COMPARISON OF SOME PHYSIOLOGICAL VARIABLES OF FOUR SPECIES OF *CYTOPHAGA/FLEXIBACTER*-LIKE BACTERIA (CFLB) AND THE PATHOGENESIS AND CHEMOTHERAPY OF DISEASES CAUSED BY SOME OF THESE PATHOGENS

By

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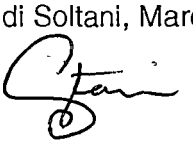
Declaration

I hereby declare that this thesis is the result of my own investigation. It has neither been accepted nor is being submitted for any other degree. All sources have been duly acknowledged.

Mehdi Soltani, March, 1995



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Species of fish with their Latin names used in this thesis.

Common name	Latin name	Common name	Latin name
African cichlid	*Oreochromis mossambicus*	Largemouth bass	*Micropterus salmoides*
Atlantic cod	*Gadus morhua*	Plaice	*Pleuronectes platessa*
Atlantic halibut	*Hippoglossus hippoglossus*	Rainbow trout	*Oncorhynchus mykiss*
Atlantic salmon	*Salmo salar*	Red sea bream	*Pagrus major*
Barramundi	*Lates calcarifer*	Rock bream	*Oplegnathus fasciatus*
Black sea bream	*Acanthopagrus schlegeli*	Sea bass	*Dicentrarchus labrax*
Black bream	*Acanthopagrus butcheri*	Sea urchin	*Pseudocentrotus depressus*
Carp	*Cyprinus carpio*	Sockeye salmon	*Oncorhynchus nerka*
Catfish	*Ictalurus* sp	Steelhead trout	*Oncorhynchus mykiss*
Channel catfish	*Ictalurus punctatus*	Striped bass	*Morone saxatilis*
Chinook salmon	*Oncorhynchus tshawytscha*	Striped trumpeter	*Latris lineata*
Coho salmon	*Oncorhynchus kisutch*	Suckers	*Catosformus* sp.
Crucian carp	*Carassius carassius*	Tench	*Tinca tinca*
Dover sole	*Solea solea*	Tilapia	*Oreochromis niloticus*
European eel	*Anguilla anguilla*	Turbot	*Scophthalmus maximus*
Goldfish	*Carassius auratus*	Walleye	*Stizostedion vitreum vitreum*
Greenback flounder	*Rhombosolea tapirina*	Weatherfish (loach)	*Misgurnus anguillicaudatus*
Guppy	*Poecilia reticulata*	Windowpane flounder	*Lophopsetta maculata*
Japanese eel	*Anguilla japonica*	Yellow-eyed mullet	*Aldrichetta forsteri*
Japanese flounder	*Paralichthus olivaceus*	Yellow perch	*Perca fluviatilis*
		Yellowtail	*Seriola quinqueradiata*

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ABSTRACT

The physiological requirements of Australian isolates of four species of *Cytophaga/Flexibacter*-like bacteria(CFLB), namely *Cytophaga johnsonae* (CJ), *Cytophaga psychrophila* (CP), *Flexibacter columnaris* (FC) and *Flexibacter maritimus* (FM) were studied. Also, the pathogenesis and chemotherapy of some diseases caused by these bacteria were investigated.

In vitro responses of the organisms to environmental conditions, including temperature, salinity and pH, showed that all species have psychrotrophic tendencies with CJ and CP growing at the lowest temperature, FC the highest and CP having the narrowest range of temperature for growth. FM preferred full salinity (seawater) for growth with no growth in the presence of NaCl alone, whereas the other three strains preferred no salinity for growth. All species grew well over a similar pH range.

In vivo assessment of susceptibility of a number of freshwater species of fish including barramundi (*Lates calcarifer*), goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*) and rainbow trout (*Oncorhynchus mykiss*) to infections by CJ and CP resulted in occurrence of infection by CJ only in barramundi. This occurred during bath exposure of fish to the organism when it was coupled with thermal stress and was achieved by rapidly reducing the maintenance temperature. No infection could be induced in the other species tested.

Barramundi were more susceptible to FC infection than goldfish and the disease was more severe at higher water temperatures than at lower ones. Pathological features were similar in both species with acute necrosis of epithelial surfaces.

Experimentally, FM induced infection in Atlantic salmon (*Salmo salar*), rainbow trout and greenback flounder (*Rhombosolea tapirina*) only by bath immersion at full or 15‰ salinity. Distribution of lesions, level of susceptibility and temporal pattern of infection were similar in both salmonids, and larger Atlantic salmon were more resistant to infection than smaller sizes. There was a great consistency in histopathological features of experimental infection in salmonids and in natural outbreaks in a number of captured species, including striped trumpeter (*Latris lineata*), flounder, yellow-eyed mullet (*Aldrichetta forsteri*) and commercial salmonids. A remarkable lack of inflammatory response, consistent fragmentation and degeneration of the epithelium, with infiltration of amorphous protein-like materials and occasional intra-epithelial inflammatory cells, congestion and haemorrhage were also observed, with invading bacterial cells colonizing dense connective tissue and occasionally the underlying musculature. Scale loss, odema and low degree of inflammation in scale pockets were evident, but the remaining scales were intact.

In vitro antimicrobial activity of skin mucus obtained from naive fish against these organisms gave variable results.

In vitro and *in vivo* efficacies of commonly-used chemotherapeutants were determined for these pathogens. Treatment of barramundi with oxolinic acid (OA) as a bath (50 ppm) or by mouth (10 mg/kg b w) resulted in serum levels above the minimum inhibitory concentration (MIC) for FC and produced significant clinical efficacy ($P<0.05$). Amoxycillin (AM) was found to produce adequate serum levels against FM, when used as a bath (200 ppm) or given orally (80 mg/kg b w) to Atlantic salmon and rainbow trout, respectively. At these dose rates this antibiotic was also clinically efficacious against this pathogen ($P<0.05$). Trimethoprim (TMP) produced more than adequate serum levels for the control of FM when given as a bath (50 ppm) or orally (10 mg/kg b w) to Atlantic salmon and rainbow trout, respectively. TMP was significantly more protective than AM when tested *in vivo* ($P<0.05$). For CJ and CP the MIC values for OA and oxytetracycline were low, whereas that for TMP was high. MIC values indicated CP strains were more sensitive to AM and norfloxacin than was CJ.

CHAPTER I

Introduction and Literature Review in Relation to Diseases Caused by Fish-Pathogenic *Cytophaga/Flexibacter*-like Bacteria (CFLB)

1.1. Introduction

The nomenclature for the *Cytophaga/Flexibacter*-like bacteria (CFLB) has been the subject of debate for many years. In deciding upon the names to be used in this thesis the candidate consulted the standard texts and discussed the contemporary situation with a number of microbiologists in Australia. It was realised whatever decision was made, not all readers would be in agreement, but, hopefully, they would appreciate the difficulties confronting the writer.

The causative agent of columnaris disease (saddleback disease or cottonmouth disease) was named *Bacillus columnaris* (Davis, 1922), *Chondrococcus columnaris* (Ordal & Rucker, 1944) and *Cytophaga columnaris* in both Bergey's Manuals of Systematic and Determinative Bacteriology (Reichenbach, 1989; Holt *et al.*, 1994), although an alternative name, *Flexibacter columnaris*, is currently used. However, it has been proposed that *F. columnaris* be included in the genus *Cytophaga* (Reichenbach, 1989; Holt *et al.*, 1994). Hereinafter these two names are considered to be synonymous and *F. columnaris* will be used as it appears widely in the scientific literature. *F. columnaris* causes disease in a wide range of both cold- and warm freshwater fish such as species of Cyprinidae, Salmonidae, Centrachidae, Ictaluridae and Percidae.

The causative agent of cold-water disease (low-temperature disease, peduncle disease or saddle-like lesions) was named as *Cytophaga psychrophila* by Stanier (1942), Borg (1948), and Pacha (1968), but Lewin and Lounsbery (1969) believed that this species of bacterium should be included in the genus *Flexibacter.* However, the current name is *C. psychrophila* as described in both Bergey's Manuals of Systematic and Determinative Bacteriology (Reichenbach, 1989; Holt *et*

al., 1994). Hereinafter these two names are considered to be synonymous and *C. psychrophila* will be used. Until 1988, *C. psychrophila* infection was believed to be restricted to salmonid culture in the USA and Canada. However, recently the infection has been reported in other regions such as Europe and Japan, and it was diagnosed as the causative agent of a systemic disease, not only in salmonids such as rainbow trout (*Oncorhynchus mykiss*) (Bernardet *et al.*, 1988), but also in non-salmonids such as eel (*Anguilla anguilla*), tench (*Tinca tinca*), carp (*Cyprinus carpio*) and crucian carp (*Carassius carassius*) (Lehmann *et al.*, 1991).

Flexibacter maritimus, which was initially described from a disease in red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) by Masumura and Wakabayashi (1977), was later isolated by Hikida *et al.* (1979) in Japan from a variety of marine fish. Then, Wakabayashi *et al.* (1986) proposed the above name which will be used herein, although Reichenbach (1989) in Bergey's Manual of Systematic Bacteriology later removed it to the genus *Cytophaga* under the name of *Cytophaga marina.* However, the name of *F. maritimus* is appropriate as documented by Holmes (1992). Depending on the main external sign exhibited by the infected fish, the infection has been described as "black patch necrosis" of Dover sole (*Solea solea*) (Bernardet *et al.*, 1990), salmonid cutaneous erosion disease of sea-caged salmonids (Carson, 1992) or "eroded mouth syndrome" (Toranzo & Barja, 1993a).

Cytophaga aquatilis was first isolated as a facultative anaerobe from the gills of freshwater fish by Strohl and Tait (1978), but the role of the organism, as a fish pathogen, is still unclear. The name of this species of bacterium remains the same at present.

Cytophaga johnsonae as a cause of skin and tail rot of cultured barramundi (*Lates calcarifer*) has recently been reported with meaningful losses from Queensland, Australia (Carson, 1992; Carson *et al.*, 1993). The current and formal name of this bacterium is *C. johnsonae* (Reichenbach, 1989) which will be used hereinafter, but it has probably sometimes been misspelled as *C. johnsonii* (Holt *et al.*, 1994).

In addition, two previously unrecognised CFLB causing systemic disease in turbot (*Scophthalmus maximus*) and jaw erosion in farmed rainbow trout have been described from Scotland and England, respectively (Mudarris & Austin, 1989; Holliman *et al., 1991*).

Also, a new ulcerative flexibacteriosis-like disease "yellow pest" affecting young Atlantic cod (*Gadus morhua*) was reported from Germany (Hilger *et al.*, 1991).

Furthermore, recently a CFLB was isolated from the adherent bacterial epiflora of Atlantic halibut (*Hippoglossus hippoglossus*) eggs for which it was considered to be an opportunistic pathogen (Hansen *et al.*, 1992). The bacterium caused high mortality both during the last few days before hatching and the first few days after hatching of halibut larvae. The name *Flexibacter ovolyticus* was given for this new species of gliding bacteria by Hansen *et al.*(1992).

Again, a marine CFLB infection has been isolated from diseased cultured sea bass (*Dicentrarchus labrax*) from the French Mediterranean coast (Pepin & Emery, 1993) and an ulcerative stomatitis in salmon smolts associated with a previously unrecognised species of CFLB has been reported in the USA (Frelier *et al.*, 1994). The latter is an economically important disease.

As a result, it is understood that *F. columnaris*, first discovered in the USA, has along with many other CFLB, been found in other countries of the world and that these CFLB affect not only salmonids but also other fish species. Moreover, a recognition of more species of this group of microorganisms as fish pathogens has arisen. As well, as is discussed later, the role of predisposing factors such as temperature, crowding and water quality fluctuations are significant in leading to disease outbreaks due to these organisms. Therefore, in this chapter an overview of the characteristics of the causative agents, pathogenicity, clinical signs and gross pathology, histopathological features, diagnosis and isolation, predisposing factors and control and treatment of the diseases caused by these pathogenic gliding bacteria is provided.

1.2. Causative agents

1.2.1. Morphological and cultural characteristics

Many morphological, physiological and biochemical characteristics of fish pathogenic CFLB have been identified since Davis (1922) described columnaris disease. Nevertheless, so far the nomenclature/taxonomy of this group of microorganisms has not been fully clarified. These organisms take the form of slender flexible aerobic rods and are Gram negative, motile with unknown mechanism of gliding, and possess a DNA, G+C ratio of 30-34 mol%. On plate culture, colony colour may be yellow, orange or golden yellow and the cell dimensions of the organisms are in the range of 1.5-15 x 0.4-0.6 μm. Generally, they degrade gelatin and casein, but not chitin, starch, agar or 3% carboxymethylcellulose (CMC). However, *C. aquatilis* and *C. johnsonae* are able to degrade chitin and starch. Usually fish-pathogenic CFLB are positive for oxidase and catalase, but do not produce indole and H_2S with the exception of *F. columnaris* which is able to produce H_2S. With the exception of *F. maritimus*, others are positive for flexirubin reaction. Aerobically, acid production from carbohydrate is usually negative. Peptone and casamino are suitable nitrogen sources. They grow on peptone alone and produce NH_3. Optimum temperatures and pH are 20-30°C and 7, respectively (Christensen, 1977a; Austin & Austin, 1987; 1993, Reichenbach, 1989; Bernardet *et al.*, 1990; Carson *et al.*, 1993, Holt *et al.*, 1993; 1994; Wakabayashi, 1993).

1.2.2. Pathogenicity

In comparison with other bacterial fish pathogens, relatively few studies have been undertaken on the pathogenicity of CFLB. As will be mentioned later, the reproduction of infections by fish-pathogenic CFLB is more effective by immersion than by injection and in fish kept in static water which favours attachment of the organisms. Also, injection routes do not mimic the natural route of infection even for pathogens, which are able to produce systemic infections (Anderson, 1990).

In addition, the fluctuation of water temperatures significantly influence the pathogenesis of CFLB: in particular, *C. psychrophila* and *F. columnaris*.

(a) *F. columnaris*

Ordal and Rucker (1944) pointed out that the mortality rate due to *F. columnaris* in juvenile sockeye salmon (*Oncorhynchus nerka*) held at 16°C was 30%, while the rate was 100% for those held at 22°C. Mortality levels were 45% and 95% at 18°C and 20°C, respectively, and no mortality was found in a control (uninfected) group maintained at 22°C.

Colgrove and Wood (1966) demonstrated that the pathogenicity of *F. columnaris* in suckers (*Catosformus* sp.) was associated with temperature so that high and rapid mortality occurred at ≥15.5°C with no infection at ≤ 11.6°C. Similar findings were observed for pre-spawning sockeye salmon which were naturally exposed to the pathogen prior to the experiment (Colgrove & Wood, 1966).

There is a marked variation in the virulence of strains of *F. columnaris.* The level of virulence of 500 strains of *F. columnaris* was assayed by immersing 6-14 month-old sockeye or chinook salmon (*O. tshawytscha*) at 18-20°C for 2 minutes in a broth culture with an optical density of 0.1 (determined with Coleman-Nepho-Colorimeter using a 525 μm filter) (Pacha & Ordal, 1963; 1970). According to the time required to produce 100% mortality, these strains were classified into four categories of virulence as follows:

(1) High virulence which caused 100% mortality within 24 hours.

(2) Moderate virulence which caused 100% mortality in 48 hours but not within 24 hours.

(3) Intermediate virulence which resulted in 100% mortality in 96 hours but not within 48 hours.

(4) Low virulence which required over 96 hours to cause 100% mortality.

Pacha and Ordal (1970) reported that the outbreaks of columnaris disease in the Columbia River Basin in salmon, particularly sockeye salmon, were associated with an increase in water temperature. They demonstrated a relationship between water temperature and different strains of *F. columnaris* of varying grades of virulence. Only highly virulent strains produced the disease at lower temperatures (12.8°C), while those of low-virulence were able to initiate infection only at ≥ 20°C.

Fujihara *et al.* (1971) produced columnaris disease in sibling chinook salmon (3.9 g) with 100% mortality by bathing fish at a concentration of ca 2.5 x 10^5 cells/l of sterile river water for 25 minutes. Times to 100% mortality were about 19 and 8 hours at 10 and 22°C, respectively.

No mortalities were observed in oriental weatherfish (*Misgumus anguillicaudatus*) exposed to *F. columnaris* (10^6 cells/ml) at 5 or 10°C (Wakabayashi & Egusa,1972). In contrast, the mortality level at 20-35°C was 100%, while it reached 25% at 15°C. Mean times of mortalities occurred 7, 3, 1.8 and 1 days post-exposure at 15, 20, 25, and 35°C, respectively.

A laboratory experiment on the relationship between water temperature and pathogenicity of *F. columnaris* in steelhead trout (*O. mykiss*), coho salmon (*O. kisutch*) and chinook salmon was conducted by Holt *et al.* (1975). Lyophilised cultures of an isolate of *F. columnaris*, which were originally recovered from a gill lesion of an adult spring chinook salmon, were used. The bacterium was passaged several times in coho salmon prior to the experiment and fish of 10-33 g body weight were exposed for 10 minutes to the pathogen in a bath at an optical density of 0.1 at 525 nm of the culture bacterium. Mortality levels increased with increasing water temperature and reached 70% in chinook salmon and 100% in other two species at 20.5°C. The minimum time to death was 1.6-2.3 days at 20.5 or 23.3°C, while the maximum time to death was 7.6-12 days at 12.2°C. Mortality levels of 4-20% occurred at 12.2°C in all species with no infection at ≤ 9.4°C.

Kuo *et al.* (1981) studied the artificial infection by 55 strains of *F. columnaris* and *Flexibacter* sp., using contact and intraperitoneal injection in eel (20 g) and tilapia (10 g). Fish with mechanical injury on one side of the gill were exposed to a bath of the bacterial culture broth at 3-5 x10^7 cells/ml for 60 minutes, while intact fish were injected intraperitoneally with 2 ml/100 g body weight of a bacterial broth culture at 3-6 x10^8 cells/ml. The immersion route was more effective than injection in producing infection (Table 1.1) and the mortality rate was higher in static rather than running water (Tables 1.1 & 1.2). Higher levels of mortality by bath immersion may however, have been owing to the effect of mechanical gill injury prior to

challenge. These strains of bacteria were collected from gill and skin of cultured fish in Taiwan. They were then grown in two transfers of cytophaga broth using 5 ml and 50 ml in sequence and held at 28°C for 24 hours during each transfer. The second transfer of bacterial culture in broth was used for the challenge and the determination of the virulence of the bacterial strain was similar to those findings by Pacha and Ordal (1963), plus some strains which were identified as avirulent.

Table 1.1. Mortality rate (%) of elver (eel) artificially infected with gliding bacteria.

Infection method	Static water		Running water	
	F. columnaris	*Flexibacter* sp.	*F. columnaris*	*Flexibacter* sp.
Immersion	100 (9)**	100 (5)	91 (11)	27 (15)
IP*	100 (9)	100 (5)	64 (11)	20 (15)

* Intraperitoneal injection. **Number of strains tested in parentheses. Adapted from Kuo *et al.* (1981).

Table 1.2. Mortality rates (%) of tilapia and elver infected by immersion with gliding bacteria.

Fish	Static water		Running water	
	F. columnaris	*Flexibacter* sp.	*F. columnaris*	*Flexibacter* sp.
Tilapia	100 (9)*	100 (5)	72 (14)	77 (13)
Elver	100 (9)	100 (5)	93 (14)	38 (13)

*Number of strains tested in parentheses. Adapted from Kuo *et al.* (1981).

No infection occurred in cultured Nile tilapia (*Oreochromis niloticus*) challenged with *F. columnaris* unless the natural barriers of the fish were damaged (Amin *et al.*,1988). The severity of the disease increased and a shortened median death time (26-52 hours) occurred when infected fish with injured gills were maintained in water containing ammonia (168 mg NH_4Cl/l) which enhanced the susceptibility of the host to the pathogen.

In respect of columnaris disease, the effect of temperature on defence mechanisms of the host is unknown. However, warm water temperature may enhance bacterial multiplication in the host's tissues. This proposition is supported by Holt *et al.* (1975) who demonstrated that this bacterium rapidly grows *in vitro* at 17.8-23.3°C, while the growth rate is very limited at low temperatures i.e. ≤ 12.2°C.

(b) *F. maritimus*

Campbell and Buswell (1982) challenged two groups of 10 Dover sole with a bacterial suspension of *F. maritimus* using scarification and subdermal injection methods. No adverse effects were observed in the fish which were challenged by the scarification method, while the group injected subdermally showed 30% mortality 48 hours post-infection.

Wakabayashi *et al.* (1984) compared a number of routes for producing infection by *F. maritimus* in juvenile black and red sea bream (25-70 mm) (Table 1.3). Mortality levels, by direct contact of cultures to the tail and mouth, were highest, while mortality levels by intramuscular injection and bath exposure were lowest and similar. Black sea bream were more susceptible than red sea bream. The mean time to death was shortest (1.2 days) and longest (2.5 days) in black bream, when challenged with bacterium by mouth application and intramuscular injection, respectively. A lyophilised culture of *F. maritimus* was used for this study. It was isolated originally from natural lesions or from kidney tissues of infected fish.

Table 1.3. Comparison of different methods of infecting fish using a *F. maritimus* strain B2 with juvenile red and black sea bream.

Fish species	IM (1)	Bath (2)	Application (mouth) (3)	Application (tail) (3)
Red sea bream	10* (0)**	9 (0)	11 (4)	10 (3)
Black sea bream	10 (2)	10 (1)	10 (9)	10 (10)

* Indicating number of total fish used per trial. ** Indicating number of dead fish. Sterile saline was used for control groups. (1). Fish were intramuscularly injected with 0.02 ml of bacterial suspension per fish. (2). Fish were exposed to a bath of bacterial suspension for two hours in standing water and were then maintained in pathogen-free flowing water at 23-24°C. (3). Fish were subjected to the cultures of pathogen by direct contact to the tail or mouth. Modified from Wakabayashi *et al* (1984).

Baxa *et al.*(1987) indicated that no infection occurred when black sea bream fry were challenged with *F. maritimus* by the immersion route. The disease, however, occurred when a combination of topical application using a test-tube brush and immersion was used. Any damage or break in fish barriers, such as

scales and epithelial layers, provides a suitable way for bacterial localisation and colonisation, with the subsequent infiltration and proliferation in the host's tissues.

(c) *C. psychrophila*

In a study Borg (1960) showed that *C. psychrophila* was most pathogenic, both at 10 and 18°C, when injected in high numbers (cited by Dalsgaard, 1993). However, there were no mortalities for the injections with dilutions of the culture at 18°C.

A comparison of 22 strains of *C. psychrophila,* that were tested for their ability to produce disease in yearling coho salmon following subcutaneous injection with ca. 2.8×10^7 cells/fish, resulted in a variable mortality rate from 0 to 100% indicating wide variations in virulence. (Holt, 1988). There were low, medium and high virulent strains of the bacterium similar to those reported with *F. columnaris.* Similar observations were found by Bertolini *et al.* (1994) when juvenile coho salmon and steelhead trout were subcutaneously injected with 14-29 isolates of *C. psychrophila.*

Observations by Holt (1988) showed many more cells of *C. psychrophila* were required to achieve 50% mortality in the fish injected intraperitoneally than by the intramuscular and subcutaneous routes. This shows that virulence mechanisms of *C. psychrophila* may be better expressed in muscle tissue than in the peritoneal cavity. The above may be because the subcutaneous and intramuscular routes represent a more natural means of infection and take advantage of the proteolytic activities of the pathogen.

Holt *et al.* (1989) investigated an experimentally induced *C. psychrophila* infection and its relationship with water temperature in juvenile coho salmon, chinook salmon and rainbow trout. Infection was produced using a virulent strain of the bacterium SH3-81 subcutaneously injected (4×10^6 and 2×10^7 cells/fish or 0.02-0.05 ml/fish) between the adipose and dorsal fins of fish. The shortest mean time (2.8-5.7 days) and the longest mean time (8.6-10.1 days) from infection to death were at 12-15°C and 3-6°C, respectively, which were the temperatures associated with doubling the population of the bacterium in the culture medium.

Infection was reduced at >15°C with no evidence of disease at 23°C. There was also no disease in juvenile steelhead trout injected with 6.6 x 10^7 cells/fish at 22°C, while 100% mortality occurred when the fish were kept at 12°C.

Intraperitoneal and intramuscular routes of injection (0.0 5ml of ca. 10^7cells/ml) of a freshly isolated strain of *C. psychrophila* in rainbow trout (6 g) achieved 80% mortality at 16°C within one week post-infection (Austin, 1992).

Artificial reproduction of a previously unrecognised CFLB causing jaw erosion in rainbow trout by immersion (10^7 cells/ml), intraperitoneal (10^6 cells/fish) and intramuscular routes (10^6 cells/fish) resulted in 100% mortality within 96 hours in rainbow trout, of average weight 2-3 g, and Atlantic salmon, of average weight 10 g, when were maintained in aerated freshwater at 16°C (Holliman *et al.*, 1991).

The effect of temperature on pathogenicity of the cold-water disease may also be due in part to its effect on growth of the bacterium in the fish tissues (Holt *et al.*, 1989). The optimum temperature at which outbreaks of bacterial cold-water disease occurs is between 4.4 and 10°C, as indicated by Pacha and Ordal (1970). Generally, the disease decreases at a water temperature of 12.8°C, although some strains of this organism are capable of causing disease at higher water temperatures (Pacha & Ordal, 1970). There is also a relationship between temperature and fish immune response, so that at lower temperatures fish reaction to bacterial infection, e.g. *C. psychrophila*, is slowed or inhibited. This may occur because processing of antigen by macrophages, interactions between macrophages and T-helper precursor cells, and the final step of helper cell maturation may be inhibited at low temperatures (Avtalion, 1981; Holt *et al.*, 1989). Moreover, other immune mechanisms such as mucus modulation, interferon, ceruloplasmin, C-reactive protein and α-2 macroglobulin production are temperature-dependent with protein synthesis of the latter being reduced or stopped at low temperatures (Langdon, 1988a).

Great variations in pathogenicity test conditions (e.g. water temperature, fish size and age, challenge route and fish density) used by different researchers make it difficult to compare virulence test results quantitatively (Dalsgaard, 1993).

1.2.3. Mechanisms of pathogenicity

The exact mechanisms which are involved in the pathogenicity of CFLB in fish are unknown, although Austin and Austin (1987; 1993) indicated that it is probable that toxins have a role in *F. columnaris* infection. Release of powerful proteolytic enzymes by cell autolysis may explain the histolysis and necrosis observed in columnaris disease (Morita, 1975; Snieszko & Bullock, 1976; Thune *et al.*, 1993).

Kuo *et al.* (1981) indicated that iron may affect the pathogenicity of *F. columnaris*. Survival time of eels challenged with the bacterium by intraperitoneal injection declined from 20 days to one day when 0.35-1.4 mg iron/100 g fish was injected prior to challenge. Iron may limit the pathogenesis of the organism more in systemic infection than in superficial infections, because the iron effect was observed in only one of the two strains examined when the challenge was delivered via the bath method. Also, this suggestion is supported by data which indicates that administration of transferrin prior to challenge enhanced survival time from three to 20 days for an intraperitoneal challenge, while it had negligible effect on a bath challenge.

F. columnaris produces, in culture, a chondroitinase enzyme which is able to degrade chondroitin sulphates A and C and hyaluronic acid, the complex polysaccharides of connective tissue (Griffin,1991; Teska, 1993). Extracellular proteases have recently been identified in *F. columnaris* which may affect the pathogenicity of the disease (Bertolini & Rohovec, 1992). This organism produces two types of slime layers, an acidic polysaccharide and a galactosamine, which probably play a role in adhesion by keeping cells attached to the surface. This adhesion mechanism may result in large bacterial population at the specific site where other virulence factors, such as toxins and enzymes, can be released (Dalsgaard, 1993). Although the extracellular proteases and chondroitinase are reported to be produced by *F. columnaris* strains, none of them has been evaluated for degradative effects in the tissue of fish. Some specific bactericidal substances similar to colicins have been reported for various strains of *F. columnaris* (Anacker & Ordal, 1959b; Becker & Fujihara, 1978). It is thought that the presence of such

inhibitory substances may be of value for the survival of individual strains in an ecological environment.

Intramuscular injection of the toxin product obtained from a broth culture of *C. psychrophila* to 20 fish resulted in no mortality or lesions within 13 days (Borg, 1960). It was concluded that the infection process was due to the presence of the live bacteria and that no significant amounts of the exotoxin were released into the culture medium. However, other authors have shown that *C. psychrophila* produces extracellular proteases which may be effective in the pathogenicity of the infection (Pacha, 1968, Morita, 1975; Bertolini *et al.*, 1994). Otis (1984) suggested that extracellular products play a significant role in the disease process. Steelhead trout were injected intramuscularly, subcutaneously or intraperitoneally with either live bacterial cells or a crude extracellular product. Lesions in both groups appeared to be similar macroscopically and microscopically. It is suggested that temperature may also affect the production of extracellular proteases and other possible determinants of virulence, including the leucocytolytic factor described for other bacterial fish pathogens (Holt *et al.*, 1989; Dalsgaard, 1993).

C. aquatilis possesses an extracellular, thermostable, glucose-repressible collagenase which could be involved in pathogenicity (Austin & Austin, 1987; 1993).

Pathogenicity effects of toxins (crude and pure lipopolysaccharide and sonicated cell-free supernatant) and enzymes (protease and haemolysin) of *F. maritimus* were examined by Baxa *et al.* (1988a). They suggested that the bacterium may achieve pathogenicity by means of synergistic interaction of these toxins.

Further work should be directed at elucidating the mechanisms involved in the pathogenicity of fish pathogenic CFLB. For example, there is no evidence whether or not the unusually thick cell envelope of these organisms is involved with their pathogenicity as noted by Mudarris and Austin (1989). This scenario is raised because, in contrast to the majority of bacterial fish pathogens, experimental induction of the infection by this group is more effective by contact challenge than by injection. This is especially true in fish kept in static water, which provides an

opportunity for bacterial colonisation and localisation on superficial surfaces with subsequent proliferation into tissues.

Some elements, such as calcium, may play a significant role in expression of pathogenicity of CFLB. Calcium is a growth promotor element in different pathogenic strains of *Flexibacter* sp.(Hikida *et al.*, 1979) and, as the enameloid of teleost teeth is highly mineralized, it could serve as a source of calcium for these bacteria (Shellis & Miles, 1974). The reason why *Flexibacter* pathogens cause stomatitis may be due to this source of calcium in the oral cavity of fish.

The role of carriers on the course of infections is unclear, although CFLB are known as opportunistic fish pathogens which are common in the environment of fish. However, it is difficult to establish a carrier state for these organisms. For example, some infected rainbow trout released *F. columnaris* up to 140 days post-infection, while other did not (Thune *et al.*, 1993).

The virulence determinants consisting of adhesins, haemolysins, cytotoxins, anti-phagocytic factors, proteases, resistance to the bactericidal effect of the complement, ability to sequester iron, penetrate epithelial cells, and survive and multiply in phagocytes, may have a role in the pathogenicity of fish-pathogenic CFLB (Dalsgaard, 1993). Occurrence of plasmids, as is observed in some strains of *C. psychrophila* (Holt, 1988), lipid component in the cell membrane, and lack of inflammatory reactions by the infected host are other factors which their role in virulence of CFLB require more investigation. The recognised virulence determinants for these organisms are presented in Table 1.4.

Table 1.4. Virulence factors of the fish-pathogenic CFLB

Determinants	*F. columnaris*	*C. psychrophila*	*F. maritimus*	*F. ovolyticus*
Adhesins				
Slime layer	+	.	.	.
Exotoxins				
Proteolytic enzymes	+	+	+	+
Haemolysins	.	.	+	.
Bacteriocins	+	+	.	.
Bacteriolysis	+	+	+	.
Proteases	+	+	+	.
Endotoxin (LPS)	+	+	+	.

Adapted from Dalsgaard (1993)

1.3. The Diseases

1.3.1. Clinical signs and gross pathology

Columnaris disease may occur clinically as peracute, acute or chronic disease, while other fish-pathogenic CFLB usually cause subacute to chronic disease. Strain virulence and water temperature are the most important factors in determining the course of infections.

The type of lesions caused by *F. columnaris* varies depending on the fish species (Snieszko & Bullock, 1976). In young fish, gills are often the major site of colonisation and destruction by bacteria (Austin & Austin, 1987; 1993; Carson, 1990). Congestion of blood vessels in gills and disassociation of the surface epithelium of the lamellae from the capillary bed result in scattered points of haemorrhage, as observed by Pacha and Ordal (1967).

In adult fish, the lesions can be found on gills, skin and in musculature. Gills show yellow orange areas of necrosis (Austin & Austin, 1987; 1993) containing long parallel rows of the organisms (Carson, 1990) and sometimes the primary lamellae are destroyed entirely (Pacha & Ordal, 1970). Snieszko and Bullock (1976) believed that in scalefish such as Pacific salmonids, the disease often causes gill necrosis which commences in the filaments and extends to the arches. On the body, the grey-white or blue plaques of lesions, with active erythematous edges on the mid-dorsal surface of the body (saddle back disease), are known as a major sign of infection (Langdon, 1988b; Carson, 1990). Other signs which may be found clinically are as follows (Austin & Austin ,1987, 1993; Thune *et al.*, 1993, Wakabayashi, 1993):

(1) Appearance of white spots on the head, gills, fin or body.

(2) Swarms of bacteria covering the lesions with a yellowish-white mucoid exudate resembling cotton wool.

(3) Destruction of tissues between fin rays, or complete loss of rays.

(4) Appearance of large lesions (3-4 cm in diameter) on the body and exposure of the underlying musculature.

(5) Oral lesions with extensive degeneration of the oral cavity and gills.

In scaleless fish, such as catfish (*Ictalurus* sp.) small-spherical lesions containing grey-blue necrotic centres with red margins and surrounded by a ring of inflamed skin may be found (Snieszko & Bullock, 1976).

It is probable that the disease develops as a systemic infection through external skin or gill damage (Wood, 1968; 1974; Wolke, 1975; Wakabayashi, 1991; 1993). It would appear that the organisms enter the circulatory system through external lesions which, in themselves would not directly cause death. The internal gross pathology in salmonids is limited to glomerular lesions in kidney tissue (Bullock *et al.*, 1986). In channel catfish (*I. punctatus*), swelling of the trunk kidney occurs in some cases of systemic columnaris disease, but gross internal signs are often lacking (Hawke & Thune, 1992).

Usually, death occurs due to ulceration in the muscles, which leads to osmoregulatory failure or severe gill epithelial destruction (Langdon, 1988b), which interferes with oxygen supply with the resultant increase in opercular activity.

The signs of cold-water disease are usually like those of superficial columnaris disease, although it occurs as a systemic disease in advanced stages (Ferguson,1988; Bruno, 1992). Infection may appear as cutaneous ulcers on the peduncle region in older fish and ulceration of the yolk sac in fry (Langdon,1988b). Saddle-like lesions near the dorsal fin consist of multitudes of bacteria (Austin & Austin, 1987; 1993). Sometimes lesions, associate with caudal peduncle or fins, may lead to exposure of the spine (Reddacliff, 1988). The chronic infection causes hyperplasia of skin and fins which can be similar to papillomatosis (Ferguson,1988). Weakness, anorexia, melanosis, exophthalmia, reddening of the vent area and occasional raised epidermal lesions, spleen hypertrophy, haemorrhaging and discolouration of liver, swollen and greyish kidney with haemorrhaging of posterior and anterior parts, swelling of abdomen, accumulation of ascites in the peritoneal cavity and severe anaemia are associated with rainbow trout fry syndrome caused by *C. psychrophila* in Europe (Bernardet *et al.*, 1988; Austin, 1992; Bruno, 1992; Rangdale *et al.*, 1993). Ataxia, spiral swimming behaviour along the longitudinal axis, loss of equilibrium, remaining motionless on the side at the water surface,

dorsal swelling posterior to the skull and dark pigmentation on either the right or left longitudinal half of the body were reported from underyearling coho salmon, rainbow trout and steelhead trout which were affected by *C. psychrophila* at hatcheries in Washington and Oregon, USA (Kent *et al.*, 1989). Recently *C. psychrophila* strains were isolated from eel and cyprinids with skin lesions and acute septicaemia in Germany (Lehmann *et al.*, 1991).

Thirteen isolates of *C. aquatilis* were recovered from the gills of diseased hatchery-reared salmon, suckers and trout in Michigan, USA, but it could no be demonstrated that this organism was capable of producing the observed disease (Strohl & Tait, 1978). This organism is thought to cause swollen gill lamellae, clubbing and gill-rot in the host (Austin & Austin, 1987; 1993).

The clinical observations of *C. johnsonae* infection in barramundi are similar to those of columnaris disease, with posterior superficial skin lesions extended somewhat to the anterior body, pectoral fins and lower jaws (Carson *et al.*, 1993).

In a freshwater environment fish infected by freshwater species of pathogenic CFLB are unable to absorb salts actively through damaged gills. This osmoregulatory imbalance and blocked respiration may cause the death of affected animals. Ulceration of the skin also interrupts normal osmoregulation with the result that infected animals become overhydrated. Blood electrolyte concentrations change and death can follow.

Infections associated with marine *Flexibacter* species are usually similar to those of other CFLB. In red and black sea bream infection by *F. maritimus* caused mouth erosion, frayed fins and tail-rot with a pale yellow appearance leading to ulcers particularly in young animals (Hikida *et al.*, 1979; Wakabayashi *et al.*, 1984). In adult fish an initial development of lesions, including grey-white cutaneous areas on the fins, head and trunk, has been reported (Austin & Austin, 1987, 1993). In Dover sole, slight blisters on the skin surface or dark areas between the caudal and marginal fins occur and lead to loss of the epithelial surface and disclose haemorrhagic dermal tissues. These are major clinical signs caused by marine *Flexibacter* infections (Bernardet *et al.*, 1990).

In the marine environment, fish infected by *F. maritimus* lose cellular water through the eroded tissues and so become dehydrated. This dehydration causes the death of affected fish. Interference with the excretion of chloride and sodium ions through the affected gills may lead to osmoregulatory imbalance and exacerbate mortality levels.

In general, CFLB invade the integument of fish and seldom affect host internal organs. Even so, in recent years, *C. psychrophila* has caused a systemic disease in rainbow trout and fingerlings in Europe (Santos *et al.*, 1992; Rangdale *et al.*, 1993; Wiklund *et al.*, 1994). Also, an unusual systemic disease caused by a peviously undescribed CFLB has been reported as a causative agent of gill hyperplasia and generalised haemorrhagic septicaemia in reared turbot from Scotland (Mudarris & Austin, 1989; 1992). In addition a CFLB has been recently reported from the USA as a causative agent of salmonid stomatitis (Frelier *et al.*, 1994).

1.3.2. Histopathology

The pathology associated with columnaris disease consists of acute necrotizing loss of epidermis with ulceration which expands into the muscle in severe cases (Ferguson, 1989). Severe spongiosis (intercellular oedema) probably causes failure of cell-to-cell contact, resulting in the formation of vesicles or bullae which may enhance the loss of epidermis.

Necrotic stomatitis, owing to columnaris-like infections, is often observed especially at warmer temperatures. This lesion, which is named cotton-wool mouth (cottonmouth) in aquarium species, is caused by *F. columnaris* and secondarily becomes mixed with fungal infections (Ferguson, 1989).

Columnaris disease causes widespread gill necrosis, degeneration and necrosis of the pseudobranchial epithelium in yellow perch (*Perca fluviatilis*) and young cultured walleye (*Stizostedion vitreum vitreum*) (Ferguson, 1989).

There is a remarkable lack of an inflammatory response in columnaris disease (Wolke, 1975; Morrison *et al.*, 1981; Thune *et al.*, 1993). Although, phagocytosis may be observed in early lesions, it is not apparent in advanced

stages of the infection (Snieszko & Bullock, 1976). There is a possibility that an early inflammatory response may occur that is subsequently destroyed by the action of proteolytic enzymes produced by the organism as the infection progresses, or the enzymes may act on the inflammatory response mediators at the site of infection to inhibit the chemotactic response of the inflammatory cells (Thune *et al.*, 1993).

There is little or no internal microscopic pathology or host inflammatory response associated with columnaris disease. Enlargement of Bowman's capsule and the appearance of an eosinophilic material surrounding the glomerular capillaries in the kidney of young salmon may be associated with the systemic infection (Pacha & Ordal,1967). However, these reactions may be owing to osmoregulatory disturbances associated with damage to the gills.

C. psychrophila causes an acute necrotizing dermatitis with epidermal hyperplasia in chronic cases (Ferguson, 1988; 1989). Subacute and chronic periostitis, osteitis, meningitis and ganglioneuritis containing mononuclear cells and polymorphonuclear leucocytes have been reported in salmonids including coho salmon, rainbow trout and steelhead trout with the causative agent being *C. psychrophila* (Kent *et al.*, 1989).

The following histopathologic changes are known in fish skin due to both *F. columnaris* and *C. psychrophila* infections (Ferguson, 1989):

(1) Superficial dermal oedema.

(2) Vascular dilation (sometimes haemorrhage).

(3) Pavementing and emigration of leucocytes.

(4) Rupture of the pigment cells with dispersion of granules.

(5) Dermal oedema if the osmotic barriers are broken.

(6) Severe necrosis with rapid sloughing of the epidermis.

The histopathological characters due to *C. johnsonae* infection in barramundi are similar to those of columnaris disease, but the thinning of scales in varying degrees occurs (Carson *et al.*, 1993) presumably because of the chitinobiase activity produced by *C. johnsonae*.

An inflammatory reaction has been decribed in sea-pen salmon smolts infected with a previously unrecognised CFLB (Frelier, *et al.*, 1994). It consists of mononuclear and polymorphonuclear cells which, surround the underlying oedematous areas and extend to the underlying bone and osteonecrosis of the jaw bone.

1.3.3. Isolation and identification of causative organisms

CFLB, especially the fish pathogenic species, can be isolated on low-nutrient media incubated at 10-25°C for a few days up to two weeks (Austin & Austin, 1993). Many useful media have been formulated to isolate these organisms from the tissues of diseased fish. Some of these media are listed in Table 1 within Appendix I. *Cytophaga* agar (Anacker & Ordal's medium, 1959a) is the most commonly used medium for this purpose. it is a selective medium when it contains antibiotic additives including one or more of neomycin (5 µg/ml), polymyxin B (10-200 IU/ml), erythromycin (10 µg/ml), oxolinic acid (2 µg/ml) (for isolation of *F. maritimus*) (Carson, 1990; Hawke & Thune, 1992). The medium must be made up with at least 30% sterile seawater for cultivation of *F. maritimus* because this species does not grow in the presence of NaCl alone.

Since the isolation and subcultivation of *C. psychrophila* in a *Cytophaga* medium is sometimes unsuccessful (Lorenzen, 1993), the medium has been improved by increasing the amount of tryptone to 0.5% (Bernardet & Grimont, 1989), adding 10% foetal calf serum (Obach & Baudin-Laurencin, 1991) or 5% new-born calf serum (Lorenzen & Karas, 1992; Lorenzen, 1993). The brand of beef extract in the medium also seems to be important for the growth of this bacterium as Lorenzen (1993) observed that the organism did not grow in the medium containing Oxoid beef extract and/or Gibco products, while it grew well in Difco products, which is a semifluid preparation. The author's own experience shows that the primary and subculture of Australian isolates of *C. psychrophila* are achieved in each of *Cytophaga* agar/broth using BBL beef extract, modified *Cytophaga* agar containing 0.5% tryptone, tryptone yeast-extract salts (TYES) (Holt *et al.*, 1993) or Sheih's medium (Song *et al.*, 1988). The effects of medium composition on the

growth of some isolates of *C. psychrophila* and *F. columnaris* has been recently studied by Cipriano and Teska (1994), and none of the media enhanced the growth.

A presumptive identification can be made from wet preparations of infected material and examining microscopically for the presence of filamentous bacterial rods. Austin and Austin (1993) believe that an effective diagnosis is best achieved by isolation in pure culture, followed by biochemical verification (e.g. Wakabayashi *et al.*, 1986; Reichenbach, 1989; Bernardet *et al.*, 1990; Austin & Austin, 1993; Holt *et al.*, 1994). However, isolation of these organisms in culture media is not always successful.

Serological tests are becoming more popular. Cell-agglutination reactions may be an effective test for differentiating *C. psychrophila, F. columnaris* and *F. maritimus* from other myxobacteria (Pacha, 1968; Morrison *et al.*, 1981; Wakabayashi *et al.*, 1984). However, some Australian isolates of *F. columnaris* and *F. maritimus* produce rough suspensions, and it is not possible to undertake serological tests which require homogeneous suspensions (Carson, pers comm, 1993). Therefore, immunofluorescent antibody tests (IFAT) are required for serological assays of these isolates. Griffin (1987) developed an IFAT procedure for identification of *F. columnaris*. Baxa *et al.* (1988b) found IFAT to be a suitable technique for *F. maritimus* diagnosis. Lorenzen and Karas (1992) developed a method based on immunofluorescene analysis of spleen imprints from diseased rainbow trout and utilized rabbit antiserum prepared against *C. psychrophila*.

1.3.4. Control and treatment

It is important to avoid every condition which causes stress to fish. Higher and lower temperature than optimal are the most important stressors which affect the incidence of columnaris and peduncle diseases, respectively. Therefore, it is advisable to decrease the temperature in order to control columnaris disease and to increase the temperature for the control of peduncle disease. In addition, increased dissolved oxygen, a suitable pH, reduced organic substances, the avoidance of unnecessary handling and overcrowding are all recommended. The use of a layer of sand on the bottom of tanks reduces the number of bacterial cells on the surface

of the skin or fin through the abrasive action of the sand in removing the cells and reducing the stress level in flatfish (McVicar & White, 1981/1982; McVicar, 1986). Such a device is, however, applicable only to flatfish species because their external topographical structure is different from other species. Therefore, abrasions, due to sand, may sometimes encourage the establishment of infection because of scale loss and "port-entry" induced on the fish skin through the abrasion on sand.

At present there is no commercially available vaccine against CFLB. So far only a few studies have been undertaken to investigate the immunity against these infections in fish. Daily administration of vaccine of heat-killed *F. columnaris* cells in feed for seven weeks reduced mortalities down from 48-8% in three-month-old coho salmon with an antibody production of 1:168 comparable with 1:17 for control survivors (Fujihara & Nakatani, 1971). A study of antigenicity of *F. columnaris* in channel catfish resulted in an agglutination antibody with a mean titre of 1:4337 when fish were injected either subcutaneously or intramuscularly by heat-inactivated *F. columnaris* cells (Schachte & Mora, 1973).The protective results of vaccination were inconclusive. Oral immunisation (10 mg/g feed of the formalin-killed *F. columnaris* cells) against columnaris disease resulted in 35% survival in vaccinated coho salmon one month post-immunisation compared with 100% mortality in the unvaccinated group (Ransom, 1975; cited by Newman, 1993). No protection was observed when vaccinated fish were challenged with the pathogen three months following vaccination. Field studies by Schachte (1978) and Moore *et al.* (1990) revealed the feasibility of vaccination of channel catfish against columnaris disease. Immersion vaccination against *F. columnaris* resulted in 59.3-89.8% protection in vaccinated rainbow trout compared with no protection in unvaccinated fish (Song, 1986, cited by Newman, 1993).

Vaccination of yearling coho salmon against *C. psychrophila* infection resulted in complete protection by intraperitoneal injection of the vaccine compared with 43% loss in control groups (Holt, 1988; Holt *et al.*, 1993). Vaccination by immersion gave a reduced level of protection (11%) compared with the intraperitoneal route. The efficacy of a heat-inactivated strain of *C. psychrophila*

vaccine which has been tested in rainbow trout resulted in 80% protection by intraperitoneal injection of the vaccine compared with no protection for control groups (Obach & Baudin-Laurencin, 1991). Bath vaccination provided a lower, but still significant, protection in fish compared with controls.

In a study by Carson *et al.* (1993), no protection was observed for immersion vaccination of rainbow trout and Atlantic salmon (*Salmo salar*) against *F. maritimus* infection. In a later attempt only immersion vaccination gave a low to moderate level of protection in Atlantic salmon against the infection (Carson *et al.*, 1994).

It is notable that a suitable vaccine against CFLB should be able to protect the outside surface of fish. This is so because these organisms usually give rise to superficial infections rather than systemic ones.

Since these pathogens primarily affect external surfaces of fish, chemicals for treatment are often added directly to the water as a dip, flush, bath or indefinitely prolonged treatment. Field observations show that oxolinic acid and oxytetracycline could be more useful than other antibiotics to treat columnaris disease (Austin & Austin, 1993; Munday, 1988; 1994; Carson, 1990; Holt *et al.*, 1993). Sulphonamides such as sulfisoxazole and sulphadimidine are known as successful drugs for the treatment of *C. psychrophila* and other *Cytophaga* spp. (Munday, 1988; Reddacliff,1988). Although egg disinfection by organic iodine compounds (Amend, 1974) against *C. psychrophila* was recommended by Holt (1972) and Schachte (1983), Holt *et al.* (1993) indicated that iodophor treatment of eggs did not prevent the disease in the resultant fry. *F. maritimus* infection may be treated by oxytetracycline, Tribrissen, trimethoprim and tiamulin (Carson, 1990; Wakabayashi, 1993; Munday, 1994). Nalidixic acid, sulphonamides and tetracycline may be used for treatment of *C. aquatilis* (Austin & Austin, 1993). In Appendix I (Table 2) a list of antimicrobial agents and chemical substances which have been utilized against fish pathogenic CFLB so far is provided.

1.4. Predisposing factors

As mentioned earlier, fluctuation in water temperature plays a significant role on the progress of infections caused by CFLB in particular *C. psychrophila* and *F. columnaris* (Fish & Rucker, 1943; Colgrove & Wood, 1966; Pacha & Ordal, 1970; Holt *et al.*, 1975; 1989; 1993; Austin & Austin, 1993; Wakabayashi, 1993). In addition to water temperature, the severity of the infections may also be influenced by a multiplicity of other environmental stressors and host-related factors.

