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Biofilm formation of *Flavobacterium psychrophilum* on various substrates

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Abstract

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

Key words

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Bacterial culture

F. psychrophilum, strain NCIMB 1947^T, was obtained from a stock of cryopreservation beads (Cryoprotect; Technical Service Consultants Service Ltd. Lancashire, UK) stored at -70°C. The bacterium was grown in TYES broth (tryptone, 4.0 g; yeast extract, 0.4 g; $MgS0_4.7H_2O$, 0.5 g; $CaCl_2.2H_2O$, 0.2 g; distilled water, 1000 mL; pH 7.2; autoclaved for 20 min at 121°C) under constant agitation at 140 rpm (Kühner Shaker LT-W, Adolf Kühner AG, Switzerland) for 72-96 h at 15°C. This culture was subsequently cultured on TYES agar plates (TYES broth with 15.0 g/L bacteriological agar) and incubated for 96 h at 15°C, from which bacterial colonies were taken and cultivated in TYES broth under agitation for 72-96 h at 15°C to obtain the bacterial culture used in the analysis.

2.2. Test materials

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

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 µL/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 x 10^6 live cells/cm^2 and 4.79 x 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 ≥ 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^{o}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 x 10^6 of viable cells/cm^2 and 7.41 x 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:

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

2.4. Assessing the number of live bacteria by fluorescence microscopy

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

The stainless steel discs were stained with 20 µL and plastic, glass, wood and the antibacterial plastic with 100 µL. Stained surfaces were left in the dark for 15 min at 22ºC to allow the stains to penetrate. Eight images were acquired from every surface evaluated using a fluorescence microscope IX70 (Olympus Optical, Tokyo, Japan) equipped with a mercury lamp, and two filters: (1) filter A (excitation 470–490 nm, emission 515–550 nm) and (2) filter B (excitation 510–550 nm, emission > 590 nm). The same microscopic parameters, input calibration and image acquisition were used throughout and images were analysed using Cytovision® software, version 2.51 (Applied Imaging, Sunderland, Tyne & Wear, UK). Cell 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***

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

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

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

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

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

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

4. Discussion

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

In our study, where *F. psychrophilum* cells were incubated on the various supports in the humidity chamber, some of the dead/injured bacteria became rounded in appearance. The morphological changes in *F. psychrophilum* cells observed here have also been reported by 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

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

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

As established from the genome analysis of *F. psychrophilum*, the bacterium has the ability to form biofilms and store cyanophicin, which could explain the bacterium's prolonged survival outside its host (Duchaud et al., 2007) and the spread of disease by this bacterium through the aquatic environment (Madetoja et al., 2002; Nematollahi et al., 2003). The ability of this bacterium to adhere to surfaces and form biofilms may explain why it is less susceptible to antimicrobial treatment. Sundell & Wiklund (2011) observed an increased antimicrobial resistance in *F. psychrophilum* biofilms containing high bacterial cell densities ($> 10^7$ CFU/mL). These characteristics, together with adherent properties of *F. psychrophilum* 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 (Nematollahi et al., 2003; Barnes & Brown, 2011). Zinc pyrithione, is widely used as an antifouling agent in paints and exhibit a high antimicrobial effects against biofilm bacteria (Konstantinou & Albanis, 2004; Ciriminna, Bright & Pagliaro, 2015). The commercial cost of zinc pyrithione, used in a concentration of 2.0% as biocide and antifouling is approximately US$ 2.50 - US$ 3.50 to cover each 100 m^2 of fish-farm environments. 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 ≥ 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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469

Tables

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

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

[†]Initial cells count/cm^2: 2.01×10^6 live cells/cm^2 and 4.79×10^1 dead/injured cells/cm^2.

[a,b] Values in columns for each surface are significantly different if the letters are different ($p \leq 0.05$).

479 **Table 2.** Adherence and biofilm formation of *Flavobacterium psychrophilum* (cells/cm^2) 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	$8.68 \times 10^4 \pm 0.12^{a\,A}$	$1.46 \times 10^5 \pm 0.12^{ab}$	$2.30 \times 10^5 \pm 0.13^{a\,A}$	$1.54 \times 10^5 \pm 0.39^{a}$
Plastic	$1.09 \times 10^5 \pm 0.08^{a\,B}$	$5.87 \times 10^4 \pm 0.11^{ab}$	$2.98 \times 10^5 \pm 0.08^{a\,A}$	$3.09 \times 10^5 \pm 0.50^{a}$
Glass	$8.52 \times 10^4 \pm 0.11^{a\,A}$	$1.25 \times 10^5 \pm 0.12^{ab}$	$2.41 \times 10^5 \pm 0.09^{a\,B}$	$2.32 \times 10^5 \pm 0.28^{a}$
Wood	$1.11 \times 10^5 \pm 0.10^{a\,A}$	$2.80 \times 10^5 \pm 0.39^{a}$	$1.38 \times 10^5 \pm 0.07^{b\,A}$	$1.56 \times 10^5 \pm 0.40^{a}$
Antibacterial plastic	$2.88 \times 10^2 \pm 0.13^{b\,B}$	$1.39 \times 10^4 \pm 0.34^{c}$	$6.03 \times 10^3 \pm 0.17^{c\,A}$	$2.25 \times 10^4 \pm 0.19^{b}$

483

484 [†] Initial cells count/cm^2: 2.14×10^6 live cells and 7.41×10^1 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

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

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

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

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.

