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Highlight

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

Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

2.1. Bacterial strains and solid surfaces

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

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

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

2.4. Biofilms resistance to cleaning

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

2.5. Statistical analysis

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

3. Results

3.1. Enumeration of the biofilms formed at ALW interface

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

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

3.2. Structure of the biofilms formed at ALW interface

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

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

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

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

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

3.3. Ease of removal of biofilms during a cleaning procedure

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

As it is highly likely that the cleaning procedure would affect bacterial viability, the coupons were 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

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

4. Discussion

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

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

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

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

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

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

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

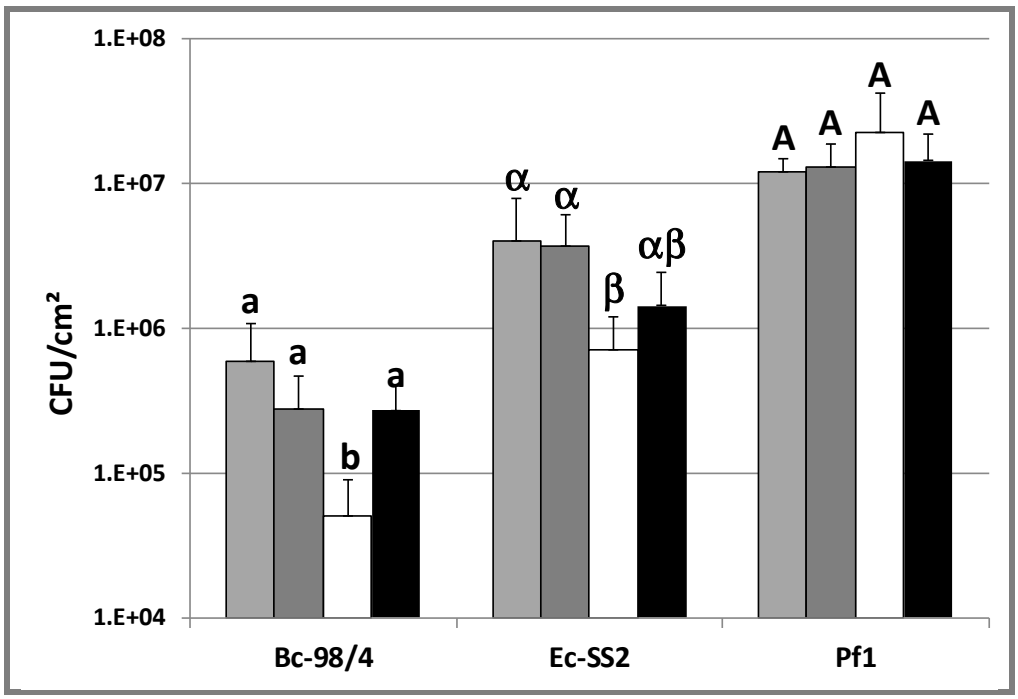

Figure 2. Biofilms at ALW interfaces after staining with orange acridine (views of 1x2 cm surfaces). The black arrows indicate the top of biofilms at ALW interfaces.

