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

Running Title: Wastewater Treating Biofilms in Polypropylene Media Reactors

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Abstract

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

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

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

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

1. INTRODUCTION

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

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

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

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

2. MATERIALS AND METHODS

2.1 Evaluation of support media

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

2.2 Experimental setup and operation

The biofilm was allowed to develop on sterilized polypropylene (PP) balls using municipal wastewater as a seed (300 mL) in small reactors (500 mL) under aerobic conditions, using a continuous airflow rate of 4 L/min (**Fig. 1)**. All experiments were conducted in continuous mode in triplicate, with the addition of freshly collected municipal wastewater (300 ml) to each experimental setup thrice, with hydraulic loading rate (HRT) of 4.6 days, organic loading rate (OLR) of 81.2 gBOD m^3.d, and influent flow rate of 2.7 mL/h, for 14 days, in order to ensure the growth of a metabolically active biofilm at three different temperatures (10, 20 and 30°C).[10] Finally, effluent samples were collected from all the reactors. Various physico-chemical parameters of the influent and effluent samples were analyzed during the experiment to check the physiological activity of the developing biofilms. pH was determined using a pH-meter (D-25 Horiba Water Quality Meter, Horiba Ltd, Japan); Dissolved oxygen (DO) levels were measured using a MM-60R Multi – Function Water Quality Meter, TOA-DKK, Japan) and biochemical oxygen demand (BOD_5) was evaluated by a 5-day BOD test according to 5210-B Standard Methods.[11] Characteristics of municipal wastewater are available in the supplementary data in **Table S1**.

2.3 Analysis of biofilm microbial communities

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

2.4 Post-run analysis of nucleotide sequences.

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

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

3. RESULTS

3.1 Characterization of support media by XPS

The intensity of photoelectrons as a function of binding energy is shown in **Fig. 2**. Different peaks corresponding to various elements were observed in the XPS survey of the surface of PP medium. It was found that PP medium contains C Is (53.04%), Ca 2p (0.98%), N Is (3.05%), O Is (39.96%), S 2p (1.64%), Si 2p (0.14%) and Zn 2p3 (1.22%).

3.2 Sequencing and metagenomic assembly

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

3.3 Biofilm community composition and taxonomic profiling

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

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

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

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

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

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

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

3.4 Treatment efficiency of the aerobic FBR

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

except BOD removal efficiency with the number of OTUs and temperature. Prevailing temperature conditions and operational taxonomic units (OTUs at 97% similarity cut-off) recovered on PP- media were positively significantly correlated with each other ($P < 0.01$). While all other parameters have shown non-significant correlation ($P > 0.05$) with each other and also with OTUs and Invisimpson in case of all media reactors (**Table 1).** The values of BOD_5, DO, pH obtained from the influent and effluent of the FBRs are shown in **Fig. 9**. The BOD of the influent for all three reactors was 378.9 mg L^{-1}. A highly significant BOD removal (66.6%) was shown by reactors operating at 30°C, decreasing from 378.9 to 126.36 mg L^{-1}. The efficiencies of the reactors operated at 10°C and 20°C were comparable, with values of BOD of 269.13 and 267.34 mg L^{-1} respectively, corresponding to wastewater treatment efficiency of approximately 28%. Another important parameter used to detect the performance of FBR was the increase in dissolved oxygen (DO). The DO of the influent was 1.9 mg L^{-1}, and the highest DO increase (70.5%) was observed in the 30°C reactors, followed by the reactors at 20 and 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 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.

