The effect of keeping Atlantic salmon smolts in low salinity seawater in reducing mouthrot after seawater transfer

- and the effect of freshwater as a mitigation measure against mouthrot

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"Even a stopped clock gives the right time twice a day." ~ I, in Withnail and I (1987)

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ABSTRACT

Mouthrot is a significant cause of mortality in Atlantic salmon (*Salmo salar*) smolts recently transferred to seawater in the Western Canada Atlantic salmon aquaculture industry. Mouthrot, associated with *Tenacibaculum maritimum* infections, is the main reason the Atlantic salmon farming industry in the British Columbia (BC) region continues to use antibiotics.

The main objective of this study was to investigate the effect of keeping Atlantic salmon smolts in low salinity seawater at different time periods prior to seawater transfer in reducing the severity of *T. maritimum* infections in BC and to take steps against reducing the use of antibiotic treatments by implementing freshwater treatment as a mitigation measure.

A bath challenge study was conducted to investigate the effect of keeping smolts in LSS for 4 weeks and 8 weeks prior to seawater transfer. An *in vitro* survival test of the *T. maritimum* strain TmarCan15-1 was developed to investigate the survival of *T. maritimum* in freshwater and a freshwater treatment was conducted on newly smoltified smolts directly exposed with *T. maritimum* strain TmarCan15-1.

Based on the results from this study, it is beneficial to keep the smolts in LSS for 8 weeks after smoltification prior to seawater transfer in reducing the severity of mouthrot. The results of this study also suggest that freshwater treatments can be a mitigation measure against mouthrot outbreaks and reducing the need for antibiotic treatments.

Further research needs to focus on the effect of keeping smolts in different water salinities and at different time periods prior to seawater transfer in order to establish at which salinities and time periods the beneficial effects demonstrated in this study are *T. maritimum* effectuated.

ABBREVATIONS

16s rRNA	16S (Svedberg) ribosomal ribonucleic acid genes
BAMA	Cermaq marine blood agar
BC	British Columbia
CMB	Cermaq Marine Broth
FW	Freshwater
HK	Housekeeping
KA-BAMA	Cermaq kanamycin marine blood agar
LSS	Low salinity seawater (26 ppt)
MB	Marine broth (Difco 2216)
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
PBC	Primary bacterial culture
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SBC	Secondary bacterial culture

Bath Challenge Study: refers to the entire study and includes all three bath challenge trials.

Bath Challenge Trial: refers to one specific bath challenge trial which is part of the bath challenge study.

1. INTRODUCTION

1.1. Aquaculture on the Pacific side of North America

Salmon farming in Canada began in the 1970s with a small number of entrepreneurs that used ocean net pens to raise native Coho salmon (*Oncorhynchus kisutch*) and Chinook salmon (*Oncorhynchus tshawytscha*) in the Pacific northwest. However, the industry soon shifted to Atlantic salmon farming, deeming Atlantic salmon a more suitable species for domestication due to its higher growth rate and better adaption to high density cage farming (Olin, 2012). The first success in Atlantic salmon farming in Canada took place in 1979 in New Brunswick (NB) and the industry has expanded ever since. Atlantic salmon is currently the main farmed species farmed, in seawater net-pens, albeit Coho and Chinook farming continues on a much smaller scale. Canada is the fourth-largest producer of farmed salmon worldwide, behind Norway, Chile and the UK. The British Columbia (BC) region, is the farming region in Canada that has experienced the largest success with domestication of Atlantic salmon (Frisch, 2018; Olin, 2012). In terms of production, Canada has produced an annual average of 177,000 tonnes in the period 2013-2017, with BC alone producing an average of 91419 tonnes over the same period (just above 50% of the total production). 60% of the Atlantic salmon production is exported, with United States as the largest receiver and farmed salmon is the third most valuable seafood export in Canada. As a result the aquaculture industry contributes significantly to the costal and rural communities both in terms of economics and jobs, especially in BC and NB where farmed salmon is the largest export and largest crop in the agri-food sector (Fisheries and Oceans Canada, 2016; Olin, 2012). Over the years as the industry has evolved, large multinational companies have made their presence in BC and a general movement towards assembling and combining the hatchery, grow-out, processing, and marketing components of the value chain in one single business is seen (Olin, 2012).

1.1.1. Fish pathogens and the fish health situation on the Pacific Coast of Northwest America

All aquaculture facility operators in Canada are obliged to report to the Fisheries and Ocean Canada (DFO) on a regular basis. The reports should contain information about the health status of their stocks and if any treatments have been used. This, in addition to inspections on-site, helps the DFO veterinarians to determine whether appropriate measures have been taken and it helps uncover any diseases at an early stage. Since 2011, DFO has issued reports regarding the fish health situation in BC with statistics under the section "Fish Mortality". A "Mortality event" is defined as either a) mortalities equivalent to 4000kg or more, or losses reaching 2%

of the current facility inventory within a 24h period; or b) losses reaching 5% of the current inventory, or mortalities equivalent to 10,000 kg within a five day period (DFO, 2017). The number of events varies from year to year, but, the most frequent cause of mortality events reported by the DFO has been due to harmful algal blooms (HABs) and low dissolved oxygen (DFO, 2014, 2017).

Losses due to HABs rapidly reached into the millions of dollars (During the 80s and 90s salmon farms in the province of BC and Washington State experienced losses exceeding US $35 million). Because of the large losses associated with the HABs the industry initiated the Harmful Algae Monitoring Program (HAMP) in 1999. This extensive monitoring program aims to decrease economic losses in the province. HAMP includes daily monitoring and identification of potential harmful plankton at every farm during periods of a high risk of HABs, (Frisch, 2018; Trainer & Yoshida, 2014).

The Pacific Northwest situation is different from other Atlantic salmon farming regions of the world. Endemic diseases are controlled with effective vaccines against pathogens such as Infectious haematopoietic necrosis (*Salmonid novirhabdovirus*), furunculosis (*Aeromonas salmonicida*) and vibriosis (*Vibrio anguillarum*). Other endemic diseases, Viral haemorrhagic septicaemia (*Piscine novirhabdovirus*) and bacterial kidney disease (*Renibacterium salmoninarum*) are well managed through broodstock screening combined with proper management practices. This results in a small, overall, percentage loss during the saltwater phase of production (Frisch, 2018; Kent, 1992; Traxler *et al*., 1999).

However, there is still a small number of marine bacteria that continue to give rise to disease outbreaks in the region. *Moritella viscosa* (Winter ulcer disease) and *Tenacibaculum* spp. are among them, the latter being associated with two types of clinical presentations. One type results in skin ulcers/lesions, mouth erosion, tail rot, and frayed fins, while the other causes lesion primarily in the mouth (mouthrot or bacterial stomatitis). Fish at any stage are susceptible to these diseases during the saltwater phase of production, although there is a higher prevalence of mouthrot in smolts recently transferred to saltwater. On the other hand, skin lesions, due to either *Tenacibaculum* spp., *M. viscosa* or both, are considered a greater problem, because of significant economic losses due to downgrading fish at processing, in harvest size fish (Frisch, 2018; Kent, 1992).

1.2. *Tenacibaculum maritimum*

Tenacibaculum maritimum (originally *Flexibacter maritimus*), (family Flavobacteriacae, genus *Tenacibaculum*) is a marine gram-negative bacterium. It was first identified in 1976 from diseased hatchery-born Red Seabream (*Pagrus major*) and Black Seabream (*Acanthopagus schlegelii*) reared in seawater net-pens in Japan and described as an aetiological agent of an opportunistic "marine flexibacteriosis" (Masumura & Wakabayashi, 1977; Wakabayashi *et al.*, 1986; Suzuki *et al.*, 2001; Bruno *et al.*, 2013a; Frisch, 2018). Since then, *T. maritimum* has been isolated from a wide range of diseased farmed and wild fish species all over the world (Table 1.1):

Table 1.1 - *Tenacibaculum maritimum*: geographic origin and host species identified to date.

Host Species	Country	Source
Asia:		
Acanthopagrus schlegelii – Black seabream	Japan	(Masumura & Wakabayashi, 1977)
Conger myriaster – Whitespotted conger	Japan	(Kawato *et al*., 2020)
Lates calcarifer - Barramundi	Singapore	(Labrie *et al*., 2008)
Lethrinus haematopterus – Chinese emperor	Japan	(Kawato *et al*., 2020)
Oplegnathus fasciatus – Rock bream	Japan	(Wakabayashi *et al.*, 1986)
Pagrus major – Red seabream	Japan	(Masumura & Wakabayashi, 1977)
Paralichthys olivaceus – Olive flounder	Japan Korea	(Baxa *et al.*, 1986) (Jang *et al.*, 2009)
Seriola dumerili – Greater amberjack	Japan	(Kawato *et al*., 2020)
Seriola quinqueradiata – Yellowtail	Japan	(Baxa *et al.*, 1988)
Takifugu rubripes – Puffer fish	Japan	(Rahman *et al.*, 2014)
Oceania:		
Acanthopagrus butcheri – Black bream	Australia	(Handlinger *et al.*, 1997)
Aldrichetta forsteri – Yellow-eye mullet	Australia	(Handlinger *et al.*, 1997)
L. calcarifer – Barramundi	Australia	(Soltani *et al.*, 1996)
Latris lineata – Striped trumpeter	Australia	(Carson *et al.*, 1992)
Oncorhynchus mykiss – Rainbow trout	Australia	(Carson *et al.*, 1992)
Oncorhynchus tschawytscha – Chinook salmon	New Zealand	(Fischer & Appleby, 2017)
Platax orbicularis – Orbicular batfish	French Polynesia	(Bardon-Albaret *et al*., 2016)
Rhombosolea tapiriña – Greenback flounder	Australia	(Handlinger *et al.*, 1997)
Salmo salar – Atlantic salmon	Australia	(Carson *et al.*, 1992)
America:		
Atractoscion nobilis – White seabass	USA (West Coast)	(Chen *et al.*, 1995)
Engraulis mordax – Northern anchovy	USA (West Coast)	(Chen *et al.*, 1995)
O. tschawytscha – Chinook salmon	USA (West Coast)	(Chen *et al.*, 1995)
S. salar – Atlantic salmon	Canada (West Coast) Chile	(Ostland *et al.*, 1999) (Apablaza *et al*., 2017)
Sardinops sagax – Pacific sardine	USA (West Coast)	(Chen *et al.*, 1995)
Scophthalmus maximus - Turbot	Chile	(Habib *et al*., 2014)
Europe:		
Carcharias taurus – Sand tiger shark	Italy	(Florio *et al*., 2016)

Host species	Country	Source
Chelidonichthys lucernus – Tub gurnard	Italy	(Magi *et al.*, 2007)
Cyclopterus lumpus – Lumpsucker	Norway	(Småge *et al.*, 2016)
Dentex dentex – Common dentex	Italy	(Salati *et al.*, 2005)
Dicentrarchus labrax – Sea bass	France	(Bernardet *et al.*, 1994)
	Greece	(Kolygas *et al.*, 2012)
	Italy	(Salati *et al.*, 2005)
	Malta	(Bernardet, 1998)
	Turkey	(Yardımcı & Timur, 2015)
Dicologoglossa cuneate – Wedge sole	Spain	(López *et al.*, 2009)
Diplodus puntazzo – Sharp-snout seabream	Italy	(Salati *et al.*, 2005)
Diplodus sargus – White seabream	Italy	(Salati *et al.*, 2005)
Oncorhynchus kisutch – Coho salmon	Spain	(Habib *et al.*, 2014)
Pagellus bogaraveo – Blackspot seabream	Spain	(Castro *et al.*, 2007)
S. salar – Atlantic salmon	Ireland	(Downes *et al.*, 2018)
	Norway	(PHARMAQ Analytiq, 2017)
	Spain	(Pazos *et al.*, 1993)
S. maximus - Turbot	France	(Habib *et al.*, 2014)
	Italy	(Magi *et al.*, 2007)
	Norway	(Frisch, 2018)
	Spain	(Alsina & Blanch, 1993)
Solea senegalensis – Senegelese sole	Portugal	(Avendaño-Herrera *et al.*,2005)
	Spain	(Cepeda & Santos, 2002)
Solea solea – Common sole	Netherlands	(Habib *et al.*, 2014)
	Scotland	(Bernardet *et al.*, 1990)
	Spain	(Avendaño-Herrera *et al.*, 2004)
Sparus aurata – Gilt-head bream	Greece	(Kolygas *et al.*, 2012)
	Italy	(Salati *et al.*, 2005)
	Spain	(Avendaño-Herrera *et al.*, 2004)
Africa:		
Cheilinus lunulatus – Broomtail wrasse	Egypt	(Abd El-Galil & Hasheim, 2012)
Neoglyphidodon melas – Black damsel	Egypt	(Abd El-Galil & Hasheim, 2011)
Rhinecanthus aculeatus – Picasso triggerfish	Egypt	(Abd El-Galil & Hasheim, 2011)

Outbreaks of tenacibaculosis due to *T. maritimum* infections can result in significant economic losses that consequently limits the culturing of commercially valuable marine fish species around the world (Avendaño-Herrera *et al.*, 2006). The most common clinical signs of tenacibaculosis caused by *T. maritimum* are ulcerative and/or necrotic skin lesions, frayed fins, tail rot and eroded and haemorrhagic mouth and this is referred to as tenacibaculosis (Toranzo *et al.*, 2005). A similar clinical presentation has been reported in Atlantic salmon smolts in Tasmania, Australia (Carson *et al.*, 1992). *Tenacibaculum maritimum* has been isolated from sea lice found on farmed Atlantic salmon from BC and detected through molecular testing from jellyfish found on gills of Atlantic salmon farmed on the Shetland Islands (Barker *et al.,* 2009;

Ferguson *et al.*,2010; Frisch, 2018). This suggests that sea lice and jellyfish may act as vector for *T. maritimum.*

1.2.1. Phenotypic characterisation

Tenacibaculum maritimum is a gram-negative, filamentous, long slender rod (0.5μm wide by 2-30μm long), that appears shorter and become spherical as cultures get older. The bacterium is strictly aerobic, exhibits gliding motility without flagella presence, and grows only on agar containing seawater or synthetic seawater (NaCl alone is not sufficient) (Avendaño-Herrera *et al.*, 2006). After incubation, pale-yellow, flat, and irregular colonies with uneven edges appear, which rarely exceed 5 mm in diameter (Wakabayashi *et al.*, 1986). Bacterial growth has been reported as low as 8 ^{o}C, and the optimal growth has been published to be 30 ^{o}C; however, growth can occur between 15-34 ^{o}C (Småge *et al*., 2016; Suzuki *et al*., 2001). The bacterium is negative for flexirubin type pigments and the production of H_2S and is positive for oxidase and catalase (Wakabayashi *et al.*, 1986; Suzuki *et al.*, 2001).

1.2.2. Genetic characterisation

Tenacibaculum maritimum is the type species of the genus *Tenacibaculum*. Multilocus sequence typing (MLST) has described several *T. maritimum* sequence types (STs) by collections of strains from a variety of host species worldwide (Habib *et al*., 2014). Through the MLST database (http://pubmlst.org/tenacibaculum/) one can access the genetic data as well as adding to this work, which was done by Frisch (2017) (Maiden, 2006). The same STs of *T. maritimum* is found to infect multiple fish species in the same geographical area, indicating cross-species contamination from the same bacterial lineage, and also suggesting, based on population structure, an endemic colonisation of fish farms by local strains indicating no long-distance contamination through fish movements. In contrast, closely related strains may be found on the same host species in different regions (e.g. Atlantic salmon in Norway, Chile, and Western Canada), suggesting the above not always to be the case (Frisch *et al.*, 2017; Habib *et al*., 2014).

Two other species, *Tenacibaculum dicentrarchi,* and *Tenacibaculum finnmarkense*, have also been associated with disease in Atlantic salmon (Avendaño-Herrera *et al*., 2016; Småge *et al*., 2015, 2017, 2018). By using 16S rRNA gene sequencing, *T. finnamrkense* has been identified and connected to tenacibaculosis in BC (Frisch, 2018). In addition, a genome comparison between certain *Tenacibaculum* species (*T. maritimum, T. dicentrarchi, Tenacibaculum ovolyticum,* and *Tenacibaculum soleae*) revealed significant differences between the in virulence factor in *T. maritimum* and other closely related *Tenacibaculum* spp. (Bridel *et al*.,

2018; Pérez-Pascual et al., 2017). Habib *et al.* (2014) suggest a parallel evolution of pathogenicity in the species encompassed in the *Tenacibaculum* genus.

MLST has established bacterial pathogen nomenclature schemes which allow researchers tools to perform epidemiological studies due to the schemes being both uniform and reproducible. The schemes are nucleotide-based because of MLST characterization of isolates within a microbial species using allelic mismatch of a small number of housekeeping (HK) genes and assigning these isolates a sequence type (ST). This makes the schemes easy to transfer and reproduce in different laboratories (Gevers *et al*., 2005; Maiden, 2006). Multilocus sequence analysis (MLSA) is currently the method of choice to explore phylogenetic relationships, both at species and subspecies levels. And the sequence data provided by MLST can be concatenated and used for phylogenetic analysis by MLSA (Gevers *et al*., 2005; Glaeser & Kämpfer, 2015).

1.2.3. Diagnosis

Observations of thin rod-shaped bacteria in wet mounts, together with cell and colony morphologies coinciding with *Tenacibaculum maritimum* observed from cultures constitute the identification of *T. maritimum* in BC. Marine agar (MA) and *Flexibacter maritimus* medium (FMM) are routinely used for primary isolation since the bacterium requires sea salt in the growth medium (Pazos *et al.*, 1996). *Tenacibaculum maritimum* does not grow on agar commonly used in routine diagnostics in salmon farming; blood agar containing NaCl (BAS) thus *T. maritimum* is likely underreported in BC (Frisch, 2018). *Tenacibaculum maritimum* may be difficult to distinguish from phenotypically similar bacteria, particularly other yellow-pigmented Flavobacteriaceae (Suzuki *et al.*, 2001; Toranzo *et al.*, 2005). Obtaining pure culture from external lesions may be troublesome since *T. maritimum* grows slower compared to other environmental bacteria commonly isolated from external lesions (e.g. *Vibrio* spp.) (Pazos *et al*., 1996). Adding antimicrobial compounds to the agar that select for *T. maritimum* has been suggested by several authors to overcome this problem (Baxa *et al.*, 1986; Chen *et al.*, 1995; Kolygas *et al.*, 2012).

With the development of PCR and sequencing nearly replacing traditional biochemical tests for identification, identifying *T. maritimum* from a pure culture is done by sequencing the 16S rRNA gene and matching it against reference gene sequences (16S rRNA (MAR and Mar), *atpA, dnaK, glyA, gyrB, ileS, infB, rlmN, tht, trpB, tuf, yqfO*) (Toyama *et al.*, 1996; Bader & Shotts, 1998; Habib *et al.*, 2014).

Real-time RT-PCR assays based on the 16S rRNA gene tend to be less specific than other assays due to the low phylogenetic resolution compared to other genes at species level (Janda & Abbott, 2007). However, two real-time RT-PCR assays based on the 16S rRNA gene for the detection of *T. maritimum* have been published (Fringuelli *et al.*, 2012; Fernández-Álvarez *et al.*, 2019). In Vallestad (2017) two genes were targeted for real-time RT-PCR assay development; the internal transcribed spacer (*ITS*) gene and *infB* (HK gene). In addition, a real-time RT-PCR assay targeting the outer membrane protein (*OmpA*) was developed by Frisch *et al.,* (2018b). The *OmpA* gene is highly conserved, throughout evolution, among the Enterobacteriaceae family. Unlike other surface-exposed components of the bacterial cell envelope, the outer membrane protein is involved in bacterial virulence and growth (Pautsch & Schulz, 1998; Koebnik *et al.*, 2000; Jeannin *et al.*, 2002). The *T. maritimum* 16S rRNA gene is used as the standard for classification and identification of microbes due to its unchanged function over time and by being present in most bacteria. The ITS lies within a region (16S-23S) which is part of the 16S rRNA gene (Janda & Abbott, 2007). In addition, two assays with Locked Nucleic Acid (LNA) developed by Vallestad, (2017) was used based on two of the previously mentioned assays. This was the qPCR_Tmar_OmpA_LNA and the qPCR_Tmar_infB3_LNA probe.

1.2.4. Pathology

Tenacibaculum maritimum has previously fulfilled koch's postulates in experiments to induce mouthrot on its own in Atlantic salmon smolts in the Pacific northwest, in contrast of it been thought of as a secondary invader which would require already damaged tissue to invade (Ostland *et al.*, 1999; Frisch *et al.*, 2018b). However, information is scarce on the pathology and pathogenesis of mouthrot in this region, which appears different compared to tenacibaculosis. Tenacibaculosis caused by *T. maritimum* in other areas has since its discovery been able to induce disease on its own in a number of species (Avendaño-Herrera *et al.*, 2006). Tenacibaculosis is generally more severe in juvenile fish and the associated pathology in marine fish has been well described macroscopically and microscopically (Toranzo *et al.*, 2005; Avendaño-Herrera *et al.*, 2006).

Lesions associated with tenacibaculosis (from Tasmanian farmed Atlantic salmon) are usually found on the external surfaces; gills, head, fins, and dorsal and lateral skin, with the flank just behind the pectoral fins being the most commonly affected area (Handlinger *et al.*, 1997). The destruction and loss of epithelium with a subsequent invasion of bacteria into the underlying connecting tissue with little inflammation are typical for the disease (Handlinger *et al.*, 1997;

van Gelderen *et al.*, 2011b). Histopathological assessments of mouthrot from field cases display affected smolts with lesions from the field being generally located in the oral cavity that usually involves the dentition and surrounding gingiva, which resembles gingival disease in mammals (Frelier *et al.*, 1994; Frisch *et al.*, 2018b).

1.2.5. Challenge model

As with *T. maritimum* and other fish pathogens of the Flavobacteriaceae, the main challenge is the difficulty of developing a reproducible bath challenge model. For other bacterial pathogens injecting the fish with the causative agent provides more control of experimental conditions and these are the most successful challenge models for fish. Economically important fish species have been subject to challenges attempting to reproduce disease caused by *T. maritimum*. However, both intra-peritoneally (IP) and subcutaneously injection have given repeatedly poor results (Yamamoto *et al.*, 2010; Faílde *et al.*, 2014).

The most effective way to induce disease with *T. maritimum* seems to be by using a bath infection model. Numerous attempts have been conducted at optimizing the method in a number of fish species (Avendaño-Herrera *et al.*, 2006). However, scarification and abrasion prior to exposure to the bacteria in these studies were subsequently proven not to be necessary (Baxa *et al.*, 1987; Mabrok *et al.*, 2016). In addition, a range of exposure times and bath bacterial concentrations have been tested along with varying water quality which includes salinity and temperature (Avendaño-Herrera *et al.*, 2006). Frisch *et al.,* (2018a) used a high dose bath model in their study of *T. maritimum* which was based on finding researchers in Tasmania did when they infected smolt for one hour at a high dose. This high dose concentration gave them the most replicable results (Soltani *et al.*, 1996; Handlinger *et al.*, 1997).

1.2.6. Treatments and vaccination

Whenever possible, the aquaculture industry uses non-therapeutic treatments against diseases. Non-therapeutic methods have been used in BC for managing mouthrot, including; improved smolt quality and functional feeds. In addition, targeting smolt entries based on site history and environmental conditions. These methods have shown not to be as effective as desired (Frisch, 2018). Vaccines have reduced the use of antibiotics in Pacific Northwest for predominant bacterial diseases (bacterial kidney disease, vibriosis, and furunculosis). However, there is currently no commercial mouthrot vaccine available (Morrison & Saksida, 2013). Fish suffering from mouthrot needs to be treated with antibiotics in order to maintain fish welfare and reduce mortality at site. Florfenicol is frequently used to treat mouthrot in BC salmon farms. Though florfenicol has low toxicity and its palatability, the rapid metabolism by the fish means

multiple treatments are required during a mouthrot outbreak (Morrison & Saksida, 2013). However, *T. maritimum* is susceptible to other known antibiotics used in aquaculture including; amoxicillin, nitrofurantoin, oxytetracycline and trimethoprim-sulphamethoxazole (Avendaño-Herrera *et al.*, 2008; Frisch, 2018).

Numerous studies indicate that *T. maritimum* is not as homogenous as previously believed and three serotypes have been identified based on their O-antigen (Ostland *et al.*, 1999; Avendaño-Herrera *et al.*, 2004; Romalde *et al.*, 2005; Castro *et al.*, 2007). The formulation and development of appropriate and effective vaccines may be hampered by some major discrepancies regarding differences in the antigens, antisera, and techniques used. This impedes comparisons between laboratories (Frisch, 2018).

Prototype vaccines have been tested in Atlantic salmon in Tasmania. However, rearing conditions of Atlantic salmon in Tasmania do not reflect rearing conditions of Atlantic salmon in BC (in regards to water temperature and disease progression) and the vaccine provided little to no level of protection (Carson *et al.*, 1992; Carson *et al.*, 1993; Carson *et al.*, 1994; Van Gelderen *et al.*, 2009). An adequate and protective vaccine that targets *T. maritimum* is therefore of high interest to the Atlantic salmon farming industry in BC. There is only one available vaccine against *T. maritimum* which is for turbot in Spain (Santos *et al.*, 1999).

1.3. Mouthrot

Mouthrot was identified in the late 80s as being a significant disease and welfare problem in Atlantic salmon farming in the Pacific Northwest (Frelier *et al.*, 1994; Ostland *et al.*, 1999). Mouthrot, which has been reported at 15% mortality, has a significant impact on the industry both in terms of direct costs of mortality and therapeutic treatment as well as indirect costs of production from poor performance (Ostland *et al.*, 1999). 90% of the total use of antibiotics in the region is used to control mouthrot. These treatments are very effective thus, the mortality numbers due to mouthrot are low. During the saltwater phase of production at the present time, mouthrot accounts for approximately 1 to 3 % mortality during the seawater phase with the majority occurring during the first 4 to 5 months after transfer to saltwater. However, gaining acceptance for the industry to operate in the region from stakeholders and the general public becomes harder due to the continued use of antibiotics to treat mouthrot (Frisch, 2018).

