Analyses of bacteriophages to Yersinia ruckeri and the salmon (*Salmo salar* L.) antibody response to the bacteriophages.

Thesis for the degree

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Abstract

New technologies for producing Atlantic salmon like the recirculation aquaculture systems (RAS) have gained popularity, and has overthrown the traditional flow through systems used in the freshwater stages of the Atlantic salmon production. This is a closed system with less water consumption, but extended water treatment. Despite the many advantages, opportunistic bacteria seem to thrive in in such closed and biological system. Since 2007 there have been an increase in the number of yersiniosis outbreaks. Many of these outbreaks have been in RAS, or in fish in sea cages that originated from infected RAS. The causative bacterium *Yersinia ruckeri* is capable of forming biofilm, thus making it hard to remove during disinfection. The bacterium can be treated with antibiotics, but facing the worldwide problem of antibiotic resistance, other alternatives are highly needed. One of the alternatives suggested is phage therapy. The long-forgotten method of using bacteria infecting viruses for biocontrol is now being studied again in aquaculture.

Bacteriophages infecting *Y. ruckeri* were investigated in this study. Four different lytic phages and a cocktail including the four were delivered by ACD Pharmaceuticals. They were all tested in growing cultures of *Y. ruckeri,* where they all showed a good bacteriostatic effect. This indicates that the bacteriophages have a god potential for application in bio treatment of water and filters used in rearing of fish larva and fish.

We studied the ability of salmon to produce anti-phage specific antibodies. This was done by immunization of salmon with phage as antigen, and the fish was injected three times with inactivated bacteriophages. The fish antisera were tested with enzyme-linked immunosorbent assay (ELISA), and all sera showed a high production of anti-phage antibodies in the fish given injection with phages alone or combined with adjuvance. The non-vaccinated (no phage antigen) control fish groups did not show any anti-phage antibody production. A neutralization test was performed using sera containing anti-phage antibodies, which proved that antibodies are highly neutralizing the bacteriolytic ability of the phages.

In summary the results showed that the phages used alone or in combination were highly bacteriostatic for *Y. ruckeri*. The ELISA test using bacteriophage as antigen can be used for screening of salmon sera after various exposure to bacteriophages. The immunsera can serve as positive controls. Thus, a screening method for anti-phage antibodies has been established.

Sammendrag

Store fremskritt innen Norsk akvakultur har ført til nye teknologier innenfor produksjon av Atlantisk laks. De siste 10 årene har resirkuleringsanlegg økt massivt i popularitet, og har erstattet de tradisjonelle gjennomstrømsanleggene som brukes i ferskvannsfasen av lakse produksjonen. Dette er lukkede systemer med mindre forbruk av vann men krever mer vannbehandling. Til tross for mange fordeler, ser det ut til at opportunistiske bakterier trives godt i slike lukkede biologiske systemer. Siden 2007 har det vært en økning i antall utbrudd av yersiniose hvor de fleste har vært i resirkuleringsanlegg, eller i sjøanlegg hvor fisken kommer fra et infisert resirkuleringsanlegg. *Yersinia ruckeri* er den forårsakende bakterien til yersiniose, og den er i stand til å danne biofilm, noe som gjør den i stand til å overleve desinfeksjon. Bakterien kan behandles med antibiotika, men nå som verden står ovenfor problemer rundt antibiotikaresistens, er andre alternativer ønsket. Et av disse alternativene er fag terapi. Den gamle metoden der bakterie infiserende virus brukes til biokontroll blir nå forsket på igjen.

Bakteriofager mot *Y. ruckeri* ble i dette studiet undersøkt. Fire forskjellige lytiske fag samt en cocktail som inneholdt alle fire ble levert av ACD Pharmaceuticals. De ble alle testet i voksende kulturer av *Y. ruckeri,* hvor de alle viste en god bakteriostatisk effekt. Dette indikerer en at bakteriofager har et stort potensiale for bruk innen biokontroll av vann og filter brukt i akvakultur av fisk og fiskelarver. I dette studiet ble laksens evne til å produsere bakteriofag spesifikke antistoff studert. Dette ble gjort ved å immunisere laks med fag som antigen. For å oppnå en høy antistoffrespons, ble fisken injisert tre ganger med inaktiverte bakteriofager. Fiskenes antiserum ble deretter testet med enzyme-linked immunosorbent assay (ELISA), hvor alle fiskene som hadde blitt injisert med inaktiverte fag viste en høy anti-fag antistoffrespons. De uinjiserte kontrollfiskene viste ingen antistoff produksjon. En nøytralisasjonstest ble gjort på antistoffene fra fisken med høy antistoffrespons, som viste at antistoffene er svært nøytraliserende på den bakteriolytiske effekten til bakteriofagene. Resultatene viste at fag brukt alene eller i kombinasjon er svært bakteriostatisk for *Y. ruckeri.* ELISA testen med bakteriofager som antigen kan brukes for screening av lakseserum etter ulike eksponeringer for fag. Immunserumet kan brukes som en positiv kontroll. Dermed har en screening metode for anti-fag antistoff blitt etablert.

Content

Abbreviations

AGD	Amoebic gill disease
CFU	Colony forming units
CMS	Cardiomyopathy syndrome
ELISA	Enzyme-linked immunosorbent assays
ERM	Enteric Redmouth Disease
HD	High dose
HSMI	Heart and skeletal muscle inflammation
ISA	Infectious salmon anemia
LB	Luria Bertani
LD	Low dose
MOI	Multiplicity of infection
OD	Optical density
PBS	Phosphate buffered saline
PD	Pancreas disease
PFU	Plaque forming units
RAS	Recirculation aquaculture system
Rpm.	Revolutions per minute
UV	Ultra violet
WW2	World War 2

1 Introduction

Norwegian aquaculture is today experiencing an increased interest of new production methods due to technological advances. More and more Atlantic salmon (*Salmo salar L.*) producers are building recirculation aquaculture systems (RAS), instead of the old traditional flow through systems for salmonid production in freshwater (Hjeltnes et al., 2012). These RAS are well known systems based on technology made to reduce the usage of water and electricity. Likewise, infection pressure and problems caused by the salmon lice have opened for salmon production in closed systems instead of traditional open sea cages. This, in addition to a new trend of producing big smolt in closed sea water facilities, have led to an overall increase of Atlantic salmon production in closed and recirculation systems. New disease challenges will naturally follow these new production systems (Hjeltnes et al., 2017). Opportunistic bacteria can cause severe losses if they are allowed to grow in such a system (Hjeltnes et al., 2012). This has been demonstrated by the increasing number of yersiniosis outbreaks in RAS systems the recent years (Hjeltnes et al., 2017). The causative bacterium *Yersinia ruckeri* is capable of forming biofilm on materials and surfaces in these systems, making it hard to remove during disinfection. Antibiotics are still the only tool for treating infected fish (Kumar et al., 2015). Recently, bacteriophages have been suggested as an alternative to reduce infection pressure, or directly treating bacterial infections in aquaculture. Their self-replicating nature is particularly beneficial in closed systems, and phages can be administered in different ways. More importantly, several successful phage therapy experiments on fish and shellfish infections have been completed and reviewed by Madhusudana Rao and Lalitha (2015) and Oliveira et al. (2012). This gives optimism in developing bacteriophages products as tools for bacterial control in closed systems. However, more research is needed to establish effective phage therapies.

Salmonid production in Norway

Norway is the leading producer of Atlantic salmon with a total of 1 171 200 tons produced in 2016 (Hjeltnes et al., 2017). In addition to producing a significant amount of 84 500 tons of rainbow trout (*Oncorhynchus mykiss*, Walbaum) (Hjeltnes et al., 2017). This enormous production has opened for a large-scale export of fish. In 2014, Norway was the second largest exporter of fish and fishery product. With a total value of 10 803 million US dollars (FAO, 2016). Aquaculture is an industry of major importance in Norway (Fiskeridepartementet,

2014). One of the factors making Norway so suitable for Atlantic salmon production is the long coastline including special fjord-systems. The Gulf stream feeds the coast with warm water and mixes with the nutritious freshwater from the coast. Facilitating exceptionally good conditions for aquaculture (Hansen and Mortensen, 1998, Bjerkestrand et al., 2011).

1.1 The fish production

Aquaculture is a relatively young industry in Norway. But has since its start in the 60s grown rapidly, with todays Atlantic salmon production being seven times bigger than meat production (Bjerkestrand et al., 2011). The production starts at breeding companies where the broodstock selected for further production is stripped for roe and milk. After fertilization, the roe is placed in trays at the hatchery. One and a half month later they have become eye-roe, and shipped off to the smolt facility. At the smolt-facility the roe start hatching about a month later. The newly hatched fry consumes their yolk sac before starting to eat dry feed. In the following growth phase the fish is manipulated by light to initiate the physiological transformation to adapt to the sea. When this physiological transformation called smoltification is completed, the fish is ready to be transferred to sea. The sea water farms are traditionally based on open net pens in fitting locations. In the sea cages the fish grow to become approximately 5 kg before being transported to the slaughter house (Bjerkestrand et al., 2011). Today there is ambitions of producing salmon in closed systems for multiple reasons such as less louse affection, reduced waste, and a good growth conditions (Rosten et al., 2011).

1.2 Production in closed or recirculation aquaculture systems (RAS)

1.2.1 Production of Atlantic salmon in closed aquaculture systems

There has been and increasing interest of farming Atlantic salmon in closed systems on land or in the sea. The main advantages giving rise to these interests are: Reducing the number of escapes, reducing the spread of salmon lice by making a barrier between the fish and environment, preventing spread of disease, better control over bio-waste, and the potential economic gain (Andaur et al., 2012). Several producers are applying for permission to build developmental systems for farming Atlantic salmon (Rosten et al., 2011).

1.2.2 Recirculation systems

Recirculation systems are the new systems preferred today. In 2015 the number of RAS facilities had reached 70 (Hjeltnes et al., 2016). Old flow through facilities are often changed

to RAS during upgrade or expansion. The technology behind RAS is based on reuse of water (Bjerkestrand et al., 2011). This is done by extensively treating the water after it has been in the fish tanks. The technology is optimized to reduce water consumption, waste, and give an overall better control over water quality (Verdegem et al., 2006, Martins et al., 2010). However, with such systems, comes new challenges and advanced technology. The water quality has to be closely monitored at all time. The danger of opportunistic pathogens is always present, especially if the water quality gets bad. To maintain good water quality in RAS facilities, ammonium (NH^4) and carbon dioxide (CO_2) must be removed, in addition to organic matter. Removing the ammonium is a critical measure. Today, this is done by a biofilter, which involves several species of bacteria. A new system like RAS opens up for new disease problems in the fresh water production (Martins et al., 2010, Hjeltnes et al., 2012, Bjerkestrand et al., 2011). (Bjerkestrand et al., 2011)
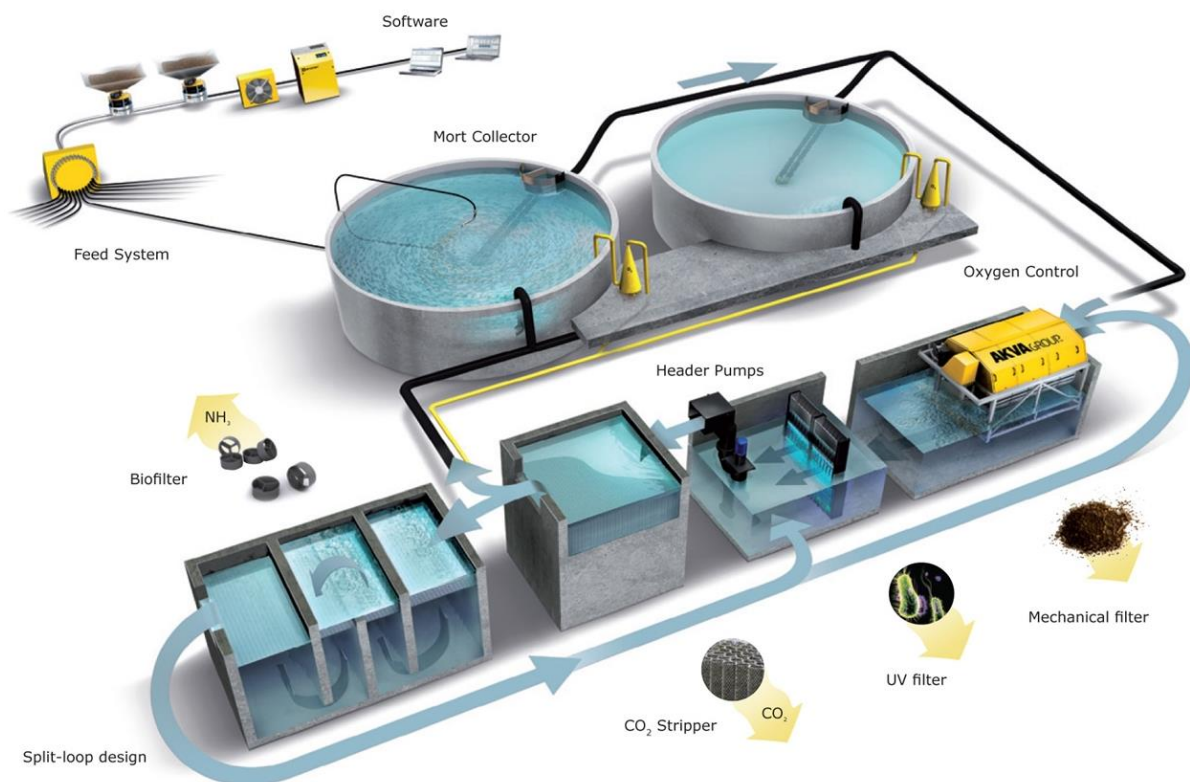
Figure 1. The concept behind of RAS facilities (Bjerkestrand et al., 2011)

1.2.3 Flow through system

Flow through is the old and traditional way to run a fresh water system. The water source is usually from a nearby lake with greater altitude. This way the water pressure is favorable (Bjerkestrand et al., 2011). The water used in production is taken from a relatively deep level to maintain a stable temperature and it contains less microorganisms. Before the water

reaches the fish tanks, the water is treated in several ways. Some producers prefer extensive water treatment, while other have a good water source, needing minimal treatment which is less expensive. Water treatment in flow through systems often consist only of particle filtration, for particle removal and UV-irradiation, for microbial removal. Oxygen is also supplemented to make sure the saturation is correct. This type of system has a higher demand of water than RAS (Bjerkestrand et al., 2011, Bjerknes and Liltved, 2007, Hjeltnes et al., 2012).

1.3 Disease in Norwegian aquaculture species

Despite the progress in fish health and welfare, there are still problems and huge economical losses due to infectious diseases in Atlantic salmon. The Norwegian coast and fjords is inhabited by large populations of wild fish, making the risk of disease transfer between wild and farmed fish greater (Johansen et al., 2011).

Today, disease caused by parasites and viruses dominate in Norwegian aquaculture. The bacterial diseases have been minimized due to effective vaccines. However, there are still some bacterial problems mainly caused by winter ulcer bacteria and lately, yersiniosis has been diagnosed in an increasing number of locations (Hjeltnes et al., 2017).

The salmon lice (*Lepeophtheirus salmonis*) represent the largest problem today. Laws and regulations impose Atlantic salmon producers to carry out lice treatment if the number of lice exceeds 0,5 adult female lice per fish. This is to protect the wild Atlantic salmon and to reduce secondary infections. However, because of the lice's ability to quickly develop resistance against effective chemical, treatments have become ineffective. The level of resistance remains high in 2016. Forcing the industry to always search for new treatment options. Thus driving the aquaculture industry to change from chemical treatment to non-chemical treatments like mechanical or biological treatment (Helgesen et al., 2017). The second most sever parasite is *Neoparamoeba perurans*, the causative agent for Amoebic gill disease (AGD). The disease was first reported in Norway in 2006 (Steinum et al., 2008). AGD has caused significant problems in the last couple of years, but was less severe in 2016 than 2014. The disease can be life-threatening and capable of giving chronical gill inflammation in sea production (Hjeltnes et al., 2017). Freshwater and hydrogenperoxide are luckily still effective in treating AGD-infected Atlantic salmon (Hytterød et al., 2017).

The most problematic viral disease is Pancreas disease (PD), with two roaming epidemics caused by different subtypes of salmonid alpha virus (SAV). The disease is slowly moving further north in Norway causing problems and discussion around the disease fighting strategy and control zones of the disease (Hjeltnes et al., 2017). For Infectious salmon anemia (ISA), the situation is more stable with 10-20 outbreaks each year. Although, still problematic when present because of the mortality and the restriction due to regulation that follow outbreaks (Hjeltnes et al., 2017). The increasing number of Heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) outbreaks, indicates a high abundance of these viruses (Hjeltnes et al., 2017). As well as the isolation of the virus associated with HSMI from wild fish and marine species (Garseth et al., 2013a, Garseth et al., 2013b, Wiik-Nielsen et al., 2012).

1.3.1 Bacterial diseases in Norwegian salmonid production

Bacteria exists everywhere in the environment. Some of them have been selected through evolution to infect certain niches. Some of these niches being tissues and cells of other organisms. These are specialized to cause disease and infect hosts, making them true obligate pathogens. There are also distinct bacteria which are abundant in the normal bacterial flora in healthy fish, that may cause disease in some cases. Conditions making this possible can be stress, genetically differences, bad water quality, malnutrition, or other factors connected to the host, bacterium or environment. These bacteria are considered facultative or opportunistic. Because their ability to create disease (virulence factors) normally would not overcome the hosts defenses. The infection route is mainly through the gill, gut or skin, before entering the circulation system and becoming systemic (Bruno et al., 2013). Closed systems and RAS systems could be fitting environments for opportunistic bacteria, and if bacteria are allowed to settle in such a system, they could be hard to get rid of.

In Norway, bacteria that cause disease in Atlantic salmon are mostly gram negative, while only a few bacteria are gram positive. Norwegian aquaculture experienced severe losses due to the diseases furuncolosis and vibriosis, caused by the respective (gram negative) bacterium *Aeromonas salmonicida ssp.* salmonicida and *Vibrio anguillarum*. The (gram-positive) bacteria *Renibacterium salmoninarum* have caused major losses in salmonid production in Canada and the Faroe Islands, but due to good routines in broodstock production, losses have been minimal in Norway (Bjerkestrand et al., 2011). Today bacterial problems in production of

Atlantic salmon are stable, with losses mainly due to infections with winter ulcer associated bacteria; *Tenacibaculum maritimum, Moritella viscosa* and *Aliivibrio voodanis*. Yersiniosis outbreaks has also increased from a few outbreaks in 2004, to over 30 outbreaks in 2015 in both fresh and seawater facilities. In 2016 the number of outbreaks have not increased, indicating a stabilization (Hjeltnes et al., 2017). The main reason for this decrease in bacterial disease problems can be explained by vaccination and preventive measures. The present use of antibiotics is therefore minimal (Folkehelseinstituttet, 2017).

