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Natural and Experimental Infections with *Flavobacterium psychrophilum* in Salmonid Fish

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Abstract

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Flavobacterium psychrophilum, the aetiological agent of rainbow trout fry syndrome (RTFS) and bacterial cold-water disease (BCWD) causes problems in salmonid aquaculture worldwide. Fry are the most seriously affected with a septicaemic disease, often with high mortalities. Skin ulcers and fin lesions are common, especially in fish infected at later life-stages.

To investigate if Swedish brood fish are infected with *F. psychrophilum,* Baltic salmon (*Salmo salar*) brood fish were sampled for bacteriological examination. Both male and female fish, without any clinical signs of disease, were found to be infected with *F. psychrophilum* at the time of spawning. The bacterium was isolated from internal organs and sexual products. This shows that the brood fish may serve as a reservoir for the bacterium and indicates vertical transmission as a route of infection.

Intraperitoneal experimental infections were performed in three species of salmonids, *i.e.* rainbow trout, Atlantic salmon (*Salmo salar*), and sea trout (*Salmo trutta*) to evaluate any species-differences in susceptibility to the bacterium. Also, macro- and microscopical pathological changes, and the distribution of the bacterium, studied by immunohistochemistry, were evaluated. No species differences in mortality were recorded. Rainbow trout showed more pronounced changes in the spleen with haemorrhages, necrosis, and with numerous free bacteria present.

A new experimental infection model, using nanoinjection of *F. psychrophilum* into newly fertilised rainbow trout eggs was performed to mimic vertical transmission of the bacterium. All infected groups showed higher mortalities compared with controls. Diseased fry showed clinical symptoms and morphological changes similar to RTFS. The nanoinjection method was also used to study the effects of exposure to polychlorinated biphenyls (PCB) on disease resistance to *F. psychrophilum* infection. Newly fertilised rainbow trout eggs were injected with a commercial blend of PCB (Clophen A50) and *F. psychrophilum*. The highest mortality was recorded in groups exposed to bacteria and the lowest dose of Clophen A50 whereas no effect on disease resistance was recorded in groups receiving the higher dose. The nanoinjection studies show that the method can be a useful tool to study vertically transmitted pathogens and that exposure to PCB might affect the disease resistance to vertically transmitted *F. psychrophilum*.

Keywords: bacterial cold-water disease, fish disease, histopathology, nanoinjection, rainbow trout fry syndrome, pathology, polychlorinated biphenyls, vertical transmission.

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Ekman, E., Börjeson, H. & Johansson, N. (1999) *Flavobacterium psychrophilum* in Baltic salmon *Salmo salar* brood fish and their offspring. *Diseases of Aquatic Organisms 37*, 159-163.

II. Ekman, E. & Norrgren, L. (2003) Pathology and immunohistochemistry in experimental infection with *Flavobacterium psychrophilum* in three species of salmonids. *Journal of Fish Diseases*. In press

III. Ekman, E., Åkerman, G., Balk, L. & Norrgren, L. (2003) Nanoinjection as a tool to mimic vertical transmission of *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms 55*, 93-99.

IV. Ekman, E., Åkerman, G., Balk, L. & Norrgren, L. Impact of PCBs on disease resistance in an experimental infection with *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss* eggs using nanoinjection technique. *Manuscript submitted for publication.*

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Abbreviations

AEC	3-amino-9-ethylcarbazole
BCWD	bacterial cold-water disease
CA	*Cytophaga* agar
CFU	colony forming units
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
EROD	ethoxyresorufin *O*-deethylase
H&E	haematoxylin and eosin
IFAT	immunofluorescence antibody technique
IHNV	infectious haematopoietic necrosis virus
IPNV	infectious pancreatic necrosis virus
ISAV	infectious salmon anaemia virus
PAH	polycyclic aromatic hydrocarbons
PAS	periodic acid-Schiff
PBS	phosphate buffered saline
PCB	polychlorinated biphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PCN	polychlorinated naphthalenes
PCR	polymerase chain reaction
RAPD	random amplified polymorphic deoxyribonucleic acid
Ribotyping	ribosomal ribonucleic acid gene restricting analysis
rRNA	ribosomal ribonucleic acid
RTFS	rainbow trout fry syndrome
SCA	selective *Cytophaga* agar
TSA	tryptone soy agar
TYES	tryptone yeast extract salt
VHSV	viral haemorrhagic septicaemia virus

Introduction

Salmonid aquaculture in Sweden

Swedish aquaculture is based on production of fish for human consumption and for stocking purposes. The annual production for human consumption, mainly rainbow trout (*Oncorhynchus mykiss*), is approximately 6000 tons. Feral Atlantic salmon (*Salmo salar*), Baltic salmon (a genetically separated subpopulation of Atlantic salmon) and sea trout (*Salmo trutta*) are important species for commercial fisheries and sport fishing. Most of the Baltic rivers are exploited for hydroelectric power production. This has ruined the possibilities for migrating fish species, including Baltic salmon and sea trout, to reach their normal spawning grounds. A compensatory rearing programme to retain genetic biodiversity and biomass of Baltic salmonids was initiated during the 1950s (Karlsson & Karlström, 1994). Brood fish are caught in traps as they return to their home river to spawn and are kept in indoor pools until stripping. The artificially produced fry are reared in hatcheries for one or two years until smoltification, before being released into the river. The annual production is approximately 2 million smolts.

Health situation in Swedish salmonid aquaculture

The health situation in Swedish salmonid aquaculture is good compared with many other countries. The inland-located fish farms are considered to be free from viruses including infectious salmon anaemia virus (ISAV), infectious haematopoietic necrosis virus (IHNV) and infectious pancreatic virus (IPNV). VHS, the marine type, has been isolated from one fish farm only, located on the west coast of Sweden, during the years 1998-2002. Infectious pancreatic necrosis virus (IPNV) serotype Ab, has sporadically been isolated from coast-located fish farms, whereas serotype Sp has not been isolated in Sweden during the last 10 years.

Bacterial diseases that have caused problems in Swedish salmonid aquaculture during recent years are infections with *Renibacterium salmoninarum* (bacterial kidney disease); *Aeromonas salmonicida* subsp. *salmonicida* (furunculosis), *Aeromonas salmonicida* subsp. *achromogenes*; *Flavobacterium columnare*; *Listonella anguillarum* (former *Vibrio anguillarum*)(vibriosis); *Yersinia ruckeri* (enteric red mouth disease), and *Aeromonas hydrophila* (Fiskhälsan FH AB, Fish-health control programme, 2003). The use of effective vaccines has dramatically decreased the problems with furunculosis and vibriosis in Sweden during the last 5 years. *Y. ruckeri* is sporadically isolated but don't cause the same dramatic symptoms and mortalities as reported in many other countries (Anders Hellström, Swedish Veterinary Institute, pers. comm.). *R. salmoninarum* is also occasionally isolated, mainly from coast-located farms. Infections with *F. psychrophilum* are considered to be one of the major problems in Swedish aquaculture today. Approximately 50 to 60% of the antibiotics used in Swedish aquaculture are used for treatment of *F. psychrophilum* and *F. columnare* infections (U.-P. Wichardt, Fiskhälsan FH AB, Fish-health control programme, pers. comm.).

Since 1974 the reproductive disorder M74 has caused major mortalities in Baltic salmon yolk-sac fry in the compensatory hatcheries (Norrgren & Amcoff, 1998). The syndrome is vertically transmitted from the female to the offspring and the whole family group is affected, often with 100% mortality. A strong correlation between low thiamine levels in the female brood fish, and M74 affected offspring has been reported and an oxidative stress in the fry has been shown (Amcoff *et al*., 1998a; Lundström *et al*., 1999). The cause of the thiamine deficiency and oxidative stress has not yet been established.

Disease caused by *F. psychrophilum*

During the last decade, disease caused by the bacterium *F. psychrophilum* has been one of the most serious problems in salmonid aquaculture all over the world. *F. psychrophilum* was first isolated in 1948 in the U.S.A. (Borg, 1960). In the mid 1980s the bacterium was isolated for the first time outside North America in Germany and France during disease outbreaks in rainbow trout (*Oncorhynchus mykiss*) (Weis, 1987; Bernardet *et al*., 1988). Since then, *F. psychrophilum* has been isolated from all over Europe (Lorenzen *et al*., 1991; Austin, 1992; Santos *et al*., 1992; Toranzo & Barja, 1993; Wiklund *et al*., 1994) as well as in Chile (Bustos *et al*., 1995), Japan (Wakabayashi *et al*., 1991), Korea (Lee & Heo, 1998) and Australia (Schmidtke & Carson, 1995).

