Bacteriophages targeting pathogenic bacteria isolated from lumpfish (*Cyclopterus lumpus* L.) and Atlantic salmon (*Salmo salar* L.)

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Anne Helene Tolås

Abstract

Bacterial diseases in aquaculture lead to reduced health and mortality of fish as well as economic consequences for the producer. Antibiotics are an important antimicrobial to control bacterial diseases, however, concerns regarding the potential development and spread of antibiotic resistance genes are rising globally. Other solutions are considered, as the prophylactic or therapeutic use of bacteriophages, natural occurring viruses that infect and kill bacteria. This study aimed to isolate bacteriophages specific to pathogenic bacteria causing problems in Norwegian aquaculture. Among the bacteria causing disease in lumpfish (*Cyclopterus lumpus* L.) farming are *Aeromonas salmonicida, Vibrio anguillarum* and *Pasteurella* sp. Pasteurellosis is also an emerging disease in salmon (*Salmo salar* L.) farming. A collection of these bacteria were used to screen water samples for bacteriophages by using plaque assays. In addition to previously characterized isolates, bacteria from clinically sick salmon and lumpfish were isolated and sequenced by 16s rRNA sequencing. Water samples were collected from the environment and aquaculture sites.

Pasteurella sp. was successfully isolated from clinically sick salmon suffering from pasteurellosis. From lumpfish diagnosed with vibriosis, isolation of *V.anguillarum* was expected, however, 16s rRNA sequencing showed the presence of *Photobacterium* spp. and *Allivibrio logei.* Isolation of bacteriophages was challenging, and target bacteriophages were not found in the majority of water samples. However, when samples from an aquaculture site with salmon diagnosed with pasteurellosis was tested, lysed bacteria were observed in plaque assays, indicating the presence of bacteriophages. Successful isolation of these bacteriophages were not achieved, and this posed a question if prophages present in the genome of *Pasteurella* sp. could have caused the plaques. Bacteriophages specific for *V.anguillarum* was not isolated, but a previously isolated bacteriophage showed an effect against pathogenic *V.anguillarum* previously isolated from lumpfish.

Populærvitenskapelig sammendrag

Bakterielle sykdommer i akvakultur medfører redusert helse og dødelighet av fisk i tillegg til økonomiske konsekvenser for produsenter. Antibiotika er et viktig virkemiddel for å kontrollere bakterielle sykdommer, men bekymringer om utvikling og spredning av antibiotikaresistente gener øker globalt. Andre løsninger blir vurdert, som forebyggende eller terapeutisk bruk av bakteriofager, naturlige virus som infiserer og dreper bakterier. Målet med dette studiet var å isolere bakteriofager spesifikke for sykdomsfremkallende bakterier som medfører utfordringer i norsk akvakultur. Sykdomsfremkallende bakterier i oppdrett av rognkjeks (*Cyclopterus lumpus* L.) er blant annet *Aeromonas salmonicida, Vibrio anguillarum* and *Pasteurella* sp. Pasteurellose er også en bekymring i oppdrett av laks (*Salmo salar* L.*)*. En samling av disse bakteriene ble benyttet i søket etter bakteriofager i ulike vannprøver. I tillegg til tidligere karakteriserte isolater, ble bakterier fra klinisk syk laks og rognkjeks isolert og sekvensert med 16s rRNA sekvensering. Vannprøvene ble hentet fra både miljøet og fra akvakulturanlegg.

Pasteurella sp. ble isolert fra klinisk syk laks diagnostisert med pasteurellose. Fra rognkjeks diagnostisert med vibriose, var det forventet å isolere *V.anguillarum*, men 16s rRNA sekvensering viste tilstedeværelse av *Photobacterium* spp. og *Aliivibrio logei.* Isolering av bakteriofager var utfordrende, og bakteriofager ble ikke funnet i flertallet av vannprøvene. Når vannprøver fra akvakulturanlegg med laks diagnostisert med pasteurellose ble testet, ble lyserte bakterier sett i plakk assays, og dette indikerte at bakteriofager var tilstede i prøven. Isolering av bakteriofagene var derimot ikke mulig, og det ble stilt spørsmål om profager tilstede i genomet til *Pasteurella* sp. kan ha forårsaket plakkene. Bakteriofager spesifikke mot *V.anguillarum* ble ikke isolert, men en tidligere isolert bakteriofag viste derimot effekt mot et sykdomsfremkallende isolat av *V.anguillarum* fra rognkjeks.

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1. Introduction

1.1 What are bacteriophages?

Bacteriophages (also referred to as phages, or Φ) are naturally occurring viruses that infect and replicate in bacteria. They are regarded as the most diverse and abundant entity on earth, and they are believed to be found in every ecosystem. Seawater is among the richest natural environment for phages and other viruses (Sharma et al., 2017). Morphologically, phages have some general characteristics. The genome, which may be double stranded (ds) or single stranded (ss) deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) is assembled in a protein capsid. The capsid may be polyhedral, filamentous, pleiomorphic or connected to a tail (Figure 1). The tailed phages are the most abundant, while the pleiomorphic phages are rare (Dias, Eller, Salgado, Da Silva, & De Paula, 2013).

Taxonomy of phages is authorized and organized by the International Committee on Taxonomy of Viruses (ICTV), which historically has characterised viruses based on characteristics such as genome, morphology and host range. To date, genomic analysis are the commonly used methods, and the classification of phages is therefore being revised (Dion, Oechslin, & Moineau, 2020). Today, the majority of phages isolated have a tailed morphology with dsDNA genome and belongs to the order *Caudovirales*. The order, which is under reclassification, consists of five phage families, *Myoviridae, Podoviridae, Siphoviridae, Ackermannviridae* and *Herelleviridae* (Dion et al., 2020).

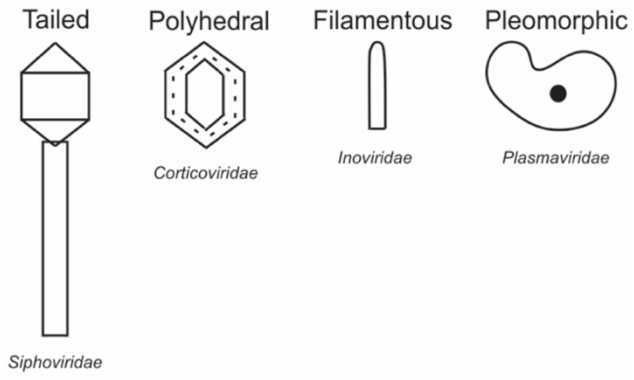

Figure 1 Graphical illustration of tailed, polyhedral, filamentous and pleiomorphic bacteriophages represented by the families *Siphoviridae, Corticovirividae, Inoviridae and Plasmaviridae.* The figure is adapted from Dias et al., 2013.

1.1.1 The life cycle of bacteriophages

Phages require a bacterial host cell for replication. Once the bacterial cell is infected, the phage will exploit the cellular machinery of the bacteria to multiply itself. The life cycle of the phage may be either lytic or lysogenic (Figure 2). In both cases, the life cycle starts with attachment to the bacterial surface. Specific targets such as lipopolysaccharides (LPS) and proteins present on the bacterial host surface serve as receptors for adhesion and entry of the bacteriophage (Simpson, Sacher, & Szymanski, 2016). Consequently, phages have a limited target range and are usually restricted to one or a few bacterial strains (Nakai & Park, 2002). Some phages have a narrow host range, and some have a broad host range. Some phages can infect one or a few bacterial strains whereas others can infect several species or bacteria from different genera. After a phage has attached to a bacteria, enzymes from the phage will permeabilize the cell wall. The genetic material of the phage will enter the host cell, whereas the capsid remains outside (Dias et al., 2013).

Phages in their lytic phase are called virulent phages. After the genetic material is inserted into the host cell, the cellular machinery of the bacterium is exploited to copy the viral genetic material (RNA or DNA) (Labrie, Samson, & Moineau, 2010). Within the cell, phage proteins are produced, synthesized and assembled together with the genetic material to produce daughter phages. After proliferation of phages, the cell wall is degraded and the bacterial cell will burst. New phages will thereafter be able to infect new bacterial cells (Sharma et al., 2017).

The lysogenic cycle differs from the lytic cycle. After phage attachment to the cell surface and insertion of viral genetic material, the genetic material will integrate into the bacterial genome. The viral genome will then continuously be replicated together with the bacterial genome. In this way, viral genes are passed on to bacterial progeny of the primary host (Sharma et al., 2017). A phage genome which is integrated into the host genome is called a prophage. Prophages will remain latent until induced to enter a lytic cycle. The induction may be caused by stressors such as antibiotics, temperature change or DNA damage (Kleppen, 2012). Some phages are also able to switch between the lysogenic and the lytic cycle. They are called temperate phages (Dias et al., 2013).

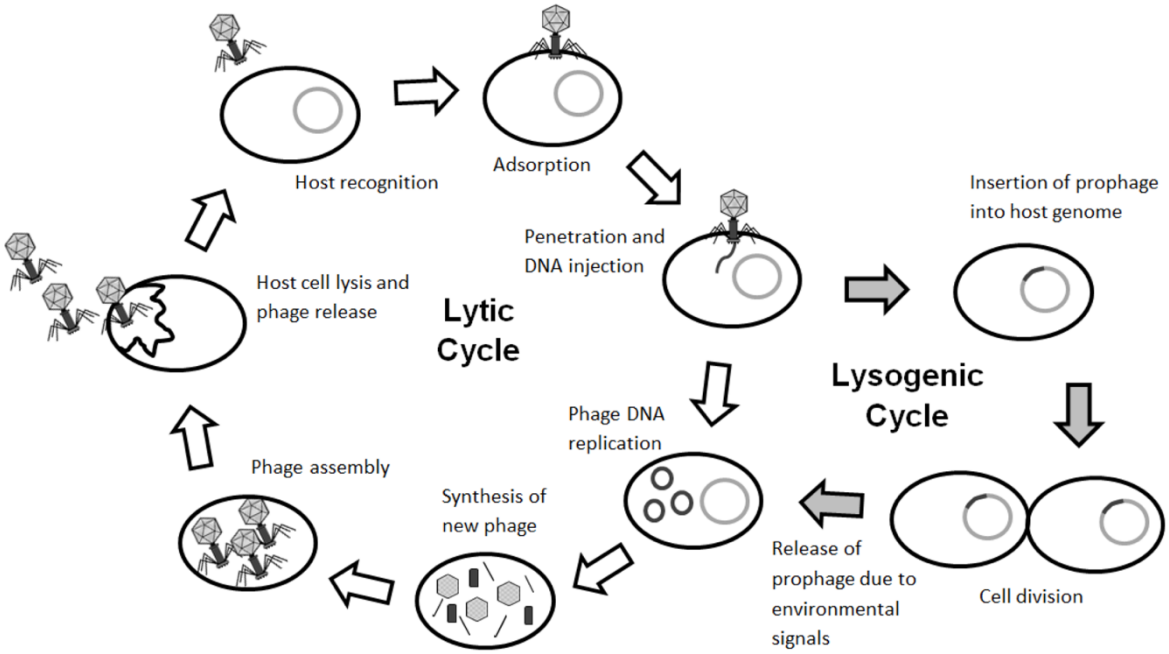

Figure 2 Life cycle of phages. The life cycle might either be a lytic cycle or a lysogenic cycle. Generally, the lytic cycle starts with host recognition, adsorption of phage to host cell, penetration of phage nucleic acid, intracellular development and final release of daughter phage particles. In the lysogenic cycle, the genetic material of the phage is inserted into the host genome. This is called a prophage. The phage genes in this state may occasionally revert to a lytic cycle, leading to release of phage particles. Temperate phages can switch between the lytic and lysogenic cycle. Figure adapted from Doss et al. (2017).

1.1.2 The history of bacteriophages

Phages were discovered independently by Frederick Twort, a British pathologist and Félix d'Hérelle, a Canadian microbiologist in 1915 and 1917, respectively (Sharma et al., 2017). The proposition of using phages to treat acute and chronic bacterial infections arose early, but after the emergence of antibiotic chemotherapy in the 1940s, the research on phages and phage therapy was reduced. However, in the former USSR, Poland, and to some extent India, phage therapy continued to be an area of research (Wittebole, De Roock, & Opal, 2014). Phage therapy for humans has remained in use in several institutes in Russia, in the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi (Georgia) and the Hirszfeld Institute in Wroclaw (Poland) (Fauconnier, 2019).

As a consequence of today's concerns regarding multidrug-resistant bacteria (MDR bacteria), bacteriophage therapy has gained new interest, and phages are again considered as a possible tool for the treatment of bacterial infections in humans (Wittebole et al., 2014). Prospect of phage therapy further exceeds its use than solely for treatments of human bacterial diseases. The successful use of phages has been described in various situations including, for example, food safety (Mahony, McAuliffe, Ross, & van Sinderen, 2011) and agriculture (Sieiro et al.,

2020). One example is phage P100 of *Listeria monocytogenes* utilised for food safety purposes. The product has been Generally Recognized As Safe (GRAS) by US food and drug Administration (FDA) and has been approved as a processing aid for all food by the US Department of Agriculture (USDA) (Sieiro et al., 2020).

1.1.3 Bacteriophages in aquaculture

In aquaculture, the use of phages has the potential to become an approach to control pathogenic bacteria and to some extent, reduce the use of antibiotics. The use of phages against pathogenic bacteria in fish was first introduced experimentally in Japan against *Lactococcus garvieae* in 1999 (Nakai & Park, 2002). In Europe, the European Union (EU) has been funding the research project "Aquaphage" (Mars 2011 – Mars 2015). The project aimed to identify and explore phages specific for bacterial pathogens that constitute severe threats for both freshwater and marine aquaculture species (https://cordis.europa.eu/project/id/269175/reporting, 2018). There is currently an interest and focus on phages for use in aquaculture.

There are several alternatives for the administration of phages to fish in aquaculture. They can be distributed directly to the water and have the advantage that they can both infect bacteria in the environment and in the farmed species. Application by immersion, feed incorporation, injection and swabbing has also been considered in the scientific literature (Culot, Grosset, & Gautier, 2019). At sea sites, administration of phages directly to the water will have a limited effect due to quick dilution of phages. However, in closed systems such as closed cages and well boats, phages can potentially be used. Effective phage therapy is dependent on the specificity of the phage, correct introduction at the right site, at the right moment and in the right concentration (Sharma et al., 2017).

In Norwegian aquaculture, bacteriophage treatment against *Yersinia ruckeri* is available as a commercial product. The product is named Custus $^{®}$**YRS**, and is developed by ACD pharmaceuticals, STIM. The product is used as a prophylactic water treatment for *Y. ruckeri* infection (yersiniosis) in Atlantic salmon (*Salmo salar* L.), mainly in recirculating aquaculture systems (RAS) (ACD Pharmaceuticals & STIM, 2020).

The use of phages can potentially become a tool to prevent disease in different situations in aquaculture. Fish in larval stages are vulnerable and prone to disease as their immune system may not be fully developed, and thus vaccination is not feasible. During the production cycle,

farmed fish are often handled due to for example sorting, transportation, vaccination, louse counting and delousing treatments. The procedures may be stressful situations for the fish and situations where the skin, the main barrier against infections, may be damaged (Takle et al., 2015). Prophylactic use of phages supplied to the water can potentially reduce the infection pressure and consequently reduce the incidence of diseases following these procedures. Some bacteria are difficult to remove from tanks and pipes in land sites due to the formation of biofilm. Some phages are shown to be effective against biofilms (Sieiro et al., 2020) and have the potential to be used as a disinfectant.

Bacterial diseases have historically been a challenge for the Norwegian aquaculture industry. Today, the incidence of bacterial diseases is low and stable in Norwegian salmonid aquaculture (*S. salar* and *Oncorhynchus mykiss*) (Sommerset et al., 2020). The reason is mainly due to effective vaccines and vaccine programs that have reduced the occurrence of diseases and prescriptions of antibiotics impressively (Brudeseth et al., 2013). Some bacterial diseases are problematic, such as *Pasteurella* spp., where an increase in prevalence of pasteurellosis is seen the recent years (2018-2020). Pasteurellosis in salmon is now considered as an emerging disease (Sommerset et al., 2020). The lumpfish (*Cyclopterus lumpus* L.) farming industry, however, is faced with various problematic bacterial diseases causing reduced welfare and high mortalities for infected fish and also economic consequences for the producer.

1.2 Lumpfish as a biological control of sea lice

Lumpfish is today the second most produced fish in Norwegian aquaculture, after Atlantic salmon (Sommerset et al., 2020). In 2019, there were 40 million farmed lumpfish produced in Norwegian aquaculture alone. Twenty-five companies held a total of 51 licences for the production and sale of cleaner fish to Atlantic salmon and rainbow trout farms (Fiskeridirektoratet, no date (n.d)).

The increasing demand for cleaner fish is a consequence of manifestations of the ectoparasites *Lepeophtheirus salmonis* K. and *Caligus elongatus*, also known as sea lice. To reduce the number of lice on wild fish and to prevent a reduction in welfare and fish health of salmonids, the Norwegian government has set a limit of 0.5 adult female salmon lice on farmed salmonids. Currently, there are limited antiparasitic therapeutics licensed for treatment and the high

consumption of chemotherapeutic treatments during recent years has resulted in increased resistance among the lice (Jansen, Grøntvedt, Tarpai, Helgesen, & Horsberg, 2016). As a consequence, alternative non-medical methods have been implemented. Mechanical, thermic and freshwater treatments are today more frequently used than chemotherapeutics in Norwegian salmonid aquaculture (Sommerset et al., 2020). Most of these treatments are regarded as effective to delouse fish. However, the current strategies used to control sea lice is considered as stressful for the fish and pose a risk of reducing welfare and also causing economic losses in salmonid aquaculture (Brooker et al., 2018; Powell et al., 2018). Consequently, alternative biological methods have been implemented to control the sea lice infestation in Norwegian aquaculture, the use of lumpfish and other cleaner fish.

Cleaner fish are defined as fish or shrimps that remove ectoparasites and dead tissue from a host (Feder, 1966). The cleaner fish are cohabitating with salmon in net pens grazing lice from it. The use of biological delousing, cleaner fish, has increased exponentially since 2008 in Norwegian aquaculture (Brooker et al., 2018; Powell et al., 2018). The most common fish used for biological delousing in Norway are different species of wrasse (mostly *Labrus bergylta* A. and *Ctenolabrus rupestris* L.) and lumpfish. To maintain sustainable harvesting of the wild populations and to meet the increased demand for cleaner fish by the farming industry, there has been a need for commercial production. Even though both wrasse and lumpfish are shown to decrease the number of sea lice when cohabiting with salmon in net pens (Imsland et al., 2018; Treasurer J., 2013), the commercial production of wrasse has increased modestly whereas the production of lumpfish has increased exponentially (Powell et al., 2018). Lumpfish are more robust to handling and transportation, have shorter production time and are eating sea lice more actively at lower temperatures compared to wrasse (Rimstad, Basic, Snorre, Hjeltnes, & Mortensen, 2017) making them also suitable for use in the northern parts of Norway.

Lumpfish are raised in land-based tanks, mostly from wild-caught broodstock, but the producers are increasingly implementing production based on farmed broodstock. The juveniles grow rapidly and will reach a desirable cleaner fish size (≥7) at approximately five months of age. However, due to the recommended size for vaccination being 8-10 g and as the recommendation of an immunisation period of 500 degree days post vaccination, the fish size at transfer to salmon net pens is commonly approximately 18-30 g (Treasurer J., 2013).

1.3 Bacterial diseases associated with the production of lumpfish

The commercial production of lumpfish has been and still is faced with bacterial, viral and parasitic diseases. Bacterial diseases are the primary challenge causing high mortalities (Brooker et al., 2018). Extensive mortalities due to bacterial diseases have been reported both in early production and after transfer to salmon cages. The numbers of outbreaks of disease caused by a selection of bacterial pathogens in Norway over the last 8 years are presented in Table 1. The data is registered by the Norwegian Veterinary Institute (Sommerset et al., 2020). As none of the bacterial diseases are subject to notification, private laboratories also diagnose diseases, thus the numbers might be even higher. The majority of disease outbreaks in recent years has been caused by Atypical *Aeromonas salmonicida*, often with high mortalities. Also, *Pasteurella* sp., bacteria in the genus *Vibrio*, and the latest year *Pseudomonas anguilliseptica* are highly prevalent. This study will focus on the bacteria *Vibrio anguillarum, A. salmonicida* and *Pasteurella* spp.

