Persistence of *Flavobacterium psychrophilum* in the aquatic environment
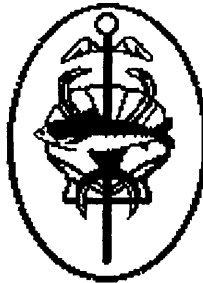
by

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to Jane...

for the strength she gave me

to my parents...

for their support and ...patience

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and has not been previously submitted for any other degree or qualification.

The work, of which it is a record, has been carried out by myself. The nature and extent of any work carried out by, or in conjunction with others, has been specifically acknowledged by reference.

Ioannis Vatsos

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thank Linton (EM) for assisting me with the electron microscopy procedures. I hope they pay him a lot for his services!

And talking about money, I would like to express my gratitude to IKY for financially supporting this project.

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And since I know that I am not perfect (!!) and I did (many, I am afraid) mistakes, I would like to say a big 'sorry' for all the troubles I caused to everybody.

ABSTRACT

Flavobacterium psychrophilum is the causative agent of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD). Despite RTFS causing significant economic losses in salmonid aquaculture, there is limited information on the epidemiology of the disease and the pathogenicity of the bacterium. An important step for the development of successful control strategies for the disease is the development of rapid, sensitive and specific diagnostic tools for the isolation and identification of the pathogen in environmental and fish samples. This study examined the potential of using a number of different methods to identify the bacterium in environmental samples. In addition the ability of the bacterium to survive under conditions of prolonged starvation and the morphological as well as functional changes that occur, were examined.

The potential of using a polymerase chain reaction (PCR) to detect and monitor *F. psychrophilum* in farming systems was investigated in this study. After validating the PCR assay in terms of sensitivity and specificity, two different surveys were performed on two fish farms with similar tank layouts, both of which had an endemic problem with RTFS. The results of both surveys indicated that the majority of areas of both farms sampled (egg incubators, hatchery, fry tanks, broodstock, farm outlet) were positive for the pathogen, while both inlets were negative. An inappropriate farm layout and possibly ineffective disinfection procedures may have led to the outbreaks of RTFS with high mortalities on these farms.

Morphological and functional changes in *F. psychrophilum*, observed under conditions of starvation were examined. Bacteria maintained in stream water stopped multiplying and became small and rounded. Their culturability declined until it was no longer possible to obtain colonies on agar plates 19 weeks after setting up of the experiment. However, even after 36 weeks, it was still possible to obtain growth of the bacterium by a resuscitation step in *Cytophaga* broth. The culturability of the bacterium did not correspond with its viability as tested with a Live/Dead kit. Bacteria maintained in distilled water or treated with a disinfectant, appeared non-viable and non-culturable 1h after setting up the experiment. No morphological changes were observed in the bacteria maintained under these conditions. Bacteria maintained in

broth were present as long, slim rods, some of which developed into 'ring' formations. Small differences were observed in the antigen profiles of the bacteria maintained under the different treatments, possibly due to a reduction in the size and metabolism of the bacteria. There was also a marked decline in the sensitivity of a PCR method used to detect bacteria 16 weeks from the onset of the study, with differences also observed in the sensitivity of the PCR between bacteria maintained under the different treatments.

The ability of *F. psychrophilum* to attach to unfertilised rainbow trout eggs and to hydrocarbon n-hexadecane was examined, whereby five different isolates of *F. psychrophilum* obtained from a variety of origins were compared. The effect of the age of the bacterium and conditions of starvation on the ability of the bacterium to adhere, were also evaluated. The different isolates were found to exhibit a similar ability to attach to both substrates. Increased surface hydrophobicity and a greater ability to attach to the surface of the eggs were observed with bacteria aged for one month, compared to bacteria cultured in *Cytophaga* agar for only three days.

The potential of four different methods to identify and enumerate *F. psychrophilum* in water samples was also examined. Semi-quantitative PCR and quantitative PCR using an internal standard appeared to be specific but lacked reproducibility. The *in situ* hybridisation technique detected bacteria from three-day old culture but no signal was observed when one-month old bacteria were tested. Further investigation is needed to validate the method in terms of specificity. The IFAT method provided positive results with aged bacteria however, there is concern over the specificity and sensitivity of the antibodies used.

The ability of *F. psychrophilum* to colonise the surface of rainbow trout eggs after a bath challenge and the effects of this colonisation on the egg was also investigated. The bacterium appeared not to penetrate within the eggs after colonisation of the surface of the eggs. Further studies are needed to confirm this, however, especially under adverse environmental conditions as often observed in commercial hatcheries during incubation (e.g. high egg densities, reduced water flow, higher water temperature).

The techniques used in this study show great potential for the investigation of the epidemiology of the disease and once the problems discussed are resolved, their application will undoubtedly assist in the successful development of control strategies for RTFS.

LIST OF PUBLICATIONS

Vatsos, I. N., Thompson, K. D. and Adams A. (2001) Starvation of *Flavobacterium psychrophilum* in stream water, broth and distilled water. *Applied and Environmental Microbiology* (submitted)

Vatsos, I. N., Thompson, K. D. and Adams A. (2001) Adhesion of the fish pathogen *Flavobacterium psychrophilum* to unfertilised eggs of rainbow trout (*Oncorhynchus mykiss*) and n-hexadecane. *Letters in Applied Microbiology* (submitted)

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ABBREVIATIONS

AODCM	acridine orange direct counting method
APES	3-aminopropyltriethoxysilane
APS	ammonium persulphate
BCWD	bacterial cold water disease
bp	base pair
BSA	bovine serum albumin
CFU	colony forming unit
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
h	hour
HMDS	hexamethyldisilazane
HPLC	high performance liquid chromatography
IFAT	immunofluoresent antibody technique
IgG	immunoglobulin G
IHC	immunohistochemistry
IS	internal standard
kbp	1000 bp
kD	kilodalton
M	molar
min	minutes
ml	millilitres
µl	microlitres
µm	micrometer
mM	millimolar
mRNA	messenger RNA

NCIMB	national collections of industrial and marine bacteria
NCTC	national collection of type cultures
OS	original sequence
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RAPD	random amplified polymorphic DNA
RTFS	rainbow trout fry syndrome
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	saline salt citrate
TBE	tris borate EDTA
TBS	tris buffered saline
TEMED	N,N,N,N-tetramethylethylenediamine
TSB	tryptone soya broth
TTBS	tween 20-TBS
TYE	tryptone yeast extract
UK	United Kingdom
USA	United States of America

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CHAPTER 1

GENERAL BACKGROUND

1.1 DISEASES ASSOCIATED WITH *FLAVOBACTERIUM PSYCHROPHILUM*

1.1.1 History

Flavobacterium psychrophilum was originally named *Cytophaga psychrophila* and was initially isolated from an outbreak of bacterial cold water disease (BCWD) in coho salmon (*Oncorhynchus kisutch*) in Washington State, USA in 1948 (Wood and Yasutake, 1956; Borg, 1960). Since then the organism has been re-classified twice, firstly as *Flexibacter psychrophilus* (Bernardet and Grimont, 1989) and more recently as *Flavobacterium psychrophilum* (Bernardet *et al.,* 1996). Since 1986, *F. psychrophilum* has also been associated with another syndrome that has caused great problems and high mortalities in rainbow trout (*Oncorhynchus mykiss)* fry and fingerlings in many countries, including Germany, Italy, UK, France, Denmark, Finland, Spain (Weis, 1987; Bernardet *et al.,* 1988; Lorenzen *et al.,* 1991; Bruno, 1992; Sarti *et al.,* 1992; Toranzo and Barja, 1993; Wiklund *et al.,* 1994), Tasmania and Japan (Chakroun *et al.,* 1997). The disease was known by a variety of names in these countries, such as Fry Anaemia, Fry Mortality Syndrome and Visceral Myxobacteriosis. However, the name that is most widely used today is rainbow

trout fry syndrome (RTFS). Since 1993, the disease has also been reported in Chile, causing important losses (Bustos *et al.,* 1995)

Over the last decade *F. psychroplilum* has also been reported in other species, such as wild ayu (*Plecoglossus altivelis*) and pale chub (*Zacco platypus*) (Iida and Mizokami, 1996), eel (*Anguilla anguilla*), carp (*Cyprinus carpio*), tench (*Tinca tinca*) and crucian carp (*Carassius carassius*) (Lehmann *et al.,* 1991) and more recently in Baltic salmon brood fish (Ekman *et al.,* 1999).

1.1.2 Causative Agent

F. psychrophilum is a Gram negative, weakly refractile slender rod with rounded ends (Holt *et al.,* 1993). Its size ranges from 0.3 to 7 μm, although some filamentous (10-40 μm) and some pleomorphic forms also exist.

Gliding motility is always reported, although some times it is slow and difficult to observe (Bernardet and Grimont, 1989; Lehmann *et al.,* 1991; Schmidtke and Carson, 1995).

Cytophaga agar (Anacker and Ordal, 1959) is the most widely used medium for the isolation of *F. psychrophilum* from infected fish (note: in this thesis, the *Cytophaga* agar used is the modified *Cytophaga* agar developed by Bernardet and Kerouault, 1989, Appendix 3.1). Some authors also suggested various modifications of this medium for improved results (Iida and Mizokami, 1996; Daskalov *et al.,* 1999). On this medium, yellowish, smooth, raised, convex colonies with spreading irregular edges appear, after 48-96 hours at 15-20° C (Lehmann *et al.,* 1991; Holt *et al.,* 1993). Colonies do not adhere to the agar (Lehmann *et al.,* 1991; Toranzo and Barja, 1993). On the other hand, the

bacterium shows poor or no growth on tryptone soya agar, brain heart infusion agar and Columbia blood agar (Santos *et al.,* 1992). In liquid media, improved growth is obtained if *Cytophaga* broth and tryptone yeast extract salts are used (Holt *et al.,* 1993). An interesting feature of the cultures is a characteristic cheese-like odour (Lorenzen *et al.,* 1997). It appears, however, that the culturability of *F. psychrophilum* is not always related to its viability, as Michel *et al.,* (1999) observed. They found that there was a difference between the viability obtained from colony forming units (CFU's) and the viability observed using the Live/Dead viability kit (Molecular Probes). They also concluded that the bacterium appeared to be susceptible to osmotic change.

F. psychrophilum is strictly aerobic and grows at temperatures ranging from 4 to 26° C (the bacterium does not grow when the temperature is 26° C or above, Pacha, 1968) and the optimal growth temperature is approximately 19.6^O C (Uddin and Wakabayashi, 1997). However, the optimal temperature for protease production appears to be much lower, around 13.3 $\pm$ 1.9^O C. With respect to the salt tolerance of the bacterium, Holt *et al.,* (1993) found that the organism could tolerate up to 1% NaCl.

Many authors using different techniques (serological analysis, genetic analysis, biochemical profiles) investigated the variation among isolates. Strains of *F. psychrophilum* generally display little variation (Lorenzen *et al.,* 1997). However, Pacha (1968) found a variation in phenotypic characteristics of various strains. He also suggested that this variation may be directly related to the pathogenicity of the bacterium. Schmidtke and Carson, (1995) also confirmed this variation. In an other study, Bertolini *et al.,* (1994b) studying the protease patterns of several isolates concluded that according to the absence or presence of certain proteases,

there was a correlation between proteases expressed and the host from which the bacterium was isolated. Thus, they divided the strains they tested into 4 groups. Madsen and Dalsgaard, (1999) reported that the elastin-degrading stains of *F. psychrophilum* appeared to be most virulent. Chakroun *et al.,* (1997) using RAPD to screen 60 isolates also found that there is a strong correlation between some patterns and the fish host.

Holt *et al.,* (1993) using a single agglutination method found 28 strains of the bacterium shared common antigens. However, they concluded that it would be necessary to examine more strains, since some appeared to possess additional antigens. Wakabayashi *et al.,* (1994) also came to the same conclusion. In addition, these authors managed to divide the *F. psychrophilum* strains into two distinct serotypes, O-1 and O-2. Bertolini *et al.,* (1994a) after studying the variability in the protein and lipopolysaccharide components, found that the species-specific common protein antigen of *F. psychrophilum,* which is the basis of the serological confirmation. However, they only studied a limited number of strains and thus further investigation is needed to confirm this finding. On the other hand, distinct antigenic groups exist among the various strains of the bacterium based on the LPS components.

Bernardet and Kerouault, (1989) studied the DNA base composition (G+C content) of several strains of the bacterium and found that this content ranged from 32.5 to 33.8 mol%. Lorenzen *et al.,* (1997) conducting a plasmid analysis of several strains, found that isolates from clinical outbreaks of RTFS/BCWD had one plasmid of approximately 3.2 kbp and isolates from chronically infected fish had an additional plasmid of 2.6 kbp. Isolates without such plasmids or with two plasmids of different size generally originated from fish without classical signs of

RTFS/BCWD. However, they concluded that challenge experiments including isolates with different plasmid profiles are needed to determine the role of these plasmids. In contrast, Madsen and Dalsgaard, (2000) found no clear correlation between the existence of the 3.3 kb plasmid and virulence.

1.1.3 Ecology-Pathogenesis

Concerning RTFS, heavy mortalities (up to 80 %) occur when water temperatures are 10° C or below (Bernardet et al., 1988; Austin, 1992; Santos et al., 1992). In some cases the disease also occurs at higher temperatures, but above 15° C mortality declines progressively (Dalsgaard, 1993). Rainbow trout fry are the most vulnerable although brown trout (Salmo trutta L.) fry also appear to be susceptible (Sarti et al., 1992). Fish weighing 0.2-1 g, approximately 7 weeks after the start of the feeding, are most usually affected (Bernardet et al., 1988; Lorenzen et al., 1991; Sarti et al., 1992; Rangdale, 1995). The pathogen can also affect larger fish, but in this case only skin and muscles are affected (Bruno, 1992; Santos et al., 1992).

Coagulated-yolk disease often proceeds outbreaks of BCWD and mortality as high as 20-50% may occur (Holt et al., 1993) when the water temperature is 4-10° C. If the disease is delayed until alevins are placed in rearing units then the mortality is around 20% and destruction on the peducle is the most characteristic external lesion (Holt et al., 1993).

There is little information regarding the route by which the organism enters the fish and how it spreads within the body. However, many experimental trials have been conducted to investigate possible routes. So far, under experimental

conditions, only intraperitoneal and intramuscular injections have been used to successfully transmit RTFS, since they provided satisfactory and reproducible results (Madsen and Dalsgaard, 1999, Garcia *et al.,* 2000). An interesting observation by Holt (1987 cited by Dalsgaard 1993) was the fact that the virulence mechanisms of the pathogen were better expressed in the muscle tissue than in the peritoneal cavity. Bath challenge and cohabitation failed to provide reproducible results (Austin, 1992; Iida and Mizokami, 1996; Madsen and Dalsgaard, 1999), although Lorenzen *et al.,* (1991) demonstrated that RTFS can be transmitted by cohabitation as well as by intraperitoneal injection of isolated bacteria. Borg (1960) demonstrated that horizontal transmission of BCWD was feasible only when the mucus and epidermis of the experimental fish were damaged. Lumsden *et al.,* (1996) also suggested that necrotic myositis, observed in many *F. psychrophilum* infections, begins as a superficial infection as a result of excessive abrasion, after fish are placed in the cages.

F. psychrophilum has been isolated from eggs, ovarian fluids and milt of sexually mature females and males respectively (Holt *et al.,* 1993; Cipriano *et al.,* 1995; Rangdale *et al.,* 1996; Brown *et al.,* 1997). The source of the contamination of the eggs was found to be either the broodstock or the water (Brown *et al.,* 1997). Kumagai and Takahashi, (1997) reported that eggs imported from the USA were responsible for outbreaks of BCWD in Japan. Bustos *et al.,* (1995) also suggested that imported eggs were responsible for serious epidemics in Chile. The localisation of the pathogen on/in the egg, however, is still open to debate. Brown *et al.,* (1997) after disinfecting the surface of infected eggs, homogenised the eggs and placed them on TYE (Tryptone Yeast Extract) agar. Although they

identified *F. psychrophilum* colonies suggesting that the pathogen was localised inside the egg, this still needs to be confirmed.

Lorenzen *et al.,* (1991) suggested that additional factors are involved in RTFS. The intestinal parasite *Hexamita salmonis* and the ectoparasite *Costia necatrix* have been isolated in many cases of RTFS. The bacterium does not produce microcysts but possibly maintains itself in a vegetative state throughout the year (Pach and Ordal, 1970 cited by Holt *et al.,* 1993). Brown *et al.,* (1997) suggested that it is possible amphibians, insects and other animals may act as reservoir of the bacterium. Wild fish, or fish escaping from farms may also contribute to the spread of the disease, as observed by Wiklund *et al.,* (1994). Nutritional factors, such as elevated levels of oxidised lipids in the diet, also appear to play a role in the occurrence of RTFS (Daskalov *et al.,* 2000). Finally, stress also contributes to the appearance of RTFS as Rangdale *et al.,* (1997a) suggested.

The organism appears to possess substances that can cause lysis of dead bacterial cells, such as *E. coli* and *A. hydrophila* (Dalsgaard, 1993). This feature is believed to give an additional advantage over other bacteria. In addition, proteases and endotoxines (LPS) were also found and probably play an important role in pathogenesis of the disease.

1.1.4 Clinical Signs and Pathology

The gross signs of the disease are the same as in many other pathological conditions of fish. Thus, diseased fish exhibit weakness, loss of appetite, melanosis, ascites and exophthalmia (Bernardet *et al.,* 1988). In addition, the

affected fish sometimes appear lethargic and swim close to the water surface, the sides and the outlet of the troughs (Lorenzen *et al.,* 1991). In some hatcheries where BCWD is endemic, juvenile fish exhibit abnormal spiral swimming behavior due to inflammation of the cranial cavity and anterior vertebrae (Kent *et al.,* 1989). The gills are pale due to anaemia with a severe reduction in the number of erythrocytes. In some cases haemorrhages are also observed in the gills (Sarti *et al.,* 1992). Larger fish show convex lesions associated with the lateral flank (Bruno, 1992) and in severe cases muscle damage at one or more sites on the body surface (Santos *et al.,* 1992; Lumsden *et al.,* 1996). Some authors also reported the existence of blisters containing watery blood and occasionally ulcers on the skin (Weis, 1987 cited by Dalsgaard, 1993). In some chronic forms of BCWD lordosis and scoliosis were also observed in many salmonid species (Kent *et al.,* 1989).

Evensen and Lorenzen (1996) studied the course of the disease in experimentally infected fish and reported that the infected fish showed signs of infection 1 day after intraperitoneal injection of the pathogen. A peak in the clinical signs was observed 36 to 48 hours post-infection and 94 hours post-infection, with survivors showing only a few distinguishable clinical signs or gross pathological changes.

Internally, the liver is pale, the head kidney atrophied, the ventricle is often distended, the intestine is empty, fragile and white, except for the caudal part, which is reddish and the spleen is greyish, enlarged with soft texture and haemorrhagic petecchiae and in later stages completely necrotic (Lorenzen *et al.,* 1991; Bruno, 1992; Sarti *et al.,* 1992). There is also ascitic fluid in the abdomen.

The histopathological signs include focal necrosis in the liver, kidney and heart, hyaline droplet degeneration in the tubules of the secretory part of the kidney and degeneration of the muscles (Lorenzen *et al.,* 1991). In addition, weakly-stained Gram negative rods are found in almost all the organs and especially in the vascular system, the head kidney, the heart and spleen. The lateral skin lesions, which are prominent findings in more chronic stages, include oedema, necrosis, collapse, pyknosis and lymphocyte infiltration of the dermis and underlying muscles (Bruno, 1992). Muscle fibres appear necrotic without striation and many of them were undergoing myophagy by large activated macrophages (Lumsden *et al.,* 1996). In many BCWD outbreaks, Ostland *et al.,* (1997b) described intraocular swelling and thickening of the corneal epithelium and in a later stage necrosis and rupture of the epithelium. Evensen and Lorenzen, (1996) also detected the pathogen in the eyes and the choroid gland and in addition, they also observed inflammatory cells containing bacteria and bacterial products in basal parts of the retina as well as in other organs. Thus, they concluded that RTFS is a severe septicemic infection that heavily involves the monocyte-macrophage system.

In many BCWD epizootics, major changes in the developing bone and cartilage, including the entire head region, such as cephalic osteochondritis and necrotic scleritis, spinal column, elements of the pectorals and ribs have been observed (Ostland *et al.,* 1997b). Kent *et al.,* (1989) observed osteitis, meningitis and ganglioneuritis in juvenile coho salmon, infected with *F. psychrophilum.* Evensen and Lorenzen (1996) also found bacteria/bacterial products in the vicinity of the vertebrae.

1.1.5 Detection of *F. psychrophilum*

A variety of *Cytophaga*-like bacteria are normally found in healthy fish, or on the surfaces of fish eggs (Barker, 1990). In many cases, simultaneous infections by infectious pancreatic necrosis (IPN) virus and *F. psychrophilum* have been observed. Thus, in each case, it is essential to establish whether *F. psychrophilum* is the actual cause of the problem or not. This is difficult, as no case definition for RTFS exists. Moreover, it appears that there is no reliable method to detect the pathogen in fish or environmental samples. At present, the diagnosis of RTFS is based on the clinical signs in combination with the successful isolation and identification of *F. psychrophilum*. However, in many cases the isolation and identification of *F. psychrophilum* are very problematic, since there is no antibody-based technique that can identify all isolates (Lorenzen and Karas, 1992; Lorenzen and Olesen, 1997) and culture-based methods appear very problematic (Daskalov *et al.,* 1999; Michel *et al.,* 1999). In addition, the pathogenicity of *F. psychrophilum* is not fully understood and the disease appears in many forms in different countries (Bernardet, personal communication). Finally, the presence of the pathogen in a farm system does not always result in a disease outbreak, since stress seem to play also an important role in the occurrence of the disease (Rangdale *et al.,* 1997a).

So far, the detection of the bacterium is based on the isolation of the pathogen from the internal organs and skin lesions on *Cytophaga* agar. The morphology of the colonies and the lack of growth on tryptic soya agar and brain heart infusion agar provide some information for the identification of the bacterium. The isolates are subsequently subjected to morphological, physiological,

biochemical and enzymatic tests (e.g. API 50CH and API ZYM systems) (Santos *et al.,* 1992). Such methods are time consuming and culture of the pathogen is not always successful (Chapter 2).

Sarti *et al.,* (1992) attempted to develop a simple method for the rapid detection of *F. psychrophilum*. For this purpose they used fuchsin to stain spleen smears from infected fish.

An immunofluoerescence technique was suggested by Lorenzen and Karas (1992) as a rapid diagnostic tool. The authors used rabbit antisera and prepared serial dilutions of it. At low dilution, there was a low-level cross reactivity with *Flavobacterium columnare.* The biggest advantage of this method, however, apart from the fact that it is a very quick one, is that it can detect viable as well as dead cells.

Toyama *et al.,* (1994) using primers developed from the 16S rRNA region, produced a polymerase chain reaction (PCR) test to detect the bacterium. Izumi and Wakabayashi (1997) used this technique to screen apparently healthy juvenile ayu and coho salmon eggs. This method has a great advantage over conventional methods of detection, since it is very specific and it can be performed in one day. More recently, Urdaci *et al.,* (1998) developed another PCR method using primers also designed from 16S rRNA.

Evensen and Lorenzen (1996) demonstrated that an immunohistochemical technique could be of use for the detection of *F. psychrophilum* in paraffin-wax-embedded sections. However, the antisera they used cross-reacted with *Aeromonas salmonicida* in the lumen of the intestine and in the pancreas. Nevertheless, this technique, in addition to being an important pathogen identification tool, can offer great assistance in a variety of pathogenesis studies.

1.1.6 Control

For the time being, the control of RTFS depends on the oral administration of a wide range of anti-microbial compounds, some of which have proven ineffective. Soltani *et al.,* (1995) studied the minimum inhibitory concentrations of several anti-microbial agents and recommended amoxycillin, norfloxacin, oxolinic acid and oxytetracycline for the control of the disease. In a similar study, Rangdale *et al.,* (1997b) found that the new generation 4-quinolones (e.g. sarafloxacin and enrofloxacin), oxolinic acid and florfenicol when given orally can be very effective against *F. psychrophilum.* Brown *et al.,* (1997) suggested that injection of antibiotics to the broodstock may help to reduce mortality due to BCWD. However, there is an increasing concern about the excessive use of antibiotics due to build-up of resistance. Rangdale (1994) discussing routes to successful treatments of RTFS, speculated that this problem could be due to either plasmid-mediated or chromosomal resistance.

With respect to the disinfection of the eggs, Kumagai and Takahashi (1997) found that 50 ppm of iodine for 15 minutes failed to prevent outbreaks of the disease in some lots of eggs. Holt *et al.,* (1993) also reported the ineffectiveness of such treatments. On the other hand, Brown *et al.,* (1997) reported that 100 ppm povidine/iodine for 30 minutes resulted in 98 % reduction in the number of culturable *F. psychrophilum* from eggs. However, the killing effect was not complete, suggesting that longer exposure may be more effective.

Wood (1974 cited by Holt *et al.,* 1993) observed that if eggs were incubated in shallow troughs, then the BCWD epidemics were less severe. In addition, when

the water flow through vertical incubators was increased, this resulted in more serious BCWD epidemics.

Holt (1988) reported that vaccination by intraperitoneal injection of formalin-killed cells and adjuvant, or by direct immersion in formalin-killed cells, effectively protected coho salmon yearlings against experimental challenge with the pathogen. On the other hand, Obach and Baudin-Laurencin (1991) after a vaccination trial of rainbow trout against the visceral form of BCWD, found that the vaccine was effective only if the size of the fish is >0.5 g (50 days post hatching). Intraperitoneal injection as the vaccination route can be effective, although in practical terms is not feasible in this case due to the small size of fish (the critical size for this disease is around 0.5 g). Immersion, on the other hand, appears to be a more advantageous way of vaccination. However, as Johnson *et al., (*1982) pointed out, in case of salmonids, the fish should weigh at least 1 g, or even better 2.5 g, for bath vaccination to be effective. At present, an effective commercial RTFS vaccine does not exist.

1.2 RESEARCH OBJECTIVES

1. Investigate the potential of using PCR to monitor *F. psychrophilum* in the aquatic environment of a farm. The primers designed by Toyama *et al.,* (1994) were used for the PCR assay, but a different extraction method was employed. The aim of this study was to identify the pathogen in water, egg and spleen samples in order to obtain information on the persistence of the bacterium in the different parts of the farm. The importance of the layout of the farm and the general management procedures in relation to the spread of the pathogen within the farm was examined.

2. Problems with the isolation and identification of the pathogen using culture-based methods appear to exist. Many of these appear to be related to the ability of *F. psychrophilum* to develop a viable but non-culturable state (the bacteria are viable but not able to form colonies on agar plates). Therefore, the ability of *F. psychrophilum* to survive under starvation conditions in stream water, distilled water, *Cytophaga* broth and after disinfection was investigated. Changes in the general morphology of the bacterium as well as its culturability and viability, using cultures and a commercially available viability kit respectively, was examined. Changes in the protein and antigenic profiles were also investigated.

3. Development of a quantitative method to enumerate *F. psychrophilum* in water samples. Such a method, once developed and standardised, could provide useful information on the levels of the pathogen in the aquatic environment before, during and after a disease outbreak. In addition, it could be used to test the efficacy of disinfection or antibiotic treatments.

4. The first step in successful colonisation leading to infection is the ability of the pathogen to adhere to its host. In the case of *F. psychrophilum*, there is a lack of information on the pathogenicity of the organism, especially during the first stages of infection. This can partially explain why the cohabitation or bath challenges have so far provided poor results. Thus the ability of *F. psychrophilum* to attach to surfaces was examined. Two substrates, unfertilised rainbow trout eggs and n-hexadecane were used. Different *F. psychrophilum* isolates were tested and the effect of the age of the bacterial cells on their ability to attach was evaluated.

5. The importance of the eggs as a source of the disease has already been established. However, there is limited information on the localisation of *F. psychrophilum* in/on the eggs. This is important, since disinfection procedures can only be effective if the bacterium remains on the surface of the eggs. Therefore, the effect of successful colonisation of rainbow trout eggs by *F. psychrophilum* on the development of the eggs was examined by experimentally infecting unfertilised rainbow trout eggs with *F. psychrophilum* using a bath challenge. Bacterium was identified on/in the eggs using electron microscopy and immunofluorescent antibody technique (IFAT).

CHAPTER 2

DETECTION OF *F. PSYCHROPHILUM* USING POLYMERASE CHAIN REACTION: A FARM SURVEY

2.1 INTRODUCTION

Little is known of the distribution of *F. psychrophilum* in the environment, especially in the water systems of fish farms. *F. psychrophilum* is a rather fastidious species, often difficult to isolate on agar. In addition, no antibody-based techniques are available that recognise all *F. psychrophilum* strains. Successful control of the disease depends on good management for which reliable methods of detection are needed to assess the presence and level of the pathogen within the fish farm environment. These are necessary to provide information on the distribution of the pathogen before, during and after an outbreak of RTFS.

