



**Biofilm formation of *Flavobacterium psychrophilum* on various substrates**

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1 **Biofilm formation of *Flavobacterium psychrophilum* on various substrates**

2

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16

17 **Abstract**

18 The ability of *Flavobacterium psychrophilum* to adhere to and form biofilms on different  
19 types of materials used on rainbow trout (*Oncorhynchus mykiss*) farms was evaluated in this  
20 study. *F. psychrophilum* NCIMB 1947<sup>T</sup>, was inoculated onto a variety of different surfaces,  
21 including stainless steel, plastic, glass, wood and zinc pyrithione encapsulated antibacterial  
22 plastic. The samples were then cultured in a humidified chamber or transferred into fish tanks  
23 containing either (1) freshwater or (2) filtered lake water. The formation of biofilms was  
24 quantified by fluorescent microscopy. *F. psychrophilum* formed biofilms on all of the surfaces  
25 tested, however, the adherence of the bacterium to the antibacterial plastic was much lower  
26 than the attachment observed on the other surfaces, illustrating the bacteriostatic properties of  
27 this material for *F. psychrophilum*. Moreover, bacterial numbers were greater on the surfaces  
28 maintained in lake water compared to those maintained in freshwater. The mineral  
29 composition of the lake water may have been responsible for the increased bacterial  
30 adherence observed between the two types of water. Treatment of the water, regular cleaning  
31 of equipment and the use of antimicrobial material to house the fish may help reduce biofilm  
32 formation by *F. psychrophilum* in fish farming systems.

33

34 **Key words**

35 *Flavobacterium psychrophilum*, biofilm formation, fluorescent microscopy, substrates, fish  
36 farming systems, rainbow trout farms

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## 42 **1. Introduction**

43 The continuous use of water within a fish farm can act as a reservoir for pathogenic bacteria,  
44 and this can be a factor in the spread of disease within the farm (King, 2001; Cai & Arias,  
45 2017). In the aquatic environment, bacteria rarely occur in a planktonic form, and their  
46 presence is more likely to be associated with surface-associated microbial communities  
47 known as biofilms (Huq, Whitehouse, Grim, Alam & Colwell, 2008; Nocker, Burr & Camper,  
48 2014; Satpathy, Sen, Pattanaik & Raut, 2016). Biofilms are structured communities of  
49 bacterial cells adhered to inert or living surfaces and enclosed in a polymeric matrix produced  
50 by the bacteria, referred to as an extracellular polymer substance (EPS). Biofilm formation is  
51 beneficial to the bacteria, providing them with protection against desiccation, increased  
52 nutrient availability and is more resistant to antimicrobial agents than planktonic bacteria  
53 (Costerton, Stewart & Greenberg, 1999; Srey, Jahid & Ha, 2013; Satpathy et al., 2016).  
54 Biofilms are composed of a variety of microflora present in the water, capable of colonizing  
55 surfaces, which can then act as a reservoir for pathogenic bacteria. Pathogenic  
56 microorganisms within the biofilm can be shed from the biofilm and are able to cause a  
57 reoccurrence of disease in fish (King, 2001; Branda, Vik, Friedman & Kolter, 2005; Nocker et  
58 al., 2014). Biofilm formation is important to many pathogenic bacterial species, especially  
59 those living in water, giving them a selective advantage by increasing their ability to persist  
60 under adverse environmental conditions (Duchaud et al., 2007). They can form on many of  
61 the materials found within aquaculture systems, appearing on the surfaces of water pipes and  
62 fish tanks, suspended matter, incubators, bio-filtration systems and even on the internal and  
63 external surfaces of the fish (King et al., 2004).

64 Bacteria belonging to genus *Flavobacterium* have been identified as a group of  
65 bacteria able to persist in a latent form in the aquatic environment (Waśkiewicz &  
66 Irzykowska, 2014). *F. psychrophilum* is a Gram–negative, yellow-pigmented bacterium,

67 responsible for causing cold water disease (CWD) (Borg, 1948; Holt, Rohovec & Fryer,  
68 1993), or rainbow trout fry syndrome (RTFS) (Lorenzen, 1994; Rangdale, 1995) in salmonids  
69 and other freshwater fish. The bacterium not only infects farmed fish, but can also affect wild  
70 fish, although disease outbreaks are apparently less severe in non-salmonids (Nematollahi,  
71 Decostere, Pasmans & Haesebrouck, 2003). It is, currently, one of the main bacterial  
72 pathogens in reared and wild salmonids, causing substantial economic losses in salmonid fish  
73 farms worldwide, and hindering expansion of the salmonid aquaculture industry (Nematollahi  
74 et al., 2003; Bernardet & Bowman, 2006). The bacterium grows in the aquatic environment in  
75 temperatures ranging between 4°C to 23°C (Holt, 1988). It has the ability to adhere to the  
76 skin, gut and eggs of fish and disease transmission studies suggest that reservoirs of the  
77 bacterium can be found within the water system of the fish farm (Madetoja, Dalsgaard &  
78 Wiklund, 2002). It also has the ability to adhere to surfaces forming biofilms, and has been  
79 detected in sediment, river water, especially near outlet water from infected fish farms  
80 (Amita, Hoshino, Honma & Wakabayashi, 2000; Álvarez, Secades, Prieto, McBride &  
81 Guijarro, 2006; Sundell & Wiklund, 2011).

