

1 Title: Investigation of the Active Biofilm Communities on Polypropylene
2 Filter Media in a Fixed Biofilm Reactor for Wastewater Treatment

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4 **Running Title: Wastewater Treating Biofilms in Polypropylene Media Reactors**

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61 **Abstract**

62 **BACKGROUND:** This research is focused on the effect of temperature on the growth of active biofilms on
63 polypropylene (PP) filter media in aerobic fixed biofilm reactors (FBR) for wastewater treatment.

64 **RESULTS:** High-throughput sequencing was used to explore the composition and diversity of the microbial
65 community of 14-days old (starting phase) biofilms grown at 10, 20 and 30°C. Members of the classes
66 *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were predominant in all the biofilm samples retrieved from PP-
67 FBRs. A total of 108 genera of bacteria were identified, with some of them present in all three reactors,
68 including *Trichococcus*, *Zoogloea*, *Aeromonas*, *Acidovorax*, and *Malikias*, among others. Besides these shared
69 populations, certain genera were abundantly found in individual biofilm samples, like *Brevundimonas* (17.1%),
70 *Chitinimonas* (10.3%) and *Roseateles* (39.3%), at 10, 20, and 30°C, respectively. The metabolic capabilities of
71 active microbial communities in PP-FBRs were estimated by assessing the changes in different variables (BOD,
72 DO, and pH) in the influent and effluent during operation. A noteworthy BOD removal (66.6%) was shown by
73 PP-FBRs operating at 30°C, as compared to 20°C (28.3%) and 10°C (28.8%), consistent with the DO levels
74 recorded in the effluents, highest at 30°C (70.5%), and decreasing with the declining temperatures. Substantial
75 wastewater treatment efficiencies were observed in the reactors at 30°C, attributable to the higher relative and
76 diversity of microbial biofilms.

77 **CONCLUSIONS:** The development of physiologically active biofilms in PP at all prevailing temperatures
78 strongly suggests that the material is suitable to be employed in FBRs for wastewater treatment at different
79 operational temperatures.

80 **Key Words:** Biofilm technologies; Microbial community composition; Municipal wastewater; Polypropylene
81 filter media; Fixed biofilm reactor

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87 **1. INTRODUCTION**

88 The challenges associated with wastewater treatment, such as rising energy costs, increasingly stringent effluent
89 requirements, quality controls, and limited land for treatment plants, have led to the development of innovative
90 and efficient technologies with high capacity. Biological methods play a crucial role in wastewater treatment .¹
91 Biofilm-based technologies for the treatment of municipal and industrial wastewater were developed to
92 overcome several disadvantages faced by conventional activated sludge systems and often produce higher
93 effluent quality.² The performance of biological wastewater treatment processes are determined by the activity
94 of microorganisms.^{3,4} Therefore, it is essential to gain a detailed insight into the structure and function of the
95 microbial community to explore its relation with the system performance, and to assist in the design of tailored
96 systems for the treatment of municipal wastewater. Culture-based and culture-independent methods were the
97 first technologies for analyses of bacterial communities in the water treatment process, but often provide
98 unrepresentative results. Molecular techniques like clone library, microarray, fluorescent in situ hybridization,
99 and real-time polymerase chain reaction based on 16S rRNA gene analysis have expanded and improved our
100 understanding of microbial communities in wastewater treatment.^{4,5}

101 However, high-throughput next generation sequencing (NGS) methods provide a more powerful tool
102 for high taxonomic resolution of complex microbial communities.^{6,7} Recently, NGS technology has been
103 applied for the metagenomic characterization of microbial communities in domestic wastewater treatment
104 processes,⁸ activated sludge in different WWTPs as well as in full-scale bioreactors.⁵ This technology has been
105 effectively used to disclose the relations between the microbial community and pollutant removal in various
106 wastewater treatment processes.^{5,9}

107 The present study aims to investigate the taxonomic structure of metabolically active biofilms grown on
108 polypropylene (PP) media and find a correlation with the efficiency of the aerobic treatment of wastewater in
109 fixed biofilm reactors (FBRs) at different temperatures. The PP media have been used in the FBRs in this
110 research because of its availability, cost-effectiveness and durability.³ This research uses a novel approach of
111 utilizing NGS to systematically characterizing and assessing the microbial communities in the biofilms grown
112 on the PP media under varying temperature conditions in an engineered bioreactor system for wastewater
113 treatment. To the best of our knowledge, this research study is the first application of NGS for characterization
114 of biofilm samples on PP media, used in an FBR system for wastewater treatment. Such information is
115 significant for the better operation, transformed engineering design, and management of the FBRs for

116 wastewater treatment in areas with large seasonal temperature variation, especially in many developing
117 countries.

118 2. MATERIALS AND METHODS

119 2.1 Evaluation of support media

120 Discarded polypropylene ping pong balls, with a surface area ($4\pi r^2$) of 50.24 cm², were selected as biofilm
121 supporting media in an aerobic fixed biofilm reactor (FBR) for treatment of municipal wastewater. X-ray
122 Photoelectron Spectroscopy (XPS) analysis was performed using a Theta Probe Spectrometer (Thermo Fisher
123 Scientific, East Grinstead, UK) for elemental quantification of the surfaces. The XPS spectra were acquired
124 using a mono-chromated Al K α X-ray source ($h\nu = 1486.6$ eV), and analyzed using Avantage software (Thermo
125 Fisher Scientific, East Grinstead, UK).