An imbalance of water quality, such as pH changes, low levels of dissolved oxygen, unfavorable changes in nitrite and ammonia levels, causes stress in fish, thus, increasing the susceptibility of fish to these organisms. Observations by Chen *et al.* (1982) showed the highest mortality levels due to *F. columnaris* infection in eel were associated with stagnant water. As expected, the lowest losses occurred in running water. There was an inverse correlation between the dissolved oxygen level and the mortality rate. A condition of concomitant rise in the level of ammonia resulted in the enhancing of the mortality rate, even in the presence of adequate dissolved oxygen. Obviously a reduction in oxygen level or an increase in ammonia causes severe stress in fish and increases the host's susceptibility to infection.

A spontaneous infection of *F. columnaris* can be induced in channel catfish when fish are exposed to 5 mg/l nitrite for seven days (Hanson & Grizzle, 1985). Arsenic increased the susceptibility of striped bass (*Morone saxatilis*) to *F. columnaris*, when fish were exposed to four and 10 times the average environmental concentrations of 1-3 µg/l of this heavy metal (MacFarlane *et al.*, 1986). Nitrite or heavy metals can depress the immunity of fish so that the host's susceptibility to pathogens is increased (Rougier *et al.*, 1994).

Work by Chowdhury and Wakabayashi (1988b) showed that infectivity of *F. columnaris* in loach (weatherfish) and common carp was established in water supplemented with four cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and in tap water in contrast with no infection in distilled water. Although distilled water is more stressful to fish than tap water or supplemented water because of osmoregulatory difficulty in distilled water, distilled water also lacks substances which are required for bacterial

survival and growth. The progress of infection in individual salt solutions was also lower than in those of either the supplemented water or tap water. The presence of fish meal in water also seems to enhance the growth and infectivity of some of these pathogens as the study by Sugimoto *et al.* (1981) showed that infectivity due to *F. columnaris* in juvenile eels (*Anguilla japonica*) increased in the presence of the particulate feed matter compared with no effect in control fish.

Other factors such as crowding of fish, handling and physical injury also enhance the susceptibiltiy of fish to these infections. For instance, observations by Wakabayashi and Egusa (1972) suggest that the higher initial density of *F. columnaris* or the concentration of fish not only cause more infection in weatherfish, but also that mortality commenced earlier (Table 1.5). Similarly, no infection or mortality occurred in a trough (30.5 x 30.5 x 305 cm) containing 50 juvenile chinook salmon when they were challenged with *F. columnaris* at 17.5-21°C, while a mortality level of 1.3%, 10.2% and 12% occurred in troughs containing 150, 450 and 500-fish, respectively (Becker & Fujihara,1978).

The effect of crowding may not only decrease the resistance of fish to the organisms through depressing the host immune system (Wakabayashi, 1991), but it also increases the chances of the bacteria meeting the host.

Table 1.5. Effects of stocking density of fish and bacterial concentration of *F. columnaris* on columnaris disease of weatherfish at 22°C and 26 °C.

Number of fish*	Number of dead fish and lethal time (day)					
	Initial density of *F. columnaris* (CFU/ml)					
	0**	10^1	10^3	10^4	10^5	10^6
1	0	0	0	0	1 (2)	0
1	0	0	1 (2)	-	1 (2)	-
5	0	0	5 (3)	1 (4)	5 (1-2)	5 (1-2)
10	0	6 (4)	10(2-4)	10 (3-4)	10 (1-2)	10 (1-2)

Fish of average weight 10 g were maintained in glass jars containing 3 l of bacterial suspension prepared in sterile water with aeration. **Control group. Modified from Wakabayashi (1991).

1.5. Conclusion

Despite extensive bacteriological data on several species of CFLB, many of the others are poorly characterised. Also, taxonomy of this group of fish pathogens is

still not clear. Recent reports of new species of this group as fish pathogens demands a clarification of the classification for CFLB. It is also difficult to ascertain whether the recovered isolates are pathogenic or not. For instance, isolates of *C. aquatilis* were recovered from salmon, suckers and trout (Strohl &Tait, 1978); and strains of *C. johnsonae* have been reported from diseased barramundi (Carson *et al.* 1993), but it is not clear that whether these organisms are true pathgens. Therefore, although the identification of CFLB is readily possible, their classitication is still the subject of debate.

Although the pathogenesis of many infections in fish is still poorly understood, it has been suggested that the pathogenicity of CFLB can be explained by the release of powerful proteolytic enzymes by cell autolysis (e.g. Austin & Austin, 1993, Thune *et al.*, 1993). Release of such enzymes would explain the histolysis and necrosis that are characteristic of CFLB infections. Even so, the exact mechanisms of pathogenicity of these organisms are poorly understood (Dalsgaard, 1993). Although, specific adhesins have not been described, many of CFLB are surrounded by a thin slime layer that attaches the cells to a surface and is actively involved in the process of gliding motility (Dalsgaard, 1993). Mechanisms of gliding motility with predictive role in the bacterial pathogenesis, however, is not understood in this group, although Beatson and Marshall (1994) recently suggested a common helical mechanism for gliding motility of some gliding bacteria.

There is a variation of virulence between species and even among strains of the one species, especially *F. columnaris* and *C. psychrophila*. Many of pathological characters due to CFLB are similar both macroscopically and microscopically. Even so, there are some significant differences in this regard which make the type of infections distinguishable. For instance, *C. johnsonae* attacks scales, while other species do not probably because of the lack of chitinobiase (Bernardet & Grimont, 1989; Carson *et al.*, 1993). In addition, unusual forms of columnaris disease caused by an atypical *F. columnaris* have been reported in rainbow trout (Backmann & Speare, 1989). The affected fish showed focally severe destruction of areas of the ventral body wall leading to herniation of internal organs and lesions closely

resembled those of furunculosis. Also, an unusual CFLB infection caused gill hyperplasia and systemic haemorrhagic septicaemia has been reported in turbot (Mudarris & Austin, 1989).

Apart from fish, there are some reports indicating that CFLB may affect other aquatic animals. For instance, Dungan *et al.* (1989) suggested that strains of a *Cytophaga*-like bacterium were the agents of a degenerative disease affecting juvenile cultured Pacific oysters (*Crassostrea gigas*).

Expression of infections by CFLB in fish is greatly influenced by predisposing factors especially fluctuations in water temperature, the presence of organic substances and stocking density. Parameters of water quality such as ammonia, nitrite, dissolved oxygen and pH, any break in external barriers such as scales and surface epithelial layers, handling and probably ultraviolet irradiation affect the course of infections by these organisms in aquatic animals, in particular fish.

General Introduction to Study

From the Literature Review (Chapter I) it will be appreciated that the interactions between water parameters such as temperature, hardness and salinity greatly influence the pathogenicity of *Cytophaga/Flexibacter*-like bacteria (CFLB). Also, poor water quality and the presence of pollutants are likely to enhance these infections. Despite a significant increase in losses owing to these organisms in aquaculture, not enough attention has been paid to their pathophysiology and chemotherapy. This may be owing to the difficulties associated with the experimental reproduction of some of the infections and the fastidious cultural conditions required by these organisms (Vasquez-Branas, 1991; Toranzo & Barja, 1993a) or it may be because these organisms have not attracted researchers until recent years, when there has been significant increase in their importance in fish medicine concomitant with the recent rapid growth in the aquaculture industry.

During the last decade the intensive hatchery and extensive cage culture technology for breeding and rearing of a number of catadromous and anadromous species of fish in Australia have been associated with an increase in the number of CFLB infections (Schmidtke *et al.*, 1991; Anderson & Norton, 1991; Carson *et al.*, 1993; Munday & Nakai, 1994; Schmidtke & Carson, 1995). In warm water culture, *Cytophaga johnsonae* and *Flexibacter columnaris* have been isolated from diseased barramundi in northern Australian freshwater impoundments. In some instances outbreaks have been associated with seasonal fluctuations in weather conditions leading to water temperature variations. In Tasmania, after the establishment of cage culture salmonid farming in 1985, an epizootic due to *Flexibacter* infection was implicated as a potential threat to the industry. *Flexibacter maritimus* then was identified as the causative agent of salmonid cutaneous erosion disease. So far *F. maritimus* has been recovered not only from commercially grown salmonids, Atlantic salmon and rainbow trout, but also from a number of captured species, including striped trumpeter (*Latris lineata*), greenback flounder (*Rhombosolea tapirina*) and yellow-eyed mullet (*Aldrichetta forsteri*). More recently, another

freshwater species of CFLB, *Cytophaga psychrophila*, has been recovered from Atlantic salmon with ulcerated skin in some freshwater ponds (Schmidtke & Carson, in press).

Usually, the pathogenic potential of a microorganism as the causative agent of a specific infection requires to be confirmed by evaluating the pathogenicity of the isolated organism in the appropriate host under laboratory conditions. This provides data which permits the potential importance of the disease to be assessed. This is even more relevant because of recent reports on "new" fish-pathogenic organisms which may confuse the differentiation between the pathogenic and non-pathogenic CFLB. Although comprehensive studies have been undertaken on the microbiological aspects of the causative organisms, there are minimal data concerning the pathogenesis of the diseases they produce.

This work was initiated to provide a comparative study of the physiological requirements, pathogenesis and chemotherapy of selected Australian isolates of CFLB including *C. johnsonae*, *C. psychrophila*, *F. columnaris* and *F. maritimus* recovered from a number of tropical and coldwater fish. More attention has been paid to *C. johnsonae* and *F. maritimus* because of their roles as "new" fish pathogens.

The effect of environmental variables (Chapter II) was an *in vitro* study to identify responses of these organisms to a variation in temperature, salinity and pH and discusses the practical implication of these findings, as fluctuations in environmental factors greatly influence infections by CFLB.

Chapter III describes an *in vivo* investigation of pathogenesis of infections due to these organisms. The intention was to:

(i) Evaluate susceptibility of a number of species of fish including barramundi, goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*) and rainbow trout to *C. johnsonae* and *C. psychrophila* infections, and to identify difficulties associated with experimental induction of infections by these isolates.

(ii) Assess susceptibility of barramundi to *F. columnaris* and compare it with a known susceptible species, namely goldfish.

(iii) Study natural and experimental disease caused by *F. maritimus* in a number of commercial and captured species including Atlantic salmon, rainbow trout, greenback flounder, striped trumpeter and yellow-eyed mullet.

In Chapter IV an *in vitro* study was undertaken to assess the antibacterial activity of skin mucus of a number of species of naive fish against the causative agents. The aim was to identify that there was interaction between these organisms with fish surfaces (skin mucus), which is the first line of non-specific defence against the pathogens.

Chapter V describes the *in vitro* and an *in vivo* studies of chemotherapy of these infections. In the *in vitro* study, the minimum inhibitory concentrations of five commonly recommended antimicrobial agents were determined. The serum or mucus levels for some of these compounds were also determined in barramundi, Atlantic salmon and rainbow trout using different dosages and different routes of administration. These data were then correlated to the *in vivo* efficacy of selected compounds to validate the clinical efficacy of the antimicrobial agents for treatment of CFLB.

A general discussion and concluding remarks on the study are provided in Chapter VI.

CHAPTER II

The Effect of Environmental Variables on the Growth of *Cytophaga/Flexibacter*-like Bacteria (CFLB) Pathogenic to Fish

Introduction

The activities of microorganisms are greatly affected by the chemical and physical conditions of their environment. These environmental influences help investigators to explain the distribution of microorganisms in nature and make it possible for them to devise methods for controlling microbial activities, and to destroy undesirable organisms such as pathogens. Bacteria respond differently to environmental factors such as temperature. In other words, an environmental condition, e.g. a particular temperature, may be suitable for one organism while it is harmful to another, although bacteria can tolerate some adverse conditions under which they are unable to grow, but can still survive. Researchers therefore, need to distinguish between the effects of environmental conditions on the viability of an organism and effects on growth, differentiation, reproduction and pathogenicity.

Temperature, salinity and the pH of water are three of the important environmental factors influencing the growth, survival and pathogenicity of fish pathogenic *Cytophaga/Flexibacter*-like bacteria (CFLB) in aquatic environments (e.g. Fijan, 1968; Becker & Fujihara, 1978; Holt *et al.*, 1975; 1989; 1993; Chowdhury & Wakabayashi, 1988a; 1988b; 1991; Wakabayashi, 1991; 1993). These organisms are mesophiles, often with a strong psychrotrophic tendency. Usually soil and marine species of these organisms have an optimum temperature between 30 and 35°C, while the freshwater species show a considerably lower temperature optimum, usually between 20 and 25°C (e.g. Stanier, 1941; Veldkamp, 1961; Lewin & Lounsbery, 1969; Oyaizu *et al.*, 1982). The maximum temperature for growth of these bacteria is generally below 40°C and thermal death occurs at about this temperature. For instance, investigations on a collection of species of CFLB found

that the thermal death point occurred at 48 and 50°C after 10 and four minutes, respectively (Warke & Dahala, 1966; Reichenbach, 1989). Many strains of this group grow fairly well at 30°C when kept on agar plates, but when they are grown in liquid media, they prefer lower temperatures. Furthermore, it is possible to have a wide temperature range for near optimal growth. For example, one strain of *Cytophaga johnsonae* has a range of 22-30°C for near optimal growth (Reichardt & Morita, 1982b) .

Organisms have a pH range within which growth is possible, and usually have a well defined pH optimum for best growth. Most natural environments have pH values between 5 and 9, and organisms with optima in this range are common (Brock & Madigan, 1991). Most bacteria grow best at neutral pH, although there are some species of bacteria which require particular acidic or alkaline conditions. The optimal pH for CFLB is around 7.0 and usually no growth occurs above pH 8-9 or below 5.5 (Reichenbach, 1989).

Other factors of water quality such as salinity and hardness could affect the survival and growth rate of fish pathogenic CFLB. For example, Fijan (1968) indicated that *F. columnaris* could live for long periods in very hard water with high organic matter content at 25°C, while the survival time of the organism decreased significantly in water with a pH of 6. He also suggested that soft water of about 10 ppm $CaCO_3$, particularly when acidic or with low organic matter content, was unable to provide a proper environment for the organism's survival. Another trial by Wakabayashi and Egusa (1972) showed that *F. columnaris* could survive for a long time in autoclaved tap water containing 2.2% Ca^{2+} and 1.4% Mg^{2+} at pH 8. The survival of this organism was also studied in a variety of waters containing different concentrations of Na^{+}, K^{+}, Ca^{2+} and Mg^{2+} (Chowdhury & Wakabayashi, 1988a). It was found that the bacterium could survive for the longest period in water with 0.03 % NaCl, 0.01%KCl, 0.002% $CaCl_2.H_2O$ and 0.004% $MgCl_2$, while the survival of the organism in water containing the salts at 10 times or greater concentrations was significantly reduced. Chowdhury & Wakabayashi (1990b) demonstrated that some strains of *F. columnaris* could survive for above 10 days in tap water and diluted sea

water containing 0.5% NaCl, while total loss of viability occurred within three days in water containing 0.5% NaCl and no other salts. Results of these findings show that the presence of other components such as Ca, Mg, K and Na ions may augment the stabilisation of the freshwater CFLB in low salinity conditions or hard water resulting in long-term survival.

Therefore, environmental factors are of crucial importance in the expression of the pathogenic potentials of disease-producing microorganisms, particularly in the aquatic environment where both the host and the pathogen are greatly influenced by fluctuations of ambient water conditions. The present study was undertaken to investigate the effects of variations in temperature, salinity and pH on the *in vitro* growth of four species of fish-pathogenic CFLB, and it discusses the practical implication of these findings.

Materials and Methods

Materials

1. Bacteria

Flexibacter maritimus 89/4762, *Cytophaga psychrophila* 91/4043-17, *Cytophaga johnsonae* 91/0262-10, (Department of Primary Industry and Fisheries, Tasmania) and *Flexibacter columnaris* 1468 (Animal Health Laboratory, Department of Agriculture, Western Australia) were used in this study. The first two were isolated in Tasmania, Australia from a case of salmonid cutaneous erosion disease and from fin-rot in Atlantic salmon smolts, respectively (Carson, 1990; 1992; Schmidtke & Carson, in press). The latter two have been reported as a putative agent of an erosive skin disease and of columnaris disease, respectively of reared juvenile barramundi in the Northern Territory and Western Australia (Carson *et al.*,1993; C. Mifsud, pers comm, 1993).

2. Media

Marine Ordal broth (MOB) (Anacker & Ordal, 1959a) containing tryptone 0.05% (w/v) (Difco, Detroit, Michigan, USA), yeast extract 0.05% (w/v) (BBL, Becton Dickinson Cockeysville, MD, USA), sodium acetate hydrated (Univar) 0.02% (w/v) (Ajax

Chemicals) and beef extract 0.02% (w/v) (BBL) prepared in natural seawater was used for the culture of *F. maritimus*. The medium was autoclaved at 121°C for 15 minutes, held for 24 hours and then filtered (0.22 µm Millipore filter) to remove the particulates precipitated from the sea water. Appropriate volumes were then placed in 70 or 200 ml sterilized rectangular culture bottles. For growing *F. columnaris, C. johnsonae*, and *C. psychrophila,* the medium was prepared in dechlorinated tap water (freshwater Ordal broth, FOB).

Methods

Frozen ampoules of *F. maritimus* and *C. johnsonae*, and lyophilised ampoules of *F. columnaris* and *C. psychrophila* were reconstituted and grown in marine Ordal's agar (MOA) or freshwater Ordal's agar (FOA) at 25 or 15°C for two to five days under aerobic conditions. Bacterial cultures were then subcultured in 50 ml MOB or FOB at 25 or 15°C for up to 48 hours. Following purification on MOA or FOA, 0.5 ml of the exponentially growing cultures of *F. maritimus* and *C. johnsonae* were inoculated into the culture bottles containing 50-55 ml fresh MOB or FOB. For other bacterial species, the FOB bottles were inoculated to give an optical density of 0.030 at time zero.

The bacterial growth was measured turbidmetrically at 550 nm with a Spectronic 20D spectrophotometer (Milton Roy Company). This was used because cells changed size during culture. The absorbance of *F. maritimus* and *C. johnsonae* were measured daily for nine days (temperature) and five days (pH and salinity). The absorbance of *F. columnaris* and *C. psychrophila* was measured for up to nine hours. The morphology, cell length, motility and contamination were checked by wet mount, modified Hucker's Gram stain (Hendrickson & Krenz, 1991) and culture using two random replicates. The organisms were inoculated in MOB or FOB and incubated at temperatures of 4, 8, 10, 15-16, 20, 25, 30, 35, 37-38 and 42°C (± 0.5°C) with five replicates per temperature at pH 7.2 ± 0.2. The effect of pH was observed over the range 3 to 10 (± 0.1) at intervals of 0.4-0.7 with three or four replicates for each pH value at the optimum temperature determined in the first trial. NaOH or HCl (1M) was used to adjust the pH of the broths. The fresh water broths

were buffered to each pH value with 0.1M phosphate buffer. The bacteria were cultured at salinities of 0 to 3% NaCl at intervals of 0.3-0.5% with three or four replicates per salinity at optimum temperatures and pHs determined in the first and second trials. As well, *F. maritimus* was inoculated at 0, 10, 30, 70 and 100% natural sea water with 3 replicates each. Cultures of *F. maritimus* at 4 and 8°C were measured every second day for 22 days and those of *C. psychrophila* at 4°C were measured over 72 hours.

To verify the survival of the organisms at low and high values of temperature, pH and salinity, samples of these cultures were subcultured on to MOA or FOA at optimum temperature, salinity and pH for up to 10 days following inoculation.

The length of bacteria was measured using an ocular micrometer calibrated against a stage micrometer at x 40 magnification of phase contrast microscope.

Doubling times for *F. columnaris* and *C. psychrophila* were calculated at different temperatures, salinities and pH values. The log of absorbance was plotted against time (hours) and the line of best fit was then computed by regression analysis. The doubling times for the organisms were then calculated according to the following formula (McMeekin *et al.*, 1993):

Generation time = $\log^2$/slope of steepest tangent of exponential growth phase $\text{Log}^2 = 0.301$

Results

Trial 1. Temperature

The results of growth at different temperatures are shown in Figures 2.1 to 2.4. (Details in Tables 1 to 4 in Appendix II). Where growth is shown to be zero, this was after two to three weeks of incubation.

F. maritimus grew at temperatures ranging between 8 and 35°C (Fig. 2.1). Up to six days the optimum temperature for growth was 30°C. The bacterium grew faster at high temperatures, e.g. 20-30°C, but after eight to nine days the absorbance of cultures decreased indicating a decrease in biomass. Subcultures taken from the cultures grown at 4°C were positive, while those of 37-38°C and 42°C

were negative (i.e. there was survival at 4°C, but not at 37-38 and 42°C). Microscopic observation showed gliding motility was present at temperatures 4-35°C, although it appeared that the bacteria were more motile at temperatures 16-30°C inclusive.

C. johnsonae grew at temperatures ranging between 4 and 35°C (Fig. 2.2). After eight days inoculation, highest absorbance occurred in cultures incubated at 20°C. At temperatures below 15°C the absorbance was still increasing on day nine. Maximum absorbance at other temperatures occurred on day seven, or earlier, and was less than the maximum seen for 20°C. Absorbance for all temperatures ≥ 15°C decreased with further incubation past the time of maximum absorbance, indicating a decrease in biomass. Growth rates for cultures held at 15-30°C were similar and higher than cultures held at lower temperatures. No growth occurred at ≥ 37°C. Subcultures of the samples grown at 37 and 42°C gave no growth. Observation of gliding motility indicated that the bacteria appeared to be more motile at 4-20°C than 25-30°C.

The relationship between temperature and the growth shows that on days eight to nine post-inoculation, the growth increased with an increase in temperature up to 25°C for *F. maritimus* and up to 20°C for *C. johnsonae* and, then, at higher temperatures it decreased (Fig. 2.5). While cells initially appeared to get longer, after six days cells in cultures at 30-35°C became shorter. This may explain the decrease in absorbance after eight days for these cultures.

Growth of *F. columnaris* occurred at temperatures ranging between 15 and 37°C (Fig. 2.3). The growth rate increased with an increase in temperature up to 30°C and then it decreased at higher temperatures. *C. psychrophila* grew at temperatures ranging between 4 and 20°C with no growth at 25°C and above (Fig. 2.4). The doubling time of *F. columnaris* was shortest (two hours) at 25-30°C and longest (about 4.6 hours) at 15°C, while that of *C. psychrophila* was 3.7 hours at 20°C, 4.4 hours at 15°C and 34 hours at 4°C. Optimum, maximum and minimum temperatures for growth of *F. columnaris* were estimated to be 25-30°C, 37°C and about 13°C respectively, while those of *C. psychrophila* were estimated at 20°C

(optimum) and about 3°C (minimum), respectively (Fig. 2.5). Subcultures of *F. columnaris* taken from the cultures grown at 5-10°C were positive, while those at 42°C were negative. Subcultures of *C. psychrophila* at ≥ 25°C were negative.

Fig. 2.1. Comparison of growth of *F. maritimus* at different temperatures.
(Mean ± SE, n = 5)

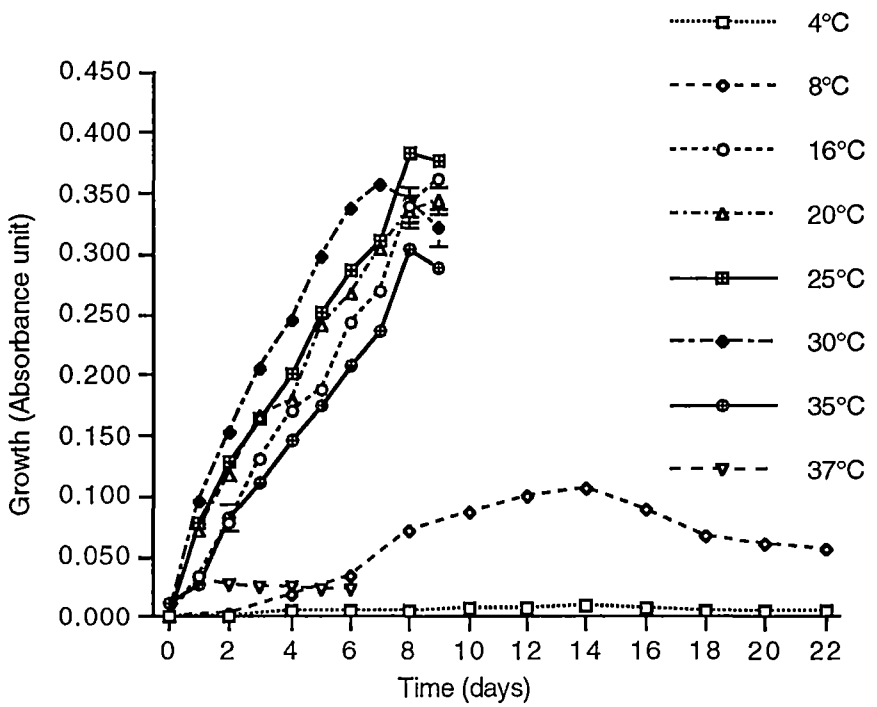


Fig. 2.2. Comparison of growth of *C. johnsonae* at different temperatures.
(Mean ± SE, n = 5)

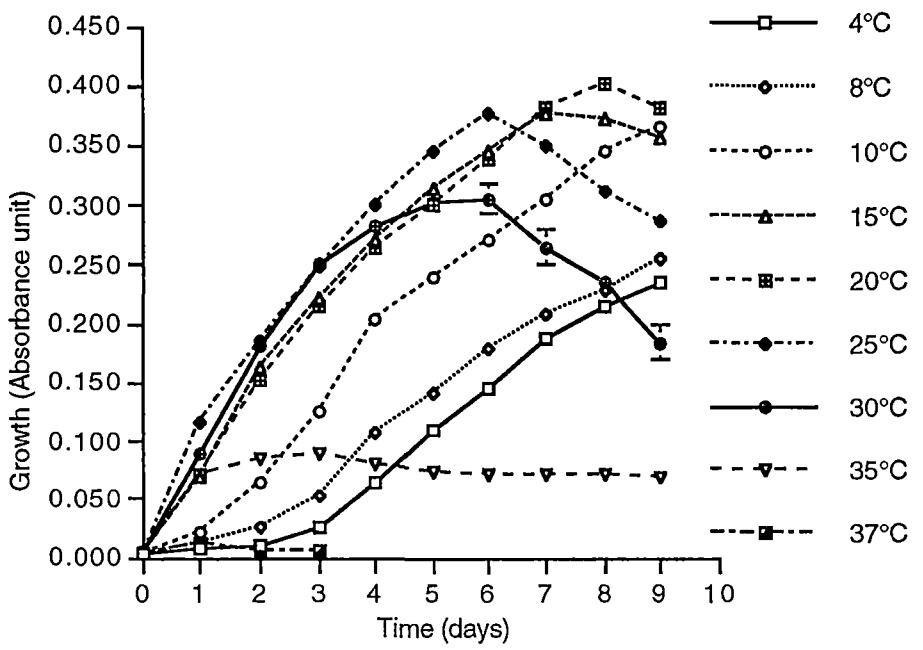


Fig. 2.3. Comparison of growth rate of *F. columnaris* at different temperatures.
(Mean ± SE, n = 5)

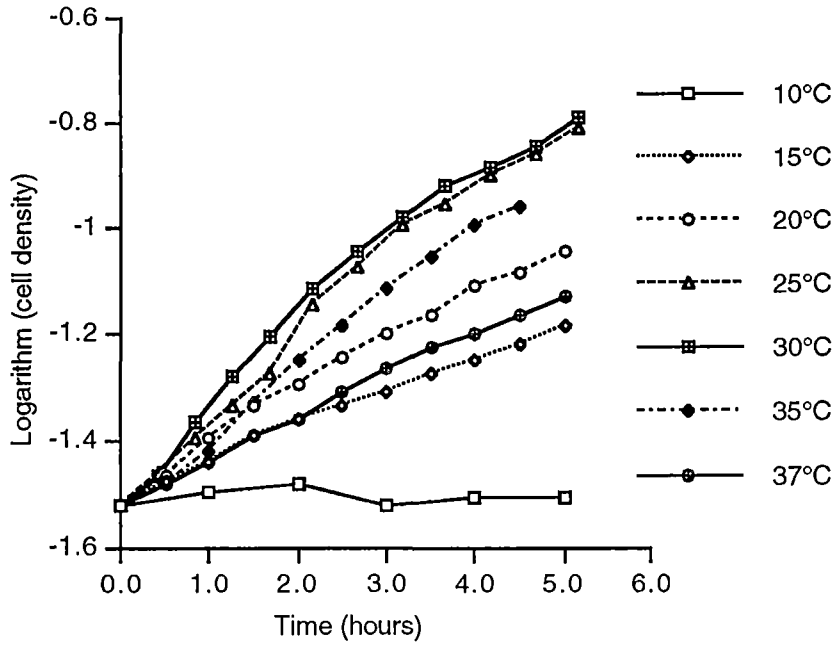


Fig. 2.4. Comparison of growth rate of *C. psychrophila* at different temperatures.
(Mean ± SE, n = 5)

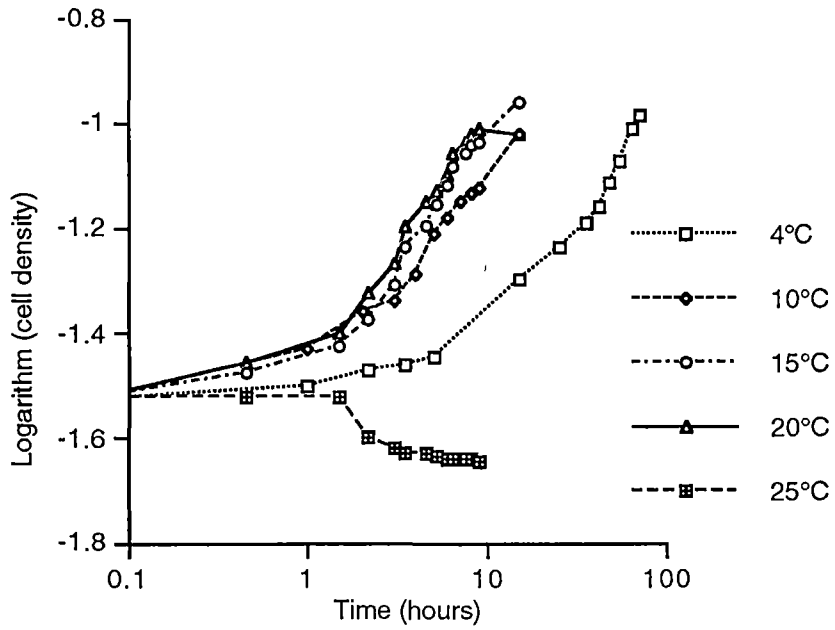
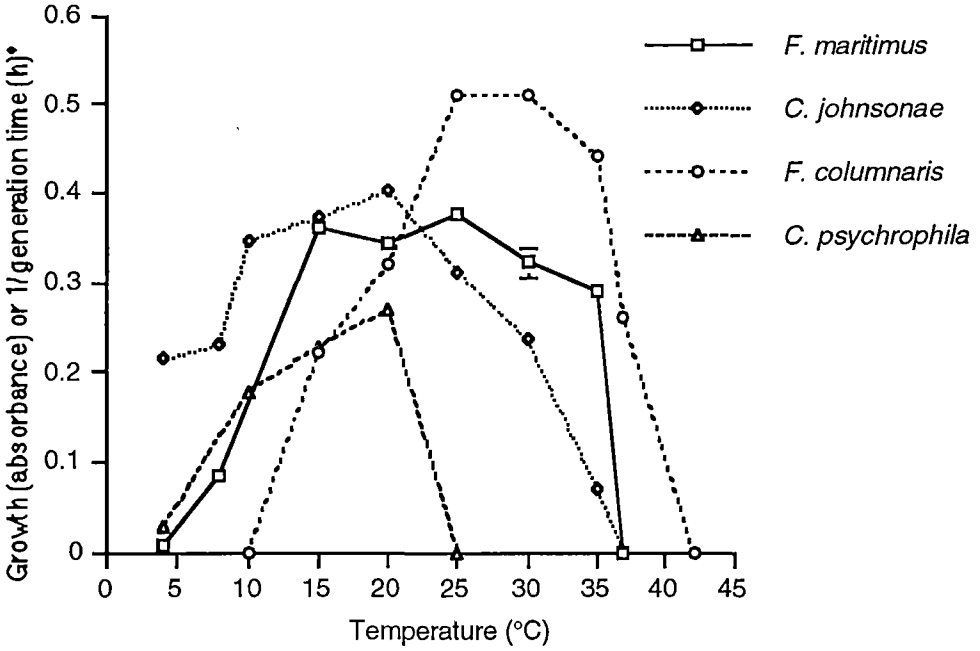


Fig. 2.5. Relationship between temperature and growth/growth rate of *Cytophaga/Flexibacter.*

*The value for *F. maritimus* and *C. johnsonae* is based on absorbance units (eight to nine days post-incubation) and for other species is 1/generation time (h) (five to nine hours post-incubation). (Mean ± SE, n = 5)



Trial 2. pH

The results of growth at pH values are shown in Figures 2.6 to 2.9 (Details in Tables 5 to 8 in Appendix II).

F. maritimus was able to grow at pHs between 6 to 8.6 (Fig. 2.6). No growth occurred at pHs ≤ 5 or > 8.6.The fastest and maximum growth occurred at a pH value of 7.2, while the minimum growth occurred at pH 8.6. Furthermore, the cells of these cultures remained viable only at pH ≥ 5.

C. johnsonae grew at pHs ranging between 5 to 9 (Fig. 2.7). Following five days of inoculation, optimum growth occurred at pH 7.5. The maximum and minimum of growth occurred at pHs of 7.5 and 5, respectively. The bacterium grew better in alkaline conditions than it did at acidic pH (Fig. 2.10). Wet mount preparation indicated that the organism was alive (gliding motility) at pH values of 3, 4, 9.5 and 10 five days following inoculation, but subcultures of these only grew from pH 4. Gram staining showed that the organism was elongated at all pH values, except for pHs 3-4 and 9.5-10. The length of the bacteria at pHs 3 and 4 was approximately the same as the original size, while the organisms appeared as short

rod to coccoid shapes at pHs of 9.5 and 10. Failure to elongate is further evidence of no growth occurring.

The pH values ranging from 6 to 8 were found to be suitable for *F. columnaris* and *C. psychrophila* growth (Figs. 2.8 & 2.9). No growth was observed at pHs of ≤ 5 and ≥ 9. The growth of *F. columnaris* at pH 8.5 started a few hours post-inoculation, while *C. psychrophila* did not grow at this pH. The shortest (about two hours) and longest (about three hours) generation times for *F. columnaris* were estimated to occur at pHs of 7 and 8.2, respectively (Fig. 2.10), while those of *C. psychrophila* were three and eight hours at pHs of 7-7.5 and 6, respectively. Subcultures taken from the cultures grown at pHs 3 and 4 resulted in no growth, while those of pHs 5, 9 and 9.5 were positive.

Fig. 2.6. Comparison of growth of *F. maritimus* at different pH values and incubated at 30°C. (Mean ± SE, n = 3)

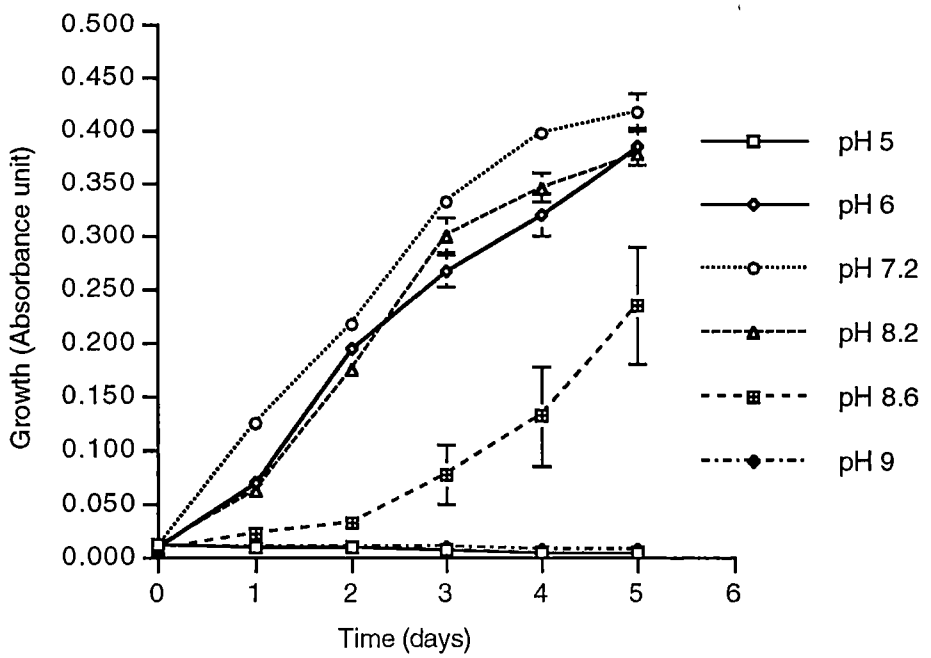


Fig. 2.7. Comparison of growth of *C. johnsonae* at different pH values and incubated at 20°C. (Mean ± SE, n = 3)

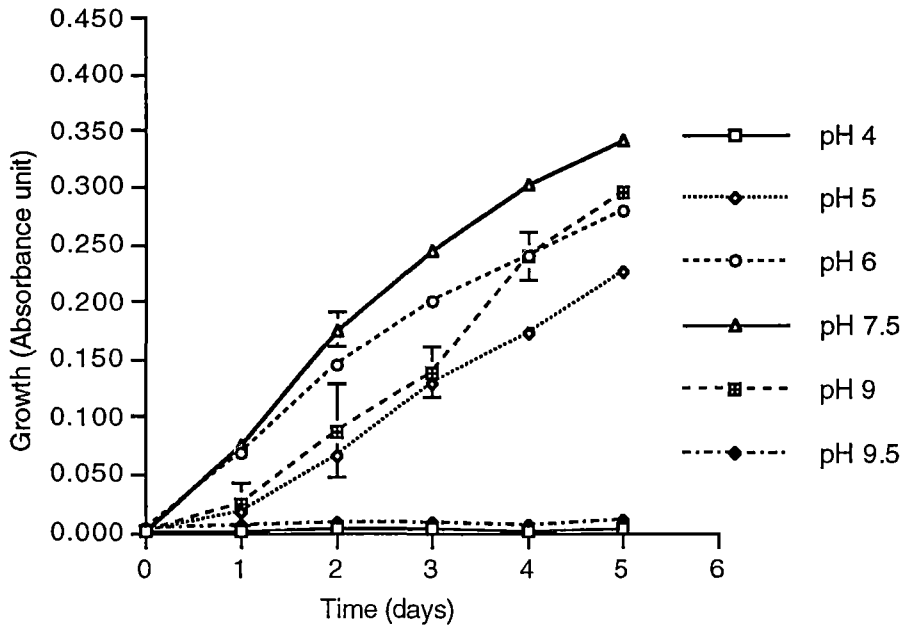


Fig. 2.8. Comparison of growth rate of *F. columnaris* at different pH values and incubated at 30°C. (Mean ± SE, n = 4)

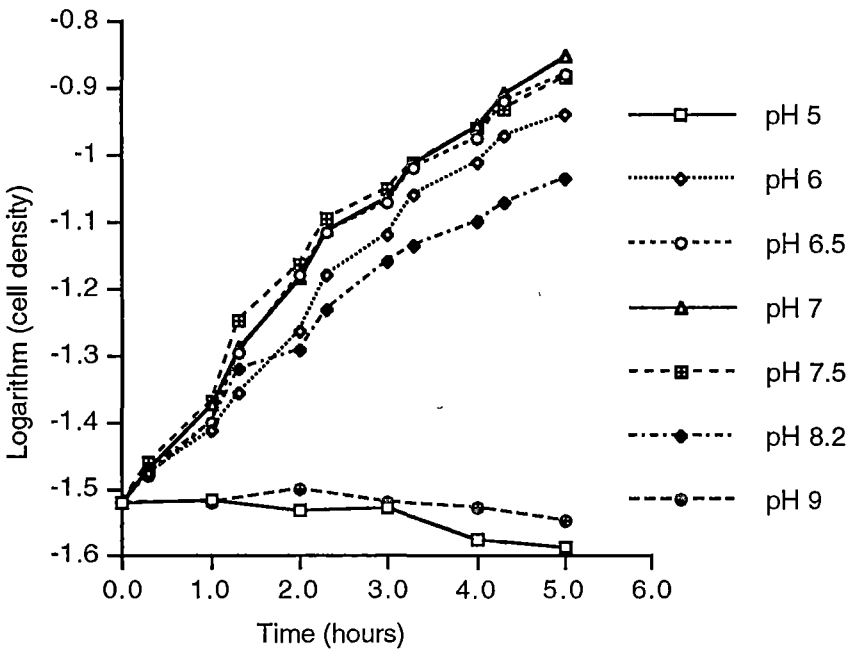


Fig. 2.9. Comparison of growth rate of *C. psychrophila* at different pH values and incubated at 20°C. (Mean ± SE, n = 4)

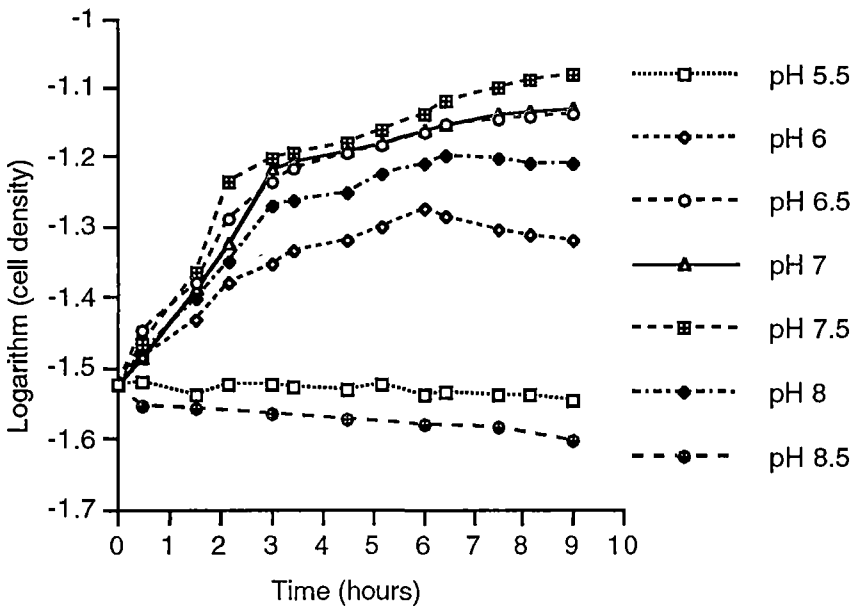
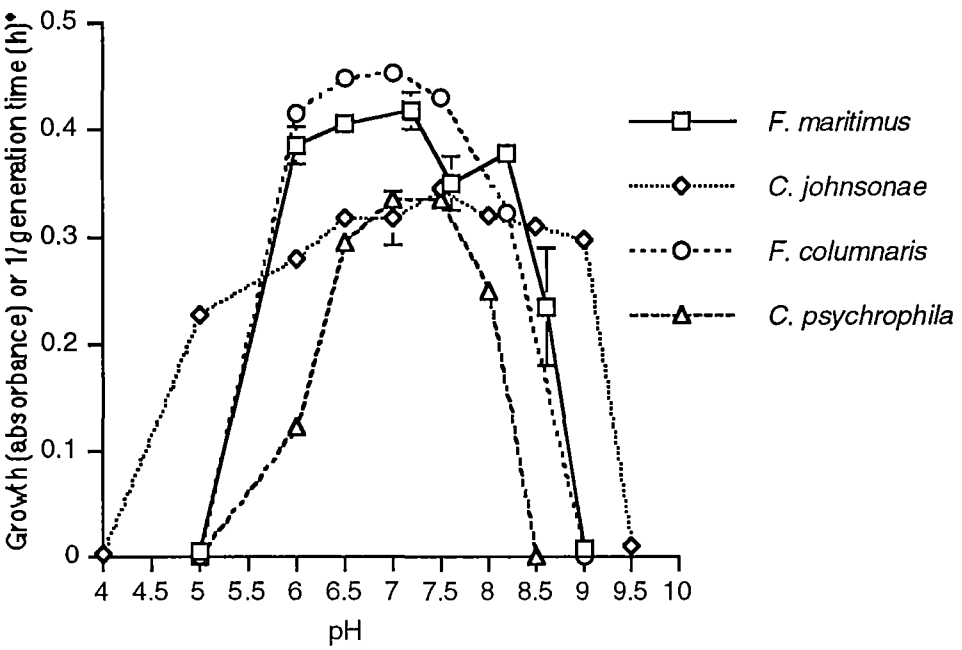


Fig. 2.10. Relationship between pH and growth/growth rate of *Flexibacter/Cytophaga.* (Mean ± SE, n = 3-4). *The value for *F. maritimus* and *C. johnsonae* is based on absorbance units (five days post-incubation) and for other species is 1/generation time (h) (five to nine hours post-incubation).



Trial 3. Salinity

The growth at different salinities is shown in Figures 2.11 to 2.14 (Details in Tables 9 to 13 in Appendix II).

F. maritimus is a fastidious organism which requires seawater or a complex salt solution for growth. Even so, subcultures on MOA made with NaCl only indicated that *F. maritimus* remained viable in media containing NaCl at the concentrations of 1 to 2.5% for three days. No growth occurred at 0% and 10% natural sea water, while maximum and optimum growth occurred in media containing 100% sea water (Fig. 2.11). The results of subcultures of 0% and 10% sea water were positive. There is a coincidental relationship between the ratio of sea water provided in the media and the growth of the organism (Fig. 2.15). When the sea water percentage was increased, the growth increased.

C. johnsonae was able to grow at salinities of 0% to 2% NaCl. No growth occurred at salinities above 2% NaCl (Fig. 2.12). The optimum growth occurred at 0% salinity. There was an inverse relationship between salinity and growth. When salinity was increased, the growth decreased (Fig. 2.15). The bacteria were alive (gliding motility) at salinities of 2.5% and 3% NaCl, five days following inoculation. This was confirmed by subcultures on FOA. The Gram stain results indicated that, with an increase in the salinity up to 2% NaCl, the length of the cells appeared to decrease. However, the size of the cells at salinities of 2.5% and 3% was the same as the original inoculum.

Optimum and minimum growth for *F. columnaris* and *C. psychrophila* were demonstrated to be 0% and 0.7% NaCl, respectively (Figs. 2.13 & 2.14). The doubling times for bacterial population of *F. columnaris* at salinities of 0% and 0.7% NaCl were estimated to be about two and 3.9 hours, respectively, while those of *C. psychrophila* were 3.7 and 6.8 hours, respectively (Fig. 2.15). No growth was observed at salinities of 1%, 1.5% and 2% NaCl. Subcultures taken from samples at 1% NaCl grew, while those from above 1% NaCl did not.

Fig. 2.11. Comparison of growth of *F. maritimus* at different percentages of natural sea water and incubated at 30°C. (Mean ± SE, n = 3).