82 As aquaculture facilities are particularly prone to the development of biofilms by *F.*  
83 *psychrophilum*, understanding the factors that influence biofilm formation could reduce the  
84 presence of this pathogenic bacterium within the fish farming system (Huq et al., 2008;  
85 Wietz, Hall & Høj, 2009; Srey et al., 2013). Thus, the purpose of this study was to gain a  
86 better understanding of the survival of this bacterium in the aquatic environment and to  
87 examine the ability of *F. psychrophilum* to adhere to and form biofilms on different types of  
88 materials used by the salmonid aquaculture industry. Biofilm formation by *F. psychrophilum*  
89 was examined in the presence of tryptone yeast extract salts (TYES) broth, freshwater taken  
90 from the aquarium or water from a freshwater lake.

91

## 92 **2. Materials and methods**

### 93 **2.1. Bacterial culture**

94 *F. psychrophilum*, strain NCIMB 1947<sup>T</sup>, was obtained from a stock of cryopreservation beads  
95 (Cryoprotect; Technical Service Consultants Service Ltd. Lancashire, UK) stored at -70°C.  
96 The bacterium was grown in TYES broth (tryptone, 4.0 g; yeast extract, 0.4 g; MgSO<sub>4</sub>.7H<sub>2</sub>O,  
97 0.5 g; CaCl<sub>2</sub>.2H<sub>2</sub>O, 0.2 g; distilled water, 1000 mL; pH 7.2; autoclaved for 20 min at 121°C)  
98 under constant agitation at 140 rpm (Kühner Shaker LT-W, Adolf Kühner AG, Switzerland)  
99 for 72-96 h at 15°C. This culture was subsequently cultured on TYES agar plates (TYES  
100 broth with 15.0 g/L bacteriological agar) and incubated for 96 h at 15°C, from which bacterial  
101 colonies were taken and cultivated in TYES broth under agitation for 72-96 h at 15°C to  
102 obtain the bacterial culture used in the analysis.

### 104 **2.2. Test materials**

105 Four different types of material, e.g. stainless steel (type 1.4301, also known as grade 304)  
106 used as positive control (Fuster-Valls, Hernández, Marín de Mateo & Rodríguez-Jerez, 2008),  
107 polyethylene (PE) plastic, silica glass, wood (*Pinus* sp.) and an antibacterial plastic [poly-  
108 propylene (PP) containing micro-encapsulated zinc pyrithione] (Microlitix, Sant Cugat del  
109 Valles, Spain) were selected to assess their ability to support *F. psychrophilum* biofilm  
110 formation. The size of the material used was 4.0 x 4.0 cm<sup>2</sup>, while the stainless steel surfaces  
111 consisted of discs with a diameter of 2.0 cm and a thickness of 1.2 mm. Prior to performing  
112 the study, the surfaces were cleaned, disinfected and autoclaved at 121°C for 15 min (Ríos-  
113 Castillo, González-Rivas & Rodríguez-Jerez, 2017), except the antibacterial plastic, which  
114 was only cleaned with 70% isopropyl alcohol (2-propanol) before use so as not to interfere  
115 with its antibacterial properties.

116

### 117 2.3. Experimental design

118 The formation of biofilms on the different test materials was performed by incubating the  
119 supports in a *F. psychrophilum* suspension under two different sets of test conditions.

120 (a) In the first test, **all surfaces were inoculated with 16  $\mu\text{L}/\text{cm}^2$** . The number of live and dead  
121 bacteria was determined using the LIVE/DEAD staining kit with a bacterial concentration on  
122 the surfaces of  $2.01 \times 10^6$  live cells/ $\text{cm}^2$  and  $4.79 \times 10^1$  of dead/damaged cells/ $\text{cm}^2$ . The  
123 surfaces inoculated were placed in Petri dishes, which were then placed into a humidified  
124 chamber (30 x 22 x 14 cm) maintained at a saturated relative humidity of  $\geq 90\%$  using pieces  
125 of paper towel moistened with sterile distilled water (Wiklund & Dalsgaard, 2003; Fuster-  
126 Valls et al., 2008; International Organization for Standardization, 2011). The bacteria were  
127 incubated on these surfaces for 48 h at  $15^\circ\text{C}$ . After this time, the excess liquid was removed  
128 from each surface and 50  $\mu\text{L}$  of TYES broth was added to the surface of the stainless steel  
129 disc and 255  $\mu\text{L}$  to the other surfaces with a sterile pipette. These were then incubated for a  
130 further 48 h at  $15^\circ\text{C}$  before the degree of biofilm formation on each support was assessed  
131 using fluorescence microscopy.