1.3 Yersiniosis

Yersiniosis, also known as enteric redmouth disease (ERM) in rainbow trout, is a contagious bacterial disease caused by the bacterium *Yersinia ruckeri*. *Y. ruckeri* was first isolated from rainbow trout in Hagerman Alley, Idaho, USA, in the 1950s. Later described by Ross et al. (1966). Several species are susceptible to the bacteria, but it mainly infects salmonids, where rainbow trout are the most susceptible to the acute ERM disease. Yersiniosis is a term often referred to as a less severe condition, often affecting Atlantic salmon, involving a different serotypes of *Y. ruckeri*. Besides from Europe, the bacteria has been found in Australia, Asia, South America, North America and South Africa (Carson and Wilson, 2009).

Yersiniosis has usually either acute or chronical development, and the clinical and pathological symptoms are often similar to other Gram negative bacterial diseases. The acute or peracute outbreaks of the disease often occur in freshwater stage, causing high mortality in fry and fingerling populations, with only a few external clinical signs of disease. The more chronic form often causes changes in pigmentation, disturbance of balance, and lethargy. While other signs of disease are exophthalmia, ascites, cutaneous petechiae and heamorrhage in the gill filaments. The popular name enteric redmouth disease comes from the hyperaemia on the jaw and oral cavity, that is often, but not always present with ERM in rainbow trout. In Norway, this is not normally observed as yersiniosis is mainly a problem on Atlantic salmon. Internal signs can be intestinal heamorrhage, petechiae on serosa membranes, swollen kidney and an enlarged spleen. Histologically; congestion, oedema and petechiae are frequent. The bacteria can be found in several organs, often affecting the gills and brain, while necrosis is common in spleen and kidney tissue, especially in the glomeruli (Bruno et al., 2013).

1.3.1 Yersiniosis in Norwegian aquaculture

The first isolation of *Y. ruckeri* was in 1985 from an Atlantic salmon sea farm close to the island of Senja, northern Norway (Sparboe et al., 1986). After the first isolation, there was about 10-30 outbreaks each year from 1986-1992. In 1987 there was a top, with a total of 58 outbreaks. So far the bacterium has mostly been connected to disease in Atlantic salmon. It usually aoccurs in the early freshwater stages, but the disease has now also been observed after transfer to sea as well, including fish with no signs of yersiniosis earlier (Bornø and Linaker, 2015).

From 2007-2008, the number of yersiniosis outbreaks doubled again, with an increase in the following years. In 2016, all 34 reported outbreaks were north of Sogn and Fjordane, except one in Hordaland. Of these, 27 was from sea locations, and seven from freshwater hatcheries (Hjeltnes et al., 2017). All confirmed isolations of the bacteria were also serotyped, placing all isolates in serotype O1 and O2 (Hjeltnes et al., 2017).

As an explanation of this increase of *Yersinia* outbreaks, the conversion to new recirculation systems is blamed. Instead of the old flow through systems, a more environmental friendly closed system with more water treatment is used during the freshwater phase. This gives opportunistic bacteria an advantage. It is well known that *Y. ruckeri* can form biofilm which promote its presence on production surfaces like tanks (Coquet et al., 2002a, Coquet et al., 2002b). Biofilm formation also promotes the bacteria's ability to survive disinfection, this is reflected when looking at the reported outbreaks each
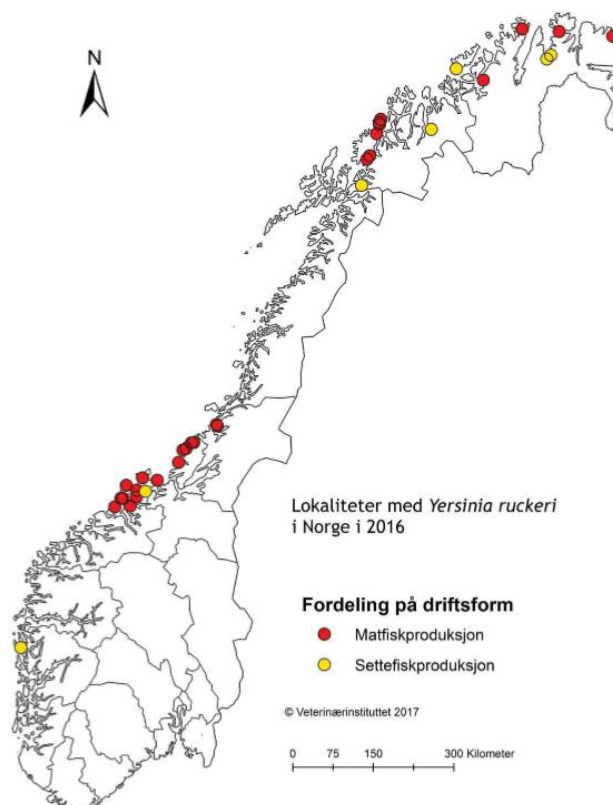

Figure 2. Localities experiencing *Y. ruckeri* outbreaks in 2016 (Hjeltnes et al., 2017)

year. Half of the freshwater locations that had an outbreak in 2007, had yearly outbreaks the following years (Hjeltnes et al., 2017). This can indicate that infections are caused by house

strains of *Y. ruckeri*. The number of yersiniosis outbreaks might also be under-reported, due to the producers not always reporting yearly outbreaks of house strains. (Hjeltnes et al., 2017, Hjeltnes et al., 2016)

1.3.2 *Yersinia ruckeri*

The causative agent of yersiniosis and ERM is the flagellated rod shaped Gram negative bacterium, *Y. ruckeri.* Reaching a length of 1-3 µm and 0,75 µm in diameter. It was first described to be fermentative, catalase positive and oxidase negative bacterium in the 1950s (Adams, 1959). The taxonomy of the bacterium was first difficult to determine due to differences from the *Yersinia* genus in biochemical tests. Although it was confirmed and included to the family Enteriobacteriaceae and genus *Yersinia* by Kumar et al. (2015). A family and genus that includes many non-pathogenic and pathogenic bacteria in both mammals and fish. The most known *Yersinia* bacteria infecting humans, is *Yersinia pestis,* responsible for causing millions of deaths in the last two millennia (Raoult et al., 2008). There are other *Yersinia* species such as *Yersinia enterocolitica* that commonly cause sickness in humans (Hering et al., 2016).The bacterium is classified into several serotypes and two biotypes. The bacterium does not form bacterial spores, but some strains have shown to form biofilm (Coquet et al., 2002a, Coquet et al., 2002b).

1.3.3 Current treatment and prevention of Yersiniosis

Today vaccination is considered necessary to maintain production of Atlantic salmon as increasing number of producers experience infections of yersiniosis every year (Hjeltnes et al., 2017). The bacterium appears to survive disinfection and other cleaning methods by forming biofilm, this may create house strains of *Y. ruckeri* in freshwater facilities (Coquet et al., 2002a, Coquet et al., 2002b). Several methods are presently used to prevent, control and treat *Yersinia* infections in fish and production sites. Due to the fear of antibiotic resistance, scientists are especially looking for preventive measures against yersiniosis. The different measures tried out are probiotics, vaccination, antibiotics and phage therapy (Kumar et al., 2015).

1.3.3.1 Probiotics

There is a great concern about the development of antibiotic resistance. Therefore, alternative measures for treating or preferably preventive measures are likely appreciated or needed. Several studies have been successful in bio-controlling of *Y. ruckeri* by orally administering

probiotic bacteria (Raida et al., 2003, Robertson et al., 2000, Capkin and Altinok, 2009, Balcázar et al., 2008). However, the protection gained was most likely from immune stimulation (Robertson et al., 2000, Abbass et al., 2010). In addition to bacterial probiotics, bacteriophages can be used as natural viral probiotics in bio-controlling bacteria (Madhusudana Rao and Lalitha, 2015, Pereira et al., 2011) .

1.3.3.2 Vaccination

Vaccination is the most effective way to prevent bacterial infections, it has solved some of the biggest disease challenges in Norwegian aquaculture. In Norway, vaccines have removed the bacterial infections furunculosis and vibriosis in farmed Atlantic salmon (Hjeltnes et al., 2017). A vaccine against *Yesinia ruckeri* was one of the first effective vaccines made for fish, and has been available for over 40 years, and was initially produced against the EMR disease in rainbow trout (Busch, 1978, Gudding et al., 2014). The vaccine was a monovalent vaccine, containing inactivated whole cells of *Y. ruckeri* serotype O1, biotype 2, and it could be administered by injection, immersion, or orally. It provided good protection against biotype 1 strains (Raida and Buchmann, 2008, Costa et al., 2011). The same level of protection was also shown in a new vaccine containing several intracellular and extracellular *Yersinia* products (Ispir and Dorucu, 2010). Biotype 2 strain have been more difficult to defeat, and this strain has emerged since the late 1980s and has been shown to cause the yersiniosis in Atlantic salmon, and thus, causing disease in fish vaccinated against biotype 1 (Austin et al., 2003, Gudding et al., 2014). There has now been developed a bivalent vaccine and it gives good protection against both, biotype 1 and 2 strains (Deshmukh et al., 2011). Since yersiniosis often appear at the hatchery, vaccination by immersion is the normally used method, and a second vaccine boost is usually necessary for further protection (Gudding et al., 2014).

1.3.3.3 Antibiotics

After a bacterial disease occurs, the fish are often treated with antibiotics. Today, there is only a few effective chemicals licensed for fishery use that are available. These are oxolonic acid, oxytetracycline, amoxicillin, sulphadiazine, trimethroprim, and the more recent florfenicol. With such a limited selection of compounds, the risk of developing resistance increases (Alderman and Hastings, 1998). Studies indicate that European isolates of *Y. ruckeri* are still susceptible to antibiotic therapy (Calvez et al., 2014). However, in vitro experiments show *Y. ruckeri* to easily develop resistance against oxolinic acid, oxytetracyline and possibly

suphonamide (Rodgers, 2001). *Y. ruckeri* produces holomycin and are thus naturally resistant to it (Qin et al., 2013).

1.3.3.4 Phage therapy

There have been found bacteriophages causing lysis of *Yersinia* species, indicating the potential of phage therapy against *Y. ruckeri* (Stevenson and Airdrie, 1984). There are no present phage-based preparation available for aquaculture use (Madhusudana Rao and Lalitha, 2015). However, a Norwegian pharmaceutical company is in a late stage of developing a bacteriophage product against yersiniosis. (http://www.acdpharma.com/?page_id=17).

1.4 Bacteriophages

The word phage derives from the Greek and translate to eat or devour. Translating bacteriophages to bacteria eaters (Stent, 1963). Bacteriophages are small, bacteria-infecting viruses. First discovered independently in 1915 by Twort (1915) and d'Herelle (1917), but first described by d'Herelle (1917), d'Hérelle and Smith (1926). Bacteriophages can be found everywhere, and are the most abundant organisms in the world. There can be as much as in ~10^7 virus particles per ml of seawater (Suttle, 2005). Bacteriophages as the name implies only infect bacteria and can be species-specific or strain-specific. Bacteriophages can vary in both size and morphology. After the discovery of antibiotics, the research on phages has been limited to the Soviet Union and eastern Europe. While the western countries focused their research on the favored antibiotics with a much broader spectrum in killing bacteria. The phages are simple organisms with a very small genome, which is easy to manipulate, therefore, they have a big potential for application using modern technology in production, molecular analysis and manipulation. Other applications of phages can be as biocontrol, vectors/targeted delivery vehicles, lytic phage typing, phage therapy, and they can also produce lytic enzymes (Drulis-Kawa et al., 2015). Phage typing is a method used to classify unknown bacteria by their susceptibility to various bacteriophages (Cammack et al., 2006). Phage therapy is using bacteriophages to defeat a specific bacterial infection by applying the phage in different ways (Kutter and Sulakvelidze, 2005).

1.4.1 Bacteriophage lifestyles

Bacteriophages are divided into two different groups by the characteristic lifestyles they adopt when infecting a bacterium, which is the lytic and lysogenic lifesyles. The most common lifestyle of phages is the lytic lifestyle which is a virulent infection form that results in

destruction of the infected bacteria. The lytic lifestyle consists of four steps ending in lysis of the bacteria. These steps are: Attachment of phage to bacterium, injection of phage nucleic acid, intracellular development, and a final lysis of the cell with a release of progeny phage particles (Madhusudana Rao and Lalitha, 2015). Lytic phages are natural killers of bacteria, and a lytic cycle can be as short as 20 minutes, this quality makes them suitable for controlling bacteria. They will also increase in number as long as a there are host cells to infect (Breitbart, 2012). The other lifestyle is a more latent one. After injection of phage nucleic acid into the host, the phage genes are replicated with the host genes. The phage genome can stay in this state for several generations with no impact on host metabolism. The phage genes in this state can change into a lytic cycle, followed by natural release of phage particles. This ability is known as lysogeny. Phages capable of both lifestyles are called temperate phages. Lysogenic phages can be triggered to enter a lytic life style by different chemical or physical stimuli (Madhusudana Rao and Lalitha, 2015).

1.4.2 Phage classification and morphology

Bacteriophages differ considerably in structure, biological characteristics, and physiochemically. Making them a very heterogeneous group of viruses. This also indicates apolyphyletic origin. Their genome is primarily consisting of dsDNA, although some dsRNA, ssRNA, and ssDNA occur in some small groups. All DNA phages only have a singular DNA molecule. The phage morphology can also be very different, the virions can be tailed, filamentous, polyhedral, and pleomorphic. Thus, bacteriophage families are often listed after morphology for convenience. Some may also include a lipid-containing envelope or vesicle. Many phage species have been examined since the discovery of the electron microscope, at least 5500 in total (Ackermann, 2001). Making them the largest virus category studied by electron microscopy, and largest existing virus group (Ackermann, 2012). Bacteriophages are categorized into 1 order, 13 families, and 31 genera. Over 95% of the described bacteriophages are from the family Caudovirales, which are tailed phages (Kutter and Sulakvelidze, 2005). The classification of phage families is mainly based on virion morphology and nucleic acid, however there are many more criteria to consider for classification. When it comes to genera and species there are no common standard so far (Kutter and Sulakvelidze,
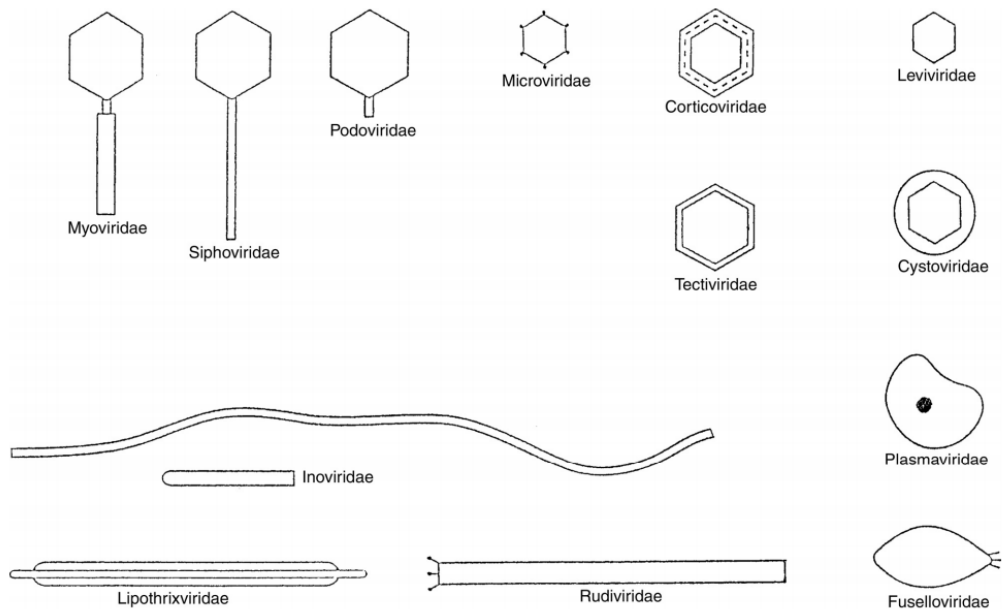
2005, Van Regenmortel and Viruses, 2000).

Figure 3. Basic bacteriophage morphotypes (Kutter and Sulakvelidze, 2005).

1.4.3 Isolation

Isolation of bacteriophages can be done by collecting samples from any source material abundant in the desired target bacterium, and following specific methods for isolation (Pereira et al., 2011, Park et al., 2000). These samples are often added to a soft top agar supplemented with the respective bacteria you want to find phages for. If any plates show signs of lysed areas after incubation, the liquid formed in these areas is collected for centrifugation. Before being tested on homogenous plates of the bacterium to test ability of lysis. If plaques are formed they are transferred to liquid cultures and purified (Stevenson and Airdrie, 1984).

1.4.4 Phage therapy

Today, bacterial resistance against antibiotics have become a worldwide problem, this is a result of uncontrolled use antibiotics. Antibiotics have been our first-line, and last tool to control pathogenic bacteria (Center for Disease Dynamics, 2015). This have made scientist open their eyes again for a long forgotten weapon in the fight against pathogenic bacteria. More precisely bacteriophages, bacteria-killing viruses, and the method of using them to kill specific bacterial pathogens. These phages were first applied as therapeutic agents in treating cholera in by d'Herelle (1929), and the method was named bacteriophage therapy. The discovery of these bacteria killing viruses quickly became overthrown by the discovery of

antibiotics, but was used and considered a valuable resource in fighting infections in the Eastern Europe and Soviet. The Soviet Union frequently used bacteriophages for treating infection like dysentery and gangrene during world war 2 (WW2) (Summers, 2012). After WW2, the research on phages continued at several institutes, with the center of activity at the Eliava institute of bacteriophage, microbiology, and Virology in Tbilisi, Georgia. The institute is still today a leading facility in treating infections with multi resistant bacteria. Today they have the biggest archive on phages in the world. Containing phages against many human pathogenic bacteria (Sulakvelidze et al., 2001). Western scientist now got a renewed hope in phage therapy as antibiotic no longer are effective against some bacterial strains. The search for new weapons is critical, and phages has shown to be effective in several animal trials. More importantly, all the successful treatment at the Eliava institute shows the potential of phage therapy. Another reason for this optimistic interest in phages is due to the progress in molecular and biochemical science since last time phage therapy was tried out. This interest is especially reflected in the increasing research on the topic of phages the last decades (Adams, 1959, Sabour et al., 2010, Pereira et al., 2011, Richards, 2014, Borysowski et al., 2014, Madhusudana Rao and Lalitha, 2015).

