

Tenacibaculosis in Norwegian farmed Atlantic salmon

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Thesis for the Degree of Philosophiae Doctor (PhD)
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ABSTRACT

Tenacibaculosis is a bacterial ulcerative skin disease of many economically important farmed fish species worldwide caused by members of genus *Tenacibaculum*. For the Norwegian salmon farming industry, however, tenacibaculosis was not recognized as an important disease until 2010. Since then, the increased use of media supporting *Tenacibaculum* growth has resulted in the identification of *Tenacibaculum* spp. from a large number of outbreaks of skin lesions/ulcers. Bacterial skin infections that cause skin lesions/ulcers in farmed Atlantic salmon are not only important fish health and welfare issues that needs to be solved, but also cause significant economic losses.

From investigations of tenacibaculosis outbreaks in the northernmost parts of Norway, a *Tenacibaculum* sp. strain was repeatedly identified over several years at multiple farms. Sequence similarity analysis showed that this *Tenacibaculum* sp. strain was most closely related to *Tenacibaculum dicentrarchi*, but different enough to constitute a novel *Tenacibaculum* species. A polyphasic investigation showed that this was indeed a novel species in the genus *Tenacibaculum*, for which the name *Tenacibaculum finnmarkense* was proposed. The delineation of *T. finnmarkense* and *T. dicentrarchi* has since been further demonstrated by whole genome analysis.

In September 2015, two separate outbreaks of tenacibaculosis occurred at two Atlantic salmon farms in Northern Norway. The events resulted in major losses of smolts newly transferred into seawater. Prior to, and during the outbreaks, large numbers of small jellyfish, identified as *Dipleurosoma typicum* (Boeck) were observed in the vicinity of the farms and inside the net-pens. During these outbreaks, *T. finnmarkense* was found to be the dominant bacteria associated with the outbreaks. Moreover it was shown that *D. typicum* were unlikely to be a vector for *T. finnmarkense*. However, it was shown that the jellyfish caused direct damage to the fish's skin which likely exacerbated the bacterial infection by allowing an entry point for *T. finnmarkense*.

Bath challenges conducted during this study using *T. finnmarkense* and Atlantic salmon smolts showed that for the first time this bacterium was able to induce

tenacibaculosis as it presents in the field without pre-stressors or co-infection. The study also showed that there is pathogenic variation between *T. finnmarkense* strains, with *T. finnmarkense* strain HFJ^T being the most pathogenic. A cohabitation experiment showed that *T. finnmarkense* does not appear to spread horizontally from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors for this bacterium.

Due to the success of vaccines targeting other bacterial pathogens in Norwegian farmed Atlantic salmon, the efficacy of a whole cell inactivated vaccine targeting *T. finnmarkense* was tested. Despite the vaccines inducing an antibody response, they did not manage to give any protection against tenacibaculosis induced through a bath infection. Future research needs to focus on mitigation tools, which will need to include refining the challenge model necessary for testing such tools.

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LIST OF PUBLICATIONS

These papers are referred to by their Roman numerals in this thesis.

PAPER I

Småge, S.B., Brevik, Ø.J., Duesund, H., Ottem, K.F., Watanabe, K., Nylund, A. (2015) *Tenacibaculum finnmarkense* sp. nov., a fish pathogenic bacterium of the family *Flavobacteriaceae* isolated from Atlantic salmon. *Antonie van Leeuwenhoek*, 109, 273-285.

PAPER II

Småge, S.B., Brevik, Ø.J., Frisch, K., Duesund, H., Watanabe, K., Nylund, A. (2017) Concurrent jellyfish blooms and tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon (*Salmo salar*) farms. *PLOS ONE*. 12(11): e0187476.

PAPER III

Småge, S.B., Frisch, K., Vold, V., Duesund, H., Brevik, Ø.J. Hetlelid Olsen, R., Sjaatil, S.T., Klevan, A., Brudeseth, B., Watanabe, K., Nylund, A. (2018) Experimentally induced tenacibaculosis in Atlantic salmon smolts using *Tenacibaculum finnmarkense*. (*Manuscript, submitted for publication*)

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ABBREVIATIONS

16S rRNA	16S (Svedberg) ribosomal ribonucleic acid genes
ANI	Average Nucleotide Identity
AMPs	Antimicrobial peptides
BAS	Blood agar supplemented with 1.5-2% NaCl
BLAST	Basic Local Alignment Search Tool
DDH	DNA-DNA Hybridization
DIFC	Desmosome-intermediate filament complex
FDRG	Fish Disease Research Group
FMM	<i>Flexibacter maritimus</i> medium
TEM	Transmission Electron Microscopy
H&E	Hematoxylin and eosin
IP	Intraperitoneally
MA	Marine agar (Difco 2216)
MB	Marine broth (Difco 2216)
MCMC	Markov chain Monte Carlo
MLSA	Multilocus Sequence Analysis
MLST	Multilocus Sequence Typing
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction
Pers.com	Personal communication
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
sp.	Species (singular)
spp.	Species (plural)
subsp.	Subspecies (singular)
WGS	Whole Genome Sequencing

1. INTRODUCTION

1.1 Norwegian salmon farming

There has been a substantial growth in the Norwegian Atlantic salmon farming industry during the last 50 years. From the first successful production of Atlantic salmon in 1969 (Gjendrem, 1995) the industry has grown to become the largest producer of salmon in the world, producing 1.2 million tonnes with a total first hand value of 60 billion NOK in 2016 (Statistics Norway). The salmon farming industry is therefore of major importance to the Norwegian economy and for employment in rural regions along the Norwegian coast. However, the rise to become the world's largest producer of Atlantic salmon has not been free of challenges. In the 1980s and early 1990s, the fish diseases cold-water vibriosis (*Aliivibrio salmonicida*), vibriosis (*Vibrio (Listonella) anguillarum*) and furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) had a devastating impact on the industry. These bacterial diseases caused mass mortalities and led to the massive use of antimicrobial drugs that constrained further expansion of the industry. It was not until the advent of effective commercial oil-adjuvant injection vaccines in the early 1990s that the industry managed to gain control of the situation (Figure 1) (Grave et al., 1996; Sommerset et al., 2005). Since then, extensive vaccination together with better husbandry and management practices has improved fish welfare by reducing diseases and clinical interventions (Bricknell et al., 2006). This has allowed for a steady increase in the production of salmon, while the use of antimicrobial drugs has more or less stabled at very low levels (Figure 1) (WHO, 2015; NORM/NORM-VET-2016, 2017). As a result, the current Norwegian aquaculture legislation (§63) requires that Atlantic salmon are at a minimum vaccinated against the three above mentioned bacterial diseases (Lovdata, 2017). In 2016, the use of antimicrobial drugs in Norwegian aquaculture was at the lowest level recorded since 1981 (NORM/NORM-VET-2016, 2017).

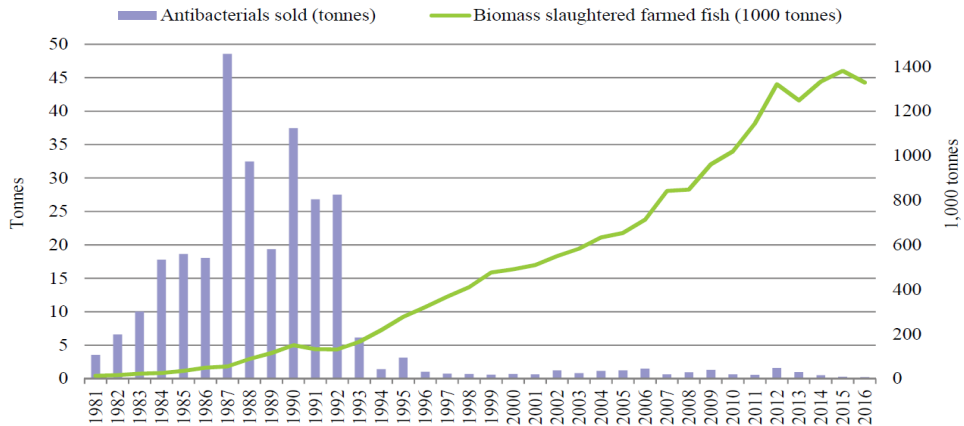


Figure 1 “Total sales, in tonnes of active substance, of antimicrobial veterinary medicinal products for therapeutic use in farmed fish in Norway in the period 1981-2016 versus produced biomass (slaughtered) farmed fish in 1,000 tonnes” (NORM/NORM-VET-2016, 2017).

Despite the fact that many of the main bacterial diseases in farmed Atlantic salmon have been effectively controlled by vaccines, ulcerative bacterial skin diseases, mainly associated with *Moritella viscosa* and *Tenacibaculum* spp., and yersinosis (*Yersinia ruckerii*), still constitute significant problems for the industry and need to be addressed (**Paper-II**; Shah et al., 2012; Hjeltnes et al., 2017; Karlsen et al., 2017a).

1.2 Bacterial skin diseases

Skin lesions/ulcers are common in intensively farmed fish due to the delicate nature of the fish’s skin and the intimate contact with the environment (Ferguson, 2006). Under intensive farming conditions several stressors may interact and influence the extent of bacterial skin diseases (e.g. de-lousing operations, handling, and transport). Bacteria that cause skin lesions/ulcers in farmed Atlantic salmon are therefore important fish health and welfare problems for the Norwegian industry. There are also significant financial losses associated with skin lesions/ulcers due to mortalities and downgrading at harvest, as ulcers will affect the flesh quality (Gudmundsdóttir and Björnsdóttir, 2007; Olsen et al., 2011). In a nationwide survey investigating total

losses of sea pen-reared Atlantic salmon in Norway, it was found that 16 % of all Atlantic salmon put to sea are lost during the grow out phase (Bleie and Skrudland, 2014). A large part of these losses are due to skin ulcers (Aunsmo et al., 2006; Aunsmo et al., 2008). In Norway there are also geographical variations with regard to the impact of skin lesions/ulcers as they are more prevalent in the northernmost parts of Norway, likely due to lower seawater temperatures (Bleie and Skrudland, 2014).

Due to the impact on fish welfare and the economic losses associated with skin lesions/ulcers, this issue is currently regarded as the main bacteriological problem for the Norwegian salmon farming industry. However, skin lesions/ulcers in sea-pen reared Atlantic salmon in Norway have been described since the 1980s (Lillehaug et al., 2003; Coyne et al., 2006; Olsen et al., 2011). As skin lesions/ulcers are most prevalent at cold seawater temperatures during the winter months, the condition is commonly referred to as the “winter ulcer disease” by veterinary personnel and salmon farmers. However, skin lesions/ulcers may occur all year, especially in Northern Norway (**Paper-II**). A symptomatic diagnosis “fin rot/ulcers” with undetermined etiological agent(s) has also been used to describe skin lesions/ulcers in Atlantic salmon during the 1990s (Lillehaug et al., 2003). Different from the “winter ulcer disease”, the “fin rot/ulcers” condition was primarily diagnosed in smaller fish (<100 g). Because several different bacteria are recurrently identified during bacteriological investigations, determining the etiological agent of skin lesions/ulcers has proven to be difficult (Karlsen et al., 2017a).

1.2.1 “Winter ulcer disease”

Following bacteriological investigations into the condition “winter ulcer disease” in the early 1990s, two bacteria were predominantly identified, *M. viscosa* (*Vibrio viscosus*) and *Aliivibrio wodanis* (*Vibrio wodanis*) (Lunder, 1992). Of these two, only *M. viscosa* has been shown to recurrently replicate the disease in challenge experiments and successfully fulfill Koch’s Postulates (Lunder et al., 1995; Bruno et al., 1998; Løvoll et al., 2009; Karlsen et al., 2017a). Although found to be non-pathogenic, *A. wodanis* seems to have an antagonistic effect on the progression of *M. viscosa* infections, leading to a chronic condition (Karlsen et al., 2014). “Winter ulcer

disease” associated with *M. viscosa* have been described as a systemic disease that is characterized by ulcers and skin lesions that occur on the scale covered parts of the fish, and most frequently along the flanks (Figure 2) (Salte et al., 1994; Lunder et al., 2000; Gudmundsdóttir and Björnsdóttir, 2007). The condition has been reported from all North Atlantic salmon farming regions (Benediktsdóttir et al., 1998; Bruno et al., 1998; Whitman et al., 2001; Grove et al., 2010; Takle et al., 2015). During outbreaks of “winter ulcer disease” the mortality rates are usually low (< 10 %), even though a large part of the fish may be affected (Løvoll et al., 2009). The disease appears to subside at lower salinities and at water temperatures above 8 °C (Løvoll et al., 2009; Olsen et al., 2011). As ulcers may be present for long periods, it constitutes a serious fish welfare problem (Løvoll et al., 2009; Olsen et al., 2011). Approximately half of the current antimicrobial usage in Norwegian aquaculture is used to control this disease, even though antimicrobials have been shown to not effectively control it (Coyne et al., 2006; Løvoll et al., 2009).



Figure 2 Salmon showing a typical “winter ulcer” associated with *M. viscosa*.

An antigen component of *M. viscosa* is included in commercially available polyvalent vaccines that are routinely used in Norwegian salmon aquaculture (Gudmundsdóttir

and Björnsdóttir, 2007; Karlsen et al., 2017a). Both experimental studies and field surveys have reported that vaccines targeting *M. viscosa* offers significant protection against both mortalities and ulcer development (Bleie and Skrudland, 2014; Karlsen et al., 2017a), and fish health enterprises report that primary outbreaks of “winter ulcer disease” without any pre-existing skin damages is basically non-existing (Marin-Helse, 2016).

It has been shown that isolates of *M. viscosa* can be divided into two genetically different clusters (Grove et al., 2010). A “typical” cluster that consists mainly of isolates from diseased Atlantic salmon, and a “atypical” cluster that have been obtained from variety of fish species (Takle et al., 2015). In recent years, however, there has been an emergence of *M. viscosa* isolates from the “atypical” cluster recovered from outbreaks of lesions/ulcers. This might reflect the widespread use of the *M. viscosa* vaccines that are likely developed using isolates in the “typical” cluster (Takle et al., 2015).

Historically, blood agar supplemented with 1.5-2.0 % NaCl (BAS) has been used to isolate bacteria from skin lesions/ulcers during outbreaks of “winter ulcer disease” in Norway. This medium allows for easy identification of *M. viscosa* by several typical phenotypic and morphological traits like colony colour, colony viscosity and haemolytic properties (Takle et al., 2015). However, bacterial investigations into the “winter ulcer disease” when using both BAS and marine agar (Difco 2216) (MA) often reveal *M. viscosa* in mixed cultures with other bacteria (Bornø and Lie, 2015). Recently, it has therefore been suggested to separate the “winter ulcers disease” condition into two conditions: “classical winter ulcers” associated with *M. viscosa* and tenacibaculosis associated with *Tenacibaculum* spp. (**Paper-II; Paper-III**). The term “atypical winter ulcers” has sometimes been used as a synonym for tenacibaculosis (Hjeltnes et al., 2017). The separation between the two conditions is based on both the clinical presentation of the disease and the bacteria recovered from the skin lesions/ulcers.

1.2.2 Tenacibaculosis in Norway

In 2010, several high mortality outbreaks of skin lesions/ulcers in sea-reared Atlantic salmon occurred in all regions along the Norwegian coast at low seawater temperatures. The fish displayed severe skin lesions/ulcers, mainly in the head region, but clinical signs as described for the “winter ulcer disease” were also recorded (Bornø and Sviland, 2011). Microscopy of wet mounts preparations from the lesions revealed large amounts of thin long rod-shaped bacteria that were subsequently grown on MA and identified as members of the genus *Tenacibaculum*. In many cases the outbreaks were linked to panic behaviour and mechanical injury of smolts following sea transfer at low seawater temperature (Bornø and Sviland, 2011). This was the first time, that the author is aware of, that *Tenacibaculum* spp. were reported to be associated with high mortality outbreaks of skin lesions/ulcers in Norwegian farmed Atlantic salmon .

Following 2010, the use of MA has become more common and is now routinely used in bacteriological investigations during outbreaks of skin lesions/ulcers. As a consequence, *Tenacibaculum* spp. have been increasingly identified (**Paper-II**; Olsen, 2012; Takle et al., 2015). In the autumn of 2014 and 2015, several outbreaks occurred one to three weeks post sea transfer among smolts at fish farms in Finnmark County where entire pens had to be culled due to fish welfare issues (personal observations)(**Paper-II**). The clinical signs matched what is described for tenacibaculosis; a disease characterized by scale loss, frayed fins, fin rot, skin lesions/ulcers and mouth erosion (Toranzo et al., 2005; Bruno et al., 2013; McBride, 2014). The sea water temperatures measured during the outbreaks were above 8 °C, which is above the temperatures when “winter ulcer disease” usually decreases. Following these significant outbreaks there was renewed interest in tenacibaculosis in Norway, which motivated this study.

1.3 *Tenacibaculum* spp. associated with skin lesions/ulcers

The increased identification of *Tenacibaculum* spp. from skin lesions/ulcers, led to a bath challenge study conducted by Olsen et al. (2011) in order to elucidate their role

in the “winter ulcer disease” pathogenesis. The results from these experiments were inconclusive as the salmon only developed ulcers when co-infected with *M. viscosa* or when the skin was scarified prior to bath challenge. One of the *Tenacibaculum* sp. isolates used (Group 1 isolate F95B/98 = TNO010 (Olsen et al., 2017)) was, however, detected in skin ulcers immunohistochemically which suggests a potential role in the “winter ulcer disease” pathogenesis. Conversely, another isolate (Group 2 isolate F95C/98) was not detected, suggesting that it may not be of importance for the development of skin lesions/ulcers. Due to the results from this study, *Tenacibaculum* spp. isolated from skin lesions/ulcers have been commonly regarded as secondary invaders, only able to infect salmon following primary infection with *M. viscosa* or physical trauma to the skin.

In the 2015 tenacibaculosis outbreaks, *Tenacibaculum finnmarkense*, a newly identified species of *Tenacibaculum* (**Paper-I**) was recurrently found in apparent pure culture from the skin lesions, especially from mouth lesions, whereas *M. viscosa* was not isolated (**Paper-II**). This triggered further investigations which resulted in the demonstration that this bacterium is able to cause tenacibaculosis as it presents in Northern Norwegian farmed Atlantic salmon smolts (**Paper-III**).

Co-infections with *Tenacibaculum finnmarkense* and *M. viscosa* have been reported from suspected outbreaks of tenacibaculosis in larger farmed Atlantic salmon (> 2 kg) vaccinated against *M. viscosa* at low seawater temperatures (3-6 °C) (Ø. Brevik, Cermaq, pers.com). Interestingly, the *M. viscosa* isolates recovered from the skin lesions/ulcers in these cases were found to belong to the “atypical” cluster of *M. viscosa* isolates. As *Tenacibaculum* spp. are frequently observed in histopathological assessment of skin lesions from outbreaks of “winter ulcer disease” in Norwegian farmed salmon, it has been suggested that a *Tenacibaculum* sp. may be an important factor in the pathogenesis of the “winter ulcer disease” (Olsen et al., 2011). This notion is further supported by the findings in a study of the microbiota of farmed Atlantic salmon in Northern Norway with ulcerative disorders (Karlsen et al., 2017b). In that study, the amplicon from skin lesions/ulcers revealed that the skin lesions/ulcers were dominated by *Tenacibaculum* spp. followed by *Arcobacter*, whereas very limited levels of *M. viscosa* were detected.

1.4 The genus *Tenacibaculum*

1.4.1 Taxonomy

The taxonomy of bacteria affiliated with the genera *Cytophaga* and *Flexibacter* (*Cytophaga-Flexibacter-Bacteroides* complex) was a matter of confusion for several decades (Lewin, 1969; Bernardet et al., 1996; Hahnke et al., 2016). However, based on revisions made to the family Flavobacteriaceae in early 2000s, bacteria that were previously named *Cytophaga* and *Flexibacter* had to be renamed (Bernardet et al., 2002; Pinhassi et al., 2004). The genus *Tenacibaculum* was proposed by Suzuki et al. (2001), who found that two bacterial isolates retrieved from sponge and green algae were closely related to two previously characterized marine fish pathogenic *Flexibacter* species: *Flexibacter maritimus* (*Cytophaga marina* (Holmes, 1992)) and *Flexibacter ovolyticus*. A phylogenetic analysis of these bacteria based on their *gyrB* nucleotide sequences and 16S rRNA gene sequences, revealed that they were distantly related to the type strain in genus *Flexibacter*. These findings prompted the transfer of the two *Flexibacter* species to a new genus named *Tenacibaculum*: *Tenacibaculum maritimum* and *Tenacibaculum ovolyticum*, together with the novel species *Tenacibaculum mesophilum* and *Tenacibaculum amylolyticum*.

Currently, the genus *Tenacibaculum* is placed in the *Tenacibaculum-Polaribacter* clade within the family Flavobacteriaceae in phylum Bacteroidetes (McBride, 2014). Even though members of genus *Tenacibaculum* belongs to the family Flavobacteriaceae, they are not closely related to the genus *Flavobacterium*, which holds several important pathogens of freshwater fish (Barnes and Brown, 2011; Nilsen et al., 2011; Declercq et al., 2013; McBride, 2014). Genus *Tenacibaculum* holds 29 species isolated from a wide variety of marine organisms and environments (**Paper-I**; Kim et al., 2013; Kim et al., 2017; Park et al., 2017; Xu et al., 2017; LPSN, 2018; Park et al., 2018). It has also been suggested that *Flexibacter aurantiacus* subsp. *copepodarum* and *Flexibacter echinica* should be included as members of genus *Tenacibaculum* (Nakagawa et al., 2002). A phylogenetic tree based on the 16S rRNA gene sequences of all current members of genus *Tenacibaculum* including *F. aurantiacus* subsp. *copepodarum* and *F. echinica* is presented in Figure 3.

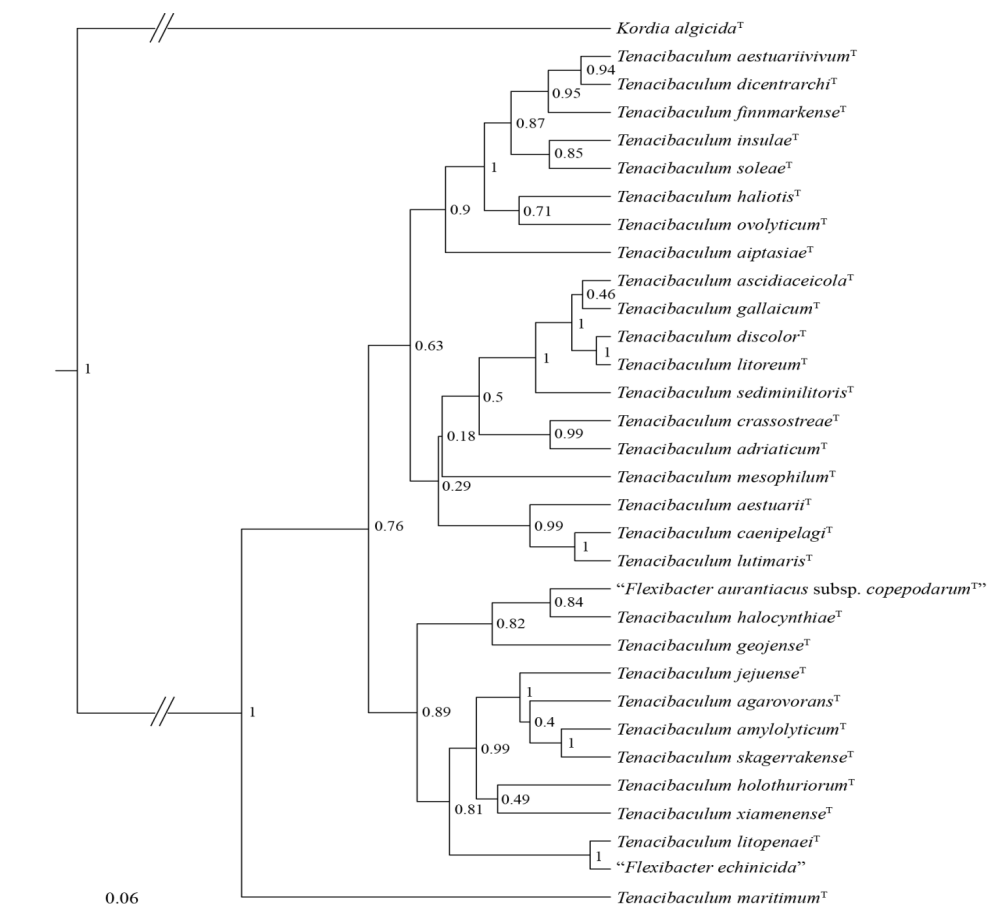


Figure 3 A phylogenetic tree showing the phylogenetic placement of the type strains in genus *Tenacibaculum* including *F. echinica* and *F. aurantiacus* subsp. *copepodarum*. The tree was inferred using an alignment of 1349 base positions of the 16S rRNA gene from 32 taxa. The alignment was constructed in AlignX in Vector NTI (Invitrogen) before being 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). The phylogenetic analysis was performed using BEAST (Drummond and Rambaut, 2007). For the Bayesian analysis a relaxed lognormal molecular clock and a MCMC of 150,000,000 generations was applied, using *Kordia algicida*^T (AB681152) as the outgroup. Sample size values (ESS) were inspected using Tracer ver. 1.6 (Rambaut et al., 2014), and found to be above recommended range (above 200) for all parameters. A maximum clade credibility tree was obtained using a 10% burn-In in Tree-Annotator and viewed using FigTree (Drummond et al., 2012).

1.4.2 Ecology

Little is known about the natural reservoir(s) and/or vectors of *Tenacibaculum* spp. commonly recovered from skin lesions/ulcers in farmed Atlantic salmon in Norway. However, members of the family Flavobacteriaceae (including genus *Tenacibaculum*) are ubiquitous in the marine environment (Buchan et al., 2014). Interestingly, their abundance has been shown to be closely linked to phytoplankton blooms, which can be explained by their ability to utilize a broad range of biopolymers (protein and polysaccharides) as primary carbon and energy sources (Pinhassi et al., 2004; Buchan et al., 2014). This ability may also explain why *Tenacibaculum* spp. can be found associated with the microbiome of marine vertebrates and mammals (Johnson et al., 2009; Thomas et al., 2011; Apprill et al., 2014; Lokesh and Kiron, 2016; Godoy-Vitorino et al., 2017; Karlsen et al., 2017b; Llewellyn et al., 2017). The fish pathogen *T. maritimum* have been found to be associated with jellyfish and sea lice which suggests that they may act as vectors for this bacterium (Barker et al., 2009; Ferguson et al., 2010; Delannoy et al., 2011; Fringuelli et al., 2012). This may be relevant considering that *T. maritimum* has limited survival in natural seawater (Avendaño-Herrera et al., 2006b). However, the role of jellyfish as vectors for *Tenacibaculum* spp. commonly isolated from tenacibaculosis outbreaks in Norway has not been established (**Paper-II**).

1.4.3 Fish pathogenic species

Several *Tenacibaculum* species are well recognized pathogens or associated with disease in many economically important species of farmed marine fish (**Paper-II**; **Paper-III**; Wakabayashi et al., 1986; Hansen et al., 1992; Avendaño-Herrera et al., 2006a; Avendaño-Herrera et al., 2016; Frisch et al., 2017). With regards to fish diseases, it is important to note that members of *Cytophaga* and *Flexibacter* were associated with outbreaks of skin lesions in seawater-reared salmonids several decades prior to the proposal of genus *Tenacibaculum* (Anderson, 1969; Sawyer, 1976; Kent et al., 1988). These bacteria were then collectively referred to as “Myxobacteria” or “Gliding-bacteria” by fish health workers and fish farmers (Kent

et al., 1988; Kent, 1992; Kent and Poppe, 2002). The term “yellow-pigmented bacteria” has also been used to describe such bacteria (Ferguson, 2006). Based on their histological and phenotypic characteristic and their association with skin lesions/ulcers in seawater farmed salmonids, it is likely that the majority, if not all, of these “myxobacteria” would have been affiliated with genus *Tenacibaculum*.

The most studied fish pathogen in the genus is *T. maritimum*. In 2016, this bacterium was isolated and identified for the first time in Norway in connection with an episode of increased mortality of lumpfish in a land based production facility (Småge et al., 2016). Recently, *T. maritimum* have also been identified by real-time RT-PCR during investigations into gills disorders in farmed Atlantic salmon (PHARMAQ-Analytiq, 2017). However, *T. maritimum* has never been isolated or associated with skin lesions/ulcers in farmed Atlantic salmon in Norway (Olsen et al., 2011). As *T. maritimum* growth is very slow or non-existent at temperatures below 10 °C (**Paper-I**; Wakabayashi et al., 1986; Apablaza et al., 2017), the problems associated with this bacterium in Norway may be limited to certain areas and times of the year when seawater temperatures are above 10 °C. Accordingly, this bacterium would likely not be associated with outbreaks of skin lesions/ulcers at low seawater temperatures and in the northernmost parts of Norway. This has also been shown to be the case in Chile, where outbreaks of skin lesions/ulcers in farmed Atlantic salmon have been associated with *T. dicentrarchi* (Avendaño-Herrera et al., 2016) and *T. finnmarkense* (Grothusen et al., 2016; Bridel et al., 2018), while *T. maritimum* has not been detected in connection with such outbreaks (Grothusen et al., 2016; Apablaza et al., 2017).

The *Tenacibaculum* spp. associated with outbreaks of skin lesions/ulcers in farmed Atlantic salmon in Norway, have been shown to be closely related to the species *T. dicentrarchi* and *T. finnmarkense* (**Paper-I**; **Paper-II**; Habib et al., 2014; Karlsen et al., 2017a; Olsen et al., 2017; Bridel et al., 2018). In a recent multilocus sequence analysis (MLSA: See section 1.5) of a large number of *Tenacibaculum* spp. isolates retrieved from Norwegian aquaculture (Olsen et al., 2017), it was shown that the isolates belonged to four major clades. The species *T. finnmarkense* was shown to belong to clade I and III, *T. dicentrarchi* to clade II, whilst clade IV constituted a

novel yet undescribed *Tenacibaculum* species (Olsen et al., 2017; Bridel et al., 2018). Less commonly isolated *Tenacibaculum* spp. from diseased fish in Norway are mostly related to *Tenacibaculum soleae* and *T. ovolyticum* (**Paper-I; Paper-II**; Habib et al., 2014; Olsen et al., 2017). However, as three new members of *Tenacibaculum* closely related to the above mentioned species recently have been suggested: *Tenacibaculum haliotis* (Kim et al., 2017), *Tenacibaculum insulae* (Park et al., 2018) and *Tenacibaculum aestuariivivum* (Park et al., 2017), the phylogenetic affiliation of *Tenacibaculum* spp. associated with skin lesion/ulcers in Norwegian farmed Atlantic salmon has somewhat changed. As shown in the phylogeny presented in Figure 3, they all share common ancestry with *Tenacibaculum aiptasiae* and are phylogenetically distantly related to *T. maritimum*.

Recently, it has been shown from full genome sequences that *T. maritimum* possesses genes that codes for strikingly different virulence strategies than what is identified in the genomes of *T. ovolyticum*, *T. soleae*, *T. dicentrarchi* and *T. finnmarkense* (Pérez-Pascual et al., 2017; Bridel et al., 2018). These findings are also reflected in the phylogenetic placement of the fish pathogenic species and suggest different paths in the evolution of virulence for *T. maritimum* in relation to the other members of the genus associated with fish diseases (Pérez-Pascual et al., 2017; Bridel et al., 2018).

Little is currently known about the virulence factors involved in *T. finnmarkense* and *T. dicentrarchi* pathogenesis. However, genes that codes for possible virulence factors like metallopeptidases, collagen binding proteins, haemolysins and T9SS mediated secretion system have been identified in their genome sequences (Grothusen et al., 2016; Bridel et al., 2018). The T9SS system is important for both locomotion (gliding motility) and secretion of the previously mentioned virulence factors (McBride and Nakane, 2015; Lasica et al., 2017).

1.4.4 Diagnostics

Microscopy of wet mount preparations from skin lesion/ulcers during suspected tenacibaculosis typically reveals large amounts of long rod-shaped bacteria (Figure 4), but still the recovery of *Tenacibaculum* may prove challenging (Kent, 1992; Pazos

et al., 1996; Olsen et al., 2011; Kolygas et al., 2012). Isolation of *Tenacibaculum* spp. from skin lesions/ulcers in Norwegian farmed Atlantic salmon are usually performed on MA as this growth medium provides the sufficient nutrients and sea salts for growth. The inclusion of sea salts in the growth media used for bacteriological investigation of skin lesions/ulcers are important as several *Tenacibaculum* spp. have an absolute requirement for sufficient sea salt (**Paper-I**; Suzuki et al., 2001; Olsen et al., 2011; Karlsen et al., 2017b).

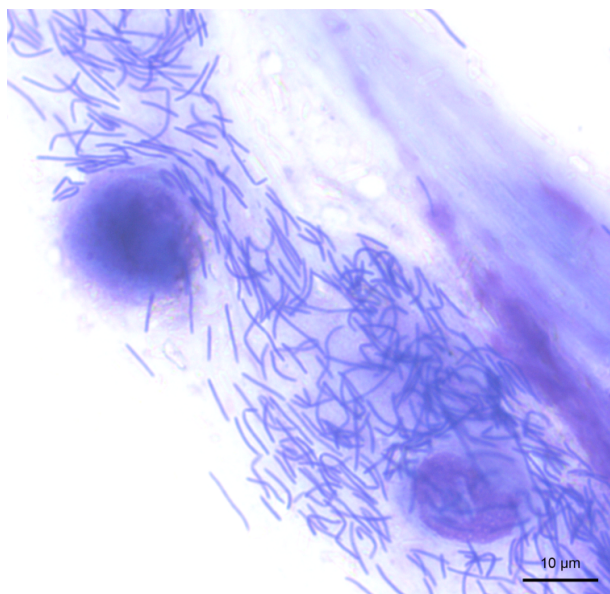


Figure 4 Wet mount of a skin smear from a tenacibaculosis affected smolt.

As *Tenacibaculum* spp. can be found in mixed cultures with other marine bacteria when grown on MA, the recovery can sometimes be difficult as they may be outcompeted, inhibited, or overgrown by bacteria like *Vibrio*, *Alteromonas*, and *Pseudoalteromonas* (Kent et al., 1988; Hahnke and Harder, 2013; Toranzo, 2015; Frisch et al., 2017). For this reason disinfection of the surface of the lesion/ulcer with ethanol prior to sampling has been suggested as a method for improving bacterial recovery (Kent et al., 1988). As members of genus *Tenacibaculum* are generally resistant to aminoglycoside antibiotics, the addition of such antibiotics to the growth media may also aid in the recovery of *Tenacibaculum* spp. (Hahnke and Harder,

2013; Frisch et al., 2017). Another commonly used growth medium for growing *Tenacibaculum* spp. is the *Flexibacter maritimus* medium (FMM), initially developed for the isolation of *T. maritimum* (Pazos et al., 1996). However, *Tenacibaculum* spp. isolated from skin lesions/ulcers in farmed Atlantic salmon in Norway generally show slower and/or less growth when grown on this medium (personal observations), which has also been noted by Toranzo (2015). Still, FMM can be useful when isolating *T. maritimum* as this medium can indicate which serotype the isolate belongs to and that it generally reduces the growth of extraneous bacteria (Pazos et al., 1996; Toranzo, 2015).

Another problem that can occur when isolating *Tenacibaculum* is that they may display gliding motility. This can make colonies grow into each and hamper the acquirement of bacterial clones. As a result of the above mentioned challenges, several sub-cultivations may be required in order to obtain clones, which is very labour-intensive, and may affect bacterial properties like virulence (Kent, 1992; Somerville et al., 2002; Chapuis et al., 2011). For this reason, fish health personnel that provide samples to the Fish Disease Research Group (UiB) are encouraged to perform the primary isolation on one MA plate per sample using the plate-streak method as this usually allows for an easy and swift recovery of bacterial clones. Moreover, as lesions usually contain large amounts of *Tenacibaculum*-like bacteria (**Paper-II; Paper-III**; Kent et al., 1988; Olsen et al., 2011), making serial dilutions of affected tissue using seawater may be helpful in obtaining bacterial clones (Kent and Poppe, 2002). It should also be noted that when performing serial dilutions of *Tenacibaculum* spp. for example when determining CFU by direct plate counting, the transition from a liquid medium to a solid medium can make certain *Tenacibaculum* spp. display swarming colonies (Figure 5B) which makes determining CFU virtually impossible.

The colony morphology of commonly recovered *Tenacibaculum* spp. from skin lesions/ulcers is round with smooth edges when grown on MA (Figure 5A) and displays varying degree of yellow pigmentation ranging from pale to bright yellow (**Paper-I**; Olsen et al., 2011). There are generally three types of cell morphologies observed microscopically for *Tenacibaculum* spp. isolated from fish in Norway (in

exponential growth phase): short rod-shaped cells with occasional longer filamentous cells (2-10 μm) (e.g. *T. ovolyticum*, *T. maritimum* and *T. soleae*), medium sized rod-shaped cells (2-25 μm) (e.g. *T. finnmarkense*) (Figure 6.A1-2), and longer flexible filamentous cells (2-40 μm) (e.g. *T. dicentrarchi*) (**Paper-I**). From outbreaks of tenacibaculosis in Finnmark County, medium sized rod-shaped cells (5-15 μm) are most frequently observed (e.g. Figure 4) (**Paper-II**). As a concurrent mixture of heterogeneous *Tenacibaculum* isolates may be identified from the same tenacibaculosis outbreak (**Paper-II**; Karlsen et al., 2017b; Olsen et al., 2017), it is important to examine more than one colony in order to gain insight into the genetic variation of *Tenacibaculum* isolates present.

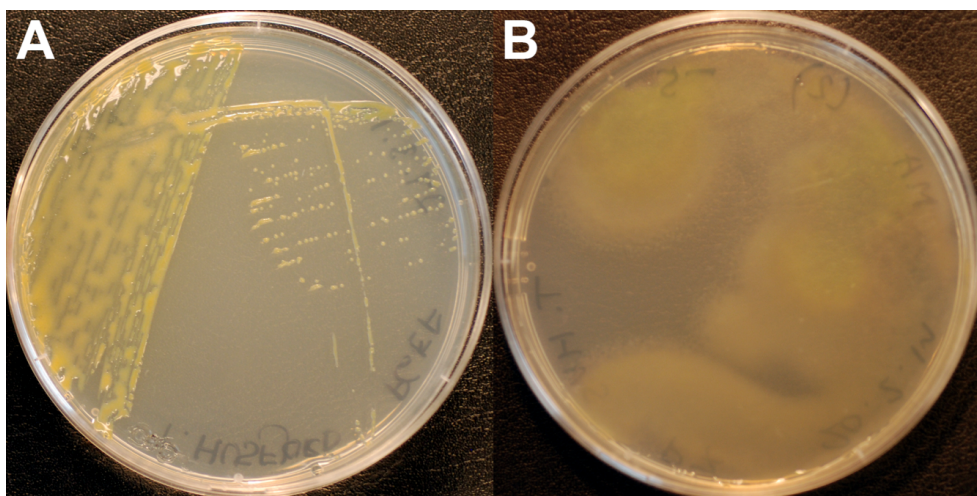


Figure 5 MA plates displaying the colony morphology of *T. finnmarkense* strain HFJ^T when: (A) subcultured from a solid medium (MA) and (B) when subcultured from a liquid medium (MB). Notice the swarming colonies (B) resulting from the gliding motility of the *T. finnmarkense* strain HFJ^T.