132 (b) The ability of *F. psychrophilum* to form biofilms on the different surfaces (stainless steel,  
133 plastic, glass, antibacterial plastic and wood) was also assessed using freshwater collected  
134 from either a freshwater aquarium [Aquaculture Research Facility (ARF), Institute of  
135 Aquaculture, University of Stirling], or from a freshwater lake (Airthrey Loch, University of  
136 Stirling). The water samples taken from the aquarium and the lake were filtered through a  
137 0.45  $\mu\text{m}$  filter (Millipore Co., Billerica, Ma, U.S.A.) prior to use. The various supports were  
138 incubated with *F. psychrophilum* ( $2.14 \times 10^6$  of viable cells/ $\text{cm}^2$  and  $7.41 \times 10^1$  cells/ $\text{cm}^2$  of  
139 dead or injured cells) for 72 h. After this time, the excess liquid was removed from each  
140 surface and the surfaces were attached onto the side of plastic fish tanks (20 x 40 x 22 cm)  
141 with Blu-Tack™ malleable rubber adhesive (Bostik Ltd, Leicester, UK). **The tanks contained:**

142 (i) 100 mL of TYES broth with 10 litres of freshwater from the aquarium (dechlorinated,  
143 mains water), or (ii) 100 mL of TYES broth with 10 litres of freshlake water. The surfaces  
144 were maintained in the tanks for 96 h at 15-16°C. After this incubation period, the test  
145 materials were removed from the tanks, washed carefully with distilled water, taking care not  
146 to disturb the biofilm on the surfaces and these were then examined by fluorescence  
147 microscopy using the LIVE/DEAD staining kit.

148

#### 149 **2.4. Assessing the number of live bacteria by fluorescence microscopy**

150 **Live/Dead BacLight Bacteria Viability Kit** (Molecular Probes, Europe BV) was used to  
151 determine the initial number of live bacteria present in the bacterial cultures used for the  
152 studies and to assess the number of live bacteria present on surfaces after 96 h of incubation  
153 with *F. psychrophilum*. The LIVE/DEAD staining kit is composed of two nucleic acid-  
154 binding stains: SYTO<sup>®</sup> 9, penetrating all bacterial membranes and stains the cells green, and  
155 propidium iodide which only penetrates cells with damaged membranes, producing red  
156 fluorescing cells when the cells are damaged or dead. The kit was used according to  
157 manufacturer's instructions.

158 The stainless steel discs were stained with 20 µL and plastic, glass, wood and the antibacterial  
159 plastic with 100 µL. Stained surfaces were left in the dark for 15 min at 22°C to allow the  
160 stains to penetrate. Eight images were acquired from every surface evaluated using a  
161 fluorescence microscope IX70 (Olympus Optical, Tokyo, Japan) equipped with a mercury  
162 lamp, and two filters: (1) filter A (excitation 470–490 nm, emission 515–550 nm) and (2)  
163 filter B (excitation 510–550 nm, emission > 590 nm). The same **microscopic** parameters,  
164 input calibration and image acquisition were used throughout and images were analysed using  
165 Cytovision<sup>®</sup> software, version 2.51 (Applied Imaging, Sunderland, Tyne & Wear, UK). Cell  
166 counts and bacteria size (i.e. minimum and maximum diameter and area) were automatically



167 measured as a colour scale interpretation using Soft Imaging System™ program, AnalySIS®  
168 version 3.2 (GmbH, Munich, Germany).

169

## 170 **2.5. Water analysis**

171 Prior to performing the fresh water studies, the mineral composition of the water was  
172 analysed from both water sources. To do this, 10 litres of freshwater was obtained from the  
173 aquarium facility and directly from the **freshwater lake**, both of which were filtered through a  
174 0.45 µm filter. The samples of water were then analysed by inductively coupled plasma mass  
175 spectrometry (ICP-MS) to determine the concentration of their mineral content. This analysis  
176 was performed by the Water Quality Laboratory, Institute of Aquaculture, University of  
177 Stirling.

178

## 179 **2.6. Statistical analysis**

180 Each analysis comparing biofilm formation on the various supports was repeated three times  
181 and each test material surface was analysed in triplicate (n = 9). The statistical software  
182 package SAS® v 9.1.3.4 (Institute Inc, North Carolina, USA) was used for the statistical  
183 analysis. The assumption of normality of the data was carried out using the Shapiro–Wilk test.  
184 Statistical analysis was performed on data between cells counted on the various surfaces  
185 under the different test condition using an analysis of variance (ANOVA). Student-Newman-  
186 Keuls *post hoc* test was used to test the significance of differences between live and dead or  
187 injured bacteria on the surfaces, where  $p \leq 0.05$  was considered significant.

