EPIDEMIOLOGICAL ASPECTS OF *Aeromonas salmonicida* IN THE MARINE ENVIRONMENT

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by

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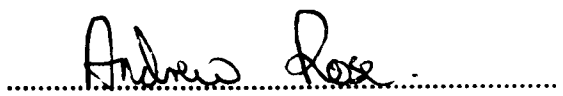
DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It has neither been accepted, nor submitted, for any other degrees. All sources of information have been duly acknowledged.



Andrew Rose

LIST OF PUBLICATIONS

a) Rose, A.S., Ellis, A.E. and Munro, A.L.S. (1989). The infectivity by different routes of exposure and shedding rates of *Aeromonas salmonicida* subsp. *salmonicida* in Atlantic salmon, *Salmo salar* L., held in seawater. *Journal of Fish Diseases*, **12**, 573-578.

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d) Rose, A.S., Ellis, A.E. and Munro, A.L.S. (1990). The survival of *Aeromonas salmonicida* subsp. *salmonicida* in seawater. *Journal of Fish Diseases*, 13 (in press).

ABSTRACT

The epidemiology of *Aeromonas salmonicida* subsp. *salmonicida* in the marine environment was investigated.

Nutrient resuscitation and infectivity studies did not support a previous claim of dormancy in *A. salmonicida* and validated the use of colony-forming units (cfu) in survival studies.

Survival of *A. salmonicida* in seawater was assessed and found to be of short duration (<10 days). Survival of the bacterium in non-sterile sediment, obtained from beneath a salmon cage, appeared to be limited.

The minimum infective dose of *A. salmonicida* to Atlantic salmon in short duration (1-3 days) bath exposure in sea water was 10^4 cfu ml^{-1}. Prolonged exposure for three weeks resulted in infection with 10^2 cfu ml^{-1}. Intragastric intubation of the bacterium established infection with doses $>10^5$ cfu. Shedding of *A. salmonicida* from infected salmon was 10^5-10^8 cfu/fish/hr.

Survival and shedding results were combined in a computer model. *A. salmonicida* was predicted to travel >6 km suspended within the water column of a sea loch.

Covert infection in freshwater farmed salmon was assessed by ELISA and the standard stress test. Results indicated that ELISA may be useful as a routine monitor of furunculosis infection. The efficacy of dot-blot immunoassay was found to be 10^3 cfu *A. salmonicida* in fish kidney tissue.

Rainbow trout (*Oncorhynchus mykiss*) and salmon mucus were not found to inhibit the growth of *A. salmonicida* supporting recent evidence that fish skin is a site of carriage. *In vitro* studies suggested that trout serum proteins do not confer protection from fish antibody on *A. salmonicida* in covert infections.

Preliminary work was undertaken to develop a specific DNA probe for *A. salmonicida* which will allow its detection in environmental samples and carrier fish. A gene library of *A. salmonicida* was constructed in lambda gt11 and screened for "A"-protein with antibodies.

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COMMON ABBREVIATIONS

BHIA	brain-heart infusion agar
BSM	basal salts medium
cfu	colony-forming unit
cm	centimetre
DAB	diaminobenzidine tetrahydrochloride
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
g	gram
hr	hour
HRP	horse-radish peroxidase
i.m.	intramuscular
IPTG	isopropyl-β-thiogalactoside
l	litre
lacZ	gene coding for β-galactosidase
LB	Luria-Bertani medium
Ln	natural logarithm
mAb	monoclonal antibody
ml	millilitre
µg	microgram
µl	microlitre
µm	micrometre
NC	nitrocellulose
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
s	second
SDS	sodium dodecyl sulphate
TBS	tris-buffered saline
TE	tris/EDTA buffer
TSA	tryptone soya agar
TSB	tryptone soya broth
TTBS	tween/tris-buffered saline
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER 1

THE EPIDEMIOLOGY OF *Aeromonas salmonicida*

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1.1 INTRODUCTION

Furunculosis is a bacterial disease of salmonid fish and is a major constraint on fish cultivation, especially of Atlantic salmon in Scotland. The causative agent, *Aeromonas salmonicida* subsp. *salmonicida* is a focus for research in disease prevention and control including vaccine production (Hastings, 1988). A greater understanding of its transmission biology may lead to the development of cheap and effective management practices to combat disease. The epidemiology of furunculosis in the marine environment has been relatively neglected in comparison with studies in freshwater (eg Austin and Austin, 1987). Recent evidence suggests that furunculosis may be able to cross infect widely separated (*ca* 10-20 km) commercial marine salmon farms both in Scotland (Munro, 1988) and North America (Novotny, 1975, 1978). This may restrict the availability, and size, of suitable farm sites along a coastline.

In the light of these considerations the work presented in this thesis was funded by the Crown Estate Commissioners, Scotland, to study the epidemiology of *A. salmonicida* subsp. *salmonicida* (ie "typical" strains) in relation to marine salmon farming. The following areas were studied: disease transmission (including survival and infectivity) in seawater; detection of covertly infected (carrier) fish; and preliminary development towards a specific DNA probe against the pathogen to aid detection in the environment. The rationale and aims of the study are explained below.

Disease transmission is considered to be a complex, multi-factorial process dependent on the characteristics of the host, pathogen and their effective contact (Snieszko, 1974; Gordon-Smith, 1982). These factors were investigated through a study of the pathogen's

ability to survive in seawater and produce dormant forms (characteristics of the pathogen); the infectivity of the pathogen and routes of infection (characteristics of host/pathogen/effective contact); and infectiousness, or amount of pathogen a host can transmit (characteristic of host/pathogen). These results were then used to predict the potential spread of the free-living pathogen in the marine environment using the computer-simulation model of Turrell and Munro (1988).

Transmission of *A. salmonicida* is believed to occur via a water-borne route (McCarthy, 1977; Austin and Austin, 1987). Hence its ability to survive, and remain infective, in the external environment is a major factor in the epidemiology of furunculosis (Austin and Austin, 1987). Allen-Austin *et al.* (1984) proposed that *A. salmonicida* could occur in a dormant form similar to the unculturable-but-viable "somni-cells" described for enteric pathogens (Roszak and Colwell, 1987a). The existence of these dormant cells may invalidate previous survival studies on *A. salmonicida.* Allen-Austin *et al.* (1984) reported that in survival experiments conducted in riverwater microcosms bacterial cells could still be observed microscopically after colony-forming units (cfu) in 1 ml microcosm samples had reached zero. Furthermore, following the addition of nutrients to microcosms at this stage, cfu could once again be detected. The existence of dormant *A. salmonicida* in the aquatic environment was re-examined because in the previous study (Allen-Austin *et al.*, 1984) the possibility that resuscitation of dormant cells may have been an artefact caused by the presence of small numbers of viable cells (cfu) too few to detect, was not considered. In this study (Chapter 2) the survival of two strains of *A. salmonicida*

were monitored in riverwater and freshwater or seawater basal salts media and nutrient resuscitation and infectivity studies in Atlantic salmon (*Salmo salar* L.) were performed to investigate the presence, or otherwise, of dormant *A. salmonicida.*

Although the survival of *A. salmonicida* in the external aquatic environment has been extensively studied (reviewed by McCarthy, 1977; Austin and Austin, 1987) several factors which could influence survival have not been considered. These factors include: water quality eg nutrient levels (Evelyn, 1971); possible changes in the bacterium during storage and *in vitro* culture (McCarthy, 1983); and absence of a selective medium in mixed culture studies (McCarthy, 1977). These factors were investigated in this study for the pathogen in seawater (Chapter 3). Survival was assessed in different types of microcosm (glass-flask or dialysis bag); of bacteria grown *in vivo* (shed from infected fish) and *in vitro* (broth-culture); in sterile seawater with added nutrients; and in non-sterile (mixed culture) seawater, and sediments, obtained from commercial salmon farms. Enumeration of *A. salmonicida* in non-sterile seawater was achieved using an antibiotic containing selective medium following the earlier study of McCarthy (1977).

The number of *A. salmonicida* required to infect fish (ie the infectivity of the pathogen) is an important determinant of disease transmission. Lateral transmission between fish is believed to occur in seawater (Scott, 1968; Novotny, 1978; Smith *et al.*, 1982). However, its infectivity in this environment, unlike freshwater (Adams *et al.*, 1987) is unknown. In this study (Chapter 4) the susceptibility of Atlantic salmon (*Salmo salar* L.) to *A. salmonicida* was investigated by both bath and intragastric (oral) challenge.

A computer-simulation model (Turrell and Munro, 1988) has been developed at the DAFS Marine Laboratory, Aberdeen which models the spread of bacteria or antibiotics in a sea-loch. The potential for free-living *A. salmonicida* to spread in the marine environment was estimated with this model (Chapter 5) using the results obtained from the survival and infectivity studies and *A. salmonicida* concentrations at infected sites were estimated from an assessment of the numbers of the pathogen shed from experimentally infected Atlantic salmon.

Covertly infected (carrier) fish are considered to be an important reservoir of infection (McCarthy, 1977; Austin and Austin, 1987) and may be responsible for furunculosis spread by movement between fish farms (McCarthy, 1977; Munro, 1988). The most effective method of carrier detection (Bullock and Stuckey, 1975) available involves injection of corticosteroids and heat stress. The method is time consuming and requires large holding facilities. In this study (Chapter 6) two alternative methods (dot-blot and ELISA) for carrier detection were assessed. These methods are based on the identification of *A. salmonicida* antigen in fish tissue. In addition, the ability of *A. salmonicida* to survive in fish mucus was investigated. It has recently been suggested (Hiney *et al.*, 1989) that *A. salmonicida* is carried on the external surface of covertly infected fish.

Although ELISA was found to be useful in carrier detection the technique was too insensitive to detect *A. salmonicida* in the environment. Improved detection methods

may be provided by gene probe technology. DNA probes have been shown to be sensitive and specific means of microbial detection (eg Tenover, 1988; Walker and Dougan, 1989). Preliminary development of a specific DNA probe for *A. salmonicida* was initiated (Chapter 7). A genomic library was constructed using the bacteriophage lambda gt11 which was then screened for *A. salmonicida* "A"-protein. The "A"-protein gene was chosen as a putative gene probe because it has been cloned previously and is thought to be specific for *A. salmonicida* (Belland and Trust, 1987).

1.2 LITERATURE REVIEW - THE ECOLOGY OF *Aeromonas salmonicida*

This review is concerned with the ecology of the bacterial fish pathogen *Aeromonas salmonicida*, causative agent of furunculosis in salmonids, and the methodological difficulties involved in this field of research. The majority of experimental problems arise from difficulties in the detection and isolation of the bacterium (McCarthy, 1977; Austin and Austin, 1987). The ecology of the pathogen is discussed within the context of studies on the persistence of the pathogen in the environment; its transmission and the carrier state in covertly infected fish. The molecular genetic approach to bacterial detection is briefly reviewed which may greatly contribute to *A. salmonicida* research in the future. Several reviews covering the general biology and control of the pathogen are available (Mackie *et al.*, 1930, 1933, 1935; McCraw, 1952; Herman, 1968; McCarthy, 1977; McCarthy and Roberts, 1980; Paterson, 1982; Bullock *et al.*, 1983; Munro, 1984, 1988 and Hastings, 1988).

1.2.1 *Aeromonas salmonicida*

Aeromonas salmonicida is an asporogenous, non-motile, pigment producing Gram negative coccobaccilli (Trust *et al.*, 1980; Popoff, 1984; Bohm *et al.*, 1986). The bacterium has a near cosmopolitan distribution within temperate regions. Recently, atypical *A. salmonicida* has been detected in non-salmonid fish stocks in Australia (Langdon, 1987; Humphrey *et al.*, 1987; Whittington *et al.*, 1987). The distribution and historical dissemination of the disease are reviewed by Mackie *et al.* (1930, 1933, 1935), McCraw (1952), Herman (1968), McCarthy and Roberts (1980) and Austin and Austin (1987).

A variety of pathologies arising from infection with typical and atypical *A. salmonicida* have been described in addition to furunculosis of salmonids eg post-traumatic septicaemia in centrarchids and carp erythrodermatitis. These pathologies are described in McCarthy and Roberts (1980) and Austin and Austin (1987).

The classification of the bacterium is confused and has recently been reviewed by McCarthy and Roberts (1980) and Austin and Austin (1987). The nomenclature adopted here is that described by Belland and Trust (1988) who confirmed the earlier classification proposed by McCarthy and Roberts (1980). In this system *Aeromonas salmonicida* subsp. *salmonicida* refers to "typical" strains which are generally isolated from salmonids; *Aeromonas salmonicida* subsp. *achromogenes* for "atypical" isolates from non-salmonids; and *Aeromonas salmonicida* subsp. *nova* for field isolates from non-salmonids. This classification does not correspond to that given by Popoff (1984) in Bergey's Manual of Systematic Bacteriology. The present study is restricted to "typical" strains of the pathogen, *Aeromonas salmonicida* subsp. *salmonicida.*

1.2.2 The Persistence of *A. salmonicida* in the Environment

In this section studies on the ability of *A. salmonicida* to survive in the aquatic environment are described and factors which may potentially influence these studies are discussed. The subject has been reviewed by several authors including McCraw (1952), Smith (1962), McCarthy (1977) and Austin and Austin (1987).

A. salmonicida is thought to be an obligate pathogen (Popoff, 1984) of salmonids in both fresh and seawater with limited ability to survive outside the fish host. The pathogen, both "typical" and "atypical" strains, possesses a broad host-range (Table 1.1) which may help to maintain the bacterium within fish communities and be responsible for introduction of the disease at commercial salmon farms (Munro, 1982b; Smith *et al.*, 1982; Ostland *et al.*, 1987). Covertly infected, carrier, fish are also considered to be important reservoirs for the pathogen (McCarthy, 1977).

a) Experimental design in survival studies

The results obtained from bacterial survival studies are often dependent on experimental protocol (McFeters and Stuart, 1972).

i) Experimental Microcosms

The survival of *A. salmonicida* has been assessed in experimental microcosms and been found to be of relatively short duration (Austin and Austin, 1987). However, the design of microcosms may influence the results of survival studies. For example, Buck (1978) found that the yeast *Candida albicans* survived longer in membrane-bound chambers *in situ* than in laboratory flask systems. Most survival studies involving *A. salmonicida* have been undertaken in glass-flask microcosms. These systems present several potential drawbacks: the enclosure of small volumes of water may effect resident bacterial

TABLE 1.1

Examples of non-salmonid host-range of *Aeromonas salmonicida*

Host species	Reference
Typical *A. salmonicida* strains	
Sculpin (*Cottus bairdi*)	Rabb and McDermott (1962)
Eel (*Anguilla rostrata*)	Hayasaka and Sullivan (1981)
Sablefish (*Anoplopoma fimbria*)	Klontz and Wood (1972)
Lamprey (*Ichthyomyzon castaneus*)	Hall (1963)
Shiner (*Notropis cornutus*)	Ostland et al (1987)
Sucker (*Catostomus commersoni*)	
Atypical *A. salmonicida* strains	
Goldfish (*Carassius auratus*)	Elliott and Shotts (1980), Trust *et al.* (1980)
Eel (*Anguilla japonica*)	Kitao *et al.* (1984)
Carp (*Cyprinus carpio*)	Bootsma *et al.* (1977)
Bream (*Blicca bjoerkna*)	McCarthy (1975a)
Sablefish (*Anoplopoma fimbria*)	Evelyn (1971)
Minnow (*Phoxinus phoxinus*)	Hastein *et al.* (1978)
Cod (*Gadus morhua*)	Cornick *et al.* (1984)
Bass (*Morone mississipiensis*)	Buckley (1969)

populations (Jones, 1973); may result in bacterial multiplication due to nutrient concentration on glass surfaces, the so called "bottle effect" (Porter, 1984; Atlas and Bartha, 1987) and inoculation of bacteria into enclosed microcosms may also give rise to cryptic growth ie survivors are able to persist on the remains of dead bacteria (Postgate and Hunter, 1963).

A limited number of studies on *A. salmonicida* have attempted to provide more natural conditions for assessment of the pathogens ability to persist in the aquatic environment. Smith (1962) constructed a free-flowing laboratory stream into which *A. salmonicida* was inoculated. Several investigators (McFeters and Stuart, 1972; Buck, 1978) have utilised semi-permeable chambers, which were then placed *in situ*, to assess survival of enteric bacteria. These methods are not appropriate for *A. salmonicida* because of the danger of the accidental escape of this pathogenic bacterium into the environment. However, McCarthy (1977) investigated the survival of *A. salmonicida* under semi-natural conditions by inoculation into dialysis bags which were then placed into open-circulation tanks of rainbow trout.

ii) The Physiological Status of Bacteria

The growth conditions of bacteria, prior to introduction into microcosms, may influence survival. For example Gauthier *et al.* (1989) found that the survival of *Escherichia coli* in seawater was influenced by the salinity of culture medium used to grow the bacteria prior to inoculation into experimental microcosms. The phase at which cells are harvested from culture medium for inoculation into microcosms may also influence survival (Postgate and Hunter, 1962; Gauthier *et al.*, 1989). These factors have not been studied with *A. salmonicida.* However, McCarthy (1983) has presented evidence that suggests that prior growth conditions influence the virulence of the pathogen.

iii) The Enumeration of *A. salmonicida*

An inability to isolate *A. salmonicida* has restricted research in many areas of its ecology. For example, the pathogen is generally not isolated from covertly infected fish unless they have been severely stressed (Bullock and Stuckey, 1975; McCarthy, 1977). Power *et al.* (1987) and Bernoth and Artz (1989) found that tryptone soya agar and furunculosis agar may occasionally prove inadequate media even for the detection of *A. salmonicida* from fish with clinical disease. Plehn (cited by Williamson, 1928) reported isolation of *Bacillus salmonicida* (*A. salmonicida*) from stream water during a disease outbreak, and Scallan (1983) detected the pathogen in a freshwater stream supplying a fish hatchery. However, other authors have not been able to isolate the bacterium from water even during disease outbreaks (Cornick *et al.*, 1969; Kimura, 1970, Allen, 1982; Allen *et al.*, 1983). This is, in part, due to the presence of contaminating bacteria which inhibit the growth of *A. salmonicida* (McCarthy, 1977; Austin and Austin, 1987). The differential media available for culture of the bacterium eg furunculosis agar or coomassie-blue (eg Wilson and Horne, 1986) and congo-red agar (Ishiguro *et al.*, 1985) merely aid identification of *A. salmonicida* and are not selective. McCarthy (1977), in survival studies in mixed-culture, overcame these difficulties by using an antibiotic-resistant strain of the pathogen which could be selectively isolated with an antibiotic-containing agar. Cunliffe and Adcock (1989) have recently demonstrated that motile aeromonads can be selectively isolated under anaerobic conditions. This method may also prove useful in the isolation of *A. salmonicida.*

The enumeration of *A. salmonicida* populations in survival experiments has consistently been estimated by growth of colony-forming units on nutrient agar. However, growth on agar or in broth is regarded to underestimate the number of active microorganisms (Brock, 1971; Buck, 1979). For example, culture on solid media may yield only 0.1%

of viable bacterial cells present in seawater (Kogure *et al.*, 1979). Poor efficiency of isolation has been attributed to lack of a single universal growth medium (Mason *et al.*, 1986); sublethally stressed cells with fastidious growth requirements (Bissonnette *et al.*, 1975; Dawe and Penrose, 1978; Gurijala and Alexander, 1988); and physiologically "dormant" cells (Roszak and Colwell, 1987a). Allen-Austin *et al.* (1984) have suggested that *A. salmonicida* enters an unculturable dormant state in riverwater which would have important implications for the spread of the disease (Austin, 1987; Austin and Austin, 1987). Dormancy in *A. salmonicida* is further discussed in Section 1.2.2d).

Direct microscopic counts of bacteria provide an alternative to culture methods of enumeration but can lead to over-estimation because of lack of selectivity between species and living/dead bacteria. Direct counts of *A. salmonicida* were assessed in the Allen-Austin *et al.* (1984) study of dormancy (see 1.2.2.d) and were found to be significantly greater than viable counts obtained from agar plates. Austin (1987) reported that *A. salmonicida* cells could be detected on river particles by immunofluorescent staining. These cells could not be isolated with culture techniques. There are many problems associated with immunofluorescent methods (Bohlool and Schmidt, 1980) not least of which is cross-reactivity of anti-sera with antigenically related contaminants which may give rise to false positives and the method gives no indication of viability.

b) Survival of *A. salmonicida* in water

Studies on the survival of *A. salmonicida* suggest that it may persist in freshwater for 2-3 weeks and in seawater for 1-2 weeks under normal conditions (McCraw, 1952; McCarthy, 1977; Austin and Austin, 1987). In general, survival under sterile conditions is greater than in mixed communities (non-sterile); appears to be prolonged at low temperatures (Dubois-Darnaudpeys, 1977a); and is dependent on mineral content

(Dubois-Darnaudpeys, 1977a; Wedemeyer and Nelson, 1977) and initial inoculum size. A summary of the major studies are provided in Table 1.2. This table does not include investigations undertaken in tapwater or distilled water (Williamson, 1928; Evelyn, 1971) which may not adequately represent the natural environment because of addition of chemical disinfectants and lack of nutrients/minerals respectively. McCarthy (1977) has argued that survival studies conducted in sterile media are of limited value. However, such studies are included here as they represent the maximum survival potential in the absence of competition/inhibition of the pathogen and may be useful in disease risk assessment.

Studies in mixed culture (non-sterile conditions) arguably provide the most accurate assessment of survival ability (McCarthy, 1977). However, community structure within microcosms may be susceptible to random changes and results should be interpreted with care (Brock, 1987). Survival studies in mixed culture with *A. salmonicida* have been limited by the poor efficacy of techniques to isolate the pathogen (McCarthy, 1977). McCarthy (1977) overcame the problem of selectivity with an antibiotic resistant strain of *A. salmonicida* which could then be isolated from mixed culture on antibiotic containing nutrient agar. Survival was found to be eight days in seawater, 20 days in freshwater, 26 days in brackish water (McCarthy, 1977).

Several studies have described competitive effects upon *A. salmonicida* by other bacteria including *Pseudomonas* sp. and *Flavobacterium* sp. (Cornick *et al.*, 1969; Dubois-Darnaudpeys, 1977b; Michel and Dubois-Darnaudpeys, 1980; Allen, 1982). Bacteria may also be predated upon by protozoa (McCambridge and McMeekin, 1980); influenced by zooplankton extracellular products (Porter, 1984) and antibiotics; infected by bacteriophage (Rodgers *et al.*, 1981 for details of *A. salmonicida* "phage") and are

TABLE 1.2

Survival of *A. salmonicida* in water

Aquatic system	Inoculum (bacteria/ml)	Survival
Autoclaved water		
Riverwater	-	4-5 days Williamson (1928)
Riverwater (20°C)	7.5×10^8	Eight days Lund (1967)
Riverwater (21.1°C) (15.6°C)	- -	Four days >14 days Ross and Smith (1974)
Soft Lakewater (20°C) Hard Lakewater (20°C)	10^3 10^3	Two days *ca* 14 days Wedemeyer and Nelson (1977)
Riverwater (4°C) (14°C) (22°C)	10^6 10^6 10^6	50 days 30 days 20 days Dubois-Darnaudpeys (1977a)
Riverwater (15°C)	10^2	*ca* 18 days Allen-Austin *et al.* (1984)
Estuarine water (0.91%, 20°C) (2.41%, 20°C) Seawater (3.31%, 20°C)	7.5×10^8 7.5×10^8 7.5×10^8	16 days 16 days 24 days Lund (1967)
Seawater (3.1%, 15°C)	10^6	13 days Evelyn (1971)
Filter-sterilised water		
Riverwater (21.1°C) (15.6°C)	- -	Three days Six days Ross and Smith (1974)

TABLE 1.2 (continued)

Aquatic system	Inoculum (bacteria/ml)	Survival
Non-sterile water		
Riverwater	-	Two days Williamson (1928)
Riverwater	-	12 weeks Slack (1937)
Riverwater (artificial stream)	10^5-10^{10}	8-19 days Smith (1962)
Riverwater (20°C)	7.5×10^8	Two days Lund (1967)
Freshwater (11-13°C)	10^7	20 days* McCarthy (1977)
Estuarine water (0.9%, 20°C) (2.4%, 20°C)	7.5×10^8 7.5×10^8	Two days Two days Lund (1967)
Brackish water (2.34%, 11-13°C)	10^7	26 days* McCarthy (1977)
Seawater	-	Two days Williamson (1928)
Seawater	-	21-26 days Smith (cited by McCarthy, 1977)
Seawater (3.31%, 20°C)	7.5×10^8	Five days Lund (1967)
Seawater (3.4%, 15°C)	10^7	10 days* McCarthy (1977)

* enumerated on antibiotic containing agar

influenced by a number of abiotic effects (eg ultraviolet radiation, heavy metals etc). Factors involved in bacterial mortality have been reviewed by Mitchell and Chamberlin (1975), Walker and Guarraia (1975) and Mason *et al.* (1986).

The ability of *Aeromonas salmonicida* to survive in the external environment may be influenced by the presence of other organisms. King and Shotts (1988) have shown that the survival of the bacterium in nutrient poor conditions may be enhanced by interactions with protozoa. They found increased survival, and multiplication, of *A. salmonicida* co-cultured with the ciliated protozoan *Tetrahymena pyriformis*. This phenomenon was suggested to be due to the uptake of nutrients by bacteria which survived protozoan digestion. A similar role for protozoa has also been made for *Legionella pneumophila* (Fields *et al.*, 1984) and *Escherichia coli* (Sambanis and Fredrickson, 1988). The importance of this apparent symbiosis for *A. salmonicida* in the natural environment is unknown. Enhancement of survival has also recently been shown for *Vibrio cholerae* associated with crustacean zooplankton (Huq *et al.*, 1983; 1984). Similar interactions may occur with *A. salmonicida* and plankton but have not been investigated.

c) Survival of *A. salmonicida* in nutrient enriched aquatic environments

The survival of *A. salmonicida* in the external environment has been shown to be extended in the presence of available nutrient or sediment (Table 1.3). For example, Evelyn (1971) and Allen (1982) have described multiplication of the pathogen in sterile sea and freshwater respectively on the addition of nutrient. McCarthy (1977) described extended survival (>29 days), but not multiplication, in unsterilised freshwater in the presence of detritus/sediment. Dubois-Darnaudpeys (1977b) was able to detect specific *A. salmonicida* bacteriophage in river sediments throughout the year and believed that

TABLE 1.3

Survival of *A. salmonicida* in nutrient-enriched environments

Aquatic system	Inoculum (bacteria/ml)	Survival
Sterile		
ether-treated sewage	-	67 days Duff *et al.* (1940)
moist soil (4-30°C)	-	>40 days Cornick *et al.* (1969)
river sediment (4-22°C)	10^4	>21 days Dubois-Darnaudpeys (1977b)
river sediment (15°C)	-	Nine months (progressive loss of virulence after six months) Michel and Dubois-Darnaudpeys (1980)
Sand/humic acid/ tryptone/distilled water	10^7	>15 weeks Sakai (1986)
Seawater + 0.1% peptone	-	>80 days Evelyn (1971)
Non-sterile water		
Sewage	-	Two days Horne (1928)
Freshwater silt	-	<7 days Slack (1937)
Pond water + detritus (11-13°C)	10^7	>29 days* McCarthy (1977)

* enumerated on antibiotic containing agar

this indicated the presence of the pathogen. That the phage may persist for a longer period than *A. salmonicida* was not considered. Bacteriophage specific for *A. salmonicida* was also detected by Hidaka and Kawaguchi (1986) in water samples obtained from fish farms, rivers, lakes and marshes in Japan. These studies indicate that under certain conditions, eg nutrient excess, the ability of *A. salmonicida* to survive may be considerably extended and that sediments may act as reservoirs for the pathogen. The survival of the bacterium in marine sediments has not been studied. However, Smith *et al.* (1982) reported that an *A. salmonicida* strain had persisted at a fallow seawater farm site for six months but, it was not known whether the strain had survived in wild fish or in sediments beneath cages.

Michel and Dubois-Darnaudpeys (1980) consider that the activities of benthic fauna and fish may resuspend the bacterium within the water column where it may be able to cause infection. However, a reduction in virulence of *A. salmonicida* maintained in natural (Michel and Dubois-Darnaudpeys, 1980) or artificial (Sakai, 1986) sterile sediments have been recorded which questions the importance of these substrates to the pathogen.

d) The existence of dormant forms of *A. salmonicida*

Allen-Austin *et al.* (1984) suggested that *A. salmonicida* entered a dormant state in sterile riverwater. Similarly, dormant states ("somni-cells") have been proposed for some enteric pathogens, such as *Escherichia coli* and *Vibrio cholerae*, which are unculturable on solid media but are capable of infecting rabbit ileal loops (Roszak and Colwell, 1987a). In the Allen-Austin *et al.* (1984) study *A. salmonicida* could be re-isolated seven days after viable counts, iè colony-forming units on solid media, had reached zero on the addition of 0.1-0.01% tryptic soy broth to experimental flasks. Austin

(1987) later suggested that these "dormant" cells could account for the spread of furunculosis between segregated populations of fish. Their existence may have important consequences for the control of the pathogen and its obligate nature may have to be reassessed. However, Allen-Austin *et al.* (1984) did not consider that a small number of viable cells may be present in microcosms in numbers too few to detect by the sampling regime and that it was these cells that were stimulated to grow after addition of nutrient broth and not unculturable "dormant" cells. The existence of dormant forms of *A. salmonicida* remains undecided.

1.2.3 Transmission Studies on *A. salmonicida* Within Fish Populations

a) Variation in susceptibility of salmonids to furunculosis

A range of susceptibilities to furunculosis are exhibited within the Salmonidae although all members are believed to be susceptible to some degree (Mackie *et al.*, 1930, 1933, 1935; McCraw, 1952). In general, Atlantic salmon (*Salmo salar* L.), brown and sea-trout (*Salmo trutta* L.) and brook trout (*Salvelinus fontinalis*) have been shown to be very susceptible whereas rainbow trout (*Salmo gairdneri* Richardson = *Oncorhynchus mykiss*) are relatively resistant to infection (McCarthy, 1977).

The sex and age of salmonids have been reported to influence resistance to infection. Smith (1962) found disproportionate numbers of specific mortalities due to *A. salmonicida* in female Atlantic salmon in Summer river stocks. However, mortalities may have reflected the sex ratio of the stock rather than variation in susceptibility to furunculosis. A number of studies have suggested that susceptibility increases with age of fish (Plehn, 1924; Blake and Clarke, 1931; Mackie and Menzies, 1938; Scallan, 1983). Conversely, McCarthy (1977) considered that *A. salmonicida* affects all age groups.

b) Environmental influence on furunculosis

Furunculosis occurs in wild fish under a wide set of environmental conditions but in the UK is most predominant between May and October (Horne, 1928; Mackie *et al.*, 1930, 1933, 1935). A number of environmental factors are believed to enhance disease outbreaks. In brief, development of furunculosis appears to be enhanced under conditions of crowding and low water levels (Williamson, 1928; Mackie *et al.*, 1930, 1933, 1935); temperature of 15-21°C (Mackie *et al.*, 1930, 1933, 1935; Groberg *et al.*, 1978); and increased organic matter (Plehn, 1924; Duff *et al.*, 1940). This literature is further reviewed by McCraw (1952), McCarthy, (1977) and McCarthy and Roberts (1980) and Austin and Austin (1987).

c) Furunculosis in the marine environment

Early investigators (Mackie *et al.*, 1930, 1933, 1935) considered that furunculosis was solely a disease of freshwater fish. However, *A. salmonicida* is now regularly isolated from both freshwater and marine salmon farms (Novotny, 1975, 1978; Smith *et al.*, 1982; Munro and Waddell, 1984). Scott (1968) was able to infect sea and brown trout (*Salmo trutta* L.) maintained in seawater on exposure to dead infected fish. However, susceptible fish were not tested for asymptomatic infection and may have therefore developed clinical furunculosis from previous contact with the pathogen in freshwater. In this respect, Ezura *et al.* (1984) recorded high mortalities due to *A. salmonicida* after transfer of coho salmon (*Oncorhynchus kisutch*) to seawater and concluded that fish had been infected prior to transfer because the pathogen could be isolated from fish retained in freshwater. Novotny (1978) also believed that fish often carry furunculosis in a latent state from fresh to seawater. Transmission in seawater does, however, appear likely as the pathogen has been isolated from non-salmonid marine fish (Evelyn, 1971; Klontz and Wood, 1972; Novotny, 1975, 1978) which must have become infected in

seawater. Finally, Smith *et al.* (1982) reported that an *A. salmonicida* strain, identifiable by a resistance plasmid, was transmitted at a marine site between infected Atlantic salmon and recently introduced fish with no previous record of infection.

d) Transmission studies via water

The routes and mechanisms of infection of *A. salmonicida* are little understood (McCarthy, 1977; McCarthy and Roberts, 1980; Hastings, 1988). However, it is generally accepted that transmission between fish (lateral or horizontal transmission) occurs via a water-borne route (McCarthy, 1977; Munro, 1982a). A number of transmission studies have been performed in freshwater between infected and susceptible co-habitees; from water effluent of tanks containing infected fish; and bath challenge in which fish are exposed to a suspension of broth-grown bacteria. Although anecdotal evidence is available for the transmission of *A. salmonicida* in seawater (Novotny, 1975, 1978) there are no reports of its infectivity in the marine environment. Several transmission studies are described in Table 1.4. Scallan (1983) has criticised transmission studies, with the exception of McCarthy (1977), because absence of covert infection in susceptible fish was not established prior to experimentation.

Bath challenge with *A. salmonicida* is assumed to adequately reflect the natural infection process and is the method of choice for vaccine studies (McCarthy, 1983; Adams *et al.*, 1987). However, several factors may influence the outcome of these challenges and further investigation is required. The physiological status of *A. salmonicida* under natural challenge is unknown and prior culture conditions may influence experimental challenge. McCarthy (1983) is the only worker to investigate prior growth conditions and found decreasing virulence with increased incubation time in brain heart infusion broth. The bacterial concentrations, 10^4-10^9 bacteria/ml (Cipriano, 1982; McCarthy,

TABLE 1.4

Selected transmission studies on *A. salmonicida* via water

Experimental infection/fish species	Reference
Freshwater	
Tank effluent exposure/brown trout (*Salmo trutta* L.)	Blake and Clarke (1931)
Bath + effluent exposure/Atlantic salmon (*Salmo salar* L.)	Bullock *et al.* (1976)
Cohabitation/scarified rainbow trout (*Oncorhynchus mykiss*)	McCarthy (1977)
Cohabitation/infection from carrier to non-carrier salmon (*Salmo salar* L.)	Scallan (1983)
Cohabitation/effluent/infection from Common Shiner (*Notropis cornutus*) to coho salmon (*O. kisutch*) and brook trout (*Salvelinus fontinalis*)	Ostland *et al.* (1987)
Seawater	
Cohabitation with infected dead fish/sea + brown trout (*Salmo trutta*)	Scott (1968)

NB In all cases specific mortalities were recorded in susceptible fish

1983; Adams *et al.*, 1987), used in bath challenges may not represent the natural levels of the pathogen. Environmental levels of the pathogen are not known and most workers (Cornick *et al.*, 1969; Kimura, 1970; Allen, 1982, Allen *et al.*, 1983) have been unable to recover the bacterium from water samples even during clinical furunculosis outbreaks. Austin and Austin (1987) predicted that during disease outbreaks *A. salmonicida* concentrations may be in the region of 10^2 bacteria/ml allowing for dilution effects. Assessment of release of *A. salmonicida* from infected fish may provide estimates of bacterial density in the environment but there is little information on the release of the bacterium from infected fish (see Table 1.5). It may, however, be difficult to extrapolate the available data to the wild because of restricted water flow and confinement of fish in experimental facilities.

e) Alternative routes of infection

It is likely that infection with *A. salmonicida* can occur via other routes than direct contact with the pathogen in water. For instance, McCarthy (1977) and Sakai (1979) were unable to produce mortalities in rainbow trout (*Salmo gairdneri* Richardson = *Oncorhynchus mykiss*) and masu salmon (*Oncorhynchus masou*) respectively after bath challenge unless the skin had been previously abraded indicating that infection across normal skin may be a rare occurrence in these fish species. However, Sakai (1979) was able to infect masu salmon by anal and oral intubation of *A. salmonicida.* A number of workers have investigated oral, and transovarian (vertical transmission) routes of infection for the pathogen with conflicting results. These studies are summarised in Table 1.6. The role of vertical transmission (transovarian), from parents to progeny, has also been investigated (summarised in Table 1.7) and is believed not to occur with this fish pathogen.

