Adherence and biofilm formation of *Flavobacterium psychrophilum* in the presence of aquarium or loch water

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Abstract

The continuous use of water for various purposes can be a source for spreading disease by pathogenic bacteria. In aquatic environments, bacteria rarely occur in planktonic form, however their presence are associated with surface microbial communities known as biofilms [1]. Biofilm formation is of importance to several pathogenic bacterial species, especially those living in water, conferring a selective advantage by increasing their ability to persist under adverse environmental conditions [2]. In aquaculture, biofilms can form on many of the components of the aquaculture system, and these are composed of various microflora present in the water. Pathogenic microorganisms incorporated into the biofilm can be shed from the biofilm, and can cause the reoccurrence of disease in fish [3].

Bacteria belonging to the *Flavobacterium* genus have been identified as a group of bacteria able to persist in a latent form in the aquatic environment, and *F. psychrophilum*, a Gram-negative, yellow-pigmented bacterium, is responsible for causing Bacterial Cold Water Disease (CWD) [4–5], or Rainbow Trout Fry Syndrome (RTFS) [6–7] in salmonid and other freshwater fishes. This bacterium is currently, one of the main bacterial pathogens in reared and wild salmonids, causing substantial economic losses in salmonid fish farms worldwide and hindering the rapid expansion of the salmonid aquaculture industry [8–9]. *F. psychrophilum* grows under natural conditions in rivers and lakes in a temperature range from 4 ºC to 23 °C [10]. It has been demonstrate that *F. psychrophilum* has the ability to adhere to surfaces forming biofilms, and has been detected in sediments, samples of river water, and biofilms from rivers receiving outlet water from infected fish farms [2,11–13].

The objective of the present study was to evaluate the ability of *F. psychrophilum* to adhere to and form biofilms on different types of materials used by the salmonid aquaculture industry so as to gain a better understanding of the survival of this bacterium in the aquaculture environment.

F. psychrophilum NCIMB 1947 was grown in Tryptone Yeast Extract Salts (TYES) broth under a constant agitation by a shaker at 140 rpm (Kühner Shaker®) with a temperature of 15°C for 72 h. After this time, the broth culture was inoculated at a concentration of 2.1 x10^6 viable cells/cm^2 onto stainless steel, plastic, glass, wood, and antibacterial plastic surfaces. Subsequently, the inoculated surfaces were transferred to fish tanks containing 10 litres of water supplied from two different sources: (i) aquarium water (dechlorinated, mains water),

or (ii) water recovered directly from the Airthrey Loch, University of Stirling, Scotland, UK, and filtered though a membrane (pore size, 0.45 µm) before using in the analysis. The water used in the study was first analyzed in the Water Quality Laboratory, Institute of Aquaculture, University of Stirling for mineral composition. The inoculated surfaces were placed in the fish tanks together with an aliquot of the bacterium culture added directly to the tanks. The surfaces were incubated in these conditions for 96 h at 15°C. After this time, the surfaces were removed and the formation of biofilms on the different surfaces monitored and quantified using fluorescence microscopy (Olympus IX70) using a Live/Dead® staining kit, with which live cells appearing green and dead or injured cells appeared red. Micrographs were obtained with the program Cytovision® 2.51 and analyzed with AnalySIS®.

After 96 h at 15 ºC, *F. psychrophilum* in aquarium water and loch water had adhered to the various supports (stainless steel, plastic, glass, wood, and antibacterial plastic) forming biofilms on these surfaces (Table 1). The results for the aquarium water showed high levels of live bacteria adhering to the stainless steel (8.68×10^4), plastic (1.09×10^5), glass surfaces (8.52×10^4) and wood (1.11×10^5). Significantly more bacteria had adhered to stainless steel ($P<0.05$) compared with the antibacterial plastic surface (3.22×10^3). For the water obtained from Airthrey Loch, there was statistical significance higher levels ($P<0.05$) of living cells attached to stainless steel (2.30×10^5), plastic (3.09×10^5) and glass (2.32×10^5) compared with the wood or the antibacterial plastic surface. As with the aquarium water, the adherence of *F. psychrophilum* to the antibacterial surfaces in the loch water was again lower than with the others surfaces.

Table 1. Adherence of *Flavobacterium psychrophilum* (cells/cm^2) on surfaces in aquarium water and loch water.

