Mouthrot in farmed Atlantic salmon

Kathleen Frisch

Thesis for the Degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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SCIENTIFIC ENVIRONMENT

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"Somewhere, something incredible is waiting to be known." ~ Carl Sagan

ABSTRACT

Mouthrot is a major health and welfare problem in farmed Atlantic salmon (*Salmo salar*) smolts in the Pacific Northwest (West Coast of North America), particularly in the first few months following saltwater transfer. This disease, associated with the bacterium *Tenacibaculum maritimum*, is the main reason the Atlantic salmon farming industry in this region continues to use antibiotics. Mouthrot results in large economic losses due to direct fish mortality, as well as the cost of treatments and poor performance of treated fish. Affected smolts die with very little external or internal clinical signs other than characteristic small yellow plaques in the mouth. This clinical presentation is visibly different to that of tenacibaculosis, the disease commonly associated with *T. maritimum* in other regions of the world or in other marine fish species. *T. maritimum* is the most extensively studied member of the *Tenacibaculum* genus; however, its role in causing mouthrot in British Columbia (BC) has not been the focus.

The main objective of this study was to gain more knowledge about *T. maritimum* in BC and its connection to mouthrot in the Pacific Northwest, and to make steps towards developing management tools that would help decrease the use of antibiotic treatments and improve fish welfare.

Genotyping of *T. maritimum* isolates collected from natural outbreaks of mouthrot on Atlantic salmon farms in BC showed the presence of two genetic strains of the bacterium based on 11 housekeeping genes. These strains are most closely related genetically to strains collected from lumpsuckers (*Cyclopterus lumpus*) with skin lesions and Atlantic salmon in Norway, as well as Atlantic salmon gills in Chile. The division of the BC isolates into two genetic groups is further supported by a serological analysis that showed that there are two serological groups that match the genetic strains.

Representative isolates from the two identified BC genetic strains were used to develop a bath challenge model with Atlantic salmon smolts, which is necessary for testing

management tools such as treatments and vaccines. Through these experiments, it was demonstrated that *T. maritimum* is the causative agent of mouthrot in BC without the need for other stressors or co-infections. The main pathology in Atlantic salmon smolts infected with BC *T. maritimum* strains are mouth lesions that damage the tissues surrounding the teeth causing a disease that is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with very little associated inflammation. A cohabitation experiment also showed that *T. maritimum* readily transfers from infected smolts to naïve ones. As mouthrot mainly affects smolts, further studies are needed to investigate the link between smolt status and susceptibility to developing mouthrot.

With the knowledge gained from the genetic characterisation and serological analysis of the BC *T. maritimum* isolates, whole cell inactivated adjuvanted vaccines were created and tested using the developed challenge model. Despite giving an antibody response in immunised fish, the vaccines did not protect the smolts against mouthrot induced through a bath infection. Future research needs to focus on preventative tools, including other types of vaccines such as immersion or live-attenuated. This will require a better understanding of the pathogenesis of mouthrot.

TABLE OF CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, hereafter referred to in the text by their Roman numerals:

PAPER I

Frisch, K., Småge, S.B., Brevik, Ø.J., Duesund, H., Nylund, A., 2017. Genotyping of *Tenacibaculum maritimum* isolates from farmed Atlantic salmon in Western Canada. Journal of Fish Diseases 41, 131-137.

PAPER II

Frisch, K., Småge, S.B., Vallestad, C., Duesund, H., Brevik, Ø.J., Klevan, A., Olsen, R.H., Sjaatil, S.T., Gauthier, D., Brudeseth, B., Nylund, A., 2018. Experimental induction of mouthrot in Atlantic salmon smolts using *Tenacibaculum maritimum* from Western Canada. Journal of Fish Diseases 41, 1247-1258.

PAPER III

Frisch, K., Småge, S.B., Johansen, R., Duesund, H., Brevik, Ø.J., Nylund, A., 2018. Pathology of experimentally induced mouthrot caused by *Tenacibaculum maritimum* in Atlantic salmon smolts. (*manuscript, submitted for publication*)

KEY DEFINITIONS AND ABBREVIATIONS

Mouthrot: bacterial stomatitis caused by *Tenacibaculum maritimum* in farmed Atlantic smolts of the Pacific Northwest and characterised by the presence of small yellow plaques in the mouth and no other internal or external clinical signs

Tenacibaculosis: infection caused by *Tenacibaculum* spp. (generally *T. maritimum* or *T. finnmarkense*) in a number of marine fish species characterised by frayed fins, tail rot, mouth erosion and skin lesions/ulcers

BAS	Blood agar with added 1.5-2.0 % NaCl
BC	British Columbia
ECP	extracellular products
ELISA	Enzyme-linked immunosorbent assay
FMM	*Flexibacter* maritimus medium
HK	Housekeeping
IP	Intra-peritoneal
MA	Marine agar (Difco 2216)
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
PCR	Polymerase chain reaction
ST	Sequence type
sp.	Species (singular)
spp.	Species (plural)
T9SS	Type IX secretion system

1. INTRODUCTION

1.1 Aquaculture on the West Coast of North America

Salmon farming on the West Coast of North America (Pacific Northwest) started in the 1970s when wild salmon numbers began to diminish. The industry began with the raising of native Coho (O*ncorhynchus kisutch*) and Chinook (*Oncorhynchus tshawytscha*) salmon that were already being produced by fisheries enhancement hatcheries. Over the following decades, the farming focus shifted to Atlantic salmon (*Salmo salar*) due to its better suitability for domestication and because it grows faster than the Pacific species of salmon (Olin, 2012). The main species farmed is now Atlantic salmon in open net-pens; concurrently, Chinook and Coho salmon continue to be farmed on a much smaller scale. Canada is the fourth largest producer of farmed salmon in the world, after Norway, Chile and the UK, with British Columbia (BC) representing over 75% of the total production in the country (about 80,000 tonnes annually) (Statistics Canada, 2017). It is the largest agri-food export from BC, with the United States being the largest market, and is a significant economic contributor to coastal and rural communities (Fisheries and Oceans Canada, 2016). The distribution of saltwater farms in the Pacific Northwest can be seen in **Figure 1**.

1.1.1 Fish health of farmed salmon in the Pacific Northwest

Unlike many Atlantic salmon regions, the Pacific Northwest has very few marine pathogens that impact the industry. Endemic diseases such as furunculosis (*Aeromonas salmonicida*), vibriosis (*Vibrio anguillarum*) and infectious haematopoietic necrosis (*Salmonid novirhabdovirus*) are well controlled through the use of effective vaccines (Kent, 1992; Traxler et al., 1999). Viral haemorrhagic septicaemia (*Piscine novirhabdovirus*) and bacterial kidney disease (*Renibacterium salmoninarum*) are also endemic but are well managed through screening of broodstock and/or good management practices; and therefore, account for only a small percentage of the overall losses during the saltwater phase of production.

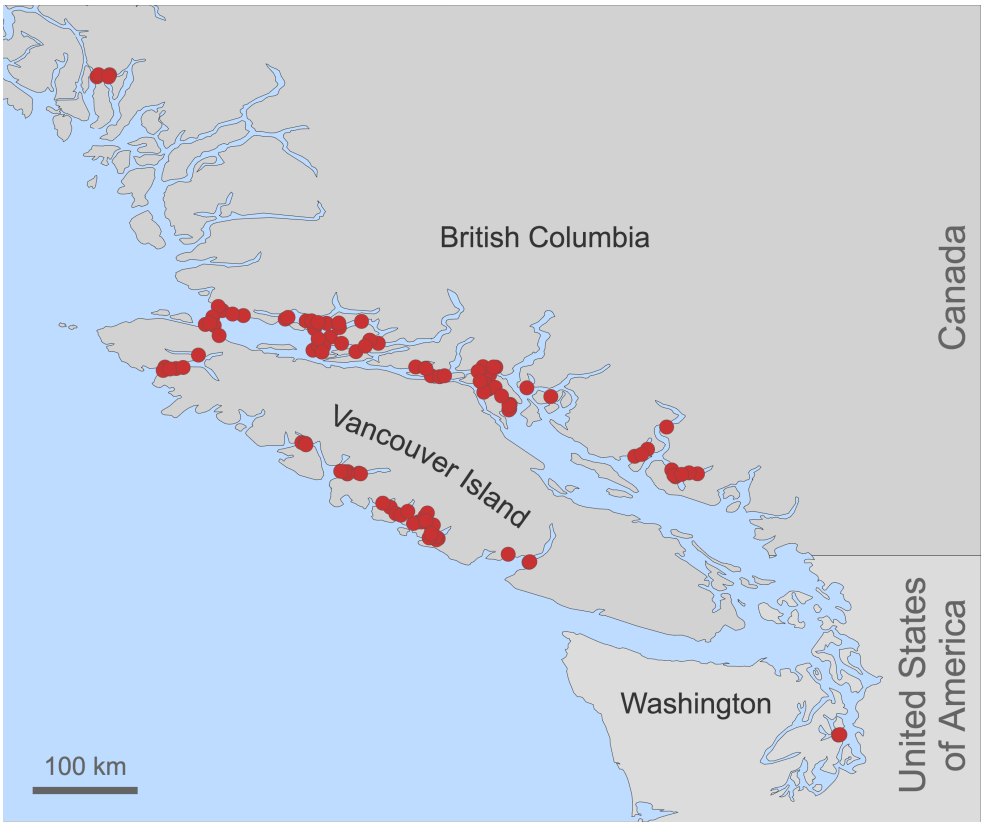

Figure 1 – Map of British Columbia, Canada, and Washington State, USA, showing salmon farm locations (red dots).

The largest cause of mortality to farmed salmon in the region are harmful algal blooms that have been occurring since the start of the industry (Haigh et al., 2014). As a result, extensive monitoring programs have been put in place with mitigation methods to decrease economic losses. These include daily monitoring and identification of potential harmful plankton at every farm, as well as regional coordinated monitoring during riskier periods of the year.

Although fish health issues are fewer than in other farming regions, there are a small number of marine bacteria that continue to give rise to disease outbreaks. These include *Moritella viscosa*, the cause of winter ulcers, and *Tenacibaculum* spp. that have been associated with two types of clinical presentations. One type results in skin lesions/ulcers, mouth erosion, frayed fins and tail rot. The other, bacterial stomatitis or

mouthrot, causes lesions primarily in the mouth (Kent, 1992). These diseases can affect fish at any stage during the saltwater phase of the production cycle; however, mouthrot is more prevalent in smolts recently entered into saltwater, and lesions, due to either *Tenacibaculum* spp. or *M. viscosa*, or both, are a greater problem in harvest size fish due to significant economic losses as a result of downgrades at processing.

1.2 Mouthrot

Mouthrot is a significant fish welfare problem in the Pacific Northwest (Ostland et al., 1999). The disease has been identified in Atlantic salmon farms in the region as early as the late 80s and is generally associated with losses in smolts recently transferred into saltwater (Frelier et al., 1994). The impact on the industry is significant due to direct costs of mortality and antibiotic treatments, as well as indirect costs of loss of production from poor performance. Mouthrot has been reported to cause up to 15 % mortality (Ostland et al., 1999); however, due to the effectiveness of antibiotic

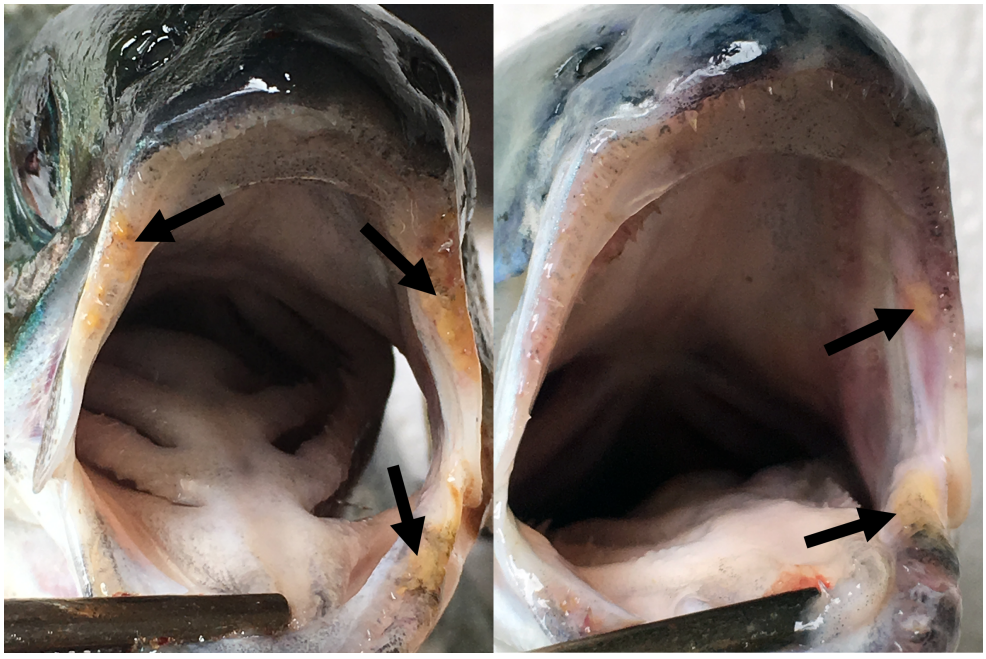

Figure 2 – Mouths from mouthrot affected smolts from an Atlantic salmon farm in BC showing the distinct yellow plaques (arrows).

treatments, mortality numbers are now much lower. At present, mouthrot accounts for around 1 to 3 % mortality during the saltwater phase of production with the majority occurring during the first 4 to 5 months in saltwater. Antibiotic treatments used to control the disease account for over 90 % of total use in the region (personal observations, Cermaq Canada). The continued use of antibiotics to treat this disease also impacts the acceptance of the industry to operate in the region by stakeholders and the general public.

Diseased fish during a mouthrot outbreak die with small yellow plaques in the mouth (**Figure 2**) and little or no other clinical signs both externally and internally (Frelier et al., 1994; Ostland et al., 1999). Occasionally, the characteristic yellow plaques are seen on the gills (**Figure 3**), a finding that has also been noted in Europe (Mitchell et al., 2011). The disease course in smolts is very rapid as surveys of the live population of a farm during an outbreak reveal a prevalence of clinical signs below 1 % (personal communication, Peter McKenzie).

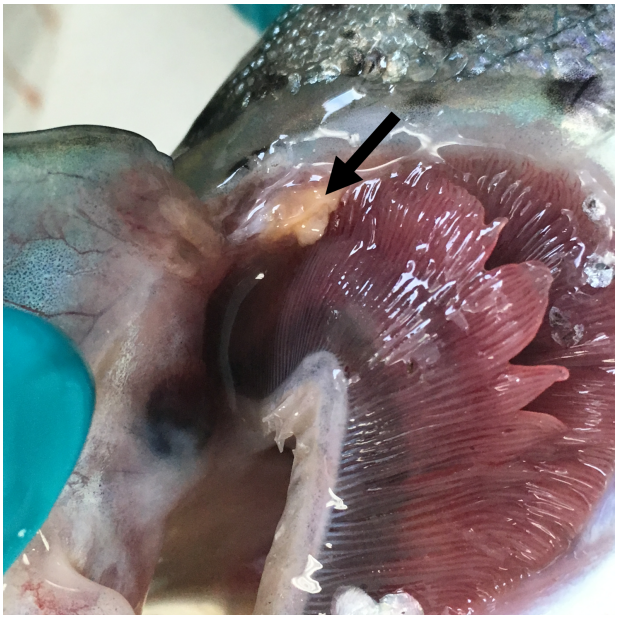

Figure 3 – Gills from a mouthrot affected smolt from an Atlantic salmon farm in BC showing a distinct yellow plaque (arrow).

The severity of a mouthrot outbreak is influenced by a number of factors that include smoltification quality, previous handling, and water quality parameters such as temperature, salinity and the presence of algal blooms (Frelier et al., 1994; Hewison et al., 2015). In general, susceptibility to outbreaks decreases after a certain amount of time in saltwater (3 to 6 months post-transfer); however there appears to be a trend

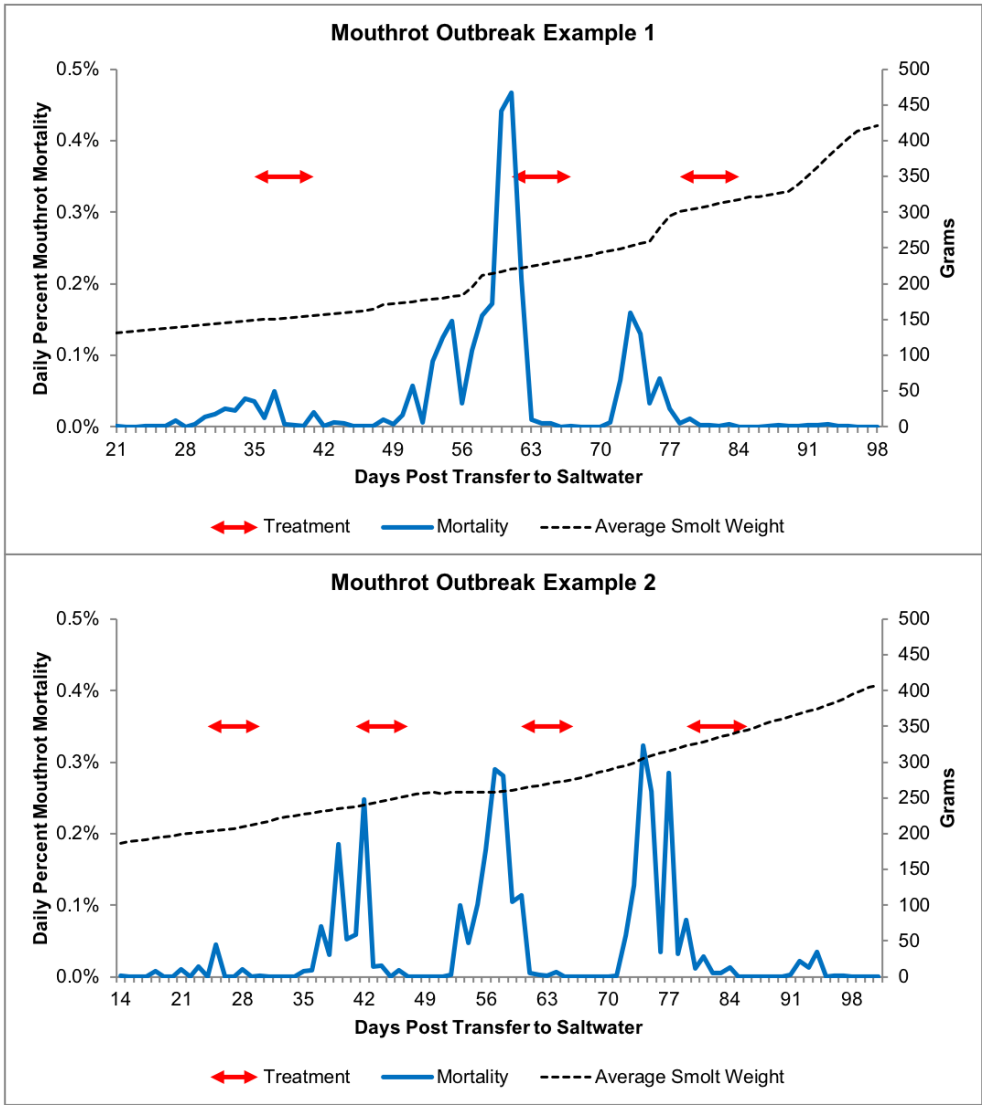

Figure 4 – Two examples of mouthrot outbreaks on BC Atlantic salmon farms showing the daily percent mortality attributed to mouthrot and the effect of treatments with florfenicol (7 days at 10 mg kg-1).

towards more severe cases with outbreaks occurring in larger and better quality fish in lower salinities, and lasting longer than historical cases (Hewison et al., 2015).

Mortalities showing clinical signs of mouthrot can occur as early as two days post-transfer to saltwater and most commonly occur within the first few weeks. **Figure 4** shows examples of mortality curves from typical outbreaks on Atlantic salmon farms in BC, showing repeated spikes in mortality and treatments with florfenicol (Aquaflor, Merck). Florfenicol is the most commonly used antibiotic for this purpose due to its palatability and low toxicity; however, its rapid metabolism by the fish means that a mouthrot outbreak often requires multiple treatments (Morrison et al., 2013).

The bacterium isolated from the mouth lesions of affected smolts is *Tenacibaculum maritimum* (Ostland et al., 1999) and is now known to be the causative agent of the disease (**Paper II**). Most commonly, *T. maritimum* is associated with tenacibaculosis, a disease characterised by ulcerative skin lesions, mouth erosion, frayed fins and tail rot (Toranzo et al., 2005). This is a disease that is clinically different from mouthrot as seen in BC and Washington state.

1.3 *Tenacibaculum maritimum*

Tenacibaculum maritimum (originally *Flexibacter maritimus*) is a marine gram negative bacterium in the family Flavobacteriaceae that was first identified in 1976 from diseased Japanese farmed juvenile red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegelii*) reared in seawater net-pens (Masumura et al., 1977; Wakabayashi et al., 1986; Suzuki et al., 2001). The pathogen has since been isolated from a large range of wild and farmed fish species all over the world (Table 1).

Table 1 – *T. maritimum*: geographic origin and host species identified to date.

Host Species	Country	Source
Asia:		
Acanthopagrus schlegelii - Black sea bream	Japan	(Masumura et al., 1977)
Lates calcarifer - Barramundi	Singapore	(Labrie et al., 2008)
Oplegnathus fasciatus - Rock bream	Japan	(Wakabayashi et al., 1986)

Host Species	Country	Source
Pagrus major - Red sea bream	Japan	(Masumura et al., 1977)
Paralichthys olivaceus - Olive flounder	Japan Korea	(Baxa et al., 1986) (Jang et al., 2009)
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	(Ministry for Primary Industries, 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)
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 Greece Italy Malta Turkey	(Bernardet et al., 1994) (Kolygas et al., 2012) (Salati et al., 2005) (Bernardet, 1998) (Yardımcı et al., 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 Norway Spain	(Downes et al., 2018) (PHARMAQ Analytiq, 2017) (Pazos et al., 1993)
S. maximus - Turbot	France Italy Norway Spain	(Habib et al., 2014) (Magi et al., 2007) (Olsen et al., 2017) (Alsina et al., 1993)
Solea senegalensis - Senegelese sole	Portugal	(Avendaño-Herrera et al., 2005)

Host Species	Country	Source
	Spain	(Cepeda et al., 2002)
Solea solea - Common sole	Netherlands Scotland Spain	(Habib et al., 2014) (Bernardet et al., 1990) (Avendaño-Herrera et al., 2004)
Sparus aurata - Gilt-head bream	Greece Italy Spain	(Kolygas et al., 2012) (Salati et al., 2005) (Avendaño-Herrera et al., 2004)
Africa:		
Cheilinus lunulatus - Broomtail wrasse	Egypt	(Abd El-Galil et al., 2012)
Neoglyphidodon melas - Black damsel	Egypt	(Abd El-Galil et al., 2011)
Rhinecanthus aculeatus - Picasso triggerfish	Egypt	(Abd El-Galil et al., 2011)

In most reported cases, the disease associated with *T. maritimum* results in ulcerative skin lesions, mouth erosions, frayed fins and tail rot, called tenacibaculosis (Toranzo et al., 2005). This type of clinical presentation is what has been reported in Atlantic salmon smolts in Tasmania, Australia (Carson et al., 1992). Outbreaks can result in significant economic losses and therefore limit the culturing of many commercially valuable marine fish species around the world (Avendaño-Herrera et al., 2006b). The bacterium has also been isolated from sea lice (*Lepeophtheirus salmonis*) found on farmed Atlantic salmon from BC (Barker et al., 2009), and has been detected through molecular testing from jellyfish found on the gills of farmed Atlantic salmon from the Shetland Islands (Ferguson et al., 2010). These organisms may be acting as vectors for the bacteria.

1.3.1 Phenotypic characterisation

The bacterium, *T. maritimum*, is a gram-negative long slender rod (0.5 μm by 2 to 30 μm) (**Figure 5**) that gets shorter and eventually spherical as cultures get older, is strictly aerobic, exhibits gliding motility without the presence of flagella, and only grows on agar containing sea water or a synthetic sea water (they do not grow on NaCl alone) (Avendaño-Herrera et al., 2006b). Colonies are pale-yellow and flat with uneven edges, and rarely exceed 5 mm in diameter (Wakabayashi et al., 1986). The optimal growth for *T. maritimum* has been published to be 30 ºC; however, bacterial growth can occur between 15 to 34 ºC and has been reported as low as 8 ºC (Suzuki et al., 2001; Småge et al., 2016). The bacterium is positive for oxidase and catalase, and negative for

flexirubin type pigments and the production of H_2S (Wakabayashi et al., 1986; Suzuki et al., 2001).

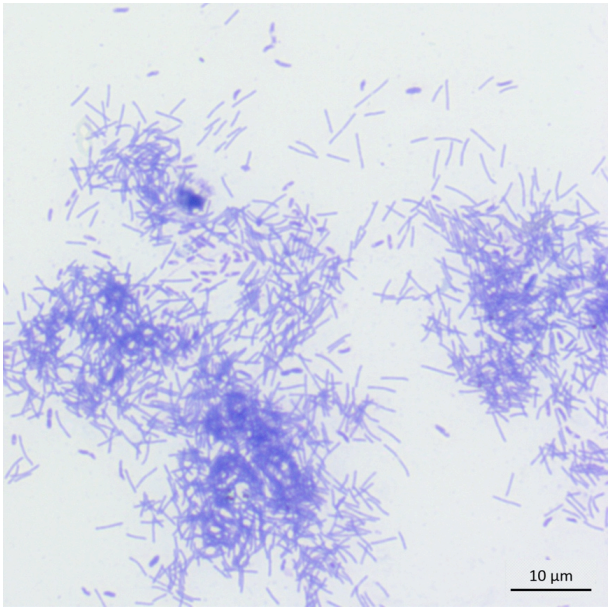

Figure 5 – Smear of a skin lesion on a diseased smolt showing a large amount of *T. maritimum*. (DifQuik stained)

1.3.2 Genotypic characterisation

Tenacibaculum maritimum is the type species of the *Tenacibaculum* genus, which is depicted in **Figure 6**. Two other species, *Tenacibaculum dicentrarchi* and *Tenacibaculum finnmarkense* have also been associated with disease in Atlantic salmon (Småge et al., 2015; Avendaño-Herrera et al., 2016; Småge et al., 2017; 2018). This latter one has been isolated, identified through 16S rRNA gene sequencing and connected to tenacibaculosis in BC (personal observations) (see section 4.1.2). A recent genome comparison between certain *Tenacibaculum* species (*T. maritimum*, *T. dicentrarchi*, *Tenacibaculum ovolyticum* and *T. soleae*) reveals significant differences between them, particularly with respect to virulence factors (Pérez-Pascual et al., 2017).

Figure 6 – Phylogenetic tree showing all the identified type species of *Tenacibaculum*. Two species of *Flexibacter* spp. are included due to their close genetic relationship to the *Tenacibaculum* genus. *Kordia algicida*^{T} (AB681152) is used as an outgroup. The tree was inferred using an alignment of 1348 base positions of the 16S rRNA gene from 33 taxa. The alignment was constructed in AlignX in Vector NTI (Invitrogen) and then adjusted to equal length in GeneDoc (Nicholas et al., 1997). The best fitted evolutionary model (GTR+G+I) was calculated in Mega6 (Tamura et al., 2013). A Bayesian analysis (relaxed lognormal molecular clock and a MCMC of 150,000,000 generations) was performed using BEAST (Drummond and Rambaut, 2007). Sample size values (ESS) were inspected using Tracer ver. 1.6 (Rambaut et al., 2014), and found to be above the recommended range for all parameters (> 200). A maximum clade credibility tree was obtained using a 10% burn-in in TreeAnnotator and viewed using FigTree (Drummond et al., 2012).

Molecular typing methods (e.g. multilocus sequence typing (MLST)) have led to the establishment of bacterial pathogen nomenclature schemes that are both uniform and reproducible, which give researchers the tools to perform epidemiological studies (Gevers et al., 2005). MLST characterises isolates within a microbial species using the allelic mismatch of a small number of housekeeping (HK) genes, and assigns these isolates a sequence type (ST). As these schemes are nucleotide sequence based, they are easy to transfer and reproduce in different laboratories (Maiden, 2006). The sequence data provided by MLST can be concatenated and used for phylogenetic analysis, called multilocus sequence analysis (MLSA). MLSA is the current method of choice to explore phylogenetic relationships at the species and subspecies levels (Gevers et al., 2005; Glaeser et al., 2015).

A number of *T. maritimum* STs have been described through MLSA and MLST using strains collected from a variety of host species worldwide (Habib et al., 2014) (HK genes listed in Table 2). The genetic data from this work is available through the *Tenacibaculum* MLST database (http://pubmlst.org/tenacibaculum/) and allow researchers to use and add to this work (Maiden, 2006), as was performed in **Paper I**. The population structure of *T. maritimum* suggests an endemic colonisation of fish farms by local strains with little or no indication of long distance contamination through fish movements (Habib et al., 2014). The same sequence type is found to infect multiple fish species in the same geographical area, which indicates cross-species contamination from the same bacterial lineage (Habib et al., 2014). However, this may not always be the case; for example, closely related strains can be found on the same host species (Atlantic salmon) in three different geographical regions (Norway, Western Canada and Chile) (**Paper I**).

1.3.3 Diagnosis

The identification of *T. maritimum* in BC is generally based on the observation of thin rod-shaped bacteria in wet mounts, and the colony and cell morphologies observed from cultures. Primary isolation of the bacterium requires sea salt in the growth medium and two agars are routinely used for this purpose: marine agar (MA) and

Flexibacter maritimus medium (FMM) (Pazos et al., 1996). It does not grow on blood agar that contains NaCl (BAS) that is traditionally used in routine diagnostics in salmon farming, and therefore may be the reason it has not been frequently recovered in BC. *T. maritimum* can be difficult to distinguish from other phenotypically similar bacteria, particularly from other yellow-pigmented Flavobacteriaceae (Suzuki et al., 2001; Toranzo et al., 2005). *T. maritimum* grows slower than other environmental bacteria such as *Vibrio* spp. commonly isolated from external lesions, which can make pure cultures from external lesions difficult to obtain (Pazos et al., 1996). To resolve this issue several authors have suggested adding antimicrobials compounds to the agar that select for *T. maritimum* (Baxa et al., 1986; Chen et al., 1995; Kolygas et al., 2012). The addition of kanamycin to the agar was used in this study (**Paper I**, **II**).

Nowadays, the most common and specific way to identify the species of bacterium from a pure culture is PCR and sequencing, and these methods have now nearly completely replaced the traditional biochemical tests for bacterial identification. *T. maritimum* is no exception and two primer sets based on the 16S rRNA gene have been developed to identify the species (Toyama et al., 1996) (Table 2). The identification is confirmed by sequencing the 16S rRNA gene and matched against reference gene sequences.

Table 2 – Primers used for PCR and sequencing of *T. maritimum*.

Gene	Primer	Sequence (5'-3')	Source
16S rRNA	MAR1	AATGGCATCGTTTTAAA	(Toyama et al., 1996)
	MAR2	CGCTCTCTGTTGCCAGA	
	Mar1	TGTAGCTTGCTACAGATGA	(Bader et al., 1998)
	Mar2	AAATACCTACTCGTAGGTACG	
atpA	forward	ATTGGWGAYCGTCAAACWGG	(Habib et al., 2014)
	reverse	CCAAAYTTAGCRAAHGCTTC	
dnaK	forward	GGWACYACNAAYTCDTGTGT	(Habib et al., 2014)
	reverse	TCWATCTTMGCTTTYTCAGC	
glyA	forward	CAYTTAACWCAYGGWTCDCC	(Habib et al., 2014)
	reverse	ACCATRTTTTTRTTTACHGT	
gyrB	forward	AGTATYCARGCRCTRGAAGG	(Habib et al., 2014)
	reverse	GTWCCTCCTTCRTGYGTRTT	
ileS	forward	CCWACHTTTGGWGCHGAYGA	(Habib et al., 2014)
	reverse	GAATCRAACCAWACATCAAT	

Gene	Primer	Sequence (5'-3')	Source
infB	forward	ATGCCDCAAACWAAAGARGC	(Habib et al., 2014)
	reverse	GTAATHGCTCCAACYCCTTT	
rlmN	forward	GCKTGTGTDTCDAGYCARGT	(Habib et al., 2014)
	reverse	CCRCADGCDGCATCWATRTC	
tgt	forward	GAAACWCCWATWTTYATGCC	(Habib et al., 2014)
	reverse	TAYAWYTCTTCNGCWGGTTC	
trpB	forward	GTWGCNCGWATGAAAATGYT	(Habib et al., 2014)
	reverse	CCWGGRTARTCYAATCCTGC	
tuf	forward	AGAGAWTTATTRTCTTTCTA	(Habib et al., 2014)
	reverse	GTTACCTGACCWGCWCCWAC	
yqfO	forward	GCBGAARRTTTTGAYAAYGT	(Habib et al., 2014)
	reverse	AYTTCRTARGCDACYTCTTC	

There are two published real-time RT-PCR assays for the detection of *T. maritimum*, both based on the 16S rRNA gene (Fringuelli et al., 2012; Fernández-Álvarez et al., 2018). However, the 16S rRNA gene has lower phylogenetic resolution than other genes at the species level (Janda et al., 2007), and assays based on this gene tend to be less specific than other assays. As a result, these assays were not used in this study (**Paper III**).

