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1 **Formation and resistance to cleaning of biofilms at air-liquid-wall interface. Influence of bacterial**
2 **strain and material**

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20

21 **Highlight**

- 22 • Some bacteria form large amounts of biofilm at air-liquid-wall interface
- 23 • Material properties affect biofilm formation at air-liquid-wall interface
- 24 • Biofilms at air-liquid-wall interfaces are highly resistant to cleaning
- 25 • A close relationship exist between the shapes of meniscus at wall and biofilms

26 **Abstract**

27 Interfaces between air, liquid and walls (ALW interfaces) are known to be conducive to the formation
28 of biofilms, at least in some bacteria, yet little information is available on the influence of material
29 properties on the amount of biofilms formed and their resistance to a cleaning procedure. In this
30 study, we investigated the ability of four bacterial strains (*Pseudomonas fluorescens* [Pf1], *Escherichia*
31 *coli* [Ec-SS2], *Bacillus cereus* [Bc-98/4] and *B. subtilis* [Bs-PY79]) to form biofilms in static conditions at
32 the ALW interface on four materials with very different topographic and hydrophilic/hydrophobic
33 properties (stainless steels with 2R or 2B finishes, polypropylene and glass). Biofilms were observed
34 after staining with orange acridine visually, by epifluorescence microscopy and by confocal scanner
35 laser microscopy. The number of culturable cells within biofilms was also estimated after growth on
36 agar. After one-day of incubation in a bacterial suspension, three strains (except Bc-PY79) were
37 found to form large amounts of biofilm, easily observable to the naked eye. However, great
38 differences were observed between strains in the number of CFU (between 4.7 and 7.4 log CFU cm⁻²),
39 as well in the biofilm structure. Furthermore, the material also affected the amount and/or structure
40 of biofilms, and a 3D-biofilm organization was only observed for two of the four tested strains (Bc-
41 98/4 and Pf1) on PP, a hydrophobic material. After a standard cleaning-in-place treatment involving
42 NaOH 0.5% at 60°C, cultivable cells were only detected from Bc-98/4 biofilms (growth on agar), while
43 biofilms were also still visible on coupons contaminated with Pf1. Furthermore, most residual
44 biofilms after cleaning appeared orange by epifluorescence microscopy after staining with orange
45 acridine suggesting the presence of many viable but non-culturable cells within the residual biofilms.
46 In Bc-98/4 biofilms, spores were also clearly observed by epifluorescence microscopy. Knowing their
47 ability to survive the conditions encountered during cleaning procedures, this could account for the
48 high level of CFU enumerated after cleaning. Lastly, Bc-98/4 biofilms formed on stainless steel 2R
49 were more resistant to cleaning than on PP and glass. All of these results highlighted the importance
50 of biofilms at the ALW interfaces in the control of surface hygiene, particularly in the food industry.
51 We then investigated whether the shape of the menisci at the interfaces (convex vs concave, kinetics
52 over time) could at least partly explain the shape or even the resistance to detachment of the ALW
53 biofilms.

54

55 **Keywords:** biofilm; air-liquid-wall interface; meniscus; cleaning in place; VBNC; *Bacillus* spores

56 1. Introduction

57 Despite the attention currently being paid to hygiene procedures in the food industries, it remains
58 difficult or impossible to produce food free of micro-organisms. Thus, the involvement in food
59 contamination of adherent bacteria, spores, or biofilms on the surface of food processing equipment
60 is widely acknowledged. For the record, biofilms are communities of micro-organisms attached to
61 biotic or abiotic surfaces and embedded in a matrix made of self-produced extracellular polymeric
62 substances (EPS). These biofilms represent a serious challenge to the food industry since their
63 formation is possible on every material, including polymers, stainless steel, thereby compromising
64 food safety and quality. For example, cross-contamination of food by *Listeria monocytogenes* could
65 result from biofilms on food-contact surfaces including slicing machines and cutting boards, as well
66 as on non-food contact surfaces such as floors or drain sinks (Rodríguez-López et al., 2018).
67 Furthermore, bacteria in biofilms are 100 to 1000 times more resistant to cleaning (Bénézech &
68 Faille, 2018; Cunault et al., 2019) and disinfection (Maes et al., 2019) processes than are planktonic
69 cells, rendering them difficult to control them. The slow or incomplete penetration of antimicrobial
70 agents through the EPS matrix of the biofilm, the slow growth rate and the increased number of
71 efflux pumps of cells within biofilms are thought to play a role in this increase in resistance (Soto,
72 2013). The presence of cells in the non-viable but culturable (VBNC) state, widespread in biofilms,
73 could also explain this augmented resistance. Indeed, bacteria can even enter a VBNC state within
74 biofilms, as has already been shown for some pathogenic bacteria including *Campylobacter jejuni*
75 (Duffy & Dykes, 2009) or *Listeria monocytogenes* (Gião & Keevil, 2014).

76 Most of the data available in the literature on biofilms in the food industries relate to fully
77 immersed biofilms, formed under static or dynamic conditions. Indeed, the role of air-liquid-wall
78 (ALW) interfaces on surface contamination has been the subject of relatively few publications, in
79 spite of the warnings issued by some authors about the risk due to these areas on the control of
80 surface hygiene (Faille et al., 2018). In fact, ALW interfaces can be found in many environments,
81 including the medical sector or the food industry. This is notably the case for surfaces of partly-filled
82 devices such as tanks, sinks, or washing units as well as industrial storage and piping systems used
83 either during process or after the cleaning procedure in areas where some residual liquid has
84 remained after operations. Unfortunately, it would appear that these areas are particularly
85 conducive to bacterial adhesion, as well as to the formation and/or the persistence of biofilms (Faille
86 et al., 2018). Indeed, when biofilms are produced in tubes or in the wells of microtitre plates
87 (laboratory conditions), a ring-like structure often forms at the air-liquid interface on the surface of
88 the tube or wells and it is reasonable to assume that these biofilms might provide an attachment site
89 for subsequent pellicle formation at the liquid surface (Fagerlund et al., 2014; Koza et al., 2009), even

90 though controversial data have also been reported on *Salmonella typhimurium* (Römling et al., 2000).
91 Other authors have also shown differences between psychrotrophic *Pseudomonas* isolates
92 concerning their ability to form biofilms at the various interfaces (Robertson et al., 2013). Some
93 isolates only produced biofilms on solid surfaces, while others only produced floating biofilms and
94 still others were able to produce both biofilms. The presence of a meniscus forming a more or less
95 acute angle with the vial wall would be a major element in the formation of the biofilm at the ALW
96 interface (Kuśmierska & Spiers, 2016).

97 In food industries, phenomena that occur at ALW interfaces have received only little attention
98 despite the challenge in terms of surface hygiene. However, these interfaces provide an ecological
99 opportunity for bacteria because of the joint presence of high levels of both oxygen and nutrients.
100 Biofilms at ALW interfaces are produced by a wide range of bacteria including foodborne pathogens
101 such as *Salmonella spp.* (Giaouris & Nychas, 2006; Sutrina et al., 2019) or *Bacillus spp.* (Constantin,
102 2009), as well as non-pathogenic bacteria widespread in food environments such as *Pseudomonas*
103 *spp.* (Mosharaf et al., 2018; Robertson et al., 2013). Marked differences in the ability to form ALW
104 biofilms have been also reported between strains belonging to a single species, e.g. *Bacillus cereus*
105 (Wijman et al., 2007). This ability to form biofilms at ALW interfaces would be strain- or species-
106 dependent, at least in part because of differences in motility (Magana et al., 2018; Majed et al.,
107 2016) or of the presence of aggregative fimbriae (Römling et al., 2000) or curli (Weiss-Muszkat et al.,
108 2010). Environmental conditions such as the source of carbon or energy (Sutrina et al., 2019) or the
109 growth medium (Mosharaf et al., 2018) would also greatly affect the biofilm formation at these
110 interfaces.