Commonly used incubation temperatures for *Tenacibaculum* spp. are between 15-20 °C (**Paper-I**; Olsen et al., 2011). Temperatures above 20 °C are not recommended as they will not sustain growth for psychrophilic i.e. “cold loving” *Tenacibaculum* strains commonly isolated in the northernmost parts of Norway (**Paper-I**). Subcultivation should be performed every 48-72 hours (15-20 °C) as there is a rapid decrease in viability, indicated by the observation of spherical degenerative cells

(Figure 6B1-B2) with prolonged incubation (**Paper-I**; Olsen et al., 2011). Lower incubation temperatures (2-10 °C) can be useful when there is a need to extend the viability of the bacterial cells for example during transportation. In this regard it is important to note that several *Tenacibaculum* spp. isolated in Norway are psychrophilic, able to grow quite rapidly at very low temperatures, which entails that the viability in any case will be limited. On the other hand, isolation and cultivation of *T. maritimum* must be performed at temperatures above 10 °C (Småge et al., 2016; Apablaza et al., 2017; Olsen et al., 2017). Storage of bacterial cultures is usually performed by freezing at -80 °C or in liquid nitrogen for long time storage. Before freezing, the cultures are either suspended in marine broth (MB) (Difco 2216) or grown in MB, and then mixed with a standard mixture proportion of glycerol or 50/50 MB and Biofreeze (Biochrom AG). As thawing time may be important for successful revival of bacterial cells, the total volume in each vial should not exceed 0.5 ml.

BAS is routinely used as a standard growth medium in the bacteriological investigation of skin lesions/ulcers, as well as other bacterial diseases in Norwegian aquaculture. Because this medium does not support growth for several *Tenacibaculum* spp. (**Paper-I**; Olsen et al., 2011; Karlsen et al., 2017b), it is evident that BAS should not be used as the only growth medium in bacteriological investigations of skin lesions/ulcers, as highlighted in a study by Karlsen et al. (2017b). However, BAS has proven useful for isolating other bacteria associated with skin lesions/ulcers like *A. wodanis* and *M. viscosa*.

When successfully isolated, *Tenacibaculum* spp. can be identified by sequencing the 16S rRNA gene using universal primers for bacteria, and then uploading their sequences to BLAST. As the 16S rRNA gene may lack the resolution needed to separate closely related strains within a species, a multilocus sequence typing (MLST) (Maiden et al., 1998) scheme has been developed for *Tenacibaculum* (Habib et al., 2014). A single gene alternative to MLST, using the gene *rlmN* has been proposed as a rapid, reliable and less costly diagnostic typing approach for *Tenacibaculum* spp. isolated from farmed fish in Norway (Olsen et al., 2017).

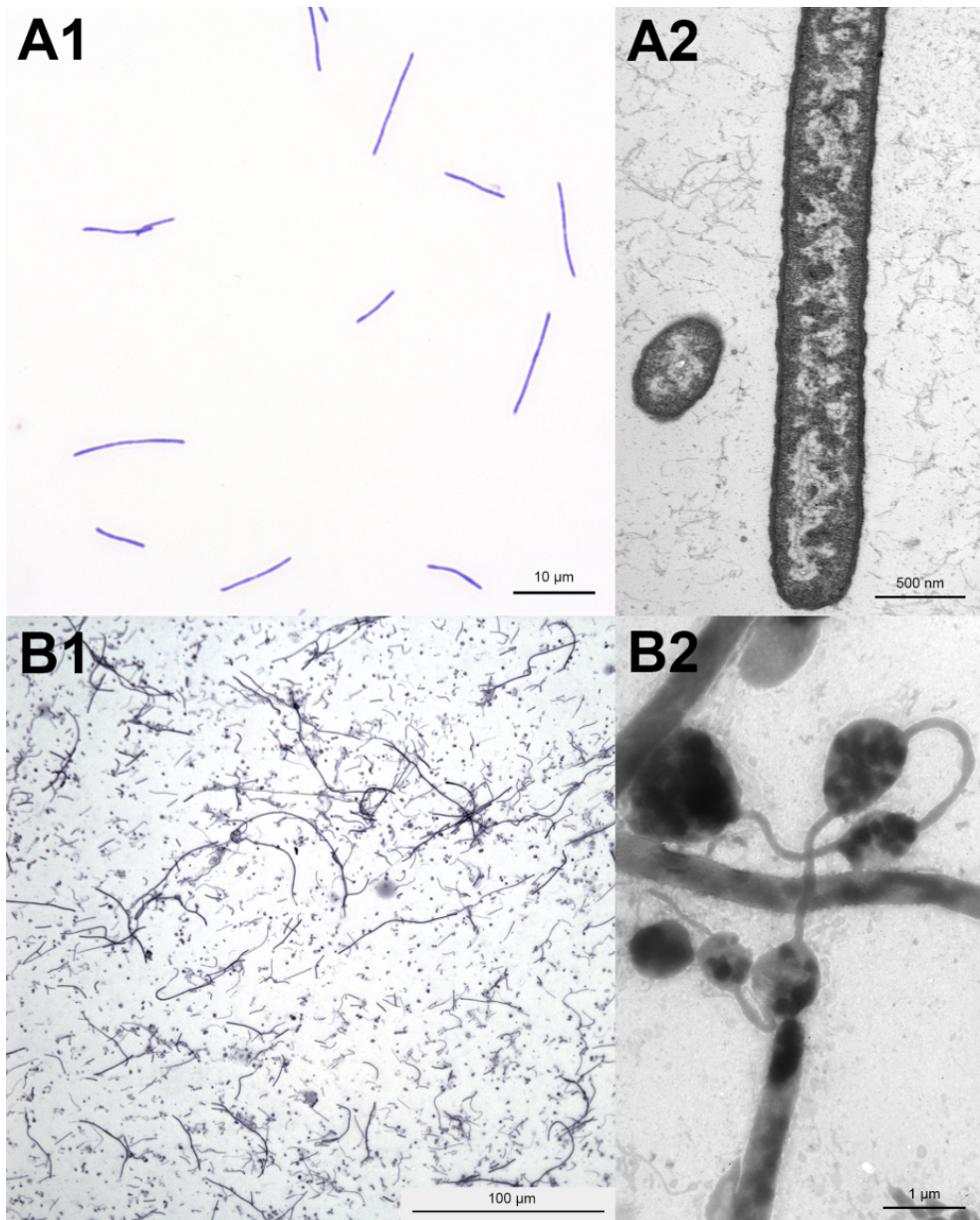


Figure 6 Light microscopy and TEM micrographs showing the cell morphology of *T. finnmarkense* strain HFJ^T at 2 days post incubation (**A1-A2**) and at 6 days post incubation (**B1-B2**). The bacterium was grown on MA plates at 16 °C. Notice the accumulation of spherical degenerative cells after 6 days of incubation (**B1**). Highly condensed bacterial remnants within spherical compartments inside a degenerative cell can be seen (**B2**).

For routine diagnostic purposes, species specific PCR-assays have been developed for the rapid detection of *T. maritimum* (Toyama et al., 1996; Bader and Shotts Jr, 1998; Avendano-Herrera et al., 2004), *T. soleae* (García-González et al., 2011) and *T. dicentrarchi* (Avendaño-Herrera et al., 2018). Recently, the use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has been suggested as a tool for rapid differentiation of *Tenacibaculum* spp. in routine diagnostics (Fernández-Álvarez et al., 2017).

Few differentiating biochemical tests exists for *Tenacibaculum* spp., but it should be noted that *T. maritimum* has a strong catalase reaction whereas other *Tenacibaculum* spp. isolated from skin lesions/ulcers in Norwegian farmed Atlantic salmon typically present weak reactions (personal observations) (Olsen et al., 2011).

1.5 Describing novel bacterial species

Although there is no widely accepted concept of what constitutes a prokaryotic species (i.e. the species concept) (Gevers et al., 2005; Doolittle and Zhaxybayeva, 2009), there is a general agreement that the delineation of a species for taxonomic purposes must be based on a set of parameters that will guarantee an understanding of its uniqueness (Rosselló-Móra and Amann, 2015). This is commonly achieved by using the “polyphasic approach” to bacterial taxonomy, which aims to integrate the phenotypic, chemotaxonomic, genetic and phylogenetic data as a basis for the delineation of bacterial species. This approach is generally advocated as a best practice (Tindall et al., 2010) and is recommended in the “proposed minimal standards for describing new taxa in family Flavobacteriaceae” (Bernardet et al., 2002). According to Rosselló-Móra and Amann (2015), an accurate classification of a novel species should be based on the fulfilment of three major premises based on the polyphasic data: (i) monophyly, (ii) genomic coherence, and (iii) phenotypic coherence.

From genome comparison by means of measuring mol % G+C content and DNA-DNA- hybridization (DDH) reassociation values back in the 1960s, it was established that phenotypically coherent bacteria that shared DDH values above 70 % belonged to the same species (Rosselló-Móra and Amann, 2015). The DDH reassociation value of 70 % thus became the cut-off criterion when describing novel species (Wayne et al., 1987; Gevers et al., 2006). Following the advent of sequence analysis of the 16S rRNA gene, it became clear that it could be used as a tool to determine the phylogenetic position of bacteria. It was found that a 16S rRNA gene sequence similarity between two bacteria of less than 97 % corresponded to a DDH reassociation value of less than 70 % (Stackebrandt and Goebel, 1994). This meant that DDH analysis, which is known to be labour-intensive, expensive and error prone, only needed to be performed when 16S rRNA gene sequence similarities were above 97 % (Stackebrandt and Goebel, 1994). The 97 % threshold value has since then been widely used and resulted in a large increase in species descriptions, especially ones based on single strains. Recently, however, this threshold value has been adjusted to 98.6-98.7 % 16S rRNA gene sequence similarity for when DDH analysis are needed to establish if a strain belongs to the same species (Stackebrandt and Ebers, 2006; Kim et al., 2014).

MLST is a tool intended for epidemiological studies which can be used to recognize distinct strains within named species by using the allelic mismatches of a small number of (most commonly seven) housekeeping (HK) genes (Gevers et al., 2005). However, by concatenating the HK gene sequences obtained for MLST, the sequence data can be used to construct phylogenetic trees which can help in the separation of closely related species and thus be used for species demarcation (Gevers et al., 2005). When used for this purpose it is referred to as MLSA. Currently, MLSA is a widely used method to investigate phylogenetic relationship at the genus and species level (Glaeser and Kämpfer, 2015) and has been used to investigate relationships within and between species in genus *Tenacibaculum* (**Paper-I**; Habib et al., 2014; Avendaño-Herrera et al., 2016; Småge et al., 2016; Apablaza et al., 2017; Frisch et al., 2017; Olsen et al., 2017). It is clear that MLSA can provide the phylogenetic

resolution needed for species delineation as it avoids possible erroneous results based on single genes, which may be affected by recombination (Didelot et al., 2009; Cody et al., 2014).

Modern advances in DNA sequencing technology, i.e. Next Generation Sequencing (NGS), have greatly reduced the costs of whole-genome sequencing (WGS) and have led to a rapid increase of available complete bacterial genomes. Even prior to genomics being introduced as a tool for prokaryotic species circumscriptions, it was stated by the *ad hoc committee* that “the complete deoxyribonucleic acid (DNA) sequence would be the reference standard to determine phylogeny and that phylogeny should determine taxonomy” (Wayne et al., 1987). This implies that instead of using DNA reassociation for species demarcation, as at that time was considered the best applicable procedure, a comparative genome analysis approach can be used. However, this approach needed to be validated in light of the corresponding recommended DDH value of 70 % for species demarcation. For this purpose, it is evident that average nucleotide identity (ANI), which is a category of computational analysis, has its advantages.

The calculation of ANI usually involves the fragmentation of genome sequences, followed by nucleotide sequence search, alignment and identity calculations (Yoon et al., 2017). Because ANI provides an overall description of the genetic relatedness derived from lineage-specific genes, it increases the robustness and resolution of phylogenetic signals (Arahal, 2014). As ANI is based on a large number of genes, it provides a better measure of relatedness than what can be obtained by single gene derived data such as 16S rRNA gene sequences. Moreover it has the advantage of not being affected by horizontal gene transfer or varied evolutionary rates of a single or a few genes (Arahal, 2014). Several studies have shown that an ANI value of 95–96 % corresponds to the DDH reassociation value of 70 % (Goris et al., 2007; Richter and Rosselló-Móra, 2009).

In a study by Kim et al. (2014), it was further established that a 16S rRNA gene sequence similarity of 98.65 % corresponded to the 95-96 % ANI cut-off value, which is in accordance with the 98.6-98.7 % 16S rRNA gene sequence similarity

suggested for when DDH analysis are needed (Stakebrandt and Ebers, 2006). As a consequence, ANI values can be used with confidence to replace DDH values when the full genome of the strain under study is available; although these values need to be in agreement with the corresponding phenotypic and phylogenetic data (Arahal, 2014). Recently, comparative ANI analysis have been used to separate the closely related fish pathogenic *T. finnmarkense* and *T. dicentrarchi* into two distinct species with an ANI value of 93-94 % (Bridel et al., 2018). This separation was also strongly supported by bootstrap values obtained in the phylogenetic tree inferred using 895 core genome genes in the same study.

It is also worth mentioning that from genome sequences, important taxonomic information can be easily derived such as G+C content and sequences of genetic markers (Arahal, 2014). Labour-intensive chemotaxonomic and phenotypic tests can therefore be omitted (Gevers et al., 2005), and their role in bacterial species description in the genomic era becomes less relevant (Sutcliffe et al., 2012; Vandamme and Peeters, 2014). In conclusion, WGS provides a superior taxonomic tool that would greatly affect future taxonomic studies and has already proven useful in taxonomic studies of bacteria in the genus *Tenacibaculum* (Pérez-Pascual et al., 2017; Bridel et al., 2018).

1.6 Fish skin

The fish skin is very important in several disease processes (Ferguson, 2006; Roberts, 2012), especially for a skin disease such as tenacibaculosis. The fish skin is the vital barrier that separates the fish from the aquatic environment and which allows for normal internal physiology (Kryvi and Totland, 1997; Le Guellec et al., 2004; Roberts, 2012). Due to the intimate contact with the environment, this metabolically active organ serves vital functions in for example respiration, osmoregulation, excretion and immunity (Esteban, 2012). There are two main differences between the fish skin and the skin of other vertebrates. Firstly, the fish skin possesses scales and secondly, it does not possess a keratinized outer layer of dead cells (Kryvi and Totland, 1997; Le Guellec et al., 2004). The results and discussions in this thesis have

many references to fish skin anatomy and function, therefore more information about this organ has been attached to this thesis, see Appendix - The Fish Skin.

2. PROJECT AIMS

The overall aim of this study was to gain knowledge about *Tenacibaculum* spp. isolated from skin lesions/ulcers of farmed Atlantic salmon in Northern Norway with the following specific objectives:

1. Describe tenacibaculosis as it presents in Atlantic salmon smolts in Northern Norway, including clinical presentations, pathology, bacteria involved and disease factors.
2. Describe a novel *Tenacibaculum* sp. recurrently recovered from outbreaks of tenacibaculosis by using a polyphasic approach.
3. Investigate possible vectors and reservoirs, as well as environmental factors associated with tenacibaculosis outbreaks.
4. Develop a challenge model in order to reproduce the disease in the laboratory.
5. Using the developed challenge model to test differences in pathogenicity between *Tenacibaculum* sp. strains, investigate modes of transmission and test “proof of concept” vaccines.

3. PAPER ABSTRACTS

Paper I: A novel Gram-stain negative, aerobic, non-flagellated, rod-shaped gliding bacterial strain, designated HFJ^T, was isolated from a skin lesion of a diseased Atlantic salmon (*Salmo salar* L.) in Finnmark, Norway. Colonies were observed to be yellow pigmented with entire and/or undulating margins and did not adhere to the agar. The 16S rRNA gene sequence showed that the strain belongs to the genus *Tenacibaculum* (family *Flavobacteriaceae*, phylum '*Bacteroidetes*'). Strain HFJ^T exhibits high 16S rRNA gene sequence similarity values to *Tenacibaculum dicentrarchi* NCIMB 14598^T (97.2 %). The strain was found to grow at 2–20 °C and only in the presence of sea salts. The respiratory quinone was identified as menaquinone 6 and the major fatty acids were identified as summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{15:1} and iso-C_{15:0} 3-OH. The DNA G+C content was determined to be 34.1 mol%. DNA–DNA hybridization and comparative phenotypic and genetic tests were performed with the phylogenetically closely related type strains, *T. dicentrarchi* NCIMB 14598^T and *Tenacibaculum ovolyticum* NCIMB 13127^T. These data, as well as phylogenetic analyses, suggest that strain HFJ^T should be classified as a representative of a novel species in the genus *Tenacibaculum*, for which the name *Tenacibaculum finnmarkense* sp. nov. is proposed; the type strain is HFJ^T = (DSM 28541^T = NCIMB 42386^T).

Paper II: Tenacibaculosis is an increasing problem in the Norwegian Atlantic salmon aquaculture industry causing significant economic losses. In September 2015, two separate outbreaks of suspected tenacibaculosis occurred at two Atlantic salmon farms in Finnmark County in Northern Norway. The events resulted in major losses of smolts newly transferred into seawater. Prior to, and during the outbreaks, large numbers of small jellyfish, identified as *Dipleurosoma typicum* (Boeck) were observed in the vicinity of the farms and inside the net-pens. This study investigates the possible link between the jellyfish, *Tenacibaculum* spp. and the tenacibaculosis

outbreaks. Bacteriology, histology, scanning and transmission electron microscopy, and real-time RT-PCR screening were performed on both fish and jellyfish samples. Based on the findings, *Tenacibaculum finnmarkense* was found to be the dominant bacteria associated with the tenacibaculosis outbreaks at both sites and that *D. typicum* is unlikely to be a vector for this fish pathogenic bacterium. However, results do show that the jellyfish caused direct damage to the fish's skin and may have exacerbated the bacterial infection by allowing an entry point for bacteria.

Paper III: Tenacibaculosis, an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins and tail rot, is a significant fish welfare issue in marine aquaculture worldwide. The disease is a concern to the Atlantic salmon aquaculture industry due to mortality losses and the use of antibiotics. The most commonly isolated bacterium from Northern Norwegian salmon farms during tenacibaculosis outbreaks is *Tenacibaculum finnmarkense*; however it has not been proved to be the causative agent. This study investigates the role of *T. finnmarkense* and closely related strains as the possible agent of tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarkense* was also investigated using the developed challenge model. The results show that *T. finnmarkense* is a causative agent of tenacibaculosis as it presents in Northern Norway without the need for any pre-stressor or co-infection. The pathogen does not appear to spread horizontally from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors. Even though a whole cell inactivated vaccine induces an antibody response, this study did not manage to show any protection against tenacibaculosis induced through a bath infection. Future studies need to include elucidating the pathogen-host interactions and identifying possible antigens for vaccine candidates.

4. DISCUSSION

The major aim of the present study was to increase the knowledge about *Tenacibaculum* spp. and their role in causing skin lesions/ulcers in farmed Atlantic salmon in Northern Norway. Historically, skin lesions/ulcers in farmed Atlantic salmon have been associated with *M. viscosa* infections (i.e. “winter ulcers disease”). Following the increased use of suitable growth media since 2010, the identification of *Tenacibaculum* spp. associated with skin lesions/ulcers in Norwegian farmed Atlantic salmon has risen. Despite the increased identification, *Tenacibaculum* spp. have generally been regarded as secondary invaders that are only able to infect salmon following primary infection with *M. viscosa* or physical trauma to the skin. As a result, a low number of scientific papers concerning this topic were available at the initiation of the present study in autumn 2014. Through this current study, the identification, description and demonstration of *Tenacibaculum finnmarkense* as the etiological agent of tenacibaculosis as it presents in Northern Norwegian farmed Atlantic salmon smolts has been given (**Paper-I**; **Paper-II**; **Paper-III**). As these aspects are discussed in **Papers I-III**, they will not be discussed in this thesis.

4.1 Genetic diversity of Norwegian *Tenacibaculum* spp.

Results from a recent MLSA study including 89 Norwegian *Tenacibaculum* spp. isolates recovered from farmed fish along the entire Norwegian coastline revealed considerable genetic diversity (Olsen et al., 2017). It was further shown that the majority of these isolates could be separated into four distinct clades (I-IV) (Olsen et al., 2017). Due to the lack of comparable gene sequences available for *T. finnmarkense* strain HFJ^T when Olsen et al. (2017) did their MLSA analysis, the phylogenetic placement and clade affiliation of *T. finnmarkense* strain HFJ^T was not addressed. However, by inferring the phylogenetic placement of *T. finnmarkense* strain HFJ^T and 20 representative strains from the four clades using the *rlmN* gene, it is clear that *T. finnmarkense* strain HFJ^T belongs to clade III (Figure 7); and that all

isolates included in the Olsen et al. (2017) MLSA study are closely related to *T. finnmarkense* and *T. dicentrarchi*.

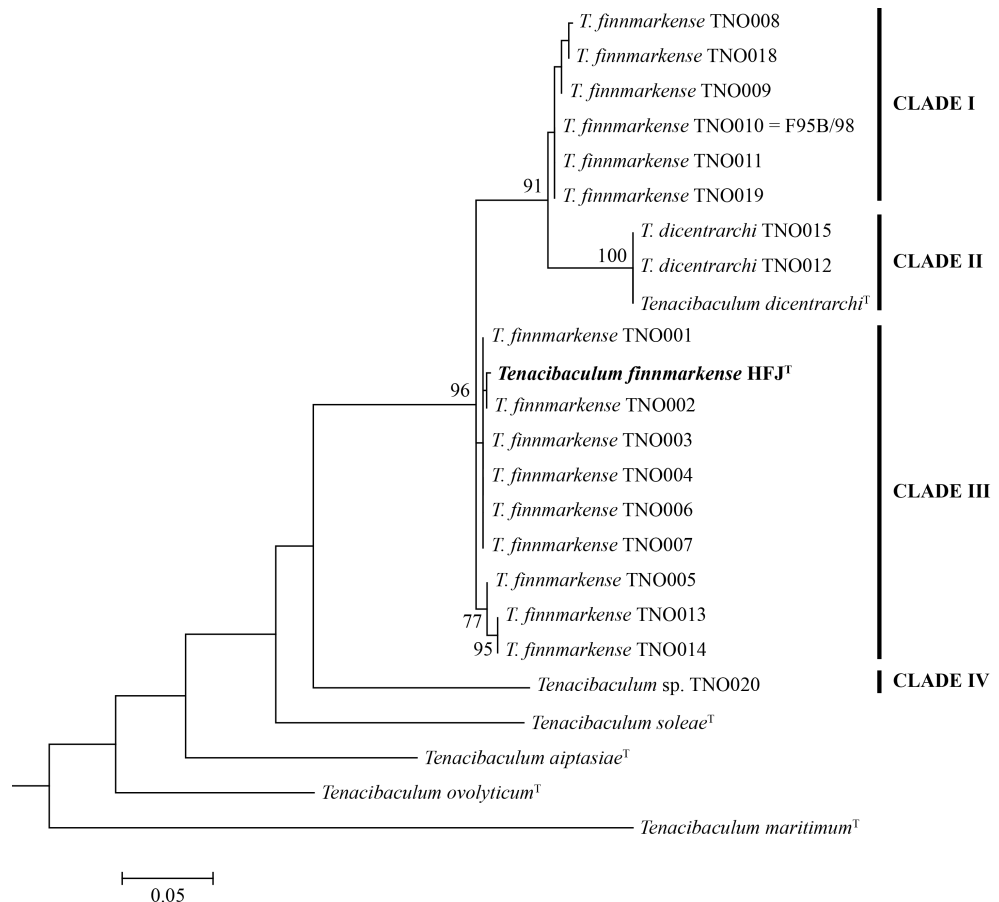


Figure 7 The phylogenetic placement of Norwegian *Tenacibaculum* spp. strains inferred using the *rlnN* gene sequences available in GenBank. The analysis was performed in Mega6 using the T92+I model in a Maximum Likelihood analysis (support values below 75 have been removed). The analysis reveal the separation of the strains into four clades as proposed by Olsen et al. (2017). The phylogenetic placement of the isolates show that the fish pathogenic *T. finnmarkense* HFJ^T belongs to clade III, while *T. finnmarkense* strain TNO010 (=F95B/98) used in the challenge study conducted by Olsen et al. (2011) belongs to clade I.

Recently, a genomic phylogenetic analysis which included *T. finnmarkense* strain HFJ^T, *T. dicentrarchi* strain 35/09^T, in addition to six *Tenacibaculum* sp. field isolates (mainly from farmed salmon) from both Chilean and Norwegian fish farms, showed

that they all could be placed within the four groups (I-IV) suggested by Olsen et al. (2017) (Bridel et al., 2018). This study further revealed that *T. finnmarkense* had been isolated from outbreaks of tenacibaculosis in farmed Atlantic salmon in Region XI (Region de Aysèn) in Chile, expanding the geographic distribution of this bacterium (Grothusen et al., 2016; Bridel et al., 2018). Moreover, from comparative ANI calculations and from genome phylogenetic analysis it was shown that *T. finnmarkense* constitutes two sub-clusters where *T. finnmarkense* strain HFJ^T belongs to clade III, while *T. finnmarkense* strain TNO010 used in the challenge study conducted by Olsen et al. (2011) belongs to clade I.

Interestingly, the data from the MLSA study conducted by Olsen et al. (2017) revealed that the majority of the *T. finnmarkense* isolates recovered from Atlantic salmon in Northern Norway belonged to clade III (10/13), whereas Atlantic salmon isolates that affiliated with clade I were from both Western Norway (9/18) and Northern Norway (6/18). Because the majority of tenacibaculosis outbreaks occur in the northernmost parts of Norway (**Paper-I**; **Paper-II**; Hjeltnes et al., 2017; Karlsen et al., 2017b), these results indicate that the *T. finnmarkense* strains belonging to clade III are predominantly associated with tenacibaculosis outbreaks in this region. This is further supported by the findings in **Paper-II**, which revealed that *T. finnmarkense* strain HFJ^T was the predominant strain recovered from Atlantic salmon sampled during tenacibaculosis outbreaks in Northern Norway.

In the challenge study conducted by Olsen et al. (2011) it was shown that one of the two strains used was unable to induce an infection (Isolate F95C/98), presumably due to poor invasive properties; whereas strain TNO010 was able to induce infection, but only following skin scarification or co-infection with *M. viscosa*. This indicates that there are differences in pathogenicity between the strains. As the clade affiliation of the non-pathogenic isolate F95C/98 is not known, it is difficult to determine if this variation in pathogenicity of the strains is found within the same clade or not. However, based on the results from the challenge study conducted in **Paper-III**, there seems to be a difference in pathogenicity between strains belonging to the same clade (clade III): *T. finnmarkense* strain HFJ^T appears to be more pathogenic than *T.*

finnmarkense strain Tsp.2. Moreover, differences in pathogenicity between strains belonging to clade I compared to clade III is supported by the fact that strain TNO010 was only able to cause disease following skin scarification or co-infection with *M. viscosa*, whereas no such pre-stressors were needed for *T. finnmarkense* strains HFJ^T and Tsp.2 (**Paper-III**). These results may indicate a similar clade difference in pathogenicity as observed for *M. viscosa*, where the “typical” cluster holds strains mostly associated with the “winter ulcers disease” in Atlantic salmon, whereas the “atypical” cluster holds strains obtained from a variety of fish hosts and which appear to be less pathogenic to Atlantic salmon in Norway.

The *T. dicentrarchi* strain 35/09^T, which belongs to clade II, appears to be less pathogenic to Atlantic salmon smolts than *T. finnmarkense* strain HFJ^T and Tsp.2 (**Paper-III**). The notion that *T. dicentrarchi* is less pathogenic to Atlantic salmon is further supported by the results from the MLSA study conducted by Olsen et al. (2017), which revealed that *T. dicentrarchi* is mainly associated with skin lesions/ulcers in non-salmonid fish host (mainly wrasse). However, Avendaño-Herrera et al. (2016) found that a Chilean *T. dicentrarchi* strain obtained from a tail ulcer of an Atlantic salmon to be pathogenic to salmonids in a challenge study. The experimental conditions in that study were very different to the condition used in the challenge study conducted in **Paper-III**, therefore, the results may have been affected.

As shown in **Paper-II**, *T. finnmarkense* strains belonging to clade III that are identical or closely related to strain HFJ^T are predominantly identified from skin lesions/ulcers in Northern Norwegian salmon farms. However, it has been shown that *Tenacibaculum* spp. likely constitutes members of a healthy microbiota of Atlantic salmon (Karlsen et al., 2017b; Llewellyn et al., 2017); some of which are found to be closely related to *T. finnmarkense* strain HFJ^T (Karlsen et al., 2017b). The likely simultaneous presence of both pathogenic and non-pathogenic *T. finnmarkense* strains in diseased fish may confound the prevention and control of tenacibaculosis. This has also been noted for *F. psychrophilum* infections in salmonids (Dalsgaard, 1993; Ngo et al., 2017). In-depth studies of the variation in the *Tenacibaculum* spp.

present in healthy skin and in skin lesions/ulcers of Atlantic salmon are therefore needed in order to separate clinically important isolates from commensal or environmental ones. These studies also need to include the significance of this variation in the prevention of tenacibaculosis.

Since the proposal of *T. finnmarkense* as a novel fish pathogenic species in genus *Tenacibaculum* in 2015 (**Paper-I**), several new members of the genus have been described. The recently described species *T. aestuariivivum* and *T. insulae*, both isolated from tidal flats, and *T. haliotis* isolated from Abalone, are found to be closely related to *T. finnmarkense*, *T. dicentrarchi*, *T. ovolyticum* and *T. soleae*, all of which are associated with fish diseases (Figure 3). This shows that strains isolated from non-diseased marine organisms and sediments are intertwined with the fish pathogenic strains, suggesting a parallel evolution of fish pathogenicity within this cluster of *Tenacibaculum* type strains (all sharing common ancestry with *T. aiptasiae*). When considering the 16S rRNA gene sequence similarity between the *Tenacibaculum* strains belonging to this cluster of type strains (Table 1), it is clear that they all constitute distinct species when applying the 98.6-98.7 % threshold.

Table 1 The calculated 16S rRNA gene sequence similarities (%) between members of the *Tenacibaculum* cluster of type strains (all sharing common ancestry with *T. aiptasiae*) that are closely related to *T. finnmarkense* and *T. dicentrarchi*. The sequence similarities were calculated using the DNADIST program in PHYLIP (Felsenstein, 2013).

	<i>T. aestuariivivum</i>	<i>T. dicentrarchi</i>	<i>T. finnmarkense</i>	<i>T. haliotis</i>	<i>T. insulae</i>	<i>T. soleae</i>	<i>T. ovolyticum</i>
<i>T. aestuariivivum</i>	100%	98.3%	97.1%	96.3%	96.5%	96.1%	97.3%
<i>T. dicentrarchi</i>	98.3%	100%	97.4%	95.9%	96.7%	96.2%	97.0%
<i>T. finnmarkense</i>	97.1%	97.4%	100%	96.4%	96.8%	96.5%	97.0%
<i>T. haliotis</i>	96.3%	95.9%	96.4%	100%	97.6%	96.5%	97.0%
<i>T. insulae</i>	96.5%	96.7%	96.8%	97.6%	100%	98.2%	97.0%
<i>T. soleae</i>	96.1%	96.2%	96.5%	96.5%	98.2%	100%	96.7%
<i>T. ovolyticum</i>	97.3%	97.0%	97.0%	97.0%	97.0%	96.7%	100%

Recently, the separation of *T. finnmarkense* and *T. dicentrarchi* into two distinct species has been definitely demonstrated in a comparative full genome analysis study (Bridel et al., 2018). This study also revealed that the genome sizes of *T. finnmarkense* and *T. dicentrarchi* were about half of that of the environmental species

T. agarivorans and *T. jejuense*, and that they both lack pathways encoding for the degradation of marine carbohydrates. The reduced genomes sizes of *T. finnmarkense* and *T. dicentrarchi* supports the notion that pathogenic bacteria typically have smaller genomes and possess fewer genes compared to that of their non-pathogenic or less pathogenic relatives (Weinert and Welch, 2017).

4.2 A description of tenacibaculosis in Northern Norway

Skin lesions/ulcers of farmed Atlantic salmon in Norway can manifest in different ways and share several pathological signs (Lillehaug et al., 2003). Thus, it may be difficult to distinguish the “winter ulcer disease” condition from tenacibaculosis based on general descriptions given in the literature. Presently there is some confusion over what constitutes tenacibaculosis in Norwegian farmed Atlantic salmon. Inasmuch as a description and a definition have not been previously given, it is the purpose of the following section to give a description of the disease as it presents in Northern Norwegian salmon farms. This description will be given based on field observations and results obtained in **Paper I, II and III** supplemented with data acquired in the course of this PhD-project, and which will be further compared and discussed in light of relevant scientific literature.

4.2.1 Clinical observation and gross pathology

Outbreaks of tenacibaculosis in Northern Norwegian Atlantic salmon farms mainly occur in late winter and spring (February-April) at low seawater temperatures (3-6 °C) and in late summer and autumn (August-October) at higher seawater temperatures (8-12 °C) (**Paper-II**; Bornø and Sviland, 2011; Karlsen et al., 2017b); whereas smolts introduced to seawater during the summer appear less affected by the disease (**Paper-II**). There seems to be a seasonality to outbreaks, which is similar to what has been noted for outbreaks of skin lesions/ulcers in farmed Atlantic salmon smolts in British Columbia (BC), Canada (Kent et al., 1988). The disease commonly affects smolts shortly following introduction to seawater (1-3 weeks post transfer) and typically presents with an acute progression. Although larger salmon may be

affected by tenacibaculosis, it appears that smolts are particularly susceptible to infections (**Paper-II**; Bornø and Sviland, 2011; Avendaño-Herrera et al., 2016). The higher susceptibility of juvenile fish to tenacibaculosis has been noted in several farmed fish species worldwide (Wakabayashi et al., 1986; Kent, 1992; Frelie et al., 1994; Toranzo et al., 2005; Småge et al., 2016).



Figure 8 Tenacibaculosis in a Northern Norwegian Atlantic salmon farm. Affected smolts are lethargic, swimming high in the water and positioned towards the current.

Both environmental factors (e.g. jellyfish, seawater temperature, UV) and factors associated with intensive farming (e.g. transport, handling, smolt size, smolt quality) may exacerbate the outbreaks; for example, causing skin abrasions which can allow the bacteria to establish an infection (**Paper-II**; Bornø and Sviland, 2011). Interestingly, the most severe outbreaks of tenacibaculosis reported from Northern Norwegian salmon farms have occurred in autumn at seawater temperatures higher ($> 8^{\circ}\text{C}$) than when the “winter ulcer disease” typically lessens or subsides (**Paper-II**).

This matches what is seen in farmed salmonids in BC, where outbreaks of skin lesions/ulcers has been noted to be more severe at temperatures above 10 °C (Hicks, 1989).

During tenacibaculosis outbreaks in Northern Norwegian salmon farms, affected smolts typically show abnormal swimming behaviour (e.g. lethargy and swimming high in the water) (Figure 8). The most common pathological signs are mouth lesion/erosion, lesions/erosion of the fins and tail and lesions/ulcers at the caudal peduncle-tailfin junction (Figure 9). The striking appearance of the mouth lesion/erosion has led to the disease sometimes being referred to as “mouth rot” by fish farmers. However, the term is not to be confused with “mouthrot” used to describe *T. maritimum* tenacibaculosis in farmed Atlantic salmon in BC, which has a different clinical presentation (Kent and Poppe, 2002; Frisch et al., 2017; Frisch et al., 2018). Even though lesions/ulcers frequently occur along the abdomen and posterior flanks, the smolts may also show few or no pathological signs on the scale covered parts of the skin (Figure 9). Interestingly, the dorsal fin and adipose fin are rarely affected, which suggest that these areas of the skin are not the preferred site of infection. The lesions/ulcers observed in moribund fish during tenacibaculosis outbreaks are typically yellow tinged; however, the lesions/ulcers are not covered in a thick yellowish coloured slimy biofilm as seen in *T. maritimum* tenacibaculosis (personal observations).

In scaled skin, the lesions/ulcers typically have uneven margins accompanied by a wide adjacent zone of scale loss (Figure 9A3, 9B3). This is different from what is commonly described for “winter ulcers” associated with *M. viscosa*, as these ulcers have a more defined, rounder form and a narrower zone of scale loss (Figure 2). Moreover, tenacibaculosis in Northern Norwegian farmed Atlantic salmon smolts is a condition in which the unscaled parts of the skin (i.e. mouth and fins) are predominantly affected. The “winter ulcer disease” mainly affects the scale covered parts of the skin. The apparent affinity for unscaled skin has also been noted for other fish pathogenic members of family Flavobacteriaceae infecting Atlantic salmon (Martínez et al., 2004; Barnes and Brown, 2011).

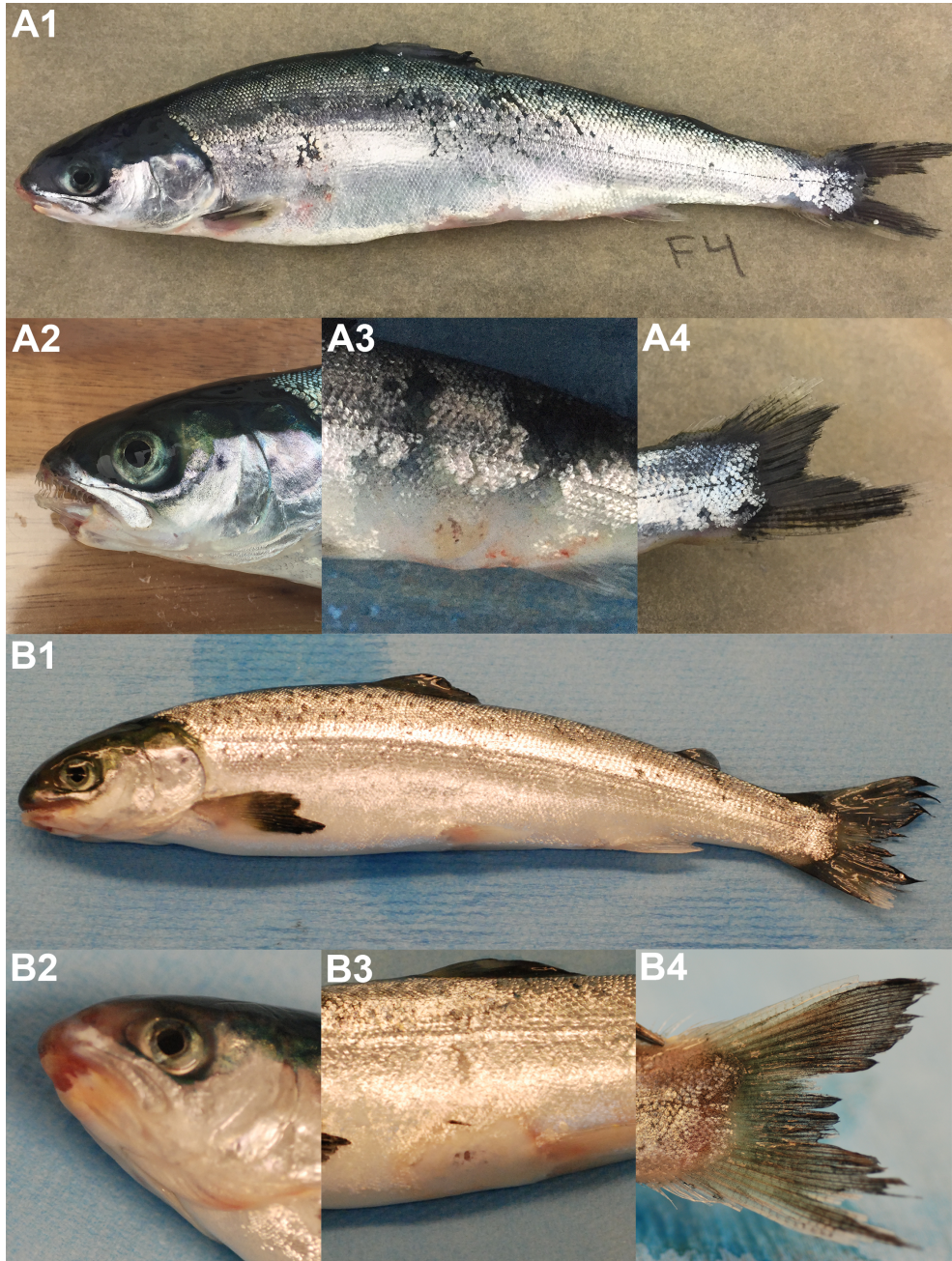


Figure 9 Moribund Atlantic salmon smolts from field outbreaks (A1-4) and challenge studies (B1-4). The smolts are displaying the typical pathological signs of tenacibaculosis: erosion of the jaws and fins (except adipose and dorsal fin) and lesions/ulcers at the abdomen and peduncle-tailfin junction. Figure obtained from Paper III.

