Challenge model for *Pasteurella atlantica* genomovar *salmonicida* in Atlantic salmon (*Salmo salar* L.)

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Abstract

Pasteurellosis in Atlantic salmon (*Salmo salar* L.*)* has become a problem in Norway the last couple of years. In the fish health report of 2021, the Norwegian Veterinary Institute (NVI) classified pasteurellosis in Atlantic salmon as an established bacterial disease for the first time. In 2021 45 outbreaks of pasteurellosis in Atlantic salmon was reported. The disease has also been diagnosed in lumpsucker (*Cyclopterus lumpus* L.) since 2012. Though the causative agent of disease in the two fish species has been classified as belonging to two different genomovars and the NVI have suggested the working nomenclature *Pasteurella atlantica* genomovar *salmonicida* for the salmon isolates and *Pasteurella atlantica* genomovar *cyclopteri* for the lumpsucker isolates.

In this project the main aim was to perform a challenge experiment to establish a reproducible challenge model for pasteurellosis in Atlantic salmon. Progression of disease was evaluated through observations of gross pathology, histopathology, qPCR and re-isolation of the bacteria. Possible difference in virulence between three isolates of *P. atlantica* genomovar *salmonicida* was evaluated and if an acute stress exposure influenced the mortalities or disease progression.

For the experimental challenge different infection routes were examined, administration through intraperitoneal (i.p.) injection and cohabitation and challenge by bath also including cohabitation. Mortality was obtained in all groups but with varying results on cumulative percent mortality, and in general the mortality rates obtained were low.

During the challenge experiment *P. atlantica* genomovar *salmonicida* was reisolated from all challenge groups. A qPCR assay for identification of *P. atlantica* geomovar *salmonicida* was established and used to analyse anterior kidney samples collected throughout the experimental period. This analysis identified the presence of *P. atlantica* genomovar *salmonicida* in both dead fish and in sampled fish. Gross pathology previously described for fish diagnosed with pasteurellosis in fish farms was reproduced after experimental challenge, and histopathological analysis confirmed the presence of bacteria in tissue samples form areas with macroscopical signs of disease.

No difference in virulence between the isolates tested were seen in the present study, and the acute stress exposure did not influence the mortality of the Atlantic salmon.

Sammendrag

Pasteurellose i Atlantisk laks (*Salmo salar* L.) har blitt et problem i Norge de siste årene. I fiskehelserapporten fra 2021 karakteriserte Veterinærinstituttet (VI) for første gang pasteurellose i Atlantisk laks som en etablert bakteriell sykdom. I 2021 var det innrapportert 45 utbrudd av pasteurellose i Atlantisk laks. Pasteurellose har blitt diagnostisert i rognkjeks (*Cyclopterus lumpus* L.) siden 2012. Bakteriene som forårsaker sykdom i Atlantisk laks og rognkjeks er blitt karakterisert som ulike genomovarer og VI har foreslått arbeidsnomenklaturen *Pasteurella atlantica* genomovar *salmonicida* for lakseisolatene og *Pasteurella atlantica* genomovar *cyclopteri* for rognkjeks-isolatene.

I dette prosjektet var hovedmålet å utføre et smitteforsøk for å etablere en reproduserbar smittemodell for pasteurellose hos Atlantisk laks. Progresjon av sykdom ble evaluert gjennom observasjoner av patologi, histopatologi, qPCR og re-isolering av bakteriene. Mulig forskjell i virulens mellom tre isolater av *P. atlantica* genomovar *salmonicida* ble evaluert i tillegg til om en akutt stresseksponering påvirket dødeligheten eller sykdomsprogresjonen.

Ulike infeksjonsveier ble undersøkt, administrering gjennom intraperitoneal (i.p.) injeksjon og kohabitering og administrering via bad også inkludert kohabitering. Dødelighet ble oppnådd i alle grupper, men med varierende resultater på kumulativ prosent dødelighet, og generelt var dødeligheten som ble oppnådd lav.

Under smitteforsøket ble *P. atlantica* genomovar *salmonicida* reisolert fra alle smittegruppene. En qPCR-analyse for identifikasjon av *P. atlantica* geomovar *salmonicida* ble etablert og brukt til å analysere vev fra fornyre samlet gjennom forsøksperioden. Denne analysen identifiserte tilstedeværelsen av *P. atlantica* genomovar *salmonicida* i både død fisk og i prøvetatt fisk. Patologi som tidligere er beskrevet for fisk diagnostisert med pasteurellose i oppdrettsanlegg ble reprodusert etter eksperimentell smitte, og histopatologisk analyse bekreftet tilstedeværelsen av bakterier i vevsprøver fra områder med makroskopiske tegn på sykdom.

Det ble ikke funnet forskjell i virulens mellom isolatene som ble testet i denne studien, og den akutte stresseksponeringen påvirket ikke dødeligheten til Atlantisk laks.

Abbrevations

Abbreviation	Clarification:
°C/sec	Degrees Celsius per second
BA	blood agar
bact/fish	Bacteria per fish
bact/mL	Bacteria per millilitre
bp	Base pear
cfu	Colony forming units
cfu/mL	Colony forming units per millilitre
Cohab	Cohabitant
Ct	Cycle time
DNA	Deoxyribonucleic acid
FCS	Foetal calf sera
g	Gravitational force
h	hour
HE	Hematoxylin and eosin
i.p.	Intraperitoneal
ILAB	Industrial and Aquatic Laboratory
MLSA	Multilocus sequence analysis
NaCl	natrium chloride
NTC	Non template control
NVI	Norwegian Veterinary Institute
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PO	Production sone
qPCR	Quantitative Polymerase chain reaction
R^2	Coefficient of determination
rpm	Round per minute
SD	Standard deviation
sec	Seconds
TSB	tryptic soy broth
VIE	Visible implant elastomer
dpi	days past infection
dpc	days past challenge
BLAST	Basic Local Alignment Search Tool
O_2	Dioxygen
µL/fish	Microliter per fish
ng/ µL	Nanogram per microliter
rRNA	Ribosomal ribonucleic acid
log	Common logarithm
i.p.-S	Intraperitoneal shedder
i.p.-C	Intraperitoneal cohabitant
B-S	Bath shedder
B-C	Bath cohabitant

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1. Introduction

1.1 Aquaculture in Norway

Pasteurellosis has in recent years been established as a disease occurring in Atlantic salmon farmed in production sone (PO) 2 – 5 (Figure 1) in Norway. Currently there are no licenced vaccines to protect against pasteurellosis in Atlantic salmon (*Salmo salar* L.) making it a huge welfare and economical issue for the farming industry (Sommerset *et al.*, 2021, 2022).

Over the decades the Aquaculture industry has faced threats from different infectious diseases which has challenged its sustainability. The nature of salmon farming with many fish concentrated within a small area has created the possibility of having large outbreaks of disease and thus may lead to a change in the virulence of pathogens, making them more pathogenic (Fraslin *et al.*, 2020).

Historically, outbreaks of disease led to an unsustainable use of antibiotics to combat bacterial pathogens. In the late 1980s and early 1990s the amount of active substances of antibiotics prescribed each year varied from around 20 000 kg to 50 000 kg (Sommerset *et al.*, 2005). The use of antibiotic has since the early 1990s been drastically reduced, and in 2020 the total amount of active substance of antibiotics prescribed was 230 kg (Sommerset *et al.*, 2021). This remarkable reduction has largely been credited to the development of highly effective vaccines.

Knowledge is needed on the pathogenesis and pathology of fish suffering from pasteurellosis, and on possible virulence differences across isolates of the causative agent *Pasteurella atlantica* genomovar *salmonicida.* A challenge model must be established to answer these questions and to enable the efficacy of future vaccines, which was the major scope of this thesis.

1.2 Pasteurellosis

Pasteurellosis in Atlantic salmon has gone from being considered an emerging disease in 2020 (Sommerset et al., 2021) to become an established bacterial disease in 2021 by the Norwegian Veterinary institute (NVI) (Sommerset *et al.*, 2022). The disease is caused by the bacterium *P. atlantica* genomovar *salmonicida.* It is distinct from pasteurellosis caused by *Photobacterium damselae* subsp. *piscicida,* a disease found in fish in warmer climates, such as yellowtail (*Seriola quinqueradiata,* (Temminck and Schlegel)) and rudd (*Scardinius erythrophthalmus* L.) (Romalde, 2002). The term pasteurellosis also describes diseases generally caused by

Pasteurella bacteria such as *Pasteurella multocida* in humans, and *Pasteurella skyensis* in Atlantic salmon (Birkbeck *et al.*, 2002)

P. atlantica genomovar *salmonicida* is not the only relevant *Pasteurella* bacterium for the Norwegian aquaculture industry. The two other relevant bacteria are *P. atlantica* genomovar *cyclopteri* affecting lumpsucker and *P. skyensis* affecting Atlantic salmon. These bacteria are genetically similar, but distinct from each other (Alarcón *et al.*, 2016; Ellul *et al.*, 2021).

The bacterium *P. atlantica* genomovar *salmonicida* has been morphologically described, as a gram-negative small rod, with a size between 1.7 to 3.5 μm, that is non-motile without a flagellum. On blood agar (BA) with 2 % NaCl, the colonies are small with a size of about 1 mm, convex in shape and are grey or appear colourless (Valheim *et al.*, 2000; Legård and Strøm, 2020).

1.3 Pasteurellosis in farmed fish in Norway

The first known case of pasteurellosis in farmed Atlantic salmon in Norway was recorded in 1989. At the time the causative pathogen for the disease was unknown. The disease outbreak lasted for the winter of 1989 and 1990 and reoccurred at the farm the following two winters. The pathology of the disease gave inspiration for the suggested name “Varracalbmi”, a Sami name for bloody eye. This pathology included panophthalmitis that left the eyes haemorrhagic and with necrotizing inflammation (Valheim *et al.*, 2000). After this, pasteurellosis in Atlantic salmon occurred sporadically until the spring of 2018, when the number of cases started to increase (Legård and Strøm, 2020; Sandlund *et al.*, 2021; Sommerset *et al.*, 2021). In total 7 cases of pasteurellosis were identified in 2018, the affected fish had a weight ranging from 1.5 to 4 kg, and the farms experienced varied mortality levels and gross pathology during disease outbreaks (Hjeltnes *et al.*, 2019).

Pasteurellosis is not a disease notifiable to the authorities, meaning the true extent of the problem cannot be truly verified. Nevertheless, the NVI has tried to get more reliable data of the scope of the problem. In their Fish Health Report of 2020 (Sommerset *et al.*, 2021) and 2021 (Sommerset *et al.*, 2022) numbers of reported outbreaks from private laboratories are also included, thereby getting more reliable information on disease outbreaks for 2020 and 2021 compared to previous years. A total of 45 registered outbreaks of pasteurellosis were reported

in farmed Atlantic salmon in 2021, while in 2020 the number of registered outbreaks were 57, as compared to 14 outbreaks in 2019.

The report from 2020 presents a summary of a questionnaire answered by aquamedicine biologists and veterinarians involved in farmed fish welfare. Results of the questionnaire show that pasteurellosis was ranked as the 10th greatest problem nationally, with around 25 % of the participants indicating that pasteurellosis gave reduced welfare and increased mortality, respectively. Further 12.7 % and 30.9 % indicated that the disease resulted in reduced fish growth and that the prevalence of pasteurellosis had an increased. The disease has been established in PO 2 to 5, see Figure 1, from Ryfylket to Hustadvika, with most cases detected in PO 3 from Karmøy to Sotra. PO 3 was also identified as a problem area in the questionnaire, reflecting the severity of this disease and following welfare issues if allowed to spread along the Norwegian cost (Sommerset *et al.*, 2021).

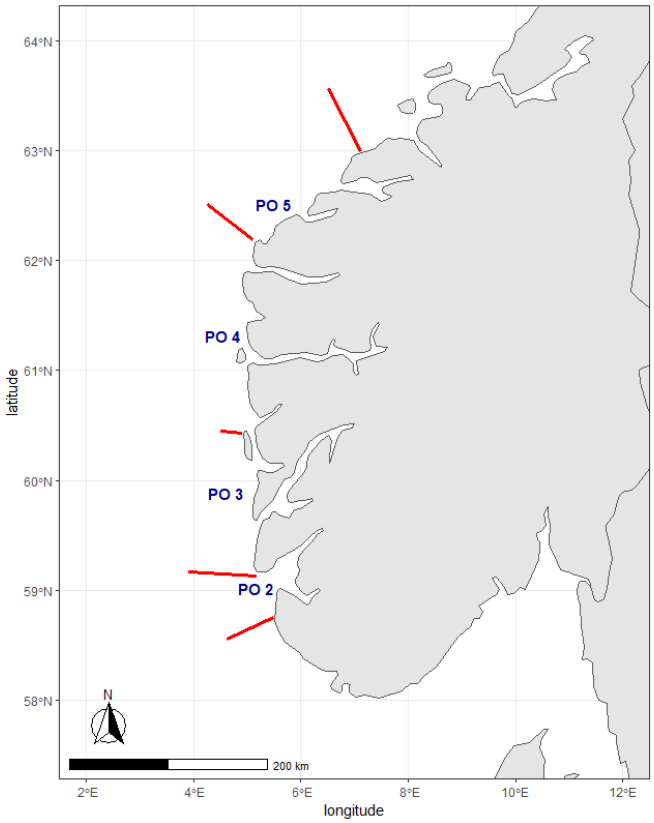

Figure 1 Map showing production sone 2 to 5 at the west cost of Norway. The red lines on the map reflects the borders between the production sones for Atlantic salmon farming. The different sones are marked PO 2-5.

Pasteurellosis has for some years been a major problem for farming of lumpsucker (*Cyclopterus Lumpus* L.) with the causative agent being *P. atlantica* genomovar *cyclopteri*. The first identification of pasteurellosis in lumpsucker was registered in 2012, with the number of confirmed cases steadily increasing over time (Sommerset *et al.*, 2021, 2022). The NVI reposted in the Fish Health Report of 2020 (Sommerset *et al.*, 2021) 36 registered cases from lumpsucker in 2020 as compared to 10 only in 2019. In 2021, only 6 cases were confirmed (Sommerset *et al.*, 2022). It has been shown that lumpsucker also can contract the salmon variant of *P. atlantica* genomovar *salmonicida* (Sandlund *et al.*, 2021).

1.4 Cohabitation and bacterial genetic similarities

From the start of the salmon aquaculture industry in Norway in the 1970s the salmon louse (*Lepeophtheirus salmonis,* (Kroyer)) has been a problem. The high intensity method of farming within the industry has inadvertently created good conditions for salmon lice (Torrissen *et al.*, 2013). The high number of delousing treatments using chemotherapeutics carried out each year has created resistance in the salmon lice to the chemicals in use. This has led to a need for new methods for fighting salmon lice, including biological delousing by using cleaner fish (Torrissen *et al.*, 2013; Sandlund *et al.*, 2021) and mechanical delousing (Torrissen *et al.*, 2013; Legård and Strøm, 2020). The efficiency of mechanical treatments on fish has not always been documented properly as the treatment is not medical and thus not governed by the same regulations. Experimental data from thermal delousing has brought up questions on the treatment and potential harm the fish are exposed to (Moltumyr *et al.*, 2021).

Biological delousing using cleaner fish are used as a prophylactic approach to keep the number of salmon lice down (Imsland *et al.*, 2014). The cleaner fish typically used are lumpsucker, and different species of wrasse (Alarcón *et al.*, 2016). According to the Directorate of Fisheries official statistics regarding the use of cleaner fish in the salmonid aquaculture, a total of 51 million cleaner fish were used in 2020. Out of this, 34 million were lumpsuckers (Fiskeridirektoratet, 2021).

The first applications of biological delousing in Norwegian fish farming were back in the 1980s with wrasses. However, the use of cleaner fish did not become more prevalent until the onset of more widespread resistance in salmon lice against chemotherapeutics. Lumpsucker was later introduced to aquaculture as a more favourable cleaner fish species compared to wrasses and a

shift in focus towards this fish species occurred in the early 2010s (Sveier and Breck, 2018). After the introduction of lumpsucker several bacterial diseases that affected the species were identified. Amongst them were pasteurellosis caused by a then unknown *Pasteurella* species (Alarcón *et al.*, 2016; Ellul *et al.*, 2019).