During a mouthrot outbreak, diseased fish die with small yellow plaques in the mouth and little to no other clinical signs both externally and internally; however, the characteristic yellow plaques have been found on gills (Frelier *et al*., 1994; Mitchell & Rodger, 2011). Frisch (2018)

noted that mouthrot has a very rapid course as the prevalence of clinical signs was below 1 % from surveys of live populations of a farm during an outbreak.

Several factors may affect the severity of a mouthrot outbreak. This includes smoltification quality, handling, presence of algal blooms, and water quality parameters such as temperature and salinity (Frelier *et al*., 1994; Frisch, 2018). In general, the susceptibility to mouthrot decrease 3 to 6 months post-transfer to saltwater. However, more severe cases with outbreaks occurring in larger and higher quality fish in lower salinities, and longer lasting than historical outbreaks appear to become more common (Frisch, 2018).

Clinical signs of mouthrot during outbreaks can occur as early as two days post-transfer to saltwater and a majority occur within the first few weeks. Frisch *et al.* (2018a) proved Koch's Postulates for *T. maritimum* as being the causative agent of mouthrot. Tenacibaculosis is commonly associated with *T. maritimum*, and characterized by ulcerative skin lesions, mouth erosion, frayed fins and tail rot (Toranzo *et al.*, 2005). The diseases, both caused by *T. maritimum* infections, are clinically different from each other with mouthrot being distinct for BC.

In 2019 the government of Canada released a report regarding the adoption of new salmon aquaculture technology with the ambition of minimize environmental impacts in BC whilst still support development of rural economy, employment and Canada's food supply (DFO, 2019). Antibiotics are used as a treatment against mouthrot and this use of treatment sort under environmental impacts (and costly for the company).

1.4. Smolt production strategies

Technological advances and an increasing pressure on freshwater resources have altered the production strategy for smolts in recent years along with the lack of space for expansion and concerns over pollution. Recirculating aquaculture systems (RAS) are promoted in aquaculture producing countries like Norway, Ireland, UK, 100 % of production is carried out by RAS on the Faroe Islands and there are some advantages. Originally RAS was developed for fish farming when freshwater availability is restricted thus making requirement for freshwater decreases along with energy consumption due to heating of running water. It enables 90-99 % of the water to be recycled and RAS allows a greater control over water qualities (Badiola *et al.,* 2012) New implementations of production strategies for smolt can be utilized (e.g. sea water transfer could possible other times of the year) and better utilization of personnel- and tanks. However, the use of RAS requires higher competence in the company and it increases the need

for alarm systems and proper back-up solutions to work immediately along with difficulties treating diseases (Badiola *et al.*, 2012; Fjellheim *et al.*, 2016). Flow-through systems (FTS) as opposed to RAS depends on water exchange to maintain suitable water quality for fish production including water flow for the collection and removal of metabolic wastes (Fornshell & Hinshaw, 2009). In addition, there is little to no water treatment and limiting water parameters in FTS is oxygen which often is added to ensure the water quality, fish welfare and growth. Moreover, depending on the location and local environmental legislation disinfection of the intake and effluent water could be required (Aarhus *et al.*, 2011).

1.5. Skin and oral cavity of Atlantic salmon

The fish's skin is the primary barrier against the environment. Epidermis, dermis, and hypodermis are the three main layers of the salmon skin. The outermost layer: epidermis is covered by mucus.

The epidermis, in contrast to land mammals, is made up of non-keratinising stratified squamous epithelium. The parts of the skin that do not contain scales like the head and fins have a thicker epidermis (Elliott, 2011). The epidermis contains several cell types like epithelial cells and mucous cells. The mucus cells produce the mucus layer (Elliott, 2011; Kryvi & Poppe, 2016). Even the outermost layer retain their ability to divide (unlike mammals), and the apical cells in epidermis have a microridge pattern (Kryvi & Poppe, 2016).

The dermis consists of two layers. The upper layer, called *stratum spongiosum*, is a loose network of collagenous connective tissue, whereas the lower layer, *stratum compactum*, is denser and consists of orthogonal collagen bands. The latter layers give fish skin its strength (Elliott, 2011). The basal lamina (or adepidermal membrane) is an acellular basement membrane that separates the epidermis from the dermis. One main physical protection is the scales, which locates in scale-pockets in the dermis and tilts backward towards the outside and are covered by the epidermis. In practice, scale-loss means that the epidermis is also damaged and the physical and/or osmotic barriers in the fish have been breached, consequently leaving the fish more vulnerable to infections (Elliott, 2011; Frisch, 2018; Kryvi & Poppe, 2016). Separating the subcutaneous muscle and the dense *stratum compactum* of the dermis is a layer comprised of well-vascularised loose adipose and loosely organised collagen fibers tissue called hypodermis (Elliott, 2011; Kryvi & Poppe, 2016). However, in the non-scaled parts of the fish, the dermis layer is highly reduced while the hypodermis is increased (Sverre Småge personal communication).

The mucus plays a vital role in capturing foreign particles, including bacteria and viruses and is therefore an important first line of defence against pathogens entering through the skin. In addition, mucus is constantly secreted and replaced which prevents colonisation of microorganisms (Esteban & Cerezuela, 2015). Epidermis in the head region and lower jaw appears to be devoid of mucosal cells (Småge, 2018). Microridges of apical epithelial cells of the epidermis provide an extensive surface area for the secreted mucus. This helps in both maintaining the mucous layer that covers the epithelium as well as adhering to the mucus to the skin (Kryvi & Poppe, 2016; Peterson, 2015). However, the mucus does not seem to contain any growth-inhibiting compounds against *T. maritimum*. This, combined with the strong binding capacity of *T. maritimum* to the skin are mechanisms that likely the enable *T. maritimum* to effectively colonise its hosts (Magarinos *et al.*, 1995).

With some differences, the integument of the oral cavity is made up of the same layers as the skin. The epidermis lies on a thick membrane with a very condensed dermis binding it to the bone or muscle and contains an abundance of mucous cells (Frisch, 2018). In the oral cavity of salmon, teeth are attached to the jawbones by an acrodont connection made up of dense connective tissue. Gingival pockets are formed surrounding the area where the epidermis and teeth connects (gingival-enameloid interface). Salmon are homodont: meaning all their teeth have the same shape and the teeth are continously formed throughout their lives. The teeth consist of an inner pulp with an outer dentin layer which is covered by enameloid on the crown, the exposed part (Kryvi & Poppe, 2016).

1.6. Aims of the study

The main aim of this study is to investigate the effect of keeping smolts in low salinity seawater (LSS) for two periods (4 weeks and 8 weeks) prior to seawater transfer, on the development of mouthrot. The challenge study will focus on the effects of different production strategies of smolt with the goal to reduce infections caused by *Tenacibaculum maritimum* in seawater. Two water qualities, freshwater and low salinity seawater (26‰), are to be compared. Smolts are held in LSS before exposing the fish to *T. maritimum* TmarCan15-1 strain by using bath challenge (bath challenge trail 2 and bath challenge trial 3). Smolts representing the freshwater groups in this study are smoltified prior to each of the challenge trails to avoid desmoltification which can occur in smolts kept in freshwater for a prolonged time post smoltification. Preliminary data from a similar study indicate a positive effect of keeping smolts on LSS, compared to freshwater, in reducing *Tenacibaculum finnmarkense* infections. Therefore, the results from the current study may be of importance for future production of smolts and design

of smolt/RAS facilities in order to prevent and mitigate the negative impact of *Tenacibaculum maritimum* infections (mouthrot) in Atlantic salmon farming in BC.

A challenge study consisting of three challenge trials (one pre challenge trial and two main challenge trials) was designed in order to investigate the effect of LSS versus freshwater on *T. maritimum* infection in Atlantic salmon smolts.

In addition, as a secondary study a freshwater survival test was designed. This *in vitro* freshwater survival test is to be conducted to investigate the survival of *T. maritimum* in freshwater with varying parameters and the same time frame as an on-site freshwater treatment of Atlantic salmon.

2. MATERIAL AND METHODS

2.1. Fish husbandry

The challenge study was conducted using flow-through tanks at the Stiftelsen Industrilaboratoriet (ILAB) facilities, Bergen, Norway. The freshwater at ILAB is supplied from Bergens main water source Svartediket. The freshwater is filtered through several filters which functions as chemical settling plants with kitoflokk (https://www.chitosan.no/product/kitoflokk/) as the precipitant. The filters contain layers with gravel, sand and anthracite that filters out humus particles and bacteria from the freshwater. After filtration, the freshwater is UV treated to eliminate any remaining microbes. Silicate is added at the end of the filtering process to neutralize the toxicity of potential metals in the water. Seawater is supplied from an intake at Nordnes (Bergen) at 105m depth. The seawater is initially filtrated with a drum filter at 20 µm before being treated by ultraviolet (UV) light.

ILAB supplied all the Atlantic salmon used in this study. They get their eggs from the Icelandic company StofnFiskur. The fish were smoltified using standard ILAB procedures with 4 weeks of 24:0 (L:D) light ar 12 ºC. The parent fish for the population of fish used were screened and found negative for *Piscine orthoreovirus* (PRV), *Infectious Pancreatic necrosis virus* (IPNV), *Salmon isavirus* (ISAV), Piscine myocarditis virus (PMCV), Salmon gill poxvirus (SGPV), and *Salmon pancreas disease virus* (SPDV) with real-time RT-PCR prior to the start of the challenge trials. In addition, the fish are screened for *Tenacibaculum maritimum*, *Tenacibaculum finnmarkense*, *Moritella viscosa*, *Yersinia ruckeri,* Costia (*Ichtyobodo* spp.), *Flavobacterium psychrophilum* and *Branchiomonas cycticola*.

Fish were fed ad libitum with feed from Skretting (Nutra Olympic) and all experiments were conducted in 12 ºC seawater. Water flow was 300 L per kg fish per minute with a minimum oxygen saturation of 80 % in the outlet water of all the tanks. Fish used in each challenge trial were checked at least twice a day on weekdays and at least once a day during weekends.

All fish were starved for at least 24 hours before any handling. In the aquahall-2 facilities the fish used in bath challenge trial 1 (C1) all came from the same tank containing freshwater (Figure 2.2). Bath challenge trials 2 (C2) and 3 (C3) were assigned two tanks each with one tank containing freshwater (0 ppt) and one tank containing low salinity seawater (26 ppt). The fish used came either from the tank containing freshwater or the tank containing low salinity seawater. Fish used in C2 and C3 were transferred from aquahall-2 to the challenge facility where they went into the same water quality as they came from (freshwater or 26 ppt).

All fish were anaesthetised when transferred from the aquahall to the challenge facilities. The salinity was gradually increased to 34 ppt over a 24 hours period prior to exposure of the bacteria. Oxygen was supplied using air diffusors in each challenge container during bath infection. The *T. maritimum* strain TmarCan15-1 was used as the challenge material throughout the challenge study.

Fish showing signs of either illness (e.g., ulcerative lesions) and/or abnormal behaviour (e.g., loss of equilibrium and erratic swimming) in the challenge trials were removed from the tanks and euthanized with a powerful blow to the head. However, due to the rapid development of the disease this was not always possible. Hence, the term mortality, in this study, includes both moribund fish and fish that were found dead in the tanks. At the termination of each of the trials the remaining fish were euthanized by using an overdose of Finquel vet.

This study was approved by The Norwegian Food Safety Authority (Mattilsynet) in 2019 under the FOTS ID 21057.

2.2. Bacterial Cultures for Bath Challenge Study

Cermaq marine broth (CMB) or Difco 2216 marine broth (MB) were used as a growth medium when the bacteria were inoculated (see Appendix 1).

The *T. maritimum* isolate selected to be used in this study was chosen based on studies performed by Frisch *et al.,* (2018a) that showed that the *T. maritimum* TmarCan15-1 isolate produced the most reproducible challenge model. Two stocks of *T. maritimum* isolate 15-1 were considered as challenge material. Cultures were prepared by inoculating each stock into separate 2 L Erlenmeyer flasks containing CMB or MB. The cultures were incubated in a shaking incubator (Infors-HT Multitron Standard) at 230 rpm and 16 ^{o}C for a period of 80.5 hours. Sampling of the cultures was conducted every second hour from 70 hours post inoculation (hpi) until 80 hpi in order to determine the optical density (OD) and the Most Probable Number (MPN) for both stocks. Sampling of Stock-2 started at 70 hpi and continued until 80 hpi, whilst sampling of Stock-1 started at 70.5 hpi and continued until 80.5 hpi. Based on the corresponding OD and MPN results and a microscopic assessment of both stocks at every sampling point, Stock-2 was chosen as the preferred inoculum for the challenge material. In addition, the OD and MPN results helped determine at what time Stock-2 was at its most desirable in terms of cell concentration as well as cell morphology (i.e. no or few dead spherical degenerative cells) for use in the challenge (infection).

A new stock of the challenge material was prepared prior to the bath challenge trials with 1.2 ml of Stock-2 added to two 2.0 L Erlenmeyer flasks containing 1000 ml MB. The flasks were further inoculated while shaking at 16 ºC at 230 rpm for 72 hours. After inoculation, a visual examination of the bacterial cultures was performed in order to determine which of the two culture to use as stock for the challenges. A total of 81 tubes of stock solution were made (Each tube contained 200 µl Biofreeze and 200 µl MB with bacteria). To determine the optimal OD and MPN combined with desired cell morphology the same approach was taken towards the new stock as described above.

2.3. Preparation of challenge material

Bacterial cultures were prepared by inoculating 1.2 mL of the *T. maritimum* strain TmarCan15-1 stock in 1000 mL CMB or MB in a 2.0 L Erlenmeyer flask. In total 8 Erlenmeyer flasks were made. The flasks were inoculated at 16 ºC at 230 rpm for 76 hours. The volume of the challenge material for each trial was determined prior to the start of each bath challenge trial based on the OD and the MPN results of the new *T. maritimum* TmarCan15-1 stock.

OD was measured for all 8 flasks shortly before exposing the fish to the challenge material. The flasks that showed slow and/or elevated growth, were eliminated from the challenge to ensure that viable bacteria were used. In addition to OD, all the cultures were examined microscopically for deviant cell morphology. After incubation and selection of which bacterial cultures to be used, all selected flasks with viable bacteria were poured into a pre-autoclaved 10 L bottle using an autoclaved funnel to avoid spills. The culture was then thoroughly mixed before a sample was obtained for MPN. The culture was then partitioned by using an autoclaved measuring cylinder before the cultures were poured separate pre-autoclaved 1.0 L bottles. Each flask was assigned a number corresponding with the volume of the challenge material to be used in each tank. The 1.0 L bottles were then transported in a sealed container to the infection cell were the fish eventually was exposed to the challenge material.

The MPN was used to calculate the bath bacterial concentration of *T. maritimum* used in each bath challenge trial. In essence, the MPN method dilutes the sample to a degree that bacterial cultures in the tubes will sometimes, but not always, contain viable organisms (Blodgett, 2010; Oblinger & Koburger, 1975). The estimation of the original, undiluted concentration of bacteria in a sample, is retrospectively calculated based on the number of wells containing growth at each dilution.

Four rows with 12 tubes each are set up on a stand, making a two parallel dilution series. 1000 µl of the challenge material is added to the first tube in each row while 900 µl of marine broth is added to the remaining 11 tubes on each row. The last 11 tubes with 900 µl marine broth (CMB or MB) were prepared and placed in an incubator and kept at 16 °C. The reason for keeping the prepared tubes with marine broth at 16 °C was to match the temperature which the bacterial cultures were incubated at. 100 µl of the bacterial culture in the first tube was subsequently added into the second tube in each row and mixed well by pipetting the medium 10 times. 100 µl was then transferred from the second tube into the third tube, and mixed. This was repeated until all the tubes had been diluted with the bacterial culture creating a dilution series. Dilutions 10^{-6} – 10^{-11} were transferred to a sterile 96-well titer plate (Thermo scientific – Nunclon Delta Surface) (8 wells per dilution in quadruplicate) and incubated at 20 °C for minimum 48 hours. The exception was one series which consisted of the 10^{-7} – 10^{-11} dilutions leaving one row of wells for measuring the OD. The OD was measured from the undiluted first row of wells using SparkControl Magellan 2.2 (Tecan - Spark®) spectrophotometer. Growth was observed as a precipitate at the bottom of the wells of the 96-well titer plate. Numbers were obtained from a modified version of a MPN 8 table which was originally published by the U.S. Food & Drug Administration in "Bacteriological Analytical Manual" *Online*, February 2006.

In addition, samples were transferred from the bacterial culture onto 3 blood marine agar (BAMA) by using a bacterial loop (see Appendix 1). The plates were incubated at 16 °C for 72 hours or more to check for contamination.

2.4. Challenge procedures

Fish in all challenge trials will be exposed to the *T. maritimum* in designated plastic containers (figure 2.1 A) with various water volume in the containers, depending on the challenge trial. The lid of each container had two holes (figure 2.1 B). One hole for air diffusors and one hole for measuring temperature and oxygen throughout the exposure period. Exposure time varied between the challenge trials. After subjecting the fish to the *T. maritimum* bath culture the fish will be transferred back into their main 150 L fiberglass tanks (figure 2.1 C).

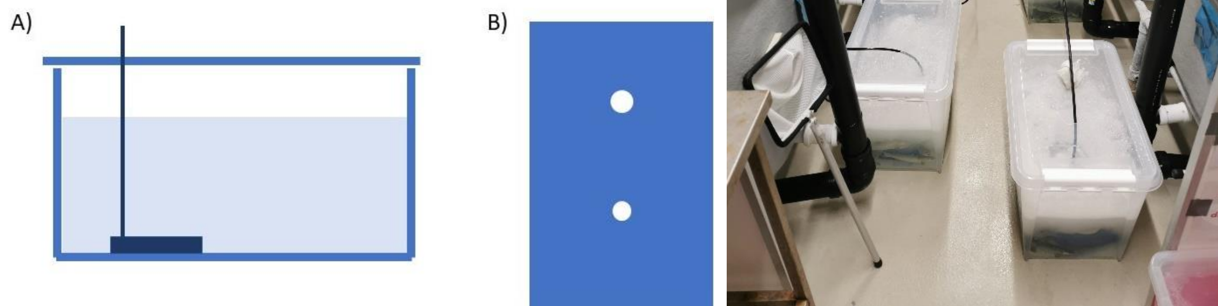

Figure 2.1 - A) container with air diffusor, B) lid with two holes, one for ait diffusor and one for measuring oxygen and temperature during exposure, C) overview of infection cell 7 with fish and air diffusors in containers (floor) and tanks (green lids)

2.5. Bath Challenge Study

The challenge procedures used in this study were based on a study by Frisch *et al*., (2018a) that found that a bath infection using separate infection tanks and a high concentration (dependent on pathogenicity of the isolate) for a short duration gives the most reproducible challenge model for Atlantic salmon smolts. The use of a cohabitation model was deemed to be the most appropriate model for infecting smolts with *T. maritimum*.

The bath challenge study was designed with 3 separate trials (see table 2.1). Bath challenge trial 1 (C1) used seven tanks that were further split into two groups. In tank-1, tank-2 and tank-3, a cohabitation model was used with a total of 30 Atlantic salmon smolts in each tank. In tank-5, tank-6, tank-7 and tank-8, 20 Atlantic salmon smolts and all fish were directly exposed with the *T. maritimum* bath culture. In bath challenge trial 2 (C2) and trial 3 (C3) eight tanks were used with 20 Atlantic salmon smolt in each tank. In C2 and C3 fish from two different groups (Low salinity seawater and freshwater) were directly infected with the *T. maritimum* bath culture. The

LSS group was transferred to tank-1 – tank-4 and the FW group was transferred to tank-5 – tank-8 (see table 2.1 for overview).

Fish used in C1 was produced in freshwater in the same tank in Aquahall-2 (figure 2.2). C2 and C3 used fish kept in LSS and fish kept in FW from aquahall-2. The LSS fish used in C2 (figure 2.3) and C3 (2.4) came from a designated tank in aquahall-2 which only contained LSS. The FW fish used in C2 (figure 2.3) and C3 (figure 2.4) came from a designated tank in aquahall-2 which only contained freshwater.

Since the control groups used in bath challenge trial 2 and 3 are not in triplicates, they serve as negative control groups for detection of any unwanted effects which could have influenced the bath challenge study (e.g. underlying disease).

Table 2.1 - Overview of the bath challenge study showing the amount of fish used, isolate used and treatment when applicable. N/A: not applicable, CO: cohabitant model, FW: freshwater, DIR: directly infection model, FWT: freshwater treatment, LSS: low salinity seawater (26 ppt), cohab: cohabitant fish, shed: shedder fish, NO.: number, C1: bath challenge trial 1, C2: bath challenge trial 2, C3: bath challenge trial 3.

Challenge period	Fish no. screening	Experiment	Group	Directly infected	No. of cohab	Total no. of fish	Isolate	Treatment
1	10	C1-CO-FW	1 CO	10 (shed)	20	30	TmarCan 15-1	N/A
			2 CO	10 (shed)	20	30	TmarCan 15-1	N/A
			3 CO	10 (shed)	20	30	TmarCan 15-1	N/A
		C1-DIR-FWT	5 DIR	20	N/A	20	TmarCan 15-1	N/A
			6 DIR	20	N/A	20	TmarCan 15-1	N/A
			7 DIR	20	N/A	20	TmarCan 15-1	Freshwater
			8 DIR	20	N/A	20	TmarCan 15-1	Freshwater
2	10	C2-DIR-LSS	1 LSS	20	N/A	20	TmarCan 15-1	N/A
			2 LSS	20	N/A	20	TmarCan 15-1	N/A
			3 LSS	20	N/A	20	TmarCan 15-1	N/A
			4 LSS	20	N/A	20	Control	N/A
	10	C2-DIR-FW	5 FW	20	N/A	20	TmarCan 15-1	N/A
			6 FW	20	N/A	20	TmarCan 15-1	N/A
			7 FW	20	N/A	20	TmarCan 15-1	N/A
			8 FW	20	N/A	20	Control	N/A
3	10	C3-DIR-LSS	1 LSS	20	N/A	20	TmarCan 15-1	N/A
			2 LSS	20	N/A	20	TmarCan 15-1	N/A
			3 LSS	20	N/A	20	TmarCan 15-1	N/A
			4 LSS	20	N/A	20	Control	N/A
	10	C3-DIR-FW	5 FW	20	N/A	20	TmarCan 15-1	N/A
			6 FW	20	N/A	20	TmarCan 15-1	N/A
			7 FW	20	N/A	20	TmarCan 15-1	N/A
			8 FW	20	N/A	20	Control	N/A

2.5.1. Bath Challenge Trial 1 (Pre-challenge)

A pilot experiment was conducted in order to determine which challenge model and what bath concentration of the challenge material was suitable for the remaining two challenge trials. C1 was further split into a shedder/cohab group (C1-CO-FW) in tank-1 – tank-3 and a direct exposed group (C1-DIR-FWT) in tank-5 – tank-8 (table 2.2). Both groups of fish were exposed to the challenge material for 2 hours. Oxygen and temperature were measured three times during exposure; before transfer, half-way, and right before return transfer. Fish behaviour was checked along with the measurements. The duration of bath challenge trial 1 was 20 days.

2.5.1.1. Cohabitation pre-challenge model

C1-CO-FW groups in tank-1, tank-2 and tank-3 had each a total of 30 fish (N = 90), 10 fish being shedder fish and 20 fish being cohabitation fish. The shedder fish were transferred concurrent with the direct exposure fish. The shedder fish were also adipose fin clipped. Further, shedder fish in tank-1 – tank-3 were exposed with three different concentrations of the challenge material. Tank-1 was the "low concentration" ($1*10^7$ cfu/ml), tank-2 "medium concentration" ($3*10^7$ cfu/ml) and tank-3 "high concentration" ($5*10^7$ cfu/ml). The cohabitation fish were transferred the day after exposure with 20 fish added to each tank.

2.5.1.2. Freshwater treatment (bath challenge trial 1)

In C1-DIR-FWT, tank-5, tank-6, tank-7 and tank-8 contained 20 fish each (total number of fish = 80). All four tanks (tank-5 – tank-8) were directly exposed to the same dose of the *T. maritimum* TmarCan15-1 bath culture. At first observation of clinical signs resembeling *T. maritimum* infection in any of the four tanks (tank-5 – tank-8), tank-7 and tank-8 were to receive freshwater treatment for 5 hours the following morning. The treatment was carried out the following morning after first sign of disease. All tanks-5 – tank-8 were exposed to the same bath concentration. Freshwater treatment against Amoebic gill disease (AGD) is regarded benign and has been shown to have reducing effect on the pathogenicity caused by *Paramoeba perurans*. *T. maritimum*, like the causative agent of AGD, is a strict marine species. It is important to examine if FWT will have similar reducing effect on mouthrot development. If FWT has the desired reducing effect on *T. maritimum* it will affect the fish welfare and reducing the need for antibiotic treatments.

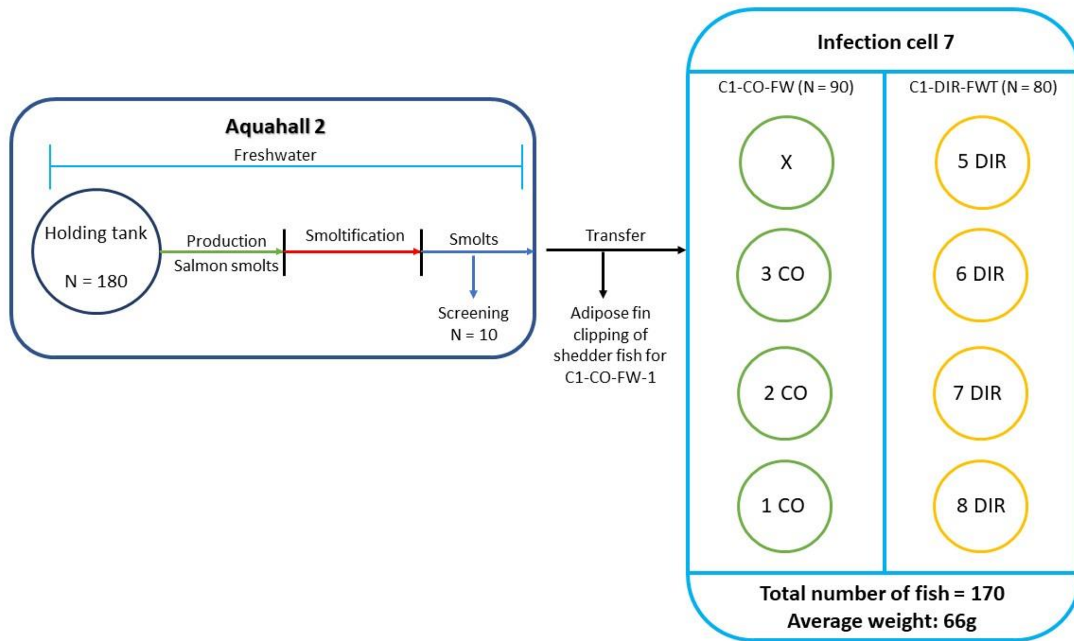

Figure 2.2 – The figure displays the production and transfer of smolts from Aquahall 2 to infection cell 7, the distribution of the fish in each tank, the total number of fish and the average weight in bath challenge trial 1. CO: shedder/cohabitation tanks, DIR: directly exposed tanks.