Polymerase Chain Reaction (PCR) is a very sensitive technique, which can detect small amounts of DNA or RNA by amplifying specific sequences more than a million fold. Exact replicas of the target sequence are rapidly produced in large numbers making them available for further analysis. Well-designed sets of primers can lead to a very sensitive and specific method, enabling the rapid detection of various pathogens in different samples. This technique overcomes the sensitivity constraints of other detection methods, such as antibody-based techniques or culture. The potential of using PCR as a tool for monitoring *F. psychrophilum* in water, eggs and fish tissues, was examined in this study. The primers developed

by Toyama *et al.,* (1994) were used for the assay. However, a different extraction method to the one used by Toyama *et al.,* was employed. For the PCR reaction Ready To Go PCR beads (Amersham Pharmacia Biotech Inc) were used. Once the assay was validated in terms of sensitivity and specificity, a survey on two farms with endemic RTFS was conducted in order to collect information on the persistence of the pathogen in the different areas of the farms.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial cultures

F. psychrophilum isolate B97026 was used to establish the sensitivity of the PCR, while a range of *F. psychrophilum* isolates obtained from a variety of geographical regions (Table 2.1, page 25) was used to examine its specificity. A selection of other bacterial strains was also included in the analysis. Three-day old cultures of different *F. psychriphilum* strains were cultured at 15^0 C on modified *Cytophaga* Broth (Bernardet and Kerouault 1989, 1959), while all the other species were cultured in TSB (Tryptone Soya Broth), with the exception of *Vibrio salmonicida*, for which TSB was supplemented with 2% NaCl. The bacteria were collected by centrifugation (3000 g for 15 min) and re-suspended in 0.02 M phosphate buffered saline (PBS, pH 7.2). The total number of the bacterial cells in each suspension was estimated using the acridine orange direct counting method (AODCM) described by Hobbie *et al.,* (1977) (Appendix 1). The bacterial suspensions were counted using a Leitz Orthoplan fluorescence microscope, fitted with a 200W ultra high pressure mercury lamp, using the following set of filters: diffusing disc N, exciting filters BG38 and BG12 and suppression filter K430.

In order to establish the sensitivity threshold of the PCR, serial dilutions of bacterial suspensions were prepared with PBS (using AODCM), ranging from 10^8 bacteria ml^{-1} to 1 bacteria ml^{-1}. To establish the specificity of the PCR, bacterial suspensions of each of the strains listed in Table 2.1 were prepared and the concentration of each suspension adjusted to 10^7-10^8 bacteria ml^{-1} (using AODCM).

The presence of various inhibitors in the different samples (eggs, spleen and milt) was also examined. For spleen samples, homogenates were prepared and mixed with *F. psychrophilum* as described below. Three-day old cultures of *F. psychrophilum*, isolate B97026, in *Cytophaga* broth were used in this study. After centrifugation (3000 g for 15 min) the bacteria were re-suspended in PBS and serial dilution prepared using PBS (10^3 to 10 bacteria ml^{-1}). Three 1 ml aliquots from each dilution were placed into nine 1.5 ml tubes (three groups, each group containing three serial dilutions). Spleen tissue (400 mg) was homogenised using 4 ml of reagent A. Aliquots of the homogenate (500 µl) were added to the tubes of the two of the above groups and the tubes were then centrifuged (3000 g for 15 min). In one group of the two groups, the resulting pellets (bacteria+spleen) were washed as described above, while in the other group, the remaining pellets were not washed but re-suspended in reagent B, as will be described below.

For the egg and milt samples, 10^7 bacteria (determined by AODCM) collected as above were placed into four 1.5 ml tubes. Milt (100 µl) was added to two tubes, while in the third 500 µl of egg homogenate was added. The tube containing only bacteria was used as positive control. After briefly mixing, the tubes were centrifuged. DNA was extracted as described below (basic method). For milt samples, an additional washing step, as described for spleen samples, was also used.

2.2.2 DNA Extraction

Extraction of DNA from the various samples was performed using a DNA Extraction Kit (BACC2, Nucleon) with the omission of RNAase treatment.

Modifications in the extraction procedure depended on the type of sample extracted and these are outlined below.

Bacterial suspensions (basic method)

Bacterial suspension (1 ml) was added to 1.5 ml sterile tubes and centrifuged at 3000 g for 15 min. The supernatants were discarded and 500 µl of reagent B was then added to the pellets and the samples were incubated at 37° C for 30 min before adding 150 µl of 5 M sodium perchlorate. The samples were mixed by inverting the tubes several times before adding 500 µl of chloroform. The tubes were mixed again before 150 µl of Nucleon resin was added to the tubes. The samples were mixed and centrifuged at 350 g for 1 min and the supernatants (650-680 µl) transferred to new 2 ml tubes. A double volume of cold (-20° C) absolute ethanol was then added and the samples were left at -20° C for at least 30 min to allow DNA to precipitate. The samples were centrifuged for 5 min at 5000 g and the pellets finally washed with 1 ml of cold 70% v/v ethanol. The samples were centrifuged again at 5000 g for 5 min and the pellets were left to air dry for 10 min. Finally, each pellet was re-suspended in 100 µl of sterile nano-pure water.

 Water samples

Water samples (200 ml or 50 ml) were concentrated to 10 to 20 µl by successive centrifugations at 3000 g for 15 min, prior to extraction. To this, 500 µl of reagent B was added and the method described in the previous section followed.

Spleen samples

Whole spleens were aseptically removed from fry and homogenised in 2 ml of reagent A. Samples were centrifuged at 3000 g for 15 min and pellets washed with 1.5 ml Tris- ethylenediaminetetraacetic acid (EDTA) (10 mM Tris, 1 mM EDTA, pH 8) to remove endogenous inhibitors, before extraction of DNA from the resulting pellets according to the basic method.

Egg samples

Between 10 and 15 unfertilised eggs were collected directly from the fish during stripping (in every case the first few eggs were discarded to prevent contamination from water). The same number of disinfected eyed eggs was also collected. The eggs were homogenised in a sterile 1.5 ml tube with a sterile plastic pestle. The aqueous part of the sample was then collected and centrifuged at 3000 g for 15 min. Resulting pellets were extracted using the method outlined in the basic method.

2.2.3 PCR

For the PCR, Ready To Go PCR beads (Amersham Pharmacia Biotech Inc) were used and the reaction was performed according to the parameters described by Izumi and Wakabayashi (1997): preheating cycle at 94° C followed by 35 amplification cycles of 94° C for 30 sec, 51° C for 90 sec and 72° C for 120 sec and a final cycle of 72° C for 5 min. Both a nested PCR and a one-round PCR, (using the second set of primers PSY1 & PSY2), were evaluated in this study using the same amplification parameters.

When one round PCR was used, 2.5 µl of the extracted DNA samples was added to 22.5 µl of reaction mixture containing the PSY1/PSY2 primers. When a nested PCR was performed, after an initial amplification of the extracted DNA (2.5 µl) using the 20F/1500R primers, 2.5 µl of the PCR products from the first round was added to a fresh tube containing 22.5 µl of reaction mixture containing PSY1/PSY2 primers.

2.2.4 Farm surveys

Two farms with endemic problems of RTFS, located in South England, were selected as models to evaluate PCR as a tool for monitoring the presence of *F. psychrophilum* within fish farming systems. The layout of the first farm (Farm 1) is shown in Figure 2.1. Although the second farm (Farm 2), had a similar layout, there were also differences between the two sites. The stream that provided Farm 1 with water was underground water and the source was just before the farm. An artificial channel was used to bring water to the farm. It should be noted that no wild fish lived in the stream. Farm 2 on the other hand had a bore-hole as source of water and a trough was used to distribute the water to the farm. In case of rain due to outflows, the bore-hole was contaminated with water from the farm. Farm 1 had one pipe as outlet, that carried the water from the sediment tank off the farm, while Farm 2 had many different small outlets.

It can be seen from Figure 2.1 that the hatchery and the fry tanks (fish weight 0.5-5 g) in Farm 1, received water straight from the stream. The brood stock received water from the fry tanks, because of a problem with the water flow. The broodstock (approximately 2100 fish) were not treated with antibiotics,

however, fry tanks that showed signs of the disease were treated with Vetremox (100% amoxycillin) and Aquaflor (50% florfenicol). For the disinfection of the equipment, Limox (5% H_2O_2 & peroxyacetic acid) was used (4 ml l^{-1} =400 ppm H_2O_2 for 20 min) and for the eyed eggs, Vetroxyl (H_2O_2) was used (2% Vetroxyl v/v, 10 min immersion). Prior to the disinfection of the equipment, everything was washed thoroughly with water and the tanks were cleaned using a big brush, to remove the organic matter that adhered to the walls.

The first samples were collected from Farm 1 in early March 1999 and an extensive survey on this farm site then followed and samples were taken from all the parts of the site. The dates of collecting the various samples (eggs, water, and spleens) and the results of the PCR from Farm 1 are summarised in Table 2.2 (page 28).

Routinely, 50 ml of water was collected for analysis. However, when the extensive survey from the farm was performed, 200 ml of water was sampled from each sampling site (Figure 2.1).

Samples from Farm 2 were collected over a period of one month (mid May until mid June 2000). In the case of this farm, water samples (200 ml) from different parts of the farm as well as fry that showed signs of the disease were collected and analysed (Table 2.3, page 29).
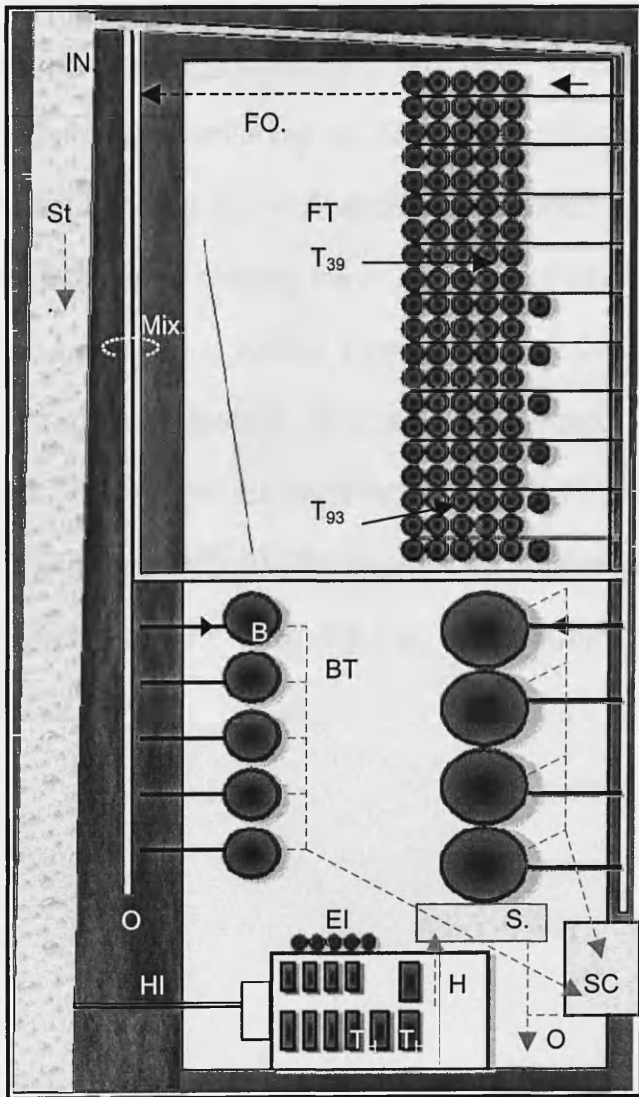
Figure 2.1 Farm layout of the first farm site. Numbers indicate the different sampling sites of the farm and the sites from which the water samples were taken. FT. Fry tanks, BT. Broodstock tanks, H. Hatchery, EI. Incubators, HI. Hatchery inlet (separate). St. Stream, SC. Farm settlement channel, supplies farm outlet, T_{39}. Tank 39, T_{93}. Tank 93, B. Broodstock tank, T_H. Hatchery tanks, S. Hatchery settlement channel, O. Farm outlet, IN. Stream (inlet), FO. Fry tank outlets: the fry tanks drain into the broodstock supply channels, Mix. Mixing of the water in the two channels.

2.2.5 Sequencing PCR products

PCR products from two egg samples (10-15 eggs each) collected during the first survey (unfertilised eggs collected on 3/3/99 and 18/3/99, see Table 2.2), were sequenced to confirm that the PCR products obtained resulted from amplified *F. psychrophilum* DNA. After running the PCR products on an agarose gel, the bands of the expected length were purified, using GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia Biotech Inc) and sequenced using a BigDye DNA Sequencing Kit (PE Applied Biosystems) on a ABI PRISM 377 DNA Sequencer (*PERKIN ELMER*) (Appendix 6). The sequences obtained were compared with the sequence previously obtained from the type strain NCIMB1947.

2.3 RESULTS

2.3.1 Validation of PCR method

The extraction method yielded a final concentration of approximately 0.2 ±0.01 µg µl^{-1} genomic DNA from 10^8 bacterial cells with a 260nm/280nm ratio of 1.1-1.2. Addition of RNAase increased this ratio to 1.5-1.7, although it did not affect the PCR test and was therefore omitted from the assay.

A number of *F. psychrophilum* isolates from different origins (Table 2.1) were tested with the PSY1/PSY2 primers using nested PCR (Figure 2.2). Some other bacterial species, not previously tested by Toyama, *et al.,* (1994), were also included in the analysis. All of the bacterial species tested in the nested PCR produced bands larger than 1230 base pairs (bp) because of the first pair of primers (1500R-20F), which are considered to act as universal primers (Toyama *et al.,* 1994). However, only strains of *F. psychrophilum* produced a band of 1078 bp.

The sensitivity of the nested PCR method was determined to be approximately 10 bacterial cells ml^{-1} (Figure 2.3), using AODCM to count the bacteria, since the PCR technique can detect both live and dead bacteria. When only the second pair of primers (PSY1/PSY2) was used the sensitivity decreased to 10^5 bacterial cells ml^{-1}.

There appeared to be complete inhibition of the PCR reaction, when spleen homogenates or milt samples were mixed with *F. psychrophilum* (Figure 2.4, Lanes 4-6 and 13-14) were used as source of DNA. Addition of a washing step prior to DNA extraction improved the results obtained with the spleen, although the

sensitivity of the reaction decreased from 10 to 10^3 bacteria ml^{-1} (Figure 2.4, Lanes 7-9). On the other hand, a similar washing step did not appear to improve the inhibition observed with the milt samples (Figure 2.4, Lane 14). No inhibition was observed when egg homogenates were mixed with the pathogen (Figure 2.4, Lane 12).

Table 2.1 Bacteria tested to determine the specificity of the PSY1 and PSY2 primers.

	STRAIN	HOST	ORIGIN	PCR
	NCIMB 2282	SS	USA	+
	NCIMB 1947 (type strain)	CS	USA	+
	B97026	RT	UK[t]	+
	UP96107P1	RT	UK[t]	+
	B97211	RT	UK[t]	+
	B95076A7	RT	UK[t]	+
	LVDL1456/91	RT	France*	+
	LVDL1829/91	RT	Spain*	+
F.psychrophilum	LVDL3077/91	RT	Spain*	+
	LVDJG62215	BT	France*	+
	LVDJD2172	Tench	France*	+
	JIP30/98	Eel	France*	+
	FIAC	RT	Canada[t]	+
	F314	RT	Canada[t]	+
	B398C	RT	Canada[t]	+
	32/97	RT	Chile**	+
	34/97	RT	Chile**	+
	35/97	RT	Chile **	+
	89/97	RT	Chile**	+
	59/95	-	Chile **	+
	R104	CS	Chile**	+
	R107	CS	Chile**	+
Microbacterium marinotypicum			UK[t]	-
Aeromonas.sobria B96152		G	UK[t]	-
Edwarsiella ictaluri		Sn	Thailand[t]	-
Edwarsiella tarda[1] B88308		RB	UK[t]	-
E.coli[1] NCTC 10418				-
Aeromonas salmonicida[1] NCIMB 2020				-
Yersinia ruckeri[1] Finland'84		Salmon	Finland[t]	-
Pseudomonas fluorescens[1] NCIMB 1953				-
Aeromonas hydrophila[1] T4			Bangladesh[t]	-
Micrococcus lutens NCIMB 8553				-
Serratia sp.		Salmon	Ireland[t]	-
Vibrio salmonicida 488		Salmon	AVL	-

Note: RT: rainbow trout, BT: brown trout, SS; silver salmon, CS: coho salmon, G: goldfish, Sn: snakehead, RB: red bream, AVL: Aquaculture Vaccine Ltd, NCTC: National Collection of Type Cultures, NCIMB: national collections of industrial and marine bacteria [1]Species also tested by Toyama *et al.,* (1994), *: Institut National de la Recherche Agronomique, France, **: Universidal Austral de Chile [t]: Institute of Aquaculture, University of Stirling,
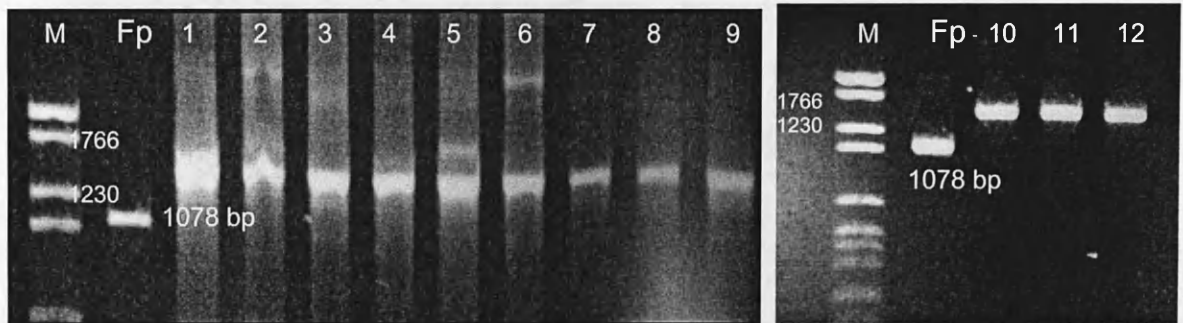
Figure 2.2 Specificity of PCR. 1% agarose gels showing the results of nested PCR. Lanes M: marker (DNA Molecular weight marker VI, Ross), Lanes Fp: *F. psychrophilum*. Lanes 1-12: *E. ictaluri Y. ruckeri, E. coli, A. salmonicida, E. tarda, P. fluorescens, A. hydrophila, M. luteus, Serratia* sp. *A. sobria, F. marinotypicum, V. salmonicida.*
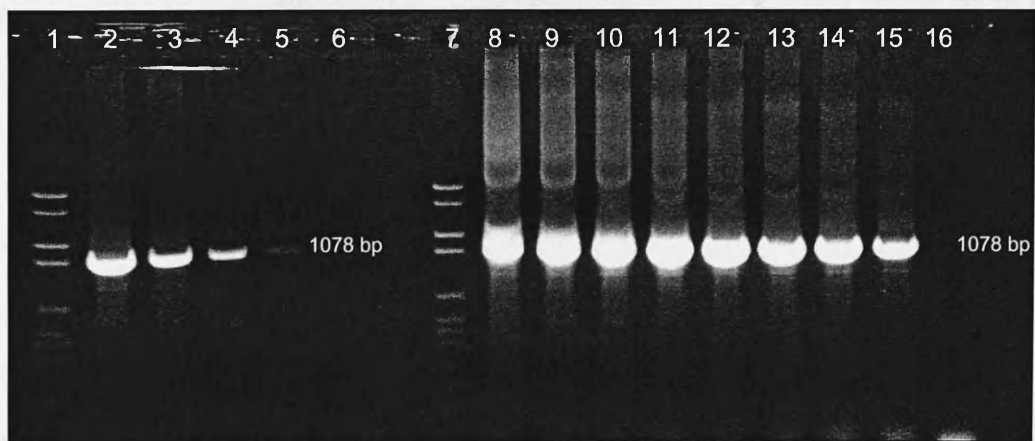
Figure 2.3 Sensitivity of PCR. 1% agarose gel. Lanes 1,7 marker (DNA Molecular weight marker VI, Ross), Lanes 2-6: *F. psychrophilum* suspensions 10^8-10^4 cells ml^{-1}. Only the primers PSY1 and PSY2 were used. Lanes 8-16: *F. psychrophilum* suspensions 10^8-1 cells ml^{-1}. Both pairs of primers were used.
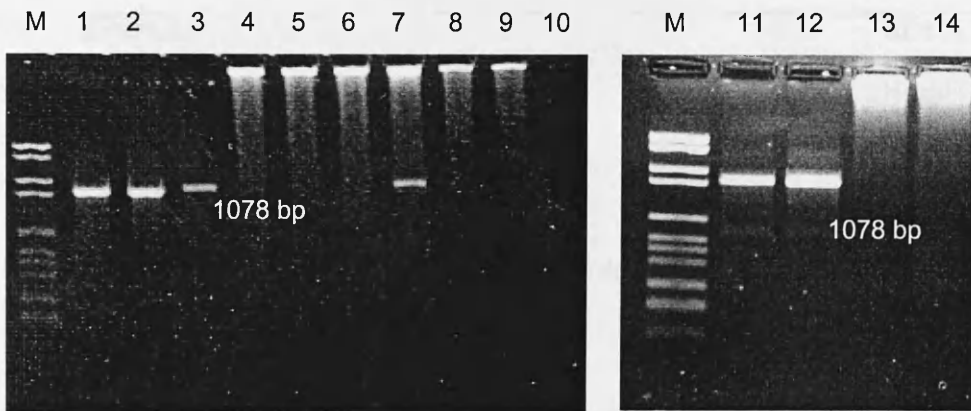
Figure 2.4 Inhibition of the PCR assay by spleen and milt samples. 1% agarose gel. Lanes 1-3: *F. psychrophilum* suspensions 10^3-10 cells (total number), Lanes 4-6: Spleen homogenate mixed with *F. psychrophilum* suspensions (10^3-10 cells total number), Lanes 7-9: spleen homogenate mixed with *F. psychrophilum* suspensions (10^3-10 cells total number), a washing step was included prior to DNA extraction, Lane 10: negative control (water), Lanes 11: *F. psychrophilum*, 10^7 bacteria, Lane 12: *F. psychrophilum*, (10^7 bacteria) mixed with egg homogenate, Lane 13: *F. psychrophilum*, (10^7 bacteria) mixed with milt, Lane 14: *F. psychrophilum*, (10^7 bacteria) mixed with milt, a washing step was included prior to DNA extraction, Lanes M: marker (DNA Molecular weight marker VI, Ross),

2.3.2 Farm surveys

Once the specificity and sensitivity of the PCR test had been evaluated, the PCR method was used to screen samples collected from two farms with endemic RTFS. The results of the first survey (Table 2.2) indicated that all sites of the farm (egg incubators, hatchery, fry tanks, broodstock tanks, hatchery and farm outlet) were positive for the pathogen. The water inlet, however appeared to be negative. Similarly, in the second survey, all parts of the second farm were also found to be positive for the pathogen, with the exception of the farm inlet (bore hole) and the steam water downstream of the farm (Table 2.3).

Table 2.2 PCR analysis of samples collected from the first farm site.

DATE	SAMPLES	RESULTS
3/3/99	S_1. Water sample from hatchery inlet.	Negative
	S_2. Water sample from hatchery outlet.	Positive
	S_3. Eyed eggs (undisinfected).	Positive
	S_4. Eyed eggs (disinfected).	Positive
	S_5. 23 samples of unfertilized eggs in ovarian fluid.	7 positive
	S_6. 10 live alevins in water.	The water containing the fish found positive.
18/3/99	S_1. Water sample from Farm Inlet.	Negative
	S_2. Water sample from Farm outlet.	Positive
	S_3. Water sample from Hatchery outlet.	Positive
	S_4. Water sample from Tank F1.	Positive
	S_5. Water sample from Tank F11.	Positive
	S_6. Water sample from Egg incubators.	Positive
	S_7. Water sample from Tank 93.	Negative
	S_8. Water sample from Tank 39.	Positive
	S_9. 11 samples of unfertilized eggs in ovarian fluid.	3 positive
	S_{10}. Disinfected eggs.	Negative.
26/5/99	$S_{1,2}$. Water from hatchery troughs with apparently healthy fry ready to move out.	Positive
	$S_{3,4}$. The same troughs ($S_{1,2}$) 16h after disinfection.	Positive
	S_5. Isolated/disinfected rearing tank before introduction of fish.	Positive
7/6/99	$S_{1,2}$. Water from tanks 2,3 with fish showing signs of the disease.	All positive
17/6/99	S_1. Water sample from Farm inlet	Negative
	S_2. Water from Tank 2 (inlet tap).	Negative
	S_3. Tank 2 10d after florfenicol treatment.	Positive
	S_4. Tank 3 10d after florfenicol treatment	Positive
	S_5. Water from tank previously disinfected and left empty for 1 week.	Positive
	S_6. 3 spleen samples from fry showing signs of the disease.	Positive
8/7/99	S_{1-5}. Water from 5 tanks with fish recovered after 12d florfenicol treatment	All positive

Table 2.3 PCR analysis of samples collected from the second farm site.

DATE	SAMPLES	RESULTS
19/5/00	S_1. Water sample from trough which supplies water to the farm	Negative
	S_2. Water from hatchery inlet	Negative
	S_3. Water samples from steam water, downstream of the farm	Negative
	S_4. Water sample from egg incubators	Positive
	S_5. Water sample from broodstock tank	Positive
	S_6. Water sample from fry tank containing fish showing signs of the disease	Positive
	S_7. Water from fry tank containing fish that had recovered (2 days after the end of the treat with antibiotics)	Positive
	$S_{8,9}$. Water samples from two hatchery tanks	Positive
	S_{10-13}. Spleens from 4 fry showing signs of the disease	3 positive
30/5/00	S_1. Water from trough which supplies water to the farm	Negative
	S_2. Water from the actual bore hole	Negative
	S_3. Water from the tank containing the broodstock	Positive
	S_4. Water from the egg incubators	Positive
10/6/00	S_1. Water from trough which supplies water to the farm	Negative
	S_2. Water from the actual bore hole	Negative
	S_{3-4}. Water from the two fry tanks that were tested on the 19th of May	Positive

2.3.3 Sequencing PCR products

Only partial sequences of the two samples were obtained for the two PCR products sequenced, due to the low quantity of PCR products purified from the gel. However, from the sequences obtained the PCR products were found to correspond to sequence obtained for the *F. psychrophilum* type strain NCIMB1947 (Figure 2.5).

```
                     PSY 2
Sample A    CGATCCTACTTGCGTAG TGGCTGCTCTCTGTACCGGCCATTGTAGCACGTGTGTAGCCCAAGGCGT
Sample B    CGATCCTACTTGCGTAGTGGCTGCTCTCTGTACCGGCCATTGTAGCACGTGTGTAGCCCAAGGCGT
NCIMB1947   CGATCCTACTTGCGTAGTGGCTGCTCTCTGTACCGGCCATTGTAGCACGTGTGTAGCCCAAGGCGT

Sample A    AAGGGGCCGTGATGATTTGACGTCATCCCCACCTTCCTCACAGTTTACACTGGCAGTCTTGCTAGAG
Sample B    AAGGG---CCGTGATGATTTGACGTCATCCCCACCTTCTTCACAGTTTACACTGGCAGTCTTGCTAGAG
NCIMB1947   AAGGG---CCGTGATGATTTGACGTCATCCCCACCTTCCTCACAGTTTACACTGGCAGTCTTGCTAGAG

Sample A    TTCCCGACATGACTCGCTGGCAACTAACAACAGGGGTTGCGCTCGTTATAGGACTTAACCTGACACC
Sample B    TTCCCGACATGACTCGCTGGCAACTAACAACAGGGGTTGCGCTCGTTATAGGACTTAACCTGACACC
NCIMB1947   TTCCCGACATGACTCGCTGGCAACTAACAACAGGGGTTGCGCTCGTTATAGGACTTAACCTGACACC

Sample A    TCACGGCACGAGCTGACGACAACCATGCAGCACCTTGTAAATTGTCTTGCGAAAAGTCTGTTTCCAA
Sample B    TCACGGCACGAGCTGACGACAACCATGCAGCACCTTGTAAATTGACTTGCGAAAAGTCTGTTTCCAA
NCIMB1947   TCACGGCACGAGCTGACGACAACCATGCAGCACCTTGTAAATTGTCTTGCGAAAAGTCTGTTTCCAA

Sample A    ACCGGTCAATCTACATTTAAGCCTTGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCA
Sample B    ACCGGTCAATCTACATTTAAGCCTTGGTAAGGTTCCTCGCG-----------------------------
NCIMB1947   ACCGGTCAATCTACATTTAAGCCTTGGTAAGGTTCCTCGCGTATCATCGAATTAAACCACATGCTCCA

Sample A    CCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGGAT
Sample B    -------------------------------------------------------------------
NCIMB1947   CCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCATTCTTGCGAACGTACTCCCCAGGTGGGAT

Sample A    ACTTATCACTTTCGCTTAGCCACTGAGATTGCTCCCAACAGCTAGTATCCATCGTTTACGGCGTGGAC
Sample B    -------------------------------------------------------------------
NCIMB1947   ACTTATCACTTTCGCTTAGCCACTGAGATTGCTCCCAACAGCTAGTATCCATCGTTTACGGCGTGGAC

Sample A    TACCAGGGTATCTAATCCTGTTCGCTAC--------------------------------------
Sample B    -------------------------------------------------------------------
NCIMB1947   TACCAGGGTATCTAATCCTGTTCGCTACCCAGCTTTCGTCCATCAGCGTCAATCAATTTGTAGTACCTG

Sample A    -------------------------------------------------------------------
Sample B    -------------------------------------------------------------------
NCIMB1947   GCTTTCGCAATTGGATTCCATGTAATCTCTAAGCATTTCACCGCTACACTCATATTCTAAGTTACTTTC

Sample A    -------------------------------------------------------------------
Sample B    -------------------------------------------------------------------
NCIMB1947   CAAATAATTCAAGCCCAACAGTATCAATGGCCGTTCCATCGTTGAGCGATGGGCTTTCACCACTGAC

Sample A    -------------------------------------------------------------------
Sample B    -------------------------------------------------------------------
NCIMB1947   TTATCTGGCCGCCTACGGACCCTTTAAACCCAATGATTCCGGATAACGCTTGGATCCTCCGTATTAC

Sample A    -------------------------------------------------------------------
Sample B    -------------------------------------------------------C AC---CGTG
NCIMB1947   CGCGGCTGCTGGCACGGAGTTAGCCGATCCTTATTCTCACAGTACCGTCAAGCTACCTCACGA-GTG

Sample A    --------------------------AGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCGGCATGGCTG
Sample B    AGTGTTTCTTCCTGTGCAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCGGCATGGCTG
NCIMB1947   AGTGTTTCTTCCTGTGCAAAAGCAGTTTACAATCCATAGGACCGTCATCCTGCACGCGGCATGGCTG

Sample A    GTTCAGGCTTGCGCCCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCT
Sample B    GTTCAGGCTTGCGCCCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCT
NCIMB1947   GTTCAGGCTTGCGCCCATTGACCAATATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGTCCGTGTCT

Sample A    CAGTACCAGTGTGGGGGATCTCCCTCTCAGGACCCCTACCCATCGTTGCCTTGGTAAGCCGTTACCT
Sample B    CAGTACCAGTGTGGGGGATCTCCCTCTCAGGAC--CCTACCAATCGTTGCCTTGGTAAGCCGTTACCT
NCIMB1947   CAGTACCAGTGTGGGGGATCTCCCTCTCAGGACCCCTACCCATCGTTGCCTTGGTAAGCCGTTACCT

Sample A    TACCAACTAGCTAATGGGACGCATGCTCATC TTTA--G---G-G--------------TGTTGATGCCAAC
Sample B    TACCAACTAGCTAATGGGACGCATGCTCATCGGTACTG----TGACTTT-----AGTGTGTTGATGCCAAC
NCIMB1947   TACCAACTAGCTAATGGGACGCATGCTCATC TTTACTGTTGTGACTTTAAT AGTGTGTTGATGCCAAC
                                                                 PSY 1
```

Figure 2.5 Comparison of the partial sequences obtained from two egg samples positive for *F. psychrophilum* by PCR. The two primer sites can be seen at the ends.