188

## 189 **3. Results**

### 190 **3.1. Biofilm formation by *F. psychrophilum***

191 In the first study, in which a suspension of *F. psychrophilum* in TYES was incubated onto the  
192 various supports in a humidified chamber, the live-cell counts obtained using the  
193 LIVE/DEAD kit showed a similar amount of live and dead bacteria attached to the various  
194 supports (Table 1). There was no statistical difference obtained in the level of adherence or  
195 biofilm formation on stainless steel ( $1.41 \times 10^6$  live cells/cm<sup>2</sup>), plastic ( $9.12 \times 10^5$  live  
196 cells/cm<sup>2</sup>) or glass ( $8.32 \times 10^5$  live cells/cm<sup>2</sup>) after 96 h of incubation. Whereas adherence of  
197 the bacterium to the antibacterial plastic surface was significantly lower ( $p < 0.05$ ) than  
198 observed on the other materials with respect to both live ( $6.46 \times 10^3$  cells/cm<sup>2</sup>) and  
199 dead/injured cells ( $1.10 \times 10^4$  cells/cm<sup>2</sup>). Also, an increase in the number of cells/cm<sup>2</sup> of dead  
200 or injured cells was observed on all test materials, increasing from an initial concentration of  
201  $4.79 \times 10^1$  cells/cm<sup>2</sup> to  $10^4$  or  $10^5$  cells/cm<sup>2</sup> after 96 h of being introduced on to the support.

### 203 3.2. Biofilm formation of *F. psychrophilum* in aquarium or lake water

204 The degree of bacterial attachment to the various supports (stainless steel, plastic, glass, wood  
205 and antibacterial plastic) after 96 h at 15 - 16°C, in either the aquarium water or the lake  
206 water, is presented in Table 2. Higher levels of live bacteria ( $p < 0.05$ ) were present in the  
207 freshwater from the aquarium adhering to stainless steel ( $8.68 \times 10^4$  cells/cm<sup>2</sup>), plastic ( $1.09 \times$   
208  $10^5$  cells/cm<sup>2</sup>) (Figure 1c), glass surfaces ( $8.52 \times 10^4$  cells/cm<sup>2</sup>) (Figure 1d) and wood ( $1.11 \times$   
209  $10^5$  cells/cm<sup>2</sup>) compared to the antibacterial plastic ( $2.88 \times 10^2$  cells/cm<sup>2</sup>). The results with the  
210 water obtained from freshwater lake showed significantly higher levels of live-cells ( $p < 0.05$ )  
211 attached to stainless steel ( $2.30 \times 10^5$  cells/cm<sup>2</sup>), plastic ( $2.98 \times 10^5$  cells/cm<sup>2</sup>) and glass ( $2.41$   
212  $\times 10^5$  cells/cm<sup>2</sup>) compared with the wood ( $1.38 \times 10^5$  cells/cm<sup>2</sup>) (Figure 1e) or the  
213 antibacterial plastic surface ( $6.03 \times 10^3$  cells/cm<sup>2</sup>) (Figure 1f). Figure 2 shows the values in  
214 parts per billion (ppb) of sodium, magnesium, potassium and calcium of freshwater aquarium  
215 and in lake water used to evaluate the biofilm formation of *P. psychrophilum*. According to

216 these results, the concentrations of all minerals analysed in the lake water were higher than  
217 those obtained from the freshwater aquarium. The highest mineral element concentration for  
218 the lake water was calcium (13760.0 ppb), and the lowest magnesium (1022.0 ppb). In the  
219 case of aquarium water, the highest value, although lower than that found in the lake water  
220 was also calcium (7118.5 ppb), while the potassium concentration was the lowest (211.0 ppb).

221

### 222 **3.3. Antimicrobial properties of antibacterial plastic [poly-propylene (PP) containing** 223 **micro-encapsulated zinc pyrithione]**

224 The results of this study indicate that the antibacterial properties of zinc pyrithione under  
225 saturated relative humidity of  $\geq 90\%$  had  $2.34 \log_{10}$  cells/cm<sup>2</sup> fewer bacteria attached relative  
226 to the positive control,  $2.48 \log_{10}$  cells/cm<sup>2</sup> in freshwater and  $1.58 \log_{10}$  cells/cm<sup>2</sup> in lake  
227 water, while only a minimal reduction was observed for other surfaces under the various  
228 conditions.

229

## 230 **4. Discussion**

231 When environmental conditions are unfavourable, aquatic bacteria are subjected to a  
232 rapid change in nutrient availability and must therefore adapt accordingly in order to be able  
233 to survive under these adverse conditions. For example, cells undergo reduced cell division,  
234 with the resulting cells having an overall reduction in size and typically become rounder and  
235 coccus in morphology, in what is known as a 'rounding up' strategy (Arias, LaFrentz, Cai &  
236 Olivares-Fuster, 2012).

237 In our study, where *F. psychrophilum* cells were incubated on the various supports in  
238 the humidity chamber, some of the dead/injured bacteria became rounded in appearance. The  
239 morphological changes in *F. psychrophilum* cells observed here have also been reported by  
240 Vatsos, Thompson & Adams (2003), for bacteria maintained in a broth culture for four weeks.