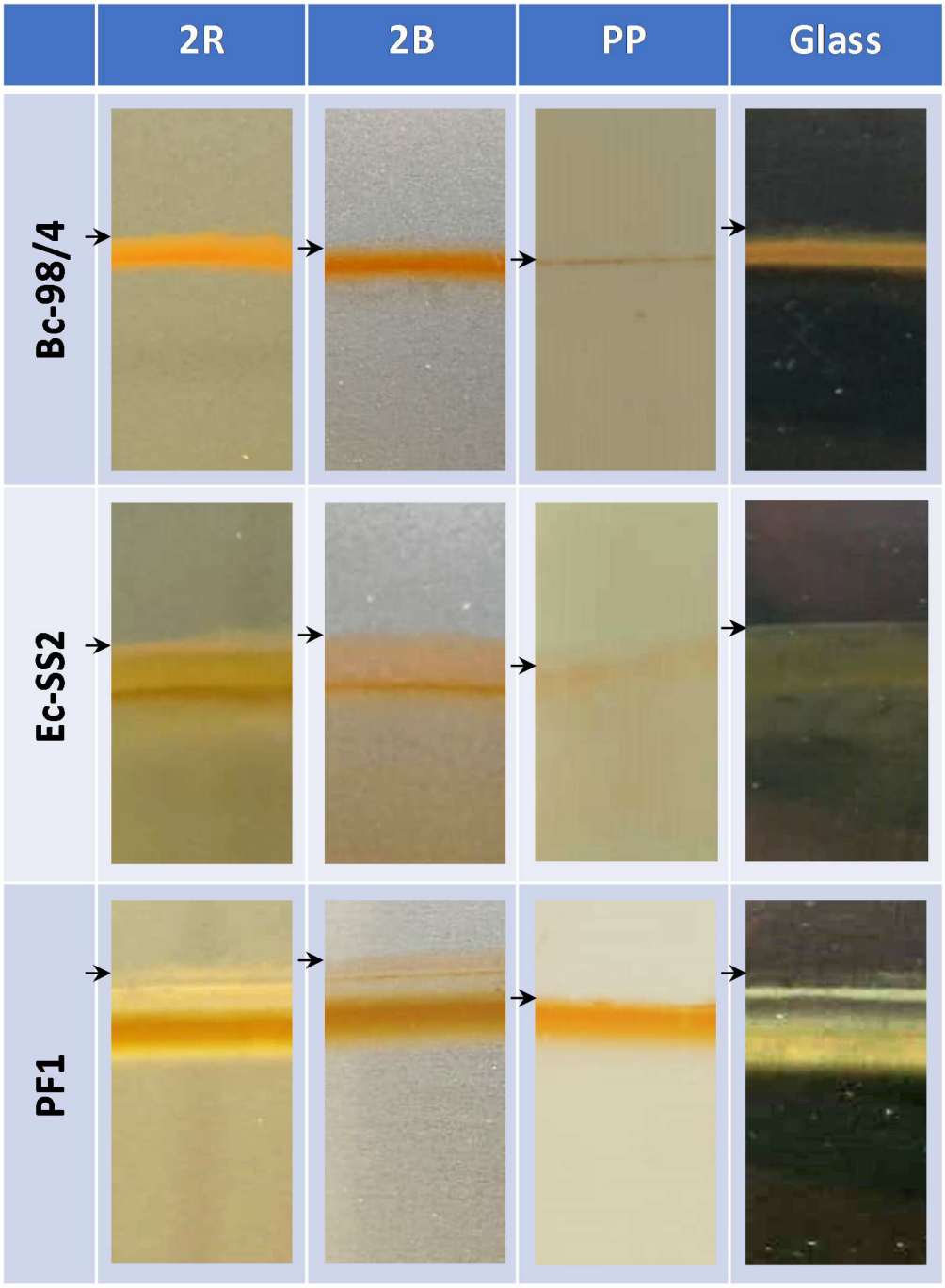

Figure 3. Microscopic images of biofilms at ALW interface of Bc-98/4, Ec-SS2 and Pf1 on the four materials. Observation by epifluorescence microscopy after staining with orange acridine. The black arrows indicate the top of biofilms at ALW interfaces. Scale bar = 500 µm.