Table 2.2 – Table showing the bacterial isolate used, the duration of the bath challenge and the bacterial bath concentration of culture added to the bath volume in bath challenge trial 1. Challenge times are given in hours (hr) and inoculum in millilitres (mL). S = shedders, C = cohabitants.

Tank	Group	Shedder/Cohab	Isolate	Challenge time (hr)	Bacterial culture (ml)	Bath volume (L)	Bath concentration (cells/ml)
1	1s	S	TmarCan 15-1	2	400	30	$1.64*10^7$
1	1c	C	TmarCan 15-1	-	-	-	-
2	2s	S	TmarCan 15-1	2	700	30	$2.86*10^7$
2	2c	C	TmarCan 15-1	-	-	-	-
3	3s	S	TmarCan 15-1	2	900	30	$3.68*10^7$
3	3c	C	TmarCan 15-1	-	-	-	-
5	5	Direct	TmarCan 15-1	2	900	40	$2.76*10^7$
6	6	Direct	TmarCan 15-1	2	900	40	$2.76*10^7$
7	7	Direct	TmarCan 15-1	2	900	40	$2.76*10^7$
8	8	Direct	TmarCan 15-1	2	900	40	$2.76*10^7$

2.5.2. Bath Challenge Trial 2

Based on the results from cohabitation model in bath challenge trial 1, it was decided to use the direct infection model for bath challenge trials 2 (C2) and 3 (C3). This was due low mortality from C1 in combination with having to few fish in order to reproduce the cohabitation model described by Frisch *et al.,* (2018a) (performed using 20 shedder and 40 cohabitation fish). In the C2 trial, each tank contained 20 fish (table 2.3). The fish was trasferred directly from either freshwater (FW) or low salinity seawater (LSS) from aquahall-2 and subsequent exposed to *T. maritimum* TmarCan15-1 (figure 2.3). The fish from LSS were smoltified and kept in LSS for 4 weeks prior transfer and subsequent exposure. The fish in FW were smoltified the last 4 weeks

before transfer and subsequent exposure. Tank-4 and tank-8 served as control tanks. In C2, two factors from C1 were considered altered; either extend the challenge time or change the growth medium. It was decided that the exposure time was extended by 3 hours to last for 5 hours.

The challenge procedure in C2 was the same as for C1 except for the increased exposure time. Oxygen and temperature were measured every hour for the first 3 hours and then every half hour for the remaining hours of the exposure time. Fish behaviour was checked concurrent with the oxygen and the temperature measurements. The duration of bath challenge trial 2 was 22 days.

Table 2.3 - Table showing the bacterial isolate used, the duration of the bath challenge and the bacterial bath concentration of culture added to the bath volume in bath challenge trial 2. Challenge times are given in hours (hr) and inoculum in millilitres (mL).

Tank	Group	Isolate	Challenge time (hr)	Bacterial culture (ml)	Bath volume (L)	Bath concentration (cells/ml)
1	1	TmarCan 15-1	5	1000	30	$2.42*10^7$
2	2	TmarCan 15-1	5	1000	30	$2.42*10^7$
3	3	TmarCan 15-1	5	1000	30	$2.42*10^7$
4	4	TmarCan 15-1	5	1000	30	CMB
5	5	TmarCan 15-1	5	1000	30	$2.42*10^7$
6	6	TmarCan 15-1	5	1000	30	$2.42*10^7$
7	7	TmarCan 15-1	5	1000	30	$2.42*10^7$
8	8	TmarCan 15-1	5	1000	30	CMB

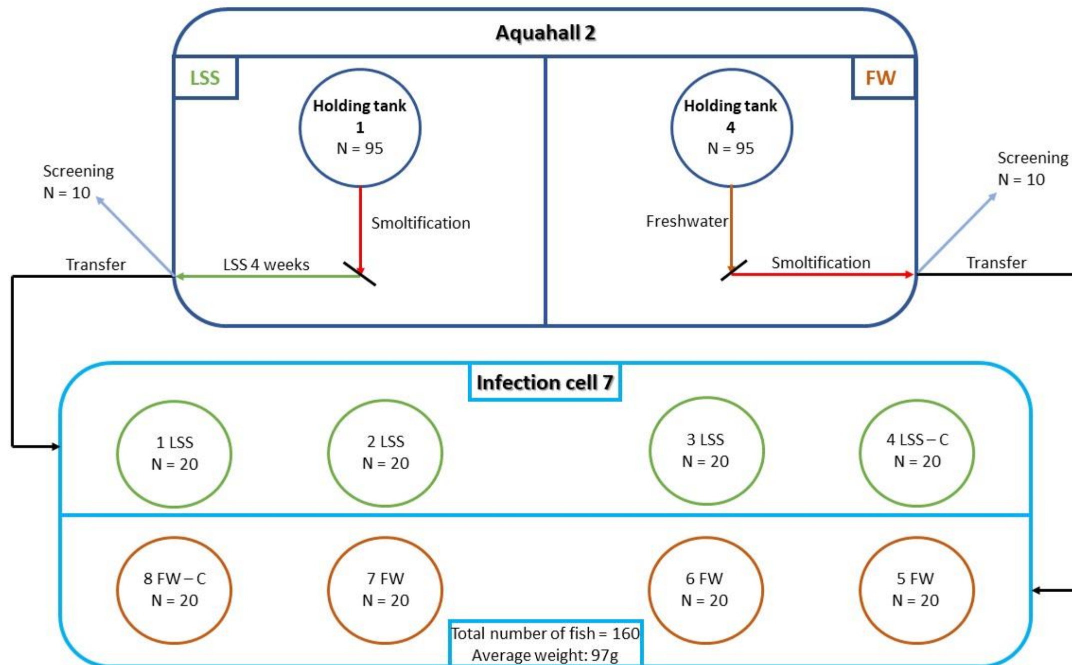

Figure 2.3 - The figure displays the production and transfer of smolts from Aquahall 2 to infection cell 7, the distribution of fish in each tank, the total number of fish and the average weight in bath challenge trial 2. LSS: low salinity seawater, FW: freshwater, C: control, N: number of fish.

2.5.3. Bath Challenge Trial 3

The bath challenge 3 was conducted as described in the bath challenge trial 2 (figure 2.4 for detailed overview). In bath challenge trial 3, the growth medium was changed from CMB to Difco 2216 marine broth (MB) based on the results from C2. The challenge time was kept at 5 hours. Oxygen and temperature were measured every hour for the first 3 hours, and every half hour the remaining hours of the exposure time. The bacterial bath concentration was increased (table 2.4) Fish behaviour was checked concurrent with the oxygen and temperature measurements. The duration of bath challenge trial 3 was 20 days.

Table 2.4 – Table showing the bacterial isolate used, the duration of the bath challenge and the bacterial bath concentration of culture added to the bath volume in bath challenge trial 3. Challenge times are given in hours (hr) and inoculum in millilitres (mL).

Tank	Group	Isolate	Challenge time (hr)	Bacterial culture (ml)	Bath volume (L)	Bath concentration (cells/ml)
1	1	TmarCan 15-1	5	1000	30	$3.95*10^7$
2	2	TmarCan 15-1	5	1000	30	$3.95*10^7$
3	3	TmarCan 15-1	5	1000	30	$3.95*10^7$
4	4	TmarCan 15-1	5	1000	30	Broth
5	5	TmarCan 15-1	5	1000	30	$3.95*10^7$
6	6	TmarCan 15-1	5	1000	30	$3.95*10^7$
7	7	TmarCan 15-1	5	1000	30	$3.95*10^7$
8	8	TmarCan 15-1	5	1000	30	Broth

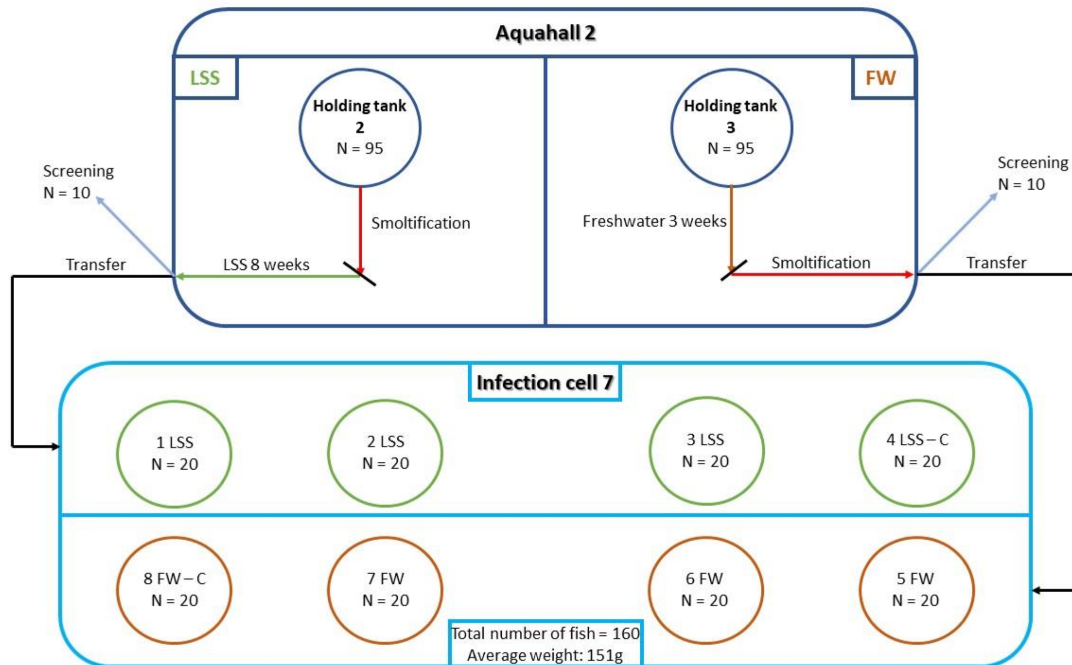

Figure 2.4 - The figure displays the production and transfer of smolts from Aquahall 2 to infection cell 7, the distribution of the fish in each tank, the total number of fish and the average weight in bath challenge trial 3. LSS: low salinity seawater, FW: freshwater, C: control, N: number of fish.

2.6. Bath Challenge Sampling

Sampling was continuously performed on moribund or dead fish removed from the tanks. Precautions were taken to avoid contact of fish and water between tanks and all fish were sampled with designated hand nets for each tank. Moribund fish was euthanized with a swift blow to the head, transported to the ILAB dissection laboratory in separate plastic bags, before being placed on ice prior to sampling. Tissue scraping of infected areas was smeared onto glass slides and examined at 40x magnification using a light microscope. Length and weight were recorded for each individual fish before sampling. Any fish with clinical and macroscopic pathology was photographed and scored. A scoring scheme (table 2.5) was used in all bath challenge trials to determine the degree of pathology of mouth, gills and skin lesions in order to assess the fish welfare.

Table 2.5 - Scoring scheme used when sampling fish in all three bath challenge trials. Mouth and skin lesions were scored from 0 - 3, gills were scored from 0 - 2.

Score	Mouth	Gills	Skin Lesions
0	No abnormality	No abnormality	No abnormality
1	*Mild* – tiny plaques and/or small haemorrhage	Lesions on one side	*Mild* – some scale loss and/or small haemorrhage
2	*Moderate* – small lesions and/or haemorrhage	Lesions on both sides	*Moderate* – lesions(s) with scale loss through to skin
3	*Severe* – large plaques and/or large lesions (mouthrot)	N/A	*Severe* – lesion(s) through to muscle and/or many lesions

Tissue samples of skin and kidney were collected for real-time RT-PCR analyses from all fish sampled during the challenge study. Skin, gill, heart and kidney tissues were sampled intended for histology from one moribund fish from each tank. In all bath challenge trials, tissue samples intended for histology were taken from each tank as described in section 2.15. One fish from each tank displaying clinical signs present during the trials was sampled for bacteriology. Bacteriological samples recovered from diseased fish were transferred onto Cermaq kanamycin marine blood agar (KA-BAMA) with a bacterial loop (see Appendix 1).

The KA-BAMA plates containing the recovered bacterial samples were incubated for a minimum of 72 hours at 16 ^oC. The bacteria were then sub-cultured by selecting a single colony displaying the characteristic iridescent colourisation and uneven colony margins of *T. maritimum* and trasferred onto Cermaq marine blood agar (BAMA) plates and incubated for an additional 72 hours at 16 ^oC. After incubation the cultures were sampled in 1.8 ml Nunc Cryotube vials (Thermo scientific) in a 50/50 solution of biofreeze (Biochrom GmbH) and Cermaq Marine broth (CMB) (i.e. 200 µl Biofreeze and 200 µl CMB) and stored at -80 ^oC. In addition, a scoop of the bacterial culture was placed into a 1.8 mL Eppendorf tube containing 200 µL dH_2O and stored at -80 ^oC for subsequent PCR and sequencing.

2.7. Histology and scanning electron microscopy (SEM)

Skin, gills, spleen, kidney and heart tissues intended for histology and SEM were sampled in all three bath challenge trials. Histology samples were placed in a modified Karnovsky's fixative. They were fixed at 4 ^oC by immersion (Nylund *et al.*, 1995), except for bath challenge trial 3 which used buffered formalin. In C3 the disease progressed with different clinical signs depending on days post exposure of *T. maritimum* strain TmarCan15-1. Therefore, tissues from

one fish were sampled on formalin shortly following infection and one fish was sampled closer to the termination date from each of the LSS-tanks.

Tissues intended for SEM were post-fixed for 60 minutes in 2.0 % OsO_4, washed in PBS, dehydrated through acetone and critical point dried by using liquid CO_2 as a transitional fluid. The dried tissues were mounted on double-stick carbon tape on SEM stubs before being sputter-coated with gold/palladium (Småge *et al.*, 2016).

2.8. Microscopic pathology

Tissues from lesions of diseased Atlantic salmon smolts from the challenge trials were fixed in 10% neutral buffered formalin solution (provided by PHARMAQ Analytiq) and kept at 4ºC upon processing. Representative tissues were skin, gills, kidney and mouth. Processing of tissues and sectioning for histology were conducted by PHARMAQ Analytiq. Histology sections were stained with hematoxylin and eosin (H&E).

2.9. Statistical analyses

Statistics are applied in the Bath Challenge Study. A t-test is applied to determine the statistical significance between the means of two groups in each bath challenge study trial and is used to compare the average values of two data sets selected from bath challenge 1, 2 or 3. In this study a null hypothesis (H_0) is assumed for the LSS and FW groups (or the treated vs non-treated or shedder- vs cohabitation-fish both in bath challenge study 1) to be no difference and the t-test assumes mathematically a null hypothesis that the means of the two selected data sets are equal. The values are calculated and compared against standard values based on formulas applied. Accordingly, the mathematically assumed null hypothesis is either accepted or rejected determined by the p-value. If the H_0 is rejected, then the sample gives support to the alternative hypothesis (H_1) which is the hypothesis set out to investigate (Gillard, 2020). P-value is the probability of the H_0 either being accepted or rejected. In this study p-values of $p \leq .05$ are considered statistically significant thus giving support to the H_1 (Goodman, 1999).

Based on the statistical parameters provided by Rosner (2016), this could give some indications regarding the effect of keeping smolts in low salinity seawater versus freshwater before transfer to seawater on the development of mouthrot. The formula (Formula 2) is used to calculate adequate sample size for group 1 (e.g. LSS group):

Formula 2 - p_1, p_2 = incidence of groups 1 and group 2; $\Delta = |p_2 - p_1|$ = absolute difference between two proportions; $q_x = 1 - p_x$, $\overline{p} = \frac{p_1 + kp_2}{1+K}$; $\overline{q} = 1 - \overline{p}$; α = probability of a type-I error. Type-I error (probability of finding a difference when a difference does not exist). α, are set to 0.05 meaning there is a 5% chance of a significant difference due to randomness; β = probability of a type-II error. Type-II error (not able to detect a difference when it does exist), β, use a cut-off value of 0.2 meaning there is a 20% chance of missing a significant difference; z = critical Z value for a given α or β; K = ratio of sample size for group 2 to group 1; N_1, N_2 = sample size for group 1 and group 2.

$$N_1 = \left\{ z_{1-\alpha/2} * \sqrt{\bar{p} * \bar{q} * \left(1 + \frac{1}{k}\right)} + z_{1-\beta} * \sqrt{p_1 - q_1 + (\frac{p_2 - q_2}{k})} \right\}^2 / \Delta^2$$

While the following formula (formula 3) is used to calculate adequate sample size for group 2 (e.g. FW group):

Formel 3 - K = ratio of sample size for group 2 to group 1; N_1, N_2 = sample size for group 1 and group 2.

$$N_2 = K * N_1$$

2.10. DNA Extraction

DNA was extracted by heating the culture samples for 5 minutes at 95 °C with shaking at 1400 rpm (Thermomixer comfort, Eppendorf) followed by 5 minutes of centrifugation at 14800 rpm Heraeus Fresco 21 Centrifuge, Thermo Scientific). The supernatants containing the DNA were collected and transferred to a new 1.5 ml Eppendorf tube. All DNA extractions were stored at -18 °C until further use.

2.11. RNA Extraction

A modified TRIzol extraction protocol from Sigma-Aldrich was followed to extract RNA from fish tissue. 1000 µl TRI Reagent and a steel bead (5 mm) were added to each sample before homogenisation in a TissueLyser II (Qiagen) for 2 minutes and 30.0 Hz. After homogenization the samples were kept at room temperature for 5 minutes to further dissociate nucleoprotein complexes. 200 µl of chloroform (Sigma-Aldrich) was then added to each sample and vortexed for 15 seconds before being left at room temperature for 10 minutes. The samples were then centrifuged at 12.000 x *g* for 15 minutes at 4 °C in order to separate the content into 3 phases:

a red organic phase containing proteins, an interphase containing DNA and a colourless upper aqueous phase containing RNA. Approximately 500 µl of the aqueous phase was then transferred to new 1.5 ml tubes and 500 µl of isopropanol (isopropanol added whilst centrifuging the samples). The new samples were then left on a stand for 10 minutes at room temperature before centrifuged at 12,000 x *g* at 4 °C for 15 minutes that formed a pellet at the bottom of the tube. The supernatant was removed and 1000 µl of a 75 % ethanol solution was added to wash the pellets. The samples were then centrifuged at 12,000 x *g* at 4 °C for 5 minutes. The 75 % ethanol solution poured out after centrifugation and the same procedure was repeated with 1000 µl 100 % ethanol solution. The ethanol was then discarded, leaving only the RNA-contain pellet left in the bottom of the tube. The tubes were then left to air dry for 10 minutes before 50 µl of dH_2O heated to 70 °C was added to dilute the extracted RNA pellet. All RNA samples are stored at -80 °C.

2.12. Polymerase Chain Reaction (PCR) and Gel Electrophoresis

The PCR's performed in this study were based on a standard reaction mixture. One reaction consists of: 2.5 µl 10x Extra Buffer, 1.5 µl (1.25 mM) dNTP (Deoxynucleotide Triphosphate), 1.0 µl (10µM) forward primer, 1.0 µl (10µM) reverse primer, 0.15 µl (5 units/µl) Taq polymerase, 2.0 µl DNA template and 16.85 µl dH_2O. This makes 25 µl the total reaction volume for one volume. All PCR's were performed in Applied Biosystems GeneAmp® PCR systems 9700 machines with following settings: 5 minutes initialization at 95 °C, 35 cycles of denaturation at 94 °C for 0.30 minutes, annealing at 55 °C for 0.30 minutes, elongation at 72 °C, and the extension steps at 72 °C for 10 minutes. The primers used were the *dnaK* (fwd: GGWACYACNAAYTCDTGTGT, rev: TCWATCTTMGCTTTYTCAGC) (Habib *et al.*, 2014).

Agarose gel electrophoresis was performed on the PCR products (amplicon) in order to check whether the expected DNA fragments (*dnaK*: 573 basepairs (Habib *et al.*, 2014)) were generated in the PCR process. Agarose gel was prepared by mixing 4.0 gram agarose with 400 ml Tris-acetate-EDTA (TAE) buffer. This mixture makes up an 1 % agarose solution. 25 µl 1 % agarose solution and 1.0 µl of GelRed™ (fluorescent nucleic acid dye) was used for gel moulding. When the gel had set, it was submerged in 1 X TAE-buffer. 2.5 µl of SmartLadder was added to first well in the gel serving as a PCR product length reference. A mixture of 5.0 µl PCR product and 1.0 µl of loading dye was added to the remaining wells. The container containing the gel was connected to a Ps500x DC Power Supply (HIS Hoefer Scientific

instrument) set to 80 volts for 30 minutes. Carestream MI GelLogic 212 Pro was used to visualize the results under UV-light.

2.13. Sequencing

Only PCR products confirmed by gel electrophoresis were sequenced. The PCR products were cleaned with USB ExoSAP-IT PCR Product Cleanup before sequencing was conducted. The ExoSAP-IT PCR clean-up was carried out as described in the protocol (Appendix 3). The ExoSAP-IT reagent removes unused primers and nucleotides that might interfere with the sequencing reaction without risking loss of samples of PCR products. The PCR products treated with ExoSAP-IT were used as a template in the sequencing.

The sequencing amplification was based on a standard reaction mixture consisting of: 1.0 µl BigDye® Terminator 3.1 version sequencing buffer, 1.0 µl (2.5X) BigDye Terminator 3.1 Ready Reaction Premix, 1.5 µl template DNA, 1.5 µl (3.2 pmol) primer (*dnaK*: forward or reverse), and 5.0 µl dH_2O making the total volume 10 µL of the mixture. Sequencing was conducted in Veriti thermal cycler (Applied Biosystems) using the following settings: 95 ^oC for 5 minutes, 35 cycles for 10 seconds at 95 ^oC, 55 ^oC for 5 minutes, and 4 minutes at 60 ^oC. 10 µl dH_2O was subsequently added to the tubes containing the sequencing products before delivered to the sequencing laboratory facilities located at Høyteknologisenteret in Bergen.

2.14. Genetic analysis

Alignments of recovered sequences were constructed in AlignX in Vector NTI v.9.0 (Invitrogen) before the sequences were adjusted. The *dnaK* sequence available in the GenBank for Canadian *T. maritimum* strain TmarCan15-1 was included as this was the isolate used to infect the fish in the current bath challenge studies.

2.15. real-time RT-PCR primer and probe selection

Several real-time RT-PCR assays were tested to detect the presence of *T. maritimum* strains in pure cultures (see table 2.7 and table 2.9). Finguelli *et al.,* (2012) had previously developed an assay using DNA to detect the 16S gene in *T. maritimum*, but it has been shown that this cross-reaction with *T. soleae* and *T. dicentrarchi* (Fringuelli *et al.*, 2012; Vallestad, 2017). Assays targeting the *T. maritimum* 16S rRNA gene had previously shown to be inferior to assays targeting the *T. maritimum* House Keeping (HK) genes (Vallestad, 2017). As a result, all the real-time RT-PCR assays in table 2.7 were tested in order to determine which assay to use in the current study.

RNA extraction was conducted as described in section 2.11 on pure cultures of *T. maritimum* and other *Tenacibaculum* spp. isolates listed in table 2.8. The specificity of each assay (table 2.6) was tested against the isolates in table 2.8 using optimised primers and probe concentrations (table 2.7) from Vallestad (2017). Each reaction was performed in triplicates.

Table 2.6 – Lists showing the different primers and probes used in the real-time RT-PCR assay selection, their target and associated sequence. [+] indicates LNA modified nucleotides.

Target	Primer/Probe	Direction	Sequence (5-3')	Origin
T. maritimum	Tmar_OmpA_F	Forward	GCCAATAGCAACGGGATACC	(Frisch *et al.*, 2018b)
-	Tmar_OmpA_R	Reverse	TCGTGCGACCATCTTTGGT	(Frisch *et al.*, 2018b)
-	Tmar_OmpA_P	Probe	TGAATCAAATGCGATCTT	(Frisch *et al.*, 2018b)
-	qPCR_Tmar_ITS_F	Forward	CACTGATAATGTAGAGGTC	(Vallestad, 2017)
-	qPCR_Tmar_ITS_R	Reverse	CCTCAATCTTGTAATGTTG	(Vallestad, 2017)
-	qPCR_Tmar_ITS_P	Probe	[6FAM]TCC[+T]C[+T]A[+A]C[+T]T[+C]CAGAATT[BHQ1]	(Vallestad, 2017)
-	qPCR_Tmar_OmpA F_LNA	Forward	AGAGCAATTTACTTCAACTC	(Vallestad, 2017)
-	qPCR_Tmar_OmpA R_LNA	Reverse	GTAGCAATTAAGTCTAATTTACC	(Vallestad, 2017)
-	qPCR_Tmar_OmpA P_LNA	Probe	[6FAM]TCA[+T]T[+C]A[+G]A[+C] CAGG[+A]GT[BHQ1]	(Vallestad, 2017)
-	qPCR_Tmar_infB F	Forward	CAGGAGCAGTTGTAGAAG	(Vallestad, 2017)
-	qPCR_Tmar_infB R	Reverse	TCCAGCCAATAAGTAATCTC	(Vallestad, 2017)
-	qPCR_Tmar_infB P	Probe	[6FAM]TCA[+A]TG[+T]TC[+C] TG[+C]CTG[BHQ1]	(Vallestad, 2017)
-	qPCR_Tmar_infB2 F	Forward	GAGGGTATGTATCTACAA	(Vallestad, 2017)
-	qPCR_Tmar_infB2 R	Reverse	CAGCCAATAAGTAATCTC	(Vallestad, 2017)
-	qPCR_Tmar_infB3 P_LNA	Probe	[6FAM]CAA[+T]GT[+T]CC[+T]GC [+C]TGT[BHQ1]	(Vallestad, 2017)
-	qPCR_Tmar_MAR F	Forward	TGCCTTCTACAGAGGGATAGCC	(Fringuelli *et al.*, 2012)
-	qPCR_Tmar_MAR R	Reverse	CTATCGTTGCCATGGTAAGCCG	(Fringuelli *et al.*, 2012)
-	qPCR_Tmar_MAR P	Probe	FAM-CAGTTTGGAATGGCATCG	(Fringuelli *et al.*, 2012)
Salmon elongation factor 1 Alpha A	EF1A F	Forward	CCCCTCCAGGACGTTTACAAA	(Olsvik *et al.*, 2005)
-	EF1A R	Reverse	CACACGGCCCACAGGTACA	(Olsvik *et al.*, 2005)
-	EF1A P	Probe	6-FAM-ATC GGT GGT ATT GGA ACMGB	(Olsvik *et al.*, 2005)

Table 2.7 - The different assays used in the specificity test and their optimized combination of forward- and reverse primer and probe. The concentrations are given in nanomolar (nM) (Vallestad, 2017).