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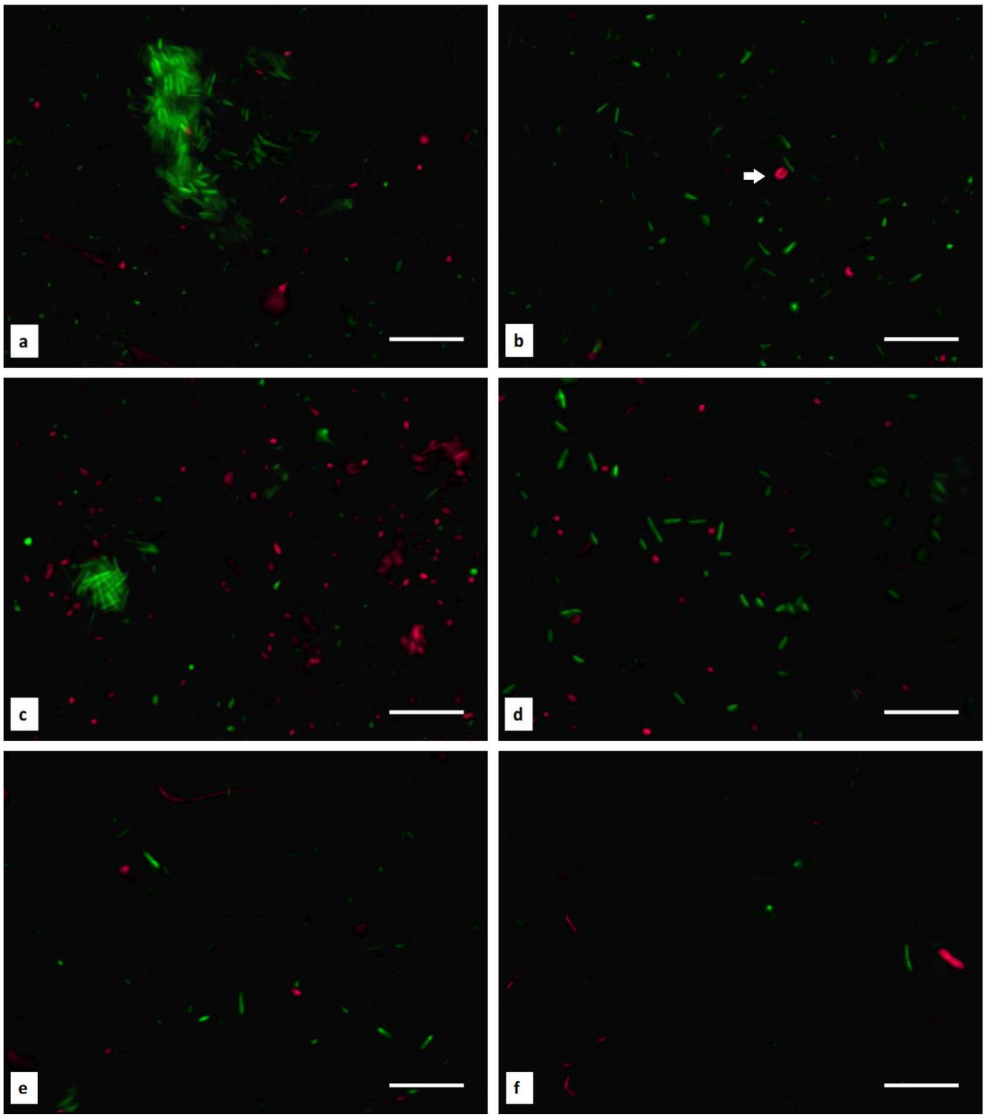

Figure 1. Examples of the fluorescence microscopy images of Flavobacterium psychrophilum cells stained with the LIVE/DEAD® kit after 96 h of incubation. Live cells appeared green in colour and dead or injured cell appeared red. In humidity condition: (a) live cells forming biofilm, (b) round–shaped appearance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

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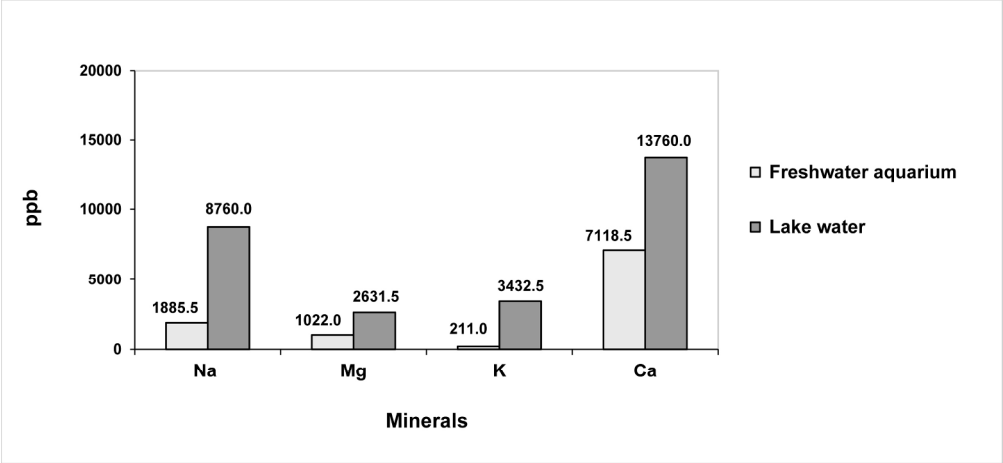

Figure 2. Mineral concentration in parts per billion (ppb) of freshwater aquarium and lake water used to examine biofilm formation by F. psychrophilum. Abbreviations: Na-sodium, Mg-magnesium, K-potassium and Ca-calcium.

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