STUDIES ON
RAINBOW TROUT FRY SYNDROME (RTFS)

by

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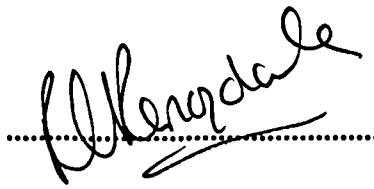
DECLARATION

I hereby declare that this thesis has been composed by myself, that it has not been accepted in any previous application for a higher degree, that the work of which it is a record has been performed by myself, and that all sources of information have been specifically acknowledged.



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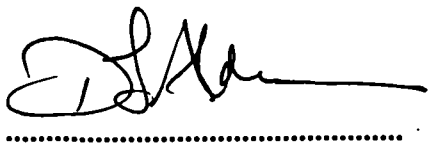

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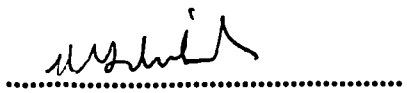

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ABSTRACT

A comprehensive survey of representative rainbow trout (*Oncorhynchus mykiss*) hatcheries revealed that the Gram negative, yellow-pigmented, filamentous bacterium *Cytophaga psychrophila* was implicated in a single disease in the U.K. and other European states. The involvement of *C. psychrophila* as the aetiological agent of the syndrome was substantiated by the fulfilment of Koch's postulates.

Infectivity studies with isolates of *C. psychrophila,* carried out under natural and laboratory conditions successfully reproduced clinical signs and gross pathological changes analogous to those observed during field outbreaks of the condition. Histopathological examination of artificially and naturally infected fish tissues demonstrated several features that were considered to be pathognomonic for RTFS. Preliminary electron microscopic studies described the ultra-structure of *C. psychrophila* and partially elucidated the cellular response to the pathogen.

Environmental sampling across selected hatchery sites demonstrated that members of the family Cytophagaceae formed a substantial element of the bacterial flora from natural waters, although recovery of *C. psychrophila* was restricted to areas where the substantial mortalities in fry attributable to RTFS had occurred. *C. psychrophila* was isolated from the sexual fluids of broodstock and additionally the bacterium was demonstrated associated with surfaces of eyed ova following various disinfection regimes.

The minimum inhibitory concentrations of a range of antimicrobial agents both existing in, and novel to, aquaculture were examined, revealing compounds which would potentially mitigate losses attributable to RTFS during field outbreaks. The emergence of bacterial resistance to chemotherapeutants was discussed. The minimum inhibitory and bactericidal concentrations and required exposure times to a number of disinfecting agents were demonstrated. The efficacy of these agents as disinfectants of egg surfaces and equipment associated with fish production was assessed.

The potential of a number of serodiagnostic techniques were evaluated as a means of rapid detection of *C. psychrophila* in diseased fish.

Parts of this work have been published or presented at conferences:

1. R.E. Rangdale (1993) Investigations into Rainbow Trout Fry Syndrome (RTFS). In: Proceedings of the European Association of Fish Pathologists (E.A.F.P.) Sixth International Conference "Diseases of Fish and Shellfish" Brest, France.

2. R.E. Rangdale (1994) Rainbow Trout Fry Syndrome- routes to successful treatment. Fish Farmer **17** 14-15 (March/ April).

3. R.E. Rangdale (1994) Research update on the rainbow trout fry syndrome project. Trout News **18** 23-26 June.

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I would like to dedicate this thesis to my parents
for their encouragement and support over the years- thanks

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Cytophaga / Flexibacter spp. as pathogens of farmed fish

Interest in the relationships between chromogenic Gram-negative gliding bacteria and infectious disease in fish began in the early 1920's. Davis (1922) reported serious mortalities amongst small mouth bass (*Micropterus dolomieui*) and the common perch (*Perca fluviatilis*) from the St Lawrence river at Ogdensburg in the USA. Affected animals exhibited a mould-like growth on the skin resulting in the development of epidermal lesions extending through into the underlying tissues. He noted that when material was scraped from a lesion and examined microscopically as a wet mount, the bacteria possessed the peculiar characteristic of collecting together to form column shaped structures on pieces of fish tissue. Because of this ability, the organism was assigned the name *Bacillus columnaris* and the condition was referred to as columnaris disease (Anacker and Ordal, 1955). Further reports of a comparable condition were not forthcoming until 1943 when Nigrelli isolated a similar organism from catfishes (*Ameiurus platycephalus, A. nebulosus and Opladelus olivaris*) and in 1945 from killifish (*Fundulus heteroclitus*) (Nigrelli and Hunter, 1945). Bacteriological studies were undertaken on a number of isolates from these cases and it was ascertained that the organism was a myxobacterium with a complex developmental lifecycle. On the basis of the properties observed the bacterium was renamed as *Chrondrococcus columnaris* (Anacker and Ordal, 1959). In 1945 Garnjobst reassigned the organism to the family Cytophagaceae due to its inability to produce either fruiting bodies or microcysts. The family Cytophagaceae includes the genera *Cytophaga, Flexibacter, Herpetosiphon, Flexithrix, Saprospira, and Sporocytophaga* and, initially, *C. columnaris* was placed in the genus *Flexibacter* (Leadbetter, 1974). However, subsequently, it has been reclassified as a member of the genus *Cytophaga* (Reichenbach, 1989). Differentiation between *Flexibacter* and *Cytophaga* is complex and detailed characterisation studies are required before bacterial isolates can be accurately assigned between these genera. *Cytophaga columnaris* has been implicated in diseases of a wide range of freshwater fish species

including bass (*Micropterus sp.*), black bullheads (*Ictalurus melas*), carp (*Cyprinus sp.*), channel catfish (*Ictalurus punctatus*), eel (*Anguilla anguilla*), goldfish (*Carassius auratus*), killifish (*Fundulus spp.*), loach (*Cobitus spp.*), perch (*Perca spp.*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*), Atlantic salmon (*Salmo salar*), chinook salmon (*O. tshawytscha*), sockeye salmon (*O. nerka*), sheatfish (*Silurus glanis*), *Tilapia* sp., lake whitefish (*Coregonus clupeaformis*) and whitesuckers (*Catastomus commersoni*) (Austin and Austin, 1993).

Other fish pathogenic members of the family Cytophagaceae have been reported, typically causing external lesions (Anderson and Conroy, 1969; Pacha and Ordal, 1970) or septicaemic infections in both fresh water (Pacha and Ordal, 1970; Snieszko and Bullock, 1976; Becker and Fujihara, 1978) and marine fish species (Masumura and Wakabayashi, 1977; Hikida, Waka, Egusa and Masumura, 1979; Baxa, Kawai and Kusuda, 1986; Baxa, 1987; Kent, Dungan, Elston and Holt, 1988). The pathology of an infection of juvenile rainbow trout (*Oncorhynchus mykiss*) at ambient water temperatures below 10°C was first described by Davis (1946) in the USA. The aetiological agent of this condition was later identified by Borg (1960), who isolated a non-fruiting myxobacterium. This isolate from open lesions anterior to the tail in coho salmon (*Oncorhynchus kisutch*) was unable to grow at temperatures above 25°C and was described as *Cytophaga psychrophila* (Borg, 1960). Due to the higher prevalence of disease at low water temperatures, the condition was named "cold water disease" (Wood and Yasutake, 1956). Further work has shown that a "cold water disease" appears to affect all species of salmonid fish, causing up to 50% mortalities in coho salmon (Lorenzen, 1994), but the severity and clinical signs are dependent on the developmental stage and species of the fish (Wood, 1974). Epizootics affecting populations of alevins are characterised by erosion of the membrane covering the yolk, often preceded by coagulated yolk disease (Holt, Rohevec and Fryer, 1993). Pacha and Ordal (1970) reported that darkening of the peduncle was a sign of the onset of infection, whereas Bullock, Conroy and Snieszko (1971) indicated that the adipose fin was the site from which the infection progressed in fingerlings and larger fish. Complete necrosis of the peduncle fin was commonly observed, resulting in the alternative epithet of "peduncle disease" (Wood and Yasutake, 1956). Wood and Yasutake (1956) isolated a bacterium with the

characteristics of *C. psychrophila* from the kidney, eye, gills, heart, peritoneum and spleen of fish exhibiting severe anaemia. They postulated that the condition had the characteristics of a general systemic infection. Fish showing signs of anaemia and haemorrhage of the gills were reported by Holt *et al.*, (1993) who also cited the presence of an erythrocytic inclusion body syndrome (EIBS) as a complicating factor in the successful diagnosis of cold water disease. Originally thought to be solely associated with marine species, EIBS, or viral erythrocytic-necrosis virus (VEN) has also been identified in freshwater reared juvenile chinook salmon (*Oncorhynchus tshawytscha*) (Leek, 1986). Its role as the primary causal agent of disease which can predispose young salmonids to secondary infections with *C. psychrophila* has not yet been fully elucidated (Holt *et al.*, 1993). *C. psychrophila* shows a lack of host specificity and has also been demonstrated in a range of non-salmonid fish including eel (*Anguilla anguilla*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), tench (*Tinca tinca*) (Lehmann, Mock, Sturenburg and Bernardet, 1991) and ayu (*Plecoglossus altivelis*) (Lorenzen, 1994).

In the marine environment members of the family Cytophagaceae, principally *Flexibacter maritimus,* have been described as pathogens in a variety of cultured fish species (Wakabayashi, Hikida and Masumura, 1986). Infections with *F. maritimus* present common problems in fry and adult stages of cultured black sea bream (*Acanthopagurus schlegeli*), red sea bream (*Pagrus major*), Japanese flounder (*Paralichthys olivaceous*) (Baxa, Kawai and Kusuda, 1988) and in white seabass (*Atractoscion nobilis*), Pacific sardine (*Sardinops sagax*) and northern anchovy (*Engraulis mordax*) (Chen, Henry-Ford and Groff, 1994); all are economically important farmed marine fish species. *F. maritimus* has also been implicated presumptively in disease in several other marine fish and shellfish in the marine environment including salmonids (Kent *et al.*, 1988) and oysters (Dungan, Elston and Schiewe, 1989). Additionally, an incompletely described species of *Sporocytophaga* has been associated with heavy mortalities amongst salmon and trout in sea water (Amend, 1970).

Other myxobacterial species have generally been considered to be non-pathogenic, but opportunistic infections can occur when the fish is weakened by poor environmental conditions or other stress factors. Carson and Schmidtke (1993)

isolated pure cultures of *C. johnsonae* from skin lesions in juvenile barramundi (*Lates calcifer*) from a Tasmanian fishery. They suggested that infection was probably endogenous in view of the fact that the normal flora of freshwater fish included a variety of myxobacterial species (Pacha, 1968), and that environmental factors such as adverse water temperature or stress-inducing management practices had increased the host susceptibility to infection. Another example is the facultative anaerobe, *Cytophaga aquatilis* which can be isolated from the gills of freshwater fish displaying signs of bacterial gill disease (Strohl and Tait, 1978). Historically, bacterial gill disease is a condition associated with a diversity of yellow pigmented, filamentous, Gram-negative bacteria of the phylogenetic branch that includes *Cytophaga, Flexibacter* and *Flavobacter* (Turnbull, 1993). As with similar diseases it is possible that these organisms are pathogenic to fish only under a number of predisposing environmental conditions. Hansen, Bergh, Michaelsen and Knappskog (1992) showed that *Flexibacter ovolyticus* was an opportunistic pathogen for Atlantic halibut (*Hippoglossus hippoglossus* L.) eggs and larvae in which infections cause high levels of mortality impeding commercial success in the early stages of fish production.

The increase in the intensity of production and variety of farmed fish species over the last century has been paralleled by an increase in infectious disease (Alderman and Michel, 1992). Members of the family Cytophagaceae form one of the most important groups of pathogens in both hatchery reared and natural populations of fish (Pacha 1968). In the USA myxobacterioses have been the most regularly diagnosed diseases in freshwater fishes and epizootics have also frequently occurred in both brackish and seawaters (Schaperclaus, 1992). In recent years significant mortalities attributable to myxobacterial infections have been reported in Canada (Daoust and Ferguson, 1983), Europe, Japan, New Zealand (Schaperclaus, 1992), Australia (Carson and Schmidkte, 1993), USA (Chen *et al.*, 1994) and Chile (Bustos, Calbuyahue, Montana, Opazo, Entrala and Solvervincens, 1994).

Hatchery production of rainbow trout (*Oncorhynchus mykiss*)

The practise of fish culture is ancient, Egyptian bas-reliefs depicting fishing scenes and the conservation of fish raised in ponds date back to the pre-Roman era (Huet, 1986). Salmonid culture rapidly expanded in Europe during the mid nineteenth

century due predominantly to the discovery of effective transport methods for eyed rainbow trout eggs from the USA and partially to improved means of communication, the discovery of artificial reproduction and the development of rearing techniques. Technical advances in aquaculture now mean that fresh, marketable fish are available to the consumer all year round.

The production of rainbow trout falls broadly into two categories; the "on-growing" site where young stock are brought in and grown up for either the table or re-stocking market and hatcheries specialising in the supply of eggs, fry and fingerlings. In the UK, eggs are either home produced i.e. stripped from specifically kept broodstock, or imported at the "eyed" stage. The demands of the UK trout industry for widened production seasons, faster growing strains and increased genetic diversity require that ova are imported from several other salmonid producing countries throughout the world. Despite health certification and widely practised disinfection, the single factor which most of all encourages the spread of trout diseases is the importation of eggs or live fish from one country to another (Hill and Purdom, 1979).

Successful incubation of salmonid eggs requires adequate water flow rates, good water quality, constant temperatures, neutral or slightly alkaline pH, dissolved oxygen levels of between 5-9 ppm and low levels of suspended solids (Barker, 1989). During incubation most hatcheries treat all of their eggs with a 3-5 mg/l solution of malachite green to minimise fungal colonisation. After hatching, yolk sac fry slip through perforations in the egg trays and continue their development, deriving nourishment from the yolk sac. Once the content of the yolk sac is completely absorbed the fry swim up to the water surface to begin feeding. It is vital that the timing and frequency of feeding and feed formulation are optimised, as inadequately nourished fry rarely recover their health or feeding response (Bromage, Shepherd and Roberts, 1988). Rainbow trout fry develop very quickly at this stage, doubling their weight every week. Management practises vary between sites but generally fry are moved out of the hatchery building when they reach 800-500/lb (approximately 0.5-0.9g) and transferred to larger outdoor tanks or raceways. Prior to this time, providing that high standards of hygiene and husbandry are maintained, the most significant mortalities are due to either genetic disorders or maladies connected with water

quality problems, such as blue sac and gas bubble disease. Once transported to the outside, fry are very susceptible to a wide range of disorders, including: costiasis, saprolegniasis, infectious pancreatic necrosis, and bacterial gill disease (Chua, 1991). Optimisation of the nutritional requirements and surrounding environment enable production of 4-5g fry within approximately 130 days post fertilisation at 10°C (Bromage *et al.*, 1988), and at this stage a large proportion of hatcheries sell their stock to the "on-growing" sites.

Rainbow Trout Fry Syndrome

Increasingly as the 1980`s progressed, a novel systemic disease of unknown aetiology was recognised in rainbow trout hatcheries across the UK. The condition was characterised by certain behavioural traits including lethargy, disorientation, cessation of feeding, swimming close to water inlets/ outlets, and hanging at the sides of the tanks. External gross clinical signs comprised exophthalmia, swollen abdomen, darkening of skin, reddening of the vent and occasional raised epidermal lesions. Internally, clinical signs comprised enlarged friable spleen, haemorrhaging of the liver, posterior and anterior kidney, severe anaemia and ascites. Losses attributable to this epizootic were commonly responsible for mortalities of 10-30% in a single batch of fry (Scott, 1989a), rising to 70%, as rapidly the outbreaks appeared to become more widespread and persistent (Chua, 1991; Santos, Huntly, Turnbull and Hastings, 1992).

Due to the gill pallor of affected fish, and the inability to detect any known viral, bacterial, fungal, or parasitic causal agent with any consistency, the condition became known as rainbow trout fry anaemia syndrome. Such a subjective diagnosis created confusion throughout the industry where initially both husbandry losses and mortalities caused by nutritional deficiencies were also attributed to the syndrome. The situation was further compounded in 1989, when similar histopathological changes to those seen in preliminary studies on fry apparently affected by the syndrome were identified. This second condition was not responsive to antimicrobial therapy but was ameliorated by increased vitamin E levels. McCloughlin, Kennedy and Kennedy (1992) reported that vitamin E inclusion of 484mg/kg was required to alleviate skeletal and cardiac myopathies. Furthermore the current recommendation of 50mg/kg (Halver, 1989) and a commercial diet containing 134mg/kg resulted in

myopathy and mortality. Possibly due to modification of the vitamin levels in commercially produced diets, the incidence of this condition has subsequently decreased (Chua, 1991). Several other proposals for the cause(s) of the syndrome or potential aetiologies were forthcoming, including infection through contaminated commercial salmonid diets (Scott, 1989a). Scott (1989a) suggested, that because in certain pet foods *Clostridium perfringens* was not killed by the pelleting process, a similar effect might result in outbreaks of clostridial enteritis. Austin and Stobie (1991) reported that yellow pigmented coryneforms, *Cytophaga columnaris* and *Janthinobacterium* sp. were recovered from a minority (47%) of two discrete populations of fry showing clinical disease similar to rainbow trout fry anaemia syndrome. Pathogenicity studies showed that all three taxa produced mortality and morbidity in 2.0g rainbow trout. However the inability to isolate any microbial pathogen from the majority of the dead and moribund fish in the field suggested that the bacteria were merely opportunists, colonising already unhealthy hosts. In a further investigation Austin and Stobie (1992) isolated the Gram positive cocci, *Micrococcus luteus* and *Planococcus* sp. from the kidney, spleen and ascitic fluid of clinically diseased fry. Inocula containing 10^5 cells/fish of both bacteria produced mortalities in 2.0g fry when given via the intraperitoneal and intramuscular route. It was not however suggested that the condition which had now become known as rainbow trout fry syndrome (RTFS) was caused exclusively by Gram positive cocci, but that their possible involvement in fish pathology should not be disregarded (Austin and Stobie, 1992). During 1991, pure cultures of the purple pigmented Gram negative rod, *Janthinobacterium lividum* were isolated from two populations of fry showing clinical RTFS. Laboratory infectivity experiments resulted in morbidity and mortality in 2.0g rainbow trout. *Janthinobacterium lividum* is, however, ubiquitous within the aquatic environment, and its precise role in RTFS was not fully elucidated (Austin, Stobie, Gonzalez, Curry and McLoughlin, 1992).

Concurrently, in France, Denmark, Scotland, Spain, Germany, Italy, Finland and Chile, a comparable condition, known variously as "visceral cold water disease", "fry mortality syndrome", "visceral myxobacteriosis", "rainbow trout mortality" and "rainbow trout fry syndrome", had been reported in both farmed (Baudin-Laurencin Castric, Vigneulle, and Tixerant, 1989; Weis 1989; Lorenzen, Dalsgaard, From,

Hansen, Horlyck, Korsholm, Mellergaard and Olesen, 1991; Santos *et al.*, 1992; Sarti, Giorgetti and Manfrin, 1992; Toranzo and Barja, 1993; Bustos *et al.*, 1994) and wild populations of rainbow trout (Wiklund, Kaas, Lonnstrom and Dalsgaard, 1994). Gross pathological signs of disease appeared to agree with those observed in England, including lethargy, swimming close to water surface, dark colouration, bilateral exophthalmia and poor appetite (Lorenzen *et al.*, 1991), abdominal distension, periocular haemorrhaging (Toranzo and Barja, 1993), pale gills, and yellow edged skin lesions, particularly in larger fish (Santos *et al.*, 1992). In the above investigations *Cytophaga psychrophila* (syn. *Flexibacter psychrophilus*) was repeatedly isolated from external lesions, liver, spleen and kidney tissue of affected fry and fingerlings, in the weight range 0.2-6.0g. In 1992 mixed cultures of *C. psychrophila* and a *Cytophaga*-like bacterium (CLB) were recovered from infected groups of rainbow trout at two hatcheries in the UK. Infectivity experiments with field isolates produced gross clinical signs consistent with RTFS and 80% mortality within 7 days (Austin, 1992).

Characteristics of *Cytophaga psychrophila*

Taxonomic Position

The taxonomy of the phylogenetically heterogenous group of gliding bacteria, which includes *Cytophaga psychrophila,* remains in a state of confusion (Holt *et al.*, 1993). Isolates of *C. psychrophila* were initially considered to be homogenous with *Cytophaga aurantiaca* recovered from garden soil (Lewin, 1969). However on the basis of its pathogenicity and low optimal growth temperature (Pacha, 1968), the organism was later considered to be a new species of myxobacterium and was given the name *Cytophaga psychrophila.* The description of this organism was however incomplete and as a result was not included in *Bergey's Manual of Determinative Bacteriology,* 7th Edition (Pacha, 1968). Latterly, the order Myxobacteriales has been defined as containing only those fruiting myxobacteria that exhibit a DNA G+C ratio of between 67-71% (Buchanan and Gibbons, 1974), and members of the genus *Cytophaga*, with characteristically low G+C ratios, were placed in the family Cytophagaceae (Holt *et al.*, 1993). In 1974, Leadbetter proposed that *C. psychrophila*

was *incertae sedis* and that the organism was more closely related to members of the genus *Flexibacter*. He suggested that the *Flexibacter* and *Cytophaga* groups should be distinguished on the basis of their differing abilities to degrade polysaccharides. In an attempt to validate this nomenclature, Bernardet and Grimont (1989) reported that presumptive *C. psychrophila,* isolated from rainbow trout with cold water disease, did not possess the ability to degrade polysaccharides, and was consequently a member of the genus *Flexibacter.* Reichenbach and Dworkin (1981) disputed genomic separation based on this characteristic, proposing that more significance should be placed on the morphological properties of the organism and the G+C contents of their DNA. In accordance with Christensen (1977) they recommended that *C. psychrophila* should remain in the genus *Cytophaga* pending further reorganisation of the entire group. This approach has been followed in this review.

Morphological Characteristics

Cells of *C. psychrophila* are generally considered to be weakly refractile, slender, weakly Gram-negative, strictly aerobic, flexible rods. Actively growing cells of the organisms are approximately 0.75μm (Pacha, 1968), 0.3-0.5μm (Bernardet and Grimont, 1989) in diameter and between 1.5 and 7.5μm (Pacha, 1968), 1.0-5.0μm (Bernardet and Grimont, 1989) in length. In 48 hour liquid cultures, pleomorphic forms, including cells that are longer (8.0-12μm), involuted, S and V shaped, have rounded ends, or are branched may be present (Holt *et al.*, 1993; Lorenzen, 1994). No microcysts or resting stage have been observed. Electron micrographs of negatively stained *C. psychrophila* show a granular, crenated, rough appearance (Holt, 1993; Lorenzen, 1994), similar in their ultrastructure to *C. johnsonae* (Gorski, Godchaux, Leadbetter and Wagner, 1992) and *C. aquatilis* (Strohl and Tait, 1978).

Gliding motility is present in broth grown cultures and, although there is some strain variation in this ability, it is generally considered to be very slow and difficult to see, even after prolonged observation (Bernardet and Kerouault, 1989). The nature of the machinery responsible for gliding or spreading motility is not known (Gorski *et al.*, 1992). However features associated with the cell envelope in the genera *Cytophaga* and *Flexibacter* have been correlated with the ability to translocate over solid surfaces, without evidence of visible mechanism (Chang, Pate and Betzig, 1984;

Wolkin and Pate, 1985). Pate (1988), postulated that moving components of the cell envelope were responsible for maintaining the movement of an amorphous "slime" over the cell surface, and that interaction of this moving material with the substratum enabled the cell to translocate (Sorongon, Bloodgood and Burchard, 1991; Gorski *et al.,* 1992). The production of copious amounts of extracellular polysaccharides or "slime", potentially facilitating gliding motion and adhesion, has been demonstrated for many *Cytophaga*-like bacteria (Dalsgaard, 1993).

On *Cytophaga* agar (Anacker and Ordal, 1959), after incubation for 48-96 hours at 15-18°C, most colonies appear as yellow, glossy and convex, with regular margins (Bernardet, 1989). Occasionally colonies with thin, spreading edges are apparent (Holt *et al.*, 1993). *C. psychrophila* will grow within a temperature range of 4-23°C. Optimum generation times of 2 hours were achieved at approximately 15°C (Holt *et al.*, 1993). No growth can be observed in the presence of 2.0% NaCl, although the majority of isolates appeared to have a salt tolerance of between 0.8-1.0% (Pacha, 1968). According to Bernardet (1989) good growth occurs in *Cytophaga* broth (Anacker and Ordal, 1959) enriched with 0.5% tryptone. Several authors have reported difficulties in sub-cultivation of *C. psychrophila.* These problems have been attributed to the brand of beef extract used in the medium formulation. Lorenzen (1993), reported that the incorporation of semi-solid beef extract, supplied from Difco (Cat. No. 0126-01) into standard Anacker and Ordal (1959) *Cytophaga* agar resulted in augmented growth rates. It is far from clear why the lyophilization process should inhibit bacterial isolation, and the role of other factors such as strain variation and the nutritional requirements of this fastidious organism deserve investigation.

Biochemical Properties

Flexirubins can be demonstrated in most species of freshwater and soil *Flexibacter, Cytophaga, Sporocytophaga* and flavobacteria with low G+C content (Reichenbach and Dworkin, 1981), whereas they are absent in Gram-positive bacteria, flagellated bacteria, and marine *Cytophaga*-like bacteria, thus detection of these distinctive pigments forms reliable chemosynthetic markers for members of this group (Holt *et al.*, 1993). Holt (1987) considered that flexirubin-type pigments were not produced, but in a description of the species, Bernardet and Grimont (1989), showed

that non-diffusible flexirubin-type pigments were present in *C. psychrophila.* Lorenzen (1994), confirmed these findings, but suggested that young cultures should be used in these tests, as older populations of the bacterium (more than 3 days) produced weak and inconclusive reactions. Unlike *C. columnaris* and *F. maritimus,* colonies of *C. psychrophila* do not absorb Congo red, suggesting possible differences in the surface layers (Dalsgaard, 1993). Colonies are weakly catalase positive and nitrate is not reduced (Pacha, 1968; Bernardet and Kerouault, 1989; Lorenzen, 1994). The apparent decrease in reactivity of older bacterial cultures may explain the inconstant reports of cytochrome oxidase activity. Pacha (1968), Pacha and Porter (1968) and Holt *et al.*, (1993) reported that *C. psychrophila* was devoid of cytochrome oxidase, but Bernardet and Grimont (1989), Bernardet and Kerouault (1989), Bustos *et al.*, (1994), Lorenzen (1994) and Schmidkte and Carson (1994) reported varying degrees of activity from weakly positive (Bernardet and Kerouault, 1989) to readily detectable (Schmidkte and Carson, 1994). In accordance with Pacha (1968), Bernardet and Grimont (1989), Bernardet and Kerouault (1989) and Holt *et al.* (1993), reported that isolates did not possess the ability to produce hydrogen sulphide (H_2S). Conversely, in a characterisation study of the organism, Lorenzen (1994) described H_2S production. She suggested that this variability may be due to the increased sensitivity of filter papers impregnated with 10% lead acetate as recommended by the Public Health Laboratory Service compared with 1% incorporation endorsed by Pyle and Shotts (1980).

Isolates of *C. psychrophila* do not utilise either simple or complex carbohydrates (Holt *et al.,* 1993). Hydrolysis of cellulose, carboxymethyl cellulose, chitin, starch, esculin and agar is not evident, whereas gelatin, casein and tyrosine are hydrolysed (Bernardet and Grimont, 1989). Otis (1984) (cited in Lorenzen, 1994) described strains of *C. psychrophila* that produced extracellular enzymes capable of degrading collagen, fibrinogen, chrondroitin sulphate and fish muscle extract (Dalsgaard, 1993), although some strain variation in the proteolytic ability of the organism has been reported (Holt *et al.,* 1993). Griffin (1992) identified enzymes capable of degrading the complex connective tissue polysaccharides, chrondroitin sulphates A and C, and hyaluronic acid from pathogenic, but not from non-pathogenic strains of *C. columnaris.* Pacha (1968) described the actively proteolytic nature of *C.*

psychrophila, suggesting that this ability played an important role in the pathogenicity of the organism. The extensive tissue destruction and necrosis associated with infections of *Cytophaga*-like bacteria indicate that tissue destroying enzymes may be utilised by other fish pathogenic species (Dalsgaard, 1993).

Antimicrobial susceptibility tests carried out on 7 strains of *C. psychrophila*, isolated from freshwater fish suffering from a cold water type disease in North America and France, revealed a comparable sensitivity pattern. Isolates were resistant to gentamycin (15μg), neomycin (30μg), polymyxin B (30μg), and trimethoprim (5μg). Strains that exhibited inhibition zones of greater than 9mm were considered to be sensitive, and these were demonstrated with vibriostatic compound O/129 (500μg), ampicillin (10μg), cephalothin (30μg), streptomycin (10 IU), tetracycline (30 IU), chloramphenicol (30μg), erythromycin (15 IU), novobiocin (30 IU), and furans (300μg), (Bernardet and Grimont, 1989).

Pacha (1968) examined the antibiotic sensitivity profiles of 10 strains of *C. psychrophila* from silver and chinook salmon in the Northwest Pacific. Homogeneous susceptibilities were displayed for chlortetracycline, bacitracin, chloramphenicol, dihydrostreptomycin, erythromycin, neomycin, penicillin and tetracycline. Disparate sensitivity was seen to polymyxin B. The efficacy of antimicrobials against aquatic bacterial pathogens is frequently compromised by the rapid emergence of bacterial resistance (Alderman and Michel, 1992). More recently, antibiograms have indicated the appearance of resistance to sulphadiazine/ trimethoprim (Bustos *et al.*, 1994; Lorenzen, 1994; Schmidkte and Carson, 1994), phosphomycin, penicillin (Bustos *et al.*, 1994), and have shown considerable variation in the potential efficacy of oxytetracycline, oxolinic acid (Lorenzen, 1994) and ampicillin (Schmidkte and Carson, 1994).

Occurrence of Plasmids

Plasmid genes regularly encode "optional" characteristics which are selectively beneficial to bacteria in some environments but not others (Eberhard, 1989). The presence of plasmids in the fish pathogenic species *Vibrio anguillarum* (Crosa *et al.*, 1977), *Aeromonas salmonicida, A. hydrophila, Pasteurella piscicida, Yersinia ruckeri, Edwardsiella tarda* and *Renibacterium salmoninarum* have been

demonstrated (Toranzo, Barja, Colwell and Hetrick, 1983). However there is a paucity of information on the occurrence of plasmids in *Cytophaga*-like bacteria (Dalsgaard, 1993). In 1987, Holt described a medium weight plasmid in approximately 37% of strains of *C. psychrophila* isolated from coho salmon, cutthroat and steelhead trout exhibiting characteristic signs of bacterial coldwater disease (Lorenzen, 1994). In an examination of the plasmid profiles of Danish, North American and French isolates, Lorenzen (1994) visualised a similar molecular weight plasmid in strains from rainbow trout exhibiting typical signs of disease, but curiously found either two or a total absence of plasmids in isolates originating from fish without classical clinical signs. Evaluation of the possible relationships between virulence or antibiotic resistance and the presence of these plasmids was not undertaken.

Serological Studies

Pacha (1968) and Pacha and Porter (1968), using a slide agglutination technique, demonstrated that isolates of *C. psychrophila* shared common antigens, and were serologically distinct from other pathogenic *Cytophaga*-like bacteria and non-pathogenic myxobacteria. Holt *et al.* (1993), also using slide agglutination, indicated the presence of shared antigens in strains from New Hampshire, Michigan, Alaska and Oregon. However further experiments with absorbed sera revealed differences between some strains (Dalsgaard, 1993). In another study, *C. psychrophila* from diseased rainbow trout fry in Chile and Spain appeared to be serologically different from morphologically and biochemically analogous isolates from the USA. It was proposed that these strains belonged to a second serotype, expressing different surface antigen(s) to that of the type strain NCIMB 1947^{T}, which is characteristic of cold water disease-type infections in North America (Bustos *et al.*, 1994). In accordance with these findings Wakabayashi, Toyama and Iida (1994) found that *C. psychrophila* isolated from coho salmon, rainbow trout and ayu shared common antigen(s), however serological analysis with absorbed antisera and heat-stable antigens divided the strains into two distinct serotypes. Additional serogrouping studies were carried out by Lorenzen (1994) using both rapid slide agglutination and enzyme linked immunosorbent assay (ELISA) techniques, with which she determined that 3 serotypes were representative amongst 44 isolates of *C. psychrophila* from a variety of

freshwater fish species. No information currently exists in the published literature on inherent correlations between pathogenicity or epizootiology and serotype.

Research Objectives

The main objectives of this study were:

(1) To define rainbow trout fry syndrome (RTFS), establishing whether or not the disease was of a single aetiology or was a complex multi-factorial syndrome.

(2) To describe the histopathology of the condition at light and electron microscopic level.

(3) To establish minimum inhibitory concentrations of antimicrobial compounds against the organism(s) responsible, enabling a reduction in the reliance on, and misuse of, chemotherapeutic agents.

(4) To assess the epizootiology of RTFS.

(5) To evaluate alternative methods of mitigating losses from RTFS (vaccines, disinfection procedures, etc.) lessening the need for imported eggs which bring the concurrent risks of the introduction of exotic diseases and thus hazard the UK`s fish disease status.

CHAPTER 2

SURVEY OF RAINBOW TROUT FRY SYNDROME AT UK HATCHERIES

INTRODUCTION

As a result of the uncertainty surrounding the definition of RTFS in the U.K. the initial phase of this study commenced with a survey of representative co-operative rainbow trout farms to assess epidemiological aspects of the condition and to examine potential aetiologies. A range of preliminary short term investigations had previously been undertaken funded by the trout industry and feed manufacturers. The results of these studies implicated *Cytophaga* sp., however their findings were inconclusive and definite information was required to confirm the causal agent and to estimate the impact on future production of the trout industry. Consequently, with the co-operation of the British Trout Association (B.T.A.), the health status of rainbow trout fry at nine representative hatcheries across the U.K. was examined during 1992.

As clinical observations and gross pathological signs of disease are common to a number of bacterial infections (Austin and Austin, 1993) fry were screened for a range of bacterial fish pathogens including *Cytophaga psychrophila, Aeromonas hydrophila, Aeromonas salmonicida, Yersinia ruckeri* and *Renibacterium salmoninarum*. Additionally examinations for the presence of viral haemorrhagic septicaemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), and infectious haematopoietic necrosis virus (IHNV) were made using tissue culture methods. The possibility of the involvement of unknown viral inclusions was investigated using electron microscopy and the occurrence of any primary or opportunistic fungal or parasitic agents was recorded. The incidence of behavioural and physical changes potentially associated with the onset of disease was reported. Condition indices were also calculated as an indication of the nutritional status of the fish. In addition, to elucidate any commonality between factors predisposing fry to outbreaks of the disease, the managers/ owners of participating sites were asked to complete a detailed questionnaire (Figure 2.1) to gather information relating not only to the occurrence of presumptive RTFS, but also to the use of therapeutic treatments for the control of the disease.

Figure 2.1: Details of the questions contained in the Rainbow Trout Fry Syndrome (RTFS) questionnaire

Sample Site: **Date:**

1. Farm information:

a. What is the origin of the eggs?

b. Are the eggs from a photoperiod system?

c. Are incoming eggs disinfected on arrival?

d. Disinfectant used?

e. Is the egg incubation system, through-flow or recirculation?

f. What is the stocking density of fry?

g. Water source?

h. Are there any problems with water supply, i.e.

high suspended solids

pollutants

low dissolved oxygen content

other

i. What is the estimated water temperature?

j. Feed type?

k. Are the fish fed by hand or by automatic feeder?

l. Feeding rate?

2. History of rainbow trout fry syndrome:

a. Estimated first appearance of syndrome on farm?

b. Does every batch of fry show disease signs?

c. What is the frequency of the outbreaks?

3. Epizootiology:

a. Is the problem seasonal?

b. If so, when are the fry most noticeably affected?

c. At what size/age are the stock affected?

Figure 2.1 continued: Details of the questions contained Rainbow Trout Fry Syndrome (RTFS) questionnaire

d. What is the estimated water temperature during outbreaks?

e. Are there any indications of predisposing factors precipitating outbreaks?

f. Are there any other disease problems on the farm?

e. Do survivors exhibit resistance to further outbreaks?

4. Clinical signs:

a. What in your opinion are the typical behavioural disease signs:

lethargy

lurking at water outlets

reduced appetite

swimming close to intlets

other

b. What in your opinion are typical external clinical disease signs?

swollen abdomen

pop-eye

pale gills

death

other

c. What is the estimated time between the onset of these disease signs and death?

5. Prevention/ management of outbreaks:

a. What general methods are employed to prevent disease outbreaks:

use of prophylactic antibiotics, chloramine-T, disinfection, etc.

b. If an outbreak does occur what strategies are employed to reduce fry mortalities?

c. Any additional information.

MATERIALS AND METHODS

Participating Farms

The survey comprised nine rainbow trout hatcheries located in England, Wales and Scotland. Mortalities attributable to RTFS had been recorded at eight of these sites, whilst the ninth had no history of the condition. To respect the confidentiality of the farms involved they will be referred to as farms 1 to 9. Selection of the sites was made on the basis of their geographical location, and the owners/managers' willingness to submit voluntarily to an extensive examination of stock for both notifiable and non-notifiable diseases.

Fish

A total of 110 rainbow trout fry in the weight range 1.2-7.0g were randomly selected from each site between May and August 1992 and transported to the Fish Diseases Laboratory in aerated tanks. Bacteriological samples were taken from fifty fish, 30 were examined for virology and the remaining 30 were retained for light and electron microscopic analysis. The ambient water temperature was recorded at the time of sampling. Fork length and weights were recorded in order that condition index (CI) could be calculated according to the following equation: CI = weight/length^3. An examination was carried out to assess the frequency of external signs of disease. Fish were killed immediately prior to necropsy by severance of the spinal cord adjacent to the head and the entire surface of the fish was disinfected with 70% ethanol. The occurrence of ascites, splenomegaly and evidence of recent feeding was also recorded on ventral midline incision with a sterile scalpel blade.

Bacteriology

Loopfuls of kidney and spleen tissue were removed aseptically, inoculated and incubated as follows:- Anacker and Ordal agar (Anacker and Ordal, 1959), 15°C for 5-7 days for the enhanced recovery of *C. psychrophila*, ROD agar (Rodgers, 1992), at 22°C for 48 hours for *Y. ruckeri*, *Aeromonas* agar (Oxoid, Basingstoke, UK) at 37°C for 24 hours for the recovery of *A. hydrophila*, SKDM agar (Austin, Embley and Goodfellow, 1983) at 15°C for 6 weeks for the recovery of *R. salmoninarum,* and tryptone soya agar (TSA), (Oxoid, Basingstoke, UK), at 22°C for

48 hours, a general purpose medium capable of supporting growth of *A. salmonicida.* Resulting colonies were purified by streaking and re-streaking on fresh media before identification.

Identification of bacterial isolates

1. Gram reaction

Two to three colony forming units (cfu) were emulsified with 50µl of sterile distilled water on a microscope slide. The slide was then heat fixed by passing across a bunsen flame three times. It was then allowed to cool before the addition of 10% crystal violet in 95% ethanol for 1 minute. The residual stain was washed off with iodine, then flooded with fresh iodine (iodine crystals 3.3g, potassium iodide 6.6g, distilled water 1l) (Difco) for a further 1 minute. The iodine was removed by covering the slide with decolorizer (acetone 250ml, isopropanol 750ml) (Difco) followed by two washes in sterile distilled water. The slide was then flooded with the counterstain safranin (safranin O powder (pure dye) 4g, denatured ethanol 200ml, distilled water 800ml) (Difco) and left for 2-3 minutes. Excess stain was removed by washes in three changes of sterile distilled water. The slide was mounted in D.P.X. and left to dry before viewing under oil at 1000x magnification.

2. Motility test

Motility was studied using the hanging drop method. Soft paraffin was first applied to the four corners of a coverslip. Two colony forming units (cfu) were emulsified with 50µl of phosphate buffered saline (PBS) (pH 7.2) and the resultant suspension was dropped onto the coverslip. A microscope slide was then lowered onto the coverslip, and quickly inverted allowing the drop to hang from the coverslip. Bacterial motility was then observed using 400x bright field magnification.

3. Oxidase test

An oxidase strip (Unipath, Basingstoke) was placed in a clean petri dish and a heavy bacterial inoculum directly from a plate grown culture was smeared onto the end of the strip containing the oxidase reagent (N,N-dimethyl-1,4-phenylene

diammonium chloride). The strip was left for 30 seconds and examined for any colour change, deep purple/ blue indicated oxidation of the reagent and a positive.

4. Catalase test

The presence of catalase was tested for by the addition of 100µl-200µl of 15% H_2O_2 to 72-96 hour cultures.

5. Presence of flexirubin-type pigments

The presence of flexirubin-type pigments was demonstrated by flooding 72-96 hour plate grown cultures with 20% KOH (Reichenbach and Dworkin, 1981).

6. Absorption of congo red

The congo red reaction was performed by the method of McCurdy (1969), using 0.01% w/v congo red.

7. Enzymatic activity

The ability to hydrolyse 19 substrates was tested using API ZYM galleries (API-bioMerieux, U.K. ltd.) incubated at 17°C for 24 hours. Hydrolysis of any given substrate resulted in production of a given colour the intensity of which was scored on a scale from 1 to 5.

8. Whole cell slide agglutination

A whole cell agglutination test was performed on selected isolates using rabbit anti-*C. psychrophila* antisera (Chapter 8) and anti-*C.psychrophila* anti-sera provided by the National Serum Laboratory, Aarhus, Denmark. Isolates were tested after 72-96 hours incubation at 17°C. Fifty microlitres of a suspension containing $1x10^9$ cells/ml (od 0.8±0.02 at 520nm) was prepared in PBS and mixed with 10-20µl of antiserum diluted 1:8 in PBS, on a clean glass slide. The slide was observed over a black background, and agglutination within 10 sec was recorded as a positive result. *C. psychrophila* (isolate U239, Table 5.1, Chapter 5) and *Yersinia ruckeri* (isolate 1315, Table 5.1, Chapter 5) were used as comparative positive and negative controls in each case.

Virology

To examine for underlying infections with IPN, VHS and/or IHN, pooled samples of anterior kidney, spleen and encephalon (1.0g) from 30 fry were homogenised with sterile sand. This homogenate was diluted 1/10 in transport medium (Glasgow Modified Eagles Medium, (Glasgow MEM) MacPherson and Stoker, 1961) containing 10% new-born calf serum, penicillin (1000 IU/ml), streptomycin (1000 IU/ml) and nystatin (15 IU/ml)) at 1/10 and centrifuged at 2000g for 20min at 4°C. The supernatant was further diluted 1/10 and 1/100 in growth medium (Glasgow MEM containing 10% foetal calf serum, penicillin (1000 IU/ ml) streptomycin (1000 IU/ml) and amphotericin B (0.25μg/ml), was added to the supernatant to give final dilutions of 1/100 and 1/1000. Then 1.0ml aliquots of these final dilutions were inoculated onto bluegill fry caudal trunk (BF-2) and epithelioma papilloma carpio (EPC) cells and incubated at 15°C for 7 days, the appearance of viral cytopathic effect (CPE) was monitored daily. After 7 days the tissue cultures were frozen at -20°C then thawed to lyse the cells. Samples were pooled and re-inoculated onto fresh BF-2 and EPC cell lines at 1/100 and 1/1000 dilutions. The trays were incubated for a further 7 days, then if no viral CPE was detected after a total of 14 days the test was considered negative.

Histopathology

Light microscopic preparations were made to investigate any involvement of parasites. The preparation details are given in Chapter 7. Fresh spleen, kidney and blood smears were air dried, fixed in 5% acetic-methanol for 5 minutes and stained with haemotoxylin and eosin (H/E) (Appendix 3).

Material for light microscopic examination was fixed in 10% neutral buffered formalin (NBF). After fixation, samples were dehydrated via a ascending alcohol gradient, and embedded in paraffin wax with the aid of a vacuum infiltration processor (VIP 2000, Miles Laboratories Equipment). Blocks were sectioned at a thickness of 4.0-5.0μm using a Leitz rotary microtome, and sections were stained with haematoxylin (Shandon Activity No. 3) and eosin (H&E) or May-Grunwald Giemsa.

Primary fixation of tissues for electron microscopy was in 4.0% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 2 hours at 4°C, followed by several washes in 0.1M cacodylate buffer. Secondary fixation was carried out overnight in 1.0% osmium-tetroxide in 0.1M cacodylate buffer. The fixed tissues were then washed thoroughly in 0.1M cacodylate buffer and dehydrated through an ascending gradient of alcohols from 50% through 100%. After two changes of propylene oxide, as an intermediate solvent used to achieve miscibility with the resin, the tissues were embedded in an epoxy resin and cured at 60°C for a minimum of 24 hours.

Semi-thin sections (>1.0μm) were cut using a glass knife and stained with 1% toluidine blue in 1% aqueous borax. Selected tissue blocks were re-trimmed and ultra-thin sections were collected on uncoated copper grids, double stained with saturated aqueous uranyl acetate and Fahmy's lead citrate (Lewis and Knight 1977). The stained grids were examined in a 100CX electron microscope at 100kV.

Questionnaire to farmers

At the time of sampling each selected farm received a questionnaire comprised of 5 main questions (Figure 2.1). Managers/ owners were asked to complete the form and return it by post to the Fish Disease Laboratory. All of the participating farms returned the completed questionnaires within 14 days.

Statistical analysis

A students t-test was used to establish any correlation between the condition indices and recovery rates of bacteria from fish in the initial survey. The possible association between incidence of clinical signs and isolation of *C. psychrophila* was assessed using χ^2 tests. To determine significant differences amongst mortality in the infectivity experiments further chi- square tests were performed. All statistical analysis was carried out with the use of UNISTAT® version 3.0 for Windows.

RESULTS

Clinical and gross pathological signs

Only farms 1, 2 and 3 were suffering from significant mortalities attributable to RTFS during the sampling period. Farms 4, 5, 6, 7 and 8 had previously reported outbreaks of the disease, farm 9 had no record of the condition. The ambient water temperatures at the time of sampling ranged from 10.5 °C to 13.7 °C.

Despite the frequent emaciated appearance of diseased fry, post mortem recordings frequently revealed extensive deposits of visceral fat. There was no correlation between the condition indices and isolation of *C. psychrophila* from spleen tissue (Table 2.1). (overall incidence $t = 0.424$, $P > 0.01$; *** $t = 0.108$, $P > 0.01$; ** $t = 0.123$, $P > 0.01$; * $t = 0.112$, $P > 0.01$).

Table 2.1 Condition indices (± standard deviation (sd)) with isolation of *C. psychrophila* from spleen tissues of sampled fish

Sample site	Isolation of *C. psychrophila*				
	***	**	*	no detection	overall
1	1.16±0.17	1.12±0.21	1.22±0.16	1.26±0.34	1.21±0.19
2	1.17±0.24	1.09±0.13	1.15±0.1	1.17±0.21	1.14±0.17
3	0	1.15±0.11	1.11±0.08	1.06±0.11	1.09±0.1
4	0	0	1.36±0.36	1.15±0.22	1.17±0.23
5	0	0.87±0.11	0	0.91±0.08	0.91±0.08
6	0	0	1.17±0.00	1.21±0.24	1.21±0.19
7	0	1.36±0.00	1.19±0.00	1.06±0.25	1.06±0.24
8	0	1.02±0.05	1.09±0.00	1.06±0.1	1.07±0.1
9	0	0	0	1.21±0.31	1.21±0.31

***=confluent growth, **>12cfu, *<12cfu

Infected fish exhibited several consistent clinical disease signs (Table 2.2). Pale gills ($\chi^2=12.37$ $p<0.01$) and swollen abdomen ($\chi^2=17.1$ $p<0.01$) were highly significantly correlated with the recovery of *C. psychrophila* from spleen tissues. Exophthalmia ($\chi^2=2.47$ $p>0.01$), ascites ($\chi^2=4.73$ $p>0.01$) and darkening of the skin

(χ^2=2.94 p>0.01) were commonly observed but were not always associated with recovery of the bacterium. Internal changes such as splenomegaly (χ^2=29.57 p<0.01) and to a lesser extent anorexia (χ^2=8.25 p<0.01) were positively correlated with isolation of pure cultures of *C. psychrophila* from the spleens of fry.

Table 2.2: Incidence of disease signs against isolation of *C. psychrophila*

Disease signs	Farms								
	1	2	3	4	5	6	7	8	9
none	0,0	0,2	8,3	1,35	0,1	0,12	0,13	1,16	0,49
pale gills	3,8	26,7	0,0	1,2	2,33	0,5	22,2	3,13	0,0
swollen abdomen	23,4	7,4	1,0	0,1	0,4	0,4	0,1	0,0	0,0
exophthalmia	11,1	16,3	2,5	0,3	0,1	0,1	0,3	0,2	0,0
darkened skin	7,3	3,6	17,17	1,2	2,33	0,9	0,10	1,4	0,0
swollen spleen	32,10	36,5	6,4	3,2	2,30	1,24	1,17	2,15	0,1
ascites	31,8	6,1	0,0	0,1	0,10	1,3	0,3	0,1	0,0
not feeding	24,6	8,3	1,1	0,0	0,12	1,6	1,3	0,3	0,0

(isolation of bacterium, no isolation)

Bacteriological examination

No *Cytophaga*-like bacteria were recoverable from the kidneys of fry sampled in the present study. Yellow pigmented bacteria producing mainly smooth, glossy, compact colonies with regular edges but occasionally with uneven spreading margins were seen after 72-96 hours' incubation at 17°C from spleen inocula. Cells were filamentous and rod-shaped approximately 0.5µm in width and 3.5-7.5µm in length. They were Gram negative and exhibited weak gliding motility in hanging drop preparations. No growth was observed on TSA, ROD or *Aeromonas* agar, although scant growth was infrequently seen on SKDM. Bacteria were weakly catalase and cytochrome oxidase positive. Non-diffusible flexirubin-type pigments were produced and colonies did not absorb congo red. Hydrolysis of the following substrates was observed to a greater or lesser extent using API ZYM galleries: 2-naphthyl phosphate, 2-naphthyl butyrate, 2-naphthyl caprylate, 2-naphthyl myristate, L-leucyl-

2-naphthylamide, L-valyl-2-naphthylamide, 2-naphthyl phosphate and naphthol-AS-BI-phosphate. These characteristics were identical to Bernardet and Grimont`-s characterisation of *C. psychrophila* (Bernardet and Grimont, 1989). Agglutination was achieved for presumptive *C. psychrophila* against whole serum (See Chapter 8 for details of anti-*C.psychrophila* anti-sera production) with little evidence of cross reactivity with other members of the Cytophagaceae or *A. hydrophila.* No other bacteria of known fish pathological significance were isolated from the fish sampled. Results of the bacterial examination of fish spleens and morphological and biochemical characterisation of *C. psychrophila* are summarised in Tables 2.3 and 2.4.

Table 2.3: Isolation of *C. psychrophila* from the spleen of fry/ fingerlings

Farm	**Isolation of *C. psychrophila***			
	***	**	*	**no detection**
1	12	14	9	15
2	14	7	18	11
3	0	8	22	20
4	0	0	4	46
5	0	2	0	48
6	0	0	1	49
7	0	1	1	48
8	0	3	1	46
9	0	0	0	50

***=confluent growth, **>12cfu, *<12cfu

Table 2.4: Morphological and biochemical characterisation of *C. psychrophila*

Gram reaction	-
Gliding Motility	+
Catalase	+/-
Cytochrome oxidase	+/-
Presence of flexirubin-type pigments	+
Absorption of congo red	-
Hydrolysis of the following substrates: 2-naphthyl phosphate; 2-naphthyl butyrate; 2-naphthyl caprylate; 2-naphthyl myristate; L-leucyl-2-naphthylamide; L-valyl-2-naphthylamide; 2-naphthyl phosphate; naphthol-AS-BI-phosphate	
Resulting in the API ZYM profile:	523150003300000000

- negative reaction, + positive reaction +/- weakly positive

Virological examination

No cytopathic effects were seen in any of the cell lines used. Electron microscopic examination revealed no viral inclusion bodies within host cells.

Histopathological examination

Costia spp. was occasionally identified but no parasites of any pathological significance were seen. Light microscopic studies of fish from which *C. psychrophila* could be isolated showed identical pathology (Chapter 7). Electron microscopic examination of the spleen revealed numerous filamentous bacteria. Details of histopathological changes associated with RTFS are discussed later in this thesis (Chapter 7).

Results of questionnaire supplied to participating farms

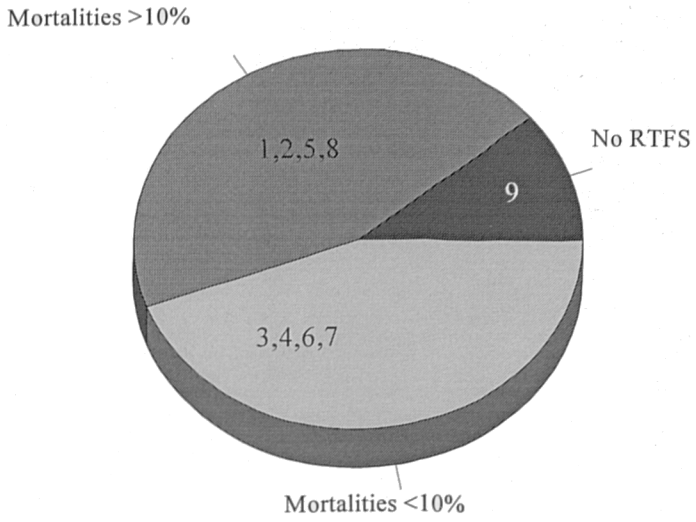
On the basis of the information gathered from the questionnaire survey the farms could essentially be divided into three categories (Figure 2.2):

(a) Those with no recorded history of RTFS

(b) Those with annual estimated mortality attributable to RTFS of< 10%

(c) Those with annual estimated mortality attributable to RTFS of >10%

Figure 2.2: Distribution of mortality across surveyed farms



Farm information

Farms 1,2,5,and 8 had experienced heavy recurrent losses attributable to RTFS in recent years, whereas farms 3,4,6 and 7 had recorded low level "grumbling" mortality. Farm 9 had no reported history of the condition. The sources of eggs for the surveyed farms are detailed in Table 2.5.

Table 2.5 : Egg sources for surveyed farms

Own (UK)	Other(UK)	Other (Europe)	USA	Southern hemisphere
1	1	1	1	2
7	2	8		4
9	3			
	5			
	6			

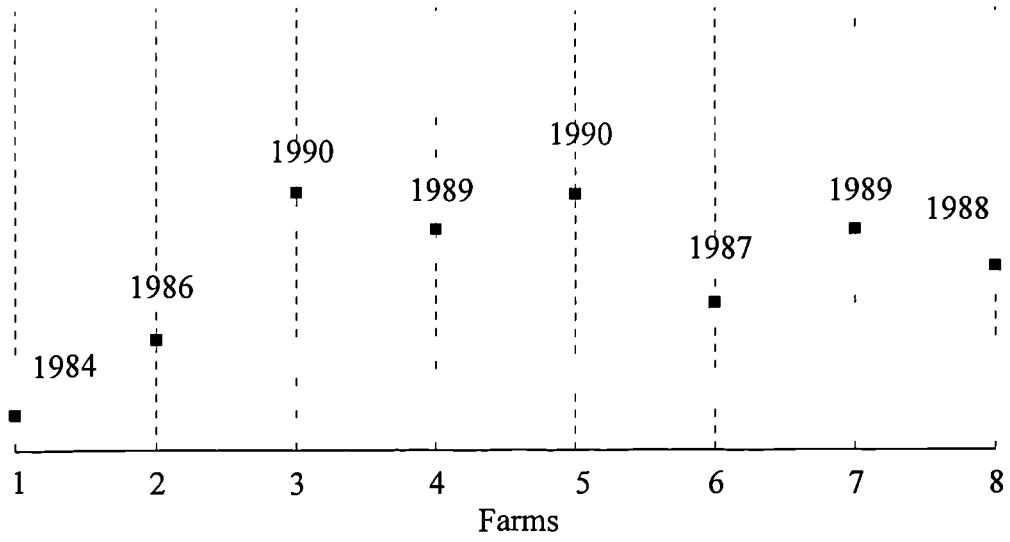
Farms 7 and 9 had been self sufficient in eggs over the last 10 years. Farm 1 had gradually been reducing its reliance on importations by using home produced broodstock, but due to the demand for year round fry output, had operated a photo-

period system to extend the production cycle. Farm 1 had previously received imported eggs from Denmark, Northern Ireland, North America and elsewhere in the UK to make up short falls in numbers. Farms 2,3,4,5,6 and 8 had obtained eggs from Denmark, Northern Ireland, Isle of Man, South Africa and Tasmania. All farms that received imports used an iodophor based compound such as Buffodine® or Wescodyne® to disinfect the egg surfaces on arrival according to MAFF instructions (MAFF, 1985). Farms 1 through 8 operated throughflow egg trough incubation systems, whilst farm 9 used upwelling incubators. Estimates of egg mortality to the eyed stage varied considerably from farm to farm, ranging from over 50% at farm 1 to negligible at farm 2. In general, approximately 30% losses were reported depending upon water temperatures, flow rates and egg densities. The egg holding facilities on all but 2 sites were spring fed, farms 5 and 9 received water from a borehole. No serious water quality problems were reported, although farm 1 recorded high $CaCO_3$ content and farm 4 recognised that low dissolved oxygen levels were an occasional problem. Water temperatures ranged from between 5°C and 16°C depending on the time of year and the distance from water source. Fry on all farms received a commercial diet fed according to the manufacturers' instructions. Fish were fed by hand at all sites except farm 4 where automatic feeders were employed.

History of RTFS on survey sites

The first appearance of RTFS on the surveyed farms is summarised in Figure 2.3. In all cases initial diagnosis was made by a veterinary surgeon, however the fastidious nature of the causal agent and uncertainties over its aetiology make accurate information on the emergence of the syndrome difficult to obtain. *Cytophaga psychrophila* was first isolated from fry showing clinical signs of RTFS in 1984 at farm 1, but on reflection it was felt that the disease had probably been present on this site since 1980. Farm 2 initially recognised the condition in 1986 but the remaining farms, 3 through 8, did not experience RTFS until the late eighties/ early nineties. There appeared to be little agreement between farms concerning frequency of outbreaks. Several reported that disease had occurred in every batch of fry whilst others believed that infection was more sporadic on their sites.

Figure 2.3 : First emergence of presumptive RTFS on surveyed farms



Epizootiology

Consideration of the results suggested that RTFS was not a seasonal problem insofar as it was not associated with a set of specific environmental factors, but that the increased frequency of outbreaks in the late spring was related to the increased numbers of fish at that time of year. Fish regularly became infected from <0.5g to 5.0g, farms 3 and 5 reported that outbreaks did not reoccur after an initial epidemic, but on farms 1, 2, 4, 6, 7 and 8 recurrence of the disease was apparent and the same batch of fry could be affected on 2 or 3 separate occasions. Generally, heaviest mortalities with fewer signs of disease were reported in the smaller fry (<0.5g), whereas larger fry and fingerlings appeared to exhibit a more chronic condition. One farm revealed that fingerlings sold on to ongrowing sites subsequently presented with severe external lesions which persisted in the fish up to market size. Water temperature appeared to play no significant role in either the frequency of outbreaks or the severity of the condition, although epizootics occurred more commonly in the spring and early summer when ambient temperatures were between 10.5°C and 15°C. Six of the farms felt that there were no identifiable elements that predisposed fry to disease. Farms 5 and 6 cited overcrowding, the presence of varied fish sizes,

increased suspended solid levels and general stress as factors influencing the onset of infection. No relevant concurrent disease problems were evident at any of the farms during the period of this study.

Clinical signs

The gross lesions and clinical signs described by all of the 8 sites that suffered from RTFS were consistent. Certain behavioural traits emerged as characteristic of the condition including disorientation, lethargy, increased respiration rate, swimming close to the water inlets/outlets and reduced appetite. Bilateral exophthalmia, pale gills, swollen abdomen, necrotic lesions extending through the epidermis to the muscular tissue and poor fin quality were reported as common indications of disease. Typically the estimated time from the onset of clinical signs to death was between 1 and 3 days.

Prevention and management of outbreaks

Farms recognised that successful disinfection could help mitigate the losses caused by a communicable disease, but in reality there was little evidence of effective sanitation. A number of preventative measures were however in operation at the affected sites. Where time allowed, tanks were pressure washed, and treated with super-saturated salt, hypochlorite solution or were sun dried between the introduction of batches of fry. However most farms admitted that frequently, during the busiest time of the year, it was not possible to clean the facilities adequately due to lack of time and space. None of the farms questioned had dedicated equipment for specific areas and although footbaths containing either iodophor or sodium hypochlorite existed at the entrance to the hatchery buildings at farms 1,4, 7 and 8 they were replenished so infrequently that it was unlikely that they were effective.

Chloramine-T (10ppm every 48 hours for 10 minutes) was used by all the farms that suffered from RTFS. In addition one (farm 5) occasionally treated with benzalkonium chloride, whilst another (farm 7) employed regular treatments of formalin (2.0ppm for 12 hours) and copper sulphate (0.01ppm for 12 hours). On 2 farms (farms 1 and 6), amoxycillin (75mg/kg/fish/day), oxytetracycline (300mg/kg/fish/day) and/or oxolinic acid (10mg/kg/fish/day) was administered

prophylactically in feed, although generally the dangers associated with prophylactic use of chemotherapeutics were well understood. Other measures employed by farms to prevent initial outbreaks included removal of moribund and dead fry, and reduction of feeding rates, fish handling, and stocking densities.

