DEVELOPMENT AND ANALYSIS OF ANAEROBIC BIOFILMS ONTO HYDROPHOBIC AND HYDROPHILIC SURFACES

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ABSTRACT

Fluorescent in situ hybridization (FISH) with domain and group specific probes that target intracellular 16S rRNA were used to investigate microbial composition of anaerobic biofilms developed on polypropylene (hydrophobic) and glass (hydrophilic) surfaces fitted inside a Modified Robbins Device (MRD). Crushed anaerobic granular sludge was used as inoculum for biofilm development in the MRD. The inoculum and biofilms formed showed nearly the same microbial composition, both were dominated by hydrogenotrophic methanogenic Archaea related to the Methanobacteriaceae as detected by the specific probe (MB1174). This group accounted for 44 to 90% of the DAPI-stained cells. Cells which hybridized to the Bacteria specific probe (EUB338) accounted for 3-18% of the DAPI-stained cells. After the first day of the biofilm formation experiment, a larger number of cells, 4.6 x 10^5 cells mm^-2, could be seen colonizing the polypropylene coupon compared to the glass, 8.2 x 10^5 cells mm^-2. However, after 9 days these numbers were very similar, i.e. 6.3 x 10^5 cells mm^-2 and 7.2 x 10^5 cells mm^-2, for the glass and polypropylene coupons, respectively. Our data suggest that the hydrophobicity of the support material did not influence the initial development and the microbial composition of anaerobic biofilms developed in the MRD.

Keywords: Anaerobic biofilms, hydrophobic and hydrophilic surfaces, “in situ” hybridization, modified robbins device, oligonucleotide probe

INTRODUCTION

Microbial biofilms occur in virtually any natural or engineered system. Hence, scientists working in different fields (biofouling, biocorrosion, bioconversion, medicine, limnology, among others) have been studying biofilm growth [1]. In environmental biotechnology, biofilm formation inside reactors is very important to uncouple solid and liquid retention times enabling treatment of more waste in a smaller volume and a shorter time. The performance of the reactors has been shown to be partly dependent on the support material [2, 3]. When the substrate to be treated is diluted or contains low concentration of suspended solids (e.g. pharmaceutical, chemical, pulp and paper wastewaters), the support material has always a large influence on the start-up and on the efficiency of the process, because the first bacterial layer may be of major importance for further biofilm growth and stability [4,5]. Some factors such as bacterial cell wall hydrophobicity, electrophoretic mobility, electrostatic surface charges, among others, which could have an effect on initial adhesion of bacteria to supports have been investigated [6,7,8]. The influence of the hydrophobicity of the support material on adhesion of pure cultures of methanogenic Archaea and initial development of anaerobic biofilms has been shown [4,5]. These authors showed that in continuous culture, the colonization of hydrophobic surfaces such as polypropylene and polyethylene was faster than more hydrophilic ones such as PVC and Polyacetal.

Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has become an appropriate tool to detect and study microorganisms in their natural habitats [9]. The use of 16S and 23S rRNA sequences has been especially valuable since ribosomes may be present in high numbers and sequence divergences reflect phylogenetic relationships. FISH has been previously used to detect specific groups of Bacteria and Archaea in order to characterize microbial populations in anaerobic biofilms [10, 11] and granular sludges [12, 13].

This paper aims to develop and compare anaerobic biofilm formation and the number of attached cells on...
hydrophilic (glass) and hydrophobic (polypropylene) surfaces, through the use of cells from a granular sludge. The question to be answered is “Does surface hydrophobicity have any influence on adhesion of anaerobic cells and initial biofilm formation ?”. The non-destructive sampling of biofilm material was achieved by removable polypropylene and glass coupons inserted into the wall of the Modified Robbins Device (MRD) [14]. The development and structure of the anaerobic biofilms, in terms of number of attached cells over time, were examined by microscopic techniques, through the use of phase-contrast, epifluorescence and confocal laser scanning microscope (CLSM). FISH was used to quantify the microbial community of anaerobic biofilms formed on both surfaces. Furthermore, we provided the first example of the MRD use to develop anaerobic biofilms.