1.3.4 Pathology

Since its discovery, *T. maritimum* has been shown to be able to produce disease on its own in a number of fish species, including Atlantic salmon (Avendaño-Herrera et al., 2006b). The pathology associated with tenacibaculosis in marine fish has been fairly well described macroscopically and microscopically (Avendaño-Herrera et al., 2006b), and in general the disease is more severe in younger fish (Toranzo et al., 2005). In Tasmanian farmed Atlantic salmon, tenacibaculosis is predominantly associated with erosive lesions on the external surfaces (dorsal and lateral skin, gills, head and fins) with the most commonly affected area being the flank just behind the pectoral fins (Handlinger et al., 1997). Histologically, lesions are associated with bacterial mats of long thin rod-shaped *T. maritimum*-like bacteria. Destruction and loss of the epithelium with bacterial invasion of the underlying connective tissues is typical of the disease, and there are seldom any signs of inflammatory processes (Handlinger et al., 1997; van Gelderen et al., 2011).

There is scarce information on the pathology and pathogenesis of mouthrot in Atlantic salmon in the Pacific Northwest, which appears to be different from tenacibaculosis (see section 1.2). In this region, *T. maritimum* was thought to be a secondary invader, and would therefore require damaged tissue to invade (Ostland et al., 1999); however, the pathogen has been shown in this study to be able to induce mouthrot on its own in Atlantic salmon smolts (**Paper II**). Lesions of mouthrot affected smolts from the field are generally located in the oral cavity and usually involve the dentition and surrounding gingiva, and histopathology show changes in the mouth that may be similar to gingival diseases in humans (Frelier et al., 1994, **Paper III**). Interestingly, a pathology similar to mouthrot has been described in juvenile Atlantic cod (*Gadus morhua*) that the researchers called "yellow pest" (Hilger et al., 1991). The associated bacteria were not fully identified, but were described as *T. maritimum*-like; however, these isolates deviated phenotypically from *T. maritimum*.

1.3.5 Challenge model

As is the case with other fish pathogens of the Flavobacteriaceae family (Decostere et al., 2000), the main challenge in investigating *T. maritimum* is the difficulty in developing a reproducible bath challenge model. The most successful fish challenge models involve injecting fish with the causative pathogen as this provides more control of experimental conditions. Many experiments have been conducted in economically important fish species attempting to reproduce disease caused by *T. maritimum*. The injectable route has given repeatedly poor results, both intra-peritoneally (IP) and subcutaneously (Yamamoto et al., 2010; Faílde et al., 2014).

Using a bath infection model seems to be the most effective way to induce disease with *T. maritimum* with many attempts at optimising this method in a number of fish species (Avendaño-Herrera et al., 2006b). These studies have included scarification or abrasion pre-exposure to the bacteria that was subsequently proven not to be necessary (Baxa et al., 1987; Mabrok et al., 2016). A range of bath bacterial concentrations and exposure times have been tested, as well as varying water parameters including salinity and temperature (Avendaño-Herrera et al., 2006b). In Atlantic salmon in Tasmania,

researchers found that bath infecting smolts for one hour at a high concentration gave them the most replicable results (Soltani et al., 1996; Handlinger et al., 1997). This high dose bath model was used to bath infect smolts in this study (**Paper II**).

1.3.6 Treatments and vaccination

There is an increasing move towards non-therapeutic disease management in the aquaculture industry whenever possible. Non-therapeutic methods of managing mouthrot have been attempted by the industry in BC including improving smolt quality, using functional feeds, and targeting smolt entries based on site history and environmental conditions; however, none of these methods have been shown to be wholly effective (Hewison et al., 2015). While vaccination has reduced antibiotic treatments to almost zero for the predominant bacterial diseases (bacterial kidney disease, vibriosis, and furunculosis) in the Pacific Northwest, there is no commercial vaccine available for *T. maritimum* (Morrison et al., 2013). Without effective non-therapeutic measures against mouthrot, affected fish need to be treated with antibiotics to maintain fish welfare and reduce mortality. *T. maritimum* is susceptible to many of the known antibiotics in use in aquaculture including amoxicillin, nitrofurantoin, florfenicol, oxytetracycline and trimethoprim-sulphamethoxazole (Avendaño-Herrera et al., 2008), and florfenicol is the most frequently used antibiotic against mouthrot in BC.

At least three serotypes based on O-antigen have been identified for *T. maritimum* and the many studies indicate that this bacterium may not be as homogenous as previously believed (Ostland et al., 1999; Avendaño-Herrera et al., 2004; Castro et al., 2007). This suggests that different vaccine formulations may be required for different aquaculture regions or different fish species (Romalde et al., 2005). There are some major discrepancies between serological studies (e.g. differences in the antigens, antisera and techniques used) that impede comparisons between laboratories, and may therefore hamper the development and formulation of appropriate and effective vaccines.

Prototype vaccines have been tested in Atlantic salmon in Tasmania with poor results from little to no level of protection (Carson et al., 1992; 1993; 1994; van Gelderen et

al., 2009b). However, the tested conditions do not reflect the ones in BC in regards to water temperature and disease progression. Currently there is only one vaccine available for use against *T. maritimum*, and it is for turbot in Spain (Santos et al., 1999). A protective vaccine against mouthrot is of high interest to the BC salmon farming industry to improve fish welfare (by decreasing mortality) and sustainability (by decreasing antibiotic use).

1.4 The skin and oral cavity of salmonids

The skin is the primary barrier against the environment, and fish are no exception. There are three main layers (epidermis, dermis and hypodermis) in the skin of salmon with a cuticle layer (or mucus layer) covering the whole structure (**Figure 7**).

The epidermis, which is thicker in scale-less areas such as the head and fins, is made up of non-keratinising stratified squamous epithelium. This layer contains the mucous (or goblet) cells that are vital in the production of the mucus layer (Elliott, 2011). The epithelial cells retain the capacity to divide even in the outermost layer (unlike mammals) and the outer cells have a fingerprint-like microridge pattern (Ferguson, 2006). The dermis is composed of two layers: the upper *stratum spongiosum*, a loose collagenous network, and the lower *stratum compactum*, a dense matrix of collagen fibres. This latter layer is what gives the skin its strength (Roberts et al., 2012). The epidermis and dermis are separated by an acellular basement membrane. Scales, some

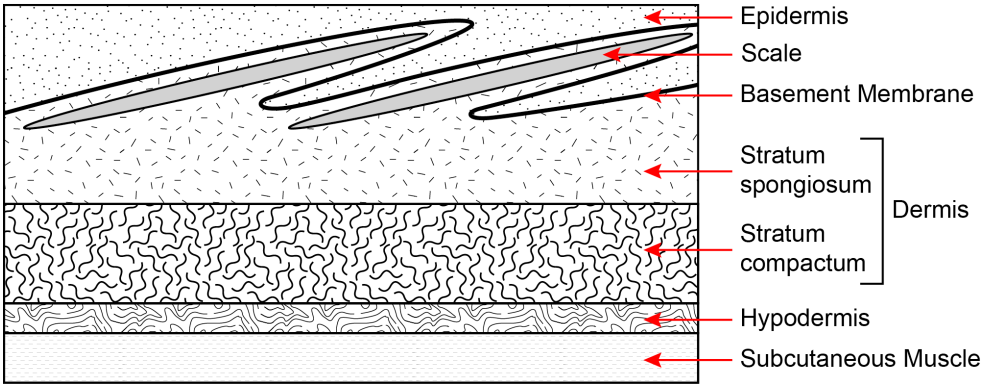

Figure 7 – Structure of the skin of salmon showing the different layers.

of the main physical protectors of the skin originate in the dermis in scale-pockets and are covered in the epidermis. Scale-loss is therefore a significant breach in the physical and/or osmotic barriers of the fish (Ferguson, 2006). The hypodermis is comprised of vascularised loose adipose tissue between the dense *stratum compactum* of the dermis and the subcutaneous muscle (Roberts et al., 2012).

The outer surface of epithelial cells of the epidermis has microridges that provide an extensive surface area for the secreted mucus to adhere to that helps maintain the mucous layer that covers the epithelium (Peterson, 2015). Mucus is considered the first line of defence against pathogens entering though the skin, as it captures foreign particles, including bacteria and viruses that are then removed by the surrounding water. Additionally, mucus is constantly secreted and replaced, which prevents colonisation by microorganisms (Esteban et al., 2015). *T. maritimum* has the advantage that it is able to strongly bind to this protective mucous layer, and that the mucus does not seem to contain compounds that inhibits this bacterium's growth (Magariños et al., 1995). This mechanism is likely the reason why *T. maritimum* is able to colonise the host so effectively.

The integument of the oral cavity is made up of the same layers as the skin, however there are some differences. The epidermis contains an abundance of mucous cells and lies on a thick membrane with a very condensed dermis binding it to the bone or muscle (Roberts et al., 2012). Filiform and fungiform papillae may be found (Ferguson, 2006). Salmon are homodont: all their teeth have the same shape. These teeth are formed throughout the fish's life. Teeth have an inner pulp with an outer dentine layer that is covered by enameloid on the crown, or exposed part. In salmon, teeth are attached to the jaw bones by an acrodont connection made of dense connective tissue. Where the epidermis connects with the teeth (gingival-enameloid interface), gingival pockets are formed surrounding them (Kryvi et al., 2016).

2. AIMS OF THE STUDY

The overall aim of the study was to increase the knowledge of mouthrot as it is seen in Atlantic salmon farms in the Pacific Northwest and make steps towards developing management tools that would help decrease the use of antibiotic treatments. This was accomplished through the following goals:

- Characterising the bacteria isolated from field cases of mouthrot through the use of genotyping tools. (**Paper I**)
- Testing if *T. maritimum* causes mouthrot on its own in Atlantic salmon smolts without other stressors or co-infections and thereby proving Koch's Postulates. (**Paper II**)
- Developing a bath challenge model for *T. maritimum* using Atlantic salmon smolts that is necessary for testing management tools. (**Paper II**)
- Testing whole cell inactivated adjuvanted vaccines using the developed challenge model. (**Paper II**)
- Investigating the pathology of the disease. (**Paper III**)

3. PAPER ABSTRACTS

Paper I

Mouthrot infections (bacterial stomatitis) have a significant impact on the Atlantic salmon aquaculture industry in Western Canada due to economic losses and fish welfare. Bacteria isolated from lesions in the field have been identified as *Tenacibaculum maritimum*. Mouthrot is different to classical tenacibaculosis, which is most commonly associated with ulcerative lesions, frayed fins and tail rot. The marine fish pathogen *T. maritimum* is found worldwide; however, in Western Canada, the knowledge of the genetic profile of *T. maritimum* is limited. This study looked at increasing this knowledge by genotyping *T. maritimum* isolates collected from Atlantic salmon from farms in Western Canada. These genotypes were compared to other species of the genus *Tenacibaculum*, as well as other known sequence types within the species. The Western Canadian isolates belong to two new sequence types within the *T. maritimum* species. Phylogenetic analysis shows that the isolates form a distinct branch together with *T. maritimum* NCIMB 2154^{T} separate from other *Tenacibaculum* type strains, and they are most closely related to strains from Norway and Chile.

Paper II

Mouthrot, or bacterial stomatitis, is a disease which mainly affects farmed Atlantic salmon, (*Salmo salar*, L.), smolts recently transferred into salt water in both British Columbia (BC), Canada, and Washington State, USA. It is a significant fish welfare issue which results in economic losses due to mortality and antibiotic treatments. The associated pathogen is *Tenacibaculum maritimum*, a bacterium which causes significant losses in many species of farmed fish worldwide. This bacterium has not been proven to be the causative agent of mouthrot in BC despite being isolated from affected Atlantic salmon. In this study, challenge experiments were performed to determine whether mouthrot could be induced with *T. maritimum* isolates collected from outbreaks in Western Canada and to attempt to develop a bath challenge model. A secondary objective was to use this model to test inactivated whole-cell vaccines for *T. maritimum* in Atlantic salmon smolts. This study shows that *T. maritimum* is the causative agent of mouthrot and that the bacteria can readily transfer horizontally

within the population. Although the whole-cell oil-adjuvanted vaccines produced an antibody response that was partially cross-reactive with several of the *T. maritimum* isolates, the vaccines did not protect the fish under the study's conditions.

Paper III

Mouthrot, caused by *Tenacibaculum maritimum* is a significant disease of farmed Atlantic salmon, *Salmo salar* on the West Coast of North America. Smolts recently transferred into saltwater are the most susceptible and affected fish die with little internal or external clinical signs other than the characteristic small (usually < 5 mm) yellow plaques that are present inside the mouth. The mechanism by which these smolts die is unknown. This study investigated the microscopic pathology (histology and scanning electron microscopy) of bath infected smolts with Western Canadian *T. maritimum* isolates TmarCan15-1, TmarCan16-1 and TmarCan16-5 and compared the findings to what is seen in a natural outbreak of mouthrot. A real-time RT-PCR assay based on the outer membrane protein A specific for *T. maritimum* was designed and used to investigate the tissue tropism of the bacteria. The results from this showed that *T. maritimum* is detectable internally by real-time RT-PCR. This combined with the fact that the bacteria can be isolated from the kidney suggests that *T. maritimum* becomes systemic. The pathology in the infected smolts is primarily mouth lesions, including damaged tissues surrounding the teeth; the disease is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with little associated inflammation. Skin lesions are more common in experimentally infected smolts than in natural outbreaks, but this could be an artefact of the challenge dose, handling and tank use during the experiments.

4. DISCUSSION

Mouthrot in the Pacific Northwest has been a significant smolt disease since the start of the industry in the region. The disease has been controlled through the use of antibiotics; however, this is not a solution as the aquaculture industry, like other animal production industries, strives to go antibiotic free. In addition, antibiotic treatments are expensive, as well as resulting in the poorer performance of treated fish. One of the conundrums when comparing to other production regions (in particular Tasmania) is that *T. maritimum* is associated with a different clinical presentation in the Pacific Northwest (mouthrot) than what is observed in Atlantic salmon in Spain and Tasmania (tenacibaculosis). This study has been able to give an insight into the strains of *T. maritimum* found during mouthrot outbreaks at BC salmon farms and their genetic relationship to *T. maritimum* strains found in other parts of the world (**Paper I**), as well as a definitive connection between the bacterium and the disease (**Paper II**). Whole cell oil adjuvanted vaccines were tested with the established bath challenge model (**Paper II**) and a description of the associated pathology was given (**Paper III**). As such, significant steps have been taken into the potential of a vaccine against mouthrot.

4.1 *Tenacibaculum maritimum* in farmed Atlantic salmon

The *T. maritimum* isolates collected from natural outbreaks of mouthrot in BC form a homologous group with only two different genetic strains based on the concatenated sequences of 11 HK genes (**Paper I**). The separation of the isolates into these two groups is further supported by the serological assessment (ELISA) that was performed showing strong cross-reaction between isolates from the same genetic strain and weaker cross-reactions with ones from the other genetic strain (**Paper II**). These findings are in line with the fact that despite *T. maritimum* having a very broad host range with a worldwide geographical distribution, it is a homologous species, exhibiting a low level of genetic diversity (Habib et al., 2014). The BC *T. maritimum* genetic strains are most closely related with strain NLF-15 isolated from diseased lumpsuckers in Norway and strain Ch-2402 isolated from Atlantic salmon gills in Chile

during a harmful algal bloom (Småge et al., 2016; Apablaza et al., 2017, **Paper I**). This wide geographic distribution between closely related strains is in contradiction with what has been described for other *T. maritimum* strains that suggest an endemic colonisation of fish farms by local strains with little or no indication of long distance contamination (Habib et al., 2014).

The main concern with finding closely related strains to the BC ones in Norway and Chile, is the potential risk it presents to the Atlantic salmon industry of both these regions. This concern is increased by the fact that isolates closely related to the BC strains were recently isolated from the gills and oral cavity of Atlantic salmon in Western Norway experiencing gill health problems in autumn 2017 (personal communication, Are Nylund). In addition, *T. maritimum* NLF-15 was found linked to a disease outbreak in lumpsuckers (Småge et al., 2016), which have increased in usage as biological lice controls in recent years (Bornø et al., 2016). These lumpsuckers are kept in cohabitation with Atlantic salmon in saltwater net-pens, and therefore the potential risk of pathogen transference is quite high, particularly when considering the ease in which *T. maritimum* appears to spread horizontally between fish (**Paper II**). This potential risk is exacerbated by the fact that the water temperatures in Western Norway are similar to what are seen in Western Canada. This is also the case for Chile, where the water temperatures in region X (Los Lagos) are in a similar range. In Chile, the high level of antibiotics (with the vast majority being florfenicol) used to combat salmonid rickettsial syndrome (*Piscirickettsia salmonis*) (Miranda et al., 2018) may be masking the effects of *T. maritimum*. It is possible that reducing the usage of these drugs may result in an increased prevalence of *Tenacibaculum* associated infections in Chile.

4.1.1 Why mouthrot?

The differences in clinical presentations (mouthrot versus tenacibaculosis) observed in Atlantic salmon in Tasmania and BC may be attributed to a number of factors, such as genetic differences between the strains, host factors, and environmental conditions. The genetic relationship between the isolates used to reproduce mouthrot in this study

(**Paper II**) and the ones used to reproduce tenacibaculosis in Tasmania (Handlinger et al., 1997; van Gelderen et al., 2010) is not known, as these latter isolates were not included in the MLST scheme (Habib et al., 2014). It is therefore impossible to know how closely or distantly related the BC and Tasmanian strains are and whether or not this may be contributing to the differences in clinical presentations.

The most significant difference between the outbreaks in Tasmania and BC appear to be the water temperature. Outbreaks of mouthrot occur in temperatures as low as 8 ºC and up to 16 ºC (**Paper I**). The tenacibaculosis outbreaks in Tasmania reported temperatures above 16 ºC (Handlinger et al., 1997). The 'yellow pest' syndrome described in Atlantic cod, which resembles mouthrot, was only found during the colder months (2 to 8 ºC) (Hilger et al., 1991) suggesting that there is a possibility that different temperatures may result in a variance in clinical presentations.

Mouthrot has historically been described as a seasonal disease with smolts introduced later in spring and summer being most severely affected during their first summer in saltwater (Kent et al., 2002). Seasonality in the levels of *T. maritimum* associated with gill health problems has been reported in Ireland and Norway, with the highest levels of the bacteria being reported during the times of the year with the highest seawater temperatures (PHARMAQ Analytiq, 2017; Downes et al., 2018). The seasonality of mouthrot outbreaks in BC has gradually changed over time with outbreaks now occurring all year round, not only during the warmest times of the year. The higher frequency of harmful algal blooms in the warmer half of the year further leads to poorer control of mouthrot related mortality as antibiotic treatments are administered through feed and the halting of feed is one of the primary mitigation tools in the face of harmful plankton (personal observations).

With warming sea water temperatures, the coastal areas of the Pacific Northwest where salmon farms operate can be expected to increase in biological productivity (Beamish et al., 2008). This results in higher levels of phytoplankton in the region. These elevated levels of organic material in the water will presumably lead to increased levels of polysaccharide and protein degraders including *Flavobacteriaceae*, the family to which

Tenacibaculum belongs (Buchan et al., 2014; Teeling et al., 2016; Bohórquez et al., 2017). The Pacific Northwest is particularly productive, with high abundance of phytoplankton in the region for at least six months of the year, and sometimes more, particularly in warmer years or years with significant freshwater runoff (Haigh et al., 2014; Zalzal, 2017). These environmental conditions may therefore lead to high levels of *T. maritimum* for a significant part of the year and may contribute to the reason why there no longer appears to be a seasonality to mouthrot outbreaks. The warmer waters and high organic load seen in the region have also been connected to jellyfish blooms and increased levels of sea lice, which have both been linked to *T. maritimum* (Barker et al., 2009; Ferguson et al., 2010).

4.1.2 *T. maritimum* versus other *Tenacibaculum* spp.

Although *T. maritimum* is the type species of the *Tenacibaculum* genus, phylogenetic analyses show that it branches out first and is quite different from the other species in the genus (**Figure 6**). There has therefore been some debate as to whether *T. maritimum* should be separated out; however, based on average amino acid identity analyses this species belongs in this genus (above the 55-60 % threshold) (Rodriguez-R et al., 2014; Pérez-Pascual et al., 2018). This "outgroup" phenomenon at the phylogenetic level is also reflected in the fact that *T. maritimum* is very different to the other pathogenic *Tenacibaculum* spp. (Bridel et al., 2018; Pérez-Pascual et al., 2018), including *T. dicentrarchi* and *T. finnmarkense* that have both been shown to cause disease in Atlantic salmon (Avendaño-Herrera et al., 2016; Småge et al., 2017; 2018). Whole genome sequencing has shown that the virulence factors of *T. maritimum* are strikingly different to the other pathogenic *Tenacibaculum* spp. (*T. dicentrarchi*, *T. finnmarkense*, *T. ovolyticum*, *T. soleae*) (Bridel et al., 2018; Pérez-Pascual et al., 2018). These differences in virulence factors (as deduced from their genes) likely contribute to the clinical presentation and pathogenic differences, as well as phenotypic characteristics, observed between the *Tenacibaculum* spp.

During this study, outbreaks of tenacibaculosis were observed at two different Atlantic salmon farms in BC and were characterised by mouth erosion (or dropped jaw), frayed

fins and skin lesions. The affected smolts resembled what has been described for tenacibaculosis in Norway (Olsen et al., 2011; Småge et al., 2017; 2018). At both sites, the predominant *Tenacibaculum* sp. recovered was *T. finnmarkense* (*T. finnmarkense* L441-3 and *T. finnmarkense* L556-6 in **Figure 8**), and no *T. maritimum* was found. Both isolates belonged to clade III, one of the two *T. finnmarkense* clades (**Figure 8**) (Olsen et al., 2017; Bridel et al., 2018). Tenacibaculosis outbreaks in Atlantic salmon smolts in Norway have also been associated with *T. finnmarkense* strains belonging to clade III and appear to be more pathogenic to Atlantic salmon than the ones belonging to clade I. This finding is supported by infection experiments that have shown that clade III strains are able to produce disease in smolts with no prior handling or stressor, whereas it was difficult to do so with isolates from clade I (Olsen et al., 2011; Småge et al., 2018). Strains belonging to clade I are therefore thought to be less pathogenic and most frequently found in association with other diseases. A *T. finnmarkense* isolate (*T. finnmarkense* L293-3 in **Figure 8**) belonging to clade III was also recovered from a smolt during a mouthrot outbreak from which *T. maritimum* strain TmarCan15-1 was recovered (**Paper I**). These findings indicate that tenacibaculosis in farmed Atlantic salmon in BC is associated with *T. finnmarkense*, and not *T. maritimum* like in Tasmania. However, it seems that *Tenacibaculum* spp. other than *T. maritimum* are sometimes recovered from mouthrot outbreaks.

The *Tenacibaculum* sp. strains belonging to clade IV appear to be a novel species (Bridel et al., 2018). Furthermore, *Tenacibaculum* sp. TNO020 (= F95C/98) that belongs to clade IV is not pathogenic in challenge experiments (Olsen et al., 2011; 2018). Isolates belonging to clade I and clade IV (data not shown) have also been found in BC; however, these are likely accidental findings as they were recovered during mouthrot outbreaks. Clade II contains *T. dicentrarchi* strains. There is no confirmed report of *T. dicentrarchi* being present in the Pacific Northwest, but as *T. dicentrarchi*, *T. finnmarkense* and *T. maritimum* are found in Norway and Chile, it is likely that *T. dicentrarchi* also exists in BC.

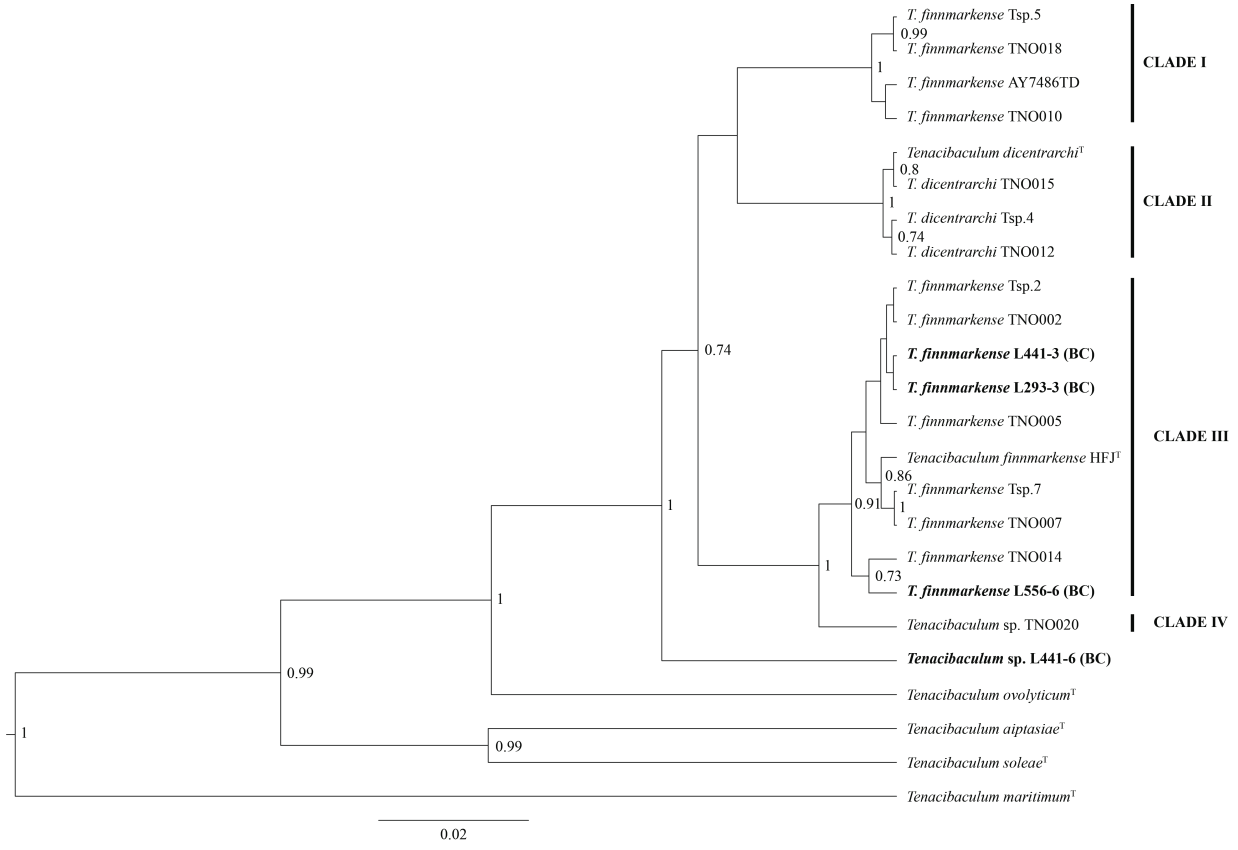

Figure 8 – The phylogenetic placement of non-*T. maritimum Tenacibaculum* spp. isolates from outbreaks of mouthrot and tenacibaculosis in BC inferred using *tuf* gene sequences available in GenBank. The tree was inferred using an alignment of 533 base positions of the tuf gene from 24 taxa. The alignment was constructed in AlignX in Vector NTI (Invitrogen) and then adjusted to equal length in GeneDoc (Nicholas et al., 1997). The best fitted evolutionary model (HKY+I) was calculated in Mega6 (Tamura et al., 2013). A Bayesian analysis (relaxed lognormal molecular clock and a MCMC of 50,000,000 generations) was performed using BEAST (Drummond et al., 2007). Sample size values (ESS) were inspected using Tracer ver. 1.6 (Rambaut et al., 2014), and found to be above the recommended range for all parameters (> 200). A maximum clade credibility tree was obtained using a 10% burn-in in TreeAnnotator and viewed using FigTree (Drummond et al., 2012). Support values below 0.70 have been removed.

In Norway, tenacibaculosis outbreaks in smolts appear to be caused by *T. finnmarkense* with the highest mortality in fish recently transferred into saltwater (Småge et al., 2017). In the above-mentioned BC cases, the observed tenacibaculosis outbreaks were connected to prior stressors; one was following a poor smolt transport from freshwater to saltwater and the other was following a harmful plankton bloom shortly after transfer to saltwater. These observations may suggest that *T. finnmarkense* isolates found in Canada requires some form of stressor to result in a significant outbreak similar to what has been reported from severe outbreaks in Norway (Småge et al., 2017).

4.2 Virulence mechanisms and pathogenesis

Despite the significance of mouthrot to the aquaculture industry in BC, little information is available regarding how *T. maritimum* is able to establish an infection and the mechanism by which it kills Atlantic salmon smolts. Several studies have looked into the virulence factors of *T. maritimum*, including recent whole genome work identifying encoding genes, that have provided insights into the mechanisms by which it causes pathological changes in host tissues (Avendaño-Herrera et al., 2006b; Pérez-Pascual et al., 2017). The success of a pathogenic bacterium such as *T. maritimum* to infect the host is dependent on its ability to attach to and colonise the host, and to grow to pathogenic levels within host tissues whilst avoiding host defence mechanisms (Ribet et al., 2015).

Interestingly, *T. maritimum* lacks external structures, such as pili, fimbriae and flagella that are known to be involved in adhesion and colonisation in other fish pathogenic bacteria (Avendaño-Herrera et al., 2006b; Ribet et al., 2015). Despite lacking these structures, *T. maritimum* possesses a type IX secretion system (T9SS) that secretes a variety of extracellular products (ECP) onto the cell surface (Pérez-Pascual et al., 2018). This includes adhesins that likely gives the bacterium its strong adhesive properties (McBride et al., 2015). *T. maritimum* is able to strongly adhere to hydrophobic surfaces (Burchard et al., 1990) thereby allowing the bacterium to non-specifically adhere to surfaces such as fish mucus (Ofek et al., 1994; Magariños et al., 1995). Strong adhesion appears to be associated with virulence as *T. maritimum* strains

that are more adherent correlate with increased virulence (van Gelderen et al., 2009b). This was also the case with BC strains: TmarCan16-1 is the most adhesive of the isolates tested in the challenge experiments and the most virulent (**Paper II**).

The T9SS is also associated with the motility of the bacterium by releasing adhesins that move rapidly along the cell surface, enabling *T. maritimum* to glide while firmly attached to the host surface (McBride et al., 2015). The ability to glide appears to be associated with virulence, as a non-gliding strain of *T. maritimum* has been shown to be less adhesive and avirulent (Rahman et al., 2014). This is likely due to the inability of a non-gliding strain to spread across surfaces, a key phenotypic characteristic of pathogenic *Tenacibaculum* spp. (Suzuki et al., 2001). Interestingly, genes that code for signaling molecules normally involved in quorum sensing (a bacterial communication process) in gram negative bacteria are lacking in the *T. maritimum* genome (Pérez-Pascual et al., 2018), and therefore the mechanisms by which the bacterium functions at the population level is not known.

In addition to creating strong adhesive colonies, *T. maritimum* has the ability to create a matrix of exopolysaccharides (slime), resulting in a biofilm formation (Avendaño-Herrera et al., 2006a). This is visible macroscopically as a yellow-tinged slime that is very distinct in mouthrot (**Figure 2**). In histopathological assessment, it is seen as large aggregates of *T. maritimum*-like bacteria (**Paper III**). Biofilm formation constitutes a protective mode of growth that makes *T. maritimum* more resistant to host defence mechanisms (Ribet et al., 2015) and likely enables the bacterium to grow to pathogenic levels (Dalsgaard, 1993). The production of this slimy biofilm is distinct for *T. maritimum* and has not been reported in other pathogenic *Tenacibaculum* spp.

Tenacibaculum maritimum appears to liquify and destroy the tissues surrounding its biofilm (**Paper III**); this is different to the infiltration associated with *T. finnmarkense* where bacteria appear to be able to infiltrate laterally into the dermis below the epidermis (Småge et al., 2018). These differences in infection mechanisms are likely due to the differences in virulence genes detected in their genomes (Pérez-Pascual et al., 2018). *T. maritimum* produces a number of toxins that are delivered through the

T9SS, some of which have high proteolytic activity facilitating the destruction of host tissues (Baxa et al., 1988; Handlinger et al., 1997; van Gelderen et al., 2009a; Vilar et al., 2012; Pérez-Pascual et al., 2018). An example of this is sphingomyelinase that has been shown to be present in vitro and has the ability to be cytotoxic to host cells by acting as a haemolytic factor (Pérez-Pascual et al., 2017). Besides this, the bacterium possesses multiple mechanisms for iron sequestration including the synthesis of siderophores and the utilization of haem groups as iron sources by direct binding (Avendaño-Herrera et al., 2005; Pérez-Pascual et al., 2017). The ability to acquire external iron allows *T. maritimum* to survive under poor iron conditions such as saltwater. Another virulence factor that has been demonstrated in vitro is a chondroitin AC lyase that has the ability to degrade chondroitin sulfate A and C, an important component of cartilage and fish connective tissue (Rahman et al., 2014; Pérez-Pascual et al., 2017). This is reflected by the large quantities of *T. maritimum* present in the dermis of the lining of the mouth in mouthrot affected smolts (**Paper III**). These tissues are also rich in collagen, another protein for which *T. maritimum* produces a proteolytic enzyme (Baxa et al., 1988; Pérez-Pascual et al., 2017).