In North America, disease caused by *F. psychrophilum* is known as bacterial cold-water disease (BCWD) because of its occurrence in low water temperatures, often below 10 ºC (Holt, 1987). When *F. psychrophilum* infections spread to Europe the aetiology was not known at first, and the disease was often referred to as fry mortality syndrome or rainbow trout fry syndrome (RTFS). Young life stages are most seriously afflicted by *F. psychrophilum* infections. Coho salmon (*Oncorhynchus kisutch*) yolk-sac fry suffering from BCWD develop lesions on the yolk sac and mortalities up to 50% have been reported (Holt, 1987). Severe BCWD outbreaks in yolk-sac fry are often preceded by coagulated yolk disease (Holt, 1987). In feeding fry suffering from BCWD the mortality is lower, often around 20% (Holt, 1987). Fry and fingerlings with BCWD often have skin ulcerations on the peduncle, anterior to the dorsal fin, at the anus, or on the lower jaw (Holt, 1987). Muscle lesions can also occur (Holt, 1987; Lumsden *et al*., 1996). RTFS usually occurs during the first two months of feeding (Lorenzen *et al*., 1991) and mortalities up to 70% have been reported (Lorenzen *et al*., 1991; Bruno, 1992). Disease signs are anorexia, lethargy, dark pigmentation of the skin, ascites, and exophthalmia (Baudin-Laurencin *et al*., 1989; Lorenzen *et al*., 1991; Bruno, 1992). At necropsy, an enlarged spleen, pale gills, liver and kidney, as well as a hemorrhagic protruding anus are typical findings (Lorenzen *et al*., 1991; Bruno, 1992). Diseased fingerlings and larger fish usually exhibit skin ulcerations. Vertebral deformations, periostitis, osteitis and osteochondritis in cranial parts of the skeleton have been described in connection with chronic *F. psychrophilum* infections (Kent *et al*., 1989; Ostland *et al*., 1997; Madsen & Dalsgaard, 1999a; Madsen *et al*., 2001) as well as eye disorders with necrotic scleritis and blindness (Ostland *et al*., 1997; Lorenzen, 1994).

Histopathological changes due to acute *F. psychrophilum* infections are similar in BCWD and RTFS. Infected fish suffer from an acute septicaemic infection with bacteria present in almost all organs (Wood & Yasutake, 1957; Lorenzen *et al*., 1991; Bruno, 1992). Necrosis in spleen, kidney (both tubular epithelium and haematopoietic tissue), heart, and liver are often present (Wood & Yasutake, 1957; Lorenzen *et al*., 1991; Bruno, 1992). Changes in the spleen of rainbow trout suffering from RTFS, consisting of oedema, congestion, haemorrhages and necrosis, often totally destroying the normal architecture of the organ are considered to be typical of the disease (Lorenzen, 1994; Rangdale *et al*., 1999). In acute BCWD, ulcerations of the skin on the peduncle and lower jaw, as well as inside the mouth, are often present (Wood & Yasutake, 1956). Skin ulcers extending down to the subcutaneous tissue or the musculature with a polymorphic inflammatory response can be seen in chronic RTFS infection. Also eye changes with congestion in the choroid gland and inflammation in the retina have been reported (Lorenzen, 1994). Another finding in chronic RTFS infections is accumulation of eosinophilic material in the kidney tubular epithelium, sometimes accompanied by degenerative lesions (Lorenzen, 1994).

Host susceptibility

F. psychrophilum probably affects all salmonid species. Coho salmon, rainbow trout and ayu (*Plecoglossus altivelis*) seem to be particularly susceptible (Holt, 1987; Lorenzen *et al*., 1991; Wakabayashi *et al*., 1994; LaFrentz *et al*., 2002). Chinook salmon (*Oncorhynchus tshawytscha*) has been reported to be less susceptible (Rucker *et al*., 1953; Wood & Yasutake, 1956). *F. psychrophilum* has also been isolated from diseased non-salmonid species, *i.e.* eel (*Anguilla anguilla*), carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*), tench (*Tinca tinca*) and pale chub (*Zacco platypus*) (Lehmann *et al*., 1991; Iida & Mizokami, 1996).

In Sweden, as in other European countries, rainbow trout is the most common species affected. The bacterium has also been isolated from disease outbreaks in Atlantic/Baltic salmon, sea trout, and Arctic char (*Salvelinus alpinus*) (Fiskhälsan FHAB, Fish-health control programme 2003). Rainbow trout fry are often afflicted by RTFS whereas Atlantic salmon and sea trout more often are affected in later life-stages, with skin ulcers and fin lesions.

Taxonomy

The taxonomy of *F. psychrophilum* was initially based on phenotypic characteristics and has been revised several times during the years. When first isolated in 1948 (Borg, 1960) the bacterium was considered to belong to the order *Myxobacteriales* and named *Cytophaga psychrophila*. In the 8^{Th} edition of Bergey's Manual it was placed in the order *Cytophagales* (Reichenbach, 1989). Bernardet & Grimont (1986) studied the deoxyribonucleic acid (DNA) relatedness in addition to phenotypic characteristics and suggested that the bacterium should be placed in the genus *Flexibacter* with species name *Flexibacter psychrophilus*. Bernardet *et al*. (1996) made the latest reclassification based on G+C content,

DNA-ribosomal ribonucleic acid (rRNA) hybridisation, fatty acid and protein profiles and *F. psychrophilum* now belongs to the phylum/division *Cytophaga-Flavobacterium-Bacteroides*, family *Flavobacteriaceae*, genus *Flavobacterium*. Other fish pathogenic species in this genus are *F. columnare* and *Flavobacterium branchiophilum*. Additionally, *Flavobacterium hydatis* (former *Cytophaga aquatilis*), *Flavobacterium johnsoniae* (former *Cytophaga johnsonae*) and *Flavobacterium succinicans* (former *Cytophaga succinicans*) have occasionally been isolated from diseased fresh-water fish (Bernardet *et al*., 1996).

Isolation and identification

Like other members of the *Cytophaga-Flexibacter-Flavobacterium* group, *F. psychrophilum* requires a low nutrient medium and cannot be isolated on blood agar. One of the most used media for isolation of *F. psychrophilum* has been *Cytophaga* agar (CA) (Anacker & Ordal, 1955) composed of tryptone, yeast extract, beef extract and sodium acetate. In order to enhance bacterial growth, several improvements of the media have been made during the years, such as a higher tryptone concentration (Bernardet & Kerouault, 1989), addition of different salts (Shieh medium, tryptone yeast extract salt (TYES) medium) (Shieh, 1980; Holt *et al*., 1993), calf serum (Obach & Baudin-Laurencin, 1991; Lorenzen *et al*., 1997), carbohydrates and skimmed milk (Dalaskov *et al*., 1999), horse serum and trace elements (Michel *et al*., 1999). Selective media with addition of antibiotics, like selective *Cytophaga* agar (SCA) with neomycin and polymyxin B added (Fijan, 1969) and Shieh medium with Tobramycin (Decostere *et al*., 1997), have also been used for isolation of members in the *Cytophaga-Flexibacter-Flavobacterium* group in order to prevent overgrowth of other bacteria.

Identification of isolated *F. psychrophilum* is routinely made through morphological, biochemical, and physiological characteristics. Additionally, agglutination tests with anti-*F. psychrophilum* serum is often used. Identification of isolated bacteria can also be performed with molecular-based methods like the polymerase chain reaction (PCR) (Bruun *et al*., 2000; Madetoja & Wiklund, 2002). The bacterium can be identified in fish tissues without time-consuming culturing. Techniques that have been used are immunofluorescence antibody technique (IFAT) (Lorenzen & Karas, 1992; Madetoja *et al*., 2000), enzyme linked immunosorbent assay (ELISA) (Lorenzen & Karas, 1992; Rangdale & Way, 1995), immunohistochemistry (Evensen & Lorenzen, 1996) and in situ hybridization (Liu *et al*., 2001). PCR technique has also been used to identify the bacteria in fish tissues, ovarian fluid, eggs and water samples (Izumi & Wakabayashi, 1997; Urdaci *et al*., 1998; Wiklund *et al*., 2000; Baliarda *et al*., 2002; Madetoja & Wiklund, 2002).

Phenotypic and genotypic characteristics

Several studies on phenotypical characteristics of *F. psychrophilum*, isolated from different fish species and geographic areas, have shown that the isolates form a homogeneous group with only minor differences in biochemical properties (Pacha,

1968; Bernardet & Kerouault, 1989; Cipriano *et al*., 1996; Lorenzen *et al*., 1997; Madetoja *et al*., 2001). *F. psychrophilum* is a Gram-negative slender rod. The size of cells from a 10-24h broth-culture varies from 0.3 to 0.75µm x 2 to 7 µm (Holt *et al*., 1993). *F. psychrophilum* is strictly aerobic and shows poor gliding motility, which often is difficult to observe (Bernardet & Kerouault, 1989). The colony morphology on agar plates varies. Commonly, 1-5mm in diameter, yellow, raised colonies with a spreading irregular margin is seen. Colonies with sharp non-spreading margins are sometimes also observed, alone or together with spreading colonies (Pacha, 1968; Bernardet & Kerouault, 1989; Holt *et al*., 1993). Some significant biochemical properties of *F. psychrophilum* are presence of flexirubin-type pigment, weak positive cytochrome oxidase and catalase activities, and no ability to hydrolyse esculin or absorb Congo-red (Bernardet & Kerouault, 1989; Lorenzen *et al*., 1997; Madetoja *et al*., 2001). The bacterium has no ability to degrade simple or complex carbohydrates but is lipolytic and highly proteolytic, degrading albumin, casein, collagen, fibrinogen, gelatin, haemoglobin and tyrosine (Bernardet & Kerouault, 1989; Holt *et al*., 1993; Bertolini *et al*., 1994; Dalsgaard & Madsen, 2000). The ability to degrade elastin varies among strains (Holt *et al*., 1993; Bertolini *et al*., 1994; Dalsgaard & Madsen, 2000; Madetoja *et al*., 2001). The bacterium grows between 5 and 25 ºC with an optimal growth at 15 ºC (Holt *et al*., 1993). The tolerance to NaCl varies among strains but the upper limit seems to be 1% (Pasha, 1968; Holt *et al*., 1993).