111 Some authors have then questioned whether the structure of the biofilms at ALW interface
112 differed from those on submerged surfaces. First, some bacteria including pathogens such as
113 *Salmonella enteritidis* (Giaouris & Nychas, 2006) or *B. cereus* (Wijman et al., 2007) produced greater
114 amounts of biofilm at interfaces than on the submerged zones. Similarly, in conditions mimicking
115 those encountered in fresh-cut food processing equipment, the ALW interface was shown to be
116 particularly favorable to surface contamination by *Pseudomonas fluorescens* (Cunault et al., 2015).
117 The same phenomenon has been observed in dairy factories, where *B. cereus* biofilms have been
118 reported to occur mainly at the air-liquid interface with a typical ring attached to the wall (Fagerlund
119 et al., 2014), while strains from other dairy-associated *Bacillus* species formed both robust fully
120 immersed and ALW interface biofilms in milk (Ostrov et al., 2019). The structure of ALW biofilm could
121 also differ greatly from that produced in fully-submerged conditions, as shown on *Pseudomonas*
122 *fluorescens* which produced thick patches, which were easily observed under the microscope in

123 biofilms at the ALW interface, but not in fully immersed biofilms (Cunault et al., 2018). Their
124 resistance to rinsing and cleaning procedures was also greater (Cunault et al., 2019).

125 The objective of the current study was to investigate the ability of bacteria often found in biofilms
126 on surfaces of food processing equipment to form biofilms at ALW interfaces. We first analyzed the
127 formation (amount and structure) of biofilms on various materials and their further resistance to a
128 standard cleaning-in-place procedure. We then investigated the relationship between the ability to
129 form biofilms at ALW interfaces, the biofilm properties and the shape of the liquid meniscus near the
130 solid wall.

131

132 **2. Materials and Methods**

133

134 **2.1. Bacterial strains and solid surfaces**

135 For this study, a total of four strains and four solid surfaces were selected. The bacterial strains
136 used were *E. coli* SS2 (Ec-SS2) (Gomes et al., 2017), *P. fluorescens* Pf1 (Pf1), isolated by ANSES from
137 cleaning-in-place effluent (Cunault et al., 2015), *B. cereus* CUETM 98/4 (Bc-98/4), isolated from a
138 dairy processing line (Lequette et al., 2010), and lastly *B. subtilis* PY79 (Bs-PY79), a laboratory strain,
139 frequently used for studying cellular pathways (Schroeder & Simmons, 2013). These bacterial species
140 were chosen mainly because of their ability to contaminate the surfaces of equipment in the food
141 industry. *P. fluorescens* is an important spoilage organism widespread in many environments and is
142 able to form biofilms on food processing surfaces (Meliani & Bensoltane, 2015). Concerning *E. coli*,
143 many strains including Shiga toxin-producing (STEC) isolates of different origins can form strong
144 biofilms on various food-contact surfaces (Adator et al., 2018). Lastly, *B. cereus* strains, often
145 associated with foodborne outbreaks, but also with food spoilage are capable of forming greater or
146 smaller amounts of biofilms which may contain, after several days, up to 90% spores, making them
147 extremely difficult to control (Majed et al., 2016). Conversely, *B. subtilis*, also prevalent in low acidic
148 food products such as meat (Soni et al., 2016), is rather noted for the production of enzymes.

149 Three materials, relevant to food processing equipment design, used in the form of rectangular
150 coupons (45 mm x 15 mm), were compared to glass (Glasatelier Aillart, Meerhout, Belgium), which
151 was included in this study because of its highly hydrophilic nature. The three materials were AISI 316
152 stainless steel with pickled (2B) and bright annealed (2R) finishes (kindly provided by APERAM,
153 Isbergues, France), and polypropylene (PP) (API Plastiques, Brenelles, France). Prior to each
154 experiment, the coupons were cleaned and disinfected using a standard protocol used at PIHM. Each
155 coupon was first cleaned using pure alkaline detergent (RBS T105, Traitements Chimiques des
156 Surfaces, France). They were then subjected to a 10 min immersion in a 5% RBS T105 at 60 °C,

157 followed by thorough rinsing with tap water, then with softened water (reverse osmosis water) for 5
158 min each. 24 h prior to the experiments, the coupons were disinfected as follows. Stainless steel
159 coupons were treated in a dry heat oven at 180°C for 1 h, while PP and glass were autoclaved at
160 121°C for 30 min.

161

162 **2.2. Biofilm production**

163 The biofilms at ALW interfaces were formed in static conditions on sterilised coupons placed
164 vertically, but only partially immersed in polypropylene vials (3.4 cm x 7 cm) containing 27 mL of 1/10
165 TSB inoculated at around 10^6 CFU mL⁻¹ (Bc-98/4 and Bs-PY79) or 10^7 CFU mL⁻¹ (Pf1 and Ec-SS2) with a
166 24 h-culture of each strain. The biofilms were analysed after a 24 h incubation at 30°C in order to
167 observe and/or measure surface microbial load (CFU), biofilm structure (observation under
168 microscope). In order to determine the effectiveness of a cleaning procedure on the biofilms formed
169 on the different materials, biofilms at ALW interfaces were subjected to cleaning in place using NaOH
170 and then analysed as described above. Before analysis, coupons were removed from the fouling
171 suspension and for each coupon, the side meant-to-be analysed was rinsed gently using drip rinsing
172 with 15 mL of sterilised ultrapure water.

173

174 **2.3. Analysis of the biofilms grown at the ALW interface**

175 Just after rinsing, the biofilms present at the ALW interface were easily observable to the naked
176 eye. The biofilms (surfaces of one centimetre width) were sampled using a dry cotton swab (Copan,
177 Brescia, Italy). The swab was then placed in tubes containing 2.5 mL of sterile saline solution and
178 then vortexed (VELP Scientifica, Italy) in automatic mode for 25 s (at maximal speed, 2400 rpm). In
179 parallel, some rinsed coupons were kept for observation under the microscope.

180 In order to enumerate the cultivable bacteria, the suspensions containing the detached bacteria
181 were plated on TSA (Tryptone Soy Agar, Biokar, France) and counted after 48 h incubation at 30°C.

182 For microstructure examination, the rinsed coupons were firstly dried at 20 °C for 1 hour. Next,
183 the coupons were stained with acridine orange (0.01%) for 15 min at 20 °C, gently rinsed with
184 softened water and allowed to dry before observation. For the record, acridine orange is a cell-
185 permeant nucleic acid binding dye that emits green fluorescence when bound to double-stranded
186 DNA and red/orange fluorescence when bound to single-stranded DNA or RNA. Finally, the biofilm
187 structure was observed using an epifluorescence microscope (Zeiss Axioskop 2 Plus, Oberkochen,
188 Germany, x50 magnification). In order to detect a potential 3-D organisation of biofilms at ALW
189 interface, confocal laser scanning microscopy (Zeiss, LSM780, Oberkochen, Germany) at the x400
190 magnification (40x oil objective NA 1.3) was performed. These observations focused on the densest

191 region of ALW biofilms, previously revealed by the epifluorescence microscopy. Using this technique,
192 multiple two-dimensional images (Z stacks) were obtained at different depths in the samples,
193 enabling the reconstruction of 3D structures.

194

195 **2.4. Biofilms resistance to cleaning**

196 In order to determine the bacterial biofilm resistance to cleaning, the rinsed coupons were left to
197 dry at room temperature. The dried coupons were placed in rectangular test tubes, connected to a
198 CIP pilot rig (Jullien et al., 2008). A three-step CIP procedure was then carried out: (i) rinsing for 5 min
199 with softened water at 20 °C at a wall shear stress of 1.34 Pa, (ii) cleaning for 10 min with 0.5% NaOH
200 water at 60 °C at a wall shear stress of 3.60 Pa, and finally (iii) rinsing with softened water at 20 °C at
201 a wall shear stress of 1.34 Pa. After the CIP process, the coupons were taken out of the test tubes
202 and rinsed by dipping in a beaker containing one litre of sterile ultrapure water. The residual biofilms
203 were then analysed as described in Section 2.3.