126 2.2 Experimental setup and operation

127 The biofilm was allowed to develop on sterilized polypropylene (PP) balls using municipal wastewater as a seed
128 (300 mL) in small reactors (500 mL) under aerobic conditions, using a continuous airflow rate of 4 L/min (**Fig.**
129 **1**). All experiments were conducted in continuous mode in triplicate, with the addition of freshly collected
130 municipal wastewater (300 ml) to each experimental setup thrice, with hydraulic loading rate (HRT) of 4.6 days,
131 organic loading rate (OLR) of 81.2 gBOD m³.d, and influent flow rate of 2.7 mL/h, for 14 days, in order to
132 ensure the growth of a metabolically active biofilm at three different temperatures (10, 20 and 30°C).¹⁰ Finally,
133 effluent samples were collected from all the reactors. Various physico-chemical parameters of the influent and
134 effluent samples were analyzed during the experiment to check the physiological activity of the developing
135 biofilms. pH was determined using a pH-meter (D-25 Horiba Water Quality Meter, Horiba Ltd, Japan);
136 Dissolved oxygen (DO) levels were measured using a MM-60R Multi – Function Water Quality Meter, TOA-
137 DKK, Japan) and biochemical oxygen demand (BOD₅) was evaluated by a 5-day BOD test according to 5210-B
138 Standard Methods.¹¹ Characteristics of municipal wastewater are available in the supplementary data in **Table**
139 **S1**.

140 2.3 Analysis of biofilm microbial communities

141 Biofilms were removed by scraping the PP media surfaces and then washed with phosphate buffer (PBS). Then
142 these biofilms were resuspended in the PBS, vortexed and centrifuged at 10,000 ×g for 5 min. Cell pellets were
143 resuspended in 100 µL of sterile DNase and RNase free water (Promochem LGC) for DNA extraction using a
144 Fast DNA SPIN Kit for Soil (MP Biomedicals).¹² The quantity and purity of the extracted DNA were assessed
145 with a NanoDrop ND-1000 spectrophotometer (NanoDrop). For the amplification of bacterial 16S rRNA gene
146 fragments, the PCR primers GAGTTTGATCNTGGCTCAG (forward) and GTNTTACNGCGGCKGCTG
147 (reverse) were used. Different barcodes (**Table S2**) were incorporated between the 454 adapter and the forward
148 primers to sort each biofilm sample from the mixed pyrosequencing outcomes. Each 50-µl reaction mixture
149 included 1X EF-Taq buffer (Solgent, Daejeon, South Korea), 2.5 units of EF-Taq polymerase (Solgent), 0.2
150 mMdNTP mix, 0.1 µM of each primer and 100 ng of template DNA. The temperature profile used was as
151 follows: 95°C for 10 min; 35 cycles at 94°C for 45 sec, 55°C for 1 min and 72°C for 1 min, with a final
152 extension at 72°C for 10 min. The duplicate PCR products were pooled and purified using the QIA quick gel
153 extraction kit (Qiagen, Hilden, Germany), and the purified products were used for pyrosequencing.

154 **2.4 Post-run analysis of nucleotide sequences.**

155 All partial 16S rRNA gene sequences were preprocessed initially using the Pyro-pipeline at the Ribosomal
156 Database Project (RDP) to sort by barcode and remove primers and barcodes from the partial ribotags, and
157 discard low quality and short (< 250-bp long) sequences. These sequences were denoised and assembled into
158 clusters using the precluster command to generate the fasta files datasets. These sequences were further
159 analyzed through Mothur. The processed-sequences were clustered into Operational Taxonomic Units (OTUs)
160 based on 0.97 sequence similarity with the Uclust algorithm. Representative OTUs were selected based on the
161 most abundant sequences and taxonomic assignment was conducted using the RDP classifier. The software
162 STAMP was used to calculate the *P*-values (ANOVA) for multiple groups/samples within the datasets. FastTree
163 was used to create phylogenetic trees for UniFrac distance matrix construction in Mothur. Bacterial community
164 richness and diversity indices (observed OTUs, Chao1 estimator and ACE) and rarefaction curves were
165 estimated at a cut-off set at 0.97. For determination of beta-diversity (OTU based analysis) and Clustering (e.g.
166 Heat maps), samples were rarefacted to reduce sequence heterogeneity. For the evaluation of the similarity in
167 bacterial community composition among all three samples, the relative sequence at class and genus level for
168 each sample was used to calculate pairwise similarities. All data were transformed by square root calculations
169 and Bray Curtis similarity matrixes were generated using the software Primer v6 (PRIMER-E, Plymouth, UK).

170 Pyrosequencing data were deposited to the European Nucleotide Archive (ENA) under secondary study
171 accession number of ERP004725. To investigate the relationships between water chemical variables (BOD, pH
172 and DO) and relative sequence at genera level within biofilm samples, Pearson's correlation coefficients (r)
173 were calculated using PASW[®] Statistics 18.SPSS.

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175 3. RESULTS

176 3.1 Characterization of support media by XPS

177 The intensity of photoelectrons as a function of binding energy is shown in **Fig. 2**. Different peaks
178 corresponding to various elements were observed in the XPS survey of the surface of PP medium. It was found
179 that PP medium contains C 1s (53.04%), Ca 2p (0.98%), N 1s (3.05%), O 1s (39.96%), S 2p (1.64%), Si 2p
180 (0.14%) and Zn 2p_{3/2} (1.22%).