After the initial identification of pasteurellosis in lumpsuckers in May 2012, the number of confirmed outbreaks increased. It was investigated whether the causative agent was not only in the same genus, but also the same species as the previously known salmon isolate causing "Varracalbmi". When compared, the isolates from lumpsucker appeared phenotypically similar, though appeared biochemically less reactive. There were also pathological similarities between infected Atlantic salmon and lumpsucker, however lumpsucker did not show severe eye pathology as could be seen for Atlantic salmon. Genetical differences were present between the lumpsucker isolates and "Varracalbmi" isolates, though in the initial investigations it was not possible to determine if the isolates were host specific or not (Alarcón *et al.*, 2016).

A genetic difference between the different variants has been confirmed by the NVI by whole genome sequencing of more than 80 different isolates of *Pasteurella*. When genetic variants for Norwegian Atlantic salmon and lumpsucker were compared, it was concluded that the genetic differences were enough to differentiate between the salmon variants and the lumpsucker variants. Nevertheless, the genetic differences were not large enough to categorise the lumpsucker variants and the Norwegian Atlantic salmon variants into different species. Based on this analysis nomenclature was proposed to differentiate the variants: *P. atlantica* genomovar *salmonicida* for the Atlantic salmon isolates and *P. atlantica* genomovar *cyclopteri* for the lumpsucker isolates. The *P. atlantica* genomovar *salmonicida* variants are distinct from *P. skyensis*, manly found in farmed salmon in Scotland however this bacterium caused one outbreak of pasteurellosis in Norwegian salmon in 2020. The same whole genome sequencing analysis determined that the salmon variant from 2018 onwards could be found in one cluster based on multilocus sequence analysis (MLSA), however the isolates that caused "Varracalbmi" showed more diversity (Gulla *et al.*, 2020).

Furthermore, in an experiment by Sandlund *et al.*, 2021 they tested the suseptebility of Atlantic salmon and lumpsucker to *P. atlantica* genomovar *salmonicida* isolates and a *P. atlantica* genomovar *cyclopterid* isolate. In this trial the lumpsucker showed clinical signs of pasteurellosis from infection with both isolates, showing that lumpsucker was susceptible to both isolates. Clinical signs or pathology, however, was not found for Atlantic salmon.

1.5 Pasteurellosis in Atlantic salmon

The clinical manifestations of pasteurellosis in Atlantic salmon may vary from non to exophthalmia and abscesses in the skin. Furthermore, both the prevalence of affected fish and the severity of disease can vary to a great extent between affected farms (Legård and Strøm, 2020; Sandlund *et al.*, 2021).

From the first known outbreak of pasteurellosis in Atlantic salmon in 1989, the recorded mortality was 2.5 % and the fish typically died 4 to 6 weeks after the first clinical signs appeared (Valheim *et al.*, 2000). For the outbreaks recorded from 2018 onwards, the mortality levels have varied from outbreak to outbreak. This variation ranges from pasteurellosis found under routine necropsy to cases with significant increase in mortality in the net pens (Legård and Strøm, 2020).

In addition to the variation in mortality, there is also a variation in the reported clinical signs and the number and severity of pathology from outbreak to outbreak. The reason for this variation is unknown with a possibility of virulence differences from strain to strain (Legård and Strøm, 2020; Sandlund *et al.*, 2021).

Although clinical and patological signs assosiated with pasteurellosis in Atlantic salmon at fish farms has been reported to differ from outbreak to outbreak Legård and Strøm, 2020, reported that purulent peritonitis, ascites in the cardiac cavity and exophthalmos were typical gross pathology. Although this has been the most frequent findings in the field, other signs such as skin wounds and abscesses in the muscle have also been reported. The fins of the fish have also been affected by the disease with observations of wounds on the base of the pectoral fins and fistula at the same spot.

1.6 Challenge models

The establishment of reliable and reproducible challenge models for diseases is important for pathogenesis studies and for development of prophylactic and treatment methods. One of the important prophylactic methods that needs a challenge model to show effectiveness is vaccines. There are different types of challenge protocols including injection-based, bath -based and cohabitation-based models. Injection-based protocols are easier to control and standardise,

though bath and cohabitation-based protocols are considered better protocols as these mimics a natural exposure and infection route for pathogens (Gudding, 1997; Adams, 2019).

Using injection protocols, either intra-peritoneal or intra-muscular injection, has the benefit of being easy to control regarding infection dose and they are often reproducible. Every fish gets the same infection dose, so the method ensures that all fish gets infected. The disadvantage with this type of protocol is that it does not represent a natural route of infection for fish and the method evades the immune responses in the skin and mucus membranes. Bath- and cohabitation protocols mimic a more natural way of infection and ensures that the mucosal and other outer surfaces have a chance to protect the fish. In a bath challenge fish is exposed to the pathogen through the water for a designated amount of time. A cohabitation challenge consists of artificially infecting fish, shedders, that are housed together with non-infected or naïve fish. These types of protocols are more difficult to standardise, and the infection dose that each individual cohabitant fish is exposed to is harder to control (Gudding, 1997).

1.7 Ethical reflections

The challenge experiment conducted to establish a challenge model for pasteurellosis in Atlantic Salmon was applied for to the Norwegian Food Safety Authorities and designated the approval identification Id: 27748.

As described in "Forskrift om bruk av dyr i forsøk" all animals used for research shall be protected against unnecessary suffering. One of the guiding principles in the regulations that promises to uphold the protection against unnecessary suffering can be found in § 9, this is the 3-R principals, replace, reduce, and refine (Lovdata, 2015). Early humane endpoints were implicated for the fish in the challenge trial.

As Pasteurellosis of Atlantic salmon has increasingly become a problem in the last couple of years, this has sparked an interest in research on the disease and possible prophylactic measures. By using utilitarian ethics, doing this type of research can therefore be justified for the betterment of the health of farmed Atlantic salmon. Nevertheless, the suffering of each individual fish used in the challenge should not be ignored and should be minimised.

The goal of the challenge experiment was to evaluate the susceptibility of Atlantic salmon to the different isolates of *P. atlantica* genomovar *salmonicida*. Therefore, it was not possible to

replace live animals with alternative methods not utilizing animals. To study the susceptibility to a pathogen and the disease progression in a specific animal the animal has to be exposed to the pathogen. The number of fish used in the challenge experiment was reduced to the minimum level that was necessary to secure a normal dynamic between the fish in the tanks, and to have enough fish for the planned samplings. In addition, it was important to secure an equal infection pressure between the tanks and a sufficient infection dynamic in the tanks.

To refine the model, it was ensured that qualified personnel performed daily monitoring of the fish. Dead/moribund fish, and fish having reached the predefined humane endpoints were removed from the tanks and euthanized. Water quality parameters such as temperature, salinity, water flow and oxygen levels, were monitored daily.

1.8 Amins for the study

With the increased numbers of outbreaks of pasteurellosis in Atlantic salmon, the need for more knowledge on the disease is essential. Among the knowledge needed is route of infection and the disease progression in the fish. Furthermore, it is important to uncover potential differences in virulence of different isolates of the bacteria (Legård and Strøm, 2020; Sandlund *et al.*, 2021; Sommerset *et al.*, 2021).

Development of methods to control the spread of pasteurellosis such as vaccines and other prophylactic or treatments depends on the establishment of a reproducible challenge model. This will make it possible to potentially develop a vaccine and measure its effectiveness in preventing disease and possibly prevent spread of infection.

In this master project the aims were:

- To perform an experimental challenge study to establish a reproducible challenge model for pasteurellosis in Atlantic salmon.
- To study the disease progression after experimental challenge, using real-time PCR, histopathology and bacteria re-isolation.
- To evaluate possible differences in virulence among different isolates of *P. atlantica* genomovar *salmonicida.*
- To evaluate if exposure to stress will influence on disease progression and mortality.

2. Materials and methods

2.1 Bacteria

For this challenge experiment three different bacteria isolates of *P. atlantica* genomovar *salmonicida* was used. These isolates had previously been isolated from clinically sick Atlantic salmon during outbreaks of pasteurellosis. An overview over the three different isolates is shown in Table 1.

Table 1 *P. atlantica* genomovar *salmonicida* isolates used in the challenge experiments, showing year of isolation, name, and county of origin and abbreviation.

Isolate name	Isolated from	Year	Origin	Abbreviation
PaL-1UiB2019	Atlantic salmon	2019	Vestland county	PaL1
PaL-2UiB2020	Atlantic salmon	2020	Vestland county	PaL2
PaL-3UiB2020	Atlantic salmon	2020	Vestland county	PaL3

2.1.1 Cultivation of bacteria

The bacteria were cultured in tryptic soy broth (TSB, BD Bacto™ BD Diagnostic Systems, Lot No 9302047) supplemented with 0.5 % NaCl (Honeywell, Fluka, LotNo L2180) and 10 % (v/v) Foetal Calf Sera (FCS) (Gibco, Lot No 2094466RP).

Cultures of bacteria were grown in 50 ml tubes (Sarstedt AG & CO. AS) incubated in a shaking incubator (INFORS AG CH-4103 BOTTMINGEN) at 200 rpm and 20 °C for 24h. For this purpose, 1 mL glycerol stock bacteria were supplied to 40 mL liquid growth medium. These bacteria stocks had previously been grown under identical conditions. They had been incubated in growth medium over night at 20 °C with 200 rpm and had been harvested in the late exponential phase. The glycerol stock consisted of bacterial culture and glycerol (>99 %) in a 4 to 1 ratio and were stored at – 80°C.

2.2 Bath and i.p. challenge

2.2.1 Fish and rearing conditions

The salmon, produced at the Industrial and Aquatic Laboratory (ILAB), were transported to the challenge unit at ILAB and given a period of two weeks for acclimatization prior to onset of the challenge. The water in the tanks had a temperature of 12 °C, salinity was set to 34 ‰, the O_2 level was set to above 77 % saturation in the outlet water. The light regime was 12:12 light on:off. The fish were fed a commercial dry feed "Nutra Olympic 3 mm" (Skretting) according to appetite by automatic feeders. The fish were starved for 24h prior to challenge and prior to sampling.

The fish were monitored daily, and after challenge they were monitored twice a day. At start of the experiment average weight of the fish was 99.1 g (±17.3 SD) and the length was 20.7 cm (±1.0 SD). Each tank held 50 fish and had a water volume of 150 L.

2.2.2 Preparation of challenge material

Bacteria cultures for use in the experiment was grown as described in section 2.1.1. The bacteria cultures were harvested after 24h, at late exponential growth phase, and centrifuged at 2 500 x *g* for 15 min at 4 °C in a Beckmann coulter allegra x-15R centrifuge.

The supernatant was gently pipetted off, leaving the pellet consisting of bacteria in the 50 mL tubes (Sarstedt AG & CO. AS). The bacteria were resuspended in in 30 mL PBS (Lonza, BioWhittaker, Lot N° 7MB119). All bacterial resuspensions for each isolate were collected in one sterile collecting bottle per isolate.

The concentrations of bacteria were measured in a CASY cell counter (Inovatis) in a suspension of 10 mL CASY-ton (Inovatis, LOT 177001) supplemented with 10 µL of the bacterial suspension.

The concentration of bacteria for the bath-challenge was $5*10^6$ bact/mL in 75 L water. The PSB (Lonza) suspensions with the different bacterial isolates were diluted in PBS (Lonza) to a concentration of $1*10^9$ bact/mL. A volume of 375 mL bacterial suspension was supplied to the fish tanks subject to bath challenge (calculated using Formula 1).

$$c_1 \cdot v_1 = c_2 \cdot v_2 \qquad \text{Formula 1}$$

The bacterial suspension for intraperitoneal i.p. injection had a concentration of $5*10^5$ bact/mL, of this material 100 µL were injected to each fish giving a dose of $5*10^4$ bact/fish. The PBS (Lonza) suspension of all three isolates were first diluted to a concentration of $1*10^7$ bact/mL through tenfold dilution of the $1*10^9$ bact/mL suspension. Further, the $1*10^7$ bact/mL suspensions were diluted to $5*10^5$ bact/mL using Formula 1.

To control the number of living bacteria a 100 µL sample of each of the three isolates were taken from the PBS suspension at a concentration of $1*10^9$ bact/mL and used in a dilution series with a tenfold per step. From dilution -6 with a concentration of 10^3 bact/mL and dilution -7 (10^2 bact/mL), (Figure 2), 100 µL was extracted and plated on blood agar (BA) (2 % NaCl) and incubated at 15 °C until colonies formed before colony forming units / ml (cfu/mL) were calculated.

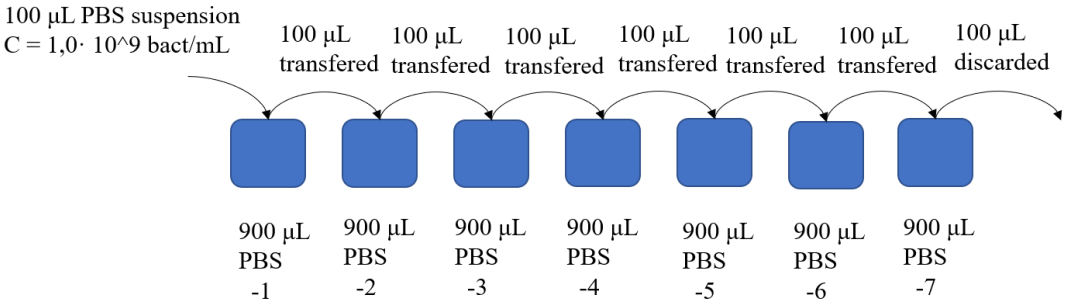

Figure 2 Dilution steps of bacterial suspensions in PBS for control of growth and plating on BA to calculate cfu/mL of bacteria used for experimental challenge of the salmon.

For the tank containing control fish for the bath-challenge 375 mL sterile PBS (Lonza) was prepared and supplied to the tank (75 L). For fish in control tanks for i.p. injection sterile PBS (Lonza) was used for injection in the i.p. injected fish group, 100 µL/fish.

2.2.3 Challenge by bath and cohabitation

Prior to challenge half of the fish in the tanks (n=25) were transferred to separate holding tanks. The bath-challenge was conducted by lowering the water level in the tanks to 75 L. Tanks 1 to

4 was used for the bath-challenge (Figure 3). Fish in tank 1 was exposed to PaL3, fish in tank 2 was exposed to PaL2, fish in tank 3 was exposed to PaL1, and fish in tank 4 was exposed to sterile PBS (Lonza) as a control. The bath-challenge fish was exposed to the bacterial suspension for one hour. During this time compressed air was constantly supplied to the water to ensure a sufficient level of oxygen in the water. To the tanks holding the cohabitants, to be supplied to the challenge tanks after 1h, 0.5 mL Aqui-S (MSD Animal Health) was used as a sedative to calm the fish. The holding tanks of cohabitant fish contained a water volume of 121 L in holding tank 1 and 109 L in holding tank 2.

After one hour the water supply was re-instated and the infectious material was washed out. The fish held in the holding tanks were identified using Visible Implant Elastomer (VIE) tags set subcutaneously above the left eye before they were returned to their respective tanks.

Figure 3 Challenge and tank setup. The figure shows the setup of the challenge experiment. Each tank (blue square) is marked with a tank number (Tank 1-8), the model of challenge and isolate used for challenge, the number of fish in each group and which (if any) markings the fish group had.

2.2.4 Challenge by i.p. injection and cohabitation

In tanks 5 to 8 (Figure 3), the fish was divided into groups; half of the fish were naïve cohabitants that during the procedure did not leave the tanks. Fish that were to be i.p. injected with challenge material (shedders) were gently scooped out of the water with a hand net and anesthetised in 15 L water using 1.5 g Finquel Vet (MSD Animal Health). The fish were i.p. injected with a dose of 100 µL of the infectious material and then marked with a green VIE mark set subcutaneously over the eye. Control fish from tank 5 were injected with 100 µL of sterile PBS (Lonza).

2.2.5 Exposure of the fish to acute stress

Fish form tanks 1 to 4 were subject to experimental stress by handling 6 weeks post challenge. The stressor used were confinement by reducing water levels to 75 L and netting the fish using a fine-masked net for 2 min, repeated 2 times.

2.3 Water samples

Pre challenge a water sample was collected form the excess water hose to serve as a control. One litre was sampled into a sterilized glass flask.

Post challenge, water samples were collected Wednesday mornings before feeding, between 08:00 and 8.15 am. From each tank one litre of water was sampled into sterilized glass flasks. Water was sampled from the upper part of the water column, including surface water, from the opposite side of the water inlet. The sampled water was filtered using vacuum filters with pore size 0.45 µL (Thermo Fisher Scientific). The filters were stored in 5 mL tubes (Axygen, Corning Incorporated) at – 20 °C until further processing.