204

205 **2.5. Statistical analysis**

206 The statistical analyses were performed using SAS V9.4 software (SAS Institute, Gary, NC, USA).
207 Each experiment was repeated three or four times. Variance analyses and Tukey's grouping (Alpha
208 level=0.05) were performed to determine the fouling capability of strains, the respective role of
209 bacterial strains and materials on biofilm formation (in terms of CFU) and the role of materials on the
210 resistance to the cleaning procedure.

211

212 **3. Results**

213

214 **3.1. Enumeration of the biofilms formed at ALW interface**

215 Biofilms were first analysed for the number of cells able to form colonies on agar (cultivable cells).
216 First of all, whatever the material, no colony could be counted from coupons contaminated with Bs-
217 PY79 after a 48-h incubation at 30°C. Figure 1 shows the number of cultivable cells within the
218 biofilms formed by the three other strains at the ALW interface. The three strains were capable of
219 forming biofilms on each of the materials. However, the amount of cultivable cells within biofilms
220 significantly depended on the strains. The amount of biofilm was the highest for Pf1 with over 7 log
221 CFU cm⁻², whatever the material, followed by Ec-SS2 with around 5 to 6 log CFU cm⁻², depending on
222 the material. Lastly, Bc-98/4 only produced relatively small amounts of biofilm, less than 6 log CFU
223 cm⁻², on the four materials. The variance analysis confirmed that the amount of biofilms at ALW
224 interface was significantly affected by the strain ($p < 0.0001$), and the Tukey's grouping showed that

225 the three strains belonged to different groups (A, B, and C for Pf1, Ec-SS2 and Bc-98/4, respectively).
226 The amount also of biofilms was also influenced not only by the materials, but their role in biofilm
227 formation varied with the bacterial strain. Indeed, no clear difference was observed with Pf1 ($p =$
228 0.9047). Conversely, 0.8 and 1.1 log differences were observed in the number of CFU cm^{-2}
229 enumerated from the biofilms produced by Ec-SS2 and Bc-98/4, respectively. For these two strains,
230 PP was the least contaminated material, stainless steel with a 2R finish the most contaminated.
231 Tukey's grouping confirmed that PP (group b) was significantly less contaminated by the Bc-98/4
232 strain than the three other materials (group a). A roughly similar grouping was obtained with Ec-SS2,
233 except for glass, which was not significantly different to the other materials (group ab).

234

235 **3.2. Structure of the biofilms formed at ALW interface**

236 After staining the contaminated coupons with orange acridine, biofilms at ALW interfaces were
237 readily apparent to the naked eye in the form of a more or less thick orange strip (Figure 2).
238 Concerning the influence of the material on the amounts of biofilm, PP appeared to be clearly less
239 conducive to the formation of Bc-98/4 and Ec-SS2 (but not of Pf1) biofilms than the three other
240 materials, in accordance with the number of colonies counted on agar. Furthermore, Bc-98/4
241 produced the thinnest biofilms followed by Ec-SS2, thereby suggesting that the differences in the
242 CFU numbers were related to the surface of the contaminated area. It is also interesting to note the
243 heterogeneous coverage (presence of a second strip, differences in colour intensity within biofilms)
244 observed very clearly on stainless steels contaminated with Pf1 and Ec-SS2.

245 In order to observe the biofilm distribution or structure on the different materials, the upper parts
246 of the ALW biofilms were observed under microscope at a x50 magnification. As shown in Figure 3 on
247 biofilms stained with orange acridine, first and foremost, the orange staining of the biofilms revealed
248 that most of the bacterial cells were viable, whatever the strain or the material. However, in spite of
249 the presence of an area with a high bacterial density (more strongly-stained strip) in all ALW
250 interface biofilms, the biofilm organization was influenced by the bacterial strain and material. First,
251 in accordance with the number of colonies enumerated from the different biofilms, the
252 contamination level on top of the biofilms depended on the bacterial strain. This was the highest for
253 Pf1, the lowest for Bc-98/4. Furthermore, Bc-98/4 biofilms were the only ones with areas devoid of
254 cells (black zones), while the other biofilms were more homogeneously distributed on the surfaces.

255 On the other hand, material properties also seemed to have affected the contamination level and
256 the structural organisation of the upper part of the biofilms. The most visible effect due to material
257 properties was observed in the biofilm of Bc-98/4, followed by Ec-SS2 and PF1. Bc-98/4 biofilms
258 formed on PP were completely different to the other biofilms, not only from a quantitative but also
259 from a qualitative point of view. Indeed, along with a low contamination level, Bc-98/4 biofilms on PP

260 consisted mainly of two parts, an upward, very narrow, yet dense strip and just underneath, a broad
261 sparser biofilm, in which the contamination level decreased with depth. On the three other
262 materials, the biofilms exhibited a roughly similar spatial distribution, i.e. a broad, highly
263 contaminated strip surmounted by a less contaminated area, and below the dense strip, a rapid
264 decrease in the contamination level. The differences between materials were less pronounced for
265 the two other strains. On Ec-SS2 biofilms, a more coloured area within the biofilm was observed on
266 both stainless steels, but not on PP and glass. A large coloured strip was also observed on Pf1
267 biofilms whatever the material, in lower areas of the biofilm, except on PP.

268 To go further in the analysis of the structure of the biofilms, in particular their 3-D structure,
269 particularly dense areas of each biofilm were observed by CSLM at a x400 magnification (Figure 4). As
270 expected, great differences were observed between strains. For example, Ec-SS2 biofilms were
271 organized in a net-like/reticular pattern visible on weakly contaminated areas of the four materials. A
272 similar organisation was also observed in some places in biofilms of the other strains. On the other
273 views, materials were covered with more or less dense and thick cell clusters, separated by darker
274 areas which reflected the presence of few if any cells. Some diffuse staining was also observed,
275 possibly indicative of the presence of secreted exopolymers. This is particularly true not only for Pf1
276 biofilms whatever the material, but also for Bc-98/4 biofilms on glass. Surprisingly, despite the
277 presence of very dense clusters, most biofilms were thin. A clear 3-D organization was only visible on
278 Bc-98/4 biofilms grown on PP (hydrophobic) and to a lesser extent on Pf1 biofilms on the same
279 material.

280

281 ***3.3. Ease of removal of biofilms during a cleaning procedure***

282 The biofilms grown at the ALW interface were then subjected to a standard CIP procedure to
283 determine their resistance to cleaning. After the CIP treatment, out of three strains, only Bc-98/4
284 grew on TSA after a 48-h incubation period, while the other two strains were unable to form
285 colonies, even after five days of incubation, indicating the absence of cultivable cells on the surfaces.
286 Data concerning Bc-98/4 are shown in Figure 5. The greater the reduction in the number of CFU logs
287 after CIP, the less resistant the biofilms are to the cleaning procedure. The resistance to cleaning was
288 highly dependent on the material and the reduction in the number of CFU logs ranged from around
289 1.7 (on stainless steel with 2B and 2R finishes) to 3.0 (on glass). The influence of the materials was
290 thus significant as confirmed by the variance analysis ($p < 0.0001$). According to the Tukey's grouping,
291 Bc-98/4 biofilms formed on stainless steel 2R were more resistant to cleaning than on PP, but more
292 importantly than on glass, while only the 2B finish differed significantly from glass.

