

# Experimentally induced marine flexibacteriosis in Atlantic salmon smolts *Salmo salar*. II. Pathology

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**ABSTRACT:** The fish disease marine flexibacteriosis is characterised by necrotic lesions on the body, head, fins, and occasionally gills, with erosive lesions on the external surface as the prominent clinical sign. In Australia, the main species affected are Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in sea-cage culture in Tasmania. Using a dose-dependent trial to determine pathology, 2 forms of the disease were noted in Atlantic salmon. The acute form occurs within 2 to 3 d after inoculation at high doses ( $1 \times 10^8$  cells ml<sup>-1</sup>) and is characterised by the disintegration of the epithelium. The chronic form of the disease began as small superficial blisters of the epidermis, which develop into ulcerative lesions that leave musculature exposed. The predominant lesion sites were the dorsum and pectoral fins. Jaws were commonly affected, and gill necrosis was also noted. Behaviour of Atlantic salmon as well as the conditions under which they were kept contribute to the size and distribution of lesions observed. Lack of an inflammatory response in pathology and rapid and destructive mortalities observed in higher inoculum doses suggested a role of toxins in the pathogenesis of *Tenacibaculum maritimum*. This is the first study to examine the development of marine flexibacteriosis lesions and to utilise immunohistochemistry to verify that the bacteria observed in histology was *T. maritimum*.

**KEY WORDS:** Atlantic salmon · Marine flexibacteriosis · *Tenacibaculum maritimum* · Lesion formation · Immunohistochemistry · Necrosis

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## INTRODUCTION

*Tenacibaculum maritimum* is a fish pathogen causing significant mortalities in the commercial production of many fish species and therefore economic loss to the aquaculture industry. It was first described from a gliding bacterial infection affecting intensively cultured sea breams in Japan (Masumura & Wakabayashi 1977). Erosive skin disease was noted as the cause of mortalities (20 to 30%) among fry, a couple of weeks after transfer from hatchery tanks to inshore net cages. After identification of the causative organism (Hikida et al. 1979, Wakabayashi et al. 1984), subsequent isolation in other marine fish species were reported including Japanese flounder *Paralichthys olivaceus*, yellowtail *Seriola quinqueradiata*, Dover sole

*Solea solea*, turbot *Psetta maxima*, sole *Solea senegalensis*, white seabass *Atractoscion nobilis*, Pacific sardine *Sardinops sagax*, and northern anchovy *Engraulis mordax* (Baxa et al. 1986, Wakabayashi et al. 1986, Devesa et al. 1989, Bernardet et al. 1990, Alsina & Blanch 1993, Chen et al. 1995).

In Australia, the main species affected are Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in sea-cage culture in Tasmania (Handler et al. 1997). After salmonid farming was established in 1985, the appearance of ulcerated skin lesions was observed, although these early cases were assumed to be a mixed *Flexibacter/Vibrio* spp. infection (Handler et al. 1997). The first major outbreak of a *Tenacibaculum maritimum* infection occurred in the summer of 1988/89. Fish in marine cage sites in south-

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eastern Tasmania, which were up to 30 km apart, were affected simultaneously. Morbidity levels were reported as high as 30% with significant losses to farms (Handlinger et al. 1997). Since that time, the disease has occurred in varying degrees of severity, but not to the same extent as the initial outbreak (Handlinger et al. 1997).

Gross pathology of fish affected with a *Tenacibaculum maritimum* infection has been described for many fish species, and the findings show similarities (McVicar & White 1979, Campbell & Buswell 1982, Wakabayashi et al. 1984, Baxa et al. 1986, Devesa et al. 1989, Alsina & Blanch 1993, Pazos et al. 1993, Chen et al. 1995, Handlinger et al. 1997, Ostland et al. 1999). Areas commonly affected are the head, mouth, fins, and flanks, with no internal signs of disease reported. Necrotic gill lesions have been reported in Chinook salmon (Chen et al. 1995), Atlantic salmon, and rainbow trout (Handlinger et al. 1997), but are seldom observed.

Some species-specific differences in pathology caused by *Tenacibaculum maritimum* have been noted. Pacific sardine did not show any lesions or gross pathological signs, but large areas of the body were covered by a tan-coloured pseudomembrane of gliding bacteria (Chen et al. 1995). Corneal lesions and eye rupture have been reported in salmonids in Australia (Handlinger et al. 1997), while in Atlantic salmon in British Columbia, the disease is usually associated with the mouth ('mouth-rot') with no other clinical signs apparent (Ostland et al. 1999).

The aims of this study were to determine the gross and histological pathology of a marine flexibacteriosis infection in Atlantic salmon and to verify the presence of *Tenacibaculum maritimum* associated with the lesions using histology and immunohistochemistry.

## MATERIALS AND METHODS

**Experimental setup.** A recirculation system was set up with 12 × 200 l tanks including a header, footer, and reservoir with a total water capacity of 4500 l. All tanks were fitted with Perspex lids so that airborne transmission was minimised. The system was fitted with a filter bank down to 0.8 µm for incoming water and 2 UV lights (400 J m<sup>-2</sup>, 4000 l h<sup>-1</sup>) to reduce bacterial load. Each treatment was duplicated, with 15 fish assigned to each tank. There were 2 different control treatments. One control treatment was within the recirculation system (control in) and the second, which was still part of the system, had a separate water source (control out). The different control groups were included because of the known high level of infectivity of *Tenacibaculum maritimum*.

**Experimental fish.** Naive, disease-free Atlantic salmon smolts were obtained from a commercial hatchery. Smolt weight ranged from 47.6 to 138.3 g with a mean of 88.1 g, while length varied from 16.2 to 23.9 cm with a mean of 20.9 cm. The fish were initially kept in fresh water at 15°C; salinity was increased to 35 (seawater) over the course of 1 wk, and temperature was raised to 18°C over the following week. They were fed on a commercial diet (Atlantic HP-Skretting) at maintenance (1% body weight) during acclimation, and feeding ceased 48 h before challenge.

