Vaccination of Atlantic salmon (*Salmo salar*) against marine flexibacteriosis

By

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ABSTRACT

Tenacibaculum maritimum (formerly *Flexibacter maritimus*) is a well known pathogen in a number of cultured fish species worldwide. It is a marine bacterium that causes necrotic lesions on the body, head, fins and gills, with erosive lesions on the external surface as the prominent clinical sign. A large outbreak of the disease occurred in Tasmania over 15 years ago causing significant mortalities. Management strategies have reduced the incidence of outbreaks, however smaller outbreaks continue to occur. There is limited information on the biological factors that could initiate further outbreaks of the disease and patterns of infection are variable. Chemotherapeutants such as antibiotics are undesirable for use with problems of bacterial resistance, residence times in tissues and effects on other flora and fauna in the surrounding marine environment. Therefore, development of a safe and effective vaccine against *T. maritimum* is the focus of this research.

To characterise and to understand the similarities and differences between *T. maritimum* isolates, several physical characterisation tests were carried out. The Tasmanian isolates of *T. maritimum* appeared relatively homogeneous physically, but were antigentically different. All isolates were hydrophobic and produce a variety of extracellular product (ECP) profiles. There were two isolates that stood out in all assays (89/4747 and 01/0356-7) and were different from the other isolates. Based on their physical characteristics, three isolates (89/4747, 89/4762, 00/3280) were chosen for further investigation in pathogenicity trials.

Pathogenicity trials were carried out to test the effects of culture media, virulence of strains, confirm Koch's postulates and develop a model of infection so that the efficacy of the experimental vaccines could be established. Culture media did not affect the pathogenicity of *T.*

maritimum, but the presence of aeration within the culture broth revealed a dose effect caused by the cohesive nature of the bacteria. Isolate 89/4747 was found to be non-pathogenic, while the other isolates (89/4762, 00/3280) were highly pathogenic causing 100% mortalities within 3 days. The more moderate isolate 89/4762 was chosen to develop the model of infection and to be used in the subsequent vaccination trial. A model of infection using immersion as the challenge method was established for Atlantic salmon (*Salmo salar*). An LD_{60} of approximately 1×10^6 cells/mL was calculated for the experimental period and this was the dose used for the vaccination trial.

The possible role of extracellular products (ECP) in the pathogenicity of *T. maritimum* and its toxicity was investigated *in vivo* by injecting different doses of ECP into the peritoneal cavity of Atlantic salmon. The ECP was found to be toxic and caused internal necrosis and haemorrhaging. An LD_{50} calculated for the experimental period was 125 µg/fish.

A vaccination trial was conducted using a formalin de-activated vaccine and the vaccine plus an adjuvant (Freund's incomplete). Some protection was provided with the vaccine alone (RPS = 22.0 – 27.7%) but significant protection was achieved with the vaccine plus adjuvant (RPS = 78.0 – 79.6%). The adjuvant caused pigmentation and inflammation in the pyloric caeca of the fish.

For future work it is suggested that further characterisation of isolates are required, that toxins such as extracellular products (ECP) and lipopolysaccharide (LPS) be investigated in more depth and that duration of protection for the vaccine and alternative adjuvants be examined. In the short-term, the vaccine preparation from this study may be useful to the salmonid aquaculture industry in Tasmania, Australia.

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General Introduction

1.1 Nomenclature

The nomenclature within the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group has undergone significant changes over many years. *Tenacibaculum maritimum*, the subject of this thesis, has also previously been known as *Flexibacter maritimus* and *Cytophaga marina*. The bacteria were initially described from a disease affecting red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) (Masumura and Wakabayashi 1977). Wakabayashi *et al.* (1986) proposed the name *Flexibacter maritimus* although it was later placed in the *Cytophaga* genus and given the name *Cytophaga marina* (Reichenbach 1989). However in 1992, the name *Flexibacter maritimus* was restored (Holmes 1992). Recently, more changes have occurred within the CFB group with the introduction of a new genus *Tenacibaculum* (meaning 'holding fast' 'stick'). Through phylogenetic, chemotaxonomic and phenotypic characterisation, it was proposed that *Flexibacter maritimus* should be transferred to the genus *Tenacibaculum* and given the name *Tenacibaculum maritimum* (Suzuki *et al.* 2001). This is the current status of the taxonomy of this bacterium and the name which will be used herein. However, flexibacteriosis will be used to describe the disease rather than tenacibacteriosis since this is the term still used in Tasmania, Australia.

1.2 *Tenacibaculum maritimum* as a pathogen of farmed fish

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 and Wakabayashi 1977). Erosive skin disease was noted as the cause of mortalities (20-30%) 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; Japanese flounder (*Paralichthus olivaceus*), leather jacket (*Aluterus monoceros*), rock bream (*Oplegnathus fasciatus*), plaice (*Cleisthenes pinetorum herzenstenini*), puffer (*Takifugu rubripes*), and yellowtail (*Seriola quinqueradiata*) (Baxa *et al.* 1986, Wakabayashi *et al.* 1986).

Subsequently, an infectious disease, 'black patch necrosis', appeared in cultured Dover sole (*Solea solea*) in Scotland causing mortalities of up to 10 % per day (McVicar and White 1979, McVicar and White 1982). *Flavobacterium columnare*-like bacteria were isolated from the diseased fish (Campbell and Buswell 1981, Campbell and Buswell 1982); these bacteria were later confirmed as *T. maritimum* (Bernardet *et al.* 1990). This was the first report of *T. maritimum* outside of Japan and in Europe. Further isolation of the same bacterium was reported from diseased sea bass (*Dicentrarchus labrax*) from farms along the Mediterranean coast of France, Malta and Greece (Pepin and Emery 1993, Bernardet *et al.* 1994, Bernardet 1998). It has also become a significant pathogen of cultured turbot (*Psetta maxima*), sole (*Solea senegalensis*), gilthead sea bream (*Sparus aurata*), Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*) on the Atlantic coast of Spain (Devesa *et al.* 1989, Alsina and Blanch 1993, Pazos *et al.* 1993, Pazos *et al.* 1996, Avendaño-Herrera *et al.* 2004). Isolation of *T. maritimum* has continued outside of Europe in northern America, including in net-penned Atlantic salmon in British Columbia (Ostland *et al.* 1999) as well as chinook salmon (*Oncorhynchus tshawytscha*), white seabass (*Atractoscion nobilis*), Pacific sardine (*Sardinops sagax*) and the northern anchovy (*Engraulis mordax*) along the southern coast of California (Chen *et al.* 1995). Culturing of chinook salmon ceased at one site along the coast as a result of severe outbreaks (Chen *et al.* 1995).

In Australia, the main species affected are Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) in sea-cage culture in Tasmania (Handlinger *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 (Handlinger *et al.* 1997). The first major outbreak of *T. maritimum* occurred in the summer of 1988/89. Fish in marine cage sites in south-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). There have been no studies on the prevalence of *T. maritimum* on Tasmanian farms and what is known is based on anecdotal evidence (J. Carson, *pers. comm.*).

No patterns of disease have been established other than fish are more susceptible in the summer months. There is a lack of epidemiological information and no ability to predict the nature and severity of an outbreak on a year-to-year basis. A factor which could affect the detection of a disease pattern could be a chronic outbreak of amoebic gill disease (AGD) in Tasmanian salmonid farms. The main form of treatment for AGD is freshwater bathing which occurs on a regular basis throughout the year, but increases during summer (Parsons *et al.* 2001). Contact with freshwater kills *T. maritimum* within minutes (R. van Gelderen, unpublished) and treatment baths are used hours at a time which is likely to reduce the levels of *T. maritimum* associated with fish. The incidence of *T. maritimum* might be higher without the freshwater treatment against AGD. There have been no studies in this area and due to the lack of surveillance of *T. maritimum* there is no information available regarding the prevalence of *T. maritimum* on sites where AGD is also present. *Tenacibaculum maritimum* is an exclusively saltwater organism and

therefore detection at farms where they use freshwater bathing may mask any appearance of the organism.

Striped trumpeter (*Latris lineata*), greenback flounder (*Rhombosolea tapirina*) and yellow-eyed mullet (*Aldrichetta forsteri*), have also been affected with this disease in Tasmania when kept in captivity (Carson *et al.* 1993).

It is apparent that *T. maritimum* is an important pathogen to different cultured fish species worldwide. In Japan, Australia and Spain in particular it is still a persistent problem, hindering the culture of fish species and causing significant economic losses for aquaculture (Wakabayashi *et al.* 1986, Magariños *et al.* 1995, Romalde *et al.* 2005, Avendaño-Herrera *et al.* 2006, J. Carson *pers. comm.*).

1.3 The causative agent - *Tenacibaculum maritimum*

1.3.1 Morphological and culture characteristics

The morphological and culture characteristics of *T. maritimum* were defined by Wakabayashi *et al.* (1984) and are found to be consistent with other authors (Baxa *et al.* 1986, Wakabayashi *et al.* 1986, Bernardet and Grimont 1989, Bernardet *et al.* 1990, Schmidtke *et al.* 1991, Alsina and Blanch 1993, Chen *et al.* 1995). *Tenacibaculum maritimum* are Gram negative, flexible, slender aerobic rods measuring 0.5 μm x 2-30 μm in length (occasionally observed up to 100 μm long) which do not posses flagella but display gliding motility. On plate agar, colonies are flat, thin, pale yellow with a translucent appearance, uneven margin and rarely exceeding 5 mm in

diameter. The bacteria can be cultured between 15 and 34°C with the optimal temperature being 30°C. Colonies will not grow in Cytophaga agar with NaCl as a seawater supplement; they have an absolute requirement for seawater with at least 30 ppt seawater needed in the media for growth. Different combinations of salts in growth media revealed a requirement of KCl and NaCl for growth, Ca^{2+} for enhanced growth and SO_4^{2-} to inhibit growth. In addition to these results, Soltani and Burke (1994) reported that Tasmanian isolates can grow between 8-35°C, with an optimal temperature range of 20-30°C and that growth is restricted to a pH range of 6-8.5. Results of physiological tests between exotic strains (Wakabayashi *et al.* 1984) and local strains (Schmidtke *et al.* 1991) are summarised in Table 1.1. Some discrepancies exist between individual strains in nitrate reduction and production of hydrogen sulphide for which there are contrasting results in the literature (Bernardet *et al.* 1990, Chen *et al.* 1995, Ostland *et al.* 1999, Avendano-Hererra *et al.* 2004). A small percentage of Tasmanian isolates were able to utilise sodium glutamate, while other reported strains do not (Wakabayashi *et al.* 1984, Wakabayashi *et al.* 1986). DNA base-compositions for *T. maritimum* ranged from 31.3 to 32.5 mol % G+C (Wakabayashi *et al.* 1984).

1.3.2 Pathogenicity

There have been many experimental infections of *T. maritimum* reported (Masumura and Wakabayashi 1977, Campbell and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1987, Carson *et al.* 1992, Alsina and Blanch 1993, Bernardet *et al.* 1994, Soltani 1995, Soltani *et al.* 1996, Handlinger *et al.* 1997, Powell *et al.* 2004, Avendaño-Herrera *et al.* 2006). However, due to differences in the route of challenge, size of fish, species of fish and inoculum dose, comparisons between studies are difficult. What is apparent is that intramuscular and

intraperitoneal injections are not efficacious methods of inducing disease (Tables 1.2, 1.3). Challenge via immersion has provided mixed results with salmonids in particular being susceptible to this method of challenge (Tables 1.2, 1.3). Disease can be induced with varying degrees of success through subcutaneous injection, direct application as well as scarification and smearing (Tables 1.2, 1.3).

Table 1.1 Physiological characteristics of *Tenacibaculum maritimum*. + = positive (100% of strains), - = negative (100% of strains), nt = not tested, and % = % of strains that were positive for a specified attribute. Modified from Wakabayashi *et al.* (1984) and Schmidtke *et al.* (1991).

Physiological characteristic	**Wakabayashi *et al.* 1984**	**Schmidtke *et al.* 1991**
Degradation of:		
Agar	-	-
Cellulose	-	Nt
Chitin	-	-
Starch	-	-
Esculin	-	-
Casein	+	+
Gelatin	+	+
Tributyrin	+	Nt
Tyrosine	+	+
Carbohydrate utilisation:		
Glucose	-	-
Galactose	-	-
Lactose	-	-
Sucrose	-	-
Sorbose	-	Nt
Inulin	-	Nt
Sorbitol	-	Nt
Inositol	-	Nt
Nitrogen source:		
Tryptone	+	+
Casamino acids	+	+
Yeast extract	+	+
Sodium glutamate	-	10%
Ammonium sulfide	-	Nt
Potassium nitrate	-	Nt
Production of:		
Ammonium	+	Nt
Hydrogen sulfide	-	6%
Indole	-	-
Catalase	+	+
Cytochrome oxidase	+	+
Reduction of nitrate	+	96%
Congo red test	+	+

Table 1.2 Summary of *Tenacibaculum maritimum* pathogenicity studies undertaken in salmonids

Fish species	Size of fish	Temperature	No. of fish per group	Method of challenge	Concentration/dose of challenge	% mortalities	Reference
Rainbow trout	63-218g	16-18°C	3	Immersion	1×10^7 cells/mL	100[a]	Carson *et al*. 1992
			3	Scarification	1×10^8 cells/mL	100[a]	
			3	Subcutaneous injection	1×10^7 cells/mL	na[a]	
Atlantic salmon	avg. 225g	18°C	50	Immersion	2.5×10^6 CFU/mL	2-4	Soltani 1995
	20-50g	16°C	15	Immersion	5.6×10^6 CFU/mL	100	
	20-50g	18°C	10	Intramuscular injection	2.3×10^6 CFU/mL	0	
	20-50g	18°C	10	Intraperitoneal injection	2.3×10^6 CFU/mL	0	
Atlantic salmon	20-40g	17-18°C	12	Immersion	1.6×10^6 CFU/mL	74.9	Soltani *et al*. 1996
Rainbow trout	50-140g	17-18°C	12	Immersion	1.6×10^6 CFU/mL	50	
Atlantic salmon	20-50g	18°C	83	Immersion	2.5×10^6 cells/mL	7.2	Handlinger *et al*. 1997
Atlantic salmon	20-50g	20°C	25	Immersion	5.3×10^7 CFU/mL	10[b]	
Atlantic salmon	20-50g	15-16°C	20	Immersion after passaging	4.3×10^6 CFU/mL	97.5	
Rainbow trout	50-140g	15-16°C	23	Immersion after passaging	4.7×10^6 CFU/mL	84.8	
Atlantic salmon	avg. 77g	18°C	12	Direct application to gills	4×10^{11} cells/fish	50	Powell *et al*. 2004
			12	Application to gills with abrasion	4×10^{11} cells/fish	70	

a= control fish were also affected by the disease in this experiment
b= experiments were conducted in water with a salinity of 15ppt
c= the aim of this experiment was to determine and LD_{50}
na= information not available

Table 1.3 Summary of *Tenacibaculum maritimum* pathogenicity studies undertaken in marine fish species

Fish species	Size of fish	Temperature	No. of fish per group	Method of challenge	Concentration/dose of challenge	% mortalities	Reference
Black sea bream	20-75 mm	23-24°C	10	Intramuscular injection	na	20	Masumura and Wakabayashi 1977, Wakabayashi *et al*. 1984
			10	Immersion		10	
			10	Direct application to mouth		90	
			10	Direct application to tail		100	
Red sea bream	20-75 mm	23-24°C	10	Intramuscular injection	na	0	Masumura and Wakabayashi 1977, Wakabayashi *et al*. 1984
			10	Immersion		0	
			10	Direct application to mouth		36.4	
			10	Direct application to tail		30	
Black sea bream	1-2 g	26-28°C	10-20	Immersion	1×10^4 - 1×10^8 cells/mL	0	Baxa *et al*. 1987
			20	Subcutaneous injection	1×10^4 - 1×10^8 cells/mL	40.8 - 52.6[a]	
			20	Smearing with immersion	1×10^4 - 1×10^8 cells/mL	43.7 - 75.0	
Sea bass	na	na	10	Intraperitoneal injection	1×10^7 & 1×10^8 cells/mL	0	Bernardet *et al*. 1994
			10	Scarification	1×10^7 & 1×10^8 cells/mL	0	
			10	Scarification with smearing	1×10^7 & 1×10^8 cells/mL	100	
Dover sole	165 days	17.5°C	10	Scarification	5.95×10^7 cells/mL	0	Campbell and Buswell 1982
			10	Subdermal injection	5.95×10^7 cells/mL	30	
Greenback flounder	10-25g	20°C	25	Immersion	5.3×10^7 CFU/mL	2[b]	Handlinger *et al*. 1997
Turbot	na	na	na	Intramuscular injection	na	0	Alsina and Blanch 1993
				Intracoelomatic injection		0	
Turbot	4-6g	18-20°C	12	Intraperitoneal injection	5×10^3 - 5×10^8 cells/mL	0	Avendaño-Herrera *et al*. 2006
			12	Immersion	5×10^3 - 5×10^8 cells/mL	50[c]	

a= control fish were also affected by the disease in this experiment
b= experiments were conducted in water with a salinity of 15ppt
c= the aim of this experiment was to determine and LD_{50}
na= information not available

1.3.3 Mechanisms of pathogenicity

The exact mechanisms of pathogenicity of *T. maritimum* are not known; however, most of the speculation is centred on toxins since these are suggested to play an important role in the pathogenesis of other CFB organisms in fish (Austin and Austin 1993, Dalsgaard 1993). Lack of inflammatory cells in the histological findings of diseased Atlantic salmon challenged with *T. maritimum* was suggested to be the result of toxins which prevented a host response (Soltani 1995). Ostland *et al.* (1999) speculated on the role of toxins due to the presence of bacteria in connective tissue in the oral folds of Atlantic salmon. *Flavobacterium columnare* bacteria are also found in the connective tissue of oral folds and produce several extracellular proteases (Ostland *et al.* 1999). Evidence for the role of toxins in the pathogenesis of *T. maritimum* was provided through *in vitro* and *in vivo* study of a variety of toxins and enzymes in red and black sea breams (Baxa *et al.* 1988c). Intraperitoneal injection of precipitated extracellular product (ECP) and hemolysin caused mortalities, severe internal pathological signs and had the lowest LD_{50} and mean time to death in comparison with other bacterial toxins. In contrast, both ECP and hemolysin showed insignificant *in vitro* activity. No definitive toxic factor was identified although ECP and hemolysin were suggested to play a part in the pathogenesis of *T. maritimum* (Baxa *et al.* 1988c).

1.4 The disease – flexibacteriosis

1.4.1 Clinical signs and pathology

Gross pathology of fish affected with a *T. maritimum* infection has been described for many fish species with remarkable similarity between descriptions (McVicar and White 1979, Campbell

and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Devesa *et al.* 1989, Alsina and Blanch 1993, Pazos *et al.* 1993, Chen *et al.* 1995, Handlinger *et al.* 1997, Ostland *et al.* 1999). The development of lesions begins with the appearance of white/yellow patches or blisters. There is local scale loss in discrete areas which progresses (quite rapidly) into necrotic erosion of the dermis which expose underlying musculature. The lesions can further develop into eroded ulcers on the body, jaw erosion around the mouth, or tail rot; mortalities usually follow. 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 differences have been noted in relation to fish species. Pacific sardine did not show any lesions or gross pathological signs, but large areas of the body were covered in 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).

1.4.2 Isolation and identification of the causative organism

Characteristic lesions caused by flexibacteriosis are usually associated with secondary infections and other opportunistic pathogens, in particular *Vibrio* spp. which produces a mixed infection (Kimura and Kusuda 1983, Carson *et al.* 1992, Chen *et al.* 1995, Handlinger *et al.* 1997, Kusuda and Kawai 1998). These other bacteria can overgrow *T. maritimum* during plate culture by producing substances that inhibit its growth, making confirmation of the disease difficult

(Carson *et al.* 1992, Pazos *et al.* 1996). However, a culture media, *Flexibacter maritimus* medium (FMM) was designed to inhibit other marine bacteria and therefore enhance the recovery of *T. maritimum* from diseased fish (Pazos *et al.* 1996). It has also proven effective in susceptibility testing (Avendaño-Herrera *et al.* 2005a). Media routinely used for the culture of *T. maritimum* in Australia are marine Ordal's and Shieh's with the addition of oxolinic acid or neomycin sulfate to enhance selectivity (Carson *et al.* 1992, Pazos *et al.* 1996).

Isolation is frequently made from the external surfaces of the fish, although there have been reports of isolating bacteria from the kidney in severely affected fish (Alsina and Blanch 1993, Avendaño-Herrera *et al.* 2004). Identification of the causative organism can be achieved through several methods, the most basic being Gram stain and selective agar cultivation. Phenotypic characterisation and DNA-relatedness studies based on bacteria from culture (Hikida *et al.* 1979, Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Wakabayashi *et al.* 1986, Bernardet and Grimont 1989, Bernardet *et al.* 1990, Schmidtke *et al.* 1991, Alsina and Blanch 1993, Pazos *et al.* 1993, Bernardet *et al.* 1994, Chen *et al.* 1995) as well as immunofluorescent antibody test (IFAT) (Baxa *et al.* 1988a) and PCR analysis from plate culture and fish tissues (Toyama *et al.* 1996, Cepeda *et al.* 2003, Avendaño-Herrera *et al.* 2004) have all been employed to identify *T. maritimum* in diseased fish.

Based mainly on the phenotypic characterisation, the species *T. maritimum* was considered to be a homogeneous taxon. Initial serological data using slide agglutination also indicated a homogeneous group (Wakabayashi *et al.* 1984); however, further investigation proved antigenic heterogeneity within the species (Pazos *et al.* 1993, Ostland *et al.* 1999, Santos *et al.* 1999), with two major serotypes described from turbot and sole isolates (Avendaño-Herrera *et al.* 2004).

1.4.3 Predisposing factors of flexibacteriosis

Several risk factors have been identified for flexibacteriosis and the management of these factors will affect the severity of losses and influence outbreaks (Bernardet 1998). As *T. maritimum* is an external pathogen, a portal of entry appears to be required on the external surfaces of fish. Physical trauma provides an entry point for the bacteria into the host and optimal conditions for *T. maritimum* growth increase the risk of infection. Higher than average water temperature is a consistent risk factor during outbreaks (Wakabayashi *et al.* 1984, Bernardet *et al.* 1994, Handlinger *et al.* 1997). Not only temperature, but in the case of salmonids in Tasmania, weather conditions such as cloudless sunny days were believed to influence an outbreak by causing skin damage through excess UV exposure (Handlinger *et al.* 1997). Weather conditions had an indirect effect on an outbreak in sea bass by interrupting their feeding. Sea bass are cannibalistic and display antagonism at feeding times causing physical trauma to the fish (Chen *et al.* 1995). Similarly, poor feeding management in Atlantic salmon also contributed to the appearance of the disease through competition which caused scale loss (Handlinger *et al.* 1997). Other risk factors reported include physical trauma such as net and tank abrasions, wounding by birds and the presence of parasites such as amoeba or marine trichodinids in salmonids (Chen *et al.* 1995, Handlinger *et al.* 1997). Physical trauma and stress are also caused by excessive and traumatic handling (Alsina and Blanch 1993, Handlinger *et al.* 1997). Handling stress can occur when fish are transferred from a hatchery to a sea location (Wakabayashi *et al.* 1984, Pepin and Emery 1993, Carson *et al.* 1994).

Another consistent factor is the prevalence of the disease in younger fish which may be attributed to the relative pliability of scales and skin in comparison to larger, older fish

(Wakabayashi *et al.* 1984, Bernardet *et al.* 1994, Handlinger *et al.* 1997, Romalde *et al.* 2005). In Dover sole, control of the disease in young fish was achieved by including a layer of sand in the rearing tanks (McVicar and White 1982). The authors have suggested that the addition of sand may have reduced stress in the fish and aided in the sloughing of moribund and dead skin cells from the body.

1.4.4 Treatment and control

Improved husbandry practices and/or chemotherapy are the main ways of controlling disease. Better management provides a natural defence for fish that can aid in reducing losses, but this is not always practicable in commercial production (Alderman and Michael 1992). Chemotherapy is an alternative strategy for treatment of disease that is necessary without a viable vaccine. Many substances have been used to treat *T. maritimum*, from chemicals such as formalin, copper sulphate, potassium permanganate as well as drugs/antibiotics such as nitrofurans, sulphonamides, furazolidone, oxytetracycline and oxolinic acid (McVicar and White 1979, Baxa *et al.* 1988b, Carson 1990, Vázquez-Brañas *et al.* 1991, Alsina and Blanch 1993, Bernardet *et al.* 1994, Kusuda and Kawai 1998).

In Australia, the most common treatment recommendation is trimethoprim, to be used at 10 mg/kg of body weight/day for 10 days (Cameron 1993a). However, it also has an unacceptably long withholding period of 500 degree days (S. Percival *pers. comm.*). Amoxycillin (80 mg/kg of body weight/day for 10 days) is suggested as an alternative closer to harvest as it has a much shorter withdrawal time, but is not as efficacious (Cameron 1993b).

There are also many considerations and variables when using chemotherapeutants, such as the effect of prolonged use and bacterial resistance for antibiotics, toxicity and side effects to fish, administration routes, efficacy under outbreak conditions and whether the substance is available and registered for use (Toranzo *et al.* 1984, Baxa *et al.* 1988b, Danayadol *et al.* 1999). Chemotherapeutants are also used after the appearance of the disease, where a more desirable outcome would be a vaccine that would prevent the disease from initially occurring. A vaccine eliminates the problems and resource limitations of dealing with a bacterial outbreak once it has begun.

1.4.5 Vaccine development

Tenacibaculum maritimum is a significant pathogen to many fish species worldwide and a potential threat to the salmonid aquaculture industry in Tasmania, Australia. A safe and protective vaccine is desirable for disease prevention as it eliminates the threat before it becomes a reality. Vaccination has become an important tool in the prevention of bacterial diseases in farmed fish (Håstein *et al.* 2005). For salmonids, vaccines are already used for the prevention of vibriosis, cold water vibriosis, yersiniosis, and furunculosis among others (Press and Lillehaug 1995, Håstein *et al.* 2005). Vaccination studies of fish species against *T. maritimum* have presented contrasting results (Table 1.4). Recently, a vaccine for flexibacteriosis in turbot has been developed, patented and is commercially available in Spain (Romalde *et al.* 2005, Toranzo *et al.* 2005). This vaccine is delivered initially by immersion when the fish are 1-2 g, and then a booster injection is given when the fish are 20-30 g. Protection reaches 85% with the booster injection compared to 50% when fish are vaccinated via immersion only (Toranzo *et al.* 2005). In sole, a vaccine administered via intraperitoneal

injection gave a relative percent survival (RPS) value of 94% in laboratory trials (Romalde *et al.* 2005).

While salmonids in Australia are vaccinated against yersiniosis and vibriosis (Håstein *et al.* 2005), there is currently no vaccine for Atlantic salmon against marine flexibacteriosis. Initial attempts using dip immersion for 30 seconds did not provide protection for Atlantic salmon or rainbow trout, even when an immersion booster was used (Carson *et al.* 1993). Further work using various immersion and injection vaccines in Atlantic salmon provided moderate levels of protection, but the results were not reproducible (Carson *et al.* 1994).

All of the vaccines described above were produced from killed bacterial whole cells. Alternative vaccine preparations for *T. maritimum* using other antigens are currently being investigated in sea bass where lipopolysaccharide (LPS) is considered to be a potential vaccine component (Salati *et al.* 2005). Toxins have also been identified as a possible constituent in vaccine preparations (as a toxoid) due to their suspected role in pathogenesis and prevention of the host response (Handlinger *et al.* 1997).

Table 1.4 Summary of vaccination studies of *Tenacibaculum maritimum* in different fish species

Fish species	Size of fish	Method of vaccination	Concentration/dose of vaccination	Method of challenge	Concentration/dose of challenge	RPS (%)	Reference
Rainbow trout Atlantic salmon	27-74g	Dip (repeated twice)	1.41×10^9 cells/mL	Immersion	2.3×10^5 cells/mL	0-9	Carson *et al*. 1993
Atlantic salmon	40-60g	Immersion Injection	1×10^8 cells/mL 7.5×10^6 cells/mL	Immersion Immersion	2.5×10^4 cells/mL and 3.5×10^4 cells/mL	5-77 0-23	Carson *et al*. 1994
Turbot	1-2g 20-30g	Immersion Booster injection	na na	na na	na na	50 >80	Hastein *et al*. 2005, Romalde *et al*. 2005
Sole	na	Intraperitoneal injection	na	Intraperitoneal injection	1.1×10^8 cells/mL	94.2	Romalde *et al*. 2005

na=not available

1.5 Aim and outline of the thesis

The aim of this thesis is to develop a vaccine for Atlantic salmon against marine flexibacteriosis. The following approach was adopted to investigate this aim:

Chapter 2: Identify similarities and differences in the physical characterisation of *T. maritimum* isolates and in particular, examine antigenic aspects and select isolates for further investigation.

Chapter 3: Conduct a series of *in vivo* trials to study different aspects of the pathogenicity of *T. maritimum* in Atlantic salmon, confirm Koch's postulates and develop a model of infection.

Chapter 4: Investigate the extracellular product (ECP) of *T. maritimum* for its toxicity in Atlantic salmon and possible role as a mechanism of pathogenicity and therefore potential incorporation into a vaccine.

Chapter 5: Conduct a vaccine trial using experimental vaccine preparations and evaluate their efficacy under laboratory conditions.

1.6 Ethics requirements

All investigations carried out were under the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 1997 and the Tasmanian Animal Welfare Act 1993. Approval was given by the University of Tasmania's Animal Ethics Committee under the project No. A0007722. This approval was given under the stipulation that fish should be collected for examination when the first signs of physiological disturbance were evident. Physiological disturbances were defined to occur when a fish was unable to control its position in the water column and/or when erosive lesions were apparent. This is a state from which fish were unlikely to recover. For the most part, fish were euthanized before mortality occurred but this was not always possible. For the purpose of this thesis the term mortality is used to coincide with the literature, even though morbidity would be a more accurate description.

Chapter 2

Physical characterisation of *Tenacibaculum maritimum*

This chapter is modified from a paper to be submitted to Veterinary Microbiology.

2.1 Introduction

In microbiology, a strain is the basic unit of taxonomy and is comprised of successive generations produced from a single isolation in pure culture (Garrity 2001). Strains having similar characteristics are then grouped as a species. One strain of a species is designated as the type strain (or reference), to which all others can be compared (Garrity 2001). The basis of this classification comes from knowledge of morphological, biochemical, physiological and genetic characteristics (Garrity 2001). In addition to species classification, a second approach is required when investigating pathogens. Strains of the same species can exhibit differences in virulence and therefore pathogenicity (Alcamo 1983). These pathotypes and potentially different subspecies need to be characterised and compared with each other and in the case of *Tenacibaculum maritimum* in Australia this has not been done. Selection of isolates for later pathogenicity and vaccine trials also requires the determination of similarities and differences below the species level, particularly properties that enable production of the disease in the host (antigenic differences) and those that can affect vaccine efficacy.

Analysis of whole cell protein profiles is useful for determining taxonomic relationships at or below the species level (Schmidtke and Carson 1994). The separation and comparison of cellular proteins is used to determine the relatedness of strains and the degree of heterogeneity of phenotypes (Carson *et al.* 1993). Variation of strains at this level can equate to antigenic differences, which in turn can affect pathogenesis and vaccine efficacy (Perez-Perez *et al.* 1987).

Pathogenesis, or the ability of bacterial pathogens to produce disease, is dependent on several mechanisms. One of these is the production of toxins, which are complex molecules that activate and stimulate the host response (Dalsgaard 1993). Toxins are examples of antigens that can be divided into particulate antigens (endotoxin such as lipopolysaccharide) and soluble antigens (exotoxin such as extracellular products). Both play significant roles in the virulence of bacterial strains and therefore pathogenesis of infections (Dalsgaard 1993). A vaccine can be developed from either component or a combination of both (Ellis 1997a).

The lipopolysaccharide (LPS) molecules of Gram-negative bacterial cell walls are the main antigen, a source of endotoxic activity, and are thought to be responsible for some of the characteristic signs of disease caused by Gram-negative bacteria (Dalsgaard 1993). These molecules are associated with the outer membrane of cell walls and consist of three covalently linked components: the core polysaccharide, polysaccharide side chains and Lipid A (Dalsgaard 1993). Lipid A is the main endotoxic determinant, that is, the component of LPS that is responsible for the toxic activities (Bennett-Guerrero *et al.* 2000). Differences in toxicity have been related to the antigenicity of the pathogen, which is linked to the lipid component of the cell membrane (Dalsgaard 1993). Presence of the lipid component was associated with highly virulent strains, while absence or low abundance was correlated to low virulence isolates for *Flavobacterium columnare* (formerly *'Flexibacter columnaris'*) (Amend 1982). Detection of differences of the antigentically active LPS will indicate different serotypes. Therefore not only will pathogenicity be affected but also vaccine development. A vaccine produced for one serotype may not be effective against another (Romalde *et al.* 2005).

Extracellular products (ECP) are compounds released from bacterial cells and are thought to have a role in the virulence of some bacteria (Gudmundsdóttir *et al.* 1990, Zorrilla *et al.* 2003). They can act in two different ways: either by acting extracellularly or on cell membranes; or by entering cells and disrupting cellular processes through enzymes. Therefore, they can affect pathogenicity by directly causing tissue damage or by enhancing invasiveness (Dalsgaard 1993). Strain variation of bacterial species is related to differences in cytotoxic activity between isolates from diverse geographical locations and fish species (Madetoja *et al.* 2003).

Another mechanism that facilitates bacterial pathogens to cause disease is their ability to adhere to cell or tissue surfaces. Adhesion of the bacterium takes two forms: non-specific or specific attachment (Ofek and Doyle 1994). Non-specific adherence involves the reversible attachment of the bacterium to the cell surface. One possible cause of this attachment is hydrophobic interactions between the host and bacterial cell. This reversible attachment and hydrophobic nature enables bacteria to interact with other living cells and avoid host defence mechanisms while adhesion to the host tissue facilitates bacterial antigen uptake and processing (Beachery *et al.* 1980, Daly and Stevenson 1987). Determination of hydrophobicity is seen as necessary to understand the pathogenicity of the bacteria, particularly in the initial stages of infection (Vatsos *et al.* 2001). It will also be useful in determining possible vaccine administration routes. If the bacteria are found to be hydrophilic, then its use as an immersion vaccine may be limited.

Some physical characterisation of different strains of *Tenacibaculum maritimum* has been done in Spain where flexibacteriosis is an emerging disease in the culture of sole (*Solea solea* and *S. senegalensis*) (Avendaño-Herrera *et al.* 2004). Toxic activities and hydrophobicity of strains of *T. maritimum* from yellowtail (*Seriola quinqueradiata*) and red sea bream (*Pagrus major*) have

also been investigated (Baxa *et al.* 1988c, Kawahara and Kusuda 1998). However, to understand pathogenicity of strains from a defined geographic region and develop an efficacious vaccine for the Tasmanian Aquaculture industry, a study of local strains is necessary.

The main aims of this study were to:

1. Define and characterise various local isolates of *T. maritimum*,
2. Characterise the major antigenic components of *T. maritimum*.

2.2 Materials and Methods

2.2.1 Bacterial strains

Eighteen *T. maritimum* isolates were cultured from diseased Atlantic salmon and rainbow trout in Tasmania during 1989-2001 (Table 2.1). Isolates were cultured on marine Shieh's agar (Song *et al.* 1988) or marine Ordal's medium (Anacker and Ordal 1959) and biochemically identified (Schmidtke *et al.* 1991). Two other strains were used for comparison: the type strain of *T. maritimum* (NCIMB 2154^T) and a reference strain Baxa lyl-1 (Table 2.1). Stock cultures were stored frozen in peptone water with 8% glycerol (Ward and Watt 1971) at –80°C in single use cryovials. Bacteria were routinely cultured on marine Shieh's agar (MSA) at 25°C or in marine Shieh's broth (MSB) at room temperature (20°C).

