature were highly regulated. Since infections only occur if a phage encounters a host, phages must be added in the right amount and at the right time. This was demonstrated by Skurnik and Strauch (2006), whom despite successful trials *in vitro*, were unable to reproduce the results once applied to mice *in vivo*. These observations should also be taken into consideration when evaluating phage resistance development among bacteria. According to Sharma et al. (2017) and Ly-Chatain (2014) the mutation rate among bacteria is lower in nature than in laboratories. A possible explanation to this is that, under laboratory conditions, phages are the main factor posing a selective pressure towards bacteria. In the environment, other factors such as competitors, nutrients and other viruses all contribute to form a higher and more diverse pressure to which bacteria much adapt.

5.5 Concluding remarks and future prospects

In a report from 2014 regarding global antibiotic resistance, the World Health Organization (WHO) stated that *"antibiotic resistance is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country"* (WHO 2014). In the same report, the need for developing new tools to combat the emerging resistance was pointed out. The best would probably be to prevent disease outbreaks by vaccination, but in aquaculture there is a limited selection of vaccines available. Additionally, vaccination of fish larvae can be difficult due to their small size and high number, and the fact that some fish larvae are unable to develop specific immunity during their short lifetime (Silva et al. 2014). An alternative to vaccination could be phage therapy, where phages are applied directly into the water, preventing colonization of pathogens inside the fish and on the surface (Silva et al. 2014). Phage therapy will probably never fully replace antibiotics, but may help reduce todays consumption, and hopefully, contribute to reduce antibiotic resistance development.

Throughout this work, environmental samples were processed in search for *Vibrio* species and vibriophages. A library of *Vibrio* spp. was established and used for isolating vibriophages. Isolates were characterized based on information derived from their genotype, combined with their observed phenotype. From a total of 24 isolated vibriophages, 11 different phages were successfully identified, covering all three families within the order *Caudovirales*. Of these, two showed potential for infecting more than one *Vibrio* species. This shows that wide host ranged vibriophages are more demanding to find than narrow ranged phages. After considering results from this study together with observations made by others, a theory was developed for how to isolate wide host ranged vibriophages more efficiently in the future. Overall, one must consider the host of interest, the sample material, time of sampling and method of propagation. It is believed that better results can be obtained by isolating vibriophages from large volumes of water during spring or late autumn. These samples should further be enriched with species spanning a wide phylogenetic range within the *Vibrio* genus, so that a more systematic and efficient screening for wide host ranged phages can be achieved. Should phages be successfully isolated through this method, the next step could be to develop them further by the HRE method, and ultimately employ them for therapeutic use in aquaculture.

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7. APPENDIX

Appendix A: Growth media, agars and buffers

Table A.1: Recipes for growth media, agars and buffers used during the study.

Medium/buffer	Ingredients/L	Supplier
6sYP		
NaCl	19.45g	Oxoid
$MgCl_2x6H_2O$	18.7g	Oxoid
Na_2SO_4	3.24g	Merck
$CaCl_2x2H_2O$	2.40g	Oxoid
KCl	0.55g	Merck
$NaHCO_3$	0.16g	Merck
Yeast Extract	1g	Oxoid
Bacto Peptone	10g	Difco
	Dissolve ingredients in dH_2O and bring the volume to 1L. If medium is to be used for agar plates, add 15g of agar powder (VWR Chemicals) prior to autoclaving. For soft agars, add 0,5% SeaPlaque™ Agarose (Lonza, USA).	
Marine Agar (MA)		
Marine Agar powder	55.1g	Difco
	Dissolve powder in dH_2O and bring the volume to 1L. Prior to autoclaving, make sure to boil medium for 1min to ensure completely dissolved powder. Precipitations is expected and may cause cloudy agar plates.	
Thiosulfate citrate bile salt sucrose (TCBS)		
TCBS growth powder	88g	Oxoid
	Dissolve powder in 1L dH_2O. Heat until boiling while stirring, but do not autoclave. Cast plates straight away and let them cool properly before storing at 4°C to avoid condensation.	
YP (concentrated 4x)		
Yeast extract	0.4g	Oxoid
Bacto peptone	2.0g	Difco
	Dissolve ingredients in dH_2O and bring the volume to 100ml. Autoclave.	
6s(T) buffer		
NaCl	19.45g	Oxoid
$MgCl_2x6H_2O$	18.7g	Oxoid
Na_2SO_4	3.24g	Merck
$CaCl_2x2H_2O$	2.40g	Oxoid
KCl	0.55g	Merck
$NaHCO_3$	0.16g	Merck
1M Tris-HCl, pH 7.4	10 ml	
	Dissolve ingredients in dH_2O and bring the volume to 1L. Add 10ml of 1M Tris-HCl and autoclave	