In mouthrot, the destruction caused by *T. maritimum* is limited to the oral cavity and is most likely initiated in the periodontal tissue (**Paper III**). It was originally thought to be linked to abrasion of these tissues (e.g. feeding on spiny crustaceans such as crab larvae) (Kent et al., 2002), but disease can occur without a pre-abrasion. This pathology is different to what is reported for *T. maritimum* associated tenacibaculosis seen in other regions of the world or other host species, in which the bacteria seem to infect any exterior surface of the host (Avendaño-Herrera et al., 2006b). It appears that with tenacibaculosis in Atlantic salmon smolts, the fish die as a result of osmoregulatory imbalances due to a significant breach of the protective skin barrier caused by the lesions or ulcers. However, for mouthrot, this does not seem to be the case as smolt die with only the smallest lesion in their mouths (Ostland et al., 1999). *T. maritimum* must therefore have other mechanisms, such as the production of toxins that can affect vital organs or regulatory processes, and may play a role in killing the host. In addition, as

mouthrot appears to only affect smolts, these mechanisms may be linked to processes that are vital to this stage of the salmon's life cycle.

Histopathological assessments of mouthrot lesions usually show little immune response associated with the lesions and an abundance of bacteria (**Paper III**). This indicates that *T. maritimum* is able to avoid detection by the host's defence systems. Inflammatory processes such as tissue oedema occur at the lesion site and low levels of cellular infiltration are typically present (**Paper III**). This is either a specific immune response against *T. maritimum* or a response to the destruction of the host tissue. *T. maritimum* is likely able to escape immune surveillance through the formation of biofilms, and it is also likely to avoid the activation of the immune system through the production of extracellular proteases that can directly interact with the host defence mechanisms and tissue components (Finlay et al., 2006; Koziel et al., 2013; Pérez-Pascual et al., 2018). The genome of *T. maritimum* also contains genes encoding multiple enzymes able to deal with stressors such as reactive oxygen species produced by host macrophages (Pérez-Pascual et al., 2018). This bacterium therefore possesses many mechanisms that likely help it adapt to different environments.

4.3 Potential for a vaccine

The greatest successes in controlling bacterial diseases in aquaculture has been through the use of vaccines. The development and widespread use of water-in-oil emulsion adjuvanted vaccines allowed the Atlantic salmon farming industry to bloom by significantly decreasing the mortality resulting from furunculosis and vibriosis and the associated antibiotic treatments. These vaccines are frequently thought to have been essential for the growth of the industry. As with other bacterial diseases, the most economically viable tool for the mitigation of mouthrot is believed to be a protective vaccine. The duration of protection is generally higher in an injectable vaccine versus immersion or oral ones and the development of water-in-oil emulsions allowed these injectable vaccines to induce an even longer duration of protection when compared to other types of formulations (Brudeseth et al., 2013). For these reasons, whole cell oil adjuvanted vaccines were tested in this study (**Paper II**). However, for mouthrot this

approach did not protect the smolts against the disease despite giving a plasma antibody response in immunised fish. This was the case for immunised smolts bath challenged with homologous or heterologous strains.

The challenging part in vaccine development is how to determine the efficacy of a vaccine. In fish, the common method is to expose immunised individuals to the pathogen through a standardised and effective challenge model; thereby allowing the effect of the vaccine to be noticeable. In this study, a bath challenge model was developed and used to test the whole cell inactivated adjuvanted vaccines (**Paper II**). An immersion type challenge model reflects a natural transmission of *T. maritimum* from the environment; whereas an injectable type challenge model would bypass the integument that *T. maritimum* targets. However, with a bath infection, the fish are exposed to one high dose of bacteria that may not reflect the natural infection pressure during a mouthrot outbreak, which is more likely to be a low continuous pressure. This high dose may overwhelm the immune system of the host, making it difficult to determine the effectiveness of a vaccine, as suggested for *M. viscosa* (Karlsen et al., 2017). Despite this, a bath challenge model appears to be the best way to reliably reproduce disease with *T. maritimum* when compared to injection methods (Avendaño-Herrera et al., 2006b).

As previously mentioned, a *T. maritimum* whole cell inactivated vaccine for Atlantic salmon in Tasmania has been attempted a few times (Carson et al., 1993; van Gelderen et al., 2009b). The latter attempt showed promising results with an adjuvanted vaccine giving partial protection against tenacibaculosis (van Gelderen et al., 2009b). However, the challenge conditions (including water temperature) and *T. maritimum* strains used were different, as was the clinical presentation when compared to the experiments conducted in this study (**Paper II**). Another potential difference is in the vaccine preparation.

Although *T. maritimum* seems to become systemic, based on the detection through real-time RT-PCR and bacteriology, it is likely that the systemic immune response induced by an IP vaccine will not give protection due to the acuteness of mouthrot **(Paper II,**

III). The immune system does not appear to notice the infection until it is too late and the damage has already occurred. A localised mucosal immune response is therefore likely needed for the host to combat the pathogen. For *Flavobacterium psychrophilum*, the causative agent of bacterial coldwater disease in rainbow trout, the stimulation of a strong serum antibody response with a whole cell inactivated adjuvanted IP vaccine does not always infer a detectable mucosal antibody response (LaFrentz et al., 2002; Madetoja et al., 2006). Therefore, as for *F. psychrophilum*, it is likely that for *T. maritimum* a different approach is required that would stimulate a localised mucosal immune response.

Another possibility that may be hampering the effectiveness of a whole cell inactivated vaccine is that this method (IP) does not allow the pathogen to express virulence factors and that it is possible that it is these virulence factors that are antigens in stimulating the host's immune response. If this is the case, then the antibodies created in response to a vaccine like this would not protect the host against the disease. The identification of predicted virulence factors may hence lead to the development of attenuated *T. maritimum* variants for vaccine development (Pérez-Pascual et al., 2017). An option would be to test an apparently avirulent strain in a live vaccine such as *T. maritimum* TmarCan16-2 that did not cause disease in challenge experiments (**Paper II**). In addition, a protective vaccine against mouthrot would likely need to specifically stimulate a localised mucosal immune response. If effective, this may allow the smolt to stop *T. maritimum* from establishing an infection by targeting one the mechanisms it requires to invade and colonise host tissues. As the disease only seems to affect smolts during the first few months in saltwater (section 1.2), it is possible that a different delivery method may be more suitable, such as oral or immersion, as these are more likely to induce a mucosal immune response, but a shorter lived one (Soto et al., 2015).

5. CONCLUSION

The main objective of this study was to make steps towards developing management tools that would help improve the welfare of the smolts and decrease the use of antibiotic treatments. Unfortunately, despite giving a plasma antibody response in the immunised fish, the whole cell oil adjuvanted vaccines tested in this study did not give protection against *T. maritimum* infections under the tested conditions. Nonetheless a number of findings were discovered that will aid in future mouthrot research.

Based on a MLST scheme and serological analyses, *T. maritimum* isolates collected from natural outbreaks of mouthrot on BC Atlantic salmon farms belong to two different strains that are most closely related to strains isolated from diseased cultured lumpsuckers and Atlantic salmon in Norway and a strain isolated from Atlantic salmon gills during a harmful algal bloom in Chile.

Mouthrot in Atlantic salmon smolts was reproduced in the laboratory using BC *T. maritimum* strains. The main pathological signs in experimentally infected smolts are mouth lesions that damage the tissues surrounding the teeth causing a disease that is similar to periodontal disease in mammals. The changes are focal, severe, and occur very rapidly with very little associated inflammation. *T. maritimum* is detectable internally by real-time RT-PCR and bacteriology, and one possible point of entry would be the teeth. However, the mechanism by which *T. maritimum* kills Atlantic salmon smolts remains a mystery.

The reproduction of the disease in the laboratory with *T. maritimum* isolates collected from mouthrot outbreaks in BC, as well as the re-isolation of the bacteria from these diseased individuals fulfils Koch's Postulates, which is the preferred method for proving disease causation (Fredricks et al., 1996). This study therefore shows that *T. maritimum* is the causative agent of mouthrot in BC. Horizontal transmission of the pathogen was shown to readily occur between infected smolts to naïve ones. Also, there are major differences in pathogenicity between isolates, from avirulent ones (TmarCan16-5) to highly virulent ones (Tmar16-1).

6. FUTURE PERSPECTIVES

A better understanding of the relationship between stressors and the development of mouthrot is required. This should include water temperature, as well as other environmental factors such as the presence of plankton, jellyfish, or crustacean larval blooms, and the effect of these on the risk and severity of disease. The fact that mouthrot mainly affects Atlantic salmon smolts recently transferred into saltwater suggests that investigations should include the significance of the smolt's transition from freshwater to saltwater and its impact on the fish's microbiota, immune function and homeostasis.

Continued research is needed to determine the mechanism by which smolts die while only having minor clinical pathology. Is this the result of a toxin released by *T. maritimum*? If it is in fact a toxin, this may point towards a potential target for a vaccine antigen. A better understanding of the course of the disease may shed some light as to why the clinical presentation is different in Atlantic salmon smolts in Tasmania. Improved knowledge of host/pathogen interactions would also provide a better understanding of the potential risks of finding closely related *T. maritimum* strains to the BC ones in the Norwegian and Chilean salmon farming industry.

Future vaccine development should focus on identifying important virulence factors and potentially using these to develop live attenuated *T. maritimum* variants that may be used in live vaccines. Investigations should include other vaccine methods such as immersion or oral vaccines, as these are likely to stimulate a better mucosal immune response than injectable vaccines. Further improvements to the challenge model would help with the testing of vaccines and other mitigation tools. The use of a cohabitation challenge model for testing vaccines may help with better mimicking the infection pressure of natural outbreaks of mouthrot.

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ORIGINAL ARTICLE

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Genotyping of *Tenacibaculum maritimum* isolates from farmed Atlantic salmon in Western Canada

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Abstract

Mouthrot infections (bacterial stomatitis) have a significant impact on the Atlantic salmon aquaculture industry in Western Canada due to economic losses and fish welfare. Bacteria isolated from lesions in the field have been identified as *Tenacibaculum maritimum*. Mouthrot is different to classical tenacibaculosis, which is most commonly associated with ulcerative lesions, frayed fins and tail rot. The marine fish pathogen *T. maritimum* is found worldwide; however, in Western Canada, the knowledge of the genetic profile of *T. maritimum* is limited. This study looked at increasing this knowledge by genotyping *T. maritimum* isolates collected from Atlantic salmon from farms in Western Canada. These genotypes were compared to other species of the genus *Tenacibaculum*, as well as other known sequence types within the species. The Western Canadian isolates belong to two new sequence types within the *T. maritimum* species. Phylogenetic analysis shows that the isolates form a distinct branch together with *T. maritimum* NCIMB 2154^T separate from other *Tenacibaculum* type strains, and they are most closely related to strains from Norway and Chile.

KEYWORDS
mouthrot, multilocus sequence analysis, phylogenetic, *Salmo salar*

1 | INTRODUCTION

Tenacibaculum maritimum is a marine Gram-negative bacterium in the family Flavobacteriaceae (Suzuki, Nakagawa, Harayama, & Yamamoto, 2001; Wakabayashi, Hikida, & Masumura, 1986). It is a pathogen found worldwide causing infections in a wide variety of farmed marine fish. The disease, tenacibaculosis, is described as causing mouth erosion, ulcerative skin lesions, frayed fins and tail rot (Toranzo, Magariños, & Romalde, 2005). However, in British Columbia (BC), Canada, the bacterium is most commonly associated with yellow plaques in the mouth (mouthrot) and is a significant issue for Atlantic salmon, *Salmo salar* (L.), smolts newly transferred into saltwater (Ostland, Morrison, & Ferguson, 1999). Mouthrot outbreaks are associated with major economic losses in the Western Canadian aquaculture industry and cause significant fish welfare problems (Hewison & Ness, 2015). There are also reports of mouthrot affecting farmed Atlantic salmon in Washington State (Frelier, Elston, Loy, & Mincher, 1994).

On the west coast of North America, the bacterium has also been isolated in California from white seabass, *Atractoscion nobilis* (Ayres), northern anchovy, *Engraulis mordax* (Girard), Pacific sardine, *Sardinops sagax* (Jenyns) and farmed Chinook salmon, *Oncorhynchus tschawytscha* (Walbaum) (Chen, Henry-Ford, & Groff, 1995), and in these fish species, the pathogen has been associated with gill lesions and skin ulceration. Mouthrot has been recorded since the 1990s in BC, however little research has been conducted into the disease and

the role of *T. maritimum* as a causative agent. There are no publications confirming, through genetic identification, that the bacteria isolated from mouthrot lesions are *T. maritimum*.

Molecular typing methods such as multilocus sequence typing (MLST) have allowed the development of uniform and reproducible nomenclature schemes for bacterial pathogens (Maiden, 2006). MLST characterizes prokaryotes within a species using the allelic mismatches of a small number of housekeeping (HK) genes (Gevers et al., 2005) and allows researchers to perform epidemiological studies and define strains within named species (Gevers et al., 2005). Because MLST schemes are based on nucleotide sequences, they are intrinsically reproducible and portable between laboratories (Maiden, 2006). The sequence data from MLST can be used for phylogenetic analyses, called multilocus sequence analysis (MLSA) and defined as the sequence analysis of multiple protein-coding genes for the genotypic characterization of a diverse group of prokaryotes including entire genera (Gevers et al., 2005). The usual approach to MLSA is to concatenate the sequences of several housekeeping genes and then use these to assess clustering patterns. MLSA is the current method of choice to explore phylogenetic relationships at the genera and species levels (Glaeser & Kämpfer, 2015). Habib et al. (2014) used MLST and MLSA to describe 47 sequence types (STs) of 73 *T. maritimum* strains from a variety of host species worldwide. The genetic data from their study were uploaded into the *Tenacibaculum* MLST database (http://pubmlst.org/tenacibaculum/) and allow others to use and add to this work as was suggested by (Maiden, 2006).

Although a lot of genetic research has been completed on *T. maritimum* strains from European, Asian and Australian waters (Habib et al., 2014), nothing is known about the genetic profile of *T. maritimum* from Western Canada. This study provides this knowledge and adds to the genotyping of *T. maritimum* worldwide.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolation

The *T. maritimum* isolates included in this study were collected from Atlantic salmon smolts showing clinical signs of mouthrot (yellow plaques in the mouth) from saltwater farms in BC, Canada from 2011 to 2016 as shown in Figure 1. A marine agar (Difco 2216) was modified to include 50 μg/ml kanamycin (MKA) to improve the recovery of *T. maritimum* from primary cultures. MKA was used for the primary isolation from diseased fish, and the plates were incubated for a minimum of 5 days at 16°C. Cultures that matched the phenotypic description of the *Tenacibaculum* genus (Suzuki et al., 2001) were subcultured on marine agar (MA) and incubated at 16°C.

2.2 | PCR and sequencing

Genomic DNA was extracted from isolates of interest using an E.Z.N.A Tissue DNA Kit (Omega Bio-tek). A PCR was performed using the 16S rRNA primers 27F and 1518R (Giovannoni, Rappé, Vergin, & Adair, 1996). Amplification was based on a standard reaction mixture containing 2.5 μl Extra buffer, 1.25 mM deoxyribonucleotide triphosphates, 0.75 units (0.15 μl) Taq DNA polymerase (VWR), 5 μM (1 μl) of forward and reverse primers, and then DNase-RNase free water was added to a final volume of 25 μl (16.85 μl H_2O). The amplification was performed at 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 90 s, followed by 72°C for 10 min in a Veriti Thermal Cycler (Thermo Fisher Scientific). The PCR product was confirmed using gel electrophoresis and then enzymatically purified using ExoSAP-IT PCR Product Cleanup (Thermo Fisher Scientific) in a Veriti Thermal Cycler at 37°C for 15 min then heated to 80°C for 15 min. Sequencing was performed on PCR products using both the forward and reverse PCR primers. Analyses of the sequences were carried out by the Sequencing Facility at the University of Bergen (http://www.uib.no/seqlab) using big dye termination chemistry. Vector NTI (Invitrogen) software suite was used to assemble and align the obtained sequences. A BLAST search was performed to determine the identity of each isolate. Pure cultures of *T. maritimum* were cryopreserved at −80°C.

PCRs were performed on the *T. maritimum* isolates using primers designed by Habib et al. (2014) for 11 HK genes. Amplifications were performed at 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C (50°C for primers: *glyA*, *infB*, *tgt*, *tuf* and *yqfO*) for 30 s and 72°C for 1 min, followed by 72°C for 10 min using the same reaction mixture and machine as above. The PCR products were confirmed and sequenced as above.

2.3 | Genetic analysis

To determine whether the Canadian *T. maritimum* isolates from this study belonged to a known ST of *T. maritimum*, the MLST profiles that consisted of seven HK gene sequences (*atpA*, *gyrB*, *dnaK*, *glyA*, *infB*, *rlmN* and *tgt*) were uploaded and analysed in the *Tenacibaculum* MLST database. The Canadian isolates found to be genetically identical were assigned one unique ST name (e.g., STCan1, STCan2) and a unique strain name (e.g., TmarCan1, TmarCan2) for the phylogenetic analyses.

In this study, one 16S rRNA and two concatenated HK gene sequence alignments were constructed for phylogenetic analysis. All alignments were constructed in AlignX in Vector NTI before sequences were adjusted to equal length and correct reading frames in GeneDoc (Nicholas, Nicholas, & Deerfield, 1997). The 16S rRNA gene sequence alignment included the Canadian *T. maritimum* strains from this study and sequences from all known type strains in genus *Tenacibaculum*. The length of the 16S rRNA gene alignment was 1351 bp. Concatenation of the HK gene alignments was performed using Kakusan4 (Tanabe, 2011). The length of the concatenated HK gene alignment was 5811 bp (*atpA* 1-567, *dnaK* 568-1140, *glyA* 1141-1698, *gyrB* 1699-2295, *ileS* 2299-2841, *infB* 2842-3405, *rlmN* 3406-3954, *tgt* 3955-4440, *trpB* 4441-4809, *tuf* 4810-5364 and *yqfO* 5365-5811). The first HK gene sequence alignment included concatenated sequences of the Canadian strains found in this study and 19 type strains in genus *Tenacibaculum*. The second HK gene sequence alignment included concatenated sequences of the Canadian strains found

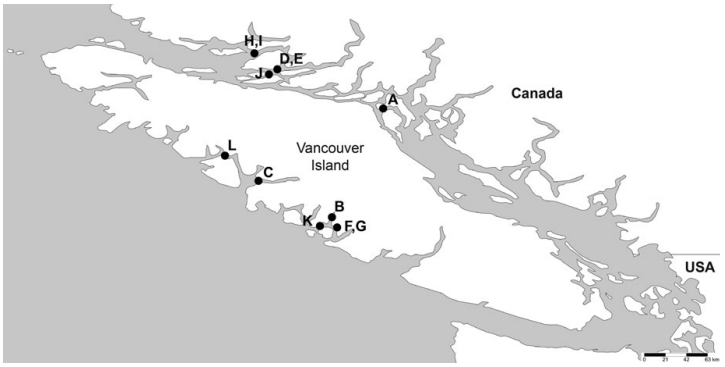

FIGURE 1 Location of the origin of each isolate listed in Table 1

TABLE 1 Canadian *Tenacibaculum maritimum* isolates used in this study

Isolate	Map reference (Figure 1)	Sampling date	Strain (sequence type)	Seawater temperature (°C)
TmarCan11-1	A	Jun. 2011	TmarCan1 (STCan1)	8.9
TmarCan15-1	B	Sep. 2015	TmarCan2 (STCan2)	14.7
TmarCan16-1	C	Feb. 2016	TmarCan1 (STCan1)	8.7
TmarCan16-2	D	Apr. 2016	TmarCan1 (STCan1)	9.0
TmarCan16-3	E	Apr. 2016	TmarCan1 (STCan1)	9.0
TmarCan16-4	F	Apr. 2016	TmarCan1 (STCan1)	12.0
TmarCan16-5	G	Apr. 2016	TmarCan2 (STCan2)	12.0
TmarCan16-6	H	May 2016	TmarCan1 (STCan1)	11.3
TmarCan16-7	I	May 2016	TmarCan1 (STCan1)	11.3
TmarCan16-8	J	May 2006	TmarCan2 (STCan2)	10.7
TmarCan16-9	K	Jun. 2016	TmarCan1 (STCan1)	11.1
TmarCan16-10	L	Oct. 2016	TmarCan2 (STCan2)	12.1

in this study (Table 1), the Norwegian *T. maritimum* strain NLF-15 isolated from lumpsuckers, *Cyclopterus lumpus* (L.) (Småge, Frisch, Brevik, Watanabe, & Nylund, 2016), the Chilean *T. maritimum* strain Ch-2402 isolated from Atlantic salmon (Apablaza et al., 2017), as well as a single strain from each of the 47 *T. maritimum* STs (identified by their ST number) described in Habib et al. (2014).

All gene sequences, except the isolates from this study and the *Tenacibaculum finnmarkense*^{T} HK genes sequences, were obtained from GenBank (Table S1). The *T. finnmarkense*^{T} HK gene sequences were obtained as described for the Canadian *T. maritimum* isolates.

For the 16S rRNA gene data set, the best fitted evolutionary model was calculated using Mega 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The BEAST package v1.8 (Drummond, Suchard, Xie, & Rambaut, 2012) was used for Bayesian analysis using the K2 + G + I model, a relaxed lognormal molecular clock and a mcmc of 100,000,000 generations. *Kordia algicida*^{T} was used as the outgroup.

For the MLSA of the two concatenated HK alignments, Kakusan4 was used to calculate the substitution rate and best fit model for the individual loci and codon positions for the Bayesian analysis performed in MrBayes (Ronquist et al., 2012) using the data block with the proportional codon proportional model from Kakusan4 and a mcmc of 100,000,000 generations. The effective sample size (ESS) values in the Bayesian analysis were inspected using Tracer ver. 1.6 (Rambaut, Suchard, Xie, & Drummond, 2014). Due to the size of the output file of the *T. maritimum* HK analysis, the sump command in MrBayes was used to summarize the ESS values.

A maximum clade credibility tree was obtained for each analysis using a 10% burn-in in Tree-Annotator and viewed using FigTree (Drummond et al., 2012). Posterior probability values above 95% were regarded as accurate (Huelsenbeck & Rannala, 2004). All sequences obtained in the current study are available in GenBank with accession numbers KY428880 to KY428914 and MF421902 to MF422021.

3 | RESULTS

The MKA media supported growth for all Canadian isolates from this study, as well as the Norwegian *T. maritimum* strain NLF-15, the Chilean *T. maritimum* strain Ch-2402 and *T. maritimum*^{T}. Field experience generated during the isolation of the Canadian *T. maritimum* showed that MKA improved the success of isolating the bacterium when compared to MA. The added kanamycin decreased the amount of overgrowth by other bacteria.

The Western Canadian isolates collected in this study were found to belong to two distinct ST (STCan1 and STCan2) based on the *Tenacibaculum* MLST database (Table 1). This was supported by the fact that the alignment of the 11 HK gene sequences for the MLSA showed only two distinct genetic strains. The two strains were isolated on both the east and west coast of Vancouver Island, and for one outbreak, they co-occurred (isolates F and G) (Figure 1). The results of the 16S rRNA phylogenetic analysis (Figure 2) show that the *T. maritimum* isolates found in Western Canada form a distinct branch together with *T. maritimum*^{T} separate from other *Tenacibaculum* type strains. This is further supported by the *Tenacibaculum* HK phylogenetic analysis (Figure 3). Figure 4 shows that the *T. maritimum* strains found in this study form a branch with strains NLF-15 and Ch-2402.

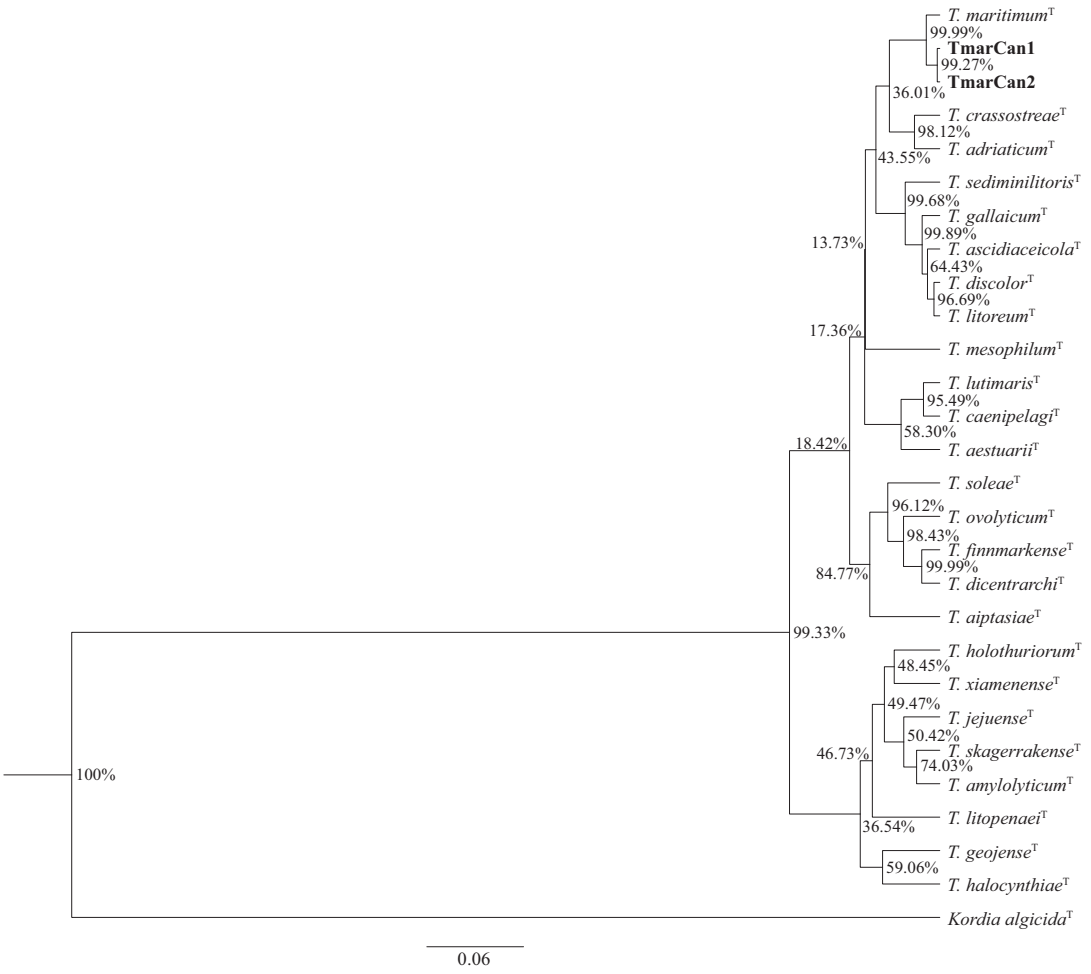

FIGURE 2 The 16S rRNA phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains and all type strains in genus *Tenacibaculum*. *Kordia algicida*^{T} was used as an outgroup. The Bayesian analysis was performed on 1351 bp using the K2 + G + I model. The posterior probability is presented next to each node. Scale bar = 0.06 substitutions per site

4 | DISCUSSION

The use of MKA improved the success of isolating *T. maritimum* from the field when compared to only using MA. MKA inhibited the growth of other faster growing bacteria that routinely outcompete *T. maritimum* on primary cultures from skin or ulcers grown on MA. The idea of adding kanamycin to the growth medium was based on previous work with Flavobacteria that showed that adding kanamycin to growth media, such as TYS or SYL agar, allowed for selective isolation of certain genera due to growth inhibition of others (Hahnke & Harder, 2013; Rahman, Suga, Kanai, & Sugihara, 2014).

The *T. maritimum* isolates described in this study belonged to two distinct STs (STCan1 and STCan2) different from all other previously published STs (Habib et al., 2014), and the MLSA shows that the Western Canadian strains are most closely related with strain NLF-15 isolated from lumpsuckers in Norway (Småge et al., 2016) and strain Ch-2402 isolated from Atlantic salmon in Chile (Apablaza et al., 2017). These four strains are grouped together in subgroup C (designated C in Habib et al. (2014)) with three strains from Spain (salmonids and turbot, *Scophthalmus maximus*, L.) and two from Tasmania (Atlantic salmon and striped trumpeter, *Latris lineata*, Forster). Strains NLF-15 and Ch-2402 were isolated from fish reared in waters of approximately 12°C and 14 °C, respectively (Apablaza et al., 2017; Småge et al., 2016), which is in the same range as the Canadian isolates (Table 1). Temperatures from the other strains in subgroup C are unknown to the authors of this study, but thought

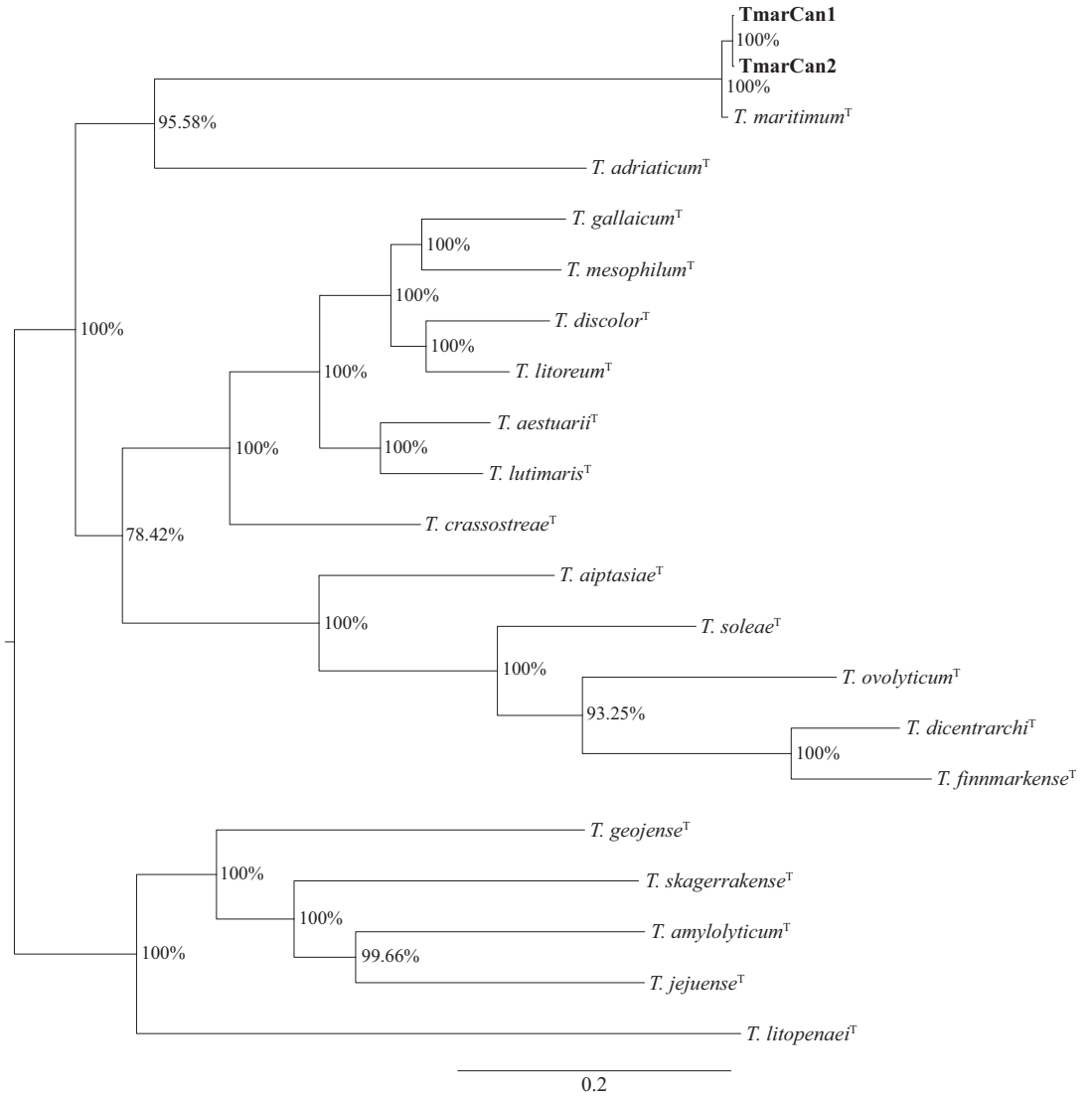

FIGURE 3 The phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains and 19 type strains in genus *Tenacibaculum* based on the concatenated HK gene sequences. The Bayesian analysis was performed on 5811 bp. The posterior probability is presented next to each node. Scale bar = 0.2 substitutions per site

to be in the same temperature range (9–16°C), which is in the lower range of *T. maritimum* growth (Suzuki et al., 2001). This suggests that there may be a temperature distribution, which would fit with the geographic relationship suggested by Habib et al. (2014). Increased data regarding the host environment of the other strains in subgroup C are required to test this hypothesis. The isolation of different *T. maritimum* strains from the same mouthrot outbreak at the same site is likely due to the bacterium being present in the marine environment.