**Bacterial culture.** *Tenacibaculum maritimum* strain 89/4762 isolated from infected Atlantic salmon was used in this study. Stock cultures were stored frozen at -80°C in single-use cryovials. Bacteria were initially cultured on marine Shieh's agar at 25°C for 24 h and used as inoculum in 500 ml (in a 2 l glass conical flask) marine Shieh's broth at room temperature (20°C) for another 48 h. The culture was decanted and washed 3 times in 0.2 µm sterile filtered seawater by centrifugation at 3000 × g for 30 min. Cell concentration was estimated using a spectrophotometer at 550 nm.

**Challenge procedures.** Fish were bath challenged in 100 l semi-static tanks in seawater (salinity 35) for 1 h before being randomly distributed into assigned tanks and were held at salinity 35 and 18°C for the duration of the experiment. The doses used in this study were 1 × 10<sup>5</sup>, 1 × 10<sup>6</sup>, 1 × 10<sup>7</sup>, 1 × 10<sup>8</sup> cells ml<sup>-1</sup>. Controls were bathed for 1 h in seawater (salinity 35).

**Sampling procedures.** Fish were checked 3 times a day for morbidity and those affected were anaesthetised using clove oil (0.03 ml l<sup>-1</sup> seawater). The experiment was concluded when there were 3 consecutive days without mortalities. Gross lesion distribution and lesion size were documented. The numbers of lesions recorded at each body site were divided by the total number of lesions recorded for each treatment, thereby indicating differences in areas being affected. Lesion size was measured as length of lesion along the longest axis and was recorded to the nearest 0.5 cm. Pectoral and pelvic fins as well as the immediate surrounding skin area were considered a single site (i.e. pectoral site) since one was not affected without the other. Photographs were taken of the fish to document gross pathology and samples of skin lesions or sites of erosion were taken for histological examination.

**Histological examination.** Initially the samples were fixed in seawater Davidson's (Shaw & Battle 1957, Speilberg et al. 1993) for 72 h before being transferred into 70% ethanol. Samples were embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin (H&E). Sections were viewed under a light microscope at magnification 40 to 1000× for examination of lesion necrosis, and the results were compared with gross lesion photographs.

**Survivors.** Any fish surviving at the end of the challenge period were euthanised using an overdose of clove oil ( $0.06 \text{ ml l}^{-1}$  seawater) and lesions or sites of erosion examined as described.

**Immunofluorescence (IFAT).** The procedure described by Carson et al. (1992) was used. Smears taken from samples were initially air-dried and heat-fixed. The smears were overlaid with  $40 \mu\text{l}$  of rabbit anti-*Tenacibaculum maritimum* 89/0329-5 (strain sourced from the Department of Primary Industries and Water, DPIW) diluted 1:100 in phosphate buffered saline (PBS) (pH 7.2, 0.1 M) and incubated in a moist chamber for 30 min at  $37^\circ\text{C}$  before rinsing in PBS for 15 min. After the removal of excess buffer by blotting,  $20 \mu\text{l}$  of anti-rabbit fluorescein isothiocyanate (Silenus) diluted 1:60 in PBS was added to each slide. Smears were incubated at  $37^\circ\text{C}$  for 30 min and rinsed for 30 min in PBS, which was changed every 10 min. Slides were mounted using alkaline glycerol buffer (Johnson & Munday 1993), coverslipped, and examined at  $40\times$  magnification with epifluorescent microscopy using UV illumination.

**Immunohistochemistry.** Histological sections were probed immunohistochemically based on the method by Adams & Nowak (2003). Tissue used in this procedure was fixed as described in 'Histological examination', and sections were cut ( $5 \mu\text{m}$ ) and mounted on polylysine coated slides (Menzel-Glaser). Sections were hydrated and heat-induced epitope retrieval (HIER) performed: slides were placed into citrate buffer solution (pH 6) and microwaved on high (700 W) for 10 min then allowed to stand for a further 20 min. Following HIER and a brief rinse in deionized water ( $\text{diH}_2\text{O}$ ), sections were blocked for endogenous peroxidase (10 min in 3%  $\text{H}_2\text{O}_2$  in 100% methanol), washed in PBS, and incubated ( $20^\circ\text{C}$ ) with 0.1% bovine serum

albumin (BSA) for 20 min. Sections were then incubated at room temperature with a primary antibody in 0.1% BSA (rabbit anti-*Tenacibaculum maritimum* 89/0329-5; DPIW) at a concentration of 1:100. Sections were rinsed in PBS, incubated with anti-rabbit IgG (Sigma) in PBS (concentration 1:1000 for 30 min) at room temperature, and then washed again in PBS. Slides were flooded with 3,3'-diaminobenzidine in peroxide buffer (Zymed) for 1.5 min, then rinsed in  $\text{diH}_2\text{O}$ , counterstained with Mayer's haematoxylin for 30 s, rinsed in  $\text{H}_2\text{O}$ , differentiated in PBS, dehydrated, cleared, and mounted. A plug made from  $200 \mu\text{l}$  of 2% agarose (w/v) in PBS (pH 7.4) containing  $200 \mu\text{l}$  of *T. maritimum* cell pellet (fixed and processed as above) was used as a positive control (Fig. 1A). The use of normal rabbit serum served as a negative control (Fig. 1B).

## RESULTS

Pathogenicity results for this experiment have already been published under Expt 3 in van Gelderen et al. (2010). All fish in the control (out) group were unaffected by the disease (Fig. 2A) and returned negative results for immunological tests (see Fig. 6H) and culture. Histological examination of the control (out) group showed normal pathology (see Fig. 6C).

Progression of the disease was clearly defined, and 2 different forms of infection were evident. The first was the acute form, which occurred, in the first 2 to 3 d of the experiment. The whole body of the fish was affected, and the main clinical sign was disintegration of the epithelial layer. Other signs included extensive scale loss, areas of raised skin, and superficial cutaneous erosions (Fig. 2B).

