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Survival and virulence of *Flavobacterium psychrophilum* in water microcosms

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Abstract

Flavobacterium psychrophilum, the causative agent of rainbow trout fry syndrome and cold water disease in salmonids, causes serious disease outbreaks in fish farms worldwide. The aim of the present study was to examine the survival capacity of *F. psychrophilum* in laboratory microcosms containing sterilised water under different environmental conditions and to examine the virulence of starving *F. psychrophilum* cells. The results showed that *F. psychrophilum* survived for very long time in sterilised fresh water at 15°C and the cells were still culturable after starvation for 300 days. A high salinity of the water (30‰) drastically reduced the number of culturable cells below detection limit after incubation for 1 day. A water salinity of approximately 6‰ initially reduced the number of culturable cells below the detection limit, but cells were again recovered on agar plates at the end of the experiment. The presence of sediment containing nutrients in the experimental water microcosms increased the survival of *F. psychrophilum*. The challenge experiments indicated that the virulence of starving *F. psychrophilum* is maintained for at least seven days after the transfer of the bacterial cells to fresh water.
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Keywords: *Flavobacterium psychrophilum*; Survival; Fish pathogen; Virulence

1. Introduction

The isolation of *Flavobacterium psychrophilum*, causing rainbow trout fry syndrome (RTFS) and cold water disease in salmonids (family Salmoniformes), has since the end of 1980's frequently been reported by several European countries [1–5]. *F. psychrophilum* was initially described in USA [6], and the pathogen has subsequently been isolated also in Australia, Canada, Chile and Japan [7–10]. *F. psychrophilum* has in Europe mainly been isolated from rainbow trout (*Oncorhynchus mykiss*) but occasionally also from cyprinids and eel (*Anguilla anguilla*) [11]. In the USA the pathogen has been isolated from different salmonids and in Japan also from ayu (*Plecoglossus altivelis*) and pale chub (*Zacco platypus*) [12].

Although *F. psychrophilum* has been associated with significant mortalities in fish culture, the ecology and biology of this bacterial species has been poorly examined. Gliding bacteria consists of several different bacterial species, including *F. psychrophilum*. These species have mainly a degradative metabolism and they play a considerable role in the turnover of organic material in the nature [13]. The fact that *F. psychrophilum* often affect the skin of larger rainbow trout indicates that the bacteria are transmitted through the water, and that the reservoir could be the water column, including water organisms, debris or the sediment. In previous studies, *F. psychrophilum* has been detected and isolated from fresh and brackish water associated with fish farms [14–16]. However, to our knowledge so far there is limited information available about the survival capacity of *F. psychrophilum* in the environment [17] and the maintenance of the virulence of *F. psychrophilum* in the water environment.

The aims of the present study were to examine the survival capacity of *F. psychrophilum* in water under different environmental conditions and to examine the virulence of starving cells of *F. psychrophilum*.

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2. Materials and methods

2.1. Experimental design

Three independent experiments were done to examine the survival of the fish pathogen *F. psychrophilum* in water microcosms under different physiological conditions. (1) The influence of temperature on its survival was tested in sterilised fresh water without sediment at 5, 10 and 15°C. (2) The influence of salinity on its survival was tested in sterilised fresh, brackish and artificial salt water. (3) The effect of two different sediments on its survival was examined using microcosms with sterilised sediment and fresh water. The inoculated microcosms were incubated at 15°C (except for the experiment with different temperatures) for 50–60 days, and in addition microcosms from one treatment of the experiments were incubated for more than 300 days to determine the long-term survival capacity of *F. psychrophilum*.

Additionally, in order to examine the ability of starving *F. psychrophilum* cells to survive and maintain their virulence in fresh water, three different fish challenge experiments were done. For injection experiments bacteria were inoculated in high and low concentrations (see below) into microcosms in order to examine the influence of cell concentration on the invasiveness of the bacteria. Water samples from the microcosms containing bacteria were after certain time intervals injected into rainbow trout. For bath challenge experiments bacteria were inoculated into a small aquarium containing 1 l sterilised water. Rainbow trout were subsequently transferred to the aquarium after 2 weeks incubation.