	Aquarium water		Loch water	
	Live	Injured or dead	Live	Injured or dead
Stainless steel	$8.68 \times 10^{4\ a}$	$1.46 \times 10^{5\ ab}$	$2.30 \times 10^{5\ a}$	$1.54 \times 10^{5\ a}$
Plastic	$1.09 \times 10^{5\ a}$	$5.87 \times 10^{4\ ab}$	$2.98 \times 10^{5\ a}$	$3.09 \times 10^{5\ a}$
Glass	$8.52 \times 10^{4\ a}$	$1.25 \times 10^{5\ ab}$	$2.41 \times 10^{5\ a}$	$2.32 \times 10^{5\ a}$
Wood	$1.11 \times 10^{5\ a}$	$2.80 \times 10^{5\ a}$	$1.38 \times 10^{5\ b}$	$1.56 \times 10^{5\ a}$
Antibacterial plastic	$3.22 \times 10^{3\ b}$	$1.39 \times 10^{4\ c}$	$6.43 \times 10^{3\ c}$	$2.25 \times 10^{4\ b}$

[a-c] Statistical significant values ($P<0.05$, Student-Newman-Keuls).

The mineral composition of the loch water showed a higher concentration of sodium, magnesium, potassium and calcium (ppb) compared to the dechlorinated mains water supply (Fig. 1), however the presence of minerals in the water used in the aquarium may have an influence on the bacterial adherence observed. In fact, a deficiency of certain nutrients may increase the ability of bacteria such as *Flavobacterium* to form biofilms [14].

The genus *Flavobacterium* is one of the most common biofilm producers and it has previously been shown that organic and inorganic sediments can influence its ability to form biofilms. Using confocal microscopy, Staroscik & Hunnicutt [15] observed that the addition of Ca^{2+} and Mg^{2+}, mucus from skin salmon or glucose induced the formation of biofilms of *Flavobacterium columnare*.

The ability of *F. psychrophilum* to adhere to surfaces could explain the bacterium's survival under adverse conditions like starvation. The fact that water can act as a source of infection implies that *F. psychrophilum* is able to survive outside its host for a period of time under conditions of starvation [16]. Madetoja et al. [17] observed that the virulence of *F. psychrophilum* was maintained for at least seven days after transferring the bacteria to fresh water, and increasing the bacterium's survival by the addition of nutrient-containing sediments; thereby, highly virulent *F. psychrophilum* can readily spread from infected fish to uninfected ones in recirculating aquaculture systems

The adhesion and biofilm formation properties of this bacterium may explain why it is less susceptible to antimicrobial treatment. Sundell & Wiklund [18] showed increased antimicrobial resistance in biofilms containing high bacterial cell densities (>10^7 CFU mL^{-1}). These properties may explain the subsequent transmission of this bacterium to fish, and probably contribute to its dissemination in salmonid fish farms, representing a significant risk in the development of the salmonid aquaculture [8,19].

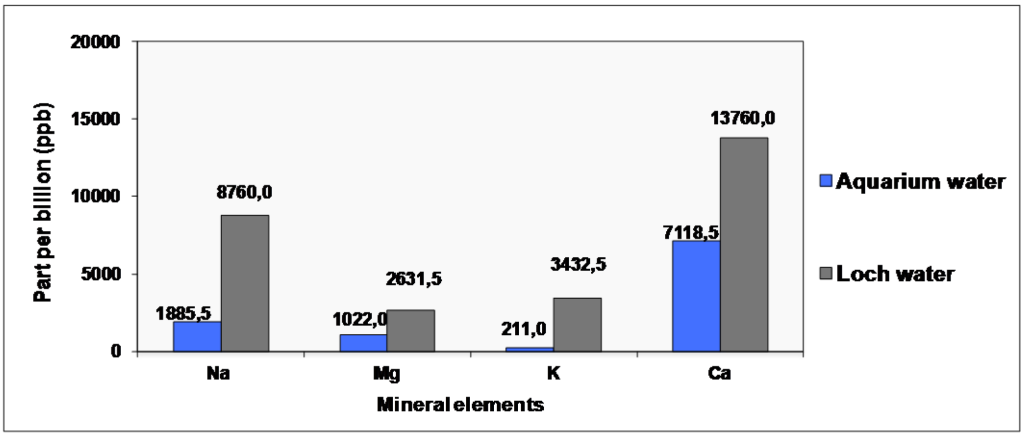

Figure 1. Mineral concentration of the aquarium water and loch water (ppb).

The results suggest that this bacterium has the ability to form biofilms on surfaces used in aquaculture systems. Procedures such as water treatment, regular equipment sanitation, and the use of antimicrobial surfaces may be useful in preventing biofilm formation in fish farming systems, and in turn preventing disease outbreaks caused by this bacterium.

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