Medium/buffer	Ingredients/L	Supplier
TM buffer, pH 7.4		
NaCl	5.84g	Oxoid
$MgCl_2x6H_2O$	2.03g	Oxoid
$CaCl_2x2H_2O$	1.47g	Oxoid
1M Tris-HCl, pH 7.4	10ml	
	Dissolve ingredients in dH_2O and bring the volume to 1L. Add 10ml of 1M Tris-HCl and autoclave	
1M Tris-HCl, pH 7.4		
Trizma® base	121.14g	Sigma-Aldrich
HCl (37%)	Until pH 7.4	VWR Chemicals
	Dissolve Trizma base in in dH_2O and adjust pH to 7.4 with concentrated HCl. Bring volume to 1L and control the pH after. Autoclave when pH once is OK.	
50x Tris-acetate-EDTA (TAE)		
Tris base	242g	Sigma-Aldrich
CH_3COOH	57.1 ml	Sigma-Aldrich
0.5M EDTA, pH 8.0	100 ml	Merck
	Mix ingredients and add dH_2O to a final volume of 1L. Dilute to 1x for use as buffer in gel electrophoresis.	
6x loading buffer		
bromophenol blue	0.25%	Sigma-Aldrich
Xylene cyanol FF	0.25%	Sigma-Aldrich
sucrose	40% (w/v) in H_2O	Sigma-Aldrich

Appendix B: Kits

Table A.2: Laboratory kits used during the study. The protocol accompanying the different kits were followed as described by the manufacturer, unless otherwise specified in material and methods (section 3).

Kit	**Supplier**
DNeasy Blood & Tissue kit (69506)	**Qiagen**
Proteinase K	
Buffer AW1 and AW2	
Buffer AE	
Lysis Buffer ATL	
Lysis buffer AL	
2ml collection tubes	
DNeasy Mini Spin Columns in 2ml collection tubes	
MiSeq Reagent Kit v3 (600-cycles) (MS-102-3003)	**Illumina**
Hybridization Buffer HT1	
Incorporation Buffer	
PR2 Bottle	
MiSeq Flow Cell (signle use)	
Reagent Cartridge (single use)	
Nextera XT DNA Sample Preparation Kit (FC-131-1096)	**Illumina**
Amplicon Tagment Mix, 24 RXN (ATM)	
Tagment DNA Buffer (TD)	
Nextera PCR Master Mix (NPM)	
Resuspension Buffer (RSB)	
Library Normalization Additives 1 (LNA1)	
Library Normalization Wash 1 (LNW1)	
Hybridization Buffer (HT1)	
Neutralize Tagment Buffer (NT)	
Library Normalization Beads 1 (LNB1)	
Library Normalization Storage Buffer 1 (LNS1)	
Nextera XT Indexing Kit (96-indeces) (FC-131-1002)	**Illumina**
Index Primers (S501-S508)	
Index Primers (N701-N712)	
PhiX control V3 kit (FC-110-3001)	**Illumina**
PhiX control	
Qubit® dsDNA HS Kit (Q32854)	**Invitrogen**
dsDNA HS dye reagent concentrate	
dsDNA HS Buffer	
dsDNA HS Standard 1	
dsDNA HS Standard 2	

Qubit® dsDNA BR Kit (Q32853)	**Invitrogen**
dsDNA BR dye reagent concentrate	
dsDNA BR Buffer	
dsDNA BR Standard 1	
dsDNA BR Standard 2	
Nucleospin® PCR Clean-Up and Gel Extraction Kit (740609.250)	**Macherey-Nagel**
Binding buffer NT1	
Wash Buffer NT3	
Elution Buffer	
Nucleospin® Gel and PCR Clean-Up Columns	
2ml collection tubes	

Appendix C: Plaque assay of positive control

Figure A.1: Plaque assay of positive control. *V. anguillarum* was infected with phage KVP40 at an OD of 0.3. Plaque formation was inspected after ON incubation.

Appendix D: Identified *Vibrio* spp.

Table A.3: Overview of bacteria isolated in this study. Isolates were identified according to NCBI BLAST analysis of the v3-v6 region of the 16S rDNA sequences.