2.2. Bacterial strains and growth conditions

Two strains of *F. psychrophilum* were used in the survival tests in different microcosms: strain V9/93 isolated from an ulcer of rainbow trout in 1993 in Finland [18] and the type strain NCIMB 1947^T. Strain V9/93 was chosen as it represents the common serotype Th of *F. psychrophilum* and a common genotype [18]. The type strain NCIMB 1947^T, which is serotype Fp^T and represents a different genotype compared to strains V9/93 [18], was included for comparison. The strains were stored at −70°C in *Cytophaga* broth [19] supplemented with 12% glycerol. For the experiments, the bacteria were thawed, inoculated into *Cytophaga* broth and incubated at 15°C for 36 h. The cells were collected by centrifugation (2200×*g*, 20 min) and washed twice in sterile fresh water and subsequently introduced into the microcosms.

For the survival and virulence experiments strain T1-1 of *F. psychrophilum* was used. The strain was used because its virulence has previously been thoroughly studied [18,20]. Strain T1-1 was stored in tryptone yeast extract salts (TYES) broth [8] supplemented with 15% (v/v) sterile glycerol at −70°C. For the experiments, the bacteria were thawed and inoculated on TYES agar (TYES broth+1.5% agar) and 1–3 colonies were transferred into TYES broth, incubated for 2 days at 15°C with shaking. The cells were harvested and washed once with sterile phosphate buffered saline (PBS, pH 7.2) by centrifugation (2500×*g*, 20 min). After the centrifugation the cells were re-suspended into sterilised lake water (pH adjusted to 7.0–7.2) and added into two microcosms in two different concentrations (high concentration: initial concentration 2.0×10^8 CFU ml^{-1}; and low concentration: initial concentration 5.0×10^6 CFU ml^{-1}) and into an aquarium (initial concentration 1.0×10^5 CFU ml^{-1}).

2.3. Microcosms

The microcosms for the survival experiments were prepared using 100 ml Erlenmeyer flasks with metal caps containing 50 ml water and in one experiment also sediment (25 g) in addition to the water. After inoculation with bacterial cells the microcosms were statically maintained in the dark at 15°C unless otherwise stated. The experiments were done with three parallel microcosms for each treatment. For the virulence tests one microcosm (containing fresh water) per bacterial concentration was used and these were maintained at 11°C in the dark.

2.4. Water

Natural fresh water (taken from Kakskerta lake in SW Finland, water conductance = 12.3 mS m^{-1}), natural brackish water (taken from the Archipelago Sea, SW Finland, salinity (S) = 6.0‰ after sterilisation) and artificial salt water (S = 30.5‰) were used in the experiments. The fresh and brackish water were collected into clean plastic bottles, transported to the laboratory and kept at 4°C until filtered through membrane filters (Sartorius, 0.45-μm pore size), to remove all particles and sterilised by autoclaving at 121°C for 20 min. The artificial salt water was made from brackish water supplemented with chemically defined sea salt (Natura sea salt) and adjusted to 30‰. The salinity of the water samples was measured with a salinometer (Limnos, YSI model 33). The pH of the waters was adjusted to 7.0 ± 0.2 with 1.0 M HCl after autoclaving.

2.5. Sediment

Two different types of sediments were used: 'natural' and 'refined'. The 'natural' sediment consisted of sand collected from the shore of a sand beach on the south coast of Finland. The sand was cleaned from large debris by eye and it contained microorganisms, which could serve as a nutrient source. The 'refined' sediment was obtained from Merck Eurolab (Sea sand, extra pure, Germany). In order to stabilise the salinity and pH, both sediments were washed in filtered fresh water 10 times and dried before

use. The sediments for the experiment were sterilised together with the water in the microcosms by autoclaving.