The gills of moribund smolts sampled from tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon farms typically reveal few or no pathological signs (**Paper-II**); despite the fact that large numbers of *Tenacibaculum*-like bacteria can be present in gill mucus, and that both *T. dicentrarchi* and *T. finnmarkense* have been retrieved from the gills (Are Nylund, UiB, pers.com.). This indicates that gills are not the preferred site of infection. However, a *Tenacibaculum* sp. has been associated with the proliferative gill inflammation (PGI) condition in Norwegian farmed Atlantic salmon (Steinum et al., 2009). Internally, there are typically no pathological signs associated with the disease, which reflects that *T. finnmarkense* and *T. dicentrarchi* are rarely isolated from internal organs (Olsen et al., 2011; Avendaño-Herrera et al., 2016). These findings are in accordance with what Kent et al. (1988) associated with outbreaks of skin lesions/ulcers in farmed Atlantic salmon smolts in BC: no *Tenacibaculum*-like bacteria or pathological signs in the gills and internal organs. However, this is opposed to what has been shown for *T. maritimum* tenacibaculosis, as this bacterium can cause severe damage to the gills and can be isolated from both gills and internal organs (personal observations) (Chen et al., 1995; Mitchell and Rodger, 2011; Toranzo, 2015). Similarly, *M. viscosa* is also readily recovered from kidney in bacteriological investigation of “winter ulcer disease” outbreaks.

Tenacibaculum spp. have also been associated with a high mortality outbreak in a Northern Norwegian salmon farm where the bacteria infected the eyes (Berg, 2017). This is an uncommon manifestation of tenacibaculosis, although occasional smolts can present with eyes that have a cloudy appearance during field outbreaks (**Paper-II**). In the challenge study conducted by Olsen et al. (2011), infections of the cornea of the eye were described as a pathological sign of tenacibaculosis, but this has not been demonstrated in other challenge studies using *T. finnmarkense* (**Paper-III**) and *T. dicentrarchi* (**Paper-III**; Avendaño-Herrera et al., 2016). The ability to cause eye infections can be related to strain and/or clade: the *T. finnmarkense* strain used in the Olsen et al. (2011) belongs to clade I, whilst the *T. finnmarkense* strains used in **Paper-III** belongs to clade III, and *T. dicentrarchi* to clade II. However,

demonstration of a *Tenacibaculum* spp. strain/clade specific association with eye infections remains to be tested.

The gross pathological signs of tenacibaculosis as it presents in Norwegian farmed Atlantic salmon smolts generally match what has been described from outbreaks of tenacibaculosis associated with *T. dicentrarchi* and *T. finnmarkense* in other salmonid farming regions of the world (Avendaño-Herrera et al., 2016; Grothusen et al., 2016; Bridel et al., 2018). However, the mouth lesion/erosion typically associated with tenacibaculosis in Northern Norwegian farmed salmon smolts has not been described. This is an interesting finding as the *Tenacibaculum* spp. associated with the outbreaks in the different regions are the same (**Paper-I; Paper-II**; Habib et al., 2014; Karlsen et al., 2017b; Olsen et al., 2017).

The ultimate cause of mortality of smolts during tenacibaculosis outbreaks in Northern Norwegian salmon farms is currently not known. It is likely that osmotic perturbation following destruction of the osmotic barriers of the skin is an important factor (Zydlowski et al., 2010). The less favourable surface/volume ratio of smolts, compared to that of larger salmon, may partly explain why smolts appears to be more affected by tenacibaculosis than larger fish. However, the mortality during outbreaks may also be attributed to the release of potent exotoxins as experimentally shown for *T. maritimum* (Van Gelderen et al., 2009).

4.2.2 Microscopic pathology

Histopathological assessments of advanced jaw lesions from moribund smolts sampled during tenacibaculosis outbreaks in Northern Norwegian salmon farms, generally reveal large numbers of *Tenacibaculum*-like bacteria infiltrating the dermis, with a complete loss of the epidermis. The loosely organized dermis of unscaled skin typically holds large numbers of *Tenacibaculum*-like bacteria. Large numbers of *Tenacibaculum*-like bacteria are also typically observed in the *stratum spongiosum* of scaled skin, which has a similar tissue organization as the dermis of unscaled skin. A gradual decrease in tissue destruction and bacterial numbers with distance away from the centre of the lesion is typically observed (Figure 10B1).

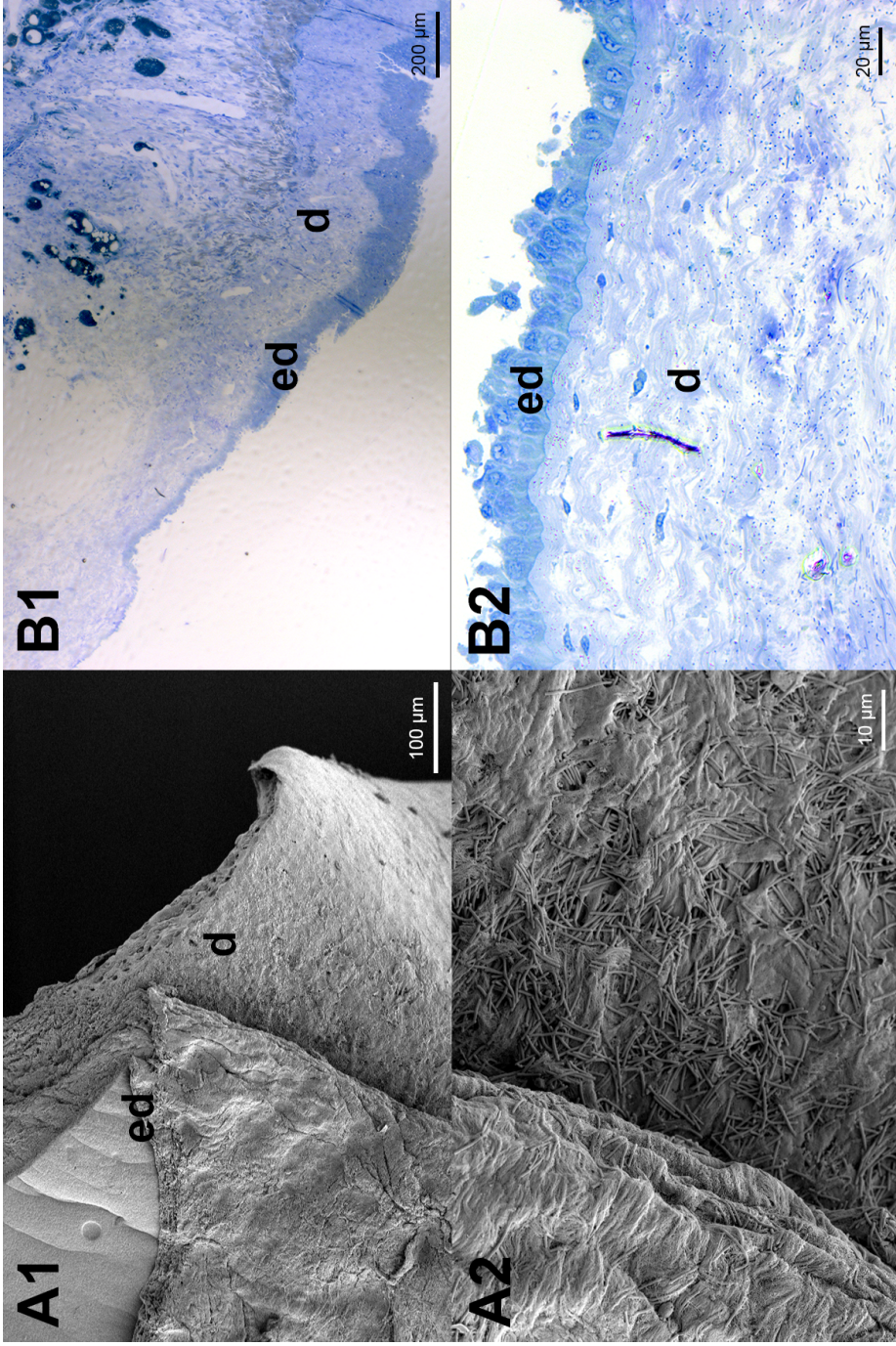


Figure 10 Histopathology and SEM of unscaled skin of Atlantic salmon smolts from tenacibaculosis outbreaks. **A1:** A SEM micrograph of a lesion of unscalded skin showing epidermis and dermis. **A2:** Massive infiltration of *Tenacibaculum*-like bacteria in the dermis layer. **B1:** Histopathological section of a lesion of the lower jaw. **B2:** Infiltration of *Tenacibaculum*-like bacteria in the dermis layer with a partly intact epidermis present. (ed: epidermis; d: dermis)

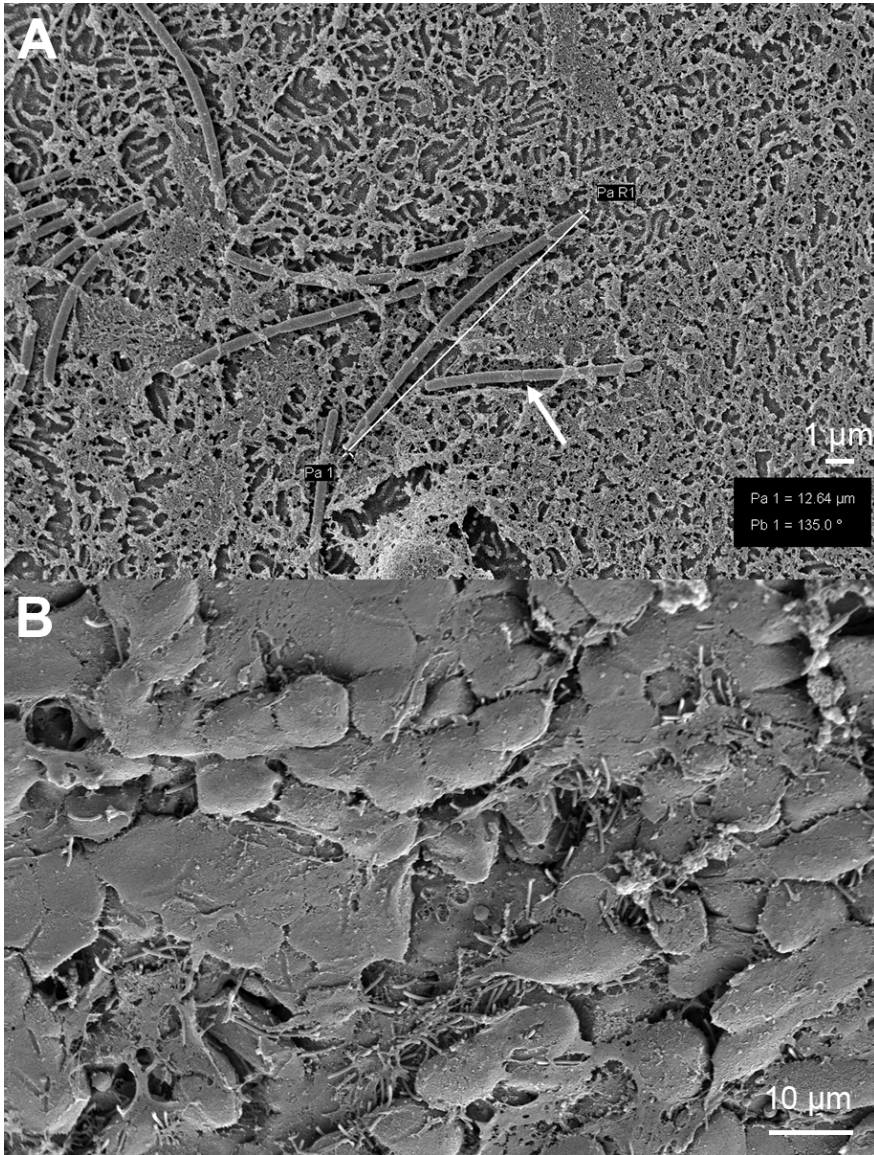


Figure 11 (A) SEM micrograph showing *Tenacibaculum*-like bacteria present in the mucus layer of the cornea of a salmon smolt. This smolt was experimentally bath challenged using *T. fimmarkense* strain HFJ^T. Intact microridges of the malpighian cells can be observed underneath the mucus. Bacterial growth is indicated by the presence of transversely diving bacterial cells (arrow). Courtesy of Are Nylund, UiB. (B) SEM micrograph of the epidermis of moribund Atlantic salmon smolt from a natural outbreak of tenacibaculosis. *Tenacibaculum*-like bacteria are shown infiltrating the epidermis with the associated disassociation of the malpighian cells. The microridges as observed in normal skin are not present which indicates tissue damage.

In less advanced lesions, and in the periphery of severe lesions/ulcers, the epidermis may be partly intact with *Tenacibaculum*-like bacteria situated in the dermis below (Figure 10B2). *Tenacibaculum*-like bacteria are rarely observed in the epidermis in histopathological sections, which also has been noted in other studies of skin lesions associated with tenacibaculosis in Norway (Olsen et al., 2011). On the other hand, in SEM micrographs of skin with partly intact epidermis, large numbers of *Tenacibaculum*-like bacteria can be observed infiltrating the epidermis (Figure 11B) with the associated disassociation of the malpighian cells. The lack of visible *Tenacibaculum*-like bacteria on the surface of the skin in histological sections is likely due to the mucus layer typically not being preserved using standard histological preparation techniques as noted by Handler et al. (1997). In SEM however, *Tenacibaculum*-like bacteria can be observed proliferating in the mucus, with intact microridges of malpighian cells present (Figure 11A).

In less affected tissue or in the periphery of more severely affected tissue of scaled skin, the *Tenacibaculum*-like bacteria are frequently observed in *stratum spongiosum* following the lining of the basement membrane and scale pockets with partially intact epidermis (Figure 12B1). Scale pocket oedema is usually not observed in histopathological assessments of scaled skin from outbreaks of tenacibaculosis in Northern Norway. However, histopathological observations consistent with oedema have been reported from outbreaks of skin lesions/ulcers in Norway (Olsen et al., 2011). Scale pocket oedema is also described for *T. maritimum* tenacibaculosis (Handler et al., 1997).

In sections of severely affected tissue of scaled skin, the complete loss of epidermis and scales, concurrent with a large number of *Tenacibaculum*-like bacteria replacing the *stratum spongiosum* and the distal parts of *stratum compactum* is frequently observed. The loss of epidermis associated with similar lesions has also been noted for tenacibaculosis associated with *T. dicentrarchi* (Avenidaño-Herrera et al., 2016). In scaled skin, *Tenacibaculum*-like bacteria are also found infiltrating deep into the *stratum compactum* where the bacteria can be observed aligned with the collagen fibres following in the direction of their plywood-like organization (Figure 12B2).

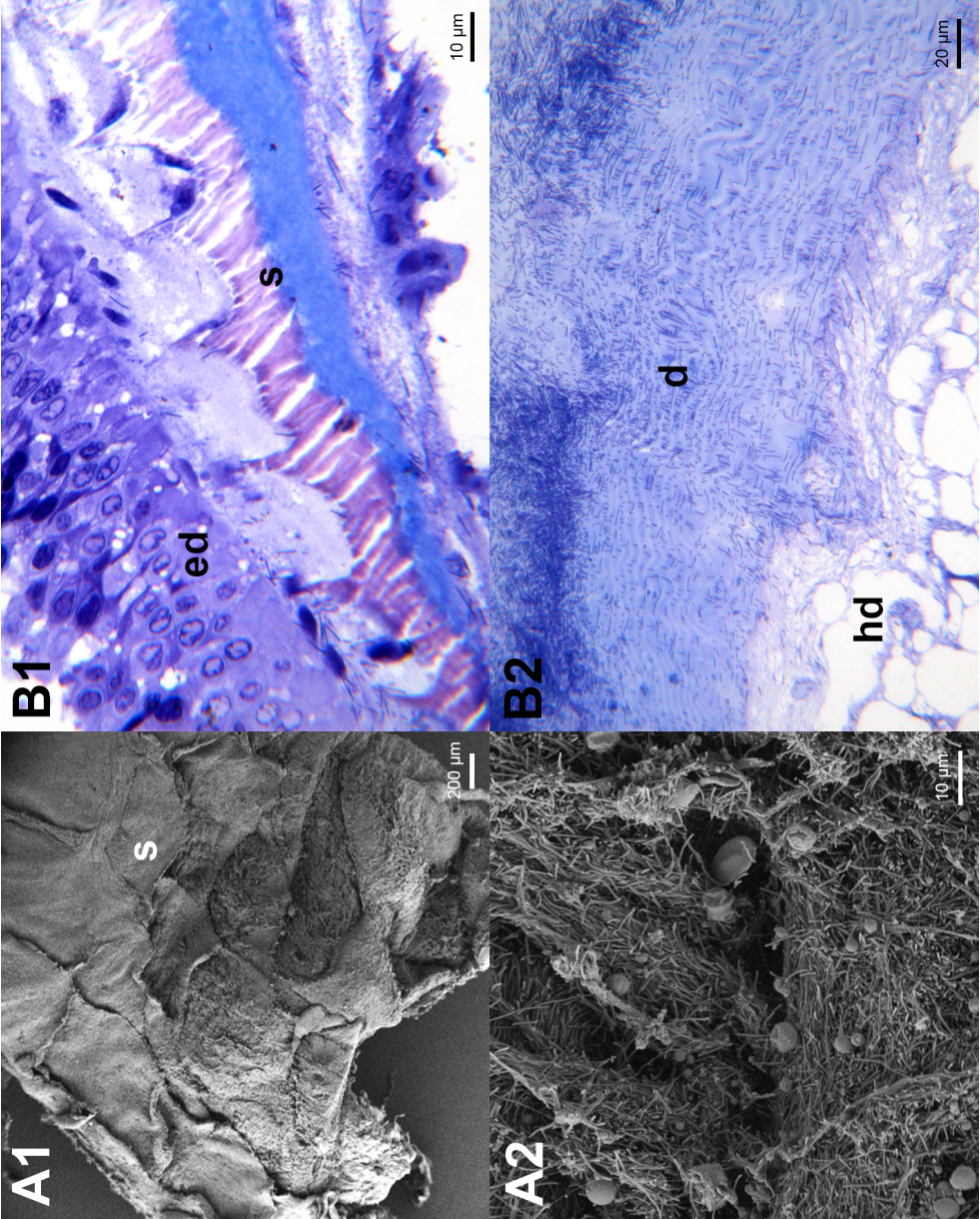


Figure 12 Histopathology and SEM of scaled skin of Atlantic salmon smolts sampled during outbreaks of tenacibaculosis. Picture **B1** and **B2** courtesy of Are Nylund, UiB. (ed: epidermis; d: dermis; hd: hypodermis; s: scale)

This makes the bacterial infiltration into the *stratum compactum* appear highly organized compared to that of *stratum spongiosum* and the dermis of unscaled skin. In severely affected tissue, the *Tenacibaculum*-like bacteria can also be observed invading deep into the connective tissue of the hypodermis and adjacent to muscle tissue. Degeneration of entire muscle fibres and granulation of the sarcoplasm may be observed in muscle tissue associated with the bacteria. A minor inflammatory host response is sometimes observed associated with muscle degeneration. SEM observations of ulcers reveal only the presence of *Tenacibaculum*-like bacteria (Figure 10A2), similar to what can be observed in unscaled skin. The associated loss of scales can also be observed in SEM micrographs (Figure 12A1).

Generally, no other bacterial morphologies other than *Tenacibaculum*-like are observed in lesions/ulcers of both unscaled and scaled skin (Figure 10A2, Figure 12A2). Microscopic evidence shows a distinct lack of host immune response. The restriction of visible *Tenacibaculum*-like bacteria to the skin and musculature, suggests that the bacteria are primarily surface pathogens. The infiltration of large numbers of *Tenacibaculum*-like bacteria into the dermis and underlying musculature with little or no associated host inflammatory response has also been noted in histological assessments from skin lesions/ulcers from field outbreaks associated with *Tenacibaculum* spp. (Kent et al., 1988).

4.3 Disease factors

A large part of the losses in Norwegian salmon farms occur in the first few months following seawater transfer (Aunsmo et al., 2008). This period is therefore considered critical in the production cycle of Atlantic salmon. Smoltification and seawater adaptation of Atlantic salmon is associated with large alterations in endocrine status, osmoregulation, immune system function and behaviour (Johansson et al., 2016). These physiological alterations likely have an effect on the smolts' ability to withstand skin infection following seawater transfer (**Paper-II**; Aunsmo et al., 2008; Bornø and Sviland, 2011; Johansson et al., 2016). Additional factors such as greater pathogen pressure in seawater compared to that of freshwater, stress, and physical

trauma following loading, transport and offloading likely increase the risk of infectious diseases in these already compromised smolts (Iversen et al., 2005).

The transition from freshwater to saltwater is shown to significantly destabilize and alter the skin microbiota of salmon smolts (Lokesh and Kiron, 2016). This suggests that the skin microbiota shortly after sea transfer may be more influenced by the microorganisms (e.g. *Tenacibaculum* spp.) present in the environment than later on once the microbiota has stabilized. The microbiota of larger salmon has, however, been shown to be very different from that of the surrounding water (Karlsen et al., 2017b). The commensal microorganisms present in healthy microbiota act as a barrier against colonization of harmful pathogens or opportunistic pathogens by for example depleting available nutrients, preventing adherence and colonization, releasing toxic metabolites and degrading virulence factors by releasing proteases (Dalsgaard, 1993; Koziel and Potempa, 2013). Thus, the changes in the microbiota following sea transfer likely increases the smolts' susceptibility to pathogenic or opportunistic pathogens like *T. finnmarkense*.

4.3.1 Adherence and colonization of the skin

The ability of pathogenic bacteria to adhere to the epithelial surfaces of the skin is an important initial step for the successful colonization and infection of the host (Dalsgaard, 1993; Ribet and Cossart, 2015). *T. finnmarkense* lacks external structures like pili, fimbria or flagella (Figure 6A2) that are known to be important for adhesion and colonization in other fish pathogenic bacteria (Toranzo and Barja, 1993; Toranzo et al., 2005). Instead, *T. finnmarkense* likely produces extracellular biopolymers or “slime” in order to adhere to the surface of the fish skin as shown for *T. maritimum* (Avendaño-Herrera et al., 2006a). This “slime”, which is secreted via the T9SS secretion system, makes adhesion possible even when the bacteria are gliding, and which can allow for their proliferation at specific sites of the skin (Dalsgaard, 1993; McBride and Nakane, 2015; Lasica et al., 2017). Moreover, this “slime” may serve as a protective coat against innate immune defences and enhance *T. finnmarkense*'s ability to survive within the host.

As shown in Figure 11A, *T. finnmarkense* appears to be able to withstand the harsh environment of the mucus which contains an array of antibacterial innate host immune components. This ability has also been demonstrated for *T. maritimum* (Magariños et al., 1995). Bacterial proliferation can further initiate biofilm formation, which provides resistance to host immune defence systems (Koziel and Potempa, 2013).

4.3.2 Tissue invasion and destruction

The unscaled skin areas of Atlantic salmon smolts (the tip of the fins, the tail and certain parts of the head region), which are virtually devoid of mucous cells (Appendix), are most frequently affected during outbreaks of tenacibaculosis. This indicates that *T. finnmarkense* has a preference for these unscaled regions. This may be because scaled skin contains numerous mucous cells which would therefore result in a thicker layer of mucus that may prevent the colonization and proliferation of *T. finnmarkense*. The role of mucus needs to be further investigated.

WGS has shown that both *T. finnmarkense* and *T. dicentrarchi* possess genes encoding haemolysin, several peptidases, collagenases and collagen-binding proteins (Grothusen et al., 2016; Bridel et al., 2018). These putative virulence factors are likely responsible for the severe tissue destruction associated with tenacibaculosis. Although, *T. finnmarkense* is shown to be able to cause tenacibaculosis without skin abrasions (**Paper-III**), it is likely that when the integrity of the skin is compromised (e.g. handling, transport, scale loss, and jellyfish stings), the severity of outbreaks would be significantly increased as described in **Paper-II**. The mechanical strength of the epidermis is provided by the desmosome-intermediate filament complex (DIFC) (Appendix, Figure 1) which links the malpighian cells together. As seen in Figure 10B, the malpighian cells are disassociating which indicates that *T. finnmarkense* is able to destroy the DIFC. Considering that the strength of any structure is dependent upon the strength of its individual parts, the destruction of the DIFC can make the epidermis simply fall apart when subjected to mechanical stress (Garrod and Chidgey, 2008). Once the malpighian cells of the epidermis are

disrupted, the underlying dermis can be penetrated by *T. finnmarkense*. Moreover, the destruction of the epidermis renders the malpighian cells unable to rapidly migrate in order to close the injured areas and restore the protective barrier of the skin.

The ability to grow in host tissue is a common trait for pathogenic bacteria (Dalsgaard, 1993). As seen in histopathological skin section from tenacibaculosis outbreaks, the *Tenacibaculum*-like bacteria are able to multiply within the dermis with little or no host immune response. This indicates that *T. finnmarkense* is able to neutralize or elude the host's defence mechanisms. The severe destruction of the dermis seen in *T. finnmarkense* infections is likely a result of the collagen binding protein and collagenases that enable the destruction of the collagen fibres. The infiltration of *Tenacibaculum* spp. and unidentified *Tenacibaculum*-like bacteria in the connective tissue of the dermis of fish has been reported from several outbreaks of ulcerative skin diseases in marine fishes (Miyazaki et al., 1975; Kent et al., 1988; Olsen et al., 2011; Avendaño-Herrera et al., 2016). This has also been noted for the hinge ligament of shellfish (Handlinger et al., 1996; Burioli et al., 2017). This further underpins the notion that these bacteria have a high affinity for connective tissues rich in collagen. As observed in histopathological section, *T. finnmarkense* appears to spread laterally in the dermis (Figure 10, Figure 12). This likely enables *T. finnmarkense* to bypass the epidermal defences of the host. This may explain why there is typically little or no host immune response associated with tenacibaculosis caused by *T. finnmarkense*. Moreover, the destruction of the dermis which provides the mechanical integrity of the skin, can explain the rapid development of skin lesions/ulcers once the smolt is infected.

Because *Tenacibaculum* spp. likely constitute members of the normal microbiota of farmed Atlantic salmon, it indicates that some strains are opportunistic pathogens that can become pathogenic when they escape their original niche, outcompete other members of the microbiota and start to colonize deeper tissues (Ribet and Cossart, 2015). The result of bacterial infection is tightly dependent on host susceptibility (Ribet and Cossart, 2015); therefore, the higher susceptibility of smolt to infectious

diseases may explain why tenacibaculosis mainly affects Atlantic salmon smolts shortly following sea transfer.

4.4 Host-pathogen-environment interactions

As previously mentioned, it appears to be a seasonality to the tenacibaculosis outbreaks in the northernmost parts of Norway. These outbreaks do to some extent coincide with the occurrence of phytoplankton blooms in these areas. In the North Atlantic and in the Barents sea (the seas that surrounds the northernmost parts of Norway), phytoplankton blooms typically occur in both spring and autumn (Bratbak et al., 1990; Larsen et al., 2004; Martinez et al., 2011; Sigler et al., 2014; Sundby et al., 2016; Oziel et al., 2017). Members of the family Flavobacteriaceae constitute one of a few dominating bacterial lineages in phytoplankton-associated bacterial communities (Teeling et al., 2012). Moreover, there are swift successions of distinct Flavobacteriaceae lineages with associated abundance peaks, as have been observed for *Polaribacter* and *Tenacibaculum* (Teeling et al., 2012; Teeling et al., 2016; Bohórquez et al., 2017). This indicates that members of the family Flavobacteriaceae have particular niches related to the transformation of phytoplankton derived organic matter. A large part of the phytoplankton cellular material is carbohydrates (Biersmith and Benner, 1998), but phytoplankton is also high in protein (30-50 %) (Sakshaug et al., 2009). Genomic studies have shown that *T. finnmarkense* and *T. dicentrarchi* lack the genes found in “environmental” *Tenacibaculum* species that enables the degradation of marine carbohydrates (Bridel et al., 2018). This predicts a restricted ecological niche and a solely protein based regimen for *T. finnmarkense* and *T. dicentrarchi* (Bridel et al., 2018). The ability to degrade protein may therefore be linked to *T. finnmarkense*'s and *T. dicentrarchi*'s niche as degraders of phytoplankton derived protein. Their association with tenacibaculosis in farmed Atlantic salmon may therefore be coincidental; these bacteria may simply have escaped their original environmental niche. The seasonality of the phytoplankton blooms seems to correlate with the apparent seasonality of the tenacibaculosis outbreaks. As *Tenacibaculum* spp. are expected to be high following phytoplankton

blooms, it may explain the higher incidences of tenacibaculosis outbreaks in spring and autumn in the northernmost parts of Norway. However, further studies are needed to address this potential link.

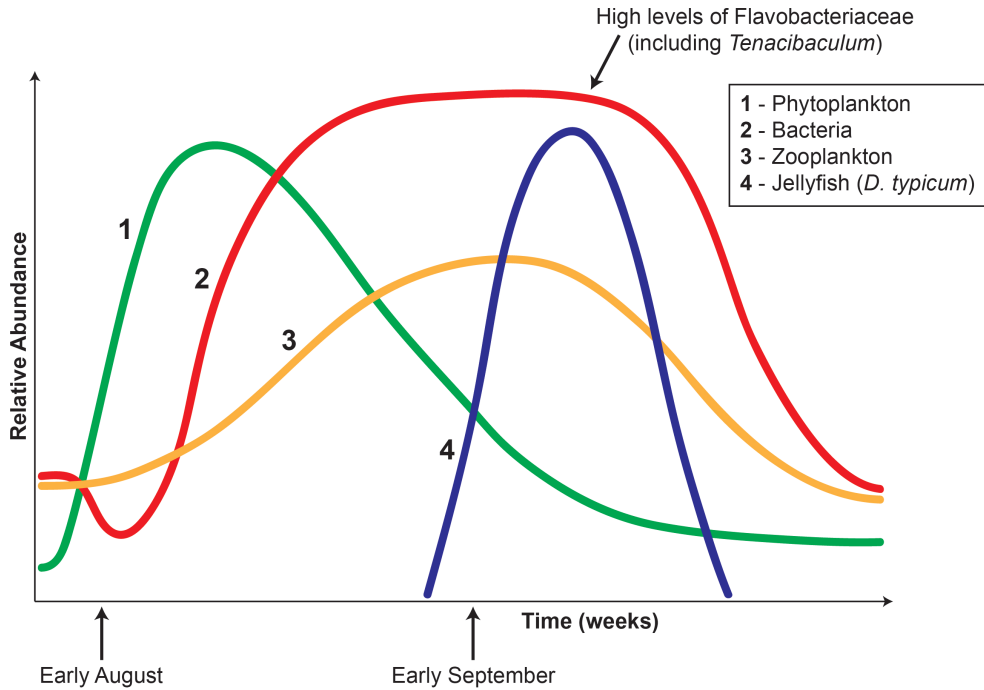


Figure 13 The hypothetical relative abundance of phytoplankton, zooplankton, bacteria and jellyfish before and during the tenacibaculosis outbreaks described in **Paper-II**.

There is however not just a complex relationship between phytoplankton and heterotrophic bacteria in the marine environment following phytoplankton blooms, but also between phytoplankton, zooplankton and jellyfish. An increase in zooplankton levels typically lags that of phytoplankton blooms by approximately a month (Boero et al., 2008; Eiane and Tande, 2009). Because jellyfish (Cnidaria) forage on zooplankton, their levels also increase in response to increased zooplankton levels (Boero et al., 2008). Interestingly, the sequence of events described in **Paper-II** fits with the expected abundance of phytoplankton, zooplankton, bacteria and jellyfish prior to and during tenacibaculosis outbreaks (Figure 13): the tenacibaculosis outbreaks occurred approximately 4-6 weeks following an autumn phytoplankton

bloom and large numbers of jellyfish were observed in the vicinity and inside the net-pens.

It has been suggested that if there are a large number of infected fish (e.g. in one net-pen), the infection pressure may increase and result in the spread of the disease to the remainder of the net-pens at the site (Hjeltnes, 2014). From the results obtained during the cohabitation experiment in **Paper-III**, it was however shown that neither *T. finnmarkense* nor *T. dicentrarchi* easily transmits from diseased shedder fish to naïve fish, opposed to what has been shown for *T. maritimum* (Frisch et al., 2018). *T. finnmarkense* and *T. dicentrarchi* are likely mainly transmitted through free dissemination in the water. Therefore, vectors such as jellyfish may not be needed for their transmission, which is supported by the results from **Paper-II**. The lack of transmission between infected and non-infected fish has also been noted in *F. psychrophilium* cohabitation experiments (Madsen and Dalsgaard, 1999).

4.5 Vaccine development

There are currently no mitigation tools for tenacibaculosis in Norway. Antibiotics have been used by the industry with mixed results (Takle et al., 2015). This is likely due to the fact that tenacibaculosis caused by *T. finnmarkense* is an acute disease (**Paper-II**) and that fish are likely too sick and therefore do not eat by the time the treatment is distributed to the population. As with other bacterial diseases, the most economically viable tool would be a vaccine. However, as with other Flavobacteriaceae, *T. finnmarkense* attacks the skin of the fish and therefore, the development of a vaccine has proved difficult. The use of inactivated bacterial whole-cell vaccines as tested in the current vaccine challenge study (**Paper-III**), are the most commonly used form of vaccines for inducing immunity in fish due to their safety and effectiveness when administered via IP injection (Gómez et al., 2014). However, in this case the whole cell inactivated vaccines tested did not appear to give any protection, even when containing a very high level of bacterin and inducing a high serum antibody response (**Paper-III**). This was true for both homologous and heterologous bath challenges.

Tenacibaculum spp. isolated from farmed fish in Norway are rarely retrieved from or detected in internal organs (**Paper-II**; Olsen et al., 2011; Olsen et al., 2017), suggesting that these bacteria do not cause a systemic infection. Because of this, the effect of the specific humoral antibodies is likely limited and an effective vaccine would need to target the skin's immune response. It is well known that the innate mucosal immune factors in the skin are important for activation of adaptive immune responses (e.g. complement activation) (Esteban, 2012; Gomez et al., 2013; Salinas, 2015; Salinas and Magadán, 2017). When considering adaptive immune factors of the skin mucosal surfaces, a high level of antibodies in serum may not reflect the presence of antibodies in the skin, as seen for *F. psychrophilum* after IP vaccination (Madetoja et al., 2006). This may have been the case in the current *T. finnmarkense* vaccine challenge study (**Paper-III**), and may explain the lack of protection induced by the tested vaccines.

Part of the difficulties with the development of an effective vaccine is the need for standardised and effective challenge model. As previously mentioned the spread of the *T. finnmarkense* is likely occurring through free dissemination in water; thus a bath challenge model reflects the natural transmission of the bacterium from the water into the fish through the skin (**Paper-III**). However, the high numbers of bacteria that the smolts are exposed to during a bath challenge, due to the logistical constraints of having a continual exposure may not reflect the natural infection pressure during tenacibaculosis outbreaks. Therefore, the immune system may have been overwhelmed. This makes determining the effect of vaccines when using a bath infection model difficult as suggested by Karlsen et al. (2017a) for *M. viscosa*. Despite this potential drawback, the bath challenge model has the advantage of not bypassing the mucosal immune defence of the salmon, as is the case when using subcutaneous or IP-injection procedures.

The low seawater temperatures used in the vaccine challenge study (**Paper-III**) may have affected the efficacy of the vaccines, as low temperatures compromise adaptive immune responses in poikilothermic animal like fish (Abram et al., 2017). Therefore, the vaccine challenge study (**Paper-III**) should perhaps have been conducted at

seawater temperatures above 4 °C. Still, it is important to note that Atlantic salmon naturally encounter water temperatures as low as 0 °C, and for the majority of their life post-smoltification experience seawater temperatures below 8 °C (Reddin, 1985; Valiente et al., 2004; Lacroix, 2013). As previously discussed, little or no host immune response can be seen in histological sections of both experimentally-induced and natural outbreaks of tenacibaculosis (**Paper-II**; **Paper-III**). In the Olsen et al. (2011) study, however, inflammatory cells were found to be present in the dermis in close proximity to *Tenacibaculum*-like bacteria. A similar inflammatory cell infiltration into the dermis has also been reported from outbreaks of tenacibaculosis caused by *T. dicentrarchi* in Chile (Avendaño-Herrera et al., 2016). These differences in host immune responses may be due to differences in the water temperatures during these two studies (9.5-12 °C and 18 °C respectively); however little to no immune cells infiltration was observed in the **Paper-II** study that occurred in water temperatures of 8-10 °C. The differences in immune responses may be attributed to the infiltration of extraneous bacteria that could have induced the observed immune response. All of these factors indicate that the developed challenge model needs to be further developed to be effective and reproducible at higher seawater temperatures.

5. CONCLUSION

The differential genetic, phylogenetic, phenotypic and chemotaxonomic data presented in this thesis show that strain HFJ^T isolated from tenacibaculosis outbreaks in Northern Norway should be classified as a novel species in genus *Tenacibaculum*, for which the name *Tenacibaculum finnmarkense* sp. nov. is proposed. This species delineation has been confirmed through a recent WGS study.

T. finnmarkense strain HFJ^T is a causative agent of tenacibaculosis as it presents in Northern Norway without the need for any scarification, pre-handling or co-infection. Based on WGS, *T. finnmarkense* can be subdivided into two clades (clade I and III) where strains belonging to clade III (including *T. finnmarkense* strain HFJ^T) appear to be more pathogenic.

Tenacibaculosis caused by *T. finnmarkense* is an acute disease that targets the unscaled regions of the smolt (i.e. fins, tail, head) and appears to cause very little to no host immune response. Possible factors that increase the severity of the disease include handling, smolt size, and environmental stressors such as jellyfish.

In opposition to *T. maritimum*, *T. finnmarkense* does not appear to spread easily from fish to fish. The bacteria are instead thought to infect smolts directly from the environment. The abundance of *Tenacibaculum* spp. in Northern Norway seems to be seasonal and therefore salmon producers should target smolt entries accordingly.

Even though a whole cell inactivated vaccine induces an antibody response, it does not appear to offer protection against tenacibaculosis induced through a bath infection.