Habib et al. (2014) found that the relative positions of the strains in subgroup B seemed correlated with fish host and geographic origin. Based on support values in the MLSA, the authors of this study decided to divide subgroup B into two (Figure 4), one of which consists solely of strains from Japanese waters (B1) including the type strain, which would fit with the proposed geographic distribution. A possible hypothesis as to the global distribution of closely related strains is the movement of organisms between locations; for example, the transference of ballast waters

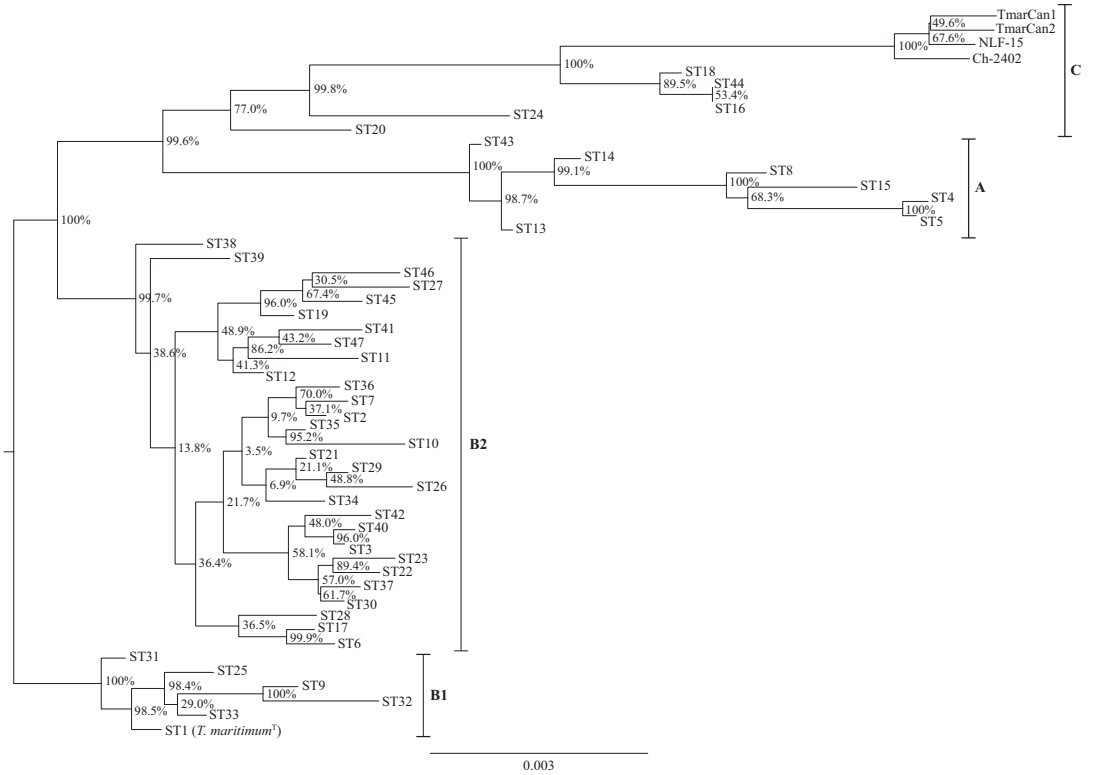

FIGURE 4 The phylogenetic relationship of the two *Tenacibaculum maritimum* Canadian strains, the Norwegian *T. maritimum* strain NLF-15 (Småge et al., 2016), the Chilean *T. maritimum* strain Ch-2402 (Apablaza et al., 2017), as well as a single strain from each of the 47 *T. maritimum* STs (identified by their ST number) (Habib et al., 2014) based on the concatenated HK gene sequences. The Bayesian analysis was performed on 5811 bp. The posterior probability is presented next to each node. Scale bar = 0.003 substitutions per site

when ships travel from one region to another. A wide range of bacteria have been shown to be present in these waters including *Tenacbaculum* spp. and more specifically *T. maritimum* (Brinkmeyer, 2016).

Other information, not looked at by Habib et al. (2014), such as environmental conditions (e.g., temperature, salinity), clinical signs and tissue tropism of the different *T. maritimum* strains would enable further investigations into the pathogenic differences seen worldwide, even within one host species. This variation is particularly noticeable in Atlantic salmon, where typical tenacibaculosis infections have been described in Tasmania, Australia (Handlinger, Soltani, & Percival, 1997) and Spain (Toranzo, 2015), whereas the infections seen in Western Canada present as mouthrot (small yellow plaques in the mouth) (Ostland et al., 1999). Understanding the pathogenic differences between strains could allow for better management of the disease, and studies of genetic variation, as presented in this paper, may provide the needed genetic markers.

The strain most closely related to the two Canadian ones is the one found in lumpsuckers in Norway. This finding should be a concern to the Norwegian Atlantic salmon aquaculture industry as the use of lumpsuckers as biological lice controls has increased in recent years (Bornø et al., 2016). However, the average rearing water temperatures for Atlantic salmon in Norway are lower than the ones seen in Western Canada, which may decrease the risk of the development of mouthrot or *T. maritimum* tenacibaculosis.

Most of the research conducted on *T. maritimum* has been on warmer water (more than 16°C) strains. The authors suggest that more research is needed on strains from Atlantic salmon in colder regions. Furthering the knowledge is essential for the development of diagnostic tools for the pathogen such as real-time PCR and preventative measures against the disease such as vaccine development.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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ORIGINAL ARTICLE

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Experimental induction of mouthrot in Atlantic salmon smolts using *Tenacibaculum maritimum* from Western Canada

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Abstract

Mouthrot, or bacterial stomatitis, is a disease which mainly affects farmed Atlantic salmon, (*Salmo salar*, L.), smolts recently transferred into salt water in both British Columbia (BC), Canada, and Washington State, USA. It is a significant fish welfare issue which results in economic losses due to mortality and antibiotic treatments. The associated pathogen is *Tenacibaculum maritimum*, a bacterium which causes significant losses in many species of farmed fish worldwide. This bacterium has not been proven to be the causative agent of mouthrot in BC despite being isolated from affected Atlantic salmon. In this study, challenge experiments were performed to determine whether mouthrot could be induced with *T. maritimum* isolates collected from outbreaks in Western Canada and to attempt to develop a bath challenge model. A secondary objective was to use this model to test inactivated whole-cell vaccines for *T. maritimum* in Atlantic salmon smolts. This study shows that *T. maritimum* is the causative agent of mouthrot and that the bacteria can readily transfer horizontally within the population. Although the whole-cell oil-adjuvanted vaccines produced an antibody response that was partially cross-reactive with several of the *T. maritimum* isolates, the vaccines did not protect the fish under the study's conditions.

KEYWORDS
challenge model, cohabitation, experimental model, Pacific Northwest, *Salmo salar*

1 | INTRODUCTION

Mouthrot, or bacterial stomatitis, is a significant fish welfare problem in Atlantic salmon (*Salmo salar*, L.) farming in both British Columbia (BC), Canada, and Washington State, USA (Frelier, Elston, Loy, & Mincher, 1994; Ostland, Morrison, & Ferguson, 1999). The disease mainly affects smolts recently transferred into salt water and results in economic losses due to mortality and antibiotic treatments. Diseased fish show little or no clinical signs, with small yellow plaques in the mouth as the only visible abnormality (Frelier et al., 1994). The bacterium isolated from these lesions is *Tenacibaculum maritimum*, a fish pathogen found worldwide on many marine fish species (Frisch, Småge, Brevik, Duesund, & Nylund, 2017; Ostland et al., 1999; Toranzo, Magariños, & Romalde, 2005). Most commonly, *T. maritimum* is associated with tenacibaculosis, characterized by ulcerative skin lesions, mouth erosion, frayed fins and tail rot (Toranzo et al., 2005); a disease which is clinically different from mouthrot as seen in BC. Although *T. maritimum* has been isolated from mouthrot-affected fish, the bacterium has not been identified to be solely responsible for this disease.

One of the challenges in *T. maritimum* research is the difficulty to develop a replicable challenge model. A number of experiments have been conducted attempting to reproduce tenacibaculosis in economically important fish species around the world (Avendaño-Herrera, Toranzo, & Magariños, 2006a; Baxa, Kawai, & Kusuda, 1987; Carson, McCosh, & Schmidtke, 1992; Handlinger, Soltani, & Percival, 1997; Mabrok, Afonso, Valente, & Costas, 2015; Mabrok et al., 2016; Nishioka, Watanabe, & Sano, 2009; Powell, Carson, & van Gelderen, 2004; Soltani, Munday, & Burke, 1996; van Gelderen, Carson, & Nowak, 2010; Wakabayashi, Hikida, & Masumura, 1984; Yamamoto, Kawai, & Oshima, 2010). Injection models are frequently used in fish as they allow for more control and therefore reproducibility; for example, *Infectious pancreatic necrosis virus* or *Piscirickettsia salmonis* in Atlantic salmon (Rozas & Enríquez, 2014; Taksdal, Ramstad, Stangeland, & Dannevig, 1998). However, their main shortcoming is that they do not reproduce a natural route of transmission. Intraperitoneal (IP) injection of *T. maritimum* in Japanese flounder (*Paralichthys olivaceus*, Temminck & Schlegel) (Yamamoto et al., 2010) has not resulted in disease, and subcutaneous injection has only given disease in turbot (*Scophthalmus maximus*, L.) (Faílde, Losada, Bermúdez, Santos, & Quiroga, 2014). Bath infection has been shown to be the most effective way to induce tenacibaculosis with *T. maritimum* (Avendaño-Herrera, Toranzo, & Magariños, 2006b). Scarification or abrasion pre-exposure was originally thought to be a prerequisite for positive results, as was performed in Black Sea bream (*Acanthopagrus schlegelii*, Bleeker) (Baxa et al., 1987). However, this is not necessary, and prolonged bath exposure without scarification has induced tenacibaculosis in Senegalese sole (*Solea senegalensis*, Kaup) (Mabrok et al., 2016) and turbot (Avendaño-Herrera et al., 2006a). More recently, a shorter bath immersion has also been successful in Japanese flounder (Nishioka et al., 2009), and a short immersion followed by a dilution of the bath over time produced more stable mortality rates in this species than immersion followed by transfer into a new tank (Yamamoto et al., 2010).

In Tasmania, Australia, *T. maritimum* has been linked to tenacibaculosis in Atlantic salmon smolts. This disease has been reproduced in the laboratory by bath infecting fish for a short period of time (1 hr) at a high concentration (Handlinger et al., 1997; Soltani, Shanker, & Munday, 1995; Soltani et al., 1996; van Gelderen et al., 2010; van Gelderen, Carson, & Nowak, 2011). These experiments also showed that Atlantic salmon was more susceptible than rainbow trout (*Oncorhynchus mykiss*, Walbaum) and that exposure at lower salinities (15 ppt) gave very low mortality (Handlinger et al., 1997; Soltani et al., 1996). This finding is consistent with the fact that *T. maritimum* has a strict requirement of sufficient sea salt to grow (Suzuki, Nakagawa, Harayama, & Yamamoto, 2001). In Atlantic salmon, it has also been shown that *T. maritimum* can cause necrotic bronchitis by directly inoculating high concentrations of the bacteria on the gills and that this can be exacerbated by prior abrasion (Powell et al., 2004).

Currently, mouthrot in BC is managed through antibiotic treatments and is the main reason the industry in this region continues to use antibiotics in the production of Atlantic salmon (Morrison & Saksida, 2013). This is mainly due to the fact that no commercial vaccine is currently available for *T. maritimum* in this fish species. Attempts to create a vaccine in Atlantic salmon against tenacibaculosis have given mixed results, from no protection to partial protection (Carson, Schmidtke, & Lewis, 1994; Carson, Schmidtke, & McCosh, 1993; van Gelderen, Carson, & Nowak, 2009).The only protective vaccine used commercially for *T. maritimum* is in turbot (Toranzo et al., 2005). A reproducible challenge model is required for the development of vaccines, including determining the pathogenicity of isolates, virulence factors and the testing of novel vaccines.

In this study, challenge experiments were performed to determine whether mouthrot could be induced with *T. maritimum* isolates collected from outbreaks in Western Canada (Frisch et al., 2017) and to develop a reproducible bath challenge model. A secondary objective was to use this model to test whole-cell adjuvanted vaccines against *T. maritimum* in Atlantic salmon smolts.

2 | MATERIALS AND METHODS

2.1 | Challenge material

The *T. maritimum* isolates used in this study were collected from diseased fish during mouthrot outbreaks on Atlantic salmon farms in BC (Frisch et al., 2017). *Tenacibaculum maritimum* type species NCIMB 2154^T was also used in the first challenge experiment as a comparison. The choice of isolates for the challenge experiments was based on geographic distribution and genotyping results (Frisch et al., 2017).

Aliquots of each of the isolates used in this study were created by inoculating 500 ml of marine broth, Difco, 2216 (MB) with a small amount of culture grown at 16°C on marine agar, Difco, 2216 (MA) in a 2-L flask which was then incubated at 16°C and 230 rpm. After 48–72 hr, when a large quantity of active bacteria could be seen microscopically, the culture material was cryopreserved in 1 ml aliquots containing 20% glycerol at −80°C. To produce the challenge material, one of these 1 ml aliquots was added to 500 ml of MB in a 2-L flask which was incubated at 16°C and 230 rpm for 72 hr. The cell concentration of the bacterial cultures was determined using the most probable number (MPN) method: 10-fold dilutions in duplicate with eight replicates per dilution (Blodgett, 2010; Cochran, 1950). The quantity of challenge culture needed to achieve the required bath concentrations (Table 1) was based on growth curves (data not shown) performed for each isolate prior to the start of this study using the MPN method.

In one of the challenge experiments (experiment 2), a group of fish was challenged with supernatant alone. The supernatant was acquired by centrifuging a culture of isolate TmarCan15-1 at 3,000 *g* for 30 min, decanting out the supernatant, and filtering this liquid through a 5.0-μm syringe filter, followed by a 0.2-μm syringe filter.

2.2 | Fish husbandry

All live fish experiments were conducted at the Aquatic and Industrial Laboratory (ILAB), Bergen, Norway, using flow-through tanks.

TABLE 1 Experimental groups showing number of smolts and challenge bath isolate, concentration and duration (shed refers to shedders, and cohab refers to cohabitants)

Experiment	Group	Number of fish	Isolate	Bacteria bath concentration (cells/ml)	Bath duration (hr)	Accumulated per cent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
Experiment 1	1-1a	10	TmarCan15-1	3.80×10^6	1.5	0	—	—
	1-1b	10	TmarCan15-1	3.80×10^6	5.0	0	—	—
	1-2a	10	TmarCan15-1	1.90×10^7	1.5	30	6	11
	1-2b	10	TmarCan15-1	1.90×10^7	5.0	60	4	10
	1-3a	10	*Tenacibaculum maritimum*^{T}	5.10×10^6	1.5	0	—	—
	1-3b	10	*T. maritimum*^{T}	5.10×10^6	5.0	0	—	—
	1-4a	10	*T. maritimum*^{T}	2.55×10^7	1.5	0	—	—
	1-4b	10	*T. maritimum*^{T}	2.55×10^7	5.0	30	1	6
Experiment 2	2-1a	10	TmarCan16-5	7.30×10^6	5.0	0	—	—
	2-1b	10	TmarCan16-5	7.30×10^6	7.5	0	—	—
	2-2a	10	TmarCan16-1	2.25×10^6	5.0	100	3	6
	2-2b	10	TmarCan16-1	2.25×10^6	7.5	100	4	8
	2-3a	10	TmarCan16-6	1.52×10^7	5.0	100	5	13
	2-3b	10	TmarCan16-6	1.52×10^7	7.5	100	5	10
	2-4a	10	TmarCan15-1	1.87×10^7	5.0	70	7	16
	2-4b	10	TmarCan15-1	1.87×10^7	7.5	40	7	14
	2-5a	10	TmarCan15-1	3.74×10^7	1.5	40	5	10
	2-5b	10	TmarCan15-1	3.74×10^7	5.0	50	3	14
	2-6a	10	Control (marine broth)	500 ml	7.5	0	—	—
	2-6b	10	Control (supernatant)	500 ml	7.5	0	—	—
Experiment 3	3-1	20	TmarCan16-5	1.81×10^7	5.0	5	11	11
	3-2	20	TmarCan16-5	1.81×10^7	5.0	55	3	12
	3-3	20	TmarCan15-1	5.74×10^6	5.0	75	4	8
	3-4	20	TmarCan15-1	5.74×10^6	5.0	90	3	12
	3-5	20	TmarCan16-2	1.28×10^7	5.0	0	—	—
	3-6	20	TmarCan16-2	1.28×10^7	5.0	0	—	—
	3-7	20	TmarCan16-1	6.36×10^5	5.0	100	3	6
	3-8	20	TmarCan16-1	2.45×10^6	5.0	100	4	10
Cohabitation experiment	4-1	20 shed 40 cohab	TmarCan15-1	1.68×10^7	5.0	Shed: 100 Cohab: 75	Shed: 2 Cohab: 9	Shed: 7 Cohab: 20
	4-2	20 shed 40 cohab	TmarCan15-1	1.68×10^7	5.0	Shed: 100 Cohab: 76	Shed: 3 Cohab: 7	Shed: 7 Cohab: 17
	4-3	20 shed 40 cohab	TmarCan16-5	1.78×10^7	5.0	Shed: 95 Cohab: 27	Shed: 2 Cohab: 12	Shed: 16 Cohab: 17
	4-4	20 shed 40 cohab	TmarCan16-5	1.78×10^7	5.0	Shed: 84 Cohab: 31	Shed: 3 Cohab: 10	Shed: 10 Cohab: 20
	4-5	20 shed 40 cohab	TmarCan16-1	8.75×10^5	5.0	Shed: 100 Cohab: 100	Shed: 3 Cohab: 6	Shed: 5 Cohab: 11
	4-6	20 shed 40 cohab	TmarCan16-1	8.75×10^5	5.0	Shed: 100 Cohab: 100	Shed: 3 Cohab: 6	Shed: 6 Cohab: 9
	4-7	20 shed 40 cohab	Control (marine broth)	1 L	5.0	Shed: 0 Cohab: 0	—	—
	4-8	20 shed 40 cohab	Control (no exposure)	N/A	N/A	Shed: 0 Cohab: 0	—	—

Accumulated per cent mortality is shown for each group in the challenge experiments, as well as the time period post-exposure that mortality occurred. In general, the mortality curve for each group had a sigmoid shape.

The Atlantic salmon used in the experiments were supplied by ILAB. For the duration of each experiment, fish were checked at least twice a day on weekdays and once on weekend days and fed ad libitum with the commercial dry feed Nutra Olympic, Skretting AS, Norway. All experiments were conducted in 12°C water. The outlet water in all tanks had a minimum oxygen saturation of 77%, and the water flow was 300 L per hour per tank (regardless of tank size). Except during smoltification, the fish were kept on a 12-hr photoperiod. Whenever smolts were transferred from freshwater to salt water prior to being bath challenged, the salinity was gradually increased to 34 ppt over the first 24-hr period. Prior to all handling, fish were starved for 48 hr, and before vaccination and marking, fish were anaesthetized with tricaine methanesulphonate, Tricaine, PHARMAQ (TMS).

The population of fish were screened and found negative for *Infectious salmon anaemia virus*, *Infectious pancreatic necrosis virus*, salmonid alphavirus, *Piscine orthoreovirus*, *Tenacibaculum* spp. (including *T. maritimum*) and *Moritella viscosa* with real-time RT-PCR prior to the start of the experiments.

Fish showing signs of illness (e.g., ulcerative lesions) and/or abnormal behaviour (e.g., erratic swimming and loss of equilibrium) during the experiments were killed due to the low expectation of fish showing these signs to recover. However, due to the rapidity in the development of the disease, this was not always possible. In this study, the term mortality includes both killed morbid fish and mortality. All morbidity, as well as any fish surviving at the end of each of the experiments, was killed using an overdose of TMS or a swift blow to the head.

The animal experiments were approved by the Norwegian Food Safety Authority (Mattilsynet) in 2016 and 2017 under the identification codes 16/33868, 16/174198, 16/207694 and 17/106558.

2.3 | Challenge model development (experiments 1–3)

Three initial challenge experiments were conducted with three different aims: determine if mouthrot could be experimentally replicated (experiment 1), ascertain whether or not there are differences in pathogenicity between isolates and refine the challenge model (experiment 2) and ensure that the challenge model is replicable (experiment 3). Because of this, the protocols varied between these experiments with details given in Table 1.

The smolts supplied for the challenge experiments were of an average weight of 38 g for experiment 1, 45 g for experiment 2 and 70 g for experiment 3. The smolts were transferred from freshwater and distributed into the 150-L experiment tanks containing salt water (Table 1). The fish were maintained in these tanks for the duration of the experimental infections. After 24 hr of acclimatization, the smolts were transferred into aerated 40-L infection containers with 12°C salt water (34 ppt) and the challenge material as described in Table 1. After the desired exposure time (Table 1), the fish were transferred back into their respective experiment tanks. There were two subgroups, differentiated by exposure times, for each of the main groups in experiments 1 and 2, designated "a" and "b" (Table 1). These were kept in the same experiment tank after the bath infection. To differentiate between the two subgroups, half the smolts were adipose fin-clipped at the time of transfer into salt water. In experiment 2, 10 fish were exposed to MB alone (group 2-6a) and 10 fish to supernatant alone (group 2-6b) for the longest duration as a control. In experiment 3, TmarCan16-2 was included even though it was not tested in experiments 1 or 2. This was done because it grew better compared to the other Western Canadian isolates and would therefore make an ideal vaccine candidate. All experiments were concluded 3 weeks post-bath infection.

2.4 | Cohabitation and horizontal transmission (experiment 4)

The supplied smolts for the cohabitation experiment were of an average weight of 40 g. In the cohabitation experiment, each group comprised 20 shedders (fish that were directly exposed to the bacteria through a bath infection) and 40 cohabitants (naïve fish that were added to the shedder population). At the time of transfer into the 150-L experiment tanks containing salt water, the shedders were labelled by adipose fin clipping. After 24 hr of acclimatization, the shedders were bath infected (Table 1) as described for the challenge experiments. One control group (4-7) was exposed to MB for the same duration as the other groups, and the other control group (4-8) was not handled. The cohabitants (40 per group) were added to their respective tanks 24 hr after the shedders were exposed to the bacteria. The experiment was ended 3 weeks later.

2.5 | Fish sampling

Smolts removed from the tanks were examined for internal and external clinical signs. The gills, mouths and skin of all fish in the cohabitation experiment were scored (Table 2). Scrapings from

TABLE 2 Scoring scheme used in the cohabitation experiment to characterize external clinical signs seen in mortality

Organ	Score	Clinical signs
Gills	0	No abnormality on either side of fish
	1	Lesion on one side of fish
	2	Lesion on both sides of fish
Mouth	0	No abnormality
	1	Mild change—tiny plaque and/or small haemorrhage
	2	Moderate change—small lesion and/or haemorrhage
	3	Severe change (mouthrot)—large plaques and/or large lesion
Skin	0	No abnormality
	1	Mild change—some scale loss and/or small haemorrhage
	2	Moderate change—lesion(s) with scale loss through to skin
	3	Severe change—lesions(s) through to muscle and/or many lesions

external lesions were examined with light microscopy. Reisolation of the bacteria was performed by streaking mucus scraped off these lesions onto Marine Kanamycin Agar (MKA) (Frisch et al., 2017). Kidneys from affected fish were streaked on MA. Cultures were incubated at 16°C for a minimum of 3 days after which colony and cell morphology was recorded. Colonies typical of *T. maritimum* were subcultured on MA and a minimum of two cultures per group were cryopreserved at −80°C, and subsequently, two isolates per group were sequenced to confirm genetic identity with the challenge isolates to support Koch's Postulates (Fredricks & Relman, 1996). Genomic DNA was acquired by placing single colonies into nuclease-free water, heating at 95°C for 5 min, then centrifuging at 9,600 g for 5 min and transferring the DNA-containing supernatant into a new tube. The resulting supernatant was stored at −20°C. PCR was performed using the 16S rRNA gene primers 27F and 1518R (Giovannoni, Rappé, Vergin, & Adair, 1996), as well as the housekeeping gene, *atpA* primers (Habib et al., 2014). The amplification and sequencing were performed as described in Frisch et al. (2017). Obtained sequences were compared to the ones from the challenge material by aligning them using AlignX in Vector NTI, Invitrogen.

The skin of the lower jaw was sampled from five cohabitant mortalities from groups 4-1 to 4-6 in the cohabitation experiment for *T. maritimum* screening with real-time RT-PCR as a confirmation of the presence of the bacteria. Another four "healthy" fish were sampled from the control group 4-7 as a comparison. The samples were sent to a commercial laboratory for analysis.

2.6 | Vaccine formulation

Monovalent oil-adjuvanted vaccines were produced for the isolates TmarCan15-1, TmarCan16-2 and TmarCan16-5. Unfortunately, due to extensive aggregate formation, TmarCan16-1 was deemed unsuitable for vaccine formulation. The bacterial isolates were cultured by inoculating 10 ml of preculture into 400 ml MB in 2-L baffled shaker flasks at 90 rpm for 48 hr at 15°C. The cultures were inactivated with formalin before being concentrated 10 times by sedimentation. Prior to inactivation, the cultures were plated out on blood agar containing 2% NaCl and MA and subject to prolonged incubation at 15°C to verify purity. The concentrated bacterin suspensions were homogenized by pressing between two syringes back and forth 50 times, before formulation into three monovalent oil-adjuvanted vaccines using mineral oil and prepared by a Silverson LR5 rotor–stator mixer according to standard procedures for PHARMAQ vaccines.

The vaccines for the vaccine challenge experiment were produced as described above; however, because a larger volume was required, the protocol had to be adjusted. 1 L of bacterin culture (instead of 400 ml) was incubated in 2-L baffled shaker flasks at 240 rpm, and the formalin-inactivated bacterins were concentrated approximately 30 times by centrifugation at 3,000 *g* for 10 min. The supernatants were removed and pellets were resuspended in 30 ml PBS before homogenization and formulation as described above.

2.7 | Antibody cross-reaction

Antibodies were produced in parr to check their cross-reaction to homologous and heterologous isolates. Parr of an average weight of 32 g were separated into three groups of 15 fish. The fish were marked by fin clipping before being IP injected with 0.1 ml of one of the three formulated vaccines. An unmarked fourth group, in which the fish were injected with 0.1 ml of PBS, was included as control. All the fish were kept in one 500-L tank in freshwater. Twelve weeks post-vaccination (approximately 1,000 day-degrees), the fish received an overdose of TMS prior to blood sampling with heparinized syringes from the caudal vein. The blood samples were centrifuged, and blood plasma was collected. The plasma was then stored at −20°C.

Microtiter plates, Maxisorp™, Nunc prepared with 5 μg/ml Poly-L-lysine, Sigma were coated by adding 100 μl of inactivated bacteria, diluted twofold starting with an $OD_{600\ nm}$ of 0.1. After washing with PBS containing 0.05% Tween-20, Merck (PBST), the plates were blocked for 2 hr at room temperature with 5% skimmed milk in PBST. Plasma was added in 1:100 dilution, and the plates were incubated overnight at 4°C. A monoclonal antibody, mouse anti-Rainbow Trout Immunoglobulin (cross-reacting with Atlantic salmon, produced in-house), was diluted 1:3500 in PBST with 1% skimmed milk, and 100 μl was added to each well and incubated for 1 hr at room temperature. The secondary anti-mouse immunoglobulin conjugated to alkaline phosphatase, Dako, was diluted 1:500 in PBST with 1% skimmed milk, and 100 μl was added to each well followed by 1-hr incubation at room temperature. The plates were washed three times with PBST between the incubations. Bound antibodies were detected by adding 100 μl substrate p-nitrophenyl-phosphate, Sigma in 10% diethanolamine buffer, pH 9.8, Sigma-Aldrich to each well, and the colour reaction was read at $OD_{405\ nm}$ after 60 min.

2.8 | Vaccine challenge (experiment 5)

Three groups of 190 parr (average weight 16 g) were vaccinated with a 0.1 ml dose of one of the three formulated vaccines containing the isolates TmarCan15-1, TmarCan16-2 or TmarCan16-5. A fourth group of 190 parr was vaccinated with 0.1 ml PBS. The fish were marked by adipose fin clipping or maxilla trimming to identify each group. The fish were kept in 500-L freshwater tanks. The parr were triggered to smoltify 4 weeks prior to transfer to salt water by increasing their photoperiod to 24 hr. At 8 weeks post-immunization (approximately 675 degree days), 240 fish (60 per vaccine group) were sorted into their respective groups (Table 3) and transferred into 150-L saltwater tanks. The remaining fish (100 per vaccine group) were sorted and transferred at 12 weeks post-immunization (approximately 1,000 degree days) (Table 3).

Only isolate TmarCan15-1 was used to challenge the vaccinated fish as the other two isolates used in the vaccines (TmarCan16-2 and TmarCan16-5) were not causing reproducible mortality. The challenge material was produced as previously described, except that 1 L of MB (instead of 500 ml) was inoculated in each 2-L flask. The

8-week groups (5-1 to 5-3) were bath challenged 24 hr post-transfer as described in the challenge experiments using isolate TmarCan15-1 at a concentration of 4.35×10^7 cells/ml for 5 hr. The 12-week groups (5-4 to 5-8) were bath challenged for 2 hr 48 hr post-transfer into salt water with isolate TmarCan15-1 at a concentration of 3.24×10^7 for groups 5-4 and 5-5 and 3.50×10^7 for groups 5-6 and 5-7. Group 5-8 was bath challenged with marine broth as a control. The vaccine experiment was concluded 3 weeks post-bath infection.

At 0 (unvaccinated fish), 8 and 12 weeks (approximately 675 and 1,000 degree days), 10 fish per vaccine group were killed with an overdose of TMS and blood sampled with heparinized syringes from the caudal vein to measure the antibody response at the time of challenge. For the 8-week group, 15 fish were sampled instead of 10 with the exception of the group vaccinated with the TmarCan16-5 vaccine, where only four fish were sampled. The blood samples were centrifuged and the blood plasma stored at −20°C for subsequent ELISA analysis. The ELISA was performed as described above with the exception that the bacterial coat had an $OD_{600\ nm}$ of about 0.05, and the plasma was diluted twofold starting with 1:50, for the purpose of estimating plasma antibody response against the homologous vaccine isolate.

3 | RESULTS

3.1 | Experiment 1: Koch's postulates

Moribund fish showed loss of equilibrium and circling behaviour at the surface. Externally, there was a mix of abnormalities ranging from small mouth lesions (Figure 1a), to gill lesions (Figure 1c) and small skin lesions. These lesions resemble what is seen during outbreaks on farms (Figure 1b,d). Smears from these lesions showed large amounts of long thin rod-shaped bacteria, matching the phenotypic description of *T. maritimum* (Suzuki et al., 2001). The isolated bacteria were shown to be genetically identical with both the 16S and *atpA* genes to the challenge material (data not shown). The accumulated mortality percentage for each experimental group is shown in Table 1.

The first challenge experiment demonstrated that the disease could be replicated in the laboratory setting and that *T. maritimum* NCIMB 2154^T is not as pathogenic as the Western Canadian strain TmarCan15-1. Disease was mainly seen in the group exposed to the highest concentration of bacteria for the longer duration.

3.2 | Experiment 2: Virulence differences between isolates

Based on the results from experiment 1, four isolates were used in experiment 2 including TmarCan15-1. Also, because of the gill lesions present in some of the affected fish, one group was exposed to MB, and one to the supernatant alone to rule these out as the cause of the damage. Two of the strains, TmarCan16-1 (groups 2-2a and 2-2b) and TmarCan16-6 (groups 2-3a and 2-3b) resulted in 100% mortality with the former acting faster at a lower bacterial bath concentration (2.25×10^6 cells/ml versus 1.52×10^7 cells/ml). The more acutely affected fish showed fewer gross clinical signs than the more chronically affected ones. TmarCan16-5 (2-1a and 2-1b) resulted in no mortality in experiment 2 regardless of exposure time; however, the bath concentration was lower at 7.30×10^6 cells/ml. No difference was observed between using 5 or seven and a half hours for the bath duration; however, one and a half hours seemed too short as it gave more varied results. Neither of the control groups in experiment 2 (2-6a and 2-6b) had mortality, and none of the fish showed signs of disease.