2.2.2 Indirect immunofluorescent antibody test (IFAT)

All isolates were tested by IFAT as this technique would be used later in other trials as a confirmatory test for *T. maritimum*. The procedure described by Carson *et al.* (1992) was used. Smears were produced using bacteria from plate culture that were then air-dried and heat fixed. The smears were overlaid with 40 µL of rabbit anti-*T. maritimum* 89/0329-5 (DPIW) diluted 1:100 in PBS (pH 7.2, 0.1M) and incubated in a moist chamber for 30 minutes at 37°C before rinsing in PBS for 15 minutes. After the removal of excess buffer by blotting, 20 µL of anti-rabbit FITC (Silenus) diluted 1:60 in PBS was added to each slide. Smears were incubated at 37°C for 30 minutes and rinsed for 30 minutes in PBS, which was changed every 10 minutes. Slides were mounted using alkaline glycerol buffer (Johnson and Munday 1993), coverslipped and examined at x 40 magnification with epifluorescent microscopy using UV illumination. A

marine *Flavobacterium* sp. 99/1972-4b isolated from Atlantic salmon (DPIW), the primary antibody only and the anti-rabbit FITC only were used as negative controls. All isolates were tested in triplicate.

Table 2.1 *Tenacibaculum maritimum* isolates used in this study. DPIW = Department of Primary Industries and Water.

Accession No.	Source	Host	Year	Collection location
00/0400-3	DPIW	Atlantic salmon	2000	Pillings Bay, Tasmania
00/0422-3	DPIW	Atlantic salmon	2000	Brabazon Point, Tasmania
00/0813	DPIW	Atlantic salmon	2000	Nubeena, Tasmania
00/1793-1	DPIW	Atlantic salmon	2000	Port Arthur, Tasmania
00/3280	DPIW	Rainbow trout	2000	Rowella, Tasmania
01/0356-7	DPIW	Atlantic salmon	2001	Tinderbox, Tasmania
89/0329-1	DPIW	Atlantic salmon	1989	Dover, Tasmania
89/0329-5	DPIW	Atlantic salmon	1989	Dover, Tasmania
89/1579-2G	DPIW	Atlantic salmon	1989	Recherche Bay, Tasmania
89/4747	DPIW	Atlantic salmon	1989	Nubeena, Tasmania
89/4762	DPIW	Atlantic salmon	1989	Penguin, Tasmania
89/4913-6	DPIW	Atlantic salmon	1989	Tinderbox, Tasmania
90/1123	DPIW	Atlantic salmon	1990	Port Arthur, Tasmania
90/1445	DPIW	Atlantic salmon	1990	Port Arthur, Tasmania
91/0126	DPIW	Atlantic salmon	1991	Nubeena, Tasmania
91/0247	DPIW	Atlantic salmon	1991	Nubeena, Tasmania
96/0457-4	DPIW	Atlantic salmon	1996	Tinderbox, Tasmania
98/3186	DPIW	Atlantic salmon	1998	Satellite Island, Tasmania
Baxa lyl-1	DPIW	Japanese flounder	1988	Japan
NCIMB 2154^T	DPIW	Sea bream	1988	Japan

2.2.3 Whole cell protein and lipopolysaccharide (LPS) profiles

Whole cell and lipopolysaccharide (LPS) samples were prepared using a 48 h broth culture. After harvesting, whole cell suspensions were washed twice and re-suspended in PBS (pH 7.2) at 2900 x *g* for 30 minutes. The suspensions were only washed twice because agglutination would occur in most isolates in subsequent washes or re-suspension. Each suspension was standardised to an absorbance of 1.0 at 550 nm in a spectrophotometer. The main objective of protein staining is to determine the presence/absence of bands, not the intensity. Different absorbances were trialled and it was determined that an absorbance of 1.0 produced the best results and was more easily resolved. It is apparent that standardising absorbances made no difference in determining the presence or absence of bands. Aliquots of 1.5 mL were centrifuged at 6500 x *g* for 3 minutes and the supernatant removed. Whole cell lysates were produced by resuspending the cell pellet in 500 µL of SDS solubilisation buffer (Laemmli 1970) and heating for 10 minutes at 100°C. Cell debris from extracts were centrifuged at 6500 x *g* for 3 minutes; the supernatant removed and stored at -20°C.

Lipopolysaccharide analysis was based on Hitchcock and Brown (1983) with minor modifications. Cells were harvested at 16,500 x *g* for 5 minutes and samples were washed once in PBSA (0.14 M NaCl, 0.01 M Na_2HPO_4, pH 7.4, 1 mg of bovine serum albumin per mL) and adjusted to 1 x 10^9 cells/mL. The cell pellet was re-suspended in 100 µL of 2% SDS and mixed thoroughly. Glass beads (Sigma) were added to break up the bacterial pellet as *T. maritimum* appeared to aggregate when pelleted forming a clump that is difficult to disperse. To assist solubilisation of the pellet, in addition to the glass beads, samples were heated to 60°C. Proteinase K (Amresco) at a concentration of 20 mg/mL was added to each sample to give a

final concentration of 5 mg/mL. Samples were then incubated in a thermomixer for an hour at 60°C. The temperature was increased to 99°C for 30 minutes, after which the samples were stored at -20°C. Prior to loading, samples were thawed and mixed with 134 µL of 2 x SDS solubilisation buffer. Each sample was heated in a water bath at 100°C for 10 minutes and then centrifuged at 16,000 x *g* for 5 minutes. Controls for LPS analysis were *Escherichia coli* and *Lactococcus garvieae*.

2.2.4 Extracellular products (ECP)

Extracellular products (ECP) were produced by the cellophane overlay method (Liu, 1957). For each isolate, three plates were incubated for 72 h at 25°C. After incubation the culture was washed off the cellophane with 1 mL of PBS (pH 7.2) and centrifuged at 4000 x *g* for 20 minutes. The supernatant fluid (ECP) was sterile filtered (0.2 µm) and stored in aliquots at -80°C. Protein content was determined using the bicinchoninic acid (BCA) assay kit (Pierce).

2.2.5 Analysis of proteins and lipopolysaccharide (LPS)

ECP and whole cell proteins were separated by SDS-PAGE (Laemmli 1970) with a 5% stacking gel and 12% resolving gel. Samples were heated to 100°C for 10 minutes prior to loading. Electrophoresis was carried out for 1 h at 40 mA and 200 V. Gels were stained with 0.125% Coomassie Blue R-250 (Sigma).

LPS was run on a NuPAGE ™ 12% Bis-Tris Gel for an hour at 200 V. Gels were originally silver stained according to the method of Tsai and Frasch (1982). However, as attempts at silver

staining were deemed unsatisfactory to distinguish differences between isolates (Figure 2.3), western blotting was used to detect LPS bands.

Western blot was run using the method of Towbin *et al.* (1979) with a single modification. As NuPAGE ™ Bis-Tris gels were used, 10% methanol was added in the transfer buffer instead of 20% methanol. LPS was transferred onto nitrocellulose membranes using an Invitrogen Novex XCell II Blot Module (semi-dry transfer) for an hour at 30 V. After transfer, the nitrocellulose membranes were rinsed in PBSA and then blocked for 30 minutes with 3% skim milk powder in Tris-saline (Ab SM). Membranes were washed with Tris-saline and incubated for 30 minutes with antisera against *T. maritimum* SFC 419 (FHU 89/0578-4) in rabbit (dilution, 1:100) in Ab SM. After being washed twice in Tris-saline-0.05% Tween20, and once in Tris-saline, the membranes were incubated for 1 h with anti-rabbit Horseradish Peroxidase (HRPO) diluted 1:1000 in Ab SM. Membranes were washed as previously described above. Bands were visualized by incubating the membranes in 30% w/v H_2O_2 in Tris-saline and a 4-chloro-1-napthol tablet (Sigma) solubilised in 100% methanol which were combined immediately prior to use. The reaction was stopped with the addition of water.

2.2.6 Hydrophobicity

The hydrophobicity assay was modified from the method described by Rosenberg *et al.* (1980). Twenty-four hour broth cultures of *T. maritimum* were centrifuged for 30 minutes at 2000 x *g*. Cell pellets were resuspended and washed twice in PBS (pH 7.2) and diluted to an initial OD (OD_I) of 0.6 within a spectrophotometer (λ = 600 nm). In a glass test tube, 1.2 mL of each bacterial suspension was added along with 0.2 mL of the hydrocarbons *n*-hexadecane (Merck),

n-octane (Sigma) or *p*-xylene (Merck). Each bacterial suspension-hydrocarbon test tube was prepared and assayed in triplicate. The suspension was agitated vigorously for 2 minutes and then allowed to separate for 20 minutes into the hydrocarbon and aqueous phases.

From each test tube, 1 mL of the aqueous phase was transferred to a cuvette and the final OD (OD_F) of each suspension measured (λ = 600 nm). The hydrophobicity of each bacterial suspension to each hydrocarbon was expressed as the percentage partitioning into the hydrocarbon phase:

$$\text{Partitioning into hydrocarbon (\%)} = [(OD_I - OD_F)/ OD_I] \times 100$$

For each hydrocarbon of each isolate the percentage partitioning was ranked. Each rank was added together and then divided by three (i.e. three hydrocarbons). This gave a final rank that could be sorted in order of greatest to lowest overall hydrophobicity.

2.3 Results

Out of the eighteen local isolates, 16 produced the same whole cell protein profile as the type strain and Baxa lyl-1 (Figure 2.1). These isolates showed banding patterns from 118.4 kDa down to 9.2 kDa. Major single bands were located around 12 kDa, 15.5 kDa, 25 kDa, 45.6 kDa, and 99.5 kDa with a triplet at 20kDa. The two local isolates that varied (89/4747 and 01/0356-7) showed similarities to the other isolates, however, there was a distinct band at 17.6 kDa for 89/4747 and bands at 135 kDa, 22 kDa and 12.7 kDa for isolate 01/0356-7 which also indicated that they are also different from each other (Figure 2.1).

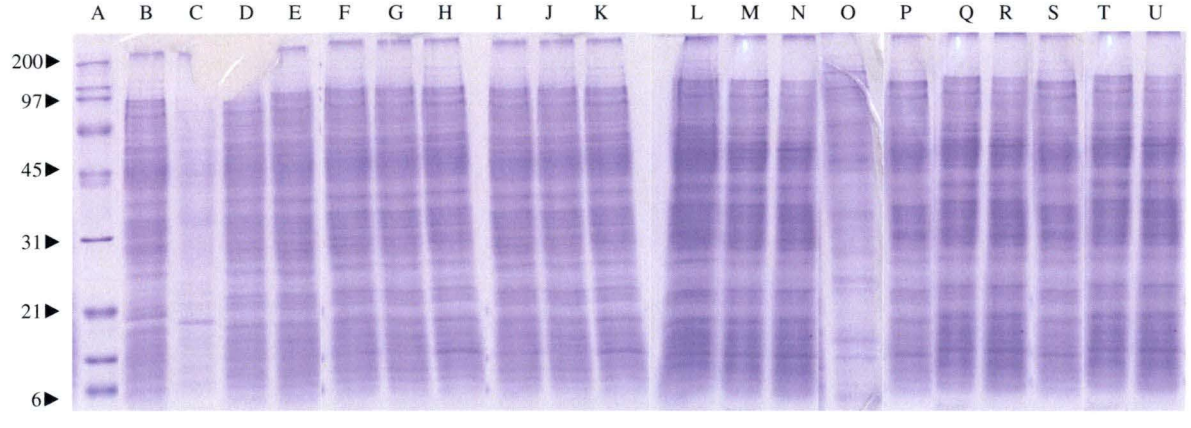

Figure 2.1 SDS-PAGE whole cell protein profile of *Tenacibaculum maritimum* isolates stained with Comassie Blue-R. Lanes: A, Broad-range molecular weight markers (kDa); B, 89/4762; C, 89/4747; D, 00/0400-3; E, 00/0422-3; F, 00/0813; G, 00/1793-1; H, 89/0329-1; I, 89/0329-5; J, 89/1579-2G; K, 89/4913-6; L, 90/1123; M, 90/1445; N, 91/0126; O, 91/0247; P, 01/0356-7; Q, 96/0457-4; R, 98/3186; S, Baxa lyl-1; T, 00/3280; U, NCIMB 2154^{T}. Numbers on the left indicate molecular sizes (in kilo Daltons).

Eight different ECP groupings were detected from all 20 isolates (Table 2.2). Bands that all isolates have in common occurred at 46.5 kDa and 73 kDa. Isolates 01/0356-7 and 89/4747 showed the greatest variation, making up individual groups. Strains used for comparison, NCIMB 2154^T and Baxa lyl-1, showed identical profiles to each other, which was again different from the local isolates (Figure 2.2).

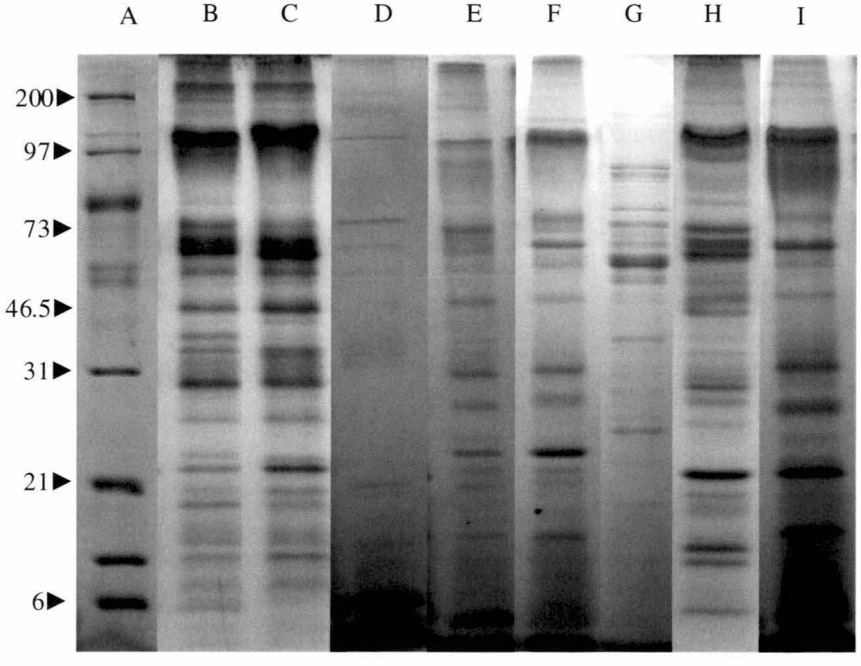

Figure 2.2 SDS-PAGE of ECP profiles of *Tenacibaculum maritimum* isolates stained with Comassie Blue R. Greyscale analysis of gels was used to establish differences. Lanes: A, Broad-range molecular size markers (kDa); B, Group 1; C, Group 2; D, Group 3; E, Group 4; F, Group 5; G, Group 6; H, Group 7; I, Group 8. Numbers on the left indicate molecular sizes (in kilo Daltons).

Table 2.2 ECP groupings of *Tenacibaculum maritimum* isolates based on SDS-PAGE analysis.

Groups	Isolates accession numbers
1	89/1579-2G, 89/0329-5, 89/4762, 96/0457-4
2	89/4913-6, 90/1445, 90/1123,91/0126, 91/0247
3	89/4747
4	89/0329-1, 00/0813
5	00/0422-3, 00/3280
6	01/0356-7
7	00/0400-3, 00/1793-1, 98/3186
8	Baxa lyl-1, NCIMB 2154^T

LPS profiles indicated that there are at least two serotypes amongst the isolates; 89/4747 and 01/0356-7 did not show any banding (Figure 2.4). It was not determined if these isolates had a shared serotype. Laddering appeared uniform across most other isolates; the major exceptions were 89/0329-5, Baxa lyl-1, 89/4762 and 91/0126 which had a distinct band at 55kDa and isolates 00/1793, 00/0400-3 and 98/3186 showed a different LPS profile. Based on differences in the profiles, isolates were grouped together (Table 2.3).

Table 2.3 LPS groupings of *Tenacibaculum maritimum* isolates based on Western blot analysis.

Groups	Isolates accession numbers
1	89/0329-1, 891579-2G, 89/4913-6, 96/0457-4, NCIMB 2154^T, 00/3280, 91/0247, 00/0813, 00/0422-3, 90/1445, 90/1123
2	98/3186, 00/1793, 00/0400-3, 89/0329-5
3	Baxa lyl-1, 89/4762, 91/0126
4	89/4747, 01/0356-7

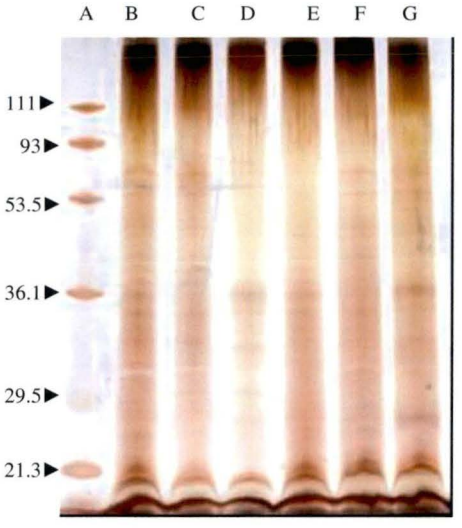

Figure 2.3 SDS-PAGE of silver stained LPS profiles of *Tenacibaculum maritimum* isolates. Lanes: A, Low-range molecular size markers (kDa); B, 89/0329-1; C, 89/0329-5; D, 89/1579-2G; E, 89/4913-6; F, 98/3186; G, 96/0457-4;. Numbers on the left indicate molecular sizes (in kilo Daltons).

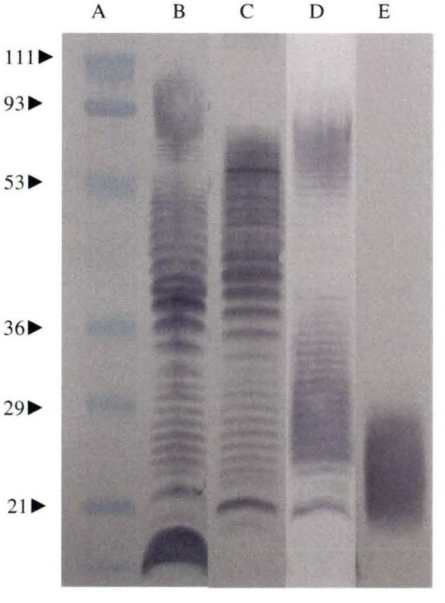

Figure 2.4 Nu-PAGE™ western blot of LPS profiles of *Tenacibaculum maritimum* isolates. Lanes: A, Low-range molecular size markers (kDa); B, Group 1; C, Group 2; D, Group 3; E, Group 4. Numbers on the left indicate molecular sizes (in kilo Daltons).

All isolates of *T. maritimum* are hydrophobic (Figure 2.6). In all cases, isolates showed the lowest hydrophobicity towards *n*-hexadecane and the highest to *p*-xylene, with moderate hydrophobicity to *n*-octane. After final rankings, isolate 89/4762 was found to be the most hydrophobic. Isolates 89/4747 and 01/0356-7 were IFAT negative; all other 18 isolates were IFAT positive (Figure 2.5).

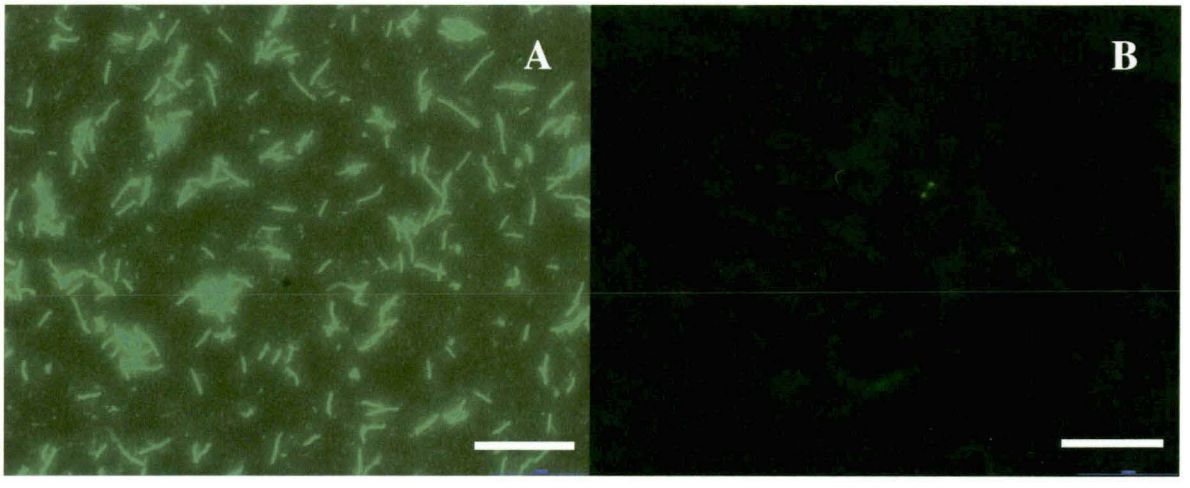

Figure 2.5 IFAT for *Tenacibaculum maritimum*; A, positive response, B, negative response. Scale bars 30 μm.

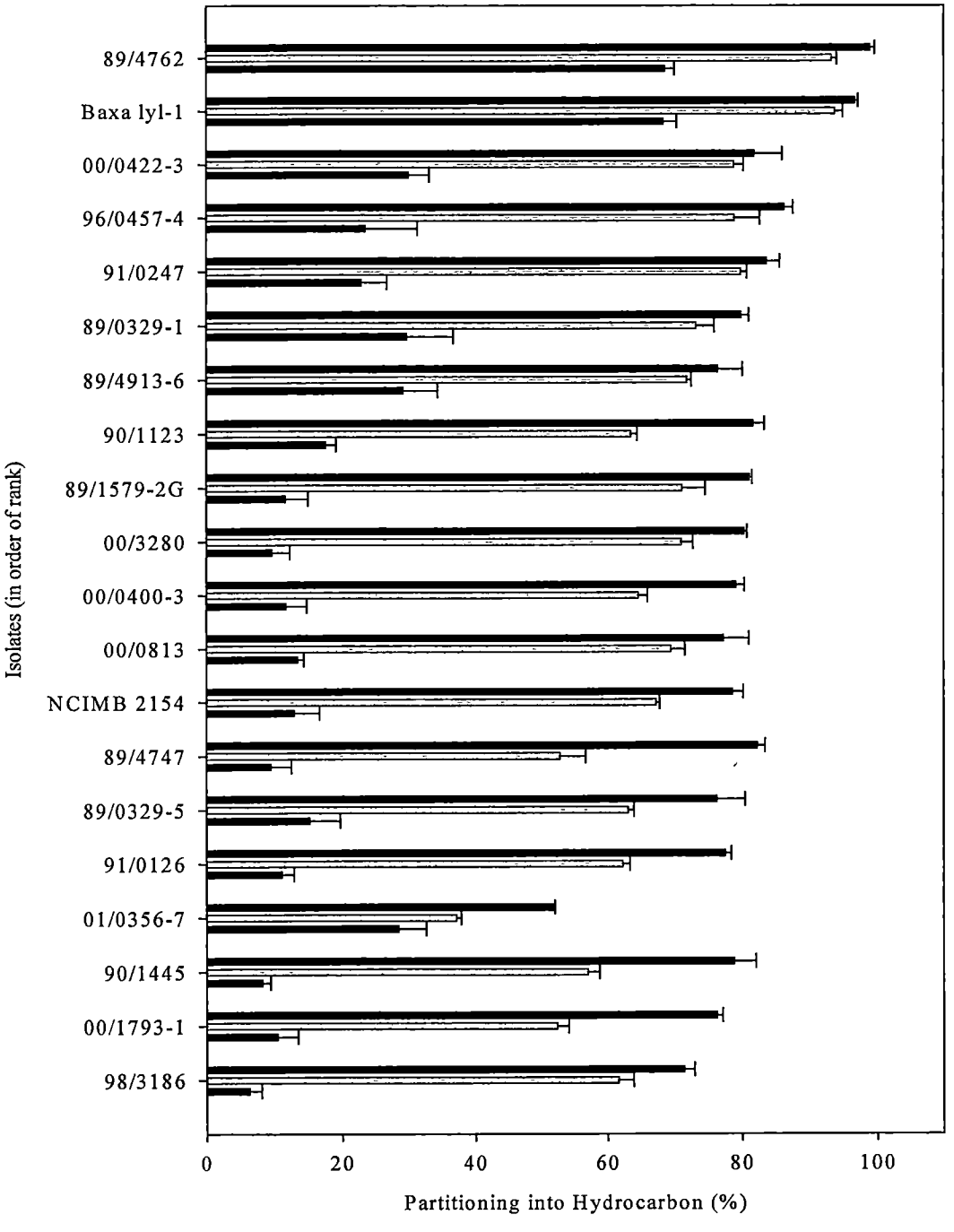

Figure 2.6 Graph showing the partitioning of each *Tenacibaculum maritimum* isolate into the three hydrocarbons (± S.E):

p-xylene *n*-octane *n*-hexadecane

2.4 Discussion

Based on the cell envelope, including whole cell analysis, *T. maritimum* has been described as a homogeneous species (Bernardet *et al.* 1994, Avendaño-Herrera *et al.* 2004). Initial observations of the *T. maritimum* isolates using whole cell protein profiles indicate a relatively homogeneous group. These findings are similar to that of Pazos (1997), who reported *T. maritimum* isolates with a large amount of similarity between the isolates studied and a molecular mass between 14.4 and 97.4 kDa. While light banding does appear above 100 kDa with the isolates, major bands appear at 99.5 kDa down to 12 kDa. With the exception of two isolates (89/4747 and 01/0356-7), all other local isolates showed the same profile to that of the type strain NCIMB 2154^T. Department of Primary Industries and Water (DPIW) records indicate that both 89/4747 and 01/0356-7 are PCR positive for *T. maritimum* and have been characterised using morphological and physiological tests (Schmidtke *et al.* 1991, Carson 1998). Therefore, we have detected differences below the species level, possibly different strains or at least different serotypes. The results from IFAT and LPS indicate these isolates have different outer membrane components to which both antigens were unable to bind; a result that is reflected in the whole cell protein profiles. Whether these two isolates are the same or different serotypes is yet to be determined.

Avendaño-Herrera *et al.* (2004) reported different serotypes according to the source of the isolation. For example, strains isolated from turbot reacted only with the antiserum derived from turbot isolates. Similar findings were noted for sole and gilthead sea bream strains against the antiserum obtained against the sole isolate. No such pattern was found in this investigation. The serum used in the current study was raised against an Atlantic salmon strain. Isolate 00/3280

was collected from rainbow trout, Baxa lyl-1 from Japanese flounder and NCIMB 2154^T strain from red sea bream, all of which reacted to the antiserum.

LPS analysis revealed further antigenic heterogeneity within the species, with three other patterns evident. Antigenic differences have previously been demonstrated among *T. maritimum* isolates from Atlantic salmon using immunoblot analysis of LPS (Ostland *et al.* 1999) where differences were detected among isolates and between the isolates and reference strains (NCIMB 2153, 2154^T). Although initially described as antigenically homogeneous (Wakabayashi *et al.* 1984), *T. maritimum* is antigentically heterogenous (Ostland *et al.* 1999, Avendaño-Herrera *et al.* 2004). In addition, these differences have been attributed to the O-antigen side chains which may be associated with different strains from outbreaks on both temporal and geographical aspects (Ostland *et al.* 1999). The current study supports these findings; however, further investigation is required, particularly into defining serotypes.

Agglutination studies were not attempted due to the cohesive nature of *T. maritimum.* It has been consistently found that isolates from this species clump together regardless of the method in which they were grown (broth or agar). Producing a homogeneous suspension proved difficult and when spun down, the pellet formed at the bottom of the microcentrifuge tube would adhere to itself (become 'sticky') and required additional physical agitation (e.g., glass beads) to ensure dispersal into suspension. An interesting point to note is that initially there was an attempt to determine LPS profiles using silver staining. While this method produced clear laddering for *Escherichia coli* and *Lactococcus garvieae* isolates, it proved unsatisfactory for *T. maritimum* isolates as banding was undistinguishable within the profile (Figure 2.3). Some proteins are thought to be resistant to digestion with proteinase K (Sørensen *et al.* 1992) and *T.*

maritimum could contain such proteins. Probing the LPS profiles with antiserum was required to reveal the detail of the banding pattern.

Extracellular products (ECP) are compounds secreted by bacteria and thought to facilitate invasion of the host (Dalsgaard 1993). Although toxic activities of *T. maritimum* have been investigated previously (Baxa *et al.* 1988c), this is the first account of ECP protein profiles for *T. maritimum*. In the present study, all isolates were harvested in the stationary phase (72 hours), as this yielded the greatest quantity of ECP (R. van Gelderen, unpublished). This also allowed minimal growth-phase effects as different proteases are produced at different times of the growth curve (Gudmundsdóttir 1996). Duplicate strains of bacteria to produce ECP were used to confirm reproducibility of results. The ECP findings suggest significant heterogeneity within the species. Exotic isolates NCIMB 2154^T and Baxa lyl-1 produced the same profile, while all local isolates were divided into seven groups separate from the exotic isolates. The two isolates which consistently differed from all others (89/4747 and 01/0356-7) also showed individual profiles different from each other. There was no correlation between year groups or geographical locations.

Heterogeneity of ECP is present in other marine pathogenic bacterial species including *Aeromonas salmonicida* (Gudmundsdóttir 1996). Out of 32 strains analysed, six different protease groups were detected. All typical strains belonged to a single group, while atypical strains showed significant differences from the typical strains and between each other. It was suggested that ECP production was more strongly associated with geographic locations than the host fish species. Our results indicate that host is not an important factor as isolate 00/3280 (from rainbow trout) grouped with 00/0422-3 (from Atlantic salmon), although both fish species

are closely related. The type strain NCIMB 2154^T and Baxa lyl-1 are also from different host species, yet are grouped together having identical protein profiles. However, from a geographical perspective, they are distinct from our local isolates.

The diversity of profiles brings up an interesting question: what does this variation mean? This is not a topic discussed in the literature and is based on more than just protein profiles. All isolates share proteins at 46.5 and 73 kDa. These two proteins may be important and require further investigation to determine their significance for pathogenicity. It may be that there is no significance at all; isolate 89/4747 contains these two proteins but is believed to be non-pathogenic (Powell *et al.* 2004). It has been previously stated that ECP may contribute to the pathogenicity of *T. maritimum* in red and black sea bream although there were no strong correlations between *in vivo* and *in vitro* effects (Baxa *et al.* 1988c). The association between ECP and toxicity *in vivo* is reported in Chapter 4 of this thesis.

Attachment by bacterial pathogens to different substrates is considered to be an important step in successful colonisation of host cells as this has the potential to lead to a disease outbreak (Vatsos *et al.* 2001). A number of techniques are employed to assess non-specific and specific attachment to different substrates e.g., salt aggregation test (SAT), phase partitioning with hydrocarbon solvents, and adherence to nitrocellulose filters (NCF). In the present study, adherence to three hydrocarbons was used to assess the surface hydrophobicity of *T. maritimum.* It is recommended that more than one assay be used to obtain a reliable measurement of hydrophobicity (Dillon *et al.* 1986, Santos *et al.* 1990). Only one assay was used here because the focus of this study was to assess similarities and differences between isolates. The one assay

allowed a comparison of isolates not variations of the one isolate. There was also no attempt to link hydrophobicity to attachment and virulence *in vivo*.

In the current study, most of the *T. maritimum* isolates possessed a weak hydrophobicity when tested with *n*-hexadecane. Moderate to strong hydrophobicity was apparent with *n*-octane, while isolates showed strong hydrophobicity when tested with *p*-xylene. Two exceptions were isolates 89/4762 and Baxa lyl-1 that possessed strong hydrophobic tendencies with all three hydrocarbons. Overall, all isolates were hydrophobic and as surface area of the alkane molecule decreased, hydrophobicity increased. Variations in assay type, isolate and growth media used will invariably provide disparity in results. Previous work by Kawahara and Kusuda (1998) on the hydrophobicity of *T. maritimum* described their isolate as weakly hydrophobic while, as with this study, there was an increase in hydrophobic tendencies related to the surface area of the hydrocarbon used. In another study, while there was variation in tendencies between assays and isolates, it was concluded that all *T. maritimum* isolates used were hydrophobic (Sorongon *et al.* 1991). Hydrophobicity of bacterial isolates can also be affected by different growth media (Magariños *et al.* 1996). In the current study marine Shieh's broth (MSB) was used as the culture medium. Kawahara and Kusuda (1998) did not state which growth medium they used, while Sorongon *et al.* (1991) used Chrome Azurol S (CAS) medium in different forms. However, in general, *T. maritimum* can be described as hydrophobic.

However, does the hydrophobicity of isolates translate to attachment to the host or virulence? Measures of hydrophobicity do not always correlate well to either attachment or virulence. López-Cortes *et al.* (1999) reported no significant relationship between hydrophobicity and cell attachment with *Vibrio tapetis* in clam cells. It was concluded that there were other attachment

specific mechanisms that play a role in cell adhesion. There also needs to be consideration as to whether attachment equates to virulence as well. The virulence of the bacterium *Photobacterium damselae* subsp. *piscicida* (formerly '*Pasteurella piscicida*') was found not to correlate with its ability to attach to host cells. Attachment was related to the presence of a capsule layer around the bacterial cells of virulent strains (Magariños *et al.* 1996). In contrast, a strong association was found between virulence and surface hydrophobicity of *Renibacterium salmoninarum* (Grayson *et al.* 1995). Association between surface hydrophobicity, attachment and virulence has not been investigated for *T. maritimum*. A comparison of surface hydrophobicity and virulence using three of the isolates used in this study are reported in a pathogenicity trial in Chapter 3. For the purpose of this study, the isolates were ranked to determine their selection in this experiment.

In conclusion, the Tasmanian strains of *T. maritimum* appear relatively homogeneous physically, but antigentically different. All isolates are hydrophobic and produce a variety of ECP profiles. There are two isolates that stand out in all assays (89/4747 and 01/0356-7) and show great variation from the other isolates. All isolates have been confirmed as *T. maritimum*. Three isolates have been chosen for subsequent pathogenicity trials: 89/4747, 89/4762, 00/3280. Isolate 89/4747 was chosen due to its differences with other isolates from the characterisation tests. While not yet confirmed, it is likely that this strain is a different serotype to the others. Strain 89/4747 appeared only moderately hydrophobic, ranked 14th out of the 20 isolates, with similar properties to the 10th ranked strain 00/3280, also considered moderately hydrophobic. Both 00/3280 and 89/4762, however, showed similarities to each other and most other isolates in whole cell protein profile and serotype. In the ECP groupings, 89/4762 occurred within a

common group, whereas 00/3280 was grouped with only one other isolate with a distinct electropherotype. This contrasts with the LPS groupings, where 00/3280 was similar to most other isolates while conversely 89/4762 was different and placed into a minor group. Isolate 89/4762 was the most hydrophobic of all the isolates and was included to determine if this characteristic was a marker of virulence.

However, there is an interesting quandary concerning isolate 89/4747. Carson *et al.* (1992) used this isolate as part of its investigation into pathogenicity of *T. maritimum* in rainbow trout. They employed IFAT as a confirmatory test for the presence of the pathogen with the same anti-serum used in the present study. Isolate 89/4747 in this case was IFAT positive and pathogenic. Over ten years later and the same isolate is conclusively IFAT negative based on current findings. This change has also appeared to have affected pathogenicity; use of the same isolate and serum for an experimental induction of gill disease in Atlantic salmon reported an IFAT negative result and a non-pathogenic response (Powell *et al.* 2004). It is unknown wether pathogenicity can be restored and how many passages it took to cause the non-pathogenic effect. It is likely that an outer membrane component has changed, but what part/s and the mechanisms involved are unknown. Age of isolate, preservation, attenuation and culture media are possible causes and require further investigation.

Chapter 3

Pathogenicity of *Tenacibaculum maritimum* to Atlantic salmon

3.1 Introduction

Pathogenicity relates to the ability of bacteria to produce disease in a host organism. Bacteria express pathogenicity via virulence, which refers to the degree of damage to the host (Alcamo, 1983). The interaction between a pathogen and its host is dynamic as both are able to modify the activities and functions of the other. Infection of the host will depend not only on virulence of the pathogen, but also infectivity of the pathogen and susceptibility of the host (Madigan and Martinko, 2006). Understanding the pathogenicity of *Tenacibaculum maritimum* could result in prevention of the disease, improved treatment or development of a vaccine.

The development of a vaccine against *T. maritimum* requires the pathogenicity of the organism to be confirmed by Koch's postulates. These state that a disease causing organism isolated from their host must be able to be grown *in vitro* and then reintroduced to a naive susceptible host. The resulting challenge should produce similar disease signs and the organism must be recovered from the host. Additionally, a model of infection should be developed in order to determine the efficacy of experimental vaccines and in particular to establish an LD_{60} for challenge in the vaccine trial (Chapter 5). The development of infection models under different parameters (growth conditions, strains and doses) would also provide insight into the nature of the pathogen.