6. FUTURE PERSPECTIVES

Continued work is required to determine the pathogenicity of different *Tenacibaculum* spp. strains (intra- and inter-species) in Norway, as several isolates are generally found during investigations of skin lesions/ulcers outbreaks. This would allow for the identification of clinically important strains that are necessary for the development of management tools.

Future developments need to include investigating the host-pathogen interactions, as well as finding the right protective antigen for a vaccine candidate. Co-factors that increase the severity of disease need to be identified, such as the transition from freshwater to saltwater and its impact on the microbiota and immune function of the smolt's skin. Smoltification and the following shift in environment is likely the most significant stressor in the production of salmon and needs to be central to any future work.

Reservoirs and/or vectors of *Tenacibaculum* spp. and their role in the development and spread of tenacibaculosis need to be further investigated. Semi-closed or closed post-smolt facilities need to be included in these studies due to their increased use in the Norwegian salmon farming industry. Using treated saltwater could be a mitigation tool for these facilities, but needs further research.

To be able to test mitigation tools, further refinement of challenge models for *T. finnmarkense* needs to be conducted. In particular, methods to standardise the protocols are needed, such as accurate real-time bacterial cell counts and determination of smolt quality.

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APPENDIX - THE FISH SKIN

Epidermis

The fish skin consists of two basic layers: the epidermis and the dermis (Figure 2). The outermost layer, the epidermis, is an avascular stratified squamous epithelium that is mainly built up of fibrous malpighian cells which forms the fundamental unit of the epidermis. Intercellular junctions called desmosomes provide strong adhesion between the cells. Because the desmosomes also link to the intermediate filament cytoskeleton, they form adhesive bonds in a network of scaffolding, called the desmosome-intermediate filament complex (DIFC) (Figure 1) (Garrod and Chidgey, 2008). The DIFC provides mechanical strength and maintains the integrity of the epidermis (Kryvi and Totland, 1997; Garrod and Chidgey, 2008). In addition to the malpighian cells, other cell types like mucus-secreting cells (mucous cells), club cells (only in some species), wandering leukocytes, macrophages and eosinophilic granular cells are found in the epidermis (Ferguson, 2006). Cell mitosis occurs throughout the epidermis, and in contrast to mammals, the outermost cells are viable and only replaced when they are injured or dead (Ferguson, 2006; Takle et al., 2015). The thickness of the epithelium varies with species, age and body site, and can vary from more than 20 cells in exposed areas to only a few in the olfactory epithelium (Kryvi and Totland, 1997; Roberts, 2012). In salmon, for instance, the thickness of the epithelium is greatest in the head region and fins where there are no scales present (Figure 3, Figure 4, Figure 5) (Ferguson, 2006).

The epidermis comprises three strata: *stratum superficiale*, *stratum spinosum* and *stratum basale* (Rakers et al., 2010). The outermost *stratum superficiale* consists mainly of malpighian cells that are flattened horizontally and have microridges on the surface that presumably aid in better attachment for the mucus (Kryvi and Totland, 1997; Ferguson, 2006). These cells have the ability to rapidly migrate in order to close injured areas and restore the protective barrier of the skin, as well as identify and neutralize pathogens (Bullock et al., 1978; Karlsen et al., 2012; Roberts, 2012). In addition to the malpighian cells, there are mucous cells present, which are largely

responsible for secreting the mucus layer that lines the epithelium and form the first physical barrier from the environment (Esteban, 2012; Roberts, 2012).

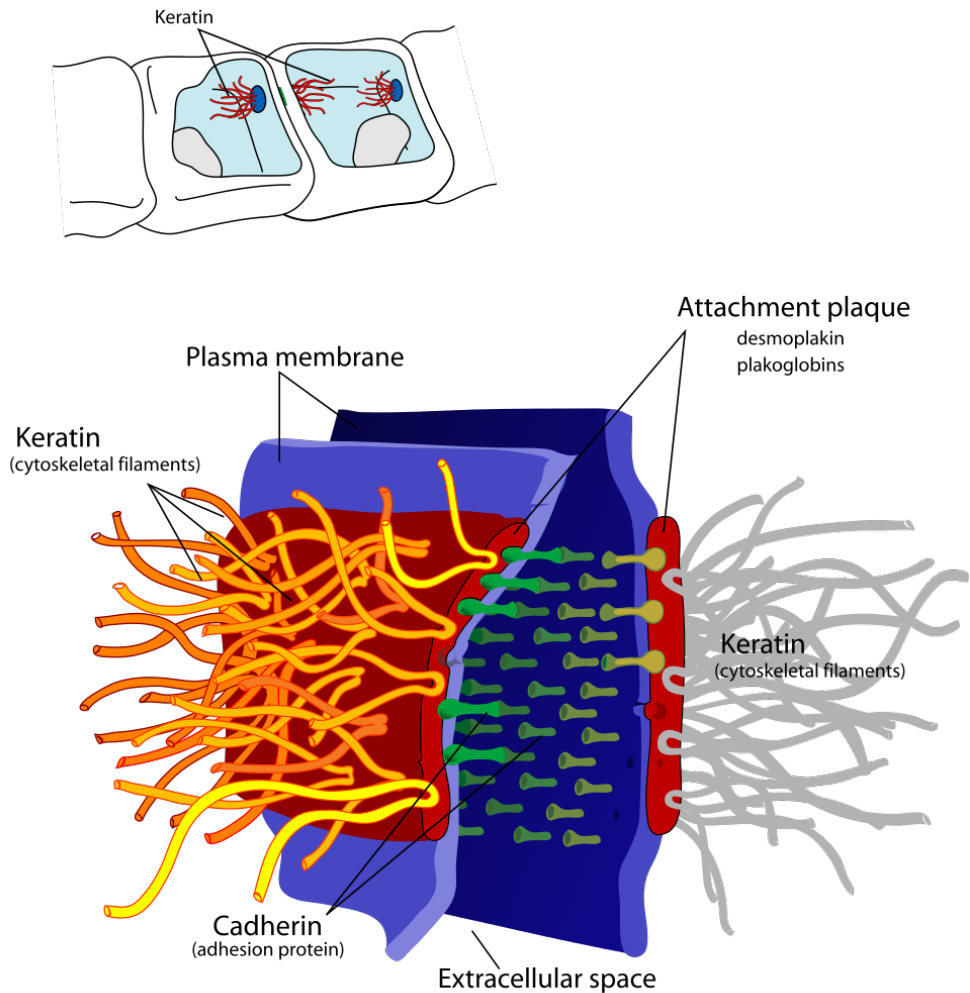


Figure 1 Desmosome. Cadherin adhering the malpighian cells together and forming links with the intermediate filament cytoskeleton. Figure by Mariana Ruiz (2007). Released to the Public Domain.

The medial *stratum spinosus* of the epidermis consists mainly of mucous cells and undifferentiated epidermal progenitor cells which can be differentiated and subsequently recruited to the *stratum superficiale* when needed (Roberts, 2012). These cells emerge from the proximal *stratum basale* that contains basal cells and a

layer of filamentous proteins that make up the basement membrane. The basement membrane separates the epidermis from the underlying dermis.

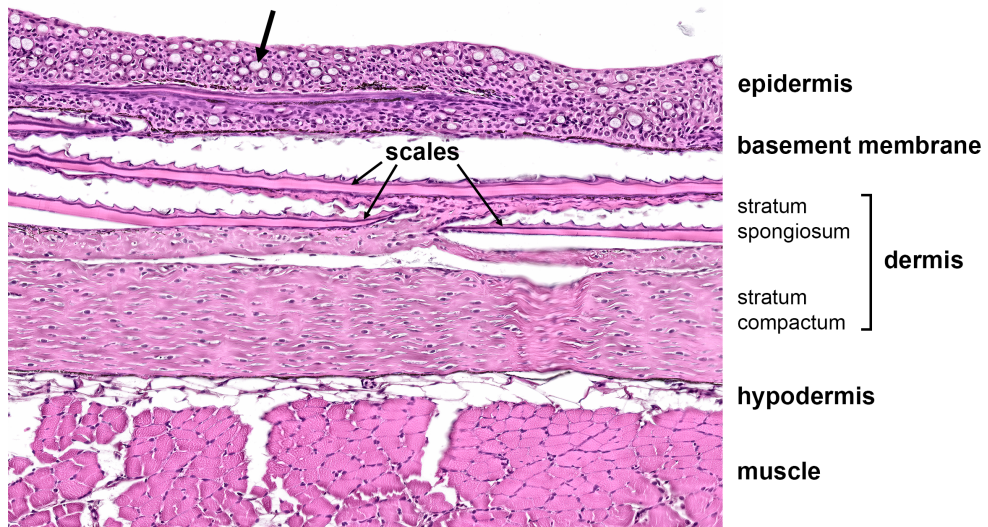


Figure 2 Longitudinal histological section of scaled skin of Atlantic salmon showing the normal anatomy. The different layers of the skin are indicated at the right hand side of the image. Numerous mucus cells (bold arrow) are present in the epidermis. Haematoxylin and eosin (H&E) stain. Original image courtesy of Renate Johansen, PHARMAQ-Analytiq.

Dermis

The dermis of scaled skin consists of two strata that are most commonly organized in an upper *stratum spongiosum* and a deeper *stratum compactum* (Figure 2) (Kryvi and Totland, 1997). The *stratum spongiosum* is highly vascularized and consists of a loosely organized network of collagen fibres adjoining the basement membrane (Roberts, 2012). It contains cells like fibroblasts, mast cells and a variety of pigment cells (chromatophores), as well as blood vessels, nerves and scales (Kryvi and Totland, 1997; Roberts, 2012). The scales are localized inside scale-pockets, which are separated from the *stratum compactum* by an uninterrupted cellular sheet called the scale pocket-lining (Sire, 1989). As the scales are situated in the *stratum spongiosum*, the loss of scales, as frequently occurring in newly smoltified salmon

smolts, represents a significant breach in the protective barrier (i.e. the epidermis and the basal membrane) of the fish (Zydlowski et al., 2010).

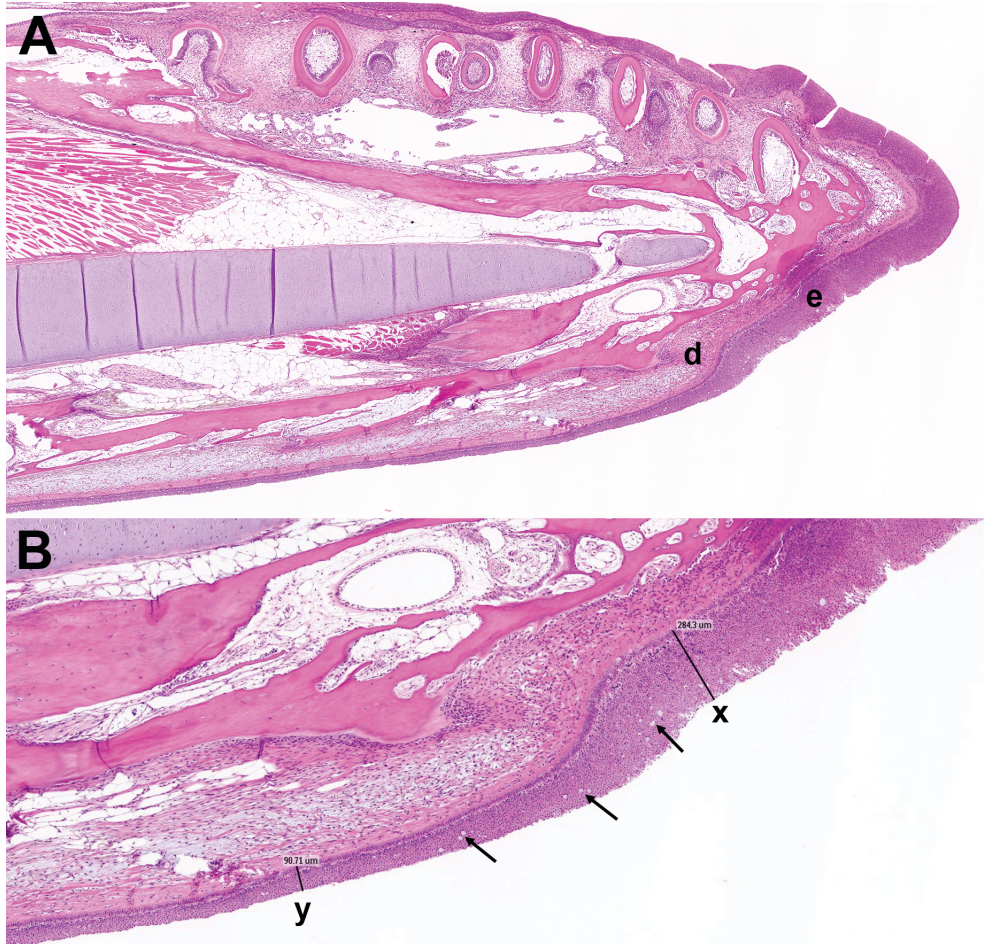


Figure 3 Horizontal histological section of unscaled skin of the lower jaw of an Atlantic salmon smolt showing the normal anatomy. **(A)** Overview of lower jaw showing the epidermis (e) and the dermis (d). **(B)** A thick epidermis layer (x) is present at the tip of the jaw which is gradually reduced posteriorly (y). The dermis is mainly loosely organized, but a thin strip of dense connective tissue is present directly below the epidermis. The epidermis is almost devoid of mucous cells (arrows). H&E stain.

The *stratum compactum* is recognized by a dense plywood-like organization of collagen bands (alternating directions, rotated up to 90 degrees to one another) interspersed with the occasional fibroblast between the bands (Le Guellec et al.,

2004). The primary function of the *stratum compactum* is to strengthen the skin and protect it from splitting under tensile forces (Kryvi and Totland, 1997; Le Guellec et al., 2004). In addition to collagen fibres, a clear, colourless, and viscous fluid consisting of glycosaminoglycans and/or proteoglycans called ground substance constitutes a part of the intercellular substance in both strata (Kryvi and Poppe, 2016). There is generally very little ground substance in the dense connective tissue of the *stratum compactum* compared to that of the loosely organized connective tissue of the *stratum spongiosum* (Kryvi and Poppe, 2016). The *stratum compactum* is reduced in unscaled skin for example in the head region and fins of Atlantic salmon (Figure 3, Figure 4, Figure 5). The separation of the dermal layers (including the hypodermis) may therefore be indistinct in unscaled skin (Ferguson, 2006; Elliott, 2011). In contrast to the epidermis, healing of the dermis is a much slower process and may be completely halted at low temperatures (Roberts, 2012).

Hypodermis

The hypodermis is found subjacent to the dermis in well-differentiated skin and separates the dermis from the underlying skeletal muscle. It is more vascularized than the *stratum compactum* and consists of loosely organized collagen fibres, pigment cells and adipocytes (fat storage cells). It is separated from the dermis by a thin layer of fibrocyte-like cells joined by cell junctions called the *dermal endothelium* (Kryvi and Totland, 1997; Le Guellec et al., 2004; Elliott, 2011). The hypodermis is a frequent site for lateral movement of pathogens and inflammatory processes (Ferguson, 2006).



Figure 4 Longitudinal histological sections of the pectoral fin of Atlantic salmon smolts showing the normal anatomy. **(A)** Tip of fin. A thick epidermis layer is present at the tip of the fin which is devoid of mucous cells. The dermis is loosely organized and separated into two layers by the bones (lepidotrichia). The joints of the bones are connected by dense connective tissue. **(B)** Base of fin. The epidermis is thinner and interspersed with occasional mucous cells. H&E stain.



Figure 5 Horizontal histological section of the tailfin of an Atlantic salmon smolt showing the normal anatomy. The epidermis contains numerous mucous cells at the scaled/unscaled junction (arrow). The number of mucous cells is gradually reduced towards the tail tip concurrent with an increase of the thickness of the epidermis. The dermis is highly organized in the scaled skin but is loosely organized in the unscaled parts of the skin (posteriorly). Notice the large number of adipocytes present in the hypodermis at the basis of the tail. H&E stain.

tail tip

Skin mucus, mucosal immune system and the microbiota

The epidermis is covered by an approximately 1 μm thick layer of mucus that consists predominantly of large gel-forming glycoproteins called mucins (Shephard, 1994). Mucus serves important functions and is not only important for immobilizing or inhibiting pathogen binding by being constantly produced and slough off, but also serves a role as a barrier against the external environment (Shephard, 1994; Salinas and Magadán, 2017). The number and size of mucus cells in Atlantic salmon have been shown to vary significantly with body site, but the lowest number and size are found in the head region, whilst the dorsolateral skin contains the highest densities (Pittman et al., 2013). The smaller size and number of mucous cells in the head region indicates that it is an area of little mucus production (Pittman et al., 2013). It has also been shown that the number of mucous cells present in the skin of Atlantic salmon is reduced by approximately 50 % during smoltification (O'Byrne-Ring et al., 2003). The consistency of the mucus layer may also be thickened or altered through exposure to pathogens or pollutants (Esteban, 2012; Roberts and Rodger, 2012).

The mucosal immune system of the skin consists of innate humoral (e.g. complement protein, lysozyme, protease, esterases and AMPs), cellular (e.g. mast cells, macrophages and granulocytes), adaptive humoral (e.g. IgT, IgD and IgM), and cellular (B-cells and T-cells) immune components whose function is to protect the fish from harmful microorganisms (Gomez et al., 2013; Brinchmann, 2016). Some of these components are secreted into the mucus layer (Roberts and Rodger, 2012). Compared to terrestrial mammals, fish have a greater challenge coping with high microbial loads that constantly interact with their mucosal epithelial barriers (Salinas, 2015). Therefore, when mucosal barriers sense a danger signal (e.g. the presence of a harmful microorganism, toxins, physical trauma), an immediate immune response will be triggered. This initial innate immune response is essential for the subsequent establishment of specific adaptive immunity (Salinas, 2015).

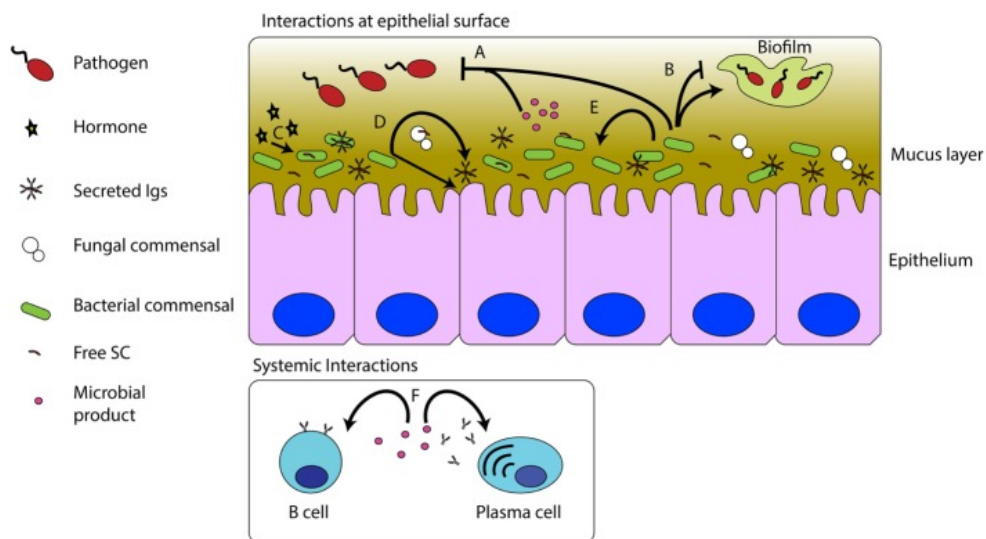


Figure 6 Interactions between commensal microbes, pathogens, and the host immune system. (A) Commensal bacteria and their products can inhibit pathogenic infections. (B) Commensal microbes can promote or inhibit biofilm formation. (C) Host hormones, such as cortisol, can be sensed by, and have effects on commensals. (D) The presence of the microbiota stimulates S-Ig production and epithelial turnover. (E) Commensal microbes and their products can affect other commensal microbes. (F) Commensal products, such as sphingolipids, can modulate B cell numbers and antibody titres in mucosal and systemic compartments. This figure and figure text were obtained from Kelly and Salinas (2017). (Igs: Immunoglobulins; S-Ig: Secreted immunoglobulin; Free SC: Free Secretory Component (unbound cleaved portion of pIgR)).

Within the mucus and at the mucosal surfaces of the skin, complex interactions between the microbiota, pathogenic microorganisms, the host, and the surrounding environment occur (Figure 6) (Boutin et al., 2013; Foster et al., 2017; Kelly and Salinas, 2017). The microorganisms that reside in mucosal surfaces consist mostly of commensal microorganisms (Foster et al., 2017). The microbiota is important for the development of the immune system as well as protecting the host from pathogens by colonization resistance (Llewellyn et al., 2014; Foster et al., 2017; Guven-Maiorov et al., 2017). Any disturbance to the commensal normal microbiota can therefore enhance the susceptibility to disease (Llewellyn et al., 2014).

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Tenacibaculum finnmarkense sp. nov., a fish pathogenic bacterium of the family *Flavobacteriaceae* isolated from Atlantic salmon

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Abstract A novel Gram-stain negative, aerobic, non-flagellated, rod-shaped gliding bacterial strain, designated HFJ^T, was isolated from a skin lesion of a diseased Atlantic salmon (*Salmo salar* L.) in Finnmark, Norway. Colonies were observed to be yellow pigmented with entire and/or undulating margins and did not adhere to the agar. The 16S rRNA gene sequence showed that the strain belongs to the genus *Tenacibaculum* (family *Flavobacteriaceae*, phylum '*Bacteroidetes*'). Strain HFJ^T exhibits high 16S rRNA gene sequence similarity values to *Tenacibaculum dicentrarchi* NCIMB 14598^T (97.2 %). The strain was found to grow at 2–20 °C and only in the presence of sea salts. The respiratory quinone was identified as menaquinone 6 and the major fatty acids were identified as summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{15:1} and iso-C_{15:0} 3-OH. The DNA G+C content was determined to be 34.1 mol%. DNA–DNA hybridization and comparative phenotypic and genetic tests were

performed with the phylogenetically closely related type strains, *T. dicentrarchi* NCIMB 14598^T and *Tenacibaculum ovolyticum* NCIMB 13127^T. These data, as well as phylogenetic analyses, suggest that strain HFJ^T should be classified as a representative of a novel species in the genus *Tenacibaculum*, for which the name *Tenacibaculum finnmarkense* sp. nov. is proposed; the type strain is HFJ^T = (DSM 28541^T = NCIMB 42386^T).

Keywords Norway · Polyphasic taxonomy · Salmon farming · Skin lesions · Ulcerative disease · Winter ulcers

Introduction

During an outbreak of an ulcerative disease in Atlantic salmon at a seawater site in Finnmark, Norway, long rod shaped bacteria were found to predominate in the skin lesions. One strain of these isolates designated HFJ^T is described in the present study. The 16S rRNA gene sequence showed that it belongs to the genus *Tenacibaculum* (family *Flavobacteriaceae*, phylum '*Bacteroidetes*') described by Suzuki et al. (2001). To date the genus comprises 21 species derived from a variety of marine environments and marine organisms (Kim et al. 2013; LPSN 2015). Several of the type strains in genus *Tenacibaculum* have been reported as pathogenic for fish or associated with disease in cultured marine fish (Wakabayashi et al. 1986; Hansen

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et al. 1992; Piñeiro-Vidal et al. 2008a, b; López et al. 2010; Piñeiro-Vidal et al. 2012). *Tenacibaculum maritimum*, the causative agent of marine tenacibaculosis, is the best known and most extensively studied fish pathogenic bacterium in the genus (Wakabayashi et al. 1986; Suzuki et al. 2001). The disease has been reported from Europe, Japan, North America and Australia and affects both wild and cultured fish, including Rainbow trout and Atlantic salmon (Toranzo et al. 2005; Avendaño-Herrera et al. 2006; Bruno et al. 2013). *T. maritimum* has never been isolated in cases of ulcerative disease in Norway (Olsen et al. 2011).

There has been a growing attention regarding the potential role of Norwegian *Tenacibaculum* spp. in causing ulcerative disease in sea-reared Atlantic salmon, as they are commonly identified from skin lesion in mixed cultures with the bacterium *Moritella viscosa* or as the apparent sole agent (Olsen et al. 2011; Bornø and Lie 2015). The aim of the present study was to determine the taxonomic position of the fish pathogenic *Tenacibaculum* strain HFJ^T using genetic, phenotypic and chemotaxonomic characterisations, a detailed phylogenetic investigation based on 16S rRNA gene sequences and concatenated housekeeping (HK) gene sequences, and DNA–DNA hybridization (DDH).

Materials and methods

A total of 11 isolates from genus *Tenacibaculum* were included in the present study (Table 1). Strain HFJ^T was isolated in spring 2013 from a skin lesion of a

diseased Atlantic salmon at a seawater site in Finnmark, Norway. *Tenacibaculum* sp. strains Tsp. 2–7 were collected from skin or gill of Atlantic salmon and cod in Norway. The type strains *Tenacibaculum dicentrarchi* NCIMB 14598^T, *Tenacibaculum ovolyticum* NCIMB 13127^T, *Tenacibaculum soleae* NCIMB 14368^T and *T. maritimum* NCIMB 2154^T were obtained from *The National Collection of Industrial, Marine and Food Bacteria* (NCIMB). Subcultivation was performed on Marine agar (MA) (Difco 2216) plates at 16 °C for 48 h. The strains were preserved in CryoTubeTM vials (Thermo scientific) at –80 °C.

Draft genome sequencing of strain HFJ^T was carried out by BaseClear B.V (Leiden, The Netherlands.) using Illumina next generation sequencing on a HiSeq 2500TM platform. Extraction of the required concentration (>100 ng/μl) of genomic DNA was performed using an E.Z.N.A. tissue DNA kitTM (Omega Bio-Tek) following the cultured cells protocol. The draft genome sequence obtained for strain HFJ^T was used for the PCR primer design using primer-BLAST (Ye et al. 2012) and to verify obtained sequences for strain HFJ^T.

Genomic DNA from all *Tenacibaculum* sp. strains listed in Table 1 was extracted using an E.Z.N.A. tissue DNA kitTM (Omega Bio-Tek) following the cultured cells protocol. PCR was performed using the 16S rRNA primers 27F and 1518R (Giovannoni et al. 1996) and specific primers for five HK genes (*atpD*, *fusA*, *pgk*, *rpoB*, and *tuf*) (Table 2). Amplification was based on a standard reaction mixture containing 2.5 μl

Table 1 List of *Tenacibaculum* strains included in the present study

Bacterial species	Strain	Origin	Host	Tissue	Year
<i>Tenacibaculum</i> sp.	HFJ ^T	Norway	Atlantic salmon	Skin	2013
<i>Tenacibaculum</i> sp.	Tsp.2	Norway	Atlantic salmon	Skin	2013
<i>Tenacibaculum</i> sp.	Tsp.3	Norway	Atlantic salmon	Gill	2014
<i>Tenacibaculum</i> sp.	Tsp.4	Norway	Atlantic salmon	Skin	2013
<i>Tenacibaculum</i> sp.	Tsp.5	Norway	Atlantic salmon	Skin	2014
<i>Tenacibaculum</i> sp.	Tsp.6	Norway	Atlantic salmon	Skin	2009
<i>Tenacibaculum</i> sp.	Tsp.7	Norway	Farmed Atlantic cod	Skin	2009
<i>Tenacibaculum maritimum</i>	NCIMB 2154 ^T	Japan	Red sea bream fingerling	Kidney	1977
<i>Tenacibaculum soleae</i>	NCIMB 14368 ^T	Spain	Senegalese sole	Unknown	2007
<i>Tenacibaculum ovolyticum</i>	NCIMB 13127 ^T	Norway	Atlantic halibut eggs	Eggs	1989
<i>Tenacibaculum dicentrarchi</i>	NCIMB 14598 ^T	Spain	European sea bass	Skin	2009

The *Tenacibaculum* sp. strains were collected from Norwegian field cases, whereas the type strains were obtained from NCIMB

Table 2 List of PCR primers used in present study

Target gene	Name	Sequence (5′–3′)	Source
16S rRNA	B27F	AGAGTTTGATCMTGGCTCAG	Giovannoni et al. (1996)
16S rRNA	A1518R	AAGGAGGTGATCCANCCRCA	Giovannoni et al. (1996)
<i>tuf</i>	Tb_ <i>tuf</i> F1	ACCTCCTTCACGGATAGC	Present study
<i>tuf</i>	Tb_ <i>tuf</i> R1	TTACGATCGTTTGAAGCCCC	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> F1	ATYTCTCCAAAACGCTGACC	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> R1	AAAACGAATCAAGGWACGAAYA	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> F2	ACCCTTTCCAAGGCATAAAGG	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> R2	GAGCCATYGGTTTTGAAAGAGA	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> F3	CTCTTGCTGTCTCCTCATCTG	Present study
<i>rpoB</i>	Tb_ <i>rpoB</i> R3	ATCCACCAAGATATAGCATCCA	Present study
<i>pgk</i>	Tb_ <i>pgk</i> F1	GCTCCWCCACCWGTAGAAAC	Present study
<i>pgk</i>	Tb_ <i>pgk</i> R1	TYCGTGTAGATTTAATGTGCCT	Present study
<i>atpD</i>	Tb_ <i>atpD</i> F1	TGGYCCAGTWATCGATGTTGA	Present study
<i>atpD</i>	Tb_ <i>atpD</i> R1	AATACGYTCTTGCAATTGCTC	Present study
<i>fusA</i>	Tb_ <i>fusA</i> F1	ATGGTAACTCACCCATTCAGAA	Present study
<i>fusA</i>	Tb_ <i>fusA</i> R1	TGGCATGATGCAACACAAGG	Present study

Extra buffer, 1.25 mM deoxyribonucleotide triphosphates, 0.75 units (0.15 μ L) *Taq* DNA polymerase (BioLabs, New England), 5 μ M (1 μ L) of forward and reverse primers; DNase-RNase free water was added to a final volume of 25 μ L (16.85 μ L H₂O). Amplification was performed in a GeneAmp PCR system 2700 (Applied Biosystems) at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 60–100 s, followed by 72 °C for 10 min. The PCR product was confirmed by gel electrophoresis and enzymatically purified using ExoStar 1-Step[®] (GE Healthcare Bio-Sciences Corp) in an Artik Thermal Cycler (Thermo Scientific) at 37 °C for 15 min and at 80 °C for 15 min. The sequencing reaction was performed using a BigDye[®] version 3.1 reaction in an Arktik Thermal Cycler, at 96 °C for 5 min; 30 cycles of 96 °C for 10 s, 58 °C for 5 s and 60 °C for 4 min. The reaction was composed of a mixture of 5.5 μ L deionized water, 1 μ L BigDye[®] Terminator 3.1 version sequencing buffer, 1 μ L BigDye Terminator 3.1 version Ready Reaction Premix (2.5X) (Invitrogen), 3.2 pmol (1 μ L) forward and reverse primers and 1.5 μ L purified PCR product. Sequencing was carried out by the Sequencing Facility at Høyteknologisenteret i Bergen (<http://www.uib.no/seqlab>). Samples were cleaned with Agencourt CleanSeq (Beckman Coulter, Inc.) before being sequenced in a 96-capillary 3730xl DNA Analyzer (Applied Biosystems). The software Vector NTI[®] v.9.0 (Invitrogen)

was used to assemble and align the obtained sequences.

Three alignments were constructed for phylogenetic analysis. The first, 16S rRNA gene sequence alignment, consisted of 1341 positions and included sequences of HFJ^T and the 21 published type strains in the genus *Tenacibaculum*. In this alignment, all sequences were obtained from GenBank with the exception of strain HFJ^T, *T. dicentrarchi* NCIMB 14598^T, *T. ovolyticum* NCIMB 13127^T, *T. soleae* NCIMB 14368^T and *T. maritimum* NCIMB 2154^T. The second 16S rRNA gene sequence alignment of 1349 positions contained sequences from all strains listed in Table 1. A third alignment, of 6750 positions, was constructed using concatenated sequences of the five HK genes of the strains listed in Table 1; *atpD* at position 1–807, *fusA* at position 808–1575, *pgk* at position 1576–2511, *rpoB* at position 2512–5778 and *tuf* at position 5779–6750. All sequences obtained in the present study are available in GenBank with accession numbers presented in Table 3. Alignments were constructed in AlignX in the Vector NTI[®] v.9.0 (Invitrogen) software package before sequences were adjusted to equal length and correct reading frames in GeneDoc (Nicholas et al. 1997). Concatenation of the HK alignments was performed using KAKUSAN4 (Tanabe 2011). The best fitted evolutionary model for each alignment was calculated using Mega 6 (Tamura et al. 2013). For the Bayesian analysis of the

Table 3 List of GenBank accession numbers of sequences obtained in the present study

Bacterial species/strain	16S rRNA	<i>atpD</i>	<i>fusA</i>	<i>pgk</i>	<i>rpoB</i>	<i>tuf</i>
<i>T. finnmarkense</i> sp.nov HFJ ^T	KT270385	KT270377	KT270369	KT270424	KT270410	KT270399
<i>T. dicentrarchi</i> NCIMB 14598 ^T	KT270381	KT270375	KT270364	KT270421	KT270408	KT270402
<i>T. maritimum</i> NCIMB 2154 ^T	KT270382	KT270378	KT270366	KT270416	KT270411	KT270393
<i>T. ovolyticum</i> NCIMB 13127 ^T	KT270383	KT270379	KT270367	KT270423	KT270412	KT270395
<i>T. soleae</i> NCIMB 14368 ^T	KT270384	KT270380	KT270368	KT270417	KT270413	KT270394
<i>Tenacibaculum</i> sp. Tsp.2	KT270386	KT270376	KT270365	KT270420	KT270409	KT270400
<i>Tenacibaculum</i> sp. Tsp.3	KT270387	KT270373	KT270362	KT270422	KT270406	KT270397
<i>Tenacibaculum</i> sp. Tsp.4	KT270388	KT270370	KT270359	KT270414	KT270403	KT270392
<i>Tenacibaculum</i> sp. Tsp.5	KT270389	KT270371	KT270360	KT270418	KT270404	KT270398
<i>Tenacibaculum</i> sp. Tsp.6	KT270390	KT270372	KT270361	KT270415	KT270405	KT270396
<i>Tenacibaculum</i> sp. Tsp.7	KT270391	KT270374	KT270363	KT270419	KT270407	KT270401

concatenated HK alignment, KAKUSAN4 was used for calculation of substitution rate and the best fit model for the individual loci and codon positions and exported into a Mr. Bayes-block. A phylogenetic analysis of all three alignments were conducted using the Maximum Likelihood (ML) method with the best fitted evolutionary model, 1000 bootstrap replications and default settings in Mega 6. The BEAST package v1.8 (Drummond and Rambaut 2007) was used for Bayesian analysis of the two 16S rRNA gene datasets using the best fitted model, relaxed lognormal molecular clock and a mcmc of 100 000 000 generations. The Bayesian phylogenetic analysis of the HK gene dataset was conducted in Mr.Bayes V.3.2.2 (Ronquist et al. 2012) using the data block with the proportional codon proportional, model from KAKUSAN4 and a mcmc of 67 000 000 generations. *Kordia algicida* (GenBank accession nr: AB681152) was used as outgroup in the 16S rRNA phylogenetic analysis that included all the type strains, while *T. maritimum* NCIMB 2154^T was used as outgroup in the other phylogenetic analysis. The phylograms for the ML analysis were constructed in Mega 6. The Effective sample size values (ESS) in the Bayesian analysis were inspected using Tracer ver. 1.6 (Rambaut et al. 2014). All ESS values were within the recommended range (above 200) for all parameters. A maximum clade credibility tree was obtained using a 10 % burn-in in Tree-Annotator and viewed using FigTree (Drummond et al. 2012). For 16S rRNA gene sequence similarity analysis, Percent Nucleotide Identity (PNI) was calculated using the distance matrix option in BioEdit (Hall 2011). In the Average

Nucleotide Identity (ANI) calculations, the sequences from the concatenated HK gene alignment for all strains listed in Table 1 were uploaded and analysed using the Average Nucleotide Identify option in EzGenome (Kim et al. 2012).

Morphological, physiological and biochemical tests were performed as described by Bernardet et al. (2002) for strain HFJ^T and the phylogenetically closely related type strains *T. dicentrarchi* NCIMB 14598^T and *T. ovolyticum* NCIMB 13127^T as reference strains. All tests were performed on cultures incubated at 16 °C for 48 h unless otherwise stated. Colony shape, margin, elevation, size, texture, appearance, pigmentation and optical property were examined as described by Smibert and Krieg (1994). The ability to stick to agar and viscosity of the colonies was also investigated. Cell morphology was investigated using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and light microscopy. Gliding motility was determined by phase contrast microscopic examination of a Marine broth (MB) (Difco 2216) culture by the hanging drop technique as recommended by Bernardet et al. (2002). Presence of flexirubin type pigments was determined by the bathochromic shift test using a 20 % (w/v) KOH solution (Fautz and Reichenbach 1980). Congo red absorption was tested as described by Bernardet et al. (2002). The Gram reaction were performed with a Fluka 77730 Gram Staining Kit (Fluka[®] analytical) following the manufacturer's protocol and the non-staining KOH method (Buck 1982). The Voges-Proskauer reaction was performed as described by Piñeiro-Vidal et al. (2012). Oxidase

activity and ability to split indole from tryptophan was tested using BBL™ DrySlide Oxidase and BBL™ DrySlide Indole (BD BBL™, U.S.A), following the manufacturer's protocol. Catalase activity was examined using the slide (drop) method following the protocol by Reiner (2010). Growth under anaerobic conditions was tested on MA using the GasPak anaerobic system (BBL). Production of H₂S was detected by taping a lead acetate impregnated paper strip (Sigma) to the inside of the lid of MA plates, using Parafilm to seal lid and plate. The plates were incubated at 16 °C for 6 days. Growth on blood agar was tested using blood agar containing 2 % NaCl (BAS) (Microbial laboratory, Haukeland University Hospital, Bergen). Degradation of starch (1 % w/v), casein (1 % w/v), and Tween 80 (1 % v/v) was investigated on MA. MB supplemented with gelatin (1 % w/v) was used to investigate degradation of gelatin. Utilisation of carbon sources was tested on basal agar medium [0.2 g NaNO₃, 0.2 g NH₄Cl, 0.05 g yeast extract, 15 g agar and 36 g red sea salt (Red Sea)] per liter distilled water containing 0.4 % carbon source [D(+)-sucrose, D(-)-ribose, D(+)-galactose, D-glucose, L-proline, L-glutamate, L-tyrosine] as described by (Suzuki et al. 2001). Absence of growth after one month of incubation was recorded as negative. Other enzymatic reactions were evaluated in the API ZYM system (bioMerieux) following the manufacturer's instructions, except that sterile seawater was used as suspension medium. Growth at pH 4–10 (at unit intervals) was assessed in MB; pH was adjusted using 1 M NaOH and 1 M HCl. The temperature range for growth was determined on MA plates incubated at 2, 4, 8, 16, 20, 25, 30 and 37 °C for 7 days. Salinity requirement was determined with saltless MA [per liter distilled water: 5.0 g peptone, 1 g yeast extract and 0.1 g ferric citrate] containing 10, 20, 30, 50, 70 and 100 % strength seawater (100 % seawater = 38.2 g/L red sea salt) or 0.8, 1.0, 3.0, 5.0, 7.0 and 10.0 % (w/v) NaCl (Sigma). Sensitivity to antimicrobials was evaluated by the disc diffusion method following the procedures of *The Clinical and Laboratory Standards Institute* (CLSI 2005), except that the plates were incubated at 16 °C for 10 days on MA plates due to reduced growth for some strains on the recommended Flexibacter Maritimus Medium (FMM). The tests were performed using commercial discs (Neo-sensitabs™ and Sensi-disc™) containing kanamycin (500 µg), streptomycin (10 µg),

gentamicin (30 µg), trimethoprim + sulfamethoxazole (125 + 2375 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), piperidic acid (30 µg), cefuroxime (30 µg), penicillin G (1U), ampicillin (2 µg), tetracycline (30 µg), erythromycin (15 µg), florfenicol (30 µg), oxolinic acid (10 µg) and oxytetracycline (30 µg). Several of the tests described above were also performed for the other strains included in the present study, except Tsp.7. Strain Tsp.7 was uncultivable after prolonged cryo-storage and was therefore not included in the phenotypic tests.