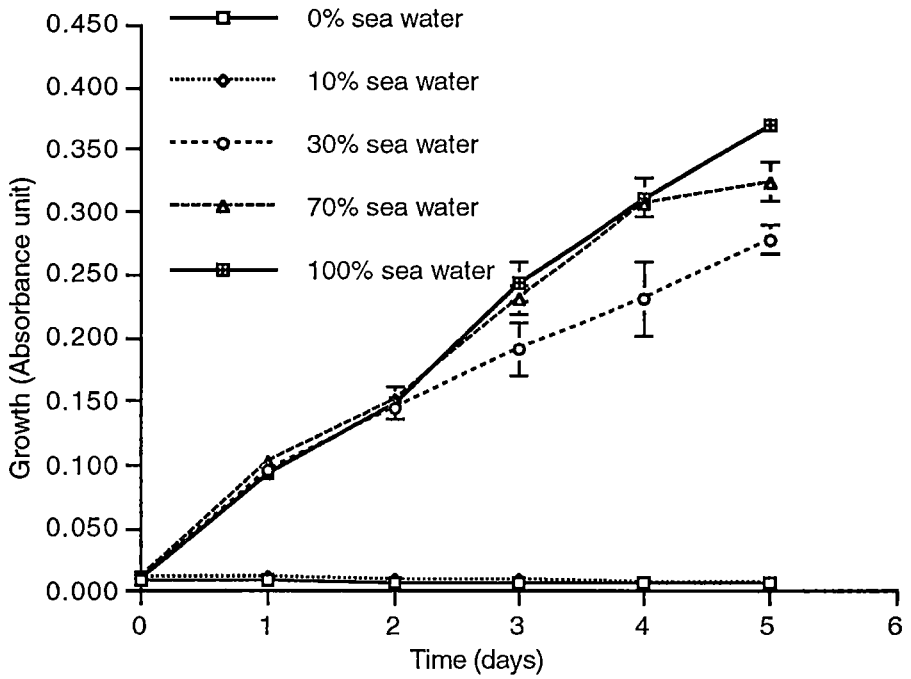


Fig. 2.12. Comparison of growth of *C. johnsonae* at different salinities (NaCl%) and incubated at 20°C. (Mean ± SE, n = 3)

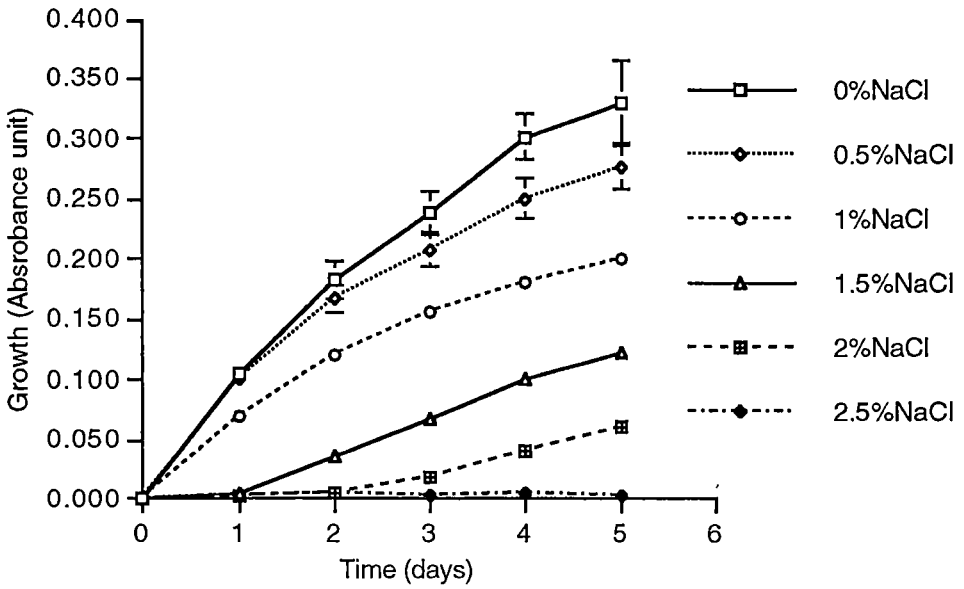


Fig. 2.13. Comparison of growth rate of *F. columnaris* at different salinities (NaCl%) and incubated at 30°C. (Mean ± SE, n = 4)

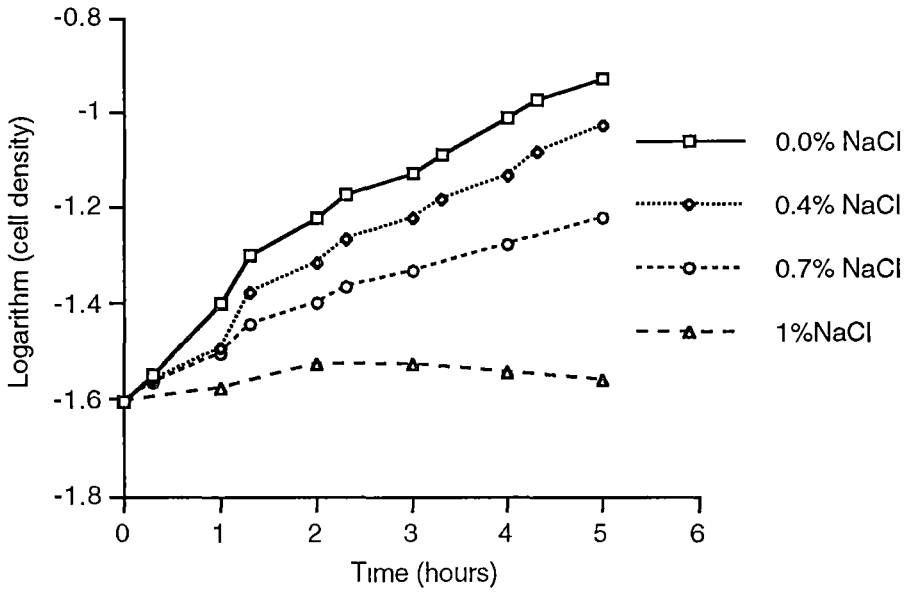


Fig. 2.14. Growth rate of *C. psychrophila* at different salinities (NaCl%) and incubated at 20°C. (Mean ± SE, n = 4)

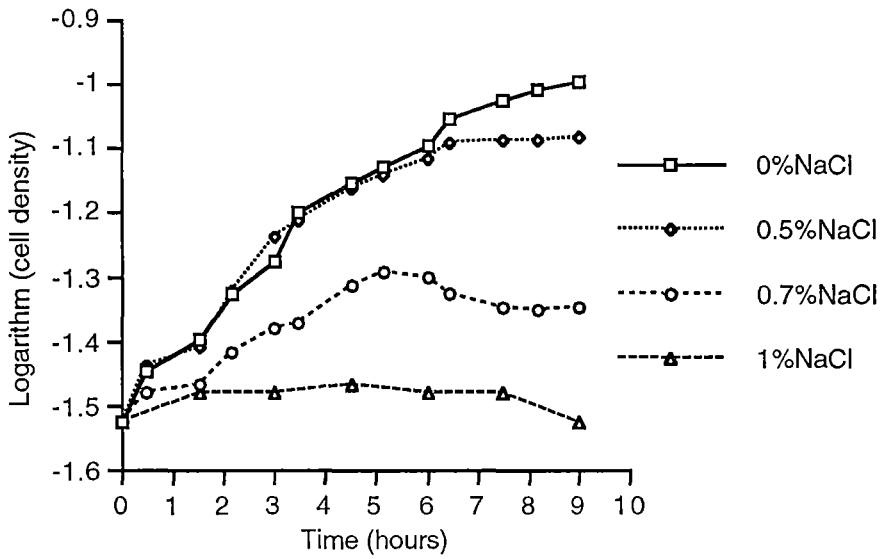
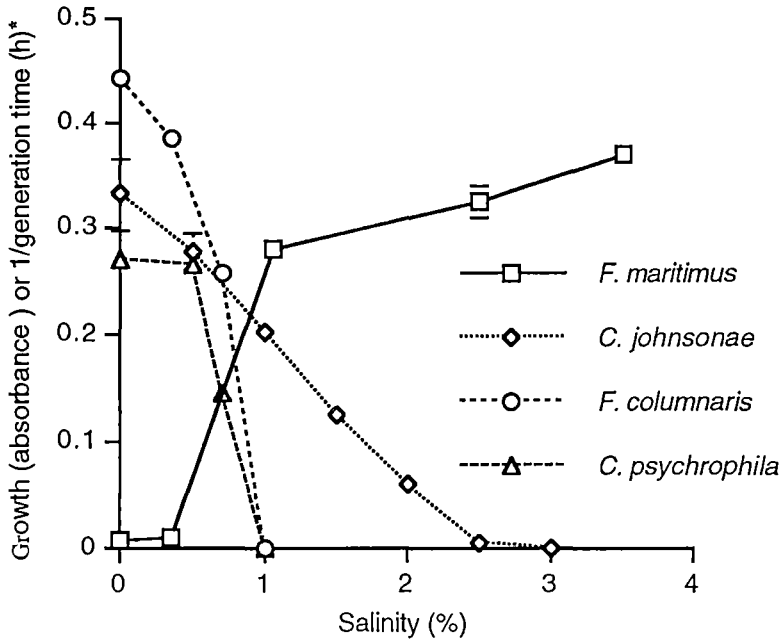


Fig. 2.15. Relationship between salinity and growth/growth rate of *Flexibacter/Cytophaga* (Mean ± SE, n = 3-4). * The value for *F. maritimus* and *C. johnsonae* is based on absorbance units (five days post-incubation) and for other species is 1/generation time (h) (five to nine hours post-incubation).



Discussion

These strains of *Cytophaga/Flexibacter* should be considered as psychrotrophic bacteria having an optima of 20-30°C, except for *F. columnaris* which had mesophilic tendencies (Fig. 2.5). This is supported by the fact that the organisms grow well at 20°C and by the minimum temperatures for growth being ≤ 8°C except for *F. columnaris* in which the minimum temperature is >10°C.

Although the Japanese and Scottish strains of *F. maritimus* grow at temperatures of 15-34°C (Wakabayashi *et al.*, 1986; Austin & Austin,1987; Bernardet & Grimont, 1989), this Australian strain is able to grow at 8 and 35°C. A temperature range from 4-35°C was found for *C. johnsonae* strain with an optimum of about 20°C. This concurs with Canadian strains of this organism obtained from diseased freshwater fish, moose dung and soil (Christensen, 1977b).

Sanders and Fryer (1988) found that some strains of *F. columnaris* grew at temperatures from 10-37°C. The study of eight strains of this organism isolated in Europe, the USA and Japan revealed that growth occurred in Ordal broth at 10-33°C (Bernardet & Grimont, 1989; Wakabayashi, 1993). This Australian isolate of *F. columnaris* grew at temperature range from 10°C > to 37°C but not at 42°C.

Comparison of *C. psychrophila* strains obtained from different hosts or geographical locations shows that the activity of this bacterial species is restricted to cold to temperate geographical regions with a temperature range from about 4-20°C. Some isolates may have a scant growth at below 4°C or at 25°C (Pacha, 1968; Bernardet & Kerouault, 1989; Holt *et al.*, 1989; 1993). In tryptone -yeast extract broth (TYEB; 0.4% tryptone and 0.04% yeast extract) a generation time of about two hours at 15°C, and 20 and 26 hours at 3°C were estimated for two strains (Holt *et al.*, 1989), compared with a doubling time of three to four hours at 15-20°C and 34 hours at 4°C in this study. This is probably because FOB has less nutrients than TYEB. Therefore, FOB is likely to give a better estimate of growth rate in the natural environment. Fry rainbow trout syndrome caused by *C. psychrophila* , has a higher incidence at lower temperatures (4-12°C), partly because the host's immune system is less responsive at this low temperature (see pathogenicity, Chapter I).

These Australian isolates of *F. maritimus* and *F. columnaris* were not able to grow at 4 and 10°C respectively, even after three weeks incubation, while *C. johnsonae* and *C. psychrophila* started to grow at 4°C with 45 minutes and two days, respectively. The growth was slow at low temperatures of 4-15°C as psychrotrophic organisms do not grow very quickly at low temperatures, and it may take several weeks before visible growth is seen in culture media at 0°C (Brock & Madigan, 1991).

Brock and Madigan (1991) found that the catalytic reactions of psychrotrophic bacteria are more active to some degree at low temperatures, with the result that their enzymes are inactivated at higher temperatures of 30-40°C. Efficient low temperature function is due to high content of unsaturated fatty acids in the psychrotrophs cell membrane so the membrane remains semifluid at low temperatures. This enables the organisms to concentrate effectively essential nutrients at low temperatures. A higher metabolic versatility of these organisms at lower temperatures was also observed by Reichardt & Morita (1982a) and Reichenbach (1989).

The decrease in absorbance at higher temperatures, 25 and 30°C after growth, shows that the bacteria have shrunk or possibly lysed, indicating that culture is in a death phase. The death phase of *C. johnsonae* occurred at temperatures of 25 and 30°C six days following inoculation, while it occurred on day eight for *F. maritimus*. Death could be due to lack of nutrients and accumulation of waste products which cause stress to the bacteria.

While *C. johnsonae* grew over the pH range of 5 to 9, *F. maritimus* and *F. columnaris* and *C. psychrophila* were restricted to pH 6 to 8.5. Therefore, none of the organisms are acidophilic or alkalinophilic. The strains of *C. johnsonae* studied by Christensen (1977b) were able to grow on skim milk acetate plates over a similar wide pH range from 5 to 10. Change in the pH of seawater may affect the concentrations of the seawater elements thus resulting in direct and indirect effects on the growth/survival of *F. maritimus*. In this study *F. maritimus* had a narrow range of pH for growth.

Wakabayashi *et al.* (1986) pointed out that the Japanese strains of *F. maritimus* did not grow in cytophaga medium prepared in NaCl alone. They found that these strains required K^+ and Na^+ for growth. Also Ca^{2+} and Mg^{2+} enhanced growth, while SO_4^{2-} had a slightly inhibitory effect. These results show that this strain of *F. maritimus* required other salts than just NaCl, therefore, it will be restricted to an estuarine or marine environment. *F. columnaris, C. psychrophila* and *C. johnsonae* are considered to be freshwater organisms which do not require any NaCl for growth (Reichenbach, 1989) and the results here concur with this.These freshwater organisms grew at higher sodium chloride concentrations (up to 0.7% for *F. columnaris* and *C. psychrophila* and 2% for *C. johnsonae*), than the highest salinity concentrations quoted by Reichenbach (1989). Using TYEB all examined isolates of *C. psychrophila* were able to grow in the presence of 1% NaCl (Holt *et al.*, 1993), while only some of those isolates which were tested in *Cytophaga* broth grew at 1% NaCl (Pacha, 1968). However, the isolate examined here did not grow at 1% NaCl even four weeks post-inoculation.

With regard to mariculture conditions, seawater usually has a pH of between 7.5 and 8.5 and a salinity of 32 - 38‰ (Austin, 1988). Many freshwater environments usually have a pH ranging between 6 and 8 and a salinity up to 3‰. The results of *in vitro* culture predict that these organisms may grow/survive well in such marine or freshwater conditions. These strains were shown to grow at temperatures ranging from 4-35°C. Therefore, it appears that the organisms will be found in many aquatic environments of temperate or tropical regions. Consequently, understanding of the environmental characteristics observed in this study can be useful as a guideline to preventing the incidence of disease in fish farms.

The environmental conditions, including temperatures of 15-30°C, pH values of 6 to 8 and 3.5% salinity, are significant physiological requirements which increase the growth of *F. maritimus*. The requirements for *C. johnsonae* consist of a temperature of 5-30°C, a pH of 5-9 and preferably no NaCl. The significant parameters for growth of *F. columnaris* and *C. psychrophila* consist of a temperature ranging from 15-35°C (for *F. columnaris*) and 4-20°C (for *C. psychrophila*), pH values of 6 -8 and zero salinity. Therefore, within such conditions the possibility of the incidence of the disease will be increased, particularly if the host becomes stressed. Outside of these physiological requirements, the incidence of the disease is likely to be less because, according to this study, the bacteria grow more slowly or are unable to grow at all. Furthermore, a decrease in salinity (seawater dilution) to below 10‰ for *F. maritimus* infection and an increase in salinity to 10-15‰ for columnaris disease or *C. johnsonae* infection should be a reasonable approach for the control and treatment of these bacterial infections in fish. These methods are not only economic procedures, but are also relatively harmless to the environment.

CHAPTER III

In vivo Study of the Pathogenesis of *Cytophaga/Flexibacter*-like Bacteria (CFLB) Infections

3.1. Susceptibility of Some Freshwater Species of Fish to Infection by *Cytophaga johnsonae* and *Cytophaga psychrophila*

Introduction

There are very little data concerning the role of *C. johnsonae* as a pathogenic bacterium for fish. Christensen (1977a) isolated strains of *C. johnsonae* from diseased fish, probably rainbow trout, but the exact role of the bacterium was unknown. Some strains of this organism have been isolated from external lesions of fish including carp, eel, crucian carp and tench, where these organisms have acted as opportunistic pathogens (Lehmann *et al.*, 1991). Also, Rintamäki and Bernardet (1993) reported some isolates of this organism from the head and tail lesions of Atlantic salmon in Finland. More direct evidence of pathogenicity has been provided by the isolate of this organism from juvenile barramundi with severe cutaneous erosion in Australia (Carson *et al.*, 1993). However, neither these authors nor Lehmann *et al.*, (1991) and Rintamäki and Bernardet (1993), have attempted to reproduce the various disease conditions which have been associated with *C. johnsonae*.

The geographical distribution of *C. psychrophila* infection was thought to be limited to the North American continent until 1984, when the disease was recognised in rainbow trout in Europe (Wiklund *et al.*, 1994). Since then, the disease has frequently been reported with the mortality of farmed fry and fingerling rainbow trout in several European countries (Bernardet, *et al.*, 1988; Dalsgaard & Hørlyck, 1990; Lorenzen *et al.*, 1991; Austin, 1992; Santos *et al.*, 1992; Toranzo *&* Barja, 1993b; Wiklund *et al.*, 1994). Recently, infection was also reported from coho salmon and Atlantic salmon in Japan and Australia, respectively (Wakabayashi *et al.*, 1991; Wakabayashi & Toyama, 1993; Schmidtke & Carson, 1995). In Australia, the report of *C. psychrophila* infection was documented by recovering the organism from Atlantic salmon smolts of 45 g body weight at 5°C. The affected fish had fin and tail erosion with a mortality level of < 0.01% week and no bacterium was recovered from

the internal organs. The pathogenesis of this southern hemisphere strain of *C. psychrophila* has not been evaluated so far.

Therefore, the initial objective of this study was to assess the susceptibility of a number of freshwater species of fish to infections by *C. johnsonae* and *C. psychrophila.*

Material and Methods

1. Cultures

Strain 91/0262-10 of *C. johnsonae* (Chapter II) was used for all experiments. The bacterium was passaged in goldfish (*Carassius auratus*) and barramundi three times to ensure there was no loss of virulence. For passaging, fish were bathed in bacteria at a concentration of 1.0×10^7 cells/ml for one hour or injected intraperitoneally with 1.0×10^6 cells/fish. A lyophilised ampoule of *C. psychrophila* 91/4043-17 (Chapter II) with one passage (intraperitoneal injection with 5×10^6 cells/fish) in rainbow trout was used. In addition, the strain of *C. johnsonae* 3550-4 and one isolate of *Cytophaga* sp. (Fish Health Unit, Department of Primary Industry and Fisheries, DPIF) recovered from rainbow trout with tail-rot were included in this study. The cultures used for challenge were grown into 1000 or 2500 ml volumes of *Cytophaga* broth (CB) (Anacker & Ordal, 1959a) in 2 or 3 liter conical flasks. The cultures were grown with shaking using an orbital shaker (30-40 rpm), at 15 or 25°C for *C. johnsonae* and *Cytophaga* sp. (Table 3.1.1) and at 16°C for *C. psychrophila* (Table 3.1.2) for up to 96 hours. The cells for challenge were counted on *Cytophaga* agar (CA) in triplicate using the spread plate method, and viable count were estimated as colony forming unit (CFU) per ml of water tank or fish (Table 3.1).

2. Fish stocks

Barramundi, goldfish and guppies (*Poecilia reticulata*) were maintained in aquaria and rainbow trout in a recirculation tank with biofiltration. Water was exchanged 5-10%/day; pH ranged between 7.0-7.5. Juvenile barramundi, 2-5 g body weight were maintained at 28°C; goldfish 5-30 g body weight and guppies 2-5 g body weight were maintained at 15-20°C. Rainbow trout 4-9 g body weight (challeged

with *C. psychrophila*) and 100-300 g body weight (challenged with *C. johnsonae*) were maintained at 10 and 12-15°C, respectively. Also, goldfish 1-4 g body weight were maintained at 10°C. Fish were fed once daily using Tropical Fish Flakes (Wardley's) or Salmon Feed (Gibson's Feed Mill).

3. Purification and confirmation of cultures prior to challenge

The purification tests were wet mounts, Gram stains and subcultures on CA, blood agar and nutrient agar or CA only before inoculation. A specific *C. johnsonae* antisera was also prepared in a white New Zealand rabbit and slide agglutination test was then used as a confirmatory test.

The preparation of anti-*C. johnsonae* antisera was as follows:

A lyophilised ampoule of *C. johnsonae* 91/0262-10 was cultured on CA at 25°C for 48 hours. Subcultures were introduced into CB at room temperature (20-22°C) for 48 hours with gentle agitation. Bacterial cells were harvested (1500 g for 25 min). The cells were washed three times with sterile phosphate buffered saline (PBS, pH 7.3, 0.1M) and subcultures were made on blood agar and CA to a verify purity of the cells. The cells were inactivated by formalin 1.4 v/v% and left at 4°C for 24 hours. Inactivated cells were then washed once with sterile PBS and re-suspended in PBS to McFarland standard 1 (ca. 3×10^8 cells/ml). Incremental doses of 0.1 ml were given intravenously to a New Zealand white rabbit at two day intervals commencing at 0.1 ml and finishing at 1 ml; repeat 1 ml doses were given after two and six days. Antibody titre was assessed by slide agglutination test; and the rabbit was exsanguinated five days later; blood was left at 4°C overnight and the antiserum separated and stored at -20°C.

4. Challenge

Challenge methods are given in Table 3.1. An unchallenge control group was used for each experiment. Control groups were kept in separated tanks provided in an isolated unit but in the same area. The fish were observed twice daily for 21 days and any moribund or dead fish removed. Following Gram smear preparations from lesions, all fish with skin or fin lesions were cultured on CA; the presence of *C.*

johnsonae or *C. psychrophila* was confirmed by the appearance of a characteristic cellular and colonial morphology and/ or by slide agglutination with rabbit raised polyclonal antiserum to *C. johnsonae* .

Table 3.1. Methods used to assess the pathogenicity of *C. johnsona* e and *C. psychrophila*

3.1.1. *C. johnsonae*

No.	Method of challenge	Cell no. as CFU	Water temp. (°C)	Fish species	No. fish
1	Bath	7.9 x 10^7/ml	25	Goldfish	20 (2)*
2	Scarification & bath	5.2 x 107/ml	30	Goldfish, Guppy	20 (1)
3	Scarifying and swabbing	-	15	Goldfish, Guppy, Rainbow trout	5 (3)
4	I. M. injection	4.85 x1 0^6/fish	25	Goldfish	20 (1)
5	I. P. injection	1.4 x 10^4-1.4 x 10^7/fish	21	Goldfish	10 (2)
6	Immunosuppression and bath	1.4 x 10^7/ml	25	Guppy	20 (4)
7	Bath	9.7 x 10^7/ml	25	Barramundi	25 (2)
8	Bath	9.4 x 10^7/ml	20	Barramundi	20 (2)

An unchallenged control group was used for each method. Challenge time for bath method was 60 minutes except for methods 2 and 8 where fish were exposed to bacteria for 90 minutes.

(1) Following anaesthetization, a few scales were removed and the skin lightly scraped with a sterile scalpel blade.

(2) Scarifications 10 - 20 x 5 - 10 mm were made on the sides of the fish. Rainbow trout and goldfish were also challenged with *C. johnsonae* 3550-4 and *Cytophaga* sp.

(3) Scarifications were directly swabbed with plate cultures. Rainbow trout and goldfish were also challenged with *C. johnsonae* 3550-4 and *Cytophaga* sp.

(4) Injections were given into the dorsal muscles.

(5) Doses used for intraperitoneal injection were 1.4 x 10^4, 1.4 x 10^5, 1.4 x 10^6 and 1.4 x 10^7 CFU/fish.

(6) A week prior to challenge, fish were intraperitoneally injected by a single dose (100 µg/g body weight) of the corticosteroid triamcinolone acetonide (TA) (Sigma), and an unchallenged control group treated with TA.

(8) Fish were taken from 28°C, immediately challenged at 20°C and then held at this temperature.

*Number of replicates in parentheses.

3.1.2. *C. psychrophila*

No.	Method of challenge	Cell no. as CFU	Water temp.(°C)	Fish species*	No. fish
1	Bath	1.2 x 10^7/ml 5.6 x 10^6/ml	10	Goldfish Rainbow trout	13 (2)**
2	Scarifying and bath	1.2 x 10^7/ml 5.6 x 10^6/ml	10	Goldfish Rainbow trout	13 (2)
3	I.M. injection	5.8.x 10^6/fish	10	Goldfish Rainbow trout	13 (2)
4	I.P. injection	5.8 x 10^6/fish	10	Goldfish Rainbow trout	13 (2)

An unchallenged control group was used for each trial.

(2) Scarifications (5-20 mm in length) were produced on the sides of the fish.

* Goldfish 1-4 g body weight and rainbow trout 4-9 g body weight were used. **Number of replicates in parentheses.

Results

No significant lesions were detected in any fish other than barramundi and then only in the fish challenged after the water temperature was dropped rapidly from 28-20°C (Method 8, Table 3.1.1). The lesions induced were not as severe as those described by Carson *et al.* (1993) and consisted of fin rot, involving especially dorsal fins and tail (Fig. 3.1). Clinically, 45% of fish showed some lesions and exhibited a peculiar swimming behaviour with flexion of the body and rapid rippling of the fins with little actual movement of the fish. Mortality to 21 days after exposure was 25%, which were confirmed by Gram stain, plate culture or slide agglutination.

The goldfish did not show any external signs or internal abnormality in this study. From Methods 2 and 3 (Table 3.1.1), some of the guppies (8 and 5 fish respectively) died within a few days following challenge. This was considered to be due to the effect of low or high temperatures, i.e. 15 and 30°C. The bacterial examinations were negative except for one (Method 2), which showed haemorrhages around the scar site, unilateral swimming, sluggishness and hovering below the surface of the water, as well as a positive result of the bacterial identification taken from this scar site. The rest did not show any signs. From rainbow

trout, only two of the fish infected with the isolate 3550-4 (Method 3) showed large lesions on the caudal site as well as tail rot. The wet mount and Gram stain preparations from these lesions showed the existence of ectoparasites including *Trichodina* and *Ichthyophthirius* as well as the filamentous rod bacterium. Results of cultures and slide agglutination from these lesions were positive for *C. johnsonae.*

Goldfish and rainbow trout exposed to *C. psychrophila* did not succumb to infection. In the other words, no mortality or lesions were attributable to *C. psychrophila.* Two rainbow trout (Method 2, Table 3.1.2) died 11 days post-exposure with no sign of the infection. Gram smears and plate cultures obtained from external surfaces and internal organs (kidney and spleen) were negative for *C. psychrophila.* There were no mortalities or lesions recorded in the control group.

Fig. 3.1. (A) Caudal, dorsal and ventral fin-rot in barramundi infected by *C. johnsonae.* (B) Control fish.

Discussion

Barramundi were infected by *C. johnsonae* 91/0262-10 only when there was a sudden drop in water temperature from 28 to 20°C. Goldfish, guppy and rainbow trout were resistant to *C. johnsonae.* Goldfish and guppy were resistant even at high and low temperatures, i.e. 30 and 15°C. No effect was observed when guppy

were injected by the immunosuppressor (triamcinolone acetonide) and then challenged with the organism. The bacterium was also unable to affect rainbow trout even with the existence of large and deep scars on the saddle sites. The tail rot and caudal lesions observed on two dead fish were probably due to ectoparasites such as *Trichodina* and *Ichthyophthirius*, although Gram stain preparation of the lesions revealed numerous *C. johnsonae* which was confirmed by lesion culture and slide agglutination test. Therefore, this species may be affected by this organism in the presence of predisposing factors such as high temperature and poor water quality.

None of the rainbow trout or goldfish challenged with *C. psychrophila* were affected even three weeks post-challenge. This is not altogether surprising as this isolate was recovered from an instance of low grade fin and tail rot in stressed Atlantic salmon held at 5°C (Schmidtke & Carson, 1995). It is well-established that rainbow trout are susceptible to *C. psychrophila* (e.g. Lorenzen *et al.*, 1991; Austin, 1992; Wiklund *et al.*, 1994) and, therefore, it is probable that the isolate used is of low virulence and only able to produce disease under conditions of stress for the host. Another possibility is that this strain may require a very low temperature, e.g. 4-6°C, to invade the host as the natural infection was reported at 5°C (Schmidtke & Carson, 1995). However, the *in vitro* growth of the organism showed that it grows very slowly at low temperature, 4°C, compared with higher temperature (10-20°C) (Figs. 2.4 & 2.5, Chapter II).

Many factors are involved in predisposing fish to infection with CFLB and, particularly, to the production of morbidity and mortality associated with these organisms. These factors include fluctuation in environmental temperature, overcrowding, immunity of previously exposed fish, species differences, age, size and condition of fish, differences in strain virulence of the pathogen and interaction with other fish diseases (e.g. Fujihara *et al.*, 1971; Holt *et al.*, 1975; 1989; Wakabayashi, 1991).

The effect of temperature and crowding on the resistance or susceptibility to the diseases caused by these organisms have been previously discussed (Pathogenicity and Predisposing factors, Chapter I). The outbreak of natural disease

in barramundi described by Carson *et al.* (1993) was associated with a dramatic drop in water temperature from 35-27°C, a situation not dissimilar to that reported for temperature-mediated immunosuppression in channel catfish (Bly & Clem, 1991). It is probably pertinent that disease could be experimentally produced only in barramundi when they were subjected to a sudden drop in water temperature.

The host non-specific factors such as lysozyme, complement, protease, agglutinins may affect the organisms during the challenge or later. This is investigated in Chapter IV.

The age and size of fish are also related to the pathogenicity of these organisms. As an example, in a study by Fujihara *et al.* (1971) the older and larger rainbow trout and chinook salmon were less susceptible to *F. columnaris* compared to the younger and smaller fish. At similar weights of about 1.25 g, the rainbow trout were resistant to a virulent strain of the bacterium, whereas 90% of the chinook salmon were killed in less than 24 hours. The fish which were used in this study were at the age range of approximately eight months to two years. Such fish have already developed non-specific defence, such as thickening of epithelial layers of skin and scales, and specific immune system with the result that they become more resistant to infections compared to younger and smaller sizes.

The interaction of these organisms with other fish diseases may also be important in pathogenicity of these organisms Fujihara *et al.* (1971). For example, fish ectoparasites or gas bubble disease may have a synergistic effect on the course of the infections and mortality caused by these organisms. The stress induced by the parasites or gas bubble disease, and particularly the presence of ruptured gas blisters or damage in the superficial epithelial layers of the epidermis by the parasites that open "portals of entry", makes the host particularly susceptible to these organisms as in this study it was observed in the case of the infected rainbow trout with the parasites.

In addition, it is important to emphasise that for the experimental reproduction of a disease the use of a virulent bacterium is necessary. There is a wide variation in virulence among the strains of fish-pathogenic CFLB. For example, the virulence of

eight strains of *F. columnaris* obtained from different sources indicated high and moderate virulence strains caused 70-80% and 10-20% infection at the same concentration of 4-6 x 10^6 CFU/ml in loach (*Misgurmus anguillicaudatus*) after three and seven days, respectively post-challenge (Chowdhury, 1993). The low virulence strains affected the fish when the challenge doses were increased by 10-100 times. The virulence of the organism was also studied by Pacha and Ordal (1963; 1970) using yearling sockeye or chinook salmon. High, moderate and intermediate strains resulted in 100% mortalities within 24, 24-48 and 48-96 hours respectively, while low virulence strains were only able to cause 100% mortality over 96 hours.

The environment greatly influences the properties of a pathogen. For example, often one or more of the factors including temperature, growth rate, availability of nutrients, ionic strength/osmolarity, oxygen tension, pH and host factors, such as complement and phagocytes, are important in determining the expression of virulence. Therefore, the growth of a bacterium in a rich nutrient medium in the laboratory is very different from the bacterium in an infection (Poxton & Arbuthnott, 1990). Therefore, the virulence factor of these strains of *C. johnsonae* and *C. psychrophila* used in this study may be affected by *in vitro* conditions or during storage time. However, the organisms used in this study were passaged in the appropriate host prior to challenge in an attempt to restore any virulence properties.

Carson *et al.* (1993) histopathologically demonstrated that fish scales were attacked by the *C. johnsonae* during a natural outbreak. This shows that chitinase and chtinobiase (Sundarraj & Bhat, 1971; 1972) may play an important role in the infections by *C. johnsonae*. None of these chitinobiases is reported for other fish-pathogenic CFLB. There is also no data regarding the effects of other enzymes produced by *C. johnsonae* on the process of infections in fish. These enzymes include extracellular enzymes (endo- lyase (Sundarraj & Bhat, 1971; 1972), end-ß-(1-3)-glucanases, ß-(1-6)-glucanases (Bacon *et al.*, 1970) and endoenzyme, DNase, (Reichenbach, 1989). Unlike other chitinolytic bacteria some strains of *C.*

johnsonae do not liberate/release an extracellular chitinase, but need close contact with the chitin particles to hydrolyse them (Christensen, 1977a).

These studies confirm *C. johnsonae* 91/0262-10 as a potential pathogen of barramundi. Also, they illustrate the difficulty of reproducing diseases caused by CFLB, and the need to use freshly-isolated cultures on fish exposed to environmental conditions similar to those encountered by fish in the wild or held under commercial aquaculture conditions (Ferguson *et al.*, 1991). This is demonstrated experimentally in the following study where a fresh isolate (recently recovered) of *F. columnaris* was used.

3.2. A Study of the Pathogenesis of Diseases Produced by Two *Flexibacter* Species, *Flexibacter maritimus* and *Flexibacter columnaris*

3.2.1. Introduction

Since the original reports of erosive skin disease due to *F. maritimus* in Japanese aquaculture species such as red sea bream, black sea bream, rock bream (*Oplegnathus fasciatus*) and flounder (*Paralichthus olivaceus*) (Baxa *et al.*, 1986; Wakabayashi *et al.*, 1986), infections have been recorded in Dover sole (Bernardet *et al.*, 1990), turbot (Alsina & Blanch, 1993) and more recently sea urchin (*Pseudocentrotus depressus*) (Hamaguchi *et al.*, 1994) in the northern hemisphere.

In the southern hemisphere, *F. maritimus* has been identified as a pathogen of sea-caged Atlantic salmon and rainbow trout and, recently, captured greenback flounder (*Rhombosolea tapirina*), striped trumpeter (*Latris lineata*), yellow-eyed mullet (*Aldrichetta forsteri*) and black bream (*Acanthopagrus butcheri*) in Australia (Schmidtke *et al.*, 1991; B. Munday, pers comm).

A low incidence of ulcerated skin lesions was observed in sea caged salmonids in Tasmania during the first few years following the industry's establishment in 1985. While these early cases were assumed caused by mixed infections of *Flexibacter* and *Vibrio* spp., the first severe outbreak of *F. maritimus* infection as a distinct entity occurred in the summer of 1988-89 (J. Handlinger & J. Carson, pers comm, 1994). During this episode, ulcerated fish were observed virtually simultaneously at most of the marine cage sites in south eastern Tasmania, with distances of up to 30 km between farms. There were also significant numbers of fish showing necrotic gill lesions, although these were more sporadic. The outbreak occurred during an extended period (months) of cloud-free days and water temperatures as high as 21°C. High levels of skin lesions and increased mortalities were observed in individual cages on most farms from late November, through December, and into January, with sporadic cases diagnosed thereafter into July.

During December virtually all cages on some farms were affected to a varying degree. *F. maritimus* was isolated from the surface of lesions in all groups of fish, with variable levels of mixed *Vibrio* spp. also being involved (J. Carson, pers comm, 1993).

In the following summer (1989-90), the incidence of the disease was much lower, with sporadic cases being linked to predisposing traumatic factors, including rough weather, poor handling and bird attack. The number and severity of outbreaks have continued to decline, probably owing to more sophisticated husbandry on the farms, improved treatment and milder weather patterns. Though morbidity varied between individual cages, post-smolts were much more severely affected than fish in their second summer at sea. The disease was also seen in rainbow trout at marine sites.

The incidence of *F. maritimus* have also been recently observed by Dr Barry Munday and the author, not only in salmonids but also in a number of captured non-salmonid species in the National Key Centre for Aquaculture, University of Tasmania. These consisted of greenback flounder, striped trumpeter, yellow-eyed mullet and black bream. Although a comprehensive and detailed bacteriological description of the causative agent has been provided by Schmidtke *et al.* (1991), minimum data is available on the pathogenesis of this disease in fish.

The application of intensive hatchery and cage culture technology for breeding and rearing of barramundi has been associated with an increase in the number of diseases of this species in Australia (Anderson & Norton, 1991). A condition resembling columnaris disease has been reported in barramundi raised in freshwater, and the organism used in this study was isolated in 1993 (Anderson & Norton, 1991; C. Mifsud, pers comm, 1993). Water temperature at the time of outbreaks has ranged from 18-24°C, with only isolated cases occurring at above 24°C.

There were five initial objectives in this study.

(1) Study of pathology due to *F. maritimus* in natural infections.

(2) Successful experimental reproduction of the diseases conditions caused by *F. maritimus* and *F. columnaris* with the evaluation of a number of methods or conditions inclusive.

(3) Assessment of fish susceptibility to the infections by these organisms using a number of species of fish.

(4) Comparison of the macroscopic and microscopic pathology of the produced diseases by the organisms, and between naturally and experimentally infections of *F. maritimus*.

(5) Comparison of virulence level and determination of the lethal concentration dose to achieve 50% (LC_{50}) mortality in population with the consideration of the temporal pattern of the infections in fish.

3.2.2. *Flexibacter maritimus*

3.2.2.1. Field Studies

Materials and Methods

Materials

Natural infection of *F. maritimus* was studied in a number of commercial and captured species of fish. These included:

1. Atlantic salmon and rainbow trout

Fish and specimens from affected fish were submitted to the Fish Health Unit, DPIF from marine salmonid farms in southern Tasmania, especially during 1988 and 1989. Retrospective histopathological examination of selected samples was undertaken by the author in conjunction with Dr Judith Handlinger of the DPIF. Specimens were also collected from infected Atlantic salmon at the Key Centre for Aquaculture, University of Tasmania.

2. Striped trumpeter

Striped trumpeter were wild-caught broodfish. Most fish with lesions were recently captured, but affected fish were likely to be seen after any period of prolonged or traumatic handling.

3. Greenback flounder

Natural infection was noted in commercial batches of fish, especially when being weaned from live (artemia) to artificial diets.

4. Yellow-eyed mullet

Yellow-eyed mullet were 10-40 g body weight obtained by netting from the Brid River at Bridport, Tasmania.

Methods

Initial examination of lesions was by taking smears from the periphery of lesions and staining with Gram stain. Lesion materials were cultured on CA prepared in seawater (SCA); the presence of the bacteria was confirmed by the appearance of the characteristic cell morphology of the organisms and the colonial morphology on the

medium. Biochemical characteristics of the causative agent for the samples submitted to the DPIF was undertaken by Schmidtke *et al.* (1991). For the other samples, the confirmation of the causative agent was undertaken by a slide agglutination test using rabbit raised polyclonal antisera to *F. maritimus* made by Carson *et al.* (1992).

Selected lesions including skin and gill tissues were collected and fixed in 10% formal saline or Bouin's fixative. After trimming, these blocks of tissue were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin.

Results

Bacteriology

In most instances, the infections were confirmed bacteriologically and some of these have been reported previously by Schmidtke *et al.* (1991).

1. Salmonids

1.1. Gross pathology

Lesions occurring more commonly as circular shallow erosions were observed in salmonids most typically on the flank at the point of pectoral fish brushing, but less commonly on the dorsum, as "fin-rot" erosions, or as "gill-rot" erosions of the free ends of one to several primary lamellae which were reduced to white bare exposed cartilages. Skin erosions involving the head as the predominant lesion were seen on two occasions, in which irritant pre-disposing factors leading to cage ramming were identified. Eye lesions were identified on two occasions, and at least once in conjunction with head lesions. Rupture of affected eyes was variable.

Advanced flank or dorsal lesions were most obvious and were similar on all occasions, and in both species, though lesion distribution varied with predisposing factors. These lesions showed shallow erosions up to 5 cm diameter, exposing the white dense dermal connective tissue often with central exposure of muscle, giving a substantially white appearance, with variable incomplete central reddening. Occasional gross lesions of deep extensive muscle reddening were shown histologically and bacteriologically to be mixed infections, with superficial *F. maritimus* and deep secondary vibriosis especially *V. splendidus*.

Early lesions were seldom identified, but those noted during outbreaks included scale loss, or pale sub-epithelial raised blisters up to 1 cm in diameter. Scale loss was often seen at the point of pectoral fin brushing, or at other sites consistent with identified pre-disposing trauma. Raised blisters were seen to progress rapidly to typical erosive lesions.

Gill lesions were more sporadic, and were seldom seen when frank skin trauma was identified as the pre-disposing cause.

Additional gross finding in severely affected fish included petechial haemorrhage of abdominal peritoneum, occasionally inflammation and erosion of the buccal cavity and jaws, some congestion of meninges and peri-orbital tissue, and of the lower intestine, and sanguineous abdominal fluid. Spleens were not enlarged, gill pathology was variable between farms and cages, some having concurrent amoebic gill disease, others being unaffected.

1.2. Histopathology

1.2.1. Skin lesions

Advanced skin lesions generally showed an abrupt marginal cessation of the epithelium, large numbers of long filamentous *F. maritimus* rods formed adherent bacterial mats of varying thickness over the eroded surface, and extended deep into the dense connective tissue layer and associated fibrin. *Flexibacter* were not seen on, within, or under marginal epithelium. *Flexibacter* type rods were never seen within the muscle, though deep bacterial invasion with plump bacterial rods of *Vibrio* or mixed morphology were a common, but variable, finding.

Much of the dense connective tissue layer infiltrated by *Flexibacter* showed necrosis and loss of nuclear detail, with degeneration extending well beyond the area of visible bacteria. However, the degenerate collagen retained much of its gross morphology, serving in most cases as a barrier to deep bacterial invasion.

Exposed or underlying muscle usually showed congestion only, though fibre lysis occurred where little dense layer remained, apparently owing to osmotic effects. A variable acute inflammation was sometimes seen in conjunction with other types of bacteria deep within the lesions. On two occasions filamentous rods had invaded

into the connective tissue of exposed blood vessels within the muscle layer, but not into the surrounding muscle fibres. Similar invasion of vessels was seen occasionally in gills.

Inflammatory reaction was generally negligible in the invaded dense connective tissue layer. Inflammation was more common, but not invariably present, at the residual epithelial margins and in the epidermis and superficial dermis of the early lesions.

Early lesions showed variable scale loss, oedema and inflammation of scale pockets. Small numbers of inflammatory cells were variably seen in the epithelium adjacent to scale pocket inflammation. Those with the gross appearance of blisters generally showed more intense scale pocket oedema, with a more intense inflammation and sometimes haemorrhage only where scales had been lost (Fig. 3.2). Despite these changes, an intact epithelium was often seen even in early lesions associated with known trauma, for example from storms, sometimes even over lost scales, suggesting probable rapid repair. Filamentous bacteria were seldom identified in early lesions, either of scale loss or blisters. Where epithelium remained, *Flexibacter* were only seen in connective tissue or fibrin deposits, where these were directly exposed to the exterior through small fissures. Only in one fish was *Flexibacter* seen within the surface epithelium, and once extending beneath intact epithelium.

In early lesions of some outbreaks in which prolonged sunlight was suspected to be the predisposing factor, intact epithelium over inflamed scale pockets or at the margins of early erosions showed variable changes of intra-epithelial oedema, vacuolation or vesiculation; irregular ragged or occasionally thin and flattened surface; separation from basement membrane or splitting of the epithelium parallel and near to the basement membrane; and variable intra-epithelial pycnotic cells. The latter could not be confirmed as typical of "sunburn cells" as described by Bullock (1988).

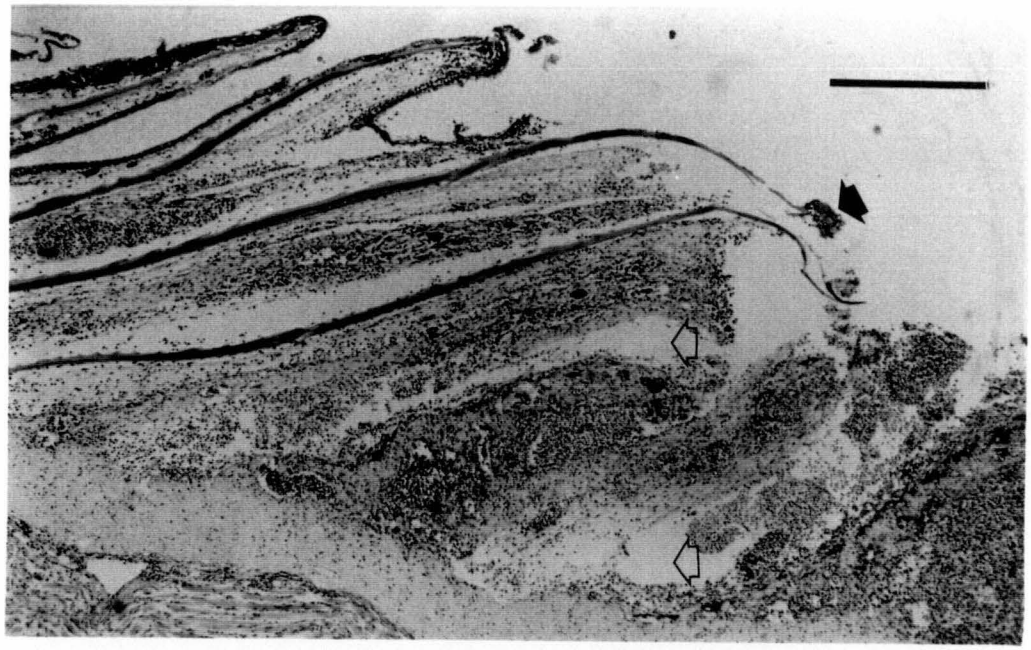


Fig. 3.2. Section through the margin of a blister lesion with early erosion, showing denuded scales with peri-scale oedema and adherent filamentous bacteria (solid arrow), and empty scale pockets (open arrow) surrounded by intense inflammation. (H & E, bar = 0.43 mm)

1.2.2. Head and fin lesions

Head and fin lesions showed erosion to expose superficial bone, with sharp demarcation of the margin of epithelial cover as for flank skin. With fully affected fins showing lesions on both surfaces, there was total necrosis with neither congestion nor inflammatory cells in areas of fin distal to the advancing margins, but where only one surface of the fin was affected, a variably intense inflammation of the deep structures was seen. *Flexibacter* mats over necrotic surfaces were as for flank skin lesions.

1.2.3. Gill lesions

All gill lesions were erosive in character, with total necrosis distal to heavy encroaching filamentous bacterial mats (Fig. 3.3). There was usually minimal reaction of affected lamellae except in a small zone of congestion deep to the bacterial masses, suggesting rapid expansion exceeding the ability of the host to mobilise responses to tissue damage or bacteria. Adjacent lamellae showed either minimal reaction or a very marked acute inflammation.

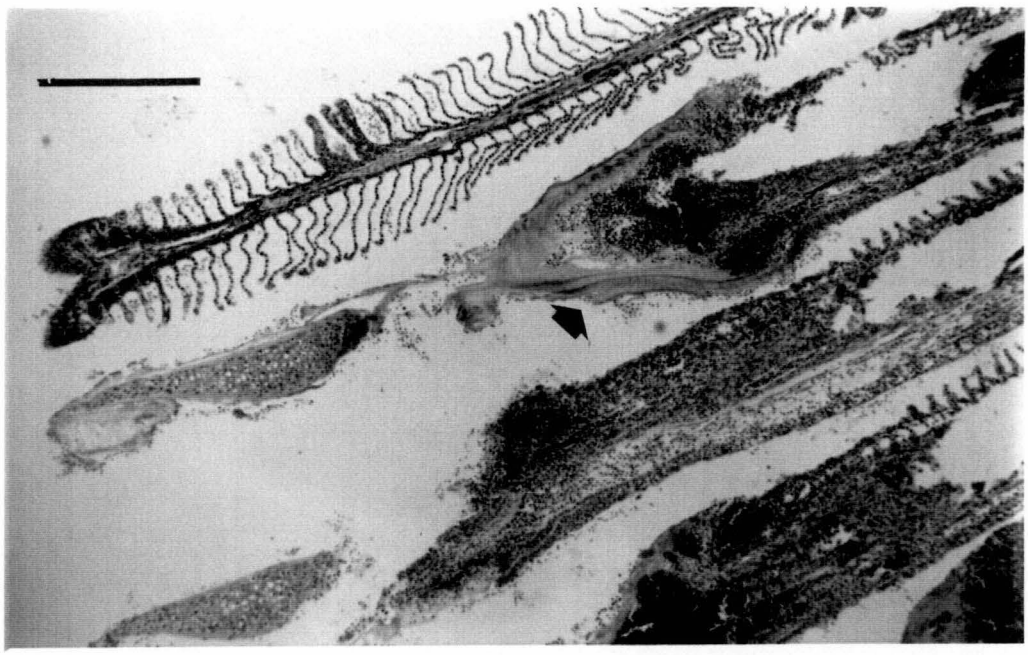


Fig. 3.3. Gill section from a natural infection, showing an eroded primary lamella with adherent filamentous bacteria (arrow). (H & E, bar = 0.43 mm)

Occasional lesions showed *Flexibacter* in association with overwhelming acute inflammation with oedema, marked congestion, and large number of polymorph leucocytes. Often such cases were associated with other insults such as jelly fish stings or lightning flashes, suggesting the *F. maritimus* infection may have been secondary to, rather than the cause of, the intense inflammation. Indeed, occasionally very small filamentous mats were seen in small surface fibrin aggregates of intense acute inflammatory lesions, but it was not possible to determine if these were at the edge of much larger lesions.

1.2.4. Eye lesions

Outbreaks of affected eyes were seen on only two occasions, both with filamentous rods within the cornea of eyes with corneal epithelial erosion and choroid congestion, sub-choroid haemorrhage, or complete eye collapse and corneal rupture. *Flexibacter* cells were also sporadically seen at margins of ruptured eyes where rupture was suspected to be due to other causes.

2. Striped trumpeter

2.1. Gross pathology

Lesions in striped trumpeter were usually associated with sites of trauma e.g. caudal peduncle owing to manual restraint at this point during capture. However, all fins and body surfaces were found to be affected and nercotising branchialitis occurred in some instances.

The skin lesions usually commenced as small areas of redding and scale loss with rapid progression to ulceration and, finally, the presence of large (up to 70 mm in length) ulcers exposing the underlying muscle and having a pale periphery of eroded skin (Fig. 3.4).

Fin lesions were essentially erosive leading to raggedness and, in some instances, complete loss of individual fins or tail elements.

The gill lesions were typical of *Flexibacter* infection being aggressively necrotising.

2.2. Histopathology

Histologically, the early lesions in striped trumpeter were similar to those in salmonids, but more advanced cases were invaded by *Uronema*-like ciliates which provoked an intense inflammatory reaction. The parasites invaded between, and into, the muscles causing myolysis and provoking a pseudograulomatous reaction dominated by macrophages with granular cytoplasm.