2.4 DISCUSSION

PCR is a very powerful tool and proper design of the primers is crucial for the development of a sensitive and specific technique. A detection method is only of value if it measures/detects what it is supposed to (Thrusfield, 1986 in Hiney and Smith, 1998). Quantitativity, qualitativity and reliability are important criteria for evaluation of any detection techniques. PCR aims to overcome obstacles, such as small numbers of bacteria in water samples and differing antigenic profiles of the bacteria, making it difficult to use conventional or antibody-based methods to identify pathogens, especially if they are unculturable. On the other hand, PCR also has disadvantages, such as the existence of possible inhibitors in the samples, cross-reactivity, contamination and inability to distinguish between dead and live bacteria. However, in cases where it is necessary to establish the presence of the pathogen, this provides useful information on the distribution of the organism within the aquatic environment.

Toyama *et al.,* (1994) developed a PCR method to detect *F. psychrophilum*, the causative agent of BCWD. This was later used by Kumagai and Takahashi (1997) to detect the pathogen in eggs imported to Japan, which were found to be the source of the BCWD outbreaks occurring in Japan. The method used was based on nested PCR and although the first pair of primers used (1500R and 20F) were universal (all the bacterial strains produced a band larger than 1230 bp), the second pair appeared to be specific for the pathogen. So far over 42 different strains of *F. psychrophilum* and 21 other bacterial species have been tested with the PCR (including those of this study) (Toyama *et al.,* 1994; Wiklund *et al.,* 2000)

and only *F. psychrophilum* strains result in a 1078 bp band. Bader and Shotts (1998) also found that the second pair of primers can be used to distinguish *F. psychrophilum* from other *Flavobacterium* and *Flexibacter* species. However, Urdaci *et al.,* (1998) reported that the primers used by Toyama *et. al.* (1994) produced some non-specific products, although specific details were not given.

Izumi and Wakabayashi (1997) reported that the sensitivity threshold of the nested PCR method was between 1.1 and 1.5 colony forming units (CFU's) per PCR tube. The sensitivity of the nested PCR using the same primers (20F/1500R and PSY1/PSY2) using slightly different amplification conditions was also evaluated by Wiklund *et al.,* (2000). They reported that the sensitivity threshold for spiked fish brain tissue was 0.4 CFU's per PCR tube and 1.7 CFU's per PCR tube for spiked non-sterile fresh water. However, because of the difficulty in determining levels of *F. psychrophilum* by culture (Daskalov *et al.,* 1999; Michel *et al.,* 1999), in the current study the total number of bacteria in a sample (based on AODCM) was used to evaluate the sensitivity of the PCR rather than the CFU's. In addition PCR is able to detect both dead and live bacteria and thus, the PCR was found to detect a total of approximately 10 bacteria per PCR tube.

Inhibition of the reaction was observed using DNA extracted from both spleen and milt spiked *in vitro* with *F. psychrophilum*. Although the inhibitors were not identified in this study, for the spleen, haemoglobin or other serum proteins may acted as strong inhibitors, as suggested by Wilson *et al.,* (1997). Inclusion of a washing step prior to DNA extraction minimised the inhibition.

Problems are known to exist with the culture of *F. psychrophilum*, and therefore a quick and reliable method, such as PCR would be ideal for screening environmental samples. Two farms with endemic problems of RTFS were

monitored using PCR, whereby water samples, eggs and spleen were collected. The findings of the monitoring, especially of the water samples, indicated that the pathogen possibly did not come from the water source supplying the farm, since the farm inlet was always found to be negative. It is possible that *F. psychrophilum* was present in very low numbers in the inlet water. However, because the source of the inlet was just upstream from the farm and because there were no wild fish upstream of the farm that could act as carriers of the pathogen, it is unlikely that the pathogen arrived through the water inlet. Fish of all ages carried the pathogen and appeared to be a possible reservoir for the pathogen.

The association of the pathogen with eggs has already been reported (Holt *et al.,* 1993; Bustos *et al.,* 1995; Cipriano *et al.,* 1995; Rangdale *et al.,* 1996; Brown *et al.,* 1997; Kumagai and Takahashi, 1997). The source of this transmission was found to be either the broodstock or the water (Brown *et al.,* 1997). In the present study, approximately 30 % of the broodstock on the Farm 1 were found to be positive for the pathogen by PCR and since different batches of the eggs were mixed together, eventually all eggs in the incubators were prone to infection and water samples from the incubators were also found to be positive. As a result, small fry were infected (water samples from different tanks within the hatchery were found to be positive), but did not show high levels of mortality while in the hatchery. Over the past three years only two incidences of RTFS outbreaks associated with high hatchery mortalities were reported at this farm. When fry were transferred to larger tanks outside the hatchery the fish, however, sometimes died in high numbers, suggesting that the fish were carrying *F. psychrophilum.* Transportation stress and greatly increased light intensity from direct sun exposure possibly made the fish succumb to the disease. Generally, samples from

both disinfected and undisinfected eggs appeared positive (although one test in mid March showed a disinfected batch to be negative). This was not surprising, since PCR is able to detect both live and dead bacteria, as long as there is no degradation of the DNA. Therefore, it was not possible to determine whether the disinfection procedure used by the farm (as described in section 2.2.4, page 23) was effective or not using PCR.

The localisation of the pathogen within the eggs is still open to debate. To study the vertical transmission of the disease Brown *et al.,* (1997) cultured homogenised eggs, which had been previously surface-disinfected, on TYE (Tryptone Yeast Extract) agar. Although the cultures proved positive for *F. psychrophilum,* further studies need to be performed to confirm the presence of the pathogen inside the eggs. This issue will be discussed in Chapter 6.

Another interesting finding, which questions the efficacy of the disinfection procedure used on the farm, comes from the fact that water samples taken from tanks previously disinfected and left empty for some time were still positive upon sampling. Although it was not possible to determine whether the bacteria were still alive or dead, this indicated that the bacteria were not successfully removed during disinfection. Eginton *et al.,* (1998), studying the attachment of micro-organisms to surfaces following disinfection, found that the treatment with the disinfectant resulted in the removal of loosely attached cells but not cells located deeper within the biofilms. Studies performed *in vitro* and *in vivo* in the laboratory by Ziddah (1997) showed disinfectants to be effective when used to treat eggs and equipment. However, under farm conditions, the pathogen may survive such procedures, possibly hidden in biofilms formed on the surfaces of the tanks.

Branson (1998) emphasised that thorough cleanliness of the equipment prior to disinfection is very important for a successful procedure.

In some cases, water samples taken from tanks containing fish that had recovered from infection after treatment with antibiotics (9-10 days of treatment) appeared positive. However, because the water coming into the fry tanks was clean (no bacteria were ever detected in the inlet stream water samples), the bacteria either managed to survive by adhering to the surfaces of the tanks, as suggested by Eginton *et al.,* (1998), or the bacteria either dead or alive, were being shed by the fish. However, considering the constant water flow within the tank, the latter is more likely to be the case. The fact that fry may still be able to shed bacteria after treatment with antibiotics, raises the question whether the fry are acting as carriers for the pathogen. A reliable quantitative method to enumerate the pathogen in water samples could provide useful information on the shedding rate of the bacterium. Although such a method is not able to distinguish the live bacteria from the dead ones, fluctuations in the numbers of the pathogen present in the water of the tank would indicate the period during which the fish shed the pathogen. For example, Madetoja *et al.,* 2000 examining the shedding rate of *F. psychrophilum* after subcutaneous (s.c.) or intraperitonal injection (i.p.) and bath challenge, using a double-staining technique, found that after three days for i.p. injection and 14 days after s.c. injection *F. psychrophilum* were detectable in the water containing the infected fish.

Both the hatchery and the farm outlets were always found to be positive, indicating that these sites would be ideal for monitoring the disease status of the farm.

The second survey, conducted one year later, on a farm with a similar layout, also confirmed the same scenario in that the pathogen existed in all parts of a farm and fish of all ages carried the bacterium. It also confirmed that inappropriate farm layout (i.e. the brood stock receiving water from the fry tanks) for this site, making disease control within the farm difficult. Interestingly, the water downstream of the farm was found to be negative. In this case there was no one specific outlet of the farm, as in case of the first farm and thus, the bacteria were probably below the detectable level in the samples collected.

Knowledge on the epidemiology and pathogenesis of RTFS is still limited. The results from both surveys clearly showed that the problem of tackling the disease within the farm is very complex. Thorough disinfection procedures, good farm management and careful design of the farm appear to be key factors in disease control. The survey presented here indicated that PCR is a very useful tool for investigating and monitoring disease. However, further investigations are needed to establish how the bacterium survives in the environment, how levels of the bacterium change throughout the year and how environmental parameters can affect these levels. A method, which quantifies the numbers of bacteria in samples is necessary to establish the levels of the pathogen at different sites throughout the farm and to establish changes in the numbers prior to and after treatment, hence indicating indirectly the effectiveness of the treatment. A development of such a method will be discussed in Chapter 4.

CHAPTER 3

STARVATION OF *F. PSYCHROPHILUM*

3.1 INTRODUCTION

So far, little is known of the epidemiology of RTFS or the survival of *F. psychrophilum* outside its host. There is also little information relating to the portal of entry of the organism or its spread within the body of the fish, although a number of infectivity trials have been conducted by a variety of authors, each with differing results. For example, Lorenzen *et al.,* (1991) demonstrated that the disease could be transmitted by cohabitation as well as by intraperitoneal injection of isolated bacteria. On the other hand, Iida and Mizokami (1996) failed to transmit the pathogen by immersion and Holt (1987) found that the virulence mechanisms of the pathogen were better expressed in muscle tissue of the animal rather than in the peritoneal cavity. Brown *et al.,* (1997) suggested that amphibians, insects or other animals, may act as a reservoir for the bacterium. Wild fish, or fish escaping from farms, may also contribute to the spread of the disease, as observed by Wiklund *et al.,* (1994). The possibility of the disease being transferred by bacteria suspended in the water column or in sediment also can not be excluded. However, for this mode of transmission *F. psychrophilum* must be able to survive outside the fish for a period of time.

F. psychrophilum is considered a rather fastidious organism and problems with the culture of the bacterium have been reported by many authors (Daskalov *et al.,* 1999; Michel *et al.,* 1999). Under adverse conditions it may be possible that the bacterium enters a viable, but non-culturable state (i.e. not able to form colonies on agar plates) as in the case of many other bacterial species, such as *Salmonella* spp. (Dupray *et al.,* 1997) and *Yersinia ruckeri* (Thorsen *et al.,* 1992). This characteristic results in an inability to detect the pathogen in environmental samples when culture is used as the only means of identification.

The ability of *F. psychrophilum* to survive under conditions of starvation was examined in this study and changes in the organism so as to adapt to its environment described. *F. psychrophilum* cells were maintained under different environmental conditions; in stream water, broth and distilled water. Changes in the general morphology of the cells and their culturability and viability under the different conditions were examined. The sensitivity of a PCR was also evaluated with respect to its ability to detect possible DNA degradation within the starved cells.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial preparation

F. psychrophilum, strain B97026, isolated from an outbreak of RTFS in UK in 1997 was used in the present study. A three-day old culture, grown in modified *Cytophaga* broth (Bernardet and Kerouault 1989) at 14° C, was used as the stock culture from which a series of four treatments were prepared. Before preparing these, the bacterial concentration of the stock culture was determined (total number of bacteria per ml) using an acridine orange direct counting method (AODCM) as described by Hobbie *et al.,* (1977).

The concentration of the stock culture was approximately 2.8×10^8 cells ml^{-1}, as determined by AODCM, which corresponded to a CFU count of approximately 2.8×10^8 CFU ml^{-1}. All bacteria stained 'green' with the Live/Dead Kit described below, indicating that all the bacteria were alive. The four treatments involved placing stock bacteria in a) stream water, b) *Cytophaga* broth, c) distilled water and d) killing bacteria with Limox (400 ppm H_2O_2 & peroxyacetic acid, DiverseyLever Ltd), a disinfectant used for farm equipment.

An aliquot of 3.5 ml of the stock culture, equivalent to 10^9 bacteria, was placed into eight universals. The universals were centrifuged at 3000 g for 15 min and the supernatants discarded. Five ml of distilled water, filtered through a 0.2 µm filter, was added to 6 of the tubes, while 5 ml of Limox was added to the remaining two tubes. The universals were left for 10 min at 20° C before centrifuging, as described above, and the supernatants were then discarded. Five

ml of *Cytophaga* broth was added to the two universals containing the bacteria treated with the disinfectant. Five ml of filter-sterilised distilled water was added to another two of the universals, 5 ml of filter-sterilised stream water to another two of the universals, while 5 ml of *Cytophaga* broth was added to the remaining two universals. After preparing the bacteria in the different treatments, the total number of bacteria in each universal was determined using the AODCM. Approximately half of the bacteria were lost during the two centrifugations, with yields of bacteria ranging from 4.4 to 4.7×10^8 bacteria per tube. The concentration of the bacteria was finally adjusted to 4.5×10^7 bacterial cells ml^{-1} for each universal by adding stream water, *Cytophaga* broth or distilled water as appropriate. The bacterial preparations were maintained in the dark at 14° C for the duration of the experiment.

The number of culturable cells within the stock culture and the four experimental cultures was determined on a weekly basis for the duration of the experimental period, which lasted for nineteen weeks. These were expressed as CFUs per ml for samples cultured on *Cytophaga* agar plates. Plates were prepared in triplicate for each count and bacterial colonies were counted 3 to 4 days after inoculating the plates.

In addition to the CFU counts, viability and culturability was also tested by adding a 100 μl aliquot of each bacterial suspension to fresh *Cytophaga* broth. This was tested after setting up the experiment and again after 36 weeks.

3.2.2 Live/Dead kit

As well as determining the number of viable cells by culture, the number of viable cells within the stock culture and each treatment was also estimated using a Live/Dead kit (Molecular Probes). This kit is based on the ability of certain fluorescent dyes (e.g. propidium iodide and ethidium homodimer-1) to penetrate damaged cell membranes. Only when the integrity of the cell membrane is compromised, as for example when the cells are dead, can the dyes penetrate the cell membranes and stain nucleic acids.

A 100 µl aliquot of each bacterial suspension, prepared in triplicate, was added to 400 µl of filtered PBS and 1.5 µl of a 1:1 mixture of the two components of the kit was added to each bacterial suspension. The bacterial suspensions were mixed thoroughly and incubated in the dark for 10 min. Three drops (5 µl) from the replicates of each treatment, were placed onto a coverslip, the corners of which had been covered with wax. A slide was then placed on top of the coverslip. The slides were inverted and examined using a Leitz Orthoplan fluorescence microscope, as described in Section 2.2.1.

Bacterial suspensions were examined weekly with the Live/Dead Kit. The first set of samples was examined one hour after setting up the experiment and the last set was examined 10 weeks later. Statistical analysis of the results was carried out using Minitab 10.1 (Minitab Inc) software. Results for bacteria maintained in broth were compared with the results for bacteria maintained in stream water, by a one way analysis of variance (ANOVA) following tests for normality (Anderson-Darling test). Tukey test was employed to identify significantly different, where $p < 0.01$ was considered significant.

3.2.3 Processing of samples for Transmission Electron Microscopy.

The first samples for transmission and scanning electron microscopy were taken approximately 4 weeks after setting up the experiment and the last ones after 14 weeks (three and a half months). In addition, a sample from the stock culture (three- day old bacteria) was also collected when the experiment was set up. Bacterial samples were fixed in 2.5 % gluteraldehyde for 2 h. One ml of each bacterial suspension was added to an equal volume of 5% gluteraldehyde in 0.1 M sodium cacodylate buffer to give a final concentration of gluteraldehyde at 2.5% v/v. The samples were centrifuged at 3000 g for 15 min and the supernatants discarded, taking care not to disturb the pellet, before adding 500 μl of 0.1 M sodium cacodylate buffer to each tube. The samples were incubated overnight at 4° C. The buffer was carefully removed to prevent disturbing the pellet and 50 μl of 2% (w/v) agar prepared in filter-sterilised distilled water, at a temperature of 45° C, was added to the pellet. The agar was thoroughly mixed with the pellet by pippeting up and down several times and was then allowed to cool. The agar plugs were removed from the tubes and cut into 6-8 smaller pieces. The small pieces were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer. The osmium was removed and 2% uranyl acetate in 30% acetone was added for 1 h in the dark. After dehydration through a series of acetone at 20° C (60% for 40 min, 90% for 40 min, 100 % for 40 min and 100 % acetone for 1 h), the samples were infiltrated with Spurrs Resin [10 ml 4-Vinylcyclohexene Dioxide, 6 ml DER Resin, 26 ml (2-Nonen-1-yl) succinic anhydride, 0.4 ml 2-Dimethylaminoethanol] using a rotator mixer. A mixture of 1:1 acetone:Spurrs was added overnight, followed by

further infiltration with 100 % Spurrs Resin for 24 h. Finally, the small pieces were embedded in green block moulds and left to polymerise at 60° C for 48 h.

3.2.4 Processing of samples for Scanning Electron microscopy.

The samples were fixed with 2.5 % glutaraldehyde for 2 h as described above, then passed through a 0.2 μm polycarbonate filter membrane (Millipore) to collect bacterial cells on the membrane. The membranes were then placed into small plastic Petri dishes, to which 0.1 M sodium cacodylate buffer was added so as to cover the membranes. The membranes were incubated in the buffer overnight at 4° C. The samples were then post-fixed in 1 % osmium tetroxide in sodium cacodylate buffer for 2 h. After dehydration through an ethanol series (30% for 30 min, 60% for 30 min, 90% for 30 min, 100% for 30 min and 100% for 1 h), a mixture of 100% ethanol: hexamethyldisilazane (HMDS) was added to the membranes at a ration of 1:1 for 30 min. Two changes of HDMS were made at 30 min intervals, and the membrane filters were then left to air-dry overnight. Small pieces of the filter membranes were cut, mounted on stubs and sputter coated.

3.2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of bacteria.

An aliquot of each bacterial suspension was sampled eight weeks after setting up the experiment, each containing 6.8×10^8 bacterial cells. These were placed in 1.5 ml microcentrifuge tubes, which were centrifuged for 15 min at 3000 g. Bacterial pellets were re-suspended in 100 μl of deionised water. Sample buffer (25 μl) was

added to each of the samples, which were then boiled for 10 min. Finally, the samples were centrifuged at 13,000 g for 5 min and the supernatant used for the analysis. An aliquot of the stock culture containing the same number of the bacteria as the experimental samples was also collected after the set up of the experiment, processed and used for a comparison.

A 12% polyacrylamide gel was prepared according to the method of Laemmli, (1970). Using a microsyringe, 10 μl of broad range molecular weight marker (BioRad) was added in the first well and 15 μl of each sample added to the remaining wells. The gel was run at 70 V for approximately 3 h at 4° C. It was then stained using a Silver Staining Kit (Sigma, Appendix 5.2).

3.2.6 Western Blot analysis of bacteria

Bacterial components separated by SDS-PAGE as described above, were transferred onto nitrocellulose membranes as described by Wiens *et al.,* (1990) by applying 60 V for 70 min. The nitrocellulose membranes were then incubated in 1 % w/v bovine serum albumin (BSA) in Tris buffered saline (TBS) overnight at 4° C. After washing 3 times with TTBS (TBS+0.1 % v/v Tween 20) for 10 min at each wash, the membranes were incubated with a 1/1000 dilution of rabbit anti-*F. psychrophilum* sera raised against strains B97026 (Faruk, 2000) for 2 h at 20° C. The membranes were washed three times as previously described and incubated with a 1/100 dilution of biotin anti-rabbit IgG (Diagnostics Scotland) for 1h at 20° C. The membranes were again washed and incubated with a 1/100 dilution of streptavidin peroxidase (Diagnostics Scotland) for 1 h at 20° C. After washing 3 times with TTBS, once with TBS and once with PBS, bands were visualised by

adding chromogen and substrate (Appendix 2.4). The reaction was stopped by soaking the membranes in distilled water for 10 min.

3.2.7 PCR analysis

Approximately 2×10^7 bacteria was added to 1.5 ml sterile tubes and centrifuged at 3000 g for 15 min. Extraction of DNA from bacterial samples was performed according to the method described in Section 2.2.2. After the extraction, the DNA pellets were re-suspended in 100 µl of sterile nano-pure water.

A nested PCR was used for the amplification of *F. psychrophilum* DNA, using primers described by Toyama *et al.,* (1994). Ready To Go PCR beads (Amersham Pharmacia Biotech Inc) were used for the reaction, and this was performed according to the parameters described in Section 2.2.3.

3.3 RESULTS

3.3.1 Total number and number of viable bacteria present in each treatment

The total number of bacteria present in each bacterial suspension maintained under the different conditions was determined weekly, throughout the 19-week experimental period using AODCM, while at the same time the numbers of culturable cells were determined by counting CFU's on *Cytophaga* agar. The percentage of viable cells was also determined using the Live/Dead kit during the first 10 weeks. The viability of the bacteria kept under different conditions, using the Live/Dead kit and the culturability using CFU's on *Cytophaga* agar are summarised in Table 3.1.

All the bacteria present in the stock culture used to set up the experiment appeared culturable on agar plates and viable with the Live/Dead kit.

The first sampling of the bacteria maintained under the different experimental conditions was performed one hour after setting up the treatments. Culturable cells were present at approximately 1.8×10^7 (40%) CFU's ml^{-1} in universals containing bacteria in stream water (Table 3.1), while approximately 4×10^7 CFU's ml^{-1} (90%) were present in the universals containing bacteria in broth. All bacteria in the samples appeared viable with the kit at this time. No bacterial growth was obtained from universals either containing bacteria treated with the disinfectant or bacteria placed into distilled water. Using the Live/Dead kit, all bacteria present in these treatments also appeared non-viable. When bacteria from all different treatments were placed into fresh *Cytophaga* broth, growth was

obtained only from bacteria transferred from the universals containing bacteria in stream water or broth.

The total number of bacteria determined by AODCM remained relatively constant throughout the experimental period for all treatments, except the bacteria maintained in broth. The total number of bacteria present in the stream water maintained constant for the first 16 weeks and started to decline slightly thereafter. The number of bacteria kept in broth reached a peak number at Week 8 of 8.5×10^8 cells ml^{-1} and the number started to decline slightly thereafter. Cells gradually became more difficult to stain with acridine orange, making cell number determination difficult. Thus, no further samples were counted after Week 16.

The number of culturable cells present in stream water showed a steady decline, and by Week 19, very few bacterial colonies (and in some cases no colonies at all) were obtained on *Cytophaga* agar and thus the experiment was terminated. When bacteria were placed directly to broth, however, growth of the bacterium could be observed, even after 9 months suggesting that broth was a more suitable environment for them than agar to grow.

The viability of the bacteria under the different conditions was assessed for the first 10 weeks using the Live/Dead kit. After the end of this period, 51.6 % of the bacteria maintained in broth appeared 'live' with the kit, while 94.5% of the bacteria in stream water were still viable. After this time the non-viable ('dead') bacteria were not clearly visible and thus no further samples were taken.

Table 3.1 Total number of cells using AODCM and assessment of the viability of *F. psychrophilum* under the different experimental conditions using culture on *Cytophaga* agar and a Live/Dead kit. All values represent the means of six samples ± standard deviation

Sampling periods	Bacterial sample	Concentration of bacteria ($\times 10^7$ cells/ml)[a]	Culturable cells (%)[b]	LIVE/DEAD Kit (% of 'live' cells)
1 h	Broth	4.5±0.71	90*±1.4	100±0
	Stream water	4.5±0.82	40*±2.1	100±0
	dH₂O	4.5±1.1	no growth**	0±0
	Disinfected	4.5±0.66	no growth**	0±0
1 week	Broth	53.0±0.54	-[c]	99.5±0.8
	Stream water	4.5±1.2	40±2.23	99.8±04
	dH₂O	4.5±1	-	0±0
	Disinfected	4.5±0.75	-	0±0
2 weeks	Broth	55.0±0.4	-	98.3±0.75
	Stream water	4.5±0.78	31±3.2	99.6±0.51
	dH₂O	4.5±0.9	-	0±0
	Disinfected	4.5±1.1	-	0±0
4 weeks	Broth	63.0±2.3	-	97.1±0.89
	Stream water	4.5±1.65	4.4±1.5	97.8±0.75
	dH₂O	4.5±1.2	-	-
	Disinfected	4.5±2.6	-	-
8 weeks	Broth	85.0±0.94	-	54.5±3.44[d]
	Stream water	4.5±1.12	2.7±1.95	97±0.75
	dH₂O	4.5±1.55	-	-
	Disinfected	4.5±0.75	-	-
10 weeks	Broth	79.0±1.44	-	51.6±2.1[d]
	Stream water	4.5±2.4	2.2±2.1	94.5±1.37
	dH₂O	4.5±2.22	-	-
	Disinfected	4.5±0.89	-	-
16 weeks	Broth	60.0±02.66	-	-
	Stream water	2.5±1.7	1.6±3.2	-
	dH₂O	4.5±1.1	-	-
	Disinfected	4.5±2.3	-	-
19 weeks	Broth	-	-	-
	Stream water	2.2±0.97	0.07±2.7	-
	dH₂O	4.5±0.87	-	-
	Disinfected	4.5±1.2	-	-
36 weeks	Broth	-	-*	-
	Stream water	-	-*	-
	DH₂O	-	-	-
	Disinfected	-	-	-

Note: A stock culture of 2×10^8 bacteria ml⁻¹ was used to set up the experiment. All bacteria in the stock culture were 100% viable assessed by both CFU's and LIVE/DEAD Kit. The concentrations in the different treatments were adjusted to 4.5×10^7 bacteria ml⁻¹ with the appropriate dilutent. [a] Assessed by Acridine Orange Direct Counting Method (AODCM), [b] Assessed by CFU's on *Cytophaga* plates, -[c] not determined, [d] significally different values ($p<0.01$), *resuscitation in *Cytophaga* broth occurred, ** bacteria were placed in broth but no growth was observed

3.3.2 Scanning electron microscopy.

Bacteria from the stock culture (3-day-old) appeared as long straight rods, 5-8 μm in length (Figure 3.1a) and a few were found to be 10-12 μm in length.