Assay	Forward primer (nM)	Reverse primer (nM)	Probe (nM)
pPCR_Tmar_OmpA	600	600	175
qPCR_Tmar_ITS	600	600	200
qPCR_Tmar_OmpA_LNA	600	600	175
qPCR_Tmar_infB	600	600	150
qPCR_Tmar_infB2	600	600	
qPCR_Tmar_infB3_LNA			150
qPCR_Tmar_MAR	400	400	175

Table 2.8 - The *T. maritimum* isolates used in the current study to test the specificity for the selected real-time RT-PCR assays.

Isolate name	Species
M106	*T. maritimum*
L293-2	*T. maritimum*
M218-2	*T. maritimum*
NS-17	*T. maritimum*
NLF-15	*T. maritimum*
Ch-2402	*T. maritimum*
SWH210918	*T. maritimum*
NFL-15 (positive control)	*T. maritimum*
T. maritimum type strain (NCIMB 2154)	*T. maritimum*
T. ovolyticum (NCIMB 13127)	*T. ovolyticum*
T. soleae (NCIMB 14368)	*T. soleae*
T. dicentrarchi (NCIMB 14598)	*T. dicentrarchi*
T. finnmarkense (NCIMB 42386)	*T. finnmarkense*
T. adriaticum (DSM 18961)	*T. adriaticum*

Based on the initial specificity test, a final specificity test was conducted using the qPCR_Tmar_*OmpA*_LNA and qPCR_Tmar_MAR assays. This was performed in order to determine which assay was best suited for this study. In the first test there was some concern

that the *T. maritimum* specific assay qPCR_Tmar_*OmpA*_LNA assay detected *T. soleae* (strain NCIMB 14368^T). To eliminate any possibility of contamination, a new RNA extraction was performed using only a pure culture of *T. soleae* with a following test between the qPCR_Tmar_*OmpA*_LNA and qPCR_Tmar_MAR assays to determine what assay was best suited for this study. The test revealed that the qPCR_Tmar_*OmpA*_LNA assay was not specific enough and the qPCR_Tmar_MAR assay ended up being the preferred real-time RT-PCR assay in the current study.

2.16. real-time RT-PCR analyses of tissue samples from bath challenge study

Skin and kidney tissues were selected as the most relevant tissues for the real-time RT-PCR analysis in the current study. Before fish was transferred from aquahall-2, 10 fish from each group (e.g. 10 fish from LSS and 10 fish from FW) were sampled for skin, gill, heart and kidney tissue. The tissues sampled were screened with real-time RT-PCR analyses for other pathogens (see section 2.1). RNA-extraction controls (Rensekontroll, RK) and Non-Template-Controls (NTC) were included in all real-time RT-PCR analyses. These were included in order to reveal any contamination of the mastermix (MM), and that no contamination had occurred during the RNA extractions. An endogenous control assay targeting the elongation factor 1 alpha (ELA1α) was used on the tissue samples (forward primer: CCCCTCCAGGACGTTTACAAA, reverse primer: CACACGGCCCACAGGTACA, probe: ATCG GTGGTATTGGAAC) (Olsvik *et al.*, 2005) in all real-time RT-PCR analyses.

2.17. *In vitro T. maritimum* freshwater survival test

As previously mentioned in section 1.6, *T.maritimum* is a strictly marine bacterium relying on sufficient sea salts to grow. Based on this fact, a series of *in vitro* freshwater survival tests were designed. These survival tests are performed to examine the survival of the bacteria after a 5 hour exposure to freshwater and to seawater. The *T. maritimum* isolate TmarCan15-1 was chosen since the isolate already were selected for the bath challenge study. Treatment time of freshwater on Atlantic salmon lasts a maximum of 5 hours. The incubation time on the secondary bacterial cultures of 5 hours is directly related to treatment time to best mimic actual conditions faced in the aquaculture industry (Parsons *et al.*, 2001; Clark *et al.*, 2003; Roberts & Powell, 2003; Adams & Nowak, 2004; Powell *et al.*, 2015).

An aliquot of *T. maritimum* strain TmarCan15-1 is added to 1.0 L of Cermaq marine broth (CMB) in a 2.0 L pre-autoclaved Erlenmeyer flask (see figure 2.5 A). The bacterial culture will be incubated at 16 °C for 72 hours while shaking at 230 rpm. After incubation of the primary

bacterial culture (PBC), both MPN and OD will be performed in order to determine the concentration of bacterial cells added to the two groups. MPN of the PBC will serve as a reference for the subsequent MPN and OD measurements performed on the fresh- and seawater bacterial after incubation. While the PBC is incubating a total of six 2.0 L Erlenmeyer flasks containing 1.0 L CMB will be prepared and divided into two groups; freshwater and seawater. Each of the groups contains three flasks that will constitute the secondary bacterial cultures (SBC). The freshwater flasks contain water from the Mili-Q system, peptone from animal tissue and yeast extract. The seawater flasks contain all ingredients from the Cermaq marine broth recipe (see Appendix 1).

50 mL will be removed from each of the flasks making up the SBC, making the total volume 950 mL of these flasks (see figure 2.5 B). This is because 50 mL from the PBC will be added into each of the six flasks thus making them secondary bacterial cultures (SBC) (see figure 2.5). However, since the PBC (50 mL) added to the freshwater flasks contains 34‰ seawater, a salt concentration of 1.7 ‰ is retained in each of the freshwater flasks. By the following equation, 1.7 ‰ equals 5 % of 34 ‰:

$$\left(\frac{1.7\ ‰}{34\ ‰}\right) * 100 = 5\ \%$$

When MPN is carried out for the freshwater flasks post-treatment, a separate solution for the freshwater flasks must be made containing 95 % of the total salt concentration which is 34 ‰. This solution is added to the first tube of the dilution series in the MPN and contain, by the following equation, a concentration of 32.3 ‰:

$$\frac{(95\ \% * 34\ ‰)}{100\ \%} = 32.3\ ‰$$

The reason being to equalize the growth conditions for both groups thus not interfering with the MPN results. The saltwater flasks already contain the standard 34 ‰ concentration.

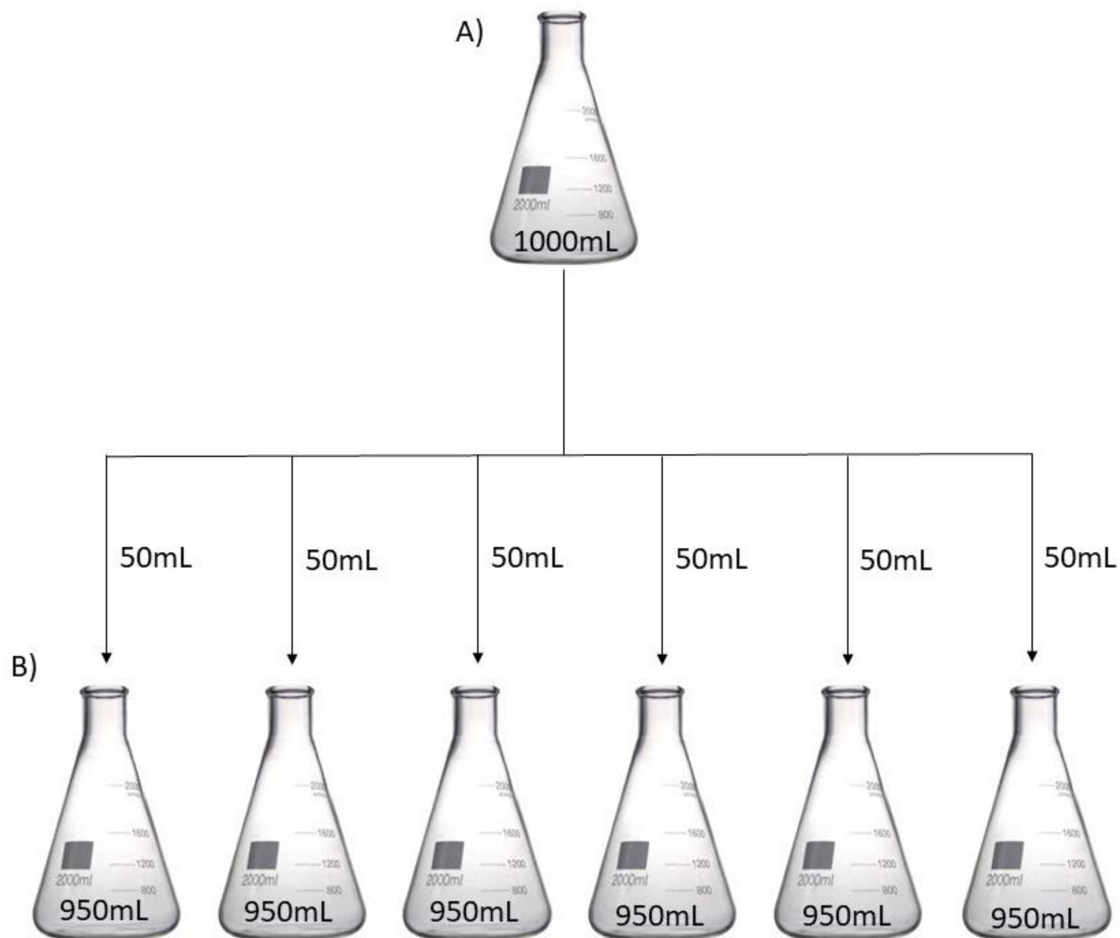

Figure 2.5 - Survival test 1, A) Erlenmeyer flask containing 1000 mL of the primary bacterial culture distributing 50 mL of the culture to, B) the secondary bacterial cultures.

The secondary bacterial cultures (both freshwater and seawater flasks) will be incubated for 5 hours at 16 ^{o}C at 230 rpm. After the 5 hours, freshwater flasks samples and completion of MPN will be taken first in order to prevent the bacteria from being exposed to the freshwater for too long to minimise any effect on MPN estimations, such as exaggerated cell death.

Dilutions in the MPN were from 10^{-2}-10^{-7} and 10^{-4}-10^{-9} for all bacteria subjected to freshwater, 10^{-3}-10^{-8} and 10^{-5}-10^{-10} for bacteria subjected to seawater, and 10^{-4}-10^{-9} and 10^{-5}-10^{-10} for the PBC.

2.17.1. *T. maritimum* freshwater survival test no. 1

The first *T. maritimum* survival test was conducted as described in the section above (see section 2.17) with some adjustments. Due to a technical issue with the shaking incubator, the secondary cultures were incubated at 150 rpm instead of 230 rpm to avoid spills. Ingredients in the SBC are listed in table 2.9.

Table 2.9 - Table displays the water source used and nutrients included in the secondary bacterial cultures in *T. maritimum* freshwater survival test no. 1.

Water source	Mili-Q
Peptone from animal tissue	Yes
Yeast extract	Yes
Sea salt	Non

As a result of handling the freshwater flasks first in order to measure the MPN, the seawater flasks were incubated for 1.5 hours longer than the freshwater flasks.

2.17.2. *T. maritimum* freshwater survival test no. 2

The setup was the same for both the freshwater and saltwater flaks (the secondary bacterial culture) that they were performed in triplicates. However, the water used was from ILAB and not the Mili-Q and the volume in the flasks were 500 ml instead of 1000 ml. In addition, no peptone from animal tissue, yeast extract or salt were added to the freshwater (see table 2.10 for ingredients).

Table 2.10 - Table displays the water source used and nutrients included in the secondary bacterial cultures in *T. maritimum* freshwater survival test no. 2.

Water source	ILAB, freshwater from Svartediket
Peptone from animal tissue	Non
Yeast extract	Non
Sea salt	Non

Based on the results from *T. maritimum* freshwater survival test no. 1 some alterations were made before the *T. maritimum* freshwater survival test no. 2. The PBC was grown on Cermaq marine blood agar (BAMA) (see figure 2.6 A). The *T. maritimum* strain TmarCan15-1 were grown on BAMA and incubated for 72 hours at 16 °C. After incubation, an inoculation loop was used to gather *T. maritimum* from the plate and transfer it onto a Cell Strainer (VWR) placed on top of a 50ml falcon tube. The use of a cell strainer was deemed necessary due to the sticky nature of *T. maritimum* (see figure 2.6 B). The bacteria were worked through the 100 µm grid of the cell strainer by using two bacterial loops simultaneously. A total of 300 µL of RNase-free water in several steps was done to allow the bacteria to more easily seep through the cell strainer. Bacteria that sept through the grid was collected at the bottom of the 50 mL tube. When a sufficient amount of *T. maritimum* had been collected, CMB was added to adjust the final

volume to a total of 33 ml. In order to calculate the MPN of the bacterial mixture (BM = CMB + *T. maritimum*) 2 ml was pipetted out and used in the MPN measurement. The remaining 31 ml of the BM in the falcon tube was then divided into six new falcon tubes (5 ml in each new falcon tube). 25 mL of either 500 ml of freshwater or 500 ml of CMB was removed from the Erlenmeyer flasks, leaving a total of 450 ml in each flask. 20 ml out of the 25 ml of freshwater or CMB was then transferred into the falcon tubes containing 5 ml of bacterial mixture, making a total volume of 25 ml in each falcon tube. The falcon tubes were then mixed by pipetting up and down several times to make a homogenous bacterial suspension. The 25 ml of bacterial suspension was then transferred back to the corresponding Erlenmeyer flask, making a total of 500 ml. As a preventive measure each falcon tube contained water from one distinct Erlenmeyer flask and both the falcon tubes, and the Erlenmeyer flasks had numbers corresponding with each other so that no mixing freshwater with freshwater or freshwater with saltwater occurred See figure 2.6 C, D). A final volume of 25 mL was achieved by adding 5 mL of the bacterial culture to the 20 mL contained in each 50ml tube. The 25 mL bacterial culture was transferred back into their respective flasks to a total volume of 500 mL (see figure 2.6 D). Each flask was incubated while shaking at 150 rpm at 16°C for 5 hours. After incubation, MPN was performed for each of the flasks, starting with the freshwater flasks. As with the first survival test, the saltwater flasks were incubated for a longer time than the freshwater flasks.

To calculate the final concentration added to the SBC the following formula (formula 1) was used:

Formula 1 - The modified formula is used to calculate the final concentration in the SBC after the bacterial mixture was added to the SBC. C_1 = concentration in solution 1. V_1 = volume of concentration 1. C_2 = concentration of solution 2. V_2 = volume of solution 2.

$$C_2 = \frac{C_1 * V_1}{V_2}$$

The formula was applied in two steps. The first step calculated the bacterial concentration in the 25 mL in the falcon tube after adding *T. maritimum*. The second step is used to calculate the final *T. maritimum* concentration of the 500 mL in the Erlenmeyer flasks after adding the concentration from the first step. The calculation of *T. maritimum* in each SBC was performed in order to determine the effect of the subsequent treatment.

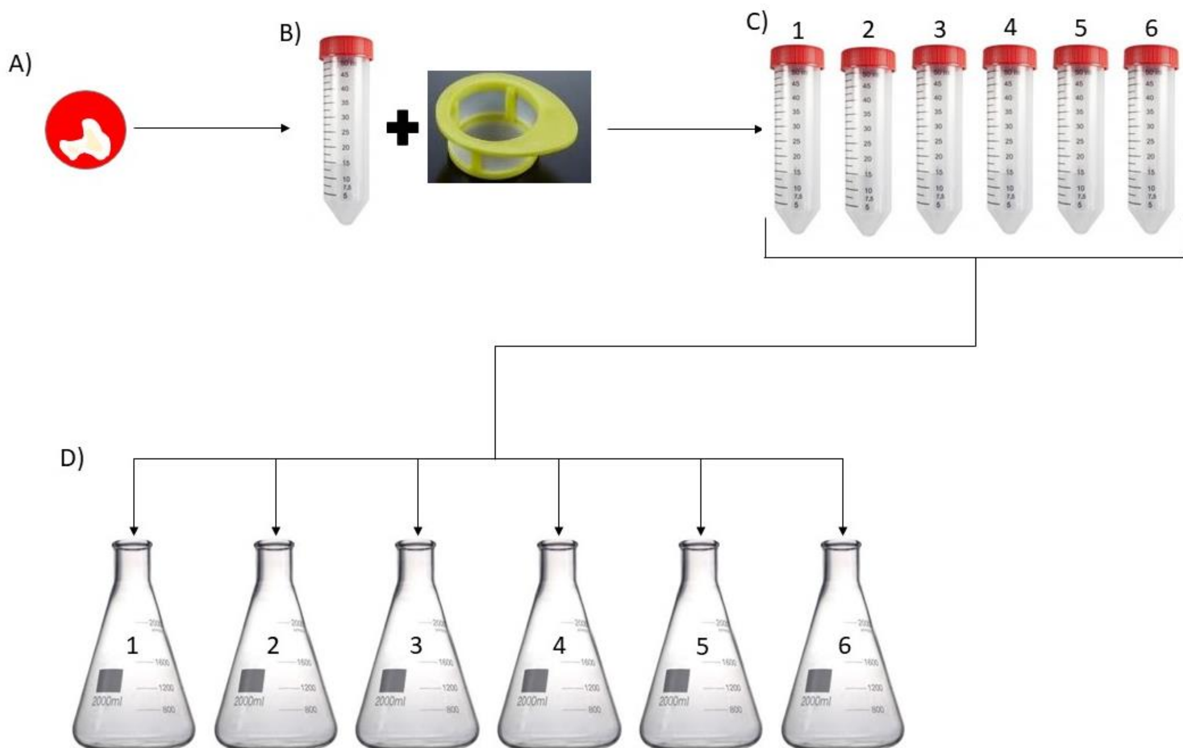

Figure 2.6 - Survival test 2: A) An agar plate containing biomass of *T. maritimum* TmarCan15-1 isolate, B) 50 mL Falcontube and cell strainer used to collect *T. maritimum*, C) six 50 mL falcon tubes in which the bacteria were distributed, D) 2 L Erlenmeyer flasks with corresponding numbers to the falcon tubes in C) which the bacterial cultures from the falcon tubes were distributed.

2.17.3. *T. maritimum* freshwater survival test no. 3

The third survival test was conducted as described in section 2.17.2 and figure 2.6. However, in this test both peptone from animal tissue and yeast extract were added to the freshwater flasks to investigate the effect of survival in freshwater with nutrients present (see table 2.11 for ingredients).

Table 2.11 - Table displays the water source used and nutrients included in the secondary bacterial cultures in *T. maritimum* freshwater survival test no. 3.

Water source	ILAB, freshwater from Svartediket
Peptone from animal tissue	Yes
Yeast extract	Yes
Sea salt	Non

3. RESULTS

3.1. Bath Challenge Trial 1

Prior to the start of bath challenge trial 1, the OD and the MPN were measured for the two *T. maritimum* TmarCan15-1 stocks which were considered to be used to produce the challenge material for use in the bath challenge study. Both stocks were grown in CMB. In general, the *T. maritimum* TmarCan15-1 stock 2 (figure 3.2) had elevated OD and MPN values compared to that of *T. maritimum* TmarCan15-1 stock 1 (figure 3.1). However, both stocks displayed uneven values of OD and MPN throughout the measuring period, but *T. maritimum* TmarCan15-1 stock 2 had a better MPN to OD compared to *T. maritimum* TmarCan15-1 stock 1. Based on the two measurements *T. maritimum* TmarCan15-1 stock 2 was chosen as the preferred challenge material inoculum to use in the bath challenge study.

T. maritimum strain TmarCan15-1 stock 2 was grown for 76 hours before being used in the bath challenge trial 1. This incubation period produced a sufficient bacterial concentration and OD value and at the same time had not reached the stationary growth phase (see figure 3.2).

Two challenge models were used in bath challenge trial 1: a cohabitation model (tank-1 – tank-3) and a direct infection model (tank-5 – tank-8). Both groups were exposed by using *T. maritimum* strain TmarCan15-1. The fish were challenged for 2 hours. Fish from tank-1 were infected with the lowest concentration ($1.64*10^7$ cfu/ml) and the first mortality was recorded as late as 11 days post exposure (dpe) in a shedder fish. In total, the shedder fish (group-1s) had a mortality of 10 % and the cohabitation fish (group-1c) had a mortality of 5 % (see figure 3.3). Fish from tank-2, which was infected with the medium concentration ($2.86*10^7$ cfu/ml), experienced mortality 13 dpe and 15 dpe. Interestingly, the first fish was a cohabitation fish. Mortality in group-2s was 10 % and 5 % in group-2c (see figure 3.5) which is the same as recorded in group 1s and 1c. Fish from tank-3, which had the highest infection concentration ($3.86*10^7$ cfu/ml), recorded the first mortality 6 dpe and the mortalities continued until 16 dpe. The same number of fish mortalities was recorded in both groups. Making a total mortality of 40 % in group-3s and 20 % in group-3c (see figure 3.7).

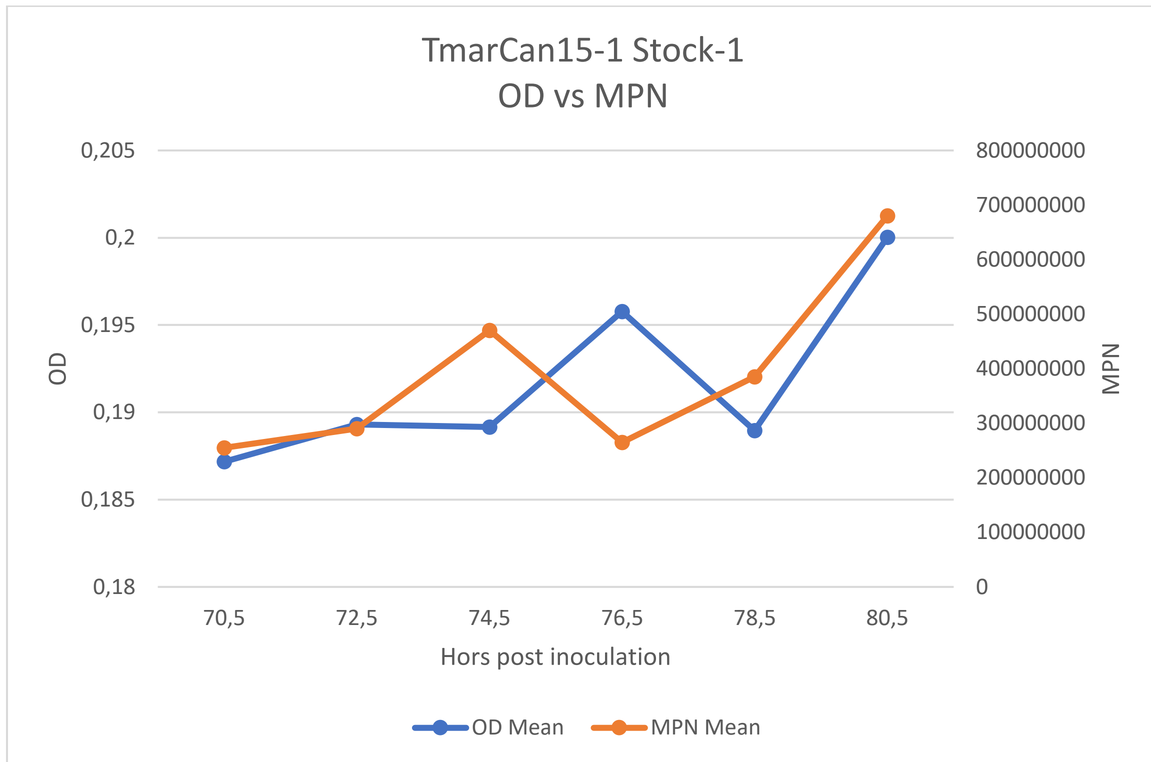

Figure 3.1 - Figure displays the OD (y-axis) and the calculated MPN (z-axis) measurements at certain hours post inoculation (x-axis) for Canadian *T. maritimum* TmarCan15-1 Stock 1 isolate. OD: blue line, MPN: orange line.

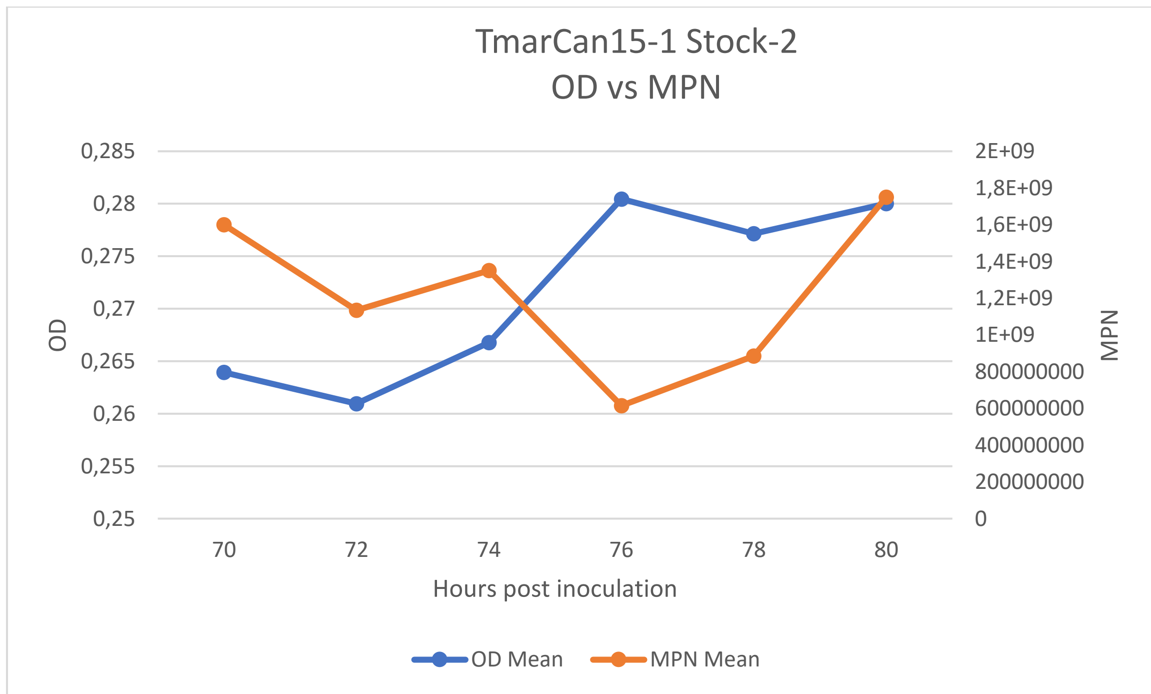

Figure 3.1 - Figure displays the OD (y-axis) and the calculated MPN (z-axis) measurements at certain hours post inoculation (x-axis) for Canadian *T. maritimum* TmarCan15-1 Stock 2 isolate. OD: blue line, MPN: orange line.