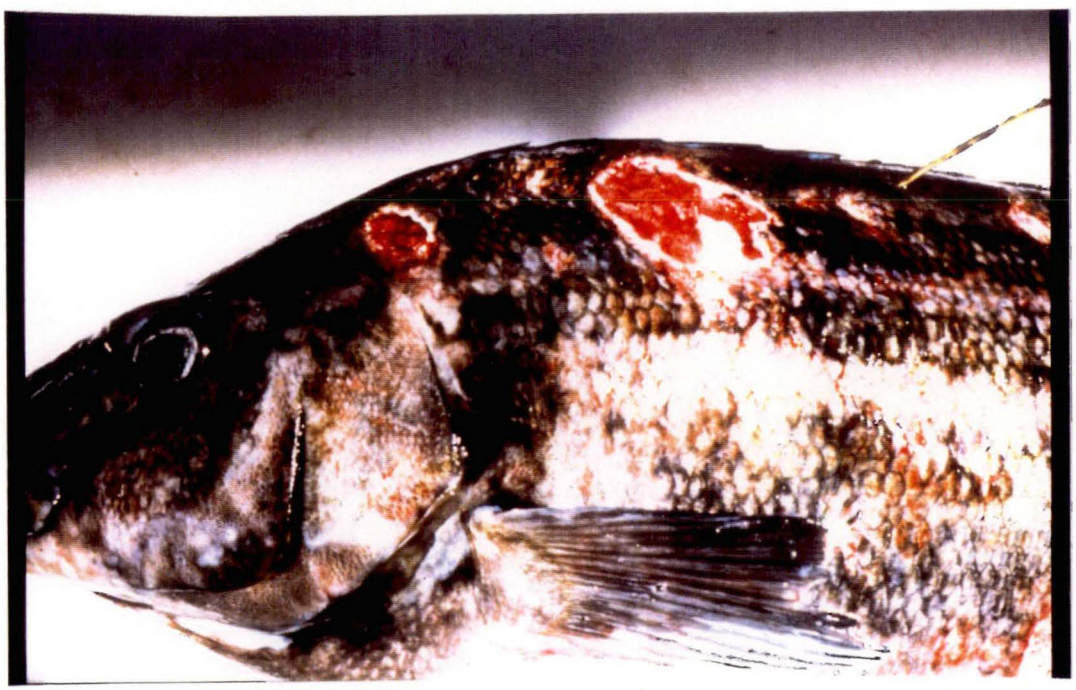


Fig. 3.4. Infected striped trumpeter showing head and trunk lesions caused by *F. maritimus*.

3. Greenback flounder

3.1. Gross pathology

Infected flounder were relatively mildly affected, exhibiting mild to moderate erosion of fin and tail margins with no lesion on the head and trunk. The eroded edges of these elements were blanched.

3.2. Histophathology

Histological lesions were quite muted. Affected areas of epithelium were eroded and filamentous bacteria were adherent to the damaged surface in numbers varying from occasional to dense mats. There was practically no discernible inflammatory response to the presence of the bacteria.

4. Yellow-eyed mullet

4.1. Gross pathology

Gross pathology in mullet consisted of melanosis of skin, haemorrhagic spots on the base of fins, and on the jaws, mouth and head erosions, tail rot, and small pale to somewhat redded blisters on the lateral, pectoral and ventral sites. Loss of scales at variable sites of the body, in particular the pectoral and lateral sites was observed. Observation of internal organs did not show any particular gross pathology.

4.2. Histopathology

Histological changes due to *F. maritimus* in naturally infected mullet were similar to those of early lesions in salmonids. A mild and low degree of inflammatory response in epithelial layers was evident in some sections, while condensed connective tissue and scales were intact. There was mild congestion in soft connective tissues. Fragmentation and degeneration of superficial epithelial layers were seen. In some samples amorphous granular protein-like materials occupied the fragmented epithelial fissures and empty scale pockets. Filamentous bacteria were seldom observed in sections, although, the bacteriological work confirmed the infection.

3.2.2.2. Experimental Studies

Materials and methods

1. Materials

1.1. Cultures

Frozen ampoules of *F. maritimus* 89/4762 (Chapter II) originally isolated from infected Atlantic salmon in southern Tasmania (Schmidtke *et al.*, 1991) were used. The bacterium was passaged twice in Atlantic salmon by bath immersion at 1 x 10^8 cells/ml to enhance the virulent factor prior to challenge. The passaged strain was used for Trials 4, 5, 6, 7 & 8 (Table 3.2).

1.2. Fish stocks

Atlantic salmon 20-50 g body weight and 150-300 g body weight, greenback flounder 10-25 g body weight, goldfish 5-30 g body weight and rainbow trout 50-140 g body weight were used in this study. Fish were maintained at the National Key Centre for Aquaculture, University of Tasmania using a re-circulation with biofiltration system, aeration and a temperature of 24-26°C, 12-15°C and 8°C for goldfish, salmonids and flounder, respectively. Fish were acclimatised to seawater (Table 3.2) by increasing 4-5‰ seawater each day interval, and they were then held in seawater 3-4 weeks prior to challenge. Fish were fed twice daily using Tropical Fish Crumble or Salmon Feed (Gibson's Feed Mill). Yellow-eyed mullet 10-40 g body weight, which were naturally infected with *F. maritimus*, were obtained from Brid River at Bridport, Tasmania, and they were used for the direct transmission of the infection (Trial 9, Table 3.2).

2. Methods

2.1. Inoculum preparation

Inoculums were prepared according to the method described by Carson *et al.* (1992). Bacteria were grown on SCA at 25°C for up to four days. Subcultures for challenge were inoculated into 1000-2500 ml volumes of CB prepared with seawater in 2-3 litre conical flasks. The cultures were grown at 22 or 25° for 48-96 hours with or without

agitation using an orbital shaker (30-40 rmp). The cells were counted in triplicate using the spread plate method on SCA, and cell concentrations for challenge were estimated based on viable count (CFU/ml of water tank), except for Trials 1 and 2 (Table 3.2), which the cell numbers were estimated microscopically using a Helber counting chamber. Broth culture or harvested cells in phosphate buffered saline (PBS) were used for parenteral inoculation. Culture purity tests included the use of wet mount and Gram stain preparations and subcultures on SCA to check for contamination of the cultures prior to challenge.

2.2. Methods of inoculum

Challenge methods are given in Table 3.2. Control groups were kept in separated tanks with separated biofilter system.

Table 3.2. Methods used to assess pathogenicity of *F. maritimus* in a number of fish species.

No	Method of challenge	Cell no. as CFU	Water temp. °C and (salinity ‰)	Fish density g/l and (average weight g)	Fish species	No. fish and (No.rep.)
1	Bath	2.5 x 10^6/ml	18 (35)	0.8 (35) 0.8 (35)	Atlantic salmon Atlantic salmon	83(1) 83(1)
2	Bath	2.5 x 10^6/ml	18 (35)	3.2 (225)	Atlantic salmon	50 (2)
3	Bath	5.3 x 10^7/ml	20 (15)	2.9 (35) 1.5 (17.5) 1.5 (17.5)	Atlantic salmon Flounder Goldfish	25 (2) 25 (2) 25 (2)
4	Bath	1.8 x 10^3- 1.6 x 10^7/ml	18 (35)	1.4 (35) 3.8 (95)	Atlantic salmon Rainbow trout	12 (2) 12 (2)
5	Bath	2.3 x 10^6/ml 4.7 x 10^6/ml	15-16 (35)	2.3 (35) 7.3 (95)	Atlantic salmon Rainbow trout	20 (2) 23 (2)
6	Bath	5.6 x 10^6	16	1.8 (35)	Atlantic salmon	15 (2)
7	I.M. injection	2.3x10^6/fish	18 (35)	1.2 (35)	Atlantic salmon	10 (2)
8	I.P. injection	2.3x10^6/fish	18 (35)	1.2 (35)	Atlantic salmon	10 (2)
9	Scarification & swabbing	-	18 (35)	1 (35)	Atlantic salmon	100(1)

◀— Legend shown on page opposite (71a)

2.3. Collection and processing of samples

2.3.1. Bacteriology

Bacteriological techniques of experimental studies were similar to those utilised for natural infections as previously described (3.2.2.1). Samples from lesion materials were cultured on SCA supplemented by 4 µg/ml of oxolinic acid at 25°C for up to five days. In some instances the slide agglutination test was also used as a confirmatory test.

2.3.2. Detection of the bacterium by indirect immunofluorescent antibody test (IFAT) in infected salmonids

IFAT was used as a confirmatory test for *F. maritimus* and was compared with wet mount, Gram smears and lesion cultures. For this purpose, the smears (10 x 10 mm) from the edge of skin lesions and gills of 30 infected fish (15 Atlantic salmon and 15 rainbow trout) were taken at random. By plate culture, isolations were made simultaneously from the same organ sites used for the smear preparations and cultured on fresh SCA at 24°C for up to five days. The technique of indirect IFAT described by Carson *et al.* (1992) was used. The smears were air dried, overlaid by 40 µl of rabbit antiserum 89/0329-5 of *F. maritimus* diluted 1:100 in PBS (pH 7.2 0.1 M) incubated in a moist chamber at 37°C for 30 minutes, and rinsed for 15 minutes in PBS. Following the removal of the excess buffer by blotting the slides, 20 µl of anti-rabbit FITC conjugate (Silenus Laboratories) diluted 1: 60 in PBS was added to each sector of the slide. Smears were incubated at 37°C for 30 minutes and rinsed for 30 minutes in PBS. Smears were then blotted to remove the excess buffer. Smears were mounted using alkaline glycerol buffer ($NaHCO_3$ 0.0729g, Na_2CO_3 0.016 g, distilled water 10 ml, glycerol 90 ml, pH 9.0), coverslipped and were examined at x 40 magnification with epifluorescent microscopy (Nikon) using UV illumination.

2.3.3. Gross pathology

Clinical signs and mortality were checked daily. Lesion distribution, and lesion size were recorded. The distribution of lesions on the body of fish were recorded in fish of

trials one, two, three and five (Table 3.2), and lesion size was measured using a metric ruler in fish of trial one.

2.3.4. Histopathology

Histological techniques of experimental studies were similar to those utilised for field studies as previously described (3.2.2.1). In addition, Geimsa and Gram stains (Vacca, 1985; Stevens, 1990) were used for staining the selected lesions. At the end of the trials, the fish were anaesthetised by carbon dioxide and appropriate samples were collected from survivors.

2.3.5. Serology

To monitor whether the infected animals develop a specific immune response or not, blood samples were taken from surviving Atlantic salmon (Trial 1, Table 3.2), left at 37°C for one hour, transferred at 4°C overnight and sera separated, which were then kept at -20°C until tested by the slide agglutination test. One drop of serum was put on the clean slide and one drop of antigen homogenised in PBS (10^6 cells/ml of 24-hour cultured *F. maritimus*) was added. The slide was rotated gently for 2-3 minutes and closely watched for the evidence of an agglutination. The sera of 20 uninfected Atlantic salmon were taken as a control group. *F. columnaris* 1468, *C. johnsonae* 91/0262 and *Vibrio anguillarum* 85-3954-1 (DPIF) were also included to check any cross reaction.

2.4. Statistical analysis

Data were analysed by Student t-test. Pairwise comparisons of means were conducted using Fisher's Least Significant Difference (LSD) test.

Results

1. Bacteriology

The results of all smears taken for Gram stain resulted in Gram negative, slender, flexuous, rods with a dimension of 2-6 x 0.4 µm (occasionally longer to 30 µm) (Fig. 3.5). In general, over 60% of the plate cultures revealed concave, spreading, white to pale yellow colonies with an irregular edge and relatively sticky. These colonies appeared after two days at 25°C and developed on the surface of the plates within

seven days. The slide agglutination resulted in a positive reaction using specific antisera against *F. maritimus*.

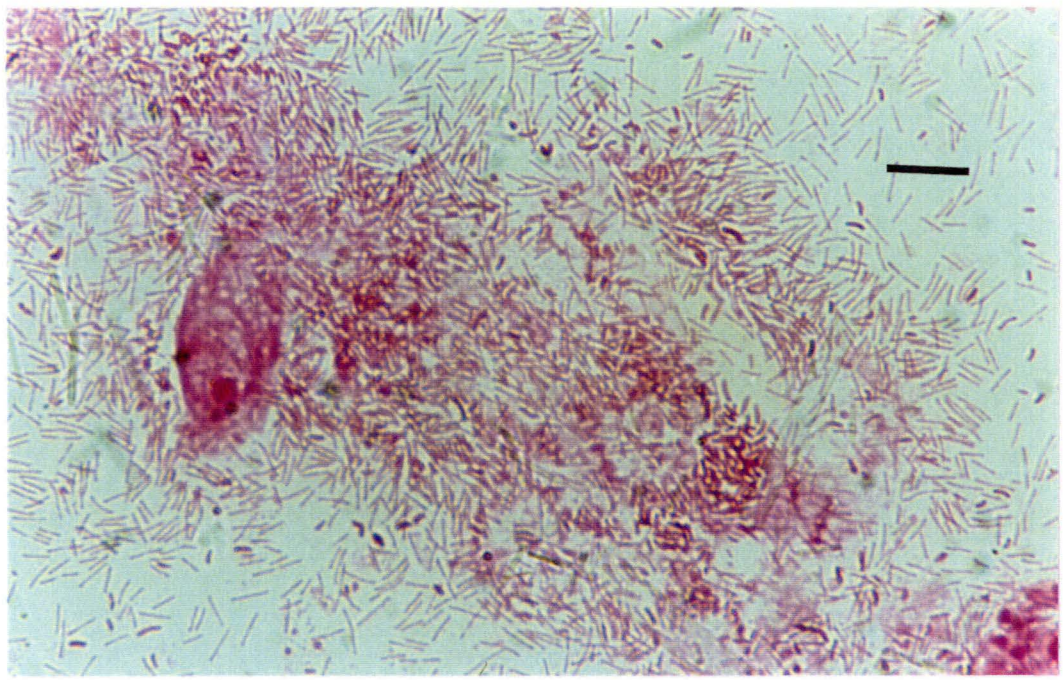
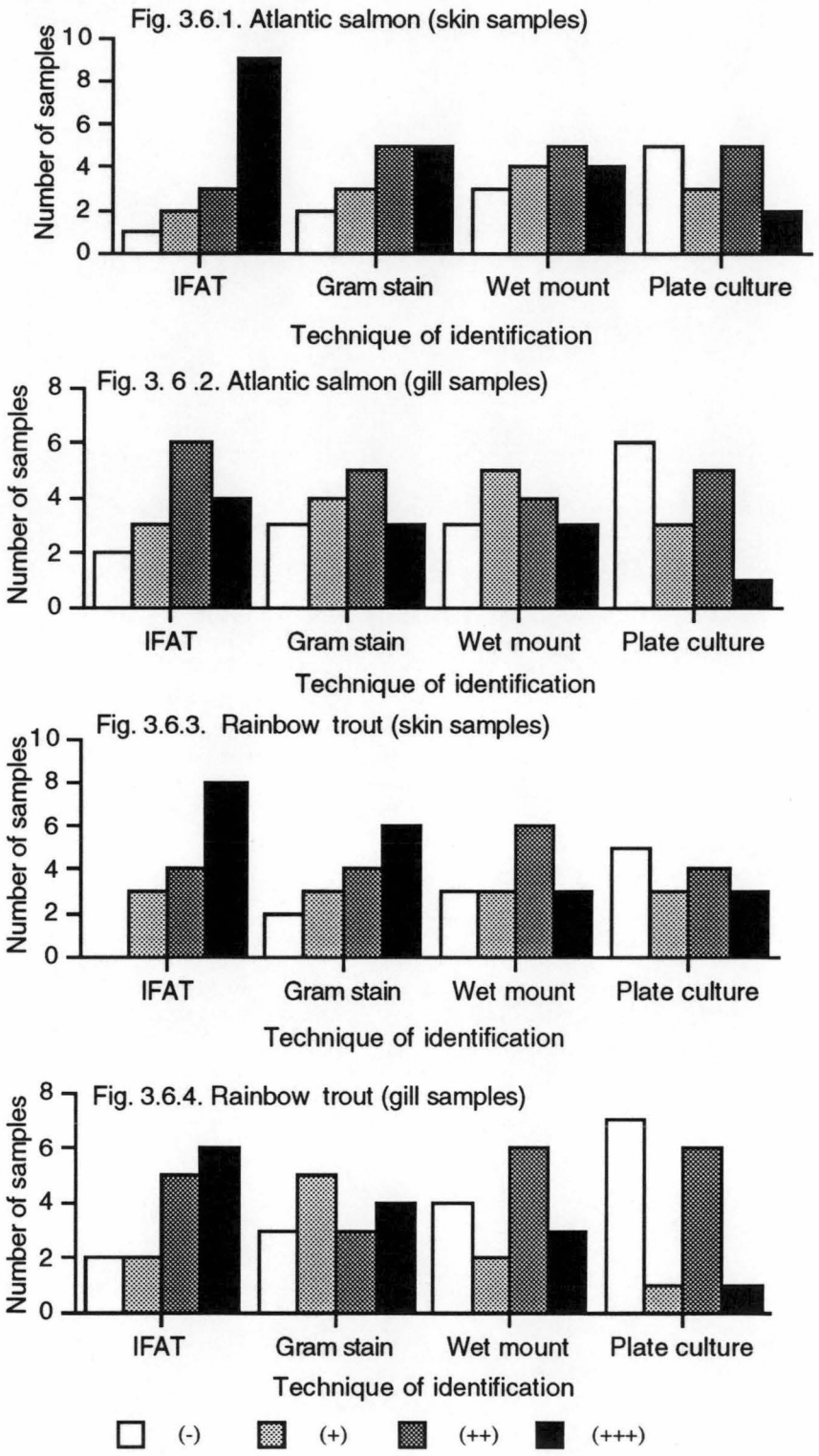


Fig. 3.5. Gram smear from the lesions of infected Atalantic salmon with *F. maritimus*. (Bar = 8 μm).

2. Detection of *F. maritimus* by IFAT

As data shown in Figure 3.6, bacterial identification by IFAT was more sensitive than other methods with two-third of smears diagnosed as positive for *F. maritimus* (Fig. 3.7). Plate cultures showed less sensitivity than Gram smears and wet mount slides indicating a suppression of *F. maritimus* by other opportunist organisms on the plate agar. Gram stain was relatively more applicable as an assumptive identification procedure than wet mount preparations. Detection value in both species of fish was similar, but bacterial detection was more successful in skin samples than gill samples.

Fig. 3.6. Comparison of IFAT, media culture, Gram stain and wet mount preparations for detection of *F. maritimus* in Atlantic salmon (15 samples) and rainbow trout (15 samples) challenged with the bacteria by bath immersion.



(-): No positive bacterium or growth, (+): Positive bacterium per field or a few *F. maritimus* colonies per plate, (++): More than one but less than ten positive bacteria per field or distinct *F. maritimus* colonies per plate, (+++): More than ten bacteria per field or numerous *F. maritimus* colonies per plate. Identity of colonies was confirmed by slide agglutination test against anti-*F. maritimus* serum.

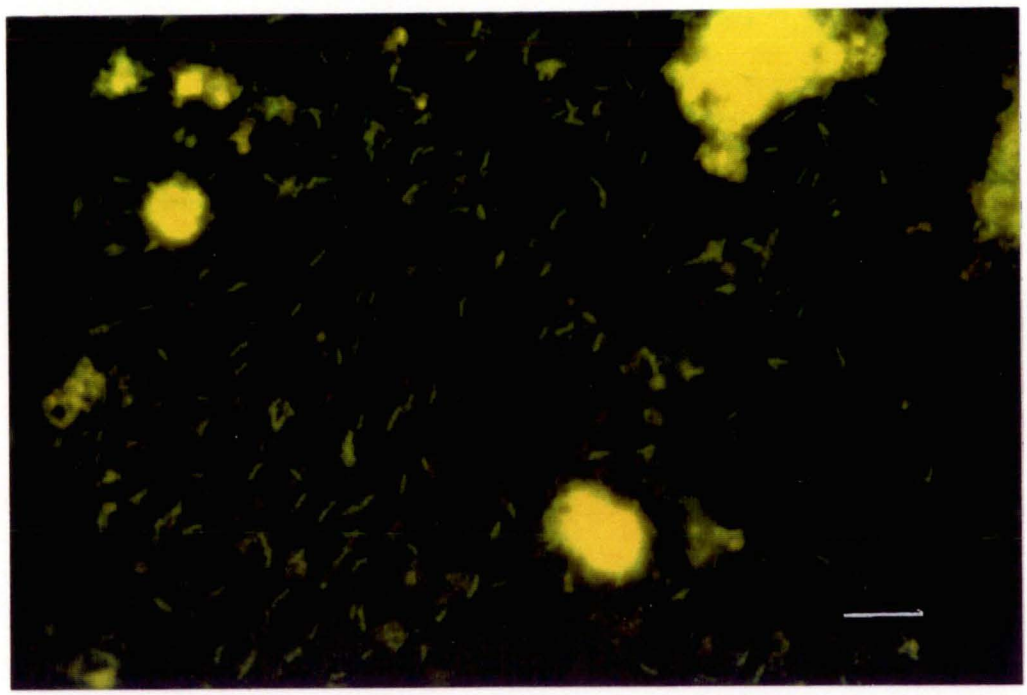


Fig. 3.7. An IFA staining of smear obtained from a lesion of an experimentally infected Atlantic salmon with *F. maritimus*. Note the smears obtained from the same lesion site for wet mount and plate culture gave negative results, and for Gram stain, a small numbers of *Flexibacter*-type filamentous Gram negatives were detected. (Bar = 17 μm).

3. Serology

The sera from 30 of the surviving fish were positive using the rapid slide agglutination test, while 45 fish showed a negative reaction. A control test, which included the sera of 20 healthy Atlantic salmon (approximately the same age as the test group) were examined to avoid mistakes in reading the results of the test and all these proved negative. No cross-reaction was observed with *F. columnaris* , *C. johnsonae.* and *V. anguillarum.*

4. Mortality and gross pathology

In general, clinical signs of experimental induction of disease by *F. maritimus* was similar in Atlantic salmon, rainbow trout and flounder and were similar to those described for natural infections.

In all trials fish became anorexic within one to three days after exposure. Fish developed melanosis and showed flashing swimming behaviour. The loss of scales at different sites of body in particular at the pectoral and lateral areas was evident.

Congestion and haemorrhagic spots appeared on the jaws and fins especially on the base of pectoral and ventral fins. Individual details for each trial were as follows:

4.1. Atlantic salmon (Trial 1)

Shaken culture

Mortalities commenced one week post challenge, and reached 7.2% within two weeks of exposure. The gross pathology of these fish included, skin erosion around the jaw and at sites adjacent to the fins, congestion and haemorrhagic spots on the jaw and skin, erosion and hyperaemia of the fins and tail rot. The largest lesions (0.3-6 x 0.5-3.5 cm) occurred on the ventral surfaces. Of the survivors, a third of the fish showed macroscopic lesions with the largest lesions found on the jaws (0.5 x 0.5-1 cm) and the ventral fins (0.5-1 x 0.5 cm). Most fish (both mortalities and survivors) displayed lesions on jaws (12%), pelvic fins (15.6%) as well as tail rot (14.5%), while the smallest lesions occurred on the dorsum (1.2%), caudal sites and head (3.6%), and on the pectoral fins and lateral sites (4.8%). The characteristics of lesions were similar to those described for natural infections. Gill-rot was observed in two (2.4%) cases of the infected fish.

Static culture

The infection was very similar to that of the shaken culture group, but with a lower mortality level of 6% which occurred within two weeks following exposure.

4.2. Effect of stocking density and fish size on the course of infection (Atlantic salmon, Trial 2)

Trial 2 was carried out to compare the effect of fish size, fish weight and fish density with Trial 1. Atlantic salmon challenged in this trial were six times in weight, approximately 2.5 times in size and about one year older than fish used in Trial 1. Fish density in this trial was also four times that of Trial 1.

Clinically the infection condition in this trial was almost similar to that of Trial 1. However, development of infection and mortality level were less than Trial 1. Mortality levels of 2 and 4% occurred in the different replicates. Of the survivors,

one-fourth of the fish in each replicate showed macroscopic lesions indicating larger animals were more resistant to infection than smaller ones.

4.3. Effect of salinity on the course of infection (Atlantic salmon, flounder and goldfish, Trial 3)

Atlantic salmon and flounder were challenged with *F. maritimus* to assess the course of infection at a low level salinity (15‰) as a relatively minimum salinity required for growth of bacterium *in vitro* condition (Chapter II). Goldfish was used to evaluate whether the bacterium is able to affect some freshwater species.

Atlantic salmon had a mortality level of 10% for each replicate two weeks after challenge. Of the survivors, 20% showed lesions, haemorrhagic spots, congestion and/ or hyperaemia on the skin or the fins. The gross signs and the type of lesions were similar to those described in first trial.

A total mortality level of 2% occurred in flounder. No gross signs of disease were seen in the survivors. In comparison with natural infection, the disease in experimentally-infected flounder was somewhat more severe with a greater degree of "fin-rot" with congestion and haemorrhage at the bases of the fins. This difference may have resulted from the relatively elevated water temperature (20°C) used in the experiment.

There was no mortality or lesion in goldfish challenged with organism at this salinity.

4.4. Determination of the lethal concentration dose to achieve 50% (LC_{50}) mortality in population and temporal pattern of infection in salmonids (Trial 4)

The lethal concentration dose (LC_{50}), temporal pattern of infection and comparison of the susceptible level between Atlantic salmon and rainbow trout were evaluated in this trial. Results are shown in Table 3.3. In general the temporal pattern of infection caused by *F. maritimus* was similar in both salmonid species (Fig. 3.8). Both species showed a relatively similar level of susceptibility to infection with a slightly higher level of resistance indicated by rainbow trout (Fig. 3.9). However, there was no

significant difference between the susceptibility level of salmonids ($P > 0.05$). A bacterial concentration of 2.3×10^5 and 1.6×10^6 CFU/ml was required to achieve 50% mortality in Atlantic salmon and rainbow trout populations, respectively indicating a higher concentration of about 10 times required to achieve the LC_{50} in rainbow trout. However, a mortality level of 100% occurred in both species when they were challenged with higher bacterial concentration of 1.6×10^7 CFU/ml.

The temporal pattern of infection in both species showed that virulent strains of *F. maritimus* are able to cause early mortality in susceptible species three days following challenge at concentrations 2.3×10^5-1.6×10^7 CFU/ml, while the development of lesions was prolonged, up to one week, when fish were subjected to lower concentrations. Cutaneous erosions were superficial in early mortality, with some fish showing white blisters on the skin. In late mortality, erosions developed into eroded ulcers.

Table 3.3. Determination of LC_{50} in salmonids by bath immersion of *F. maritimus* over 10 days post-infection.

Fish species	Dose CFU/ml	Rep.	No. of dead fish	Mortality per replicate(%)	Mean of total mortality (%) ± SD
Atlantic salmon	1.8×10^3	1	2	16.7	12.5 ± 5.9
		2	1	8.3	
Rainbow trout		1	0	0	4.15 ± 5.9
		2	1	8.3	
Atlantic salmon	2.3×10^4	1	4	33.3	33.3 ± 0.0
		2	4	33.3	
Rainbow trout		1	3	25	29.15 ± 5.9
		2	4	33.3	
Atlantic salmon	2.3×10^5	1	6	50	45.9 ± 5.9
		2	5	41.7	
Rainbow trout		1	3	25	33.3 ± 11.8
		2	5	41.7	
Atlantic salmon	1.6×10^6	1	10	83.3	74.95 ± 11.8
		2	8	66.7	
Rainbow trout		1	7	58.3	50 ± 11.8
		2	5	41.7	
Atlantic salmon	1.6×10^7	1	12	100	100 ± 0.0
		2	12	100	
Rainbow trout		1	11	91.7	95.85 ± 5.8
		2	12	100	

Each replicate originally contained 12 fish.

Fig. 3.8 Percentage survival of Atlantic salmon and rainbow trout subjected to various concentrations of *F. maritimus*. (Mean ± SD, n = 2 replicates of 12 fish)

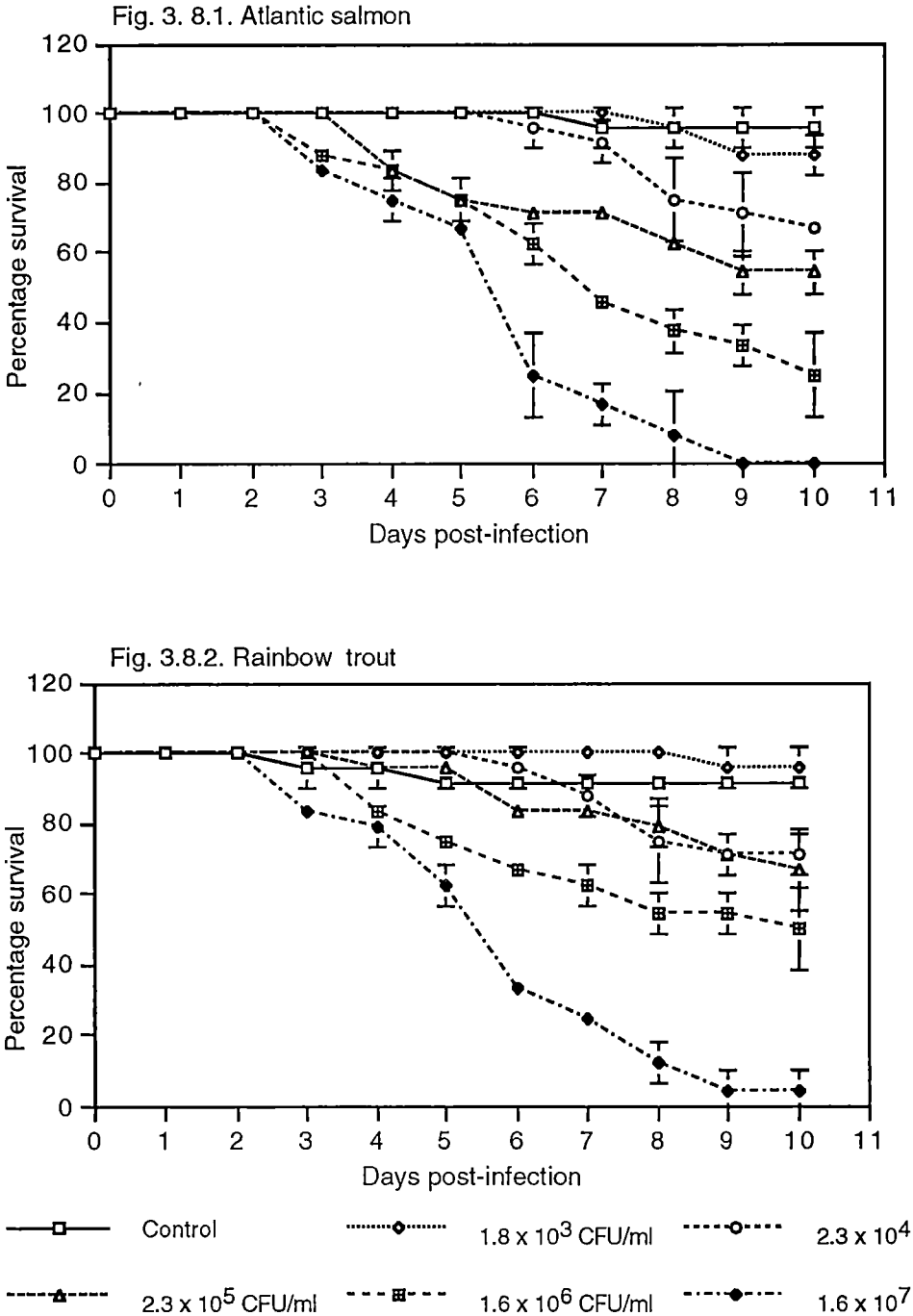
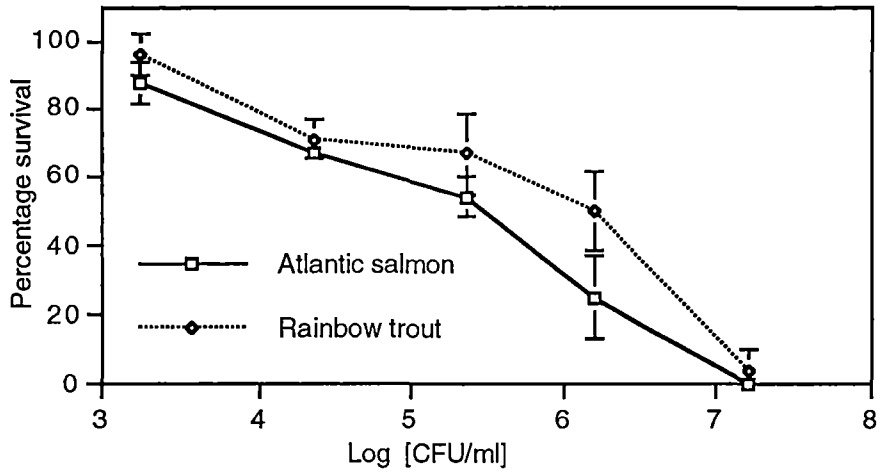


Fig. 3.9. Comparison of percentage survival of Atlantic salmon and rainbow trout exposed to *F. maritimus* infection over 10 days post-infection. (Mean ± SD, n= 2 replicates of 12 fish).



4.5. Comparison of distribution of lesions between Atlantic salmon and rainbow trout (Trial 5)

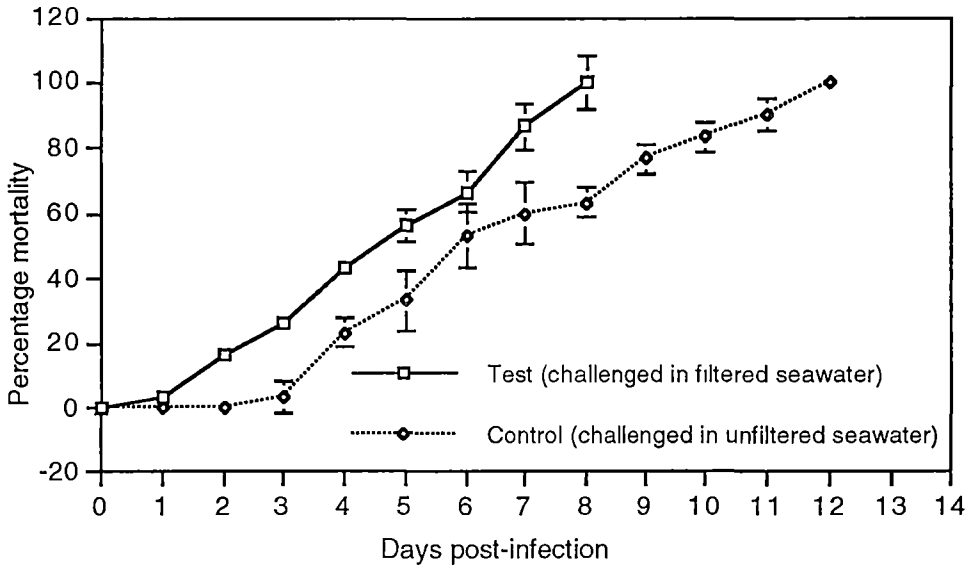
Mortality levels of 100 and 95% (with mean of total mortality equal to 97.5 ± 2.5%) and 87 and 82.7% (with mean of total mortality equal to 84.8 ± 2.18%) occurred in Atlantic salmon and rainbow trout, respectively. The distribution of lesions in rainbow trout was similar to those of Atlantic salmon (Table 3.4, Fig. 3.11). However, the lesions were more extensive in trout, especially in that they showed pale white blisters of 0.5-1 x 0.5-1 cm on caudal, lateral or pectoral sites as well as at the bases of fins (Fig. 3.12) Such blisters rapidly developed into typical lesions and observations revealed that these fish could survive for up to 48 hours after becoming infected.

4.6. Effect of predictive competitive bacteria on the course of infection (Atlantic salmon, Trial 6)

This trial was undertaken to investigate whether the normal microflora in natural seawater influenced the progress of infection and the gross pathology. All Atlantic salmon challenged with *F. maritimus* in filtered seawater (test group) and unfiltered seawater (control group) died by the time that the the experiment was terminated (Fig. 3.10). The process of infection was found to be faster in the test group and 100% mortality was reached four days earlier than in the control group. Fish in the

test group showed signs of infection one day post-challenge, while lesions in control groups appeared three days later. Clinically, lesions in the test group were more superficial, uniform in shape (circular) and smaller in size than lesions in the control fish, but distribution of the lesions in both groups were mainly similar

Fig. 3.10. Comparison of the progress of *F. maritimus* infection in Atlantic salmon exposed to the bacteria in filtered seawater and unfiltered seawater. (Mean ± SD, n = 2 replicates of 15 fish).



4.7. Evaluation of the parenteral routes (IP and IM) of disease induction (Atlantic salmon, Trials 7 & 8)

No infection occurred in fish injected IP or IM by *F. maritimus*. However, 20% fish injected IM developed small lesions on the site of injection. Gram smears of the lesions showed that the organisms were morphologically similar to *F. maritimus*, in most instancies mixed with some Gram negative rods. *F. maritimus*, however, was not isolated from these lesion cultures probably because of overcrowding by opportunistic bacteria in the culture media. There were no abnormalities in internal organs, except that some IP injected fish showed a low degree of hyperaemia/congestion in the peritoneal cavity and on the liver surface.

4.8. Direct transmission of infection (Atlantic salmon, Trial 9)

Direct transmission of mullet strain of *F. maritimus* did not affect Atlantic salmon even after scraping the skin. Fish did not show any lesions or mortality at the termination of the experiment.

5. Distribution of lesions

The distribution of lesions due to *F. maritimus* are shown in Table 3.4 and Figure 3.11. The following points were noted:

(1) Generally, the pattern of lesions distribution was similar in all experiments and with the two species of salmonids.

(2) The incidence of dorsal lesions and gill-rot were minimum, while lateral and pectoral lesions, pectoral and pelvic fin-rot, tail-rot, jaw erosions and haemorrhage or congestion spots were predominant in all experiments.

(3) The incidence of lesions was higher when the virulent strain (passaged strain) of *F. maritimus* was used for challenge (Trials 4 & 5).

(4) Outbreak of lesions was higher in the smaller size of Atlantic salmon (Trial 1) than in the larger size ones (Trial 2).

(5) Lesions were fewer and less severe when the Atlantic salmon were challenged at 15‰ of seawater (Trial 3).

(6) The pattern and outbreak of lesions in both species of salmonids were similar (Trials 4 & 5).

Table 3.4. Distribution of lesions in Atlantic salmon and rainbow trout experimentally infected by *F. maritimus* at different conditions of fish density, fish size (age) and salinity.

Fish species	Replicate	Pectoral site	Head	Jaws	Lateral site	Caudal site	Dorsum	Ventral site	Pectoral fin rot	Pelvic fin rot	Tail rot	Gill rot	Haemorrhage /hyperaemia /congestion
Atlantic	1 (a)	5(6)*	3(3.6)	10(12)	4(4.8)	3(3.6)	1(1.2)	3(3.6)	4(4.8)	13(15.6)	12(14.5)	2(2.4)	13(15.6)
salmon(1)	2 (b)	4(4.8)	2(2.4)	11(13.2)	3(3.6)	2(2.4)	0.0	1(1.2)	5(4.8)	10(12)	8(9.6)	1(1.2)	11(13.3)
Atlantic	1	3(6)	1(2)	8(16)	2(4)	3(6)	1(2)	2(4)	3(6)	8(16)	7(14)	0.0	8(16)
salmon(2)	2	3(6)	2(4)	7(14)	3(6)	2(4)	0.0	0.0	4(8)	10(20)	6(12)	1(2)	7(14)
Atlantic	1	4(16)	1(4)	5(20)	4(16)	2(8)	0.0	2(8)	2(8)	2(8)	2(8)	1(4)	2(8)
salmon(3)	2	3(12)	1(4)	4(16)	2(8)	3(12)	1(4)	4(16)	5(20)	4(16)	3(12)	0.0	4(16)
Atlantic	1	13(65)	2(10)	8(40)	16(80)	11(55)	3(15)	10(50)	9(45)	10(50)	7(35)	4(20)	12(60)
salmon(4)	2	11(55)	3(15)	9(45)	14(70)	13(65)	5(25)	7(35)	12(60)	8(40)	10(50)	6(30)	9(45)
Rainbow	1	11(47.8)	1(4.35)	3(13.1)	10(43.5)	11(47.8)	0.0	5(21.7)	9(39.1)	4(17.4)	13(56.5)	2(8.7)	5(21.7)
trout(5)	2	9(39.1)	2(8.7)	4(17.4)	13(56.5)	9(39.1)	2(8.7)	5(21.7)	10(43.5)	3(13)	11(47.8)	4(17.4)	3(13)

*: Indicating percentage. (a): Shaken culture and (b) static culture were used. (1): Trial 1 in Table 3.2. (2): Trial 2 in Table 3.2. (3): Trial 3 in Table 3.2. (4) and (5): Trial 5 in Table 3.2.

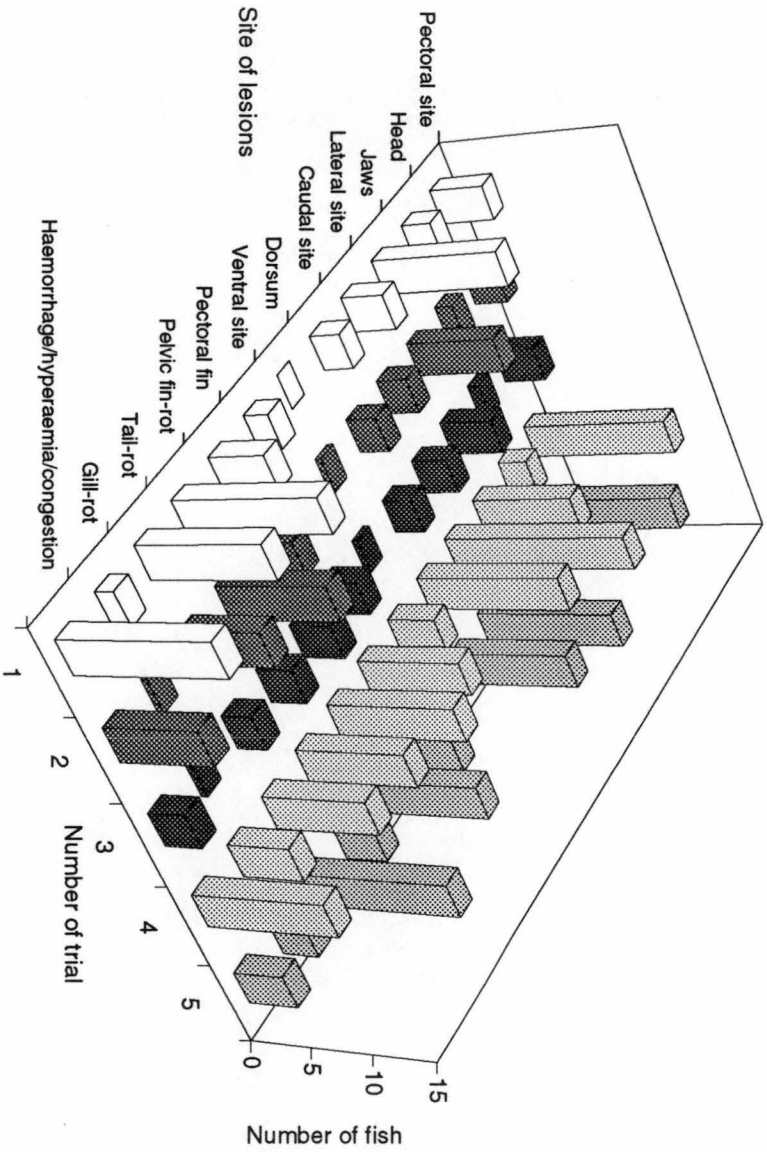
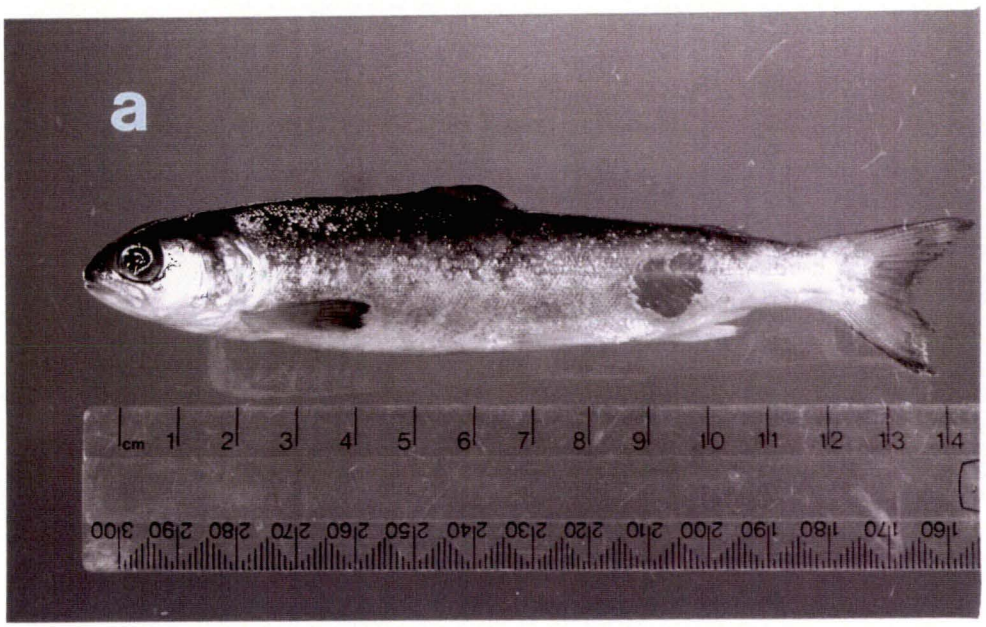


Fig. 3.11. Distribution of lesions in Atlantic salmon (Trials 1 to 4) and rainbow trout (Trial 5) experimentally infected with F. maritimus. (Mean +/- SE= 0.0-1.5, n = 2 replicates).

Fig. 3.12. Classic lesion of salmonid cutaneous erosion disease caused by *F. maritimus* in experimentally infected Atlantic salmon and rainbow trout. (a) Atlantic salmon with lateral lesion and (b) gill-erosion (arrow); (c) Rainbow trout with early erosion showing white blisters on the pectoral (solid arrow), lateral and dorsal (open arrow) and (d) tail erosion.

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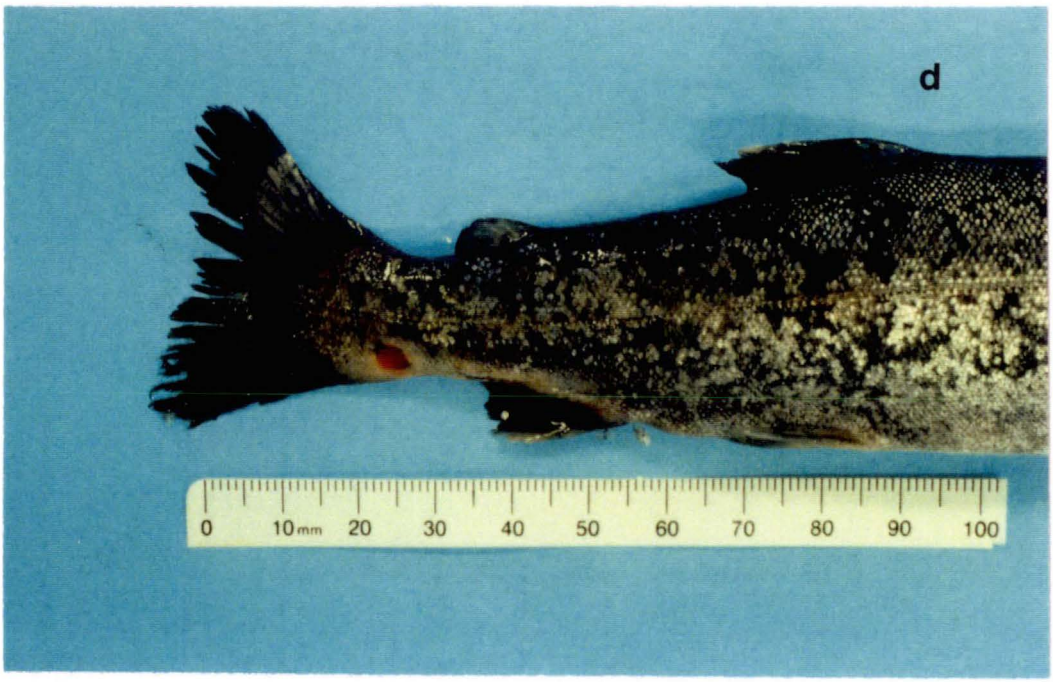
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6. Histopathology

6.1. Histopathology of greenback flounder

The histopathological changes in experimentally-infected greenback flounder were similar to those of natural infection (3.2.2.1, Field Studies), but lesions were somewhat more severe (possibly temperature related), with a greater degree of "fin-rot" with congestion and haemorrhage at the bases of the fins. Epithelial erosions with adhered filamentous bacteria, and lack of inflammatory response were evident.

6.2. Histopathology of salmonids

Histologically, lesions due to *F. maritimus* in salmonids may be categorised as early (superficial) lesions and late (deep) lesions.

6.2.1. Histopathology of early lesions

Early lesions were collected from the infected survivors soon after their appearance. Histopathological changes observed in the epidermis included a relatively mild exudative inflammation containing amorphous granular basophilic material, intra-epithelial inflammatory cells, occasional hyperplasia and necrosis of the epithelium (Fig. 3.13).

There was a consistency of fragmentation of the epithelium in some or all areas of the epidermis, with amorphous proteinaceous material between epithelial layers, and scattered intra-epithelial inflammatory cells (Figs. 3.14 & 3.15). Thinning of epithelial layers was evident in some samples, especially in infected rainbow trout. An accumulation of exudative fluid was seen on the edges of the scales, but the scales themselves remained intact. Where epithelial erosion was complete, small foci of filamentous bacteria were occasionally seen adhering to the most superficial, amorphous, fibrin-like layer. In some samples there was scale loss and scale pockets showed a variable odema and inflammation. Acute dermal and intra-epithelial cellular inflammatory infiltrates were seen in all fish which retained epithelium, but were not seen in the dermis of eroded lesions. Necrosis and hyperplasia in fibroblast cells, hyperaemia and haemorrhage were also occasionally observed. Inflammatory response or haemorrhage was seldom detected in the hypodermis.

6.2.2. Histopathology of late lesions

Late lesions were collected from fish which were moribund or had died overnight (12-15 hours dead). In most samples examined, there was a loss of the epidermis with replacement by mats of filamentous rods bacteria sometimes mixed with amorphous material. Inflammatory response was largely restricted to serous protein rich fluid over the fragmented or absent epithelium. Bacterial cells invaded the dense connective tissue layer (dermis) with necrosis and loss of nuclei (Figs. 3.16 & 3.17). Bacterial cells were not seen on and within the remaining epithelial layers. In some samples bacterial cells invaded underlying muscle and there was a congestion with some necrosis of muscle fibres (Fig. 3.18). In some sections, the connective tissue or fibroblast cells, had increased between the skeletal muscle cells. Other histological changes were similar to those described for natural infections.