Bacteria kept in broth for one month (Figure 3.1b) appeared as long slim rods, 8-12μm in length. Some were enlarged either at the end or along the length of cells (Figure 3.1b, black arrow). Many cells, which formed rings, were also evident (Figure 3.1b, white arrows).

Bacterial cells kept in distilled water for one month (Figure 3.1c) appeared relatively shorter than bacteria kept in broth and appeared about the same size as the bacteria taken from the stock culture (Figure 3.1a), although a few long rods could also be observed. Bacteria maintained under this condition generally appeared as straight rods, although some enlarged segments (Figure 3.1c, black arrow), and ring forms (Figure 3.1c, white arrow) could also be observed. These were less frequently observed compared to bacteria maintained in broth. Bacteria maintained in distilled water retained their general morphology, even after 16 weeks.

Bacteria treated with disinfectant appeared similar to the bacteria kept in distilled water when examined one month after the treatment (Figure 3.1d).

After keeping bacteria in stream water for one month, cells appeared shorter compared to the bacteria kept in broth, distilled water, or after treating with disinfectant (Figure 3.1e). The enlargements, seen in Figure 3.1c with bacteria maintained in distilled water, were also observed (Figure 3.1e, black arrows) in many cells. The bacteria were generally straight but a few curved cells were also

observed (Figure 3.1e, white arrow). Enlarged sections within the bacterial cells are highlighted in Figure 3.1f.

Bacteria kept in broth for 16 weeks were generally seen as long or ring cells with an anomalous surface; a lot of cell debris was also observed (Figure 3.1g). Bacteria of the same age kept in stream water appeared either as small rods, approximately 1-2 μm long, or as small rings or small spheres (Figure 3.1h).

Figure 3.1 Scanning electron microscopy of *F. psychrophilum* maintained in: a) broth for 3 days, Bar=1μm, b) broth for approximately 4 weeks, Bar=1μm, c) distilled water for 4 days, Bar=1μm, d) broth after disinfection for 4 weeks, Bar=1μm, e) stream water for 4 weeks, Bar=1μm, f) stream water for 4 weeks, Bar=1μm, g) broth for 16 weeks, Bar=1μm, h) stream water for 16 weeks, Bar=1μm
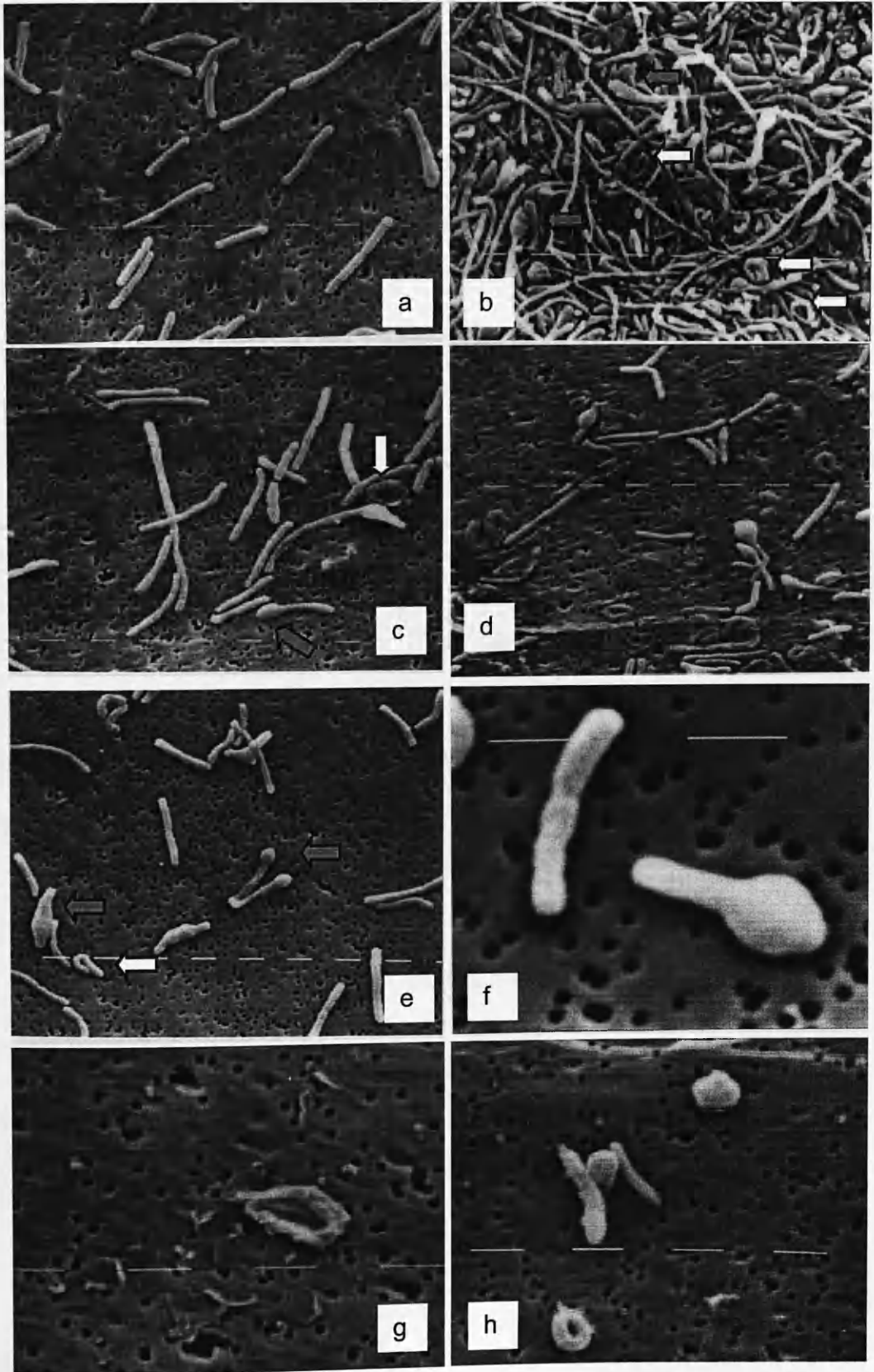
3.3.3 Transmission Electron Microscopy

Regardless of conditions under which the bacteria were maintained, four weeks after setting up the experiment, all bacteria generally had the same general morphology as the bacteria taken from the stock culture (3-day-old culture) (Figure 3.2a). A glycocalyx could be seen around the cells (black arrows, Figure 3.2a,c), although the glycocalyx in bacteria maintained in broth and stream water for 16 weeks appeared not to be as thick and well-defined as in 3-day-old bacteria. Bacteria maintained in stream water, distilled water or treated with disinfectant retained this morphology even after 16 weeks (Figure 3.2b). Bacteria kept in broth for the same period, however, appeared irregular in shape and deep indentations could be seen in the surface of the cell (white arrows, Figure 3.2c,d). Many cells appeared 'empty' with large vacuoles within the cell (Figure 3.2d). An example of an enlarged segment within the bacteria kept in stream water is shown in Figure 3.2e.
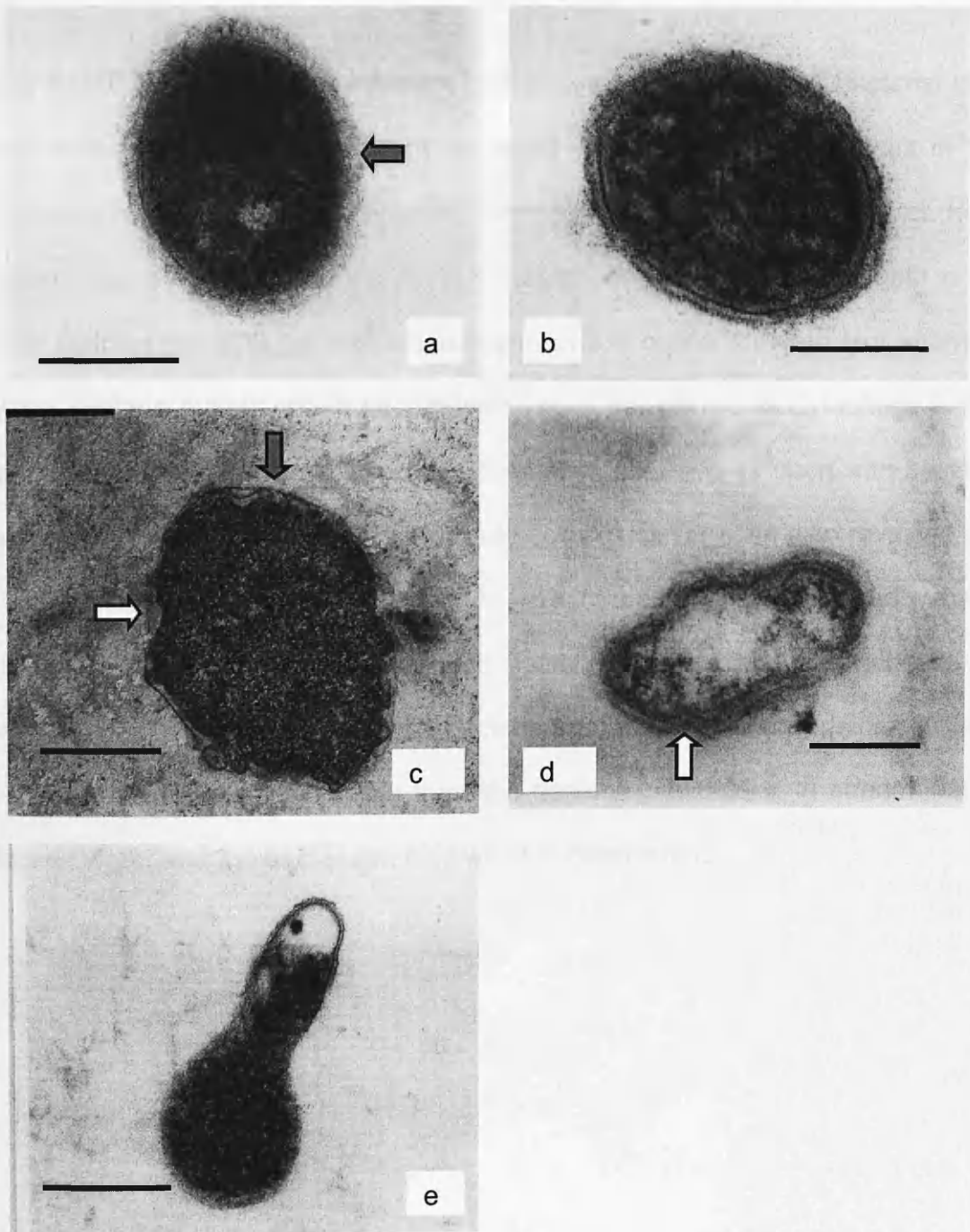
Figure 3.2 Transmission Electron Microscopy of *F. psychrophilum* maintained in: a)broth, for 3 days, bar=0.2 μm, b) stream water for 16 weeks, bar=0.2 μm, c) broth for 16 weeks, bar=0.5 μm, d) broth for 16 weeks, bar=0.2 μm, e) stream water, for 16 weeks, bar=0.2 μm. Black arrows indicate the presence of glycocalx (the glycocalx in Figure 3.2c appears to be not as thick and well defined as in Figure 3.2a or b). White arrows indicate indentations

3.3.4 SDS-PAGE and Western blot analysis of whole bacterial cells

SDS-PAGE (Figure 3.3) and Western blot analysis (Figures 3.4) of bacterial cells kept under the different conditions for eight weeks showed differences in the profiles between treatments, especially bacteria maintained in stream water. Less material was evident around the 20-40 kD region, under this treatment, both in the silver stain of the SDS gel and the antigenic profile of the Western blot analysis. When bacteria maintained in stream water were transferred to *Cytophaga* broth, and cultured for three days, similar profiles were obtained as seen with bacteria taken from the stock culture (three-day-old culture). Silver stain also revealed the disappearance of a band at approximately 45 kD from the bacteria treated with the disinfectant. In addition, a band of approximately 75 kD, which was evident in the bacteria maintained in broth and the bacteria from the fresh culture (stock culture) also appeared to be less visible from the bacteria maintained in stream water, distilled water and the bacteria treated with the disinfectant.
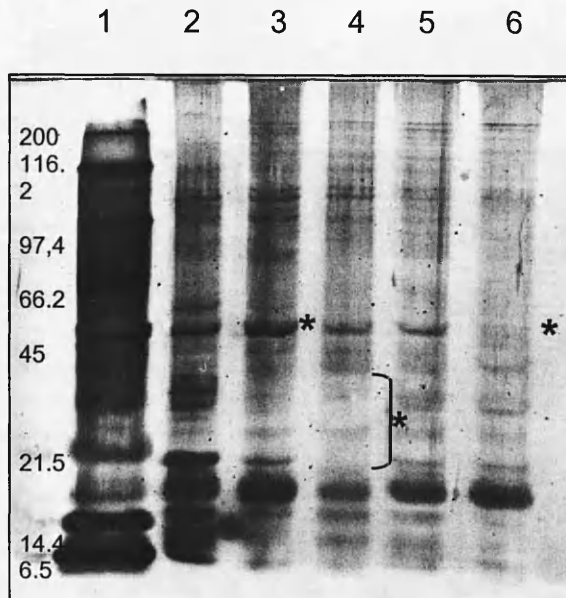
Figure 3.3 Silver stain of 12% SDS-PAGE of *F. psychrophilum* maintained under different experimental conditions. Lanes 1: marker, 2: bacteria from the stock culture, 3: bacteria maintained in broth for eight weeks, 4: bacteria kept in stream water for eight weeks, 5: bacteria kept in distilled water for eight weeks, 6: disinfected bacteria maintained in broth for eight weeks. * indicates regions of difference in the protein profile
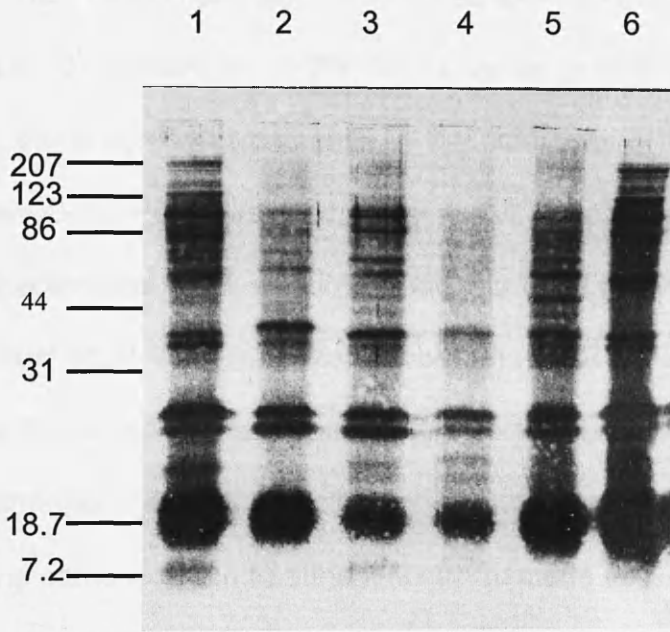
Figure 3.4 Western Blot analysis of *F. psychrophilum*, maintained under experimental conditions, using rabbit anti *F. psychrophilum* B97026 serum. Lanes 1: bacteria maintained in stream water for eight weeks and transferred to fresh broth for 3 days, 2: disinfected bacteria maintained in broth for eight weeks, 3: bacteria kept in distilled water for eight weeks, 4: bacteria kept in stream water for eight weeks, 5: bacteria maintained in broth for eight weeks, 6: bacteria from the stock culture

3.3.5 PCR analysis.

The sensitivity threshold of the one-round PCR method (when only the PSY1/PSY2 pair of primers were used) had been previously estimated to be approximately 10^5 bacteria ml^{-1} (Chapter 2). In the present study however, there appeared to be a significant decrease in the sensitivity of the PCR (Figure 3.5). When the one-round PCR was used, the sensitivity threshold appeared to be more than $2x10^7$ bacteria ml^{-1}, the amount of bacteria used in the assay (Figure 3.5a). When a nested PCR was performed (two-round PCR), there appeared to be a difference in the intensity of the bands obtained with bacteria taken from the four different treatments (Figure 3.5b), although the number of the bacteria used in the assay was the same for each treatment ($2x10^7$ bacteria ml^{-1}). A region of smearing close to the expected band of 1078 bp was also observed with the bacteria maintained in broth and stream water (Lanes 1 and 4).

Figure 3.5 A 1% agarose gel of polymerase chain reaction (PCR) products of DNA extracted from bacteria kept for fourteen weeks under different experimental conditions. a): one round PCR (PSY1/PSY2 primers) b) nested PCR (using 2OF/1500R and PSY1/PSY2 primers), Lanes (M): marker (DNA Molecular weight marker VI, Ross), (1): in broth, (2): in dH$_2$O, (3) treated with disinfectant, (4): in stream water, (5) negative control (water)

3.4 DISCUSSION

All microbes at some point in their life cycle encounter starvation to various degrees, and therefore starvation may be considered as a 'normal' stage of their life. There appears to be only a few exceptions to this rule, such as the bacteria living in the intestine of their host or when grown in artificially enriched media (Morita, 1985). The present study investigated the ability of *F. psychrophilum* to survive under starvation in different media for a period of 9 months. Morphological changes as well as changes in the viability and culturability were examined. In addition, the sensitivity of a PCR test to detect the starved cells was also evaluated.

All bacteria have different abilities to survive under adverse conditions. Some can survive for a few hours only, while others can survive for many years. Many factors are involved in the survival of microbes under starvation and because of the complex interactions between bacteria and their environment it is difficult, if not impossible, to mimic states of starvation under laboratory conditions. A number of internal factors can also affect the ability of the cells to survive under different environmental conditions, such as the endogenous metabolism of the bacterium, the presence of material stored within the bacterial cell, the energy requirement of the cell, adenylate energy charge and the preservation of membrane potential (Dawes, 1985). However, since no bacterial species exists solely in one particular environment, it has to be able to cope with a variety of external factors to be able to survive. Competition, predation, light, temperature and pH fluctuations have significant influence on the ability of the bacterium to survive in its environment (Panicker *et al.,* 1994).

It is well established that *F. psychrophilum* is a rather fastidious organism and that many problems exist with its detection using antibody-based techniques (Lorenzen and Karas, 1992; Lorenzen and Olesen, 1997) or culture methods (Daskalov *et al.,* 1999; Michel *et al.,* 1999). The kit used in the present study to test the viability of *F. psychrophilum* has been used by many authors to test the viability and especially the permeability of starved microbes. Joux *et al.,* (1997) for example, used the same kit in flow cytometry to test the membrane permeability of starved *Salmonella typhimurium*. Michel *et al.,* (1999) also using the same kit reported differences in viability of cells between plate enumeration and fluorescent staining methods. In the present study, the level of viability of *F. psychrophilum* obtained with the kit and by culture were examined at weekly intervals over a 10 week period. However, slight modifications were made here to the kit protocol; one component, the propidium iodide, was replaced with ethidium homodimer-1 a large molecule (almost double the size of propidium iodide). Preliminary analysis with propidium iodide showed that when 3-4 day old cultures of *F. psychrophilum* cells were subjected to any form of pressure, such as centrifugation, filtration or placed between microscope slides and coverslips, cells appeared 'red', indicating that the bacteria were 'dead', while this was obviously not the case. Many of the bacterial cells still appeared 'dead' after subjecting the cells to any kind of pressure, even after substituting propidium iodide with ethidium homodimer-1. However, preliminary analysis using bacteria of different age as well as dead and the method proposed in this study provided the expected results.

One drawback with the kit seemed to be an over-estimation of live cells, since after the first ten weeks, dead cells started to become less visible. Despite this, the method can provide a rapid and easy alternative to flow cytometry, for

determining cell numbers. Another consideration when using the kit is that cells under starvation can be permeable to normally membrane-impermeant stains (Kell *et al.,* 1994).

The results of the study showed that growth of the bacterium on *Cytophaga* agar did not correspond to the viability of the bacterium obtained with the Live/Dead kit and that more bacteria appeared viable than culturable. The differences observed in this study were greater than those seen by Michel *et al.,* (1999), possibly due to the fact that the bacteria entered a viable but not culturable state under starvation. The ability of the microbes to enter a viable but non-culturable state is known for many species. This is a very important issue for accurate determination of cell numbers or detection of the bacterium in different environmental samples by culture, if culture is the only method used to quantify or identify the bacterium (Huq and Colwell, 1994).

Starved cells appear to exhibit an enhanced resistance to heat, oxidative and osmotic shock (Barcina *et al.,* 1997) and in some cases increased surface hydrophobicity occurs, as in the case of *Photobacterium damselae* subsp. *piscicida* (Magariños *et al.,* 1994). The metabolic activity of the cells is reduced and the cells stop multiplying (Magariños *et al.,* 1994). This was also found to be the case with *F. psychrophilum* in this study; the total number of bacteria maintained in stream water was the same throughout the duration of the experiment. Comparison of viability between bacteria maintained in stream water and broth, assessed using the kit, revealed that the percentage of viable bacteria in stream water was substantially higher than bacteria kept in broth. Perhaps, a lower metabolic activity of the cells kept in stream water, possibly due to the

limited nutrients in the medium, delayed the death of the cells, while an opposite situation occurred with bacteria maintained in broth.

Although CFU's were not determined after 19 weeks from the onset of the study, it was possible to resuscitate bacteria maintained in stream water by transferring them to *Cytophaga* broth, and such bacteria were able to multiply even after 9 months, suggesting that some of the cells were still alive and placing the bacteria in broth helped to resuscitate them. This phenomenon has also been observed by Kell *et al.,* (1994) for *Micrococcus luteus*. They found that starved bacteria were incapable of growing when placed on agar plates, but could be recovered by a resuscitation step in a weak nutrient broth.

Although the bacterium can survive in stream water for more than four months, its survival in distilled water is very short. It was found that one hour in distilled water resulted in the death of all the cells (both cultures and Live/Dead kit showed cells to be non-viable). Michel *et al.,* (1999) found that *F. psychrophilum* appears to be highly susceptible to osmotic conditions.

Changes in the general morphology of cells during starvation were also of interest. Some bacterial species form specific structures, such as spores (e.g. *Bacillus cereus*), while others assume a vegetative state, which enables them to survive under adverse conditions. *F. psychrophilum* maintained in stream water adopts the latter option. During starvation in stream water bacterial cells tend to shrink and become round. The formation of dwarf cells is a very common feature for many bacterial species to help them survive long periods under starvation conditions. For example, *Vibrio parahaemolyticus* has been observed to change from rods to spheres under starvation (Jiang and Chai, 1996). *F. psychrophilum* maintained in broth on the other hand, tends to form long slender filamentous

cells. When maintained in stream water and broth for long periods of time, the cells appear to develop areas of swelling across the cell. The function of these swellings is still unknown, since it is a non spore-forming species. They may possibly be an intermediate stage in the development of the dwarf cells. Cells of *F. psychrophilum* also tend to 'curl' and even develop 'ring' forms, especially those maintained in broth. Similar types of formation have also been reported by Ostland *et al.,* (1994) for *F. branchiophilum.* Little information is available as to the causes of this change in morphology. Round bacterial cells, which are usually found in aged liquid cultures of other *Cytophaga*-like bacteria, such as *Flavobacterium columnare* and *Flexibacter maritimus* (Garnjobst 1945; Reichenbach 1989), were never observed in the present study. These cells are called sheroplasts and are degenerative forms that cannot be revived when transferred to fresh medium.

Interestingly, the bacterium is able to retain the glycocalyx around its cell, regardless of the environmental conditions in which it was maintained, although slight differences are apparent in the thickness of this layer between the different treatments.

Some species of bacteria have been reported to produce either less or different substances during starvation. Papapetropoulou *et al.,* (1993), studying metabolic and structural changes of starved *E. coli* cells, found that some strains of the bacterium lost some of their cytoplasmic proteins during starvation and the levels of many enzymes increased, while others dramatically decreased. Rahman *et al.,* (1998) found that starved *Aeromonas hydrophila* produced different outer membrane proteins. In the present study, both silver staining of SDS-PAGE gels and Western Blot analysis revealed little differences between treatments, although it would appear that bacteria kept in stream water had less lower molecular weight

material (20-40 kD) than bacteria maintained in broth, compared to bacteria from the stock culture (a three day old culture). Faruk (2000) using different staining techniques identified glycoproteins and carbohydrates in this region. Roslev and King, (1995) reported a similar loss of low molecular weight material for starved methanotrophic bacteria, suggesting that this was due to either low metabolism of cells or a substantial reduction in their size. When *F. psychrophilum* maintained in stream water was transferred to fresh *Cytophaga* broth, they regained both their original morphology as well as the SDS-PAGE and Western Blot profiles as exhibited by fresh cultures. Analysis of substrate gels could provide information on the changes in the proteolytic activity of *F. psychrophilum*, maintained under prolonged periods of starvation.

Finally, a decrease in the sensitivity of the PCR and an area of smearing around the expected region for the PCR products were observed, indicating a possible deterioration of the bacterial DNA, as suggested by Morgan *et al.*, (1991) in the case of starved *Aeromonas salmonicida*. The decrease seen in the fluorescence signal of the Live/Dead kit may also be as a result of this deterioration. Environmental factors, such as water temperature or the existence of other microbes that are able to destroy and consume dead cells, can also affect the persistence of DNA within the cells, as observed by Dupray *et al.*, (1997) and Joux *et al.*, (1997).

Our knowledge of the survival of *F. psychrophilum* outside the fish is currently very limited. This study examined the changes which occurred in starved *F. psychrophilum* when maintained under different conditions. The bacterium was found to survive for long periods of time in stream water, and became smaller retaining its characteristic glycocalyx, which could be seen around the bacterial

cell, while the bacterial cells became long and formed rings in *Cytophaga* broth. On the other hand, the bacterium could not survive for more than an hour in distilled water. A decrease in culturability was also observed, making identification of the pathogen by culture very difficult, especially for environmental samples. Problems were also observed with the PCR used to detect the starved cells. The sensitivity of the reaction appeared to decrease possibly due to degradation of DNA within the cells. No significant changes were observed in the antigenic profile of whole cells, but the bacterium appeared to lose some of the low molecular weight material associated with the bacterium cultured in *Cytophaga* broth, when it was maintained in stream water.

Although this study demonstrated some of the changes that occur in *F. psychrophilum* during its the starvation, more general aspects of the bacterial function still need to be investigated under periods of starvation. Virulence mechanisms and resistance to various antimicrobial agents are also properties that may be altered during starvation. Other aspects, associated with the changes that occur during the starvation of *F. psychrophilum*, such as changes in the surface hydrophobicity and ability of the bacterium to attach to eggs, or problems associated with the used of some diagnostic techniques are discussed in the following chapters.

CHAPTER 4

DEVELOPMENT OF A QUANTITATIVE METHOD FOR THE DETECTION OF *F. PSYCHROPHILUM*

4.1 INTRODUCTION

The presence of a particular bacterial pathogen in the aquatic environment of a fish farm does not always mean that the bacterium will cause disease, nor does the identification of the pathogen in environmental samples, such as water, eggs, sediment or even in fish indicate that it is the causative agent of the disease. Barker (1990) identified the presence of *Cytophaga* sp., *Pseudomonas fluorescens* and *Aeromonas hydrophila* on the surface of live eggs of rainbow trout. However, the author concluded that these bacteria were only likely to be responsible for egg deaths when other environmental factors were present in the hatchery, such as high water temperature, slow flow rates or high egg densities. In many cases, a number of other factors need to be present in order for the disease to manifest itself in fish, particularly those of stress and poor farm management.

The level of bacteria present in the aquatic environment seems to vary throughout the year for a number of different reasons. Crabill *et al.,* (1999) found a deterioration in the water quality of Oak Creek in Arizona during summer months, together with an increase in faecal coliform counts. They believed this change to be due to sediment agitation caused by recreation activities and summer storms. Levels of some other pathogens seem to follow the variations seen with other

microbes present in the aquatic environment. For example, Padmakumar and Ayyakkannu (1997), reported variations in the number of *Staphylococcus aureus* and *Vibrio* spp. present in the water. They believed these variations to be due to fluctuations in the number of marine algae, which exhibit antimicrobial activity.

Although the presence and the level of the pathogen in the aquatic environment is not always directly related to a disease outbreak, enumeration of the pathogen in environmental samples, especially in water samples, can be very useful for disease monitoring. For instance, the efficacy of water treatment can be established by counting bacteria in the water before and after treatment. Ozone, for example, effectively reduces the number of microbes present in the water (Austin, 1983). On the other hand, the level of a particular pathogen tends to increase in the water when a disease occurs. Also, important information can be obtained on the mode of transmission, especially relating to the shedding rate of the pathogen, as seen in the case of *Renibacterium salmoninarum*, as determined by McKibben and Pascho (1999).

A variety of techniques have been used to determine bacterial levels in environmental samples. These included conventional microbiology methods, such as colony forming units (CFU's), epifluorescence microscopy, antibody-based techniques (e.g. IFAT) and more recently DNA or RNA-based techniques, including quantitative PCR and *in situ* hybridisation. Such techniques are very useful for epidemiological studies before, during and after a disease outbreak, as well as for testing the efficacy of disinfection or antimicrobial treatments.