Growth media can affect the expression of bacterial characteristics including those that determine virulence. Marine Shieh's (Song *et al.* 1987) and Ordal's (Anacker and Ordal 1959) are the two growth media used to previously investigate *T. maritimum* in Tasmania. Pilot studies using marine Shieh's (MS) as the growth medium initially yielded non-pathogenic results (R

van Gelderen, unpublished). It is believed that attenuation of the bacteria through routine bacteriology caused the non-pathogenic results observed in earlier trials of the current study. As previous studies used marine Ordal's (MO) as the growth medium (Soltani 1995, Carson *et al.* 1992), it was determined that a comparison of media using the one strain (89/4762) was worth investigating. In addition, anecdotal evidence from the current study growing *T. maritimum* in broth revealed a difference in the 'clumping' of strains. Aeration of broth cultures appeared to assist in obtaining a homogeneous suspension. This has the potential to affect pathogenicity as it is suspected that 'clumping' is associated with virulence; passaged strains became stickier on agar while attenuated strains lost adherence (R van Gelderen, unpublished). Therefore, the presence of aeration during broth culture was also investigated.

Differences in pathogenicity can also be observed between isolates or strains of the same species depending on the presence or absence of different virulence factors (Alcamo, 1983). In the previous chapter, 18 local isolates were characterised. Three of these strains were chosen for comparison of pathogenicity (89/4747, 89/4762, 00/3280). This was based on their physical characterisation (Chapter 2). Strain 89/4747 was chosen due to its differences with other isolates from the characterisation tests. While not yet confirmed, it is likely that this strain is a different serotype to the others. Strain 89/4747 appeared only moderately hydrophobic, ranked 14th out of the 20 isolates, with similar properties to the 10th ranked strain 00/3280, also considered moderately hydrophobic. However, both 00/3280 and 89/4762 showed similarities to each other and most other isolates in whole cell protein profile and serotype. In the ECP groupings, 89/4762 occurred within a common group, whereas 00/3280 was grouped with only one other isolate with a distinct electropherotype. This contrasts with the LPS groupings, where 00/3280

was similar to most other isolates while conversely 89/4762 was different and placed into a minor group. Strain 89/4762 was the most hydrophobic of all the isolates and was included to determine if this characteristic was a marker of virulence. One strain will be selected to compare doses since virulence can be better understood by investigating dose and comparing the number of cells required to kill different proportions of the population (Madigan and Martinko, 2006). The comparison will be used to establish an LD_{60} which will subsequently be used in a vaccine trial.

There has been previous work investigating the pathogenicity of *T. maritimum* in several fish species in Australia (Carson *et al.* 1992, Soltani 1995, Handlinger *et al.* 1997). In particular, Soltani (1995) challenged Atlantic salmon under various conditions including culture media (shaken vs. stirred), age of fish, seawater salinity, dose, exposure, and methods of challenge. However, questions in the current study relate to anecdotal evidence regarding the growth of the pathogen and its relationship to pathogenicity and a single goal; to produce an experimental vaccine.

The aims of this study were to:

1. Determine the pathogenicity of *T. maritimum* under different culture conditions;
    a) using different culture media,
    b) using aeration,
2. Assess differences in pathogenicity between three strains,
3. Assess virulence by measuring differences in challenge doses for one strain,
4. Describe the pathogenesis of the disease using an experimental infection,
5. Assess the pathology due to *T. maritimum* under experimental conditions,
6. Determine an LD_{60} for future assessment of the efficacy of an experimental vaccine.

3.2 Materials and Methods

3.2.1 Pathogenicity experiments

Three different methods were used to assess the pathogenicity of *T. maritimum* in Atlantic salmon (Table 3.1). The first experiment was a comparison of methods not only of different growth media, marine Shieh's (MSB) and marine Ordal's (MOB), but also with (+A) or without (-A) aeration during culture of bacteria; the assessment was made with strain 89/4762. The second was a comparison of pathogenicity of strains (89/4762, 89/4747, 00/3280) and the third compared effects of dose for the one strain (89/4762). Assessment of culture media and strains on pathogenicity was made in a semi-static experiment. As a recirculation system was used in the third experiment there were two different control treatments. One control treatment was within the recirculation system (control in) and another, which was still part of the system, but had a separate water source (control out). The system was set up with a filter bank down to 0.8 μm for incoming water and two UV lights (400 J/m^2, 4000 L/h) to reduce bacterial load.

Table 3.1 Methods used to assess pathogenicity of *Tenacibaculum maritimum* using bath challenge.

Experiment	Isolates	Dose (cells/mL)	Method of challenge	Tanks
1	89/4762	1×10^7	Bath	10 x 100 L semi-static
2	89/4762 89/4747 00/3280	1×10^7	Bath	10 x 200 L semi-static
3	89/4762	1×10^5, 1×10^6, 1×10^7, 1×10^8	Bath	12 x 200 L recirculation system

3.2.2 Experimental fish

Atlantic salmon (*Salmo salar*) smolts were obtained from a commercial hatchery. Fish were not graded prior to experimentation due to the limited availability of fish supplied by commercial hatcheries. However, fish were assigned to tanks in order to keep density consistent between tanks. The fish were initially kept in fresh water and then acclimated to seawater (35 ppt) over a week starting at 5 ppt. They were fed on a commercial diet (Atlantic HP - Skretting) at maintenance (1% body weight) during acclimation and feeding ceased 48 hours before challenge. Numbers of fish, replicates, weight, lengths and water conditions for each experiment are summarised in Table 3.2.

Table 3.2 Fish information for each pathogenicity experiment and water parameters.

Experiment	Fish No. and (tanks)	Weight range (g) (mean)	Length range (cm) (mean)	Temp. (°C) and Salinity (ppt)
1	8 (2)	29.2 – 150.8 (86.2)	14.5 – 25.2 (23.5)	16.5, 35
2	15 (2)	14.6 – 214.6 (72.4)	11.7 – 27.6 (19.1)	18.5, 35
3	15 (3)	47.6 – 138.3 (88.1)	16.2 – 23.9 (20.9)	18.0, 35

3.2.3 Bacteria cultures

Three strains of *T. maritimum*, two collected from Atlantic salmon (89/4762, 89/4747) and one from rainbow trout (00/3280) were 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 (MSA) at 25°C for 24 hours and used as inoculum in 500 mL (in a 2 L glass conical flask) marine Shieh's broth (MSB) at room temperature (20°C) for another 48 hours. The culture was decanted and washed three times in filtered seawater by centrifugation at 3000 x *g* for 30 minutes and 0.2 µm sterile filtered with seawater. Cell concentration was estimated using a spectrophotometer at

550 nm. The only variation to this method was in experiment 1 where marine Ordal's broth (MOB) was used in addition to MSB to grow strain 89/4762. Each medium was also divided into two treatments: with and without aeration. With aeration meant that air was supplied by an air pump without a diffuser through a 0.2 μm filter at a rate of 120 L/hr. Without aeration meant the 2 L conical flask was placed on an orbital shaker (90 rpm) with no addition of air.

3.2.4 Challenge procedures

Fish were bath challenged in 100 L semi-static tanks in 35 ppt seawater for 1 hour before being randomly distributed into assigned tanks.

3.2.5 Sampling procedures

Fish were checked 3 times a day for morbidity/mortality and those affected were anaesthetised using clove oil (0.03 mL/L seawater). The experiment was concluded when there were 3 consecutive days of no mortalities. All fish were examined bacteriologically by culturing skin lesions or sites of erosion (no internal organs were sampled) on plates of MSA (Figure 3.1b) containing 4 μg/mL neomycin sulfate (Pazos *et al.* 1996). Smears (1 cm x 1 cm) were prepared for staining by Gram's method and by immunofluorescence (IFAT) (Figure 3.1a, c). Cultures were incubated at 25°C for 2 days and the colonial morphology typical of *T. maritimum* was recorded. Flexibacteriosis lesion distribution and lesion size were also documented, except for experiment 1 where only lesion distribution was recorded. 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 half centimetre. 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.

3.2.6 Case definition

The criteria for accepting that a fish was diseased involved two parts: recovery and quantification of colonies and cells. *Tenacibaculum maritimum* had to be recovered from mortalities by culture and in smears by IFAT as these methods are more specific than Gram stain. The minimum abundance of cells required for a positive result was 10-50 bacteria per 25 fields for IFAT and Gram stain. The minimum abundance required to record a positive result in culture was growth of colonies extending to the first streaking lines.

3.2.7 Histological examination

Initially the samples were fixed in seawater Davidson's (Shaw and Battle 1957, Speilberg *et al.* 1993) for 72 hours 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 x40 – x1000 for examination of lesion necrosis and the results were compared with gross lesion photographs.

3.2.8 Survivors

Any fish surviving at the end of the challenge period were euthanised using an overdose of clove oil (0.06 mL/L seawater) and lesions or sites of erosion examined bacteriologically as described.

3.2.9 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 µL of rabbit anti- *T. maritimum* 89/0329-5 (DPIW) diluted 1:100 in phosphate buffered saline (PBS) (pH 7.2, 0.1M) and incubated in a moist chamber for 30 minutes at 37°C before rinsing in PBS for 15 minutes. After the removal of excess buffer by blotting, 20 µL of anti-rabbit FITC (Silenus) diluted 1:60 in PBS was added to each slide. Smears were incubated at 37°C for 30 minutes and rinsed for 30 minutes in PBS, which was changed every 10 minutes. Slides were mounted using alkaline glycerol buffer (Johnson and Munday 1993), coverslipped and examined at x 40 magnification with epifluorescent microscopy using UV illumination.

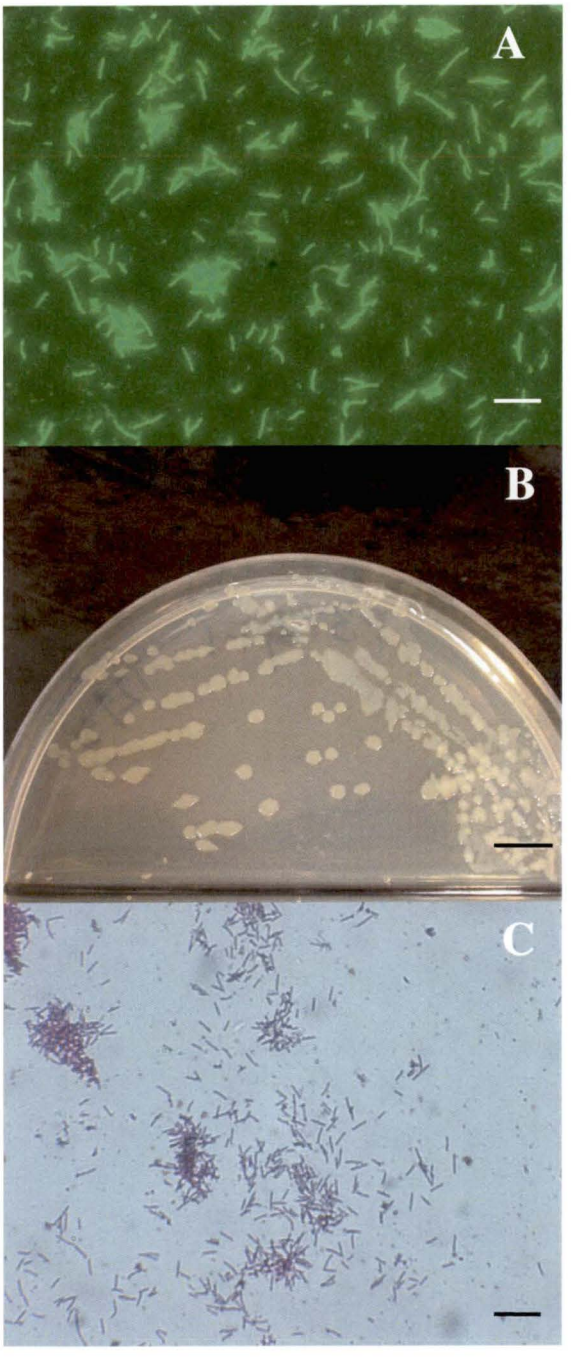

Figure 3.1 Methods of confirmation for the presence of *Tenacibaculum maritimum*: A, IFAT (scale bar 10 μm); B, colony morphology grown on marine Shieh's at 25°C (scale bar 1 cm); C, Gram stain at x40 magnification (scale bar 20 μm).

3.2.10 Immunohistochemistry

Histological sections were probed immunhistochemically based on the method by Adams and Nowak (2003). Tissue used in this procedure was fixed as previously described and sections were cut (5 μm) and mounted on polylysine coated slides (Menzel-Glaser, Braunschweig, Germany). 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 minutes then allowed to stand for a further 20 minutes. Following HIER and a brief rinse in deionized water (DIH_2O), sections were blocked for endogenous peroxidase (10 min in 3% H_2O_2 in 100% methanol) washed in PBS and incubated (20°C) with 0.1% bovine serum albumin (BSA) for 20 minutes. Sections were then incubated at room temperature with a primary antibody in 0.1% BSA (rabbit anti-*T. 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 (DAB) in peroxide buffer (Zymed) for 1.5 minutes, then rinsed in DIH_2O, counterstained with Mayer's haematoxylin for 30 seconds, rinsed in H_2O, differentiated in PBS, dehydrated, cleared and mounted. The use of normal rabbit serum served as a negative control (Figure 3.2b). A plug made from 200 μL of 2% agarose (w/v) in PBS (pH 7.4) containing 200 μL of *T. maritimum* cell pellet (fixed and processed as above) was used as a positive control (Figure 3.2a).

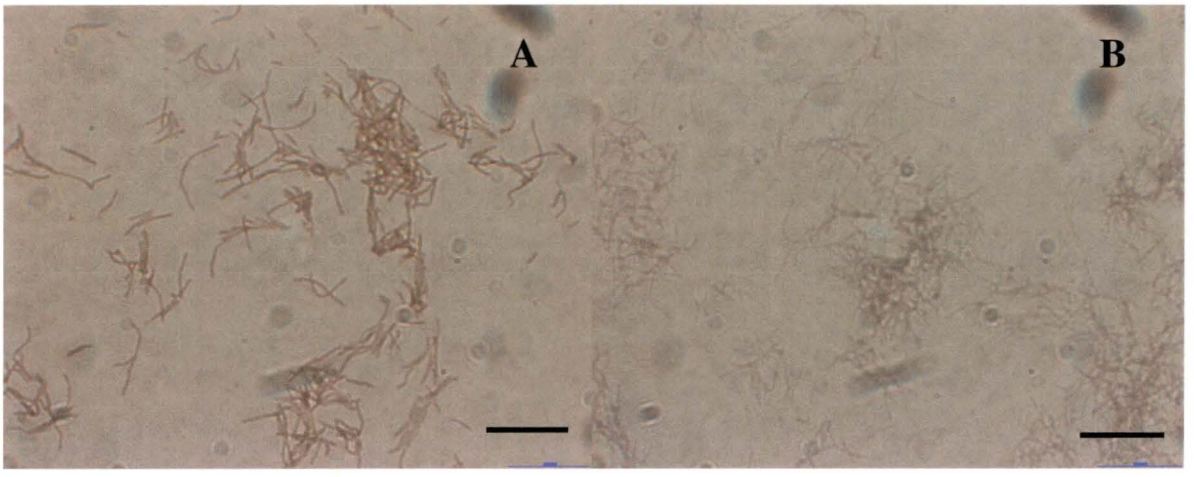

Figure 3.2 Immunohistochemistry for *Tenacibaculum maritimum*; A, positive control; B, negative control. Scale bars 10 μm.

3.2.11 Statistical analysis

Data were analysed using Kaplan-Meier survival analysis and a pair-wise comparison over strata with a Bonferroni correction of *p*-values using SPSS 11.5 for Windows. The LD_{60} for the experimental period was calculated based on the work of Reed and Muench (1938). An LD_{60} was determined rather than an LD_{50} since it has become a standard in testing vaccines against a higher mortality rate to ensure efficacy of vaccines.

3.3 Results

3.3.1 Experiment 1

Fish challenged with *T. maritimum* grown in either MO or MS with aeration had cumulative mortalities of 93.75 % and 87.5 %, respectively (Figure 3.3). The MS grown bacteria produced mortalities in a shorter amount of time than MO after the ten day experimental period. The MO infected fish, however, had a higher mortality rate. *Tenacibaculum maritimum* grown in either MO or MS without aeration caused similar mortality profiles to each other and achieved cumulative mortality rates of 68.75 % and 73.3 %, respectively within the 10 day experimental period. There were no mortalities in the control treatment. Kaplan-Meier survival analysis showed significant differences between curves ($p \leq 0.0001$). Pair-wise comparison showed that a statistical difference occurred between the control treatment and all challenge treatments ($p \leq 0.005$). There was no significant difference between all challenge treatments (i.e. no significant difference between all MO and MS cultures) even though a distinct divergence in mortalities appeared between bacteria grown with and without aeration.

The gross pathology of these fish included skin erosion around the jaw and sites adjacent to the fins, discrete areas of raised skin, erosion on the skin and fraying and hyperaemia of the fins. The percentage of gross pathology at the various sites (e.g. jaw) was the same between all treatments and therefore the data were pooled together (Figure 3.4). Most fish displayed skin erosion on the jaw (16%), pectoral (20.8%) and pelvic (14.2%) sites as well as the dorsum (19.8%). Pectoral and pelvic sites include the fins and the skin area surrounding them since both areas were affected. Lesions on the ventral and lateral sites were prevalent (7.5% and 8.5%, respectively) as was gill necrosis (8.5%). Rarer cases of absent or ruptured eyes (1.9%) and

lesions on the caudal site (2.8%) were also noted (Figure 3.4). All sites of erosion were consistent with lesions associated with natural infections. Although lesion size was not recorded for this experiment it was apparent that larger lesions developed on fin sites while smaller lesions occurred on the body, in particular the dorsum. It was also noted that infected fish darkened (mainly fin margins) and became listless. Of the survivors, no lesions were present, only small breaks in the skin in isolated areas.

Methods to detect the presence of *T. maritimum* showed that plate culture was less sensitive than Gram stain or IFAT (Table 3.3). MS culture plates containing 4 µg/mL neomycin sulfate was used to select for *T. maritimum* and while growth of other organisms were rarely observed, a lack of growth on the plates was more common.

Table 3.3 Comparison of media culture, Gram stain and IFAT preparations for the detection of *Tenacibaculum maritimum* in Atlantic salmon. MS = marine Shieh's medium, MO = marine Ordal's medium, +A = with aeration, -A = without aeration.

Treatment	Culture (%) mean (± S.E.)	Gram (%) mean(± S.E.)	IFAT (%) mean (± S.E.)	Lesion
MS-A	58.3 (8.3)	83.3 (16.6)	100 (0)	+
MO-A	78.6 (7.1)	92.8 (7.1)	92.8 (7.1)	+
MS+A	66.9 (4.6)	100 (0)	93.3 (6.2)	+
MO+A	78.6 (7.1)	92.8 (7.1)	100 (0)	+
Control	0 (0)	0 (0)	0 (0)	-

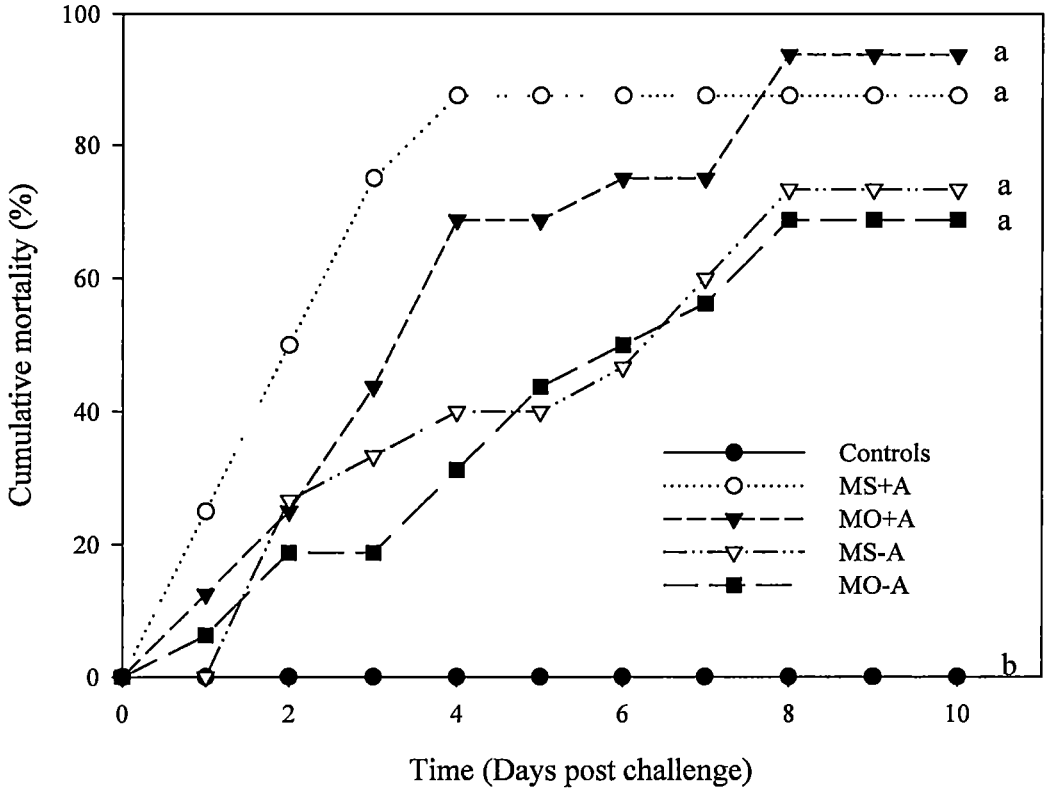

Figure 3.3 Cumulative mortality (%) of Atlantic salmon challenged with *Tenacibaculum maritimum* strain 89/4762 grown on different media and with different aeration conditions. Lines with different letters are significant ($p \leq 0.005$).

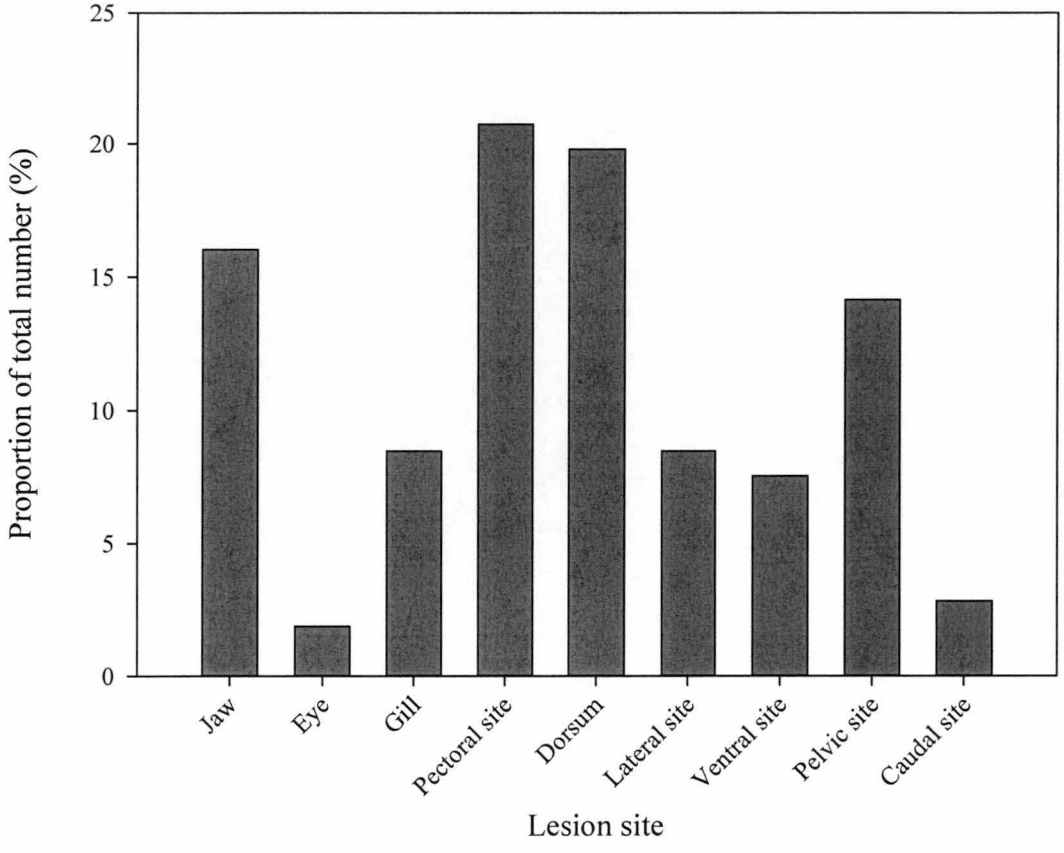

Figure 3.4 Distribution of lesions in Atlantic salmon challenged with *Tenacibaculum maritimum* strain 89/4762 grown on different media and with different aeration conditions.

3.3.2 Experiment 2

Mortalities reached 100% for strains 00/3280 and 89/4762 within three days of challenge (Figure 3.5). On the second day, strain 00/3280 had mortalities of 81.4% while strain 89/4762 only had mortalities of 31.1%. In contrast, the control treatment and strain 89/4747 had no mortalities within the experimental period. No statistical operations were performed on this data as the differences between treatments were obvious. Results for detection of *T. maritimum* were consistent between treatments and with challenge results, showing high re-isolation values except in the non-pathogenic strain 89/4747 (Table 3.4). At the end of day three, water samples from all tanks were serially diluted in filtered seawater, plated onto Shieh's medium, incubated at 25°C for three days and then counted. The results revealed that the bacterial concentration of *T. maritimum* in the tanks with 00/3280 and 89/4762 were approximately 3.1×10^8 and 2.8×10^8 cells/mL, respectively.

Table 3.4 Comparison of media culture, Gram stain and IFAT preparations for the detection of *Tenacibaculum maritimum* in Atlantic salmon.

Treatment	Culture (%) mean (± S.E.)	Gram (%) mean (± S.E.)	IFAT (%) mean (± S.E.)	Lesion
00/3280	100 (0)	100 (0)	100 (0)	+
89/4762	100 (0)	96.6 (3.3)	100 (0)	+
89/4747	43.3 (10.0)	23.3 (10.0)	0 (0)	-
Controls	0 (0)	0 (0)	0 (0)	-

In terms of gross pathology, the main clinical sign was disintegration of the epithelial layer. Tail necrosis was found in all cases ranging from moderate tail necrosis to absence of caudal fin. All fish had extensive scale loss, areas of raised skin and erosion sites. The distribution of lesions was similar between the different strains (Figure 3.6). The pectoral site and dorsum were the main areas affected. There was a higher proportion of pectoral sites affected in the fish challenged with 89/4762 (29.3%) than 00/3280 (22.8%). It was also noted that there was a greater proportion of gill necrosis, particularly with strain 89/4762 (12.1%), while the number of lesions around the jaw area and pelvic site was lower in this experiment. Most of the lesions were 1.5 cm in size or less for both strains (Figure 3.7). Few lesions appeared above that size. For strain 00/3280, the majority of lesions appeared between 0.5-1.0cm (43.8%), while for 89/4762, most were found between 1.0-1.5cm (40.0%).

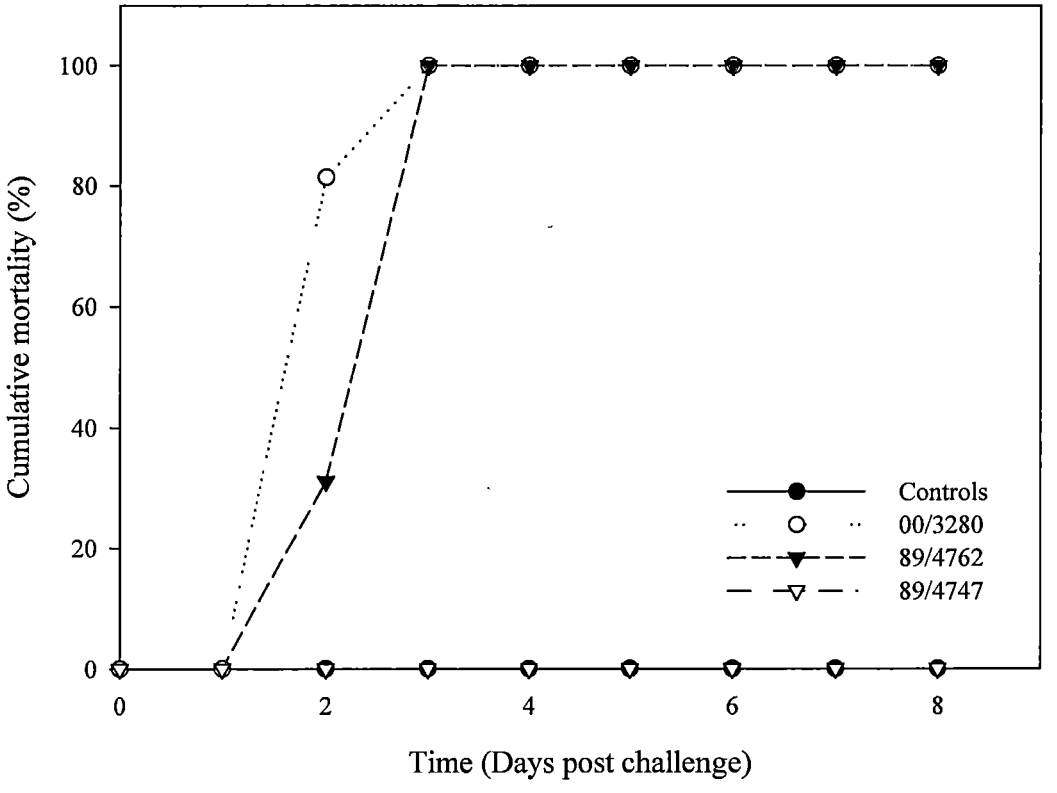

Figure 3.5 Cumulative mortality (%) of Atlantic salmon challenged with different strains of *Tenacibaculum maritimum*.

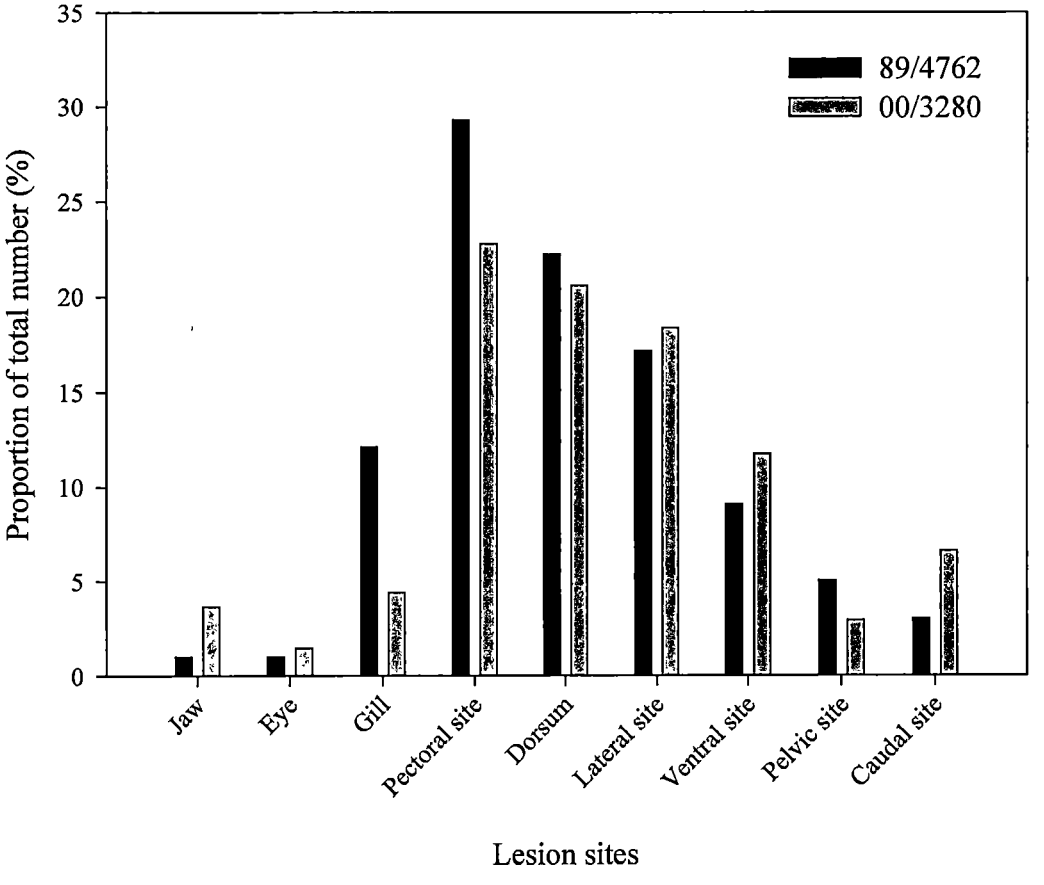

Figure 3.6 Distribution of lesions in Atlantic salmon challenged with *Tenacibaculum maritimum* strains 89/4762 and 00/3280.

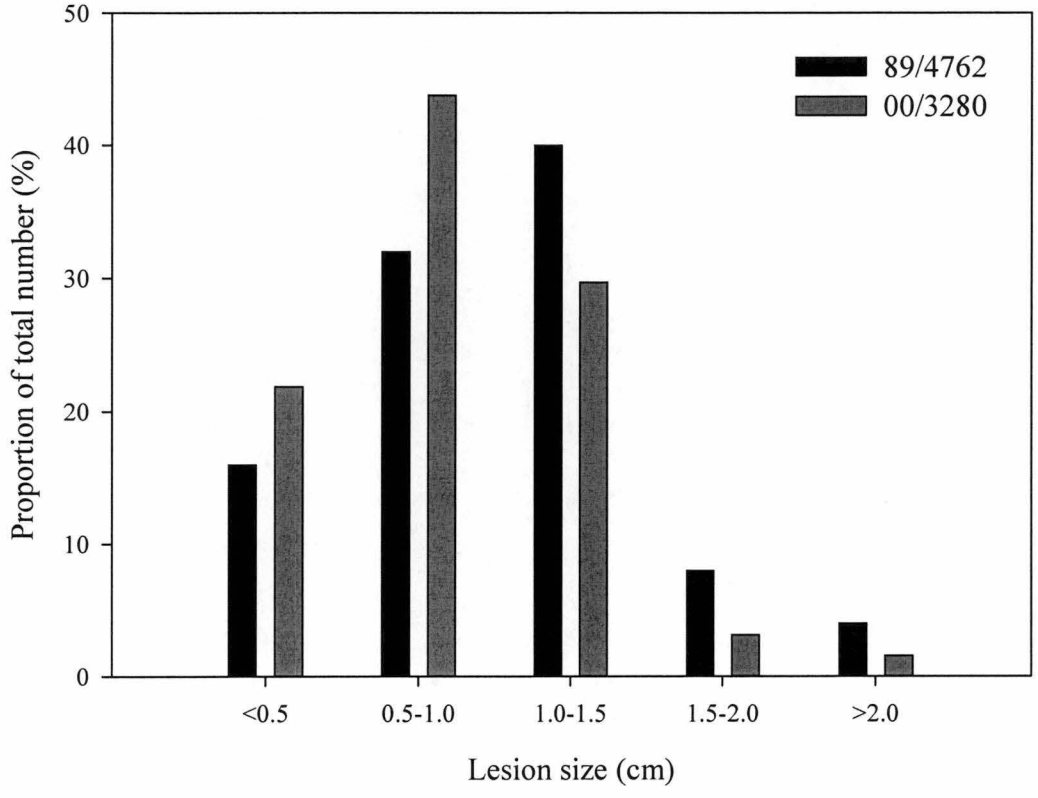

Figure 3.7 Lesion size (%) in Atlantic salmon challenged with *Tenacibaculum maritimum* strains 89/4762 and 00/3280.

3.3.3 Experiment 3

Fish challenged with the highest dose of 1 x 10^8 cells/mL had mortalities that reached 100% within 4 days of the experiment commencing (Figure 3.8). The percent mortality decreased with decreasing dose size and no mortalities were recorded in the control treatment that was outside of the immediate experimental system. Controls within the system recorded mortalities of 17%, however, only one mortality was recorded for this treatment before day sixteen; 7 fish subsequently died within the last 5 days of the experiment. A dose of 1 x 10^6 cells/mL yielded a 60% mortality rate and therefore established an LD_{60} for the vaccine trial based on this experimental system. Kaplan-Meier survival analysis showed significant differences between curves ($p \leq 0.0001$). Pair-wise comparisons showed that significant differences occurred between the highest dose (1 x 10^8 cells/mL) and all other doses as well as both control treatments. There was no difference between doses 1 x 10^7 cells/mL and 1 x 10^6 cells/mL. Similarly there was no significant difference between the 1 x 10^5 cells/mL dose, controls (in) and controls (out). All other combinations were statistically significant ($p \leq 0.0033$). Methods used to detect the presence of *T. maritimum* showed that the bacteria could be easily re-isolated from the fish with most of the tests achieving at or just below 100% (Table 3.5).