TABLE 1.5

Release (shedding) of *A. salmonicida* from experimentally infected fish

Species	Infection route	Shed bacteria* (bacteria/ml)
Rainbow trout (*Onchorynchus mykiss*)	intramuscular injection	10^1-10^4 Michel (1979)
Brown trout (*Salmo trutta*)	effluent from infected brown trout (*Salmo trutta* L.)	10^3-10^5 Bullock and Stuckey (1977)
Rainbow trout (dead)	intramuscular injection	10^5-10^7 McCarthy (1977)

* concentration of *A. salmonicida* in tankwater

TABLE 1.6

A. salmonicida transmission studies via the oral route

Reference	Description
Plehn (1911), Clayton (1927), Blake and Clarke (1931)	Successful infection via *A. salmonicida* contaminated food
Krantz *et al.* (1964)	Failed to infect fish with 10^8 bacteria/g in feed
Klontz and Wood (1972)	Observed *A. salmonicida* infection in marine sablefish apparently due to ingestion of infected coho salmon
McCarthy (1977)	Oral uptake in rainbow trout of 10^8 bacteria/g feed but no mortalities recorded
Sakai (1979)	Infected masu salmon by oral and anal intubation of *A. salmonicida*
Markwardt and Klontz (1989)	Infected chinook salmon by gastric and intubation of *A. salmonicida* and broth-culture coated feed

TABLE 1.7

Transmission studies via the transovarian route

Reference	Description
Plehn (1911), Mackie *et al.* (1930, 1933, 1935)	Failed to infect fish eggs
Smith (1939, cited by McFadden, 1969)	Claimed *A. salmonicida* carried on egg surface
Lund (1967); McDermott and Berst (1968)	Isolated *A. salmonicida* from gonads of mature fish
McCarthy (1977)	Isolated pathogen on eggs of experimentally infected rainbow trout but not carrier fish. Pathogen rapidly lost (five days) from infected eggs. Considered transovarian route not significant in transmission
Bullock and Stuckey (1987)	Unable to infect brook/rainbow trout eggs with 10^7-10^8 bacteria with a four hour exposure. Unable to isolate *A. salmonicida* from progeny of covertly infected brook/rainbow trout

1.2.4 The Carrier State

a) The role of carrier infections in furunculosis

Covert, carrier, infections may be defined as a stage of persistent infection in which the pathogen causes no disease, but remains capable of activation and disease production (Mims, 1987). The *A. salmonicida* carrier is considered to be an important reservoir of infection. The introduction of asymptomatic carriers to both freshwater and marine fish farms may be the main cause of furunculosis spread (Klontz and Wood, 1972; McCarthy, 1977; Drinan *et al.*, 1978; Novotny, 1978; Munro, 1982b, 1988). A number of workers have suggested that carriers may be able to transmit the pathogen to susceptible fish in freshwater (Plehn, 1911; Blake and Clarke, 1931). However, in these studies susceptible fish were not assessed for carrier status prior to experimentation and the reported transmission may have actually resulted from development of clinical disease from the carrier state in these fish. McCarthy (1977) and Scallan (1983) have emphasised the problems of undetected carriers in experimental transmission studies and reported experimental transmission of furunculosis between carrier and *A. salmonicida*-free fish in freshwater. In addition, Scallan (1983) reported the isolation of *A. salmonicida* from tankwater in which furunculosis-carriers were maintained. Markwardt and Klontz (1989) have recently shown that carrier status in juvenile chinook salmon (*Oncorhynchus tshawytscha* (Walbaum)) may be established by gastric intubation; broth culture bath; ingestion of broth-culture coated feed; and cohabitation with intraperitoneally infected fish. However, carrier infection was only monitored over a 16 day period and was detected by presence of culturable *A. salmonicida* in intestinal tissue and anal swabs. These fish may have developed clinical furunculosis if monitored for a longer period of time. Clearly, a consensus is required for the definition of covert (carrier) infection with *A. salmonicida* to prevent confusion between workers.

b) Seasonal variation and transient nature of furunculosis carrier status

In contrast to earlier workers (Mackie *et al.*, 1930,1933, 1935), McCarthy (1977) considered that clinical furunculosis need not be the outcome of latent, carrier, infection. Similarly, Andrews (1981) speculated that the carrier state could be lost. The transient nature of covert furunculosis is supported by reports of seasonal variation in carrier rates at fish farms. For example, Jensen and Larsen (1981) reported carrier rates of 90% during summer months but were unable to detect carriers throughout winter. Conversely, Scallan (1983) found that carrier rates peaked during Spring and Winter. In further studies Scallan (1983) found that carrier rates in fish held in individual tanks dropped more rapidly than those held in groups. It was concluded that the carrier state was not stable and was maintained by continual re-infection within fish populations.

c) Detection of furunculosis carrier fish

The efficient detection of covertly infected fish is an important component of disease control. Earlier workers (Horne, 1928; Blake and Clarke, 1931; McDermott and Berst, 1968) identified covert, carrier, infections by bacteriological isolation from internal organs or by hind-sight after disease outbreaks. Both of these methods are unreliable (Mackie *et al.*, 1930, 1933, 1935; Bullock and Stuckey, 1975): the number of *A. salmonicida* in infected fish may be too few to detect (Mackie *et al.*, 1930, 1933, 1935); or in an unculturable form (McIntosh and Austin, 1988). Hivela-Koski *et al.* (1988) have reported increased detection with prior enrichment of carrier kidney in tryptic soy broth. The present practice of solely examining kidney tissue may, however, not be sufficient. Daly and Stevenson (1985) found improved detection of the pathogen in clinically infected fish when all the internal organs were bacteriologically examined. McIntosh and Austin (1988) were able to induce L-forms of *A. salmonicida*. These cell wall deficient bacteria required a nutrient rich medium containing an osmotic support for their propagation.

The L-phase variants were recoverable in low numbers from naturally infected fish. Their existence in the carrier state may explain the poor efficacy of conventional culture methods of detection.

The most sensitive method of carrier detection available is considered to be the heat-stress method (Bullock and Stuckey, 1975). The combination of increased temperature (18°C) and injection of corticosteroid (triamcinolone acetonide or prednisalone acetate, McCarthy, 1977) precipitated clinical disease in carrier fish and was found to significantly increase detection levels compared with direct sampling of fish tissue by conventional culture methods. The main disadvantage of the heat-stress technique of carrier detection is the length of time, generally 10-14 days, with which fish must be maintained in experimental holding facilities to enable clinical disease to develop. The detection of *A. salmonicida* antigen in fish tissues presents a less time consuming alternative to the Bullock and Stuckey (1975) method. However, the detection of antigen is always restricted by the possibility of antibody cross-reaction with unrelated antigen; and detection of dead antigen. McCarthy (1975b) was able to detect *A. salmonicida* antigen in infected kidney or furuncle material using a latex agglutination test down to titres of 1:80 and 1:4,000 respectively and suggested that the technique may be useful in carrier detection. Immunoassay can prove extremely sensitive, for instance Bernoth and Bohm (1987) were able to detect *A. salmonicida* antigen, in saline, at levels below 1 cfu/ml with ELISA but unfortunately experienced problems in detecting the antigen in fish tissue. Scallan (1983) found that anti- *A. salmonicida* antibody conjugated to a fluorescent dye (FAM); or enzyme-linked immunosorbent assay (ELISA) could detect carrier fish which were negative by conventional culture. In the hope of finding a non-lethal method of carrier detection McCarthy (1977) measured serum antibody levels of carrier/non-carrier fish but could find no significant differences.

d) Site of carriage of *A. salmonicida* in carrier fish

Early workers considered that, on the basis of conventional culture techniques, the kidney was the primary site of carriage in carrier fish (Blake and Clarke, 1931) and that the intestine was of secondary importance (Mackie *et al.*, 1930, 1933, 1935). Conversely, Klontz (1968) detected *A. salmonicida* antigen in the gut wall of apparently healthy fish by immunoflourescence and considered that this was the primary site. Scallan (1983), using both FAM and ELISA, found that out of 27 fish examined antigen could be detected in the kidney of 16 fish and to a lesser extent heart (4), intestine (2) and gills (2). Antigen was always present in the kidney of these fish if detected elsewhere suggesting that this organ is a primary site.

McArdle *et al.* (1988) proposed that superficial branchial colonies (eg Bruno, 1986) observed on gills were *A. salmonicida*, and possibly a site of carriage in carrier fish, but Turnbull *et al.* (1989) were unable to support this claim after screening the colonies using an indirect immunoperoxidase technique. Recently, Hiney *et al.* (1989) have suggested that *A. salmonicida* is predominantly found on the outer surface of carrier fish. They found that injection of surface material from carriers into healthy fish resulted in 78% infections compared with only 21% after injection of blood.

1.2.5 Molecular Microbial Ecology: Bacterial Detection with DNA Probes

The inadequacy of detection methods involving culture are well known (1.2.2). Recent advances in molecular biology have produced sensitive and specific detection methods which often circumvent the need for cultivation and enable detection of organisms with fastidious growth requirements and/or which are normally undetectable because of competitive contaminants. However, molecular methods have several drawbacks including an inability to predict the activity of a detected bacterium and may also detect

extracellular DNA, which can serve as a template for hybridisation, if not rapidly degraded in the environment. These techniques should prove useful in the study of the epidemiology of *Aeromonas salmonicida* eg detection of the pathogen within the environment and carrier fish. Several probes have been constructed for other fish pathogens including *Vibrio anguillarum* (Rehnstam *et al.*, 1989; Aoki *et al.*, 1989) and *Pasteurella piscicida* (Zhao and Aoki, 1989), causative agent of pseudotuberculosis in yellowtail (*Seriola quinqueradiata*).

These genetic methods detect specific DNA sequences with appropriately labelled (eg radioactive isotopes, biotin-avidin, enzymes) DNA or RNA probes which hybridise to their complementary sequences. The molecular methods available for detection of microorganisms are briefly reviewed below. Techniques requiring cultivation of target organisms (Steffan *et al.*, 1989-colony hybridisation; most probable number-filter hybridisation) are not considered because of their limited applicability to *A. salmonicida* which has proven difficult to isolate from environmental samples by culture methods (Austin and Austin , 1987). Recent reviews of these and other techniques mentioned in this text include Trevors (1985), Ford and Olson (1988), Tenover (1988) and Walker and Dougan (1989).

a) Detection of specific DNA sequences from total community DNA

An alternate approach to DNA methodologies requiring growth of the target organism is to extract the total community genomic DNA and then apply gene probe technology (Steffan *et al.*, 1989). The success of detection and quantification of target organisms is dependent on the efficiency of DNA extraction; purity of sample; specificity and sensitivity of probes (Trevors and van Elsas, 1989). DNA extraction methods are still in the developmental stage (Holben and Tiedje, 1988) and are not efficient, eg yields

of 25-50% (Fuhrman *et al.*, 1988), 34% (Holben *et al.*, 1988) have been recorded. Recently, improved methods for DNA extraction in soils and sediments (Steffan *et al.*, 1988) and from the aquatic environment (Somerville *et al.*, 1989) have been described. The size of oligonucleotide probes should be 0.5-1.5 kb (kilobases) which is a compromise between maximum specificity (optimum 20 basepairs) and sensitivity (optimum >2 kb) (Trevors and van Elsas, 1989). The number of copies of a sequence within the target genome may also influence sensitivity. For example, Steffan *et al.* (1989) found that a 1.3 kb gene probe for *Pseudomonas cepacia* AC1100 was more sensitive than a 53 kb probe for Acaligenes A5. The 1.3 kb sequence was present in 15-20 copies per cell whereas the 53 Kb sequence was solely found on a single-copy plasmid.

b) Polymerase Chain Reaction (PCR)

The gene probe technology briefly reviewed above is unfortunately relatively insensitive. For example, the DNA probe described for the fish pathogen *Pasteurella piscicida* has a detection limit of 10^5 bacterial cells (Zhao and Aoki, 1989). Similarly, the *Vibrio anguillarum* probe (Rehnstam *et al.*, 1989) has a limit of 5×10^3 bacteria/ml of rainbow trout kidney. Fortunately, the polymerase chain reaction enables a high degree of sensitivity compared with standard DNA hybridisation. For example, Steffan and Atlas (1988) were able to detect one cell of *Pseudomonas cepacia* per gram of river sediment. This level of detection is 10^3 greater than that obtained from unamplified samples. Chaudry *et al.* (1989) were able to detect an *Escherichia coli* strain inoculated into sterile lake water/sewage water for 6-10 days respectively by plate counts. The bacterium could be detected for 10-14 days with an 0.3 kb oligonucleotide probe in conjunction with PCR. The PCR technique presents an enormous potential for ecological research

on *Aeromonas salmonicida*. Successful PCR hybridisation has been achieved with tissue sections (Impraim *et al.*, 1987) which may prove useful in investigations into the furunculosis carrier state.

PCR is an *in vitro* technique which increases the number of copies of a target sequence which, subsequently can be detected by normal hybridisation methods. Briefly, two oligonucleotide DNA primers are produced which hybridise to adjacent regions flanking the target region. The region between the two primers is then amplified with thermostable *Thermus aquaticus* DNA polymerase (Taq I). Sample DNA is heat denatured (melted) at 90°C and primers are annealed at moderate temperatures (40-60°C). Taq I is then used to extend primers (55-70°C) and duplicate the target sequence. A 10^6-fold increase in target sequence is minimally obtained after 25-30 cycles (Trevors and van Elsas, 1989) of melting, annealing and primer extension. However, there are several drawbacks with PCR: a knowledge of the DNA sequence of the target and optimum melting/annealing temperatures are required; and there may be a limit to the length of sequence which can be detected (Trevors and van Elsas, 1989). The technique is further described in Scharf *et al.* (1986), Mullis and Faloona (1987), Saiki *et al.* (1988), Erlich *et al.* (1988) and Innis *et al.* (1989).

CHAPTER 2

A RE-EXAMINATION OF "DORMANCY"

IN *Aeromonas salmonicida*

CHAPTER 2 A RE-EXAMINATION OF "DORMANCY" IN *Aeromonas salmonicida*

2.1 INTRODUCTION

Aeromonas salmonicida is believed to be transmitted via a water-borne route (McCarthy, 1977; Austin and Austin, 1987). Its ability to survive in the external aquatic environment is, therefore, a major factor in the epidemiology of furunculosis. Allen-Austin *et al.* (1984) suggested that the pathogen entered a "dormant" state in riverwater, which could explain outbreaks of the disease in fish populations without apparent contact with the pathogen (Austin, 1987) and strongly imply that reports of its ability to survive in the environment, based upon detecting colony-forming units (cfu), may be greatly underestimated.

Sussman and Halvorson (1966) defined dormancy as "any rest period or reversible interruption of the phenotypic development of an organism" and delineated two types: constitutive which is an innate property of an organism and is under genetic regulation eg endospores of bacilli, myxospores of myxobacteria (NB *A. salmonicida* is asporogenous); and exogenous "a condition in which development is delayed because of unfavourable chemical or physical conditions of the environment". Stevenson (1978) has argued that exogenous dormancy is an important physiological adaptation contributing to the survival of bacteria in the aquatic environment. Subsequently, the existence of unculturable-but-viable "somni-cells" have been described for some enteric bacterial pathogens (reviewed by Grimes *et al.*, 1986; Roszak and Colwell, 1987a) including *Escherichia coli* (Xu *et al.*, 1982) and *Vibrio cholerae* (Xu *et al.*, 1982; Colwell *et al.*, 1985). "Somni-cells" may be resuscitated, ie become culturable, with nutrient broth or by inoculation into rabbit ileal loops (eg Colwell *et al.*, 1985; Roszak and Colwell, 1987a).

An alternative means of survival, starvation-survival, has been described for marine bacteria (reviewed by Kjelleberg and Hermansson, 1987; Kjelleberg *et al.*, 1987; Morita, 1988). For example, Hood *et al.* (1986) reported that they could isolate *Vibrio cholerae* for as long as five years from a carbon-free basal salt (1.5-2.0%) microcosm into which the pathogen had been inoculated. Kjelleberg and Hermansson (1987) and Kjelleberg *et al.* (1987) have emphasised that the term "dormancy" is misleading because non-growing bacteria are in fact in an active state eg both exogenous and endogenous substrates are used for maintenance and reorganisation of cellular processes. Starvation-survival is characterised by a reduction in cell volume (Novitsky and Morita, 1978) and often cell fragmentation (Morita, 1982, 1988). These bacteria are generally culturable on solid media (Lappin-Scott *et al.*, 1988). Recently, long-term survival, but not cell fragmentation and diminution, have been described (Hoff, 1989a) for two bacterial fish pathogens, *Vibrio anguillarum* (>50 months) and *Vibrio salmonicida* (>14 months) in seawater.

The evidence for "dormancy" in *A. salmonicida* (Allen-Austin *et al.*, 1984) was based on the re-isolation of the pathogen upon the addition of nutrient broth to riverwater microcosms in which viable counts had reached zero. However, the possibility that resuscitation of these "dormant" cells may have been an artefact caused by the presence of small numbers of viable cells too few to detect was not considered.

The concept of "dormancy" in *A. salmonicida* was re-examined in both fresh and seawater microcosms. The study was similar to that described by Allen-Austin *et al.* (1984) except that, in addition, nutrient broth was added to serially diluted 1 ml microcosm aliquots to control for the effect of small numbers of viable cells in the main microcosm;

and microcosm aliquots were injected into Atlantic salmon (*Salmo salar* L.) as an alternative method of resuscitation. Some of the work presented in this chapter has been published previously (Rose *et al.*, 1990a,b).

2.2 MATERIALS AND METHODS

2.2.1 Bacteria

Three strains of *Aeromonas salmonicida* subsp. *salmonicida* were used, MT432, MT212 and MT393, which were isolated from Atlantic salmon, *Salmo salar* L., with clinical furunculosis. The biochemical characteristics of the strains were determined with API20E identification kits (Montalieu) which were incubated at 22°C for 48 hr prior to reading. The characteristics are summarised in Table 2.1. The strains were routinely maintained on tryptone soya agar (TSA, Oxoid) plates (short-term) or slants (medium-term) at 4°C. For long-term storage, bacteria were maintained in a 9:1 mixture of 3% TSB and glycerol at -20°C.

Strains were grown overnight in 100 ml 3% or 0.1% tryptone soya broth (TSB, Oxoid) at 22°C. Cells were washed twice in filter-sterilised (0.22 μm Millex-GV, Millipore) riverwater or basal salts media (BSM) and suspended to an optical density of one at 540 nm which is equivalent to 10^9 colony-forming units per ml (cfu ml^{-1}). This bacterial suspension was serially diluted 10-fold and 100 μl inoculated into microcosms.

2.2.2 Microcosms

Prior to use, 150 ml screw-topped glass flasks were washed with 2% potassium dichromate (Sigma) and 50% (v/v) hydrochloric acid (Analar) for 12 hours respectively and rinsed at least 10 times with double distilled, deionised water. Experimental microcosms

TABLE 2.1

Characteristics of *A. salmonicida* strains

Character	MT432	MT212	MT393
Coccobaccilli	+	+	+
Gram Stain	-	-	-
Autoagglutination	+	+	+
Brown, diffusible pigment	+	+	+
Motility	-	-	-
API20E			
ONPG	+	-	+
ADH	-	-	-
LDC	(+)	-	-
ODC	-	-	-
CIT	-	-	-
H_2S	-	-	-
URE	-	-	-
TDA	-	-	-
IND	-	-	-
VP	-	-	-
GEL	+	+	+
GLU	+	+	+
MAN	+	+	+
INO	-	-	-
SOR	-	-	-
RHA	-	-	-
SAC	-	-	-
MEL	-	-	-
AMY	(+)	+	(+)
ARA	-	-	-
Oxidase	+	+	+
OF/F (Oxidative/fermentative)	F	F	F
Gas from glucose	+	+	+

\+ positive reaction
(+) partial positive reaction
\- negative reaction
F Fermentative

contained 100 ml filter-sterilised riverwater (pH 7.3) or BSM and *A. salmonicida* was added to a concentration of 10^4-10^5 cfu ml^{-1}. All flasks were wrapped in tin-foil and statically incubated at 15°C.

2.2.3 Basal Salts Media (BSM)

The basal salts media (BSM) were prepared in double-distilled deionised water and consisted of the following in g/l: freshwater BSM NaCl 0.159, $CaCl_2 2H_2O$ 0.243, $MgSO_4 7H_2O$ 0.103 adjusted to pH 7 with 0.1 M HCl and 0.1 M NaOH. Seawater BSM NaCl 23.926, $MgCl_2 2H_20$ 10.83, Na_2SO_4 4.008, $CaCl_2 2H_20$ 1.533, KCl 0.677, $NaHCO_3$ 0.196, KBr 0.098, NaF 0.003, $SrCl_2$ 0.024 and HBO_3 0.026 (after Singleton *et al.*, 1982) to give a salinity of 3.3% and pH 8.3. All chemicals were of Analar grade (BDH). 100 ml BSM was added to experimental microcosms which were then autoclaved for 20 minutes at 121°C, 15 psi prior to inoculation with *A. salmonicida.*

2.2.4 Sampling Regime and Nutrient Resuscitation

Survival of viable, culturable cells was monitored by counting cfu after spreading either 100 µl or 1 ml from experimental microcosms (three microcosms per strain *A. salmonicida*) onto TSA plates which were then incubated at 22°C.

i) Addition of nutrient broth

Six days after viable counts had reached zero, 1 ml aliquots from microcosms were 10-fold serially diluted to 10^{-3} in sterile riverwater or BSM containing 0.1% (w/v) TSB. TSB was also added to the microcosms to achieve the same final concentration. In the case of seawater BSM TSB was added to a concentration of 0.01% and microcosms and dilutions incubated overnight at 22°C and then TSB was added to give 0.1% TSB. After

seven days incubation at 22°C, swabs were taken from the microcosms and the diluted aliquots, spread onto TSA and incubated at 22°C to detect the presence of culturable *A. salmonicida.*

ii) Resuscitation in Atlantic salmon serum and sucrose

Atlantic salmon sera and sucrose, which may provide stimulatory factors and osmotic support respectively, were used in an attempt to resuscitate putative "dormant" cells from seawater BSM microcosms.

Sera were obtained from anaesthetised Atlantic salmon. Blood was collected by hypodermic syringe (Terumo, 25G) from the caudal vein and left to clot at room temperature in a glass specimen bottle for 3-4 hours after which the serum was decanted.

Ten days after viable counts had reached zero in seawater BSM microcosm 1ml aliquots were spread on agar plates and incubated for four days at 22°C. The agar (TSA) plates contained either 2% salmon serum, 17% (w/v) sucrose, or a combination of 2% serum and 17% sucrose. At the same time 100 µl aliquots were added to either 20 µl serum plus 20 µl 17% sucrose, or 20 µl 17% sucrose alone and incubated as described above.

2.2.5 Experimental Infection

Atlantic salmon (*Salmo salar* L.), 3-10 g for the riverwater study and 30 g seawater acclimated fish for the seawater study, were obtained from the DAFS Marine Laboratory Cultivation Unit. Fish were routinely maintained in 150 l plastic tanks at 8-10°C (freshwater) or 13-14°C (seawater). During the experimental period fish were maintained in 3l tanks at 17°C (riverwater trial) or in 20l plastic tanks at 13-14°C supplied with running water (seawater trial). Aliquots (0.1 ml) were taken from microcosms (one

microcosm per strain *A. salmonicida*) at various time intervals and injected into fish anaesthetised with benzocaine (Sigma). For the riverwater study these were: before culturable counts reached zero (culturable bacteria); six days after culturable counts had reached zero ("dormant" bacteria); and following resuscitation of microcosms ("resuscitated" bacteria). For the BSM study fish were only inoculated with microcosm aliquots six days after culturable counts had reached zero but four fish were injected with 10^2 culturable cells (cfu), grown overnight in 3% TSB at 22°C, to act as a positive control. In both seawater BSM and riverwater trials saline negative controls were included and riverwater aliquots were adjusted to 0.15 M NaCl prior to injection. Individual fish were maintained in separate tanks to prevent cross infection. Mortalities were monitored for 14 days (riverwater) or 21 days (BSM). Surviving fish were assessed for covert infection (carrier status) using the modified protocol of McCarthy (1977).

2.2.6 Anaesthetisation of Fish

Fish were anaesthetised with benzocaine (Sigma). A saturated solution of benzocaine in ethanol was added to water to give a final concentration of 25 ppm benzocaine (Roberts and Shepherd, 1986). Individual fish were placed into the solution for 1-2 min until anaesthetised. After experimental procedure fish were placed into vigorously aerated water to recover.

2.2.7 Carrier Test (after McCarthy, 1977)

Fish were anaesthetised with benzocaine (Sigma) and injected with 20 mg kg^{-1} prednisalone acetate (Deltastab, Boot's) in phosphate buffered saline (Gibco) and water temperature raised to 17-18°C. Fish were monitored for 10 days after which kidney loops (10 µl) were taken from all survivors and spread on TSA to monitor for *A. salmonicida.*

2.3 RESULTS

The existence of "dormant" forms of *Aeromonas salmonicida* subsp. *salmonicida* in riverwater and freshwater or seawater basal salts media (BSM) was examined by both nutrient resuscitation and experimental infection of Atlantic salmon.

2.3.1 Survival in Experimental Microcosms

In these survival studies *A. salmonicida* was grown overnight in TSB, washed, and added to riverwater, freshwater BSM or seawater BSM to give a final concentration of 10^4-10^5 cfuml^{-1}. Survival was monitored by the enumeration of cfu. Results have been expressed graphically as the natural logarithm (Ln) of median counts of three TSA plates per time interval obtained from three microcosms per treatment ie nine plate counts per interval.

The survival characteristics, monitored by cfu, of both strains of *A. salmonicida* (MT212 and MT432) in filter-sterilised riverwater were similar. Survival of bacteria cultured in 3% TSB (Fig. 2.1) prior to inoculation into microcosms survived slightly longer (29-34 days) than those cultured in 0.1% TSB (Fig. 2.2; 25-26 days).

The survival of the two strains in autoclaved freshwater BSM (Fig. 2.3) was slightly less than that described for filter-sterilised riverwater above. MT212 could be isolated on TSA for 20 days whereas MT393 was isolated for 16 days. During the first 0-7 days there was little diminution in population size, as determined by cfu, but cfu rapidly decreased from then on. The reduction in survival, compared with riverwater, may be due to the presence of low levels of nutrients in riverwater.

The survival of MT212, MT432 and MT393 was shorter in autoclaved seawater BSM (Fig. 2.4) than for the previously described freshwater BSM and riverwater microcosms.

Fig 2.1: Survival of A.salmonicida (grown in 3% TSB) in Riverwater.

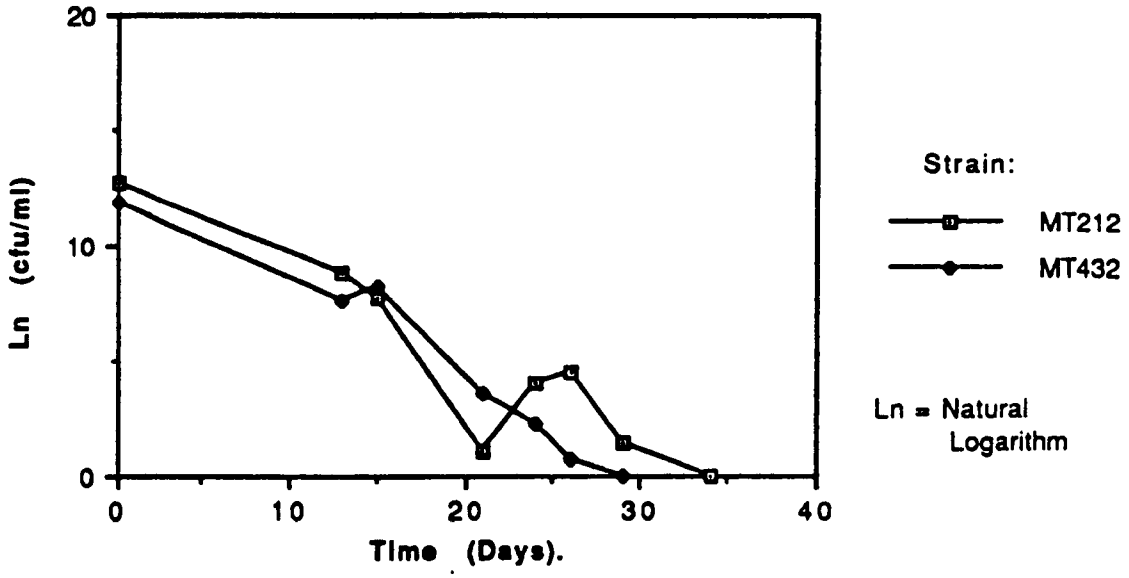


Fig 2.2: Survival of A.salmonicida (grown in 0.1%TSB) in Riverwater.

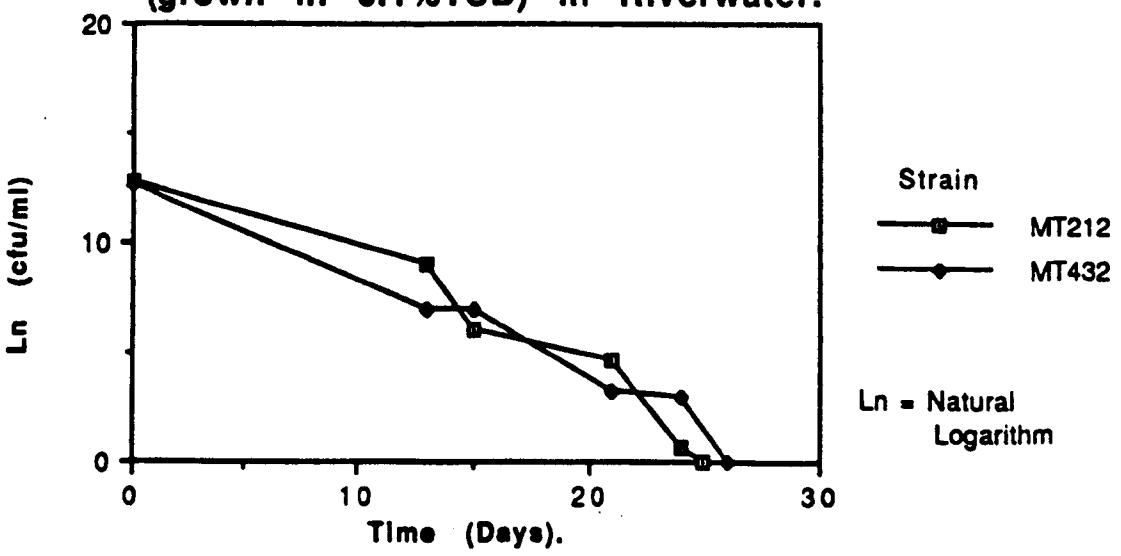


Fig 2.3 Survival of A.salmonicida in Freshwater BSM.

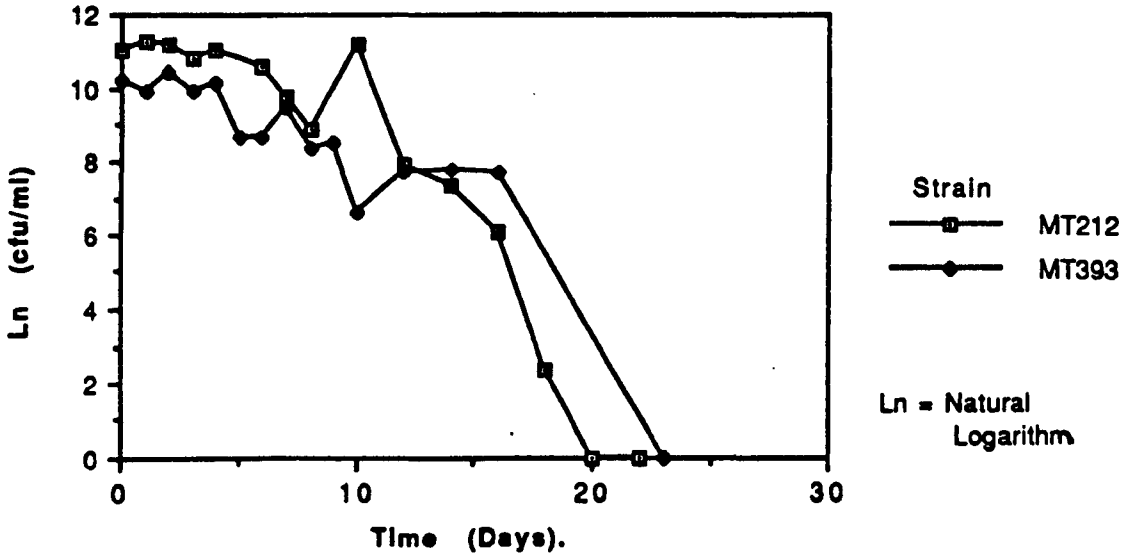
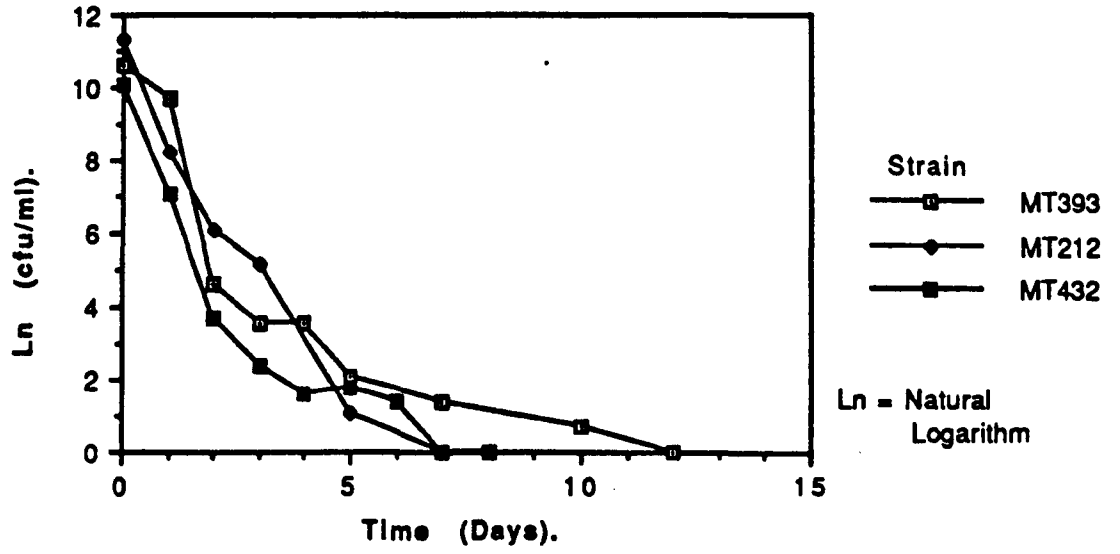


Fig 2.4: Survival of A.salmonicida in Seawater BSM.



In seawater BSM initial numbers of bacteria diminished rapidly (24-48 hrs) but small numbers of viable cells were detectable for up to 12 days post-inoculation. The shorter survival time reported for *A. salmonicida* in seawater BSM may be a consequence of an increased dissipation of metabolic energy. An increase in external sodium ions disturbs the cellular pH which then has to be maintained by the active uptake of H+ ions (Konings and Veldkamp, 1980).

The survival times observed here are within the range previously reported for *A. salmonicida* in fresh and seawater (reviewed by Austin and Austin, 1987).

In all cases, six days after viable counts had reached zero, bacterial cells could still be observed microscopically in water samples taken from microcosms (approximately 10^4/ml). These cells were observed either singly or aggregated in groups of *ca* 2-4 cells in contrast to Evenberg *et al.* (1985) who described aggregates of about 50 cells in a low ionic strength solution. In view of the limited clumping described here the source of error in using cfu as a method of enumeration of viable cells has been assumed to be limited.

2.3.2 Nutrient Resuscitation

i) Addition of nutrient broth

Nutrient broth was added to microcosms (riverwater, freshwater BSM and seawater BSM) following the procedure of Allen-Austin *et al.* (1984). In addition, nutrient was added to undiluted and serially diluted aliquots of the microcosms. Absence of cfu from these aliquots, after the addition of nutrient, would suggest that the reappearance of *A. salmonicida* in the microcosms may be due to the presence of a small number of viable cells too few to detect by the sampling procedure.

Addition of nutrient broth (to 0.1% TSB) to microcosms, after viable counts had reached zero, resulted in the reappearance of viable, culturable cells within 48 hrs at 22°C. In contrast, addition of nutrient to a concentration of 0.1% TSB to 1 ml aliquots of the microcosms only occasionally resulted in re-isolation of the pathogen and the bacterium was never detected in serial dilutions of microcosm aliquots.

ii) Resuscitation with Atlantic salmon serum and sucrose

Atlantic salmon serum and sucrose, both in combination and singly, were used as enhancement media for putative "dormant" cells. Microcosm aliquots (100 µl) were either spread on TSA containing serum/sucrose or incubated in serum/sucrose directly. In all cases, culturable, viable *A. salmonicida* could not be isolated from microcosms.

2.3.3 Experimental Infection

Fish were injected with aliquots from microcosms (riverwater or seawater BSM), after viable counts (MT212 or MT432) had reached zero, as it was thought that exposure to the natural host may provide stimulatory factors for the bacterial cells visible by light microscopy. Similar techniques have been used to resuscitate dormant ("somni-cells") enteric bacteria by inoculation into rabbit ileal loops (Roszak and Colwell, 1987a).