293 As it is highly likely that the cleaning procedure would affect bacterial viability, the coupons were
294 viewed after staining with orange acridine to observe the amount of residual biofilm after cleaning

295 and the possible presence of VBNC and dead cells. The residual biofilms stained with acridine orange
296 were still readily apparent to the naked eye on all materials for Pf1, on all materials except PP for Bc-
297 98/4, while nothing was apparent for Ec-SS2 (data not shown). This discrepancy in the results
298 obtained by CFU enumeration, at least for Pf1, suggested the presence of a large number of VBNC or
299 dead cells within Pf1 biofilms. The red colour after staining with orange acridine (Figure 6) would
300 indicate that a non-negligible portion of the remaining cells would be in a VBNC state. Higher
301 magnification images also revealed the presence of spores within the Bc-98/4 biofilms
302 (Supplementary Figure S1). The presence of spores probably at least partially explained why it was
303 possible to count a significant number of bacteria having retained their ability to form colonies on
304 agar after CIP. Elsewhere, Ec-SS2 biofilms were found to be highly susceptible to the cleaning process
305 (Figure 6), as they underwent the most significant changes in both structure and contamination level,
306 and their ease of cleaning was even greater on PP and glass. For the two other strains, a clear strip
307 was still observed, but the covered areas were smaller than those of the untreated biofilms.
308 Conversely, large areas were now devoid of any clear contamination. Furthermore, while only one
309 coloured strip was observed on Bc-98/4 biofilms, a second thinner strip was also observed above or
310 below the larger one on Pf1 biofilms. The biofilm organisation was affected by the material, both on
311 the size of the stained strips and on the density. For example, in the case of Bc-98/4, stainless steel
312 surfaces showed larger contaminated surfaces than the two other materials.

313

314 ***3.4. Measurement of meniscus at the ALW interface***

315 At the ALW interfaces, we observed clear menisci which shape depended on the material, and the
316 presence of these menisci is likely to play a major role in the amount and structure of biofilms
317 formed at the interfaces (Figure 7). In order to investigate the possible role of the meniscus at the
318 ALW interface on the amount and organisation of the biofilm, the coupons were half-immersed in
319 TSB and incubated for up to one day at 30°C, before measuring of the meniscus. Data are given in the
320 Table 1. At T0, as expected, a convex meniscus (negative value) was observed on PP, due to its
321 marked hydrophobic character. A concave meniscus was observed on the three other materials
322 (more or less hydrophilic), whose height differed on the different materials. As suggested by the
323 Tukey's grouping, this was significantly smaller on PP, although marked differences were also
324 observed between the meniscus on the hydrophilic materials (smaller on 2R than on glass and 2B).
325 Then, over time, the size and even the convex/concave shape of the meniscus evolved. After only
326 one hour in TSB, the meniscus had already become convex on PP and its height continued to increase
327 over time, although even after 24 h incubation, this was still much smaller than that of other
328 materials. Concerning the three hydrophilic materials, the height decreased slowly with time on 2R

329 and glass, but remained stable on 2B, perhaps because of its roughness. As indicated by the Tukey's
330 grouping, the largest meniscus after 24 h in TSB was observed on 2B (group A), followed by glass
331 (group B), then 2R (group C) and lastly PP (group D).

332

333 4. Discussion

334 in this study, we looked for the ability of strains to form biofilms at ALW interfaces on materials
335 with different surface properties, but also for their further resistance to a standard cleaning
336 procedure. In the experimental conditions used throughout this study, in particular static conditions,
337 we first observed a strong ability of all strains except Bs-PY79 to form biofilms at ALW interfaces,
338 easily observed by the naked eye, once stained with orange acridine. On the other hand, for the
339 other three strains, very significant differences were observed both in the surface covered and in the
340 number of cultivable cells between strains as well as between materials, except for Pf1. It can be also
341 noted that the fully-immersed surfaces (below the colored strip) were rather heavily contaminated
342 by Ec-SS2, and much less by Bc-98/4 and Pf1. The observation on the latter strain is consistent with
343 previously reported results on Pf1, grown at 10°C in ten times diluted TSB (Cunault et al., 2019). In
344 these conditions, Pf1 produced around 300 times more biofilm (estimated by the number of CFU cm⁻²)
345 at ALW interfaces than on fully immersed surfaces. The small amount of Bc-98/4 biofilm at ALW
346 interface suggests that ALW interface would not be particularly conducive to the formation of
347 biofilms by this strain. Indeed, it has already been shown that some *B. cereus* strains produce only
348 small amounts of biofilms at ALW interfaces (Wijman et al., 2007). However, similar differences in
349 the ability to form biofilms have been previously reported between Bc-98/4 (Faille et al., 2014) and
350 Pf1 (Cunault et al., 2018), when biofilms were formed on coupons placed horizontally in bacterial
351 suspensions. It can therefore be assumed that the differences observed here between the two
352 strains simply reflect different abilities to produce biofilms in any condition. Furthermore, the
353 structure of Bc-98/4 biofilms also clearly differed from those produced by the other strains, with a
354 heterogeneous distribution of cells on the surface and the presence of areas with little or no
355 contamination on all materials. Elsewhere, on the less contaminated zones, the classical net-like
356 pattern was observed for all three strains. Astonishingly, as all biofilms were very flat, except perhaps
357 on PP. it is conceivable that large amounts of biofilms were present at the meniscus but when the
358 coupons were rinsed off, most of the biofilm was removed. This could be explained if the cohesive
359 forces within the biofilm are significantly weaker than the interaction forces between the biofilm and
360 the material.

361 Concerning the role of materials on the ability of strains to form biofilms at ALW interfaces, the
362 most striking result was the relatively low amount of biofilm at ALW interface on PP, except for the

363 best biofilm former, i.e. Pf1. It can be assumed that, for strains capable of forming thick biofilms,
364 material properties play a major role (if at all) only in the early stages. In any case, the major
365 difference of PP compared to the other three materials, is its marked hydrophobic character.
366 However, on the basis of the information reported in the literature, it is difficult to conclude as to the
367 major role of the hydrophobic character of materials on biofilm formation. Indeed, conflicting results
368 have been reported on biofilm formation in submerged condition as shown by the following few
369 examples. *L. monocytogenes* (Bonsaglia et al., 2014) and *S. aureus* (Lee et al., 2015) strains would
370 form biofilms more effectively on stainless steel and glass (hydrophilic) than on polystyrene that is
371 hydrophobic. Conversely, higher populations of *Cronobacter sakazakii* were found after 24 h-biofilm
372 formation on silicone and polycarbonate surfaces than on stainless steel (Jo et al., 2010). As is often
373 the case, differences could also have been observed between strains from the same species, as
374 shown on *E. coli* biofilms (Uhlich et al., 2006). That being said, a previous study on *B. cereus* biofilms
375 gave results in line with those obtained here at the level of ALW interfaces. Indeed, the biofilm
376 amount was significantly lower on polystyrene than on stainless steel (Hayrapetyan et al., 2015).

377 The resistance of these biofilms to a standard cleaning procedure was then investigated. It is
378 worth noting that a drying step was introduced between the rinsing and clean-in-place (CIP) process,
379 as it was observed during preliminary trials (data not shown) that biofilms were easily removed
380 otherwise (by a CIP process, but also by a rinsing step). Indeed, as we had previously shown that a
381 drying step after surface contamination would reinforce interaction between spores and materials
382 (Faille et al., 2016), it was highly likely that biofilms would also be concerned by this phenomenon.
383 Moreover, since a drying step often occur under most real-world conditions, the presence or absence
384 of residual biofilms after cleaning demonstrated in this study must be relevant to what would be
385 produced in agro-industrial environments, for example. Despite this drying step, no CFU could be
386 enumerated from biofilms of Pf1 and Ec-SS2 after cleaning. Taking into account the detection
387 threshold of the procedure used (around 10^2 CFU cm^{-2}), this reflects a decrease of at least 5 log of the
388 culturable cells from Pf1 biofilms and at least 4 log for Ec-SS2 biofilm. This result is not surprising
389 since such decreases in the number of CFU following a cleaning procedure have already been
390 observed on fully immersed biofilms of *E. coli* (Furukawa et al., 2010) and *P. fluorescens* (Bénézech &
391 Faille, 2018). As a result, the biofilms at ALW interfaces do not appear to be significantly more
392 resistant than those formed on fully immersed materials. Conversely, Bc-98/4 biofilms were more
393 resistant to cleaning, with only between a 1.5 to 3.5 log reduction in the number of culturable cells,
394 depending on the material, with the least cleanable materials being the two stainless steels. This
395 result is in line with a previous work performed on four bacterial species. In this study, a similar
396 classification of materials in terms of resistance was obtained i.e. stainless steel > polypropylene >
397 glass (Hyde et al., 1997). It is thus difficult to relate the cleaning resistance of different materials to

398 any of their surface parameters (topographic or hydrophobic property) i) since polypropylene and
399 glass are respectively strongly hydrophobic/hydrophilic, ii) stainless steel with a 2B finish is
400 significantly rougher than the three other materials. Elsewhere, it is interesting to note that
401 discrepancies exist between the results obtained by counting and by direct observation of coupons.
402 Indeed, biofilms were still visible on coupons with Bc-98/4 but also with Pf1 biofilms (yet no residual
403 biofilm when coupons were contaminated with Ec-SS2). The resistance to cleaning of Pf1 biofilms
404 formed at ALW interfaces has already been observed (Cunault et al., 2019) on Pf1 biofilms formed at
405 different locations in a pilot-scale washing tank, and further subjected to a cleaning procedure
406 involving an enzymatic cocktail. In these conditions that have probably little effect on bacterial
407 viability as compared to NaOH at 60°C, the resistance to cleaning of Pf1 biofilms formed at ALW
408 interface was higher than that of biofilms formed on the vertical wall of the tank.