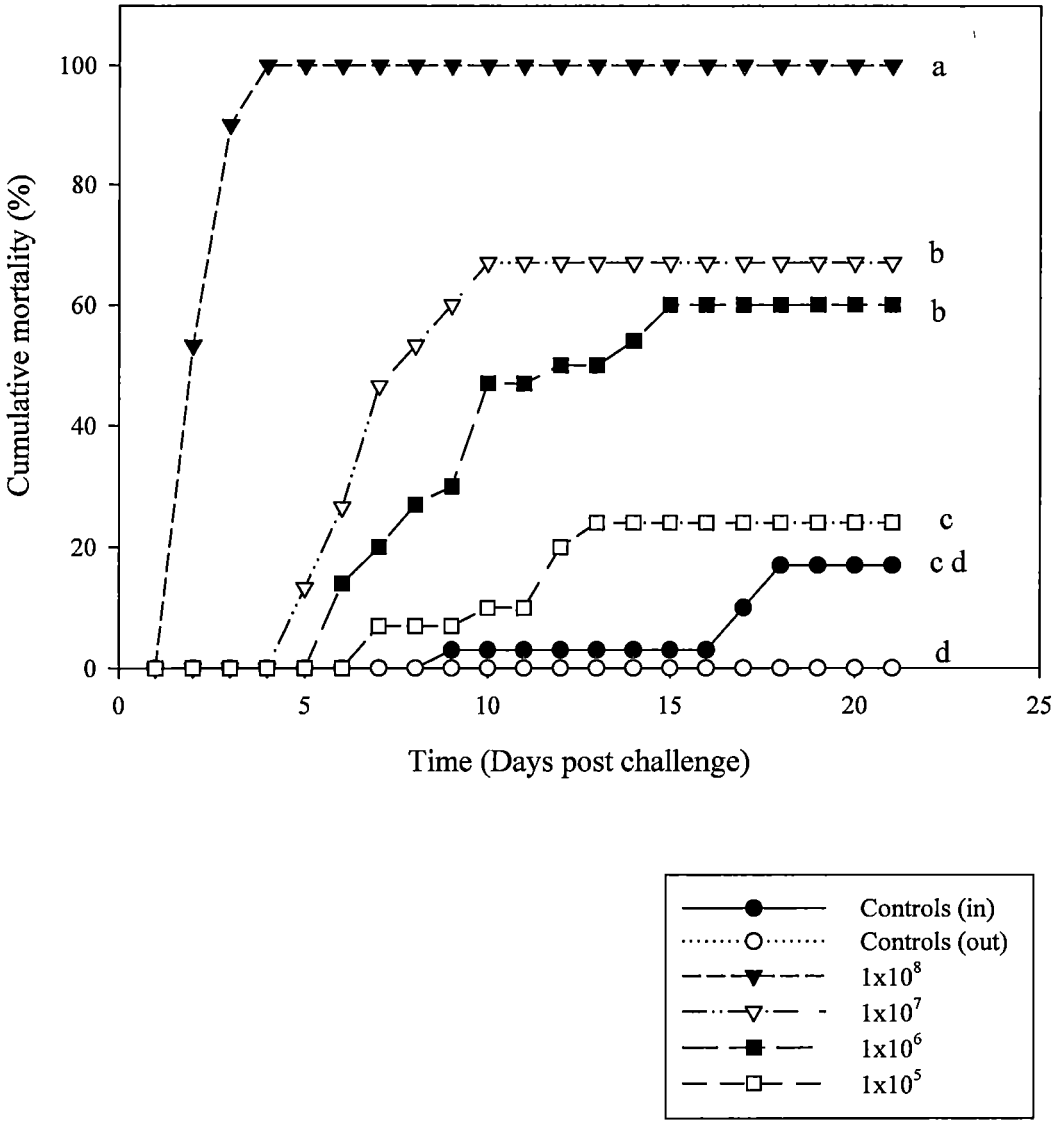

Figure 3.8 Cumulative mortality (%) of Atlantic salmon challenged with different doses of *Tenacibaculum maritimum* strain 89/4762. Lines with different letters are significantly different ($p \leq 0.0033$).

Table 3.5 Comparison of media culture, Gram stain and IFAT preparations for the detection of *Tenacibaculum maritimum* in Atlantic salmon.

Treatment	Culture (%) mean (± S.E.)	Gram (%) mean (± S.E.)	IFAT (%) mean (± S.E.)	Lesion
1×10^5	100 (0)	97.8 (2.1)	100 (0)	+
1×10^6	100 (0)	97.8 (2.1)	100 (0)	+
1×10^7	100 (0)	100 (0)	100 (0)	+
1×10^8	100 (0)	100 (0)	100 (0)	+
Control (in)	100 (0)	97.8 (2.1)	95.6 (4.3)	+
Control (out)	0 (0)	0 (0)	0 (0)	-

In this experiment, progression of the disease was clearly defined and two different effects evident. The first was the acute effect which occurs in the first few days of the experiment (Figure 3.9). The entire body of the fish was affected and the same response occurred in experiment 2. Disintegration of the epithelial layer was a consistent condition. There was extensive scale loss, areas of raised skin and some fish had erosion sites present. Cutaneous erosions were superficial in early mortalities, with some fish showing white areas of scale loss.

The second effect was chronic, took longer to develop and resulted in large lesions. It started with scale loss in discrete areas (Figure 3.10a). Swelling began to appear over the site and this swelling appeared to be of epithelial origin (Figure 3.10b). The destruction of the epithelium continued within a discrete area until the necrotic tissue was shed to reveal a lesion which continued to erode (Figures 3.10c, d). The underlying musculature was exposed and necrosis and oedema were evident (Figure 3.10e).

Pectoral, dorsal and lateral sites were the main areas where lesions developed (Figure 3.11). In the highest dose (1 x 10^8 cells/mL), 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 x 10^5 cells/mL treatment where the lateral site was the dominant area (28.9 %). The percentage of 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 two highest doses as well as the control (in) group. Lesion size was smaller in the higher doses (Figure 3.12). There is a shift to larger lesions as dose decreases; however, a peak in growth of lesion was apparent in the group challenged with 1 x 10^6 cells/mL.

Figure 3.9 An acute effect of a *Tenacibaculum maritimum* infection; A, control Atlantic salmon; B, Atlantic salmon exposed to 1 x 10^8 cells/mL where mortality occurred within 48 hours. The control fish were euthanized at the end of the experiment. Any scale loss would have been a result of handling the fish prior to taking the photograph. Scale bars 2 cm.

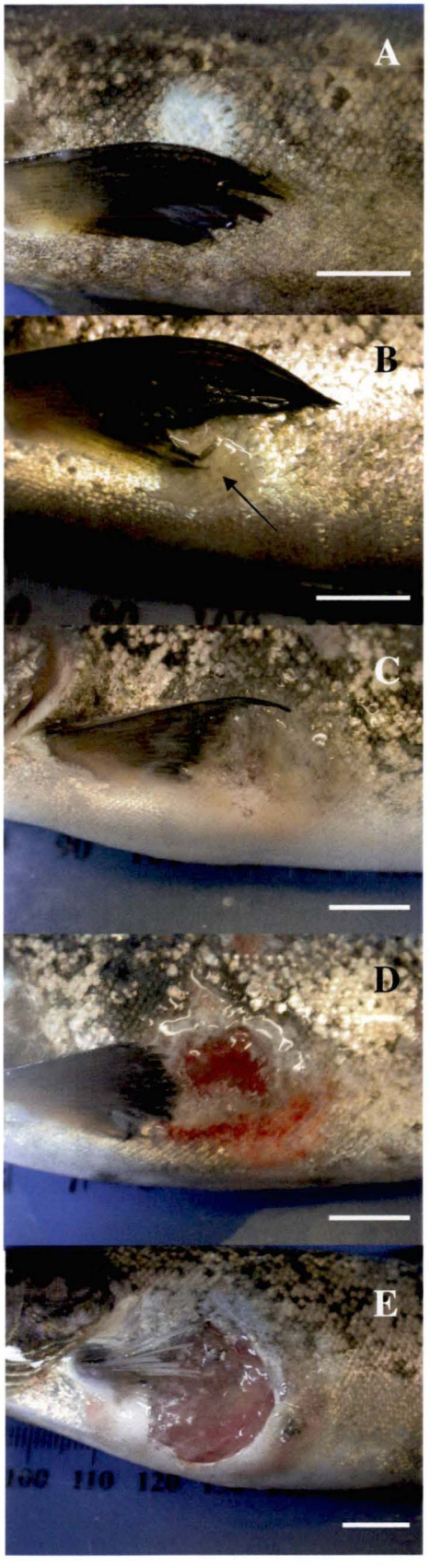

Figure 3.10 Formation of lesions in Atlantic salmon caused by *Tenacibaculum maritimum*: A, scales loss in discrete areas; B, swelling appears over site (arrow); C, destruction of the epithelium continues within a discrete area; D, necrotic tissue is shed to reveal lesion; E, lesion continues to erode, musculature is exposed and necrosis and oedema are evident. Scale bars 1 cm.

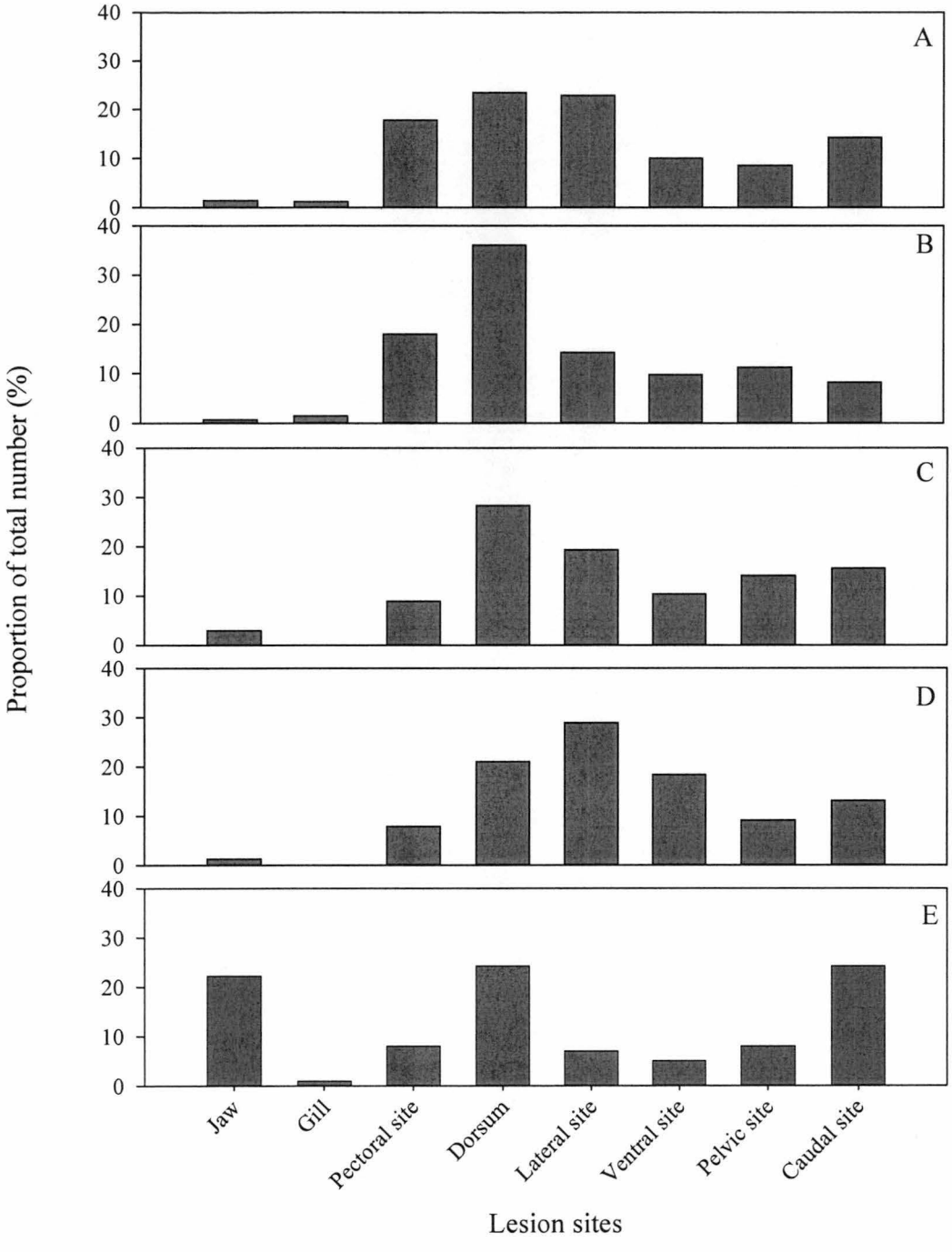

Figure 3.11 Distribution of lesions in Atlantic salmon challenged with different doses of *Tenacibaculum maritimum* strain 89/4762: A; 1 x 10^8 cells/mL; B, 1 x 10^7 cells/mL; C; 1 x 10^6 cells/mL; D, 1 x 10^5 cells/mL; E, control (in).

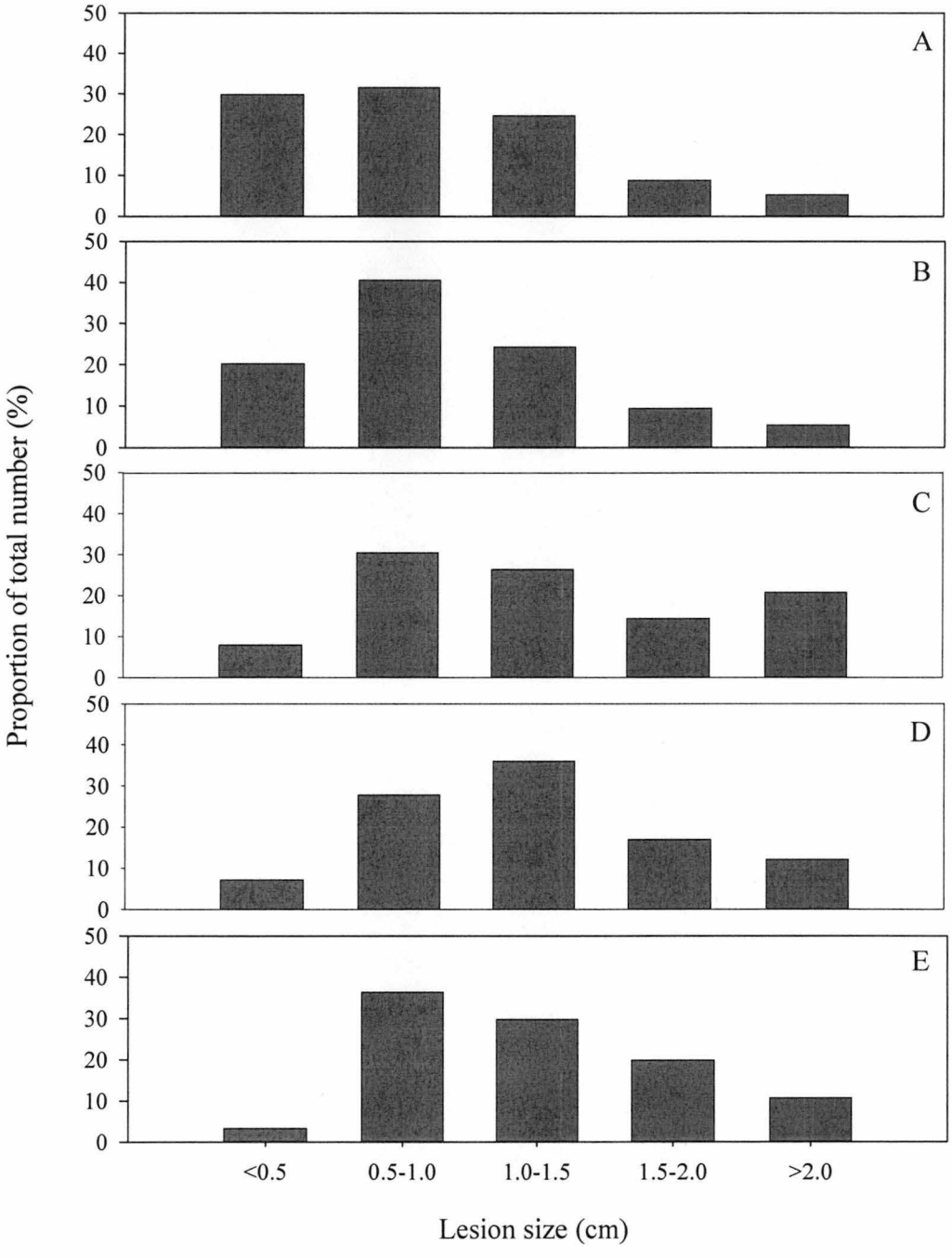

Figure 3.12 Lesion size (%) in Atlantic salmon challenged with different doses of *Tenacibaculum maritimum* strain 89/4762: A; 1 x 10^8 cells/mL; B, 1 x 10^7 cells/mL; C; 1 x 10^6 cells/mL; D, 1 x 10^5 cells/mL; E, control (in).

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 (Figure 3.13d) with instances of heavy filamentous bacterial mats encompassing several lamellae (Figure 3.13b). The presence of *T. maritimum* filamentous rods was confirmed by immunohistochemistry (Figure 3.13g) and Gram stain (Figure 3.13f). Adjacent uninfected lamellae showed minimal to no reaction.

Control fish skin showed normal skin structure of an epidermis with mucous cells and scales, the dermis, underlying fat and musculature (Figure 3.14a). The first step in the progression of a lesion began with scale loss and disintegration of the epidermis with scale pockets evident (Figure 3.14b). Loss of the epidermis (Figure 3.14c) preceded erosion of the dermis and exposure of the underlying musculature (Figure 3.14d). Muscle fibres appeared to be replaced by fat in the latter stages of lesion development (Figure 3.14d). Bacteria were seldom observed in skin samples and were never seen invading the dermis or muscle. Due to the lack of bacterial numbers in these samples, confirmation by immunohistochemistry and 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 (Figure 3.15a). Immunohistochemistry confirmed the bacteria to be *T. maritimum* (Figure 3.15c).

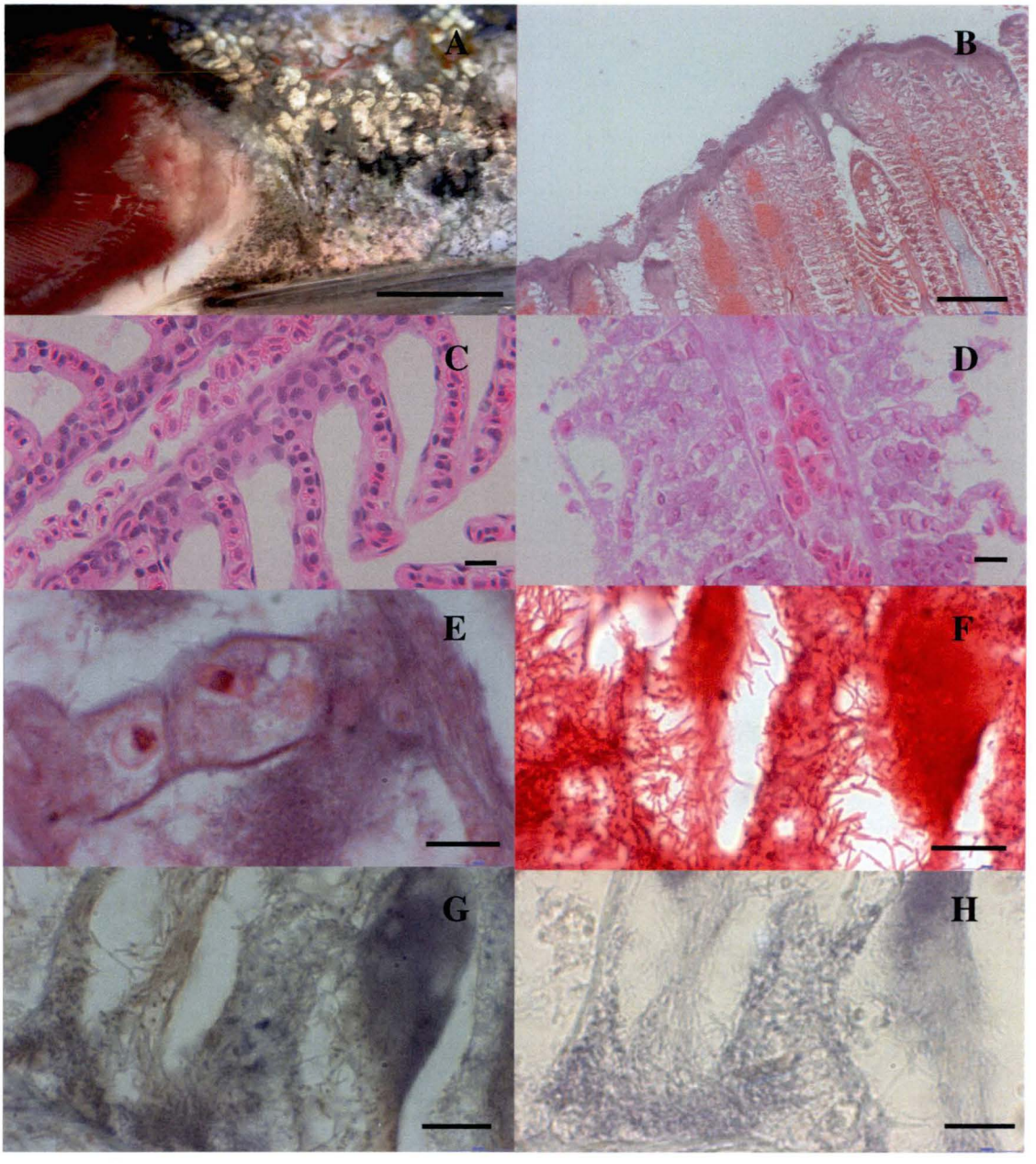

Figure 3.13 Necrosis of the gill in Atlantic salmon; A, gross pathology of gill necrosis (scale bar 1 cm); B, necrosis of several lamellae (scale bar 100 μm); C, control gill; D, necrosis of an individual filament; E, H&E stained section of gill showing the large mass of bacteria associated with the necrosis; F, Gram stain; G, positive immunohistochemistry result; H, negative immunohistochemistry result. All other scale bars 10 μm.

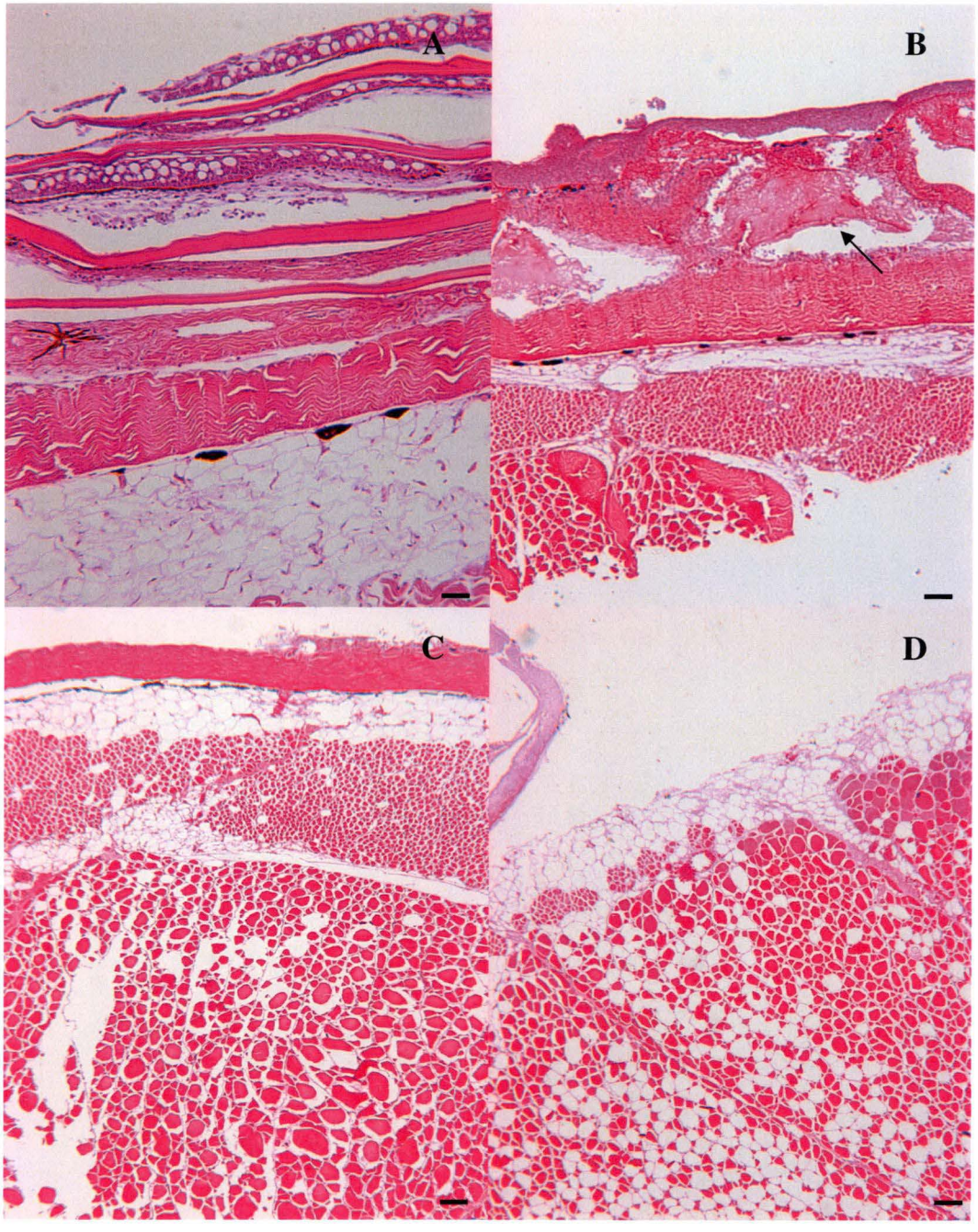

Figure 3.14 Histological examination of the progression of lesion development; A, control skin; B, scales were lost leaving scale pockets (arrow) underneath the epidermis; C, epidermis was eroded away by bacteria; D, dermis was eroded leaving the underlying musculature exposed. All scale bars 100 μm.

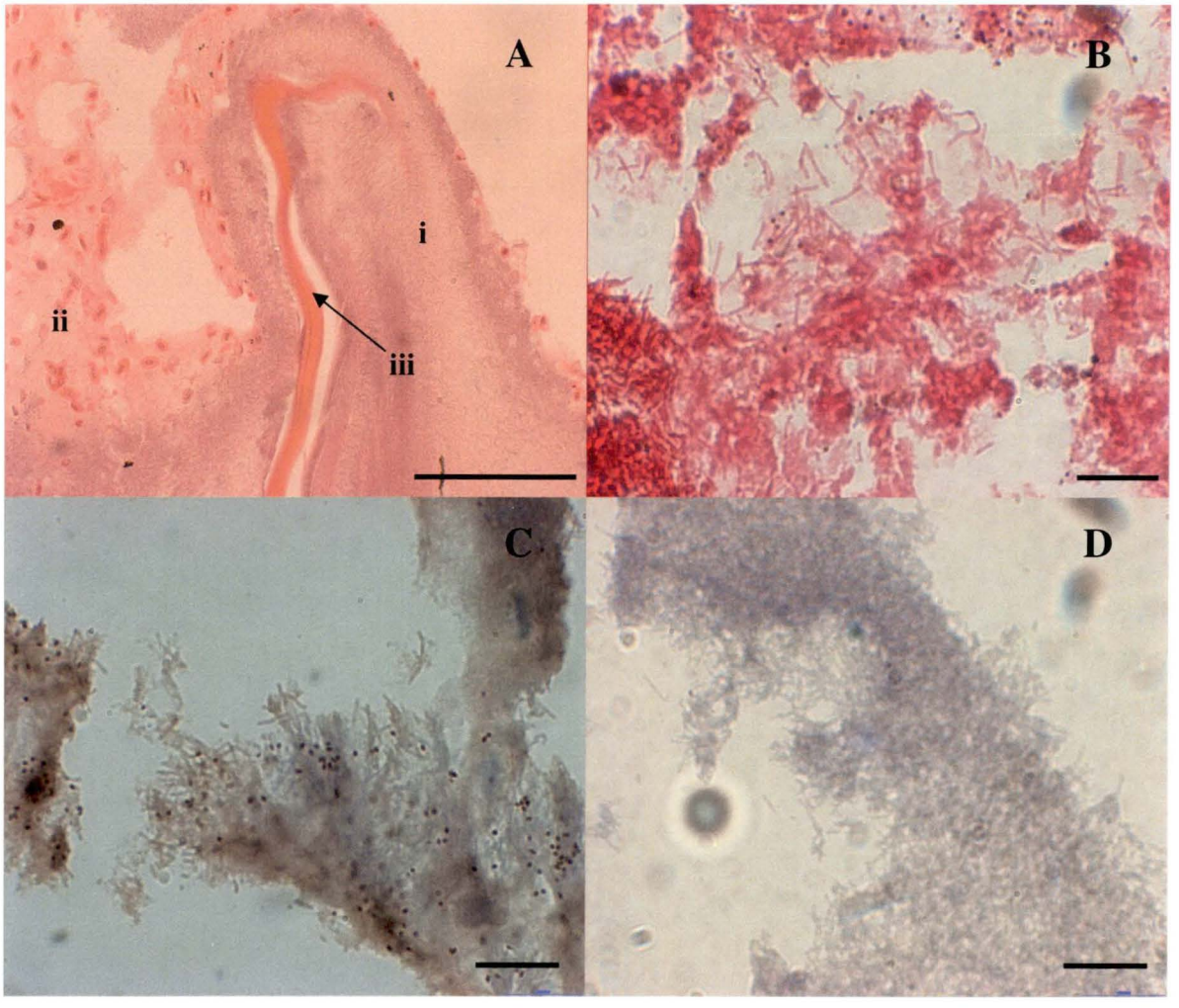

Figure 3.15 Necrotic material from *Tenacibaculum maritimum* infection; A, necrotic material is made up from (i) bacteria, (ii) necrotic tissue and (iii) scales (scale bar 100 μm); B, Gram stain; C, positive immunohistochemistry result; D, negative immunohistochemistry result. All other scale bars 10 μm.

3.4 Discussion

Different aspects of pathogenicity were studied not only to confirm Koch's postulates but also to compare the effects of bacterial culture conditions, virulence of strains and to establish a model of infection.

Marine Shieh's (MS) and Ordal's (MO) are the two growth media used previously to investigate *T. maritimum* in Tasmania. Bacteria cultured from both media were able to cause infection and mortalities in Atlantic salmon (*Salmo salar*) within the 10 day experimental period thereby confirming Koch's postulates. No significant difference in cumulative mortality was detected between the two media at the end of the experimental period. The nutrient concentration is the major difference between both media with the addition of citric acid and sodium pyruvate in marine Shieh's medium. This apparently did not significantly affect the pathogenicity of *T. maritimum* strain 89/4762. A comparison of marine Ordal's broth culture with natural seawater and synthetic seawater (Schmidtke *et al.* 1991) showed no significant effect on the expression of virulence (Carson *et al.* 1992). The authors recommended more sensitive assay procedures to confirm this observation. In contrast, *T. maritimum* cultured on marine Ordal's broth with a limited essential inorganic ion supplement (Schmidtke *et al.* 1991), failed to infect fish (Carson *et al.* 1993). The broth using the supplement produced a more homogeneous suspension than that produced with marine Ordal's medium. The authors speculated that there was a connection between agglutination in broth and expression of virulence factors (Carson *et al.* 1993).

There was however an effect of aeration during bacterial culture on the pathogenicity, even if the effects were not considered statistically significant. During broth culture, without aeration,

the bacteria form a dense layer at the air-water interface. The addition of constant aeration allows a thinner layer of bacteria at the air-water interface with long thread-like masses moving throughout the broth as well as a fine film apparent on the inside flask wall. Clumping of bacteria was also evident around the inlet of aeration. *Tenacibaculum maritimum* is an obligate aerobic bacterium (Wakabayashi *et al.* 1984), and is therefore likely to grow best where oxygen is readily available, that is, at the air-water interface and where there is an input of oxygen. It appears, however, that the difference observed between aeration and non-aeration may simply be a dose effect. While care was taken to accurately estimate cell concentration using a spectrophotometer, the clumping nature of the bacteria proved problematic. Physical agitation was employed to break up aggregated material, but this was not completely successful. It was observed that during the culture process, those bacteria grown without aeration formed stickier, more cohesive cell masses while growing at the air-medium interface. These cell masses could not be broken apart during re-suspension as those grown with aeration. Differences between challenge doses prepared with and without aeration may simply represent differences in cell concentrations derived from the two different culture methods. Carson *et al.* (1992) noted the presence of adherent macroscopic clumps in broth culture made up with marine Ordal's with natural seawater. The use of artificial seawater produced a homogeneous suspension and this did not appear to affect the pathogenicity of *T. maritimum* in rainbow trout (Carson *et al.* 1992).

Also of interest was the effect that the anaesthetic, clove oil, had on the re-isolation of *T. maritimum*. The use of culture was far less sensitive than Gram and IFAT with little or no bacterial growth on many plates for the first experiment in which clove oil was used as an anaesthetic. It was not only the absence of *T. maritimum* colonies on the plates but a lack of growth of bacteria that was interesting. Chemical components of anaesthetics could potentially

kill off bacteria if the dosage is too high or the exposure time too long. This problem has been observed with field investigations of *T. maritimum* in farmed salmonids (J. Carson *pers. comm.*). Therefore, the use of carbon dioxide or another non-chemical anaesthetic may be more suitable to ensure better isolation of *T. maritimum.* A subsequent decrease in dosage (0.025 mL/L seawater) and time (<2 minutes) in the anaesthetic provided better and more consistent results in the following two experiments.

The second experiment was undertaken to observe differences in the pathogenicity of strains. Strain 89/4747 was non-pathogenic causing no infection or mortalities throughout the experimental period, which is consistent with the findings of Powell *et al.* (2004). In contrast, Carson *et al.* (1992) described this strain as highly virulent with all fish becoming infected and developing lesions, including controls. All fish were kept in a single flow through tank and were identified using branding and tagging techniques. The high level of infectivity was suggested to be the result of the use of a single tank, insufficient water turnover and a virulent strain. In the current study, pilot studies of pathogenicity using strain 89/4762 found non-pathogenic results. It is suggested that attenuation was the probable cause. Loss of virulence due to subculturing occurs during routine bacteriology, storage and preservation (Michel and Garcia 2003, O'Keefe *et al.* 2006). Anecdotal evidence from this study suggests that attenuation occurs quickly in *T. maritimum* isolates (R van Gelderen, unpublished). It is most likely that attenuation is the cause of the current non-pathogenic results of strain 89/4747, but this requires further investigation. Preservation techniques, age of isolate, culture media and storage could affect the pathogenicity of bacteria (Michel and Garcia 2003, O'Keefe *et al.* 2006).

In contrast, strains 00/3280 and 89/4762 were highly virulent reaching 100% mortalities in three days. Both strains have previously been demonstrated as highly pathogenic (Carson *et al.* 1992, Handlinger *et al.* 1997, Powell *et al.* 2004). Experimental application of *T. maritimum* to the gills of Atlantic salmon using strain 00/3280 caused high levels of mortalities within 48 hours (Powell *et al.* 2004). Strain 89/4762 was highly pathogenic in rainbow trout and Atlantic salmon when challenged by immersion (Carson *et al.* 1992, Soltani 1995, Handlinger *et al.* 1997). The study undertaken by Soltani (1995) also noted that strains of *T. maritimum* could be host specific and found that a strain isolated from mullet (*Aldrichetta forsteri*) did not cause disease in Atlantic salmon. The strain 00/3280 (from rainbow trout) was highly virulent in Atlantic salmon but as both are salmonids, cross species susceptibility was more likely.