Different genotyping methods have been used to study *F. psychrophilum* isolates originating from different fish species and geographic regions. Studies on the DNA base composition have shown a G+C content ranging from 32.5 to 35.3% (Holt, 1987; Bernardet & Kerouault, 1989; Bernardet *et al*., 1996). Ribosomal ribonucleic acid gene restricting analysis (ribotyping) of *F. psychrophilum* isolates has been performed in several studies (Cipriano *et al*., 1996; Chakroun *et al*., 1998; Madsen & Dalsgaard, 2000; Madetoja *et al*., 2001). A correlation between ribotype and the fish species from which the strains were isolated has been indicated (Chakroun *et al*., 1998). Random amplified polymorphic DNA (RAPD) has been used to detect genetic diversity among strains isolated from different fish species and geographic areas. No correlation between isolates and geographic region has been shown but some primers resulted in profiles that clearly showed an association between strains and their fish host (Chakroun *et al*., 1997). The presence of plasmids in *F. psychrophilum* isolates has also been investigated (Holt, 1987; Lorenzen *et al*., 1997; Chakroun *et al*., 1998; Kroon & Wiklund, 1998; Madsen & Dalsgaard, 2000). *F. psychrophilum* isolates have been shown to contain 0 to 3 different plasmids (Lorenzen *et al*., 1997; Chakroun *et al*., 1998; Madsen & Lorenzen, 2000). However, the use of plasmid profiles in epizootiological investigations has been reported to have limited value (Chakroun *et al*., 1998; Madsen & Dalsgaard, 2000).

Serology

Different strains of *F. psychrophilum* share common antigens making it possible to distinguish *F. psychrophilum* from other members in the *Cytophaga-Flexibacter-Flavobacterium* group by serological methods (Pacha, 1968; Holt, 1987; Cipriano

et al., 1996). Holt (1987) was the first to identify the presence of at least two different serotypes in American isolates of *F. psychrophilum* using absorbed antisera. Wakabayashi *et al*. (1994) and Izumi & Wakabayashi (1999) recognized three different serotypes among isolates from Japan and the U.S.A., O-1, O-2 and O-3. Serotype O-1 included the type strain NCMB 1947^T and isolates from coho salmon. Serotype O-2 included isolates from ayu, and O-3 isolates from rainbow trout. Lorenzen & Olesen (1997) also identified three different serotypes called Th (subtypes Th-1 and Th-2), Fd and Fp^T. The type strain NCMB 1947^T belonged to serotype Fp^T together with isolates from fish without clinical signs of disease and isolates from fish species other than rainbow trout. Most of the isolates from diseased rainbow trout belonged to serotype Th. An attempt to harmonize the serological typing system for *F. psychrophilum* has been made by Mata *et al*. (2002), resulting in 7 host-depending serotypes (1: salmon; 2: trout; 3: trout; 4:eel; 5:carp; 6:tench and 7:ayu). The type strain NCMB 1947^T belongs to serotype 1.

Virulence factors

F. psychrophilum is highly proteolytic, producing enzymes that cause direct tissue damage or enhanced invasiveness. This production of proteases has been suggested to be one virulence factor of the bacterium (Pacha, 1968; Bertolini *et al*., 1994; Madsen & Dalsgaard, 1998; Dalsgaard & Madsen, 2000). The ability to degrade elastin varies among different *F. psychrophilum* strains and it has been indicated that elastin-degrading isolates are more virulent than isolates not capable of degrading elastin (Madsen & Dalsgaard, 1998, 1999b). However, later studies have not been able to confirm this (Madsen & Dalsgaard, 2000; Madetoja, 2002).

The serotype and ribotype seems to be involved in the virulence of the bacterium (Madsen & Dalsgaard, 2000; Madetoja, 2002). Lorenzen *et al*. (1997) indicated that isolates containing a small plasmid (3.7 kb) were more virulent than isolates without plasmids or with plasmids of other sizes. In contrast, Chakroun *et al*. (1998) and Madsen & Dalsgaard (2000) were unable to show any correlation between plasmid content and virulence.

Transmission

Infectious diseases in fish can be horizontally transmitted, *i.e.* spread from individual to individual by direct contact, through water, food, or vectors, or vertically transmitted, *i.e.* spread from parents to offspring via infected milt or eggs. *F. psychrophilum* has been isolated from diseased and apparently healthy wild fish (Lehmann *et al*., 1991; Wiklund *et al*., 1994; Iida & Mizokami, 1996; Amita *et al*., 2000; Wichardt, 2000; Madetoja, 2002) as well as from healthy farmed rainbow trout (Dalsgaard & Madsen, 2000; Baliarda *et al*., 2002). Wild fish and latent carriers in the fish farms might serve as a reservoir for the pathogen. Stressful events could trigger the infection in latent carriers and start an outbreak of disease (Dalsgaard & Madsen, 2000). *F. psychrophilum* has been detected in fish farm water (Bruun *et al*., 2000; Schmidt *et al*., 2000; Wiklund *et al*., 2000; Madetoja & Wiklund, 2002) and the bacterium is capable of surviving

for a long time in sterile water outside its host (Madetoja *et al*., 2003). Large numbers of bacteria are shed from moribund and dead fish (Madetoja *et al*., 2000) and the virulence of bacteria in the water can be maintained for at least 7 days (Madetoja *et al*., 2003). Subsequently, recirculation of water in the fish-farm might be a source of infection to uninfected fish.

F. psychrophilum has been isolated from surface disinfected, homogenized eggs, strongly indicating that vertical transmission from brood fish to offspring is present (Brown *et al*., 1997; Kumagai *et al*., 1998; Kumagai *et al*., 2000). The bacterium has also been isolated from internal organs, ovarian fluid, egg-surfaces and milt from different species of salmonid brood fish (Holt, 1987; Rangdale *et al*., 1996; Brown *et al*., 1997; Baliarda *et al*., 2002; Madetoja, 2002). It is most likely that the bacterium can be introduced to fish farms via transported eggs and live fish. (Borg, 1960; Bustos *et al*., 1995; Izumi & Wakabayashi, 1997; Madetoja, 2002).

Treatment and prevention

Fish suffering from RTFS or BCWD are often treated with antibiotics in the food. Commonly used substances are oxytetracycline, florfenicol and amoxicillin (Holt *et al*., 1993; Bruun *et al*., 2000). During the last decade increasing problems with resistance to oxytetracycline and amoxicillin have been reported (Rangdale *et al*., 1997a; Bruun *et al*., 2000; Dalsgaard & Madsen, 2000). *F. psychrophilum* is resistant to trimethoprim/sulfadiazin and often to oxolinic acid (Rangdale *et al*., 1997a; Bruun *et al*., 2000; Dalsgaard & Madsen, 2000). In Sweden, florfenicol is the first choice in treating *F. psychrophilum* infections. Oxytetracycline is only occasionally used and resistance is sporadically recorded (Fiskhälsan FH AB, Fish-health control programme, 2003).

Bath-treatments with anti-bacteriological chemicals like quaternary ammonium compounds (Shotts & Starliper, 1999), sodium chloride and Chloramine-T (Fiskhälsan FH AB, Fish-health control programme, 2003) are sometimes used to treat mild outbreaks of disease with primarily external lesions. Elevation of the water temperature has been suggested as a method to control disease outbreaks (Holt *et al*., 1989; Lorenzen, 1994). This has proven to be efficient in an experimental infection of juvenile steelhead trout (*Oncorhynchus mykiss*) where the water temperature was raised from 12 to 22 °C (Holt *et al*., 1989). On the other hand, Lorenzen (1994) did not see any effect of a rise in water temperature from 15 to 21 °C in an experimental infection using rainbow trout fry. Nevertheless, the costs involved in raising the water temperature and the risk of other bacterial and parasitic infections make this method less useful (Holt *et al*., 1989).