The second form was chronic, took longer to develop, and resulted in large lesions. It started with

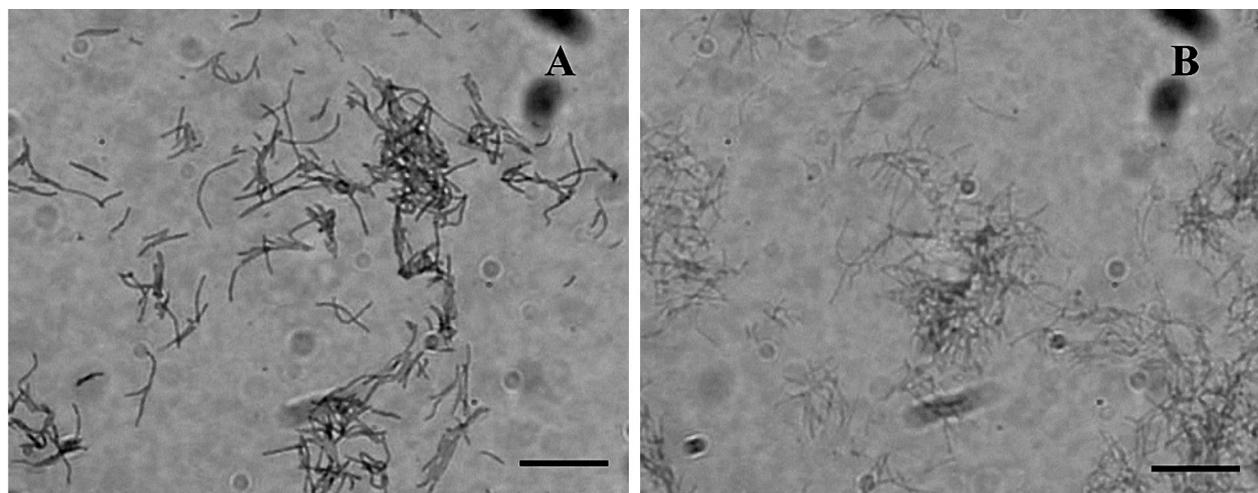


Fig. 1. *Tenacibaculum maritimum*. Immunohistochemistry. (A) Positive control; (B) negative control. Scale bars =  $10 \mu\text{m}$

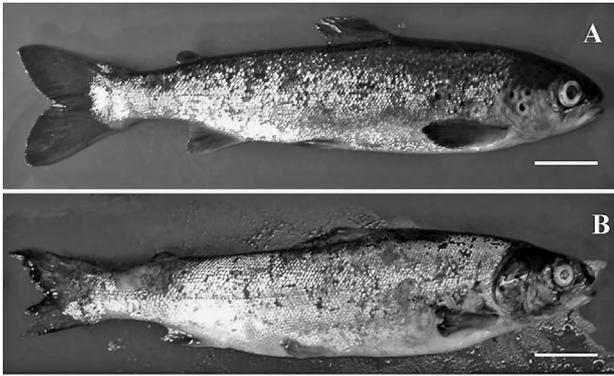


Fig. 2 *Tenacibaculum maritimum* infecting *Salmo salar*. Effect of an acute infection. (A) control Atlantic salmon; (B) Atlantic salmon exposed to  $1 \times 10^8$  cells  $\text{ml}^{-1}$  where mortality occurred within 48 h. Scale bars = 2 cm

scale loss in discrete areas (Fig. 3A). Swelling began to appear over the site, and this swelling appeared to be of epithelial origin (Fig. 3B). The destruction of the epithelium continued within a discrete area until the necrotic tissue was shed to reveal a lesion, which continued to erode (Fig. 3C,D). The underlying musculature was exposed, and necrosis and oedema were evident (Fig. 3E).

Pectoral, dorsal, and lateral sites were the main areas where lesions developed (Fig. 4). In the highest dose ( $1 \times 10^8$  cells  $\text{ml}^{-1}$ ), the pectoral, dorsal, and lateral sites showed similar percentages compared to the other doses. In most cases, the dorsum was the predominant lesion site in each treatment except in the  $1 \times 10^5$  cells  $\text{ml}^{-1}$  treatment where the lateral site was the dominant area (28.9%). The percentage of