The following chemotaxonomic and genetic analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany): DNA G+C content, DDH, menaquinone and fatty acid methyl ester analysis. All strains were grown in MB at 16 °C for 48 h, except for the DDH test. For DDH, cells were grown in MB at 16 °C for 72 h and the obtained bacterial biomass washed twice in 1× Phosphate Buffered Saline. The cells were disrupted using a Constant Systems TS 0.75 KW (IUL Instruments, Germany) and the DNA in the crude lysate purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DDH was carried out as described by Ley et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian). Extraction of fatty acid methyl esters, washing of extracts and GC analysis were performed by using the Sherlock MIS (MIDI Inc, Newark, USA) system using the MIDI Sherlock version 6.1 and TSBA40 database.

Results and discussion

A polyphasic approach that integrates phenotypic data with genetic and phylogenetic data was performed in the current study. This approach is recommended by several authors for bacterial taxonomic investigations (Bernardet et al. 2002; Tindall et al. 2010). As it has been regarded as best practice to include more than one representative strain when describing a novel taxon, several *Tenacibaculum* sp. strains (Tsp.2–7) obtained from Norwegian mariculture were included in the present study. Strains HFJ^T and Tsp.2 have been shown to be pathogenic to Atlantic salmon

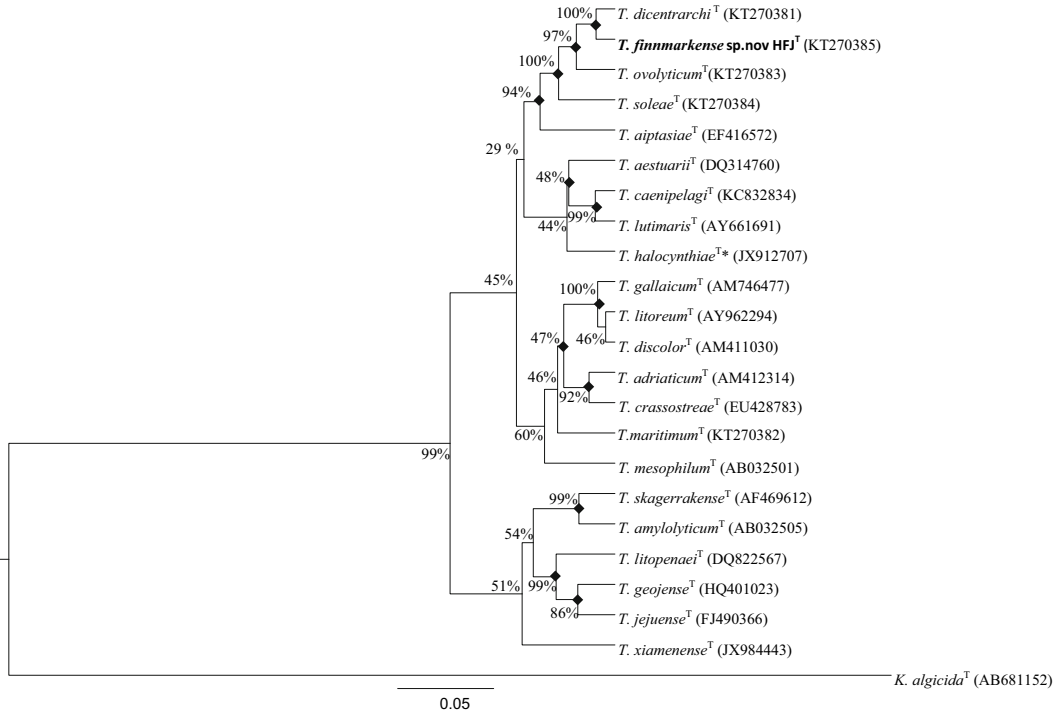


Fig. 1 The relationship of the novel species *T. finnmarkense* sp. nov. HFJ^T and the 21 type strains in genus *Tenacibaculum* (* = quotation marks denote names that have not been validly published) based on the 16S rRNA gene sequences, using *Kordia algicida*^T as outgroup. The phylogenetic analysis was inferred using the Bayesian method with the best fitted evolutionary

model (GTR + G + I). The posterior probability is presented next to each node in percentage. There were a total of 1341 positions in the dataset. Evolutionary analyses were conducted using BEAST package v1.8. Shared nodes identified in corresponding ML analysis are marked with filled squares. Accession numbers are in parentheses. Scale bar 0.05 substitutions per site

reproducing the clinical signs in a challenge study in 2013 (Vold 2014). The bacteria were re-isolated and their identity confirmed by sequencing of the 16S rRNA gene, thus fulfilling Koch's postulates.

The phylogenetic analysis based on the 16S rRNA gene sequences and the concatenated HK gene sequences (Fig. 1, 2, 3) showed that strains HFJ^T, Tsp.2, Tsp.5 and Tsp.7 belong to a distinct clade separate from the closely related type strains in the genus *Tenacibaculum*. Moreover, the analysis showed that strain Tsp.4 forms a clade with *T. dicentrarchi* NCIMB 14598^T and Tsp.6 forms a clade with *T. ovolyticum* NCIMB 13127^T, while the phylogenetic placement of strain Tsp.3 is uncertain. These clades were evident in all phylogenetic trees using both the Bayesian and ML method. All phylogenetic trees

showed that strain HFJ^T is closely related to *T. dicentrarchi* NCIMB 14598^T, both belonging to distinct clades.

As strain HFJ^T showed more than 97 % 16S rRNA gene sequence similarity (PNI) to *T. dicentrarchi* NCIMB 14598^T (Table 4), DDH was performed as recommended (Stackebrandt and Goebel 1994; Tindall et al. 2010). The DDH tests revealed that the DNA relatedness of strain HFJ^T was 54.8 (52.0) % to *T. dicentrarchi* NCIMB 14598^T and 36.6 (39.7) % to *T. ovolyticum* NCIMB 13127^T. Results from repeated tests are shown in parentheses. When considering the threshold value of 70 % DNA–DNA similarity for delineation of bacterial species proposed by the *ad hoc* committee (Wayne et al. 1987), strain HFJ^T does not belong to the species *T. dicentrarchi* NCIMB 14598^T

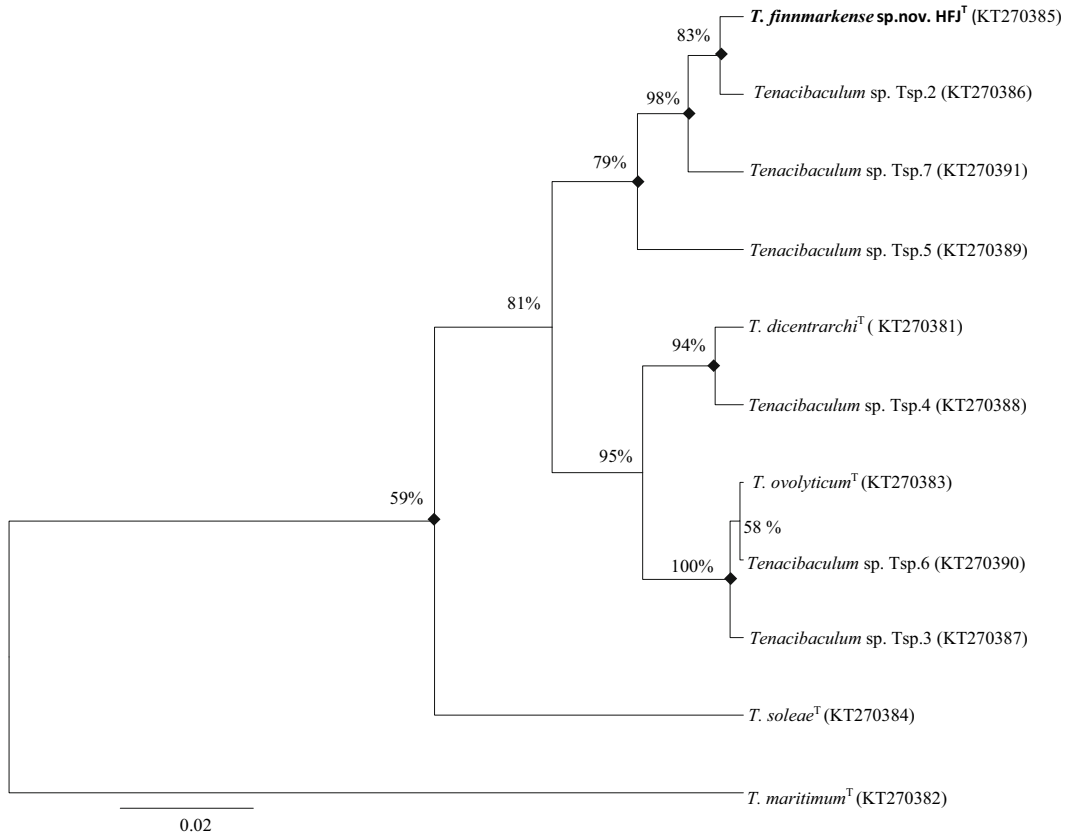


Fig. 2 The relationship of the novel species *T. finnmarkense* sp.nov HFJ^T, *Tenacibaculum* sp. strains Tsp. 2–7 and the three closest related type strains based on 16S rRNA gene sequences, using *T. maritimum* NCIMB 2154^T as outgroup. The phylogenetic analysis was inferred using the Bayesian method with the best fitted evolutionary model (HKY + G + I). The posterior

probability is presented next to each node in percentage. There were a total of 1349 positions in the dataset. Evolutionary analyses were conducted using BEAST package v1.8. Shared nodes identified in corresponding ML analysis are marked with filled squares. Accession numbers are in parentheses. Scale bar 0.02 substitutions per site

or *T. ovolyticum* NCIMB 13127^T. It is generally accepted that an ANI value of 95–96 % corresponds to a DDH threshold value of 70 % and can be used as a boundary for species delineation (Goris et al. 2007; Richter and Rosselló-Móra 2009). Furthermore, a study by Kim et al. (2014) revealed that a PNI of 98.65 % corresponded to an ANI value of 95–96 %. The calculated ANI and PNI values between strain HFJ^T and *T. dicentrarchi* NCIMB 14598^T were 94.6 and 97.2 % respectively. By applying both the ANI and PNI threshold on all 11 strains included in this study (Table 4) we found that strains HFJ^T, Tsp.2,

Tsp.5 and Tsp.7 belong to the same species; Tsp.4 belongs to the species *T. dicentrarchi*, while Tsp.6 belongs to the species *T. ovolyticum*. These findings correspond to results from the phylogenetic analysis and underpin that strain HFJ^T represents a novel species in genus *Tenacibaculum*.

Cells of strain HFJ^T were observed to be rod-shaped, 0.5 µm wide and 5–25 µm in length and Gram-stain negative. Considerably longer filamentous cells and spherical degenerative cells were frequently observed in older cultures. A rapid decrease in viability was found to occur with prolonged

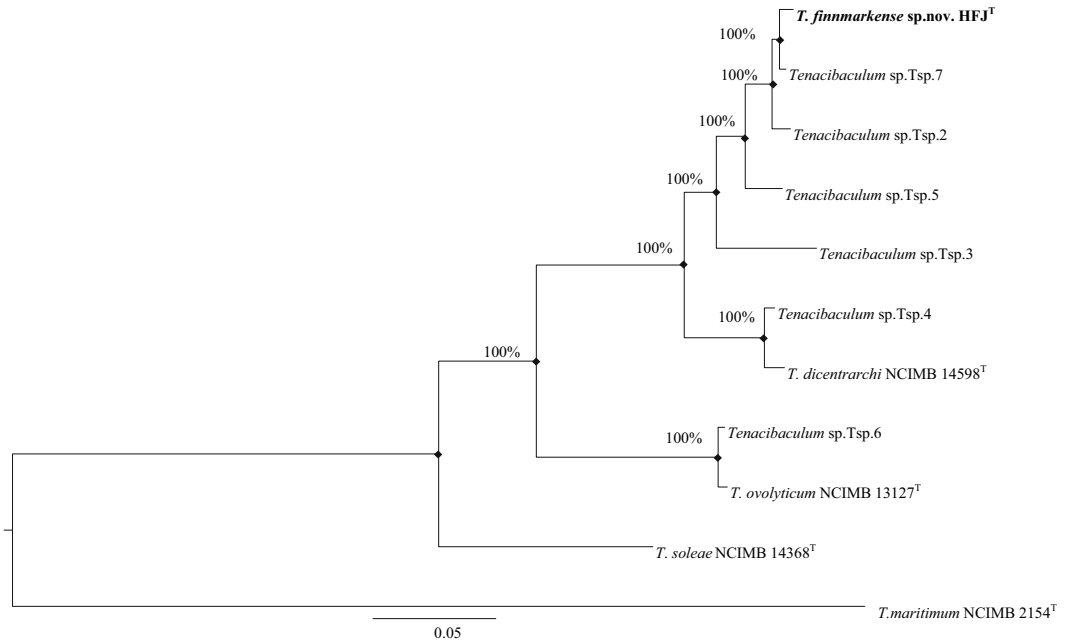


Fig. 3 The relationship of the novel species *T. finnmarkense* sp.nov HFJ^T, *Tenacibaculum* sp. strains Tsp. 2–7 and the three closest related type strains based on a concatenated sequences of five HK genes (*atpD* at position 1–807, *fusA* at position 808–1575, *pgk* at position 1576–2511, *rpoB* at position 2512–5778 and *tuf* at position 5779–6750), using *T. maritimum*^T as outgroup. The accession numbers for the HK genes used in the concatenated dataset are presented in Table 3. The phylogenetic

analysis was inferred using the Bayesian method with the best fitted evolutionary model. The posterior probability is presented next to each node in percentage. There were a total of 6750 positions in the dataset. Evolutionary analyses were conducted using KAKUSAN4 and Mr.Bayes. Shared nodes identified in corresponding ML analysis are marked with filled squares. Scale bar 0.05 substitutions per site

incubation (>96 h). Differential phenotypic characteristics between all strains listed in Table 1, except strain Tsp.7, are summarised in Table 5 and are included in the species description. The G+C content of strain HFJ^T was determined to be 34.1 mol% which is within the range reported for other type strains in the genus *Tenacibaculum* (29.8–35.2 mol%). The major fatty acids (>5 % of the total fatty acids) for strain HFJ^T were identified as summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, anteiso-C_{15:0}, Iso-C_{15:1} and iso-C_{15:0} 3-OH. Results from the fatty acid analysis for strain HFJ^T and *T. dicentrarchi* NCIMB 14598^T are listed in Table 6. The respiratory quinone was identified as menaquinone 6 (100 %) while flexirubin-type pigments were found to be absent. This is in accordance with the chemotaxonomic characteristics of the members of the genus *Tenacibaculum* (Suzuki et al. 2001). In the API ZYM

system, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase were found to be present. Lipase (C14), trypsin, α-chymotrypsin and all enzymes related to the metabolism of carbohydrates were found to be absent. Strain HFT^T was found to be susceptible to trimethoprim-sulfamethoxazole, cef-tazidime, ciprofloxacin, pipemidic acid, cefuroxime, penicillin G, ampicillin, tetracycline, erythromycin, florfenicol, oxytetracycline and oxolinic acid, but resistant to kanamycin, gentamicin and streptomycin.

Results from the phenotypic and chemotaxonomic tests show that strain HFJ^T differs significantly from *T. dicentrarchi* NCIMB 14598^T and *T. ovolyticum* NCIMB 13127^T (Table 5). The fatty acid composition analysis (Table 6) shows that strain HFJ^T has a very similar profile compared to that of *T. dicentrarchi*

Table 4 Results from the PNI and ANI analysis performed for all strains listed in Table 1

	Tsp.2	Tsp.3	Tsp.4	Tsp.5	Tsp.6	Tsp.7	Strain HFJ ^T	<i>T.dicentrarchi</i> ^T	<i>T.ovolyticum</i> ^T	<i>T.soleae</i> ^T	<i>T.maritimum</i> ^T
Strain HFJ ^T	99.3 (98.7)	95.7 (95.4)	97.3 (94.8)	98.6 (97.4)	96.0 (91.2)	98.8 (99.3)		97.2 (94.6)	96.0 (91.1)	96.4 (89.5)	93.6 (83.7)
<i>T. dicentrarchi</i> ^T	97.1 (94.6)	96.8 (94.0)	99.6 (99.0)	98.2 (95.2)	97.0 (90.7)	97.0 (94.6)	97.2 (94.6)		97.0 (90.6)	96.5 (89.3)	93.1 (84.4)
<i>T. ovolyticum</i> ^T	96.6 (91.1)	99.7 (91.0)	97.0 (90.6)	96.3 (90.9)	100 (99.4)	96.8 (91.0)	96.0 (91.1)	97.0 (90.6)		96.7 (89.8)	93.2 (85.2)
<i>T. soleae</i> ^T	96.6 (89.6)	96.5 (89.4)	96.3 (89.3)	96.9 (89.5)	96.7 (89.8)	97.1 (89.5)	96.4 (89.5)	96.5 (89.3)	96.7 (89.8)		94.0 (84.3)
<i>T. maritimum</i> ^T	93.7 (83.9)	92.9 (83.9)	93.1 (84.2)	93.3 (84.0)	93.2 (85.3)	93.8 (84.0)	93.6 (83.7)	93.1 (84.4)	93.2 (85.2)	94.0 (84.3)	

The pairwise 16S rRNA sequence identities and ANI values are presented as percent (%) similarity. ANI values are shown in parentheses

NCIMB 14598^T. Moreover, the G+C content of strain HFJ^T is higher than those reported for *T. dicentrarchi* NCIMB 14598^T and *T. ovolyticum* NCIMB 13127^T. Strain HFJ^T and *T. ovolyticum* NCIMB 13127^T do not grow on BAS, in contrast to *T. dicentrarchi* NCIMB 14598^T. *T. ovolyticum* NCIMB 13127^T is positive for the enzymes trypsin and N-acetyl-glucosaminidase, while strain HFJ^T and *T. dicentrarchi* NCIMB 14598^T are negative. *T. ovolyticum* NCIMB 13127^T was unique in being resistant to the antimicrobial drugs ceftazidime, penicillin G and ampicillin. The above mentioned differences further support strain HFJ^T as representative of a novel species in the genus *Tenacibaculum*. Cell length was the only characteristic shown to correspond to the three clades inferred in the phylogenetic analysis. Results showed a length of 2–40 μm for strain Tsp.4 and *T. dicentrarchi* NCIMB 14598^T, 2–30 μm for strains HFJ^T, Tsp.2, and Tsp.5, and 2–15 μm for strain Tsp.6 and *T. ovolyticum* NCIMB 13127^T.

In conclusion, the differential genetic, phylogenetic, phenotypic and chemotaxonomic data presented shows that strain HFJ^T should be classified as a novel species in genus *Tenacibaculum*, for which the name *Tenacibaculum finnmarkense* sp.nov. is proposed. This novel species also includes strains Tsp.2 and Tsp.5.

Description of *Tenacibaculum finnmarkense* sp. nov.

Tenacibaculum finnmarkense (finn.mark.en'se. N.L. neut.adj. *finnmarkense* of Finnmark, Norway, referring to the place of isolation).

Cells are strictly aerobic, Gram-stain negative, straight rods, 0.5 μm in diameter and 2–30 μm in length (filamentous cells >100 μm long may occur in older cultures) and motile by gliding. Degenerative spherical cells are observed in ageing cultures. Colonies on MA are circular, convex, pale yellow or yellow pigmented with translucent edges, have entire and/or undulating margins and a smooth texture with a shiny and sometimes nacreous appearance. The colonies are slightly viscous and do not stick to agar. Congo red absorption is negative. Growth occurs in media containing 50–100 % strength seawater but not in media supplemented with NaCl only. No growth occurs on BAS. Growth occurs at 2, 4, 8, 16 and 20 °C, but not at 25, 30 and 37 °C. Growth occurs at pH

Table 5 Differential characteristics of all strains listed in Table 1, except strain Tsp.7

Characteristic	Tsp.2	Tsp.3	Tsp.4	Tsp.5	Tsp.6	HFJ ^T	<i>T.dicentrarchi</i> ^T	<i>T.ovolyticum</i> ^T	<i>T.soleae</i> ^T	<i>T.maritimum</i> ^T
Cell size	3–30 µm	2–20 µm	2–40 µm	2–25 µm	2–15 µm	5–25 × 0.5 µm	2–40 µm	2–10 µm	2–25 µm	2–30 µm
Gram stain	–	–	–	–	–	–	–	–	–	–
Gliding motility	+	+	+	+	+	+	+	+	+	+
Colony morphology	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Color	Pale yellow	Bright Yellow	Pale yellow	Yellow	Pale yellow	Yellow	Brownish yellow	Pale yellow	Yellow	Pale yellow
Growth temp °C	8–16 ^b	8–16 ^b	8–16 ^b	8–16 ^b	8–16 ^b	2–20	2–25	8–25	8–16 ^b	8–16 ^b
Salinity range										
NaCl	Nt	Nt	Nt	Nt	Nt	Ng	Ng	Ng	Nt	Nt
Seawater % ^a	Nt	Nt	Nt	Nt	Nt	50–100	50–100	70–100	Nt	Nt
pH range	Nt	Nt	Nt	Nt	Nt	4–9	5–10	Nt	Nt	Nt
Catalase	w	w	w	w	w	w	w	w	w	+
H ₂ S	Nt	Nt	Nt	Nt	Nt	–	+	+	Nt	Nt
Antimicrobial drugs										
Ceftazidime	s	r	s	s	r	s	s	r	r	s
Pipemidic acid	s	s	s	s	s	s	s	s	r	s
Penicillin G	s	r	s	s	r	s	s	r	r	s
Ampicillin	s	s	s	s	r	s	s	r	r	s
Growth on										
L-tyrosin	Nt	Nt	Nt	Nt	Nt	–	–	+	Nt	Nt
D-glucose	Nt	Nt	Nt	Nt	Nt	–	–	+	Nt	Nt
Blood agar (2 % NaCl)	–	+	–	–	–	–	+	–	+	+
Hydrolyzation of										
Tween 80	Nt	Nt	Nt	Nt	Nt	–	+	+	Nt	Nt
API-ZYM										
Esterase (C4)	+	+	+	+	+	+	+	–	+	–
Cystein arylamidase	+	+	+	+	+	+	+	–	–	–
Trypsin	–	+	–	–	+	–	–	+	–	–
N-acetyl-β-glucosaminidase	–	+	–	–	+	–	–	+	–	–
G+C (mol%)	Nt	Nt	Nt	Nt	Nt	34.1	31.3	30.3	Nt	Nt

All data is from this study, except the DNA G+C contents of the two reference strains taken from [Pineiro-Vidal et al. \(2012\)](#) and [Suzuki et al. \(2001\)](#) + positive, – negative, w weakly positive, Nt not tested, Ng no growth, r resistant, s susceptible. All strains are oxidase positive and indole negative

^a Percent calculated using a relation of 100 % seawater = 38.2 g red sea salt L⁻¹

^b Only tested at 8 and 16 °C

^c Induces β-hemolysis or hemedigestion (CDC 2013) on blood agar containing 2 % NaCl

^d Induces α-hemolysis on blood agar containing 2 % NaCl

Table 6 Cellular fatty acid composition (%) of strain HFJ^T and *T. dicentrarchi* NCIMB 14598^T

Fatty acid	1	2
Straight chain		
C _{14:0}	1.3	1.0
C _{15:0}	1.8	3.8
Branched chain		
iso-C _{13:0}	1.3	1.3
iso-C _{14:0}	1.6	2.5
iso-C _{15:0}	17.1	15.2
anteiso-C _{15:1}	17.7	13.3
iso-C _{15:1}	9.5	9.0
anteiso-C _{15:1}	1.9	1.9
iso-C _{16:0}	Tr	1.0
iso-C _{16:1}	Tr	2.8
Unsaturated		
C _{15:1} ω6c	3.3	3.1
C _{16:1} ω5c	1.8	1.6
C _{17:1} ω6c	Tr	2.0
Hydroxylated		
iso-C _{15:0} 3-OH	12.4	11.6
C _{15:0} 2-OH	1.0	1.3
C _{15:0} 3-OH	1.5	2.2
iso-C _{16:0} 3-OH	2.9	5.3
C _{16:0} 3-OH	4.0	3.8
iso-C _{17:0} 3-OH	2.4	2.7
Summed feature 3 ^a	9.5	10.3

Strains 1 HFJ^T, 2 *T. dicentrarchi* NCIMB 14598^T

All data are from this study. Fatty acids amounting to <1 % of the total fatty acids in all strains are not shown. Tr, Trace (<1 %)

^a Summed feature are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 comprises C_{16:1} ω7c and/or iso-C_{15:0} 2-OH

4.0–9.0 (optimum pH 6–8). Catalase and cytochrome oxidase activities are present. Gelatin and casein are hydrolysed, but Tween 80 and starch are not. The Voges–Proskauer and flexirubin tests are negative. No anaerobic growth is observed. H₂S and indole are not produced. L-Proline and L-glutamate are utilised but D(+)-sucrose, D(–)-ribose, D(+)-galactose, D(+)-glucose and L-tyrosine are not. The major fatty acids (>5 % of the total fatty acids) are summed feature 3 (comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, anteiso-C_{15:0}, Iso-C_{15:1} and iso-C_{15:0} 3-OH. The

respiratory quinone is menaquinone 6. The DNA G+C content of the type strain is 34.1 mol%.

The type strain is HFJ^T (=DSM 28541^T = NCIMB 42386^T), isolated from diseased Atlantic salmon (*Salmo salar* L.) in Norway. The GenBank accession number for the 16S rRNA gene sequence of strain HFJ^T is KT270385.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Concurrent jellyfish blooms and tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon (*Salmo salar*) farms

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Abstract

Tenacibaculosis is an increasing problem in the Norwegian Atlantic salmon aquaculture industry causing significant economic losses. In September 2015, two separate outbreaks of suspected tenacibaculosis occurred at two Atlantic salmon farms in Finnmark County in Northern Norway. The events resulted in major losses of smolts newly transferred into seawater. Prior to, and during the outbreaks, large numbers of small jellyfish, identified as *Dipleurosoma typicum* (Boeck) were observed in the vicinity of the farms and inside the netpens. This study investigates the possible link between the jellyfish, *Tenacibaculum* spp. and the tenacibaculosis outbreaks. Bacteriology, histology, scanning and transmission electron microscopy, and real-time RT-PCR screening were performed on both fish and jellyfish samples. Based on the findings, *Tenacibaculum finnmarkense* was found to be the dominant bacteria associated with the tenacibaculosis outbreaks at both sites and that *D. typicum* is unlikely to be a vector for this fish pathogenic bacterium. However, results do show that the jellyfish caused direct damage to the fish's skin and may have exacerbated the bacterial infection by allowing an entry point for bacteria.

Introduction

Jellyfish blooms are a rising concern to the marine aquaculture industry because they have been associated with an increasingly large number of mortality events in Atlantic salmon farming, which have resulted in economic losses and fish welfare issues [1,2]. This phenomenon is not fully understood but is possibly due to changing ocean conditions, including anthropogenic causes, and/or that production is now occurring in areas with naturally high occurrences [3–5]. High levels of *Pelagia noctiluca* in Northern Ireland have been linked with several mortality events with Atlantic salmon exhibiting skin and gill lesions, as well as abnormal behaviour such as increased jumping [2,6,7]. This species has also been linked to a mortality event in Atlantic Coastal France [8]. In Scotland and Norway, mass mortality events showing mainly gill lesions have been linked to blooms of *Phialella quadrata* [9,10]. Mortality events in Norway have also been associated with blooms of: *Aurelia aurita*, *Muggiaea atlantica*, *Apolemia uvaria*,

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Competing interests: The authors have declared that no competing interests exist.

and *Bolinopsis infundibulum* [11–13]. Some of these jellyfish (e.g. *Aurelia aurita* and *Pelagia noctiluca*) have been experimentally shown to cause gill and skin damage to marine-farmed fish without the need for other stressors or pathogens [14,15].

The ability of jellyfish (in this paper, members of Phylum Cnidaria are referred to as “jellyfish”) to form blooms under favourable environmental conditions is due to them having both asexual and sexual reproduction [4]. All jellyfish have the potential to be toxic due to having cnidocytes mainly found on the tentacles, which contain stinging nematocysts [16]. These are highly specialised organelles that fire a venom containing structure when triggered in response to a chemical or physical stimuli such as contact with a fish’s skin [17,18]. There are variations in the composition of the venoms between jellyfish species and some have been shown to be cytotoxic or haemolytic [15,19]. The severity of mechanical and toxic injury caused by jellyfish is exacerbated by certain factors such as increased temperatures and exposure [15].

Studies have shown that many environmental bacteria, including pathogenic ones such as *Tenacibaculum* spp. (Family Flavobacteriaceae) and *Moritella viscosa* (Family Moritellaceae) are found on jellyfish and these could therefore act as vectors [10,20–22]. *Tenacibaculum* spp. are found worldwide with some species causing tenacibaculosis in marine aquaculture; a disease mainly characterised by ulcerative lesions, frayed fins and mouth erosion [23–27]. The bacteria have also been linked to gill lesions [28,29]. Tenacibaculosis has been reported with increased frequency in the last few years in the Norwegian salmon farming industry [30,31]. In Norway, affected fish most commonly have mouth erosion, frayed fins (pectoral, pelvic and anal), and tail rot and these lesions have been associated with *Tenacibaculum finnmarkense* [25]. Lesions are often characterised by skin ulcers with yellow margins that are surrounded by wide areas of scale loss (personal observations). Similar lesions have been associated with jellyfish mortality events [6]. The literature would therefore suggest that such mortality events are a result of the direct damage by the jellyfish, as well as associated filamentous bacterial infections [2,13,32].

In September 2015, two separate outbreaks of suspected tenacibaculosis occurred at two Atlantic salmon farms in Finnmark County in Northern Norway. The events resulted in major losses of smolts newly transferred into seawater. Prior to, and during the outbreaks, large numbers of small jellyfish (approximately 10 mm in diameter) were observed in the vicinity of the farms and inside the net-pens. This study investigates the possible link between the jellyfish, *Tenacibaculum* spp. and the tenacibaculosis outbreaks.

Materials and methods

Field sampling

Jellyfish. Several specimens of the jellyfish were collected at each sampling point as shown in Fig 1 and placed into individual tubes containing 100% ethanol. Some specimens were also placed directly in a modified Karnovsky fixative [33] for histology, Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Single jellyfish specimens were placed onto Marine Kanamycin Agar (MKA) [34] to look into the *Tenacibaculum* community present on the jellyfish as kanamycin is reported to prevent overgrowth by other environmental bacteria [34–37]. Bacterial samples were identified by their site number, followed by their environmental sampling point if not at the site, and then the jellyfish number (E.g. isolate S1E2J3 was sampled from site 1 at environmental sampling point 2 from jellyfish 3).

Fish. All fish samples were collected from two licensed (Norwegian Directorate of Fisheries) Atlantic salmon saltwater farms during two outbreaks of tenacibaculosis. Cermaq Norway who runs both farms, gave permission for licensed aqua medicine biologists to collect samples from their sites during the outbreaks. Both commercial sites receive smolts and grow them to

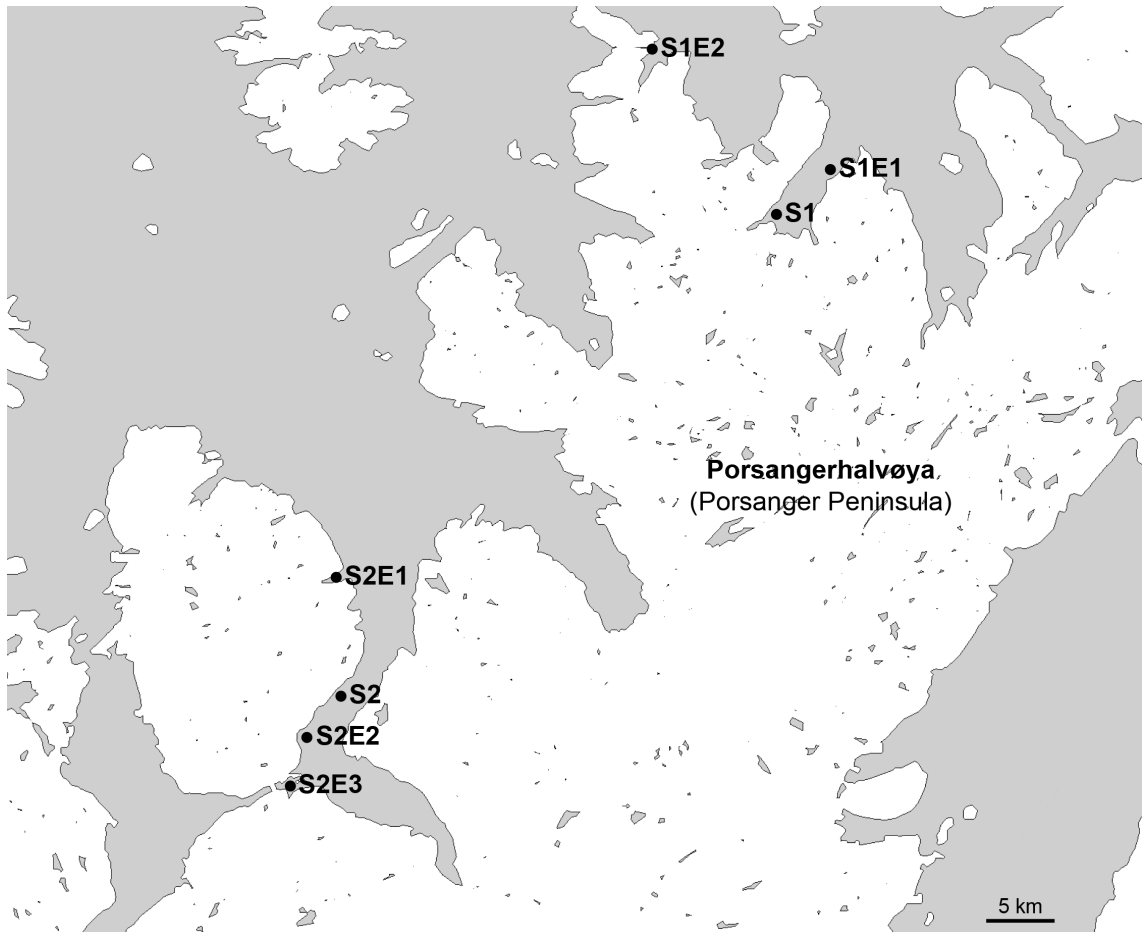


Fig 1. Map of sampling points. Map showing the area surrounding Porsangerhalvøya in Finnmark in the northernmost part of Norway. Farm sites are marked as S1 and S2 and environmental sampling points are marked with the site number, followed by the environmental sampling point number (e.g. S1E2).

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market size using best aquaculture practices following the requirements of the Norwegian Aquaculture Act. Randomly sampled fish were euthanized with an overdose (100 mg L^{-1}) of benzocaine (Benzoak vet—Europharma). Moribund fish were euthanized with a swift blow to the head to avoid contamination between fish.

Skin, gill, and some eye samples from moribund fish were immediately placed in a modified Karnovsky fixative for histology and SEM. Bacterial samples from the skin lesions of these fish were collected and grown on Marine Agar (Difco 2216) (MA) and identified by their sample point (Fig 1) followed by the fish number (E.g. isolate S1F3 was sampled from site 1 from fish 3). Skin tissue (margin of lesion and if not present, the skin under the mandible) were collected for *Tenacibaculum* spp. and *M. viscosa* real-time RT-PCR screening from 10 moribund fish

from affected pens at site 1 and 30 randomly caught 'healthy' fish and 5 moribund fish from each net-pen at site 2 (Fig 1).

Jellyfish identification

One jellyfish specimen from each farm site was removed from the ethanol and lysed in a mixture of OB Protease solution and TL buffer (OMEGA bio-tek) prior to DNA extraction. The DNA was extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (24:25:1), then once with chloroform (24:1), followed by precipitation using 1:10 volume of 3M Sodium Acetate (pH 5.5) and two volumes of ice cold 100% ethanol. The quality and concentration of the eluted DNA was measured by using a Nanodrop (Thermo Scientific) spectrophotometer. For use in PCR reactions, the eluted DNA was diluted using nuclease free water to a concentration of approximately 50 ng μl^{-1} . The DNA was stored at -20°C .

PCR of the jellyfish mitochondrial large ribosomal subunit (MLRS) DNA sequence was performed using the forward and reverse primers described in Cunningham and Buss [38]. The amplification was performed using the standard reaction mixture described in Småge, Frisch [39] at 94°C for 5 min, 35 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min, 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 followed by 80°C for 15 min. Sequencing was performed on PCR products using the internal primer described in Cunningham and Buss [38]. The analysis was carried out by the Sequencing Facility at the University of Bergen (<http://www.uib.no/seqlab>) using Big Dye termination chemistry. Obtained sequences were uploaded to The Basic Local Alignment Search Tool (BLAST) (NCBI) to determine the identity of the jellyfish.

A morphological identification of the sampled jellyfish was also performed using the identification keys and notes for identification of thecate hydroids and their medusae [40] and the description given by Russell [41].

Bacteriology

All primary cultures were incubated for a minimum of 5 days at 16°C and sub-cultivation was performed on MA and incubated at 16°C for 48–72 hours. Bacteria were identified by uploading their DNA sequences to the BLAST. In order to do so, genomic DNA was obtained by heating single colonies obtained from bacterial clones in tubes containing nuclease free water at 95°C for 5 min. The samples were then centrifuged at 10,000 rpm for 5 min and the DNA-containing supernatant was transferred to new tubes and stored at -20°C . PCR was performed using the 16S rRNA primers 27F and 1518R [42] and the amplification and sequencing were performed as described in Småge, Frisch [39].

Jellyfish real-time RT-PCR analysis

The ethanol in which the jellyfish samples were preserved was removed by centrifuging the tubes at 8,000 rpm for 5 min before discarding the ethanol. Cultured *Halobacterium salinarum* DSM 3754^T cells suspended in PBS were added to each of the tubes prior to RNA extraction as an exogenous control in the real-time RT-PCR analysis [43]. The jellyfish samples were then homogenized by adding 1ml of Isol-RNA Lysis Reagent (5 Prime) to each sample and then placed in a TissueLyser LT (Qiagen) at 50 Hz for 5 min followed by 5 min sitting at room temperature. The RNA was extracted following the manufacturer's protocol (5 Prime), except that a second washing step using 100% ethanol was performed prior to air drying of the RNA pellet.

Table 1. Real-time RT-PCR assays.

Assay	Assay Efficacy	Primer/Probe	Sequence
Tb tuf	1.9397	Forward	AGTGTGACGTCCACCTT
		Reverse	CTGTAAGCCAGGTCTGT
		Probe	TTTCAATACATACACCTCAGC
Mv ompA	1.8826	Forward	GATGATAACGCAACAGCAG
		Reverse	CGGAAACTTACACCAGATAATG
		Probe	TCTTGGAGCAGGTCTAGAATATACACCAG
SAL	1.8501	Forward	GGGAAATCTGTCCGCTTAACG
		Reverse	CCGGTCCCAAGCTGAACA
		Probe	AGGCGTCCAGCGGA
ELF1A	1.9800	Forward	CCCCTCCAGGACGTTTACAAA
		Reverse	CACACGGCCACAGGTACA
		Probe	ATCGTGGTATTGGAAC

The sequences of the primers and probes and the efficacy of each real-time RT-PCR assay used in this study [43–45].

