The effect of using different salinities in smolt production on the susceptibility to tenacibaculosis in Atlantic salmon smolts after seawater transfer

– Establishing a challenge model for Tenacibaculum finnmarkense strain HFJ^{T} at 8 °C

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Abstract

The aim of this study was to establish a challenge model for *Tenacibaculum finnmarkense* strain HFJ^T at 8 °C and investigate the possible effect of using different salinities used in smolt production facilities on the susceptibility of the smolt to tenacibaculosis after transfer into sea water net-pens. Tenacibaculosis is an increasing problem in the Norwegian Atlantic salmon aquaculture causing significant economic losses and reduced fish welfare. The disease normally affects smolts 1-3 weeks post transfer into sea water and are associated with low sea water temperatures. It has been observed that after a period of exposure to sea water, the smolts appear to become less susceptible to tenacibaculosis. A challenge study was conducted in order to study the effect of exposing the smolts to two different water qualities; freshwater (F) and low salinity sea water (LSS) (26 ppt).

Smolts kept in the two different water qualities for four or eight weeks were challenge with *Tenacibaculum finnmarkense* strain HFJ^T for 2 hours at 8 °C. Bacteriology, histology, real-time RT-PCR and scanning electron microscopy (SEM) were performed on tissue samples collected during the challenge study. A tenacibaculosis welfare scoring scheme was used to assess the fish welfare. The average percent mortality in each group was used to compare the susceptibility to tenacibaculosis. The study shows that a higher bath concentration is needed to induce tenacibaculosis at 8 °C compared to studies at lower temperatures. Results from the challenge study show that keeping the smolts in LSS for four or eight weeks prior to sea water transfer has a positive effect on the smolts susceptibility to tenacibaculosis. These findings are important when developing new smolt production facilities, such as sea water recirculation aquaculture systems (RAS). Further investigations are needed to study the effect of LSS at lower temperatures and to determine what salinity (ppt) smolts need to be produced in order to obtain the beneficial effects of reduced susceptibility to tenacibaculosis as demonstrated in this study.

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Abbreviation

F	Freshwater
LSS	Low Salinity Saltwater
ILAB	Industrilaboratoriet
RAS	Recirculating Aquaculture System
mL	Milliliter
L	Liter
MPN	Most Probable Number
OD	Optic Density
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
Ct	Cycle threshold
NTC	Non-template Control
BAMA	Blood agar with sea salt
BAS	Blood agar supplemented with 1.5 – 2 % NaCl
MA	Marine agar (Difco 2216)
MB	Marine Broth (Difco 2216)
Pers.com	Personal communication
h	Hour

Table of content

1. Introduction

1.1 Norwegian salmon farming

The Norwegian Atlantic salmon farming industry has experienced significant growth since its beginning in the 1970s. In 2018, 1 281 872 tons of Atlantic salmon were produced in Norway (Statistics Norway (SSB) 2019). This enormous growth in such a short period of time facilitates increased infection pressure and new challenges concerning fish health and welfare. Today, sea lice, viral diseases, gill-problems and skin ulcers represents the main challenges for the Norwegian Atlantic salmon farming industry (Sommerset et al., 2020). Some years back, the situation was different and bacterial diseases dominated. In the 1980s to early 1990s, several bacterial diseases such as cold-water vibriosis (*Aliivibrio salmonicida*), vibriosis (*Vibrio anguillarum*) and furunculosis (*Aeromonas salmonicida subsp. salmonicida*) caused mass mortalities of farmed Atlantic salmon in Norway. This resulted in the use of a vast amount of antibiotics (Sommerset et al., 2005). In the early 1990s, a commercial oil-adjuvant injection vaccine was introduced to the industry, which led to control over the bacterial diseases mentioned above without the use of antibiotics. Even though many of the bacterial diseases in farmed Atlantic salmon is managed with vaccines today, it has been difficult to produce vaccines for ulcerative bacterial skin diseases.

1.2 Bacterial ulcer disease

Bacterial skin ulcers represent a significant welfare problem in the Norwegian salmon farming industry (Colquhoun & Olsen, 2019; Olsen et al., 2011). The mortality is usually below 10 % but the amount of fish affected at one site will lead to large economic losses, due to downgrading at slaughter. The development of ulcers typically occurs during the autumn and the winter months at low seawater temperatures. However, the ulcer can also occur throughout the year along the entire Norwegian coastline, especially in the northernmost parts of the country (Colquhoun & Olsen, 2019; Bleie & Skrudland, 2014). Due to the higher incidents of skin ulcers during the cold winter months, it is commonly referred to as the "winter ulcer disease".

There are typically two different types of bacteria isolated from salmon with ulcers when using BAS (blood agar supplemented with 1.5-2% NaCl): *Moritella viscosa (Vibrio viscosus)* and *Allivibrio wodanis (Vibrio wodanis)* (Lunder, 1992). However, in a transmission

experiment performed by Lunder (1992) using these isolates, it was shown that only *Moritella viscosa* reproduced the clinical signs of winter ulcers and successfully fulfilled Koch`s Postulates (Bruno et al., 1998; Lunder et al., 1995). Based on this experiment it was established that *M. viscosa* was the most important factor in causing winter ulcer disease. The disease has been described as a systemic disease characterized by ulcers that appears as circular, epidermal lesions affecting the scale covered part of the body, and degeneration in the underlying muscle (Gudmundsdóttir & Björnsdóttir, 2007; Salte et al., 1994). The bacteria infect Atlantic salmon in saltwater at temperatures below 10 °C. The disease is mainly a problem in Norway and Iceland, but is also reported from the Faroe Islands, Denmark, Ireland and Canada (Gudmundsdóttir & Björnsdóttir, 2007). Vaccines targeting *M. viscosa* have significant protection against ulcer development and mortality (Bleie & Skrudland, 2014). Despite this, winter ulcer disease still represents a threat (Olsen et al., 2011). This may be linked to the appearance of a variant type of *M. viscosa* isolates (Takle et al., 2015).

Routine histopathological examination of winter ulcer cases at the National Veterinary Institute over the years revealed long, rod-shaped bacteria frequently present in the ulcers, either alone or in mixed culture with *M. viscosa* and other bacteria. Further examination of these bacteria showed yellow-pigmented colonies with long rods when cultivated on marine agar (MA) (Olsen et al., 2011). Based on phenotypic characterization and 16S rRNA sequencing, the isolates could be separated into two groups, both belonging to the genus *Tenacibaculum*. These types of bacteria were observed at histopathological examination already at the end of 1980s but have probably been underdiagnosed because the bacteria could not be grown on the standard growth medium (blood agar supplemented with 1.5 – 2 % NaCl (BAS)). The bacteria do not grow on BAS, which is still used in routine bacteriological investigations of skin lesions/ulcers in Norway (Olsen et al., 2011; Småge et al., 2016a; Takle et al., 2015).

In 2010, *Tenacibaculum* was for the first time associated with high mortality of Norwegian farmed Atlantic salmon (Bornø & Sviland, 2011). The disease was reported along the coast of Norway from seawater-reared Atlantic salmon at low sea-temperatures in the spring and early summer. Affected fish were newly transferred to sea and showed lesions/erosions mainly in the head region, but also skin and fins were affected during the outbreak. The bacteria were identified as isolates closely related but not identical to *Tenacibaculum* spp. isolates that have earlier been observed in association with winter ulcer disease in Norway (Bornø & Sviland,

2011). Although this is the first time *Tenacibaculum* is mentioned, there has been reports of large losses in Norwegian farmed Atlantic salmon farms occurring in the first few months after seawater transfer (Aunsmo et al., 2008). In the fall of 2014 and 2015, several separate outbreaks of suspected tenacibaculosis occurred at Atlantic salmon farming sites in Finnmark in Northern Norway. This resulted in major losses of smolt newly transferred to seawater. The seawater temperature during these outbreaks were above 8°C, which is higher than the normal temperature for winter ulcer disease and the clinical signs matched what is described for tenacibaculosis (Småge et al., 2017). In these cases, a stressor (jellyfish) was present and may have been a contributing factor.

Over the past few years, there has been a growing interest focusing on the *Tenacibaculum* bacteria. In 2019, fish health personal in Norway ranked infections caused by *Tenacibaculum* sp. as one of the top 10 problems for Atlantic salmon in sea water net-pens, causing high mortality and reduced welfare (Sommerset et al., 2020). It has been showed that a whole cell inactivated vaccine using *T. finnmarkense* strain HFJ^{T} induces an antibody response but do not protect against tenacibaculosis induced through a bath infection performed at low seawater temperatures (Småge et al., 2018). The most common production strategy of smolt today represents newly smoltified smolt that is direct transferred from freshwater to seawater shortly following smoltification. The productions strategies are in constant development and new strategies, including RAS and production of "post-smolt" in closed facilities is under development (Bergheim et al., 2009; Ytrestøyl et al., 2019). The new strategies may facilitate new challenges concerning fish health but may also contribute to new strategies help solve big challenges the industry is facing today, such as tenacibaculosis.

1.3 Genus Tenacibaculum

The bacteria genus *Tenacibaculum* belongs to the family Flavobacteriaceae, which is the largest family in the phylum Bacteroidetes (McBride, 2014). The family consists of gram-negative, non-spore forming, rod-shaped bacteria that are chemoorganotrophic, with primarily respiratory metabolism.

Tenacibaculum spp. are common members of the marine environment and found worldwide. The bacteria are isolated from organic environment like fish skin, inorganic environment such as sediments and freely in the water (Takle et al., 2015). Several species are reported to be pathogenic to fish; *Tenacibaculum maritimum, Tenacibaculum soleae, Tenacibaculum*

ovolyticum, and in recent years *Tenacibaculum finnmarkense* and *Tenacibaculum dicentrarchi* have been isolated from skin lesions of fish. *Tenacibaculum* spp. have been associated with ulcers of farmed salmonids in Norway since 1980s, but changes in farming practice and introduction of new farming species to aquaculture may partly explain the increasing impact of these infections the recent years (Olsen et al., 2017, 2011). Several *Tenacibaculum* spp. are isolated from different fish species in Norway over the years.

1.3.1 Tenacibaculum finnmarkense

T. finnmarkense isolated in Norway is a gram-negative, non-flagellate, aerobic, rod-shaped bacterium that is motile by gliding. The rod-shaped cells are 0.5 μm in diameter and 2-30 μm in length. Aging cultures have frequently shown longer filamentous cells and degenerative spherical cells. A rapid decrease in viability occurs with prolonged incubation (>96 h) (Småge et al., 2016a). The incubation temperature is 15-20 °C and the bacterium should be sub-cultivated 48-72 hours after incubation to avoid reduced viability (Olsen et al., 2011; Småge et al., 2016a). *T. finnmarkense* is a marine bacterium that requires media containing 50-100 % strength seawater in order to grow. It is not sufficient to only add NaCl to the agar and the bacteria do not grow on BAS medium (Karlsen et al., 2017; Olsen et al., 2011; Småge et al., 2016a). Growth occurs at temperatures between 2-20 °C, but not at temperatures above 25 °C. The pH-optimum for growth is pH 7-8. *T. finnmarkense* colonies grown at marine agar (MA) medium are circular, convex with a varying degree of yellow pigmenting and translucent edges. The colonies have smooth texture with shiny and sometimes iridescent appearance, are slightly viscous and do not stick to agar.

1.4 Tenacibaculosis in Norway

Tenacibaculosis usually occur in Northern Norway Atlantic salmon farms in late winter and spring (February – April) at low seawater temperatures (3-6 °C) and in late summer and autumn (August – October) at higher seawater temperatures (Karlsen et al., 2017; Småge et al., 2017). The disease normally affects smolts 1-3 weeks post-transfer to seawater and is typically presented with an acute progression (Bornø & Sviland, 2011). While outbreaks at low seawater temperatures occur without any stressors, outbreaks during the autumn at higher seawater temperatures are associated with stressors like jellyfish (Småge et al., 2017).

Isolates of *Tenacibaculum* spp. from the Norwegian coast can be divided into four different clades (Olsen et al., 2017). *T. finnmarkense* consists of two clades, clade I and III. The type strain HFJ^T belongs to clade III, while the isolate TNO010 used in a challenge study by Olsen et al. (2011) and in Habib et al. (2014) belongs to clade I. *T. dicentrarchi* belongs to clade II and appears to be less pathogenic to Atlantic salmon smolts, mainly associated with skin lesions/ulcers in non-salmonid fish in Norway (Olsen et al., 2017; Småge et al., 2018). Isolate TNO020 belongs to clade IV and is shown to be non-pathogenic in a study conducted by Olsen et al. (2011). Based on results from challenge studies conducted using *T. finnmarkense* isolates from both clade I and III and MLSA studies, it appears that isolates belonging to clade III are more pathogenic to Atlantic salmon smolt than isolates from clade I (Olsen et al., 2017; Småge et al., 2016a). *T. finnmarkense* strain HFJ^T is the dominant strain recovered from Atlantic salmon sampled during tenacibaculosis outbreaks in Northern Norway (Småge et al., 2017).

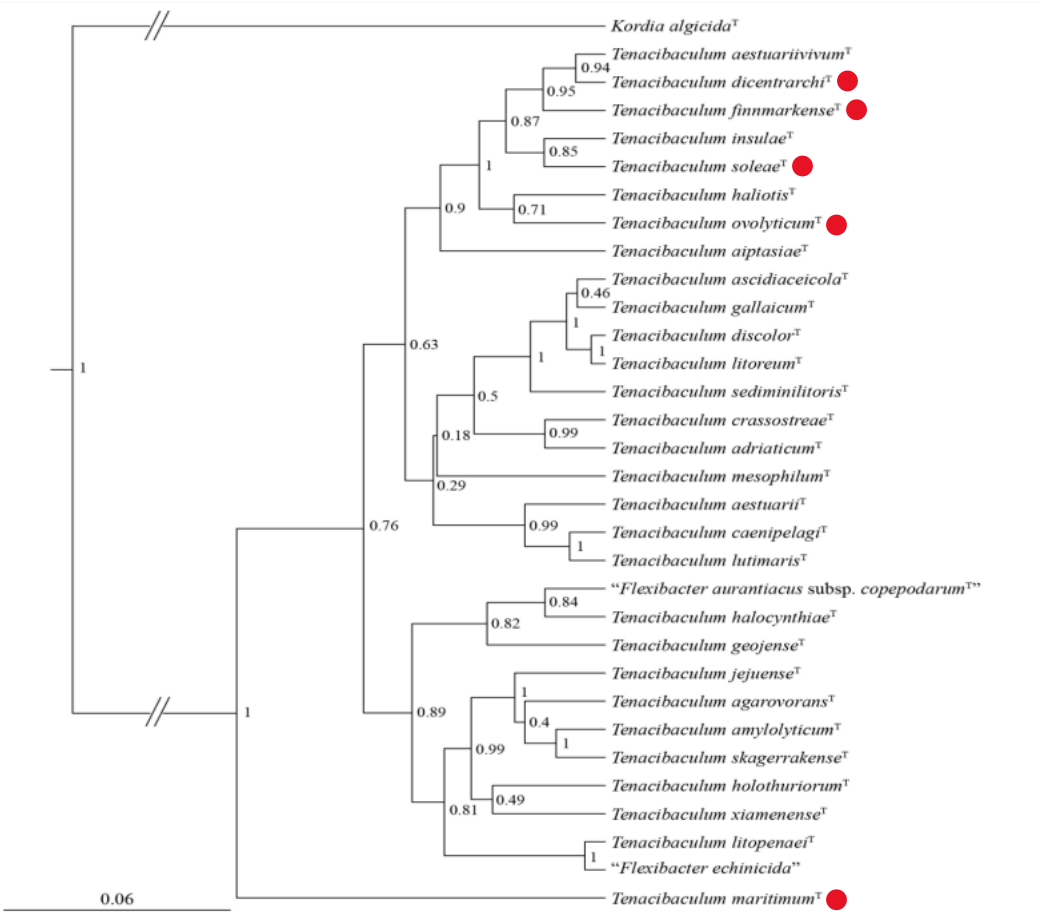

Figure 1. A phylogenetic tree based on 16S rRNA gene showing the phylogenetic placement of the type strains in genus *Tenacibaculum*. Both fish pathogenic species and environmental species are showed in the tree (Småge, 2018). The fish pathogenic species *T. dicentrarchi, T. finnmarkense, T. soleae, T. ovolyticum and T. maritimum* are indicated by a red dot.

The type-species *Tenacibaculum maritimum* is the best-known fish pathogen from the genus *Tenacibaculum*. In Norway, the bacterium has mostly been associated with disease in lumpfish but is also found in framed Atlantic farmed salmon in the recent years (PHARMAQ-Analytiq, 2017; Småge et al., 2016b). Unlike *T. finnmarkense*, *T. maritimum* is not associated with ulcer disease in farmed salmon in Norway, but it affects the gills. The bacterium mainly poses a threat at seawater temperatures higher than 10 °C and a reduction in gill-problems due to this species are observed at sinking seawater temperatures (PHARMAQ-Analytiq, 2017).

1.4.1 Clinical signs and gross pathology

Smolts affected by tenacibaculosis caused by *T. finnmarkense* in Northern Norway typically show abnormal swimming behavior. The unscaled part of the skin is usually most affected where fish most commonly shows mouth erosion, lesion/erosion on the fins and lesion/erosion on the caudal peduncle-tailfin (Småge et al., 2017). Lesions/ulcers also frequently occur along the abdomen. However, the smolt may show few or no pathological signs in the scale-covered parts of the skin. In scaled skin, lesions are often characterized by skin ulcers with uneven and yellow margins that are surrounded by a wide area of scale loss (Småge et al., 2017). This is different from ulcers described from *M. viscosa,* as these ulcers tend to have a more defined and rounder form with a narrower zone of scale loss (Salte et al., 1994). The fact that *T. finnmarkense* have a high affinity for unscaled skin is also a characteristic that distinguishes the two bacteria from each other as *M. viscosa* mainly affects the scaled part of the skin (Salte et al., 1994). The ultimate cause of death of smolts affected with tenacibaculosis in Northern Norway is not known, but the destruction of the osmotic barriers of the skin is likely an important factor (Zydlewski et al., 2010). Smolts have a less favorable surface/volume ratio compared to that of larger salmon, which may partly be why smolt are more affected by tenacibaculosis than larger fish. Mortality during outbreaks may also be associated with the release of potent exotoxins as experimentally shown for *T. maritimum* (Van Gelderen et al., 2009).

1.4.2 Microscopic pathology

Histopathological assessments of advanced jaw lesions from smolt affected with tenacibaculosis in Northern Norway with complete loss of epidermis reveals a large number

of bacteria with *Tenacibaculum* morphology in the loosely organized dermis of unscaled skin. In scaled skin, a large number of bacteria with *Tenacibaculum* morphology is typically found in the *stratum spongiosum*, which has a similar tissue organization as the dermis of unscaled skin (Småge, 2018). In cases where the epidermis is partly intact, bacteria with *Tenacibaculum* morphology are normally found in the dermis below. In histological sections, *Tenacibaculum*-like bacteria are rarely observed in the epidermis (Olsen et al., 2011). In less affected tissue where the epidermis is intact, the bacteria with *Tenacibaculum* morphology are typically observed in *stratum spongiosum* following the lining of the basement membrane and scale pockets (Småge, 2018). Scale covered areas in the skin that are heavily affected typically have a large number of bacteria with *Tenacibaculum* morphology replacing the *stratum spongiosum*. Bacteria are also found infiltrating the *stratum compactum*, where they are observed infiltrate the collagen fibers. In severe cases, bacteria with *Tenacibaculum* morphology can be observed invading into the connective tissue of the hypodermis and associated with muscle tissue. At this stage, degenerative muscle fibers may be observed (Småge, 2018).

1.4.3 Diagnostics

Diagnostics of tenacibaculosis involves microscopy of wet mount preparations from diseased fish for a large amount of long, gliding rod-shaped bacteria at the site of infection (McBride, 2014). For isolation, the bacteria should be grown on a suitable medium. BAS is routinely used as a standard growth medium in the bacteriological investigation of skin lesions/ulcers in Norwegian aquaculture but this medium does not support growth for several *Tenacibaculum* spp. and should not be the only medium used as growth media in bacteriological investigations of skin lesions/ulcers (Olsen et al., 2011; Småge et al., 2016a). Marin agar (MA) is a medium that provides the nutrients and sea salts necessary for growth of *Tenacibaculum* spp., and should be used in bacteriological investigations of skin lesions/ulcers in Norway.

When *Tenacibaculum* spp. are successfully isolated, the bacteria can be identified by sequencing the 16S rRNA gene using universal primers for bacteria and then upload their sequence to the BLAST. To separate closely related strains within a species, a multilocus sequence typing (MLST) scheme has been developed for *Tenacibaculum* (Habib et al., 2014). As an alternative to MLST, it has been proposed to use a single housekeeping gene as a rapid,

reliable and less costly diagnostic typing approach for *Tenacibaculum* spp. The gene suggested to be used is *rlm*N, as it reflects the result from MLSA studies using 7 or more HK-genes (Olsen et al., 2017). Recently, other identification tools like MALDI-TOF MS has been advocated as a rapid method reliable for identification of *Tenacibaculum* to the subgroup level (Olsen et al., 2019). With the recent events in whole genome sequencing, this method would likely become an important tool in the future (Bridel et al., 2018).

1.5 The project and aim of the study

This study is part of a larger project financed by FHF: *Begrense effekten av tenacibakulose i norsk lakseoppdrett (LimiT).* The main goal for this project is to reduce the significance of tenacibaculosis in the Norwegian Atlantic salmon farming industry. This includes improving fish-health, reduce mortality and downgrading of slaughtered salmon. A specific goal for this project was to improve the existing challenge model for *T. finnmarkense* and to investigate mitigation measures. A major focus of this master thesis was to study the effect of using different salinities in smolt production on the susceptibility of the smolt to tenacibaculosis during early production in sea water net-pens. The working 0-hypothesis for this current study was: Different salinities in smolt production facilities do not affect the smolts susceptibility to tenacibaculosis in seawater net-pens.

2. Materials and methods

2.1 Challenge design

The main aim of this study was to investigate different smolt production strategies to reduce infections caused by *Tenacibaculum finnmarkense* in seawater. A challenge study was conducted from May to July 2019 at the Aquatic and Industrial Laboratory (ILAB), Bergen, Norway, where Atlantic salmon smolts were bath infected with *Tenacibaculum finnmarkense* strain HFJ^T. The challenge study was divided into three challenge trials designated; Challenge 1F, Challenge 2F and 2LSS and Challenge 3F and 3LSS. The purpose of these challenge trials was to investigate the effect of keeping smolts in either freshwater (F) or low salinity seawater (LSS), before seawater transfer and exposure to the challenge material, on their susceptibility to tenacibaculosis. A pre-challenge was conducted in order to determine the appropriate challenge dose for the main challenge study.