Table 1 Number of positive sites of selected bacterial agents reported from lumpfish to the Norwegian Veterinary institute from 2012-2019. The bacterial agents presented in this table are Atypical *A. salmonicida*, Classical *A. salmonicida*, *Pasteurella sp*., *P.anguilliseptica*, *V. anguillarum* and *V.ordalii* (Sommerseth et al., 2019).

	Number of positive sites from 2012-2019							
Disease/agent	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Atypical *A. salmonicida*	1	8	5	51	27	24	20	27
Classical *A.salmonicida*	0	0	0	1	4	0	0	0
***Pasteurella* sp.**	1	16	8	14	28	23	14	10
P. anguilliseptica	0	0	1	4	8	15	17	7
V. anguillarum	7	6	8	12	12	7	7	3
V. ordalii	3	4	1	3	1	6	3	2

1.3.1 *Vibrio*'s and vibriosis

The genus *Vibrio* compromises a broad range of Gram-negative, curved, rod-shaped, bacteria causing disease in fish inhabiting marine, coastal and estuarine water and may cause severe losses in biomass, with significant economic consequences for the aquaculture industry (Sharma et al., 2017). *V. anguillarum* is causing the disease classical vibriosis and was first isolated from infected eels during 1909 (Naka & Crosa, 2010). The disease has since then been reported from several fish species introduced to farming, including salmon and lumpfish. After

the implementation of routine vaccination of Atlantic salmon, vibriosis is not highly prevalent in salmon farming. In Norwegian lumpfish farming however, the bacteria *V. anguillarum* and *V. ordalii* are regularly isolated from diseased fish (Sommerset et al., 2020).

Serotyping, based on the O antigen, has so far identified 23 distinct serotype of *V.anguillarum* from fish. Both serotype O1 and O2a has been isolated from lumpfish suffering from vibriosis, but O1 causes the majority of disease outbreaks. In addition, a presently un-characterised O2b/x variant has been identified in lumpfish (Erkinharju, Dalmo, Hansen, & Seternes, 2020). *V.ordalii* is sporadically isolated from lumpfish (Bornø et al., 2016). Pathology seen in both cases is external lesions, ulcers, oedema and haemorrhages, enlargement of the caudal peduncle due to fluid accumulation and lastly necrosis in internal organs (Brooker et al., 2018). Other bacteria in genus *Vibrio* have been isolated from lumpfish, however, their pathogenicity is unclear (Erkinharju et al., 2020).

1.3.2 *Pasteurella* spp. and pasteurellosis

The disease pasteurellosis is characterised by bacterial septicaemia of Gram-negative coccobacillus. The disease was first described in wild populations of white perch (*Morone americana*) and striped bass (*Morone saxatilis*) in the Chesapeake Bay (USA) in 1963 (Snieszko, Bullock, Hollis, & Boone, 1964). Based on the first morphological and biochemical characteristics of the bacteria, the bacteria were placed in genus *Pasteurella*. This bacteria was later, based on genomic analysis, found to be related to bacteria in the genus *Photobacterium*. The new name for the bacteria became *Photobacterium damselae* subsp. *piscicida* (Romalde, 2002).

Pasteurellosis was not reported from salmonids before the beginning of the 1990s. In Scotland, high mortality was reported from farmed Atlantic salmon. The disease was found to be caused by a *Pasteurella*-like bacteria which was later reported from four separate disease outbreaks in Atlantic salmon. The bacteria was given the name *Pasteurella skyensis* and is, in contrast to *Photobacterium damselae* subsp. *Piscicida*, a true member of the genus *Pasteurella* (Reid & Birkbeck, 2015). *P. skyensis* has later been reported sporadically from Atlantic salmon in Scotland. Pasteurellosis was first reported from farmed salmon between 1989 and 1992 in northern parts of Norway. The disease was named “Varracalmbi” which means bloody eye in Lappish (Valheim, Håstein, Myhr, Speilberg, & Ferguson, 2000). Pasteurellosis has later been

reported sporadically from salmon. In 2018 and 2019, however, an increase in infections with *Pasteurella* sp. was registered as respectively 7 and 14 outbreaks were registered (Sommerset et al., 2020). In addition, in October 2020, *P. skyensis* was reported from Norway for the first time (Norwegian Veterinary Institute, 2020). The disease thus represents an emerging disease in Norwegian aquaculture. The bacteria are not yet speciated and is presently named *Pasteurella* sp. (Sommerset et al., 2020).

Bacteria in genus *Pasteurella* is also a huge concern in lumpfish farming. The bacteria isolated from lumpfish are poorly described and is still not characterised on a species level, at present it is named *Pasteurella* sp. (Rimstad et al., 2017). The disease was first reported by the Norwegian Veterinary Institute from a farm in southern Norway in 2012 (Sommerset et al., 2020). The incidence of the disease has been steadily increasing, but in recent years a reduction is seen (Table 1). The disease is reported from all life stages of lumpfish, both in hatcheries and in salmon cages (Alarcón et al., 2016).

As mentioned, *Pasteurella* spp. isolated from lumpfish and salmon in Norway are not yet speciated, but they are believed to be found in different, but related groups. *P. skyensis* from Scotland is proposed by 16S gene annotation to represent different species or subspecies from *Pasteurella* spp. isolates from Norway (Alarcón et al., 2016). For the first time, in 2018, the same genotype was identified in both clinically diseased salmon and clinically diseased lumpfish held in the same farm (Hjeltnes, Bang Jensen, Bornø, Haukaas, & Walde, 2019). The mortality was low for both species. It is not known if the disease spreads from the lumpfish to the salmon or the other way (Sommerset et al., 2020).

The infection leads to bacterial septicaemia, and mortality might be as high as 100% for lumpfish (Alarcón et al., 2016; Erkinharju et al., 2020). Gross pathology observed includes tail rot, white lesions in the skin and around the eyes and bleedings in the gills and at the base of the fins. In the internal organs, haemorrhages and white nodules are commonly observed (Ellul, Walde, Haugland, Wergeland, & Rønneseth, 2019).

1.3.3 Atypical *Aeromonas salmonicida* and atypical furunculosis

A. salmonicida is a Gram-negative, rod-shaped bacteria that causes furunculosis in several fish species in fresh- and seawater, including salmonids and lumpfish. *A. salmonicida* strains are generally characterised as typical and atypical strains. *A. salmonicida* ssp. *salmonicida* which mainly causes classic furunculosis in Atlantic salmon is termed typical whereas other subspecies, often causing disease in marine species, are abbreviated as atypical. The atypical subspecies registered are *achromogenes, masoucida, pectinolytica* and *smithia* (Gulla, Lund, Kristoffersen, Sørum, & Colquhoun, 2016). After the implementation of vaccine programs for Atlantic salmon, furunculosis is not highly prevalent in Norwegian salmon farming (Sommerset et al., 2020). In lumpfish farming, typical *A. salmonicida* has been reported in 2015 and 2016. Both cases were reported from the same farm site and these are the only reported cases of typical furunculosis in cleaner fish (Sommerset et al., 2020). Atypical *A. salmonicida*, however, is regularly reported from cleaner fish. The disease is associated with mortality in farmed lumpfish (Gulla et al., 2016).

The *A. salmonicida* isolates can be grouped into subtypes based on the sequence of the vapA gene encoding the A-layer protein. The publications suggest that there are 14 subtypes where most of the Atypical *A. salmonicida* isolates from lumpfish belong to A-layer group V and VI (Gulla et al., 2016).

The progression of atypical furunculosis in lumpfish is usually chronic and often result in high mortalities (Rønneseth, Haugland, Colquhoun, Brudal, & Wergeland, 2017; Sommerset et al., 2020). The disease cause infection with ulcers in the skin, inflammation, granulomas in internal organs and fluid accumulation in the abdominal cavity. Outbreaks of disease occur in both hatcheries and at cage sites. Most disease outbreaks are during the summer months when the water temperatures are high (Brooker et al., 2018).

1.4 Treatment and prevention of bacterial diseases in Norwegian aquaculture

1.4.1 Treatment

There is a need for effective methods to treat or prevent bacterial disease in aquaculture. Due to severe bacterial infections, the amount of antibiotics prescribed to treat farmed fish in Norway today is mainly to cleaner fish, mostly lumpfish (Figure 3) (Sommerset et al., 2020). In 2019, a total of only 18 treatments for Atlantic Salmon (juveniles, smolt, food and broodstock) with antibiotics was done compared to 78 treatments for lumpfish. Lumpfish are treated with antibiotics in hatcheries. In sea cages, however, treatment is not possible due to cohabitation with Atlantic salmon for human consumption (Ellul et al., 2019).

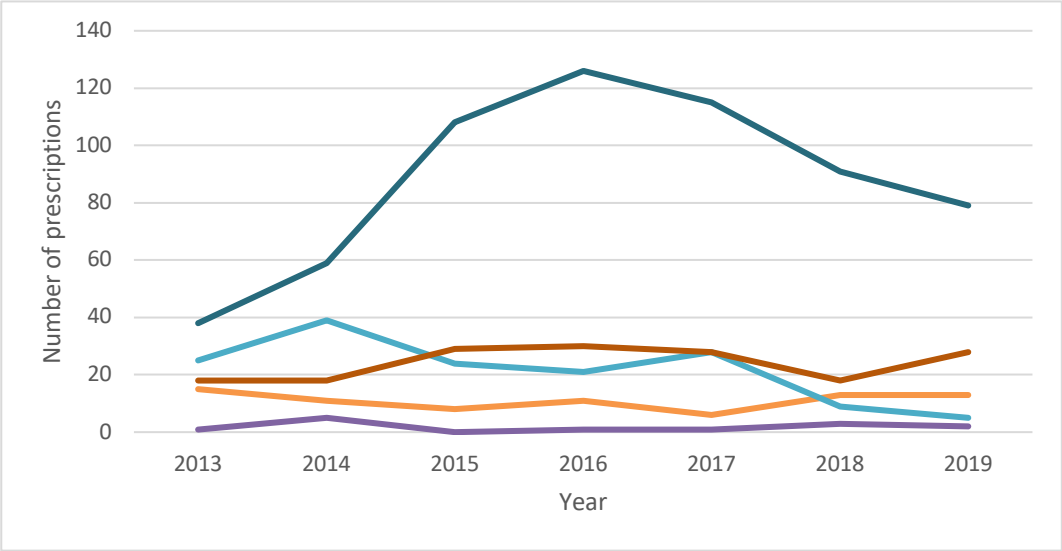

Figure 3 Number of prescriptions of antibiotics from 2013-2019 to different categories of farmed fish in Norwegian aquaculture. The categories of farmed fish presented in this figure are Atlantic salmon, food and broodstock (orange), Atlantic salmon, juveniles and smolt (light blue), Rainbow trout and trout (purple), marine fish (red) and other species (cleaner fish (dark blue)). The data was registered in 2019 from Veterinært legemiddelregister (Sommerseth et al., 2020).

Antibiotics are an important antimicrobial for controlling bacterial diseases, however, concerns regarding the use of antibiotics are rising globally. The wide use of antibiotics has globally resulted in a selective pressure towards antibiotic-resistant bacteria. Negative environmental impact from the wide use of antibiotics is a concern. Antibiotic-resistant genes and non-biodegradable antimicrobials can be spread to the environment (Cabello, 2006). In Norwegian aquaculture, there is a low prevalence of bacterial fish pathogens resistant to antibiotics, both in salmon farming and from cleaner fish (Sommerset et al., 2020). Even though antibiotic resistance in Norwegian aquaculture not a major problem to date, it is essential to uphold this situation and important to develop new tools to combat disease.

1.4.2 Vaccines

Vaccines are the most important tool to prevent diseases in farmed fish. Historically vaccination has reduced the occurrence of diseases and prescriptions of antibiotics impressively (Brudeseth et al., 2013). Sustainability in intensive aquaculture of salmonids is today dependent on effective vaccines. The majority of commercial vaccines are heat- or formalin-inactivated microorganisms formulated with oil adjuvants and delivered by intraperitoneal (i.p) injection.

Vaccination of lumpfish can reduce the prevalence of diseases in lumpfish farming. It has been shown that i.p vaccination of lumpfish leads to increased production of pathogen-specific antibodies and protection against disease in lumpfish (Rønneseth et al., 2017). Commercially produced vaccines for lumpfish are available in Norway under special consumption, and vaccine programs are implemented. Most of the farmed lumpfish are today vaccinated against vibriosis and atypical furunculosis. However, there are no available vaccines immunizing lumpfish or salmon against pasteurellosis (Ellul et al., 2019).

Even though efforts are put towards reducing the incidence of diseases in lumpfish farming through routine programs, bacteria also cause disease before the lumpfish reaches the necessary size of vaccination. The high prevalence of bacterial infections in lumpfish farming, also in hatcheries, clearly demonstrates the need for effective methods to prevent disease. Due to a proportion of infections coincides before the lumpfish can be vaccinated i.p, water treatment using highly specific and effective phages could reduce the infection pressure, consequently reducing the occurrence of infectious disease and improving fish welfare. *Pasteurella* sp. is an emerging disease in Norwegian salmonid aquaculture, and phages represent a possible tool to reduce the incidence of disease. Using phages directly in sea cages are expected to have a limited effect due to the quick dilution of phages in open seawater. On the other hand, phages can be supplied in closed systems such as well boats. Outbreaks of disease in salmon farming are often seen after delousing treatment of salmonids as the external barriers to infectious agents is damaged and stress might suppress the immune system.

1.5 Bacteriophages - a future antimicrobial in aquaculture?

Phages have advantages compared to antibiotics. Phages have a narrow antibacterial spectrum often limited to one or two strains within a species. Consequently, phages will have limited impact on the naturally occurring microflora compared to non-specific antibiotics. The specificity will be an advantage if phages are to be used in fish farms that apply probiotic cultures of bacteria or in RAS where the system is dependent on the biofilter. In addition, the fact that phages can only replicate in the presence of a host makes them both self-replicating and self-limiting (Doss, Culbertson, Hahn, Camacho, & Barekzi, 2017).

The effectiveness of phages in therapy or prophylaxis relies on the ability of phages to bind to a host bacteria. In cases where phages are used in solid matrixes, the diffusion of phages is limited (Ly-Chatain, 2014). In a liquid environment, however, phages might reach their host more easily (Culot et al., 2019). This makes phages attractive to aquaculture settings.

Experimental results with marine animal models have demonstrated a therapeutic effect of phage therapy against bacteria such as *V. anguillarum* and *A. salmonicida* (Imbeault, Parent, Lagacé, Uhland, & Blais, 2006). Silva et al. showed in 2014 that phage therapy could prevent *V. anguillarum* infection in zebrafish (*Danio rerio*) larvae. The fish group treated with phages showed significantly lower larvae mortality than the untreated group. This proposed that the direct supply of phages to water in larvae cultures might be an effective and inexpensive way of preventing disease by *V. anguillarum* (Silva et al., 2014).

However, there are several challenges to overcome before phage therapy can be used broadly in aquaculture. Some of the concerns are bacterial resistance to phages, the high specificity of phages, lysogenic phages and anti-bacteriophage immune response by the fish.

1.5.1 Concerns regarding phage therapy

Just as bacteria can develop resistance against antibiotics, they can also do so against phages (Skurnik & Strauch, 2006). Bacteria can become resistant, for example, by changing their surface receptors, preventing DNA injection or digest extrinsic DNA/RNA and thereby preventing phages from completing their life cycle. However, in contrast to antibiotics, phages can undergo mutations and adapt to the host bacterial change. As a result of this, there is a

continuous competition between bacterial resistance mechanisms and phages adapting to overcome them (Labrie et al., 2010). Furthermore, it is shown that mutations in bacteria which makes them resistant to phages, might also affect the pathogenicity of the bacteria (León & Bastías, 2015). Development of resistance often comes at a cost for the bacteria. Loss of virulence and gliding motility has been observed in phage resistant bacteria, for example *Flavobacterium coulmnare* (Laanto, Bamford, Laakso, & Sundberg, 2012) and phage resistant *Flavobacterium psychrophilum*. The latter showed decreased haemolytic and biofilm-forming activity, change in cell walls as well as mutations in significant virulence genes (Kalatzis, Castillo, Katharios, & Middelboe, 2018).

As mentioned, the high specificity of phages is a strengthening effect of phage therapy compared to broad-spectra antibiotics. However, the high specificity can also be a limiting property. The narrow host range makes identification of the pathogenic bacteria mandatory before applying phage therapy (Gon Choudhury, Tharabenahalli Nagaraju, Gita, Paria, & Parhi, 2017). One possible solution to this difficulty is to use phages with a wider host range or using a phage cocktail containing multiple phages. A cocktail of phages with different adsorption receptors would also make resistance development harder for the bacteria (Chan, Abedon, & Loc-Carrillo, 2013; Doss et al., 2017).

Phages to be used prophylactically or in disease therapy are required to be virulent, as only virulent phages can guarantee the death of the host cell (Almeida et al., 2009). Lytic phages are predators of bacteria resulting in the destruction of the bacterial host. Phages in a lysogen life cycle possess a risk of transduction of genetic material between hosts. In some cases, phages can convert their host from non-pathogenic to a pathogenic strain by providing the host with phage virulence genes. This is termed phage conversion (Brussow, Canchaya, & Hardt, 2004). Transferred genes might be encoding antibiotic resistance, virulence factors and toxins (Skurnik & Strauch, 2006). The toxins CTX and Zot in the human pathogen *Vibrio cholera* are examples of prophage generated virulence in pathogens (Mathur & Waldor, 2004). It is therefore important that phages to be allowed for application in aquatic food production systems are screened for the presence of virulence and antibiotic resistance genes.

Anti-bacteriophages immune response has been identified in fish (Neill, 1979). There is little information on the subject, and the impact on phage therapy is uncertain (Culot et al., 2019). If phages naturally present in the aquatic environment are going to be used as water treatment, an

immune response in fish is not likely. However, if phages are given in high doses by oral administration, or by i.p injection production of phage specific antibodies might be expected. Investigation of anti-bacteriophage immune response is necessary before application of phages to aquaculture systems.

The ideal phage for preventive use would be an obligated lytic phage with low resistance development. It should be unable to perform transduction, free of bacterial pathogenic virulence genes, and last but not least, easily produced and stored (Larsen, 2017).

Aims of the study

- Isolate pathogenic bacteria from fish and bacteriophages targeting them

    - Collect pathogenic bacteria from diseased lumpfish and salmon in Norwegian aquaculture, find optimal growth conditions and compile growth curves.
    - Collect water samples and screen for bacteriophages using collected bacterial isolates.
    - Propagation, isolation and purification of bacteriophages and explore the effect of a known vibriophage

2. Materials and methods

A schematic workflow chart of initial work identifying a phage-based product for aquaculture is presented in Figure 4. The first step is to isolate the bacterial strains responsible for disease and phages specifically targeting these strains. For isolation of phages, water from aquaculture farms, seawater, sediments, diseased animals or contaminated ecosystems such as sewage water can be used (Culot et al., 2019). As Culot et al. 2019 states, phages are likely to be found in the environment where their host bacteria are present. In order to study the process of exposure of host bacteria to phages, it is an advantage if optimal growth conditions and growth curves of the bacteria has been compiled. After isolation and purification of phages, the phages should be amplified before genome sequencing, characterization by transmission electron miscopy and analysis of host range assay.