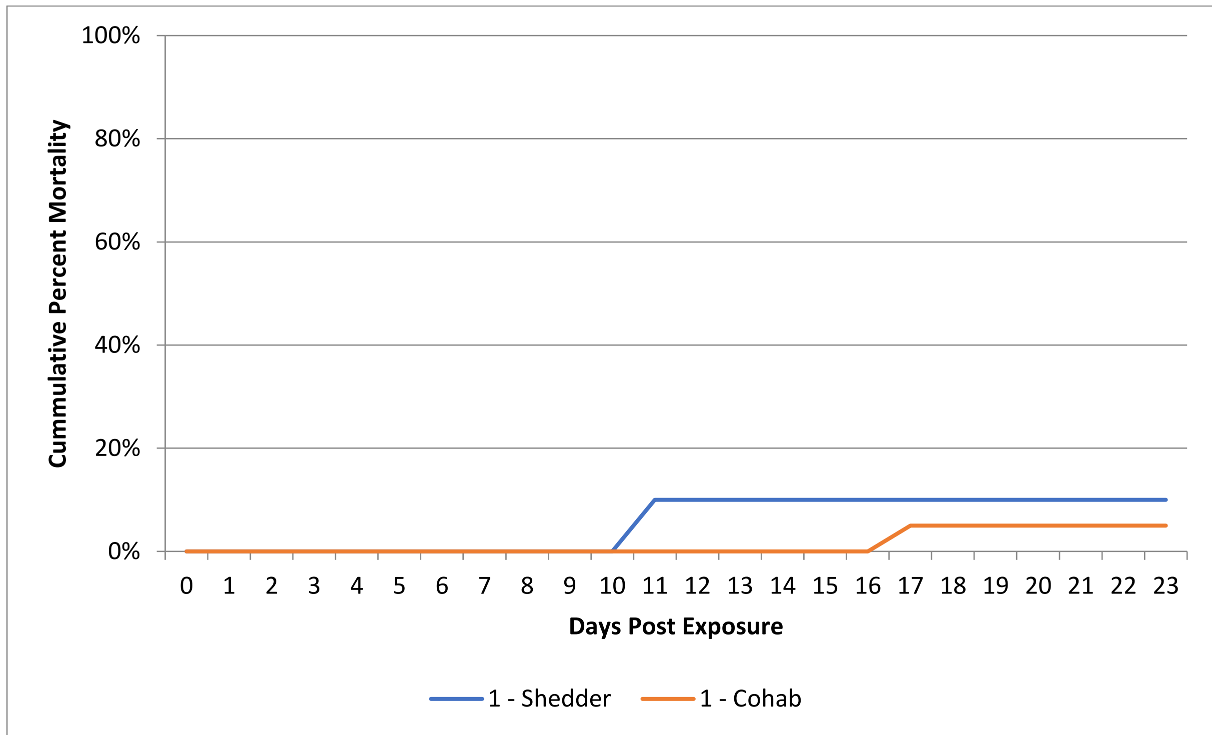

Figure 3.3 – The figure displays the cumulative mortality (y-axis) and days post exposure (x-axis) for tank-1 in bath challenge trial 1. The blue line represents shedder fish with a mortality of 10 %. The orange line represents cohabitation fish with a mortality of 5 %.

Figure 3.4 – Figure shows fish sampled from bath challenge trial 1. A) Displays fish no. 4 from tank-1 (shedder fish). The fish survived until the termination day and represent a fish with no lesion on the skin or gills and no haemorrhage in the mouth (received score 0 on all parameters); B) Displaying fish 2 from tank-2 (shedder fish). This fish were sampled 15 days post exposure and represent a fish with lesions on the skin (blue arrowheads) (score 3 of 3) and haemorrhage associated with the mouth region (red circle) (score 3 of 3).

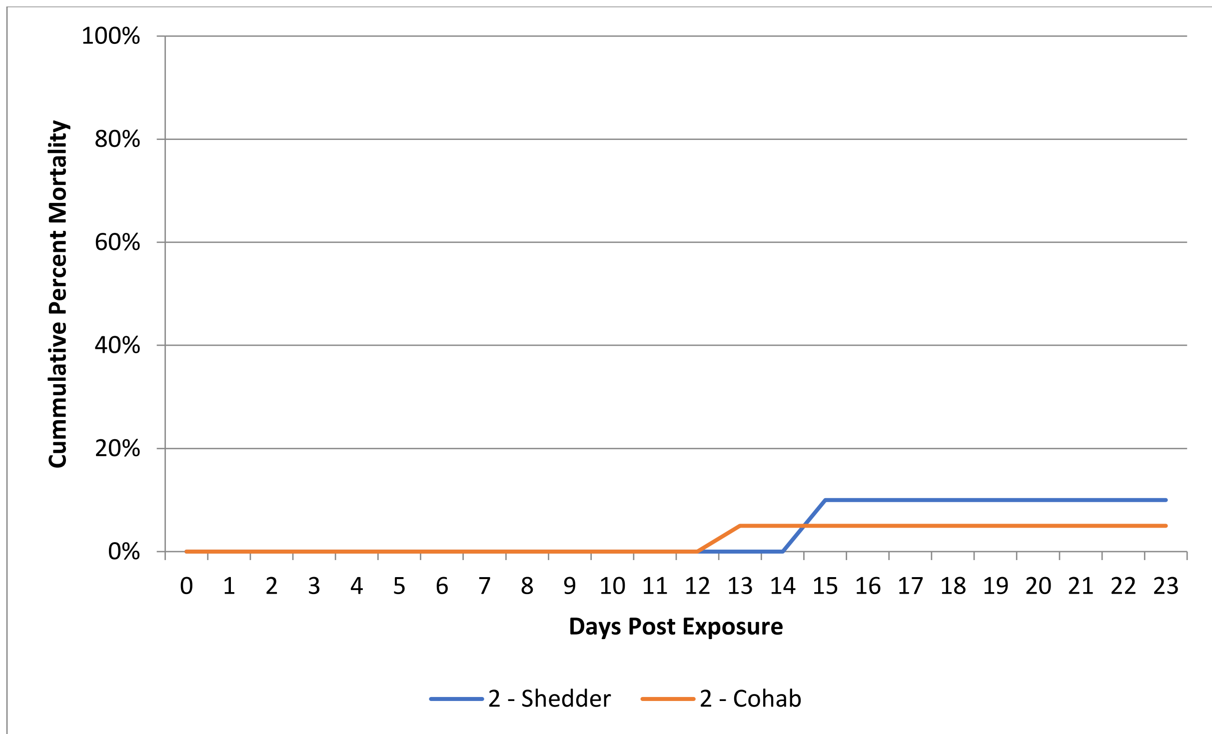

Figure 3.5 - The figure displays the cumulative mortality (y-axis) and days post exposure (x-axis) for tank-2 in bath challenge trial 2. The blue line represents shedder fish with a mortality of 10 %. The orange line represents cohabitation fish with a mortality of 5 %.

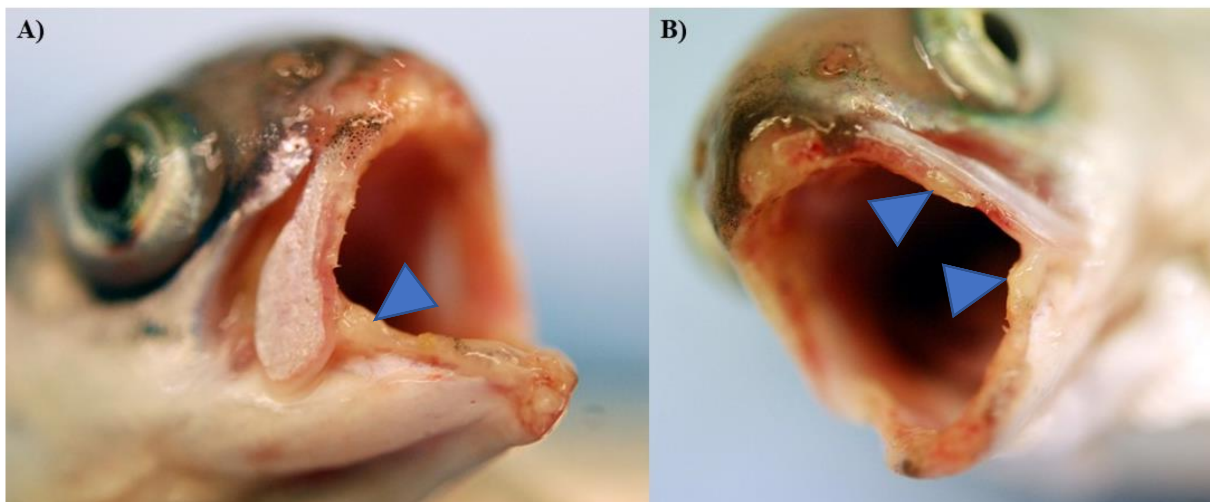

Figure 3.6 – Fish sampled in bath challenge trial 1. A) Displaying fish 2 from tank-2. This was a shedder fish and was sampled 15 days post exposure. The fish displays characteristics plaques in the mouth associated with mouthrot (blue arrowhead). This fish was scored 3 out of 3 in the mouth; B) Displaying fish 5 from tank-3. This fish was a cohabitation fish and was sampled 13 days post exposure and plaques associated with mouthrot (blue arrowheads) is clearly visible in the mouth indicating horizontal transfer of *T. maritimum* from a shedder fish in tabk-3. Fish was graded 2 out of 3 in the mouth.

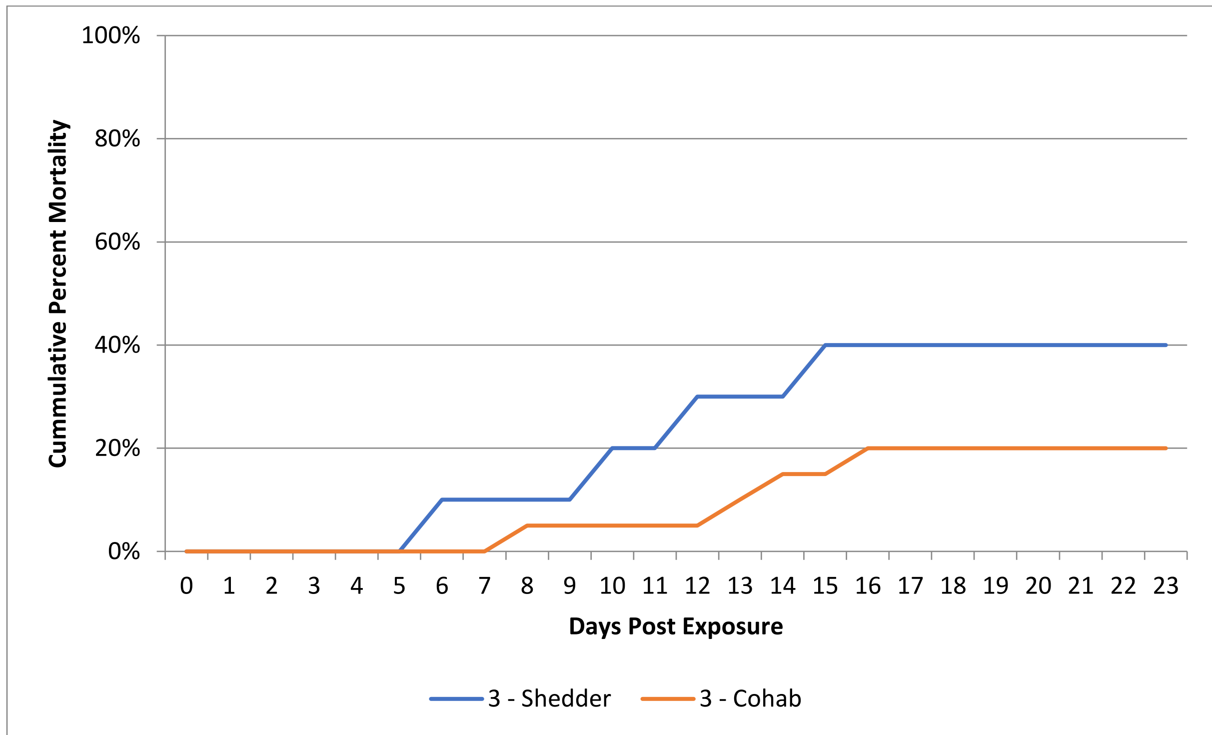

Figure 3.7 - The figure displays the cumulative mortality (y-axis) and days post exposure (x-axis) for tank-3 in bath challenge trial 1. The blue line represents shedder fish with a mortality of 40 %. The orange line represents cohabitation fish with a mortality of 20 %. The graphs show a dose-response trend in tank-3 of bath challenge trial 3.

3.1.1. Freshwater treatment trial

All fish in tanks-5 – tank-8 in bath challenge trial 1 were exposed to the same concentration of *T. maritimum* strain TmarCan15-1 ($2.76*10^7$ cfu/ml). Prior to the bath challenge it was decided that tank-7 and tank-8 would receive freshwater treatment at first sign of disease in either of the tanks-5 – tank-8. The first sign of disease was recorded at 7 dpe in tank-5. As a result, the 5 hour freshwater treatment was initiated in tank-7 and tank-8 on day 8 post exposure. The cumulative mortality in tank-5 was 30%, with the first recorded mortality 7 dpe, while tank-6 had a cumulative mortality of 20 % with the first mortality being recorded 8 dpe. The two tanks that received freshwater treatment had no mortality in tank-7 and 5 % in tank-8. For tank-8 the mortality of a single fish was recorded at 14 dpe (see figure 3.8).

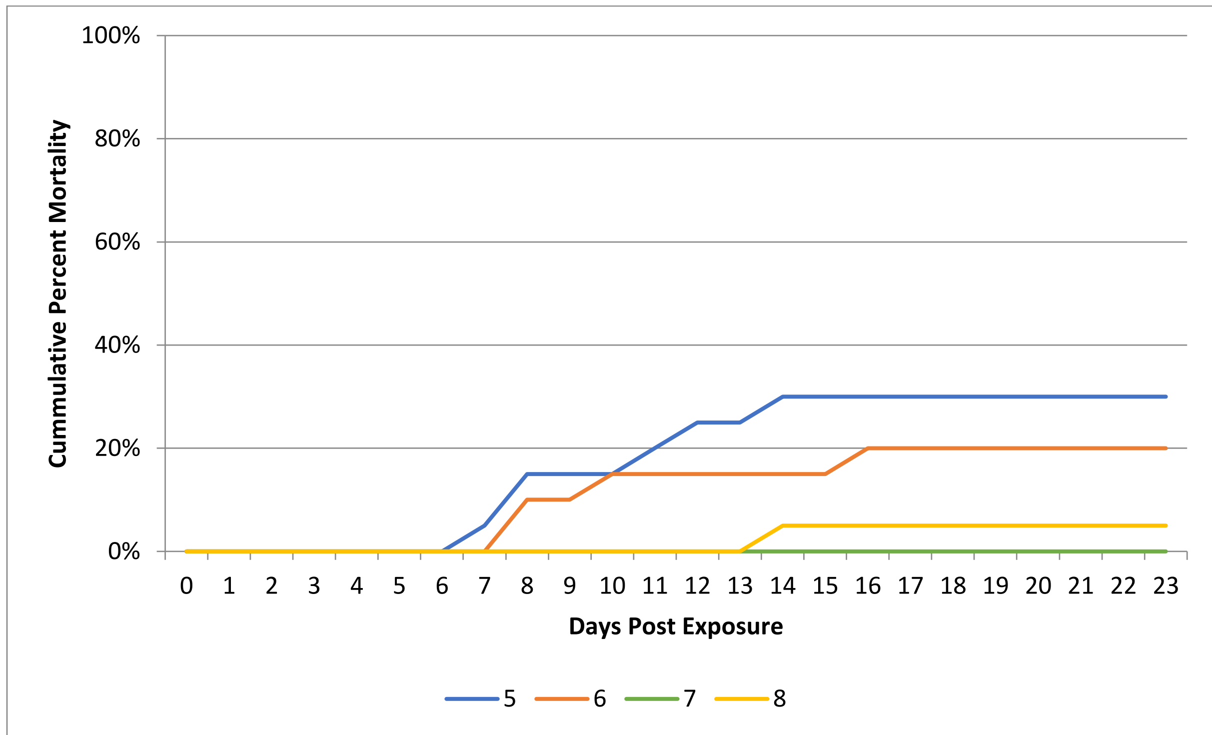

Figure 3.8 – Freshwater treatment: The figure displays the cumulative mortality (y-axis) and days post exposure (x-axis) for tank-5 – tank-8 in bath challenge trial 1 (freshwater treatment trial). Tank-5: blue line (mortality 30 %). Tank-6: orange line (mortality 20 %). Tank-7: green line (mortality 0 %). Tank-8: yellow line (mortality 5 %).

Figure 3.9 –Freshwater treatment in the bath challenge trial 1 (direct exposure), both fish are sampled tanks that did not receive freshwater treatment. A) Displaying fish 4 in tank-5, the fish was sampled out 11 days post exposure and was scored 2 out of 3 for the lesions on the skin (red circle); B) Displaying fish 7 from tank-6. This fish survived until the termination day and received a score of 1 out of 3 for the lesion on the skin (blue arrowhead).

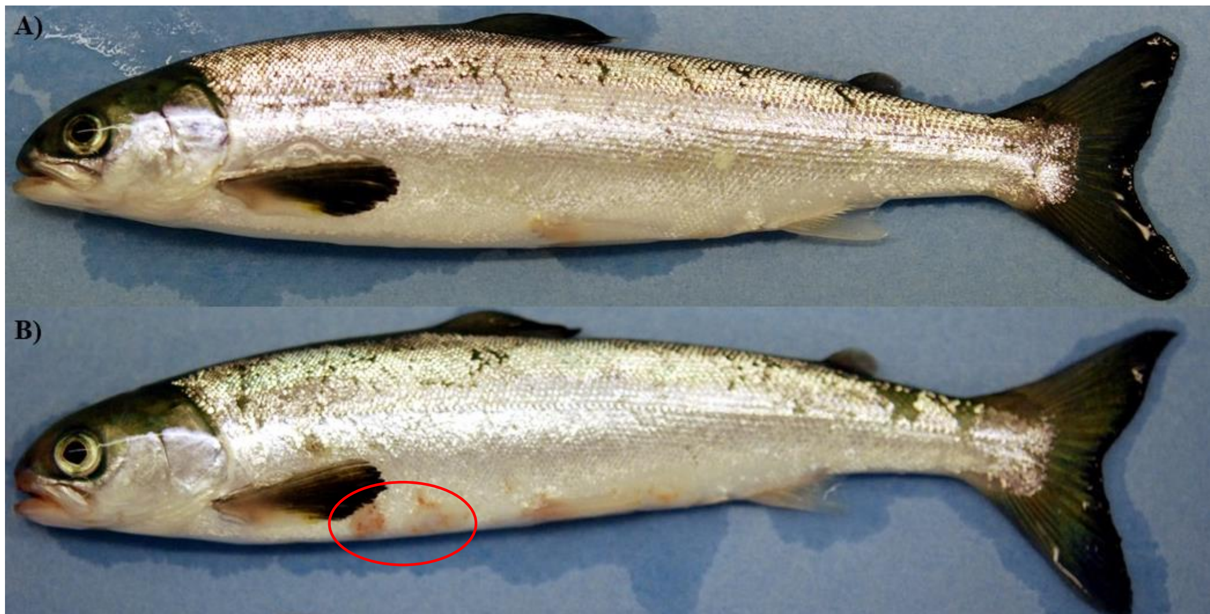

Figure 3.10 – Freshwater treatment in bath challenge trial 1 (freshwater treated fish). A) Displaying fish 2 from tank-7. This fish survived until the termination day and was scored 0 out of 3 as it had no lesions; B) Displaying fish 2 from tank-8. The fish survived until the termination day and was scored 1 out of 3 for the lesions on the skin (red circle).

In the freshwater treatment trial, of bath challenge trial 3, a greater number of the fish from tank-5 and tank-6 displayed clinical signs, than fish from tank-7 and tank-8. Fish removed from the tanks during the freshwater treatment trial of bath challenge trial 1 were removed based on the presence of lesions on the skin and fins or by displaying deviant behavioural traits (e.g. standing high in the water). Only six out of 170 fish were recorded with clinical signs on the gills. 41 fish were recorded with clinical signs in the mouth region. Two fish were graded 3 out of 3 (see grading of "mouth" given in table 2.5) with the characteristic yellow plaques associated with mouthrot. A greater part of the fish had lesions on the skin and bacterial aggregates (yellow pigmented slime) behind the pectoral fins (figure 3.10 B). No internal pathological sings were found in all the fish examined.

Bacteria isolated from tanks-1 – tank-3 and tank-5 – tank-8 on KA-BAMA and BAMA displayed the characteristic growth pattern and morphology of *T. maritimum*. It is most likely that the bacteria isolated on the plates are *T. maritimum*. Analyses using histology and SEM of the sampled tissues, sequencing of the recovered bacterial clones and real-time RT-PCR analyses were planned to be conducted, but due to the SARS-CoV-2 outbreak this has not been performed.

3.2. Bath Challenge Trial 2

All fish groups in bath challenge trial 2 were exposed using the direct exposure model to the *T. maritimum* strain TmarCan15-1 with a bath concentration of $2.42*10^7$ cfu/ml. All tanks holding the LSS fish had 0 % mortality during the bath challenge. Tanks holding FW fish had an overall mortality of 8 % (see figure 3.11) with initial mortality (one fish) occurring 7 dpe, followed by a second mortality at 12 dpe. At 15 dpe there was a peak in the mortality with three fish removed from the FW-group (two fish from tank-7 and one fish from tank-5). The total cumulative mortality in percent for each of the tanks in bath challenge trial 2 is displayed in Appendix 7 table VIII.

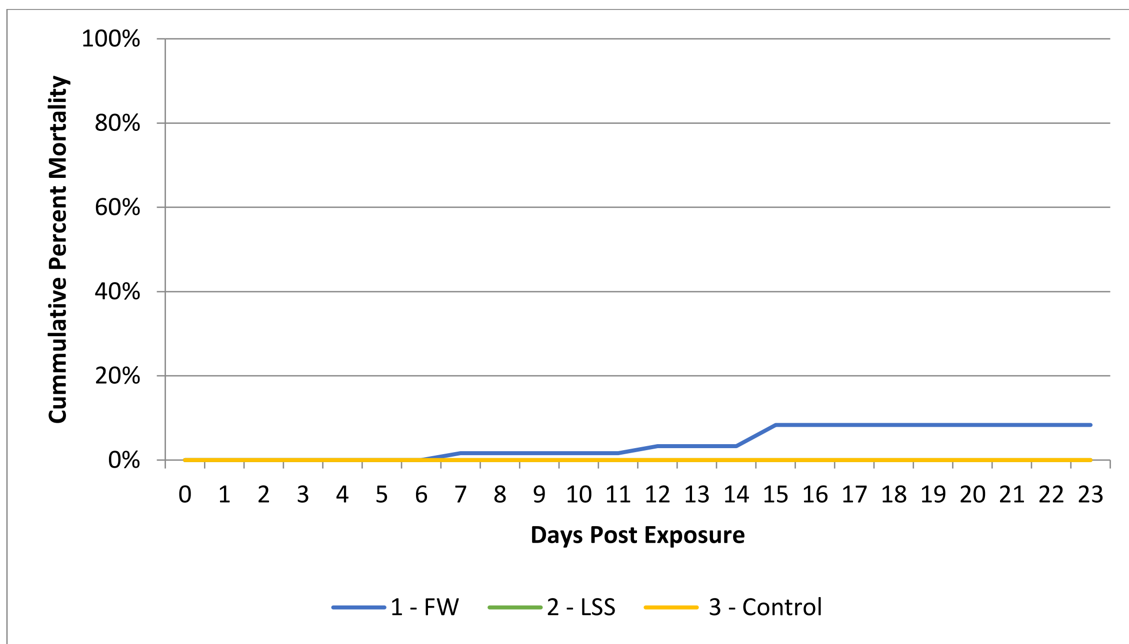

Figure 3.11 - Figure displays the combined cumulative mortality in percent (y-axis) for the LSS-, FW- and Control-group (Both the LSS- and Control-group had 0% mortality) and days post exposure (x-axis) for FW, LSS and Control group of bath challenge trial 2. FW: blue line (mortality 8 %). LSS: green line (mortality 0 %). Control: yellow line mortality 0 %).