The development of a number of methods to quantify *F. psychrophilum* in water samples is described in this chapter. Four different approaches were used; (a) semi-quantitative PCR, (b) quantitative PCR, using an internal standard (c) *in*

situ hybridisation on polycarbonate filter membranes using the primers PSY1/PSY2 described in Chapter 2 as probes and (d) IFAT on polycarbonate filter membranes. The advantages and disadvantages of each method are also discussed.

4.2 MATERIALS AND METHODS

4.2.1 Semi-quantitative PCR

<u>Preparation of bacterial DNA</u>

A culture of *F. psychrophilum*, isolate B97026, was prepared by growing the bacterium in modified *Cytophaga* broth in the dark for 3-4 days at 14° C. The concentration of bacterial suspensions were determined using AODCM (Appendix 1). Serial dilutions of the bacteria, from 10^8 to 1 bacterial cells ml^{-1}, were prepared in PBS in triplicate, in order to establish a standard curve to determine bacterial concentrations in water samples. Extraction of bacterial DNA was performed according to the method described in Section 2.2.2. The extracted DNA was subjected to a PCR using the primer set PSY1/PSY2, as described in Chapter 2.2.3, however, primer PSY1 was labelled at the 5'-end with biotin and primer PSY2 with fluorescein isothiocyanate (FITC). As a result, the PCR products were labelled at one end with biotin and at the other end with FITC. The PCR products (25 μl) were diluted 1/10 (final volume 250 μl) with dilution buffer (Appendix 2.5), ready to be added to the ELISA plates.

<u>ELISA detection of PCR products</u>

Wells of a 96-well ELISA plate were coated with 100 μl $well^{-1}$ of streptavidin (Sigma, 5 μg ml^{-1} in 0.1 M sodium carbonate buffer, pH 9.6). The plates were covered and left to incubate overnight at 20° C or for 2 h at 37° C. The plates were then washed three times with washing buffer (Appendix 2.5).

A 100 µl aliquot of each diluted PCR sample was added to wells of the plates and this was incubated at 20° C for 2 h under constant, but gentle agitation in order to allow the biotin end of the PCR products to bind to the streptavidin. Wells were prepared containing re-suspended PCR beads without bacterial DNA for use as negative controls. After incubation, the wells were washed 5 times with washing buffer before adding 100 µl of 1/2000 dilution of anti-FITC monoclonal antibody labelled with alkaline phosphatase (Sigma) for 2 h at 20° C, diluted in antibody dilution buffer, to detect the FITC end of the PCR products. The wells were washed 5 times as before and 100 µl of 2.5 mg ml^{-1} p-nitrophenyl phosphate (Sigma) in 1 M diethanolamine 0.5 mM $MgCl_2$ buffer pH 9.6, was added to each well. The development of colour was monitored spectophotometrically at 405 nm over a 3 h period.

4.2.2 Quantitative PCR

The principle of the quantitative PCR is described in Figure 4.1. The various steps involved in the development of the method include the production of an internal standard (IS) by modifying the original PCR product sequence (OS) (Figure 4.2), co-amplifying the OS and the IS together in a tube, then applying the PCR products of the reaction to an ELISA, in which labelled oligonucleotide probes are used to detect the OS and IS sequences.

DEVELOPMENT OF THE INTERNAL STANDARD (IS)

The IS is produced from the Original Sequence (OS) of the PCR product. It has the same terminal regions as the OS, however one region is modified so as to allow it to be distinguished from the OS product (Figure 4.4). The IS contains almost the same number of bases as the OS.

CO-AMPLIFICATION OF THE *IS* AND THE *OS* BY PCR

A standard curve is prepared by co-amplifying constant amounts of IS with different amounts of OS. One primer is biotin-labeled.

ELISA

Streptavidin-coated plates are used to capture the PCR products according to the following procedure:

IS
OS
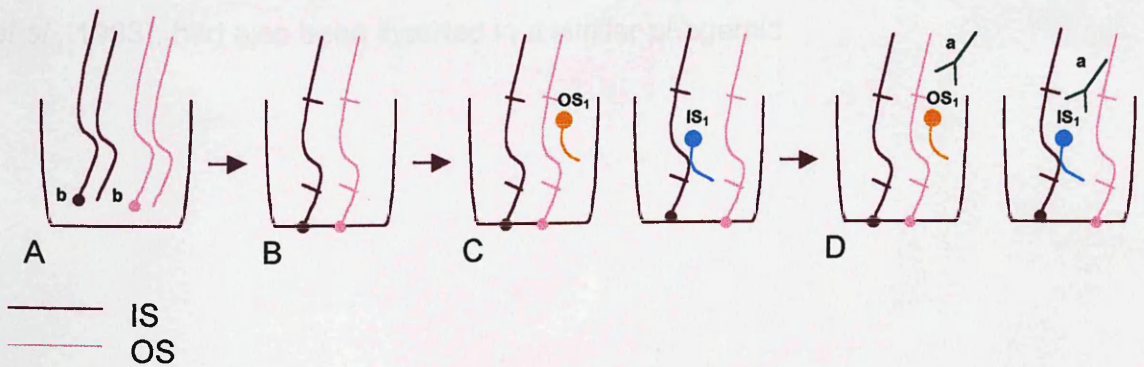
A. Capturing of the PCR products with the biotin labeled end of the products (b).
B. Denaturation of the products using NaOH.
C. Addition of two different FITC-labeled oligonucleotide probes (OS$_1$ and IS$_1$) to detect IS and OS respectively (hybridization step).
D. Use anti-FITC alkaline phosphatase antibody (a) to detect the probes. The ratio between the two signals is used to plot a standard curve for known bacterial concentrations and this is used then to quantify amount of pathogen in unknown samples.

Figure 4.1 Flow diagram of the various steps used in the development of the internal standard.

Development of the internal standard

The original sequence of the PCR products was determined by amplifying DNA obtained from *F. psychrophilum*, strain NCIMB1947, using the PSY1/PSY2 set of primers described in Chapter 2.2.3. The PCR products which resulted were 1078 bp in size. An internal standard was then produced by modifying the original sequence as shown in Figure 4.2, according to the procedures described by Sambrook *et al.,* (1989) (Appendix 4), after inserting it into a pBluescript II KS (+/-) phagemid.

In order to modify the original sequence, a 328 bp fragment was removed from the original sequence and replaced by another fragment of 300 bp with suitable restriction sites at the ends of the fragment (Figure 4.2). The new sequence, obtained from plaice, *Pleuronectus platessa*, P450 1A1 cDNA (Leaver *et al.,* 1993), had also been inserted in a similar phagemid.
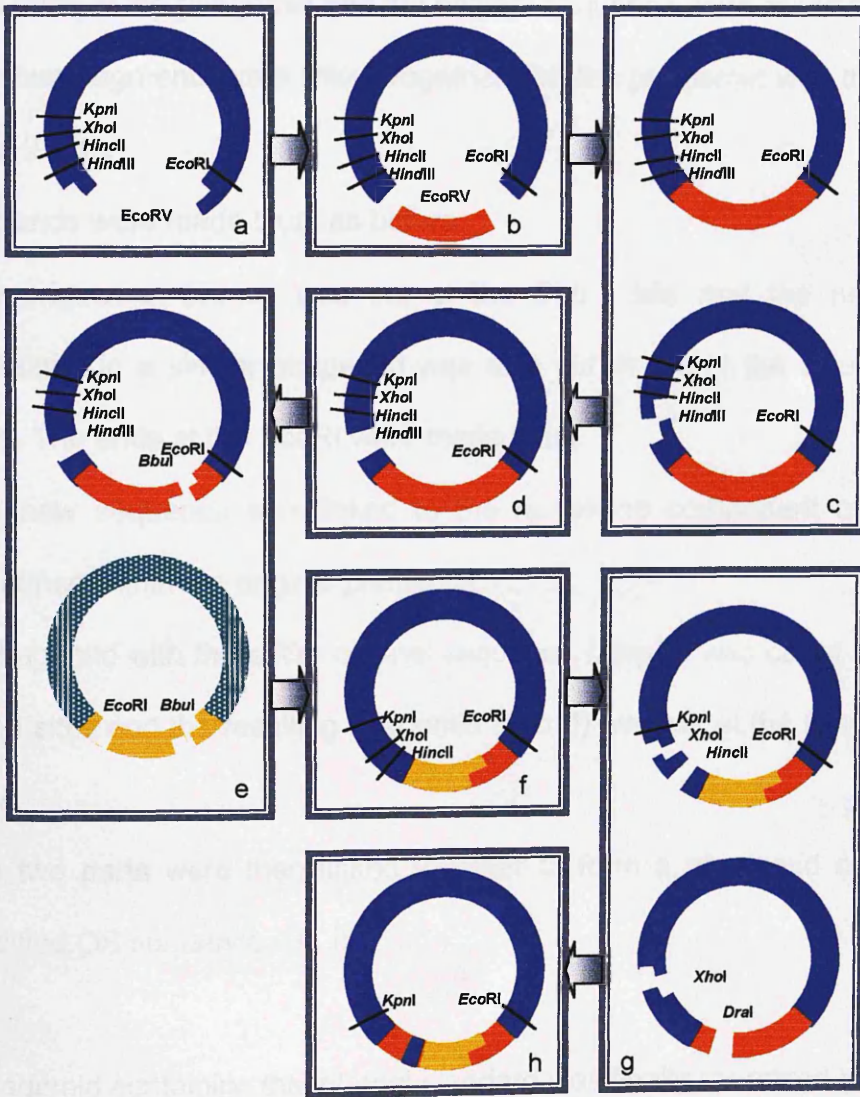
Figure 4.2 Development of the Internal Standard (IS) by inserting the original sequence (OS) into a phagemid and then modifying it.

a. The pBluescript II KS +/- phagemid (STRATAGENE) was cut at the *EcoRV* site.

b. The ends of the phagemid and the original sequence were made blunt.

c. The two fragments were linked together and the phagemid was then cut at the *Hind* III site.

d. The ends were made blunt as before.

e. The original sequence was cut at the *Bbu* I site and the new sequence contained in a similar phagemid was also cut at the at the *Bbu* I and *Eco*RI sites. The ends at the *Eco*RI were made blunt.

f. The new sequence was linked to the remaining component of the original sequence within the original phagemid.

g. A phagemid with the entire original sequence (step b) was cut at the *Dra* I and *Xho* I sites and the resulting phagemid from (f) was cut at the *Hinc* II and *Xho* I sites.

h. The two parts were then linked together to form a phagemid containing the modified OS sequence, i.e. IS.

The phagemid containing the internal standard was finally linearized by cutting it at the *Kpn* I site. Serial dilutions of the phagemids were then prepared and the highest dilution that provided a visible band by PCR was used in the ELISA assay. Aliquots of this dilution were prepared and stored at -20° C.

Competitive PCR

Bacterial suspensions containing between 1 and 10^8 bacteria (total number, estimated by AODCM) were prepared in triplicate. The DNA was extracted from

these samples and co-amplified by PCR with the linearized phagemid containing

the IS. The same amount of IS was added to each reaction: 2.5 µl of the highest

dilution that gave a visible band by PCR according to the method and parameters

described in Chapter 2.2.3. Primer PSY1 was labelled at the 5'-end with biotin

while PSY2 remained unlabelled.

Detection of the PCR products by ELISA

ELISA plates were coated with streptavidin as described in Section 4.2.1 to

capture the PCR products. The protocol used followed that described by

Kancharla and Hanson (1996) with modifications. Firstly, 100 µl of a 1/10 dilution

of the PCR products, diluted in dilution buffer, was added to two parallel wells and

the plates were incubated at 20° C for 3 h with constant shaking. Wells containing

only re-suspended PCR beads and primers were used as negative controls. The

solution was then removed and 100 µl of 250 mM NaOH was added to the wells to

denature the PCR products. The plates were incubated for 15 min and then

washed 3 times with NaOH, and 5 times with washing buffer (Appendix 2.5). Next,

10 pmol of OS_1 (5'-TGTGAGAATAAGGATCGGCTA-3') and IS_1 (5'-

TGTCCTTGTTAAAAGTGGTGT5-3') fluorescein–labelled probes in hybridisation

buffer was added to each well respectively. The T_m for the hybridisation step was

calculated for each probe using the following formula (Keller and Manak 1989):

$$T_m=81.5 + 16.6 \log M + 0.41 (\%G+C) - 500\ n^{-1}$$

where $M=[Na^+]$ in moles l^{-1} in the hybridisation buffer and n= length of the probes

From the above formula, the T_m for IS_1 and OS_1 were estimated to be 71.1^O C and 73° C respectively. The plates however, were incubated at a variety of temperatures between 37° C and 60° C for different periods of times (2 to 4h), since the actual hybridisation temperature is usually 25 degrees below that of T_m and should be determined empirically (Keller and Manak 1989). The plates were then washed 5 times with washing buffer and 100 µl of a 1/2000 dilution of anti-FITC monoclonal antibody labelled with alkaline phosphatase (Sigma), diluted in antibody dilution buffer, was added to the wells to detect the FITC-end of the products. The plates were incubated for 2 h at 20° C and then washed 5 times with washing buffer. Finally 100 µl of 2.5 mg ml^{-1} p-nitrophenyl phosphate in diethanolamine-MgCl$_2$ buffer (pH 9.6) was added to each well. The development of colour was read spectophotometrically at 405 nm over a 3 h period.

Sequencing of the OS and IS

After amplifying OS and IS by PCR using the PSY1 and PSY2 primers, PCR products were run on 1% agarose gels. Slices from the agarose gels containing the expected bands were purified using a GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia Biotech Inc) (Appendix 6.1). The sequencing was performed according to the procedure described in Appendix 6.

4.2.3 Quantifying bacterial samples on polycarbonate filter membranes using *in situ* hybridisation or IFAT

<u>Preparation of bacterial samples</u>

Before performing *in situ* hybridisation and IFAT on bacteria captured on filter membranes, the effect of the age of the bacterium on the performance of the two assays was examined. *F. psychrophilum*, isolate B97026, was grown in *Cytophaga* broth for three days at 15° C or bacteria were maintained in either broth or stream water for one month at 15° C in the dark.

Cytospin preparations were made for *in situ* hybridisation, whereby bacteria were collected onto microscope slides for the IFAT.

Bacterial samples from each of the three treatments were collected by centrifugation (3000 g for 15 min) and fixed with 10% neutral buffered formalin solution for 3 h at 20° C. They were then washed twice with PBS and re-suspended in PBS. For *in situ* hybridisation, bacteria were centrifuged for 10 min at 2000 g using a cytospin (Cytospin3, SHANDON) to collect the bacteria on slides coated with 3-aminopropyltriethoxysilane (APES, Sigma) (Appendix 2.2). A 100 µl aliquot of each of the bacterial suspension, corresponding to approximately 10^6 bacteria, by AODCM, was used for the cytospin preparations.

For the IFAT, a drop of each of the three treatments (approximately 5 µl) was placed in the wells of a 21-well microscope slide (DYNEX). They were then left to dry in an oven at 45° C for 5 min.

To perform the *in situ* hybridisation and IFAT on polycarbonate membrane filters (Millipore) samples containing a range of bacterial concentrations (10^3 to 10^8 bacterial cells, as determined by AODCM), were collected on the 2 µm filters using

the filtration apparatus shown in Figure 4.3; each sample was prepared in triplicate. The bacteria were fixed onto the membrane with 10% neutral buffered formalin as described above.

The effect of the filtration process on the performance of the IFAT was also examined using transmission electron microscopy. Since the antibodies used appeared to bind to sites on the glycocalyx and/or the cell membrane, the effect of filtration on the integrity of these moieties was examined. Bacteria grown in *Cytophaga* broth for three days at 15° C were either collected on polycarbonate membranes as described above, or embedded in agar pellets, fixed with 2.5 % gluteraldehyde for 2 h and processed as described in Section 3.2.3. The polycarbonate membranes and the agar pellets containing the bacteria were finally cut into small pieces and embedded in Spurrs resin.

Figure 4.3. Filtration apparatus. (a) bacterial suspension, (b) filter membrane.

In situ hybridisation

In situ hybridisation was performed on polycarbonate membranes according to the method described by Lim, *et al.,* (1996) with slight modifications. The same method was also used for *in situ* hybridisation on cytospin preparations with the omission of the filtration step. Preparations in which bacteria were not treated with proteinase K or where the probes were omitted from the *in situ* hybridisation were used as negative controls.

After filtration, the polycarbonate membrane was left within the filtration apparatus, and the bacteria were washed twice for 5 min with TBS (Appendix 2.1).

Bacteria were treated with proteinase K (10 μg ml^{-1} in TBS pH 8) for 30 min at 37° C. The membranes were then washed twice with 0.2% w/v glycine in PBS, for 5 min each time and then post-fixed with 0.4% v/v paraformaldehyde in PBS for 15 min at 20° C. Finally, the membranes were washed twice with distilled water (5 min for each washing), removed carefully from the filtration apparatus and put into small metallic containers and left to dry for the hybridisation step.

After adding 1 ml of hybridisation buffer (Appendix 2.2) containing 10 μg of both FITC-labelled PSY1 and PSY2, the container was sealed with nescofilm (Bando Chemicals Ltd) and put into the hybridisation apparatus. The temperature of the hybridisation apparatus was increased to 95° C for 10 min to denature the DNA of the bacteria and subsequently lowered to 41° C for 4 h. After the hybridisation step, the membranes were returned to the filtration apparatus and washed once with 2 x SSC (saline salt citrate, Appendix 2.2) and once with 0.1 x SSC (Appendix 1.2) for 20 min each time. The membranes were finally removed from the filtration apparatus and after air-drying they were placed onto glass slides as described for AODCM. Bacteria present on the membranes were counted using a fluorescent microscope, as described for AODCM (Appendix 1).

<u>IFAT on polycarbonate filter membranes</u>

The method used was the same as that described by Marco-Noales *et al.,* (2000) with slight modifications. An anti *F. psychrophilum* B97026 rabbit serum (Faruk, 2000), diluted to 1/1000 with PBS, was added to the membranes and these were then incubated for 30 min at 20° C. Bacterial preparations incubated with only PBS were used as negative controls. The membranes were washed three times with PBS; the membranes were left in the PBS for 5 min during the last wash. Anti

rabbit IgG FITC-labelled (Diagnostics Scotland) diluted 1/100 with PBS was then placed onto each membrane and these were incubated for 30 min at 20° C. The membranes were washed as described above, removed from the filtration apparatus and mounted onto a microscope slide. Bacteria were then counted using a fluorescent microscope, as described for AODCM (Appendix 1).

4.3 RESULTS

4.3.1 Semi-quantitative PCR

DNA extracted from samples of *F. psychrophilum* was amplified by PCR in order to establish a standard curve from known samples, concentrations of between 10^8 and 1 bacteria, total number. Differences in absorbencies were observed between the replicates of DNA extracted from samples with the same bacterial numbers. Overlapping absorbencies were also obtained with DNA extracted from different bacterial concentrations. Thus, it was impossible to construct an accurate standard curve from the results of the semi-quantitative PCR, due to the variations obtained between and within the different dilutions of bacteria.

4.3.2 Quantitative PCR using an internal standard

The original sequence of the PCR products obtained with primers PSY1 and PSY2 from *F. psychrophilum*, NCIMB1947, strain was successfully inserted into the pBluescript II KS +/- phagemid, as shown in Figure 4.4a. The two fragments (phagemid and OS) can be seen when purified phagemid, after being cut at the *Eco*RI and *Hind*III sites, was run on a 1% agarose gel. The resulting bands corresponded one to the phagemid and to the OS.

The successful development of the internal standard was confirmed when phagemids obtained from different *E. coli* colonies were tested by PCR using primers PSY1 and PSY2 (Figure 4.4b). A band was obtained at 1050 bp,

corresponding to the IS product. The position of the fragment inserted into the OS so as to produce the IS, is shown in Figure 4.5.

In order to prepare a standard curve, DNA extracted from samples of *F. psychrophilum* containing between 10^8 and 1 bacteria were co-amplified with the IS in the same reaction tubes together as described above. The hybridisation step using probes OS_1 and IS_1 to detect the PCR products prior to ELISA, was unsuccessful, since no difference could be detected between wells containing bacterial samples and those containing the negative controls. A variety of different hybridisation temperatures and different incubation periods were used to try to improve the hybridisation step, but this did not increase the signal obtained in the ELISA. It was therefore, not possible to establish an effective standard curve for the quantitative PCR assay.
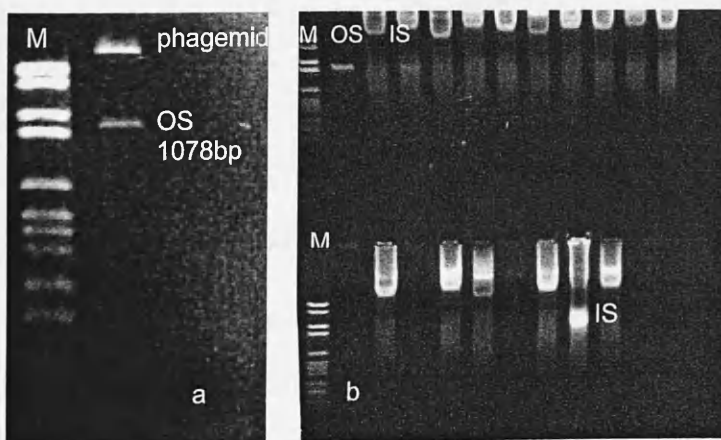
Figure 4.4 Verification of the production of the internal standard for the quantitative PCR. a) 1% agarose gel. Insertion of OS into a pBluescript II KS +/- phagemid. Purified phagemid was cut at the *Eco*RI and *Hind* III sites to separate the phagemid from the OS. b) Final screening of *E. coli* colonies using one round PCR with primers PSY1/PSY2 to determine colonies containing the phagemids with IS. M: marker (DNA Molecular weight marker VI, Ross), OS: original sequence, IS: internal standard.

```
        PSY1
OS  GTTGGCATCAACACACT-33bp-TCCCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCA----ACGAT
IS                          -ATCCAG-ACCCACTTTGTCCTCTATTTCTTGATAAAGTCTCTCCTGTAT

OS  GGGTAGGGGTCCTGAGAGGGAGATCCCCCACACTGGTACTGAGACACGGACCAGACTCCT
IS  CTCTGGGTGCGCCACAAGGTACATGACCGACCATG--ACAGGG---CAGTAGAGACGG-T

OS  ACGGGAGGCAGCAGTGAGGAA-TATTGGTCAATGGGCGCAAGCCTGAACCAGCCATGCCG
IS  ATCGAAACCAGCTCCAAACAGGTCATTGACAATTCCTACAA---TTTTCTCGTC-TGACA

OS  CGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGCATAGGAAGAAACACTACCT
IS  TCTGGACATTCGAGTTCTCATCCAGCTTCCTGTCCT----CACAGTGATCAATAAGGGAG

OS  CGTGAGGTAGCTTGACGGTACTGTGAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCG
IS  TCTGTGATGTCTCGAATGT--TGTCCTTGTTAAAAGTGGTGTA----GTGC--TCGGTGA

OS  CGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTT-683bp-CTACGCAAGTAGGATCG
IS  CGATCTTTTGCACGAA--TAGC-TT-------ATCGATACCGTC-          PSY2
```

Figure 4.5 A comparison of the original DNA sequence (OS) obtained with primers PSY1/PSY2 to that of the modified internal standard (IS). The position of the modified sequence within the PCR product is indicated.

4.3.3 *In situ* hybridisation

Bacteria were clearly visible when *in situ* hybridisation was used to examine cytospin preparations of 3-day old cultures of *F. psychrophilum*, (Figure 4.6). Similarly, bacteria could be clearly seen by *in situ* hybridisation when trapped on the polycarbonate filter membranes. It was possible to determine numbers of bacteria in the samples using a similar counting technique to that described for the AODCM. *In situ* hybridisation appeared to give similar results to those obtained with AODCM for bacterial numbers between 10^5 filter^{-1} (corresponding to approximately 3 bacteria grid area^{-1}) to approximately 10^7 filter^{-1} (corresponding to approximately 350 bacteria grid area^{-1}).

On the other hand, there was almost a complete lack of signal when *in situ* hybridisation was performed on cytospin preparations of bacteria maintained for one month in either stream water or *Cytophaga* broth.
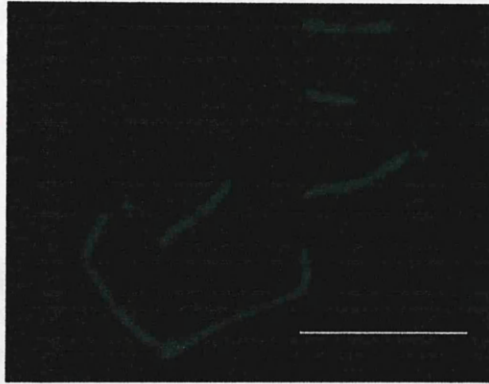
Figure 4.6 Detection of three-day-old *F. psychrophilum* by *in situ* hybridisation on cytospin preparations of bacteria. Bar= 10 μm

4.3.4 IFAT

F. psychrophilum cultured in *Cytophaga* broth for three days (Figure 4.7a), or maintained for one month in either broth (Figure 4.7b) or stream water (Figure 4.7c), were clearly visible by IFAT when collected and fixed onto microscope slides. However, when IFAT was performed on bacteria collected onto polycarbonate membranes, the resulting signal was substantially reduced (Figure 4.7d, white arrows).

The antibody used in the IFAT appeared to bind to sites on the glycocalyx and/or the cell membrane (Figure 4.7a, white arrow). The glycocalyx appeared to be severely damaged after filtration of bacteria onto the polycarbonate

membranes, as observed by transmission electron microscopy (Figure 4.8a, black arrow). The glycocalyx of bacteria collected by centrifugation and embedded in agar pellets appeared unaffected (Figure 4.8b, black arrow). Thus, it was not easy to count numbers of *F. psychrophilum* collected onto polycarbonate membranes by IFAT.
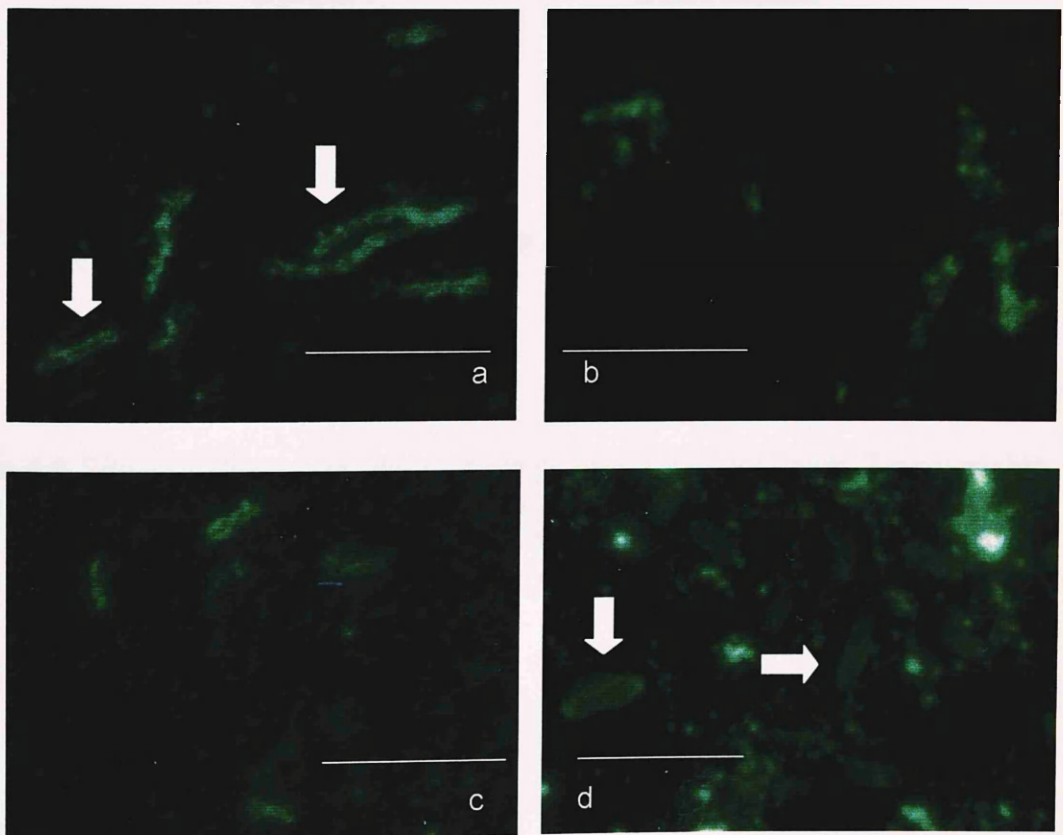
Figure 4.7 Detection of *F. psychrophilum* by IFAT using microscope slides (a,b,c) and polycarbonate filter membranes (d). Bacteria were maintained in broth for either three days (a,d), or one month (b), or in stream water for one month (c). Bar= 10 μm
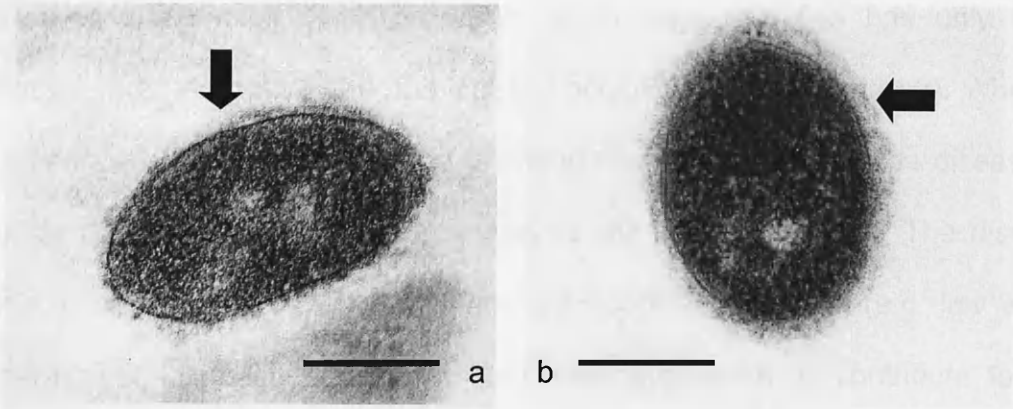
Figure 4.8 Effect of filtration on the glycocalyx of *F. psychrophilum*. Transmission microscopy of three-day-old *F. psychrophilum*. a) bacteria collected after filtration. Black arrows indicate the glycocalyx around the cells, b) bacteria collected without filtration. Black arrows indicate the disruption of the glycocalyx. Bars=0.2 μm

4.4 DISCUSSION

Little is known of the epidemiology and distribution of the pathogen *F. psychrophilum* in the aquatic environment, especially in farm systems before, during and after a disease outbreak. So far the presence of the pathogen in environmental samples, such as water, or in eggs and fish has only been confirmed when an outbreak of the disease actually occurs (Chapter 2). Whether the presence of the pathogen alone is enough for an outbreak of the disease to occur or other factors are also necessary is still being discussed. The disease seems to appear when certain environmental conditions prevail (e.g. low water temperatures), although stress has also been suggested to contribute to the appearance of the disease (Rangdale *et al.,* 1997). There is limited information regarding the survival and levels of the bacterium in the aquatic system and whether certain environmental factors are able to influence these levels. Such information is necessary for the development of successful control strategies against the disease. The development of quantitative methods to establish levels of *F. psychrophilum* in the environment, especially in water samples, was therefore examined in this chapter.