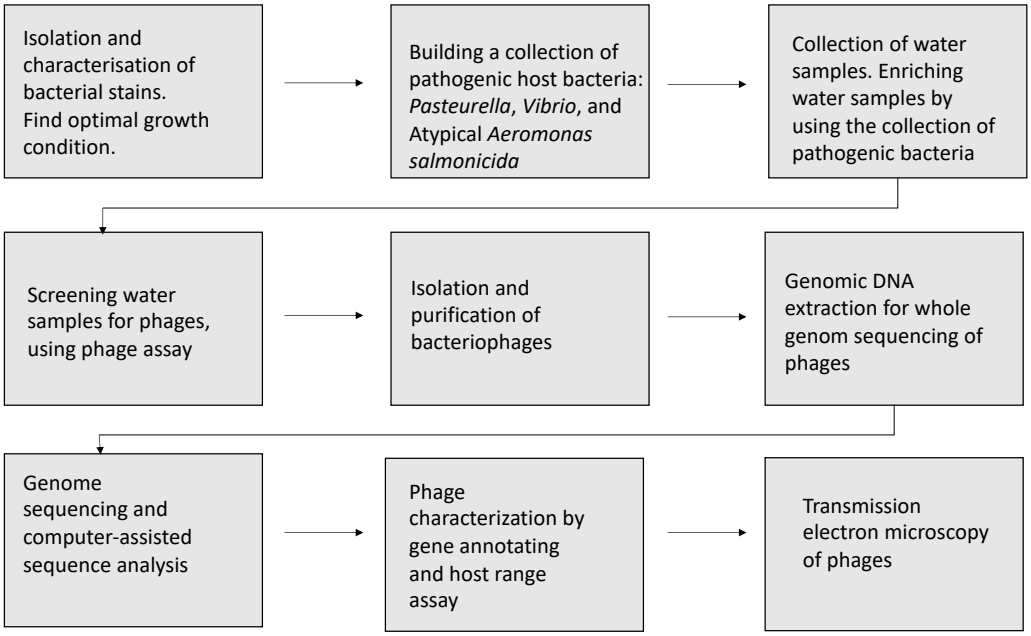

Figure 4 Schematic workflow of initial work identifying a phage-based product for aquaculture. These steps lead to the identification of bacteria responsible for disease and bacteriophages specific for the bacteria. Figure adapted and modified from ACD pharmaceuticals, STIM.

2.1 General procedures used through the study

Sterile technique was used when working with bacteria and phages. Gloves disinfected with 70% ethanol were used and all equipment were sterile. Prior to and after use of growth material, the bottle tops were burned off with a gas-burner. Pipettes used in the study were always filter tips to avoid contamination. The recipes for the solutions used in this study are presented in Appendix A. The growth media and buffers were sterilized by autoclavation. The exception was 10x$CaCl_2$ which was sterilized by using a 0.2 µm syringe filter when supplying it to the growth material. This was done due to precipitation of $CaCl_2x2H_2O$ when added directly into the growth media prior to autoclaving. Agar plates and broth were stored at 4°C. The agar plates were inverted to prevent condensation from dripping on the agar surface.

2.2 Building a collection of host bacteria

The current study included bacteria previously characterised, and these bacteria were *Pasteurella* sp. isolated from salmon (later termed *P*-S-1), *Pasteurella* sp. isolated from lumpfish (later termed *P*-L), *V. anguillarum* serotype O1 isolated from lumpfish (later termed *V.a*-L) and Atypical *A. salmonicida* isolated from lumpfish (later termed *A.s*-L). The latter has been identified to belong to A – layer type VI. All of the bacteria were isolated from clinically sick fish at farms located in Vestland County, except for *P*-L which were isolated from Rogaland County. In addition, one strain of *V. anguillarum*, isolated from Sea bass (*Dicentrarchus labrax)* in Hellenic Centre for Marine Research (HCMR), Crete, Greece (later termed *V.a*-SB), was provided by STIM and included in this study. A summary of the details of the included isolates is presented in Table 2.

Pasteurella sp. from salmon (later termed *P*-S-2) was isolated from clinically sick salmon (slaughter weight) at a farm with an ongoing diagnosed case of pasteurellosis (Vestland county). Three representative fish were examined. Bacterial samples were aseptically collected from the head kidney at the farm site and inoculated on blood agar supplemented with 2% NaCl. The bacteria samples were incubated for growth at 20°C (Panasonic MIR-154-PE, PHC Europe BV). The formed colonies were subcultured on agar plates until pure cultures were obtained. Single colonies were selected for 16s rRNA sequencing to identify the bacteria. Colonies selected for sequencing were further subcultured and subsequently transferred to Tryptic soy

broth (TSB) supplied with 2% NaCl (Appendix A) and foetal calf serum (FCS) % (FBS, Gibco by life technologies^{TM}) as *Pasteurella* spp. require an additional supplement of FCS to grow. The bacteria were harvested to glycerol stocks and frozen at -80 °C (see section 2.3.6).

In addition, bacteria were harvested from clinically sick lumpfish (8 g) at a farm with an ongoing diagnosed case of vibriosis (Vestland county). Previous to the disease outbreak the fish had been handled due to sorting according to size. Some of the fish had been transferred from a tank where the temperature was 8-9°C to a tank holding 11°C. In these tanks increased mortality was observed. Antibiotic feed treatment with oxolinic acid was started for fish in one tank one day prior to sampling. Samples from frozen dead fish from both antibiotic treated lumpfish and non-treated lumpfish were aseptically collected from the head kidney and inoculated on Tryptic soy agar (TSA) supplied with 2% NaCl. The bacteria were incubated at 15°C (Panasonic MIR-154-PE, PHC Europe BV). After growth until colonies were formed, a selection of colonies were sub cultured and grown until pure culture before they were characterised by 16s rRNA sequencing. After sequencing, a pure culture was transferred to TSB (2% NaCl), harvested to glycerol stocks and frozen at -80°C (see section 2.3.6). As the first attempt of isolation of bacteria lead to isolation of *Aliivibrio logei* (later termed *A.l*-L), not *V. anguillarum*, another sampling was performed. This time samples were taken aseptically harvested from the head kidney and inoculated on TCBS (Appendix A) which is selective for bacteria in genus *Vibrio*, TSA (2% NaCl) and blood agar (2% NaCl). Selected colonies were checked by an agglutination test specific for *V. anguillarum* (MONO-Va, 50 test, Appendix B – Table A.2). One positive colony were sequenced by 16s rRNA. Neither did this attempt lead to isolation of *V.anguillarum,* but *Photobacterium* spp. (Later termed *Pb*-L).

Table 2 Different bacteria isolates used in this study. The table presents the origin of the bacteria, abbreviation used in this study, year of isolation and serotype/group.

Bacteria	Origin	Abbreviation	Year of isolation	Serotype/Group
***Pasteurella* sp.**	Salmon, Vestland	*P*-S-1	2019	
***Pasteurella* sp.**	Salmon, Vestland	*P*-S-2*	2020	
***Pasteurella* sp.**	Lumpfish, Rogaland	*P*-L	2012	
Atypical A.*salmonicida*	Lumpfish, Vestland	*A.s*-L	2015	A-layer type VI
V.anguillarum	Lumpfish, Vestland	*V.a*-L	2018	01
V.anguillarum	Sea Bass, Crete	*V.a*-SB	2013	01
A.logeii	Lumpfish, Vestland	*A.l*-L*	2020	
***Photobacterium* spp.**	Lumpfish, Vestland	*Pb*-L*	2020	

*Isolated in the current study

2.2.1 Identification of bacteria by 16S ribosomal RNA (16S rRNA sequencing)

The 16S rRNA gene of bacteria isolated in this study was amplified from a colony growing on agar. One colony was suspended in 50 µl RNA and DNase free water and heat treated at 98°C for 10 minutes (min). The tube was centrifuged at 13 000 g for 2 min, and the supernatant was collected as a template. Before amplification by polymerase chain reaction (PRC), different dilutions were made of the supernatant. The 16S rRNA gene was amplified by PCR using universal primers 27F: 5′-AGAGTTTGATCMTGGCTCAG-3′, 1525R: 5′-AAG-GAGGTGWTCCARCC-3′ (Collins et al., 1991). The template reaction mixture had a final volume of 50 µl consisting of 5× Phusion buffer, 0.5 U of Phusion DNA polymerase (Phusion DNA polymerase, Thermo Scientific), 10 mM dNTP, 2.5 µl of 10 µM of each primer and 0.1 µl template. The cycle conditions were as follows: 1 cycle at 98°C for 3 min; 30 cycles- 98°C for 30 s, 58°C for 30 s and 72°C for 1 min; and a final cycle at 72°C for 10 min.

The PCR products were visualized on 1% agarose gel run in 1xTAE buffer at 80V for approximately 45 min. Samples were traced by adding 5x loading buffer (BlueJuice^{TM} Gel Loading Buffer (10X), Thermo Fisher, Scientific) and was visualized under UV-light (G:Box Gel imaging for fluorescence and visible applications, Syngende). The PCR products were purified using GenElute^{TM} PCR Clean-Up Kit (GenElute^{TM} PCR Clean-Up Kit, Appendix B, Table A.3). If multiple bands showed up, a new gel with wider wells was made. The gel was visualized under UV-light (UVP, Ultra Violet Product), and the band of interest was cut out. The band of interest was purified by using EZNA Gel Extraction kit (EZNA gel Extraction kit, Appendix B, Table A.4). Sequencing was performed by the DNA sequencing facility at the High-Technology Centre, Bergen, Norway.

2.3 Bacteriology, general methods

2.3.1 Cultivation of bacteria

During the study, the bacteria were cultured in media according to their specific growth requirements (Table 3). For cultivation of *A.s*-L , TSB (0.5 % NaCl) (Appendix A) was used. For cultivation of *Pasteurella* spp. and *V. anguillarum*, TSB supplied with 1.5 % NaCl (Appendix A) was used. *Pasteurella* spp. grows best under anaerobic conditions and therefore 40 ml of TSB supplemented with 2% NaCl and 4ml FCS was supplied to a 50 ml tube (Falcon,

Sigma). *A.s*-L were grown aerobically in 250 ml Erlend Meyer bulbs with 100 ml TSB 0.5% NaCl. *V.a*-L was grown aerobically in 250 ml Erlend Meyer bulbs with 50 ml TSB 2% NaCl, in 5 ml in 15 ml tubes (15 ml Falcon, Sigma), or 25 ml in 50 ml tubes. All the bacteria, when grown in broth, were incubated at 20°C at 200 RPM (Infors HT minitron, INFORS-HT). As both *A.l*-L and *Pb*-L were assumed to be *V.anguillarum,* similar growth condition as *V.anguillarum* was used during the study.

Table 3: Summary of growth media and growth conditions for the different bacteria included in this study.

Bacteria	Growth media	Growth condition	
Pasteurella spp.	TSB 2% NaCl + 10%FCS	20°C and 200 RPM	Anaerobically
V. anguillarum	TSB 2% NaCl	20°C and 200 RPM	Aerobically
Atypical *A. salmonicida*	TSB 0.5% NaCl	20°C and 200 RPM	Aerobically

2.3.2 Growth curves

Growth curves were compiled for isolates included in this study. First, a pre culture was made by transferring frozen glycerol stocks of bacteria to the growth media. 1ml of *V.a*-L was supplied to 50 ml TSB 2% in a 250 ml Erlend Meyer bulb. 1ml of *Pasteurella* spp. was supplied to 40 ml of TSB 2% NaCl supplied with 10% FCS tube (50ml Falcon, Sigma). 1ml *A.s*-L was supplied to 250 ml Erlend Meyer bulb with 100 ml TSB 0.5% NaCl. Once the pre culture had reached the late exponential growth phase, the bacteria were transferred to new growth media which became the main culture. 2.5 ml of the culture with *V.a*-L was supplied to 250 ml TSB 2% in a 1000 ml Erlend Meyer bulb. 1ml of *Pasteurella* spp. was transferred to 40 ml TSB 2% with 10% FCS. 1 ml of the *A.s*-L culture was supplied to a 250 ml Erlend Meyer bulb containing 100 ml TSB 0.5% NaCl. To measure the growth, Optical density (OD) was measured. The measurements were done until the stationary phase was reached.

2.3.3 Measurements of OD of bacterial cultures

Measurements of OD is used to measure the density of a bacteria in a suspension. In this study, OD was measured in a spectrophotometer (Spectroquant® Pharo 300, Merck) at 600 nm. Prior to a measurement, a zero measurement of the growth media used to cultivate the bacteria was used to calibrate the instrument. For all of the bacteria isolates, OD was measured by transferring 1.5 ml of bacterial suspension to a cuvette of plastic (BRAND UV cuvettes, 1.5-

3ml, Brand). The cuvettes were inserted to the spectrophotometer and measured. The exception was Atypical *A. salmonicida* that aggregates. These bacteria were centrifuged (Beckman microfuge lite centrifuge, Beckman) at 13000 g for 1 min in 1ml in tubes (1.5 ml microcentrifuge Tube, Axygen). The supernatant was discarded, and the pellet was re-suspended in 0.1M NaOH (v/v) (Appendix A). After 3 min, OD was measured at 340 nm in a 10 mm cuvette of quarts.

2.3.4 Bacterial cell counting

A CASY bacterial cell counter was used to count bacterial cells in the solutions (CASY TT, Inovatis). The instrument provides the number of viable and dead bacteria/ml in the solution and the aggregation factor. Counting was performed by supplying 10 µl bacterial suspension to 10 ml of filtered (0.2 µm syringe filter) degassed CASY ton (CASY ton, CASYton, Omni Life Sciences GmbH). This was loaded in triplicate volumes of 300 µl to the instrument. To correlate OD and cells/ml, a twofold serial dilution was made of the bacteria included in this study. OD and cells/ml were measured for each dilution. The results were combined to find how many cells that were present at a given OD.

2.3.5 Correlation of OD and Colony Forming Units (cfu)

Another approach to measure cells in relation to OD is to make an OD-cfu correlation. When cfu are measured, the viable cells in a bacterial suspension is measured by counting colonies on agar plates. The bacteria were harvested in their exponential growth phase and a twofold dilution series was made to measure OD. To measure cfu/ml, bacteria were diluted in a tenfold series in PBS (Appendix A) before plating. 100 µl of diluted bacterial suspension was transferred to agar plates. The bacterial suspension was spread by using sterile bend glass pipettes. Two parallels of each dilution were made. *Pasteurella* spp. was grown on blood agar with 2% NaCl, *V. anguillarum* was grown on TSA with 2% NaCl (Appendix A), atypical *A. salmonicida* on TSA with 0.5% NaCl (Appendix A). The plates were incubated at 15 °C (Panasonic MIR-154-PE, PHC Europe BV) until colonies were formed. After incubation, the plates which had formed a countable number of colonies were selected and counted. Plates containing between 50-300 colonies where therefore preferred to get the most accurate numbers of colonies.

Colonies formed on the agar plates were counted and cfu/ml was calculated by using the formula:

$$\text{Number of cells(cfu)pr. ml} = \frac{\text{Number of colonies pr plate}}{\text{Dillution factor}}$$

2.3.6 Preparation of bacterial glycerol stocks

Glycerol stocks were made of all the included bacterial strains. These stocks were used in all the included experiments, thus ensuring that the same passage of bacteria were used in subsequent experiments. The glycerol stocks were prepared by harvesting the bacteria in the late exponential growth phase, supplementing them with 12% glycerol (v/v) (Glycerol≥ 99.5%, Sigma-Aldrich), and from this mixture 1 ml was allocated to Cryo tubes (CryoTubeTM 1.8 ml, Nunc, Denmark). The tubes were marked with bacteria name and date of freezing before stored at -80 °C.

2.3.7 SDS-PAGE and silver staining

Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to investigate the protein profiles of some of the bacteria included in this study. Bacteria were harvested in the late exponential growth phase and centrifuged at 2.500 g (Beckmann Coulter Allegra X-15R) for 15 min at 4°C. Cells were washed once by re-suspending in sterile phosphate-buffered saline (PBS), followed by centrifugation and resuspension in PBS prior to use. The bacterial solutions were heat-treated (96 ˚C for 5 min) in sample-buffer containing β-mercaptoethanol. Protein profiles were analysed by SDS-PAGE (12% acrylamide) according to the method of Laemmli (1970). A Mini Protean Tetra Cell (Bio-Rad) was used to perform electrophoreses. Samples (10 µl) were loaded onto each well and electrophoresed at 190V for 1 hour. Unstained low range SDG-PAGE standard (#161-0304, Bio-Rad) diluted at 1/20 in the gel-loading buffer was used as ladder (5 µl). After protein separation the proteins were fixed for 20 min using Fixative solution (Appendix) and washed (2x10 min) using deionised water. Protein bands were visualised using the Silver Stain plus kit (Bio-Rad). When clear bands were formed, the staining solution was replaced by 5% acetic acid and the gels were photographed (Molecular imager chemidoc XRS^{+}, Bio-Rad).

2.4 Sample material for isolation of bacteriophages

Different sample material for the isolation of phages was used in this study. For isolation of phages targeting *Pasteurella* spp. water samples were collected from the same farm as where *P*-S-2 was isolated (Vestland county). To increase the probability of isolating phages targeting *Pasteurella* spp. three clinical sick fish were incubated in a tank containing 20L seawater for 3 days in outdoor temperature, approximately 4°C. After harvesting the water, a drop of chloroform was supplied to the 50 ml sample tubes and they were stored in the fridge at 4°C.

For isolation of phages targeting *V.a*-L several sea water samples and samples containing mussels were collected from various locations (Table 4). Mussels were crashed and the mussel juice was used for enrichment. At each sampling location, information was recorded. This included a geographical description, sampling date and sample material (Table 4). In addition, samples harvested from outlet water from tanks with salmon at The Aquatic and Industrial Laboratory (ILAB) and samples from seawater incubated (24 hours at room temperature (RT)) with juvenile lumpfish diseased from vibriosis (*V.a*-L) were included. Water samples were also collected at the lumpfish farming site where *Pb*-L and *A.l*-L was isolated (Vestland county). At this site, one sample was collected from the outlet water, and one sample was taken directly from the tank. If the water samples were not to be analysed shortly after harvesting, a small drop of chloroform was supplied. The water samples were also filtrated by using a 0.2 µm syringe filter before used in the enrichment process.

Table 4 Water samples collected in this study. Location, the isolate used for enrichment of samples, material, description and sampling date of the included samples.

Location	Isolate used in enrichment	Material	Description of sample location	Sampling date
Fish farm Vestland County Salmon aquaculture site	*P-S-1* *P-S-2*	Ocean water	Water from a fish tank where three clinical fish had incubated for three days.	02.02.2020
Kyrkjetangen, Bergen Location 1	*V.a-L*	Ocean water	Sandy bottom with some algae vegetation.	04.05.2020
Kyrkjetangen, Bergen Location 2	*V.a-L*	Ocean water Mussels	Sandy bottom. The sample included one mussel (*Mytilus edulis).*	04.05.2020
Kyrkjetangen, Bergen Location 3	*V.a-L*	Ocean water Mussels	Sandy bottom. The sample included two mussels (*Mytilus edulis*).	04.05.2020
Solheimsviken, Bergen Marineholmen Beach	*V.a-L*	Ocean water	Water sample taken from under the pier in Marineholmen Beach.	05.05.2020
ILAB, Bergen Water sample	*V.a-L*	Ocean water	Water sample from outlet water from ILAB.	29.05.2020
ILAB, Bergen Water sample	*V.a-L*	Ocean water	Water sample from ILAB with sea water from a tank with *Moritella Viscosa* trials.	29.05.2020
Clinical sick fish	*V.a-L*	Ocean water	Sea water incubated with frozen lumpfish (24h) that had been diagnosed with *V. anguillarum* (*V.a*-L).	05.06.2020
Kadettangen, Bærum Water sample	*V.a-L*	Ocean water	Sandy bottom with low algae vegetation	13.07.2020
Solvikbukta, Bærum	*V.a-L*	Ocean water with one Mussels (Mytilus edulis)	Muddy bottom with high algae vegetation.	13.07.2020
Høvik harbour, Bærum	*V.a-L*	Ocean water	Stony bottom with high algae vegetation	13.07.2020
Slependen, Asker	*V.a-L*	Ocean water	Sandy bottom, a lot of algae and high biological activity	07.08.2020
Holmen strand, Asker	*V.a-L*	Ocean water	Under floating dock with algae growth	07.08.2020
Hvalstrand, Asker	*V.a-L*	Ocean water	Rocky bottom, some algae growth	07.08.2020
Leangbukta, Asker	*V.a-L*	Ocean water	Rocky bottom, algae growth and several mussels	07.08.2020
Spirebukta, Asker	*V.a-L*	Ocean water	Rocky bottom, algae growth and several mussels	07.08.2020
Gamlehaugen, Bergen	*V.a-L*	Ocean water	Rock bottom, some algae growth	12.08.2020
Solheimsviken, Bergen	*V.a-L*	Mussels (Mytilus edulis)	Mussels (*Mytilus edulis)* from under a pier in Solheimsviken.	13.08.2020
Solheimsviken, Bergen Marineholmen Beach	*V.a-L*	Mussel and ocean water	Under the pier in Marineholmen Beach. Many small mussels(*Mytilus edulis)* and ocean water	13.08.2020
Fish farm Vestland County Lumpfish aquaculture site	*V.a-L* *V.a-SB* *A.l*-L *Pb*-L	Ocean water	Two water samples were taken. One was taken from the tank and one sample was taken from the outlet water.	26.08.2020

2.5 General methods concerning work with bacteriophages

Phages require a bacterial host to complete their life cycle, and proliferation of phages is therefore dependent on available host bacteria. Growth media, incubation temperatures and agitation were thus optimized according to the requirements of the different bacteria used in this study. Phages are dependent on divalent cations (Sambrook & Russel, 2001) for host absorption, and therefore $CaCl_2x2H_2O$ were supplied to the bacterial growth media to enable phage proliferation. The optimal time point for phage introduction to the bacterial culture is when the host bacteria are in early- to mid-exponential growth phase (Russell & Sambrook, 2001). Bacteria in their stationary phase are growing slowly or not growing at all, giving the phages little time to proliferate. The growth curves compiled for the bacteria were therefore used to identify when the bacteria were in the exponential growth phase. The OD and the time for harvesting are presented in Table 5.