1.4.5 Phage therapy in aquaculture

The intensive production of fish and shellfish is increasing worldwide, although pathogenic bacteria are responsible for major losses. This in addition to a global market demanding fish not treated with antibiotics, have led scientists to look for other methods to use in aquaculture.

Because of the high presence of bacteriophages in aquatic environment the phage therapy is naturally suggested a possible alternative to antibiotics. The viruses are the natural enemies of the aquatic bacteria. The administration methods in aquaculture are multiple, equally important is the replicating infection nature of these viruses. When bacteria infected with one or more phage undergo lysis, it will release more infectious phages until there are no more bacterial host to infect. This is a quality especially useful in viscous aquatic environments. Many successful experiment have been completed on fish pathogens like *Aeromonas salmonicida, Flavobacterium psychrophilum, Flavobacterium columnare, Pseudomonas plecoglossicida* and *Edwardiella tarda* (Richards, 2014, Madhusudana Rao and Lalitha, 2015, Oliveira et al., 2012).

Background

Bacteria are responsible for large disease outbreaks and can cause major losses in the farming of fish and shellfish. As a result of this, massive over-use of antibiotics has been practiced in an effort to control the situation. The use of antibiotics is disputed due to side-effects, its impact on the necessary natural microflora, risk of resistance development and an increasing demand for food free for antibiotics. There is a need for alternatives to antibiotics and bacteriophages, viruses that specifically infect bacteria, is one such alternative. A major research and development project in ACD Pharmaceuticals AS has, since 2011, been the development of bacteriophage based products for use in reducing bacterial infections in aquaculture. Immunization of fish after receiving phage treatment could potentially reduce efficacy of subsequent treatments. Due to the ubiquitous nature of aquatic phages, no additional immunization effect is expected from phage treatment, however, it is necessary to determine and document this.

Aim for the study

The first aim was to test the bacteriolytic activities of four phages to *Y. ruckeri* to obtain information useful for various later planned experiments on applications of the phages in aquaculture.

The next aim was to investigate the immunogenicity of the phages in salmon and to establish an ELISA to measure specific anti phage antibodies.

2 Materials and Methods

2.1 Bacteriophages

The different phages and the phage cocktail used in the present study, were obtained from ACD Pharmaceuticals AS, Norway. There was a total of four different phages, and a cocktail containing all four. The phage solution was filtrated (0,22 μm) and diluted in TM-buffer (see appendix) to reach a concentration of 1x10^{11} pfu/mL each. All four phages were lytic infective for the bacterium *Yersinia ruckeri.* The phages were stored at 4°C during the whole study.

2.2 Bacterium *Yersinia ruckeri*

The only *Y. ruckeri* strain used throughout the study was delivered by ACD Pharmaceuticals AS, and was cultivated in Luria-Bertani (LB) broth (see appendix 7.1), and stored frozen at -80°C in ampoules, ready to be used later. For colony growing either LB-agar (see appendix 7.1) or Tryptone-Yeast-Sodium-Glucose-Agar (See appendix 7.1) were used. All cultures used were initially grown as liquid cultures, by adding a freezing culture to an Erlenmeyer flask (250 ml) containing 50 ml LB-medium, incubated for 12 hours at 25°C, with shaking in an Minitron (Infors HT) incubator.

2.2.1 Preparation of frozen bacteria stock

Bacterial cultures were harvested at the exponential growth phase, supplemented with 12 % glycerol, and distributed into Cryo tubes (CryoTube^{TM} 1,8 ml, Nunc, Denmark). The glycerol stocks were marked with the name of bacteria, passage, and date of freezing, before being frozen at -80°C to maintain access to bacteria for inoculation of starter cultures throughout the study.

2.2.2 Freeze drying of *Y. ruckeri*

One Cryo tube (CryoTube^{TM} 1,8 ml, Nunc, Denmark), containing 1 ml, of frozen *Y. ruckeri* culture was added to an Erlenmeyer flask containing 50 ml LB-medium. The flask was incubated at 25°C for approximately 16 hours, without shaking. When the OD had reached 0.7, 2ml of the preculture was supplied to a 1000 ml Erlenmeyer flask containing 200 ml LB-medium, and left for 16 hours of incubation at 25°C. The main culture was equally divided into four 50 ml tubes. The tubes were centrifuged for 15 minutes at 3000xg at 4°C in a centrifuge (Allegra® X-15R, Beckman Coulter). The supernatant was poured of, and the bacterial pellet dissolved in Phosphate Buffered Saline (PBS, Lonza, Bio Wittaker®) before centrifuging again as described earlier. The supernatant was again poured of, and the pellet

dissolved in 1 ml RNAse free water (Sigma-Aldrich), and frozen at -20°C, laying obliquely for the liquid to get as much surface as possible. They were then freeze dried with an Alpha 1-2 LDplus (Martin Christ Gefriertrocknungsnlagen GmbH) freezedrier.

2.3 Optical density

Optical density (OD) can be used to measure the density of bacteria in a suspension. The spectrophotometer sends beams of light at a chosen wavelength through a suspension and the amount of light absorbed will give a measurement of the optical density (OD) in the solution. In the present study *Y. ruckeri* cultures were red at wavelength at A_{620nm} using plastic cuvettes (MBH) in a Spectroquant Pharo 300 (MERCK) Spectrophotometer.

2.3.1 *Y. ruckeri* growth curve

A growth curve was established for *Y. ruckeri* to identify the time when the bacteria was in the lag, log and stationary growth phases. This information is important for knowing when to harvest the bacterium at the exponential phase. The phase where they are most viable. All *Y. ruckeri* cultures were grown in LB-medium. Thus, a sterile 1 ml suspension of LB-medium was used for calibrating the spectrophotometer for background absorption by the growth media. A_{620nm} was red on a spectrophotometer (Spectroquant Pharo 300, MERCK). To make a pre-culture one freezing culture of *Y. ruckeri* was added to a 250 ml Erlenmeyer bulb containing 50 ml LB medium and left overnight at 25°C. The next day 2 ml of the pre-culture was added to two new 1000 ml Erlenmeyer bulbs, containing 200 mL LB medium to make duplicate main cultures. The optical density at A_{620nm} was red on a spectrophotometer (Spectroquant Pharo 300, MERCK) in both main cultures every 60 minutes until stationary phase was reached.

2.3.2 Colony forming units and OD-CFU/ml calculation

Colony forming units (CFU) are the number of viable bacteria capable of forming colonies in a suspension. The suspension tested for CFU was diluted to make sure the number of colonies on the plates was countable. Hence the bacterial culture was diluted and plated in triplicates from ten-fold dilution on LB-agar. The agar plates with bacteria were incubated over night at 25°C. The next day colonies formed was counted, and CFU calculated by the formula below.

$$\text{Number of CFU pr. ml} = \frac{\text{number of colonies pr. plate}}{\text{Dilution factor}}$$

2.4 Inactivation of phages with UV-light

The effect of UV-C (200-280 nm) radiation was tested on bacteriophage φ 2. Bacteriophage φ 2, diluted to a concentration of $2x10^9$ PFU/ml in TM-buffer, was supplied as aliquots of 60 µl to each of 18 wells in 96-well Nunc plates (Nunclon^{TM} Surface). Two plates were included, one plate treated with UV-C radiation, and one as a non-treated control. There were five sample points, 15, 30, 60, 90 minutes and 24 hours post UV-treatment. At each sampling, triplicate samples of a combined volume of 120 µl were collected from both plates. The exposure to UV-radiation was done in an airflow cabinet made for microbiology with the plate lid of. The phages inactivated for the immunization experiment were exposed to UV-radiation in a petri dish. Hence, the volume of phage suspension was calculated to be corresponding to the volume tested in the wells of the inactivation trial.

2.4.1 Phage survival spot assay

Soft agar was melted, and aliquots of 6780 µl were put in individual sterile 15 ml tubes (SARSTEDT). The tubes were left in a water bath (GFL) at 30°C, for 10 minutes to temperate, before 678 µl of *Y. ruckeri* culture (at OD=0.6) was supplied to each tube, the suspension vortexed and poured onto quadratic petri dishes containing TYSG agar. The plates were left to dry with the lid on.

Every 120 µl phage samples at each 30, 60 and 90 minutes were diluted by tenfold dilutions, in TM-buffer. One drop of 5 µl of every dilution of UV-C treated phage samples were then carefully supplied on to TYSG agar plates containing softagar/*Y. ruckeri* top layer. From the non-UV treated control samples only the last four dilutions were supplied to the agar plates. The plates were left to dry for 30 minutes before they were incubated overnight at 25C°. The following day plaques in the bacterial lawn were counted and plaque forming units (PFU) calculated by the formula below. Skriv inn formel her.

$$\text{Number of PFU pr. ml} = \frac{\text{number of plaques pr. plate}}{\text{Dilution factor}}$$

2.5 Phage activity curves

To test the phages bactericidal abilities, phages were added to a growing culture of the respective bacterium *Y. ruckeri* at two different optical densities, OD 0.3 and OD 0.6. In parallels for each OD, phage stock, diluted in TM-buffer, was added at multiplicity of infection (MOI) at 1:1. For each culture 1 ml *Y. ruckeri* culture was added to each well in 24-well plates (Nunclon™ Surface), and one 24-well plate was included for each OD. A suspension of 100 µl phage dilution suspension was then added to every well, achieving a final MOI of 1.

The OD was measured in the first two wells immediately after adding the phage suspension, and was then measured every 10 minutes, from duplicate wells from each plate. The measurements ended after 160 minutes with a total of 17 sampling times.

2.5.1 Phage activity assay

Supplement of two different phage concentrations were tested on *Y. ruckeri*, for each phage alone and for the cocktail. For each phage and for the cocktail one 50 ml tube (Greiner) was supplied with 1 ml undiluted phage sample, and one tube was supplied with a 1 ml phage sample diluted with TM-buffer to a MOI=1 with the number of bacterium at the optical density used. A third tube containing 1 mL of sterile TM-buffer was included as a none-phage control. A volume of 1 mL *Y. ruckeri* at OD 0.5 was then applied to each of the tubes before incubating the tubes for 30 minutes at 25°C to allow adherence of the phage onto the bacteria.

After incubation, 24 ml LB-medium was supplied to each tube, and the tubes gently mixed, before distributing the contents of the tubes into e 24-well plates (Nunclon™ Surface), 1 ml in each well. At sampling time one (t_0) out of seven samplings, the two first wells in each plate were harvested. Sampling continued every hour, except for t_6 which was sampled after 24 hours. At each sampling 100 µl harvested probe was supplied with 2 µl of 2% chloroform (Sigma-Aldrich) to kill the bacteria, before being placed in the fridge at 4°C for later PFU testing.

2.5.2 Colony forming units

The 100 µl of samples for CFU calculations were first diluted by ten-fold dilutions in LB-medium before aliquots of 100 µl suspensions of the dilutions were plated on duplicate LB-agar dishes. The agar-plates were incubated at 25 °C in a Mir.154-PE (Panasonic) incubator. After 24 hours, the colonies that formed on the plates were counted.

2.5.3 Bacterial cell number

At each sampling the number of bacteria/ml was measured using a CASY-TT cell counter^{TM} (INOVATIS AG). In addition to cell number, size and aggregation factor of the bacterial suspensions were determined.

2.5.4 Determination of phage titers

The twenty-one 100 µl probes for each phage were first diluted with a series of tenfold dilution to reach a concentration that would give countable plaques on a petri dish. For each sampling time, duplicate samples of 100 µl were supplied with 300 µl *Y. ruckeri* suspensions from a main culture with a OD at 0.5. The bacteria-phage mixture was incubated for 20 minutes before every probe were transferred to a tube containing 3 ml melted soft agar (30°C) and gently mixed before they were overlaid on TYSG agar plates. After 24 hours of incubation, the phage plaques were counted and PFU was calculated.

2.7 Immunization of Atlantic salmon with bacteriophages
2.7.1 The fish and rearing conditions

The Atlantic salmon used in the immunization experiment was delivered and reared by the Industry laboratory, Marineholmen Bergen. During the whole study the fish were kept in 500 liter tanks with a salinity of 25 ppt and a minimum oxygen saturation of 87% in the outlet water. The light manipulation was set to 12:12 light:dark, and the fish were fed by automatic feeders. In the trial, three treatment groups of fish were included. The treatment groups were identified by different colors of Visible Implant Elastomer (VIE) tags placed subcutaneously in the transparent epithelia around the eyes. One group (n=15) injected with TM-buffer was identified by a yellow stipe. One group (n=15) injected with only phage was identified by an orange stripes on the right side, while the fish injected with phage and adjuvant (n=15) was identified by orange stripes around both eyes.

2.7.2 Vaccine preparation

The vaccine injected into the treatment fish during the immunization trial, were made by inactivating the bacteriophage cocktail ($7,2 \times 10^{11}$ PFU/ml) using UV radiation. One fish group received inactivated bacteriophage cocktail emulsified in Freunds adjuvant, incomplete (DIFCO) and one group received inactivated bacteriophages in TM-buffer. Preparation of vaccine with adjuvant was performed by adding the adjuvant to a final concentration of 5.0 mg/ml vaccine and thoroughly mixing using a shaking machine (FastPrep-24^{TM}5G).

2.7.3 Immunization

To test immunogenicity and production of bacteriophage reactive antibodies, Atlantic salmon was injected with inactivated phages. Inactivation was confirmed by plaque test performed on phage suspension used in the immunization experiment. A total of 60 fish, separated into four groups of 15 fish, were used in the immunization experiment. In addition sera from fish in one group, non-vaccinated, were sampled at the time of vaccination of the other three groups. This was the non-immunized baseline control group. Fish in one group was injected with sterile TM-buffer as a negative non-phage control. The Last two groups were injected with the UV-inactivated phage cocktail, one group received cocktail mixed with adjuvant and one cocktail in TM-buffer. The injections were done by intraperitoneal injections in doses of 100 µl using vaccination pistols (Socorex) supplied with 0.6 x 5 mm needles. The fish were injected three times. For the fish group receiving bacteriophage cocktail in adjuvant, the adjuvant was only incorporated at the first vaccination. The second injection was 30 days after the first injection, and the third was 15 days after the second injection. The fish were starved for 24h prior to vaccination. At vaccination, the fish were anaesthetized using Tricain Pharmaq® (metacain, MS-222).

2.7.4 Blood sampling

At time of the first immunization, blood was collected from the non-injected negative control fish. At termination of the experiment, 60 days post the first vaccination, blood samples were drawn and sera collected were from the remaining 45 fish. Hematocrit levels were measured in all fish. Length and weight of the fish was measured at all three injection times, and at termination of the experiment.

The fish were starved for 24h prior termination of the experiment and anaesthetized using Tricain Pharmaq® (metacain, MS-222) before blood was drawn. Blood was sampled from the caudal vein, *Vena caudalis*, and allowed to coagulate at 4°C for 12h before centrifugation at 1300 x g (Allegra® X-15R, Beckman coulter) for 5 min. The serum fractions were collected and stored in aliquots at -20°C.

2.7.5 Enzyme-linked immunosorbent assay

Presence of specific antibodies reactive to the bacteriophage cocktail was measured by enzyme-linked immunosorbent assay (ELISA). Three 96 well immunoplates (Nunc MaxiSorp™) were coated with bacteriophage cocktail ($7,2x10^{11}$ PFU/ml) by adding 150 µl of the antigen

suspension to each well except for the outermost wells. The plates where then wrapped in plastic with lids on before being incubated at 4°C overnight.

After washing three times in PBS-T (PBS containing 0,05% Tween 20), 200 µl blocking solution (3% (w/v) skimmed milk powder in PBS-T) was added to each well and the plates incubated for 1 hour at room temperature. The plates were washed as described earlier, and 100 µl of the salmon sera diluted in PBS-T was then supplied to the wells. Two parallel wells were included for all dilution of sera and wells containing PBS instead of salmon serum were used as blank control. After overnight incubation at 4°C. The plates were washed as described earlier and supplied with 50 µl of rabbit anti-salmon IgM diluted 1:2000 in PBS-T. The plates were incubated for 2 hour at room temperature and washed. Before 50 µl of peroxidase conjugated goat anti-rabbit Ig diluted 1:2000 in PBS-T was added to each well and the plates were incubated for one hour at room temperature. After washing, 50 µl of peroxidase substrate solution (o-Phenylenediamine, OPD) was supplied to each well to initiate the reaction. After 6 minutes the reaction was stopped by adding 50 µl of 2,5M H_2SO_4 to each well. The OD was read at 492 nm in a Sunrise microplate reader (Tecan Group Ltd.).

Two-tailed unpaired t tests were performed on the results using GraphPad Prism 5. The results were considered significant when $p<0.05$. In Prism 5, $P>0.05$ =n.s.,*$p≤0.05$, **$P≤0.01$, ***$P≤0.001$

2.7.6 Antibody opsonization test

To investigate the antibodies ability to neutralize the bacteriophages, a neutralization test was completed. This was done by performing a plaque assay where antisera from the fish group injected with inactivated phage cocktail were premixed with the phages before mixing phages with the bacteria culture at OD 0.5, and PFU calculations was read as described earlier. The antisera were diluted in TM-buffer to learn at what dilutions neutralization would be effective. There was included a control where phages were premixed with the sera from the fish group injected with sterile TM buffer.

2.7.7 SDS-PAGE of bacteriophage components

Protein profile of the bacteriophage cocktail were analyzed by SDS-PAGE (12% acrylamide) according to the method of Laemmli U.K. (1970). SDS-PAGE were performed on the bacteriophage cocktail used for coating 96-well plates (Nunc MaxiSorp^{TM}) and on bacteriophages harvested after coating and during the first washing step on 96-well plates (Nunc MaxiSorp^{TM}). Harvest of bacteriophages from 96-well plates was performed by gently scraping the wells using a bent Pasteur pipette and re-suspending in TM buffer. Electrophoresis was performed using a Mini Protean Tetra Cell (Bio-Rad). The antigens were heat-treated (96°C for 5 min) in sample buffer containing β-mercaptoethanol. Samples of 5 μl were loaded onto each well, electrophoresed at 190 V for 45 min, followed by staining of proteins using Silver Stain Plus kit (Bio-Rad) according to Switzer et al.