In Challenge 1F, newly smoltified salmon were directly transferred to seawater before being challenged with *Tenacibaculum finnmarkense* strain HFJ^T. This strategy of transferring newly smoltified salmon directly from freshwater to seawater represents the most common production strategy in Norwegian Atlantic salmon farming. In Challenge 2F and 2LSS, smolts that were kept at two different water qualities for 4 weeks post smoltification were transferred into seawater and challenged with *Tenacibaculum finnmarkense* strain HFJ^T. The purpose of this challenge trail was to investigate whether an increase in fish size or water quality affected the smolts susceptibility to tenacibaculosis. In Challenge 3F and 3LSS, the smolts were kept in two different water qualities for 8 weeks post smoltification before being transferred into seawater and challenged with *Tenacibaculum finnmarkense* strain HFJ^T. At this stage, the smolts are considered to be what is typically referred to as “post-smolts” (Ytrestøyl et al., 2019). The purpose of this challenge trail was to investigate whether a further increase in fish size or further exposure for the different water qualities affected the smolts susceptibility of tenacibaculosis.

A separate master thesis conducted at Nofima uses this challenge study to investigate the development in the fish skin over time in the different water qualities. Skin was therefore sampled for histology prior to challenge and three days post challenge from both water qualities. The samples were used to compare the histology of non-infected skin to infected skin from fish from both water qualities. Data from this study is not included in the present

master thesis. However, a few relevant findings from this study has been mentioned in the discussion.

The challenge study was approved by the Norwegian Food Safety Authority (Mattilsynet) under the identification code 19450.

2.2 Fish husbandry

All Atlantic salmon smolts used in this study were provided by ILAB and originated from the same batch of salmon (Stofnfiskur). The provided salmon (25-30 g) were transferred to the production facility (PF) into four 500 L tanks containing 12 °C freshwater. Fish in two of the tanks was subjected for low oxygen which resulted in mortality in one of the tanks. The fish was replaced by fish from the same fish batch and all four tanks were remixed so that any bias was eliminated. The fish used in the challenge study were not vaccinated. Smoltification was initiated approximately five weeks prior to challenge 1F. In the smoltification period the photoperiod was increased from 12:12 to 24:0. After smoltification, two of the four tanks were set to 26 ppt (LSS), whilst the other two were set on freshwater (F). When smolts were transferred from the PF to the challenge facility (CF), the temperature was gradually lowered from 12 °C in the PF to 10 °C in CF, and further lowered to 8 °C two days before exposure to the challenge material. The smolts were starved for 48 hours prior to handling. The smolts were always transferred to the same water quality as they originated from. Full-strength seawater (34 ppt) was turned on 24 h before the fish was challenged. All challenge procedures were conducted at a seawater temperature of 8 °C and the fish were fed with the commercial dry feed Nutra Olympic, Skretting AS, Norway. Flow-through tanks of 150 L were used in all challenge trials, with a water flow of 300 L per hour per tank. The water temperature, salinity and oxygen levels were measured daily. The minimum oxygen saturation was 85 %. The fish were kept on a 24 hours photoperiod during all challenge trials.

Freshwater was supplied from lake Svartediket in Bergen, approximately 2 km from ILAB`s facilities. The freshwater is filtrated through seven filters. Kitoflokk™ Chitosan is added as a precipitant and humus particle was filtrated out through sand filters of different mesh sizes. After filtration, the freshwater is UV-treated in order to remove any bacteria or viruses present in the water. Lastly, silicate is added to neutralize the toxicity of any metals in the water. The freshwater is pH-adjusted by going through a sand filter and by the added silicate.

The seawater at ILAB is retrieved from 105 meters deep 3 km from ILAB`s facilities. The seawater is first filtrated through a drum filter of 20 μm and then UV-treated before reaching the fish tanks.

The smolts were screened and found negative for *Piscine orthoreovirus*, *Piscine myocarditis virus*, *Infectious pancreatic necrosis virus*, *Infectious salmon anemia virus* and salmonid alphavirus by ILAB. In addition to ILAB`s screening, 0-samples of ten fish were sampled prior to each challenge trial in order to screen for *Tenacibaculum* spp., *Moritella viscosa, Flavobacterium psychrophilum, Yersinia ruckerii, Branchiomonas cysticola* and Costia (*Ichthyobodo spp.*).

SmoltVision was used to monitor the smoltification status of the salmon before each challenge trail. Samples from the second gill arch were put into RNA-later and delivered to PHARMAQ Analytiq for analyses. SmoltVision is a real-time RT-PCR method where gene expression of the genes that are active during smoltification is measured. The method gives an indication of the enzyme activity in the gill tissue if it is mainly freshwater-ATPase, seawater-ATPase or a combination. The results from these analyses can be used to indicate the smoltification status i.e. if the smolt is ready to be transferred to seawater or at risk of de-smoltification. A fully de-smoltified status would indicate that the study was not to be performed.

2.3 Challenge material

2.3.1 Challenge isolate

The *T. finnmarkense* strain HFJ^T has been shown to recurrently cause tenacibaculosis in challenge studies using Atlantic salmon smolts (Småge et al., 2018). The *T. finnmarkense* strain HFJ^T used in this challenge study was isolated from a mouth lesion of salmon (approx. 2.5 kg) suffering from tenacibaculosis in April 2013 at low seawater temperature (4-5 °C) at a seawater site in Finnmark County in northern Norway. The fish at the site had developed large ulcers 14 months after sea transfer and the ulcers appeared after periods of handling.

2.3.2 Stock

A pre-made bacterial stock with *T. finnmarkense* isolate HFJ^T from 2015 passage 4 (P4) was used as the inoculum for a new bacterial stock of *T. finnmarkense* isolate HFJ^T (P5) to be used

in current study. This new stock was incubated in a shaking incubator at 140 rpm and 16 °C for 54 h. The stock was stored at -80 °C in a 50/50 mixture of marine broth (Difco2216) (MB) (200µL) and biofreeze (BioChrom™) (200µL).

2.3.3 Growth of the *T. finnmarkense* isolate HFJ^T (P5)

Different stocks of *T. finnmarkense* strains HFJ^T can grow differently using the same broth medium. A growth curve of the challenge isolate *T. finnmarkense* strain HFJ^T (P5) grown in the specific Difco 2216, Marine Broth (MB) batch intended for the challenge study, was therefore made prior to the challenge study. This made it possible to find out how many hours post-incubation the *T. finnmarkense* strain HFJ^T (P5) is in its exponential growth phase and when it reaches the stationary phase. It also made it possible to calculate the number of bacteria at certain time intervals post-incubation by using the Most Probable Number (MPN) and by measuring the Optic Density (OD) (methods described in section below designated MPN and OD). The MPN was then correlated with the OD measurement. When measuring MPN and OD, the morphology of the bacteria was checked under a light microscope to look for any contamination or dead bacterial cells.

Growth medium preparation and inoculation the bacterium:

The growth medium (Difco 2216, Marine Broth (MB)) was made by mixing 37.4 g MB in 1 L distilled water (Milli-Q®) in a 2 L Erlenmeyer flask before being autoclaved. 400 µL of frozen inoculum (bacterial stock solution of *T. finnmarkense* strain HFJ^T (P5)) was gently thawed and added directly into the MB. The flask was then placed in a shaking incubator at 140 rpm and 16 °C for a total of 264 hours.

Most Probable Number (MPN) and Optical Density (OD):

MPN and OD were measured at certain time intervals post-incubation. The MPN method is a dilution method used for estimating the number of viable cells in a sample. The method is based on series of tubes containing a progressively more diluted bacterial cell suspension that are inoculated in a broth, incubated, and examined for growth (Blodgett, 2010; Cochran, 1950; Hogg, 2005). The method is used in this study to estimate the number *T. finnmarkense* strain HFJ^T cells grown in MB. The growth of the bacteria was monitored by measuring OD at 600 nm (OD_{600}) (TECAN Spark®). The OD of a material is a logarithmic intensity ratio of

the light falling upon the material, to the light transmitted through the material (Meyers et al., 2018).

The MPN method was performed by using 10-fold dilutions in duplicate (quadruplicate for challenge) with 8 replicates per dilution. The 10-fold dilution was made by transferring 900 µl MB into 11 x 2 2 mL Eppendorf tubes, using a multi pipette. 1000 µl of the bacterial suspension was transferred from the 2 L Erlenmeyer flask to an empty 2 mL Eppendorf tube after carefully mixing the content. 100 µl of the bacteria was then transferred to the first Eppendorf tube containing MB and mixed by pipetting up and down 10 times. Next, 100 µl of the content in Eppendorf tube number one was transferred to Eppendorf tube number two containing MB, and so on until this was performed for all 11 Eppendorf tubes (Figure 2). 100 µl of the chosen dilutions was then added to a 96-well plate with 8 replicates per dilution. The dilutions chosen for the growth curve is listed in Table 1.

X	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
X	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}

Figure 2. Illustration of two parallels of the dilution series of bacteria in MB. X represents a non-diluted sample of the bacterial culture.

	OD	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	OD	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Figure 3. Illustration of a 96-well plate with 8 replicates (A-H) per dilution. The dilutions are marked over the plate. Well 1-6 contains dilutions from parallel one, while well 7-12 contains dilutions from parallel two. Well 1 A-H and well 7 A-H contains undiluted bacterial culture used for measuring OD.

The plates were incubated at 16 °C for at least 48 h before growth could be observed in the wells. The plates were read from the underside by holding them up against the light. Growth appeared as precipitation in the wells. The degree of dilution where the growth stops indicate that the sample has been diluted as much as possible for growth. The number of positive and negative samples is used to estimate the original concentration of the bacterium in the sample. The number of positive wells were counted for each dilution, with a maximum of eight positive or negative wells. The figure shows an example of results of a 96-well plate incubated at 16 °C for 48 h. X represents growth in the wells and from this plate the reading results for parallel 1 is 8 8 8 3 0 0 (Figure 4). Three numbers, with the last number preferably being 0, are chosen: 8 3 0. The results for parallel 2 is 8 8 8 6 4 0 and the three numbers chosen are 6 4 0.

	OD	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	OD	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	X	X	X	X			X	X	X		X	
B	X	X	X				X	X	X	X	X	
C	X	X	X				X	X	X	X		
D	X	X	X	X			X	X	X			
E	X	X	X				X	X	X	X		
F	X	X	X	X			X	X	X	X	X	
G	X	X	X				X	X	X	X	X	
H	X	X	X				X	X	X	X		

Figure 4. Illustration of an example of how to read the results of a plate incubated at 16 °C for 48 h. The wells marked X illustrates growth of bacteria. Parallel one is well 1-6 A-H, while parallel two is well 7-12 A-H. For this example, the result from parallel 1 is 8 - 3 - 0, where the growth stops at dilution 10^{-10}. The result from parallel 2 is 6 - 4 - 0, where the growth stops at dilution 10^{-11}.

In order to determine the MPN value of the three numbers from the reading of the plate, an MPN-reference table for 8 replicates was used. The MPN value of the three numbers in the table was multiplied with the middle 10^{X} value of the three numbers. By applying this to the figure above where it is two parallels, this would be first parallel plus the second parallel and

divide that number by two. This number was than multiplied by 10 because the bacterial suspension had been diluted with 100 µl bacterium in 900 µl MB. The result was given in cells/mL. OD (600 nm) was measured using a TECAN Spark® machine by adding 100 µl of undiluted bacteria to a 96-well plate in 8 replicates.

Table 1. Hours post incubation for each OD and MPN measurement and the dilution used for measuring the MPN in the growth curve made for *T. finnmarkense* HFJ^T.

Hours post incubation	**Dilution (MPN)**
0	-
24	1-11 x2
35	1-11x2
46	3-7, 5-9
48	6-10, 7-11
50	6-10, 7-11
52	6-10, 7-11
54	6-10, 7-11
56	6-10, 7-11
58	6-10, 7-11
60	6-10, 7-11
72	6-10, 7-11
78	6-10, 7-11
96	6-10, 7-11
120	6-10, 7-11
144	6-10, 7-11
264	6-10, 7-11

2.3.4 Challenge material preparation

The stock solutions were stored at -80 °C and the challenge material was produced by inoculating 400 µL of the inoculum (frozen *T. finnmarkense* strain HFJ^T (P5)) into 1.0 L of MB in 5 x 2L Erlenmeyer flasks. A non-inoculated flask containing 1 L of MB served as mock challenge material for the control fish. The inoculated flasks were incubated in a shaking incubator at 140 rpm between 48-52 hours at 16 °C. The flask containing only MB (mock) was kept at 4 °C but put at room temperature half an hour before infection in order to bring it to the same temperature as the challenge material (16 °C). The number of viable bacteria in the challenge trails were retrospectively calculated by using the MPN method with 10-fold dilutions using eight replicates per dilution. As a standard, dilution 10^{-6}-10^{-10} and 10^{-7}-

10^{-11} was used to measure the MPN, with two parallels of each dilutions (i.e. quadruplicate). OD was measured using a TECAN Spark® machine as described in the section above. The amount of challenge material to be used for the challenge trials was based on the pre-challenge trial, and the desired OD was based on the established growth curve for *T. finnmarkense* strain HFJ^T (P5) (see section 2.2.3). The *T. finnmarkense* strain HFJ^T bacteria were always checked under a light microscopy before the challenge trials to investigate the bacterial morphology (included dead cells) and reveal possible contamination. This was performed by aseptically removing a small sample of the bacterial culture and transfer it onto a glass before putting a coverslip onto the sample. The OD of the bacterial culture in all flasks were measured prior to being used as challenge material. 1-2 flasks were selected as challenge material based on the measured OD and bacterial morphology, one flask for Challenge 1F and Challenge 2F and 2LSS, and two flasks were mixed for Challenge 3F and 3LSS to get enough challenge material (volume (mL)). A small sample from the bacterial culture in the selected flasks selected was streaked out onto Blood Agar with sea salt (BAMA) plates and incubated at 16 °C for 48 h. A bacterial sample from the agar plates was frozen after 48 hours for later identification (sequencing of 16S rRNA gene and the housekeeping gene *rlmN*). This was performed by transferring a single bacterial colony from BAMA plates into an Eppendorf tube containing 400 µl MB. 200 µl of this mixture was transferred to two cryo tubes containing 200 µl Biofreeze (BioChrom™) and placed in -80 °C. The BAMA plates were further incubated at 16 °C for two weeks to reveal any potential contamination.

2.4 Challenge study

As described in section 2.1, the challenge study was divided into three challenge trails designated Challenge 1F, Challenge 2F and 2LSS, and Challenge 3F and 3LSS, in addition to a pre-challenge where the dose of infection was investigated. The total number of salmon smolt used in this study was N = 568 (Table 2).

Table 2. The number (N) of fish used in the challenge study. The total number and the number of fish used for sampling and for each challenge.

Screening/challenge	Number of fish (N)
Screening	10
Pre-challenge 1	10
Pre-challenge 2	10
0-sample 1F	15
1F	95
0-sample 2F	12
2F	95
0-sample 2LSS	12
2LSS	95
0-sample 3F	12
3F	95
0-sample 3LSS	12
3LSS	95
TOTAL	**568**

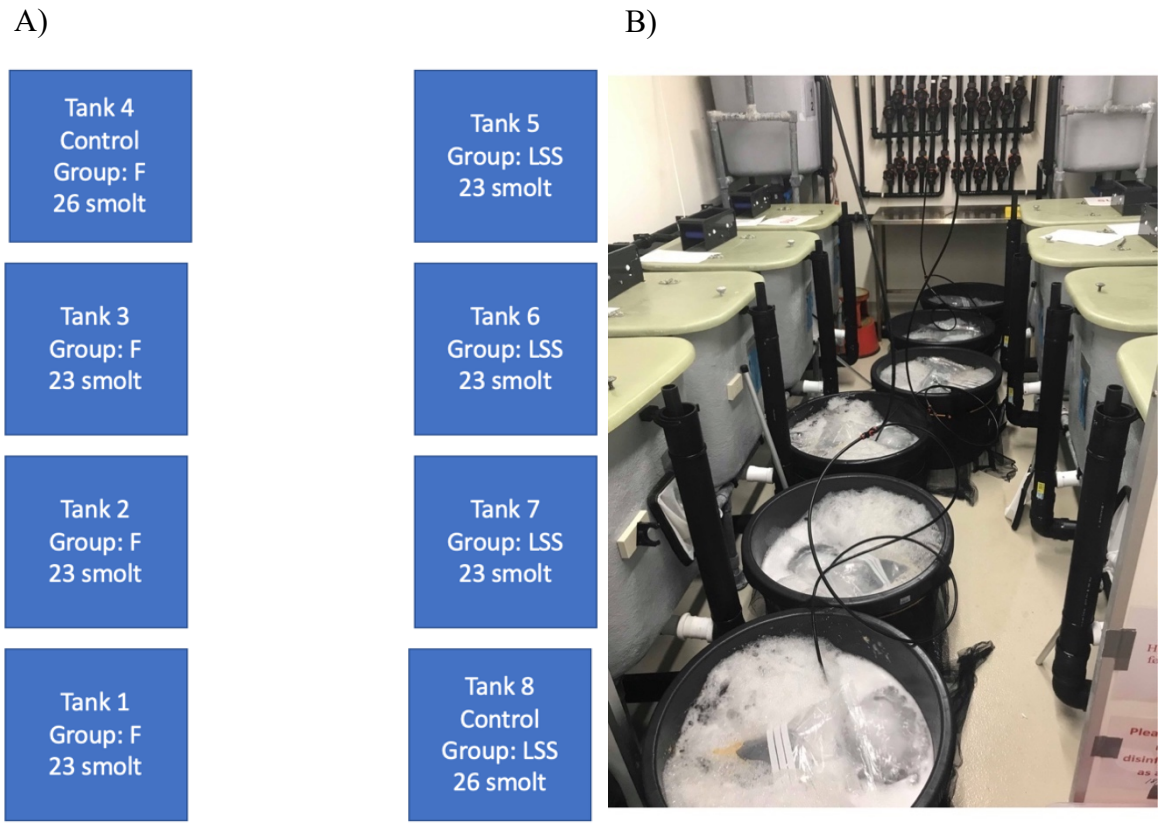

Figure 5. **A)** Overview of the tanks in the challenge facility (CF) and how the smolts were distributed. The figure shows the exact layout of tanks in Challenge 2F and 2LSS and Challenge 3F and 3LSS. In Challenge 1F only tank 1-4 were used. **B)** Overview of challenge facility where the challenges were conducted. Picture was taken during the challenge period of 2 hours where the smolt was bath infected with *T. finnmarkense* strain HFJ^{T}.

2.4.1 Pre-challenge

In the pre-challenge trial, a total of 20 smolts were challenged with *T. finnmarkense* strain HFJ^T. The smolts were newly smoltified and had only been exposed to freshwater prior to the challenge trial. The smolts were divided into two 150 L tanks with 10 smolts in each tank in the CF. Two volumes of challenge material were tested; a high dose and a low dose. The exposure time was 2 h for both tanks and the bath concentration for the high dose group was 2.2 x 10^6 cells/mL in 60 L seawater and the bath concentration for the low dose group was 7.75 x 10^5 cells/mL in 60 L seawater (Table 3). The mortality from this pre-challenge trial was used as a guide for determining the challenge dose to be used in the main challenge trials. An important goal was to avoid getting 100 % acute mortality as this would indicate a too high dose. Therefore, we aimed to establish a dose that would allow for a normal progression of the disease, as well as being high enough to measure potential differences in susceptibility to tenacibaculosis between the two fish groups (F vs LSS).

2.4.2 Experiment 1F

Challenge 1F aimed to test the susceptibility for tenacibaculosis of newly smoltified Atlantic salmon with an average weight of 70 g shortly after transfer from freshwater to seawater. A total of 110 smolts were used in the challenge trial, in which 15 smolts were sampled a week prior to the challenge and 95 smolts were used for challenge. The 95 smolts used for the challenge were transferred from freshwater and distributed into four 150 L tanks, with 23 smolts in tank-1 – tank-3 and 26 smolts in tank 4 (Figure 5). Tank-4 served as a control tank, with smolts mock challenged with 200 mL of MB. The bath concentration was 1.57 x 10^6 cells/mL in 60 L of seawater and the exposure time was 2 h (Table 3). The total amount of challenge material added to the 60 L of seawater was 200 mL.

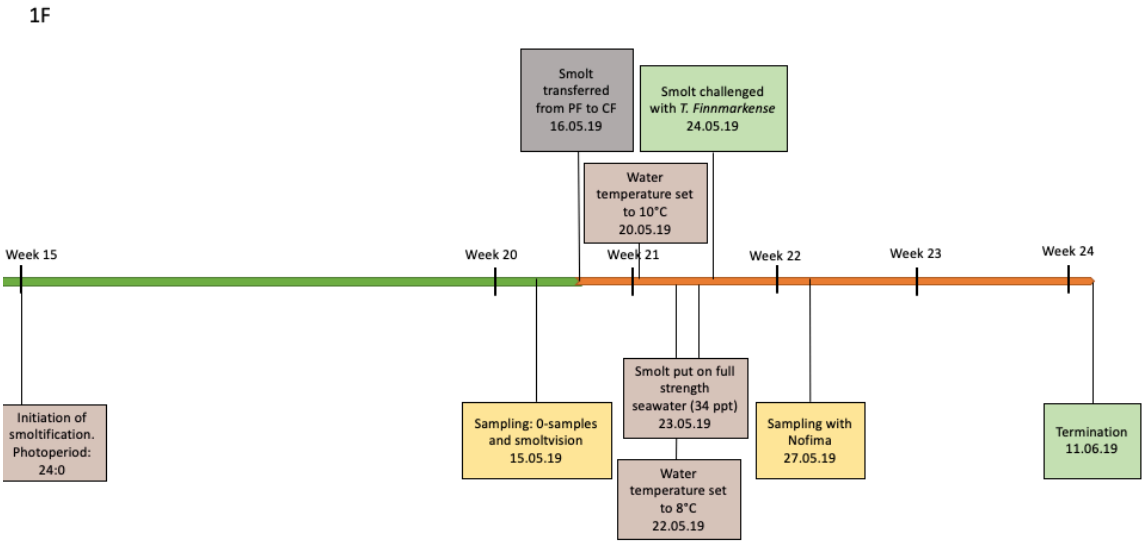

Figure 6. A detailed overview of the timeline for Challenge 1F. Initiating of smoltification started 5 weeks before the smolt was transferred from PF to CF and challenged with *T. finnmarkense* strain HFJ^{T}. The water temperature was gradually lowered to 8 °C two days before challenge. The challenge trial was terminated 11.06.19, 19 days post challenge.

The remaining smolts in the PF after the pre-challenge and challenge 1F were set on two different water qualities. Two tanks continued using freshwater while two tanks were set to 26 ppt (LSS) (Figure 9).

2.4.3 Experiment 2F and 2LSS

In Challenge 2F, a total of 107 smolts with an average weight of 105 g were used. 12 smolts were sampled a week prior to challenge and 95 smolts were used for challenge. The smolts were transferred from freshwater and distributed into four 150 L tanks in the CF, with 23 smolts in each of tank-1 – tank-3 and 26 smolts in tank-4 (Figure 5). The bath concentration was 1.69 x 10^{6} cells/mL in 60 L seawater and the exposure time was 2 h (Table 3). The total amount of challenge material added to the 60 L of seawater was 250 mL. Tank-4 served as a control tank and the smolts were mock challenged with 250 mL of MB.

