



Biofilm formation of *Flavobacterium psychrophilum* on various substrates

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

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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^T, 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^T, 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; MgSO₄.7H₂O,
97 0.5 g; CaCl₂.2H₂O, 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 cm², 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 2.01×10^6 live cells/ cm^2 and 4.79×10^1 of dead/damaged cells/ 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°C . After this time, the excess liquid was removed
128 from each surface and 50 μL of TYES broth was added to the surface of the stainless steel
129 disc and 255 μL to the other surfaces with a sterile pipette. These were then incubated for a
130 further 48 h at 15°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 μm filter (Millipore Co., Billerica, Ma, U.S.A.) prior to use. The various supports were
138 incubated with *F. psychrophilum* (2.14×10^6 of viable cells/ cm^2 and 7.41×10^1 cells/ 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[®] 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[®] 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 (1.41×10^6 live cells/cm²), plastic (9.12×10^5 live
196 cells/cm²) or glass (8.32×10^5 live cells/cm²) 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 (6.46×10^3 cells/cm²) and
199 dead/injured cells (1.10×10^4 cells/cm²). Also, an increase in the number of cells/cm² of dead
200 or injured cells was observed on all test materials, increasing from an initial concentration of
201 4.79×10^1 cells/cm² to 10^4 or 10^5 cells/cm² 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 (8.68×10^4 cells/cm²), plastic ($1.09 \times$
208 10^5 cells/cm²) (Figure 1c), glass surfaces (8.52×10^4 cells/cm²) (Figure 1d) and wood ($1.11 \times$
209 10^5 cells/cm²) compared to the antibacterial plastic (2.88×10^2 cells/cm²). 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 (2.30×10^5 cells/cm²), plastic (2.98×10^5 cells/cm²) and glass (2.41
212 $\times 10^5$ cells/cm²) compared with the wood (1.38×10^5 cells/cm²) (Figure 1e) or the
213 antibacterial plastic surface (6.03×10^3 cells/cm²) (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/cm² fewer bacteria attached relative
226 to the positive control, $2.48 \log_{10}$ cells/cm² in freshwater and $1.58 \log_{10}$ cells/cm² 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 Ca^{2+} and 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 m² 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.

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For Review Only

470 **Tables**

471 **Table 1.** Adherence and biofilm formation of *Flavobacterium psychrophilum* (cells/cm²) on
 472 stainless steel, plastic, glass, and antibacterial plastic surfaces after 96 h of incubation in
 473 humidity test condition.

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

474

475 [†]Initial cells count/cm²: 2.01 x 10⁶ live cells/cm² and 4.79 x 10¹ dead/injured cells/cm².

476 ^{a,b} 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/cm²) 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 [†]			
	Live	Injured or dead	Live	Injured or dead
Stainless steel	8.68 x 10 ⁴ ± 0.12 ^{aA}	1.46 x 10 ⁵ ± 0.12 ^{ab}	2.30 x 10 ⁵ ± 0.13 ^{aA}	1.54 x 10 ⁵ ± 0.39 ^a
Plastic	1.09 x 10 ⁵ ± 0.08 ^{aB}	5.87 x 10 ⁴ ± 0.11 ^{ab}	2.98 x 10 ⁵ ± 0.08 ^{aA}	3.09 x 10 ⁵ ± 0.50 ^a
Glass	8.52 x 10 ⁴ ± 0.11 ^{aA}	1.25 x 10 ⁵ ± 0.12 ^{ab}	2.41 x 10 ⁵ ± 0.09 ^{aB}	2.32 x 10 ⁵ ± 0.28 ^a
Wood	1.11 x 10 ⁵ ± 0.10 ^{aA}	2.80 x 10 ⁵ ± 0.39 ^a	1.38 x 10 ⁵ ± 0.07 ^{bA}	1.56 x 10 ⁵ ± 0.40 ^a
Antibacterial plastic	2.88 x 10 ² ± 0.13 ^{bB}	1.39 x 10 ⁴ ± 0.34 ^c	6.03 x 10 ³ ± 0.17 ^{cA}	2.25 x 10 ⁴ ± 0.19 ^b

483

484 [†] Initial cells count/cm²: 2.14 x 10⁶ live cells and 7.41 x 10¹ dead or injured cells. ^{a-c} Values in
 485 columns for each surface are significantly different if the letters are different ($p \leq 0.05$). ^{A-B}
 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

489

490

491 **Table 3.** The differences (represented in \log_{10} cells/cm²) 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

502

503

504 **Figure legends**

505

506 **Figure 1.** Examples of the fluorescence microscopy images of *Flavobacterium*
507 *psychrophilum* cells stained with the LIVE/DEAD[®] 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.