Table 5 OD at harvesting and time for harvesting of bacteria included in this study when the bacteria were used in phage assays and enrichment of water sample.

Bacteria	OD for harvesting	Time for harvesting
***Pasteurella* sp. salmon (*P*-S-1)**	A (600 nm) 0.4-1.0	9-12 hours
***Pasteurella* sp. salmon (*P*-S-2)**	A (600 nm) 0.4-1.0	9-12 hours
***Pasteurella* sp. lumpfish (*P*-L)**	A (600 nm) 0.3-0.8	14-17 hours
***V. anguillarum* lumpfish (*V.a*-L)**	A (600 nm) 0.6-1.2	3-4.5 hours
***V. anguillarum* sea bass (*V.a*-SB)**	A (600 nm) 0.3-0.9	3-4.5 hours
Atypical *A. salmonicida* (*A.s*-L)	A (340 nm) 0.1-0.3	15-20 hours

2.5.1 Enrichment of water samples

The process of isolating phages started with the enrichment of the search material with the target bacteria. This was done to increase the likelihood of finding phages targeting the specific bacterium. In sterile flasks, search material, 5 x growth medium in relation to the requirement to the bacteria supplied with 10% $10xCaCl_2xH_2O$ and host bacteria in exponential growth (1%) was mixed. As *Pasteurella* spp. is dependent of FCS, 10% FCS was supplied to these enrichments. The mixed solution was incubated at 20°C for 24-72 at hours depending on the growth of the bacteria. Agitation and growth condition was adapted to each the bacteria. After incubation, the enrichment was centrifuged at 1500 g for 15 min (Allegra X15R centrifuge,

Beckman Coulter), the supernatant harvested and sterilized by filtering through a 0.2 µm syringe filter. The resulting bacteriophage lysate was tested against the bacteria used in the enrichment process by plaque or spot assays.

2.5.2 Plaque assay

To isolate and to determine the number of phages in a solution, plaque assays are often used. Specific phages will infect specific bacteria, release daughter viral particles which in turn will infect neighbouring bacteria. Growth in a semisolid medium will give limited diffusion of viruses, creating a small zone of bacterial lysis. This zone is named a plaque and contains phages that are genetically identical to one another (Sambrook and Russel, 2001). The concentration of plaques is measured and calculated as plaque-forming units per ml (PFU/ml).

Plaque assays were obtained by using a soft agar method, consisting of two layers of agar. Both layers contain growth media in relation to the requirements for the bacteria. The bottom layer is serving as a nutrient supplier for the bacteria and contains 1.5% of agar. The upper layer contains the bacteria, growth media with 0.5% soft agar (Sea plaque agarose, Lonza), $CaCl_2x2H_2O$ and phage lysate. The soft agar will improve the diffusion of phages.

For plaque assays, 150 µl of bacteria in their exponential growth phase was gently mixed with 100 µl of bacteriophage lysate in a sterile tube after which 10% of 10x $CaCl_2x2H_2O$ was added. As *Pasteurella* spp. is dependent of FCS, 10% FCS was supplied. Soft agar solution was melted and stored in a water bath at 37°C for *V. anguillarum* and 25°C for *Pasteurella* spp. After incubation of bacteria and phage for 15-30 min, 3ml of soft agar was added and the mixture was gently poured on top of the bottom layer. The plates were incubated inverted in sealed boxes at 20°C and inspected for plaques the following days. If the concentration of a phage solution was too high to visualise single plaques, tenfold serial dilutions were prepared, and the assays repeated.

2.5.3 Spot plaque assay

Spot assay is a quick way of checking the activity range of phages. The bacteria were plated on a thin agar plate by adding 150 µl bacteria to 3 ml soft agar in addition to 100 µl $CaCl_2xH_2O$. The plate was left 20 min to stiffen and subsequently, drops of lysate (5µl) were added to the plate.

2.5.4 Positive control bacteriophage (Φ 53)

In this study, a positive control, bacteriophage 53 (Φ 53), was included. The bacteriophage solution was supplied by ACD pharma, STIM. The phage (Φ 53) was isolated from a harbour in Drøkbaksfjorden in Eastern Norway. *V.a*-SB had previously been identified as a host for this bacteriophage. The concentration of the bacteriophage suspension was 10^8 PFU/ml.

2.5.5 Phage plaque isolation and purification

Positive results from the plaque assays needed to be investigated for the presence and further isolation of different phage strains in the enriched samples. Single plaques that showed different plaque morphology and size could be isolated from agar plates using sterile pipette tips. The agar piece (plaque) was transferred to an Eppendorf tube containing 1ml TM-buffer (Appendix A). The tubes were left over night (ON) at 4°C to allow phages to diffuse from the agar. After diffusion of phages ON, the buffer solution was mixed with 9 ml of bacteria in the exponential growth phase and incubated at 20°C. *V. anguillarum* was incubated ON, and *Pasteurella* sp. was incubated for 2 days. After incubation, the bacteria were removed by filtering the suspension through a 0.2 µm syringe filter, and the lysate was subsequently used in plaque assays. This procedure was repeated three times to purify single specific phage isolates.

2.5.6 Re-isolation of bacteriophages from top agar

To isolate phages directly from a plate, not using the method described in section 2.5.2 phage plaque isolation and purification, 5ml of TM buffer was added directly to each plate and incubated at 4°C for 10-18 hours. The buffer was collected and transferred to a 50 ml tube (50ml Falcon, Sigma). Chloroform was then added to a final concentration of 10%. The solution was thereafter centrifuged at 9000 g for 15 min. The supernatant was harvested and filtered through a 0.2 µm syringe filter. This lysate was stored at 4°C and used in plaque assays.

2.6 Investigating phage activity of Φ53 on *V.a*-L and *V.a*-SB

To investigate the effect of the positive control bacteriophage against *V.a*-L and *V.a*-SB another approach than plaque and spot assays was additionally attempted. 100 µl bacteriophage solution and 100 µl bacteria in exponential growth phase were mixed in a 1.5 ml Eppendorf tube. The first tube contained bacteria and bacteriophage at a multiplicity of infection (MOI) 1:1 and the second tube phages and bacteria at MOI 2:1. A third tube containing 1 ml of sterile TM-buffer and bacteria was included as a none-phage control. The tubes were incubated for 1 hour at RT before 800 µl of TSB 2% NaCl was added. Subsequently, sampling was done each hour for 5 hours. At each sampling, the number of bacteria/ml was measured using a CASY-TT cell counter™ (INOVATIS AG). In addition to this, bacterial cell size was analysed. To make sure that only viable cells were counted in the CASY cell counter, only cells between 1 and 2 µm was measured as positive counts. In addition, CFU was measured as described in section 2.3.5. This was done both immediately after adding TSB 2% NaCl and 3 hours later.

3. Results

3.1 Isolation of *Pasteurella* sp. (*P*-S-2) from salmon

P-S-*2* was isolated from a clinically sick salmon at a salmon farm site in Vestland County. Characteristic gross pathology was observed and varied between representative examined fish (Figure 5). Pathology included exophthalmia and lesions in and around the eyes. Haemorrhages were observed into the iris. In addition, the cornea showed opacity. Abscesses were observed in the head region. Bloody ascites was observed in the abdominal cavity. The fish behaviour in the net pens was abnormal as some of the fish were swimming close to the surface and stationary to one area in the net pen.

The bacteria sample from the head kidney gave growth of numerous bacteria when grown on blood agar supplemented with 2% NaCl.

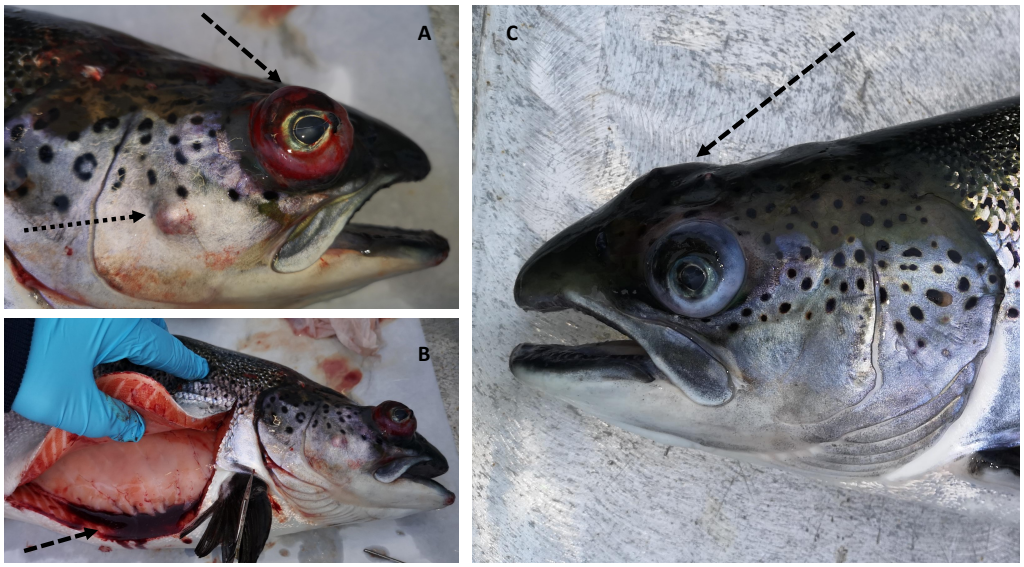

Figure 5 Gross pathology of Atlantic salmon diagnosed with infection with *Pasteurella* sp. at a fish farm in Vestland County. Picture A shows salmon with exophthalmia with haemorrhage (big stapled arrow) and an abscess in the head region (small stapled arrow). B shows salmon with bloody ascites in the abdominal cavity (big stapled arrow). C shows abscess in head region (big stapled arrow).

3.1.1 16s rRNA sequencing of *Pasteurella* sp.

The 16s rRNA gene sequence analysis identified the bacteria isolated from salmon (Figure 6) as *Pasteurella* sp. The isolate was found to be 98.48 % similar by 16S rRNA to *Pasteurella* sp. NIVO-1993 by Basic Local Alignment Search Tool (BLAST). The PCR products of amplified *Pasteurella* sp. were visualised on agarose gel (1%) (Appendix C – Figure A.1).

Figure 6 Picture of colony morphology of *P*-S-2 on blood agar with salt (2% NaCl). The colonies are small and identified as *Pasteurella* sp.

3.1.2 SDS-PAGE protein profiles of *Pasteurella* spp.

The protein profile of *P*-S-1 was compared to the protein profile of *P*-S-2 and the protein profile of a *P*-L. The two salmon isolates have similar protein profiles. The protein profile of the *P*-L however, is different from the *Pasteurella* species isolated from salmon (Figure 7). The major differences are as follows. Between 14.4 and 21.5 kilo Dalton (kDa), a protein band is more clearly expressed from the two salmon isolates (Black arrow, Figure 7). Between 21,5 and 31,0 kDA, two major differences are seen (Figure 7, Black arrows). Proteins are present in the salmon isolates but not in the lumpfish isolate.

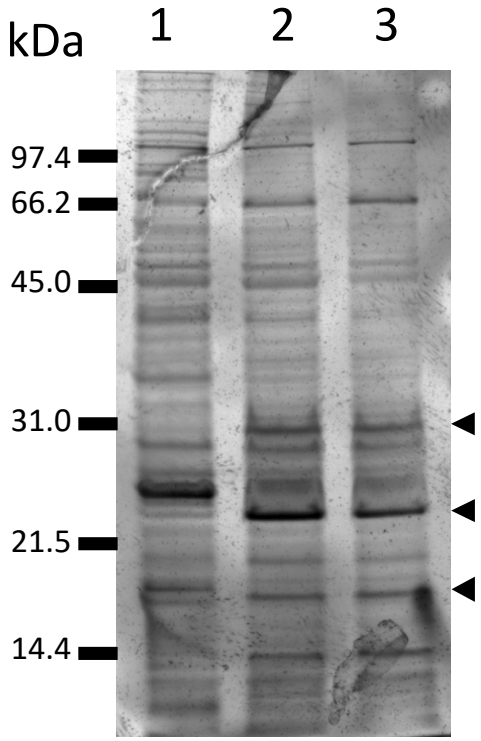

Figure 7 Silver stained Sodium-dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) showing protein profiles of various *Pasteurella* isolates. 1: shows *Pasteurella* sp. isolated from lumpfish (*P*-L). 2: shows *Pasteurella* sp. from salmon (*P*-S-1). 3 shows *Pasteurella* from salmon (*P*-S-2). The black arrows show some of the major differences between the isolates. The ladder was unstained low range SDG-PAGE standard (#161-0304, Bio-Rad) diluted at 1/20 in gel-loading buffer.

3.2 Isolation of bacteria from lumpfish with an ongoing diagnosed case of vibriosis

Bacteria were isolated from diseased lumpfish (app. 10g) from a fish farm in Vestland County. Gross pathology was examined and included haemorrhages in the skin, pale internal organs, ascites in the body cavity and an empty gut. The vent was swollen in some of the fish (Figure 8). The behaviour of the fish was abnormal during the outbreak, as the fish were found close to the water surface gasping for air. The first sampling gave growth of multiple bacterial species, identified by morphology (Figure 9A), on TSA 2% NaCl. The second sampling also gave bacterial growth on TSA 2% NaCl and blood agar with 2 % NaCl. Different colony morphology was observed. Bacterial growth was not observed on TCBS. On blood agar, some colonies

showed beta-haemolysis (ß-haemolysis) after 7 days of incubation (Figure 9B). Selected colonies of these were positive by the agglutination test.

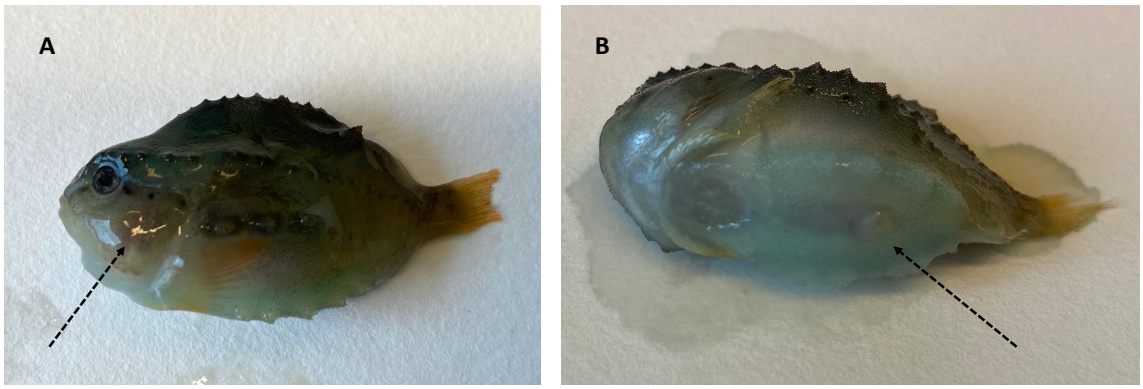

Figure 8 Lumpfish with macroscopic lesions associated with disease. The stapled arrow shows an area with red discolouring, potential haemorrhage (A). The stapled arrow shows swollen vent due to accumulation of ascites fluid in the abdomen (B).

3.2.1 16s rRNA sequencing of bacteria

16s rRNA gene sequence analysis from the first sampling identified the bacteria isolated from lumpfish as *Aliivibrio logei*. The PCR products of amplified *A.logei*. were visualised on agarose gel (1%) (Appendix C – Figure A.1). The isolate was found to be 98.43% similar by 16S rRNA to *Aliivibrio logei* (MT111586) by using BLAST. The 16s rRNA gene analysis from the second sampling identified the bacteria isolated from lumpfish as *Photobacterium* spp. The PCR products of amplified *Photobacterium* spp. were visualised on agarose gel (1%) (Appendix C – Figure A.1). The isolate was found to be 100% similar to *Photobacterium* spp. by BLAST.

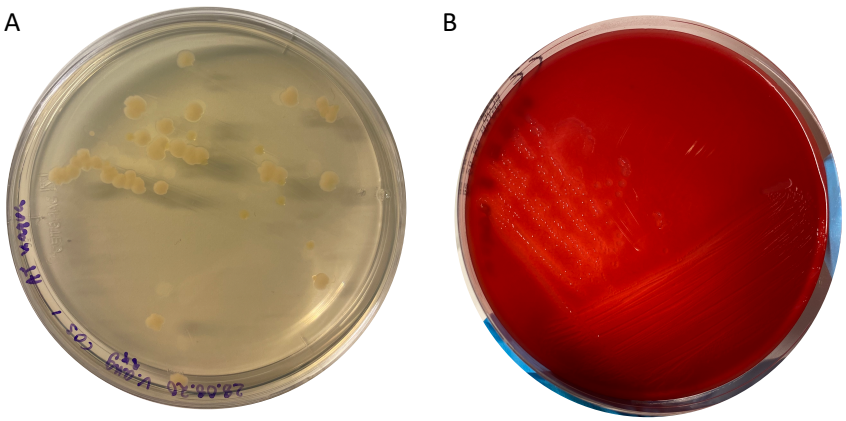

Figure 9 Colony morphology of primary sampling from lumpfish diagnosed with vibriosis on TSA with salt (2% NaCl) (A). Different colony morphology where observed. Picture of colony morphology of *Photobacterium* spp. on blood agar with salt (2% NaCl) after approximately 7 days of incubation. The colonies are small and ß-haemolytic (B).

3.2.2 SDS-PAGE protein profiles of isolates in family *Vibrionaceae*

The protein profile of *V.a*-L, *V.a*-SB and *A.l*-L was visualized by silver staining and compared to each other. The protein profile of *V.a*-L and *V.a*-SB shows clearly a high similarity. Isolate *A.l*-L is however clearly different from the *V.anguillarum* isolates. Several expressed proteins are different, among them are bands between 14.4 kDa and 21.5 kDa, between 31.0 and 31.0 kDa and 45.0 kDa, and between 66.2 kDA and 97.4 kDa (Figure 10, black arrows).