181 3.2 Sequencing and metagenomic assembly

182 As shown in **Table S3**, a total of 2205 16S rRNA gene sequences were obtained, corresponding to 1016, 2050
183 and 1139 sequences reads at 10, 20, and 30°C respectively. After quality analysis, filtering and trimming, 610
184 sequences were annotated, corresponding to 163 high quality V4-V6 tags of the 16S rRNA-genes in library PP
185 10°C; 224 in library PP 20°C; and 223 in PP 30°C. The numbers of OTUs, Chao 1, and ACE at a cutoff level of
186 3%, are shown in **Table S3**. The number of OTUs ranged from 163 (PP 10°C) to 224 (PP 20°C) and the patterns
187 of Chao 1 and ACE values were very similar to the OTU numbers. The alpha diversity indices ranged from
188 8.6720 (PP 30°C) to 33.4449 (PP 10°C) and Good's coverage values varied from 0.898156 (PP 30°C) to
189 0.94878 (PP 20°C). Additionally, for a comparison of species' richness among the three samples, rarefaction
190 curves were generated using a 3% cutoff, indicated a large number of sequences in the biofilm retrieved from PP
191 carriers at 20°C (**Fig. 3**).

192 3.3 Biofilm community composition and taxonomic profiling

193 Ribosomal Database Project (RDP) classifier was employed to assign the effective bacterial sequences to
194 different phylogenetic taxa. In total, ten phyla were observed (**Fig. 4**). The phylum *Proteobacteria* accounted for
195 the largest number of sequences (59.0%) detected from all samples, accounting for 67.4%, 50.0%, and 40.0% at

196 10, 20 and 30°C biofilms respectively (**Fig. 5**). The other two dominant phyla were *Bacteroidetes* (20.0-26.6%)
197 and *Firmicutes* (3.6-20.0%) of the entire community. These three groups, viz., *Proteobacteria*, *Bacteroidetes*,
198 and *Firmicutes* were predominant in all samples (~91%), with other bacterial phyla only accounting for ~8%.
199 The phyla *Actinobacteria*, *Acidobacteria*, *Planctomycetes* and *Gemmatimonadetes* were detected in the
200 biofilms developed at 10°C accounting for 1.8%, while at 30°C all these phyla accounted for 17.7, 1.2, 2.0 and
201 3.8 respectively. Moreover, *Verrucomicrobia* were found in biofilm samples at 20°C (6.7%) and 30 °C (1.6%)
202 (**Fig. 5**).

203 Within the phylum *Proteobacteria*, the *Betaproteobacteria* was the dominant class (47.0%), followed by
204 *Gammaproteobacteria* (21.0%), *Alphaproteobacteria* (20.0%), *Deltaproteobacteria* (8%) and
205 *Epsilonproteobacteria* (4%) in all biofilm samples (**Fig. 6A**). Within *Betaproteobacteria*, four orders were
206 identified: *Burkholderiales* was the most abundant order, accounting for 4.6% – 44.5%, followed by
207 *Rhodocyclales* (3.6 – 24.3%), while the orders *Rhodobacteriales* (0.05- 0.7%) and *Nitrosomonadales* (0- 0.4%)
208 were present at much lower abundances (**Fig. 7A**). Within *Bacteroidetes*, the relative abundance of classes
209 *Sphingobacteria*, *Flavobacteria*, and *Bacteroidetes* were 16.0% – 63.9%, 8.9% – 63.0%, and 13.7% – 75.0%,
210 respectively, in all samples (**Fig. 6B**). *Sphingobacteria* dominated the biofilms developed at 30°C, with a
211 relative abundance of 63.9%, while *Flavobacteria* was prominent in the 10°C biofilms (63.0%) and
212 *Bacteroidetes* at 20°C (75.0%) biofilms (**Fig. 6B**). The phylum *Firmicutes* was the third most abundant,
213 comprised of three classes *Clostridia* (21.0 – 62.0%), *Bacilli* (23.0 – 38.0%), and *Erysipelotrichia* (38.0-42.0%).
214 However, *Erysipelotrichia* were not found in the biofilm at 30°C (**Fig. 6C**).

215 A total of 32 known orders and 1 unknown order were identified, using the RDP classifier (**Table S3**). From
216 those, 13 orders were present in all biofilm samples (**Fig 7A**), dominated by the orders *Burkholderiales*,
217 *Rhodocyclales*, *Lactobacillales*, and *Caulobacterales*. The dominant orders accounted for 26.9%, 20.9%, 17.4%
218 and 6.2% respectively. Several orders were distinctly detected in biofilms at 30°C, such as *Flavobacteriales*,
219 *Myxococcales*, *Sphingobacteriales*, *Chlamydiales*, *Acidimicrobiales*, *Holophagales*, *Herpetosiphonales*,
220 *Nitrosomonadales*, *Gemmatimonadales*, and *Planctomycetales* and varied from 0.1-3.5% (**Fig 7A**). However,
221 some orders like *Actinomycetales* (0.15%), and *Alteromonadales* (0.21%) were distinctly restricted to 10°C
222 biofilms. While, *Desulfobacteriales* was found only at 20°C with relative of 0.21% (**Fig 7A**).