The currently licensed antimicrobial compounds were orally administered to fish on all farms where outbreaks of RTFS were seen. All the farms questioned that suffered disease problems attributable to RTFS (i.e. except farm 9) stated that they treated with oxytetracycline (up to 300mg/kg/fish/day for 10 days), and most believed that if treatments were initiated immediately at the onset of clinical signs they were successful. Only farm 1 had noticed a diminishing efficacy in oxytetracycline and had as a result recently switched to amoxycillin (up to 150mg/kg/fish/day for 7 days). Oxolinic acid (up to 50mg/kg/fish/day for 10 days) was used on 4 farms (farms 1,3 5 and 6) and potentiated sulphonamides (up to 150mg/kg/live weight/day) on one (farm 1) but with little discernible therapeutic benefit. The on farm orally administered chemotherapeutic dose levels are summarised in Figures 2.4 through 2.7.

Figure 2.4: On farm treatments with potentiated sulphonamides

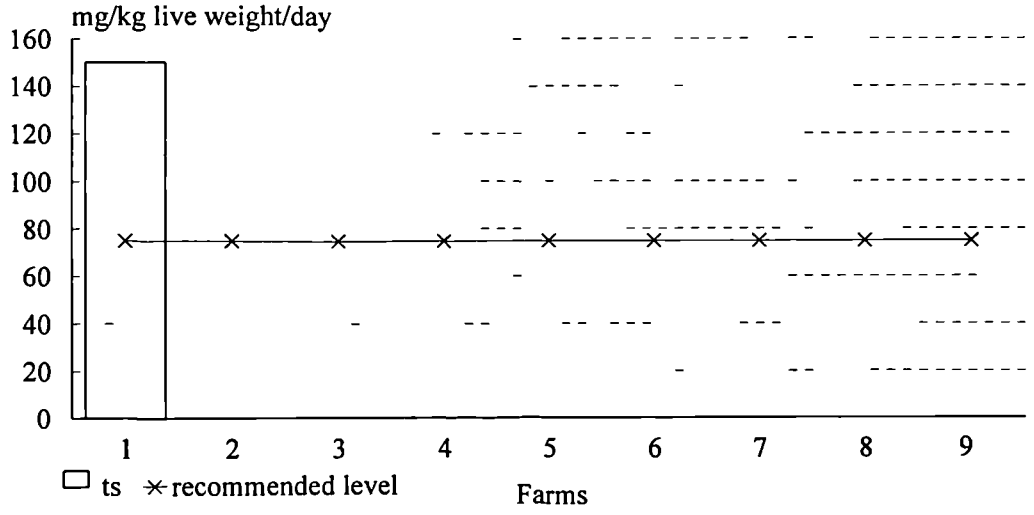


Figure 2.5 : On farm treatment with oxolinic acid

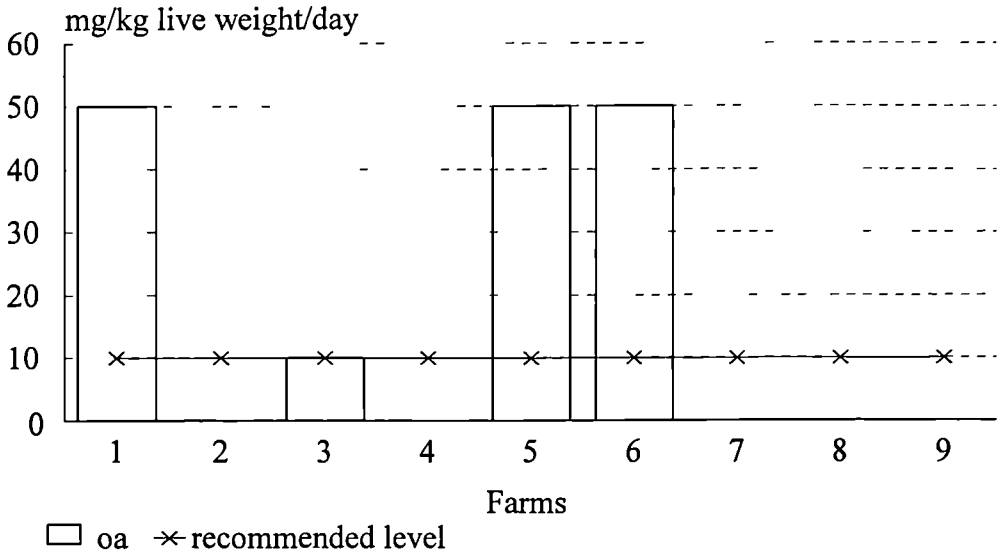


Figure 2.6 : On farm treatments with oxytetracycline

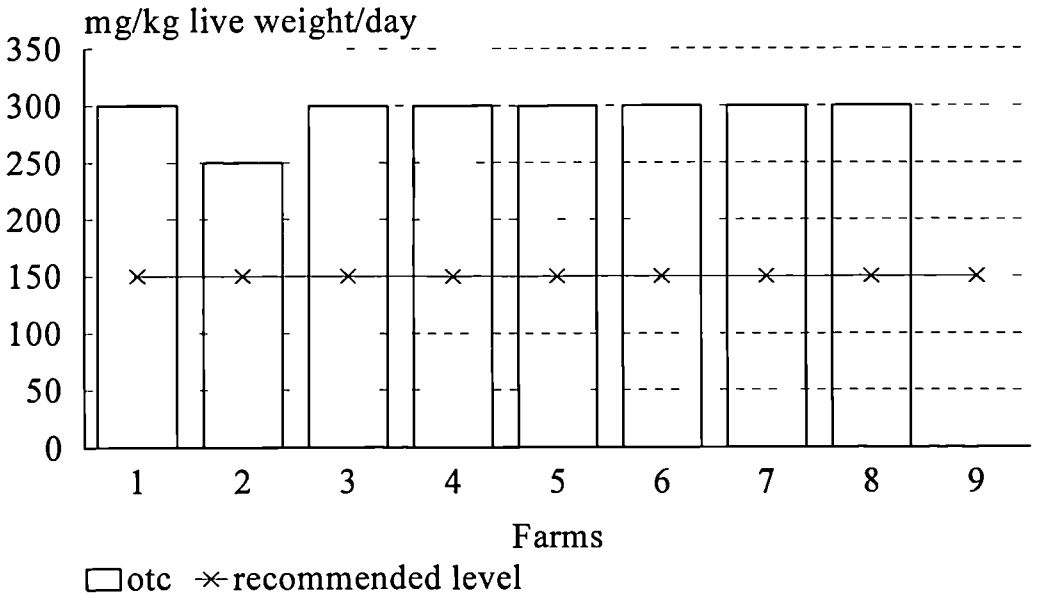
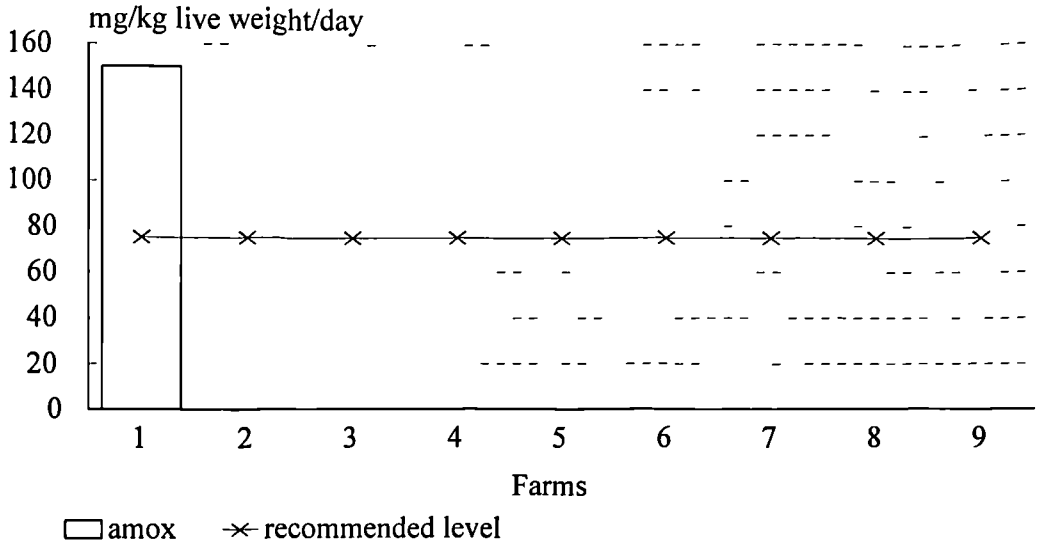


Figure 2.7: On farm treatments with amoxycillin



DISCUSSION

Bernardet, Baudin-Laurencin and Tixerant (1988) first described infections with *C. psychrophila* outside North America. Chronic mortality was recorded in juvenile farmed rainbow trout from several geographical locations in France. Typically in 0.2-1.0g fish, lethargy, inappetance, melanosis, ascites and exophthalmia were most commonly recorded, but clinical disease signs were inconsistent, with a variable degree of association between behavioural and physical changes and isolation of the bacterium. The most consistent gross pathological sign was reported to be severe hypertrophy of the spleen, often allied with discolouration of the liver. Numerous slender, filamentous bacteria were observed in spleen, kidney and less frequently liver tissues. Bernardet and Kerouault (1989) identified the bacterial isolates as morphologically, physiologically and biochemically similar to *C. psychrophila* (NCIMB 1947^T) reference strain from the National Collection of Marine and Industrial Bacteria (N.C.I.M.B., Aberdeen, Scotland) and to a strain of *C. psychrophila* from a diseased coho salmon in 1981. During the early 1980s an apparently comparable condition characterised by abdominal distension, melanosis, hyperplasia of the spleen and ascites was reported in rainbow trout fry in Germany causing 40 to 100% mortality in 2.0-5.0g fish (Weis, 1989). Additionally, Lorenzen,

Dalsgaard, From, Hansen, Horlyck, Korsholm, Mellergaard and Olesen, (1991) described an analogous disorder in fry and fingerlings in Danish fisheries implicating *C. psychrophila* in outbreaks of disease, and citing anaemia, exophthalmia, distension of the abdomen and melanosis as typical clinical signs.

In the present study anaemia, indicated by gill pallor, and distension of the abdomen were highly significantly related to recovery of *C. psychrophila* from spleen tissue. However, anaemias are frequently associated with diseases of fishes caused by a variety of aetiologies, including dietary deficiencies and heavy metal poisoning (Doimi, Bovo, Ceshia, Giorgetti and Saroglia, 1985) as well as a number of bacterial and viral infections (Schlotfeldt and Alderman, 1995). Distension of the abdomen, usually due to the accumulation of ascitic fluid, is a common clinical sign of numerous diseases of salmonids including infections with *Aeromonas hydrophila*, *A. salmonicida* (Austin and Austin, 1992), *Yersinia ruckeri* and VHSV (Schlotfeldt and Alderman, 1995). To a lesser extent bilateral exophthalmia and melanosis (darkening of the skin) were also closely correlated to isolation of the bacterium. Again both physical changes are associated with a range of bacterial and viral diseases (Rodgers, 1991; Schlotfeldt and Alderman, 1995) and therefore diagnosis of rainbow trout fry syndrome (RTFS) cannot be made on the basis of the presence of non-specific clinical signs and bacteriological identification of isolates is always required.

Characterisation of the isolated rod-shaped, yellow pigmented bacteria recovered from spleen tissue during the current survey showed them to be a very homogenous group, morphologically and biochemically identical to Bernardet and Grimonts (1989) description of *C. psychrophila* from outbreaks of disease in rainbow trout in France and closely related to *C. psychrophila* (NCIMB 1947^T). In accordance with this, *C. psychrophila* has been recovered from both cultured (Baudin-Laurencin *et al.*, 1989; Bernardet and Kerouault, 1989; Weis 1989; Lorenzen *et al.*, 1991; Santos *et al.*, 1992; Sarti *et al.*, 1992; Toranzo and Barja, 1993; Bustos *et al.*, 1994) and wild salmonid fish (Wiklund *et al.*, 1994) exhibiting similar disease signs. These findings indicate that an analogous condition, termed "cold-water disease", "low temperature disease", "visceral cold water disease", "fry mortality syndrome", "visceral myxobacteriosis", "rainbow trout mortality" and "rainbow trout fry

syndrome" (Baudin-Laurencin *et al.*, 1989; Bernardet and Kerouault, 1989; Lorenzen *et al.*, 1991; Santos *et al.*, 1992) is present throughout the major trout producing countries.

Primary isolation of *C. psychrophila* from the spleens of moribund fish in the present survey and subsequent sub-cultivation for several generations fulfil Koch's first and second postulates (Wilson and Miles, 1966) which state that:

1. A pathogen should be found in every instance of the disease and its occurrence in the host should be in coincidence with the lesions observed.

2. The organism should be grown outside the body of the host in pure culture for several generations.

However, reproduction of the disease in other susceptible fish is required before it can be definitely established that *C. psychrophila* is the primary causal agent of RTFS rather than an opportunistic secondary pathogen. Whilst no other bacterial, viral, parasitic or fungal causal agents could be identified, additional aetiologies or extraneous factors predisposing fish to disease outbreaks cannot be entirely discounted. As an example, chronic anaemia and myopathy caused by infections with *Vibrio salmonicida* in salmon were initially misdiagnosed as vitamin E, selenium deficiencies and/ or the result of too high a level of polyunsaturated fats in the diet. The *Vibrio* spp., isolated from moribund fish were frequently classified as secondary, opportunistic invaders. As a result "Hitra disease" in farmed Atlantic salmon (*Salmo salar*) was considered to be a more complex multi-factorial condition (Poppe, Hastein, Froslie, Koppang and Norheim, 1986). Nutritional deficiencies have been implicated in outbreaks of presumptive RTFS (Scott, 1989a; McLoughlin *et al.*, 1992) but extensive studies on feed formulation have failed to prevent outbreaks of disease in Denmark (Lorenzen, pers. comm.) and it is unlikely that a direct association between dietary deficiency and disease signs exists. Several farms reported that the presence of various stressors accelerated the onset of disease. Stress-mediated infections are common in hatchery production of salmonid fish where population densities are regularly high. Opportunistic fish pathogens such as *Aeromonas hydrophila*, *Pseudomonas fluorescens* and members of the family Cytophagaceae are common in water but infrequently cause disease unless the fish are stressed by excessive handling or crowding. Consequently, under over-crowded,

conditions bacterial gill disease (BGD) is widespread. However, if the stocking densities are reduced, BGD often disappears without recourse to therapy (Wedemeyer, 1970). Although two farms cited overcrowding as a contributory factor to the onset of disease no conclusive evidence of a positive relationship was forthcoming. Stocking densities at the disease free site were exceptionally high, with low water levels and rapid water exchange, as it was felt that this system reduced exposure to toxic metabolic products and suppressed territorial aggression which consequently reduced susceptibility to disease outbreaks. The susceptibility of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) to infections with *C. columnaris* are influenced by the age of fish, crowding and water temperature (Fujihara, Olson and Nakatani, 1971).

In the present survey, mortalities attributable to RTFS were related to the size or age of fry, and all affected farms reported that losses were most serious in the smallest fish (<0.5g). Fujihara *et al.*, (1971) stated that age rather than size was a major factor in resistance to *C. columnaris*, however Ellis (1988) reported that protective immunity in salmonids is a function of the size rather than the age of fry. It is not currently known if size/ age of fish plays any significance role in the onset of RTFS although all farms recorded a reduction in mortality in larger/ older fish.

Interrelations with other diseases may have synergistic effects upon mortality levels. Lorenzen (1994) reported that the gut parasite *Hexamita salmonis* was detected in over 50% of cases of RTFS in Denmark, suggesting as a result that more attention should be paid to prophylactic treatments to reduce the prevalence of potential interactive infections. In approximately 30% of RTFS outbreaks first diagnosed in France, IPNV was also diagnosed. In the current study no detection of other significant fish pathogens was possible. However, Fujihara *et al.* (1971) reported that gas bubble disease frequently resulted in secondary infections with *C. columnaris* which rapidly became the primary cause of mortality. Whilst infections with opportunistic pathogens may not be a prerequisite of outbreaks of RTFS the synergistic effects of subordinate infectious agents even at a sub-clinical level cannot be disregarded.

Results of the survey suggested that the initial outbreaks of RTFS corresponded with the importation of eyed ova from Europe and the U.S.A. which

farms reported during the early 1980s. *C. psychrophila* has been recovered from coelomic fluid and milt of sexually mature salmonid fish (Holt *et al.*, 1993) suggesting that broodstock may function as reservoirs of the pathogen. In addition, in an investigation into the effects of bacteria colonising rainbow trout eggs, Barker, (1989) demonstrated numerous *Cytophaga* sp. adhering to the surface. However no speciation of the group was attempted, and currently it is not known if *C. psychrophila* colonises the surface of salmonid eggs under hatchery conditions. Some authors (Borg, 1960; Bernardet, 1989, Holt *et al.*, 1993) have however postulated that *C. psychrophila* may be found attached to salmonid eggs. Disinfection of eyed ova on arrival at a hatchery should negate the risk of the transfer of communicable diseases. However, despite the awareness that iodophor disinfection of imported eggs could reduce the problems associated with infectious agents, this survey revealed that methods currently employed on farms had not prevented RTFS epizootics. Holt *et al.*, (1993) stated that exposure of ova to iodophors did not prevent CWD in hatching fry and furthermore the biocidal properties of the iodophors *in vivo* has been queried by various workers (McFadden, 1969; Ellis, Cotton and Khowaja, 1993). The intense traffic of salmonid eggs between USA and Europe may therefore be responsible for the onset of RTFS in the U.K.. Evaluation of the effectiveness of disinfection of eyed salmonid ova is required to assess potential problems associated with existing methods.

Information gathered from the present survey indicated that high levels of chemotherapeutic agents were frequently employed to mitigate losses from RTFS. In the U.K. only a potentiated sulphonamide, oxolinic acid, oxytetracycline and amoxycillin are licensed for use in aquaculture. The sulphonamides have been frequently recommended in the control of bacterial cold water disease and non-specific myxobacterial infections (Amend, Fryer and Pilcher, 1965 (cited in Amend, 1970); Wood, 1968 (cited in Amend 1970)). However no therapeutic benefit was reported for the potentiated sulphonamides in the current study. Equally oral administration of oxolinic acid was not successful in reducing mortalities. Oxytetracycline, incorporated into the diet at levels of up to 300mg/kg/fish, was the most frequently administered compound, but one farm reported a marked decrease in effectiveness of this agent in recent years. This is potentially indicative of an increase

in resistance to the compound and further studies are required to elucidate both the mechanisms and frequency of distribution of these isolates. Lorenzen (1994), showed that amoxycillin incorporated into the diet at 200g/25kg feed for 10 days reduced mortality levels in artificially induced outbreaks of RTFS. However, one farm in her study reported that outbreaks had not responded to treatment, again possibly due to the development of resistance within the bacterial population as a result of long term high dosage use of the compound. More data are required on antibiotic sensitivity profiles of *C. psychrophila* in order to minimise proliferation of resistance, and to prolong the life span of currently available drugs,

This survey confirms that in the UK., RTFS is a serious systemic disease of hatchery reared rainbow trout (*O. mykiss*), caused by the Gram negative, pigmented, filamentous bacterium, *C. psychrophila.* Information from other countries indicates that the same disease affects rainbow trout fry and fingerlings throughout Europe. It is difficult to assess the exact economic impact of RTFS on the British rainbow trout industry. Estimates of actual mortality levels vary from between 5 to in excess of 70% depending on the developmental stage of the affected fish. Despite the relatively low value of fry, the hidden costs of the syndrome are considerable. Problems associated with epizootics include the apparent increased susceptibility of fingerlings to other common diseases, shortages of fry causing the inability to meet customer demand, reduced customer confidence, inflated labour costs and greater expenditure on chemotherapy.

CHAPTER 3

ISOLATION OF *C. PSYCHROPHILA* FROM TWO FISH FARMS

INTRODUCTION

Aeromonas salmonicida is considered to be a true fish pathogen as it does not occur in the aquatic environment when fish are not present. In contrast many of the common saprophytic water bacteria including *Aeromonas hydrophila, Pseudomonas* spp., *Vibrio* spp., and certain *Cytophaga*-like bacteria may produce disease when fishes are compromised by adverse environmental conditions which affect the susceptibility of the fish, growth rate and pathogenicity of the bacteria (Snieszko, 1964). Members of the family Cytophagaceae, which include the *Cytophaga*-like bacteria, are known to form part of the normal aerobic flora of salmonid skin, gills (Pacha and Porter, 1968; Horsley, 1973; Trust, 1975), egg surfaces (Trust, 1975; Barker, 1989) and aquatic systems (Reichenbach, 1989). *C. aquatilis* and *C. johnsonae,* though considered part of the natural aquatic flora can also cause opportunistic infections in fish weakened by deleterious environmental conditions or other stress factors. Conversely, *C. columnaris* and *C. psychrophila* have been viewed as primary pathogens of fish as outbreaks of disease can occur with limited evidence of stress. Few bacteria that cause disease in fish are obligatory pathogens, and most are capable of independent existence outside the host (Roberts, 1991). Chowdhury and Wakabayashi (1988) showed that *C. columnaris* remained viable in variously formulated waters devoid of nutrients and, despite the absence of microcyst formation Pacha and Porter (1968) highlighted the ability of *C. psychrophila* to persist in a vegetative state throughout the year.

Whilst it is likely that the emergence of RTFS coincided with the introduction of exotic infectious agents from abroad, the natural reservoirs of *C. psychrophila* have not been determined, and in order that management practices can be maximised an understanding of the bacterium in the context of its environment is required. It would for example be of little benefit to disinfect premises thoroughly, to alleviate a disease

problem, if the causal organism was a ubiquitous environmental bacterium constantly replenished via the water supply.

Little information on the mode of transmission of *C. psychrophila* infections exists in the published literature. Borg (1960) was unable to reproduce horizontal transmission of cold water disease (CWD) unless the mucus or epidermis of experimental fish was damaged. He proposed that *C. psychrophila* was probably transported via eggs. In a survey of sexually mature salmonid fish, Holt *et al.* (1991) revealed the presence of large numbers of the organism in sexual fluids (38% of broodstock). They suggested that *C. psychrophila* may be associated with the salmonid egg and that CWD may be transmitted vertically from adult fish via contaminated eggs to alevins.

During the current investigation a detailed examination of water, sediment and surfaces was carried out across two spring fed fish farms in the south west of England. One of these had experienced heavy recurrent losses attributable to RTFS and one had had no history of the condition. Additionally, the incidence of *C. psychrophila* in the sexual fluids and on egg surfaces collected from fish at the infected site was investigated. The aims of the exercise were:

(1) To determine whether or not *C. psychrophila* was ubiquitous in aquatic systems or required introduction via either eggs, fish or equipment and to identify persistent populations of the organism in the fish farm environs.

(2) To examine the incidence and occurrence of *C. psychrophila* in the sexual fluids and on the egg surfaces.

Elevated levels of microbial flora occur naturally within the fish farm environment. At the outset of this work, it was recognised that, due to the fastidious nature and slow growth rate of *C. psychrophila,* detection of the organism *in situ* would be difficult. The current study represents a first attempt at environmental sampling and detection of *C. psychrophila* in the field. The problems associated with the isolation of a specific bacterium from areas that have intrinsically high microbial populations are discussed.

MATERIALS AND METHODS

Environmental sampling for *C. psychrophila*

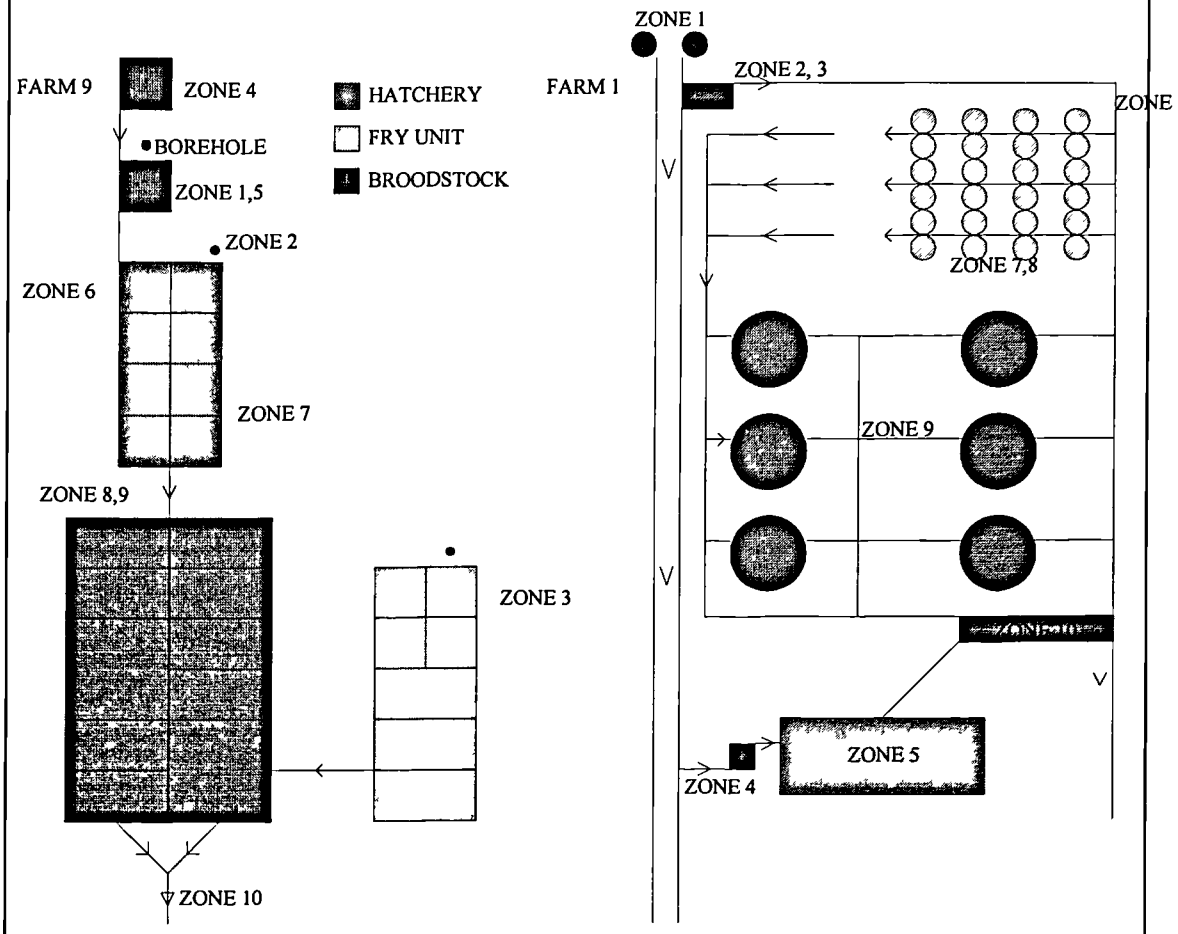
Participating farms

Two rainbow trout hatcheries in the south-west of the UK were selected for the initial phase of this study, and are known hereafter as farms 1 and 9 (Chapter 2). A survey of farm 9 had revealed neither presence of *C.psychrophila* nor any history of rainbow trout fry syndrome amongst stock. Conversely, bacteriological testing of fry and examination of records at farm 1, confirmed that the extensive losses experienced over recent years could be attributed to RTFS. Each farm was divided into 10 zones, (Figure 3.1), and from each zone samples of water, sediment and surface swabs were collected. Samples of ovarian fluid, milt and eggs were collected from farm 1.

Water sampling

A membrane filtration system was employed to detect *C. psychrophila* from water samples. The membrane filtration apparatus consisted of a base supporting 3 porous discs. The filter funnels, graduated at 50ml and 100ml were secured to the base by magnetic action. Three replicate aliquots of 5 and 50ml from each zone were filtered through pre-sterilised 0.45μm, grid-marked cellulose nitrate membranes (Whatman, England) using the three port filtration manifold connected to a vacuum source. Resultant membranes were inverted onto modified Anacker and Ordal agar (MAOA) (Lorenzen, 1993) (Appendix 1) and incubated at 17°C for 7 days. Additionally, undiluted and serial ten fold dilutions to 10^{-4}, of water from each zone were made and inoculated onto MAOA using the drop count method of Miles and Misra (1938). Subcultures of all yellow pigmented bacteria were purified by streaking and re-streaking onto fresh media.

Figure 3.1: Schematic diagram of the two farm sites



Zone	farm 9	farm 1
1	hatchery/fry tanks water source (borehole)	water source (spring)
2	ongrowing pond 1 water source (borehole)	inflow channel
3	ongrowing pond water source (borehole)	inflow channel
4	hatchery/ fry tanks	hatchery inflow
5	hatchery outflow	hatchery outflow
6	ongrowing pond 1	fry tanks inflow
7	ongrowing pond 2	fry tanks
8	ongrowing/ broodstock pond 1 outflow	fry tanks outflow
9	ongrowing/ broodstock pond 2 outflow	broodstock inflow
10	farm outflow	farm outflow

Sediment sampling

Three replicates of 1.0g sediment was shaken vigorously with 5.0ml of sterile river water onto a vortex stirrer for 3 minutes. Suspensions were centrifuged at 6000g for 5mins. Using the drop count method (Miles and Misra, 1938), undiluted and ten fold serial dilutions to 10^{-5} of the supernatant were inoculated onto MAOA. Again, subcultures of all yellow pigmented bacteria were purified by streaking and re-streaking onto fresh media.

Surface sampling

Where reasonable access was available, tank and pipe surfaces were sampled with cotton wool swabs (Urethral swab, Medical and Wire Equipment Company Ltd., England). Material was plated directly onto MAOA on the site. Plates were returned to the laboratory and treated as above.

Sampling of sexual fluids and egg surfaces for *C. psychrophila*

Ovarian fluid and milt sampling

Aliquots of ovarian fluid from 15 hen fish at the time of stripping, and milt from 3 cock fish, were serially diluted and inoculated using the drop inoculation method in 0.12ml amounts onto MAOA for the enhanced recovery of *Cytophaga* sp.. Plates were incubated for 7 days at 17°C. Resulting colonies were purified by streaking and re-streaking on fresh media before identification.

Egg sampling

Eggs were sampled from farm 1 at the green (unfertilised) stage and during incubation at 7, 14, 21 and 28 day intervals post-fertilisation. The first eggs of each fish were discarded to avoid contamination from the surrounding environment and ventrolateral surfaces of the female. The remaining eggs were maintained in up-welling incubators until the eyed stage (approximately 2 weeks post-fertilisation at 10°C), whereupon they were transferred to trays in the hatchery building. Water supplied directly from a spring was diverted through a system of baffles and

oxygenated by means of an air blower. The ambient water temperature remained at 10±0.1°C. At each sampling time, three separate batches of 10 eggs were collected at random and removed to the laboratory. Samples were rinsed in 4 changes PBS and added to a further 5ml of PBS, before shaking vigorously on a vortex stirrer for two minutes. The number of viable cells transferred to the media was determined by plate count, dividing by 10 gave the average count per egg and by taking into account the dilution factor and volume of the diluent, the number of cfu/egg could be estimated. Colonies with the main features of *Cytophaga*-like bacteria were purified by streaking and re-streaking on fresh media before identification.

Bacterial identification

A battery of primary tests was employed to identify presumptive members of the *Cytophaga/Flexibacter/Flavobacterium* complex, including morphology and pigmentation of the colonies, morphology and motility of the bacteria in wet mount preparations, growth at 32°C, Gram reaction, reactivity of the colonies with 10% KOH, catalase and oxidase reaction, and absorption of congo red. Isolates exhibiting the main characteristics of the selection criteria were further separated by the API ZYM system (API BioMerieux, Basingstoke) inoculated according to the manufacturers' instructions. Isolates giving the enzymatic activity patterns corresponding closely to Bernardet and Grimonts (1989), characterisation of *C. psychrophila* (Chapter 2) were re-streaked onto fresh media before confirmatory serological tests were carried out.

Slide agglutination test

Simple whole cell agglutination was carried out using antisera previously prepared in New Zealand white rabbits (Chapter 8) and anti-*C.psychrophila* provided by the National Serum Laboratory, Aarhus, Denmark. Colonies were collected from a 72-96 hour AOA plate and a suspension containing $1x10^9$ cells/ml (od 0.8±0.02 at 520nm) was prepared in PBS. A small amount (10-20µl) of antiserum was diluted 1:8 in PBS, and mixed with 50µl of the bacterial test suspension on a clean glass slide. The slide was observed over a black background and agglutination within 10 sec was

recorded as a positive result. *C. psychrophila* and *Yersinia ruckeri* were used as comparative positive and negative controls in each case.

Fluorescent antibody test

Confirmatory indirect fluorescence tests were carried out on presumptive isolates of *C. psychrophila.* One or two colonies from a 72-96 hour plate were suspended in PBS, 50µl of the suspension was fixed in acetic methanol on a Cookes slide (BDH, Poole Dorset, England) and air dried. The slides were then overlaid with 50µl of rabbit antiserum diluted 1:8 with PBS (either antisera prepared at the Fish Diseases Laboratory (Chapter 8) or anti-*C.psychrophila* provided by the National Serum Laboratory, Aarhus, Denmark) and incubated in a humid chamber for 30 minutes. Following a rinsing cycle (3x5min. in PBS), slides were incubated for a further 30min. In a humid chamber with fluorescein isothiocyanate isomer I (FII) (Sigma Chemical Company, Poole, Dorset, England) conjugated goat anti-rabbit IgG, at a working dilution of 1:40. After a second rinsing cycle (3x5min. in PBS), slides were blotted dry, mounted in glycerol (pH 9.0) and examined with a Leitz Dialux 20 microscope equipped with a 100w halogen lamp, at x40 and x100 fluorescence oil objective.

Additional Sampling of Ovarian fluid and egg surfaces

With the co-operation of the management and staff at farm 1, further weekly sampling of ovarian fluid and egg surfaces was carried out where possible between January 1993 and January 1995. Ovarian fluid was routinely collected from `ripe` hen fish immediately prior to manual egg stripping. Using sterile cotton wool swabs (Urethral swab, Medical and Wire Equipment Company Ltd., England) material was plated directly onto MAOA. Plates were returned to the laboratory, where they were incubated for 7 days at 17°C. Resulting colonies were purified by streaking and re-streaking on fresh media before identification as detailed above.

Eggs were sampled two weeks post fertilisation at the `eyed` stage, when `picking` took place (removal of non-fertile/dead eggs). Ten eggs were removed with sterile forceps and placed directly onto the surface of MAOA. The plates were either

refrigerated, returned to the laboratory immediately, or incubated on site at 17°C for 7 days. Colonies with the main features of *Cytophaga*-like bacteria were purified by streaking and re-streaking on fresh media before identification.

RESULTS

Environmental sampling for *C. psychrophila*

Water, sediment and swab samples

Numerous bacteria were isolated from the various zones across the two farms. Genera recovered included; *Aeromonas*, *Pseudomonas*, *Janithobacterium*, *Flavobacterium* and *Cytophaga/Flexibacter*. Of the original 446 yellow pigmented bacteria, 50 isolates (25 from each site) satisfied the primary selection criteria and were selected for inclusion in API ZYM galleries. Details of the zones from which the bacteria were recovered are shown in Tables 3.1 and 3.2. The enzymatic profiles did not conclusively speciate the 50 isolates evaluated, although 7(14%) appeared to be very closely related to Bernardet and Grimont's (1989) characterisation of *C. psychrophila*.

Table 3.1: Identification of 25 *Cytophaga*-like bacteria from farm 9

	Gliding motility	Catalase	Oxidase	Growth at 32°C
Zone 2. 1(sw)	+	+	+	+
2(sw)	+	+	+	+
Zone 3 1(sw)	+	+	+	+
Zone 4. 1(w)(10^{-1})	+	+	+	+
2(w)(10^{-2})	+	+	+	+
3(sw)	+	+	+	+
Zone 5. 1(w)(10^{-1})	+/-	-	+	+
2(w)(10^{-2})	+	+	+/-	-
Zone 6. 1(se)(10^{-2})	+	+	+	+
2(se)(10^{-1})	+	+	+	+
3(se)(10^{-1})	+	+	+	+
4(w)(10^{-1})	+	+	+	+
Zone 7. 1(se)(10^{-3})	+	+	+	+
2(w)(10^{-1})	+	+	+	+
3(w)(10^{-1})	+	+	+	+
Zone 8. 1(w)(10^{-2})	+	+	+	+
2(se)(10^{-3})	-	+	+	+
3(w)(10^{-1})	-	+	+	-
4(w)(10^{-1})	+	+	+	-
5(sw) -	+/-	+	+	+
Zone 9. 1(se)(10^{-3})	+	+	+	+
2(sw)	+	+	+	+
3(w)(10^{-1})	+/-	+	+	-
Zone 10.1(se)(10^{-3})	-	+ -	+/-	+
2(se)(10^{-4})	+	+ -	+/-	+

se=sediment sample, sw=swab sample, w=water sample

+ positive reaction - negative reaction +/- weakly positive reaction

Table 3.1 (continued): Identification of 25 *Cytophaga*-like bacteria from farm 9

	API ZYM profile	absorption of congo red	flexirubin pigment	Gram reaction
Zone 2. 1(sw)	4331332114221031200	+	-	-
2(sw)	433132210340000001	-	-	-
Zone 3. 1(sw)	4121322112201022201	+	+	-
Zone 4. 1(w)(10^{-1})	4221432124311043401	+	+	-
2(w)(10^{-2})	4111333104101240211	+	+	-
3(sw)	4331443214310043500	+	+	-
Zone 5. 1(w)(10^{-1})	4331443214310043500	-	-	-
2(w)(10^{-2})	4333443124300040000	-	-	-
Zone 6. 1(se)(10^{-2})	4232434234300020000	-	-	-
2(se)(10^{-1})	4233432243200030000	+	-	-
3(se)(10^{-1})	3223422324301020000	+	-	-
4(w)(10^{-1})	4342343233323032301	+	+	-
Zone 7. 1(se)(10^{-3})	5441432112310031300	+	-	-
2(w)(10^{-1})	4321432213312013000	-	-	-
3(w)(10^{-1})	4232532325401041401	-	+	-
Zone 8. 1(w)(10^{-2})	3221432113102012310	+	-	-
2(se)(10^{-3})	3221432113102012310	+	-	-
3(w)(10^{-1})	4332432113410140411	+	-	-
4(w)(10^{-1})	1221411111100000000	+	-	-
5(sw)	3321232122310200411	+	+	-
Zone 9. 1(se)(10^{-3})	4232333123201141300	+	+	-
2(sw)	4232333123201141300	+	+	-
3(w)(10^{-1})	2211222112100010000	-	+	-
Zone 10.1(se)(10^{-3})	3123334322200141000	+	-	-
2(se)(10^{-4})	3211232322100010300	+	-	-

se=sediment sample, sw=swab sample, w=water sample

+ positive reaction - negative reaction +/- weakly positive reaction

Table 3.2: Identification of 25 *Cytophaga*-like bacteria from farm 1

		Gliding motility	Catalase	Oxidase	Growth at 32°C
Zone 1.	1(w)(5ml)	+	-	+	+
	1(se)	+	-	+	+
Zone 3.	1(w)(5ml)	+	-	+	+
	2(sw)	+	-	+	+
	3(se)(10^{-1})	-/+	-	+	+
Zone 4.	1(w)(50ml)	-/+	+	+	-
	2(se)(10^{-2})	+	+	+	+
Zone 5.	1(w)(10^{-2})	+	-	+	-
	2(se)(10^{-2})	+	+	+	-
	3(se)(10^{-1})	+	-	+	-
Zone 6.	1(sw)	+	-	+	-
	2(w)(10^{-1})	-/+	-	+	-
Zone 7.	1(sw)	-/+	-	+	+
	2(w)(10^{-2})	-/+	-	+	-
Zone 8.	1(sw)	+	-	+	-
	2(sw)	+	+	+	-
	3(se)(10^{-2})	+	+	+	-
Zone 9.	1(se)(10^{-3})	-/+	-/+	+	-
	2(w)(10^{-2})	-/+	-/+	+	+
	3(w)(10^{-3})	+	-/+	+	-
Zone 10.	1(se)(10^{-3})	+	-/+	+	-
	2(se)(10^{-2})	+	-/+	+	+
	3(se)(10^{-4})	+	-/+	+	-
	4(w)(10^{-1})	+	-/+	+	-
	5(sw)	+	-/+	+	-

se=sediment sample, sw=swab sample, w=water sample

+ positive reaction - negative reaction +/- weakly positive reaction

Table 3.2 (continued): Identification of 25 *Cytophaga*-like bacteria from farm 1

Origin of isolate	API ZYM profile	absorption of congo red	flexirubin pigment	Gram reaction
Zone 1. 1(w)(5ml)	5330410205300030100	+	-	-
1(se)	4121442102100000300	-	+	-
Zone 3. 1(w)(5ml)	4131332314300000300	-	-	-
2(sw)	3121443003400031200	+	+	-
3(se)(10^{-1})	4131432104400000400	-	-	-
Zone 4. 1(w)(50ml)	4331445004501032300	+	+	-
2(se)(10^{-2})	4221231004212041401	-	+	-
Zone 5. 1(w)(10^{-2})	4331322103400000001	-	-	- *
2(se)(10^{-2})	4232434234300020000	-	+	-
3(se)(10^{-1})	4121432003311030300	+	+	-
Zone 6. 1(sw)	4330443004400030300	-	+	-
2(w)(10^{-1})	4441442004111030001	-	-	- *
Zone 7. 1(sw)	4231332423400040000	-	-	-
2(w)(10^{-2})	43313220124000000000	-	+	- *
Zone 8. 1(sw)	5110110011101010010	+	+	-
2(sw)	5120331004410000010	-	+	-
3(se)(10^{-2})	4120332112300010000	-	+	- *
Zone 9. 1(se)(10^{-3})	5121321224300000000	-	-	- *
2(w)(10^{-2})	4131443013400000000	-	-	- *
3(w)(10^{-3})	3231332011203022100	+	+	-
Zone 10. 1(se)(10^{-3})	4221551004400000000	-	+	- *
2(se)(10^{-2})	5341510044010000100	+	+	-
3(se)(10^{-4})	5230543005503000000	-	+	-
4(w)(10^{-1})	5341554024400030000	-	-	-
5(sw)	3330110001100020000	-	-	-

se=sediment sample, sw=swab sample, w=water sample

+ positive reaction - negative reaction +/- weakly positive reaction

*=isolates found to be positive for FAT

In the current study, results of whole cell agglutination (WCA) tests were inconclusive, however 7 isolates from 6 zones on farm 1 showed some evidence of agglutination, when compared to positive controls. No sign of cross-reactivity with other members of the *Cytophaga-Flexibacter* complex, used as negative controls was evident. The complementary FAT used in the present investigation indicated that the isolates from farm 1 shared common antigens with *C. psychrophila* from clinical cases of RTFS. The results of the WCA and FAT tests are summarised in Table 3.3.

Table 3.3: Identification of *C. psychrophila* on the two farms by WCA and FAT

Origin of isolate	farm 9		farm 1	
	WCA	FAT	WCA	FAT
Zone 1	-	-	-	-
Zone 2	-	-	-	-
Zone 3	-	-	-	-
Zone 4	-	-	-	-
Zone 5	-/+	-	-/+	+(1 isolate)
Zone 6	-	-	+	+(1 isolate)
Zone 7	-	-	+	+(1 isolate)
Zone 8	-	-	+	+(1 isolate)
Zone 9	-	-	+	+(2 isolates)
Zone 10	-	-	+	+(1 isolate)

- negative reaction +positive reaction +/- weakly positive

Sampling of sexual fluids and egg surfaces for *C. psychrophila*

Ovarian fluid and milt sampling

Bacteria were isolated from the ovarian fluid of 12 (80%) of the 15 fish during this trial. A total of 179cfu were isolated from the samples taken. There were pronounced differences in the bacterial loading between individual fish, however the genera isolated appeared fairly uniform, with *Aeromonas* sp. and *Pseudomonas* sp. presumptively identified as the most frequently occurring groups. A total of 28 *Cytophaga*-like bacteria was recovered from 5 (33.3%) of the fish sampled, generally

from more dilute aliquots of ovarian fluid. Isolates were purified by streaking and re-streaking on fresh media before identification. Two isolates corresponded exactly to Bernardet and Grimont's (1989) characterisation of *C. psychrophila,* and were biochemically and serologically identical to field isolates of *C. psychrophila* recovered from fry during naturally occurring outbreaks. Details of the characteristics of *C. psychrophila* are given in Chapter 2.

Numerous bacteria representing a variety of genera were recovered from the milt of fish in this study. Again *Pseudomonas* sp. and *Aeromonas* sp. were the most frequently isolated. However other species were identified, including *Streptococcus acidominus* and *Staphylococcus* sp. The fish pathogenic species *Aeromonas salmonicida* was isolated from one milt sample, although no clinical signs of furunculosis had been recognised in fish at this site. Problems with fungal contamination and bacterial overgrowth that potentially inhibited slower growing, more fastidious organisms such as *C. psychrophila* were observed in samples even with the highest dilution factors. The greater concentration of organisms recovered from milt samples compared to ovarian fluid may be due to contamination as a result of removal and subsequent handling of the testes necessary with sex-reversed fish (Barker, 1989), there was however little evidence of *Cytophaga*-like bacteria in any of the sample material, and no *C. psychrophila* was identified.

Egg sampling

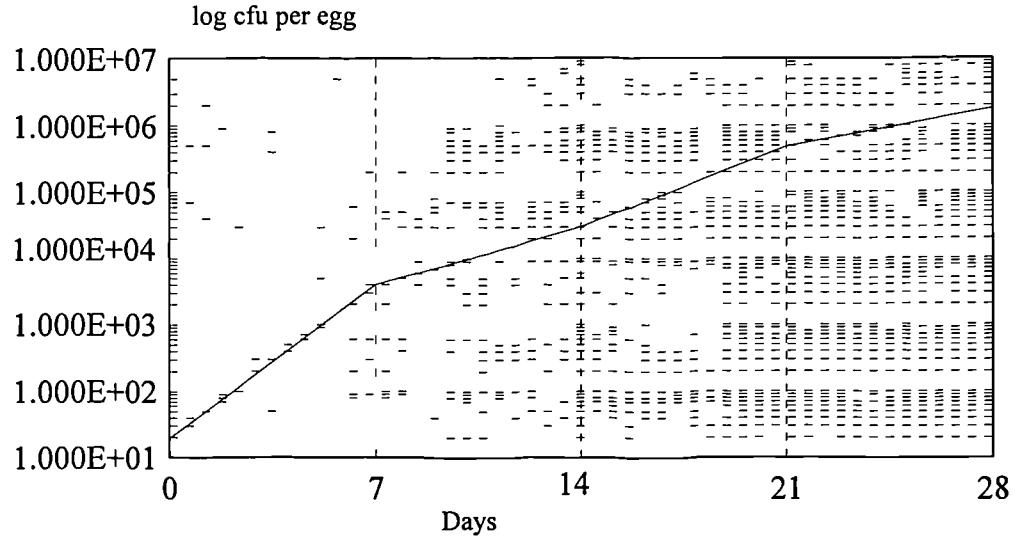
Analysis of the genera inhabiting the egg surfaces revealed that *Pseudomonas* sp., *Pseudomonas fluorescens* and *Cytophaga*-like bacteria formed the dominant flora. Few bacteria of any genera were found adhering to green (unfertilised) eggs but during incubation bacterial populations increased dramatically with time. The total numbers of bacteria isolated from the incubating egg surfaces at the 5 sampling times are given in Table 3.4.

Table 3.4 Estimated numbers of bacteria isolated from incubating egg surfaces

Day	*Cytophaga*-like bacteria	Others	Total
0	24	60	84
7	276	1979	2255
14	3196	4514	7710
21	5302	2850	8152
28	1879	1792	3671

The estimated numbers of colony forming units of *Cytophaga*-like bacteria recovered per egg over the 28 day hatching cycle are summarised in Figure 3.2. Isolates satisfying the primary selection criteria for *C. psychrophila* were purified as above, prior to confirmatory biochemical and serological testing. Speciation of the *Cytophaga*-like bacteria revealed that *C. aquatilis* dominated the egg surface. Only one isolate, recovered 14 days into incubation, corresponded to Bernardet and Grimont's (1989) characterisation of *C. psychrophila*, this represented just 0.0033% of the total *Cytophaga*-like-bacteria isolated from the 3rd sampling point. No further recovery of *C. psychrophila* was made during the study.

Figure 3.2: Estimation of log colony forming units of *Cytophaga*-like bacteria per egg during incubation under farm conditions



Additional sampling of ovarian fluid and egg surfaces

During the period January 1993-January 1995 ovarian fluid and eggs were taken from 360 fish. *C. psychrophila* was successfully isolated on 7 occasions, 4 isolates were recovered from ovarian fluid, whilst 3 originated from the egg surfaces. This low level of recovery represents just 1.9% of the total sampled group. In accordance with the studies described above, *Cytophaga*-like bacteria and *Pseudomonas* spp., predominated on the surfaces of eggs, whilst low levels of both *Aeromonas* sp., and *Pseudomonas* sp., were commonly recovered from ovarian fluid.

DISCUSSION

Salmonid eggs and fry require waters of the highest quality. For fish farming, ground water sources are normally preferred because of the constancy of temperature and freedom from disease or pollution (Bromage *et al.*, 1988). Information on the numbers and types of bacteria in the freshwater environment is essential if abnormal conditions, such as adverse water quality or the onset of favourable disease conditions, are to be recognised and corrected (Allen, Austin and Colwell, 1983).

Ground and spring waters generally contain fewer organic nutrients than surface waters because of the filter effect as the water seeps through the soil layers. As a result there are also low numbers of bacteria. Due to the close relationship between the bacterial flora of inland waters and soil it is not always possible to distinguish between limnic and terrestrial organisms, and most bacteria that occur in soils can also be recovered from spring and stream water.

In an evaluation of the numbers and diversity of bacterial species in ground waters, Rheinheimer (1992) isolated numerous organisms belonging to the genera *Achromobacter* and *Flavobacterium,* and smaller numbers of *Micrococcus* sp., *Nocardia* sp., and *Cytophaga* spp.. In a subsequent study of the aerobic heterotrophic bacterial flora of natural spring waters, Quevedo-Samiento, Ramos-Cormenzana and Gonzalez-Lopez (1986) confirmed that members of the phylogenetic group including the *Cytophaga-Flexibacter-Flavobacterium* complex were frequently present. However, as no speciation of the complex was attempted and as members of this group can also be frequently recovered from soil, it was not confirmed that they form part of an autochthonous aquatic population.

As water flows through the fish farm, the levels of available nutrients increase as a result of the metabolic process of the fish and from uneaten food. Rheinheimer (1992) stated that with eutrophication, the proportion of *Flavobacteria* and *Achromobacter* species diminished in favour of representatives of the Pseudomonadaceae, Bacillaceae and Enterobacteriaceae. Bell, Holder-Franklin and Franklin (1980) reported that the dominant component of the aerobic, heterotrophic bacterial flora in streams and rivers comprised Gram-negative, asporogenous rods, representatives of *Aeromonas, Pseudomonas* and Enterobacteriaceae, and the Gram-positive spore bearing rods, namely *Bacillus* spp..

In the present study no attempt was made to determine fluctuations in bacterial flora across the two sites, although in accordance with previous studies, members of the *Cytophaga-Flexibacter-Flavobacterium* complex, *Pseudomonas* sp. and *Aeromonas* sp. formed the dominant group of bacteria isolated from the two hatcheries. *Cytophaga*-like bacteria were recovered in large numbers from all zones tested, however, water samples from zones containing fish and consequently having the higher organic loading revealed an increased prevalence of *Pseudomonas* sp. and

other saprophytic bacteria. Overgrowth by these dominant organisms and by Oomycete fungi may have out-competed *C. psychrophila* and thus reduced the recovery rates.

In a study of myxobacterial populations of aquatic systems Carlson and Pacha, (1968) used antibiotic additives (Neomycin 5μg/ml and Cycloheximide 10μg/ml) in modified peptonised milk agar to suppress the growth of contaminating bacteria and aquatic moulds. By air-drying the agar plates (37°C for 24-72 hours) prior to inoculation Carlson and Pacha (1968) reported that they were able to reduce the tendency of pseudomonad and eubacterial colonies to spread over the surface of the agar and obscure myxobacterial growth. No attempt was made to speciate the *Cytophaga*-like bacteria during that study and therefore the frequency and occurrence of opportunistic and/or primary fish pathogenic *Cytophaga* sp. from environmental waters was not established.

Whilst the addition of antimicrobial compounds to suppress contaminants is widespread for diagnostic purposes, extensive use of chemotherapeutic agents in aquaculture has encouraged the localised proliferation of multi-resistant strains of both fish pathogenic and innocuous endemic bacteria. The addition of antimicrobial agents to media, have been used to enhance the recovery of specific *Cytophaga* species. By incorporating Neomycin (5μg/ml) and Polymyxin B (10units/ml) both singly and in combination into *Cytophaga* agar (Anacker and Ordal, 1959), Fijan (1969) increased the numbers of discrete colonies of *C. columnaris* recoverable from the gills of healthy channel catfish (*Ictalurus punctatus* Raf.). However minimum inhibitory concentrations of antimicrobials required for numerous strains of *C. psychrophila* revealed that, due to the substantial variation in resistance profiles, selective isolation by means of antibiotic additives to growth media was unlikely to be successful in separating individual species of the genera in this instance.

Confirmatory biochemical and serological tests were carried out on presumptive isolates of *C. psychrophila* from both sites. The API ZYM system for detection of enzymatic activity on various substrates has been used for identification of *C. psychrophila* from infected fish tissues (Bernardet and Kerouault, 1989; Lorenzen, 1994). The inability of either *C. psychrophila* or *C. columnaris* to degrade simple or complex carbohydrates separates the two primary freshwater pathogenic

Cytophaga species from other members of the genera, which lack pathogenicity or only cause opportunistic infections in immuno-compromised fish. However, the tests were originally designed for use in the medical field, and the strips of micro-tubules require incubation at a temperature of 37°C for 4 hours. Generally, Gram-negative bacteria isolated from fish have an optimum growth temperature of less than 30°C (Rodgers, 1991). *C. psychrophila* will not tolerate temperatures in excess of 25°C (Pacha, 1968; Holt, *et al.* 1993; Lorenzen, 1994) and consequently the manufacturers' criteria for using API ZYM enzymatic galleries are not satisfied. Accordingly, careful interpretation of the profiles is required.

Purification of the Cytophagaceae has presented great difficulties and whilst studies on morphology and general biological observations do not necessitate the use of single species (Stainer, 1942), investigations into nutritional and metabolic characteristics requires true breeding, pure cultures (Cowan and Steel, 1974). The inconclusive results obtained in the work presented here may therefore be explained by the only partial fulfilment of the test criteria and the presence of mixed cultures.

The sensitivity and specificity of serodiagnostic methods varies according to the quality of the anti-sera and diversity of expressed antigenic components of the test organism. Although techniques such as whole cell agglutination (WCA) and fluorescent antibody technique (FAT) are commonly used for the identification of fish pathogens, absolute reliance upon these methods is not advisable (Frerichs, 1991). Whilst for the most part the results of WCA and FAT were complementary, cross reactions with ubiquitous *Cytophaga* sp., or other saprophytic bacteria, and in the case of FAT, non-specific fluorescence, could not entirely be excluded. Additionally, Toranzo and Barja (1993), reported that serological analysis conducted by slide agglutination on *C. psychrophila* from Spain showed that both the whole cells and somatic "O" antigens failed to produce positive reactions with antiserum raised against the type strain NCIMB 1947^T. Further studies, on Danish isolates of *C. psychrophila* from fish with clinical signs of RTFS and CWD, demonstrated the presence of 3 serogroups, one comprising the majority of isolates, a second smaller group, and a third defined by the type strain NCIMB 1947^T (Lorenzen, 1994). In contrast Wakabayashi *et al.* (1994) indicated the presence of common antigens in *C. psychrophila* from coho salmon (*Oncorhynchus kisutch*), rainbow trout

(*Oncorhynchus mykiss*) and ayu (*Plecoglossus altivelis*) from Japan. However, on the basis of absorption analysis with thermostable antigens, two distinct serotypes were recognised.

In this study, polyclonal antiserum raised against a clinical field isolate of *C. psychrophila* from the UK and anti-*C. psychrophila* anti-sera supplied by E. Lorenzen (National Serum Laboratories, Aarhus, Denmark) was utilised in both serodiagnostic procedures. The reported variation in expressed antigens of *C. psychrophila* may have resulted in failure to identify every serogroup present.

In summary, the results of this study do not unequivocally demonstrate that *C. psychrophila* is not ubiquitous in aquatic systems but do show that detectable levels were found only at the site where RTFS caused persistent and significant mortalities in fry. Furthermore, identification of *C. psychrophila* was only possible in zones of this farm that either held or had been in contact with infected fish. On the basis of the available tests and in the absence of more specific screening techniques such as use of enzyme-linked-immunosorbent assay (ELISA) or polymerase chain reaction (PCR) it was concluded that cells of *C. psychrophila* did not enter the farms via the water supply. It is therefore tentatively suggested that in common with the majority of true fish pathogenic conditions, RTFS has been introduced into UK aquaculture and that, potentially, introduction was as a result of the transport of eggs and fry.

Holt *et al.*, (1993) reported that viable cells of *C. psychrophila* were recoverable from the milt of salmonid fish. It was not stated whether samples were taken from natural or sex-reversed females but, in the present investigation there was no evidence of *C. psychrophila* in milt sampled from sex-reversed fish. No natural males were available for study.

Ovarian fluid obtained with eggs from 12 out of 15 fish utilised during this trial was found to contain bacteria, predominantly representative of the genera *Aeromonas* and *Pseudomonas*, although from 5 (33.3%) *Cytophaga*-like bacteria were also recovered. Evelyn, Ketcheson and Prosperi-Porta (1984) demonstrated the presence of two species of Aeromonad in ovarian fluid from ripe coho salmon (*Oncorhynchus kisutch*) at the time of spawning. In a later study, Sauter, Williams, Meyers, Celnik, Banks and Leith (1987) found that *Aeromonas hydrophila* was the most abundant species present in the ovarian fluid of chinook salmon (*Oncorhynchus*

tshawytscha). Barker (1989) enumerated the bacterial populations present within ovarian fluid from ostensibly healthy rainbow trout. He found concentrations in the range of 10^1 to 10^2 cfu/ml and in accordance with previous studies, the dominant genera were *Aeromonas* and *Pseudomonas* though *Staphylococcus*, *Corynebacterium* and *Flavobacterium* were also identified. *A. hydrophila* and *P. fluorescens* are common inhabitants of freshwater and marine environments. Both species have been associated with mortalities in salmonid fish (Richards and Roberts, 1978), however in an examination of the potential aetiologies of unexplained fry mortalities Sauter *et al.* (1987), found little evidence that either bacterium was implicated.

Outbreaks of bacterial gill disease (BGD) in salmonid fish have been attributed to colonisation by yellow-pigmented *Cytophaga*-like bacteria, including *Flavobacterium* sp.. Whilst there exists some debate as to the relative significance of the environmental versus bacterial components of this disease, it is generally considered that the onset of BGD is commensurate with adverse conditions and that the *Cytophaga*-like bacteria found in association with opercular damage are opportunistic colonisers as opposed to primary pathogens.

Sauter *et al.*, (1987) were unable to isolate the fish pathogenic species *C. columnaris* or *C. psychrophila* from egg yolk and body fluid samples. They concluded that as no single microbial pathogen was recoverable, the causation of early lifestage disease in salmonids was multi-factorial. Whilst this is probably true in many cases, in the current study *C. psychrophila* was successfully cultured from the ovarian fluid of spawning fish. As previously discussed lack of selective, highly specific detection methods potentially limited the recovery rates but presence of the organism at detectable levels provided evidence for a possible mechanism of vertical transmission. In a survey of female chinook and coho salmon, Holt *et al.*, (1993) reported that the incidence of *C. psychrophila* within ovarian fluid was 38%. In contrast Lorenzen (1994) was unable to isolate the organism from farmed rainbow trout in Denmark. Conversely, by artificially infecting adult female rainbow trout 3 months prior to spawning, *C. psychrophila* was detectable in 20% of ovarian fluids sampled at the time of egg collection. Cone (1982) observed erythrocytes and unidentified phagocytic cells that he later defined as macrophages within the ovarian fluid of spawning rainbow trout. The low numbers of organisms in the ovarian fluid observed

in the present study may in part be attributable to the existence of phagocytic activity suppressing the bacterial populations in healthy fish. Extensive variation in mortalities from RTFS between batches of fry, observed in the field, may be partially attributable to the numbers of *C. psychrophila* within the ovarian fluid at the time of spawning, and consequently be a reflection on the health status of individual broodstock.

Currently, ovarian fluid from salmonid broodstock can be screened for *Renibacterium salmoninarum* by the use of ELISA and FAT (Elliott, 1987), and fish culturists have attempted to eliminate vertical transmission of bacterial kidney disease (BKD) by discarding eggs from broodstock that prove to be carriers of the pathogen (Brown, Ricks, Evelyn and Albright, 1990). However, due to its small size *R. salmoninarum* can be taken up by developing eggs during the process of vitellogenin incorporation by micropinocytosis (Evelyn *et al.*, 1984). Whilst it is theoretically possible that *C. psychrophila* (0.5μm-3.5-7.5μm) could enter the micropyle of rainbow trout eggs (the diameter of which is estimated at between 3.3-4.3μm (Riehl 1980)), and thus become internalised within the egg, Trust (1972) estimated that approximately $1.5x10^8$ bacterial cells were required to cover the entire surface of a single rainbow trout egg (and therefore the micropyle). Whilst conceivably, a few bacteria could penetrate the developing ova, the infective dose is not presently known and no information exists on generation times within host tissues. There is however no suggestion or evidence that *C. psychrophila* occurs in high numbers within the ovarian fluid of salmonid fish.

