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MOLECULAR EVALUATION OF BIOFILMS FORMED ON PIPES OF DIFFERENT MATERIALS DEPICTS *CLOSTRIDIUM* AND *AEROMONAS* SPP. DOMINATION

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ABSTRACT

The materials of water supply pipes exhibit differential tendencies to develop biofilms on the pipe surface. To evaluate the vulnerability of five types of commonly used pipe materials (HDPE, PC, PVC, PP and ABS) in water supply pipes, to biofilms formation, an annular reactor (AR) was operated. Twenty slides including 4 slides of each material were mounted in the AR. The source water (SW) bacteria were grown in TSB medium (100ml SW and 900ml TSB media) and were inoculated in the AR, followed by operation of the AR for 24 hours with the inoculum and 12 hours with continuous flow of SW. The genomic DNA was isolated from the biofilms formed on the slides, and amplicon libraries targeting V3 to V4 region of the 16S rRNA bacterial gene were prepared and sequenced using the Illumina MiSeq sequencer. Bioinformatic analysis with QIIME2 tools showed difference in bacterial composition in inlet water and biofilms formed on different pipe materials. At phylum level. *Firmicutes* (48 to 54%), *Bacteroidetes* (29 to 34%) and *Proteobacteria* (15 to 18 %) were the most abundant phyla in biofilms formed on different pipe materials. At genus level, *Clostridium* was the most abundant genus in biofilms of each plastic slide. In PICRUSt analysis biofilm formation pathways were mostly contributed by *Aeromonas* and *Clostridium*. Beside the deep insight into bacterial composition of biofilms, PVC pipe material was found the least prone to biofilm formation while the ABS material had the highest affinity to biofilm formation.

Keywords: 16S rDNA, biofilms, Drinking water distribution system, Pipe materials, QIIME2, Water quality

INTRODUCTION

As the relationship between low quality drinking-water and disease causation is well established, water managing authorities are investing significant amount of resources to improve the water quality. Several methods are already in practice to disinfect the water and produce good quality effluent. For example, physical disinfection is carried out by flocculation and sedimentation, filtration, ultraviolet (UV) radiation and pasteurization. Likewise, chemical disinfection methods include the addition of chemicals e.g. chlorine (gas and hypochlorite solution), chloramine, chlorine dioxide, and ozone (Pichel *et al.*, 2019).

However, some microbes survive even after getting adequate water treatment (Mahapatra *et al.*, 2015). Additionally, transporting the water from a treatment facility to an end-user through a water distribution system allows the entry and subsequent re-growth of undesirable microbes during pipe refitting and regular maintenance of the system (Besner *et al.*, 2011; Mahapatra *et al.*, 2015). However, due to the presence of harsh conditions such as scarce nutrients, changes in flow, and pressure fluctuations, survival of microbes within the distribution network becomes difficult. To cope up with this challenging environment, microbes get themselves attached to pipe surfaces (Henne *et al.*, 2012) and generate extracellular polymeric substances (EPS) that enables the microbial cells to stick together and form a complex matrix of biofilm (Branda *et al.*, 2005). This matrix acts as a physical barrier for the microbes against biocidal agents and adverse environmental conditions within the drinking water distribution system (Davey and O'toole, 2000). The biofilms harbor bacteria, fungi, archaea, and viruses. These microbes could be pathogenic and may cause water-borne diseases including typhoid, diarrhea, giardiasis, dysentery, hepatitis A etc. Most of the waterborne diseases are caused by bacterial infections (https://lifewater.org/blog/7-most-common-waterborne-diseases-and-how-to-prevent-them/).

Owing to the complex structure of the biofilms and their resistance to disinfection, it is of utmost importance to characterize the potential biofilm forming bacteria, as they do not only decrease the efficacy of disinfectants but also make a home for other pathogens. Their attachment to various surfaces is dependent on various factors including

inner surface of the pipes, water flow rate, temperature, and pH etc. (Hyun-Jung *et al.*, 2011; Rożej *et al.,* 2015). The composition of the pipe material is important because it defines attachment possibility for the bacteria (LeChevallier *et al.*, 1990). Various pipe materials were tested previous studies For example among steel, copper, stainless steel, and polyvinyl chloride (PVC), stainless steel was found most suitable due to its rust resistance (Hyun-Jung *et al*., 2011), in PVC, silane cross-linked polyethylene (PEX) and high density polyethylene (HDPE), PVC was least suseptable to biofilm formation (Rożej *et al.*, 2015).

In this context, this research is designed to investigate potential bacterial genera that form biofilms on commonly used pipes of different materials in drinking water distribution systems (DWDS). Also, the correlation of biofilm bacteriome with inlet water and outlet water was evaluated in order to find the source of planktonic cells going out of the reactor and which could possibly reach the end users of the DWDS. Functional capabilities of found bacterial genera, 16S rDNA based functional prediction were carried by using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) in order to determine the bacterial genera that contribute to the biofilm formation pathways.

MATERIALS AND METHODS

Source water and its physicochemical characteristics

The source water (SW) to the annular reactor came from the Mehran University treatment plant which had coagulation and sedimentation unit processes. The treatment plant is located at the right bank of the Indus River and gets water from Kalri Baghar (KB) Feeder. The current distribution system of this treatment plant has a capacity to supply 1 million gallons of water per day. No disinfectants (i.e. hypochlorite or chloramine) were being used at the treatment plant due to limitation of resources.