223 At the family level, a total of 53 families were found (**Table S4**), with *Comamonadaceae*, *Rhodocyclaceae*,
224 *Carnobacteriaceae*, *Caulobacteraceae*, and *Aeromonadaceae* being the most abundant, accounting for 57.2,

225 48.8, 41.8, 17.8, 14.9% respectively in all the samples (**Fig. 7B**). The relative abundance of *Caulobacteraceae*
226 and *Aeromonadaceae* were higher at 10°C, accounting for 17.1% and 13.2%, respectively, as compared to their
227 at 20°C (0.4 and 1.1 % respectively) and 30°C (0.4 and 0.6% respectively). However, the relative abundance of
228 *Comamonadaceae* was much higher at 30°C (40.5%), as compared to 10°C (13.8%) and 20°C (2.9%). Some
229 families, like *Nocardiaceae* and *Holophagaceae* were shared by all biofilm samples, at relative abundances
230 below 1.0% (**Fig. 7B**).

231 On a finer scale, microbial communities of all samples were distributed in 108 genera of bacteria (**Table S4**).
232 The top abundant genera in each biofilm sample were selected for generating the heatmap, which illustrated
233 shared genera (**Fig. 8**). Among shared genera, the dominating genera were *Trichococcus*, *Zoogloea*, *Aeromonas*,
234 *Acidovorax*, and *Malikias* (**Fig. 8**). The relative of these genera varies from 3.1-36.8%, 2.3-9.4%, 0.6-13.2%,
235 0.4-9.1% and 0.2-1.9% respectively in all three biofilm samples (**Fig. 7C**). The genera found only distinctly in
236 the biofilms developed at 10°C were *Undibacterium*, *Janthinobacterium*, *Bosea*, *Devosia*, *Gemmobacter*,
237 *Paracoccus*, *Nubsella*, *Pedobacter*, *Microbacterium*, and *Shewanella*, ranged from 0.1-1.7%. Additionally,
238 several genera were found to be abundant only in some biofilm samples. *Aquincola*, *Brachymona*,
239 *Diaphorobacter*, *Rhodoferax*, *Achromobacter*, *Camelimonas*, and *Dysgonomonas* (relative < 0.1%) were
240 restricted to the biofilms retrieved from 20°C, while 32 genera were distinctly identified only in the biofilm
241 samples removed from the reactors operating at 30°C, and was dominated by *Roseateles* (39.3%), *Filimonas*
242 (5.5%), *Aquimonas* (5.1%), *Fluviicola* (2.7%), *Runella* (2.5%), *Sediminibacterium* (2.0%), *Mycobacterium*
243 (2.0%), *Byssovorax* (2.0%), *Algoriphagus* (1.9%), *Neochlamydia* (1.0%), *Segetibacter* (1.0%). The relative
244 abundance of the remaining 21 genera at 30°C were below 1%. Some genera like *Hydrogenophaga* (0.1-0.2%),
245 *Paludibacter* (0.9-0.1%), *Phenylobacterium* (0.2-0.3%), *Clostridium* (0.1-1.4%), were found in two samples of
246 biofilms retrieved from 10 and 20°C. A total 15 genera were found to be shared by 10 and 20°C biofilms,
247 dominated by *Brevundimonas* (17.3%), *Dechloromonas* (11.0%), *Propionivibrio* (9.4%), *Quatronicoccus*
248 (5.5%), *Sulfurospirillum* (2.5%), *Stenotrophomonas* (2.1%), *Uliginosibacterium* (1.5%), however other were
249 much less (<1.0% relative abundance). The genera *Erythromicrobium* was also detected in the biofilms
250 developed at 10 and 30°C, but not in the 20°C (**Fig. 7C**).

251 **3.4 Treatment efficiency of the aerobic FBR**

252 The correlation of the physico-chemical parameters of influent and effluent and number of species observed on
253 PP media in the 10, 20 and 30°C FBRs is shown in **Table 1**. All parameters showed non-significant correlation,

254 except BOD removal efficiency with the number of OTUs and temperature. Prevailing temperature conditions
255 and operational taxonomic units (OTUs at 97% similarity cut-off) recovered on PP- media were positively
256 significantly correlated with each other ($P < 0.01$). While all other parameters have shown non-significant
257 correlation ($P > 0.05$) with each other and also with OTUs and Invisimpson in case of all media reactors (**Table**
258 **1**). The values of BOD₅, DO, pH obtained from the influent and effluent of the FBRs are shown in **Fig. 9**. The
259 BOD of the influent for all three reactors was 378.9 mg L⁻¹. A highly significant BOD removal (66.6%) was
260 shown by reactors operating at 30°C, decreasing from 378.9 to 126.36 mg L⁻¹. The efficiencies of the reactors
261 operated at 10°C and 20°C were comparable, with values of BOD of 269.13 and 267.34 mg L⁻¹ respectively,
262 corresponding to wastewater treatment efficiency of approximately 28%. Another important parameter used to
263 detect the performance of FBR was the increase in dissolved oxygen (DO). The DO of the influent was 1.9 mg
264 L⁻¹, and the highest DO increase (70.5%) was observed in the 30°C reactors, followed by the reactors at 20 and
265 10°C. The pH in the 30°C reactors increased from 7.3 in the influent to 7.4 in the effluent. A small change was
266 observed at 10°C, with a value of 7.2 in the effluent, while the pH of the effluent at 20°C dropped to 7.0.