The low detectable levels of *C. psychrophila* from egg surfaces in the present study may have been another indication of the difficulties encountered in the isolation of fastidious organisms and in the speciation of the *Cytophaga-Flexibacter-Flavobacterium* complex. In concurrence with several authors, the dominant flora adhering to the egg shell were representatives of the Cytophagaceae and Pseudomonaceae. Adherence is a crucial but not exclusive prerequisite for successful colonisation of the egg surfaces (Barker, 1989). Significant differences in the cell surface hydrophobicity which in turn influence the adhesive properties of the *Cytophaga-Flexibacter-Flavobacterium* complex, have been demonstrated (Sorongon *et al.*, 1991). As no information on the specific capacity of *C. psychrophila* to bind to substrates compared with other *Cytophaga* sp. exists, no assumption that the

techniques utilised in this study removed all adhering cells of the organisms could be made.

Gee and Sarles (1942) reported that the causal agent of furunculosis in salmonid fish could be isolated from the shell but not from inside trout eggs. They concluded that eggs served as mechanical carriers of the bacterium, and that the transfer of contaminated eggs was one of the ways by which the disease spreads. In 1969, McFadden stated that sterilisation of hatchery water did not eliminate infections, passed from parents to eggs at the time of spawning, caused by *Aeromonas hydrophila* and he postulated that viable bacterial cells were carried on the outer surface of the egg shell. More recently, a newly defined *Flexibacter* species, *Flexibacter ovolyticus* has been isolated from the adherent bacterial epiflora of Atlantic halibut (*Hippoglossus hippoglossus* L.). Dramatic increases in mortalities at the hatching stage have been correlated to elevated levels of the bacterium on the egg surface (Hansen *et al.*, 1992).

Borg (1960) first referred to the conceivable relationship between *C. psychrophila* and salmonid eggs in the USA. He suggested that bacterial cold water disease was probably transported between States via eggs. Holt *et al.*, (1993), implied that the presence of the bacterium in large numbers in the sexual fluids of adult coho and chinook salmon indicated that it was associated with the egg. Lorenzen (1994) demonstrated the presence of viable cells of *C. psychrophila* from eggs collected from artificially infected rainbow trout broodstock, however the eggs in this study were homogenised and no information on the location of the bacteria was available.

This study provides the first suggestion that *C. psychrophila* can be isolated from rainbow trout egg surfaces incubated under farm conditions. Extensive further investigations are required to ascertain whether *C. psychrophila* harboured on the egg surface plays a significant role in either the mortality in hatching fry or transport of the RTFS. However the results of the present study together with the relatively recent emergence of RTFS in the UK and the intense trade of eggs between Europe and the USA indicate that *C. psychrophila* is an exotic fish pathogen and question the efficacy of on farm egg disinfection procedures.

CHAPTER 4

TRANSMISSION STUDIES ON *C. PSYCHROPHILA*

INTRODUCTION

Initial transmission studies had demonstrated that intraperitoneal challenge of rainbow trout with field isolates of *C. psychrophila* from outbreaks of presumptive RTFS in Scotland resulted in the reisolation of *C. psychrophila* and produced some typical gross lesions (Chua, 1991). In a concurrent study, Chua (1991) was unable to demonstrate significant mortalities or morbidities in 3.4-4.3g rainbow trout fry via bath challenge, but found that histological examination of the spleens of experimental fish revealed tissue disruption, intercellular oedema and evidence of resolution. He concluded that disease was established in surviving fish, and that they were in the process of defeating infection at the time of sampling.

Several authors have shown the ability of *C. psychrophila* isolated from clinical outbreaks of RTFS to produce similar disease signs in rainbow trout fry via the intraperitoneal route (Lorenzen *et al.,* 1991; Austin and Stobie, 1992; Bustos *et al.,* 1994). In addition, Lorenzen *et al.* (1991) indicated that intraperitoneal injection of homogenised spleen, kidney and heart tissues of infected fry produced clinical signs of RTFS in naive fry. However in accordance with Borg (1960), they were unable to produce evidence of horizontal transmission in a series of cohabitation experiments.

Little information exists in the published literature relating to the virulence of strains of *C. psychrophila.* Dalsgaard (1993) noted that the difference in the severity of the signs of cold water and peduncle disease depended upon the virulence of the pathogen. She suggested that in fish dying from infection with highly virulent strains less extensive lesions were evident. Pacha and Ordal (1970) reported similar findings from their studies on the pathology of columnaris disease in salmonid fish. Highly virulent strains of *C. columnaris* produced death without macroscopic evidence of gill damage, suggesting that the occurrence of gill lesions was not always indicative of the extent of infection in a population of fish. The route of infection was also found to be

important in determining the disease producing capacity of strains of *C .columnaris.* Strains displaying high virulence were found to produce disease more readily by the contact method than by either intramuscular or intraperitoneal routes, whilst intramuscular and intraperitoneal routes were more effective at causing disease in strains with low virulence (Pacha and Ordal, 1970). In a review paper, Holt *et al.* (1993) reported on the ability of 19 strains of *C. psychrophila* to produce disease in yearling coho salmon given subcutaneous injection of viable cells. The mortalities produced by the different strains varied from 0 to 100% but unfortunately no information on the original isolation or geographical location of the strains used was available.

In the present study, a series of transmission experiments was undertaken in an attempt to experimentally reproduce RTFS under laboratory and natural conditions. The initial aim of the study was to replicate the disease in susceptible animals i.e. rainbow trout fry, thus partially fulfilling Koch's postulates. Secondly, a series of transmission experiments utilising *C. psychrophila* originating from various geographical locations and representing the different serotypes of the bacterium (Chapter 8) was carried out. The aim of this second study was to establish that *C. psychrophila* from a range of geographical regions possessed the ability to produce a disease analogous to RTFS in susceptible animals, and additionally to determine potential contrasts in the virulence of isolates from distinct areas. Thirdly an attempt was made to infect fish with RTFS from the water via the in- and outflow of an infected site, farm 1 (Chapter 2), in order to provide further information on the ubiquity of the bacterium and its ability to infect fish under farm conditions.

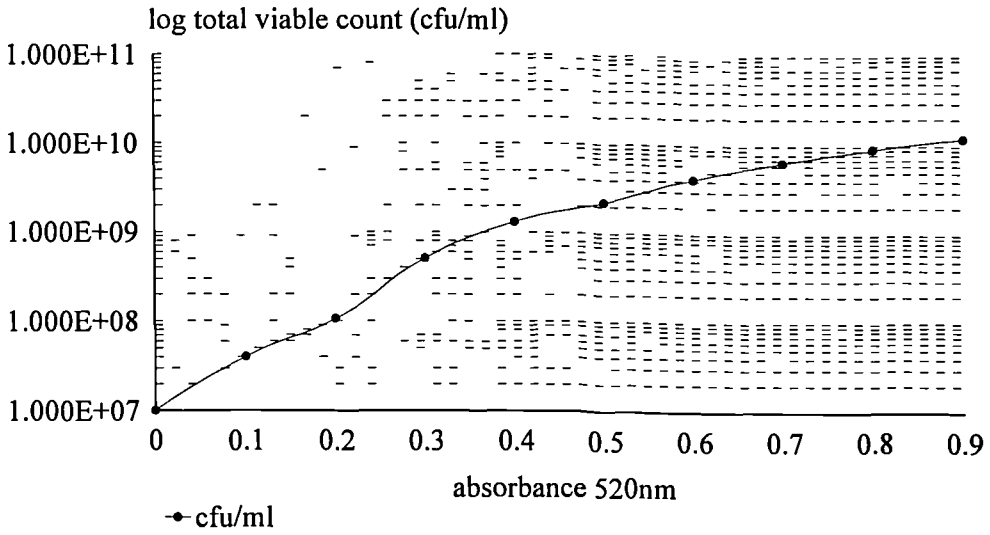
MATERIALS AND METHODS

Growth conditions for *C. psychrophila*

Modification of *Cytophaga* agar (Anacker and Ordal, 1959) (Appendix 1) by the incorporation of non-lyophilised beef extract (Difco, Cat. No. 0126-01) (Lorenzen, 1993), was used to produce good growth rates of *C. psychrophila* throughout this study. The medium is subsequently referred to as modified Anacker and Ordal agar/broth (MAOA/B).

A calibration curve from absorbance values and cell counts, using the counting chamber method (Collins and Lyne, 1985) (17°C, broth grown cultures) of several strains of *C. psychrophila* was calculated (Figure 4.1). This calibration curve enabled estimations of cell concentration from exponential phase broth grown cultures.

Figure 4.1 Standard curve for *C. psychrophila* optical density against viable cell count



Transmission experiments

The experimental protocols for the transmission studies in this work are summarised in Table 4.1.

Table 4.1: Summary of transmission experiments

Experiment no.	No. of fish	Average weight (g)	Source of fish	Inoculum	route	isolate
1	2x50	0.75g	farm 9	30µl of $1x10^7$cells/ml	IP	U239
2	2x50	0.75g	farm 9	30µl of $1x10^9$cells/ml	IP	U239
3	2x50	0.75g	farm 9	$2.5x10^5$cells/ml	5hr bath	U239
4	2x50	0.75g	farm 9	$3.2x10^5$cells/ml	10hr bath	U239
5	2x50	0.7g	Isle	20µl of	IP	1344

Table 4.1 (continued): Summary of transmission experiments

Experiment no.	No. of fish	Average weight (g)	Source of fish	Inoculum	route	isolate
6	2x50	0.7g	of Man Isle of Man	$1x10^8$cells/ml 20µl of $1x10^8$cells/ml	IP	FEL2
7	2x50	0.7g	Isle of Man	20µl of $1x10^8$cells/ml	IP	JO121
8	2x50	0.7g	Isle of Man	20µl of $1x10^8$cells/ml	IP	NL193
9	2x50	1.5g	Isle of Man	30µl of $1x10^8$cells/ml	IP	0273
10	2x50	1.5g	Isle of Man	30µl of $1x10^8$cells/ml	IP	1947
11	2x50	1.5g	farm 9	outflow water farm 1		
12	2x50	1.5g	farm 1	outflow water farm 1		
13	2x50	1.5g	farm 9	inflow water farm 1		
14	2x50	1.5g	farm 1	inflow water farm 1		

Statistical analysis

To determine significant differences amongst mortalities in the infectivity experiments chi-square tests were performed (Wardlaw, 1985). Statistical tests and methods are given in Appendix 4. All statistical analysis was carried out with the use of UNISTAT® version 3.0 for Windows.

Initial laboratory challenge studies (Experiments 1-4)

Experimental animals

Four hundred rainbow trout fry (weight range 0.5-1.0g) were randomly selected from farm 9, the site with no known history of RTFS. Fifty fish were assigned to each of eight 12 l tanks with through-flow water supply at a rate of 1 l/min, and were acclimatised for 4 days. The water temperature was maintained at between 10-12°C.

Preparation of Inocula

C. psychrophila isolated from spleen tissue of moribund fry at farm 1 (isolate no. U239) was grown on MAOA. Five colonies from a 96 hour plate were used to inoculate 100ml AO broth in 500ml flasks. After 72-96 hours at 17°C, cells were harvested by centrifugation (2000rpm (≈800g) 20 minutes at 4°C) and washed thoroughly in phosphate buffered saline (PBS). Bacteria were re-suspended in PBS to a final concentration of $5x10^9$ cells/ml. The number of cells in the inocula was adjusted to approximately $1x10^7$ cells/ml and $1x10^9$ cells/ml.

Intraperitoneal Challenge

Fish were anaesthetised with an alcoholic benzocaine solution (0.15g benzocaine in 3ml ethanol, added to 10l water). Two groups of 50 fry were intraperitoneally injected with 20µl of $1x10^7$ cells/ml, whilst 2 identical batches received 30µl of $1x10^9$ cells/ml. All injections were carried out using 25 gauge luer sterile disposable hypodermic needles with 1ml sterile single use syringes (BS5081) Four corresponding control groups received 30µl of PBS. Mortality and morbidity were monitored throughout the course of the trial.

Bath Challenge

The throughflow system was turned off and the tanks left static for 10 hours to allow deterioration of the water quality. The volume of water was reduced to 5 l. Twenty ml of bacterial suspension was added to 2 tanks each holding 50 fry. The throughflow system was re-started after 5 hours. The final concentration of bacteria in the bath immediately prior to re-starting the water circulation was calculated to be $2.5x10^5$ cfu/ml.

In a second bath challenge the protocol described above was followed but the throughflow system was re-started 10 hours after the introduction of 20ml of bacterial suspension. The final cell concentration was determined to be $3.2x10^5$ cfu/ml. In each case 2x50 fry received 20ml of PBS as control groups.

Further laboratory challenge studies (Experiments 5-10)

Experimental animals

To ensure that the rainbow trout fry used in the present study were free of RTFS, 15000 eyed ova were transported on ice from a disease free salmonid hatchery in the Isle of Man. On arrival at the laboratory all eggs were disinfected with iodophor at 100ppm for 10mins. Eggs were incubated in aluminium egg trays at low densities (a single monolayer of non touching eggs), the ambient water temperature during incubation was 10°C. After hatching, fry were fed a commercial diet (TROUW, UK) *ad libitum* until they were required for the trials. Two hundred fry were equally distributed into 4, 50l., throughflow tanks and acclimatised for 7 to 10 days prior to the start of each procedure. The water temperature was maintained at 10 ±0.6°C.

Preparation of Inocula

Information on the origin of the isolates of *C. psychrophila* used in the present study is given in Table 4.2 and in Chapter 5. Freeze dried cultures were resuspended in 100ml MAOB in 250ml conical flasks and incubated at 17°C for 72-96 hours. Cells were harvested by centrifugation at 2000rpm (≈800g) for 20mins. Pellets of bacteria were washed in 2 changes of 10ml PBS and resuspended in PBS to a final concentration of $1x10^8$cells/ml. Bacterial numbers in each inoculum were estimated by spectrophotometry, according to the standard curve for *C. psychrophila* (Figure 4.2). Fresh cultures isolated directly from infected fry were purified by streaking and re-streaking onto fresh MAOA. Several colonies from a 72-96 hour plate were then inoculated into MAOB incubated for a further 72-96 hours. Inocula were prepared as described above. Purity checks on the inocula were carried out in every case on MAOA incubated at 17°C for 7 days.

Table 4.2 Origin of isolates of *C. psychrophila* used in further laboratory challenge experiments

Isolate number	Origin of isolate	Source of isolate
1344	T. Wiklund, Institute of Parasitology, Abo Akademi University, Abo, Finland	*O. mykiss*, skin lesions (Finland)
0273	J. Carson, Fish Health Unit, Dept. of Primary Industry and Fisheries, Tasmania , Australia	*O. mykiss*, spleen (Tasmania)
NL193	N. Auchterlonie	*Salmo salar* parr spleen (Scotland)
1947^T	National Collection of Marine and Industrial Bacteria, Aberdeen	*O. kisutch* (USA)
JO121	Fish Disease Laboratory isolate Weymouth, England	*O. mykiss* fingerling (England)
FEL2	E. Lorenzen, National Serum Laboratory, Aarhus, Denmark	*O. mykiss* adult fish spleen (Denmark)

Intraperitoneal Challenge

Fish were anaesthetised with MS222 (Sigma, UK) diluted 1:10000 (w/v) in tank water and were injected whilst under the effect of the anaesthetic. In each case 2 groups of 50 fry received 20µl or 30µl of $1x10^8$cells/ml by parenteral delivery (Table 4.1). Two replicate control tanks were set up for each of the different experiments. Control fish were treated in an identical manner to the experimental animals receiving intraperitoneal injection of either 20µl or 30µl sterile PBS. All injections were carried out using 25 gauge luer sterile disposable hypodermic needles with 1ml sterile single use syringes. After challenge, ambient water temperatures and mortality/morbidity amongst fry were monitored twice daily. Dead and moribund fish were collected and examined for clinical signs of RTFS and the presence of the pathogen.

Sampling of control groups

At the end of each experimental procedure a representative 20% of the control fish were randomly chosen from each of the control tanks. Fish were killed by severing the spinal cord immediately behind the head with a scapel blade. The spleens were removed and plated onto MAOA, the plates were incubated at 17°C for 7 days.

Natural challenge of *C. psychrophila* (Experiments 11-14)

A field experiment was carried out in order to determine if RTFS could be transmitted to disease free rainbow trout fry via either the water in- or outflows of a heavily infected farm site (farm 1) (Chapter 2). Firstly, 110 fry (group A) (mean weight 1.5g) were transferred from a local disease free hatchery (farm 9) to the field site. On arrival fry were evenly distributed between two, 8.5l., fibreglass troughs. Additionally 110 rainbow trout (mean weight 1.5g) exhibiting no external signs of RTFS but reared at farm 1 (group B), were transported on a "dummy" journey under the same conditions as group A, to ensure that any stress associated with movement was replicated in the `home` produced fry. Prior to the start of the experimental procedure 10 fry from groups A and B were killed and spleen samples were inoculated onto MAOA. Plates were returned to the laboratory and incubated at 17°C for 7 days to check for the presence of *C. psychrophila*. On their return to the farm, 2 replicates of 50 fry were introduced into 2 identical fibreglass troughs. Water was pumped into the troughs directly from the outflow, which was situated immediately beyond the farm settlement area and received effluent from the entire farm. The water flow rate was approximately 0.2 litres/second. The ambient water temperature, mortality and morbidity amongst fry were recorded daily throughout the course of the trial. Dead and moribund fry were removed to the laboratory and examined for clinical signs of RTFS and sampled for the presence of *C. psychrophila*.

The experimental conditions of the inflow natural challenge trial were identical to the outflow trial except that troughs were fed with water pumped directly from the spring which supplied the farm. Any dead and moribund fry were returned to the laboratory and examined for clinical signs of RTFS and sampled for the presence of *C. psychrophila*. The trials took place over a 60 day period, during which time RTFS was frequently diagnosed in commercial fish across the site. On termination of

the trial surviving fish from each experimental category were killed and sampled for the presence of the pathogen.

RESULTS

Initial laboratory challenge studies (Experiments 1-4)

Experiments 1-2 were designed in order to partially fulfil Koch's postulates and as such represent a preliminary study into the transmission of RTFS. A highly significant 76% and 94% ($p<0.001$) fry died throughout the course of the trial. The results are expressed graphically in Figures 4.2-4.3.

Prior to day 3, moribund fry exhibited no gross pathological or behavioural disease signs. Subsequently, throughout the course of the experiment, post mortems of dead fry revealed severe splenomegaly, ascites and haemorrhage around the vent. Invariably the site of injection showed little evidence of healing. In one instance the viscera were observed protruding through the body wall. From day 5 onwards, all fry in the experimentally infected groups were lethargic, showed a tendency to congregate at the water inlets and air supplies, and exhibited little evidence of recent feeding. Towards the termination of the trial, two fish presented with saddleback type lesions on the dorsal surfaces. Pure cultures of *C. psychrophila* were culturable from the spleens and ascitic fluid of dead and moribund fry and post mortems of random samples of control groups revealed no evidence of either disease signs or bacteriological involvement.

A lower level, but significant ($p<0.01$; $p<0.001$), mortality was observed in the 2 bath challenged groups when compared with their corresponding controls, 30% (Figure 4.4) and 38% (Figure 4.5), but differences between the 2 challenges were not found to be significant ($p>0.1$). As in the initial intraperitoneal injection experiments, fish that died within the first 3-4 days of the challenge showed no clinical disease signs. Whilst most fry remained ostensibly healthy throughout, as the trial progressed, several showed little propensity to take food and were generally listless. Darkening of the skin, exophthalmia, reddening of the vent and swollen abdomens were manifest in the challenged groups. Spleens of dead and moribund fry were frequently enlarged and friable. Pure cultures of *C. psychrophila* were isolated from organs in accordance

with the observed lesions. After 15 days, 10 of the surviving fish were killed, and their spleens inoculated onto MAOA. Colonies of *C. psychrophila* were isolated from 6 of the fry sampled. No disease signs were observed in the control groups.

Figure 4.2: Cumulative % mortality resulting from IP challenge $1x10^7$ cells/ml

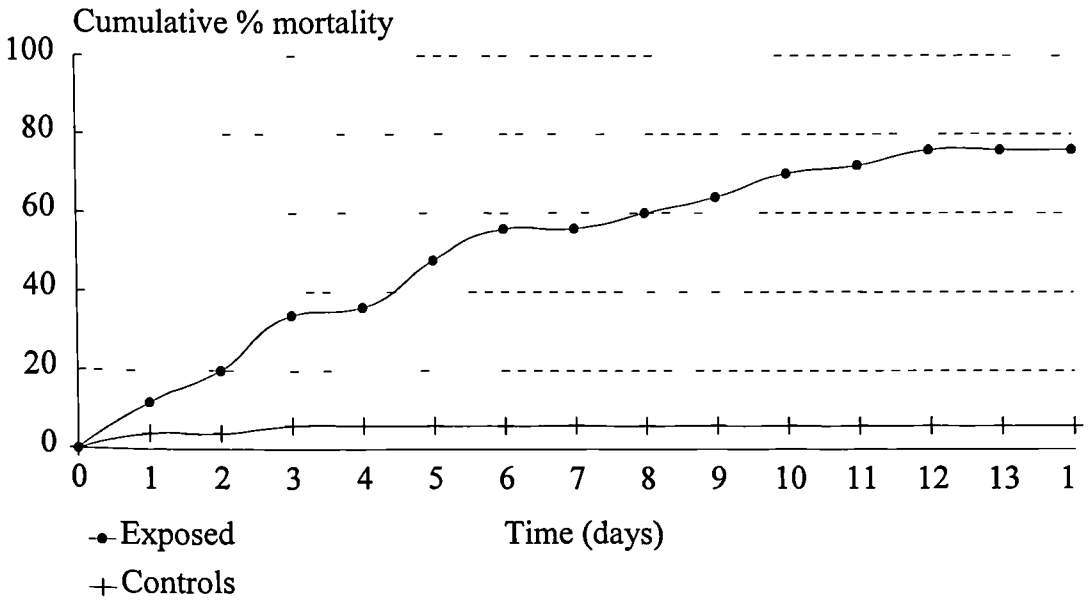


Figure 4.3: Cumulative % mortality resulting from IP challenge $1x10^9$ cells/ml

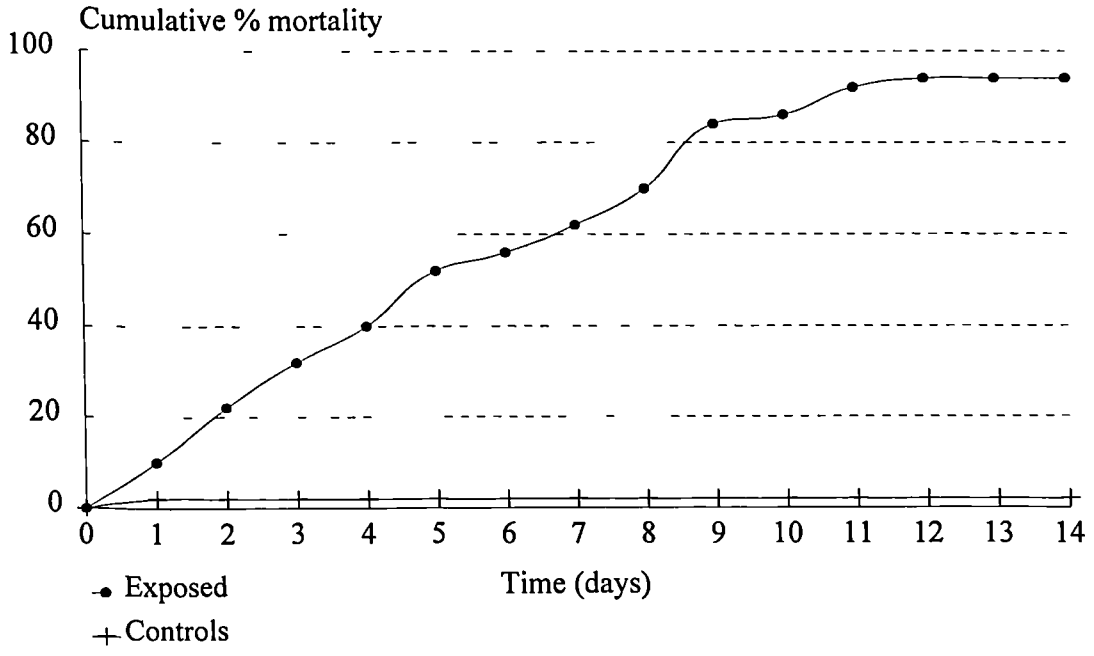


Figure 4.4: Cumulative % mortality resulting from 5 hours exposure to bath challenge

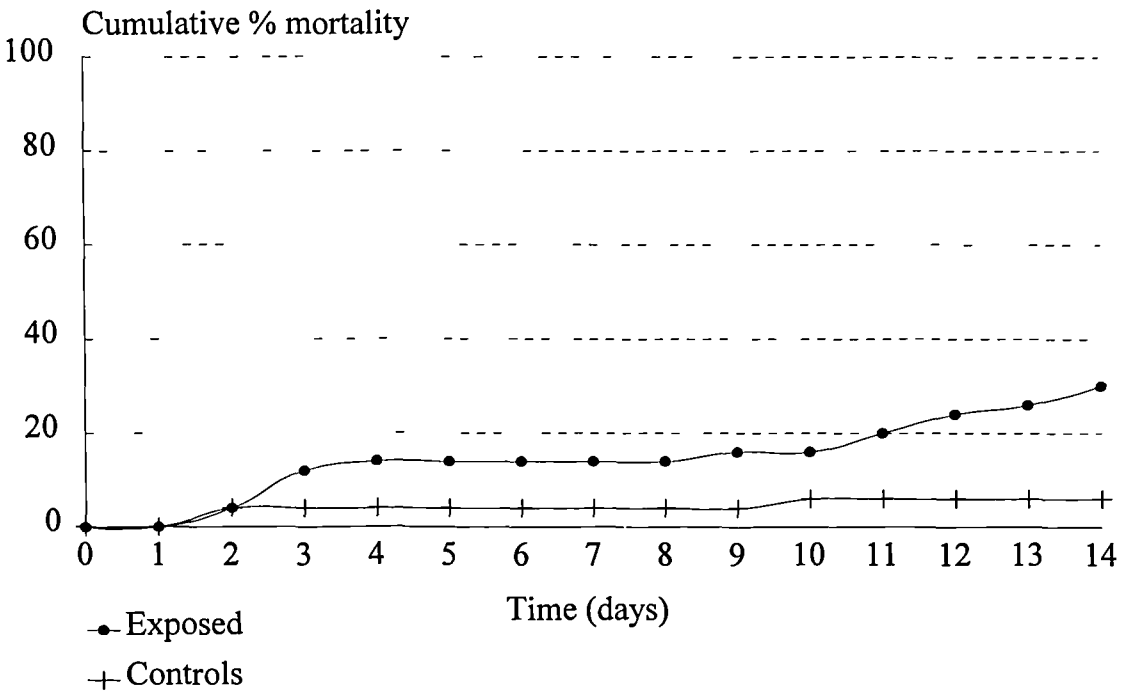
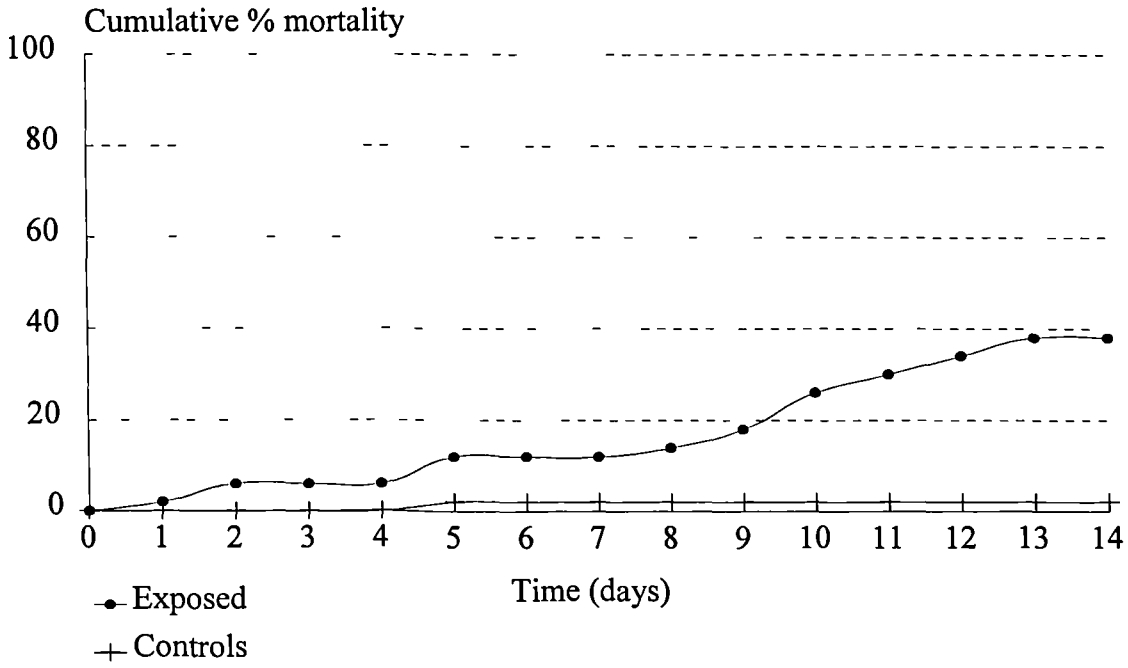


Figure 4.5: Cumulative % mortality resulting from 10 hours exposure to bath challenge



Further laboratory challenge studies (Experiments 5-10)

The percentage mortality caused by the 6 strains of *C. psychrophila* from the additional transmission studies (Experiments 5-10) varied from 17 to 74%. Analysis of differences in mortality between the experimental groups was highly significant ($p<0.001$). Fry injected with isolate 1344 exhibited the highest levels of mortality with over 50 % recorded within 6 days (Figure 4.6). The time between the onset of clinical disease signs and death varied from a few hours to 2 days. The rapidity of deaths in this trial necessitated that fish were monitored 4-5 times daily. Challenged fry showed classic clinical signs of RTFS, including lethargy, erratic swimming behaviour, absence of the feeding response, darkening of skin, bilateral exophthalmia and severe anaemia. One characteristic behavioural sign observed with this strain, but apparently absent in other IP challenges was the tendency of moribund fry to remain motionless on the bottom of the tanks. By day 20, 1 fish demonstrated erratic, spiral swimming behaviour.

Isolate 0273 produced 60% mortality over 25 days ($p<0.001$) (Figure 4.7). In this group, deaths occurred more gradually with just 12% recorded within the first 6 days post challenge. Clinical signs of disease were evident in fry dying after day 3. The time between the onset of clinical signs and death was approximately 2-3 days. Externally fish appeared dark in colour, showed severe bilateral exophthalmia and pale gills. Gill pallor was only recorded as a disease sign in moribund fry, as pale gills may have been associated with post mortem changes in experimental animals that had been dead for some time. The site of IP injection showed no evidence of healing and had become ulcerated in several surviving fry at the end of the experiment. It was however not possible to isolate *C. psychrophila* from these lesions.

Approximately 60% mortality was observed in fry injected with isolate NL193 over the course of the 25 day trial ($p<0.001$) (Figure 4.8) with an average of 28% within 6 days of challenge. Again no clinical signs of RTFS were observed in fish dying before day 3. Generally the time between the appearance of disease signs and death was 3-4 days. However, several fry developed signs of RTFS 3-4 days post challenge but survived to the termination of the experiment. Internally these surviving fry exhibited enlarged friable spleens, swollen, grey kidneys and severe anaemia. *C. psychrophila* was culturable from the viscera.

The NCIMB 1947^T type strain produced a lower level of mortality (mean 31%) ($p<0.001$) (Figure 4.9). Some clinical signs of disease analogous to those observed in fry diagnosed with RTFS were evident after day 3, however recovery of *C. psychrophila* from dead and moribund fry was not possible in every case.

Intraperitoneal injection of isolate JO121 caused mortality of 21% in each of the two replicate tanks ($p<0.01$) (Figure 4.10) over the 25 day trial. Behavioural and external signs of RTFS were evident in moribund fry 3 days post challenge, although no clinical disease signs were recorded in fish dying during 1 or 2 days after inoculation. Two fry became dark in colour within 72-96 hours of injection and exhibited behavioural signs of disease including lethargy and hanging at the sides of the tanks, however they survived until days 18 and 24 respectively.

The Danish isolate (FEL2) produced low level of mortality and morbidity in the experimental group (17%) ($p<0.01$) (Figure 4.11). Few internal or external signs of RTFS were evident, with the majority of fry dying with no evidence of disease.

Necropsies were carried out where possible on all dead and moribund fish. Internally, fish showing external disease signs indicative of RTFS presented with enlarged friable spleens, swollen, grey kidneys, whitish yellow mucoid discharge within the lower intestine and extensive hyperaemia of the posterior gut. In many cases, fatty adhesions extending throughout the viscera were evident. The livers of affected fish were often pale, with occasional haemorrhage on the surface of the organ. Petechial haemorrhage was frequently observed on the heart muscle. Virtually pure cultures of *C. psychrophila* were recoverable from swollen spleens and ascitic fluid after incubation on MAOA for 4-7 days from dead and moribund fish. However, bacteriological examination of dead and moribund from challenge groups infected with 1947 and FEL2 did not consistently reveal colonies of *C. psychrophila.* Post mortem examinations were carried out on 10 surviving fish from each experimental group and on 10 control fish. Examination of surviving fry in experimental groups 1344, NL193, 0273 and 1947^T revealed slight enlargement and congestion of the spleen, but no other indication of disease was apparent. Bacteriological sampling of the spleen revealed the presence of *C. psychrophila* from a number (1344, 9/10; NL193, 6/10; 0273, 6/10; 1947^T, 2/10) of these fish. Surviving fish from groups

JO121 and FEL2 showed no sign of disease and no isolation of the organism was possible. At necropsy there was no evidence of disease in the control groups.

Natural challenge of *C. psychrophila* (Experiments 11-14)

Isolation of C. psychrophila prior to natural challenge trial

Results of the ten fish sample from experimental groups A and B are detailed in Table 4.3. No isolation of *C. psychrophila* was possible from the spleens of fry taken from the disease free site (farm 9). *C. psychrophila* was recoverable from spleen samples from 4 of 10 fry used in the outflow trial and 3 of 10 used in the inflow trial.

Table 4.3 Isolation of *C. psychrophila* prior to the natural challenge experiments

	Fish									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Group A outflow farm 9	-	-	-	-	-	-	-	-	-	-
Group A inflow farm 9	-	-	-	-	-	-	-	-	-	-
Group B outflow farm 1	+	+	+	+	-	-	-	-	-	-
Group B inflow farm 1	+	+	+	-	-	-	-	-	-	-

Figure 4.6: Cumulative % mortality resulting from IP challenge with isolate 1344

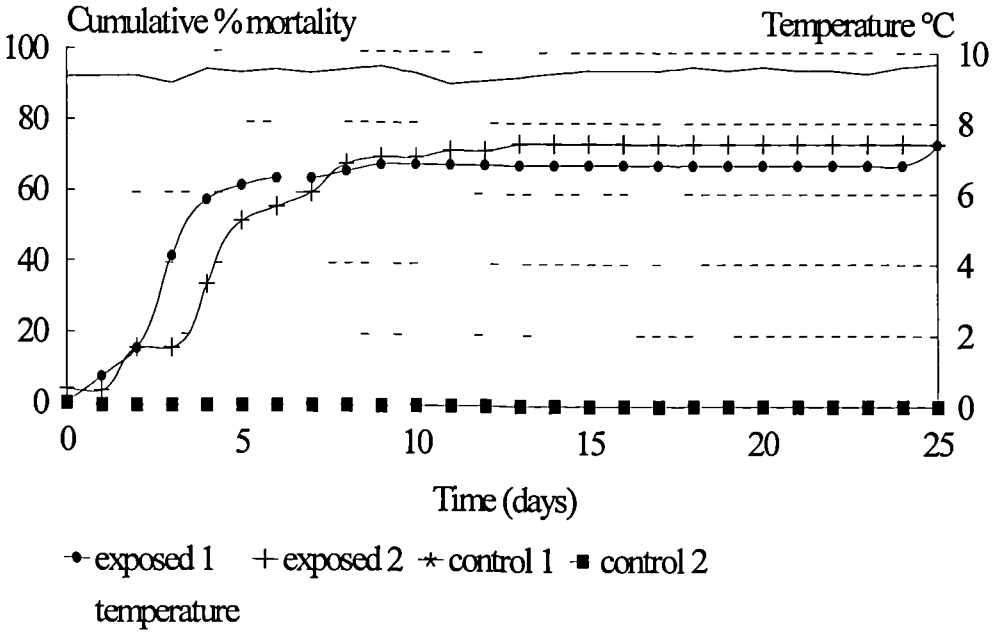


Figure 4.7: Cumulative % mortality resulting from IP challenge with isolate 0273

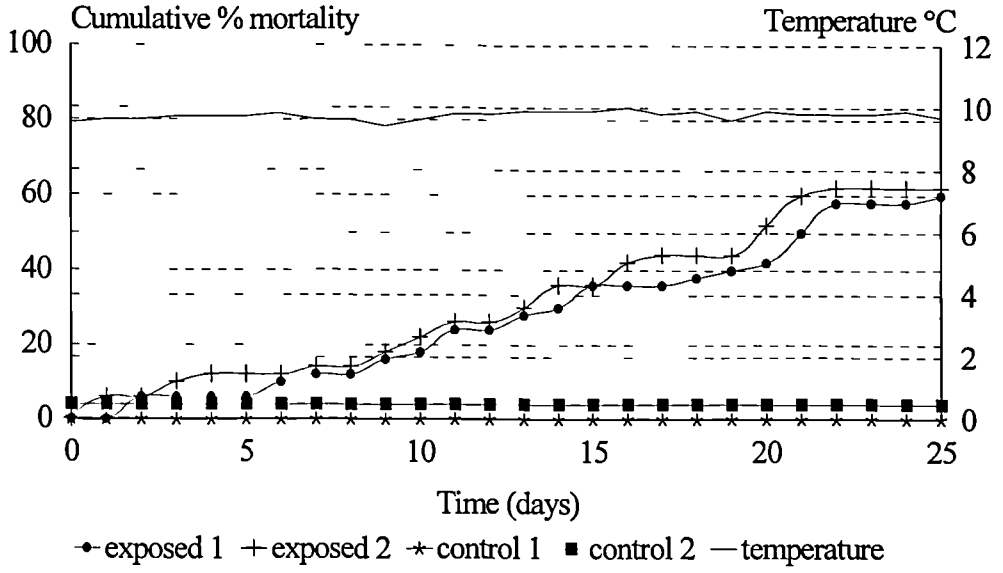


Figure 4.8: Cumulative % mortality resulting from IP challenge with isolate NL193

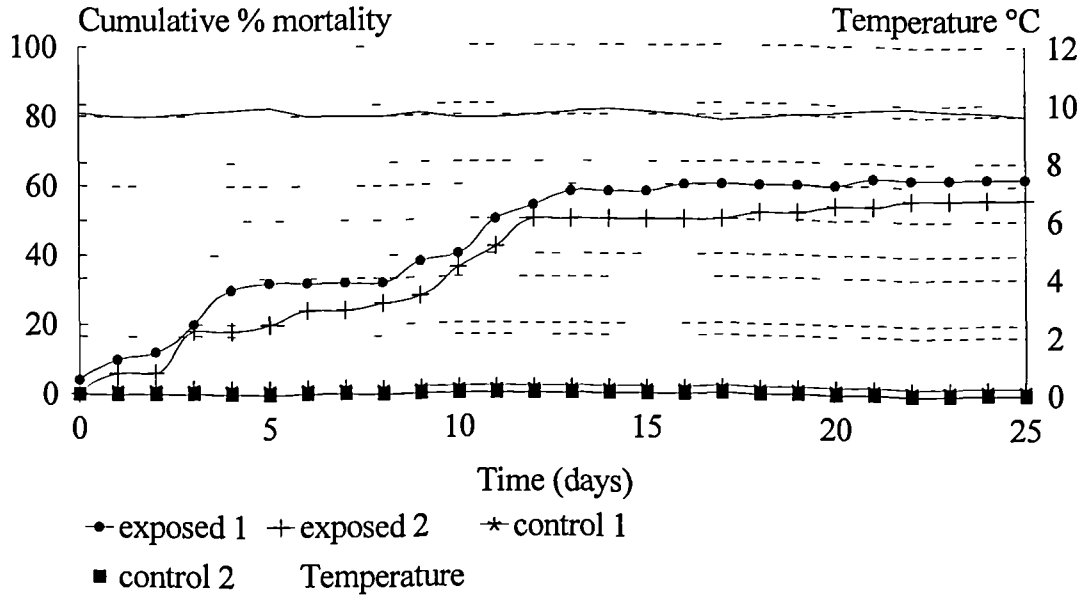


Figure 4.9: Cumulative % mortality resulting from IP challenge with isolate 1947

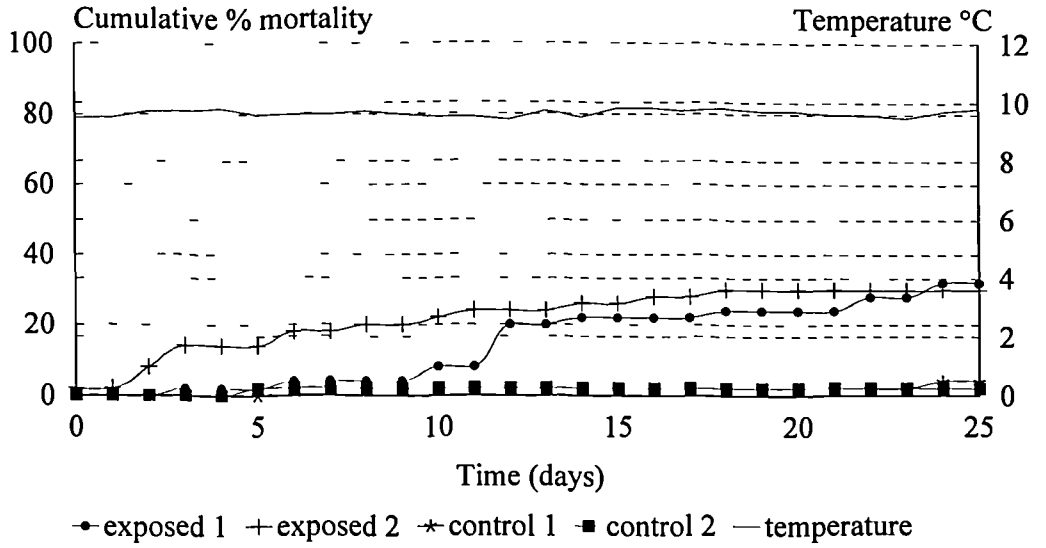


Figure 4.10: Cumulative % mortality resulting from IP challenge with isolate JO121

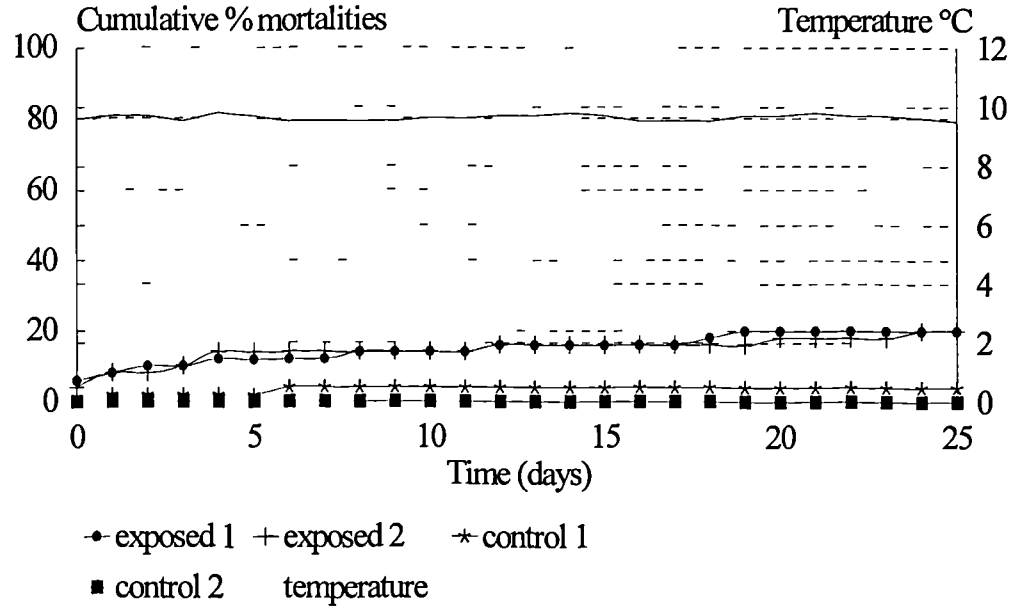
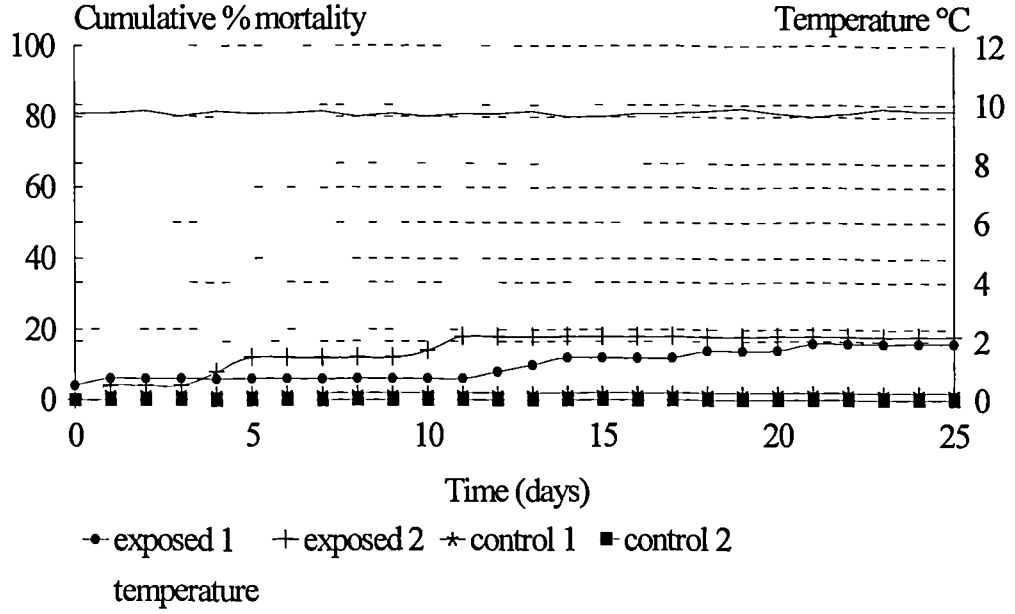


Figure 4.11: Cumulative % mortality resulting from IP challenge with isolate FEL2



Fish confined in outflow water

RTFS was transmitted to experimental fish originating from both farms 1 and 9 via the outflow of farm 1. In total, 9 from 100 (9%) naive fry (ie. those from farm 9 that had not previously been exposed to the pathogen, and 23 from 100 (23%) fry from farm 1, died during the 60 day trial. The results of the trial are expressed graphically in Figure 4.12. On MAOA, discrete colonies of C. *psychrophila* were recovered from viscera, particularly spleen tissue of all experimental animals exhibiting clinical disease signs, confirming that it was possible to transmit RTFS from the outflow water of farm 1.

Externally, clinical signs of disease included pale gills, with occasional melanisation, darkening of the dorsum, exophthalmia and distension of the abdomen. Elevated grey nodules were visible on the external dorsal and ventral surfaces of some affected fish. Localised haemorrhage and minor ulceration around the base of the anal fins was observed. Internally the fish were anaemic in appearance. The organ most consistently affected was the spleen, which showed varied gross pathology including congestion and enlargement. Spleens were haemorrhagic and friable with petechial haemorrhaging in adjacent viscera and extensive adhesions to visceral fat. The liver was pale in colour with localised "pin prick" haemorrhages on the surface. Severe petechial haemorrhage was infrequently observed on the heart muscle. The kidneys of affected fish showed minor pathological changes but were occasionally grey in appearance.

Fish confined in inflow water

Fish originating from farm 1 and therefore potentially carrying sub clinical levels of the pathogen prior to the start of the experiment showed signs of RTFS from day 11. *C. psychrophila* was recoverable from the spleens of dead and moribund fry in this experimental group. Conversely, RTFS was not diagnosed in naive fry that died in tanks supplied with inflow water from farm 1. The mortalities of both naive and pre-exposed fry over the course of the 60 day trial are expressed graphically in Figure 4.13. Although mortalities occurred within the naive fry held in inflow water, no isolation of *C. psychrophila* was possible. Fry were of poor initial quality and the cause of death was not discovered. Highly significant isolation of *C. psychrophila*

($p<0.01$) was possible from dead and moribund fry from the naive fry on exposure to outflow water compared to fish kept at the inflow. The percentage mortality with recovery of the bacterium amongst fry during the 2 trials is expressed in Figure 4.14.

Figure 4.12: Cumulative % mortality of fry exposed to outflow water of farm 1

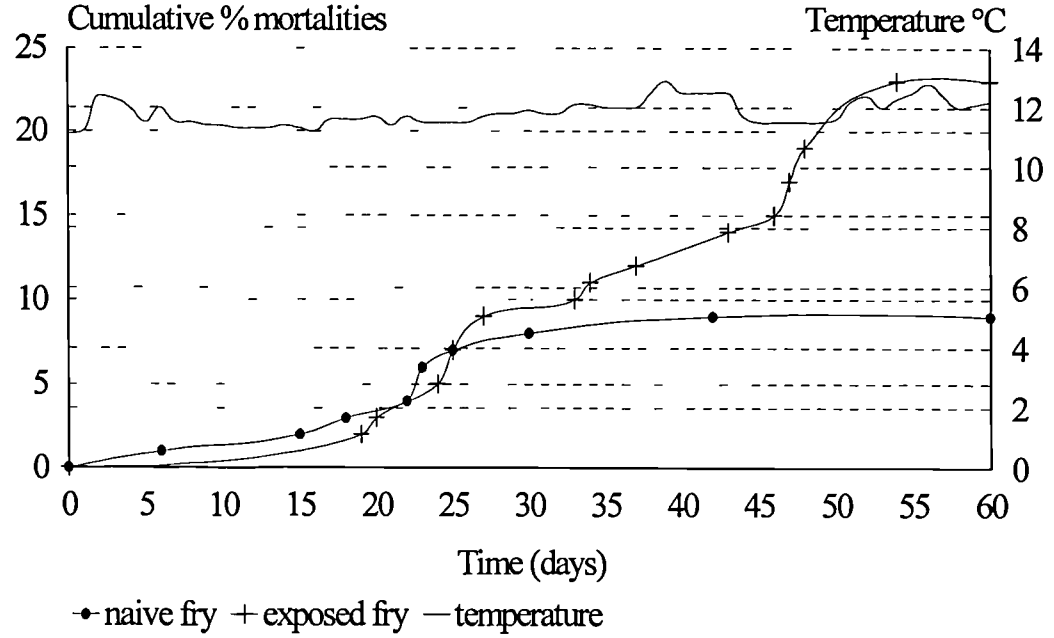


Figure 4.13: Cumulative % mortalities of fry exposed to inflow water of farm 1

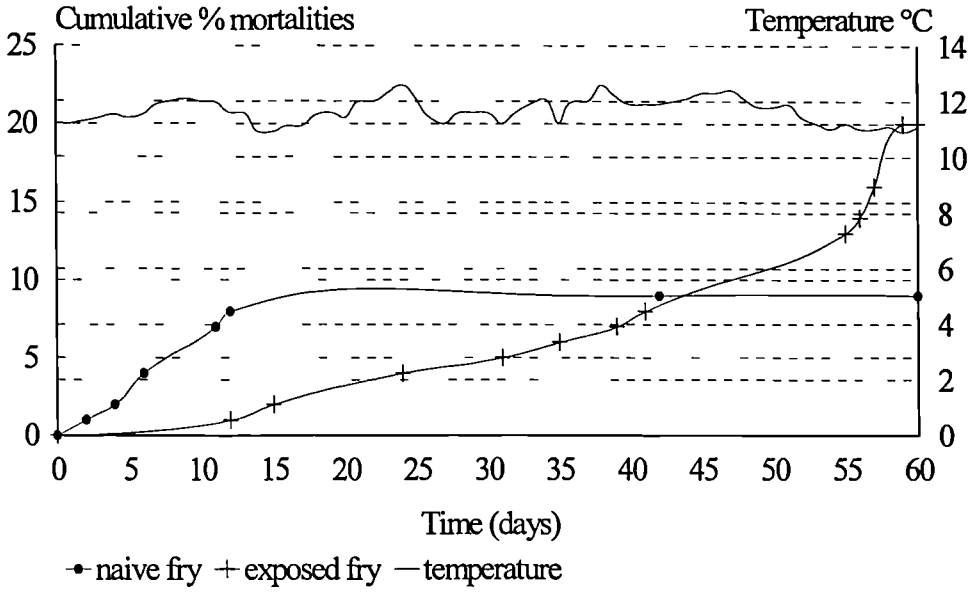
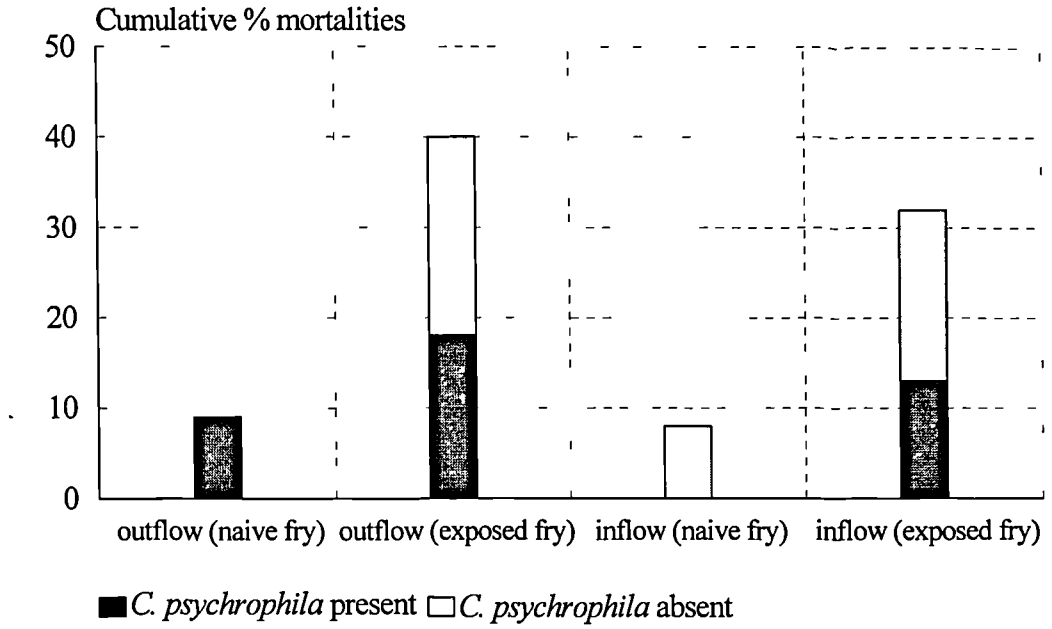


Figure 4.14: Cumulative % mortalities amongst fry with recovery of *C. psychrophila* from outflow and inflow water supply



DISCUSSION

The work presented here demonstrated that intraperitoneal and immersion challenge with viable cells of *C. psychrophila* isolated from diseased salmonid fish, caused clinical signs analogous to those seen in field outbreaks of RTFS in rainbow trout fry under laboratory conditions. Furthermore *C. psychrophila* was successfully cultivated from the spleens and ascitic fluid of experimental fish that became moribund or died over the course of the transmission trials. These findings fulfil Koch's postulates (Wilson and Miles, 1966), the criteria required in order to establish a causal relationship between a potential pathogen and specific infection. In general terms Koch postulated that:

(1) The organism should be found in all cases of the disease in question and its distribution in the body should be in accordance with the lesions observed.

(2) The organism should be cultivated outside the body of the host in pure culture for several generations.

(3) The organism so isolated should reproduce the disease in other susceptible animals.

Despite the difficulties in substantiating the causal role of a particular organism in a specific disease, especially in the aquatic environment where ubiquitous bacteria can frequently cause secondary infections, the results of this study indicate that RTFS is a single disease condition rather than a multi-factorial syndrome.

In accordance with this, Chua (1991) produced some typical gross lesions of RTFS in rainbow trout fry by intraperitoneal injection of $8x10^7$ cfu/fish of a field isolate of *C. psychrophila*, but was unable to demonstrate significant mortality using an immersion challenge. He did however observe tissue disruption, intercellular oedema and the presence of viable blast cells and macrophages, and fewer pyknotic cells in 50% of the spleens of bath challenged fry, suggesting that fry may have been compromised by the presence of the bacterium and were in the process of overcoming infection. In a subsequent study, intraperitoneal and intramuscular injection of 0.05ml of 10^7 cfu/ml of *C. psychrophila* isolated from the kidneys, spleens and gills of moribund fry at two UK rainbow trout farms produced 80% mortality within 7 days in 6.0g rainbow trout fry (Austin, 1992). In a series of infectivity experiments, Lorenzen *et al.* (1991) showed that parenteral inoculation of $5x10^6$ cfu/fish of *C. psychrophila,* isolated from spleen and kidneys of diseased fry from two different fish farms, produced a similar infection in rainbow trout in the laboratory to that observed over the previous 5 years in Danish hatcheries. However, the intestinal parasite *Hexamita salmonis* and the ectoparasite *Costia necatrix* were cited as potential additional factors contributing to the overall aetiology of the syndrome.

The behavioural, external and internal signs of disease in this study observed in fish after both intraperitoneal and immersion challenge were closely comparable to those observed in natural outbreaks, although mortality rates and numbers varied considerably with challenge method and bacterial origin. The distribution of mortality amongst the bath challenged fish was closely allied to the numbers of deaths recorded during epizootics of RTFS in the field. On affected hatchery sites, mortality levels can frequently peak at 70%, but are more usually between 2-30% (Chapter 2). Deaths in the bath challenged groups were not dependent upon either, the concentration of cells that the fry were exposed to, or to the exposure time. Furones (1990) stated that a

positive correlation existed between the length of exposure time and establishment of *Yersinia ruckeri* in the intestine of rainbow trout. Conversely, in bath vaccination, which relies on a similar route of uptake, via gills and to a lesser extent by imbibition (Horne and Ellis, 1988), several authors have noted that increased exposure time has no effect upon efficacy of the vaccine above the critical minimum (Tatner and Horne, 1983; Johnson, Flynn and Amend, 1982). The pattern of mortalities in both bath challenge groups was similar, with several fish dying on days 3, 4 and 5 followed by a period of 4-5 days during which time few fish died, then in each case further mortalities were observed between days 9 and 14. This may result from massive shedding of bacterial cells into the tanks from fish infected early in the trial. Expulsion of bacterial cells from affected fish has been demonstrated in infection caused by *Aeromonas salmonicida*, where 10^5-10^8 cfu/fish/hour can be released from dead and moribund animals (Rose, Ellis and Munro, 1989). The potential spread of infection from dead and moribund fish to healthy individuals in a population highlights the need for prudent monitoring and removal of dead stock both in an experimental situation and on the fish farm. In both intraperitoneal and immersion challenge trials fish that died up to 3 days post challenge exhibited no gross external signs of disease, whilst fish that died or became moribund 3-4 days after challenge showed classic gross clinical signs of the condition. Negligible mortality levels in the control groups indicated that no deaths should be attributed to post inoculation trauma. However this early mortality could have been associated with exposure to massive quantities of foreign proteins (R.H. Richards pers. comm.), and not to infection with *C. psychrophila per se*. *C. psychrophila* is a slow growing fastidious organism *in vitro* and whilst no information is available on its ability to grow *in vivo*, the development of clinical signs in the later cases could be allied to the division and subsequent increase in bacterial numbers within the host tissues.