2.4 Sampling of fish

2.4.1 Scheduled sampling of fish

Sampling of fish was done on a regular basis (Table 2). At each sampling the weight and length of the fish were measured. Any clinical and pathological signs were registered. Tissue from heart and anterior kidney was sampled for qPCR analysis and stored at – 20°C on RNAlater (Sigma, Lot MKCB4095) for analyses at a later stage. Histology samples was collected from the kidney, heart, and spleen and preserved on formalin, 4 % formaldehyde (VWR chemicals, Lot 20K164130) for further processing on a later stage. Bacteriological sampling for growth on BA with 2 % NaCl was aseptically taken from the anterior kidney using an inoculation loop. The plates were incubated for up to 14 days and examined for growth after 4 days, 7 days and 14 days at 15°C. In case of severe pathology affected organs were also sampled.

For each sampling, 2 fish per group in each tank was sampled. Except for sampling 4, were no i.p. shedders were sampled.

Table 2 Timeline of the challenge experiment and samplings, presenting an overview of when different procedures were caried out.

Week post challenge	Wednesday	Thursday
0	Water sample, -1 dpc	Challenge
1	Water sample, 6 dpc	Sampling 1, 7 dpc
2	Water sample, 13 dpc	Sampling 2, 14 dpc
3	Water sample, 20 dpc	
4	Water sample, 27 dpc	Sampling 3, 28 dpc
5	Water sample, 34 dpc	
6	Water sample, 41 dpc	Sampling 4, stressing fish and water sampling, 42 dpc
7	Water sample, 48 dpc	Sampling 5, 49 dpc
8	Water sample, 55 dpc	
9	Water sample, 62 dpc	Sampling 6, end challenge, 63 dpc

2.4.2 Sampling of moribund and dead fish

Moribund fish and fish that died throughout the experiment, was either stored at – 20 °C until further processing or necropsied and sampled immediately. For immediate sampling the weight and length of the fish was registered. Any clinical and pathological signs were registered. Tissue from heart and anterior kidney was sampled for qPCR analysis and stored on RNAlater (Sigma,

Lot MKCB4095). Bacteriological sampling for growth on BA (2 % NaCl) was collected from the anterior kidney. For moribund fish histology samples were also collected, this included heart, kidney, and spleen, if other organs showed pathology, these were also sampled and stored on formalin, 4 % formaldehyde (VWR chemicals, Lot 20K164130).

Fish, initially stored at – 20 °C, was defrosted at room temperature for approximately 60 min and bacteriology samples was taken from the anterior kidney for growth on BA (2 % NaCl).

2.4.3 Termination of the challenge trial

For the termination of the challenge trial the remaining fish in each group, after end sampling, was control counted and up to 10 fish per group were sampled for bacteriology from anterior kidney. The fish was euthanised with an overdose of Finquel Vet. (MSD Animal Health).

2.4.4 Histopathology

Samples collected for histology from scheduled sampling dates and from moribund fish was fixed in formalin, 4 % formaldehyde (VWR chemicals, Lot 20K164130). Spleen was sampled as a whole organ, gill arch number 2 on the left side was sampled for gills, liver was sampled as a cube of 0.5 mm on each side, for kidney a rectangular sample of 0.5 mm x 0.2 mm was sampled. For other tissues samples were always less than 1 cm thick. This resulted in 196 fish sampled at regular samplings and 6 moribund fish sampled for histology. The samples collected from fish before onset of the challenge experiment ("zero-samples"/ background samples, n=10) were gills, liver, and heart. For sampled fish throughout the challenge experiment heart, kidney and spleen were collected. Furthermore, due to observed gross pathology, fins were sampled from two fish, gills from one fish, liver from 4 fish, abscess in muscle from 4 fish, and from one fish the nasal cavity, gills, jawbone and pseudobranch were sampled. Samples taken from moribund fish were heart, spleen, and anterior kidney. In addition, muscle was sampled from one moribund fish, and from two fish hind gut and liver were sampled.

Collected samples were sent to the NVI in Ås for further processing of the tissue following standard procedures (Culling, Allison and Barr, 1985), to enable samples to be cast in paraffin and coloured with HE-colour. A light microscope (Olympus BX43) was used to examine the

tissue for pathology, and to compare this to non-infected fish. Olympus software cellSens Entery 2 was used to take pictures.

2.5 Identification of bacteria by 16s ribosomal RNA (16s rRNA) sequencing

Bacteria isolated from sampled fish was plated on BA (2 % NaCl) and one colony was isolated and suspended in 50 µL RNase and DNase free water. The suspension was heat treated at 98 °C for 10 min and then centrifuged at 13 000 *g* for 2 min in a Beckman Microfuge Lite centrifuge. The supernatant was collected for further processing and stored at – 20 °C.

For the PCR reaction the supernatant was diluted in DNase and RNase free water in a ratio of 1 to 9 for use as template. The reagents added to the reactions were 31.5 µL DNase and RNase free water 10 µL Phusion buffer x5, 1 µL dNTP 10mM, 2.5 µL F universal primers 27_univ (5'-AGAGTTTGATCMTGGCTCAG-3'), R and 1525R_univ (5'-AAGGAGGTGWTCCARCC-3') (Collins *et al.*, 1991), 1.5 µL Phusion DNA polymerase (Phusion DNA polymerase, Thermo Scientific) and 2 µL Template. The PCR-reaction was caried out in Applied biosystems 2720 thermal cycler at the following program one cycle at 98 °C for 3 min, 30 cycles of 98 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 1 min, followed with one cycle on 72 °C for 10 min. A 1 % agarose gel was used to confirm products after the PCR reaction.

For purification of the PCR-product the protocol for GenElute^{TM} PCR Clean-Up Kitwas used. Quantification of DNA in the clean PCR product carried out using with Nanodrop (Thermo Scientific NanoProp 2000). For Sanger sequencing two solutions per bacteria were prepared, one with forward primer and one with reverse primer. The reagents added to the reactions were 1 µL Big-Dye Version 3.1, 1 µL sequencing buffer, 1 µL primer, 1.5 µL template, and 5.5 µL Dnase and RNase free water. The sequencing reaction was caried out in Applied biosystems 2720 thermal cycler at the following program one cycle at 96 °C for 5 min, 35 cycles of 96 °C for 10 sec, 50 °C for 15 sec, and 60 °C for 4 min. After the reaction, 10 µL DNase and RNase free water was added to the sample. The reading of sequencing result was done at the DNA sequencing facility at the High-Technology Center, Bergen, Norway.

2.6 Primer validation and optimalisation

The DNA template for testing of the qPCR assay were made from both *P. atlantica* genomovar *salmonicida* and *P. atlantica* genomovar *cyclopteri* and anterior kidney from naïve fish mixed with bacteria. The bacteria were grown as described in section 2.1.1.

The bacterial cultures were harvested after 24h and centrifuged at 2 500 *g* for 15 min at 4 °C in a Beckmann coulter allegra x-15R centrifuge. The supernatant was discarded, and the bacteria pellet was left in the tube. Next, the pellet was resuspended in in 5 mL sterile PBS and the bacteria cell concentrations were measured in a CASY cell counter (Inovatis) in a suspension of 10 mL CASY-ton (Inovatis) and 10 µL bacterial suspension. The bacterial cultures had a concentration before extraction on $4.7*10^9$ bact/mL from PaL1 and $1.3*10^8$ bact/fish for the lumpsucker isolated, used previously in Ellul et. al. 2019. One mL bacterial suspension was collected to be used as the bacterial template.

For the kidney samples exposed to bacteria, the bacterial suspension was resuspended in 10 mL sterile PBS (Lonza). Anterior kidney samples from naïve fish were taken and stored in RNA-later (Sigma) at 4°C. Kidney samples of 25 mg were cut into smaller pieces and placed in 1.5 mL Microtubes (Axygene®, Corning korpoated), 5 tubes per isolate were prepared. In each tube, 25 µL prepared bacterial suspension was added with a concentration of $2.5*10^7$ bact/mL for lumpsucker isolate and $1.4*10^8$ bact/mL for PaL1. These tubes were set to incubate at room temperature for 1h.

For DNA extraction the DNeasy® Blood & Tissue Kit, Qiagen GmbH, was used and the protocol pretreatment for Gram-Negative Bacteria was used to prepare the bacterial template and the infection material for the kidney samples. Furthermore, DNA extraction from spiked anterior kidney was done as described from templates in 2.7. The primer assays for use in qPCR analysis were designed using the software Primer Premier version 6.24 (PREMIER Biosoft). The assays were tested using real time qPCR and the most stable assays was selected and standard curves compiled to determine the stability of the primers and the qPCR protocol, in addition to be able to pick the best assay to analyse the samples. For assay testing, each assay was tested against templates of the bacteria *P. atlantica* genomovar *salmonicida* and *P. atlantica* genomovar *cyclopterid* and samples of spiked anterior kidney for both bacteria in addition to NTC as a negative control.

qPCR analysis for validation was carried out with the same program and volumes described in 2.7 for both initial testing of assays and standard curve tests.

2.7 qPCR analysis of tissue samples from challenged fish

Samples for real time qPCR were taken from the anterior kidney and hearts. The samples from anterior kidneys were processed in this thesis.

DNA extraction was done using the DNeasy® Blood & Tissue Kit, (Qiagen GmbH) using protocol Purification of Total DNA form Animal Tissues (spin-Column Protocol) with some modifications. In point 2, samples were incubated overnight in a water bath at 56 °C or a heating block (Eppendorf Thermomixer comfort) at 56 °C with a centrifugation program of 10:50 sec at 600:0 rpm and in point 7, 100 µL Buffer AE were used. The quantification of DNA was measured using a Thermo Scientific NanoDrop 2000. Samples were stored at – 20 °C.

For qPCR each well contained a total volume of 10 µL, consisting of 5 µL 2x SYBR Green JumpStart Taq Ready Mix (Thermo-Fisher Scientific), 0.4 µL Forward primer, 0.4 µL Reverse primer and 0.2 µL, (primer information detailed in table 3) DNase and RNase free water (Sigma-Aldrich), in addition to 4 µL of DNA with a concentration of 25 ng/µL. The PCR reaction was caried out in a C1000 Touch thermal cycler with CFX96 Real-Time System (Bio-Rad) with the following program one cycle at 94 °C for 5 min, 40 cycles of 94 °C for 15 sec and 60 °C of 1 min. Melting curves were generated using temperatures from 60 °C to 92 °C with a rate of 1 °C/sec.

Table 3 Assay used for qPCR analysis, details about the primers are shown in the table

Assay	Target	Type	Primer name	Sequence 5' – 3'	Primer length (bp)
B-75_PAgDNAiLK_9	PAgDNAiLK	Forward	#99_PAgDNAiLK_R4	GACTCTTGCCGCCGTAGAGATTGAT	25
		Reverse	#103_PAgDNAiLK_F8	GCTAAGTTGTCGCCATTCGCCTTG	24

Pipetting the qPCR samples into plates was done by Gibson pipetmax 268. Plates were then spun down by Beckmann coulter allegra x-15R centrifuge for 1 min at 1 000 *g* before qPCR analysis.

3. Results

3.1 General fish health during the challenge experiment

Deformities of the dorsal fin, and of the opercula such as curved operculum was observed for a few individuals. Furthermore, the dorsal fin of one i.p. shedder fish (PaL3) were severely eroded, exposing the fin rays.

Generally, about a month into the challenge experiment it was noted that many fish had small bleedings on their pectoral fins. This was observed irrespective of challenge isolate and mode of challenge, though a possible slight difference in prevalence was observed where bleedings was more prevalent in tanks with bath challenged fish.

3.2 Water parameters

The water parameters described in section was measured daily. The mean temperature was 11.92 °C, the temperature range was from 11.4 °C to 12.2 °C. The mean salinity was 34.55 ‰ with a range from 33 ‰ to 39.8 ‰. O_2 levels was above 77 % saturation in the outlet water throughout the experiment.

3.3 Cell count of challenge material

The concentration of cells in the bacterial stocks used as challenge material was measured in a CASY cell counter. The measured concentrations after resuspension in sterile PBS (Lonza) before further dilutions were:

- PaL1: $1.5*10^9$ bact/mL
- PaL2: $1.5*10^9$ bact/mL
- PaL3: $1.6*10^9$ bact/mL

The presence of live bacteria was confirmed in all bacterial stocks, and the amount of colony forming units was registered by calculating cfu/ml (Table 4).

Table 4 Bacterial counts of colonies formed after plating of the infection material used for challenge of shedder fish. The table shows the number of bacterial colonies on agar plates (BA, 2 % NaCl) from the 10^6 dilution and 10^7 dilution of the original stock for each isolate and the calculated number of colony forming units per mL in the stock, including an average calculation.

	CFU		**CFU/mL**		
Isolate	Dilution - 6	Dilution - 7	Dilution - 6	Dilution - 7	Average
PaL1	85	11	$8.5*10^8$	$1.1*10^9$	$9.75*10^8$
PaL2	75	9	$7.5*10^8$	$9*10^8$	$8.25*10^8$
PaL3	71	3	$7.1*10^8$	$3*10^8$	$5.05*10^8$

3.4 Mortality throughout the challenge experiment

Throughout the challenge i.p. injected shedders from all challenge isolates had a higher mortality rate compared to bath shedders and all groups of cohabitants. The total cumulative percent mortality for i.p. shedders were 32 % for PaL1, 32 % for PaL2 and for PaL3 it was 44 %. For bath shedders mortalities were registered from PaL1 and PaL3, in both 1 fish per isolate died (4 %). However, no mortality was registered for PaL2 bath shedders. For cohabitants, with i.p. infected fish, mortalities were registered in challenge group PaL1 (4 %) and PaL2 (4%). For cohabitants with bath challenged fish, mortalities were registered in challenge group PaL3 (8 %). One control fish, i.p. injected, died post injection, no other mortalities were registered in the control groups. I.p. shedders had the highest mortality rate in the experiment, the other challenge groups had lower mortality rates and some fish groups as referenced had no mortality.

In the first three weeks post challenge, all but one registered mortality was i.p. injected shedders, one was a bath (PaL3) shedder fish. In this period 8 PaL3 i.p. shedders, 5 PaL2 i.p. shedders, and 5 PaL1 i.p. shedders died. The first cohabitant (bath PaL3) died three weeks post challenge. Cohabitants with i.p. infected fish from PaL1 and PaL2 died five (PaL2) and seven (PaL1) weeks post challenge. See Figure 4 for overview over when fish for i.p. groups died and Figure 5 for an overview over when fish from bath groups died.

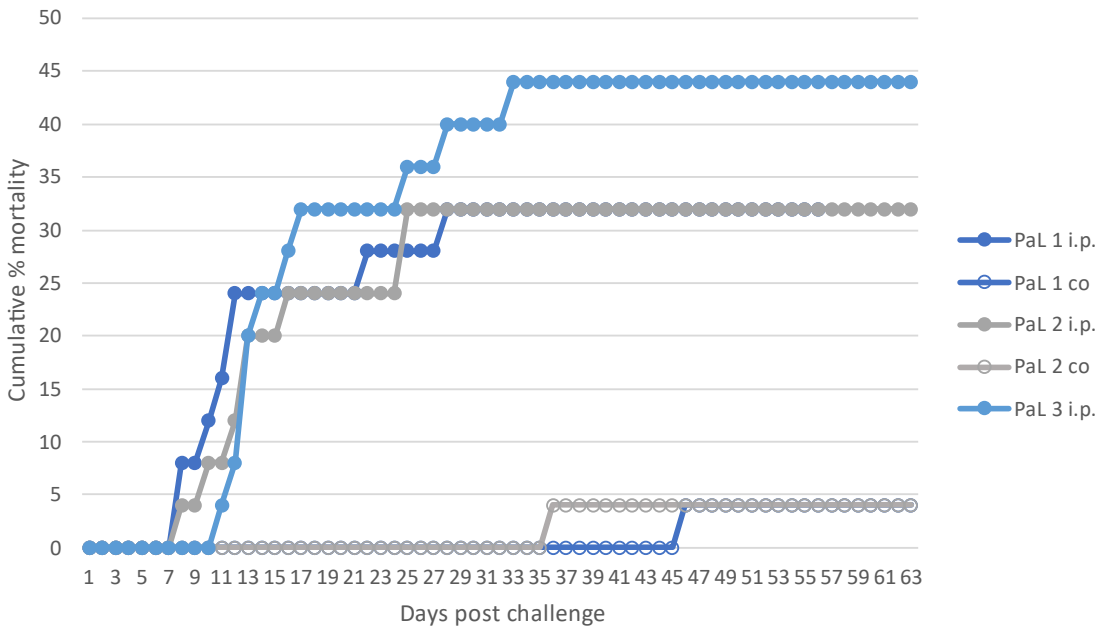

Figure 4 Cumulative percent mortality of i.p. infected shedders and cohabitants throughout the experimental period. Blue line closed circles = i.p. infected shedders PaL1. Blue line open circles = cohabitants PaL1. Gray line closed circles = i.p. infected shedders PaL2. Gray line open circles = cohabitants PaL2. Light blue line closed circles = i.p. infected shedders PaL3.