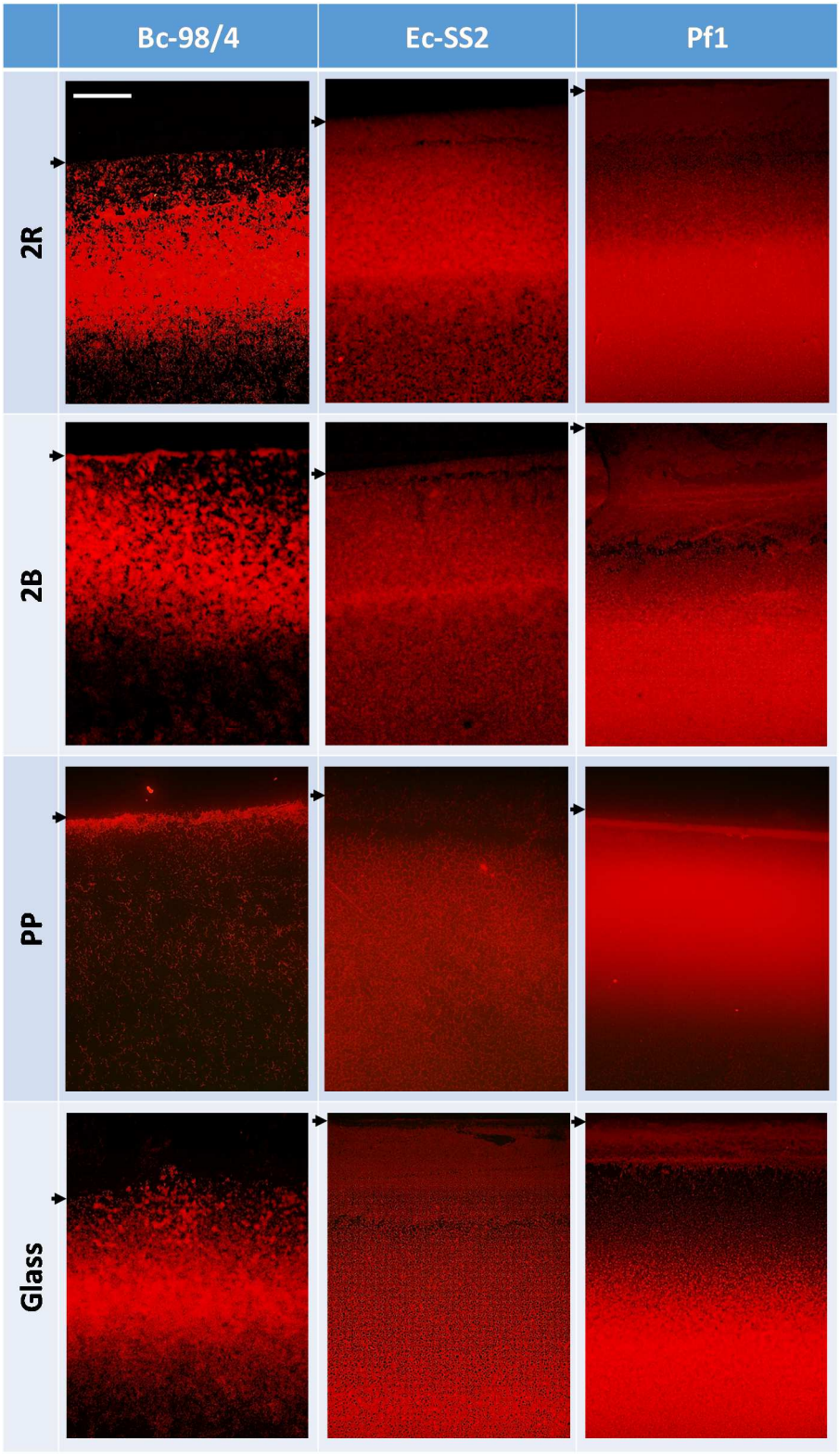

Figure 4. Confocal laser scanning microscopic images of Bc-98/4, Ec-SS2 and Pf1 biofilms formed on the different materials. For all the images, the central picture [A] represents the horizontal projection (xy) and the flanking pictures represent cross-sections taken along transects indicated by green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