Atlantic salmon (*Salmo salar* L.) were injected with 0.1 ml aliquots of either strain MT212 or MT432 obtained from riverwater or seawater BSM microcosms six days after viable counts had reached zero. *A. salmonicida* could not be isolated from any fish even after a standard test for carrier infection. In contrast, for riverwater microcosms, aliquots containing viable bacteria (10^2 cfu) of either strain, both before and after nutrient resuscitation, produced clinical furunculosis within 1-2 weeks when injected

into fish. Similarly, for the seawater BSM microcosm, all fish (4/4) injected with culturable cells (10^2 cfu) of either strain died within seven days. These results are summarised in Table 2.2.

2.4 DISCUSSION

The present nutrient resuscitation and infectivity studies provide no positive evidence for the existence of "dormant" forms of *A. salmonicida* subsp. *salmonicida* in contrast to the previous report of Allen-Austin *et al.* (1984). These authors claimed that "dormant" *A. salmonicida* could give rise to culturable, fully viable cells after incubation in low levels of nutrient broth. In contrast, on the basis of the results presented here, the simplest interpretation is that the reappearance of the pathogen in the microcosms was, in fact, due to the presence of a few culturable bacteria in numbers too low to be detectable by the sampling methods employed. These culturable cells may be attached to the walls of the glass-flask microcosms. Some bacteria have been shown to exhibit increased hydrophobicity and adhesion to inanimate surfaces during starvation (Hermansson *et al.*, 1987). MacDonnell *et al.* (1984) have calculated that *ca* 17% of an inoculum of *Vibrio cholerae* may be expected to be lost from the culturable count in glass-flask microcosms through association with the glass walls.

The absence of "dormant" *A. salmonicida* in microcosms was further supported by the inability to isolate culturable bacteria after inoculation into Atlantic salmon, which was thought could provide stimulatory factors for these putative "dormant" pathogenic bacteria of fish.

TABLE 2.2

Experimental infection of Atlantic salmon (*Salmo salar* L.) with riverwater or seawater BSM microcosm aliquots

Treatment	Strain	Mortality (week post-injection)			Carrier test
		1	2	3	
Riverwater					
"dormant" cells					
(0 cfu)	MT212	0/6	0/6	-	-ve
(0 cfu)	MT432	0/6	0/6	-	-ve
Positive controls: "culturable" cells					
(670 cfu)	MT212	0/5	5/5	-	NT
(96 cfu)	MT432	0/5	5/5	-	NT
"resuscitated" cells					
(128 cfu)	MT212	2/2	-	-	NT
(293 cfu)	MT432	2/2	-	-	NT
Negative control: Saline	MT212	0/2	0/2	-	NT
	MT432	0/2	0/2	-	NT
Seawater BSM					
"dormant" cells					
(0 cfu)	MT212	0/6	0/6	0/6	-ve
(0 cfu)	MT432	0/5	0/5	0/5	-ve
Positive control: "culturable" cells					
(10^2 cfu)	MT212	4/4	-	-	NT
(10^2 cfu)	MT432	4/4	-	-	NT
Negative control: Saline	MT212	0/5	0/5	0/5	NT

Mortalities represented as fraction of deaths per group of fish
NT not tested
-ve negative

The presence of small numbers of undetected culturable, viable cells may also explain the *in vitro* (nutrient broth) and *in vivo* (rabbit ileal loops) resuscitation described for unculturable-but-viable ("somni-cells") enteric bacteria (Grimes *et al.*, 1986; Roszak and Colwell, 1987a). For example, Roszak *et al.* (1984) were able to resuscitate unculturable *Salmonella entiritidis* in sterile riverwater upon addition of heart-infusion broth but attempts to resuscitate cells in this manner after 21 days were unsuccessful. These results may also be explained by the presence of a few undetectable culturable (normal) cells which were finally depleted 21 days after viable counts had apparently reached zero. As for the Allen-Austin *et al.* (1984) study controls were not included to preclude this possibility. Similarly, no controls were included in rabbit ileal loop assays (Colwell *et al.*, 1985) for which the alternative explanation of undetected culturable cells cannot be excluded. In this latter study cells were concentrated by centrifugation (10^8 bacteria) before inoculation which would increase the possibility of including culturable cells in the inoculum.

Despite the criticism of the enteric pathogen "dormancy" studies above, additional evidence has been provided to support the existence of enteric "somni-cells". The "somni-cells" have shown positive responses (Grimes *et al.*, 1986; Roszak and Colwell, 1987a) with the so-called direct viable count of Kogure *et al.* (1979). In this method reproduction of bacterial cells is inhibited by nalidixic acid, which disrupts DNA synthesis, and nutrient is supplied with which other cellular constituents may be synthesised resulting in enlargement of viable cells. The direct viable count is dependent on the bacteria being sensitive to nalidixic acid and responsive to the added nutrient (Peele and Colwell, 1981; Hoff, 1989a). Recently, Roszak and Colwell (1987b) presented evidence, using a microautoradiographic method, which suggested that *ca* 90% of cells

positive by the direct viable count were metabolically active. The method of Kogure *et al.* (1979) has not been applied to *A. salmonicida* but could provide supporting evidence for, or against, "dormancy" in this pathogen.

Microbial death is a gradual process which may be confused with non-reproductive and dormant states. Furthermore, it is impossible to attribute death to loss of any cellular function (Mason *et al.*, 1986). Enumeration of bacteria by their ability to grow, ie viable counts on solid media or most-probable number methods, are considered to underestimate bacterial population sizes (eg Buck, 1979; Mason *et al.*, 1986). However, infectivity experiments described in this chapter suggest that cfu's should adequately describe the persistence of *A. salmonicida* in the aquatic environment.

CHAPTER 3

THE SURVIVAL OF *Aeromonas salmonicida* subsp. *salmonicida* THE SEAWATER

CHAPTER 3 THE SURVIVAL OF *Aeromonas salmonicida* subsp. *salmonicida* IN SEAWATER

3.1 INTRODUCTION

The ability of a pathogen to survive outside its host is an important determinant in the transmission of disease (Thrusfield, 1986). The survival of *A. salmonicida* in the external environment has been extensively studied (reviewed by McCarthy, 1977; Austin and Austin, 1987). However, survival was considered to be worthy of further investigation because previous studies have been limited in scope. For example, most studies have only considered survival of broth-cultured bacteria contained within glass-flask microcosms.

In this study several factors which may influence the survival of *A. salmonicida* were studied. Survival was monitored in alternative microcosm vessels (dialysis bags after McCarthy, 1977). In addition, the survival of the bacteria grown in nutrient broth or excreted by infected Atlantic salmon was compared. The survival of *A. salmonicida* in fresh and seawater may be extended in the presence of nutrients (Evelyn, 1971; Allen, 1982). In this study, the influence of the addition of nutrient to sterile basal salts medium (BSM) on the survival of the bacterium and its survival in waters obtained from beneath marine salmon cage farms, which may possess heightened nutrient loads, was assessed. Finally, the ability of the pathogen to survive in marine sediments, which has not been studied previously, was investigated. Some of the work presented here has been published (Rose *et al.*, 1990b).

The bacterium is not easily isolated from mixed-cultures and only one study (McCarthy, 1977) has utilised a selective medium to overcome this problem. McCarthy (1977) found

that *A. salmonicida* (initial inoculum 10^8 cfu) could be isolated for eight days in seawater at 11-13°C with an antibiotic containing selective medium. In the present study an antibiotic-containing selective medium was also used to isolate the bacterium from mixed-culture.

3.2 MATERIALS AND METHODS

3.2.1 Bacteria

Four strains of *A. salmonicida* subsp. *salmonicida* were used, MT212, MT432, MT393 and MT423. MT212, MT432 and MT212 have previously been described in Chapter 2 (2.2.1). The characteristics of MT423, which has been described by Adams *et al.* (1987) as 184/86, are presented in Table 3.1.

3.2.2 Antibiotic Sensitivity of *A. salmonicida* Strains

The antibiotic sensitivity patterns of MT212, MT432, MT393 and MT423 were determined with sensitivity discs (Masts Laboratory Ltd). Three colonies of *A. salmonicida* from a TSA plate were suspended in phosphate buffered saline (pH 7.2, Difco) to an optical density of one at 540 nm. A 10 µl loop of this suspension was spread on a TSA plate and sensitivity discs carefully placed on the resultant bacterial lawn. Plates were incubated at 22°C and zones of inhibition measured after 48 hrs. The antibiotic sensitivity patterns of the strains are given in Table 3.2.

3.2.3 Bacterial Inocula

Strains were grown overnight in 100ml 3%TSB shaken cultures at 22°C and then centrifuged for 5 min at 3,000 g. Cells were washed twice in sterile seawater, or basal salts media (BSM), and suspended to an optical density of one at 540 nm (equivalent to

TABLE 3.1

Characteristics of *A. salmonicida* strain MT423

Character	MT423	Character	MT423
Coccobaccilli	+	API20E (continued)	
Gram Stain	-		
Autoagglutination	+	IND	-
Brown, Diffusible		VP	-
Pigment	+	GEL	+
Motility	-	GLU	+
		MAN	+
API20E		INO	-
		SOR	-
ONPG	+	RHA	-
ADH	-	SAC	-
LDC	-	MEL	-
ODC	-	AMY	-
CIT	-	ARA	-
H2S	-	Oxidase	+
URE	-	OF/F (oxidative/	F
TDA	-	fermentative)	
		Gas from Glucose	+

\+ positive reaction
\- negative reaction
F fermentative

TABLE 3.2

Antibiotic sensitivity of *A. salmonicida* strains

Antibiotic	Concentration $\mu g\ ml^{-1}$	Sensitivity strain			
		MT432	MT423	MT212	MT393
Erythromycin	5	(+)	(+)	(+)	(+)
	15	(+)	(+)	+	(+)
Sulphadiazine	200	-	-	-	-
Trimethoprim	2.5	-	-*2	-	-
Oxolinic Acid	2	(+)*0.2	(+)	+	(+)*0.2
Oxytetracycline	25	-*2	(-)	-	-*2
Furazolidone	200	(+)	(+)	(+)	(+)

Strains were considered sensitive, +, if the zone of inhibition surrounding discs was >25 mm; partially sensitive <25 mm, (+); no zone of inhibition resistant, -. Antibiotics used in selective media indicated by asterisk followed by concentration in $\mu g\ ml^{-1}$

10^9 cfu ml^{-1}) and serially diluted 10-fold. This suspension (100 µl) was inoculated into experimental microcosms (150 ml screw-topped glass flasks or into *ca* 100 ml Visking dialysis bags) to give a final concentration of 10^4-10^5 cfu ml^{-1}.

Shed bacteria were obtained from a 100 g Atlantic salmon (*Salmo salar* L.) bath challenged with *A. salmonicida* (MT423) in a 50 l plastic tank for 12 hr. *A. salmonicida* was grown overnight, shaken, at 22°C in 3% TSB. The bacterium was washed in PBS and suspended to an optical density of one at 540 nm (= 10^9 cfu ml^{-1}). This suspension was added to seawater to give a challenge dose of 10^5 cfu ml^{-1}. Nine days after exposure the fish was placed into 2 l of seawater for one hr and then 100 ml aliquots used in survival studies.

3.2.4 Survival Microcosms and Sampling Regime

All studies were performed in microcosms containing 100 ml seawater. Sediment studies were performed in glass flasks containing 100 ml seawater and 6-10 g of marine sediment. Glass-flask microcosms were prepared as described in 2.2.2. These microcosms were wrapped in tin-foil and incubated at 15°C. Glass-flask microcosms were performed at least in duplicate with each flask sampled on triplicate agar plates. Dialysis bags were immersed in a plastic container through which seawater was allowed to flow. Survival studies in dialysis bags were performed by triplicate plating of one bag at each time interval.

Bacteria in non-sterile water (mixed-culture) were enumerated on antibiotic-containing tryptone soya agar (TSA, Oxoid) spread plates. The antibiotics used were based on the respective antibiotic sensitivity patterns of the strains investigated. These antibiotics, and their respective concentrations, are given in Table 3.2. Antibiotics were dissolved

in 10 ml phosphate buffered saline (PBS, pH 7.2, Difco), plus 100 µl dimethyl sulfoxide (Sigma) for oxolinic acid, before addition to cool (*ca* 40°C) TSA. These selective media gave similar results to TSA alone when used to isolate *A. salmonicida* inoculated into sterile PBS (see Table 3.3). *A. salmonicida* strains were grown on TSA at 22°C for 48 hr and suspended in PBS to about 10^3 cfu ml^{-1}. 100 µl was spread on TSA plates, with and without antibiotics, and incubated at 22°C for 48 hrs after which cfu were determined.

In survival studies, bacteria were enumerated on TSA, with or without antibiotics, on 100 µl or 1 ml aliquots diluted in sterile seawater where necessary. In sediment studies 100 µl aliquots were diluted in one or 10 ml filter sterilised seawater, which was then vortexed for 5 sec, and 100 µl was spread on agar plates. Total viable heterotrophic bacterial counts were performed on marine agar (Difco). Plates were air-dried in a sterile flow cabinet and then incubated at 22°C. Survival values shown in figures are expressed as the natural logarithm (Ln) of the median of cfu's per ml from replicate samples. Presumptive identification of *A. salmonicida* was based on cream coloured, convex, friable colonies which produced brown pigment. Representative colonies isolated from mixed-culture were subcultured and identification confirmed by biochemical analysis (API20E).

3.2.5 Water and Sediment Samples

Seawater samples were obtained from 1 m beneath commercial salmon cages (Marine Harvest Ltd, Loch Ewe, Scotland) using a standard water sampler (Nansen bottle) and transported in 500 ml sterile glass-flask. These samples were held at ambient temperature and used within 24 hr of collection. Water samples were either left untreated (mixed-cultures in non-sterile seawater), autoclaved at 121°C, 15 psi for 15 min; or filter-sterilised (0.22 µm Millex, Millipore). Seawater was also obtained from the

TABLE 3.3

Comparison of TSA and antibiotic selective media for isolation of *A. salmonicida*

Antibiotic selective medium (in TSA)	*A. salmonicida* strain		
	MT432	MT423	MT393
no antibiotics	261 ± 15	74 ± 13	118 ± 7
0.2 μg ml^{-1} oxolinic acid and 2 μg ml^{-1} oxytetracycline	261 ± 19	-	91 ± 8
2 μg ml^{-1} oxytetracycline	213 ± 12	-	-
2 μg ml^{-1} trimethoprim	-	40 ± 12	-

Results presented as $\bar{x}$ (± SD) of cfu's from four agar plates

recirculating seawater aquarium at the DAFS Marine Laboratory, Aberdeen and Institute of Aquaculture, University of Stirling. The seawater basal salts medium (BSM) was prepared as described in Chapter 2 (2.2.3). TSB was added to BSM to give a range of nutrient concentrations.

Marine sediment was kindly collected by Dr J Overnell (Scottish Marine Biological Association, Dunnstaffnage) on 30 May 1989 from below commercial Atlantic salmon marine cages (Golden Sea Produce Ltd, Loch Creran, Scotland). Samples were collected using a ship-borne coring apparatus. These samples were stored at 4°C and used within three days of collection.

3.2.6 Experimental Infection

After viable counts had reached zero (strain MT432) aliquots (0.1 ml) were inoculated intramuscularly (i.m.) into five 25-30 g seawater-acclimated Atlantic salmon (*Salmo salar* L.). Fish were anaesthetised with benzocaine before injection as described in Chapter 2 (2.2.6). Fish were maintained in 20 l plastic tank (13-14°C) for 14 days and survivors were then carrier tested (2.2.7) following the protocol of McCarthy (1977). Virulence of the strain was confirmed by injecting non-sterile seawater containing culturable *A. salmonicida* (10^4 cfu) i.m. into three Atlantic salmon which resulted in specific mortalities (3/3) within seven days.

3.3 RESULTS

3.3.1 Survival in Different Microcosm Vessels

The survival of broth-cultured *A. salmonicida* strain MT432 was monitored in 100 ml non-sterile seawater (pH 7.9; salinity 3.3%), obtained from the DAFS Marine Laboratory,

Fig 3.1: Survival of A.salmonicida MT432 in Glass-flask and Dialysis Bag Seawater Microcosms.

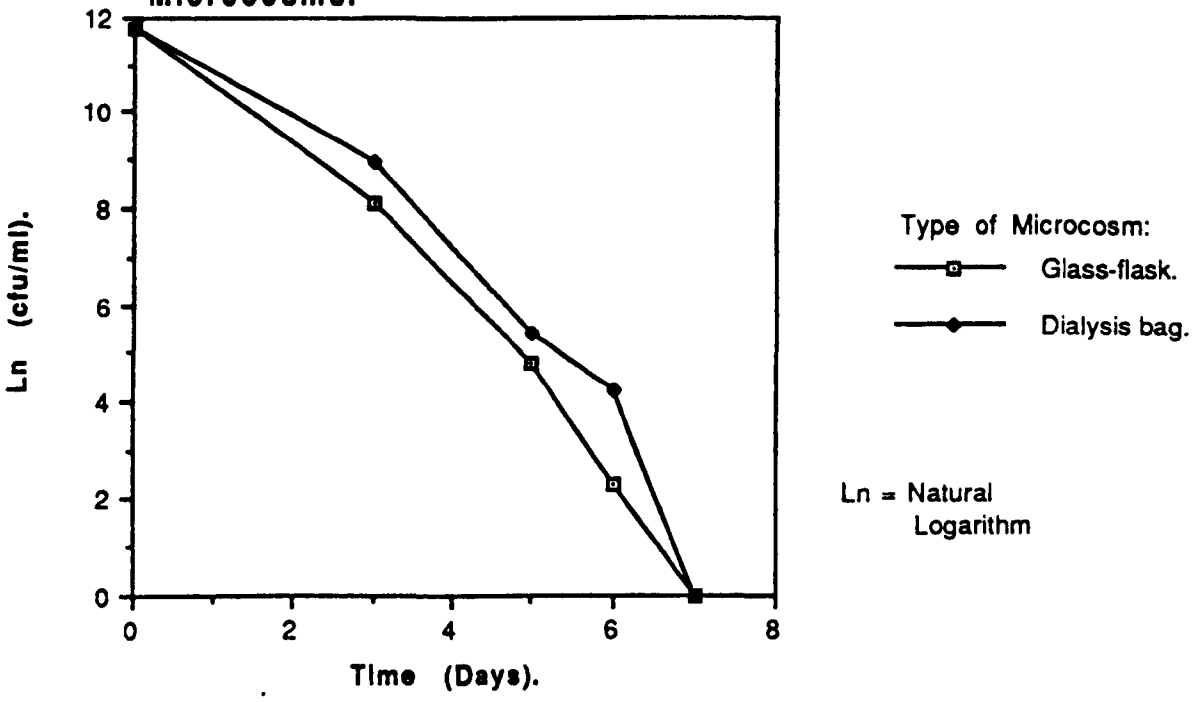
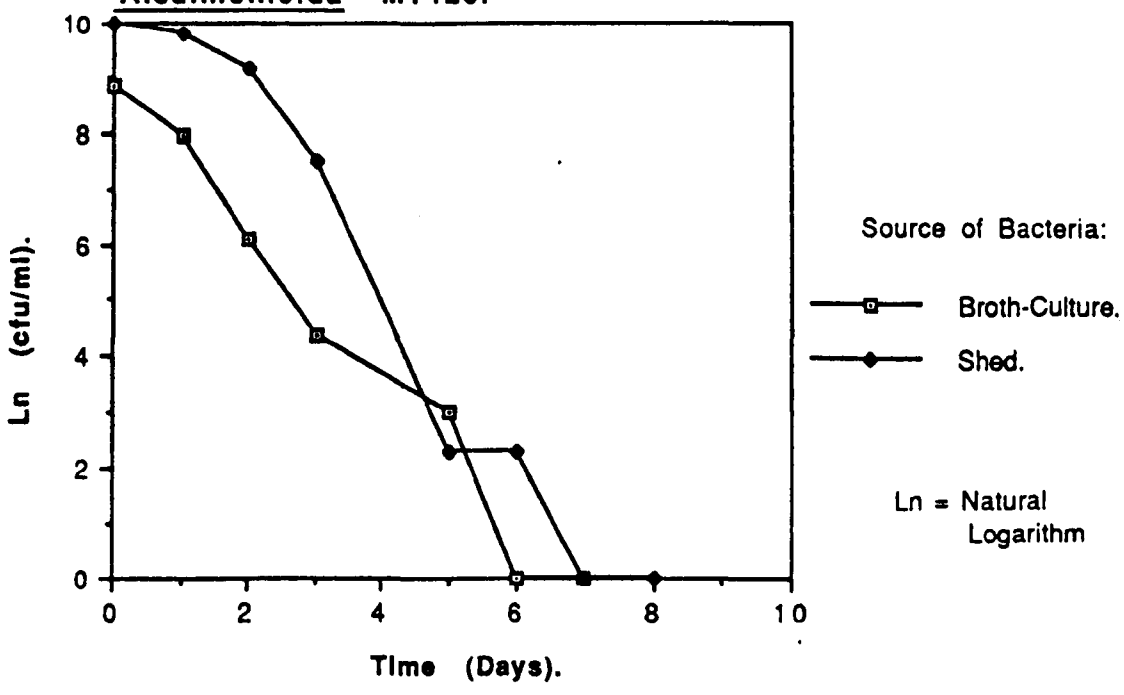


Fig 3.2: Survival of Shed and Cultured A.salmonicida MT423.



contained either within 150 ml glass-flasks or dialysis bags at 14°C. The pathogen was isolated on antibiotic-containing selective medium for up to seven days and no significant differences were observed between the two types of microcosm (Fig. 3.1). Total heterotrophic viable bacteria, enumerated on marine agar, varied from 33,500-137,000 cfu ml^{-1} throughout the experimental period.

3.3.2 Survival of Shed (*In Vivo*) and Broth-Cultured (*In Vitro*) Bacteria

A. salmonicida strain MT423, either broth-grown or obtained from infected fish was inoculated into dialysis bags containing 100 ml non-sterile seawater (pH 7.9; salinity 3.1%, 14°C) obtained from the DAFS Marine Laboratory. No differences in survival were observed between bacteria of different culture histories. An inoculum of 10^3 cfu ml^{-1} of broth-grown bacteria could no longer be detected on antibiotic-containing agar after five days and 10^4 cfu ml^{-1} of shed bacteria were undetectable after six days (Fig. 3.2). Total heterotrophic bacteria varied between 1,000-645,000 cfu ml^{-1}.

3.3.3 Survival in Sterile Seawater and the Effects of Nutrients

a) BSM with nutrient

Survival of *A. salmonicida* strain MT393 was monitored in BSM containing graded levels of nutrient, 0.01 μg TSB ml^{-1}-100 μg TSB ml^{-1} (Nutrient levels are expressed as the dry weight of TSB in 1 ml of BSM). Autoclaved BSM (10 ml) plus nutrient was inoculated with 100 μl of an *A. salmonicida* suspension in BSM (OD 540 nm = 1) and incubated at 15°C. Samples (100 μl) were taken from microcosms every week for seven weeks and spread on TSA. The bacterium could not be isolated from control BSM, without nutrient, or in BSM+ 0.01 μg TSB ml^{-1} after seven days of incubation. However, survival increased from 2-7 weeks with increasing levels of nutrient (0.1 μg TSB ml^{-1}-10 μg TSB ml^{-1})

Fig 3.3: Survival of A.salmonicida in Sterile BSM + Nutrient.

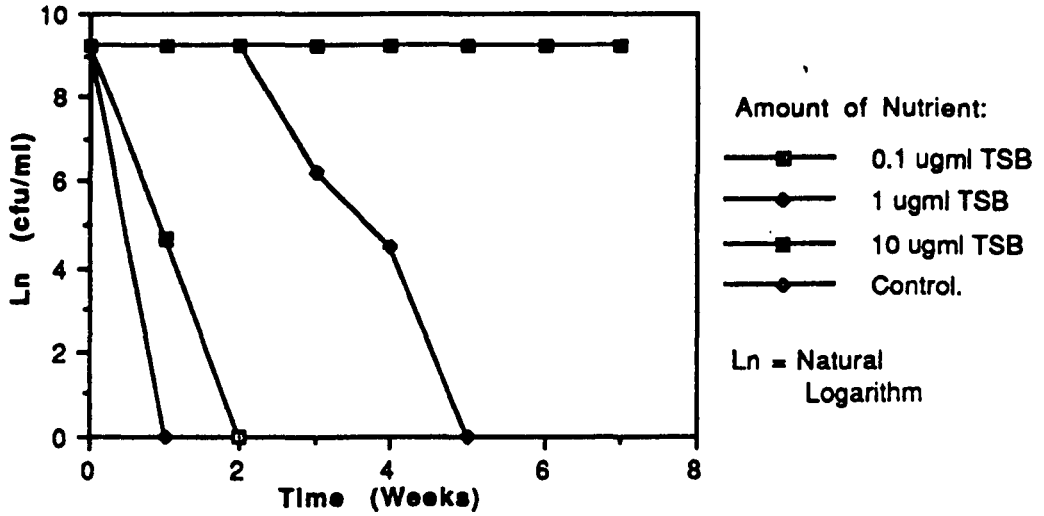
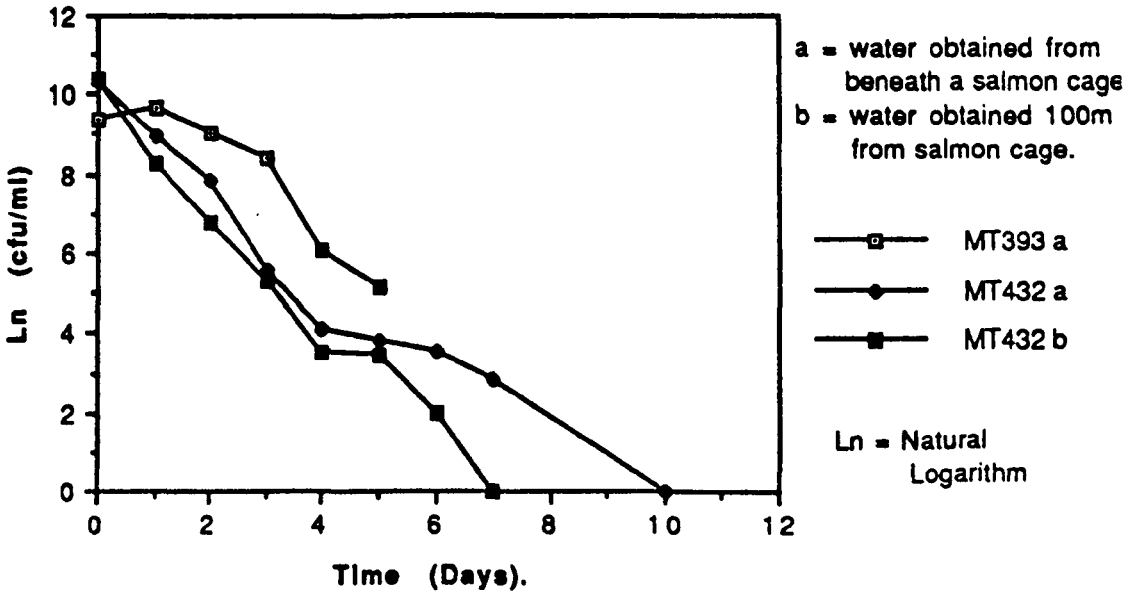


Fig 3.4 Survival of A.salmonicida in Filter-Sterilised Seawater.



(Fig. 3.3). Reproductive growth of the pathogen, as indicated by development of turbidity and bacterial lawns on TSA spread plates, was observed in BSM containing at least 10 μg TSB ml^{-1}. The experiment was terminated after seven weeks.

b) Cage water (autoclaved or filter-sterilised)

Survival of two strains, MT393 and MT432, was monitored in autoclaved or filter-sterilised seawater obtained from a commercial salmon farm (Marine Harvest Ltd). Strain MT393 was isolated for up to five days in filter-sterilised water obtained during winter (sea temperature 7°C, salinity 3.3%, pH 8.1). Survival of strain MT432 was investigated in filter-sterilised water obtained during summer (sea temperature 13°C, salinity 3.3%, pH 8.0) and the survival was found to be slightly extended, 7 and 10 days in water obtained 100 m from cage and beneath cage respectively (Fig. 3.4).

The pathogen could be isolated for only 24-96 hrs in autoclaved water (salinity 3.4%, pH 8.5).

3.3.4 Survival in Non-sterile Cage Water

The survival of strains MT393 and MT432 was similarly monitored in non-sterile seawater obtained from the commercial site during winter and summer. The strains could only be isolated for 3-4 days (Fig. 3.5). Total heterotrophic counts, enumerated on marine agar, varied from 2,700-32,400 cfu ml^{-1} in experimental microcosms.

3.3.5 Survival in Marine Sediment from Beneath a Salmon Cage

The survival of *A. salmonicida* strain MT432 was monitored in non-sterile marine sediment obtained either immediately beneath an established salmon cage or 10m distant (approximately 1/3 between two cages).

Fig 3.5 Survival of A.salmonicida in Non-Sterile Seawater.

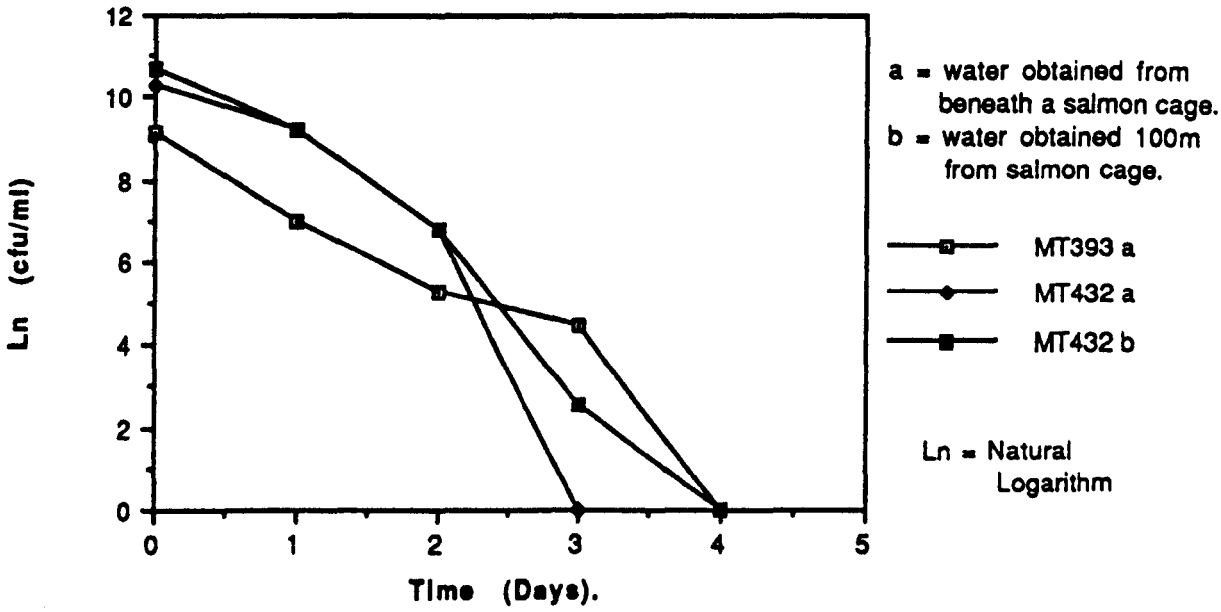
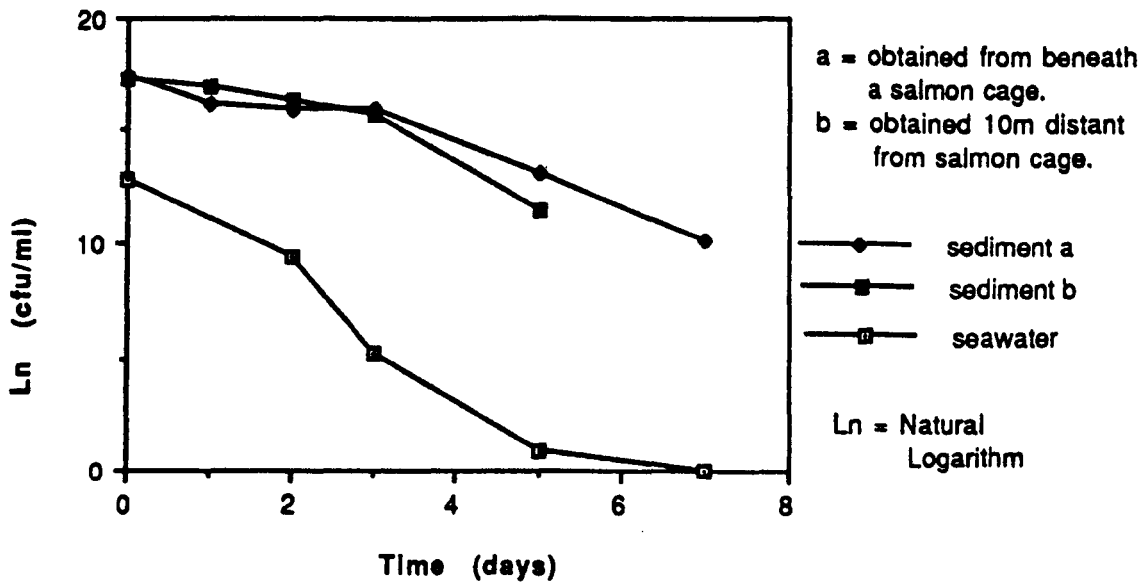


Fig 3.6: Survival of A.salmonicida MT 432 in seawater and farm sediment.



The sediment from beneath a cage was brown/black, flocculent and was covered with a white fibrous mat, possibly the bacterium *Beggiatoa* spp. There were remains of food on the surface of the sediment. The other sediment was brown, becoming grey/black at 2 cm depth, flocculent and contained annelid worms. The sediments had an *in situ* temperature of 11°C. Microcosms consisted of 6-10 g sediment plus 100 ml non-sterile seawater obtained from the Institute of Aquaculture, University of Stirling. Microcosms were inoculated with *A. salmonicida* (10^7 cfu ml^{-1}), wrapped in tin-foil and statically incubated at 15°C in the dark. Before sampling, microcosms were shaken to suspend the sediment.

The detection limit of the selective medium (TSA + oxytetracycline) was found to be only 10^4 cfu ml^{-1} and below this level the experiment had to be terminated. This level of detection was considered to be a consequence of contaminating bacteria, resistant to the antibiotic and considered to be *Pseudomonas* spp., which overgrew *A. salmonicida* below 10^4 cfu ml^{-1}. Nevertheless, using 10^7 cfu ml^{-1} inoculated into the microcosms *A. salmonicida* could be isolated for five days (farm sediment) and seven days (sediment 10 m distant) (Fig. 3.6). For comparison, survival in non-sterile seawater of MT432 was assessed at the same time and found to be seven days. Total heterotrophic viable counts varied as follows: cage sediment 2.27×10^7-4.28×10^8 cfu ml^{-1}, adjacent (10 m) sediment 3.1×10^7-4.7×10^8 cfu ml^{-1}, seawater 4.7×10^5-4.85×10^5 cfu ml^{-1}.

3.3.6 Experimental Infection

Injection i.m. of aliquots of strain MT432 from microcosms containing non-sterile seawater from salmon cages six days after viable counts had reached zero did not produce clinical disease in five Atlantic salmon and *A. salmonicida* could not be isolated from the kidney of these experimental fish following carrier testing for covert infection. In

comparison, fish (n = 3) injected with non-sterile seawater containing viable *A. salmonicida* (10^4 cfu) resulted in mortalities within seven days confirming that strain MT432 was virulent.

These negative infectivity results support the use of cfu to monitor the survival of this bacterial pathogen and provide further evidence for the conclusions of Chapter 2.

3.4 DISCUSSION

The survival studies reported here do not conflict with the description of *A. salmonicida* subsp. *salmonicida* as an obligate pathogen with a limited ability to survive in the external aquatic environment (Popoff, 1984). The survival values described in this chapter, for both sterile and non-sterile seawater, are within the range of previously published values (Austin and Austin, 1987). A number of factors which could potentially influence survival were investigated in the present study but were not found to be of major importance. These were type of microcosm, past nutritional history, competition and nutrient availability. These factors are further discussed below:

Microcosms are a simplification of the natural environment (Trevors, 1988) and extrapolation from them must be made with care. The enclosure of small volumes of water may effect resident bacterial populations (Jones, 1973). For example, Buck (1978) found that the yeast *Candida albicans* died off rapidly when introduced into dialysis bag microcosms compared with glass-flask or plexiglass chambers closed with Nucleopore membranes. To overcome this drawback survival studies on microorganisms have been performed in a variety of microcosm systems eg membrane filter chambers (McFeters

and Stuart, 1972) or dialysis bags (Slanetz and Bartley, 1965). In this study the survival of *A. salmonicida* in non-sterile (mixed culture) seawater was compared in glass-flask and dialysis bag microcosms and was found to be of similar, short, duration in each.