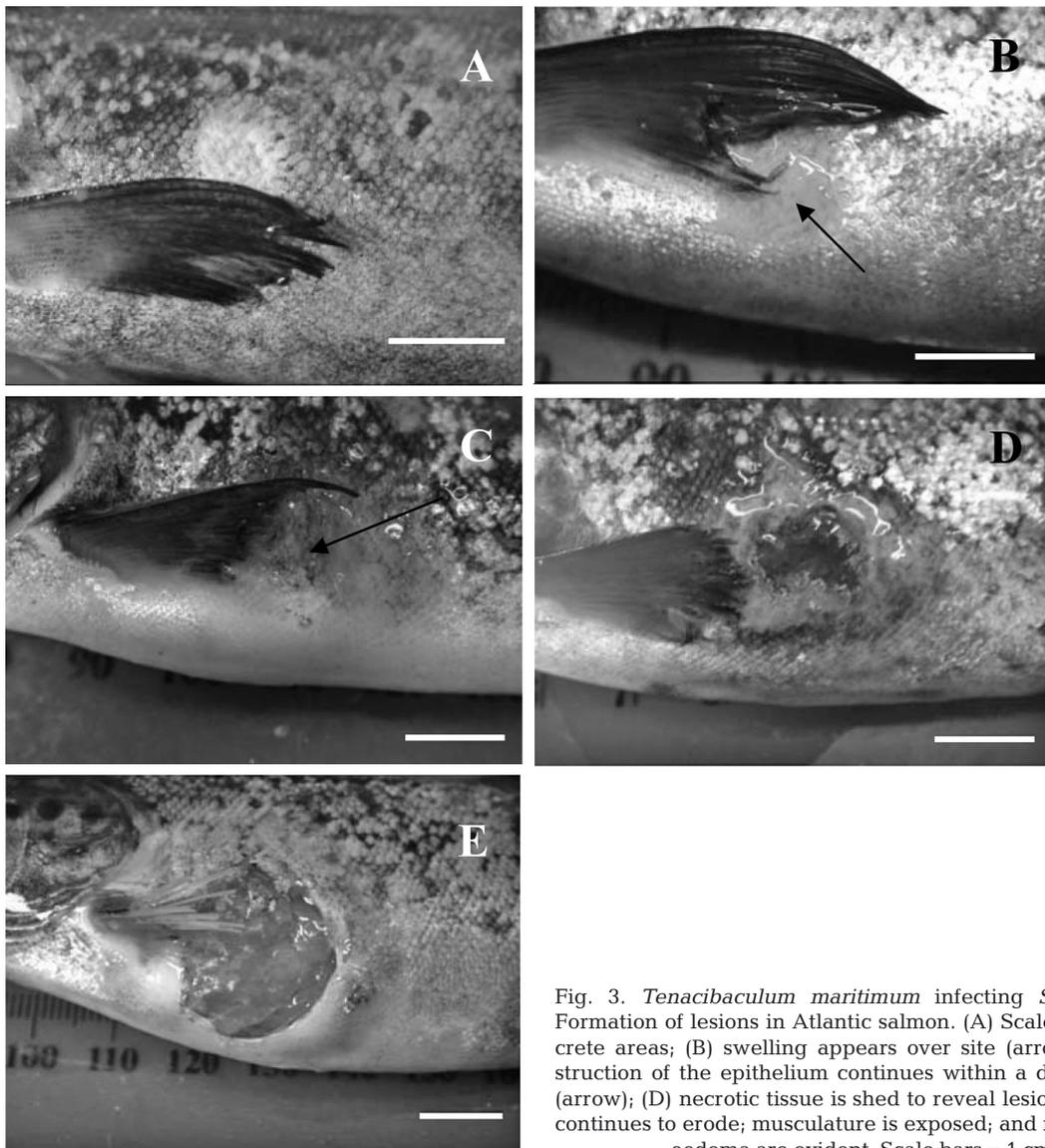


Fig. 3. *Tenacibaculum maritimum* infecting *Salmo salar*. Formation of lesions in Atlantic salmon. (A) Scale loss in discrete areas; (B) swelling appears over site (arrow); (C) destruction of the epithelium continues within a discrete area (arrow); (D) necrotic tissue is shed to reveal lesion; (E) lesion continues to erode; musculature is exposed, and necrosis and oedema are evident. Scale bars = 1 cm

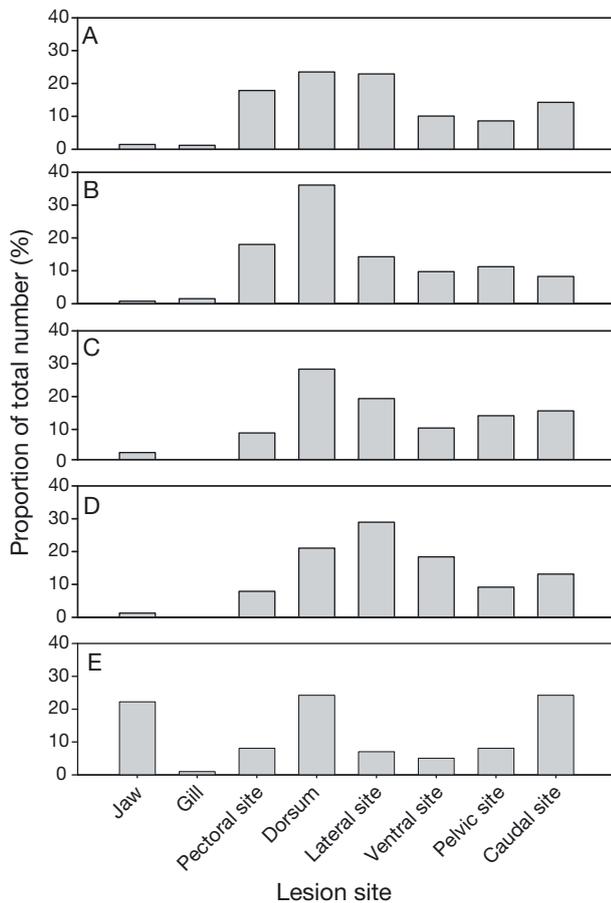


Fig. 4. *Tenacibaculum maritimum* infecting *Salmo salar*. Distribution of lesions in Atlantic salmon challenged with different doses of strain 89/4762. (A)  $1 \times 10^8$  cells  $\text{ml}^{-1}$ ; (B)  $1 \times 10^7$  cells  $\text{ml}^{-1}$ ; (C)  $1 \times 10^6$  cells  $\text{ml}^{-1}$ ; (D)  $1 \times 10^5$  cells  $\text{ml}^{-1}$ ; (E) control (in)

lesions in the pectoral sites decreased with dose. Of interest is the increase in jaw and caudal site lesions in the control (in) group. Gill necrosis was also present in small numbers in the 2 highest doses as well as the control (in) group. Lesion size was smaller in the higher doses, and there was a shift to larger lesions as dose decreased (Fig. 5). Rare cases of absent or ruptured eyes were also noted.

Gross examination of the gills showed discolouration and disintegration on the outer edges (Fig. 6A). Histological examination of infected gills showed diffuse necrosis starting from the tips of the filaments progressing towards the gill arch, and there was no inflammatory response in affected lamellae. All gill lesions were erosive with necrosis affecting distal areas of individual lamellae (Fig. 6D) with instances of heavy filamentous bacterial mats encompassing several lamellae (Fig. 6B). The presence of *Tenacibaculum maritimum* filamentous rods (Fig. 6E) was confirmed by immunohistochemistry (Fig. 6G) and Gram stain