3. Results

The study of the bacteriophages, and its respective bacterium *Y. ruckeri* began with determining the bacterial growth and phage characteristics. Results from phage activity experiments, will be presented individually for each phage and the cocktail. The study of anti-phage antibody production in Atlantic salmon will be presented finally.

3.1 *Y. ruckeri* growth curve

To study the growth of the bacteria, OD was used to measure two parallel cultures over 14 hours. After the addition of 2 ml *Y. ruckeri* pre-culture to 200 ml LB-medium, a slow increase in OD was observed. This increase was enhanced after about four hours. A rapid growth continued until 10 hours' post addition of bacteria. After 12 hours, the nutrition in the LB-medium seem to be exploited and growth slowed. The growth was measured to be similar in both parallel flasks (Fig.4). The *Y. ruckeri* cultures was grown with or without shaking, however the culture stopped growing at OD 0.5 without shaking.
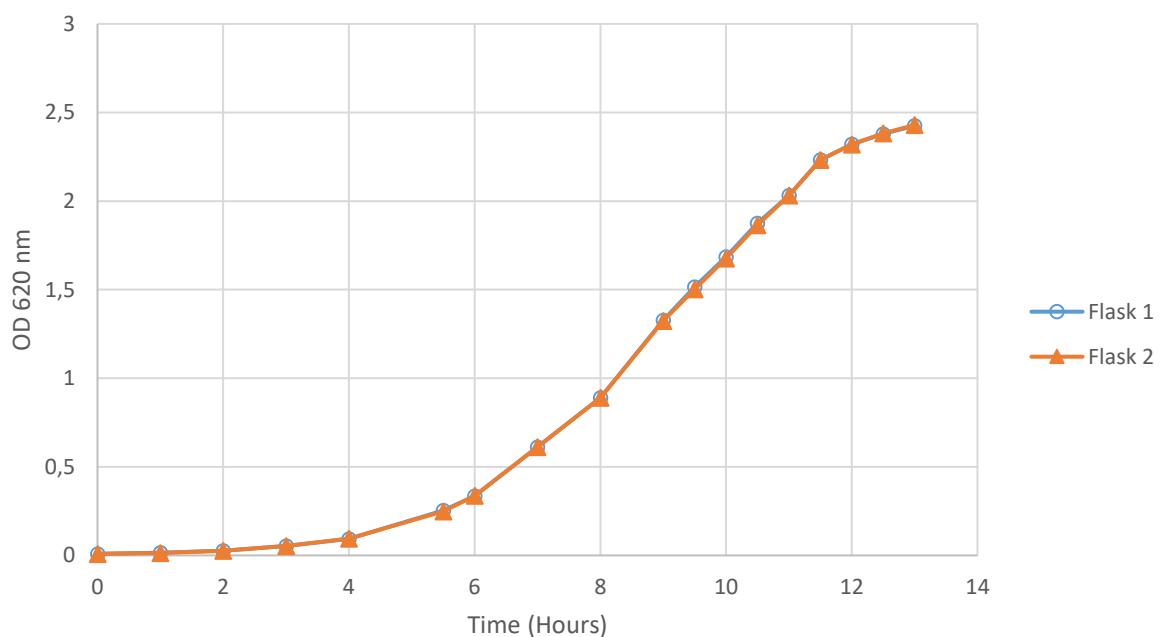

Figure 4. Growth of *Y. ruckeri* in LB-medium at 25°C and 200 rpm. The growth in two parallel cultures (orange and blue) are measured by absorbance at 620 nm over time.

3.1.1 OD and CFU correlation

To get an overview of the correlation between the OD and the CFU, a correlation curve (Fig. 5) was made by plating out dilutions of *Y. ruckeri* cultures at an given OD. The plates which had formed a countable number of colonies were chosen. Plates containing between 50-300 colonies are therefore preferred to get the most accurate number of colonies. These plates were then counted and the total numbers of CFU (table 1) was calculated by the formula shown in material and methods (section 2.4.1). The curve shows a that after the OD reach 0.2 the number of CFU increase rapidly until OD 0.5 when the CFU/ml is $1x10^8$.
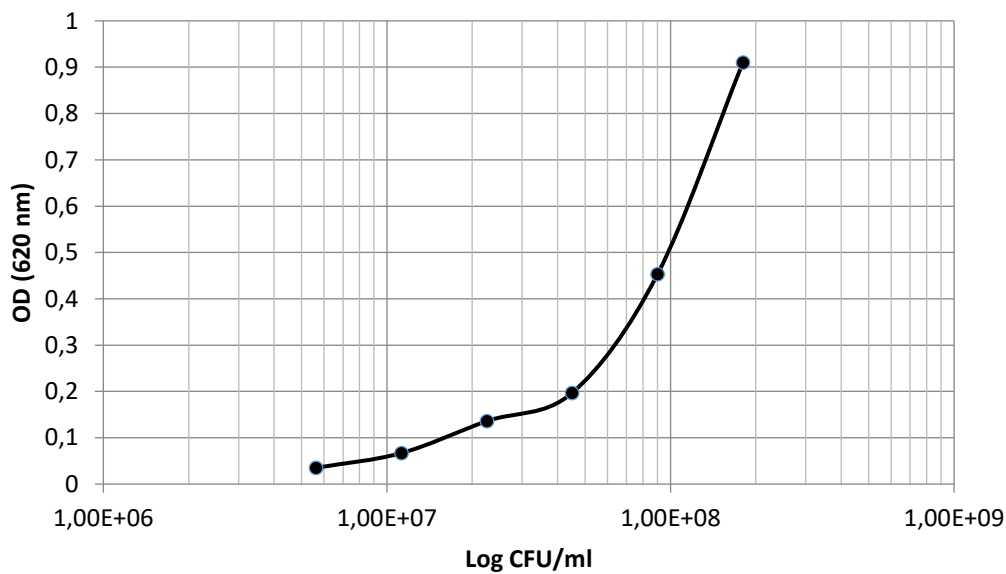

Figure 5. Correlation between optical density and CFU for *Y. ruckeri* incubated in LB-medium at 25°C and 200 rpm.

Table 1. An overview of CFU with correlation to OD measured at the different dilutions. The optical density was measured at 620nm.

Dilution	CFU/ml	OD620nm
0	1,80E+08	0,910
2	9,00E+07	0,453
4	4,50E+07	0,197
8	2,55E+07	0,136
16	1,125E+07	0,067
32	5,625E+06	0,035

3.2 Spot survival assay of phages inactivated with UV-light

To make sure full inactivation of the bacteriophages was achieved prior vaccination of salmon, a plaque assay was performed after UV-C treatment. Two replicates trials were performed. The first one had 30 minutes as the lowest time for UV treatment (Fig. 6), and the second had 15 minutes (fig. 7). In both trials a Plaque assay was also performed after 24 hours to ensure that the phages were still inactivated. A non-UV treated control was included. For the non-UV treated control the phage dilution providing a clear and countable number of visible plaques, preferably 1-40, was used for counting. The plate treated with UV-radiation did not form any plaques after 15 minutes in any of the dilutions (fig. 7). Furthermore, no plaque was observed at the later sample points. The control plates without inactivation gave a high density of plaques at the low dilutions and a few at the three highest dilutions as seen in figure 6 and 7.
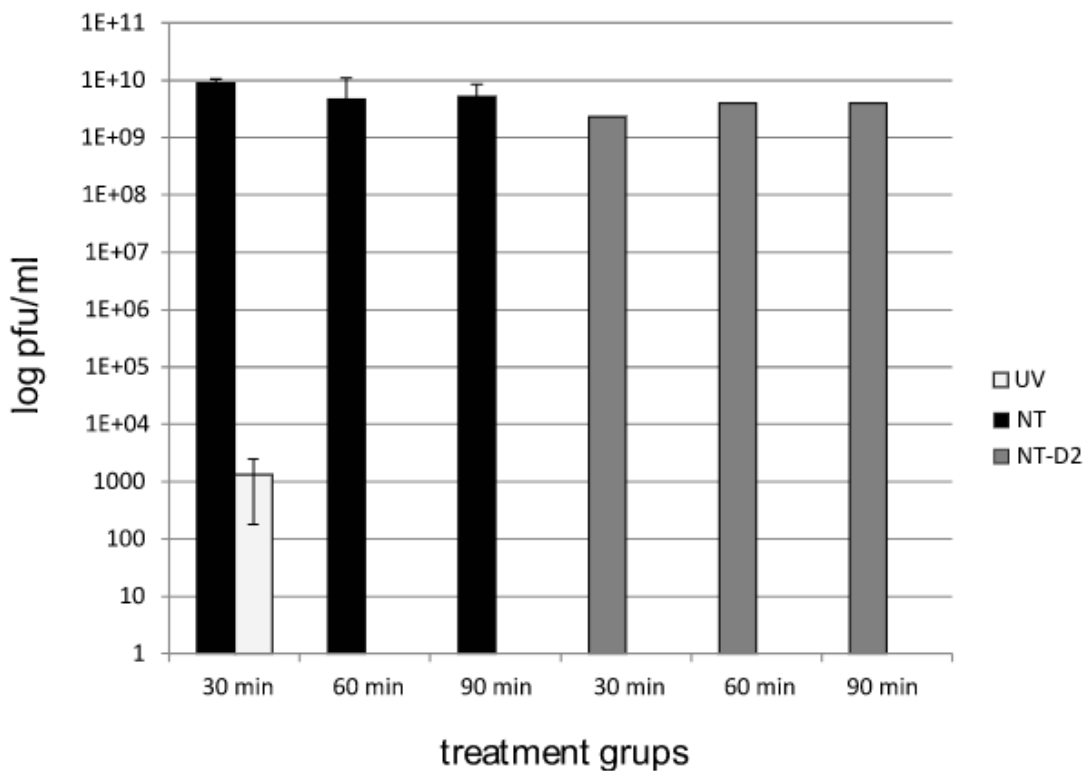

Figure 6. Results from plaque assay performed on UV-C treated (UV) and control non-treated phages (NT) in the pilot trial. Showing PFU counts of phage samples taken after 30, 60, and 90 minutes of exposure of UV-C and PFU counts after 24 hours treatment (NT-D2).
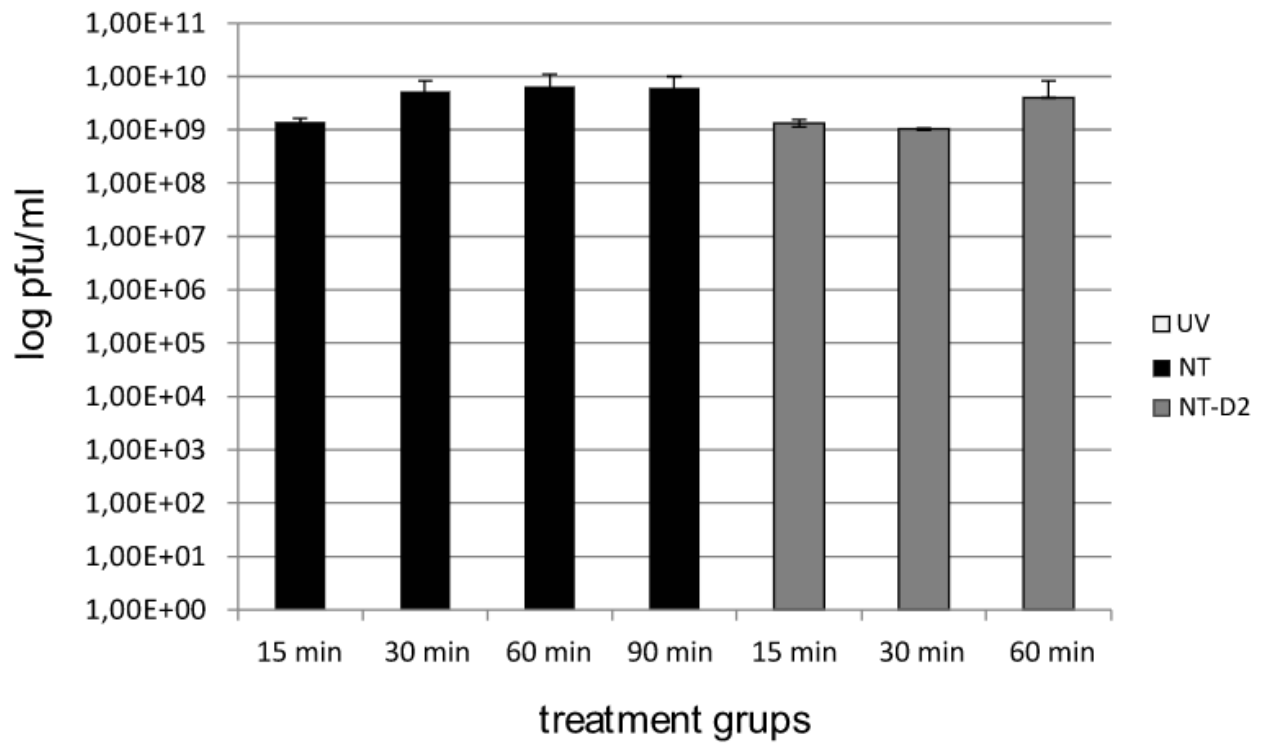
Figure 7. Results from plaque assay performed on UV-C treated (UV) and control non-treated phages (NT). Showing PFU counts of phage samples taken after 15, 30, 60, and 90 minutes of exposure of UV-C and PFU counts after 24 hours treatment (NT-D2).

3.2.1 *Y. ruckeri* growth curves with added bacteriophage

To investigate the effect of phages on growing liquid bacteria cultures, a controlled phage titer was added to two different amounts of bacteria in cultures, one at OD 0.58 and one at 0.36. For both cultures bacteriophage was supplied at a MOI of 1. For the growth curve with start OD 0.54 the growth continued for 20 minutes, where there was a slight increase from there to an OD of 0,62 at 120 minutes. At 20 minutes the OD declined. This decline in OD was also observed at in the culture with start OD 0,36, only 10 minutes later (Fig. 8). The OD in the culture with start OD 0.36 had a similar growth pattern with slightly variating growth until 120 minutes, before the OD started to decline at an OD of 0,52 at 120 minutes.
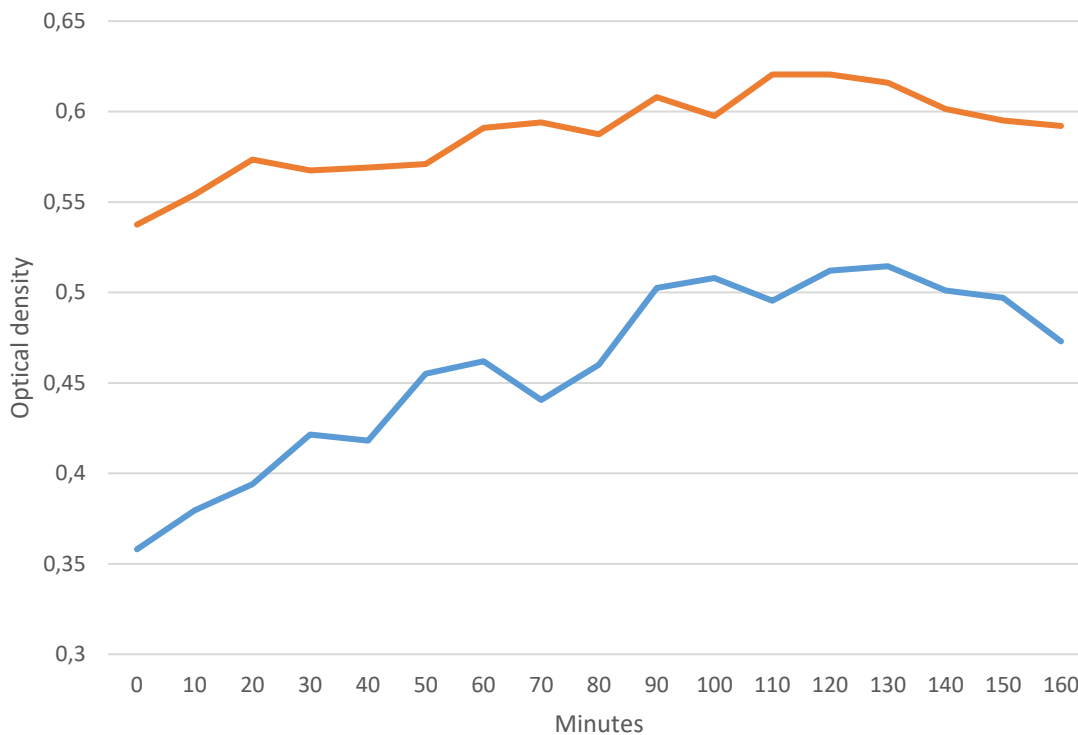

Figure 8. *Y. ruckeri* growth as a function of OD over time in two cultures with different start OD's. One culture with start OD 0.36 (blue) and one culture with start OD 0.54 (orange). To both cultures bacteriophages were supplied at a MOI of 1. Optical density was measured every ten minutes for 160 minute

3.3 Phage activity

To study how different titers of phages affects *Y. ruckeri*, two different phage doses (undiluted phage stock and phage stock diluted to a MOI of 1 with the bacteria) were premixed and incubated with to two parallel *Y. ruckeri* cultures with OD 0.5, before adding growth medium. In order to examine the production of phages and bacteria, CFU and PFU samples was collected every sample point. A control group without phages was included to see the natural growth of *Y. ruckeri*. All agar plates used for either CFU or PFU counting, were observed and inspected for contamination or other problems before and after incubation.

3.3.1 Colony forming units

To see how phages affect the *Y. ruckeri* cultures, CFU was calculated and compared to the control. This was done by plating aliquots of 100 µl of the cultures on LB-agar. The bacterium formed small singular colonies, that were easy to count. All phages showed a bacteriostatic effect as the bacterial growth was inhibited. There was also observed a significant difference in colony size on the control plates with only bacteria and the plates which had phages added to the bacteria before being overlaid. The colonies from plates with phages had a generally bigger size range, while some where rather small and some colonies were of similar size as the control *Y. ruckeri* colonies without phages.