In Challenge 2LSS, a total of 107 smolts with an average weight of 90 g were used. 12 smolts were sampled a week before the challenge trail and 95 smolts were used for challenge. The smolts were put on 26 ppt four weeks before transferred to four 150 L tanks in the CF. 23

smolts in each of tank-5 – tank-7 and 26 smolts in tank-8 (Figure 5). The bath concentration was 1.69 x 10^6 cells/mL in 60 L seawater (Table 3). The total amount of challenge material added to the 60 L of seawater was 250 mL. Tank-8 served as a control tank and the smolts were mock challenged with 250 mL of MB.

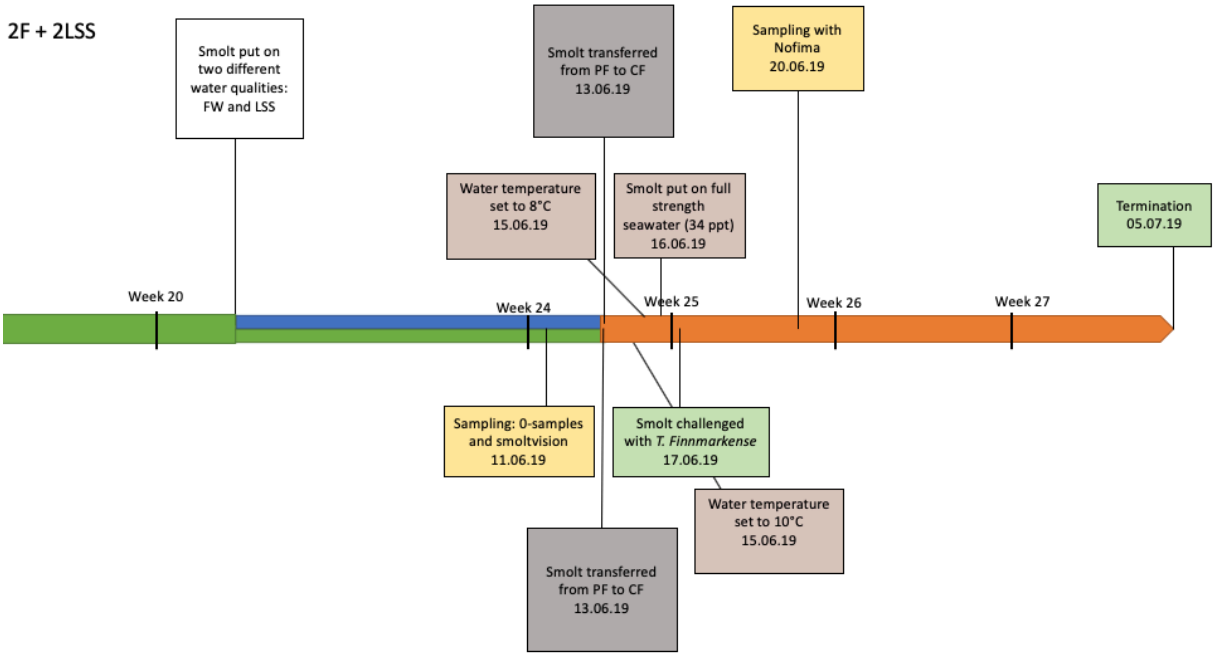

Figure 7. A detailed overview of the timeline for Challenge 2F and 2LSS. The smolts were put on two different water qualities four weeks prior to being transferred from the PF to the CF. The water temperature was lowered to 8 °C and the smolt were put-on full-strength seawater prior to being challenged with *T. finnmarkense* strain HFJ^T. The challenge trial was terminated 05.07.19, 19 days post challenge.

2.4.4 Experiment 3F and 3LSS

In Challenge 3F, a total of 107 smolts with an average weight of 150 g were used, in which 12 smolts were sampled a week before challenge and 95 smolts were used for the challenge. The smolts were transferred from freshwater and distributed to four 150 L experiment tanks in CF with 23 smolts in each of tank-1 – tank-3 and 26 smolts in tank-4 (Figure 5). The bath concentration was 1.71 x 10^6 cells/mL in 60 L seawater and the exposure time was 2 h (Table 3). The total amount of challenge material added to the 60 L of seawater was 280 mL. Tank-4 served as a control tank and the smolts were mock challenged with 280 mL of MB.

In Challenge 3LSS, a total of 107 smolts with an average weight of 124 g were used. 12 smolts were sampled a week before challenge and 95 smolts were used for the challenge. The smolts were put on 26 ppt eight weeks before distributed to four 150 L tanks in the CF. 23 smolt in each of tank-5 – tank-7 and 26 smolt in tank-8 (Figure 5). The bath concentration was 1.71 x 10^6 cells/mL in 60 L seawater and the exposure time was 2 h (Table 3). The total amount of challenge material added to the 60 L of seawater was 280 mL. Tank-8 served as a control tank and the smolts were mock challenged with 280 mL of MB.

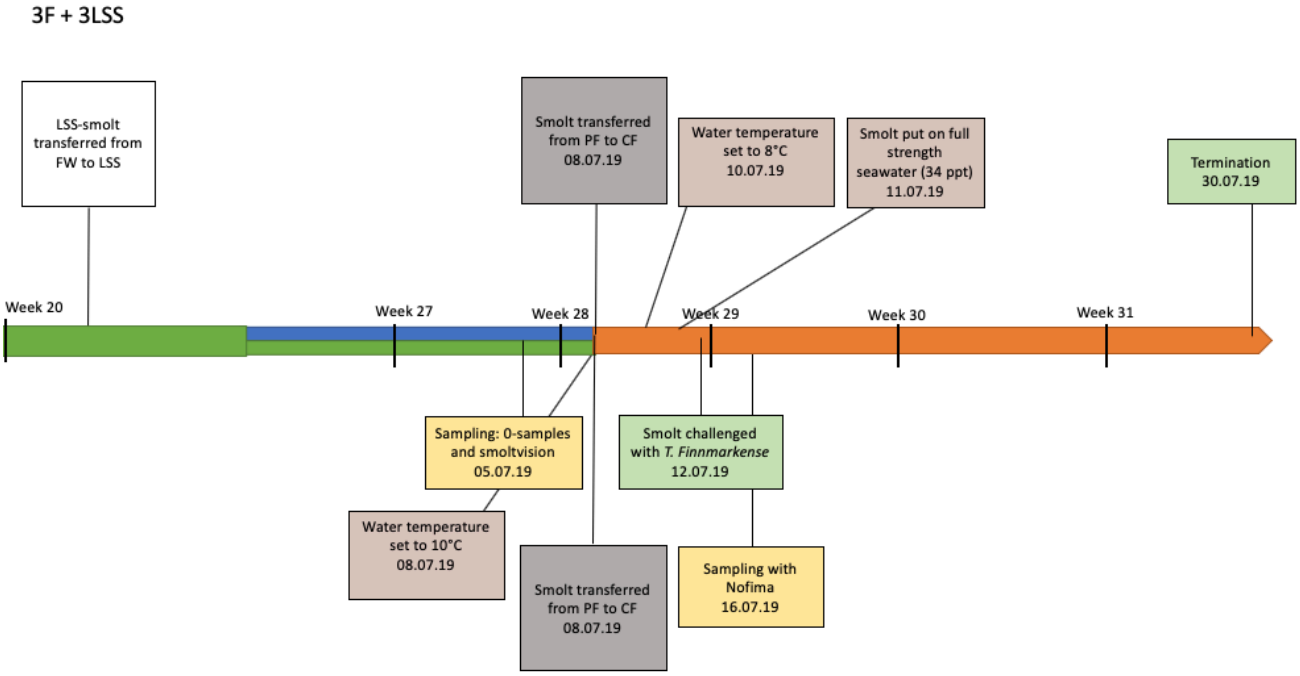

Figure 8. A detailed overview of the timeline for Challenge 3F and 3LSS. The smolts were put on two different water qualities eight weeks prior to transfer from the PF to the CF. The water temperature was gradually lowered to 8 °C and the smolts were put-on full-strength sea water prior to being challenged with *T. finnmarkense* strain HFJ^T. The challenge trial was terminated 30.07.19, 18 days post challenge.

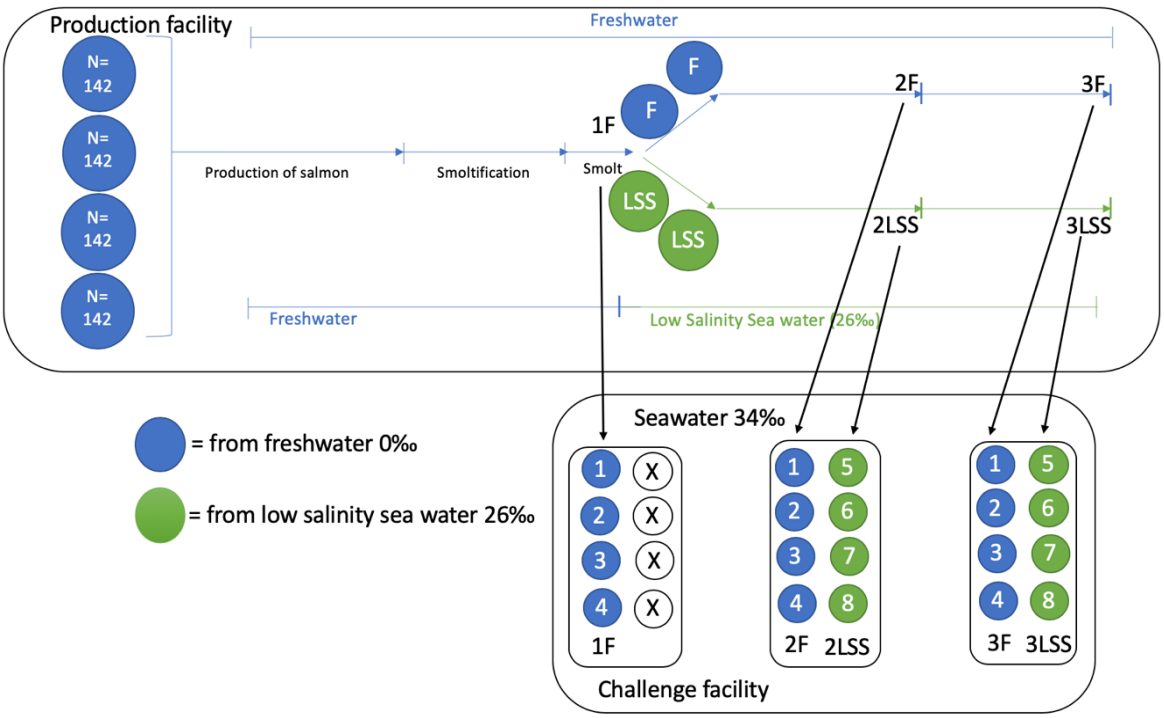

Figure 9. Schematic overview of the challenge study, with an overview of the fish tanks in both the production facility (PF) and the challenge facility (CF). The arrows marks when smolt is transferred from the PF to the CF. Freshwater (F) tanks are marked blue and low salinity sea water (LSS) tanks are marked green.

2.4 Challenge procedure

After four days of acclimatization in the 150 L experimental tanks the fish was transferred by using a net into separate challenge containers containing 60 L of 8 °C saltwater (34 ppt) and the challenge material (Figure 5). The fish were exposed to the challenge material (*T. finnmarkense* strain HFJ^{T} (P5)) for two hours. The control fish were also transferred to challenge containers but were only exposed to MB (mock). The same challenge procedure was performed for the control fish as for the fish exposed to *T. finnmarkense* strain HFJ^{T}. Temperature and oxygen were monitored during the challenge period: one measurement shortly following the challenge, one measurement an hour post challenge and one measurement after two hours at the end of the challenge period. Oxygen was provided through compressed air diffusers. In order to keep the temperature at 8 °C throughout the challenge period, the addition of a bag of ice to the containers were sometimes needed towards the end of the challenge period (Figure 5). After two hours, the fish were transferred back into their respective tanks. The control fish were transferred first to avoid any contamination from the challenge material.

The bath concentration (cells/mL) for each challenge trial was calculated by using this equation:

$$Bath\ concentration\ \left(\frac{cells}{mL}\right) = \frac{Volume\ challenge\ material\ (L) \times\ MPN\ challenge\ material\ (\frac{cells}{mL})}{Volume\ challenge\ container\ (L)}$$

Table 3. An overview of the challenge trials, the isolate used for challenge, the amount challenge material added, the bath concentration, the infection time, the OD and the incubation time for the challenge material and the number of fish used in each challenge trial.

Challenge	Isolate	Amount added (mL) to 60L bath	Bath concentration (cells/mL)	Infection time (h)	OD	Incubation time (h)	Number of fish (N)
Pre-challenge High dose	*T. finnmarkense* HFJ^T	200	2.2x10^6	2	0.73	53 h 40 min	10
Pre-challenge Low dose	*T. finnmarkense* HFJ^T	75	7.75x10^5	2	0.73	53 h 40 min	10
1F	*T. finnmarkense* HFJ^T	200	1.57x10^6	2	0.74	53 h 15 min	95
2F	*T. finnmarkense* HFJ^T	250	1.69x10^6	2	0.75	54	95
2LSS	*T. finnmarkense* HFJ^T	250	1.69x10^6	2	0.75	54	95
3F	*T. finnmarkense* HFJ^T	280	1.71x10^6	2	0.73	53.5	95
3LSS	*T. finnmarkense* HFJ^T	280	1.71x10^6	2	0.73	53.5	95

2.5 Sampling

2.5.1 Sampling – production facility

About a week prior to each challenge trial, skin and kidney tissues were sampled for real-time RT-PCR analyses. Backup samples from gill and heart were also sampled. This was performed in order to check that the fish were not infected with any known pathogens (0-samples) that could compromise the validity of the results. At the same time, the second gill-arch were sampled from 10 fish from each water quality and transferred to tubes containing

RNA-later provided by PHARMAQ Analytiq. The sample was sent to PHARMAQ Analytiq for SmoltVision analysis of the Na^+ K^+ ATPase enzyme activity in the gills. The result of this analysis indicates the smolt status of the salmon. Weight and length were measured, in addition to sampling performed by Nofima. Nofima sampled skin tissues (caudal fin, two skin samples with muscle and lower jaw) for histology and microarray. The fish were randomly sampled using the same number of fish from each tank in the production facility (PF).

2.5.2 Sampling – challenge facility

Random sampling post challenge:

A random sampling of challenged fish was conducted four days post challenge, together with Nofima. Three fish were randomly sampled from tank-1- tank-3, three fish tank-5 – tank-7, as well as six fish from tank-4 and tank-8. Weight and length of the fish were measured, and each individual fish evaluated for external welfare indicators by using a scoring scheme developed for tenacibaculosis caused by *T. finnmarkense* (Table 4). Tissue samples from the lower jaw or edge of lesions and kidney were sampled for real-time RT-PCR. Nofima followed the same procedure for sampling as described in section 2.5.1.

Table 4. Scoring scheme used in the challenge experiments to characterize external signs of tenacibaculosis caused by *T. finnmarkense*.

Jaw	0	No abnormality
	1	Mild – a little hemorrhage
	2	Moderate – hemorrhage/lesion
	3	Severe – jaw erosion
Fin	0	No lesions
	1	Mild – splitting
	2	Severe – shredded
Skin	0	No lesions
	1	Mild – some scale loss and/or hemorrhage
	2	Moderate – skin lesion(s), scale loss through skin
	3	Severe – skin lesion(s) through to muscle and/or many lesions

Sampling of moribund fish:

The fish were checked at a minimum of two times per day during the entire challenge trail period. Any fish that showed signs of disease or moribund behavior during the challenge trials

were removed from the tanks and euthanized with either an overdose of Finquel vet. (Scan Aqua) or by a swift blow to the head. No fish were found dead in the tanks during this study; hence, the wording "mortality" refers to fish removed from the tank due to animal welfare considerations. Moribund fish removed from the tanks during all the challenge trials were examined for external clinical signs and photographed. The jaw, skin, and fins were scored using the designated scoring scheme (Table 4), and weight and length of each individual fish were measured. As a standard, tissue samples for real-time RT-PCR were taken from the lower jaw and kidney from each fish and transferred into individual Eppendorf tubes and kept on ice. The size of the sample was approximately 3 x 33 x 1.5 mm. If the fish had lesions, a tissue sample was sampled from the edge of the lesion and from the kidney. All samples were stored at -20 °C.

Mucus from skin and lesions of 1-2 fish from each experiment were smeared onto glass slides and colored with Hemacolor® Rapid. The slides were then examined by light microscopy to look for bacteria resembling *T. finnmarkense* morphology. Isolation of the bacteria was performed on 1-2 fish per tank by streaking a sample from lesions or the lower jaw of the fish and a kidney sample onto BAMA-plates by using a bacterial loop (VWR). The plate was incubated at 16 °C for at least 48 hours before sub-cultivation. Tissue samples from the margin of the lesions were sampled for histology and Scanning Electron Microscopy (SEM) from 1-2 affected fish per tank. The samples were placed in a modified Karnovsky Fixative (Småge et al., 2018), in order to perform both Scanning Electron Microscopy (SEM) as well as histology. These samples were stored at 4 °C before processing. All sampling of infected fish in this study was conducted at ILAB.

Termination of the challenge trials:

Remaining fish at the termination of each challenge trial were euthanized with an overdose of Finquel vet. (Scan Aqua) (> 80-135 mg/L). Length and weight were measured and all fish were scored according to the designated scoring scheme (Table 4), before being transferred into individual plastic bags and stored at -20 °C. Sampling of these fish were performed by carefully defrosting the fish and follow the same sampling protocol as for the fish sampled during the challenge trials.

2.7 Bacteriology

2.7.1 Hemacolor ® Rapid staining

Air-dried skin smears from lesions of infected fish were colored using Hemacolor® Rapid staining (Sigma-Aldrich). The coloring set consist of three solutions: fixative, solution A and solution B. A plastic pipette was used to transfer each solution from a container to the slides, starting with the fixative, second solution A and last solution B. Each solution was held on the slides for approximately one minute before the next solution was added. Finally, the slide was washed with water and air-dried before a cover glass was fitted. The scrapings were then examined for bacteria with *T. finnmarkense* morphology by using a light microscope.

2.8 DNA extraction and sequencing

Primary bacterial cultures were incubated at 16 °C for at least 48 hours. If growth was detected, each colony type was examined using a light microscope and colonies containing bacterial cells matching *T. finnmarkense* morphology were sub-cultivated on BAMA and incubated at 16 °C for 48 h. Bacterial clones were frozen in nuclease-free water and stored at -80 °C, before genomic DNA was extracted to sequence parts of the 16S rRNA and the *rlmN* gene of the bacteria.

2.8.1 DNA extraction

Method 1: boiling

Genomic DNA was obtained by heating bacterial clones in tubes containing nuclease-free water at 95 °C for 5 minutes. The samples were then centrifuged at 15.000 x g for 5 minutes and the DNA-containing supernatant (250 µl) was transferred into new tubes. The DNA was stored at -20 °C.

Method 2: E.Z.N.A® Tissue DNA Kit (Omega Bio-tek)

The manufacturers protocol was followed.

Bacterial suspension was added to the tubes containing 220 µl BL-buffer. The tubes were incubated at 70°C for 10 minutes and briefly vortexed after 5 minutes. 220 µl of 100 % ethanol was added, and the tubes were briefly vortexed. The tubes were centrifuged at 12.000 x g for 5 minutes. The supernatant was then transferred to a HiBind®DNA Mini Column that was inserted into a collection tube. The tubes were centrifuged at 12.000 x g for 1 minute, before the filtrate was discarded. The collection tube was reused, and 500 µl HBC buffer was

added to the tubes. The tubes were centrifuged at 12.000 x g for 30 seconds before the filtrate and collection tubes were discarded. The HiBind®DNA Mini Columns were inserted into new collection tubes and 700 µl DNA wash buffer was added. The tubes were centrifuged at 12,000 x g for 30 seconds. The filtrate was discarded, and the collection tube reused. The washings steps were repeated for a second DNA wash. The empty HiBind®DNA Mini Columns were centrifuged at maximum speed for two minutes to dry the column. The HiBind®DNA Mini Columns were then transferred into a 1.5 mL Eppendorf tube and 100 µl of Elution Buffer heated to 70 °C was added to the tubes. The tubes sat in room temperature for two minutes, before they were centrifuged at maximum speed for one minute. The eluted DNA was stored at -20 °C.

2.8.2 Polymerase chain reaction (PCR)

PCR was performed using the universal 16S rRNA gene primers 27F and 1492R and a specific primer pair for the *rlmN* gene (Table 5). The PCR method amplifies a certain DNA-sequence *in vitro* and was performed on bacterial colonies collected from fish during the challenge study and from the challenge material. A master mix containing the following ingrediencies: 16.85 µl nuclease-free water, 2.5 µl Taq DNA Polymerase 10 x buffer, 1.25 mM dNTP, 1µl forward and reverse primer and 0.75 units (0.15 µl) Taq DNA Polymerase (VWR) was made and added to PCR-tubes (23 µl per tube). 2 µl extracted DNA was then added to the master mix making a total volume per reaction of 25 µl.

The samples were transferred to an Applied Biosystems Vereti 96 well Thermal cycler where the PCR was conducted after these terms: denaturation for 5 minutes at 95 °C, 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C (*rlmN*) / 58 °C (16S rRNA) for 30 seconds and 1.5 minutes of elongation at 72 °C followed by the final extension for 5 minutes at 72 °C.

Table 5. Overview of PCR primers used in this study.

Target gene	**Primer**	**Sequence (5`- 3`)**	**Reference**
rlmN	Forward Reverse	GCKTGTGTDTCDAGYCARGT CCRCADGCDGCATCWATRTC	(Habib et al., 2014)
16S rRNA	27 Forward 1492 Reverse	AGAGTTTGATCCTGGCTCAG TACCTTGTTACGACTT	(Frank et al., 2008)

2.8.3 Gel electrophoresis

Gel electrophoresis was performed to visually confirm the presence of PCR product. It was performed using a gel containing 1,5 % agarose solved in 1 X Tris-acetate-EDTA (TAE) buffer. 1 µl of the fluorescent dye GelRed™ was added to the gel to stain the nucleic acids. The gel hardened for 10-15 minutes before it was covered in 1X TAE-buffer. 2 µl GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific) was added to the first well to be used as a molecular weight marker. 2.5 µl of the PCR product was mixed with about 1 µl loading dye (6x TriTrack DNA Loading Dye (Thermo Fisher Scientific)) before being added to the wells in the gel. The gels were run for 30 minutes (15 min) at 80 Volts, before being examined by UV-light (Gel Logic 212 Pro, Fisher Scientific) using the program Carestream MI.