Disease signs analogous to field outbreaks of RTFS were exhibited in 0.7-1.5g fry following injection with 6 strains of *C. psychrophila*, although mortalities between experimental groups varied significantly and marked differences in the virulence of isolates was demonstrated. Injection of infectious agents may not be an entirely appropriate way of testing for pathogenicity as the method by-passes the integumental defence mechanisms of the host. However, this study revealed that via parenteral

inoculation, the most virulent strain of *C. psychrophila* was that originally isolated from deep, external skin lesions of >10g rainbow trout in Finland. Serologically and biochemically, this isolate was identical to members of serotype 1, but plasmid profiling revealed the presence of an additional plasmid band (Chapter 5). Crosa *et al.* (1977) demonstrated clear correlation between the occurrence of a plasmid and virulence in *Vibrio anguillarum.* The different plasmid profiles observed in the characterisation of the isolates may be related to their virulence but further investigations are required before the functions of plasmids found in *C. psychrophila* can be elucidated. The isolates from diseased Atlantic salmon from a commercial hatchery in Tasmania (0273) and from Atlantic salmon parr from a Scottish fishery (NL193) produced similar levels of mortality in rainbow trout fry. The type strain for *C. psychrophila* NCIMB 1947^T produced fewer deaths with little evidence of clinical signs. Analysis by ELISA and slide agglutination revealed that this isolate may have belonged to a different serological group. Additionally, plasmid profiling of isolate 1947 showed a novel configuration of bands. It was originally isolated from diseased coho salmon in North America and is possibly less pathogenic for rainbow trout. Low levels of mortality were observed after inoculation with isolate FEL2 and JO121. The former originated from a Danish hatchery and is representative of the second minor serotype of *C. psychrophila* as demonstrated by Lorenzen (1994). The low pathogenicity of this organism may be related to the expression of different surface antigens, however both serogroups have been found in fry suffering from RTFS in Danish hatcheries. In this study, intraperitoneal injection with isolate JO121 resulted in only a low level of mortality, although the organism had originally been isolated from diseased fry at a heavily affected rainbow trout hatchery in the UK. With the exception of this isolate, all of the bacteria used in the present study had been either freeze-dried or stored at -20°C immediately after recovery from fish tissues using a microbiological bead storage system (Protect®, Technical Service Consultants Limited, Heywood, Lancashire). Isolate JO121 was old and had been sub-cultured for many generations outside of a fish in artificial media. Although Furones (1990) reported that virulence of pathogenic microbes is not always lost by either progressive cultivation or storage, Smith, (1988) stated that successive isolation on artificial media resulted in a decrease in pathogenicity. It is speculated that serial sub-cultivation of

isolate JO121 in artificial growth media reduced its ability to produce mortality in rainbow trout fry.

In all experimental groups, fish inoculated with the pathogen frequently exhibited acute disease signs, typically found internally, and fish died within hours or a very few days of injection. Whilst this type of mortality pattern can be attributable to non-specific factors as discussed above, extensive mortalities with little evidence of clinical disease can be associated with the production of extracellular toxins. A number of extracellular toxins and enzymes are associated with the virulence of other fish pathogens, including adhesins, haemolysins, cytotoxins, antiphagocytic factors, proteases resistant to the bactericidal effect of complement, ability to sequester iron, ability to penetrate epithelial cells, and ability to survive and multiply in phagocytes (Dalsgaard, 1993). No investigation into the production of toxic substances was attempted in the current work, however previous studies have failed to demonstrate mortalities or lesions following intramuscular injection of cell free filtrate from a broth culture (Borg, 1960). These findings implied that disease caused by *C. psychrophila* was due to the presence of live organisms and that no significant amounts of exotoxin were liberated. Other factors that can be related to the pathogenicity of bacteria are extra protein layers, O antigens, fimbriae and other non-specific adherence mechanisms, however no attempts were made to identify specific factors relating to the virulence mechanisms in the strains of *C. psychrophila* utilised in this study.

Spiral swimming behaviour observed in one fish from the 1344 challenge group and the tendency of fry to remain motionless on the bottom of the tanks was possibly due to the formation of neural lesions. Kent, Groff, Morrison, Yasutake and Holt, (1989) reported similar behavioural signs in coho salmon suffering from cold water disease. Histological examination revealed localisation of *C. psychrophila* within the posterior vertebral column. They suggested that the ataxic swimming behaviour was due to chronic inflammation of the cranial cavity and anterior vertebrae as a result of bacterial cells being transported via the cerebrospinal fluid. Spinning behaviour, posterior paralysis, spinal deformities, and neural lesions have been observed by several authors following outbreaks of cold water disease in salmonid fish (Meyers, 1989). However, in the reported cases, development of neurological

disorders occurred 2-3 months after the initial deaths, whilst the clinical signs noticed in artificially infected rainbow trout fry in this study occurred within 20 days of exposure to the pathogen. In general, recorded observations of outbreaks of cold water disease and the signs of disease noted in artificially infected rainbow trout fry in this study are remarkably consistent. Further investigations into the localisation and role of the organism are needed to elucidate the similarities between the two conditions.

Bustos *et al.* (1994) achieved 73.3% mortality in 10-22g rainbow trout inoculated intraperitoneally with 0.1ml of $1x10^7$-$1x10^9$ bacteria/ml. Signs of disease were identical to clinical field outbreaks of presumptive RTFS in Chilean salmonid farms. Additionally they isolated *C. psychrophila* from spleens and gills of fish surviving the trials, but exhibiting no clinical signs of disease. In the current study, bacteriological sampling of surviving fry revealed that viable cells of *C. psychrophila* were recoverable from internal organs of challenged animals indicating the possibility of carrier states within populations of fish. The presence of asymptomatic carriers is not uncommon with other bacterial fish diseases and the existence of fish harbouring sub clinical levels of *C. psychrophila* in farmed populations which could serve as additional reservoirs of disease is worthy of investigation.

Bacterial shedding by asymptomatically infected carrier fish and shedding of micro-organisms from dead and moribund fish are important mechanisms in the spread of infectious diseases. Horizontal transmission via the water has been shown for numerous pathogenic fish bacteria including *Yersinia ruckeri* (Hunter, Knittel and Fryer, 1980) and *Aeromonas salmonicida* (Rose *et al.*, 1989). Borg (1960), however, was unable to reproduce clinical signs of cold water disease in silver salmon without prior scarification of the skin. He concluded that failure of healthy fish to develop the disease when placed in the same water with infected fish indicated that either transmission of *C. psychrophila* was poor under these conditions or that uninjured fish were not readily affected. Conversely, Lorenzen *et al.*, (1991) achieved between 20% and 60% mortality over a 20 day period in disease free rainbow trout fry in cohabitation experiments with naturally infected fish exhibiting typical signs of RTFS.

In the present study *Cytophaga psychrophila* was successfully transmitted to naive rainbow trout fry, held in tanks supplied with outflow water from farm 1. The

mortality pattern and rate was similar to that seen in naturally occurring epizootics, with deaths frequently occurring rapidly within hours or a very few days of the onset of clinical disease signs. During the 60 day trials, RTFS was diagnosed in 0.5-5.0g fry in the main hatchery site, with average mortalities over the period running at approximately 20,000/week. It is suggested that viable cells of *C. psychrophila* were discharged from affected fry in raceways/tanks and passed to the trial facility, initiating identical disease signs in the experimental fish.

In contrast, although RTFS was diagnosed in the fry obtained from farm 1, the naive fry held in the inflow water did not become infected with *C. psychrophila* and no signs of RTFS were apparent. Mortality attributable to RTFS in the fry which originated on farm 1 can be explained by the presence of sub-clinical levels of the pathogen carried by the fish prior to the start of the experiment. Immediately before these fry were removed from the affected hatchery tanks and introduced into the experimental unit, a representative number of fish were sampled for the presence of the pathogen. *C. psychrophila* was found to be present in up to 40% of spleens tested. Therefore, the appearance of clinical signs of the disease and the isolation of the pathogen from this experimental group is not indicative of horizontal transmission, but of the establishment of the organism within the host and subsequent development of the condition. The inability to transmit the bacterium to naive fry via the inflow water provides further evidence that *C. psychrophila* is not endemic in natural waters in this UK farm.

CHAPTER 5

THE EFFECTS OF ANTIMICROBIAL COMPOUNDS ON *C. PSYCHROPHILA*

INTRODUCTION

Currently only four antimicrobial compounds are licensed for use against outbreaks of infectious diseases in the aquaculture industry in the UK. These comprise a potentiated sulphonamide, oxolinic acid, oxytetracycline and amoxycillin.

The potentiated sulphonamide combination of sulphadiazine and trimethoprim (Tribrissen) has been frequently used in the treatment of salmonid fish, notably against the aetiological agent of enteric red mouth disease, *Yersinia ruckeri.* Amend, Fryer and Pilcher (1965) (cited in Amend, 1970) compared the efficacy of starter diets medicated with sulphamethazine, sulphisoxazole, and sulphaethoxypyridazine, fed at either 220mg/kg/day for 10 days or administered prophylactically at 88mg/kg/day for 26 days, against *C. psychrophila* infections in juvenile salmonid fish. They concluded that sulphisoxazole gave the best control of bacterial cold water disease at both dosage levels. In addition, Wood (1968) (cited in Amend 1970) recommended sulphamethazine levels of 220-440mg/kg/day in starter diets and 110mg/kg/day in pelleted feeds against myxobacterial infections. However no evidence of efficacious action of the potentiated sulphonamides has been observed in the treatment of RTFS in the field, and several authors have reported on the *in vitro* resistance of *C. psychrophila* isolated from diseased fish (Chua, 1991; Schmidtke and Carson, 1994). Conversely, Wiklund, Kaas, Lonnstrom and Dalsgaard (1994) reported that *C. psychrophila* isolated from wild and farmed rainbow trout in Finland was sensitive to sulphonamides but resistant to trimethoprim, but no reports on the effectiveness of the compound *in vivo* were available.

Despite indications that growth of *C. psychrophila* is inhibited by oxolinic acid in concentrations in excess of 0.5μg/ml (Schmidtke and Carson, 1994), there has been little evidence that outbreaks of RTFS can be successfully controlled by oral administration of the compound during naturally occurring outbreaks of disease.

However there is a dearth of information in the published literature on either the *in vitro* or *in vivo* action of oxolinic acid against *C. psychrophila.*

Several authors have reported that oral administration of oxytetracycline at 50-75mg/kg fish /day for 10 days in the form of a medicated fish feed is effective in reducing mortalities attributable to bacterial cold water disease (Amend, 1970; Holt *et al.*, 1993). Oxytetracycline incorporated into the diet at levels of up to 300mg/kg/fish, administered for 10-14 days has been used in field outbreaks of RTFS with some success (Chua, 1991; Lorenzen *et al.*, 1991; Bustos *et al.*, 1994), but the decreasing effectiveness noticed on several hatchery sites in recent years is indicative of the rapid emergence of bacterial resistance to the compound.

The β lactam, amoxycillin, was first licensed for use in aquaculture in the UK in 1990 (Richards, 1992). Lorenzen (1994) demonstrated that administration of amoxycillin at 200g/25kg feed for 10 days reduced mortality in laboratory induced infections with *C. psychrophila.* Since its introduction in the UK, amoxycillin has been prescribed to control outbreaks of RTFS, although normally only where previous treatment with oxytetracycline has proved ineffectual. However, more recently, outbreaks have failed to respond to therapy at a number of sites where persistent, recurring mortalities attributable to RTFS are common and drug usage is correspondingly high (R. de W. Harrison, pers. comm.)

The commercial development of "new" drugs for use in aquaculture is limited by the relatively small size of the industry and the high cost of licensing novel compounds. Due to the limited number of compounds available to the veterinarian in the treatment of epizootics in fish and the rapid emergence of multiple resistant strains, pathogenic bacteria threaten to compromise commercial viability of the aquaculture industry and have considerable implications for animal welfare.

The recent emergence of RTFS means that there is a paucity of published information on drug sensitivity profiles of *C. psychrophila* isolated from clinical outbreaks of the syndrome. Consequently, drugs have frequently been prescribed on a "trial and error" basis, often resulting in inappropriate, ineffectual treatments and massively increased dose levels. Primarily, the present investigation attempted to determine the minimum inhibitory concentrations (MIC) of both licensed and non-licensed antimicrobials against *C. psychrophila* isolated from clinical cases of RTFS

and bacterial cold water disease (BCWD) throughout the world. The objective of this was to evaluate the sensitivities to currently available compounds in order that the success of on farm treatments could be maximised, and to assess the potential efficacy of novel chemotherapeutic agents that may become accessible to the aquaculture industry in the future. Secondly, the plasmid profiles of the strains were examined in an effort to identify possible inherent correlations between multiple resistance to antimicrobial compounds and resistance factors.

MATERIALS AND METHODS

Bacterial Inocula

The field isolates used in this investigation were recovered from clinical cases of RTFS/BCWD occurring in hatcheries worldwide (Table 5.1). The NCIMB 1947^T reference strain of *C. psychrophila* was supplied by the National Collection of Marine and Industrial Bacteria, Aberdeen, Scotland.

Forty eight strains of *C. psychrophila* (Table 5.1) were cultivated in modified Anacker and Ordal broth (MAOB) (Chapter 4) at 17°C. The 72-96 hour broth grown cells, were washed twice in phosphate buffered saline (PBS) then resuspended in PBS. The optical density of the suspensions was adjusted to 0.2±0.01 at 520nm, corresponding to $1x10^8$ cells/ml.

Table 5.1: Origin of bacterial strains used in this study

Laboratory accession no.	Origin	Source of isolate	Chapter No.
Cytophaga aquatilis			
2215	NCIMB	Salmonid species gills (USA)	5,8
Cytophaga columnaris			
2248	NCIMB	Diseased salmonid fish (USA)	5,8
Flexibacter aurantiacus			
8628	NCIMB	Source unknown	5
FA1a	FDL isolate	Fish farm effluent	5

Cytophaga psychrophila			
1342	T Wiklund	*Oncorhynchus mykiss* fry spleen (Finland)	5
1344	T. Wiklund	*Oncorhynchus mykiss* fry skin lesions (Finland)	4,5
0278	J. Carson	*Oncorhynchus mykiss* fry spleen (Tasmania)	5
0273	J. Carson	*Oncorhynchus mykiss* fry spleen (Tasmania)	4,5
FEL62	E. Lorenzen	*Oncorhynchus mykiss* adult fish spleen (France)	5
FEL10	E. Lorenzen	*Oncorhynchus mykiss* fry spleen (France)	5
FEL16	E. Lorenzen	*Oncorhynchus mykiss* fry spleen (Denmark)	5
U648	FDL isolate	*Oncorhynchus mykiss* fingerling spleen (England)	5
FEL5	E. Lorenzen	*Oncorhynchus mykiss* fingerling spleen (Denmark)	5
1947^T	NCIMB	*Oncorhynchus kisutch* (USA)	8,4,5
FEL2	E. Lorenzen	*Oncorhynchus mykiss* adult fish spleen (Denmark)	4,5,8
C292	FDL isolate	*Oncorhynchus mykiss* fry spleen (Wales)	5
R151	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
A1	FDL isolate	source unknown	5
D593	FDL isolate	*Oncorhynchus mykiss* fingerling spleen (England)	5
U147	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
C193	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
W693	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
CO11	FDL isolate	*Oncorhynchus mykiss* adult ovarian fluid (England)	5
U249	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
W593	FDL isolate	*Oncorhynchus mykiss* fingerling spleen (England)	5
W1293	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
S5793	FDL isolate	*Oncorhynchus mykiss* fry spleen/kidney (Scotland)	5
S7793	FDL isolate	*Oncorhynchus mykiss* fry spleen/kidney (Scotland)	5
B593	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
T20	FDL isolate	*Oncorhynchus mykiss* adult fish spleen (England)	5
B393	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
SH221	FDL isolate	*Salmo salar* parr spleen (Scotland)	5
MM221	FDL isolate	*Oncorhynchus mykiss* fry spleen (Wales)	5
BW222	FDL isolate	source unknown	5
RB192	FDL isolate	*Oncorhynchus mykiss* fry spleen (Wales)	5
BB693	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
U842	FDL isolate	*Oncorhynchus mykiss* fingerling (England)	5,6
BB5	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
TVT20	FDL isolate	*Oncorhynchus mykiss* fingerlingspleen (England)	5
U239	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	2,5,6
F163	FDL isolate	source unknown	5

BB8	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
NL193	FDL isolate	*Salmo salar* parr spleen (Scotland)	4,5
P183	FDL isolate	*Oncorhynchus mykiss* yolk-sac fry viscera (Scotland)	5
D693	FDL isolate	*Oncorhynchus mykiss* fingerling (England)	4
U148	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
SH222	FDL isolate	*Salmo salar* parr spleen (Scotland)	5
JO121	FDL isolate	*Oncorhynchus mykiss* fingerling (England)	4,5
VT121	FDL isolate	*Oncorhynchus mykiss* egg surface (England)	5
OV812	FDL isolate	*Oncorhynchus mykiss* ovarian fluid (England)	5
F167	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
M249	FDL isolate	*Oncorhynchus mykiss* fry spleen (England)	5
Aeromonas hydrophila			
72	NCIMB	Moribund goldfish (*Carassius auratus*)	8
Yersinia ruckeri			
1315	NCIMB	Red mouth disease in rainbow trout	2,3

Addresses of donors are listed in appendix 5

Antibiotic Dilutions

Soluble antimicrobial compounds were dissolved in distilled water to stock concentrations of 1024ppm. Antibiotics which needed a shift of pH to pH 10 in order to be dissolved were made up to a final concentration of 4096ppm. This increased concentration was necessary to enable greater subsequent dilution to prevent the inhibitory effect of the alkaline pH on the bacterial growth and to avoid interference with the sensitivity of the assay. For the potentiated sulphonamide assay, trimethoprim was first dissolved in ethanol and then added to the sulphadiazine, which had already been dissolved in distilled water. Antimicrobial dilutions were sterilised by filtration through 0.45μm filters (Whatman). Details of the sources and purity of the antimicrobials used are given in Appendix 4. Serial two fold dilutions in distilled water gave a final concentration gradient for all compounds from 1024ppm to 0.000122ppm. Ninety six well, microtitre assay trays (Sterilin) were filled with 100μl double strength MAO broth and 100μl of the appropriate antibiotic dilution and inoculated with 10μl of bacterial suspension. The trays were sealed in plastic bags to maintain humidity and incubated for 7 days at 17°C. Trays were read over a black

background to increase the contrast. Bacterial growth was evident as opaque spots on the bottom of each well. The MIC value was taken as the lowest antimicrobial concentration where growth did not occur.

Plasmid Analysis

Isolates were grown for 72-96 hours in MAOB. Cultures were centrifuged for 20min at 800g in 50ml centrifuge tubes (Becton Dickinson Labware). The pellets obtained were suspended in 200µl TE buffer (25mM Tris HCl + 1mM EDTA (Ethylenediamine-tetra-acetic acid)) (Analar BDH). To 0.2ml of the resuspended cells, 400µl of 1% SDS (Sodium dodecyl sulphate, Sigma, Poole) in 2M NaOH was added. The mixture was inverted and allowed to stand for 5 min to ensure that cell lysis had occurred. On completion of cell lysis, 300µl of 3M potassium acetate, 300ul of 2M acetic acid and 300µl of 1mM EDTA were added. The potassium acetate neutralised chromosomal DNA, whilst acetic acid and EDTA aided the precipitation of chromosomal material and cell walls from the lysate. After further centrifugation the supernatant was recovered and 500µl of buffered phenol (pH 8.0) was used to separate proteinaceous material. The mixture was recentrifuged and the supernatant decanted into Eppendorf tubes (BDH). Using fresh tubes, 100µl of 4M sodium acetate (pH 6.0) (Analar, BDH) was added to the supernatant. This stage ensured removal of impurities. Plasmid DNA was precipitated with 500µl of isopropanol (BDH). The resulting pellet was resuspended in absolute ethanol (BDH) to remove any residual salts and to complete precipitation. Traces of ethanol were then displaced with ether (BDH). The ether was discarded and the pellet was allowed to dry. Plasmid pellets obtained were dissolved in 100µl 10mM TE buffer. The addition of 5µl RNAase (1mg/ml) (Ribonuclease A, Sigma Chemicals) facilitated removal of RNA. The density of the solution was increased by the inclusion of 25µl of loading dye (0.15% bromo cresol green (Sigma), 0.25% xylene cyanol (Sigma), 1.8% ficoll (Sigma), 6mM EDTA). DNA samples (10µl) were electrophoresed through 0.8%(w/v) agarose + 25µl ethidium bromide (Sigma, Poole) in Tris borate buffer (TBE) (pH 8.0) at 80mV for 60 min.

RESULTS

Minimum inhibitory concentrations (MIC)

Minimum inhibitory concentration (μg ml^{-1}) values for all the antimicrobial compounds against all isolates of *C. psychrophila* tested in this study are given in Appendix 4.1. To facilitate the interpretation of the results gathered in the present study MIC values obtained for clinical isolates of *C. psychrophila* have been separated into 3 categories according to arbitrary assessments made on their relative sensitivities: (1) susceptible (2) moderately susceptible and (3) resistant. Thus Table 5.2 describes guidelines for categorising strains of *C. psychrophila* for susceptibility to antimicrobial compounds. Estimations of probable clinical effect were made with reference to the comparative reactions of the type strain NCIMB 1947^T (Table 5.3) previous published information on MIC values for the organism, achievable drug serum levels and experience of therapeutic benefit of specific compounds in the field. The approaches of Thrupp (1986) and Tsoumas, Alderman and Rodgers (1989) were followed to produce these estimates.

Table 5.2: Guidelines used for assessing strains of *C. psychrophila* for sensitivity to antimicrobial compounds (μg ml^{-1})

Drug class	Susceptible	Moderately susceptible	Resistant
4- Quinolones	≤0.5	>0.5-3.0	>3.0
Tetracyclines	≤1.0	>1.0-4.0	>4.0
β lactams	≤2.0	>2.0-5.0	>5.0
Nitrofurans	≤0.5	>0.5-1.0	>1.0
Aminoglycosides	≤8.0	>8.0-16.0	>12.0
Sulphonamides	≤2.0		>2.0
"Phenicols"	≤20.0	>20.0-32.0	>32.0
Macrolides	≤2.0	>2.0-4.0	>4.0

Table 5.3: Comparisons between the MIC scores for NCIMB 1947^T and isolates of *C. psychrophila*

	MIC (μg ml^{-1}) 1947^T	MIC (μg ml^{-1}) for 47 isolates of *C. psychrophila*				
		mean	mode	range	MIC_{50}	MIC_{90}
Enrofloxacin	0.000977	0.05	0.03125	0.00098-0.25	0.03125	0.125
Ciprofloxacin	0.00391	0.05	0.0078	0.0005-8	0.0078	0.25
Sarafloxacin	0.00195	0.05	0.000195	0.000195-8	0.0625	2
Flumequine	0.5	2.4	2	0.03125-16	1	8
Sodium oxolinate	0.25	1.65	0.25	0.00098-16	0.5	8
Oxolinic acid	0.25	1.3	1	0.03125-16	1	2
Doxycycline	0.125	0.5	0.25	0.03125-8	0.25	0.5
Oxytetracycline hydrochloride	0.125	10.9	8	0.03125-64	8	32
Amoxycillin	0.0625	2.1	0.125	0.00195-64	0.125	1
Furazolidone	0.5	29.4	16	0.03125-512	16	64
Gentamycin	16	98.7	128	0.25-512	64	256
Neomycin	1	169.2	128	1-512	128	512
Streptomycin	4	4.25	4	0.25-32	4	8
Sulphadiazine/ Trimethoprim (5/1)	16	92.1	128	8-512	64	256
Chloramphenicol	0.125	27.6	1	0.03125-512	2	64
Thiamphenicol	0.25	9.7	2	0.0156-64	2	32
Florfenicol	0.5	2.9	8	0.00098-16	1	8
Vancomycin	16	23.1	16	2-512	8	32
Erythromycin	0.03125	12.4	1	0.03125-64	2	64

Characterisations of the antimicrobial sensitivities of the bacterial strains included in the present study are given in Table 5.4. The results of these evaluations were subsequently used to produce graphical representations of the susceptibility of *C. psychrophila* to the 19 antibacterial agents used in this study (Figures 5.1 -5.8). Additionally, the mean, modal, median and range values for the MICs together with MIC_{50} and MIC_{90} values are given for each compound in Table 5.5.

Table 5.4: Relative susceptibilities (%) of *C. psychrophila* (48 isolates)

Drug class	Susceptible	Moderately susceptible	Resistant
Enrofloxacin	100	0	0
Ciprofloxacin	100	0	0
Sarafloxacin	85	10	5
Flumequine	42	39	19
Sodium oxolinate	70	14	16
Oxolinic acid	54	35	11
Doxycycline	96	2	2
Oxytetracycline	27	23	50
Amoxycillin	94	0	6
Furazolidone	12	4	84
Gentamycin	21	10	69
Neomycin	6	10	84
Streptomycin	92	6	2
Sulphadiazine/ Trimethoprim	0	0	100
Chloramphenicol	77	6	17
Florfenicol	100	0	0
Thiamphenicol	79	19	2
Vancomycin	4	27	69
Erythromycin	64	15	21

Figure 5.1-5.9: Histograms describing MIC ranges and distribution for 48 isolates of *C. psychrophila* against 8 classes of antimicrobial compounds

Figure 5.1: Susceptibility ranges for the new and older generation 4-quinlones

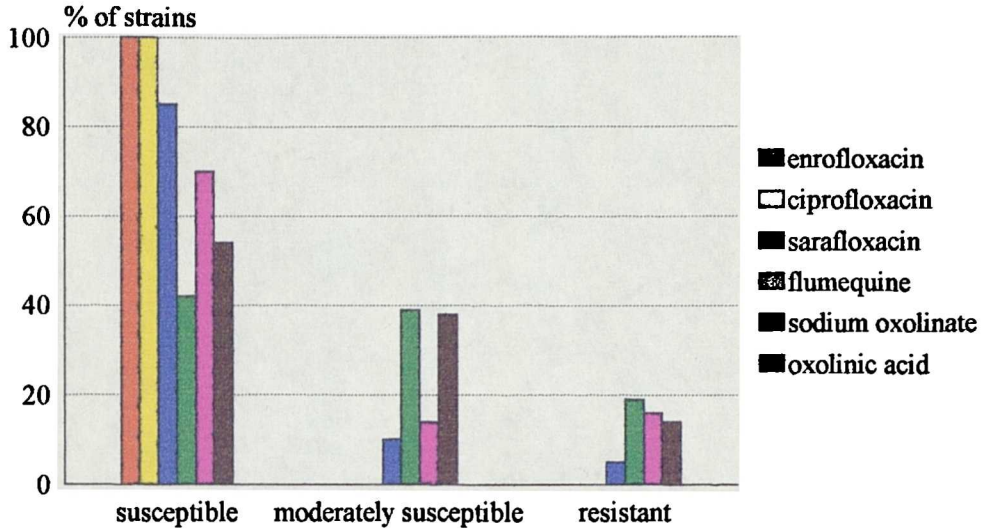


Figure 5.2: Susceptibility ranges for the tetracyclines

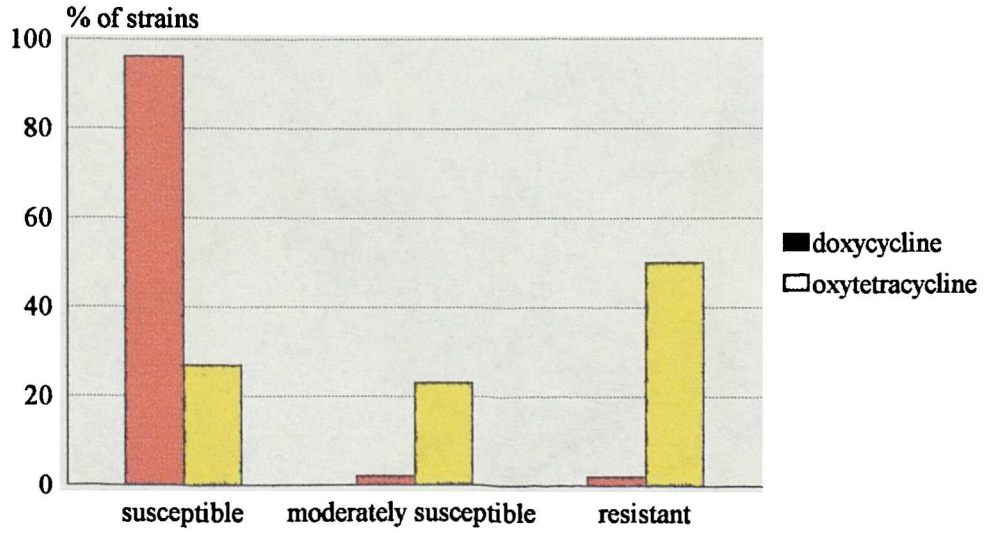


Figure 5.3: Susceptibility ranges for the β-lactams

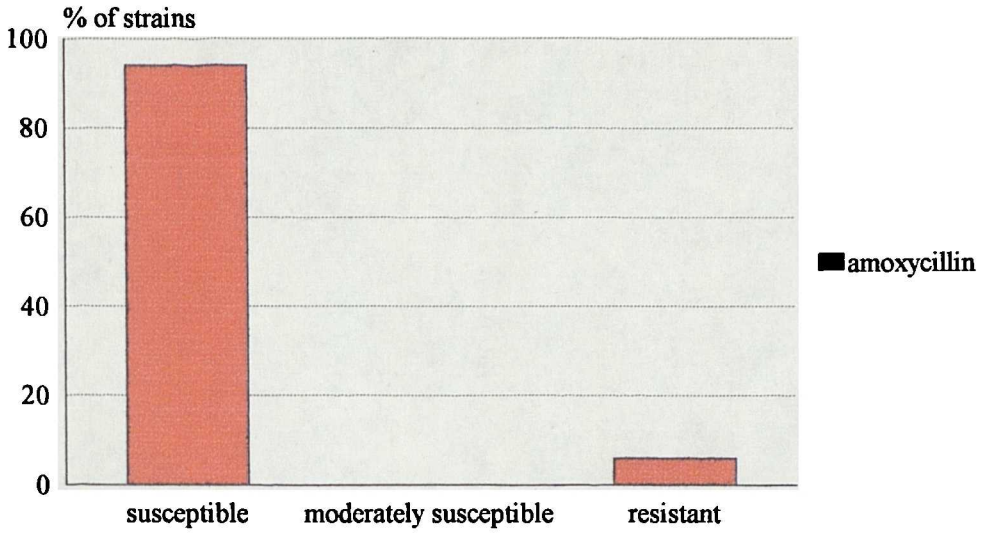


Figure 5.4: Susceptibility ranges for the nitrofurans

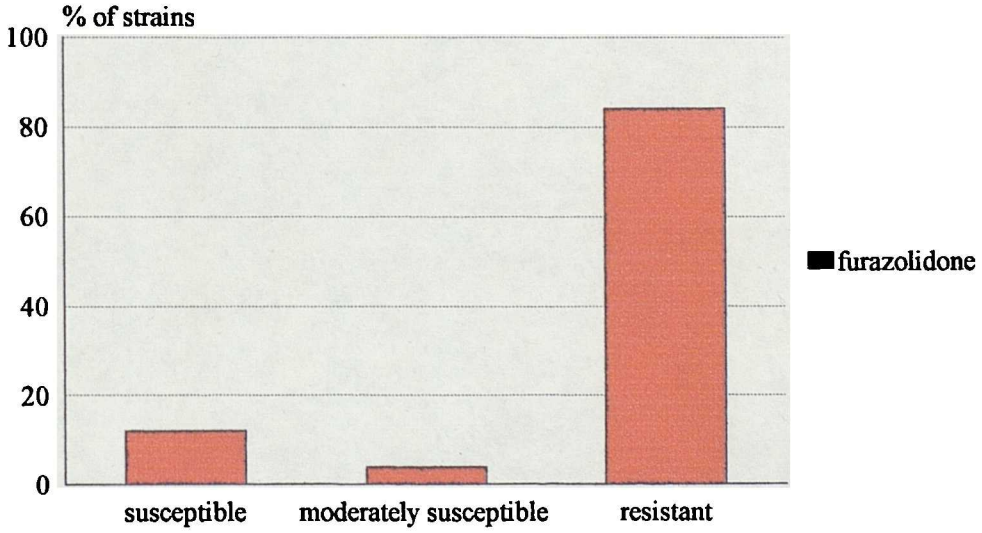


Figure 5.5: Susceptibility ranges for the aminoglycosides

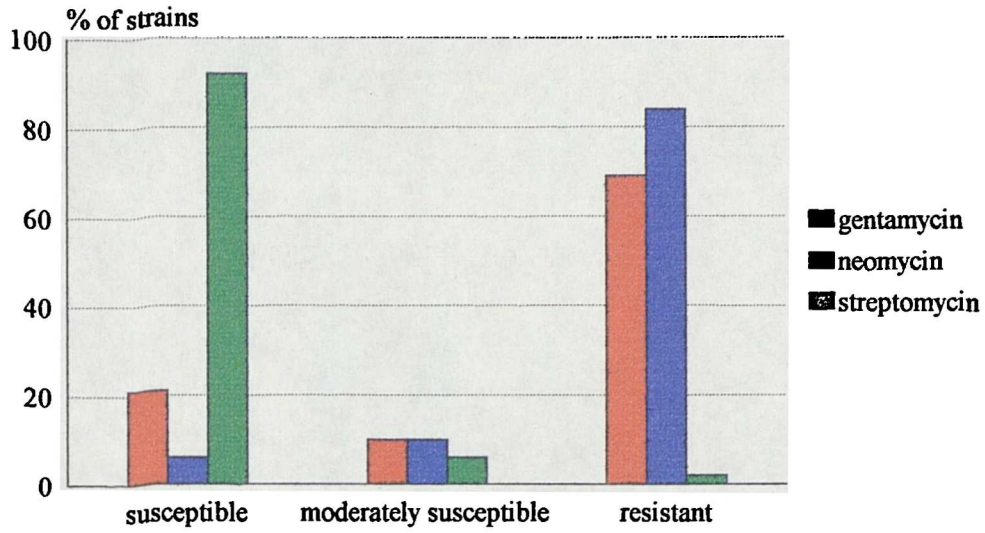


Figure 5.6: Susceptibility ranges for the potentiated sulphonamides

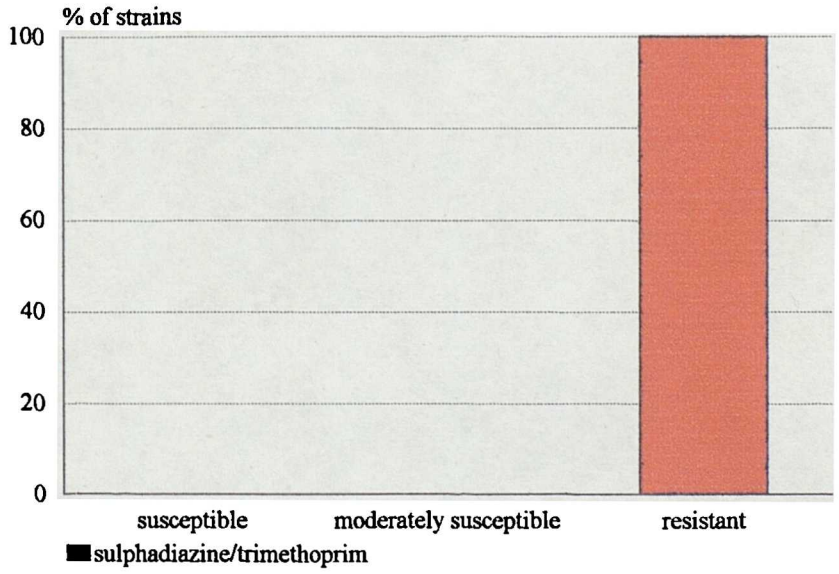


Figure 5.7: Susceptibility ranges for the phenicols

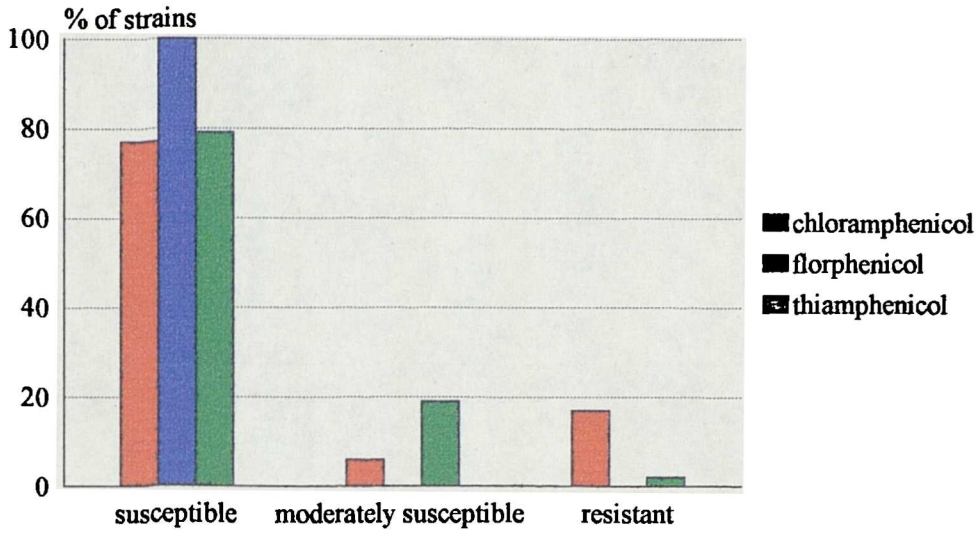


Figure 5.8: Susceptibility ranges for the macrolides

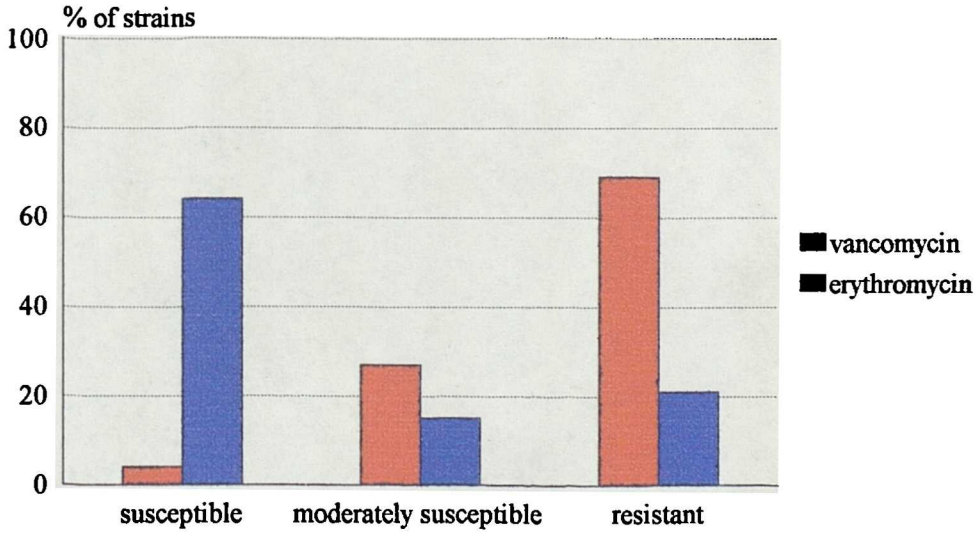


Table 5.5: **Mean, median, mode MICs, MIC_{50} and MIC_{90} values of antimicrobial compounds for *C. psychrophila* with range.**

Compound	mean (μg ml^{-1})	median (μg ml^{-1})	mode (μg ml^{-1})	MIC_{50} (μg ml^{-1})	MIC_{90} (μg ml^{-1})	range (μg ml^{-1})
Enrofloxacin	0.05	0.0078	0.0078	0.03125	0.125	0.00048-0.5
Ciprofloxacin	0.05	0.03125	0.03125	0.0078	0.25	0.000977-0.25
Sarafloxacin	0.5	0.03125	0.00195	0.03125	2	0.00195-8
Flumequine	2.3	1	2	1	8	0.03125-16
Sodium oxolinate	1.6	0.25	0.25	0.25	8	0.000977-16
Oxolinic acid	1.3	0.5	1	0.5	2	0.03125-16
Doxycycline	0.48	0.25	0.25	0.25	0.5	0.03125-8
Oxytetracycline	10.7	4	8	4	32	0.03125-64
Amoxycillin	2.0	0.0625	0.125	0.0625	1	0.00195-64
Furazolidone	28.8	8	16	8	64	0.03125-512
Gentamycin	96.8	64	128	64	256	0.25-512
Neomycin	166	128	128	128	512	1-512
Streptomycin	4.2	2	4	2	8	0.03125-32
Sulphadiazine / Trimethoprim	90.8	64	128	64	256	8-512
Chloramphenicol	27	1	1	1	1	0.03125-512
Florfenicol	2.6	0.5	0.5	0.5	8	0.0625-32
Thiamphenicol	9.5	2	2	2	32	0.01563-64
Vancomycin	22.7	8	16	8	32	2-512
Erythromycin	12.1	2	2	2	64	0.03125-64

The MIC values for all compounds tested were distributed over a wide range, with isolates giving between a 6 and 14 fold difference in drug sensitivity. The new generation 4-quinolones, enrofloxacin, ciprofloxacin and sarafloxacin demonstrated excellent antibacterial activities, between 100% and 85% of the isolates tested giving values of $\leq 0.5\mu g ml^{-1}$. Ten percent of isolates fell within the range > 0.5-3.0, and were considered to be moderately susceptible to sarafloxacin. Five percent of isolates tested exhibited MIC values in excess of $3.0\mu g\ ml^{-1}$. The older generation 4-quinolones, flumequine, sodium oxolinate and oxolinic acid gave sensitive inhibitory concentrations of $\leq 0.5\mu g\ ml^{-1}$ for 42%, 70% and 54%, moderate sensitivities were obtained for 39%, 14% and 35% and resistance (>4.0μg ml-1) was observed in 19%, 16% and 11%, respectively.

Inhibitory concentrations of $\leq 1.0\mu g\ ml^{-1}$ were achieved for 96% of bacterial strains against doxycycline, with just 2% of isolates giving values >2.0-4.0$\mu g\ ml^{-1}$ and 2% >4.0$\mu g\ ml^{-1}$. Twenty seven percent of strains showed sensitivity ($\leq 1.0\mu g\ ml^{-1}$) to oxytetracycline, with 23% falling within the moderately susceptible group (>1.0-4.0$\mu g\ ml^{-1}$), however comparatively resistant strains of the bacterium were present and the mode value for the compound was $8.0\mu g\ ml^{-1}$ with 50% of isolates giving MIC values >4.0$\mu g\ ml^{-1}$.

Amoxycillin demonstrated good antimicrobial activity with 94% of strains showing values $\leq 2.0\mu g\ ml^{-1}$, however 3 outlying values of 8, 16 and 64$\mu g\ ml^{-1}$ skewed the mean MIC value to $2.0\mu g\ ml^{-1}$ and resulted in 6% of isolates showing relative resistance to the compound with inhibitory levels in excess of $5.0\mu g\ ml^{-1}$.

The strains of *C. psychrophila* used in the current study exhibited a high percentage which were sensitive ($\leq 20.0\mu g\ ml^{-1}$) to chloramphenicol and thiamphenicol with 100% of isolates $\leq 20.0\mu g\ ml^{-1}$ for the novel fluorinated analogue, florfenicol. Six percent and 19% fell within a MIC range of >20.0-32.0$\mu g\ ml^{-1}$ for chloramphenicol and thiamphenicol respectively, whilst 17% of isolates demonstrated resistance to chloramphenicol compared with just 2% for thiamphenicol.

Of the aminoglycosides, streptomycin was the most effective *in vitro* with 92% of strains showing susceptibility to the drug of $\leq 8.0\mu g\ ml^{-1}$, 6% in the range >8.0-16.0$\mu g\ ml^{-1}$ and just 2% demonstrating resistance in excess of $16.0\mu g\ ml^{-1}$. Gentamycin and neomycin exhibited poor antibacterial activities, with 69% and 84%

respectively, of strains giving MICs >16.0μg ml^{-1} and MIC_{50} scores of 64μg ml^{-1} and 128μg ml^{-1}. Just 31% and 16% fell within the susceptible or moderately susceptible brackets.

It was considered that the macrolide drug, vancomycin, would be relatively ineffective in inhibiting growth of *C. psychrophila* with only 4% giving values less than 2.0μg ml^{-1}, 27% and 33% of isolates showing MICs of <4.0μg ml^{-1} respectively, and modal values of 16μg ml^{-1}. Conversely, 64% of isolates exhibited MIC values of ≤2.0μg ml^{-1}, with a further 15% of values falling within the moderately susceptible range (2.0-4.0μg ml^{-1}) and 21% of bacterial strains exhibited resistance (>4.0μg ml^{-1}) to the macrolide antimicrobial, erythromycin.

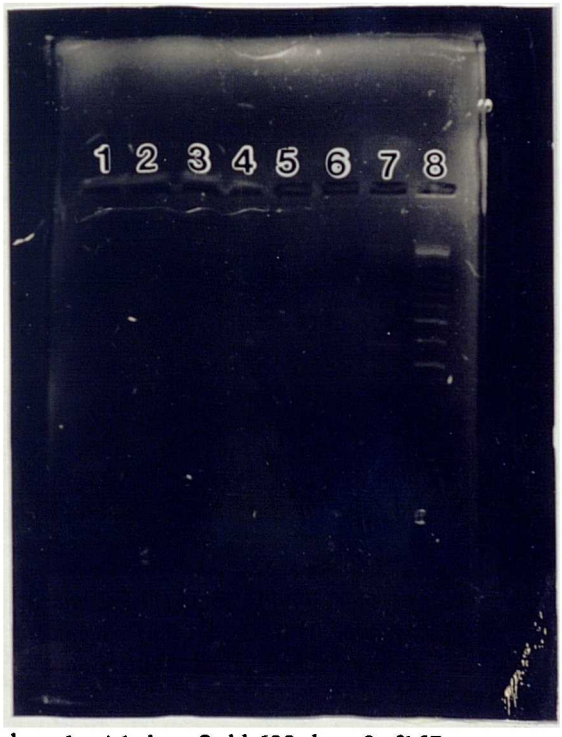
Low antibacterial activity was demonstrated by furazolidone with 84% of isolates giving MIC values of >1.0μg ml^{-1} and just 16% showing any degree of susceptibility *in vitro*. Finally 100% of isolates of *C. psychrophila* examined in the present study showed resistance to the sulphadiazine/ trimethoprim combination with a modal score of 128μg ml^{-1}.

Plasmid Analysis

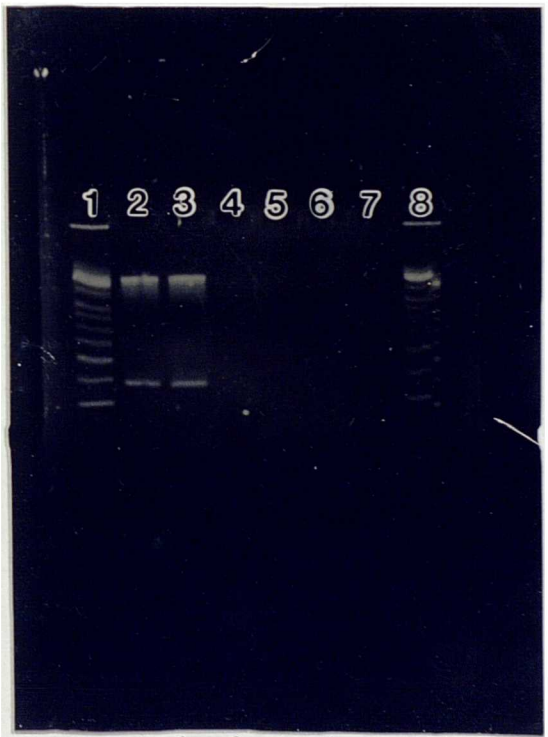
Analysis of the strains of *C. psychrophila* for the presence of plasmids revealed 4 distinct profiles from 42 of the 48 original strains used in this study (Figure 5.9 and Table 5.6). Unfortunately 6 isolates utilised in the determination of MICs were contaminated during the storage process and consequently had to be discarded. No plasmids were present in the majority of isolates tested (24/42 (57%)), whilst 15/42 (35.7%) of isolates including all of those from outbreaks of RTFS and cold water disease (CWD) in Denmark and Scotland, had one small plasmid of approximately 2.9kb. This profile was frequently visualised in isolates from outbreaks of RTFS in the UK where mortality levels were persistent and consistently high. It was also demonstrated in cells of *C. psychrophila* isolated from the viscera of dead and moribund fry from farm 1 (Chapter 2), and subsequently found in larger fish (up to table size) suffering from a condition analogous to cold water disease on farms that had previously received fingerlings from farm 1. Two isolates (4.7%) both originating from Finland had 2 plasmid bands, one at a similar molecular length to the previous profile at 2.9kb and a second at approximately 2.0kb. Finally the fourth plasmid

profile was found exclusively in the NCIMB 1947^T strain of *C. psychrophila,* where again just one plasmid band was visualised, the molecular length of which was estimated at between 2.9kb and 2.0kb. Details of the plasmid profiles demonstrated in individual isolates are given in Appendix 4.2. No plasmids were visualised in the NCIMB type strains, 2248 *Cytophaga columnaris*, 2215 *Cytophaga aquatilis*, 8628 *Flexibacter aurantiacus* or from *C. aquatilis* and *F. aurantiacus* isolated from fish and water samples during previous studies and routine bacteriological sampling at the laboratory. These isolates were included in the analysis for reference purposes only. All plasmid molecular weights were estimated by comparison with a supercoiled DNA ladder (2-16kb) (Sigma, Poole, UK), and therefore represent approximate molecular weights.

Figure 5.9 Agarose gel electrophoresis of selected strains of *C. psychrophila*



lane 1= A1; lane 2=bb693; lane 3=f167;
lane 4=f163; lane 5=fel10; lane 6=0278;
lane 7=1344; lane 8=ladder



lane 1=ladder; lane 2=u147; lane 3=u147;
lane 4=fel62; lane 5=jo121 lane 6=1947;
lane 7=bb8; lane 8=ladder

Table 5.6: Plasmid analysis of 42 isolates of *C. psychrophila* from outbreaks of RTFS and CWD from various geographical locations.

Plasmid kb	No. of Isolates							
	England	Scotland	Wales	Denmark	Finland	Tasmania	France	USA
C. psychrophila								
None	16	0	2	0	0	1	2	0
2.9	10	5	0	3	0	0	0	0
2.0-2.9	0	0	0	0	0	0	0	1
2.9+2.0	0	0	0	0	2	0	0	0
C. aquatilis								
None	1	0	0	0	0	0	0	1
F. aurantiacus								
None	1	0	0	0	0	0	0	1
C .columnaris								
None	0	0	0	0	0	0	0	1

Relationship between the occurrence of plasmids and resistance

No curing experiments were carried out on specific isolates giving high MIC values for any compounds as no correlation between the presence of plasmids and an increase in relative resistance to the chemotherapeutic agents screened in the current study was demonstrated. Details of the plasmid profiles for the isolates of *C. psychrophila* and other members of the Cytophagaceae tested in the present study are given in Appendix 4.2.

DISCUSSION

Resistance is always a relative term (Smith, Hiney, Samuelson and Lunestad, 1994), and minimum inhibitory concentrations obtained from *in vitro* testing do not unconditionally reflect an antimicrobial compound's potential therapeutic benefit in the field. However several techniques exist for assessing the *in vitro* efficacy of antimicrobials, each providing indications of a drug's possible effect. Frequently in both medical and veterinary laboratories, the disc diffusion method is favoured as it can rapidly provide information on the sensitivities of specific isolates. The success of

this method depends upon effective standardisation of the composition, pH, humidity and volume of the media, the incubation temperature, the method of production of the inoculum and the size and method of inoculation (Smith *et al.*, 1994). Slow growing, fastidious organisms such as *C. psychrophila* are not really suited to this method, due firstly to their inability to grow under the prerequisite conditions and secondly to the instability of the antimicrobial gradient, which becomes increasingly evident during extended incubation time. Testing of individual isolates from discrete outbreaks of disease provides information on the susceptibility profiles of specific bacterial populations. This enables recommendations for therapy to be made, but does not account for differences in subspecies or biotypes within a species, and hence may not reflect the sensitivity of a bacterial pathogen *per se*.

Another method of susceptibility testing which has recently received some interest from workers involved with fish pathogenic bacteria is the E-test. This method, which utilises a graduated strip containing predetermined levels of the compound is both versatile and easy to use, however currently it is predominantly designed for use with bacteria of medical significance and few of the antimicrobial agents available in the range are applicable to aquaculture (Barker, Page and Kehoe, 1995). Furthermore, the slow growth rate of cells of *C. psychrophila* means that, in common with the disc diffusion method, the E-test is not an entirely efficient method for assaying the antibiotic sensitivity of this organism.

An alternative procedure used to assay the *in vitro* efficacy of chemotherapeutants is the serial dilution technique using either liquid or solid media. Although more labour intensive and time consuming, both methods serve to provide immediate numerical values for MICs, and are considered to constitute a more accurate approach when the sensitivity of a strain is uncertain (Smith *et al.*, 1994). In this study, a broth microdilution technique was developed due to the lack of previous information on susceptibilities to antimicrobial compounds and the slow growth rate and stringent incubation requirements of *C. psychrophila*. Furthermore the use of 96 well microtitre trays reduced the practical difficulties involved with testing the large number of isolates examined.

To be of practical use, data generated by laboratory sensitivity tests must be related to the probable clinical efficacy of therapy (Smith *et al.*, 1994). In order that

values can be assigned to pertinent categories; i.e. susceptible, moderately susceptible or resistant, critical breakpoint levels need to be established for the specific organism-antimicrobial interaction. *C. psychrophila* has not been studied extensively and therefore no data on proposed breakpoints has been published. Standardised breakpoint concentrations which would distinguish susceptibility from resistance to antimicrobial compounds are not available for any bacterial fish pathogens. Therefore, to facilitate the interpretation of the results gathered in the present study MIC values (mean, mode, range, MIC_{50} and MIC_{90}) obtained for clinical isolates of *C. psychrophila* have been related to values demonstrated for the NCIMB 1947 type strain *C. psychrophila* (Table 5.3). Although this isolate is thought to be atypical, and may form a serotype (Chapter 8) distinct from the majority of *C. psychrophila* isolated from epizootics of RTFS and CWD, it represents a strain of the bacterium that has not been exposed to the levels and range of antimicrobial compounds experienced by *C. psychrophila* isolated more recently from clinical outbreaks of RTFS and CWD. Consequently, comparisons with the antibiograms obtained for the NCIMB 1947 and strains of the bacterium isolated from naturally occurring outbreaks of the disease, were used to help distinguish between the emergence of bacterial resistance as a result of antibiotic use and the inherent lack of susceptibility of the organism to particular compounds. Furthermore, comparisons between MIC data gathered in the present study and antibiotic susceptibility profiles for *C. psychrophila* in the published literature (Bernardet and Grimont, 1989; Schmidtke and Carson, 1994; Lorenzen, 1994) have been made. Finally, to aid interpretation of the results, published information on achieved serum and tissue levels of the seven classes of drugs in fish was taken into consideration (Katae, 1982; Alderman, 1988) and reports on clinical efficacy of the compounds were, where possible, also included in the appraisal.

The modal and range MIC values obtained for the strains of *C. psychrophila* predicted that the new generation 4-quinolones would prove effective in controlling outbreaks of RTFS and CWD. These agents are reported to have a broad spectrum antibacterial activity against both gram positive and gram negative bacteria, bactericidal activity at low concentrations, good absorption after oral or parenteral administration and a large volume of distribution (Bauditz, 1987, cited in Hsu,

Stroffregen, Wooster and Bowser, 1993). In a target animal safety study of enrofloxacin in fingerling rainbow trout Hsu *et al.* (1993) demonstrated no deleterious effects after oral administration of the compound at either 400mg/kg as a single dose or 50mg/kg/day for 30 days. Martinsen, Horsberg, Sohlberg and Burke (1993) showed that peak plasma concentrations, after oral delivery of sarafloxacin to Atlantic salmon, varied within a range of 0.08μg ml^{-1} to 0.70μg ml^{-1} and were dependent on the type of oil used as a drug vehicle. The modal MIC values obtained in the current study fall below the achievable serum levels and are indicative of the potential therapeutic benefits of these compounds in the field. However, the location of the pathogen in the host fish where the concentration of drug is required has not been determined for infections with *C. psychrophila* and therefore assumptions that plasma or serum levels of a compound form a reliable index of therapeutic efficacy should not be made. For example in CWD and the chronic form of RTFS found in larger fish, lesions are typically found superficially. In such cases the concentration of antimicrobial in the skin and muscle may be more appropriate than serum concentration.

The relatively low MIC values demonstrated for the older 4-quinolones including flumequine, oxolinic acid and a soluble salt of oxolinic acid, sodium oxolinate, suggested that they could be effective against infections caused by *C. psychrophila.* These findings are in accordance with several authors who have reported *in vitro* sensitivity of *C. psychrophila* to oxolinic acid (Wiklund *et al.*, 1994: Bustos *et al.*, 1994). Under fish farming conditions, achievable serum levels for oxolinic acid following oral administration of the drug are between 1.0-2.0μg ml^{-1} (O`Grady, Smith, Palmer and Hickey, 1986, cited in Smith *et al.*, 1994). However, despite the good absorption rates, a high degree of palatability and the bactericidal action against Gram negative organisms of oxolinic acid, very little success has been achieved in controlling naturally occurring outbreaks of RTFS. The reasons for this lack of efficacy in the field are far from clear, although a common characteristic behavioural sign of RTFS is inappetance which consequently reduces the intake of the drug by the fish and potentially results in lower plasma levels than would be expected in a fish that was feeding normally. Another factor that may possibly influence the apparent sensitivity or resistance, is unequal distribution of the compound on the feed during the medication process. This problem is particularly associated with relatively

low dosage rates for oxolinic acid (10mg/kg live fish weight/day) compared to other chemotherapeutic agents commonly used in aquaculture. The use of inert premixes may therefore increase the success of treatments. Variation in the chemical or physical state e.g. by altering the particle size, modifying the incorporation methods into diets and examining the various salts of oxolinic acid are topics worthy of investigation. Weis (1989) reporting on outbreaks of RTFS in Germany, acknowledged that effective treatment of the condition was difficult, but suggested that the 4-quinolone Flumequine was the most efficacious drug. Flumequine is not licensed in the UK for use in aquaculture and consequently no published evidence of its therapeutic effect is available.

Inglis, Soliman, Higuera Ciapara and Richards (1992) reported that plasma levels of 1.25μg ml^{-1} were achieved in Atlantic salmon parr after feeding amoxycillin coated diets at 80mg/kg body weight. In the same trial, artificially induced infections of furunculosis were effectively controlled by oral delivery of amoxycillin. The MIC value for the challenge isolate of *Aeromonas salmonicida* was 0.6μg ml^{-1}. The modal MIC values obtained for the majority of strains of *C. psychrophila* with this compound of less than 0.5μg ml^{-1} concur with the results achieved in the field, where oral administration of amoxycillin at 75-80mg/kg live fish/day for 7 days is generally clinically effective at controlling RTFS. However the extended range of MIC values observed in the current investigation may be indicative of the emergence of resistance to the compound and indeed the high MIC values obtained for isolates in this study corresponded closely with an apparent inefficacy of amoxycillin as a therapeutic agent on the farm from which they were isolated. Although relatively new to aquaculture and therefore unlikely to have been exposed to selection pressure, β-lactams such as amoxycillin have been used extensively in both veterinary and human medicine for many years (Inglis, Millar and Richards, 1993). It might be speculated that the transfer of resistance factors from bacteria of agricultural or human origin could have accounted for the perceived decrease in its therapeutic effect in the field, although the absence of any positive correlation between the occurrence of plasmids and elevated MIC values suggest that the apparent resistance is not plasmid related. Nusbaum and Shotts (1981) examined the action of selected antibiotics on four common bacteria associated with diseases of fish. They reported that resistance plasmids were

demonstrated in *A. hydrophila* and *A. salmonicida* having high minimum bactericidal concentrations (MBC) against oxytetracycline, but were not noticeable in the MIC studies. It was concluded from this that the R-factor involved in plasmid resistance was distributed throughout the population at a low frequency. The broth microdilution technique can easily be adapted to provide MBC values which may provide more information on the mechanisms of resistance observed for *C. psychrophila.*

Oxytetracycline and one of the newer longer-acting tetracyclines, doxycycline were selected for inclusion in this study as they have both been investigated for use in therapy of fish diseases. Doxycycline has shown clinical efficacy during epizootics in France (F. Desalle, pers. comm.). On the basis of the MIC values from this study it could be predicted that doxycycline would be of therapeutic benefit against RTFS, but again it is not licensed in the UK and no published information on its effect on clinical infections with *C. psychrophila* exists. Conversely, oxytetracycline has become the most commonly used therapeutic agent during outbreaks of RTFS and CWD. Snieszko (1964) reported that oxytetracycline incorporated into the diet at 50-75mg/kg fish/day for 10 days was effective at controlling CWD. Subsequently several authors have described the beneficial effects of treatments against both CWD (Holt *et al.*, 1993) and RTFS (Lorenzen, 1994). Despite these findings, the compound is now frequently administered at up to 300mg/ kg fish/ day for 10-14 days (Chua, 1991) often producing little discernible reduction in mortality. Only a minority of isolates showed sensitivity to the drug of less than 1.0μg ml^{-1}. The majority of MIC values were high and this, together with reports of the compounds declining efficacy against outbreaks of RTFS in the field over the last three years (R. de W. Harrison pers. comm.), indicate that acquired bacterial resistance to the compound is now presenting major difficulties. However, no association between the presence of plasmids and *in vitro* sensitivity was detected. Apparent reductions in bacterial susceptibility accompanying prolonged usage of antimicrobial compounds are not solely associated with the acquisition of R-factors and can be a result of selection of chromosomal mutation (Christofilogiannis, 1992). These changes in sensitivity are believed to occur due to alterations in the selective permeability of the membrane porins, present on the outer membrane of the cell envelope, in Gram negative bacteria. The selective pressure exerted by repetitive therapy with the same antimicrobial agent or

prophylaxis can therefore, increase the frequency of these "resistant" organisms within a population and diminish the response to chemotherapy. One further factor that may affect the action of oxytetracycline is the water hardness at individual fish farm sites. The tetracyclines are chelating agents, and as such may be inactivated by exposure to calcium and magnesium ions in the water and then in the bowel of the fish (Alderman, 1988). Whilst this probably has little relevance in the treatment of terrestrial animals it may have a detrimental effect upon the action of the compound in fish and partially explain some anomalies between *in vitro* susceptibilities and clinical efficacy of this class of drugs.