The influence of the past nutritional environment of *A. salmonicida* on its ability to survive was studied. McCarthy (1983) has presented evidence which suggests that the outcome of infectivity experiments (bath challenges) with *A. salmonicida* are influenced by the prior culture conditions of the pathogen. The environment can have an important influence on the chemical composition of bacteria (Herbert, 1961) and their ability to persist in microcosms. Populations grown under conditions more closely approximating the natural aquatic environment, eg low temperature, submaximal growth rate caused by nutrient limitation, are more resistant to antibacterial agents, including seawater (Harakeh *et al.*, 1985; Moyer and Morita, 1989). Similarly, the ability of *Escherichia coli* to survive in nutrient-free seawater has been shown to be extended if grown in a medium with increased osmotic pressure due to sodium chloride, lithium chloride or saccharose (Gauthier *et al.*, 1987; Munro *et al.*, 1989). The stage at which growing cells are harvested also appears to influence survival. For example, Gauthier *et al.* (1989) found that survival of *E. coli* was reduced if exposed to seawater during the early exponential phase of growth.

In these studies on *A. salmonicida* the survival ability of bacteria grown in nutrient broth and *in vivo*, released from infected Atlantic salmon, were compared in dialysis bags containing non-sterile (mixed culture) seawater. Growth of the pathogen in host fish could be expected to provide the optimum conditions for its culture and perhaps enable it to survive longer in the external environment. The survival of *A. salmonicida*

released from infected fish has not been examined previously. Surprisingly, in view of studies described above, the pathogen had the same survival times independent of source (ie broth culture or by passage through fish).

The presence of organic matter has a protective influence on the ability of enteric bacteria to survive in marine water (Singleton *et al.*, 1982; Munro *et al.*, 1989). For example, Venkateswaran *et al.* (1989) suggested that high amounts of nutrients in the water column increased the survival of *Salmonella* spp. in saline waters because a higher incidence of serotypes were isolated in the polluted Fukuyama port than in clean marine waters. More direct evidence has been provided by Lopez-Torres *et al.* (1988) who found that *Escherichia coli*, in *in situ* membrane diffusion chambers, could overcome the bactericidal effect of tropical marine waters when sufficient organic loading was present. Similarly, in studies reported here for sterile BSM with varied levels of nutrients, survival of *A. salmonicida* was prolonged. High levels of nutrient resulted in reproductive growth of the pathogen. This supports earlier work by Evelyn (1971) and Allen (1982) who reported that addition of nutrient to sterile seawater or freshwater respectively could increase the survival of this bacterium.

On the basis of the results obtained with BSM plus nutrients it was considered that survival in seawater obtained from commercial marine salmon cages was worthy of study. Organic enrichment is often found at commercial cage sites (Phillips *et al.*, 1985; Gowen and Bradbury, 1987). For example, Enell (1986), reporting on the environmental impact of cages in a freshwater lake, found that the nutrient loading of nitrogen and phosphorous was 86-91 kg and 12-13 kg respectively per year per tonne of produced fish. It, therefore, was important to examine whether this level of nutrient input may influence the survival of the pathogen in the marine environment and thereby the

potential for disease spread. However, survival in both non-sterile and sterile seawater, obtained from beneath a cage at two different times (summer and winter) of the year, did not extend survival of *A. salmonicida* enumerated by cfu. This may indicate that nutrients at fish farms are unavailable to the pathogen; are rapidly dispersed; and/or are released at insignificant levels for the bacterium.

McCarthy (1977) has argued that studies of *A. salmonicida* under sterile conditions are of limited value because the competitive effect of natural bacterial communities are absent. However, studies in sterile media may provide the maximum survival values for the pathogen and as such may be useful in the evaluation of disease spread. In the present study survival was extended in sterile seawater, compared with non-sterile. The reduced survival of *A. salmonicida* in non-sterile seawater suggests that bacteria present in the latter exerted a competitive effect and is consistent with previous studies showing the poor competitive ability of the pathogen (Cornick *et al.*, 1969, Dubois-Darnaudpeys, 1977 ; Michel and Dubois-Darnaudpeys, 1980). However, in this study, the method of sterilisation appears important. There was a reduction in survival time in autoclaved seawater compared with filter-sterilised seawater, non-sterile seawater and even nutrient-free BSM. Autoclaving appears to have rendered the water somewhat toxic to the bacterium.

In this study *A. salmonicida* was isolated from mixed culture (non-sterile conditions) with an antibiotic-containing selective medium. It is important to note that Henis *et al.* (1989) found that *Escherichia coli* inoculated into lake water often failed to grow on antibiotic amended eosin-methylene blue agar (EMB) compared with half-strength TSA. The inability of *E. coli* to grow on EMB was attributed to "bacterial stress". Similar effects cannot be ruled out in the present study.

The survival of bacteria has been shown to be extended in sediments (Perez-Rosas *et al.*, 1988). For example, Gerba and McLeod (1976) found that *Escherichia coli* survived longer in glass-flask microcosms containing unsterile natural seawater supplemented with sediment than with seawater alone. In part extended survival of bacteria in sediments in microcosms is due to the greater surface area available (Faust *et al.*, 1975). Nutrients (Kjelleberg *et al.*, 1982) and bacteria (Marshall, 1985) tend to accumulate at interfaces and particles are important microniches of organic matter (2.9 g organic matter per litre in surface microlayers, Norkrans, 1980) allowing bacteria to persist which would not otherwise (Kirchman and Ducklow, 1987). In this respect, Sakai (1986) has shown that *A. salmonicida* survives longer in riverwater microcosms containing sand particles and Michel and Dubois-Darnaudpeys (1980) found that two strains of the pathogen could survive in autoclaved river sediment for at least nine months although virulence steadily declined after six months. McCarthy (1977) was able to isolate *A. salmonicida* (initial inoculum 10^9 cfu ml^{-1}) from dialysis bags containing either water or mud/detritus for 29 and 10 days respectively at which time 10^5-10^6 cfu ml^{-1} were present. The experiment had to be prematurely terminated because of decomposition of the dialysis bags. Sakai (1986) considers that the pathogen persists in river sediments but in a considerably starved form. Austin (1987) has reported the presence of *A. salmonicida* cells, detected by immunofluorescence, on river particles. Smith *et al.* (1982) found that the pathogen could persist at fallow marine cages for up to 6 months and suggested that the bacterium may have survived in sediments.

The survival of *A. salmonicida* in marine sediment has not previously been studied. In this study, survival was assessed in accumulated sediments obtained from beneath a commercial salmon cage farm and in sediments adjacent (10 m) to a cage. The presence of putative *Beggiatoa* spp. on the surface of the sediment suggest that the bottom

water/sediment was anoxic and hydrogen sulphide may have been present (Earll *et al.*, 1984). Thus the sediment microcosms used here, which were statically incubated, may have been anaerobic, adversely influencing the survival of the pathogen. Pfeiffer *et al.* (1988) found that the survival of *E. coli* in sediment/lakewater microcosms was dependent on redox conditions. Nevertheless, in this study *A. salmonicida* could be isolated for 5-7 days at which time *ca* 10^4 cfu ml^{-1} were present and aerobic heterotrophic bacteria were detectable, at similar levels throughout the experimental period, with marine agar. Assuming that the "die-off" of the pathogen remained constant below this level then, by extrapolation, the results indicate its survival in these sediments is limited (*ca* 10-14 days). It would, however, be useful to repeat these experiments with an alternative means of detection, eg immunofluorescence, and in semi-open microcosms, eg seawater/sediment aquaria which may be aerated.

Recently, King and Shotts (1988) have reported increased survival, *in vitro*, of both *Edwardsiella tarda* and *A. salmonicida* in the presence of the protozoan *Tetrahymena pyriformis*. Similar persistence in the presence of bacterivorous ciliates has been described for other bacteria (Sambanis and Fredrickson, 1988). Huq *et al.* (1983) have shown that *Vibrio cholerae* associate with planktonic crustacean copepods. *In vitro* survival studies with this bacterium in the presence of copepods (Huq *et al.*, 1984) indicated that the zooplankton may significantly contribute to the survival and distribution of *V. cholerae* in the aquatic environment. Colonisation of copepods may also represent the first stage in concentration of cells, which are normally at low levels in suspension, needed to constitute an infective dose to initiate disease in human populations. The potential role of vectors in the transmission of *A. salmonicida* are further discussed in Chapter 5. Future work on the persistence and dispersion of *A. salmonicida* may profitably be conducted on these novel means of survival.

This study has confirmed that *A. salmonicida* has a limited potential for survival in the marine environment. Nevertheless, anecdotal information continues to accumulate that it may rapidly spread between fish cages at a sea site and even between sites at some considerable distance apart (Novotny, 1975, 1978; Munro, 1988). The reasons for this spread, despite these observations on survival, may be more connected with the high levels of shedding from infected fish (Rose *et al.*, 1989b) and by hydrographic conditions such as rapid water movements combined with limited dispersion (Turrell and Munro, 1988). These factors are considered in more depth in Chapter 5. Alternatively, the movement of infected wild and feral (escapees from cage sites) fish between sites (Phillips *et al.*, 1985) may promote disease spread. In this respect, Munro and Waddell (1984) have reported that disease occurrences are increased at freshwater sites in contact with wild migrating anadromous populations of salmon.

Although the use of *in vitro* microcosms for assessment of *A. salmonicida* survival is convenient, extrapolation to natural conditions must be made with reservations. The aquatic environment is subjected to large spatial and temporal nutrient fluxes and adjacent microhabitats may be very heterogenous (Roszak and Colwell, 1987a) which cannot be modelled in simple microcosms. The decline of non-indigenous bacterial populations on introduction into a model system appears to be a general phenomenon (Strauss *et al.*, 1984). Several authors (Liang *et al.*, 1982) have interpreted this to indicate microbial communities resist invasions by introduced exogenous organisms. However, although an organism may not be established in a microcosm, it does not follow that it will not be established in a natural ecosystem that has a greater degree of physical and biological heterogeneity (Suter, 1985). These considerations regarding microcosm studies suggest that the best method of assessing the survival of *A. salmonicida* can be achieved by monitoring its persistence in the natural environment.

Although the experimental infections described in this chapter and in Chapter 2 validate cfu methodology in monitoring the survival of *A. salmonicida* in seawater there are no reports of its isolation from the aquatic environment even during disease outbreaks (Cornick *et al.*, 1969; Allen *et al.*, 1983) apart from Plehn (cited by Williamson, 1928) and Scallan (1983) both of whom isolated the pathogen from freshwater. This indicates that the technique is insensitive for accurately monitoring the spread of *A. salmonicida* in the environment and future work may profitably be directed at developing more sensitive detection methods, such as DNA probes (Chapter 7).

CHAPTER 4

THE INFECTIVITY OF *A. salmonicida* subsp. *salmonicida* IN SEAWATER

CHAPTER 4 THE INFECTIVITY OF *A. salmonicida* subsp. *salmonicida* IN SEAWATER

4.1 INTRODUCTION

The mode of transmission, and infectivity (ie number of bacteria required to initiate infection), of a pathogen are important factors in its epidemiology (Thrusfield, 1986). *Aeromonas salmonicida* subsp. *salmonicida* may be carried from fresh to seawater by transmission of covertly infected (carrier) fish (Novotny, 1978; Ezura *et al.*, 1984). Lateral transmission between fish in seawater is believed to occur (Scott, 1968; Smith *et al.*, 1982) but it's infectivity has not been investigated. The infectivity of *A. salmonicida* in seawater was considered worthy of study because it may differ significantly from that recorded for freshwater (Adams *et al.*, 1987). For instance, the limited ability of *A. salmonicida* to survive in the marine environment (Chapter 3) suggests that its infectivity may be reduced in seawater. Some of the work presented in this chapter has been published previously (Rose *et al.*, 1989a).

The transmission of *A. salmonicida* is believed to occur via a water-borne route (McCarthy, 1977). A number of workers (Bullock *et al.*, 1976; Cipriano, 1982; McCarthy, 1983; Adams *et al.*, 1987) have reported infection of fish in freshwater using relatively high numbers, ie 10^4-10^9 cfu ml^{-1} (Bullock *et al.*, 1976; Cipriano, 1982; McCarthy, 1983), of bacteria suspended in tank water (ie bath challenge). It is likely that in the natural environment, including fish farms, fish are continuously exposed to lower levels of the pathogen than in these challenge experiments, due to dilution and dispersion of the bacterium in large bodies of water. For this reason, exposure of fish to low levels of *A. salmonicida* was studied here.

The infectivity of *A. salmonicida* subsp. *salmonicida* to Atlantic salmon (*Salmo salar* L.) was investigated in seawater. A challenge dose of 10^2-10^5 cfu ml^{-1} over a 1-3 day period was examined. In addition the effect of continuous exposure of fish to 10^2 cfu ml^{-1} over a 1-3 week period was investigated.

Several workers (Blake and Clarke, 1931; Klontz and Wood, 1972; Sakai, 1979) have reported that *A. salmonicida* can cause infection in salmonids by ingestion. To assess the infectivity of the pathogen via this route, furuncle material (ie the necrotic lesion) from experimentally infected fish was serially diluted and intragastrically intubated into Atlantic salmon. This is the first time that the infectivity, by ingestion, of *A. salmonicida* in furuncle material has been examined.

4.2 MATERIALS AND METHODS

4.2.1 Bacteria

Aeromonas salmonicida subsp. *salmonicida* strain MT423 (strain 184/86 of Adams *et al.*, 1987) was obtained from the Aquatic Vaccine Unit, Institute of Aquaculture. This strain is described in 3.2.1. The antibiotic resistance pattern of the strain is given in Table 3.2.

4.2.2 Fish

Atlantic salmon, 20-32 g and 70-115 g, were obtained from the DAFS Marine Laboratory's own furunculosis-free site and held in tanks supplied with a continuous flow of seawater (3-4 l min^{-1}). Absence of *A. salmonicida* in this population of fish was confirmed by carrier test following the protocol described in 2.2.7. Challenges were performed on groups of 8-10 fish in 80l plastic tanks at 11-14°C.

4.2.3 Bath Challenge

Prior to challenge, the strain was grown in tryptone soya broth (Oxoid) at 22°C overnight for short exposure regimes (1-3 days). Cells were harvested by centrifugation at 3,000 g for 5 min and washed in physiological phosphate buffered saline (PBS pH 7.2, Difco). Bacteria for extended challenges (1-3 weeks) were cultured on tryptone soya agar (TSA, Oxoid) for 48 hr at 22°C. Bacteria were suspended to 10^9 cfu ml^{-1} in PBS (optical density at 540 nm = 1), serially diluted to obtain the required challenge dose and cfu were then confirmed on TSA plates. Throughout the challenge period, which was overnight (*ca* 12hr/day) water flow was discontinued and tanks aerated. Fish were exposed to 10^2-10^5 cfu ml^{-1} of bacteria on each day of challenge. Fish were monitored for up to 19 days post-challenge and surviving fish were then carrier tested. Kidney inocula were prepared from all dead fish on TSA plates to provide presumptive evidence for the presence of *A. salmonicida.*

4.2.4 Intragastric Challenge

Furuncle material (wet weight 0.4 g) was obtained from an Atlantic salmon (35 g) injected intramuscularly (i.m.) with 10^5 cfu ml^{-1} of broth-grown (TSB) *A. salmonicida.* The number of *A. salmonicida* present in the furuncle was estimated by serially diluting in sterile PBS and found to be approximately 10^{10} cfu g^{-1}. Atlantic salmon, eight fish per dose, were anaesthetised with benzocaine (Sigma), as described in 2.2.6, and 10-fold dilutions of furuncle material in PBS were intubated (dose range 10^1-10^7 cfu). A soft silicone plastic tube (Altec, Alton, Hampshire, UK), diameter 3 mm, attached to a syringe, was pushed 3-5 cm into the oesophagus so that the end of the tube was positioned in the stomach and 0.1 ml furuncle suspension was then inoculated. Mortalities were monitored for 14 days and surviving fish were carrier tested.

4.2.5 Detection of *A. salmonicida* in Tank Water

Release of *A. salmonicida* from groups of fish infected via a bath-challenge were monitored. 100 µl aliquots of seawater were removed from tanks and spread on a selective medium (TSA) containing antibiotics. Plates were incubated at 15°C for 24 hr and then transferred to 22°C for 48 hr prior to enumeration of cfu. The selective medium was based on the antibiotic sensitivity pattern of strain MT423 and comprised trimethoprim (Sigma) 2 µg ml^{-1} TSA. The antibiotic was added to cool (40°C) TSA as a suspension in PBS. This medium yielded viable counts similar to TSA alone when used to isolate *A. salmonicida* strain MT423 inoculated into sterile PBS (see Table 3.3).

4.3 RESULTS

4.3.1 Short-duration Bath Challenge

Fish (20-32 g) were exposed to 10^2-10^5 cfu ml^{-1} of *A. salmonicida* over a challenge period of 1-3 days. Mortalities were monitored for 19 days post-challenge. The exposure regime and resultant mortalities for these bath-challenge experiments are summarised in Table 4.1. No mortalities were recorded at a bacterial dose of 3 x 10^3 cfu ml^{-1} day^{-1} or below, and no covertly infected (carrier fish) were found amongst the survivors after injection with prednisalone acetate and heat stress. Specific mortalities were observed when the bacterial dose was at least 3 x 10^4 cfu ml^{-1} day^{-1}, but no dose-dependent mortality, both in terms of total percentage mortality (Fig. 4.1) or mean time to death (Fig. 4.2), was evident over the 19 day monitoring period. Initial mortalities occurred 8-9 days post-exposure in all groups. The cumulative mortalities for each group are presented in Figures 4.3 and 4.4. Approximately 6% of fish that died developed typical furuncle lesions along their flanks before death in these challenges. *A. salmonicida* was isolated from the kidney of all specific mortalities. Atlantic salmon (4.5-9.5 g) in

TABLE 4.1

Short-duration bath exposure of Atlantic salmon (20-32 g) to *A. salmonicida* (MT423) in seawater

Duration of exposure (days)	Bacterial dose (cfu ml^{-1} day^{-1})	Cumulative dose cfu ml^{-1}	No fish	Mortality (%)	Mean time to death (days)	Carrier test
1	3×10^4	3×10^4	10	90	16 (9-19)	-ve
2	3×10^4	6×10^4	10	0	-	-ve
3	3×10^4	9×10^4	10	70	12(8-19)	-ve
1	3×10^5	3×10^5	10	80	16(12-19)	-ve
2	3×10^5	6×10^5	10	70	13(9-19)	-ve
3	3×10^5	9×10^5	10	50	13(8-19)	-ve
1 (freshwater)	3×10^5	3×10^5	7	57	11(7-14)	NT
control (seawater)	-	-	10	0	-	-ve

Carrier test: -ve, *A. salmonicida* not isolated; NT, not tested

No mortalities were recorded in batches of 10 fish exposed to 3×10^2 or 3×10^3 cfu ml^{-1} day^{-1} for 1-3 days

Time to death measured from onset of exposure, range in brackets

Fig 4.1 Dose-Mortality Response for Short-Duration Challenge.

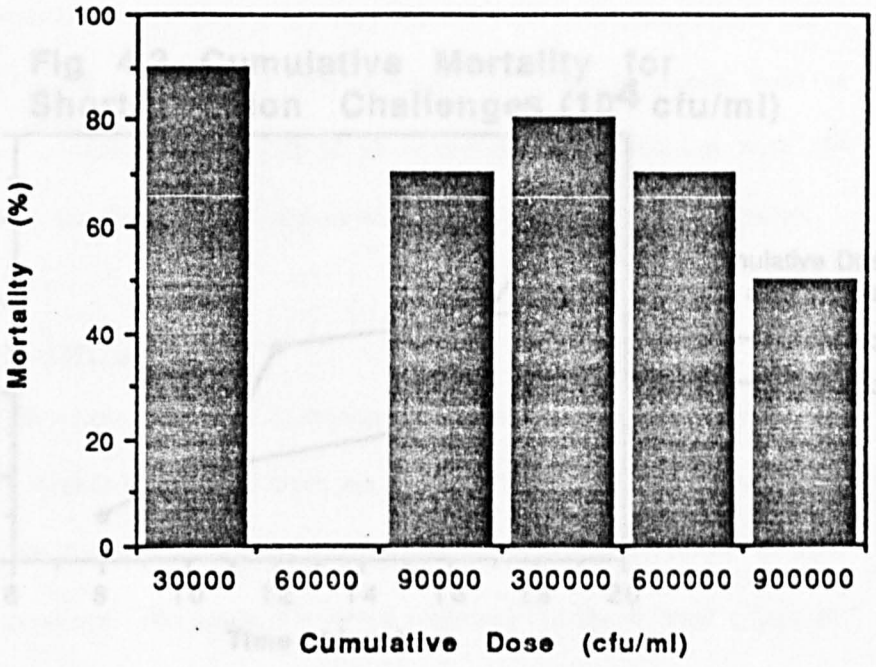


Fig 4.2 Mean Time to Death For Short-Duration Challenge.

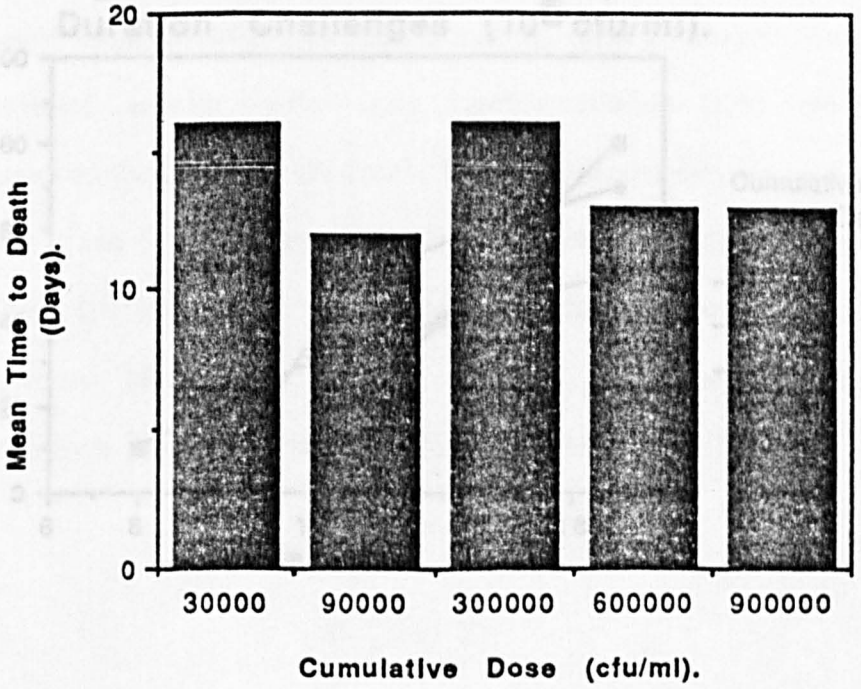


Fig 4.3 Cumulative Mortality for Short-Duration Challenges (10^4 cfu/ml)

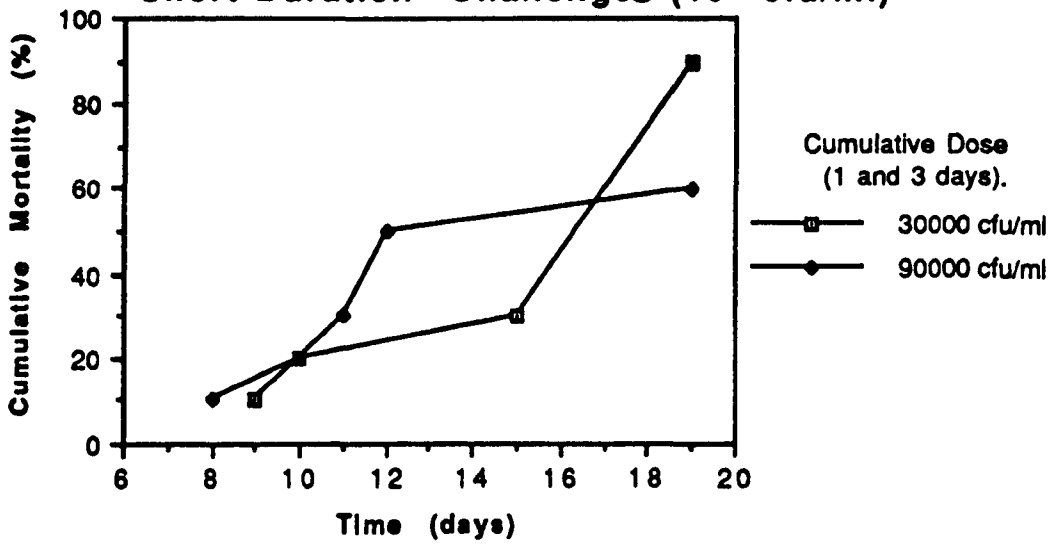
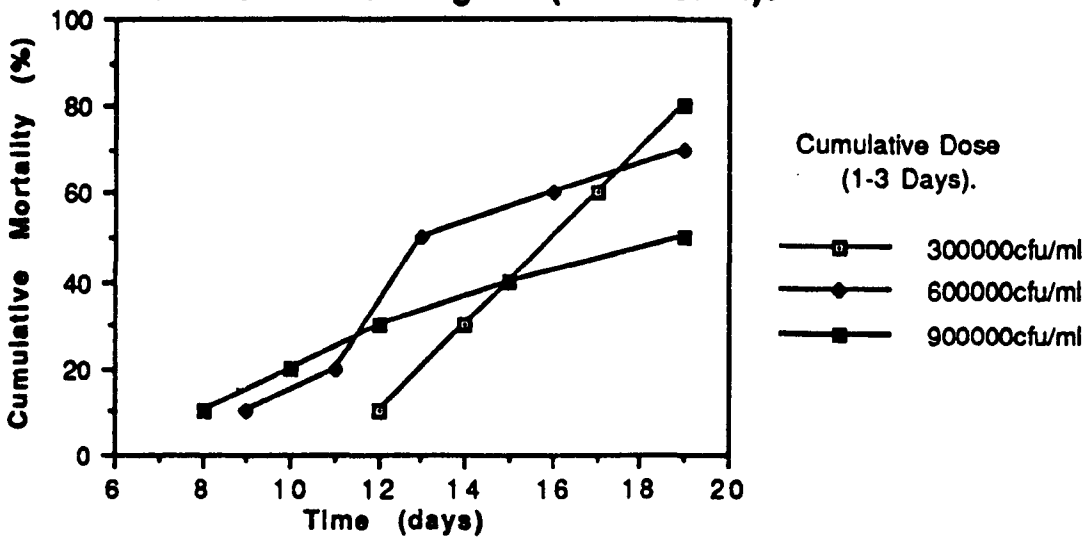


Fig 4.4 Cumulative Mortality for Short-Duration Challenges (10^5 cfu/ml).



freshwater were exposed to the bacterium for comparison with the previous study with this strain (Adams *et al.*, 1987). A dose of 3 x 10^5 cfu ml^{-1} for one day produced 57% mortality over the 19 day experimental period. This would indicate that the results reported here are comparable to the previous study with this strain (Adams *et al.*, 1987) where 10^4 cfu ml^{-1} for 20 hr produced 96% mortality in Atlantic salmon held in freshwater. A group of Atlantic salmon (20-32 g), in seawater, injected i.m. with 10^4 cfu bacteria died within six days of inoculation with the development of furuncles.

4.3.2 Long-duration Bath Challenge

The duration of exposure to low doses of *A. salmonicida* in bath-challenge was prolonged. Groups of 10 Atlantic salmon (75-115 g) were exposed to 10^2 cfu ml^{-1} *A. salmonicida* every day for either one or three weeks and surviving fish were carrier tested 10 days after the last day of exposure. No mortalities were recorded for the 1 week exposure. In contrast, 2/10 fish exposed for three weeks died three and five days after termination of challenge. No carriers were detected amongst surviving fish of either experimental group. These results are summarised in Table 4.2.

4.3.3 Intragastric Challenge

Fish were challenged intragastrically with diluted material from a furuncle from an Atlantic salmon infected i.m. with *A. salmonicida*. Specific mortalities (1/8) were only recorded in the group administered 10^7 cfu $fish^{-1}$. Following carrier test, the pathogen was isolated from 4/8 and 3/7 fish exposed to 10^5 and 10^7 cfu, respectively. Results are presented in Table 4.3. Infected fish were often observed to exhibit an accumulation of fluid, and distension, of the lower intestine. However, *A. salmonicida* was not consistently isolated from this region, in contrast to the kidney.

TABLE 4.2

Long-duration bath exposure of Atlantic salmon (75-115 g) to *A. salmonicida* (MT423) in seawater

Duration of exposure	Bacterial dose (cfu ml^{-1} day^{-1})	No fish	Mortality (%)	Carrier test
One day	10^4	10	0	+ve(3/10)
One week	10^2	10	0	-ve
Three weeks	10^2	10	20	-ve
Control	-	10	0	NT

Carrier test: +ve, *A. salmonicida* isolated; -ve, *A. salmonicida* not isolated; NT, not tested

TABLE 4.3

Intragastric challenge with *A. salmonicida* (MT423)

Furuncle dilution	Bacterial dose (cfu)	Mortality	Carrier test
10^{-1}	8.53×10^{7}	1/8	+ve (2/7)
10^{-3}	8.53×10^{5}	0/8	+ve (4/8)
10^{-5}	8.53×10^{3}	0/8	-ve
10^{-7}	8.53×10^{1}	0/8	-ve
Control (PBS)	-	0/8	-ve

Carrier test: +ve, *A. salmonicida* isolated; -ve, *A. salmonicida* not isolated

TABLE 4.4

Detection of *A. salmonicida* (MT423) in water during short-duration bath exposure

Bacterial dose (cfu ml^{-1} day^{-1})	Duration (days)	Days post-exposure					
		1	3	5	7	9	11
3×10^4	3	0	0	0	50	0	0
3×10^5	1	260	0	0	260	0	40
3×10^5	2	0	0	0	160	0	200
3×10^5	3	300	0	0	20	380	30

A. salmonicida was not isolated from other challenge groups. Results expressed as cfu ml^{-1} of *A. salmonicida*

4.3.4 Shedding of Bacteria from Infected Fish

Throughout the short-duration bath challenges, tank water (100 µl per challenge group) was monitored for the presence of *A. salmonicida* with the antibiotic medium. The pathogen could be detected at least 12 hrs after initial exposure in some tanks at a level of 10^2-10^3 cfu ml^{-1}. Thereafter *A. salmonicida* was not detectable until at least seven days post-challenge, ie not more than two days before first mortalities, in those groups in which specific mortalities occurred. At this time, 20-380 cfu ml^{-1} were detectable. This data is presented in Table 4.4. *A. salmonicida* was not detected amongst groups in which no mortalities occurred. Shedding from infected Atlantic salmon is considered in more detail in Chapter 5.

4.4 DISCUSSION

This study has examined the infectivity of *A. salmonicida* in seawater which has not previously been investigated. Infectivity was measured by bath challenge, as opposed to parenteral inoculation, because it is believed that the former procedure provides a more realistic assessment of the multi-faceted host-pathogen relationship (McCarthy, 1983). Bath challenge accounts not only for the virulence factors of the pathogen once localised in the fish host, but also the initial phases of the disease process such as adhesion and penetration of the host surface defence mechanisms (Carson and Handlinger, 1988). In addition, the enteric route of infection was examined for which there have been conflicting reports in the past.

On the basis of the bath exposure experiments described here the infectivity of *A. salmonicida* in seawater appears similar to that observed under freshwater conditions (Adams *et al.*, 1987). Mortalities were observed in Atlantic salmon exposed to at least

10^4 cfu ml^{-1}. However, in the present work, at a bacterial dose of 10^2-10^3 cfu ml^{-1}, no infections were evident, even after carrier test, over a three day exposure period. In contrast, Adams *et al.* (1987) were able to infect Atlantic salmon in freshwater at these lower concentrations albeit at higher temperatures (15-16°C).

The absence of mortalities in seawater at lower levels of *A. salmonicida* described in this study may be a consequence of several factors. Atlantic salmon in seawater may exhibit increased resistance to the pathogen. This seems unlikely however, the experimental fish had only recently been introduced to seawater which suggests that they may have been stressed and susceptible to infection. In this respect, Pickering and Duston (1983) found increased susceptibility to *A. salmonicida* in brown trout, *Salmo trutta* L., when cortisol, a stress hormone, is raised to levels within the physiological range found during low level chronic stress such as crowding, pollution and confinement. A more likely explanation for the differences may be found in the experimental design of the respective studies. The study of Adams *et al.* (1987) used a recirculating system in which *A. salmonicida* may have been able to persist, or even grow, within the filter system. Whereas in this study exposures to *A. salmonicida* were periodic (12 hr per 24 hr) throughout the challenge period and seawater was allowed to flow through the experimental holding facilities during the remaining 12 hr period each day. This latter explanation is supported by the extended exposure challenges, at 10^2 cfu ml^{-1}, which resulted in mortalities only after three weeks exposure to *A. salmonicida.*

A. salmonicida was detectable in tank water in which specific mortalities were recorded. Similarly, Hoff (1989b) reports that Wiik *et al.* (1989) were able to detect 45-670 bacteria/ml in seawater after injection of the fish bacterial pathogen *Vibrio salmonicida* into fish; and Kanno *et al.* (1989) could detect *Vibrio anguillarum* at concentrations up

to 10^4 cfu ml^{-1} in tank water containing infected ayu (*Plecoglossus altivelis*). Shedding of *A. salmonicida* from infected fish has been reported previously. Bullock and Stuckey (1977) and Michel (1979) recorded 10^3-10^5 cfu ml^{-1} and 6.5 x 10^3 cfu ml^{-1} in water in which infected populations of brown trout, *Salmo trutta* L., and rainbow trout, *Oncorhynchus mykiss*, were held, respectively. McCarthy (1977) found that the release of the pathogen from dead rainbow trout in freshwater reached 10^8 cfu ml^{-1} after 12-36 days but, in contrast to this study, shedding prior to death was not apparent.

In this study, and others (Michel, 1982), there was no correlation between fish mortalities and the concentration of bacteria added to tank water. The absence of a dose-mortality response may be due to the shedding (excretion?) of the pathogen from infected fish, which resulted in superinfection. Diseased fish may shed more bacteria than were in the original challenge and result in extended exposure of susceptible fish to the pathogen. The absence of dose dependent mortality may also be explained by natural variability in bath challenge and may be influenced by the design and position of experimental tanks. In bath challenge systems the number of bacteria entering fish is not precisely known, unlike parenteral injection (Michel, 1980), and may vary from fish to fish thereby masking any dose-mortality response.

The shedding of bacteria from infected Atlantic salmon reported here indicates that during epizootics large numbers of *A. salmonicida* may be released into the environment and present a significant risk of disease spread between farm sites. The possibility of infection at farm sites indicates the importance of hygiene in restricting the build up of bacteria within a cage. The shedding of *A. salmonicida* from infected Atlantic salmon in seawater is considered in more detail in Chapter 5.

Invasion of fish hosts by *A. salmonicida* is assumed to occur at each of the principal body surfaces, gill, gut, and skin (Munro, 1984; Hodgkinson *et al.*, 1987). Several studies have indicated the importance of gills (Alexander *et al.*, 1981) and skin (Kanno *et al.*, 1989) as sites of bacterial uptake. In this study the enteric route has been shown to be a possible route of entry of *A. salmonicida* in Atlantic salmon. Intragastric intubation of furuncle material resulted in mortalities, or covert (carrier) infection, with high doses of bacteria ($>10^5$). Infection at lower doses was unsuccessful possibly because of inactivation and excretion of the pathogen from the gut which has been described for rainbow trout (*Oncorhynchus mykiss*) by McCarthy (1977) and Tatner *et al.* (1984) for relatively high levels (10^6-10^7 cfu) of *A. salmonicida* intubated.

The role of the oral route of infection with *A. salmonicida* is controversial. Blake and Clarke (1931), Klontz and Wood (1972), Sakai (1979) and Markwardt and Klontz (1989) have described infection, in various salmonids, via this route whilst other workers (Krantz *et al.*, 1964; McCarthy, 1977) have been unable to establish infection through oral presentation of the pathogen. The successful infections described in this study may be a consequence of intubating *A. salmonicida* in the presence of digested muscle (furuncle) which may have protected the bacterium from the acidic environment of the stomach. All previous authors have either introduced *A. salmonicida* directly (McCarthy, 1977; Markwardt and Klontz, 1989) or coated on fish feed (Krantz *et al.*, 1964; McCarthy, 1977; Markwardt and Klontz, 1989). The growth of the pathogen *in vivo*, injected i.m. into Atlantic salmon, may have also increased its virulence, but this was not examined, thereby increasing the probability of establishing infection by ingestion.

In view of the fact that undiluted furuncle material contains *ca* 10^{10} cfu ml^{-1}, ingestion of only 1 µl of furuncle by a fish would be sufficient to establish infection, assuming

a minimum lethal dose of 10^7 cfu. This implies that cage hygiene at a farm, by removal of dead fish, especially those with visible furuncles, is particularly important in limiting the spread of furunculosis.