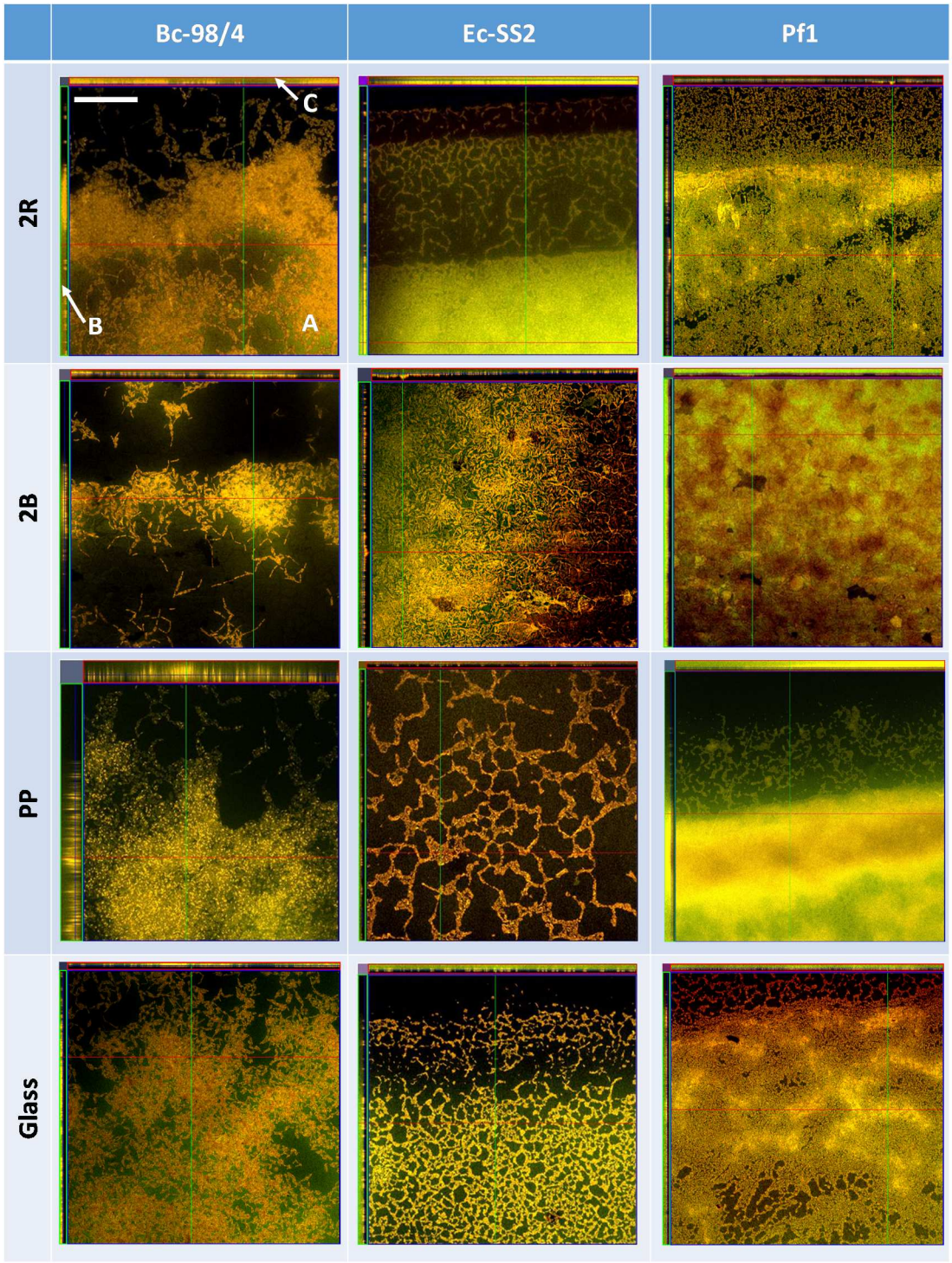

Figure 5. Reduction of the number of log CFU induced by the CIP procedure on Bc-98/4 biofilms. Following Tukey's grouping, conditions with no common letter are significantly different.