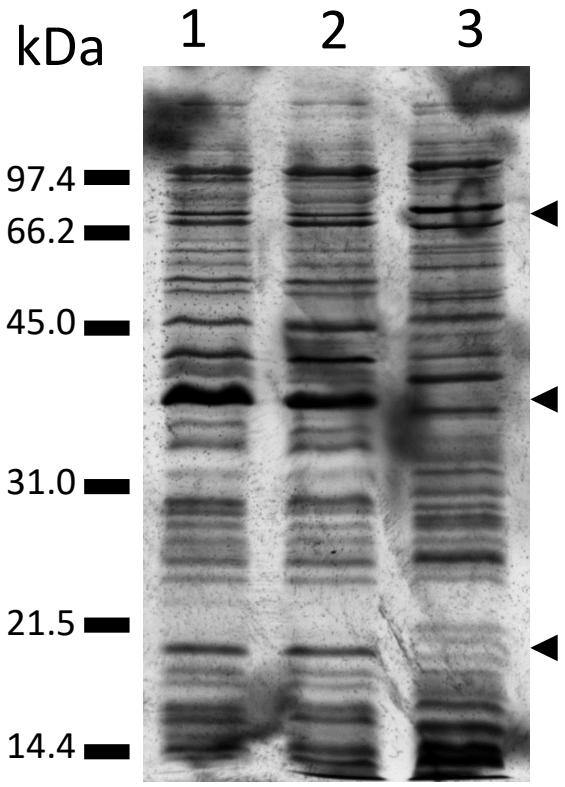

Figure 10 Silver stained Sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing protein profiles of various isolates in family *Vibrionaceae*. 1: shows *V.anguillarum* from lumpfish (*V.a*-L), 2: shows *V.anguillarum* from Sea Brass (*V.a-SB)*, 3: shows *A.logei* (*A.l*-L) from lumpfish. The ladder was unstained low range SDG-PAGE standard (#161-0304, Bio-Rad) diluted at 1/20 in gel-loading buffer.

3.3 Growth Curves

3.3.1 *V. anguillarum*

V.a-L is reaching the start of the exponential growth phase after 2 hours. The stationary phase is reached after 11 hours. The optimal time for phage introduction to a bacterial culture is when the host bacteria are in early- to mid-exponential growth phase. The bacteria were therefore harvested at OD 0.6-1.2 when used in enrichment or plaque assays (Figure 11).

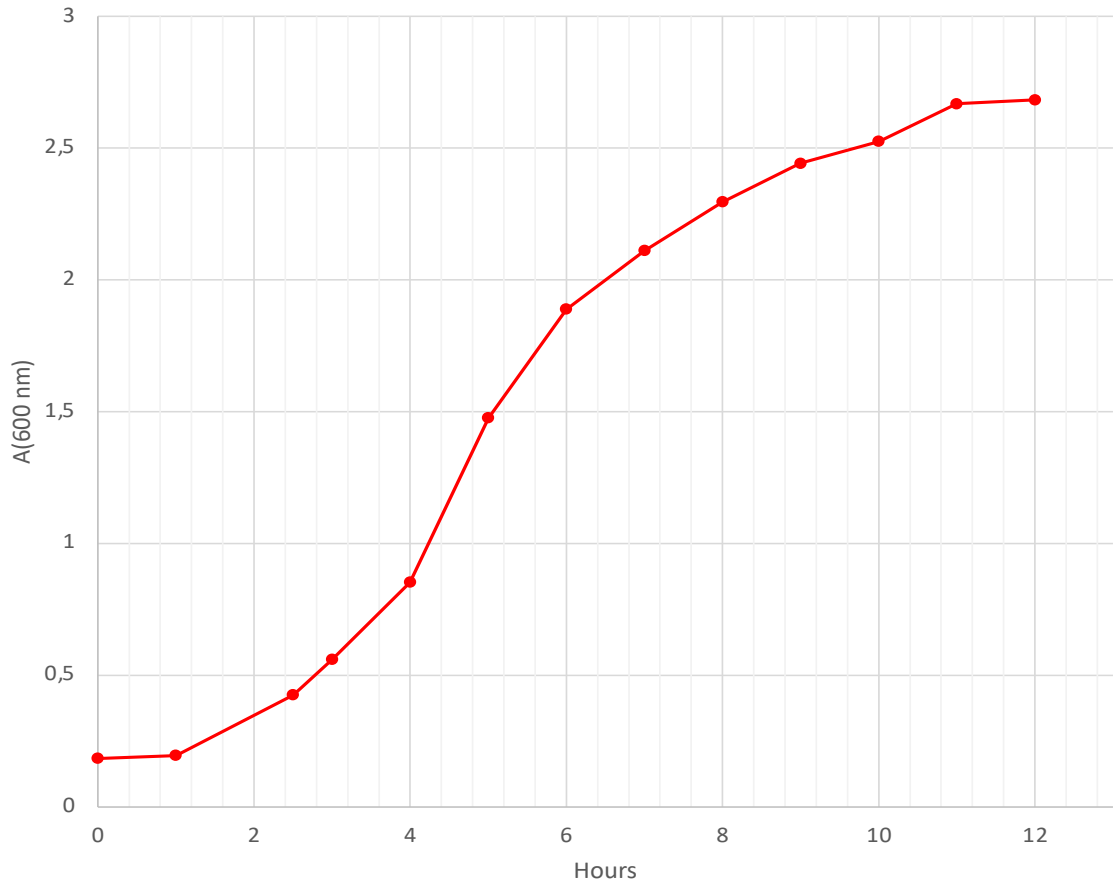

Figure 11 Growth curve for *V. anguillarum* isolated from lumpfish (*V.a*-L) grown in Tryptic Soy Broth (TSB) with 2% NaCl at 20°C at 200 RPM. The x-axis shows hours post inoculation and the y-axis shows OD measured at 600nm. The figure shows that the exponential growth phase is reached after 2 hours and the stationary phase is reached after 11 hours.

3.3.2 *Pasteurella* sp.

P-S-1 (Figure 12, yellow line) and *P*-S-2 (Figure 12, green line) are growing modestly until reaching the early exponential growth phase after 6 hours. The stationary phase is reached after 20 hours and. *P*-L (Figure 12, orange line) is growing modestly until reaching the exponential growth phase after 11 hours. The stationary phase is reached after approximately 23 hours. The *Pasteurella* isolates from salmon are reaching a higher OD than *Pasteurella* sp. isolated from lumpfish. *P*-S-1 and *P*-S-2 were harvested at OD 0.4-1.0 and *P*-L were harvested at OD 0.3-0.8 when used in enrichment or plaque assays.

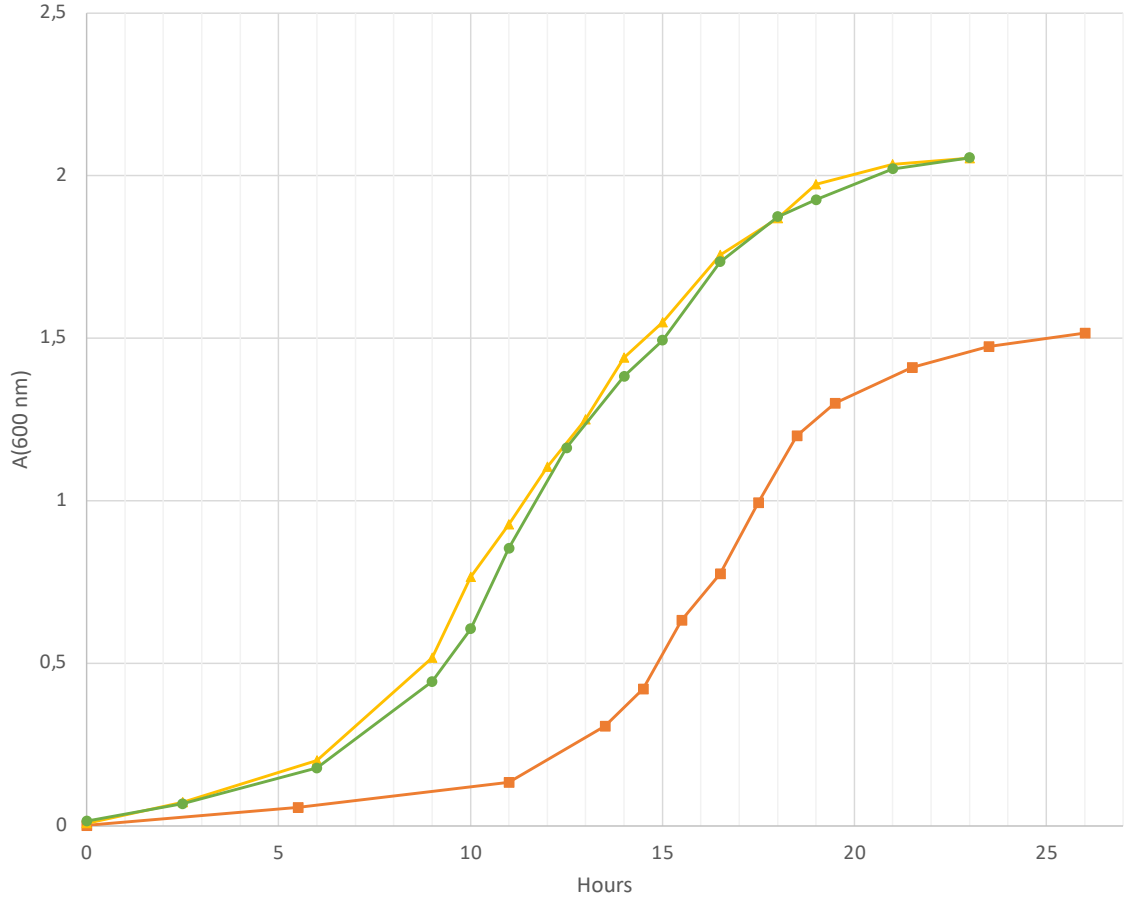

Figure 12 Growth curve for *Pasteurella* sp. isolated from lumpfish and salmon grown in Tryptic Soy Broth (TSB) with 2% NaCl supplemented with 10% Foetal Calf serum (FCS) at 20°C at 200 RPM. The growth curve shows P-S-1 (Yellow line), P-S-2 (Green line) and P-L (Orange line). The x-axis shows hours post inoculation and the y-axis shows OD_{A600}. The exponential growth phase is reached after 6 hours and the stationary phase is reached after 20 hours for P-S-1 and P-S-2. The exponential growth phase is reached after 11 hours and the stationary phase is reached after 23 hours for P-L.

3.3.3 Atypical *Aeromonas salmonicida*

A.s-L is growing modestly until reaching the start of the exponential growth phase after approximately 14 hours. The stationary phase was not found and is reached at a point after 27 hours (Figure 13).

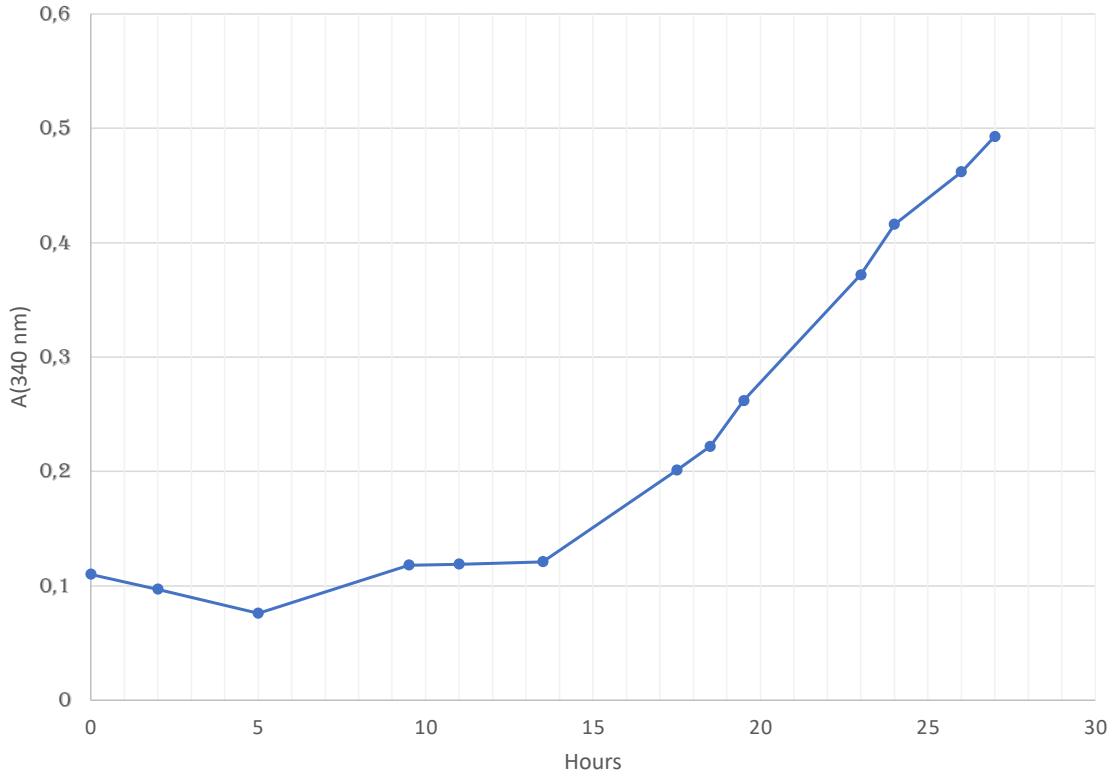

Figure 13 Growth curve for Atypical *A. salmonicida* isolated from lumpfish grown in Tryptic Soy Broth (TSB) 0.5% NaCl. The x-axis shows hours post inoculation and the y-axis shows OD_{A340}. The figure shows that the exponential growth phase is reached after 14 hours. The stationary phase is not found and is reached at a point after 27 hours.

3.4 OD/cfu/ml and OD/cells/ml

To give an estimate of how many living cells that are present at a given OD, OD/cfu/mL and OD/cells/ml correlation curves were made for isolates included in this study. When measuring cfu/ml, the viable cells are measured by counting colonies on agar plates. When measuring cells/ml both viable and dead cells are measured by using a cell counter.

3.4.1 *V. anguillarum*

Results from measurement (*V.a*-L) of OD, cells/ml and cfu (Table 6) were used to make OD/cfu/ml and OD/cells/ml correlation curves (Figure 14). To calculate cfu/ml of *V.a*-L, the plates inoculated with different dilutions. Dilution 10^{-7} of bacterial suspension was selected. The number of colonies were 118 colonies in parallel one and 145 in parallel two. The average was therefore 132. Cfu/ml was found by using the formula presented in section 2.2.5:

$$\text{Number of cells(cfu)pr. mL} = \frac{(132)}{10^{-7}} = 1.32 \text{ x } 10^{9}\text{cfu/mL}$$

Table 6 Results from measurements of OD_{A600}, cells/ml, cfu/ml of different dilutions of *V.anguillarum* isolated from lumpfish (*V.a*-L).

Dilution factor	$OD_{A(600nm)}$	Cells/ml	Cfu/ml
0	2.32	$4.0x10^9$	$1.3x10^9$
1/2	1.96	$2.0x10^9$	$6.6 x10^8$
1/4	1.38	$1.0x10^9$	$3.3 x10^8$
1/8	0.88	$7.1x10^8$	$1.6 x10^8$
1/16	0.50	$4.4x10^8$	$8.3 x10^8$
1/32	0.28	$2.3x10^8$	$4.1 x10^7$
1/64	0.12	$1.2x10^8$	$2.0 x10^7$
1/128	0.06	$6.8x10^7$	$1.0x10^7$
1/256	0.03	$3.8x10^7$	$5.0x10^7$
1/512	0.01	$2.4x10^7$	$2.5x10^7$
1/1024	0.00	$2.7x10^7$	$1.3x10^7$

The results from Table 6 were combined and shows the correlation between OD and cells/ml and OD and cfu/ml of *V.a*-L (Figure 14). The number of cells are increasing rapidly after OD 0.4. When OD 1 is measured, the number of cells is estimated as $8x10^8$ from cells/ml and $2x10^8$ from cfu/ml. The number of cells are higher when estimated from cells/ml than cfu/ml.

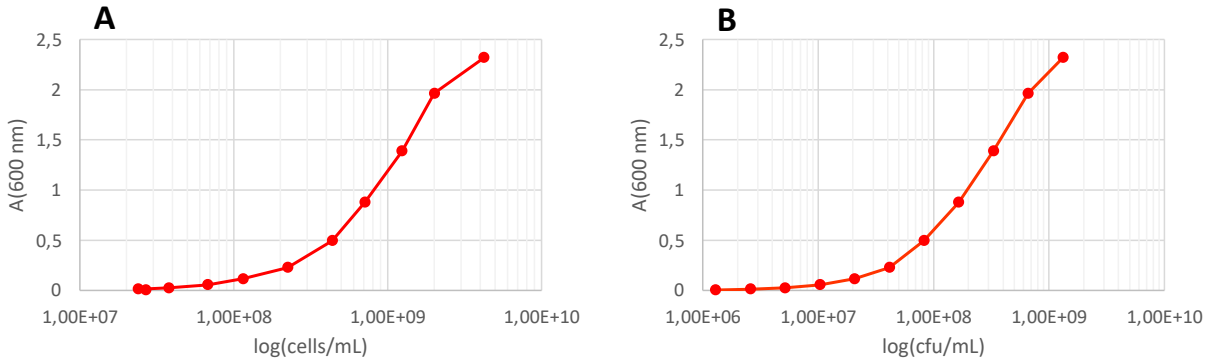

Figure 14 Correlation between OD_{A600} and cells/ml (A) and OD_{A600} and cfu/ml (B) for *V. anguillarum* isolated from lumpfish (*V.a*-L).

3.4.2 *Pasteurella* sp.

The same method as presented in section 3.4.1 was used to calculate cfu/ml for *Pasteurella* spp. To simplify, the results are only presented as figures. When OD of bacteria culture *P*-S-1 is reaching 0.2 both cfu/ml and cells/ml are increasing rapidly (Figure 15, A and B). At OD 1 numbers of cells measured as cells/ml are approximately $5x10^8$ cells/ml and numbers of bacteria measured by cfu/ml are approximately $3x10^8$ cfu/ml. The estimated numbers of cells are higher when estimated from cells/ml than from cfu/ml.

The curve which shows *P*-S-2 (Figure 15, C and D) shows that when OD is reaching 0.2, the number of cells measured by both cfu/ml and cells/ml is increasing rapidly. When OD is 1 the cells/ml is approximately $9x10^8$ and cfu/ml is measured as $2x10^8$. The estimated numbers of cells are higher when estimated from cells/ml than from cfu/ml.

The curve which shows *P*-L (Figure 15, E and F) shows that after the OD is reaching 0.1 both cfu/ml and cells/ml are increasing rapidly. At OD 1 numbers of cells measured as cells/ml are $6x10^8$ cells/ml and numbers of cells measured by cfu/ml are approximately $3x10^8$ cfu/ml. The estimated number of cells is higher when estimated by cells/ml than cfu/ml.