In the U.K. the use of chloramphenicol has been severely restricted with the intention of maintaining its medical availability (Alderman, 1988). Although the compound has been prescribed during outbreaks of RTFS in France, with reasonable clinical effect (Desalle, pers. comm.), its use is now prohibited in food producing animals under recent EC legislation (Commission regulation 1430/94/EC, MAVIS, 1994) which has placed chloramphenicol in Annex IV of regulation 2377/90/EC. No reports of *in vitro* or *in vivo* studies of the effects of thiamphenicol on *C. psychrophila* exist in the published literature. In this study the majority of isolates exhibited moderate sensitivity to both compounds, but relatively resistant strains of the organism were detected and high MIC values were shown by *C. psychrophila* originating from epizootics in Denmark. Bacteria resistant to chloramphenicol and thiamphenicol ordinarily produce a plasmid-mediated acetyltransferase which inactivates the compounds by the acetylation of their 3-hydroxyl groups (Shaw, 1967 cited by Fukui, Fujihara and Kano, 1987). However, no plasmids were visualised in isolates of the bacterium isolated from diseased fish in Danish fisheries.

The fluorinated analogue of thiamphenicol, florfenicol, is undergoing development for animal health use and has been marketed in Japan for the treatment of pseudotuberculosis and streptococcosis in yellowtail fish (Nordmo, Varma, Sutherland and Brokken, 1994). Unlike thiamphenicol and chloramphenicol, 3-fluoro derivatives are rarely inactivated by acetyltransferase and may as such prove potentially useful in the treatment of diseases in farmed fish where resistant bacteria are becoming increasingly prevalent (Fukui *et al.*, 1987). Studies *in vitro* have demonstrated the potent antibacterial activity of florfenicol against *Pasteurella*

piscidia, *Vibrio anguillarum* and *Edwardsiella tarda* and single oral administration of the compound at 25mg/kg was clinically successful in reducing mortalities in laboratory induced infections with these pathogens in eels (*Anguilla anguilla*) and yellowtails (*Seriola quinqueradiata)* (Fukui *et al.*, 1987). The results of the previous study reveal a high degree of correlation between *in vitro* and *in vivo* results; of the three "phenicols" examined in the present investigation, florfenicol gave the lowest modal MIC value, with all isolates demonstrating susceptibility, indicating that if it becomes available it promises to have utility on farms where outbreaks of RTFS are unresponsive to existing therapeutic regimes.

The macrolide antibiotics are principally active against Gram positive bacteria. Erythromycin has been found to have practical fisheries potential (Alderman, 1988), where it has been used in attempts to alleviate losses due to bacterial kidney disease (BKD) in salmonid fish and against streptococcosis in yellowtails, and vancomycin has been used in the therapeutic control of gaffkemia. The majority of strains examined in this study showed moderate sensitivity to erythromycin, whilst vancomycin displayed low bacteriostatic ability against the organism. It is however unlikely that this class of antimicrobials would provide any conspicuous therapeutic benefit against infections with Gram negative bacteria.

Holt and Conrad (1975) reported that Furanace (nifurpirinol), a nitrofuran derivative, administered by bath at 0.5μg ml^{-1} for 1 hour on every third day to fry followed by twice weekly treatments to the fingerlings provided protection against the CWD bacterium. Additionally Amend (1972) showed that immersion in 1.0μg ml^{-1} Furanace was sufficient for control of infections caused by *C. psychrophila.* In this study modal MIC values were obtained for the nitrofuran derivative, furazolidone of 16.0μg ml^{-1}. These inhibitory levels do not compare favourably with the figures of either Holt and Conrad (1975) or Ross and Smith (1972) who described MIC values for nitrofurans in the range 0.78-3.1 μg ml^{-1}. One distinct advantage however, of the nitroheterocycles, is that they are effective by bath application and therefore may have therapeutic effect in infections of yolk-sac fry prior to the development of the feeding response. Despite this property, on the basis of the current study, treatment of RTFS with nitrofurans is not recommended and interest in this class of chemotherapeutants

has fallen due to their oncogenic potential (Alderman, 1988) and most are or may be expected to be placed in Annex IV of EC Regulation 2377/90/EC.

Neomycin and gentamycin were included in this study, not for their therapeutic clinical properties, but for their potential as constituents in selective media development for *C. psychrophila*. Isolation of the organism on bacteriological media is often hampered by overgrowth of faster growing, less fastidious organisms, which frequently prevent accurate detection of the pathogen. Fijan (1969) demonstrated that inclusion of a combination of neomycin (5μg ml^{-1}) and polymyxin B (10 units ml^{-1}) into *Cytophaga* agar (Anacker and Ordal, 1959), revealed a higher incidence of discrete colonies of *C. columnaris* from ostensibly healthy channel catfish (*Ictalurus punctatus* Raf.). Unfortunately, the wide range of sensitivities apparent for the strains of *C. psychrophila* tested in this work implied that these compounds would not produce acceptable selectivity if incorporated as antibiotic additives to the growth media.

The final antimicrobial combination tested was the sulphadiazine/ trimethoprim combination sold under the trade name, Tribrissen. Wiklund *et al.*, (1994) reported *in vitro* sensitivity to the sulphonamides, but resistance to trimethoprim. Wood (1968) (cited in Amend, 1970) recommended oral administration of sulphonamides at between 220mg and 440mg/kg/day for the therapeutic control of CWD in salmonid fish. In the USA, sulphonamide-resistant strains of *A. salmonicida* were recognised within ten years of the start of its regular use to control outbreaks of furunculosis (Alderman, 1988). All strains of *C. psychrophila* examined, including the NCIMB 1947^T type strain, were considered to be resistant to this antimicrobial combination, and no evidence of the field efficacy of the sulphonamides could be substantiated. The sulphonamides are antagonists of dihydrofolic acid biosynthesis competing with the natural metabolite p-aminobenzoic acid. Bacteria that show sensitivity to the compounds are unable to absorb dihydrofolic acid (Alderman, 1988). The results of the current study may indicate an inherent lack of susceptibility to this class of compounds, potentially due to an omission of the biochemical pathways on which the drugs act. However, the sulphonamides were first used in aquaculture in the 1930s (Wolf, 1939), and historically several authors have reported on the therapeutic benefits of the sulphonamides against outbreaks of CWD in the USA (Amend, 1970;

Holt *et al.*, 1993). Consequently the type strain, which was originally isolated from diseased coho salmon in the USA, may have been exposed to selection pressure from use of the compound in fisheries prior to its deposition in the culture collection.

In summary, minimum inhibitory concentrations can be useful indicators of the probable clinical efficacy of particular therapy and the results of this study provide indications of antimicrobial compounds worthy of further examination for their use against outbreaks of RTFS. The emergence of bacterial "resistance" to the currently available compounds has become a serious problem within the rainbow trout industry and a wider range of therapeutic agents are required to help mitigate losses attributable to the condition. However, the administration of antibiotics forms a palliative rather than absolute solution and careful stock management, disinfection and vaccination will ultimately provide a more effective control strategy.

CHAPTER 6

THE USE OF DISINFECTANTS AGAINST *C. PSYCHROPHILA*

INTRODUCTION

It is always better to attempt to prevent rather than to treat an infection and as inappetance is a common behavioural sign of infectious disease in fish, and under commercial fish farming conditions most antimicrobial agents are administered orally, epizootics are often difficult to treat. Currently no commercially available vaccine exists against RTFS or CWD, and as infections with *C. psychrophila* often begin when fish are alevins and incapable of taking medicated food (Holt *et al.*, 1993), the practicality of a cure for both conditions is frequently problematic. Disinfection is therefore an attractive approach to attempt to prevent infection.

In natural waters, bacteria are commonly associated with surfaces where they can be attached to sediment particles and to suspended particulate matter. Bacterial extracellular polymeric substances (EPS) can occur as slime loosely bound to the adhering cell surfaces. These accumulations, known as microbial biofilms, can be found on virtually every surface that comes into contact with natural waters (Hintelmann, Ebinghaus and Wilken, 1993). Within a biofilm, bacterial cells are shielded from exogenous antibacterial compounds. Furthermore, particularly in areas of hard water, lime and other scale deposits on tank surfaces obstruct the effective removal of viable cells. Successful sanitation and disinfection of facilities associated with fish production would reduce the numbers of pathogenic bacteria in the fish environment, potentially mitigating losses in young non-immunocompetent stock.

Several authors (Borg, 1960; Bernardet, 1989; Holt *et al.*, 1993) have suggested that *C. psychrophila* may be found associated with the surface of the salmonid egg. The trade of eggs that takes place between USA, Europe and the Southern hemisphere may thus have facilitated the spread of the disease. Therefore effective disinfection of egg surfaces may both decrease transmission of this infectious condition and limit the exposure of newly hatched fry to the pathogen. Ross

and Smith (1972) demonstrated that 5 minute contact with $25mgl^{-1}$ available iodine was sufficient to kill $5x10^3$ cells ml^{-1} of *C. psychrophila.* In an examination of the *in vitro* activity of a neutrally reacting iodophor (Aktomar K30®) against *C. psychrophila,* Lorenzen (1994) showed that 20-80ppm available iodine effectively killed $1.4x10^7$cfu ml^{-1} during 10 min incubation at 15°C. However Holt *et al.* (1993) reported that iodophor treatment of ova did not prevent CWD in subsequent fry and the bactericidal action of the iodophors *in vivo* has been questioned by several authors (McFadden, 1969; Ellis, Cotton and Khowaja, 1993).

The aim of the present study was:

(1) To determine the minimum inhibitory and bactericidal concentrations for a range of antibacterial compounds, including a selection of those commonly utilised as aquacultural disinfectants and a variety of products exotic to fish production, but currently used in other areas of intensive farming.

(2) To establish realistic concentrations and contact times for these compounds for use against *C. psychrophila* on both equipment associated with fish production and on the external surfaces of eyed rainbow trout eggs.

(3) Then, on the basis of the results obtained, to make recommendations to the industry on additional methods of on site hygiene that may facilitate reduction in the mortality attributable to infections with *C. psychrophila,* and help prevent the spread of RTFS to currently disease free farm sites.

MATERIALS AND METHODS

Test organism and inocula

C. psychrophila (isolate U842) was isolated from the spleens of diseased fry immediately prior to the start of the trial to ensure that the study was made on a virulent fish-adapted (rather than cultured) strain. Cells were grown on MAOA, at 17°C for 4-5 days, purified by streaking and re-streaking onto fresh media. Three to four colonies were then inoculated into 100ml of MAOB in 250ml conical flasks and grown for a further 72-96 hours. Cells were separated by centrifugation and washed twice in 2 changes of PBS. The bacteria then were resuspended in fresh PBS, and the

concentration of the inocula was adjusted to an optical density of 0.2±0.01 at 520nm which corresponded to $1x10^8$ cells ml^{-1}.

Preparation of test compounds

Lifeline®, Macrokill®, Microquat®, formalin, chloramine-T, malachite green, Macrodyne® and compound A were supplied by C-Vet Livestock Ltd.. Compound B was provided by Vetrepharm Ltd.. Limox® was supplied by Interox Chemicals Ltd.. Wescodyne® was supplied by Th. Goldschmidt Ltd.. The glutaraldehyde used in the current study was obtained from Sigma Chemicals Ltd..

Test compounds were made up as aqueous solutions in 10ml of MAOB in glass universal bottles. Four replicate test concentrations were prepared for each disinfectant dilution. Eight dilutions were made for each compound. Where appropriate these corresponded to the manufacturers' approved levels of application for use in aquaculture, with 3 to 4 dilutions either side of the recommended dosage. Where compounds to be tested had no recorded application within the fish farming industry, standard concentrations were taken from the use of the product in other examples of agriculture e.g. its use in poultry, pig production, and again a series of dilutions was prepared to give a broad range of concentrations. To assess the effect of pH on the growth of the organism, solutions of MAOB were formulated in the range pH1.0 to pH13.0, with intervals of 0.5. The pH gradient was achieved by the addition of 10M NaOH to increase the alkalinity or by the addition of 2M HCl to raise the acidity.

The active ingredients contained in the base formulation of the products used in the current study are given in Table 6.1. The concentrations and types of disinfectant used in the current study are tabulated in Table 6.2.

Table 6.1: Active ingredient contained in base formulation of product used in this study

Test compound	Active ingredient(s)	Content in product
formalin	formaldehyde	38%w/v
Lifeline®	formaldehyde	8.73%w/v
	glutaraldehyde	5.03%w/v
	quaternary ammonium	13.58%w/v
glutaraldehyde	glutaraldehyde	25.0%w/v
Macrokill®	glutaraldehyde	12.0%w/v
	mono-propylene glycol	ns
Microquat®	benzalkonium chloride	45%w/v
Chloros®	free chlorine	10%w/v
Chloramine-T	free chlorine	0.1%w/v
compound A	2-bromo-2-nitropropane-1,3-diol	99.0%w/v
compound B	hydrogen peroxide	50%w/v
Limox®	peracetic acid	5.0%w/v
Macrodyne® (iodophor)	available iodine	4.5%w/v
Wescodyne® (iodophor)	available iodine	2.5%w/v
malachite green	malachite green	50.0%w/v

ns; not stated

Table 6.2: Concentration and types of disinfectants used in the current study

Test agent	Units and active ingredient	Concentrations of agents used in experimental protocols		
		protocol 1	protocol 2	protocol 3
pH		1,1.5,2,2.5,3,3.5,4,4.5,5,5.5,6,6.5,7,7.5,8,8.5,9,9.5,10,10.5,11,11.5, 12,12.5,13,	2,4,10,13	none
formalin	$\mu g\ ml^{-1}$ formaldehyde	0.4,4,20,40,380,760,1900,3800,7600,11400	3800,7600,11400	none
Chloros®	$\mu g\ ml^{-1}$ free chlorine	1,5,10,50,100,200,500	100,500,1000,2000	none
Wescodyne®	$\mu g\ ml^{-1}$ available iodine	25,50,100,200	100,200,400,1000,2000	200,2000
glutaraldehyde	$\mu g\ ml^{-1}$ glutaraldehyde	50,100,400,800	100,200,400	200,400
Lifeline®	% dilution of stock soln. formaldehyde, glutaraldehyde, quaternary ammonium	0.1,0.2,0.5,1,2,3,5,6	1.5,3,6	none
Limox®	$\mu g\ ml^{-1}$ peracetic acid	25,50,100,150	50,100,200	none
malachite green	$\mu g\ ml^{-1}$ active dye	.25,.5,1,5,25	1,25,50	none
chloramine-T	$\mu g\ ml^{-1}$ free chlorine	.5,1,2,3,10,50,100,500,1000	2,500,1000	none
Macrodyne®	$\mu g\ ml^{-1}$ available iodine	5,50,100,200	200,400,800,1600	200,1600
Macrokill®	% dilution of stock soln. benzalkonium chloride, glutaraldehyde, mono-propylene glycol	0.01,0.02,0.03,0.05,0.1,0.2,	0.05,0.1,0.2	0.05,0.1
Microquat®	$\mu g\ ml^{-1}$ benzalkonium chloride	45,450,900,1350,2250	225,450,900	450,900
compound A	$\mu g\ ml^{-1}$ 2-bromo-2-nitropropane-1,3-diol	0.25,0.5,1,5,10,20,25,50,100,200, 300,500,1000,2000,5000,10000	50,100,200,400, 1000,2000	100,1000
compound B	$\mu g\ ml^{-1}$ H_2O_2	1,50,3000, 10000	100,200,400,1000,2000, 4000,5000,	100,400

Experimental protocol (1): Determination of minimum inhibitory (MIC) and bactericidal concentrations (MBC) for *C. psychrophila* against a range of commercially available compounds

Two ml of the bacterial inocula were added to 3 replicates of each test dilution, 2ml of PBS were introduced into the fourth replicate concentrations, these were retained for standardisation of the optical density measurements and as a control to assess sterility of the system. Tubes were incubated at 17°C for 7 days, the culture suspensions were shaken three to four times a day to prevent the cells settling on the bottom of the bottles. Changes in the optical density of the liquids were monitored daily using a Shimidazu UV-2101PC scanning spectrophotometer, with reference to a standard curve for *C. psychrophila* (Figure 4.2; Chapter 4) so that changes in cell concentration could be determined. After 7 days incubation, a membrane filtration system (Chapter 3) was employed to detect viable cells of *C. psychrophila* from each disinfectant dilution. The contents of the tubes were filtered through pre-sterilised 0.45μm, grid-marked cellulose nitrate membranes (Whatman, England) using the three port filtration manifold connected to a vacuum source. Filters were washed *in situ* three times with three changes of PBS to remove residual disinfectant. The resultant membranes were inverted onto modified Anacker and Ordal agar (MAOA) (Lorenzen, 1993), and incubated at 17°C for a further 7 days. The minimum inhibitory concentration was determined as the highest dilution of antibacterial compound showing no growth. The minimum bactericidal concentration was determined as the concentration from which no viable cells were recoverable.

Experimental protocol (2): Determination of minimum lethal concentrations and exposure times for *C. psychrophila* against a range of commercially available compounds

The second experimental protocol was based upon an improved Kelsey-Sykes test for disinfectants (Kelsey and Maurer, 1974). Three replicates of 3 doubling dilution test concentrations were prepared in sterile, distilled water, for each disinfectant, based upon the MIC and MBC values obtained from the first trial and the

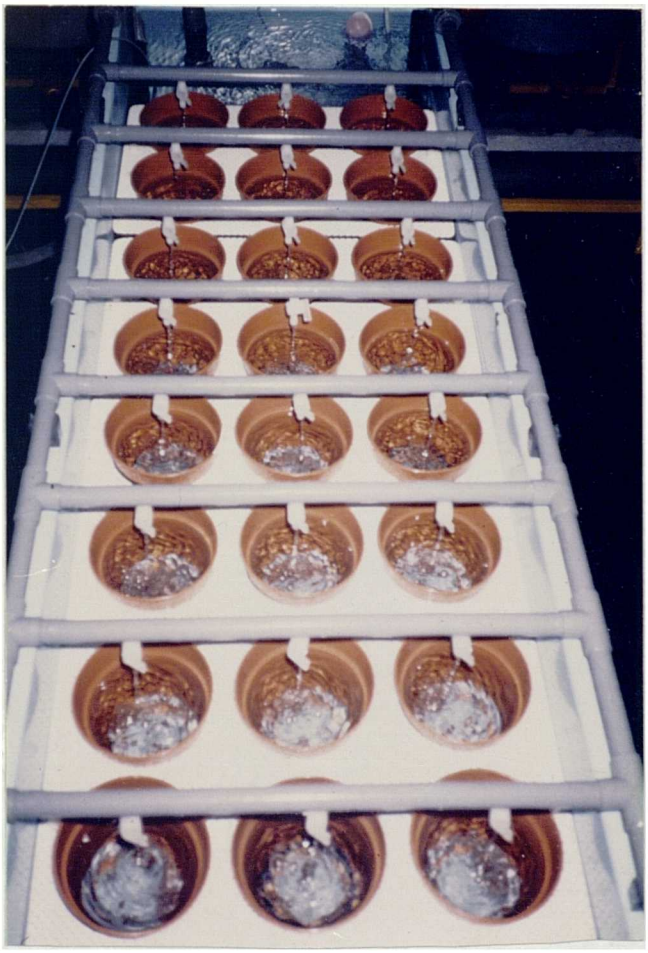
manufacturers' instructions. Two ml of the bacterial inocula were exposed to 10ml of the disinfectant solution for 2, 4, 8, 16, 20 or 40 min. At the end of the individual exposure time, test solutions were filtered using the filtered through pre-sterilised 0.45μm, grid-marked cellulose nitrate membranes (Whatman, England) using the three port filtration manifold system described in Chapter 3. As in experimental protocol 1, filters were washed in three changes of PBS to remove any residual disinfectant that may have inhibited growth. The resultant membranes were inverted onto modified Anacker and Ordal agar (MAOA) (Lorenzen, 1993), and incubated at 17°C for a further 7 days. After the incubation period colonies of yellow pigmented bacteria were enumerated.

Experimental protocol (3): Artificial infection of rainbow trout eggs with *C. psychrophila* and subsequent disinfection with a range of commercially available compounds

Nine thousand eyed rainbow trout ova were transported on ice from a disease free salmonid hatchery in the Isle of Man. On arrival at the laboratory, all eggs were disinfected with Wescodyne® at 200ppm for 10min. Three replicate samples of 10 eggs were removed immediately and checked for sterility by vigorous shaking in 5ml of PBS, and plating out the resultant diluent onto MAOA and tryptone soya agar (TSA) (Chapter 2), (detail of this method is described in Chapter 3 under egg sampling). The remaining eggs were immersed and gently agitated in a suspension of $1x10^8$cells ml^{-1} *C. psychrophila* in PBS for 1 hour at 10°C. At the end of the exposure time, all of the eggs were transferred from the bacterial suspension and rinsed thoroughly in PBS, a 3 replicate sample of 10 eggs was removed from the group and screened as before for the presence of bacteria colonising the egg surface. The remaining eggs were equally distributed throughout an egg incubation system (Figure 6.1) (100 ova/ pot). Compounds were chosen for inclusion in this study on the basis of their performance in the previous 2 trials and on information on their likely application as egg disinfectants. For each compound selected, 2 concentrations were made up in sterile, distilled water. Estimations of suitable concentrations and exposure times were made after analysis of the results obtained in the previous trials and

additionally on information supplied by the manufacturers of the various products. Batches of 100 ova were exposed to the disinfectants in 50mm stainless steel tea strainers for either 10 or 20min, by immersing the strainer in a plastic weigh boat containing the appropriate dilution. Eggs were agitated during the procedure to ensure uniform exposure to the compounds and thoroughly rinsed in 3 changes of PBS before being replaced in the incubating containers. Three aliquots of 10 ova were removed immediately and screened for the presence of viable cells of *C. psychrophila* using the egg sampling method. Eggs were maintained at 10°C for 10 days until hatching began. Eggs were monitored daily for mortality and the hatching success of each experimental group was recorded.

Figure 6.1: Experimental system used in protocol 3



RESULTS

The change in optical density, and therefore approximate alteration in cell concentration are expressed graphically in Figures 6.2 through 6.15.

Figure 6.2: Change in optical density with pH variation

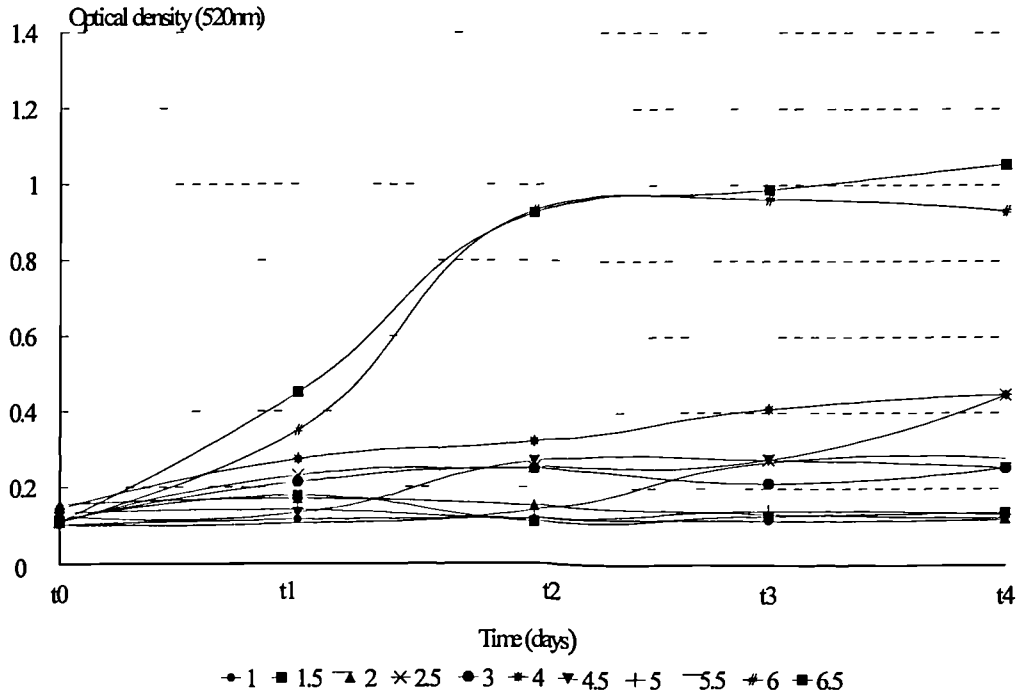


Figure 6.3: Change in optical density with pH variation

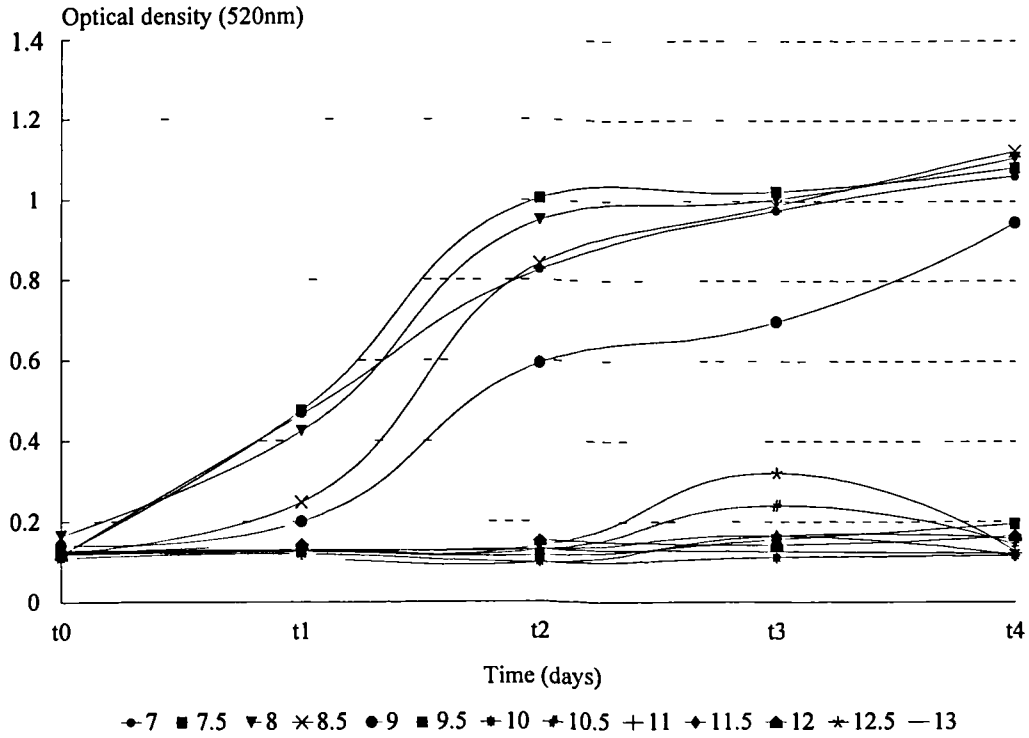


Figure 6.4: Change in optical density with variation in formaldehyde concentration

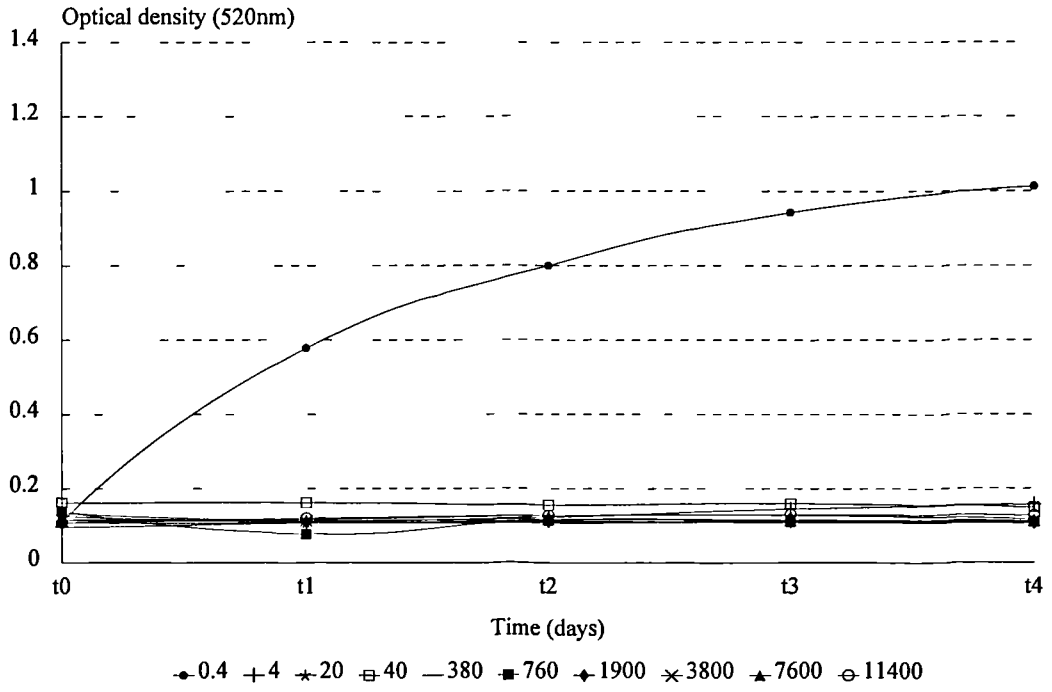


Figure 6.5: Change in optical density with variation in Chloros® concentration

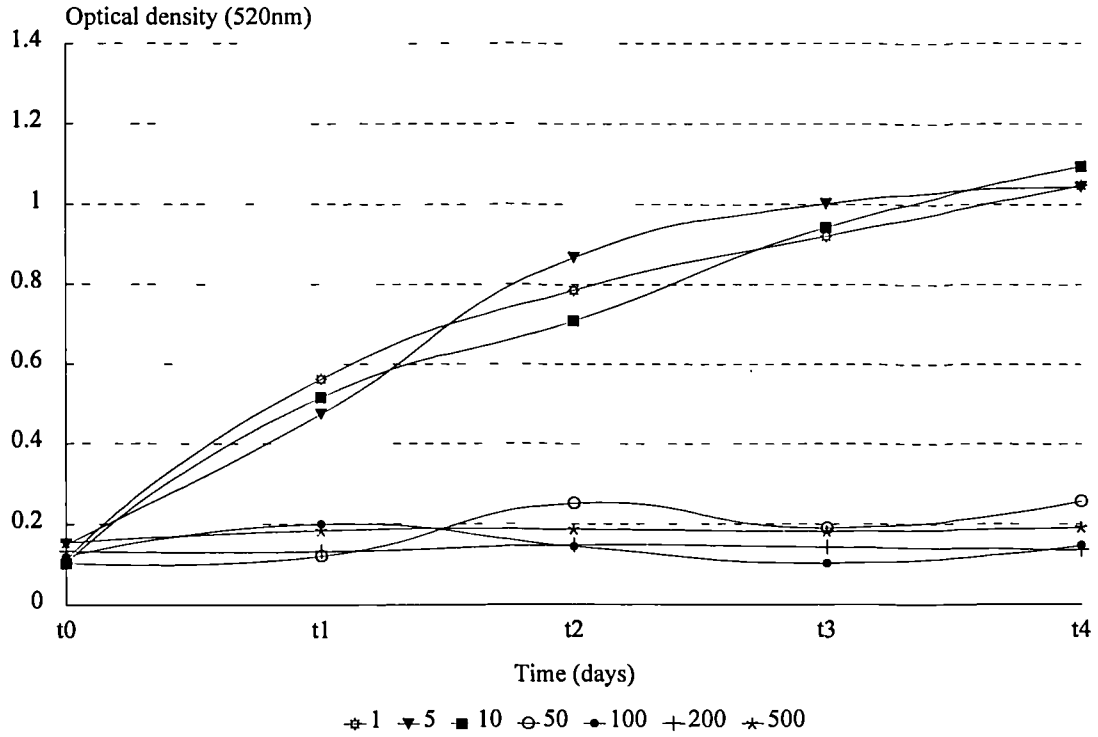


Figure 6.6: Change in optical density with variation in Wescodyne® concentration

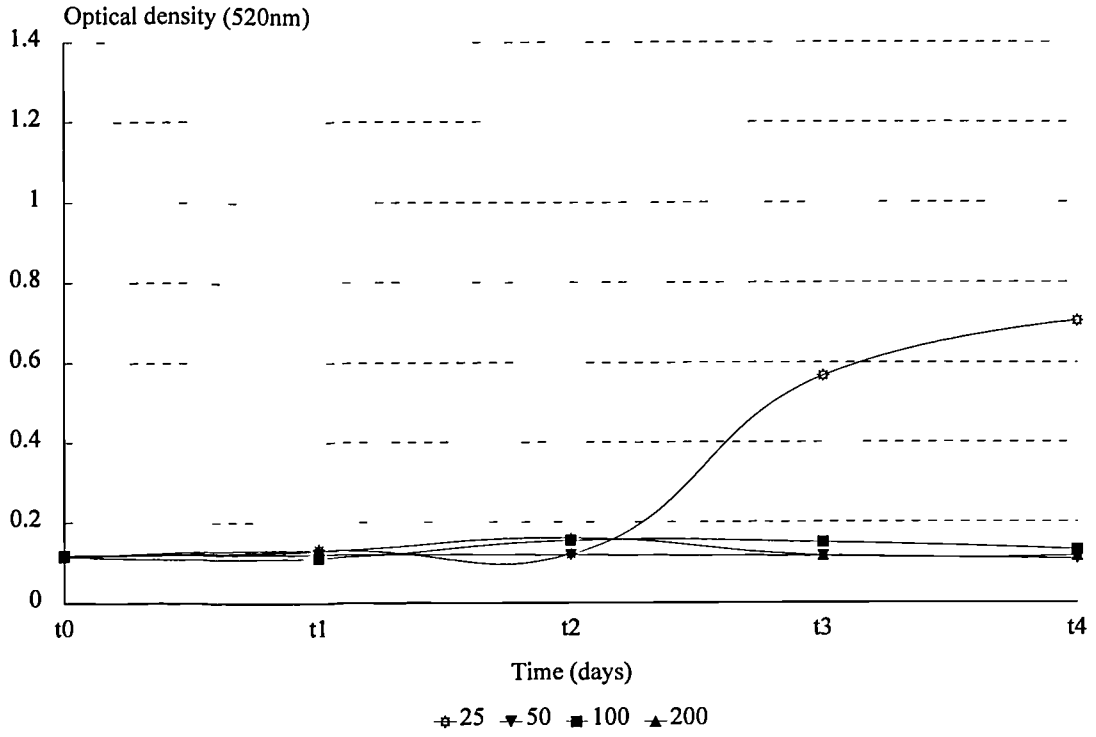


Figure 6.7: Change in optical density with variation in glutaraldehyde concentration

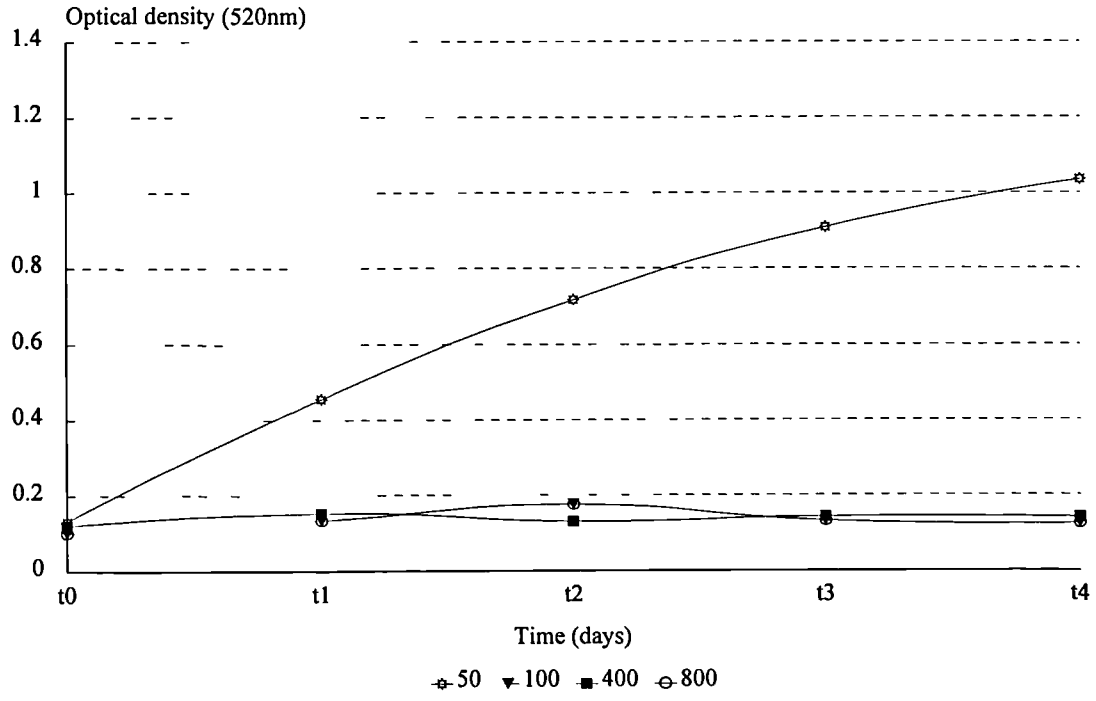


Figure 6.8: Change in optical density with variation in Limox® concentration

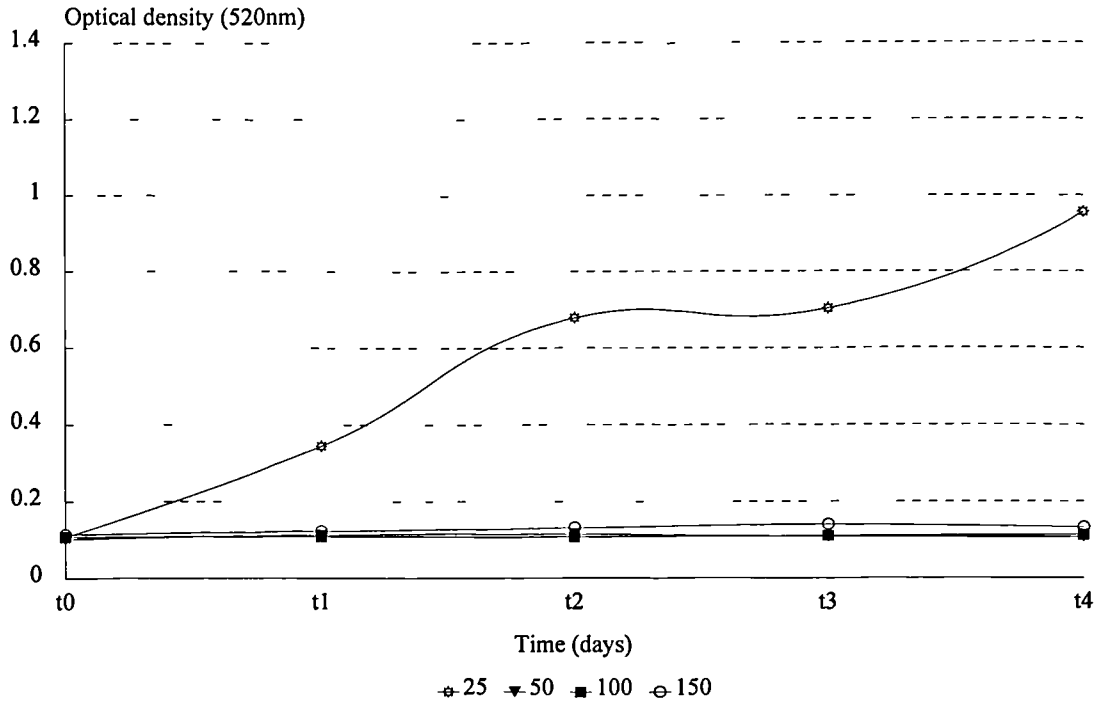


Figure 6.9: Change in optical density with variation in malachite green concentration

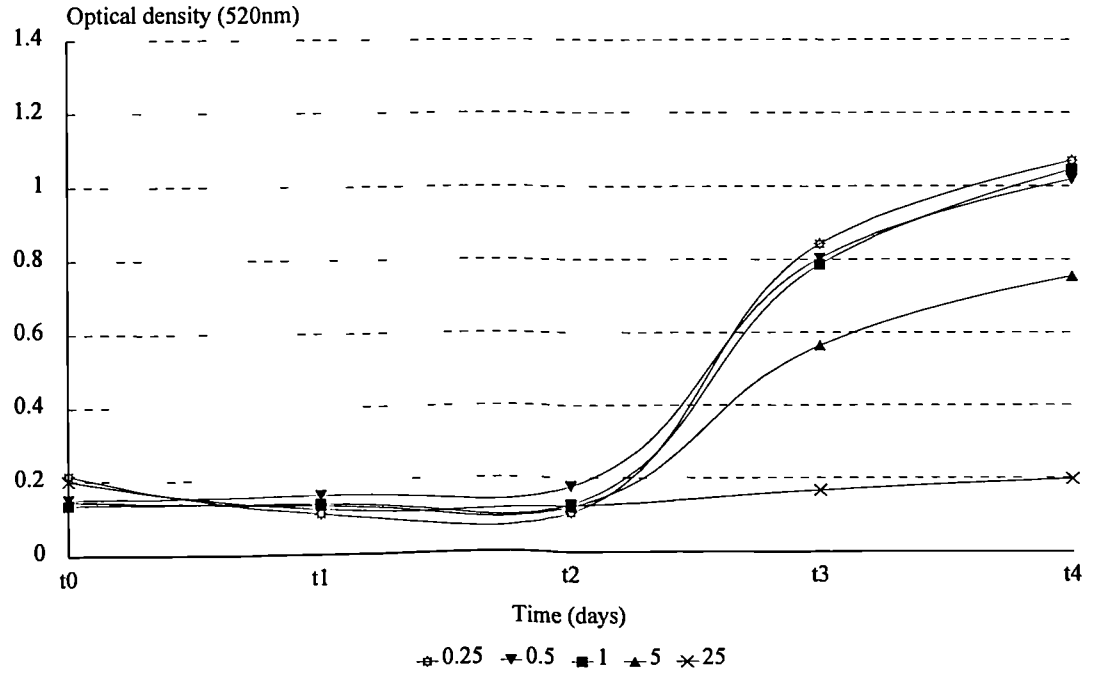


Figure 6.10: Change in optical density with variation in chloramine- T concentration

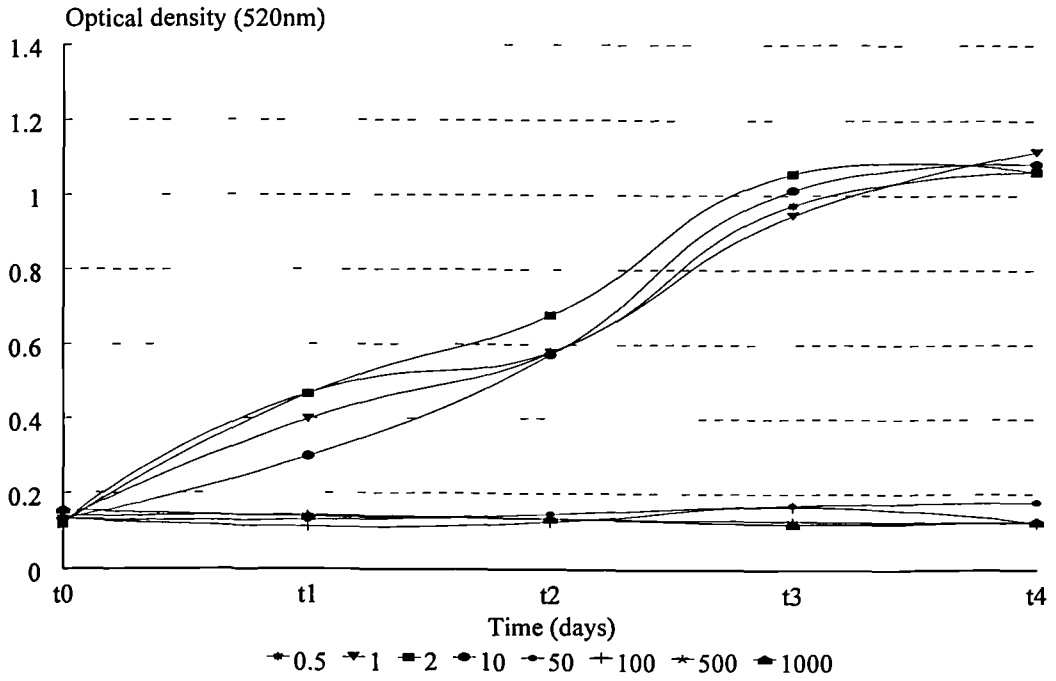


Figure 6.11: Change in optical density with variation in Macrodyne® concentration

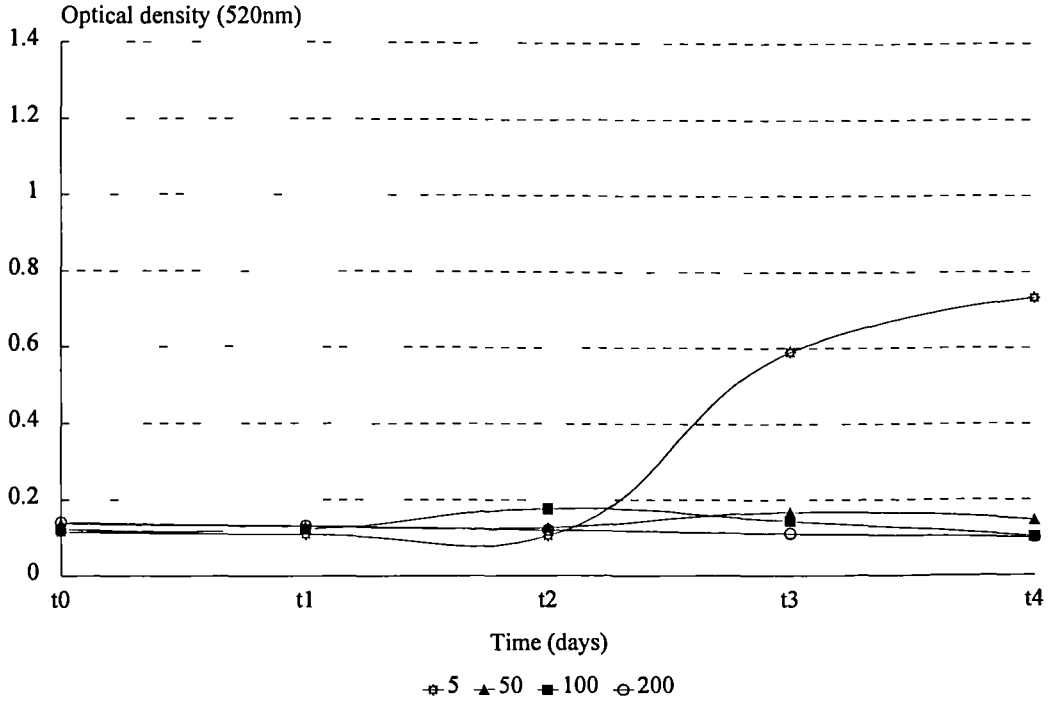


Figure 6.12: Change in optical density with variation in Microquat® concentration

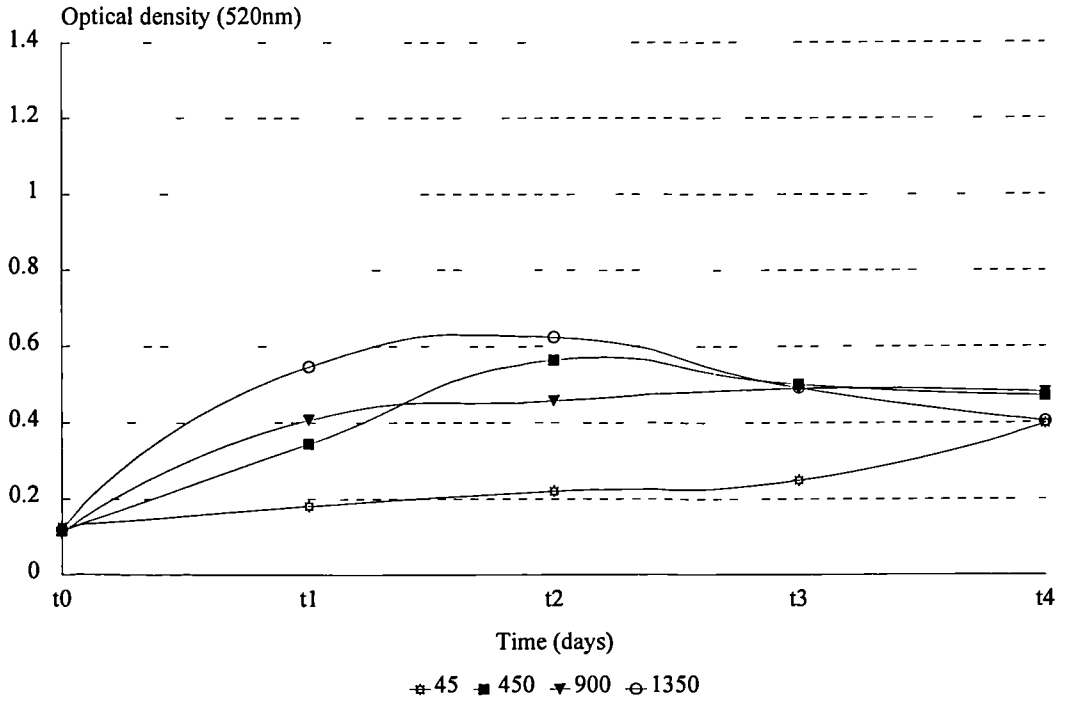


Figure 6.13: Change in optical density with variation in compound A concentration

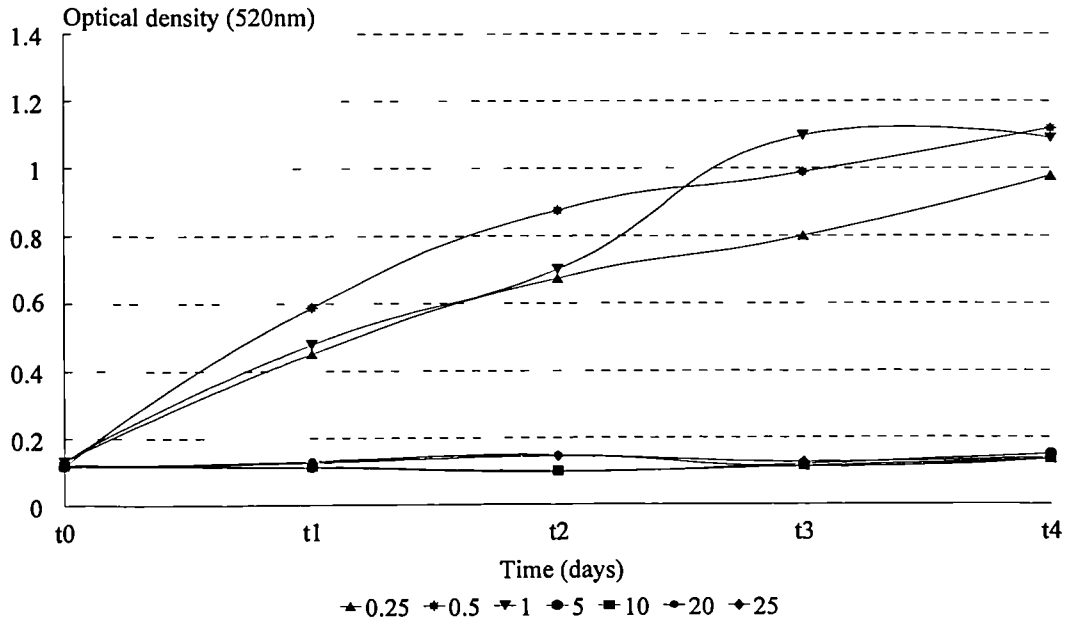


Figure 6.14: Change in optical density with variation in compound B concentration

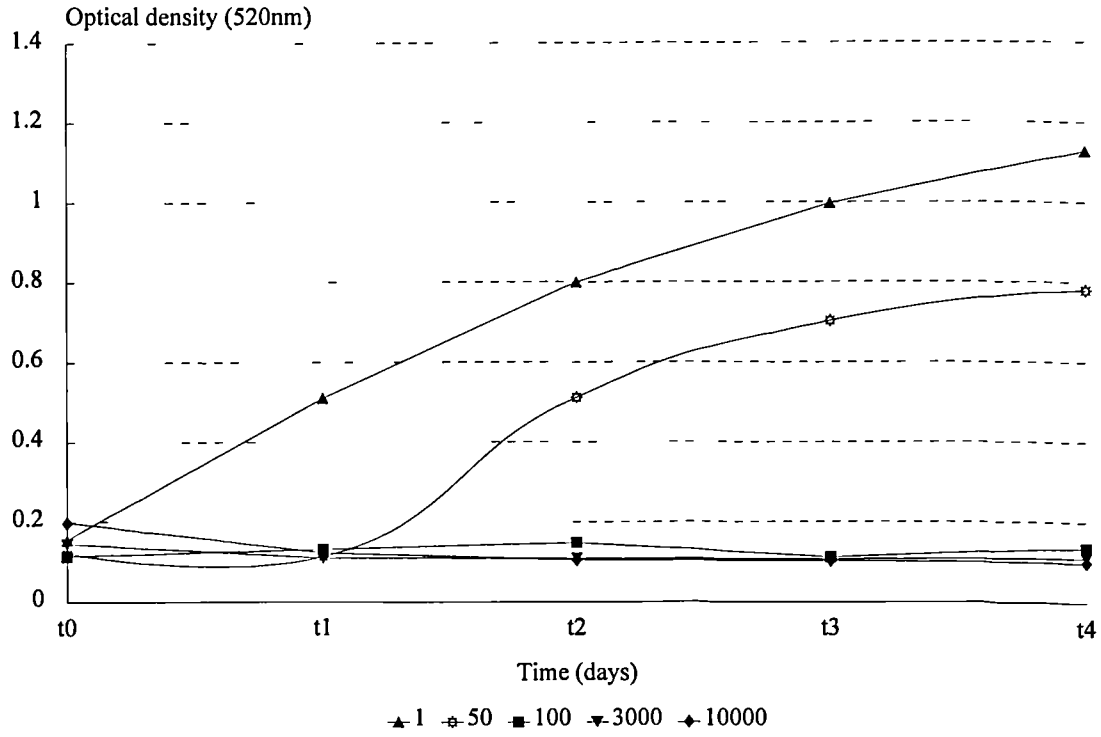
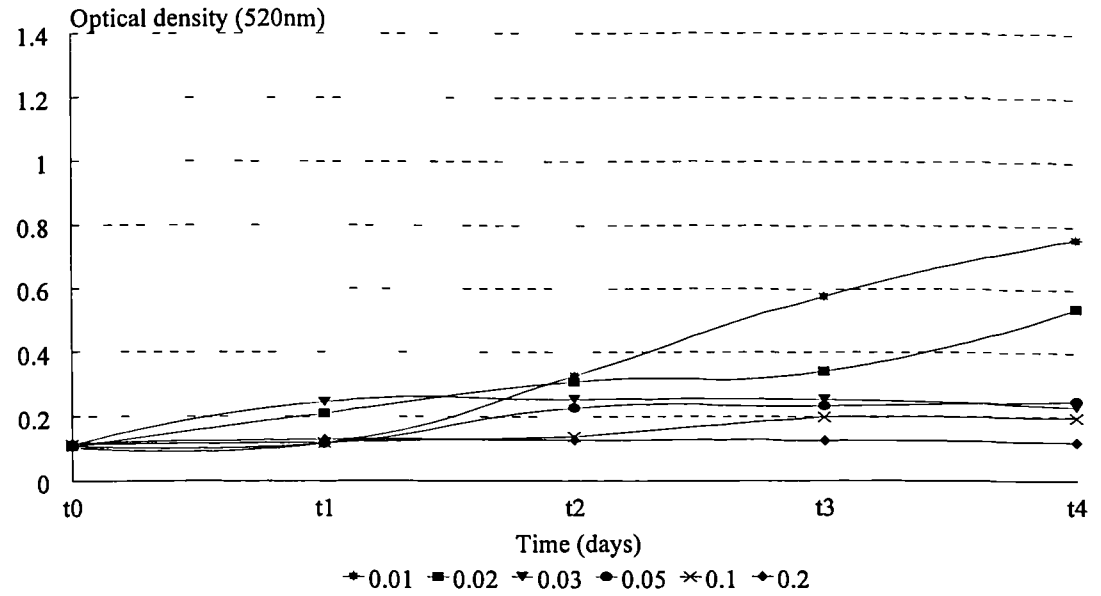


Figure 6.15: Change in optical density with variation in Macrokill® concentration



The minimum inhibitory concentration (MIC) level was taken as the highest dilution of antibacterial compound where growth did not occur. In this way MIC values for the 14 candidate compounds against *C. psychrophila* could be estimated. Additionally, using protocol 1 the viability of cells incubated with the various disinfectants was assessed. The results of these viability studies, on recovered cells following exposure, provided minimum bactericidal concentrations (MBC) for each compound against *C. psychrophila.* Table 6.3 shows the MIC and MBC values for each test substance. Where possible the manufacturer's recommended treatment regimes are included for comparison.

Table 6.3: Minimum inhibitory (MIC) and bactericidal (MBC) concentrations of antibacterial compounds by protocol 1 with recommended levels (RL).

Compound	MIC	MBC	RL
	($\mu g\ ml^{-1}$ active ingredient)		
formaldehyde (formalin)	4	20	7600≡2%formalin[1]
free chlorine (Chloros®)	50	100	1000-2000[1]
available I_2 (Wescodyne®)	50	200	250[1] 100[2]
available I_2 (Macrodyne®)	100	200	50-60[3]
glutaraldehyde	100	100	*na*
pH (acid)	5.5	3.5	*na*
pH (alkali)	9.5	10.5	>11[1]
Lifeline®	nt	30000	10000[1]
Microquat®	450	2250	500-600[1]
malachite green	25	25	1-2[2]
chloramine-T	50	500	1-2[4]
compound A	5	25	12.5-50[3]
compound B	100	200	100[4]
peracetic acid	50	50	15-20[5]
Macrokill ®(stock dilution)	0.03%	0.05%	0.5%[1]

[1] recommended level of use for equipment, [2] recommended level of use for eyed ova, [3] recommended level of use against *Pseudomonas* sp.,[4] recommended level of use for treatment of adult salmonids, [5]recommended level of use as bactericide, *na* no relevant information available

Under protocol 2, concentrations and contact times were established (Table 6.4), this data enabled the minimum lethal times at various usable concentrations to be determined for each of the test agents.

Table 6.4: Numbers of viable cfu following exposure to antibacterial compounds by protocol 2 (* confluent growth, + =no cfus)

Compound (ppm)	Exposure time (min)					
	2	4	8	16	30	40
formalin						
3800μgml^{-1}formaldehyde	*	29	+	+	+	+
7600	+	+	+	+	+	+
11400	+	+	+	+	+	+
chloros						
100μgml^{-1} free chlorine	43	+	+	+	+	+
500	+	+	+	+	+	+
1000	+	+	+	+	+	+
2000	+	+	+	+	+	+
Wescodyne®						
100μgml^{-1} available I_2	*	*	1540	770	1485	1650
200	*	880	935	550	342	825
400	275	440	165	330	285	220
1000	*	*	*	9081	1850	220
2000	129	64	12	+	+	+
glutaraldehyde						
100μgml^{-1}glutaraldehyde	*	1008	119	15	35	+
200	1428	714	1124	+	+	+
400	160	145	612	+	+	+
pH						
13	+	+	+	+	+	+
10	*	*	*	*	*	*
4	*	*	*	*	*	*
2	+	+	+	+	+	+
Lifeline®						
1.5% dilution	120	32	+	15	4	3
3.0%	+	+	+	+	+	+
6.0%	+	+	+	+	+	+

Table 6.4: continued **Numbers of viable cfus following exposure to antibacterial compounds by protocol 2** (* confluent growth, + =no cfu)

Compound (ppm)	Exposure time (min)					
	2	4	8	16	30	40
Microquat®						
225ppm	45	23	19	+	27	8
450ppm	+	+	+	+	+	+
900ppm	+	+	+	+	+	+
malachite green						
1μgml^{-1}malachite green	*	*	209	220	*	*
25	*	1530	189	1122	25	1024
50	*	612	+	+	21	+
Macrodyne®						
200μgml^{-1} I_2	223	34	109	343	79	17
400	+	+	+	+	+	+
800	+	+	+	+	+	+
1600	+	+	+	+	+	+
chloramine-T						
2μgml^{-1} free chlorine	*	*	*	*	*	*
500	+	4	+	+	+	+
1000	+	+	+	+	+	+
compound A						
50μgml^{-1} active	*	*	*	*	*	*
100	*	*	*	*	*	*
200	*	*	*	*	*	*
400	58	104	+	7	+	1
1000	15	+	+	+	+	+
2000	+	+	+	+	+	+
Macrokill®						
0.05% dilution	54	+	+	12	2	+
0.1%	+	+	+	+	+	+
0.2%	+	+	+	+	+	+
Limox®						
50μgml^{-1} paa	*	*	*	*	+	+
100	+	+	+	+	+	+
200	+	+	+	+	+	+

Table 6.4: continued **Numbers of viable cfus following exposure to antibacterial compounds by protocol 2** (* confluent growth, + =no cfus)

Compound (ppm)	Exposure time (min)					
	2	4	8	16	30	40
compound B						
100μgml^{-1} H_2O_2	*	*	*	*	*	*
200	*	*	*	*	*	*
400	*	*	*	*	*	*
1000	749	924	384	565	51	64
2000	22	334	285	180	34	65
4000	+	+	+	10	+	19
5000	+	+	+	+	+	+

From the results obtained in protocols 1 and 2, and from published information on previous studies with eyed salmon eggs (Gee and Sarles, 1942; Mcfadden, 1969; Alderman, 1984) seven of the original candidate compounds were selected for inclusion in protocol 3. Survival of eyed rainbow trout eggs after 10 days, following exposure to these disinfectants tested under protocol 3, is tabulated in Table 6.5, additionally the mean colony forming units representing the viable cells of *C. psychrophila* recovered from the egg surfaces post exposure to the compounds are included.