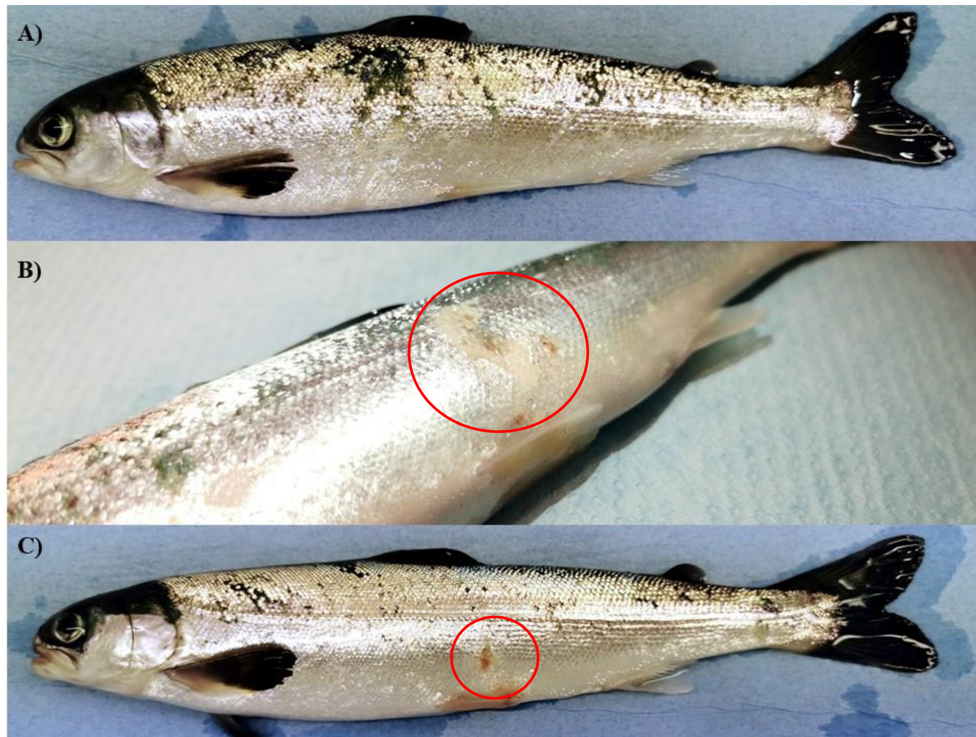

Figure 3.12 - All fish are from bath challenge trial 2. A) Displaying fish 3 from tank-1. This fish was in the low salinity seawater group and survived until termination day. The fish had no lesions on the skin and scored 0 out of 3.; B) Dysplaying fish 2 from tank-5. This fish was from the freshwater group and was sampled 12 days post exposure. The fish was scored 2 out of 3 for the lesions on the skin (red circle).; C) Displaying fish 8 from tank-6. This fish was from the freshwater group and survived until the termination day.The fish scored 1 out of 3 for the lesions on the skin (red circle).

Bath challenge trial 2 had the lowest registered incidences of clinical signs present on the fish upon examination. Only 0.6 % of the fish (from a total of 162) was registered with clinical signs on the gills, 3.1 % of the fish were registered with clinical signs around the mouth (none of the fish was given more than a score of 1 out of 3). A total of 18.1 % of the fish had clinical signs on the skin, while 78.8 % of the fish showed no clinical signs during or at the end of the bath challenge. The most severely affected fish had petechial spots surrounding the mouth and lesions on the abdomen (figure 3.12), at the base of the caudal fin, and between the skin and the pectoral fins. None of the examined fish showed any clinical signs on internal organs.

Bacteria isolated from tanks-1 – tank-3 and tank-5 – tank-7 on KA-BAMA and BAMA displayed the characteristic growth pattern and morphology of *T. maritimum*. It is most likely that the bacteria isolated on the plates are *T. maritimum*. Analyses using histology and SEM of the sampled tissues, sequencing of the recovered bacterial clones and real-time RT-PCR analyses were planned to be conducted, but due to the SARS-CoV-2 outbreak this has not been performed.

3.3. Bath Challenge Trail 3

Before bath challenge trial 3 the bacterial growth medium was changed from CMB to Difco 2216 marine broth (MB). This was done as this was the same growth medium used in the challenge study conducted by Frisch *et al.* (2018a). From the OD and MPN measurements from MB and CMB, the values were higher with MB compared to CMB. The *T. maritimum* strain TmarCan15-1 was incubated for 76 hours before exposure as this period had the best observed cell morphology, a sufficient bacterial concentration and OD value and at the same time had not reached its stationary growth phase based on the results from figure 3.13.

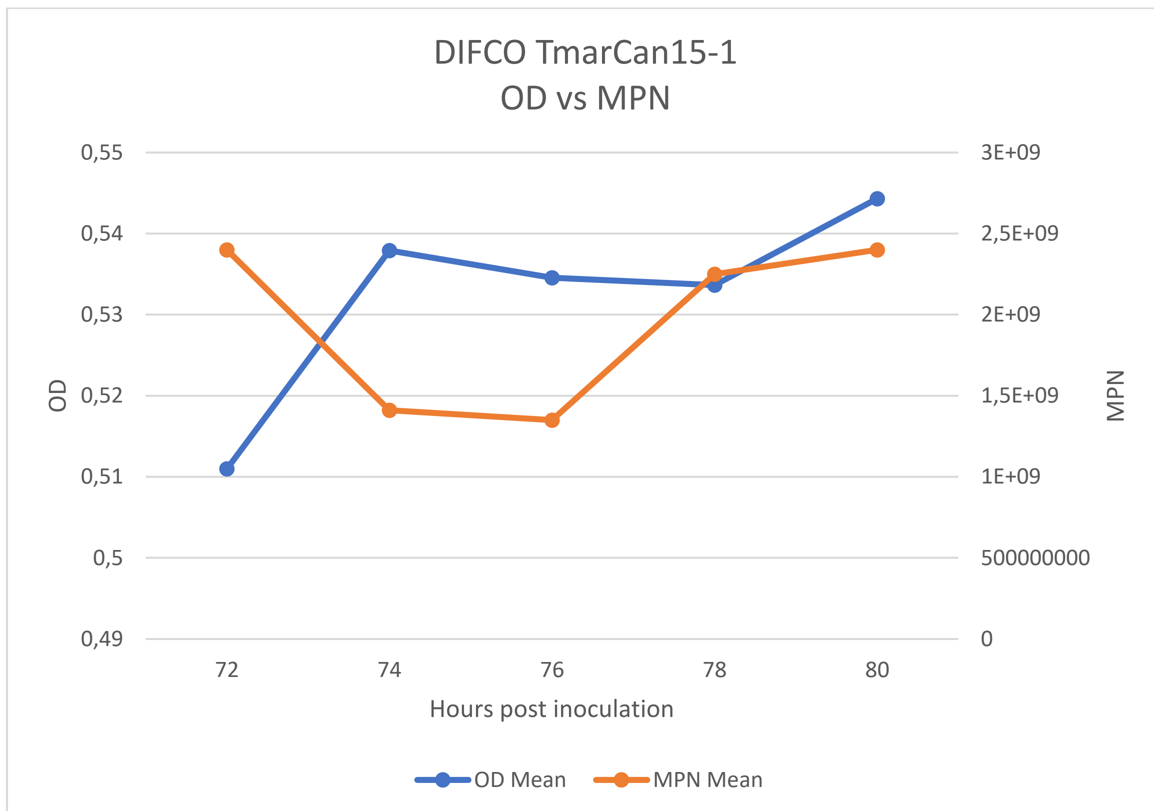

Figure 2.13 - Figure displays the OD (y-axis) and the calculated MPN (z-axis) at certain hours post inoculation (x-axis) for Canadian *T. maritimum* TmarCan15-1 Difco 2216 isolate. OD: blue line, MPN: orange line.

Both the LSS and FW group in the bath challenge trial 3 registered the highest mortality of any of the bath challenges trials, with the highest calculated bacterial dose $3.95*10^7$ cfu/ml using the Difco 2216 marine broth (MB).

The overall mortality for the FW-group was 85 %, but only 20 % for the LSS-group (see figure 3.15). The t-test was applied to the mortality data and the difference in mortality between the LSS group and FW group statistically significant difference between the groups ($p < .0013$) (see Appendix 6). Sufficient number of individuals were used in bath challenge trial 3 in order for this bath challenge trial to have sufficient statistical power to detect a treatment effect. No

mortality occurred in the control groups (tank-4 and tank-8). Total cumulative mortality in percent for each of the tanks in bath challenge trial 3 is displayed in Appendix 7 table IX.

Figure 3.14 – Both fish were sampled from the LSS group in bath challenge trial 3. A) Displaying fish 3 from tank-3. This fish was from the low salinity seawater group and was sampled 16 days post exposure. The fish displays severe lesions on the tail (blue arrowhead) and lesions on the skin (red circle) and scored 3 out of 3 on skin which is the most severe.; B) Displaying fish 2 from tank-2. This fish was from the low salinity seawater group and was sampled 6 days post exposure. This fish scored 2 out of 3 for the lesions on the skin (red circle). Bacterial aggregates can be observed at the basis of the tail (green circle).

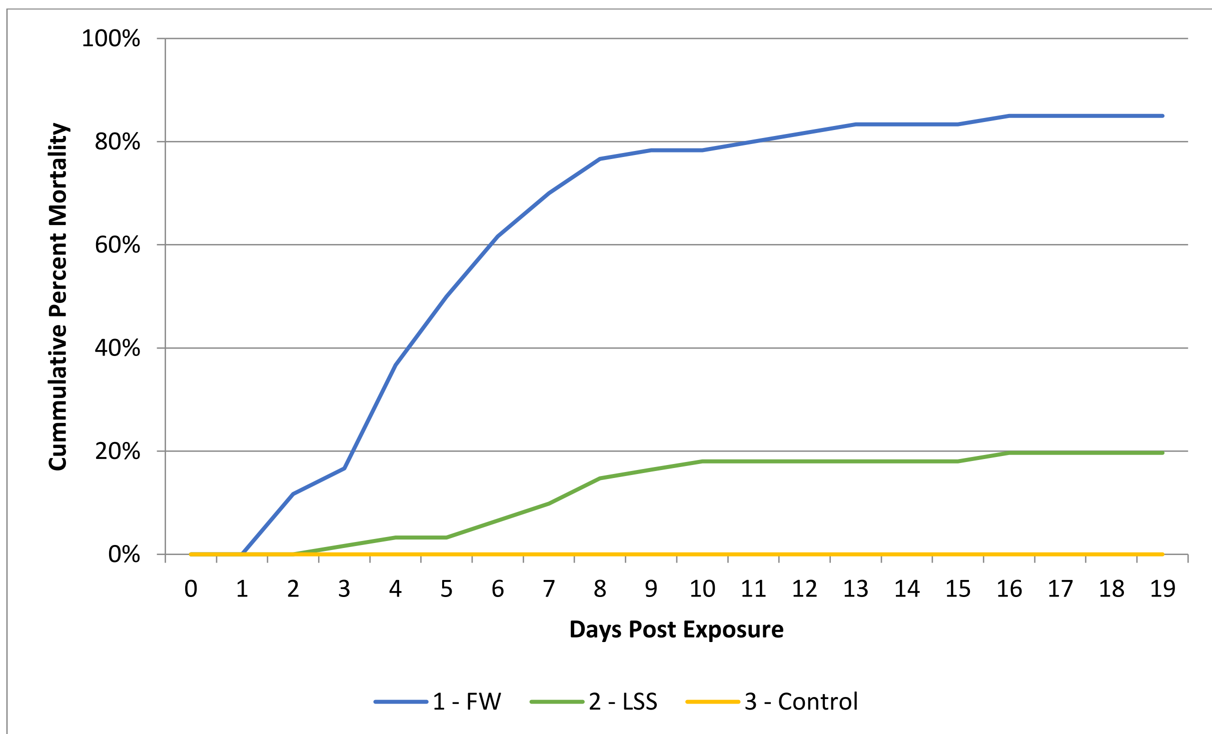

Figure 3.15 - Figure displays combined cumulative mortality in percent (y-axis) and days post exposure (x-axis) for the LSS-, FW- and Control-group in bath challenge trial 3. FW: blue line, LSS: green line, Control: yellow line. Mortality for the FW-group was 85 %. Mortality for the LSS-group was 20 %. Mortality for the Control-group was 0 %.

Figure 3.16 - Both fish is from the LSS group in bath challenge trial 3. A) Displaying fish 6 from tank-3. This fish survived until the termination day. This fish scored 1 out of 3 for the lesions on the skin (red circle).; B) Displaying fish 2 from tank-4 (control). This fish survived until the termination day. This fish did not display any lesions on the skin and scored 0 out of 3.

Bath challenge trial 3 had the highest incidence of clinical signs present on the fish during the challenge. Necrotic patches on the gills (figure 3.17 A) and lesions on the skin were most frequently observed (figure 3.14, 3.18 A), but haemorrhage surrounding the mouth with, occasionally with yellow plaques, was also observed in a number of fish. Microscopic examination of tissue scraping from the infected areas revealed large number of thin rod-shaped bacteria similarly to the *T. maritimum* morphology.

Figure 3.17 - Both fish are from bath challenge trial 3. A) Displaying fish 1 from tank-5. This fish is from the freshwater group and was sampled 2 days post exposure. This fish displays patches both gills (red circle) and scored 2 out of 2; B) Displaying fish 18 from tank-6. This fish was from the freshwater group and survived until the termination day. The fish scored 1 out of 3 for the lesions on the skin (red circle).

Figure 3.18 - Both fish are from bath challenge trial 3. A) Displaying fish 20 from tank-7. This fish was in the freshwater group and was sampled 13 days post exposure. This fish scored 3 out of 3 for the lesion on the tail (blue arrowhead).; B) Displaying fish 1 from tank-8 (control). This fish was in the freshwater group and survived until the termination day. This fish scored 0 out of 3 as it had no lesions on the skin.

No signs of disease were observed in the internal organs and muscle by dissection in any of the examined fish. Histology revealed bacterial maths associated with the epithelia of the skin and on filaments of the gill (see figure 3.19 and figure 3.20 A). In addition, a number of melano-

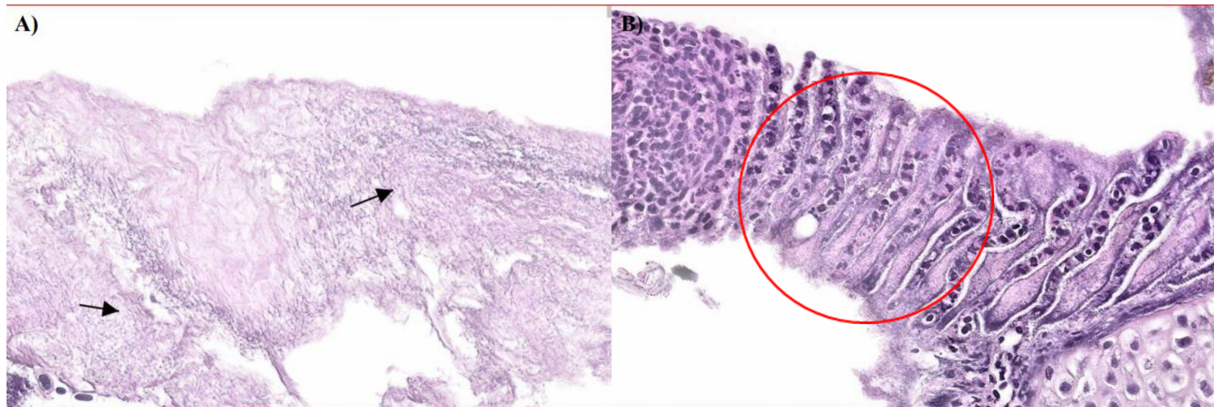

Figure 3.19 – Figure displays histology sections from fish no. 8 from tank-6 (FW group). A) Display histology section from skin tissue with bacterial mats (black arrows); B) Display histology section of the apex part of the gill lamella with necrotic patches surrounded by bacterial maths (red circle).

macrophages were observed in the kidney tissue without the observation of bacteria (see figure 3.21). Immunohistochemistry performed on tissue samples from fish no. 8 in tank-6 (diseased fish from the FW group) showed bacterial aggregates on the epidermis of the skin (figure 3.22 C) and at the basis of the gills (figure 3.22 D). Hyperplasia and lifting on the secondary filaments on the gills were also observed (figure 3.22 B). Interestingly, a number of melano-

macrophages were observed in the kidney tissue, but no associated bacteria were observed with the staining for *T. maritimum* (figure 3.22 A).

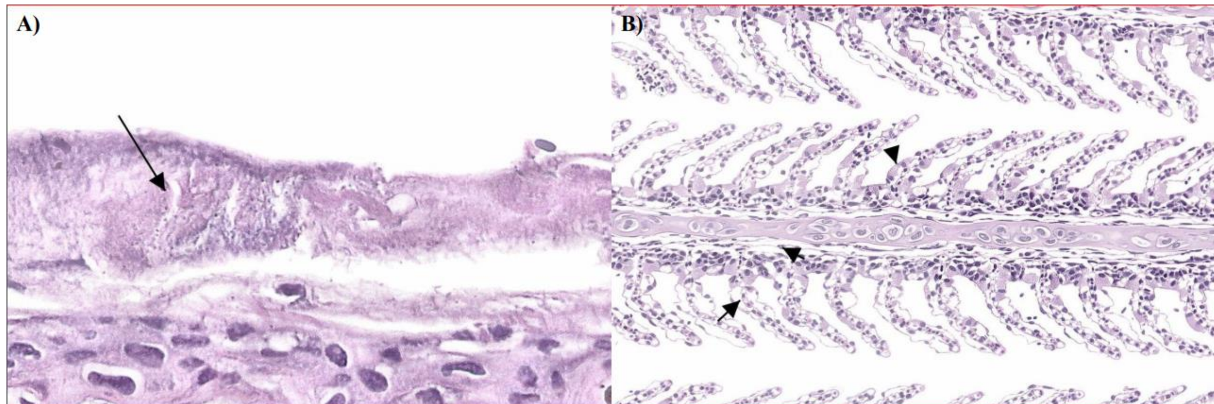

Figure 3.20 - Figure displays histology sections from fish no. 1 from tank-1 (LSS group). A) Display a histology section of skin tissue with a bacterial math (black arrows); B) Display histology section of the gill with pink cells (black arrowhead) which is either a chloride cell or an epithelia cell. Black arrows display lifting of the gill epithelia.

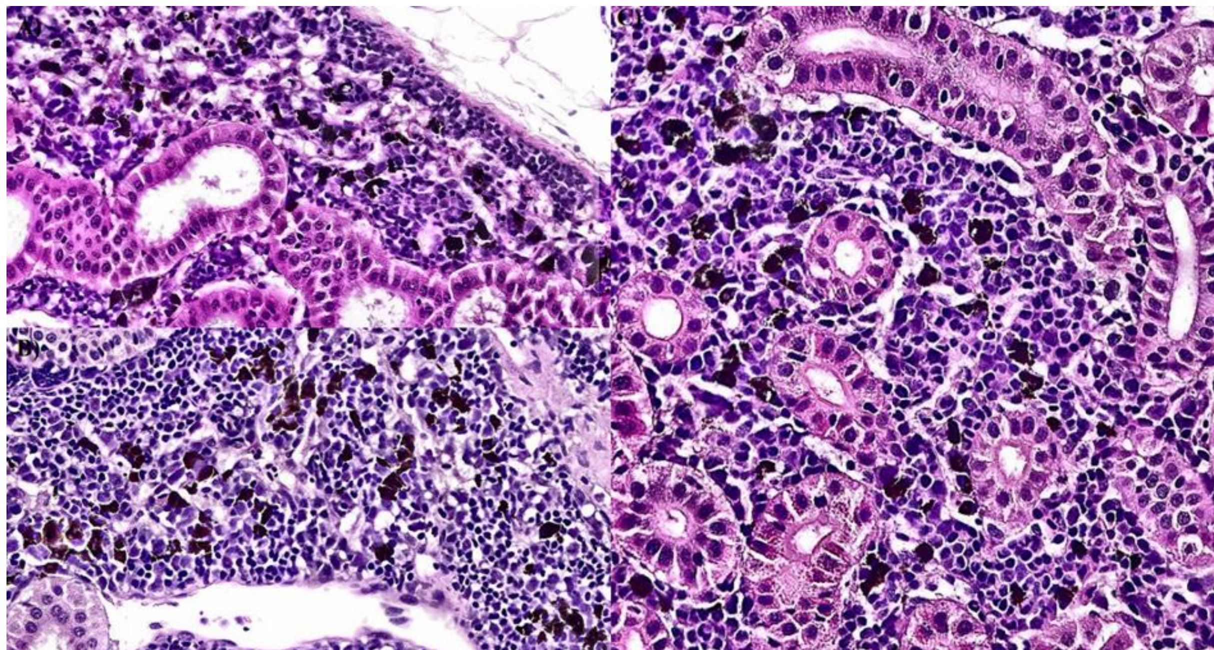

Figure 3.21 – Figure displays kidney tissue from three different fish from bath challenge trial 3. A) Display histology section of the kidney tissue from fish no. 8 from tank-6 (FW group) with melano-macrophages; B) Display histology section of the kidney tissue from fish no. 1 from tank-1 (LSS group) with melano-macrophages; C) Display histology section of the kidney tissue from fish no. 4 from tank-1 (LSS group) with melano-macrophages.

Immunohistochemistry was performed on tissue samples from fish no. 1 from tank-1 from the LSS group. Bacteria were observed on the epidermis of the skin (figure 3.23 A) and at the basis of the gills (figure 3.23 B). The heart tissue displayed normal tissue without any signs of infection (figure 3.23 D). In addition, a number of melano-macrophages were observed in the kidney tissue without any associated bacteria present positive with the staining for *T. maritimum* (figure 3.23 C).

Immunohistochemistry performed on fish no. 4 from tank-4 sampled form the LSS-group displayed no clinical signs. Clubbing and lifting of the epithelia on the secondary filaments of the gills were observed (figure 3.24 B). Melano-macrophages were also observed in the kidney tissue without associated bacteria observed with the staining for *T. maritimum* (figure 3.24 A).

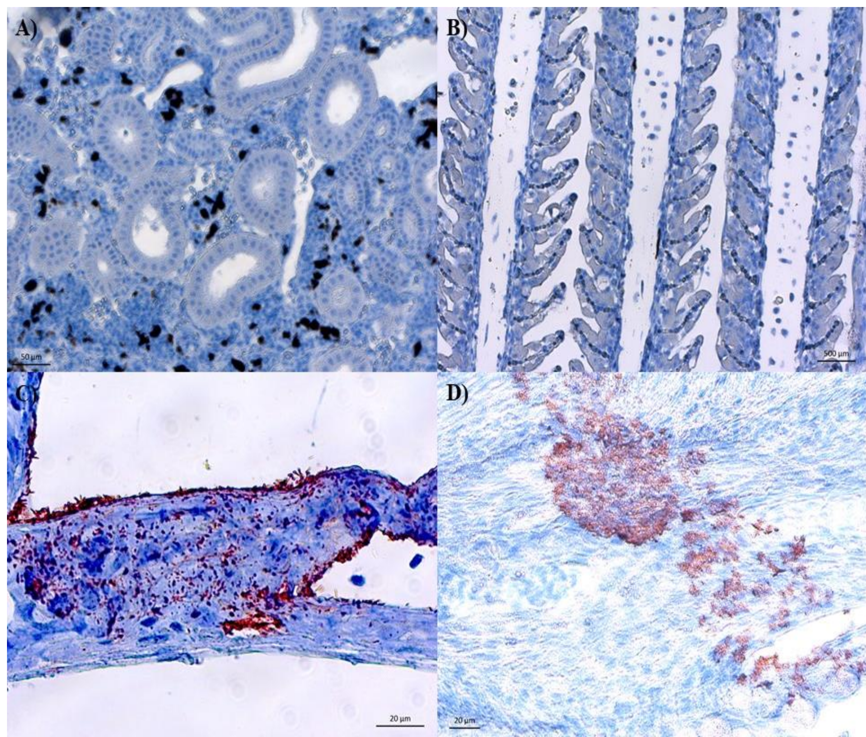

Figure 3.22 - The fish (fish no. 8 tank-6) is from the freshwater group and were sampled 6 days post exposure due to observations of clinical signs. Figure displays 3 tissues; kidney (A), gills (B, D) and skin (C) from fish 8 in tank-6 from bath challenge trial 3. A) kidney with melano- macrophages (black spots), B) hyperplasia and lifting in gills, C) epidermis of the skin with bacteria (red colour), D) bacteria (red colour) on the basis of the gill.

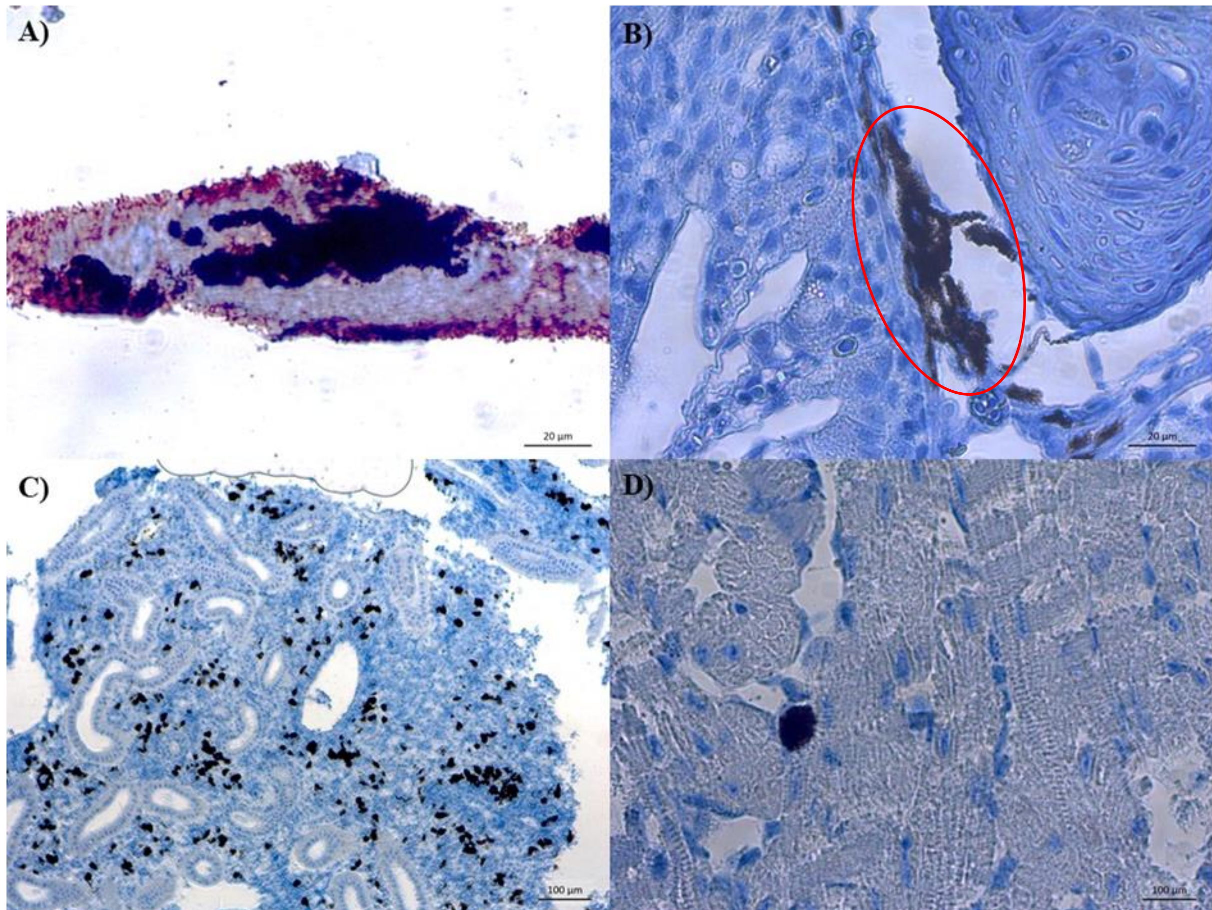

Figure 3.23 - This fish (fish no. 1, tank-1) is from the low salinity seawater group and was sampled 6 days post exposure due to observations of clinical signs. Figure displays histological sections from skin (A), gills (B), kidney (C) and heart (D) from fish 1 in tank-1 from bath challenge trial 3. A) epidermis on the skin loosened from the dermis with associated bacteria and positive staining for *T. maritimum* (red colour), B) basis of the gill with bacteria (red circle), C) kidney with melano-macrophages (black spots), D) heart tissue (spongiosum) revealed no pathological changes.