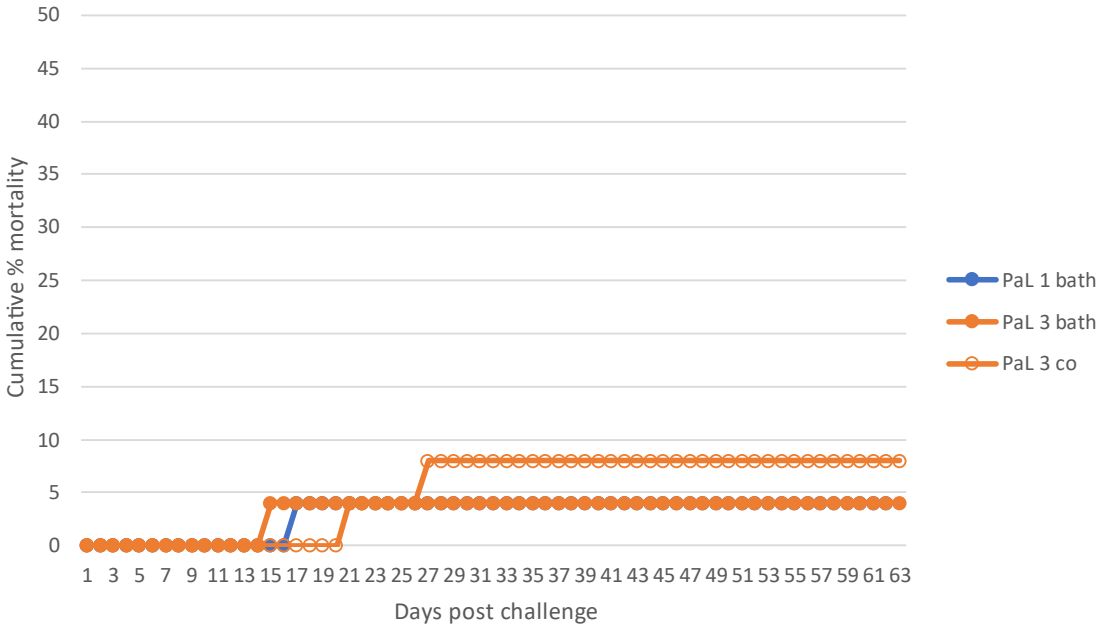

Figure 5 Cumulative percent mortality of bath infected shedders and cohabitants throughout the experimental period. Blue line closed circles = bath infected shedders PaL1. Orange line closed circles = bath infected shedders PaL3. Orange line open circles = bath cohabitants PaL3.

The stress inflicted on the fish in the bath challenged fish 42 days post challenge did not induce increased mortality in the fish group (Figure 5).

3.5 Gross pathology of challenged fish

3.5.1 Dead/moribund fish throughout the challenge

Fish that were i.p. shedders from all isolates had wounds, abscesses and/or inflammation at the injection point. Ascites in the body cavity were identified in all challenge groups expect in cohabitant fish from i.p. challenge with isolate PaL1 and PaL2. Enlarged spleen was also observed in several challenge groups, expect in PaL1 bath shedders and PaL2 cohabitants in tanks with i.p. infected fish. Fin bleedings was observed for cohabitants and bath shedders, but not from i.p. shedders. Petechial bleeding in the pseudobranch was seen for one fish (PaL2 cohabitant with i.p. shedders) and a fistula was seen in one fish PaL1 (cohabitant with i.p. shedders) under the left pectoral fin (Figure 6), which penetrated the skin and into the heart cavity.

Figure 6 Dead cohabitant fish from the group challenge with PaL1 (Tank 6). The left picture shows a fistula under the left pectoral fin that penetrated the skin and into the heart cavity (marked with 1). Around the same pectoral fin are bleedings marked with 2 (right picture). Fish died 46 dpc.

Abscess and inflammation in muscle were seen from one fish in each of the challenge group PaL1 i.p. shedders, PaL2 i.p. shedder and PaL3 bath shedder. One PaL3 bath shedder had a

swelling on its left lateral side just anterior of the anal fin (Figure 7). When the swelling was examined using a scalpel and forceps an abscess in the underlying muscle was identified.

Figure 7. Dead fish from group PaL3 bath shedders with an abscess in muscle, 1 marks the swelling in the muscle, 2 marks the bloody abscess found when the swelling were examined during necropsy. Fish died 15 dpc.

For a more complete overview over gross pathology seen in the different groups of fish with dead/moribund fish, see appendix.

3.5.2 Fish sampled during scheduled samplings

The fish used for sampling were randomly collected from the tanks.

Gross pathology was registered in sampled fish, the most common findings were fin lesions, such as fin erosion that could be bleeding and a bacterial layer on the fins (Figure 8). Eroded lower jaws were also commonly observed starting at sampling 3 (28 dpi) for PaL1 i.p. shedders and at later samplings for the other groups, this eroded lower jaws was in some cases also bloody. A more comprehensive overview of gross pathology and time point of registration see Table 10 for i.p. groups and Table 11 for bath groups.

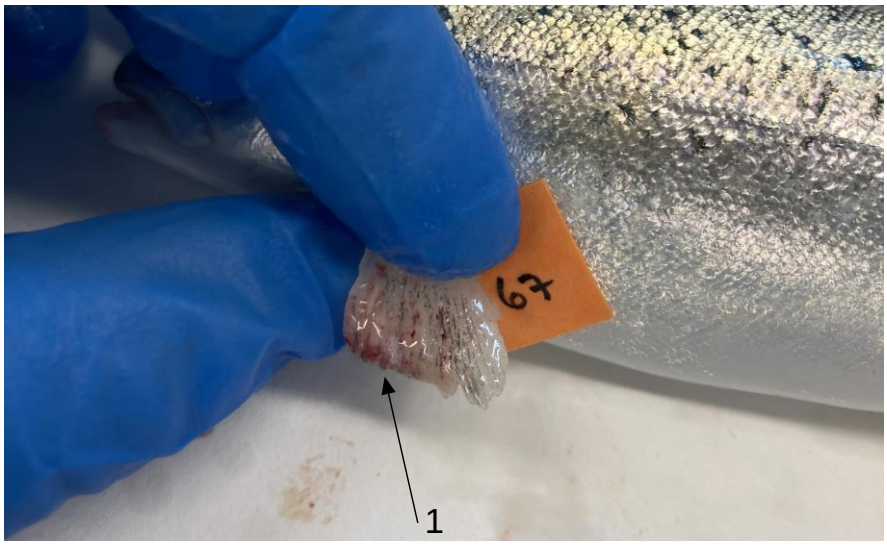

Figure 8 Fish (PaL3 bath cohabitant sampling 2) with bloody fin erosion with a bacterial layer on its left pectoral fin. Sampled 14 dpc.

For i.p. injected fish bacterial layers on internal organs were observed at sampling 2 (14 dpi) (Figure 9).

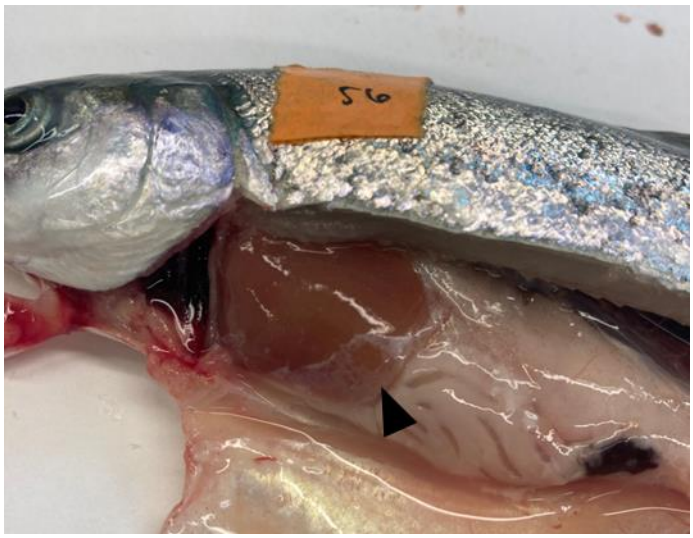

Figure 9 Fish (PaL1 i.p. shedder, sampling 2) with bacterial layer on the liver, see pointer. Sampled 14 dpi.

Severe abscesses on the lower jaw (Figure 10) were observed in three fish, from different challenge groups at sampling 6, 63 dpc. Abscesses were also found inside the mouth of fish belonging to group PaL1 i.p. cohabitant at sampling 6 (Table 10). The tissue of the lower jaw was soft, and the muscle was exposed. When the abscess tissue was examined, it was noted that the soft tissue was looser on the right side of the lower jaw compared to the left side.

Figure 10 Sampled fish with abscess on the lower jaw fish number 192 (PaL3 Bath shedder sampling 6) and fish number 179 (PaL1 i.p. cohabitant sampling 6). The abscesses are marked with 1 and 2. Sampled 63 dpc.

An example of a fish with eroded fins was PaL2 i.p. shedder at sampling 2, 14 dpi. This fish had an eroded caudal fin, an open wound anterior of the pelvic fins at the injection point with what looked like a bacterial layer on top (Figure 11). Bleedings at the base of the pelvic fins and the anal fin were also seen. When the body cavity was opened a bacterial layer was identified on the hind gut.

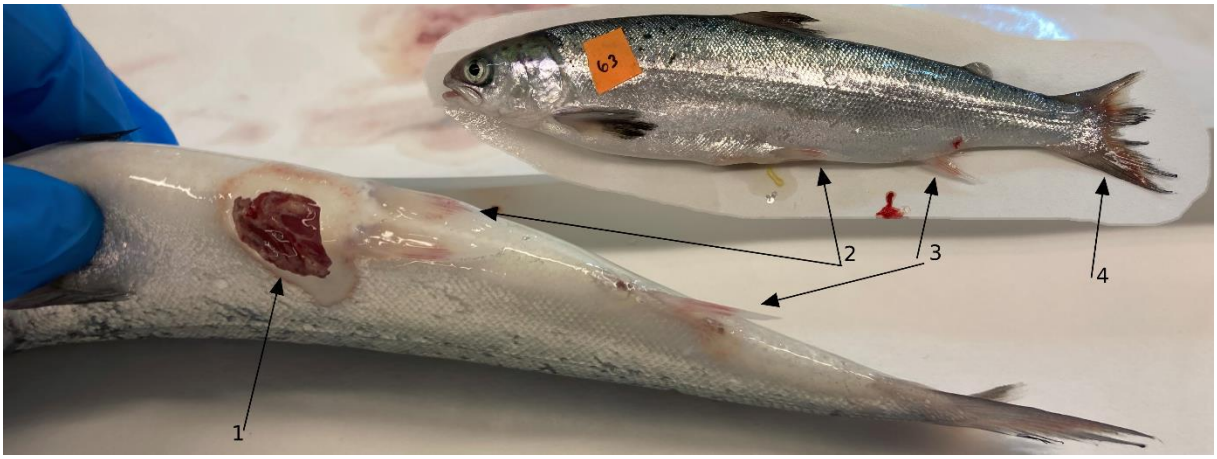

Figure 11 Fish (from sampling 2 PaL2 shedder) with severe lesions. 1 points to a wound with bacterial layer at the injection point, 2 points to bleeding at the base of the pelvic fins, 3 points to bleeding at the base of the anal fin, and 4 point to an eroded caudal fin. Sampled 14 dpi.

Abscesses on the skin/muscle was observed in PaL1 bath cohabitant challenged fish from 49 dpi (sampling 5) and onwards (Figure 12).

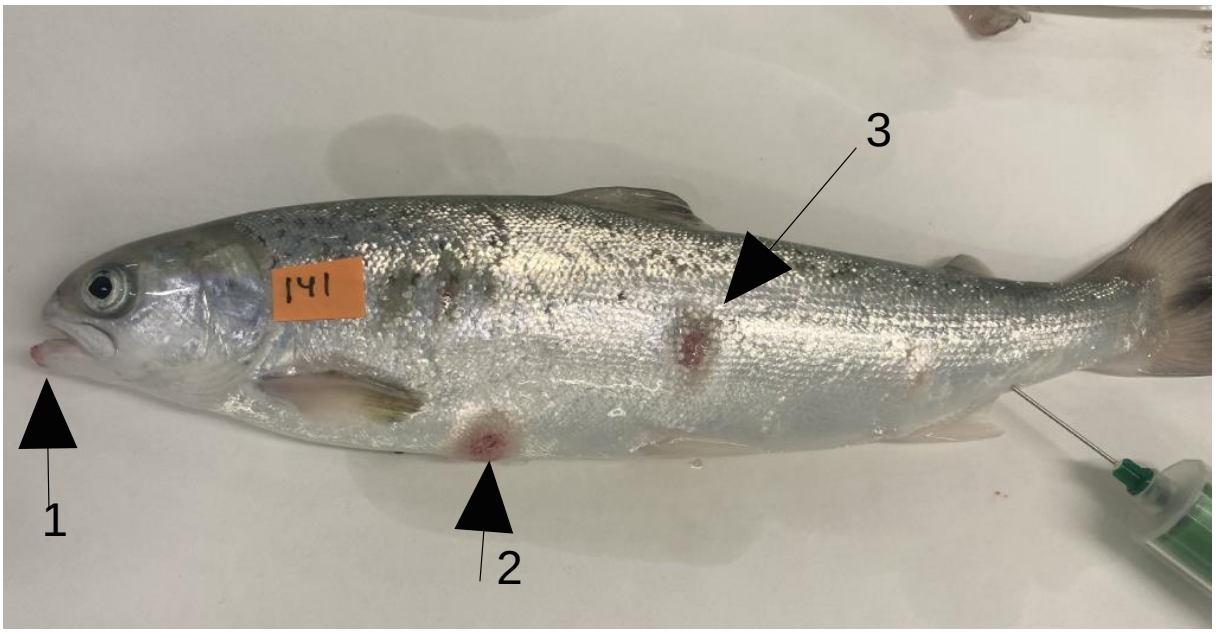

Figure 12 Fish (PaL1 bath cohabitant sampling 5) with (1) eroded lower jaw, and (2 and 3) wounds/abscess with oedema over the abscess. One abscess was located ventrally on the left side midway between the pectoral fin and the pelvic fin. The other was located ventral of the lateral line lateral for the posterior end of the dorsal fin. Sampled 49 dpc.

3.6 Bacteriology

3.6.1 Overview of bacteriology from sampled fish

The number of samples with growth for *P. atlantica* genomovar *salmonicida* growth on BA varied between the different groups of fish (Table 5). PaL1 i.p. shedders and PaL2 i.p. shedders had the highest prevalence of positive samples through the duration of the challenge, respectively 50 % and 40 %. PaL1 i.p. cohabitants, PaL2 i.p. cohabitants, PaL1 bath cohabitants, PaL2 bath cohabitants, PaL3 bath shedders, and PaL3 bath cohabitants all had a prevalence of 16.7 % positive samples. PaL3 i.p. shedders had a prevalence of 10 % and PaL3 i.p. cohabitants, PaL1 bath shedders, and PaL2 bath shedders had no positive bacterial samples of *P. atlantica*. No control fish tested positive for *P. atlantica.*

Table 5 Sampled fish, divided into groups based on isolate and mode of infection. The table shows growth of *P. atlantica* genomovar *salmonicida* on BA after sampling, number, and the percentage of samples where bacterial growth on agar were seen.