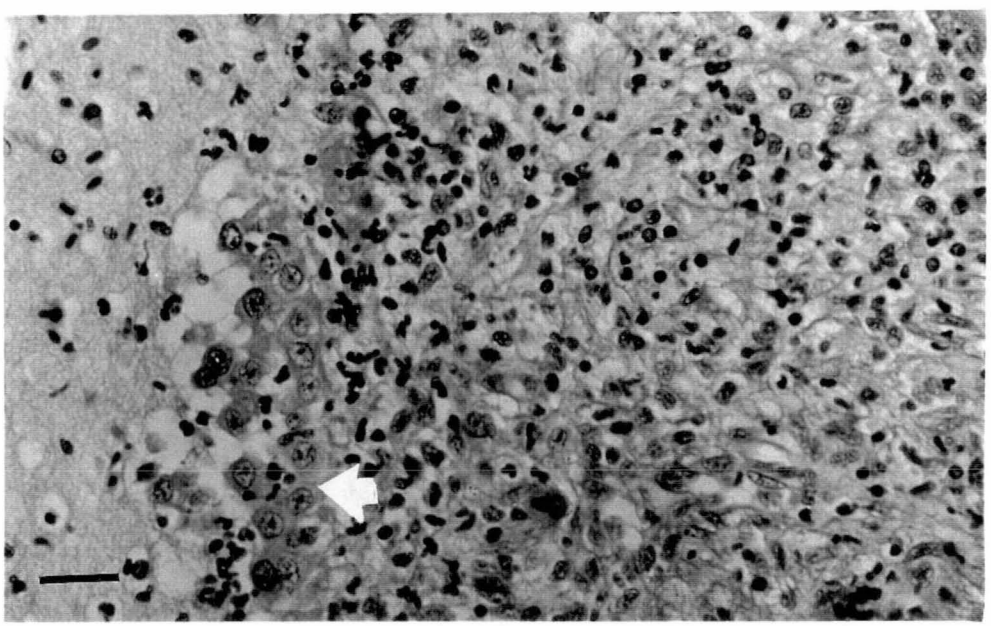


Fig. 3.13. Superficial lesion in Atlantic salmon experimentally infected by *F. maritimus* showing remnants of epithelial cells (arrow), superficial proteinaceous exudate and early fibroblastic reaction infiltrated by lymphocytes, macophages and the occasional neutrophil. (H & E, bar = 21 μm).

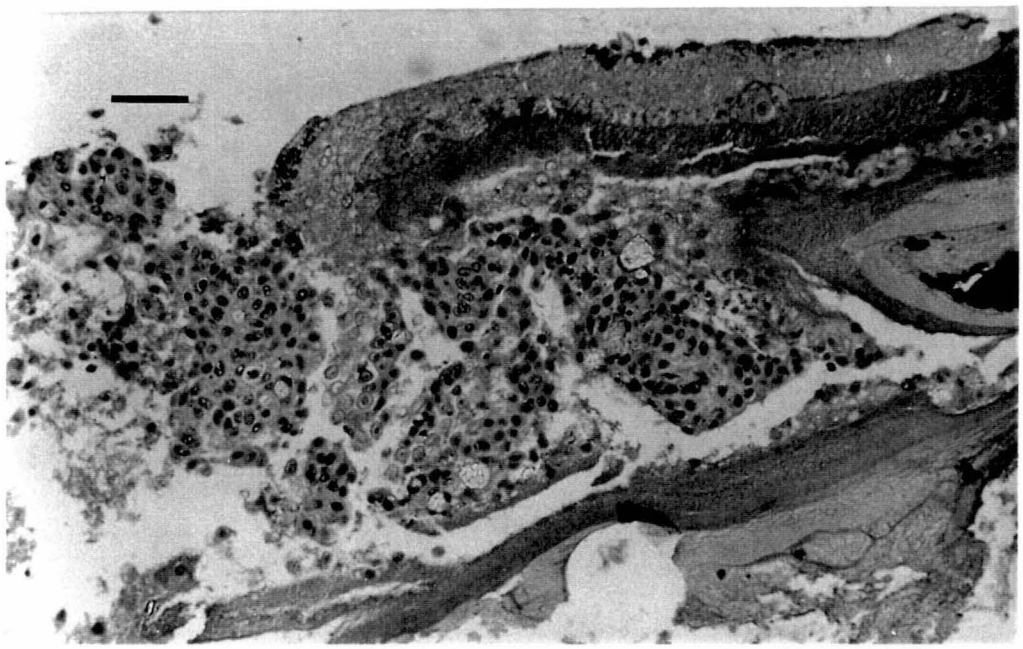


Fig. 3.14. Early mouth lesion in Atlantic salmon experimentally infected with *F. maritimus* showing fragmentation and degeneration of epithelial cells. (H & E, bar = 34 μm)

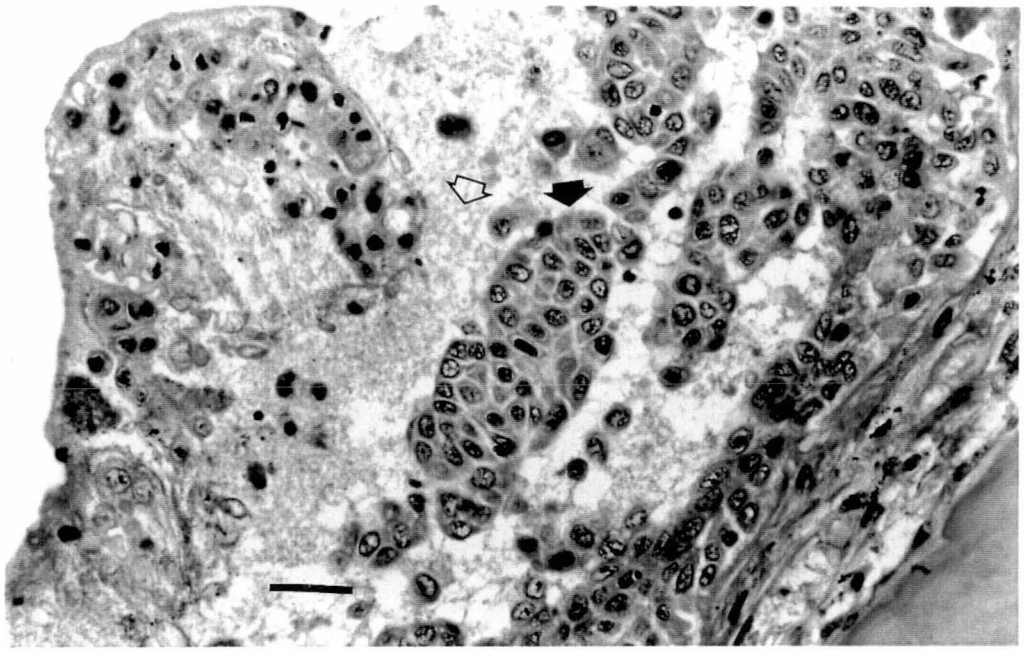


Fig. 3.15. Early skin lesion in an Atlantic salmon experimentally infected with *F. maritimus* showing detached epithelial cells (solid arrow) embedded in a matrix of proteinaceous exudate (open arrow). (H & E, bar = 21 μm)

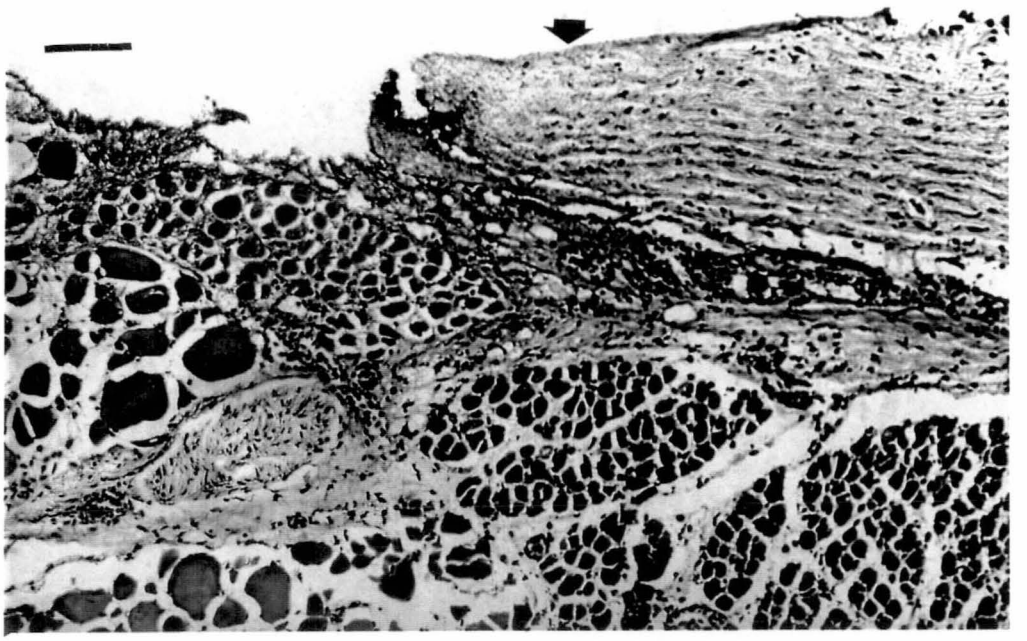


Fig. 3.16. View of a mature lesion in a rainbow trout experimentally exposed to *F. maritimus*. Epithelial layers are completely missing exposing collagen fibres of dermis (arrow) (Gram stain, bar = 75 μm)

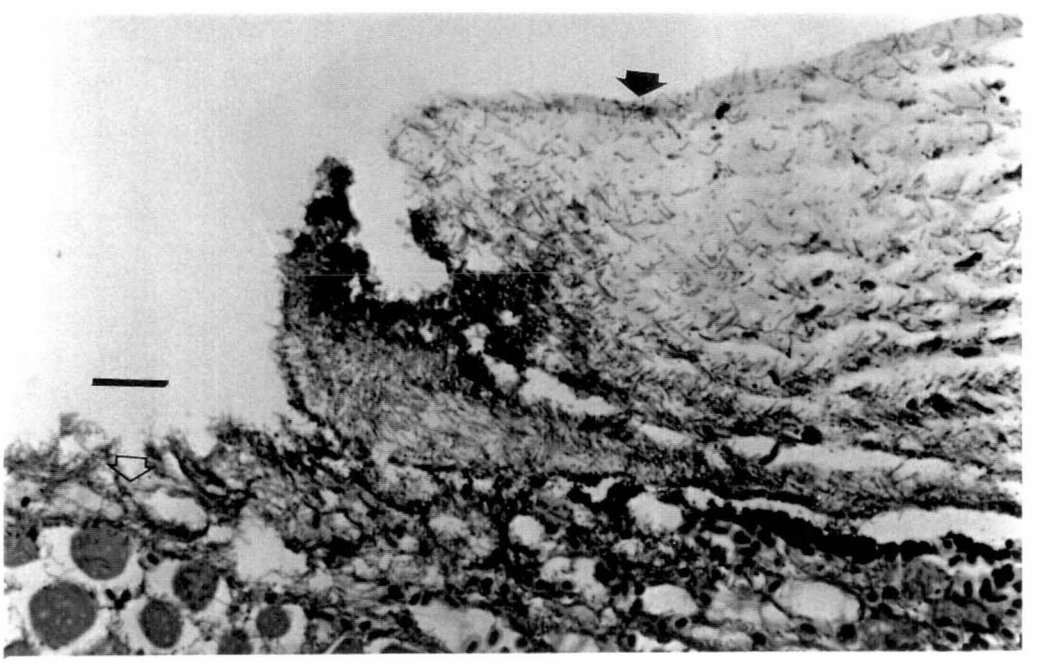


Fig. 3.17. Higher magnification of Figure 3.16 showing loss of epidermis and mats of long filamentous *F. maritimus* cells invading collagen fibres of dermis (solid arrow) and underlying musculature (open arrow). (Gram stain, bar = 25 μm).

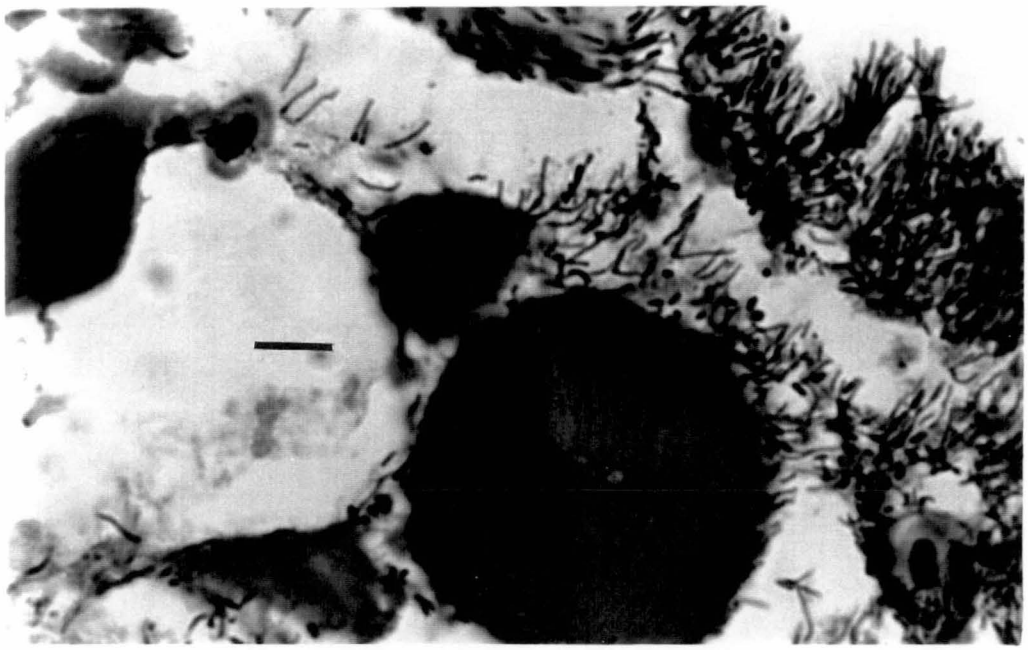


Fig. 3.18. Higher magnification of an eroded lesion in rainbow trout experimentally infected by *F. maritimus* showing invasion of long filamentous bacteria into underlying musculature. (Gram stain, bar = 8 μm).

3.2.3. *Flexibacter columnaris*

Materials and Methods

1. Test organism

A fresh lyophilised (recently isolated and freeze-dried) ampoule of *F. columnaris* 1468 (Chapter II) was used. The bacterial isolate was originally recovered from freshwater caged barramundi (30-100 mm in length, seven month old) at Lake Argyle in Western Australia in August-September, 1993 (C. Mifsud, pers comm, 1993).

2. Fish stocks

Barramundi 2-6 g body weight and goldfish 2-7 g body weight were used. Barramundi were obtained from West Beach Aquaculture in South Australia and goldfsh were obtained from a pet shop. Fish were maintained in the National Key Centre for Aquaculture, University of Tasmania using a re-circulation with biofiltration system, aeration and 5-10% water exchange daily at 25 or 28°C. Fish were fed twice daily using Tropical Fish Flakes (Wardley's).

3. Challenge

Bacteria were grown on CA at 25°C for up to 4 days. Cultures for challenge were prepared by inoculating the bacterium into 1000 or 2500 volumes of CB in 2 or 3 liter conical flasks at 25°C for 48-96 hours with gentle agitation using an orbital shaker. Cell counting technique, preparation of the inoculum for parenteral inoculation and validation tests prior to challenge were similar to those previously described for *C. johnsonae* and *F. maritimus*.

Challenge methods are given in Table 3.5. One week prior to challenge, fish were transfered to the 40 l aquaria. Fish were exposed to different doses of bacteria separately, and they were then kept in separated aquaria for 10 days post-exposure. Control groups were exposed to sterile CB, and they were kept in separated tanks provided in an isolated box, but in the same area. A box filter was provided for each tank. Water exchange was provided about 10% daily using 24-hour stored tap water.

Table 3.5. Methods used to assess the susceptibility of barramundi and goldfish to *F. columnaris*.

No.	Method of challenge	Cell no. as CFU	Water temp. (°C)	Fish species	No. fish
1	Bath	1.3×10^2 - 1.7×10^7/ml	25	Barramundi Goldfish	15 (2)*
2	Bath	2.4×10^5/ml	20	Barramundi Goldfish	15 (2)
3	Intraperitoneal injection (IP)	10^5 - 10^7 cells/fish	25	Barramundi Goldfish	10 (2)

Two unchallenged control groups were used for each method. Challenge time for bath methods was 60 minutes.

(1) Fish were bath challenged at concentrations of 1.3×10^2, 1.3×10^3, 1.3×10^4, 1.7×10^5, 1.7×10^6, 1.7×10^7 CFU/ml, and were then maintained in 40 l aquaria for up to 10 days.

(2) A week prior to challenge, fish were adapted at 20°C and then were challenged at this temperature. Fish were then maintained in 40 l aquaria for up to 10 days.

(3) Doses used were 1×10^5, 1×10^6 and 1×10^7 cells/fish of direct count. Control groups were injected with normal sterile saline.

*Number of replicates in parentheses.

4. Collection and processing of samples

Initial examination of lesions was by making smears from the periphery of the lesions and staining with Gram stain. Lesion materials were cultured on selective CA (Hawke & Thune, 1992); the presence of *F. columnaris* was confirmed by the appearance of the characteristic cell morphology of the organism and colonial morphology on the medium. Clinical signs and mortality were checked 2-3 times a day. Material obtained from the selected lesions was processed for histopathological examination as described for *F. maritimus* (3.2.2.2).

Results

1. Bacteriology

The bacterial examination including wet mount and Gram smears from the skin and fins of dead or surviving fish resulted in typical mats of filamentous, Gram negative, rod and flexuous bacteria (0.45-10 µm or occasionally longer) (Fig. 3.19). The cultures of these smears resulted in yellow, rhizoid, convex, sticky colonies with mild spreading on the plate surface on CA at 25° for up 72 hours.

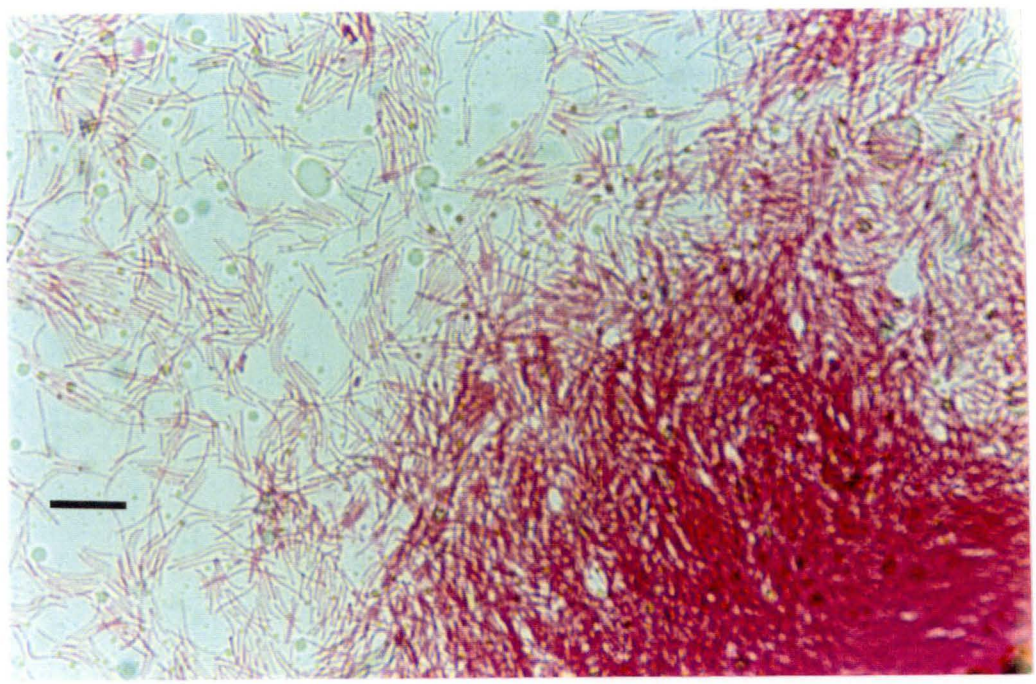


Fig. 3.19. Gram smear obtained from a lesion (white spot on the body surface, Fig. 3.23) of experimentally infected barramundi with *F. columnaris*. Note filamentous rods are quite dominant in the smear indicating a minimum secondary infection. (Bar = 8 μm).

2. Mortality and gross pathology

At a temperature of 25°C, a mortality level of 100% occurred in barramundi at concentration range from 1.3×10^4-1.7×10^7 CFU/ml, while this mortality level in goldfish resulted from concentrations of 1.7×10^6-1.7×10^7 CFU/ml (Fig. 3.20). The bacterium caused 100% mortality in barramundi with a dose rate at least 20 times less than what was needed to achieve this level in goldfish (Fig. 3.21). The LC_{50} for barramundi was $< 1.3 \times 10^3$ and $> 1.3 \times 10^2$ CFU/ml, while that of goldfish was 1.7×10^5 CFU/ml. Minimum time to death in barramundi was eight hours while that of goldfish was up to 18 hours. At a temperature of 20°C, mortality levels of 60% and 30% occurred in barramundi and goldfish, respectively (Fig. 3.22). All fish injected intraperitoneally died within 24 hours post-injection, with some fish showing eroded skin and fins containing mats of long filamentous *F. columnaris* cells conifrmed by wet mount, Gram smears or plate culture.

Fig. 3.20. Temporal pattern of *F. columnaris* infection in barramundi and goldfish subjected to various concentration of bacterium at 25°C. (Mean ± SD, n = 2 replicates of 15 fish).

Fig. 3.20.1. Barramundi

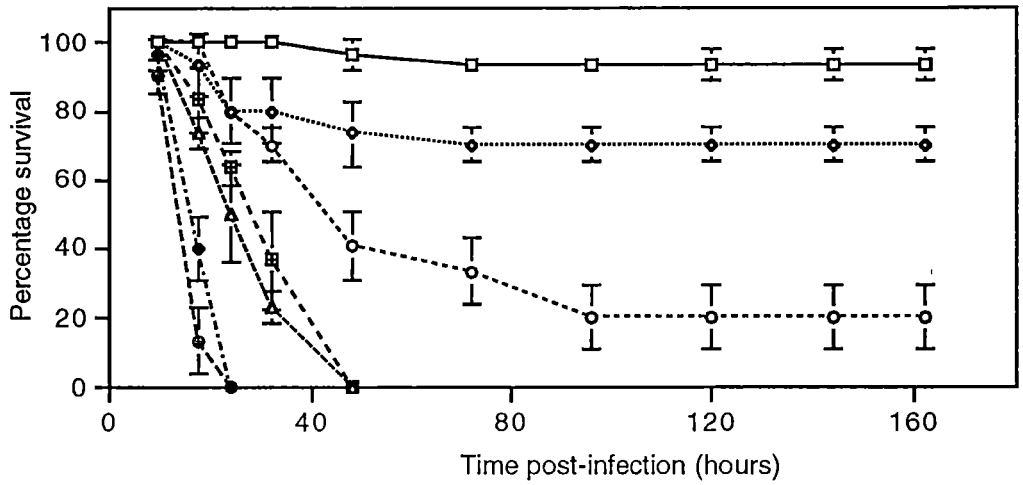
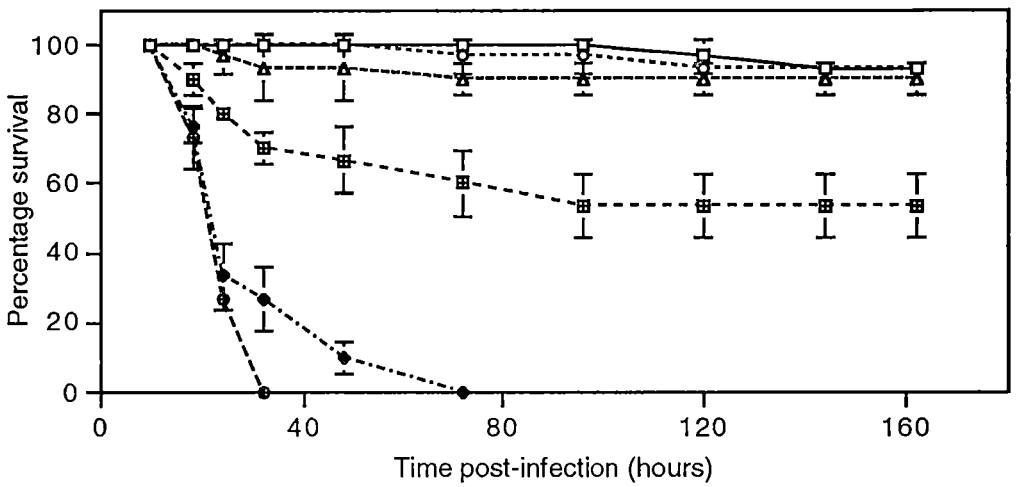


Fig. 3.20.2. Goldfish



10^0 CFU/ml
1.3 x 10^2 CFU/ml
1.3 x 10^3 CFU/ml
1.3 x 10^4 CFU/ml
1.7 x 10^5 CFU/ml
1.7 x 10^6 CFU/ml
1.7 x 10^7 CFU/ml

Fig. 3.21. Comparison of percentage survival in barramundi and goldfish subjected to various concentrations of *F. columnaris* at 25°C over 160 hours post-challenge. (Mean ± SD, n= 2 replicates of 15 fish).

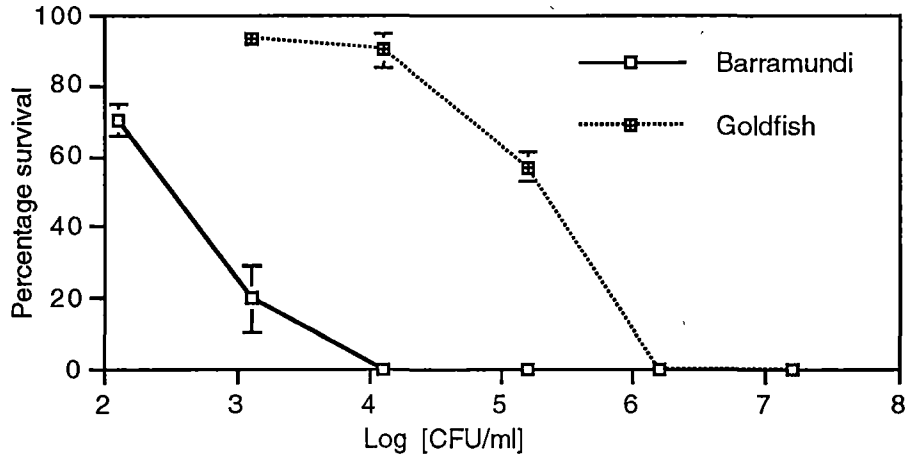
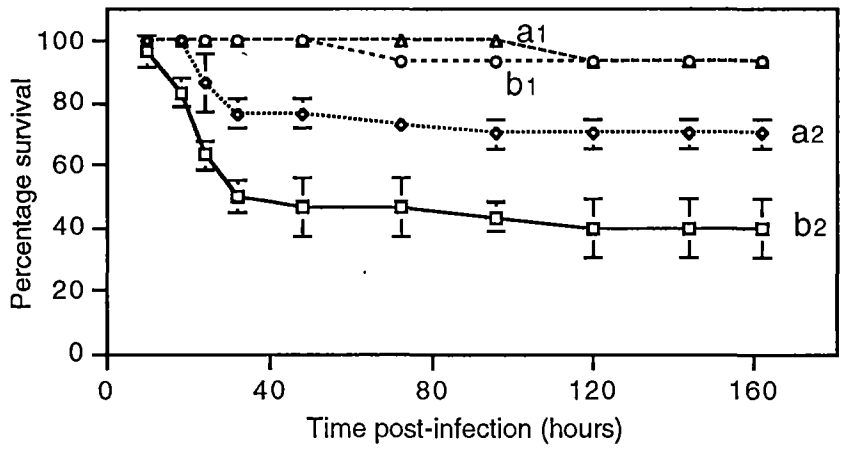


Fig. 3.22. Comparison of percentage survival of barramundi and goldfish subjected to bath immersion (2.4 x 10^5 CFU/ml) of *F. columnaris* at 20°C. (Mean ± SD, n= 2 replicates of 15 fish). a1 = Control (goldfish), a2 = Infected goldfish, b1 = Control (barramundi), b2 = Infected barramundi.



Clinically, the signs of "cotton-wool" including small to large white spots on the head , around the mouth, on the fins and tail as well as fin and tail-rot were apparent on infected fish (Fig. 3.23). The sign of cottonmouth was typically observable on the both species especially at lower concentrations of bacteria. Typical gross pathology was usually not observable in fish infected at higher concentrations of 1.7 x 10^6-1.7 x 10^7 CFU/ml at the time of death. These fish showed only "sudden death" Any survivors showed melanosis, tiny haemorrhagic spots, rippling of the fins and the body, hovering, fin nipping and flashing swimming behaviour.

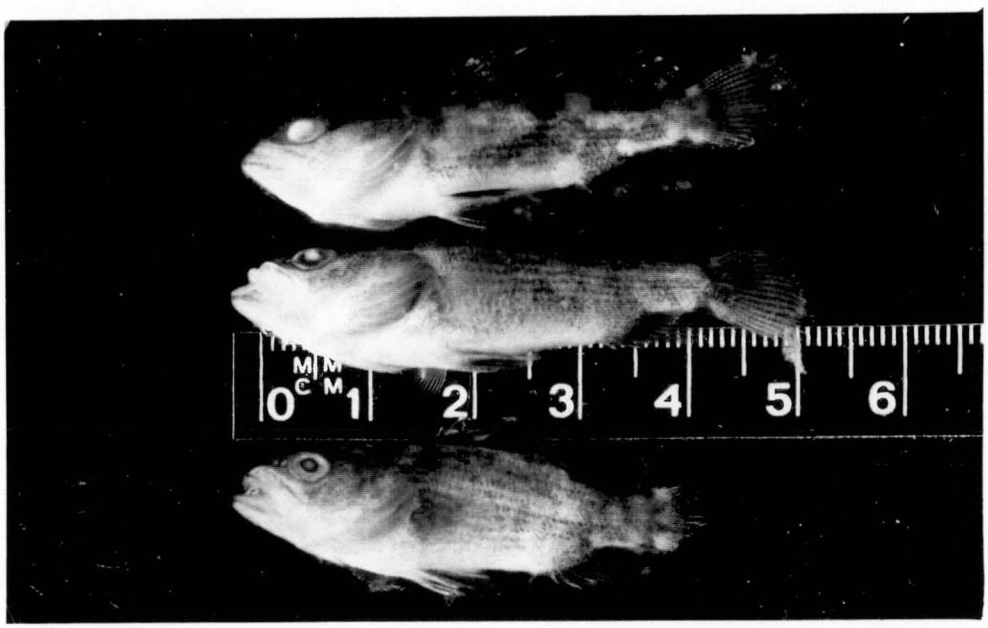


Fig. 3.23. Experimental columnaris disease in barramundi showing typical signs of cottonmouth disease, tail-rot, and white spots (bacterial clusters) covering skin surfaces or attached to fins.

3. Histopathology

All lesions examined showed acute necrosis of epithelial layers with minimum inflammatory reactions (Figs. 3.24 & 3.25). An accumulation of filamentous bacteria on the surface of the skin and throughout the epidermis and dermis was observed. In severely involved sites such as fins and mouth, large mats of the bacterial cells replaced the dense connective tissue (dermis) and bacteria also occurred deep in the underlying musculature. Affected muscles were necrotic and sometimes inflammation was observed. Scale loss was evident but the remainding scales were intact, with odema and inflammation in scale pockets. Most of the samples showed a severe bacterial invasion into epithelial layers, resulting in complete loss of the epidermis and replacement by filamentous cells and amorphous granular material. Internal organs, including liver, spleen and kidney were histologically intact and the bacterium was not observed in these organs.

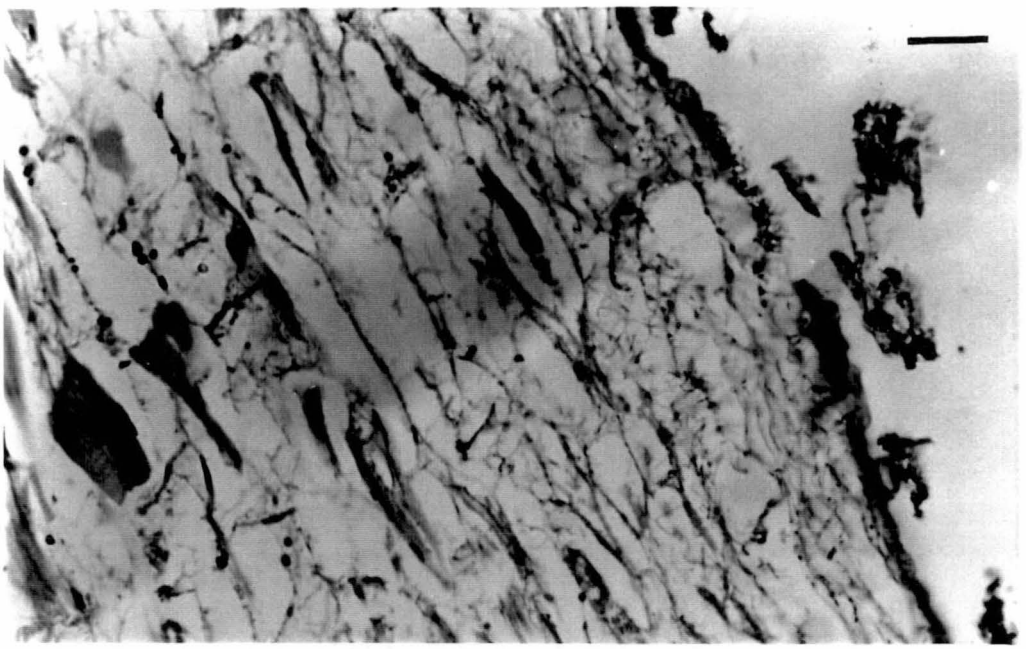


Fig. 3.24. Columnaris disease in barramundi. Epidermis is completely missing, and mats of filamentous bacteria have infiltrated into the necrotic and oedematous dermal collagen. (Giemsa, bar = 21 μm).

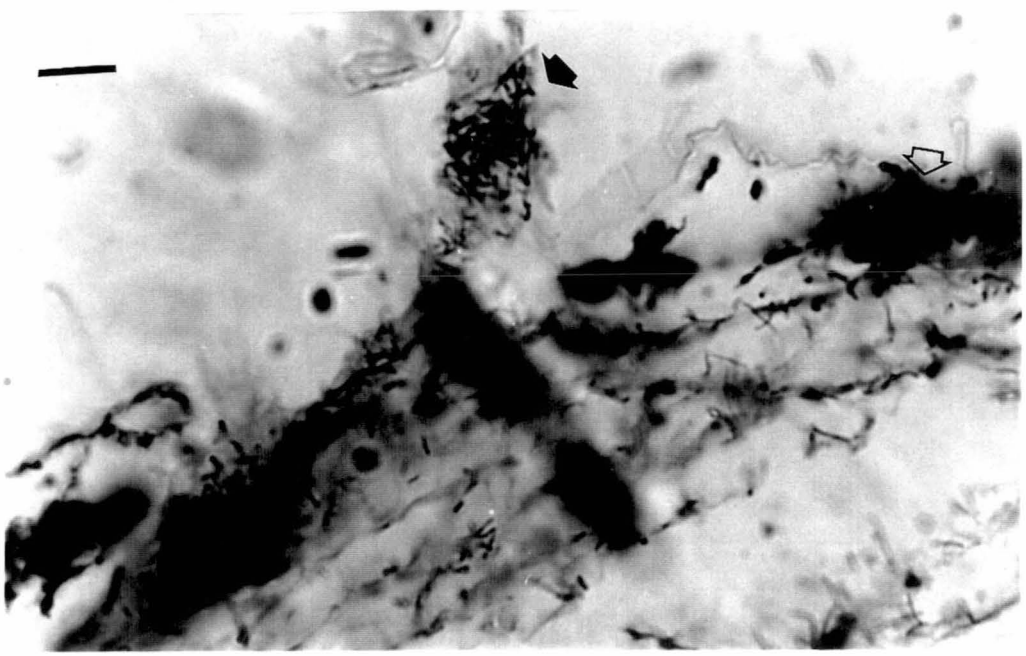


Fig. 3.25. Higher magnification of Figure 3.24 showing clumps of *F. columnaris* cells (solid arrow) and remnant pigment cells (open arrow) in necrotic connective tissue. (Giemsa, bar = 8 μm).

3.2.4. Discussion

The findings of this study provide a comparative study on the pathogenesis of freshwater and marine *Flexibacteria* pathogens for fish.

Despite the similarity of histopathological characters owing to *F. columnaris* and *F. maritimus*, the gross pathology was somewhat dissimilar. *F. columnaris* clinically caused "cotton-wool" covered lesions, especially anterior ones (cottonmouth disease), on the fins and to a lesser degree at caudal sites with the seldom occurrence of erosive skin disease characteristic of marine *Flexibacter* infection. The disease produced by *F. maritimus* began with small white-grey blisters at the infected sites and developed into cutaneous erosion. Therefore, the name of cutaneous erosion disease, given by Carson (1992), may describe the condition of *F. maritimus* infection, especially in salmonids, better than saltwater columnaris given by Wakabayashi (1993).

The progress of columnaris infection at higher concentrations of bacterium was usually rapid and highly aggressive resulting in a rapid death about eight hours in barramundi and 18 hours in goldfish post-in fection (Figs. 3.20.1 & 3.20.2). In contrast, marine *Flexibacter* infection required at least 48 hours to cause death in the host (Figs. 3.8.1 & 3.8.2).

Studies of gross pathology and histological changes in Atlantic salmon, rainbow trout and flounder infected with *F. maritimus* highlighted the similarity of the disease conditions, both natural and experimental. Fin erosions owing to experimental infection in greenback flounder were slightly more severe than those of natural infections. This may possibly be owing to the effect of temperature at which fish were exposed to the bacteria. There was a great consistency of lesions in experimentally infected salmonids in all trials (Fig. 3.11). The severity of infection was enhanced when fish were subjected to the virulent strain (passaged strain) of the bacterium (Trials 4 & 5, Fig. 3.11). The lesions mainly occurred on the jaws, ventral sites, tail; and pectoral and ventral fins. This distribution of lesions is similar to that observed in natural outbreaks of the infection in caged Atlantic salmon, rainbow

trout (3.2.2.1. Field Studies) and sea bream (Hikida *et al.*, 1979; Wakabayashi *et al.*, 1984).

Oral cavity/jaws and fin erosions were consistently dominant in all trials. The reason why *Flexibacteria* prefer to attack the oral cavity, jaws and fins is not entirely clear. The primary physical abrasions on the fins and mouth may provide a suitable place for bacterial localisation and colonisation with subsequent proliferation and infiltration into epithelial tissues. Teeth and fins of teleost fish are usually rich in Ca ions which are important elements in promoting the growth of *Flexibacteria* (Hikida *et al.*, 1979). In the recent report by Frelier *et al.* (1994) they demonstrated the enameloid erosion due to a *Cytophaga*-like bacterium in Atlantic salmon.

The main common histological findings in this study were a remarkable lack of an inflammatory response in advanced lesions in both *Flexibacter* infections. Although there were differences between the species of salmonids and non-salmonids studied in regard to the siting and extent of *F. maritimus* lesions, there was a great consistency in the histopathology, with usually minimal inflammatory reaction to the bacterium which produces a necrotising, expanding lesion with bacteria in the most superficial layers and extending into but not beyond the necrotic tissue. Interference with the host immune response may occur in different ways, such as immune tolerance, production of immunosuppresive factors, the use of molecular mimicry and interference with immune factors at the site of infection (Thune *et al.*, 1993). Histopathological findings in this study, however, showed a low level of inflammatory cells, i.e. macrophages and lymphocytes, in the early lesions, in particular for *F. maritimus* infection. This suggests that powerful exotoxins are produced by the organisms, effectively preventing a host response. The studies of Baxa *et al.* (1988a) and Bertolini & Rohovec (1992) support this proposition. It is likely that any efficacious vaccine would need to include this toxin. Thinning or damage of the scales could not be seen for flexibacterial infections, probably because of a lack of chitinobiase production which is involved in *C. johnsonae* infection (Bernardet & Grimont, 1989; Carson *et al.*, 1993).

The restriction of visible *F. maritimus* in sections to necrotic tissue, connective tissue, or fibrin, suggests that bacterium is unable to survive in well vascularised living tissue, or with possible specific activity such as collagenase activity. If the former, the occasional visualisation of the bacteria within blood vessel walls is surprising. The epithelium appears normally to be an effective barrier to establishment of the bacterium.

In natural infections, factors damaging to the epithelium were identified as predisposing to *F. maritimus* infection. In experimental infection erosion and degeneration of surface layers were seen to occur following exposure to the organism, without localisation within the epithelium. It is possible that the presence of this presumptive toxin in the dense cell suspensions used for experimental challenge may have led to superficial skin damage, thus initiating the cascade of changes typical of the disease. However, the lack of visible *Flexibacter* in early lesions may be due to early localisation of the bacteria within the mucus layer, which was not preserved by standard histopathology techniques. These findings are in part consistent with variable bacteriological findings of early lesions and have been interpreted as probably reflecting uneven distribution of the bacteria over the lesion.

In contrast to early lesions, there were mats of *F. maritimus* invading dense connective tissue and they were occasionally in the underlying musculature in late lesions. In such sections there was usually a loss of epidermis.

At a salinity of 15‰ seawater disease induction by *F. maritimus* was successful in Atlantic salmon and greenback flounder. This concurs with the *in vitro* physiology of the organism (Chapter II), which is able to grow well at this salinity. The process of infection was similar at full salinity resulted in slightly less intense infections. Infection may, however, become more severe at full salinity which enhances the growth of the bacterium (Fig. 2.11, Chapter II). The reason why goldfish adapted at this salinity were not affected by *F. maritimus* is not entirely understood. It may be due to host immunity or host specificity.

F. columnaris achieved 100% mortality in fish subjected by IP challenge. This highlights that infection may occur both as a superficial and systemic disease and

the existence of previously injury on skin, fins or gills for bacterial invasion of the blood circulation system as suggested by Wakabayashi (1991; 1993) may not be necessary. Even so, experimental induction of infection by CFLB is generally more effective by bath immersion rather than by parenteral routes. Also, pathogenicity of many of bacterial fish infections should be assessed by immersion rather than an injection route, because bath challenge mimics natural routes of infection (Anderson, 1990).

Both Atlantic salmon and rainbow trout showed a similar level of susceptibility and temporal pattern of infection to *F. maritimus*. A relative LC_{50} could be achieved in both species at concentrations ranging from 2.3×10^5-1.6×10^6 CFU/ml at a temperature range 16-20°C at which bacterium grows well (Chapter II). However, environmental variables may affect the obtained LC_{50} when facing natural outbreaks.

The mullet strain of the organism did not affect Atlantic salmon even when scarification was used. There are two possible explanations. First, low salinity of PBS may affect the organism's virulence, as this strain is more susceptible to lower salinity than the Atlantic salmon strain (Schmidtke *et al.*, 1991; B. Munday, unpublished data). The cultures provided from affected mullet and PBS suspension also support this, because there was no growth from the PBS suspension, while there was positive growth for samples cultured from mullet. Secondly, there may be a different host susceptibilities in relation to various strains of *F. maritimus*.

This study clearly shows that the Atlantic salmon strain of *F. maritimus* affects rainbow trout and greenback flounder. Greenback flounder appear to be relatively resistant to marine *Flexibacter* infection, although more numbers and replicates would be required to confirm this. However, infection should not create any significant impediment to commercial farming of this species. In contrast, striped trumpeter are easily infected with *F. maritimus* and the disease could be significant in this species if appropriate strategies such as shading tanks for juvenile fish are not instituted, once this fish becomes a viable commercial prospect. However, more work needs to be directed to the inter-host susceptibility among the isolated strains of *F. maritimus*, e.g.

whether mullet, striped trumpeter, sea bream, and flounder isolates are able to invade successfully salmonids or not.

This study also shows that barramundi is a very susceptible species to *F. columnaris* infection and should therefore be included in the list of susceptible species such as that given by Wakabayashi (1991) and Austin & Austin (1993). The fish were more susceptible to the bacterium than goldfish and, when challenged with high concentrations of bacteria, they showed no gross tissue damage at the time of death, especially at the higher temperature of 25°C. Probably, death occurred before external signs of the infection appeared. This event was observed in young salmon (sockeye or chinnok salmon) infected by a high virulent strain of the organism (Pacha & Ordal, 1967). According to Pacha & Ordal (1967), the virulence of this isolate of *F. columnaris* may be categorised as being high. This is so because of its ability to produce 100% mortality within 24 hours at 1.7×10^5-1.7×10^7 CFU/ml. Natural outbreaks of infection in barramundi occur at temperatures above 24°C (C. Mifsud, pers comm). Comparison of survival levels at two different temperatures of 25 and 20°C showed that bacterial invasion to the host at higher temperatures (25-35°C) is more rapid than lower water temperatures of < 20°C. One main reason for this is because bacterial growth rate is faster at higher temperatures. The *in vitro* study proved that optimum temperature for growth of *F. columnaris* is between 25 and 30°C, with a doubling time of 0.6 and 0.4 those at 20 and 15°C, respectively (Chapter II).

Data on the actual mechanisms involved in pathogenesis due to *Flexibacteria* are limited. So far, the role of exotoxins such as proteases have been suggested to play an important part in the pathogenesis of these organisms in fish (e.g. Pacha, 1961; Baxa *et al.*, 1988a, Bertolini & Rohovec, 1992; Austin & Austin, 1993; Dalsgaard, 1993).

Long survival of *F. columnaris* in water was reported by some workers (Fijan, 1968; Ross & Smith, 1974; Becker & Fujihara, 1978; Chowdhury & Wakabayashi, 1988a; 1990b). From practical observations undertaken in this study, it was notable that in immersion trials bacterial proliferation in the water containing infected fish

rapidly resulted in slight water turbidity and an induction of a slime/sticky layer covering the water surface within 24 hours. This slime layer degraded within 48 hours. Wet mounts and Gram stain smears prepared from water sample showed mats of filamentous bacteria. The production of such slime layers was also relatively observed with virulent strains of *F. maritimus*. Interestingly, there were no such observations for other less or non-virulent CFLB isolates of *F. columnaris, F. maritimus, C. johnsonae* and *C. psychrophila*. In broth culture the comparison of virulent and non-virulent strains of *F. columnaris* and *F. maritimus* showed that virulent strains were capable of producing this slime cluster layers in broth much more than non-virulent strains. Therefore, an adhesion mechanism, due to this sticky production, may play a role in the pathogenicity process of virulent strains of gliding bacteria leading to the attachment of cells to the host surface, resulting in large bacterial populations at the site where other virulence factors such as toxins and enzymes can be released. This is supported by Johnson and Chilton (1966) and Pate and Ordal (1967), who demonstrated the production of two types of slime layers, an acidic polysaccharide and a galactosamine by *F. columnaris*. Also, work by McEldowney and Fletcher (1988) suggested that differences in the adhesion of the gliding bacteria (*Flexibacter* sp.) may be associated with their adaptation to different ecological niches. They found progressive adhesion levels of a gliding *Flexibacter* sp. were with increased growth rates in a continuous culture until the death phase, and an increase in either temperature or pH caused a decline in adhesion level, probably through a decrease in viscosity.

The bacteriocin and bacteriolytic activities of CFLB may also give them a competitive advantage over other commensal bacteria resulting in the improvement of the attachment and colonisation on the host surface (Dalsgaard, 1993). The work by Chowdhury and Wakabayashi (1989a; 1989b; 1990a) showed variable effects of other bacterial species on the course of columnaris infection. For instance, the bacterium successfully invaded the fish in the presence of *Streptococcus* sp. or each of two species of *Flavobacterium flavescens* and *F. fuscum,* even when the numbers of these bacteria were 1000 times that of *F. columnaris*. Observations of

Gram smears and cultures taken from lesions on infected fish in this study indicated massive mats of *F. columnaris* (Fig. 3.19). This was also observed by histological examination of the tissues from infected fish. However, the progress of marine *Flexibacter* infection in Atlantic salmon was faster in filtered (0.2 μm) than unfiltered seawater (Trial 6, Fig. 3.10) indicating a possible interaction between natural microflora in seawater and the infection.

Apart from temperature fluctuations as a crucial important factor influencing the progress of infections by *Flexibacter* spp (Chapters I, II & Section 3.1 in this Chapter), there are some other variables which may be involved in the natural outbreaks of these infections in Australian aquaculture, in particularly marine *Flexibacter* infection in salmonids. These may include:

(1) Effect of ultraviolet (UV) irradiation

Atlantic salmon parr are usually transferred to marine habitats during spring/summer when the water temperature is above 15°C. The highest amount of UV-B arrives on the earth's surface at this time, water at the site where the fish are located is of variable clarity and fish are fed at frequent intervals to optimise the growth rates. These conditions have given rise to a scenario in which UV-B irradiation may act as a predisposing factor on the course of *F. maritimus* infection. Recent reports show that the so-called "hole in the ozone layer" has dramatically increased the amount of UV-B irradiation during spring/summer in the Australian region (Munday, 1993). For example, in the summer of 1990-1991 the biologically-weighted UV irradiances in New Zealand were about twice those of northern Germany. The hypothesis is also supported, because at the time of natural outbreaks the fish are under rapid growth, having actively-dividing cells which are most susceptible to UV-B damage (Munday, 1993). Occasional eye damage and dorsum lesions in natural outbreaks have also been described. In contrast there were no such lesions in experimental animals.

(2) Effect of translocation

Smolting salmon are transferred from freshwater to seawater and the process of transferring fish may suppress the animal's immune system and they may become susceptible to infections.

(3) Softness of scales and skin in small sized fish

Most naturally affected Atlantic salmon were post-smolts which were more severely affected than fish in their second summer at sea. The higher incidence of the disease in post-smolts almost certainly relates to the softness of the scales and the ease with which they are lost. Comparison of results taken in experiments 1 and 2 (Table 3.2) support this proposition, because the incidence of infection and lesions were fewer in larger size animals than smaller size fish, even though the stocking density in larger sizes was four times that of smaller sizes.