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The obtained RNA was subsequently diluted 1:10 in order to avoid inhibition in the real-time RT-PCR reaction and stored at -20°C.

The extracted RNA was tested for the presence of *Tenacibaculum* spp. commonly recovered from tenacibaculosis outbreaks in Norwegian farmed Atlantic salmon using the Tb tuf real-time RT-PCR assay (Table 1) [44]. The exogenous control (*H. salinarum*) was detected using the assay (SAL) developed by Andersen, Hodneland [43] (Table 1). All assays were run using an AgPath-ID kit (Thermo Scientific) with 2 µl of RNA and the standard AgPath-ID concentrations of primers (400 nM) and probe (120nM). Each run consisted of 45 cycles.

Atlantic salmon real-time RT-PCR analysis

The RNA from the site 1 samples was extracted using Isol-RNA Lysis Reagent (5 Prime) following the manufacturer’s protocol and the RNA from the site 2 samples was extracted by a commercial laboratory. The RNA was screened using real-time RT-PCR with the Tb tuf assay, an assay targeting *M. viscosa*: Mv ompA, and an assay targeting the elongation factor 1 alpha (EF1A) [45] (Table 1). All assays were run using the same procedure as for the jellyfish real-time RT-PCR analysis.

Phylogenetic analysis

Sequences were assembled and aligned using Vector NTI v.9.0 (Invitrogen) software and adjusted to equal lengths using GeneDoc [46]. The alignment included all 16S rRNA gene sequences obtained in this study and for all known type strains in genus *Tenacibaculum*. In addition, the 16S rRNA gene sequences of *Flexibacter echinica* and *Flexibacter aurantiacus* subsp. *copepodarum*^T [47] were included to improve the phylogenetic resolution. The bacterium *Kordia algicida*^T AB681152 was used as an outgroup as suggested by Habib, Houel [48]. All sequences not from this study were obtained from the GenBank. The phylogenetic relationship was calculated using BEAST 1.8 [49] using the best fitted model, a relaxed lognormal molecular clock and a mcmc of 150,000,000 generations. The GTR+G+I model was found to be the best fitted model by using Mega6 [50]. All ESS values were above 200 for the Bayesian analysis and a maximum clade credibility tree was created using a 10% burn-in in Tree-Annotator [49] and viewed using FigTree v1.4.0 [49].

Histology, scanning and transmission electron microscopy

Preparation of fish and jellyfish tissues for histology and SEM was performed as described in Småge, Frisch [39]. Jellyfish samples for TEM were prepared as described in Nylund, Hovland [33]. Ten jellyfish were prepared for histological examination: five were pre-embedded in 0.4% agarose before embedding in EMBED 812 resin and five were directly embedded in the EMBED 812 resin.

Results

Field sampling

At site 1, the tenacibaculosis outbreak started at the beginning of September. Five of the six net-pens were newly stocked with smolts, one to three weeks prior, and had an average weight of 87 grams at the start of the outbreak. The sixth net-pen had been stocked six weeks prior, and the fish were significantly larger (average of 280 grams) at the time of the outbreak. The accumulated mortality in that pen was 2.1% for the duration of the event. The other five pens had to be culled due to fish welfare reasons as a result of the disease.

At site 2 there were two separately stocked smolt populations: six net-pens were stocked at the start of summer with spring smolts and the other six net-pens were stocked at the end of the summer with autumn smolts. In the middle of September, the spring smolts started showing signs of tenacibaculosis followed by the newly transferred autumn smolts. At this point the average weight was 155 grams for the spring smolts and 68 grams for the autumn smolts. Mortality spiked towards the end of the month and continued into the middle of October. All net-pens were treated with florfenicol at 10 mg kg⁻¹ for 10 days. The accumulated mortality for the outbreak differed between net-pens and generations. The accumulative mortality per net-pen for the tenacibaculosis outbreak, which lasted approximately one month, was 2.5 to 8% for the spring smolts and 17 to 40% for the autumn smolts.

The water temperatures measured at five meters at both farm sites were between 8 and 10°C at time of sampling. Sampling occurred at the start of the outbreak for both sites. At site 2, three of the net-pens containing spring smolts had already been treated with florfenicol at 10 mg kg⁻¹ for 10 days.

During the sampling, large blooms of small (approximately 10 mm) transparent jellyfish (Fig 2) were present in and around the net-pens at both sites, as well as throughout the surrounding fjords. In certain areas aggregations of the jellyfish gave a greyish cloudy appearance to the sea. The individual jellyfish were found to be very friable thus hampering the sampling. Fish in the net-pens showed abnormal behaviour, including swimming high in the water column and increased jumping activity. Moribund fish showed signs of tenacibaculosis: frayed fins, scale loss, and skin ulcers and mouth erosion with yellow pigmentation of the margins (Fig 3). Some fish had eyes with a cloudy appearance. No gross pathology was observed internally.

Large numbers of small pollock (*Pollachius virens*) were observed feeding on the jellyfish at both sites. One of these fish was caught and found to have jellyfish remains in the stomach on necropsy.

Jellyfish identification

Based on the morphological description, the dominating jellyfish found at both sites were leptomedusae of *Dipleurosoma typicum* [51] (Phylum Cnidaria; Class Hydrozoa; Subclass Hydroidolina; Order Leptothecata; Family Dipleurosomatidae). The BLAST search of the MLRS DNA sequences did not yield any matches as there are no sequences of this organism in the database; therefore, the identification of the jellyfish had to be purely based on the morphological features.

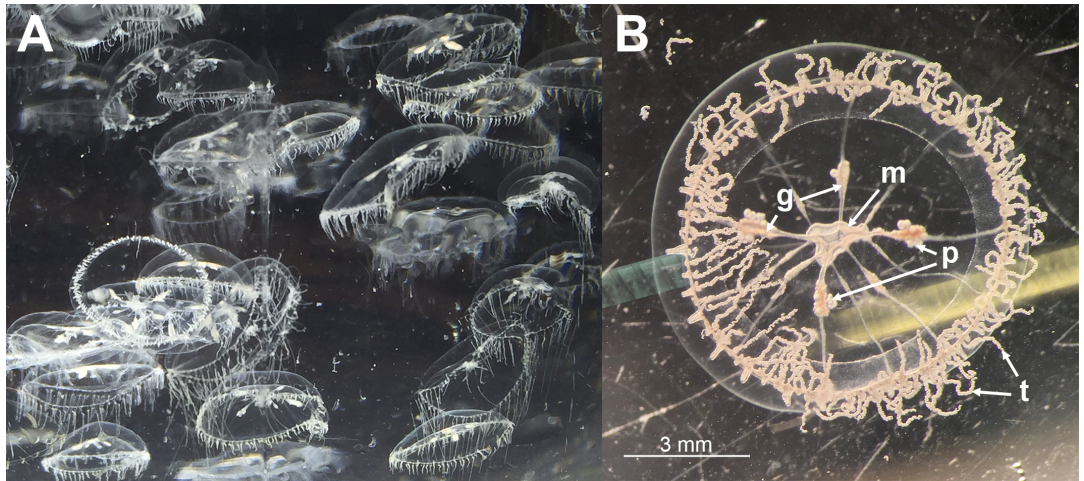


Fig 2. Jellyfish morphology. (A) A water sample collected outside one of the net-pens at site 1. A high concentration of *D. typicum* was present in the sample. (B) One of these specimens under a stereomicroscope showing the characteristics typical for this species [40,41]. m: the mouth, t: tentacles, g: gonads, p: planula larva (Note: the planula larva was found to be ciliated and highly motile, which differs from previous descriptions given for this species).

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Both macroscopic (Fig 2) and microscopic examinations of the sampled jellyfish showed the features classically described for *D. typicum* (i.e. size, number of radial canals, and the position and characteristics of the tentacles and gonads). The obtained *D. typicum* sequences from site 1 and site 2 were identical to each other except for one nucleotide. These sequences have been made available in the GenBank (accession number: MF192883 from site 1 and MF192884 from site 2).

Bacteriology

Bacterial cultures from the fish revealed mostly yellow colonies containing filamentous bacteria that were identified based on the obtained 16S rRNA gene sequence to be *Tenacibaculum*



Fig 3. Moribund fish showing signs of tenacibaculosis. (A) Fish representing moribunds from both site 1 and 2. Skin lesions have the typical scale loss and yellow margins seen with tenacibaculosis. Frayed fins are also clearly visible. (B) A close up of an affected head showing a mouth lesion that has the typical yellow margin associated with tenacibaculosis.

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finnmarkense^T or closely related strains (Fig 4). The bacteria recovered from the jellyfish revealed a wide range of Flavobacteriaceae: *Tenacibaculum* spp. (Fig 4), *Winogradskyella* sp., *Lacinutix* sp. *Krokinobacter* sp., *Polaribacter* sp., *Olleya* sp. and *Cellulophaga algicola*. *Tenacibaculum* spp. were only recovered from a small number of the jellyfish sampled. No *M. viscosa* was recovered from the fish or from the jellyfish.

Jellyfish real-time RT-PCR analysis

A low number of jellyfish were positive to *Tenacibaculum* spp. with real-time RT-PCR analysis, which was reflected in the bacteriology results. However, the initial results showed inhibition, determined by the exogenous control; therefore the analysis was repeated by diluting the RNA 1:10. The resulting Ct values were close to the detection limit.

Fish real-time RT-PCR analysis

All sampled moribund fish at both site 1 and 2 were positive with the Tb *tuf* assay indicating that they are positive for *Tenacibaculum* spp. Only two of these fish were positive for *M. viscosa* and both had high Ct values indicating a very low load. Of the randomly sampled fish from site 2, none were positive for *M. viscosa* and only a proportion of them were positive for *Tenacibaculum* spp. using the Tb *tuf* assay as indicated in Fig 5. The average load for the moribund fish was higher than that of the randomly sampled fish (Fig 5).

The RNA obtained from the randomly sampled fish, determined by the EF1A results, was within the acceptable quality range. However, some of the samples from the moribund fish were outside this quality range. The loss of quality is most likely due to sampling lesions with a high probability of containing necrotic and therefore degraded tissue. For this reason normalized Ct values were not used for analysis of results in this study.

Phylogenetic analysis

A total of 57 *Tenacibaculum* isolates were recovered in this study: 23 from fish and 34 from jellyfish (GenBank accession numbers: MF192899 to MF192955). The phylogenetic analysis (Fig 4) showed that the majority of isolates recovered from fish were identical to the type strain or other strains of *T. finnmarkense*. Three isolates were more closely related to *Tenacibaculum dicentrarchi*^T and one isolate was more closely related to *Tenacibaculum ovolyticum*^T. The recovered *Tenacibaculum* isolates from the *D. typicum* samples revealed a greater phylogenetic diversity than seen in the isolates recovered from the fish; however, none were shown to be identical to the type strain of *T. finnmarkense*. A small group of jellyfish isolates were closely related to *T. ovolyticum*^T and another group were closely related to *Flexibacter aurantiacus subsp. copepodarum*^T.

Histology, SEM, and TEM

Jellyfish. No bacteria were observed on the histologically examined jellyfish. Thread-like structures were observed on the surface of the mouth region on histology, TEM (Fig 6) and SEM (Fig 7) sections that were identified to be surface cilia based on the descriptions given by Fisch and Dupuis-Williams [52], Mariscal [53] and Martin and Archer [54]. Structures were observed on both histology and TEM sections that correspond to what is described for nematocysts. These structures were observed in the epidermis of the mouth and in abundance in the tentacle regions.

Fish. On histology, sampled gills showed little tissue changes with the exception of some lamellae that had epithelial lifting (Fig 8A) with a few foci of hypertrophy and necrosis (Fig 8B). SEM examination of the gills revealed that the microridges were intact and no nematocysts or

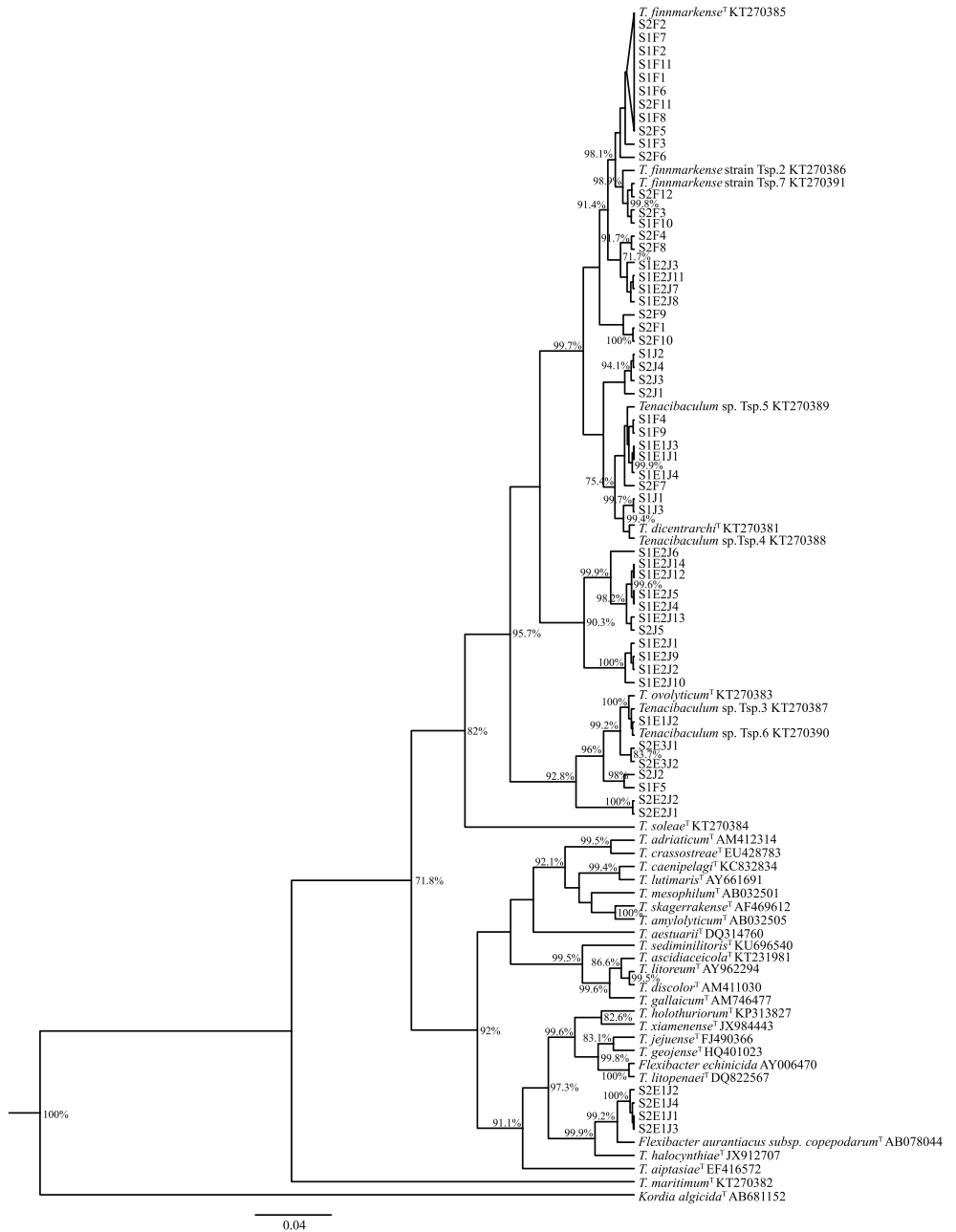


Fig 4. Phylogenetic analysis of recovered *Tenacibaculum* spp. isolates. Phylogenetic tree of the 16S rRNA gene sequences obtained in this study and all known type strains in genus *Tenacibaculum*. In addition, *Flexibacter echinidica* and *Flexibacter aurantiacus* subsp. *copepodarum*[†] were included. A Bayesian analysis was performed using the GTR+G+I model.

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pathogens were found (S1 Fig). Filamentous bacteria were seen on the surface of some sampled eyes during SEM examination; however there was no associated tissue damage with the exception of a few nemocysts penetrating the cornea (S2 Fig).

The skin from all of the sampled fish revealed significant damage to the tissue associated with large numbers of *Tenacibaculum*-like bacteria (Fig 9 and Fig 10), that looked like mats in some areas. The bacteria were not only present in areas of epidermis loss, but were also seen infiltrating the dermis causing degradation of the stratum compactum (Fig 9A). In a few cases the bacteria had penetrated all the way down to the hypodermis and caused damage to the connective tissue (Fig 9B). Bacterial cells could be seen to be in the process of dividing and were closely entangled with the damaged collagen layer (Fig 10B). Nematocysts were observed

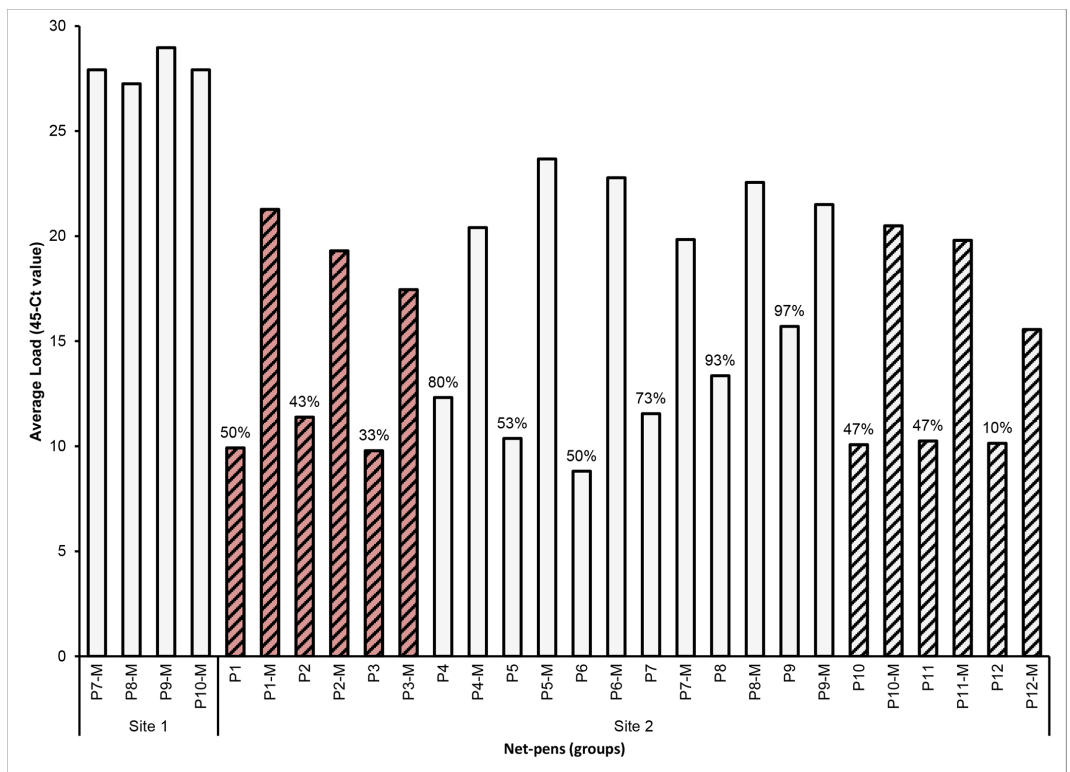


Fig 5. Real-time RT-PCR results from sampled fish. Average load of *Tenacibaculum* spp. positive fish for each sampled group measured with the *Tb* *tuf* real-time RT-PCR assay. Dataset includes both randomly sampled fish from each pen at site 2 (n = 30) and moribunds (-M) from each affected pen (n = 10 for site 1 and n = 5 for site 2). The prevalence in the moribunds was 100% and the prevalence in the randomly sampled fish is indicated as % above the respective bar. Striped columns are spring smolts and non-striped columns are autumn smolts. Red columns were treated with florfenicol prior to sampling.

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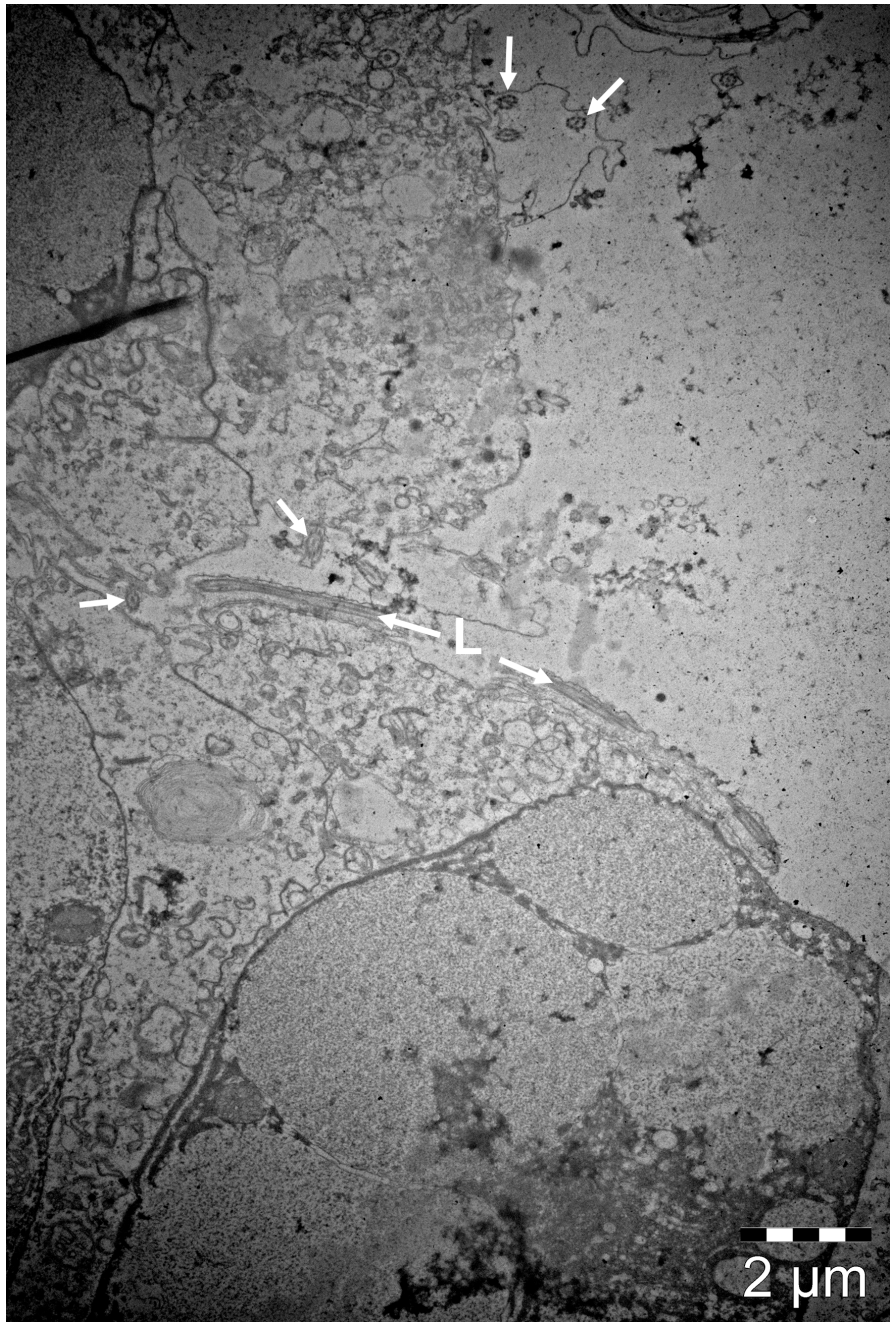


Fig 6. TEM micrograph of the mouth region of *D. typicum*. The typical 9+2 pattern (arrows) of motile ciliary axonemes are clearly visible. A cilium can be seen longitudinally (L).

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on some of the sections from these fish and were seen to be embedded in the skin (Fig 11B) accompanied by large numbers of *Tenacibaculum*-like bacteria (Fig 11A). Holes, the same size as the nematocysts' shafts were observed, and are likely a result of them being ripped out (S3 Fig).

Discussion

This is the first case study of a tenacibaculosis outbreak in Atlantic salmon in Norway. Based on the results, the authors suspect that the tenacibaculosis was primarily caused by *T. finnmarkense*, but exacerbated by the presence of large amounts of jellyfish. The phylogenetic analysis showed that 19 of the 23 isolates recovered from diseased fish belonged to the *T. finnmarkense* clade and approximately half were identical to the type strain. The dominance of this species during both outbreaks strongly supports it being the causative agent of tenacibaculosis in Atlantic salmon in Northern Norway. The very low prevalence of *M. viscosa* detected in this study (0.4%) is similar to another published case of ulcerative disease in Northern Norway [55]. Therefore, *M. viscosa* does not seem to have an important role in causing disease in these particular cases.

Fish behaviour (lethargy, swimming close to the surface and excessive jumping) during the jellyfish bloom matched what is described in the literature [6,9]. Unlike other described jellyfish blooms, the fish showed few gill lesions both grossly and microscopically leading to the conclusion that the fish did not directly die from their exposure [14,15]. The affected fish showed signs of tenacibaculosis which has been observed during other jellyfish related mortality events [6,32] and which has been known to cause large mortality events in Atlantic salmon without the presence of jellyfish (personal observations). Also, tenacibaculosis related mortalities at both sites seemed to have started before the jellyfish bloom. The difference in the presentation of the jellyfish damage in this case, when compared to other cases in the literature could

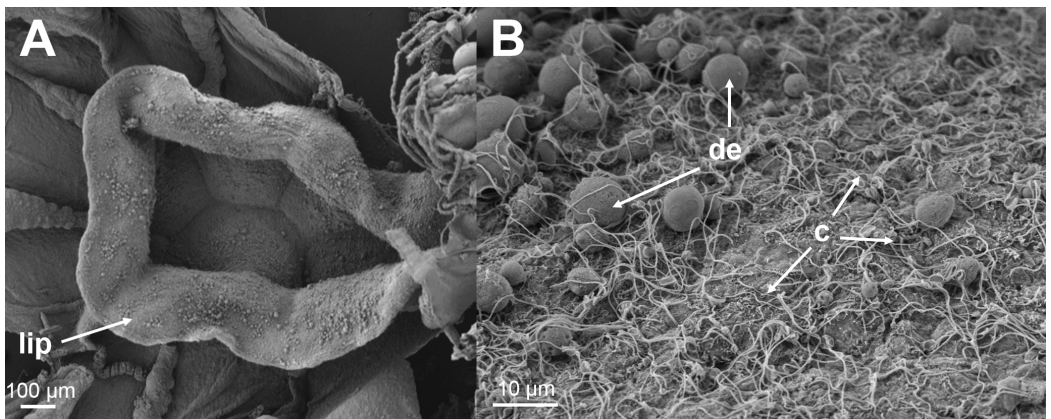


Fig 7. SEM micrographs of *D. typicum* mouth region. (A) A SEM micrograph showing the lip of the mouth of *D. typicum*. (B) A higher magnification of the inside of the mouth showing an abundance of cilia (c) and spherical structures that are likely droplets of exoenzymes (de) important in the digestion of food [10].

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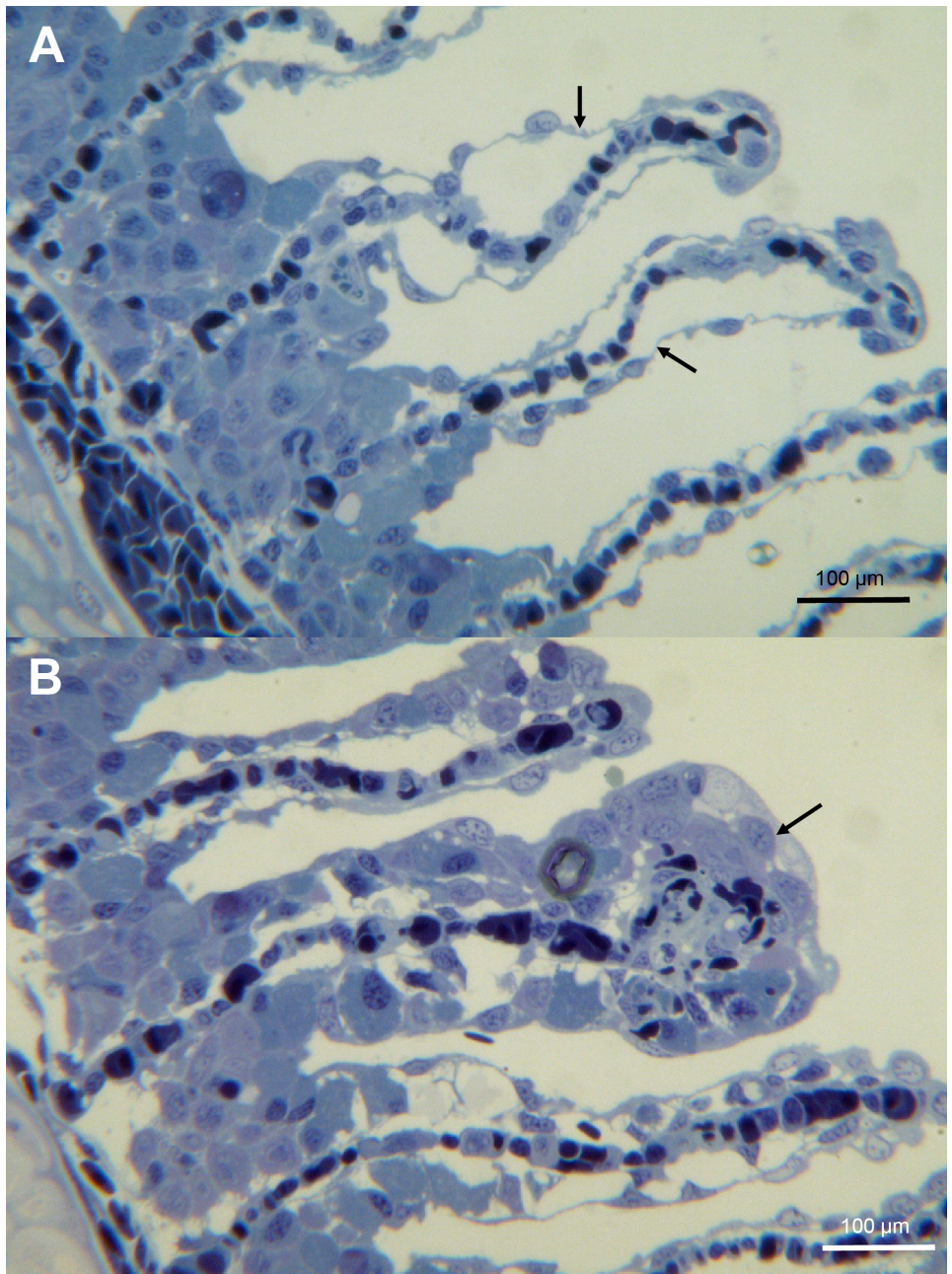


Fig 8. Histology of gills from moribund fish. (A) A toluidine blue stained section of gills showing epithelial lifting (arrow). (B) A toluidine blue stained section of gills showing foci of hypertrophy (arrow).

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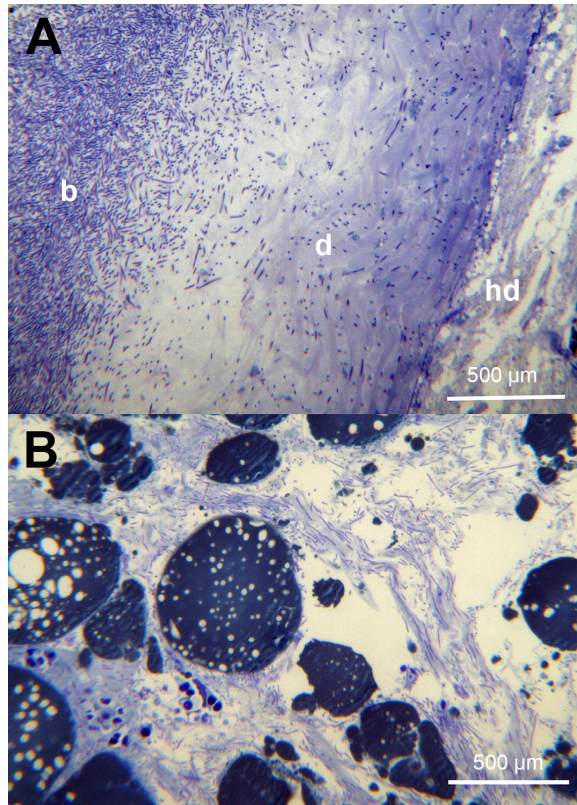


Fig 9. Histology of skin from moribund fish. (A) A toluidine blue stained section of skin showing complete loss of epidermis where the bacteria (b) are present and infiltration of the stratum compactum of the dermis (d). This section does not appear to have bacteria infiltrating the hypodermis (hd). (B) A toluidine blue stained section of the skin hypodermis showing large number of bacteria.

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be due to this being a *D. typicum* bloom and its potential for causing damage to fish has not been studied.

The histological, SEM and TEM investigations into the presence of bacteria on the jellyfish revealed that the thread-like structures observed in the mouth region in our samples were cilia and not bacteria (*Tenacibaculum* spp.), in contrast with what is concluded by Delannoy, Houghton [21] and Ferguson, Christian [10]. The lack of bacteria on the surface of the jellyfish, including the mouth region could be due to the preparation of the samples; however, the authors do not think so due to the fact that identical results were found with histology, SEM and TEM, and that bacteria were observed on similarly prepared samples of fish tissues. A recent study by Viver, Orellana [56] revealed *Tenacibaculum*-like cells in the gastric filaments of the jellyfish *Cotylorhiza tuberculata*. This indicates that the bacteria might be present internally and not on the surface of the jellyfish.

The nematocysts present in some of the examined fish tissue and the associated pathological changes match what is known about their mechanism of action [18]. The authors observed

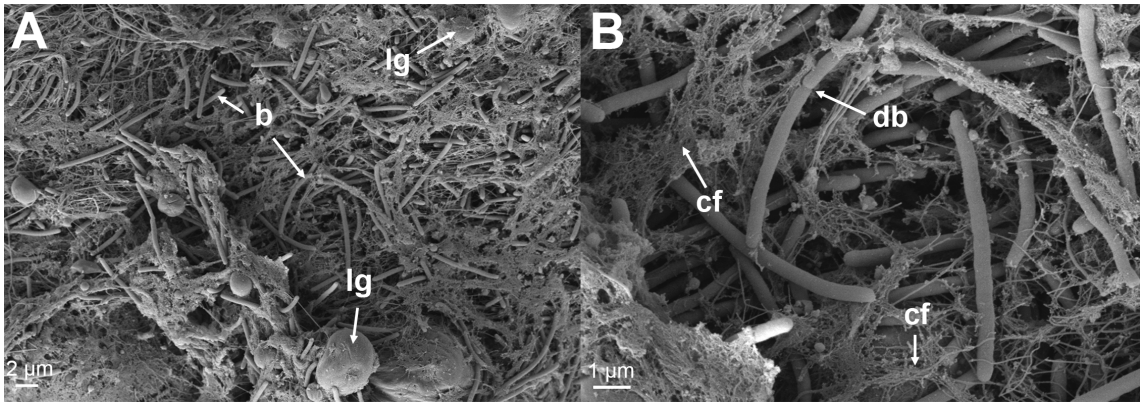


Fig 10. SEM micrographs of lesions from moribund fish. (A) A SEM micrograph from the margin of a jaw lesion showing *Tenacibaculum*-like bacteria (b) infiltrating the dermis layer of the skin. Lipids globules (lg) can be seen and are likely a result of the destruction of the dermis layer as seen histologically. (B) A higher magnification showing a *Tenacibaculum*-like bacterial cell replicating in the dermis (db). The breakdown of the collagen fibers (cf) of the dermis can be seen associated with the bacteria.

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embedded nematocysts with *Tenacibaculum*-like bacteria close to or attached to them (Fig 11B), as well as holes likely to be a result of nematocysts being ripped out. It is natural to assume that the holes provide an entry point for pathogens; in this case *Tenacibaculum*, which seems to have a high affinity for the underlying collagen rich dermis layer [31]. There is evidence of this in the histology sections and the SEM sections show dividing *Tenacibaculum*-like bacteria within the collagen layer of the skin (Fig 10B).

In this study, the smolts that had been recently transferred to saltwater (five net-pens at site 1 and six net-pens at site 2) were more affected in terms of total mortality when compared to the smolts that had been in saltwater for longer. This is likely a result of smolts going through a

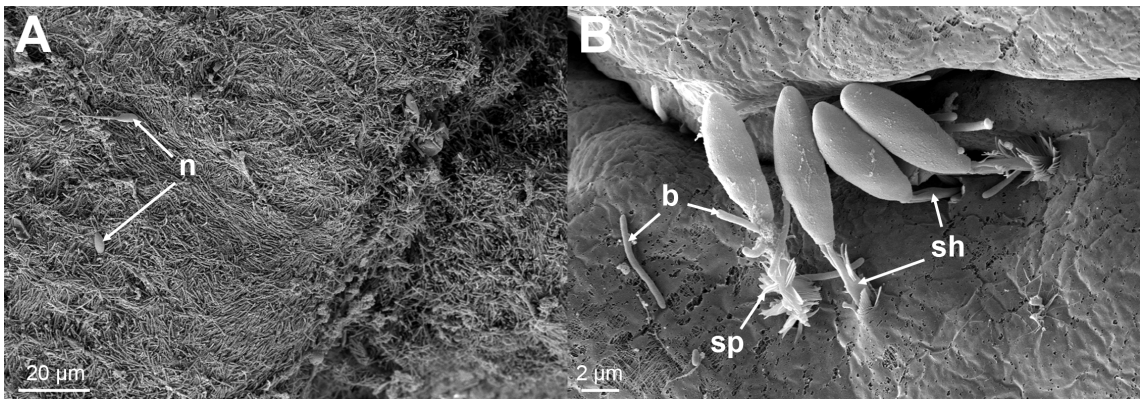


Fig 11. SEM micrographs. (A) A SEM micrograph from the margin of a lesion showing a mat of *Tenacibaculum*-like bacteria with a few attached nematocysts (n). (B) A higher magnification of one of these areas showing the nematocysts with their spiked (sp) covered shafts (sh) embedded in the skin. Bacteria (b) can be seen in close proximity with some seeming to be entering the holes created by the nematocysts.

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transition phase from freshwater, which has a significant impact on the physiology and immunology of the fish, and also the skin microbiota [57]. Interestingly, it looks like 'healthy' smolts acquire a certain level of *Tenacibaculum* spp. in their skin microbiota after only a few weeks in saltwater [57], and these bacteria seem to be part of the 'normal' skin microbiota of Atlantic salmon [55,58]. The real-time RT-PCR results showed that there was a higher bacterial load between moribund and randomly sampled 'healthy' fish, which may indicate that a certain level of the bacteria is required to cause disease.