In this study few covertly infected (carrier) fish were detectable by the heat/stress test (Bullock and Stuckey, 1975) amongst survivors exposed to the pathogen in water (bath-challenge). This may be a consequence of stress-inducing conditions in experimental tanks leading to immunosuppression of susceptible fish and development of clinical disease rather than establishment of covert infection. Alternatively, bath exposure intrinsically may not be an efficient means of producing carrier fish. In this study, carrier fish were only detected after oral challenge with the pathogen. In this respect Markwardt and Klontz (1989) found that gastric intubation of broth-culture (TSB) containing 10^8 cfu *A. salmonicida* produced greater (65%) carrier rates in chinook salmon (*Oncoryhnchus tshawytscha*) than bath exposure (40%). However, their method of assessing carrier status is questionable. Fish were regarded as covertly infected if *A. salmonicida* was isolated from anal swabs 16 days after challenge. These authors did not consider that fish so detected could have developed overt clinical disease if left for a longer period. Further work is clearly required to develop a reliable method of establishing, and defining, carrier infection which will enable experimentation on this important aspect of furunculosis epidemiology.

This study has shown that transmission of *A. salmonicida* between Atlantic salmon may potentially occur by both ingestion of infected fish tissue and extended contact with the pathogen in water and confirms that there should be efficient removal of dead fish from commercial salmon cages. Although infection via bacteria in water has been demonstrated experimentally the role of shed bacteria in infection at cage sites, where

dispersal and dilution of bacteria will occur, is less certain. Nevertheless, the stocking density at fish farms (in excess of 50,000 fish per site), compared with the small groups of fish used here, may increase the probability of contact of fish with *A. salmonicida* present in water. This topic is considered in more detail in Chapter 5.

CHAPTER 5

THE TRANSMISSION OF *Aeromonas salmonicida* subsp. *salmonicida* IN THE MARINE ENVIRONMENT

CHAPTER 5 THE TRANSMISSION OF *Aeromonas salmonicida* subsp. *salmonicida* IN THE MARINE ENVIRONMENT

5.1 INTRODUCTION

The epidemiology of furunculosis in the marine environment has been relatively neglected compared with studies in freshwater (Austin and Austin, 1987). Furunculosis is, however, one of the main causes of mortality in marine salmon farming and *A. salmonicida* is regularly isolated from seawater fish (Munro, 1988). It is considered that the introduction of asymptomatic carriers from freshwater to marine fish farms is one of the main causes of furunculosis spread (Klontz and Wood, 1972; Novotny, 1978; Drinan *et al.*, 1978; Ezura *et al.*, 1984; Munro, 1982b, 1988). The pathogen has been isolated from non-salmonid marine fish (Klontz and Wood, 1972; Novotny, 1975, 1978) and infection in seawater has been demonstrated experimentally (Chapter 4; Scott, 1968). Smith *et al.* (1982) reported that an *A. salmonicida* strain, identifiable by a resistance plasmid, was transmitted at a marine site between infected Atlantic salmon and recently introduced fish with no history of furunculosis.

Recent evidence suggests that furunculosis may be able to cross-infect widely separated (*ca* 10-20 km) commercial marine salmon farms (Novotny, 1975, 1978; Turrell and Munro, 1988). The mechanism of transmission is unknown. In this chapter the information available on the survival of *A. salmonicida* (see Chapter 3) and its infectivity in seawater (see Chapter 4) were used to assess whether the evidence for long distance transmission could be accounted by simple movement of bacteria in coastal currents before their eventual death. A computer particle-tracking model has been developed at the DAFS Marine Laboratory, Aberdeen (Turrell and Munro, 1988) which follows

bacterial dispersion and decay and simulates advection and diffusion in a hypothetical Scottish fjordic loch typical of many coastal salmon farming sites. The potential of *A. salmonicida* to spread in the marine environment was estimated with this model.

The simulation requires, as a starting condition, the *A. salmonicida* concentration at a farm site. Water was obtained from farm cages experiencing active disease outbreaks and attempts were made to isolate the pathogen with an antibiotic-containing, agar selective medium. Unfortunately, *A. salmonicida* was not detected. Therefore, *A. salmonicida* concentrations at infected sites were estimated from an assessment of the numbers of the pathogen shed from experimentally infected Atlantic salmon.

Some of the material presented in this chapter has previously been published (Rose *et al.*, 1989a). The computer model was designed by Turrell and Munro (1988) and is used with their permission.

5.2 MATERIALS AND METHODS

5.2.1 Bacteria

Aeromonas salmonicida subsp. *salmonicida* strain MT423 was used in shedding experiments and strain MT212 was used as a control in the ELISA assay. These strains are described in 2.2.1 and 3.2.1.

5.2.2 Water Samples

a) Collection of samples

Seawater samples were obtained 1 m beneath commercial salmon cages using a standard water sampler (Nansen bottle) and transported in 500 ml sterile glass bottles. Water samples were taken from these bottles for further analysis within one hour of collection.

b) Farm sites

Samples were taken from two sites which contained fish infected with *A. salmonicida*. Site 1 was sampled in February and May 1988 when the sea temperature was 7 and 9°C respectively. The site contained *ca* 86,000 Atlantic salmon and between September 1987 and February 1988 14 out of 89 fish examined were found to be infected with *A. salmonicida*. However no major disease outbreak had occurred at this site and no antibiotics had been fed to fish. Site 2 was sampled in May 1989 (sea temperature 8°C). The site had two flotillas of cages. The first (flotilla A) had three groups of six cages (9 m x 9 m x 4 m) containing 3,500 fish per cage. At this flotilla mortalities, due to furunculosis, BKD and sea-lice, were 5-200 fish/cage/week and antibiotic treatment had been started four days prior to water samples being taken. The second flotilla (B) consisted of two groups of eight cages (12 m x 12 m x 8 m) with 11,000 fish per cage and mortalities were 10-70 fish/cage/week. The nets at this flotilla were covered in algae and water flow through the cages was severely restricted. Large numbers of sea-lice, on salmon, and crustacean zooplankton were visible at both flotillas.

5.2.3 Enumeration of *A. salmonicida*

a) Antibiotic selective media

Antibiotic containing selective media were used to isolate *A. salmonicida* from disease outbreaks and in experimental shedding studies. The selective media were based on the

antibiotic sensitivity patterns of strains isolated from fish experiencing furunculosis at sites sampled (data supplied by farm site owners). At site 1 the medium used was TSA plus oxytetracyline (2 μg ml^{-1}) or oxolinic acid (0.2 μg ml^{-1}, Parke, Davis and Co) and oxytetracycline (2 μg ml^{-1},). At site 2 this medium consisted of tryptone soya agar (TSA, Oxoid) or brain-heart infusion agar (BHIA, Oxoid) containing oxytetracycline (Terramycin, Pfizer Ltd) at 2 μg ml^{-1}. The selective medium used to isolate MT423 in shedding experiments consisted of TSA plus trimethoprim (2 μg ml^{-1}, Sigma) and gave similar viable counts to TSA alone when used to isolate MT423 inoculated into sterile PBS (see Table 3.3).

Antibiotics were dissolved in 10 ml PBS (+ 100 μl dimethyl sulphoxide (Sigma) for oxolinic acid) and added to cool (40°C) TSA. These selective media have been used succesfully to isolate other *A. salmonicida* strains, with similar antibiotic resistance patterns, in previous studies (see Chapter 3).

100 μl water samples were spread onto antibiotic selective medium to evaluate the numbers of *A. salmonicida.* Plates were incubated at 15°C for seven days for farm site samples. Samples obtained from shedding experiments were incubated at 15°C for 24 hr and then transferred to 22°C for 48 hr prior to enumeration of colony-forming units (cfu). At site 1 (February 1988) 100 ml seawater samples were filtered through sterile 0.22 μm Sartorius membrane filters (diameter 45 mm) which were then placed onto antibiotic selective media and incubated as described above. Putative identification of *A. salmonicida* was based on Gram negative coccobaccilli, oxidase positive, friable cream coloured colonies and production of diffusible brown pigment.

b) ELISA (Enzyme-Linked Immunosorbent Assay)

The sensitivity of ELISA (Aquakit Furunculosis, Stirling Diagnostic Ltd) in detecting *A. salmonicida* in seawater was assessed and used to measure the concentration of the pathogen at site 2. The ELISA has been developed to detect *A. salmonicida* antigen in fish kidney samples.

100 ml of seawater from site 2 or seawater obtained from a source at the DAFS Marine Laboratory inoculated with broth grown (3% TSB, shaken overnight at 22°C) *A. salmonicida* MT 212 was filtered through a 0.22 μm Sterivex-GV filter (Millipore). The filter was then opened at one end with a hacksaw and placed in a sample bottle containing concentrated ELISA extraction buffer for one hour at room temperature. Diluent was then added from the dilution dispenser and the sample bottle inverted several times.

The immunoassay was performed within seven days of sampling and is briefly described below. The assay is based on a specific sheep anti-*Aeromonas salmonicida* antibody which is attached to a plastic support (microwells). 100 μl water samples were incubated in individual wells for 30 min at room temperature. Wells were then washed four times with wash buffer and 100 μl conjugate (Sheep anti-*Aeromonas salmonicida* conjugated to horse-radish peroxidase) added and incubated for a further 30 min, to form a sandwich with *A. salmonicida* antigen present, followed by four washes as before. The enzyme peroxidase was detected by its activity on 100 μl of the chromogen tetramethyl benzidine (120 μl in 12 ml sodium acetate/citric acid buffer containing hydrogen peroxide) for 5 min. The reaction was stopped by adding 100 μl stop solution (2 M sulphuric acid). The colour change produced is a direct measure of the *A. salmonicida* concentration initially present in the sample. In this case the optical density was read on a Dynatech

II Minireader at 410 nm. A negative control (1 ml PBS) and positive controls containing known concentrations of *A. salmonicida* were included in the assay.

5.2.4 Shedding Experiments

The release of *A. salmonicida* from experimentally infected Atlantic salmon was assessed. Fish were infected either by bath-challenge or intramuscular (i.m.) injection. Atlantic salmon (20-32 g), held in seawater, were bath challenged with 3 x 10^4 cfu ml^{-1} of broth-grown (3% TSB, shaken 24 hr, 22°C) *A. salmonicida* MT423 for 1-3 days. For comparison, fish (*ca* 6.9 g), held in freshwater, were bath-challenged with 10^5 cfu ml^{-1}. Fish (20-32 g or 1,200 g), maintained in seawater, were anaesthetised with benzocaine as described in 2.2.6 and injected i.m. with either 10^3 cfu or 10^5 cfu *A. salmonicida* MT423. Individual fish were placed in known volumes of water for one hour after which samples (100 µl) of the water (three plates per fish) were spread onto antibiotic selective medium (2 µg ml^{-1} trimethoprim).

5.2.5 Mathematical Models of Disease Transmission

a) Particle tracking model

This model was developed to assess over what time and distance *A. salmonicida*, released from infected fish at a commercial marine site, remains alive to infect other farms. The model was designed by W R Turrell at the DAFS Marine Laboratory and is further described in Turrell and Munro (1988). A brief description of the model is provided below.

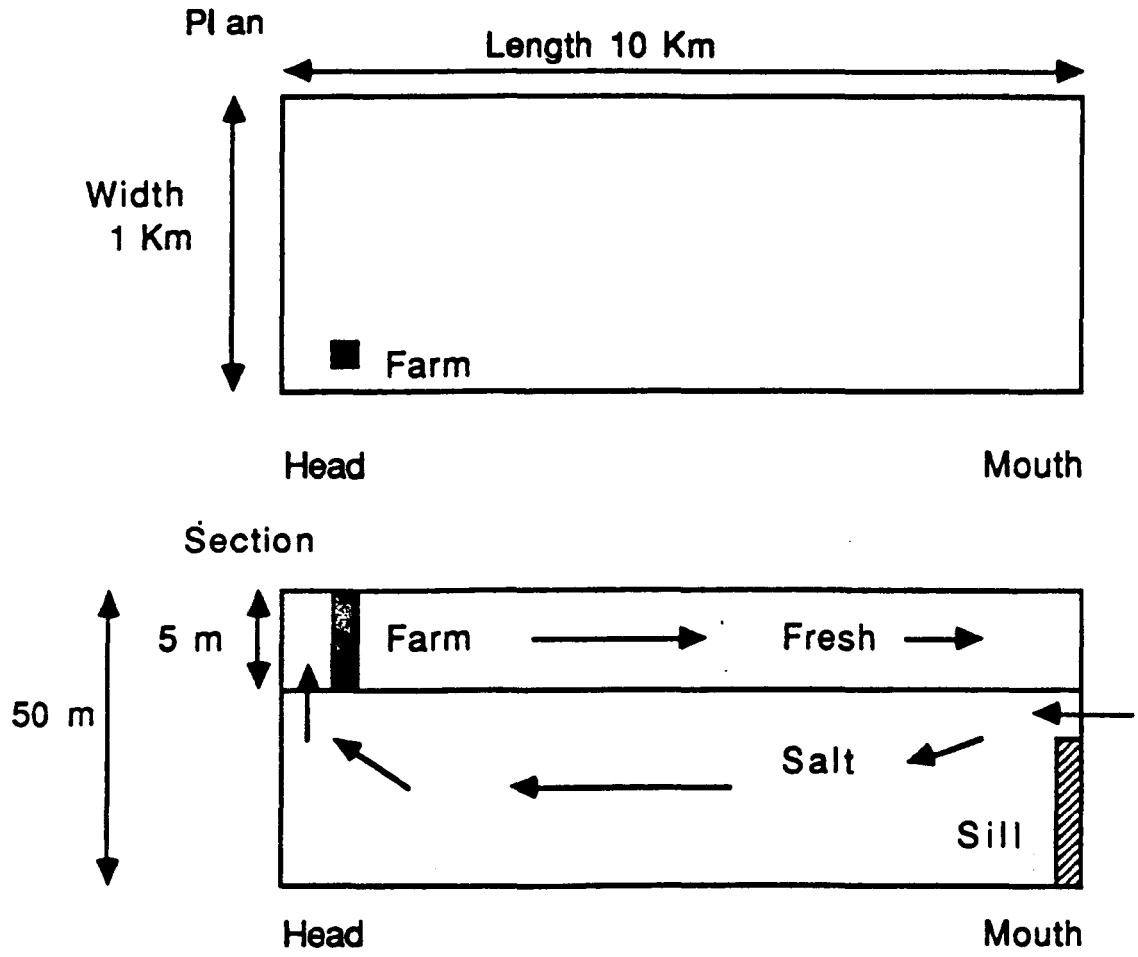
i) Description

The particle tracking model consists of a "two-box" model of a hypothetical Scottish fjordic loch. The dimension of the loch are shown in vertical and horizontal profile in Figure 5.1. Freshwater run-off results in a 5 m deep layer of lower salinity lying on the loch surface. The upper layer is given an assumed flushing time of seven days resulting in a residual seawards velocity of 0.02 m s^{-1}. The model neglects any mixing between layers. The fish farm is envisaged in multiples of a flotilla (total volume 5,780 m^3) each containing several cages (depth 5 m). The farm is sited at 500 m from the head of the loch and at 100 m and 900 m from the opposite shore lines. It is assumed tidal effects are operating, amplitude of oscillating current 0.05 m s^{-1}, but that currents within the loch have no vertical or horizontal structure. Fickian diffusion (random motions) is included in the model at a a level of 0.1 m^2 s^{-1}, which is within the range observed by Curran (1986). The model iterates through time with a step of five minutes. Each time step a particle, representing a set number of bacteria, is released at the centre of the model source (flotilla). The position of the particle is calculated throughout the subsequent iterations. Particles are reflected from the three solid boundaries of the loch, and from the surface and bottom of the 5 m deep layer. The particles are allowed to leave the model through the open sea end.

ii) Starting Conditions

The initial number of *A. salmonicida* at the fish farm was calculated from shedding rates observed in experimentally infected Atlantic salmon. Bacterial death rates were derived from applying a first-order decay relationship, $Nt = No\ e^{-kt}$ (Crane and Moore, 1986) (Nt = population size at time t; No = population size at t = 0; k = bacterial decay constant) to the survival curves described in Chapter 2 and previously published material.

Fig 5.1 Plan and Section of Model Loch Basin.



(not to scale).

redrawn from Turrell and Munro (1988).

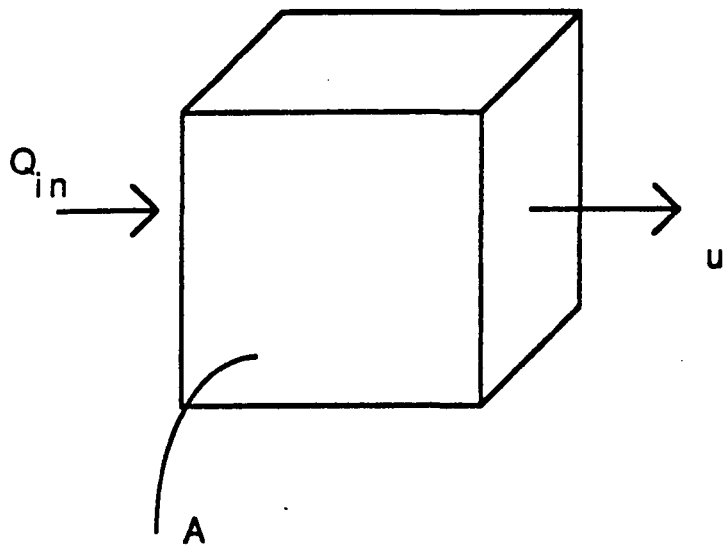
This relationship is equivalent to a first order reaction in chemical kinetics and has been found to adequately provide bacterial "die-off" coefficients for enteric bacteria (Crane and Moore, 1986).

Survival data was plotted as the natural logarithmn of bacterial concentration (cfu ml^{-1}) against time (days) and the "die-off" constant derived from the slope of the curves by linear regression. Some of the curves analysed could not be fitted to this simple equation and were, therefore, subdivided and k was calculated for the resultant pair of curves. The published data supplied without survival curves but with initial inoculum size were assumed to fit the equation and k calculated accordingly. Published data not providing initial inoculum sizes (N_o) could not be interpreted.

b) A simple model of infection in a salmon cage

This model was used to assess whether shedding from infected fish can account for the spread of furunculosis at a hypothetical marine cage. The model is represented in Figure 5.2. The dimensions of the cage are 4 m x 5 m x 5 m (area of one side, A, 20 m^2). The water flow through the cage is 0.02 m s^{-1} (u), the water volume entering the cage is Q m^3 s^{-1}, and the *A. salmonicida* concentration in the cage is C bacteria/m^3. If Qin = Qout = uA then the numbers of *A. salmonicida* leaving the cage equals uAC. The number of *A. salmonicida* entering the cage equals the shedding rate (bacteria/second/fish) Sh, multiplied by the number (n) of infected fish actively shedding. If the number of bacteria entering the cage equals the number leaving then Sh.n = uAC. By rearrangement the bacterial concentration within the cage (c) is given by $\frac{Sh.n}{uA}$ (equation 5.2).

Fig 5.2 A Simple Model of Infection at a Salmon Cage.



Q in=water volume entering cage (m^3).
A=area of one side of cage (m^2).
u=water flow (m/s).
C=bacterial concentration within cage (bacteria/m^3).

Q in = u.A
Q out = u.A
Bacteria out = u.A.C
Bacteria in = No fish x shedding rate
(bacteria/sec/fish)

therefore:

$$C = \frac{\text{(no fish x shedding rate)}}{\text{(water flow x area of one side)}}$$ ..... equation 5.2

5.3 RESULTS

5.3.1 Detection of *A. salmonicida* in the External Environment at Infected Marine Farm Sites

a) Isolation with antibiotic selective media

In total 2 ml (20 x 100 µl) was sampled at each site but *A. salmonicida* was not detected at any of the farm sites examined. The membrane filters used at site 1 were completely overgrown with contaminating bacteria which were resistant to the antibiotics (oxolinic acid and oxytetracycline) in the agar selective media. In contrast, broth-grown *A. salmonicida* MT393 (described in 2.1.1), suspended in PBS and inoculated into 100ml of sterile seawater, grew on the membrane filters. The contaminating bacteria were putatively identified as *Pseudomonas* spp. (whitish mucoid colonies of Gram negative, oxidase positive, rods incapable of anaerobic fermentation).

b) Detection of *A. salmonicida* in seawater by ELISA

The ELISA immunoassay was tested at site 2 and no *A. salmonicida* antigen was detected in 2 x 100 ml water samples at flotilla A and 6 x 100 ml samples from flotilla B. In contrast, positive controls ,non-sterile seawater to which known concentrations of broth grown *A. salmonicida* had been added, were detectable down to 5.61×10^5 cfu which validates the use of ELISA.

5.3.2 Shedding of *A. salmonicida* from Experimentally Infected Atlantic Salmon

The release of *A. salmonicida* from experimentally infected Atlantic salmon was assessed using antibiotic selective media. Groups of fish, in fresh and seawater, were bath challenged and the number of bacteria shed from infected fish examined within 12 hrs of their death. Fish (6.9 g) in freshwater shed 4.1×10^4 cfu/fish/hr whereas those in seawater (25.8 g) were found to shed 7.1×10^7 cfu/fish/hr. None of these dead fish had developed furuncles.

Calculation of *A. salmonicida* Shedding Rates from Fish Farm

Shedding rates were converted to bacteria released per second to allow input into the particle tracking model. It was assumed that 231 fish (Turrell and Munro, 1988) were shedding *A. salmonicida*.

a) Shedding rates per fish:

   i) 6.7×10^6 cfu/fish/hr = 1.86×10^3 bacteria/sec

   ii) 6.4×10^8 cfu/fish/hr = 1.78×10^5 bacteria/sec

b) Shedding rates per farm:

   i) $1.86 \times 10^3 \times 231 = \underline{4.3 \times 10^5}$ bacteria/sec

   ii) $1.78 \times 10^5 \times 231 = \underline{4.1 \times 10^7}$ bacteria/sec

To assess shedding from furuncles fish were injected i.m. with the pathogen. Shedding from 23 g fish was monitored on day 5 post-inoculation of 10^3 cfu i.m. and found to have a median of 1.3 x 10^7 cfu/fish/hr. These fish died on day 6. Shedding from two larger fish (1,200 g) injected i.m. with 10^5 cfu was monitored on day of death (5 and 13 days) post-challenge and found to be 5.4 x 10^7 cfu/fish/hr. These results are graphically presented in Figure 5.3 and summarised in Table 5.1.

A. salmonicida could be isolated, on TSA + antibiotics, from both the gills and rectum of dead fish used in this study suggesting that these are sites of bacterial shedding. In comparison, Hunter *et al.* (1980) and Hoff (1989b) state that the bacterial fish pathogens *Yersinia ruckeri* and *Vibrio salmonicida*, respectively, are shed in faeces.

5.3.3 Mathematical Models of Disease Transmission

a) Particle tracking model

The particle tracking model predicts the dispersion of *A. salmonicida* released from infected fish at a marine fish farm. The variables used in the model are bacterial decay rate ("die-off") and shedding rate of *A. salmonicida* from infected fish. These variables are described below with their respective dispersions in the hypothetical loch.

i) Shedding Rates of Infected Fish

Two different shedding rates were chosen based on the results of 5.3.2. These were 6.7 x 10^6 cfu/fish/hr (= 4.3 x 10^5 bacteria/second) and 6.4 x 10^8 cfu/fish/hr (= 4.1 x 10^7 bacteria/second).

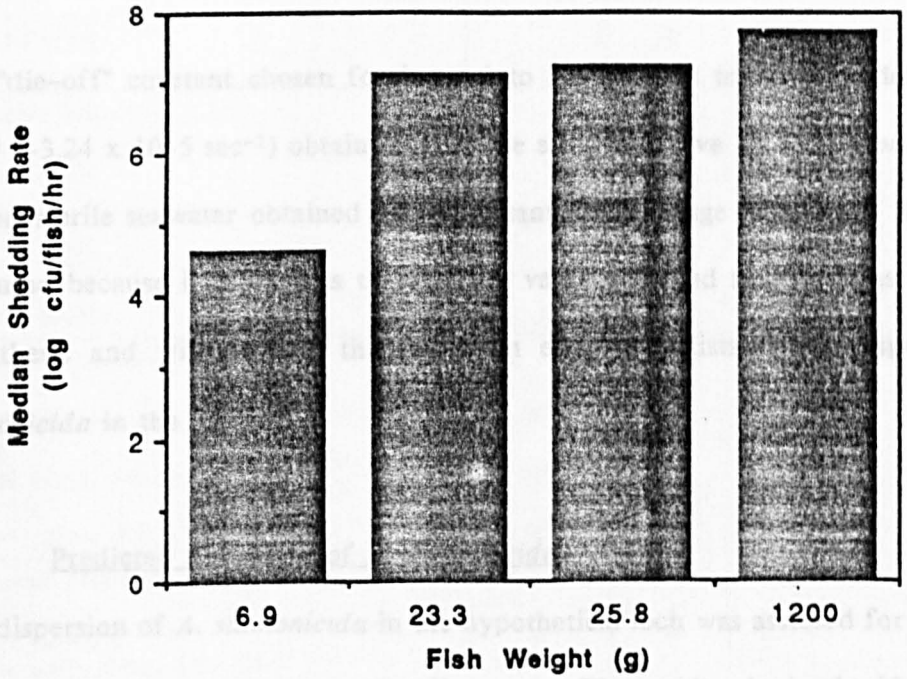
TABLE 5.1

A. salmonicida shedding rates from infected Atlantic salmon

Treatment	No fish	Mean fish weight (g)	Bacterial shedding rate* (cfu/fish/hr)
Seawater			
Bath challenge (10^5 cfu/ml) dead	6	25.8	$1.7x10^6$ ($1.7x10^5$-$1.1x10^7$)
i.m. challenge (10^3 cfu) live	4	23.3	$1.3x10^7$ ($5.7x10^5$-$2.1x10^7$)
i.m. challenge (10^5 cfu) dead	2	1,200	$5.4x10^7$ ($9.0x10^6$-$6.4x10^8$)
Freshwater			
Bath challenge (10^5 cfu/ml) dead	3	6.9	$4.1x10^4$ ($1.7x10^4$-$7.0x10^4$)

* median values, range in brackets
i.m., intra muscular

Fig 5.3 Shedding Rate of A.salmonicida From Experimentally Infected Fish.



ii) Analysis of Survival Curves

The survival curves obtained in Chapters 2 and 3, and previously published curves, were analysed to produce "die-off" constants (k) which could then be used in the particle tracking model. It was assumed that all curves were of the form $N_t = N_{o.e}^{-kt}$. The results of this analysis are provided in Tables 5.2 and 5.3. It is interesting to note the similar magnitude in many of the "die-off" constants.

The "die-off" constant chosen for input into the particle tracking model was -2.803 day^{-1} (-3.24 x 10-5 sec^{-1}) obtained from the survival curve of *A. salmonicida* MT432 in non-sterile seawater obtained 100 m distant from a cage (Fig. 3.5). This constant was used because it represents the smallest value obtained from studies presented in this thesis and will provide the minimum estimated distance of dispersion of *A. salmonicida* in the model.

b) Predicted dispersion of *A. salmonicida*

The dispersion of *A. salmonicida* in the hypothetical loch was assessed for one value of decay constant and two seperate shedding rates. The results obtained with these values are presented in Figures 5.4 (shedding rate = 4.3 x 10^6 bacteria/second) and 5.5 (shedding rate = 4.1 x 10^7 bacteria/second). The maximum dispersion obtained from the two sets of data are approximately 6 km and 9 km respectively.

5.3.4 Simple Model of *A. salmonicida* Infection at a Salmon Cage

The bacterial concentration at a cage was calculated using equation 5.1. The cage was assumed to contain 5,000 Atlantic salmon (1 kg) and three different levels of infection (2, 10 and 20%) were examined. Each infected fish was assumed to be shedding *A.*

TABLE 5.2

Bacterial decay rates of survival curves (Chapter 2 and 3) of *A. salmonicida*

Survival curve	Temperature (°C)	Initial inoculum (cfu ml^{-1})	Survival (days)	Decay constant (k day^{-1})
Freshwater BSM (Fig. 2.3)				
Strain MT393	15	10^4	23	day 0-16; 0.187*** day 16-23; -1.09[a]
Strain MT212			20	day 0-14; -0.297*** day 15-20; -1.284*
Seawater BSM (Fig. 2.4)				
Strain MT393	15	10^4	12	day 0-4; -2.03* day 4-12; -0.287***
Strain MT212			7	-0.834*
Strain MT432			10	-0.986**
Seawater (non-sterile)				
MT432 (dialysis bag, Fig. 3.1)	14	10^5	7	-1.660**
MT432 (shed, Fig. 3.2)		10^4	7	-1.522**
MT432 (glass-flask, Fig. 3.1)		10^5	7	-1.564**
Seawater (from fish cage)				
MT393 (winter) non-sterile (Fig. 3.5)	15	10^4	4	-2.308*
filter-sterilised (Fig. 3.4)			6	-0.936**
MT432 (summer) non-sterile (Fig. 3.5)			3	-3.38 ns
filter-sterilised (Fig. 3.4)			8	-1.167***
Seawater (100 m distant from cage) MT432 non-sterile (Fig. 3.5)	15	10^4	4	-2.803*** (used in particle tracking model)
filter-sterilised (Fig. 3.4)			7	-1.389***

Levels of significance

*** - $p<0.001$; ** - $p<0.01$; * - $p<0.5$; ns - not significant

[a], decay constant (k) calculated from initial inoculum No; and survival time t, assuming $\text{Ln } N_t - L_n N_o = -Kt$

TABLE 5.3

Bacterial decay rates of selected published data

Aquatic system	Inoculum (cfu ml^{-1})	Survival (days)	Decay constant (day^{-1})	Reference
Sterile freshwater:	10^6			Evelyn (1971)
Typical strain		1	-3.341[a]	
Atypical strain		2	-4.375*	
Sterile seawater:				
Typical strain		13	-1.047***	
Atypical strain		8	-1.287*	
Sterile riverwater:	7.5×10^8	8	-2.555[a]	Lund (1967)
Seawater (3.31%)		24	-0.852[a]	
Estuarine (0.9%)		16	-1.277[a]	
Estuarine (2.41%)		16	-1.277[a]	
Riverwater 4°C	10^6	50	0-37 days, -0.083*** 37-48 days, -0.973*	Dubois-Darnaudpeys (1977a)
14°C		30	0.555***	
22°C		20	0.909***	
Non-sterile:	7.5×10^8			Lund (1967)
Riverwater		2	-10.28[a]	
Seawater (3.31%)		5	-4.089[a]	
Estuarine (0.9%)		2	-10.28[a]	
(2.41%)		2	-10.28[a]	
Non-sterile:	10^7			McCarthy (1977)
Freshwater		20	-0.475**	
Brackish (2.34%)		26	-0.693***	
Seawater (3.46%)		10	-1.987**	

Levels of significance

*** - $p<0.001$; ** - $p<0.01$; * - $p<0.5$; ns - not significant

[a], decay constant (k) calculated from initial inoculum No; and survival time t, assuming $\text{Ln } N_t - L_n N_o = -Kt$

Fig 5.4 Dispersion of *A. salmonicida* in hypothetical loch.

Bacterial decay rate (K) = -3.24×10^{-5}/second and Shedding rate = 4.3×10^{5} bacteria/second.

i) Concentration of bacteria/m^3 ($\log_{10}$) averaged along a strip y = 0 to 250 m (width), x = 0 to 10 km (length).

ii) Concentration of bacteria averaged over 100 m^2 boxes. Empty boxes indicate concentrations between 1 to 1,000 bacteria/m^3. Hatched boxes indicate concentrations greater than 1,000 bacteria/m^3.

Fig 5.4

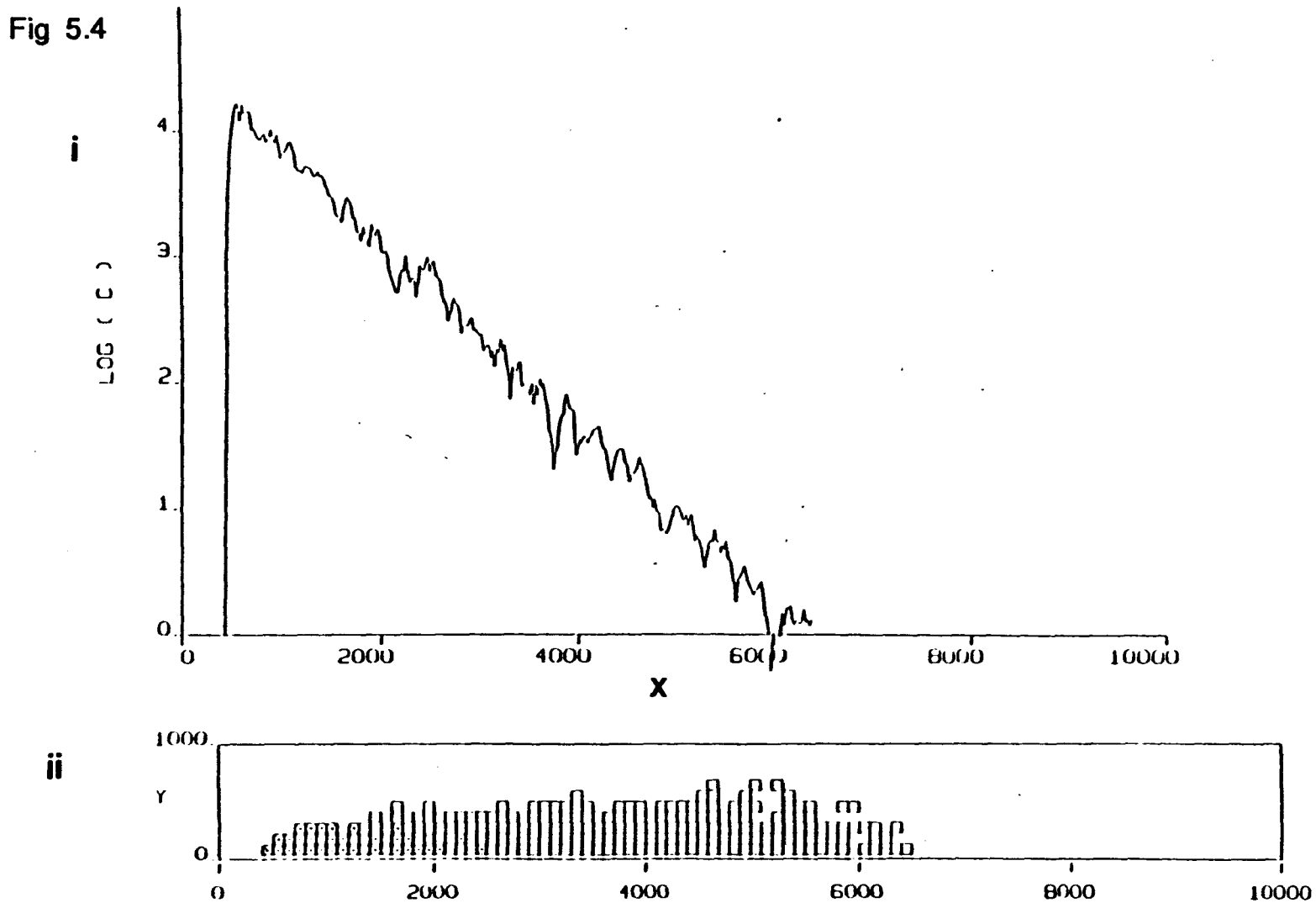

i
LOG (C)
4
3
2
1
0
0
2000
4000
6000
8000
10000
X
ii
1000
Y
0
0
2000
4000
6000
8000
10000

Fig 5.5 Dispersion of *A. salmonicida* in a hypothetical loch.