241 In this study, these changes were observed after only 96 h incubation suggesting that this  
242 adaptation may be accelerated during the growth of the bacteria on the surfaces compared to  
243 growth in TYES broth, reflecting the environment stress experience by the bacteria during  
244 biofilm formation. This reduction in bacterial size during biofilm formation, which in mature  
245 stages of biofilm contained more damaged cells (dead or non-viable) than live cells, has also  
246 been reported by Roszak & Colwell (1987); Boulos, Prevost, Barbeau, Coallier & Desjardins,  
247 (1999); Chmielewski & Frank (2003); and Fuster-Valls et al. (2008). The results also suggest  
248 that high levels of humidity and the use of TYES broth could favour the adhesion and biofilm  
249 formation of *F. psychrophilum*. According to Ehrlich, Miller & Walker (1970), the survival of  
250 *Flavobacterium* sp. is not affected by high conditions of humidity (up to 99%), but can be  
251 affected by a lack of nutrients. Under dry conditions, Fuster-Valls et al. (2008) observed a  
252 considerable reduction in the level of bacterial attachment by the cells, with some cells  
253 appearing injured, and non-culturable in culture medium. They were still considered to have  
254 the potential to cause disease outbreaks, however. Humid areas within the fish farming  
255 system, ideal for bacterial growth, can favour the adhesion and biofilm formation by *F.*  
256 *psychrophilum*, and microorganisms present on equipment and surfaces within the fish farm,  
257 may survive there for prolonged periods of time (Lee Wong, 2004).

258 The results of the mineral analysis may explain the high levels of bacteria seen  
259 adhering to the surfaces in the presence of the lake water compared to the aquarium water  
260 (Figure 2). These values were statistically different ( $p < 0.05$ ) for the live cell counts attached  
261 to the stainless steel, glass and antibacterial zinc pyrithione surfaces. These results are in  
262 accord with Fletcher (1988), who observed that cationic metal concentrations of sodium,  
263 calcium, magnesium minimize the repulsive forces between the bacterial cell and surfaces,  
264 having an influence on the ability of bacteria to adhere to surfaces and form biofilms. The  
265 lower concentration of minerals in the aquarium freshwater may also influence bacterial

266 attachment; in fact, a deficiency of certain nutrients may increase the ability of bacteria to  
267 form biofilms, though the concentration of nutrients necessary for bacterial development is  
268 low (Mattila-Sandholm & Wirtanen, 1992; Percival & Walker, 1999). The presence of  
269 organic and inorganic material can also influence biofilm formation by bacteria within the  
270 *Flavobacterium* genus. Staroscik, Hunnicutt & Nelson (2007) observed that the addition of  
271  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  or glucose to the culture medium, or the presence of mucus from salmon skin  
272 induced the formation of biofilms by *Flavobacterium columnare*. Likewise, the environment  
273 can represent a reservoir of *F. psychrophilum*, since the ability of this microorganism to  
274 adhere to surfaces could explain the bacterium's survival under adverse conditions. The fact  
275 that water can act as a source of infection implies that *F. psychrophilum* is able to survive  
276 outside its host under conditions of starvation (Vatsos, Thompson & Adams, 2001). Madetoja,  
277 Nystedt & Wiklund (2003) found that the virulence of *F. psychrophilum* was maintained for  
278 at least seven days after transferring the bacteria to freshwater, and the bacterium's survival  
279 increased with the addition of nutrient-containing sediments; thereby *F. psychrophilum* can  
280 readily spread from infected fish to uninfected ones in recirculating aquaculture systems.  
281 The differences in bacterial counts (expressed in decimal logarithms,  $\log_{10}$ ) of live-cells  
282 adhered to plastic, glass, wood or antibacterial plastic were compared with the number of live-  
283 cells attached to the stainless steel surfaces, used as a control (Table 3). According to  
284 Japanese Standard JIS Z 2801 (Japanese Standards Association, 2010) and ISO 22196  
285 (International Organization for Standardization, 2011) surfaces with antibacterial properties  
286 must demonstrate a reduction in bacterial attachment equal to or higher than  $2 \log_{10}$  of that  
287 determined for the control surface. The zinc pyrithione antibacterial plastic showed a high  
288 efficiency in preventing bacterial adherence when it was tested under the humidity conditions  
289 (2.34 log) or under the aquarium water condition (2.48 log). On the other hand, when it was  
290 tested under the lake water condition, the efficiency was lower (1.58 log) (Table 3). This

291 could be explained because the high mineral concentration of sodium, magnesium, potassium,  
292 and calcium in the lake water may prevent the adequate action of zinc-pyrithione. It has been  
293 earlier reported that higher level of minerals favour the adherence of *Flavobacterium* and  
294 biofilm formation (Madetoja et al., 2003; Staroscik et al., 2007). The antibacterial action of  
295 zinc pyrithione in preventing the adherence of cells is favoured by the use freshwater used in  
296 fish farms and is partly inhibited by the presence of water with a high mineral content. Zinc  
297 pyrithione interacts with the membrane phospholipids in bacteria, inhibiting membrane  
298 transport of substrates and decreasing intracellular ATP levels by inhibiting ATP synthesis  
299 causing a lethal toxicity of bacterial cells (Qian, Chen & Xu, 2013).