Of interest was the growth of the strains in culture. The virulent strains 89/4762 and 00/3280 were highly adherent to the agar in initial culture and produced a highly aggregated suspension in broth culture. Strain 89/4747 was easy to remove from agar plates and also produced a far more homogeneous suspension in broth. This phenomenon has also noted by others (Carson *et al.* 1992, Carson *et al.* 1993, Soltani 1995) who, like the current study, suggest that this 'sticky' clumping nature may facilitate an adhesion mechanism that plays a role in the pathogenicity of *T. maritimum*. Attachment to the host surface could result in increasing bacterial populations (Horne and Baxendale 1983, Ostland *et al.* 1995) at a site where other virulence factors such as toxins and enzymes can be released (Soltani 1995).

In the previous chapter, hydrophobicity was used as a test for the selection of isolates for the pathogenicity studies. One of the aspects of this was to test hydrophobicity and whether it is a good measure of virulence. Strain 89/4762 was ranked highest and therefore was deemed to be

the most hydrophobic. Strains 89/4747 and 00/3280 were ranked mid-range and were considered moderately hydrophobic compared to all other isolates. The most virulent strain was 00/3280 followed by 89/4762 and 89/4747 which was non-pathogenic. The results from the pathogenicity trial suggest that for *T. maritimum*, hydrophobicity is not a good measure of virulence. However, this should be confirmed with more isolates of known virulence and employing other methods of measurement for hydrophobicity (Santos *et al.* 1990).

The virulence of these strains does need to be put into perspective. The available experimental systems for the initial experiments were semi-static with a full water change every 24 hours. Water used during these experiments was filtered down to 0.8 μm. The inoculum was approximately 1×10^7 cells/mL. After three days (when mortalities had reached 100% in two groups) the concentration of *T. maritimum* cells was determined to be 3.1×10^8 cells/mL for 00/3280 and 2.8×10^8 cells/mL for 89/4762 per tank. It is possible that the fish were subjected to an increasing dose over time, which may have contributed to the high mortality rate observed. Semi-static conditions would have assisted in the attachment of the pathogen to the host by providing greater opportunity for contact with the host. However, all strains were under the same conditions and therefore virulence differences can be observed. The semi-static conditions would also account for the high levels of mortality (68.75 - 93.75%) observed in the first experiment (comparison of culture methods). However, mortalities did not reach 100% in the first experiment and this was probably due to a colder water temperature and a less dense fish population (Table 3.2) (Carson *et al.* 1992, Soltani 1995).

Virulence of strains was compared to select a strain for use in the vaccine trial. Strain 00/3280 reached mortalities of 81.4% within 48 hours while 89/4762 only had mortalities of 31.1%. The

highly pathogenic nature of strain 00/3280 that can cause mortalities within 24 h post-inoculation has been noted previously (Powell *et al.* 2004, R van Gelderen, unpublished). It seemed more appropriate to use a strain that was mild in its capacity to cause disease rather than using a strain that was obviously highly pathogenic and could cause mortalities in a shorter amount of time (a possible 'hot strain'). It may also be more difficult to assess dose effect and vaccine efficacy using a highly virulent strain. Therefore the more moderately virulent strain 89/4762 was selected for the dose experiment and subsequent vaccine trial.

The comparison of different doses of the strain 89/4762 of *T. maritimum* was carried out to develop a model of infection and to determine an LD_{60} for a subsequent vaccine trial. Death of fish occurred between 48 hours and 18 days post-challenge depending on the dose used. Controls that were kept within the system had a cumulative mortality of 17%. The LD_{60} for the experimental period worked out to be 1×10^6 cells/mL, the dose required as the challenge dose for the vaccine trial.

It is difficult to compare the current findings with other pathogenicity investigations due to differences in routes of challenge, size of fish and inoculum dose. What is apparent is that a challenge model of infection has been established for Atlantic salmon. The current study is further supported by the work of Soltani (1995) who produced a similar model of infection with LC_{50} values calculated at 2.3×10^5 CFU/mL for rainbow trout and 1.6×10^6 CFU/mL for Atlantic salmon at the same salinity and similar temperatures.

Another model of infection using immersion has been produced for turbot (Avendaño-Herrera *et al.* 2006). Fish weighing 4-6 g required 18 hours of inoculum (5×10^3 to 5×10^8 cells/mL)

exposure time to generate a successful infection. The authors reported that they were unable to infect turbot with only a 1 or 2 hour bath. The immersion challenge was repeated with 15-20 g fish and similar findings were noted. They suggested that the exposure time is not sufficient for the development of a biofilm on the skin/gills which is required for successful infection. In contrast, the current study, Carson *et al.* (1992) and Soltani (1995) only required a one hour bath to produce an infection using challenge inoculum within the same range as Avendaño-Herrera *et al.* (2006). This could be the result of either using a more virulent strain or that salmonids are more susceptible to *T. maritimum* than turbot.

While immersion challenge provided a suitable method of infection of *T. maritimum* in Atlantic salmon, it was deemed unsuitable in red and black sea bream (Wakabayashi *et al.* 1984, Baxa *et al.* 1987). Indeed many authors have found that successful disease induction occurs only following scarification and smearing (Masumura and Wakabayashi 1977, Wakabayashi *et al.* 1984, Baxa *et al.* 1987, Bernardet *et al.* 1994). Flexibacteriosis could also be induced by subcutaneous injection of the pathogen in Dover sole and red and black sea bream (Campbell and Buswell 1982, Baxa *et al.* 1987), but this was not demonstrated when tested in Atlantic salmon (Soltani 1995). However, there is agreement with the use of intraperitoneal injection as a method of inducing experimental infection. There is strong evidence from a number of fish species that this method does not induce disease (Wakabayashi *et al.* 1984, Alsina and Blanch 1993, Bernardet *et al.* 1994, Soltani 1995, Avendaño-Herrera *et al.* 2006). Through the use of nested-PCR, Avendaño-Herrera *et al.* (2006) demonstrated that most of the intraperitoneal injection is released into the water within the first 6 hours post-injection. This is suggested as the reason why intraperitoneal injection is not an efficient method of inducing disease. It is also likely that as an external pathogen, the conditions within the peritoneal cavity do not support the

growth and proliferation of *T. maritimum*. The bacteria have a requirement for NaCl for growth and at least 30 ppt seawater is needed in culture media (Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Wakabayashi *et al.* 1986, Bernardet and Grimont 1989, Bernardet *et al.* 1990).

From the work of Carson *et al.* (1992) it was apparent that horizontal transmission occurred, and in the current study as well as Soltani (1995) controls were kept separate from the main system, even using a separate biofilter system. Another set of controls was used within the system to evaluate the level of horizontal transmission in the current study. The system was set up with a filter bank down to 0.8 μm for incoming water and two UV lights to reduce bacterial load. After 16 days, fish with lesions appeared and consequently some mortalities occurred. Considering the experiment only lasted 21 days and 3 days of no mortalities had to occur before terminating the experiment, the level of horizontal transmission was quite low.

The development of a lesion started with small discrete areas of scale loss which progressed into cutaneous erosion. At high concentrations the progress of the disease was rapid and highly aggressive resulting in 100% mortalities within 2-3 days post-challenge. In contrast, lower concentrations required several days to weeks to cause host mortalities. 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). The main clinical signs of the disease from the current study are 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, Handlinger

et al. 1997). There are also similarities to the gross pathology described in other affected fish species (Masumura and Wakabayashi 1977, Campbell and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Alsina and Blanch 1993, Pazos *et al.* 1993, Bernardet *et al.* 1994, Handlinger *et al.* 1997, Avendaño-Herrera *et al.* 2006).

In natural infections, damage to the epithelium is seen as a pre-disposing factor to an infection caused by *T. maritimum* (Handlinger *et al.* 1997). Experimental infection in the current study occurred without the addition of scarification or deliberate damage. Handlinger *et al.* (1997) suggested the presence of a toxin which causes superficial skin damage thereby initiating further progression of the disease. Indeed, at high concentrations infection, erosion and death occurred within 72 hours 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 could be able to exist and proliferate in the mucus of the host fish. In the current study, *T. maritimum* could be re-isolated from surviving fish in all groups with no clinical signs present. 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 (Avendaño-Herrera *et al.* 2006). The mucus of turbot does not contain 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 (Avendaño-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 tissue (Handlinger

et al. 1997). The teeth and fins of teleost fish contain calcium ions which are considered important in the growth of flavobacteria (Hikida *et al.* 1979). Pectoral fins cause constant irritation to the surrounding area, and along with the jaw are the two areas that come into frequent contact with the tank walls which allows damage to occur (e.g. cherry fin). In all experimental treatments the pectoral and dorsal sites predominately showed signs of erosion. The jaw was consistently affected except in experiment 2 where there was a decrease in jaw erosion. This was most likely the result of the short experimental time frame (3 days). It is also possible that under experimental conditions, behavioural traits such as aggression play a role in the susceptibility of Atlantic salmon to *T. maritimum*. Atlantic salmon are known to charge and bite each other (Mork *et al.* 1999). Fin damage, in particular dorsal fin damage, is primarily cause 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). Larger lesions observed with fin sites can be attributed to the irritation and spreading nature of fin movement.

Lesions are formed in Atlantic salmon through scale loss and the build up of necrotic material, which sloughs away to reveal 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 as though *T. maritimum* acts to dislodge the scales from the dermis and breakdown 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. It is suggested 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 reason why the lesions can continue to erode outwards is most likely the result of continuing the process of breakdown in tissues that are not vascularised. Soltani (1995) suggested that *T. maritimum* is not able to survive in well vascularised living tissue, probably because of low 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.

Through immunohistochemistry, *T. maritimum* has been identified within the slough material and gills. Gills showed diffuse necrosis that can affect individual or encompass several lamellae. Bacteria were not observed in the connective tissue or musculature in skin lesions. It is suggested that this is the result of the preference of *T. maritimum* bacteria to exist within the necrotic material (slough). Handlinger *et al.* (1997) noted large mats of *T. maritimum* that invaded the connective tissue and occasionally in the musculature of late lesions which is inconsistent with current findings. A possible explanation for the discrepancies observed was that late lesions were collected from fish that could have been dead for between 12-15 hours. Fish were not dead for more than a couple of hours in the current 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 it is proposed that colonisation of the tissue and musculature had not taken place in the tissue of fish in the current 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. Baxa *et al.* (1988c) suggested extracellular products (ECP) play an important role in the pathogenesis of *T. maritimum*, which is investigated in the next chapter.

In conclusion, *T. maritimum* is pathogenic to Atlantic salmon using both marine Shieh's and marine Ordal's media for culture. The use of aeration in broth culture produced a dose effect in challenge. Differences in virulence between three strains was apparent with one of the strains (89/4747) being non-pathogenic and unable to produce disease in the host. The two other strains (89/4762, 00/3280) were highly virulent recording 100% mortalities within three days. The high mortality rate may have been assisted by the use of semi-static water tanks. Koch's postulates were confirmed, a model of infection established and an LD_{60} was determined using strain 89/4762. Measurements of hydrophobicity did not appear to be a good indicator of virulence for *T. maritimum* strains.

The progression 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 *T. maritimum.*

Chapter 4

Toxicity of extracellular products produced by *Tenacibaculum maritimum* in Atlantic salmon

4.1 Introduction

Toxins are believed to play an important role in the pathogenesis of bacterial diseases in a number of fish species (Austin and Austin 1993, Dalsgaard 1993). Extracellular products (ECP) are toxins that are excreted into the external environment by bacterial cells (Alcamo 1983, Madigan and Martinko 2003), and are considered to be virulence factors and contribute to the pathogenesis of bacterial disease (Dalsgaard 1993, Liu *et al.* 1996, Zorrilla *et al.* 2003). These microbial products are types of aggressins that directly affect the tissues, organs and physiological processes of the host (Alcamo 1983, Madigan and Martinko 2003). For pathogenic bacteria they can cause tissue damage and enhance invasiveness (Dalsgaard 1993).

Toxigenesis, or the ability of bacteria to produce toxins, may be an important mechanism in the ability of bacterial pathogens to produce disease (Liu *et al.* 1996, Zorrilla *et al.* 2003). Exotoxins are products of metabolism and their production can be species specific (e.g., *Bacillus anthracis* produces the anthrax toxin) (Griffiths 2005). The virulence of a bacterial species can be affected by the production of exotoxins. Virulent strains produce exotoxins and non-virulent strains do not (Griffiths 2005). Exotoxins are highly antigenic which means they have the capacity to stimulate an immune response by a host (Ellis 1997b). In addition, ECP are heat-labile proteins and therefore can be denatured (Dalsgaard 1993). The resulting toxoid may retain its ability to induce an immune response and therefore can be incorporated into vaccines (Magariños *et al.* 1994, Santos *et al.* 1995).

Results of an earlier pathogenesis experiment (Chapter 3) showed that challenge with *Tenacibaculum maritimum* at higher doses (approximately $1x10^8$ cells/mL) had an acute lethal

effect on Atlantic salmon (*Salmo salar*). Mortalities occurred within days, and the clinical sign was the disintegration of the epithelium. *In vitro*, *T. maritimum* is relatively slow growing with at least 48 hours at 25°C required before growth was evident in culture (R van Gelderen, unpublished). In addition, a lack of an inflammatory response is characteristic of early flexibacteriosis lesions. Handlinger *et al.* (1997) suggested that this was the result of powerful exotoxins that prevent a host response. These findings pointed to a possible role of toxins in the pathogenicity of *T. maritimum* in Atlantic salmon.

Activities of *T. maritimum* toxins have been explored in red and black sea bream (*Pagrus major* and *Acanthopagrus schlegeli*) (Baxa *et al.* 1988c). In both fish species, ECP showed insignificant *in vitro* activity, however, this did not correspond with the toxic effects observed *in vivo* with ECP recording the lowest LD_{50}. Baxa *et al.* (1988c) did indicate that the pathogenicity of *T. maritimum* in black and red sea bream may be ascribed in part to ECP. Extracellular products produced by *T. maritimum* have also been studied *in vitro* to determine immune response in sea bass (*Dicentrarchus labrax*) and to investigate the potential use of ECP in vaccine preparations (Salati *et al.* 2005). While ECP were immunogenic and stimulated a secondary immune response, lipopolysaccharide (LPS) showed increased antibody titres and enhanced phagocytosis. Lipopolysaccharide was identified by the authors as the antigen requiring greater attention for further vaccine investigations.

From previous work (Baxa *et al.* 1988c), it is apparent that *in vitro* activity and *in vivo* effects do not always correlate. The current study investigated ECP toxicity *in vivo* to observe direct effects rather than *in vitro* activity of different toxins. Further, this study provides the first observations of *T. maritimum* ECP toxicity in Atlantic salmon.

The aims of this study were:

1. Determine toxicity and the LD_{50} (for the experimental period) of *T. maritimum* ECP in Atlantic salmon by injection with different ECP doses,
2. Describe the pathology in Atlantic salmon due to *T. maritimum* ECP under experimental conditions.

4.2 Materials and Methods

4.2.1 Bacteria and ECP

The ECP of *T. maritimum* strain 89/4762 was prepared following the cellophane overlay method of Liu (1957) using marine Shieh's agar (23 cm x 23 cm) at 25°C. Bacteria were washed off the cellophane using minimal amounts of phosphate buffered saline (PBS, pH 7.4) after 72 hours incubation. The harvested bacterial suspension was then centrifuged at 4000 x *g* for 30 mins at 4°C; the pellet was discarded. The supernatant fluid was sterilised using 0.2 μm filters and the resulting ECP was aliquotted and stored at -80°C until used. To ensure that any toxic effect observed was from the ECP and not from either the cellophane overlay or agar medium, fluid that dialysed across the cellophane overlay from marine Shieh's medium alone (MSF) was harvested using PBS. Protein concentration was determined with the bicinchoninic acid (BCA) assay kit (Pierce) using bovine serum albumin as a standard.

4.2.2 Experimental design

Seventy Atlantic salmon averaging 40 g (30.4 g – 56.5 g) were held in 14 aerated static freshwater circular tanks (100 L) at 15-16°C. Each treatment had 2 replicate tanks with 5 fish per tank.

4.2.3 ECP injection

Fish were anesthetised using 0.03 mL/L clove oil in seawater. Ten fish per treatment were injected intraperitoneally with 0.1 mL of ECP of five different doses: 1000, 500, 250, 125 and 62.5 μg protein/fish. There were also two control treatments: a group of unhandled controls

(Control) and a group of controls (Control IP) that were injected with 0.1 mL of MSF. Intraperitoneal injection was used for the route of challenge as the production of ECP was insufficient for bath challenge. Immersion would have been the preferred method of challenge as it reflects the natural course of the disease. Subcutaneous injection of ECP could also be used as a method to trial in the future. However, the test via injection allows a basis for comparison with previous work (Baxa *et al.* 1988c) and ensures an accurate dose.

4.2.4 Sampling procedure

Gross pathology of each fish was determined using the method of Baxa *et al.* (1988c). The gill, liver, spleen, heart, kidney, stomach and intestine were examined and each organ was assigned a classification based on its gross pathology: Discoloured; Suppurated; Hypertrophied; Haemorrhaged; Normal. Pathology was also graded according to the severity of the changes. Histopathology was assessed by collecting organ samples and fixing in seawater Davidson's for an initial fixation of 72 hours 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 x40 – x1000 for examination and interpretation.

4.2.5 Fish sampling and determination of LD_{50}

Fish were removed from the tanks when there were clear signs of morbidity (fish were unable to control position in the water column). Survivors were euthanased 24 hours post-injection with an overdose of clove oil (0.06 mL/L seawater). The LD_{50} for the 24 hour experimental period was calculated according to Reed and Muench (1938).

4.3 Results

Within the 24 hour experimental period, the three highest doses resulted in 100% mortality with the time taken to morbidity increasing with a decrease in dose (Figure 4.1). No mortalities were observed in the 61.2 μg and control groups. A 50% mortality rate was recorded in fish injected with 125 μg, which is 3.125 μg of protein/g of fish body weight. This was determined to be the LD_{50} for the experimental time period. Severity of pathological signs appeared to be related to the dose of the ECP toxin; the lower the dose, the less severe the gross pathology.

The prevalent sign of the reaction to the ECP was hemorrhaging and ascites in the peritoneal cavity (Figure 4.2). Fish in both control groups did not show any pathological signs. The gills, heart and liver were all discoloured, appearing to be drained of blood (Figures 4.3 and 4.4). In three cases the liver of surviving fish in the 125 μg group appeared yellow, indicating jaundice (Figure 4.4). The spleen was haemorrhagic, hypertrophic, and dark in colour (Figure 4.5), but moreover was abnormally firm at the highest dose and became more pliable (similar to controls) with decreasing dose. Blood was present in the stomach which together with the intestines was hypertrophied (Figure 4.5a). This enlargement caused the swim bladder to appear smaller and in some cases, particularly in the higher doses, the swim bladder appeared to be 'bubbled' (Figure 4.5b). The kidney was haemorrhagic (Figure 4.5b) and the anus inflamed in most cases while bleeding from the anus occurred in the two highest doses (Figure 4.5c). The severity of pathological signs reduced with decreasing dose; minor signs were present in fish injected with 61.2 μg that survived the 24 hour experimental period.

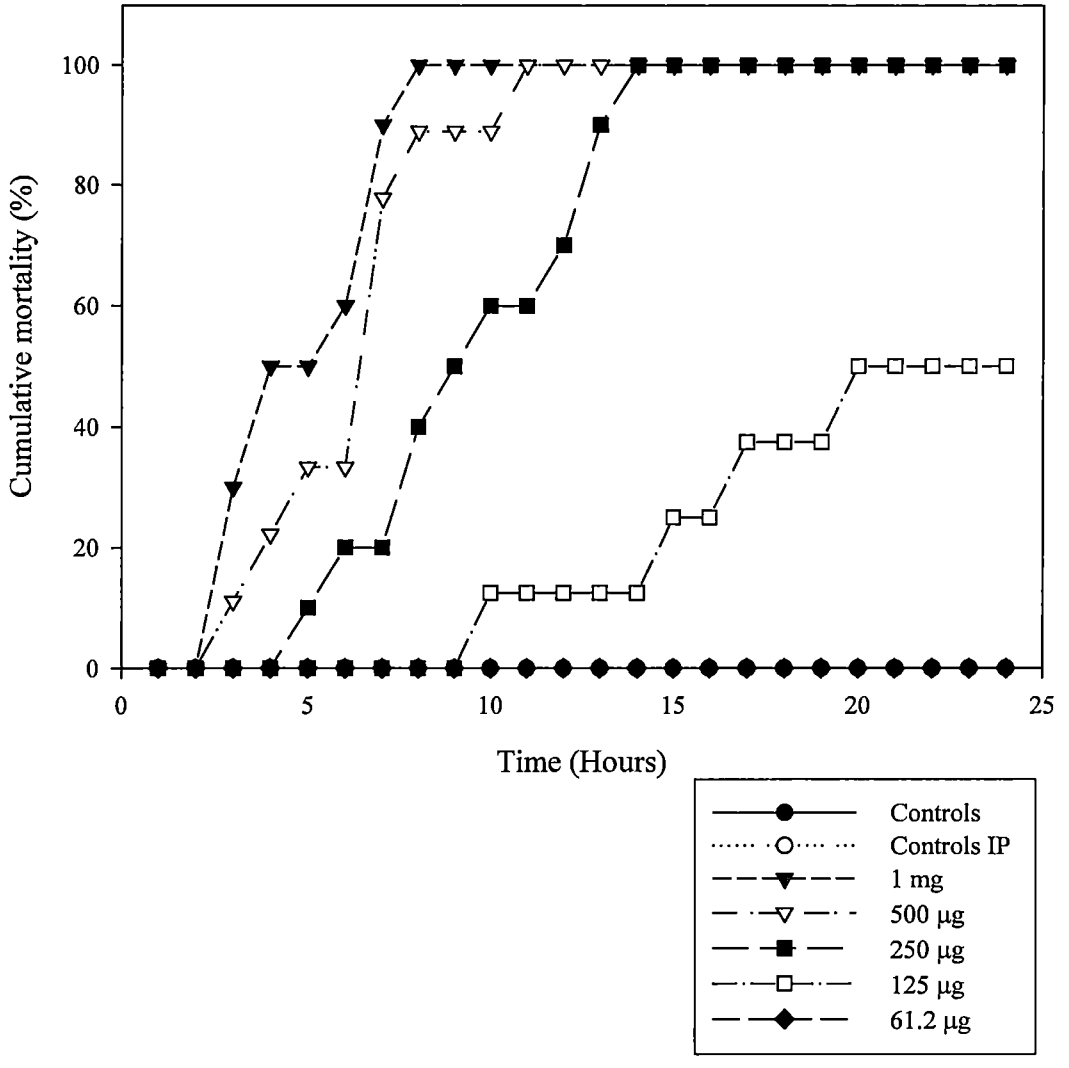

Figure 4.1 Cumulative mortality (%) of Atlantic salmon injected with *Tenacibaculum maritimum* ECP toxin.

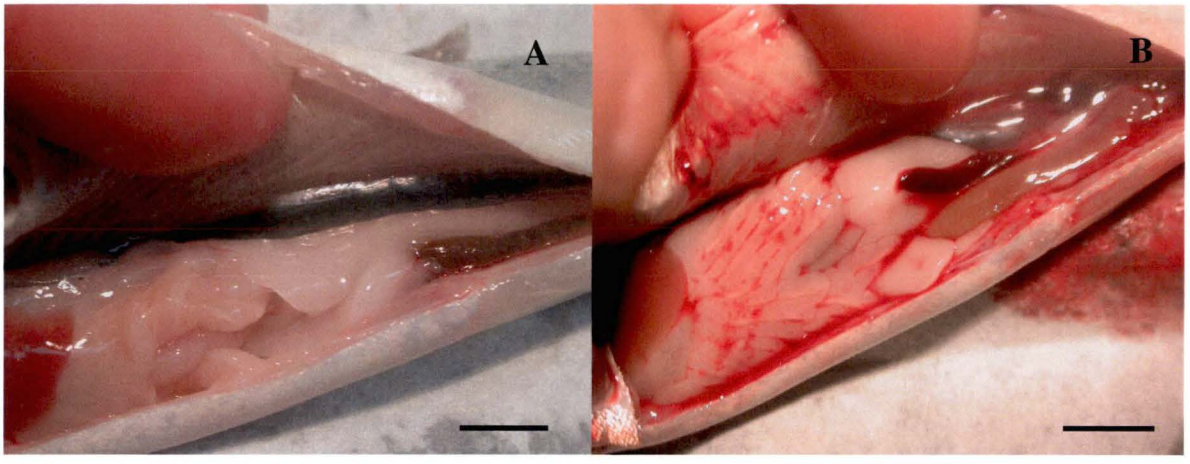

Figure 4.2 Internal organs of Atlantic salmon: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 3 hours post injection. Note the haemorrhaging inside and outside of the peritoneal cavity. The spleen is firm and rigid and all organs are enlarged. Scale bars 1 cm.

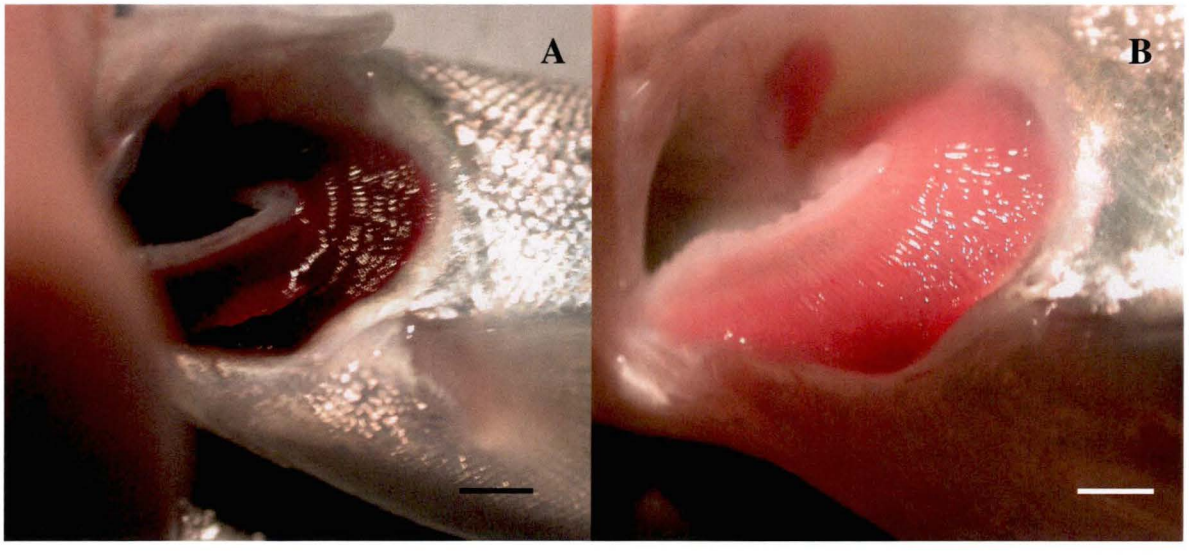

Figure 4.3 Gills of Atlantic salmon: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the lack of colour in the gill, appearing to be drained of blood. Scale bars 0.5 cm.

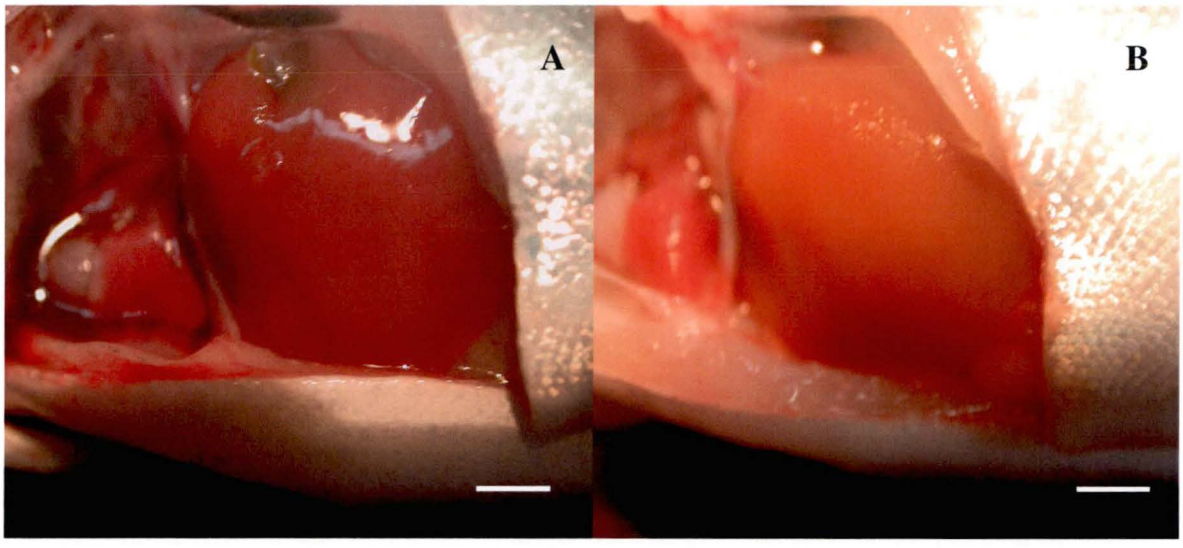

Figure 4.4 Heart and liver of Atlantic salmon: A, control; B, injected with 125 µg of *Tenacibaculum maritimum* ECP 24 hours after injection. Note the discolouration of the heart and the yellow appearance of the liver. Scale bars 0.5 cm.

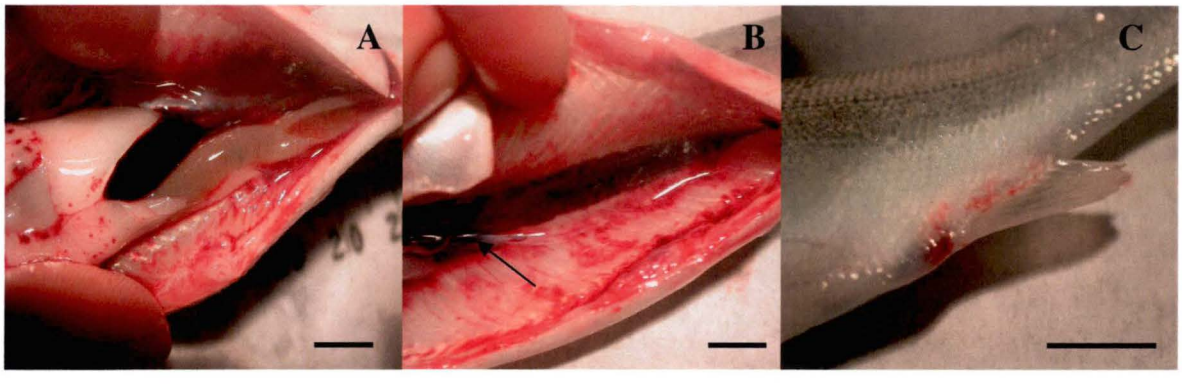

Figure 4.5 Internal organs of Atlantic salmon injected with 1 mg of *Tenacibaculum maritimum* ECP 3 hours post injection: A, hypertrophied stomach, intestine and spleen with haemorrhaging in the stomach; B, haemorrhagic kidney with 'bubbled' swim bladder (arrow); C, bleeding anus. Scale bars 1 cm.

Histological examination of the gills showed epithelial lifting, hypertrophy of cells, necrotic epithelium and there appeared to be an increase in the number of chloride cells (Figure 4.6). The cardiac muscle was necrotic and vacuolated and an infiltration of inflammatory cells was observed in the heart (Figure 4.7). There was a degeneration of kidney tubules (Figure 4.8), an increase of red blood cells in the spleen (Figure 4.9), and focal inflammation and necrosis in the liver (Figure 4.10). The lumen of the pyloric caeca was not visible due to hyperplasia and the central mucosal epithelium appeared necrotic (Figure 4.11). The intestine showed infiltration of submucosa with inflammatory cells (Figure 4.12b, d), a thickening of the muscularis circularis with separation of the smooth muscle by inflammatory infiltrate (Figure 4.12b, c). Pancreatic tissue and fat appeared normal.

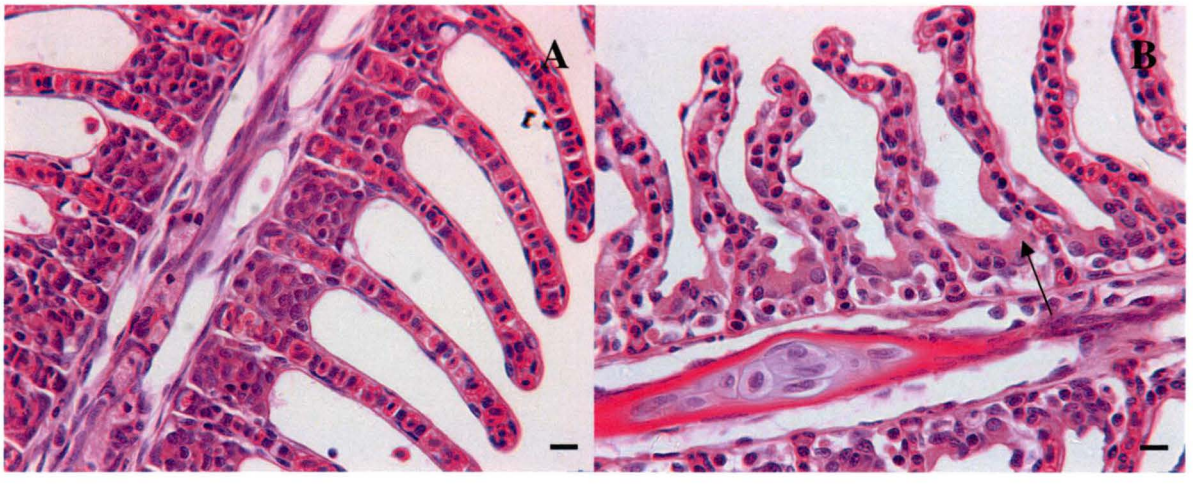

Figure 4.6 Atlantic salmon gill section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the apparent increase in chloride cells, seen as large eosinophilic cells (arrow) and epithelial lifting and necrosis. Scale bars 10 μm.

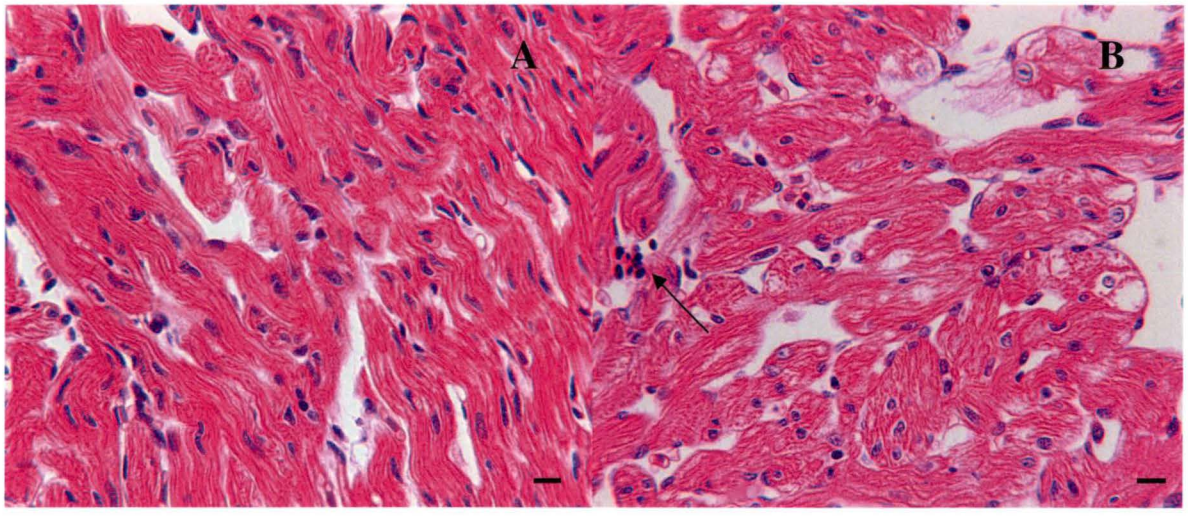

Figure 4.7 Atlantic salmon heart (cardiac muscle) section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the necrotic and vacuolated nature of the tissue and aggregation of inflammatory cells (arrow). Scale bars 10 µm.