3.3 | Experiment 3: Replicable challenge model

The isolates used in experiment 3 were chosen as these were the most promising in regards to vaccine development. Again, TmarCan16-1 (groups 3-7 and 3-8) gave 100% mortality even at the lower bath concentration tested. TmarCan16-2 (groups 3-5 and 3-6) gave 0% mortality and no fish showed signs of disease. TmarCan16-5 (groups 3-1 and 3-2) gave variable results when comparing the duplicate groups.

From experiments 1-3, isolate TmarCan15-1 produces the most reproducible challenge model. The variation in mortality between the isolates at similar bath concentrations in experiment 2 and 3 shows that there are differences in pathogenicity between isolates.

3.4 | Experiment 4: Cohabitation and horizontal transmission

Figure 2 shows the accumulated mortality in both shedders and cohabitants for each group. Fish in the control groups exhibited no signs of clinical disease or mortality. For groups 4-1 and 4-2 (TmarCan15-1), the shedders had 100% mortality within 7 days of bath infection (Figure 2a), which is higher than the previous challenge experiments for this isolate. The cohabitants in these two groups started showing signs of disease on day 6 post-transfer into the tanks and resulted in around 75% mortality in both groups (Figure 2a). Shedder mortality in groups 4-3 and 4-4 (TmarCan16-5) was between 80% and 95% (Figure 2b), which is also higher than in the challenge experiments for this isolate. Disease in the shedders started on day 2 post-exposure and continued to day 16. Cohabitants started to show signs of disease 9 days post-transfer into the tanks and accumulated mortality at the end of the experiment was about 30% (Figure 2b). The third isolate (TmarCan16-1) used in the cohabitation experiments caused 100% mortality in both shedders and cohabitants within 9 days of exposure (Figure 2c). Mortality in the shedders started 2 days post-exposure and mortality in the cohabitants started 3 days later (4 days post-transfer).

Both shedders and cohabitants in groups 4-5 and 4-6 (TmarCan16-1) presented with less external lesions when compared to the other groups. Affected fish in groups 4-1 and 4-2 (TmarCan15-1) had more gill lesions in the shedders than the other two isolates, but less severe mouth and skin lesions than in groups 4-3 and 4-4

TABLE 3 Experimental groups for the vaccine experiment showing number of smolts per group

Group	Vaccine	Number of fish	Bacteria bath concentration (cells/ml)	Bath duration (hr)	Accumulated per cent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
5-1	TmarCan15-1	20	4.35×10^7	5.0	80	5	12
	TmarCan16-2	20			75	4	10
	TmarCan16-5	20			90	5	10
	PBS (control)	19			74	5	10
5-2	TmarCan15-1	20	4.35×10^7	5.0	75	5	17
	TmarCan16-2	20			60	5	16
	TmarCan16-5	20			85	5	10
	PBS (control)	20			50	5	10
5-3	TmarCan15-1	19	4.35×10^7	5.0	84	6	16
	TmarCan16-2	20			60	5	13
	TmarCan16-5	20			80	4	13
	PBS (control)	20			75	4	18
5-4	TmarCan15-1	20	3.24×10^7	2.0	70	5	11
	TmarCan16-2	20			80	4	12
	TmarCan16-5	20			75	4	13
	PBS (control)	20			80	4	17
5-5	TmarCan15-1	20	3.24×10^7	2.0	85	4	12
	TmarCan16-2	20			65	5	14
	TmarCan16-5	20			60	5	10
	PBS (control)	20			60	5	10
5-6	TmarCan15-1	20	3.50×10^7	2.0	85	4	18
	TmarCan16-2	20			90	6	13
	TmarCan16-5	20			75	5	14
	PBS (control)	20			75	4	13
5-7	TmarCan15-1	20	3.50×10^7	2.0	95	5	17
	TmarCan16-2	20			75	5	13
	TmarCan16-5	20			95	5	17
	PBS (control)	20			60	6	14
5-8	TmarCan15-1	20	Control (marine broth)	2.0	0	—	—
	TmarCan16-2	20			0	—	—
	TmarCan16-5	20			0	—	—
	PBS (control)	20			0	—	—

Groups 5-1 to 5-3 were bath challenged 8 weeks post-immunization, and groups 5-4 to 5-8 were bath challenged 12 weeks post-immunization. All groups were challenged with TmarCan15-1. Accumulated per cent mortality is shown for each group, as well as the time period post-exposure that mortality occurred. In general, the mortality curve for each group had a sigmoid shape.

(TmarCan16-5). Affected cohabitants in groups 4-1 to 4-4 showed little gill lesions, but nearly all fish showed signs of mouth and skin lesions. The percentage of mortality showing clinical signs and their severity is shown in Figure S1. In general, fish that had an acute disease (within the first week post-exposure) exhibited less external lesions than ones that had a more chronic presentation.

All fish sampled for *T. maritimum* screening with real-time RT-PCR in groups 4-1 to 4-6 were positive for the bacteria, and the control fish were negative.

3.5 | Antibody cross-reaction

The antibody responses against the three vaccinated bacterial antigens were strong against the homologous bacteria, which demonstrate that the immune system of the host was capable of producing antibodies recognizing *T. maritimum* (Figure 3). More importantly, the results showed that plasma from fish vaccinated against TmarCan15-1 produced antibodies with the same specificity against TmarCan16-5, demonstrated by the comparable binding pattern of

FIGURE 1 (a) Mouth lesion showing the typical yellow plaque, and (c) gill lesion from a mouthrot-affected fish from a farm in British Columbia, Canada. (b) Mouth lesion and (d) gill lesion from a diseased fish in the challenge experiments [Colour figure can be viewed at wileyonlinelibrary.com]

the homologous isolate (Figure 3a). The same pattern was observed using plasma from fish vaccinated against TmarCan16-5 that bound to TmarCan15-1 (Figure 3c). Vaccinating against TmarCan16-2 induced plasma antibodies with the same specificity towards TmarCan16-1 as to the homologous strain (Figure 3b). Some cross-reaction was also observed against TmarCan15-1 and TmarCan16-5.

3.6 | Experiment 5: Vaccine challenge

No difference was seen between vaccine groups (both 8-week and 12-week groups) and the controls vaccinated with PBS. Mortality in all groups ranged from 50% to 95%, starting 4–6 days post-exposure and followed a sigmoid pattern which plateaued around day 14 post-exposure. The groups bath challenged with MB showed no sign of disease or mortality for the duration of the experiment. Mortality results for the vaccine challenge experiment are presented in Table 3. ELISA analysis of the plasma samples from vaccinated fish (both at 8 and 12 weeks) taken just prior to being bath challenged demonstrated that smolts in all three vaccine groups had developed antibodies towards the vaccine isolates.

4 | DISCUSSION

Western Canadian *T. maritimum* isolates have been shown to produce disease, which resembles mouthrot as it is seen in the field, in smolts without prestressors or coinfection. Differences in virulence between the isolates were observed. A variation in virulence among *T. maritimum* strains has also been shown in other studies (Avendaño-Herrera et al., 2006a; Rahman, Suga, Kanai, & Sugihara, 2014; van Gelderen et al., 2010). Interestingly, these differences seem to be evident within Western Canadian genotypic strains. For example, TmarCan16-1 and TmarCan16-2 are genetically identical on 16S rRNA gene sequence and 11 housekeeping genes sequences (Frisch et al., 2017). This study has shown that TmarCan16-1 causes 100% mortality with a bath concentration as low as 8.75×10^5 cells/ml, whereas disease has not been induced with TmarCan16-2, even at concentrations as high as 1.28×10^7 cells/ml. This highlights the importance of full genomic studies of *T. maritimum* to identify the genes responsible for virulence markers, as has been undertaken for this species type strain (Pérez-Pascual et al., 2017).

Based on the ELISA results, the four *T. maritimum* strains (TmarCan15-1, TmarCan16-2, TmarCan16-2 and TmarCan16-5) seem to form two different serogroups, which coincides with the genotyping observed for the same four strains (Frisch et al., 2017). TmarCan15-1 and TmarCan16-5 are recognized by the immune system as one serogroup, with some degree of cross-reaction against TmarCan16-2. The other two strains, TmarCan16-1 and TmarCan16-2, can be grouped in another serogroup with cross-reactivity against TmarCan15-1 and TmarCan16-5. The development of improved serotype-specific antibodies is needed to fully determine the serological mapping of *T. maritimum* strains in the future.

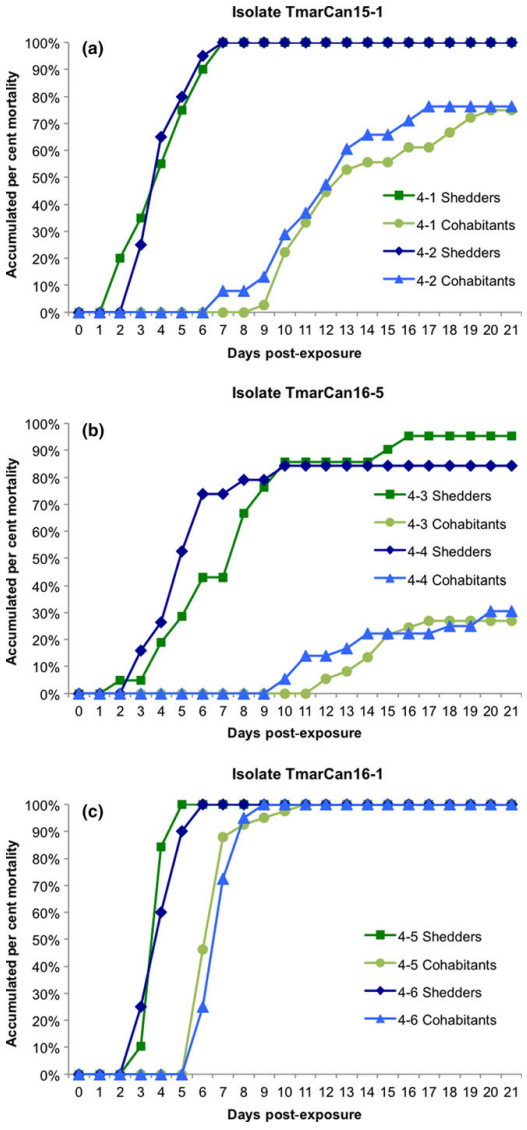

FIGURE 2 Accumulated per cent mortality from the cohabitant experiment [Colour figure can be viewed at wileyonlinelibrary.com]

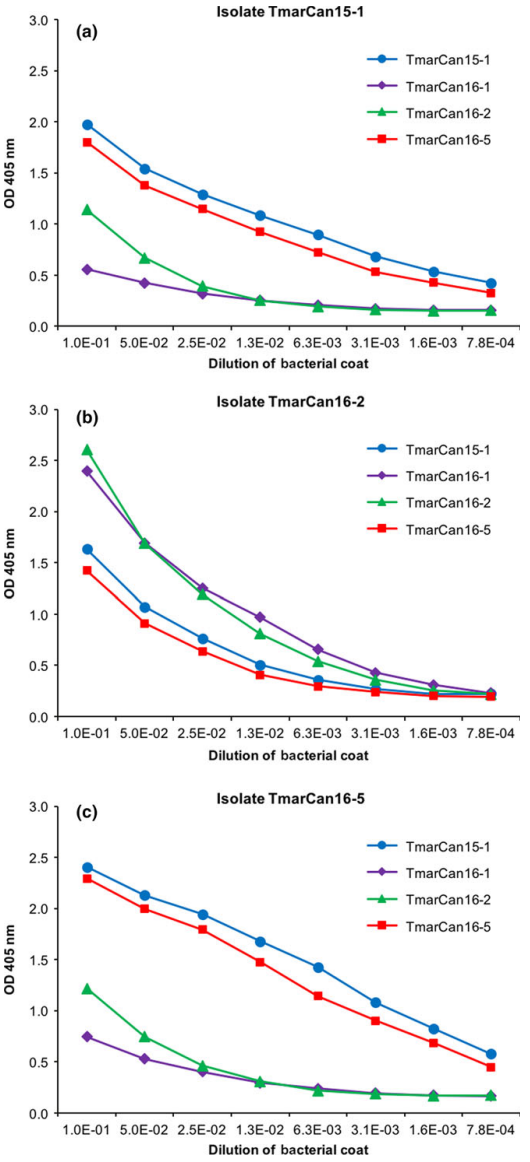

FIGURE 3 ELISA results showing the antibody binding from the plasma of fish injected with a monovalent vaccine containing isolate (a) TmarCan15-1, (b) TmarCan16-2 or (c) TmarCan16-5 to plates coated with a dilution series of four isolates: TmarCan15-1, TmarCan16-2, TmarCan16-5 and TmarCan16-1. Plasma was collected 12 weeks post-immunization [Colour figure can be viewed at wileyonlinelibrary.com]

The monovalent adjuvanted vaccines tested in this study did not give protection against the disease in the conditions tested. The absence of vaccine efficacy cannot be attributed to the lack of a plasma antibody response. A study using Australian *T. maritimum* strains and performed in Tasmania showed that a whole-cell inactivated vaccine gave Atlantic salmon smolts partial protection against the disease when adjuvanted (van Gelderen et al., 2009). However, the challenge conditions and isolates used were different, as were the clinical signs observed in the diseased individuals which showed higher levels of skin lesions when compared to this study (van Gelderen et al., 2009). One of the challenges with the model used for testing these vaccines is that the fish are exposed to one high dose of bacteria which may not reflect the natural infection

pressure as suggested by Karlsen, Thorarinsson, Wallace, Salonius, and Midtlyng (2017).

Creating a challenge model for *T. maritimum* is difficult primarily due to the highly adhesive property of the bacteria (Magariños, Pazos, Santos, Romalde, & Toranzo, 1995). Because of this, we found that estimating bacterial growth and cell concentrations was challenging. The use of surfactants or detergents to avoid bacterial aggregates in broth culture has been unsuccessful, and the best technique for growing the bacteria is rigorous shaking (Mabrok et al., 2016), which is the method we used. Regular methods of bacterial cell concentration measurements such as optical density (OD) and cell chamber counting were found to be unreliable due to poor repeatability. The clumping nature of *T. maritimum* is likely the reason for OD variations, and the gliding motility of the bacteria hampers cell counting. The McFarland standard has also been used in estimating *T. maritimum* concentration (Faílde et al., 2014); however, we found that the scale did not give precise enough estimates for their purposes. MPN was therefore used to estimate culture cell concentrations in these experiments; this has the drawback of giving retrospective counts and making the challenge model difficult to replicate.

The adhesive nature of *T. maritimum* means that the bacteria create a biofilm on surfaces, including plastic ones, which can make it challenging to target specific doses in bath infections. We tried to challenge the fish in the same tank that they were kept in during a pre-experiment; however, there was a visible biofilm formation on the tank wall at the water surface which lingered for at least 24 hr after the water flow was turned back on. The presence of a biofilm could possibly extend the challenge duration.

The water quality parameters used in this study were chosen so that the challenge model, if successful, could be used for vaccine development and therefore need to follow regulatory guidelines, for example the European Medicines Agency (EMA) guidelines (CVMP 2012). As such, temperature and salinity needed to reflect the environment under which the vaccine would be used for commercial purposes. The temperature of 12°C used in all the experiments is much lower than previously performed challenge studies involving *T. maritimum*. This is particularly relevant when comparing to other experiments previously performed with Atlantic salmon smolts, where temperatures up to 20°C were used (Handlinger et al., 1997; Soltani et al., 1996; van Gelderen et al., 2010, 2011). These higher temperatures are at the upper range of optimal rearing conditions for Atlantic salmon smolts, which may have had an influence on results (Jonsson, Forseth, Jensen, & Næsje, 2001). Based on this study, a bath infection using a separate infection tank and a high concentration (dependent on isolate pathogenicity) for a short duration gives the most reproducible challenge model in Atlantic salmon smolts. Improving the evaluation of bacterial culture concentration would allow for the improvement of this method.

The cohabitation experiment demonstrates that horizontal transfer occurs easily for Western Canadian *T. maritimum* strains. This is an interesting finding in view of the fact that a previous study has shown that the bacteria does not survive well in sea water (Avendaño-Herrera, Irgang, Magariños, Romalde, & Toranzo, 2006). The transmission between fish of *T. maritimum* may be of concern to the Norwegian salmon farming industry, as a closely related strain to the ones found in BC was associated with disease on lumpsuckers, which are frequently used as biological sea lice controls (Frisch et al., 2017; Småge, Frisch, Brevik, Watanabe, & Nylund, 2016). Further studies are needed to determine whether or not this is a valid concern.

5 | CONCLUSION

The reproduction of the disease in the laboratory with isolates collected from mouthrot outbreaks, as well as the reisolation of the bacteria from these diseased individuals, fulfils Koch's Postulates, which is the preferred method for proving disease causation (Fredricks & Relman, 1996). This study therefore shows that *T. maritimum* is the causative agent of mouthrot in BC. Despite giving an antibody response in the immunized fish, the trialled whole-cell oil-adjuvanted vaccines did not give protection under the tested conditions. The results from the cohabitation experiment show that *T. maritimum* readily transfers from fish to fish.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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III

Pathology of experimentally induced mouthrot caused by *Tenacibaculum maritimum* in Atlantic salmon smolts

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Short Title: Pathology of experimentally induced mouthrot

Abstract

Mouthrot, caused by *Tenacibaculum maritimum* is a significant disease of farmed Atlantic salmon, *Salmo salar* on the West Coast of North America. Smolts recently transferred into saltwater are the most susceptible and affected fish die with little internal or external clinical signs other than the characteristic small (usually < 5 mm) yellow plaques that are present inside the mouth. The mechanism by which these smolts die is unknown. This study investigated the microscopic pathology (histology and scanning electron microscopy) of bath infected smolts with Western Canadian *T. maritimum* isolates TmarCan15-1, TmarCan16-1 and TmarCan16-5 and compared the findings to what is seen in a natural outbreak of mouthrot. A real-time RT-PCR assay based on the outer membrane protein A specific for *T. maritimum* was designed and used to investigate the tissue tropism of the bacteria. The results from this showed that *T. maritimum* is detectable internally by real-time RT-PCR. This combined with the fact that the bacteria can be isolated from the kidney suggests that *T. maritimum* becomes systemic. The pathology in the infected smolts is primarily mouth lesions, including damaged tissues surrounding the teeth; the disease is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with little associated inflammation. Skin lesions are more common in experimentally infected smolts than in natural outbreaks, but this could be an artefact of the challenge dose, handling and tank use during the experiments.

Introduction

Tenacibaculosis is a disease characterized by frayed fins, tail rot, mouth erosion, and skin lesions that are often ulcerative; it causes significant losses in a number of economically important marine fish species worldwide [1,2]. Three species belonging to the genus *Tenacibaculum* have been associated with this clinical presentation in farmed Atlantic salmon (*Salmo salar*): *Tenacibaculum dicentrarchi* [3], *Tenacibaculum finnmarkense* [4-6], and *Tenacibaculum maritimum* [7]. However, the clinical presentation of *T. maritimum* infections in Atlantic salmon smolts in the Pacific Northwest (British Columbia (BC), Canada and Washington, USA) is different from classical tenacibaculosis (as described above) and is commonly referred to as mouthrot [8-11]. Cultured Pacific salmon species (e.g. Chinook salmon, *Oncorhynchus tshawytscha*) in the Pacific Northwest appear to be resistant to developing mouthrot [9].

Mouthrot typically affects smolts recently transferred into saltwater, and has been present in the Pacific Northwest since the late 80s [12]. Due to a lack of preventative measures against this disease, mouthrot continues to be the main reason that antibiotics are used in the production of Atlantic salmon in the region [13]. Mouthrot is generally diagnosed by the presence of distinctive yellow plaques associated primarily with the teeth of affected smolts [10,14]. This clinical manifestation of *T. maritimum* infections has not been reported in any other Atlantic salmon farming region even in areas where *T. maritimum* is present.

The pathology of mouthrot in the Pacific Northwest was first described in the early 90s, before the bacterial agent was identified [12,14]. Gross pathology includes focal yellow bacterial mats around the palate and teeth. The lesions range from small and hardly visible to multiple with erosion of the upper and/or lower jaw in severe cases [14]. Microscopic

examination of these lesions were described as “mats of *Cytophaga*-like filamentous bacteria associated with areas of ulceration and necrosis often extending into the underlying bone" [12]. Major taxonomical revisions have since identified these “*Cytophaga*-like” bacteria as *T. maritimum* [15,16]. Diseased individuals die with little or no other gross external or internal lesions other than these typical “yellow plaques” in the mouth, and there is no evidence of concurrent disease [10].

When Atlantic salmon smolts are experimentally bath infected with one high dose of Western Canadian *T. maritimum*, clinical signs are not exclusive to the mouth; the gills and skin can also be affected [11]. Necrotic gill lesions have sometimes been observed in mouthrot affected smolts in BC (personal observations, Frisch); however, this is not a common finding. Gill lesions associated with this bacterium have also been noted in naturally and experimentally infected Atlantic salmon smolts in Tasmania [7,17] and Chinook salmon in California [18]. Skin lesions are also more common in experimentally infected smolts than in natural outbreaks, but this could be an artefact of the experiments [11].

The mechanism by which *T. maritimum* kills Atlantic salmon smolts in the Pacific Northwest while only causing very small mouth lesions continues to be a mystery. This study describes for the first time the pathology associated with experimentally induced mouthrot and compares it to what is normally seen in natural outbreaks of this disease. Tissue tropism of the bacteria, using the newly developed real-time RT-PCR is also investigated.

Materials and Methods

Real-time RT-PCR for *T. maritimum*

Prior to this publication, there was only one published real-time RT-PCR assay specific for *T. maritimum* [19]. The assay targets the 16S rRNA gene and was tested using DNA [19]. However, the 16S rRNA gene has low phylogenic resolution at the species level when compared to other genes [20], and real-time RT-PCR assays based on this gene may not be very specific. The new real-time RT-PCR assay (Tmar_ompA) targets the outer membrane protein A (*ompA*) gene (forward primer: GCCAATAGCAACGGGATACC, reverse primer: TCGTGCGACCATCTTTGGT, probe: TGAATCAAATGCGATCTT). An alignment of the *ompA* gene using available *Tenacibaculum* spp. sequences in the GenBank and from the *T. maritimum* strains TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402 [16,21,22] (also available in the GenBank) was used during the design of the assay.

The specificity of Tmar_ompA, based on this alignment, was tested using RNA extracted from clonal cultures of *Tenacibaculum* spp. The aim for the assay was to amplify *T. maritimum* strains NCIMB 2154^{T}, TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402 [16,21,22], and not to amplify *Tenacibaculum adriaticum* DSM18961^{T}, *Tenacibaculum dicentrarchi* USC35/09^{T}, *Tenacibaculum finnmarkense* HFJ^{T}, *Tenacibaculum ovolyticum* EKD-002^{T} and *Tenacibaculum soleae* LL0412.1.7^{T}. To compare this new assay to the already published one, these RNA samples were also tested using the assay developed by Fringuelli, Savage [19]. Tmar_ompA was optimized and the efficiency determined using 10-fold dilutions of RNA extracted from TmarCan15-1 [16] and from known positive skin tissue samples from the cohabitation experiment described in Frisch, Småge [11].

All RNA was extracted using TRI Reagent (Sigma-Aldrich) following the manufacturer's protocol, except that an additional washing step using 100% ethanol was performed prior to air drying the RNA pellet. Extracted RNA was stored at -80 ºC. All assays were run using an

AgPath-ID kit (Thermo Fisher Scientific) with 2 µL of RNA and the standard concentrations of primers (400 nM) and probe (120 nM). Each run consisted of 45 cycles.

Cohabitation experiment

Tissue samples from a previously published cohabitation experiment [11] were used to investigate the tissue tropism of the bacteria through real-time RT-PCR screening. In this experiment six groups of 20 Atlantic salmon smolts (shedders) were bath infected with three different isolates of *T. maritimum* (TmarCan15-1, TmarCan16-1 and TmarCan16-5) that came from natural mouthrot outbreaks on BC Atlantic salmon farms [16]. The shedders were bath infected for 5 hours in 12 ºC saltwater (34 ppt) using one of the above isolates (groups 4-1 and 4-2 with 1.68 x 10^7 cells mL^{-1} TmarCan15-1, groups 4-3 and 4-4 with 1.78 x 10^7 cells mL^{-1} TmarCan16-5 and groups 4-5 and 4-6 with 8.75 x 10^5 cells mL^{-1} TmarCan16-1). Two additional groups of 20 shedders were used as controls (4-7 and 4-8), one bath infected with 1 L marine broth (Difco 2216) (MB) and the other untouched. 24 hours post-bath infection, 40 smolts (cohabitants) were added to each group. The husbandry conditions are described in Frisch, Småge [11] and results are summarized in Table 1. The mouth, gill and skin lesions visible macroscopically on mortality were scored as described in Frisch, Småge [11] and are summarized in Table 2.

The cohabitation experiment was approved by the Norwegian Food Safety Authority (Mattilsynet) under the identification code 16/207694.

Cohabitation experiment tissue screening

The mouth and gills of five diseased cohabitants from each group were sampled with the exception of the 2 control groups that had no mortality. The brain, heart, kidney and skin

mucus were also sampled from two smolts of each of these groups. At days 7 and 14 post-infection, two randomly selected cohabitants were sampled (mouth, gills, brain, heart, kidney and skin mucus) from each group. However, due to the rapid mortality in groups 4-5 and 4-6, this was not possible in these groups. The day 7 samples in group 4-2 were also missed. All samples were collected aseptically and kept on ice and then stored at -20 ºC. Moribund smolts and randomly selected cohabitants were euthanized with a swift blow to the head.

RNA was extracted from each of these samples and screened using the Tmar_ompA assay using the above protocol. An assay targeting the elongation factor 1 alpha (EF1A) was used on the mouth, gills, brain, heart and kidney samples as an endogenous control (forward primer: CCCCTCCAGGACGTTTACAAA, reverse primer: CACACGGCCCACAGGTACA, probe: ATCGGTGGTATTGGAAC) [23]. Due to the variability of an endogenous control such as EF1A in skin mucus, these samples were spiked with cultured *Halobacterium salinarum* DSM 3754^{T} cells suspended in PBS prior to the RNA extraction. This exogenous control was detected using the Sal assay (forward primer: GGGAAATCTGTCCGCTTAACG, reverse primer: CCGGTCCCAAGCTGAACA, probe: AGGCGTCCAGCGGA) [24].

Microscopic pathology

Representative tissues from the lesions (mouth, skin and gills) of diseased fish sampled from Atlantic salmon smolts bath infected with BC strains of *T. maritimum* [11] were fixed in 10 % neutral buffered formalin solution and kept at 4 ºC until processing. The tissue processing and sectioning for histology were performed by a commercial laboratory. Histology sections were stained with hematoxylin and eosin (H&E). Histology sections from a diseased smolt from a natural outbreak of mouthrot at a BC farm were used as a reference (Fig 1).

Tissues (mouth and skin) from experimentally infected smolts were also selected for scanning electron microscopy (SEM) examination. Preparation of tissues for SEM was performed as described in Småge, Frisch [21].

Results

Real-time RT-PCR for *T. maritimum*

The Tmar_ompA assay is specific to *T. maritimum* based on the testing of RNA extracted from the *T. maritimum* strains (*T. maritimum* strains NCIMB 2154^T, TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402) and RNA extracted from other *Tenacibaculum* species (*T. adriaticum* DSM18961^T, *T. dicentrarchi* USC35/09^T, *T. finnmarkense* HFJ^T, *T. ovolyticum* EKD-002^T, and *T. soleae* LL0412.1.7^T). When compared to assay developed by Fringuelli, Savage [19], Tmar_ompA is less sensitive (S1 Table). The efficiency of Tmar_ompA is 1.9138 for pure *T. maritimum* culture (TmarCan15-1) and 1.9386 for *T. maritimum* positive skin tissue (S1 Table).

Cohabitation experiment tissue screening

All samples from diseased cohabitants were positive for *T. maritimum* using the newly developed Tmar_ompA assay (S2 Table). Bacterial loads were higher in the gills and mouth of the groups exposed to the two less pathogenic isolates (TmarCan15-1 and TmarCan16-5). Results from the heart, brain and kidney samples showed that *T. maritimum* was in all three of these tissues in clinically affected cohabitant fish, indicating that the bacteria or the detected segments become systemic. *T. maritimum* was also detected in most of the sampled tissues in the randomly sampled non-diseased cohabitants (S2 Table). Although a majority of

these were positive, not all internal tissues were positive in all individuals. Cohabitants from the control groups were screened by Frisch, Småge [11] and were negative for *T. maritimum*.

Clinical signs

As described in Frisch, Småge [11], Atlantic salmon smolts bath infected BC *T. maritimum* strains from BC presented with very few external (Fig 2) or internal clinical signs. Mouth lesions were the most common finding, with some fish also having skin and/or gill lesions. Mouth lesions were usually on or surrounding the teeth and tongue (Fig 2B) and were associated with a slime layer that generally had a yellow tinge. This slime contained a large quantity of long thin rod-shaped bacteria with *T. maritimum* morphology [11]. When lesions were on the skin (Fig 2A) or gills (Fig 2C), these were also linked with a slime layer containing large amounts of bacteria with *T. maritimum* morphology.

Microscopic pathology

In the experimentally infected smolts, histopathological changes are mainly present in the mouth, and some fish have gill and/or skin lesions. Generally, these changes are associated with the gross lesions (Fig 2). The gross oral lesions (Fig 2B) are microscopically associated with mats of long thin rod-shaped bacteria matching what is described for *T. maritimum* (Fig 3 and 4). The severity of the histopathology varies between individuals. The distance between intact epidermis with no signs of structural damage to an open ulcer with large quantities of bacteria is very short (Fig 3). In most cases, little or no inflammation surrounds lesions (Fig 3B). Large quantities of bacteria with *T. maritimum* morphology are present in the gingival pockets surrounding the teeth and these are often loose and, in some cases, falling out or completely missing (Fig 3 and 4). In severe cases, normal tissue structures are replaced by a structureless mass of large amounts of bacteria and cellular debris (Fig 4).

Most of the examined gills from the experimentally infected smolts have no microscopic changes associated with disease and were deemed “healthy”; however, gills with macroscopic lesions have significant microscopic changes (Fig 5). As with the mouth lesions, there is a total loss of cell and tissue structure linked to these lesions with little or no inflammation and large amounts of bacteria with *T. maritimum* morphology. Most of the gill lesions occurred at the curve of the gill arch (Fig 2C and 5A). The tip of the filaments in affected areas is completely destroyed and replaced by a thick layer of bacteria with *T. maritimum* morphology (Fig 5A). The distance between the ulcer and the intact filaments of the gills is very short (Fig 5A and 5B). Only remnants of the lamellae are within the ulcer (Fig 5B and 5C).

The skin lesions that developed during the experiments were associated with scale pocket edema. Total destruction of the underlying tissue is replaced with mats of bacteria with *T. maritimum* morphology. The SEM micrographs support the histopathological findings. Large aggregates of bacteria with *T. maritimum* morphology are in the areas of tissue destruction and surrounding the teeth (Fig 6). Cellular debris is clearly visible within these bacterial mats (Fig 6C and 6D). The bacteria are embedded in the surface of some of the teeth (Fig 7). Some teeth are fractured and bacterial aggregates are within the exposed pulp of these teeth (Fig 8). Bacterial mats and aggregates with associated tissue destruction are also in the skin lesions (Fig 9).

Discussion

The macroscopic and microscopic findings of experimentally induced mouthrot described in this study match the pathology in field cases (Fig 1), as well as what is described in the

literature [12,14]. Comparing our findings to publications is difficult as most of these were written in the 1980s and 1990s before the *Tenacibaculum* genus was described and it is therefore difficult to make a meaningful comparison. Bacterial mats with *T. maritimum* morphology typically surround the teeth, and bacterial cells are seen within the gingival epithelium invading the tissues below. This suggests that the bacteria proliferate in the gingival pockets surrounding the teeth and spread to the surrounding tissues as was described by Frelier, Elston [12]. The SEM micrographs (Fig 6) add to the picture by showing that the bacteria adhere to the tooth surface and epithelium, creating large aggregates. This is associated with destruction of the surrounding tissues.

Skin lesions with associated scale pocket edema that matched the description by Handlinger, Soltani [7] occurred in a subset of Western Canadian *T. maritimum* experimentally infected smolts, particularly ones with a more chronic presentation [11]. Skin lesions, which are not common in natural outbreaks of mouthrot, may be attributed to the use of tanks that result in a greater potential for physical skin abrasions than saltwater net-pens. The use of dip nets to transfer the smolts in and out of the challenge tanks may also have contributed to this by disrupting the protective mucus layer and causing scale loss. The greater prevalence of gill lesions in experimentally infected smolts might be due to the clumping nature of *T. maritimum* that may create bacterial aggregates capable of lodging themselves in the gill filaments during respiration. This hypothesis is supported by the finding in the cohabitation experiment that fewer cohabitants had gill lesions than the shedders that were directly exposed to the bacterial culture during the bath infection (Table 2) [11].