300 As established from the genome analysis of *F. psychrophilum*, the bacterium has the  
301 ability to form biofilms and store cyanophycin, which could explain the bacterium's prolonged  
302 survival outside its host (Duchaud et al., 2007) and the spread of disease by this bacterium  
303 through the aquatic environment (Madetoja et al., 2002; Nematollahi et al., 2003). The ability  
304 of this bacterium to adhere to surfaces and form biofilms may explain why it is less  
305 susceptible to antimicrobial treatment. Sundell & Wiklund (2011) observed an increased  
306 antimicrobial resistance in *F. psychrophilum* biofilms containing high bacterial cell densities  
307 ( $> 10^7$  CFU/mL). These characteristics, together with adherent properties of *F. psychrophilum*  
308 may explain the subsequent transmission of this bacterium to fish, and probably contribute to  
309 its dissemination in salmonid fish farms, representing a significant risk in the development of  
310 the salmonid aquaculture (Nematollahi et al., 2003; Barnes & Brown, 2011). Zinc pyrithione,  
311 is widely used as an antifouling agent in paints and exhibit a high antimicrobial effects against  
312 biofilm bacteria (Konstantinou & Albanis, 2004; Ciriminna, Bright & Pagliaro, 2015). The  
313 commercial cost of zinc pyrithione, used in a concentration of 2.0% as biocide and antifouling  
314 is approximately US\$ 2.50 - US\$ 3.50 to cover each 100 m<sup>2</sup> of fish-farm environments.  
315 Although this is an added expense to the fish farms, the reduced mortality caused by disease

316 from this bacterium justifies the investment. Thus, the use of materials that inhibit bacterial  
317 growth such as zinc pyrithione may offer alternative ways to reduce the spread of *F.*  
318 *psychrophilum* within the fish farming system as well as other bacterial species involved in  
319 disease outbreaks.

320

## 321 **5. Conclusions**

322 This study suggests that *F. psychrophilum* has the ability to adhere to and form biofilms on  
323 materials used within aquaculture systems such as stainless steel, plastic, glass and wood at  
324 saturated relative humidity levels of  $\geq 90\%$  and in freshwater aquarium or lake water.

325 Procedures such as water treatment, regular sanitation of equipment, and the use of  
326 antimicrobial surfaces may be useful in preventing biofilm formation in fish farming systems,  
327 and in turn preventing disease outbreaks caused by this bacterium.

328

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333

## 334 **Conflict of interest**

335 The authors declare no conflicts of interest.

336

337

338

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For Review Only

470 **Tables**

471 **Table 1.** Adherence and biofilm formation of *Flavobacterium psychrophilum* (cells/cm<sup>2</sup>) on  
 472 stainless steel, plastic, glass, and antibacterial plastic surfaces after 96 h of incubation in  
 473 humidity test condition.

Surfaces	Cells <sup>†</sup>	
	Live	Injured or dead
Stainless steel	1.41 x 10 <sup>6</sup> ± 0.37 <sup>a</sup>	3.47 x 10 <sup>5</sup> ± 0.45 <sup>a</sup>
Plastic	9.12 x 10 <sup>5</sup> ± 0.06 <sup>a</sup>	9.33 x 10 <sup>5</sup> ± 0.48 <sup>a</sup>
Glass	8.32 x 10 <sup>5</sup> ± 0.02 <sup>a</sup>	5.62 x 10 <sup>5</sup> ± 0.05 <sup>a</sup>
Antibacterial plastic	6.46 x 10 <sup>3</sup> ± 0.17 <sup>b</sup>	1.10 x 10 <sup>4</sup> ± 0.28 <sup>b</sup>

474

475 <sup>†</sup>Initial cells count/cm<sup>2</sup>: 2.01 x 10<sup>6</sup> live cells/cm<sup>2</sup> and 4.79 x 10<sup>1</sup> dead/injured cells/cm<sup>2</sup>.

476 <sup>a,b</sup> Values in columns for each surface are significantly different if the letters are  
 477 different ( $p \leq 0.05$ ).

478

479 **Table 2.** Adherence and biofilm formation of *Flavobacterium psychrophilum* (cells/cm<sup>2</sup>) to  
 480 stainless steel, plastic, glass, wood, and antibacterial plastic surfaces after 96 hours in  
 481 aquarium freshwater and lake water conditions.