MATERIALS AND METHODS

Inoculum Preparation for Biofilm Development

A mesophilic granular sludge sampled from an anaerobic sequential batch reactor was used as inoculum. Granular sludge (150 ml) was crushed before cultivation and added to a serum bottle (2 l) containing 850 ml of sterile culture medium as described elsewhere [15, 16]. The medium was supplemented with 2.7 g l⁻¹ sodium acetate, 1.10 g l⁻¹ sodium butyrate, 0.68 g l⁻¹ sodium formate, 0.96 g l⁻¹ sodium propionate, 0.46 g l⁻¹ ethanol and 0.32 g l⁻¹ methanol as carbon sources. The inoculum was pregrown at 33°C in a sealed bottle, gassed with an O₂-free N₂ -CO₂ mixture (70/30, vol/vol) for 143 days without shaking. These cells were fed once a week by adding the carbon sources (same concentrations mentioned before) by using sterile syringes. Cell growth and activity were checked through the analysis of the gas phase for methane production. On the day before the beginning of the experiment, the inoculum (720.0 ml) was transferred, under a N₂ -CO₂ atmosphere (70/30, vol/vol), to a sterile culture vessel (1 liter).

Surfaces

Two surfaces were tested: polypropylene and glass (glass microscope slides). The first is a hydrophobic surface with the water contact angle ranging from 93 ± 2° [4,5] to 100.3° [17]. The latter is a hydrophilic surface with a water contact angle of 12 ± 2° [18]. They were cut to obtain round coupons of polypropylene and glass with diameters of 8 and 9 mm, respectively. Coupons were cleaned with ethanol (96%) and rinsed with Millipore-Q water.

Biofilm Reactor and Biofilm Generation

Biofilms were grown on the coupons inserted into the wall of a modified Robbins Device [14, 19]. The MRD had 120 ml volume, 80 cm length and 5.2 cm width. It was made of acrylate and was connected in a closed loop to a culture vessel containing the inoculum (Figure 1). Prior to the start of the experiment, the MRD with the coupons was sterilized.
The MRD was seeded with $3.3 \times 10^{11}$ cells in a total volume (MRD+culture vessel) of 720 ml. Media was pumped through the MRD at 9.6 ml min$^{-1}$, for a period of 22 days. The culture vessel was stirred continuously, gassed periodically with N$_2$ and CO$_2$ (70:30) to maintain anoxic conditions, and carbon sources (2.7 g l$^{-1}$ sodium acetate, 1.10 g l$^{-1}$ sodium butyrate, 0.68 g l$^{-1}$ sodium formate, 0.96 g l$^{-1}$ sodium propionate, 0.46 g l$^{-1}$ ethanol and 0.32 g l$^{-1}$ methanol) were added as sterile syringes every 4 days. Both MRD and culture vessel were maintained at 33 °C. Glass coupons were removed from the MRD and analysed on the 1st, 5th, 6th and 9th days, while polypropylene coupons were taken on the 1st, 5th, 9th, 14th, 15th and 22nd days. At each sampling day, two coupons were removed from the MRD and analysed; one biofilm was left intact and the other was removed from the coupon surface. Planktonic cells from the culture vessel were sampled at time zero and on the 1st, 9th, 15th, 20th and 22nd days.

Oligonucleotides

The oligonucleotide probes used for FISH are listed in Table 1. Probes were 5’ end labeled with rhodamine (Gibco Life Technologies, MD, USA), except for the MSMX860 probe, specific for Methanosaetales, which was labeled with Cy3 (Bio-Synthesis, Inc., USA). For each sample, two negative controls were used, one of these controls was performed to assess nonspecific binding with rhodamine-labelled probe NON338, and the other, leaving out of the probe, was used to monitor autofluorescence.

Fluorescent in situ Hybridization (FISH)