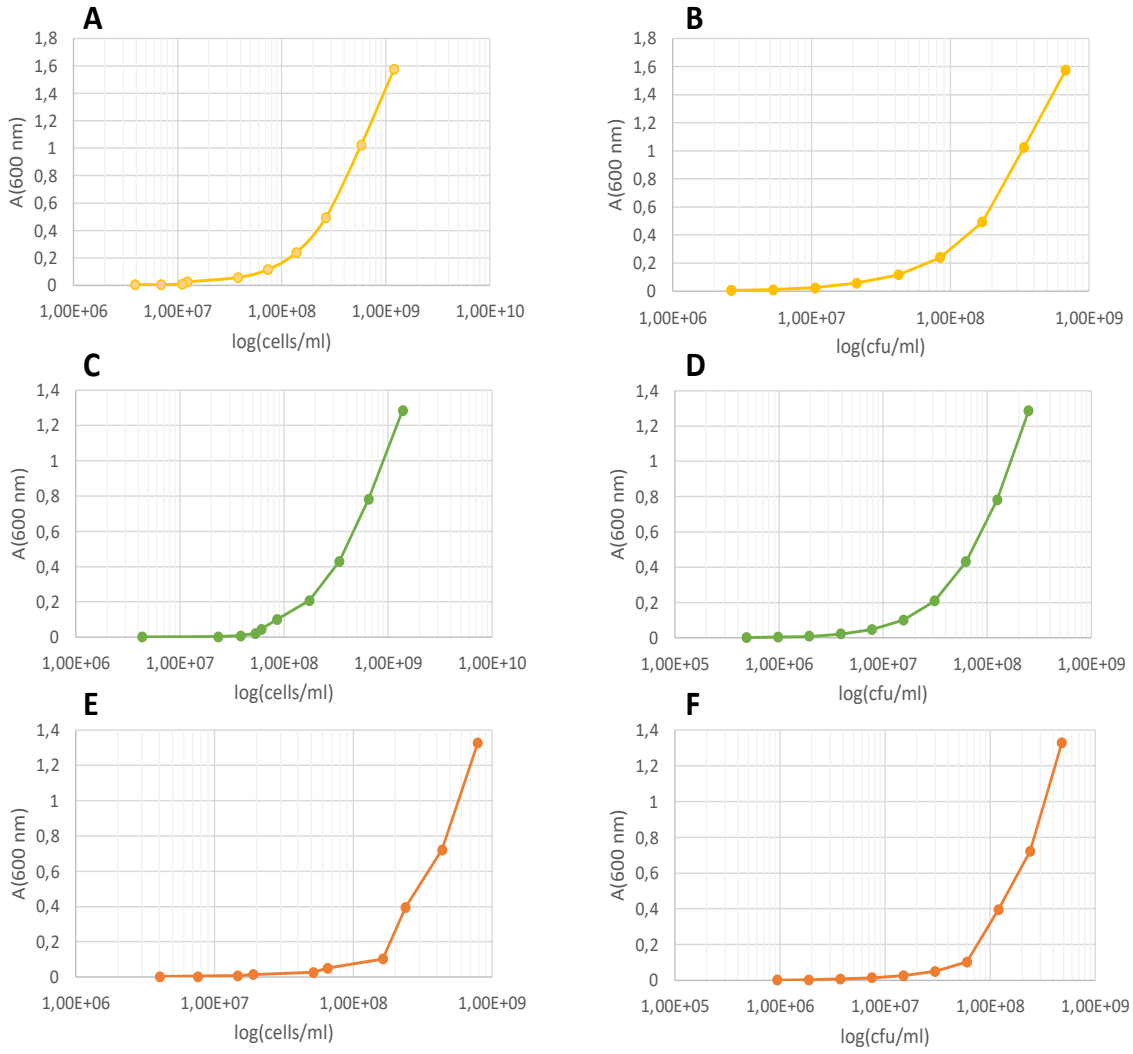

Figure 15 Correlation between OD_{A600} and cells/ml (A, C, E) and OD_{A600} and cfu/ml (B, D, F) for *Pasteurella* spp. isolated from lumpfish and salmon. OD/cells/ml and OD/cfu/ml was measured for *P*-S-1 (Yellow lines, A and B), *P*-S-2 (Green lines, C and D) and *P*-L (Orange lines, E and F).

3.4.3 Atypical *Aeromonas salmonicida*

The same method as presented in section 3.4.1 was used to calculate cfu/ml for atypical *A. salmonicida*. To simplify, the results are only presented as figures. The correlation seen between OD and cells/ml and OD and cfu/ml is that number of cells is increasing rapidly after OD is 0.1 (Figure 16). When OD is measured as 0.3, the number of cells is estimated as approximately $6x10^8$ from cells/ml and $1.0x10^8$ from cfu/ml. The number of cells estimated from cells/ml are higher than number of cells estimated from cfu/ml.

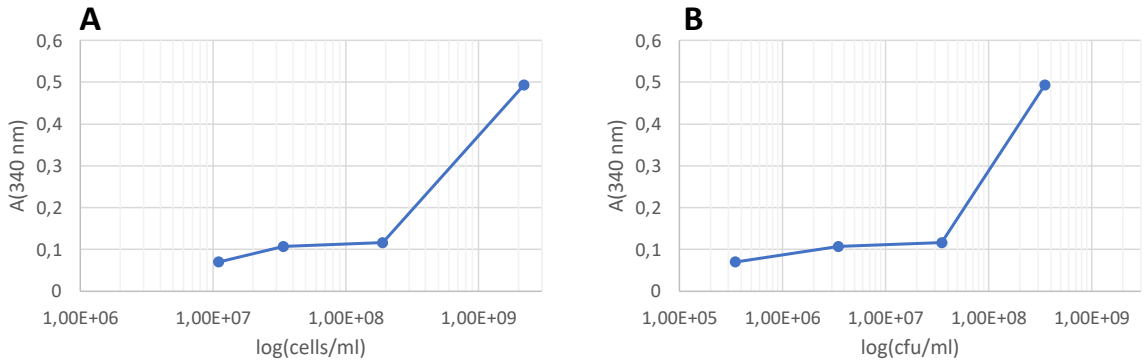

Figure 16 Correlation between OD_{A340} and cells/ml (A) and OD_{A340} and cfu/ml (B) for Atypical *A. salmonicida* isolated from lumpfish.

To summarize for all the isolates, the estimates cells are higher when measured as cells/ml than cfu/ml. When OD has reached a given number, the log phase is entered and the number of cells is increasing rapidly.

3.5 Isolation of bacteriophages

The majority of water samples did not contain phages targeting the test bacterial strains when used in plaque assays. However, water sampled at the salmon farm in Vestland county enriched with *P*-S-1 and *P*-S-2 tested positive for presence of phages.

3.5.1 Plaque assay *Pasteurella* sp.

Enrichment of water samples with *P*-S-2 and using the lysates harvested in plaque assay led to formation of morphologically different types of plaques (Figure 17). In the first trial with *P*-S-2, eight assumed plaques were transferred to TM buffer for further purification. After purification, two out of eight assays showed plaques. After the third purification however, new plaques were not formed. Neither did re-isolation of plaques from the agar as described in section 2.5.4 lead to formation of plaques.

The trial was repeated by enriching new water samples from the same site and the samples were subsequently used in plaque assays. The lysate again led to morphologically different types of plaques which was purified. New plaques were formed in 3 out of 18 assays. The plaques were purified and subsequently tested in a plaque assay. Plaques were not formed after the third purification round. The same result was observed when *P*-S-1 was used in plaque assays. Plaques were formed in some plaque assays, but complete isolation was not successful.

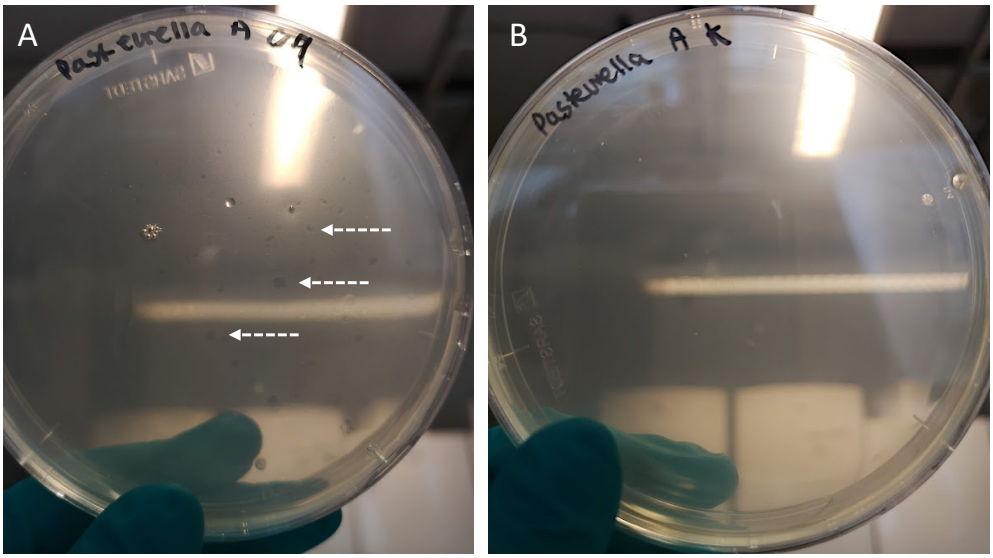

Figure 17 Result from plaque assay. Plaques with different morphology were formed (white stapled arrows) after addition of lysate enriched water samples (*P*-S-2) from an aquaculture site with clinical sick salmon (*P*-S-2) (A). Control bacterial, without phage supplement, showed bacterial growth (B). The results were inspected after ON incubation.

3.5.2 Plaque assay *V. anguillarum*

Lysate from enrichment with *V.a*-L of water samples presented in Table 4 did not lead to formation of plaques. Water samples from the fish farm with an ongoing case of vibriosis did neither lead to formation of plaques when enriched with *V.a*-L, *V.a*-SB, *A.l*-L nor *Pb*-L.

Φ53 and *V.a*-SB was included as a positive control in plaque assays, and plaques were formed in these assays (Figure 18 A). When Φ53 was tested with *V.a*-L plaques were not formed, but a reduced bacterial growth compared to control bacteria without the addition of bacteriophage was however observed. In spot assays, the bacteriophage showed bacteriolytic effect against *V.a*-L (Figure 18 B). It was therefore hypothesised that Φ53 had an effect on both *V.a*-SB and V.*a*-L.

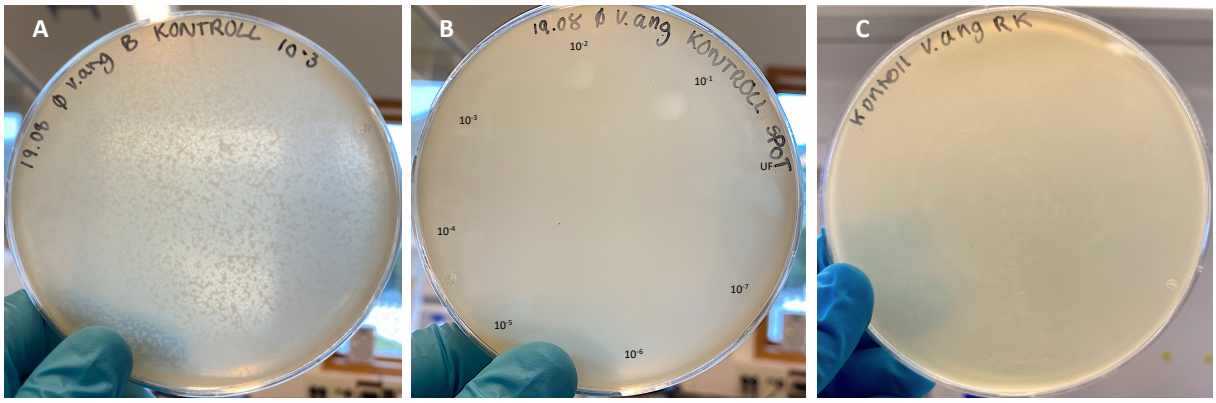

Figure 18 Results from plaque and spot assays. A shows plaque formation of *V.a*-SB infected with Φ 53 after ON incubation. Plaques were formed in these assays. B shows the result from spot assay of *V.a*-L infected with Φ 53. Φ 53 was spotted on to top agar in tenfold dilutions. The picture show clear zones at dilutions where Φ 53 had a bacteriocidic effect on *V.a*-L. C shows bacterial growth of *V.a*-L after ON incubation.

3.6 Phage activity and effect of Φ 53 on *V.a*-L and *V.a*-SB

The effect of Φ 53 on *V.a*-L and *V.a*-SB was investigated in another approach than plaque and spot assays. Bacterial counts as a function of cell size analysed in a CASY cell counter after incubation of different ratios of bacteria and phages was investigated (Figure 19). In addition, bacterial growth of *V.a*-L and *V.a*-SB measured by cfu/ml and cells/ml after incubation with different doses of Φ53 was investigated (Figure 20).

In function of cell size analysed in a CASY cell counter, there is a difference between *V.a*-L without bacteriophage supplement (Figure 19 A), *V.a*-L with Φ53 supplemented at MOI 1:1 (Figure 19 C) and Φ53 supplemented to *V.a*-L at MOI 2:1 (Figure 19 E). *V.a*-L without addition of phage is increasing over time and most of the cells are between 1 and 2 µm of size. When *V.a*-L is incubated with Φ 53 at a MOI 1:1 the number of bacteria also increases over time. However, a clear peak of cells between 1 µm and 2 µm is not seen as cells are present in a range between 0.5 µm and 2 µm of size. When *V.a*-L is incubated with Φ 53 at a MOI 2:1, a peak of cells between 1 µm and 2 µm is not seen as most of the cells present in the solution are between the 0.5 µm and 1 µm in size.

In function of cell size analysed in a CASY cell counter, there is also a difference between *V.a*-SB without bacteriophage supplement (Figure 19 B), *V.*a- L with Φ53 supplemented at MOI 1:1 (Figure 19 D) and Φ53 supplemented at MOI 2:1 (Figure 19 F). Without supplement of phages, *V.a*-SB show bacterial growth over time. The bacteria present are between 1 µm and 2 µm in cell size (Figure 19 B). When incubating *V.a*-SB with the addition of Φ 53 at MOI 1:1 the number of bacteria also increase over time. The first two hours, there is an increase in cells both in size range 0.5 µm to 1 µm and 1µm to 2 µm. After three hours, an increase in cells between 0.5 µm to 1 µm is seen, and some cells between 1 µm and 2. This is followed by a reduction in numbers of cells within both size ranges. The last measurement (at 5 hours) shows an increase in cells between 1 µm and 2 µm (Figure 19D). When *V.a*-SB is incubated with Φ 53 at a MOI 2:1, the number of cells increases over time. The bacterial counts of cells between 1 µm and 2 µm is low until 5 hours after the addition of the bacteriophage. The cell counts of bacteria between 0.5 µm and 1 µm vary over time (Figure 19, F).

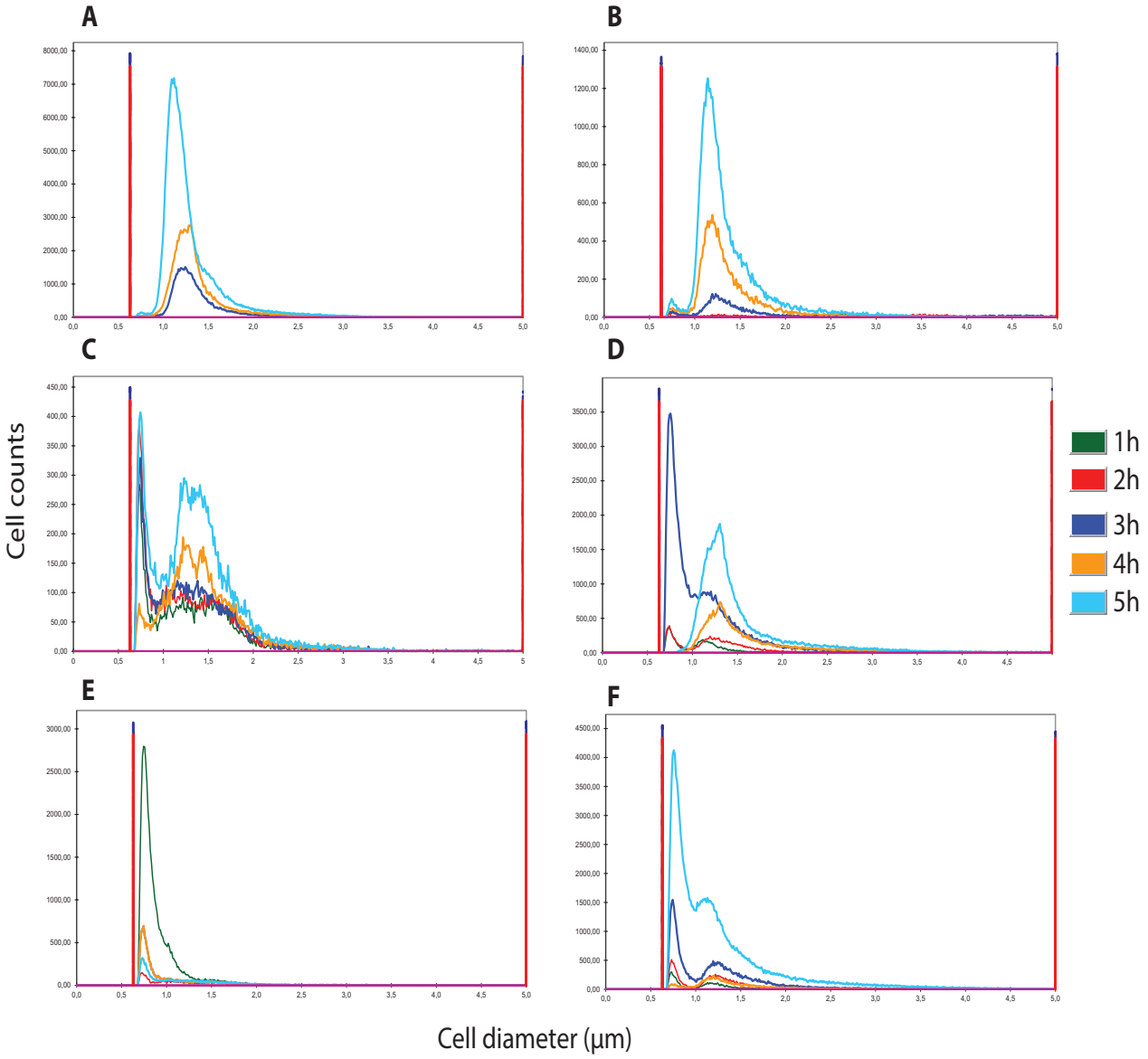

Figure 19 Bacterial counts over time as function of size analysed in CASY cell counter after incubation of *V.a*-L (A,C,E) and *V.a*-SB (B,D,F) with different doses of Φ 53. The growth of *V.a*-L and *V.a*-SB was measured without addition of phage (A,B), phage supplemented at MOI 1:1 (C,D), and phage supplemented at MOI 2:1 (E,F). The measurements were conducted each hour for 5 hours. The bacteria were grown in TSB 2% NaCl and were incubated at room temperature. There is a clear difference in bacterial counts as a function of cell size.

In function of number of cells measured by cfu/ml (Figure 20 A) and cells/ml (Figure 20 B), there is a difference between *V.a*-L without bacteriophage supplement (Figure 20, dark red columns and lines), *V.*a- L with Φ53 supplemented at MOI 1:1 (Figure 20, middle red columns and lines) and Φ53 supplemented at MOI 2:1 (Figure 20 light red columns and lines). Without phage supplement, the number of bacteria is increasing both in function of cfu/ml and cells/ml. The number of bacteria has increased from $8x10^7$ cfu/ml to $3x10^8$ cfu/ml. Measured as cells/ml

there is continuous growth each hour . When Φ53 is supplemented at MOI 1:1, the number of bacteria increases less. Cfu/ml has increased from $2x10^6$ to $6x10^6$. Based on cells/ml, the bacteria are growing modestly the first hour, and after 2 hours a reduction in bacterial count is seen. After 3 hours a slight increase in bacterial count is measured. When Φ53 is supplemented at MOI 2:1, the number of bacteria is not increasing both function of cfu/ml and cells/ml. In function of cfu/ml, the number of bacteria is reduced from the start to the end from $1x10^6$ cfu/ml to $6x10^5$ cfu/ml. This reduction is also seen when measured as cells/ml. After 2 hours a small increase of bacterial counts is measured, but this is followed by an reduction the two following hours.

In function of number of cells measured by cfu/ml (Figure 20 C) and cells/ml (Figure 20 D), there is a difference between *V.a*-SB without bacteriophage supplement (Figure 20, dark green columns and lines), *V.*a- L with Φ53 supplemented at MOI 1:1 (Figure 20, middle green columns and lines) and Φ53 supplemented at MOI 2:1 (Figure 20 light green columns and lines). Without addition of phage to *V.*a-SB, the number of bacteria has increased in function of cfu/ml from $3x10^6$ cfu/ml to $2x10^8$ cfu/ml. Measured as cells/ml, the number of cells has increased enormously the second hour. The aggregation factor from this measurement was almost 10 times higher than from other measurements. This measurement is followed by a reduction and thereafter continuous growth each hour. When phage Φ53 was supplemented at MOI 1:1, the number of bacteria measured by cfu/ml has increased, respectively from $3x10^6$ cfu/ml to $1x10^8$ cfu/ml. It has increased less than *V.a*-SB without phage supplement. The number of bacteria measured as cells/ml is reduced the first hour, and followed by an increase after the second hour. After the third hour, an reduction in cell numbers is seen, and this is followed by an increase in cell numbers. When Φ53 is supplemented at MOI 2:1, the number of bacteria has increased less when measured by cfu/ml, from $2x10^6$ to $2x10^7$ compared to bacteria without phage and with Φ53 at MOI 1:1. In function of cells/ml , the number of bacteria is increasing until the third hour, when a reduction in cell numbers is seen. This is followed by an increase in cells/ml.