517

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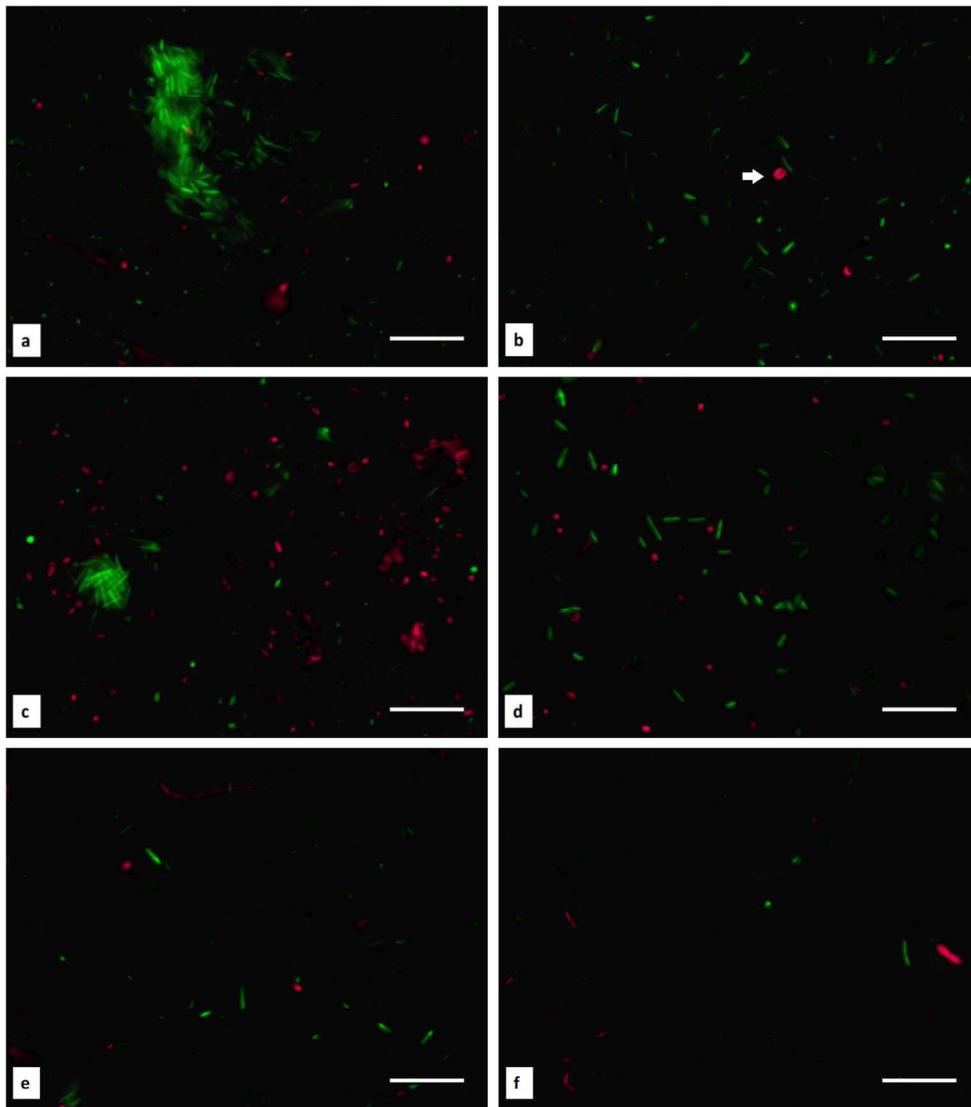


Figure 1. Examples of the fluorescence microscopy images of *Flavobacterium psychrophilum* cells stained with the LIVE/DEAD® kit after 96 h of incubation. Live cells appeared green in colour and dead or injured cell appeared red. In humidity condition: (a) live cells forming biofilm, (b) round-shaped 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 µm.

162x183mm (300 x 300 DPI)

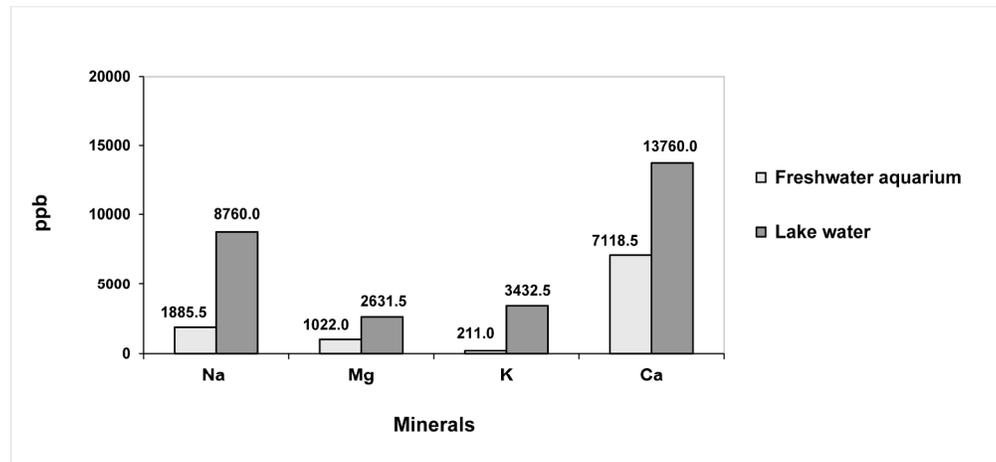


Figure 2. Mineral concentration in parts per billion (ppb) of freshwater aquarium and lake water used to examine biofilm formation by *F. psychrophilum*. Abbreviations: Na-sodium, Mg-magnesium, K-potassium and Ca-calcium.

209x96mm (300 x 300 DPI)