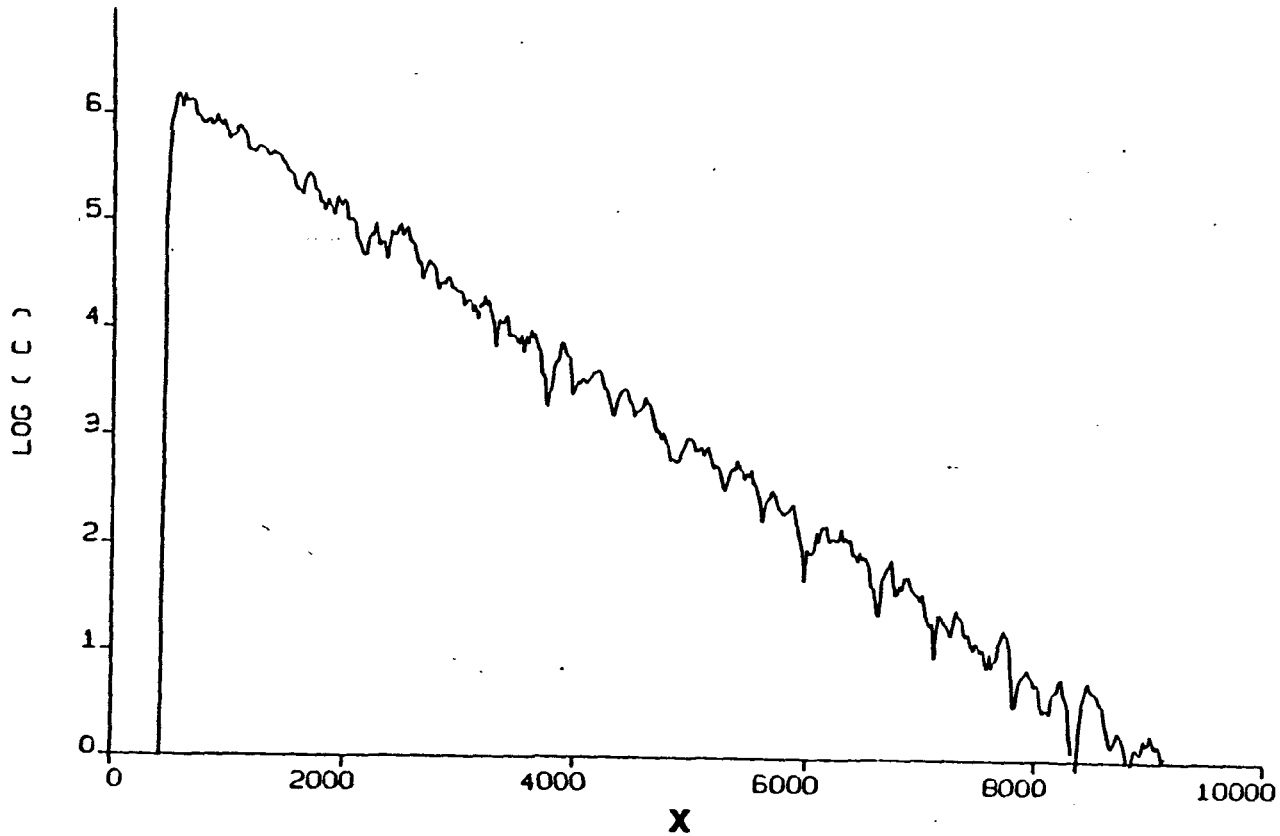
Bacterial decay rate (k) = -3.24×10^{-5}/second and Shedding rate = 4.1×10^{7} bacteria/second.

i) Concentration of bacteria/m^3 ($\log_{10}$) averaged along a strip y = 0 to 250 m (width), x = 0 to 10 km (length).

ii) Concentration of bacteria averaged over 100 m^2 boxes. Empty boxes indicate concentrations between 1 to 1,000 bacteria/m^3. Hatched boxes indicate concentrations greater that 1,000 bacteria/m^3.

Fig 5.5

i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ii

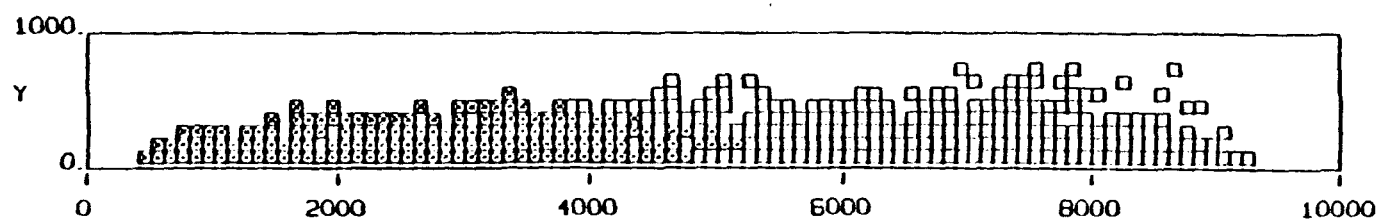
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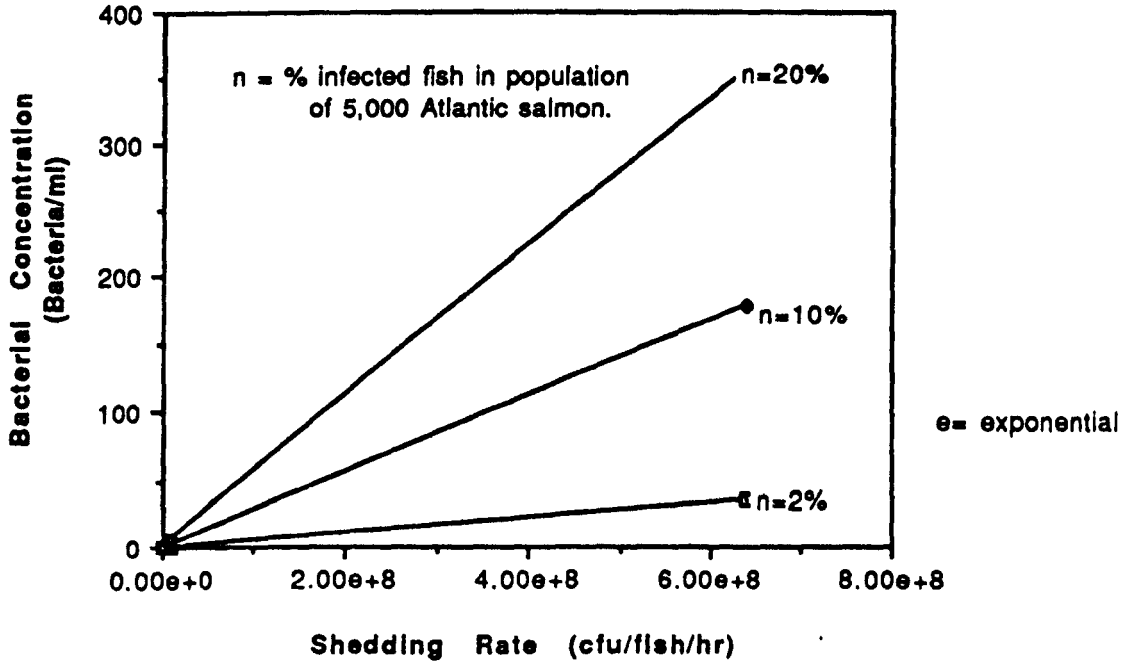
Fig 5.6 Simple Model of Infection at a Cage.



salmonicida at a constant rate. The results are presented in Figure 5.6, which shows the effect of shedding rate on *A. salmonicida* concentration within the cage at the chosen levels of infection.

5.4 DISCUSSION

The extent of furunculosis transmission in the marine environment is an important factor in the epidemiology of *Aeromonas salmonicida* subsp. *salmonicida*. However, there are no reports of detection of the bacterium in the free-living state in seawater. It is, therefore, difficult to assess the ability of the pathogen to spread within the environment and cause disease. In this study seawater at fish farms experiencing active outbreaks of furunculosis were examined for *A. salmonicida*. In addition, the release of the pathogen from experimentally infected fish was assessed. This data was used to predict the potential spread of the disease in a hypothetical loch.

Water samples from marine fish farms were examined for the presence of the pathogen using antibiotic containing agar and also ELISA. Unfortunately, both methods proved unsuccessful and *A. salmonicida* was not detected. The inability to isolate *A. salmonicida* on antibiotic selective media suggests that the pathogen was absent from the samples of water tested because the media reproducibly isolated bacteria shed from experimentally infected fish. The absence of the bacterium in water samples may be due to small numbers of *A. salmonicida* being shed from infected fish, in contrast to the experimental studies described here; rapid dispersion and dilution of shed bacteria; and/or non-homogeneous distribution of bacteria within the water column. In the future larger volumes of water should be examined.

Antibiotic selective media do not appear to be useful tools for detection of *A. salmonicida* because of the presence of antibiotic resistant contaminating bacteria which overgrow the pathogen. However, the method of Cunliffe and Adcock (1989) may prove more useful in isolating the bacterium. These workers were able to selectively isolate motile aeromonads under anaerobic conditions, which inhibits the growth of contaminating Pseudomonads. Pseudomonads appeared to be the major contaminants encountered in the present study and prevented the examination of large volumes of water. A more feasible means of detection is offered by ELISA, which when combined with membrane filters, can analyse large volumes of water.

The number of *A. salmonicida* released into the water column by infected fish is obviously an important determinant of disease spread. To estimate the numbers of *A. salmonicida* at an infected farm site Atlantic salmon were experimentally infected and the number of *A. salmonicida* shed detected on antibiotic containing agar. Previously, *A. salmonicida* has been isolated from freshwater containing clinically infected experimental fish (Bullock and Stuckey, 1977; Michel, 1979; McCarthy, 1977) and carrier fish (Scallan, 1983) but the rate of shedding has not been investigated. In the present study 10^5-10^8 cfu/fish/hr were estimated to be shed from Atlantic salmon in seawater and could be detected prior to death of the fish. These values are comparable to those reported by Hoff (1989b) for *Vibrio salmonicida* in Atlantic salmon faeces (1.5×10^5 bacteria/g wet faeces).

The release of large numbers of *A. salmonicida* from infected fish indicate that during disease outbreaks there may be a significant risk of disease spread between farm sites. To assess the potential for furunculosis spread in coastal environments a particle tracking model has been developed (Turrell and Munro, 1988). Data obtained from the present

study on the shedding and survival of *A. salmonicida* were analysed in the model and the extent of disease transmission in seawater predicted. Similar models have been developed to predict aerial dispersion of bacteria (Knudsen, 1989). The model tracks the fate of particles within a hypothetical Scottish loch. The model may help to assess the ecological impact of fish farming at specific coastal sites and has previously been used to predict antibiotic dispersion from fish farms (Turrell and Munro, 1988). These models of dispersion may also help in the design of sampling regimes and the collection and interpretation of field data (Thrusfield, 1986; Knudsen, 1989).

On the basis of the data supplied to the model *A. salmonicida* was predicted to travel for at least 6,000-9,000 m downstream from the fish farm. These distances are not insignificant and show that any other farm in the loch is at risk of suffering infection. The values for shedding rate and bacterial decay used in the study were not the maximum available. However, for the purposes of disease risk assessment the worst combinations of circumstances should always be assumed (Turrell and Munro, 1988). In this respect, Turrell and Munro (1988) found that with greater values of shedding rate and lower die-off constants there was minimal reduction in the bacterial population over the 10 km of study. These studies support the circumstantial evidence for long distance (10-20 km) transmission described between fish farms in the marine environment in Scotland, Norway and N.America (Novotny, 1975, 1978; Turrell and Munro, 1988).

In experimental studies dose rates of thousands of bacteria per ml are required to establish infection (see Chapter 4). However, the simple mathematical study of infection at a cage (5.3.3b) indicate that these bacterial densities are only achieved at high infection and shedding rates. Obviously this study is simplistic and the situation in the field will be more complex. For instance, the movement of fish within the cage will produce

vortices and eddies which may lead to the retention of *A. salmonicida* in the cage. In any case, for purposes of disease risk assessment the presence of only one bacterium contacting a fish should be considered hazardous. In the field situation the infection of one fish in a cage may be sufficient to establish a disease outbreak (Turrell and Munro, 1988). The stocking densities at farms, often in excess of 50,000 fish per site, will increase the probability of infection with *A. salmonicida* present in the water column.

The assumption that *A. salmonicida* is directly transferred in water suspension may be incorrect. It would be unwise to dismiss alternative mechanisms of transmission which may increase the potential for disease spread. For instance, the movement of infected feral (Phillips *et al.*, 1985) and wild fish (Munro, 1982b) between sites may promote disease. Munro and Waddell (1984) have reported that incidence of disease is increased at farm sites in contact with migrating anadromous populations of Atlantic salmon. Alternatively, *A. salmonicida* may be transmitted by birds moving between farm sites. Birds have been inplicated in the spread of other important bacterial diseases. Willumsen (1989) isolated *Yersinia ruckeri* from gulls, *Larus* spp., and suggested that birds contaminate uninfected areas with bacteria from their faeces. Aquatic birds have also been identified as potential carriers of *Vibrio cholerae* and are believed to disseminate the pathogen over a large area (Ogg *et al.*, 1989).

Transmission of *A. salmonicida* may be facilitated by the activities of ectoparasites. Parasites may act as vectors of disease, for example, Griffin (1953) suggested that leeches and fish lice (*Argulus* spp.) may contribute to outbreaks of infectious dropsy, caused by *Aeromonas liquefaciens* (= *A. hydrophila*), in carp. Horne (1928) reported the isolation of *A. salmonicida* from sea-lice (*Lepeophthirius salmonis*) obtained from Atlantic salmon

with furunculosis. This suggests that sea-lice may act as mechanical vectors of furunculosis. The presence of parasites may also increase the susceptibility of fish to infection, by an increase in general stress levels and mechanical damage. For example, Hastein and Bergsjo (1976) reported that the lesions caused by sea-lice on Atlantic salmon facilitated access to tissues for the fish pathogen *Vibrio anguillarum.*

Protozoa have been reported to increase the ability of some bacteria to persist in the aquatic environment, eg *Legionella pneumophila* (Fields *et al.*, 1984) and *Escherichia coli* (Sambanis and Fredrickson, 1988). King and Shotts (1988) have described enhancement of *A. salmonicida* in the presence of the ciliated protozoan *Tetrahymena pyriformis.* Accumulation of *A. salmonicida* within protozoa may lead to the establishment of a minimum infective dose and an easy means of infecting fish by ingestion. Other planktonic organisms may be important in the transmission of *A. salmonicida.* For instance, zooplanktonic crustacea are believed to have a major role in the ecology of *Vibrio cholerae* (Huq *et al.*, 1983; Brayton *et al.*, 1987). The crustacea are believed to provide a source of nutrient for the bacterium and act as vehicles of transmission to humans.

This study has raised many questions concerning the transmission of *A. salmonicida* in the aquatic environment. For example, at what level is the pathogen present in the water column during disease outbreaks? To what extent is it dispersed and diluted in the coastal environment? Are biological vectors important in the spread of furunculosis? Answers to these questions will aid the development of modelling risks of infection. However, studies are dependent on the development of more sensitive detection methods. Immunofluorescent methods allow large volumes of water to be sampled and may prove useful in detecting *A. salmonicida.* For example, Enger *et al.* (1989) have recently

studied the persistence of the fish pathogen *Vibrio salmonicida* in farm sediments by use of a flourescent antibody technique. *V. salmonicida* was detected at levels of 10^4-10^7 cells/g sediment and viability was demonstrated by staining with 4', 6-diamino-2-phenylindole (DAPI) which detects intact DNA (Hoff, 1988, 1989a). However, the bacterium was detected at disease-free fish farms, as well as at diseased farms. This suggests that the antibodies (monoclonal and rabbit polyclonal) used to detect the pathogen may cross-react with other bacteria, which is always a problem with immunofluorescent techniques (Bohlool and Schmidt, 1980), and over estimate the presence of the pathogen. Alternatively, DNA probes, in conjunction with polymerase chain reaction (PCR), may provide sensitive and specific means of detecting *A. salmonicida.*

CHAPTER 6

ASPECTS OF THE FURUNCULOSIS CARRIER STATE

CHAPTER 6 ASPECTS OF THE FURUNCULOSIS CARRIER STATE

6.1 INTRODUCTION

Carrier, covertly infected, fish are believed to be a significant reservoir of furunculosis infection (McCarthy, 1977; Austin and Austin, 1987) and play a major role in the spread of the disease. In particular, the introduction of asymptomatic carriers to both fresh and seawater fish farms may be the main cause of furunculosis spread (Klontz and Wood, 1972; McCarthy, 1977; Drinan *et al.*, 1978; Novotny, 1978; Munro, 1982a, 1988). Several authors have experimentally demonstrated transmission of *A. salmonicida* between carrier and susceptible fish (Blake and Clarke, 1931; McCarthy, 1977; Scallan, 1983).

Unfortunately, research on the carrier state has been neglected in comparison with other aspects of furunculosis, eg vaccine production. In part, this is due to inadequate means of detecting covert infection in fish. The detection of covertly infected fish is important in fish husbandry. It can influence decisions on the movement of fish between sites and preliminary treatment with appropriate antibiotics. In this study two methods of carrier detection, which are based on the identification of *A. salmonicida* antigen, were investigated. In addition, preliminary studies related to the site of carriage of the pathogen in covertly infected fish were undertaken and are described below.

At present the most effective furunculosis carrier detection method is that developed by Bullock and Stuckey (1975) which involves injection of corticosteroids and heat stress. The aim of the technique is to precipitate clinical disease. The method is both time consuming and, if a carrier rate is needed requires large holding facilities. Direct culture on solid medium from kidney samples is considered ineffective, although

enrichment by prior culture of organs in broth (Daly and Stevenson, 1985; Hirvela-Koski *et al.*, 1988) has been shown to enhance detection rates. There is therefore a requirement for an effective, simple and rapid means of carrier detection.

The present study examined the relative effectiveness of detecting carrier fish by direct plating, corticosteroid/heat stress (modified protocol of McCarthy, 1977) and a commercial ELISA detection kit (Stirling Diagnostics Ltd) specific for *A. salmonicida*. This work has been previously published (Rose *et al.*, 1989b). The ELISA (enzyme-linked immunosorbent assay) detects antigens using specific antibodies which are bound to a solid support. The antigen is sandwiched between the bound antibody and a second antibody which is conjugated to an enzyme. The presence of the antigen is indicated by a colour change mediated through the activity of the conjugated enzyme on a chromogenic substrate. In addition to ELISA, the sensitivity of the dot-blot method, for detection of furunculosis carriers, was evaluated. The dot-blot is also an enzyme immunoassay which detects specific antigen. In this case, however, the antigen is bound to a solid support (nitrocellulose) and then detected with antibody.

The nature of furunculosis covert infection, eg site of carriage and physiological status of the bacterium, largely remains unknown. This is due to the ineffective detection methods which do not allow precise identification of *A. salmonicida*. The pathogen may be present within fish tissues or, as recently suggested by Hiney *et al.* (1989) on the external surface of the fish. In the present study, investigations related to the site of carriage were performed. If *A. salmonicida* is present within fish tissues it must be able to counteract the immune system of its host. Phipps and Kay (1988) have shown that *A. salmonicida* can non-specifically bind immunoglobulin (rabbit IgG and human

IgM). In the present study, the ability of fish serum proteins and IgM to mask mammalian IgG sites on *A. salmonicida* was investigated.

Hiney *et al.* (1989) have shown that in the furunculosis carrier state the pathogen is present, but perhaps not exclusively, on the surface of the host fish. In the present study, the potential antibacterial activity of rainbow trout and Atlantic salmon mucus to *A. salmonicida* were investigated. If *A. salmonicida* is sensitive to fish mucus it would suggest that the role of the skin surface in covert infection is limited.

6.2 MATERIAL AND METHODS

6.2.1 Bacteria

Aeromonas salmonicida subsp. *salmonicida* MT048, MT004 and MT423 were used and are further described in Table 6.1 and 3.2.1 respectively. *A. salmonicida* subsp. *salmonicida* B89498 was provided by the Institute of Aquaculture, University of Stirling. A strain of *Aeromonas hydrophila* and *Escherichia coli* were obtained from the DAFS Marine Laboratory, Aberdeen and Institute of Aquaculture, respectively.

6.2.2 Fish

Atlantic salmon (*Salmo salar* L.; 30-50 g) were obtained from a commercial freshwater site with a known history of both clinical and carrier furunculosis. Fish were held in a 100 l plastic tank at 11°C prior to examination of carrier status by ELISA and stress/heat test (6.2.3 a,b,c).

TABLE 6.1

Characteristics of *A. salmonicida* MT048 and MT004

Character	MT048	MT004	Character	MT048	MT004
Coccobaccilli	+	+	API20E (continued)		
Gram stain	-	-			
Autoagglutination	+	-	IND	-	-
Brown Diffusible			VP	-	-
Pigment	+	+	GEL	+	+
Motility	-	-	GLU	+	+
			MAN	+	+
API20E			INO	-	-
			SOR	-	-
ONPG	+	+	RHA	-	-
ADH	+	-	SAC	-	-
LDC	-	-	MEL	-	-
ODC	-	-	AMY	-	+
CIT	-	-	ARA	-	-
H2S	-	-	Oxidase	+	+
URE	-	-	OF/F	F	F
TDA	-	-	(Oxidative/fermentative)		
			Gas from glucose	+	+

+, positive; -,negative; F, fermentative

Atlantic salmon (10-20 g), used in assessing the dot-blot method (6.2.3 d) of *A. salmonicida* detection, were obtained from the DAFS Marine Laboratory furunculosis-free site and held in plastic tanks containing 100 l freshwater (11°C).

Atlantic salmon (20-30 g) and rainbow trout (*Oncorhynchus mykiss*; 20-30 g), for mucus (6.2.4) and serum binding experiments (6.2.5), were obtained from the Aquatic Vaccine Unit, Institute of Aquaculture.

6.2.3 Carrier Detection

a) Direct streaking

Atlantic salmon were sacrificed by benzocaine (Sigma) overdose and 10 µl loops taken from both the rectum and kidney on tryptone soya agar (TSA, Oxoid) and brain-heart infusion agar (BHIA, Oxoid). Plates were incubated at 22°C for seven days to detect the presence of *A. salmonicida*. At the same time both the kidney and intestine of these fish were removed and analysed by ELISA (6.2.3d)

b) Stress/heat test

Atlantic salmon were anaethetised with benzocaine (2.2.6) and injected intramuscularly with prednisalone acetate at 20 mg/kg. Fish were then transferred to 100 l plastic tanks containing freshwater at 18°C. Mortalities were monitored and surviving fish killed after 10 days. Kidney samples were taken with 10 µl loops from all fish on TSA to confirm the presence of *A. salmonicida*. *A. salmonicida* infection was confirmed by biochemical analysis (API20E) of representative isolates.

c) ELISA

Atlantic salmon were sacrificed by benzocaine overdose and the kidney and intestine removed. These organs were placed into antigen extraction buffer and the presence of *A. salmonicida* detected by ELISA (Aquakit Furunculosis, Stirling Diagnostics Ltd). The ELISA method has been described in 5.2.3b. The number of bacteria in tissues was estimated by comparison with the optical density of ELISA results using standard bacterial concentrations. The ELISA was performed by Stirling Diagnostics Ltd.

d) Dot-blot

i) Antigen Extraction

A number of methods for extracting *A. salmonicida* antigen were compared. *A. salmonicida* MT048 was grown for 72 hr on TSA at 22°C. Cells were suspended in phosphate buffered saline (pH 7.2, PBS, Gibco) to a concentration of 10^9 cfu ml^{-1} (optical density at 540 nm = 1). 1 ml aliquots of this bacterial suspension were centrifuged at 6,000 g for 10 min and the antigen extract decanted. The following antigen extraction procedures were performed on the *A. salmonicida* pellets:

1. Boiled at 100°C for 20 min and sonicated in a Decon Sonication Bath for 60 s.
2. Incubated in 1 ml 2% sodium dodecyl sulphate (SDS, BDH) for one hour at room temperature.
3. Incubated in 1 ml 2% SDS for one hour (room temperature) and dialysed against distilled water (*ca* 500 ml) overnight (4°C).
4. Incubated in 1 ml 2% SDS for one hour (room temperature) and boiled at 100°C for 20 min.
5. Incubated in 1 ml 2% SDS for one hour (room temperature), boiled for 20 min and dialysed against distilled water (*ca* 500 ml) overnight (4°C).

6. Lysozyme (Sigma) added to 1 mg ml^{-1}, incubated at room temperature for 30 min, and then boiled for 20 min.

All treatments were centrifuged at 6,000 g for 10 min and frozen at -20°C. Extracts were defrosted and 10-fold serially diluted in PBS and *A. salmonicida* antigen detected by dot-blot.

Studies were performed to assess the sensitivity of detection of *A. salmonicida*, by dot-blot, in the presence of Atlantic salmon kidney. The bacterium was diluted to 10^3-10^6 cfu ml^{-1} in PBS and 1 ml of each dilution centrifuged at 6,000 g for 15 min. The bacterial pellets were either resuspended in 100 µl kidney homogenate (macerated in PBS) or PBS alone. 1 ml PBS or 2% SDS was added to samples which were incubated for one hour at room temperature and then boiled for 20 min. The PBS treated samples, with and without kidney homogenate, were also sonicated for 60 s. All antigen extracts were centrifuged at 6,000 g for 10 min, to remove kidney fragments and pigment, and frozen at -20°C.

ii) Dot-blot Immunoassay

Antigen extracts (100 µl) were applied to a 0.45 µm nitrocellulose (NC) membrane (Schleicher and Schuell) using the Bio-Rad Dot-Blot apparatus. The NC was pre-wetted with 100 µl tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5) which was then drawn through the membrane by vacuum. 100 µl of antigen extract was added to NC and allowed to filter through the membrane under the action of gravity for 1-2 hr. The NC was then removed from the dot-blot apparatus and immersed in blocking solution (3% gelatin-TBS) for 60 min and then washed twice in TBS for 5 min, followed by two washes (5 min) in TTBS (1% gelatin-TBS + 0.005% Tween-20, Sigma). The NC

was then incubated, for 40 min, in two consecutive antibody solutions. NC was washed twice (5 min) in TTBS between each antibody incubation. The first antibody was a rabbit anti-*A. salmonicida* MT048 (diluted 1:1,000 in TTBS) kindly provided by Dr T S Hastings, DAFS Marine Laboratory. The second antibody was a Goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1:3,000 in TTBS. NC was then washed in TTBS for 5 min (twice) and finally TBS (5 min). The Goat antibody was detected using BCIP/NBT alkaline phosphatase colour development solution. 30 mg of nitro-blue tetrazolium (NBT, Sigma) was added to 1 ml 70% N,N-dimethyl-formamide solution (solution A). 15 mg bromochloroindoyl phosphate (BCIP, Sigma) was added to 1 ml N,N-dimethyl-formamide. (solution B). Solution A and solution B were added to 100 ml carbonate solution (0.1 m $NaHCO_3$, 1.0 M $MgCl_2.6H_20$, pH 9.8) and used immediately. NC was incubated in NBT/BCIP for up to 30 minutes and development was then stopped by two washes in distilled water (10 min). The developed membrane showed the antigens as purple dots against a white background.

6.2.4 Mucus Experiments

a) Collection of mucus

Skin mucus was obtained from Atlantic salmon and rainbow trout by carefully sliding the edge of a glass slide in an anterior to posterior direction along the flanks of the fish. Mucus (*ca* 0.1 ml) from each fish was suspended in 1 ml filter-sterilised (0.22 μm Millex, Millipore) freshwater from fish holding tanks. The mucus was suspended by repeated passage through a hypodermic needle (25G, Terumo). Mucus was used within one hour of collection and was not pooled.

b) Anti-bacterial activity of fish mucus

The anti-bacterial activity of fish mucus was investigated by assessing its ability to inhibit the growth of bacteria on solid media, using sensitivity discs, and in broth (ie minimum inhibitory concentration).

i) Sensitivity Discs

Bacteria (*A. salmonicida* strains MT423 and B89498, *A. hydrophila* and *E. coli*) were grown overnight, shaken at 22°C, in 3% brain-heart infusion broth (BHI, Oxoid) and then suspended to a concentration of 10^9 cfu ml^{-1} in PBS (optical density at 540 nm = 1). The bacterial suspension was diluted 10-fold and 100 µl spread on TSA and air-dried. Four 6 mm AA assay discs (Whatman) were carefully applied to the surface of the agar using a sterile hypodermic needle. 10 µl mucus suspension was then pipetted onto assay discs. Filter-sterilised water (10 µl) was added to one assay disc per TSA plate to act as a negative control. TSA plates were incubated for 48 hr at 22°C after which the zone of inhibition around discs was measured.

ii) Minimum Inhibitory Concentration

Mucus suspensions (duplicates per fish) were serially diluted 2-fold in 90 µl sensitivity broth (Oxoid) in a sterile microtitre plate (Flow Lab's). *A. salmonicida* strain B89498 was grown on TSA at 22°C for 48 hrs. Two colonies were suspended in 10 ml 3% TSB and incubated for 4-5 hrs, shaken, at 22°C until logarithmic phase. The broth was diluted 10-fold in sterile TSB and 10 µl added to microtitre wells. A sterility control, no *A. salmonicida* added, was included. The samples were incubated, statically, for 48 hrs at 22°C after which they were examined for bacterial growth.

6.2.5 Fish Serum Protein/IgM Binding to *A. salmonicida*

The binding of fish serum proteins and IgM to *A. salmonicida* was examined. Rabbit IgG binds to *A. salmonicida* non-specifically (Phipps and Kay, 1988), therefore, serum proteins/IgM could not be directly detected on the pathogen using rabbit antibody. An indirect method was followed to find whether fish serum/IgM could prevent non-specific binding of rabbit antibody to *A. salmonicida.*

a) Fish serum

Blood was obtained from the caudal vein of a benzocaine anaesthetised (see 2.2.6) rainbow trout using a hypodermic syringe (25G, Terumo). Blood was left to clot in a glass specimen bottle for 2-3 hrs at room temperature. Serum was collected from the clot with a Gilson pipette and remaining blood cells were removed by centrifugation. Fish serum was stored at 4°C and was used within one hour.

b) Binding experiments

A. salmonicida MT004 and MT423 were grown overnight in 3% TSB, shaken, at 22°C. Cells were suspended to an optical density at 540 nm of one in PBS. 10 µl bacterial suspensions were carefully applied to a nitrocellulose (NC) membrane and air-dried and incubated for 40 min in blocking solution (2% bovine serum albumen, BSA Sigma, in PBS) at room temperature. NC was then either incubated in rainbow trout sera, diluted 1:10 in PBS, or undiluted rabbit serum (Scottish Antibody Production Unit) each for 40 min. NC was then washed (twice) in antibody buffer (PBS + 0.2% BSA + 0.1% Triton X-100, Sigma) for 5 min. A second group of NC was incubated in fish sera followed by rabbit serum. All groups were incubated in a donkey anti-rabbit IgG conjugated to horse-radish peroxidase (HRP), diluted 1:200 in antibody buffer, for 40 min. NC were washed, twice, for 5 min in antibody buffer and then PBS. Donkey antibody was

detected by incubating NC in HRP substrate ie 50 mg diamino benzidine tetrahydrochloride (Sigma) in 0.1 m Tris HCl (pH 7.4) plus 100 µl 30% hydrogen peroxide (Sigma). The reaction was completed by washing four times in distilled water. A positive reaction was identified by development of brown colouration.

6.3 RESULTS

6.3.1 Comparison of Direct-plating, Stress/heat Test and ELISA for Detection of Furunculosis Carrier Infection

A. salmonicida colonies were not isolated on either TSA or BHIA swabbed with material from kidney and rectum of test fish. However, in the ELISA of these same fish, 56.1% were found to be carriers when the results for both kidney and intestine samples were combined. The results of the corticosteroid/heat stress carrier test, on fish from the same population, indicated that 26.4% of the fish sampled were covertly infected with *A. salmonicida*. A chi-squared comparison showed a significant difference ($\chi^2 = 9.97$, 1 df, $p<0.01$) between the carrier rates obtained from corticosteroid (stress)/heat test and ELISA (see Table 6.2). The number of bacteria ($x \pm sd$) in the kidney and intestine was estimated to be $1.2 \times 10^4 \pm 1.0 \times 10^3$ bacteria/ml ($n = 27$) and $1.2 \times 10^4 \pm 1.7 \times 10^3$ bacteria/ml ($n = 9$) respectively.

A comparison of ELISA results on the 26 fish in which both kidney and intestine were examined showed that positive tests for these organs did not always occur in the same fish. The combined carrier rate was found to be 53.8% (14/26). However, only 4/14 of carrier fish detected were positive for both tissues. Of the remainder, 5/14 were positive for kidney only, and 5/14 were positive for intestine only.

TABLE 6.2

Comparison of corticosteroid/stress and ELISA carrier test

Carrier test	Number of fish	Number of carriers detected	% carrier rate
Corticosteroid/heat	53	14	26.4
ELISA	57	32	56.1

ELISA results performed by Stirling Diagnostics Ltd

6.3.2 Detection of *A. salmonicida* Antigen by Dot-blot

Antigen from *A. salmonicida* was extracted by a variety of procedures and the sensitivity of the dot-blot methodology assessed. *A. salmonicida* alone could be detected down to 10^3 cfu (= 10^4 cfu ml^{-1}) in all the extraction regimes, except the lysozyme treatment (10^4 cfu), examined. These results are presented in Plate 6.1a.

Two treatments were chosen for further investigation of sensitivity in the presence of fish kidney. These antigen extraction regimes were i) heat (100°C, 20 min)/sonicate (60 sec) and ii) 2% SDS (one hour)/heat (100°C, 20 min), with and without subsequent dialysis. The heat/sonicate treatment could detect *A. salmonicida* antigen at 10^3 cfu (= 10^4 cfu ml^{-1}) in the presence of kidney, compared with 10^3 cfu with bacterial antigen alone. However, in contrast to the study described above, the SDS treatment could only weakly detect bacteria alone at 10^5 cfu and could not be detected in the presence of kidney. These results are presented in Plate 6.1b. The poor result for the SDS treatment was attributed to the presence of SDS which can inhibit the binding of antigen to nitrocellulose. This did not occur in the previous study because antigen was extracted from 10^9 bacteria and the resultant extract serially diluted. This would have diluted the SDS present in the extract.

6.3.3 Antibacterial Studies with Fish Mucus

a) Sensitivity discs

In total mucus from 10 rainbow trout and three Atlantic salmon was used in sensitivity experiments with *A. salmonicida* strain MT423 and three trout and three salmon were used with *A. salmonicida* strain B89498. Mucus (0.1 ml in 1 ml water) from each fish was applied to six assay discs (10 µl per disc). However, in all cases no zone of inhibition could be detected around the assay discs, of either bacterial strain studied, after incubation

Plate 6.1

a) Dot-blot of *A. salmonicida* antigen extracts

Antigen extraction procedure

a) Positive control (rabbit anti-*A. salmonicida* MT048 applied directly to NC at row 4)
b) PBS negative control
c) heat/sonicate
d) SDS treatment
e) SDS/dialysis
f) SDS/heat
g) SDS/dialysis/heat
h) lysozyme treatment

A. salmonicida concentration 1: 10^6 cfu, 2: 10^5 cfu, 3: 10^4 cfu, 4: 10^3 cfu, 5: 10^2 cfu.

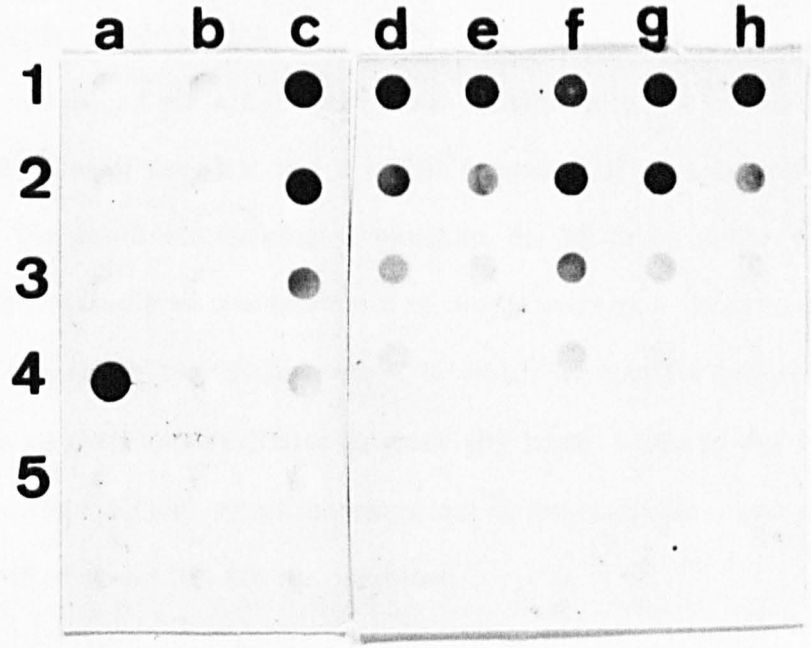
b) Dot-blot of *A. salmonicida* with fish kidney

a) SDS negative control (no bacterial antigen)
b) SDS/dialysis
c) SDS
d) Heat/sonicate
e) heat/sonicate negative control (no bacterial antigen)

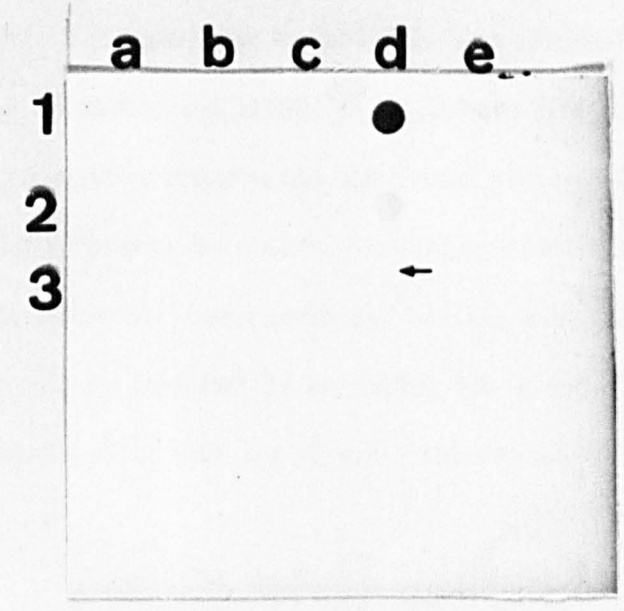
A. salmonicida concentration 1: 10^5 cfu, 2: 10^4 cfu, 3: 10^3 cfu. Arrow indicates positive blot not visible on photograph.