Counting bacteria in any environmental sample is not an easy task. Many factors can affect the results obtained, depending on the method used to quantify the levels of bacteria. Culture, for example, has the major disadvantage in that only culturable forms of the bacteria are detected. Many problems have been reported with the culture of *F. psychrophilum* (Daskalov *et al.,* 1999; Michel *et al.,* 1999), thus making the use of culture-based methods unsuitable for quantifying bacteria in the aquatic environment. Also, it has been found that under conditions

of starvation, bacteria develop into a viable but non-culturable state (Kell *et al.,* 1994; Barcina *et al.,* 1997; Baleux *et al.,* 1998). Four different techniques were described here to try to quantify the numbers of *F. psychrophilum* in water samples.

Advanced techniques such as *in situ* hybridisation and PCR-based methods have recently been employed by a number of researchers to detect and quantify microbes in environmental samples (Harmsen *et al.,* 2000; Oda *et al.,* 2000; Tortorello and Reineke 2000). These methods have the advantage of being both sensitive and more specific. However, they offer little information on the viability of the organism, since such methods detect both live and dead bacteria. In the present study, attempts were made to develop a semi-quantitative and a quantitative PCR to try to identify and quantify the pathogen in environmental samples. However, both methods gave poor results and proved unsatisfactory for detecting *F. psychrophilum* in water samples.

Semi-quantitative PCR, without the use of any internal standard, has been used by a number of authors (e.g. Stevens *et al.,* 1995; Gutiérrez *et al.,* 1997), and is a simple and rapid technique to perform. Due to minor variations in the reagents and/or the amplification conditions, however, tube-to-tube differences can be quite substantial resulting in a variation in the yields of the PCR products obtained (Zhang *et al* .1996). Thus, the results are not reproducible and a standard curve cannot easily be constructed. This was found to be the case in the present study.

A similar assay to that of the semi-quantitative PCR, which includes an internal standard so as to allow a quantification of the assay, has been developed and this is known as a quantitative PCR using internal standard. Co-amplification

of the original sequence together with an internal standard with a similar amplification efficiency, assures that even if small variations in the reaction do occur, in theory, the ratio between the two amplified sequences should remain the same. This allows tube-to-tube and to some extend sample-to-sample variations to be eliminated (Zhang *et al.,* 1996; Reischl and Kochanowski 1999). A critical stage in the assay is the hybridisation step, where an internal, labelled probe is used to increase the specificity of the assay. It was not possible to optimise the hybridisation step for the assay system described in the present study. Different hybridisation conditions were tried but all failed to improve the signal obtained in the ELISA. Only two probes (i.e. OS_1 and IS_1) were used in this assay. It is possible that under the assay conditions tested here, the probes did not bind to the PCR products or if they did, the binding was weak, so that the probes were removed during the washing.

A completely different method for detecting the original sequence and the internal standard, which avoids the use of the hybridisation step, may produce better results, for example direct high performance liquid chromatography (HPLC) (Reischl and Kochanowski, 1999). In some other cases, a quantitative method using an internal standard, appeared to work well *in vitro*, but when using field samples it provided overlapping values, as reported by Shammas *et al.,* 1999. The existence of various inhibitors in the samples can possibly cause variations between samples, thus resulting in different yields of PCR products (Reischl and Kochanowski, 1999).

Internal Standards have also been used to detect false negatives in samples. Incorporation of the standard into the PCR reaction can help to detect any inhibition in the reaction caused by various contaminants present in the

samples and thus inhibit the amplification of the sequence during the PCR (Cone, *et al.,* 1992; Brightwell *et al.,* 1998).

Oligonucleotide probes have been used in many *in situ* hybridisation techniques to identify and directly enumerate microbes in environmental samples. When these techniques are compared to conventional culture-based techniques they appear to provide more reliable results, similar those obtained with AODCM (Kleessen, *et al.,*1999; Harmsen, *et al.,* 2000; Tortorello and Reineke, 2000). Lim *et al.,* (1996) developed a quantitative method to count protists in water samples using cell culture inserts to collect the microbes. A similar technique was employed in the present study. The PSY1/PSY2 primers, used for the PCR method described in Section 2.2.3, were used here as probes to identify the bacteria. *In situ* hybridisation appeared to work well for bacteria collected from fresh cultures (3-4 days old bacteria) but when the bacteria were maintained in broth or stream water for one month, the signal appeared significantly reduced, especially for the bacteria maintained in broth. Oda *et al.,* (2000), reported a similar finding for starved *Rhodopseudomonas palustris.* They found a total lack of hybridisation, 20 days after setting up the experiment, although more than 50% of the bacteria were still detectable by culture. The authors concluded that quantitative detection of the bacterium using 16S rRNA-targeted probes (as in the case of PSY1/PSY2 with *F. psychrophilum*) should be used with extreme caution, especially in the case of starved cells.

Using IFAT, Marco-Noales *et al.,* (2000) was able to identify and enumerate both starved and viable *Vibrio vulnificus* which appeared to be non-culturable when the bacterium was captured onto polycarbonate membranes. In the present study, IFAT appeared to work well when applied to *F. psychrophilum*

fixed onto slides, with either fresh or one month old bacteria. However, when filtration was used to collect the bacteria, many of the bacteria appeared poorly stained. The polyclonal antibodies used appeared to bind to sites on the cell membrane and/or glycocalyx. The glycocalyx appeared to be partially destroyed, either due to the filtration or the fixation process, making it difficult to distinguish the weakly stained bacteria from background staining. Potentially, this method is very useful, since it is rapid and simple to perform. However it may be necessary to use different antibodies, which bind to epitopes that are not affected by the filtration process.

Another problem with antibody-based detection methods is the possibility of cross-reacting antibodies which react with other bacterial species. Faruk (2000) for example, reported that the anti B97026 serum used in this assay had low reactivity with a number of *F. psychrophilum* isolates and high cross-reactivity with some non-*F. psychrophilum* species. On the other hand, the use of primers PSY1/PSY2 as probes to detect *F. psychrophilum* appears to be more promising, since no false positive results have been reported with the PCR using these primers.

This chapter investigated the potential of using four different methods to enumerate *F. psychrophilum* in water samples. Such methods could provide important information on the epidemiology and pathogenicity of the disease. Although further development is needed to establish a suitable quantitative method for environmental monitoring of *F. psychrophilum*, the problems associated with these methods were discussed. The PCR-based methods appeared to be specific but lacked reproducibility. Possibly a different detection method, as the direct HPLC method or more advanced methods, such as quantitative real time PCR can provide better results (Haugland *et al.,* 1999). On

the other hand, the *in situ* hybridisation technique appeared very useful for conditions where the bacteria remain in the environment for short periods of starvation, although further investigation is needed to validate the method in terms of specificity. The IFAT method provided better results with the aged bacteria, however, there is concern over the specificity and sensitivity of the antibodies used. This technique is useful in cases where experiments are conducted in closed, controlled systems, where other bacterial species are less likely to exist.

CHAPTER 5

ATTACHMENT OF *F. PSYCHROPHILUM* TO UNFERTILISED EGGS AND N-HEXADECANE

5.1 INTRODUCTION

The first step in successful bacterial colonisation for a bacterium on a particular surface, be it the walls of an aquarium tank or the live cells of its host, is its ability to adhere to the surface. Adhesion of bacterium to a support can be divided into either specific or non-specific attachment (Ofek and Doyle, 1994). Specific adherence is mediated through specific compounds on the surface of the bacterium, which bind via rigid stereochemical bonds to particular molecules on the support to which it is adhering. Non-specific adherence on the other hand, depends on hydrophobic or ionic interactions between certain structures on the surface of the bacterium and on the support (Ofek and Doyle, 1994).

Surface hydrophobicity appears to be important for the adherence of some bacterial species to hydrophobic surfaces, although no generalisation between surface hydrophobicity and ability to adhere can be made. Hydrophobicity appears to enable the bacterium to interact with other living cells and even helps it to avoid host defence mechanisms (Santos *et al.,* 1990). However, when adherence is selective, as in the case of attachment of oligotrophic and copiotrophic bacteria to immersed glass surfaces in seawater (Kjelleberg *et al.,* 1985), cell surface hydrophobicity does not appear to be as important for adherence. Other factors,

such as salinity and pH also appear to influence the ability of microbes to adhere to fish as in the case of adhesion of pathogenic strains of *Vibrio* to skin mucus of sea bream (*Sparus aurata*) (Balebona *et al.*, 1995).

Hydrophobicity tends to increase with the age of the bacterium, as in the case of *Acinetobacter calcoaceticus* (Rosenberg *et al.,* 1981). Differences between strains of the same species have also been observed, for example in the case of different strains of *Flavobacterium johnsoniae* (Sorongon *et al.,* 1991) or *Vibrio* spp. (Lee and Yii, 1996).

A variety of techniques and substrates have been used to investigate the surface hydrophobicity of bacteria and their ability to adhere (Ofek and Doyle 1994). In the current study, the ability of five different strains of *F. psychrophilum* to attach to two different substrates were compared. The substrates used were n-hexadecane (a hydrocarbon), a substrate not normally colonised by the bacterium, and unfertilised rainbow trout eggs, a substrate frequently found to be colonised by the bacterium in commercial trout hatcheries. The effect of age and starvation of *F. psychrophilum* on its ability to adhere to the two substrates was also assessed. Such information is necessary in understanding the pathogenicity of the bacterium during the initial stages of infection.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial preparation

Five different isolates of *F. psychrophilum* were used in the study (Table 1). Isolate B97026 was used to examine the effect of age and starvation on the surface hydrophobicity of the bacterium and also to examine the ability of the bacterium to attach to unfertilised rainbow trout eggs.

Isolates of *F. psychrophilum* were cultured in *Cytophaga* broth at 15° C for three days in the dark. Samples of bacteria were collected from each bacterial suspension after three days and surface hydrophobicity and attachment to unfertilised eggs assessed according to the methods described below.

A total of 10^7 bacteria from each bacterial suspension determined by AODCM were placed in 2 ml microcentrifuge tubes. The final volume of each sample was adjusted to 1 ml with filter-sterilised PBS. Five replicates were prepared for each bacterial suspension.

In order to examine the effects of age and starvation on the surface hydrophobicity of the bacterium and on its ability to attach to unfertilised eggs, 10^9 bacteria (isolate B97026) were placed into two universals. After centrifuging for 15 min at 3000g, bacterial pellets were washed twice with sterile PBS by again centrifuging for 15 min at 3000 g. One pellet was re-suspended in 5 ml of filter-sterile stream water (filtered through a 0.2 μm filter) and the other pellet in 5 ml of *Cytophaga* broth. After maintaining the bacteria at 15° C for 1 month in the dark, bacterial samples were prepared from each universal as described above and the surface hydrophobicity and the ability of *F. psychrophilum* to attach to unfertilised

eggs assessed according to the methods described below. The adherence of these bacteria were compared to fresh *F. psychrophilum* cultured for three days in *Cytophaga* broth as described above.

5.2.2 Attachment of *F. psychrophilum* to n-hexadecane

Assessment of the attachment of *F. psychrophilum* to n-hexadecane followed the method described by Rosenberg *et al.,* (1980) with slight modifications. A 200 µl aliquot of 100% n-hexadecane (Sigma) was added to the bacterial suspensions (10^7 bacteria total number). The tubes were vortexed for 2.5 min at 20° C on a low setting. The tubes were then left to stand for 15 min to allow the aqueous and organic phases to separate. Eight hundred µl of the aqueous phase (bottom layer) was carefully removed and placed into a fresh tube, to which was added 80 µl of 0.1% w/v acridine orange in distilled water. Bacterial numbers present in the aqueous phase were determined using the method described by Hobbie *et al.,* (1977).

5.2.3 Attachment of *F. psychrophilum* to unfertilised rainbow trout eggs

Unfertilised rainbow trout eggs, free of RTFS as determined by PCR (Section 2.2.2 and 2.2.3) were obtained from Houghton Springs Hatchery in Dorset, UK. After washing briefly in filter-sterilised PBS to remove excess ovarian fluid, the eggs were placed in the 2 ml microcentrifuge tubes containing the bacterial suspensions (one egg per tube containing 10^7 bacteria, total number). The tubes were vortexed at 20° C for 30 min on the lowest setting. The PBS was carefully

removed and transferred into a new 2 ml tube. One ml of fresh PBS was added to the tube containing the eggs in order to remove any loosely attached bacteria and this was then added to the first sample of PBS. One hundred µl of 0.1% w/v acridine orange solution was added to the PBS and the number of bacteria present determined as above.

5.2.4 Statistical analysis

For the analysis, Minitab 10.1 (Minitab Inc) software was used. Results were compared by a one way analysis of variance (ANOVA) following tests for normality (Anderson-Darling test). Tukey test was employed to identify significantly different groups, where $p < 0.01$ was considered significant.

5.3 RESULTS

No significant difference was evident in the surface hydrophobicity of the different isolates of *F. psychrophilum* examined in the present study, as assessed using either n-hexadecane or their ability to attach to unfertilised eggs (Table 5.1).

There was a marked increase in the ability of *F. psychrophilum* isolate B97026) to attach to both n-hexadecane and unfertilised eggs after the bacteria had been maintained in either stream water or *Cytophaga* broth for one month compared to bacteria cultured in *Cytophaga* broth for three days (Figure 5.1). Approximately, 18% of the bacteria from the 3-day-old culture attached to n-hexadecane and 25% to unfertilised eggs, while 49% and 66% of the bacteria maintained in *Cytophaga* broth for one month and 52% and 67% of the bacteria maintained in stream water for one month attached to n-hexadecane and unfertilised eggs respectively. No significant difference in the percentage of the bacteria attached to the two supports was observed however, between bacteria maintained in broth or stream water for one month.

Table 5.1 Attachment of *F. psychrophilum* to n-hexadecane and unfertilised rainbow trout eggs. The values represent the mean of five replicates ± SD.

Isolate	Origin	Host	Bacteria attached (%)	
			n-hexadecane	Unfertilised trout eggs
B97026	UK	*Oncorhynchus mykiss*	17.6±3.0	25.0±2.7
NCIMB1947	USA	*Oncorhynchus kisutch*	17.6±2.3	27.0±2.0
JIP30/98	France	*Anguilla anguilla*	16.8±2.1	25.6±2.4
32/97	Chile	*Oncorhynchus mykiss*	17.2±1.9	25.8±3.0
34/97	Chile	*Oncorhynchus mykiss*	16.6±2.6	24.6±2.8
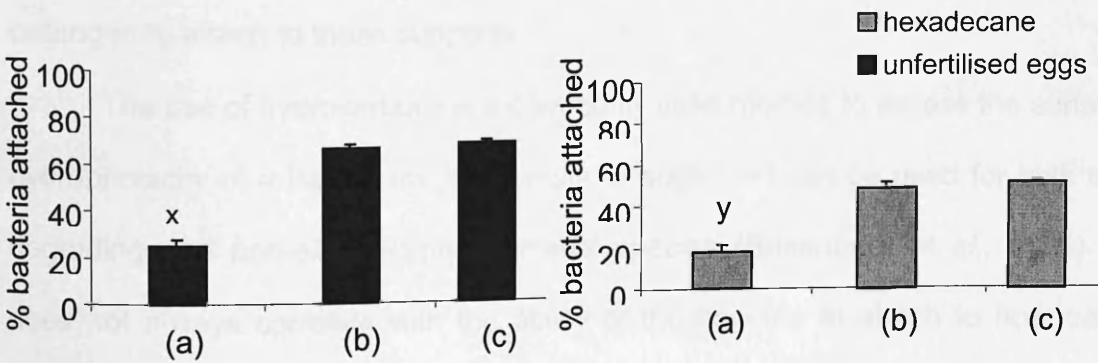
Figure 5.1 Attachment of *F. psychrophilum*, isolate B97026, to n-hexadecane and unfertilised rainbow trout eggs after culturing for (a) 3 days and (b) one month in *Cytophaga* broth and (c) one month in stream water. Values represent the mean of five replicates +SD. ^{x,y} indicate significantly different values (p<0.01) within treatments.

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5.4 DISCUSSION

The attachment of a bacterial pathogen to different substrates, particularly to host cells, is considered to be the first step in successful colonisation. This colonisation may in turn, lead to disease. A variety of different techniques have been used to assess the ability of bacteria to adhere specifically or non-specifically to different substrates (Ofek and Doyle, 1994). These techniques involve the use of hydrocarbons, solid surfaces coated with different materials, aggregation of particles coated with different substances (e.g. hemagglutination, aggregation of latex beads etc.), binding to tissues, cells or tissues excised after inoculation *in vivo* with bacteria. In the present study, adherence to n-hexadecane (a hydrocarbon) and unfertilised eggs, were used to assess differences in attachment between the five different isolates of *F. psychrophilum*. One of these isolates was then chosen to examine the effect of age and starvation on the ability of the pathogen to attach to these supports.

The use of hydrocarbons is a commonly used method to assess the surface hydrophobicity of a bacterium. It is simple to apply and can be used for both oil-degrading and non-oil-degrading bacterial species (Rosenberg *et al.,* 1980). It does not always correlate with the ability of the bacteria to attach to host cells (Lopez-Cortez, *et al.,* 1999), however, and thus, only a combination of techniques can offer an accurate assessment of the ability of the bacteria to adhere.

In many cases, surface hydrophobicity is not always the most important characteristic involved in mediating bacterial adherence (Vanhaecke *et al.,* 1990; Balebona *et al.,* 1995; Lopez-Cortez *et al.,* 1999). Therefore, attachment of *F. psychrophilum* to unfertilised rainbow trout eggs was also used here to evaluate

the ability of the bacterium to adhere. The presence of the pathogen on rainbow trout eggs was established several years ago by Holt *et al.,* (1993) and is considered the most important source of infection in RTFS outbreaks experienced in many hatcheries worldwide. The source of the pathogen has been identified as either the broodstock (vertical transmission) or water (Brown *et al.,* 1997), or both. The fact that water can act as a source of infection implies that the bacterium is able to survive outside its host for a period of time under conditions of starvation. We have previously shown that the bacterium is able to survive in stream water for many months and adopts a different morphology to cope with conditions of starvation (Chapter 3). Little is known of other characteristics of the pathogen under these conditions, however, such as virulence, or resistance to adverse environments. It has been demonstrated that many bacterial species exhibit a change in such characteristics under conditions of starvation (Kell *et al.,* 1994; Barcina *et al.,* 1997).

Barker (1991) used a similar method to the one discussed here to compare the ability of *Pseudomonas fluorescens*, *Cytophaga* spp. and *Serratia* spp. to attach to rainbow trout eggs. The method described was based on the percentage of bacteria which attached to the surface of the eggs over a 24 h period. It is possible that additional bacterial growth occurred during this 24 h period. In the present study, the method used was based on the percentage of bacteria which remained in the PBS rather than counting the number that attached to the eggs. A short incubation period of half an hour was used here to ensure that no additional bacterial growth occurred. Also, the eggs were washed prior to incubation to remove excess ovarian fluid. These precautions helped to eliminate possible bacterial growth during the assay.

The five isolates of *F. psychrophilum,* which were examined here exhibited similar levels of bacterial attachment to n-hexadecane and unfertilised eggs. These bacteria had been cultured in *Cytophaga* medium prior to the test. No significant difference in the attachment was found between the bacteria maintained in broth and in stream water for one month. It has been shown that when bacteria are maintained in artificial media, some of their properties, including their ability to attach to supports might be altered, as reported by Dewanti and Wong (1995). The authors found that *Escherichia coli* produced poorer biofilms on stainless steel when the bacteria were grown in low nutrient medium. Magariños *et al.,* (1996) also found that different culture media affected the hydrophobicity of *Pasteurella piscicida* (=*Photobacterium damselae* subsp. *piscicida*) and the ability of the bacterium to adhere to cell lines. However, they found that the virulence of the bacterium was not correlated with its ability to attach; the presence of a capsule layer around the bacterial cells of the virulent strains was reported as the key factor in attachment. In contrast, Grayson, *et al.,* (1995) reported that virulence was strongly associated with the surface hydrophobicity of *Renibacterium salmoninarum.* The same was also reported for *Flavobacterium columnare* by Decostere *et al.,* (1999b). The authors also reported that a number of factors, such as immersion of the gills in bivalent ion-rich water, the presence of nitrite or organic matter, increased the ability of the virulent bacterium to attach to gills. In an other study, Decostere *et al.,* (1999a) reported that a lectin-like carbohydrate-binding substance incorporated in the capsule is responsible for the attachment of *F. columnare* to gill tissue. Ostland *et al.,* (1997a) also suggested that an outer membrane component is associated with the adherence of *F. branchiophilum* to gills.

In the present study, an elastin-degrading isolate (B97026) was compared with non-elastin-degrading isolates (NCIMB1947, 32/97 and 34/97) (Faruk, 2000). Although in the present study the virulence of the isolates used was not tested prior to the experiment, Madsen and Dalsgaard (1998) found that elastin-degrading isolates of *F. psychrophilum* appeared more virulent than the non-elastin-degrading isolates. All of the *F. psychrophilum* isolates used here exhibited similar levels of attachment, however, indicating that for *F. psychrophilum*, the virulence of a strain is probably not related to its ability to attach to the host.

Bacteria taken from a one-month-old culture and also bacteria starved in stream water for one month exhibited increased surface hydrophobicity, tested by their ability to adhere to n-hexadecane and an increased ability to attach to the surface of the eggs, with higher numbers of the bacterium attaching in each case compared with the three-day old bacteria. These findings offer a possible explanation why until now it has not been possible to reproduce a bath or cohabitation challenge as a method of experimentally infecting fish. The challenge method used has been based mainly on intraperitoneal or intramuscular injection of the pathogen, collected during fresh outbreaks of the disease and then freshly cultured *in vitro*. Bath challenge and cohabitation challenge methods with such isolates have resulted in poor levels of infection (Madsen and Dalsgaard 1999; Garcia *et al.*, 2000). Future bath and cohabitation challenge studies should compare aged bacteria with fresh bacteria.

Other methods used to examine bacterial attachment have been based on attachment of bacteria to various cell lines, using an antibody-based detection technique (ELISA) to determine levels of attachment (Jung, 1999). This method was not used in the present study, however, because of differences in the size

and morphology of the bacterial cells during starvation (Chapter 3). Bacteria maintained in broth for one month tend to become long and slim, while bacteria maintained in stream water for the same period of time become shorter and round. Differences in the antigenic profiles between the different bacterial preparations were also observed (Chapter 3), which could lead in misinterpretation of the ELISA results.

The five different isolates of *F. psychrophilum* examined here were found to exhibit similar attachment to n-hexadecane and eggs. More strains need to be examined to confirm this finding and it would be interesting to examine the effect of culturing them in different culture media. It has also been suggested from this study that the ability of *F. psychrophilum* to attach to supports may not be related to the virulence of the bacterium. Again this needs to be confirmed with more isolates of known virulence. Increased attachment was observed with one-month old bacteria maintained in either stream water or *Cytophaga* broth. The physiological state of the bacterium, thus appears to be an important factor in the attachment of the organism to host cells. Establishing inhibitors of bacterial attachment would also provide useful information as to the nature of the adherence. These results provide important information regarding the attachment of *F. psychrophilum* to its host, a necessary first step for infection.

CHAPTER 6

COLONISATION OF RAINBOW TROUT EGGS BY
F. PSYCHROPHILUM

6.1 INTRODUCTION

The increasing demand for fish eggs and fry worldwide has led to the development of a variety of different intensive incubation techniques. Intensification, however, often results in a number of bacterial overgrowth-related problems. The mucous surface of eggs appears to be a good substance for adhesion and colonisation by many different bacterial species (Nelson and Ghiorse, 1999). Even under normal conditions, many bacterial species appear able to colonise the surface of the egg. The most common bacterial species that appear to dominate the microflora of the surface of the fish eggs belong to the genera *Pseudomonas*, *Aeromonas*, *Alteromonas*, *Vibrio*, *Flavobacterium*, *Acinetobacter* and *Cytophaga* (Hansen and Olafsen, 1989; Ogbondeminu, 1994; Nelson and Ghiorse, 1999). The surface of dead eggs appears especially good as a breeding ground for bacteria (Keskin and Rosenthal, 1993). Thus, one way of minimising bacterial overgrowth of the eggs is early removal of dead eggs.

Problems associated with bacterial growth usually occur when the relationship between the indigenous microflora present on the surface of the egg and a pathogen present in the aquatic environment is disturbed (Hansen and Olafsen, 1989). Environmental conditions, such as high water temperatures, slow

water flow or high egg densities can all lead to increased bacterial growth on the surface of the eggs and subsequently potential disease problems (Barker, 1989).

Bacterial pathogens of the eggs can either be transmitted horizontally through the water or vertically, from the broodstock. In the first case, most bacterial species appear to colonise the surface of the eggs, but some can also penetrate the eggshell, as in the case of *Flexibacter ovolyticus* (Bergh *et al.,* 1994), causing high egg mortalities. During vertical transmission, bacteria enter the eggs while the eggs are still in the body of the fish. *Renibacterium salmoninarum* for example, can infect eggs when the eggs are released into the body cavity of the fish via heavily infected coelomic fluid (Evelyn *et al.,* 1986).

The eggs themselves appear to possess some antimicrobial agents. The most important one appears to be lysozyme, which catalyzes the hydrolysis of beta-1,4-glycosidic linkage between N-acetyl muramic acid and N-acetylglucosamine of peptidoglycan in the bacterial cell wall (Takemura 1996). All pathogens are not equally susceptible to the antimicrobial action of lysozyme; *Renibacterium salmoninarum*, causative agent of bacterial kidney disease, for example does not appear to be affected by lysozyme and can survive within the eggs (Yousif *et al.,* 1994). A variety of other antimicrobial substances are also present in fish eggs, such as lectin-like agglutinins, C-reactive protein (Takemura 1996) and in some cases maternal antibodies (e.g. against *Vibrio* spp.), (Tanaka *et al.,* 1999), although protection by the latter is still questionable.

Common strategies to control bacterial growth on the surface of fish eggs include the use of disinfectants, introduction of beneficial probiotic bacteria either directly to the water system or via food to the larvae, or the use of microbial mature water, which selects non-opportunistic microflora that inhibits the

proliferation of pathogenic bacteria (Skjermo and Vadstein, 1999). Injection of the broodstock with antibiotics has also had some success in preventing a number of diseases (Lee and Evelyn, 1994).

Rainbow trout eggs as a source of transmission for RTFS has been known for a number of years (Chapter 1). It has been suggested that the eggs can either be infected by the water and/or from the broodstock (Brown *et al.,* 1997). It has been demonstrated from studies in Chapter 2 that *F. psychrophilum* is associated with eggs. However, little is known about the horizontal transmission of the bacterium, especially localisation of the bacterium on the eggs or its ability to affect the development of eggs. If *F. psychrophilum* is capable of penetrating the eggs, then it could seriously affect the development of the eggs and also make disinfection procedures ineffective as reported to be the case for *Renibacterium salmoninarum* (Elliott *et al.,* 1991).

