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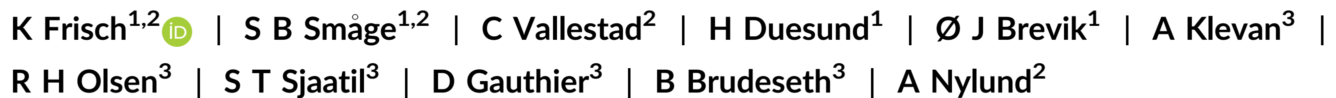
# Experimental induction of mouthrot in Atlantic salmon smolts using *Tenacibaculum maritimum* from Western Canada



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## Abstract

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

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

## 1 | INTRODUCTION

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

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

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

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

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

## 2 | MATERIALS AND METHODS

### 2.1 | Challenge material

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

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

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

### 2.2 | Fish husbandry

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

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

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

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

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

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

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

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

## 2.3 | Challenge model development (experiments 1–3)

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

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

## 2.4 | Cohabitation and horizontal transmission (experiment 4)

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

## 2.5 | Fish sampling

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

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

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

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

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

## 2.6 | Vaccine formulation

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

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

## 2.7 | Antibody cross-reaction

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

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

## 2.8 | Vaccine challenge (experiment 5)

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

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

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

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

## 3 | RESULTS

### 3.1 | Experiment 1: Koch's postulates

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

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

### 3.2 | Experiment 2: Virulence differences between isolates

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

### 3.3 | Experiment 3: Replicable challenge model

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

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

### 3.4 | Experiment 4: Cohabitation and horizontal transmission

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

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

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

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

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

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

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

### 3.5 | Antibody cross-reaction

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

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

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

### 3.6 | Experiment 5: Vaccine challenge

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

## 4 | DISCUSSION

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

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

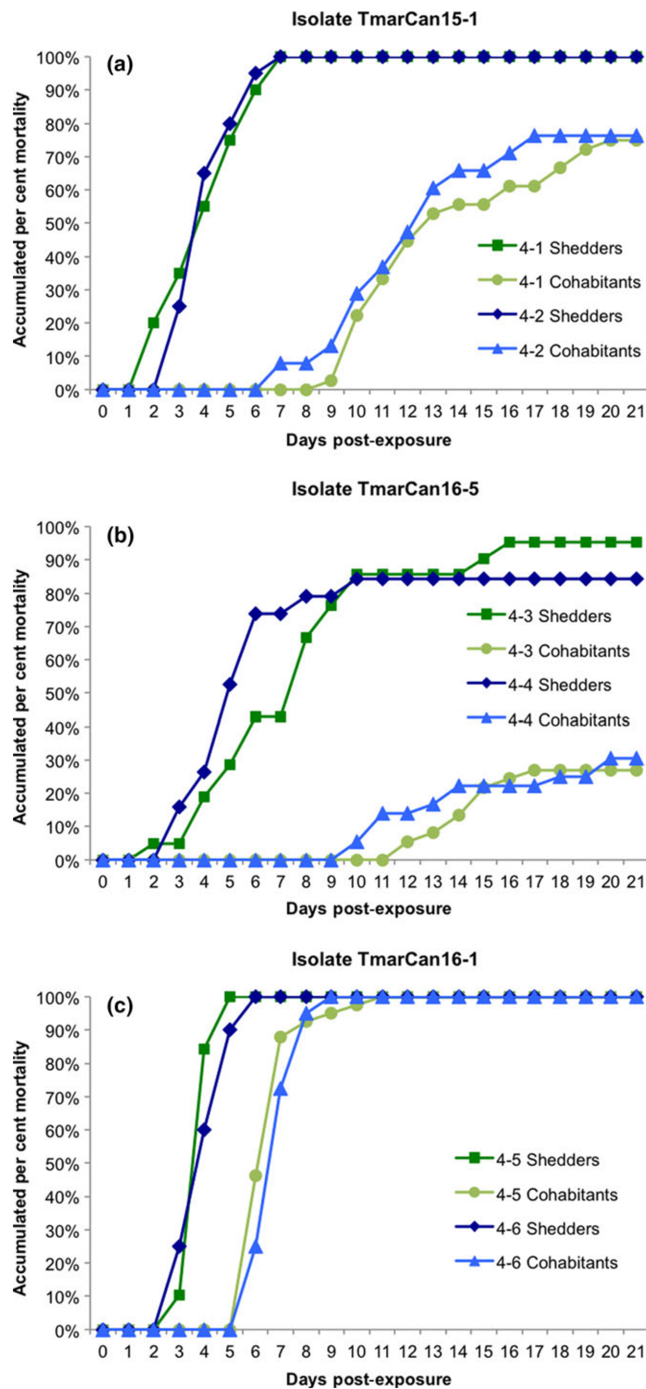


**FIGURE 2** Accumulated per cent mortality from the cohabitant experiment [Colour figure can be viewed at wileyonlinelibrary.com]

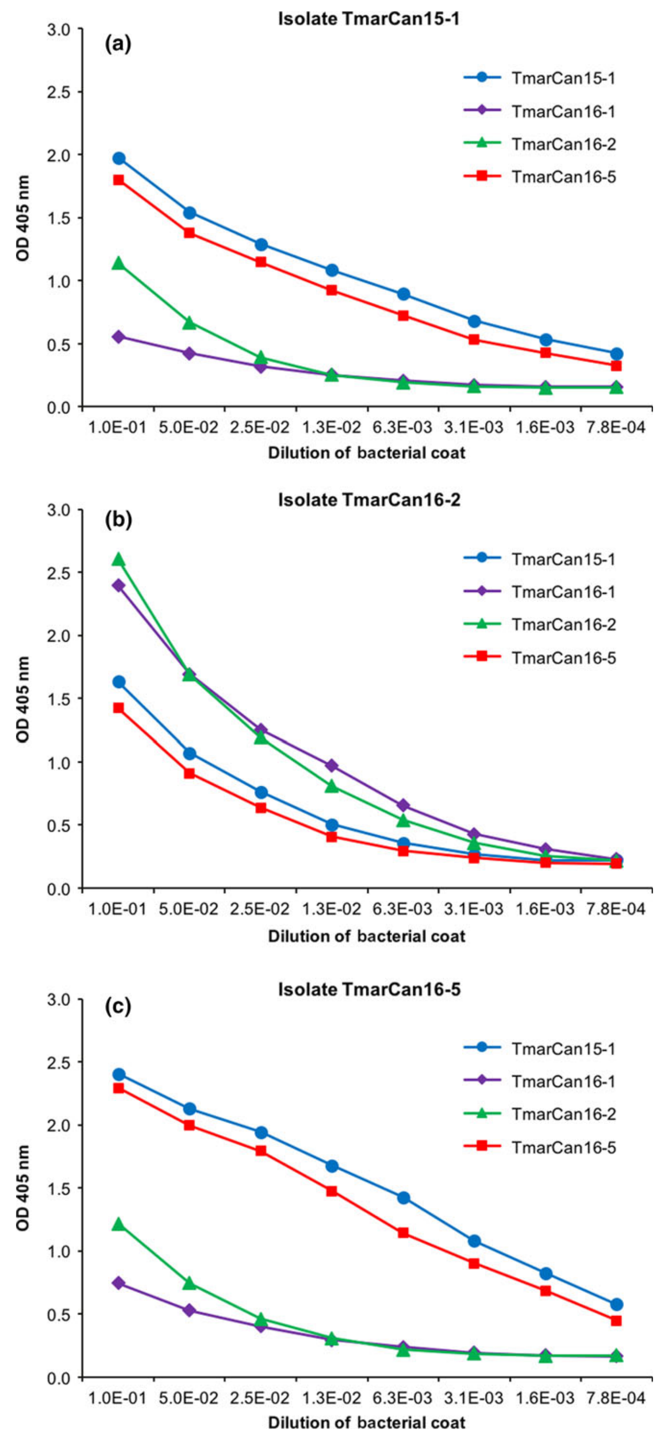


**FIGURE 3** ELISA results showing the antibody binding from the plasma of fish injected with a monovalent vaccine containing isolate (a) TmarCan15-1, (b) TmarCan16-2 or (c) TmarCan16-5 to plates coated with a dilution series of four isolates: TmarCan15-1, TmarCan16-2, TmarCan16-5 and TmarCan16-1. Plasma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

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

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

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

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

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

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

## 5 | CONCLUSION

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


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## SUPPORTING INFORMATION

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

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