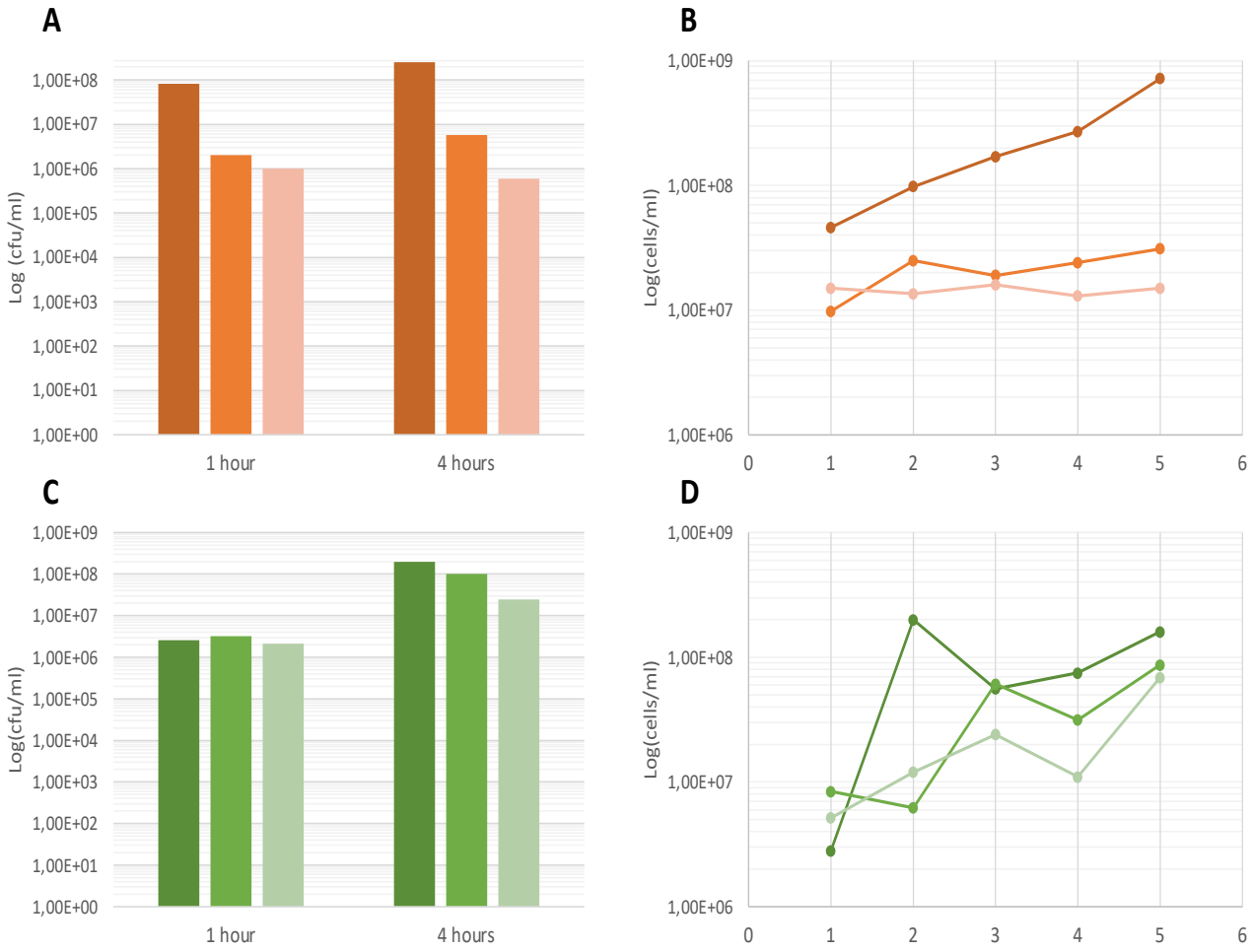

Figure 20 Growth of *V.a*-L (A and B) and *V.a*-SB (C and D) measured over time by cfu/ml (A,C) and cells/ml (B,D) after incubation with different doses of Φ 53. The bacteria was incubated without supplementation of Φ 53 (Dark green and dark red columns and lines), with supplementation of Φ 53 at a MOI 1:1 (Medium green and medium red columns and lines), and supplementation of Φ 53 at MOI 2:1 (Light green and light medium columns and lines). Compared to *V.a*-L without phage addition, reduced growth of *V.a*-L incubated with Φ 53 at MOI 1:1 and 2:1 is seen. A slight reduction of *V.a*-SB incubated with Φ 53 at MOI 1:1 and MOI 2:1 is seen. The bacteria were grown in TSB 2% NaCl in room temperature, and the control bacteria included the addition of sterile TM buffer.

4. Discussion

The commercial production of lumpfish has been and still is faced with bacterial diseases. Among the pathogens that infect lumpfish are *Pasteurella* sp., *V. anguillarum*, and Atypical *A. salmonicida*. The high prevalence of bacterial infections demonstrates the need for effective methods to prevent disease. Vaccination is an important tool to prevent disease, however, bacteria might cause disease before the fish have reached a size when they can be vaccinated and there might be limited vaccines available. In addition, *Pasteurella* spp. is reported as an emerging disease in Norwegian salmonid aquaculture. As there are no commercially available vaccines immunizing against pasteurellosis, neither for lumpfish nor for salmon, pasteurellosis is a severe threat to the industry. To prevent mortality and suffering of fish, antibiotic treatment must be used. Controlling diseases by using antibiotics is a concern due to the potential development and spread of antibiotic resistance. Phage therapy or prophylactic use of phages is suggested as an alternative to reduce the occurrence of bacteria, and to some extent, reduce the use of antibiotics.

In this study, an effort was put towards isolating the bacteria *Pasteurella* spp., *V. anguillarum* and Atypical *A. salmonicida* and bacteriophages targeting them. *Pasteurella* sp. was successfully isolated and characterized from clinically sick Atlantic salmon. By investigation and comparison of protein profiles, *Pasteurella* sp.(*P*-S-2) from salmon showed high similarity to a previously characterized salmon *Pasteurella* sp. isolate (*P*-S-1) and these isolates were different from the protein profile of *Pasteurella* sp. from lumpfish (*P*-L). From clinically sick lumpfish diagnosed with vibriosis, *Photobacterium* spp. and *A.logei* was isolated, not *V.anguillarum* as expected. The protein profiles of two previously characterized *V.anguillarum* were shown to be different from *A.logei.* The growth curves made showed that the included isolates grew differently, and were necessary in further work of bacteriophages. Complete isolation of lytic bacteriophages was not achieved even though plaques were shown in plaque assays with *Pasteurella* sp. (*P*-S-1 and *P*-S-2). Preliminary results show that the included control bacteriophage showed an effect against *V.anguillarum* isolated from lumpfish.

4.1 General work with bacteria

Compiling growth curves for the included bacteria isolates in this study was necessary before the enrichment of water samples with target bacterial strains. Single-celled organisms divide by binary fission, the bacteria will copy their genetic material and divide into two daughter cells which will result in exponential growth. The growth of bacteria will go through lag, log and stationary phase. The lag phase is when the bacteria are metabolically active but not multiplying. The log phase is the exponential growth phase. In the stationary phase, the growth reaches a plateau with no growth in the culture, and eventually the cells will die due to starvation. The bacteria are most active in the log phases and the growth curves are compiled to determine when the bacteria are in their log phases. During this phase, supplement of phages will provide high yield of phage progeny while if supplemented in the lag phase the phage will not multiply (Sambrook & Russel, 2001). The bacteria were harvested at the same stage of the log phase for each of the experiments to make sure that the results were repeatable and verifiable.

The bacterial isolates included in this study formed different growth curves. The *V. anguillarum* isolates grew quickly in contrast to *Pasteurella* spp. and Atypical *A. salmonicida*. As most *Vibrio* spp. are opportunistic (Suttle, 2007), they respond to favourable conditions by rapid growth and this is consistent with the results from the growth curves. Growth of the *Pasteurella* sp. isolated from lumpfish used in this study has been described by Ellul et al., 2018. They found that *Pasteurella* sp. grew slowly, and the stationary phase was not reached until 18-19 hours post-inoculation when TSB was supplied with 10% FCS. Likely due to the presence of the A-layer the atypical *A. salmonicida* strain used in this study is aggregating, thus the OD was measured after suspension in NaOH. These OD measurements are thus most likely less precise than the measurements done for *Pasteurella* spp. and *V. anguillarum*.

To determine the number of cells in the solutions, OD/cfu and OD/cells/ml correlation curves were made. OD will not give an estimate of how many living bacteria that are present in the suspension, but rather an estimate of biomass and the concentration of bacteria. OD is used as a quick way of controlling bacterial growth (Sutton, 2011). To make sure that viable cells were present in the exponential growth phase and to know the concentration of cells, OD-cfu and OD-cells/ml correlation curves were made. In the performed correlations, the estimated numbers of cells were higher when estimated as cells/ml than cfu/ml. This is most likely due to

only viable cells are counted when measuring cfu/ml and both viable cells and dead cells are measured as cells/ml in the cell counter. However, the number of dead cells present in the exponential growth phase is expected to be low. On the other hand, when cfu/ml is measured, it is estimated that one bacterial cell will form one colony. The forming unit might be one cell or it might be more.

4.2 *Pasteurella* sp. and bacteriophages targeting *Pasteurella* spp.

In this study, *Pasteurella* sp. (*P*-S-2) was isolated from clinical sick salmon from a sea site in Vestland County. In addition to severe eye lesions, pathological changes included bloody ascites and abscesses. Similar gross pathology as observed in this study has been observed in other cases with infection of *Pasteurella* sp. In 2000 Valheim et al., described the disease Varracalbmi. Gross pathology included severe eye lesions and blindness in addition to pathological changes in kidney, liver, gills and pseudobranch. The causative bacterium was later found to belong to the genus *Pasteurella* sp. (Johansen, 2013). The following years, outbreaks of pasteurellosis were regularly reported from salmon. In recent years (2018-2020), however, incidences of pasteurellosis in salmon have increased drastically (Sommerset et al., 2020). In 2020, Legård and Strøm described pasteurellosis in Atlantic salmon in Western Norway. Gross pathology from different cases of pasteurellosis varied, but the most frequent characteristics were purulent peritonitis, opalescent fluid in the cardiac cavity and exophthalmos (Legård & Strøm, 2020).

Identification of *Pasteurella* sp. was done by 16s rRNA sequencing. 16s rRNA is widely used to determine and assign bacterial species to their respective genera (Martens et al., 2008). The 16S rRNA gene fulfils the requirements of a suitable phylogenetic marker. The gene occurs ubiquitously in bacteria and archaea, has a fundamental function in protein synthesis and is hence evolutionary conserved. Even though the gene has a high evolutionary conservation, it has still enough sequence variation to discriminate between taxa. At an intraspecific level, 16S rRNA sequencing has limitations. Studies conducted on diverse bacterial isolates have discovered similar 16S rRNA gene sequences (Jaspers & Overmann, 2004). Investigation of the relationship to other *Pasteurella* spp. isolates such as the bacteria causing pasteurellosis in lumpfish, the salmon Varracalmbi isolate and *P. skyensis*, must be investigated by whole genome sequencing.

In addition to *P*-S-2, earlier characterized isolate *P*-S-1 and *P*-L were included in this study. Analysis by SDS-PAGE and silver staining was performed to investigate the differences at the level of expressed proteins. The results show that the relative abundance of major proteins and the distribution of other proteins is different between the salmon isolates and the lumpfish isolate. The difference in protein profile might indicate differences in virulence or antigenicity. In order to detect specific immunogenic proteins, enzyme-linked immunosorbent assay (ELISA) or western blot can be used.

An interesting aspect is that the same genotype of *Pasteurella* sp. was isolated from clinically sick salmon and lumpfish held on the same farm in 2018 (Hjeltnes et al., 2019). There is an ongoing project led by the Institute of Marine Research in collaboration with Pharmaq Analytic and UiB aiming to investigate how and if the disease spreads between the fish species (https://www.hi.no/hi/nyheter/2020/juli/rognkjeks-utviklet-sykdom-etter-smitte-mens-smittet-laks-ble-ikke-syk). Preliminary results from the study show that the lumpfish were infected by both isolates, and developed pasteurellosis. The salmon developed no clinical signs and no disease after challenge with either of the isolates.

Water samples from the salmon farm site in Vestland county with an ongoing outbreak of pasteurellosis were collected aiming to find phages targeting *Pasteurella* sp. from salmon and potentially *Pasteurella* sp. isolated from lumpfish. To increase the probability of collecting phages targeting *Pasteurella* spp. clinical sick fish were incubated in a tank, and hypothetically the fish could have shredded phages against the isolated bacteria. Complete isolation and purification of phages against *Pasteurella* spp. was not achieved, and there might be several reasons to this. Some phages are stable, other phages are unstable. Phages consist of nucleic acid and proteins and different physio-chemical conditions might influence the phage particles. If the phage is supposed to be used in therapy or for prophylactic use, it is however important that the phage is stable. The phages might also have been lost during filtration and handling of the water samples. This is not uncommon and has been reported previously (Tan, Gram, & Middelboe, 2014). Some phages, particularly the largest viruses, may aggregate significantly and consequently reducing their titre after centrifugation or membrane filtration (Serwer, Hayes, Thomas, & Hardies, 2007).

The plaque formation observed could also have been caused by a prophage or different prophages present in the genome of *P*-S-1 and *P*-S-2. As presented in the introduction, a

prophage is a phage genome integrated in the host genome. Prophages are latent until induced into a lytic cycle. A stressor could have induced the prophage into a lytic cycle and thereby making plaques in the agar. Smaller changes in pH and temperature (Capra, Mercanti, Reinheimer, & Quiberoni, 2010), or growing a host bacteria for several passages might lead to the release of virions. It was shown by the growth curves for *P*-S-1 and *P*-S-2 that the growth of the bacteria is slow. Hypothetically prophages induced to a lytic cycle may contribute to the slow growth. Whether or not temperate phages are present in the genome can be investigated by whole genome sequencing.

It is likely that phages isolated from the tank could have been a phage with a narrow host range. The water was sampled from the same environment as the host *Pasteurella* sp. isolate. Phages isolated from the same environment as their host will often have a narrow host range specific for the same strain as they were isolated with (Ross, Ward, & Hyman, 2016). Phages isolated from an environment with salmon might therefore only target *Pasteurella* sp. isolates from salmon and not *Pasteurella* sp. isolates from lumpfish. This is dependent on whether or not the phage binds specifically to targets only present on *Pasteurella* isolates from salmon or targets present on both isolates. As presented by the protein profile, the *Pasteurella* bacteria infecting salmon and lumpfish in this study are different. Proteins expressed on the cell surface that serves as receptors for phages might be different between isolates. Consequently, phages might be specific for the different strains.

However, isolating phages targeting salmon *Pasteurella* sp. could potentially be used in salmon farming. Using phages directly in sea cages are expected to have a limited effect due to the quick dilution of phages in open seawater. On the other hand, phages can be supplied in closed systems such as well boats. Outbreaks of disease in salmon farming are often seen after thermic treatment of salmonids to remove sea lice. Phages can potentially become a tool to reduce the infection pressure of bacteria in well-boats and potentially reduce the outbreaks of diseases that have been seen following treatments in well-boats.

Even though isolation of phages targeting *Pasteurella* sp., was not fulfilled in this study, growing *Pasteurella* spp. successfully in a double agar was successful. Before the start of this study, there were no established protocols on how to grow *Pasteurella* sp. isolated from fish in a double agar. By adding FCS 10% the growth of the bacteria was ensured, and by adding divalent cations the requirements of the phages were fulfilled. The method can further be used to investigate water samples for phages.

4.3 *V. anguillarum* and vibriophages

In this study, bacteria were isolated from a lumpfish farm with an ongoing outbreak of vibriosis in Vestland County. Previous to the disease outbreak, the fish had been handled due to sorting according to size. Some of the fish were transferred from a tank holding 8°C to a tank holding 11°C. Production management, changes and fluctuations in environmental conditions and treatments are potentially stressful for the fish. This might again lead to a weakened defence system making the lumpfish more susceptible to infection by pathogens (Erkinharju et al., 2020). In addition, stressful situations might amplify the severity of pathogen-related issues (Noble et al., 2018). The rapid change in temperature in addition to the sorting process might have made the lumpfish more susceptible to infection.

None of the bacterial isolates sampled from clinical sick fish diagnosed with vibriosis were identified as *V.anguillarum*. In the first isolation, a relative *A. logei* was isolated. As the first attempt of isolating *V.anguillarum* was unsuccessful, another effort was conducted. As TCBS is selective for bacteria in genus *Vibrio*, TCBS was used aiming to identify *V.anguillarum.* However, bacterial colonies were not present after sampling when grown on TCBS. The effectiveness of TCBS for *V.anguillarum* has been questioned. Another medium (VAM), has been suggested as a medium for presumptive identification of *V.anguillarum* (Alsina, Martinez-Picado, Jofre, & Blanch, 1994). Swabs from the head kidney were also grown on blood agar. After a week of incubation, haemolytic activity was shown. Haemolytic activity has been suggested to be a virulence factor for *V. anguillarum* (Li, Mou, & Nelson, 2011). Even though the agglutination kit showed positive agglutination test, the bacteria isolate were found to be *Photobacterium* spp. The genus *Photobacterium* belongs to the family *Vibrionaceae* and are a group of Gram-negative, rod-shaped bacteria that require NaCl for growth. They have been isolated from various marine habitats (Lo, Jin, & Jeon, 2014). The antibody used in the agglutination kit is likely targeting an antigen present on both bacterial surfaces and consequently a cross reaction is seen from the agglutination test.

V. anguillarum is a part of a large family of bacteria, *Vibrionaceae*, including several bacteria causing vibriosis. Genus *Vibrio* and *Aliivibrio* is known from fish farming and has been reported to cause severe disease in several species (Tan et al., 2014). Many marine wild and farmed fish are susceptible to vibriosis, and outbreaks normally result in significant mortalities (Austin & Austin, 2007). As the bacteria are present in the environment, and as it is an important pathogen

to many fish species, an effort was made to isolate vibriophages from environmental water samples.

In an attempt to isolate phages, potentially wide host range phages, environmental samples from various locations were enriched with *V.a*-L isolated from lumpfish. Water samples were collected before the summer months and during late summer 2020. No phages were isolated from the water samples collected before the summer months. *Vibrio* spp. are often growing when the water temperatures are high. The water samples collected during the summer-months did neither lead to the isolation of vibriophages. It is likely to believe that phages should easily be isolated from an aquatic ecosystem as phages occupy all those habitats where bacteria thrive. *V.a*-L used in this study was isolated from an aquaculture environment, and broad-ranged phages targeting this strain was not likely present in the water samples. Another explanation could be that phages were present in low concentration and contact between the phage and bacteria was not obtained.

Phages targeting *V. anguillarum* has previously been successfully isolated from environmental samples. So far, vibriophages have been found in marine and freshwaters, sediments, plankton, molluscs, crustaceans, sewage as well as aquaculture environment (Xu, 2016). In Chile, phages against *V. anguillarum* serotype O3 has been isolated. The phages were isolated from bivalve samples that were purchased in the central market of Santiago, Chile (Higuera, Bastías, Tsertsvadze, Romero, & Espejo, 2013). The phage was able to infect both *V. anguillarum* serotype O3 and *V. ordalii* but not *V. parahaemolyticus* strains. As mussels are filter feeders, it is likely to believe that bacteria and specific phages will be present in the organs of the mussels. Even though soft tissue from mussels was included in this study, this did not lead to isolation of phages targeting the test bacterial strains.