409 These discrepancies could be ascribed to the presence of VBNC (which would explain why the
410 biofilms are still colored red instead of green), but it is also very likely that a large number of residual
411 cells have died as a result of treatment with NaOH at 60°C during the CIP procedure. Nevertheless, it
412 is widely admitted that bacteria can enter a VBNC state within biofilms and the VBNC state has been
413 described in at least 85 species of bacteria including 35 foodborne pathogens such as *Salmonella spp.*
414 (Highmore et al., 2018). The presence of VBNC bacteria on surfaces raises significant concern for the
415 food industry because it is now known that many of these are capable of recovering and
416 contaminating new foods, but above all, this means they are able to evade detection by routine
417 laboratory culture, and tolerate stressful environments including food pasteurization processes. In
418 the literature, strains of *E. coli* (Ayrapetyan & Oliver, 2016), *P. fluorescens* (Mascher et al., 2000) and
419 *B. cereus* (Rowan, 2004) have been shown to enter VBNC state when subjected to various stresses.
420 That being said, even if most residual cells are dead, biofilms still present on surfaces can serve as the
421 site for secondary adhesion of microorganisms, thereby facilitating the redevelopment of biofilms
422 (Ohsumi et al., 2015). This could be due to the presence of EPS, which is a rich source of nutrient and
423 possesses electron donor or acceptor properties (Flemming & Wingender, 2010).

424 One can also wonder why many Bc-98/4 cells were still able to form colonies on agar. Two
425 hypotheses have been put forward. First Bc-98/4 cells within biofilms could be highly resistant to
426 NaOH, but due to the structure of the biofilms (lack of EPS matrix), the cells appear to have little
427 protection from environmental stresses. It is also possible that Bc-98/4 produced spores within the
428 biofilms, with these spores being much more resistant to inactivation (Faille et al., 2010) and
429 detachment (Faille et al., 2014) than their vegetative cell counterparts. Indeed, *B. cereus* is able to
430 form spores within established biofilms and the high sporulation efficiency often observed may be
431 explained by the high cell density and nutrient limitation in biofilms (Huang et al., 2020). Exposure of
432 the biofilm to the air, therefore under conditions close to those encountered at the ALW interfaces,

433 would also promote spore production (Hayrapetyan et al., 2016). Here, on observations by
434 microscopy of the Bc-98/4 biofilms after cleaning, one can easily observe the presence of spores
435 more or less easily identifiable by their oblong form. This result is in line with our previous study on
436 fully immersed biofilms (Faille et al., 2014) in which up to 90% spores were obtained on stainless
437 steel after a 48 h-incubation.

438 We finally investigated whether the shape of the meniscus at the wall could be a determining
439 factor in the formation of biofilms at the ALW interfaces, as previously suggested (Kuśmierska &
440 Spiers, 2016). Due to the marked differences in the hydrophilic/hydrophobic character of the
441 different materials used in this study, convex to highly concave menisci were observed as soon as the
442 coupon is partially immersed in the liquid. Surface wetting along with meniscus formation and shape
443 depend on both surface energy and topography of the wall, as recently studied when working with
444 nanostructured surfaces (Kim et al., 2020). This wetting zone allowing bacteria to grow under highly
445 aerobic conditions as previously stated would be likely to induce some easing in cell transfer,
446 enhanced by the evaporation along the curved liquid vapor interface at the wetting meniscus. Such
447 strongly multiscale phenomena were thus recently modeled (Bellur et al., 2020). We also
448 demonstrated that the shape of the meniscus changed over time, except on stainless steel with a 2B
449 finish, possibly because of its greater roughness. The meniscus changed from convex to slightly
450 concave on PP, while its height remained the same or decreased on the hydrophilic materials. A
451 similar phenomenon, which has been called "wall climbing", has already been observed on *B. subtilis*
452 pellicles (Angelini et al., 2009). The wall climbing of the bacterial biofilm was observed after a 12-h
453 delay, at a speed of 1.39 mm h^{-1} . In this work, it is interesting to note that the small meniscus on PP
454 was accompanied first by a narrow biofilm for the three strains, but also by a 3-D structure on Bc-
455 98/4 and Ec-SS2 biofilms. It is also highly likely that the biofilm formed by the different strains would
456 mask the properties of the materials, in turn inducing changes in the meniscus shape. This could
457 explain the complex structures of Pf1 and Ec-SS2 biofilms, including the presence of several very
458 dense areas, especially in Pf1 biofilms. In order to investigate the relative role of physical vs.
459 biological phenomena at ALW interfaces, additional studies will therefore be carried out using inert
460 microspheres or bacterial spores to observe the meniscus kinetics on the different materials and the
461 shape/structure of the final deposit.

462 In conclusion, biofilm formation at ALW interfaces is clearly affected not only by the strain
463 (intrinsic ability to form biofilms, preference for this specific environment), but also by the properties
464 of the material. It is likely that the shape and size of the meniscus formed at the wall plays a major
465 role in dictating the structure and density of the biofilm formed. It is also remarkable that some
466 biofilms formed at the ALW interfaces are highly resistant to detachment during a cleaning
467 procedure, even though the bacteria sometimes lose their ability to form colonies on agar. Thus,

468 these biofilms probably represent a major problem in terms of surface hygiene and great vigilance
469 will be required to limit their installation, especially in food industries.

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Figure 1. Number of cultivable cells within the ALW biofilms formed by Bc-98/4, Ec-SS2 and Pf1 on the four materials (from left to right: Stainless steels 2R and 2B, PP, and Glass). Following Tukey's groupings performed for each strain, conditions with no common letter are significantly different.

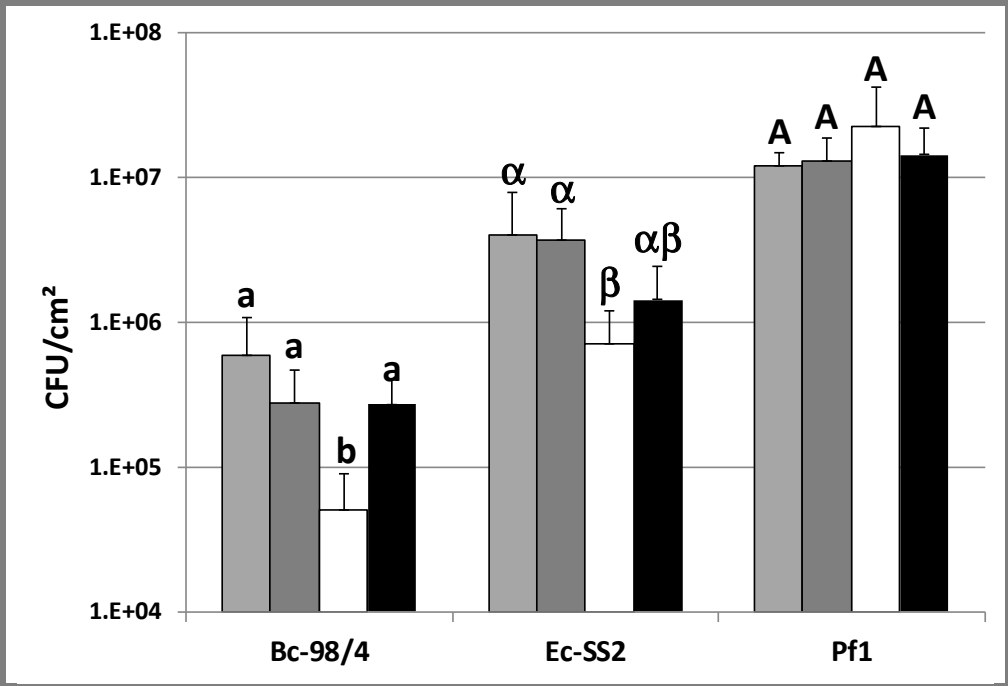


Figure 2. Biofilms at ALW interfaces after staining with orange acridine (views of 1x2 cm surfaces). The black arrows indicate the top of biofilms at ALW interfaces.