Bacterial isolate	Most related sequence in BLAST	Identity score (%)	Accession number	Morphology[1]	Source[2]
V.1	*V. tasmaniensis*	99,9	KR270128	TCBS, yellow	Water, Drøbak
V.2	*V. tasmaniensis*	99,9	KR270128	TCBS, green	Water, Drøbak
V.5	*V. crassostreae*	99,9	CP016228	TCBS, yellow	Mussels, Ulvøya
V.6	*V. rumoiensis*	99,7	NR_024680	MA, milky white[3]	Mussels, Ulvøya
V.8	*V. toranzoniae*	99,7	LN832986	TCBS, yellow	Mussels, Malmøya
V.9	*V. penaeicida/ V. rumoiensis*	99,9/ 99,8	EU073023/ KY474375	TCBS, green	Mussels, Malmøya
V.10	*V. toranzoniae*	99,9	LN832984	TCBS, yellow	Mussels, Malmøya
V.17	*V. rumoiensis*	99,7	KY474375	MA, transparent[3]	Mussels, Sildevika
V.18	*V. tasmaniensis*	99,7	KR270128	TCBS, yellow	Water, Hellvik
V.21	*V. splendidus*	99,9	KT792721	TCBS, green	Water, Hellvik
V.23	*Vibrio sp.* hMe10-1	100	KX453229	TCBS, green	Water, Hellvik
V.24	*V. tasmaniensis*	99,9	KR270128	TCBS, green	Water, Hellvik
V.25	*V. tasmaniensis*	99,9	KR270128	TCBS, yellow	Water, Drøbak
V.28	*V. tasmaniensis*	99,7	KR270128	TCBS, green	Water, Drøbak
V.30	*V. lentus*	100	LN555642	TCBS, green	Water, Drøbak
V.32	*Vibrio sp.* hMe10-1	100	KX453229	TCBS, yellow	Water, Drøbak
V.35	*Vibrio sp.* hMe10-1	100	KX453229	TCBS, green	Water, Drøbak
V.36	*V. tasmaniensis*	99,6	KR270128	TCBS, green	Mussels, Hellvik
V.38	*V. splendidus*	99,9	KF444398	TCBS, green	Mussels, Hellvik
V.40	*V. tasmaniensis*	99,8	KR270211	TCBS, green	Mussels, Hellvik
V.41	*V. rumoiensis*	100	NR_024680	TCBS, yellow	Mussels, Drøbak
V.v1	*V. vulnificus*	99,7	KX966525	TCBS, yellow	Shrimp liver, Vietnam pond 1
V.v4	*Photobacterium ganghwense*	99,8	JQ394838	TCBS, green	Shrimp liver, Vietnam pond 2

[1] Growth media used for isolation, and color of colonies.
[2] Sample material used for isolating bacteria, and sample location
[3] Unable to grow on TCBS