2.6. Long-term survival of F. psychrophilum

In order to determine the long-term survival of *F. psychrophilum*, the following microcosms were incubated for more than 300 days: (1) microcosms containing sediment, inoculated with strain V9/93 and incubated at 15°C; (2) microcosms containing fresh water, incubated at 15°C and inoculated with strain NCIMB 1947^T (2 experiments).

2.7. Cell count

Culturable bacterial counts, from the temperature, salinity and sediment experiments, were obtained by spreading serially 10-fold diluted (in filtered, sterilised fresh water) subsamples (0.1 ml) from the microcosms onto *Cytophaga* agar plates. The agar plates were incubated for seven days at 15°C. The viable number of cells in the microcosms was calculated from the number of colonies, the volume of the inoculum used, and the dilution factor.

2.8. Fish

Rainbow trout for the challenge experiments were obtained from a commercial freshwater fish farm in Finland without previous history of infection with *F. psychrophilum*. The fish were kept in a 0.225 m^3 fibreglass tank with continuously flowing well water [16] of 10°C. Prior to each experiment the fish were transferred to aquaria containing 10 l of static, aerated well water and acclimatised for a minimum of three days. Fish were fed ad libitum with a commercial (Ewos) feed throughout the experiments. The water in the aerated aquaria was changed regularly during the experiment. The water temperature during the experiment was 11–12°C.

2.9. Virulence of starving F. psychrophilum

In order to examine the virulence of starving *F. psychrophilum*, rainbow trout were challenged in three experiments. In two experiments, water (50 µl) from the microcosms containing high (initial cell number: 2.0×10^8 CFU ml−1) and low (initial cell number: 5.0×10^6 CFU ml^{-1}) concentrations of maintained cells of *F. psychrophilum* was injected (s.c.) into rainbow trout ($n=20$ fish $group^{-1}$, average weight 0.5 g with high and 0.7 g with low concentration of cells), 1, 7, 14, 21, 28 and 49 days post-inoculation. As positive control groups served 2×20 fish injected s.c. with 50 µl PBS containing 5.0×10^4 (low concentration) and 5.5×10^5 (high concentration) CFU $fish^{-1}$ of a fresh culture (cultivated in TYES) of *F. psychrophilum* strain T1-1. As negative control groups served 2×20 fish injected with only PBS.

In a third experiment rainbow trout (average weight 0.7 g) were exposed in a bath challenge for 1 h to *F. psychrophilum* strain T1-1, which had been maintained for two weeks in sterilised lake water and using artificially wounded fish as described by Madetoja et al. [20]. The number of *F. psychrophilum* was estimated to be 2.0×10^4 CFU ml^{-1} at the bath challenge. As a negative control group served 20 skin-wounded rainbow trout bathed in identical water as the challenged fish, but without *F. psychrophilum*. The mortality of the fish was monitored for up to 15 days after injection or bath challenge. All dead fish were collected daily and tissue samples from their spleen, kidney and skin ulcer (if any) were inoculated onto TYES agar plates, which subsequently were incubated at 15°C for at least seven days. Bacteria forming yellow colonies on the agar plates, typical to *F. psychrophilum*, were identified using IFAT [21].

2.10. Presentation of the results

The numbers of viable bacterial cells in the microcosms were calculated from the dilution that gave between three and 150 colonies on the agar plates. The bacterial numbers are indicated as an average of the bacterial numbers in the three parallel microcosms ± S.D.