Group of fish	Positive for growth of *P. atlantica* genomovar *salmonicida* on agar	Number of positive samples	Precent positive samples
PaL1 – i.p. shedders	Yes	5/10	50 %
PaL1 – i.p. cohabitant	Yes	2/12	16.7 %
PaL2 – i.p. shedders	Yes	4/10	40 %
PaL2 – i.p. cohabitant	Yes	2/12	16.7 %
PaL3 – i.p. shedders	Yes	1/10	10 %
PaL3 – i.p. cohabitant	No	0/12	0 %
PaL1 – Bath shedders	No	0/12	0 %
PaL1 – Bath cohabitant	Yes	2/12	16.7 %
PaL2 – Bath shedders	No	0/12	0 %
PaL2 – Bath cohabitant	Yes	2/12	16.7 %
PaL3 – Bath shedders	Yes	2/12	16.7 %
PaL3 – Bath cohabitant	Yes	2/12	16.7 %

From both fish shown in Figure 6, bacteriological samples were taken from the abscess. When grown on BA (2 % NaCl), bacterial colonies of *P. atlantica* genomovar *salmonicida* were identified (Figure 13).

Figure 13 bacterial sample streaks from an abscess on the lower jaw of PaL1 i.p. cohabitant at sampling 6 (63 dpi).

3.6.2 Bacteriology from dead/moribund fish

The presence of *P. atlantica* genomovar *salmonicida* on BA was confirmed for all but two samples of dead/moribund fish (Table 6). Two dead fish from PaL3 i.p. shedders did not test positive for *P. atlantica* genomovar *salmonicida* by bacteriology sampling.

The one control fish that died during the experiment, was not positive for *P. atlantica* genomovar *salmonicida* on BA. From the wound and anterior kidney bacterial growth was detected on BA and the bacterium *Photobacterium phosphoreum* was identified by 16S rRNA sequencing.

Table 6 Dead/moribund fish, divided into groups based on isolate and mode of infection. The table shows growth of *P. atlantica* genomovar *salmonicida* on BA, number, and the percentage of samples where bacterial growth on agar were seen.

Group of fish with mortality	Positive for growth of *P. atlantica* genomovar *salmonicida* on BA	Number of positive samples	Precent positive samples
PaL1 – i.p. shedders	Yes	8/8	100 %
PaL1 – i.p. cohabitant	Yes	1/1	100 %
PaL2 – i.p. shedders	Yes	7/7	100 %
PaL2 – i.p. cohabitant	Yes	1/1	100 %
PaL3 – i.p. shedders	Yes	9*/11	81.8 %
PaL1 – Bath shedders	Yes	1/1	100 %
PaL3 – Bath shedders	Yes	1/1	100 %
PaL3 – Bath cohabitant	Yes	2/2	100 %

* The anterior kidney was still frozen in two fish from this fish group when the fish was dissected for bacteriology.

3.6.3 Bacteriology at termination of the challenge

At termination of the challenge experiment, swabs from anterior kidney on BA were performed from up to ten fish per challenge group. For the i.p. shedders, fewer than 10 fish remained in these groups, thus all the remaining fish was sampled. The prevalence varied between the fish groups as shown in Table 7. PaL2 i.p. cohabitants and PaL3 bath cohabitants had the highest prevalence of positive samples at 30 %, PaL1 i.p. shedders had a 25 % positive prevalence, PaL1 bath cohabitants had a prevalence of 20 % positive samples PaL1 i.p. cohabitants, PaL3

i.p. shedders, PaL1 bath shedders, PaL2 bath shedders, and PaL3 bath shedders all had a positive prevalence of *P. atlantica* genomovar *salmonicida* of 10 %. PaL2 i.p. shedders, PaL3 i.p. shedders, and PaL2 bath cohabitants all had no positive samples of *P. atlantica* genomovar *salmonicida* at termination sampling. *P. atlantica* genomovar *salmonicida* was not identified in control fish at termination of the trial.

Table 7 End sampling of fish, divided in groups based on isolate and mode of infection. The table shows growth of *P. atlantica genomovar salmonicida* on BA after sampling, numbers, and the percentage of samples where bacterial growth on agar were seen.

Group of fish	Positive for growth of *P. atlantica* genomovar *salmonicida* on BA	Number of positive samples	Precent positive samples
PaL1 – i.p. shedders	Yes	1/4	25 %
PaL1 – i.p. cohabitant	Yes	1/10	10 %
PaL2 – i.p. shedders	No	0/7	0 %
PaL2 – i.p. cohabitant	Yes	3/10	30 %
PaL3 – i.p. shedders	No	0/1	0 %
PaL3 – i.p. cohabitant	Yes	1/10	10 %
PaL1 – Bath shedders	Yes	1/10	10 %
PaL1 – Bath cohabitant	Yes	2/10	20 %
PaL2 – Bath shedders	Yes	1/10	10 %
PaL2 – Bath cohabitant	No	0/10	0 %
PaL3 – Bath shedders	Yes	1/10	10 %
PaL3 – Bath cohabitant	Yes	3/10	30 %

3.7 Primer validation and optimalisation

For primer validation 21 different assays were evaluated with a standard qPCR as described in section 2.6. out of these eleven assays were designed to be specific for *P. atlantica* genomovar *salmonicida*, whereas 9 were designed to be specific for *P. atlantica* genomovar *cyclopteri*, and 1 was designed to be able to detect both genomovars of *P. atlantica*. Out of the 21 assays 4 assays were further evaluated with a standard curve analysis, based on the Ct value from the qPCR analysis and whether the assay was specific to *P. atlantica genomovar salmonicida*. The best of these primers was then used to analyse the samples from challenged fish (Table 8).

Table 8 Technical details about the assays that were tested by standard curves in preparation for qPCR. The details about the different assays such as primers, product length, correlation, and efficiency are given.

Assay	D	1	SodA	C
Target gene	protein phosphatase 2C domain-containing protein	OmpA family protein	SodA gene	ADP-ribosylglycohydrolase family protein
Forward primer,5'-3'	GACTCTTGCCGCC-GTAGAGATTGAT	TCTAATATTGA-TGATCTTGTTTG	TTCACCATTCAA-AGCACCATCAAG	GTTGCGAGGCTC-ATACTGGTGTCAT
Reverse primer,5'-3'	GCTAAGTTGTCG-CCATTCGCCTTG	ATTTCCTAAATT-AGGAAAGATAC	CTTCTAAAGCAG-CATTGGCATTAT	GCGATGCTTTAG-GTGTACCCGTTGA
Product length bp	112	78	*	139
Correlation, R^2	0.9835	0.9826	0.998	0.996
Efficiency	2.19	2.08	2.02	2.07
Design	This thesis	Sandlund et. al. 2021	Ellul et. al. 2019	This thesis

*Product length not measured in the referenced paper

The assay selected for analysing of samples after testing was Assay D. The results from testing are presented in two standard curves one with bacterial culture as template (Figure 14), and one with bacterial culture mixed with anterior kidney from naïve fish as template (Figure 15).

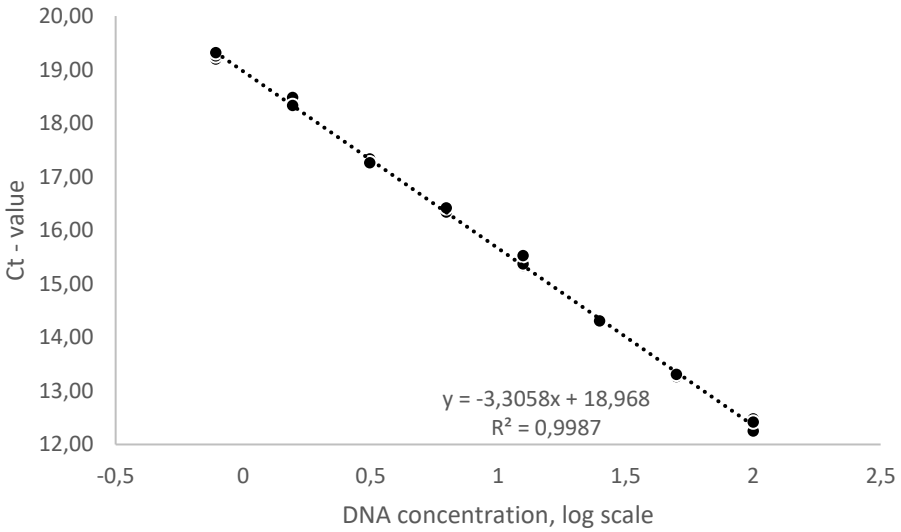

Figure 14 Standard curve for test of assay against *P. atlantica* genomovar *salmonicida* with DNA extracted from bacterial culture. The x-axis shows concentration of DNA as logarithmic transformed (log). The y-axis shows Ct – values that the different reactions produced.

Figure 14 above shows the standard curve produced from Ct values from a qPCR analysis of DNA isolated from a bacterial stock. This resulted in a R^2 of 0.9989 and an effect of 2.0067.

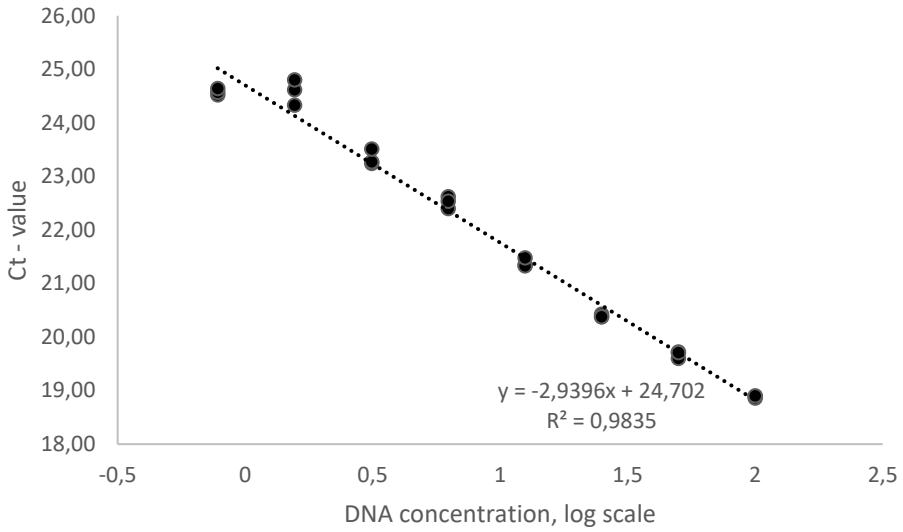

Figure 15 Standard curve for test of assay against *P. atlantica* genomovar *salmonicida* with DNA extracted from naïve anterior kidney spiked with bacterial culture. The x-axis shows concentration of DNA as logarithmic transformed (log). The y-axis shows Ct – values that the different reactions produced.

Figure 15 above shows the standard curve produced from Ct values from a qPCR analysis of DNA isolated from anterior kidney from naïve fish, spiked with bacterial stock. This resulted in a R^2 of 0.9835 and an effect of 2.1887.

3.8 Calculation curve, *P. atlantica* genomovar *salmonicida*

To enable quantification of bacteria in the samples a calculation curve was made, with tenfold dilutions per step (Figure 16).

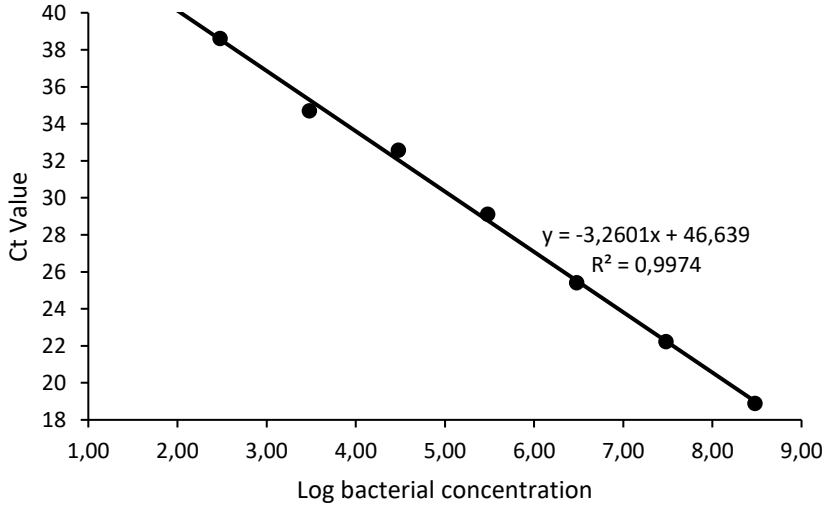

Figure 16 Calculation curve, *P. atlantica* genomovar *salmonicida* DNA extracted from anterior kidney from naïve fish spiked with bacterial culture. The x-axis shows concentration of DNA as logarithmic transformed (Log). The y-axis shows Ct – values that the different reactions produced.

Figure 16 above shows the concentration curve produced from Ct values from a qPCR analysis of DNA isolated from naïve anterior kidney spiked with bacterial stock. This resulted in a R^2 of 0.9974 and an effect of 2.0264.

3.9 qPCR analysis

The qPCR results for i.p. injected groups collected at scheduled samplings are summarised in Figure 17. The qPCR results for bath challenged groups collected at scheduled samplings are summarised in Figure 18. The qPCR results for dead/moribund are summarised in Figure 19. The figures show average Ct values of three replicates from each fish from qPCR analysis.

Tissue from non-challenged control fish were also analysed, and i.p. controls and cohabitants of this group were negative. Some zero samples and bath control samples did show presence of *P. atlantica* genomovar *salmonicida.*

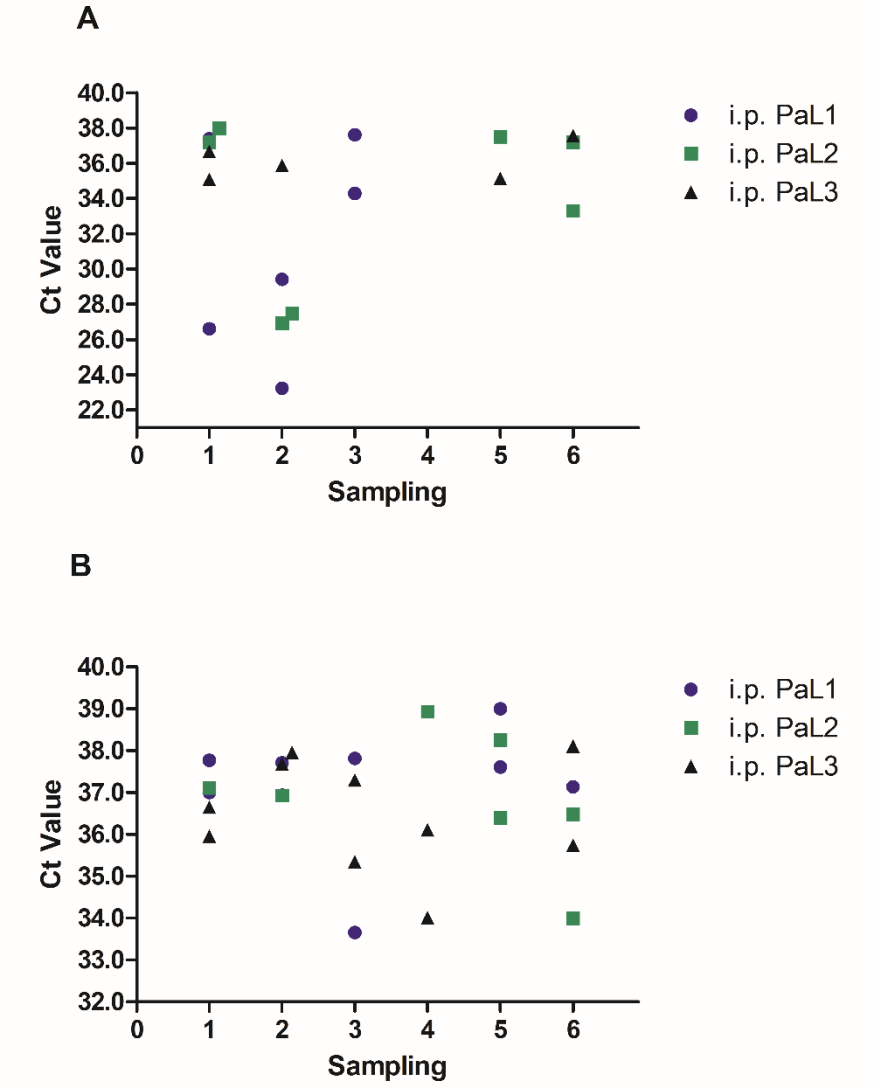

Figure 17 qPCR results from anterior kidney in the i.p. infected groups (A), PaL1 fish are marked in the colour blue, PaL2 are marked in the colour green and PaL3 are marked in the colour black. qPCR results from cohabitants (B), PaL1 fish are marked in the colour blue, PaL2 are marked in the colour green and PaL3 are marked in the colour black.