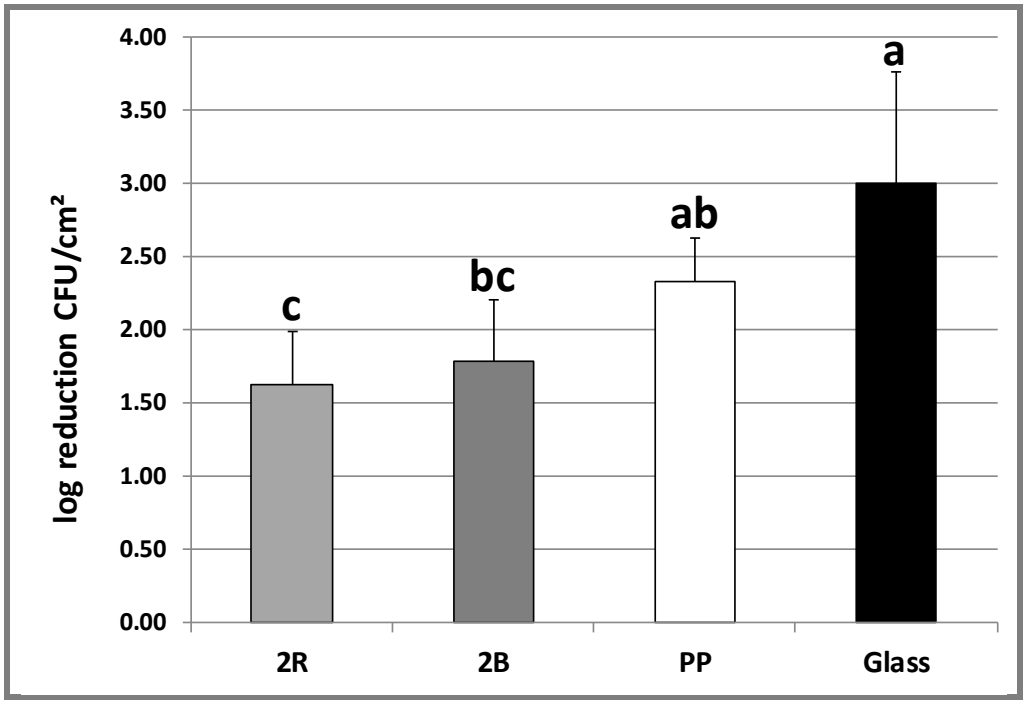

Figure 6. Microscopic images of biofilms at ALW interface of Bc-98/4, Ec-SS2 and Pf1 on the four materials after cleaning in place. Observation by epifluorescence microscopy after staining with orange acridine. Scale bar = 500 µm.