3. Results

3.1. Effect of the temperature on the survival of F. psychrophilum cells

The survival capacity of the two strains (V9/93 and NCIMB 1947^T) tested at different temperatures showed similar trends (Fig. 1A,B). In general, in the beginning of the experiment a gradual reduction of the number of culturable bacteria was observed, with a subsequent slight increase in CFU ml^{-1} at the end of the experiment (50–60 days). No large variations were observed between the three different temperatures, although the number of culturable cells of strain NCIMB 1947^T at 5°C decreased constantly until the end of the experiment. Viable and culturable bacteria were present in all microcosms at the end of the experiment after 56 days of incubation. The extended incubation of strain NCIMB 1947^T showed that the cells were able to survive for a very long time, and the bacteria were culturable 300 days after inoculation into fresh water at 15°C (Fig. 1B).

3.2. Effect of salinity on the survival of F. psychrophilum cells

The survival patterns of the two examined strains (V9/93 and NCIMB 1947^T), inoculated into microcosms with sterile fresh-, brackish- and artificial salt water, are shown in Fig. 2A,B. One day after inoculation into artificial salt water the bacteria of both strains were not recovered on

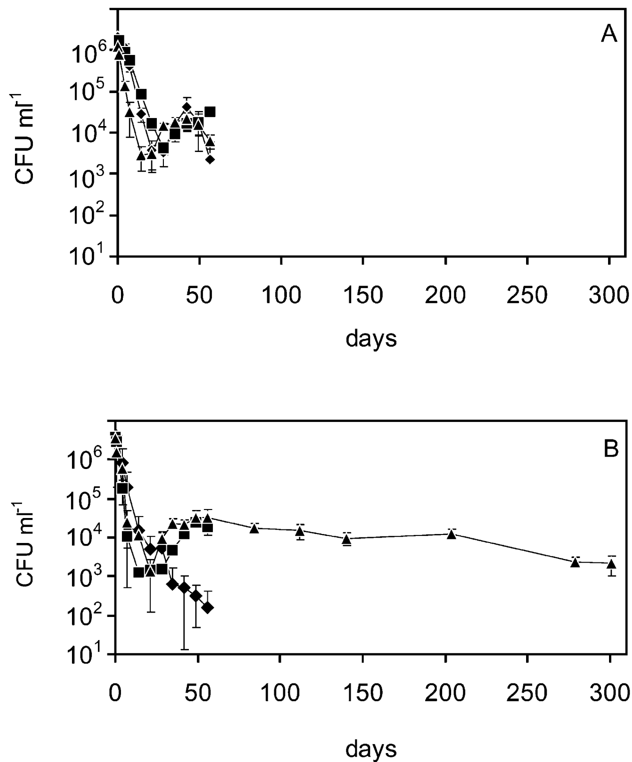

Fig. 1. Survival patterns of *F. psychrophilum* strains V9/93 (A) and NCIMB 1947^T (B) in microcosm containing sterilised fresh water at different temperatures, 5°C (◆), 10°C (■) and 15°C (▲). Bars indicate standard deviation.

Cytophaga agar. In fresh water, both strains showed a similar survival pattern with an initial reduction of number of culturable cells followed by a stabilisation around 10^4 CFU ml^{-1}. In brackish water the number of culturable cells was initially drastically reduced after inoculation, with a subsequent increase in the number of culturable cells. The extended incubation of strain NCIMB 1947^T in fresh water showed that the cells were able to survive for very long time and the bacteria were viable 300 days after inoculation into fresh water at 15°C (Fig. 2B).

3.3. Effect of sediment on the survival of F. psychrophilum cells

The survival of strains 13–24 and NCIMB 1947^T in microcosms containing fresh water and different sediments are shown in Fig. 3A,B. The cells of both strains showed an improved survival in microcosms containing sterile 'natural' sediment compared to the microcosms without sediment and those with 'laboratory' sediment. In fact, the number of culturable bacteria were lowest in the microcosms containing 'laboratory' sediment for both examined strains.