4. DISCUSSIONS

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

4.1 Biofilm community composition and diversity

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

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

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

4.2 Shared taxonomic genera on polypropylene media material

Genus level analysis can provide further detailed information on microbial adaptation to external conditions, such as temperature. The heatmap shows some core genera in all biofilms (**Fig. 8**). Among the commonly abundant genera, many have been identified in wastewater treatment processes. For instance, *Trichococcus* was a dominant microorganism of all pyrotags in sewage and appeared to be well adapted to the sewer infrastructure environment. [22] Members of the genus exhibit various features that may have potential for biotechnological applications such as environmental bioremediation, extracellular polysaccharide production, lactic acid production from various carbohydrates, etc..[23] Dethlefsen *et al* [24] also reported *Trichococcus* sp. from a wastewater treatment plant with the capability of precipitating crystals of calcium carbonate and struvite. *Zoogloea* was found in all samples (15.8%) and was reported that fast-growing species resulted in the formation of biofim granules.[25] Previously, species of the genus *Zoogloea* were recognized to form zoogloeal matrices ,[26] and are the main mediator for the flocculation of activated sludge processes.[27] *Zoogloea* was also identified to be potential phosphate accumulating organisms (PAOs).[28] The genus *Aeromonas* (14.95%) was present in all samples, but surprisingly most abundant in the 10°C biofilms. However, an increase of its strain (*Aeromonas hydrophila*) was observed in summer in raw sewage, treated wastewater and effluent-carrying canal. In summer, *Aeromonas* sp. demonstrated multiple resistance patterns towards antimicrobials,[29] resistant to nalidixic acid in the wastewater [30] , and are recognized carriers of antibiotic resistance in wastewater habitats.[31] Antimicrobial residues found in municipal wastewater may increase selective pressure on microorganisms for development of resistance. However, *Aeromonas* was reported for exoprotease production or biofilm formation though quorum sensing via N-acylated-l-homoserine lactones (AHLs) in activated sludge.[32] *Aeromonas* sp. can grow both aerobically and anaerobically in a mesophilic environment by using a wide range of carbohydrate sources. [33] *Acidovorax* (10.67%) was present in all biofilm samples with high abundance at 10°C. It was reported

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

4.3 Distinct taxonomic genera on polypropylene filter media

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

Other genus found at high abundances was *Acinetobacter*, is a strictly an aerobic chemoorganotrophic bacterium with an oxidative metabolism that plays a significant role in the detoxification of different pollutants, [43] and has been identified as a potential PAO. [44] *Mycobacteria* have been previously isolated from wastewater and sludge, and its hydrophobicity is linked to the removal of insoluble compounds. [45] The genus *Aquimonas* has been reported to be involved in nitrification processes in warm springs.[42] *Filimonas* is an exopolymer-producing bacterium, previously isolated from fresh water. The genera *Sediminibacterium* and *Fluvicola* (*Bacteroidetes*) and a genus *Byssovorax* (*Deltaproteobacteria*) were also found in biofilms samples at this temperature. Members of the genus *Sediminibacterium* are reported to inhabit eutrophic reservoirs.[46] *Fluvicola* and *Aquimonas*, were previousely reported that forming biofilms with greater microbial diversity . [47]

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

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

Some genera like *Hydrogenophaga* and *Clostridium* were found in two samples of biofilms retrieved from 20 and 30°C, PP-FBRs (**Fig. 7C**; **Table S4**). *Hydrogenophaga* was shown to play an important role in autohydrogenotrophic denitrification in a hollow fiber membrane biofilm reactor for nitrate removal from drinking water.[5] Genera such as *Aquincola*, *Brachymona*, *Diaphorobacter*, *Rhodoferax*, *Achromobacter*, *Camelimonas*, and *Dysgonomonas* (relative <0.1%) were restricted to the biofilms retrieved from 20°C (**Fig. 7C**; **Table S4**). The strain *Aquincola* is strictly an aerobic, previouly isolated from methyl tert-butyl ether (MTBE)-contaminated aquifer [54], and a wastewater treatment plant [55] and is one of the most efficient aerobic MTBE degraders.[56] The genus *Diaphorobacter* has the capability of carrying out simultaneous nitrification and denitrification.[57] *Diaphorobacter* sp. were previously isolated from an industrial wastewater treatment plant utilizing 3-nitrotoluene (3-NT) as a sole source of carbon, nitrogen and energy,[58] through the dihydroxylation of the benzene ring. [59] *Achromobacter* sp. were isolated from wastewater reported to degrade di-n-Butylphthalate[60].

At the genus level, some species, including *Undibacterium*, *Janthinobacterium*, *Bosea*, *Devosia*, *Gemmobacter*, *Paracoccus*, *Nubsella*, *Pedobacter*, *Microbacterium*, and *Shewanella* were distinctly observed with very low abundances (<0.1%) (**Table S4; Fig. 7C**). Surprisingly, some of them, like *Microbacterium* sp. was isolated from activated sludge as ethylhexyl phthalate (DEHP)-degradation strain and reported to have an optimal temperature of 25–35°C.[61] Other genera such a, *Flavobacterium* was found at 10 and 30°C. However, its relative abundance was distinctly high at 10°C **(Fig. 7C**). These results were in accordance with Biswas *et al*.[62], who observed elevated levels of *Flavobacterium* in the winter in treatment plants. Recently, the strictly aerobic *Flavobacterium* was also isolated from a municipal wastewater treatment plant.[63] *Flavobacteria* has been found

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

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

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

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

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

5. CONCLUSIONS

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

Conflict of interest

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.

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

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

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

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

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

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

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

Supporting Information

Table S1. Barcodes used for different biofilm samples

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

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