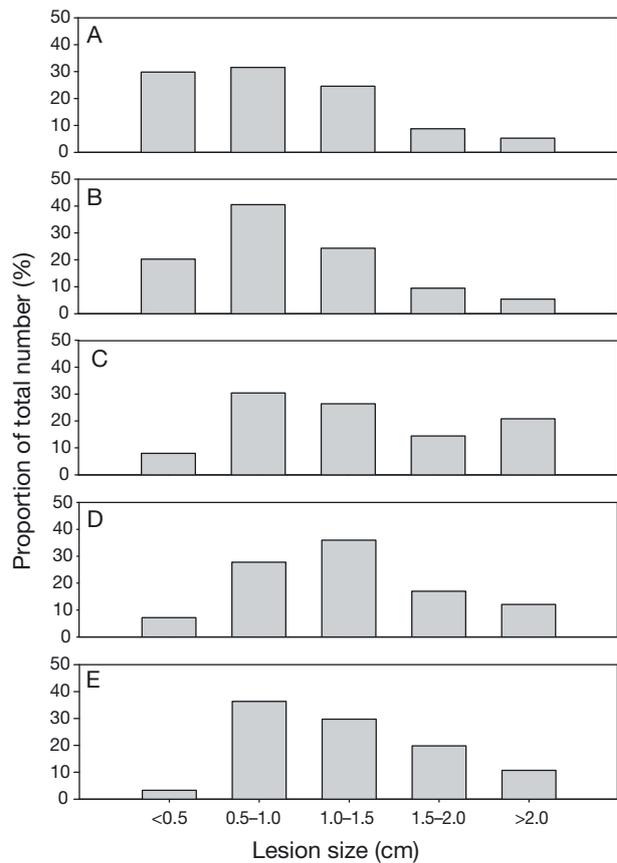


Fig. 5. *Tenacibaculum maritimum* infecting *Salmo salar*. Lesion size (%) in Atlantic salmon challenged with different doses of strain 89/4762. (A)  $1 \times 10^8$  cells  $\text{ml}^{-1}$ ; (B)  $1 \times 10^7$  cells  $\text{ml}^{-1}$ ; (C)  $1 \times 10^6$  cells  $\text{ml}^{-1}$ ; (D)  $1 \times 10^5$  cells  $\text{ml}^{-1}$ ; (E) control (in)

(Fig. 6f). Adjacent uninfected lamellae showed minimal to no reaction.

Control fish showed normal skin structure: an epidermis with mucous cells and scales, the dermis, underlying fat, and musculature (Fig. 7A). The first step in the progression of a lesion began with scale loss and disintegration of the epidermis with scale pockets evident (Fig. 7B). Loss of the epidermis (Fig. 7C) preceded erosion of the dermis and exposure of the underlying musculature (Fig. 7D). Muscle fibres appeared to be replaced by fat in the latter stages of lesion development (Fig. 7D). Bacteria were seldom observed in skin sections and were never seen invading the dermis or muscle. Due to the lack of bacteria in the sections, confirmation by immunohistochemistry or Gram stain was impractical. The material that sloughed off the eroded area to reveal underlying musculature was made up of scales, necrotic material, and bacteria (Fig. 8A,B). Immunohistochemistry confirmed the bacteria to be *Tenacibaculum maritimum* (Fig. 8C,D).

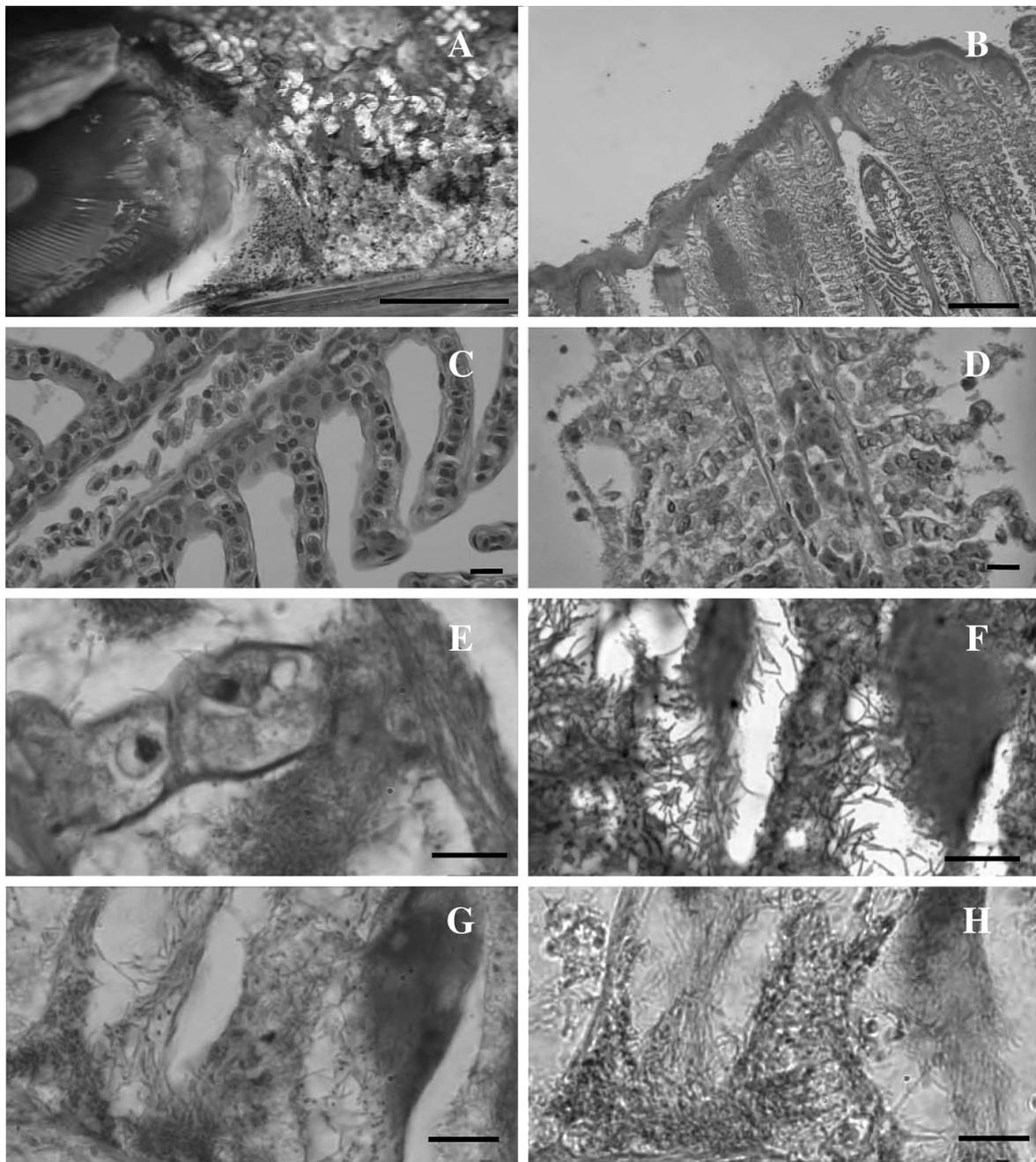


Fig. 6. *Tenacibaculum maritimum* infecting *Salmo salar*. Necrosis of the gill in Atlantic salmon. (A) Gross pathology of gill damage; (B) histology of several necrotic lamellae showing bacterial association; (C) control gill; (D) necrosis of an individual filament; (E) haematoxylin and eosin (H&E)-stained section of gill showing the large mass of bacteria associated with the necrosis; (F) Gram stain of gill lamellae; (G) positive immunohistochemistry result of gill lamellae; (H) negative immunohistochemistry result. Scale bars = (A) 1 cm, (B) 100  $\mu$ m, (C–H) 10  $\mu$ m