The reasons why *T. maritimum* targets the teeth and surrounding mucosa in mouthrot are not fully understood. However, the teeth are a high source of calcium that has been shown to

promote the growth of *T. maritimum* [26] and thus may contribute to the affinity for this particular tissue. Also, a gene encoding a collagenase has been identified in the whole genome sequence of *T. maritimum* [27] and likely the reason why high levels of *T. maritimum* are present in the collagen-rich submucosa (Fig 3 and 4). *T. maritimum* is also strongly adhesive to hydrophobic surfaces, including fish mucus [28,29]. This ability to adhere and colonize is an important first step for pathogenic bacteria to invade the host [30]. This is likely the main mechanism by which *T. maritimum* is able to create biofilms so effectively. Biofilms, created by many pathogenic bacteria including *Staphylococcus aureus*, provide resistance against many host defense mechanisms [31], and may explain the low level of immune response in mouthrot.

We developed a new real-time RT-PCR assay based on the *ompA* gene that is as specific but less sensitive than the published assay based on the 16S rRNA gene [19]. The results from the real-time RT-PCR tissue screening performed in this study and the recovery of the bacteria from kidneys of experimentally diseased fish [11] provide evidence that mouthrot is a systemic disease. However, no significant pathology occurred in internal organs [11]. This is further supported by the fact that when examining mouthrot affected smolts from the field, lesions in other organs are not obviously associated with mouthrot but further research is required to determine if such a link exists (personal communication, Gary Marty). The microscopic pathology of the mouth suggests that *T. maritimum* might be entering the highly vascular tooth pulp (Fig 8) that might provide an entry point to the bloodstream, to then become systemic. This hypothesis matches what is described for periodontal disease in mammals. The lack of visible internal pathology, as well as the lack of observable inflammatory response may reflect the acuteness of the disease and resulting rapid tissue destruction. This is likely due to toxins with high proteolytic activity produced by *T.*

maritimum [7,27,32-34].

The real-time RT-PCR screening of the cohabitants showed that the external tissues (gills, mouth and mucus) of the fish infected with TmarCan16-1 had a lower load of *T. maritimum* than TmarCan15-1 and TmarCan16-5. This is interesting in view of the fact that this isolate results in a more rapid and severe disease (Table 1) with less severe gross clinical signs (Table 2). This relationship between highly pathogenic strains and a lack of severe lesions has previously been noted before for *Flavobacteria* [30]. The real-time RT-PCR results are therefore not an indicator of pathogenicity. Variation in pathogenicity between *T. maritimum* strains has been shown in other studies, including other fish species [2,35,36]. Differences in pathogenicity also occur between isolates belonging to the same multilocus sequence type (genetically identical on 11 housekeeping gene sequences) as was the case for TmarCan16-1 and TmarCan16-2 [11].

The pathology in this study is different to what has been described in both experimentally and naturally infected farmed Atlantic salmon smolts in Tasmania, Australia with *T. maritimum* [7]. In Tasmania, the pathology has to a greater extent resembled what is described for typical tenacibaculosis: frayed fins, tail rot, skin lesions/ulcer and mouth erosion [7,17]. The reason behind these pathological differences is not known. It could be due to a difference in the *T. maritimum* strains associated with the different pathological presentations, but it could be due to other factors, including host and environment. One possibility is that the experiments were conducted at different temperatures, 12 ºC in our study and around 18-20 ºC in the experiments in Tasmania [7,17,36,37]. Pathogenicity differences associated with temperature has been shown *in vitro* with *M. viscosa*, a different skin pathogen of Atlantic salmon [38].

Conclusion

The mechanism by which *T. maritimum* kills smolts in the Pacific Northwest still remains a mystery. The main pathology in experimentally infected smolts with Western Canadian *T. maritimum* strains are mouth lesions that damage the tissues surrounding the teeth causing a disease that is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with very little associated inflammation. *T. maritimum* is detectable internally by real-time RT-PCR and bacteriology, and one possible point of entry would be the teeth.

Acknowledgments

Gary D. Marty reviewed the manuscript and he prepared the micrographs for Fig 1 from sections of a fish that was sampled as part of a regulatory auditing program operated by Fisheries and Oceans Canada.

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Table 1. Cohabitation experiment groups.

Group	Number of Fish	Isolate	Bacterial Bath Concentration (cells mL^{-1})	Accumulated Percent Mortality	Start of Mortality (days post-exposure)	End of Mortality (days post-exposure)
4-1	20 shed 40 cohab	TmarCan15-1	1.68×10^7	shed: 100 cohab: 75	shed: 2 cohab: 9	shed: 7 cohab: 20
4-2	20 shed 40 cohab	TmarCan15-1	1.68×10^7	shed: 100 cohab: 76	shed: 3 cohab: 7	shed: 7 cohab: 17
4-3	20 shed 40 cohab	TmarCan16-5	1.78×10^7	shed: 95 cohab: 27	shed: 2 cohab: 12	shed: 16 cohab: 17
4-4	20 shed 40 cohab	TmarCan16-5	1.78×10^7	shed: 84 cohab: 31	shed: 3 cohab: 10	shed: 10 cohab: 20
4-5	20 shed 40 cohab	TmarCan16-1	8.75×10^5	shed: 100 cohab: 100	shed: 3 cohab: 6	shed: 5 cohab: 11
4-6	20 shed 40 cohab	TmarCan16-1	8.75×10^5	shed: 100 cohab: 100	shed: 3 cohab: 6	shed: 6 cohab: 9
4-7	20 shed 40 cohab	Control (Marine Broth)	1 L	shed: 0 cohab: 0	-	-
4-8	20 shed 40 cohab	Control (no exposure)	N/A	shed: 0 cohab: 0	-	-

This table is a summary of the group descriptions and results from the cohabitation experiment in Frisch, Småge [11] (shed refers to shedders and cohab refers to cohabitants). The isolates used were collected from natural outbreaks of mouthrot on Atlantic salmon farms in BC, Canada [16]. Accumulated percent mortality is shown for each group, as well as the time period post-exposure that mortality occurred. In general, the mortality curve for each group had a sigmoid shape.

Table 2. Cohabitation experiment gross lesion scoring of mortality.

		Shedders (% of total mortality)			Cohabitants (% of total mortality)		
Tissue	**Score**	**TmarCan15-1**	**TmarCan16-1**	**TmarCan16-5**	**TmarCan15-1**	**TmarCan16-1**	**TmarCan16-5**
Mouth	0	62.5	94.9	27.8	-	82.7	-
	1	30.0	5.1	19.4	13.0	13.6	19.0
	2	2.5	-	33.3	35.2	3.7	57.1
	3	5.0	-	19.4	51.9	-	23.8
Skin	0	47.5	97.4	36.1	9.3	88.9	28.6
	1	45.0	2.6	22.2	42.6	11.1	28.6
	2	5.0	-	33.3	33.3	-	33.3
	3	2.5	-	8.3	14.8	-	9.5
Gills	0	32.5	100.0	88.9	94.4	100.0	95.2
	1	35.0	-	5.6	5.6	-	4.8
	2	32.5	-	5.6	-	-	-

Scoring of external clinical signs seen in mortality in the cohabitation experiment as a percentage of total mortality. Duplicate groups are combined. Scores were 0 to 3 for mouth and skin lesions, and 0 to 2 for gill lesions as described in Frisch, Småge [11], with 0 being no visible abnormalities and 2 or 3, the most severe.

Fig 1. Histopathology of the jaw of a smolt from a natural outbreak of mouthrot.

Histopathology of the jaw from a farmed Atlantic salmon that died 2 months after it was transferred from freshwater into a saltwater net-pen in BC; H&E stain. (A) The mucosal epithelium on the left side of the section is ulcerated and covered by a layer of deeply basophilic bacteria (arrowheads). The black box surrounds the transition from the bacteria-covered ulcer (left) to intact epithelium (right), and it outlines the area included in B. (B) Higher magnification of the transition between the ulcer covered by filamentous bacteria (arrowheads) and intact epithelium (right of right arrow); black box outlines the area included in C. (C) Higher magnification of abundant filamentous bacteria streaming in a proteinaceous matrix. (Optimization of photomicrograph illumination and color balance followed published methods [25]).

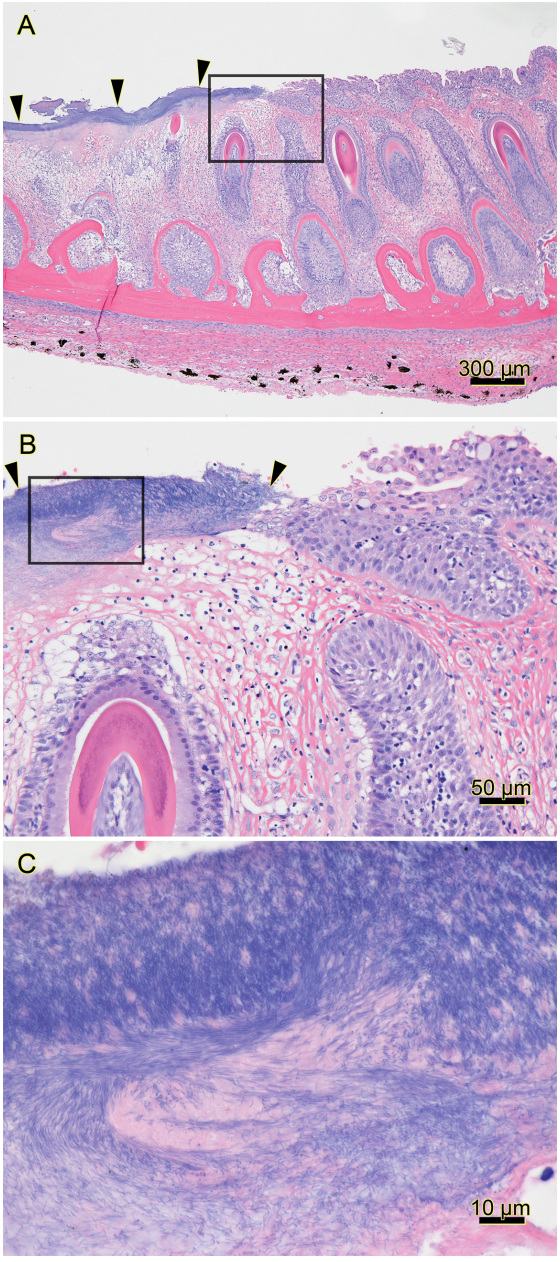


Fig 2. Gross clinical signs of an experimentally infected smolt. A moribund Atlantic salmon smolt that was bath infected with *T. maritimum* strain TmarCan16-1. Gross lesion scoring [11]: mouth = 2 out of 3, skin = 1 out of 3, gills = 1 out of 2. (**A**) Very few clinical signs are on the body surface other than some scale loss at the base of the peduncle and dorsal-lateral surface (arrows). (**B**) The gingiva is swollen (arrow). (**C**) A gill lesion (arrow).

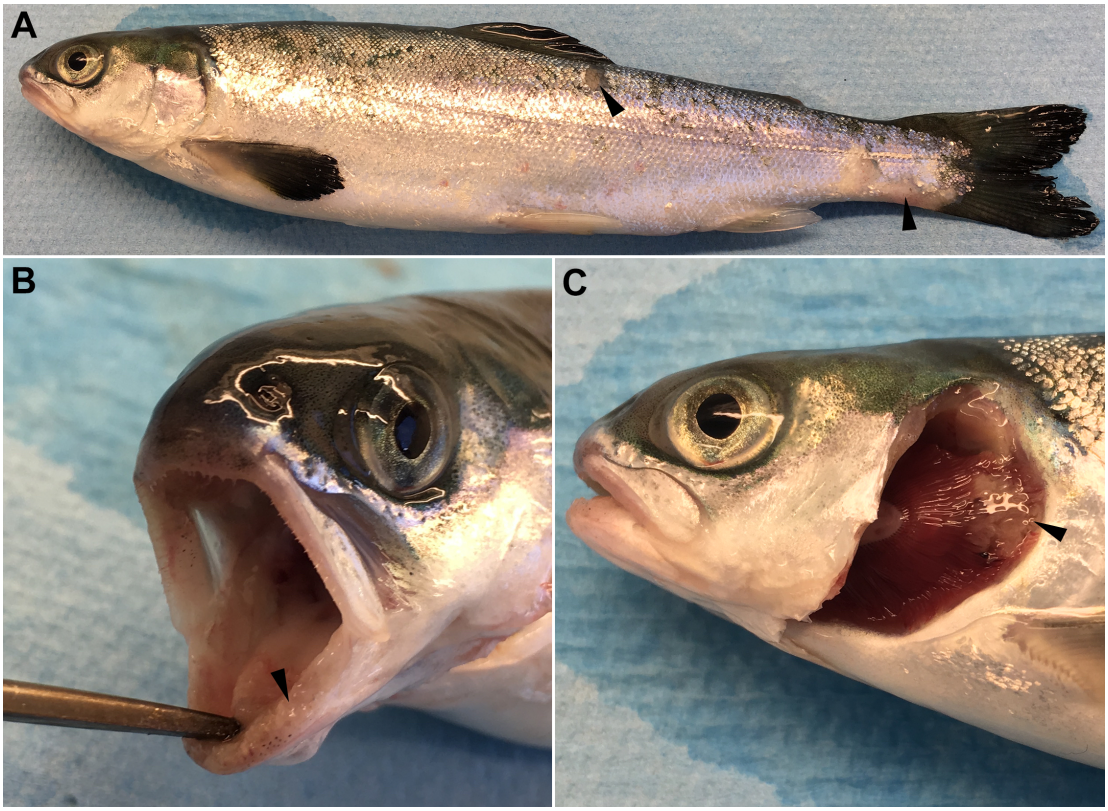


Fig 3. Histopathology of the jaw from an experimentally infected smolt. Histopathology of the gills from a moribund Atlantic salmon smolt experimentally bath infected with *T. maritimum* strain TmarCan15-1; H&E stain. (**A**) Oblique section of the jaw with mouthrot and loose teeth (arrowheads) with only a few of them connected to the jaw. The top is the inside of the oral cavity and the bottom the outside. The epidermis on the outside is intact. The black box outlines the area included in B and represents the transition at the edge of the ulcer. (**B**) The distance between intact mucosal epithelium (arrow "a") and the ulcer (arrow "b") is very short. Large quantities of bacteria with *T. maritimum* morphology are within the ulcer (arrow "c"). No signs of inflammation at the edge of the ulcer.

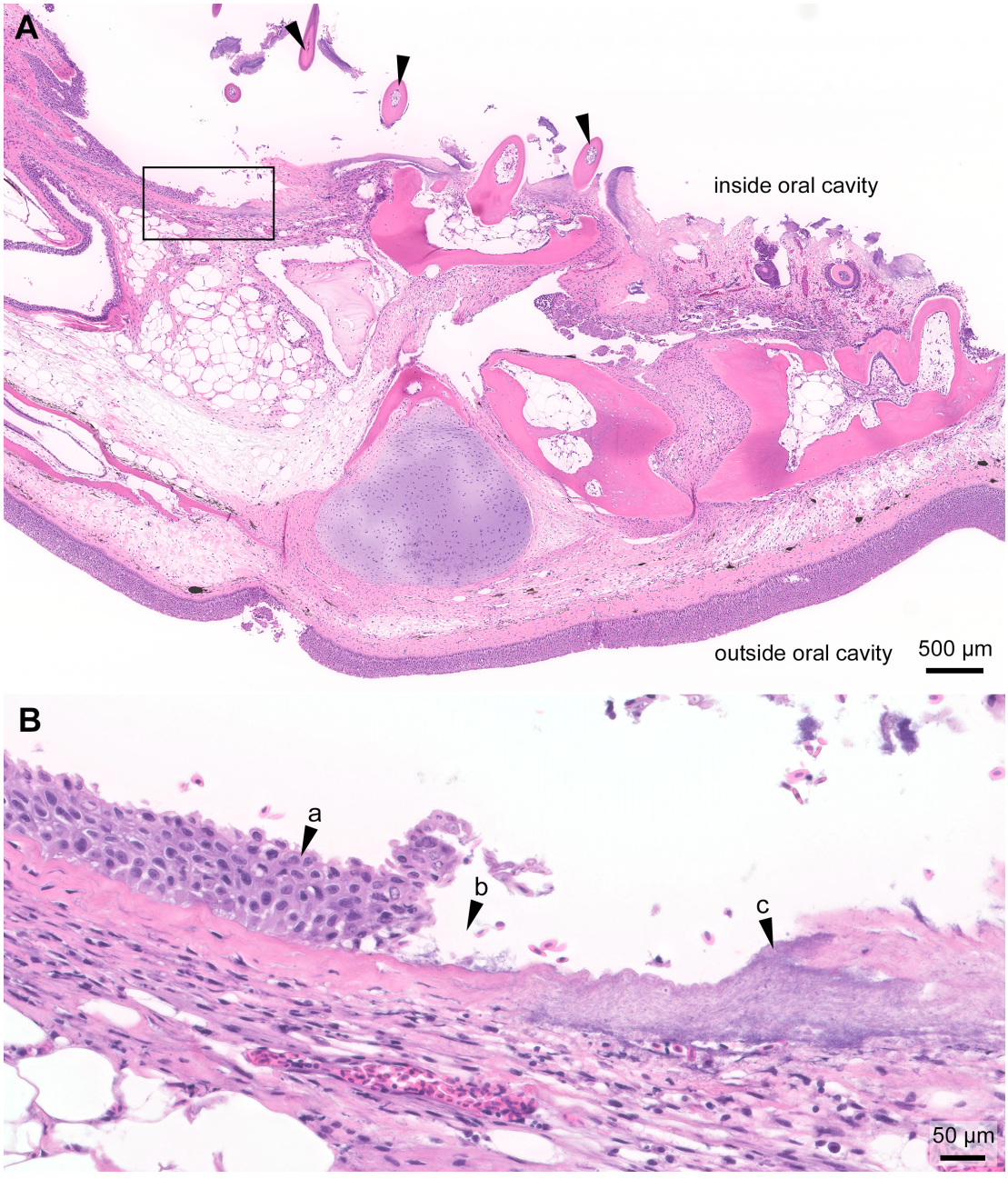


Fig 4. Histopathology of the jaw from an experimentally infected smolt. Histopathology of the jaw from the smolt in Fig 2; H&E stain. (**A**) Oblique section of the jaw. The epidermis is completely missing and the outer surface is covered with a thick mat of long thin rod-shaped *T. maritimum*-like bacteria that have infiltrated the submucosa (arrow "a"). Only one tooth (arrow "b") remains and holes are present where there used to be more teeth (arrow "c"). The black boxes labelled "B" and "C" outline the areas included in Fig 4 B and C. (**B**) A mat of bacteria with *T. maritimum* morphology is on the outer surface (arrow "d") and the bacteria have infiltrated the underlying submucosa. (**C**) Large quantities of bacteria with *T. maritimum* morphology are within the destructed submucosa surrounding the tooth (arrow "e"). Some intact red blood cells (arrow "f") are within the mass of bacteria and remnants of tissue.

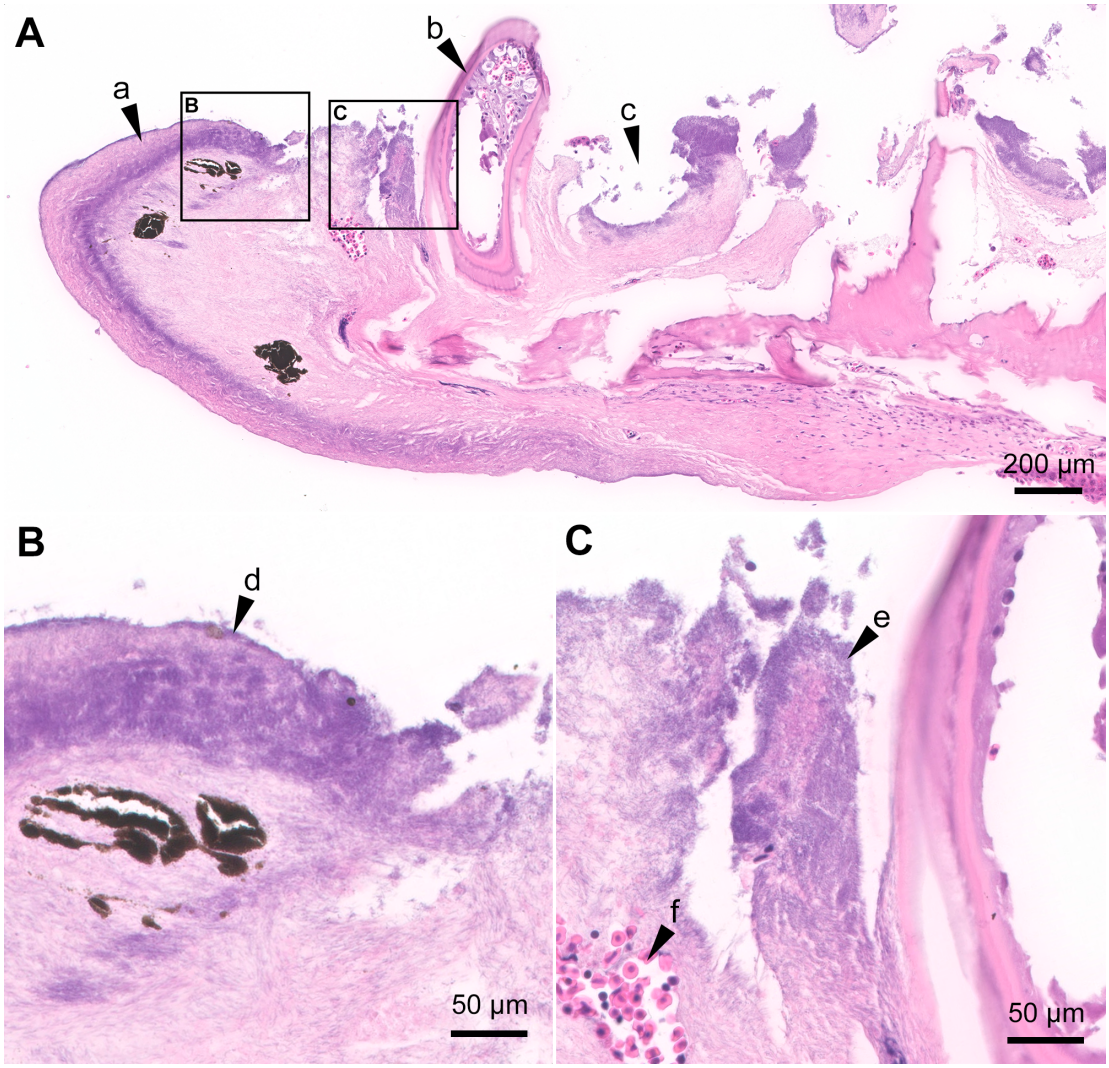


Fig 5. Histopathology of the gills from an experimentally infected smolt. Histopathology of the gills from the smolt in Fig 2; H&E stain. (**A**) Section of the gills with a distinct lesion on the top of the curve of the gill arch. The tips of the filaments are missing in the center of the lesion, and the remaining distal end of the filament is necrotic. The tissue is replaced by a thick layer of bacteria with *T. maritimum* morphology. The black box includes the transition between the lesion and normal tissue and outlines the area included in B. (**B**) The distance between the lesion and normal gill filaments is very short. In the damaged area, only the blood vessels remain in some of the lamellae. The black box outlines the area included in C. (**C**) Abundant bacteria with *T. maritimum* morphology cover the destroyed region of the gills. Only remnants of the lamellae are within the ulcer.

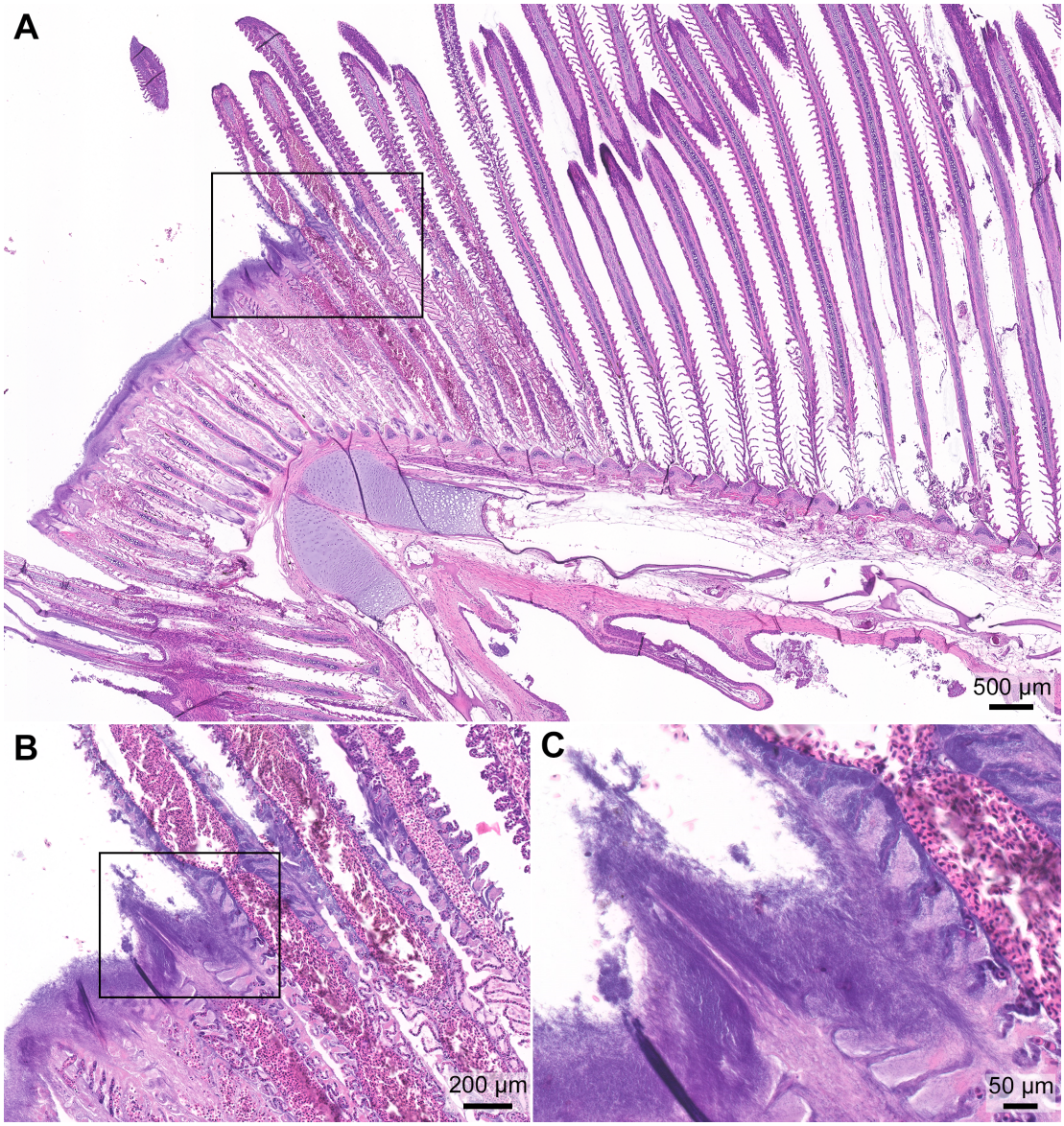


Fig 6. SEM of teeth from an experimentally infected smolt. Micrographs of teeth and the surrounding tissue from the mouth of a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. (**A**) Teeth and surrounding gingiva are covered by mats of bacteria with *T. maritimum* morphology (arrowheads) and the associated tissue is damaged. (**B**) Zoomed in view of a tooth showing bacterial growth on the surface of the tooth (arrow "a") as well as the surrounding gingival tissue (arrow "b"). (**C**) The dentin-enameloid interface with associated tissue destruction. White box indicates area in D. (**D**) Cellular debris within the bacterial mats.

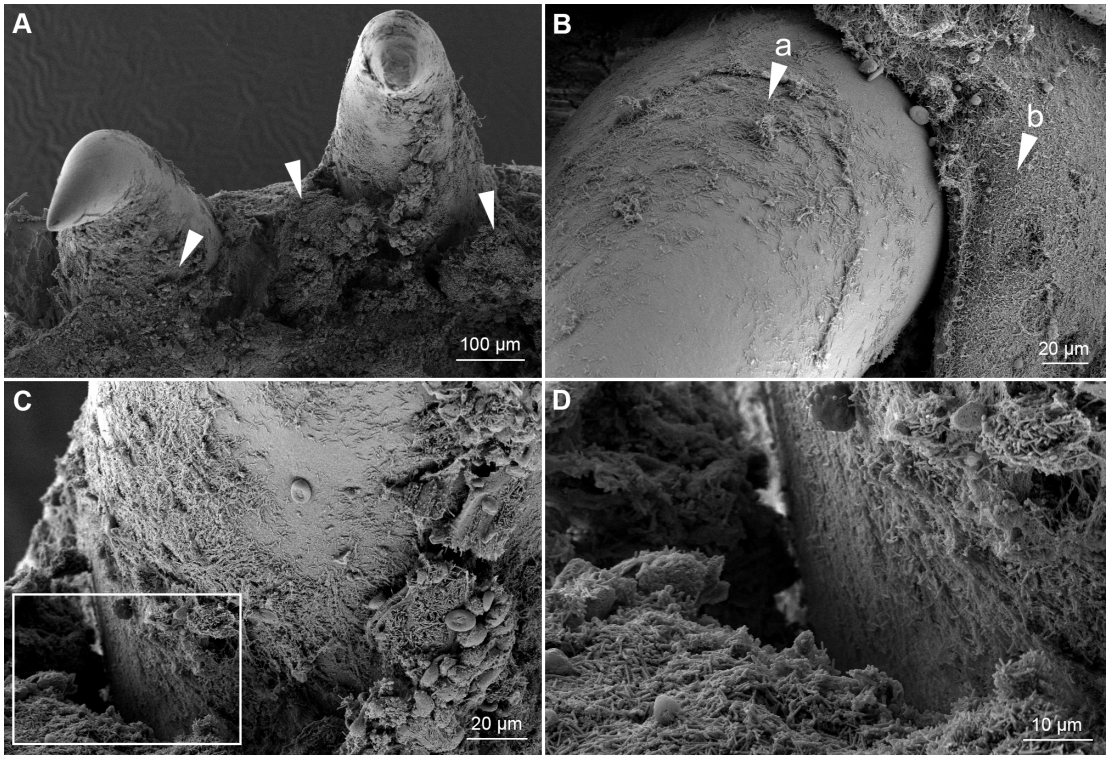


Fig 7. SEM of a tooth surface from an experimentally infected smolt. Micrograph of a tooth surface from a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. Bacteria with *T. maritimum* morphology are within the enameloid of the tooth (arrowheads).

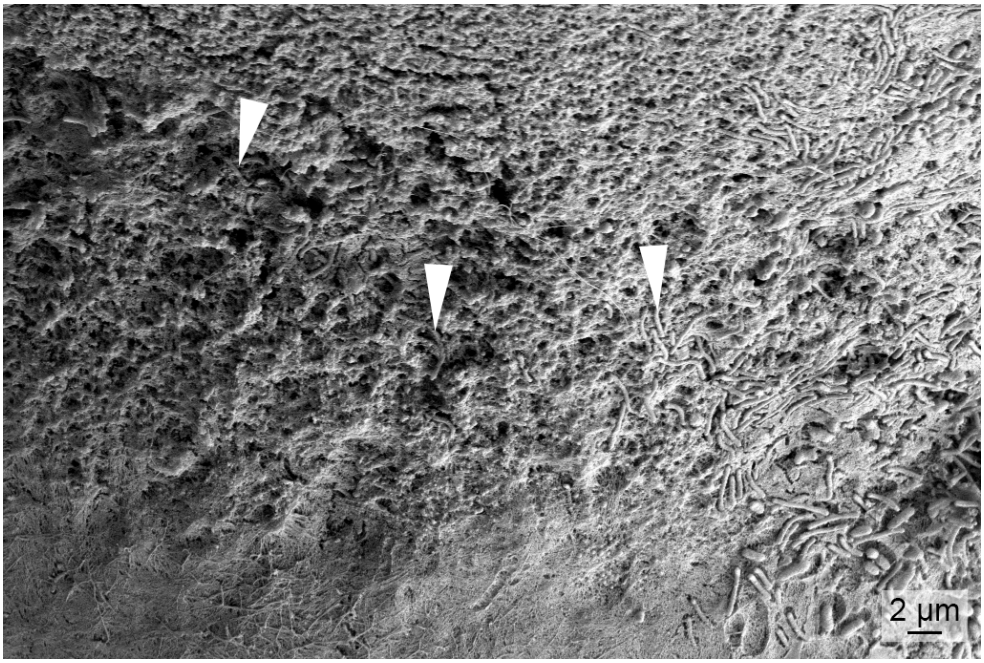


Fig 8. SEM of a fractured tooth from an experimentally infected smolt. Micrograph of a fractured tooth from a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. Large aggregates of bacteria with *T. maritimum* morphology are on the outside of the tooth (white arrowheads) as well as within the exposed pulp (black arrowheads) of the tooth.