An effort to isolate bacteria and phages from the same environment was done as bacteria and water samples were collected from an aquaculture site with an ongoing outbreak of vibriosis. Isolation of *V.anguillarum* was not achieved, but *A.l*-L and *Pb*-L was used in enrichment. There is a chance that a broad-ranged phages targeting *Photobacterium* spp. and *A.logei* also can infect *V.anguillarum*. This is seen for example in broad ranged bacteriophage KVP40 that infects at least eight species of *Vibrio* spp. and one *Photobacterium* sp. All of the bacteria has an outer membrane protein that serves as a receptor for KVP40 (Rørbo et al., 2017). In this study, phages were not isolated from the fish farm.

Successful isolation of phages from fish farms has previously been conducted. Tan et al., 2014 isolated several vibriophages from farm sites with rainbow trout. In this study 20 *V. anguillarum* strains were included as test hosts. Important to mention is that they showed that phages could not be isolated without previous propagation in enrichment cultures and this could indicate that the density of specific vibriophages were low in the fish farm. Enrichment of the water samples was done prior to all assays in this study. Phages targeting other bacteria, such as *Flavobacterium psycrophilum*, has been isolated from water and water-sediment samples from freshwater rainbow trout farms (Stenholm, Dalsgaard, & Middelboe, 2008). A total of 25 isolates of *F. psycrophilum* were included. Common for the mentioned studies is that several isolates were included in the search for phages. The probability of finding phages is higher when many isolates are included.

In contrast to phages targeting different species of *Vibrios*, phages targeting *Pasteurella* sp. has not yet been isolated. An explanation might be that it is easier to isolate phages targeting *Vibrios* as the bacteria are regularly present in the environment. In addition, *Vibrio*s are opportunistic bacteria. Favourable conditions will therefore lead to a rapid growth of bacteria, and possibly this will be followed by increase of phages targeting them.

4.3.1 Investigating the effect of bacteriophage Φ 53 on *V.a*-L and *V.a*-SB

The control bacteriophage provided by ACD pharma originally isolated using *V.a-SB* as a target was tested against *V.a*-L and *V.a*-SB. It was hypothesised that the bacteriophage is lytic against both bacterial isolates. A greater reduction in the number of cells was observed when Φ 53 was incubated with *V.a*-L than with *V.a*-SB, despite the fact that the bacteriophage originally was isolated by enrichment of *V.a*-SB. The measurements done to study the effect of bacteriophage Φ 53 was cells/ml, cfu/ml and cell counts as a function of cell size. The cell size was measured to investigate if the bacteriophage had an effect cell burst. Cells/ml and cfu/ml was measured to determine if the number of bacteria were reduced after supplementing the bacteria with phages.

Bacteriophage Φ 53 seems to have an effect on *V.a*-L. The control bacteria, *V.a*-L shows growth over time. After 4 hours, an exponential growth is observed. This is consistent with the results presented in the growth curve of *V.a*-L (Figure 11). This exponential growth of *V.a*-L is not seen neither when the bacteriophage is supplemented at MOI 1:1 nor at MOI 2:1 both in

function of cells/ml and cfu/ml. Several bacteria in size range 0.5 µm to 1 µm is observed, and this might indicate bacterial cell bursts. There is also a reduction in bacterial growth when Φ 53 is supplied to *V.a-SB*. An effect is seen in function of cell size and bacterial growth measured as cfu/ml and cells/ml. The protein profiles of *V.a*-L and *V.a*-SB shows that the expressed proteins are similar for the two *V.anguillarum* isolates. Hypothetically, proteins serving as a receptor for Φ 53 is present on both bacterial surfaces.

When Φ 53 is supplemented to both bacterial cultures, it seems like the number of bacteria is increasing and followed by a reduction. This can be explained by the growth dynamic of bacteria and phages. Phage propagations lag behind the host in time, as they need the bacteria to propagate. A high number of host cells leads to a high number of phages. When the phages lyse their host, the number of bacteria is reduced (Parracho, Burrowes, & Enright, 2012).

It would have been interesting to include PFU/ml in this study. PFU/ml is widely used to investigate if the number of phages increases over time in contact with the bacteria. As presented in section 4.6 it was difficult to measure PFU due to that the phages did not make clean plaques when mixed with *V.a*-L even though lysis was observed in spot assays. The reason why *V.a*-L did not give clean plaques but only a zone of lysis when bacteriophage Φ 53 was added is uncertain. A possible explanation is that the plaques are very small, and thus are difficult to see in plaque assays. Furthermore, it is shown that the concentration of agar used in the top agar will affect plaque formation. Small phages are not able to form plaques in high agarose concentration (Mullan, 2002). Other studies have shown that plaques were not formed when a high concentration of agarose was used. Serwer et al., 2007 found that *Bacillus thuringiensis* phage on initial isolation made small (<1mm) plaques in a 0.4% agarose overlay but the plaques became progressively larger as the agarose gel concentration decreased to 0.2%. Another phage included in this study did not form plaques if the concentration of agarose where higher than 0.25%. In this study, the concentration of soft agar was 0.5%. Using a lower concentration of agarose should be considered for further search for phages.

In the present study, phages was supplemented at MOI 1:1 or MOI 2:1. MOI represents the ratio of the numbers of virus particles to the numbers of the host cells in a given infection medium. A value of MOI 1:1 implies that one phage will be absorbed by one bacterium. However, multiple phage particles can be adsorbed on a single host cell, whereas some of the host cell remain uninfected (Abedon, 2016).

It is important to mention that these studies were only replicated once, and several replicates are needed to investigate the effect of the bacteriophage. If several replicates and measurements are done, statistics can be used to investigate if the effect of the bacteriophage is significant. The preliminary results suggest that the bacteriophage can the reduce growth of both *V.a*-L and *V.a*-SB, but is most efficient towards isolate *V.a*-L. This is despite the fact that the bacteriophage was isolated using *V.a-SB* as the host.

4.4 *A. salmonicida* and bacteriophages

Water samples were not enriched with atypical *A. salmonicida*. No fish farms reported outbreaks of atypical furunculosis during this study. A growth curve showing the early exponential growth phase, OD/cfu and OD/cells/ml are established for this strain and can in a later stage be used in search of phages targeting this bacterium.

Phages targeting both atypical and typical *A. salmonicida* has been isolated in previous studies. Compared with vibriophages, the number of *Aeromonas* phages is relatively small (Xu, 2016). *Aeromonas* phages have been isolated, characterised and genome sequenced aiming to find the best candidate to phage therapy in aquaculture. The phages have been isolated from, for example, sediment samples of rainbow trout in Korea or environmental river waters in Korea. A few attempts have been made on the experimental applications of phage therapy to control *A. salmonicida* infections (Imbeault et al., 2006; Xu, 2016).

4.5 The use of phages in aquaculture

Phages have the potential to become an important tool in various situations in aquaculture. The use of phages is highly relevant for the larval stage when the immune system is not fully developed and therefore vaccination is not possible. Similarly, in the period after the immune system is fully developed before the fish has reached a size where it is able to tolerate the vaccine and the process of vaccination. Phages can furthermore be of relevant use in various situations when the fish is handled. As fish become more vulnerable to infections during stressful situations such as handling, sorting, sea lice treatment and with rapid temperature

changes, phages can be used prophylactically when it is important to keep the infection pressure low, mainly in closed environments. Phages are advantageous in fish farms that apply probiotic cultures of bacteria or in RAS where the system is dependent on the biofilter due to the high specificity compared to antibiotics. At last, the use of phages is highly relevant for aquaculture of shellfish. Invertebrates do not have an adaptive specific antibody immune response (Gudding, Lillehaug, & Evensen, 2014), and therefore they cannot respond by memory and antibody production after vaccination.

After finding phages targeting pathogenic bacteria, models of phage therapy using aquaculture animals must successfully be set up and tested. Results from *in vitro* studies may differ from what can be achieved in field (Rørbo et al., 2017; Sieiro et al., 2020). A phage showing a broad host range and high efficiency of infection in lab conditions, might not do the same in aquaculture settings. It is important to establish a suitable protocol of the use of the phages in the field. Independent of which method of administration of phages, preventive or therapeutic, it is important that contact between phage particles and the bacterial host in the water, as well as on the surface or inside fish is obtained. Furthermore, the actual ratio of infecting phage particles to bacterial prey is central in terms of effective treatment (Zaczek-Moczydłowska et al., 2020).

In vitro results may also differ from *in vivo* results in terms of other limiting factors of phages. As presented in the introduction, there is a risk that bacteria will develop resistance to phages. Different solutions are considered to reduce the resistance rate, like the use of phage cocktails. It is however important to distinguish between mutation rate in laboratories and in the environment. According to Sharma et al., 2017 and Ly-Chatain, 2014 the mutation rate among bacteria is lower in nature than in laboratories. In laboratory conditions, the phages are the main factor posing a selective pressure towards the bacteria. In the environment, other factors such as competitor bacteria, nutrients and other viruses contribute to form a selective pressure to which the bacteria much adapt.

Last but not least, legislation on the use of phages must be adapted. Today, the legislation for phages to be applied for bio sanitation and disinfection is different from the legislation for phages used as therapeutics. Therapeutic phage products are today regarded as pharmaceuticals product, but there are difficulties in legislating phages as a pharmaceutical (Fauconnier, 2019). One of the reasons is that pharmaceuticals must be highly defined. Phages are not stable and

will continuously develop in contact with the bacteria. Pharmaceuticals, such as antibiotics, are passive as the concentration of the pharmaceutical will decline by a combination of metabolism or excretion processes. Phages will in contrast increase depending on the bacterial density. Today, different countries regulate and gives different guidelines for phage therapy in aquaculture, according to their respective national legislation (Xu, 2016). After all, the scientific criteria that is needed to be met by the phage products are quality, safety and efficacy (Henein, 2013).

4.6 Concluding remarks and future perspectives

The prophylactic or therapeutic use of bacteriophages represents a potential tool to reduce the infection pressure of pathogenic bacteria in Norwegian aquaculture. Among the pathogens causing problems in lumpfish farming industry are *V.anguillarum,* Atypical *A.salmonicida* and *Pasteurella* sp. In salmonid farming, pasteurellosis is considered as an emerging disease. Vaccination is the most important tool to prevent bacterial diseases, however, a vaccine immunizing salmon or lumpfish against *Pasteurella* spp. is not yet commercially available. In various situations, for instance before vaccination is feasible and in handling procedures, fish might be vulnerable to disease. Phages can potentially reduce the infection pressure in these situations, and possibly reduce disease outbreaks.

Throughout this work, a collection of bacteria pathogenic to both lumpfish and salmon were used in search for bacteriophages. *Pasteurella* sp. was isolated from clinically sick salmon, and *Photobacterium* sp. and *Aliivibrio logei* were isolated from clinically sick lumpfish diagnosed with vibriosis. Water samples from aquaculture sites and environmental water samples were collected, processed and used in the search for specific bacteriophages. Successful isolation were not fulfilled, however plaques were observed in samples from an aquaculture location with an ongoing diagnosed case of pasteurellosis. This posed a question if prophages are present in the genome. Bacteriophages specific for *V.anguillarum* was not isolated, but a previously isolated bacteriophage showed an effect against pathogenic *V.anguillarum* previously isolated from lumpfish. This study has shown that isolation of phages specific for pathogens from an aquaculture environment might be challenging. In future search for phages, a wider collection of bacterial isolates and water samples should be considered.

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7. Appendix

Appendix A

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

Medium/Buffer	Ingredients	Supplier
Tryptic Soy Broth (TSB) 0.5 %		
I: Tryptic Soy Broth (TSB)	30 g	Dickinson and company
II: Distilled H_2O	1000 ml	
	I is added to II. Mix well and heath to dissolve the powder. Sterilize by autoclavation at 121 °C in 15 min.	
Tryptic Soy Broth (TSB) 2 %		
I: Tryptic Soy Broth (TSB)	30 g	Dickinson and company
II: NaCl	15 g	VWR international
III: Distilled H_2O	1000 ml	
	Add I and II to III. Mix well and heath to dissolve the powder. Sterilize by autoclavation at 121 °C in 15 min.	
Tryptic Soy Agar (TSA) 0.5 %		
I: Tryptic Soy Broth (TSA)	30 g	Dickinson and company
II: Distilled H_2O	0.5 L	
	I is added to II. Mix well and heath to dissolve the powder. Sterilize by autoclavation at 121 °C in 15 min. After autoclavation, the agar is distributed on approximately 20 agar plates.	
Tryptic Soy Agar (TSA) 2 %		
I: Tryptic Soy Broth (TSA)	30 g	Dickinson and company
II: NaCl	7.5 g	VWR international
III: Distilled H_2O	500 ml	
	I and II is added to III. Mix well and heath to dissolve the powder. Sterilize by autoclavation at 121 °C in 15 min. After autoclavation, the agar is distributed on approximately 20 agar plates.	

Medium/Buffer	Ingredients	Supplier
Thiosulfate-citrate-bile salts-sucrose agar (TCBS)		
I: TCBS agar	89 g	Dickinson and Company
II: Distilled H_2O	1000 ml	
	I is added to II. The solutions is mixed well and boiled for 1 min.	
TM buffer, pH 7.4		
I: NaCl	5.84 g	VWR international
II: $MgCl_2x6H_2O$	2.03 g	Sigma-Aldrich
III: $CaCl_2x2H_2O$	1.47 g	Sigma-Aldrich
IV: 1M Tris-HCl, pH 7.4	10 ml	
V: Distilled H_2O	1000ml	
	Dissolve I, II, II in 900mlV and bring volume to 1000ml. Add 10ml IV and autoclave.	
1M TRIS-HCL, pH 7.4		
I: Trisma® base	121.14 g	Sigma-Aldrich
II: HCl (37%)		VWR chemicals
	Dissolve I in II and adjust pH to 7.4 with concentrated HCl. Bring volume to 1000 ml and control the pH after. Autoclave when pH once is OK.	
10x $CaCl_2x2H_2O$		
I: $CaCl_2x2H_2O$	24.00 g	Sigma-Aldrich
II: Distilled H_2O	500 ml	
	Dissolve I in II. Sterilize trough a 0.2 µm filter before use.	
0.1 M NaOH		
I: NaOH	40g	Fluka Chemie AG
II: Distilled H_2O	1L	
	I is added to II and mixed well.	

Medium/Buffer	Ingredients	Supplier
Phosphate buffered saline (PBS)		
I: Na_2HPO_4 x H_2O	0.72 g	Sigma-Aldrich
II: KH2PO4	0.27 g	Fluka Chemie AG
II: NaCl	8.5 g	VWR international
IV: Distilled H_2O	1000ml	
	Add I, II and III to 900 ml IV. Mix well and heath the solution gently. Adjust pH to 7.3 and add dH_2O until 1000ml. Sterilize by autoclavation at 121 °C in 15 min.	
50x Tris-acetate-EDTA (TAE)		
I: Tris base	242 g	Sigma-Aldrich
II: Acetat (100%)	57.1 ml	Sigma-Aldrich
III: EDTA(0.5M)	100ml	
IV: Distilled H_2O	600 ml	
	I, II and III is added to IV and mixed well. Before use the solution is diluted to x1.	
10 x TGS		
I: Trisbase	30.3 g	Sigma-Aldrich
II: Glycine	144 g	Sigma-Aldrich
III: SDS	20 g	Sigma-Aldrich
IV: Distilled H_2O	1 L	
	I, II and III is added to IV.	
1.5 M Tris- HCL, pH 8.8		
I: Tris base	27.23 g	Sigma-Aldrich
II: Distilled H_2O	80 ml	
	I is added to II and mixed well.	
0.5 M Tris- HCL, pH 6.8		
I: Tris base	6 g	Sigma-Aldrich
II: Distilled H_2O	60 ml	
	I is added to II and mixed well	
Fixative solution		
I: Metanol	100 ml	Honywell
II: Acetic acid	20 ml	Fluka Chemie AG
III: Fixative enhancer concentrate	20 ml	Bio-Rad
IV: Distilled H_2O	20 ml	
	I, II and III is added to IV and mixed well.	

Appendix B

Kits (A.2-A.4) used in this study.

Table A.2: BIONOR™ MONO-Va, 50 tests

Kit	Supplier
BIONOR™ MONO-Va, 50 tests DE020	**TAMAR – Laboratory supplies Ltd.**
Test reagent (specific antiserum)	
Negative control reagent	

Table A.3: GenElute™ PCR Clean-Up Kit

Kit	Supplier
GenElute™ PCR Clean-Up Kit	**Sigma-Aldrich**
Column Preparation solution	
Binding Solution	
Wash Solution Concentrate	
Elution Solution	
GenElute plasmid mini spin column	
Collection Tubes, 2 ml	
Ethanol	

Table A.4: E.Z.N.A.® Gel Extraction Kit (V-spin)

Kit	Supplier
E.Z.N.A.® Gel Extraction Kit (V-spin)	**Omega Bio-Tek**
HiBind® DNA Mini Columns	
2 mL Collection Tubes	
Binding Buffer (XP2)	
Elution Buffer	
SPW Wash Buffer	

Appendix C

Gels (1% agarose) from 16s rRNA sequencing showing amplified PCR products approved for sequencing (Figure A.1).

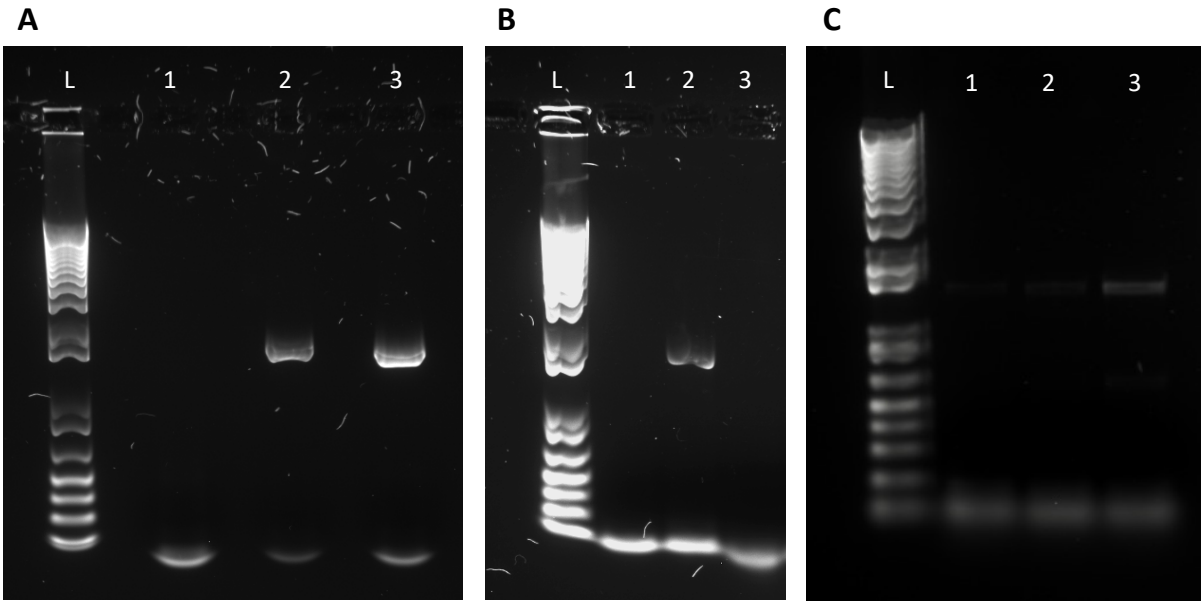

Figure A.1 Gel (1% agarose) showing PCR product of amplified 16s rRNA of *Pasteurella* sp. (A), *Aliivibrio logei* (B) and *Photobacterium* spp. (C). The isolates are indicated by a band size of approximately 1500 base pairs (bp). The outermost lane at left side (L) shows 1kb DNA ladder. Lane 1 shows undiluted sample, lane 2 shows the sample diluted 1:10 and lane 3 shows the sample diluted 1:100 (A and B). Lane 1 shows undiluted sample, lane 2 shows the sample diluted 1:5 and lane 3 shows the sample diluted 1:25 (C).