Plate 6.1

a)



b)



at 22°C for 48 hrs. Similarly, preliminary experiments with rainbow trout mucus showed no inhibitory effects on the *A. hydrophila* and *E. coli* strains used. No inhibition was found with the filter-sterilised freshwater negative controls. Positive controls of 0.3 μg oxytetracycline (Sigma) in 10 μl autoclaved distilled water produced 3-4 mm zones of inhibition with *A. salmonicida* strain B89498.

b) Minimum inhibitory concentration

Rainbow trout (n = 3) mucus (0.1 ml in 1 ml water) was serially diluted in sensitivity broth, duplicate microtitre wells per fish, and a known inoculum of *A. salmonicida* strain B89498 added. The broth was incubated, statically, for 48 hr at 22°C. The bacterial strain was able to grow in all concentrations of mucus examined. In contrast, there was no bacterial growth in the sterile control, to which no bacteria had been added. Oxytetracycline was also serially diluted in sensitivity broth, with and without fish mucus, and *A. salmonicida* added. At all concentrations of the antibiotic examined, down to 0.075 μg, growth of strain B89498 was inhibited.

6.3.4 Fish Serum/IgM Binding to *A. salmonicida*

The binding of fish serum proteins and IgM to *A. salmonicida* was investigated by examining the ability of these substances to inhibit non-specific binding of rabbit IgG to the bacterium. The results are presented in Table 6.3. Two strains of *A. salmonicida* were studied MT423 (an A+ strain) and MT004 (A-). Donkey IgG was found to bind to MT423 and MT004. A positive reaction was also found with rabbit IgG with these bacteria. However, in this latter case, the positive reaction may have been due to donkey IgG, which was used to detect the rabbit antibody, binding to *A. salmonicida*. The binding of donkey IgG was not inhibited by incubating the *A. salmonicida* strains in rainbow trout sera, diluted 1:10 in PBS, for 40 min. Incubation of Donkey IgG and

TABLE 6.3

Fish serum/IgM binding experiment

Bacteria		Incubation treatment			Colour reaction
A. salmonicida	"A" protein	Fish serum	Rabbit serum	Donkey anti-rabbit IgG	
MT004	-	-	-	-	-ve
		-	-	+	+ve
		-	+	+	+ve
		+	-	+	+ve
		+	+	+	+ve
MT423	+	-	-	-	-ve
		-	-	+	+ve
		-	+	+	+ve
		+	-	+	+ve
		+	+	+	+ve

-, negative; +, positive

rabbit IgG with rainbow trout serum, on nitrocellulose, did not produce a positive reaction. This indicates that the mammalian IgG used did not cross-react with the fish serum. These studies have not demonstrated that fish protein/IgM was present on the *A. salmonicida* strains investigated. However, if they are present the results suggest that they do not prevent non-specific binding of mammalian IgG.

6.4 DISCUSSION

In this study two immunologically based methods of detecting *A. salmonicida*, dot-blot and ELISA, were investigated. The detection of pathogens by immunoassay is advantageous in that it is both rapid and does not involve maintenance of fish. In addition, studies related to the nature of *A. salmonicida* in the carrier state were performed. These were: i) an assessment of the potential antibacterial activity of mucus on *A. salmonicida*. Recent evidence (Hiney *et al.*, 1989) suggests that the pathogen is present on the outer surface of carrier fish; and ii) an investigation of the binding of fish serum proteins or IgM to the outer surface of *A. salmonicida* which may mask important bacterial epitopes.

There are three limitations with immunologically based tests. Firstly, immunoassays do not differentiate between live and dead antigen. This may not necessarily present a problem as the presence of dead antigen is an indicator that a fish, and hence fish farm, has been exposed to infection and is probably still infected. Secondly, immunoassays do not give information on the antibiotic sensitivity of the strain of *A. salmonicida* which is essential if the infected fish are to be treated. And thirdly, there is always the possibility, however remote, of cross-reaction with other bacteria present, resulting in false positives. In this respect the specificity of the ELISA kit used here has been

extensively examined and found only to cross-react with *Haemophilus piscium* (Stirling Diagnostics Ltd) which is now considered to be an atypical *A. salmonicida* (Belland and Trust, 1988). It may be expedient, however, to consider immunoassays for use in rapid diagnosis of clinically infected fish and routine monitoring of fish populations for carrier status. The latter may then be confirmed by standard carrier test, corticosteroid/heat, where direct culture will enable biochemical and antibiogram identification to be carried out.

The dot-blot method has previously been used to detect the bacterial fish pathogens *Renibacterium salmoninarum* (Sakai *et al.*, 1987a,b) and *Vibrio anguillarum* (Cipriano *et al.*, 1985). In the present study *A. salmonicida* could be detected down to 10^3 cfu in the presence of fish kidney. This compares unfavourably with the studies of Sakai *et al.* (1986, 1987) who claimed to detect 100 cells *A. salmonicida* per gram of kidney using a similar method. Lee (1989) using an indirect fluorescent antibody technique (IFAT) was able to detect *R. salmoninarum* at levels as low as 100 cells/g of kidney. Samples were pre-filtered (5 μm) to remove kidney fragments which could interfere with the assay. The poor levels of detection of *A. salmonicida* reported in the present study are probably connected with inefficient antigen extraction procedures.

In contrast to dot-blot, ELISA may provide a viable alternative to the standard corticosteroid/ heat stress test (Bullock and Stuckey, 1975; McCarthy, 1977) used to identify fish populations with furunculosis-carrier status. The technique is considered to be more versatile and accurate than dot-blot, but is not as economical (Sakai *et al.*, 1987a; Cipriano *et al.*, 1985). Pascho *et al.* (1987) found ELISA to be more sensitive than fluorescent antibody and culture in detecting *R. salmoninarum*. Bernoth and Bohm (1987) were able to detect *A. salmonicida* below 1 cfu/ml in water but encountered

problems in recognising antigen in kidney samples. However, in the present study, Atlantic salmon from a known furunculosis carrier population were tested for *A. salmonicida* infection by either ELISA or standard carrier test (corticosteroid/heat). The standard carrier test detected 14/53 carriers whereas 32/57 carrier fish were detected by ELISA. Scallan (1983) also found good correlation between immunological studies on kidney material and corticosteroid/heat stress test for furunculosis carriers.

A. salmonicida was not isolated from any of the fish by direct plating of kidney and rectal material. Similarly, Scallan (1983) in one experiment was able to detect Atlantic salmon carriers by ELISA or fluorescent antibody but was unable to isolate *A. salmonicida* by culture. Evelyn *et al.* (1981) detected *R. salmoninarum* by fluorescent antibody in sockeye salmon (*Oncorhynchus nerka*) but not by culture even after corticosteroid/heat stress. With respect to *A. salmonicida* Bernoth and Artz (1989) and Power *et al.* (1987) reported that the presence of the pathogen may be overlooked by sole reliance on furunculosis agar or TSA. The absence of the bacterium by culture reported here, and by Scallan (1983), may be due to a requirement of the bacterium for specific growth conditions acquired within the carrier fish. For example, McIntosh and Austin (1988) have reported inducing cell wall deficient (L-phase variants) of *A. salmonicida* which require osmotic support for growth. If these cells are present within carrier fish they will be undetectable by conventional culture methods.

In the ELISA study reported here the presence of *A. salmonicida* antigen in both intestinal and kidney samples of Atlantic salmon but not necessarily in the same individual fish is in agreement with McDermott and Berst (1968) and Daly and Stevenson (1985) who detected the pathogen in a range of organs by culture. This confirms the notion that for complete assessment of carrier rates in populations sampling must not be restricted

to the kidney. The number of bacteria detectable in the intestine by ELISA (1.2×10^4 bacteria/ml) compares with that reported by Markwardt and Klontz (1989) who detected 1.7×10^6 cfu/g intestinal tissue nine days after gastric intubation of *A. salmonicida* into chinook salmon (*Oncorhynchus tshawytscha*).

Early workers (Mackie *et al.*, 1930, 1933, 1935; Blake and Clarke, 1931) considered that the kidney is the primary site of infection in carrier fish. In contrast, McDermott and Berst (1968) were able to isolate *A. salmonicida* from a variety of organs in carrier fish. Klontz (1968) identified the intestine as the principal site by immunofluorescence but, McCarthy (1977) was unable to repeat these findings. Scallan (1983) was able to detect *A. salmonicida* by ELISA and fluorescent antibody in the heart, gills and intestine, but only if the pathogen was also present in the kidney. A problem with these studies is that fish examined may have been stressed prior to examination and covert infection may have developed into clinical disease. In this respect, McCarthy (1977) corticosteroid/heat stressed rainbow trout carriers and sampled organs at various times afterwards. *A. salmonicida* was isolated at first only from the kidney but was later found in the intestine.

Recent evidence (Hiney *et al.*, 1989) indicates that fish skin may be an important site of carriage in furunculosis covert infection. Munro (1984) suggested that isolation from the kidney may result from a slow persistent skin infection which could otherwise pass unnoticed. Supporting the role of the external surface in covert infection Willumsen (1990) was only able to isolate *A. salmonicida* from the gills or intestine, but not from the kidney, of saithe (*Pollachius virens*) and cod (*Gadus morhua*) found in the vicinity of sea cages with furunculosis diseased Atlantic salmon.

To examine the role of the body surface as a site of carriage the potential antibacterial activity of Atlantic salmon and rainbow trout mucus against *A. salmonicida* was investigated. Previous workers have suggested that fish mucus may have antibacterial properties. For example, Itami *et al.* (1986) found high bacteriolytic activity in mucus of ayu (*Plecoglossus altivelis*) using a turbidometric method with *Micrococcus lysodeikticus* as substrate. Austin and McIntosh (1988) have presented evidence which suggests that the skin mucus of rainbow trout is inhibitory to resident freshwater bacteria, including *Aeromonas hydrophila.* Harrell *et al.* (1976) demonstrated anti-*Vibrio anguillarum* activity in rainbow trout mucus. Fouz *et al.* (1990) have shown that turbot (*Scophthalmus maximus*) mucus has a wide spectrum of antibacterial activity against both Gram positive and Gram negative bacteria, including *A. salmonicida.* However, the present study could find no antibacterial activity in rainbow trout and salmon mucus against *A. salmonicida*, *A. hydrophila* and *E. coli.* These results have been supported by preliminary studies by Fouz *et al.* (personal communication).

Alternatively, *A. salmonicida* may be found internally, but perhaps not exclusively, in covertly infected fish. If this is the case they must be able to counteract the fish's immune system. There are a number of possibilities available to account for their persistence in fish. There may be limited numbers of *A. salmonicida* in the carrier which exist in balanced pathogenicity causing a minimal amount of damage to the host. However, Scallan (1983) found no reduction in virulence of "carrier" strains of the pathogen. Phipps and Kay (1988) found that rabbit IgG and human IgM could non-specifically bind to the outer protein layer ("A" layer) of *A. salmonicida.* In the present study the ability of fish serum proteins and IgM to non-specifically bind and mask immunologically important epitopes on the bacterium was investigated. Studies on human bacterial pathogens have shown that serum proteins may interact with the

surface of bacteria and interfere with the defence of the host (eg Lammler *et al.*, 1988). However, incubation of *A. salmonicida* in fish serum did not prevent donkey IgG from binding to cells. This indicates that antigen masking may not occur in the fish host. In contrast to Phipps and Kay (1988) mammalian IgG was found to bind to both "A"-layer positive and "A"-layer negative strains of *A. salmonicida.* Finally, Graham *et al.* (1988) have shown that fish macrophages are only able to kill "A"-layer positive strains of *A. salmonicida* if activated. Non-activated macrophages may, therefore, represent an immunologically privileged site for *A. salmonicida* in the non-immune host (Trust *et al.*, 1983).

The study of covert infection with *A. salmonicida* has been restricted by the lack of a sensitive and rapid method of detection. The advent of molecular biology should provide extremely sensitive and specific means of detecting *A. salmonicida* and will allow the nature of the carrier state to be investigated more fully.

CHAPTER 7

TOWARDS A SPECIFIC DNA PROBE FOR *Aeromonas salmonicida*

CHAPTER 7 TOWARDS A SPECIFIC DNA PROBE FOR *Aeromonas salmonicida*

7.1 INTRODUCTION

A major constraint on investigations into the epidemiology of furunculosis is the lack of an effective means of detecting the causative agent, *A. salmonicida* subsp. *salmonicida*. For example, *A. salmonicida* has rarely been isolated from the external aquatic environment and is difficult to detect in covertly infected fish. Immunological methods, eg ELISA, fluorescent antibody, are available but only have detection limits of 10^3-10^4 bacteria/ml and may cross-react with non-*A. salmonicida* antigens.

The new DNA technologies should provide sensitive and specific probes for the detection of *A. salmonicida*. DNA probes will allow examination of large volumes of water to enable detection of the pathogen in the aquatic environment. *In situ* hybridisation of fish tissues will allow further investigations into the furunculosis carrier state. Hennigan *et al.* (1989) have described two DNA plasmid probes for *A. salmonicida* containing 9 and 2.5 kb fragments respectively, the gene products of which were haemolytic. These probes were used to analyse interstrain DNA sequence heterogeneity, but have not been applied to the detection of the pathogen. To date, few DNA probes have been developed for the detection of bacterial fish pathogens. These include *Pasteurella piscicida* (Zhao and Aoki, 1989) and *Vibrio anguillarum* (Rehnstam *et al.*, 1989) which have detection limits of 10^3-10^5 bacteria. Fortunately, the polymerase chain reaction (PCR) should improve the sensitivity of these and other probes (Innis *et al.*, 1989).

In this chapter preliminary development of a specific DNA probe, which in conjunction with PCR, should allow the detection of *A. salmonicida* in environmental samples is described. The probe was to be based on the "A"-protein, the crystalline surface array,

gene of *A. salmonicida.* A gene library was constructed in lambda gt11 and clones identified with rabbit polyclonal and mouse monoclonal antibodies against the "A"-protein.

The "A"-protein gene was chosen as a potential probe because it shares little homology with the corresponding gene in *Aeromonas hydrophila* (Belland and Trust, 1987). This suggests that the gene may be specific for *A. salmonicida* and hence is a likely candidate for a DNA probe. The gene has previously been cloned, on a 4.0 Kilobase fragment in lambda gt 11 by Belland and Trust (1987). Their study indicated that the loss of expression of the "A" layer, resulting from growth of *A. salmonicida* at 30°C, was accompanied by genetic rearrangement in which the N-terminal sequence of the gene was lost be deletion. A portion of the gene is, therefore, present in *A. salmonicida* strains lacking the "A"layer (Belland and Trust, 1987). Therefore, if the probe is constructed from this part of the gene, both A+ and A- strains may be detected.

7.2 MATERIALS AND METHODS

7.2.1 Bacteria

Aeromonas salmonicida subsp. *salmonicida* MT423 was used to construct the gene library in lambda gt11. This strain is described in 3.2.1. *A. salmonicida* MT004 was used to pre-absorb rabbit antiserum and is described in 6.2.1.

Escherichia coli strain Y1090 (r-) was used to plate bacteriophage and was obtained from Promega (Protoclone Lambda gt11 system). Y1090 (r-) is a restriction minus derivative of the original strain of Young and Davis (1983). The genotype of the strain is listed below:

Y1090 (r-): *E. coli lacU169 proA+ lon ara*D139 *str*A *sup*F [*trp*C22::Tn10] *hsdR hsdM+* (pMC9) pMC9 = pBR322 - *lac*I+.

Y1090 (r-) allows direct plating of bacteriophage DNA and does not require prior modification in Y1088 as required for other variants of Y1090 (Huynh *et al.*, 1985). The strain was stored at -20°C in Luria-Bertani (LB) broth with 20% glycerol. The strain was routinely maintained at 4°C on LB plates containing 100 μg ml^{-1} ampicillin, to maintain the plasmid.

Luria-Bertani (LB) medium:	
per litre	adjusted to pH 7.5 with NaOH
Bacto-tryptone	10 grams
Bacto-yeast	5 grams
NaCl	5 grams
Agar	15 grams (for plates)

7.2.2 Chemicals

Chemicals were obtained from Sigma or BDH (Analar grade) unless stated otherwise. Restriction enzymes and buffers were obtained from Boehringer-Mannheim.

7.2.3 Construction of gt11 Gene Library

An *A. salmonicida* gene library was constructed in the bacteriophage Lambda gt11 using the Promega Protoclone Lambda GT11 System. Lambda gt11 is a cloning and expression vector (Huynh *et al.*, 1985). The *Eco RI* insertion site is located within the lacZ (β-galactosidase) gene. Recombinant phage are recognised by their ability to form colourless plaques when plated on a lac-minus bacterial host (eg Y1090) in the presence of X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). DNA sequences

inserted into the cloning site may be expressed as fusion proteins, in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) which induces the lacZ gene, and screened with antibodies.

a) Extraction of *A. salmonicida* MT423 DNA

The DNA extraction method was adapted from that provided by L M Vaughan (University of Dublin, personal communication). MT423 was grown for 24 hr in 100 ml 3% tryptone soya broth (TSB, Oxoid), shaken at 22°C. The bacterial cells were pelleted by centrifugation (3,000 g, 10 min) and suspended in 20 ml TE1 (10 mM Tris, 25 mM EDTA, 150 mM NaCl, pH 7.5). This procedure was repeated twice and then the pellet was frozen at -20°C, quickly thawed and refrozen. The pellet was then washed in 20 ml TE1 and resuspended in 2 ml 1 M NaCl and stored on ice for one hour with periodic vortexing. 1 ml of TE10 (10 mM tris, 25 mM EDTA, pH 7.5) was added along with lysozyme (Sigma) to 5 mg ml^{-1} and the mixture incubated at 37°C for one hour. 200 µl 25% (w/v) sodium dodecyl sulphate (SDS, Sigma) was added and incubated at 60°C for 5 min with shaking. The SDS lysed the bacteria as indicated by a dramatic increase in viscosity of the bacterial suspension.

The lysed bacteria were repeatedly extracted with phenol, phenol/chloroform (1:1), and finally chloroform following the method of Maniatis *et al.* (1982) to purify DNA and remove protein/lipid/carbohydrate contaminants. Briefly, the aqueous DNA solution was mixed with the organic solvent for 5-10 min and centrifuged (6,000 g, 10 min) to separate the aqueous and organic phases. The aqueous layer was carefully removed with a wide-bore pipette and re-extracted. The phenol was pre-equilibrated with 0.1 M Tris (pH 8.0) and the chloroform contained isoamyl alcohol at a ratio of 24:1 (after Maniatis *et al.*, 1982).

DNA was precipitated from the aqueous layer with 2-3 volumes cold ethanol (-20°C) for 30-60 min. The DNA precipitate was collected by centrifugation at 5,000 g, 5 min, and washed in 75% ethanol. The DNA was air-dried overnight at 4°C and resuspended in 250 µl TE (10 mM tris, 1 mM EDTA, pH 8).

The method described by Belland and Trust (1987), which they adapted from Stern *et al.* (1984), was also used to extract DNA from *A. salmonicida* MT048 and MT423. Briefly, this method involves suspension of bacterial cells in 20 mM Tris (pH 7.5), 100 mM NaCl and 1 mM EDTA with lysozyme (Sigma) to 10 µg ml^{-1} and Triton X-100 (Sigma) to a final concentration of 2% (v/v). The suspension was incubated at 50°C for one hour. Proteinase K (Sigma) was then added (5 µg ml^{-1}) and incubation continued for a further 2 hrs. However, bacteria were not lysed by this method unless SDS to 5% (w/v) was added.

These extraction methods produced DNA which was resistant to digestion with the restriction enzyme *Eco RI* under normal conditions. However, a modification of the Marmur (1961) method was later found to isolate DNA which could easily be restricted under normal conditions. Briefly, the Marmur (1961) method involves suspension of bacterial cells in saline-EDTA (0.15 m Nacl, 0.1 M EDTA, pH 8) which were lysed with 25% (w/v) SDS at 60°C, 10 min. 5 M sodium perchlorate was added to a final concentration of 1 M, to dissociate protein from DNA, and repeatedly extracted with chloroform/isoamyl alcohol to deproteinise the DNA solution. After ethanol precipitation of DNA it was resuspended in saline-citrate (0.015 M NaCl, 0.0015 M trisodium citrate, pH 7.0) and then made up to 0.15 M NaCl/0.015 M trisodium citrate with concentrated saline/citrate (1.5 M NaCl, 0.15 M trisodium citrate). The solution was re-extracted

with chloroform/isoamyl alcohol and DNA precipitated from the aqueous layer with 2-3 volumes cold ethanol (-20°C).

b) Removal of RNA from isolated DNA

The isolated DNA was treated with RNAase to remove contaminating RNA (Maniatis *et al.*, 1982). RNAase was prepared as a stock solution (20 mg ml^{-1}) in 0.2M sodium acetate (pH 5.0) and heated to 100°C for 15 min to remove contaminating DNAase activity. 10 µl of RNAase was added to the DNA in TE and incubated for 30-45 min at 37°C. 10 µl 5 M ammonium acetate was added and DNA precipitated with two volumes isopropanol for 10 min at room temperature. DNA was collected by centrifugation (10,000 g, 10 min), washed in 75% ethanol, and resuspended in 400 µl TE. 40 µl 3M sodium acetate (pH 5.6) was added and DNA was precipitated in 2-3 volumes cold ethanol (-20°C), collected by centrifugation and dissolved in TE to a concentration of 1 µg ml^{-1}. This DNA solution was stored at -20°C.

c) Estimation of DNA concentration

The concentration of DNA isolated was estimated from the absorbance of the DNA solution at 260 nm. An A260 of 1.0 corresponds to 50 µg of double stranded DNA per ml (Maniatis *et al.*, 1982).

d) *Eco RI* "star" digestion of *A. salmonicida* MT423 DNA

A. salmonicida MT423 DNA was digested with *Eco RI* under conditions that are known to give rise to *Eco RI* "star" activity. Belland and Trust (1987) used an *A. salmonicida* DNA *Eco RI* "star" partial digest when constructing a lambda gt11 library.

DNA was digested in a series of 10 μl aliquots following the conditions described by Boehringer-Mannheim (Biochemicals for Molecular Biology, 1987) and Mayer (1978) for *Eco RI* "star" activity. The digestion mixture was as follows:

i) 10 μl DNA solution (10 μg)

ii) 10 μl Star buffer (25 mM Tris, pH 8.5, 2 mM M_gCl_2)

iii) 30 μl *Eco RI* (10 units/ul)

iv) 50 μl Double distilled water

Glycerol, at 7.5%, was provided by restriction enzyme buffer.

The digestion mixture was incubated at 37°C for 20 hrs and then the enzyme was inactivated by incubation at 60°C for 5 min. The extent of digestion was assessed by electrophoresis of 5 μl aliquots of digestion mixture in a 0.6% agarose mini-gel. The digestion products after 5 hr and 20 hr are shown on Plate 7.2a). Digestion mixtures were pooled and DNA precipitated with 2-3 volumes cold ethanol (-20°C). DNA was recoverd by centrifugation, washed in 75% ethanol, air-dried and dissolved in TE to give a DNA concentration of 0.1 μg μl^{-1}.

e) Agarose mini-gels

Agarose mini-gels were prepared following the protocol of Maniatis *et al.* (1982). Agarose (Gibco) was added to Tris-borate electrophoresis buffer (0.089M Tris-borate, pH 8.0, 0.089 M boric acid, 0.002 M EDTA). The agarose was dissolved in a microwave oven and cooled to 50°C and ethidium bromide (10 mg ml^{-1} in water) was added to a final concentration of 0.5 μg ml^{-1}. The liquid agarose was poured into a gel mould and a plastic comb inserted. The gel was then left to set at room temperature for one hour.

The gel was then placed in a Mini sub-cell (Bio-Rad) electrophoresis unit and electrophoresis buffer, containing 0.5 μg ml^{-1} ethidium bromide, added to cover the gel to a depth of 1-2 mm. Samples were mixed with 5 μl loading buffer (0.25% bromophenol blue, 25% Ficoll-type 400 in distilled water) and slowly applied to the gel with a Gilson pipette. Gels were run at 50 volts (DC Power Supply Model PAB, Kikusui Electronics Corp) for 1.5-2 hr. Gels were visualised using a UV transilluminator and photographed with a Polaroid camera (Polaroid type 667).

f) Ligation of MT423 DNA into lambda gt11

DNA fragments of *A. salmonicida* MT423, obtained from *Eco RI* "star" digestion of genomic DNA, were ligated in lambda gt11 using the Promega Protoclone lambda gt11 system.

The following ligation reaction was prepared:

i) 1 μl gt11 DNA (0.5 μg)

ii) 2 μl MT423 DNA (0.2 μg)

iii) 0.5 μl double distilled water

iv) 1 μl 5x ligation buffer (BRL)

v) 0.5 μl T4 DNA ligase (0.5 Weiss units, BRL)

(5x ligation buffer (BRL): 66 mM Tris-HCl, pH 7.6, 6.6 mM M_gCl_2, 10 mM dithiothreitol, 0.4 mM ATP). The ligation mixture was incubated for 2 hr at 16°C.

g) *In vitro* packaging of bacteriophage DNA

The products of ligation prepared in 7.2.3f above, was packaged into bacteriophage heads using the Promega Packagene *in vitro* packaging system. The extract was thawed on ice and briefly microfuged to the bottom of the Eppendorf tube. Ligation reaction mixture (5 µl) containing the substrate DNA, prepared in 7.2.3f, was added and the solutions mixed by gently tapping the bottom of the tube. After incubating the packaging mixture at 22°C for 2 hrs, 0.5 ml phage dilution buffer (0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.01 M $MgSO_4$) and 25 µl chloroform were added. The solutions were mixed by gentle inversion. The prepared gene library was stored at 4°C.

i) Estimation of gene library size

A culture of *E. coli* Y1090 (r-) was prepared by inoculating a single colony into LB medium. The culture was grown in a shaking incubator at 37°C overnight to an approximate optical density of 0.7-0.9 at 550 nm. After packaging, the phage was plated on X-gal plates (LB plates with X-gal top agar). The packaged phage (gene library) prepared in 7.2.3g was diluted in phage dilution buffer to 10^{-2}-10^{-4}. 100 µl of the prepared *E. coli* Y1090 (r-) culture was added and phage was allowed to adsorb for 20 min at room temperature. The adsorbed phage/Y1090 (r-) was added to 2.5 ml melted top agar (LB medium plus 0.8% agarose) containing 10 mM M_gCl_2, 20 µl IPTG (Boehringer-Mannheim) (20 mg ml^{-1} stock solution) and 50 µl X-gal (Boehringer-Mannheim) (20 mg ml^{-1} stock solution). The top agar was then immediately poured onto LB plates and allowed to set at room temperature. Plates were incubated overnight at 42°C and the number of colourless (recombinants, containing *A. salmonicida* MT423 DNA) and blue plaques (non-recombinants) counted.

7.2.4 Immunological Screening

The lambda gt11 gene library prepared above, 7.2.3, was screened using antibody probes. Rabbit IgG anti-*A. salmonicida* MT048 and mouse monoclonal (mAb) antibody (SB6) against *A. salmonicida* "A" protein were kindly given by T S Hastings, DAFS Marine Laboratory and Stirling Diagnostics Ltd. The rabbit anti-*A. salmonicida* MT048 was absorbed with *A. salmonicida* MT004 (an "A" protein minus mutant) and *E. coli* Y1090 (r-) to remove antibodies not specific for the "A" layer. The specificities of the antibodies were assessed against surface antigen extracts of *A. salmonicida* MT423 ("A" layer positive), MT004 ("A" layer negative) and Y1090 (r-). Huynh *et al.* (1985) state that it is reasonable to assume that antibodies which produce good signals with an immunoblot ("Western" blot) will produce good signals in the lambda gt11 screening procedure.

a) Bacterial antigen extracts

A. salmonicida MT423 and MT004 were grown on tryptone soya agar (TSA, Oxoid) for 48-72 hr at 22°C. *E. coli* Y1090 (r-) was grown on LB agar at 37°C for 24 hr. Bacterial colonies were removed and suspended in PBS to an optical density of one at 540 nm. 1 ml of this suspension was centrifuged at 6,000 g, 5 min and resuspended in 0.5 ml SDS-PAGE sample buffer (2x sample buffer: 30 ml 2% (w/v) SDS, 12.5 ml stacking gel buffer, 10 ml glycerol, pH 6.8 with HCl; stacking gel buffer: 0.05 g Tris-HCl, 0.4 g SDS in 100 ml distilled water at pH 6.8) and placed in a boiling waterbath for 5 min.

b) Absorption of rabbit anti-*A. salmonicida* MT048 with MT004 and Y1090 (r-)

A. salmonicida MT004 and *E. coli* Y1090 (r-) were grown overnight in 100 ml LB broth plus 10 mM MgSO4 (37°C) and 100 ml 3% tryptone soya broth (TSB, Oxoid) at 22°C respectively. Bacterial cells were pelleted by centrifugation at 3,000 g for 10 min and

resuspended in 10 ml PBS to an optical density of one at 540nm. This suspension was placed in a boiling water bath for 5 min. Cells were then pelleted by centrifugation (3,000 g, 10 min).

Rabbit anti-*A. salmonicida* MT048 antiserum was diluted 1:100 in antibody buffer (PBS, pH 7.4 + 0.2% gelatin + 0.1% Triton X-100) and mixed with *A. salmonicida* MT004 cells and incubated at room temperature for 30 min. *A. salmonicida* MT004 cells were removed by centrifugation and the rabbit antiserum absorbed with *E. coli* Y1090 (r-) cells. *E. coli* Y1090 (r-) were removed by centrifugation. The rabbit antiserum was stored in 1 ml aliquots at -20°C.

c) Western blotting

The specificity of antibodies and crude antigen preparations from selected bacteriophage clones were examined by Western blotting.

i) SDS-PAGE

Samples were resolved by SDS-PAGE (polyacrylamide gel electrophoresis) following the modified method of Laemmli (1970). SDS-PAGE was carried out using an LKB 2050 Midget Electrophoresis Unit.

SDS-PAGE gels were prepared as below:

Separating gel	12.5% gel	7.5% gel
Separating gel buffer	1,250 µl	(1,250 µl)
10% SDS	100 µl	(100 µl)
Acrylamide stock	8 ml	(2.5 ml)
TEMED	5 µl	(5 µl)
1.5% ammonium persulphate	500 µl	(500 µl)
Distilled water	to 10 ml	to 10 ml

4% stacking gel	
Stacking gel buffer	1,250 µl
10% SDS	50 µl
Acrylamide stock	625 µl
1.5% ammonium persulphate	250 µl
TEMED	5 µl
Distilled water	to 5 ml

TEMED = N N N' N'-tetramethyl ethylene diamine (Sigma). Separating gel buffer = 1.5 M Tris, 0.4% SDS, pH 8.7 with HCl. Stacking gel buffer = 0.5 M Tris, 0.4% SDS, pH 6.8 with HCl. Acrylamide stock = 30% acrylamide, 0.8% bis-acrylamide.

Stacking and resolving gels were made as above, minus TEMED and ammonium persulphate, and degassed with gentle vacuum. TEMED and ammonium persulphate were added to gels immediately before gels were poured. The resolving gel was poured and allowed to set before the stacking gel was applied.

Samples were diluted 1:1 in SDS-PAGE sample buffer containing 2 µl β-mercaptoethanol and 10% bromophenol blue and placed in a boiling waterbath for 2 mins. 10-20 µl samples were applied to SDS-PAGE gels with a Hamilton syringe which was washed with distilled water between each sample. Gels were run at 16mA for 1-2 hr using a Vokam Shandon Southern Power Supply.

ii) Immunoblot

After electrophoresis, the resolved samples were transferred from gels to 0.45 µm nitrocellulose membranes (Schleicher and Schuell) using TE series Transphor Electrophoresis unit (Hoefer Scientific Instruments) by electrophoresing in 2.5 l transfer

buffer (3 g Tris/l, 14.4 g glycine/l, 200 ml methanol/l) at 200 mA for one hour using a LKB 2197 Power Supply. Duplicate samples were transferred. One group of samples was stained with amido-black and the second group was immunostained.

The nitrocellulose was stained in 0.1% amido-black in 40% methanol/10% glacial acetic acid. The membrane was destained in the same solvent for about 10 min, followed by 10 min in 25% methanol/10% acetic acid. Destaining was stopped by repeated washes in distilled water (10 min) and the membrane dried on filter paper.

Nitrocellulose membranes for immunostaining were blocked in PBS (10 mM KPO_4, 150 mM NaCl, pH 7.4) containing 2% (w/v) gelatin for 2 hr at room temperature. The membrane was then placed in antibody buffer (PBS, pH 7.4, 0.2% (w/v) gelatin, 0.1% (v/v) Triton X-100) plus primary antibody, either absorbed rabbit anti-*A. salmonicida* MT048 or mAb, added to the appropriate dilution and incubated at room temperature for one hour. The membrane was then washed four times, 5 min each, in antibody buffer and incubated either in goat anti-rabbit antibody (for rabbit antiserum) or sheep anti-mouse antibody (for mAb) both at 1:50 dilution in antibody buffer. Sheep and goat antibodies were conjugated to horseradish peroxidase and supplied by the Scottish Antibody Production Unit. The second antibody was incubated for one hour at room temperature then the membrane was washed four times in antibody buffer and once in PBS for 5 min. The conjugated antibodies were developed with diamino benzidine tetrahydrochloride (DAB, Sigma). 50 mg DAB was added to 100 ml 0.1M Tris HCl (pH 7.4) and 100 µl 30% hydrogen peroxide. This solution was added to the nitrocellulose membrane and incubated for up to 5 min. The reaction was stopped by 4-5 washes in distilled water and the membrane was air-dried.

d) Screening gene libraries with antibody probes

Two methods were used to screen recombinant clones using antibodies:

i) Gene libraries were screened on *E. coli* Y1090 (r-) and plated on LB plates as described in 7.2.3i, but without X-gal or IPTG, and screened following a modification of the method of Boulnois (1987). Nitrocellulose membranes were soaked in 10 mM IPTG for one hour and then air dried at room temperature. LB plates were incubated at 42°C for 3 hr and then dried nitrocellulose membranes were overlaid on the top agar and incubated at 37°C for 2-3 hr. Plates were cooled to 4°C and membranes removed and screened with antibody as described for Western blots (7.2.4cii).

ii) Individual clones were screened and purified by streaking. Plaques were removed from plates in agar plugs and placed into 200 µl phage dilution buffer (plus 5 µl chloroform) and incubated at room temperature for at least one hour. Buffer (10 µl) containing eluted phage was streaked on LB plates to obtain single plaques. Melted LB top agar plus Y1090 (r-) and IPTG was poured over the surface of the plates which were then incubated at 42°C for 2-3 hrs, until plaques were visible. Nitrocellulose membrane squares (1 cm^2) were placed on the top of streaked plaques and incubated for 2-3 hr at 37°C. The membranes were screened using either the mAb or rabbit anti-*A. salmonicida* MT048 following the protocol described above for immunoblots (7.2.4cii).

7.2.5 Analysis of Clones

Immunoreactive clones were further analysed. Crude antigen was prepared from plate lysates of purified recombinant phage and characterised by Western blotting. At the same time the fragment size of clones were estimated using the polymerase chain reaction.

a) Preparation of crude antigen from lambda gt11 recombinant

Crude antigen was prepared from recombinant phage using the plate lysate method (eg Maniatis *et al.*, 1982). Phage was purified by repeated streaking of single plaques which were immunoreactive with absorbed rabbit anti-*A. salmonicida* MT048 antiserum. Purified phage was plated on *E. coli* Y1090 (r-) to give near confluent growth following the protocol given in 7.2.3i, except X-gal was omitted. Top agar was carefully removed from plates and centrifuged at 10,000 g, 10 min. Supernatant (100 µl) was added to SDS-PAGE sample buffer (plus 10% (v/v) β- mercaptoethanol/bromophenol blue) and placed in a boiling waterbath for 3-5 min. 20 µl samples were resolved on a 7.5% SDS-PAGE minigel, transferred to nitrocellulose and screened with absorbed rabbit anti-*A. salmonicida* MT048 antibody. A control, omitting the rabbit antibody, was performed to confirm that the second antibody (donkey anti-rabbit conjugated to HRP) did not cross react with the *E. coli* Y1090 (r-) antigens.

b) Size of phage insert fragments-polymerase chain reaction

The size of selected recombinant bacteriophage inserts were estimated by polymerase chain reaction (PCR) which amplifies DNA *in vitro*. The principle of PCR is described in 1.2.5b.

Oligonucleotide primers, which hybridise either side of the *Eco RI* site in the lacZ gene of lambda gt 11, were kindly donated by Dr J S Miles (Edinburgh University). The primers may be obtained from New England Bio-Lab's. The primers, FSP (358) and RSP (359), were provided at a concentration of 1 $\mu g\ ul^{-1}$ and were stored at -20°C.