Prevention of disease outbreaks is, of course, the most desirable. Several studies on the effects of vaccination have been made although commercial vaccines are not yet available. Both bath vaccination and intraperitoneal injection have been evaluated with varying results (Holt, 1987; Obach & Baudin-Laurencin, 1991; Lorenzen, 1994; LaFrentz *et al*., 2002, Rahman *et al*., 2002). One of the most important prophylactic tools to prevent disease outbreaks is probably to make the environment for the fish as optimal as possible by avoiding crowding and stressful

events, optimising water flows and water quality, and enhancing the hygiene and general management. In connection with high stocking densities, elevated cortisol levels, suppressed serum immunoglobulin M concentrations and increased susceptibility to *F. psychrophilum* infections in ayu have been shown (Iguchi *et al*., 2003). Furthermore, high levels of nitrite and the presence of organic material in the water have been reported to enhance the adhesion of the bacterium to rainbow trout gill tissue *in vitro* (Nematollahi *et al*., 2003). Large amounts of *F. psychrophilum* are shed from infected fish prior to death (Madetoja *et al*., 2000) and it is important to remove dead and moribund fish from ponds and tanks in order to decrease the infectious pressure during disease outbreaks.

Since *F. psychrophilum* can be present in ovarian fluid and on egg-surfaces (Holt, 1987; Rangdale *et al*., 1996; Madetoja, 2002) disinfection of the egg after fertilisation is important in order to reduce the infectious pressure in the hatcheries. The importance of vertical transmission of BCWD and RTFS is still unclear and further studies need be performed in order to evaluate if any other preventive strategies should be used.

Experimental infection methods

Different experimental infection methods with *F. psychrophilum* have been used during the years. Intramuscular, subcutaneous, and intraperitoneal injection of the bacterium have so far been the most used and reproducible methods (Holt, 1987; Lorenzen, 1994; Madsen & Dalsgaard, 1999b; Rangdale *et al*., 1999; Garcia *et al*., 2000). Bath infection and co-habitant infections have also been performed, but there have been problems in producing reproducible results, especially with co-habitant infections (Holt, 1987; Lorenzen, 1994; Madsen & Dalsgaard, 1999b). However, bath challenge in connections with various stress factors like formalin treatment (Madsen & Dalsgaard, 1999b) or wounding of the skin, has shown promising results (Madetoja *et al*., 2000). Oral challenge through a live vector has been tried without any success (Madetoja *et al*., 2000).

Experimental infection of eggs has been performed with *F. psychrophilum* by immersion of fertilised coho salmon, ayu and masu salmon (*Oncorhynchus masou*) eggs in a bacterial suspension just before water hardening. (Kumagai *et al*., 1998; Kumagai *et al*., 2000). The bacterium was isolated from surface disinfected eggs up to 50 days after infection. Rangdale *et al*. (1997b) immersed eyed rainbow trout eggs in a *F. psychrophilum* suspension, resulting in the development of RTFS in swim-up fry.

Egg injection techniques

The microinjection technique, based on administration of very small volumes into fish embryos, was initially developed to study hepatic carcinogenicity of different chemicals (Metcalfe & Sonstegard, 1984; Black *et al*., 1985). The method has also been used to study other toxic effects caused by different chemicals, and extracts from sediments and animal tissues, on early life stages of fish (Metcalfe *et al*., 1990; Wilson & Tillit, 1996; Norrgren *et al*., 1993a; Lundström *et al*., 1998).

Furthermore, microinjection into fertilised eggs has been used to study pathogens that can or might be vertically transmitted, *i.e. R. salmoninarum* and IHNV (Brown *et al*., 1990; Yoshimuizu *et al*., 1989). A problem with the microinjection technique is that egg mortalities in injected control eggs has been very high (30 to 40%) (Yoshimuizu *et al*., 1989; Brown *et al*., 1990; Norrgren *et al*., 1993a). In order to refine the method, a nanoinjection technique has been developed (Åkerman & Balk, 1995; Walker *et al*., 1996). Significant improvements include the use of a picoinjector and a very fine glass needle, which facilitates injection volumes down to nanolitres. Besides reducing background mortality, the nanoinjection technique makes it possible to inject very small eggs, and administration into different compartments of the egg.

Interactions between pollutants and infectious diseases

Both wild and farmed fish are exposed to many pollutants present in their aquatic environment. The developing fry can be exposed to lipophilic pollutants before ovulation by maternal transfer, or directly by uptake of compounds from the surrounding water (Guiney *et al*., 1979, Broyles & Noveck, 1979a,b). Many of these agents have been reported to interfere with the immune functions of the fish, resulting in tumour development and increased susceptibility to infectious diseases, as reviewed by Dunier & Siwick (1993). One of the most wide spread pollutants in the environment is polychlorinated biphenyls (PCB). Even though the use of PCB has been banned in most countries for several years, it still constitutes one of the major contaminants in the aquatic environment (Bignert *et al*., 1998). PCB are well known immunomodulators in mammals, as reviewed by Vos (1977) and Luster & Rosenthal (1993). Effects on the innate as well as the acquired immune system have also been shown in fish (Thuvander & Carlstein, 1991; Thuvander *et al*., 1993; Arkoosh *et al*., 1994; Rice & Schlenk, 1995; Lacroix *et al*., 2001; Regala *et al*., 2001; Duffy *et al*., 2002). Atrophy of lymphoid tissue in the spleen and toxic effects on thymocytes have been observed in PCB exposed fish (Nestel & Budd, 1975; Spitsbergen *et al*., 1988; Sweet *et al*., 1998). Several studies on the effects of PCB on disease resistance in fish have been performed with varying results. Decreased (Arkoosh *et al*., 2001), unaltered (Spitsbergen *et al*., 1988; Powell *et al*., 2003) and increased disease resistance (Snarski, 1982; Mayer *et al*., 1985) have been recorded.

Organic lipophilic compounds, such as PCB, need to be biotransformed to more water-soluble forms before they can be excreted. The cytochrome P450-monooxygenase system in the liver is one of the most important enzyme systems involved in this process. One commonly used method for measurements of the catalytic activity of the P450-monooxygenase system is the ethoxyresorufin *O*-deethylase (EROD) assay. EROD activity is induced in fish exposed to different xenobiotics, *i.e.* PCB, polychlorinated naphthalenes (PCN), polycyclic aromatic hydrocarbons (PAH) and polychlorinated dibenzo-*p*-dioxins (PCDD) (Hendricks *et al*., 1985; van der Weiden *et al*., 1992; Norrgren *et al*., 1993a) and has often been used as a biomarker for exposure to environmental pollutants (Förlin *et al*., 1985; Norrgren *et al*., 1993b, Bucheli & Fent, 1995).

Aims

The objectives of the present study were to:

● Investigate the prevalence of *F. psychrophilum* in Baltic salmon brood fish during their spawning migration and at stripping.

● Evaluate species differences in susceptibility and pathological responses after experimental infection with *F. psychrophilum*.

● Evaluate the possibility to use nanoinjection technique as an experimental infection model to mimic vertical transmission of *F. psychrophilum*.

● Study the impact of an environmental pollutant on disease resistance to *F. psychrophilum* by use of nanoinjection technique.

Materials and Methods

Fish materials

Paper I

Feral Baltic salmon brood fish, of the River Dalälven population, were captured during their spawning migration at the salmon trap at the National Board of Fisheries, Älvkarleby, and kept in indoor pools supplied with flow-through river water until spawning. The water temperature decreased from 14 to 7 °C during the sampling period. Eggs from 15 females were artificially fertilised with milt from 15 males. After water hardening and disinfection with 1% Buffodine (Evans Vanodine International Ltd., Preston, U.K.) each family group of eggs was separately incubated in flow-through water from River Dalälven at a water temperature of 0.1 – 8 °C.

Paper II

Eyed rainbow trout eggs from a commercial fish farm, and eyed eggs of feral Atlantic salmon and sea trout originating from the River Dalälven stocks were disinfected with 1% Buffodine (Evans Vanodine International Ltd., Preston, U.K.) for 10 min before incubation. Eggs and hatched fry were kept in 5 L aquaria with well-aerated flow through ground water at a temperature of 10 ± 1 °C at the Department of Pathology, SLU. After yolk sac resorption the fry were fed commercial fish food (Aller Aqua AB, Sweden) three to five times a day. The fry were used in the experimental infections at a weight of approximately 0.7 g.

Papers III-IV

Unfertilised rainbow trout eggs and milt from two different commercial fish farms were used. To immobilise the eggs during the injection and incubation, fertilised, water-hardened eggs were placed in prepared holes in a 1% agarose gel, cast in square Petri dishes, and were kept in the gel until hatching. Eggs and hatched fry were kept in 5 L aquaria with well-aerated flow-through ground water at a temperature of 10 ± 1 °C at the Department of Pathology, SLU. After yolk sac resorption the fry were fed commercial fish food (Aller Aqua AB, Sweden) three to five times a day.

Bacteriological examination and identification of *F. psychrophilum*

Samples from Baltic salmon brood fish were initially cultivated on SCA (Fijan, 1969) (paper I). Further cultivations were made on CA (Anacker & Ordal, 1955). Samples from fry in the experimental infections were cultivated on TYES agar (papers II-IV) and 5% horse blood agar (papers II and IV). All incubations were performed at 15 ºC.