Table 6.5 **Survival of eyed rainbow trout eggs after 10 days following exposure to indicated levels of disinfectants for either 10 or 20 min with mean colony forming units (cfu) isolated from egg surface immediately post disinfection**

Compound	% Survival Exposure time (min)		Mean cfu	
	10	20	10	20
Glutaraldehyde concentration (μgml^{-1})				
200	91.3	nt	4.3	
400	94.3	nt	0.3	
Macrodyne® concentration (μgml^{-1} I_2)				
200	92.7	90.6	38	24
1600	1.4	nt	1.6	
Macroquat® (μgml^{-1} benzalkonium chloride)				
450	0	nt	0	
900	3.4	nt	1.3	
Macrokill®				
0.05% dilution	0.4	nt	0	
0.1%	0.6	nt	0	
Wescodyne® concentration (μgml^{-1} I_2)				
200ppm	90.4	91.2	*	*
2000ppm	1.2	nt	10.3	
compound A concentration				
100	96.4	97.2	14.3	4
1000	98.4	nt	8	
compound B concentration				
100	93.3	92	12	11.3
400	96.3	nt	12.3	
control (no disinfection)	98.7	nt	*	

nt= not tested *-confluent growth

These results, together with published information on toxicity (Gee and Sarles, 1942; Finlay, 1978; Schachte, 1979; Alderman, 1982; Smith, Gould, Zaugg, Harrel and Mahnken, 1987) enabled candidate compounds to be assigned to arbitrary groups describing their potential efficacy as either disinfectants for equipment associated with fish production and/or as candidates for the disinfection of eyed ova. A third group contained those compounds that promised to have little utility for use in aquaculture (Table 6.6).

Table 6.6: Arbitrary grouping of the agents used in the current study according to their potential as disinfectants against *C. psychrophila* in aspects of aquaculture

Disinfectant	Application		Treatment	
	Equipment	Eyed ova	Equipment	Eyed ova
formalin	+	-	1-2% ≥8 min	
Chloros®	+	-	0.5-1% ≥2min	
Wescodyne®	+	-	*200μgml^{-1}	
Macrodyne®	+	-	*200-400μgml^{-1} ≥2min	
pH (alkali)	+	-	≥pH13 ≥2min	
pH (acid)	+	-	≥pH2 ≥2min	
glutaraldehyde	+	+	200μgml^{-1} ≥20min	200μgml^{-1} ≥20min
Lifeline®	-	-		
Macroquat®	+	-	450μgml-1≥2min	
malachite green	-	-		
chloramine-T	-	-		
Compound A	-	+	*25μgml^{-1}	100μgml^{-1} ≥10min
Compound B	+	+	4-5000μgml^{-1} ≥8min	100μgml^{-1} ≥10min
Macrokill®	+	-	0.05-0.1% ≥8 min	
Limox®	+	-	50μgml-1 ≥30 min	

*likely to be effective as continuous bath

The MIC and MBC values for all agents, (excepting the formulated formaldehyde, glutaraldehyde, quaternary ammonium with non-ionic detergent, Lifeline®, and the benzalkonium chloride, Microquat®), fell below the manufacturers recommended level of use for the disinfection of equipment. Minimum bacteriostatic and bactericidal concentrations for the fungicide malachite green indicated that it would have little efficacy against *C. psychrophila* when applied at the levels recommended for the disinfection of eyed salmonid eggs. Likewise chloramine-T (frequently used to remove myxobacteria from the gills) appeared to have no antibacterial action at levels below $50\mu gml^{-1}$. The minimum lethal times demonstrated under protocol 2 indicated that, at the concentrations suggested by the suppliers, a contact time of ≥ 40min would be necessary to effectively kill cells of *C. psychrophila* following exposure to compound A and compound B. Similarly both of the iodophor based products Wescodyne® and Macrodyne® demonstrated limited bactericidal abilities following exposure for up to 40min at the MIC and MBC level. Chloros, formalin and the glutaraldehyde, benzalkonium chloride, mono-propylene glycol combination sold as Macrokill®, exhibited excellent bactericidal activity against *C. psychrophila* with 100% reduction in cell viability after exposure for 2, 8 and 40min respectively to 50% of the recommended application levels. Cells of *C. psychrophila* showed a relatively wide tolerance to pH variation with bacteriostatic action at 5.5 and 9.5, and viable cells remaining after exposure to pH 4-10. However under protocol 2, 2 minute exposure to pH 4 and 10 efficiently killed cells. Confluent bacterial growth was observed following contact times of 2,4,8 and 16 min with peracetic acid at the MIC and MBC level of $50\mu gml^{-1}$, however exposure times of ≥30min resulted in 100% reduction in cell viability. Glutaraldehyde demonstrated good bactericidal properties, with a MIC and MBC level of $100\mu gml^{-1}$, 30 min exposure to the minimum bactericidal concentration produced an approximately 7 Log reduction in cell viability and increasing the contact time to 40 min resulting in complete cell death. However, < 8 min exposure to 200% and 300% of the MBC level only resulted in between 5 Log and 6 Log reduction in cell viability.

Glutaraldehyde at both $200\mu gml^{-1}$ and $400\mu gml^{-1}$ was non-toxic to eyed rainbow trout eggs, with mean survival rates over ten days of 91.3% and 94.3%

respectively after exposure times of 10 min (Table 6.5). The mean colony forming units recovered from the egg surfaces following disinfection was 4.3 and 0.3 respectively compared with confluent growth of *C. psychrophila* from the artificially infected but non-disinfected control groups (Table 6.5). At the MBC of 200μgml^{-1} available iodine, both iodophors tested appeared relatively non toxic to the eggs with between 90% and 93% survival after exposure to the compounds for 10 and 20 min (Table 6.5). Macrodyne® which contains 6.0% phosphoric acid and is not recommended as an egg disinfectant, reduced the numbers of recoverable cells from the disinfected egg surfaces to mean levels of 38 and 24 colony forming units dependent upon the contact time, whilst Wescodyne® did not significantly influence the viability of the cells *in situ* when compared with the non disinfected control group. Both compounds caused over 98% egg mortality within 24 hours following exposure to 1600μgml^{-1} and 2000μgml^{-1} available iodine (Table 6.5). At the concentrations required to inhibit growth of *C. psychrophila* both Microquat® and Macrokill® caused between 96% and 100% egg mortalities after exposure for 10min, and were therefore not considered as potential candidates for use as egg disinfectants. Compound A demonstrated excellent bactericidal capabilities *in situ* at the MIC and MBC level with little evidence of toxic effect over the subsequent 10 day incubation period. Similarly compound B exhibited good antibacterial activity against the organism with no apparent deleterious effect upon the developing ova.

DISCUSSION

The three protocols utilised in this study provided information on the potential efficacies of various methods of disinfection which could be applied on a commercial fish farm. However, it must be emphasised that protocols 1 and 2 represent *in vitro* tests and that extrapolation of the results to *in vivo* situations must be treated with care.

Protocol 1 was based upon the standard macrodilution method of antimicrobial testing described by Thrupp (1986), but the fastidious growth requirements of *C. psychrophila* dictated modifications in experimental design. Modified AOB was used as the test medium although in common with Mueller-Hinton broth (Mueller and

Hinton, 1941), the undefined composition of the beef extract makes it a poor "reference" medium, particularly as successful secondary and tertiary growth of *C. psychrophila* is influenced by both the brand and lyophilisation of this constituent (Lorenzen, 1993). Many disinfectants are inactivated by organic material, although in a study of *in vitro* testing of fisheries chemotherapeutics Alderman (1982) suggested that interactions between the nutrient medium and antimicrobial agent may either increase or reduce fungitoxicity. In the current study no attempt was made to establish either the presence or significance of components antagonistic to the test agents within the medium. Provided that the test agents were not inhibited by the presence of the media, growth of the organism under the conditions of continuous exposure in protocol 1 indicated that the compound would have little efficacy in the field where transient exposure is the norm. Another factor which may have influenced the results in this study was bacterial "clumping", i.e. aggregations of bacterial cells formed in suspension as a result of interplay of hydrophobic and hydrophilic surface components. Viable cells present within the "clump" may be protected from exposure by the organisms present on the surface.

The experimental design of protocol 2 was based upon a modified Kelsey-Sykes test for disinfectants (Kelsey and Maurer, 1974). This method is used to estimate concentrations of disinfectants which may be recommended for use in hospitals both in the absence and presence of organic matter. Whereas the improved Kelsey-Sykes test uses a recovery broth (Oxoid CM67) containing 3.0% Tween 80 for isolation of surviving organisms, the stringent growth requirements of *C. psychrophila* necessitated the use of fresh MAOB and therefore several washing stages were essential to remove any residual disinfectant that remained adhering to the cells and that may have inhibited growth. Under protocol 2, test agents must either have penetrated or have been absorbed rapidly by the cells, and whilst the test does not give quantitative information on relative degrees of inhibition, indications of bacteriostasis can be recorded by observations of growth rates on filters, post exposure. This procedure provided data on lethal concentrations and contact times, and as such has more application to the field situation where practical constraints often limit exposure times.

Protocol 3 was designed to provide information on both the toxicity of selected compounds to eyed rainbow trout eggs and on their bactericidal effect against *C. psychrophila in situ.* It was not felt that cross contamination of the experimental eggs or any tank effect were significant factors influencing the results, as the incubation system enabled distinct batches of eggs to be treated and maintained discretely under identical conditions. Alderman (1984) observed differential toxicity of iodophors to salmonid eggs and suggested that a significant factor was the age of the egg within the hen. No information on the variation of the individual fish used to supply the eggs was available in this study.

Formalin (38%w/v formaldehyde) is commonly used in hatcheries for the treatment of ectoparasitic infections at doses of 167-250μgml^{-1} for up to 1 hour (Scott 1989b), but currently in the UK it is not in general use as a disinfecting agent for fish farm facilities. Wood (1974) (cited by Smith, Gould, Zaugg, Harrel and Maknken, 1987) reported that formalin toxicity was infrequent, although deleterious effects were observed in young salmonid fish suffering from bacterial gill disease when exposed to a 1:4000 dilution at water temperatures above 10°C. In addition Wedemeyer (1971), demonstrated that dilutions of 1:6000 caused a significant decline in the blood levels of Cl^-, Ca^{++} and CO_2 and interfered with the uptake of dietary vitamin C. Over long periods at temperatures of below 10°C paraformaldehyde can precipitate out of the solution. Rucker, Taylor and Toney (1963) reported that the toxicity problems observed particularly in rainbow trout, and associated with formalin use, were as a direct result of paraformaldehyde precipitation and could therefore be avoided by proper storage. The results obtained under protocol 1 in the current study indicate that at concentrations of 20μgml^{-1} formaldehyde, equivalent to a 1:19000 dilution of formalin, a bactericidal effect is produced upon *C. psychrophila.* Therefore theoretically, bath treatments with the compound would reduce the numbers of viable cells present on the fish gills and skin, consequently causing a reduction in infection. However, the information yielded from protocol 2 clearly indicated that exposure to 1.0% formalin ($\equiv$3800μgml^{-1}), for at least 8 minutes, would be required to eliminate the bacterium. Whilst these concentrations would be unacceptable for therapeutic use in aquaculture, formalin is used as a disinfecting agent for equipment associated with fish production in Denmark at 1.0-2.0% (Lorenzen, pers comm.). Provided that the

manufacturers' safety recommendations are strictly adhered to, it may have some utility in the UK. As with all fish farm chemicals, concerns over the impact of discharge on aquatic life have been raised. Formaldehyde is not persistent and is unlikely to bioaccumulate. Therefore, despite a lack of chronic data and a wide variation in acute toxicity to freshwater invertebrates, a tentative environmental quality standard (EQS) for the use of formaldehyde in fish farming has been set at $5\mu gl^{-1}$, as an annual average, for the protection of freshwater life (Jerman and Wilkinson, 1993).

Lifeline® marketed by C-Vet, is a combination of formaldehyde, glutaraldehyde, quaternary ammonium and non-ionic detergent. It is a broad spectrum germicidal agent, recommended for terminal disinfection of *Escherichia coli,* salmonellae, staphylococci, *Aspergillus,* and *Mycoplasma* in livestock houses and hatcheries. In this study, Lifeline® exhibited some bactericidal activity against *C. psychrophila* at the endorsed level of application of 1:100, however a 300% increase in the concentration of the product was required to reduce cell viability. Use of Lifeline® at this level is not recommended and as a consequence it has little utility as a disinfecting agent against *C. psychrophila.*

Conversely, the results of this study demonstrated that glutaraldehyde promised to have considerable application as a disinfectant of both equipment and the surfaces of eyed ova. In a large scale field trial in Norway, Holmesfjord, Gulbrandsen, Lein, Refstie, Leger, Harboe, Huse, Sorgeloos, Bolla, Olsen, Reitan, Vadstein, Oie and Danielsburg, (1993) achieved good results for disinfection of egg surfaces, and demonstrated enhanced hatching success in Atlantic halibut (*Hippoglossus hippoglossus*). Gorman, Scott and Russel (1980), reported that glutaraldehyde destroyed Gram negative bacteria by a combination of a partial sealing or contraction of the lipoprotein components of the cell envelope and inactivation of certain cell wall associated or periplasmic enzymes which appear to be crucial to cell viability. This mechanism effectively seals the cell, inhibiting the transport of essential nutrients across the cell envelope. Furthermore, Gorman *et al.*, (1980), reported that the glutaraldehyde molecule does not require extensive penetration to achieve its effect. The inability of disinfectant molecules to penetrate the cell envelope of *C. psychrophila* may be due to the extracellular polysaccharide layer which surrounds

cells of *C. psychrophila* (Chapter 7). It is speculated that this complex surface structure may contribute to the relative ineffectiveness of some of the disinfectants tested in the current study which deactivate bacteria by the oxidation of internalised enzyme systems, and may offer a partial explanation of the apparent efficacious action of glutaraldehyde.

The active ingredients in Macrokill 200®, a synergistic surface active residual germicide manufactured by C-Vet, include glutaraldehyde. The compound is reported to be predominantly anti viral but also has bactericidal and fungicidal properties. It is marketed as a persistent agent for the disinfection of livestock accommodation following a prewash to remove gross soiling. In this study Macrokill 200® demonstrated excellent biocidal effect against *C. psychrophila* at concentrations considerably under the recommended level of use at easily obtainable contact times. It therefore has potential as a terminal disinfectant of *C. psychrophila* on equipment associated with fish production. However, as demonstrated under protocol 3, Macrokill 200® is extremely toxic to rainbow trout eggs at these concentrations. The suggested method of application for Macrokill 200® is by pressure spray and it is recommended that even well ventilated buildings treated with the compound should not be entered until the sprayed material has dispersed (2-3 hours after application). No EQS is currently available for glutaraldehyde or novel formulations of the agent, and extreme care must be taken when disposing of the product.

The quaternary ammonium compound marketed as Microquat® contains the active ingredient benzalkonium chloride at 45%w/v. It is recommended for the terminal disinfection of Gram positive and Gram negative bacteria, including *Salmonellae* and is commonly utilised as a combined detergent steriliser for pre-washing poultry houses and equipment at dilutions of between 1 in 800 and 1 in 1000. In aquaculture, benzalkonium chlorides have been used as disinfectants of nets, clothing and hands (Finlay, 1978) and in the therapeutic control of bacterial gill disease where *Cytophaga*-like bacteria multiply within a film of mucus on the gills. Treatment with between 1 and $10mgl^{-1}$ is believed to inhibit bacterial growth and lift off mucus (Scott, 1989b). Dorson and Michel (1988) reported that bacteria were completely inactivated by exposure to $100mgl^{-1}$ for 2 minutes and, although this result was not always achieved at doses of $10mgl^{-1}$, the myxobacteria tested in the study

appeared very susceptible to the compound with some inactivation observed at $1mgl^{-1}$. The results obtained using protocol 1 showed that Macroquat® had good bacteriostatic ability in continuous exposure trials, although by this method the MBC was considerably higher than the dose required to neutralise bacteria after the limited exposure conditions of protocol 2. This anomaly may have been attributable to reduction in the biocidal properties of the compound with time.

Chlorine is probably the most extensively used disinfectant in fisheries management and is conveniently available as a solution of sodium hypochlorite which is diluted with water to give a final concentration of 1.0-2.0%, corresponding to 1000-$2000\mu gml^{-1}$ free chlorine (Finlay, 1978). The mechanism of chlorine inactivation of bacteria is thought to be by oxidation of the sulphydryl groups present in enzyme systems close to the cell wall (Fauris, Danglot and Vilagines, 1986). It is suggested that resistance to chlorination observed in bacterial spores and acid fast bacteria is due to insufficient penetration by the chlorine molecule (CES report, 1988). In the present study, continuous exposure to $50\mu gml^{-1}$ free chlorine was sufficient to inhibit growth of *C. psychrophila* and $100\mu gml^{-1}$ effectively killed all bacterial cells. However, results of protocol 2 demonstrated that an exposure greater than two minutes to the bactericidal concentration of free chlorine was required to totally inactivate the organism. This may be related to the time required for the chlorine molecule to traverse the mucopolysaccharide "slime" layer and cell envelope of *C. psychrophila*. Transient exposure to the recommended levels of Chloros® produced 100% reduction in cell viability, and therefore it should be effective for use in the field. However the high chemical reactivity of chlorine and its ability to react with organic material by oxidation, substitution and/or addition (Kinman, Black, and Thomas, 1970) determine that disinfection will only be truly efficient if clean, dry surfaces are treated, ensuring satisfactory exposure of the pathogen to the compound. Chlorine and its derivatives are extremely toxic to fish, and although free chlorine is rarely detected in treated effluents, exposure to residual mono and dichloramines formed in the presence of ammonia results in severe respiratory stress and death in salmonids (Brungs, 1973). It is therefore essential that residues are thoroughly rinsed from the disinfected items before they are brought into contact with fish.

Chloramine-T is widely used as a prophylactic agent in fisheries management, predominantly aimed at infections caused by *Cytophaga*-like bacteria, *Costia, Trichodina, Ichthyophthirius multifiliis* and *Gyrodactylus* (Scott, 1989b). The action of the compound is based upon gradual breakdown to hypochlorous acid and consequent release of oxygen and free chlorine. The results of this study demonstrate that it has no bactericidal or bacteriostatic effect on *C. psychrophila* at the safe level of usage in fish and whilst it may have some therapeutic benefit by the removal of bacteria found loosely adhering to the gills, it is unlikely to have utility as a terminal disinfectant against *C. psychrophila.*

Iodophors have both bactericidal and anti-viral properties and have good pathogen/host differential of toxicity (Alderman, 1984). In the UK, iodophors are recommended for disinfecting equipment and eggs at 200μgml^{-1} and 100μgml^{-1} respectively for 10 minutes (MAFF, 1985). Unlike chlorine, iodine reacts slowly with organic material and does not form iodamines in the presence of ammonia. The efficiency of iodophor disinfection is, however, pH related. At a low pH, iodine remains predominantly in its more active elemental form, whilst as pH rises it is increasingly present in the form of hypoiodous acid which has lower penetrative ability. Therefore the relative hardness of water influences its effectiveness negatively. Several authors have advocated the use of organic iodine compounds for the disinfection of fish eggs to reduce the risk of transfer of major viral and bacterial pathogens (McFadden, 1969; Wright and Snow, 1975; Schachte, 1979; Alderman, 1984). Ross and Smith (1972), demonstrated that exposure to 25μgml^{-1} Betadine® or Wescodyne® for 1 minute neutralised $1.4x10^6$ cells of *C. psychrophila.* Additionally Lorenzen (1994) reported that 20-80μgml^{-1} available iodine deactivated $1.4x10^7$ $cellsml^{-1}$ in 10 minutes. However, Lorenzen (1994) was unable to inactivate *C. psychrophila* present on rainbow trout egg surfaces and Holt *et al.*, (1993) reported that iodophor disinfection of the surfaces of eyed Atlantic salmon eggs did not prevent outbreaks of cold water disease in the resulting fry. The results of this study are in accordance with these findings, and indicate that whilst iodine has a biocidal effect upon *C. psychrophila in vitro* and consequently is useful for surface disinfectants i.e. foot baths and wheel dips, at the doses non-toxic to eyed salmonid ova, application of iodophors to artificially infected egg surfaces does not effectively remove the bacteria.

The reasons for this apparent inefficacy are not clear although they may be related to the adhesion or location of the pathogen on the egg, poor penetrative ability of the iodine complexes and morphological and biochemical properties of the bacterial cell wall. Further studies on the *in vivo* interactions of the organism and the availability of the iodine molecule are required before this can be fully elucidated

In the brewing and dairy industry, peracetic acid (PAA) is used increasingly as a terminal disinfectant in precleaned stainless steel storage, transfer, process vessels and soak tanks. The major advantage of the peracids, which contain the -CO OOH group is that they are non-foaming water soluble liquids, which are fast acting, non-derivatising, which break down to environmentally acceptable, innocuous decomposition products. Furthermore the activity of PAA is retained in the presence of hard water, and it is little reduced by organic contamination such as blood, serum, casein, yeast extract or faeces (Fraser, 1986). The mechanism of disinfection is thought to be through disruption of the sulphydryl and disulphide bonds within enzyme systems, which form important components of the bacterial cell wall. Dislocation of the chemiosmotic function of membrane transport causes rupture of cell walls and affects intracellular solute levels and prevents cellular activity (CES Report, 1988). The results of the present study suggest that PAA has a considerable biocidal effect on *C. psychrophila* and may consequently have some utility in fisheries management. It is however extremely harmful to fish, and whilst it is reported that residual levels of the compound are highly unlikely to be encountered (CES Report, 1988) the potential for toxic effects arising from exposure to unreacted PAA exist and extreme care should be taken with its application.

The active ingredient product referred to as compound B marketed by Vetrepharm is the oxidator hydrogen peroxide (H_2O_2). The action of H_2O_2 is similar to PAA in that it attacks cell membranes, however, unlike peracids, it is detoxified by the enzyme, catalase. Quimby (1981) reported that no residues were detectable in environmental samples 4 days post treatment with between 1 and 4mM H_2O_2 and no toxic effect was observed in fish after continuous exposure to the compound. In a study on the potential efficacy of hydrogen peroxide against the salmon louse (*Lepeophtheirus salmonis*) Johnson, Constible and Richard (1993), observed epithelial lifting, tissue necrosis and congestion of the primary and secondary gill lamellae in

Atlantic and chinook salmon following 30 minute exposure to $10.0gl^{-1}$. However the toxic effect of the compound diminished with a decrease in ambient water temperature. In this study, compound B demonstrated potential as both a terminal disinfectant of *C. psychrophila* on surfaces and as a biocidal agent for use on rainbow trout eggs at below the levels of administration recommended for safe use in fish.

The arylmethane organic dye malachite green had negligible bactericidal effect against *C. psychrophila* at the recommended level of application. Currently the compound is used prophylactically and as a treatment of fungal infections in both salmonid eggs and fish. Carcinogenic and teratogenic effects associated with malachite green have been reported and consequently use of the compound may soon be prohibited (Scott, 1989b).

It is suggested that compound A, 2-bromo-2-nitropropane-1,3-diol, (C-Vet Livestock Ltd.), may form a safe, effective replacement in the event of the removal of malachite green from the list of chemicals available for use in fish. The mode of action of the compound has not been fully elucidated but research has indicated that the antibacterial activity results from blocking of thiol containing enzymes such as membrane bound dehydrogenase. Alterations to cell membrane structure have been observed associated with cell leakage and destruction (C. Muller pers. comm.). The MIC and MBC values as demonstrated under protocol 1 in the current study show excellent bacteriostatic and bactericidal action against *C. psychrophila* within the recommended safe levels of use. Additionally the product appears to be non-toxic to rainbow trout eggs at these levels and to have no deleterious effects upon the hatching success of fry. However, under protocol 2, increased concentrations were required to achieve complete neutralisation of the cells. This may be due to the antibacterial active ingredient requiring a relatively long contact time. Despite this apparent drawback the results achieved in the present study indicate that compound A would have considerable potential as a disinfectant against *C. psychrophila,* particularly on the surfaces of eyed eggs.

The results presented here suggest that exposure of *C. psychrophila* to a pH below 3.5 has an excellent biocidal effect, however no information on the use of acid conditions to remove harmful bacteria in fisheries management could be found in the published literature. In contrast the use of sodium hydroxide, especially in conjunction

with the detergent Teepol (which increases the penetration) against bacteria, viruses and protozoa is well documented. It is recommended for use in earth ponds as it is not inactivated by organic material to any great extent. Provided that the pH of the disinfecting solution is retained above 10.5 this method has good effect against the organism. Additionally it is of very low toxicity to fish and therefore would have utility in systems where fish are held downstream from holding facilities requiring disinfection.

It is important to emphasise that the results presented in this thesis have largely been obtained from *in vitro* testing of the candidate compounds and therefore can only serve to provide indications of their potential field efficacy. Proteinaceous accumulations and the formation of microbial biofilms on the surfaces of tanks, together with the deposition of limescale on the surfaces associated with fish production, limit the effectiveness of even the most promising compound tested in the laboratory. Since only a "clean" surface can be completely sanitised, careful removal of excess material is a prerequisite of successful disinfection.

CHAPTER 7

CLINICAL, HISTOPATHOLOGICAL AND ELECTRON MICROSCOPIC ASPECTS OF RTFS

INTRODUCTION

The histopathological changes of infections attributable to *C. psychrophila* were first described by Wood and Yasutake (1956) in coho salmon suffering from cold water disease (CWD). The condition was characterised by acute septicaemia with effects seen particularly in the buccal cavity and renal elements of the kidney. *C. psychrophila* was successfully isolated from vascular tissues, including secondary gill lamellar capillaries, kidney, spleen and heart. In heavily infected fish the causative organism was also identified in the peritoneum, swim bladder, liver, intestinal muscularis layers and the pancreas. Wolke (1975) reported that gross lesions attributable to infection with *C. psychrophila* were generally confined to ulceration of the skin, muscle and fins, although the causal agent could frequently be recovered from kidney and spleen tissues. Wolke (1975) also observed infiltration of lymphocytes and macrophages as the epidermal and dermal lesions progressed. In accordance with these findings Borg, (1960) noted a localised mild mono-nuclear infiltration of macrophages in tissues of fish suffering from CWD. Little information exists in the published literature on the histopathological changes associated with rainbow trout fry syndrome (RTFS). Bruno, (1992) observed weakly stained gram negative filamentous rods within the spleen, liver, kidney and trabecular layer of the heart. Focal necrosis of these organs has been described, together with increased vacuolar degeneration, pyknotic nuclei, necrotic hepatocytes and a slight increase in haemosiderin in the liver (Chua, 1991). Eosinophilia of the kidney tubules has also been observed (Bruno, 1992). Chua, (1991) described the most common and prominent changes in affected fish in the spleen, with the splenic capsule replaced by an eosinophilic layer consisting of cellular and acellular fibrin-like material. This layer was frequently seen extending into the surrounding peritoneum and pancreatic tissue. Localised fibrino-haemorrhagic peritonitis of the spleen extending into the peritoneum and abdominal fat was observed. Filamentous bacteria could be seen associated with inflammatory cells at the periphery of the spleen. Severe congestion of the splenic

stroma, depletion of the haemopoetic tissue components, pyknosis and karyorrhexis in the sinusoids and intercellular oedema in the reticular tissue were reported.

In accordance with authors' observations on CWD (Wood and Yasutake, 1956; Borg, 1960), Bruno, (1992) reported that lateral skin lesions displayed necrosis, collapse, pyknosis and lymphocytic infiltration of the dermis and underlying muscle blocks, although conversely Chua, (1991) stated that deeper layers of the skin were unaffected in cases of RTFS. Chua, (1991) described pericarditis, myocardial and endocardial degeneration in acutely infected fry, but no other information on the histological changes in the heart is available in the published literature. Additionally, several authors have noted the presence of filamentous bacteria in the blood vessels of gill tissue, or located loosely around the gill arch (Chua, 1991; Bruno, 1992).

The aim of the present study was to examine the histological changes at light microscopic level associated with natural and artificial infections of RTFS, and to undertake an ultrastructural electron microscopic examination of selected tissues.

MATERIALS AND METHODS

Moribund rainbow trout fry were collected during 1992/93 from several hatcheries throughout the UK that had experienced mortalities attributable to RTFS. Additionally, 0.5-1.5g, dead and moribund rainbow trout from intraperitoneal and immersion laboratory challenge studies were examined and post mortem examinations were made.

Fresh spleen and kidney imprints and blood smears were air dried, fixed in 5% acetic-methanol for 5 minutes and stained with May-Grunwald Giemsa and haematoxylin (Shandon activity No. 3) and eosin (H&E).

Material for light microscopic examination was fixed in 10% neutral buffered formalin (NBF) for at least 24 hours. Embedding of the tissue to paraffin wax was undertaken with the aid of a vacuum infiltration processor (VIP 2000, Miles Laboratory Equipment Inc., Mishawaka, USA.). Blocks were sectioned at a thickness of 4-5μm using a Leitz rotary microtome, sections were stained with haematoxylin (Shandon Activity No. 3) and eosin (H&E), May-Grunwald Giemsa or Martius scarlet blue (MSB)

Primary fixation of tissues for electron microscopy was achieved in 4% glutaraldehyde in 0.1M cacodylate buffer (pH7.2) for 2 hours at 4°C, followed by several

washes in 0.1M cacodylate buffer. Secondary fixation was carried out in 1% osmium tetroxide in 0.1M cacodylate buffer. The fixed tissues were then washed thoroughly in 0.1M cacodylate buffer and dehydrated through an ascending gradient of alcohols from 50% through 100%. After two changes of propylene oxide, used to achieve miscibility with the resin, the tissues were embedded in an epoxy resin and cured at 60°C for a minimum of 24 hours.

Semi-thin sections (>1μm) were cut using a glass knife and stained with 1% toluidine blue in 1% aqueous borax. Relevant tissue blocks were re-trimmed and ultra-thin sections were cut and collected on uncoated copper grids, double stained with saturated aqueous uranyl acetate and Fahmy`s lead citrate (Lewis and Knight 1977). The stained grids were examined in a JEOL 100CX electron microscope at 100kV.

RESULTS

Clinical signs

Fry affected with the syndrome appeared lethargic, and congregated close to the water inlets and outlets, often "hanging" at the water surface (Figure 7.1; 7.2). The feeding response was severely reduced or absent. In artificially infected fry, erratic, spiral swimming behaviour was occasionally recorded.

Gross pathological changes

Commonly, darkening of the skin, bilateral exophthalmia (Figure 7.3) and distension of the abdomen were observed. Subcutaneous haemorrhage was frequently observed in the region around the anus, and occasionally a mucoid discharge was visible trailing from the anus. Reddening of the mouth, tongue, jaw area and operculum was also regularly observed. The gills of affected fish were extremely pale (Figure 7.4). Ulcerated "saddleback"-like lesions were present on the dorsal and ventral surfaces of some chronically affected fish, extending through into muscle tissue (Figure 7.5; 7.6).

On post mortem the most prominent feature of affected fish was severe hypertrophy of the spleen, which was often 10 times the normal size and extremely friable (Figure 7.7). The surrounding peritoneum and fat was frequently haemorrhagic. Fry were typically very pale and occasionally enlarged kidneys, grey livers, and colourless, fragile, intestines were

observed (Figure 7.8). The caudal region of the intestine was often inflamed with petechial haemorrhage around the anus.

Histopathological lesions

The histopathology associated with infections of RTFS varied with the progression of the disease, and depended upon the method of infection i.e. natural or artificial via the intraperitoneal route. Spleen squashes from fry exhibiting clinical signs of RTFS during natural outbreaks of the disease consistently revealed numerous filamentous bacteria (Figure 7.9). The bacteria were frequently seen enclosed in vesicles within phagocytic-like cells. Conversely, little evidence of inflammatory cell infiltration or bacteria was demonstrated in squashes from the kidneys of moribund fry or from spleens and kidneys of artificially infected fry.

Natural infections in rainbow trout fry were characterised by changes in the spleen. Initially severe hypertrophy of the spleen and loss of border definition was observed (Figure 7.10). Varying degrees of peritonitis were seen, extending from the outer surface of the spleen. Within the spleen there was a loss of the dense appearance of the splenic pulp and oedematous changes in the red and white pulp gave rise to the appearance of gaps in the stroma. Typically these changes led to the irregular staining appearance of the tissue (Figure 7.11). Varying degrees of cell degeneration were apparent in more advanced cases of the infection. Deeper into the stroma severe congestion was frequently seen, additionally cells showing evidence of karyorrhexis and pyknosis were commonly present in the sinusoids, although these changes were difficult to photograph.

Using the Martius scarlet blue stain (MSB), fibrin-like material and reticulin fibres could be seen deposited throughout the splenic tissue, indicative of a red blood cell destruction and a subsequent healing response (Figure 7.12). In some cases, especially from slightly larger, more chronically affected fry and fingerlings, massive haemorrhage was observed within the spleen, with no evidence of any splenic pulp remaining. In such cases it was not possible to clearly identify individual bacteria with the haematoxylin and eosin stain (H&E) due to the accumulation of necrotic cell debris and the deposition of fibrin-like material which stained in a very similar manner to filamentous bacteria.

Figure 7.13 shows a transverse section of a rainbow trout fry 3 days after intraperitoneal injection with $1x10^5$cells ml^{-1} of *C. psychrophila*. Within the peritoneal

cavity, infiltration of red blood cells and peritonitis affecting the pancreatic tissue was observed. Pyknotic cells were observed in the pancreas together with occasional vacuolation of the pancreatic acinar cells. Material which appeared to consist of bacteria-like cells and cellular debris could be seen surrounding the spleen tissue (Figure 7.14). In experimentally infected fry the splenic capsule generally appeared normal, however an apparent accumulation of inflammatory cells was sometimes observed at the periphery of the spleen. In some cases these cells appeared to be undergoing degenerative changes. The capsule of the liver of artificially infected fry was frequently disrupted (Figure 7.15). Cellular debris possibly including bacterial cells could be seen surrounding the liver capsule. In addition necrosis of individual cells was apparent within this debris but no marked inflammatory response was observed. Few changes were seen within the liver, although in one fish suffering from a chronic infection the formation of haemopoietic tissue was evident adjacent to liver sinusoids (Figure 7.15). Bacteria-like cells were observed on the outer surfaces of the swim bladder, with adjacent bacterial spread in the kidney. Peritonitis was also evident in affected fry but no obvious inflammatory response was evident in the gut tissues. In addition bacteria-like cells were observed in major blood vessels demonstrating the potential for the organism to spread by the haematogenous route.

Degrees of pericarditis were evident in both naturally and experimentally infected fry, but frequently the changes were not severe and often limited to localised areas of cellular infiltration and vacuolation of the heart muscle. Occasionally these changes were accompanied by the deposition of fibrin-like material interlaced with degenerative inflammatory cells in the outer layers of the heart muscle.

No other organs were consistently affected although some changes in the skin were observed. Slender filamentous bacteria were sometimes seen associated with ulcerated areas of the skin although an inflammatory response was not marked. Likewise the gills showed no significant pathological changes, although an increased number of chloride cells was found in some cases.

Electron microscopic observations

Figures 7.16 and 7.17 show the ultrastructure of *C. psychrophila* within infected spleen tissue from rainbow trout fry exhibiting classical signs of disease during a natural outbreak of RTFS. The bacterial cell wall appeared to be typically that of a Gram negative organism, showing a lipopolysaccharide outer membrane, an underlying peptidoglycan layer

and a cytoplasmic membrane surrounding a densely staining nuclear region. Additionally a "fuzzy" electron-opaque region was visible surrounding the cells. Figures 7.18 and 7.19 show phagocytic activity of a granulocyte-like cell in which bacterial cells can clearly be seen enclosed within phagocytic vesicles surrounded by refractile granules. The splenic cellular response appeared to include lymphocytes, plasma cells and eosinophils with the latter present in the greatest numbers. Extensive lysosomal activity was observed in the splenic tissue although in the majority of samples little evidence of an effective phagocytic response was observed and numerous filamentous bacteria were present in the tissue with extensive cellular degeneration causing the appearance of "holes" in the cytoplasmic membrane (Figures 7.20 and 7.21).

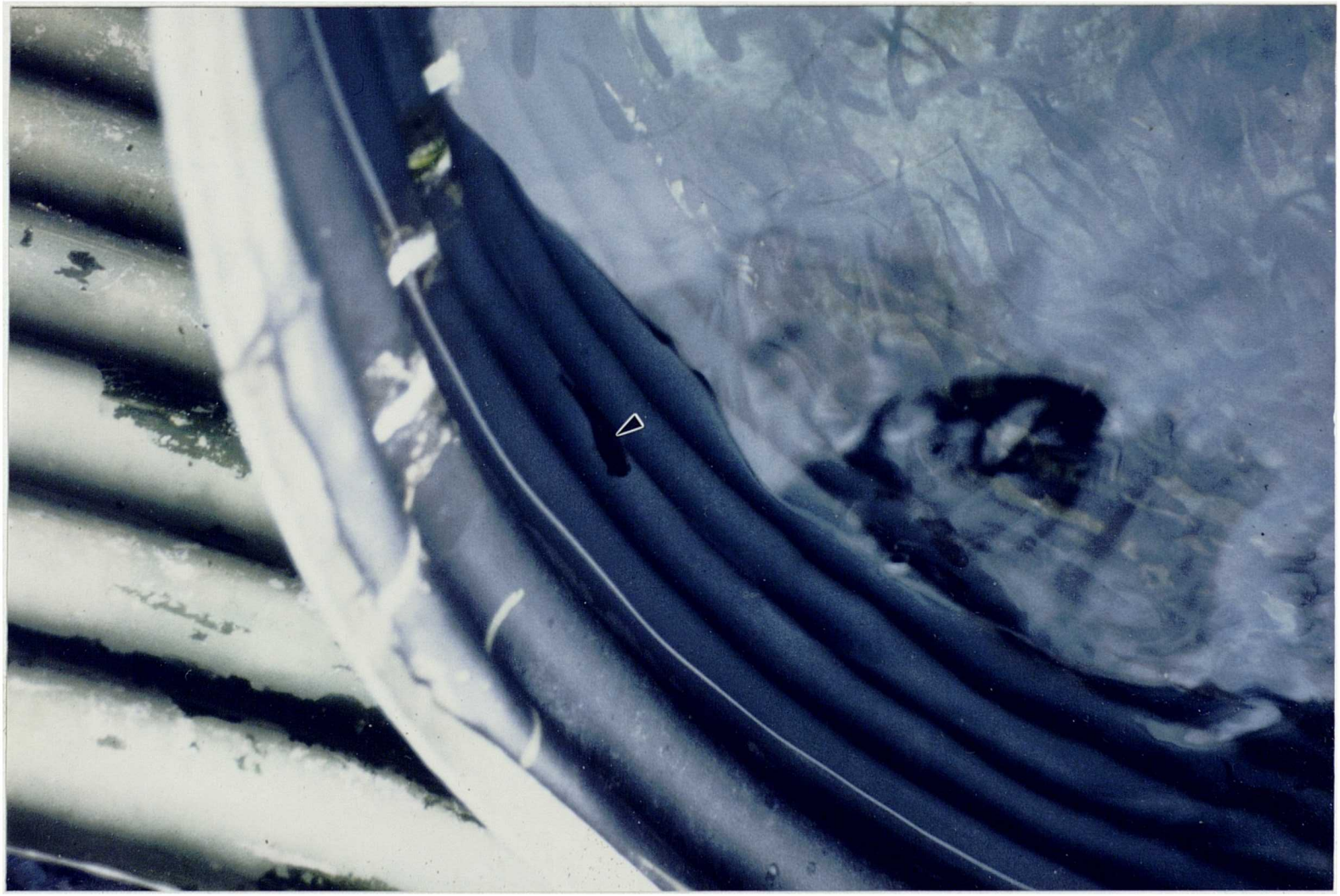


Figure 7.1: Rainbow trout fry (≈3.0g) (arrowed) showing typical behavioural signs of RTFS which include lethargy and swimming close to the sides of the tank

Figure 7.2: Rainbow trout fry (≈3.0g) (arrowed) showing darkening of skin and "hanging" at the water surface.

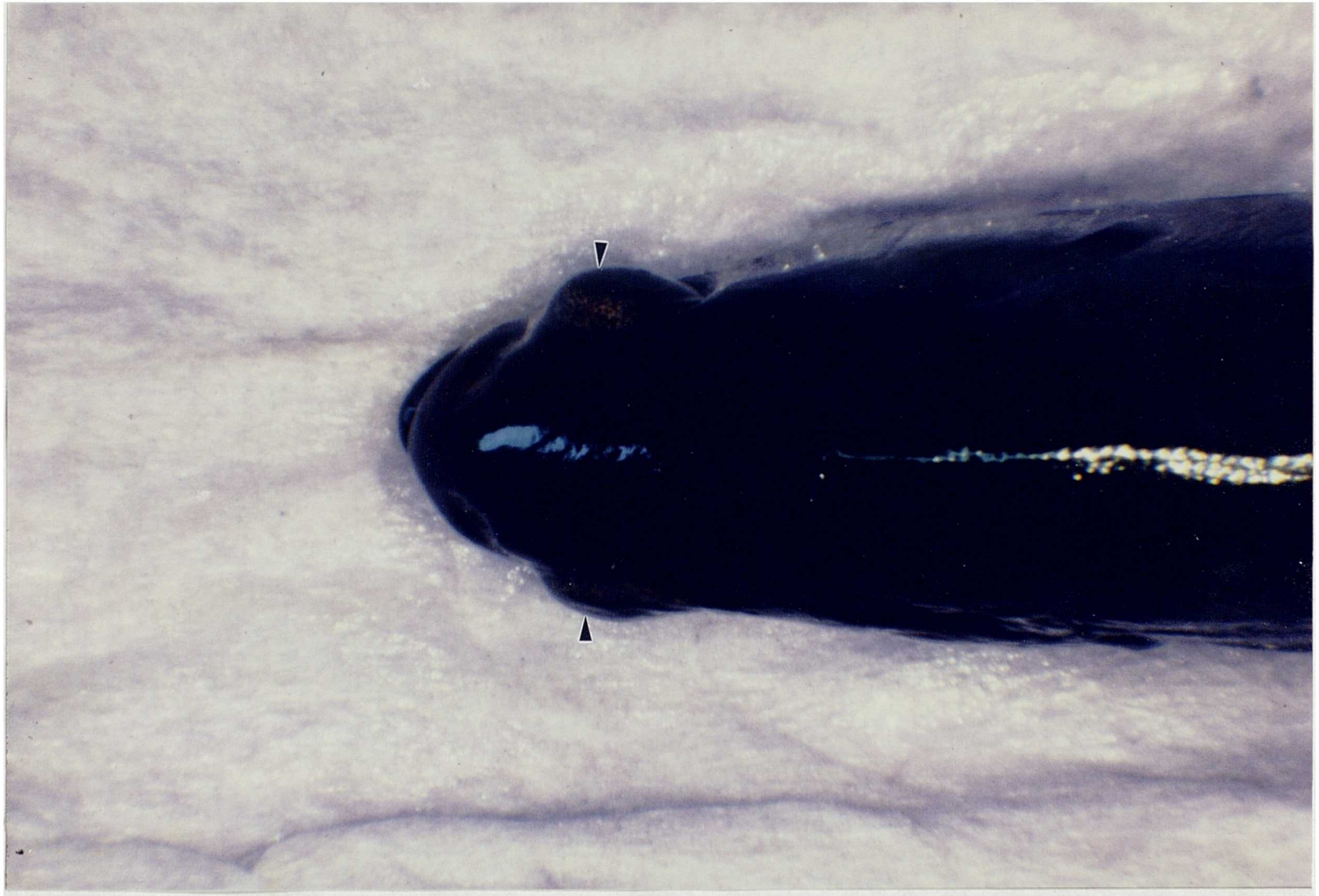


Figure 7.3: Rainbow trout fry (≈4.0g) showing bilateral exophthalmia (arrowed).

Figure 7.4: Rainbow trout fry (≈1.5-2.0g) exhibiting pale gills (arrowed).

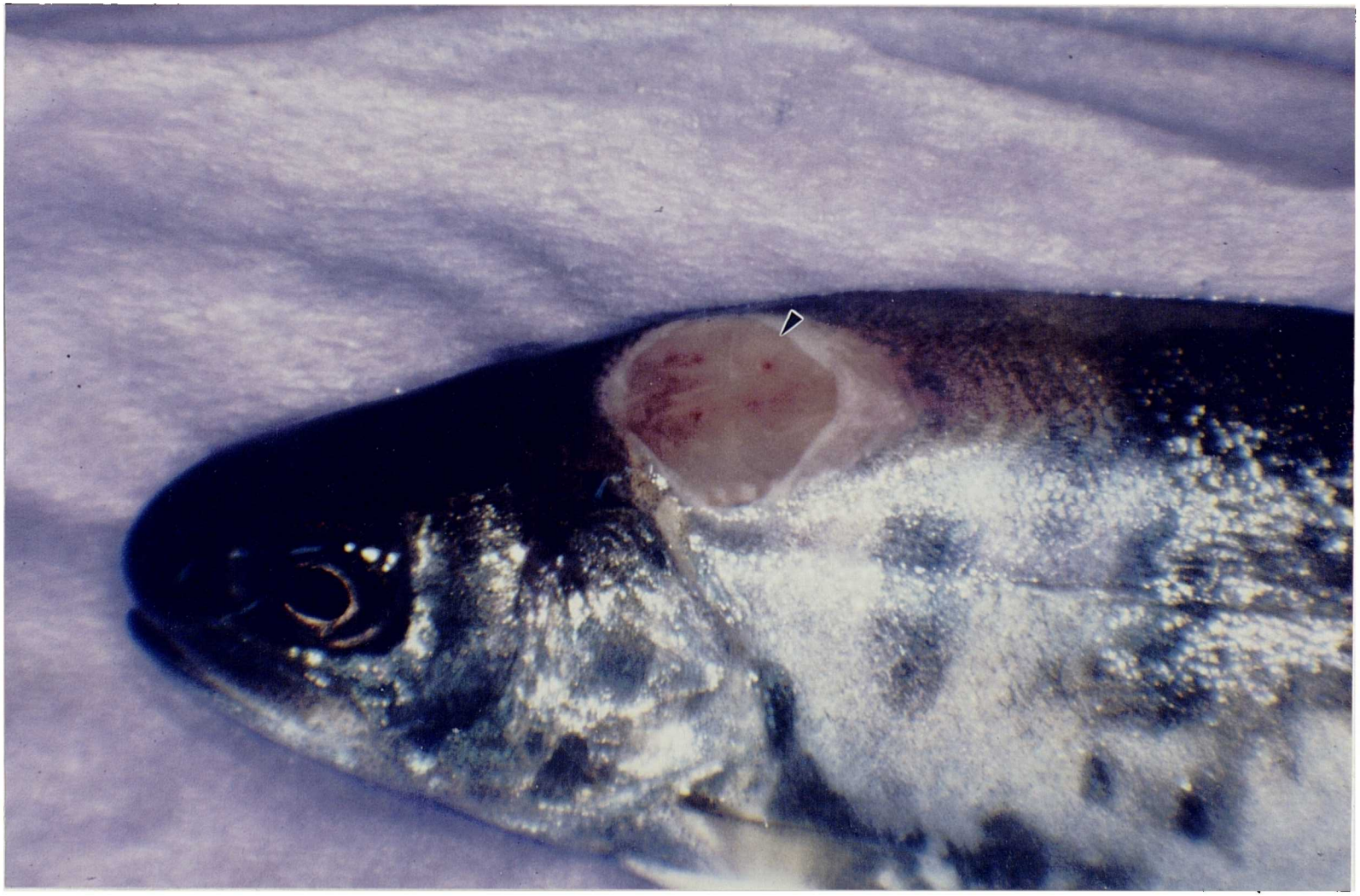


Figure 7.5: Rainbow trout fingerling (≈5.0g) showing severe skin lesion (arrowed) on the dorsal surface, exposing underlying muscle layers.

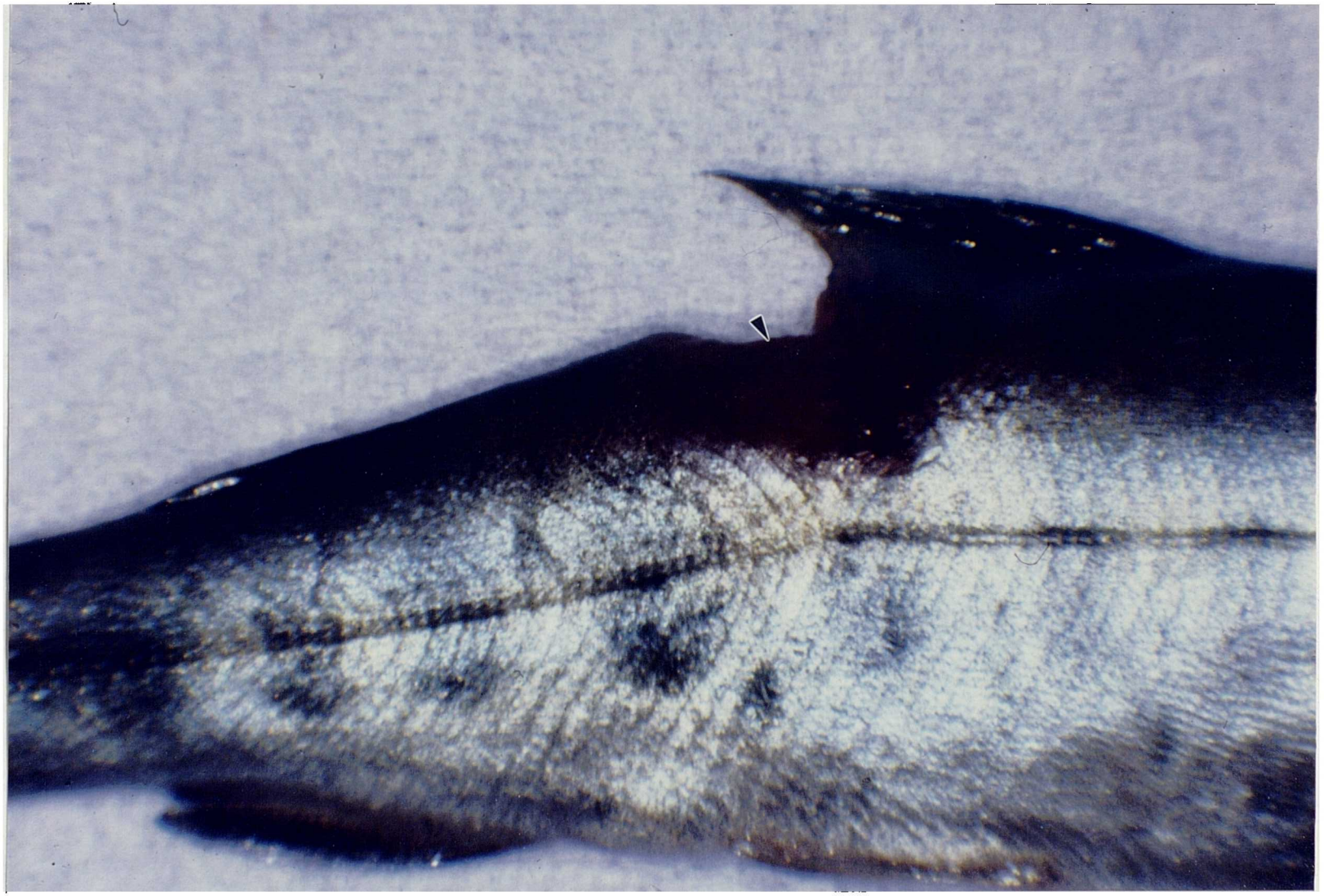


Figure 7.6: Rainbow trout fingerling (≈10.0g) showing extensive ulcerative lesion (arrowed) immediately behind the dorsal fin, extending through into the underlying muscle layers.

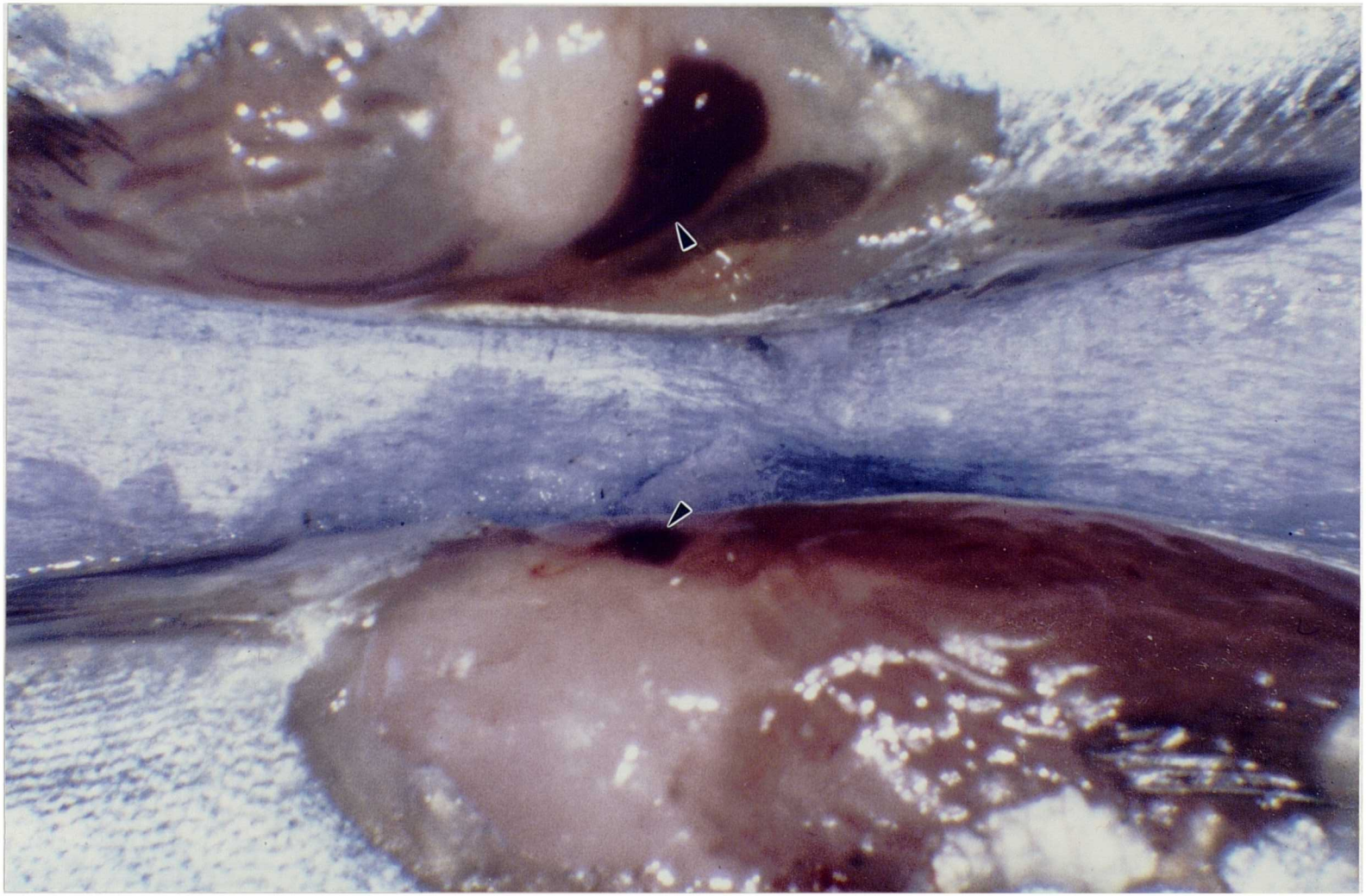


Figure 7.7: Two rainbow trout fry (≈4.0g) showing severe hypertrophied spleen (arrowed) of fry suffering from RTFS (top) compared with normal spleen (arrowed) of healthy fry (bottom).

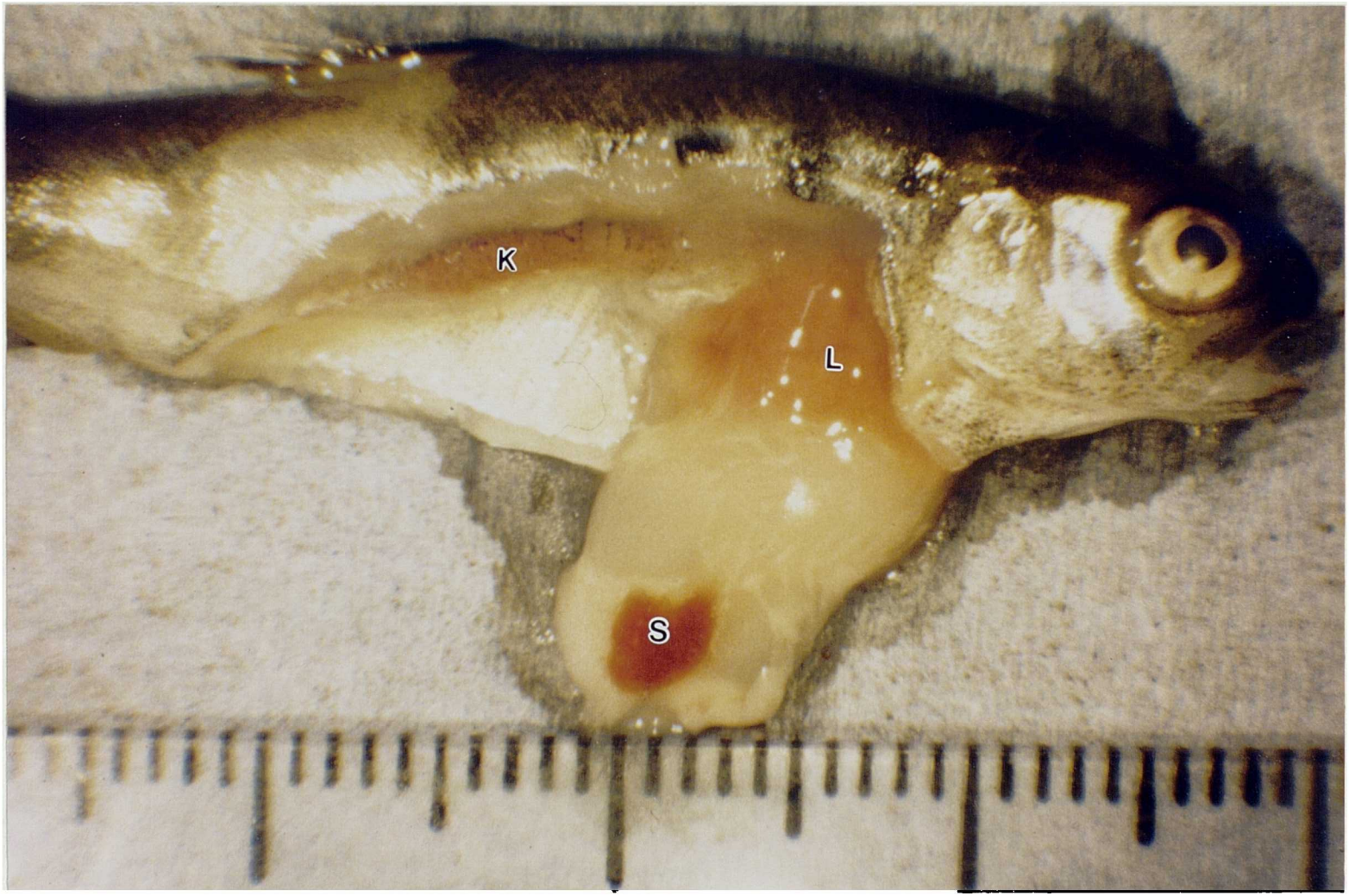


Figure 7.8: Rainbow trout fry (≈1.5-2.0g) exhibiting swollen spleen (S), pale liver (L), slightly enlarged kidney (K) and severe pallor.