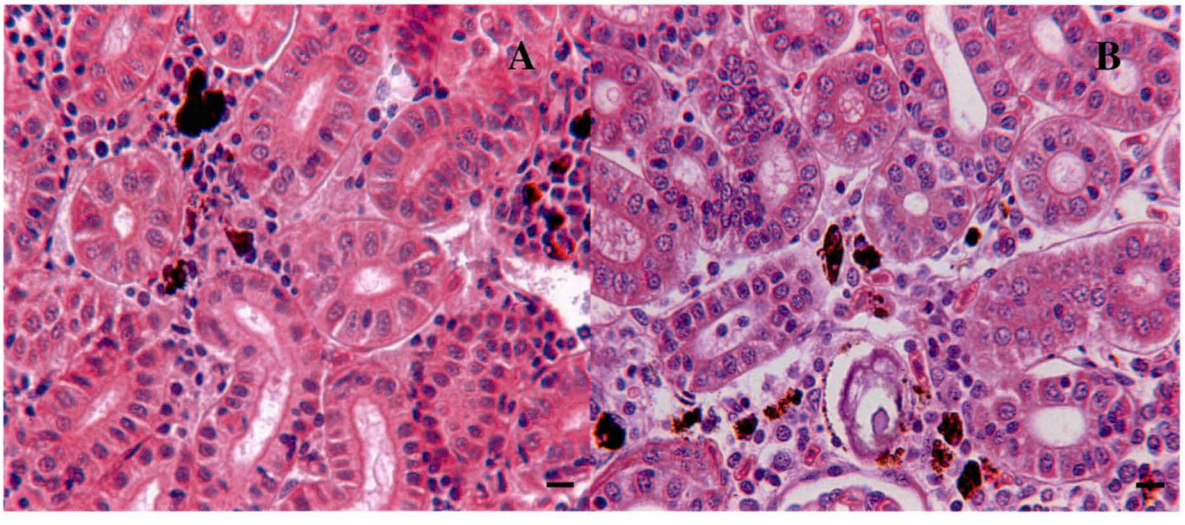

Figure 4.8 Atlantic salmon kidney section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the focal necrosis. Scale bars 10 µm.

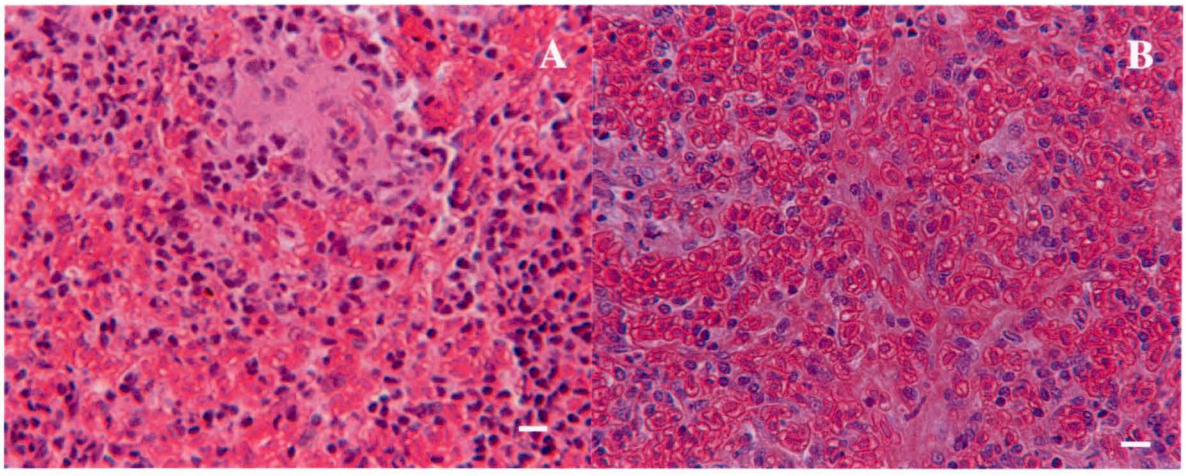

Figure 4.9 Atlantic salmon spleen section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 3 hours post injection. Note the increase in the number of red blood cells. Scale bars 10 μm.

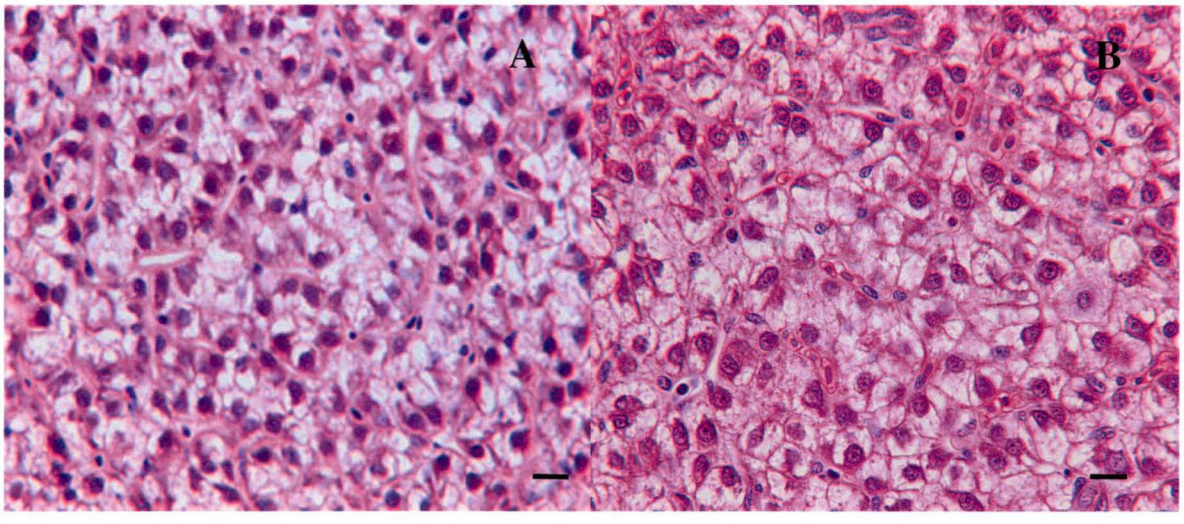

Figure 4.10 Atlantic salmon liver section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the focal necrosis. Scale bars 10 μm.

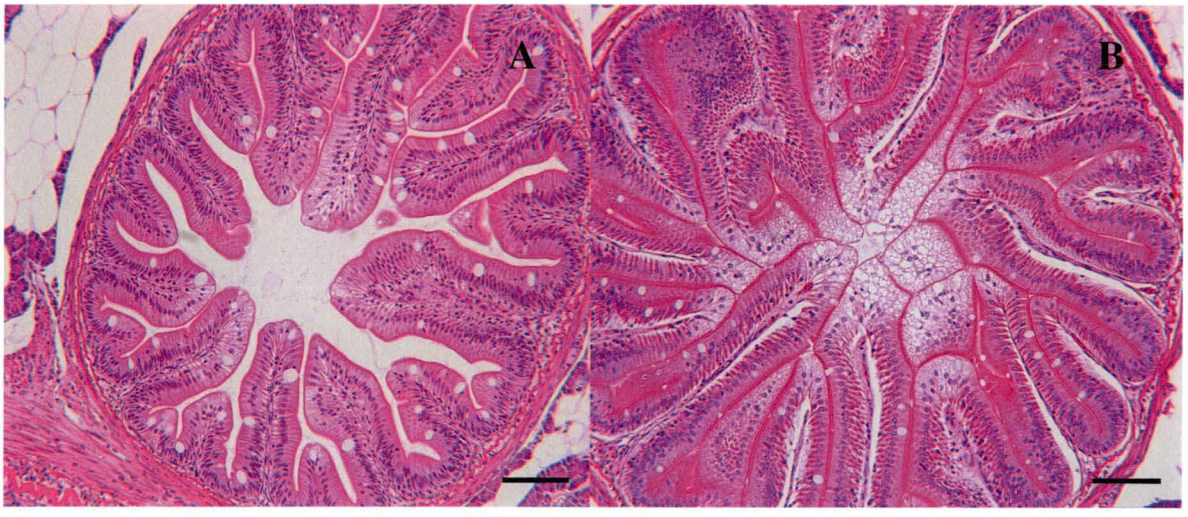

Figure 4.11 Atlantic salmon pyloric caeca section: A, control; B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection. Note the blockage of lumen and necrosis of the central mucosal epithelium. Scale bars 100 μm.

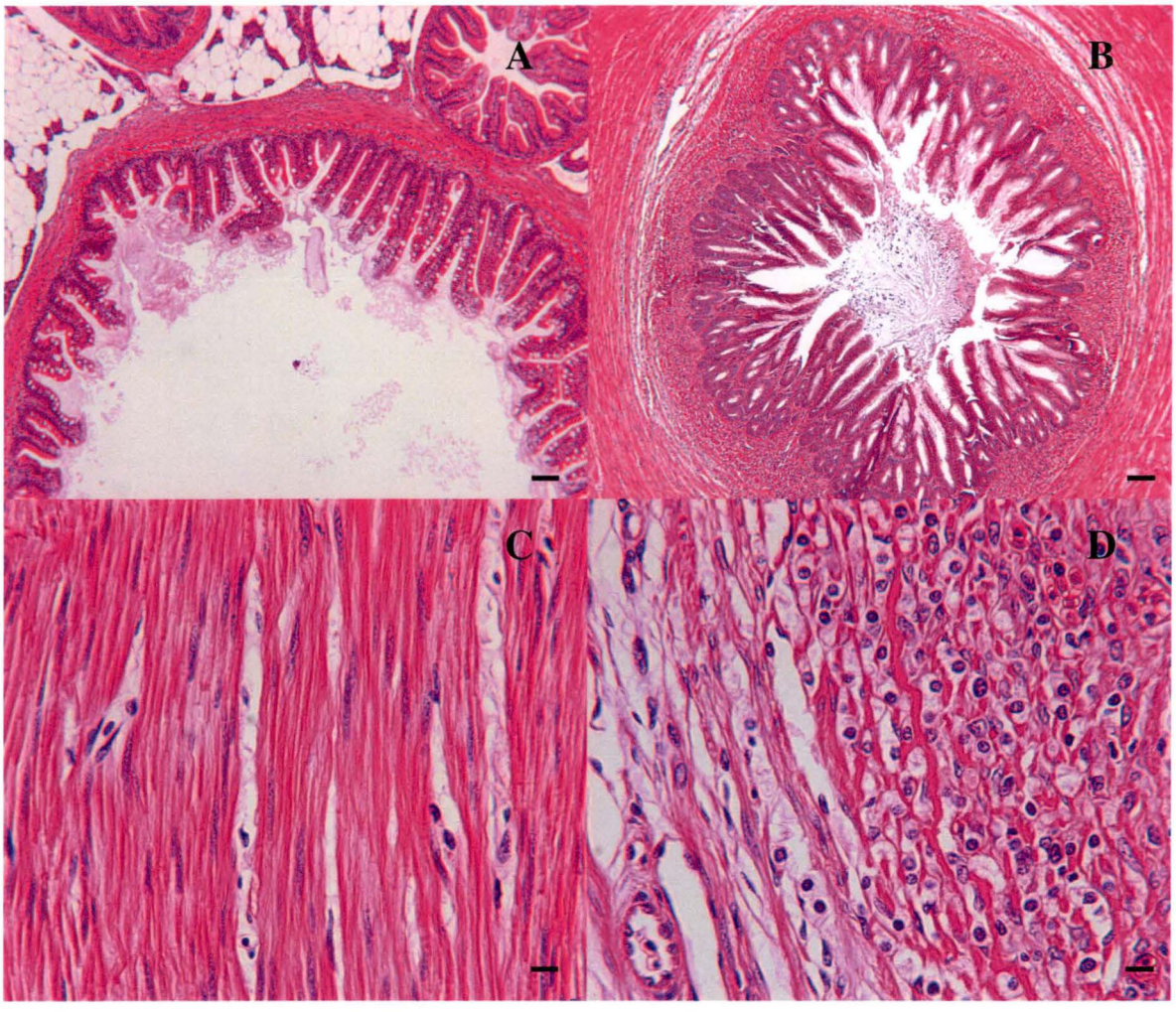

Figure 4.12 Atlantic salmon intestine sections: A, control (scale bar 100 μm); B, injected with 1 mg of *Tenacibaculum maritimum* ECP 2 hours post injection (scale bar 100 μm); C, thickening of the muscularis circularis and the presence of inflammatory infiltrate (scale bar 10 μm); D, infiltration of submucosa with inflammatory cells (scale bar 10 μm).

4.4 Discussion

Results from the current study confirm the toxicity of *T. maritimum* ECP in Atlantic salmon. Toxicity in black and red sea bream has previously been established (Baxa *et al.* 1988c). LD_{50} values were 25.5 µg/fish and 44.0 µg/fish in black and red sea bream, respectively. The current study determined an LD_{50} of 125 µg/fish for Atlantic salmon. However, the size of fish was different; 2.5 g black and red sea bream compared to 40 g Atlantic salmon. Taking the weight into account, the LD_{50} for black and red sea bream was 10.2 µg of protein/g of fish and 17.6 µg of protein/g of fish, respectively. The LD_{50} for Atlantic salmon was 3.125 µg of protein/g of fish. Therefore, the ECP produced from strain 89/4762 was more potent, or alternatively, Atlantic salmon more susceptible. Factors such as extraction method, growth media, incubation times and strain used could affect potency because they can affect the expression of virulence factors (Ellis 1991, Gunnlaugsdóttir and Gudmundsdóttir 1997, Zhang and Austin 2000). The current study used the cellophane overlay method of Liu (1957) to produce ECP while Baxa *et al.* (1988c) used ammonium sulfate precipitation. The differences observed between the studies may have been the result of using different production methods. In order to compare results more accurately between studies it would be beneficial to standardise the procedure to control variables. In addition, evaluation of the factors that have the potential to affect toxicity could be an area of future research.

Following injection of Atlantic salmon with ECP the toxic effect occurred only internally and did not induce clinical signs of flexibacteriosis. The lack of external clinical signs is consistent with Baxa *et al.* (1988c). The ECP of several pathogenic bacteria administered by injection have caused clinical signs of the disease which resembled those from an infection with live cells.

These include *Vibrio harveyi* (Zhang and Austin 2000, Zorrilla *et al.* 2003), *Aeromonas salmonicida* (Munro *et al.* 1980, Ellis *et al.* 1981) and *Vibrio alginolyticus* (Balebona *et al.* 1998). All of these bacterial pathogens cause a systemic infection unlike *T. maritimum*, which is an external pathogen. This could be a reason why the ECP of these pathogens can cause external clinical signs of the disease and *T. maritimum* could not. Gross and histopathological results from natural and experimental infections in Atlantic salmon have shown necrosis of the gills (Soltani 1995, Handlinger *et al.* 1997). Gross pathology in this study showed discoloured gills while histology showed necrosis of the gill filaments. Even though the gills do not show clinical signs of the disease, the histology provides a link between the pathology of the disease and the ECP.

Considering *T. maritimum* is a marine bacterium with an absolute requirement for seawater (Wakabayashi *et al.* 1986), it is conceivable that the use of freshwater inhibited external expression of the disease. Keeping fish in freshwater was primarily due to the use of 40 g Atlantic salmon that would not yet be able to tolerate seawater. Baxa *et al.* (1988c) did not report under what conditions their fish were held, but as red and black sea bream are marine fish, it can be assumed that their experiments used seawater. Since both studies show consistent results and no external clinical signs were reported it can be concluded that freshwater most likely had no effect on the outcome.

Another possibility is that external clinical signs of flexibacteriosis were not observed in the fish because the effect was too acute. However, there are similarities between the mortality rates in the current study and Baxa *et al.* (1988c). Mortalities began within 2-3 hours post injection at the highest doses (1 mg and 250 µg respectively) and time to death increased with decreasing

dose. Survivors showed no external clinical signs of the disease in the current study; the status of survivors in Baxa *et al.* (1988c) was not reported.

It is also necessary to consider the possibility that ECP may not be the cause of topical changes seen with naturally occurring infections. These changes may be caused by local proteolysis. It is also possible that ECP might spread systemically from the sites of the infection and into the body of the fish causing early mortalities without causing clinical signs of disease. The current study is speculating that ECP plays a role in the pathogenesis of *T. maritimum* and further research is required to fully elucidate its role.

Gross pathology was similar between the current study and Baxa *et al.* (1988c) with the prevalent sign being excessive fluid in the peritoneum. Blood associated with the ascites was also found in the current study. Most organs showed similar signs in both studies with the exception of the liver. Baxa *et al.* (1988c) reported the liver to be suppurated. In the current study, all morbid fish had a discoloured liver and in 3 of the surviving fish in the 125 µg group, the liver appeared yellow, indicating jaundice. There was no sign of pus internally in any fish. This reaction may be a host specific or age specific response. The suppurated liver was reported to be the result of a tissue reaction, which blocked blood supply causing necrosis and secretion (Baxa *et al.* 1988c). Histology was not undertaken in the study of Baxa *et al.* (1988c) and therefore the extent of tissue damage is unknown. However, the discoloured liver was consistent in fish from both studies and the current study in Atlantic salmon provides histological evidence to support liver necrosis.

Histological examination of the internal organs showed focal inflammation and necrosis in the liver. These sections were taken from fish injected with 1 mg ECP which represents the greatest toxic effect. All fish within this group were morbid within 8 hours indicating an acute effect and there was insufficient time to observe a gross response other than discolouration. In 3 of the surviving fish from the 125 µg group, a jaundiced liver was observed. The focal necrosis and inflammation in this case was enough to damage the hepatocytes and prevent the removal of bilirubin from the liver causing jaundice (Maeno *et al.* 1995). A jaundiced liver was observed in these fish because they were still alive whereas the morbid fish had succumbed to the damage caused not only to the liver but also all other internal organs.

The toxin caused necrosis of the cells within organs as observed in the gills, heart, liver and pyloric caeca. The toxin is either causing autolysis or rapid lysis of the cell (Mallatt 1985). It is suggested that the fish attempts to mount a defence response; epithelial lifting and hyperplasia could serve as a defence mechanism by increasing the diffusion distance between the toxin and the bloodstream (Mallatt 1985), and there is also the infiltration of inflammatory cells into the heart and intestine. Haemorrhaging into the peritoneum is a result of bleeding from the organs, which are breaking down. The spleen may be increasing red blood cell production in order to counteract the blood loss or alternatively to store blood cells for later use.

Overall, the ECP produced by *T. maritimum* caused necrosis in Atlantic salmon. Therefore, it has the potential to play a significant role in the formation of lesions on the external surfaces of fish. Necrotic lesions on the external surfaces of fish with flexibacteriosis are characterised by destruction of the epithelial cells and connective tissue which leaves musculature exposed to the external environment (Carson *et al.* 1992). It has been demonstrated that the ECP caused cell

necrosis in the internal organs of Atlantic salmon; the same affect that occurs on the external surface during infection. This, together with the short survival times recorded in the pathogenicity studies (Chapter 3), provides evidence that this bacterium probably utilise the ECP to facilitate cellular breakdown and by acting synergistically are an effective pathogenic system.

Chapter 5

Vaccination of Atlantic salmon against *Tenacibaculum maritimum*

This chapter is modified from a paper prepared for publication in Aquaculture.

5.1 Introduction

Tenacibaculum maritimum is a well-established fish pathogen worldwide (Wakabayashi *et al.* 1986, Devesa *et al.*1989, Bernardet *et al.* 1990, Chen *et al.* 1995, Kusuda and Kawai 1998, Ostland *et al.* 1999). Apart from a major outbreak in 1988/89, it has been a relatively minor yet persistent problem in the Tasmanian salmonid aquaculture industry. Previous management strategies such as improved handling practices have significantly decreased the incidence of the disease in Tasmania (Handlinger *et al.* 1997).

However, with seawater temperatures continuing to rise around Tasmania, the incidence of *T. maritimum* may increase from minor outbreaks to a chronic problem. There is also limited information on the biological factors that could initiate further outbreaks of the disease. Patterns of infection are variable and effective management strategies would be difficult to implement (Carson *et al.* 1992). Antibiotics are undesirable as a long term strategy with problems of bacterial resistance and tissue residence times (Cabello 2006). The most common treatment, trimethoprim, has a withholding period of 500 degree days which is unacceptable in commercial production (S. Percival *pers. comm.*). Amoxycillin is an alternative that has a short withdrawal time of 50 degree days, but is less efficacious than trimethoprim (Cameron 1993b). Oxytetracycline was efficacious during the initial outbreak of *T. maritimum,* but is known to have several long term detrimental effects (Soltani *et al.* 1996). While *in vivo* evaluation of amoxycillin and trimethoprim against *T. maritimum* has been validated by clinical trials (Soltani *et al.* 1996), there is no evidence of their efficacy under severe outbreak conditions. It is also necessary to consider the environmental impacts of antibiotic use. Antibiotic resistant bacteria, an increase in the bacterial resistance in fish pathogens, changes to the bacteria flora of the

marine environment and their flow on effect to other animals and humans are a result of using chemotherapeutants heavily or long term (Cabello 2006).

In order to reduce the reliance on chemotherapeutants and to prevent future unforseen outbreaks from occurring, development of a safe and effective vaccine against *T. maritimum* in salmonids is the focus of this study. Other bacterial diseases such as yersiniosis and vibriosis have been successfully managed with the development of a vaccine (Håstein *et al.* 2005). As yet, a vaccine is not available for *T. maritimum* for salmonids. Previous attempts to develop a vaccine for salmonids in Tasmania proved unsuccessful due to inconsistency of results (Carson *et al.* 1993, Carson *et al.* 1994), because numbers of mortalities were different between experiments that were repeated. Recently, promising results for an injectable vaccine for species of sole (*Solea solea* and *S. senegalensis*) in Spain were reported (Romalde *et al.* 2005). However, an effective vaccine for the Tasmanian salmonid aquaculture industry will likely need to be based on local strains of *T. maritimum* affecting local Atlantic salmon (*Salmo salar*) stocks. Since *T. maritimum* is an external pathogen, it may be assumed that immersion vaccination would be initially trialled. However, while immersion vaccination has shown to be effective for other external pathogens such as *Vibrio anguillarum* and *Yersinia ruckeri*, it is not effective for *Aeromonas salmonicida* which requires intraperitoneal injection with adjuvants to produce a moderate level of protection (Ellis, 1988). Intraperitoneal injection is also the industry standard for vaccination and with the development of polyvalent vaccines, intraperitoneal injection would be the most suitable method of delivery.

The aims of this study were to:

1. Determine the cumulative mortality of Atlantic salmon challenged with *T. maritimum* that have been inoculated with experimental vaccines.
2. Determine vaccine efficacy using Relative Percent Survival (RPS).
3. Determine the short-term side effects of vaccination following challenge with *T. maritimum*.

5.2 Materials and Methods

5.2.1 Vaccine preparation

A strain of *T. maritimum* (89/4762) isolated from diseased Atlantic salmon (see Table 2.1) was grown in an aerated 10 L carboy using Shieh's medium formulated with natural seawater. The bacteria were grown in 5 L of broth at room temperature (20°C) with aeration (120 L/h) and harvested after 48 hours incubation. Cell concentration reached a mean of 1.27×10^9 cells/mL as determined by direct counts using a Helber counting chamber. The bacteria were inactivated by the addition of formalin to a final concentration of 5 mL/L. After 48 hours refrigeration, the cells were collected by centrifugation at 3000 x *g* for 30 minutes and washed three times using phosphate buffered saline (PBS, pH 7.4). Cells were resuspended in PBS to a final concentration of 1.08×10^{10} cells/mL and formalin added to a final concentration of 0.1% v/v as a preservative. A vaccine preparation containing Freund's incomplete adjuvant (FIA) (Sigma) was also produced. The adjuvant was mixed 50:50 with the vaccine suspension just prior to use. Both vaccine preparations used for this study contained approximately 1.79×10^9 cells/0.1 mL inoculum.

The vaccine was tested for sterility using a modified version of the British Pharmacopoeia standard (Anon. 2005). Ten millilitres of tryptone soy broth (TSB, Oxoid) and marine Shieh's broth (MSB) and 25 mL of fluid thioglycollate medium (FTM, Oxoid) were inoculated with 500 µL of the formalin-inactivated suspension. After 7 days incubation at 25°C for TSB and MSB and 35°C for FTM, the broth media were subcultured on to tryptone soy agar (TSA, Oxoid), marine Shieh's agar (MSA) and FTM and incubated for a further 5 days at the same

temperatures as previously stated. Absence of growth on the agar plates or FTM was interpreted as absence of culturable bacteria in the vaccine preparation; five replicates for each broth were used for testing.

A fertility test of the enrichment broths was made using cultures of *Staphylococcus aureus*, (ATCC 6538) *Pseudomonas aeruginosa* (ATCC 9027) (supplied by the Department of Primary Industries and Water) and the strain 89/4762 of *T. maritimum*. Broth cultures, 24-48 h old of the test organisms were diluted to a density McFarland 0.5 and diluted 10^{-5} in sterile PBS (pH 7.4). TSB was inoculated with 100 µL of *P. aeruginosa*; the FTM with 100 µL of *S. aureus* and MSB with the *T. maritimum*. All cultures were incubated for 48 h and then *P. aeruginosa* was subcultured to TSA, *S. aureus* to FTM and *T. maritimum* to MSA for another 48 h at 25°C for TSA and MSA and 35°C for FTM. Growth on the agar plates or FTM was taken to mean that inhibitors were not present in the enrichment media and was capable of supporting the growth of bacteria; five replicates were used for testing.

5.2.2 Experimental fish and challenge conditions

Atlantic salmon (approximately 100 g) were obtained from a commercial hatchery and maintained in 12 x 200 L tanks in an aerated freshwater flow-through system. The fish were assigned to tanks to ensure consistency of density between tanks. Fish were held for one week prior to vaccination. Post vaccination, fish were held at 15°C for 6 weeks in freshwater. Acclimation to seawater was achieved by the gradual increase (5 ppt/day) of seawater over seven days and the fish acclimated to 18°C over another 7 days. The system was switched over to re-circulation once acclimation commenced. Fish were fed a commercial diet (Atlantic HP - Skretting) at maintenance (1% body weight) until challenged, 8 weeks post vaccination. The

fish were randomly assigned to tanks and divided into 4 treatments which were tested in triplicate (Table 5.1).

Table 5.1 Treatments used for *Tenacibaculum maritimum* vaccination experiment. Each treatment was undertaken in triplicate.

Treatment	Description	Fish No.
Control	Fish not injected and not handled until challenge	32
Control IP	Fish injected with sterile PBS to examine the effects of handling and injection	37
Vaccine	Fish injected with formalin inactivated bacteria vaccine	38
Vaccine + Adjuvant	Fish injected with formalin inactivated bacteria vaccine containing Freund's incomplete adjuvant	36

Water quality variables such as temperature, salinity (maximum 35 ppt), pH (max. 8.0), total ammonia (max. 2 mg/L), nitrates (max. 10 mg/L) and nitrites (max. 0.5 mg/L) were monitored throughout the experiment and controlled accordingly. Throughout the study, the freshwater pH was consistently around 6.0 and even though flow was decreased and sodium bicarbonate added, maintaining a higher pH was difficult. The problem of low pH levels was exacerbated by the high chlorine levels of the incoming water that was controlled by the use of sodium thiosulphate. The use of sodium thiosulphate caused the pH of the freshwater to decline, but not below 6.0. During maintenance in freshwater, some non-specific mortalities occurred due to the occasional decreases in water pH. Fish were unable to be moved between tanks since mortalities occurred after treatment (vaccination) had been applied. This affected the number of fish per treatment (see Table 5.1). No mortalities occurred during acclimation or holding in seawater.

5.2.3 Vaccination

Fish were anaesthetised using clove oil (0.03 mL/L of seawater) and vaccinated using Luer Lock glass syringes (2 mL capacity) with 23 gauge needles. The vaccine was injected as 0.1 mL volumes per fish into the peritoneal cavity, 1 cm anterior to the pelvic fins and approximately 0.5 cm in depth. A separate tank with aerated freshwater was used to ensure the recovery of the fish before returning them to their experimental tanks.

5.2.4 Challenge procedures

The challenge strain (89/4762) was cultured using an aerated 10 L carboy at room temperature (20°C) in MSB. The bacteria were grown in 5L of broth with aeration (120 L/h). After 48 hours the cells were collected by centrifugation at 3000 x *g* for 30 mins at 4°C and washed three times using 0.22 μm filtered seawater. Cell concentration was determined microscopically using a Helber counting chamber and adjusted to a final concentration of 1.0×10^6 cells/mL which was used as the challenge dose. This dose was calculated to give a 60% mortality rate in control treatments. Fish were placed in 100 L seawater tanks and challenged for 1 h by bath exposure. Following challenge, the fish were returned to their original tanks. Mortalities were monitored 3 times a day for the experimental period which was deemed to have ended when there were 3 consecutive days of no mortalities.

5.2.5 Sampling procedure

All fish were examined bacteriologically by culturing skin lesions or sites of erosion on plates of MSA containing 4 μg/mL neomycin sulfate (Pazos *et al.* 1996). Direct smears (1 cm x 1 cm) were prepared for staining by Gram's method and by immunofluorescence (IFAT). Cultures

were incubated at 25°C for 2 days and the colonial morphology typical of *T. maritimum* recorded. Internal organs were examined grossly since fish had been vaccinated by intraperitoneal injection. Flexibacteriosis lesion distribution and lesion size were recorded for all fish. Lesion size was measured as length of lesion along the longest axis and was recorded to the nearest half centimetre. The numbers of lesions recorded at each site were divided by the total number of lesions recorded for that treatment, thereby indicating differences in areas being affected. Any fish surviving at the end of the challenge period were euthanased with clove oil (0.025 mL/L) and lesions or sites of erosion examined bacteriologically as described. Photographs were taken of the fish to document gross pathology and samples of skin lesions or sites of erosion were taken for histological examination.

5.2.6 Case definition

The criteria for accepting that a fish was diseased involved two parts: method of recovery and quantification of colonies and cells. *Tenacibaculum maritimum* had to be recovered from mortalities by culture and in smears by IFAT as these methods are more specific than Gram stain. The minimum abundance of cells required for a positive result was 10-50 bacteria per 25 fields for IFAT and Gram stain. The minimum abundance required to record a positive result in culture was growth of colonies extending to the first streaking lines.

5.2.7 Histological examination

Initially the samples were fixed in seawater Davidson's (Shaw and Battle 1957, Speilberg *et al.* 1993) for 72 hours 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 x40 – x1000 for interpretation.

5.2.8 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 μL of rabbit anti-*T. maritimum* 89/0329-5 (DPIW) diluted 1:100 in PBS (pH 7.2, 0.1M) and incubated in a moist chamber for 30 minutes at 37°C before rinsing in PBS for 15 minutes. After the removal of excess buffer by blotting, 20 μL of anti rabbit FTIC (Silenus) diluted 1:60 in PBS was added to each slide. Smears were incubated at 37°C for 30 minutes and rinsed for 30 minutes in PBS, with a change of buffer every 10 minutes. Slides were mounted using alkaline glycerol buffer, (Johnson and Munday 1993), coverslipped and examined at x 40 magnification with epifluorescent microscopy using UV illumination.

5.2.9 Vaccine efficacy and statistical analysis

Vaccine efficacy was determined using Relative Percent Survival (RPS) (Amend 1981).

$$RPS = 1 - (\%\ \text{vaccine mortality} / \%\ \text{control mortality}) \times 100$$

Data were also analysed using Kaplan-Meier survival analysis and a pair-wise comparison over strata with a Bonferroni correction of *p*-values using SPSS 11.5 for Windows.

5.3 Results

Fish injected with Vaccine + Adjuvant showed significantly better survival than other treatments based on pair-wise comparisons (Figure 5.1). Within the experimental period, cumulative mortalities of the different treatments were: Controls IP, 54%; Controls, 50%; Vaccine, 39%; Vaccine + Adjuvant, 11% (Figure 5.1) which Kaplan-Meier survival curve analysis deemed to be significant ($p \leq 0.0010$). Mortality rates in the control treatments were lower than the expected rate of 60%. Low RPS values were found between the Vaccine and both control treatments. In contrast, against both control treatments, high RPS values were found for the Vaccine + Adjuvant treatment (Table 5.2). Methods used to confirm the presence of *T. maritimum* from mortalities were sensitive with bacteria being detected from all tests (Table 5.3).

Table 5.2 Relative percent survival of *Tenacibaculum maritimum* vaccine mixtures injected into Atlantic salmon.

Treatment	Compared to:	Relative Percent Survival (RPS)
Vaccine	Control IP	27.7%
	Control	22.0%
Vaccine + Adjuvant	Control IP	79.6%
	Control	78.0%

Table 5.3 Comparison of media culture, Gram stain and IFAT preparations for the detection of *Tenacibaculum maritimum* in Atlantic salmon.

Treatment	Culture (%) mean (± S.E.)	Gram (%) mean (± S.E.)	IFAT (%) mean (± S.E.)	Lesion
Control	100 (0)	100 (0)	100 (0)	+
Control IP	100 (0)	100 (0)	100 (0)	+
Vaccine	100 (0)	100 (0)	100 (0)	+
Vaccine + Adjuvant	100 (0)	100 (0)	100 (0)	+

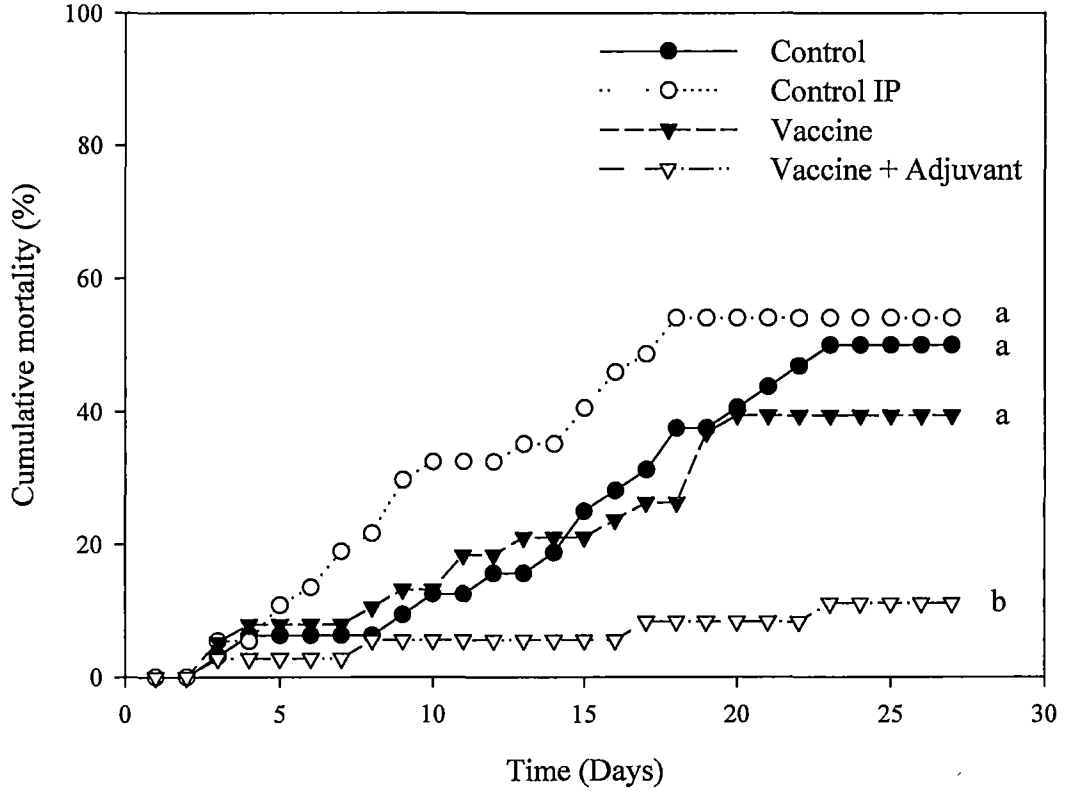

Figure 5.1 Cumulative mortality (%) of Atlantic salmon given intraperitoneal vaccination of experimental vaccine mixtures against *Tenacibaculum maritimum*. Lines with different letters are significantly different ($p \leq 0.0083$).

Gross pathology in the challenge of fish was similar to natural infections and previous pathogenicity trials. Lateral sites were dominant in lesion distribution; however, pectoral sites were more predominant in the Vaccine and Vaccine + Adjuvant treatments (Figure 5.2). Tail necrosis was common among all treatments while gill necrosis only appeared in one case (Control IP) throughout the entire study. Skin missing from the jaw area was notable in all treatments and eye damage were also present; eyes were either absent from the eye socket or had ruptured within the eye socket (Figure 5.4a). *Tenacibaculum maritimum* could be recovered from affected sites, but lack of corneal tissue prevented histological assessment to be made to discern if *T. maritimum* was involved in the eye pathology. Larger lesions tended to be found in the Vaccine and Vaccine + Adjuvant treatments, while both control treatments displayed smaller lesions (Figure 5.3). The number of lesions between vaccinated treatments and unvaccinated control treatments was 4.3 lesions/fish and 5.6 lesions/fish respectively. Similar to previous pathogenicity trials (Chapter 3), larger lesions were found around pectoral, pelvic and caudal sites. Smaller lesions tended to be found on the dorsal, lateral or ventral surfaces. The majority of surviving fish did not show any clinical signs of flexibacteriosis, with inflamed fins and small blisters noted on rare occasions.