To examine the presence of *F. psychrophilum* on egg surfaces, unfertilised eggs were rolled directly on SCA plates (paper I). Eggs sampled during incubation were rinsed in sterile water, and then shaken on a vortex stirrer for 2 min in 5 mL peptone (0.1%) -saline (0.85%) solution, and 100 µL spread on SCA agar (paper I). To examine the presence of *F. psychrophilum* inside fertilised eggs (papers III-IV), eggs were disinfected with 5% Buffodine (Evans Vanodine International Ltd, U.K.) for 20 min and rinsed with sterile water. Each egg was separately incubated in test tubes with 3 mL TYES broth. After 5-7 days the broth was visually inspected for bacterial growth and 0.1 mL was inoculated on TYES agar. Eggs yielding visual growth or growth on the agar plates were excluded from further studies. Surface-sterile eggs were crushed in the test tubes with a sterile glass rod and incubated for another 7 days before 0.1 mL was inoculated on TYES agar plates. In paper IV, eggs that died 5 days or less before expected hatching were disinfected as described above and the embryos were aseptically removed from the eggs. The whole embryo was placed in a test tube with 3 mL TYES, crushed with a sterile glass rod and incubated at 15° C for 7 days. All incubations were performed at 15 ºC (papers I-IV).

Identification of yellow-pigmented colonies on CA or TYES agar was performed by morphological and phenotypic characteristics. All isolates were examined for colony morphology, Gram staining, presence of flexirubin pigments, catalase, and cytochrome oxidase, ability to grow at 6 and 30 ºC, and reactivity in the API-zym gallery (bioMériux sa, Marcy-l'Etoile, France). Additionally, isolates in paper I were tested for growth in 0.5, 1.0 and 1.5% NaCl, growth on tryptone soy agar (TSA), ability to produce acid aerobically from glucose and saccharose, hydrolysis of starch and hydrolysis of esculin and susceptibility to the vibriostatic compound O/129. Furthermore, approximately 50% of the isolates were tested for gliding motility, and hydrolysis of starch and casein. All isolates in paper III were tested for their ability to degrade elastin on TYES agar with 0.05% elastin added.

Sampling of Baltic salmon brood fish and eggs during incubation (Paper I)

Baltic salmon brood fish were sampled for bacteriological examination at capture in the salmon trap. A total of 50 fish were killed with a blow on the head and samples from spleen, gonads and kidney were taken. During the captivity period, 19 fish showing an abnormal wiggling swimming behaviour were killed and sampled for bacteriological examination from the brain, spleen, gonads and kidney. At the time of stripping, sexual products were sampled from 272 fish (232 females and 40 males). After stripping, the fish were killed and samples from the brain and kidney were taken for bacteriology. At eyed stage, and just before hatching, 10 eggs from each from each family group were sampled for bacteriological examination.

Bacterial isolates and suspensions (Papers II-IV)

F. psychrophilum isolate 1F-97, isolated from the kidney of a diseased Atlantic salmon, was used in the experimental infections in paper II. In the experimental infections using nanoinjection technique isolates F9 (papers III and IV) and F169 (paper III), both isolated from diseased rainbow trout, were used. Isolates F9 and F169 were phenotypically similar, except for their ability to degrade elastin, where F9 was able to degrade elastin and F169 was not. The isolates were stored frozen at –70 to –80 ºC in TYES broth with 15% glycerol added. Bacteria from the frozen batches were inoculated in TYES broth and incubated on a shaker at 15 ºC for 48h. The bacteria were harvested by centrifugation at 1500*g* for 10min at 5 ºC, washed twice in phosphate buffered saline (PBS) (paper II) or 0.9% NaCl (papers III-IV) and resuspended in PBS or 0.9% NaCl. The turbidity was measured in a spectrophotometer (UV-1601 PC, Shimadzu Scientific Instruments Inc. Burlingame, USA) at 525 nm. Serial dilutions were made and the number of viable bacteria in the suspension was determined by drop inoculation on TYES. Further dilutions of the bacterial suspensions were made with PBS or 0.9% NaCl.

Clophen A50 solutions (Paper IV)

The Clophen A50 solutions were prepared by dissolving Clophen A50 in triolein (Sigma Chemical Co., St Louis, USA) to a concentration of 40 mg mL^{-1}. This solution was used for the injections in the high dose groups. The solution for low dose groups was further diluted with triolein to a concentration of 8.0 mg mL^{-1}.

Experimental infections (Papers II-IV)

Intraperitoneal infection (Paper II)

After anaesthesia in carbonate buffered MS222 (Sandoz Ltd, Basel, Switzerland) at a concentration of 100 mg L^{-1}, rainbow trout, Atlantic salmon, and sea trout fry were intraperitoneally injected with 0.05 mL of a bacterial suspension. Two different doses of *F. psychrophilum* were administered, 1 x 10^7 or 1 x 10^6 colony forming units (CFU) $fish^{-1}$. Controls were injected with PBS. Each treatment was performed in duplicate groups with 38 to 40 individuals in each duplicate. Mortality and clinical signs of disease were recorded at least 3 to 6 times every day. The experiment was ended 21 days after infection.

Nanoinjection (Papers III and IV)

Infections with *F. psychrophilum* (papers III and IV) and exposures to Clophen A50 (paper IV) were performed using nanoinjection technique (Åkerman & Balk, 1995; Walker *et al*., 1996). The injections were made with a needle of aluminium silicate glass, into the yolk of the eggs. The needle was held in a micromanipulator (WR-87, Narishige Scientific Instrument Laboratory, Japan), and the injection volume (0.05 µL egg^{-1}) was controlled with a pico-injector (PLI-100, Medical Systems Corp., USA). The whole procedure was performed under a stereomicroscope. Eggs were injected 1 to 5 days after fertilisation with a

suspension of *F. psychrophilum* in 3 different doses 10, 100 or 1000 CFU egg^{-1} (paper III). In paper IV, Clophen A50 was injected in 2 doses, 0.4 and 2 µg egg^{-1}. An injection of 100 CFU *F. psychrophilum* egg^{-1} was performed 1 to 2 days later. Some groups were injected with Clophen A50 or bacteria alone. Controls were uninjected, or injected with 0.9% NaCl, triolein or NaCl + triolein. On day 12 after fertilisation, unfertilised eggs were removed (papers III-IV) leaving 79 to 90 fertilised eggs in each group (paper III). In paper IV, all treatments were performed in duplicates with 57 to 66 fertilised eggs in each duplicate. The eggs were inspected daily and monitored for mortality. After hatching, fry were inspected 3 times a day and clinical signs of disease and mortality were recorded. The experiments were ended 70 (paper III) or 65 (paper IV) days after hatching.

Sampling

Dead eggs were examined for the presence of *F. psychrophilum* inside the eggs (papers III-IV). Bacteriological examination was also performed on apparently viable eggs at the beginning of the eyed stage (paper III). Dead and moribund fry were examined for gross pathological findings or sampled for bacteriological examination or histopathological and immunohistochemical studies. Samples for bacteriological studies were taken from the yolk and brain of yolk-sac fry (papers III-IV), and from kidney and spleen of feeding fry (papers II-IV). Livers from yolk-sac fry were sampled for EROD analyses 16 days post hatching (paper IV). At the end of the experiments all remaining fish were killed and sampled for bacteriological and morphological studies.

Histopathology (Papers II-IV)

Fry were fixed in 10% phosphate-buffered formalin (pH 7.2-7.4), embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). Periodic acid-Schiff (PAS) staining was used to evaluate presence of glycogen in the liver (paper II).

In paper II, lesions in spleen (congestion, haemorrhages, necrosis) and kidney (dilated sinusoids and peritubular capillaries, necrosis) were scored as 0 = lesions not present, 1 = weakly developed lesions, 2 = moderate developed lesions, 3 = severe developed lesions. Mean and standard deviations were calculated for each lesion in the groups. Furthermore, 100 renal tubules were randomly counted at x25 magnification and the number of tubules with intracellular eosinophilic inclusions was recorded in each group.

Immunohistochemistry (Papers II-IV)

Immunohistochemical stainings were performed on 2 to 3 µm sections of paraffin embedded tissues mounted on SuperFrost®Plus glass (Menzel, Germany). An avidin-biotin immunoperoxidase kit, VECTASTAIN®elite ABC Kit (Vector Laboratories INC, Burlingame, USA), was used according to the manufacturer's instructions. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 5 min. A polyclonal antibody, raised in rabbit against *F. psychrophilum*, anti-Th

(Evensen & Lorenzen, 1996) (paper II), or a commercial polyclonal antibody against *F. psychrophilum* (RFP01, Microtek International Ltd., Canada) (papers III and IV), diluted 1:5000 in 0.05 M Tris buffer (pH 7.6), were used as primary antibodies. The antibody was replaced with Tris buffer or non-immune rabbit serum in negative controls. As chromogen, 3-amino-9-ethylcarbazole (AEC) was used and as counterstain Mayer's haematoxylin. Evaluation was performed on encoded slides.