Biofilm coupons and planktonic cells from the culture vessel were fixed with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS; 130mM NaCl, 7mM Na$_2$HPO$_4$, 3 mM NaH$_2$PO$_4$; pH 7.2) for 12 h, at 4ºC. Coupons were washed two times by placing them into a static beaker (11229; tested with pure cultures of Desulfovibrio vulgaris, DSM 844) before the start of the experiment. The fixation protocol (with PFA 4% for 12 hours) used was sufficiently good for the permeabilization effect on the cells, because when examined under the microscope no cells were injured or lised. Aliquots of 1 µl from planktonic and dispersed biofilm samples were spotted onto glass slides and dried for 20 min at 45°C. Those biofilms which were fixed on the coupon (henceforth called “intact biofilms”), before hybridization, were dried for 50 min at 45°C in order to increase adhesion to the coupon throughout the hybridization protocol. Then, all samples were further dehydrated in 50%, 80% and 96% ethanol for three minutes each. For the intact biofilms the dehydration step was done by gently placing the coupons in a static beaker with the ethanol solution. For biofilms formed on glass and polypropylene coupons after the first day of the experiment, hybridization with EUB338 probe was done with the intact biofilms, and then hybridized cells were observed and counted directly under fluorescent microscopy. Hybridizations were performed as described previously [15, 27]. Fixation protocol, hybridization conditions and the quality of the probes were tested with pure cultures of Bacteria (Escherichia coli, ATCC 11229; Desulfovibrio vulgaris, DSM 644) and Archaea (Methanoseta concilii DSM 3671, Methanobacterium formicicum DSM 1535, Methanosarcina barkeri DSM 800) before the start of the experiment. The fixation protocol (with PFA 4% for 12 hours) used was sufficiently good for the permeabilization

<table>
<thead>
<tr>
<th>Probe name</th>
<th>OPD name*</th>
<th>Target group</th>
<th>Sequence (5’ to 3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NON338</td>
<td>S-D-bact-0338-a-S-18</td>
<td>Negative control</td>
<td>ACTCTACGCGGAGGCAGC</td>
<td>[20]</td>
</tr>
<tr>
<td>ARCA951</td>
<td>S-D-Arch-0915-a-A-20</td>
<td>Archaea domain</td>
<td>GTGCTCCCCGGCAATTCCT</td>
<td>[22]</td>
</tr>
<tr>
<td>SBS385**</td>
<td>S-Srb-0385-a-A-18</td>
<td>Gram-negative SRB</td>
<td>CGGGCTCGTGCTGAGGG</td>
<td>[23]</td>
</tr>
<tr>
<td>MC1109</td>
<td>S-F-Mcoc-1109-a-A-20</td>
<td>Methanococcaceae</td>
<td>GCAACAGGGCCACGAGGT</td>
<td>[24]</td>
</tr>
</tbody>
</table>

* OPD, oligonucleotide probe database [25]
**Several nontarget organisms share the target sequence of this probe for gram-negative sulfate-reducing bacteria [10], while some target organisms (e.g., Desulfotahdus amnigenus) have several mismatches with the probe [26].
and hybridization of the *Archaea* cells tested. For the probe NON338 (an antisense EUB338 probe), used as negative control. Hybridization was performed at 46°C for 1.5 h with the same hybridization and washing buffers as those used for ARC915 probe [21]. After hybridization, slides were stained with 10ml of a 10 mg ml⁻¹ solution of DAPI (4', 6-diamidino-2-phenylindole, Sigma), washed briefly in double distilled water, air dried, and mounted with a glycerol/PBS (20/80) solution with a pH 8.5.

Samples were examined on an Olympus BH2-RFCA fluorescent microscope using filter sets specific for DAPI or rhodamine (and Cy3). Cells were counted after image capture on a 17-inch color screen using a color digital camera set (Olympus U-PMTUC) at exposure times of 0.08 s for phase-contrast and 0.5-4 s for epifluorescence. Digital images of rhodamine and DAPI-fluorescence from the same microscopic field were taken and temporarily stored before counting. For each sample, standard errors of the mean (SE) were calculated after arcsine transformation of the data [28,29] based on enumerating ten microscopic fields per well, or from 500 to 1,000 DAPI-stained cells. Hybridization values were corrected by subtracting the values obtained from the NON338 probe.

For the intact biofilms, fluorescent microscopy enumeration of attached cells was done by direct counting after DAPI-staining, with counting of ten to twenty microscopic fields per coupon. To evaluate the number of attached cells per coupon, the mean of total cell-count in two coupons was calculated based on cell numbers found in dispersed and intact biofilm samples. The area of each coupon was 63.5 mm² and 50.24 mm², respectively for glass and polypropylene. Studies through the depth of the biofilms were performed with intact biofilms developed on polypropylene coupons. They were carried out on a BioRad MRC 1024UV confocal system coupled to a Zeiss Axiovert microscope using a 100x Planapochromatic oil immersion 1.4 NA objective (CLSM). Serial confocal images were collected by Kalman averaging 5 frames (512x512 pixels) at 0.18 – 0.36 mm steps using an aperture (pinhole) of 2.0 mm maximum. The image acquisition and processing software was lasershard (version 2.1a).