3.4. Virulence of starved F. psychrophilum

Viable counts of *F. psychrophilum* in the inoculated water used for the virulence tests decreased from 2.0×10^8 CFU ml^{-1} to 9.0×10^5 CFU ml^{-1} (high concentration) 49 days after inoculation, and from 5.0×10^6 CFU ml^{-1} to 1.5×10^5 CFU ml^{-1} (low concentration) 28 days after inoculation (Table 1). The mortality of the injected fish was 100% after 1–21 days starvation of *F. psychrophilum* in water and 50% after 49 days starvation (high concentration, Fig. 4A), and 100% after 1 day and 0% after 28 days (low concentration) starvation of *F. psychrophilum* in water (Fig. 4B). The cumulative mortality of the fish in the positive control group, injected with fresh cultures of strain T1-1, was 93% in the high (Fig. 4A) and 80% in the low (Fig. 4B) concentration experiments. Mortality among the negative control fish injected with PBS was not observed during 15 days monitoring of the fish. A significant correlation ($R^2 = 0.8917$) was observed between viable culturable bacterial cells from the experiments using high and low bacterial concentrations and corresponding fish mortality (mortality higher than 0% and lower than 100%; Fig. 5).

Viable counts of *F. psychrophilum* used for the bath challenge decreased from 1.0×10^5 CFU ml^{-1} to 2.0×10^4 CFU ml^{-1} during 14 days incubation in fresh water. Mortality of the fish bath challenged with starving *F. psychrophilum* was 50% after 21 days (Fig. 4C). Mortality of the negative control fish was 7%.

F. psychrophilum was isolated from spleen/kidney and

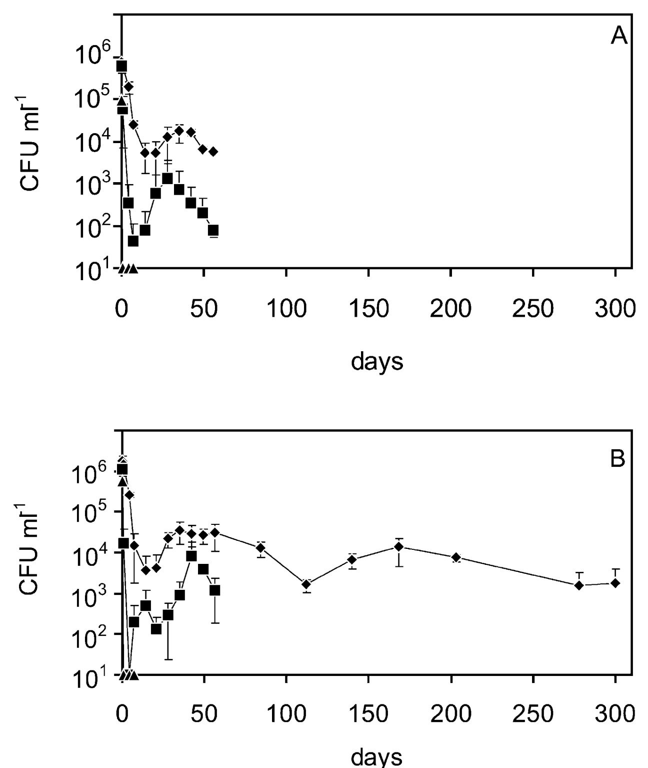

Fig. 2. Survival patterns of *F. psychrophilum* strains V9/93 (A) and NCIMB 1947^T (B) in microcosm containing sterilised fresh water (◆), brackish water (■) and salt water (▲). Bars indicate standard deviation.