EROD analysis (Paper IV)

The yolk-sac fry were decapitated and the livers removed. Three livers were pooled in 300 µL sucrose (0.25 M) and immediately homogenized at 0 °C and frozen in liquid nitrogen. Totally 15 livers from each sampled group (7 + 8 from each duplicate) were analysed. All groups except the uninfected controls were analysed. The samples were stored at –140 °C and rapidly thawed just before analysis. EROD activity was measured according to Prough *et al*. (1978) and the protein content was determined according to Lowry *et al*. (1951).

Statistics (Papers I-IV)

To evaluate significant differences in the number of *F. psychrophilum* positive fish sampled at the trap and at stripping, a chi-square (χ^2) test was performed (paper I). Fisher's exact probability test was performed to evaluate differences in mortality (papers II-III) and gross pathological findings (paper II). In paper IV, differences in mortality were calculated by comparing Kaplan-Meier survival probability curves with the Log rank (Mantel-Haenszel) test. The Mann-Whitney U test was performed to evaluate differences in microscopical findings (paper II), presence of intracytoplasmatic eosinophilic droplets in kidney tubules (paper II) and EROD activities (paper IV).

Results

Identification of *F. psychrophilum*

Yellow-pigmented colonies on CA or TYES agar, identified as *F. psychrophilum* were phenotypically very homogeneous. All isolates from Baltic salmon brood fish and offspring (paper I) as well as reisolated bacteria in the experimental infections (papers II-IV) were Gram-negative slender rods that showed positive reaction for presence of flexirubin pigments, cytochrome oxidase and a weakly positive catalase reaction. They grew slowly at 6 ºC, but not at 30 ºC, in 0.5 and 1.0% NaCl, but not in 1.5% NaCl (paper I), and not on TSA (paper I). The reactivity in the API-ZYM gallery was in accordance with results of Bernardet & Kerouault (1989) with production of lipolytic and proteolytic enzymes but not enzymes involved in the carbohydrate metabolism. Furthermore, investigated isolates from Baltic salmon brood fish and offspring showed positive reactions for gliding motility, hydrolysis of casein and susceptibility to vibriostatic compound O/129. In paper III, all re-isolated *F. psychrophilum* showed the same elastin-degrading capacity as the isolate used in the infection, *i.e*. re-isolated bacteria from F9 groups were able to degrade elastin whereas bacteria from F169 groups were not.

F. psychrophilum in Baltic salmon brood fish and on eggs during incubation (Paper I)

F. psychrophilum was isolated from 14.0% (7 of 50) of the fish sampled at capture in the salmon trap. The bacterium was found in the kidney or gonads of female fish. The only five males sampled yielded no growth of *F. psychrophilum*. During the captivity period, *F. psychrophilum* was isolated from 11% (2 of 18) of the fish showing wiggling behaviour. *F. psychrophilum* was isolated from spleen or gonads but not from the brain.

At the time of stripping, after 3 to 4 months in captivity, 23.3% (63 of 272) of the fish were positive for *F. psychrophilum* in internal organs and/or sexual products. The bacterium was isolated from 20.2% (47 of 232) of the females and 40% (16 of 40) of the males. In females, the bacterium was most often isolated from the kidney, and in males from milt and kidney. No significant differences were recorded in the prevalence of *F. psychrophilum* infected fish at capture in the trap, and at the time of stripping.

F. psychrophilum was not isolated from the egg surfaces of incubated eggs at the eyed stage. At sampling just before hatching, the bacterium was isolated from 33% (5 of 15) of the family groups.

Mortality (Papers II-IV)

Intraperitoneal infection (Paper II)

No significant differences in mortality were recorded between duplicate groups. No mortalities were recorded in the control groups. Only minor mortalities were observed in the groups infected with the lower dose *F. psychrophilum*, with the highest mean cumulative mortality of 7.5% in Atlantic salmon. In groups infected with the higher dose of bacteria, the mortality varied between 55 and 70%, with a mean mortality of 56.2% for the duplicates of rainbow trout, 56.4% for sea trout and 68.8% for Atlantic salmon. No significant species differences in mortality were recorded. The mortalities occurred from day 1 to 10 after infection.

Nanoinjection (Papers III-IV)

Mortalities in all control groups were below 5%. No significant differences in mortality between duplicates were recorded (paper IV). The total cumulative mortalities (means of duplicates) of infected and Clophen A50 exposed groups are shown in Table 1. All *F. psychrophilum* infected groups showed a significantly higher mortality compared with control groups. No differences in mortality between the two isolates, F9 and F169, were recorded in groups receiving 10 or 1000 CFU egg^{-1} (paper III). In groups receiving 100 CFU egg^{-1}, the mortality in the F169 (elastin-negative) infected groups was significantly higher than in the F9 (elastin-positive) infected groups (paper III). In the group infected with 100 CFU of isolate F9 a mortality of 24.6% (16 of 65 feeding fry) was recorded days 4 to 18 after the beginning of feeding (paper III). In groups infected with 1000 CFU *F. psychrophilum*, all mortalities occurred during the egg stage.

Table 1. *Cumulative mortalities (%) of rainbow trout eggs and fry after nanoinjection with F. psychrophilum (isolates F169 and F9) and/or Clophen A50 into newly fertilised eggs. The cumulative mortalities in all control groups were below 5%. The results from paper IV are means of duplicate groups*

	Treatment	CFU egg^{-1}	µg Clophen A50 egg^{-1}	Cumulative mortality (%)
Paper III	F169	10	-	13.5
	F9	10	-	10.6
	F169	100	-	82.9
	F9	100	-	62.7
	F169	1000	-	100
	F9	1000	-	95.6
Paper IV	F9	100	-	21.6
	F9+ Clophen A50	100	0.4	58.6
	F9+ Clophen A50	100	2	20.4
	Clophen A50	-	0.4	4.0
	Clophen A50	-	2	5.8

Bacteriology (Papers II-IV)

No *F. psychrophilum* was isolated from fertilised control eggs sampled just after water hardening, from viable or dead eggs in control groups or from dead or moribund fry in control groups. The bacterium was isolated from almost all investigated dead eggs/embryos in infected groups (papers III-IV). In viable eggs, sampled at eyed stage, the bacterium was isolated from all eggs in groups infected with 100 and 1000 CFU egg^{-1} but only in one egg from groups infected with 10 CFU egg^{-1} (papers III-IV). Furthermore, the bacterium was isolated from almost all examined dead or moribund fry (papers II-IV). No other known fish pathogenic bacteria were isolated. No *F. psychrophilum* was isolated from any fish at the termination of the experiments.

Clinical signs of disease and gross pathological findings (Papers II-IV)

Yolk-sac fry in all *F. psychrophilum* infected groups showed similar clinical signs of disease, and similar gross pathological findings were recorded in dead fry (papers III-IV). The diseased yolk-sac fry were lethargic and often showed precipitates and haemorrhages in the yolk sac, sometimes accompanied by oedema. Ulcerations of the yolk sac with leakage of yolk were present in individuals from all infected groups in paper IV. In dead yolk-sac fry from groups exposed to Clophen A50, precipitates and oedema in the yolk sac were present (paper IV). No significant gross pathological findings, besides occasionally observed precipitates in the yolk sac (paper IV), were recorded in control groups.

Diseased feeding fry from *F. psychrophilum* infected groups showed similar clinical signs of disease regardless of fish-species, bacterial dose or bacterial isolate used, or if exposed to Clophen A50 or not. Clinical signs of disease included lethargy, loss of appetite and dark pigmentation of the skin (papers II-IV). Gross pathological changes in dead or moribund feeding fry from infected groups were pale gills, liver and kidney, enlarged spleen, empty gastro-intestinal tract and a dark pigmentation of the skin (papers II-IV). In paper II, no significant species differences in gross pathology findings were recorded besides haemorrhages at the injection site that were most common in Atlantic salmon, and haemorrhages and oedema at the anus, most commonly observed in sea trout. No significant gross pathological findings were observed in dead or moribund feeding fry from control groups or groups exposed to Clophen A50 alone. Fry examined at the termination of the experiments did not show any gross pathological findings (papers II-IV).

Histopathology (Papers II-IV)

Intraperitoneal infection (Paper II)

The histopathological findings of dead and moribund fish infected with the low (1 x 10^6 CFU $fish^{-1}$) and high (1 x 10^7 CFU $fish^{-1}$) doses of bacteria were similar. However, some significant species differences in pathological changes were

recorded in the spleen and kidney. In the spleen, congestion, haemorrhages, and areas with necrosis were commonly present in rainbow trout, sometimes with a total destruction of the normal architecture of the organ. In Atlantic salmon and sea trout, congestion was often present, but haemorrhages and necrosis were rare findings. In the kidney, necrosis of the tubular epithelium was often present in rainbow trout and Atlantic salmon, whereas it was only occasionally observed in sea trout. Small areas of necrosis in the haematopoietic tissue in the kidney were present in rainbow trout and Atlantic salmon but not in sea trout. A prominent finding in dead or moribund sea trout was the presence of intracytoplasmatic eosinophilic droplets in the kidney tubular epithelium. This was also observed in sea trout and rainbow trout that survived until the termination of the experiment. In the liver, single cell necrosis was often present in dead and moribund infected fish of all three species. A depletion of glycogen in the hepatocytes was also commonly present. Lesions in the anal region and at the site of injection were often seen in all three species. Haemorrhages and necrosis in hypodermis and muscles were observed, sometimes with a mild granulocytic reaction.