For the phage activity tests *Y. ruckeri* cultures, without bacteriophage supplement, was included as controls. And for all tests the control cultures gave high and increasing CFU counts throughout the sampling period. The phage activity test performed using a low dose of phage ɸ 2, had lower CFU counts compared to the control, and the CFU counts were stable throughout the sampling period (Fig. 9). The culture supplied with a high dose of bacteriophage ɸ2 gave stable CFU counts until four and a half hours had passed, when it increased to the same CFU count as in the low dose (fig. 9). The *Y. ruckeri* culture supplied with a low dose of phage ɸ 3 had the lowest CFU counts one hour after incubation, before it slowly increased to a CFU count slightly higher than the first count (Fig. 10). For phage ɸ 4 the low dose culture has a decrease in CFU counts at every measuring. After 5 and a half hour the low dose culture have a lower CFU count than the high dose culture (Fig. 11). The High dose culture have a slight increase in CFU at the second measuring point, before it

decreases significantly at the third. At the last hours, the number of CFU increase again in the high dose culture, and has as mentioned a slightly higher number of CFU than the low dose culture (Fig. 11). The CFU measurements in the low dose culture from the phage ɸ 9 test are almost identical to those from the low dose culture in phage ɸ 4 (Fig. 12). The high dose culture in the phage ɸ 9 test, does not have much change in CFU count before three and a half hours have passed when a small increase is observed before a minor decrease follows (Fig. 12). In the low dose culture where the cocktail was added, a rapid decrease in CFU is observed from two and a half hours and until the last measuring (Fig. 13). The high dose culture with added cocktail have a slight increase in CFU count, before a small decrease is observed. At the end the CFU count increases again and is just slightly less than the low dose in CFU count after 5 and a half hour (Fig. 13).
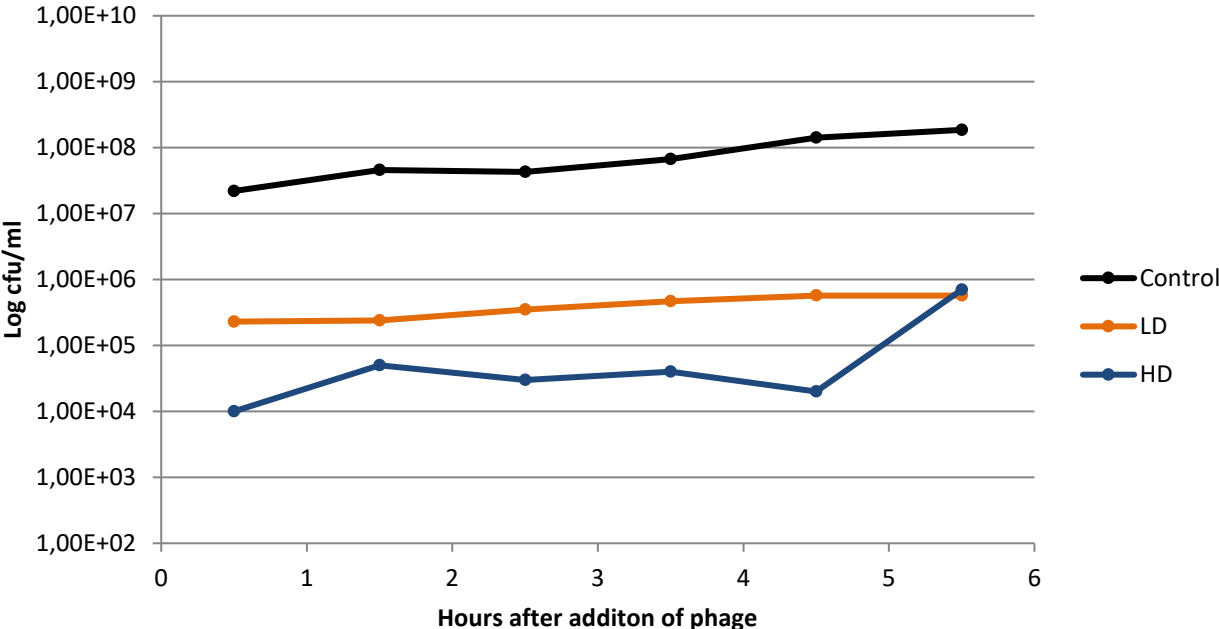

Figure 9. Bacterial growth in number of CFU of *Y. ruckeri* cultures after incubating with different doses of phage ɸ 2. The different lines show non-phage control (black line), low phage dose (LD) MOI 1:1 (orange line), and a high dose (HD) (blue line). Containing undiluted phage stock dose of $1,44x10^{11}$ PFU/ml.
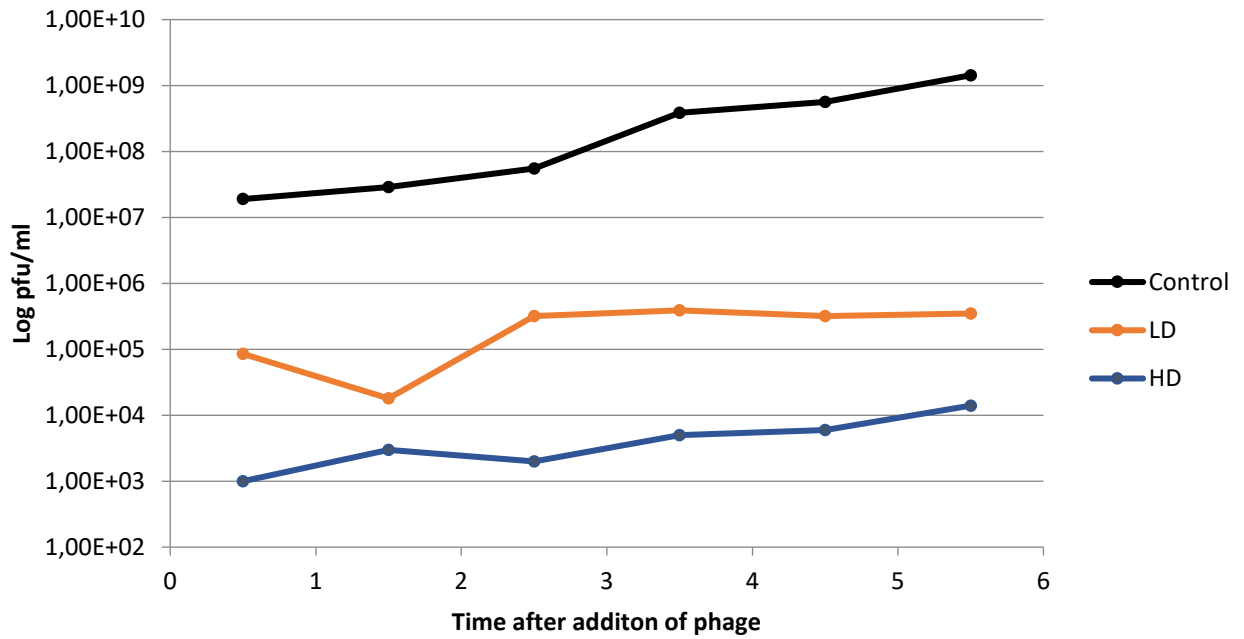
Figure 10. Bacterial growth in number of CFU of *Y. ruckeri* cultures after incubating with different doses of phage ф 3. The different lines show non-phage control (black line), low phage dose (LD) MOI 1:1 (orange line), and a high dose (HD) (blue line). Containing undiluted phage stock dose of $2,25 \times 10^{11}$ PFU/ml.
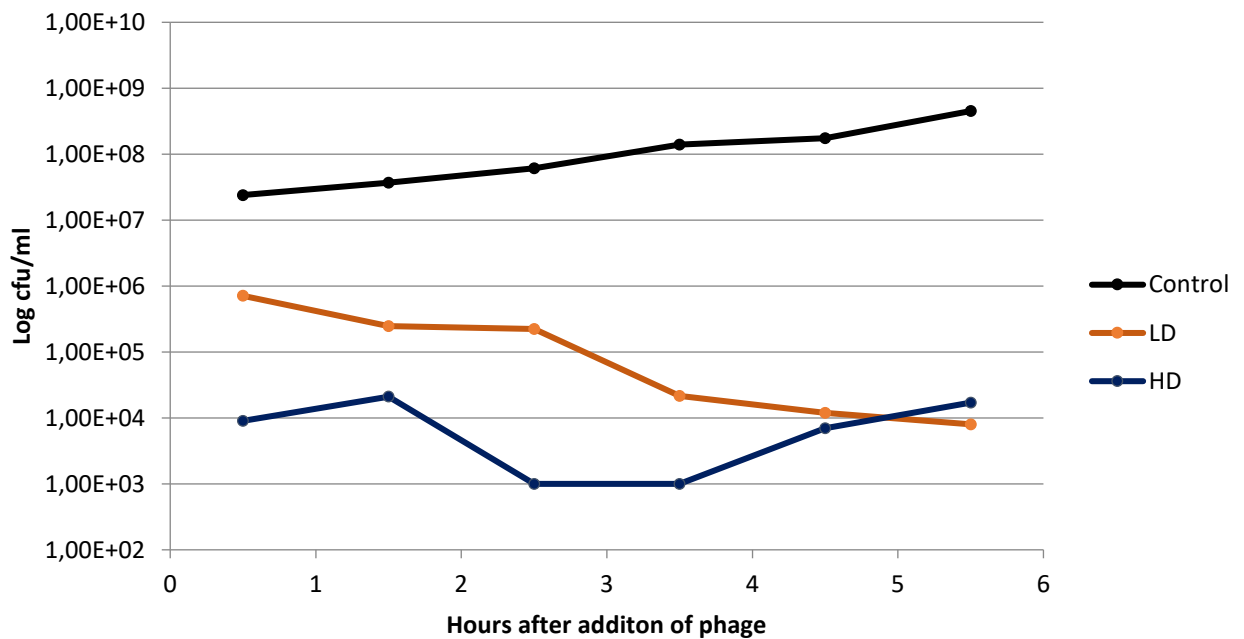

Figure 11. Bacterial growth in number of CFU of *Y. ruckeri* cultures after incubating with different doses of phage ф 4. The different lines show non-phage control (black line), low phage dose (LD) MOI 1:1 (orange line), and a high dose (HD) (blue line). Containing undiluted phage stock dose of $15,12 \times 10^{11}$ PFU/ml.
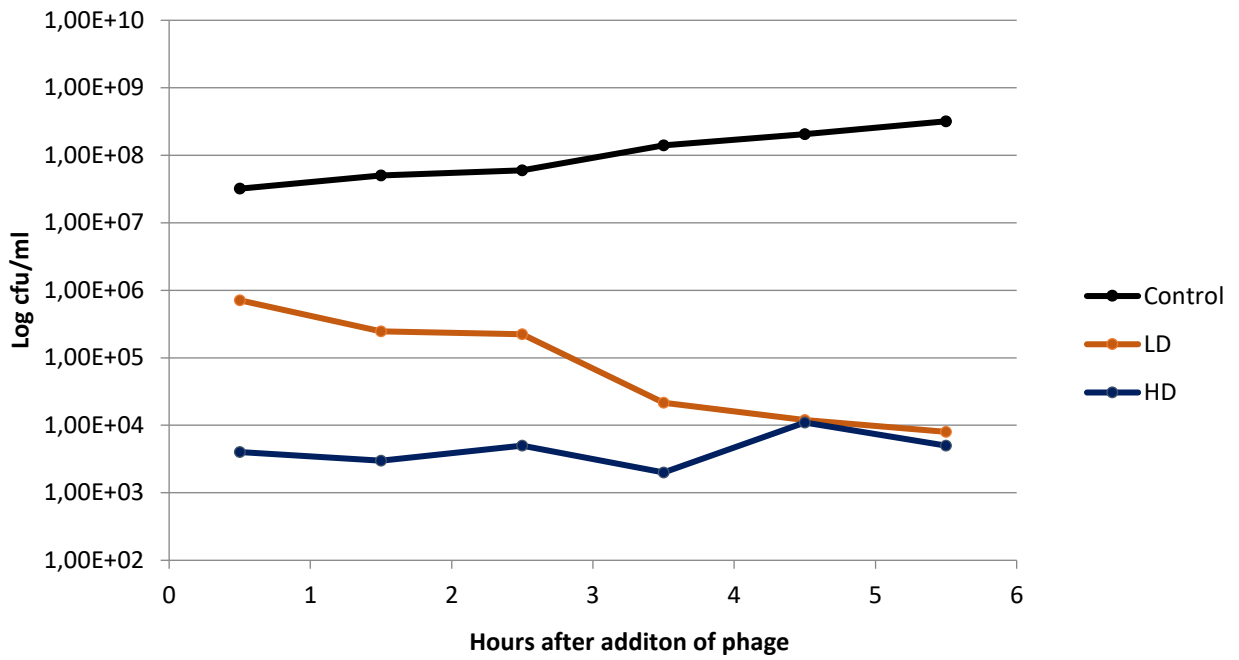
Figure 12. Bacterial growth in number of CFU of *Y. ruckeri* cultures after incubating with different doses of phage φ 9. The different lines show non-phage control (black line), low phage dose (LD) MOI 1:1 (orange line), and a high dose (HD) (blue line). Containing undiluted phage stock dose of $1,62 \times 10^{11}$ PFU/ml.

Figure 13. Bacterial growth in number of CFU of *Y. ruckeri* cultures after incubating with different doses of phage cocktail. The different lines show non-phage control (black line), low phage dose (LD) MOI 1:1 (orange line), and a high dose (HD) (blue line). Containing undiluted phage stock dose of $7,2 \times 10^{11}$ PFU/ml.
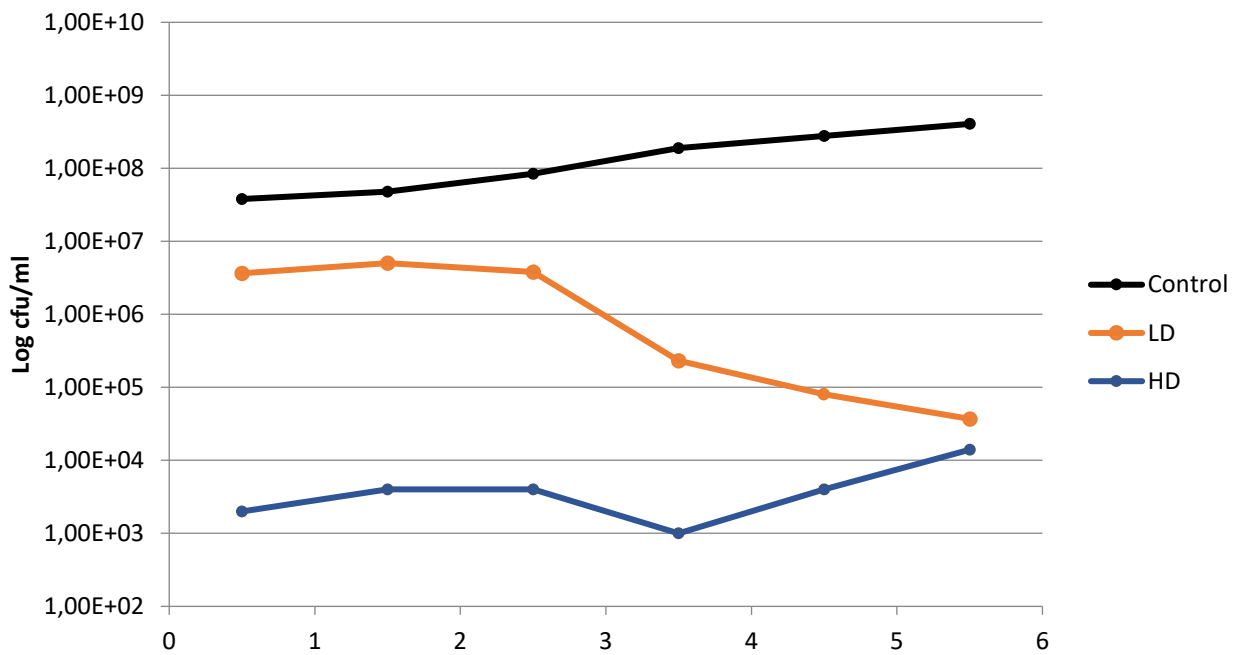
3.3.2 Bacterial cell number

The number of bacterial/ml was measured with CASY cell counter (INOVATIS) at every sampling time. With the Casy cell counter analyses, it was possible to separate infected/dead, and not infected cells visually by cell size. A portion of the cells counted would be dead, however, a good separation between the control and LD/HD is achieved. There was a significant decrease in cell count from the wells of the LD and HD culture during the time period before 4,5 hours post phage infection, as seen in figure 14.
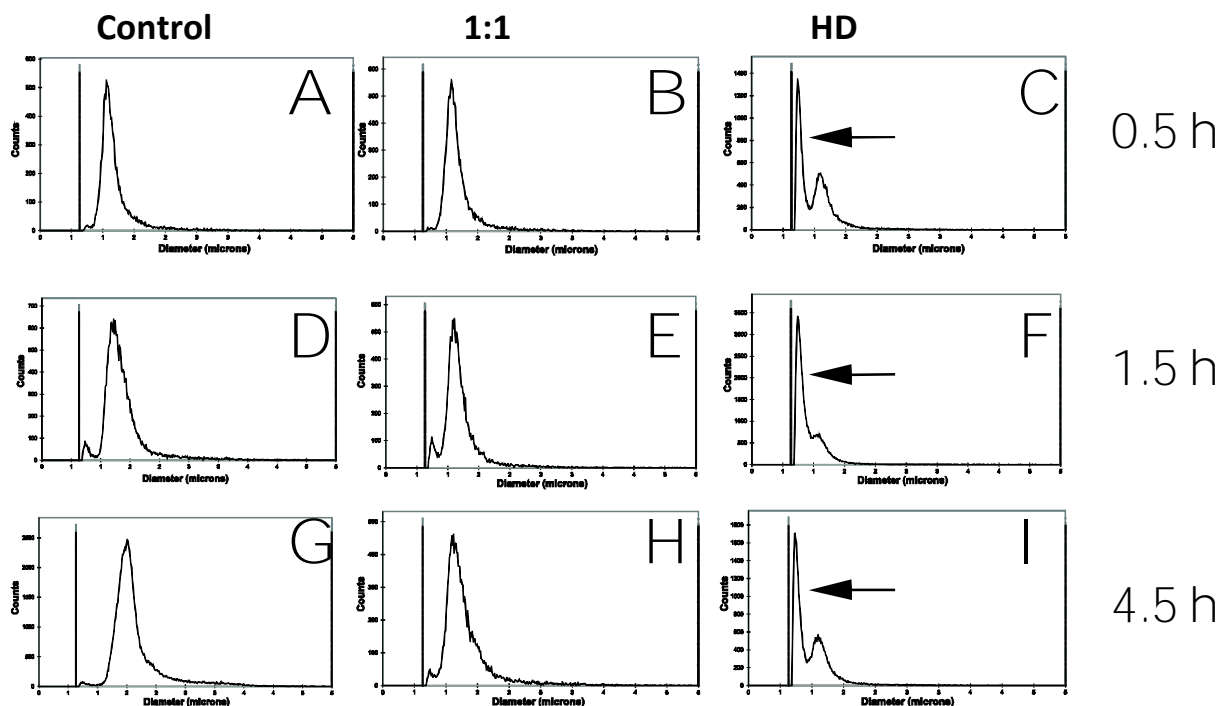

Figure 14. Bacterial counts as function size analyzed in CASY cell counter for bacteriophage ϕ1. Control (A, D and G) is bacteria without phage supplement, 1:1 (B, E and H) is phage supplied at a MOI if 1 and HD (C, F and I) is phage supplied in excess. All samples are shown at harvest after 0.5 h (A, B and C), after 1,5h (D, E and F) and after 4,5h (G, H and I).