267 **4. DISCUSSIONS**

268 We investigated the composition and diversity of physiologically active biofilms developed on polypropylene
269 (PP) media in aerobic FBRs at different temperatures. The efficiency of wastewater treatment in FBRs is highly
270 dependent on the filter medium, which provides the matrix for microbial attachment, growth and contact with
271 pollutants for removal. Biofilter media, both synthetic and natural, have shown variation in supporting biofilms
272 and their respective potential to degrade pollutants in wastewater. Synthetic media are usually preferred as they
273 are less biodegradable, and provide good support for the biofilms oxidizing contaminants in sludge and
274 wastewaters.³ To be used in the FBRs, these media need to be durable and non-reactive, and should sustain the
275 growth of metabolically active biofilms, as the wastewater treatment effectiveness depends on the microbial
276 communities of the biofilm and the filter media used as substratum. The elemental composition of the support
277 media should be compatible with microbial growth. XPS analysis of the media for the evaluation of media was
278 undertaken, which showed its compatibility with microbial growth as composed mostly of carbon, oxygen,
279 nitrogen, zinc contents (**Fig. 2**).

280 **4.1 Biofilm community composition and diversity**

281 The effect of temperature on biofilm formation within PP-FBRs was explored after 14 days of the experiment.
282 In order to investigate the composition of the bacterial community, 16S rRNA gene sequences were obtained
283 from biofilms grown at 10, 20, and 30°C. The Chao1 index estimated 290.8, 367.6842 and 398.5263 OTUs at a
284 3% cutoff for the 10, 20 and 30°C biofilm samples, respectively, demonstrating that the highest bacterial
285 diversity is observed in the biofilms grown at 30°C. The same trend was calculated using other nonparametric
286 diversity indices, such as ACE. Furthermore, the parameter Invisimpson was calculated as a measure of Alpha-
287 diversity, as it provides an indication of the richness in a community with uniform evenness that would have the
288 same level of diversity. The highest Invisimpson (dominance) of 33.4449 was found at 10°C (**Table S3**). The
289 diversity and richness of the biofilm samples were noticeably lower than that of the municipal wastewater
290 treatment systems.^{5, 13, 14} For a comparison of bacterial species richness among 3 biofilm samples, rarefaction
291 curves of all the observed OTUs were developed (**Fig. 3**). This curve persistently increased with the number of
292 sequences in the samples and did not reach a plateau, demonstrating that further increases in sample size would
293 yield more species and suggesting that minor species would have remained unidentified. The data presented here
294 was based on the evaluation of 14-days old biofilms, capable of degrading pollutants in the wastewater. The
295 biofilm sample retrieved at 30°C was the steepest, reflecting the highest species richness among the samples.
296 The highest bacterial diversity was found in the biofilms developed on 30°C, while the lowest diversity was
297 found in the biofilms developed at 10°C (**Table S3**). Although the same municipal wastewater was used as an
298 inoculating agent for biofilm development, these results show that different temperatures support biofilms with
299 different diversity.¹⁵

300 As shown in **Fig. 5**, *Proteobacteria* was the most dominant phylum in all three samples. This
301 widespread and highly diverse phylum was reported to be dominant in pharmaceutical, petroleum refinery,
302 industrial wastewater treatment plants (WWTPs), sewage,¹³ various municipal wastewater treatment plants and
303 bioreactors.^{5, 16, 17} The phylum *Proteobacteria* included a very high level of bacterial metabolic diversity related
304 to global carbon, nitrogen and sulfur cycling.¹⁸ Other phyla such as *Bacteroidetes*, *Firmicutes* and
305 *Actinobacteria* were detected abundantly in all three biofilm samples, in agreement with published results for
306 activated sludge processes.⁵ *Bacteroidetes* (24.0%) was the second largest phylum represented by classes
307 *Sphingobacteria*, *Bacteroidetes* and *Flavobacteriia*. *Sphingobacteria* have been identified as one of the main
308 bacterial genera responsible for organic pollutant removal.¹⁹ The phylum *Firmicutes*, represented by members
309 of *Bacillaceae* and *Clostridiaceae*, was the major bacterial phylum, accounting for 8.0% of the entire
310 community. In this study, however, it was notable that *Clostridiaceae* accounts for 1.5%, and was represented

311 by only single genus *Clostridium sensu stricto*, only in the biofilms developed at 20 and 30°C. A possible
312 explanation for this low abundance was that the growth of *Clostridium* strains is mediated by anaerobic
313 fermentation. The other five bacterial phyla, only accounted for 6%, with *Verrucomicrobia* (2.0%),
314 *Gemmatimonadetes* (1.0%), *Planctomycetes* (1.0%), *Acidobacteria* (1.0%) and *Cynobacteria* (1.0%) (**Fig. 5**).
315 These phyla were reported to be widespread in other wastewater treatment systems.²⁰ *Bacteroidetes* and
316 *Proteobacteria* such as *Flavobacterium*, and *Acinetobacter* are heterotrophic carbon degraders isolated from
317 municipal wastewater treatment system.²¹