Nanoinjection (Papers III-IV)

In dead fry from groups exposed to Clophen A50 alone, no significant pathological changes besides oedema in the yolk sac in some individuals were recorded (paper IV). Dead or moribund fry in all *F. psychrophilum* infected groups, with or without Clophen A50 exposure, showed similar histopathological changes. Hyperaemia and haemorrhages in the yolk were often present, as well as oedema. Focal areas of epidermal hyperplasia, often with spongiosis were present on the yolk sac (paper IV). Vesicle formations, where the epidermis had lost contact with the underlying tissue, were seen in some individuals (paper IV). In yolk-sac fry with macroscopically visible leakage of yolk, parts of the epidermis covering the yolk were sloughed off and the yolk sac was ulcerated with subsequent leakage of yolk material. Fibrosis was present in dermis and hypodermis adjacent to the lesion (paper IV).

Histopathological findings in the spleen included congestion, haemorrhages and necrosis. The distinct outlining of the spleen was often destroyed, with peritonitis on the serosa. In the kidney, necrosis of tubular epithelium, and occasionally in the haematopoietic tissue, was present. Eosinophilic droplets in the kidney tubular epithelium were often seen (paper IV). No histopathological changes were recorded in fry examined at the termination of the experiment.

Immunohistochemistry (Papers II-IV)

Intraperitoneal infection (Paper II)

No positive immunohistochemical staining was observed in dead or moribund fish in control groups. In dead and moribund fish from all infected groups, immunohistochemically positive-stained phagocytes and free bacteria were present in the vascular system in almost every organ, indicating a septicaemic disease in

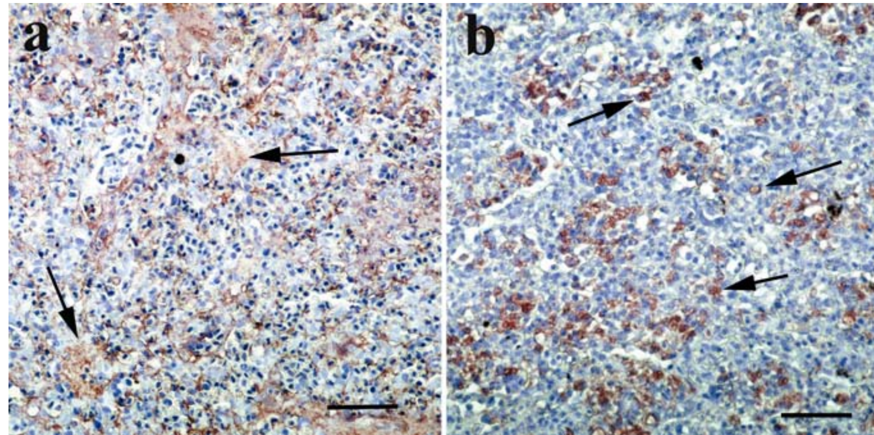

Figure 1a-b. *Immunohistochemical staining with a polyclonal F. psychrophilum antibody, avidin-biotin complex method, AEC as chromogen, and Mayer's haematoxylin as counterstain. Scale bars=0.05mm.* a) *Spleen from rainbow trout. Numerous positively stained free bacteria are present in the parenchyma (arrows)* b) *Spleen from sea trout. Positively stained phagocytes are dispersed in the parenchyma (arrows).*

all three fish species. Positively stained endothelial cells were seen in capillaries in the kidney and liver and in both the atrium and ventriculum of the heart. Positively stained bacteria and phagocytes were present in the peritoneal cavity and in the connective tissue surrounding the swim bladder and kidney. Positively stained phagocytes were seen in the haematopoietic tissue in the kidney and in the liver parenchyma. In the spleen, numerous positively stained phagocytes were present in the parenchyma, especially in sea trout (Fig. 1a) and Atlantic salmon. In rainbow trout, the number of positive phagocytes in the spleen was lower but free bacteria were commonly present (Fig. 1b).

Numerous positively stained bacteria were often outlining the vertebrae and cartilage of the anal fin. Free bacteria and positively stained phagocytes were also present in connection with the lesions in the anal region and at the injection site.

Positively stained phagocytes were occasionally observed in the spleen and at the anal region in surviving fish from the higher dose groups at the termination of the experiment (paper II). No positive immunohistochemical staining was observed in surviving fish infected with the lower dose of bacteria (paper II), or at the termination of the nanoinjection experimental infections (papers III-IV).

Nanoinjection (Papers III-IV)

No positive immunohistochemical staining was observed in any control groups or in groups exposed to Clophen A50 alone. In dead and moribund yolk-sac fry from all *F. psychrophilum* infected groups, large amounts of immunohistochemically positive-stained bacteria were present in the yolk. Positively stained phagocytes were seen inside epidermal vesicles, and dispersed in the epidermis and dermis, adjacent to the observed lesions on the yolk sac.

Immunohistochemically positively stained phagocytes and free bacteria were present in the vascular system of most internal organs including kidney, liver, heart, gills and intestine. Furthermore, positively stained phagocytes were seen in the haematopoietic tissue in the kidney, and in the spleen parenchyma together with free bacteria.

EROD induction (paper IV)

All Clophen A50 exposed groups had a significantly higher EROD activity compared with controls and groups exposed to *F. psychrophilum*. EROD activities in control groups and groups exposed to bacteria alone were in the range of 26 to 44 pmol mg protein^{-1} min^{-1}. In groups exposed to the lower dose of Clophen A50, a 10- to 20-fold increase in EROD activity was recorded and in groups exposed to high dose Clophen A50, a 40- to 60-fold increase compared with controls was observed. Exposure to *F. psychrophilum* did not have any effect on the EROD activity compared with controls.

General discussion

The fish health in Swedish aquaculture is good compared with many other European countries. This is due to a restrictive import policy, a successful fish-health control programme, and the use of effective vaccines. However, infections with *F. psychrophilum* are a problem in rainbow trout farming, and in the compensatory fish farms for Baltic salmon and sea trout. *F. psychrophilum* has been isolated from wild fish, including Baltic salmon, trout, eel, rainbow trout, vendace (*Coregonus albula*) and ruffe (*Gymnocephalus cernua*) in Sweden (Cooray *et al*., 1999; Wichardt, 2000). The present study shows that one possible route of transmission of *F. psychrophilum* to reared fish is through the feral brood fish used in the compensatory breeding programme. The bacterium was isolated from internal organs and/or sexual products of apparently healthy, female and male, brood fish at capture in the fish trap and at the time of stripping. This shows that brood fish can be systemically infected with the bacterium without any clinical signs of disease as also reported in earlier studies on rainbow trout/steelhead trout, and coho salmon brood fish (Holt, 1987; Brown *et al*., 1997; Baliarda *et al*., 2002). The presence of the bacterium in sexual products of both males and females indicates that vertical transmission can occur. Brown *et al*. (1997) isolated *F. psychrophilum* from the inside of fertilised steelhead trout eggs and the bacterium has also been isolated from the inside of fertilised coho salmon, rainbow trout and masu salmon eggs, after experimental infections (Kumagai *et al*., 1998; Kumagai *et al*., 2000). It has been suggested that the bacterium enters the egg during water hardening (Kumagai *et al*., 2000). This makes both the presence in milt and ovarian fluid a possible source of infection. The mechanisms by which the bacterium enters the egg are not yet known. The bacterium could enter the egg during oogenesis, infect the egg in connection with fertilisation, through the micropyle or have the ability to enter the egg during water hardening, as indicated by Kumagai *et al*., (2000). Several bacteria, *e.g. A. salmonicida* and *A. hydrophila,* are sensitive to lysozyme present in the egg (Yousif *et al*., 1994; Brown *et al*., 1997). However, *R. salmoninarum* and *F. psychrophilum* have been shown to be less sensitive, and consequently have the ability to survive inside the egg (Yousif *et al*., 1994; Brown *et al*., 1997). Before incubation in the hatcheries, the fertilised and water-hardened eggs are disinfected with an iodine solution. This reduces the risk of introducing pathogens present on the egg surfaces into the hatcheries. In the present study, no *F. psychrophilum* was isolated from egg surfaces at eyed stage. However, the bacterium was isolated from egg surfaces just before hatching, indicating that the bacterium is present in the environment in the hatcheries, and that reinfection of disinfected eggs occurs. *F. psychrophilum* has been isolated from fish farm water (Bruun *et al*., 2000; Wiklund *et al*., 2000; Madetoja & Wiklund, 2002) and the bacterium can survive for a long time outside its host (Madetoja *et al*., 2003). Disinfection of the eggs is, nevertheless, of crucial importance in order to minimise the infectious pressure in the hatcheries.