There is a complex relationship between bacterio-, phyto- and zooplankton and jellyfish communities in the environment, which make determining the reservoirs and vectors of pathogenic bacteria difficult. Flavobacteriaceae levels, including *Tenacibaculum*, have been shown to be associated with elevated levels of organic material (e.g. phytoplankton blooms) in the environment due to their ability to decompose complex molecules [59–61]. Phytoplankton blooms frequently occur in the Barents Sea (the sea surrounding the Northern most part of Norway) [62–65]. This was also the case in 2015 when a phytoplankton bloom occurred in close proximity to the sites approximately one month prior to the mortality events (NASA Worldview, <https://goo.gl/oq8Sb3>). As a result, levels of Flavobacteriaceae present around the farm sites are expected to be high during these blooms when there is a high organic load in the water [59,61,66,67]. Different clades of Flavobacteriaceae will dominate at different times of the phytoplankton bloom cycles [59,60,66,68,69], and *Tenacibaculum* spp. tend to be later in the cycle when the plankton are decomposing [70]. The timeline of these cycles match what was seen in this study with the tenacibaculosis outbreaks starting 4 to 6 weeks after the onset of the phytoplankton blooms.

Jellyfish levels also react to organic load, and blooms often occur as a result of increased levels of zooplankton which generally lags that of phytoplankton blooms by a month [3,71,72]. All known described jellyfish in Phylum Cnidaria from the Barents sea forage on zooplankton [72]; it is therefore likely to be the case for *D. typicum*. It is also shown that jellyfish blooms can have an effect on the bacterial community composition in the vicinity of the bloom; as was shown in a study where the presence of the jellyfish *Mnemiopsis leidy* was associated with increased levels and prevalence of Flavobacteriaceae [73].

The earliest recorded bloom of *D. typicum* was near the British Isles in the late 1800s and the presence of the species has been reported in much of the boreal-circumpolar region [40,41]. The presence of *D. typicum* in Northern Norwegian waters may not be a new finding, but with new areas being used for salmon aquaculture their presence could be a rising issue. The ability of *D. typicum* to rapidly propagate and cause massive blooms is due to its ability of repeated transverse fission [40].

D. typicum is described as being friable [41], which was confirmed during field sampling, and can therefore easily break up into pieces that are still capable of stinging fish [6]. This might be a concern in saltwater semi-enclosed and closed systems, which generally pump in saltwater that may contain pieces or whole jellyfish as was described by Hosteland [74]. The jellyfish may be accompanied by pathogens, in particular some members of Flavobacteriaceae that are expected to be high in times of blooms, which could further exacerbate the situation. The introduction of pathogens into a closed system has already been noted for *Tenacibaculum* [75]. This is likely to be an issue for well-boat fish transports as well.

Conclusions

Based on the findings of this study, *D. typicum* is unlikely to be a vector for the fish pathogenic species *T. finnmarkense*. However, these jellyfish are likely to cause enough damage physically through their nematocytes to result in a route of infection for pathogenic environmental or opportunistic skin bacteria.

Smolts go through an intense transition when transferred to saltwater, linked to a shift in skin microbiota, making them more susceptible to environmental stressors; therefore, increased knowledge is needed as to when and where to transfer fish into saltwater. Possible options, such as closed post-smolt facilities using treated saltwater could be a mitigation tool for outbreaks like the ones described in this study.

Supporting information

S1 Fig. SEM micrograph of gills from a moribund fish. Secondary lamellae showing intact microridges of the epidermis. There are no pathogens or nematocysts present.
(TIF)

S2 Fig. SEM micrograph of an eye from a moribund fish with "cloudy" eyes. (A) Filamentous bacteria present on the surface of the cornea. (B) Nematocyst penetrating the cornea of the eye.
(TIF)

S3 Fig. SEM micrograph of a skin lesion from a moribund fish. Nematocysts can be seen embedded in the skin with holes (h) present that are likely the results of them being ripped out.
(TIF)

S1 Table. Jellyfish real-time RT-PCR results. Ct values of the jellyfish real-time RT-PCR analysis.
(DOCX)

S2 Table. Fish real-time RT-PCR results. Ct values of the fish real-time RT-PCR analysis.
(DOCX)

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Writing – original draft: Sverre Bang Småge, Kathleen Frisch.

Writing – review & editing: Sverre Bang Småge, Øyvind Jakobsen Brevik, Kathleen Frisch, Are Nylund.

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1 **Experimentally induced tenacibaculosis in Atlantic salmon**
2 **smolts using *Tenacibaculum finnmarkense***

3

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12

13 **Abstract**

14 Tenacibaculosis, an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins
15 and tail rot, is a significant fish welfare issue in marine aquaculture worldwide. The disease is
16 a concern to the Atlantic salmon farming industry due to mortality losses and the use of
17 antibiotics. The most commonly isolated bacterium from Northern Norwegian salmon farms
18 during tenacibaculosis outbreaks is *Tenacibaculum finnmarkense*; however it has not been
19 shown to be the causative agent. This study investigates the role of *T. finnmarkense* and closely
20 related strains as the possible agent of tenacibaculosis in smolts recently transferred into
21 seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarkense* was also
22 investigated using the developed challenge model. The results show that *T. finnmarkense* is a
23 causative agent of tenacibaculosis as it presents in Northern Norway without the need for any
24 pre-stressor or co-infection. The pathogen does not appear to spread easily from fish to fish and
25 more work is therefore required to determine possible reservoirs and/or vectors. Even though a
26 whole cell inactivated vaccine induces an antibody response, this study did not manage to show
27 any protection against tenacibaculosis induced through a bath infection. Future studies need to
28 include determining pathogen-host interactions and identifying possible antigens for vaccine
29 candidates.

30 **Introduction**

31 Tenacibaculosis is a significant fish welfare issue in many economically important species all
32 over the world (Toranzo et al., 2005; Avendaño-Herrera et al., 2006), and has been reported in
33 all Atlantic salmon, *Salmo salar* farming regions (Kent, 1992; Frelief et al., 1994; Handlinger
34 et al., 1997; Ferguson et al., 2010; Barja, 2015; Avendaño-Herrera et al., 2016; Frisch et al.,
35 2017; Småge et al., 2017). The disease is a concern to the Atlantic salmon aquaculture industry
36 due to mortality losses and the use of antibiotics (Bornø and Lie, 2015). Three species of
37 *Tenacibaculum* (Gram-negative marine bacteria found worldwide) have been associated with
38 this disease in Atlantic salmon: *Tenacibaculum finnmarkense*, *Tenacibaculum dicentrarchi* and
39 *Tenacibaculum maritimum* (Barja, 2015; Avendaño-Herrera et al., 2016; Grothusen et al., 2016;
40 Frisch et al., 2017; Karlsen et al., 2017b; Småge et al., 2017; Bridel et al., 2018).
41 Tenacibaculosis is an ulcerative disease characterised by skin lesions, mouth erosion, frayed
42 fins and tail rot (Toranzo et al., 2005).

43 In Norway, *Tenacibaculum* spp. like bacteria have been identified histologically from skin
44 ulcers since the late 80s (Olsen et al., 2011); however the bacteria were not identified during
45 routine diagnostics due to the use of agars that do not support *Tenacibaculum* growth. The
46 increased use of marine agar, Difco, 2216 (MA) in recent years has resulted in the recovery and
47 identification of *Tenacibaculum* spp. from outbreaks of ulcerative disease (Bornø and Sviland,
48 2011; Takle et al., 2015; Småge et al., 2017). Although *Tenacibaculum* isolates recovered from
49 tenacibaculosis outbreaks in Norway are closely related to *T. finnmarkense* and *T. dicentrarchi*
50 (Habib et al., 2014; Småge et al., 2015; Karlsen et al., 2017b; Olsen et al., 2017; Småge et al.,
51 2017; Bridel et al., 2018), novel species are likely to exist among the recovered isolates due to
52 their large genetic variation found using multilocus sequence analysis (Olsen et al., 2017).

53 Outbreaks of tenacibaculosis in Northern Norwegian salmon farms has been shown to be
54 dominated by *T. finnmarkense* or closely related strains (Småge et al., 2017) and are most
55 commonly associated with mouth erosions and frayed fins (Hjeltnes et al., 2017; Småge et al.,
56 2017). Smolts in land-based saltwater recirculation facilities or ones recently transferred into
57 saltwater net-pens seem to be particularly susceptible (Bornø and Lie, 2015; Småge et al.,
58 2017). The susceptibility of salmonid smolts to *Tenacibaculum*-like bacteria has been reported
59 over several decades (Rucker, 1959; Borg, 1960; Anderson, 1969; Sawyer, 1976; Kent et al.,
60 1988; Frelief et al., 1994; Olsen et al., 2011; Bornø and Lie, 2015; Småge et al., 2017). One
61 possible explanation is the changes in skin microbiota, as well as the physiological changes
62 which occur when smolts are transferred from freshwater to saltwater (Lokesh and Kiron,
63 2016).

64 Historically, ulcerative disease in Atlantic salmon aquaculture has been associated with the
65 bacterium *Moritella viscosa*, which has been shown to cause disease on its own (Lunder et al.,
66 1995; Bruno et al., 1998). However, recent outbreaks of skin lesions, particularly in warmer
67 temperatures (above 8°C) have been associated with bacterial infections with *Tenacibaculum*
68 spp. (Småge et al., 2017). These findings are supported by the presence of *Tenacibaculum*-like
69 bacteria in historical histological samples (Olsen et al., 2011). They have been thought to be
70 secondary invaders, only causing disease with previous skin damage (e.g. scarification) or when
71 co-infected with *M. viscosa* (Olsen et al., 2011).

72 This study investigates the role of *T. finnmarkense* and closely related strains in causing
73 tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell
74 inactivated vaccines using *T. finnmarkense* was also investigated.

75 **Materials and Methods**

76 **Real-time RT-PCR assay development**

77 Two different real-time RT-PCR assays were developed during this study due to the lack of
78 published assays for *Tenacibaculum* spp. commonly found in Norway (Tb_tuf: forward
79 AGTGTGACGTCCACCTT, reverse CTGTAAGCCAGGTTCTGT, probe
80 TTTCAATACATACACCTCAGC (Småge et al., 2017) and Tb_rpoB: forward
81 GGAGCAAACATTGACCAAATT, reverse GGTATGTCCGTAACGTGGAA, probe
82 TCCTGCTTGATCAGTTAAAGCGT). The specificity of each of these assays was determined
83 by testing each one against *Vibrio splendidus*, *M. viscosa*, *Flavobacterium* spp., *Aliivibrio* spp.,
84 *Tenacibaculum ovolyticum*, *Tenacibaculum soleae*, *T. maritimum*, *T. dicentrarchi*, and
85 *Tenacibaculum* spp. Norwegian field isolates from Småge et al. (2015). The efficacy of each
86 assay was also determined.

87 **Fish husbandry**

88 All fish experiments presented in this paper were conducted at the Aquatic and Industrial
89 Laboratory (ILAB), Bergen, Norway, using Atlantic salmon (*Salmo salar* L.) smolts provided
90 by ILAB. All fish were confirmed negative by ILAB for the presence of known pathogens of
91 Atlantic salmon (heart screened for *Piscine orthoreovirus* and *Piscine myocarditis virus*; kidney
92 screened for *Infectious pancreatic necrosis virus*; gill screened for *Infectious salmon anaemia*
93 *virus*). Parent fish were screened and found negative for salmonid alpha virus. The skin was
94 screened prior to transfer to saltwater with real-time RT-PCR for the presence of
95 *Tenacibaculum* spp., *M. viscosa*, *F. psychrophilum*, and *Yersinia ruckerii*.

96 For the duration of each experiment, the photoperiod was 12 hours (i.e. 12 hours light : 12 hours
97 dark). Water flow was 300 L per hour per tank and the outlet water in all tanks had a minimum
98 of 77 % oxygen saturation. Fish were fed *ad libitum* with the commercial dry feed Nutra

99 Olympic (Skretting) and were checked a minimum two times a day on weekdays and once a
100 day during weekends. Fish were starved for 48 hours prior to any handling (e.g. transfer,
101 vaccination).

102 All fish showing signs of disease and/or behavioural changes during the experiments were
103 removed from the tanks and euthanized. However, due to the rapidity of the disease, this was
104 not always feasible. For the purpose of this study, the word mortality includes both fish that
105 were euthanized due to animal welfare and fish found dead in the tank. All fish taken out during
106 or at the termination of the experiments were euthanized with either an overdose of tricaine
107 methanesulphonate, Tricaine PHARMAQ (TMS) or a swift blow to the head. The animal
108 experiments were approved by the National Animal Research Committee (now governed by
109 the Norwegian Food Safety Authority, Mattilsynet) (Experiment ID: 2015/237969,
110 2015/30245).

111 **Challenge material**

112 The *T. finnmarkense* strains used in this study (strain HFJ^T and strain Tsp.2) were recovered
113 from tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon farms (Småge et al.,
114 2015). The *T. dicentrarchi* strain 35/09^T used in this study was obtained from NCIMB. It was
115 included as a comparison as it is the most closely related species to *T. finnmarkense* (Bridel et
116 al., 2018) and has been shown to cause disease in salmon (Avenidaño-Herrera et al., 2016). The
117 bacteria were grown on MA at 16 °C for 48 hours and stock solutions were stored at -80 °C.

118 The challenge material was produced by inoculating 1 L of Marine Broth, Difco, 2216 (MB) in
119 2 L Erlenmeyer flasks with a small amount of frozen bacterial stock solution (400 µL). These
120 were incubated between 60 and 72 hours at 16 °C and 140 rpm. Bacterial counts were calculated
121 using the most probable number (MPN) method (Cochran, 1950; Blodgett, 2010) with 10 fold
122 dilutions in duplicate using 8 replicates per dilution. The average MPN of the duplicate was
123 used as the culture bacterial concentration to calculate the bath concentration retrospectively.

124 The amount of challenge material required for the target bath concentration was based on
125 previous experiments (data not shown).

126 **Cohabitation experiment**

127 Eight groups of 50 fish with an average weight of 55 g (20 shedders and 30 cohabitants) were
128 used for this experiment (Table 1). The smolts were transferred to 4°C saltwater (34 ppt) and
129 acclimatized over six weeks to these water conditions. The temperature was chosen based on
130 the seawater temperature during the initial tenacibaculosis outbreak from which *T.*
131 *finnmarkense* strain HFJ^T was isolated. Groups 1 and 2 were exposed to *T. finnmarkense* strain
132 HFJ^T, groups 3 and 4 to *T. finnmarkense* strain Tsp.2, groups 5 and 6 to *T. dicentrarchi* strain
133 35/09^T, and groups 7 and 8 were controls, bath exposed with MB (Table 1). Each group pairs
134 were treated as duplicates. Prior to being bath challenged, the shedder fish were anaesthetised
135 with TMS and adipose fin clipped for marking purposes, and then transferred into the 150 L
136 challenge tanks. For the bath infection, the water level in each tank was lowered to a volume of
137 60 L and flow was shut off. The challenge material was added directly to each tank. After 5
138 hours, water flow was turned back on. The temperature was kept between 4 and 6 °C during
139 this time. Oxygen was provided through diffusers, and oxygen saturation and fish behaviour
140 was monitored every hour. The bath concentrations used for each isolate (Table 1) was based
141 on pilot studies (data not shown). Cohabitants were added to each tank 24 hours after the
142 shedders were challenged. The experiment was terminated after six weeks.

143 **Fish sampling**

144 Samples from the margins of mouth and skin lesions were streaked onto MA. The plates were
145 incubated at 16 °C for a minimum of 72 hours. Colonies that matched the morphological
146 characteristics of *Tenacibaculum* (pale yellow pigmented round colonies with long, thin rods
147 shaped cells) were sub-cultured onto MA and incubated for 48 hours at 16 °C. A minimum of

148 two cultures per group were cryopreserved at -80 °C. The identification of the recovered
149 bacteria was performed as described in Småge et al. (2015) using the housekeeping gene, *atpD*.
150 The recovered sequences were compared to the challenge material using AlignX in Vector NTI,
151 Invitrogen. Tissues from representative diseased individuals were sampled for histopathology
152 as described in Småge et al. (2016).

153 To determine the potential presence of bacteria in external and internal tissues of non-diseased
154 cohabitants, samples of gills, heart, kidney and skin under the jaw were collected. Five fish per
155 group (including the control groups) were randomly sampled four weeks and six weeks post-
156 transfer into the shedder tanks. Cultured *Halobacterium salinarum* DSM 3754^T cells suspended
157 in PBS were added to each sample (5 µl) prior to RNA extraction as an exogenous control in
158 the real-time RT-PCR analysis (Andersen et al., 2010). The RNA was extracted following the
159 manufacturer's protocol (5 Prime), except that a second washing step using 100% ethanol was
160 performed prior to air drying of the RNA pellet. The obtained RNA was stored at -20 °C.

161 The extracted RNA was tested for the presence of *Tenacibaculum* spp. using the Tb_rpoB real-
162 time RT-PCR assay, an assay targeting the exogenous control (*H. salinarum*) (Andersen et al.,
163 2010), and an assay targeting the elongation factor 1 alpha (EF1A) (Olsvik et al., 2005). All
164 assays were run using an AgPath-ID kit (Thermo Scientific) with 2 µl of RNA and the standard
165 AgPath-ID concentrations of primers (400 nM) and probe (120nM). Each run consisted of 45
166 cycles.

167 **Vaccination experiment**

168 Two monovalent oil adjuvanted vaccines were produced: *T. finnmarkense* HFJ^T with high
169 antigen content (1X) and *T. finnmarkense* HFJ^T with low antigen content (0.06X). The vaccine
170 with the high antigen content contained 17 times more bacterin than the vaccine with low
171 antigen content. The bacterin was prepared by growing *T. finnmarkense* HFJ^T in marine broth
172 using a 2 L fermentor (with 1.5 L growth medium, inoculated with 2 % pre-culture) for 24 hours

173 at 15 °C. Purity of the bacterial culture was verified by growing the bacteria for an extended
174 period of time on several different suitable agars. The culture was inactivated with 0.4 %
175 formaldehyde, and concentrated approximately 8 times by centrifugation. It was then
176 formulated into two monovalent oil adjuvanted vaccines (with either 1X or 0.06X bacterin
177 concentration) using mineral oil, and prepared by a Silverson LR5 rotor-stator mixer according
178 to standard procedures for PHARMAQ vaccines.

179 Two groups of 160 parr of an average weight of 26 g were intraperitoneally (IP) vaccinated
180 with a 0.1 ml dose of one of the two formulated vaccines, and a third group of 160 parr were IP
181 vaccinated with 0.1 ml PBS. For this procedure, the fish were anaesthetized using TMS and
182 marked by adipose fin clipping or maxilla trimming to identify each group. The vaccinated fish
183 were kept in freshwater in a 500 L tank.

184 After 8 weeks under these conditions, the fish were triggered to smoltify by increasing their
185 photoperiod to 24 hours. At 12 weeks post-immunisation (approximately 1000 degree days),
186 the smolts were anaesthetized with TMS, sorted into their respective groups (Table 2) and
187 transferred into saltwater in eight 150 L tanks. The salinity was increased gradually over the
188 first 24 hour period and the temperature was decreased to 4 °C over the following 2 weeks.
189 Tank conditions were the same as what was used for the cohabitation experiment. The challenge
190 material was produced as described above with the isolates and concentrations described in
191 Table 2.

192 At 0 (unvaccinated fish), 8 and 12 weeks (approximately 675 and 1000 degree days) post-
193 vaccination, 10 fish were euthanized with an overdose of TMS and blood sampled from the
194 caudal vein. The blood samples were centrifuged and the blood plasma stored at -20 °C for
195 subsequent ELISA analysis.

196 **ELISA**

197 Microtiterplates (Maxisorp™, Nunc) prepared with 5 µg ml⁻¹ Poly-L-lysine (Sigma) were
198 coated by adding 100 µl of inactivated bacteria, resulting in an OD_{600nm} of about 0.05. After
199 washing with PBS containing 0.05 % Tween-20 (Merck) (PBST), the plates were blocked at
200 room temperature for two hours with 5 % skimmed milk in PBST. The plasma samples were
201 diluted 2-fold, starting with a dilution of 1:50 in PBST with 1 % skimmed milk, and 100 µl was
202 added to each well before incubated at 4 °C overnight. A monoclonal antibody mouse anti-
203 Rainbow Trout Immunoglobulin (cross-reacting with Atlantic salmon IgM-A, produced in-
204 house) (Thuvander et al., 1990) was diluted 1:3500 in PBST with 1 % skimmed milk. 100 µl
205 was added to each well and the plates were incubated one hour at room temperature. The
206 secondary antibody rabbit anti-Mouse Immunoglobulin conjugated to alkaline phosphatase
207 (Dako) was diluted 1:500 in PBST with 1 % skimmed milk, and 100 µl was added to each well
208 followed by one hour incubation at room temperature. The plates were washed three times with
209 PBST between the incubations. Bound antibodies were detected by adding 100 µl substrate p-
210 nitrophenyl-phosphate (Sigma) in 10 % diethanolamine buffer, pH 9.8 (Sigma-Aldrich) to each
211 well and the color reaction was read at OD₄₀₅ after 50 minutes.

212 **Results**

213 **Real-time RT-PCR assays**

214 The Tb_rpoB assay was found to detect the common *Tenacibaculum* spp. strains isolated from
215 diseased salmon in Northern Norway. It also detects *Tenacibaculum soleae*. The Tb_tuf assay
216 was found to be less specific and can detect a wider range of *Tenacibaculum* spp. including
217 *Tenacibaculum ovolyticum* and *T. soleae*. Neither of these assays detects *Moritella*, *Aliivibrio*
218 and *Flavobacterium*. The efficacy of Tb_tuf and Tb_rpoB was 1.9397 and 1.9679 respectively.

219 **Cohabitation experiment**

220 Accumulated mortality during the cohabitation experiments is shown in Table 1 and Fig 1.

221 Neither *T. finnmarkense* strain Tsp.2 nor *T. dicentrarchi* strain 35/09^T caused an accumulated
222 mortality in the shedders or the cohabitants that was greater than the control groups and very
223 few showed clinical signs of tenacibaculosis. However, the bacteria that were recovered
224 matched the challenge material. *Tenacibaculum* isolates were also recovered from the diseased
225 control shedders and cohabitants, but these were different from the ones used in the experiment
226 suggesting contamination from the water source. *T. finnmarkense* strain HFJ^T caused 80 %
227 accumulated mortality in the shedders of both groups, but very low cohabitant mortality (Fig
228 1).

229 Diseased fish had classical signs of tenacibaculosis (Fig 2): mouth erosion, frayed fins, and skin
230 lesions. The smolts that died within the first few days had less clinical signs than the later ones;
231 however all mortality showed some changes, in particular frayed fins. Histopathology of mouth
232 and skin lesions showed large infiltrations of long thin rod-shaped bacteria within the dermis
233 (Fig 3). The bacteria recovered from the lesions were primarily pure cultures of *Tenacibaculum*-
234 like bacteria, which were found to be genetically identical to the challenge material (*atpD*).

235 The real-time RT-PCR screening of non-diseased cohabitants revealed that the skin and gills
236 (except one heart tissue from a control fish) were the only tissues positive for *Tenacibaculum*
237 spp. with the Tb_rpoB assay (0-40 % of screened fish). The Ct-values were high indicating low
238 levels of *Tenacibaculum* (Supplementary Data 1). None of the sampled cohabitants showed
239 clinical signs of tenacibaculosis, which may explain the lack or low presence of bacteria in the
240 sampled tissues.

241 **Vaccine challenge**

242 Accumulated mortality for each group in the vaccine challenge is presented in Table 2. There
243 were no differences in mortalities between vaccinated and control fish in any of the groups,
244 neither towards the homologous strain *T. finnmarkense* strain HFJ^T nor towards the
245 heterologous strain *T. finnmarkense* strain Tsp.2. Mortality in the control groups exposed to *T.*
246 *finnmarkense* strain HFJ^T was high, ranging from 90 % to 100 %, whereas the groups exposed
247 to *T. finnmarkense* strain Tsp.2 ranged from 30 % to 65 %.

248 **ELISA**

249 Results from the ELISA analysis are shown in Fig 4. The data are presented as antibody
250 response, visualizing the degree of binding of specific antibodies in the plasma towards the
251 homologous *T. finnmarkense* strain HFJ^T. Fish vaccinated with the high bacterial antigen
252 content displayed a higher antibody response than fish vaccinated with the vaccine containing
253 a lower content of bacterial antigen.

254 **Discussion**

255 This study is the first report that shows that both *T. finnmarkense* strains used in this study
256 induce tenacibaculosis through a bath infection of Atlantic salmon smolts without any pre-
257 stressor or co-infection. This is in contradiction to what has previously been reported in bath
258 challenge studies using Norwegian *Tenacibaculum* spp. (Olsen et al., 2011). One possible
259 reason for this is the fact that there are differences in pathogenicity as shown by the differences
260 in mortality rates between *T. finnmarkense* strain HFJ^T and *T. finnmarkense* strain Tsp.2. Olsen
261 et al. (2011) may have been testing strains that are not as pathogenic as *T. finnmarkense* strain
262 HFJ^T. Several studies have shown that *Tenacibaculum* spp. likely constitutes a part of the
263 healthy microbiota of farmed Atlantic salmon (Karlsen et al., 2017b; Llewellyn et al., 2017);
264 therefore an isolate recovered from a fish may not result in disease in challenge studies as the

265 majority of these are likely not pathogenic. Other factors that would explain the differences in
266 results between studies include the size of the fish, the smoltification status, and environmental
267 conditions, which include water temperature and salinity.

268 The general consensus is that smolts become less susceptible to pathogens the longer they have
269 been exposed to saltwater. In a recent study by Småge et al. (2017), smolts that had been in the
270 sea longer appeared to be less susceptible to tenacibaculosis. However, fish that were exposed
271 to *T. finnmarkense* strain HFJ^T in this study caused disease even after six weeks of saltwater
272 adaptation.

273 This study is not the first report of a non-*T. maritimum* species causing tenacibaculosis in
274 Atlantic salmon. In a study by Avendaño-Herrera et al. (2016) an isolate closely related to *T.*
275 *dicentrarchi* strain 35/09^T recovered from diseased Atlantic salmon in Chile was shown to
276 induce ulcerative disease in both Atlantic salmon and rainbow trout, *Oncorhynchus mykiss*. In
277 the current study, the *T. dicentrarchi* strain 35/09^T only caused disease in a low level of smolts,
278 indicating that *T. dicentrarchi* strain 35/09^T is less pathogenic than *T. finnmarkense* strain HFJ^T
279 to Atlantic salmon smolts. In Norway, *T. dicentrarchi* is mainly recovered from diseased
280 cleaner fish and asymptomatic Atlantic salmon (Olsen et al., 2017), which may indicate that
281 this species may be more pathogenic to non-salmonid fishes.

282 The diseased smolts in these experiment presented similar clinical signs to what is seen during
283 natural tenacibaculosis outbreaks in the field (Fig 2), and seem to follow the same timeline with
284 most of the mortality occurring one to three weeks post-transfer into saltwater (Kent, 1992;
285 Småge et al., 2017). The authors chose to conduct the experiments in 4 °C saltwater as this was
286 the seawater temperature during the initial outbreak from which *T. finnmarkense* strain HFJ^T
287 was isolated. The low temperature may have contributed to the results, in particular the vaccine
288 challenge as metabolic rate would have been lower and therefore the immune system slower
289 (Abram et al., 2017). Tenacibaculosis outbreaks in Northern Norway have occurred at higher

290 temperatures (8 to 10 °C) with *T. finnmarkense* strain HFJ^T being the most frequently recovered
291 bacteria from the ulcers (Småge et al., 2017). More work is required to determine if temperature
292 has a significant impact on the course of the disease.

293 In the cohabitation experiment, there was low mortality in all groups including the controls,
294 with the exception of the high mortality seen in the shedders exposed to *T. finnmarkense* strain
295 HFJ^T (Fig 1), suggesting that there was some other stressor present. One possibility is the low
296 temperature used. The MB that the control shedders were exposed to could have been the reason
297 why those fish were susceptible to environmental bacteria; however, some control cohabitants
298 were also affected and these were not exposed to MB. MB has been shown to be harmless to
299 Atlantic salmon in other studies in quantities much higher than the ones used in this study
300 (Olsen et al., 2011; Avendaño-Herrera et al., 2016).

301 The bacteria cause an external infection with very little to no systemic signs in most cases. The
302 tissue screening of non-diseased cohabitants indicates that the bacteria are not present internally
303 in asymptomatic fish. Tenacibaculosis is a very acute disease and bacteria may only be
304 detectable internally towards the end when the tissue damage is sufficient for the bacteria to
305 enter the blood vessels. Further studies are required to investigate this. The lack of bacterial
306 transfer from diseased fish to naïve ones seen in this study is also seen with *F. psychrophilum*,
307 a species from the same family (Madsen and Dalsgaard, 1999).

308 Vaccination with *T. finnmarkense* strain HFJ^T did not reduce mortality rates when challenged
309 with the homologous strain. These results may not be surprising considering that
310 *Tenacibaculum* spp. seem to induce little or no inflammatory response as seen in the
311 histopathology (Fig 3) and previously noted for the disease (Kent, 1992). The rapidity of the
312 infection may also contribute to the lack of protection, as the immune system may not have had
313 enough time to respond. The immunization period of 1000 degree days was likely sufficient as
314 the common protection period for commercial vaccines is 400 degree days and a vaccine against

315 *M. viscosa* has been shown to induce protection after 600 degree days (Karlsen et al., 2017b).
316 This study showed a good antibody response to the high antigen vaccine, further supporting a
317 sufficient immunization period, which would indicate that this type of vaccine may not work
318 for this bacterium. Although the bath challenge model represents the natural route of infection,
319 it is not the ideal way to test vaccine efficacy as the fish are exposed to one high dose of bacteria
320 which may not reflect the natural infection pressure as suggested by Karlsen et al. (2017a).
321 Tenacibaculosis could become a larger problem than it is with the increased use of smolt
322 facilities that pump in saltwater, which is unlikely to be completely sterile. This has the potential
323 of introducing the bacteria into the system as was seen in this study, where the controls of the
324 cohabitation experiment were infected with what are likely strains of *Tenacibaculum* spp. from
325 the water source.

326 **Conclusion**

327 This study shows that *T. finnmarkense* strain HFJ^T is a causative agent of tenacibaculosis as it
328 presents in Northern Norway without the need for any scarification, pre-handling or co-
329 infection. The pathogen does not appear to spread easily from fish to fish and more work is
330 therefore required to determine possible reservoirs and/or vectors. Even though a whole cell
331 inactivated vaccine induces an antibody response, it does not appear to offer protection against
332 tenacibaculosis induced through a bath infection. Future developments need to include finding
333 the right protective antigen for a vaccine candidate and investigating the pathogen-host
334 interactions.

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448

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451

452 **Table 1** Experimental groups for the cohabitation experiment showing isolate and bath bacterial
 453 concentrations used. Resulting accumulated mortality is shown. The shedder mortality in
 454 groups 1 and 2 are shown in Fig 1.

Group	Isolate	Amount added (ml) to 60 L bath	Bath concentration (cells ml ⁻¹)	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarkense</i> HFJ ^T	50	4.88 x 10 ⁵	20 shedders	80	3	10
				30 cohabitants	3.3	25	25
2	<i>T. finnmarkense</i> HFJ ^T	50	4.88 x 10 ⁵	20 shedders	80	3	18
				30 cohabitants	6.6	8	8
3	<i>T. finnmarkense</i> Tsp.2	200	9.17 x 10 ⁵	20 shedders	10	8	26
				30 cohabitants	16.6	10	35
4	<i>T. finnmarkense</i> Tsp.2	200	9.17 x 10 ⁵	20 shedders	15	5	12
				30 cohabitants	20	9	36
5	<i>T. dicentrarchi</i> 35/09 ^T	200	2.42 x 10 ⁶	20 shedders	20	5	32
				30 cohabitants	6.6	19	28
6	<i>T. dicentrarchi</i> 35/09 ^T	200	2.42 x 10 ⁶	20 shedders	25	3	41
				30 cohabitants	6.6	8	27
7	Control – Marine Broth	200	N/A	20 shedders	15	12	32
				30 cohabitants	6.6	5	5
8	Control – Marine Broth	200	N/A	20 shedders	15	8	19
				30 cohabitants	10	8	27

455

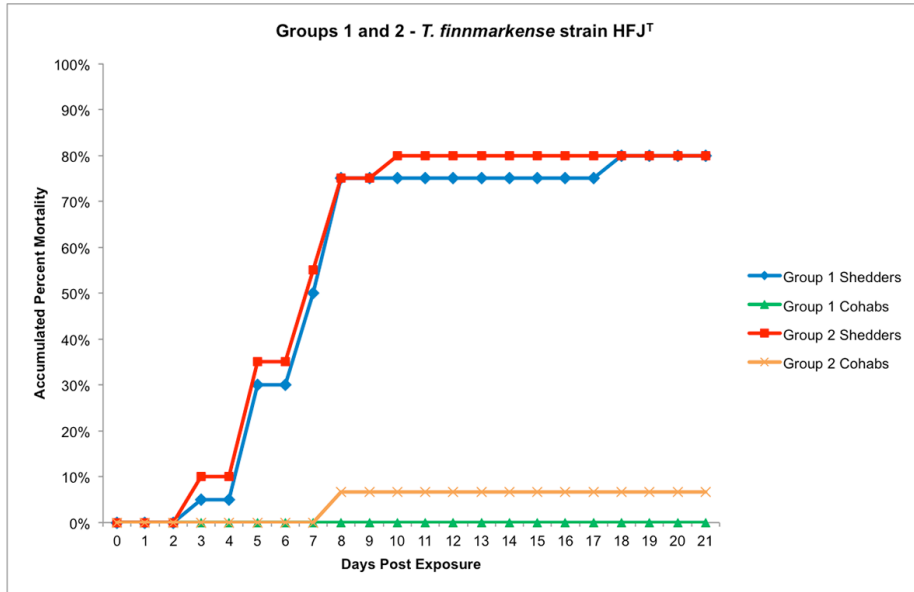
456 **Table 2** Experimental groups for the vaccine experiment indicating number of fish per vaccine
 457 in each group (A: *T. finnmarkense* strain HFJ^T (0.06X / low antigen concentration), B: *T.*
 458 *finnmarkense* strain HFJ^T (1X / high antigen concentration), C: Control (PBS)), and showing
 459 isolate and bath bacterial concentrations used. Resulting accumulated mortality is shown.

Group	Isolate	Bath concentration (cells ml ⁻¹)	Vaccine group	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarkense</i> HFJ ^T	3.5 x 10 ⁵	A	20	95	5	9
			B	20	65	5	13
			C	20	90	4	13
2	<i>T. finnmarkense</i> HFJ ^T	3.5 x 10 ⁵	A	20	95	4	8
			B	20	90	3	8
			C	20	95	4	9
3	<i>T. finnmarkense</i> HFJ ^T	7.1 x 10 ⁵	A	20	100	3	6
			B	20	100	3	7
			C	20	100	3	6
4	<i>T. finnmarkense</i> HFJ ^T	7.1 x 10 ⁵	A	21	100	3	6
			B	20	95	3	6
			C	20	100	3	7
5	<i>T. finnmarkense</i> Tsp.2	1.6 x 10 ⁶	A	20	45	6	11
			B	20	25	5	10
			C	20	30	4	9
6	<i>T. finnmarkense</i> Tsp.2	1.6 x 10 ⁶	A	20	60	6	8
			B	20	50	4	10
			C	20	45	6	12
7	<i>T. finnmarkense</i> Tsp.2	3.3 x 10 ⁶	A	20	50	5	10
			B	20	40	4	9
			C	20	35	5	9
8	<i>T. finnmarkense</i> Tsp.2	3.3 x 10 ⁶	A	19	84	4	8
			B	20	75	3	8
			C	20	65	3	8

461 **Figure 1** Accumulated percent mortality for groups 1 and 2 from the cohabitation experiment.

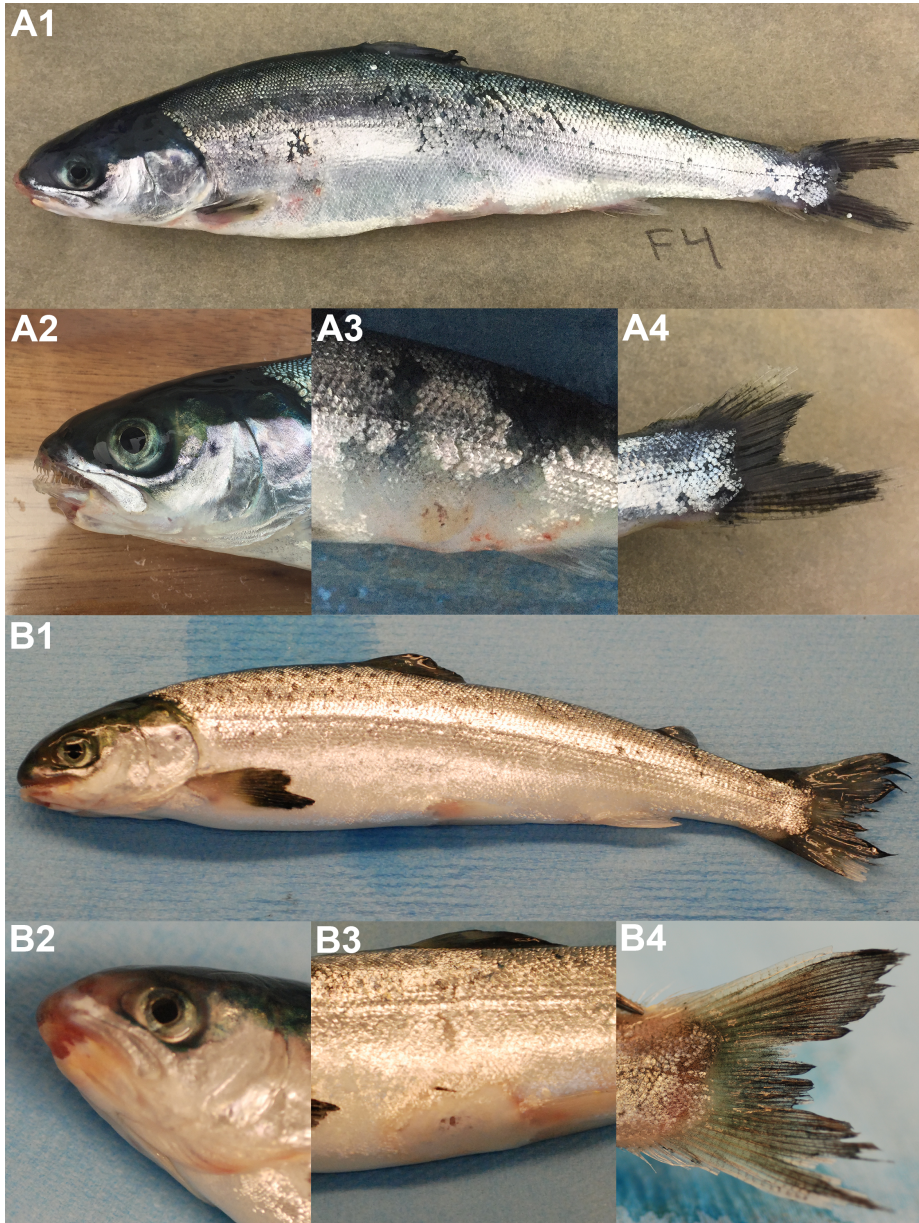
462 The shedders were bath infected with *T. finnmarkense* strain HFJ^T at a bath concentration of 4.9

463 x 10⁵ cells ml⁻¹.