2.8.4 PCR product purifying

After evaluating the PCR products on the gel, the PCR products with the correct size were purified using ExoSAP-IT (Thermo Fisher Scientific) before being used for sequencing. ExoSAP-IT is a method used to clean PCR products by enzymatic degradation of primers and dNTP`s which can interfere with the sequencing process. The PCR products were cleaned by adding 2.5 µl PCR product to 1 µl ExoSAP-IT. The samples were run in a PCR machine (Veriti™ 96-Well Thermal Cycler) at a standard program for ExoSAP-IT following these conditions: incubation for 15 minutes at 37 °C to remove primers and nucleotides, followed by 15 minutes at 80 °C to inactivate enzymes.

2.8.5 Sequencing

Sangers sequencing was performed to determine the identity of the colonies with *Tenacibaculum* morphology isolated from the fish during the challenge study and from the challenge material. 1.5 µl of the purified PCR product was used as a template and added to two PCR-tubes containing 1 µl BigDye® (version 3.1), 1µl BigDye® Terminator v3.1 5X Sequencing Buffer and 5.5 µl nuclease-free water. 1 µl forward primer was added to one of the PCR-tubes and 1 µl reverse primer was added to the other PCR-tube. The reaction was performed in a PCR machine (Veriti™ 96-Well Thermal Cycler) by an initial denaturation at 96 °C for 5 minutes, followed by 30 cycles of denaturation at 96 °C for 10 seconds, annealing at 55/58 °C for 5 seconds and 4 minutes of elongation at 60 °C. After the reaction was

completed, 10 µl nuclease-free water was added to the samples before being delivered to the sequencing facility at UiB (https://www.uib.no/en/seqlab). The gene sequences retrieved from the sequencing facility were analyzed by using the program Vector NTI® (Invitrogen).

2.9 RNA extraction:

RNA extraction was performed following the manufacturers protocol (TRIzol® Reagent, by life technologies, Invitrogen).

The tissue samples were kept on ice while adding a 5 mm steel bead (Qiagen) in the lid of the tubes and adding 5µl *Halobacterium salinarum* cells suspended in dH_2O as spike. The steel bead was sterilized by flame before it was added to the lid. 1.0 ml TRI Reagent was then added to the tissue samples, the tubes were closed, and the samples homogenized in a Qiagen tissue lyser II at 30/s for 4 minutes. The samples were then incubated for 5 minutes at room temperature before 0.2 ml of chloroform were added to the tubes. The tubes were mixed by shaking the tubes for 15 seconds by hand before being incubated for 5 minutes at room temperature. The samples were centrifuged at 12.000 x g for 15 minutes at 4 °C to separate the mixture into three phases, where the RNA is present in the uppermost colorless and aqueous layer. 370 µl of the RNA containing layer was removed and transferred to a new tube containing 0.5 ml isopropanol and mixed by turning the tube upside down three times. The samples were then incubated at room temperature for 10 minutes before being centrifuged at 12.000 x g for 15 minutes at 4 °C which makes the RNA form a white pellet on the side and bottom of the tube. The supernatant was then removed, and the pellet was washed two times: first with 1 mL of 70 % ethanol and then with 1 mL 100 % ethanol. The samples were vortexed and centrifuged for 5 minutes at 12,000 x g and 4 °C between the washing steps. The ethanol was then removed, and the pellet air-dried for 5-10 minutes until the alcohol had evaporated. The RNA pellet was resuspended in 60 µl 70 °C nuclease-free water. The samples were frozen and stored at -80 °C. Negative controls were included in all RNA extractions that followed the same protocol as the tissue samples, except that no tissue was present.

2.10 Real-time RT-PCR

The extracted RNA was analyzed by real-time RT-PCR for the detection of target RNA. Real-time RT-PCR was performed using the AgPATH-ID™ One-Step RT-PCR Kit (Applied

Biosystems) following the manufacturer`s instructions. All primers and probes used in this study are listed in Table 6. The RNA was screened using the Tb_rpoB assay targeting *Tenacibaculum* spp., the MvOmpA assay targeting *M. viscosa*, an assay targeting the elongation factor 1 alpha (EF1A) of *Salmo salar* and the Hsal assay targeting *Halobacterium salinarum* (spike).

Table 6. An overview of primer and probe sequences used in this study.

Assay	Primer	Sequence	Reference
Hsal (*Halobacterium salinarum*)	Probe Forward Reverse	AGGCGTCCAGCGGA GGGAAATCTGTCCGCTTAACG CCGGTCCCAAGCTGAACA	(Andersen, Hodneland, & Nylund, 2010)
EF1A (Elongationfactor salmon)	Probe Forward Reverse	ATCGGTGGTATTGGAAC CCCCTCCAGGACGTTTACAAA CACACGGCCCACAGGTACA	(Olsvik, Lie, Jordal, Nilsen, & Hordvik, 2005)
MvOmpA (*Moritella viscosa*)	Probe Forward Reverse	TCTTGGAGCAGGTCTAGAATATACACCAG GATGATAACGCAACAGCAG CGGAAACTTACACCAGATAATG	(Vold, 2014)
Tb_rpoB (*Tenacibaculum* spp.)	Probe Forward Reverse	TCCTGCTTGATCAGTTAAAGCGT GGAGCAAACATTGACCAAATT GGTATGTCCGTAACGTGGAA	(Vold, 2014)

MicroAmp® Optical 96-well Reaction Plates (Applied Biosystems) were used and put on ice when adding master mix and template. The total volume in each well was 12.5 µl, using 2 µl template and 10.5 µl master mix. Master mix contained 6.25 µl 2X RT-PCR Buffer, 0.50 µl Forward primer (400 nM), 0.50 µl Revers primer (400 nM), 0.15 µl TaqMan® probe (120 nM), 0.25 µl 25X RT-PCR Enzyme Mix and 2.85 µl nuclease-free water. RNA-extraction control (NC), non-template control (NTC) and a positive control (*T. finnmarkense* positive from field outbreak) were included with every run. The NC was added to detect potential contamination during RNA extraction. The plates were sealed with MicroAmp™ Optical Adhesive Film (Applied biosystems (Thermo Fisher Scientific)) and centrifuged before they were analyzed in Applied Biosystems® QuantStudio 3 Real-Time PCR Systems. The reactions were run according to Standard AgPath setup, with reverse transcription for 10 minutes at 45 °C, denaturation and activation of the DNA polymerase for 10 minutes at 95

°C, then 45 cycles of amplification at first denaturation at 95 °C for 15 seconds and then amplification at 60 °C for 45 seconds.

2.10.1 Assay optimization

Assay Hsal and assay EF1A were optimized with regard to primer and probe concentration using the AgPath-ID one-step RT-PCR kit. First, the assays were tested using different concentrations of forward and reverse primer and a recommended concentration of probe (120 nM). Concentrations tested are listed in Table 7. Each combination was tested in triplicates with a known template.

Table 7. Combinations of primer concentrations (nM) tested for assay Hsal and assay EF1A.

Forward/Reverse	**F primer 200 nM**	**F primer 400 nM**	**F primer 600 nM**	**F primer 800 nM**
R primer 200 nM	200/200	400/200	600/200	800/200
R primer 400 nM	200/400	400/400	600/400	800/400
R primer 600 nM	200/600	400/600	600/600	800/600
R primer 800 nM	200/800	400/800	600/800	800/800

After finding the optimal primer concentration, these were used to find the correct probe concentration using the same template as for primer optimization. The probe concentrations tested were 100 nM, 125 nM, 150 nM, 175 nM, 200 nM and 225 nM. The optimized concentrations for assay Hsal and assay EF1A were compared to standard concentrations recommended from AgPath-ID one-step RT-PCR kit. The concentrations used for comparison are listed in Table 8.

Table 8. Optimized concentrations (nM) and standard concentration (nM) for forward and revers primer and probe assay Hsal and assay EF1A compared.

Assay	**Optimized concentration (F/R/P)**	**Standard concentration (F/R/P)**
Hsal	600/800/175	400/400/120
EF1A	600/800/175	400/400/120

The optimization of assay Tb_rpoB and assay MvOmpA have been previously performed by Vold (2014). These optimized concentrations were compared to standard concentrations

recommended by AgPath-ID one-step RT-PCR kit. The concentrations compared are listed in Table 9. A known template was used, and the samples were analyzed with real-time RT-PCR in triplicate.

Table 9. Optimized concentrations (nM) and standard concentrations (nM) for forward and revers primer and probe for assay Tb_ropB and MvOmpA.

Assay	Optimized concentration (F/R/P)	Standard concentration (F/R/P)
Tb_rpoB	400/600/170	400/400/120
MvOmpA	400/600/170	400/400/120

The results of optimizing primer and probe concentration and comparison of optimized concentrations and standard concentrations are shown in the appendix. The optimization was conducted to make sure that the difference between mean Ct-value from the standard concentrations did not deviate significantly from the mean Ct-values from the optimized concentrations. Because of the small difference between the optimized concentrations and standard concentrations, it was decided to do the analyses with the standard concentrations recommended by AgPath-ID one-step RT-PCR kit to make the analyses most efficient.

2.10.2 Efficacy test

After the primers and probe optimization for assay Hsal and assay EF1A, an efficiency test was conducted in order to test the assay`s ability to detect the target template. Target RNA was diluted from 1 to 10^{-8} and analyzed with real-time RT-PCR in triplicates. The mean Ct-value was plotted against the dilution series in Microsoft Excel to make a standard curve and Excel was used to calculate the slope of the graph. Efficacy was calculated by the formula: $((10^{-1/slope})-1)*100$. The result is found in the appendix.

2.11 Histology and SEM

Collected tissue intended for histology and SEM were fixed by immersion, at 4 °C in modified Karnovsky fixative where the distilled water was replaced by a Ringers solution. The fixative contained 4 % sucrose. The samples were washed using a Ringer solution three times for 30 minutes while kept on ice. Tissues intended for SEM were in addition post-fixed in a 2 % osmium tetroxide solution for 1-2 hours, until the tissue turned black. The tissues

were rinsed by being washed in distilled water 3 times for 15 minutes while kept on ice. Next, the tissue was dehydrated by using acetone, with the following steps of 60 % acetone (1x15 minutes), 70 % acetone (1x15 minutes) and 90 % acetone (1x15 minutes) while kept on ice, and 100 % acetone (2x15 minutes) in room temperature. After dehydration, the tissues for histology was embedded in Epoxy resin (EPON) and polymerized by incubation at 60 °C overnight. The tissues were cut in semithin sections (1.0 µl) on a Reichert-Jung Ultracut E (Leica Microtome) (Ultramicrotome). The sections were stained using 1 % toluidine blue and examined using light microscope. After dehydration, the tissues for SEM were critical point dried using liquid CO_2 as the transitional fluid. At critical point drying, the tissue is brought to a critical temperature and pressure point at which the fluid is removed. The dried tissue was mounted by means of double-stick carbon tape on SEM stubs and coated with gold/ palladium alloy. Specimens were examined at 15kV with a ZEISS Supra 55VP scanning electron microscope.

3. Results

3.1 Growth curve of *T. finnmarkense* strain HFJ^T

The *T. finnmarkense* strain HFJ^T grown in MB used for this challenge study entered the exponential phase at approximately 36 hours post incubation (hpi) (Figure 10). The stationary phase is reached 58 hpi, before the bacterium reaches the death phase at around 72 hpi (Figure 10). Based on the results from the measurements and by checking the morphology of the bacterium under light microscope, it was decided that an OD between 0.72 – 0.75 was desired for the challenge study. This corresponded to an MPN between 2.50 x 10^8 and 4.55 x 10^8. The result from measurements conducted after 78 hpi is shown in Figure 31 in the appendix. The result showed a decrease in OD from 58 hpi to 144 hpi, before an increase in OD between 144 hpi and 264 hpi. The OD was 0.94 at 264 hpi and the reason for this is unknown. The high OD may indicate a large number of bacteria, but we did not manage to get any growth in the MPN measurement performed at this time. A sample of the bacterial culture was taken with each measurement and investigated by using light microscope. A large number of dead bacterial cells were observed from samples from 72 hpi and the further measurements.

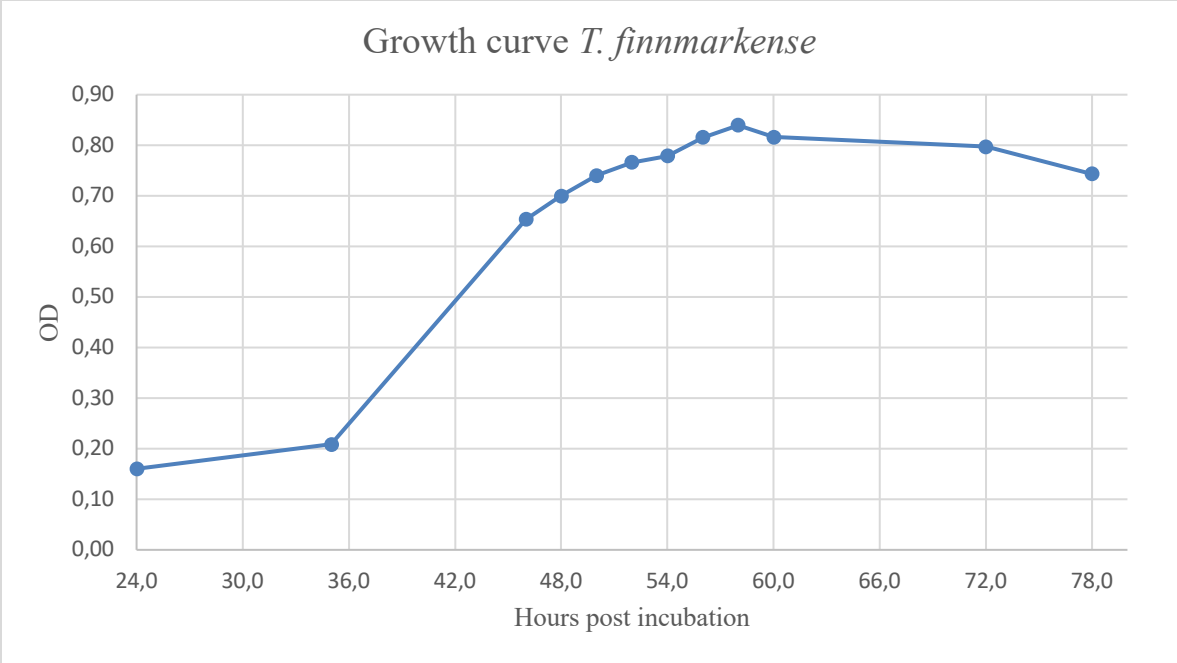

Figure 10. Growth curve for *T. Finnmarkense* strain HFJ^T grown in Difco 2216, Marine Broth (MB). The figure shows the lag phase, exponential phase and stationary phase of *T. finnmarkense* strain HFJ^T. The desired OD for challenge was decided to be between OD 0.72 - 0.75 based on this growth curve. The data is presented as average of two parallels.

0-samples of tissues from the skin (lower jaw) and kidney were sampled from smolts prior to each challenge trial. RNA extracted from the tissue samples were used for real-time RT-PCR to investigate the presence of *Moritella viscosa*, *Tenacibaculum* spp., *Flavobacterium psyhcrophilum*, *Yersinia ruckeri*, *Candidatus* Branchiomonas *cysticola* and *Ichthyobodo* spp. (costia). All samples were found negative for all these pathogens, except that one fish tested weakly positive for *F. psychrophilum* with a Ct-value of 38.9 and one fish tested weakly positive for costia with a Ct-value of 35.3.

3.3 Challenge study

3.3.1 Challenge 1F

Estimation of challenge dose:

The challenge dose for Challenge 1F was based on the dose and mortality in the pre-challenge. The high dose in the pre-challenge gave a mortality of 80 % while the low dose gave a mortality of 0 %. This result indicated that our desired dose for Challenge 1F was the same dose used as the high dose in the pre-challenge. This represented an OD of 0.73, an MPN of 6.7×10^8 and 200 mL of challenge material added to 60 L of seawater (Table 10). This resulted in a desired bath concentration of 2.2×10^6 cells/mL. The result of the measured OD and MPN from the challenge material used for Challenge 1F gave a lower bath concentration for this challenge trial with a bath concentration of 1.57×10^6 cells/mL (Table 10).

Mortality:

Moribund fish was observed from three days post challenge (dpc) to six dpc where the symptoms stopped. The percent moribund fish removed from each tank was 13 % in tank-1, 13 % in tank-2 and 17 % in tank-3 (Figure 11). No fish from tank-4 (control) was removed from the tank during the challenge trial. The average percent mortality from Challenge 1F was 14 % (Figure 12).

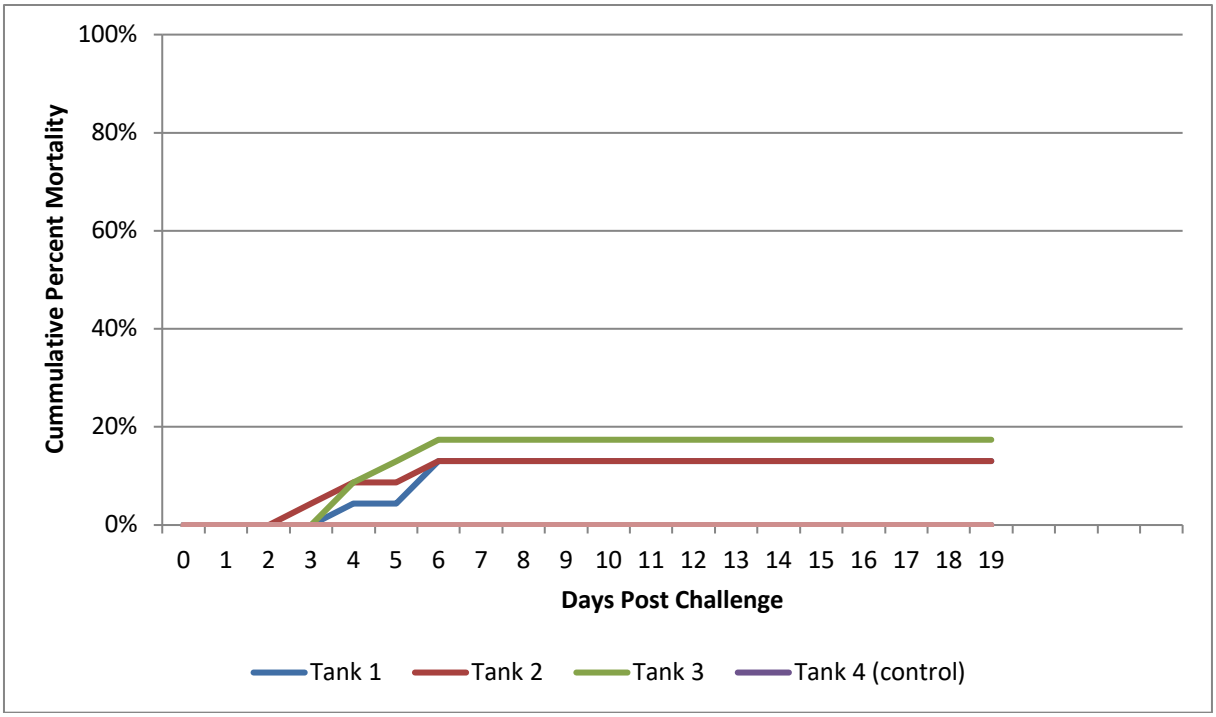

Figure 11. The figure shows the percent mortality from each tank in Challenge 1F. The percent mortality in each tank in the challenge trial was 13 % in tank-1, 13 % in tank-2 and 17 % in tank-3. No mortality was observed in tank-4.

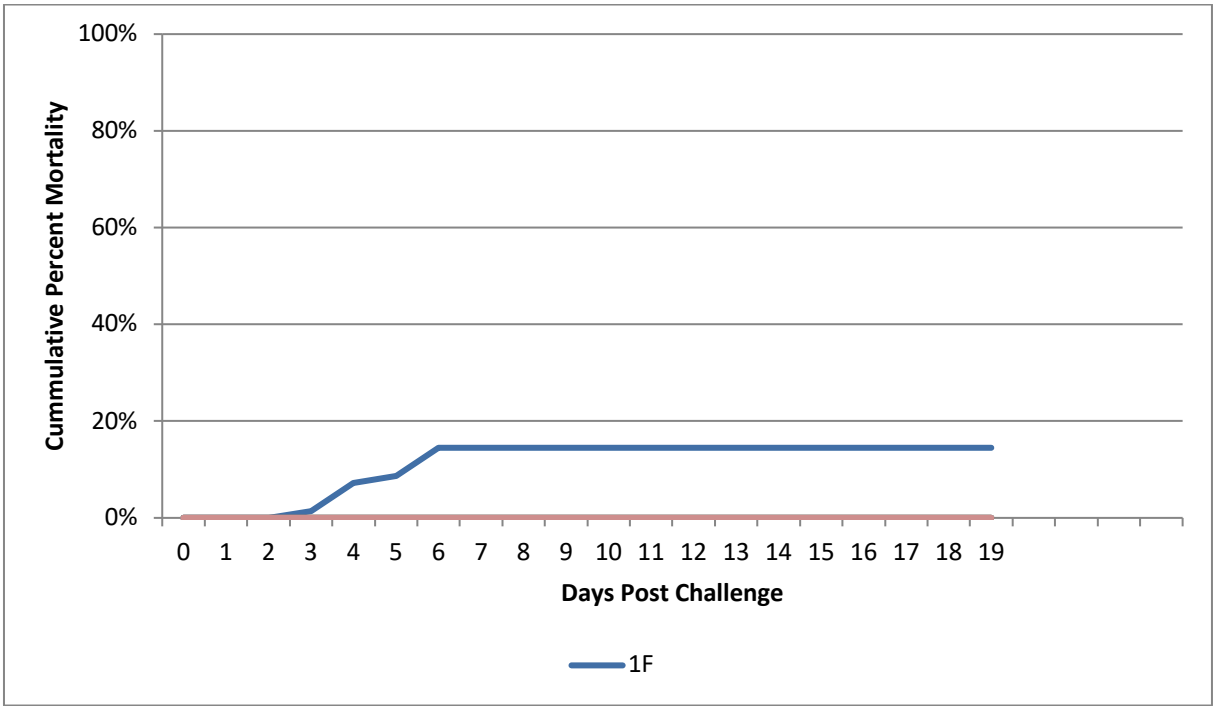

Figure 12. The average percent mortality in Challenge 1F was 14 %.

3.3.2 Challenge 2F and 2LSS

Estimation of challenge dose:

Based on the mortality and bath concentration from Challenge 1F, the amount of challenge material added to the 60 L bath was increased from 200 mL to 250 mL in Challenge 2F and 2LSS. This resulted in a bath concentration of 1.69 x 10^6 cells/mL, with an OD of 0.74 and an MPN of 4.7 x 10^8 cells/mL (Table 10).