482

Surfaces	Aquarium water		Lake water	
	Cells <sup>†</sup>			
	Live	Injured or dead	Live	Injured or dead
Stainless steel	8.68 x 10 <sup>4</sup> ± 0.12 <sup>aA</sup>	1.46 x 10 <sup>5</sup> ± 0.12 <sup>ab</sup>	2.30 x 10 <sup>5</sup> ± 0.13 <sup>aA</sup>	1.54 x 10 <sup>5</sup> ± 0.39 <sup>a</sup>
Plastic	1.09 x 10 <sup>5</sup> ± 0.08 <sup>aB</sup>	5.87 x 10 <sup>4</sup> ± 0.11 <sup>ab</sup>	2.98 x 10 <sup>5</sup> ± 0.08 <sup>aA</sup>	3.09 x 10 <sup>5</sup> ± 0.50 <sup>a</sup>
Glass	8.52 x 10 <sup>4</sup> ± 0.11 <sup>aA</sup>	1.25 x 10 <sup>5</sup> ± 0.12 <sup>ab</sup>	2.41 x 10 <sup>5</sup> ± 0.09 <sup>aB</sup>	2.32 x 10 <sup>5</sup> ± 0.28 <sup>a</sup>
Wood	1.11 x 10 <sup>5</sup> ± 0.10 <sup>aA</sup>	2.80 x 10 <sup>5</sup> ± 0.39 <sup>a</sup>	1.38 x 10 <sup>5</sup> ± 0.07 <sup>bA</sup>	1.56 x 10 <sup>5</sup> ± 0.40 <sup>a</sup>
Antibacterial plastic	2.88 x 10 <sup>2</sup> ± 0.13 <sup>bB</sup>	1.39 x 10 <sup>4</sup> ± 0.34 <sup>c</sup>	6.03 x 10 <sup>3</sup> ± 0.17 <sup>cA</sup>	2.25 x 10 <sup>4</sup> ± 0.19 <sup>b</sup>

483

484 <sup>†</sup> Initial cells count/cm<sup>2</sup>: 2.14 x 10<sup>6</sup> live cells and 7.41 x 10<sup>1</sup> dead or injured cells. <sup>a-c</sup> Values in  
 485 columns for each surface are significantly different if the letters are different ( $p \leq 0.05$ ). <sup>A-B</sup>  
 486 Values in rows for each surface for live cells results are significantly different if the letters are  
 487 different ( $p \leq 0.05$ ).

488

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490

491 **Table 3.** The differences (represented in  $\log_{10}$  cells/cm<sup>2</sup>) in the live-cell counts of *F.*  
 492 *psychrophilum* attached to plastic, glass, wood and antibacterial plastic compared with  
 493 stainless steel used as a positive control. Values higher than 2  $\log_{10}$  represent surfaces with  
 494 bacteriostatic properties according to the conditions evaluated.  
 495

Surfaces	Humidity condition	Freshwater or lake water conditions	
		Freshwater aquarium	Lake water
Stainless steel	6.15	4.94	5.36
Plastic	0.19	+ 0.1	+ 0.11
Glass	0.23	0.01	+ 0.02
Wood	-	+ 0.11	0.22
Antibacterial plastic	<u>2.34</u>	<u>2.48</u>	1.58

496  
 497 Positive signs (+) in  $\log_{10}$  values at plastic, glass or wood surfaces represent an increase of  
 498 cells count respect to the stainless steel surface. No signs before the values represent a  
 499 reduction respect the stainless steel as a control. Reductions with more than 2  $\log_{10}$  are  
 500 underlined.

501

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503



504 **Figure legends**

505

506 **Figure 1.** Examples of the fluorescence microscopy images of *Flavobacterium*  
507 *psychrophilum* cells stained with the LIVE/DEAD<sup>®</sup> kit after 96 h of incubation. Live cells  
508 appeared green in colour and dead or injured cell appeared red. In humidity condition: (a) live  
509 cells forming biofilm, (b) round-shaped appearance of a dead cell indicated by an arrow on  
510 stainless steel surfaces. In freshwater condition: (c) high density of dead or injured cells on  
511 plastic surface, (d) the presence of live and dead or injured cells on glass surface. In lake  
512 water condition: (e) wood, (f) zinc pyrithione plastic surface. All scale bars: 10 µm.

513

514 **Figure 2.** Mineral concentration in parts per billion (ppb) of freshwater aquarium and lake  
515 water used to examine biofilm formation by *F. psychrophilum*. Abbreviations: Na-sodium,  
516 Mg-magnesium, K-potassium and Ca-calcium.