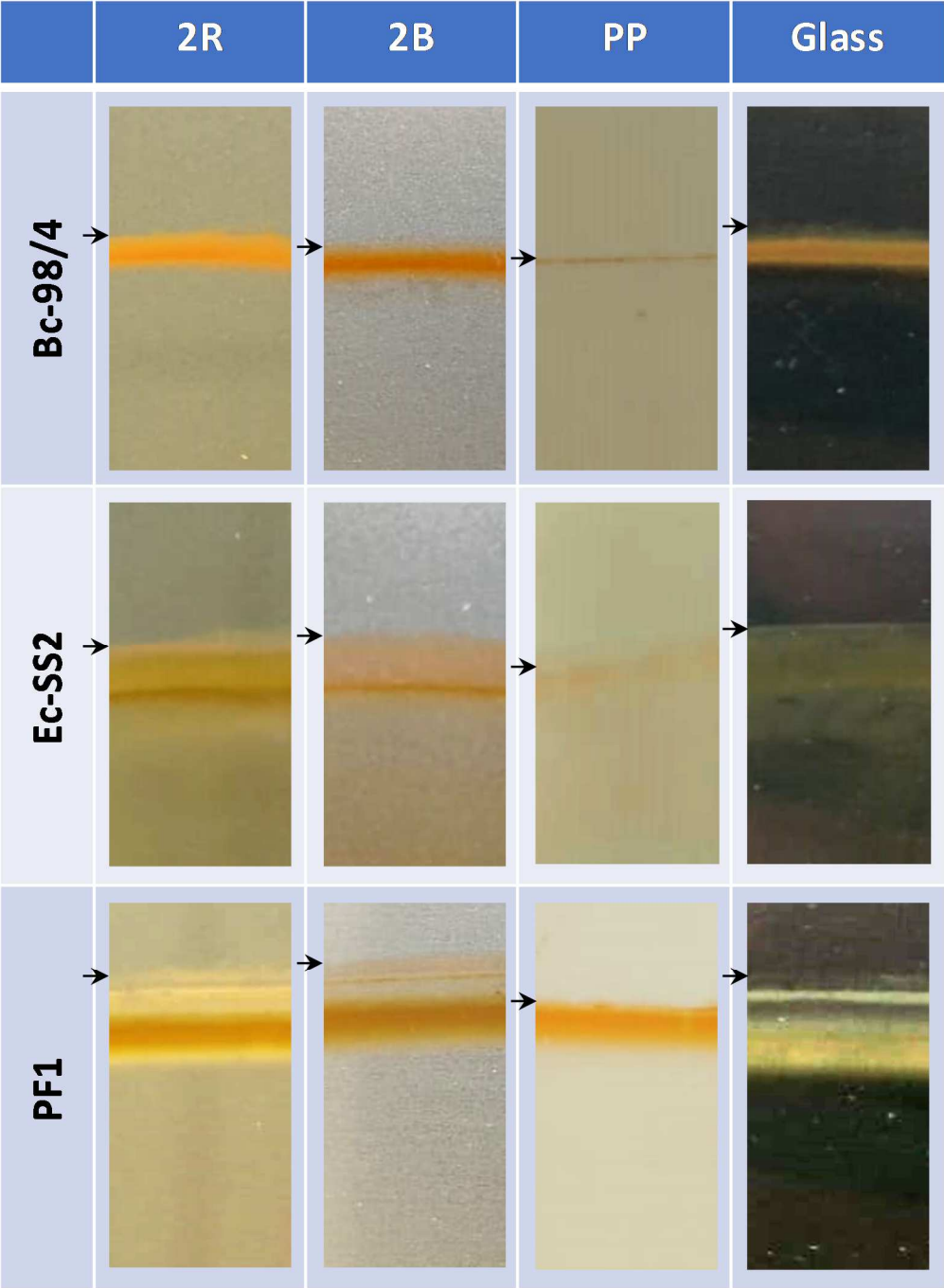


Figure 3. Microscopic images of biofilms at ALW interface of Bc-98/4, Ec-SS2 and Pf1 on the four materials. Observation by epifluorescence microscopy after staining with orange acridine. The black arrows indicate the top of biofilms at ALW interfaces. Scale bar = 500 μ m.