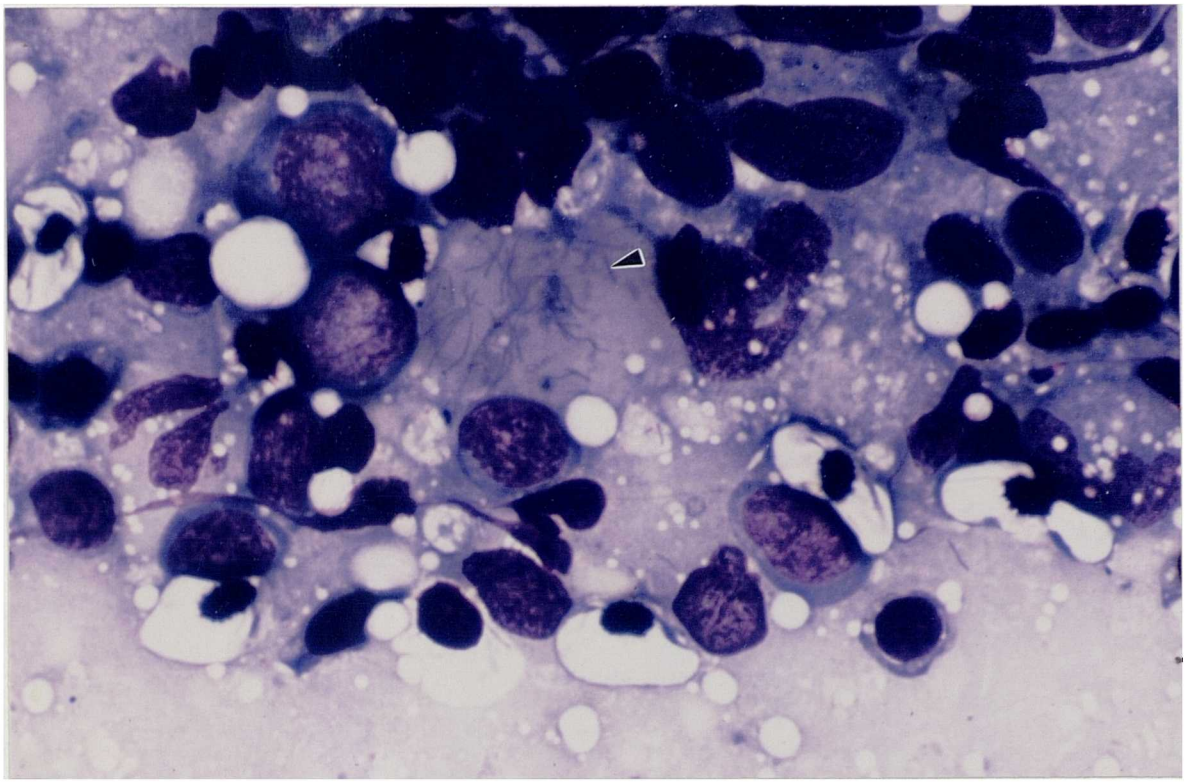


Figure 7.9: Spleen squash of rainbow trout fry (≈3.0g) sampled during natural infection of RTFS, note long, slender rod-shaped bacteria (arrowed) (toluidine blue in 1% aqueous borax x1000)

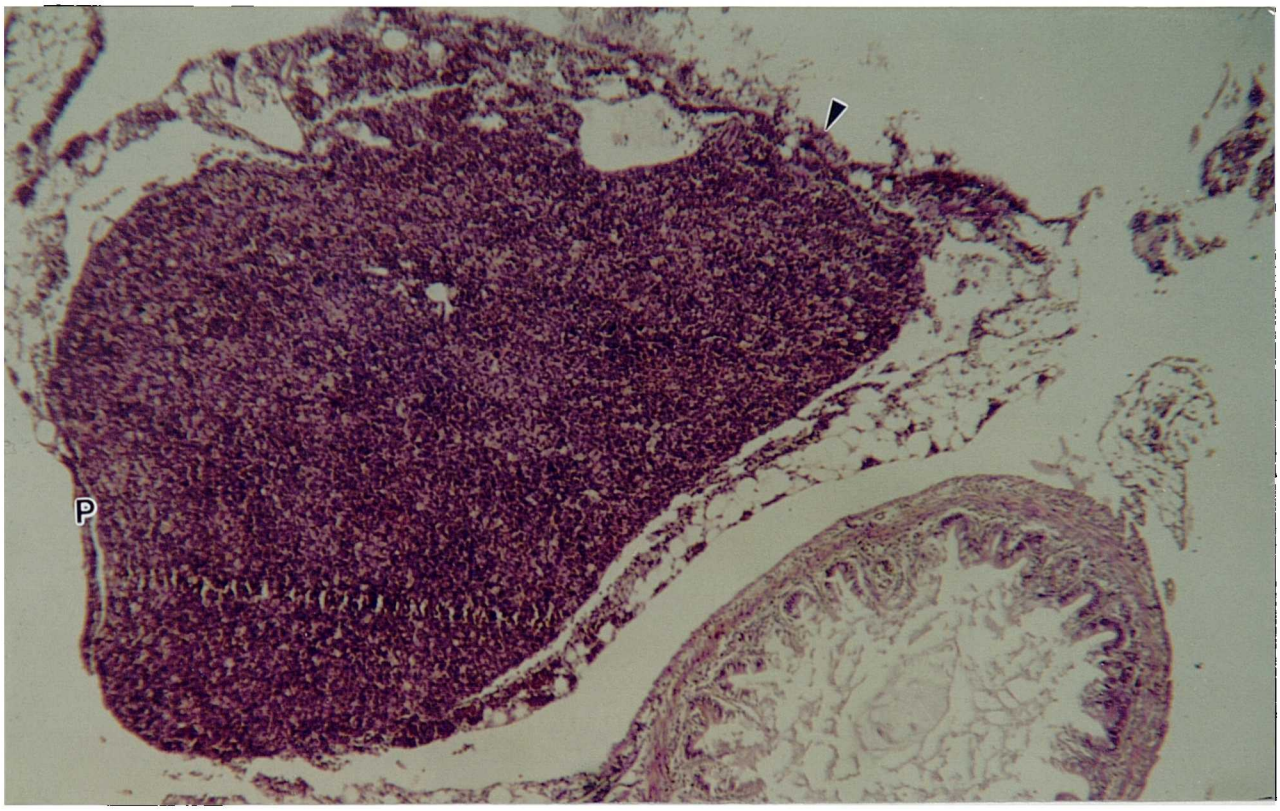


Figure 7.10: Spleen from a naturally infected rainbow trout fry (≈3.0g) showing loss of border definition (arrowed) and peritonitis (P) (H&E, x25.2)

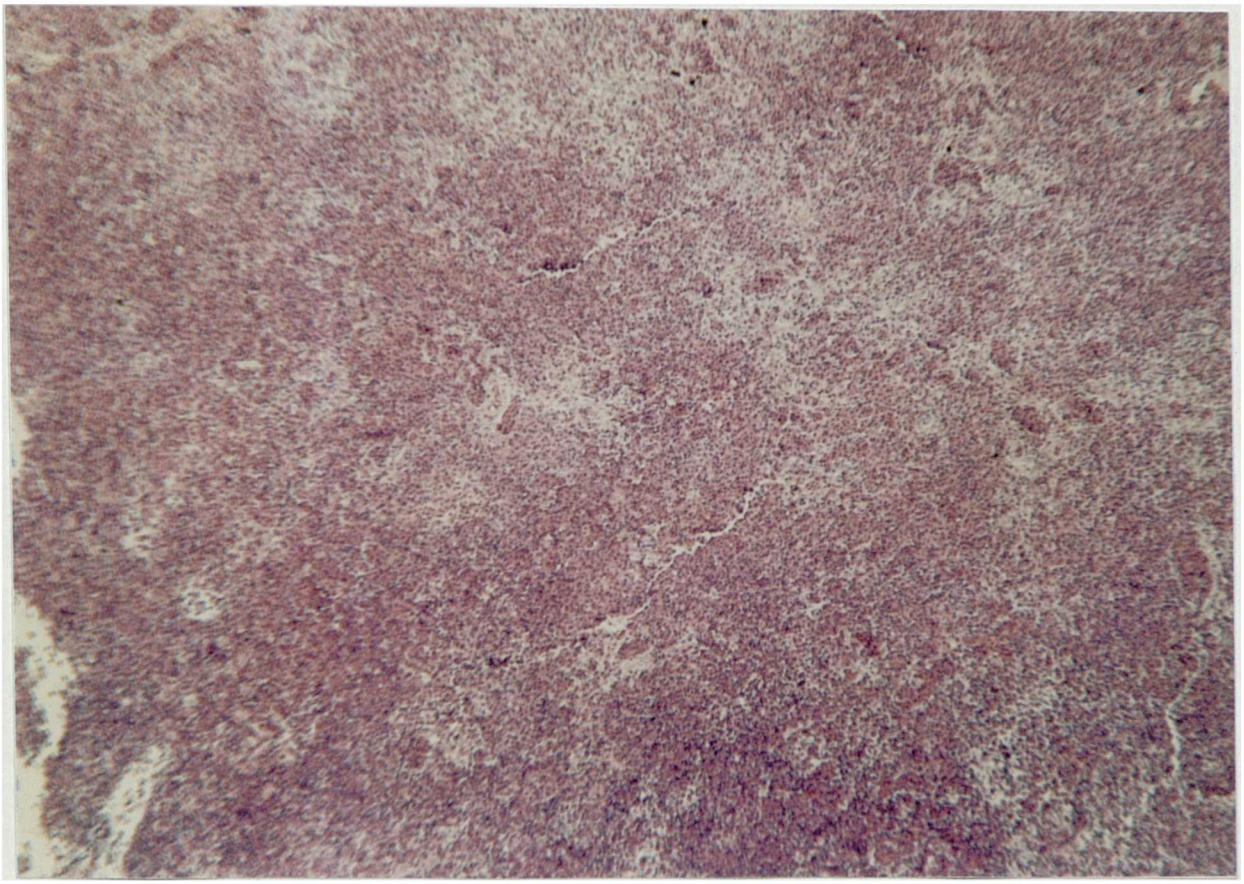


Figure 7.11: Spleen of naturally infected rainbow trout fingerling (≈ 6.0g) showing indistinct staining (H&E, x40)

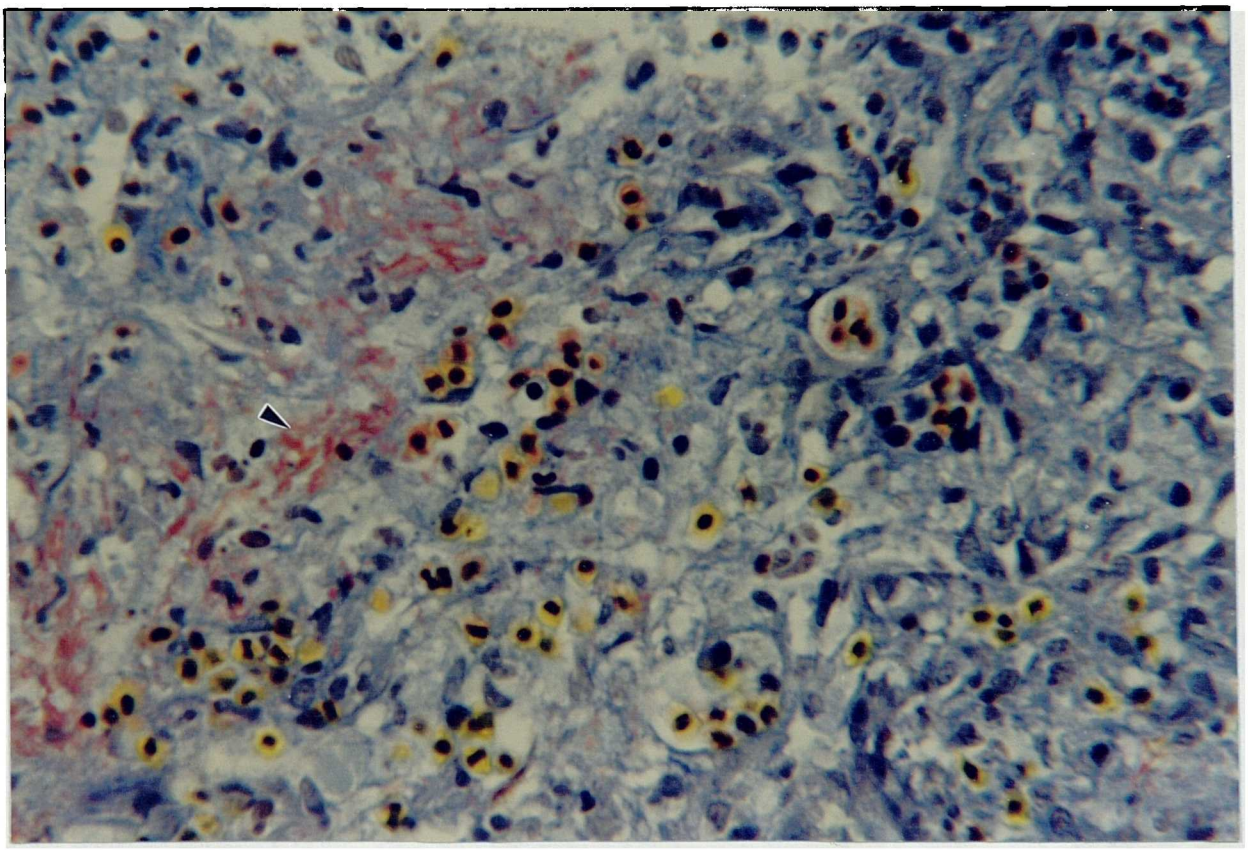


Figure 7.12 Spleen of a naturally infected rainbow trout fry stained with Martius scarlet blue (MSB), note deposition of fibrin-like material (arrowed). (MSB, x100)

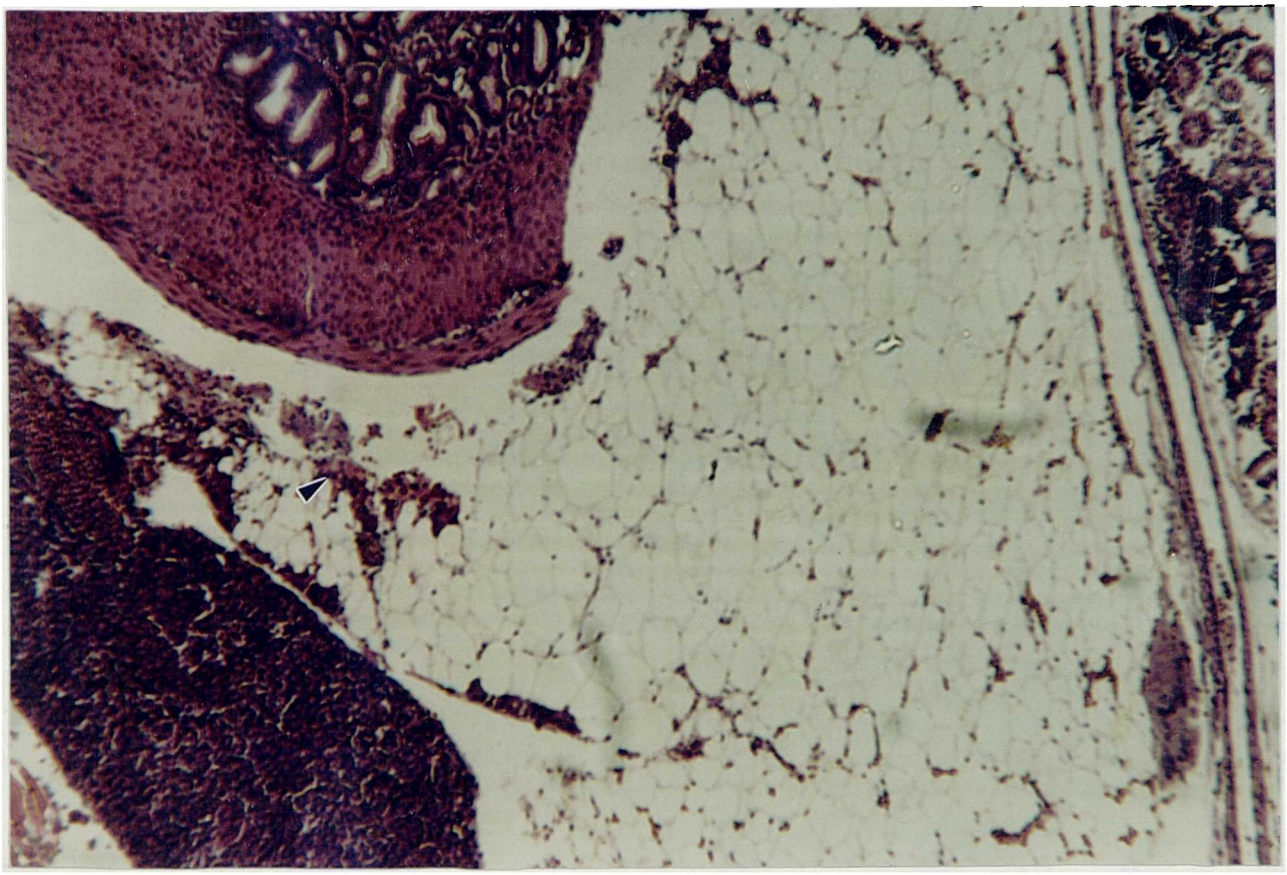


Figure 7.13 Transverse section of 1.5g rainbow trout fry intraperitoneally injected with *C. psychrophila* showing evidence of haemorrhage (arrowed) and peritonitis extending into the pancreatic tissue (H&E x25.2)

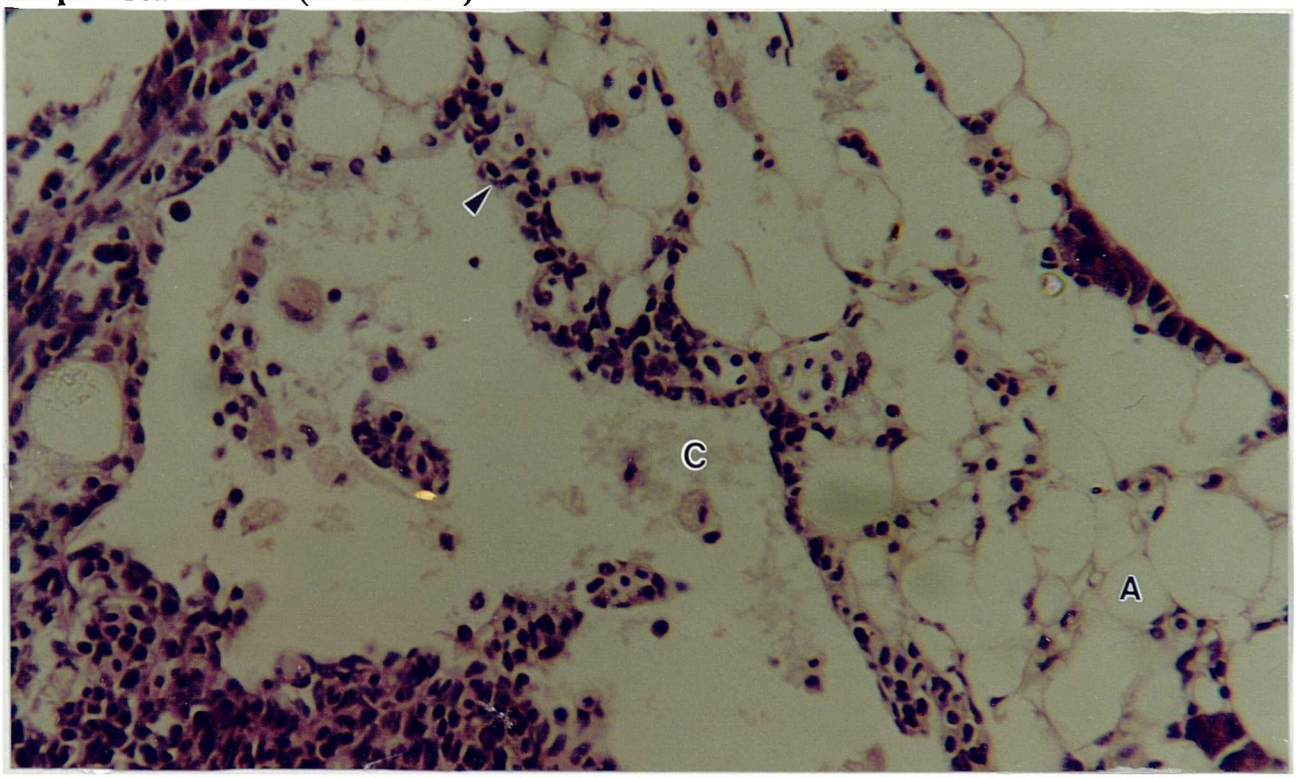


Figure 7.14 Same section at higher magnification showing erythrocytes interspersed within pancreatic tissue (arrowed) and large amounts of cellular debris or possibly bacterial cells (C) and normal appearance of peritoneal adipocytes (A) (H&E x100)

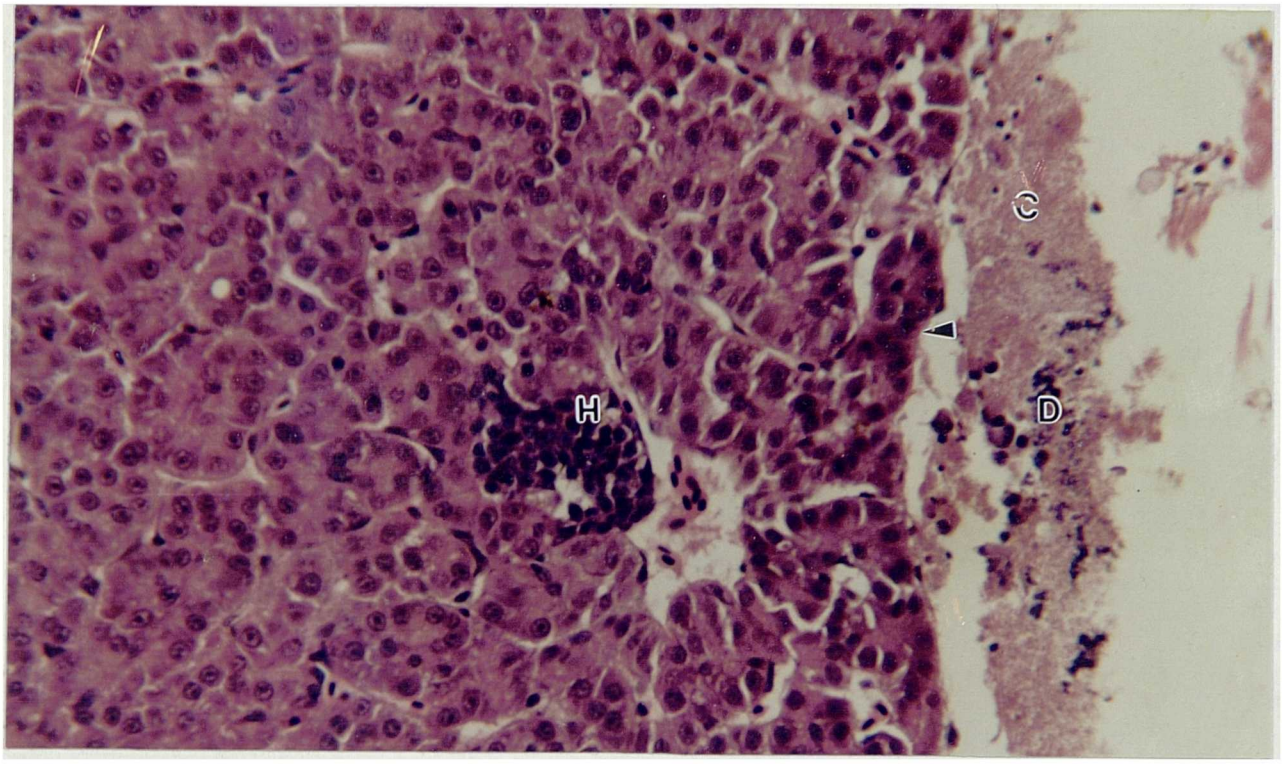


Figure 7.15 Liver of 1.5g rainbow trout fry intraperitoneally injected with *C. psychrophila* showing loss of border integrity (arrowed) and cellular debris or possibly bacterial cells (C), with cells undergoing degenerative changes within this debris (D) note also a focus of haematopoietic tissue adjacent to a liver sinusoid (H) (H&E x400)

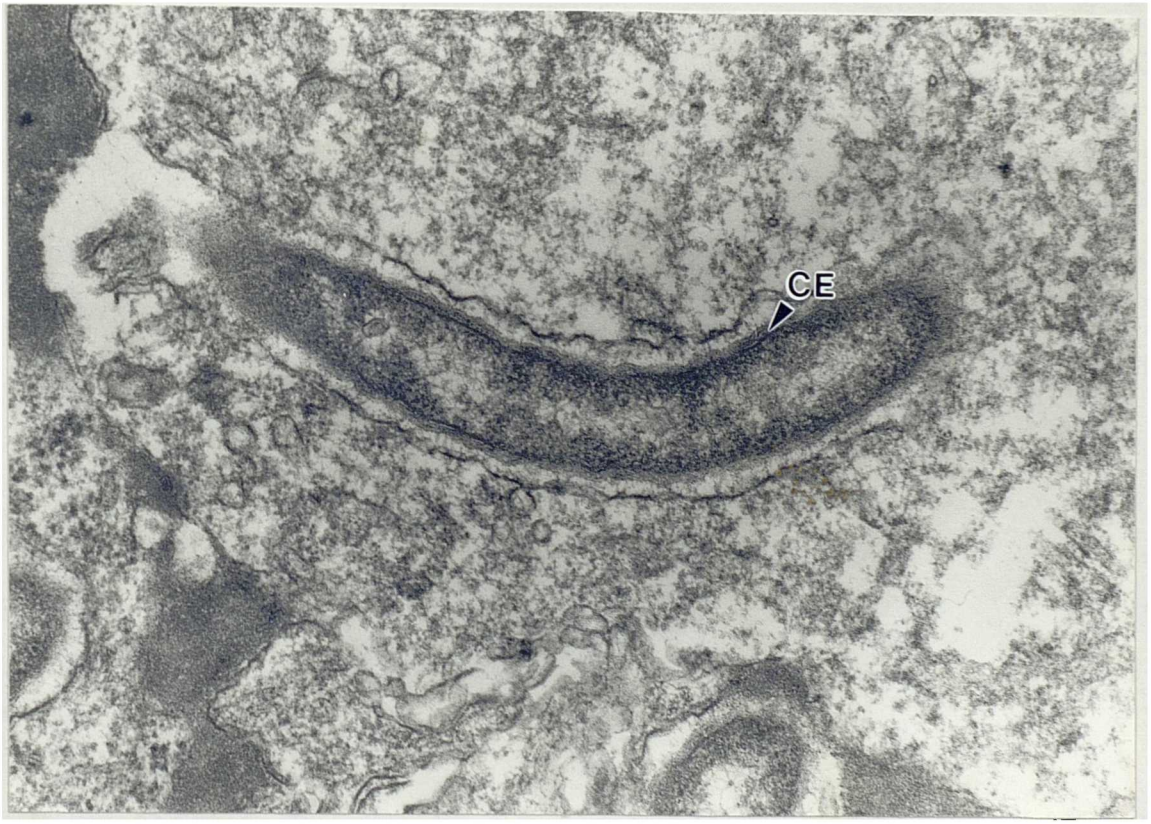


Figure 7.16: Electron micrograph showing typical Gram negative cell wall (CE) of *C. psychrophila* from spleen tissue from infected rainbow trout fry (x28 000)

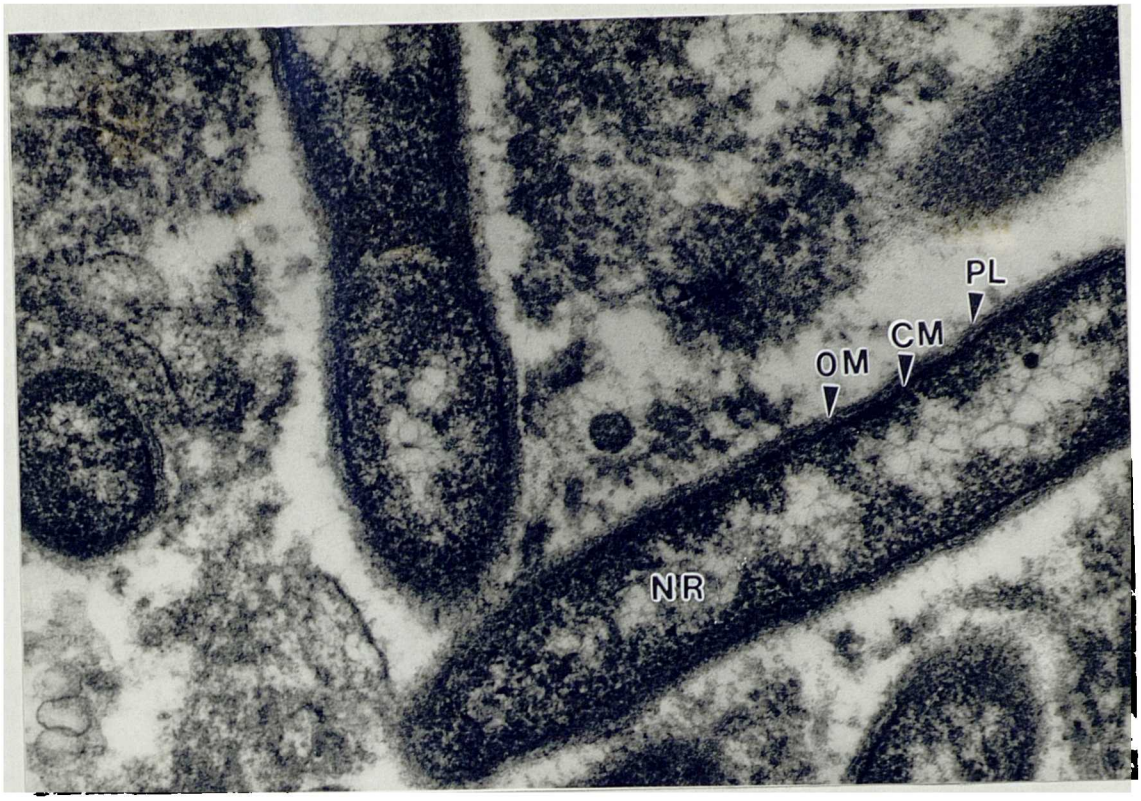


Figure 7.17: Ultrastructure of *C. psychrophila* showing lipopolysaccharide outer membrane (OM), peptidoglycan layer (PL), cytoplasmic membrane (CM) and nuclear region (NR) from spleen tissue from infected rainbow trout fry (x43 000)

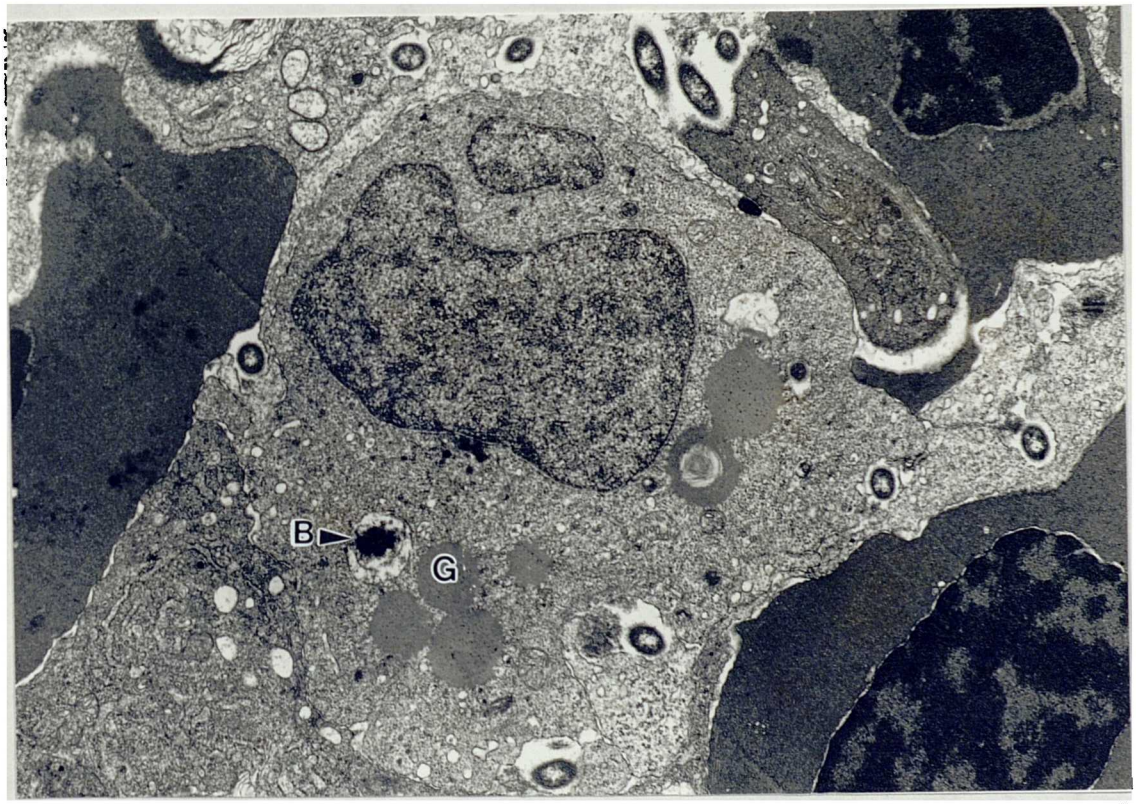


Figure 7.18: Granulocyte, probably an eosinophil or basophil, from spleen tissue from infected rainbow trout fry, note the secretory granules (G) and transverse section of bacterial cell enclosed within a phagocytic vesicle (B) (x75 00)

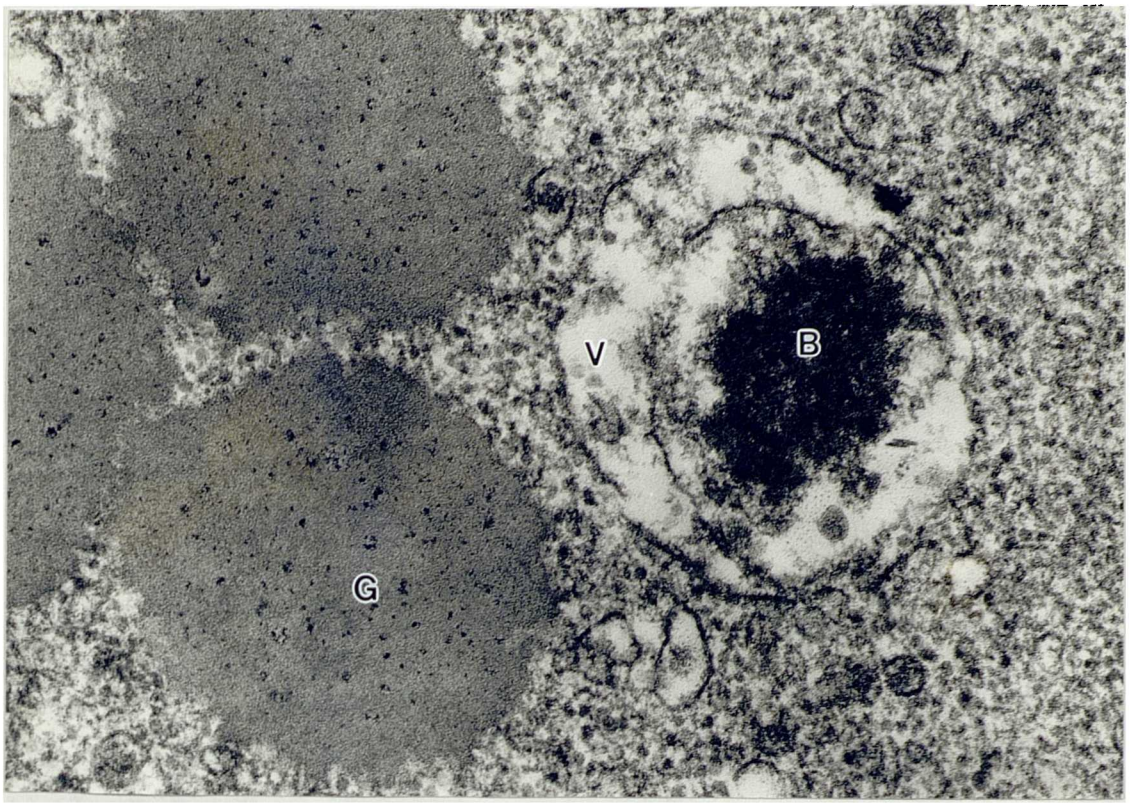


Figure 7.19: Same cell showing several refractile membrane-bound granules (G) and bacterial cell (B) a within phagocytic vesicle (V) from spleen tissue from infected rainbow trout fry (x43 000)

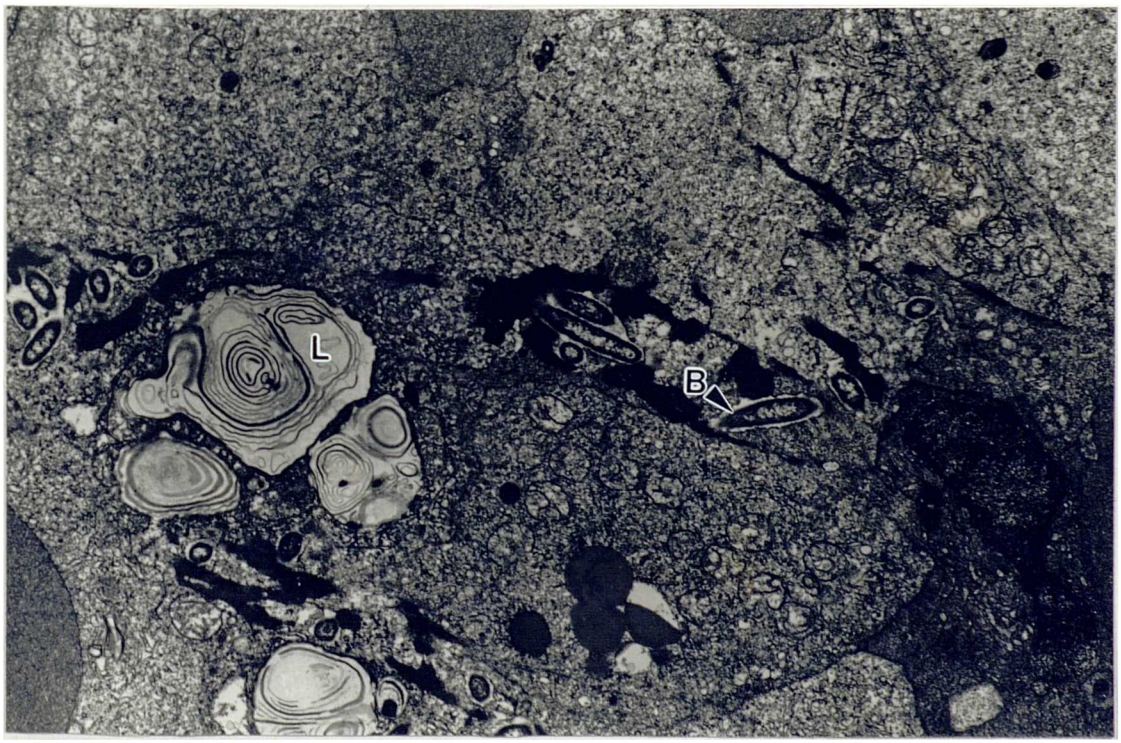


Figure 7.20: Prominent lysosomes (L), numerous filamentous bacteria (B) and evidence of cellular degeneration in spleen tissue from infected rainbow trout fry (x59 00)

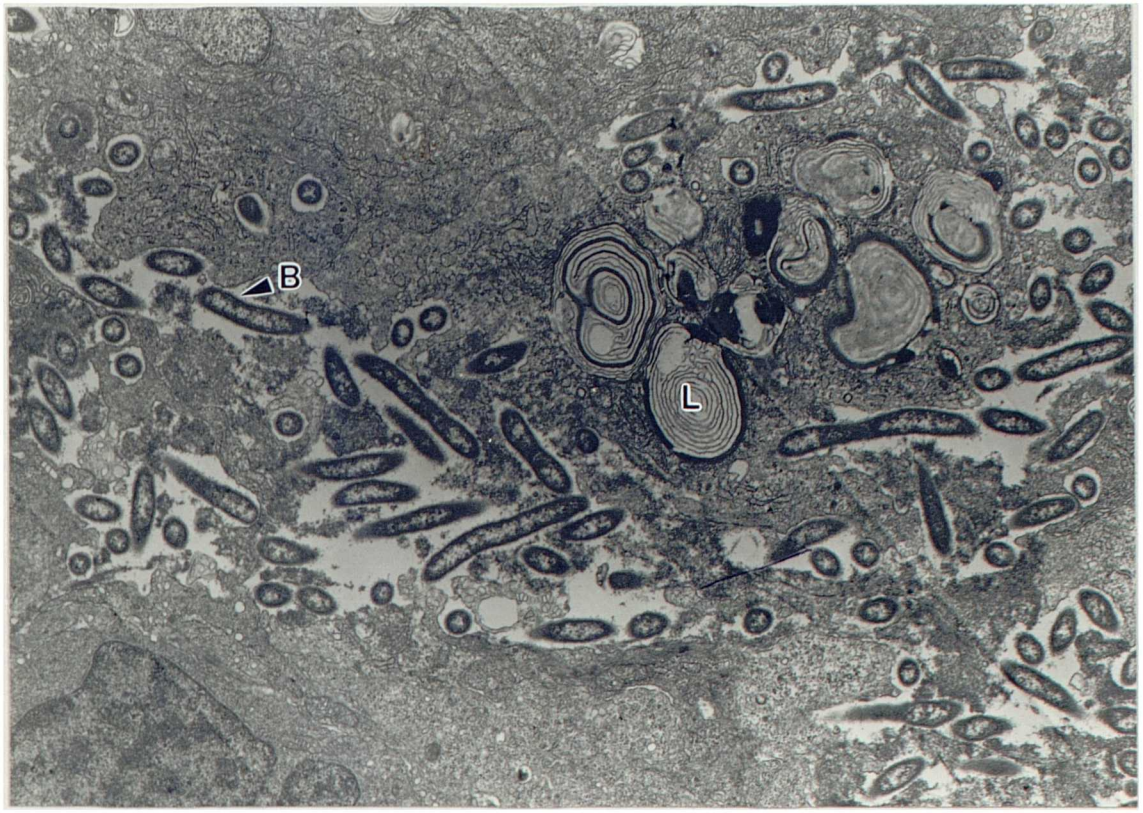


Figure 7.21: Prominent lysosomes (L), degeneration of the cytoplasmic membrane (spaces) and numerous filamentous bacteria (B) in spleen tissue from infected rainbow trout fry (x59 00)

DISCUSSION

The gross pathological signs of disease found in the work presented here are largely consistent with descriptions of clinical signs of RTFS reported by authors in France, Denmark, England, Scotland, Spain, Germany, Italy, Chile and Finland (Baudin-Laurencin *et al.*, 1989; Weis, 1989; Bucke and Barker, 1991; Chua, 1991; Lorenzen *et al.*, 1991; Santos *et al.*, 1992; Sarti *et al.*, 1992; Toranzo *et al.*, 1993; Bustos *et al.*, 1994; Wiklund *et al.*, 1994).

Anaemia, characterised by extremely pale gills, was recorded in both experimentally and naturally infected fish. Anaemias are caused by either abnormal blood loss which cannot be compensated for adequately by erythropoiesis or by a reduction in blood cell production which does not enable sufficient replacement of red cells which are lost normally (Roberts, 1978). The nature of the anaemia associated with RTFS was not investigated in this study due to the difficulty in obtaining sufficient quantities of blood without severing the dorsal aorta. This method inevitably either incised the anterior kidney and therefore, even in ostensibly healthy fry, resulted in the introduction of haemopoetic cells to the blood sample, which in turn falsely raised the reticulocyte count, or led to contamination by gut contents. Alternative methods to obtain blood from anaemic fry using severance of the caudal peduncle were unsuccessful. Despite this, low levels of circulating erythrocytes may have been a result of the sequestration of red blood cells in the spleen. The extensive congestion of the organ and massive haemorrhage and subsequent red blood cell destruction within red and white pulp seen in histological sections appeared to be responsible for its severely hypertrophied state. It would therefore suggest that initially at least, a haemorrhagic anaemia exists. Even the complete necrosis of the splenic pulp during infection does not indicate that the anaemia is entirely hypoplastic, as the kidneys which are also haematopoietic appear to be relatively uninvolved in the pathogenesis of the disease. The formation of red blood cells in the liver of fish which appeared to be in a "recovery phase" suggested that loss of the haematopoietic elements of the spleen may however be a significant factor in succumbing to infection.

Varying degrees of splenomegaly were seen in both experimentally infected and naturally infected fry. In accordance with Chua, (1991) sections revealed that this was due to congestion, intercellular oedema, infiltration with inflammatory cells and deposition of fibrin (Figure 7.12). However differences in histological findings between fry infected via the two routes existed. In intraperitoneal (IP) infections numerous filamentous bacteria-like cells could be seen in the peritoneal cavity associated with peritoneal adipocytes, and haemorrhages were recorded extending into the pancreatic tissue (Figures 7.13 and 7.14). Fewer changes were observed in the spleen where the capsule was frequently intact with little or no evidence of congestion or infiltration of inflammatory cells. The appearance of large numbers of bacteria-like cells present around the periphery of the spleen suggested that in IP infection the organism invaded the splenic tissue from the peritoneal cavity, probably due to its proteolytic ability. In contrast, during natural infections where the infective dose is likely to arrive by a different route, the infection becomes systemic and the duration of infection longer. The integrity of the capsule is gradually lost, leading to the discharge of inflammatory exudate, bacteria and red blood cells and resulting in fibrinous splenitis and peritonitis (Figure 7.10). Chua, (1991) reported that the destruction of the splenic capsule in outbreaks of RTFS was comparable to infection in the kidney by the fungus *Exophiala* sp., where fungal hyphae appear to be confined within the capsule, temporarily limiting further progression of the disease. Following breakdown of the capsule the fungus rapidly spreads causing extensive peritonitis. In a like manner, bacteria-like cells were seen in large numbers around the periphery of the spleen in both challenge groups prior to the appearance of perisplenic inflammation and peritonitis. Potentially, proliferation of the bacteria occurs in the ellipsoid sheaths as the organisms move out of the capillaries. Progressive bacterial invasion of the stroma appeared to result in the intercellular oedema and necrosis of the red and white pulp seen in advanced cases of naturally occurring disease.

Wolf (1937) described a condition affecting the swim-bladder in lake trout fingerlings involving *Pseudomonas fluorescens.* He demonstrated that the organism gained entry to the swim bladder via the pneumatic duct linking the bladder to the gut. Wood and Yasutake (1956) first recorded *C. psychrophila* in the swim-bladder of heavily infected coho salmon suffering from cold water disease. In the current study

artificially infected fry displayed haemorrhages and filamentous bacteria-like cells were seen within the bladder. In such cases, degeneration of the kidney tubules was also indicative of bacterial spread to the kidney. No evidence of the bacterium could be seen in the swim bladders of naturally infected fry, however chronically infected fingerlings showed thickening of the swim-bladder wall, possibly as a response to proliferation of the bacteria within the submucosa as seen in chronic furunculosis (Roberts, 1978).

Chua (1991) demonstrated filamentous bacteria in the blood of naturally infected fry. Although no adequate blood samples were available in the present study, bacteria were seen histologically in the major blood vessels indicating that the organism can be transported via the vascular system. Wood and Yasutake (1956) reported that in coho salmon suffering from cold water disease, mortality was almost certainly due to the formation of heart lesions. In accordance with Chua, (1991) heart pathology observed in this study was characterised by fibrinous pericarditis and endocarditis. However these changes were not consistently present and mortality could not exclusively be attributed to the development of heart lesions or myocardial necrosis. Although no precise cause of death was established during the present study, histological examination of tissues indicated that in most cases mortality could be attributable to acute anaemia (R. H. Richards, pers. comm.).

Several authors have reported on the presence of skin lesions associated with outbreaks of RTFS (Lorenzen *et al.,* 1991; Santos *et al.,* 1992; Sarti *et al.,* 1992; Toranzo *et al.,* 1993; Bustos *et al.,* 1994). Whilst ulceration of the epidermis has been recorded during outbreaks of RTFS in the U.K. (Chapter 2), in the current investigation no established skin lesions were examined. Chua (1991) reported that skin erosion was an occasional rather than constant feature of the condition. In accordance with this finding no consistent dermal or hypodermal changes were observed in fry that did not already exhibit epidermal ulceration and although filamentous bacteria-like cells were infrequently seen within the stratum compactum, skin lesions were not considered to be pathognomonic for RTFS.

The gills of affected fish showed no significant pathological changes. The slight proliferation in chloride cells demonstrated in some cases could not be attributed to specific bacterial invasion. Bruno (1992), described accumulations of *C.*

psychrophila located around the gill arch and secondary lamella and in his study, hypertrophy of the gills was recorded. Chua (1991) demonstrated the presence of bacteria associated with gill lesions, additionally Lorenzen (1994) detected bacteria within endothelial cells of the gill capillaries. Despite these observations both authors concluded that chronic gill trauma was not always implicated in the onset of disease.

In spite of suggestions that other *Cytophaga*-like bacteria gain access to the host across the skin and gills (Austin, 1993) no route of entry of the pathogen to fish tissues could be elucidated in this study. In artificially induced infection via the intraperitoneal route peritonitis was evident. Although this may have been a result of injury caused as a result of the experimental procedure, bacteria may also have been taken up by gut macrophages. Unfortunately no evidence of inflammatory response was observed in the gut of naturally infected fry and further studies are required to fully elucidate the mechanisms by which *C. psychrophila* gains access to internal organs of fish.

Transmission electron microscopy of cells of *C. psychrophila* revealed an electron-opaque region, an outer membrane, an underlying peptidoglycan layer and plasma membrane surrounding the cytoplasmic and nuclear regions. In a study on the ultrastructure of *C. columnaris* using ruthenium red ($Ru_3O_2(NH_3)_{14}Cl_6.H_2O$), Pate and Ordal (1967) described an extracellular polysaccharide layer surrounding the cells. This layer was thought to be involved in the adhesive properties of *C. columnaris* characterised by the cells' ability to aggregate into fruiting bodies and by their affinity for tissues of salmonid fish. Pate and Ordal (1967) suggested that the possession of extracellular mucopolysaccharides was a characteristic of fruiting myxobacteria and reported it absent in non-fruiting species. However in another study on the ultrastructure of Cytophagales, Reichenbach and Dworkin, (1981), demonstrated extracellular polysaccharide covering the surface of the outer membrane in the non-fruiting, non fish pathogenic species *Capnocytophaga ochracea.* In this study both uranyl acetate-lead citrate and ruthenium red fixation techniques were utilised and the layer can be seen as a "fuzzy" electron-opaque area with the former method, whilst ruthenium red allows clear visualisation. Strohl and Tate (1978) examined the non-fruiting opportunistic fish pathogen *C. aquatilis* and using frozen-surface replica preparations demonstrated the production of copious amounts of extracellular

polysaccharide or "slime", suggesting that it probably facilitated the gliding motility characteristic of the group. In the present study using uranyl acetate-lead citrate staining, an electron-opaque region was evident apparently associated with the surface of cells. This indicates that in common with both fruiting and non-fruiting and pathogenic and non-pathogenic Cytophagales an extracellular polysaccharide layer surrounds cells of *C. psychrophila.* No certain function for this layer has been established although polysaccharides on the exterior of cells are reported to have changed the morphology of colonies, altered immunological response of cells and obliterated phage receptor sites. They may also be implicated in recognition sites on erythrocytes (Kalckar 1965). Kelly (1966), demonstrated that acid mucopolysaccharides were localised in desmosomes and that the external leaflet of the plasma membrane was coated with mucopolysaccharide. He suggested that this provided morphological evidence for selective, weakly adhesive properties. Electron microscopic evidence suggests that cells of *C. psychrophila* do possess a surface extracellular "slime" layer which may function to aid motility and adhesion, furthermore this outer layer may confer additional protection to the cell by impeding the penetration of antimicrobial compounds through the cell envelope.

Transmission electron microscopic examination of spleen tissue from naturally infected rainbow trout fry revealed the presence of numerous phagosomes and residual bodies indicative of extensive lysosomal activity within the granulocytes. The splenic cellular response appeared to include lymphocytes, plasma cells, thrombocytes and eosinophil granulocytes with the latter cells present in apparently increased numbers. In mammals eosinophils are generally implicated in inflammation as well as in hypersensitivity reactions and form principal components of the cellular response to macroparasites (Feist, 1993). They are usually found at levels of between 1-3% in the blood (Ellis, Roberts and Tyler, 1978). However, eosinophilic cells are generally considered to be rare in fish blood and are most frequently found in the skin, haemopoietic and digestive tissue (Ellis *et al.* 1978). It is not known whether they demonstrate significant phagocytic ability in fish, and the appearance of numerous filamentous bacteria and extensive degeneration suggested that the response to the pathogen was not effective. The present study represents a preliminary examination

and the consequence of the cellular response to infections with *C. psychrophila* remains unclear and requires further elucidation.

CHAPTER 8

SEROLOGICAL STUDIES ON *C. PSYCHROPHILA*

INTRODUCTION

Serology and serodiagnosis of bacterial fish pathogens have played an important role in programs of fish health management in recent years (Busch, 1981). Pacha and Porter (1968) first utilised the differences in somatic antigens to distinguish between non-pathogenic and pathogenic members of the family Cytophagaceae sharing similar morphological, cultural and physiological characteristics. None of the heterologous non-pathogenic strains reacted with antisera prepared against *C. psychrophila* or *C. columnaris,* whilst homologous fish pathogenic isolates were readily agglutinated. Pacha and Porter (1968) concluded that the use of serological procedures might provide a useful and rapid means of identifying pathogenic members of the family Cytophagaceae. Using whole cell slide agglutination Bullock, (1972) confirmed that *C. psychrophila* and *C. columnaris* exhibited distinct separate serological characteristics. Furthermore, he demonstrated a high degree of cross-reactivity between isolates from outbreaks of bacterial coldwater disease (CWD) and from peduncle disease of brook trout (*Salvelinus fontinalis*), concluding that the two diseases were caused by the same organism.

Agglutination-absorption tests had demonstrated the presence of four serological groups amongst strains of *C. columnaris* isolated from fishes taken from the Columbia river system, Washington, USA (Anacker and Ordal, 1959). Holt *et al.* (1993) showed the presence of common antigen(s) in *C. psychrophila* isolated from New Hampshire, Michigan, Alaska and Oregon in the USA, but further studies revealed that some isolates from outbreaks of CWD from Oregon were antigenically different. *C. psychrophila* from diseased rainbow trout fry in Chile and Spain appeared to be serologically divergent from morphologically and biochemically analogous isolates from the USA and it was proposed that these strains belonged to a second serotype, expressing different surface antigen(s) to that of the type strain NCIMB 1947^{T}, which is characteristic of cold water disease-type infections in North

America (Bustos *et al.*, 1994). Wakabayashi, Toyama and Iida (1994) found that common antigens were shared between strains of *C. psychrophila* isolated from coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) and ayu (*Plecoglossus altivelis*). in Japan. However absorption analysis with heat-stable antigens divided the strains into two distinct serotypes. Serogrouping studies carried out by Lorenzen (1994), using both rapid slide agglutination and enzyme linked immunosorbent assay (ELISA) techniques, determined that 3 serotypes were representative amongst 44 isolates of *C. psychrophila* from a variety of freshwater fish species in Denmark.

The object of the present work was to develop diagnostic methods for the rapid and accurate detection of *C. psychrophila* based upon serological methods. The studies reported below represent an initial examination of the serology of selected isolates and an investigation into the potential for development of rapid serodiagnostic techniques for the rapid detection of *C. psychrophila.*

MATERIALS AND METHODS

Bacterial isolates and cultivation

The bacterial strains included in this study are listed in Table 5.1 (Chapter 5). Bacterial cells were grown in modified Anacker and Ordal broth (MAOB) or modified Anacker and Ordal agar (MAOA) for 72-96h at 17°C. Cells were harvested from broths by centrifugation (1400g 20min) or collected directly from plate grown cultures and washed in 3 changes of PBS and finally resuspended in PBS.

Production of rabbit antisera against *C. psychrophila*

Antisera used for the current study were raised in adult New Zealand White rabbits. A bacterial suspension containing 10^5 cfu ml^{-1} of isolate number U648 (Chapter 5, Table 5.1) was emulsified with equal volumes of Freunds complete adjuvant (FCA) and an intramuscular (i/m) injection of 1.5ml (0.5ml in each of three sites in the thigh muscle) was given. After three weeks an additional intramuscular injection of 1ml bacterial suspension (10^7cfu ml^{-1}) emulsified with an equal volume of Freunds incomplete adjuvant (FIA) (0.5ml at each of two sites, again in the thigh

muscle) was administered. A booster inoculum of 0.5ml of 10^9 cfu ml^{-1} in PBS was injected intravenously (i/v) (after a further 5 weeks) into the marginal ear vein following i/v administration of 0.5ml Promethazine-HCl (50mg ml^{-1}). After a further 5 weeks a second series of 1ml intramuscular injection (0.5ml at two sites in the thigh muscle) with Freunds incomplete adjuvant were given 14 weeks after the first i/m injection with the second a further 10 days after that. Rabbits were bled from the marginal ear vein 6-8 days after this final injection. Blood was incubated at 37°C for 1 h before being transferred to 4°C overnight, to allow a clot to form. Serum was separated by centrifugation (100g 20min 4°C) aliquoted and stored at -20°C.

Bacterial agglutination

Two-fold dilutions of whole serum were made in PBS +Thiomersal (0.01%) (PBS+Thio) + 1% bovine serum albumin (BSA). Additionally, preimmune serum at 1/2 and 1/8 in PBS +Thiomersal (0.01%) + 1% BSA was included in each case as a negative control. Twenty five µl of the serum dilutions were added to a 96 U-well microtitre plate (Bibby-Sterilin). A further 25µl of bacterial suspension (approximately $1x10^8$cells ml^{-1} ≡ optical density 0.2±0.01 at 520nm) was then added to each well and incubated at 37°C for at least 2h. The microtitre plates were then sealed to maintain the humidity and transferred to 4°C and the agglutination read after an overnight incubation.

Immuno-diffusion, Ouchterlony technique

A 1% agarose gel in 0.9% saline with 0.02% NaN_3 was melted and carefully poured onto a level glass plate and the agar allowed to cool. A pattern of holes was punched onto the plate and the agar plugs were sucked out with a Pasteur pipette connected to a vacuum pump. The diameter of the wells was 4mm and the distances between centre to centre and edge to edge were 9mm and 4mm respectively. Before addition to the wells, 1ml of TRIS HCl 25mM, EDTA 10mM, glucose 50mM followed by 1ml 1% SDS was added to 5ml of the bacterial suspension. This solution was left at room temperature for 15 min to lyse the cells. The wells were then filled with antibody or antigen until the meniscus flattened and disappeared. The plates were examined after an overnight incubation in a humid chamber. To create a permanent

record of the results the plates were stained. Plates were rinsed 3-4 times in large volumes of PBS, then any non-specific proteins were eluted out in large volumes of 0.9% saline. The plates were washed in distilled water and dried on filter paper at 30°C overnight. The dried plate was then stained with 1% Kenacid blue (CI 42660) (Coomassie brilliant blue R 250) (BDH) in 7% acetic acid. The excess stain was washed off with distilled water and destained in 7% acetic acid. After a further wash in distilled water the plate was dried.

Fluorescent antibody test (FAT)

One or two colonies from a 72-96 hour plate were suspended in 1ml of PBS, 50µl of the suspension was heat fixed on a Cookes slide (BDH, Poole, Dorset, England) and air dried. The slides were then overlaid with 50µl of rabbit antiserum (diluted 1:8 with PBS) and incubated in a humid chamber for 30 minutes. Following a rinsing cycle (3x5min in PBS), slides were incubated for a further 30min in a humid chamber with fluorescein isothiocyanate isomer I (FII) (Sigma) conjugated goat anti-rabbit IgG, at a working dilution of 1:40. After a second rinsing cycle (3x5min in PBS), slides were blotted dry, mounted in glycerol (pH 9.0) and examined with a Leitz Dialux 20 microscope equipped with a 100w halogen lamp (280nm), at x40 and x100 fluorescence oil objective.

Purification of immunoglobulin G from rabbit antisera

The immunoglobulin G (IgG) fraction was purified using protein A/ sepharose (CL-4B Pharmacia, Biotech 17-0780-01). Initially 0.75g of Prot.A/ sepharose was swollen in 5ml of PBS and packed into a 5ml column. One ml of antisera diluted 1:1 with PBS was then added to the top of the column. Any unbound proteins were flushed out with 10ml of PBS and the IgG was then eluted by applying 1ml aliquots of glycine-HCl buffer (pH 2.8) to the top of the column and the resulting fractions were collected. The pH of these fractions was adjusted to neutrality by the addition of 30µl of 1M NaOH to each 1ml volume. The IgG concentration was determined by absorbance at 278nm using a Shimidazu UV-2101PC scanning spectrophotometer. A solution containing 1mg/ml rabbit IgG has an A_{278} value of 1.4 (Harlow and Lane, 1988).

Biotinylation of immunoglobulin G

The IgG was biotinylated with a hydroxysuccinimide ester of biotin. Initially the IgG preparation was dialysed against 0.1M $NaHCO_3$ using a NAP-5 desalting column (Pharmacia, Biotech 17-0853-02). While stirring in a siliconised bijou bottle 10µl of 40mg/ml Biotin N-hydroxysuccinimide-ester (Sigma H1759) was added to 1ml of the resulting IgG solution. The mixture was then gently stirred for a further 2h at room temperature. The biotinylated IgG (BIgG) was then dialysed against PBS through a NAP 10 desalting column (Pharmacia, Biotech 17-0854-02) and the BIgG was eluted in 1.5ml of PBS. The BIgG was then stored at +4°C.

Enzyme linked immunosorbent assay (ELISA)

With slight modification, the ELISA was performed according to the double antibody sandwich method described by Voller, Bidwell and Bartlett (1979). The constituents of all the buffers used in the assay are given in Appendix 6. All washes were performed using a programmable microtitre plate washer (DYNATECH DL7000). The inner 60 wells of a 96 U-well microtitre plate (Bibby-Sterilin) were coated with 100µl anti-*C. psychrophila* protein A/ sepharose purified IgG diluted in carbonate buffer at a concentration of 3µg ml^{-1}, the plates were then covered and maintained at 4°C overnight. Following this incubation they were washed in PBS + 0.02% Sodium azide (PBSN) and blocked for 1h with 5% fish skin gelatin (FSG) (Sigma) or 1.5% bovine serum albumin (BSA) in carbonate buffer (200µl per well); after blocking, a further washing stage with PBSN was performed as above.