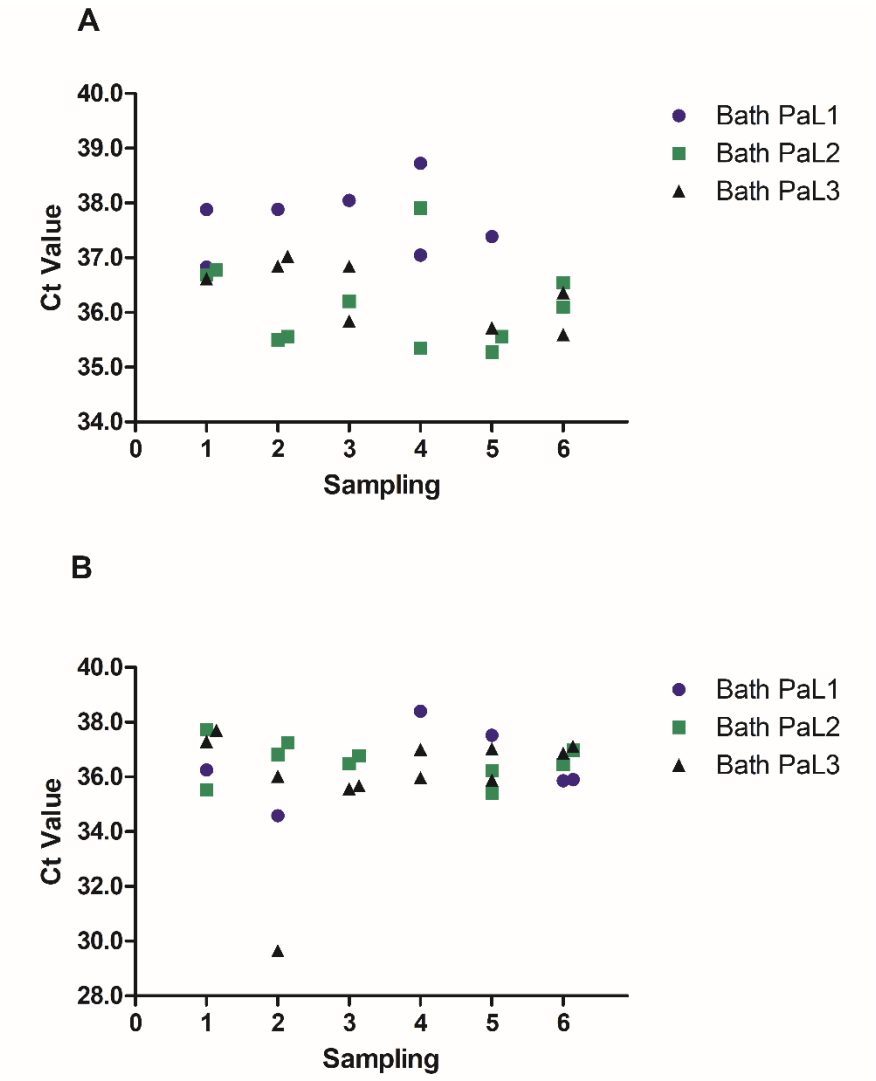

Figure 18 qPCR results from anterior kidney in the bath infected groups (A), PaL1 fish are marked in the colour blue, PaL2 are marked in the colour green and PaL3 are marked in the colour black. qPCR results from cohabitants (B), PaL1 fish are marked in the colour blue, PaL2 are marked in the colour green and PaL3 are marked in the colour black.

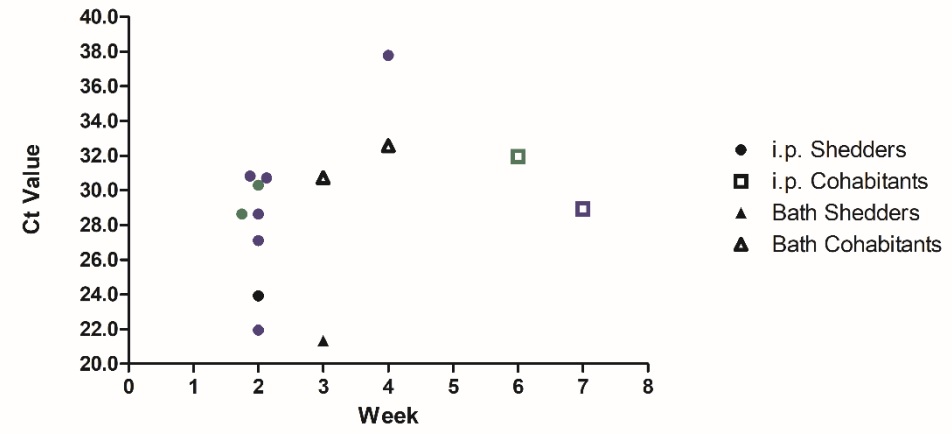

Figure 19 qPCR results from anterior kidney in the dead fish. Filled circle is i.p. shedders, open square is i.p. cohabitants, filled triangle is bath shedders, and open triangle is bath shedders. Blue colour represent a PaL1 challenged fish, green colour represent PaL2 challenged fish, and black colour represent PaL3 challenged fish.

Generally, the Ct-values from challenged fish shown in Figures 16 and 17 are high, indicating a low amount of bacteria present. Most of the values have Ct values above 33, with a few exceptions in samples from i.p. shedders form the first to samplings (Figure 16) and bath cohabitant (Figure 17). The Ct-values obtained after analysis of anterior kidney tissue was consistently high.

Samples from dead and moribund fish had varied Ct values (Figure 18), but in general the Ct – values were lower in dead compared to sampled fish (i.e. a higher amount of bacteria on dead fish). From one dead fish *P. atlantica* genomovar *salmonicida* were not detected, though bacteriology from the anterior kidney did show presence of *P. atlantica* genomovar *salmonicida*

To stipulate the infection load in the fish Ct-values were divided into three categories, where < 25 were defined as a severe infection, from 25 and up to 30 as a mild infection and 30 to 37 potentially carriers. As shown in Table 9, there are several fish were the presence of *P. atlantica* genomovar *salmonicida* were not detected.

Table 9 Number of fish included in stipulated infection groups based on qPCR results of samples from challenged fish divided based on isolate challenge with and method of challenge.

	PaL1				PaL2				PaL3			
	i.p.-S	i.p.-C	B-S	B-C	i.p.-S	i.p.-C	B-S	B-C	i.p.-S	i.p.-C	B-S	B-C
>25	1	-	-	-	-	-	-	-	-	-	-	-
25-30	2	-	-	-	2	-	-	-	-	-	-	1
30 - 37	1	2	1	4	-	4	10	8	5	6	7	6
37 - 40	2	7	6	2	3	3	1	2	2	4	1	5
N/a	4	3	5	6	5	5	1	2	3	2	4	-

The results from the qPCR analysis show that tissue from some fish from all challenge groups except PaL3 bath cohabitants, did not have presence of *P. atlantica* genomovar *salmonicida*. For many fish, where the qPCR analysis detected *P. atlantica* genomovar *salmonicida* the Ct-values were high, indicating low amounts of bacteria present.

3.10 Dead control fish

One control fish died during the challenge experiment (12 dpi), *P. atlantica* genomovar *salmonicida* was not detected from anterior kidney of this fish. Bacteriology samples were taken from wound number 1 shown in Figure 20 and from the anterior kidney. Bacterial streak from the wound is shown in Figure 21. The fish is shown in Figure 20, it had several wounds, one on its right lateral side between anal fin and pelvic fin, one wound at the basis of the dorsal fin, one at the posterior end of the right operculum, and one behind the left pectoral fin. In addition, fin erosion was seen in all fins, and some fin rays were exposed. As stated in section 3.3 the bacterium was identified as *P. phosphoreum* was identified by sanger sequencing.

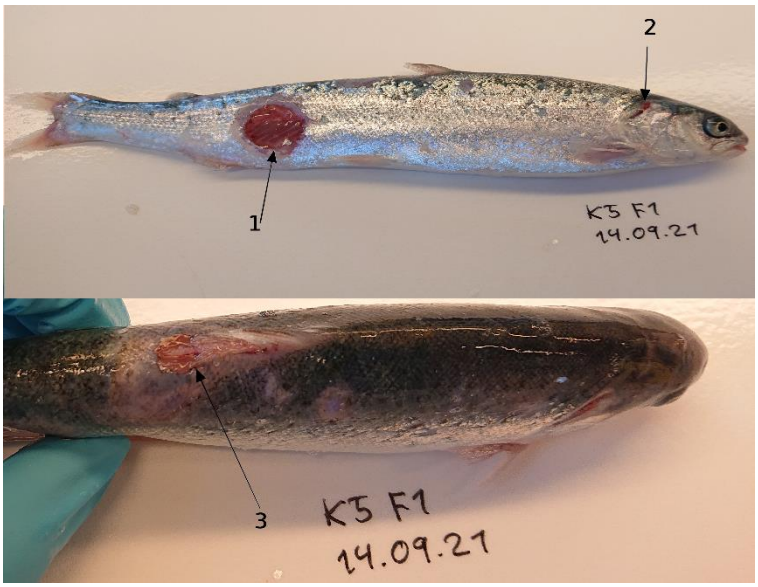

Figure 20 Dead control fish, this fish had several wounds and three of them are marked with an arrow and a number, (1) is a wound on its right lateral side between anal fin and pelvic fin, (2) is a wound at the basis of the dorsal fin, (3) is a wound at the posterior end of the right operculum. In addition, fin erosion was observed on all fins. were exposed.

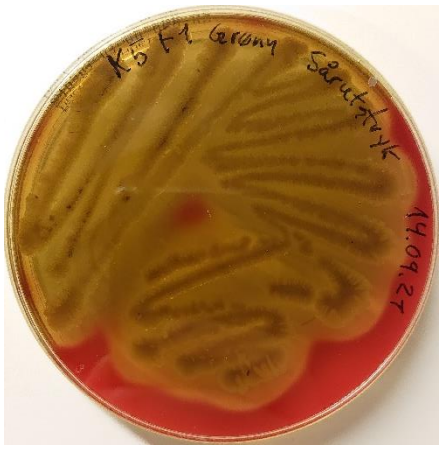

Figure 21 Bacterial streak for wound of dead control fish. Picture taken 6 days post planting on agar.

3.11 Histopathology

For histology all tissue samples from moribund fish were examined, along with histology from fish were *P. atlantica* genomovar *salmonicida* was grown from anterior kidney on BA. Further, histology from fish were *P. atlantica* genomovar *salmonicida* was not grown were examined. In i.p. infected fish at sampling one week post challenge (7 dpi) aggregates of bacteria were identified on several internal organs, including liver (Figure 22).

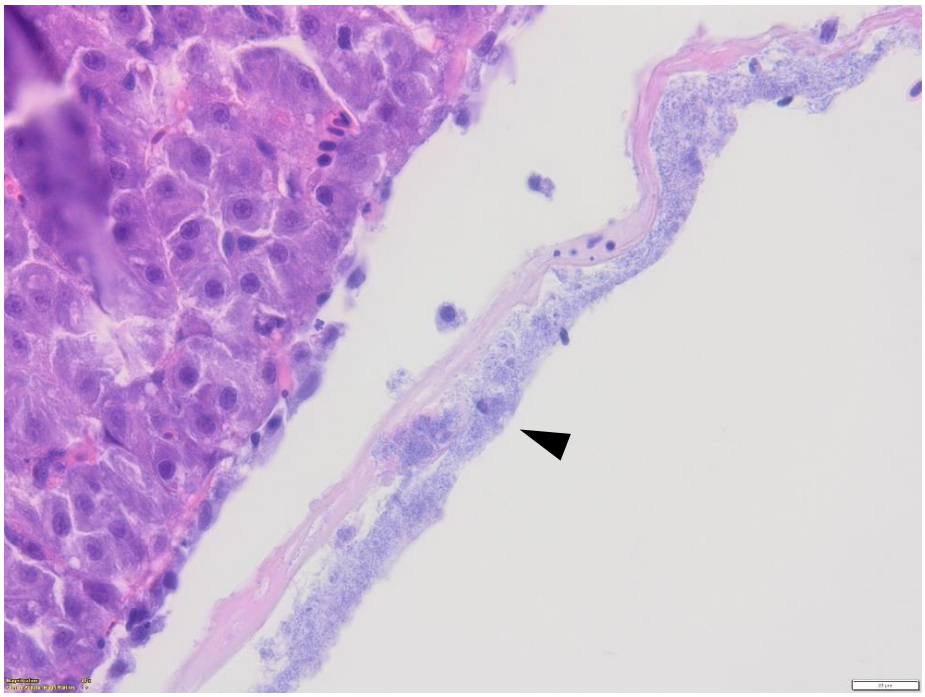

Figure 22 Layer of aggregated bacteria (arrowhead) associated with liver tissue from an i.p. injected shedder fish from the sampling conducted one week post injection (7 dpi). HE staining. Total magnification 400x, scale bar = 20 µm.

By microscopy bacteria was seen in tissues from challenged fish in areas where macroscopically clinical signs of disease were identified, such as the lower jaw of i.p. cohabitants (Figure 23), bath shedders (Figure 24), and cohabitants (Figure 25).

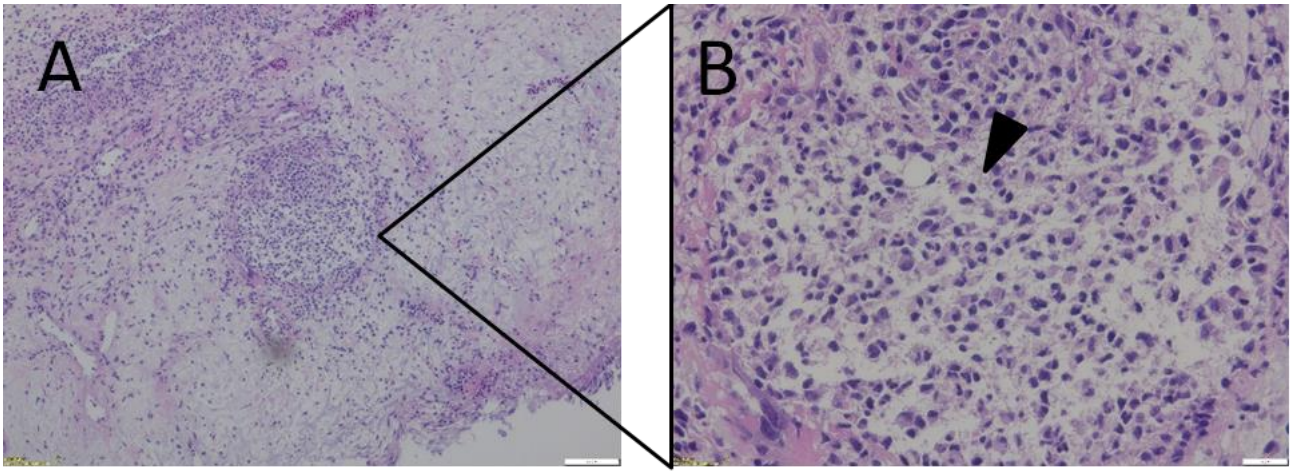

Figure 23 Abscess in lower jaw of fish cohabitating with i.p. injected shedders, from the sampling conducted eight weeks post challenge (63 dpi). HE staining. A: Abscess, total magnification 200x, Scale bar = 100 µm. B: Close up view of the abscess in A, the arrow

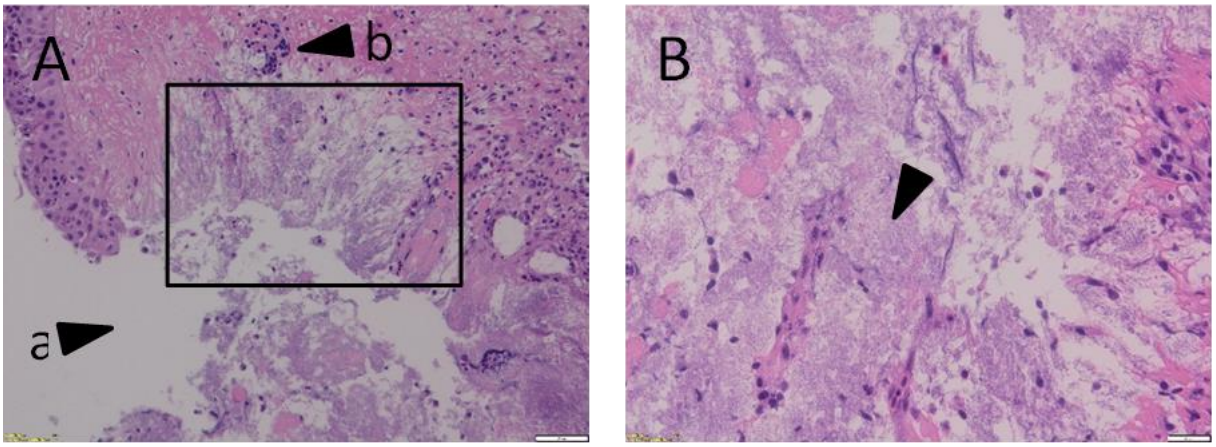

Figure 24 Histopathology of lower jaw from a bath challenged fish sampled nine weeks post challenge (63 dpc). HE staining. A: Pathological lesions in epidermis showing an ulcer (arrowhead a), and a foci with infiltrating immune cells in dermis (arrowhead b), magnification 200x, scale bar = 20 µm, B: Close up view of the area within the square of picture A showing aggregates of bacteria (arrowhead) in dermis, magnification 400x, scale bar = 20 µm.