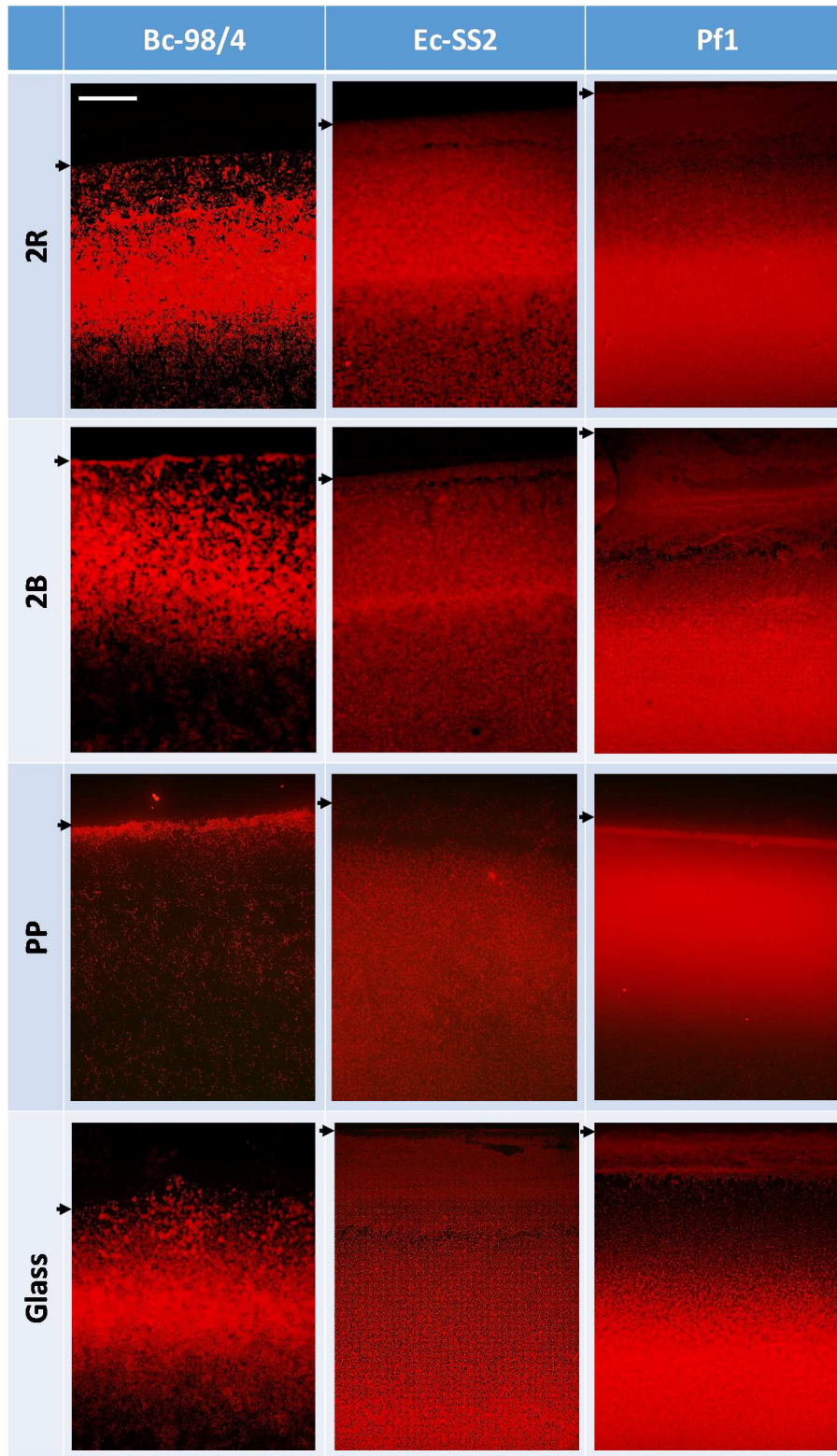


Figure 4. Confocal laser scanning microscopic images of Bc-98/4, Ec-SS2 and Pf1 biofilms formed on the different materials. For all the images, the central picture [A] represents the horizontal projection (xy) and the flanking pictures represent cross-sections taken along transects indicated by green [B] and red [C] lines in the two-dimensional image. Scale bar = 50 μ m.

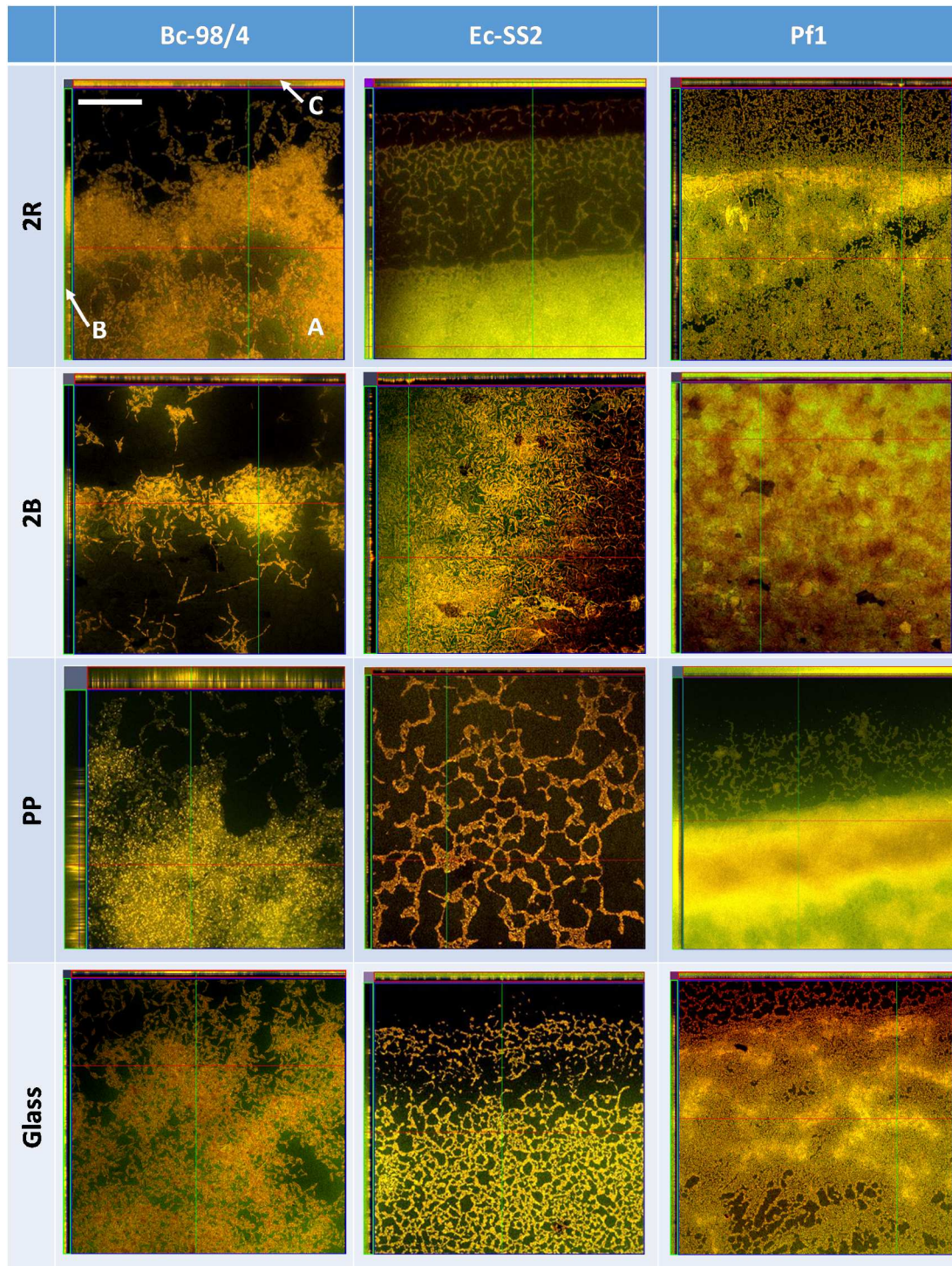


Figure 5. Reduction of the number of log CFU induced by the CIP procedure on Bc-98/4 biofilms. Following Tukey's grouping, conditions with no common letter are significantly different.

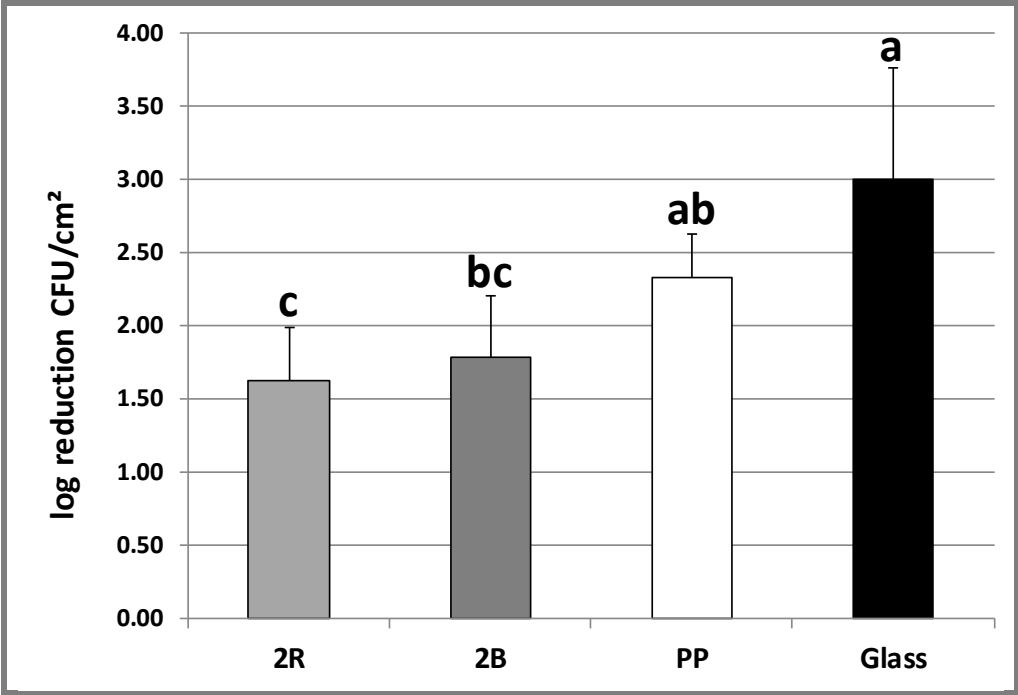


Figure 6. Microscopic images of biofilms at ALW interface of Bc-98/4, Ec-SS2 and Pf1 on the four materials after cleaning in place. Observation by epifluorescence microscopy after staining with orange acridine. Scale bar = 500 μ m.