318 **4.2 Shared taxonomic genera on polypropylene media material**

319 Genus level analysis can provide further detailed information on microbial adaptation to external conditions,
320 such as temperature. The heatmap shows some core genera in all biofilms (**Fig. 8**). Among the commonly
321 abundant genera, many have been identified in wastewater treatment processes. For instance, *Trichococcus* was
322 a dominant microorganism of all pyrotags in sewage and appeared to be well adapted to the sewer infrastructure
323 environment.²² Members of the genus exhibit various features that may have potential for biotechnological
324 applications such as environmental bioremediation, extracellular polysaccharide production, lactic acid
325 production from various carbohydrates, etc..²³ Dethlefsen *et al*²⁴ also reported *Trichococcus* sp. from a
326 wastewater treatment plant with the capability of precipitating crystals of calcium carbonate and struvite.
327 *Zoogloea* was found in all samples (15.8%) and was reported that fast-growing species resulted in the formation
328 of biofilm granules.²⁵ Previously, species of the genus *Zoogloea* were recognized to form zoogloal matrices,²⁶
329 and are the main mediator for the flocculation of activated sludge processes.²⁷ *Zoogloea* was also identified to
330 be potential phosphate accumulating organisms (PAOs).²⁸ The genus *Aeromonas* (14.95%) was present in all
331 samples, but surprisingly most abundant in the 10°C biofilms. However, an increase of its strain (*Aeromonas*
332 *hydrophila*) was observed in summer in raw sewage, treated wastewater and effluent-carrying canal. In summer,
333 *Aeromonas* sp. demonstrated multiple resistance patterns towards antimicrobials,²⁹ resistant to nalidixic acid in
334 the wastewater³⁰, and are recognized carriers of antibiotic resistance in wastewater habitats.³¹ Antimicrobial
335 residues found in municipal wastewater may increase selective pressure on microorganisms for development of
336 resistance. However, *Aeromonas* was reported for exoprotease production or biofilm formation though quorum
337 sensing via N-acylated-L-homoserine lactones (AHLs) in activated sludge.³² *Aeromonas* sp. can grow both
338 aerobically and anaerobically in a mesophilic environment by using a wide range of carbohydrate sources.³³
339 *Acidovorax* (10.67%) was present in all biofilm samples with high abundance at 10°C. It was reported

340 that *Acidovorax* sp. responsible for phosphate removal,³⁴ and is among the first colonizers of diatom micro-
341 aggregates.³⁵ It was also found in activated sludge along with other species.³⁶ *Rhodococcus* was found in all
342 biofilm samples with low relative abundance. *Rhodococcus* sp. could perform heterotrophic nitrification and
343 aerobic denitrification in wastewater treatment.³⁷ It was previously isolated from a bioreactor with extensive
344 phosphorus removal,³⁸ and are also considered to be potential PAOs.²⁸ Zhu *et al*¹⁴ studied the biodegradation
345 characteristics of quinoline (and its intermediates) by *Rhodococcus* sp. isolated from activated sludge of a coke
346 plant wastewater treatment process. A genus belonging to the family *Rhodocyclaceae*, *Malikia* sp., identified as
347 a potential PAO, was also found in all biofilms.³⁹ *Rhodocyclaceae* and *Comamonadaceae*³⁹ were the core
348 families in many wastewater treatment plants reported to be responsible for denitrifying and aromatic degrading
349 processes.⁴⁰

350 **4.3 Distinct taxonomic genera on polypropylene filter media**

351 The relatively large numbers of genera were distinctly detected in biofilm samples retrieved from 30°C. The
352 composition of bacterial community in the biofilm developed at 30°C reactors shown the presence of
353 representatives of all phyla, and a very large proportion of the genus *Roseatales* of *Betaproteobacteria*.
354 *Roseateles* sp. are aerobic, heterotrophic bacteria, able to depolymerize aliphatic as well as aliphatic–aromatic
355 co-polyesters.^{41, 42}

356 Other genus found at high abundances was *Acinetobacter*, is a strictly an aerobic chemoorganotrophic
357 bacterium with an oxidative metabolism that plays a significant role in the detoxification of different pollutants,
358⁴³ and has been identified as a potential PAO.⁴⁴ *Mycobacteria* have been previously isolated from wastewater
359 and sludge, and its hydrophobicity is linked to the removal of insoluble compounds.⁴⁵ The genus *Aquimonas*
360 has been reported to be involved in nitrification processes in warm springs.⁴² *Filimonas* is an exopolymer-
361 producing bacterium, previously isolated from fresh water. The genera *Sediminibacterium* and *Fluvicola*
362 (*Bacteroidetes*) and a genus *Byssovorax* (*Deltaproteobacteria*) were also found in biofilms samples at this
363 temperature. Members of the genus *Sediminibacterium* are reported to inhabit eutrophic reservoirs.⁴⁶
364 *Fluvicola* and *Aquimonas*, were previously reported that forming biofilms with greater microbial diversity.⁴⁷

365 *Chloroflexi* sp. and *Gordonia* sp. were found only at 30°C (**Table S4**). These genera present metabolic
366 interactions with *Cyanobacteria*. *Cyanobacteria* accumulate products of photosynthesis, which are metabolized
367 by members of *Chloroflexi*.⁴⁸ *Gordonia* were also distinctly found at 30°C, but at less relative abundance.

368 *Gordonia* sp. are known to play an important role during wastewater treatment and in biofilters.⁴⁹ It is an
369 aerobic rubber-degrading bacterium, first isolated from water accumulated inside deteriorated automobile
370 tyres.⁵⁰ *Gordoniae* are probably important in natural environments and are powerful candidates for
371 bioremediation processes because of their capacity to degrade substituted and non substituted hydrocarbons,
372 widespread toxic environmental pollutants, other xenobiotics, and natural compounds that are not readily
373 biodegradable.⁴⁹ Examples of this ability are the adhesive growth of several *Gordonia* strains during the
374 biodegradation of rubber materials⁵⁰ and the utilization of hydrophobic hydrocarbons by many species of this
375 genus.⁵¹ The genus *Erythromicrobium* was also detected in the biofilms developed at 10 and 30°C, suggesting
376 involvement in the metabolism of iron and manganese within biofilms.⁵² The genus *Erythromicrobium* has also
377 been reported to reduce heavy metals.⁵³ This trait makes the bacterium as a prospective applicant for removing
378 heavy metal ions from wastewaters.