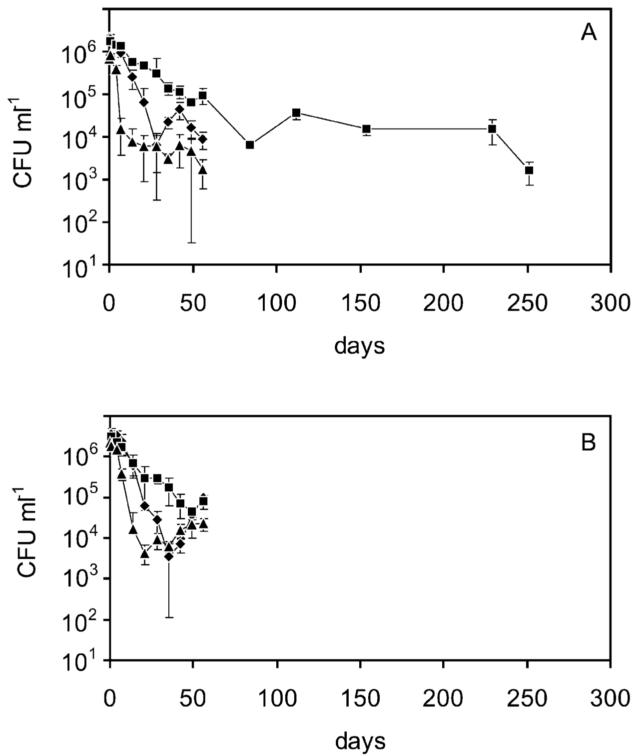

Fig. 3. Survival patterns of *F. psychrophilum* strains V9/93 (A) and NCIMB 1947^T (B) in microcosm with sterilised fresh water (◆) and with sterilised fresh water together with natural sediment (■) and laboratory sediment (▲). Bars indicate standard deviation.

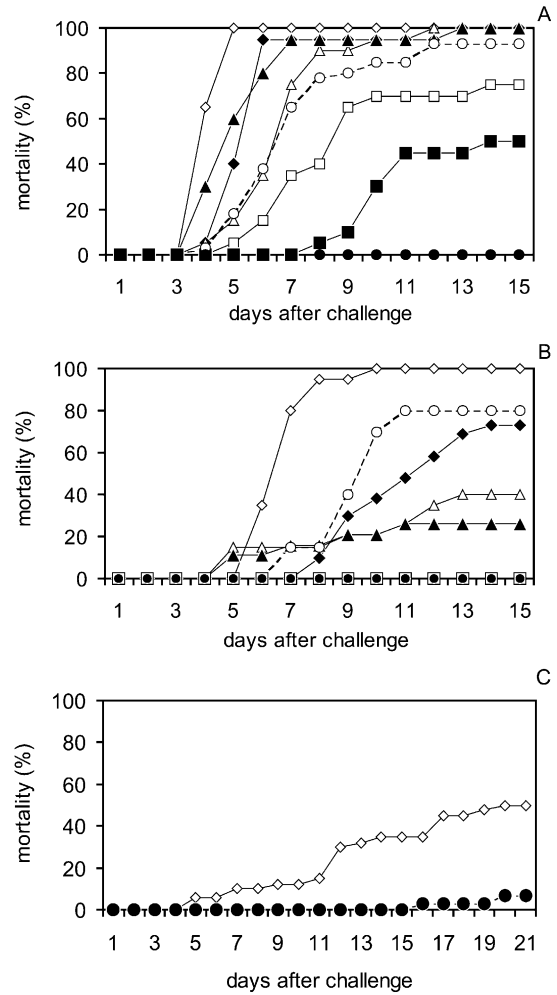

Fig. 4. Virulence of *F. psychrophilum* for rainbow trout after starvation in sterilised fresh water. Mortality of rainbow trout injected with bacteria, initially inoculated in fresh water in high concentration (A), low concentration (B) after starving for different times (◇, 1 day; ◆, 7 days; △, 14 days; ▲ 21 days; □, 28 days; ■, 49 days). ○, positive control fish injected with a fresh culture of strains T1-1; ●, negative control fish injected with sterile PBS. C: Mortality of rainbow trout exposed for *F. psychrophilum* starved for 14 days (△) and control fish not exposed for *F. psychrophilum* (●).

skin lesions of s.c. infected experimental and positive control fish, and bath infected fish, but not from dead negative control fish.