Histological examination of lesions and erosive sites showed the same pathology as those observed in chapter 3, and therefore are not repeated in this chapter.

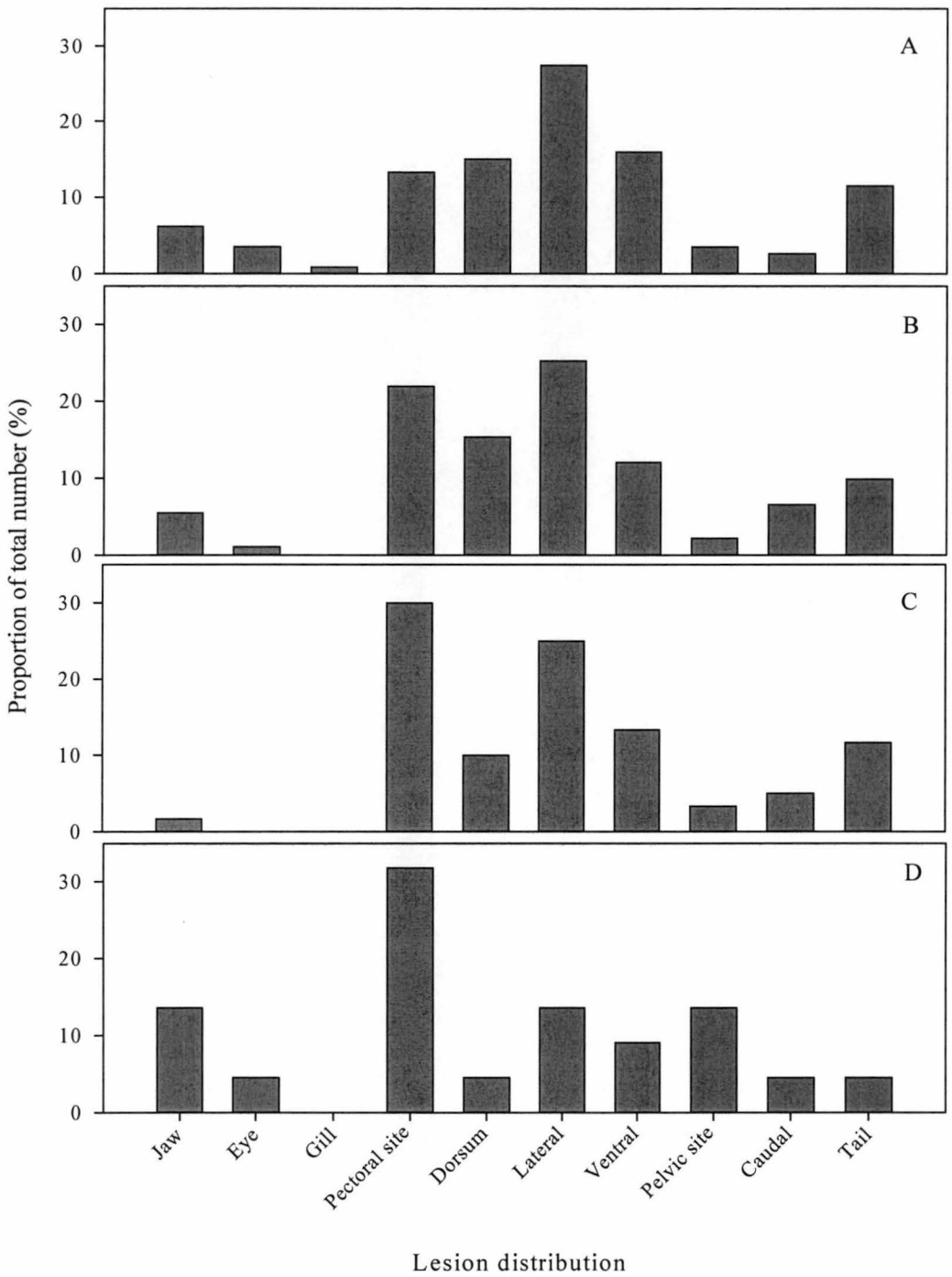

Figure 5.2 Distribution of lesions in Atlantic salmon challenged with *Tenacibaculum maritimum* strain 89/4762 post-vaccination: A, Control IP; B, Control; C, Vaccine; D, Vaccine + Adjuvant.

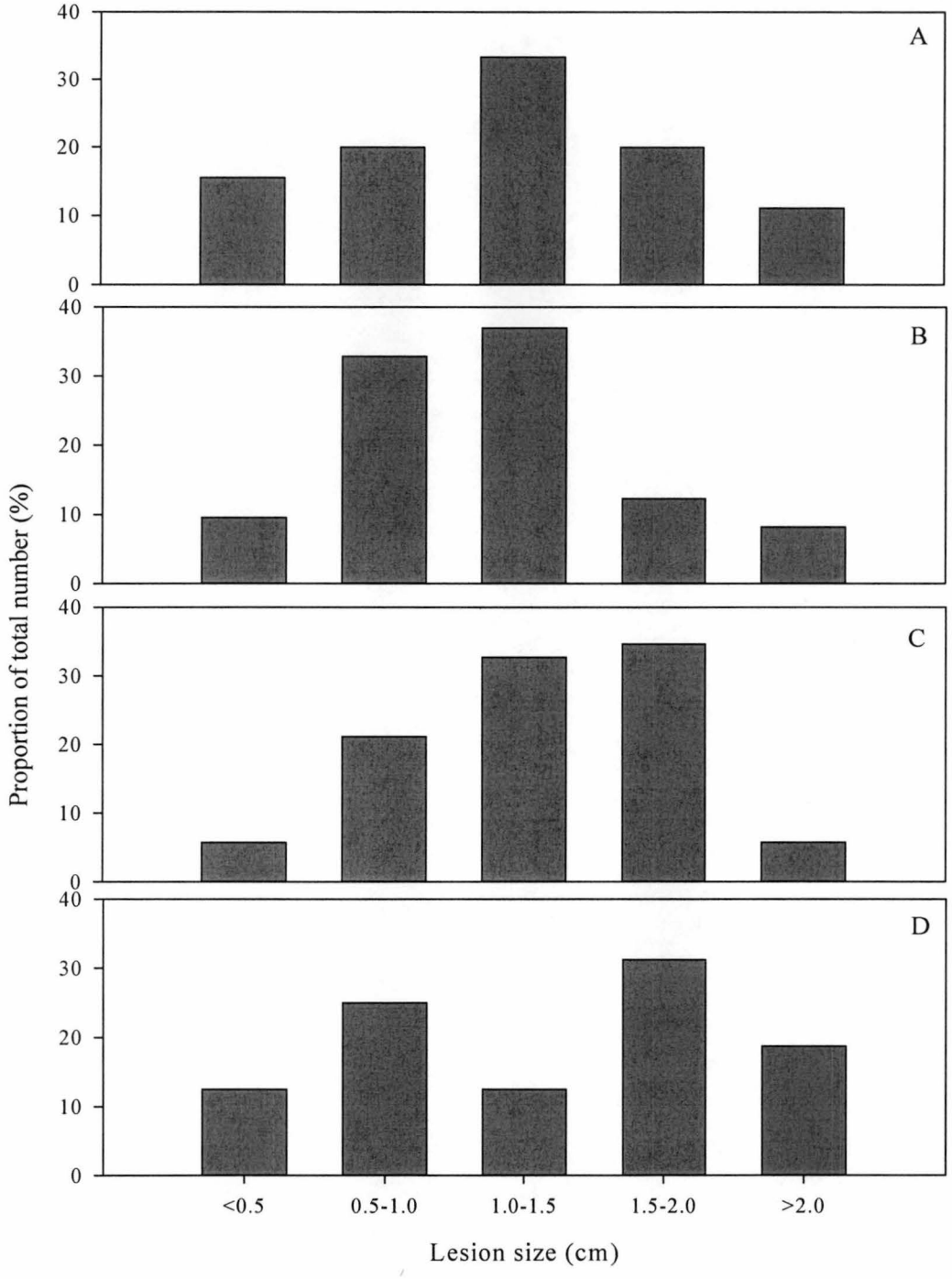

Figure 5.3 Lesion size (%) in Atlantic salmon challenged with *Tenacibaculum maritimum* strain 89/4762 post-vaccination: A, Control IP; B, Control; C, Vaccine; D, Vaccine + Adjuvant.

Internal organs were also examined to investigate any side effects due to intraperitoneal injection. A consistent finding in fish injected with the Vaccine + Adjuvant treatment was the presence of black/brown pigment associated with the stomach and an associated inflammatory response (Figure 5.5a). It was not found in fish from any other treatment. By histological examination it was apparent that the black/brown pigmentation was most likely melanin (Figure 5.5b). Associated with this pigmentation was also chronic inflammation, abnormal accumulation of fat, granulomas, inflammatory cells and cysts. Another gross clinical sign was observation of lesions through the abdominal musculature (Figure 5.4b). This only occurred when large ulcerative lesions were observed on the external surface of the fish and was not associated with sites of injection. No gross pathology was observed at injection sites in all treatments.

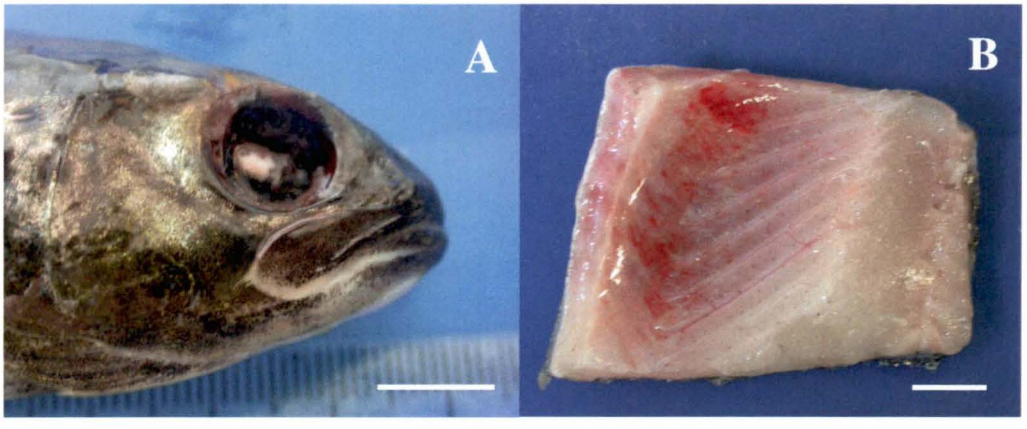

Figure 5.4 Gross pathology from the vaccine trial: A, ruptured eye of Atlantic salmon; B, flexibacteriosis lesion of the abdominal musculature. Scale bars 1 cm.

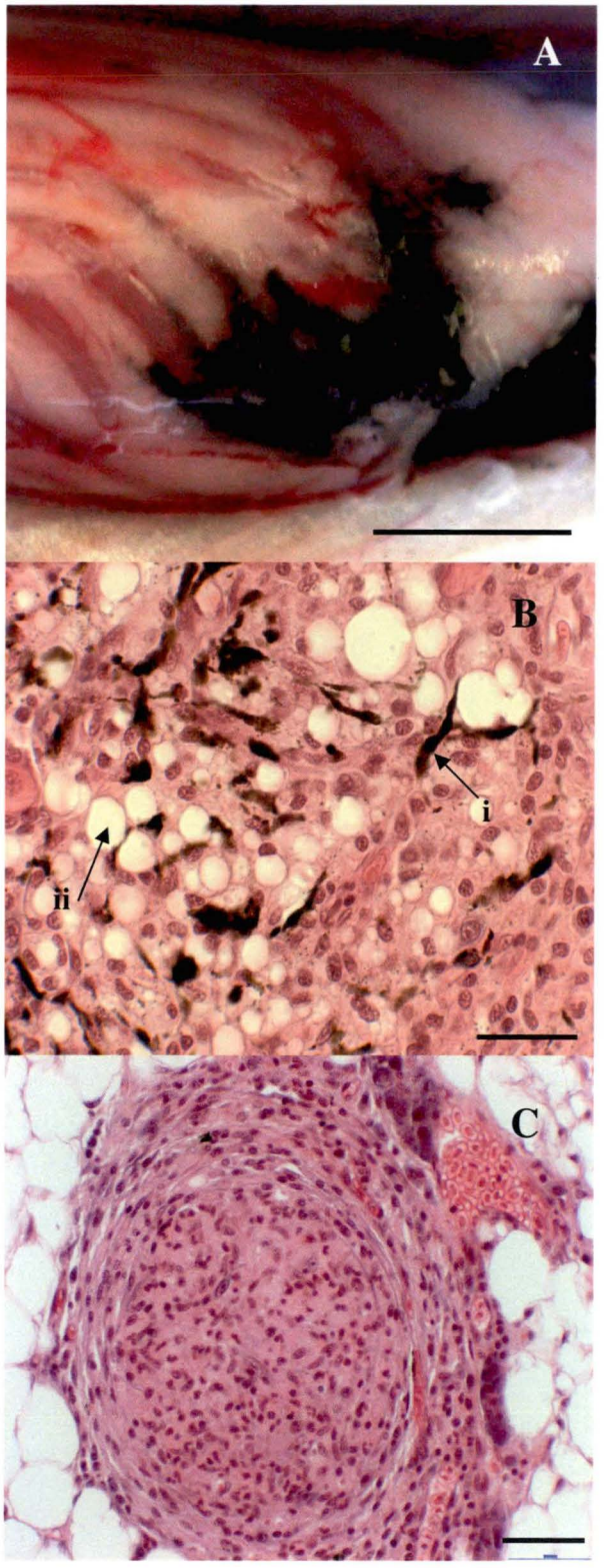

Figure 5.5 Gross and histological pathology of adjuvant induced side-effect: A, black/brown pigment associated with the stomach of fish injected with Vaccine + Adjuvant (scale bar 1 cm); B, histological section of the stomach showing (i) black/brown pigmentation (ii) vacuole (scale bar 30 μm); C, granuloma among fat tissue (scale bar 50 μm).

5.4 Discussion

Within the challenge period of 27 days, significant protection was demonstrated in fish injected with the Vaccine + Adjuvant compared to all other treatments. In fact, RPS values were calculated at 79.6% and 78.0% which according to Amend (1981) is close to the RPS value required for a vaccine to be used under practical field conditions (80%). The vaccine without adjuvant did show lower mortalities than control treatments, however, it was not significantly different from both control treatments. Low RPS values at 27.7% and 22.0% indicate that the vaccine without adjuvant could not provide sufficient protection from a moderate challenge of *T. maritimum*. It is important to note that the current study did deviate from the criteria set by Amend (1981). Treatments were set up in triplicate, not duplicate and there was not a total of 50 fish per treatment. There was also only one level of challenge. However, survival curve analysis reinforces the results obtained by RPS which reflect the comparisons of each treatment group with each control group. The Vaccine + Adjuvant treatment was significantly different, while the Vaccine treatment was not. Based on RPS values and survival curve analysis the Vaccine + Adjuvant treatment provided significant protection against *T. maritimum*.

Previous work on a vaccine for Atlantic salmon against *T. maritimum* provided inconsistent results (Carson *et al.* 1993, Carson *et al.* 1994). Initial studies using strain 89/4762 failed to protect fish against moderate to severe levels of challenge using the dip process as a vaccination technique. Further studies included a formalin inactivated injection vaccine using strain 89/4762. Based on gross clinical signs, two RPS values were calculated at 0% and 23%. The vaccination dose in this case was lower than the current study at 7.5×10^6 cells/mL, however, the fish were booster vaccinated two weeks after initial vaccination (Carson *et al.* 1994). A

booster was not provided in the current study. Another point to note was that the challenge dose used by Carson *et al.* (1994) was also at lower concentrations ($1.4 \times 10^4 - 3.9 \times 10^4$ cells/mL) than the current study and yet both studies used 60% endpoints based on previous work for the same strain (89/4762). It is clear though that in this and other studies a vaccine based on formalin inactivated bacteria is not sufficient to provide adequate protection via injection for Atlantic salmon.

In contrast, injection of sole using a formalin inactivated bacterin reported a RPS value of 94%, 6 weeks post-vaccination (Romalde *et al.* 2005). Mortality rates of 5% were recorded for vaccinated and 85% for non-vaccinated fish. However, the challenge dose of 1.1×10^8 cells/fish was injected intraperitoneally which does not reflect the natural course of the disease. Interestingly, it has been demonstrated that Atlantic salmon vaccinated against furunculosis (causative agent *Aeromonas salmonicida*) by intraperitoneal injection and then subsequently challenged via the same method resulted in high protection and RPS values (Nordmo and Ramstad 1997). The protection is suggested to be local immunity in the abdominal cavity and with a secondary water-borne infection the RPS values decrease (Nordmo and Ramstad 1997). Comparison of intraperitoneal injection and immersion challenges in Atlantic salmon using the same inoculum doses showed that immersion provided lower cumulative specific mortality (Nordmo and Ramstad 1999). The authors suggested that RPS guidelines should consider the method of challenge when evaluating vaccine efficacy. In addition, the inability to cause mortalities or induce disease using intraperitoneal injection of *T. maritimum* has been reported in Atlantic salmon (Soltani 1995); red and black sea bream (Wakabayashi *et al.* 1984) and turbot and sole (Alsina and Blanch 1993, Avendaño-Herrera *et al.* 2006). There is insufficient information from the vaccination of sole (Romalde *et al.* 2005) to determine the parameters by

which the fish were deemed to have died from an infection. Furthermore, the duration of protection after vaccination is also variable with species and size of fish, as well as bacterin concentration (Johnson *et al.* 1982). Sole were challenged 6 weeks post vaccination (Romalde *et al.* 2005) which may be a significant factor in achieving high levels of protection. Timing of challenge after vaccination is important since protection from vaccination changes over time and is dependent on variables such as fish species, water temperature and the inclusion of booster vaccinations (Bricknell *et al.* 1999). After initial vaccination, time is needed for the development of the immune response which will peak and then diminish over time (Bricknell *et al.* 1999). Given that sole were maintained in water temperatures of 20°C and that a booster was provided, a challenge 6 weeks post vaccination could achieve higher protection values than would be anticipated. Therefore, at this time conclusions about the duration of protection of the sole vaccine are uncertain

Gross pathology was consistent with previous findings in terms of development of lesions and areas affected. An important point that requires emphasis here is that vaccination gives better protection in terms of mortalities, but not necessarily in terms of lesion formation. Both vaccinated treatments showed a tendency of lesions being present at pectoral sites, with larger lesions observed compared to control treatments (Figures 5.2 and 5.3). This does not mean that the disease in vaccinated fish was worse compared to unvaccinated fish, only that the disease in these fish were focussed in these areas and that in comparison to other sites, pectoral sites constantly had larger lesions associated with them. It is suggested that sites where there is constant irritation, such as pectoral fins, facilitate the development of the lesion. Skin damage is a predisposing factor for the colonisation of *T. maritimum* (Handlinger *et al.* 1997), and the pectoral fin can scar a larger area of the body. The pectoral fin can abrade the surface of the skin

faster and affect a larger area in a shorter amount of time compared to other areas of the body. Smaller lesions were consistently found on the dorsal, ventral and lateral sites and they tended to be greater in number. Eye pathology observed throughout the experiment was either ruptured or missing eyes from which *T. maritimum* could be recovered. Handlinger *et al.* (1997) also reported complete eye collapse and corneal rupture in experimentally infected salmonids and noted the presence of *T. maritimum* at the affected sites. Eye pathology in the study of Handlinger *et al.* (1997) was reported not be the result of a *T. maritimum* infection but the result of other non-specified causes. In the current study, it is suggested that the eye pathology is an experimental artefact until further evidence is obtained.

Incorporated into this experiment was an observation into differences in handling stress by using an 'unhandled' control treatment (Control) and another that was subjected to handling, intraperitoneal injection and anaesthesia (Control IP). Previous studies have indicated stress as a significant factor for pathogenicity due to *T. maritimum* (Handlinger *et al.* 1997). However, there was only a 4% difference in the cumulative mortality between the Control treatment and the Control IP treatment over the experimental period, which was not significantly different. There was an 8 week interval between when fish were vaccinated and when they were challenged. This amount of time appears to be sufficient to negate any stress or damage that may have occurred during the initial vaccination of the Control IP treatment and therefore no differences in cumulative mortality could be detected between control treatments.

Both control treatments had mortalities of 50% and 54% which was less than the 60% mortality rate that was anticipated. While immersion challenge offers a more natural method of exposure than intraperitoneal and intramuscular injection, it is more difficult to standardise and control

(Nordmo 1997). Variables such as temperature and density affected the outcome of co-habitation challenges in Atlantic salmon infected with *Aeromonas salmonicida* and *Vibrio salmonicida* (Nordmo and Ramstad 1999). In the current study there was no difference in the temperature used in both the pathogenicity and vaccine experiments, however, there was a lower density of fish in the vaccine experiment compared to the pathogenicity trial. Further experimentation of immersion challenges would be useful to determine dose effect and vaccination efficacy.

However, it is evident that the Vaccine + Adjuvant treatment provided protection for Atlantic salmon against *T. maritimum* and warrants further research. Due to limited resources, a group of fish vaccinated with only the adjuvant was not included. It was deemed more important to have sufficient replication of tanks due to previous inconsistent results using *T. maritimum* (Carson *et al.* 1993, Carson *et al.* 1994). There was also a concern relating to handling stress and controlling for the use of injection; particularly as a portal of entry for the bacteria when the fish were challenged by bath. Carson *et al.* (1992) noted that skin damaged by scarification, branding and tagging developed into the focal point of the infection for rainbow trout challenged with *T. maritimum*. These were made a priority over an adjuvant only treatment because adjuvants are not known to provide specific immunity (Agius *et al.* 1983) and are added to vaccines in order to enhance the immune response (Evensen *et al.* 2005, Schijns and Tangerås 2005). However, there may be short term confounding effects due to non-specific factors (Olivier *et al.* 1985). Therefore, future work should include an adjuvant only treatment to fully determine its role in vaccination, thereby confirming the results of this trial.

The significant difference in cumulative mortality between the Vaccine and the Vaccine + Adjuvant treatments suggest the need for an adjuvant in the *T. maritimum* vaccine to elicit an immune response and provide protection. Vaccination of tilapia (*Oreochromis niloticus*) with *Flavobacterium columnare* required the use of Freund's complete adjuvant (FCA) to mount a humoral response in both plasma and cutaneous mucus (Grabowski *et al.* 2004). It is likely that an effective *T. maritimum* vaccine will require an adjuvant to produce an immune response. Investigations into different adjuvants will be important because even though high levels of protection were provided by Freund's incomplete adjuvant (FIA) in the current study, there were apparent side-effects.

Oil based adjuvants used in injectable vaccines are usually associated with side-effects observed in the peritoneal cavity (Evensen *et al.* 2005, Håstein *et al.* 2005). This can include inflammatory responses, the formation of granulomas, fibroblastic response and adhesions in the abdominal cavity. Long-term severe effects can include growth and spinal deformities (Evensen *et al.* 2005, Håstein *et al.* 2005). All fish injected with the Vaccine + Adjuvant had black/brown pigment associated with the stomach, which did not appear in any of the other treatments of fish. Histological examination revealed this material to be black/brown pigmentation most likely caused by melanin. An inflammatory response was noted around this material with the formations of granulomas and cysts. A granulomas inflammatory response with localised inflammatory reactions has been well described and are a common side affect of mineral oil-adjuvant vaccines (Lillehaug *et al.* 1992, Midtlyng 1996, Mutoloki *et al.* 2004). However, pathology such as this is not usually found in fish vaccinated with Freund's Incomplete Adjuvant (FIA). Currently, there are no reports in the literature that document FIA related pathology. Care was taken to ensure that the vaccine was administered in the peritoneal cavity,

just under the skin without touching any organs. The stomach is located in close proximity to the injection site and the vaccine may have been administered over the stomach which could have lead to the observed side-effects. However, Koppang *et al.* (2005) suggested that components of the adjuvant vaccine are dispersed from the injection site and cause foreign body inflammatory responses in organs and tissue throughout the body that are unpredictable and generalised. In contrast, pigmentation has been noted in pre-smolt Atlantic salmon vaccinated against furunculosis at the site of injection (Midtlyng 1997). In the current study, there was no pigmentation or any inflammatory response surrounding the injection site.

More importantly, the effect of the adjuvant being associated with the stomach could have serious consequences as growth may be retarded and/or feed intake and digestibility be impeded (Poppe and Breck 1997, Midtlyng and Lillehaug 1998, Sørum and Damsgard 2004). Side-effects of injectable vaccines with oil adjuvants have been widely reported and are undesirable as they reduce flesh quality and raise questions over animal welfare (Poppe *et al.* 2002, Koppang *et al.* 2005). The need for oil adjuvants may be reduced in the future through further investigation into the antigenic properties of *T. maritimum* and development of vaccine types and administration techniques. Other vaccine formulations to be tested could include adjuvants which do not produce side effects and the ECP toxoid.

Chapter 6

General Discussion

Before this study, there had been some attempts to produce a viable vaccine to *T. maritimum* for the Tasmanian salmonid industry; however, they had not been successful (Carson *et al.* 1993, Carson *et al.* 1994). The current study not only provided a vaccine formulation that showed significant protective value in Atlantic salmon (Chapter 5), but also provided insight into many important aspects of the nature of *T. maritimum* including its physical characterisation (Chapter 2), its pathogenicity (Chapter 3) and its ECP toxicity (Chapter 4).

Tasmanian isolates are relatively homogeneous at the whole cell level although there appear to be at least two serotypes present (Chapter 2). However, the serotypes are not host or geographically specific, unlike the Spanish isolates (Avendaño-Herrera *et al.* 2004, Avendaño-Herrera *et al.* 2005b). Initial phenotypic characterisation described *T. maritimum* as a homogeneous taxon (Wakabayashi *et al.* 1984, Bernardet *et al.* 1994), although further serological characterisation proved the existence of antigenic differences within the species (Ostland *et al.* 1999, Avendaño-Herrera *et al.* 2004). Further characterisation of isolates are required not only for Tasmanian isolates but also in comparison with European and Japanese isolates. Of particular interest are the two isolates (89/4747 and 01/0356-7) that showed not only antigenic differences but whole cell differences as well, even though they have been biochemically and phenotypically identified as *T. maritimum* (Schmidtke *et al.* 1991, Carson 1998). There is still the unanswered question of where 89/4747 and 01/0356-7 fit in and what the differences mean for the taxon and potential vaccine development. The ramifications for vaccine development are that homogeneity within the species is more likely to produce a viable vaccine across strains and fish species. Antigenic differences pose a problem as a vaccine based on one serotype may not provide protection for another serotype (Romalde *et al.* 2005). Host specific differences in serotype was the basis for the development of separate vaccines for turbot

and sole as it was believed that a vaccine produced for one would not be effective in the other (Romalde *et al.* 2005). In the current study, while differences in LPS have been detected (Chapter 2), the importance of these differences and how they relate to a protective antigen are currently unknown. So far there is only information on three Tasmanian isolates with respect to virulence of strains and heterogeneity of the molecule. Further characterisation of more isolates and investigations into the antigenic properties of *T. maritimum* LPS are areas of further investigation.

A similarity between all isolates was that they were hydrophobic in nature (Chapter 2), which is consistent with other findings for *T. maritimum* (Sorongon *et al.* 1991, Kawahara and Kusuda 1998). Pathogenic bacteria such as *Renibacterium salmoninarum* (Bruno 1988), *Vibrio anguillarum* and *Aeromonas salmonicida* (Santos *et al.* 1991) are also hydrophobic. The pathogenesis of the disease may be assisted by the hydrophobic nature of the bacteria as it would facilitate host cell attachment, which is an important step in colonisation (Ofek and Doyle 1994). Pathogenicity does not appear to be affected by the culture medium in which the bacteria are grown, but is affected by the presence of aeration within broth culture (Chapter 2). This phenomenon has also been observed between static and shaken cultures of *T. maritimum* isolate (89/4762), where the static culture produced fewer mortalities than the shaken culture (Soltani 1995). Tasmanian isolates produce rough suspensions; the cells aggregate into clumps rather than disperse into a homogeneous suspension (Chapters 2 and 3). *Tenacibaculum maritimum* is an obligate aerobe (Wakabayashi *et al.* 1984) and the presence of aeration appears to allow more dispersal within the broth rather than clumping at the air-water interface, thereby producing a more homogeneous suspension. Differences in pathogenicity may simply be related to physical conditions. The clumping of bacteria produced in a non-aerated broth would not expose fish to

the same number of bacteria as an aerated broth even though spectrophotometric measurements appear similar. It is suggested that the adhesive nature of the bacteria is associated with pathogenicity/virulence. Under natural conditions it is unlikely that the bacteria would clump together as they do in artificial broth culture. The artificial broth culture demonstrates the sticky nature of the bacteria which under natural conditions might be of benefit in attachment to the host, rather than each other. Attachment to the host is an important first step in the disease process (Ofek and Doyle 1994) and would allow proliferation on the host tissue and facilitate dispersal of any toxin the bacteria produces. The stickier the isolate the more virulent it is likely to be as it would be more adherent to the host. Agglutination would not be the only virulence factor, but on the basis of what has been found in this study, it is an extremely important one that requires greater attention. Virulence factors have been suggested to be critical for stimulating an immune response in the host and a vaccine produced from an isolate without expression of such factors can render a vaccine ineffective and therefore provide no protection of the host against disease (Carson *et al.* 1993).

Differences in the virulence of isolates were observed in the current study (Chapter 3) and have been noted in many others (Masumura and Wakabayashi 1977, Campbell and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1987, Carson *et al.* 1992, Alsina and Blanch 1993, Bernardet *et al.* 1994, Soltani 1995, Soltani *et al.* 1996, Handlinger *et al.* 1997, Powell *et al.* 2004, Avendaño-Herrera *et al.* 2006). Tasmanian isolates from salmonids appear to have a higher rate of pathogenicity than European and Japanese isolates tested in other fish species (Tables 1.2, 1.3). This leads to the question of whether the bacteria are more virulent or the host more susceptible. It is well known that salmonids are competitive and will bite and harass each other when held in captivity (Mork *et al.* 1999, MacLean *et al.* 2000). This behaviour is likely to

facilitate bacterial invasiveness as damage to the epithelium would create a portal of entry for the bacteria to colonise and proliferate (Handlinger *et al.* 1997). However, problems of aggregation in broth culture of *T. maritimum* have only been reported in Tasmanian isolates (Soltani 1995, Chapters 2 and 3). It is not known whether other non-Tasmanian *T. maritimum* isolates produce rough suspensions, which may increase pathogenicity. Some authors have used agglutination for serotyping (Wakabayashi *et al.* 1984, Vázquez Brañas 1991, Ostland *et al.* 1999, Avendaño-Herrera *et al.* 2004), which is not possible with Tasmanian isolates due to the cohesive nature of the bacteria. It appears that overseas isolates do not produce rough suspensions.

Further evidence supporting the virulent nature of rough suspensions is the fact that salmonids in Tasmania only require a 1 hour bath in a bacterial suspension to produce the disease. Bath concentrations reported by other studies are comparable with the current study (Tables 1.2, 1.3), but there was one study that had a shorter exposure time. In this case, the 1-2 g black sea bream were only exposed for 20 minutes (Baxa *et al.* 1987). No mortalities were recorded when fish were challenged via immersion, however, moderate to high levels of mortalities were achieved in the same study with direct application and subcutaneous injection. It is suggested on the basis of observing no mortalities via immersion challenge and high levels through direct contact challenge that immersion was likely to produce low levels of mortality even if the fish were exposed for a longer time. This would still not compare to the high levels of mortalities observed using the Tasmanian *T. maritimum* isolates in salmonids.

An exception to the high mortality levels observed in Tasmanian isolates was when artificial seawater was used instead of natural seawater in culture. While *T. maritimum* could grow in

media made with artificial seawater and form a homogenous suspension, the culture was avirulent (Carson *et al.* 1993). The authors suggested that the medium did not allow expression of virulence factors. The non-pathogenic isolate (89/4747) in the current study was also an exception and it should be noted that it was less adherent on plate culture and produced a far more homogeneous suspension than all other Tasmanian isolates. The association between non-pathogenic strains and the formation of homogeneous suspensions in culture provide further evidence of the connection between virulence and the cohesive nature of the *T. maritimum* isolates.

The only other study that has successfully challenged fish via bathing required immersion for 18 hours to induce disease in turbot; 1 or 2 hours duration was not sufficient (Avendaño-Herrera *et al.* 2004). Other successful methods of inducing disease are usually associated with direct application to various areas of the body and scarification through different means (Masumura and Wakabayashi 1977, Wakabayashi *et al.* 1984, Baxa *et al.* 1987, Carson *et al.* 1992, Bernardet *et al.* 1994, Powell *et al.* 2004). While intraperitoneal and intramuscular injections are ineffective (Campbell and Buswell 1982, Baxa *et al.* 1987, Carson *et al.* 1992), subcutaneous injection has worked on occasion which is probably a result of injecting into the dermal layer where bacteria are found in natural outbreaks (Masumura and Wakabayashi 1977, Wakabayashi *et al.* 1984, Alsina and Blanch 1993, Bernardet *et al.* 1994, Soltani 1995, Avendaño-Herrera *et al.* 2006). It appears as though in some fish species direct contact is required between *T. maritimum* and host so that attachment and proliferation can occur. This is not the case for Tasmanian isolates and salmonids, which could be a result of the cohesive nature that has been observed in this study; the Tasmanian isolates possess a greater adherence factor which is displayed through the 'clumping' of the bacteria in broth. Other factors are likely to be involved

in the pathogenicity of *T. maritimum*, but the cohesive nature is significant and this property should be investigated along with other non-agglutinating isolates for comparison.

A reproducible model of infection has been established in this study (Chapter 3), which will be useful for further pathogenicity and vaccination research. The most important aspect of this model is that it uses immersion to induce disease, which is comparable to the route of infection in natural outbreaks. At lower doses, the pathology exhibited under laboratory conditions was similar to that of naturally occurring infections. The progression of the disease, through gross pathology and histology, has been consistent with previous reports in other fish species including other salmonids (McVicar and White 1979, Campbell and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Devesa *et al.* 1989, Alsina and Blanch 1993, Pazos *et al.* 1993, Chen *et al.* 1995, Handlinger *et al.* 1997, Ostland *et al.* 1999): scale loss occurs in discrete areas with disintegration of the epidermis, which precedes erosion of the dermis and exposure of the underlying musculature. *Tenacibaculum maritimum* most likely uses the mucus of the fish as a nutrient reservoir and since there appears to be no antimicrobial compounds within the mucus to inhibit *T. maritimum* growth (Magariños *et al.* 1995), the bacteria can survive and proliferate. The bacteria can also be isolated from surviving fish; therefore a carrier state of the host is known to occur (Avendaño-Herrera *et al.* 2006). Damage to the skin surface through handling, excessive UV exposure or competition between fish, coupled with optimal conditions for growth, allow the bacteria to colonise and proliferate (Wakabayashi *et al.* 1984, Alsina and Blanch 1993, Bernardet *et al.* 1994, Chen *et al.* 1995, Handlinger *et al.* 1997, Ostland *et al.* 1999). Younger fish are 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 current study, not only were the fish moved from freshwater to seawater just before challenge, they were also young fish (80-100 g), handled several times and were exposed to higher than normal water temperatures. These stressful conditions could be part of the reason why the immersion challenge with salmonids was so successful.

Once the bacteria are able to invade the epidermal layer, they are able to dislodge scales from the dermis and break down the layers of the epidermis and dermis until they reach the musculature, at which time the necrotic material sloughs away and a lesion is exposed (Chapter 3). Histology from the current study suggests that the bacteria are mainly found within this necrotic material as the lesion is formed. It is unlikely that they initially invade the musculature due to its highly vascular nature in which the bacteria cannot survive (Soltani 1995). Eroded areas can continue to expand with bacteria feeding from the periphery of the lesion. Invasion of the musculature has been observed in some studies (Chen *et al.* 1995), 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 and Kusuda 1993, Chen *et al.* 1995, Handlinger *et al.* 1997, Kusuda and Kawai 1998). The current study used immunohistochemistry to verify the presence of *T. maritimum* in gill, necrotic tissue and lesions. While gill and necrotic tissue showed clearly 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 haematoxylin and eosin (H&E) stained sections. Since *T. maritimum* is

usually found with other opportunistic bacteria, the use of immunohistochemistry for histology was found to be useful.