In the present chapter uninfected rainbow trout eggs were experimentally infected with *F. psychrophilum* during fertilisation. It was shown in the previous chapter that aged *F. psychrophilum* can attach to the surface of eggs in higher numbers, compared with bacteria collected from fresh cultures. In the present study however, only fresh cultures were used, for two main reasons: a) The virulence or the proteolytic activity of the starved cells had not been previously examined. b) It was anticipated that although the initial number of the bacteria attached to eggs might have been low, a multiplication of the pathogen would occur during the incubation period. This would result in an increased population of bacteria on the egg surface. A bath challenge was used to establish whether the bacterium was able to colonise the surface of the eggs and is able to penetrate through the eggshell. Methods for screening included scanning and transmission

electron microscopy, PCR, culture, as well as IFAT on egg sections. Fry hatched from the infected eggs were also examined by IFAT in order to establish if the pathogen was present within the body of the fish after hatching.

6.2 MATERIALS AND METHODS

6.2.1 Infectivity trials

F. psychrophilum, isolate B97026, was used to artificially infect the rainbow trout eggs. The bacteria were grown in modified *Cytophaga* agar at 15° C for four days in the dark.

Approximately 900 unfertilised rainbow trout eggs and milt were collected from Houghton Springs hatchery, Dorset UK. The eggs were all collected from one female, put into a plastic bag and sent by mail. A sample of 20 eggs (selected randomly) was tested for the presence of *F. psychrophilum* by PCR (according to the methods described in Sections 2.2.2 and 2.2.3) upon arrival and prior to fertilisation (the milt was not tested by PCR or cultures). The quality of the milt was tested microscopically by mixing a drop of milt with a drop of ovarian fluid from the eggs on a microscope slide and observing the motility of the sperm. Once the motility was confirmed, the eggs were fertilised with the milt.

The eggs were split into two groups of approximately 400 eggs per group. One group was fertilised, while the second group was not. Each group was further divided into two subgroups; one group of approximately 100 eggs was used as a negative control and the other group of approximately 300 eggs was infected with *F. psychrophilum* (positive group). This was carried out by mixing the bacteria together with the eggs.

Fertilisation was performed by carefully mixing the eggs with a few ml of milt. The infection of the eggs was performed as follows: the eggs were left with the milt for 20 min. Eggs from the positive groups were carefully mixed with 10 ml

bacterial suspension (3×10^8 ml^{-1} bacteria in broth). After 30 min, the eggs were transferred to plastic tanks, where they were maintained until hatching (Figure 6.1). The group of eggs not infected with *F. psychrophilum* (negative control) was transferred to the tanks after 50 min.

Hatching of the eggs occurred 42 days post-fertilisation. Ten days after hatching fry from eggs infected with *F. psychrophilum* were divided into two further groups of approximately 50 fry per group. One of the groups was re-infected with *F. psychrophilum* by stopping the flow of the water to the fish, lowering the water level within the tank and adding 25 ml of bacterial suspension (cultured for four days in *Cytophaga* broth and adjusted to 8×10^8 bacteria ml^{-1}) to the water. The fry were bathed in the bacteria for 30 min with additional oxygenation. After this time the water level was returned to the original level. Twenty five days post-hatching fry samples were collected from all groups and the experiment terminated.

The water temperature during incubation of the eggs was 11° C and this was decreased to 7.8^O C after hatching and remained almost constant until the end of the experiment.

Samples of eggs were collected prior to fertilisation and then on day 1, 5, 10 or 30 post-fertilisation. Samples were examined by IFAT, scanning and transmission electron microscopy, culture in modified *Cytophaga* broth and PCR, to establish the presence of *F. psychrophilum* in the eggs (Table 6.1). Fry were also collected and examined by IFAT to establish the presence of the pathogen in fish tissue.

Evaluation of the sampling method was carried out using Win Episcope 2.0 software. The results are showed in Table 6.2.

Table 6.1 Sampling of eggs and fry for the detection of *F. psychrophilum* after artificially challenging them by bath with the bacterium.

	Sampling	Treatment	Number of samples per group				
			PCR	IFAT	SEM	TEM	Culture
Eggs	prior to fertilisation	Stock eggs	20				5
	after fertilisation	F-	20	5	5	5	5
		F+	20	10	10	10	5
		U-	20	5	5	5	5
		U+	20	10	10	10	5
	5 days post fertilisation	F-		5	5	5	
		F+		10	10	10	
		U-		5	5	5	
		U+		10	10	10	
	10 days post fertilisation	F-		5	5	5	5
		F+		10	10	10	5
		U-		5	5	5	5
		U+		10	10	10	5
	30 days post fertilisation	F-	20	5	5	5	5
		F+	20	10	10	10	5
		U-	20	5	5	5	5
		U+	20	10	10	10	5
Fry	10 days post hatching	F+		10			
		F-		5			
	25 days post hatching	F+nr		10			
		F+r		10			

Note: F+: fertilised infected, F-: fertilised non-infected, U+: unfertilised infected, U-: unfertilised non-infected, F+nr: from infected eggs, not re-infected, F+r: from infected eggs, re-infected
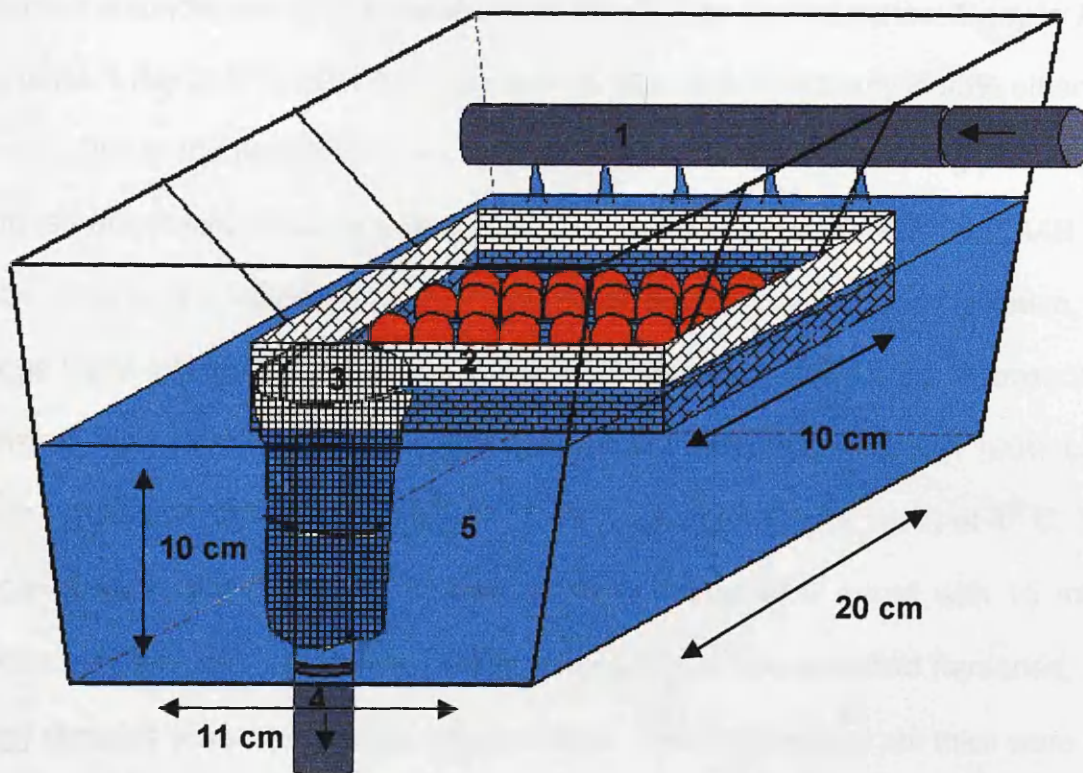
Figure 6.1 Plastic tanks used to incubate the eggs. 1: water inlet, 2: metallic tray containing the eggs, 3: mesh around the water outlet, 4: outlet, 5: plastic tank

6.2.2 IFAT

Processing of eggs

Samples of eggs were collected according to the sampling regime indicated in Table 6.1. Ten eggs were collected from each group of infected eggs and 5 from each group of non-infected eggs at each sampling. The eggs were fixed for 24 h in FAACC [10 ml glacial acetic acid, 5 ml formaldehyde (30% solution), 0.6 M

calcium chloride] and then dehydrated at 20° C in an alcohol series: 1 day in 70% ethanol, 1 day in 80% ethanol, 1 day in 90% ethanol and one day in 95% ethanol.

Due to the hardness of the eggs, normal wax-based embedding procedures proved unsuitable. Thus, a commercially available kit (Technovit 7100, TAAB Kit) was used for the infiltration and embedding of the samples. After dehydration, the eggs were infiltrated with the 'liquid' base of the kit at 20° C: 50:50 base:95% ethanol for 3 days, 75:25 base:95% ethanol for 5 days and 7 days in 100% base with hardener I (infiltration solution, 1 g hardener I per 100 ml base) at 4° C. The eggs were finally embedded in resin (1 ml of hardener II mixed with 15 ml of infiltration solution) using teflon coated moulds. Once the resin had hardened, the egg samples were mounted on wooden stubs. Three sections 2 μm thick were cut from each sampled egg using a Reichert Ultracut E (Leica) microtome fitted with a glass blade and sections were then transferred into a 50° C water bath to stretch them. Sections were finally mounted onto clean slides and left to air dry.

Processing of fry

Ten fry were sampled from each infected group and 5 from the non-infected group. The fry were killed with an overdose of benzocaine and they then placed in 10% neutral buffered formalin for 24 h. The samples were processed according to conventional histology procedures (Appendix 7), embedded in wax, and cut into 5 μm thick sections (at least three per fry).

IFAT

After re-hydrating through a series of alcohols (100% ethanol for 5 min, 70% ethanol for 3 min and distilled water for 3 min), a 1/1000 dilution of anti-*F.*

psychrophilum (isolate B97026) rabbit serum diluted in PBS, was placed onto the sections which were then incubated for 30 min at 20° C, before washing them three times with PBS. During the last wash, the sections were incubated in PBS for 5 min. A 1/100 dilution of anti-rabbit IgG-FITC (Diagnostics Scotland) was added to the slides and the sections were incubated for 30 min at 20° C. The slides were washed as before, air-dried and mounted with coverslips using a drop of immersion oil (Agar). The sections were observed under immersion oil using an Olympus fluorescence microscope.

6.2.4 Processing egg samples for scanning electron microscopy

Egg were first fixed for 1 h with 1% v/v gluteraldehyde in 0.1 M sodium cacodylate at 4° C and then overnight with 2.5% gluteralehyde buffer at 4° C. The eggs were then transferred into 0.1 M sodium cacodylate buffer for 4 h at 4° C and then into fresh sodium cacodylate buffer and incubated overnight. The following day the eggs were transferred into fresh sodium cacodylate buffer and left at 4° C until processing.

The samples were post-fixed in 1 % osmium tetroxide in sodium cacodylate buffer for 2 h. After dehydration through series of alcohols (30% for 30 min, 60% for 30 min, 90% for 30 min, 100% for 30 min and 100% for 1 h), the eggs were transferred into a 1:1 mixture of 100% ethanol: hexamethyldisilazane (HMDS) for 30 min. Two changes of 100% HDMS were made at 30 min intervals, and the eggs were then left to air-dry overnight. Finally the eggs were mounted on stubs and sputter coated.

6.2.5 Processing of eggs for transmission electron microscopy

The egg samples were fixed overnight in 2.5% v/v gluteraldehyde in 0.1 M sodium cacodylate at 4° C. The samples were then transferred into fresh sodium cacodylate buffer for 4 h at 4° C and finally transferred to fresh sodium cacodylate buffer at 4° C, where they remained until further processing.

The egg samples were post-fixed in 1% osmium tetroxide in sodium cacodylate buffer. The osmium was removed and 2% uranyl acetate in 30% acetone was added for 1 h in the dark. After dehydration through a series of acetone at 20° C (60% for 40 min, 90% for 40 min, 100 % for 40 min and 100 % acetone for 1 h), the samples were infiltrated with Spurrs Resin [10 ml 4-Vinylcyclohexene Dioxide, 6 ml DER Resin, 26 ml (2-Nonen-1-yl) succinic anhydride, 0.4 ml 2 Dimethylaminoethanol] using a rotator mixer. A mixture of 1:1 acetone:Spurrs was added overnight, followed by further infiltration with 100 % Spurrs Resin for 24 h. Finally, the eggs were embedded in green block moulds and left to polymerise at 60° C for 48 h. At least three sections were cut per egg.

6.2.6 Inoculation of eggs into *Cytophaga* broth

Egg were sampled from each treatment, whereby 5 eggs were removed from each tank (i.e. unfertilised and fertilised eggs, infected and negative controls) and briefly rinsed in sterile PBS. After squashing the eggs with a needle in a sterile tube, they were placed in universals containing *Cytophaga* broth and incubated at 15° C for 4 days in the dark. After this time, four 100 μl aliquots were sampled from each universal and spread onto *Cytophaga* agar plates. The plates were incubated until

yellowish colonies were visible at approximately 4 days. Colonies were collected and re-suspended in 500 µl PBS. Drops of each suspensions were placed on slides, left to air-dry and stained with Gram's stain (Appendix 5.1). The suspensions were also screened by PCR.

6.2.7 PCR analysis

Twenty eggs were collected from each group of eggs according to the sampling regime shown in Table 6.1.

Re-suspended bacterial colonies (450 µl) were tested by nested PCR. The procedures described in Sections 2.2.2 and 2.2.3 for DNA extraction and amplification were used.

6.3 RESULTS

6.3.1 Analysis of the sampling method

The sampling protocol was evaluated using WinEpiscope 2 software and the results are shown in Table 6.2. It appears that although the egg samples tested were negative by PCR, IFAT and TEM, when analysed for the presence of *F. psychrophilum,* there was in fact a probability that the sampling protocol used would fail to detect positive eggs (if any) in the entire population. This probability varied from 13 to 45%, according to the size of the sample in relation to the size of the entire population, as Table 6.2 indicates.

Table 6.2. Evaluation of the sampling method (assuming all samples negative and confidence level 95%).

Sample size	5	5	10	10	20	20	20
Population size	100	300	100	300	900	100	300
Maximum probable positives	45	135	25	77	124	13	41
Maximum prevalence (%)	45	45	25	25.7	13.8	13	13.7

6.3.2 Electron microscopy

The presence of a mixed microflora could be seen on the surface of all sampled eggs by scanning electron microscopy (Figure 6.2a,b). The surface of the unfertilised eggs, including both the infected and negative controls particularly

appeared to be colonised by high numbers of different microbes (Figure 6.2a). However, it was not possible to identify *F. psychrophilum* from the different bacteria morphologically.

The presence of a mixed microflora on the surface of all egg samples was also evident from the transmission electron microscopy (Figure 6.3). Egg samples, from both unfertilised (Figure 6.3c) and fertilised eggs (Figure 6.3b) collected one month after infection (just before hatching), exhibited areas of lysis within the mucous layer (Figure 6.3b,c). However it was not possible to establish whether this was due to the proteolytic activity by *F. psychrophilum*, although these areas were not found in the negative controls. Some of the cells also appeared to multiply as seen in Figure 6.3d.
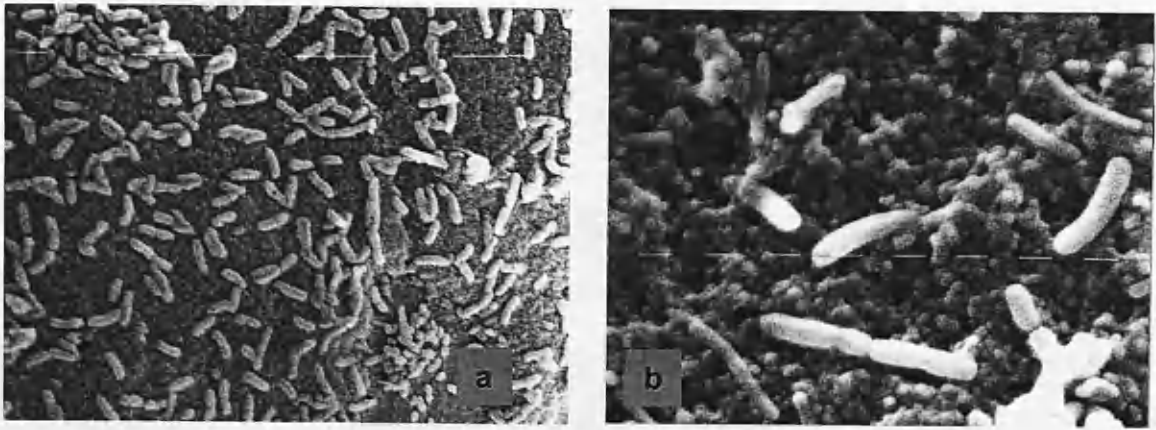
Figure 6.2 Scanning electron microscopy. (a) unfertilised eggs, negative control Bar= 10 μm, (b) infected unfertilised eggs, 5 days post infection. Bar= 1 μm
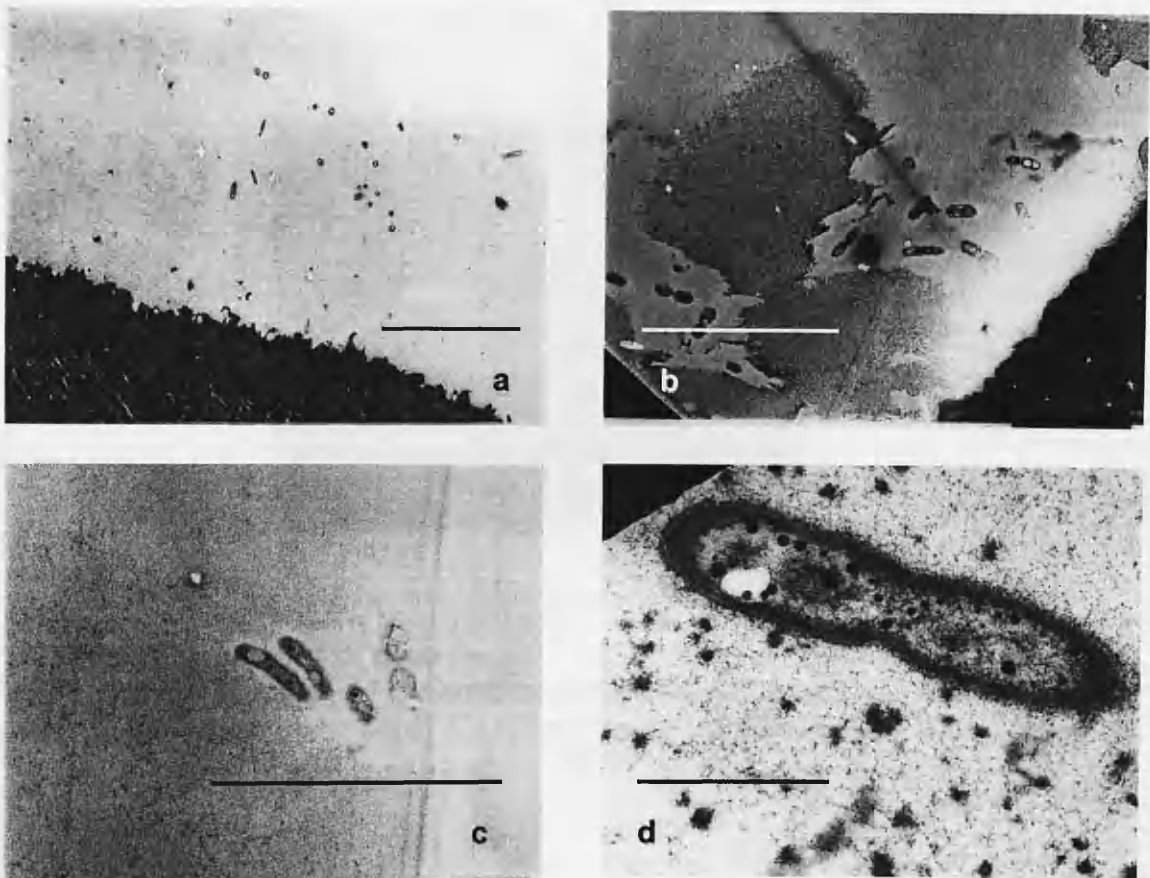
Figure 6.3 Transmission electron microscopy of a) mixed microflora on the surface of uninfected rainbow trout eggs 10 days post-infection, b) infected fertilised eggs, destruction of mucous layer by proteolytic bacteria, 30 days post-infection, c) infected unfertilised eggs, 30 days post-infection, destruction of mucous layer by proteolytic bacteria d) infected unfertilsed eggs, multiplication of bacterial cell, 30 days post-infection. Bars (a), (b), (c) =10 μm, (d)= 2μm

6.3.3 IFAT

F. psychrophilum were identified by IFAT on the surface of both infected fertilised and unfertilised eggs (Figure 6.4). The levels of the pathogen per section were quite low, ranging from between 1 and 3 bacteria. No bacteria were identified inside the eggs.

IFAT analysis on sections from sampled fry did not reveal the presence of the pathogen within the body of the fish.
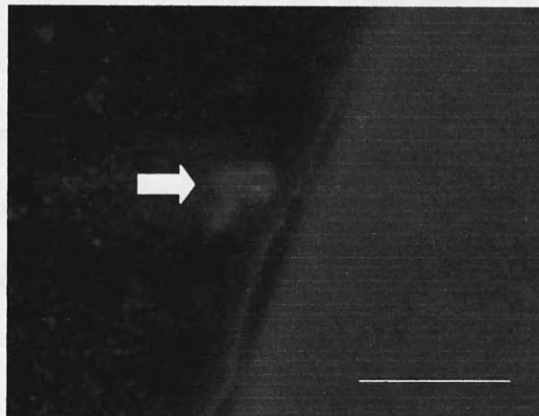
Figure 6.4 Identification of *F. psychrophilum* (white arrow) on the surface of fertilised rainbow trout egg using IFAT, one month post-fertilisation. Bar= 10 µm

6.3.4 Inoculation of sampled eggs into *Cytophaga* broth

Samples of eggs were first cultured in *Cytophaga* broth for 4 days, then aliquots of the bacterial suspensions spread onto *Cytophaga* agar. In samples of infected

eggs taken from both fertilised and unfertilised groups, a mixture of colonies containing a few yellowish ones could be seen on the agar plates. When bacteria collected from these yellowish colonies were stained with Gram stain, the bacteria appeared as long slim filamentous Gram negative rods.

6.3.5 PCR analysis of egg samples and colonies on agar plates

A band larger than 1230 bp was present in eggs collected prior to infecting the eggs. The presence of this band was due to other bacterial species on the eggs [Figure 6.5, (a)] (Chapter 2). The 1078 bp band representing the presence of *F. psychrophilum* DNA was not present in these samples. However, analysis of both the eggs collected after infection [Figure 6.5, (b)] and bacteria collected from the yellowish colonies on the *Cytophaga* agar, resulted in an expected band at 1078 bp, indicating the presence of *F. psychrophilum* DNA.
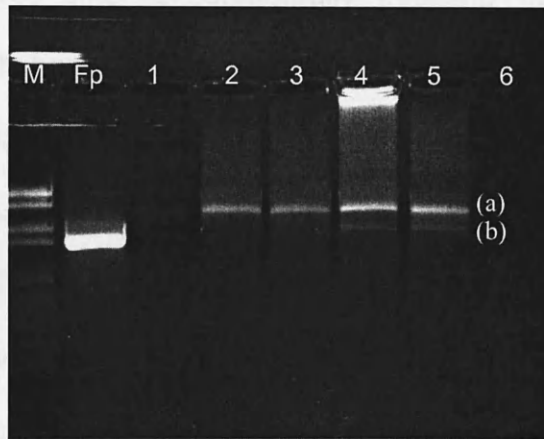
Figure 6.5 Nested PCR analysis of egg samples. 1% agarose gel. Lanes: M: marker, Fp: positive control, 1: egg sampled prior to infection, 2: unfertilised eggs 30 d post infection (negative control), 3: fertilised eggs 30 d post infection (negative control), 4: infected unfertilised eggs 30 d post infection, 5: infected fertilised eggs 30 d post infection, 6: negative control (water). (a) band at >1230 produced after the first round of the nested PCR, due to the amplification using the universal primers 1500R/20F, (b) bands at 1078 bp produced after the second round of the nested PCR using primers PSY1/PSY2 primers.

6.4 DISCUSSION

From many different studies, it has been demonstrated that the surface of the fish eggs is colonised by a variety of different bacterial species. Many of these microorganisms have adverse effects on the development of the eggs, as for example, was observed after experimentally infecting halibut (*Hippoglossus hipoglossus*) eggs with bacteria belonging to the genera *Flexibacter* and *Vibrio* (Bergh *et al.,* 1992). Bacteria are able to colonise the eggs either via the water or *intraovum*, as in the case of *R. salmoninarum* (Evelyn *et al.,* 1986), or both.

Barnes *et al.,* (2000) examining the bacterial population on the surface of rainbow trout eggs in vertical flow tray incubators, found that an initial peak in the number of bacteria occurred at the eye stage, followed by a decline during the mechanical picking of the dead eggs. A second peak was then observed during hatching. Apparently, the dead or damaged eggs can leak nutrients aiding bacterial multiplication (Barker, 1989; Ogbondeminu, 1994). Other factors, such as water temperature, egg density and poor water flow also seem to help bacteria to overgrow on the eggs (Barker, 1989).

The presence of *F. psychrophilum* in rainbow trout eggs has been confirmed from many studies (Holt *et al.,* 1993; Bustos, *et al.,* 1995; Cipriano *et al.,* 1995; Rangdale *et al.,* 1996; Brown *et al.,* 1997; Kumagai and Takahashi, 1997; Rangdale *et al.,* 1997a). It has been suggested by Brown *et al.,* (1997) that the bacterium can be transmitted to the eggs either from the broodstock or the water. Rangdale *et al.,* (1997a) reported that colonisation of eyed rainbow trout eggs with *F. psychrophilum* can in turn lead to RTFS in the fry. The ability of *F. psychrophilum* to inflict damage on rainbow trout eggs after successful

colonisation of the eggs, when artificially infected with the bacterium by a bath challenge, was examined.

All eggs originated from one female. Prior to commencing the study, the eggs were tested by PCR to confirm that the eggs were free from *F. psychrophilum* infection (it should be noted though, that it was probable to fail to detect the presence of the pathogen, as Table 6.2 shows). Although samples appeared to be negative for *F. psychrophilum*, a band was evident due to the first pair of primers (1500R/20F) used in the first round of the nested PCR. This set of primers has been found to produce a band larger than 1230 bp, for a number of other bacterial species (Chapter 2). The presence of other microbes on the surface of the eggs was also confirmed by culture and by both scanning and transmission electron microscopy. However, throughout the course of the transmission experiment, egg samples always appeared positive for *F. psychrophilum* both by PCR and by culture. IFAT performed on sections of eggs also revealed the presence of *F. psychrophilum* in low numbers only on the surface of the eggs (again it was probable to fail to detect the pathogen inside the eggs, as Table 6.2 shows), although it should be noted that the antibodies used do cross-react with other bacterial species (Faruk, 2000). In a similar experiment, Kumagai *et al.,* (2000) after immersing fertilised coho salmon eggs into broth culture of *F. psychrophilum,* 57 days post-infection, examining frozen egg sections by IFAT, found that some bacteria managed to penetrate the eggs. Since they used frozen sections to examine the eggs, it is likely that the bacteria were spread from outside to inside, thus producing a false picture.

From both scanning and transmission electron microscopy, the surface of the unfertilised eggs appeared more heavily colonised by bacteria than fertilised

eggs. In all cases bacteria were found on the surface of the eggs and never within the egg. However, it was not possible to distinguish *F. psychrophilum* from other bacterial species using these methods.

In a few instances, the mucous layer of the eggs appeared to be partially destroyed, especially in eggs collected just before hatching possibly due to enzymatic activity of the bacterium. Uddin and Wakabayashi (1997) found *F. psychrophilum* to exhibit high levels of proteolytic activity at low temperatures (around 13.3± 1.9° C), especially during the late log or stationary phase of its growth cycle. It was not possible to determine whether *F. psychrophilum* was responsible for the proteolytic activity observed in the present study, as many other bacterial species were also present.

Rangdale *et al.,* (1997a) reported that infection of eyed rainbow trout eggs with *F. psychrophilum* by bath increased the level of mortality that was observed in post-swimming-up fry. Concentrations of bacteria used by the authors for the bath challenge were 10^{10} bacteria ml^{-1} using 500 ml of a four-day old bacteria culture per 310 eggs. No significant levels of mortality were observed amongst the eggs prior to hatching and among pre-swimming-up fry in the present study. However, the number of bacteria used to infect the eggs here was much lower (10 ml inoculum of 3×10^8 bacteria ml^{-1} per 300 eggs). It was shown in the previous chapter that *F. psychrophilum* collected from fresh cultures, as in the case of the present study, do not adhere to the eggs in high numbers. Future transmission studies should therefore examine the colonisation of the eggs by starved bacteria.