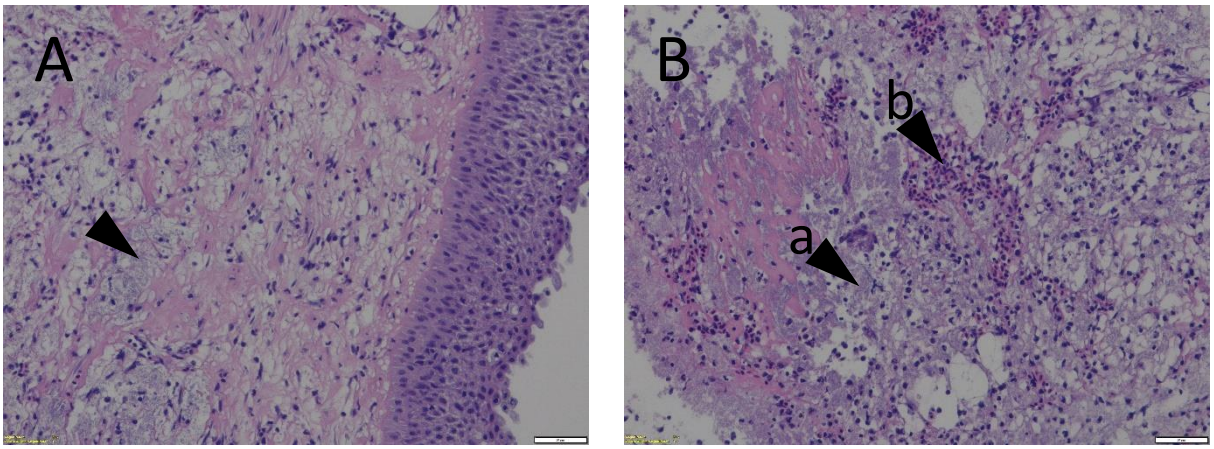

Figure 25 Aggregates of bacteria in dermis of lower jaw of cohabitant fish to bath challenged shedders sampled nine weeks post challenge (63 dpc). HE staining. Magnification 200x, scale bar 20 µm. A: Aggregates of bacteria (arrowhead) in dermis. B: Bleeding ulcer of lower jaw showing aggregates of bacteria (arrowhead a) and red blood cells (arrowhead b).

In challenged fish aggregates of bacteria were also identified in the hearts (pictures not shown).

3.12 Water samples

Water samples tanked during the challenge experiment were not processed in time to be included in this thesis.

3.13 Summary of results

Multiple diagnostic tools were used to diagnose the fish with pasteurellosis, The diagnostic tools were inspection for gross pathology, bacteriology, qPCR analysis, and histopathological analysis of selected organs. This was done for both fish groups (shedders and cohabitants) from i.p. challenge tanks (Table 10) and bath challenge tanks (Table 11).

Table 10 Overview of results from qPCR analysis, bacteriology, macroscopical changes of sampled fish in groups of i.p. injected shedders and cohabitants. Two fish are included in each sampling individually registered as positive (+) or negative (-) samples. For pathology, a number reflects a clinical sign found on the fish. If no clinical signs are found on the fish "-" is written. For clinical signs the numbers identify (1) bacterial layer on fins, (2) fin erosion, (3) bloody fin, (4) abscess lower jaw, (5) petecchia on internal organs, (6) abscess at injection point, (7) petecchia body cavity/muscle, (8) bloody swollen vent, (9) inflammation spleen, (10) ascites, (11) bloody liquid in hearth cavity, (12) exophthalmia, (13) puss on pseudobranch, (14) wound, (15) bacterial layer on internal organs, (16) eroded lower jaw, (17) yellow patches on skin, (18) abscess in muscle, and (19) bleeding in eye.

Isolate	Sampling	Group	qPCR	Bacteriology	Pathology
PaL 1	1	Shedder	+ / +	+ / +	6,7 / 6
		Cohab	+ / +	- / +	- / 2
	2	Shedder	+ / +	+ / +	8,9,10,15 / 5,8,11,23
		Cohab	+ / +	- / -	- / 3
	3	Shedder	+ / +	- / +	16 / 2,3,6,10,12,13
		Cohab	+ / +	- / -	3 / 17
	4	Cohab	- / -	- / -	16 / 2,3
	5	Shedder	- / -	- / -	- / -
		Cohab	+ / +	- / -	- / 18
	6	Shedder	- / -	- / -	16 / -
		Cohab	+ / -	+ / -	4 / 3,16
PaL 2	1	Shedder	+ / +	- / +	- / 6
		Cohab	- / +	- / -	- /-
	2	Shedder	+ / +	+ / +	2,3,6,14,15 / 2,3,5,8,10,23
		Cohab	- / +	- / +	- / -
	3	Shedder	- / -	- / -	2,3 / 1,2,3
		Cohab	- / -	- / -	2,3 / 1,2,3
	4	Cohab	- / +	+ / -	1,2,3 / 1,2,3
	5	Shedder	- / -	- / -	-/-
		Cohab	+ / +	- / -	- / 27
	6	Shedder	+ / -	- / +	1,2,3 / 1,2,3,16
		Cohab	+ / +	- / -	19 / 16
PaL 3	1	Shedder	+ / +	+ / +	7,10 / 2,7,10
		Cohab	+ / +	- / -	- / -
	2	Shedder	+ / -	- / -	2 / -
		Cohab	+ / +	- / -	2 / -
	3	Shedder	- / -	- / -	2,3 / 2
		Cohab	+ / +	- / -	- / 2,3
	4	Cohab	+ / +	- / -	16 / 2,3,16
	5	Shedder	+ / +	- / -	27 / 2,3
		Cohab	- / -	- / -	2 / 2
	6	Shedder	+ / +	- / -	2 / -
		Cohab	+ / +	- / -	1 / 16

Table 11 Overview of results from qPCR analysis, bacteriology, macroscopical changes of sampled fish in groups of bath shedders and cohabitants. Two fish are included in each sampling individually registered as positive (+) or negative (-) samples. For pathology, a number reflects a clinical sign found on the fish. If no clinical signs are found on the fish "-" is written. For clinical signs the numbers identify (1) bacterial layer fins, (2) fin erosion, (3) bloody fin, (4) abscess lower jaw, (5) abscess mouth, (6) anaemic gills, (7) anaemic blood, (8) petecchia on internal organs, (9) pale hearth, (10) pale liver, (11) ascites, (12) dotted gills, (13) wound, (14) lower jaw with wound, (15) eroded lower jaw, (16) abscess muscle, (17) white layer kidney, and (18) inflammation pseudobranch.

Isolate	Sampling	Group	Q-PCR	Bacteriology	Pathology
PaL 1	1	Shedder	+ / +	- / -	1,2,3 / 17
		Cohab	- / +	- / -	- / 12
	2	Shedder	- / +	- / -	3,14 / 2,3
		Cohab	- / +	- / -	1,2,3 / 2,3
	3	Shedder	+ / -	- / -	1,2,3,14 / 1,2,3
		Cohab	- / -	- / -	2,3 / 2,3
	4	Shedder	+ / +	- / -	2,3,13 / -
		Cohab	+ / +	- / -	15 / -
	5	Shedder	- / +	- / -	1,2,3 / 2,3
		Cohab	- / +	- / -	13,15,16 / 1,2,3
	6	Shedder	- / -	- / -	15 / -
		Cohab	+ / +	+ / -	4 / 15
PaL 2	1	Shedder	+ / +	- / -	- / 1,2,3
		Cohab	+ / +	- / +	11 / -
	2	Shedder	+ / +	- / -	- / 3
		Cohab	+ / +	- / +	- / 1,2,3
	3	Shedder	- / +	- / -	2,3 / -
		Cohab	+ / +	- / -	1,2,3 / 2,3
	4	Shedder	+ / +	- / -	1,2,3 / 1,2,3,15
		Cohab	- / -	- / -	- / 2,3
	5	Shedder	+ / +	- / -	- / 1,2,3,15
		Cohab	+ / +	- / -	1,2,3,15 / 2,3,15,16
	6	Shedder	+ / +	- / -	- / -
		Cohab	+ / +	- / -	- / 15
PaL 3	1	shedder	- / +	- / -	2,3 / -
		cohab	+ / +	- / -	- / 2,3
	2	shedder	+ / +	+ / -	1,2,3 / -
		cohab	+ / +	+ / +	1,2,3 / -
	3	shedder	+ / +	- / -	1,2,3 / 2,13
		cohab	+ / +	- / -	- / -
	4	shedder	- / -	- / -	13 / -
		cohab	+ / +	- / -	- / 15
	5	shedder	- / +	- / -	18 / 15
		cohab	+ / +	- / -	2,15 / 2,3
	6	shedder	+ / +	- / -	2,15 / 4,5,6,7,8,9,10
		cohab	+ / +	+ / -	15 / 15

4. Discussion

In this project the aim was to establish a reproducible challenge model for pasteurellosis in Atlantic salmon. Pasteurellosis in Atlantic salmon has become an increasing problem for salmon fish farming especially in PO 2 to 5 (Figure 1) since 2018, prior to this, *Pasteurella* bacteria had sporadically caused disease at different sites along the Norwegian coast since 1989 (Sommerset *et al.*, 2020).

The increase in the number of detected outbreaks since 2018 has resulted in pasteurellosis going from being regarded as an emerging disease in 2020 (Sommerset *et al.*, 2021) to an established disease in 2021. Currently there are no available treatments or prophylactic measures to prevent the disease (Sommerset *et al.*, 2022).

This has led to a need for more knowledge about the disease and possible prophylactic treatments such as a vaccine against the disease. To be able to test the efficacy of vaccines and to learn more about the disease a reliable challenge model is required, and this was the aim for the project.

In all challenged groups, except for non-challenged control fish, *P. atlantica* genomovar *salmonicida* was reisolated on BA from anterior kidney, from either dead/moribund fish, sampled fish or fish sampled at termination of the study. This shows that irrespective of challenge model (injection, bath or cohabitation) fish were infected with *P. atlantica*, and for dead/moribund fish the infection developed into disease. Additionally, individual fish from all challenge groups were positive for *P. atlantica* genomovar *salmonicida* when tissue from anterior kidney were analysed by qPCR. Notably, a higher number of fish were positive when analysed by qPCR compared to re-isolation on BA.

A higher number of i.p. infected shedder fish died throughout the challenge experiment compared to other groups (bath challenged and cohabitants). This was probably because the injected fish was given a high dose of bacteria injected into the abdomen, subverting the first line of immune defence. The relative low percentage of mortality observed for the other groups may partly be explained by that fish was regularly sampled through the challenge experiment, lowering the infection pressure and density in the tanks. By extracting clinical sick fish for sampling, the infection pressure in the tanks will be influenced.

The mortality rates obtained in the different challenge groups does not meet the proposed general requirements of use as control groups for vaccine trials, this being equal to or above 60

%, cumulative mortality (Midtlyng, 2016). As fish were regularly sampled from the tanks, it is difficult to estimate the mortality the challenge models can produce without sampling. Infection load given to the fish in challenge experiments will affect the mortality (Gudding, 1997). A higher infection load can therefore potentially increase the mortality rate in the challenge.

Mortality started at different days post challenge in the different challenge models. For i.p. injected shedders mortality started 8 days post challenge and the last i.p. shedder died 33 days post challenge. For the two bath shedder fish that died, one died 15 days post challenge (PaL3) and the other died 17 days post challenge (PaL1)

Interestingly, only low differences in mortalities were observed after challenge using the three isolates harvested at different sites in western Norway. Possible difference in virulence across isolates of *P. atlantica* genomovar *salmonicida* has been issued as one of the important unknowns that needs to be answered to elucidate background for why the disease manifestation is different in severity and in symptoms at different locations (Legård et al 2020, Sandlund et al 2021). For the current experiment, it cannot be excluded that using other isolates, or other growth media or growth conditions for the challenge material may have given other results.

Mortality rates reported from farmed Atlantic salmon varies from outbreak to outbreak (Legård and Strøm, 2020). The reported differences in mortalities caused by pasteurellosis and difference in rearing conditions under controlled laboratory experiments compared to field situations might explain why it has been difficult to replicate the diseases in Atlantic salmon under research conditions previously and can point to *P. atlantica* genomovar *salmonicida* beeing a opporunistic patogen and not a primary patogen (Sandlund *et al.*, 2021).

Field observations of mortality due to pasteurellosis in Atlantic salmon indicates that outbreaks often occur between two to three weeks after a mechanical salmon lice treatment. This type of treatment has been shown to increase stress levels in fish (Gismervik *et al.*, 2017). Thereby opening the possibility for that disease is triggered in asymptomatic carrier fish due to stress inflicted by the mechanical lice treatment. In the current experiment bath challenged fish and the cohabitants of this fish were exposed to acute stress two weeks before termination of the challenge experiment, this did not cause any additional mortality. This can be due to the handling procedure being an acute stressor, and a more chronic stress response is regarded as immunosuppressive (Webster *et al.*, 2018) and thus more likely to influence on disease progression.

The gross pathology registered during this challenge experiment had several overlaps with pathology described from clinical sick fish in fish farms. This includes abscesses in the muscle, exophthalmia, bacterial layer on the internal organs, and fistula under the pectoral fin. This shows that the challenge experiment did replicate the conditions where gross pathology is developed and confirmed that the described pathology from Atlantic salmon is caused by *P. atlantica* genomovar *salmonicida*.

In addition to the previously described gross pathology reported from fish farms (Legård and Strøm, 2020; Sommerset *et al.*, 2022) interesting not previously described pathology were observed on the challenge fish. This includes erosion and abscesses on the lower jaw of several fish, and eroded fins that for many fish was bleeding and, in some cases, had a bacterial puss layer on top. Fin erosions with or without bleeding and bacterial puss, was the most common finding on challenged fish irrespective of mode of challenge. Though the prevalence of this findings varied between the different isolates. Bacterial layer on fins was only registered in one cohabitant of i.p. PAL3, in contrast it was found in 6 fish from the PaL2 challenged group (both shedders and cohabitants). For bath challenged shedders and cohabitants, the same type of fin lesions was the most common pathology. It is notable that not all fish that despised these fin lesions, were found to have the presence of *P. atlantica* genomovar *salmonicida* in the anterior kidney after qPCR analysis. If the bacteria attack the fins first, the skin and mucus barrier need to be breached first before the infection may become systemic and reached the anterior kidney. An early stage of infection could thereby explain why the bacterium do not show up in the qPCR analysis of tissue from anterior kidney.

The gross pathology of abscesses on the lower jaw, were not registered until sampling 63 dpc (sampling 6), which was three weeks after the bath challenge groups were exposed to acute stress. The PaL1 i.p. cohabitant fish collected at sampling 6 (63 dpc) where an abscess was observed was not exposed to acute stress, confirming that the stressor did not inflict these jaw lesions. All fish displaying this pathology was exposed to the bacteria from the water, either by being cohabitants or being bath challenged.