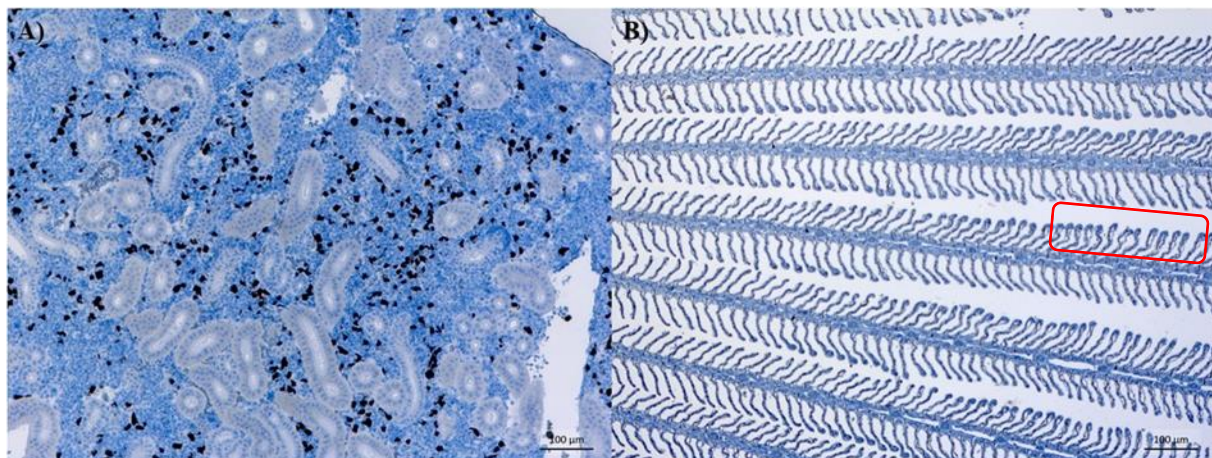

Figure 3.24 - The fish (fish no.4, tank-1) is from the low salinity group and was sampled 18 days post exposure without clinical signs for comparison with fish no. 1 and fish no. 2. Figure displays kidney (A) and gills (B) from fish 4 in tank-1 from bath challenge trial 3. A) normal kidney tissue with melano-macrophages (black spots), B) gill tissue displaying clubbing and lifting of the epithelia on the apical part of the secondary filament (red square).

Sequencing of the recovered bacteria from fish in tank-1 – tank-3 and tank-5 – tank-7 were amplificated by the primer used in the PCR and showed bonds on the gel-electrophoresis (Appendix 4 C). The comparison of the sequences obtained from bath challenge trial 3 with a known *T. maritimum* TmarCan15-1 *dnaK* sequence showed 100 % matching identity coinciding with bacteria isolated from tank-1 – tank-3 and tank-5 – tank-7 (Appendix 4 A, B). No *T. maritimum* were isolated from the control tanks (tank-4 and tank-8). However, bacteria sampled from fish in tank-4 displayed growth on KA-BAMA and BAMA but did not amplify nor showed on the gel electrophoresis. Swabs from tank-8 showed no growth on either KA-BAMA or BAMA.

A total of 162 kidney samples were analysed from bath challenge trial 3 using real-time RT-PCR using two different assays. Based on the qPCR_Tmar_MAR analyses results the qPCR_Tmar_*OmpA* was also included in the real-time RT-PCR analyses. 85% of the samples tested positive using the qPCR_Tmar_MAR assay, while 62% of the samples tested positive using the qPCR_Tmar_*OmpA* assay. There were some positive samples recorded from the control tanks with qPCR_Tmar_MAR assay. However, none of the previously positive samples were positive with qPCR_Tmar_*OmpA* assay.

Ct values obtained from the qPCR_Tmar_MAR assay ranged from 20.4 up to 37.1 for the LSS-group and 13.4 to 37.2 for the FW-group. By using the qPCR_Tmar_*OmpA* assay on the same samples, the Ct-values obtained ranged from 33.2 up to 38.4 for the LSS-group and 27.1 up to 40.1 for the FW-group (a detailed overview from all the real-time RT-PCR analyses are given in Appendix 5).

Since no mortality was recorded in the control fish during the bath challenge trial 3 only tissue sampled from skin were selected for screening. The first run used the qPCR_Tmar_MAR assay to screen for *T. maritimum* and resulted in all fish being positive with high Ct-values. A re-run with the qPCR_Tmar_*OmpA* assay to screen for *T. maritimum* were negative for all the previously positive samples.

3.4. *In vitro T. maritimum* Freshwater survival test

3.4.1. *T. maritimum* freshwater survival test no. 1

The bacterial concentration of the primary bacterial culture (PBC) that was transferred to the SBC was calculated to be $5.45*10^8$ cfu/ml. The calculated bacterial concentrations from the SBC are given in the following tables (table 3.1 and 3.2):

Table 3.1 – The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 1. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in freshwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Freshwater	$3.3*10^7$ cfu/ml	$2.58*10^7$ cfu/ml
	$2.6*10^7$ cfu/ml	
	$1.85*10^7$ cfu/ml	

Table 3.2 - The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 1. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in saltwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Saltwater	$4.15*10^7$ cfu/ml	$3.62*10^7$ cfu/ml
	$2.9*10^7$ cfu/ml	
	$3.8*10^7$ cfu/ml	

3.4.2. *T. maritimum* freshwater survival test no. 2

The calculated MPN for the primary bacterial culture in *T. maritimum* freshwater survival test no. 2 was $1,1*10^9$ cfu/ml. Bacterial concentration at the initiation of the incubation of the SBC (see section 2.14.2) was $1,1*10^7$ cfu/ml. The calculated bacterial concentration post treatment from both freshwater and saltwater inoculums are given in the tables below (table 3.3 and 3.4):

Table 3.3 - The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 2. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in freshwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Freshwater	$2.3*10^2$ cfu/ml	$3.33*10^3$ cfu/ml
	$3.0*10^3$ cfu/ml	
	$4.7*10^3$ cfu/ml	

Table 3.4 - The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 2. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in saltwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Saltwater	$6.55*10^6$ cfu/ml	$6.07*10^6$ cfu/ml
	$4.35*10^6$ cfu/ml	
	$7.3*10^6$ cfu/ml	

3.4.3. *T. maritimum* freshwater survival test no. 3

The calculated MPN for the primary bacterial culture in *T. maritimum* freshwater survival test no. 2 was $1{,}17*10^9$ cfu/ml. Bacterial concentration at the initiation of the incubation of the SBC (see section 2.14.2) was $1{,}17*10^7$ cfu/ml. The calculated bacterial concentration post treatment from both freshwater and saltwater inoculums are given in the tables below (table 3.5 and 3.6):

Table 3.5 - The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 3. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in freshwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Freshwater	$8.55*10^5$ cfu/ml	$4.23*10^5$ cfu/ml
	$1.33*10^5$ cfu/ml	
	$2.82*10^5$ cfu/ml	

Table 3.6 - The table displays the secondary bacterial cultures in the *In vitro T. maritimum* freshwater survival test no. 3. First column shows the water quality in which they were incubated in. The second column describes the calculated bacterial concentration of each secondary bacterial culture incubated in saltwater for 5 hours. The third column displays the average bacterial concentration of all three flasks after incubation.

Salinity	**CFU post 5h treatment (Flasks 1-3)**	**Average**
Saltwater	$5.45*10^6$ cfu/ml	$8.97*10^6$ cfu/ml
	$5.45*10^6$ cfu/ml	
	$1.6*10^7$ cfu/ml	

4. DISCUSSION

The main aim of this study was to investigate the effect of keeping smolts in low salinity seawater (LSS) in reducing *Tenacibaculum maritimum* infection (mouthrot) after seawater transfer. Since the start of the industry in the Pacific Northwest region, mouthrot caused by *T. maritimum* has been a significant disease in smolts recently transferred to seawater net-pens. The disease is currently controlled by the use of antibiotics, mainly florfenicol. However, like in other animal production industries, the aquaculture industry strives to become free of antibiotics. In addition, the use of antibiotics is expensive and results in poorer performing fish and affects the public perception of the industry negatively. However, without the use of antibiotics mouthrot outbreaks would likely be more severe.

4.1. Reduction of mouthrot as a result of keeping smolts in low salinity seawater

4.1.1. Establishing challenge doses

Initially, CMB were chosen over the Difco 2216 marine broth (MB) because MB tend to vary between batches, making it difficult to ensure that *T. maritimum* grows consistently. After incubation of the *T. maritimum* strain TmarCan15-1 culture for 76 hours, both the OD and the calculated bacterial concentration ($1*10^8$) of the CMB were high. No deviant cell morphology (i.e. dead spherical cells) (Avendaño-Herrera *et al*., 2006) were observed at this time point, indicating a healthy culture that had not yet reached the stationary or death phase. Based on these results the *T. maritimum* strain TmarCan15-1 seemed to be at its best potential for use as challenge material after 76 hours of incubation. CMB was used in the bath challenge trials 1 and 2, as opposed to the MB used in the challenge study conducted by Frisch *et al.* (2018a). Based on the results from bath challenge 1 and 2 which induced low mortality, the growth medium was changed from CMB to MB before bath challenge trial 3. Bath challenge trial 3 resulted in a higher accumulated mortality in the LSS group and FW group than the other two bath challenge trials. In comparison, Frisch *et al.* (2018a) obtained an accumulated mortality of 30 % with an exposure time of only 1.5 hours with *T. maritimum* strain TmarCan15-1 grown in MB and a bacterial bath concentration of $1.9*10^7$ cfu/ml. Moreover, fish exposed for 5 hours with *T. maritimum* strain TmarCan15-1 grown in MB and a bacterial bath concentration of $5.74*10^6$ cfu/ml obtained an accumulated mortality of 90 % (Frisch, *et al.*, 2018a). As only low mortality was recorded using CMB, the results from bath challenge trial 3 and the results from Frisch *et al.*, (2018a) suggest that MB is a better suited as a growth medium for inducing mouthrot in bath challenge experiments.

The reason why *T. maritimum* seems to grow better in MB than in CMB is not known. Small changes in the composition of nutrients may play a role, e.g. amino acid composition, osmotic pressure and pH, may affect the pathogenicity of *T. maritimum* strain TmarCan15-1.

4.1.2. Establishing a challenge model

Initially, a cohabitation model was chosen as the ideal challenge model for this bath challenge study. This is because a cohabitation model better mimics the infection pressure of a natural infection in field (Frisch *et al.,* 2018). However, the cohabitation model provided an overall low mortality in the cohabitation model in bath challenge trial 1. The reason for these results might be due to a lower infection pressure in the cohabitation tanks compared to similar cohabitation study conducted with *T. maritimum* strain TmarCan15-1 by Frisch *et al.* (2018a). In addition, the use of CMB to grow *T. maritimum* may have influenced the pathogenicity of the bacteria as previously discussed. However, in a challenge study conducted with Norwegian *T. maritimum* strains using lumpfish, the CMB works very well in inducing tenacibaculosis (Sverre Småge, Frisk Fisk 2018). The reason why it works when challenging lumpfish, but not salmon, is not known; albeit it may relate to differences in the skin between the two species (Patel, *et al.,* 2019).

The results from the high dose group (tank-3) in bath challenge trial 1, suggest a dose-response effect. This confirm the notion from Frisch *et al.* (2018) that *T. maritimum* can easily transfer horizontally between fish (see figure 3.6), opposed to other fish pathogenic *Tenacibaculum* spp. that does not easily transfer between fish (Småge *et al.,* 2018). In addition, the results show that *T. maritimum* strain TmarCan15-1 can induce mouthrot after a short time of exposure as previously shown by Frisch *et al.* (2018a). This is supported by the observation of sampled fish during the bath challenge trial 1, that displayed clinical signs associated with experimentally induced mouthrot (figure 3.5) (Frisch, 2018; Ostland, *et al.* 1999).

Because there were not enough fish available at ILAB to increase the number of shedder fish and cohabitation fish to match the 20 shedder fish and 40 cohabitation fish used in the cohabitation model described by Frisch *et al.* (2018a), it was decided to use a direct exposure model in the bath challenge trial 2 and 3 to infect the fish. The exposure time for bath challenge trial 2 and 3 were prolonged with 3 hours, from 2 hours of exposure to 5 hours of exposure. From the retrospectively calculated bacterial bath concentration, it was found that the bacterial concentration in bath challenge trial 2 was in accordance with the medium dose group from the cohabitation model in bath challenge trial 1. However, the overall mortality was still low, which further indicates that the CMB has a negative effect on the pathogenicity of *T. maritimum* strain

TmarCan15-1. When applying the mortality data from bath challenge trial 2 to a t-test the *p*-value comes to $p < .131$ which is higher than the threshold ($p < .05$). This means that there is no difference between the fish kept in LSS for 4 weeks and the fish kept in FW until exposure to *T. maritimum* strain TmarCan15-1 on the susceptibility to mouthrot.

The growth medium was changed from CMB to MB in bath challenge trial 3 because of the indications that MB is a better suited growth medium for inducing disease when using *T. maritimum* strain TmarCan15-1. The results from bath challenge trial 3 coincided more with the challenge study with *T. maritimum* strain TmarCan15-1 conducted by Frisch *et al.* (2018a). However, the calculated bacterial bath concentration in this trial was increased from the previously conducted bath challenge trials in this current study. By increasing the dose there is, however, a risk of obtaining 100 % mortality within a few days as shown in other studies using *Tenacibaculum dicentrarchi* (Klakegg, *et al.,* 2019). This is not a desired outcome when performing bath challenge trials, because it indicates a toxic effect. A large difference in mortality was seen between the LSS group (mortality 20 %) and the FW group (mortality 85 %). Based on the mortality data from bath challenge trial 3, there were a clear statistically significant difference ($p < .0013$) of the effect of LSS versus FW between the LSS-group and FW-group.

This supports the H_1 and demonstrates a great benefit of keeping smolts in LSS for 8 weeks after smoltification in reducing mortality due to *T. maritimum* compared to directly transfer the fish from freshwater into saltwater before exposure to *T. maritimum*. Data showing a significant statistical difference between fish kept in LSS for 4 weeks after smoltification versus newly smoltified fish prior to seawater transfer demonstrated a positive effect of fish kept in LSS for 4 weeks against *Tenacibaculum finnmarkense* infections (Kristense Solheim (2020), UiB, unpublished data). It would therefore be interesting to investigate if 4 weeks in LSS would result in the same beneficial effect on susceptibility for mouthrot, as seen in this current study for fish kept in LSS for 8 weeks.

The disease progressed similarly to what has been described in previous experimental challenge studies using *T. maritimum* strain Tmar15-1 (Frisch, 2018) for both the LSS group and the FW group in bath challenge trial 3. For both groups first mortality was recorded after a few days post exposure followed by a peak in daily mortality. After the peak in daily mortality a gradual decrease in mortality was recorded before the mortalities ceased after approximately two weeks. This demonstrates that keeping smolts in LSS for 8 weeks after smoltification reduces the total number of mortalities and delays the disease progress with a few days. This delay can

potentially result in a better manoeuvrability for the workers in the field to initiate mitigation measures when the *T. maritimum* infection first is observed.

In bath challenge trail 3, a greater number of fish had lesions on skin and patches on the gills, than what was recorded in previous trials. Interestingly, the necrotic patches on the gills seemed to be more prevalent shortly after exposure but less frequent later in the challenge. Due to the agglutinating nature of *T. maritimum* when grown in liquid media (Frisch, 2018), bacterial aggregates may have attached to the gills during exposure in this current study. This may be the reason for gills being more affected in fish removed from the tanks shortly after exposure than fish removed later in the trial. The notion that aggregates of *T. maritimum* can cause gill lesions is also reported from field cases when decaying fish tissue from other fish species containing *T. maritimum* can lodge in salmon gill filaments causing gill lesions (Chen *et al.,* 1995). In general, the FW group seemed more affected by *T. maritimum* than the LSS group in bath challenge trial 3.

When comparing the clinical signs on all fish from bath challenge trial 3, fish from both groups displayed some signs of disease. However, the FW-group had more severe clinical sings than the LSS fish. This suggest that the LSS fish is a more robust fish and can better withstand a *T. maritimum* infection than the FW group. This is demonstrated in the statistical analysis that shows a strong support for H_1 being correct. No fish in any of the groups showed signs of disease internally. The absence of internal signs of disease is in accordance with what has been described from mouthrot in field and in challenge experiments using *T. maritimum* (Frisch, 2018). The clinical signs observed on the skin in this current study are not typically observed in field today. This is most likely due the antibiotic treatments of mouthrot (Frisch, 2018). However, skin lesions have been described in other challenge studies with *T. maritimum* (Carson, *et al.,* 1992; Avendaño-Herrera, *et al.,* 2006; Van Gelderen, *et al.,* 2009; Frisch, *et al.,* 2018a; 2018b).

4.1.3. Microscopic pathology and bacteriology

Immunohistochemistry (IHC) and histology conducted in the current study reflected the pathological signs previously described for mouthrot (Frisch *et al.*, 2018b; Ostland, 1999). Little or no immune response was observed. The lack of immune response has also been noted in other studies with *Tenacibaculum* spp. and other Flavobacteriaceae (Frisch, 2018; Levipan *et al.*, 2019; Vidal, *et al.,* 2020). The reason for the lack of immune response observed in this current study may be linked to *T. maritimum* creating biofilm. The ability of *T. maritimum* to create biofilms has been demonstrated in previous studies (Avendaño-Herrera, *et al.,* 2006).

This ability contributes to resistance against the host defence mechanisms and may therefore allow for *T. maritimum* to grow to pathogenic levels (Dalsgaard, 1993). This may have resulted in the low levels of immune response observed in histological sections from gills, skin and kidney in this current study as previously hypothesised by Koziel & Potempa, (2013) and Frisch *et al.*, (2018b). The strong adhesive properties is an important first invasion step and is likely the reason it could adhere, create biofilm and invade the host so successfully (Dalsgaard, 1993; McBride *et al.,* 2015; Frisch *et al.*, 2018b). *Tenacibaculum maritimum* appears to destroy the host tissue surrounding the biofilm. This is different to infiltrations with other fish pathogenic *Tenacibaculum* species, e.g. *T. finnmarkense* (Småge *et al.*, 2018). An increased number of melano-macrophages, then what is normally found in Atlantic salmon kidney (Amin, *et al.*, 1991; Bruno, *et al.,* 2013b), was observed histologically (see figure 3.21). Interestingly, no bacteria were observed associated with the melano-macrophages in the kidney (see figure 3.22 A, figure 3.23 C and figure 3.24 A). The presence of melano-macrophages in the kidney suggest a systemic infection, however without associated bacteria it could indicate that the melano-macrophages have engulfed and destroyed the bacteria.

The findings from bath challenge trial 3 in the current study fulfil Koch's postulates (Fredricks & Relman, 1996) yet again for *T. maritimum* strain TmarCan15-1. The re-isolated bacteria were 100 % identical This is in accordance with the results from previous experimental challenge studies conducted by Vallestad (2017) and Frisch *et al.* (2018a) using *T. maritimum* strain TmarCan15-1.

4.1.4. Real-time RT-PCR

The results from the real-time RT-PCR analyses performed on kidney tissue from fish in bath challenge trial 3, supports the findings from Frisch *et al.* (2018b), that *T. maritimum* strain TmarCan15-1 becomes a systemic disease. The analyses conducted using the real-time RT-PCR assay qPCR_Tmar_MAR, suggests that a larger portion of the fish in the FW group have *T. maritimum* present systemic than the LSS-group based on the analyses with the sensitive qPCR_Tmar_MAR assay (Vallestad, 2017). The real-time RT-PCR analyses shows that when *T. maritimum* becomes systemic, the bacterium is present in large numbers and becomes systemic early in the infection progress. Moreover, the *OmpA*-gene have been shown to correlate with bacterial growth rate and is growth stage-dependent in other gram-negative bacteria (Rasmussen, *et al.,* 2005) and based on the real-time RT-PCR analyses using the qPCR_Tmar_*OmpA* assay, the fish in the FW group have greater amounts of bacteria in the growth phase systemically than the LSS group based on the lower Ct-values. In addition, the

OmpA-gene is expressed as mRNA and is present in lower quantities than the 16S rRNA (https://www.ncbi.nlm.nih.gov/nuccore/NZ_LT634361.1).

The observation of melano-macrophages in the kidney and the absence of associated *T. maritimum* from the IHC of the kidneys, may suggest that the positive real-time RT-PCR analyses for *T. maritimum* from the kidneys are not necessarily from live bacteria but may be from *T. maritimum* that has been engulfed by the melano-macrophages. Unfortunately, no bacteria were sampled from kidney that could have verified this notion.

Initially, the qPCR_Tmar_MAR assay was chosen to be used in the current study after testing several real-time RT-PCR assays. However, when analysing the results from the real-time RT-PCR analyses a few negative controls (RK) were found positive with Ct-values close to the Ct-values of the kidney tissues that were analysed from the experimentally infected smolts. Positive Ct-values were also recorded for the elongation factor. Even one non-template control (NTC) was positive which suggests a contamination of the samples. NTC being positive for *T. maritimum* may suggest that other negative samples were false positive due to contamination.

Due to the extreme sensitivity of the qPCR_Tmar_MAR assay (the assay has been shown to detect as little as 4.8 DNA copies number μL^{-1}) (Fringuelli, *et al.,* 2012), there could be false positive samples due to contamination. Because of this it was decided to use the qPCR_Tmar_*OmpA* assay developed for a study conducted by Frisch *et.al.* (2018b) on the same RNA samples. By using the qPCR_Tmar_*OmpA* assay, the analyses revealed that none of the control fish were positive and neither the NC nor the NTC were positive.

4.2. The effect of freshwater as a mitigation measure against mouthrot

From the *in vitro T. maritimum* freshwater survival test no. 3, it is clear that *T. maritimum* TmarCan15-1 strain can survive in freshwater if it has access to nutrients. The adhesive properties of *T. maritimum* towards hydrophobic surfaces, such as fish mucus, and its ability to create biofilm could indicate that freshwater treatment in field would not eliminate *T. maritimum* from the fish nor the farm once it is present (Ofek & Doyle, 1994; Magariños *et al.*, 1995; Avendaño-Herrera *et al.*, 2006; McBride & Nakane, 2015). The freshwater used in the current study was autoclaved to eliminate any inhibitors. A field study by Downes *et al.* (2018), suggests that *T. maritimum* is not affected by freshwater treatments based on positive real-time RT-PCR analyses using the qPCR_Tmar_MAR assay.

Downes *et al.* (2018) used the qPCR_Tmar_MAR real-time RT-PCR assay, which is sensitive enough to detect *T. maritimum* at very low levels (Fringuelli, *et al.,* 2012), to detect the presence

of *T. maritimum* in the field study. Since no disease associated with *T. maritimum* was recorded in the field study by Downes *et al.* (2018), the detection of *T. maritimum* on the gills by the real-time RT-PCR analyses could have been from particles in the water which could have latched onto the gills. Flavobacteriaceae have been shown to be associated with phytoplankton which also could result in the positive real-time RT-PCR analyses of the gills (Pinhassi, *et al.*, 2004; Teeling *et al.*, 2012). This could suggest that the presence of *T. maritimum* on the gills has been established, with varying amounts dependent on temperature and that *T. maritimum* could be present in the water associated with other waterborne particles.

The *in vitro T. maritimum* freshwater survival tests indicates a negative impact of freshwater on the survival of *T. maritimum* strain TmarCan15-1. Moreover, the freshwater treatment conducted in the bath challenge trial 1, further supports a positive effect of freshwater treatment against *T. maritimum* infections. The reason may be that freshwater reduces the ability of the *T. maritimum* strain TmarCan15-1 to induce disease as adjustments in salinity have shown to reduce columnaris disease caused by *Flavobacterium columnare* (Altinok & Grizzle, 2001).

The *p*-value of the freshwater treated (tank-7 and tank-8) vs non-treated (tank-5 and tank-6) in bath challenge trial 1, was found to be $p < .057$. This is only slightly over the standard threshold for *p*-value (standard *p*-value is $p < .05$). The *p*-value for statistically significant difference is a conservative value that is publicly accepted. The *p*-value of $p < .057$, may demonstrate a positive trend of freshwater treatment on *T. maritimum* infected fish which were newly smoltified and transfer to seawater. In addition, the freshwater used as treatment in the freshwater treatment part of bath challenge trial 1 came from the same water source as the freshwater in the *in vitro T. maritimum* freshwater survival test. However, the freshwater used to treat fish in the freshwater treatment part of bath challenge trial 1 was not autoclaved and thus indicates that the salinity affects the pathogenicity of *T. maritimum* regardless of the biologic material found in freshwater.