379 Some genera like *Hydrogenophaga* and *Clostridium* were found in two samples of biofilms retrieved
380 from 20 and 30°C, PP-FBRs (**Fig. 7C; Table S4**). *Hydrogenophaga* was shown to play an important role in
381 autohydrogenotrophic denitrification in a hollow fiber membrane biofilm reactor for nitrate removal from
382 drinking water.⁵ Genera such as *Aquicola*, *Brachymona*, *Diaphorobacter*, *Rhodoferax*, *Achromobacter*,
383 *Camelimonas*, and *Dysgonomonas* (relative <0.1%) were restricted to the biofilms retrieved from 20°C (**Fig.**
384 **7C; Table S4**). The strain *Aquicola* is strictly an aerobic, previously isolated from methyl tert-butyl ether
385 (MTBE)-contaminated aquifer⁵⁴, and a wastewater treatment plant⁵⁵ and is one of the most efficient aerobic
386 MTBE degraders.⁵⁶ The genus *Diaphorobacter* has the capability of carrying out simultaneous nitrification and
387 denitrification.⁵⁷ *Diaphorobacter* sp. were previously isolated from an industrial wastewater treatment plant
388 utilizing 3-nitrotoluene (3-NT) as a sole source of carbon, nitrogen and energy,⁵⁸ through the dihydroxylation of
389 the benzene ring.⁵⁹ *Achromobacter* sp. were isolated from wastewater reported to degrade di-n-Butylphthalate⁶⁰.
390 At the genus level, some species, including *Undibacterium*, *Janthinobacterium*, *Bosea*, *Devosia*, *Gemmobacter*,
391 *Paracoccus*, *Nubsella*, *Pedobacter*, *Microbacterium*, and *Shewanella* were distinctly observed with very low
392 abundances (<0.1%) (**Table S4; Fig. 7C**). Surprisingly, some of them, like *Microbacterium* sp. was isolated
393 from activated sludge as ethylhexyl phthalate (DEHP)-degradation strain and reported to have an optimal
394 temperature of 25–35°C.⁶¹ Other genera such as, *Flavobacterium* was found at 10 and 30°C. However, its
395 relative abundance was distinctly high at 10°C (**Fig. 7C**). These results were in accordance with Biswas *et al.*⁶²,
396 who observed elevated levels of *Flavobacterium* in the winter in treatment plants. Recently, the strictly aerobic
397 *Flavobacterium* was also isolated from a municipal wastewater treatment plant.⁶³ *Flavobacteria* has been found

398 to be abundant in wastewater treatment systems exhibiting good resistance to pollutants.¹⁴ They are able to low
399 temperature protein degradation through the activity of psychrophilic proteases.⁵ This suggests the capability of
400 degrading all types of protein in wastewater in the reactors at low temperatures.

401 A large number of genera (15) were found in both 10 and 20°C biofilms (**Fig. 7C**). Surprisingly,
402 *Brevundimonas* was observed in the biofilms with high relative abundances at 10 and 20°C, contrary to previous
403 research, in which an optimal growth temperature of 30°C was reported.⁶⁴ *Brevundimonas* sp. is an effective
404 extracellular polymeric substance (EPS) producer⁶⁵ that can participate in an aerobic biofilm formation.
405 *Brevundimonas* sp. participates in the biosorption of nickel, copper and lead from wastewater.^{65, 66} Wang *et al.*⁶⁷
406 isolated *Brevundimonas* sp. from activated sludge of a coking wastewater treatment plant and identified that it
407 could utilize quinoline as the sole source of carbon, nitrogen, and energy, with an optimum temperature of 30 °C
408 and pH of 9.0. Another genus, *Dechloromonas*, was abundantly present at 10 and 20°C. Previously,
409 *Dechloromonas* sp. had been observed at relative abundance in an anaerobic and aerobic zone of biofilms as
410 potential PAO.²⁸ It was also shown that certain bacteria like *Dechloromonas* were responsible for nitrate
411 reduction in wastewater.⁶⁸ The genera *Rheinheimera* and *Lactococcus* sp. were also present in both biofilm
412 samples, but with high relative abundance at 10°C. *Rheinheimera* are able to easily degrade organic matter,⁶⁹
413 while *Lactococcus* sp. can degrade organic carbon into lactate or acetate and could promote the growth of
414 sulphate reducers.⁷⁰

415 **4.4 Correlation between the Treatment efficiency of the FBR at different temperatures and bacterial** 416 **biofilms**

417 In this study, correlations were detected between bacterial diversity indices and operational/functional
418 parameters like temperature, BOD, DO, etc. (**Fig. 9**). While, a significant correlation was found between
419 microbial communities (OTUs) and the operational temperature of the FBRs, which is in agreement with
420 previous studies.¹⁶ The prevailing temperature has shown to affect ecosystem function, by influencing the
421 components of diversity such as species composition with particular traits, positive species interactions, and
422 functional redundancy.⁷¹ It has also been suggested that temperature was the most important factor affecting
423 microbial community assembly.⁷² The effect of temperature on microbial growth and metabolism is well
424 documented by Brown *et al.*⁷³ An increase in the growth rate and activity of bacterial biofilms might increase
425 with an increase in water temperatures.^{74, 75} Further, increase in temperature also accelerate microbial metabolic
426 rates, which would promote the activities of the enzymes responsible for the degradation of organic matter, thus