**RESULTS**

**Culture Vessel Composition**

On the day before the beginning of the experiment, the gas phase of the inoculum flask was analysed for methane (CH₄) production, and it was observed that 73% of the gas was CH₄. Therefore, methanogenic *Archaea* cells in the culture vessel were active and thus were able to be tested in the MRD. The inoculum (planktonic cells) was composed of 3% (SE= 1%, n=13) bacterial and 95% (SE= 7.9%, n=12) archaeal cells as detected with the EUB338 and ARC915 probes, respectively (Figure 2).

The number of bacterial cells detected with EUB338

![Figure 2](image-url)
probe ranged from 3% (SE=1%, n=13) in the beginning, to 17% (SE=2%, n=15) of the DAPI-stained cells on the 9th day and stabilized around this value until the end of the experiment. The archaeal cells, 44 to 90% of the DAPI-stained cells, were members of the family Methanobacteriaceae since most hybridized with MB1174. The remaining archaeal cells were members of the order Methanomicrobiales (Methanogenium relatives) detected by MG1200 probe, comprising approximately 10.4% (SE=2%, n=12) at the onset and decreasing to 3% (SE=1.1%, n=11) of the DAPI-stained cells on the 22nd day. These cells resembled Methanospirillum. Using Methanosarcinales specific probe (MSMX860) cells were not detected, although very few cells morphologically similar to Methanosaeta were observed in the culture vessel. Probably they occurred under the FISH detection limit which is 10⁴ cells ml⁻¹ [9].

The hybridization to the Methanococcaceae specific probe (MC1109) was negative for all samples and thus it is not shown in Figure 2. The percentage of cells detected with the SRB385 probe, specific for the delta subdivision sulphate reducing and mesophilic bacteria (SRB-group), did not change significantly during experimental time. SRB group accounted for 5% (SE=1.0%, n=10) at the onset, decreased to 3% (SE=1.0%, n=11) of the DAPI-stained cells on the 22nd day. The remaining bacterial cells which hybridized with EUB338 probe were rod-shaped cells with terminal spore. The percentage of nonspecific binding detected with NON338 probe was 18% (SE=1.1%) of the DAPI-stained cells for culture vessel samples after 9, 15, 20 and 22 days of experimental time. For another two samples, inoculum and the 1st day, the percentage of nonspecific binding was zero.

Biofilm Composition

Based on probe hybridization, the biofilms developed in the course of the experimental conditions were composed mainly of archaeal cells (Figures 3 and 4). The hydrogenotrophic methanogenic Archaea related to the Methanobacteriaceae prevailed in all the biofilms. This group accounted for 43% (SE=3.7%, n=15) to 99.6% (SE=2.8%, n=9) of the DAPI-stained cells and morphologically resembled Methanobacterium and Methanobrevibacter. Another hydrogenotrophic methanogens were detected with Methanomicrobiales specific probe (MG1200). These cells were morphologically similar to Methanospirillum and represented 0.3% (SE=0.2%, n=8) to 2.2% (SE=0.7%, n=10) of the DAPI-stained cells. No cells were detected with the Methanosarcinales specific probe (MSMX860), although very few cells resembling Methanosaeta were observed in biofilms developed after 5, 9 and 15 days of experimental time.

Figure 3. Microbial community composition in biofilms formed on polypropylene coupons. Percentages of DAPI-stained cells detected by FISH with probes for Archaea (ARC915), Bacteria (EUB338), Methanomicrobiales (MG1200), Methanobacteriaceae (MB1174) and SRB group (SRB385). Bars indicate standard error (n ranged from 9 to 15 microscopic fields).
Bacterial cell percentages detected by the EUB338 probe changed significantly in the biofilms during the experimental time. The biofilm formed after the first day on polypropylene coupon was composed of 19% (SE=1.4%, n=20) of bacterial cells whereas in the biofilm on glass were detected only 3.2% (SE=0.8%, n=16) of the DAPI-stained cells. These values were obtained with the intact biofilms and are not shown in Figures 3 and 4. Biofilms developed after 5 and 6 days of experimental time, respectively for polypropylene and glass surfaces, showed the greatest numbers for Bacteria, 16.0% (SE=2.0%, n=9) and 14.5% (SE=3.0%, n=9) of the DAPI-stained cells. For biofilms developed after 9 days, on both polypropylene and glass surfaces, and 15 days no bacterial cells were detected, whereas in the 22-day-old biofilms they accounted for 7.6% (SE=2.3%, n=14) of the DAPI-stained cells. The SRB cells detected with SRB385 probe were present in the biofilms in small amounts. They accounted for 4% of the DAPI-stained cells for biofilm developed after 6 days on glass surface (Figure 4) to 1% of the DAPI-stained cells for biofilm on polypropylene at the 22nd day (Figure 3). The percentage of nonspecific binding detected with NON338 probe was 18% (SE=1.1%) of the DAPI-stained cells for all biofilm samples developed on polypropylene and glass coupons.