## DISCUSSION

The main clinical signs of the disease from the current study were necrotic lesions of the skin and gills, jaw erosion, frayed fins, and tail necrosis. The lesions could be in various stages of development, and the

degeneration of fins was consistently associated with erosion in the surrounding areas. These gross pathological findings are consistent with natural and experimental infections previously recorded in Atlantic salmon (Carson et al. 1992, Handler et al. 1997), but this study is the first to document 2 forms of infection

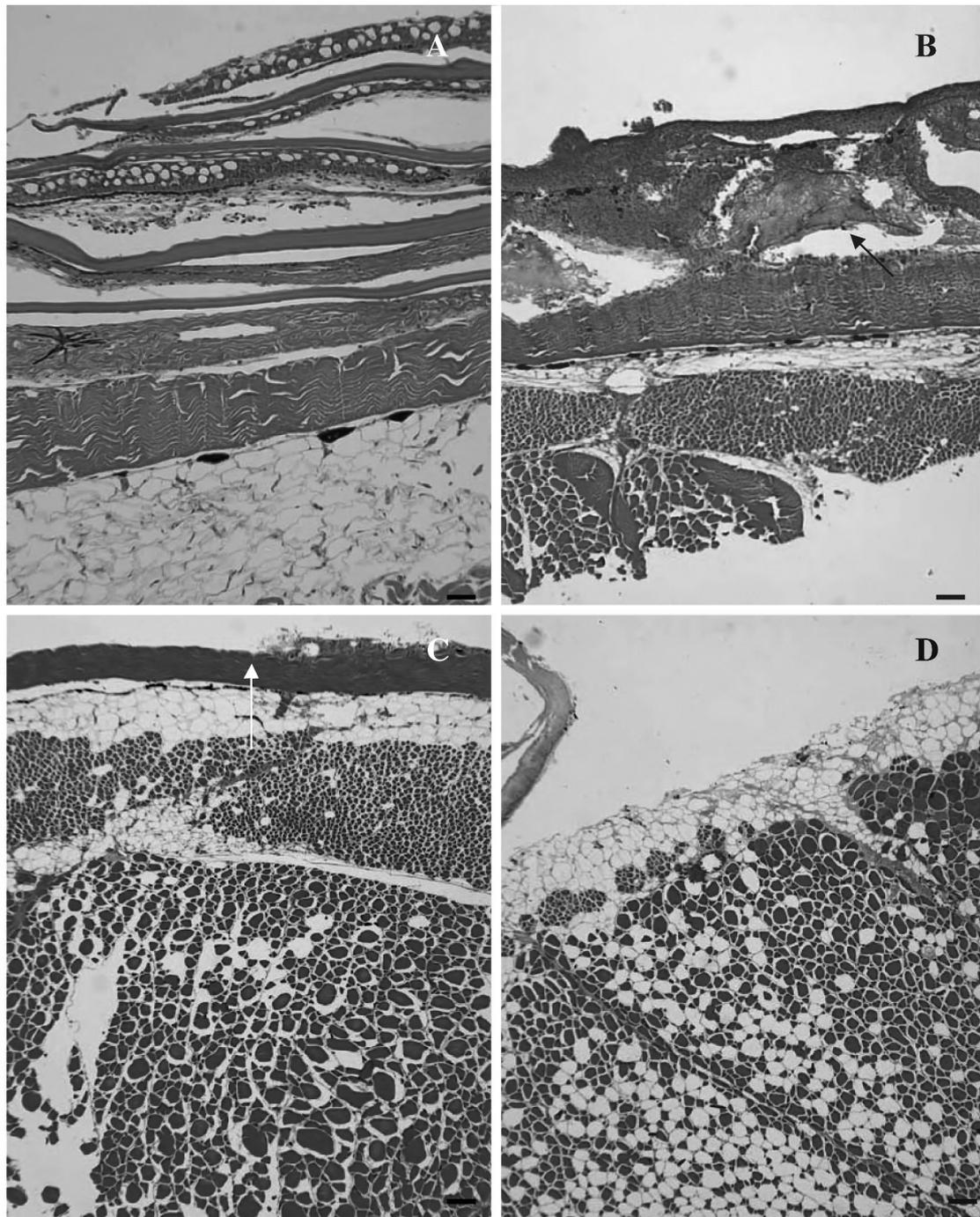


Fig. 7. *Tenacibaculum maritimum* infecting *Salmo salar*. Histological examination of the progression of lesion development. (A) Control skin; (B) scales lost leaving scale pockets (arrow) underneath the epidermis; (C) epidermis eroded away by bacteria, leaving dermis as upper layer (arrow); (D) dermis eroded leaving the underlying musculature exposed. Scale bars = 100  $\mu$ m

for any species infected with *Tenacibaculum maritimum*. There are also similarities to the gross pathology described in other affected fish species in other countries (Masumura & Wakabayashi 1977, Campbell & Buswell 1982, Wakabayashi et al. 1984, Baxa et al. 1986, Alsina & Blanch 1993, Pazos et al. 1993,

Bernardet et al. 1994, Avendaño-Herrera et al. 2006). Lesions are usually found around the head, mouth, fins, and flanks of infected fish with gill necrosis being a sporadic occurrence (McVicar & White 1979, Campbell & Buswell 1982, Wakabayashi et al. 1984, Baxa et al. 1986, Devesa et al. 1989, Alsina & Blanch 1993,