3.3.3 Phage titers

It is interesting to see how fast the bacteriophage undergoes a lytic cycle and to see the difference between the high dose (HD) and the low dose (LD) phage on a culture. To test the efficiency of the phages used in this study, PFU samples was taken out every hour after incubating phages and bacteria. After the bacteria was killed by chloroform treatment, and the samples diluted, plaque assays were used to calculate phage titers.

The phages tested showed differences in the lytic ability of *Y.* ruckeri. Phage ф 2 showed an increase in number of PFU at 4 hours in both LD and HD cultures (Fig. 15). Phage ф 3 gave relatively little change in the number of PFU over time (Fig. 16). While phage ф 4 shows minimal change at 4 hours in the HD sample series. But it rises at 5 hours (Fig. 17). In the LD culture with phage ф 9, a slow increase from one hour post addition to 4 hours past addition is observed (Fig. 18). The PFU count from the cocktail is the one that stands out most, with a clear variation in count through time (Fig. 19). Both LD and HD cultures, revealed a trend of significant increase for two hours, before a major drop at 3 hours was observed. One hour later the phage titer in both cultures have increased immensely. A 1000-fold increase is observed in low dose (Fig. 19).

Figure 15. Results from plaque assay performed phage ф 2 PFU samples. Showing phage titer growth over time after the phage addition in low dose (LD) culture with a MOI=1 to bacteria (blue line), and high dose (HD) culture with undiluted phage stock of $1,44 \times 10^{11}$ PFU/ml (black line).
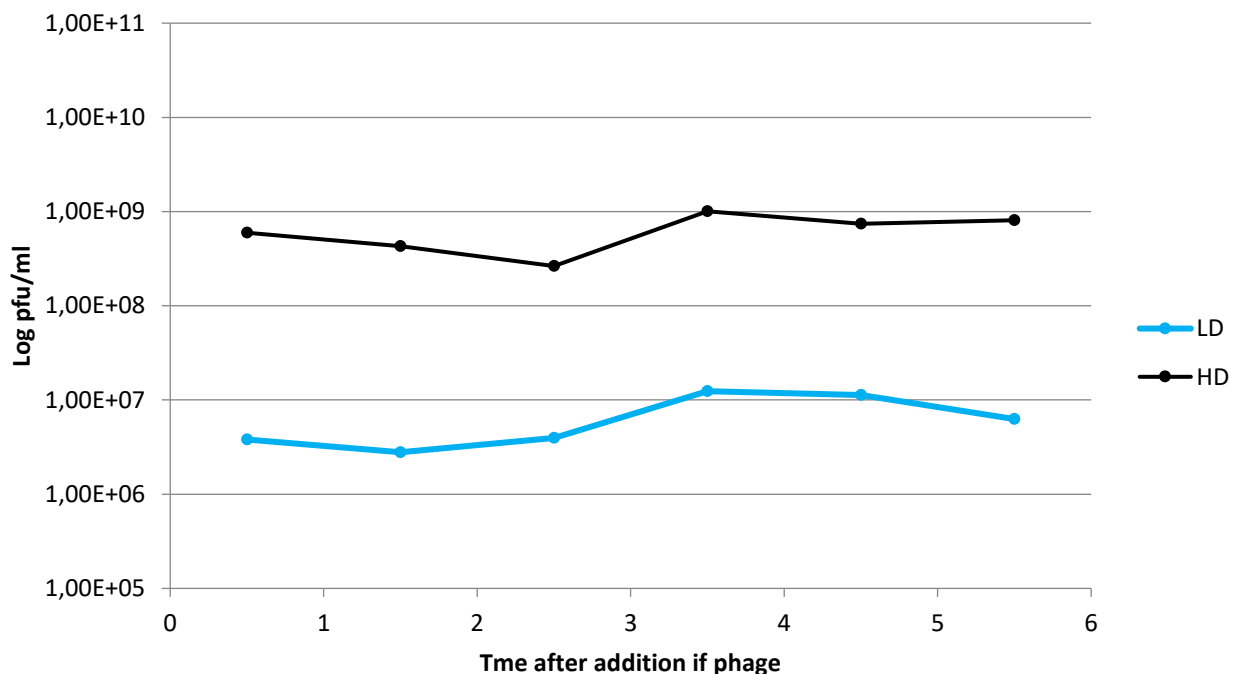
Figure 16. Results from plaque assay performed phage φ 3 PFU samples. Showing phage titer growth over time after the phage addition in low dose (LD) culture with a MOI=1 to bacteria (blue line), and high dose (HD) culture with undiluted phage stock of 2,25x10^{11} PFU/ml (black line).

Figure 17. Results from plaque assay performed phage φ 4 PFU samples. Showing phage titer growth over time after the phage addition in low dose (LD) culture with a MOI=1 to bacteria (blue line), and high dose (HD) culture with undiluted phage stock of 5,12x10^{11} PFU/ml (black line).
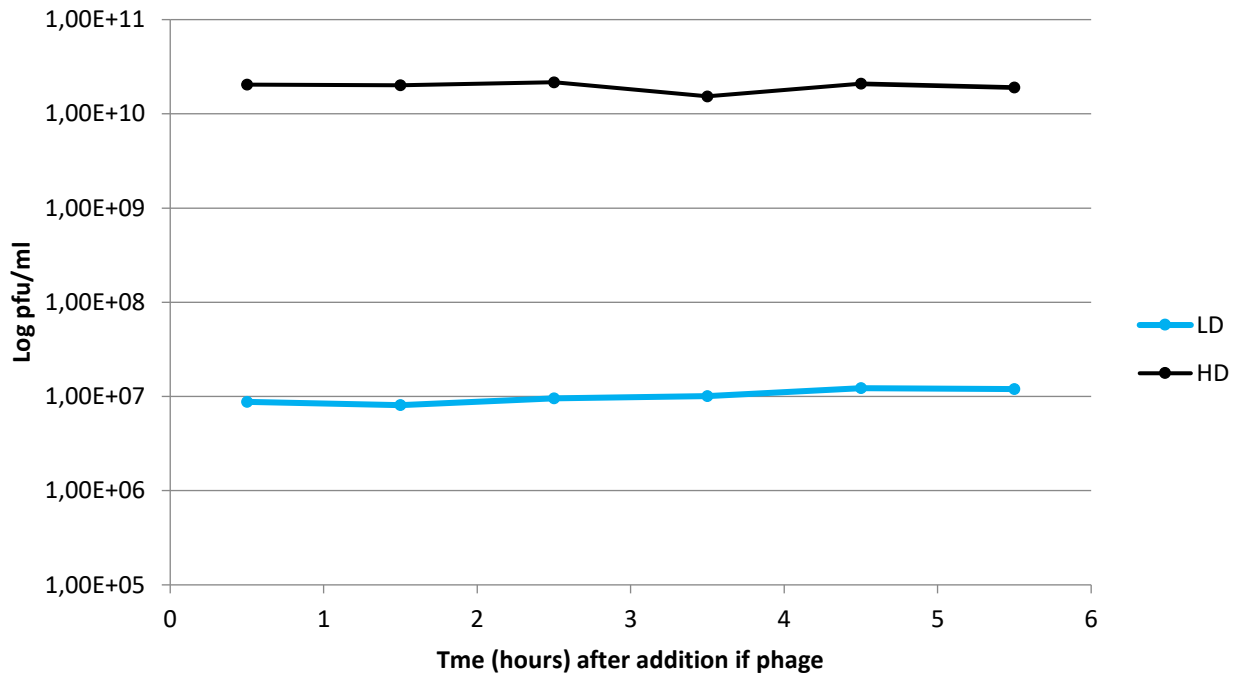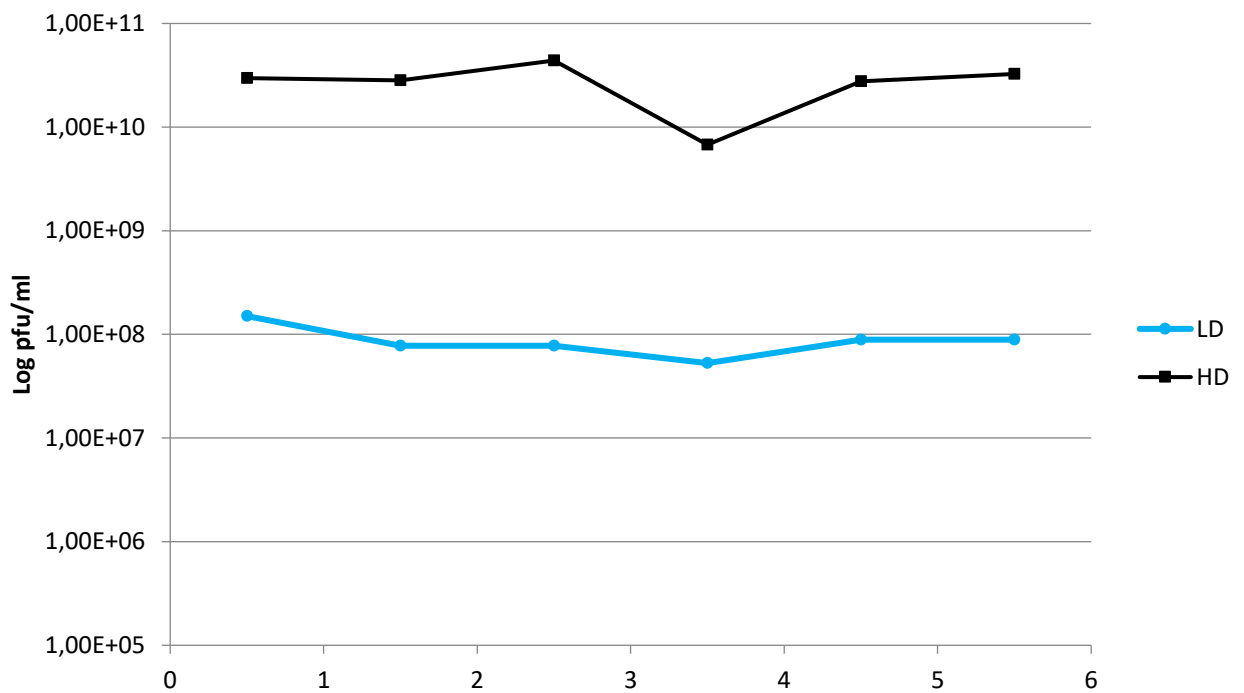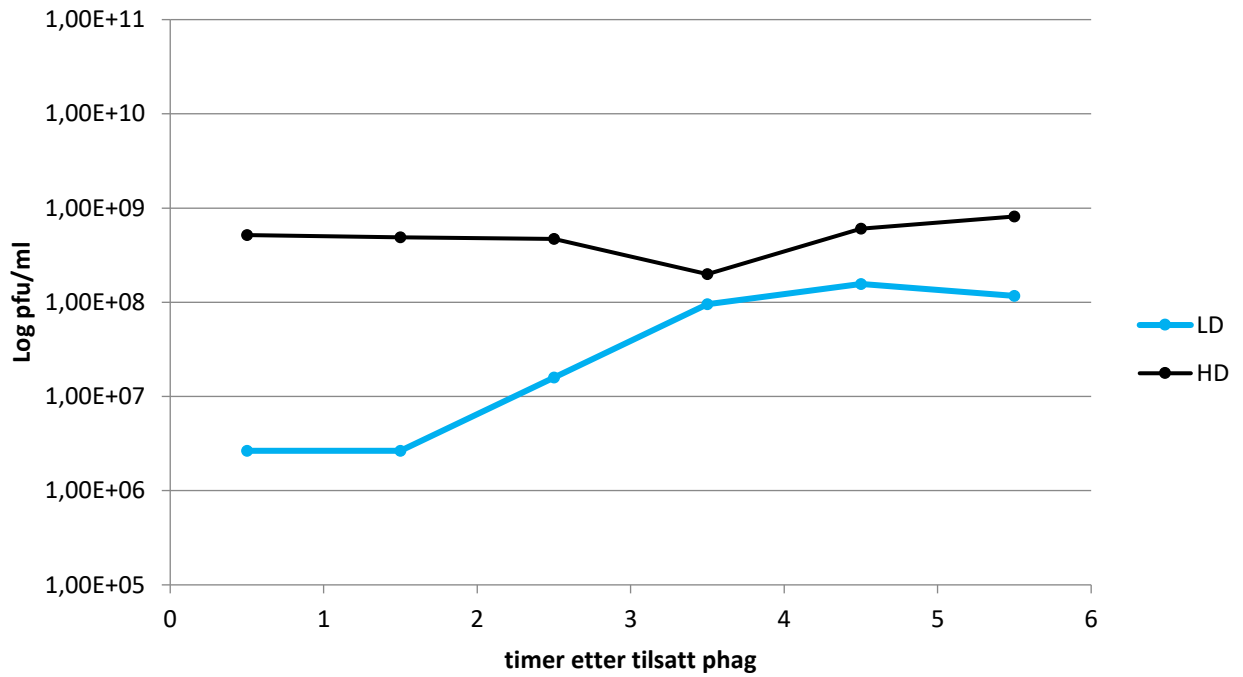
Figure 18. Results from plaque assay performed phage ϕ 9 PFU samples. Showing phage titer growth over time after the phage addition in low dose (LD) culture with a MOI=1 to bacteria (blue line), and high dose (HD) culture with undiluted phage stock of 1,62x10^{11} PFU/ml (black line).

Figure 19. Results from plaque assay performed phage ϕ cocktail PFU samples. Showing phage titer growth over time after the phage addition in low dose (LD) culture with a MOI=1 to bacteria (blue line), and high dose (HD) culture with undiluted phage stock of 7,2x10^{11} PFU/ml (black line)
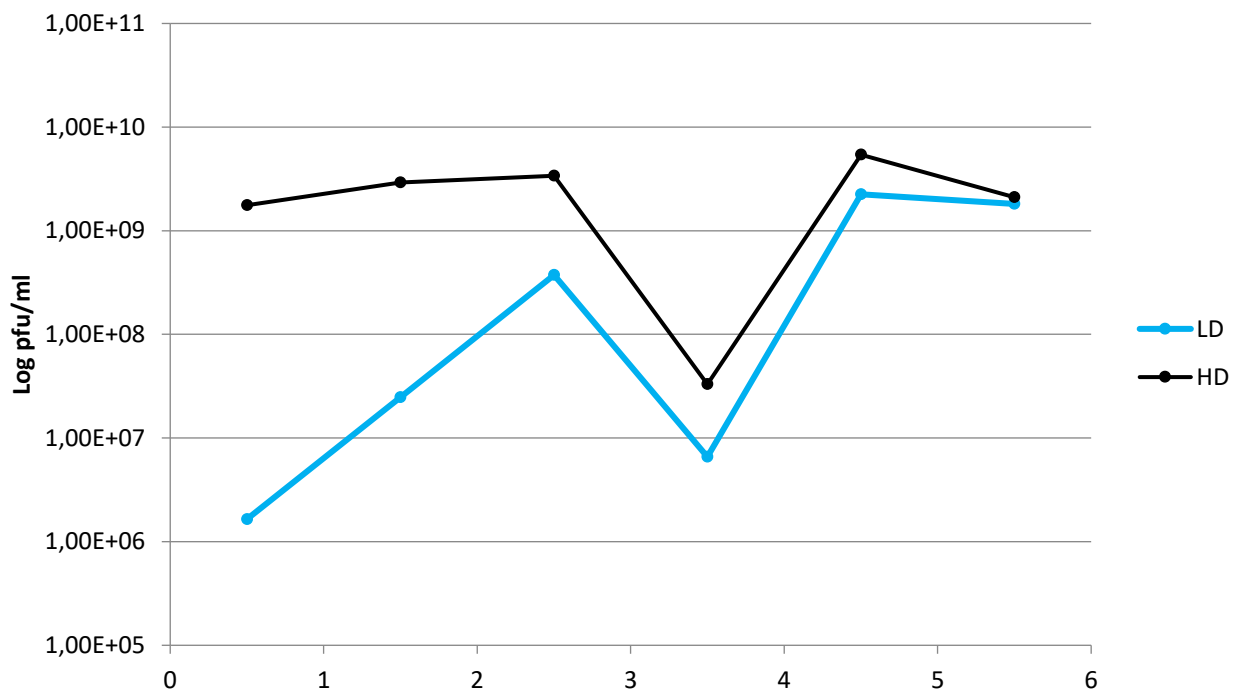
3.4 Immunization of Atlantic salmon with bacteriophages

To investigate the Atlantic salmon's ability to produce phage specific antibodies, there was made an attempt to induce a high level of specific antibody production in the salmon. Testing of sera for antibodies was done by Enzyme-linked immunosorbent assay (ELISA). In order to induce a high antibody response, the fish was injected intraperitoneal three times with cocktail stock solution $7,2 \times 10^{11}$ PFU/ml of UV-inactivated phages. If there was revealed a phage antisera production, these would be further investigated.

3.4.1 The fish and rearing conditions

The fish were examined both externally and internally, in addition to measuring length and weight shown in Table 2. This was to make sure there was no complications made by the phage injection, and to see if there was any difference between the vaccinated and non-vaccinated fish groups. When the immunization trial was finished, the fish showed a normal growth. Average weight had increased from 92,45 grams to 235,17 grams (n=45), and the average length had increased from 20,17 to 26,36 centimeters (n=45). The fish looked overall healthy with nothing indicating sickness or lethargy. There was observed no signs of disease, and none of the fish had died during the experiment. After the blood was collected form the caudal vein, an autopsy was performed on several fish. This was done to look for possible internal side effects form the injection. There was found no sign of lesions on organs in the abdominal cavity.

3.4.2 Blood data

Blood was collected from the vena caudalis to see if phage injection had any influence on hematocrit levels in the blood. The collected data showed no difference between the control fish group injected with TM-buffer, or the fish group injected with phage (Table 2). However, the group injected with phage and adjuvant did have a slightly higher hematocrit average, but it is considered to be within the normal variation.