4.3. Smolt production strategies and mitigation measures to reduce *Tenacibaculum maritimum* infections in smolts after seawater entry

Results from the current study indicates a positive effect of keeping smolts in LSS for 8 weeks prior to transfer to seawater in reducing mortality due to *T. maritimum* infections. A recent study conducted by Ytrestøyl *et al.*, (2020) suggests, in terms of fish performance, that the use of brackish water (12 ‰) combined with moderate exercise in RAS improved growth, utilization of feed and survival of smolts making the fish more robust. At 32 ‰, reduced skin

quality was reported as a negative influenced welfare factor. This indicates that there may be advantageous to keep the fish at 26 ‰ rather than 32 ‰, as a reduced skin quality likely would increase the risk of *T. maritimum* infection.

In field, the reported weight for when smolts are most susceptible towards mouthrot is from 100 g up to approximately 500g (Frisch, 2018). By using a production strategy that is based on large smolt (450 gram) production in RAS and using LSS (26 ‰) prior to seawater transfer could reduce the probability of a severe mouthrot outbreak. It would also decrease the period in which the fish are at their most susceptible to the disease (Frisch, 2018). The sum of all factors contributing to better fish welfare and robustness in general, would likely result in less mouthrot and fewer antibiotic treatments. This would not only be beneficial for reducing costs and increase fish performance, but it would also be beneficial in terms of a better public perception of the industry. In addition, the results from the freshwater treatment in bath challenge trial 1, demonstrates a reducing effect on *T. maritimum* infections and an increased chance of survival, which also would contribute to fewer antibiotic treatments during the seawater phase due to mouthrot. This coincides with the interest of the Canadian government that aims to minimize the environmental impact of fish farming by reducing antibiotics treatments (DFO, 2019).

5. CONCLUSION AND FUTURE PERSPECTIVES

The current study shows a statistically significant reducing effect of keeping smolts in LSS for 8 weeks prior to seawater transfer in reducing the mortality and severity of *T. maritimum* infections. The mortality results from the bath challenge trial 3 in the current study also demonstrate that the fish kept in LSS for 8 weeks after smoltification prior to seawater transfer, are more robust compared to newly smoltified fish. In addition, freshwater treatment shows a trend in reducing *T. maritimum* infections. This study will demonstrate that measurements can be applied to reduce the use of antibiotic treatments against mouthrot by altering the current smolts production process.

Investigating the effect of keeping smolts on different salinities and time periods after smoltification prior to seawater transfer would be an interesting future perspective in terms of fish robustness against mouthrot. In terms of production cost, it would be beneficial to compare the susceptibility to *t. maritimum* infection of fish kept on LSS for 4 weeks before transfer to seawater and the susceptibility to *T. maritimum* infection of fish kept on LSS for 8 weeks before transfer to seawater. Moreover, future studies should focus on investigation the effect of using freshwater as a treatment measure against mouthrot. This should ideally be performed on fish that have been kept in LSS prior to seawater transfer since this study have demonstrated that the LSS fish is more robust against *T. maritimum* infection.

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APPENDIX 1

Recipes

Cermaq Marine Broth (CMB)

- 37.2 g Coral Pro Salt (Red Sea)
- 5.0 g Peptone from animal tissue
- 1.0 g Yeast extract
- 1000 mL Milli-q water
- Needs to be autoclaved before inoculation

Cermaq Marine Blood Agar (BAMA)

- 37.2 g Coral Pro Salt (Red Sea)
- 15.0 g Bacteriological agar
- 5.0 g Peptone from animal tissue
- 1.0 g Yeast extract
- 950 mL Milli-q water
- 50mL Sterile defibrinated sheep blood
- Needs to be autoclaved and cooled off to 50 ^{o}C before adding sheep blood.
- Solidify for 30 minutes under UV-light.

Cermaq Kanamycin Marine Blood Agar (KA-BAMA)

- 37.2 g Coral Pro Salt (Red Sea)
- 15.0 g Bacteriological agar
- 5.0 g Peptone from animal tissue
- 1.0 g Yeast extract
- 950 mL Milli-q water
- 50mL Sterile defibrinated sheep blood
- Needs to be autoclaved.
- When cooled off to 60 ^{o}C – 70 ^{o}C add 50mg/L Kanamycin.
- When cooled off to 50 ^{o}C add sheep blood.
- Solidify for 30 minutes, do not use UV-light.

Difco 2216 Marine Broth (MB)

- 37.4 g Marine broth powder
- 1000 mL Milli-q water
- Needs to be autoclaved before inoculation

1% Agarose Gel

- SeaKem® LA Agarose (Cambrex) dissolved in 400 ml 1x TAE-buffer.
- Heat in microwave oven and store at 60 ^{o}C.

APPENDIX 2

Reagents

Reagents	Producer
Absolute ethanol	VWR®
Bacteriological agar	Sigma-Aldrich®
Chloroform	Sigma-Aldrich®
Coral Pro Salt	Red Sea
DNase/RNase-free water	Sigma-Aldrich®
Isopropanol	Sigma-Aldrich®
Kanamycin	Sigma-Aldrich®
Marine Broth	Difco™
Nuclease-Free water	Appliedbiosystems
Peptone from animal tissue	Sigma-Aldrich®
Real-time RT-PCR Primers/Probes	Invitrogen
Sheep Blood Defibrinated	ThermoFisher Scientific
Tri Reagent®	Sigma-Aldrich®
10X Extra buffer	VWR®
25X RT-PCR Enzyme	Ambion®

APPENDIX 3

Protocols

ExoSAP-IT

- The PCR cleanup protocol was followed.
- ExoSAP-IT reagent was removed from freezer and kept on ice during the procedure.
- 2.5 µL post-PCR reaction product was mixed with 1 µL of ExoSAP-IT reagent for a combined 3.5 µL reaction volume.
- Incubated at 37 ^{o}C for 15 minutes to degrade remaining primers and nucleotides.
- Incubated at 80 ^{o}C for 15 minutes to inactivate ExoSAP-IT reagent.
- The PCR product is ready for DNA sequencing.

Trizol extraction protocol from Sigma-Aldrich®

- Step 1-1 (Homogenizing samples) and step 2 (Phase separation) under sample preparation were followed, and step 1-3 under RNA Isolation were followed.

APPENDIX 4

A) PCR Results

Table I - Table shows the isolate isolated from tank which displayd bacterial growth on the agar plates, the primer pair, annealing temperature in degrees Celsius, the elongation time in minutes and whether the sequencing was positive or negative.

Tank number	Isolate name	Primer pair	Annealing	Elongation	Sequencing
1	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive
2	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive
3	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive
5	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive
6	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive
7	TmarCan15-1	*dnaK-F/dnaK-R*	55 ºC	1.00	Positive

B) Sequencing

Table II - Table shows how the bacterial sequences re-isolated from each tank match to each other and how they match with the reference gene sequence (The L293-2 dnaK gene sequence). Values are stated in %.

	Tank-2	Tank-3	Tank-5	Tank-6	Tank-7	Tank-1	L293-2 *dnaK*
Tank-2	100	100	100	100	100	100	100
Tank-3		100	100	100	100	100	100
Tank-5			100	100	100	100	100
Tank-6				100	100	100	100
Tank-7					100	100	100
Tank-1						100	100
L293-2 *dnaK*							100

C) Gel-electrophoresis

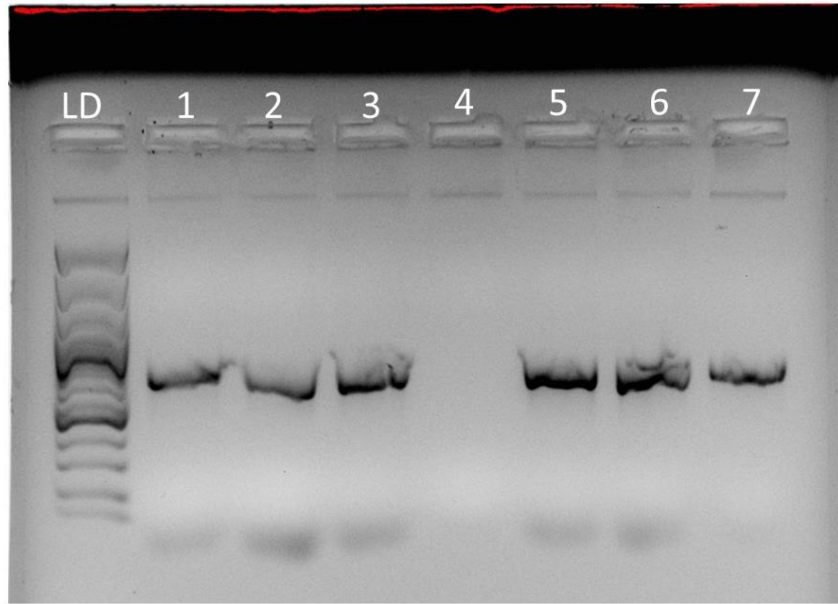

Figure I - Figure displays the result of the gel-electrophoresis conducted on the bacteriology from tank-1 - tank-7 in bath challenge 3. Numbers correspond with what tank the bacteria were isolated from, LD: ladder. Only the positive wells (1 – 3 and 5 – 7) were selected for sequencing (no amplification was shown for the non-*T. maritimum* like bacteria).

APPENDIX 5

Supplementary data for Real-time RT-PCR analysis of Kidney Tissue

Table III - The table displays results of the real-time RT-PCR from kindey tissue of each fish from low salinity seawater (LSS) in tank-1 and tank-2 in bath challenge study 3. K refers to tank number, F refers to fish number. Fish inside a red square are fish sampled before termination date of bath challenge study 3. The Ct-values of each fish for the qPCR_Tmar_MAR (green column), qPCR_Tmar_*OmpA* (yellow column) and EF1A (orange column) assays are all included.

LSS									
Tmar_16S		*OmpA*		EF1A	Tmar_16S		*OmpA*		EF1A
Tank-1					Tank-2				
K1F1	24,2	K1F1	35,2	13,1	K2F1	23,4	K2F1	37,8	13,1
K1F2	26,3	K1F2		13,8	K2F2	22,8	K2F2	33,8	14,3
K1F3	25,7	K1F3		13,5	K2F3	24,9	K2F3	37,2	12,6
K1F4	28,5	K1F4	36,2	13,6	K2F4	24,6	K2F4		13,8
K1K5	31,4	K1K5		11,2	K2F5	27,1	K2F5		12,9
K1F6	32,8	K1F6		13,4	K2F6	30,2	K2F6		12,5
K1F7	29,3	K1F7		14,6	K2F7	30,4	K2F7		14,6
K1F8	31,0	K1F8		13,3	K2F8	32,8	K2F8		14,6
K1F9	31,3	K1F9		13,4	K2F9	33,5	K2F9	38,1	15,9
K1F10	34,0	K1F10		13,6	K2F10	31,4	K2F10		14,6
K1F11	32,4	K1F11		14,5	K2F11	20,4	K2F11	33,2	14,0
K1F12	30,5	K1F12		15,3	K2F12	32,3	K2F12		14,9
K1F13	30,1	K1F13		15,1	K2F13	31,8	K2F13		13,3
K1F14	31,5	K1F14		14,1	K2F14	34,5	K2F14		13,7
K1F15	30,0	K1F15		13,7	K2F15	29,9	K2F15		14,6
K1F16	29,2	K1F16		14,1	K2F16	30,0	K2F16		14,5
K1F17	32,1	K1F17		14,3	K2F17	32,0	K2F17		15,4
K1F18	30,0	K1F18		14,6	K2F18		K2F18	38,4	15,7
K1 F19	31,0	K1 F19		15,3	K2F19	33,2	K2F19		14,7
K1F20	29,9	K1F20		14,9	K2F20	29,5	K2F20		14,0
RK	36,7	RK		36,6	K2F21	31,8	K2F21		15,3
RK	35,7	RK		33,9	RK		RK		34,3
NTC		NTC			NTC		NTC		35,4
NTC		NTC			NTC		NTC		

Table IV - The table displays results of the real-time RT-PCR from kindey tissue of each fish from low salinity seawater (LSS) in tank-3 and tank-4 (control) in bath challenge study 3. K refers to tank number, F refers to fish number. Fish inside a red square are fish sampled before termination date of bath challenge study 3. The Ct-values of each fish for the qPCR_Tmar_MAR (green column), qPCR_Tmar_*OmpA* (yellow column) and EF1A (orange column) assays are all included.

LSS									
Tmar_16S		*OmpA*		EF1A	Tmar_16S		*OmpA*		EF1A
Tank-3					Tank-4				
K3F1	25,2	K3F1	36,7	13,3	F1K4		F1K4		15,7
K3F2	25,9	K3F2	37,9	14,1	F2K4		F2K4		15,3
K3F3	24,2	K3F3	38,0	14,0	F3K4	35,7	F3K4		14,6
K3F4	24,6	K3F4	35,8	14,2	F4K4	36,1	F4K4		14,7
K3F5	34,2	K3F5			F5K4	36,8	F5K4		13,1
K3F6	34,8	K3F6		13,9	F6K4	36,4	F6K4		15,4
K3F7	30,5	K3F7		13,8	F7K4	36,6	F7K4		15,7
K3F8	35,8	K3F8		13,8	F8K4		F8K4		16,3
K3F9	34,1	K3F9		15,0	F9K4		F9K4		16,9
K3F10	35,1	K3F10	38,4	14,8	F10K4		F10K4		15,4
K3F11		K3F11		14,4	F11K4		F11K4		16,9
K3F12		K3F12		14,3	F12K4		F12K4		15,8
K3F13	29,5	K3F13	37,5	14,8	F13K4	37,0	F13K4		14,4
K3F14	34,6	K3F14		14,3	F14K4	35,1	F14K4		14,5
K3F15	34,8	K3F15		14,7	F15K4	35,2	F15K4		15,6
K3F16		K3F16		15,0	F16K4	32,4	F16K4		16,0
K3F17	34,1	K3F17		14,7	F17K4		F17K4		15,4
K3F18	33,4	K3F18		14,4	F18K4		F18K4		14,9
K3F19	30,9	K3F19		14,7	F19K4		F19K4		16,1
K3F20	26,1	K3F20		13,6	F20K4	37,1	F20K4		15,1
RK		RK		35,2	RK1		RK1		37,5
RK		RK		36,6	RK2		RK2		35,3
NTC		NTC			NTC		NTC		
NTC		NTC			NTC		NTC		

Table V - The table displays results of the real-time RT-PCR from kindey tissue of each fish from freshwater (FW) in tank-5 and tank-6 in bath challenge study 3. K refers to tank number, F refers to fish number. Fish inside a red square are fish sampled before termination date of bath challenge study 3. The Ct-values of each fish for the qPCR_Tmar_MAR (green column), qPCR_Tmar_*OmpA* (yellow column) and EF1A (orange column) assays are all included.

FW									
Tmar_16S		*OmpA*		EF1A	Tmar_16S		*OmpA*		EF1A
Tank-5					Tank-6				
K5F1	24,7	K5F1		14,1	F1K6	13,4	F1K6	27,1	16,0
K5F2	15,4	K5F2	28,5	13,1	F2K6	17,7	F2K6	33,7	14,7
K5F3	19,0	K5F3	31,5	14,3	F3K6	23,5	F3K6	34,9	16,0
K5F4	20,8	K5F4	33,1	15,0	F4K6	24,7	F4K6	35,6	15,8
K5F5	26,2	K5F5	38,0	13,9	F5K6	24,5	F5K6	36,9	
K5F6	24,1	K5F6		14,3	F6K6	24,4	F6K6	34,5	14,6
K5F7	23,2	K5F7	34,3	13,2	F7K6	22,9	F7K6	33,6	13,4
K5F8	24,9	K5F8		13,5	F8K6	22,7	F8K6	36,2	14,5
K5F9	23,9	K5F9	36,7	13,6	F9K6	26,2	F9K6	38,1	15,0
K5F10	22,6	K5F10	36,9	14,2	F10K6	22,9	F10K6	38,1	16,0
K5F11	27,8	K5F11	38,2	12,7	F11K6	22,5	F11K6	36,0	16,9
K5F12	20,3	K5F12	33,4	12,3	F12K6	22,0	F12K6	36,3	13,2
K5F13	23,1	K5F13	38,0	13,0	F13K6	16,6	F13K6	28,8	14,8
K5F14	25,0	K5F14	37,0	12,7	F14K6	23,0	F14K6	36,3	17,0
K5F15	25,0	K5F15	35,1	13,1	F15K6	25,7	F15K6		20,1
K5F16	31,3	K5F16	38,4	13,6	F16K6	26,1	F16K6	37,9	16,9
K5F17	31,9	K5F17	38,3	14,7	F17K6	30,2	F17K6		16,2
K5F18	33,3	K5F18	38,8	13,3	F18K6	30,8	F18K6		15,2
K5F19	30,2	K5F19		13,2	F19K6	30,6	F19K6		15,7
K5F20	33,2	K5F20		14,4	F20K6	30,2	F20K6		15,5
RK	33,1	RK		36,6	RK1	33,2	RK1		
RK	35,9	RK			RK2	34,8	RK2		38,2
NTC		NTC			NTC		NTC		
NTC		NTC			NTC	36,9	NTC		

Table VI - The table displays results of the real-time RT-PCR from kindey tissue of each fish from freshwater (FW) in tank-7 and tank-8 (control) in bath challenge study 3. K refers to tank number, F refers to fish number. Fish inside a red square are fish sampled before termination date of bath challenge study 3. The Ct-values of each fish for the qPCR_Tmar_MAR (green column), qPCR_Tmar_*OmpA* (yellow column) and EF1A (orange column) assays are all included.

FW										
Tmar_16S		*OmpA*		EF1A	Tmar_16S		*OmpA*		EF1A
Tank-7					Tank-8				
F1K7	22,7	F1K7	36,7	14,7	F1K8		F1K8		15,8
F2K7	25,0	F2K7		15,3	F2K8	35,1	F2K8		15,5
F3K7	25,7	F3K7	37,8	15,2	F3K8	37,0	F3K8		17,3
F4K7	25,0	F4K7	38,0	14,7	F4K8		F4K8		14,9
F5K7	26,2	F5K7	40,1	14,5	F5K8	35,4	F5K8		15,4
F6K7	23,8	F6K7	37,9	14,1	F6K8		F6K8		15,5
F7K7	24,3	F7K7		15,2	F7K8		F7K8		15,7
F8K7	21,1	F8K7	33,8	13,4	F8K8	37,2	F8K8		15,0
F9K7	18,7	F9K7	31,9	14,0	F9K8	37,0	F9K8		14,6
F10K7	17,8	F10K7	33,0	13,7	F10K8	35,3	F10K8		16,8
F11K7	21,2	F11K7	32,8	13,9	F11K8	36,1	F11K8		17,5
F12K7	17,6	F12K7	30,6	13,7	F12K8		F12K8		15,3
F13K7	23,9	F13K7	35,0	14,1	F13K8	35,9	F13K8		17,0
F14K7	26,9	F14K7	38,0	14,4	F14K8	36,3	F14K8		16,3
F15K7	26,9	F15K7	38,0	17,9	F15K8		F15K8		17,2
F16K7	28,8	F16K7	38,6	20,1	F16K8		F16K8		17,2
F17K7	23,8	F17K7	36,3	15,0	F17K8	34,6	F17K8		18,0
F18K7	22,8	F18K7	35,4	15,0	F18K8	35,9	F18K8		16,6
F19K7	28,2	F19K7		14,9	F19K8		F19K8		17,0
F20K7	26,1	F20K7	36,3	13,9	F20K8	35,8	F20K8		15,8
RK11	35,5	RK11		36,9	F21K8		F21K8		16,2
RK12	36,4	RK12		35,7	RK11		RK11		
NTC11		NTC11			RK12		RK12		
NTC11		NTC11			NTC11		NTC11		

APPENDIX 6

Supplementary data for the calculation of *p*-value

Table VII - Table displays an overview of how the p-value was calculated with the use of a t-test (hypothesis test). It includes data (mortality) from both bath challenge trail 2 and bath challenge trial 3. First section (red line) is number of mortalities each day (day post exposure) from each tank from both bath challenge trial 2 and 3. Section 2 (green line) displays the cumulative number of mortalities per day for each tank in bath challenge trial 2 and 3. Section 3 (blue line) shows cumulative mortality in % for each tank from bath challenge trial 2 and 3. Last section (yellow line) displays the calculations conducted by the formulas applied in the t-test which is represented by a p-value.

RAW DATA FROM TRIALS — Number of mortalities each day from trials

Tank number	Trial	Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
5	2	FW								1					1			1								
6	2	FW																								
7	2	FW																2								
8	2	Control																								
1	2	LSS																								
2	2	LSS																								
3	2	LSS																								
4	2	Control																								
Tank 5	3	FW			1		3	4	2	2		1		1					1							
Tank 6	3	FW			1		4	2	2	3	4															
Tank 7	3	FW			5	3	5	2	3						1	1										
Tank 8 (control)	3	Control																								
Tank 1	3	LSS							1	1									1							
Tank 2	3	LSS				1			1	1	1		1													
Tank 3	3	LSS					1				2	1														
Tank 4 (control)	3	Control																								

Raw data - cumulative — Cumulative number of mortalities per day in trials

Tank number	Trial	Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	2	FW	0	0	0	0	0	0	0	1	1	1	1	1	2	2	2	3	3	3	3	3	3	3	3	3
2	2	FW	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	2	FW	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2
4	2	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	2	LSS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	2	LSS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	2	LSS	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	2	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tank 1	3	FW	0	0	1	1	4	8	10	12	12	13	13	14	14	14	14	14	15	15	15	15	15	15	15	15
Tank 2	3	FW	0	0	1	1	5	7	9	12	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Tank 3	3	FW	0	0	5	8	13	15	18	18	18	18	18	18	19	20	20	20	20	20	20	20	20	20	20	20
Tank 4 (control)	3	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tank 5	3	LSS	0	0	0	0	0	0	1	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	3	3
Tank 6	3	LSS	0	0	0	1	1	1	2	3	4	4	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Tank 7	3	LSS	0	0	0	0	1	1	1	1	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Tank 8 (control)	3	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

STATISTICAL ANALYSIS DATA SET — Cumulative mortality (number of dead / total number) in%

Tank number	Trial	Group	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	2	FW	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,04	0,04	0,04	0,04	0,09	0,09	0,09	0,13	0,13	0,13	0,13	0,13	0,13	0,13	0,13	0,13
2	2	FW	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
3	2	FW	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,09
4	2	Control	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
5	2	LSS	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
6	2	LSS	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
7	2	LSS	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
8	2	Control	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
1	3	FW	0,00	0,00	0,04	0,04	0,17	0,35	0,43	0,52	0,52	0,57	0,57	0,61	0,61	0,61	0,61	0,61	0,65	0,65	0,65	0,65	0,65	0,65	0,65	0,65
2	3	FW	0,00	0,00	0,04	0,04	0,22	0,30	0,39	0,52	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70	0,70
3	3	FW	0,00	0,00	0,22	0,35	0,57	0,65	0,78	0,78	0,78	0,78	0,78	0,78	0,83	0,87	0,87	0,87	0,87	0,87	0,87	0,87	0,87	0,87	0,87	0,87
4	3	Control	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
5	3	LSS	0,00	0,00	0,00	0,00	0,00	0,00	0,04	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,09	0,13	0,13	0,13	0,13	0,13	0,13	0,13	0,13
6	3	LSS	0,00	0,00	0,00	0,04	0,04	0,04	0,09	0,13	0,17	0,17	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22	0,22
7	3	LSS	0,00	0,00	0,00	0,00	0,04	0,04	0,04	0,04	0,13	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17
8	3	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Simple statistics for

			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Mean	2	FW	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,01	0,01	0,01	0,01	0,01	0,03	0,03	0,03	0,07	0,07	0,07	0,07	0,07	0,07	0,07	0,07	0,07
Mean	2	LSS	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Ttest	2		#####	#####	#####	#####	#####	#####	#####	0,3739	0,3739	0,3739	0,3739	0,3739	0,3739	0,3739	0,3739	0,1318	0,1318	0,1318	0,1318	0,1318	0,1318	0,1318	0,1318	0,1318
Mean	3	FW	0,00	0,00	0,10	0,14	0,32	0,43	0,54	0,61	0,67	0,68	0,68	0,70	0,71	0,72	0,72	0,72	0,74	0,74	0,74	0,74	0,74	0,74	0,74	0,74
Mean	3	LSS	0,00	0,00	0,00	0,01	0,03	0,03	0,06	0,09	0,13	0,14	0,16	0,16	0,16	0,16	0,16	0,16	0,17	0,17	0,17	0,17	0,17	0,17	0,17	0,17
Ttest	3		#####	#####	0,16	0,27	0,08	0,02	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,0013

APPENDIX 7

Supplementary mortality data

Table VIII – Table displays the total cumulative mortality in percent for the tanks in the LSS group and the FW group in bath challenge trial 2.

BATH CHALLENGE TRIAL 2	
Tank number	**Total cumulative percent mortality**
LOW SALINITY SEAWATER	
Tank-1	0 %
Tank-2	0 %
Tank-3	0 %
Tank-4	0 %
FRESHWATER	
Tank-5	15 %
Tank-6	0 %
Tank-7	10 %
Tank-8	0 %

Table IX - Table displays the total cumulative mortality in percent for the tanks in the LSS group and the FW group in bath challenge trial 3.

BATH CHALLENGE TRIAL 3	
Tank number	**Cumulative percent mortality**
LOW SALINITY SEAWATER	
Tank-1	15 %
Tank-2	24 %
Tank-3	20 %
Tank-4	0 %
FRESHWATER	
Tank-5	75 %
Tank-6	80 %
Tank-7	100 %
Tank-8	0 %

APPENDIX 8

Calculation of the bath challenge bacterial concentration

The Bacterial bath concentration was calculated for each bath challenge trial using the following formula:

$$\frac{\boldsymbol{Bacterial\ cell\ concentration}\left(\frac{cfu}{mL}\right) * \boldsymbol{Bacterial\ bath\ volume}\ (mL)}{\boldsymbol{Bath\ volume}\ (L) * \mathbf{1000}}$$

Bacterial cell concentration: bacterial cell concentration for the optimum hours post inoculation to infect the fish from the MPN measurements.

Bacterial bath volume: how large volume of bacterial culture to be added.

Bath volume: the volume of the infection containers which the fish were kept inn during exposure (multiplied by 1000 to obtain value in mL).