The i.p. challenged shedders generally displayed more pathology compared to their respective cohabitants through the first three sampling timepoints. For sampling 5 (49 dpi) and 6 (63 dpi), the difference in observed clinical signs was reduced. For bath challenged groups there was not a noticeable difference in observed pathology between shedders and cohabitants in the first three samplings. A possible explanation for this is the infection method, i.p. shedders received a high dose of bacteria injected into the body cavity, circumventing the outer physical and

immune barriers of the fish. Injected bacteria could be observed as a layer covering internal organs at sampling seven days post injection, after this infection had to develop through colonization and propagation over time before the i.p. shedders could shed bacteria to the water and through this infect the cohabitants. The water samples collected from the challenge experiment was not a part of this study. Analysis of these can give more insight to when the bacteria was shed to the water in these groups, and if there were any differences in timepoints and levels of bacteria shed between the groups injected with the three different isolates.

The prevalence of positive bacteriology samples from sampled fish varied between the different experimental groups. *P. atlantica* genomovar *salmonicida* was not grown from streaks on BA from anterior kidney from three challenge groups, PaL3 i.p. cohabitants, PaL1 bath shedders, and PaL2 bath shedders. In addition, several of the i.p. shedders form both PaL1, PaL2, and PaL3 did not have growth of *P. atlantica* genomovar *salmonicida* on BA. For a bacterium to grow on agar, it must be alive. The results indicate that living *P. atlantica* genomovar *salmonicida* was not precent in the anterior kidney of the individual shedder fish at the time of sampling. This opens the possibility for that the fish's immune system has eliminated *P. atlanitca* from the anterior kidney, or that the bacterium was not present in the anterior kidney, but it does not exclude that the bacterium was present in the fish. At end sampling *P. atlantica* genomovar *salmonicida* were grown on BA from PaL3 i.p. cohabitants, PaL1 bath shedders, and PaL2 bath shedders.

All but three dead/moribund fish were positive for *P. atlantica* genomovar *salmonicida* on BA. One of the three fish where it was not detected was the dead non-infected control fish, this fish died of other reasons. From streaks from a wound and anterior kidney *Photobacterium phosphoreum* was confirmed present by sanger sequencing. A bacterium commonly found in the marine environment and derby also on marine fish (Dalgaard *et al.*, 1997), thought we cannot conclude if it was the causative agent of the death of this fish. For the two other fish no other analysis was done to determine the cause of death, but these fish were frozen before bacteriology sampling, and although most of the fish was fully thawed, the anterior kidney was still partly frozen when the fish was sampled. Therefore, it was not possible for sampling to be performed properly and with good sterile technique. Therefore, it is still highly possible that the fish was infected by *P. atlantica* genomovar *salmonicida* even though the bacterium was not isolated from the fish.

Generally, at all sampling points a higher number of fish were positive for the presence of *P. atlantica* genomovar *salmonicida* when analysed using qPCR compared to bacterial growth on

BA. Nevertheless, in some cases growth of *P. atlantica* genomovar *salmonicida* on BA was detected after streaks from anterior kidney for fish where the bacterium was not detected after qPCR analysis. This can be explained by that only a small sample of the kidney was collected for the qPCR analysis while a larger area is subject to swabbing for the BA growth analysis. In cases of low bacterial load, such as in carrier fish, bacterial cells may not be present in a small sample and fish sampled at scheduled samplings were randomly selected and not necessarily showing clinical signs of disease.

In general, at scheduled samplings no clear pattern was observed in detection of *P. atlantica* genomovar *salmonicida* after streaks on BA. For i.p. infected fish, irrespective of isolate used for challenge, samples were positive on BA during the first 14 days after infection whereafter the bacteria were not detected. In respective cohabitant fish most samples, irrespective of isolate used for challenge, were randomly positive for growth on BA throughout the challenge experiment. For bath challenged fish samples were negative for growth on BA throughout the experimental period, except for one fish challenged by PaL3 sampled three weeks post challenge. For their respective cohabitants only a few fish randomly tested positive for growth on BA.

For the qPCR analysis of samples collected at scheduled samplings no clear difference was detected between groups challenged with the three isolates. Most i.p. infected fish were positive in the first three weeks post challenge, whereafter the prevalence decreased. In their respective cohabitants positive fish were also detected the first three weeks of the challenge experiment, but the prevalence of positive samples was high throughout the experimental period. In bath challenged fish positive samples were registered throughout the experimental period, but most positive fish were found in the PaL2 challenged group. For their respective cohabitants most samples were positive throughout the experimental period, and all samples were positive for the PaL3 challenged groups. For all groups Ct values were mostly high, indicating that the load of bacteria present in the fish is low.

The quantitative findings of bacteria found clinically sick fish with pasteurellosis, indicates that the bacteria observed in the histological examination was *P. atlantica*. This finds are consistent with earlier described findings with large amounts of bacteria in infected areas of the fish (Legård and Strøm, 2020). This being bacterial accumulation in the lower jaws of clinical sick fish.

4.1 Primer optimalisation for real time qPCR

To analyse the collected samples by qPCR a suitable assay had to be developed. The aim was to establish an assay that only amplified *P. atlantica* genomovar *salmonicida,* and not *P. atlantica* genomovar *salmonicida*. To investigate this all assays were tested against both the Atlantic salmon isolate and lumpsucker isolate. In the initial general test, all specific primers designed for this thesis were eliminated based on attachment to both bacteria. Assays from this general test that were further analysed were the Atlantic salmon assay from (Sandlund *et al.*, 2021), referred to here as assay 1, and sodA from (Ellul *et al.*, 2019). Assay 1 was developed as a *Taq*Man assay specific to *P. atlantica* genomovar *salmonicida* and sodA were developed as a SYBR green assay for *P. atlantica* genomovar *salmonicida* generally. The stability of both assays was tested with use of SYBR green system by compiling standard curves. For assay 1 this evaluation concluded that using the SYBR green system and with the chosen running protocol, the assay was not stabile. The sodA assay was evaluated, and this assay was concluded to be stable with SYBR green system and with the chosen running protocol. Se standard curve details in Table 8.

SYBR green and *Taq*Man are different systems used to detect an amplifying product in a qPCR reaction. SYBR green molecules will not emit fluorescent light when not bound to double stranded DNA, but will form unspecific binding with doble stranded DNA that the primer in a reaction amplifies. For *Taq*Man a probe specific to a pathogen is mixed with the primers, both primers and probe binds to DNA during annealing. On the probe a reporter and a quencher are attached, and as long as both are attached, no fluorescent light is emitted. When a new DNA strand is synthesised, the polymerase destroys the probe, and the reporter will be released from the probe. When this happens, fluorescent light is emitted. The *Taq*Man probe will only bind to one DNA sequence and are therefore a more specific system that must be fitted to each pathogen. For SYBR green to be specific the assay being used has to be specific to the sequence it is design to amplify. If an analysis is performed to detect many pathogens to use a *Taq*Man system can be preferable, since one reaction can contain different assays and probes with different fluorescents light to test against different pathogens at the same time (Smith and Osborn, 2009).

When sodA was not specific to *P. atlantica* genomovar *salmonicida* new primers were designed and an initial general test of assays was evaluated, referred to here as Assay C and Assay D, see Table 8. Both assays were specific for *P. atlantica* genomovar *salmonicida* and did not react

with *P. atlantica* genomovar *cyclopteri*. Assay D was selected for analysis of tissue sampled from challenged fish based on better efficiency and stability. The target of Assay D was BLASTed and the assay picked up a protein from *Phocoenobacter uteri* (Foster *et al.*, 2000) with 80 % similarity. This bacterium was evaluated to be unlikely to be present in the water in the challenge experiment and thereby also in the fish.

Two qPCR analysis were performed on the samples from anterior kidney collected at regular samplings. First with assay C, and then with assay D. Analysis of the data with assay C was not carried out, this since further analysis of the target of the assay in BLAST indicated that the assay had an over 80 % similarity to many other sequences. Therefore, assay D was used to analyse the samples. After analysis of the assay D, melting curves were analysed and agarose gels analysed for primer dimers and no primer dimer was identified. Assay D was further analysed with a calculation curve to enable analysis of the number of bacteria in the test samples later.

As referred to in section 3.8 some of the background samples from the fish groups and bath controls were identified with the presence of *P. atlantica genomovar salmonicida genomovar salmonicida* in the qPCR analysis, this was later confirmed to be due to contamination. Samples collected from non-infected i.p. controls did not show the presence of *P. atlantica* genomovar *salmonicida* as expected. This indicates together with samples from non-infected bath control and zero samples that the fish did not have a prior infection with *P. atlantica* genomovar *salmonicida* before the challenge started. This means that fish were infected with *P. atlantica* genomovar *salmonicida* in the challenge experiment.

During processing for DNA isolation from samples from both samplings, zero samples and dead/moribund fish, anterior head kidney or in some instances heart was cut in smaller pieces. A microscope slide was used as a cutting board and a scalpel blade was used to cut the tissue. Between each sample both the microscope slide and the scalpel blade were swapped for new ones to limit cross contamination between samples. The same tweezers were used to collect the tissue form the sample tubes. To limit possible cross contamination, the tweezers were disinfected with 96 % ethanol between processing each sample. Thereby the possible cross contamination of samples during DNA isolation were if the tweezers was not sufficiently disinfected in the ethanol. Cross contamination of samples would only affect samples processed on the same day. This since tweezers were replaced between isolation days. All non-infected fish were processed alone. Background samples were processed after samples from

dead/moribund fish, so cross contamination via tweezers may have happened between dead/ moribund fish and background samples.

Other possible contamination-points for samples can be possible aerosols from infected samples. These possible aerosols contaminations could have happened when templates were diluted or plated into PCR plates before being passed the pipetting machine.

4.2 Concluding remarks and future perspectives

Mortality was obtained after experimental challenge. Highest mortality was obtained in i.p. injected fish and their respective cohabitants. No difference in mortality was observed between fish challenged with the three isolates of *P. atlantica* genomovar *salmonicida*.

During the experimental challenge *P. atlantica* genomovar *salmonicida* was isolated and grown on BA from all challenge groups, thereby confirming that the challenge model could infect Atlantic salmon with *P. atlantica* genomovar *salmonicida.* The bacterium was re-isolated from clinically sick fish, thereby the model manages to produce pasteurellosis in Atlantic salmon.

Gross pathology, previously described in Atlantic salmon suffering from pasteurellosis in field was replicated using experimental challenge. Observed histopathology was consistent with previous findings, this being presence of bacteria in areas with macroscopical change. Acute stress that bath challenge fish were exposed to did not affect the mortality rate of the fish.

For further work, the water samples taken during the challenge experiment should be analysed to examine the possible bacterial load in the water shed at different time points post challenge.

A more comprehensive qualitative analysis of histology from sampled and moribund fish will increase the knowledge about the disease itself and how it manifests in infected fish.

A challenge trial were fish are not regularly sampled can bring more knowledge on mortality induced by different routes of entery used for challenge, and from this it can be determined if the challenge model can be used to evaluate the effectiveness of a vaccine.

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6. Appendix

6.1 Gross pathology

Table 12 Pathology of dead/moribund fish throughout the challenge. If fish from a group showed the different pathology that are marked with a x in the table. Pathological that are listed are 1) Abscess /inflammation injection point, 2) Ascites body cavity, 3) Eroded fins, 4) Bleeding injection point, 5) Inflammatory gut, 6) Enlarged spleen, 7) Inflammation/abscess muscle, 8) Bleeding from internal organs, 9) Wound, 10) Ascites hart cavity, 11) Petecchia in muscle, 12) Bleeding fins, 13) Petecchia on operculum, 14) Pericardia on lip, 15) Eroded lover lip, 16) bleeding in psheudobranck, 17) Fistula, 18) Bacteria puss on internal organs, 19) bleeding in eye, 20) Liquid in swim bladder, 21) bloody vent and 22) distended abdomen. Groups are divided based on infection method and isolate.

Isolate	**PaL1**			**PaL2**		**PaL3**		
Group / Pathology	i.p. - S	i.p. - C	Bath - S	i.p. - S	i.p. - C	i.p. - S	Bath - S	Bath - C
1	x			x		x		
2	x		x	x		x	x	x
3	x			x	x	x		
4	x							
5	x			x				
6	x	x		x		x	x	x
7				x			x	
8	x			x		x	x	
9	x					x		x
10	x						x	x
11	x					x		
12		x	x		x		x	x
13							x	
14								x
15					x			
16					x			
17		x						
18		x						
19		x				x		
20	x	x				x		
21				x		x		
22				x				

Table 13 Pathology of fish from samplings where *Pasteurella*. *atlantica* genomovar *salmonicida* were grown form anterior kidney. Only infection groups that had fish with bacterial growth from anterior kidney are listed. Groups are divided based on infection method and isolate.

Isolate	**PaL1**			**PaL2**			**PaL3**		
Group / Pathology	i.p. - S	i.p. - C	Bath - C	i.p. - S	i.p. - C	Bath - C	i.p. - S	Bath - S	Bath - C
Bacteria puss fins				x		x		x	x
Eroded fins	x	x		x	x	x			
Bleeding in fins	x			x	x	x		x	x
Abscess lower jaw		x	x	x				x	
Abscess mouth								x	
Anaemic gills								x	
Anaemic blood								x	
Bleeding in internal organs	x							x	
Anaemic heart								x	
Pale liver								x	
Abscess /inflammation at injection point	x			x					
Petecchia in muscle	x						x		
Bloody vent	x								
Enlarged spleen	x								
ascites	x						x		
Bloody liquid in heart cavity	x								
exophthalmos	x								
puss pseudobranchs	x								

Table 14 Pathology of fish from sampling where bacteria was not grown form anterior kidney, though Q-PCR did show presents of *Pasteurella. atlantica genomovar salmonicida*. Only infection group that had fish with bacterial growth from anterior kidney are listed. If fish from a group pathology these are marked with a x in the table. Groups are divided based on infection method and isolate.

Isolat	**PaL 1**				**PaL 2**				**PaL 3**			
Group / Clink or pathology	i.p. - S	i.p. - C	Bath - S	Bath - C	i.p. - S	i.p. - C	Bath - S	Bath - C	i.p. - S	i.p. - C	Bath - S	Bath - C
Bacteria puss fins		x	x	x	x			x	x	x		x
Eroded fins		x	x	x	x	x	x	x	x	x	x	x
Bleeding fins		x	x	x	x	x	x	x	x	x	x	x
Abscess lower jaw								x				
Bleeding from internal organs							x				x	
bloody swollen vent											x	
ascites			x				x				x	
Pached gills			x									
Wound				x							x	
Bacterial layer on hind gut											x	
Fibrin on liver											x	
Shortened operculum					x			x				
Wound lower jaw					x							
Eroded lower jaw		x				x		x	x	x		x
Yellow area on skin									x			
Abscess muscle												x
Bleeding in eye										x		

6.2 Overview of reagents and kits

Table 15 Reagents used for bacterial growth and buffers used for resuspension and cell counting

Ingredients	Quantity	Producer
TSB 2 % NaCl		
I. TSB	30 g	BD Bacto™ BD Diagnostic Systems #LOT 9302047
II. NaCl	15 g	Honeywell Fluka™ #LOT L2180
III. Mili-Q Water	1 L	Merck Millipore, model Milli-Q® Advantage A10® System
Grow medium		
I. TSB 2 % NaCl	40 mL	Se above
II. Fetal calf serum (FCS)	4 mL	Gibco Lot 2094466RP
PBS		BioWhittaker®, Lonza Lot 7MB119
CAYS-ton		OMNI Life Science, Lot 177001

Table 16 reagents used for storage of samples before further processing

Ingredients	Producer
RNA-Latet	Sigma, Lot MKCB4095
Formalin 3.7 %	VWR chemicals, Lot 20K164130

Table 17 Reagents used for qPCR analysis

Ingredients	Producer
2x SYBR Green JumpStart Taq Ready Mix	Thermo-Fisher Scientific, Source SLCL5820
F primer	Sigma-Aldrich
R primer	Sigma-Aldrich
DNase and RNase free water	Sigma-Aldrich, Lot RNBJ6314

Table 18 GelElute™ PCR Clean-Up Kit, Sigma-Aldrich

Kit
Column Preparation solution
Binding solution
Wash Solution Concentrate
Elution Solution
GelElute plasmid mini spin column
Collection Tubes, 2 mL

Table 19 DNeasy® Blood & Tissue Kit, Qiagen

Kit
Buffet ATL
Buffer AL
Buffer AW1 concentrate
Buffer AW2 concentrate
Buffer AE
Protinase K
DNeasy Mini Spin Columns in 2 mL Collection tubes
2 mL Collection tubes