Appendix E: Sequence alignment of bacteria V.9 and V.17

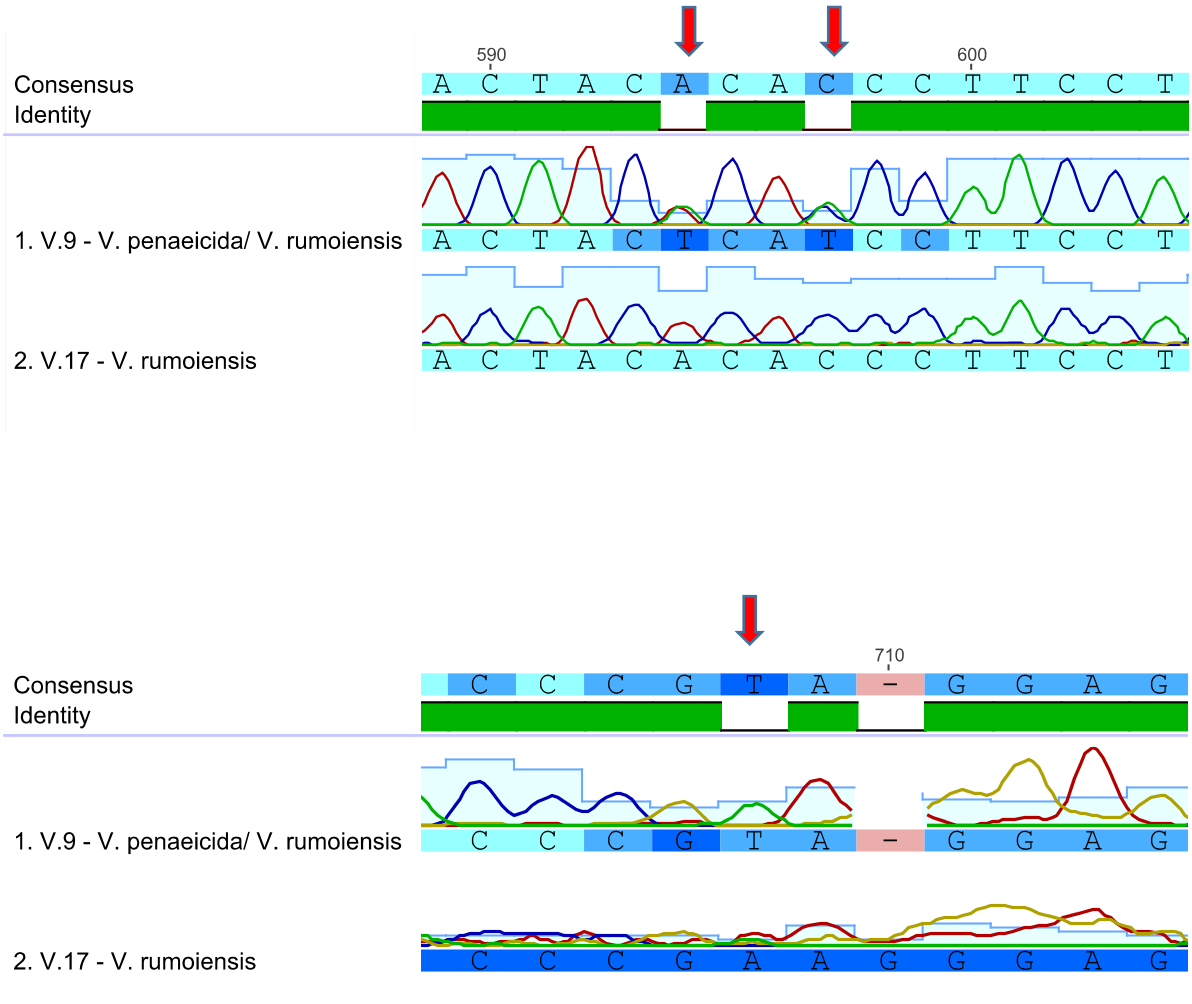

Figure A.2: Fragments from the sequence alignment of the v3-v6 region of 16S rDNA sequences for bacteria V.9 and V.17. V.9 was identified as *V.penaeicida* with 99,8% identity, but differed from *V.rumoiensis* (V.17) by only 3 possible SNPs. SNPs are marked with a red arrow.

Appendix F: Phage susceptibility pattern for bacterium V.24

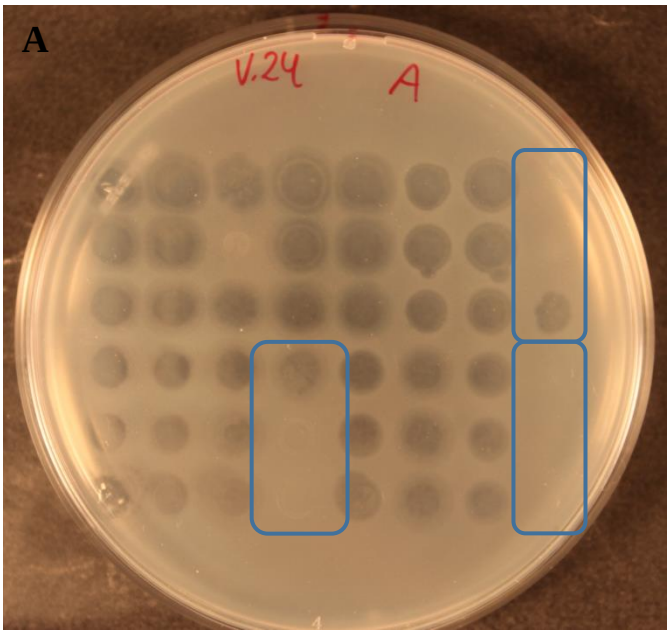

Figure A.3: Phage sensitivity pattern for bacterium V.24 –*V. tasmaniensis.* Undiluted phage lysates were spotted onto top agar containing the potential host bacteria. Phages were tested in triplicates, arranged three and three vertically. Phages were considered active if two or more plaques were formed (clear circles)**.** Of all phages tested, only three were considered inactive (encircled in blue).

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