(4) Poor management

Any condition which cause stress to fish, such as poor feeding management which results in scale loss through competition, and inappropriate treatments, e.g. use of oxolinic acid for *F. maritimus* infection, may render fish susceptible to infection. Antimicrobial activity of quinolones against CFLB will be discussed in the next Chapter.

(5) Interaction with other infections

There may also have been some predisposition created through existing gill pathology associated with amoebic gill disease (Roubal *et al.*, 1989; Howard & Carson, 1994) which could exacerbate the infection by *F. maritimus* in sea-caged salmonids.

CHAPTER IV

In Vitro Studies of the Antimicrobial Activity of Skin Mucus of Fish Against *Cytophaga/Flexibacter*-Like Bacteria (CFLB)

Introduction

The antimicrobial activity of fish mucus often acts as a first line of defence against pathogens. The mucus layer provides a significant mechanical protective function which prevents surface colonisation of some parasites, bacteria and fungi through continuous loss and replacement of this layer. Also, fish mucus contains several secretory components including agglutinins, lysins, lysozyme, non-specific precipitins, C-reactive proteins and natural antibodies (e.g. Hodgins *et al.,* 1973; Ingram, 1980; Gudkovs, 1988; Itami *et al.*, 1988; Alexander & Ingram, 1992; Rainger & Rowley, 1993; Dash *et al.*, 1993). These macromolecular mucin components act as important chemical defence barriers against pathogens.

Scant studies have been directed to evaluate the properties of antimicrobial activity of fish skin mucus, in particular non-immune skin mucus, and are summarized in Table 4.1.

In regard to immune responses, Fletcher and Grant (1969) reported an induction of hemagglutinins in the skin and intestinal mucus of plaice (*Pleuronectes platessa*) following parenteral immunisation with *Vibrio ichthyodermis* and Fletcher and White (1973) demonstrated a specific antibody for *Vibrio anguillarum* in mucus secretions of the skin of immune plaice.

Harrell *at al.* (1976) showed that the antibacterial activity of immune and non-immune skin mucus of rainbow trout comprised four significant mechanisms. Firstly, complement existed in body mucus and may, at least *in vitro,* have antimicrobial functions. Secondly, heat-stable and heat-labile components in both immune and non-immune mucus were present which, together, were able to inhibit or kill *V. anguillarum in vitro.* Thirdly, non-immune mucus, diluted in fresh normal serum

(complement source), was capable of generating plaque (growth inhibition), but its titre was lower than that of immune mucus. Finally, neither heat-inactivated immune mucus, heat-inactivated immune serum nor fresh normal serum showed anti-*Vibrio* activity.

Both serum and body mucus of rainbow trout previously vaccinated against *Aeromonas salmonicida* enhanced bactericidal/bacteriostatic activity against *A. salmonicida*, *A. punctata* and *Serratia marcescens in vitro* condition compared with control fish injected only with saline (Rainger & Rowley, 1993).

Intraperitoneal and intramuscular injections of non-absorbed and absorbed skin mucus obtained from coho salmon vaccinated against *Vibrio ordalii* did not give any protection in recipient coho salmon (passive immunisation) (Velji *et al.*, 1991). The recipient fish also contained no detectable anti-*V. ordalii* agglutinins.

The bacteriolytic activity of skin mucus obtained from yellowtail (*Seriola quinqueradiata*) was examined for its enzymatic properties against *Pasteurella piscicida* and *Micrococcus lysodeikticus* (Takahashi *et al.*, 1987). There was high bacteriolytic activity against both cells in distilled water or in low molar buffer.

Subasinghe and Sommerville (1988) did not find any antibacterial function in the skin and buccal mucus of naive mouth-brooding cichlid (*Oreochromis mossambicus*) females at different stages of their breeding cycle.

Ourth (1980) demonstrated bactericidal activity and agglutinating antibodies in the skin mucus of immune channel catfish (Table 4.1).

Kamiya & Shimizu (1980) found that mucus lectins from healthy windowpane flounder (*Lophopsetta maculata*) were capable of agglutinating a marine bacterium, *Microcyclus marinus*, but not either *V. alginolyticus* or *Pseudomonas* sp., thus indicating a possible selective activity of these mucus components.

Using skin mucus from healthy rainbow trout Austin and McIntosh (1988) demonstrated marked growth inhibition against *A. hydrophila*, while the lowest growth inhibition was observed when *Mycobacterium fortuitum* and *Nocardia asteroides* were exposed to the mucus. The mucus was also inhibitory to populations of other

freshwater bacteria, including *Cytophaga* sp., *Flavobacterium* sp., *P. fluorescens*, *Micrococcus roseus* and *Staphylococcus epidermidis*.

Work by Fouz *et al.* (1990) showed that skin mucus from healthy turbot (*Scophthalmus maximus*, Scophthalmidae) had a wide spectrum of antibacterial activity against different Gram negative and Gram positive bacterial fish pathogens including *A. hydrophila*, *A. salmonicida*, *Flavobacterium sp.*, *V. anguillarum*, *V. splendidus*, *V. damsela*, *V. tubiashii*, *V. pelagius*, *Yersinia ruckeri*, *Lactobacillus piscicola*, *Moraxella sp.*, *P. piscicida*, *P. fluorescens* and *S. aureus*

AL-Harbi and Austin (1992) showed an inhibitory action for skin and gut mucus from immune and non-immune turbot against a fish pathogenic *Cytophaga*-like bacterium. The greatest inhibitory activity was recorded in the presence of gut mucus from animals which were previously immunised with the lipopolysaccharide extracted from the bacteria, while antibacterial activities of gut mucus from non-immunised fish and skin mucus from both immune and non-immune fish were lower and of similar intensity.

Table 4.1. Antibacterial activity of fish skin mucus.

Property of mucus	Test antigen	Results (a)	(b)	Fish species
Protease (trypsin type)	*V. anguillarum*	.	+	Rainbow trout (1)
Complement components	*V. anguillarum*	+	+*	" (2)
Complement components	*V. anguillarum*	-**	.	" (2)
Enzymatic properties	*A. salmonicida, A. punctata Serratia marcescens*	+	-	" (3)
Agglutinogens	Sheep erythrocytes	+	-	" (4)
Enzymatic properties	*P. piscicida, M. lysodeikticus*	.	+	Yellowtail (5)
Enzymatic properties	*V. anguillarum*	+	.	Plaice (6)
Agglutinogens	*V. ichthyodermis*	+	-	" (7)
Enzymatic properties	*P. fluorescens, A. hydrophila, Flavobacterium sp., E. tarda Y. ruckeri.*	.	-	Cichlid (8)
Agglutinogens	Sheep erythrocytes	+	+	Gar (9)
Enzymatic properties	*Cytophaga*-like bacterium	+	+	Turbot (10)
Agglutinogens	*Salmonella paratyphi*	+	.	Catfish (11)
Agglutinogens (lectin)	*Microcyclus marinus*	.	+	Flounder (12)
Enzymatic properties	*A. hydrophila,, Cytophaga* sp., *Flavobacterium* sp., *P. fluorescens, S. epidermidis*	.	+	Rainbow trout (13)

(a): Immunised skin mucus, (b): non-immunised skin mucus. * Mucus was prepared in normal fish serum (complement source). ** Heat-inactivated mucus was used.

-: No activity/inhibitory gorwth, (+): Weak positive activity/inhibitory growth, +: Positive activity/inhibitory growth, .: Not applicable.

(1) Hjelmeland *et al.* (1983), (2) Harrell *at al.* (1976), (3) Rainger & Rowley (1993), (4) St. Louis-Cormier *et al.* (1984), (5) Takahashi *et al.* (1987), (6) Fletcher & White(1973), (7) Fletcher & Grant (1969), (8) Subasinghe & Sommerville (1988), (9) Bradshaw *et al.* (1971), (10) AL-Harbi & Austin (1992), (11) Ourth (1980), (12) Kamiya & Shimizu (1980), (13) Austin and McIntosh (1988)

Such antimicrobial activity has also been reported for other fish fluids/organs such as blood serum and kidney extracts. For instance, untreated serum of rainbow trout was bactericidal against an avirulent strain of *A. salmonicida* (Sakai, 1983). Of two lysozymes isolated from rainbow trout kidney, one was potent, having substantial antibacterial activity against a number of bacteria consisting of *V. anguillarum*, *V. salmonicida*, *A. salmonicida* subsp. *salmonicida* and *achromogenes*, *Flavobacterium* sp. and *Yersinia ruckeri* (Grinde, 1989). In trials involving goldfish

and the protozoans *Ichthyophthirius multifiliis* and *Tetrahymena pyriformis* Ling *et al.* (1993) were able to demonstrate appreciable levels of anti-parasite antibodies in the plasma and skin mucus of immunised fish.

From the above it is evident that, in certain circumstances, it is possible to demonstrate that fish skin mucus has an important external protective effect against disease microorganisms found in the aquatic environment. Especially as some CFLB show varying pathogenicity for different species of fish, it was hypothesised that this might be due, in part at least, to differing abilities of the skin mucus to inhibit colonisation by these bacteria. Consequently, the main objective of this study was to investigate whether skin mucus from a number of fish species has antimicrobial activity against a range of CFLB.

Materials and Methods

Materials

1. Test organisms

The same isolates of bacteria used for *in vivo* pathogenesis (Chapter III) were used in this study. These were: *C. johnsonae* 91/0262-10, *C. psychrophila* 91/4043-17, *F. columnaris* 1468 and *F. maritimus* 89/4762.

2. Experimental animals

Atlantic salmon 30-75 g body weight, barramundi 44-101 g or 450-1000 g body weight, greenback flounder 20-44 g body weight, goldfish 40-108 g body weight and rainbow trout 500-1000 g body weight were used to provide the skin mucus. Barramundi were held at 25°C at West Beach Aquaculture in South Australia. The other fish including goldfish, flounder and salmonids were maintained at 25-28°C, 10-12°C and 15-17°C, respectively at the Key Centre for Aquaculture, University of Tasmania.

Methods

1. Collection and processing of mucus samples

Skin mucus samples were collected by gently wiping/scraping the skin using a glass microscope slide (AL-Harbi & Austin, 1992). Care was taken not to include blood or

other body fluids in the samples. The samples were suspended in an equivalent volume of sterile phosphate buffered saline (PBS) (pH 7.4 0.1 M) and centrifuged (1500 g for 25 min at 4°C) to pellet any particulate material. The supernatants were dialyzed against PBS at 4°C for 36 hours. The sample preparations were first filtered using glass fibre prefilter (Sartorius) and, then filter-sterilised using 5 and 0.22 μm Millipore membranes in sequence and stored at -20°C until used. To precipitate any proteins present, the mucus samples were also prepared in the same way, but treated with 50% ammonium sulphate (pH 7). Mucus samples were also sonicated at 90% duty cycle (about 50 watts) for five minutes in crushed ice and stored at -20°C until used.

2. Assay for activity

Agar diffusion assay with disc and well procedures was used to demonstrate antibacterial activity of mucus against these organisms. Fish species and CFLB used for this observation are shown in Table 4.2.

Table 4.2. Test organisms and mucus sources used for antibacterial activity tests.

Bacterial species	Fish used for mucus collection
C. johnsonae	Barramundi*†, rainbow trout, goldfish
C. psychrophila	Atlantic salmon*, rainbow trout, goldfish
F. columnaris	Barramundi*†, goldfish†
F. maritimus	Atlantic salmon*†, flounder†, goldfish

* Indicates fish from which the organism was isolated.

† Indicates fish in which disease was produced.

Test organisms were grown in *Cytophaga* broth (Anacker & Ordal, 1959a) prepared in freshwater or seawater (for *F. maritimus*) at 20 or 24°C for 48 hours. The bacterial cultures were pelleted (1700 g for 15 min) and washed with PBS or sterile seawater (for *F. maritimus*). Inocula were adjusted to McCFarland 0.5 turbidity standard to give ca. 1 x 10^8 CFU/ml (Arthur, 1991). The agar plates of freshwater or seawater *Cytophaga* agar were inoculated with the standard inoculum of bacterial suspensions by swabbing the surface of the agar plates in three different directions and with a final swab around the rim. Before adding the discs, the inoculated plates

were dried in room temperature for 5-10 minutes so that excess fluid was absorbed. Sterile blank discs of 6 mm diameter (Whatman Cat No 2017 006, Maidstone, England) were then aseptically placed on the surface of the agar plate. Mucus samples of about 30 µl were added to duplicate discs in three replicate plates. Control discs were included by impregnating discs with sterile PBS or seawater. The plates were incubated upside down within 10-15 minutes. Duplicates of about 50 µl of mucus samples were also placed in 7 mm diameter wells in three replicate plates for each mucus sample. Sterile PBS or seawater was used as the control. The plates were incubated at two different temperatures of 20 and 30°C except for *C. psychrophila* samples which were incubated at 15 and 20°C. After 20-30 hours incubation, inhibitory zones were measured with a metric scale under a dissecting microscope. The experiment was performed in the same way with mucus samples treated with ammonium sulphate and sonicated mucus. The experiment was repeated twice using mucus samples collected at different times.

Results

In general, variable results were obtained in this experiment. The first set of mucus samples generally showed some antibacterial activity (Table 4.3), while the second set of samples were negative for an inhibitory zone. Similar results were found for both disc and well procedures. No reason was apparent for these anomalous results.

Mucus of goldfish and rainbow trout at 20 and 30°C were inhibitory against *C. johnsonae* having a zone of inhibition with an average of 2.5 mm. There was no zone of inhibition by barramundi mucus at 20°C, but a 1 mm zone of inhibition was evident at 30°C.

Antibacterial activity with an average inhibitory zone of 1.5 mm was obtained for skin mucus of Atlantic salmon, rainbow trout and goldfish against *C. psychrophila* at 15°C, whereas at 20°C this activity was found only for goldfish mucus with an average 1.5 mm zone of inhibition.

Antibacterial activity of barramundi and goldfish mucus against *F. columnaris* was nil at 20°C and was inhibitory with 1 mm at 30°C.

Goldfish mucus showed an anti-*F. maritimus* activity of 1.6 mm at 20°C and 30°C. Flounder mucus showed an inhibitory zone up to 1 mm at 20°C and no antibacterial activity at 30°C. No antimicrobial activity was found for Atlantic salmon mucus against this organism.

It was notable that the inhibitory zones disappeared over time of the incubation suggesting a bacteriostatic effect for the mucus samples.

There was no antibacterial activity for mucus samples treated with ammonium sulphate. Results of sonicated mucus were not detectable because of contamination by normal bacteria which were not killed by sonication procedure.

Table 4.3. Inhibitory zones of skin mucus of some species of fish against a range of CFLB.

Fish species	*C. johnsonae*		*C. psychrophila*		*F. columnaris*		*F. maritimus*	
	20°C	30°C	15°C	20°C	20°C	30°C	20°C	30°C
Atlantic salmon	-	-	1.5	0.0	-	-	0.0	0.0
Rainbow trout	2.0-3.0	2.4-2.6	1.0-2.0	-	-	-	-	-
Barramundi	0.0	1.0	-	-	0.0	1.0	-	-
Flounder	-	-	-	-	-	-	0.5-1.0	0.0
Goldfish	2.4-2.5	2.2-2.9	0.8-2.2	1.0-2.0	0.0	0.7-1.4	1.0-2.2	1.5-1.7

Values are based on annular radius (mm).

-: Not tested.

Discussion

The variable results of this study may be due to the collection of the mucus at different times. The growth inhibitory capacity of skin mucus depends on age, diet, environmental temperature, and the existence or non-existence of stressors at the time of sample collection. The fish used to collect mucus were of different age and size and received different types of diet. The antibacterial capacity of fish mucus secretions may also vary with the species being investigated (Fujihara & Hungate, 1972) as was observed in this study. Another possible explanation for the results obtained is that many of CFLB are ubiquitous in the aquatic environment, so that experimental fish may have possessed a variable degree of pre-existing immunity to these organisms.

Concentration of the proteins in the mucus could be expected to result in more antimicrobial activity. Although, the samples were dialyzed to concentrate the proteins in the mucus, this method may not have adequately concentrated the macromolecular proteins.

No growth inhibition was found when mucus samples treated with ammonium sulphate was used. This may have been due to the effect of ammonium sulphate on the structure of the proteins in the mucus.

The mode of action of the active components of skin mucus on bacterial growth is, at present, mainly unknown. It remains to be determined whether there is a bacteriostatic or bactericidal effect. One possibility is that some bacterial populations were killed when challenged with mucus (bactericidal effect) and/ or organism growth was inhibited or decreased during the inoculation time and thereafter (bacteriostatic effect). Probably the mucus factors had a bacteriostatic effect because of the observed disappearance of inhibitory zones over time. However, the type of factor or factors involved in antibacterial activity, the mode of action and the mechanisms of the effect were not a part of this study.

A common character of the skin, gills and intestines of fish is mucus secretion and these organs comprise most of the external surfaces of the animals, which is the first line of defence against potential pathogenic microorganisms. The results obtained in this study suggest that skin mucus of naive fish may have some control over the growth of CFLB, and the naturally resistant fish generally have the most inhibitory mucus. The present data are insufficient, however, to adequately assess such antimicrobial activity of mucus of fish against fish-pathogenic bacteria. Further works using more sophisticated methods need to be undertaken in this field of the study.

CHAPTER V

In vitro Antimicrobial Sensitivity and *In vivo* Chemotherapy of *Cytophaga/Flexibacter*-like Bacterial (CFLB) Pathogens of Fish

Introduction

As discussed in Chapters I and III, with the recent rapid expansion of the aquaculture industry, increased losses due to *Cytophaga/Flexibacter*-like bacteria (CFLB) in both freshwater and marine environments have been reported (e. g. Wakabayashi *et al.*, 1986; Mudarris & Austin, 1989; Holliman *et al.*, 1991; Hilger *et al.*, 1991; Schmidtke *et al.*, 1991; Hansen *et al.*, 1992; Carson *et al.*, 1993; Frelier *et al.*, 1994; Wiklund *et al.*, 1994). *F. columnaris* affects a wide range of fish, while *F. maritimus*, a marine pathogen, has been reported from Australian, Japanese and European marine aquaculture farms. *C. psychrophila* is a serious pathogen in the salmonid industry, but is now known also to affect non-salmonid species. Other newly recognised pathogenic CFLB species such as *C. johnsonae*, *F. ovolyticus* and previously unrecognised species have recently been implicated in losses in the industry (e. g. Kent *et al.*, 1988; Hilger *et al.*, 1991; Pepin *et al.*, 1993; Frelier *et al.*, 1994).

Typically, but not always, pathogens of this group cause topical infections involving the skin and gills of fish (e. g. Kent, *et al.*, 1989; Austin & Austin, 1993; Carson *et al.*, 1993; Wakabayashi, 1993). Environmental conditions are frequently crucial in the expression of pathogenicity by these species in particular, water temperature has a major influence on the occurrence of disease due to *F. columnaris*, *C. psychrophila* and *F. maritimus* (e.g. Holt *et al.*, 1975; 1989; Wakabayashi 1991; 1993; Hamaguchi *et al.*, 1994). In the case of *C. johnsonae*, sudden changes in water temperature appear to be as important as actual water temperature in precipitating clinical disease (Carson *et al.*, 1993; Chapter III). Other factors which

have been demonstrated to influence the pathogenicity of these agents include water quality and stocking density (e.g. Chowdhury & Wakabayashi 1991; Carson *et al.* 1993).

The current methods for controlling disease conditions caused by bacteria in fish are either by improving husbandry or by chemotherapy. Optimisation of culture conditions are often not completely practicable in commercial fish farms, although improving managment provides a natural defence for fish with a reduction of losses (Alderman & Michel, 1992). The limitations of such general control methods and vaccines (with the exceptions of yersiniosis, vibriosis and furunculosis) have led to chemotherapy being considered as the most effective alternative strategy against infectious disease (Herman, 1970; Rucker, 1972; Gratzek, 1983; Stuart, 1983; Alderman & Michel, 1992), particularly when faced with major epizootic disease. Unfortunately, the data on an effective vaccines against CFLB are minimal with no available commercial vaccine so far.

Little information is available on the chemotherapy of fish-pathogenic CFLB (Austin & Austin, 1993). A number of antimicrobial compounds, notably oxytetracycline, oxolinic acid and sulphonamides have been advocated for treatment of these infections in fish (Austin & Austin, 1993; Munday, 1994). The recommended dosages are most often based on field results rather than determination of minimum inhibitory concentration (MICs) of the antimicrobial agents, bioavailbility of the antibiotic in the fish's organs or the *in vivo* clinical efficacy of the therapeutant against the disease (Carson, 1990; Munday, 1994).

Sulphonamides and oxytetracycline are used for the treatment of bacterial cold-water disease caused by *C. psychrophila* and salmonid cutaneous erosion disease caused by *F. maritimus*, but not always with satisfactory results (Holt *et al.*, 1993; Wakabayashi, 1993). These antimicrobial agents and oxolinic acid are also used for columnaris disease caused by *F. columnaris* (Hawke & Thune, 1992; Austin & Austin, 1993; Scott, 1993; Wakabayashi, 1993).

The recommended treatment levels of antibiotic in Atlantic salmon and rainbow trout, two important commercial species, have been established by a number of

workers (O'Grady *et al.*, 1986; Jacobsen, 1989; Hustvedt *et al.*; 1991, Inglis *et al.*, 1992; Cameron, 1993), but there are no data to demonstrate the actual dose rates by correlation with *in vitro* MIC and *in vivo* efficacy for the treatment of CFLB.

Therefore, there were five main objectives in this field of the study.

(1) To assess a modified microdilution agar procedure in order to determine the MIC values for these pathogens. This was an aim because of a lack of such methods for fish bacterial diseases many of which have different physiological requirements from pathogens of warm-blooded animals.

(2) To determine the MIC values of five field recommended antimicrobial compounds used in aquaculture against the Australian isolates of CFLB.

(3) To evaluate the achievable levels of antibiotics in the sera of barramundi, Atlantic salmon and rainbow trout.

(4) To assess the clinical efficacy of these compounds *in vivo* condition.

(5) Finally, to correlate *in vitro* and *in vivo* results to identify the standard dosages of the compounds for treatment of this group of fish pathogens.

Materials and Methods

1. *In vitro* Studies

1.1. Materials

1.1.1. Test organisms

Nineteen Australian strains of four species of CFLB, including *C. johnsonae*, *C. psychrophila*, *F. columnaris* and *F. maritimus*, were included in this study (Table 5.1). All isolates were recovered from infected freshwater barramundi, Atlantic salmon and rainbow trout and seawater Atlantic salmon with skin, gill, fin and tail lesions.

Table 5.1. Isolates of *Cytophaga/Flexibacter*-like bacteria (CFLB) used for MIC determinations.

Bacterial sp.	Strain	Source
C. johnsonae	91/0262-10	Barramundi, Northern Australia (1)
	91/3550-3; -4; 89/1795-Y	Golden rainbow trout, Tasmania (1)
C. psychrophila	91/4043-1; -11; -12; -16 and -17	Atlantic salmon, Tasmania (1)
F. columnaris	1468; 1438	Barramundi, Western Australia (2)
	87/0023	Rainbow trout, Tasmania (1)
	93/158-1	Atlantic salmon, Tasmania (1)
F. maritimus	89/4762; 89/0148-1; 89/0239-1	Atlantic salmon, Tasmania (1)
	89/0282-1; 89/0329-2; 89/0578-5	

(1) Fish Health Unit, Department of Primary Industry and Fisheries, Tasmania.

(2) Animal Health Laboratory, South Perth, Western Australia.

1.1.2. Antimicrobial agents

The following antimicrobial agents were included in this study: Amoxycillin (AM), oxytetracycline dihydrate (OTC), oxolinic acid (OA), norfloxacin (NO) and trimethoprim (TMP) (Sigma Chemical Co. St. Louis MO, USA).

1.2. Methods

1.2.1. Determination of minimum inhibitory concentration (MIC) of antimicrobials.

Test organisms were grown in modified *Cytophaga* broth (CB) (Anacker & Ordal, 1959a) containing tryptone 0.07% (w/v) (Difco, Detroit, Michigan, USA), yeast extract 0.05% (w/v) (BBL, Becton Dickinson Cockeysville, MD, USA), sodium acetate hydrated (Univar) 0.02% (w/v) (Ajax Chemicals), beef extract 0.02% (w/v) (BBL) at 20°C for 48 hours under aerobic conditions. MIC values were determined using *Cytophaga* agar (CA) supplemented with 1.1% (w/v) agar (Difco). Media were prepared in distilled water for all organisms except *F. maritimus* for which natural seawater (SCA) was used. The pH of the media was adjusted to 7.1. Antimicrobial agents were weighed using a five decimal scale. Stock solutions of 1 mg/ml of antimicrobial agents were then prepared using appropriate solvents (PBS 0.1M pH 8, HCL 0.05M, NaOH 0.1M, and lactic acid 88% were used to dissolve AM, OTC, OA-NO and TMP, respectively) and, stored at -70°C and used within five weeks.

Final plate concentrations were in doubling dilutions in the range 0.007-64 µg/ml. A multipoint replicator (H I Clements PTY LTD, Sydney) was used to inoculate ca. 10^4 CFU/spot of test organisms. Inoculated plates (two replicates per a dilution of antimicrobial agent) were incubated at 20°C aerobically for 24 hours. The spot of lowest concentration showing no growth was interpreted as the MIC. Reference strains of *Staphylococcus aureus* (ATCC 29213) and *Escherichia coli* (ATCC 25922) were included as controls. These strains were used because of their well known MICs for most of these antimicrobials (Washington II, 1985; Sahm & Washington II, 1991). MIC of the control strains was also determined in Mueller-Hinton agar (MHA, Oxoid) at the same temperature (20°C) to compare the obtained MIC values in CA and MHA (Washington II, 1985; Arthur, 1991).

1.2.2. Determination of Serum and Mucus Levels of Antimicrobials

The serum levels of antimicrobials were determined based on general guidelines given by Garrod *et al.* (1981) and Robertson & Edberg (1991). Standard curves were prepared for AM, OA and TMP in sterile water and normal Atlantic salmon serum using 50 µl per well. In the presence of serum all zones were either the same size or up to 1 mm smaller than zones formed from antimicrobials prepared in sterile water, so serum was selected for making the standard curves. Limits of detection for these antimicrobials were determined for a number of test organisms: *Bacillus subtilis* spore suspension (ATCC 6633, BBL) *Bacillus cereus* spore suspension (NCTC 2599), *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) (Garrod *et al.*, 1981; Robertson & Edberg, 1991). Antimicrobial levels in sera and mucus were then determined by bioassay in Mueller-Hinton agar (MHA) with *B. subtilis* spore suspension as the indicator organism.

The volumes of 0.15 ml of the spore suspension (5 x10^8-9 x10^8 spore/ml) were added to 75 ml MHA at 45-50°C, mixed quickly, and poured in 140 mm diameter plates. Plates were gently rotated on a flat surface for few secends, and they were then untouched for one hour. The wells in 7 mm diameter were then induced in the agar using a cork borer. Standards of the antimicrobials were prepared in Atlantic salmon serum with the following range of concentrations: OA (0.6-10 µg/ml), TMP

(0.3-10 μg/ml) and AM (0.16-5 μg/ml). Duplicate 50 μl samples of both test and standard sera were placed in the wells in each of four replicate of plates. Plates were placed at 5°C for two hours before incubation at 35°C overnight and zones of inhibition were then measured. The log concentration of standards was plotted against zone size and the line of best fit was computed by regression analysis. The concentration of antimicrobials in sera and mucus was then calculated. The value of r^2 for all curves was 0.997 at $P<0.001$. Untreated sera obtained from Atlantic salmon and rainbow trout were aslo tested in separated plates as the controls to demonstrate any inhibitory zone when using serum without the antimicrobials.

The concentration of antimicrobial agents in water samples of bath treatments was also determined. Samples were diluted in sterile water and the level of compounds was then determined in the same procedure described for bioassay of sera and mucus, but standard dilutions of antibiotics prepared in sterile water were used.

2. *In vivo* Studies

2.1. Materials

2.1.1. Fish

Barramundi 2-5 g body weight, Atlantic salmon 18-52 g body weight and rainbow trout 53-138 g body weight were used to verify the *in vivo* efficacy of OA (nominally 100%), AM (84%) and TMP (40%). The barramundi, Atlantic salmon and rainbow trout were maintained in 40 l aquaria or 300 l tanks containing freshwater or seawater with re-circulation and biofiltration. Also, barramundi of 44-101 g body weight and rainbow trout of 220-479 g body weight were used to determine the bioassay of the antimicrobial compounds in the fish sera. Fish densities for challenged barramundi, Atlantic salmon and rainbow trout were 1.8, 2.3 and 7.3 g/l, respectively. Water exchange was maintained at 5-10% per day and water quality parameters including pH, total ammonia, dissolved oxygen and salinity were checked daily.

2.1.2. Cultures

Clinical isolates of *F. maritimus* 89/4762 and *F. columnaris* 1468 were used to challenge the fish. The former had been recovered from a case of SCED in sea-

caged Atlantic salmon in Tasmania and the latter was isolated from freshwater-caged barramundi with columnaris disease in Western Australia.

Prior to challenge, the isolates were passaged twice in appropriate hosts to enhance their pathogenicity. *F. columnaris* was passaged in barramundi by bathing (1.0×10^8 cells/ml) or intraperitoneal injection (1.0×10^7 cells/fish) and *F. maritimus* was passaged in Atlantic salmon by bathing (10^8 cells/ml). The organisms used for the challenge were three-day cultures of the last passage grown in CB at 23-24°C with gentle agitation.

2.2. Methods

2.2.1. Bath Challenge

Three replicates of 21 barramundi were challenged with *F. columnaris* at a concentration of 2.1×10^5 CFU/ml of tank water at 25°C for one hour. Two replicates of 20 Atlantic salmon and 23 rainbow trout were challenged with *F. maritimus* at concentrations of 4.3×10^6 and 4.7×10^6 CFU/ml of tank water, respectively at 16°C for one hour. Equivalent replicates of control groups (infected and unmedicated) were included for each experiment. Fish were exposed to the pathogens in their holding aquaria/tanks to reduce stress associated with the challenge procedure. At the end of the exposure period the tanks were drained as far as practicable and refilled with clean water. In addition, two replicates of 18 barramundi and two replicates of 15 Atlantic salmon and rainbow trout were used as negative controls (uninfected unmedicated) to monitor any mortality owing to handling and grading during the experiments. Negative controls fish were exposed to sterile CB for one hour. These fish were held in the isolated tanks to avoid any direct transmission of the infections. The barramundi were observed for 14 days and the Atlantic salmon and rainbow trout were observed for 16 days.

2.2.2. Treatment regimes

Treatment regimes are given in Table 5.2. Bath and feed medication treatments against *F. columnaris* were undertaken three and four hours following challenge, respectively. Treatment against *F. maritimus* was undertaken 12 hours post-

challenge. These strategies of treatments were selected based on the level of virulence of the pathogens used as the results obtained in Chapter III showed that columnaris disease is a rapid infection requiring a treatment soon after exposing the fish to the pathogen, to have left treatment longer would have led to massive mortality and negated the study. However, the progress of infection by *F. maritimus* is slower than columnaris disease and, therefore, later treatment was appropriate. The feed-medication groups were fed 0.8-1% body weight of medicated feed to encourage fish to take all feed. Medicated diets were prepared daily by surface coating pellets (Tropical Fish Crumble or Salmon Feed, Gibson's Feed Mill) with gelatin. Appropriate amounts of the antimicrobial agents were weighed and dissolved by the solvents previously mentioned and stored in small volumes at -70°C until used during the experiments. Gelatin (38g/ml) was prepared in tap water, allowed to cool to a tepid solution before adding the antibiotics. The pellets were coated using a Pasteur pipette and care was taken to coat all pellets. The control fish and bath treated groups were fed antimicrobial-free gelatin coated pellets.

Table 5.2. Bath and feed treatment regimes of antibacterial compounds for treatment of salmonid cutaneous erosion disease (SCED) and columnaris disease.

Antimicrobial agent	Treatment	Fish species
Oxolinic acid	Oral: 10 mg/kg b w/day for 10 days	Barramundi
"	Bath: 50 ppm for 1 h	"
Amoxycillin	Oral: 80 mg/kg b w/day for 10 days	Rainbow trout
"	Bath. 200 ppm for 1 h	Atlantic salmon
Trimethoprim	Oral: 10 mg/kg b w/day for 7 days	Rainbow trout
"	Bath: 50 ppm for 1 h	Atlantic salmon
"	Bath: 100 ppm for 1 h	"

2.2.3. Confirmation of infection and statistical analysis of data

Wet mounts and Gram stain preparations were made daily from lesions of each clinically affected or dead fish. Lesion material was cultured on selective CA or SCA (Carson, 1990; Hawke & Thune, 1992) and typical colonies identified. The immunofluorescent antibody test (IFAT) (Chapter III) was used as a confirmatory

test for *F. maritimus.* Twenty samples (10 from Atlantic salmon and 10 from rainbow trout) were collected at random for this purpose.

Data were analysed by one way ANOVA after confirmation of homogeneity of variance with Cochran's test. Treatment procedures were treated as a fixed factor. Pairwise comparisons of means were conducted using Fisher's Least Significant Difference (LSD) test.

2.2.4. Concentration of antimicrobial agents in fish serum and mucus

Duplicate groups of 13 barramundi were given bath and feed-medicated treatments with OA at water temperatures of 25 and 27°C, respectively. Two groups of 15 seawater-adapted Atlantic salmon were bath exposed at 15°C to AM and TMP. Serum levels of AM and TMP for feed-medication were determined in duplicate groups of five rainbow trout maintained at 14°C in freshwater. The dosages of the antimicrobial agents were similar to the treatment regimes detailed in Table 5.2. In addition, AM was administrated by feed-medication at 60 mg/kg body weight in rainbow trout.

Atlantic salmon and barramundi exposed by bath treatment were bled four and 24 hours post-dosing. Orally-treated barramundi and rainbow trout were fed medicated feed for five days and bled 12 hours after the last feeding on Day 5. Pooled blood samples were allowed to clot for 30 minutes, centrifuged at 1200 g for 10 minutes and the separated sera stored at -70°C and used within 72 hours. The sera of 10 untreated Atlantic salmon and five untreated rainbow trout were also obtained as the control.

Skin mucus samples from bath-treated fish were collected at the same time as blood collection, i.e. four hours post-dosing, by scraping lateral and dorsal areas with a glass slide. The mucus samples were diluted in sterile water (4:1, mucus: water), centrifuged and then stored at -70°C until used. Water samples of bath treatments were also collected at the commencement of treatment and one hour post-treatment to determine whether extraneous substances in freshwater or seawater affect the activity of the compounds.

Results

1. *In vitro* Studies

1.1. MIC values

MIC results are shown in Table 5.3. MIC values for control organisms were within the acceptable error, i.e. ±1 $\log_2$, of known values (Washington II, 1985; Sahm & Washington II, 1991). MIC values of reference strains were in a similar range for both CA and MHA (Table 5.3).

Quinolone compounds were in the range 0.12-4.0 and 16->64 µg/ml against freshwater and marine strains of CFLB, respectively. MIC for TMP was above 64 and 0.5 µg/ml against freshwater and marine strains, respectively. AM and OTC gave MIC in the range 0.06-4.0 and 0.12-2 µg/ml, respectively, against both freshwater and marine CFLB.

Table 5.3. Susceptibility of 19 strains of *Cytophaga/Flexibacter*-like bacteria (CFLB) to 5 antimicrobial agents.

Bacterial species	No. of strains	MIC µg/ml AM	OTC	OA	NO	TMP
F. columnaris	4	0.06	0.06-0.12	0.06-0.12	0.12	>64
F. maritimus	6	0.25-0.5	2.0	≥64	8-16	0.25-0.5
C. johnsonae	4	2.0-4.0	2.0	0.25-0.5	2.0-4.0	>64
C. psychrophila	5	0.03-0.06	0.5-2.0	0.12-0.25	0.5	≥64
E. coli ATCC 25922 (a)		4.0	1.0-2.0	0.03-0.06	0.03	4.0
" (b)		4.0-8.0	2.0	0.03-0.06	0.06	2.0-4.0
S. aureus ATCC 29213 (a)		0.25-0.5	0.25-0.5	0.5	0.5	2.0-4.0
" (b)		0.25-0.5	0.5-1.0	0.25-0.5	0.5-1.0	1.0-2.0

AM: Amoxycillin, OTC: Oxytetracycline, OA: Oxolinic acid, NO: Norfloxacin, TMP: Trimethoprim.

(a): Indicating the MIC values were obtained in CA. (b): Indicating the MIC values were obtained in MHA.

1.2. Bioassay of sera and mucus

Data obtained for serum levels of treated fish are included in Table 5.4. The smallest measurable zones in the standards occurred at 0.6, 0.3 and 0.16 µg/ml for OA, TMP and AM, respectively with no zone at the next lower concentration. For each of the

antimicrobial agents the levels in serum samples collected 24 hours post-treatment of bath-treated fish were not detectable though these agents were present in detectable quantities at four hours post-dosing. No antimicrobial agents were detected in the mucus samples collected four hours post-dosing. No inhibitory zone was recorded for untreated sera (controls). There were no significant differences between water concentrations of antimicrobial agents at time zero and one hour post-dosing ($P>0.005$).

Table 5.4. Serum levels for three antimicrobial agents in barramundi, Atlantic salmon and rainbow trout.

Dosage of antimicrobial	Fish species	Serum level (µg/ml)		
		OA	AM	TMP
Feed treatment*				
10 mg/kg b w	Barramundi	3.4	-	-
10 mg/kg b w	Rainbow trout	-	-	0.8
60 mg/kg b w	"	-	0.1	-
80 mg/kg b w	"	-	0.2	-
Bath treatment**				
50 ppm	Barramundi	0.5	-	-
50 ppm	Atlantic salmon	-	-	0.4
100 ppm	"	-	-	0.4
200 ppm	"	-	0.6	

*Blood was collected 12-hours post last feeding on day 5.

**Blood was collected four hours post-dosing. -Not applicable.

2. *In vivo* Studies

Efficacy of treatments

Mortalities were confirmed to be due to the agent of interest by Gram stains of smears, culture and IFAT of lesion materials. Of total samples (93) of barramundi checked for Gram stain and plate culture, 90 (96.8%) and 74 (79.6%) were positive for *F. columnaris*, respectively. Of 68 dead Atlantic salmon, 65 (95.6%) and 51 (75%) were positive for *F. maritimus* by using Gram stain and plate culture techniques, respectively. Also, of 58 dead rainbow trout, 58 (100%) and 44 (64.7%) were positve for *F. maritimus* by using Gram stain and plate culture techniques,

respectively. All 20 samples, which were checked by IFAT were positive for *F. maritimus*.

2.1. Efficacy of OA in barramundi

Among the replicates, mortality range of 19-38.1% and 28.6-42.9% occurred in replicates of bath and oral treatments, compared with a mortality range of 66.7-95.2% for infected nonmedicated replicates (Table 5.5). Of the negative controls (uninfected unmedicated) only three fish died by the termination of the experiment with no sign of the infection.

Table 5.5. Mortality rate in barramundi exposed to *F. columnaris* and treated with oxolinic acid by immersion and feed-medication.

Treatment	Rep.	No. of dead fish	Mortality per rep. (%)	Mortality per total fish(%)	Mean of total mortality (%) ± SE
Immersion (50 ppm)	1	6	28.6	9.5	28.5 ± 5.5
	2	4	19	6.3	
	3	8	38.1	12.7	
Feed-medication (10 mg/kg b w)	1	8	38.1	12.7	36.5 ± 4.2
	2	6	28.6	9.5	
	3	9	42.9	14.2	
Control (infected non-medicated)	1	14	66.7	22.2	82.5 ± 8.4
	2	18	85.7	28.5	
	3	20	95.2	31.7	

Each replicate originally contained 21 fish.

Mortalities in fish treated by bath and feed-medication against infection were significantly lower ($P<0.01$) than the mortalities in non-medicated fish (Fig. 5.1). Bath treatment resulted in slightly more survival than oral-medication. However, there were no significant differences ($P>0.05$) between treatment regimes. The infection pattern (Fig. 5.2) showed a rapid establishment of columnaris disease during the first 48 hours post-infection consistent with a virulent organism (Section 3.2.3., Chapter III; Wakabayashi, 1993).

Fig. 5.1. Total mortality in barramundi infected with *F. columnaris* and treated with oxolinic acid by bath (50 ppm for one hour) and feed-medication (10 mg/kg b w/day for 10 days). Means sharing a common superscript are not significantly different (P>0.05).

(Mean ± SE, n = 3 replicates of 21 fish)

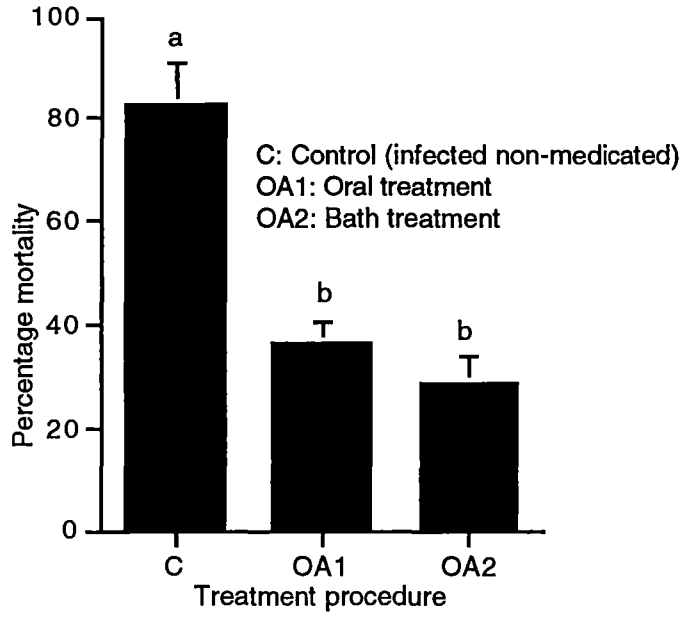
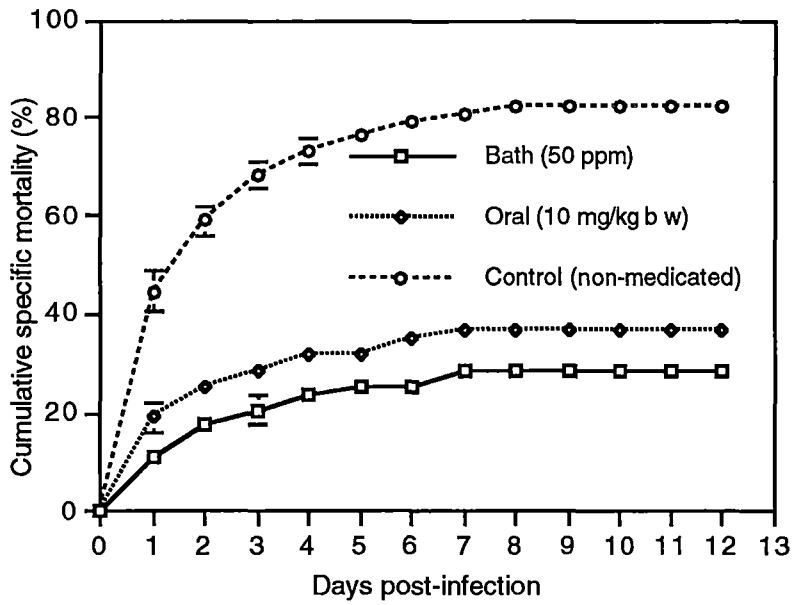


Fig. 5.2. Cumulative mortality of barramundi infected with *F. columnaris* and treated with oxolinic acid by bath and feed-medication. (Mean ± SE, n = 3 replicates of 21 fish).



2.2. Efficacy of AM and TMP in Atlantic salmon

Bath treatment of Atlantic salmon with TMP at either 50 or 100 ppm was more effective (P<0.05) than AM at 200 ppm against *F. maritimus* (Table 5.6). Treatment at these dosages resulted in 78.5%, 87.5% and 62.5% survival, respectively compared with only 2.5% survival for control groups (Fig. 5.3). Mortalities of bath

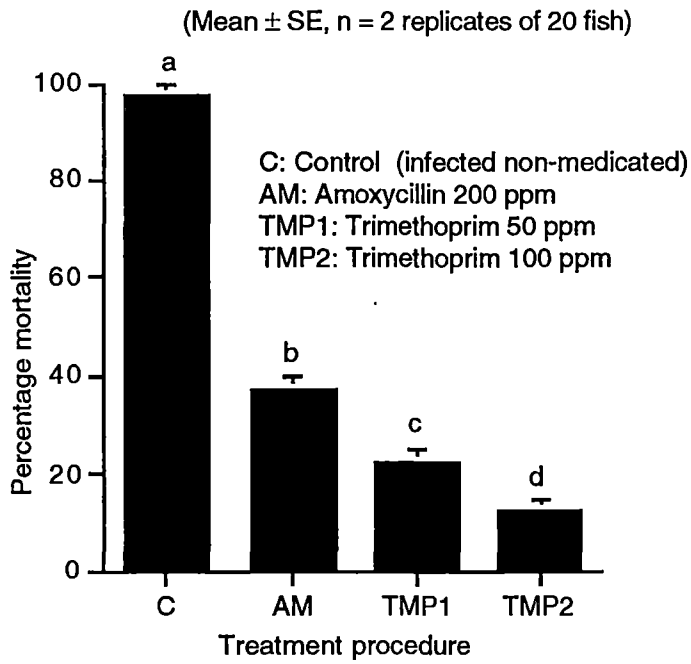
treated fish were significantly lower ($P<0.01$) than mortalities in non-medicated fish. There was also a significant ($P<0.05$) difference in clinical efficacy between the use of TMP at 50 and 100 ppm in the treatment of the infection. There were a total of two dead fish for the negative controls (uninfected unmedicated).

Table 5.6. Mortality levels in Atlantic salmon exposed to *F. maritimus* and treated by amoxycillin and trimethoprim baths.

Treatment	Rep.	No. of dead fish	Mortality per rep. (%)	Mortality per total fish (%)	Mean of total mortality (%) ± SE
Amoxycillin (200 ppm)	1	8	40	20	37.5 ± 2.5
	2	7	35	17.5	
Trimethoprim (50 ppm)	1	4	20	10	22.5 ± 2.5
	2	5	25	12.5	
Trimethoprim (100 ppm)	1	3	15	7.5	12.5 ± 2.5
	2	2	10	5	
Control (infected non-medicated)	1	20	100	50	97.5 ± 2.5
	2	19	95	47.5	

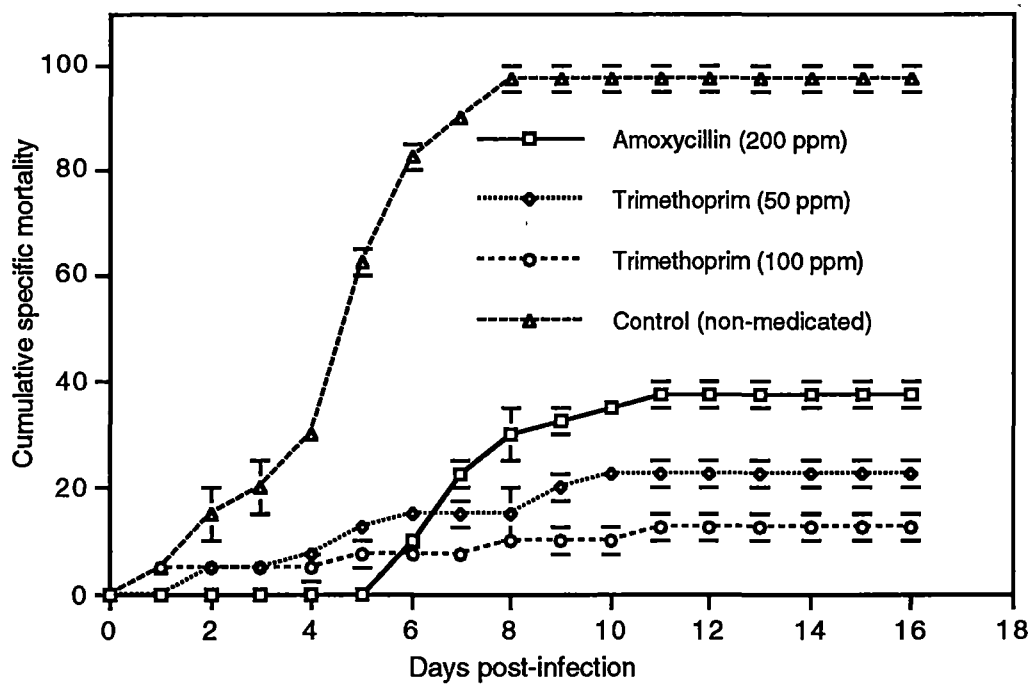
Each replicate originally contained 20 fish.

Fig. 5.3. Total mortality in Atlantic salmon infected with *F. maritimus* and treated by AM and TMP baths for one hour. There is a significant difference ($P<0.01$) between mortality levels of treatment and control groups and among different treatment regimes ($P<0.05$).



The pattern of mortality in AM treated fish (Fig. 5.4) shows that there was no infection in bath treatments during the first few days post-challenge, while mortalities commenced in control groups within 48 hours following challenge and it was significant during days four to eight post-exposure (Fig. 5.4).

Fig. 5.4. Cumulative mortality of Atlantic salmon infected with *F. maritimus* by immersion and treated by bath with AM and TMP for one hour. (Mean ± SE, n = 2 replicates of 20 fish).



2.3. Efficacy of AM and TMP in rainbow trout

The efficacy of AM and TMP incorporated in rainbow trout feed highlights the superiority ($P<0.05$) of TMP at 10 mg/kg b w (Table 5.7, Fig. 5.5). While 30.5% mortalities were observed in fish treated with AM at 80 mg/kg b w, those treated with TMP had only 13% losses. Control groups had significantly ($P<0.01$) higher mortalities than either of the treated groups. No mortality was recorded for negative controls (uninfected unmedicated).