Infection with *F. psychrophilum* in juvenile coho salmon with meningitis, and osteitis in the cranium and anterior vertebra has been associated with abnormal swimming behaviour (Kent *et al*., 1998). In Swedish compensatory hatcheries,

abnormal swimming behaviour has commonly been recorded in yolk-sac fry from certain Baltic salmon females. Furthermore, Baltic salmon brood fish occasionally show wiggling swimming behaviour. *F. psychrophilum* was not isolated from the brain of the wiggling brood fish in the present study and the bacterium has not been isolated from Baltic salmon yolk-sac fry with abnormal swimming behaviour (Cooray *et al*., 1999). The abnormal swimming behaviour in Baltic salmon brood fish and offspring is related to the M74 syndrome, and shows a strong correlation with low thiamine levels (Amcoff *et al*., 1998a). This is further supported by the fact that both brood fish and yolk-sac fry treated with thiamine generally recover (Amcoff *et al*., 1998b; Börjeson, *et al*., 1999).

Observations from Swedish aquaculture have indicated that the susceptibility differs between different species of salmonids. Rainbow trout are often affected as fry while Atlantic/Baltic salmon and sea trout are more commonly affected as parr (U.-P. Wichardt, Fiskhälsan FH AB, Fish-health control programme, pers. comm.). The experimental infections using rainbow trout, Atlantic salmon and sea trout fry did not reveal any species differences in mortality. However, some significant species differences in pathological findings were observed. Rainbow trout had more pronounced lesions in the spleen, often with total destruction of the architecture of the organ, compared with Atlantic salmon and sea trout. In rainbow trout, numerous free bacteria were present in the spleen, while the presence of phagocytes with engulfed bacteria was more common in Atlantic salmon and sea trout. Lack of an effective phagocytic response in the spleen, with the presence of numerous free bacteria, extensive degeneration and necrosis has been described previously in rainbow trout suffering from RTFS, and the changes have been suggested to be pathognomic for the disease (Lorenzen, 1994; Rangdale *et al*., 1999). It has been indicated that differences in antibacterial activity of the phagocytes contribute to the fact that young fish are more susceptible to *F. psychrophilum* infection than older fish (Decostere *et al*., 2001). This might also be true concerning differences among species.

Efficient experimental infection models are needed in order to study pathogenesis of disease, differences in virulence between bacterial strains, and to develop vaccines. In the present study, an intraperitoneal injection challenge method, similar to the one developed by Madsen & Dalsgaard (1999b) was used. Experimental infection models using injection of the pathogen are not optimal since many components of the innate immune system are overruled. However, there have been problems in obtaining reproducible results by bath and co-habitant infections with *F. psychrophilum* (Holt, 1987; Lorenzen, 1994; Decostere *et al*., 2000). In the present study, nanoinjection of *F. psychrophilum* into the newly fertilised egg caused RTFS in the fry. Nanoinjection is a new experimental infection model that might be a useful tool to study *F. psychrophilum* infections and other vertically transmitted diseases.

The localisation of *F. psychrophilum* inside naturally infected eggs is not yet finally confirmed. In an experimental infection, where eggs were immersed in a *F. psychrophilum* suspension in connection with water hardening, bacteria were demonstrated in the chorion and in the perivitelline space (Kumagai *et al*., 2000). In the present study, *F. psychrophilum* was injected into the yolk of the fertilised

egg. However, due to observations in connection with experimental infections by Kumagai *et al*. (2000) it might be more appropriate to administer the bacteria into the perivitelline space. Preliminary studies with this administration, show results similar to when the bacteria are injected into the yolk (data not shown).

Effects of exposure to toxic agents including environmental pollutants on the immune system and disease resistance in fish have been reported, as reviewed by Dunier & Siwicki (1993) and Anderson (1996). However, studies on exposure during early life stages are scarce. Egg injection methods have so far been used to study effects of either chemicals or pathogens (Metcalfe & Sonstegard, 1984; Brown *et al*., 1990; Metcalfe *et al*., 1990; Norrgren *et al*., 1993; Wilson & Tillit, 1996). In the present study, simultaneous administration of the environmental pollutant PCB and *F. psychrophilum* was applied. Exposure to Clophen A50, a commercial blend of PCB, decreased the disease resistance to *F. psychrophilum* at the lower dose (0.4 µg egg^{-1}), indicating that PCB exposure might affect the susceptibility to vertically transmitted *F. psychrophilum* in the fry. The higher dose of Clophen A50 (2 µg egg^{-1}) did not have any effect on the disease resistance to *F. psychrophilum*. PCB has been shown to have a negative impact on phagocytosis in fish with a suppressed oxidative burst activity (Rice & Schlenk, 1995; Regala *et al*., 2001) and a lowered number of active macrophages present in head kidney and the peritoneal cavity (Jones *et al*., 1979, Lacroix *et al*., 2001). This might have contributed to the lower disease resistance recorded in this study. The unaltered disease resistance in groups exposed to the higher dose of Clophen A50 is harder to explain. However, effects on the immune system might not be dose dependent. In a study by Falk *et al*. (1990), the effects on phagocytosis were evaluated after chronic exposure to the herbicide Linuron. The result showed that the phagocytic capacity of kidney macrophages did not react dose dependently to Linuron exposure. The lowest concentration had the highest suppressive effect, and the highest concentration had no effect on the phagocytic capacity compared with controls (Falk *et al*., 1990). Possible toxic effects of Clophen A50 on the bacteria might also have influenced the results. PCB levels up to 2.7 µg g^{-1} fresh weight have been reported in rainbow trout eggs (Hogan & Brauhn, 1975) whereas concentrations up to 8.3 µg g^{-1} fresh weight have been shown in chinook salmon eggs from the highly contaminated Lake Michigan (Miller, 1993). The injected doses of 0.4 and 2 µg Clophen A50 egg^{-1} theoretically correspond to concentrations of approximately 5 and 25 µg g^{-1} fresh weight, respectively. Consequently, the lower Clophen A50 dose used in this study may be environmentally relevant in contaminated areas.

Major conclusions

● Baltic salmon brood fish were found to be infected with *F. psychrophilum* during their spawning migration and at the time of stripping. The bacterium was present in internal organs and sexual products of both males and females. This shows that feral Baltic salmon may serve as a reservoir for the bacterium and also transmit the bacterium into the hatcheries. Furthermore, the presence of the bacterium in sexual products indicates that vertical transmission can occur.

● The present study could not show any major species differences in susceptibility to *F. psychrophilum* infection in rainbow trout, Atlantic salmon and sea trout. However, the study was performed using intraperitoneal injection and differences in susceptibility in fish being naturally infected cannot be excluded.

● Infection with *F. psychrophilum* using nanoinjection into newly fertilised rainbow trout egg resulted in RTFS in the fry. This shows that the nanoinjection method may be a useful, alternative, experimental infection method, mimicking vertical transmission.

● The nanoinjection technique makes it possible to study interactions between pollutants and pathogens by simultaneous exposure. Exposure to PCB during early life stages might result in decreased disease resistance to vertically transmitted *F. psychrophilum* infections.

Future perspectives

F. psychrophilum is not considered to be an obligate pathogen and the factors that trigger a disease outbreak are not yet known. In order to prevent *F. psychrophilum* infections in fish farms, prophylactic measures are desirable, both from economical and ethical points of view. As no commercial vaccines are available yet, their development must be considered to be a crucial task. To prevent disease outbreaks, efforts in optimising the environment and reducing the stress for the fish is essential.

The importance and mechanisms of vertical transmission need to be further evaluated. It is still not known how common the presence of the bacterium inside unfertilised/fertilised eggs is. Furthermore, it is not finally established how and when the bacterium enters the egg. *F. psychrophilum* is often present in the milt, and the role of the male brood fish in vertical transfer needs to be evaluated. If vertical transmission is found to be an important route of infection the possibility of immunisation of the brood fish might be a method to avoid disease in the developing fry.

The phagocytic system seems to be an important part of the immune system in connection with *F. psychrophilum* infections. Knowledge of the immune response in connection with *F. psychrophilum* infections is still scarce. Studies on the immune functions involved need to be further investigated in order to understand the pathogenesis of the disease.

The nanoinjection technique used in this study might be a useful experimental infection method, mimicking vertical transmission. However, further studies need to be performed in order to obtain more reproducible results. As mentioned previously, injection of the bacterium into the perivitelline space might be a more appropriate way of administering the bacterium.

This study showed that exposure of PCB might result in decreased disease resistance to vertically transmitted *F. psychrophilum* infections. The lack of dose response in this study needs to be further investigated. Both *in vivo* and *in vitro* tests should be used to evaluate the effects on the immune system.

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