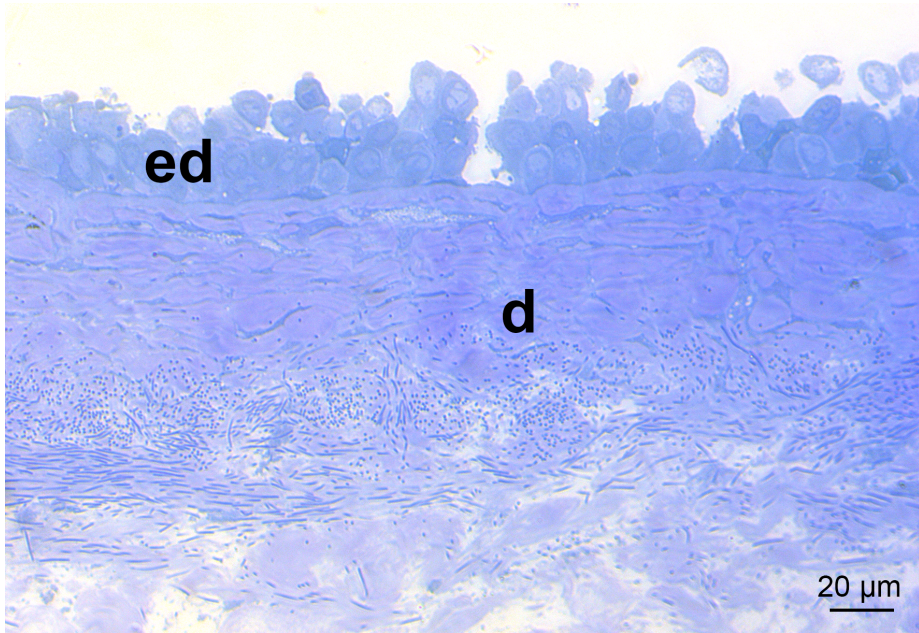


464

465 **Figure 2** A1: Diseased smolt from a natural tenacibaculosis outbreak at a Northern Norwegian
466 salmon farm. B1: Diseased smolt from experimentally induced tenacibaculosis with *T.*
467 *finnmarkense* strain HFJ^T. Both fish show the classical signs of mouth erosion (A2, B2), skin
468 lesions (A3, B3), and frayed fins (A4, B4) seen in tenacibaculosis.

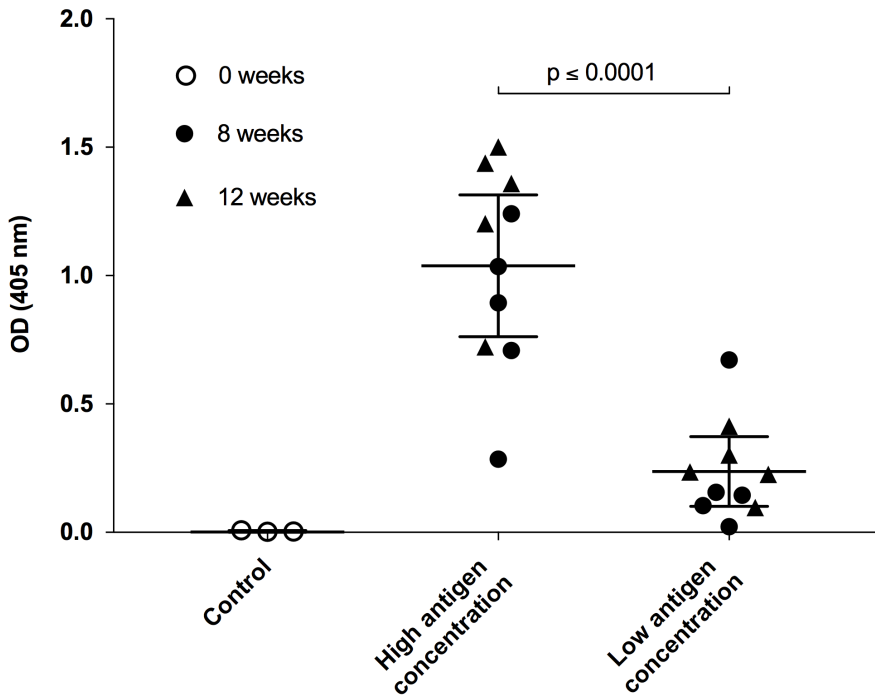


470 **Figure 3** Histopathology of a mouth lesion from a smolt experimentally infected with *T.*
471 *finnmarkense* strain HFJ^T. The epidermis (ed) is partially destroyed and an invasion of the
472 dermis (d) with *Tenacibaculum*-like bacteria can be seen. There appears to be no associated
473 host immune response. (Stained with Toluidine blue)



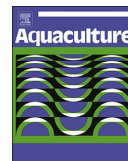
474

475 **Figure 4** Antibody response in plasma from fish vaccinated with high antigen concentration of
476 *T. finnmarkense* strain HFJ^T (1X) and low antigen concentration (0.06X) of *T. finnmarkense*
477 strain HFJ^T at 8 and 12 weeks post-vaccination, plotted as the OD (405 nm) value after 50
478 minutes incubation with substrate at the 1:100 dilution (blank, PBST, subtracted). The control
479 is plasma sampled before vaccination (1:100 dilution). Statistical analyses were performed by
480 use of GraphPad Prism 7. Antibody responses were analyzed using non-parametric Kruskal-
481 Wallis one-way analysis of variance (ANOVA) test. Error bars indicate 95% confidence
482 intervals (CI).





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Induction of tenacibaculosis in Atlantic salmon smolts using *Tenacibaculum finnmarkense* and the evaluation of a whole cell inactivated vaccine



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ABSTRACT

Tenacibaculosis, an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins and tail rot, is a significant fish welfare issue in marine aquaculture worldwide. The disease is a concern to the Atlantic salmon farming industry due to mortality losses and the use of antibiotics. The most commonly isolated bacterium from Northern Norwegian salmon farms during tenacibaculosis outbreaks is *Tenacibaculum finnmarkense*; however it has not been shown to be the causative agent. This study investigates the role of *T. finnmarkense* and closely related strains as the possible agent of tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarkense* was also investigated using the developed challenge model. The results show that *T. finnmarkense* strain HFJ^T and strain Tsp.2 are causative agents of tenacibaculosis as it presents in Northern Norway without the need for any pre-stressor or co-infection, and that different strains vary in pathogenicity. The pathogen does not appear to spread easily from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors. Even though a whole cell inactivated vaccine using *T. finnmarkense* strain HFJ^T induces an antibody response, this study did not manage to show any protection against tenacibaculosis induced through a bath infection. Future studies need to include determining pathogen-host interactions and identifying possible antigens for vaccine candidates.

1. Introduction

Tenacibaculosis is a significant fish welfare issue in many economically important species all over the world (Toranzo et al. 2005; Avendaño-Herrera et al., 2006), and has been reported in all Atlantic salmon, *Salmo salar* farming regions (Kent 1992; Frelief et al. 1994; Handlinger et al. 1997; Ferguson et al. 2010; Barja 2015; Avendaño-Herrera et al. 2016; Frisch et al. 2017; Småge et al. 2017). The disease is a concern to the Atlantic salmon aquaculture industry due to mortality losses and the use of antibiotics (Bornø and Lie 2015). Three species of *Tenacibaculum* (Gram-negative marine bacteria found worldwide) have been associated with this disease in Atlantic salmon: *Tenacibaculum finnmarkense*, *Tenacibaculum dicentrarchi* and *Tenacibaculum maritimum* (Barja 2015; Avendaño-Herrera et al. 2016; Grothusen et al. 2016; Frisch et al. 2017; Karlsen et al. 2017b; Småge et al. 2017; Bridel et al. 2018). Tenacibaculosis is an ulcerative disease characterised by skin lesions, mouth erosion, frayed fins and tail rot (Toranzo et al. 2005).

In Norway, *Tenacibaculum* spp. like bacteria have been identified histologically from skin ulcers since the late 80s (Olsen et al. 2011); however the bacteria were not identified during routine diagnostics due to the use of agars that do not support *Tenacibaculum* growth. The increased use of marine agar, Difco, 2216 (MA) in recent years has resulted in the recovery and identification of *Tenacibaculum* spp. from outbreaks of ulcerative disease (Bornø and Sviland 2011; Takle et al. 2015; Småge et al. 2017). Although *Tenacibaculum* isolates recovered from tenacibaculosis outbreaks in Norway are closely related to *T. finnmarkense* and *T. dicentrarchi* (Habib et al. 2014; Småge et al. 2015; Karlsen et al. 2017b; Olsen et al. 2017; Småge et al. 2017; Bridel et al. 2018), novel species are likely to exist among the recovered isolates due to their large genetic variation found using multilocus sequence analysis (Olsen et al. 2017).

Outbreaks of tenacibaculosis in Northern Norwegian salmon farms has been shown to be dominated by *T. finnmarkense* or closely related strains (Småge et al. 2017) and are most commonly associated with

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mouth erosions and frayed fins (Hjeltnes et al. 2017; Småge et al. 2017). Smolts in land-based saltwater recirculation facilities or ones recently transferred into saltwater net-pens seem to be particularly susceptible (Børnø and Lie 2015; Småge et al. 2017). The susceptibility of salmonid smolts to *Tenacibaculum*-like bacteria has been reported over several decades (Rucker 1959; Borg 1960; Anderson 1969; Sawyer 1976; Kent et al. 1988; Frelief et al. 1994; Olsen et al. 2011; Børnø and Lie 2015; Småge et al. 2017). One possible explanation is the changes in skin microbiota, as well as the physiological changes which occur when smolts are transferred from freshwater to saltwater (Lokesh and Kiron 2016).

Historically, ulcerative disease in Atlantic salmon aquaculture has been associated with the bacterium *Moritella viscosa*, which has been shown to cause disease on its own (Lunder et al. 1995; Bruno et al. 1998). However, recent outbreaks of skin lesions, particularly in warmer temperatures (above 8 °C) have been associated with bacterial infections with *Tenacibaculum* spp. (Småge et al. 2017). These findings are supported by the presence of *Tenacibaculum*-like bacteria in historical histological samples (Olsen et al. 2011). They have been thought to be secondary invaders, only causing disease with previous skin damage (e.g. scarification) or when co-infected with *M. viscosa* (Olsen et al. 2011).

This study investigates the role of *T. finnmarensis* and closely related strains in causing tenacibaculosis in smolts recently transferred into seawater. The efficacy of whole cell inactivated vaccines using *T. finnmarensis* was also investigated.

2. Materials and methods

2.1. Fish husbandry

All fish experiments presented in this paper were conducted at the Aquatic and Industrial Laboratory (ILAB), Bergen, Norway, using Atlantic salmon smolts provided by ILAB. All fish were confirmed negative by ILAB for the presence of known pathogens of Atlantic salmon (heart screened for *Piscine orthoreovirus* and *Piscine myocarditis virus*; kidney screened for *Infectious pancreatic necrosis virus*; gill screened for *Infectious salmon anaemia virus*). Parent fish were screened and found negative for salmonid alpha virus. The skin was screened prior to transfer to saltwater with real-time RT-PCR and found negative for the presence of *Tenacibaculum* spp., *M. viscosa*, *F. psychrophilum*, and *Yersinia ruckeri*.

For the duration of each experiment, the photoperiod was 12 h (i.e. 12 h light: 12 h dark). Water flow was 300 L per hour per tank and the outlet water in all tanks had a minimum of 77% oxygen saturation. Fish were fed ad libitum with the commercial dry feed Nutra Olympic (Skretting) and were checked a minimum two times a day on weekdays and once a day during weekends. Fish were starved for 48 h prior to any handling (e.g. transfer, vaccination).

All fish showing signs of disease and/or behavioural changes during the experiments were removed from the tanks and euthanized. However, due to the rapidity of the disease, this was not always feasible. For the purpose of this study, the word mortality includes both fish that were euthanized due to animal welfare and fish found dead in the tanks. All fish taken out during or at the termination of the experiments were euthanized with either an overdose of tricaine methanesulphonate, Tricaine PHARMAQ (TMS) or a swift blow to the head. The animal experiments were approved by the National Animal Research Committee (now governed by the Norwegian Food Safety Authority, Mattilsynet) (Experiment ID: 2015/237969, 2015/30245).

2.2. Real-time RT-PCR assay development for *Tenacibaculum* spp.

Two different real-time RT-PCR assays were developed during this study due to the lack of published assays for *Tenacibaculum* spp. commonly found in Norway. These were used to screen fish tissues prior to and during the fish experiments. One of the assays targets the

housekeeping (HK) gene, *tuf* (Tb.tuf: forward AGTGTGACGTCCACCTT, reverse CTGTAAGCCAGGTTCTGT, probe TTTCAATACATACACCTC AGC (Småge et al. 2017)), and the other targets another HK gene, *tpoB* (Tb.tpoB: forward GGAGCAACATTGACCAAATT, reverse GGATATGTC CGTAACGTGGAA, probe TCCTGCTTGATCAGTTAAAGCGT). The specificity of both assays was determined by testing each one against RNA extracted from pure cultures of *Vibrio splendidus*, *M. viscosa*, *Flavobacterium* spp., *Aliivibrio* spp., *Tenacibaculum ovolyticum*, *Tenacibaculum soleae*, *T. maritimum*, *T. dicentrarchi*, and *Tenacibaculum* spp. Norwegian field isolates from Småge et al. (2015). After the concentration of primer and probe was optimized, the efficacy of each assay was determined using a six point 10-fold dilution in triplicate of RNA extracted from a pure culture of *T. finnmarensis* strain HFJ^T. The RNA was extracted following the manufacturer's protocol (5 Prime), except that a second washing step using 100% ethanol was performed prior to air drying of the RNA pellet. The obtained RNA was stored at –80 °C. All assays were run using an AgPath-ID kit (Thermo Scientific) with 2 µL of RNA and the standard AgPath-ID concentrations of primers (400 nM) and probe (120 nM), as these were the optimal concentrations. Each run consisted of 45 cycles.

2.3. Challenge material

The *T. finnmarensis* strains used in this study (strain HFJ^T and strain Tsp.2) were recovered from tenacibaculosis outbreaks in Northern Norwegian Atlantic salmon farms (Småge et al. 2015). The *T. dicentrarchi* strain 35/09^T used in this study was obtained from NCIMB. It was included as a comparison as it is the most closely related species to *T. finnmarensis* (Bridel et al. 2018), is commonly found in Norway (Småge et al. 2015; Olsen et al. 2017; Småge et al. 2017), and has been shown to cause disease in salmon in Chile (Avendaño-Herrera et al. 2016). The bacteria were grown on MA at 16 °C for 48 h and stock solutions were stored at –80 °C.

The challenge material was produced by inoculating 1 L of Marine Broth, Difco, 2216 (MB) in 2 L Erlenmeyer flasks with a small amount of frozen bacterial stock solution (400 µL). These were incubated between 60 and 72 h at 16 °C and 140 rpm. Bacterial counts were calculated using the most probable number (MPN) method (Cochran 1950; Blodgett 2010) with 10 fold dilutions in duplicate using 8 replicates per dilution. The average MPN of the duplicate was used as the culture bacterial concentration to calculate the bath concentrations retrospectively. The amount of challenge material required for the target bath concentration was based on previous experiments (data not shown).

2.4. Cohabitation experiment

Eight groups of 50 fish with an average weight of 55 g (20 shedders and 30 cohabitants) were used for this experiment (Table 1). The smolts were transferred to 4 °C saltwater (34 ppt) and acclimatized over six weeks to these water conditions. The temperature was chosen based on the seawater temperature during the initial tenacibaculosis outbreak from which *T. finnmarensis* strain HFJ^T was isolated. Groups 1 and 2 were exposed to *T. finnmarensis* strain HFJ^T, groups 3 and 4 to *T. finnmarensis* strain Tsp.2, groups 5 and 6 to *T. dicentrarchi* strain 35/09^T, and groups 7 and 8 were controls, bath exposed with MB (Table 1). Each group pairs were treated as duplicates. Prior to being bath challenged, the shedder fish were anaesthetised with TMS and adipose fin clipped for marking purposes, and then transferred into the 150 L challenge tanks. For the bath infection, the water level in each tank was lowered to a volume of 60 L and flow was shut off. The challenge material was added directly to each tank. After 5 h, water flow was turned back on. The temperature was kept between 4 and 6 °C during this time. Oxygen was provided through diffusers, and oxygen saturation and fish behaviour were monitored every hour. The bath concentrations used for each isolate (Table 1) was based on pilot studies (data not shown).

Table 1

Experimental groups for the cohabitation experiment showing isolate and bath bacterial concentrations used. Resulting accumulated mortality is shown. The shedder mortality in groups 1 and 2 are shown in Fig. 1.

Group	Isolate	Amount added (mL) to 60 L bath	Bath concentration (cells mL ⁻¹)	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarensis</i> HFJ ^T	50	4.88 × 10 ⁵	20 shedders	80	3	10
				30 cohabitants	3.3	25	25
2	<i>T. finnmarensis</i> HFJ ^T	50	4.88 × 10 ⁵	20 shedders	80	3	18
				30 cohabitants	6.6	8	8
3	<i>T. finnmarensis</i> Tsp.2	200	9.17 × 10 ⁵	20 shedders	10	8	26
				30 cohabitants	16.6	10	35
4	<i>T. finnmarensis</i> Tsp.2	200	9.17 × 10 ⁵	20 shedders	15	5	12
				30 cohabitants	20	9	36
5	<i>T. dicentrarchi</i> 35/09 ^T	200	2.42 × 10 ⁶	20 shedders	20	5	32
				30 cohabitants	6.6	19	28
6	<i>T. dicentrarchi</i> 35/09 ^T	200	2.42 × 10 ⁶	20 shedders	25	3	41
				30 cohabitants	6.6	8	27
7	Control – Marine Broth	200	N/A	20 shedders	15	12	32
				30 cohabitants	6.6	5	5
8	Control – Marine Broth	200	N/A	20 shedders	15	8	19
				30 cohabitants	10	8	27

Cohabitants were added to each tank 24 h after the shedders were challenged. The experiment was terminated after six weeks.

2.5. Fish sampling

Samples from the margins of mouth and skin lesions were streaked onto MA. The plates were incubated at 16 °C for a minimum of 72 h. Colonies that matched the morphological characteristics of *Tenacibaculum* (pale yellow pigmented round colonies with long, thin rods shaped cells) were sub-cultured onto MA and incubated for 48 h at 16 °C. A minimum of two cultures per group were cryopreserved at –80 °C. The identification of the recovered bacteria was performed as described in Småge et al. (2015) using the housekeeping gene, *atpD*. The recovered sequences were compared to the challenge material using AlignX in Vector NTI, Invitrogen. Tissues from representative diseased individuals were sampled for histopathology as described in Småge et al. (2016).

To determine the potential presence of bacteria in external and internal tissues of non-diseased cohabitants, samples (size of a match tip) of gills, heart, kidney and skin under the jaw were collected and kept at –20 °C. Five fish per group (including the control groups) were randomly sampled four weeks and six weeks post-transfer into the shedder tanks. Cultured *Halobacterium salinarum* DSM 3754^T cells suspended in PBS were added to each sample (5 µL) prior to RNA extraction as an exogenous control in the real-time RT-PCR analysis (Andersen et al. 2010). The RNA was extracted as previously described and the obtained RNA was stored at –20 °C.

The extracted RNA was tested for the presence of *Tenacibaculum* spp. using the developed Tb_rpoB real-time RT-PCR assay, an assay targeting the exogenous control (*H. salinarum*) (Andersen et al. 2010), and an assay targeting the elongation factor 1 alpha (EF1A) (Olsvik et al. 2005). All assays were run as previously described. A positive control was included for each run using RNA from skin tissue positive for *T. finnmarensis* that was spiked with *H. salinarum* prior to RNA extraction. Negative controls for both the RNA extraction and the real-time RT-PCR were also included.

2.6. Vaccination experiment

Two monovalent oil adjuvanted vaccines were produced: *T. finnmarensis* HFJ^T with high antigen content (1 ×) and *T. finnmarensis* HFJ^T with low antigen content (0.06 ×). The vaccine with the high antigen content contained 17 times more bacterin than the vaccine with low antigen content. The bacterin was prepared by growing *T. finnmarensis* HFJ^T in MB using a 2 L fermentor (1.5 L growth medium

inoculated with 2% pre-culture) for 24 h at 15 °C to a final OD₆₀₀ of 5.2. Purity of the bacterial culture was verified by growing the bacteria for an extended period of time on several different suitable agars. The culture was inactivated with 0.4% formaldehyde and concentrated approximately 8 times by centrifugation. It was then formulated into two monovalent oil adjuvanted vaccines (with either 1 × or 0.06 × bacterin concentration) using mineral oil and prepared by a Silverson LR5 rotor-stator mixer according to standard procedures for PHARMAQ vaccines. Sterility of the vaccines was verified by standard procedures.

Two groups of 160 parr of an average weight of 26 g were intraperitoneally (IP) vaccinated with a 0.1 mL dose of one of the two formulated vaccines, and a third group (negative control) of 160 parr were IP vaccinated with 0.1 mL PBS. For this procedure, the fish were anaesthetized using TMS and marked by adipose fin clipping or maxilla trimming to identify each group. The vaccinated fish were mixed and kept in 12 °C freshwater in 500 L tanks. Fish showed no signs of adverse reactions to the vaccine during the immunization period.

After 8 weeks under these conditions, the fish were triggered to smoltify by increasing their photoperiod to 24 h. At 12 weeks post-immunization (approximately 1000 degree days), the smolts were anaesthetized with TMS, sorted into their respective groups (Table 2) and transferred into saltwater in eight 150 L tanks. The salinity was increased gradually over the first 24-h period and the temperature was decreased to 4 °C over the following 2 weeks. Tank conditions were the same as what was used for the cohabitation experiment. The challenge material was produced as described above with the isolates and concentrations described in Table 2.

At 0 (unvaccinated fish), 8 and 12 weeks (approximately 675 and 1000 degree days) post-vaccination, 10 fish were euthanized with an overdose of TMS and blood sampled from the caudal vein. The blood samples were centrifuged and the blood plasma stored at –20 °C for subsequent ELISA analysis.

2.7. ELISA

Microtiterplates (Maxisorp™, Nunc) prepared with 5 µg mL⁻¹ Poly-L-lysine (Sigma) were coated by adding 100 µL of inactivated bacteria, resulting in an OD_{600nm} of about 0.05. After washing with PBS containing 0.05% Tween-20 (Merck) (PBST), the plates were blocked at room temperature for 2 h with 5% skimmed milk in PBST. The plasma samples were diluted 2-fold, starting with a dilution of 1:50 in PBST with 1% skimmed milk, and 100 µL was added to each well before incubated at 4 °C overnight. A monoclonal antibody mouse anti-Rainbow Trout Immunoglobulin (cross-reacting with Atlantic salmon IgM-A, produced in-house) (Thuvander et al. 1990) was diluted 1:3500 in PBST

Table 2

Experimental groups for the vaccine experiment indicating number of fish per vaccine in each group (A: *T. finnmarensis* strain HFJ^T (0.06 ×/low antigen concentration), B: *T. finnmarensis* strain HFJ^T (1 ×/high antigen concentration), C: Control (PBS)), and showing isolate and bath bacterial concentrations used. Resulting accumulated mortality is shown.

Group	Isolate	Bath concentration (cells mL ⁻¹)	Vaccine group	Number of fish	Accumulated percent mortality	Start of mortality (days post-exposure)	End of mortality (days post-exposure)
1	<i>T. finnmarensis</i> HFJ ^T	3.5 × 10 ⁵	A	20	95	5	9
			B	20	65	5	13
			C	20	90	4	13
2	<i>T. finnmarensis</i> HFJ ^T	3.5 × 10 ⁵	A	20	95	4	8
			B	20	90	3	8
			C	20	95	4	9
3	<i>T. finnmarensis</i> HFJ ^T	7.1 × 10 ⁵	A	20	100	3	6
			B	20	100	3	7
			C	20	100	3	6
4	<i>T. finnmarensis</i> HFJ ^T	7.1 × 10 ⁵	A	21	100	3	6
			B	20	95	3	6
			C	20	100	3	7
5	<i>T. finnmarensis</i> Tsp.2	1.6 × 10 ⁶	A	20	45	6	11
			B	20	25	5	10
			C	20	30	4	9
6	<i>T. finnmarensis</i> Tsp.2	1.6 × 10 ⁶	A	20	60	6	8
			B	20	50	4	10
			C	20	45	6	12
7	<i>T. finnmarensis</i> Tsp.2	3.3 × 10 ⁶	A	20	50	5	10
			B	20	40	4	9
			C	20	35	5	9
8	<i>T. finnmarensis</i> Tsp.2	3.3 × 10 ⁶	A	19	84	4	8
			B	20	75	3	8
			C	20	65	3	8

with 1% skimmed milk. 100 µL was added to each well and the plates were incubated one hour at room temperature. The secondary antibody rabbit 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 one hour 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 OD₄₀₅ after 50 min.

3. Results

3.1. Real-time RT-PCR assay development for *Tenacibaculum* spp.

The *Tb_rpoB* assay was found to detect the common *Tenacibaculum* spp. strains isolated from diseased salmon in Northern Norway. It also detects *T. soleae*. The *Tb_tuf* assay was found to be less specific and can detect a wider range of *Tenacibaculum* spp. including *T. ovolyticum* and *T. soleae*. Neither of these assays detects *M. viscosa*, *Aliivibrio* spp. and *Flavobacterium* spp. The efficacy of *Tb_tuf* and *Tb_rpoB* was 1.9397 and 1.9679 respectively based on RNA from a pure culture of *T. finnmarensis* strain HFJ^T.

3.2. Cohabitation experiment

Accumulated mortality during the cohabitation experiments is shown in Table 1 and Fig. 1. *T. finnmarensis* strain HFJ^T caused 80% accumulated mortality in the shedders of both groups, but very low cohabitant mortality (Fig. 1). Diseased fish had classical signs of tenacibaculosis (Fig. 2): mouth erosion, frayed fins, and skin lesions. The smolts that died within the first few days had less clinical signs than the later ones; however all mortality showed some changes, in particular frayed fins. Histopathology of mouth and skin lesions showed large infiltrations of long thin rod-shaped bacteria within the dermis (Fig. 3). The bacteria recovered from the lesions were primarily pure cultures of *Tenacibaculum*-like bacteria, which were found to be genetically

identical to the challenge material (*atpD* gene).

Neither *T. finnmarensis* strain Tsp.2 nor *T. dicentrarchi* strain 35/09^T caused an accumulated mortality in the shedders or the cohabitants that was greater than the control groups and very few fish showed clinical signs of tenacibaculosis. However, the bacteria that were recovered on MA and sequenced from these groups matched the challenge material. *Tenacibaculum* spp. isolates were recovered from the diseased control shedders and cohabitants, but these *Tenacibaculum* spp. were different through sequencing from the challenge material used in the experiment suggesting contamination from the water source.

The real-time RT-PCR screening of non-diseased cohabitants revealed that the skin and gills (except one heart tissue from a control fish) were the only tissues positive for *Tenacibaculum* spp. with the *Tb_rpoB* assay (0–40% of screened fish). The Ct-values were high indicating low levels of *Tenacibaculum* (Supplementary Data). None of the sampled cohabitants showed clinical signs of tenacibaculosis, which may explain the lack or low presence of bacteria in the sampled tissues.

3.3. Vaccine challenge

Accumulated mortality for each group in the vaccine challenge is presented in Table 2. There were no differences in mortalities between vaccinated and control fish in any of the groups, neither towards the homologous strain *T. finnmarensis* strain HFJ^T nor towards the heterologous strain *T. finnmarensis* strain Tsp.2. Mortality in the control groups exposed to *T. finnmarensis* strain HFJ^T was high, ranging from 90 to 100%, whereas the groups exposed to *T. finnmarensis* strain Tsp.2 ranged from 30 to 65%.

3.4. ELISA

Results from the ELISA analysis are shown in Fig. 4. The data are presented as antibody response, visualizing the degree of binding of specific antibodies in the plasma towards the homologous *T. finnmarensis* strain HFJ^T. Fish vaccinated with the high bacterial antigen content displayed a higher antibody response than fish vaccinated with the vaccine containing a lower content of bacterial antigen.

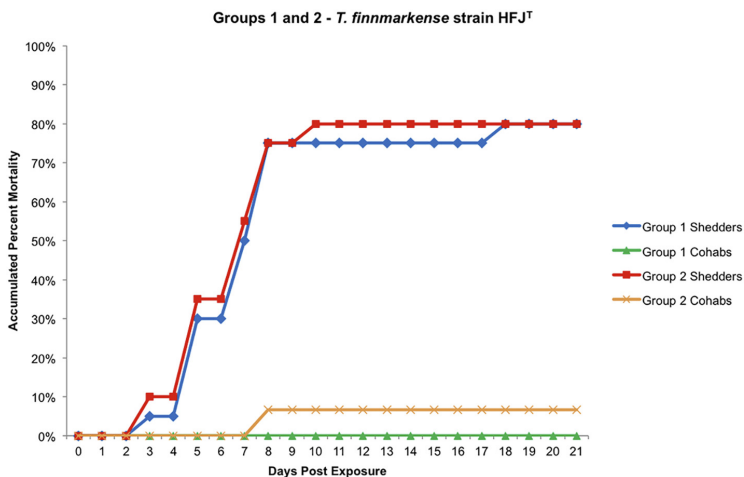


Fig. 1. Accumulated percent mortality for groups 1 and 2 from the cohobitation experiment. The shedders were bath infected with *T. finnmarkense* strain HFJ^T at a bath concentration of 4.9×10^5 cells mL⁻¹.

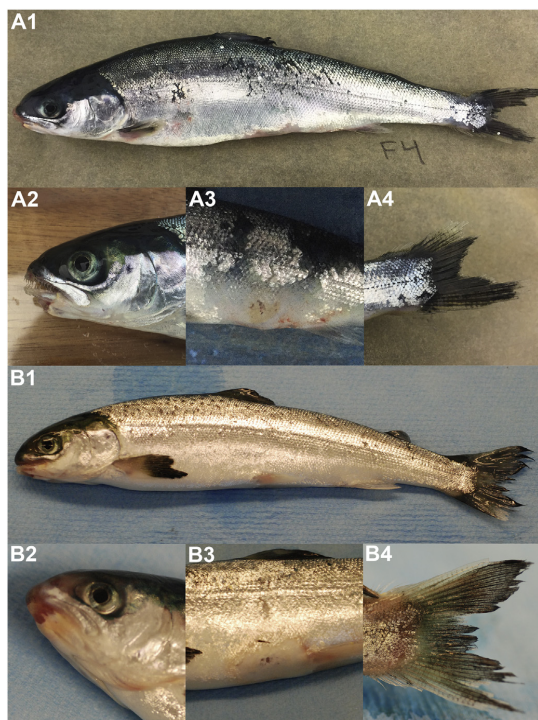


Fig. 2. A1: Diseased smolt from a natural tenacibaculosis outbreak at a Northern Norwegian salmon farm. B1: Diseased smolt from experimentally induced tenacibaculosis with *T. finnmarkense* strain HFJ^T. Both fish show the classical signs of mouth erosion (A2, B2), skin lesions (A3, B3), and frayed fins (A4, B4) seen in tenacibaculosis.

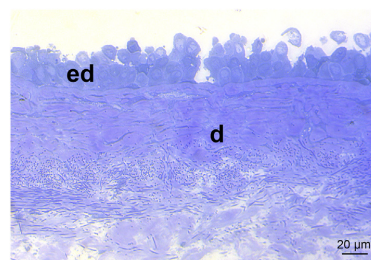


Fig. 3. Histopathology of a mouth lesion from a smolt experimentally infected with *T. finnmarkense* strain HFJ^T. The epidermis (ed) is partially destroyed and an invasion of the dermis (d) with *Tenacibaculum*-like bacteria can be seen. There appears to be no associated host immune response. (Stained with Toluidine blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

This is the first report that shows that both *T. finnmarkense* strains used in this study induce tenacibaculosis through a bath infection of Atlantic salmon smolts without any pre-stressor or co-infection. This is in contradiction to what has previously been reported in bath challenge studies using Norwegian *Tenacibaculum* spp. (Olsen et al. 2011). One possible reason for this is the fact that there are differences in pathogenicity as shown by the differences in mortality rates between *T. finnmarkense* strain HFJ^T and *T. finnmarkense* strain Tsp.2. Olsen et al. (2011) may have been testing strains that are not as pathogenic as *T. finnmarkense* strain HFJ^T. Several studies have shown that *Tenacibaculum* spp. likely constitutes a part of the healthy microbiota of farmed Atlantic salmon (Karlsen et al. 2017b; Llewellyn et al. 2017); therefore a random *Tenacibaculum* spp. isolate recovered from a fish may not result in disease in challenge studies as the majority of these are likely not pathogenic. Due to the variability in pathogenicity between strains, a bath challenge model needs to be optimized for each strain. Other factors that would explain the differences in results between studies include the size of the fish, the smoltification status, and environmental conditions, which include water temperature and salinity. The difficulties in developing an effective and reproducible challenge model are

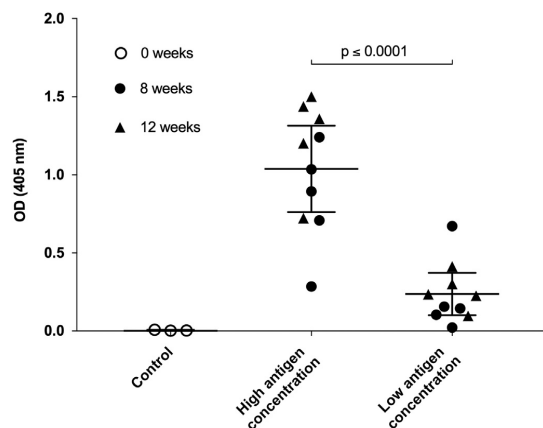


Fig. 4. Antibody response in plasma from fish vaccinated with high antigen concentration of *T. finnmarensis* strain HFJ^T (1×) and low antigen concentration (0.06×) of *T. finnmarensis* strain HFJ^T at 8 and 12 weeks post-vaccination, plotted as the OD (405 nm) value after 50 min incubation with substrate at the 1:100 dilution (blank, PBST, subtracted). The control is plasma sampled before vaccination (1:100 dilution). Statistical analyses were performed by use of GraphPad Prism 7. Antibody responses were analyzed using non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) test. Error bars indicate 95% confidence intervals (CI).

also seen with *F. psychrophilum*, a fish pathogen from the same family (Madetoja et al. 2000; Gómez et al. 2014; Long et al. 2014)

The general consensus is that smolts become less susceptible to pathogens the longer they have been exposed to saltwater. In a recent study by Småge et al. (2017), smolts that had been in the sea longer appeared to be less susceptible to tenacibaculosis. However, fish that were exposed to *T. finnmarensis* strain HFJ^T in this study caused disease even after six weeks of saltwater adaptation.

This study is not the first report of a non-*T. maritimum* species causing tenacibaculosis in Atlantic salmon. In a study by Avendaño-Herrera et al. (2016) an isolate closely related to *T. dicentrarchi* strain 35/09^T recovered from diseased Atlantic salmon in Chile was shown to induce ulcerative disease in both Atlantic salmon and rainbow trout, *Oncorhynchus mykiss*. In the current study, the *T. dicentrarchi* strain 35/09^T only caused disease in a low level of smolts, indicating that *T. dicentrarchi* strain 35/09^T is less pathogenic than *T. finnmarensis* strain HFJ^T to Atlantic salmon smolts. In Norway, *T. dicentrarchi* is mainly recovered from diseased cleaner fish and asymptomatic Atlantic salmon (Olsen et al. 2017), which may indicate that this bacterial species may be more pathogenic to non-salmonid fishes.

The diseased smolts in these experiment presented similar clinical signs to what is seen during natural tenacibaculosis outbreaks in the field (Fig. 2), and seem to follow the same timeline with most of the mortality occurring one to three weeks post-transfer into saltwater (Kent 1992; Småge et al. 2017). The authors chose to conduct the experiments in 4 °C saltwater as this was the seawater temperature during the initial outbreak from which *T. finnmarensis* strain HFJ^T was isolated. The low temperature may have contributed to the results, in particular with the vaccine challenge as metabolic rate would have been lower and therefore the immune system slower (Abram et al. 2017). Tenacibaculosis outbreaks in Northern Norway have occurred at higher temperatures (8 to 10 °C) with *T. finnmarensis* strain HFJ^T being the most frequently recovered bacteria from the ulcers (Småge et al. 2017). In this study, the bacteria were grown in 16 °C for practical reasons, and the transition from 16 to 4 °C for the bath infection may have also contributed to the results by affecting the pathogenicity. More work is required to determine if temperature of both the culturing and

bath conditions has a significant impact on the course of the disease.

In the cohabitation experiment, there was low mortality in all groups including the controls, with the exception of the high mortality seen in the shedders exposed to *T. finnmarensis* strain HFJ^T (Fig. 1), suggesting that there was some other stressor present. One possibility is the low temperature used. The MB that the control shedders were exposed to could have been the reason why those fish were susceptible to environmental bacteria; however, some control cohabitants were also affected and these were not exposed to MB. MB has been shown to be harmless to Atlantic salmon in other studies in quantities much higher than the ones used in this study (Olsen et al. 2011; Avendaño-Herrera et al. 2016).

The bacteria cause an external infection with very little to no systemic signs in most cases. The tissue screening of non-diseased cohabitants indicates that asymptomatic fish had a low prevalence of *Tenacibaculum* spp. externally and none internally. Tenacibaculosis is a very acute disease and bacteria may only be detectable internally towards the end when the tissue damage is sufficient for the bacteria to enter the blood stream. Further studies are required to investigate this. The lack of bacterial transfer from diseased fish to naïve ones seen in this study is also noted with *F. psychrophilum* (Madsen and Dalsgaard 1999).

Vaccination with *T. finnmarensis* strain HFJ^T did not reduce mortality rates when challenged with the homologous strain. These results may not be surprising considering that *Tenacibaculum* spp. seem to induce little or no inflammatory response as seen in the histopathology (Fig. 3) and previously noted for the disease (Kent 1992). The rapidity of the infection may also contribute to the lack of protection, as the immune system may not have had enough time to respond. The immunization period of 1000 degree days was likely sufficient as the common protection period for commercial vaccines is 400 degree days and a vaccine against *M. viscosa* has been shown to induce protection after 600 degree days (Karlsen et al. 2017b). This study showed a good antibody response to the high antigen vaccine, further supporting a sufficient immunization period, which would indicate that this type of vaccine may not work for this bacterium. Although not included, analysis of mucosal antibodies may have been beneficial to determine whether or not a specific immune response had been induced.

Although the bath challenge model represents the natural route of infection, it is not the ideal way to test vaccine efficacy as the fish are exposed to one high dose of bacteria which may not reflect the natural infection pressure as suggested by Karlsen et al. (2017a). One way to avoid this would be to use a more controlled challenge model, such as intramuscular or IP; however these were tested in pre-studies with very poor results (data not shown). Another possible explanation is that the challenge dose for *T. finnmarensis* strain HFJ^T may have been too high (reflected by the high mortality), which may have masked any potential effect of the vaccines.

Tenacibaculosis could become a larger problem than it is with the increased use of smolt facilities that pump in saltwater, which is unlikely to be completely sterile. This has the potential of introducing the bacteria into the facility as was seen in this study, where the controls of the cohabitation experiment were infected with what are likely strains of *Tenacibaculum* spp. (not *T. finnmarensis* strain HFJ^T or Tsp.2, or *T. dicentrarchi* strain 35/09^T) from the water source.

5. Conclusion

This study shows that *T. finnmarensis* strain HFJ^T is a causative agent of tenacibaculosis as it presents in Northern Norway without the need for any scarification, pre-handling or co-infection. The pathogen does not appear to spread easily from fish to fish and more work is therefore required to determine possible reservoirs and/or vectors. Even though a whole cell inactivated vaccine induces an antibody response, it does not appear to offer protection against tenacibaculosis induced through a bath infection. Future developments need to include

finding the right protective antigen for a vaccine candidate and investigating the pathogen-host interactions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2018.06.063>.

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