Lesions appeared in AM medicated groups at two days post-challenge and were of a low level but constant up to 10 days post-challenge, while fish treated with TMP were better protected against overt infection during the experiment (Fig. 5.6).

Table 5.7. Mortality levels in rainbow trout exposed to *F. maritimus* and treated orally with amoxycillin and trimethoprim.

Treatment	Rep.	No. of dead fish	Mortality per rep. (%)	Mortality per total fish (%)	Mean of total mortality (%) ± SE
Amoxycillin (80 mg/kg b w)	1	8	34.8	17.4	30.5 ± 4.35
	2	5	26.1	13	
Trimethoprim (10 mg/kg b w)	1	2	8.7	4.4	13 ± 4.35
	2	4	17.4	8.7	
Control (infected nonmedicated)	1	20	87	43.5	84.8 ± 2.18
	2	19	82.7	41.3	

Each replicate originally contained 23 fish.

Fig. 5.5. Total mortality in rainbow trout infected with *F. maritimus* and treated orally with AM and TMP for 10 and seven days, respectively. Mortalities of treatment groups are significantly different ($P<0.01$) from control groups. There are also significant differences ($P<0.05$) between treatment regimes. (Mean ± SE, n = 2 replicates of 23 fish).

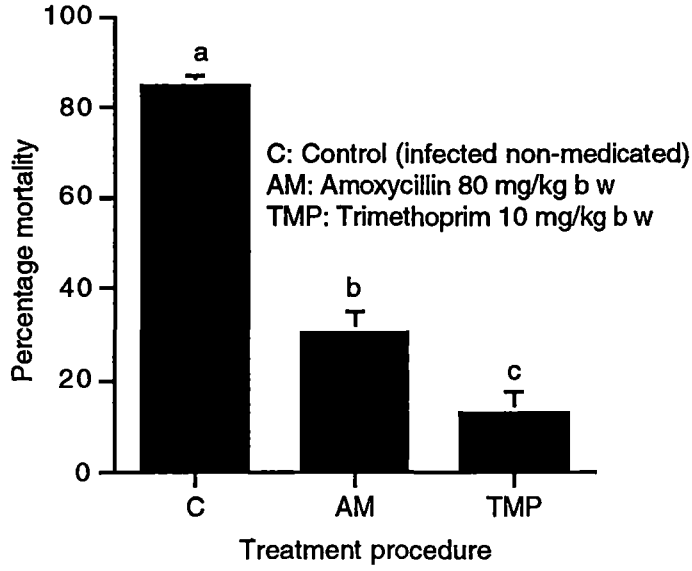
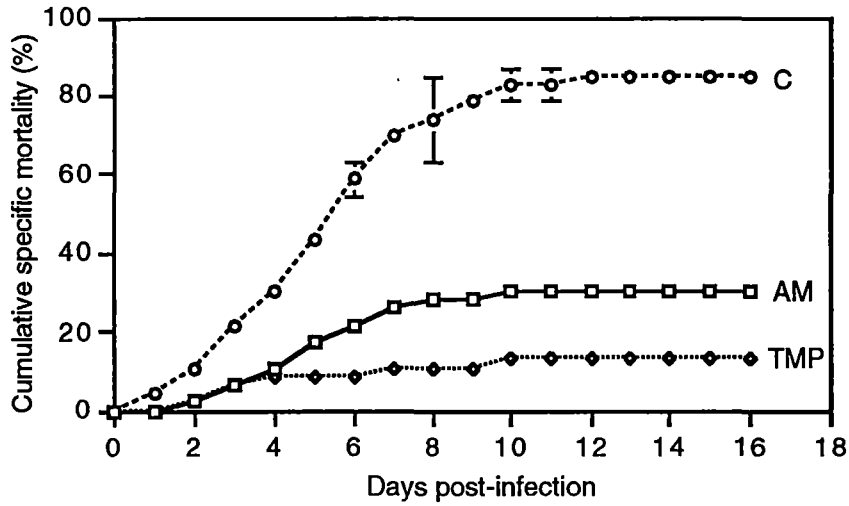


Fig. 5.6. Cumulative mortality of rainbow trout infected with *F. maritimus* by immersion and treated by feed-medication with AM and TMP. (Mean ± SE, n = 2 replicates of 23 fish).
C = Control (non-medicated), AM = Amoxycillin (80 mg/kg bw), TMP = Trimethoprim (10 mg/kg b w).



Discussion

Many of the recommended dose rates of antimicrobials used in aquaculture in Australia have been determined on the basis of clinical responses or transferred directly from recommendations for mammals, and only a minority have been fully validated under local conditions by determination of the combination of minimum inhibitory concentration (MIC), effective tissue level of the chemotherapeutic

substance and/or a critical experimental infectivity trial. Ideally, correlation between serum levels, MIC values and clinical efficacy need to be established prior to treatment strategies.

This study provides original data correlating serum antibiotic levels, MIC values and clinical efficacy. It also validates the use of CA for the determination of MIC values (Table 5.3). This is because standard Mueller-Hinton medium (MHM) inhibits adequate growth, in some instances eliminating growth completely, of many CFLB (Hawke & Thune, 1992; Thune, 1993; author's own experience) because of their fastidious character and low nutrient requirement (Austin & Austin, 1993). However, MIC value for OTC against four strains of *F. columnaris* in modified MHM was in a similar range (0.195 µg/ml) (Hawke & Thune, 1992) to those obtained in this study. Therefore, in this study the evaluation of *Cytophaga* medium as the most commonly used medium for isolation and cultivation of these bacteria showed that it may be employed as a suitable medium for the determination of MICs for the antimicrobials tested here. Furthermore, similar MIC values to those obtained in this study have been reported for some strains of CFLB using *Cytophaga* medium (Baxa *et al.*, 1988c; Carson *et al.*, 1993).

The MIC values obtained in this study showed the bactericidal quinolones, OA or NO, are suitable compounds for columnaris disease, cold-water disease and *C. johnsonae* infection, but would not be suitable for natural outbreaks of marine *Flexibacter* infection because of the high MIC (8 - ≥64 µg/ml) obtained for this organism in this study. The drug efficacy against columnaris disease highlights that OA is effective for treatment of columnaris disease at dosages of either 50 ppm (bath) or 10 mg/kg b w (oral). The bath treatment gave higher survival than feed-medication, possibly because infection may have occurred in fish which either did not take the feed-medication or became anorexic immediately post-challenge. Even so, there were no significant differences between the treatment regimes ($P>0.05$). The serum level at dosage 10 mg/kg b w was more than 50 times the MIC values, suggesting a much lower oral dosage such as 5 mg/kg b w would be efficacious when facing natural outbreaks of columnaris infection. Alternatively, the treatment

period could be reduced from 10 to five days. These findings also suggest the bath dosage may be lowered to 30 ppm for up to two hours when facing intermediate to low virulence of the outbreaks, because columnaris infection is more of a superficial disease than a systemic infection. However, although lower dosage may reduce the level of infection to the point where the fish's defence mechanisms aid in recovery, it may promote development of antibacterial resistance.

The serum level of AM by bath immersion at 200 ppm was above the MIC value for *F. maritimus* at four hours post-dosing, and fell to below the MIC after 24 hours. However, the clinical efficacy of this antibiotic in Atlantic salmon was significant with mortalities in treated fish being 37.5%, compared with 97.5 % in untreated fish ($P<0.01$). Bioassay of sera from rainbow trout given AM incorporated in feed at 80 mg/kg b w revealed that the level was below the MIC value. *In vivo* assessment of the drug efficacy, however, showed that it may be used to treat SCED at this dosage because of significant differences ($P<0.01$) between the treatment and control rainbow trout challenged with *F. maritimus*. Mortalities in fish given bath treatment with AM started about one week post-infection (Fig. 5.4). This could be due to the gradual decrease of antibiotic concentration in tissues. This interpretation is supported by the known short withdrawal period reported for this compound (Brown & Grant, 1992; Elston, 1992; Inglis *et al.*, 1992). The correlation, however, of *in vitro* and *in vivo* findings shows the efficacy of AM for control of SCED. Comparison between serum levels of AM and TMP with similar MIC values against *F. maritimus* showed a high uptake of TMP by fish tissues (Table 5.4).

Data on the use of TMP as a single chemotherapeutic substance against bacterial fish diseases are scant (Cameron, 1991). This pteridine dihydrofolic acid component analogue is usually used as one of the components of the potentiated sulphonamide combinations. However, the use of sulphonamide component, sulphadiazine, is unsuitable for the control of *F. maritimus* infection because of inherent bacterial resistance (MIC>100 µg/ml) (J. Carson, pers comm). As TMP is mobilised to, and persists in the skin (Jacobsen, 1989; Cameron, 1993), it is an ideal antibacterial agent for the control of superficial infections. The MIC values of this

compound obtained in this study indicated that TMP is not a suitable antibacterial compound for CFLB, except for *F. maritimus* infection. Serum levels of this compound at dosages of either 50 or 100 ppm (bath) and 10 mg/kg b w (oral) were generally above MIC values and in some instances up to four times of MIC values for the examined isolates of *F. maritimus* (Table 5.4). Clinical efficacy of this compound in both Atlantic salmon and rainbow trout highlights the effectiveness of TMP against SCED. TMP possesses a withdrawal period however of up to 60 days when used at five mg/kg b w for five days (Jacobsen, 1989). The correlation of *in vitro* and *in vivo* results indicates the value of a reduction in both dosage treatments from 20 to 10 mg/kg b w and treatment period from 10 to five days.

The absence of the antibiotics in mucus, at least within the sensitivity of the test used, needs comment. This apparent lack of transfer from the bloodstream to mucus could possibly be attributed to lack of transfer. However, in affected fish this may not hold as antibiotic could be transferred with serum leaking from superficial lesions.

In this study, infected barramundi were treated three to four hours post-infection and Atlantic salmon and rainbow trout were treated 12 hours post-infection: times which provided adequate opportunity for bacterial invasion of the external surfaces and render some fish moribund before the therapeutic regimes commenced. This situation occurs especially in the case of rapidly progressive infections such as columnaris disease. It is therefore pertinent to treat infected fish as soon as possible after diagnosis even though oral treatment may even then be ineffective in fish with advanced disease.

The correlation of *in vitro* and *in vivo* results indicate AM is a suitable therapeutic agent against *C. psychrophila*, *F. columnaris* and *F. maritimus*. OA is useful against *C. johnsonae*, *C. psychrophila* and *F. columnaris*, NO against *C. psychrophila* and *F. columnaris*, OTC against *F. columnaris* and *C. psychrophila* and TMP against *F. maritimus*. TMP is superior to AM for treatment of SCED caused by *F. maritimus*. These results raise important issues regarding the definition of laboratory and field studies in the evaluation of chemotherapeutic substances in aquaculture.

CHAPTER VI

General Discussion and Conclusion

The initiative for this study came from the perception that *Cytophaga/Flexibacter*-like bacteria (CFLB) are significant problems in both freshwater and marine aquaculture in Australia. The study was conducted to compare the responses of these fish-pathogenic organisms to environmental conditions *in vitro* together with the *in vitro* and *in vivo* pathogenesis and chemotherapy of the causative agents.

1. Pathogenesis

1.1. *Cytophaga johnsonae*

Carson *et al.* (1993) described an outbreak of *C. johnsonae*-associated disease in barramundi in freshwater when water temperature decreased from 35 to 27°C. The present study describes for the first time experimental reproduction of the infection by *C. johnsonae* and confirms the role of this species of gliding bacteria as a fish pathogen, especially in barramundi. Experimental induction of the infection in barramundi was achieved only through a sudden change in water temperature from 28-20°C (Chapter III). Comparison of natural and experimental infections by this organism shows that sudden changes in water temperature appear to be as important as the actual water temperature in precipitating clinical disease. This experimental condition apparently places the fish in an environment conducive to the growth of *C. johnsonae* (Fig. 2.2, Chapter II) and also probably suppresses the immune response of the host (Bly & Clem, 1991; Szalai *et al.*, 1994). In addition, based on the results of the physiological requirements of the pathogen (Figs. 2.5 & 2.15, Chapter II), maintenance of the temperature ≥ 35°C, if possible, e.g barramundi, and /or salinity above 20 g/l, may reduce the growth of *C. johnsonae* sufficiently to reduce morbidity.

The reason why barramundi are more susceptible to *C. johnsonae* infection than the other species investigated in this study is not entirely understood. The

study of skin mucus of fish showed it is less inhibitory to *C. johnsonae* than that of the other fish species investigated (Chapter IV).

Because of its role as a new fish pathogen, the pathogenicity of *C. johnsonae* may need to be assessed in other commercial, tropical or temperate species. Chitinase or chitinobiase enzymes seem to have a significant role in the progress of infection as scale thinning and scale invasion by the organism have been demonstrated in barramundi (Carson *et al.*, 1993). Strains of *C. johnsonae* do not release an extracellular chitinase, but need close contact with the chitin particles to hydrolyse them (Christensen, 1977a). Sticky adhesive character of fish mucus together with the slime layer produced by gliding bacteria may provide sufficiently close contact for a long time until the organisms become localised and proliferate *in situ*. Also, the possible roles of other biochemical, enzymatic and cell surface properties as potential indicators of virulence in *C. johnsonae* warrant further investigation.

1.2. *Cytophaga psychrophila*

The geographical distribution of *C. psychrophila*, the agent of bacterial cold-water disease, or low temperature disease was originally believed to be limited to North America (Chapter I). Recently, however, this pathogen has been reported in several kinds of fish in Europe, Japan and, more recently, in Australian aquaculture (Chapter III). The pathogenicity of the pathogen may well be related to a lowered immune response of fish at low temperatures (Holt *et al.*, 1989; 1993; Ainsworth *et al.*, 1991). Natural outbreaks are often reported in rainbow trout fry at water temperatures ranging from 4-15°C, and pathogenicity tests were often successful at similar range with the best at 12-15°C (Holt *et al.*, 1989; 1993). Virulent isolates can also infect larger fish (Wiklund *et al.*, 1994). The Australian isolate of this pathogen in this study did not affect rainbow trout fingerling or goldfish in *in vivo* tests using different routes of challenge. This shows that this strain may not possess a virulence factor. Also, natural incidence of infection in Atlantic salmon was recorded as being very low. Therefore, it is likely that this strain of *C. psychrophila* may affect fish that are only subject to a number of stressors; otherwise, its role as a pathogenic organism is

uncertain, or it may be a host specific organism. However, should disease occur or be likely to occur, maintenance of temperature above 20°C may significantly reduce mortality, particularly if salinity can be kept above 10 g/l, because the growth rate of many strains of *C. psychrophila* is also reduced at these levels (Figs. 2.5 & 2.15, Chapter II).

1.3. *Flexibacter columnaris*

The impact of water temperature on the progress of disease by *F. columnaris* has been studied in a number of commercial species of fish such as salmonids and weatherfish (Holt *et al.*, 1993; Wakabayashi, 1993). In this study higher mortality was found at 25°C than 20°C in barramundi and goldfish challenged with *F. columnaris* strain1468 (Chapter III). This may be explained to some extent by the slower growth rate of the bacterium at the lower temperatures as the doubling time at 15 and 20°C was more than twice and 1.6 times of those at 25-30°C (Chapter II). Therefore, temperature ≤15°C and, where possible, salinity ≥ 10 g/l may be useful in control of *F. columnaris* infection.

Experimentally induced disease was severe in both barramundi and goldfish with greater susceptibility for barramundi (Chapter III). There is no single valid dose to achieve 50% mortality (LC_{50}) for columnaris disease in fish. Because it will be different, depending on the level of virulence of the organism, species of fish, stocking density and water quality during the challenge time and thereafter. A valid LC_{50} for this pathogen should be obtained under appropriate conditions, especially using a freshly isolated strain and the original host from which the pathogen was recovered. In this study the LC_{50} of a barramundi isolate of *F. columnaris* was validated in the barramundi and was also compared with goldfish as a known susceptible host. The pattern of infectivity in barramundi clearly showed that this species is one of the highly susceptible fish to the pathogen. The minimum time to death in this species was less than 10 hours, indicating the high virulence of this Australian isolate similar to those strains studied by Pacha and Ordal (1970).

Histological changes were similar in both species of fish with acute necrosis of the superficial epithelium and gill necrosis, but internal organs remained intact.

These histopathological findings are similar to those previously reported in a number of coldwater or warmwater species of fish (Chapter I), and histopathological features owing to *F. maritimus* studied here. However, infection by *F. columnaris* is clinically somewhat different from marine *Flexibacter* disease. Using the appropriate host in this study, it was evident that the barramundi isolate of *F. columnaris* caused a rapid and acute infection in both barramundi and goldfish, while the progress of marine *Flexibacter* infection was relatively slow in salmonids. Without further studies the reasons are speculative, but it may be due to differences in the host, level of virulence of the powerful exotoxins, e.g. protease, and/ or the amount of these exotoxins produced by the isolates used here. Histological findings also showed the severity of these toxins produced by *F. columnaris* brought about acute necrosis in the cutaneous layer of the infected animals.

1.4. *Flexibacter maritimus*

This study describes the pathogenicity of *F. maritimus* and the histopathological features of infection with this pathogen in a number of species of fish, in particular salmonids under Australian conditions.

Comparison of the results of Trials 1 and 2 with Trials 4 and 5 (Table 3.2, Chapter III) confirms that the greater susceptibility shown in both Atlantic salmon and rainbow trout in Trials 4 and 5 was due to increased virulence as a result of passage of *F. maritimus*, as previously observed by Carson *et al.* (1992). Both natural and experimental marine *Flexibacter* infection of Atlantic salmon and rainbow trout indicated an identical range of lesions in these two species, with a slightly higher level of susceptibility of Atlantic salmon under Tasmanian conditions (Chapter III). The pattern of infection was also similar in both salmonids with LC_{50} in the range 2.3 x 10^5-1.6 x 10^6 CFU/ml at a temperature range 16-20°C and full salinity. Higher susceptibility to *F. maritimus* was found in younger and smaller sizes of Atlantic salmon than in older and larger size fish. This concurs with the higher incidence of natural outbreak in post-smolts than older fish. Work by Fujihara *et al.* (1971) showed older and larger rainbow trout and chinook salmon were less susceptible than younger and smaller sizes to *F. columnaris* infection. One likelihood is that the

fish external barriers, such as softness of scales, thinness of skin and mucus layer in smaller size fish, may provide a convenient condition for localization and colonization of the bacteria on the external surface. Other parameters such as an elevated immunity in older fish, may also affect the progress of infection.

Histological study of mature lesions showed a remarkable lack of inflammatory response with bacteria invading dense connective tissue and occasionally underlying musculature. The earliest erosions showed consistent fragmentation and degeneration of the epithelium with infiltration of amorphous protein-like materials and occasional intra-epithelial cellular inflammatory cells, congestion and haemorrhage. Scale loss, odema and low degree of inflammation in scale pockets were evident, but the remaining scales were intact. There was a great consistency in the histopathology in salmonids and non-salmonid species studied here.

Correlation of natural outbreaks of marine *Flexibacter* disease with traumatic episodes provides a logical predisposing cause. The often intact epithelium of early lesions, following trauma, may be due to successful rapid migration of the adjacent epithelium covering small lesions from scale loss, as described by Bullock & Roberts (1992). However, the most severe natural outbreaks in salmonids were related to warm water conditions and extended periods of sunny, cloudless days rather than trauma. Rodger (1991) has already reported the presence of lesions indistinguishable from those of marine *Flexibacter* disease in fish exposed to excessive solar irradiation. Although no unequivocal "sunburn" cells were seen in the material studied from natural outbreak, some early lesions of natural infections were suggestive of the spongy changes reported by Bullock (1988) as a result of ultraviolet (UV) irradiation. The likelihood of these lesions being initiated by excessive UV light is strengthened by the lack of such changes when trauma was identified as a pre-disposing cause. There was a similar epithelial finding in early lesions of experimental infections under artificial lighting. High density of bacterial cells or their toxin production in challenge may act as a predisposing factor initiating such early epithelial findings similar to natural infections, except for the virtual simultaneous appearance of the disease in widely separated farms. The importance of UV radiant

light is also supported by the lack of eye and dorsal lesions in experimental infections. However, the possible role of UV irradiation in *F. maritimus* infection of Tasmanian salmonids warrants further investigation, given the continued incidence of natural infection.

The response of *F. maritimus* to environmental factors (Chapter II) suggests that it would be necessary to reduce the salinity below 15 g/l to prevent outbreaks of salmonid cutaneous erosion disease due to *F. maritimus* 89/4762. This is supported by experimental reproduction of cutaneous erosion disease using this strain in Atlantic salmon at a salinity of 15 g/l (Chapter III). However, another (mullet origin) strain of *F. maritimus* is more sensitive to low salinities than isolate 89/4762 (Schmidtke *et al.*, 1991), and *Flexibacter* disease in mullet can be ameliorated by holding them at a salinity of 15 g/l (B. Munday, pers comm, 1994).

2. Antimicrobial activity of skin mucus

The variable inhibitory action against selected CFLB of skin mucus from presumed naive fish could be due to a wide variety of factors. Chief among these is that fish are normally exposed to a wide range of organisms, including CFLB in the water column and, as a result, may well develop varying degrees of immunity to these organisms. Although more data are required the present study suggested that such induced resistance was more apparent for low-virulence commensals such as *C. psychrophila* and *C. johnsonae* than the more pathogenic *F. columnaris* and *F. maritimus*. Findings supported this hypothesis included those of Fujihara and Hungate (1972) who demonstrated naturally-occurring agglutinin titres in the sera of 12 species of fish against *F. columnaris*, Hazen *et al.* (1981) who found high antibody titres to *Aeromonas hydrophila* among populations of wild largemouth bass (*Micropterus salmoides*) in a freshwater pond and Sakai (1983) who demonstrated a bactericidal action for naive rainbow trout serum against an avirulent strain of *Aeromonas salmonicida,* but with a lower degree for a virulent isolate. Another likelihood is that the level of natural immunity of naive fish is different, depending on the species of fish. For instance, lysozyme activity in naive rainbow trout organs such as skin mucus was recorded to be 20 times greater than Atlantic salmon (Lie *et*

al., 1989), indicating some species possess an inherent (genetically) low level of such non-specific immunity. Consequently, detailed studies of individual immunity factors of mucus, such as lysozyme activity, and other macromolecular proteins (agglutinins), are warranted in future.

3. Chemotherapy

As mentioned earlier (Chapter V), many of the recommended dose rates of antimicrobial agents used in fisheries have been based on clinical responses and not by validation of the minimum inhibitory concentrations (MIC) and effective tissue levels. Treatments should preferably be established by correlating serum levels, MIC values and clinical efficacy. This is more relevant, especially with CFLB, which are organisms associated with skin infections. There is also a great need to ascertain the sensitivity of pathogens because of increased antimicrobial resistance of organisms associated with aquaculture. Another relevant point is that the relationships between dose rate, treatment time and withdrawal time are complex. For instance, some authors have suggested that a high dose for a shorter time may lead to more rapid excretion than a low dose administered for a longer time (McCracken *et al.*, 1976; Jacobsen, 1989), although, treatment under such strategy may not always be effective because of rapid excretion of the therapeutant from the animal's tissues.

The recent worldwide severe disease outbreaks due to CFLB infections have had significant impacts on the aquaculture industry, and have led to a rational demand by industry and their advisers to establish appropriate control and treatment procedures against these infections. Immunization against these pathogens is only beginning and so far the results of those investigations have been variable (e.g. Moore *et al.*, 1990; Newman, 1993; Carson *et al.*, 1993; 1994). If possible, improving management or manipulating environmental parameters, such as water temperature and salinity as mentioned earlier, should be the first choice, otherwise use of therapeutants is justified to ameliorate the infections and improve the welfare of the fish.

These studies provided original data validating the use of some recommended antimicrobial agents against CFLB by correlating *in vitro* and *in vivo* tests.

Amoxycillin an acid stable, low protein binding, penicillinase-susceptible semisynthetic penicillin (ß-lactam) compound is suitable for oral use. This antibiotic has recently been registered for control of bacterial fish diseases, in particular *Aeromonas salmonicida*, in some countries such as the United Kingdom (Barnes *et al.*, 1994) and is under consideration for licensing in other regions such as the United States (Inglis *et al.*, 1992; Elston, 1992). Amoxycillin has been also evaluated against pseudotuberculosis (*Pasteurella piscicida*) in yellowtail (*Seriola quinqueradiata*) in Japan (Inouye *et al.*, 1992, Cameron, 1993). In the light of the resistance which has developed to the other available antimicrobial agents, the antibiotic has great potential. Resistance to this agent is plasmid mediated and involves ability to produce β-lactamase enzymes.

Correlation of the MICs data, serum levels and clinical efficacy obtained in this study provides the evidence that amoxycillin is useful for treatment of CFLB infections. Since amoxycillin has a short withdrawal time (Elston, 1992; Inglis *et al.*, 1992), bath treatments may need to be repeated. For instance, in this study a sudden mortality commenced one week post-treatment (Fig. 5.4, Chapter V), indicating a reduction of drug level in tissues to below the MIC value.

Trimethoprim is an analogue of the pteridine component of dihydrofolic acid with high structural resemblance to dihydrofolate reductase enzyme. The antibacterial action of the compound is to interfere with folic acid metabolism in the bacterial cell by competitively blocking the biosynthesis of tetrahydrofolate, resulting in blocking of the synthesis of DNA, RNA and cell wall proteins leading to the rapid death of the bacteria (Harold, 1991a; 1991b). Resistance to this compound is due to both permeability changes and to an altered dihydrofolate reductase by the mechanism of either a chromosomal mutation or a plasmid mediating a new enzyme with an altered affinity for trimethoprim.

In contrast to freshwater species of CFLB, marine *Flexibacter* infection does not respond to treatment with sulphonamides at least *in vitro* as indicated by J. Carson (pers comm, 1994). Bergsjo and Sognen (1980) reported higher uptake of trimethoprim by seawater fish compared to freshwater ones. This study has clearly demonstrated that trimethoprim at 10 mg/kg body weight/day for seven days or 50 ppm for one hour is the treatment of choice against *F. maritimus* infection, but amoxycillin at 80-100 mg/kg body weight/day for 10 days or 200 ppm for one hour would be more suitable close to harvest, when a shorter withholding period is necessary.

Oxolinic acid is commonly used in Australian aquaculture (Anderson, 1992) and has been registered for fisheries usage in some countries such as Japan (Austin *et al.*, 1983), the UK (Austin & Austin, 1987) and Norway (Lunestad, 1992). The antibiotic has been used against a number of bacterial fish pathogens including *F. columnaris, V. anguillarum, A. hydrophila, A. salmonicida* and *Y. ruckeri* (Carson, 1990; Anderson, 1992; Austin & Austin, 1993; Munday, 1994). Mechanism of antimicrobial action by these quinolone antimicrobial agents is to inhibit DNA replication through binding to DNA gyrase enzyme in the bacterial cell (Harold, 1991b). Resistance to these agents is due to two mechanisms including structural changes in the target enzyme and changes in outer membrane proteins (Hooper & Wolfson, 1991). Findings by O'Grady *et al.*, 1986 and Ishida (1990) show that this compound is more active in freshwater than seawater. The bioassay results in this study show that oxolinic acid is readily absorbed in freshwater barramundi when used orally. This may be due, in part, to the drug's stability when incorporated in a pellet (Scott, 1993; Munday, 1994), to higher absorption at higher temperatures and lower pH (6.5-7.2) as was observed for flumequine (O'Grady *et al.*, 1988; Sohlberg, *et al.*, 1994), and to drug thermostability (Lunestad, 1992; Martinsen *et al.*, 1992).

Oxolinic acid was chosen to treat *F. maritimus* infection during the initial phase of the natural outbreak in Tasmania (J. Handlinger & S. Percival, pers comm, 1994), when the causative agent was unclear, and continued once *F. maritimus* was identified as the causative agent, as recommended for treatment of freshwater

Flexibacter infections. Subsequent use of oxytetracyline proved efficacious in these outbreaks. Moreover, the consistency of high MICs values obtained in this study confirm the ineffectiveness of oxolinic acid for *F. maritimus* disease. In contrast, this study validated the usefulness of this compound against columnaris disease. MIC values, serum level and clinical efficacy data of oxolinic acid suggest a reduction in both oral and bath dosages from 10-5 mg /kg body weight/day and, with this inmind, a reduction from 50 ppm to 30 ppm against columnaris disease would be appropriate.

It is often difficult to compare the bioassay results reported by various workers because of the variability of the procedures, species of fish and conditions of treatment. For instance, levels of 1.25, 0.9-1.7, and 1 to >1.2 µg/ml have been recorded in the serum or plasma of Atlantic salmon when fish were fed at 80, 9-10 and 10 mg/kg body weight/day of amoxycillin, oxolinic acid and trimethoprim, respectively (Carson, 1990; Hustvedt *et al.*, 1991; Cameron, 1991; 1993; Inglis *et al.*, 1992). In this study at the similar oral dosages, levels of amoxycillin and trimethoprim reached 0.2 and 0.8 µg/ml, respectively in serum of rainbow trout, and oxolinic acid achieved 3.4 µg/ml in barramundi serum. Bath treatments of amoxycillin (200 ppm) and trimethoprim (either 50 or 100 ppm) produced levels of 0.6 and 0.4 µg/ml, respectively in sea water Atlantic salmon, and oxolinic acid reached 0.5 µg/ml in barramundi when given as a bath (50 ppm).

However, such procedures need to be both accurate and rapid clinically, easily reproducible and cheap in cost. Although, the use of chemical methods with high sensitivity and specificity, such as high performance liquid chromatography for assay of the reagents, have been recently increased, they are however complex with the disadvantages of the time involved, the expense and sometimes difficulties in replicating. Such physicochemical analyses do not also determine if the residues of antibiotics are either active or biologically available (Barker & Page, 1993). Therefore, it would be rational to evaluate and develop microbiological assays which provide a rapid, inexpensive and easily applied means of screening for antimicrobial agents (Inglis *et al.*, 1993; Smith *et al.*, 1993). However, the use of such physicochemical

procedures was not the aim of this study. The aim was to evaluate microbiological assays as a means of providing meaningful data.

Clearly, in a situation when rapid diagnosis and treatment of disease is imperative, quick-acting compounds with broad spectrum inhibitory activity are advantageous. The correlation of *in vitro* and *in vivo* results concludes that amoxycillin is a suitable therapeutic reagent against the CFLB; oxolinic acid and norfloxacin against freshwater species of CFLB and trimethoprim against *F. maritimus.* Oxytetracycline is a recommended compound for treatment of these infections, especially columnaris disease and this efficacy is shown by the results of MIC values obtained in this study. However, this compound suffers some disadvantages including low bioavailability (e.g. in carp), interference with osmoregulation in salmon smolts, long retention in skin and muscles with a relatively long withdrawal period of two months, production of insoluble complexes with Ca and Mg ions when it is used in sea water or hard waters and immune suppression (Rijkers *et al.*,1980; Jacobsen 1989; Lunestad & Goksøyr 1990; Lunestad, 1992; Munday 1994). In addition, results of investigations (e.g. Toranzo *et al.*, 1984) show that the long and extensive usage of this antimicrobial agent as both a therapeutant and a growth promoter (Ahmad & Matty, 1989) in aquaculture has caused a significant increase in bacterial resistance to this compound.

4. Conclusion

Substantial evidence was obtained on the pathogenesis of infections due to, and the chemotherapy of, a number of fish-pathogenic CFLB. Studies on physiological requirements of the organisms showed that temperature or salinity may well be useful as controlling agents. Conversely, it is unlikely that manipulation of pH could be used to control these bacteria because the pH range for growth of CFLB is similar to what might be expected in aquatic environments. Findings on the pathogenesis of *C. johnsonae* gave evidence that this organism may become a significant potential pathogen in freshwater or slightly brackish environment where there is a variation in the ambient water condition such as temperature. Barramundi was identified to be more susceptible to the infection by *C. johnsonae* and *F. columnaris* than other

species studied here. Infection due to *F. columnaris* was severe in barramundi and goldfish with rapid and high mortality. Another freshwater species, *C. psychrophila*, was identified as a low virulence pathogen in the instance of the Tasmanian isolate. The study also provided original data correlating pathogenesis of *F. maritimus* in a number of commercial and captured species in Tasmania. Pathological findings due to this infection suggest a significant role of the organism in natural epizootics in Atlantic salmon, rainbow trout, striped trumpeter and greenback flounder, especially under optimal environmental conditions for the organism. In addition, there was a consistent similarity between histological findings of natural and experimental infections due to this pathogen. Correlation of *in vitro* and *in vivo* findings gave a substantial and original data on the chemotherapy of these infections in fish. In addition, this study validated the clinical efficacy of oxolinic acid, amoxycillin and trimethoprim against *Flexibacter* infections in barramundi, Atlantic salmon and rainbow trout.

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Table 1. Composition of media used for the isolation of fish pathogenic *Cytophaga/Flexibacter*-like bacteria (CFLB).

No.	Ingredient	1	2	3	4	5	6	7	8	9	10	11	12
1	Agar	0.9%	0.9%	1.0%	-	1.5%	1.5%	1.5%	1.0%	1.5%	1.5%	0.9%	1.0%
2	Beef extract	0.02%	0.02%	-	-	-	0.002%	-	-	-	-	-	-
3	Casamino acids	-	-	-	0.1%	-	-	-	-	-	-	-	-
4	Casein	-	-	-	-	-	-	-	-	-	-	-	0.3%
5	Casitone	-	-	0.05%	-	-	-	-	-	-	-	-	-
6	Calcium chloride	-	-	-	0.1%	-	-	-	-	-	-	-	0.03%
7	Enzymic digest of fish muscle	-	-	-	-	-	-	-	-	-	-	5.0%	-
8	Magnesium chloride	-	-	-	1.08%	-	-	-	-	-	-	-	-
9	Potasium chloride	-	-	-	0.07%	-	-	-	-	-	-	-	-
10	Sodium chloride	-	-	-	3.13%	-	-	-	-	-	-	-	-
11	Meat broth diluted	-	-	-	-	-	-	1:10	-	-	-	-	-
12	Peptone	-	-	-	-	-	-	-	-	-	-	0.1%	-
13	Peptonized milk	-	-	-	-	-	0.05%	-	-	0.1%	-	-	-
14	Skim milk	-	-	-	-	0.5%	-	-	-	-	-	-	-
15	Sodium acetate	0.02%	0.02%	-	-	-	0.002%	-	-	0.002%	0 02%	-	-
16	Tryptone	0.05%	0.05%	-	0.1%	-	0.005%	-	0.02%	-	0.1%	-	0.2%
17	Yeast extract	0.05%	0.05%	0.05%	0.02%	0.05%	0.005%	-	-	0.02%	0.02%	0.1%	0.05%
18	Erythromycin	-	-	-	-	-	-	-	-	-	-	-	10 µg/ml*
19	Neomycin sulphate	-	5 µg/ml	-	-	-	-	-	-	-	-	-	10 µg/ml*
20	Polymyxin B	-	10 IU/ml	-	-	-	-	-	-	-	-	-	256 IU/ml*
21	pH	7.2-7.4	7.2-7.4	8.0	7-7.2							7.0	7.0

1. *Cytophaga* agar (Anacker & Ordal,1959a); for isolation of marine bacteria the medium consists of at least 30% sea water; use of 2-5 µg oxolinic acid/ml improves the isolation of *F. maritimus* (Carson, 1990, Carson *et al.*, 1992); use of 5 µg neomycin/ml and 200 IU polymyxin B/ml improves the isolation of *F. columnaris* in the systemic infection (Hawke & Thune, 1992). 2. Fijan (1969) medium ; 3. Bootsma & Clerx's (1976) medium. 4. TCY medium (Hikida *et al.*, 1979; Austin & Austin, 1987; 1993). 5. Skim milk agar (Christensen & Cook, 1972). 6. Peptonized milk agar (Carlson & Pacha,1968). 7. Mouton agar (Gräf & Stürzenhofecker, 1946; Reichenbach, 1989). 8. Tryptone agar (Lewin & Lounsbery,1969). 9. Peptonized milk yeast agar II (Christensen & Cook,1972). 10. Tryptone yeast agar (Christensen & Cook,1972). 11. Anderson & Conroy (1969); perpared in seawater. 12. Hsu *et al.* (1983). * Any one of neomycin, polymyxin or erythromycin may be used.

Table 2. Antibiotics and chemical substances which are used for control and treatment of fish pathogenic *Cytophaga/Flexibacter*-like bacteria (Austin & Austin, 1987; 1993; Munday, 1988; 1994; Reddacliff, 1988; Carson, 1990; Holt *et al.*, 1993; Noga, 1993; Thune, 1993; Wakabayashi, 1993).

Drug/detergent	Method/dosage	Pathogen/disease
Acriflavine	bath, 3-5 ppm for 3-10 days.	columnaris disease (CD)
Furanace	0.5 µg/ml for 1 hour every third day.	*Cytophaga* sp., BCWD
Nalidixic acid	oral, 40 mg/kg b w daily for 10 days.	*C. aquatilis*
Oxolinic acid[1]	oral, 10-15 mg/kg b w daily for 10 days. Bath, 1 ppm for 24 hours, 50ppm for 1 hour.	CD
Oxytetracycline[2]	oral, 75-100 mg/kg b w daily for 10 days. Bath, 20-100 ppm (100 ppm in hard water) for 5-10 days or 200 ppm for 2 hours.	bacterial cold water disease (BCWD), CD, *F. maritimus*, *C. johnsonae*
Proflavine[3]	bath, 20 ppm for 30 min.	CD
Sulphonamides:[4]		
Sulfisoxazole	oral, 100-200 mg/kg b w daily for 7-10 days.	BCWD, *Cytophaga* sp.
Sulphadimidine	oral, 100-200 mg/kg b w daily for 7-10 days. bath, 8 ppm for 5-10 days.	BCWD, *Cytophaga* sp.
Sulphamerazine	oral, 100-200 mg/kg b w daily for 7-10 days.	BCWD, *Cytophaga* sp.
Sulphamethazine	oral, 100-200 mg/kg b w daily 10-20 days.	BCWD, *Cytophaga* sp.
Sulphanilimide	bath, 200 ppm daily for 5-10 days.	BCWD, *Cytophaga* sp.
Tribrissen	oral, 50 mg/kg b w daily for 10 days. bath, 20 ppm daily for 5-7 days.	BCWD, *Cytophaga* sp., *F. maritimus*
Tetracycline	-	*C. aquatilis*
Trimethoprim	oral, 10 mg/kg b w daily for 10 days.	*F. maritimus*
Benzalkonium chloride[5]	bath, 1-2 ppm for 1 hour at water hardness 100-200 ppm.	BCWD, CD, *Cytophaga* sp.
Copper sulphate	dip, a dilution of 1:2000 for 1-2 min. bath, 1-2 mg/l for 30-60 min.	CD
Formalin	bath, 25 ppm "indefinitely"	CD, *F. maritimus*
Malachite green[6]	bath, 0.2 ppm fro 1-2 hour at 3 days intervals or 0.08 ppm indefinitely. Dip, 66 ppm (1:15000) for 30 sec.	CD, *F. maritimus*
Pyridylmercuric acetate	2 mg/l for 1 hour	CD
Sodium chloride[7]	dip. 8% NaCl for 30 sec. or to salinity of 10-15‰ seawater.	CD
Tiamulin	5-50 mg/kg b w	*F. maritimus*

(1) Good stability when incorporated into pellets. Aquarium fish should preferably be treated in a special treatment container because of drug interference with the functioning of biofilters.

(2) Poor stability in pellets. For treatment of *F. maritimus* may used at 100-200ppm. Response to therapy for BCWD is often poor because of the low temperatures at which the disease occurs.

(3) Insoluble in seawater.

(4) Must be used with extreme caution in salmonids.

(5) Reduced efficacy in seawater, can cause gill damage at higher concentration.

(6) Must be zinc free.

(7) Farkas & Olah (1980) and Austin & Austin (1993) suggested the use of sodium chloride bath is useful in order to control columnaris disease.

APPENDIX II

Table1. Change in absorbance of Flexibacter maritimus 89/4762 (5 replicates cultures) with time under different temperatures. Absorbance of blank was 0.000-0.0010.

Temperature 4°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.002	0.001	0.001	0.002	0.001	0.000245
2	0.001	0.002	0.001	0.001	0.003	0.002	0.000400
4	0.008	0.004	0.004	0.005	0.006	0.005	0.000748
6	0.007	0.005	0.005	0.005	0.006	0.006	0.000400
8	0.007	0.005	0.004	0.005	0.008	0.006	0.000735
10	0.007	0.007	0.008	0.006	0.009	0.007	0.000510
12	0.008	0.008	0.008	0.009	0.009	0.008	0.000245
14	0.008	0.009	0.011	0.008	0.007	0.009	0.000678
16	0.007	0.006	0.009	0.007	0.007	0.007	0.000490
18	0.007	0.007	0.006	0.006	0.005	0.006	0.000374
20	0.008	0.008	0.006	0.005	0.005	0.006	0.000678
22	0.008	0.007	0.006	0.005	0.004	0.006	0.000707

Temperature 8°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.002	0.001	0.001	0.001	0.001	0.000200
2	0.007	0.007	0.001	0.001	0.004	0.004	0.001342
4	0.016	0.02	0.018	0.017	0.018	0.018	0.000663
6	0.026	0.044	0.032	0.03	0.031	0.033	0.003027
8	0.056	0.073	0.078	0.072	0.074	0.071	0.003789
10	0.079	0.07	0.09	0.091	0.098	0.086	0.004946
12	0.081	0.086	0.112	0.109	0.108	0.099	0.006492
14	0.1	0.09	0.116	0.114	0.11	0.106	0.004858
16	0.08	0.07	0.097	0.1	0.095	0.088	0.005750
18	0.055	0.045	0.079	0.075	0.076	0.066	0.006753
20	0.046	0.04	0.075	0.07	0.071	0.06	0.007215
22	0.043	0.035	0.065	0.066	0.064	0.055	0.006501

Temperature 16°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.001	0.002	0.002	0.001	0.001	0.000245
1	0.033	0.038	0.035	0.034	0.036	0.035	0.000860
2	0.081	0.085	0.072	0.075	0.077	0.078	0.002280
3	0.13	0.135	0.129	0.125	0.129	0.13	0.001600
4	0.174	0.184	0.164	0.161	0.168	0.17	0.004079
5	0.181	0.2	0.18	0.182	0.192	0.187	0.003899
6	0.237	0.25	0.237	0.24	0.246	0.242	0.002588
7	0.26	0.256	0.266	0.289	0.27	0.268	0.005731
8	0.36	0.368	0.362	0.292	0.32	0.34	0.014784
9	0.369	0.374	0.369	0.319	0.369	0.36	0.010296

Table 1. continued

Temperature 20°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.003	0.002	0.001	0.002	0.001	0.002	0.000374
1	0.077	0.075	0.07	0.068	0.072	0.072	0.001631
2	0.11	0.12	0.113	0.13	0.115	0.118	0.003501
3	0.154	0.162	0.16	0.185	0.168	0.166	0.005295
4	0.176	0.17	0.172	0.193	0.178	0.178	0.004055
5	0.23	0.227	0.237	0.278	0.232	0.241	0.009441
6	0.245	0.25	0.265	0.301	0.268	0.266	0.009815
7	0.288	0.28	0.309	0.334	0.312	0.305	0.009537
8	0.318	0.305	0.338	0.382	0.33	0.335	0.013098
9	0.328	0.318	0.328	0.37	0.369	0.343	0.011134

Temperature 25°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.001	0.002	0.001	0.001	0.001	0.000200
1	0.079	0.076	0 075	0.086	0.08	0.079	0.001934
2	0.134	0.122	0.129	0.134	0.125	0.129	0.002396
3	0.165	0.163	0.164	0.166	0.162	0.164	0.000707
4	0.201	0.2	0.2	0.202	0.202	0.201	0.000447
5	0.259	0.251	0.241	0.255	0.254	0.252	0.003033
6	0.3	0.282	0.28	0.284	0.29	0.287	0.003611
7	0.316	0.296	0.311	0.318	0.31	0.31	0.003852
8	0.373	0.394	0.388	0.37	0.384	0.382	0.004521
9	0.368	0.39	0.384	0.364	0.372	0.376	0.004915

Temperature 30°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0	0.001	0.001	0.002	0.001	0.001	0.000316
1	0.108	0.102	0.1	0.083	0.085	0.096	0.004925
2	0.17	0.153	0.153	0.14	0.148	0.153	0.004913
3	0.22	0.2	0.2	0.201	0.21	0.206	0.003929
4	0.264	0.238	0.238	0.231	0.25	0.244	0.005817
5	0.325	0.286	0.285	0.28	0.316	0.298	0.009190
6	0.365	0.328	0.326	0.324	0.34	0.337	0.007626
7	0.355	0.338	0.362	0.361	0.365	0.356	0.004831
8	0.305	0.373	0.36	0.354	0.325	0.343	0.012404
9	0.278	0.35	0.343	0.342	0.29	0.321	0.015125

Temperature 35°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.012	0.01	0.01	0.01	0.011	0.011	0.000400
1	0.039	0.015	0.028	0.027	0.03	0.028	0.003839
2	0.116	0.048	0.078	0.073	0.095	0.082	0.011353
3	0.135	0.098	0.099	0.095	0.122	0.11	0.007933
4	0.159	0.144	0.135	0.14	0.145	0.145	0.004007
5	0.185	0.185	0.157	0.18	0.165	0.174	0.005689
6	0.228	0.21	0.19	0.205	0.202	0.207	0.006197
7	0.254	0.236	0.211	0.25	0.235	0.237	0.007546
8	0.325	0.307	0.286	0.302	0.296	0.303	0.006476
9	0.313	0.264	0.289	0.274	0.305	0.289	0.009171

Table 1. continued

Temperature 37°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.001	0.001	0.001	0.001	0.001	0.000000
1	0.033	0.031	0.031	0.033	0.03	0.032	0.000600
2	0.032	0.027	0.028	0.027	0.027	0.028	0.000970
3	0.03	0.025	0.025	0.025	0.025	0.026	0.001000
4	0.029	0.025	0.025	0.025	0.025	0.026	0.000800
5	0.027	0.023	0.025	0.023	0.024	0.024	0.000748
6	0.025	0.021	0.023	0.02	0.021	0.022	0.000894
9	0.01	0.006	0.005	0.008	0.007	0.0072	0.000860
14	0.004	0.003	0.005	0.006	0.007	0.005	0.000707

Temperature 38°C (absorbance of the cultures at 42°C was similar to those of at 38°C)

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.001	0.001	0.001	0.001	0.002	0.001	0.000200
1	0.006	0.006	0.006	0.007	0.007	0.006	0.000245
2	0.003	0.003	0.004	0.005	0.005	0.004	0.000447
3	0.007	0.007	0.007	0.008	0.007	0.007	0.000200
4	0.007	0.007	0.007	0.006	0.005	0.006	0.000400
5	0.007	0.007	0.003	0.005	0.004	0.005	0.000800
14	0.006	0.007	0.003	0.004	0.002	0.004	0.000927

Table 2. Change in absorbance of Cytophaga johnsonae 91/0262-10 (5 replicates cultures) with time under different temperatures Absorbance of blank was 0.000-0.0010.