The sequence of the two primers are given below:

FSP (forward):

5' - GGTGGCGACGACTCCTGGAGCCCG - 3'

RSP (reverse):

5' - TTGACACCAGACCAACTGGTAATG - 3'

The polymerase chain reaction was performed using a GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus) and Perkin-Elmer Cetus Thermal Cycler. Phage analysed by PCR were purified 3-4 times by streaking, as described in 7.2.3dii, and then agar plugs were placed in 200 µl phage dilution buffer (plus 5 µl chloroform) and left overnight at 4°C. The suspended phage (10 µl) was used directly as a sample template. A PCR reaction mixture was prepared in 0.5 ml microfuge tubes as described below:

Reaction mixture:	
double distilled sterile water	53.5 µl
10x reaction buffer	10 µl
dNTP's (1.25mM each dNTP)	16 µl
Primer 1 (FSP)	5 µl
Primer 2 (RSP)	5 µl
Sample template	10 µl
Taq polymerase	0.5 µl
Total volume:	100 µl

10x Reaction buffer: 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM M_gCl_2, 0.1% w/v gelatin). dNTP (deoxynucleotide triphosphate) mix: 125 µl of dATP, dCTP, dGTP, TTP each at 10 mM and 500 µl double distilled sterile water.

Reaction mixtures were overlaid with 100 µl Nujol mineral oil (Perkin-Elmer) and the PCR reaction was performed for 25 heat cycles. The initial template (sample DNA) denaturation step was 7.5 min at 94°C, followed by a set of three temperature steps (= one cycle). These were 2 min, 37°C (annealing); 3 min, 72°C (extension); one minute, 94°C (denaturation). The PCR samples (5 µl) were examined on a 0.7% agarose mini-gel.

7.3 RESULTS

A genomic library was prepared in the bacteriophage expression vector lambda gt11 using *A. salmonicida* MT423 DNA. The library was screened for the crystalline surface array, "A" protein produced by normal variants of *A. salmonicida*, using an absorbed rabbit anti-*A. salmonicida* MT048 antibody and a mouse monoclonal against the "A" protein.

7.3.1 The Lambda gt11 Library

A. salmonicida MT423 DNA was isolated, using a method modified from L M Vaughan, and restricted with *Eco RI* under conditions which promote "star" activity (see Plate 7.2a). *Eco RI* "star" activity recognises restriction sites which differ from the canonical *Eco RI* sequence GAATTC at least in a single position (Boehringer-Mannheim, Chemicals for Molecular Biology, 1987). There is a relaxation in specificity which results in an increase in the number of small fragments produced by digestion. The DNA isolated using this method only showed evidence of digestion, on agarose min-gels, with *Eco*

RI after 16 hr under normal conditions (37°C, Boehringer-Mannheim buffer). In contrast, partial digestion of *A. salmonicida* MT423 DNA with *Alu I* and *Bam HI* was possible within 1.5 hr at 37°C. However, *A. salmonicida* MT423, MT048 and MT004 DNA isolated, at a later date, using a modified Marmur (1961) method showed restriction with *Eco RI* within 1.5 hr.

DNA MT423 fragments obtained from *Eco RI* "star" digestion were ligated into lambda gt11 and packaged *in vitro*. The size of the gene library produced was estimated by plating an aliquot (10 µl) on *E. coli* Y1090 (r-) in the presence of X-gal and IPTG. IPTG induces the expression of the lacZ gene (β-galactosidase), and hence the DNA insert in recombinants. X-gal acts a substrate for β-galactosidase which turns non-recombinant plaques blue. The lacZ gene is split into two parts in recombinant phage and therefore β-galactosidase is not produced resulting in clear plaques. In this case 114 clear plaques (recombinants) and nine blue plaques (non-recombinants) were produced from the 10 µl gene library aliquot (see Plate 7.2b). This gives a total gene library size (500 µl) of 6,270 recombinant phage. The gene library was not amplified.

7.3.2 Immunological Screening

The gene library was screened with absorbed rabbit anti-*A. salmonicida* MT048 antibody (absorbed with *A. salmonicida* MT004 and *E. coli* Y1090 r-) and a mouse mAb (SB6) against "A" protein.

Plate 7.2

Construction of *A. salmonicida* MT423 gene library.

a) Restriction of MT423 DNA with *Eco RI* "star" activity.

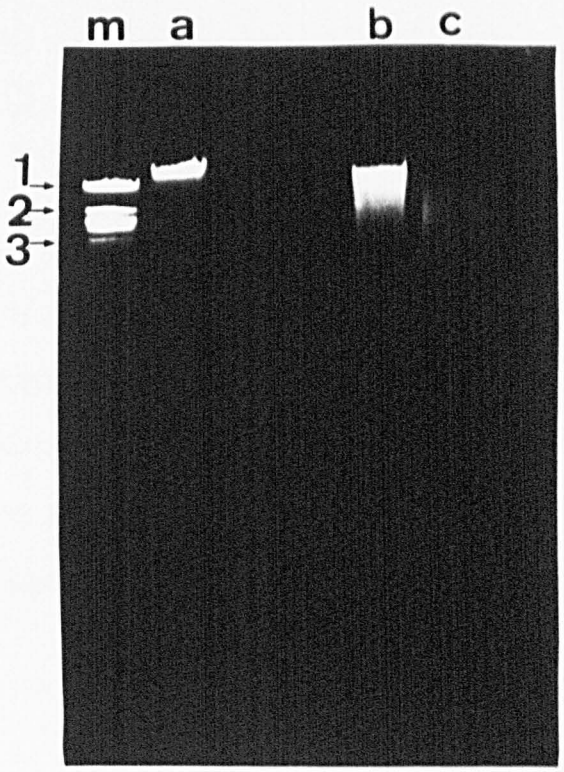
0.7% agarose gel:

m lambda/ *Eco RI* size markers (Boehringer-Mannheim) 1-21 Kilobases, 2-7 Kilobases, 3-3 Kilobases.
a *A. salmonicida* MT423 genomic DNA (undigested).
b MT423 DNA/ *Eco RI* "star" digest (5 hr, 37°C).
c MT423 DNA/ *Eco RI* "star" digest (20 hr, 37°C).

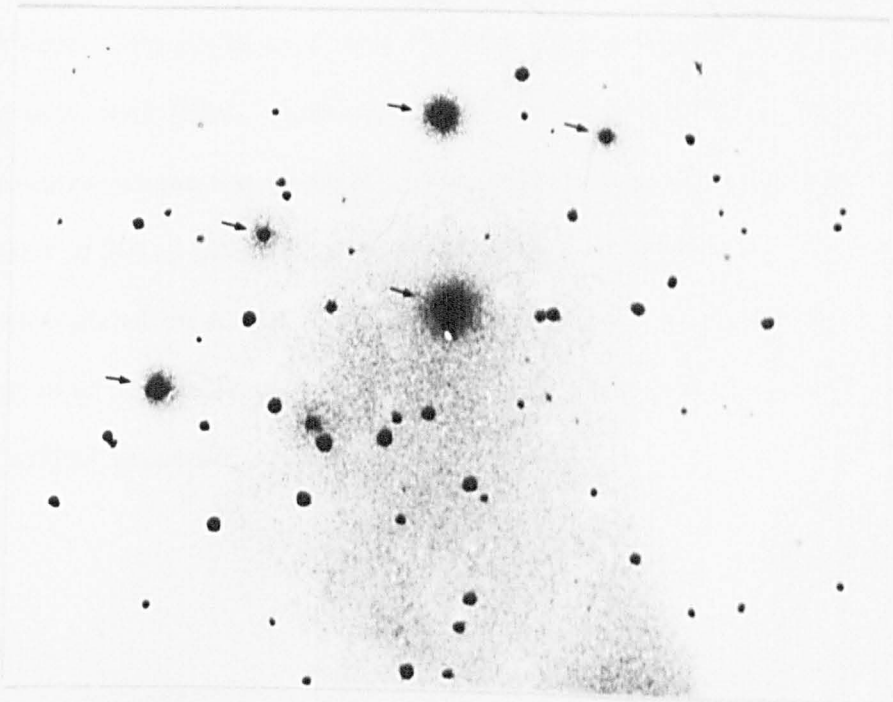
b) *A. salmonicida* MT423 gene library in lambda gt11. Library plated on *E. coli* Y1090 (r-) in the presence of IPTG and X-gal. Arrows indicate diffuse blue plaques ie non-recombinants. Discrete dark spots (clear plaques) are recombinant phage, ie contain *A. salmonicida* DNA.

Plate 7.2

a)



b)



a) Specificity of antibodies

The specificities of the rabbit antiserum and mAb (SB6) were examined against bacterial surface antigen extracts of *A. salmonicida* MT423, MT004 and *E. coli* Y1090 (r-) by Western blotting. The antibodies were used at a dilution of 1:1000 (rabbit antiserum) and 1:10000 (mAb); antigen extracts were diluted 1:10.

The antibodies did not cross-react with *A. salmonicida* MT004 ("A" minus strain) or *E. coli* Y1090 (r-). However, they both reacted with a protein of relative molecular weight approx 50,000 daltons, from *A. salmonicida* MT423, which was considered to be the "A" protein. Unfortunately, the antibodies were not mono-specific and also reacted with a series of proteins of molecular weight <50,000 daltons from MT423. These smaller proteins may have been degradation products of the "A" layer protein. These results are presented in Plate 7.1a,b.

b) Immunological screening of gene libraries

The gene library constructed from *A. salmonicida* MT423 DNA was screened directly and was not amplified. Belland and Trust (1987) previously found that "A" protein clones were unstable. It was, therefore, considered unadvisable to amplify the present library. The library was plated on *E. coli* Y1090 (r-) following the protocol in 7.2.4di and screened with mAb (SB6). Approximately 7,000 plaques were screened but only one immunoreactive plaque was identified. The positive clone was removed in an agar plug and placed in 200 µl phage dilution buffer (plus 5 µl chloroform), stored at 4°C overnight, and replated on *E. coli* Y1090 (r-). However, on re-screening the plaque was not found to be immunoreactive with either mAB (SB6) or absorbed rabbit anti-*A. salmonicida* MT048 antiserum.

Plate 7.1

Specificity of screening antibodies.

a) Immunoblot of antigen extracts developed with absorbed rabbit anti-*A. salmonicida* MT048 antibody.

b) Immunoblot of Antigen extracts developed with mAb (SB6).

Amido-black stain:

m molecular weight markers (Sigma)
a *A. salmonicida* MT423 extract
b *A. salmonicida* MT004 extract
c *E. coli* Y1090 (r-) extract
(arrow shows "A" protein).

Immunostain:

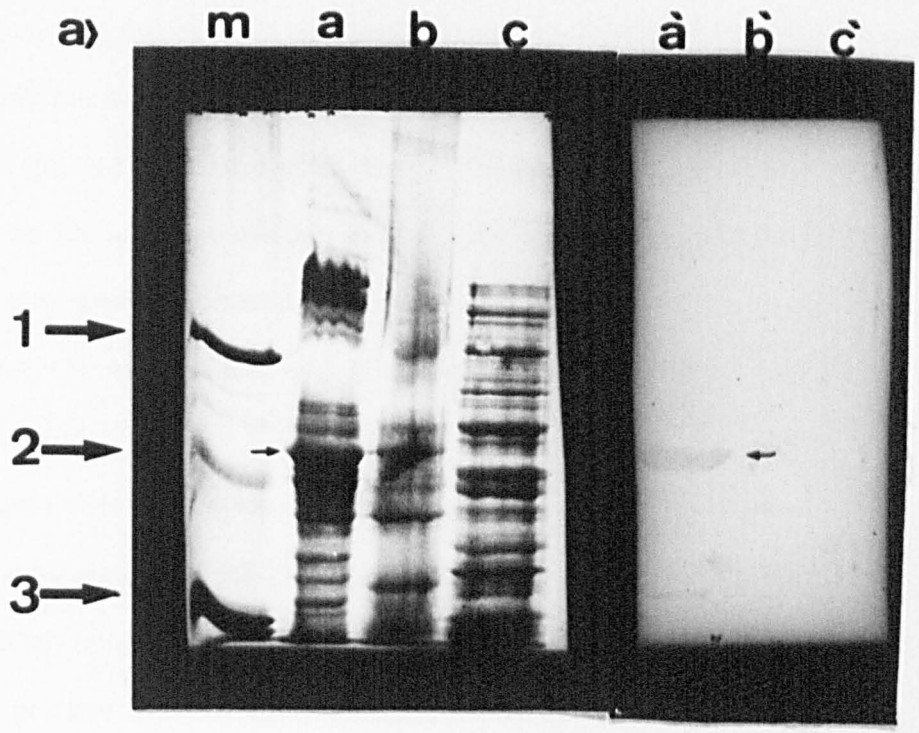
a' *A. salmonicida* MT423 extract
b' *A. salmonicida* MT004 extract
c' *E. coli* Y1090 (r-) extract

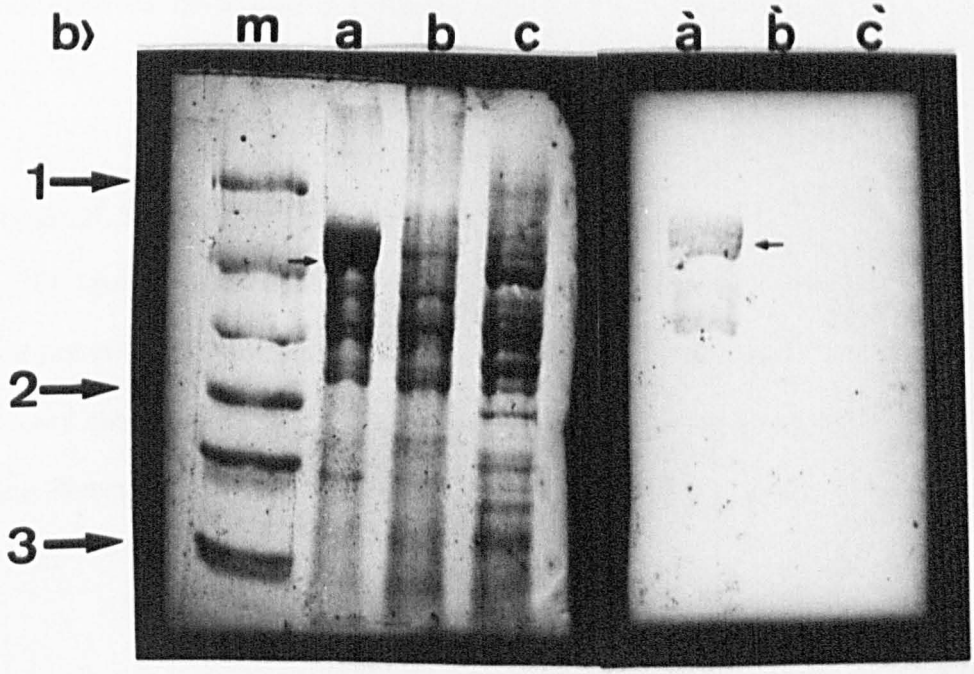
Molecular weight markers for:

a) Sigma SDS Mark VII - 1: Albumin, bovine 66,000 daltons; 2: albumen, egg 45,000 daltons; 3: trypsin inhibitor, soybean 20,100 daltons.

b) Sigma SDS Mark VII - 1: Albumin, bovine 66,000 daltons; 2: carbonic anhydrase (from bovine erythrocytes) 29,000; 3: trypsin inhibitor, soybean 20,100 daltons.

Plate 7.1





In addition, a second gene library, made by Dr M Gilpin (Polytechnic South-West) using *A. salmonicida* CM30, was screened. This library had previously been amplified. In total, approximately 90,000 plaques were plated on *E. coli* Y1090 and screened with antibody following the protocol previously described 7.2.4d. Eleven immunoreactive plaques were isolated with the absorbed rabbit anti-*A. salmonicida* MT048 antibody. The plaques did not react with mAb (SB6). The plaques were removed as agar plugs and placed in 200 µl phage dilution buffer (plus 5 µl chloroform) and stored at 4°C. Isolated plaques were re-screened, by streaking on *E. coli* Y1090 (r-), see 7.2.4dii, but only three (A1-3) were positive with absorbed rabbit anti-*A. salmonicida* MT048.

Sixteen clones (AS1-16) previously isolated from the CM30 gene library with a rabbit anti-*A. salmonicida* whole cell antibody, by M Gilpin (May 1988), were re-screened with mAb (SB6) but no immunoreactive plaques could be identified. In contrast, four clones were positive with the absorbed rabbit *A. salmonicida* MT048 antiserum.

In all immunological screenings described, a positive control (*A. salmonicida* MT423, A-layer positive cells streaked on nitrocellulose) and a negative control (lambda gt11, without *A. salmonicida* DNA insert, plaques) were used to ensure the specificity of antibody.

7.3.3 Analysis of Selected Recombinant Clones

A clone (AS20) immunoreactive with absorbed rabbit anti-*A. salmonicida* MT048 antiserum, but not mAb (SB6), was provided by Dr M Gilpin (Polytechnic South-West). For a preliminary analysis this clone, along with two recombinants (A1 and A2) from the CM30 gene library were further examined by Western blotting and PCR, to estimate DNA insert size.

a) Western blotting of crude antigen preparations from clones

Plate lysates (prepared as described in 7.2.4a) were made on Y1090 (r-) using clones AS20, A1 and A2. The antigen preparations obtained from the lysates were analysed by Western blotting using the absorbed rabbit anti-*A. salmonicida* MT048 antibody. The lambda gt11 negative control cross-reacted with the antibody at about 66,000 and 45,000 daltons. An additional protein of relative molecular weight approx. 158,000 daltons was produced by clone AS20. This protein was produced both in the presence and absence of IPTG in the top-agar of plate lysates. No additional protein bands, compared with the lambda gt11 negative control, were visible in antigens obtained from the plate lysates of clones A1 and A2. The immunoblot is presented in Plate 7.3a.

b) Size of insert in recombinant clones: polymerase chain reaction

The sizes of the inserts in the chosen recombinant phage were further analysed using polymerase chain reaction (7.2.4b). Primers, which hybridised with either side of the *Eco RI* site of the lacZ gene in lambda gt11, were used to amplify the inserts *in vitro*. An insert of approximately 500 base pairs was resolved on a 0.7% agarose gel from clone AS20. PCR analysis of A2 produced a DNA band of approximately 700 base pairs, whereas no insert was detectable from clone A1. The presence of this insert in A2 but the absence of an identifiable expressed protein by Western blot is difficult to explain. Perhaps, the expressed protein, if present coincides with one of the cross-reacting bands of *E. coli* Y1090 (r-)/lambda gt11 on the immunoblot. A negative control, using non-recombinant lambda gt11, did not produce an insert. Unfortunately, a positive control of a stable non-*Aeromonas* clone, of known insert size, was not included which could confirm that the PCR system was operating correctly. The results are presented in plate 7.3b.

Plate 7.3

Analysis of selected recombinant clones

a) Immunoblot of crude antigen produced by clones

7.5% SDS-PAGE -

amido-black stain:
m: Sigma Mark VI molecular weight markers. 1: Albumin, bovine 66,000 daltons; 2: Albumin, egg 45, 000 daltons; 3: Pepsin 34,700 daltons; 4: Trypsin, 24,000 daltons.

Immunostain:

a) *A. salmonicida* MT423 (surface antigen extract)
b) lambda gt11 (negative control)
c) Clone A1 (+IPTG)
d) Clone AS20 (+IPTG)
e) Clone AS20 (-IPTG)
f) Clone A2 (+IPTG)
g) Clone A2 (-IPTG)

arrow indicates AS20 expressed protein.

b) Insert size of clones: amplified by PCR

0.7% agarose gel -

l) 1 kilobase ladder (BRL) 1: 1,018 base pairs (bp), 2: 516 bp; 3: 394 bp; 4: 298 bp.

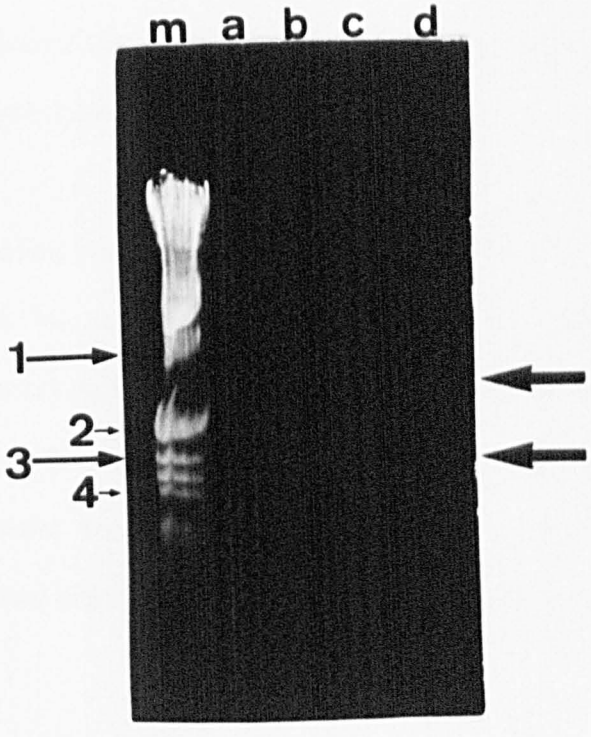
a) lambda gt11 (negative control)
b) Clone AS20
c) Clone A1
d) Clone A2

(arrows indicate position of inserts in b and d)

Plate 7.3

a)

b)



7.4 DISCUSSION

Investigations into the epidemiology of furunculosis are constrained by the absence of an efficient means of detecting the causative agent *A. salmonicida* subsp. *salmonicida.* In particular, an inability to detect the pathogen in the external aquatic environment has restricted research into its ecology. Similarly, poor detection, and isolation, of the bacterium from infected fish has hampered studies into the parasite-host relationship particularly in covert infections. The new technologies of molecular biology should provide sensitive and specific means of detecting *A. salmonicida* in the environment and infected host.

In the present study, a gene library, using DNA from *A. salmonicida* MT423, was constructed in the expression vector bacteriophage lambda gt 11. Antibodies were used to detect recombinant clones expressing *A. salmonicida* "A" protein. In total about 97,000 bacteriophage plaques, from two separate libraries (made from *A. salmonicida* MT423 and CM30) were screened for the "A" layer protein. Clones isolated from the CM30 library were immunoreactive with rabbit antiserum but not with mAB. The epitope detected by the mAB was not known. Perhaps the clones did not express the portion of the gene which possessed the mAB epitope. Alternatively, the mAB may only bind to the protein in a folded conformation.

One plaque from the MT423 library was found to be immunoreactive with the mouse monoclonal (mAB) against the "A" protein on primary screening. However, on re-screening this clone did not react with either the mAB or rabbit anti-*A. salmonicida* antiserum. Similarly, on secondary screening most of the clones isolated from the CM30 library were unreactive with these antibodies. Belland and Trust (1987) screened approximately 100,000 plaques and detected 12 immunoreactive plaques but were only

able to reisolate one of the clones. They considered that recombinant phage DNA containing the "A" protein gene was unstable. Instability of *A. salmonicida* lambda gt11 clones may be a consequence of the culture conditions required to propagate the bacteriophage. "A" protein expression in *A. salmonicida* is irreversibly lost if the bacterium is grown at high temperatures (eg Ishiguro *et al.*, 1981). Dr M Gilpin (Polytechnic South West, personal communication) has proposed that this may be the result of a temperature sensitive element within, or adjacent, to the gene. The temperature sensitive element may produce deletion of the gene at high temperatures. Unfortunately, bacteriophage lambda gt11 must be propagated at 37-42°C, to induce host bacterium lysis, which could result in the activation of the proposed temperature sensitive element within "A" gene containing clones. If this is the case, *A. salmonicida* could be cloned in plasmid expression vectors which can be grown at room temperature minimising the possibility of deletion events occurring.

The proteins expressed by three immunoreactive clones (AS20, A1 and A2) were examined by Western blot of crude antigen preparations from plate lysates on *E. coli* Y1090 (r-). AS20 was found to express a protein, of about 158,000 daltons. This protein was produced both in the presence and absence of IPTG. A1 and A2 did not appear to express proteins, even in the presence of IPTG. However, if proteins were expressed in A1 and A2 they may have co-migrated with cross-reacting lambda gt11/Y1090 (r-) antigens and were therefore unrecognised.

The AS20 immunoreactive protein does not coincide with any of the surface protein antigens of *A. salmonicida* MT423 on SDS-PAGE (see Plate 7.3a). This suggests that it is a fusion protein even though it is expressed in the absence of the lacZ inducer IPTG. In lambda gt11 cloning, foreign DNA is inserted into the lacZ gene and expressed

protein is often fused to the COOH-terminus of β-galactosidase (molecular weight 110,000 daltons, C S Gillespie, University of Stirling; personal communication), the gene product of lacZ. Assuming the AS20 protein (molecular weight 158,000 daltons) is fused with the 110,000 dalton portion of β-galactosidase then its actual molecular weight is 48,000. This is similar to the molecular weight of the "A" protein, 49-50 Kdaltons (Belland and Trust, 1987). Further analysis of protein AS20 with anti-β-galactosidase antiserum would prove whether it is a fusion protein or not.

However, the insert size of foreign DNA in AS20, estimated by PCR using primers for either side of the Eco RI site of the lacZ gene, was only about 500 base pairs (bp). An insert size of 500 bp gives a protein of 116 amino-acids (approximately molecular weight 10,000 daltons, assuming 100 daltons per amino-acid). This contradicts the results obtained by Western blot. Perhaps, amplification of the entire insert was prevented by formation of a hair-pin loop between complementary regions within the insert; or, PCR primers may have hybridised within the cloned gene, in addition to the lacZ flanking regions, resulting in premature termination of insert copies. The inser size of AS20 could be confirmed by conventional methods (eg Maniatis *et al.*, 1982).

Further work is required to produce a specific DNA probe for *A. salmonicida*. The recombinant phage AS20, described above, may provide a potential probe but the cloned gene requires characterisation. Alternatively, *A. salmonicida* gene libraries could be made in plasmid expression vectors as suggested by Gilpin (Polytechnic South West), which may be more stable than lambda gt11, and genes isolated which may be of use as a potential DNA probe. Recently, Rehnstam *et al.* (1989) have reported specific detection of *Vibrio anguillarum* using a 16S RNA oligonucleotide probe. Detection of

RNA molecules, rather than DNA, is advantageous because more copies are present within the bacterial cell. RNA probes, therefore, have a potential for high sensitivity. Investigation of RNA probes for detection of *A. salmonicida* may be profitable.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

Furunculosis, causative agent *Aeromonas salmonicida* subsp. *salmonicida*, is an important bacterial disease of salmonids and a major constraint on commercial salmon culture. The disease is regularly encountered at Scottish marine salmon farms (Munro, 1988). Infection at marine fish farms has been attributed to the introduction of covertly infected fish from freshwater sites (Drinan *et al.*, 1978; Novotny, 1978; Ezura *et al.*, 1984). However, circumstantial evidence suggests that furunculosis may spread between salmon farms in the marine environment (Novotny, 1975, 1978; Turrell and Munro, 1988; Munro, 1988) and several studies (Scott, 1968; Smith *et al.*, 1982) have suggested that fish may become infected in seawater. This thesis presents studies on the epidemiology of *A. salmonicida* in the marine environment.

In these investigations, disease transmission was studied in relation to survival of *A. salmonicida* in seawater; its infectivity in the marine environment; and the release of the pathogen from infected fish. These factors were assumed to be major determinants in the spread of furunculosis. The potential for furunculosis spread was estimated using the computer model of Turrell and Munro (1988) for tracking the fate of particles in a hypothetical sea loch.

Studies on the existence of unculturable-but-viable forms of *A. salmonicida* in the external aquatic environment did not support the previous claim of "dormancy" in this pathogen (Allen-Austin *et al.*, 1984). The re-appearance of the bacterium in microcosms, after colony-forming units (cfu) had fallen to zero, was attributed to the presence of small numbers of *A. salmonicida* rather than dormant bacteria. Microcosm aliquots

were also injected into juvenile Atlantic salmon, which may provide optimal conditions for resuscitation of putative dormant bacteria. However, *A. salmonicida* could not be isolated from these fish even after standard carrier test.

It should be emphasised that the absence of dormancy in this pathogen can never be proven beyond doubt. For example, *A. salmonicida* may only enter a dormant state under specific starvation conditions, not provided in this study. Alternatively, the method used to isolate dormant forms, ie injection into fish, may not be appropriate. In this respect, it may be useful to apply the techniques used in the study of dormancy in enteric bacteria (Roszak and Colwell, 1987a) to *A. salmonicida.* These include the direct viable count of Kogure *et al.* (1979) and microautoradiographic studies of nutrient uptake in dormant bacteria (Roszak and Colwell, 1987b).

The ability of *A. salmonicida* to survive in the marine environment is obviously an important factor in its epidemiology. The influence of several factors on the survival of the bacterium were examined. These included: type of microcosm; prior growth conditions of bacterium; sterile and non-sterile seawater; levels of nutrient and survival in sediment. Survival was generally shown to be of short duration (<10 days). However, the addition of nutrient to sterile artificial seawater (BSM) prolonged the survival of the pathogen for several weeks and, in some cases, even resulted in reproductive growth of the pathogen. The significance of this result *in situ* is not known but survival in sterile seawater obtained from beneath a salmon cage, in which there would be expected a high nutrient input, was not extended. These results confirm that *A. salmonicida* is an obligate pathogen (Popoff, 1984).

Extrapolation of these laboratory microcosm results to the natural environment should be cautious. Microcosms are closed systems and inevitably simplifications of the variable and heterogenous environment which they model. For example, survival studies on *Escherichia coli* in freshwater have found that the bacterium can persist for about a week (McFeters *et al.*, 1982) whereas others have been able to isolate cfu for up to 260 days (Flint, 1987). Suter (1985) has emphasised that introduction of an organism into an experimental system may not accurately describe its behaviour in the natural environment. In this regard, the survival of *A. salmonicida* should ideally be monitored in nature. However, few authors have been able to isolate, or detect, the pathogen in the external aquatic environment. Those workers reporting isolation of the bacterium, eg Scallan (1983), have been unable to do so quantitatively. There is, therefore, a requirement for specific and sensitive methods of detection of *A. salmonicida* which it is hoped will be provided by DNA technologies.

The release of *A. salmonicida* from experimentally infected Atlantic salmon was assessed. This allowed the number of bacteria in the water column during disease outbreaks to be estimated. Fish were found to shed between 10^5-10^8 cfu/fish/hr which is similar to recently published figures for *Vibrio anguillarum* (Kanno *et al.*, 1989) and *Vibrio salmonicida* (Hoff, 1989b).

The *A. salmonicida* shedding rates were used in combination with a typical survival value for the pathogen in non-sterile seawater and the potential for furunculosis disease spread assessed using a computer model (Turrell and Munro, 1988). The model estimated that viable *A. salmonicida* could be transmitted for at least 6 km in the hypothetical Scottish sea loch assuming relatively low levels of shedding from infected fish and short

survival of the pathogen. In the future, this model should be assessed against actual bacteriological surveys at disease outbreaks and this, once again, emphasises the requirement for an efficient means of detecting *A. salmonicida.*

The computer model described above assumes that *A. salmonicida* is solely transported in suspension through the water column. However, other modes of transmission may be important. For example, the movement of infected wild and feral fish (Phillips *et al.*, 1985; Munro, 1982; Munro and Waddell, 1984), or other animals such as birds and planktonic organisms, between farm sites may contribute to disease transmission. Recently, Willumsen (1990) has reported isolating *A. salmonicida* subsp. *salmonicida* from Atlantic cod (*Gadus morhua* L.) and saithe (*Pollachius virens* L.) caught within 200 m from sea cages containing Atlantic salmon suffering from furunculosis. Previous authors (Klontz and Wood, 1972; Novotny, 1978) have reported isolation of the pathogen from non-salmonid marine fish in the vicinity of sea cages. Further studies are required to monitor the movement of fish between farm sites. Wyche (1984) reported that juvenile saithe have restricted territories in nature suggesting that they would remain within the vicinity of a cage. However, maturing fish may migrate more widely and other coastal fish eg mackerel can travel 30-90 km/day (Dr A Johnstone, DAFS Marine Laboratory, personal communication). Phillips *et al.* (1985) found that rainbow trout released from a fish farm in a freshwater loch travelled considerable distances.

The presence of a single bacterium of *A. salmonicida* in the environment should be considered hazardous (Turrell and Munro, 1988). However, in studies on the infectivity of the pathogen in seawater, by bath challenge, Atlantic salmon had to be exposed to 10^4 cfu ml^{-1} for 1-3 days to establish infection. In contrast, with a lower challenge

dose (10^2 cfu ml^{-1}) fish had to be exposed for up to three weeks before specific mortalities occurred. These results support the immediate removal of dead/moribund fish from farm sites to minimise disease outbreaks.

Direct contact with the pathogen may not be the sole means of infection. In the present study, ingestion of 10^5 cfu, in furuncle material, resulted in specific mortalities. Alternative methods of infection, eg via ectoparasites such as sea-lice and by ingestion of plankton carrying the pathogen, should not be dismissed and, once again, monitoring the bacterium at disease outbreaks will allow the importance of different infection processes to be assessed. For example, carriage of *A. salmonicida* on sea-lice mouthparts and subsequent infection of fish during ectoparasitic feeding may be equivalent to injecting *A. salmonicida.* Intraperitoneal injection may produce clinical disease with only one cfu of a virulent strain (Dr T S Hastings, DAFS Marine Laboratory, Personal communication).

Fish disease research is handicapped by the lack of a satisfactory, standardised method of experimental replication of disease (Michel, 1980). For example, the method of challenge may be important in assessing the efficacy of vaccination (Ellis, 1988). Experimental challenge procedures may bypass host and microbial processes and not reflect natural susceptibility or infectivity (Isenberg, 1988). An understanding of the natural infection process will enable the development of laboratory challenges with *A. salmonicida.*

Fish covertly infected with *A. salmonicida* are believed to play a major role in the epidemiology of furunculosis and act as reservoirs of infection (McCarthy, 1977; Austin and Austin, 1987). The detection of carriers is increasingly important because of the

possibility of vaccinated, or antibiotic treated, fish becoming covertly infected (Munro, 1982a, 1984). Introduction of carriers to freshwater and marine fish farms may be one of the main causes of furunculosis spread (Munro 1982b, 1988). On the basis of studies presented here, routine carrier testing appears possible with ELISA. However, the standard carrier test of Bullock and Stuckey (1975) is required to isolate the pathogen for positive identification and determination of antibiotic sensitivity. Unfortunately, the standard carrier test is both time consuming and requires large aquarium facilities.

The carrier state is naturally unstable and can develop into clinical disease when fish are under stress. This is the basis of the standard carrier test (Bullock and Stuckey, 1975; McCarthy, 1977). Carrier instability may prove to be an important constraint on studies of covert infection because any movement of fish, or experimental treatment, may upset the host-parasite relationship in the carrier fish. This may explain differences between authors as to the primary site of carriage of *A. salmonicida* in the covert infection. For example, Hiney *et al.* (1989) studied a carrier population of fish and found that *A. salmonicida* could be isolated from the outer surface of the majority of these fish. However, the pathogen could also be isolated from the blood of a few fish. This may indicate that *A. salmonicida* is present in several locations in carrier fish or, alternatively, that in this experiment fish were in the process of developing clinical disease.

Studies related to the nature of the carrier state are presented in this thesis. *A. salmonicida* may be present on the outer surface of carrier fish (Hiney *et al.*, 1989) and the reported antibacterial activity of fish mucus (Austin and McIntosh, 1988) could be important in limiting infection. To test this, the antibacterial activity of fish mucus on the pathogen was examined. However, both rainbow trout and Atlantic salmon skin

mucus was found not to possess inhibitory activity against *A. salmonicida*, nor against *A. hydrophila* and *E. coli*. This result does not contradict the previous claim of carriage on the fish surface.

The internal environment cannot be dismissed as a site of covert infection. In this thesis, the ability of fish serum to mask important bacterial epitopes, and hence afford protection within the host, was examined. Mammalian IgG can non-specifically bind to *A. salmonicida* (Phipps and Kay, 1985). In this study, prior incubation in rainbow trout serum did not inhibit the binding of mammalian IgG to the pathogen. This suggests that fish serum proteins do not mask *A. salmonicida* in the host. McIntosh and Austin (1988) have demonstrated that *A. salmonicida* can form L-phase variants, ie lack a cell wall, and have claimed to isolate these forms from infected fish. L-forms can evade leucocytes and macrophages and are not recognised by the immune system (McGee, 1986). McIntosh and Austin (1989) suggest that they may be important in the carrier state.

The new DNA technologies should allow further investigation of the carrier state. For example, is covert infection merely delayed development of clinical disease due to the presence of only a few bacteria in the carrier host? *In situ* nucleic acid hybridisation has been used with success to identify human pathogens, eg *Chlamydia trachomatis* (Horn *et al.*, 1988), in biopsied tissue sections and can be used in conjunction with polymerase chain reaction (Impraim, 1987) which amplifies specific DNA sequences thereby increasing sensitivity. These techniques will allow the site of carriage of *A. salmonicida*, for instance the outer surface of fish (Hiney *et al.*, 1989) or intracellularly within macrophages, to be determined.

The work presented in this thesis has emphasised the requirement for an efficient method of detecting *A. salmonicida* in all aspects of epidemiological research. Preliminary development of a specific and sensitive DNA probe against the pathogen has been undertaken. It is hoped that in the near future a specific DNA probe will be developed which will enable the many questions raised by these investigations to be answered.

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