Mortality:

For both the F-group and the LSS-group moribund fish was observed as early as two dpc. For the F-group moribund fish was observed until 11 dpc, while for the LSS-group moribund fish was observed until 8 dpc. The percent moribund fish removed from the F-group was 48 % from tank-1, 39 % from tank-2 and 57 % from tank-3 (Figure 13). The percent moribund fish removed from the LSS-group was 13 % from tank-5, 22 % from tank-6 and 26 % from tank-7 (Figure 13). No moribund fish was observed in the control tanks (tank-4 and tank-8). The average percent mortality in the F-group in Challenge 2F and 2LSS was 48 % and the average percent mortality in the LSS-group was 20 % (Figure 14).

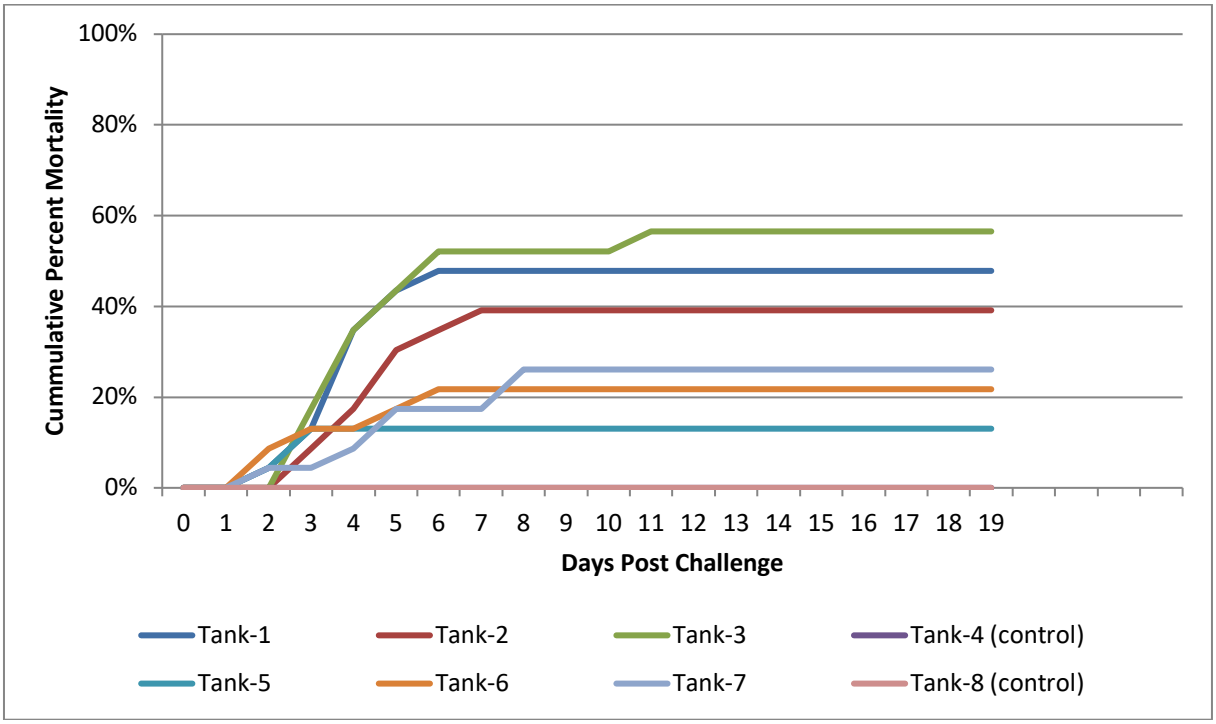

Figure 13. The percent mortality from each tank in Challenge 2F was 48 % in tank-1, 39 % in tank-2 and 57 % in tank 3. The percent mortality from each tank in Challenge 2LSS was 13 % in tank-5, 22 % in tank-6 and 26 % in tank-7. No mortality was observed in the control tanks (tank-4 and tank-8).

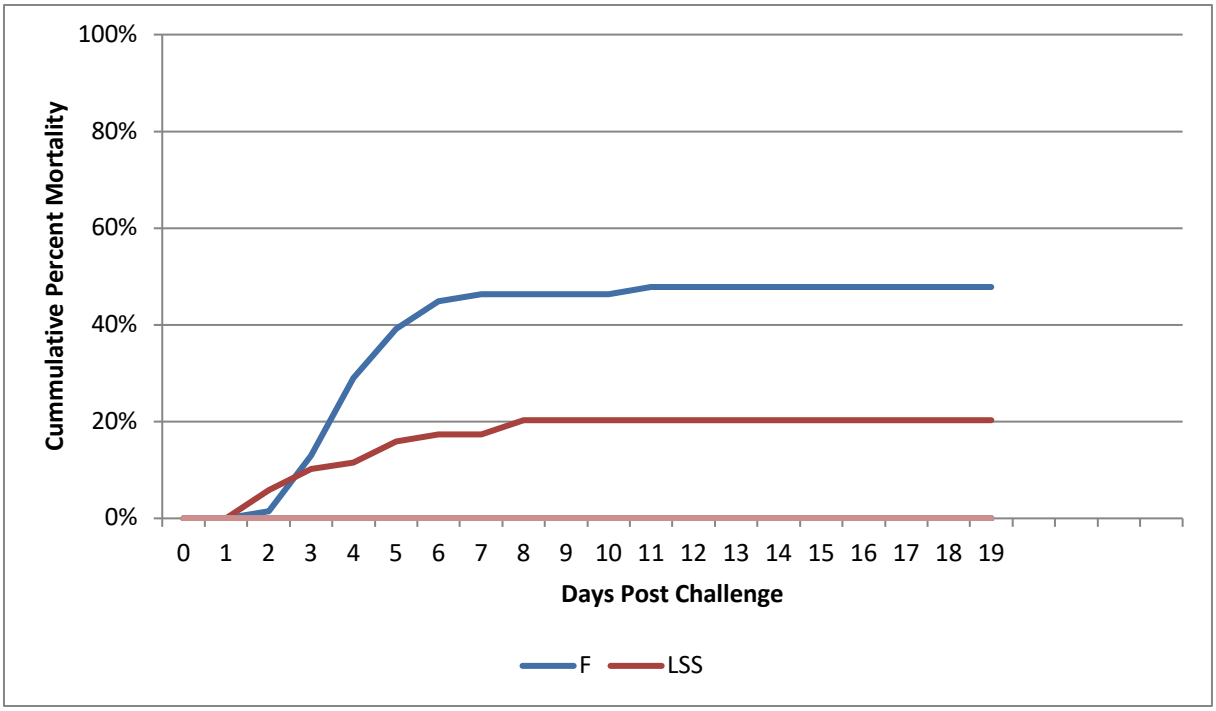

Figure 14. The average percent mortality in Challenge 2F was 48 % and the average percent mortality from Challenge 2LSS was 20 %.

3.3.3 Challenge 3F and 3LSS

Estimation of challenge dose:

For Challenge 3F and 3LSS, the amount of challenge material was increased even more, from 250 mL to 280 mL. Even though the amount of challenge material was increased, the bath concentration was almost the same for Challenge 3F and 3LSS as for Challenge 2F and 2LSS, with a bath concentration of 1.71 x 10^6 cells/mL in Challenge 3F and 3LSS (Table 10). The measured OD for the challenge material used for Challenge 3F and 3LSS was 0.73 and the measured MPN was 3.67 x 10^8 cells/mL (Table 10).

Mortality:

In the F-group, moribund fish was observed from day three post challenge until day six post challenge. The percent moribund fish removed from the F-group was 13 % from tank-1, 26 % from tank-2 and 35 % from tank-3 (Figure 15). The percent moribund fish removed from the LSS-group was 13 % from tank-5, 13 % from tank-6 and 9 % from tank-7 (Figure 15). No moribund fish was observed from the control tanks (tank-4 and tank-8). The average percent mortality for the F-group in Challenge 3F and 3LSS was 25 % and the average percent mortality in the LSS-group was 12 % (Figure 16).

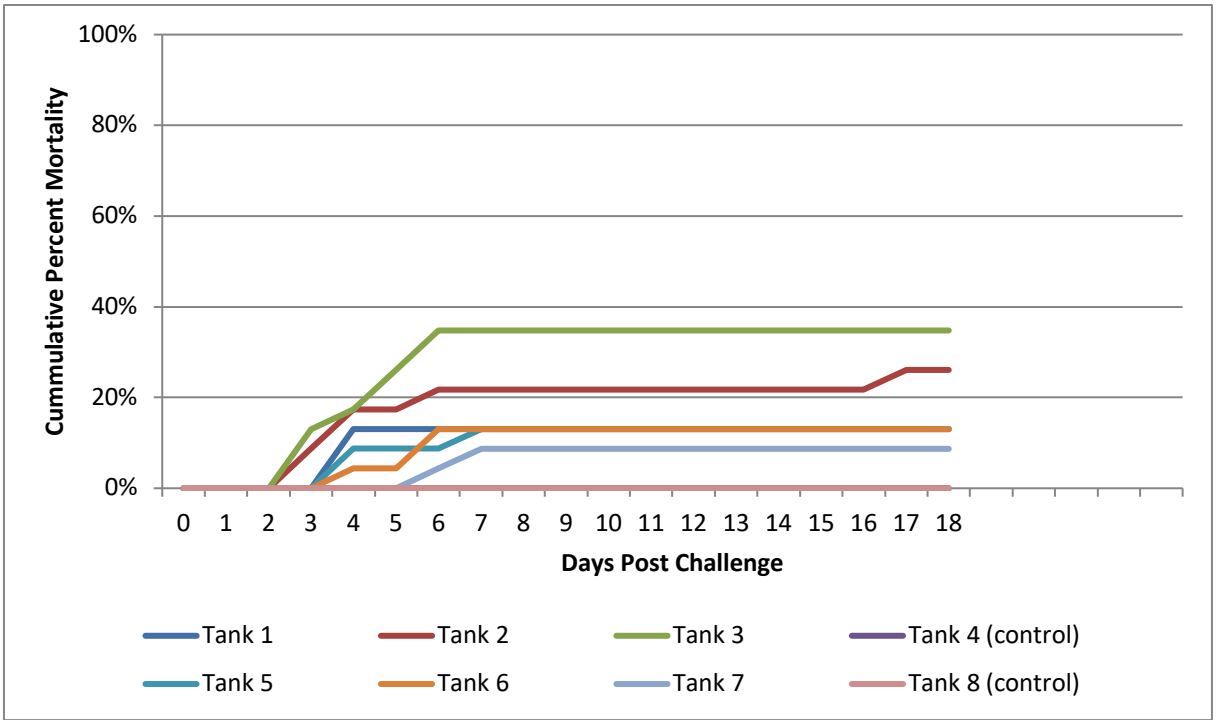

Figure 15. The percent mortality in each tank in Challenge 3F was 13 % in tank-1, 26 % in tank-2 and 35 % in tank-3. The percent mortality in each tank in Challenge 3LSS was 13 % in tank-5, 13 % in tank-6 and 9 % in tank 7. No mortality was observed in the control tanks (tank-4 and tank-8).

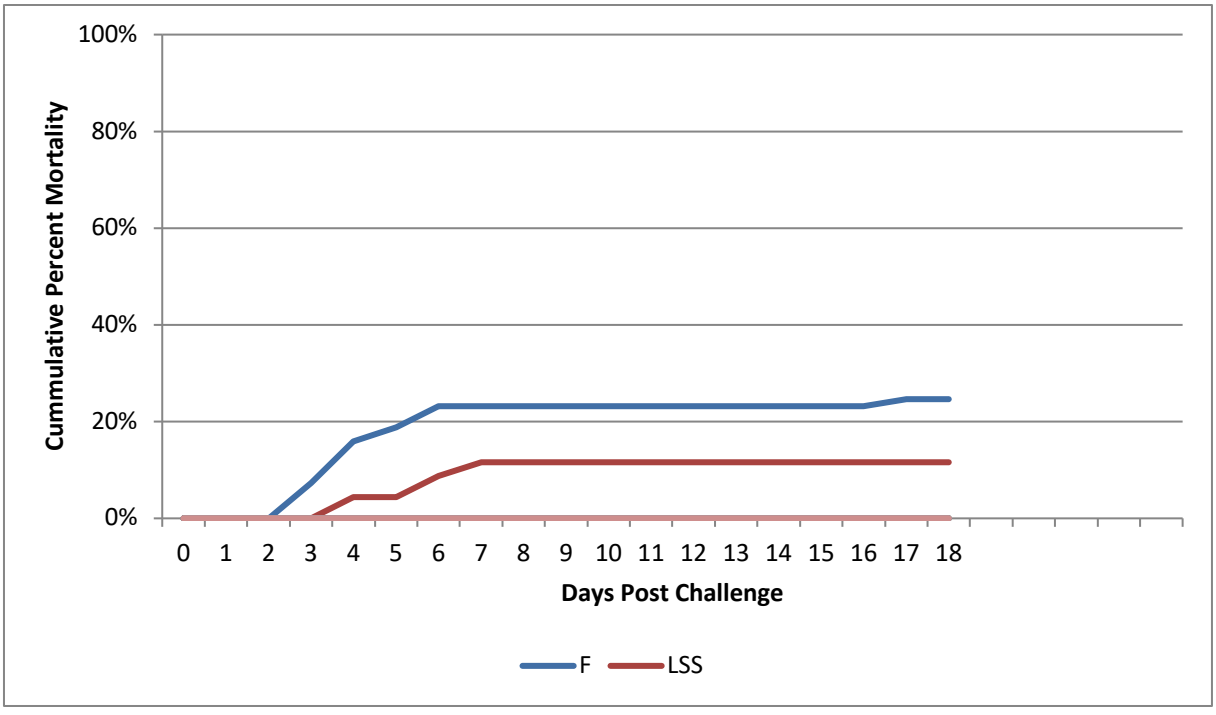

Figure 16. The average percent mortality in Challenge 3F was 25 % and the average percent mortality in Challenge 3LSS was 12 %.

The OD is almost the same for every challenge trial, but because of an increase in the amount of challenge material added to the 60 L bath for each challenge trial, the bath concentration is different for each challenge trial (Table 10).

Table 10. The amount of challenge material (mL), OD, MPN and the calculated bath concentration for each challenge trial.

Challenge trail	Isolate	Amount added (mL) to 60L bath	Bath concentration (cells/mL)	OD	MPN
Pre-challenge High	*T. finnmarkense* HFJ^T	200	$2.2x10^6$	0.73	$6.7x10^8$
Pre-challenge Low	*T. finnmarkense* HFJ^T	75	$7.75x10^5$	0.73	$6.7x10^8$
1F	*T. finnmarkense* HFJ^T	200	$1.57x10^6$	0.74	$4.7x10^8$
2F and 2LSS	*T. finnmarkense* HFJ^T	250	$1.69x10^6$	0.75	$4.05x10^8$
3F and 3LSS	*T. finnmarkense* HFJ^T	280	$1.71x10^6$	0.73	$3.67x10^8$

3.4 Status of the fish prior to the challenge trials

Prior to the challenge trials, it was observed that some LSS smolts had more fin erosion due to production, especially on the right-side pectoral fin. These were healed erosion and not active damage on the fins. The LSS smolt generally had a less pristine look compared the freshwater smolts and had a lower weight. This was observed before the smolts were challenged. One control fish in Challenge 2LSS was removed shortly following the challenge because of a high degree of fin erosion.

3.5 Weight of the fish during the challenge trials

Weight were measured for each individual fish that were removed from the tanks during each challenge trial and for each individual fish at the termination of the challenge trials. The average weight is presented in Figure 17.

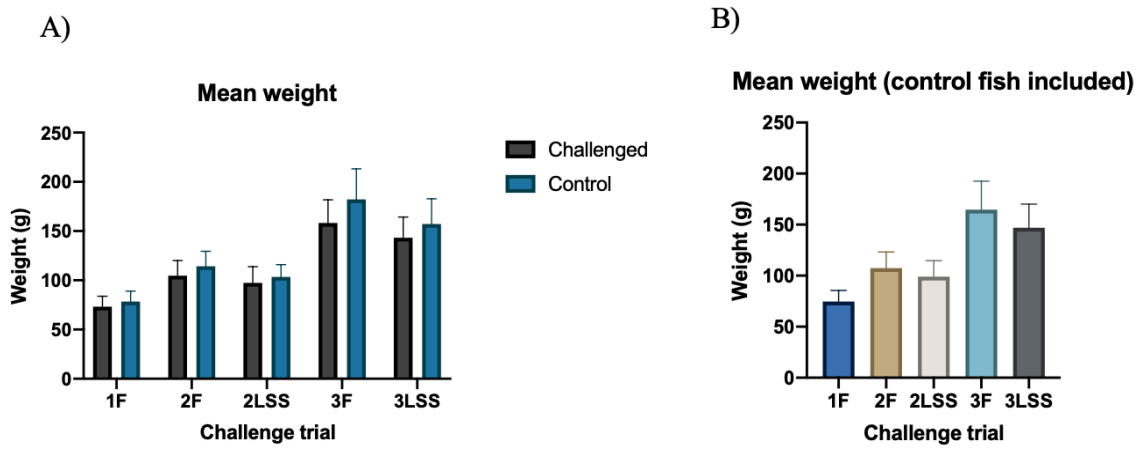

Figure 17. **A)** An overview of the average weight of all fish from each challenge trial, comparing the average weight of the control fish from each challenge trial (N = 26) to the average weight of the challenged fish (N = 69). **B)** The average weight of all fish from each challenge trial including the control fish (N = 95).

3.6 Clinic and pathology

3.3.1 Behavior

Moribund fish with reduced flight response was observed in all the tanks except in the control tanks in all the challenge trials in the current study. Some fish were observed laying on the side in the bottom of the tanks while some fish were observed swimming in the water surface. Only normal behavior was observed in the control fish in all the challenge trials.

3.3.2 Macroscopic pathological symptoms

Ulcers/lesions of varying degrees of severity were the main pathological observation during all challenges. The ulcers typically started developing 2 - 3 dpc and were mainly observed at the abdomen and jaw of the fish. Observations of moribund fish and the development of ulcers typically lasted until 7 dpc. The ulcers were scored from 0 - 3, following the scoring system shown in Table 4. The results show that a higher number of individuals in the F-group is affected by score 2 and score 3 (Table 11). These scores indicate diseased fish with the erosion of jaw and fins or ulcers through to the muscle. Score 3 was given to fish with the most extensive ulcers, in which muscular tissue was exposed.

Table 11. Table showing the different scores given for all fish in the current study, including control fish. N = number of fish with the respective scores. The table includes the dpc the fish from Challenge 2F and 2LSS and from Challenge 3F and 3LSS was observed eating.

	Welfare score				
Challenge trial	**0 N = 199**	**1 N = 180**	**2 N = 50**	**3 N = 46**	**Start eating (days post challenge)**
1F	57	25	1	12	
2F	22	40	17	16	10
2LSS	22	53	11	9	3
3F	48	27	16	4	9
3LSS	50	35	5	5	4

At the abdomen and on the side of the fish, ulcers typically started as grey areas with scale loss (Figure 20). A yellow pigmented and sometimes hemorrhagic circular margin was observed on some fish few days post challenge (Figure 21), while some fish was observed with open ulcers with a yellow margin only few days post challenge. Mouth erosion was also observed frequently only a few days post challenge (Figure 19). Erosion of tailfin tissue and the other fins were observed frequently from fish removed from the tanks during the challenge trials (Figure 18). The disease had an acute progression and a minor lesion could rapidly develop into score 3. No symptoms of tenacibaculosis were observed from approximately one week post challenge until termination of each challenge trial. Control fish showed no pathological symptoms except some healed fin erosion on especially the right-side pectoral fin in the LSS group.

Figure 18. Severe fin rot affecting the pectoral fin. The fish was samples from Challenge 2F, four dpc and scored with score 2 (the highest fin score possible).

Figure 19. Severe jaw erosion from the same fish as shown in Figure 18. The fish was sampled from Challenge 2F, four dpc. The severity of the jaw was given score 3, which is the highest score possible.

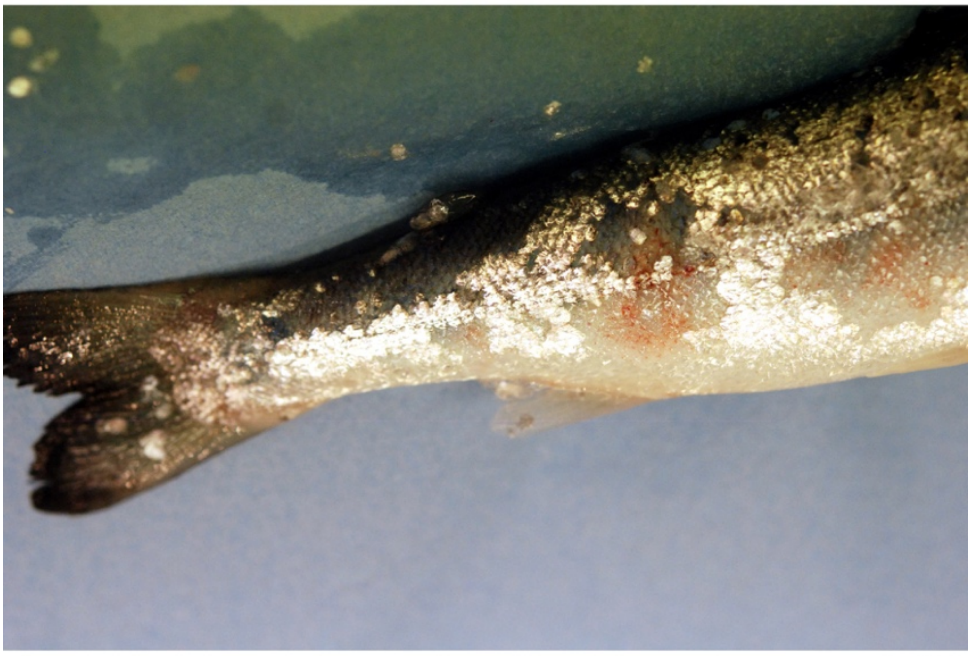

Figure 20. The skin lesion on this fish was given score 1, as the fish showed severe scale loss and small hemorrhage. Starting ulcers with uneven edges, located on the side of the fish is observed. The fish was sampled two dpc from Challenge 2F.

Figure 21. Large starting ulcer with red/yellow pigmented margin at the abdomen of the fish. The fish is sampled four dpc from Challenge 2F and was given score 2 for the skin lesion. This lesion would have rapidly developed into a score 3, if the fish had not promptly been removed from the tank.

3.4 Re-isolation of *T. finnmarkense* HFJ^T and sequencing

The *T. finnmarkense* strain HFJ^T was successfully re-isolated from ulcers and lesions from the skin, jaw, and fin from at least one fish per tank in each challenge trial. The bacterium was isolated using BAMA (blood agar with sea salt), and bacteria displaying *Tenacibaculum* cell and colony morphology were sequenced. A few colonies resembling *Tenacibaculum* colonies were grown on BAMA from three kidney samples from fish in Challenge 2F. These colonies were sequenced together with the colonies isolated from the skin of the fish. All 16S rRNA gene sequences and all *rlmN* gene sequences of bacteria with *Tenacibaculum* morphology isolated from the challenged fish had a 100 % match to each other as well as to the challenge isolate *T. finnmarkense* strain HFJ^T 100 %. This includes the colonies grown from the kidney of sampled fish in Challenge 2F. In Challenge 3F and 3LSS, a few small beige colonies were isolated from two control fish. The retrieved 16S rRNA gene sequences identified the bacteria as *Psychrobacter* sp. and *Pseudoalteromonas* sp., which are harmless bacteria normally found in seawater (sequences shown in the appendix) (Holmström & Kjelleberg, 1999; Bowman, 2006). The fish showed no signs of disease.