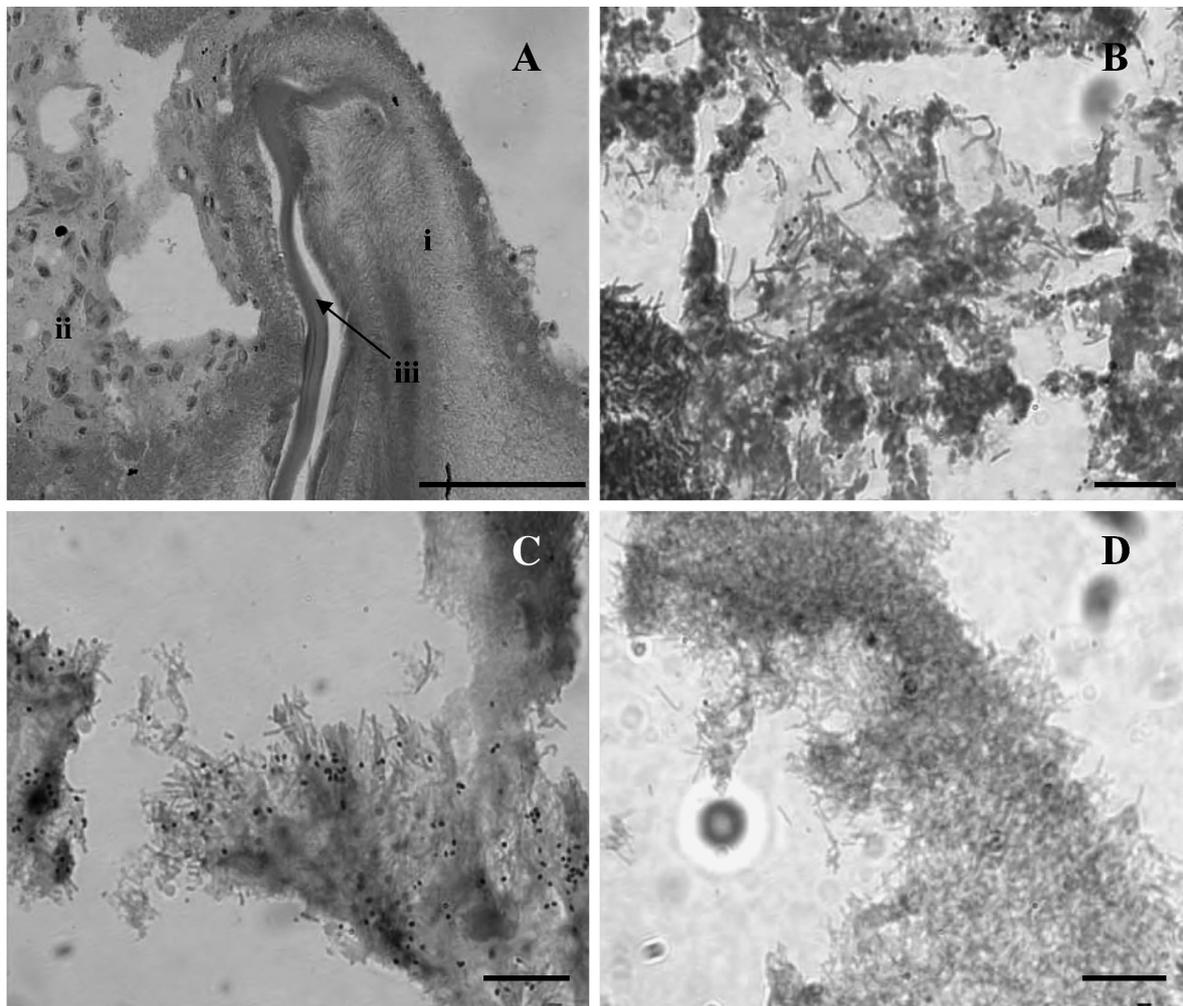


Fig. 8. *Tenacibaculum maritimum* infecting *Salmo salar*. Necrotic material from infection. (A) Necrotic material is made up of (i) bacteria, (ii) necrotic tissue, and (iii) scales; (B) Gram stain; (C) positive immunohistochemistry result; (D) negative immunohistochemistry result. Scale bars = (A) 100  $\mu$ m, (B–D) 10  $\mu$ m

Pazos et al. 1993, Chen et al. 1995, Handler et al. 1997, Ostland et al. 1999). Eye pathology was observed on rare occasions, with eye damage consisting of ruptured or missing eyes. However, the eye pathology was not definitively determined to be the result of a *T. maritimum* infection, as lesions were not associated with the eye, unlike reports of natural infections (Handler et al. 1997), and as there was no obvious sign of other or previous trauma.

At high concentrations, the progress of the disease was rapid and highly aggressive, resulting in 100% mortalities within 2 to 3 d post-challenge. In contrast, lower concentrations required several days to weeks to cause host mortalities. The development of a lesion started with small discrete areas of scale loss, which progressed into cutaneous erosion. Comparable patterns of infection were noted in turbot using similar

doses with higher concentrations causing mortalities within days and lower doses requiring days to weeks for mortalities to occur (Avendaño-Herrera et al. 2006).

In natural infections, damage to the epithelium is seen as a pre-disposing factor to an infection caused by *Tenacibaculum maritimum* (Handler et al. 1997). Experimental infection in the current study occurred without the addition of scarification or deliberate damage. Handler et al. (1997) suggested the presence of a toxin that causes superficial skin damage thereby initiating further progression of the disease. Indeed, at high concentrations, infection, erosion and death occurred within 72 h of challenge without the formation of necrotic erosive lesions. Superficial degeneration of the epithelium was apparent over the entire body. In addition, as *T. maritimum* is an external pathogen, it may be able to exist and proliferate in the mucus of the

host fish. In turbot, *T. maritimum* could be recovered from the mucus of surviving fish, which was suggested to indicate the existence of a carrier state of the bacterium (Avenidaño-Herrera et al. 2006). The mucus of turbot does not contain antimicrobial compounds that inhibit the growth of *T. maritimum* (Magariños et al. 1995), and therefore the bacteria can exist in the water while utilizing the mucus of its host as a nutrient reservoir to survive and proliferate (Avenidaño-Herrera et al. 2006). The presence of inhibitory compounds in the mucus of Atlantic salmon is unknown at this time, and an anti-microbial investigation proved inconclusive (Soltani et al. 1996).