Number of Attached Cells on Hydrophilic and Hydrophobic Surfaces

As shown in Table 2, except for samples collected on the first day, no significant differences were observed on biofilm colonization among the two surfaces tested. After the

<table>
<thead>
<tr>
<th>Time</th>
<th>Number of cells per mm² of the glass coupon</th>
<th>Number of cells per mm² of the polypropylene coupon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 day</td>
<td>8.2 x 10⁴ (SD=5.0)</td>
<td>4.6 x 10⁴</td>
</tr>
<tr>
<td>5 days</td>
<td>7.1 x 10⁴ (SD=6.0 x 10⁴)</td>
<td>9.9 x 10⁴ (SD=8.0 x 10⁴)</td>
</tr>
<tr>
<td>9 days</td>
<td>6.3 x 10⁴ (SD=4.2 x 10⁴)</td>
<td>7.2 x 10⁴ (SD=9.2 x 10⁴)</td>
</tr>
<tr>
<td>14 days</td>
<td>ND</td>
<td>1.4 x 10⁵ (SD=1.3 x 10⁵)</td>
</tr>
<tr>
<td>22 days</td>
<td>ND</td>
<td>3.0 x 10⁶ (SD=3.0 x 10⁶)</td>
</tr>
</tbody>
</table>

ND- not determined
*value of one single coupon
S.D.- standard deviation
first day, a larger number of cells (4.6 x 10^4 cells mm^-2) could be seen colonizing the polypropylene coupon compared to the glass (8.2 x 10^3 cells per mm^-2) and on the polypropylene surface cells were almost covering the entire coupon. Nevertheless, the average number of attached cells per square millimeter was almost similar after 5 days, 7.1 x 10^4 cells mm^-2 on the glass coupon and 9.9 x 10^4 cells mm^-2 on the polypropylene coupon, and after 9 days, 6.3 x 10^5 cells mm^-2 on the glass coupon and 7.2 x 10^5 cells mm^-2 of the polypropylene coupon.

Biofilm Examination

Microscopic examination revealed that biofilm development on both surfaces began as isolated patches of cells randomly distributed over the coupon. CLSM exams revealed that biofilms developed after 5, 9 and 14 days of incubation period were of uneven thickness with sites covered by a few number of cells next to the areas where the biofilm attained a thickness of 2.8 µm, 7.0 to 15.0 µm and 4.0 to 5.0 µm, respectively. Furthermore, in the 5-day-old biofilm bacterial cells, detected with probe EUB338, were present over the whole biofilm depth (data not shown). Biofilms developed on polypropylene after 22 days were the thickest, and the thickness was around 22.0 µm, but areas with 7.0 µm were also observed in the same coupon (image not shown). It was also observed that after 22 days biofilms did not cover the entire surface and were not compact empty spaces resembling pores were observed.