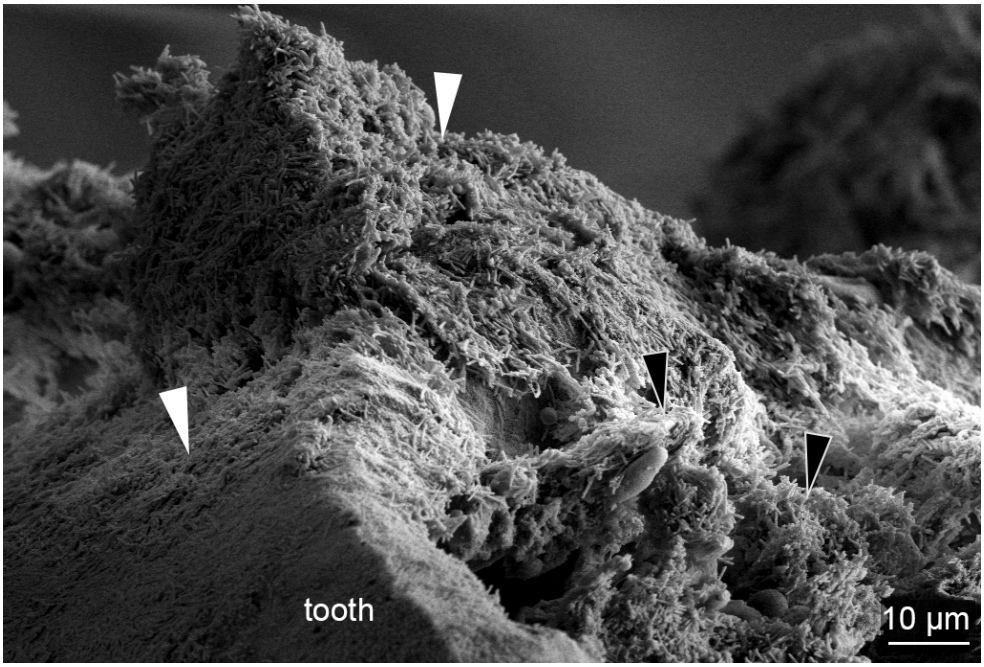
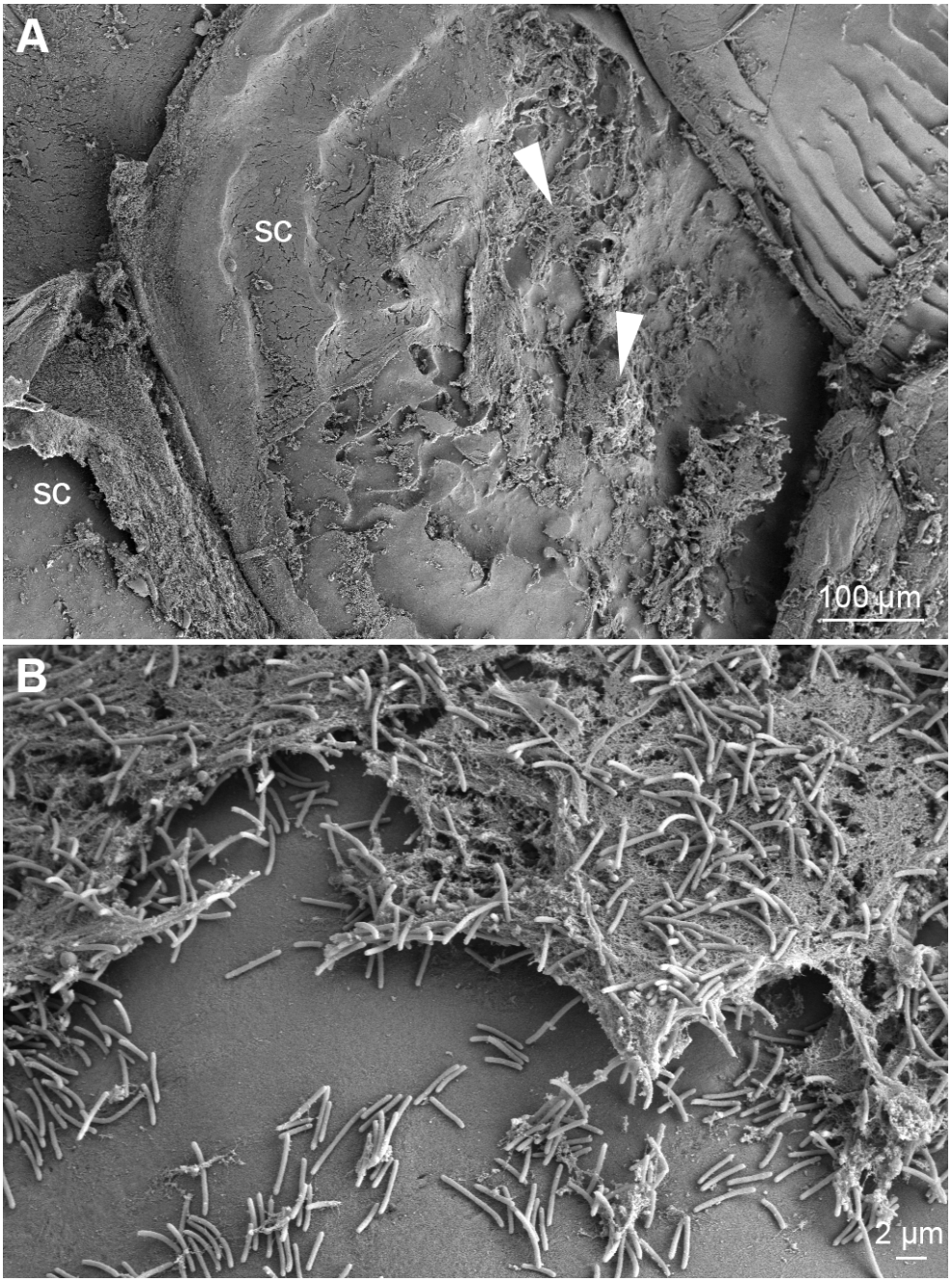


Fig 9. SEM of a skin lesion from an experimentally infected smolt. Micrographs of a skin lesion on the dorsal-lateral surface of a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. (**A**) Mats of bacteria with *T. maritimum* morphology (arrowheads) are associated with epithelial damage exposing the scales (sc). (**B**) Cellular debris with aggregates of bacteria with *T. maritimum* morphology.



Supporting information

S1 Table. Real-time RT-PCR results for the development of the Tmar_ompA assay. Ct values of the specificity and efficiency analyses performed during the development of the Tmar_ompA assay.

S2 Table. Cohabitation experiment real-time RT-PCR results. Ct values of the real-time RT-PCR analysis performed on diseased and non-diseased cohabitants.

S1 Table

	Tmar_ompA			Tmar 16S (Fringuelli)		
	Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate 1**	**Replicate 2**	**Replicate 3**
Specificity						
Tenacibaculum maritimum NCIMB 2154^{T}	16.957	16.908	17.283	5.156	4.936	4.741
T. maritimum TmarCan15-1	14.164	13.856	14.427	6.610	6.373	6.195
T. maritimum TmarCan16-1	17.322	17.531	17.384	4.486	4.530	4.365
T. maritimum TmarCan16-5	17.517	17.128	17.184	4.425	4.475	4.370
T. maritimum NLF-15	15.123	14.471	15.358	4.750	5.610	4.760
T. maritimum Ch-2402	13.714	13.690	14.256	5.699	6.968	6.371
Tenacibaculum adriaticum DSM18961^{T}	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Tenacibaculum dicentrarchi USC35/09^{T}	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Tenacibaculum finnmarkense HFJ^{T}	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Tenacibaculum ovolyticum EKD-002^{T}	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Tenacibaculum soleae LL0412.1.7^{T}	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined	Undetermined
Efficiency						
T. maritimum positive skin tissue	18.111	17.854	17.802			
T. maritimum positive skin tissue $^{-1}$	21.323	21.398	21.457			
T. maritimum positive skin tissue $^{-2}$	24.865	24.874	24.698			
T. maritimum positive skin tissue $^{-3}$	28.225	28.353	28.315			
T. maritimum positive skin tissue $^{-4}$	32.172	31.578	32.309			
T. maritimum positive skin tissue $^{-5}$	35.819	35.434	35.898			
T. maritimum positive skin tissue $^{-6}$	40.861	37.667	36.779			
T. maritimum TmarCan15-1 $^{-1}$	15.487	15.302	14.880			
T. maritimum TmarCan15-1 $^{-2}$	18.570	18.691	18.679			
T. maritimum TmarCan15-1 $^{-3}$	21.970	21.867	21.722			
T. maritimum TmarCan15-1 $^{-4}$	25.520	25.374	25.510			
T. maritimum TmarCan15-1 $^{-5}$	29.409	29.314	29.952			
T. maritimum TmarCan15-1 $^{-6}$	32.518	33.101	32.736			

S2 Table

Group	Fish number	Sample	Gills		Mouth (under jaw)		Brain		Heart		Kidney		Mucus	
			Tmar_ompA	EF1A	Tmar_ompA	EF1A	Tmar_ompA	EF1A	Tmar_ompA	EF1A	Tmar_ompA	EF1A	Tmar_ompA	Hsal
4-1	23	Mortality	26.106	13.650	27.889	15.046	33.225	16.401	33.576	17.399	20.941	16.407	18.906	22.532
4-1	31	Mortality	17.106	14.760	17.140	15.462								
4-1	33	Mortality	24.707	14.136	22.503	15.149								
4-1	34	Mortality	20.332	14.917	19.214	15.448								
4-1	41	Mortality	24.747	14.140	24.648	16.754	29.165	17.306	32.303	16.472	33.542	13.363	21.001	21.619
4-2	22	Mortality	26.262	14.614	20.745	15.825								
4-2	23	Mortality	28.703	14.297	26.453	15.610	35.553	16.986	34.659	16.674	31.677	15.701	18.515	21.905
4-2	30	Mortality	24.472	14.625	21.871	15.393								
4-2	42	Mortality	24.676	14.529	23.204	16.923	33.476	18.169	30.599	17.245	30.279	14.160	20.826	22.256
4-2	49	Mortality	25.221	14.139	18.210	15.276								
4-3	23	Mortality	21.192	14.813	18.808	17.519								
4-3	24	Mortality	26.158	16.130	17.408	16.326	32.595	17.886	33.977	16.684				
4-3	25	Mortality	24.190	14.939	22.967	16.565	33.713	17.759	34.178	16.193	33.249	13.454	22.705	21.648
4-3	28	Mortality	20.617	13.979	16.230	15.608								
4-3	34	Mortality	17.146	14.844	20.023	15.189								
4-4	21	Mortality	29.617	15.776	30.990	16.201	29.897	16.988	32.296	18.345	34.967	15.308	27.445	22.886
4-4	26	Mortality	27.583	15.472	23.702	17.090	33.337	17.806	32.853	16.736	34.710	15.118	16.645	23.789
4-4	27	Mortality	22.965	13.614	22.084	16.348								
4-4	33	Mortality	21.159	14.333	17.420	15.350								
4-4	34	Mortality	25.753	16.772	17.428	14.963								
4-5	21	Mortality	29.451	14.645	33.868	17.765								
4-5	36	Mortality	30.510	16.128	27.642	16.278	34.139	17.783	34.447	16.571	31.637	15.962	23.256	22.110
4-5	37	Mortality	31.196	14.459	31.578	14.605								
4-5	38	Mortality	29.837	14.964	31.256	15.011	31.894	17.672	33.687	17.416	31.585	15.866	27.891	23.217
4-5	41	Mortality	28.701	15.454	27.050	16.824								
4-6	26	Mortality	29.745	14.386	27.520	16.224	29.709	16.663	32.522	17.140	34.294	15.971	31.090	22.307
4-6	27	Mortality	31.357	14.714	33.380	17.509								
4-6	31	Mortality	35.024	15.823	33.018	16.348								
4-6	51	Mortality	27.956	14.514	28.574	17.914								
4-6	57	Mortality	33.427	15.378	32.003	16.795	36.708	17.193	36.684	17.960	35.915	15.743	24.239	22.449
4-1	21	Day-7 Sample	34.726	15.811	30.333	15.991	34.333	16.681	Undetermined	17.214	37.248	14.130	29.121	22.062
4-1	22	Day-7 Sample	33.631	16.172	31.405	14.986	Undetermined	17.144	33.229	15.345	Undetermined	15.358	27.531	22.694
4-1	42	Day-14 Sample	27.708	16.525	24.633	16.582	Undetermined	16.863	Undetermined	15.914	37.213	15.437	29.481	22.551
4-1	43	Day-14 Sample	32.782	15.593	30.380	16.756	Undetermined	17.135	36.756	15.926	Undetermined	13.456	21.215	22.425
4-2	46	Day-14 Sample	32.848	16.197	Undetermined	17.015	35.285	16.354	Undetermined	15.629	36.284	14.444	22.542	22.130
4-2	47	Day-14 Sample	34.811	15.147	27.987	15.583	30.773	17.390	37.041	16.082	36.185	14.191	29.117	22.402
4-3	21	Day-7 Sample	30.870	15.234	32.214	16.640	35.715	17.903	35.485	17.283	Undetermined	16.916	28.616	
4-3	22	Day-7 Sample	28.016	14.469	32.660	16.519	37.755	16.896	34.678	17.500	35.383	17.165	31.138	21.619
4-3	31	Day-14 Sample	35.171	14.947	32.793	17.553	36.668	16.820	Undetermined	15.717	37.108	14.138	28.848	21.639
4-3	32	Day-14 Sample	30.122	15.079	30.927	16.442	36.845	16.833	35.590	16.719	Undetermined	15.016	29.524	20.716
4-4	24	Day-7 Sample	26.359	14.705	21.498	14.623	32.305	17.176	34.791	16.927	34.739	16.310	19.559	22.383
4-4	25	Day-7 Sample	31.396	14.831	32.995	16.206	33.773	17.088	27.481	15.961	35.490	15.843	25.179	22.819
4-4	31	Day-14 Sample	32.221	15.375	30.071	17.527	Undetermined	17.968	Undetermined	16.134	Undetermined	14.242	22.944	21.952
4-4	32	Day-14 Sample	32.080	15.091	31.679	17.413	31.064	18.013	36.091	16.082	37.346	14.575	29.676	21.240

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RESEARCH ARTICLE

Pathology of experimentally induced mouthrot caused by *Tenacibaculum maritimum* in Atlantic salmon smolts

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Abstract

Mouthrot, caused by *Tenacibaculum maritimum* is a significant disease of farmed Atlantic salmon, *Salmo salar* on the West Coast of North America. Smolts recently transferred into saltwater are the most susceptible and affected fish die with little internal or external clinical signs other than the characteristic small (usually < 5 mm) yellow plaques that are present inside the mouth. The mechanism by which these smolts die is unknown. This study investigated the microscopic pathology (histology and scanning electron microscopy) of bath infected smolts with Western Canadian *T. maritimum* isolates TmarCan15-1, TmarCan16-1 and TmarCan16-5 and compared the findings to what is seen in a natural outbreak of mouthrot. A real-time RT-PCR assay based on the outer membrane protein A specific for *T. maritimum* was designed and used to investigate the tissue tropism of the bacteria. The results from this showed that *T. maritimum* is detectable internally by real-time RT-PCR. This combined with the fact that the bacteria can be isolated from the kidney suggests that *T. maritimum* becomes systemic. The pathology in the infected smolts is primarily mouth lesions, including damaged tissues surrounding the teeth; the disease is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with little associated inflammation. Skin lesions are more common in experimentally infected smolts than in natural outbreaks, but this could be an artefact of the challenge dose, handling and tank used during the experiments.

Introduction

Tenacibaculosis is a disease characterized by frayed fins, tail rot, mouth erosion, and skin lesions that are often ulcerative; it causes significant losses in a number of economically important marine fish species worldwide [1,2]. Three species belonging to the genus *Tenacibaculum* have been associated with this clinical presentation in farmed Atlantic salmon (*Salmo salar*): *Tenacibaculum dicentrarchi* [3], "*Tenacibaculum finnmarkense*" [4–6], and *Tenacibaculum maritimum* [7]. However, the clinical presentation of *T. maritimum* infections in Atlantic salmon smolts in the Pacific Northwest (British Columbia (BC), Canada and Washington,

for authors [KF, SS, ØB, HD], and also played a role in the study design, data collection and analysis, decision to publish, and preparation of the manuscript. One of the authors [AN] is employed by the University of Bergen (UiB) and another author [RJ] is employed by Pharmaq Analytiq AS; however neither UiB nor Pharmaq Analytiq AS had a role in study design, data collection or decision to publish. The specific roles of these authors are articulated in the "author contributions" section.

Competing interests: We have the following interests: Kathleen Frisch, Sverre Bang Småge, Henrik Duesund and Øyvind Jakobsen Brevik are employed by Cermaq Group AS, who planned and performed this study, and Renate Johansen is employed by Pharmaq Analytiq, who helped analyse the data. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

USA) is different from classical tenacibaculosis (as described above) and is commonly referred to as mouthrot [8–11]. Cultured Pacific salmon species (e.g. Chinook salmon, *Oncorhynchus tshawytscha*) in the Pacific Northwest appear to be resistant to developing mouthrot [9].

Mouthrot typically affects smolts recently transferred into saltwater, and has been present in the Pacific Northwest since the late 80s [12]. Due to a lack of preventative measures against this disease, mouthrot continues to be the main reason that antibiotics are used in the production of Atlantic salmon in the region [13]. Mouthrot is generally diagnosed by the presence of distinctive yellow plaques associated primarily with the teeth of affected smolts [10,14]. This clinical manifestation of *T. maritimum* infections has not been reported in any other Atlantic salmon farming region even in areas where *T. maritimum* is present.

The pathology of mouthrot in the Pacific Northwest was first described in the early 90s, before the bacterial agent was identified [12,14]. Gross pathology includes focal yellow bacterial mats around the palate and teeth. The lesions range from small and hardly visible to multiple with erosion of the upper and/or lower jaw in severe cases [14]. Microscopic examination of these lesions were described as "mats of *Cytophaga*-like filamentous bacteria associated with areas of ulceration and necrosis often extending into the underlying bone" [12]. Major taxonomical revisions have since identified these "*Cytophaga*-like" bacteria as *T. maritimum* [15,16]. Diseased individuals die with little or no other gross external or internal lesions other than these typical "yellow plaques" in the mouth, and there is no evidence of concurrent disease [10].

When Atlantic salmon smolts are experimentally bath infected with one high dose of Western Canadian *T. maritimum*, clinical signs are not exclusive to the mouth; the gills and skin can also be affected [11]. Necrotic gill lesions have sometimes been observed in mouthrot affected smolts in BC (personal observations, Frisch); however, this is not a common finding. Gill lesions associated with this bacterium have also been noted in naturally and experimentally infected Atlantic salmon smolts in Tasmania [7,17] and Chinook salmon in California [18]. Skin lesions are also more common in experimentally infected smolts than in natural outbreaks, but this could be an artefact of the experiments [11].

The mechanism by which *T. maritimum* kills Atlantic salmon smolts in the Pacific Northwest while only causing very small mouth lesions continues to be a mystery. This study describes for the first time the pathology associated with experimentally induced mouthrot and compares it to what is normally seen in natural outbreaks of this disease. Tissue tropism of the bacteria, using the newly developed real-time RT-PCR is also investigated.

Materials and methods

Real-time RT-PCR for *T. maritimum*

Prior to this publication, there was only one published real-time RT-PCR assay specific for *T. maritimum* [19]. The assay targets the 16S rRNA gene and was tested using DNA [19]. However, the 16S rRNA gene has low phylogenic resolution at the species level when compared to other genes [20], and real-time RT-PCR assays based on this gene may not be very specific. The new real-time RT-PCR assay (Tmar_ompA) targets the outer membrane protein A (*ompA*) gene (forward primer: `GCCAATAGCAACGGGATACC`, reverse primer: `TCGTGCGAC CATCTTTGGT`, probe: `TGAATCAAATGCGATCTT`). An alignment of the *ompA* gene using available *Tenacibaculum* spp. sequences in the GenBank and from the *T. maritimum* strains TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402 [16,21,22] (also available in the GenBank) was used during the design of the assay.

The specificity of Tmar_ompA, based on this alignment, was tested using RNA extracted from clonal cultures of *Tenacibaculum* spp. The aim for the assay was to amplify *T. maritimum*

strains NCIMB 2154^T, TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402 [16,21,22], and not to amplify *Tenacibaculum adriaticum* DSM18961^T, *Tenacibaculum dicentrarchi* USC35/09^T, "*Tenacibaculum finnmarkense*" HFJ^T, *Tenacibaculum ovolyticum* EKD-002^T and *Tenacibaculum soleae* LL0412.1.7^T. To compare this new assay to the already published one, these RNA samples were also tested using the assay developed by Fringuelli, Savage [19]. Tmar_ompA was optimized and the efficiency determined using 10-fold dilutions of RNA extracted from TmarCan15-1 [16] and from known positive skin tissue samples from the cohabitation experiment described in Frisch, Småge [11].

All RNA was extracted using TRI Reagent (Sigma-Aldrich) following the manufacturer's protocol, except that an additional washing step using 100% ethanol was performed prior to air drying the RNA pellet. Extracted RNA was stored at -80˚C. All assays were run using an AgPath-ID kit (Thermo Fisher Scientific) with 2 μL of RNA and the standard concentrations of primers (400 nM) and probe (120 nM). Each run consisted of 45 cycles.

Cohabitation experiment

Tissue samples from a previously published cohabitation experiment [11] were used to investigate the tissue tropism of the bacteria through real-time RT-PCR screening. In this experiment six groups of 20 Atlantic salmon smolts (shedders) were bath infected with three different isolates of *T. maritimum* (TmarCan15-1, TmarCan16-1 and TmarCan16-5) that came from natural mouthrot outbreaks on BC Atlantic salmon farms [16]. The shedders were bath infected for 5 hours in 12˚C saltwater (34 ppt) using one of the above isolates (groups 4–1 and 4–2 with 1.68 x 10^7 cells mL^{-1} TmarCan15-1, groups 4–3 and 4–4 with 1.78 x 10^7 cells mL^{-1} TmarCan16-5 and groups 4–5 and 4–6 with 8.75 x 10^5 cells mL^{-1} TmarCan16-1). Two additional groups of 20 shedders were used as controls (4–7 and 4–8), one bath exposed to 1 L marine broth (Difco 2216) (MB) and the other untouched. 24 hours post-bath infection, 40 smolts (cohabitants) were added to each group. The husbandry conditions are described in Frisch, Småge [11] and results are summarized in Table 1. The mouth, gill and skin lesions visible macroscopically on mortality were scored as described in Frisch, Småge [11] and are summarized in Table 2.

The cohabitation experiment was approved by the Norwegian Food Safety Authority (Mattilsynet) under the identification code 16/207694.

Cohabitation experiment tissue screening

The mouth and gills of five diseased cohabitants from each group were sampled with the exception of the 2 control groups that had no mortality. The brain, heart, kidney and skin mucus were also sampled from two smolts of each of these groups. At days 7 and 14 post-infection, two randomly selected apparently healthy cohabitants were sampled (mouth, gills, brain, heart, kidney and skin mucus) from each group. However, due to the rapid mortality in groups 4–5 and 4–6, this was not possible in these groups. The day 7 samples in group 4–2 were also missed. All samples were collected aseptically and kept on ice and then stored at -20˚C. Moribund smolts and randomly selected cohabitants were euthanized with a swift blow to the head.

RNA was extracted from each of these samples and screened using the Tmar_ompA assay using the above protocol. An assay targeting the elongation factor 1 alpha (EF1A) was used on the mouth, gills, brain, heart and kidney samples as an endogenous control (forward primer: CCCCTCCAGGACGTTTACAAA, reverse primer: CACACGGCCCACAGGTACA, probe: ATCG GTGGTATTGGAAC) [23]. Due to the variability of an endogenous control such as EF1A in skin mucus, these samples were spiked with cultured *Halobacterium salinarum* DSM 3754^T cells suspended in PBS prior to the RNA extraction. This exogenous control was detected

Table 1. Cohabitation experiment groups.

Group	Number of Fish	Isolate	Bacterial Bath Concentration (cells mL^{-1})	Accumulated Percent Mortality	Start of Mortality (days post-exposure)	End of Mortality (days post-exposure)
4–1	20 shed 40 cohab	TmarCan15-1	1.68×10^7	shed: 100 cohab: 75	shed: 2 cohab: 9	shed: 7 cohab: 20
4–2	20 shed 40 cohab	TmarCan15-1	1.68×10^7	shed: 100 cohab: 76	shed: 3 cohab: 7	shed: 7 cohab: 17
4–3	20 shed 40 cohab	TmarCan16-5	1.78×10^7	shed: 95 cohab: 27	shed: 2 cohab: 12	shed: 16 cohab: 17
4–4	20 shed 40 cohab	TmarCan16-5	1.78×10^7	shed: 84 cohab: 31	shed: 3 cohab: 10	shed: 10 cohab: 20
4–5	20 shed 40 cohab	TmarCan16-1	8.75×10^5	shed: 100 cohab: 100	shed: 3 cohab: 6	shed: 5 cohab: 11
4–6	20 shed 40 cohab	TmarCan16-1	8.75×10^5	shed: 100 cohab: 100	shed: 3 cohab: 6	shed: 6 cohab: 9
4–7	20 shed 40 cohab	Control (Marine Broth)	1 L	shed: 0 cohab: 0	-	-
4–8	20 shed 40 cohab	Control (no exposure)	N/A	shed: 0 cohab: 0	-	-

This table is a summary of the group descriptions and results from the cohabitation experiment in Frisch, Småge [11] (shed refers to shedders and cohab refers to cohabitants). The isolates used were collected from natural outbreaks of mouthrot on Atlantic salmon farms in BC, Canada [16]. Accumulated percent mortality is shown for each group, as well as the time period post-exposure that mortality occurred. In general, the mortality curve for each group had a sigmoid shape.

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using the Sal assay (forward primer: GGGAAATCTGTCCGCTTAACG, reverse primer: CCGGTCCCAAGCTGAACA, probe: AGGCGTCCAGCGGA) [24].

Microscopic pathology

Representative tissues from the lesions (mouth, skin and gills) of diseased fish sampled from Atlantic salmon smolts bath infected with BC strains of *T. maritimum* [11] were fixed in 10% neutral buffered formalin solution and kept at 4˚C until processing. The tissue processing and

Table 2. Cohabitation experiment gross lesion scoring of mortality.

Tissue	Score	Shedders (% of total mortality)			Cohabitants (% of total mortality)		
		TmarCan15-1	TmarCan16-1	TmarCan16-5	TmarCan15-1	TmarCan16-1	TmarCan16-5
Mouth	0	62.5	94.9	27.8	-	82.7	-
	1	30.0	5.1	19.4	13.0	13.6	19.0
	2	2.5	-	33.3	35.2	3.7	57.1
	3	5.0	-	19.4	51.9	-	23.8
Skin	0	47.5	97.4	36.1	9.3	88.9	28.6
	1	45.0	2.6	22.2	42.6	11.1	28.6
	2	5.0	-	33.3	33.3	-	33.3
	3	2.5	-	8.3	14.8	-	9.5
Gills	0	32.5	100.0	88.9	94.4	100.0	95.2
	1	35.0	-	5.6	5.6	-	4.8
	2	32.5	-	5.6	-	-	-

Scoring of external clinical signs seen in mortality in the cohabitation experiment as a percentage of total mortality. Duplicate groups are combined. Scores were 0 to 3 for mouth and skin lesions, and 0 to 2 for gill lesions as described in Frisch, Småge [11], with 0 being no visible abnormalities and 2 or 3, the most severe.

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sectioning for histology were performed by a commercial laboratory. Histology sections were stained with hematoxylin and eosin (H&E). Histology sections from a diseased smolt from a natural outbreak of mouthrot at a BC farm were used as a reference (Fig 1).

Tissues (mouth and skin) from experimentally infected smolts were also selected for scanning electron microscopy (SEM) examination. Preparation of tissues for SEM was performed as described in Småge, Frisch [21].

Results

Real-time RT-PCR for *T. maritimum*

The Tmar_ompA assay is specific to *T. maritimum* based on the testing of RNA extracted from the *T. maritimum* strains (*T. maritimum* strains NCIMB 2154^T, TmarCan15-1, TmarCan16-1, TmarCan16-5, NLF-15, and Ch-2402) and RNA extracted from other *Tenacibaculum* species (*T. adriaticum* DSM18961^T, *T. dicentrarchi* USC35/09^T, "*T. finnmarkense*" HFJ^T, *T. ovolyticum* EKD-002^T, and *T. soleae* LL0412.1.7^T). When compared to assay developed by Fringuelli, Savage [19], Tmar_ompA is less sensitive (S1 Table). The efficiency of Tmar_ompA is 1.9138 for pure *T. maritimum* culture (TmarCan15-1) and 1.9386 for *T. maritimum* positive skin tissue (S1 Table).

Cohabitation experiment tissue screening

All samples from diseased cohabitants were positive for *T. maritimum* using the newly developed Tmar_ompA assay (S2 Table). Bacterial loads were higher in the gills and mouth of the groups exposed to the two less pathogenic isolates (TmarCan15-1 and TmarCan16-5). Results from the heart, brain and kidney samples showed that *T. maritimum* was in all three of these tissues in clinically affected cohabitant fish, indicating that the bacteria or the detected segments become systemic. *T. maritimum* was also detected in most of the sampled tissues in the randomly sampled non-diseased cohabitants (S2 Table). Although a majority of these were positive, not all internal tissues were positive in all individuals. Cohabitants from the control groups were screened by Frisch, Småge [11] and were negative for *T. maritimum*.

Clinical signs

As described in Frisch, Småge [11], Atlantic salmon smolts bath infected with *T. maritimum* strains from BC presented with very few external (Fig 2) or internal clinical signs. Mouth lesions were the most common finding, with some fish also having skin and/or gill lesions. Mouth lesions were usually on or surrounding the teeth and tongue (Fig 2B) and were associated with a slime layer that generally had a yellow tinge. This slime contained a large quantity of long thin rod-shaped bacteria with *T. maritimum* morphology [11]. When lesions were on the skin (Fig 2A) or gills (Fig 2C), these were also linked with a slime layer containing large amounts of bacteria with *T. maritimum* morphology.

Microscopic pathology

In the experimentally infected smolts, histopathological changes are mainly present in the mouth, and some fish have gill and/or skin lesions. Generally, these changes are associated with the gross lesions (Fig 2). The gross oral lesions (Fig 2B) are microscopically associated with mats of long thin rod-shaped bacteria matching what is described for *T. maritimum* (Figs 3 and 4). The severity of the histopathology varies between individuals. The distance between intact epidermis with no signs of structural damage to an open ulcer with large quantities of bacteria is very short (Fig 3). In most cases, little or no inflammation surrounds lesions (Fig

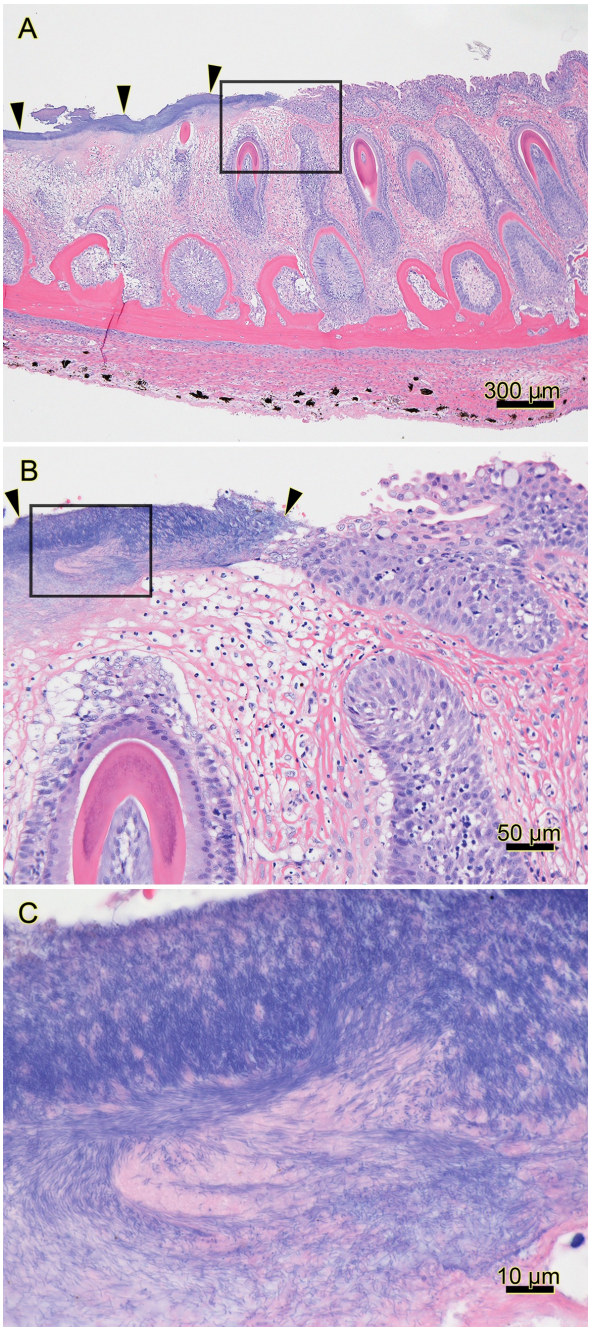
A
300 μm
B
50 μm
C
10 μm

Fig 1. Histopathology of the jaw of a smolt from a natural outbreak of mouthrot. Histopathology of the jaw from a farmed Atlantic salmon that died 2 months after it was transferred from freshwater into a saltwater net-pen in BC; H&E stain. (A) The mucosal epithelium on the left side of the section is ulcerated and covered by a layer of deeply basophilic bacteria (arrowheads). The black box surrounds the transition from the bacteria-covered ulcer (left) to intact epithelium (right), and it outlines the area included in B. (B) Higher magnification of the transition between the ulcer covered by filamentous bacteria (arrowheads) and intact epithelium (right of right arrow); black box outlines the area included in C. (C) Higher magnification of abundant filamentous bacteria streaming in a proteinaceous matrix. (Optimization of photomicrograph illumination and color balance followed published methods [25]).