Lesions are usually found around the head, mouth, fins and flanks of infected fish with gill necrosis being a sporadic occurrence (McVicar and White 1979, Campbell and Buswell 1982, Wakabayashi *et al.* 1984, Baxa *et al.* 1986, Devesa *et al.* 1989, Alsina and Blanch 1993, Pazos *et al.* 1993, Chen *et al.* 1995, Handlinger *et al.* 1997, Ostland *et al.* 1999). In the pathogenicity trials of the current study (Chapter 3), the same areas as described for naturally infected fish were affected with the addition of eye pathology on rare occasions; eye damage consisted of ruptured or missing eyes. However, the eye pathology was not definitively determined to be the result of a flexibacter infection as lesions were not associated with the eye, unlike reports of natural infections (Handlinger *et al.* 1997) and there was no obvious sign of other or previous trauma. 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. In the current study, larger lesions were found in association with pectoral and pelvic fins and it is suggested that this is the result of continual agitation of the fins against the body. 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). 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).

Extracellular products (ECP) produced by *T. maritimum* have been proven to be toxic to red and black sea bream (Baxa *et al.* 1988c) and now for the first time to Atlantic salmon (Chapter 4). The ECP was shown to cause cellular necrosis of the internal organs, a similar response to that which occurs on external surfaces during the disease process with *T. maritimum.* Since lesions occur so rapidly and infection can cause mortalities within days without an inflammatory response, toxins have been suspected to contribute to the pathogenicity of *T. maritimum* (Handlinger *et al.* 1997). From the results of this study, it is suggested that ECP play a major role in the pathogenesis of *T. maritimum.* It is also suggested that the cohesive nature of the bacteria and surface binding properties allows direct contact and penetration of the toxin to the underlying, vascularised musculature. However, more information is required concerning the nature of the ECP. In the current study, eight different ECP patterns were found by gel electrophoresis (Chapter 2). Differences in ECP patterns have been found using the same technique in other bacterial species, for example *Aeromonas salmonicida* (Gudmundsdóttir 1996), but unfortunately there is no understanding of what this variation means. A more thorough study of the exotoxins of *T. maritimum* is required including elucidating the differences between protein profiles and testing more ECP isolates, preferably using immersion. An assessment of ECP activities such as proteases and hemolysins also warrant further investigation. It is also important not to overlook the endotoxins of pathogenic bacteria. In the current study, LPS analysis revealed at least two serotypes and chimeras within the visible serotype, while recent work in sea bass showed LPS of *T. maritimum* to be an immunogenic antigen (Salati *et al.* 2005). The use of LPS as a vaccine immunogen have been effective against

other bacterial diseases such as *Vibrio anguillarum* (Bogwald *et al.* 1992), *Aeromonas salmonicida* (Ackerman *et al.* 2000) and *Aeromonas hydrophila* (Baba *et al.* 1988). This suggests further work is required to understand the nature of toxins particularly if they are to be included in vaccine development. Handlinger *et al.* (1997) suggested that preparations of a vaccine against *T. maritimum* should include a toxoid form and the current study agrees that this is an area of future research. Vaccines against *Aeromonas salmonicida* (Hastings and Ellis 1990), *Vibrio harveyi*, *Photobacterium damselae* subsp. *piscicida* (Arijo *et al.* 2005) and *Pasteurella piscicida* (Magariños *et al.* 1994) all contain ECP toxoids.

Vaccination of Atlantic salmon against flexibacteriosis using injection vaccinations with the use of an adjuvant (Freund's incomplete) achieved high levels of protection in Atlantic salmon (Chapter 5). Lesions were evident on the few mortalities that were observed in the Vaccine + Adjuvant treatment and this suggests that more work is required to produce a vaccine that is 100% effective. The use of a killed whole cell bacterial preparation was ineffective as a vaccine, which is consistent with other studies using similar formulations for salmonids (Carson *et al.* 1993, Carson *et al.* 1994). However, the same preparation in turbot and sole showed high relative percent survival (RPS) values (Romalde *et al.* 2005). There could be a difference in the host response with turbot and sole able to produce a more effective immune response than salmonids. Differences in immunity have been observed between salmon species (Johnson *et al.* 1982), and therefore could exist between species that are not as closely related. However, there are still questions regarding the vaccine produced for turbot and sole, in particular the conditions under which the vaccine was tested. Host immune responses would be of great interest because of the differences observed between salmonids, turbot and sole (Carson *et al.* 1993, Carson *et al.* 1994, Romalde *et al.* 2005).

All studies, including this thesis, investigating the vaccine efficacy against flexibacteriosis have been of a relatively short duration; no more than 8 weeks (Carson *et al.* 1993, Carson *et al.* 1994, Romalde *et al.* 2005). Duration of immunity can be affected by size of fish, the provision of a booster and the concentration of the bacterin (Johnson *et al.* 1982). There needs to be further investigation into the duration of immunity in order to produce an effective vaccine for use on a commercial farm. Also of interest are the side effects that were produced, which included a chronic inflammatory response and pigmentation in the pyloric caeca. As a short-term measure to a potential outbreak, this vaccine formulation can provide an effective means of disease protection. However, the long-term adjuvant effects of this vaccine preparation are unknown and should be an area of further investigation particularly in regards to growth deformities and animal welfare (Poppe *et al.* 2002, Koppang *et al.* 2005). The next major step from this study is to investigate the duration of protection and the use of other adjuvants, with the goal of taking the vaccine preparation to a commercial level and test efficacy under field conditions.

Vaccines for many fish species against a variety of bacterial pathogens are commonly used on commercial production fish farms around the world (Håstein *et al.* 2005) and are a preferred method of disease prevention. Understanding of the nature of the Tasmanian *T. maritimum* isolates provides a better means of developing a safe, immunogenic and viable vaccine for salmonids. By examining the similarities and differences of the isolates, in particular antigenic ones (Chapter 2), there is now further evidence of the heterogeneity of the antigenic characteristics (LPS) which has implications for further vaccine development. This study has provided greater insight into the pathogenicity of the disease in salmonids (Chapter 3), in particular the progression of the disease from a gross and histological point of view. The

cohesive nature of *T. maritimum* (Chapters 2 and 3) and the toxicity of the ECP that it produces (Chapter 4) are significant findings for pathogenicity but also for vaccine development. This study has also produced an experimental vaccine (Chapter 5), but requires further refining to confirm protection and minimise side effects. However, this thesis has been an invaluable step towards the goal of achieving a vaccine for Atlantic salmon against *T. maritimum*.

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Appendices

Appendix A

Media formulations, solutions and buffers

Marine Shieh's Medium

Song, Y.L., Fryer, J.L., Rohovec, J.S., 1988. Comparison of six media for the cultivation of *Flexibacter columnaris*. Fish Pathology 23, 91-94.

Peptone (Oxoid L37)	5.0 g
Sodium acetate	0.01 g
Sodium pyruvate	0.1 g
Citric acid	0.01 g
Yeast extract	0.5 g
Distilled water	100 mL
Seawater	900 mL
Agar	12.0 g

Autoclave at 121°C for 15 minutes, pH: 7.4 +/- 0.2 at 25°C

Ordal's Medium

Anacker, R.L., Ordal, E.J., 1959. Studies on the myxobacterium *Chondrococcus columnaris*. I. Serological typing. Journal of Bacteriology 78, 25-32.

Tryptone (Oxoid L42)	0.5 g
Yeast extract	0.5 g
Sodium acetate	0.2 g
Beef extract	0.2 g
Distilled water	100 mL
Seawater	900 mL
Agar	11.0 g

Autoclave at 121°C for 15 minutes, pH: 7.4 +/- 0.2 at 25°C

Seawater Davidson's

Shaw, B.L., Battle, H.I., 1957. The gross and microscopic anatomy of the digestive tract of the oyster, *Crassostrea virginica* (Gmelin). Canadian Journal of Zoology 35, 325–347.

Formaldehyde (37-40%)	200 mL
Glycerol	100 mL
Glacial acetic acid	100 mL
Absolute alcohol	300 mL
Seawater	300 mL

Citrate Buffer Solution

0.1 M Citric acid	9.5 mL
0.1 M Citrate	41.5 mL

Phosphate Buffer pH 7.4

0.1 M Sodium dihydrogen phosphate	34 mL
0.1 M Disodium hydrogen phosphate	166 mL

Phosphate Buffered Saline (PBS)

0.1 M Phosphate buffer pH 7.4	1000 mL
Sodium chloride	9 g

Alkaline Glycerol Mountant (pH 9)

Johnson, A.M., Munday, B.L., 1993. Toxoplasmosis: pathology, histopathology and serology. In: Corner, L.A., Bagust, T.J. (Eds.), Australian Standard Diagnostic Techniques for Animal Diseases. Standing Committee on Agriculture and Resource Management, Commonwealth Scientific and Industrial Research Organisation Publications, Melbourne.

Sodium hydrogen carbonate	0.0729 g
Sodium carbonate	0.016 g
Distilled water	10.0 mL
Glycerol	90.0 mL

Appendix B

Powell, M., Carson, J., van Gelderen, R., 2004. Experimental induction of gill disease in Atlantic salmon *Salmo salar* smolts with *Tenacibaculum maritimum*. Diseases of Aquatic Organisms 61, 179-185.

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Experimental induction of gill disease in Atlantic salmon *Salmo salar* smolts with *Tenacibaculum maritimum*

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ABSTRACT: An experimentally induced bacterial infection of marine Atlantic salmon *Salmo salar* smolt gills was developed using strains of *Tenacibaculum maritimum* originally isolated from disease outbreaks in Tasmania. The gills of salmon were inoculated with a high concentration of bacteria (4 $\times 10^{11}$ cells per fish) of either strain 00/3280 or 89/4747 *T. maritimum*. Gentle abrasion of the gills was used to enhance the progression of gill disease. One strain (00/3280) was highly pathogenic, causing morbidity and mortality within 24 h post-inoculation, and produced acute focal branchial necrosis associated with significant increases in plasma osmolality and lactate concentration compared with controls (non-inoculated) or strain 89/4747-inoculated fish. There were no differences in the whole body net ammonium flux between control (non-inoculated) and strain 00/3820-inoculated fish. Gill abrasion resulted in acute telangiectasis and focal lamellar hyperplasia in all fish regardless of bacterial inoculation. This work provides the basis of a challenge model suitable for investigating the pathophysiological processes associated with acute branchial necrosis in marine fish, suggesting that osmoregulatory and possibly respiratory dysfunction are the primary consequences of infection.

KEY WORDS: Atlantic salmon · *Tenacibaculum maritimum* · Pathophysiology · Gill disease · Osmoregulation · Respiration

INTRODUCTION

Bacterial diseases of freshwater fish epithelia have been extensively studied with the primary focus upon bacteria from the Cytophagales, such as bacterial gill disease caused by *Flavobacterium branchiophilum* (Ostland et al. 1994, 1995), and necrotizing skin diseases such as columnaris disease caused by *F. columnare* (Thomas-Jinu & Goodwin 2004) and bacterial cold-water disease caused by *F. psychrophilum* (Ciprano et al. 1996 and review by Nematollahi et al. 2003). However, in marine fishes relatively few bacterial skin and gill diseases have been characterised. *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) has been reported to cause significant skin and gill pathology in a wide variety of species including red seabream *Pagrus major*, black seabream *Acanthopagrus schegeli*, rock bream *Oplegnathus fasciatus*, Japanese flounder *Paralichthys olivaceus* (Baxa et al. 1986, Wakabayashi et al. 1986), Dover sole *Solea solea* (Bernardet et al. 1990), turbot *Scophthalmus maximus* (Alsina & Blanch 1993), Atlantic salmon *Salmo salar*, rainbow trout *Onchorynchus mykiss*, striped trumpeter *Latris lineata* and greenback flounder *Rhombosolea tapirina* (Handlinger et al. 1997). Although primarily a skin infection causing ulcerative dermatitis (Handlinger et al. 1997), gill infections where a necrotizing branchitis occurs are not uncommon (Handlinger et al. 1997).

The aim of this research was to investigate whether experimental inoculation of the gills of Atlantic salmon with *Tenacibaculum maritimum* could cause acute gill disease and to identify potential processes that may be responsible for mortality.

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MATERIALS AND METHODS

Preparation of bacterial cultures. Cultures of *Tenacibaculum maritimum* were isolated by the Department of Primary Industry, Water and Environment from the skin of farmed salmon from Tasmania, Australia, with clinical cases of cutaneous erosion disease. The cultures were designated 89/4747 (Atlantic salmon) and 00/3280 (rainbow trout) and were isolated in 1989 and 2000 respectively. The bacteria were isolated on the medium of Anacker & Ordal (1959), formulated with seawater. Isolates were identified using a 16S ribosomal RNA (rRNA) PCR primer set specific for *T. maritimum* (Carson 1998). Cultures were stored frozen at −80°C on MicroBank beads (Pro-Lab Diagnostics) until required.

Cultures for infection trials were prepared by inoculating 200 ml of Shieh's medium (Song et al. 1988) formulated with seawater (mineral salts buffer, MSB) in a 1 l conical flask and incubated with gentle agitation (30 cycles min^{-1}) at 20 to 22°C for 48 h The cell suspension was harvested by centrifugation at 2500 µg RCF (relative centrifugal force) for 20 min and the pellet washed twice with sterile seawater. Harvested cells were resuspended in 15 ml of sterile seawater.

Experimental series 1: Infection study. Atlantic salmon smolts of mean mass (±SE) 77.0 ± 2.9 g were acclimated to full strength seawater (35 ppt) over a period of 10 d, then allocated to triplicate tanks (4 fish per tank, n = 12 per treatment) and allowed to habituate for 24 h prior to anaesthetization with AQUI-S (0.04 ml l^{-1}) or clove oil (0.03 ml l^{-1}). The gill arches were then separated and all of the filaments abraded by stroking gently with a blunt spatula. Aliquots (0.5 ml) of *Tenacibaculum maritimum* culture in sterile, filtered seawater were applied to the gill arches on each side of the fish (total 1 ml *T. maritimum* culture, ~4×10^{11} cells per fish, n = 12 per treatment). Controls consisted of fish that were inoculated with filtered seawater only (n = 12, repeated twice). An additional group of fish was inoculated with 1 ml of *T. maritimum* (strain 00/3280) culture onto the gills without prior filament separation or abrasion. Fish were allowed to recover in their respective tanks and monitored over the subsequent 5 d. Moribund fish were removed and cultures made from the gills on Shieh's agar (Song et al. 1988) formulated with seawater mineral salts agar (MSA), and colonies were confirmed as *T. maritimum* by colony colour and shape, as well as indirect fluorescent antibody test (IFAT) using a rabbit antibody prepared with a whole cell formalin-inactivated cell suspension of *T. maritimum* 89/0235 (rainbow trout).

Experimental series 2: Comparative pathology of *Tenacibaculum maritimum* strains. In Experimental series 1 there was an apparent difference in the pathogenicity of the 2 strains of *T. maritimum* used. Thus, it was decided to further examine the effects of acute exposure of smolts to the 2 different strains.

Seawater-acclimated Atlantic salmon smolts were allocated to 30 l round tanks (6 fish per tank, 3 tanks per treatment) following anaesthesia, gill abrasion and inoculation with either strain 89/4747 or 00/3280 of *Tenacibaculum maritimum*. Controls consisted of fish that had the gill filaments separated but were inoculated with seawater. Fish were allowed to recover and maintained in static aerated seawater at 17.8 to 18.4°C (Fig. 1A), 79 to 95% O_2 saturation (Fig. 1B) for 24 h post-inoculation. Total ammonium concentrations were measured using the method of Verdouw et al. (1978). During the exposure, mori-

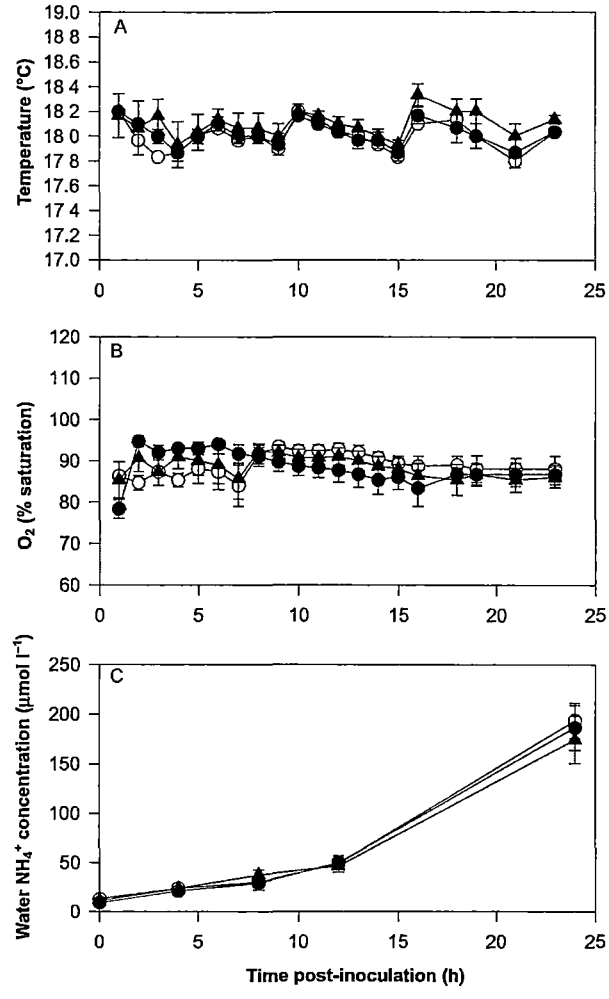

Fig. 1. (A) Mean (±SE) temperature, (B) oxygen saturation and (C) water ammonium concentration for tanks containing Atlantic salmon *Salmo salar* smolts experimentally inoculated with *Tenacibaculum maritimum* (●) strain 00/3280, (▲) strain 89/4747 or (○) controls over a 24 h static challenge

bund fish (unresponsive to touch) were removed, anaesthetised with 0.04 ml l^{-1} clove oil and bled from the caudal vein. Heparinised blood (100 IU ammonium heparin, Sigma-Aldrich) was then centrifuged at 8000 × *g*, the plasma decanted and frozen at –20°C for further analysis. The gills were swabbed onto MSA for confirmation of the presence of *T. maritimum* using colony morphology and IFAT. The gills were then removed and fixed in seawater Davidson's solution for histological examination. After 24 h of exposure, surviving fish were killed by anaesthetic overdose (clove oil >0.03 ml l^{-1}) and sampled as for moribund fish.

Plasma osmolality was determined using a Vapro 5520 vapour pressure osmometer (Wescor). Plasma chloride was determined according to the method of Zall et al. (1956) and plasma lactate concentrations were determined using a commercial lactate assay kit (Sigma Diagnostics, protocol 735). Gills were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin.

Experimental series 3: Effects of *Tenacibaculum maritimum* infection on ammonium flux. In Experimental series 2, despite the removal of moribund fish inoculated with *T. maritimum* strain 00/3280, the total ammonium concentrations in the water remained equivalent to that of fully stocked tanks that experienced no morbidity (*T. maritimum* strain 89/4747 and control tanks). Therefore, the net ammonium flux was measured for fish inoculated with *T. maritimum* strain 00/3280 and controls.

The gills of seawater-acclimated smolts were abraded and inoculated as described above with either strain 00/3280 or seawater (controls), and placed into individual black acrylic flux chambers (internal volume of 1 l) supplied with flowing seawater and constant aeration (1 fish per chamber, 9 fish per treatment). Following 24 h of recovery, the flow to the flux chambers was stopped and a water sample (5 ml) removed and immediately frozen (–20°C) for later analysis. Following a 3 h period, a second water sample was removed and similarly frozen, after which the water flow to each flux chamber was reinstated. Fish were then removed, killed by anaesthetic overdose (clove oil >0.03 ml l^{-1}) and the gills swabbed onto MSA for subsequent confirmation of the presence/absence of *Tenacibaculum maritimum* using colony morphology and IFAT.

Plasma osmolality, chloride concentrations and lactate concentrations were compared statistically between strains (00/3280 and 89/4747) and controls using analysis of variance with a Bonferroni corrected *t*-test planned contrast relative to controls. p values of less than 0.05 were considered to be significant.

Water samples were analysed for total ammonium concentration using the method of Verdouw et al. (1978). Whole body net ammonium flux was then calculated according to:

$$J_{net}\,NH_4^+ = \frac{[NH_4^+]_{t=1} - [NH_4^+]_{t=0} * V}{M * T}$$

Where $[NH_4^+]_{t=x}$ is the concentration of total ammonium (mmol l^{-1}) in the water sample at the start or end of the flux period, V is the volume of the flux chamber, M is the mass of the fish (g) and T is the duration of the flux period (h). Net whole body ammonium fluxes were compared with zero and between inoculated and control fish using a *t*-test. p values of less than 0.05 were considered to be significant.

RESULTS

Experimental series 1: Infection study

The highest rate of mortality (70%) occurred within 48 h post-inoculation in fish that had received gill abrasion and infection with strain 00/3280 (Fig. 2). Fish with non-abraded gills inoculated with strain 00/3280 had a maximum mortality rate of 50% at 96 h, whereas fish inoculated with strain 89/4747 and controls had

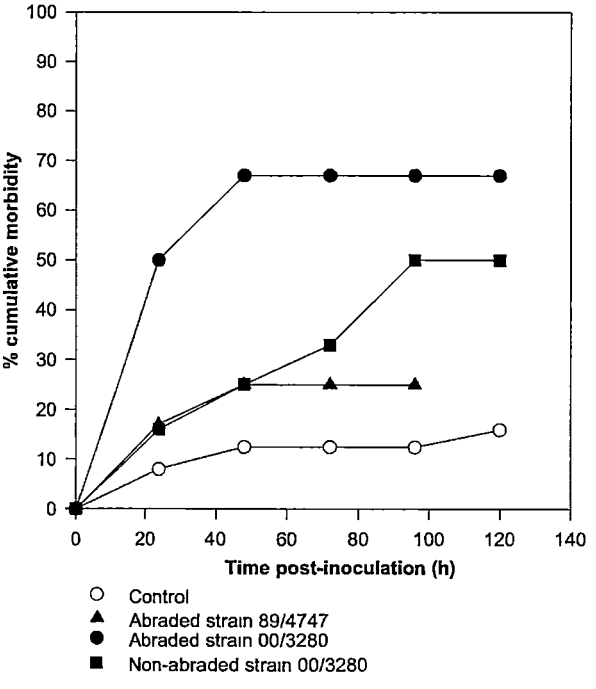

Fig. 2. *Salmo salar* infected with *Tenacibaculum maritimum*. Percent cumulative mortality of Atlantic salmon smolts experimentally inoculated with (●) strain 00/3280 following a light abrasion, or (■) no abrasion of the gills, (▲) strain 89/4747 following light abrasion or (○) controls (light abrasion, no bacteria)

maximum mortality rates of 25 and 11 % at 48 h respectively (Fig. 2). Bacterial colonies with characteristics typical of *Tenacibaculum maritimum* were isolated from all fish within those groups inoculated with bacteria. The 00/3280-inoculated fish returned heavy bacterial growth from gill smears compared with the light bacterial growth from gill smears of control (non-inoculated) fish (Table 1).

Experimental series 2 and 3: Comparative pathology and ammonium flux experiments

Fish inoculated with strain 00/3280 showed a higher rate of morbidity than fish treated with strain 89/4747 or controls over the 24 h challenge experiment (Fig. 3A). There was heavy bacterial growth from gill smears of fish inoculated with *Tenacibaculum maritimum* bacteria (both strains 00/3280 and 89/4747) and poor growth from smears taken from control fish (Table 1). Bacteria isolated from fish inoculated with strain 00/3280 returned a positive result when tested with IFAT, while bacterial smears from fish inoculated with strain 89/4747 and control fish were negative (Table 1). Water ammonium concentrations increased in all tanks over the exposure duration; however, levels remained below 200 µmol l^{-1} (Fig. 1C). Moribund fish from the strain 00/3280 groups had significantly higher plasma osmolality than fish that had been inoculated with strain 89/4747 or control animals at 24 h post-inoculation ($F_{2,19}$ = 8.61, p = 0.0022) (Fig. 3B). However, there were no significant differences in the plasma Cl^- concentration between the groups ($F_{2,19}$ = 2.99, p = 0.0742) (Fig. 3C). There were significantly elevated plasma lactate concentrations in moribund fish inoculated with strain 00/3280 compared with either strain 89/4747-inoculated or control fish after 24 h of exposure ($F_{2,19}$ = 6.24, p = 0.0082) (Fig. 3D). There was a significant negative net whole body ammonium flux in both control and strain 00/3280-inoculated fish. However, there was no significant difference in the magnitude of the net ammonium flux between control and strain 00/3280-inoculated fish (t_{31}= 1.47, p = 0.1508) (Fig. 3E). Smears from fish inoculated with strain 00/3280 showed heavy growth of bacteria typical of *T. maritimum*, whereas control (non-inoculated) fish showed no bacterial growth (Table 1).

Table 1. *Tenacibaculum maritimum* infecting *Salmo salar*. Recovery of bacteria from gills following experimental inoculation and indirect fluorescent antibody test (IFAT) confirmation. ND = not determined; – = no growth/negative; +/– = inconclusive; + = poor growth/positive; ++ = moderate growth; +++ = heavy growth

T. maritimum strain	00/3280 Scraped	00/3280 Non-Scraped	89/4747 Scraped	Control
Expt 1: Infection study				
Culture	+++	+++	+++	+
Expt 2: Comparative pathology				
Culture	+++	ND	+++	+
iFAT	+	ND	–	+/–
Expt 3: Ammonium flux	+++	ND	ND	–

Histopathology

Morbid salmon inoculated with strain 00/3280 but not 89/4747 exhibited gills with yellowish mucoid aggregations at the filament tips. Histologically, there was focal epithelial necrosis with extensive bacterial mats overlaying necrotic tissue (Fig. 4A,B). Bacterial associated necrosis of the filaments occurred in what appeared to be a distal to proximal direction. There was very limited (only seen in 1 fish out of 6 examined) evidence of bacterial associated necrosis with strain 89/4747 and no necrosis in control (non-inoculated) gills (Fig. 4C). Abraded gills often showed signs of telangiectasis and congestion, irrespective of whether fish were inoculated with *Tenacibaculum maritimum* (Fig. 4E,F).

DISCUSSION

Gill abrasion enhances the susceptibility of catfish *Ictalurus punctatus* to infection with *Flavobacterium columnare* (Bader et al. 2003) and has been used to enhance the reliability of *Tenacibaculum maritimum* challenge in salmonids (J. Carson pers. comm.). In our study, abrasion enhanced the severity and rate at which the disease progressed leading to mortality. There were, however, clear differences in the susceptibility of fish to different strains of *T. maritimum*. Strain 00/3280 was pathogenic, whereas strain 89/4747 was not. Indeed, strain 89/4747 failed to be detectable with IFAT. Failure or ambiguity of IFAT techniques have been reported with *F. psychrophilum* (Vatos et al. 2002). Both this species and its warm water counter-part *F. columnare* have a highly labile glycocalyx responsible for adhesion (Nematollahi et al. 2003) and *F. columnare* is also associated with chondroitinase (AC lyase) activity (Stringer-Roth et al. 2002).

Apart from the glycocalix, *F. columnare* adherence to gill tissue is also dependent upon the presence of D-glucose or N-acetyl-D-glucosamine (Decostere et al. 1999). It is possible that the requirements of strain 89/4747 *T. maritimum* for adherence to gills were not fully met because of the cellular microclimate of the salmon gill. Failure to detect strain 89/4747 *T. maritimum* using IFAT may also reflect a difference in the bacterial glycocalyx of this strain compared with the pathogenic strain 00/3280.

The branchial epithelial necrosis caused by strain 00/3280, and less so with strain 89/4747 *Tenacibaculum maritimum* infection was similar to that reported by Handlinger et al. (1997) for naturally infected Atlantic salmon and rainbow trout. However, in our study, telangiectasis and focal hyperplasia of the branchial epithelium due to gill abrasion was common in control fish (non-inoculated) and fish inoculated with both strains of *T. maritimum*. Interestingly, the hyperplastic lesions on the gills were similar to those seen with amoebic gill disease, but with the difference that gill abrasion lesions were associated with telangiectasis, not parasome-containing amoebal trophozoites (Adams & Nowak 2001, 2003). It appears that the branchial necrosis causes a strong osmoregulatory disturbance in Atlantic salmon with significant increases in plasma osmolality. This osmoregulatory disturbance probably involves ionoregulatory dysfunction. Although the mean plasma chloride concentrations were elevated in moribund salmon inoculated with strain 00/3280, this was not statistically different from controls. In other situations where extensive branchial necrosis occurs, such as oxidative injury, similar osmoregulatory disturbances may be present without any clear changes in plasma chloride levels (Powell & Harris 2004). Similarly, reductions in plasma osmolality have been reported with experimental *Flavobacterium branchiophilum* infections of freshwater rainbow trout, although these were in association with a reduction in plasma electrolytes including chloride (Byrne et al. 1995). Plasma lactate concentrations were clearly elevated in moribund salmon suggesting that acute respiratory failure may occur pre-mortem. It is not clear whether the probable limited oxygen transfer was due to diffusive limitations in the gill or reduced perfusion of the gill. Byrne et al (1991, 1995) did not demonstrate any reduction in blood pO_2 in freshwater brook trout *Salvelinus fontinalis* and rainbow trout experimentally infected with *F. branchiophilum*. However, Foscarini (1989) demonstrated a transient bradycardia and an acute branchial hyperplasia and oedema in *F.*

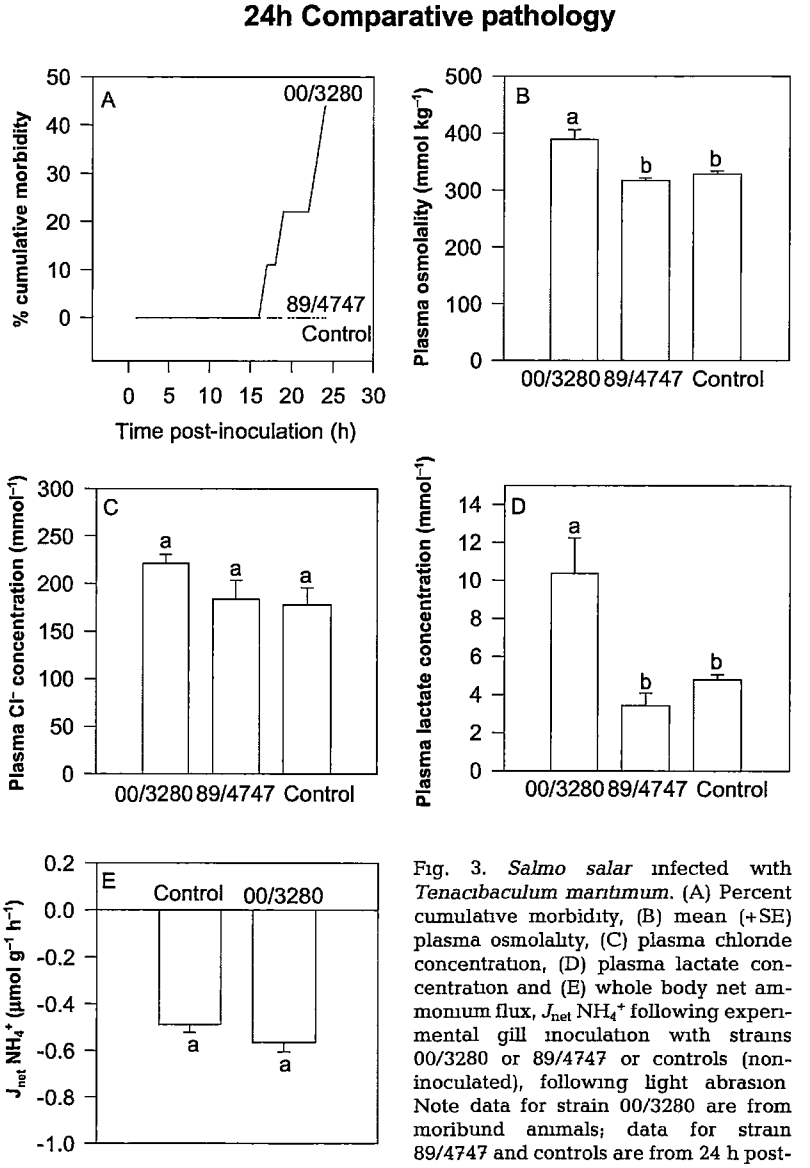

Fig. 3. *Salmo salar* infected with *Tenacibaculum maritimum*. (A) Percent cumulative morbidity, (B) mean (+SE) plasma osmolality, (C) plasma chloride concentration, (D) plasma lactate concentration and (E) whole body net ammonium flux, J_{net} NH_4^+ following experimental gill inoculation with strains 00/3280 or 89/4747 or controls (non-inoculated), following light abrasion Note data for strain 00/3280 are from moribund animals; data for strain 89/4747 and controls are from 24 h post-inoculation. Different letters indicate statistical significance

columnaris (now *F. columnare*)-exposed eel *Anguilla japonica*. It is, therefore, not possible to exclude either diffusive or perfusive limitations to gas exchange as a cause of plasma lactate elevations in *T. maritimum*-infected salmon.

Despite the apparent deterioration in water quality, even with declining fish density, in the 24 h comparative pathology trial of fish inoculated with strain 00/3280, there were no significant differences in the rate of ammonium efflux. Increased ammonium effluxes have been reported in response to acute freshwater exposures of Atlantic salmon affected by amoebic gill disease (Roberts & Powell 2003). Similarly rainbow trout experimentally infected with *Flavobacterium branchiophilum* had elevated plasma ammonium tensions (Byrne et al. 1995). It would appear that in acute *Tenacibaculum maritimum* infection of salmon, ammonium excretion rates are not significantly affected.

In conclusion, we have demonstrated that direct inoculation of high concentrations of *Tenacibaculum maritimum* directly onto the gills of Atlantic salmon smolts will induce a necrotic branchitis which appears to be enhanced by prior abrasion of the gill epithelium. Progression of the infection results in an acute and ultimately lethal osmoregulatory and potentially respiratory dysfunction.

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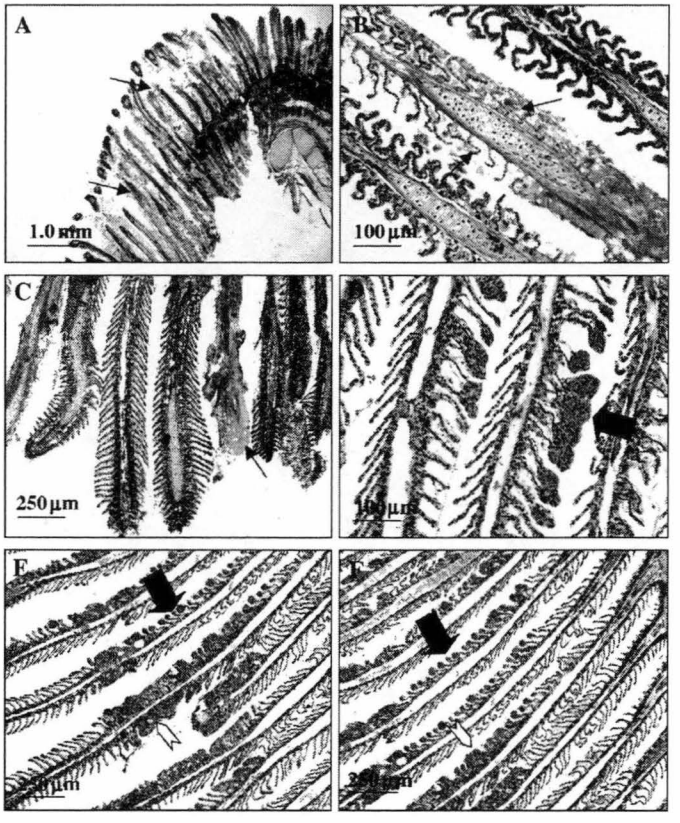

Fig. 4. *Salmo salar* infected with *Tenacibaculum maritimum*. (A,B) Extensive branchial necrosis associated with filamentous bacterial mats (arrows) on the gills of Atlantic salmon smolts inoculated with strain 00/3280. (C) Filamental necrosis associated with filamentous bacteria on the gills of salmon inoculated with *T. maritimum* strain 89/4747. (D) Telangiectasis (large black arrow) associated with the gills of control, (E) strain 00/3280-inoculated and (F) strain 89/4747-inoculated fish. Note the hyperplastic lesions also associated with gill abrasion (white arrow)

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