DISCUSSION

FISH was used to evaluate microbial community composition of the culture vessel (planktonic cells) and biofilm samples. Biofilm and culture vessel samples showed nearly the same microbial composition, both were dominated by hydrogenotrophic methanogenic *Archaea* related to the *Methanobacteriaceae* as detected by the specific probe (MB1174) (figures 2, 3 and 4). This group accounted for 44% to 90% of the DAPI-stained cells and morphologically resembled *Methanobacterium* and *Methanobrevibacter*. Cells that hybridized to the *Bacteria* specific probe (EUB338) accounted for the remaining 3 to 18% of the DAPI-stained cells. These bacterial cells which were rod-shaped with a terminal spore could be members of the genus *Clostridium*, since some methanol-utilizing homooacetogens are Clostridia and methanol was added to the system as one of the carbon sources, as mentioned in the Materials and Methods section. There are some sporeforming butyrate and propionate oxidizing bacteria which belong to the *Syntrophospora* and *Desulfotomaculum* genera, respectively (A. Stams, personal communication, October 2002). Nevertheless, since *Methanoseta* (an acetotrophic methanogen) were only present in very few numbers, this probably means that hardly any acetate was degraded in the device and it is likely that this might have interfered with the propionate degradation. Therefore, it is unlikely that *Desulfotomaculum* was present in the system. Since the MRD was dominated by hydrogenotrophic methanogens, it can be speculated that methanol, formate, ethanol and to some extent butyrate were degraded very well.

Recent reevaluations of probe EUB338, indicated that the *Planctomycetes* and *Verrucomicrobia*, as well as several green non-sulfur bacteria and members of the candidate phylum OP11 are missed by this probe [30]. In the present study, with few exceptions, the sum of the *Bacteria- and Archaea*-specific probe counts were 100% (Figures 2, 3 and 4). Therefore, it might be unlikely that members of the phyla *Verrucomicrobia* and *Planctomycetes* were present in the samples analysed in this study, since in the inoculum and in the first two days of the experiment, 100% of the DAPI stained cells were detected with probes ARC915 and EUB338.

Since previous studies [26, 31] revealed the presence of a reasonable amount of sulphate reducing bacteria (SRB) in anaerobic reactors which did not have sulphate in the influent, for this study it was decided that the probe SRB385 would be used to estimate the percentage of SRB in the inoculum and biofilms.

The flow rate applied through the MRD was 9.6 ml min^-1, which was 10 times lower than the flow rate used by other authors [32]. These authors used the MRD, with a flow rate of 100 ml min^-1 to develop heterotrophic biofilms with SRB cells on stainless steel coupons and observed the development of thick biofilms with 500 µm of thickness. Thus, it can be suggested that the flow rate used in the present study did not affect the potential of the anaerobic cells to attach and form biofilms on the coupons. Moreover, the flow rate applied to the system was far enough to cause cell washout and to avoid cell adhesion.

CLSM examinations of the anaerobic biofilms developed in this study revealed that they were of uneven thickness, heterogeneous, and had pores. These features are consistent with the water channel model described by other authors [33, 34]. Channel structures in biofilms may facilitate the transport of nutrients between the bulk solution and the deeper inner layers [35].

No significant difference was observed between hydrophobic (polypropylene) and hydrophilic (glass) surfaces in terms of biofilm microbial composition, as shown in Figures 3 and 4, respectively. The hydrogenotrophic methanogenic *Archaea* cells which predominated in the inoculum, also dominated the biofilms that developed on both surfaces. Furthermore, no significant difference between the two support materials with respect to the number of attached cells was observed. However, after 24 h of experimental time, a larger number of cells (4.6 x 10^4 cells mm^-2) could be seen colonizing the polypropylene coupon compared to the glass (8.2 x 10^3 cells mm^-2). In spite of these observations, it was not possible to presume that polypropylene is a better material, concerning anaerobic
biofilm formation, than glass. Accordingly, in the first nine days cell numbers in the biofilm formed on glass increased 77 times and in the biofilm formed on polypropylene cell numbers increased only 16 times, suggesting that after the first day glass surfaces were colonized faster than the polypropylene. In a previous study [15], which tested three pure cultures of methanogenic *Archaea*, *Methanosarcina barkeri*, *Methanosaeta concilii* and *Methanobacterium formicicum*, it was verified that all the three cultures were involved in initial biofilm formation and were colonizing both hydrophobic and hydrophilic surfaces, despite slight differences observed. In addition, in that previous study no significant differences were observed with respect to the number of attached pure culture cells on polypropylene and glass coupons. Nevertheless, more and longer experiments are needed to confirm these observations.

**REFERENCES**


**CONCLUSIONS**

No significant differences were observed between hydrophobic (polypropylene) and hydrophilic (glass) surfaces in terms of biofilm microbial composition and number of attached cells. Data suggested that the hydrophobicity of the support material did not influence the initial development and the microbial composition of anaerobic biofilms developed in the MRD.

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