3.5 Real time RT-PCR

All extracted RNA from skin samples taken during the challenge trials were analyzed using assay Tb_rpoB for detection of *T. finnmarkense*. All samples of moribund fish sampled during the challenge trials were positive for *T. finnmarkense* as well as almost all fish randomly sampled during the challenge trials, with a few exceptions of two negative samples from tank-1 and two negative samples from tank-3 in Challenge 1F (Table 19 in appendix). None of the sampled fish were positive for *M. viscosa*. The real-time RT-PCR results from all challenge trials showed a clear difference in the Ct-value between moribund fish sampled during the challenge trials and the asymptomatic fish sampled at the termination of the trials (Figure 22). Almost all fish sampled at the termination of the challenge trials that were positive for *T. finnmarkense* had a Ct-value > 30 with only a few exceptions. The results also show a difference between the F-group and the LSS-group, where there is a higher number of fish with Ct-value < 20 (strong positive samples) from the F-group than from the LSS-group in both Challenge 2F and 2LSS and Challenge 3F and 3LSS (Table 20 and Table 21 in the appendix). Ten kidney samples from Challenge 1F were screened using the Tb_rpoB assay. Eight of the ten samples tested weekly positive. 7 of the 78 skin samples from control fish also tested weekly positive (Ct-value > 30) for *Tenacibaculum* spp. when analyzing the extracted RNA using assay Tb_rpoB.

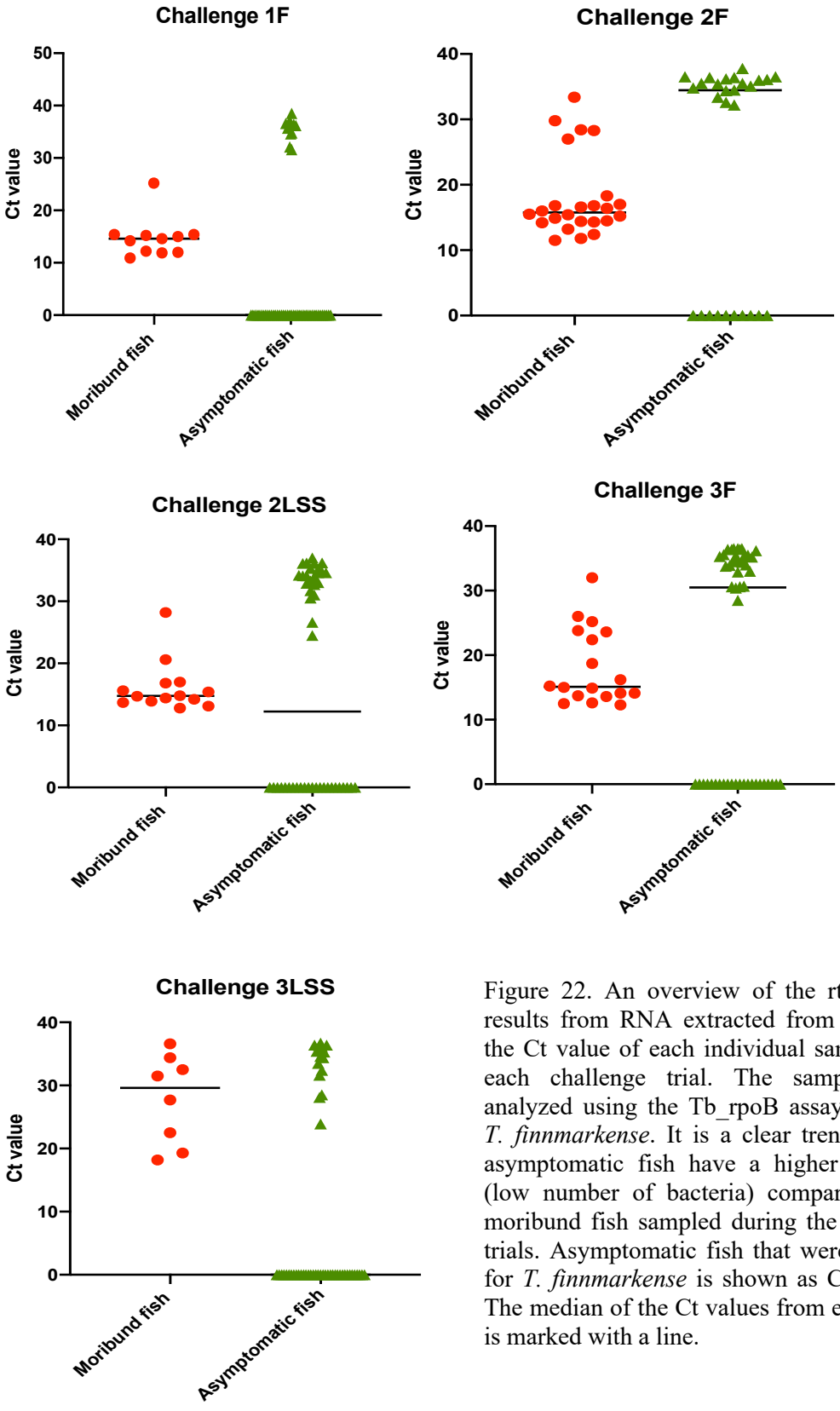

Figure 22. An overview of the rt RT-PCR results from RNA extracted from skin with the Ct value of each individual sample from each challenge trial. The samples were analyzed using the Tb_rpoB assay targeting *T. finnmarkense*. It is a clear trend that the asymptomatic fish have a higher Ct value (low number of bacteria) compared to the moribund fish sampled during the challenge trials. Asymptomatic fish that were negative for *T. finnmarkense* is shown as Ct value 0. The median of the Ct values from each group is marked with a line.

3.6 Histology and SEM

The histology samples of skin sampled from symptomatic fish during the challenge trials revealed significant tissue damage with complete loss of epidermis and a large number of bacteria with *Tenacibaculum* morphology infiltrating the dermis layer causing degradation of the *stratum spongiosum* (Figure 23). The bacteria were observed infiltrating the *stratum compactum* layer of the dermis, where the bacteria were arranged after the collagen fibers as shown in Figure 23. The bacteria were observed in the hypodermis in some of the samples, causing damage to the connective tissue. In addition, degradation of muscle fibers was observed. Bacteria were found associated with the connective tissue surrounding the muscles and could be observed inside white muscle fibers (Figure 24). SEM examination of tissue from the jaw and tail of an infected fish revealed large numbers of bacteria with *Tenacibaculum* morphology (Figure 25 and Figure 26). Degeneration of the tissue located around the bacteria was observed in the SEM sections (Figure 25 and Figure 26) and the bacteria was observed to be focal, especially at the tip of the caudal fin as shown in Figure 25.

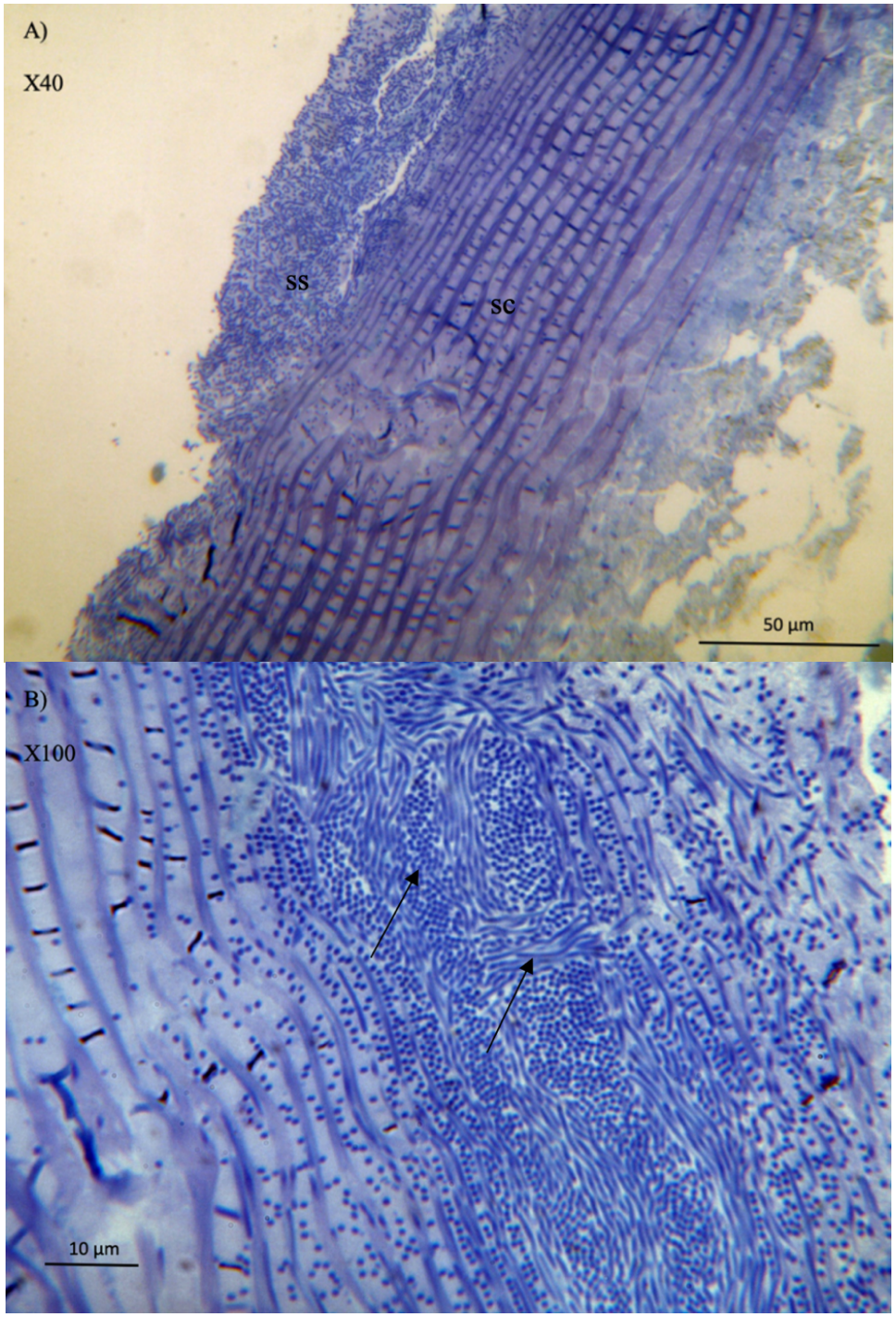

Figure 23. Histopathological section of skin with bacteria with *Tenacibaculum* morphology. **A)** Total loss of epidermis, and bacteria with *Tenacibaculum* morphology infiltrating the *stratum spongiosum* (ss). Some bacteria observed in the *stratum compactum* (sc). **B)** Bacteria with *Tenacibaculum* morphology infiltrating the *stratum compactum* layer, where the bacteria are aligned with the collagen fibers as both longitudinal section and cross section is observed (marked with arrows).

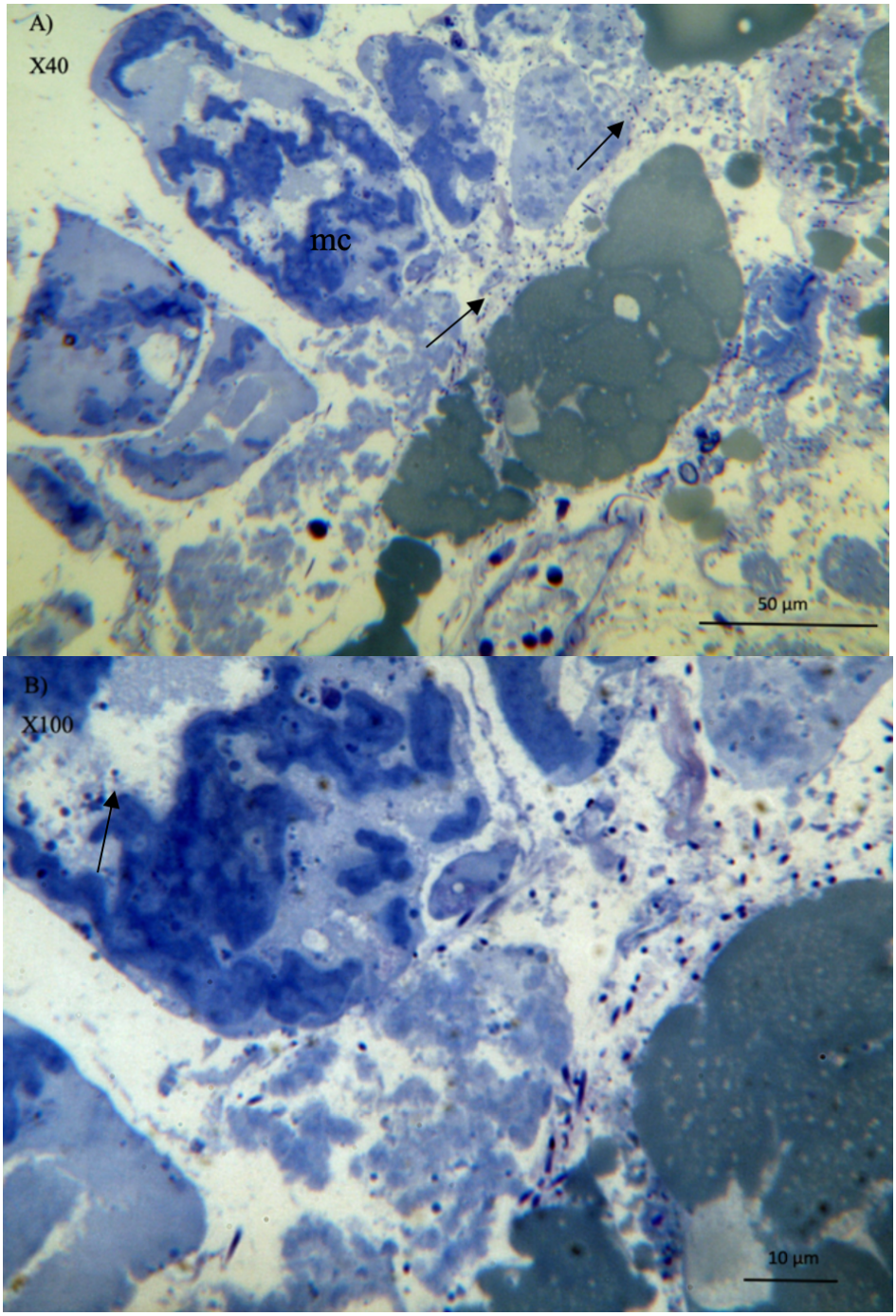

Figure 24. **A)** Histopathological section of the muscle (mc) of a moribund Atlantic salmon smolt where cross sections of bacteria with *Tenacibaculum* morphology are observed associated with the connective tissue surrounding the muscle fibers (bacteria marked with arrows). **B)** The bacteria with *Tenacibaculum* morphology observed inside the white muscle fibers (marked with arrow) and associated with the connective tissue surrounding the muscle.

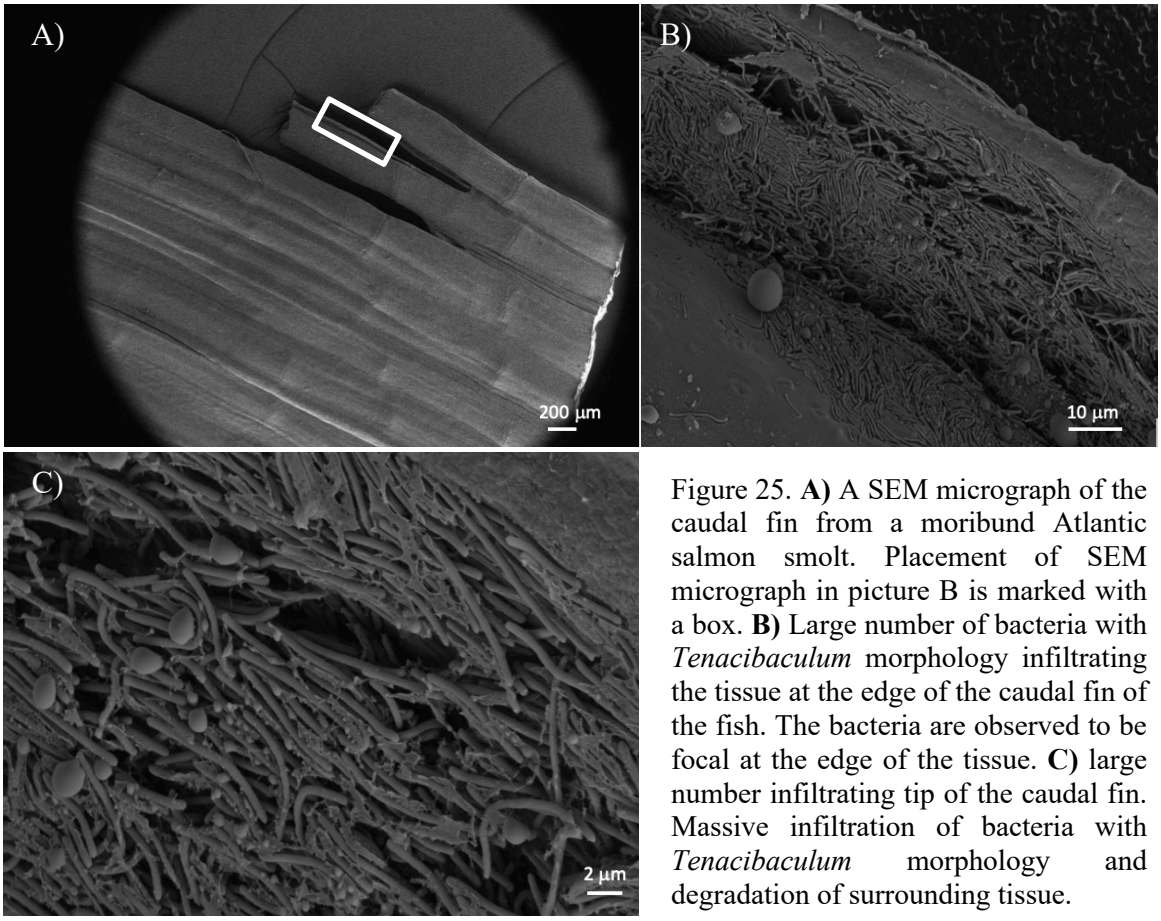

Figure 25. **A)** A SEM micrograph of the caudal fin from a moribund Atlantic salmon smolt. Placement of SEM micrograph in picture B is marked with a box. **B)** Large number of bacteria with *Tenacibaculum* morphology infiltrating the tissue at the edge of the caudal fin of the fish. The bacteria are observed to be focal at the edge of the tissue. **C)** large number infiltrating tip of the caudal fin. Massive infiltration of bacteria with *Tenacibaculum* morphology and degradation of surrounding tissue.

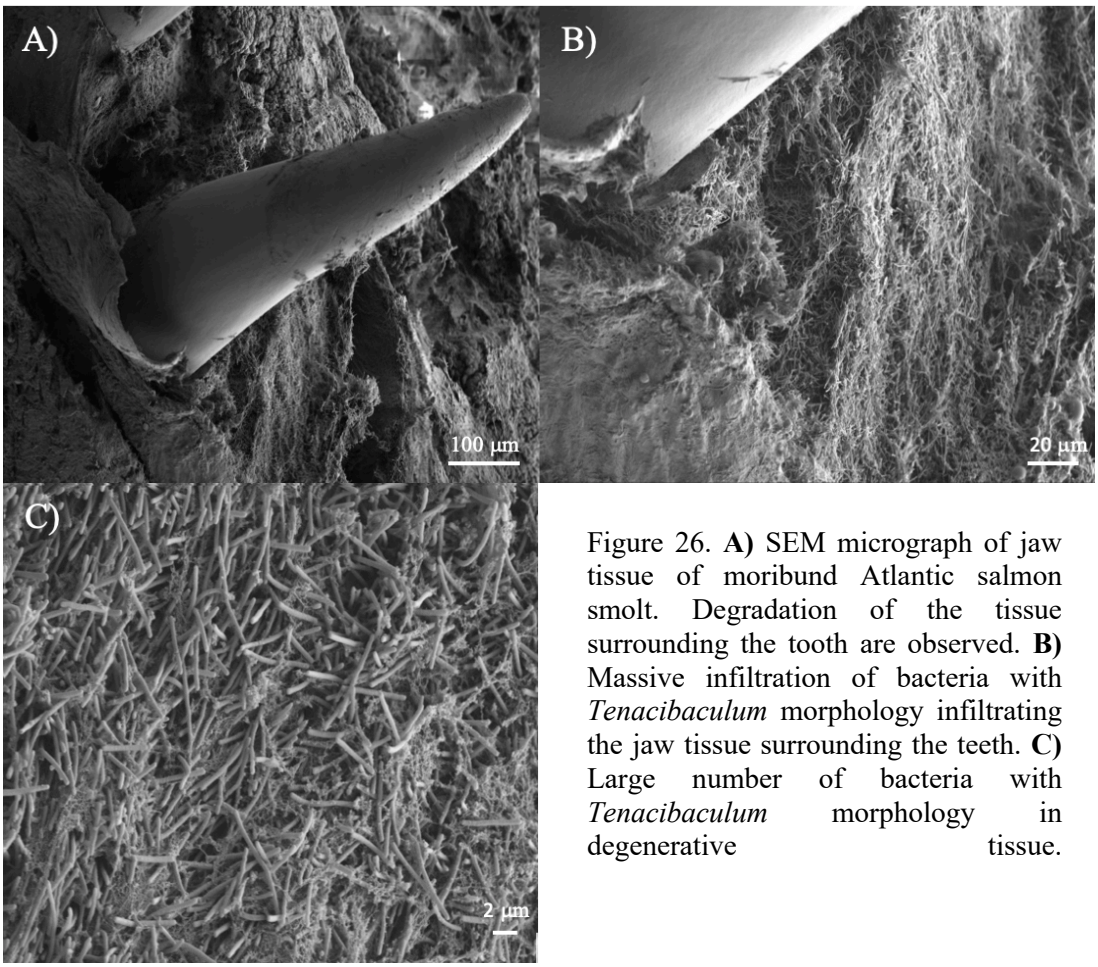

Figure 26. **A)** SEM micrograph of jaw tissue of moribund Atlantic salmon smolt. Degradation of the tissue surrounding the tooth are observed. **B)** Massive infiltration of bacteria with *Tenacibaculum* morphology infiltrating the jaw tissue surrounding the teeth. **C)** Large number of bacteria with *Tenacibaculum* morphology in degenerative tissue.