Physical abrasion can also provide a habitat for bacterial localisation and colonisation, which would allow proliferation of the bacteria and their infiltration into epithelial tissues (Handlinger et al. 1997). Skin lesions appear to occur where there is constant movement of the fins or areas where abrasion can easily arise where fish are held captive. Damage to the skin surface through handling, excessive UV exposure, or competition between fish, coupled with optimal conditions for bacterial growth, allow the bacteria to colonise and proliferate (Wakabayashi et al. 1984, Alsina & Blanch 1993, Bernardet et al. 1994, Chen et al. 1995, Handlinger et al. 1997, Ostland et al. 1999). In all experimental treatments the pectoral and dorsal sites predominantly showed signs of erosion. The fins themselves are affected, displaying signs of damage and necrosis. Captive fish and, more particularly, those kept under laboratory conditions, consistently show signs of damage or inflammation to at least one fin. This is most likely the result of contact with netting, tank walls, and possibly other fish. Mouths and heads are sites where fish can abrade themselves on nets/tanks (Chen et al. 1995) and the mouths, flanks, and fins are areas that can be affected during aggressive behaviour through competition and feeding (Chen et al. 1995, Handlinger et al. 1997).

The jaw was also consistently affected. It is possible that under experimental conditions, behavioural traits such as aggression play a role in the susceptibility of Atlantic salmon to *Tenacibaculum maritimum*. Atlantic salmon are known to charge and bite each other (Mork et al. 1999). Fin damage, in particular dorsal fin damage, is primarily caused by aggression (MacLean et al. 2000). The many small lesions observed on the dorsum of the fish along with fin damage and jaw erosion could be the direct result of biting from other fish and from net/tank abrasion (Ostland et al. 1999). The teeth and fins of teleost fish contain calcium ions, which are considered important in the growth of flavobacteria (Hikida et al. 1979). Larger lesions observed with fin sites can be attributed to the irritation and spreading nature of fin movement. Younger fish are also more susceptible to damage, as the skin and scales are softer

relative to older fish (Wakabayashi et al. 1984, Bernardet et al. 1994, Handlinger et al. 1997). Higher water temperatures would cause stress, depressing immune function and making fish more susceptible to infection (Wakabayashi et al. 1984, Bernardet et al. 1994, Handlinger et al. 1997). For salmonids, the transfer from freshwater to seawater would cause scale loss and provide stress and immunosuppression (Franklin et al. 1992). In the present study, not only were the fish moved from freshwater to seawater just before challenge, they were also young fish (80 to 100 g), handled several times, and were exposed to higher than normal water temperatures.

Management is an obvious area where aggressive behaviour may be reduced through decreasing densities and improving conditions so that infectivity is minimised. This has proven an effective strategy for Atlantic salmon in Tasmania, where improvements in the feeding management and lower stocking densities resulted in a decrease in the incidence of the disease (Handlinger et al. 1997).

Lesions formed in Atlantic salmon through scale loss in discrete areas with disintegration of the epidermis, followed by erosion of the dermis and the buildup of necrotic material, which then sloughs away to expose the underlying musculature. The necrotic material consists of scales, necrotic tissue, and bacteria. The progression of the disease has been established not only through gross pathology but also histology. It appears that *Tenacibaculum maritimum* acts to dislodge the scales from the dermis and break down the epithelial material through to the dermis. It is likely at this point that bacteria exist in the mucus layer of the host. Standard histological techniques do not preserve the mucus layer, and therefore bacteria were not observed during lesion development. We suggest that the bacteria proliferate in this necrotic material (as shown by the mat-like bacteria observed in the gills and slough material), and once through to the musculature this material detaches, leaving the musculature exposed. The lesions continue to erode outwards most likely as the result of a continuing process of breakdown in the non-vascularised epidermal tissues. Soltani (1995) suggested that *T. maritimum* is not able to survive in well-vascularised living tissue, probably because of low  $\text{Na}^+$  levels, which would hinder *T. maritimum* growth (Wakabayashi et al. 1984). This may be the reason why it does not progress past the musculature and why it is confined to the epidermis. Eroded areas can continue to expand with bacteria feeding from the periphery of the lesion.

The present study used immunohistochemistry to verify the presence of *Tenacibaculum maritimum* in gill, necrotic tissue, and lesions. Gills showed diffuse necrosis that can affect individual or encompass sev-

eral lamellae. While gill and necrotic tissue clearly showed the presence of *T. maritimum*, there were insufficient numbers of bacteria in skin lesions to determine presence of the bacteria. There was also no evidence of large numbers of bacteria present in lesions in sections stained with H&E. Since *T. maritimum* is usually found with other opportunistic bacteria, the use of immunohistochemistry for histology was found to be useful. Bacteria were not observed in the connective tissue or musculature in skin lesions. We suggest that this is the result of the preference of *T. maritimum* to exist within the necrotic material (slough). Invasion of the musculature has been observed in some studies (Chen et al. 1995, Handlinger et al. 1997), but it is possible that this was after mortality had occurred or that other bacteria, particularly *Vibrio* spp. were present (Carson et al. 1992, Kimura & Kusuda 1993, Chen et al. 1995, Handlinger et al. 1997, Kusuda & Kawai 1998). Fish were not deceased for more than 1 h in the present study, with the majority of fish euthanised before mortality occurred. As previously stated, Soltani (1995) suggested that *T. maritimum* was not able to survive in well-vascularised living tissue, and therefore we propose that colonisation of the tissue and musculature had not taken place in the tissue of fish in the present study.

Of interest was the lack of an inflammatory response, particularly in fully formed lesions. This was also observed by Handlinger et al. (1997), who proposed that the prevention of a host response was due to the production of exotoxins. The toxin may act to interfere with the host immune response as a whole or at the site of infection (Thune et al. 1993). This, together with the suggestion of toxin involvement with the initial skin damage to facilitate bacterial proliferation, makes exotoxins an area of interest. Extracellular products are considered to play an important role in the pathogenesis of *Tenacibaculum maritimum* (Baxa et al. 1988, van Gelderen et al. 2009).

The present study has provided insight on the development of marine flexibacteriosis with a description of different forms of the disease and reactions of the organism to the pathogen. It has shown that the use of immunohistochemical techniques can be used for the detection, identification, and study of *Tenacibaculum maritimum*. The sensitivity and specific nature of these methods make them suitable for use in diagnosis of the disease.

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