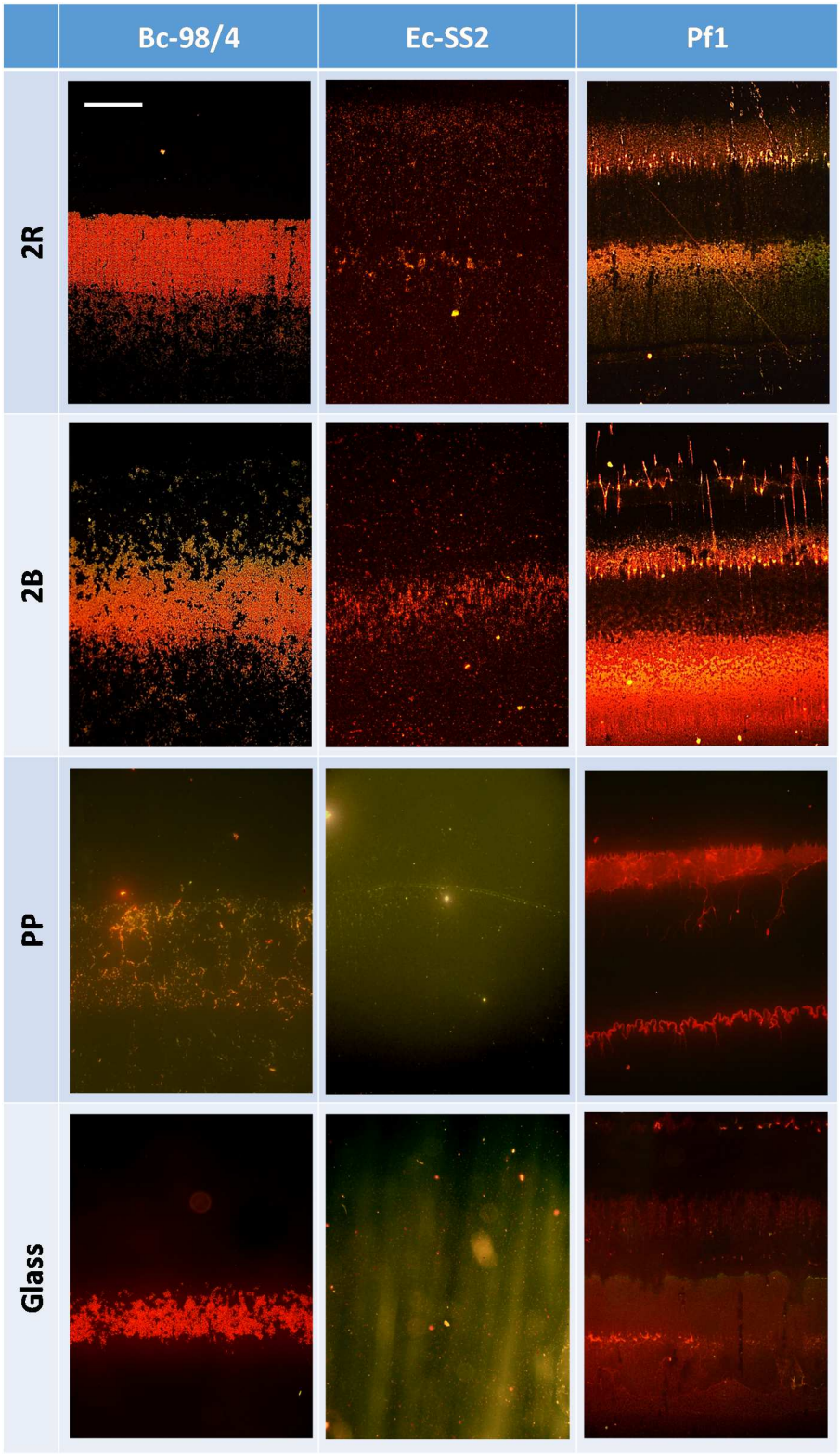

Figure 7. Organization of a Ec-SS2 biofilm no stainless steel 2R, at the ALW interface and microscopic observations after staining with orange acridine

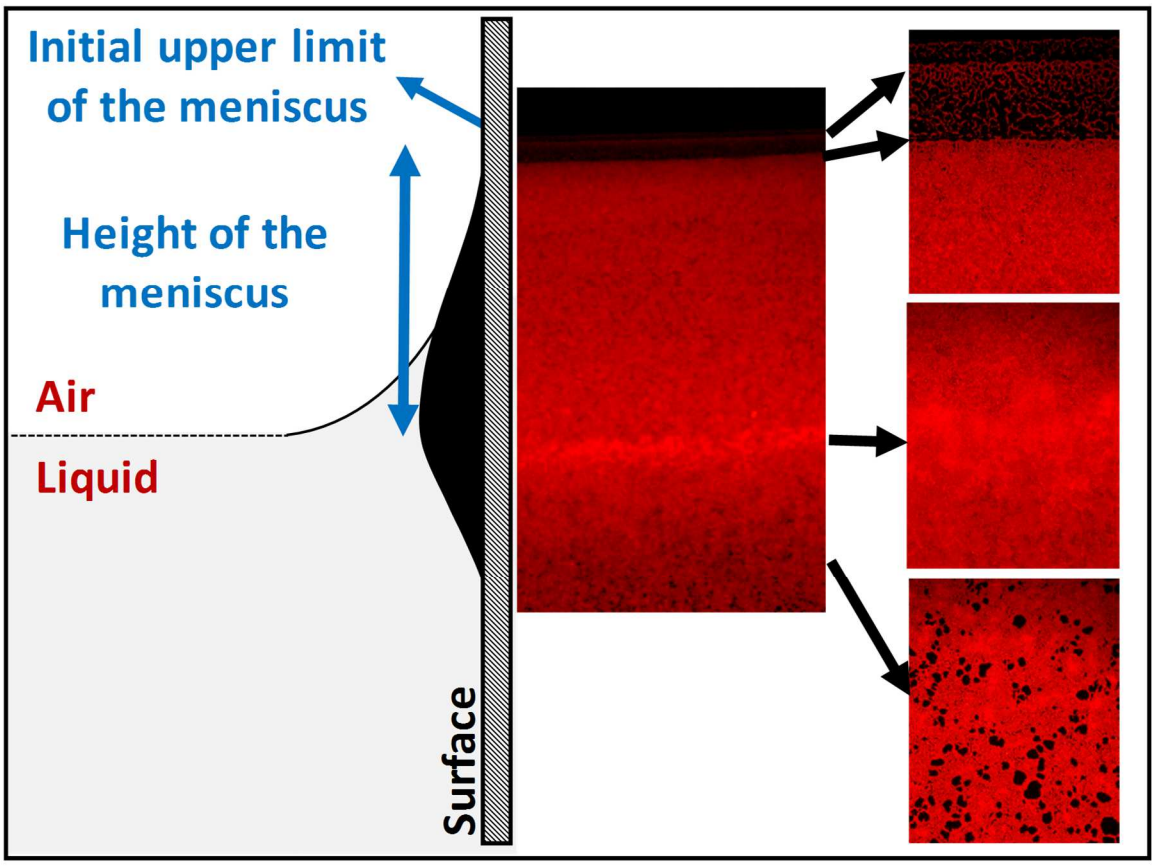

Table 1. Material properties and kinetics of the meniscus size on the four materials

	2R	2B	Glass	Polypropylene
MATERIAL PROPERTIES (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
MENISCUS HEIGHT				
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