3.7 Colored smears from skin

Bacteria with *Tenacibaculum* morphology was observed in colored smears from skin (caudal fin and starting ulcer) (Figure 27). Smears from fish skin is an efficient method to investigate for bacteria with *Tenacibaculum* morphology by light microscopy.

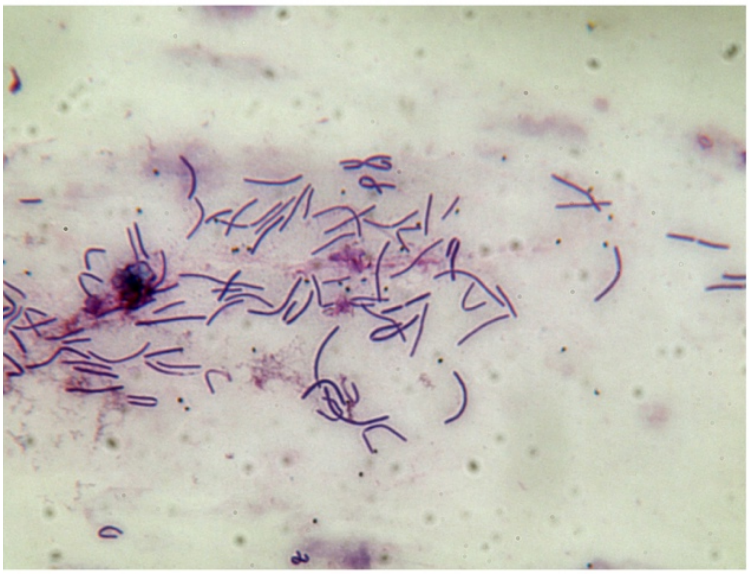

Figure 27. Colored smears from the skin from a moribund fish from Challenge 2. The smears reveal bacteria with *Tenacibaculum* morphology.

3.8 Combined mortality and statistic significant

By conducting a t – test in excel it was shown that Challenge 2F and Challenge 2LSS have a significant difference in mortality from day three post challenge. The p-value was 0.012 at the end of the challenge trial. The t – test result from Challenge 3F and Challenge 3LSS showed that there was not a significant difference between the mortality from the two different groups. The p-value was 0.11 at the end of the challenge trial and it needs to be below 0.05 for it to be a significant difference between the groups. However, Figure 28 shows the difference in mortality between the two groups in the challenge trials, where the same trend in mortality for Challenge 2F and 2LSS is observed for Challenge 3F and 3LSS (Figure 28).

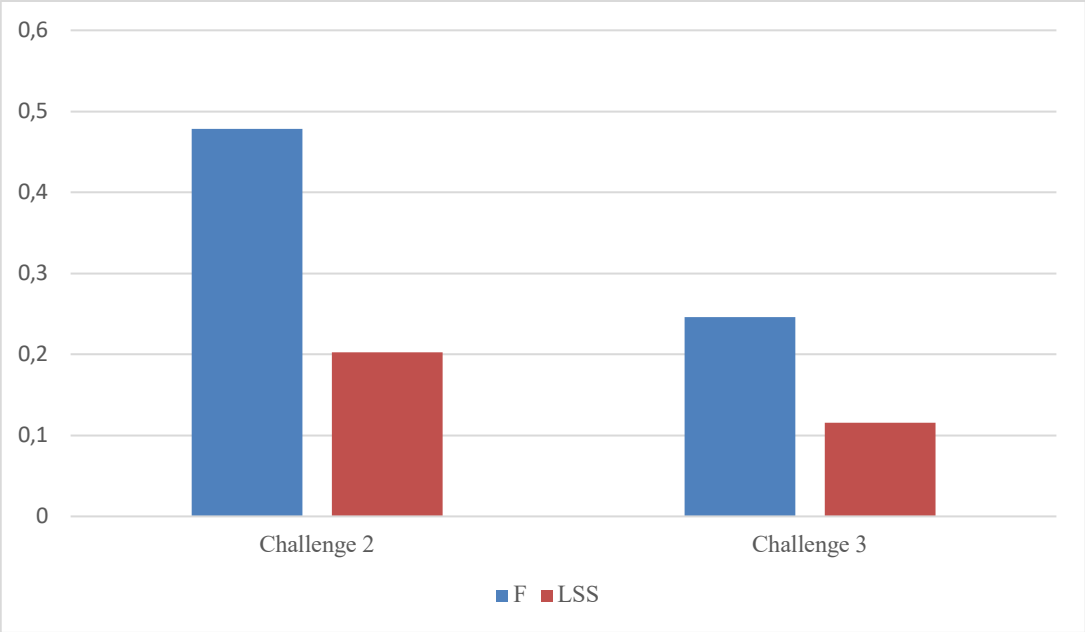

Figure 28. The figure shows the difference in mortality between the two groups (F vs LSS). Even though the difference in mortality in Challenge 3 is not significant (p-value > 0.05), this figure shows that it is the same trend in mortality for Challenge 3 as for Challenge 2.

4. Discussion

4.1 Establishing the challenge dose

An important goal in this challenge study was to establish a challenge dose at 8 °C for *T. finnmarkense* strain HFJ^T that results in a normal progression of the disease, as well as being high enough to measure potential differences in the susceptibility to tenacibaculosis between the two groups of fish. The doses selected to be used in the pre-challenge were based on previous studies on *T. finnmarkense* strain HFJ^T (Småge et al., 2018), as well as results from an unpublished study in the LimiT project performed using *T. finnmarkense* strain HFJ^T at higher temperatures (Småge pers. com). The results from the pre-challenge showed that the low dose used (7.75 x 10^5 cells/mL) did not induce tenacibaculosis at 8 °C. Interestingly, this low dose is similar to the dose used to induce tenacibaculosis, with a cumulative mortality of 80 %, at 4 °C (4.88 x 10^5 cells/mL) (Småge et al., 2018). However, in the high dose (2.2 x 10^6 cells/mL), a cumulative mortality of 80 % was recorded. This demonstrate that a higher dose is needed to induce tenacibaculosis using *T. finnmarkense* strain HFJ^T at 8 °C. Moreover, as the fish were challenged in separate containers a step involving handling was also an additional factor in the challenge procedure compared to the challenge procedure described in Småge et al. (2018), which did not involve any handling. It is known that the adaptive immune system in fish is suppressed in response to colder water temperatures (Abram et al., 2017). This may be the reason why a higher dose is needed to induce tenacibaculosis at higher temperatures and may also reflect why outbreaks of tenacibaculosis commonly occur at low seawater temperatures (< 8 °C) (Bornø & Sviland, 2011; Karlsen et al., 2017; Småge et al., 2017).

4.2 Challenge 1F

The dose for Challenge 1F was aimed to be the same as for the high dose established in the pre-challenge. The challenge material was grown to the desired OD based on the pre-challenge and growth curve. The results from the MPN measurements of the challenge material showed that the bath concentration for Challenge 1F was 1.57 x 10^6 cells/mL. This is slightly lower than the targeted bath concentration of 2.2 x 10^6 cells/mL used in the pre-challenge. The bath concentration used in Challenge 1F resulted in a mortality of 14 %, which is lower than the 80 % mortality recorded from the high dose in the pre-challenge. These results indicate that *T. finnmarkense* strain HFJ^T have a narrow threshold for inducing tenacibaculosis, which is further supported by the results from the pre-challenge where the

low dose gave no mortality, while the high dose resulted in 80 % mortality. From available literature it is evident that *Tenacibaculum*-bacteria typically have a certain threshold for inducing disease, as a challenge study using a *T. dicentrarchi* strain isolated from Atlantic salmon post-smolts in Norway, showed that a bath concentration of 3.6 x 10^6 CFU/mL resulted in no mortality, while a bath concentration of 3.0 x 10^9 CFU/mL resulted in 100 % mortality within 48 h post challenge (Klakegg et al., 2019). The notion that a certain threshold is needed to induce tenacibaculosis can make it challenging to recreate challenge models using *Tenacibaculum* spp.. This highlights the importance of making a growth curve for the specific bacterial strain intended for use in a challenge study prior to challenge and the importance of conducting a pre-challenge to establish the desired challenge dose when conducting challenge studies using *Tenacibaculum* spp..

4.3 Challenge 2F and 2LSS

The low salinity seawater (LSS) used in this study was set to 26 ppt to be able to see a difference between the effect of keeping the smolts in freshwater and keeping the smolts in LSS. It has been noticed in previous studies using *T. finnmarkense* strain HFJ^T, that fish held at 26 ppt were more difficult to infect, and that in field outbreaks, where fish had been in seawater net-pens for six weeks prior to an outbreak with tenacibaculosis, the fish appear less susceptible to tenacibaculosis compared to newly transferred smolts (Småge et al., 2017). The bath concentration in Challenge 2F and 2LSS was slightly increased from 1.57 x 10^6 to 1.69 x 10^6 cells/mL to get a higher mortality. This resulted in a higher percent mortality, with an average percent mortality in Challenge 2F of 48 % and an average percent mortality in Challenge 2LSS of 20 %. These results support the narrow threshold for inducing tenacibaculosis when using *T. finnmarkense* strain HFJ^T as discusses above, since the mortality increased even though the bath concentration were only slightly increased. The results from this challenge trial makes it possible to see a positive effect of keeping smolts in LSS for four weeks prior to sea water transfer and challenge with *T. finnmarkense* strain HFJ^T. By conducting a t – test in excel it was shown that it was a significant difference ($p < 0.05$) in mortality from day three post challenge, resulting in a p-value of 0.012 at the end of the challenge trial. Analysis performed by using a sample size calculator (https://clincalc.com/stats/samplesize.aspx) shows that based on the cumulative mortality, the number of fish used in this challenge trial was sufficient to perform statistical analysis to detect an effect of keeping the smolts in two different water qualities.

Before the challenge trial was conducted, it was observed that the LSS-fish had a less pristine look compared to the F-fish. It is commonly believed that smolt displaying a more pristine look is a more robust smolt. Interestingly, this was not the case in this study. The welfare scoring of the fish during the challenge trial showed that a higher number of the F-fish developed more severe lesions than the LSS-fish (Table 11). In addition, weight measurements of the smolts prior to the challenge trial revealed that the F-fish had an average higher weight than the LSS-fish. Hence, size and a pristine look do not seem to have had any impact on the smolts susceptibility for tenacibaculosis. Nofima investigated tissues histologically from both water qualities to look for differences in histopathological changes due to the water quality (Fredriksen, 2020). The results showed that both the LSS-fish and the F-fish had histopathological changes possibly caused by *T. finnmarkense*, but the F-fish had more severe histopathological changes than the LSS-fish. These findings indicate that transfer to sea water and challenge with *T. finnmarkense* strain HFJ^T have a more negatively effect on the F-fish than the LSS-fish. In general, the histopathological changes observed from tissue samples of affected skin resembled the observation made from histological sections of skin described both from field outbreaks of tenacibaculosis and from challenge studies using *T. finnmarkense* (Småge et al., 2017, 2018; Olsen et al., 2011). This include loss of epidermis and large number of bacteria with *Tenacibaculum* morphology infiltrating the dermis that causes degradation of the *stratum spongiosum* (Figure 23). The loss of epidermis has also been reported from similar tenacibaculosis lesions associated with *T. dicentrarchi* (Avendaño-Herrera et al., 2016). The bacteria are typically found infiltrating the *stratum compactum,* where the bacteria can be observed aligned with the collagen fibers (Figure 23). In severe cases, the bacteria infiltrate all the way down to the hypodermis and the muscle fibers, causing damage to the connective tissue (Figure 24).

4.4 Challenge 3F and 3LSS

For Challenge 3F and 3LSS, it was desired to get an even higher average percent mortality to clearly see the difference between the two groups and to assess statistical analyses. Based on the results from the SmoltVision report that indicated that the F-fish was starting to de-smoltify, it was expected to be less tolerant to the seawater. Based on the fact that *T. finnmarkense* strain HFJ^T have a narrow threshold for inducing tenacibaculosis, the bath concentration was only increased to 1.71 x 10^6 cells/mL. This resulted in an average percent

mortality of 25 % in the F-group and 12 % in the LSS group. From the t-test it is clear that these results do not demonstrate a significant difference between the two groups, with a calculated p-value of 0.11 at the end of the challenge trial. Analysis performed by using a sample size calculator (https://clincalc.com/stats/samplesize.aspx) shows that the number of fish used in this challenge trial is not sufficient to detect an effect of the two water qualities. However, by comparing the results from Challenge 2F and 2LSS to the results from Challenge 3F and 3LSS, it is a clear trend that keeping the smolts in LSS for eight weeks have the same effect as shown after four weeks. The same less pristine look of the LSS-fish as observed in Challenge 2LSS was observed also in this challenge trial, but still the F-fish were more susceptible to tenacibaculosis than the LSS-fish. The same histopathological changes observed in Challenge 2F and 2LSS was observed in this challenge trial.

4.5 Clinical signs

The diseased smolts in the challenge trials presented typical clinical signs of tenacibaculosis observed in natural field outbreaks and challenge studies, as skin ulcers, erosion of the mouth/jaw and fins were the dominant pathological finding (Figure 18 – Figure 21). *T. finnmarkense* is shown to have high affinity of the non-scaled part of the body (Bornø & Sviland, 2011; Småge et al., 2017, 2018; Takle et al., 2015). This has also been reported from other fish pathogenic members of the family Flavobacteriaceae infecting Atlantic salmon (Martínez et al., 2004). However, in this challenge study, the jaw-region of the fish seemed to be less affected compared to what has been reported from challenge studies using *T. finnmarkense* conducted at 4 °C (Småge et al., 2018). Interestingly, more fish had lesions/ulcers at the abdomen without the jaw being affected in this current study. The reason for this may be that the fish were observed "resting" at the bottom of the tanks. This can potentially lead to abrasion in the skin at the abdomen and damage the epithelial barrier of the skin which can facilitate bacterial invasion. On the other hand, lesions/ulcers along the abdomen has also been described as typically findings during field outbreaks of tenacibaculosis (MarinHelse, n.d.; Småge et al., 2017; Takle et al., 2015). This could indicate that the "resting" at the bottom of the tanks is not the reason for why the lesions were present on the abdomen.

The clinical signs observed in this challenge study differ from the clinical signs reported from outbreaks with *M. viscosa*, that affects the scale covered part of the body with circular lesions

along the flanks as the most common clinical sign. Lesions observed during this challenge study had a wide area of scale loss (Figure 20) and were most located at the abdomen. All skin samples from the fish sampled during each challenge trial were negative for *M. viscosa* when analyzed by using real-time RT-PCR.

4.6 Bacteriology

The *T. finnmarkense* strain HFJ^T used for challenge was re-isolated from lesions/ulcers on the fish from each challenge trial. The sequencing of the bacteria resulted in a 100 % match between the bacteria re-isolated from the challenge trials and the bacteria used for challenge. This fulfills Koch`s postulates for *T. finnmarkense* strain HFJ^T (Fredricks & Relman, 1996). *T. finnmarkense* strain HFJ^T was isolated from kidney samples from three fish from Challenge 2F. Two of these fish were given score 2, while the third fish was given score 3 according to the welfare score used (Table 4). This indicates that the fish were severely affected by the bacteria and that the bacteria may have managed to enter the blood stream. Interestingly, *T. finnmarkense* is rarely isolated from internal organs, and, when it does, it seems to result in very little to no systemic signs (Olsen et al., 2011; Småge et al., 2018), which is different from what is known for *T. maritimum* which is readily isolated from kidney (Frisch et al., 2018). There might also be a slight chance that the isolation of *T. finnmarkense* strain HFJ^T from the kidney is due to contamination, although great care was taken to avoid this during the bacteriological sampling from the kidney.

Bacteria identified as *Psychrobacter* sp. and *Pseudoalteromonas* sp. (sequences available in the appendix), were isolated from the skin of two control fish in Challenge 3F and 3LSS. From the literature it is clear that these are harmless bacteria normally found in the seawater (Holmström & Kjelleberg, 1999; Bowman, 2006). As no mortality was registered from the control fish, it is believed that these bacteria had no impact on the results from this challenge study.

4.7 Real time RT-PCR

When sampling lesions from challenged fish, *Tenacibaculum* was, with no exceptions, detected by using real-time RT-PCR. The results showed a clear difference in Ct-value between the moribund fish and the asymptomatic fish, where it seems like the fish either gets the disease (low Ct) or the bacteria do not manage to establish an infection in the skin (high

Ct). This further supports the notion of a threshold (sufficient infection pressure) in order to induce tenacibaculosis as previously discussed. These findings can be linked to findings from field outbreaks, where it appears to be a seasonal variation in the tenacibaculosis outbreaks in Northern Norway (Småge, 2018). The outbreaks are often linked to high phytoplankton blooms that typically occur during spring and autumn (Bratbak et al., 1990; Larsen et al., 2004; Småge, 2018). This seasonality in the outbreaks is similar to what have been reported for outbreaks of skin lesions/ulcers in farmed Atlantic salmon smolts in British Columbia, Canada (Kent et al., 1988). These findings suggest that a high concentration of the bacteria in the water during sea transfer of smolts is needed to induce tenacibaculosis. The threshold of bacteria needed to induce tenacibaculosis may be affected by several factors as for example abrasion in the skin, smolt status and temperature. These findings also support the finding from a previous study using *T. finnmarkense* strain HFJ^T that showed that the bacterium does not easily transmit from fish to fish (Småge et al., 2018). The real-time RT-PCR results from Challenge 2F and 2LSS and Challenge 3F and 3LSS shows a clear difference in the number of fish with Ct < 20 and Ct > 30, where the F-groups in both challenge trials are more heavily affected by the bacteria. The reason for this is not known, but it shows that the exposure for LSS for 4-8 weeks prior to challenge may be a contributing factor for a positive effect on the smolts susceptibility for *T. finnmarkense*.

A few control fish tested weekly positive for *T. finnmarkense* when screening skin samples using the assay Tb_rpoB. The reason for this may be that the assay is not specific for *T. finnmarkense* but can also detect other *Tenacibaculum* sp. normally present in the seawater. Based on the re-isolation of *T. finnmarkense* strain HFJ^T, it is fairly certain that it is *T. finnmarkense* strain HFJ^T that is detected in the real-time RT-PCR analyses from moribund fish sampled during the challenge trials. The *Tenacibaculum* sp. detected form the control is most likely other *Tenacibaculum* species present in the water that have been detected by the assay. This did not impact the challenge study, as no mortality were observed in the control fish and *T. finnmarkense* strain HFJ^T were not re-isolated from the control fish.

Kidney sample from ten moribund fish from Challenge 1F were screened for *Tenacibaculum* where eight of the ten samples tested weakly positive. The fish had ulcers caused by *T. finnmarkense* strain HFJ^T and the positive kidney samples may be due to the fact that the bacteria had manage to enter the bloodstream. When performing re-isolation from the kidney,

no growth from Challenge 1F were detected. As mentioned above, *T. finnmarkense* is rarely isolated from internal organs.

4.8 Other findings

By comparing the initial weight of the smolts to the average weight of the smolt at the end of the challenge trial, the LSS-smolts had a larger increase in weight during the challenge trials compared to that of the F-smolts. During Challenge 2F and 2LSS and Challenge 3F and 3LSS it was observed that the control fish and the LSS-smolts resumed to feeding approximately a week before the F-smolts (Table 11). It is known that fish have reduced or no appetite when they are sick (infected). The postponed feeding after challenge for the F-fish, may indicate that the F-smolts were more negatively affected by *T. finnmarkense* strain HFJ^T than the LSS-smolts. It is also known that the fish have an appetite drop after transfer from freshwater to seawater, but it is not evident that larger fish are resuming to feeding sooner than smaller fish (Usher et al., 1991). The control fish from both water qualities resumed to feeding within 2 days post challenge. This further indicates that it is likely that the exposure to bacterium is the reason for the appetite drop and not an effect of the water quality that the smolts were held in post smoltification.

4.9 Implementing LSS as a new production strategy for production of smolts

The most used production strategy for producing smolt is freshwater in flow-through facilities where the smolts are directly transferred from freshwater to seawater net-pens shortly following smoltification. Reports from a nationwide survey found that an average of 16 % of the Atlantic salmon put to sea is lost before they reach harvest size (Bleie & Skrudland, 2014), and most losses occur shortly after sea transfer (Aunsmo et al., 2008). It has been suggested that to produce a larger and potentially more robust smolt should improve the survival after sea transfer by making the smolt less susceptible to pathogens (Ytrestøyl et al., 2019). New production systems including land-based recirculating aquaculture systems (RAS) or closed and semi-closed facilities at sea, are being developed to investigate the possibility of producing a more robust smolt. In RAS facilities it is possible to add seawater in the production, which enable the use of LSS as a production strategy. From this current study, this production strategy could possibly be efficient for producing robust smolts. In addition, Ytrestøyl et al. (2019) performed a study where the effect of salinity and water velocity were tested on large post-smolts performance and welfare in brackish and seawater RAS. Their

study showed that exposing the smolts for brackish water (12 ppt) had a positive effect on the mortality compared to smolts exposed for full-strength sea water. This is the same as demonstrated in this current study, where it is shown that a gradually adaption to seawater is advantageous for the smolts susceptibility to tenacibaculosis. This gradually adaption to seawater resembles the natural lifecycle of Atlantic salmon smolts as they migrate from the rivers and into the sea water.

Findings from this study can be very important for future development of new production strategies and facilities. The use of brackish water RAS have higher costs than flow-through facilities and new challenges may arise. For sea water RAS it has been reported problem with accumulation of CO_2 and problems with H_2S (Sommerset et al., 2020). When pumping sea water into the facility, strict biosecurity is important to prevent pathogens from entering. Ulcers has been reported as a problem from sea water RAS (Sommerset et al., 2020). By implementing the results from this study to new productions facilities, brackish water RAS may be a better solution to prevent sea water pathogens (as *Tenacibaculum* spp.) from entering the facilities. 26 ppt have shown to have a positive effect om the smolts susceptibility for tenacibaculosis, but further studies are needed to investigate whether the salinity in the water can be lowered even more or if a salinity of approximately 26 ppt is needed.

5. Conclusion and future research

In this study, a challenge model for *T. finnmarkense* strain HFJ^T was established showing that a higher dose is needed to induce tenacibaculosis at 8 °C compared to at 4 °C. It was shown that the bacterium has a narrow threshold for inducing the disease, which highlights the importance of establishing a growth curve for the strain using the same growth media as used for the challenge trials, as well as conducting a pre-challenge. As the fish were challenged in separate containers, a step involving handling may also be important to establish infection using the developed challenge model. This study shows that there is a statistic significant effect of keeping the smolts in LSS for four weeks in reducing the susceptibility to tenacibaculosis. Therefore, this may be a good production strategy in order to reduce problems related to tenacibaculosis in the Norwegian aquaculture. The results from Challenge 3F and 3LSS indicates that keeping the smolts in LSS for eight weeks have the same reducing effect on the susceptibility to tenacibaculosis, as was shown for smolts held in LSS for four weeks. However, the difference in mortality between LSS and F were not statistically significant. The real-time RT-PCR analyses shows that tissue from fish that did not get infected had a higher Ct-value or were negative when screened for *T. finnmarkense*. This indicates that the bacterium is not able to colonize, multiply and establish an infection of the skin of the host over time. This further supports the notion that *T. finnmarkense* is not easily transmitted horizontally between the fish and that the fish that gets infected likely get the infection by a high infection pressure from the environment. Koch`s postulates were yet again fulfilled for *T. finnmarkense* strain HFJ^T, as the clinical signs and microscopical pathology were reproduced and the bacteria used for challenge were re-isolated, as well as that no disease was observed in the control fish.