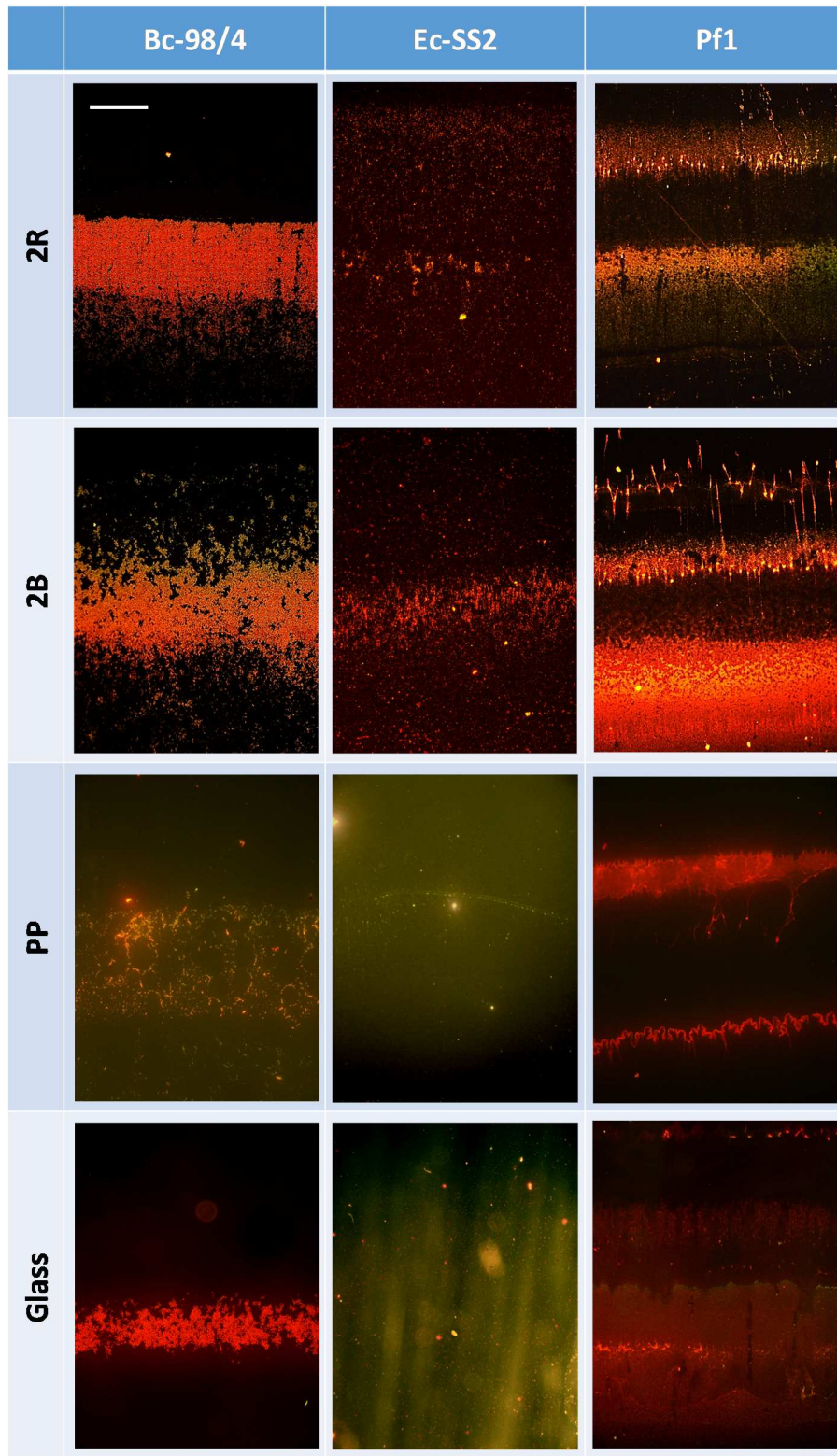


Figure 7. Organization of a Ec-SS2 biofilm on stainless steel 2R, at the ALW interface and microscopic observations after staining with orange acridine

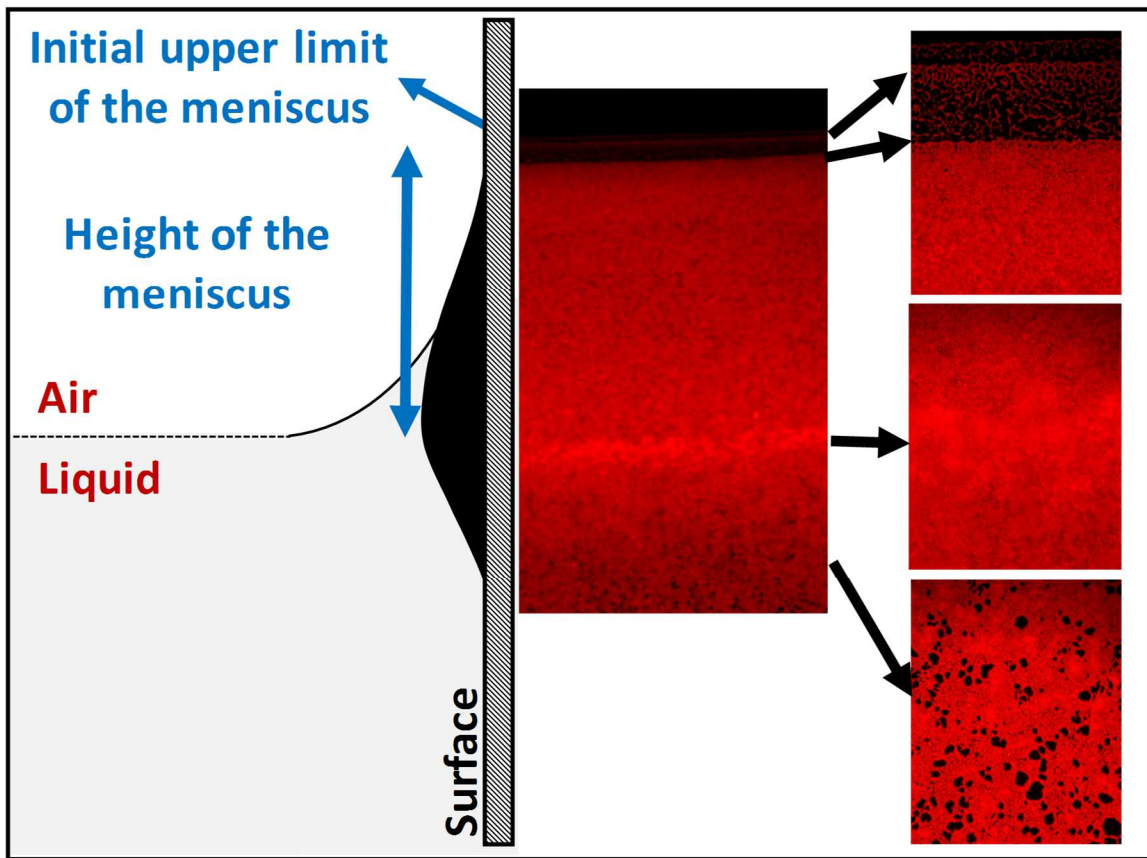


Table 1. Material properties and kinetics of the meniscus size on the four materials

	2R	2B	Glass	Polypropylene
<u>MATERIAL PROPERTIES</u> (from Richard et al., under review [Food Microbiol])				
Average roughness (μm)	0.05	0.23	0.00	0.02
Water contact angles	46.8	61.6	24.1	101.9
<u>MENISCUS HEIGHT</u>				
T0	1.7	2.2	2.4	-0.6
1 h	1.7	2.2	2.1	0.4
3 h	1.7	2.2	2.1	0.5
24 h	1.4	2.1	1.8	0.8