It is suggested in Chapter 5 that possibly the ability of *F. psychrophilum* to adhere to the eggs was not related with its virulence. In the present study and in the one conducted by Rangdale *et al.,* (1997a), no significant mortalities were

observed among the infected eggs, although different strains were used for infectivity. Since the present study was terminated prior to the post-swimming-up stage of the fry, no comparison could be made in the levels of the mortalities of post-swimming-up fry in the two studies.

Another important consideration is the conditions under which the eggs were incubated. The conditions in the present study were different from those often used in commercial hatcheries. Barker (1989) found that bacterial overgrowth on the surface of the eggs can have a negative effect on the development of the eggs, but only if other adverse environmental conditions were also present. Barnes et al., (2000) for example, found that in vertical incubators, the levels of the bacteria tend to increase from top to the bottom of the incubators. In the present study, the eggs were maintained in a single layer on the trays, under good water conditions. This may have helped to prevent the growth of the pathogen on the egg surface.

Stress also appears to play an important role in the progress of the disease. Rangdale et al., (1997a) suggested that the increased mortality observed in the swim-up fry may be associated with stress due to the onset of feeding. In Chapter 2 the survey conducted in the farms with endemic RTFS showed fry to be in contact with the pathogen from a very early stage in their life. However, they only exhibited signs of the disease when they were transferred from the hatchery to the fry tanks. Thus, stress due to transportation may have contributed to the appearance of the disease. In the present study, almost no mortalitites were observed in the fry hatched from the infected eggs or in fry re-infected with F. psychrophilum. In addition, the pathogen was not identified in the fry tested by IFAT. Rangdale et al., (1997a) also did not manage to isolate the pathogen from

pre-swim up fry by culture. The present study was terminated prior to first feeding and thus, no further fry samples were collected.

The results of this experiment indicate that *F. psychrophilum* remains on the surface of the eggs after colonisation of the surface of the eggs and does not appear to penetrate the eggs. Further studies, are needed to confirm this, especially under adverse environmental conditions as often observed in commercial hatcheries during incubation (e.g. high egg densities, reduced water flow, higher water temperature) conditions. Different isolates of *F. psychrophilum* and different physiological states of the bacterium, such as late log phase or after starvation, should also be tested, as the results from Chapter 5 show that adherence of the bacteria is higher in this state. Previous studies suggest that the broodstock is a source of contamination and may be more important for the transmission of the pathogen. It is possible that when the eggs are colonised by the pathogen *intraovum*, the pathogen can penetrate the eggs at this stage and survive within the eggs, since *F. psychrophilum* appears to be resistant to the activity of lysozyme (Brown *et al.,* 1997). This may also explain the partial ineffectiveness seen with disinfection employed at farm sites. The same was also suggested by Kumagai *et al.,* (1998). Nevertheless, colonisation of the surface of the eggs is important as it can act as an additional factor for the spread of the disease between farms.

CHAPTER 7

GENERAL DISCUSSION-CONCLUSIONS

Flavobacterium psychrophilum was first isolated in 1948 from an outbreak of bacterial cold water disease in coho salmon. Since then, it has been isolated from many other fish species worldwide, such as Canada, Chile, Australia, Japan and Europe. Since its first isolation, researchers have been faced with a number of problems relating to the pathogen, from the taxonomy of the bacterium, which has been troublesome and changed many times, to the detection of the pathogen in environment and in fish samples. Since the mid 80s, the pathogen has also been associated with a new syndrome infecting small rainbow trout fry and again the name of which has also changed many times. The name that has finally prevailed and is most commonly used today is rainbow trout fry syndrome (RTFS). This disease has been responsible for high mortalities in commercial rainbow trout hatcheries.

The present study looked at issues relating with the ability of *F. psychrophilum* to survive outside its host within the aquatic environment, especially in the environment of the farm where the disease has become endemic. The morphological and functional changes of the pathogen, such as its ability to adhere to different substrates (fish eggs and n-hexadecane) during its survival outside the fish were examined. A number of different methods were used in this study for the isolation and identification of the bacterium in environmental samples.

Problems with the application of these methods, associated with changes in the bacterium so as to enable it to survive outside its host, were identified and the implications of these changes in the diagnosis of the disease in relation to the various methods were discussed.

The first hurdle scientists had to overcome, when the bacterium was initially isolated, was the production of a suitable culture medium, in order to isolate the pathogen during a disease outbreaks. Although numerous media have been developed by a number of different authors (Iida and Mizokami, 1996; Daskalov *et al.,* 1999), the most commonly used medium today for the isolation of the pathogen is *Cytophaga* agar or *Cytophaga* broth (Anacker and Ordal, 1959). However, it would appear that problems with isolation and identification of the pathogen by culture still exist. As in the case of many other bacterial species (Kell *et al.,* 1994; Barcina, *et al.,* 1997; Baleux, *et al.,* 1998), the viability of *F. psychrophilum* is not always related to its culturability, as Michel *et al.,* (1999) first demonstrated. This was also confirmed in the present study. This is compounded by the fact that *F. psychrophilum* is capable of surviving outside the host, under conditions of starvation for many months. In this physiological starved state it is even more difficult to isolate the bacterium by culture, especially on agar plates. This has further implications, since the use of colony forming units (CFU's) as an indication of bacterial level in samples appears to be totally inadequate.

The limited success in isolating the bacterium from environmental samples by culture is one of the reasons for the lack of knowledge we have on the distribution of the pathogen in the aquatic environment, especially in the farm before, during and after a disease outbreak. Therefore, different identification methods are required. In the present study, a PCR method was used, for the first

time, to identify the presence of the pathogen in all the parts of a farm with endemic problem of RTFS. A survey was conducted at two farm sites with endemic problem of RTFS, the results of which provided useful information, especially related to the general design and management of the farm.

The layout of many hatcheries, in the UK at least, appeared to be perfect for the rapid spread of the pathogen, once it arrives on the farm. The broodstock often receives water from the fry and as a result, if the fry is infected with *F. psychrophilum*, then the broodstock also becomes infected. How the pathogen arrives on a farm in the first place is still not clear. In most cases, the eggs appear to be the source of the disease (Holt *et al.,* 1993; Bustos *et al.,* 1995; Cipriano *et al.,* 1995; Rangdale *et al.,* 1996; Brown *et al.,* 1997; Kumagai and Takahashi, 1997). An additional factor that helps the spread of the pathogen within the farm is the common policy of farms to mix different batches of eggs in the same incubator. As a result of this and increased water temperature, decreased water flow or high egg densities (Barker, 1989), the level of the pathogen inside the incubators increases dramatically. The fry that hatch from the infected eggs do not usually show signs of the disease. However, the disease appears often later, for example during first feeding or after transport to the fry tanks. This implies that stress is also important for the disease to manifest itself (Rangdale *et al.,* 1997a). Bacteria that are released from the infected and sick fry in turn infect the broodstock, which do not exhibit any sign of the disease, but act as a reservoir for the pathogen and thus the problem finally becomes endemic within the farm.

Although infected eggs have been associated with the occurrence of the disease, the localisation of the pathogen in/on the eggs, is still under investigation. In the present study, *F. psychrophilum* appeared to remain on the surface of the

eggs after successful colonisation, although it was not conclusive whether the pathogen can penetrate the egg when adverse environmental factors prevail. From previous studies (Cipriano *et al.,* 1995; Rangdale *et al.,* 1996; Brown *et al.,* 1997) and from the results of the survey of the farms using PCR, it was found that some eggs were positive when released from the female. It is possible that when the eggs are infected *intraovum*, the bacterium can survive within the eggs, escaping the disinfection procedures used by the farms, as in the case of *R. salmoninarum* (Elliott *et al.,* 1991).

The surveys of the two farms, also showed that water samples from tanks containing fry that had recovered from RTFS after being treated with antibiotics, were still positive by PCR for many days post-treatment. It is not clear whether the bacteria are being shed from the fish or if they manage to survive and possibly multiply in the water of the tanks. The latter implies that the bacteria are not removed from the tanks with the constant water flow. If the source of bacteria is the fry then this raises the question of whether the bacteria are dead or alive and thus whether the fry are actually carriers at this stage and by mixing them with other fry they can transmit the disease. A quantitative method to enumerate the pathogen in water samples could provide some information on the shedding rate. Such a method could also provide information on other antimicrobial or disinfection treatments.

The viability of *F. psychrophilum* is not related to its culturability. It has been shown that under conditions of starvation the bacterium develops into a viable but non-culturable state (i.e. not able to produce colonies on agar plates). After prolonged survival in stream water, the bacterium tends to become shorter and round. On the other hand, other properties such as the surface hydrophobicity of

the pathogen and its ability to adhere to supports increase. As it has been reported

for other bacterial species under starvation many properties, including some

virulence factors, change (Barcina *et al.,* 1997; Rahman *et al.,* 1997; Baleux *et al.,*

1998). The implication of this is that bacteria under artificial culture media probably

do not exhibit the same characteristics than those of bacteria which remain in the

environment for a period of time. This can partially explain why until now there is

no satisfactory bath challenge method, while only intramuscular or intraperitoneal

injections of bacterial suspensions can successfully transmit the disease *in vitro.*

Another issue related with the diagnosis of RTFS is the problem

encountered when either antibodies or oligonucleotides are used as probes for the

identification of the pathogen. It has been demonstrated that culture as a method

to isolate the pathogen, especially from environmental samples is not suitable, as

the bacterium can develop a viable but non-culturable state. Thus, other methods

were used in this study to identify and, if possible, enumerate the pathogen in

environmental samples, such as *in situ* hybridisation, IFAT and PCR-based

techniques. Each of these methods has a certain advantage over the other

methods. In terms of specificity for example, *in situ* hybridisation using FITC-

labelled oligonucleotide probes appears to be superior to IFAT, although further

investigation is needed to confirm this. It has been reported by Faruk (2000) that

the antibodies used in the present study cross-reacted with other bacterial species

and exhibited low reactivity with other *F. psychrophilum* strains. On the other hand,

the primers used for the PCR assay and the *in situ* hybridisation to detect the

pathogen in different samples appeared to be specific for *F. psychrophilum.* IFAT

appeared to provide better results with aged bacteria, compared with *in situ*

hybridisation method, since an almost total lack of signal was observed when one-

month old bacteria were tested by *in situ* hybridisation. Western Blot and SDS-PAGE analysis revealed that in bacteria maintained under conditions of starvation some low molecular weight bands tend to disappear compared to bacterial cells collected from fresh cultures, perhaps due to reduced metabolism or change in morphology of the bacterial cells during starvation. Additional studies using substrate gels may provide useful information on differences in the proteolytic activity between bacteria collected from fresh cultures and bacteria maintained for prolonged periods of starvation.

Four different methods (semi-quantitative PCR, quantitative PCR using internal standard and IFAT or *in situ* hybridisation on bacteria collected on membrane filters) were tried in the present study in order to produce a quantitative method to determine levels of the pathogen in water samples. Problems with the reproducibility (semi-quantitative PCR), sensitivity (quantitative PCR and IFAT on filter membranes), or problems related with the changes of *F. psychrophilum* during a prolonged survival under conditions of starvation (*in situ* hybridisation) were encountered. Therefore, further validation is needed to establish a satisfactory quantitative method to enumerate the pathogen in water samples.

Further investigation is needed to develop a successful strategy for the control of RTFS, since many aspects of the epidemiology and pathogenicity of *F. psychrophilum* have not been clarified. Good farm management and proper farm layout seem to be very important in the control of the disease. However, there is limited information on the route by which the pathogen enters the fish and whether any external environmental factors are also involved in the occurrence of the disease. This information is critical in order to develop prophylactic measures against the spread of the disease. It has been shown that both unfertilsed eggs

and milt can be positive for the pathogen (Rangdale *et al.,* 1996). In addition, Rangdale *et al.,* (1997) showed that successful colonisation of eyed eggs can lead to an increased mortality among post swim-up fry. If eggs from infected broodstock or eggs are colonised by the pathogen during incubation are the most important source for the disease, then careful screening of the broodstock and different batches of eggs may help to minimise the spread of the disease between farms. This also has important implications for the development of a vaccine against the pathogen. If fry carry the pathogen when they hatch, then a hypothetical vaccination, even at a very early stage in the life of the fry could be ineffective. For example, it has been shown that exposure of coho salmon at the egg stage to the p57 protein of *R. salmoninarum* induces partial immunosuppression (Brown *et al.,* 1996). It has been suggested that stress may also be involved in the occurrence of the disease and thus, improvement of the general management of the farm would also help to minimise the fry mortalities.

Rapid, sensitive and specific techniques, such as PCR, *in situ* hybridisation or IFAT, can significantly advance the understanding of the epidemiology of RTFS and pathogenicity of *F. psychrophilum.* This study highlighted some of the problems associated with the use of these techniques, particularly related with the bacterium's ability to survive for prolonged periods under conditions of starvation. In this physiological state the pathogen exhibited morphological as well as functional changes, making its isolation and identification problematic. The techniques used in this study show great potential for such a task and once the problems discussed are resolved, their application will undoubtedly lead to the successful development of control strategies for RTFS.

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APPENDIXES

APPENDIX 1. ACRIDINE ORANGE DIRECT COUNTING METHOD (HOBBIE *ET AL.*, 1977)

- Dilutions of bacterial cultures (10-20 µl) were prepared in filtered PBS (through a 0,2 µm filter) to a final volume of 1 ml.

- 100 µl of 0,1 % w/v acridine orange (Gurr) in distilled water was added to the diluted sample and after briefly mixing the preparations were incubated for 2 min in the dark.

- After the incubation, the preparations were put in the filtration apparatus and 1 ml of filtered PBS was also added. Black polycarbonate filter membranes were used for the filtration (Isopore Black Filter Membranes, Millipore).

- After the filtration, the membrane filters were mounted onto microscope slides using a drop of non-fluorescence immersion oil (Agar). One drop of non-fluorescence immersion oil was put on top of the filter membranes and then cover-slips were put on top of them.

- The slides were observed using a fluorescence microscope, fitted with a grid graticule using at 100x magnification with immersion oil. The grid area had previously been calculated using a calibration slide and the filtration area using a ruler. Thus, the ratio between the grid area and the filtration area had been

calculating as 2.83×10^4. In order to estimate the total number of bacteria per filter, ten grid areas were observed and the mean number of bacteria was them multiplied by the ratio (2.83×10^4).

- The method provides good results for bacterial levels between 1 bacterium grid area^{-1} (corresponding to 2.83×10^4 bacteria filter^{-1}) and 350 bacteria grid area^{-1} (corresponding to 10^7 bacteria filter^{-1}).

APPENDIX 2. BUFFERS

(All Chemicals were obtained from Sigma unless otherwise stated)

2.1 General purpose buffers

<u>PBS</u> 0.02 M (phosphate buffered saline) (per litre)

$NaH_2PO_4.2H_2O$ (Analar)	0.37 g
$Na_2 HPO_4.2H_2O$ (Analar)	2.561 g
NaCl	8.77 g
pH 7.2	

<u>SSC</u> (saline salt citrate, 20 x, stock) (per 500 ml)

NaCl	87.7 g
Sodium citrate (trisodium salt)	44.1 g

<u>TBS</u> 0.05 M (Tris buffered saline) (per litre)

Tris base	2.42 g
NaCl	29.24 g
pH 7.4	

2.2 *In situ* hybridisation buffers

<u>APES</u>

3-aminopropyltriethoxysilane (APES)   3% v/v in acetone

(5 min in APES, 5 min in 100% acetone, 5 min in dH_2O)

Hybridisation buffer (per 10 ml)

20 x SSC	2 ml
TBS	8 ml

Salmon sperm DNA (100 μg ml^{-1}) 100 μl

Aliquot and store at -20° C

2 x SSC

20 x SSC	55 ml in 495 ml distilled water

0.1 x SSC

20 x SSC	1.5 ml in 300 ml distilled water

2.3 SDS PAGE buffers

Ammonium persulfate (APS) solution

APS 10% (w/v) in distilled water, fresh

Reservoir buffer (5x)

Tris base	15 g
Glycine	43.2
SDS	5 g

pH 8.3

Separating gel buffer (per 500 ml)

Tris base	91 g
SDS	0.4% w/v

pH 8,7

<u>Stacking gel buffer</u> (per 100 ml)

Tris base	6.05 g
SDS	0.4% w/v
pH 6.8	

<u>Sample buffer</u> (5x, per 10 ml)

Tris base (1M, pH 6.8)	0.6 ml
Glycerol	2.5 ml
SDS	2 ml
2-mercaptoethanol	0.5 ml
Bromophenol blue	0.1 % w/v

Stored at -20° C. Samples were diluted 1:4 in sample buffer

<u>Separating gel</u> (12% polyacrylamide)

Separating gel buffer	5 ml
Distilled water	7 ml
Acrylamide (30% v/v)	
(Severn Biotech Ltd)	8 ml
TEMED	15 μl
APS solution	70 μl

<u>Stacking gel</u> (4% polyacrylamide)

Stacking gel buffer	2.5 ml
Distilled water	6.1 ml
Acrylamide (30%)	
(Severn Biotech Ltd)	1.34 ml
TEMED	10 μl
APS	50 μl

2.4 Western Blot buffers

<u>Transblot buffer</u> (per litre)

Tris base	14.4 g
Glycine	3.03 g
Methanol	200 ml

    pH 8.3

<u>Tween 20-TBS (TBST)</u>

1 ml Tween 20 (Sigma) in 1 litre TBS

<u>Substrate</u> (Stock solution)

4-chloro-napthol	0.3% w/v in methanol

Stored at -20° C

<u>Substrate</u> (Working solution)

Stock solution	2 ml
PBS	10 ml
H_2O_2	10 µl

2.5 Quantitative PCR buffers

<u>Antibody dilution buffer</u>

HEPES	5.95 g
NaCl	7.3 g
$MgCl_2$	0.2 g
Tween 20	1.0 ml

    pH 7.5

Dilution buffer (per 100 ml)

      Tris base   60.0 mg

      EDTA   0.372 mg

        pH 7.8

Washing buffer (per litre)

      Tris base   3.0 g

      NaCl   7.3 g

      $MgCl_2$ 0.2 g

      Tween 20   1.0 ml

        pH 7.5

Hybridisation buffer (10 ml)

      20x SSC   2.5ml

      Salmon sperm DNA (100 μg ml^{-1}) 100 μl

APPENDIX 3. CULTURE MEDIA

3.1 Modified *Cytophaga* medium (per litre) (Bernardet and Kerouault, 1989)

Tryptone (Oxoid)	5.0 g
Yeast extract (Oxoid)	0.5 g
Sodium acetate (Sigma)	0.2 g
Beef extract (Difco)	0.5 g
Agar bacteriological	
No 1 (Oxoid)	15.0 g
pH 7.2	

3.2 L.B (Luria-Bertani) broth (per 500 ml)

Tryptone (Oxoid)	10 g
Yeast extract (Oxoid)	5 g
NaCl (Sigma)	5 g
pH 7.2	

When it cools to 45° C, add 50 mg ampicillin

3.3 L.B agar (Luria-Bertani) (per 500 ml)

Tryptone (Oxoid)	10 g
Yeast extract (Oxoid)	5 g

NaCl   (Sigma)   5 g

Agar bacteriological

    No 1   (Oxoid)   5 g

    pH 7.2

When it cools to 45° C, add 50 mg ampicillin

APPENDIX 4. DEVELOPMENT OF THE INTERNAL STANDARD (BASED ON SAMBROOK *ET AL.*, 1989)

4.5 Generation of blunt ends

- PCR products were run on a 0.8% low melting agarose gel. In the case of phagemids containing inserts, after cutting the phagemids with the appropriate restriction enzymes, the fragments were run on 0.8% low melting agarose gels. Slices containing the appropriate bands were cut and melted at 65° C for 5 min.

- 10 µl of molten agar was transferred in a fresh 1.5 ml tube and mixed with 10 µl reaction mixture containing (5 units) of Klenow fragment 0.5 mmole of each dNTP and 1 µl 10 x buffer (0.5 M Tris-HCl, pH7.6, 0.5 M $MgCl_2$). The mixture was incubated for 30 min at 20° C, before 30 µl of water was added.

- 50 µl of 50:50 phenol:chloroform was added and after centrifugation at 13.000 g for 5 min the top layer was removed and placed into a fresh 1.5 ml tube.

- 6 µl of 3 M sodium acetate, pH 7) was then added to the supernatant. 150 µl of 100 ethanol was added and the tube was placed at -40° C for 40 min.

- The tubes were then centrifuged for 5 min at 13.000 g and resulting pellets were washed with 70% ethanol.

- After another centrifugation, the pellets were air-dried and re-suspended in 10 µl of nano-pure water.

4.1 Ligation reactions

- Using a clean razor blade, slices of the agarose gel containing the products of interest were cut and placed in a 1.5 ml tube.

- The agarose slices were heated to 65° C to dissolve the agarose and then cooled to 37° C.

- 8.5 µl of the melted agarose was added to a fresh 1.5 ml tube containing 1 µl of 10x ligase buffer and 0.5 µl T4 ligase (Amersham Pharmacia Biotech). In the case of ligating phagemid with PCR product, 2:1 ratio of phagemid:PCR products was used.

- The ligation mixture was left at 20° C overnight.

4.2 Transformation of *E. coli*

- The ligation mixture was melted in a 1.5 ml microcentrifuge tube at 65° C for 5 min and mixed with 200 µl of bacterial suspension containing competent *E. coli* (kept at -20° C).

- The mixture was incubated on ice for 30 min and then transferred in a water bath at 42° C for 1.5 min.

- One ml of LB medium was then added and the mixture was incubated at 37° C for 1 h. The tube was centrifuged for 10 sec at 13.000 g and the supernatant was removed.

- The pellet was re-suspended in the remaining fluid, spread on LB agar plates and incubated overnight at 37° C.

4.3 Purification of phagemid DNA

- Individual white *E. coli* colonies phagemid containing the DNA fragments were transferred in 25 ml tubes containing 5 ml of LB broth and incubated overnight at 37° C under vigorous shaking.

- The cultures were then centrifuged at 5000 g for 5 min and the supernatants discarded.

- The pellets were re-suspended in 190 μl nano-pure water and transferred into fresh 1.5 ml tubes.

- 400 μl of 1% SDS w/v+0.1 M NaOH (freshly prepared) was then added to the tubes and after briefly mixing the tubes were left on ice for 5 min.

- 300 μl of 3 M potassium acetate+2 M acetic acid was added and after briefly mixing, the tubes were left on ice for 5 min.

- The tubes were centrifuged at 13.000 g for 5 min and the supernatant was transferred to a fresh 1.5 tube.

- 600 μl of 50:50 mixture of phenol:chloroform was then added to the tubes. After further centrifugation at 13.000 g for 2 min, the top layer (approximately 750 μl) was carefully removed and placed into a fresh tube.

- 700 μl of isopropanol was added to the tubes, and then incubated at -20° C for 20 min.

- The tubes were centrifuged at 13.000 g for 10 min and after removing all traces of isopropanol, the pellets were re-suspended in 100 µl of nano-pure water.

- After adding 100 µl of 13% v/v PEG+1 M NaCl the tubes were briefly mixed and left on ice for 20 min.

- The tubes were then centrifuged at 13.000 g for 10 min and the pellets were washed with 200 µl of 70% ethanol by pippeting the ethanol into the tubes, leaving it for a minute and carefully pippeting it out without disturbing the pellet.

- The pellets were air-dried for 5 min and re-suspended in 80 µl of nano-pure water.

4.4 Digestion with restriction enzymes

- 1 µl of sample (purified phagemids or purified PCR products from agarose gels) were mixed with 9 µl of mixture containing 0.2 µl of the appropriate restriction enzyme, 1 µl of the appropriate buffer and 8.8 µl nano-pure water.

- The mixture was then incubated at 37° C overnight in order for the sample to be completely digested.

- Samples were run on a low melting agarose gel (1% w/v, 4 h at 25mV) and the regions of the gel containing fragments of the expected size were removed.

APPENDIX 5. STAINS

5.1 Gram's staining (Drury and Wallington, 1980)

- A drop of the bacterial suspension (approximately 5 µl) was placed on a clean slide and spread evenly.

- The slide was allowed to dry and then was passed slowly through a bunsen flame three times to fix the bacteria onto the slide.

- After cooling, the slide was immersed in crystal violet solution (2 g crystal violet, 20 ml 95% ethanol, 0.8 g ammonium oxalate, 300 ml dH_2O) for 1 min.

- The slide was washed with water and then immersed into iodine (1 g iodine, 2 g potassium iodide, 300 ml dH_2O) for 1 min.

- The slide was then placed in a alcohol/acetone mixture, mixed carefully for 10 sec and washed thoroughly with water.

- After drying, the slide was placed in safranine solution (0.25 g safranine, 10 ml 95% ethanol, 90 ml dH_2O) for 2 min.

- The slide was finally washed thoroughly with water and allowed to air-dry.

- Gram positive bacteria were stained blue/purple and Gram negative red/pink.

5.2 Silver staining (Sigma, catalogue number AG 25)

- The gels were fixed in 30% v/v ethanol+10% v/v glacial acetic acid+60% v/v nano-pure water (three changes, 10 min each).

- After washing three times with nano-pure water (5 min each), the gels were incubated with Silver Equilibration Solution (0.75 ml Silver Concentrate diluted to 300 ml with nano-pure water) for 30 min.

- After rinsing the gels with nano-pure water for 15 sec, the gels were immersed into development solution (30 ml of Developer 1 diluted to 300 ml with nano-pure water and then to this 0.17 ml of Developer 2 was added) twice, 8 min each time.

- The gels were then immersed into the Stop Solution (1% v/v acetic acid in nano-pure water) for 5 min.

- Finally, the gels were rinsed three times with nano-pure water, 5 min each time. The gels were stored in nano-pure water.

APPENDIX 6. CYCLE SEQUENCING

6.1 Purification of PCR products from agarose gels using GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia Biotech Inc).

- After cutting the PCR bands from gels using a clean razor blade, the agarose slices were weighed to the nearest 10 mg, cut in smaller pieces and placed in a 1.5 ml microcentrifuge tube.

- 10 μl of the Capture Buffer was added for each 10 μg of gel to microcentrifuge tube and after briefly vortexing, the tube was incubated at 60° C until the agarose was completely melted.

- After a brief centrifugation to collect the sample at the bottom of the tube, the sample was transferred to a GFX column and incubated at 20° C for 1 min.

- The tube was then centrifuged at 13.000 g for 30 sec and after discarding the flow-through from the collection tube, 500 μl of Wash Buffer was placed in the column.

- After further centrifugation, 40 μl of nano-pure water was placed on the column and the sample was incubated for 1 min at 20° C.

- The column was finally centrifuged at 13.000 g for 1 min to collect the purified PCR product.

6.2 Preparation of samples for sequencing using a ABI PRISM Dye Terminator Cycle Sequencing Kit (*PERKIN ELMER*).

- In two 0.2 ml tubes, 3 µl of either the purified PCR products or the phagemid containing the DNA fragment was mixed with 8 µl of the Terminator Reaction Mix, 3.2 pmole of either the PSY1/PSY2 primers and the appropriate amount of nano-pure water to make the final volume of the reaction mixture up to 20 µl.

- The samples were subjected to PCR amplification using a GeneAmp 9700 thermal cycler. The parameters of the reaction were: 96° C for 10 sec, 50^{C} C for 5 sec, 60° C for 4 min, 25 cycles.

- The samples were transferred to fresh 0.5 ml tubes containing 20 µl of 3 M sodium acetate, pH 4.6 and 50 µl 95% ethanol.

- After briefly mixing, the tubes were incubated on ice for 10 min.

- The tubes were then centrifuged at 13.000 g for 30 min and the supernatant was carefully removed. The pellet was first washed with 70% ethanol and then dried in a vacuum centrifuge.

6.3 Loading the samples in a ABI PRISM 377 DNA Sequencer (*PERKIN ELMER*).

- The pellets were re-suspended in 4 µl loading buffer (5:1 mixture of formamide+25 mM EDTA: Blue dextran).

- The tubes were briefly centrifuged to collect the samples.

- The tubes were heated at 90° C for 2 min and quickly placed on ice until ready to load.

6.4 Preparation of gel for ABI PRISM 377 DNA Sequencer (*PERKIN ELMER*)

Urea	18 g
Nano-pure water	26 ml
10 TBE buffer	5 ml
10% APS (fresh)	250 μl
TEMED	35 μl
Long range gel solution (50%, BMA)	5 ml

Stir to dissolve and bring to 50 ml with nano-pure water

APPENDIX 7. HISTOLOGY

7.1 Fixation

Materials for histological or IFAT examination were placed in 10% neutral buffered formalin for at least 24 h.

7.2 Cassetting

Tissue samples were trimmed to a suitable size and placed into the cassettes. Cassetted samples were left in a bowl of water until loading onto the processor.

7.3 Tissue processing

- 50% methylated spirit 1 h
- 85% methylated spirit 2h
- 100% methylated spirit 2h
- 100% methylated spirit 2h
- 100% methylated spirit 2h
- 100% ethanol 2 h
- 100% ethanol 2 h
- Chloroform 2 h
- Chloroform 2 h
- Molten wax I h

- Molten wax 2 h

- Molten wax 2 h

Cassettes were removed from the processors and placed in molten wax until ready to block out. Tissues were trimmed and sections cut by microtomy.