4. Discussion

In the present study, the survival of *F. psychrophilum* in water under different physiological conditions as well as the virulence of starved *F. psychrophilum* was examined. The results showed that *F. psychrophilum* cells might survive in sterilised water in culturable form in moderate numbers (10^3–10^4 CFU ml^{-1}) for at least 300 days with-

Table 1
Survival of *F. psychrophilum*, initially inoculated in two concentrations (low and high) into sterilised fresh water, and corresponding number of bacteria injected into rainbow trout, after starvation for certain time intervals (time after inoculation)

Time after inoculation (days)	Low concentration		High concentration	
	Survival of *F. psychrophilum* in water (CFU ml^{-1})	Number of bacteria injected (CFU fish^{-1})	Survival of *F. psychrophilum* in water (CFU ml^{-1})	Number of bacteria injected (CFU fish^{-1})
0	5.0×10^6		2.0×10^8	
1	2.3×10^6	1.2×10^5	8.9×10^7	4.5×10^6
7	2.4×10^6	1.2×10^5	3.9×10^6	2.0×10^5
14	5.0×10^5	2.5×10^4	7.5×10^6	3.8×10^5
21	3.0×10^5	1.5×10^4	1.6×10^7	7.8×10^5
28	1.5×10^5	7.5×10^3	8.6×10^6	4.3×10^5
49		N.D.	9.0×10^5	4.5×10^4

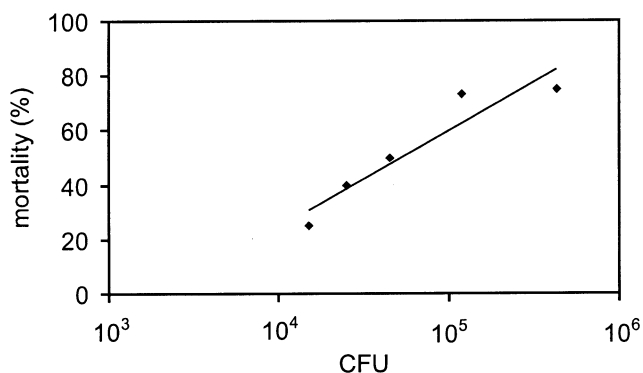

Fig. 5. Relationship between the numbers of starved *F. psychrophilum* (CFU) injected into rainbow trout and mortality of the challenged fish.

out the addition of nutrients. The present study also indicated that temperature, salinity and the presence of sediments affected the viable counts of *F. psychrophilum* cells. In general the *F. psychrophilum* cells responded to starvation in fresh water by a drastic reduction in viable cell count in the beginning of the experiments, which may be due to the transfer of the bacterial cells from nutrient (proteins) rich conditions (*Cytophaga* broth) to water, containing comparable less nutrients. After 20–30 days, only about 0.1–1% of the inoculated cell number were culturable on *Cytophaga* agar. After the adaptation of the cells to the new nutrient poor environment a subsequent increase in the cell number occurred after about 30–50 days. A similar adaptation phase may occur when bacterial cells are shed from infected fish to surrounding water. In a previous study Madetoja et al. [20] reported that high numbers of *F. psychrophilum* were shed from experimentally infected dead rainbow trout.

It has been suggested, that *F. psychrophilum* enters a viable but non-culturable stage in filtered stream water [17]. This is in contrast to our results, which clearly showed that at least some of (about 0.1–1%) the inoculated cells stayed viable and culturable for a very long time, without the addition of nutrients. However, our results do not reveal whether the majority of the inoculated cells 99–99.9% were viable but non-culturable or actually dead.