Table 2. Average data form the fish groups with standard deviation (SD).

Group	Length (cm) ± SD	Weigh (g) ± SD	Hematocrit± SD
Phage φ cocktail	26,0 ± 1,1	216,6 ± 29,7	40,7 ± 4,5
Phage φ + adjuvant	26,6 ± 1,1	244,7 ±3 7,9	44,3 ± 3,3
TM-buffer	26,5 ± 1,2	244,2 ± 30,3	41,1 ± 3,8

3.4.3 Enzyme-linked immunosorbent assay

To test the serum for phage specific antibodies an ELISA test was performed on the sera collected from the fish. A dilution test, was first performed to identify which dilution would be apropriate to use when testing sera from all fish. A dilution of 1:400 was selected (Fig. 20).

No specific antibodies were present in sera from fish in the control group injected with TM-buffer without phage. Nor in the sera sampled before injecting were there any phage reacting antibodies present. The sera from the two last groups injected with only phage, and phage together with adjuvant did however reveal a great response during the assay, indicating a high antibody production against the phages (Fig. 21).
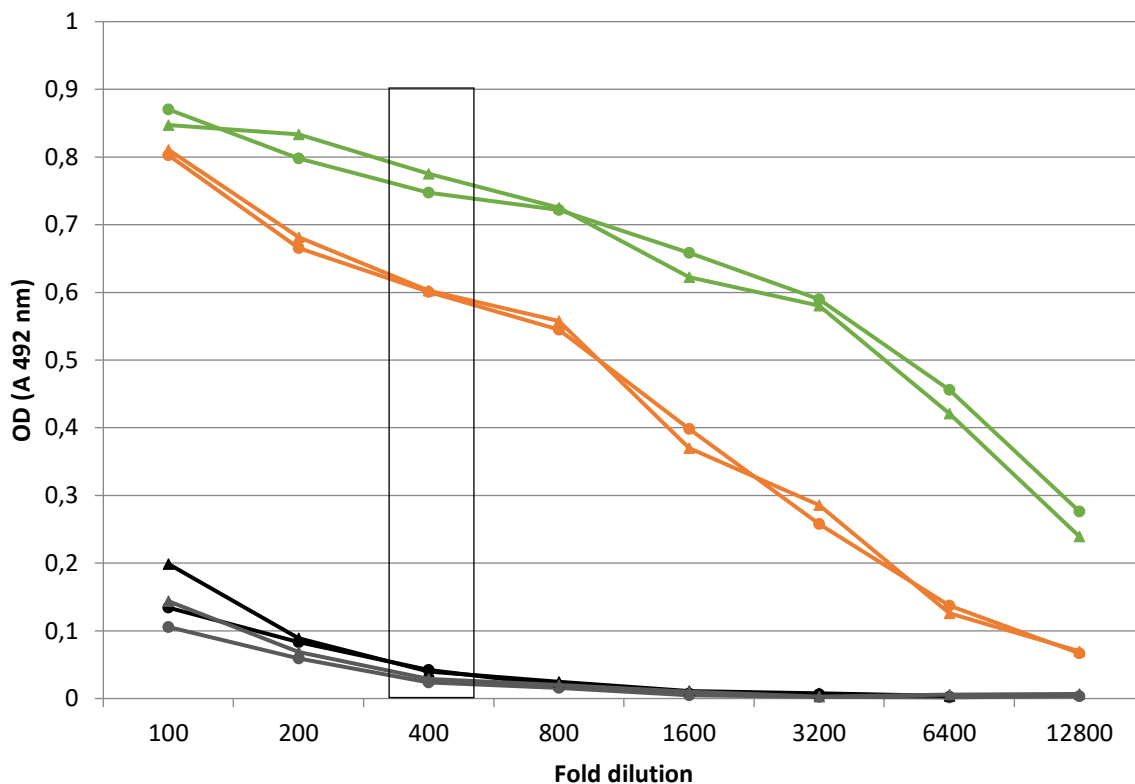
Figure 20. Dilution curves of Atlantic salmon Immune sera against bacteriophage cocktail. Injected with TM buffer (black lines), baseline sample (grey lines), sample from fish injected with bacteriophage cocktail (orange lines) and sample from fish injected with bacteriophage cocktail emulsfied in adjuvant (green lines).

Figure 21. ELISA results showing antibody response towards phage-cocktail of sera (1:400 dilution) from the 15 fish in each of the treatment groups. Means with SD are shown. Statistical significance compared to baseline samples: P>0.05 = n.s., ***=P<0.0001. Sera from individual fish in the four groups are shown by black circles.

3.4.4 Antibody opsonization test

The immunsera (salmon anti phage) was tested for the ability of the antibodies to block the lytic ability of the phage. The phages were opsonized with antibodies by incubating phage and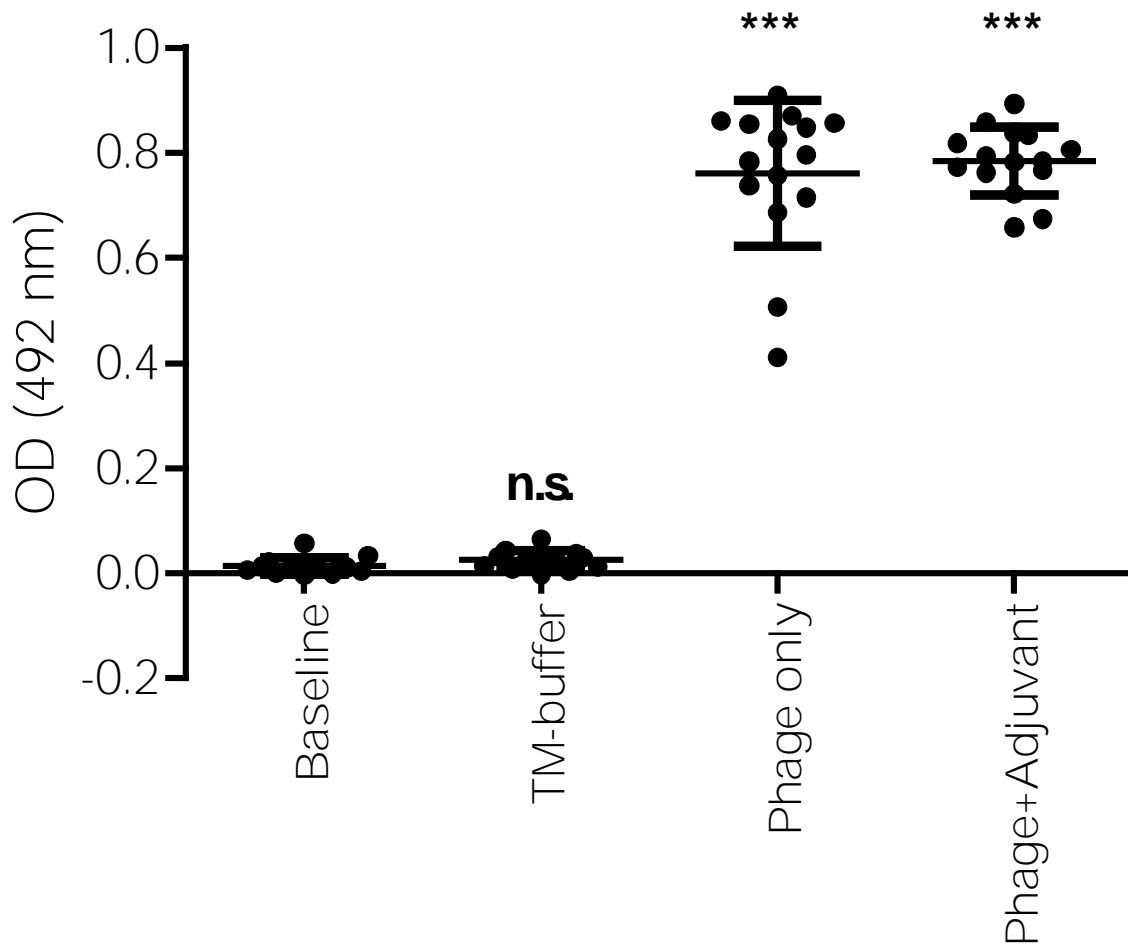 immunosera and subsequently testing the phages bacteriolytic ability of *Y. ruckeri* by. The effect was tested by PFU counts. The results showed that phages mixed with undiluted sera from fish injected with phage and adjuvant gave no plaques. While the control sera form non-vaccinated fish gave expected high PFU count. The anti-phage serum dilution experiment, revealed a good neutralizing effect on the phages until a dilution of 200 (fig. 22), before plaque count began to rise significantly.
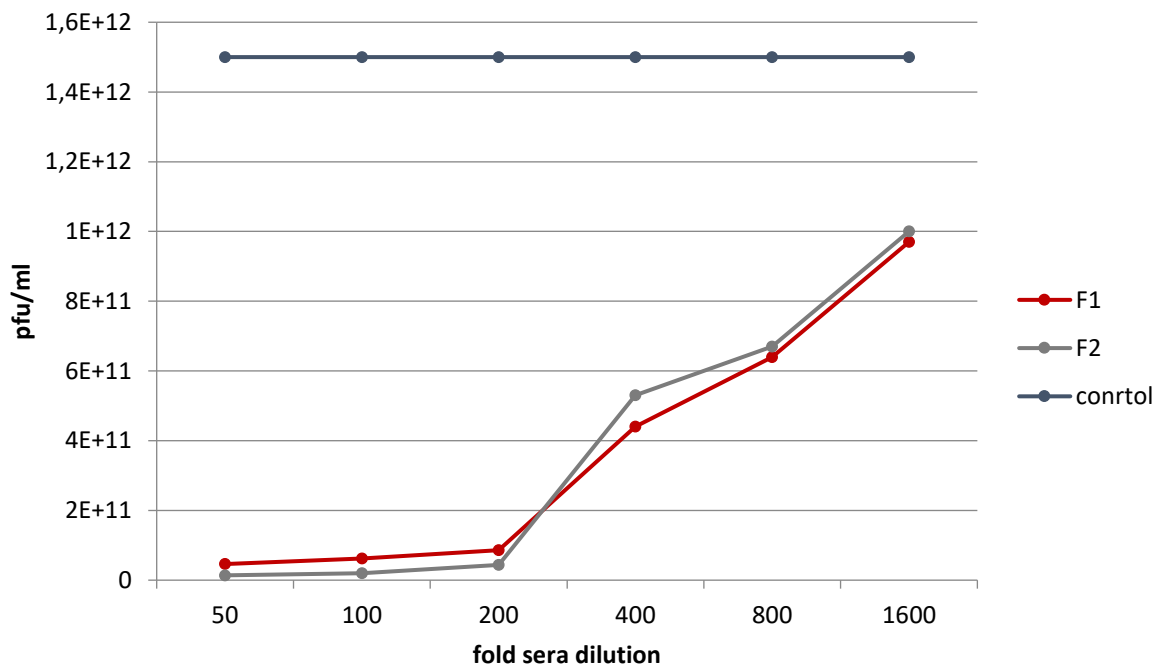
Figure 22. Showing the correlation between plaque forming units and the fold sera dilution. The two different fishers are showing as F1 (red) and F2 (grey). The control is shown as control (bl

3.4.5 SDS-PAGE of bacteriophage components

To confirm the presence of bacteriophage particles inside the wells used for ELISA, ten wells of 96 well immunoplate (nunc) were coated with the bacteriophage cocktail stock solution, washed, scraped off before being re-suspended in TM-buffer. This sample, and stock sample from the bacteriophage cocktail were used to perform a SDS-PAGE. The results were unclear, however, some of the proteins can be seen in the SDS-PAGE profile from the sample harvested from the 96-well plate (Nunc). Confirming the presence of bacteriophage in the wells after coating.

Figure 23. SDS-PAGE profiles of bacteriophage cocktail (A) and bacteriophage cocktail harvested after coating and first washing step in 96-well plate (Nunc) (B).

4. Discussion

Yersiniosis have become a major challenge problem in the Norwegian aquaculture industry. The problem now occur both in freshwater and in seawater stages of Atlanti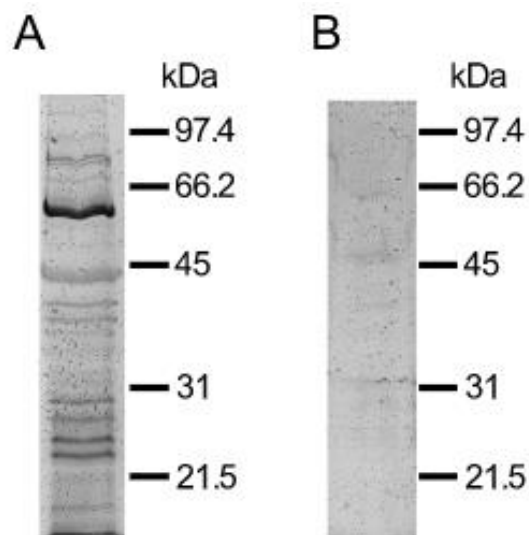c salmon production. Outbreaks often occurs at the same locations the following years, indicating that a house strain has been established, which survive in spite of the disinfection procedures used. In Norway, vaccination of salmon has been successful in preventing bacterial diseases and is considered a necessary preventive measure. The vaccines used have not included *Y. ruckeri* antigen, thus, antibiotics might be used to treat outbreaks. The current use of antibiotics in Norwegian salmon production is minimal. There is no intention to increase the consumption, and one are well aware of the risk of an increase in bacterial antibiotic resistance.

Due to the complex background and several difficulties in fighting *Y. ruckeri* infections, phage "therapy" or treatment of water, biofilms and filters are suggested as an alternative to reduce the occurrence of bacteria and also to some extent replace, or minimize, the use of antibiotics.

In this study, four different *Y. ruckeri* lytic phages and a cocktail with a combination of the four was tested. As *Y. ruckeri* form biofilm (Coquet et al., 2002a, Coquet et al., 2002b) it is relevant to test the effect of the bacteriophages on biofilm formation. Thus, we performed some pilot experiments on phage activity to obtain knowledge for application in such studies.

Inactivation of bacteriophages was examined by exposing phage stock of UV-radiation. This proved to be an effective method for inactivating bacteriophages. The radiation used was UV-C. After 30 minutes of exposure the phages were not capable of forming plaques. The following day, another plaque test was conducted to see if some phages have regained activity. No plaque was seen the following day either, indicating a sufficient inactivation. This results is consistent with the findings of Clark et al. (2012) regarding inactivation of bacteriophages in a study where they used different methods.

Inactivation of the bacteriophages with UV-C could therefore be used for preparation of inactive material for use as antigen for immunization of salmon.

UV-radiation is commonly used for disinfection of water. The effectiveness of the UV treatment is highly dependent on the murkiness of the water and particles in the water.

Therefore, UV-filters are usually located right after mechanical filters in RAS facilities (Finstad et al., 2012). As we have demonstrated, UV-radiation are capable of inactivating phages, meaning phages could be inactivated by the water UV treatment. If phages were to be added into the rearing water of fish, the addition should be right after UV- treatment. This is to keep them active for as long as possible.

Phages are naturally present in aquatic environment. In the aquatic environment, the exposure fish experience is not supposed to have any immunogenic effect. The phages are however, potentially immunogenic to Atlantic salmon if given in high doses by oral administration which is relevant for use as treatment of *Y. ruckeri* infections. A production of anti-phage antibodies could potentially lead to reduced effect in subsequent treatments. In this study the ability of salmon to produce anti bacteriophage antibodies was tested. This was done by injecting "high" amounts of phage antigen. This resulted in high anti-phage antibody levels in all fish, and worth noting is that non-immunized fish had no phage specific antibodies in serum. This indicates that there is no prior exposure, or no responses, probably because of low phage levels and exposure from the surrounding water. The specificity of the anti-phage antibodies was also shown by neutralization of phage activity. The antiserum could not be highly diluted without reducing the neutralizing effect, which indicate that likely high levels must be present to have any effect on phage activity in vivo.

The production of phage specific antibodies in Atlantic salmon was not unexpected, as an extensive immunization with three doses was used. The results from the ELISA test showed specific antibody production in the fish injected with both phage and phage with adjuvant. This was the most likely outcome after three immunizations with high titer. The antigenicity of phages was early described by Adams (1959), Kucharewicz-Krukowska and Slopek (1986) and he confirmed phage antibody production in humans receiving phage therapy. Bacteriophages are abundant and natural in aquatic environments (Breitbart, 2012). Interaction with aquatic organisms, directly or through bacteria with phages can thus occur. However, the amounts of phages and way of exposure make it not likely to stimulate any immune response in Atlantic salmon.

The results from this study confirms that non-vaccinated fish had no phage reactive antibodies. However, the inactivated phages were highly immunogenic for salmon and a good specific anti-phage antibody response was obtained in immunized salmon. After three

immunizations by injecting high titers of bacteriophages, the outcome of high antibody levels was the most probably.

The impact of these results on eventual phage therapy, is that phages used in for the second time or more treatments, can be neutralized and removed from the circulation system faster than when the fish had not experienced any phage. Thus, the first treatment will be effective, but subsequent treatments can be impaired. This can thus interfere with the clinical effect of the phage therapy in general. However, if high amounts of phages are used for a short period in subsequent treatment, this problem might be overcome.

Furthermore, how this result will affect the success of actual phage therapy of fish, will depend on several factors. The way of administration is important, and the time before the immune system removes the phage particle from circulation system will directly affect the therapy. Some bacteriophages are more potent antigens than others (Ochs et al., 1971). This is a characteristic that can be selected for. A method for selecting less antigenic phages mutants were tested by Merril et al. (1996). One must therefore take the time to find the preferred phages, as they most likely exist.

Concerning the problem regarding immunity it can be solved by the number of phage products available. If there are several different phage cocktails that are effective in treatment, they could be used in a rollover treatment system. In this way, you circumvent immune response by not using the same phage product in following treatment. However, this would not be possible if cross reactive in antibodies occurs, making the antibodies against one preparation capable of neutralizing the other ones with antigenic similarities. Given the high diversity of bacteriophages in nature, it should be possible to create phage cocktails which do not result in cross immunization. Phages with a different antigenic profile would therefore be the most preferred option in circumventing phage neutralization by serum.

The antibody opsonization of phages showed that the antibodies had a strong neutralizing effect on the phage lytic abilities of *Y. ruckeri*. The phage titers were found to be reduced when incubating phages with immune serum dilutions before tested in a plaque assay. To maintain the inhibitory effect, the sera could only be diluted at about 1:200. While the pages alone provided above $1,4*10^{12}$ PFU. This indicate that inhibitory antibodies are not realistic to obtain in a treatment regime for salmon.