427 it might further determine changes in the composition of bacterial species.⁷⁶ Another important factor affecting
428 the microbial communities is the BOD in the influent wastewater.¹³ In the present study, same wastewater was
429 used as feedstock for all the FBRs for treatment. Therefore, the influent BOD cannot be considered responsible
430 for modifying the biofilm communities in the FBRs on PP media. Oxygen was considered as the most favorable
431 electron acceptor for aerobic microbes to remove organic pollutants in the wastewater treatment processes.
432 Oxygen supply determines the bacterial growth and biomass decay rates and influences bacterial composition.⁶⁷
433 In the present study, we cannot attribute the distinct biofilm communities on PP media in the different FBRs, as
434 the same DO levels were observed in the influents.

435

436 5. CONCLUSIONS

437 In this research, a greater diversity of bacterial populations was found in the biofilms at 30°C, large number of
438 sequences was observed at 20°C, and dominance was shown by biofilms at 10°C. The dominant bacterial
439 classes within the biofilms were *Betaproteobacteria*, followed by *Gammaproteobacteria*, *Alphaproteobacteria*
440 and *Bacilli* at 10°C. While, at 20°C, *Betaproteobacteria* population was found to dominate the bacterial
441 community followed by *Bacteroidetes* and *Firmicutes*. However, the biofilm developed at 30°C constitutes
442 representatives of all the phyla. An obvious difference was observed in the diversity and richness of the bacterial
443 community composition in the biofilm samples developed at 10, 20 and 30°C. A very large proportion of genera
444 *Rosetales* and *Aeromonas* were found to dominate the communities at 30 and 10°C respectively. However, at
445 20°C, some of the genera like *Zoogloea* and *Dechloromonas* were coexisting. Further research may be carried
446 out with more sampling events (more sequences) to explain the large fraction of OTUs in the biofilm samples
447 for a detailed assessment of the abundance and the diversity. A significant reduction in BOD of the municipal
448 wastewater was observed in the reactors operating at 30°C, and to a lesser extent in the reactors operating at 10
449 and 20°C. The results show that polypropylene is a good filter media in the FBRs for wastewater treatment, with
450 temperature being the only operational parameter affecting the microbial composition in the biofilm. The results
451 indicate that a system for the biological treatment of wastewater can be constructed using inexpensive materials
452 to support the bacterial biofilms.

453 Conflict of interest

454 The authors declare that they have no competing interests.

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Table 1. Pearson correlation coefficient (r) for wastewater physico-chemical factors and number of OTUs observed (after the 3 % cutoff) on Polypropylene filter media

Parameters	OTUs	Invisimpson	BOD	DO
BOD ₅	0.00 (NS)			
DO	0.181 (NS)	0.000 (NS)		
pH	0.994 (NS)	-0.991*	-0.402 (NS)	
Temp (°C)	0.859**	-0.903 (NS)	0.000 (NS)	0.874 (NS)

Key: $n = 9$, $p < 0.01$ **, $p < 0.05$ *, NS = $p > 0.05$; a two tail test was used.

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660 **Legend of Figures**

661 **Figure1.** Schematic of the aerobic polypropylene filter media fixed biofilm reactors operating at 10, 20 and
662 30°C.

663 **Figure 2.** X-ray Photoelectron spectrums (XPS Survey) of Polypropylene filter media for wastewater treatment.

664 **Figure 3.** Rarefaction curves of OTUs at 97% of sequence similarity for three biofilm samples.

665 **Figure 4.** Taxonomic assignments of 16S rRNA gene sequences, classified at phyla level, retrieved from all
666 three the biofilm samples developed on polypropylene filter media at different temperatures.

667 **Figure 5.** Taxonomic assignments of 16S rRNA gene sequences retrieved from the biofilm samples developed
668 on polypropylene filter media at 10, 20 and 30°C in the aerobic reactors for wastewater treatment, at phyla level.

669 **Figure 6.** Microbial diversity of the dominating phyla (A) *Proteobacteria*, (B) *Bacteroidetes* and (C) *Firmicutes*
670 at class level, retrieved from biofilms developed at 10, 20 and 30°C in the Polypropylene filtermedia reactors for
671 wastewater treatment.

672 **Figure 7.** Relative (%) at (A) orders (B) families and (C) genera levels in the biofilm samples developed on
673 polypropylene filter media at 10, 20 and 30°C in an aerobic reactors.

674 **Figure 8.** Heatmap showing the most abundant species (relative $\geq 1\%$) at genus level within biofilms retrieved
675 from polypropylene filter media surfaces developed at 10, 20 and 30°C in an aerobic reactor.

676 **Figure 9.** Levels of BOD, DO and pH of the influent and effluent from Polypropylene filter media reactors at
677 different temperatures (10, 20 and 30°C)

678 **Supporting Information**

679 **Table S1.** Barcodes used for different biofilm samples

680 **Table S2.** Mothur diversity indices of bacterial communities in three aerobic biofilm samples developed on
681 polypropylene packing media for wastewater treatment

682 **Table S3.** The taxonomic classification of the bacterial communities retrieved from biofilm samples of the
683 polypropylene media aerobic reactors, operating at 10, 20, and 30°C into the Phyla, classes, orders, families, and
684 genera levels.