More work is needed to establish the effect of keeping smolts in LSS before transfer to seawater net-pens at lower temperatures. Studies have shown that to expose smolts to brackish water (12 ppt) have good effect on growth and survival of the smolts. In this current study it is shown that 26 ppt have an effect on smolts susceptibility to tenacibaculosis, however, further studies are needed to establish the optimal salinity to be used before transfer to sea water.

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Appendix

Recipes

1,5% agarose gel:

- SeaKem® LA Agarose (Cambrex) solved in 400 mL 1X TAE-buffer
- Heat in microwave oven and store at 60°

50X TAE-buffer (Tris-Acetate-EDTA-buffer):

- Tris Base (Merck) 242 g
- Glacial acetetic acid 57.1 mL
- 0.5M EDTA (pH=8.0) 100 mL
- Add dH_2O to a final volume of 1000 mL

1X TAE-buffer:

- 200 mL 50X TAE-buffer
- 10 L H_2O

Ringer`s solution:

- $NaH_2PO_4H_2O$ 1.65 g
- $Na_2HPO_{32}H_2O$ 6.76 g
- NaCl 6.75 g
- KCl 0.12 g
- $NaHCO_3$ 0.15 g
- Glucose 1.65 g
- (dd)H_2O 1000 mL

Karnovsky fixative (100 mL):

- Ringer`s solution 80 mL
- 25% glutaraldehyde 10 mL
- 10% paraformaldehyde
- Sucrose 4 gr

BAMA (Blood agar with sea salt):

- Peptone from animal tissue 5.0 g
- Yeast extract 1.0 g
- Coral pro salt (Red sea) 37.2 g
- Bacteriological agar 15.0 g
- Distilled water 950 mL
- Sterile defibrinated sheep blood 50 mL

Marine broth:

- Peptone from animal tissue 5.0 g
- Yeast extract 1.0 g
- Coral pro salt (Red Sea) 37.2 g
- Distilled water 1000 mL

Optimization and efficiency of primer and probe

Optimization:

Table 12. The optimized concentrations (nM) of forward and revers primer for assay Hsal and assay EF1A, as well as the optimized probe concentration for each assay.

Assay	**Primer (F/R)**	**Probe**
Hsal	600/800	175
EF1A	800/800	175

Table 13. Comparison of the mean Ct-value results from using the optimized concentration and standard concentration of assay Hsal and assay EF1A.

Assay	**Hsal**	**EF1A**
Mean Ct-value optimized	27,0	19,2
Mean Ct-value standard	25,6	19,6

Table 14. Comparison of the mean Ct-value results from using the optimized concentrations and standard concentrations of assay Tb_ropB and MvOmpA.

Assay	**Mean Ct-value optimized**	**Mean Ct-value standard**
Tb_rpoB	31,2	31,1
MvOmpA	18,7	18,7

Efficacy:

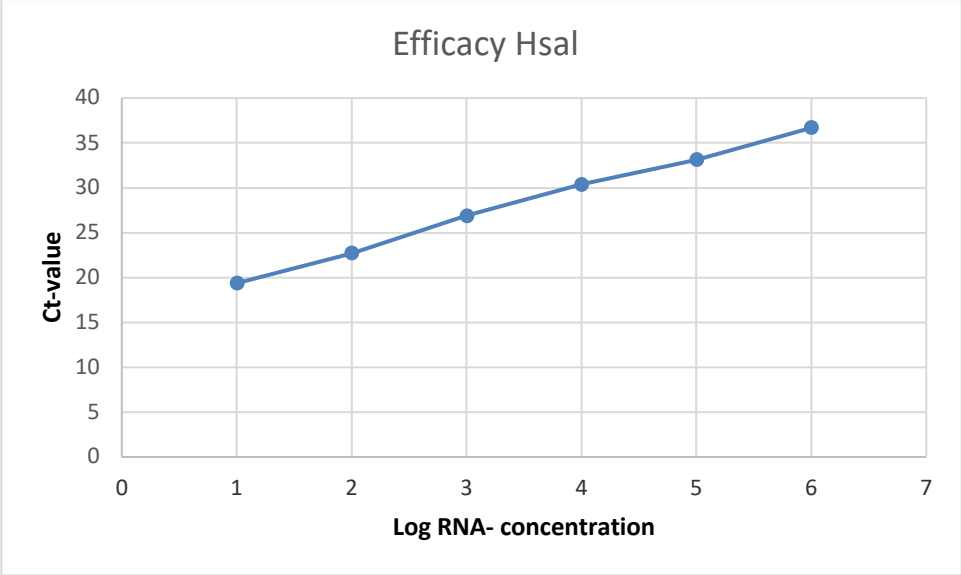

Figure 29. The standard curve of assay Hsal, with a linear trendline. The RNA dilutions are tenfold, starting from 1 to 10^{-6}. The slope is 3.466 and the efficacy is 94 %.

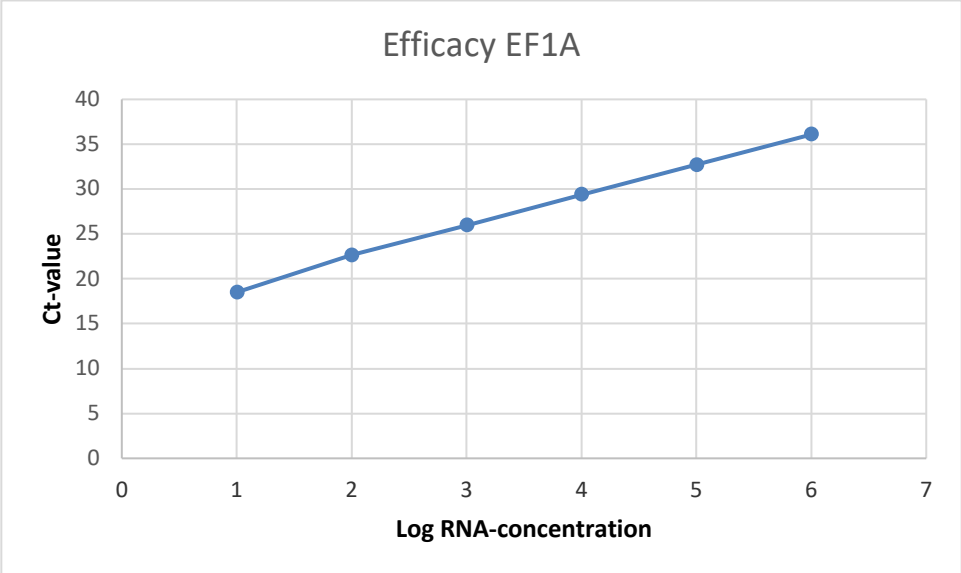

Figure 30. The standard curve of assay EF1A, with a linerar trendline. The RNA dilutions are tenfold, strating from 1 to 10^{-6}. The slope is 3.467 and the efficacy is 94 %.

Growth curve of *T. finnmarkense* strain HFJ^T:

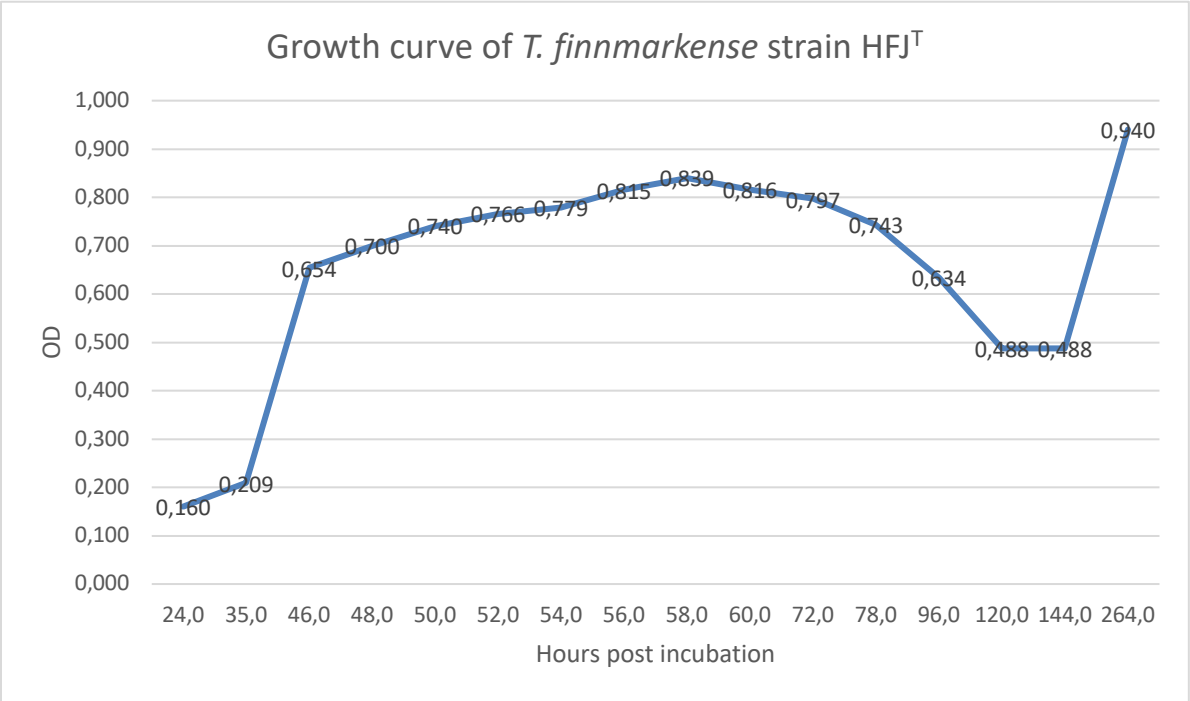

Figure 31. Growth curve for *T. Finnmarkense* strain HFJ^T grown in Difco 2216, Marine Broth (MB). The figure shows the lag phase, log phase, stationary phase and the death phase of *T. finnmarkense* strain HFJ^T. Between 144 hours post incubation (hpi) and 246 hpi, the OD increased to the higher OD measured: 0.94. The reason for this is not known.

Weight and length:

Table 15. Average weight and length of all fish sampled during the challenge trials and termination. N=95 for each challenge trial.

Challenge trial	Weight (g)	Length (cm)
1F	74,64	18,76
2F	107,29	21,39
2LSS	98,97	20,78
3F	164,78	24,42
3LSS	147,01	23,17

Sequencing

Challenge 1F:

Table 16. The PNI (Percentage Nucleotide Identity) of *Tenacibaculum* isolates collected from fish at Challenge 1F. All isolates display a 100% match to each other and to the challenge isolate, based on the 16S rRNA gene and the *RlmN* gene. The analysis was performed in the Vector NTI software.

Isolates	**Challenge**	**K1F1**	**K3F2**	**K2F4**	**K1F4**	**K3F4**	**K1F4**	**K2F1**
Challenge	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K3F2	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K2F4	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F4	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K3F4	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F4	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K2F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %

Challenge 2F and 2LSS:

Table 17. The PNI (Percentage Nucleotide Identity) of *Tenacibaculum* isolates collected from fish at Challenge 2F and 2LSS. All isolates display a 100% match to each other and to the challenge isolate, based on the 16S rRNA gene and the *rlmN* gene. The analysis was performed in the Vector NTI software.

Isolates	Challenge	K3F8	K3F8 N	K2F2 N	K5F1	K6F2	K3F2	K2F2	K1F1	K1F8	K1F8 N	K7F1	K6F1
Challenge	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K3F8	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K3F8 N	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K2F2 N	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K5F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K6F2	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K3F2	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K2F2	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F8	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K1F8 N	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K7F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
K6F1	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %

Challenge 3F and 3LSS:

Table 18. The PNI (Percentage Nucleotide Identity) of *Tenacibaculum* isolates collected from fish at Challenge 2F and 2LSS. All isolates display a 100% match to each other and to the challenge isolate, based on the 16S rRNA gene and the *RlmN* gene. The analysis was performed in the Vector NTI software.

Isolates	**Challenge**	**K3F2**	**K1F3**	**K6F4**	**K7F4**
Challenge	100 %	100 %	100 %	100 %	100 %
K3F2	100 %	100 %	100 %	100 %	100 %
K1F3	100 %	100 %	100 %	100 %	100 %
K6F4	100 %	100 %	100 %	100 %	100 %
K7F4	100 %	100 %	100 %	100 %	100 %

Sequence for *Pseudoalteromonas* sp. and *Psychrobacter* sp. isolated from control fish in Challenge 3F and 3LSS:

16S rRNA gene sequence *Pseudoalteromonas* sp.:
CTAGCTTGCTAGAAGATGACGAGCGGCGGACGGGTGAGTAATGCTTGGGAACATGCCTTGAG
GTGGGGGACAACAGTTGGAAACGACTGCTAATACCGCATAATGTCTACGGACCAAAGGGGGC
TTCGGCTCTCGCCTTTAGATTGGCCCAAGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACC
AAGGCGACGATCCCTAGCTGGTTTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
ATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCAGGAGGAAAGGTTA
GTAGTTAATACCTGCTAGCTGTGACGTTACTGACAGAANAAGCACCGGCTAACTCCGTGCCAG
CAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGTACGCA
GGCGGTTTGTTAAGCGAGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATTTCGAACTGG
CAAACTAGAGTGTGATAGAGGGTGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATC
TGAAGGAATACCGATGGCGAAGGCAGCCACCTGGGTCAACACTGACGCTCATGTACGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTAGAAG
CTCGGAACCTCGGTTCTGTTTTTCAAAGCTAACGCATTAAGTAGACCGCCTGGGGAGTACGGC
CGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGCAACGCGAAGAACCTTACCTACACTTGACATACAGAGAACTTACCAGAGATGGTT
TGGTGCCTTCGGGAACTCTGATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAGATG
TTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATCCTTAGTTGCTAGCAGGTAATGCTGAGAA
CTCTAAGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGC
CCTTACGTGTAGGGCTACACACGTGCTACAATGGCGCATACAGAGTGCTGCGAACTCGCGAG
AGTAAGCGAATCACTTAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAA
GTCGGAATCGCTAGTAATCGCGTATCAGAATGACGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGNTAG

16S rRNA gene sequence *Psychrobacter* sp.:
GATGGTAGCTTGCTACCAGGCGTCGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCT
AGTAGTGGGGGATAGCACGGGGAAACTCGTATTAATACCGCATACGACCTACGGGAGAAAGG
GGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAA
GGCCTACCATGGCGACGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAG
ACACGGCCCGGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTG
ATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCAGTGAAGA
AGACTCTTCGGTTAATACCCGGAGACGATGACATTAGCTGCAGAATAAGCACCGGCTAACTCT
GTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC
GAGCGTAGGTGGCTTGATAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATCTG
AAACTGTTAGGCTAGAGTAGGTGAGAGGGAAGTAGAATTTCAGGTGTAGCGGTGAAATGCGT
AGAGATCTGAAGGAATACCGATGGCGAAGGCAGCTTCCTGGCATCATACTGACACTGAGGCT
CGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTA
CTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGCTAACGCAATAAGTAGACCGCCTGGGG
AGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATATCTAGAATCCTGCAG
AGATGCGGGAGTGCCTTCGGGAATTAGAATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGGGTTAA
GCCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGAGGAAGGCGGGGACGACGTCAAGTC
ATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGTAGGTACAGAGGGCAGCTAC
ACAGCGATGTGATGCGAATCTCAAAAAGCCTATCGTAGTCCAGATTGGAGTCTGCAACTCGAC
TCCATGAAGTAGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACACCATGGGAGTTGATTGCACCAGAAGTGGATAGC

Real time RT-PCR: Ct-values

Challenge 1F:

Table 19. Overview of the Ct-values obtained by using the Tb_rpoB assay targeting *Tenacibaculum* spp. from each tank in Challenge 1F. Fish were sampled from the lower jaw or the margin or ulcers if present. The red box represents random sampled fish at tree dpc.

	Kar 1	Kar 2	Kar 3	Kar 4
Sample Name	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB
F1	15,4	15,4***	Neg	36,7
F2	Neg	33,6	32,5	Neg
F3	Neg	34,5	Neg	Neg
F4	11,9**	10,9***	12,2***	Neg
F5	12,0***	15,0***	25,2***	Neg
F6	14,2***	34,8	14,6***	Neg
F7	36,4	Neg	15,2***	Neg
F8	Neg	32,1	Neg	Neg
F9	Neg	Neg	Neg	Neg
F10	Neg	36,2	Neg	Neg
F11	Neg***	34,6	37,1	Neg
F12	Neg	Neg	Neg	Neg
F13	36,5	Neg	Neg	Neg
F14	Neg	Neg	Neg	Neg
F15	Neg	Neg	Neg	Neg
F16	Neg	35,7	38,5	Neg
F17	Neg	Neg	Neg	Neg
F18	Neg	Neg	Neg**	Neg

F19	Neg	Neg	Neg	Neg
F20	31,6***	Neg	Neg	31,4
F21	Neg	Neg	36,6	Neg
F22	Neg	Neg	Neg	Neg
F23	Neg	Neg	Neg	Neg
F24				Neg
F25				Neg
F26				36,5

Challenge 2F and 2LSS:

Table 20. Overview of the Ct-values for Tb_rpoB assay targeting *Tenacibaculum* spp. from each tank in Challenge 2F and 2LSS. Fish is sampled from the lower jaw or the margin or ulcers if present. The red box represents random samples fish three dpc. The * represent what welfare score the fish was given when sampled. Challenge 2F: tank-1 – tank-3. Challenge 2LSS: tank-5 – tank -7.

	Kar 1	Kar 2	Kar 3	Kar 5	Kar 6	Kar 7	Kar 4	Kar 8
Sample Name	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB
F1	17,0	35,1	15,5***	14,4	15,4**	13,9	Neg	Neg
F2	11,5***	12,4**	14,5**	33,3	17,0	28,0	Neg	Neg
F3	35,6	29,8	36,6	13,7**	36,4	14,8**	Neg	Neg
F4	33,7	14,3***	34,5	34,3	31,7	Neg	Neg	Neg
F5	13,2	15,4**	14,2	12,8***	Neg	20,6**	Neg	Neg
F6	11,8**	16,0**	16,5**	32,9	28,2	14,7***	Neg	Neg

F7	15,2**	28,4***	15,5**	31,7	16,8***	14,2***	Neg	34,6
F8	14,9**	28,3**	15,4***	24,5***	15,6***	13,1***	Neg	Neg
F9	16,8**	14,2**	14,5**	26,6	Neg	34,7**	Neg	Neg
F10	18,3**	27,0***	13,8**	35,3	Neg	Neg	Neg	Neg
F11	16,6***	33,4	29,9	31,0	30,5**	37,0***	Neg	Neg
F12	16,8	34,8***	13,7***	36,1	Neg	Neg	Neg	Neg
F13	14,4***	36,4	14,6	36,2	Neg	Neg	Neg	Neg
F14	16,4***	35,5	14,8***	34,2	35,2	Neg	Neg	Neg
F15	33,4	Neg	28,4	32,7	Neg	36,2***	Neg	Neg
F16	32,6	37,8**	21,2	34,6	Neg	Neg	Neg	Neg
F17	Neg	34,4	Neg	34,1	Neg	Neg	Neg	Neg
F18	35,4	Neg	Neg	33,0	Neg	36,1	Neg	Neg
F19	Neg***	35,5	36,4	33,3	Neg	Neg	Neg	Neg
F20	Neg	36,5	36,5	34,6	Neg	Neg	Neg	Neg
F21	Neg**	36,1***	36,0	34,0	Neg	Neg	Neg	Neg
F22	35,1	Neg	Neg	34,7	Neg	Neg	Neg	Neg
F23	32,2	34,5	36,2	34,1	Neg	Neg	Neg	Neg
F24							Neg	Neg
F25							Neg	Neg
F26							Neg	Neg

Challenge 3F and 3LSS:

Table 21. Overview of the Ct-values for Tb_rpoB assay targeting *Tenacibaculum* spp. from each tank in Challenge 3F and 3LSS. Fish is sampled from the lower jaw or the margin or ulcers if present. The red box represents random samples fish three dpc. The * represent what welfare score the fish was given when sampled. Challenge 3F: tank-1 – tank-3. Challenge 3LSS: tank-5 – tank -7.

	Kar 1	Kar 2	Kar 3	Kar 5	Kar 6	Kar 7	Kar 4	Kar 8
Sample Name	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB	Tb_rpoB
F1	35,1	32,1	36,5	34,2	Neg	35,7	Neg	Neg
F2	26,0**	34,3	12,3**	Neg	Neg	32,8	Neg	Neg
F3	15,0**	13,7**	12,5**	Neg	36,5	33,1	Neg	Neg
F4	12,6**	16,2**	14,1**	36,6***	18,2**	31,5**	Neg	Neg
F5	13,6***	14,9***	18,7***	32,5**	27,7***	19,3***	Neg	Neg
F6	23,8	15,2**	32,0**	34,4***	22,5***	35,3	Neg	Neg
F7	Neg	22,4**	14,1***	28,1	Neg	Neg	35,5	Neg
F8	35,5	30,6**	23,6	23,9	Neg	Neg	Neg	Neg
F9	Neg	35,3	25,2**	28,5	Neg	Neg	Neg	Neg
F10	Neg	34,7	Neg	Neg	Neg	Neg	Neg	Neg
F11	36,5	34,5	36,5	Neg	31,6	Neg	33,9	Neg
F12	Neg	35,6	35,2	32,4	Neg	Neg	Neg	Neg
F13	Neg	36,5	36,4	Neg	Neg	35,4	31,8	Neg
F14	Neg	30,6**	30,4	Neg	34,6	Neg	Neg	Neg
F15	Neg	33,8	Neg	Neg	Neg	Neg	Neg	Neg
F16	Neg	32,9	33,9	Neg	Neg	Neg	Neg	Neg
F17	Neg	34,4	Neg	33,5	Neg	Neg	Neg	Neg
F18	35,4	36,4	28,5	Neg	Neg	36,4	Neg	Neg
F19	34,0	33,0	30,7	34,4	36,7	Neg	Neg	Neg
F20	Neg	36,2	36,4	Neg	Neg	Neg	Neg	Neg
F21	Neg	Neg	Neg	36,4	Neg	Neg	Neg	Neg
F22	Neg	Neg	Neg	36,3	Neg	Neg	Neg	Neg

F23	Neg	35,3	Neg	Neg	36,1	Neg	Neg	Neg
F24							34,7	Neg
F25							Neg	Neg
F26							Neg	Neg