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3B). Large quantities of bacteria with *T. maritimum* morphology are present in the gingival pockets surrounding the teeth and these are often loose and, in some cases, falling out or completely missing (Figs 3 and 4). In severe cases, normal tissue structures are replaced by a structureless mass of large amounts of bacteria and cellular debris (Fig 4).

Most of the examined gills from the experimentally infected smolts have no microscopic changes associated with disease and were deemed "healthy"; however, gills with macroscopic

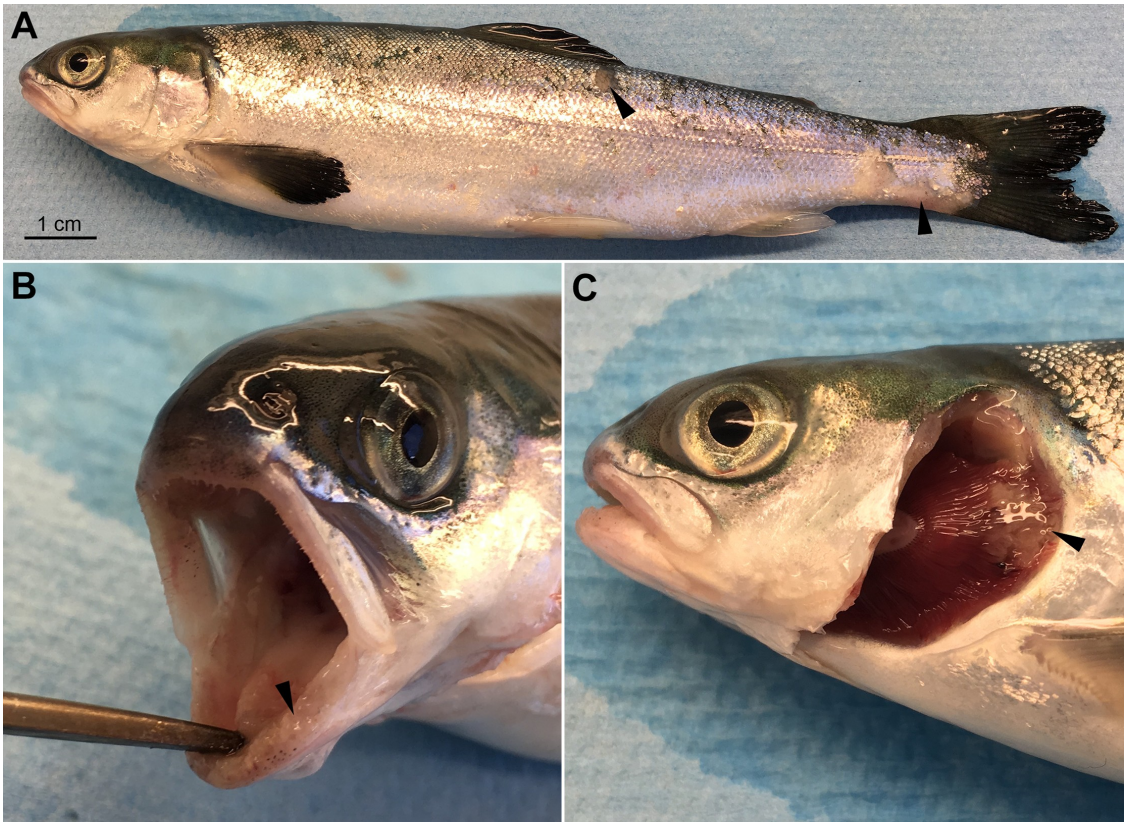

Fig 2. Gross clinical signs of an experimentally infected smolt. A moribund Atlantic salmon smolt that was bath infected with *T. maritimum* strain TmarCan16-1. Gross lesion scoring [11]: mouth = 2 out of 3, skin = 1 out of 3, gills = 1 out of 2. (**A**) Very few clinical signs are on the body surface other than some scale loss at the base of the peduncle and dorsal-lateral surface (arrows). (**B**) The gingiva is swollen (arrow). (**C**) A gill lesion (arrow).

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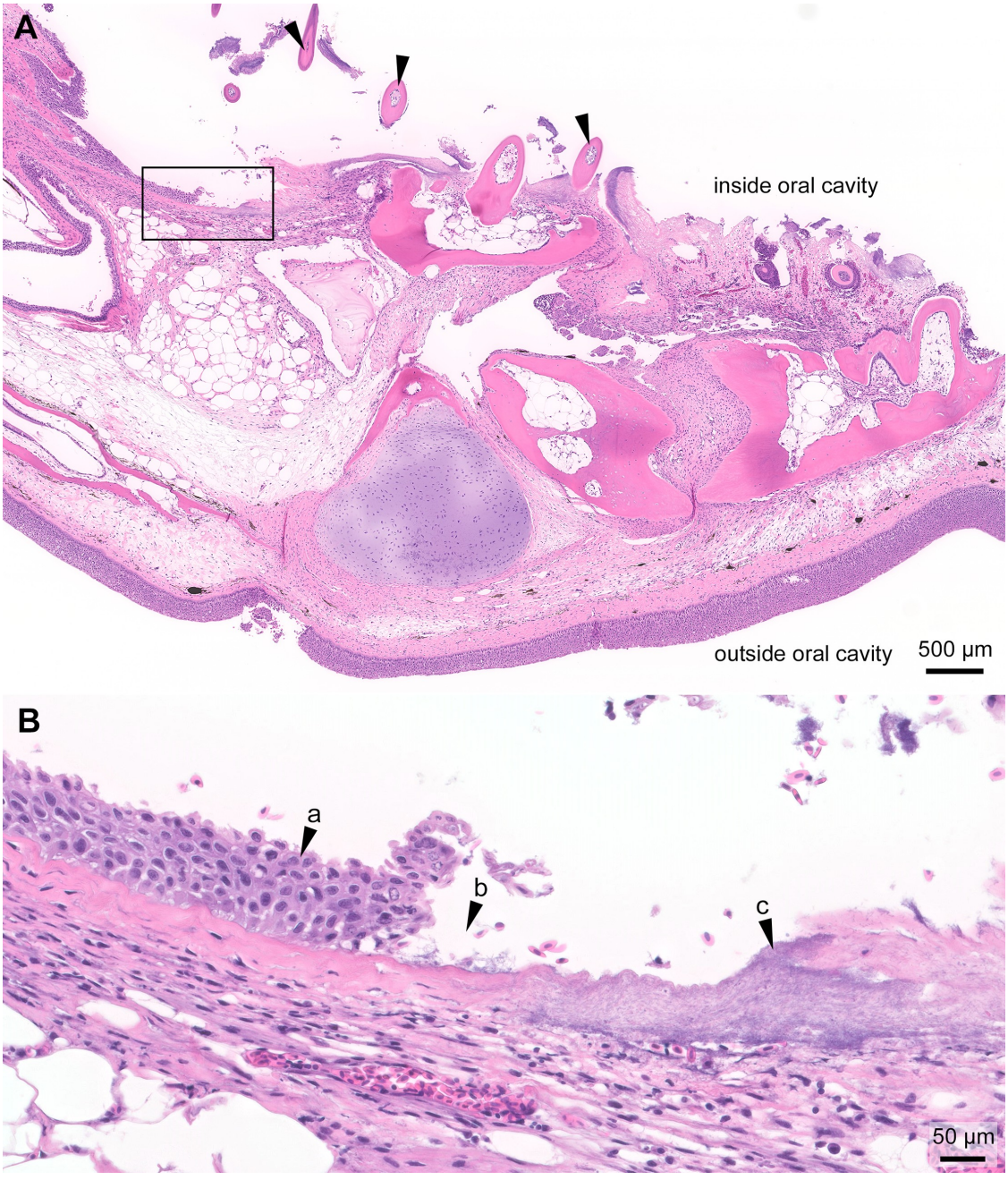
A
inside oral cavity
outside oral cavity
500 μm
B
a
b
c
50 μm

Fig 3. Histopathology of the jaw from an experimentally infected smolt. Histopathology of the gills from a moribund Atlantic salmon smolt experimentally bath infected with *T. maritimum* strain TmarCan15-1; H&E stain. (**A**) Oblique section of the jaw with mouthrot and loose teeth (arrowheads) with only a few of them connected to the jaw. The top is the inside of the oral cavity and the bottom the outside. The epidermis on the outside is intact. The black box outlines the area included in B and represents the transition at the edge of the ulcer. (**B**) The distance between intact mucosal epithelium (arrow "a") and the ulcer (arrow "b") is very short. Large quantities of bacteria with *T. maritimum* morphology are within the ulcer (arrow "c"). No signs of inflammation at the edge of the ulcer.

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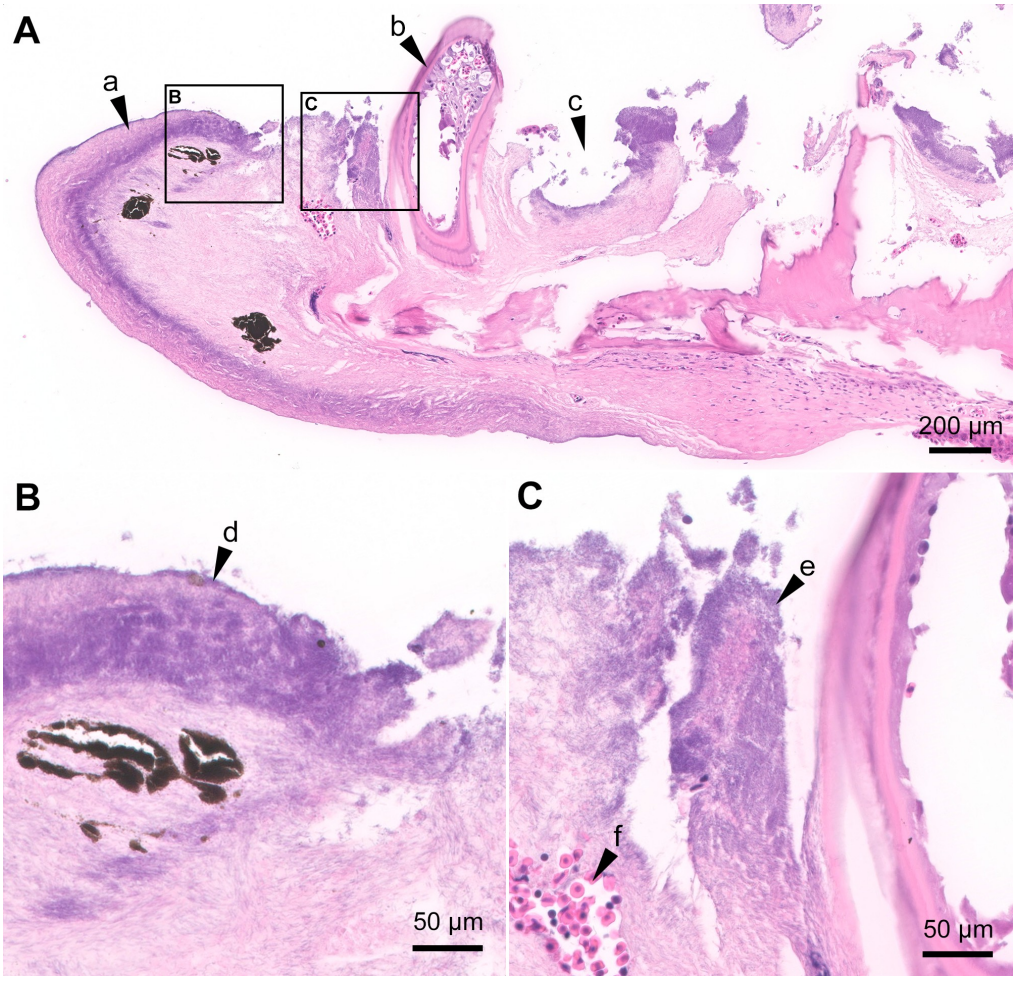

Fig 4. Histopathology of the jaw from an experimentally infected smolt. Histopathology of the jaw from the smolt in Fig 2; H&E stain. (**A**) Oblique section of the jaw. The epidermis is completely missing and the outer surface is covered with a thick mat of long thin rod-shaped *T. maritimum*-like bacteria that have infiltrated the submucosa (arrow "a"). Only one tooth (arrow "b") remains and holes are present where there used to be more teeth (arrow "c"). The black boxes labelled "B" and "C" outline the areas included in Fig 4B and 4C. (**B**) A mat of bacteria with *T. maritimum* morphology is on the outer surface (arrow "d") and the bacteria have infiltrated the underlying submucosa. (**C**) Large quantities of bacteria with *T. maritimum* morphology are within the destructed submucosa surrounding the tooth (arrow "e"). Some intact red blood cells (arrow "f") are within the mass of bacteria and remnants of tissue.

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lesions have significant microscopic changes (Fig 5). As with the mouth lesions, there is a total loss of cell and tissue structure linked to these lesions with little or no inflammation and large amounts of bacteria with *T. maritimum* morphology. Most of the gill lesions occurred at the curve of the gill arch (Figs 2C and 5A). The tip of the filaments in affected areas is completely destroyed and replaced by a thick layer of bacteria with *T. maritimum* morphology (Fig 5A). The distance between the ulcer and the intact filaments of the gills is very short (Fig 5A and 5B). Only remnants of the lamellae are within the ulcer (Fig 5B and 5C).

The skin lesions that developed during the experiments were associated with scale pocket edema. Total destruction of the underlying tissue is replaced with mats of bacteria with *T. maritimum* morphology. The SEM micrographs support the histopathological findings. Large aggregates of bacteria with *T. maritimum* morphology are in the areas of tissue destruction and surrounding the teeth (Fig 6). Cellular debris is clearly visible within these bacterial mats (Fig 6C and 6D). The bacteria are embedded in the surface of some of the teeth (Fig 7). Some teeth are fractured and bacterial aggregates are within the exposed pulp of these teeth (Fig 8). Bacterial mats and aggregates with associated tissue destruction are also in the skin lesions (Fig 9).

Discussion

The macroscopic and microscopic findings of experimentally induced mouthrot described in this study match the pathology in field cases (Fig 1), as well as what is described in the literature [12,14]. Comparing our findings to publications is difficult as most of these were written in the 1980s and 1990s before the *Tenacibaculum* genus was described and it is therefore difficult to make a meaningful comparison. Bacterial mats with *T. maritimum* morphology typically surround the teeth, and bacterial cells are seen within the gingival epithelium invading the tissues below. This suggests that the bacteria proliferate in the gingival pockets surrounding the teeth and spread to the surrounding tissues as was described by Frelier, Elston [12]. The SEM micrographs (Fig 6) add to the picture by showing that the bacteria adhere to the tooth surface and epithelium, creating large aggregates. This is associated with destruction of the surrounding tissues.

Skin lesions with associated scale pocket edema that matched the description by Handlinger, Soltani [7] occurred in a subset of Western Canadian *T. maritimum* experimentally infected smolts, particularly ones with a more chronic presentation [11]. Skin lesions, which are not common in natural outbreaks of mouthrot, may be attributed to the use of tanks that result in a greater potential for physical skin abrasions than saltwater net-pens. The use of dip nets to transfer the smolts in and out of the challenge tanks may also have contributed to this by disrupting the protective mucus layer and causing scale loss. The greater prevalence of gill lesions in experimentally infected smolts might be due to the clumping nature of *T. maritimum* that may create bacterial aggregates capable of lodging themselves in the gill filaments during respiration. This hypothesis is supported by the finding in the cohabitation experiment that fewer cohabitants had gill lesions than the shedders that were directly exposed to the bacterial culture during the bath infection (Table 2) [11].

The reasons why *T. maritimum* targets the teeth and surrounding mucosa in mouthrot are not fully understood. However, the teeth are a high source of calcium that has been shown to promote the growth of *T. maritimum* [26] and thus may contribute to the affinity for this particular tissue. Also, a gene encoding a collagenase has been identified in the whole genome sequence of *T. maritimum* [27] and likely the reason why high levels of *T. maritimum* are present in the collagen-rich submucosa (Figs 3 and 4). *T. maritimum* is also strongly adhesive to hydrophobic surfaces, including fish mucus [28,29]. This ability to adhere and colonize is an

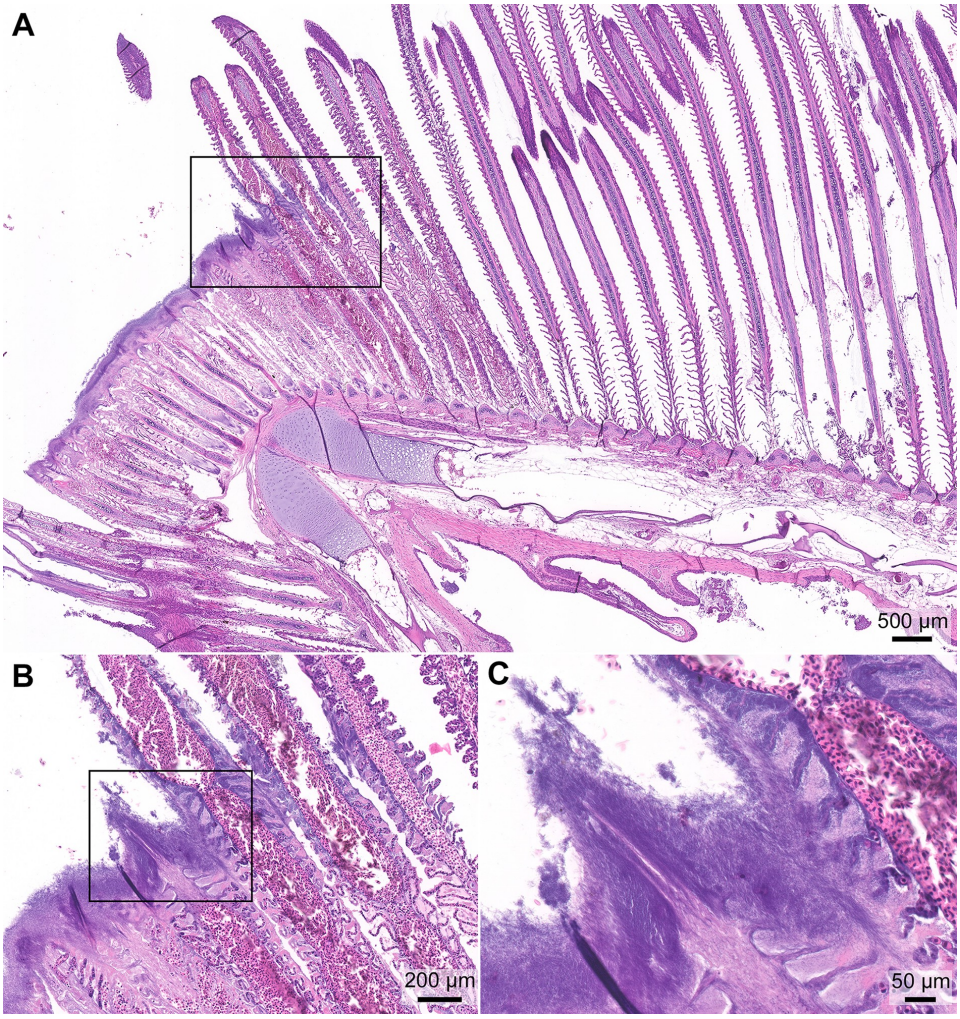

Fig 5. Histopathology of the gills from an experimentally infected smolt. Histopathology of the gills from the smolt in Fig 2; H&E stain. (**A**) Section of the gills with a distinct lesion on the top of the curve of the gill arch. The tips of the filaments are missing in the center of the lesion, and the remaining distal end of the filament is necrotic. The tissue is replaced by a thick layer of bacteria with *T. maritimum* morphology. The black box includes the transition between the lesion and normal tissue and outlines the area included in B. (**B**) The distance between the lesion and normal gill filaments is very short. In the damaged area, only the blood vessels remain in some of the lamellae. The black box outlines the area included in C. (**C**) Abundant bacteria with *T. maritimum* morphology cover the destroyed region of the gills. Only remnants of the lamellae are within the ulcer.

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important first step for pathogenic bacteria to invade the host [30]. This is likely the main mechanism by which *T. maritimum* is able to create biofilms so effectively. Biofilms, created by many pathogenic bacteria including *Staphylococcus aureus*, provide resistance against many

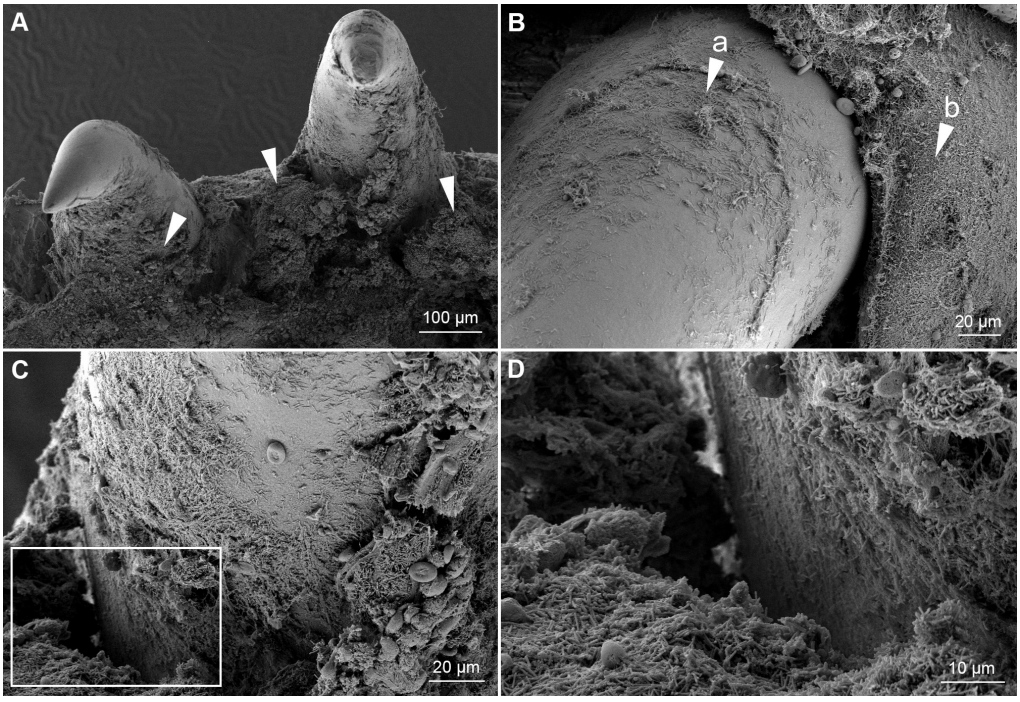

Fig 6. SEM of teeth from an experimentally infected smolt. Micrographs of teeth and the surrounding tissue from the mouth of a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. (**A**) Teeth and surrounding gingiva are covered by mats of bacteria with *T. maritimum* morphology (arrowheads) and the associated tissue is damaged. (**B**) Zoomed in view of a tooth showing bacterial growth on the surface of the tooth (arrow "a") as well as the surrounding gingival tissue (arrow "b"). (**C**) The dentin-enameloid interface with associated tissue destruction. White box indicates area in D. (**D**) Cellular debris within the bacterial mats.

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host defense mechanisms [31], and may explain the low level of immune response in mouthrot.

We developed a new real-time RT-PCR assay based on the *ompA* gene that is as specific but less sensitive than the published assay based on the 16S rRNA gene [19]. The results from the real-time RT-PCR tissue screening performed in this study and the recovery of the bacteria from kidneys of experimentally diseased fish [11] provide evidence that mouthrot is a systemic disease. However, no significant pathology occurred in internal organs [11]. This is further supported by the fact that when examining mouthrot affected smolts from the field, lesions in other organs are not obviously associated with mouthrot but further research is required to determine if such a link exists (personal communication, Gary Marty). The microscopic pathology of the mouth suggests that *T. maritimum* might be entering the highly vascular tooth pulp (Fig 8) once significant damage has occurred to the tooth and surround tissues. This may provide an entry point to the bloodstream, to then become systemic. This hypothesis matches what is described for periodontal disease in mammals. The lack of visible internal pathology, as well as the lack of observable inflammatory response may reflect the acuteness of the disease and resulting rapid tissue destruction. This is likely due to toxins with high proteolytic activity produced by *T. maritimum* [7,27,32–34].

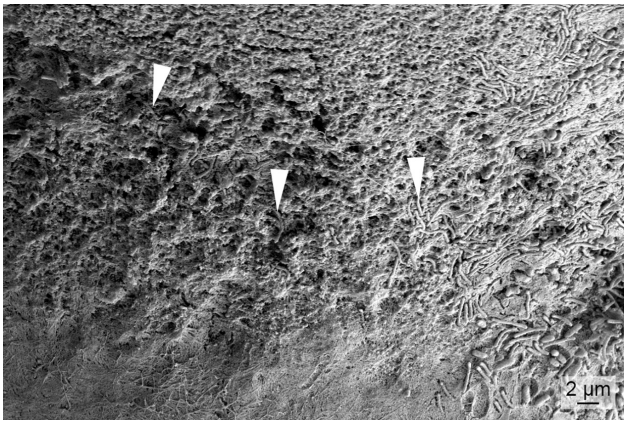

Fig 7. SEM of a tooth surface from an experimentally infected smolt. Micrograph of a tooth surface from a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. Bacteria with *T. maritimum* morphology are within the enameloid of the tooth (arrowheads).

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The real-time RT-PCR screening of the cohabitants showed that the external tissues (gills, mouth and mucus) of the fish infected with TmarCan16-1 had a lower load of *T. maritimum* than TmarCan15-1 and TmarCan16-5. This is interesting in view of the fact that this isolate results in a more rapid and severe disease (Table 1) with less severe gross clinical signs (Table 2). This relationship between highly pathogenic strains and a lack of severe lesions has previously been noted before for flavobacteria [30]. The real-time RT-PCR results are therefore not an indicator of pathogenicity. Variation in pathogenicity between *T. maritimum* strains has been shown in other studies, including other fish species [2,35,36]. Differences in

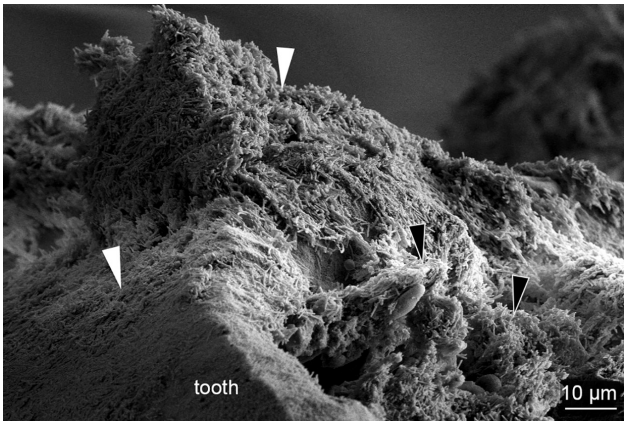

Fig 8. SEM of a fractured tooth from an experimentally infected smolt. Micrograph of a fractured tooth from a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. Large aggregates of bacteria with *T. maritimum* morphology are on the outside of the tooth (white arrowheads) as well as within the exposed pulp (black arrowheads) of the tooth.

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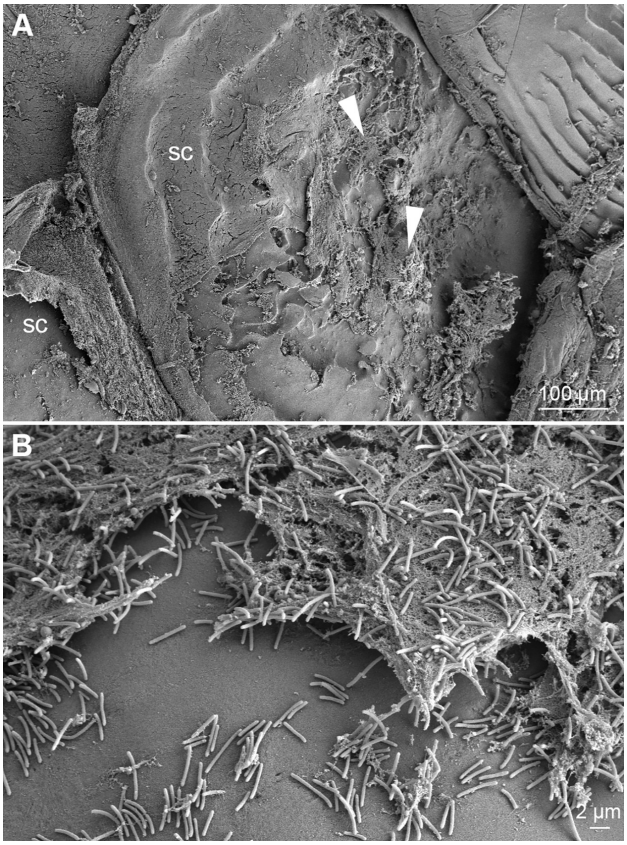

Fig 9. SEM of a skin lesion from an experimentally infected smolt. Micrographs of a skin lesion on the dorsal-lateral surface of a diseased smolt bath infected with TmarCan15-1 in the cohabitation experiment. (**A**) Mats of bacteria with *T. maritimum* morphology (arrowheads) are associated with epithelial damage exposing the scales (sc). (**B**) Cellular debris with aggregates of bacteria with *T. maritimum* morphology.

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pathogenicity also occur between isolates belonging to the same multilocus sequence type (genetically identical on 11 housekeeping gene sequences) as was the case for TmarCan16-1 and TmarCan16-2 [11]. Further analysis of the genome of TmarCan16-1 and TmarCan16-2 is required to identify the potential differences in virulence factors resulting in the observed variation in pathogenicity.

The pathology in this study is different to what has been described in both experimentally and naturally infected farmed Atlantic salmon smolts in Tasmania, Australia with *T. maritimum* [7]. In Tasmania, the pathology has to a greater extent resembled what is described for typical tenacibaculosis: frayed fins, tail rot, skin lesions/ulcer and mouth erosion [7,17]. The reason behind these pathological differences is not known. It could be due to a difference in the *T. maritimum* strains associated with the different pathological presentations, but it could be due to other factors, including host and environment. One possibility is that the

experiments were conducted at different temperatures, 12˚C in our study and around 18–20˚C in the experiments in Tasmania [7,17,36,37]. Pathogenicity differences associated with temperature has been shown *in vitro* with *M*. *viscosa*, a different skin pathogen of Atlantic salmon [38].

Conclusion

The mechanism by which *T*. *maritimum* kills smolts in the Pacific Northwest still remains a mystery. The main pathology in experimentally infected smolts with Western Canadian *T*. *maritimum* strains are mouth lesions that damage the tissues surrounding the teeth causing a disease that is similar to periodontal disease in mammals. The pathological changes are focal, severe, and occur very rapidly with very little associated inflammation. *T*. *maritimum* is detectable internally by real-time RT-PCR and bacteriology, and one possible point of entry would be the teeth.

Supporting information

S1 Table. Real-time RT-PCR results for the development of the Tmar_ompA assay. Ct values of the specificity and efficiency analyses performed during the development of the Tmar_ompA assay.
(XLSX)

S2 Table. Cohabitation experiment real-time RT-PCR results. Ct values of the real-time RT-PCR analysis performed on diseased and non-diseased cohabitants.
(XLSX)

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Gary D. Marty (Ministry of Agriculture, British Columbia, Canada) reviewed the manuscript and he prepared the micrographs for Fig 1 from sections of a fish that was sampled as part of a regulatory auditing program operated by Fisheries and Oceans Canada.

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