Rainbow trout fry were collected from two commercial fish farms and tested by the amplified ELISA technique. A survey of farm 9 (Chapter 2) had revealed neither presence of *C psychrophila* nor any history of rainbow trout fry syndrome among the stock. Bacteriological testing of fry and examination of records at farm 1, confirmed that the extensive losses experienced over recent years could be attributed to RTFS. Additionally, at the time of sampling, spleen swabs from 10 fry from each farm were inoculated onto MAOA and incubated at 17°C for 7 days to check for the presence of *C. psychrophila.* The spleen samples for the ELISA were extracted by homogenisation with a micropestle (Eppendorf). Antigen, either (a) bacterial cells harvested from broth grown cultures (Isolate U648) or (b) several colony forming

units collected directly from agar plates (Isolate U648), suspended in PBS to an initial concentration of 10^8 cells ml^{-1} (0.2±0.01 at 520nm) and heated at 55°C for 15min or, (c) homogenised fish spleens (0.1g in 500µl PBS) were diluted in PBST buffer, PBST+ 1% Tween 20 or extraction buffer (PBST + 1% NP40) and 100µl of the antigen dilutions was added to each well. The microtitre trays were incubated in a circulating waterbath at 37°C for 2h. Following incubation, trays were washed using a programme consisting of a rinse and 3x1 min washes with PBST before addition of 100µl of biotinylated anti-*C. psychrophila* protein A/ sepharose purified IgG (BIgG) diluted in PBST + 1% BSA. Finally the plates were incubated in the waterbath at 37°C for a further 1h.

Following the BIgG incubation, the plates were washed with PBST as before and incubated for 1h at 37°C in the waterbath, with 100µl ExtraAvidin Horse-Radish Peroxidase (ExAvHRPO) (Sigma) conjugate diluted with PBST +1% BSA to either 1 / 500 or 1/ 1000. Following this step the plates were again washed as before, and then rinsed in distilled water (to remove any traces of Tween). Finally the substrate/ indicator solution was prepared by dissolving one tablet of the substrate, 3,3`,5,5`,-tetramethyl-benzidine dihydrochloride (TMB) (Sigma T-3405) in 10ml of phosphate-citrate buffer with sodium perborate (Sigma P4922) and 100µl added to each well. The reaction was stopped after 5 to 10min with 25µl of 10%H_2SO_4 and read at 450nm on a multi-beam ELISA reader (DYNATECH MR7000).

RESULTS

Production of rabbit antisera against *C. psychrophila*

Although several rabbits were used in the course of the study, only one (Rabbit number 236) produced acceptable levels of antibody, which was raised against *C. psychrophila* Isolate U648. An antiserum with an agglutination titre of 1/16 was obtained 1 week after two primary intramuscular injections and one intravenous booster (236 bleed 3). The titre rose to 1/32 (236 bleed 4) 10 days after a further intramuscular booster. Ten ml and 30ml of blood were collected at 6 and 8 days after the final intramuscular booster and the antiserum agglutination titres had risen to 1/128 (236 bleed 5) and 1/256 (236 bleed 6) respectively.

Bacterial agglutination

Agglutination titres for isolates of *C. psychrophila* U648, FEL2 and 1947^T, *C. aquatilis*, *C. columnaris*, *F. auriantiacus* and *A. hydrophila* are summarised in Table 8.1.

Table 8.1: Agglutination studies on *Cytophaga* spp. and *Aeromonas hydrophila*

Antisera	Bacterium	Titre
236/6	*C. psychrophila* U648	1/128
	C. psychrophila FEL2	1/4
	C. psychrophila 1947^T	1/16
	C. aquatilis	1/64
	C. columnaris	1/8
	F. auriantiacus	1/4
	A. hydrophila	1/4

Good agglutination titres (1/128) were achieved for U648 against whole serum. Isolates FEL2 and 1947^T were biochemically and morphologically identical to Bernardet and Grimont's characterisation of *C. psychrophila* (Bernardet and Grimont, 1989). However, little evidence of cross reactivity was seen with either FEL2 (1/4) or 1947^T (1/16). A low level of agglutination was observed with isolates of *C. aquatilis*, *C. columnaris*, *F. aurantiacus* or *A. hydrophila.* The data presented in this thesis represent an early agglutination study and later attempts using stored antiserum were less successful and showed a marked decrease in specificity and sensitivity over time.

Immuno-diffusion, Ouchterlony technique

Antiserum dilutions (236/6) of 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 were used in this technique. Heat inactivated and non-inactivated antigen at concentrations of $1x10^7$ cells/ml were included. Antigen-antibody precipitation lines were visible with *C. psychrophila* (U648, FEL2 and 1947^T) at serum concentrations of 1/2 to 1/8. Cross-reactivity at dilutions of 1/4 was evident with *C. columnaris*, *C. aquatilis* and *F. aurantiacus.* Precipitation lines were formed at serum dilutions of 1/2 and 1/4 with 10^7 cells/ml *Aeromonas hydrophila.* No precipitation was evident with any bacterial

isolates at serum dilutions > 1/8. No differences between heat inactivated and non-inactivated antigen were apparent.

Fluorescent antibody test (FAT)

C. psychrophila (Isolate U648) was positive by fluorescence. Using this technique no cross-reactivity with *C. columnaris*, *C. aquatilis* or *A. hydrophila* was apparent. No attempt was made to identify different serogroups using F.A.T. methods.

Purification of immunoglobulin G from rabbit antisera

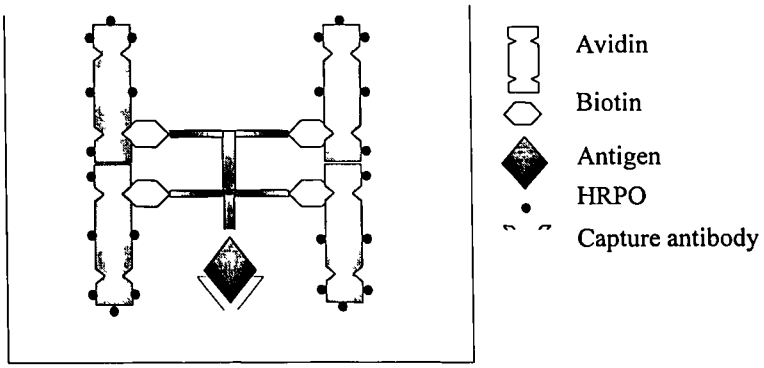
The immunoglobulin G (IgG) preparations included in the study were purified from 236 bleed 6 (agglutination titre 1/256) and ranged in concentration from 1.2mg/ml to 2.7mg/ml.

Enzyme linked immunosorbent assay (ELISA)

Amplification of ELISA technique for detection of C. psychrophila

A schematic diagram of the amplified ELISA used in the current study is shown in Figure 8.1.

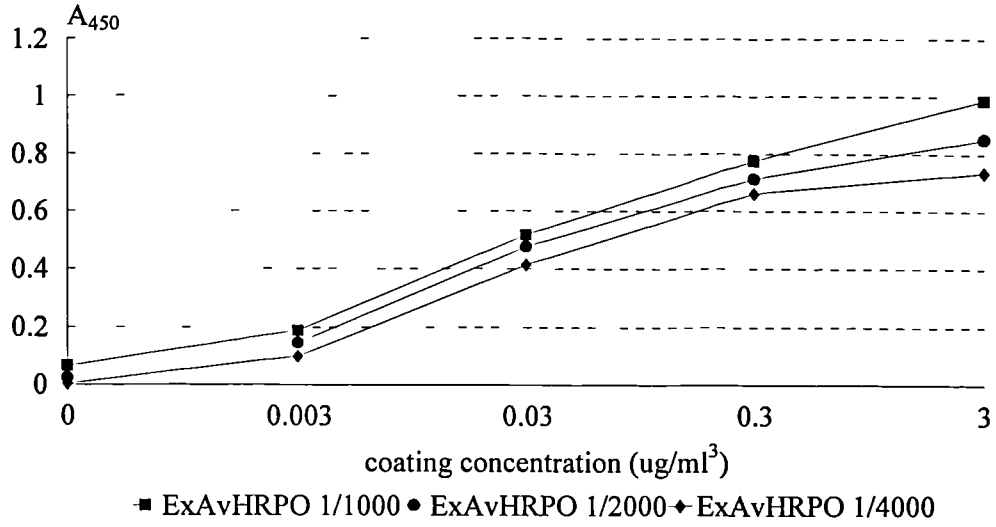
Figure 8.1: Schematic diagram of amplified ELISA technique



Chequerboard titrations were performed to establish the optimum coating concentration for IgG, biotinylated IgG dilution, antigen concentration and ExtraAvidin HPRO dilution. A series of preliminary studies showed that ideal antigen concentration was 10^8 cells/ml in PBST, preferably harvested from 72-96 hour AOA plates rather than from broth grown cultures. Working dilutions of 1/300 of the

biotinylated reagent consistently produced optimum A_{450} values and consequently were used in further ELISA studies. Figure 8.2 shows a chequerboard titration of capture and detection antibodies. A 5-fold increase in A_{450} values was observed following an increase in the coating concentration from 0.003µg/ml to 3.0µg/ml. Further studies were conducted using capture antibody concentration of 3.0µg/ml. The mean A_{450} values achieved using ExAv HRPO at concentrations of 1/1000, 1/2000 and 1/4000 were 0.74 at 1/4000, 0.86 at 1/2000 and 0.99 at 1/1000, consequently ExAv HRPO at 1/1000 was used.

Figure 8.2: Amplified ELISA for detection of *C. psychrophila*: chequerboard titration of capture and detection antibodies.(BIgG detection antibody diluted 1/300)



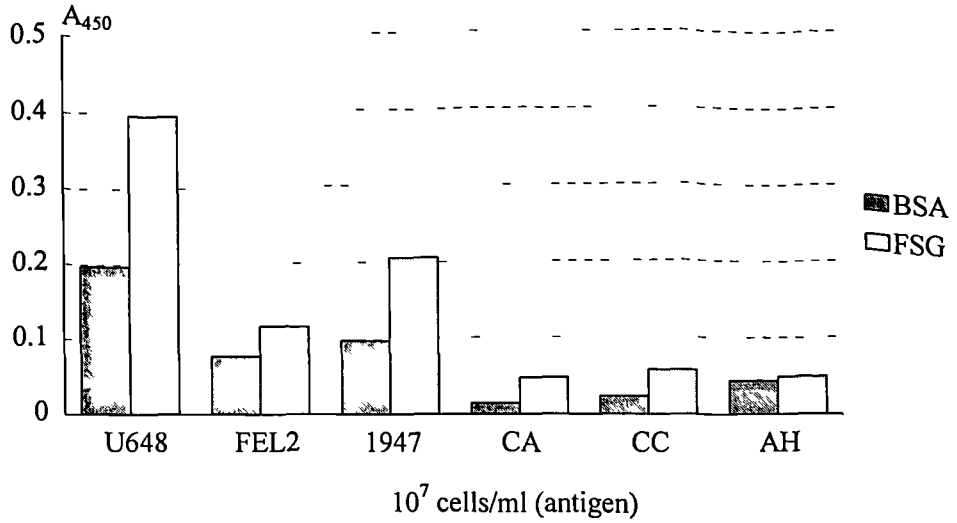
Cross reactivity amongst Cytophaga spp. and Aeromonas hydrophila

Details of the characteristics of *C. psychrophila* and the techniques used to determine these are described in Chapter 2. Figure 8.3 shows the cross-reactions amongst 3 isolates of *C. psychrophila* (U648, FEL2 and 1947^T) with identical morphological, physiological and biochemical profiles. Using the amplified ELISA differences were demonstrated between homologous antigens which separated the strains into 3 serogroups based upon their reactivity with immunoglobulin G (IgG)

236. Isolate U648 reacted strongly with IgG 236. Isolate FEL2 was donated by E. Lorenzen, National Serum Laboratories, Aarhus, Denmark, and originated from rainbow trout showing clinical signs of RTFS / CWD. No cross-reactivity was seen between FEL2 and IgG 236, but a low level of cross-reactivity was demonstrated between IgG 236 and the NCIMB *C. psychrophila* type strain 1947^T. Other representative members of the Cytophagaceae (*Cytophaga aquatilis* (CA) and *Cytophaga columnaris* (CC)) and the opportunist fish pathogen *Aeromonas hydrophila* (AH), all plate grown, were tested for cross-reaction in the ELISA technique with IgG 236. There were no cross-reactions with CA, CC or AH. On the basis of these studies it is speculated that at least three serogroups of *C. psychrophila* exist. Therefore isolates U648, FEL2 and 1947^T will be known hereafter as presumptive serotypes 1,2 and 3 and represented as CP1, CP2 and CP3 respectively.

Bovine serum albumin (BSA) and fish skin gelatin (FSG) are both routinely used to block free binding sites on microtitre plates coated with capture antibody in assays carried out at the Fish Diseases Laboratory (K. Way pers. comm.). In this study FSG was determined to be the most successful agent as A_{450} values were consistently higher with no accompanying increase in cross-reactivity.

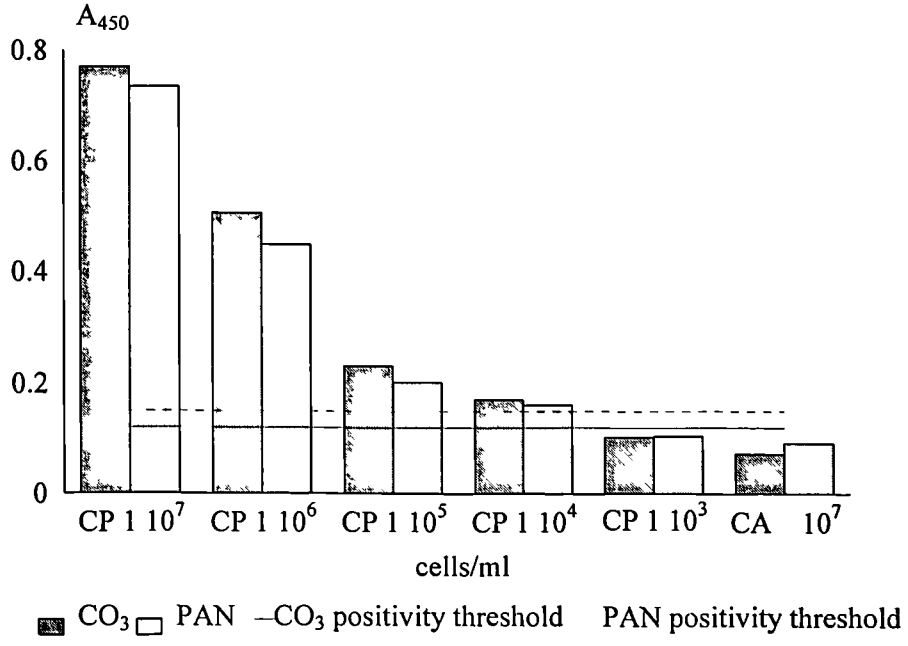
Figure 8.3: Cross reaction amongst *Cytophaga* spp. and *A. hydrophila* using an optimised ELISA technique



Sensitivity of the amplified ELISA for detection of C. psychrophila presumptive serotype 1 (CP1)

The positivity threshold used in the evaluation of the results in the current study was taken as the mean of the negative A_{450} values plus 3 standard deviations from this mean. In this case the negative wells were those incubated with *C. aquatilis* as antigens. Figure 8.4 shows the sensitivity of the ELISA technique using IgG 236 capture antibody and comparing Carbonate (CO_3) coating buffer with a commercial coating preparation, Panacoat (Biogenesis, Bournemouth, England). Panacoat is a patented solution which enhances the binding of antibodies and antigens on plastic surfaces. The manufacturers state that Panacoat is a blood platelet substitute with micronised silica particles as activators in conjunction with a low ion Tris buffer and claim that this exhibits properties similar to a biological glue as coating buffers. The positivity thresholds for CO_3 and Panacoat were calculated at 0.12 and 0.15 respectively, therefore the sensitivity of the amplified ELISA technique described here was determined to be $\geq 1x10^4$ cells/ml.

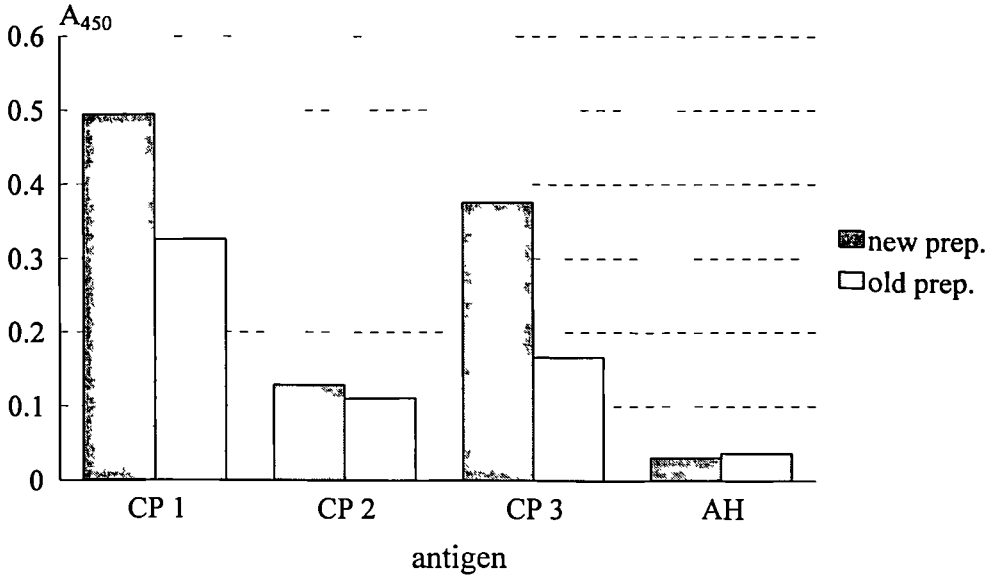
Figure 8.4: Sensitivity of the amplified ELISA for detection of *Cytophaga psychrophila* (CP 1)



The efficacy of two IgG preparations for detection of Cytophaga spp..

A number of IgG preparations were carried out during the course of the study. Figure 8.5 describes the efficacy of an original and a more recent IgG purifications prepared 6 months later. Whilst differentiation between CP 1(U648), CP 2 (FEL2) and CP 3 (1947^T) is possible using either reagent, the later preparation gave lower A_{450} values with homologous antigen and demonstrated higher levels of cross-reactivity between heterologous antigens. Neither preparation reacted with *Aeromonas hydrophila* at 10^7 cells/ml.

Figure 8.5: Efficacy of two IgG preparations for detection of *Cytophaga* sp

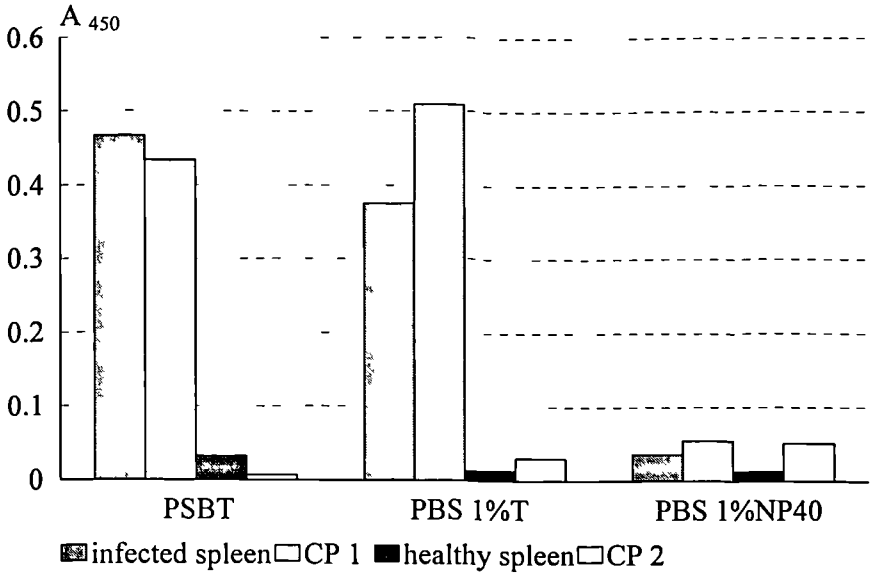


Detection of Cytophaga psychrophila (CP 1) from rainbow trout spleen

All of the samples collected from the RTFS positive site in PBS + 0.05% Tween 20 and PBS + 1.0% Tween 20 were positive by the ELISA technique (Figure 8.6). CP 1 antigen was not detected in samples diluted in PBS + 1.0% NP40. Potentially, interactions between the bacterial antigen and NP40 decreased the sensitivity of the ELISA technique. Plate grown CP 1 (Isolate U648) with 1.0% NP40

was also negative by the amplified ELISA technique, suggesting that the reduction in sensitivity was not associated with the use of fish extract. No detection of bacterial antigen was possible from fish extract from the non-infected site. Potentially this was because either no antigen was present or the level of antigen fell below the detection limit of the ELISA system. However, no clinical signs of disease were evident in the fry from the disease free site. Furthermore inoculation of spleen samples from the two sites onto MAOA revealed the presence of *C. psychrophila* in all ten fish sampled at farm 1 after 4-7 days incubation at 17°C but no evidence of the bacterium from farm 9 was seen. No cross-reactivity was demonstrated with CP 2 (Isolate FEL2) in this study.

Figure 8.6: Detection of *Cytophaga psychrophila* (CP1) from rainbow trout spleen using the amplified ELISA technique



DISCUSSION

The present study demonstrated that the ELISA technique can be used to identify isolates of *C. psychrophila* both from plate / broth grown cultures and directly from infected fish. The major modification to the double antibody sandwich ELISA was the amplification of the assay achieved by utilising biotinylated detection

antibodies and peroxidase enzyme conjugated to avidin which has a high affinity for biotin. Amplification of the test produced a high level of specificity and sensitivity, and the rapidity of the technique indicated that it could be a valuable aid to the diagnosis of RTFS. Furthermore, the ELISA technique indicated the existence of at least three distinct serological groups. Bacterial agglutination was also a satisfactory, although somewhat less sensitive or specific method of detecting *C. psychrophila* and distinguishing between the three presumptive serogroups. In accordance with this, Lorenzen (1994) had demonstrated that three serotypes were found amongst isolates of *C. psychrophila,* from a variety of freshwater fishes. *C. psychrophila* from rainbow trout fry in Chile and Spain were serologically different from morphologically and biochemically homogeneous isolates from the USA. It was proposed that these isolates made up a second serotype, expressing divergent surface antigen(s) to that of the type strain NCIMB 1947^T (Bustos *et al.*, 1994). In accordance, Wakabayashi *et al.*, (1994) found that *C. psychrophila* from coho salmon, rainbow trout and ayu shared common antigen(s), but serological analysis with absorbed antisera and heat-stable antigens separated the strains into just two serogroups. In the current study no exhaustive attempt was made to characterise the serotypical differences between isolates and those strains included in the development of the techniques all corresponded to presumptive serotype 1.

Using IgG preparations prepared from stored whole serum specificity and sensitivity of detection were diminished. In the present study, antiserum solutions and immunoglobulin preparations were stored at 4°C which possibly led to aggregation of antibodies in these solutions. Aggregation of the antibodies can cause a decrease in immunological activity by steric interference of the antigen combining site or by generating insoluble material that is then lost during centrifugation or filtration (Harlow and Lane, 1988). Another occurrence which produces a similar end result is the precipitation of cryoproteins (or cryoglobulins) on cold storage. In the work presented here, immunoglobulin G preparations were also stored at 4°C. If the proteins implicated in antigen recognition were cryoglobulins, precipitation would have effectively reduced levels of the specific antibodies and given an explanation for the decrease in specificity and sensitivity of the ELISA technique. Deterioration of IgG fraction is inherently more probable than that of whole serum as in the later

preparation other globulins are present which may serve to protect the IgG component. Although in the current study time did not allow for a full investigation of this phenomenon, future antibody preparations will be stored either at room temperature or lyophilised in an attempt to obviate these difficulties.

Although the agglutination technique did not demonstrate the specificity or sensitivity shown by the ELISA method, initially good, reproducible results were achieved. Again the quality of detection decreased with cold storage of the serum. A possible explanation for the differential sensitivity and specificity of the agglutination method compared to the ELISA technique is that in the former whole serum is utilised rather than the IgG fraction used in the latter. Mammals have five distinct classes of immunoglobulins present in their serum, differing from each other in size, charge, amino acid composition and carbohydrate content (Roitt, Brostoff and Male, 1989). Therefore, accompanying IgG, which is the major antibody of the secondary immune response and the exclusive antitoxin class, whole serum could have contained high levels of the larger IgM molecule which is designated "early" antibody (Roitt *et al.*, 1989) and may have been less specific for *C. psychrophila.* IgM is the major precipitating antibody and is also important in agglutination reactions. For successful serodiagnostics, specific and potent antisera are required and to prevent the antibody response from becoming too diffuse, immunising injections are normally carried out only over a 3 to 4 week period (Way, pers.comm.). However, the agglutination titres of antibody achieved in the current study were initially low. The poor response to the first injections necessitated the administration of a second series two months after the primary injections. These inoculations elicited a much greater response and this is likely to have led to the production of multi-specific antibody. If a large component of the antiserum comprised multi-specific antibody then this would account for the greater cross-reaction seen in the agglutination techniques compared to the ELISA which utilised IgG antibody exclusively. Equally, as the Ouchterlony technique was performed using whole serum, the lack of specificity could possibly be explained by the prevalence of non-specific immunoglobulin.

Fluorescent antibody techniques (FAT) are useful diagnostic tools as they can rapidly provide identification of disease agents and may be applied directly to histological sections (Bullock and Stuckey, 1975). Lorenzen and Karas (1992)

successfully used direct immunofluorescence analysis of spleen imprints to identify *C. psychrophila* from diseased rainbow trout fry. Initially a low level of cross reactivity with *C. columnaris* was observed but was avoided by absorption of the diagnostic antiserum. In the present study no cross-reactivity was demonstrated with *C. columnaris*, *C. aquatilis* or *A. hydrophila*. However tests were only applied to plate/ broth grown bacterial cultures and further studies are required before reliable diagnosis can be made from direct application to slides prepared from diseased specimens. Immunofluorescence is commonly used as both a presumptive and confirmatory test for the identification of bacterial fish pathogens (Smith, Goldring, and Dear 1987; Rodgers, 1991; Cvitanich, 1994). Some authors have however questioned the validity of this technique, low fluorescence intensity and the appearance of false positives particularly in the identification of *Renibacterium salmoninarum* (Evelyn, Ketcheson and Prosperi-Porta, 1981).

Under optimised conditions, the ELISA technique developed in the current study was sensitive to $1x10^4$ cells/ml allowing successful detection of *C. psychrophila* in infected spleen samples. Two non-ionic detergents were evaluated in an attempt to enhance the sensitivity of the technique. A variety of non-ionic detergents were used by Middledorp, Hooymans, Kocken, Loon, Emsbroek and Coutinho (1987) to ameliorate detection of herpes simplex virus antigens in lesion swab specimens. Frequently these are included in extraction buffers to increase the solubility and consequently the accessibility of the antigen. Way (1991) demonstrated that Spring Viraemia of Carp virus (SVCV) extracted from infected tissues with buffer containing NP40 gave consistently higher A_{405} values with than similar volumes of Tween 20. However, the inclusion of NP40 in the extraction buffers supplemented with similar levels with *C. psychrophila* in the present study had an inhibitory effect compared to Tween 20. Additionally, increasing the concentration of Tween 20 in the extraction buffer did not increase absorbance levels. In this instance Nonidet P40 may have disrupted the antigen rendering it inaccessible.

The ELISA technique has achieved popular acceptance as a quick and sensitive method for the detection and identification of a wide variety of viral and bacterial fish pathogens (Dixon, 1985). Serological demonstration of specific antigen directly from tissues of diseased fish forestalls the need to grow the organism prior to

preliminary diagnosis. The causative organism of RTFS is very slow growing and has fastidious growth requirements. Rapid, reliable serodiagnostic methods such as have been demonstrated here, could provide accurate early disease diagnosis for the farmer and veterinarian allowing considered, expeditious decisions on the administration of treatments to be made. Additionally improvements in the sensitivity of the ELISA technique may ultimately enable the detection of carrier fish.

CHAPTER 9

GENERAL CONCLUSIONS

During the initial phase of this study RTFS was shown to be a single disease and not a multi-factorial syndrome. The morphological, biochemical and serological characterisation of a large number of bacterial isolates from RTFS affected fish indicated that infection with the Gram negative, chromogenic, rod-shaped bacterium *C. psychrophila* was responsible for a distinct condition throughout the U.K. The disease was prevalent in the majority of rainbow trout producing countries, where it was known variously as Rainbow Trout Fry Syndrome, Rainbow Trout Fry Anaemia, Fry Mortality Syndrome and Visceral Myxobacteriosis. Observations on naturally and experimentally infected fry/ fingerlings revealed that lethargy, inappetance, hanging at the water surface, increased epidermal pigmentation and bilateral exophthalmia were indicative of the condition. Hypertrophy of the spleen and cellular degeneration are frequently associated with bacterial septicaemia in salmonid fish, but the changes seen in histological sections prepared from diseased fry appeared to be unique. Therefore, loss of border definition and its replacement by a loosely structured eosinophilic layer, fibrinous inflammation, intercellular oedema in the spleen together with the presence of numerous filamentous bacteria interspersed throughout the organ were considered to be pathognomonic for RTFS.

Behavioural, external and internal signs of disease analogous to natural infections were experimentally reproduced in fry by the intraperitoneal and immersion challenge routes. Considerable variation in virulence was observed between strains following intraperitoneal injection, emphasising the importance of standardisation of the infection model for future studies. Further studies on the pathogenicity mechanisms of *C. psychrophila* and effects of storage are required before a truly effective, reproducible challenge protocol can be established. The method of entry of the bacterium into fish tissues was not elucidated in the present study. It has however been reported that infective *Cytophaga*-like bacteria can gain access to the host across the skin and gills of immunosuppressed salmonids under sub-optimal environmental conditions (Roberts, 1976). Additionally, the physical damage of the skin by surface

abrasion is reported to elevate levels of bacterial uptake (Cipriano, 1982). Although *C. psychrophila* was sporadically recovered from lesions on the surface of affected fish suggesting that the bacteria may invade the skin, in the current study the use of scarification to potentially increase mortalities was not examined as the object of the immersion challenge protocol was to simulate conditions of natural infection as accurately as possible. Lorenzen (1994) reported that bacteria and/ or bacterial products were detectable in the gut lumen and mucosa/ submucosa of naturally infected fry, but in the current study filamentous bacteria were only observed in the gut of intraperitoneally infected fish. Chua (1991) and Bruno (1992) observed filamentous bacteria associated with the gills of fry suffering from RTFS. Conversely in the present investigation the gills of the majority of fish were in excellent condition showing little indication of bacterial invasion. Whilst it is conceivable that *C. psychrophila* enters the host tissues via a combination of routes, further work is required to elucidate the sequence and mode of transmission.

Evaluation of the abundance and diversity of bacterial species in ground waters has demonstrated the presence of representatives of the genera *Cytophaga* sp. (Rheinheimer, 1992). Furthermore, Quevedo-Samiento *et al.* (1986) established that members of the phylogenetic group comprising *Cytophaga-Flexibacter-Flavobacterium* regularly formed part of the aerobic heterotrophic flora of ground waters. In accordance with this, environmental sampling carried out during the present study demonstrated that members of the family Cytophagaceae formed a substantial element of the bacterial flora isolated from natural spring waters. However, speciation of the isolates obtained failed to reveal the pathogen, and recovery of *C. psychrophila* either from waters or sediments was exclusively associated with the occurrence of substantial mortalities in fry attributable to RTFS.

The presence of the bacterium in the sexual fluids of adult rainbow trout indicated that broodstock could serve as reservoirs of the pathogen. Additionally viable cells of *C. psychrophila* were demonstrated on the surfaces of eggs collected from these "carrier" fish. These results imply that *C. psychrophila* is not a ubiquitous aquatic organism, but has been imported into U.K. rainbow trout hatcheries, potentially as a consequence of the extensive worldwide trade in salmonid eggs. Transportation of both eggs and live fish of unknown health status between farms

without adequate disease precautions has unquestionably encouraged the spread of the syndrome.

Oral administration of oxytetracycline at up to 300mg/ kg fish/ day for 10-14 days and amoxycillin at 75-80mg/kg live fish/day for 7 days are frequently used on rainbow trout hatcheries during outbreaks of RTFS. Antimicrobial chemotherapy remains the most effective means of mitigating losses and, whilst in most cases judicious use of these compounds does reduce the level of mortality, increasingly "resistant" strains of the bacterium are emerging. This highlights the need for continual stock monitoring, careful drug use and cessation of prophylactic administration of the limited numbers of efficacious agents currently available to the industry. *In vitro* analysis of several novel antimicrobial compounds in the present study revealed that the new generation 4-quinolones and the fluorinated analogue of thiamphenicol, florfenicol, may have therapeutic utility against infections with *C. psychrophila.* However, assumptions on potential chemotherapeutic efficacy made on the basis of *in vitro* testing alone do not invariably equate to therapeutic benefit from particular compounds in the field. This is illustrated by the relatively low MIC level demonstrated for oxolinic acid against *C. psychrophila* compared with its slight effect against outbreaks of RTFS *in vivo*. Alterations to the chemical or physical state of currently available compounds such as varying the salts, particle sizes and analysis of the incorporation into medicated diets, may obviate some of the difficulties presently associated with the management of the syndrome. Furthermore, examination of the potential of antisepsis with antimicrobials may reduce difficulties connected with the oral administration of chemotherapeutics to fish which have either become inappetant due to the onset of disease or contracted disease prior to the development of the feeding response.

Disinfection of eyed salmonid ova and the fish holding facilities is considered to be an attractive approach to prevent the spread of infectious diseases and can potentially facilitate a reduction in the use of palliative antimicrobial therapy. Iodophors are commonly used in aquaculture to disinfect eyed ova but in accordance with Lorenzen (1994), the current study revealed that application of iodophors at the *in vitro* MIC level did not inactivate *C. psychrophila* adhering to rainbow trout egg surfaces. Iodophors are recommended for the disinfection of imported eyed salmonid

ova predominantly to reduce the risk of transmission of exotic viral diseases into the U.K.. Therefore, whilst the maintenance of rigorous disinfection procedures is vital, additional complementary methods may be needed to prevent the spread of RTFS. Evaluation of other methods of disinfection predicted that glutaraldehyde, 2-bromo-2-nitropropane-1,3-diol, and a stable form of the oxidator, hydrogen peroxide, may be efficacious as disinfectants of egg surfaces. Hydrogen peroxide and its derivatives have the additional advantage of degrading to environmentally innocuous by-products therefore reducing the problems associated with the accumulation and disposal of toxic effluent. The potential efficacy of various compounds as disinfectants of equipment associated with fish production was also examined. Most regularly utilised formulations effectively neutralised cells of *C. psychrophila* at concentrations below the recommended level of use. Therefore future work will focus on the methods of on farm application. The practice of pressure washing concrete troughs is commonplace in hatcheries, however the use of high pressure washers on unpainted concrete troughs encourages the formation of cracks and pores which are liable to make effective disinfection impossible. Ensuring adequate exposure times and application of latex or acrylic paint to cleaned, disinfected surfaces is likely to be beneficial and deserves investigation.

Currently no commercial vaccine is available against infections with *C. psychrophila*. Although world aquaculture is a rapidly expanding and significant industry it is still small relative to other forms of intensive agriculture, consequently at the moment commercial vaccines have only been produced for enteric red mouth, vibriosis and furunculosis in salmonids and erythrodermatitis in carp. This is due partly to the slow rate of development of immuno-competance in salmonid fish. Ellis (1988) reported that commercially viable levels of protective immunity in salmonids vaccinated against ERM or vibriosis are not reached until fry are 0.5-1.0g. Additionally, he established that adequate duration of protection is not reached until fry are 4.0g. It has been demonstrated that rainbow trout can succumb to infections with *C. psychrophila* as yolk-sac fry and in such cases the likely efficacy of vaccination would be negligible. However, mortality attributable to RTFS is frequent among fingerlings (>5.0g) and consequently vaccination is considered to be an attractive approach to mitigate losses in larger fish. Other limitations upon the

expansion of effective fish vaccines have been attributed to the difficulties associated with the manufacture of a marketable product which demonstrates an adequate level of protection and performs consistently according to closely defined licensing standards. Furthermore, the lack of commercial products can be traced to the absence of fish diseases which are considered sufficiently important enough to justify the scale of expenditure necessary to develop and license a commercial vaccine (Horne and Ellis, 1988). The financial impact of RTFS on world aquaculture is hard to quantify. A recent report in France (Fish Farming International, 1995) stated that since the implementation of the Commission Regulation 1430/94/EC which placed chloramphenicol in Annex IV of EC Regulation 2377/90/EC (MAVIS, 1994), in August 1994, mortalities attributable to RTFS had reached 40-80 million fingerlings. Although authentication of reports on mortality levels is problematic, considerable interest in a commercial product exists within both the industry and manufacturers of fish vaccines, and consequently future research will concentrate on standardisation of growth media, detection of protective antigens and classification of the serotypes of *C. psychrophila* to enhance the prospects of vaccine development.

In summary, the work presented in this thesis has clarified the aetiology and described the histopathology of RTFS. It has demonstrated the potential therapeutic benefits of various chemotherapeutant agents and provided methods of rapid disease diagnosis. Furthermore, it has shown that the disease can be induced in naive, susceptible fish under laboratory and farm conditions, and has elucidated possible mechanisms of transmission and transport of infections with *C. psychrophila*. Finally it has highlighted further avenues of investigation for future studies.

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APPENDIX 1: Bacterial cell culture media

Modified Anacker and Ordal Medium (MAOA)

Formula (per litre)

Tryptone	5.0g
Yeast extract	0.5g
Sodium acetate	0.2g
Beef extract	0.5g
Agar bacteriological (agar No. 1)	15.0g
(pH 7.2)	

Method

Suspend in 1 litre of distilled water. Sterilise by autoclaving at 121°C, 15mins ($0.72kg/cm^3$)

Cytophaga agar (Anacker and Ordal, 1959)

Formula (per litre)

Tryptone	0.5g
Yeast extract	0.5g
Sodium acetate	0.2g
Beef extract	0.2g
Agar no.1	9.0g
(pH 7.2)	

Method

Suspend in 1 litre of distilled water. Sterilise by autoclaving at 115°C, 20 mins ($0.72Kg/cm^3$).

Tryptone soya agar (TSA) (Oxoid)

Formula (per litre)

Tryptone	15.0g
Soya peptone	5.0g
Sodium chloride	5.0g
Agar no.1	15.0g

(pH 7.3)

Method

Suspend in 1 litre of distilled water. Sterilise by autoclaving at 115°C, 15 mins ($0.72Kg/cm^3$).

ROD agar (Rodgers, 1992)

Formula (per litre)

Yeast extract	3.0g
Ornithine	5.0g
Ribose	3.75g
Maltose	7.5g
Sodium chloride	5.0g
Sodium thiosulphate	6.8g
Ferric ammonium citrate	0.8g
Phenol red	0.08g
Agar no. 1	12.5g

(pH 7.4)

Method

Suspend in 1 litre of distilled water. Boil, cool, filter 1% sodium dodecyl sulphate into agar before pouring.

Aeromonas agar (RYAN), (Oxoid)

Formula (per litre)

59.0g Aeromonas agar

I vial rehydrated Ampicillin supplement SR136

Method

Suspend in 1 litre of distilled water. Boil, cool to 50°C add Ampicillin supplement SR136

SKDM agar (Austin, 1983)

Formula (per litre)

Tryptone	10.0g
Yeast extract	0.5g
Agar no.1	10.0g
Distilled H_2O_2	900ml
Foetal calf serum	100ml
L- Cysteine hydrochloride	12.5g
Cycloheximde (1.0g in 100ml distilled H_2O_2)	5.0ml
D-Cycloserine (0.1g in 10.0ml)	1.25ml
Polymyxin B (0.1g in 10.0ml)	2.5ml
Oxolinic acid (0.1ml in 0.5ml)	0.25ml

(pH 6.8)

APPENDIX 2

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 1:

	C1		C2		row sum
1	38	20.5	3	20.5	41
	14.939	38.0%	14.939	3.0%	41.%
	76.0%	92.7%	6.0%	7.3%	
2	12	29.5	47	29.5	59
	10.381	12.0%	10.381	47%	59.0%
	24.0%	20.3%	94.0%	79.7%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic = 50.64076

degrees of freedom = 1

right tail of probability = 0.0000

with Yates` correlation = 47.78834

degrees of freedom = 1

right tail of probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 2:

	C1		C2		row sum
1	47	24	1	24	48
	22.041	47.0%	22.041	1.0%	48.%
	94.0%	97.9%	2.0%	2.1%	
2	3	26	49	26	52
	20.346	3.0%	20.346	49%	52.0%
	6.0%	5.8%	98.0%	94.2%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =84.77564

degrees of freedom = 1

right tail probability = 0.0000

with Yates` correlation =81.12981

degrees of freedom = 1

right tail probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 3:

	C1		C2		row sum
1	15	9	3	9	18
	4	15.0%	4	3.0%	18.0%
	30.0%	83.3%	6.0%	16.7%	
2	35	41	47	41	82
	0.8780	35.0%	0.8780	47%	82.0%
	70.0%	42.7%	94.0%	57.3%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic = 9.756098

degrees of freedom = 1

right tail probability = 0.0018

with Yates` correlation = 8.197832

degrees of freedom = 1

right tail probability = 0.0042

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 4:

	C1		C2		row sum
1	19	10	1	10	20
	8.1	19.0%	8.1	1.0%	20.0%
	38.0%	95.0%	2.0%	5.0%	
2	31	40	49	40	80
	2.025	31.0%	2.025	49%	80.0%
	62.0%	48.8%	98.0%	61.3%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =20.25

degrees of freedom = 1

right tail probability = 0.0000

with Yates` correlation = 18.0625

degrees of freedom = 1

right tail probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 5 (isolate 1344)

	C1		C2		row sum
1	37	18.5	0	18.5	37
	18.5	37.0%	18.5	0.0%	37.0%
	74.0%	100.0%	0.0%	0.0%	
2	13	31.5	50	31.5	63
	10.865	13.0%	10.865	50.0%	63.0%
	26.0%	20.6%	100.0%	79.4%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic = 58.73016

degrees of freedom = 1

right tail probability = 0.0000

with Yates` correlation = 55.59846

degrees of freedom = 1

right tail probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 6 (isolate 0273)

	C1		C2		row sum
1	30.5	15.75	1	15.75	31.5
	13.813	30.5%	13.813	1.0%	31.5%
	61.0%	96.8%	2.0%	3.2%	
2	19.5	34.25	49	34.25	68.5
	6.3521	19.5%	6.3521	49.5%	68.5%
	39.0%	28.5%	98.0%	71.5%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =40.33136

degrees of freedom = 1

right tail probability = 0.0000

with Yates` correlation =37.64338

degrees of freedom = 1

right tail probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 7 (isolate NL193)

	C1		C2		row sum
1	29.5	15	0.5	15	30
	14.016	29.5%	14.016	0.5%	30.0%
	59.0%	98.3%	1.0%	1.7%	
2	20.5	35	49.5	35.5	70
	6.0071	20.5%	6.0071	49.5%	70.0%
	41.0%	29.3%	99.0%	70.7%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =40.04762

degrees of freedom = 1

right tail probability = 0.0000

with Yates` correlation =37.3333

degrees of freedom = 1

right tail probability = 0.0000

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 8 (isolate 1947)

	C1		C2		row sum
1	15.5	8.5	1.5	8.5	17
	5.7647	15.5%	5.7647	1.5%	17.0%
	31.0%	91.2%	3.0%	8.8%	
2	34.5	41.5	48.5	41.5	83
	1.1807	34.5%	1.1807	48.5%	83.0%
	69.0%	41.6%	97.0%	58.4%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =13.89086

degrees of freedom = 1

right tail probability = 0.0002

with Yates` correlation = 11.97732

degrees of freedom = 1

right tail probability = 0.0005

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 9 (isolate JO121)

	C1		C2		row sum
1	10.5	5.75	1	5.75	11.5
	3.9239	10.5%	3.9239	1.0%	11.5%
	21.0%	91.3%	2.0%	8.7%	
2	39.5	44.25	49	44.25	88.5
	0.5098	39.5%	0.5098	49.0 %	88.5%
	79.0%	44.6%	98.0%	55.4%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =8.8676

degrees of freedom = 1

right tail probability = 0.0029

with Yates` correlation = 7.098993

degrees of freedom = 1

right tail probability = 0.0077

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Experiment 10 (isolate FEL2)

	C1		C2		row sum
1	8.5	4.5	0.5	4.5	9
	3.5555	8.5%	3.5555	0.5%	9.0%
	17.0%	94.4%	1.0%	5.6%	
2	41.5	45.5	49.5	45.5	91
	0.3516	45.5%	0.3516	49.5%	91.0%
	83.0%	45.6%	99.0%	54.4%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic = 7.814408

degrees of freedom = 1

right tail probability = 0.0052

with Yates` correlation = 5.982906

degrees of freedom = 1

right tail probability = 0.0144

APPENDIX 2 cont.

CONTINGENCY TABLE (2 rows x 2 columns)

Differences between groups 5-10

	C1		C2		row sum
1	15.5	20.25	34.5	29.75	50
	1.1142	5.2%	0.7584	11.5	16.7%
	12.8%	31.0%	19.3%	69.0%	
2	20.5	20.25	29.5	29.75	50
	0.0030	6.8%	0.0021	9.8%	16.7%
	16.9%	41.0%	16.5%	59.0%	
3	37	20.25	13	29.75	50
	13.854	12.3%	9.4306	4.3%	16.7%
	30.5%	74.0%	7.3%	26.0%	
4	8.5	20.25	41.5	29.75	50
	6.8179	2.8%	4.6407	13.8%	16.7%
	7.0%	17.0%	23.2%	83.0%	
5	10.5	20.25	39.5	29.75	50
	4.6944	3.5%	3.1953	13.2%	16.7%
	8.6%	21.0%	22.1%	79.0%	
6	29.5	20.25	20.5	29.75	50
	4.2253	9.8%	2.8760	6.8%	16.7%
	24.3%	59.0%	11.5%	41.0%	
col sum	121.5		178.5		300
	40.5%		59.5%		100%

chi-squared statistic = 51.61324

degrees of freedom = 5

right tail of probability = 0.0000

APPENDIX 2

CONTINGENCY TABLE (2 rows x 2 columns)

Natural challenge trial

	C1		C2		row sum
1	0	4.5	9	4.5	9
	4.5	0.0%	4.5	9.0%	9.0%
	0.0%	0.0%	18.0%	100.0%	
2	50	45.5	41	45.5	91
	0.4450	50.0%	0.4450	41.0%	91.0%
	100.0%	54.6%	82.0%	45.1%	
col sum	50		50		100
	50.0%		50.0%		

chi-squared statistic =9.89011

degrees of freedom = 1

right tail probability = 0.0017

with Yates` correlation =7.814408

degrees of freedom = 1

right tail probability = 0.0052

APPENDIX 3

Staining Techniques

Haematoxylin and eosin stain (H&E)

1. Place slides in histoclear to remove wax (1-2min)
2. Repeat
3. Place in 100% alcohol to remove the solvent (2 min)
4. Repeat
5. Wash in running tap water (2-5 min)
6. Place in haematoxylin (BDH) (3 min)
7. Wash in running tap water 5-10 min
8. Place in aqueous eosin (BDH) (3 min)
9. Wash in running tap water (5 min)
10. Rinse in 70% alcohol
11. Place in 100% alcohol (1-2 min)
12. Repeat
13. Place in 50/50 alcohol histoclear (1-2 min)
14. Place in histoclear, mount in D.P.X. leave to dry

May-Grunwald Giemsa Stain

1. Fix in methanol or 0.5% acetic acid in methanol
2. Place in May Grunwald solution (2 parts May Grunwald stain powder (BDH) (3.0g) in methanol (1000ml) in 1 parts pH 6.8 buffer)
3. Place into Gurr's Giemsa (BDH) plus 9 parts pH 6.8 buffer for 15-20 min
4. rinse twice in pH 6.8 buffer
5. Blot dry, dry on a hot plate, dip in histoclear and mount with D.P.X.

Martius-Scarlet Blue (MSB)

1. Make up trichrome mixtures

(a) Martius yellow	0.5g
Phosphotungstic acid	2.0g
distilled H_2O	5.0ml
100% alcohol	95.0ml
(b) Brilliant crystal scarlet 6R1.0	1.0g
Glacial acetic acid	2.5ml
distilled H_2O	97.5ml
(c) Aniline blue	0.5g
Glacial acetic acid	1.0ml
distilled H_2O	99.0ml

2. Sections to water
3. Weigerts haematoxylin (15-20 min)
4. Rinse in tap water
5. Place in acid alcohol (5-10 sec)
6. Rinse in tap water
7. Rinse in alcohol
8. Place in (a) (2 min)
9. Rinse in tap water
10. Place in (b) (10 min)
11. Rinse in tap water
12. Phosphotungstic acid (5-10 min)
13. Rinse in tap water
14. Place in (c) (10 min)
15. Rinse in tap water
16. Dehydrate, clear and mount

APPENDIX 4

Purity and sources of antimicrobials used in the Chapter 5

Antimicrobial name	Supplier/ manufacturer	Lot no.	Purity
Enrofloxacin	Bayer	PT275665T	100%
Ciprofloxacin	Sigma	PT262524T	100%
Sarafloxacin	L. Brown	37788CE	100%
Flumequine	Sigma	79F0089	100%
Sodium oxolinate	Vetrepharm Pharmacy	BN4057	100%
Oxolinic acid	Sigma	20H0315	100%
Doxycycline	Vetrepharm Pharmacy	MS0083	100%
Oxytetracycline	Sigma	33H0667	100%
Vetremox (amoxycillin)	Vetrepharm Pharmacy	09368	100%
Furazolidone	Sigma	47F0405	100%
Gentamycin	Sigma	42H0610	100%
Neomycin	Sigma	92H0386	85% B 15% C
Streptomycin	Sigma	39F0749	100%
Sulphadiazine	Sigma	55H0714	100%
Trimethoprim	Sigma	114H0139	100%
Chloramphenicol	Sigma	72F0450	100%
Florfenicol	I Sutherland		100%
Thiamphenicol	Sigma	31H0465	100%
Vancomycin	Sigma		100%
Erythromycin	Sigma	31H0577	100%

APPENDIX 4.1 Minimum inhibitory concentrations of antimicrobial compounds (μg ml^{-1}) for *C. psychrophila*

Strain	eth	fur	oa	otc	strep	ts	van	amox	chlor	cip	doxy	thia	flu	neo	flor	gent	sara	so	enro
1342	0.5	0.5	0.125	1	2	32	8	0.01563	1	0.0078	0.25	2	0.063	128	0.125	32	0.00195	0.125	0.00195
1344	0.5	0.25	0.0313	1	4	32	4	0.01563	1	0.0078	0.5	2	0.125	64	0.125	32	0.00195	0.125	0.01563
o278	1	0.5	0.125	1	2	32	8	0.01563	1	0.0078	0.25	2	0.063	128	0.125	32	0.00195	0.125	0.00195
o273	1	0.25	0.0313	1	4	32	4	0.01563	1	0.0078	0.5	2	0.125	64	0.125	32	0.00195	0.125	0.125
fel62	64	64	2	8	4	64	4	0.25	64	0.0625	0.125	1	8	128	8	0.25	2	0.25	0.03125
fel10	64	64	2	4	16	64	4	0.25	64	0.125	0.125	1	1	512	8	0.25	2	0.25	0.0625
fel16	64	512	16	64	4	128	8	0.0625	32	0.25	0.5	2	8	512	8	512	0.125	0.125	0.03125
fel5	2	1	1	2	32	64	512	0.125	1	0.0625	0.0625	2	0.5	256	2	128	2	0.5	0.03125
u648	4	64	4	4	8	128	16	0.125	4	0.25	0.03125	4	0.5	128	4	8	4	0.5	0.0625
1947	0.03125	0.5	0.25	0.125	4	16	16	0.0625	0.125	0.0039	0.125	0.25	0.5	1	0.5	16	0.00195	0.25	0.00391
fel2	4	64	0.0625	32	4	64	8	0.0625	512	0.0039	0.25	1	8	512	8	128	8	0.5	0.00195
c292	1	0.03125	4	8	2	256	8	0.03125	0.25	0.5	0.25	0.0156	0.063	16	0.00098	4	0.01563	0.00098	0.01563
r151	2	16	2	4	4	256	32	0.125	4	0.0156	0.125	32	2	64	8	64	1	8	0.25
a1	4	2	4	8	2	256	16	0.125	1	0.0078	4	8	8	64	8	256	0.5	8	0.03125
d593	1	2	2	8	2	128	4	0.03125	2	0.0078	0.25	2	8	16	1	32	1	1	0.03125
u147	64	64	1	64	2	512	16	16	128	0.0156	8	8	16	128	8	128	0.25	8	0.25
c193	32	4	0.5	4	16	64	4	0.125	0.5	0.0156	0.25	32	2	256	8	256	0.5	4	0.125
w693	64	32	1	16	8	128	4	0.03125	8	0.0156	0.5	32	8	256	4	512	0.125	8	0.00098
co11	64	16	0.5	32	4	32	4	0.03125	64	0.0078	0.25	16	8	128	8	256	0.25	2	0.0625
u249	32	64	1	16	4	128	64	0.125	32	0.0625	0.5	1	0.5	256	2	256	0.0625	2	0.00195
w593	4	2	1	8	4	128	32	0.125	32	0.0078	1	2	1	256	2	64	0.125	0.125	0.00195
w1293	4	16	2	4	4	32	16	0.125	2	0.0625	0.25	2	1	128	0.125	64	0.00195	0.25	0.25
s5793	0.0625	1	0.25	16	0.25	128	16	0.03125	1	0.0078	0.25	0.25	1	128	0.25	128	0.00195	0.25	0.25
s7793	2	32	2	8	2	128	4	8	0.25	0.0156	0.03125	2	0.5	256	0.25	16	0.01563	0.25	0.00195
b593	0.25	16	0.25	16	16	32	16	0.125	0.25	0.0156	0.0625	4	2	32	0.25	32	0.01563	0.5	0.00195
t20	0.5	32	0.25	32	1	16	8	0.0625	0.125	0.0078	0.125	4	2	32	0.5	8	0.00781	0.25	0.125
b393	0.125	16	0.5	8	2	32	8	0.125	0.125	0.0156	0.125	2	2	16	0.25	32	0.00781	0.125	0.03125
sh221	1	16	0.5	16	0.25	16	16	0.0625	0.125	0.0078	0.03125	2	2	16	0.125	16	0.125	0.5	0.125
mm221	0.5	8	0.25	8	1	32	16	0.03125	0.25	0.0078	0.25	4	2	16	0.5	8	0.0625	1	0.03125
bw222	0.5	16	1	4	2	32	32	0.125	0.25	0.0156	0.25	1	1	8	1	8	0.125	0.5	0.00781
rb192	0.5	16	2	2	4	16	16	0.0625	0.0313	0.0078	0.25	2	2	8	0.5	8	0.125	0.25	0.00781

APPENDIX 4.1 continued Minimum inhibitory concentrations (μg ml^{-1}) of antimicrobial compounds for *C. psychrophila*

bb693	1	4	1	8	4	8	16	0.125	0.0313	0.0078	0.25	4	1	16	0.25	128	0.03125	4	0.03125
u842	64	64	1	16	4	16	8	0.125	128	0.0078	0.25	1	4	256	4	128	0.25	2	0.00195
bb5	4	32	0.5	4	2	64	8	0.0625	0.25	0.0078	0.125	1	2	512	2	128	0.00195	0.01563	0.00781
tvt20	1	8	0.0313	0.0625	2	128	8	1	1	0.0005	0.0625	32	0.5	128	0.01563	256	0.00195	0.01563	0.125
u239	2	16	0.0313	0.0313	4	128	16	1	0.5	0.002	0.25	32	0.5	128	0.01563	128	0.00391	4	0.03125
f163	8	4	1	1	2	64	8	64	128	0.002	0.25	32	0.5	256	4	128	0.03125	16	0.125
bb8	1	16	2	32	4	64	4	0.03125	64	0.0078	0.25	32	0.5	64	16	6	0.03125	1	0.03125
nl193	2	8	0.5	2	1	128	4	0.00195	2	0.0039	0.5	4	0.125	512	1	64	0.01565	0.25	0.00781
p183	2	8	0.25	1	1	16	2	0.00391	4	0.3913	0.25	2	0.031	256	0.25	6	0.03125	1	0.00781
d693	2	8	0.5	2	1	128	4	0.00195	2	0.0039	0.5	4	0.125	512	1	64	0.01563	0.25	0.00781
u148	2	8	0.25	1	1	16	2	0.00391	4	0.3913	0.25	2	0.031	256	0.25	64	0.01563	0.01563	0.03125
sh222	1	32	0.5	32	0.25	16	32	0.0625	0.125	0.0078	0.03125	2	2	32	0.5	128	0.00195	0.01563	0.01563
jo121	1	4	0.0313	0.0625	2	128	8	1	1	0.0005	0.0625	32	0.5	128	0.01563	128	0.00195	0.01563	0.00781
vt121	2	4	0.0625	0.0625	1	128	4	1	1	0.0005	0.0625	32	0.5	128	0.01563	128	0.00195	0.01563	0.00781
ov812	4	8	0.125	0.5	4	256	8	2	2	0.0005	0.0625	64	1	128	0.01563	64	0.125	0.125	0.03125
f167	2	8	1	4	2	32	16	0.125	2	0.0625	0.25	2	1	128	0.125	16	0.125	0.5	0.01563
m249	0.5	8	0.25	8	1	32	16	0.03125	0.25	0.0078	0.25	4	2	16	0.5	16	0.00195	0.5	0.01563

eth -erythromycin fur-furazolidone oa-oxolinic acid otc-oxytetracycline strep-streptomycin ts-sulphadiazine/ trimethoprim van-vancomycin amox-amoxycillin chlor-chloramphenicol
cip-ciprofloxacin doxy-doxycycline thia-thiamphenicol flu-flumequine neo-neomycin flor-florfenicol gent-gentamycin sara-sarafloxacin so-sodium oxolinate
enro-enrofloxacin

Appendix 4.2 Plasmid profiles of isolates of *C. psychrophila* and other members of the Cytophagaceae

Strain no.	Plasmid weight (kb)			
	≈2.9	≈2.0-2.9	≈2.0+2.9	none
1342			+	
1344			+	
0278			+	
fel62				+
fel110				+
fel5	+			
fel16	+			
u648	+			
1947		+		
fel2	+			
c292	+			
r151				+
a1				+
d593				+
u147	+			
c193				+
w693				+
u249	+			
w593				+
w1293				+
s5793	+			
b593				+
t20				+
b393				+
sh221	+			
mm221				+
bw222				+
rb192	+			
bb693				+
u842	+			
tvt20	+			
u239	+			
f163				+
bb8				+
n193	+			
p183	+			
d693	+			
u148				+
sh222	+			
jo121				+
f167				+
m249	+			
C. columnaris				+
F. aurantiacus				+
C. aquatilis				+

APPENDIX 5

Detail of donors of bacterial isolates used in thesis

E. Lorenzen, National Serum Laboratory, Hangvej 2, DK-8200 Aarhus N, Denmark.

T. Wilkund, Institute of Parasitology, Abo Akademi University, BioCity, Artillerig. 6, 20500 Abo, Finland.

J. Carson, Fish Health Unit, Department of Primary Industry & Fisheries, PO Box 46, Kings Meadows, Tasmania 7249, Australia.

APPENDIX 6

BUFFERS

PHOSPHATE BUFFERED SALINE (PBS)

Sodium chloride (NaCl)	8.0g/l
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.2g/l
DiSodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$)	1.4g/l
Potassium chloride (KCl)	0.2g/l

(pH 7.2)

CARBONATE BUFFER.

Sodium carbonate (Na_2CO_3)	1.5g/l
Sodium bicarbonate ($NaHCO_3$)	2.93g/l
Sodium azide (NaN_3)	0.2g/l

(pH 9.5)

Store at room temperature for up to 2 weeks.

BLOCKING SOLUTION.

Carbonate buffer with 5% Fish skin gelatin (Sigma G- 7765 diluted 1:8 v/v)

(pH 9.5)

P.B.S.Thio.

Sodium chloride (NaCl)	8.0g/l
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.2g/l
DiSodium hydrogen orthophosphate ($Na_2HPO_4.2H_2O$)	1.4g/l
Potassium chloride (KCl)	0.2g/l
Thiomersal ($C_2H_5.Hg.S.C_6H_4.COONa$)	0.2g/l

(pH 7.2)

P.B.S.T.

P.B.S.Thio with 0.5ml TWEEN 20 / litre

(pH 7.2)

EXTRACTION BUFFER.

P.B.S.Thio with 5ml TWEEN 20 / litre

(pH 7.2)

CONJUGATE BUFFER.

P.B.S.T. + 2.5% Fish skin gelatin (Sigma G- 7765 diluted 1:17 v/v)

(pH 7.2)

SUBSTRATE / INDICATOR SOLUTION.

One capsule of Phosphate-Citrate buffer with Sodium Perborate (Sigma P-4922) dissolved in 50ml of distilled water. To each 10ml aliquot of this solution that is required add 1 T.M.B. (Tetramethyl-benzidine dihydrochloride 1mg) tablet (Sigma T-3405).