For aquaculture use, it would be inconvenient to inject bacteriophage products, due to the large number of fish. Bath administration of phages could, however, be effective in controlling pathogenic bacteria that colonize and infect gills or skin. Bath and immersion method would be preferred for treating larvae, juveniles, and eggs in hatcheries, infected with bacterial diseases. Bacterial cold water disease and yersiniosis both affect juvenile fish, and prophylactic measures would be most wanted. It was observed an increased survival of fish larva in infected fish tanks where *Vibrio harveyi* lysing bacteriophages where present (Vinod et al., 2006). This indicates that phages have an advantageous effect for bio-controlling bacteria. The many administration methods of phages are advantageous in aquaculture from hatchery to brood stock as there are many administration routes.

The use of bacteriophages is highly relevant for aquaculture production of shellfish. Shellfish do not have an adaptive specific antibody immune response (Hovgaard et al., 2001), meaning vaccination is not an option. Therfore, bacterial disease outbreaks will be equally severe every time since no antibodies are produced. For shellfish, the fear of phage treatment being affected by antibody production is therefore not relevant.

An early study from 1943 shows that 4600 molecules of antibodies can combine with each phage used, while only 1-3 is sufficient to neutralize the infectivity of a bacteriophage. It must be noted that the antibodies are produced against different structures of the phage. In general, antibodies interacting with the tail structure were mainly responsible for phage inactivation, while 10-15% were caused by clumping, or only at high levels (Kutter and Sulakvelidze, 2005). In conclusion, this suggested antibodies are effective of inactivation of phages, but it depends highly on what structure the antibodies are interfering with. However, this is based on human experiments, and if this is the same in fish in not known.

Moreover, further investigation is needed to determine the good or bad nature an eventual antibody-phage complex. When antibodies bind to phages, they coat the phage surface. Antibodies induce phagocytosis of antigens by marking them to be more efficiently destroyed by the innate immune system, either directly or indirectly by complement activation (Ochs et al., 1993). If a phage is attached to a bacterium at the time of antibody coating an antibody-antigen complex would be made. It could be suggested that antibodies in such a bacteria-phage-antibody complex, promotes phagocytosis of the complex.

The pharmacokinetic involved in phage therapy of fish should be explored, as bacteriophage therapy of fish is a relevant alternative to use of antibiotics. For human phage therapy Payne and Jansen (2003) has developed protocols of dose and timing of treatment. They also showed that to early inoculation could result in reduced effect or total failure.

In contrast to these results, phage therapy experiments conducted by Nakai and Park gave different results. The ayu (*Plecoglossus altivelis*) treated with bacteriophages did not produce any antibodies after repeatedly receiving phage impregnated feed (Park et al., 2000, Nakai and Park, 2002, Park and Nakai, 2003). There were neither detected phage neutralizing antibodies in the ayu receiving intramuscular injections (Park and Nakai, 2003). The low immunogenicity of phages in this fish was considered an advantage for phage therapy in fish.

In humans and larger animals, we are used to think of phage therapy. However, the application of bacteriophages to water for bio sanitization purposes in closed systems for rearing of larva or in recirculation systems are highly relevant. The laws and regulations which are following each of these two ways of phage treatment are widely different. With therapeutic phage-products being regarded as a pharmaceutical product, strict laws and regulations must be followed. This is one of the main reasons for phage therapy is not being commercially available yet. When it comes to disinfection and bio sanitization the governments are more open minded. Lytic phages have already been approved and used for controlling bacterial infections in agriculture, meat and cheese production (Carlton et al., 2005, Lang, 2006, Lang et al., 2007, Balogh et al., 2008, Sabour et al., 2010, Svircev et al., 2010, Jones et al., 2012). For that reason, phage-based preparation might soon become approved and legal for use in aquaculture, maybe first as bio sanitization before therapeutically products.

Another essential point is using phages for bio control of water, or prophylaxis for infections will probably not create a significant immune response, having in mind that phages are ubiquitous in the aquatic environment. In contrast to this study where phages where injected to the fish, bacteriophages in the water will only interact with the mucosal immune system in the skin, gills or gut of the salmon. This will affect how the immune system will respond. Regarding the experiment with phage-impregnated feed, giving no antibody production (Nakai and Park, 2002). It is possible that the immune system of the fish gut, does not get immune stimulated by the ubiquitous phages. However, more research on this topic is needed.

It is important to note that phage immunized fish showed no sign of sickness or injury. The autopsy revealed no side effects or indication of a problem due to vaccine content and strong immune response. In summary, antigen administration seems to have no negative effect on fish welfare. Furthermore, there were no significant differences between the control fish and the fish injected with phages, when length, weight and hematocrit were considered.

Regarding possible changes in bacterial virulence caused by the bacteriophages. All bacteriophages and the cocktail used in this study have showed to be effective in lysing *Y. ruckeri*. It is important to use only lytic phages because lysogenic phages might induce virulence as shown by Flegel et al. (2005). Another problem is bacterial phage resistance. This can be avoided by using cocktails of several phages.

An advantage of bacteriophages is that they will not affect the necessary normal bacterial flora since highly bacteria specific phages can be used. The bacterial normal flora is especially important in closed systems where opportunistic bacteria are a big threat. Specific bacteriophages on the other hand, would not affect the normal bacterial-flora, but only infect their respective host bacteria. This makes bacteriophages a kind of beneficial probiotica that stabilizes the bacterial environment. In contrast to antibiotics that would affect a range of bacteria present.

All phages used in this study showed a bacteriostatic effect on *Y. ruckeri* cultures. This implies a high effectivity of phages to adhere to the bacterium and reducing their ability to form colonies.

The low dose (LD) cultures where bacteriophage and bacteria had a 1:1 ratio, three of the phage activity tests had a similar development in CFU. These were the LD cultures from bacteriophage φ 4, bacteriophage φ 9, and the bacteriophage cocktail. The low dose in their activity test were decreasing at every measuring time, indicating there was enough bacteriophages to lyse the bacteria and the phages where effective in lysing them. CFU of *Y. ruckeri* was much lower in HD phage exposure than the LD . This means that there were more killed bacteria in these cultures, this is logical due to the high number of phages in comparison to bacteria. When looking at the PFU counts at each of the individual phages there cannot be seen any cell bursts were the PFU count goes up like it does in the cocktail PFU curve. This might be explained by the binding epitope on the bacterium. In the cultures where there are

only one kind of bacteriophage they will bind to the bacterial phage epitope. Whereas the in the cocktail, where there are four kinds of bacteriophages, and these four phages as might not have the same epitope, all four could infect the same bacteria simultaneously. If this is the case, more phages would find a host cell to infect in a cocktail mixture of phages. The drop in the cocktail mix PFU curve is most likely due to the phages being at a stage where they are not yet able to infect new hosts, like when they have injected their genome but not yet made new infective progeny virions.

Conclusion and further perspectives

All phages tested in the study showed a bacteriolytic effect on *Yersinia ruckeri*.

The experiments where phage activity testing was performed were done in order to gain experience with this phage-bacteria system to further test the effect of bacteriophages on biofilm formation by *Y. ruckeri*. The phages tested can be used alone or in combinations as no major differences in bacteriolytic activity was observed. A bacteriostatic effect was observed by use of all phages or combinations in a certain time period.

Inactivation by UV-C irradiation was confirmed as an efficient way to inactivate the bacteriophages. If this is of any significance for the activity of bacteriophages after water treatment in the rearing system is not known.

We have produced salmon immune serum against the bacteriophage and ELISA has been established to measure specific anti phage antibodies. Immunsera have been obtained after exposure to high antigen (phage) load. The ELISA test can be used to evaluate if there is an antibody response in fish after exposure to a bacteriophage after therapeutic treatment or administration in water. An immunserum can serve as a positive control in ELISA and responses in analyzed fish can also be compared with this serum to evaluate any level of specific response.

The role of anti-phage antibodies if produced in fish after phage exposure is not known. Likely they can act in promoting opsonization of bacteria with phages and thus have a positive role. In this study we showed a phage neutralizing effect of anti-phage antibodies. It is worth to note that we used high amounts of specific antibodies. In a treatment procedure, the exposure of phages will be much lower and not injected and without adjuvant and we expect none or low levels of specific antibodies in the fish.

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7. Appenix

7.1 Recipes

- **LB- agar:**

Recipe for 1 liter Luria Bertani agar:

- *25g LB broth*
- *15g Agar-agar*
- *1 L Milli-Q water*

To make the LB-medium the LB broth powder was weight up and transferred into a 1L flask (Pyrex). The water was then poured into the flask. The powder was mixed into the water with a magnet stirrer. When the powder had dissolved in the water it was ready for autoclavation. Before the bottle was placed in the autoclave (TOMY SX-700E), the bottle cap was only half way sealed. The flask was then autoclaved for 15-21 minutes on 121°C. When the flask had cool down enough to be handled, it was poured into Petri dishes in a way that the whole bottom of the dish was covered.

- **LB-medium:**

Recipe for 1 liter Luria Bertani medium(LB-medium)

- *25g LB broth*
- *1,0L Milli-Q water*

The LB- medium was made the same way as LB-agar, only it was not poured on to petri dishes.

- **TYSG-agar:**

Recipe for 1 liter TYSG agar:

- *17g Tryptone*
- *3g Yeast extract*
- *5g Sodium Chloride*
- *2,5g Glucose*
- *15g Agar-agar*
- *1L Milli-Q water*

The TYSG-agar was made the same way as LB-agar

- **TYSG medium with SEAPlaque softagar with Ca^{2+}:**

Recipe for 1 liter TYSG medium with SEAPlaque softagar with Ca^{2+}:

- *17g Tryptone*
- *3g Yeast extract*

- *5g sodium chloride*
- *2,5g Glucose*
- *5g SEAPlaque*
- *10 ml Ca^{2+} 1M*
- *1L Milli-Q water*

The TYSG medium with SEAPlaque softagar with Ca^2 was made the same way as LB-agar.

- **TM-buffer:**

Recipe for 1L of TM-buffer solution.

- *100 mM NaCl*
- *10 mM MgCl$_2$*
- *10 mM CaCl$_2$*
- *10 mM Tris-HCl, pH 7,5*
- *1L Milli-Q water*

- ***5xPBS:***

Recipe for 4L 5xPBS

- *14,4g Na$_2$HPO$_4$x2H$_2$O*
- *5,4g KH$_2$PO$_4$*
- *170g NaCl*
- *4L Milli-Q water*

The pH was adjusted to 7,3 before it was autoclavated the same way as LB-medium.

- **1,5 M Tris-HCl**

Recipe for 150 ML 1,5 M Tris-HCl, Ph 8,8 (resolving)

- *27,23g Tris base*
- *80 ml Milli-Q*

The pH was adjusted to 8,8 with HCl, before Milli-Q water was added to a total volume of 150 ml.

0,5M Tris-HCl:

Recipe for 100 ml 0,5M Tris-HCL, Ph 6,8

- *6,0g Tris base*
- *60ml Milli-Q water*

The pH was adjusted to 6,8 with HCl, before adding Milli-Q water to a total volume of 100 ml.

10xElectrode (running) buffer

Recipe fir 1L 10xElectrode (running) buffer, pH 8,3

- ***30,3g Tris base***
- ***144g Glycine***
- ***10g SDS***

Dissolved in 1L Milli-Q water, pH was not adjusted

7.2 Poster

See page 65.

Bruk av bakteriofager i behandling mot bakterielle infeksjoner hos oppdrettsfisk

UNIVERSITY OF BERGEN

ACDPHARMA

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SAMMENDRAG

Bakterier forårsaker store sykdomsutbrudd, som medfører stor dødelighet og økonomiske tap i oppdrett av fisk og skalldyr. Den antibakterielle behandlingen som dette har medført har resultert i et massivt overforbruk av antibiotika for å få kontroll med infeksjonsproblemene og hindre spredning av smittestoff. Bruken av antibiotika er svært omstridt på grunn av bieffekter, påvirkning på den naturlige mikroflora og risiko for resistensutvikling. I tillegg er det nå en økende etterspørsel fra kunder etter «antibiotikafri» mat. Særlig utviklingen av resistente patogener har nå et stort internasjonalt fokus, både når det gjelder patogene bakterier for mennesker og dyr inkludert fisk. Det er derfor etterspørsel etter alternativer til antibiotika, og bakteriofager, virus som spesifikt infiserer bakterier, er et aktuelt alternativ. Bakteriofager infiserer bakterien, bruker den til å produsere nye bakteriofager og dreper vertsbakterien for å frigjøre nye bakteriofagpartikler. Smittestoffet blir på denne måten uskadeliggjort. Bakteriofager forekommer naturlig i alle miljøer på kloden. Vi har undersøkt bakteriolytisk effekt og reproduksjonsrate for ulike bakteriofager rettet mot den fiskepatogene bakterien *Yersinia ruckeri*.

MATERIALE OG METODER
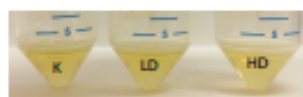
1. Vertsbakterien, *Yersinia ruckeri*, og *Y. ruckeri* bakteriofagene ble blandet ved MOI=1 (LD) eller med 100x overskudd av bakteriofager (HD). *Y. ruckeri* tilsatt sterilt TM medium ble brukt som kontroll (K). Blandede suspensjoner ble inkubert i 30 minutter i et lite volum (2 ml) for å la bakteriofagen adsorbere til vertsbakterien.

2. Bakterie/bakteriofag suspensjonene ble tilsatt 24 ml LB medium, deretter ble 1 ml suspensjon tilsatt hver brønn i 24-brønners Nunc brett. For hvert uttak av K, LD og HD samples 2 brønner. Første uttak er ved overføring til brønn, deretter uttak hver time i fem timer. Det ble også tatt ut prøver etter 24 timer.
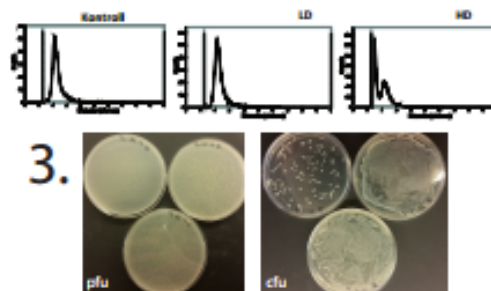
3. For hvert uttak ble bakterie antall og størrelse analysert i en Casy celleteller (Innovatis). Kolonidannende enheter (cfu) og plakkdannende enheter (pfu) ble talt i duplikater ved hvert uttak.

RESULTATER

Det var en tydelig bakteriolytisk effekt av alle testede bakteriofager (Fig. 1 A-C). Reproduksjonsraten for de tre bakteriofagene varierte i forhold til hverandre og i forhold til innblandingstiter (Fig. 2 D-F). Ved analyser 24 timer etter infeksjon var cfu/ml høyere enn ved 6 timer etter infeksjon for alle bakteriofager, her vist for bakteriofag #2 i figur 2. Ved analyser i Casy celleteller kunne infiserte/døde celler og ikke infiserte celler separeres visuelt ved at de har ulik størrelse. I celletelleren vil også en andel av cellene talt være døde, likevel oppnås god separasjon mellom K og LD/HD (Fig. 3). Det var en tydelig nedgang i antall celler for LD og HD brønner i løpet av perioden frem til 4,5t etter smitte (Fig. 4).
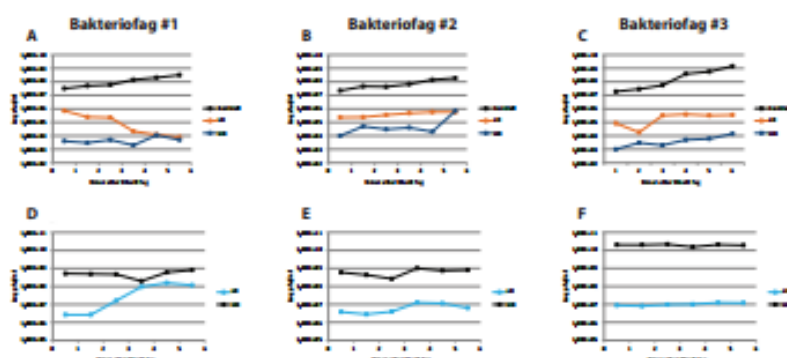

Fig 1: A-C: kolonidannende enheter over tid i brønner tilsatt bakteriofager og i kontrollbrønner. Sort= kontroll, blå = HD og orange = LD. D-F: Plakkdannende enheter over tid i brønner tilsatt ulike HD eller LD bakteriofager. Sort = HD, lys blå = LD. Kontroller tilsatt sterilt TM medium var negative.
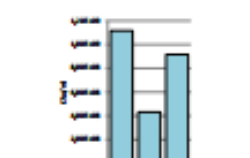
Fig 2: cfu/ml 24timer etter infeksjon med bakteriofag #2 for K, HD og LD brønner.
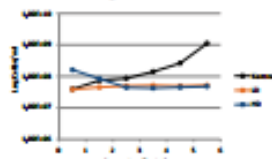
Fig 3: Antall celler/ml over tid for K (sort), HD (blå) og LD (orange) ved analyser i Casy celleteller for bakteriofag #1.
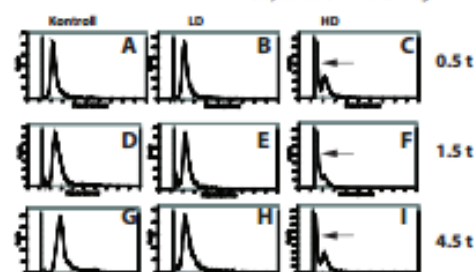
Fig 4: Antall bakterier i forhold til størrelse analysert i Casy celleteller for bakteriofag #1 ved tidspunktene 0,5t, 1,5t og 4,5t etter innokulasjon. A,D og G = Kontroll. B, E og H = LD. C, F og I = HD.

OPPSUMMERING:

De analyserte *Y. ruckeri* bakteriofagene viser god lytisk effekt på vertsbakterien. Videre analyser vil vise om en andre gangs tilsetning av bakteriofager vil hindre økningen i bakterieantall her observert etter 24 timer. Analyser av effekten av andre bakteriofager med samme vertsbakterie samt en coctail som inneholder flere bakteriofager er pågående. Når denne smittemodellen av bakterier er etablert kan den videre benyttes til å undersøke effekten av bakteriofager i kulturer med fiskelarver smittet med *Y. ruckeri* og bakteriofagenes effekt på biofilm av bakterien *Y. ruckeri*.