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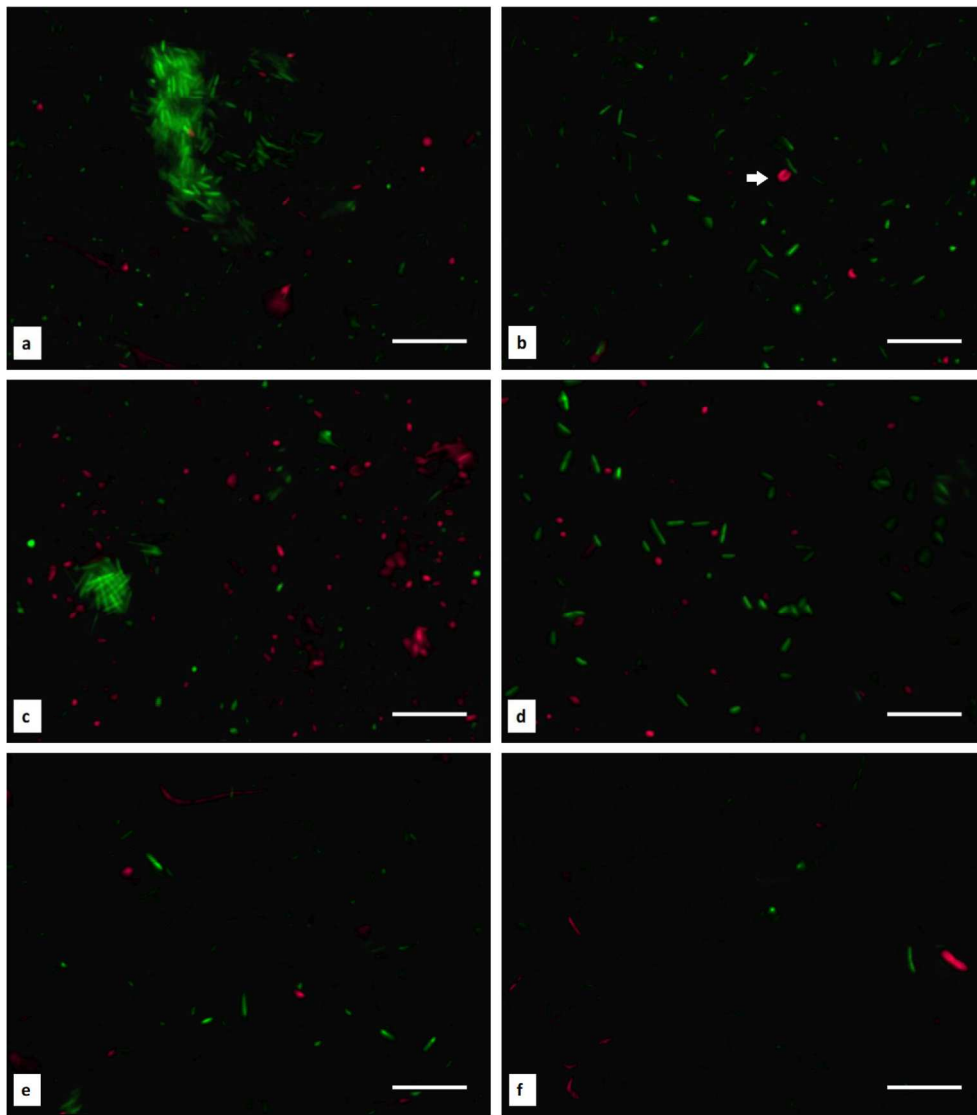


Figure 1. Examples of the fluorescence microscopy images of *Flavobacterium psychrophilum* cells stained with the LIVE/DEAD® kit after 96 h of incubation. Live cells appeared green in colour and dead or injured cell appeared red. In humidity condition: (a) live cells forming biofilm, (b) round-shaped appearance of a dead cell indicated by an arrow on stainless steel surfaces. In freshwater condition: (c) high density of dead or injured cells on plastic surface, (d) the presence of live and dead or injured cells on glass surface. In lake water condition: (e) wood, (f) zinc pyrithione plastic surface. All scale bars: 10  $\mu\text{m}$ .

162x183mm (300 x 300 DPI)

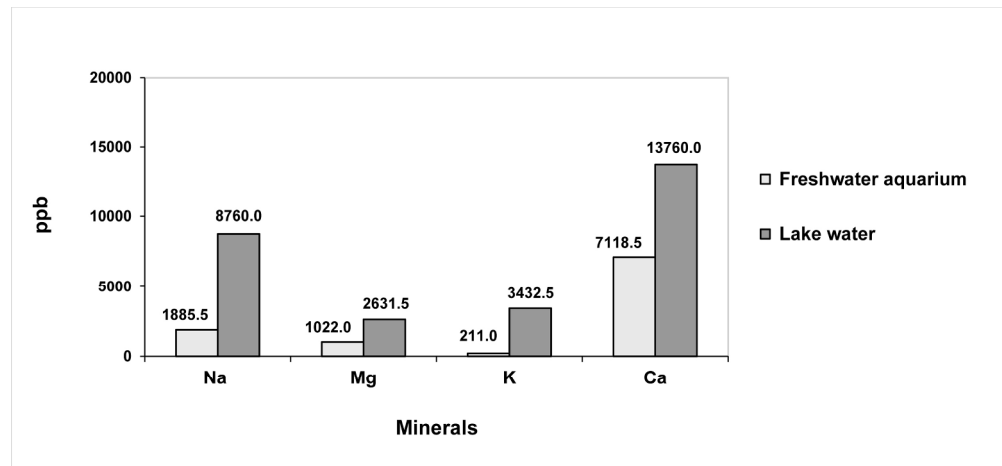


Figure 2. Mineral concentration in parts per billion (ppb) of freshwater aquarium and lake water used to examine biofilm formation by *F. psychrophilum*. Abbreviations: Na-sodium, Mg-magnesium, K-potassium and Ca-calcium.

209x96mm (300 x 300 DPI)