Temperature 4°C

Time (day)	Rep. 1	Rep 2	Rep3	Rep 4	Rep 5	Mean	St. Error
0	0.002	0.003	0.005	0.002	0.003	0.003	0.000548
1	0.011	0.005	0.009	0.01	0.005	0.008	0.001265
2	0.016	0.01	0.01	0.01	0.009	0.011	0.001265
3	0.037	0.011	0.027	0.039	0.012	0.025	0.005953
4	0.084	0.043	0.068	0.082	0.048	0.065	0.008462
5	0.127	0.078	0.115	0.132	0.095	0.109	0.010112
6	0.165	0.127	0.16	0.145	0.134	0.146	0.007290
7	0.196	0.176	0.194	0.193	0.186	0.189	0.003661
8	0.221	0.211	0.223	0.215	0.202	0.214	0.003763
9	0.245	0.231	0.238	0.237	0.231	0.236	0.002600

Temperature 8°C

Time (day)	Rep. 1	Rep 2	Rep3	Rep 4	Rep 5	Mean	St. Error
0	0.002	0.003	0.005	0.002	0.003	0.003	0.000548
1	0.012	0.013	0.014	0.011	0.012	0.012	0.000510
2	0.026	0.026	0.029	0.025	0.026	0.026	0.000678
3	0.05	0.051	0.065	0.047	0.052	0.053	0.003114
4	0.11	0.112	0.115	0.096	0.105	0.108	0.003326
5	0.137	0.139	0.16	0.139	0.132	0.141	0.004823
6	0.177	0.178	0.194	0.181	0.166	0.179	0.004488
7	0.196	0.21	0.227	0.216	0.194	0.209	0.006194
8	0.22	0.231	0.248	0.227	0.218	0.229	0.005342
9	0.239	0.243	0.293	0.254	0.245	0.255	0.009861

Table 2. continued

Temperature 10°C

Time (day)	Rep. 1	Rep 2	Rep3	Rep 4	Rep 5	Mean	St. Error
0	0.004	0.005	0.004	0.004	0.003	0.004	0.000316
1	0.021	0.018	0.022	0.022	0.021	0.021	0.000735
2	0.064	0.064	0.062	0.068	0.069	0.065	0.001327
3	0.126	0.122	0.127	0.113	0.13	0.124	0.002943
4	0.2	0.19	0.214	0.199	0.212	0.203	0.004450
5	0.226	0.232	0.258	0.242	0.236	0.239	0.005463
6	0.262	0.261	0.302	0.27	0.265	0.272	0.007662
7	0.297	0.281	0.338	0.302	0.3	0.304	0.009363
8	0.347	0.321	0.38	0.352	0.331	0.346	0.010106
9	0.362	0.367	0.37	0.37	0.36	0.366	0.002059

Temperature 15°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.004	0.003	0.003	0.004	0.003	0.003	0.000245
1	0.07	0.066	0.064	0.074	0.07	0.069	0.001744
2	0.168	0.155	0.152	0.176	0.164	0.163	0.004359
3	0.223	0.221	0.217	0.236	0.214	0.222	0.003787
4	0.266	0.269	0.262	0.3	0.256	0.271	0.007666
5	0.313	0.32	0.288	0.351	0.296	0.314	0.010966
6	0.342	0.355	0.331	0.38	0.324	0.346	0.009903
7	0.382	0 377	0.368	0.396	0.355	0.376	0.006860
8	0.379	0.35	0.372	0.374	0.393	0.374	0.006947
9	0.36	0.35	0.35	0.36	0.363	0.357	0.002750

Temperature 20°C

Time (day)	Rep. 1	Rep 2	Rep3	Rep 4	Rep 5	Mean	St. Error
0	0.005	0.004	0.004	0.004	0.004	0.004	0.000200
1	0.068	0.067	0.069	0.07	0.077	0.070	0.001772
2	0.154	0.147	0.151	0.15	0.152	0.151	0.001158
3	0.225	0.199	0.225	0.21	0.22	0.216	0.005014
4	0.271	0.256	0.284	0.248	0.264	0.265	0.006194
5	0.305	0.297	0.315	0.276	0.307	0.300	0.006648
6	0.353	0.342	0.35	0.305	0.347	0.339	0.008790
7	0.392	0.388	0.394	0.34	0.392	0.381	0.010346
8	0.414	0.42	0.404	0.375	0.397	0.402	0.007829
9	0.37	0.416	0.37	0.39	0.361	0.381	0.009867

Temperature 25°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.004	0.003	0.004	0.004	0.003	0.004	0.000245
1	0.125	0.113	0.112	0.114	0.115	0.116	0.002354
2	0.187	0.188	0.192	0.177	0.187	0.186	0.002478
3	0.25	0.23	0.268	0.245	0.252	0.249	0.006116
4	0.316	0.299	0.328	0.275	0.288	0.301	0.009494
5	0.353	0.345	0.351	0.327	0.355	0.346	0.005083
6	0.375	0.378	0.38	0.37	0.382	0.377	0.002098
7	0.368	0.35	0.338	0.355	0.343	0.351	0.005190
8	0.312	0.318	0.301	0.319	0.307	0.311	0.003385
9	0.295	0.286	0.276	0.288	0.288	0.287	0.003059

Table 2. continued

Temperature 30°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.007	0.003	0.004	0.006	0.007	0.005	0.000812
1	0.083	0.086	0.105	0.088	0.089	0.090	0.003839
2	0.18	0.175	0.195	0.18	0.181	0.182	0.003367
3	0.255	0.228	0.279	0.244	0.25	0.251	0.008303
4	0.3	0.252	0.309	0.266	0.288	0.283	0.010583
5	0.311	0.265	0.316	0.312	0.311	0.303	0.009545
6	0.335	0.29	0.268	0.313	0.326	0.306	0.012225
7	0.283	0.288	0.21	0.268	0.274	0.265	0.014084
8	0.251	0.267	0.205	0.22	0.232	0.235	0.010986
9	0.196	0.221	0.147	0.152	0.205	0.184	0.014742

Temperature 35°C

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.004	0.003	0.004	0.004	0.003	0.004	0.000245
1	0.076	0.07	0.069	0.073	0.067	0.071	0.001581
2	0.092	0.087	0.08	0.088	0.073	0.084	0.003362
3	0.093	0.094	0.087	0.095	0.077	0.089	0.003353
4	0.082	0.086	0.08	0.086	0.066	0.080	0.003688
5	0.075	0.08	0.074	0.08	0.062	0.074	0.003292
6	0.075	0.076	0.073	0.074	0.06	0.072	0.002943
7	0.074	0.075	0.07	0.074	0.058	0.070	0.003169
8	0.074	0.074	0.07	0.074	0.058	0.070	0.003098
9	0.073	0.074	0.068	0.071	0.057	0.069	0.003076

Temperature 37°C (absorbance of cultures at 42°C was similar to those of at 37°C).

Time (day)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	St. Error
0	0.003	0.01	0.003	0.002	0.003	0.004	0.001463
1	0.010	0.018	0.012	0.012	0.011	0.013	0.001400
2	0.004	0.012	0.005	0.005	0.003	0.006	0.001594
3	0.004	0.011	0.003	0.004	0.003	0.005	0.001517
4	0.003	0.010	0.003	0.005	0.002	0.005	0.001435
5	0.003	0.011	0.004	0.004	0.002	0.005	0.001594
6	0.002	0.006	0.004	0.003	0.001	0.003	0.000860
8	0.002	0.005	0.004	0.003	0.001	0.003	0.000707
14	0.003	0.007	0.005	0.002	0.002	0.004	0.000970

Table 3. Change in absorbance of Flexibacter columnaris 1468 (5 replicates cultures) with time under different temperatures. Absorbance of blank was 0.001-0.003.

Temperature 10°C (absorbance of the cultures at 4°C was similar to those of at 10°C).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
1.00	0.034	0.030	0.035	0.030	0.032	0.032	-1.495
2.00	0.034	0.030	0.038	0.030	0.035	0.033	-1.481
3.00	0.033	0.027	0.034	0.026	0.030	0.030	-1.523
4.00	0.033	0.028	0.034	0.028	0.031	0.031	-1.509
5.00	0.033	0.028	0.034	0.028	0.031	0.031	-1.509
24.00	0.029	0.022	0.034	0.020	0.030	0.027	-1.569
72.00	0.005	0.013	0.016	0.008	0.015	0.011	-1.959
336.00	0.004	0.010	0.018	0.010	0.010	0.010	-1.983

Table 3. continued

Temperature 15°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.033	0.033	0.034	0.033	0.033	0.033	-1.479
1.00	0.036	0.036	0.037	0.035	0.037	0.036	-1.441
1.30	0.039	0.040	0.043	0.039	0.041	0.040	-1.394
2.00	0.042	0.043	0.046	0.042	0.044	0.043	-1.363
2.30	0.045	0.046	0.049	0.045	0.046	0.046	-1.335
3.00	0.053	0.047	0.052	0.046	0.047	0.049	-1.310
3.30	0.057	0.051	0.056	0.049	0.050	0.053	-1.276
4.00	0.060	0.053	0.061	0.054	0.053	0.056	-1.252
4.30	0.063	0.056	0.066	0.058	0.058	0.060	-1.222
5.00	0.066	0.062	0.070	0.062	0.064	0.065	-1.187
15.00	0.086	0.082	0.106	0.085	0.094	0.091	-1.041

Temperature 20°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.035	0.032	0.037	0.032	0.033	0.034	-1.469
1.00	0.043	0.036	0.045	0.038	0.039	0.040	-1.396
1.30	0.046	0.043	0.050	0.044	0.046	0.046	-1.339
2.00	0.051	0.048	0.055	0.049	0.050	0.051	-1.296
2.30	0.057	0.056	0.060	0.055	0.055	0.057	-1.247
3.00	0.066	0.060	0.069	0.059	0.059	0.063	-1.201
3.30	0.073	0.066	0.073	0.064	0.064	0.068	-1.167
4.00	0.080	0.076	0.086	0.071	0.070	0.077	-1.114
4.30	0.088	0.081	0.088	0.078	0.076	0.082	-1.086
5.00	0.095	0.087	0.098	0.085	0.084	0.090	-1.046
15.00	0.109	0.114	0.118	0.112	0.112	0.113	-0.947

Temperature 25°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.25	0.035	0.035	0.036	0.034	0.033	0.035	-1.461
0.50	0.042	0.039	0.044	0.038	0.036	0.040	-1.398
1.15	0.050	0.044	0.050	0.044	0.041	0.046	-1.337
1.40	0.057	0.050	0.057	0.053	0.050	0.053	-1.276
2.10	0.073	0.068	0.077	0.070	0.068	0.071	-1.149
2.40	0.082	0.081	0.092	0.085	0.080	0.084	-1.076
3.10	0.101	0.101	0.106	0.103	0.095	0.101	-0.996
3.40	0.108	0.114	0.115	0.111	0.103	0.110	-0.959
4.10	0.118	0.127	0.132	0.128	0.123	0.126	-0.900
4.40	0.122	0.137	0.150	0.145	0.136	0.138	-0.860
5.10	0.139	0.151	0.160	0.165	0.155	0.154	-0.812

Table 3. continued

Temperature 30°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.25	0.034	0.035	0.034	0.033	0.035	0.034	-1.466
0.50	0.046	0.041	0.048	0.039	0.041	0.043	-1.367
1.15	0.055	0.051	0.056	0.050	0.050	0.052	-1.284
1.40	0.064	0.059	0.066	0.061	0.059	0.062	-1.208
2.10	0.081	0.075	0.077	0.075	0.074	0.076	-1.119
2.40	0.097	0.090	0.091	0.087	0.086	0.090	-1.046
3.10	0.114	0.105	0.101	0.101	0.099	0.104	-0.983
3.40	0.127	0.120	0.118	0.120	0.113	0.120	-0.921
4.10	0.143	0.131	0.126	0.130	0.120	0.130	-0.886
4.40	0.159	0.144	0.139	0.145	0.130	0.143	-0.845
5.10	0.175	0.160	0.159	0.165	0.148	0.161	-0.793

Temperature 35°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.034	0.033	0.033	0.032	0.033	0.033	-1.481
1.00	0.041	0.038	0.038	0.036	0.037	0.038	-1.420
1.30	0.050	0.047	0.046	0.044	0.045	0.046	-1.333
2.00	0.059	0.058	0.056	0.053	0.054	0.056	-1.252
2.30	0.071	0.067	0.065	0.060	0.061	0.065	-1.188
3.00	0.085	0.076	0.074	0.073	0.075	0.077	-1.116
3.30	0.098	0.085	0.086	0.084	0.085	0.088	-1.057
4.00	0.109	0.105	0.097	0.096	0.094	0.100	-0.999
4.30	0.117	0.115	0.103	0.112	0.100	0.109	-0.961
13.00	0.280	0.243	0.264	0.255	0.243	0.257	-0.590

Temperature 37°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.033	0.031	0.035	0.034	0.033	0.033	-1.481
1.00	0.037	0.035	0.036	0.035	0.037	0.036	-1.444
1.30	0.042	0.039	0.042	0.039	0.041	0.041	-1.391
2.00	0.045	0.041	0.046	0.042	0.044	0.044	-1.361
2.30	0.053	0.048	0.051	0.047	0.047	0.049	-1.310
3.00	0.058	0.054	0.054	0.050	0.053	0.054	-1.268
3.30	0.062	0.064	0.060	0.052	0.056	0.059	-1.229
4.00	0.065	0.072	0.067	0.053	0.058	0.063	-1.201
4.30	0.067	0.076	0.079	0.059	0.066	0.068	-1.167
5.00	0.070	0.090	0.084	0.064	0.068	0.074	-1.131
14.00	0.073	0.108	0.088	0.076	0.083	0.086	-1.066

Table 3. continued

Temperature 42°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.033	0.031	0.035	0.034	0.033	0.033	-1.481
1.00	0.033	0.031	0.035	0.034	0.033	0.033	-1.481
1.30	0.031	0.031	0.036	0.032	0.033	0.033	-1.487
2.00	0.030	0.031	0.035	0.034	0.036	0.033	-1.479
2.30	0.030	0.031	0.035	0.034	0.036	0.033	-1.479
3.00	0.030	0.031	0.035	0.033	0.032	0.032	-1.492
3.30	0.030	0.031	0.034	0.034	0.032	0.033	-1.479
4.00	0.029	0.031	0.033	0.034	0.031	0.032	-1.500
4.30	0.029	0.031	0.033	0.033	0.032	0.032	-1.500
5.00	0.029	0.031	0.033	0.033	0.032	0.032	-1.500
15.00	0.029	0.031	0.033	0.033	0.032	0.032	-1.500
336.00	0.032	0.031	0.034	0.034	0.036	0.033	-1.476

Table 4. Change in absorbance of Cytophaga psychrophila 91/4043-17 (5 replicates cultures) with time under different temperatures. Absorbance of blank was 0.001-0.003.

Temperature 4°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
1.00	0.033	0.032	0.030	0.030	0.033	0.032	-1.500
2.15	0.036	0.034	0.035	0.031	0.034	0.034	-1.469
3.45	0.036	0.034	0.035	0.032	0.036	0.035	-1.461
5.00	0.038	0.035	0.037	0.033	0.037	0.036	-1.444
15.00	0.046	0.048	0.052	0.053	0.052	0.050	-1.299
25.00	0.058	0.060	0.057	0.058	0.058	0.058	-1.235
35.00	0.066	0.065	0.062	0.066	0.065	0.065	-1.188
42.00	0.072	0.070	0.068	0.065	0.070	0.069	-1.161
48.00	0.082	0.080	0.077	0.070	0.077	0.077	-1.112
55.00	0.096	0.086	0.083	0.075	0.082	0.084	-1.074
64.00	0.110	0.095	0.093	0.087	0.100	0.097	-1.013
72.00	0.118	0.105	0.095	0.093	0.108	0.104	-0.984

Temperature 10°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
1.00	0.042	0.036	0.039	0.034	0.035	0.037	-1.429
2.00	0.047	0.042	0.046	0.041	0.042	0.044	-1.361
3.00	0.048	0.045	0.048	0.043	0.045	0.046	-1.339
4.00	0.054	0.052	0.056	0.047	0.049	0.052	-1.287
5.00	0.063	0.063	0.067	0.057	0.058	0.062	-1.210
6.00	0.068	0.068	0.070	0.062	0.062	0.066	-1.180
7.00	0.075	0.075	0.070	0.066	0.066	0.070	-1.152
8.00	0.078	0.079	0.073	0.069	0.068	0.073	-1.134
9.00	0.080	0.081	0.074	0.072	0.069	0.075	-1.124
15.00	0.094	0.101	0.093	0.095	0.095	0.096	-1.020

Table 4. continued

Temperature 15°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.034	0.033	0.033	0.032	0.035	0.033	-1.476
1.50	0.038	0.036	0.037	0.036	0.040	0.037	-1.427
2.15	0.047	0.038	0.042	0.039	0.045	0.042	-1.375
3.00	0.054	0.044	0.049	0.045	0.054	0.049	-1.308
3.45	0.060	0.055	0.058	0.056	0.062	0.058	-1.235
4.50	0.065	0.062	0.061	0.060	0.070	0.064	-1.197
5.15	0.076	0.065	0.068	0.064	0.076	0.070	-1.156
6.00	0.080	0.072	0.075	0.072	0.081	0.076	-1.119
6.45	0.090	0.076	0.080	0.079	0.088	0.083	-1.083
7.50	0.091	0.084	0.088	0.083	0.092	0.088	-1.057
8.15	0.093	0.087	0.091	0.087	0.095	0.091	-1.043
9.00	0.095	0.089	0.091	0.090	0.096	0.092	-1.035
15.00	0.112	0.106	0.112	0.110	0.106	0.109	-0.962

Temperature 20°C

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.036	0.034	0.034	0.034	0.036	0.035	-1.458
1.50	0.044	0.040	0.037	0.037	0.040	0.040	-1.402
2.15	0.052	0.049	0.044	0.046	0.048	0.048	-1.321
3.00	0.057	0.054	0.051	0.053	0.056	0.054	-1.266
3.45	0.066	0.060	0.063	0.065	0.064	0.064	-1.197
4.50	0.076	0.069	0.067	0.070	0.071	0.071	-1.151
5.15	0.079	0.072	0.072	0.073	0.074	0.074	-1.131
6.00	0.087	0.078	0.078	0.076	0.079	0.080	-1.099
6.45	0.096	0.085	0.088	0.082	0.085	0.087	-1.059
7.50	0.100	0.090	0.092	0.087	0.090	0.092	-1.037
8.15	0.104	0.093	0.094	0.092	0.093	0.095	-1.021
9.00	0.107	0.096	0.094	0.094	0.096	0.097	-1.011
15.00	0.102	0.100	0.093	0.095	0.096	0.097	-1.012

Temperature 25°C (absorbance of cultures at 30 and 35°C was similar to those of at 25°C).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.030	0.032	0.030	0.031	0.030	0.031	-1.514
1.50	0.031	0.032	0.029	0.031	0.030	0.031	-1.514
2.15	0.031	0.032	0.029	0.033	0.030	0.031	-1.509
3.00	0.031	0.032	0.029	0.033	0.030	0.031	-1.509
3.45	0.029	0.032	0.029	0.031	0.030	0.030	-1.520
4.50	0.029	0.032	0.029	0.031	0.030	0.030	-1.520
5.15	0.029	0.032	0.029	0.031	0.031	0.030	-1.517
6.00	0.029	0.032	0.030	0.031	0.032	0.031	-1.511
6.45	0.030	0.032	0.029	0.031	0.031	0.031	-1.514
7.50	0.029	0.031	0.029	0.031	0.031	0.030	-1.520
8.15	0.029	0.030	0.029	0.032	0.031	0.030	-1.520
9.00	0.029	0.030	0.029	0.032	0.031	0.030	-1.520
15.00	0.029	0.030	0.029	0.032	0.031	0.030	-1.520
336.00	0.019	0.028	0.025	0.020	0.031	0.025	-1.609

Table 5. Change in absorbance of Flexibacter maritimus 89/4762 (3 replicates cultures) with time under different pH values and incubated at 30°C. Absorbance of blank was 0.000-0.010.

pH 5 (Absorbance of the cultures at pHs 3 and 4 was similar to those of at pH 5).

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.012	0.011	0.010	0.011	0.000577
1	0.010	0.009	0.007	0.009	0.000882
2	0.008	0.009	0.007	0.008	0.000577
3	0.008	0.007	0.005	0.007	0.000882
4	0.005	0.006	0.005	0.005	0.000333
5	0.003	0.003	0.003	0.003	0.000000
14	0.007	0.006	0.005	0.006	0.000577

pH 6

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.010	0.009	0.010	0.010	0.000333
1	0.067	0.069	0.073	0.070	0.001764
2	0.188	0.180	0.210	0.193	0.008969
3	0.257	0.245	0.300	0.267	0.016697
4	0.330	0.280	0.347	0.319	0.020108
5	0.405	0.350	0.396	0.384	0.017033

pH 6.5

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.010	0.009	0.010	0.010	0.000333
1	0.070	0.073	0.084	0.076	0.004256
2	0.170	0.187	0.220	0.192	0.014678
3	0.265	0.274	0.320	0.286	0.017033
4	0.330	0.315	0.360	0.335	0.013229
5	0.404	0.390	0.420	0.405	0.008667

pH 7.2

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.010	0.010	0.010	0.010	0.000000
1	0.129	0.110	0.130	0.123	0.006506
2	0.231	0.198	0.220	0.216	0.009701
3	0.318	0.331	0.346	0.332	0.008090
4	0.376	0.396	0.418	0.397	0.012129
5	0.388	0.414	0.448	0.417	0.017372

pH 7.6

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.008	0.008	0.008	0.008	0.000000
1	0.099	0.096	0.101	0.099	0.001453
2	0.187	0.160	0.184	0.177	0.008544
3	0.275	0.217	0.250	0.247	0.016796
4	0.326	0.255	0.277	0.286	0.020984
5	0.396	0.310	0.340	0.349	0.025201

Table 5. continued

pH 8.2

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.010	0.010	0.010	0.010	0.000000
1	0.054	0.070	0.062	0.062	0.004619
2	0.165	0.189	0.164	0.173	0.008172
3	0.270	0.330	0.293	0.298	0.017477
4	0.320	0.370	0.346	0.345	0.014438
5	0.373	0.380	0.378	0.377	0.002082

pH 8.6

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.006	0.005	0.008	0.006	0.000882
1	0.023	0.021	0.022	0.022	0.000577
2	0.027	0.038	0.028	0.031	0.003512
3	0.060	0.130	0.040	0.077	0.027285
4	0.106	0.220	0.066	0.131	0.046135
5	0.230	0.330	0.140	0.233	0.054874

pH 9 (Absorbance of the cultures at pHs 9.5 and 10 was similar to those of at pH 9).

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.009	0.009	0.010	0.009	0.000333
1	0.010	0.010	0.011	0.010	0.000333
2	0.009	0.010	0.009	0.009	0.000333
3	0.009	0.009	0.007	0.008	0.000667
4	0.008	0.007	0.007	0.007	0.000333
5	0.008	0.006	0.006	0.007	0.000667
14	0.005	0.004	0.005	0.005	0.000333

Table 6. Change in absorbance of Cytophaga johnsonae 91/0262-10 (3 replicates cultures) with time under different pH values and incubated at 20°C. Absorbance of blank was 0.000.

pH 4 (Absorbance of the cultures at pH 3 was similar to those of at pH 4).

Time(day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.002	0.002	0.002	0.000000
1	0.003	0.000	0.003	0.002	0.001500
2	0.005	0.002	0.005	0.004	0.001500
3	0.005	0.002	0.005	0.004	0.001500
4	0.002	0.001	0.002	0.002	0.000500
5	0.004	0.001	0.004	0.003	0.001500
14	0.001	0.002	0.002	0.002	0.000333

pH 5

Time(day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.002	0.002	0.002	0.000000
1	0.017	0.017	0.017	0.017	0.000000
2	0.067	0.063	0.066	0.065	0.002000
3	0.127	0.129	0.128	0.128	0.001000
4	0.167	0.177	0.171	0.172	0.005000
5	0.225	0.229	0.226	0.227	0.002000

Table 6. continued

pH 6

Time (day)	Rep.1	Rep. 2	Rep.3	Mean	St. Error
0	0.003	0.004	0.004	0.004	0.000500
1	0.065	0.072	0.071	0.069	0.003500
2	0.146	0.142	0.144	0.144	0.002000
3	0.210	0.190	0.199	0.200	0.010000
4	0.248	0.233	0.241	0.241	0.007500
5	0.284	0.275	0.281	0.280	0.004500

pH 6.5

Time (day)	Rep.1	Rep. 2	Rep.3	Mean	St. Error
0	0.001	0.001	0.001	0.001	0.000000
1	0.079	0.078	0.079	0.079	0.000333
2	0.166	0.175	0.171	0.171	0.002603
3	0.233	0.243	0.238	0.238	0.002887
4	0.291	0.288	0.291	0.290	0.001000
5	0.318	0.317	0.318	0.318	0.000333

pH 7

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.001	0.001	0.001	0.001	0.000000
1	0.064	0.075	0.070	0.070	0.002041
2	0.132	0.154	0.144	0.143	0.004082
3	0.206	0.228	0.217	0.217	0.004491
4	0.257	0.283	0.269	0.270	0.005715
5	0.291	0.340	0.316	0.316	0.009798

pH 7.5

Time (day)	Rep.1	Rep. 2	Rep.3	Mean	St. Error
0	0.002	0.001	0.002	0.002	0.000408
1	0.071	0.081	0.075	0.076	0.002449
2	0.162	0.190	0.175	0.176	0.001400
3	0.235	0.255	0.244	0.245	0.010000
4	0.300	0.305	0.303	0.303	0.000816
5	0.342	0.343	0.342	0.343	0.000408

pH 8

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.001	0.002	0.002	0.002	0.000000
1	0.058	0.068	0.062	0.063	0.002449
2	0.151	0.159	0.154	0.155	0.002041
3	0.228	0.248	0.231	0.236	0.006940
4	0.283	0.284	0.284	0.284	0.000000
5	0.320	0.317	0.318	0.319	0.000408

pH 8.5

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.003	0.003	0.003	0.003	0.000000
1	0.024	0.025	0.025	0.025	0.000000
2	0.097	0.090	0.094	0.094	0.001633
3	0.212	0.209	0.211	0.211	0.000816
4	0.275	0.273	0.273	0.274	0.000000
5	0.314	0.307	0.309	0.310	0.000816

Table 6. continued

pH 9 (Missing culture of Rep. 3).

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.003	0.001	-	0.002	0.001000
1	0.005	0.043	-	0.024	0.019000
2	0.048	0.128	-	0.088	0.040000
3	0.118	0.160	-	0.139	0.021000
4	0.220	0.260	-	0.240	0.020000
5	0.290	0.302	-	0 296	0.006000

pH 9.5

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.004	0.005	0.005	0.005	0.000500
1	0.006	0.007	0.007	0.007	0.000500
2	0.008	0.008	0.008	0.008	0.000000
3	0.009	0.009	0.009	0.009	0.000000
4	0.007	0.007	0.007	0.007	0.000000
5	0.010	0.010	0.010	0.010	0.000000
14	0.020	0.014	0.010	0.015	0.002906

pH 10

Time (day)	Rep.1	Rep. 2	Rep. 3	Mean	St. Error
0	0.005	0.004	0.005	0.005	0.000408
1	0.006	0.007	0.007	0.007	0.000000
2	0.006	0.009	0.009	0.008	0.000000
3	0.010	0.010	0.010	0.010	0.000000
4	0.005	0.005	0.005	0.005	0.000000
5	0.007	0.007	0.007	0.007	0.000000
14	0.003	0.004	0.002	0.003	0.000577

Table 7. Change in absorbance of Flexibacter columnaris 1468 (4 replicates cultures) with time under different pH values and incubated at 30°C. Absorbance of blank was 0.000.

pH 5 (Absorbance of the cultures at pHs 3 and 4 was similar to those of at pH 5).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
1.00	0.031	0.031	0.030	0.029	0.030	-1.519
2.00	0.028	0.029	0.030	0.030	0.029	-1.534
3.00	0.030	0.029	0.030	0.029	0.030	-1.530
4.00	0.027	0.026	0.025	0.028	0.027	-1.577
5.00	0.026	0.025	0.025	0.027	0.026	-1.589
336.00	0.018	0.022	0.021	0.016	0.019	-1.716

pH 6

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.032	0.033	0.034	0.034	0.033	-1.478
1.00	0.038	0.038	0.039	0.039	0.039	-1.415
1.30	0.044	0.045	0.044	0.043	0.044	-1.357
2.00	0.053	0.054	0.057	0.053	0.054	-1.266
2.30	0.066	0.068	0.065	0.064	0.066	-1.182
3.00	0.073	0.080	0.075	0.073	0.075	-1.123
3.30	0.084	0.092	0.086	0.085	0.087	-1.062
4.00	0.094	0.101	0.095	0.097	0.097	-1.014
4.30	0.105	0.108	0.105	0.108	0.107	-0.973
5.00	0.110	0.118	0.114	0.115	0.114	-0.942

Table 7. continued

pH 6.5

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.033	0.032	0.034	0.033	0.033	-1.481
1.00	0.038	0.040	0.041	0.039	0.040	-1.403
1.30	0.048	0.051	0.054	0.049	0.051	-1.297
2.00	0.065	0.065	0.067	0.066	0.066	-1.182
2.30	0.078	0.074	0.076	0.076	0.076	-1.119
3.00	0.090	0.082	0.083	0.082	0.084	-1.074
3.30	0.101	0.092	0.093	0.094	0.095	-1.022
4.00	0.113	0.101	0.103	0.106	0.106	-0.976
4.30	0.136	0.110	0.114	0.118	0.120	-0.923
5.00	0.148	0.125	0.124	0.128	0.131	-0.882

pH 7.0

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.035	0.034	0.034	0.033	0.034	-1.469
1.00	0.045	0.044	0.041	0.039	0.042	-1.374
1.30	0.050	0.054	0.052	0.049	0.051	-1.290
2.00	0.067	0.068	0.064	0.062	0.065	-1.185
2.30	0.084	0.078	0.074	0.071	0.077	-1.115
3.00	0.098	0.085	0.080	0.082	0.086	-1.064
3.30	0.106	0.096	0.092	0.094	0.097	-1.013
4.00	0.125	0.106	0.103	0.108	0.111	-0.957
4.30	0.144	0.118	0.110	0.120	0.123	-0.910
5.00	0.164	0.129	0.127	0.138	0.140	-0.855

pH 7.5

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.034	0.033	0.035	0.036	0.035	-1.462
1.00	0.045	0.043	0.040	0.043	0.043	-1.369
1.30	0.055	0.060	0.055	0.056	0.057	-1.248
2.00	0.063	0.072	0.068	0.069	0.068	-1.167
2.30	0.082	0.081	0.077	0.079	0.080	-1.098
3.00	0.094	0.087	0.086	0.086	0.088	-1.054
3.30	0.105	0.097	0.092	0.094	0.097	-1.013
4.00	0.120	0.108	0.103	0.106	0.109	-0.962
4.30	0.131	0.113	0.109	0.115	0.117	-0.932
5.00	0.146	0.125	0.121	0.128	0.130	-0.886

Table 7. continued

pH 8.2

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.30	0.032	0.035	0.033	0.034	0.034	-1.475
1.00	0.038	0.041	0.040	0.039	0.040	-1.403
1.30	0.049	0.048	0.048	0.046	0.048	-1.321
2.00	0.054	0.054	0.045	0.051	0.051	-1.292
2.30	0.061	0.057	0.057	0.058	0.058	-1.235
3.00	0.075	0.065	0.068	0.067	0.069	-1.163
3.30	0.080	0.068	0.071	0.072	0.073	-1.138
4.00	0.087	0.074	0.077	0.079	0.079	-1.101
4.30	0.094	0.078	0.082	0.084	0.085	-1.073
5.00	0.101	0.085	0.089	0.092	0.092	-1.037

pH 9 (Absorbance of the cultures at pHs 9.5 and 10 was similar to those of at pH 9).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
1.00	0.030	0.031	0.029	0.030	0.030	-1.523
2.00	0.030	0.032	0.032	0.032	0.032	-1.502
3.00	0.028	0.030	0.030	0.032	0.030	-1.523
4.00	0.027	0.030	0.030	0.031	0.030	-1.530
5.00	0.027	0.029	0.028	0.029	0.028	-1.549
336.00	0.017	0.013	0 021	0.015	0.017	-1.783

Table 8. Change in absorbance of Cytophaga psychrophila 91/4043-17 (4 replicates cultures) with time under different pH values and incubated at 20°C. Absrobance of blank was 0.000-0.003.

pH 5.5 (Absorbance of cultures at pHs 3, 4 and 5 was similar to those of at pH 5.5)

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.028	0.031	0.030	0.032	0.030	-1.519
1.50	0.028	0.028	0.031	0.028	0.029	-1.541
2.15	0.030	0.029	0.030	0.031	0.030	-1.523
3.00	0.029	0.031	0.030	0.030	0.030	-1.523
3.45	0.030	0.030	0.030	0.029	0.030	-1.527
4.50	0.028	0.030	0.030	0.030	0.030	-1.530
5.15	0.030	0.030	0.030	0.030	0.030	-1.523
6.00	0.027	0.030	0.030	0.029	0.029	-1.538
6.45	0.030	0.030	0.029	0.028	0.029	-1.534
7.50	0.028	0.030	0.028	0.029	0.029	-1.541
8.15	0.028	0.030	0.028	0.029	0.029	-1.541
9.00	0.028	0.030	0.028	0.028	0.029	-1.545
336.00	0.020	0.015	0.013	0.021	0.017	-1.763

Table 8. continued

pH 6

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.032	0.032	0.033	0.033	0.033	-1.488
1.50	0.036	0.036	0.037	0.038	0.037	-1.435
2.15	0.040	0.040	0.042	0.044	0.042	-1.382
3.00	0.043	0.043	0.045	0.046	0.044	-1.354
3.45	0.045	0.046	0.047	0.048	0.047	-1.333
4.50	0.046	0.047	0.049	0.050	0.048	-1.319
5.15	0.048	0.048	0.051	0.053	0.050	-1.301
6.00	0.049	0.053	0.054	0.057	0.053	-1.274
6.45	0.050	0.052	0.052	0.054	0.052	-1.284
7.50	0.047	0.049	0.050	0.053	0.050	-1.303
8.15	0.047	0.048	0.048	0.051	0.049	-1.314
9.00	0.046	0.048	0.047	0.050	0.048	-1.321
15.00	0.028	0.036	0.041	0.045	0.038	-1.426

pH 6.5

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.036	0.036	0.035	0.035	0.036	-1.450
1.50	0.045	0.041	0.040	0.040	0.042	-1.382
2.15	0.055	0.055	0.049	0.047	0.052	-1.288
3.00	0.060	0.061	0.057	0.055	0.058	-1.235
3.45	0.063	0.063	0.059	0.058	0.061	-1.216
4.50	0.065	0.067	0.061	0.063	0.064	-1.194
5.15	0.066	0.068	0.063	0.066	0.066	-1.182
6.00	0.069	0.067	0.068	0.069	0.068	-1.166
6.45	0.070	0.072	0.069	0.071	0.071	-1.152
7.50	0.070	0.073	0.071	0.072	0.072	-1.146
8.15	0.071	0.073	0.072	0.073	0.072	-1.141
9.00	0.072	0.074	0.072	0.074	0.073	-1.137
15.00	0.080	0.088	0.086	0.089	0.086	-1.067

pH 7.0

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.032	0.033	0.033	0.033	0.033	-1.485
1.50	0.042	0.042	0.041	0.039	0.041	-1.387
2.15	0.050	0.048	0.047	0.045	0.048	-1.323
3.00	0.059	0.064	0.061	0.058	0.061	-1.218
3.45	0.061	0.065	0.062	0.060	0.062	-1.208
4.50	0.064	0.066	0.065	0.062	0.064	-1.192
5.15	0.064	0.068	0.067	0.065	0.066	-1.180
6.00	0.066	0.072	0.070	0.069	0.069	-1.160
6.45	0.067	0.072	0.072	0.070	0.070	-1.153
7.50	0.071	0.073	0.075	0.072	0.073	-1.138
8.15	0.072	0.074	0.075	0.073	0.074	-1.134
9.00	0.072	0.074	0.075	0.074	0.074	-1.132
15.00	0.082	0.078	0.079	0.082	0.080	-1.096

Table 8. continued

pH 7.5

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.036	0.034	0.033	0.033	0.034	-1.469
1.50	0.048	0.043	0.042	0.040	0.043	-1.364
2.15	0.062	0.057	0.058	0.056	0.058	-1.235
3.00	0.064	0.062	0.063	0.061	0.063	-1.204
3.45	0.065	0.063	0.064	0.063	0.064	-1.196
4.50	0.066	0.067	0.066	0.065	0.066	-1.180
5.15	0.071	0.068	0.069	0.068	0.069	-1.161
6.00	0.073	0.072	0.074	0.073	0.073	-1.137
6.45	0.076	0.075	0.077	0.077	0.076	-1.118
7.50	0.079	0.078	0.080	0.081	0.080	-1.100
8.15	0.080	0.080	0.082	0.083	0.081	-1.090
9.00	0.083	0.082	0.084	0.083	0.083	-1.081
15.00	0.098	0.100	0.098	0.101	0.099	-1.003

pH 8

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.034	0.033	0.033	0.033	0.033	-1.478
1.50	0.036	0.041	0.040	0.041	0.040	-1.403
2.15	0.045	0.045	0.044	0.045	0.045	-1.349
3.00	0.049	0.056	0.054	0.056	0.054	-1.270
3.45	0.050	0.056	0.056	0.057	0.055	-1.262
4.50	0.051	0.057	0.058	0.058	0.056	-1.252
5.15	0.052	0.061	0.061	0.063	0.059	-1.227
6.00	0.054	0.063	0.064	0.066	0.062	-1.209
6.45	0.055	0.064	0.065	0.069	0.063	-1.199
7.50	0.054	0.064	0.065	0.068	0.063	-1.202
8.15	0.054	0.062	0.064	0.067	0.062	-1.209
9.00	0.053	0.062	0.064	0.067	0.062	-1.211
15.00	0.031	0.032	0.035	0.035	0.033	-1.478

pH 8.5 (Absorbance of cultures at pHs 9, 9.5 and 10 was similar to those of at pH 8.5).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.028	0.028	0.028	0.028	0.028	-1.553
1.50	0.028	0.028	0.027	0.028	0.028	-1.557
2.15	0.027	0.028	0.027	0.028	0.028	-1.561
3.00	0.027	0.028	0.027	0.027	0.027	-1.565
3.45	0.027	0.027	0.027	0.027	0.027	-1.569
4.50	0.027	0.027	0.026	0.027	0.027	-1.573
5.15	0.027	0.027	0.026	0.027	0.027	-1.573
6.00	0.026	0.026	0.026	0.027	0.026	-1.581
6.45	0.026	0.026	0.026	0.027	0.026	-1.581
7.50	0.026	0.026	0.026	0.026	0.026	-1.585
8.15	0.026	0.025	0.025	0.026	0.026	-1.593
9.00	0.025	0.025	0.025	0.025	0.025	-1.602
336.00	0.020	0.012	0.020	0.015	0.017	-1.776

Table 9. Change in absorbance of Flexibacter maritimus 89/4762 (3 replicates cultures) with time under different salinities (NaCl%) and incubated at 30°C. Absorbance of blank was 0.000-0.006.

1% NaCl

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.009	0.009	0.009	0.009	0.000000
1	0.009	0.009	0.008	0.009	0.000333
2	0.008	0.008	0.008	0.008	0.000000
3	0.010	0.009	0.007	0.009	0.000882
4	0.007	0.006	0.005	0.006	0.000577
5	0.005	0.004	0.003	0.004	0.000577
14	0.003	0.004	0.003	0.003	0.000333

1.7% NaCl

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.009	0.009	0.010	0.009	0.000333
1	0.009	0.009	0.009	0.009	0.000000
2	0.008	0.009	0.008	0.008	0.000333
3	0.006	0.007	0.006	0.006	0.000333
4	0.006	0.007	0.006	0.006	0.000333
5	0.004	0.005	0.005	0.005	0.000333
14	0.004	0.005	0.007	0.005	0.000882

2.5% NaCl

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.010	0.014	0.012	0.012	0.001155
1	0.009	0.012	0.010	0.010	0.000882
2	0.008	0.010	0.009	0.009	0.000577
3	0.009	0.009	0.009	0.009	0.000000
4	0.006	0.007	0.006	0.006	0.000333
5	0.005	0.006	0.005	0.005	0.000333
14	0.005	0.002	0.005	0.004	0.001000

3.5% NaCl

Time (day)	Rep.	Rep. 2	Rep. 3	Mean	St. Error
0	0.012	0.011	0.010	0.011	0.000577
1	0.011	0.010	0.010	0.010	0.000333
2	0.011	0.010	0.010	0.010	0.000333
3	0.010	0.009	0.009	0.009	0.000333
4	0.008	0.007	0.006	0.007	0.000577
5	0.006	0.006	0.005	0.006	0.000333
21	0.010	0.008	0.009	0.009	0.000577

Table 10. Change in absorbance of Flexibacter maritimus 89/4762 (3 replicates cultures) with time under different salinities (seawater) and incubated at 30°C. Absorbance of blank was 0.000-0.008.

0% seawater

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.008	0.011	0.009	0.009	0.000882
1	0.008	0.010	0.009	0.009	0.000577
2	0.006	0.010	0.009	0.008	0.001202
3	0.004	0.009	0.008	0.007	0.001528
4	0.005	0.008	0.008	0.007	0.001000
5	0.005	0.008	0.007	0.007	0.000882
14	0.005	0.009	0.009	0.008	0.001333

10% seawater

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.012	0.012	0.010	0.011	0.000667
1	0.012	0.012	0.010	0.011	0.000667
2	0.010	0.010	0.010	0.010	0.000000
3	0.009	0.009	0.010	0.009	0.000333
4	0.008	0.008	0.009	0.008	0.000333
5	0.008	0.007	0.009	0.008	0.000577
14	0.008	0.010	0.009	0.009	0 000577

30% seawater

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.009	0.009	0.012	0.010	0.001000
1	0.098	0.089	0.101	0.096	0.003606
2	0.160	0.135	0.141	0.145	0.007535
3	0.197	0.151	0.223	0.190	0.021050
4	0.234	0.177	0.278	0.230	0.029237
5	0.275	0.260	0.299	0.278	0.011358

70% seawater

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.012	0.012	0.012	0.012	0.000000
1	0.100	0.100	0.110	0.103	0.003333
2	0.159	0.149	0.146	0.151	0.003930
3	0.251	0.230	0.210	0.230	0.011837
4	0.319	0.304	0.295	0.306	0.007000
5	0.355	0.313	0.303	0.324	0.015930

100% seawater

Time (day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.012	0.010	0.010	0.011	0.000667
1	0.083	0.086	0.113	0.094	0.009539
2	0.135	0.136	0.174	0.148	0.012837
3	0.240	0.220	0.273	0.244	0.015452
4	0.317	0.281	0.335	0.311	0.015875
5	0.360	0.374	0.372	0.369	0.004372

Table 11. Change in absorbance of Cytophaga johnsonae 91/0262-10 (3 replicates cultures) with time under different salinities (NaCl%) and incubated at 20°C. Absorbance of blank was 0.000.

0% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.002	0.003	0.002	0.000333
1	0.106	0.094	0.117	0.106	0.008134
2	0.170	0.172	0.209	0.184	0.015530
3	0.218	0.232	0.267	0.239	0.017847
4	0.284	0.288	0.334	0.302	0.019647
5	0.304	0.302	0.386	0.331	0.033892

0.5% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.002	0.003	0.002	0.000333
1	0.089	0.107	0.109	0.102	0.007789
2	0.150	0.174	0.180	0.168	0.011225
3	0.185	0.215	0.225	0.208	0.014720
4	0.224	0.259	0.268	0.250	0.016437
5	0.250	0.282	0.299	0.277	0.017593

1% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.001	0.002	0.002	0.000333
1	0.058	0.071	0.083	0.071	0.008841
2	0.116	0.119	0.125	0.120	0.003240
3	0.146	0.160	0.163	0.156	0.006416
4	0.174	0.189	0.180	0.181	0.005339
5	0.186	0.213	0.203	0.201	0.009652

1.5% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.003	0.001	0.002	0.002	0.000577
1	0.005	0.006	0.005	0.005	0.000333
2	0.025	0.041	0.042	0.036	0.005508
3	0.052	0.076	0.077	0.068	0.008172
4	0.085	0.108	0.108	0.100	0.007667
5	0.120	0.125	0.127	0.124	0.002082

2% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.002	0.001	0.002	0.000333
1	0.004	0.004	0.004	0.004	0.000000
2	0.006	0.006	0.004	0.005	0.000667
3	0.025	0.016	0.015	0.019	0.003180
4	0.056	0.032	0.032	0.040	0.008000
5	0.070	0.055	0.056	0 060	0.004842

Table 11. continued

2.5% NaCl

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.002	0.001	0.002	0.002	0.000333
1	0.004	0.003	0.003	0.003	0.000333
2	0.005	0.005	0.004	0.005	0.000333
3	0.004	0.005	0.004	0.004	0.000333
4	0.005	0.005	0.004	0.005	0.000333
5	0.005	0.004	0.004	0.004	0.000333
14	0.002	0.003	0.004	0.003	0.000577

3% NaCl (absorbance of cultures at 3.5% NaCl was similar to those of at 3% NaCl).

Time(day)	Rep. 1	Rep. 2	Rep. 3	Mean	St. Error
0	0.003	0.001	0.002	0.001	0.000577
1	0.004	0.002	0.003	0.001	0.000577
2	0.004	0.003	0.004	0.001	0.000289
3	0.004	0.003	0.004	0.001	0.000289
4	0.004	0.004	0.004	0.000	0.000000
5	0.004	0.004	0.004	0.000	0.000000
14	0.003	0.004	0.004	0.004	0.000333

Table 12. Change in absorbance of Flexibacter columnaris 1468 (4 repl. cultures) with time under different salinities (NaCl%) and incubated at 30°C. Absorbance of blank was 0.010.

0.0% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.025	0.025	0.025	0.025	0.025	-1.602
0.30	0.027	0.028	0.028	0.030	0.028	-1.549
1.00	0.038	0.042	0.040	0.040	0.040	-1.398
1.30	0.048	0.052	0.052	0.050	0.051	-1.297
2.00	0.058	0.060	0.062	0.060	0.060	-1.222
2.30	0.065	0.069	0.068	0.066	0.067	-1.174
3.00	0.074	0.073	0.076	0.073	0.074	-1.131
3.30	0.083	0.081	0.085	0.080	0.082	-1.085
4.00	0.097	0.096	0.099	0.096	0.097	-1.013
4.30	0.110	0.102	0.106	0.106	0.106	-0.975
5.00	0.120	0.113	0.118	0.119	0.117	-0.930
7.00	0.165	0.156	0.160	0.140	0.155	-0.809

0.4% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.025	0.025	0.025	0.025	0.025	-1.602
0.30	0.027	0.029	0.028	0.027	0.028	-1.557
1.00	0.031	0.033	0.033	0.032	0.032	-1.491
1.30	0.038	0.042	0.040	0.039	0.040	-1.401
2.00	0.048	0.050	0.049	0.047	0.049	-1.314
2.30	0.053	0.056	0.054	0.053	0.054	-1.268
3.00	0.060	0.062	0.060	0.059	0.060	-1.220
3.30	0.065	0.064	0.067	0.065	0.065	-1.185
4.00	0.073	0.071	0.076	0.073	0.073	-1.135
4.30	0.081	0.078	0.085	0.084	0.082	-1.086
5.00	0.093	0.094	0.095	0.093	0.094	-1.028
7.00	0.112	0.120	0.122	0.120	0.119	-0.926

Table 12. continued

0.7% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.025	0.025	0.025	0.025	0.025	-1.602
0.30	0.028	0.027	0.027	0.027	0.027	-1.565
1.00	0.031	0.031	0.032	0.031	0.031	-1.505
1.30	0.035	0.039	0.036	0.034	0.036	-1.442
2.00	0.039	0.042	0.040	0.038	0.040	-1.401
2.30	0.043	0.045	0.042	0.042	0.043	-1.367
3.00	0.046	0.047	0.047	0.046	0.047	-1.333
4.00	0.053	0.053	0.052	0.053	0.053	-1.278
5.00	0.058	0.061	0.061	0.061	0.060	-1.220
10.00	0.080	0.080	0.089	0.095	0.086	-1.066
12.00	0.093	0.094	0.106	0.101	0.099	-1.007

1.0% NaCl (absorbance of the cultures at 1.5%, 2%, 2.5% and 3% NaCl was similar to those of at 1% NaCl).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.025	0.025	0.025	0.025	0.025	-1.602
1.00	0.026	0.027	0.027	0.026	0.027	-1.577
2.00	0.030	0.030	0.031	0.028	0.030	-1.527
3.00	0.030	0.030	0.031	0.028	0.030	-1.527
4.00	0.029	0.029	0.030	0.027	0.029	-1.541
5.00	0.028	0.028	0.028	0.026	0.028	-1.561
20.00	0.019	0.018	0.025	0.022	0.021	-1.678
336.00	0.018	0.010	0.015	0.020	0.016	-1.803

Table 13. Change in absorbance of Cytophaga psychrophila 91/4043-17 (4 replicates cultures) with time under different salinities (NaCl%) and incubated at 20°C. Absorbance of blank was 0.000-0.002.

0.0% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.038	0.034	0.036	0.036	0.036	-1.444
1.50	0.042	0.039	0.040	0.040	0.040	-1.395
2.15	0.050	0.047	0.046	0.046	0.047	-1.326
3.00	0.057	0.053	0.050	0.052	0.053	-1.276
3.45	0.065	0.061	0.061	0.064	0.063	-1.202
4.50	0.074	0.070	0.066	0.071	0.070	-1.153
5.15	0.075	0.074	0.073	0.074	0.074	-1.131
6.00	0.086	0.078	0.079	0.077	0.080	-1.097
6.45	0.096	0.085	0 088	0.084	0.088	-1.054
7.50	0.101	0.094	0.093	0.087	0.094	-1.028
8.15	0.106	0.096	0.096	0.094	0.098	-1.009
9.00	0.108	0.099	0.098	0.098	0.101	-0.997
15.00	0.120	0.114	0.110	0.115	0.115	-0.940

Table 13. continued

0.5% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.038	0.034	0.038	0.036	0.037	-1.438
1.50	0.040	0.037	0.040	0.040	0.039	-1.406
2.15	0.048	0.047	0.048	0.048	0.048	-1.321
3.00	0.058	0.052	0.061	0.060	0.058	-1.238
3.45	0.062	0.059	0.062	0.063	0.062	-1.211
4.50	0.068	0.070	0.070	0.068	0.069	-1.161
5.15	0.073	0.072	0.072	0.071	0.072	-1.143
6.00	0.079	0.076	0.075	0.075	0.076	-1.118
6.45	0.085	0.078	0.080	0.081	0.081	-1.092
7.50	0.083	0.079	0.081	0.082	0.081	-1.090
8.15	0.084	0.080	0.081	0.082	0.082	-1.088
9.00	0.085	0.080	0.081	0.082	0.082	-1.086
15.00	0.088	0.081	0.083	0.085	0.084	-1.074

0.7% NaCl

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.033	0.034	0.033	0.033	0.033	-1.478
1.50	0.034	0.034	0.034	0.035	0.034	-1.465
2.15	0.038	0.041	0.037	0.038	0.039	-1.415
3.00	0.041	0.043	0.041	0.042	0.042	-1.379
3.45	0.042	0.044	0.041	0.044	0.043	-1.369
4.50	0.046	0.051	0.049	0.049	0.049	-1.312
5.15	0.047	0.053	0.052	0.053	0.051	-1.290
6.00	0.048	0.050	0.050	0.052	0 050	-1.301
6.45	0.040	0.048	0.050	0 052	0.048	-1.323
7.50	0.040	0.047	0.045	0.048	0.045	-1.347
8.15	0.040	0.047	0.045	0.047	0.045	-1.349
9.00	0.040	0.047	0.046	0.047	0.045	-1.347
15.00	0.042	0.041	0.046	0.046	0.044	-1.359

1.0% NaCl (Absorbance of the cultures at 1.5%, 2%, 2.5% and 3% NaCl was similar to those of at 1% NaCl).

Time (hr.min)	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Mean	Log
0.00	0.030	0.030	0.030	0.030	0.030	-1.523
0.45	0.033	0.034	0.033	0.033	0.033	-1.478
1.50	0.033	0.034	0.033	0.033	0.033	-1.478
3.00	0.034	0.033	0.035	0.035	0.034	-1.465
4.50	0.033	0.034	0.033	0.033	0.033	-1.478
6.00	0.033	0.034	0.033	0.033	0.033	-1.478
9.00	0.030	0.030	0.030	0.030	0.030	-1.523
15.00	0.030	0.030	0.029	0.030	0.030	-1.527
336.00	0.015	0.010	0.013	0.018	0.014	-1.854