F. psychrophilum is considered a cold water species, causing disease outbreaks at low temperatures (4–10°C) [8]. However, in the experiments at different temperatures a low temperature (5°C) did not crucially improve the viable cell count of the examined strains, due to a possible lowered metabolic activity of the experimental cells, compared to the other temperatures tested. Conversely, the type strain NCIMB 1947^T showed a reduced viable count after 30 days at 5°C compared to the other temperatures. However, the stress conditions for the bacteria when they were transferred from nutrient rich conditions and high temperature (15°C) to a nutrient deficient condition and low temperature did not give a drastic reduction in viable cell count immediately after the transfer.

The experiments with the different salinities of the water showed that a salinity of approximately 6‰ drastically reduced the number of culturable cells, below detection limit (< 10 CFU ml^{-1}), after inoculation into the water. The reduction in cell number can be explained by the salinity of the water. In previous studies it has been shown that the NaCl tolerance of *F. psychrophilum* is 0.5–1% [8,22,23,24] under nutrient rich (cultivation broth) conditions. In the present study, culturable bacteria were subsequently recovered (Fig. 2B), indicating that *F. psychrophilum* may survive in brackish water. In fact, *F. psychrophilum* has been detected in the water of brackish water (S = 4‰) fish farms in the northern Baltic Sea [16]. The (artificial) salt water (salinity approximately 30‰) was clearly unfavourable for the *F. psychrophilum* cells after a short exposure. Viable bacteria were not obtained on the agar plates for up to 7 days after inoculation and these microcosms were excluded from further sampling. Based on our results it is thus unlikely that the *F. psychrophilum* will survive in marine water under nutrient deficient and more stressful conditions.

The experiments with sediment revealed that a large number of viable *F. psychrophilum* cells were obtained from the microcosms with 'natural' sediment compared to the microcosms with 'laboratory' or without sediment. A higher nutrient load in the 'natural' sediment could explain this difference. Unfortunately the nutrient content in the natural sediment was not determined. In contrast, the laboratory sand was cleaned by heating and acid treatment, making the nutrient content rather low. The presence of particles, in this case 'laboratory' sediment, did not improve the survival of *F. psychrophilum*. In other studies of fish pathogens it has been shown that *Aeromonas salmonicida* and *Edwardsiella ictaluri* could be isolated from inoculated bottom sediment for an extended period of time after inoculation [25,26]. Sakai [27] reported that the presence of sand was essential for an extended survival of *A. salmonicida* in microcosms. Sakai [27] suggested that this extended survival in the sediment was due to electrostatic interrelationships between sand, humic acids and virulent *A. salmonicida* cells.

The main issue concerning the survival studies done with different pathogenic bacteria and especially fish pathogens are horizontal transfer from one host to another through long distance transport in the water as well as the establishment of a pathogen source in the environment outside the host. In the present study, it can be concluded from the challenge experiments that at least a certain virulence is maintained a short period after the transfer of the bacterial cells into the water. From the results it can also be concluded that under the conditions in the present experiment, virulent and viable but non-culturable *F. psychrophilum* in a significant number were not present in the microcosms. Our conclusion thus is that, although caution should be used when transferring the results from laboratory studies into field situations, *F. psychrophilum* may survive for a long time in the vicinity of fish

farms but the virulence of the bacterial cells are maintained only for a short period of time. This means that highly virulent *F. psychrophilum* can readily spread in fish farms using recirculating water from infected fish to uninfected ones.

In previous studies it was observed that non-culturable *A. salmonicida* from laboratory microcosms were non-pathogenic when injected into Atlantic salmon (*Salmo salar*) [28,29]. In contrast, viable (culturable) *A. salmonicida* were highly pathogenic for Atlantic salmon [28].

The use of microcosms for the evaluation of bacterial ecology has been questioned due to the lack of input and output of nutrients and waste products. There is often also a lack of natural variation in the studied environmental parameters. However, studies in the environment are often difficult to realise due to practical problems, and an initial knowledge of the ecology of different pathogens is most conveniently obtained by microcosm studies as reported in the present paper. Of course, these studies have to be followed up by environmental studies in farm conditions, which are in progress at our laboratory.

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