Prior to the inoculum addition reactor was operated for 2 weeks with SW of Mehran University treatment plant. The two weeks period was given to the reactor such that biofilm could grow with bacterial contributed by SW. The physicochemical parameters of fed water (SW) to annular reactor were measured in-situ twice a week, by following American Public Health Association (APHA) standard methods (Federation, 1999). The description of measured parameters and used instruments were as; the temperature, pH, electric conductivity (EC), total dissolved solids (TDS) and dissolved oxygen (DO) were measured by Multi-meter (MultiLine, Multi 3630 IDS). Alkalinity was measured by following APHA (2320B) titration method. Chloride was measured by following APHA argentometric method 4500-Cl^- B. Hardness was measured by following APHA EDTA titrimetric method 2340 C. Nitrate, Nitrite, Sulphate, Ammonia and Fluoride were measured by using portable Spectrophotometer (DR 900, HACH).

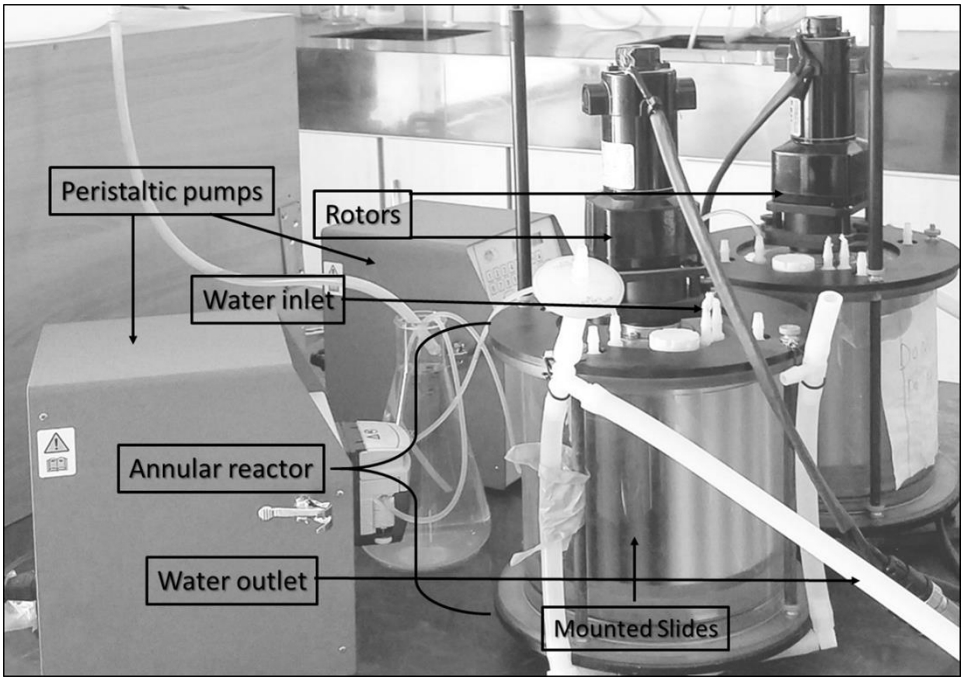

Fig. 1. Experimental set up of annular reactors for biofilm formation in pilot-scale lab showing 2 annular reactors, peristaltic pumps, Inlet, and outlet water sources.

Reactor operation and biofilm formation

An annular reactor (AR) 1320 LJ (BioSurface Technologies Corporation, USA), which consisted of a stationary cylinder and a rotating, polycarbonate made inner cylinder, was utilized in this study (Fig. **1**). The 4 slides of each 5 plastic materials i.e., High-density polyethylene (HDPE), Polycarbonate (PC), Polyvinyl chloride (PVC), Polypropylene (PP) and Acrylonitrile Butadiene Styrene (ABS) were fixed in slots in inner cylinder. Each slide had the surface area of 18.75 cm^2 (15 cm x 1.25 cm). The source water (SW) fed to the AR was the tap water from the Mehran University treatment plant. The inner cylinder could retain 1 liter of the water. 0.5 N/m^2 was the shear stress. The AR was kept at room temperature around 30-37°C. The reactor was operated in dark environment to avoid algal growth.

A bacterial culture was grown in 900 mL tryptone soy broth (TSB) by inoculating 100 mL water from Mehran University's treatment plant. The bacterial culture was grown in a shaking incubator at 37 °C and 125 rpm for 24 h. The AR was fed with the grown culture (1 L) and run for 24 h at 50 rpm. Then the reactor was fed with the source water at 8 ml/min using a peristaltic pump and operated for 12 h on continuous flow mode. After a total operation of 36 hours, the slides were scraped with a sterile metal scraper and put into 50 mL of phosphate buffer saline (PBS) solution in falcon tube. The biofilm-containing PBS solution was homogenized via a centrifuge for downstream applications. For the heterotrophic plate count (HPC),100 µL of homogenized and serially diluted suspensions were spread on R2A agar plates with a sterile spreader. The plates were incubated at 28 °C for 5 days as per standard methods (APHA, 2017).

Heterotrophic Plate Counts (HPC) in R2A agar

The serial dilutions of extracted biofilms were inoculated in R2A agar. Agar plates were incubated at 20°C for 5 days. The physical colonies were counted after 5 days of incubation. Results were calculated in CFU/cm^2 as number of CFUs per ml were calculated from which CFUs per 50 ml were obtained (Equation 1).

$$\left(\frac{\text{No of CFUs} \times 1000\ \mu l}{sample\ volume}\right) = CFUs\ per\ ml\ \times\ 50 = {}^{CFUs}/_{50\ ml} \quad \text{----------(1)}$$

CFUs/50 ml was equivalent to CFUs in total surface area of slide (whole biofilm of each slide was dissolved in 50 mL sterile PBS) from which $CFUs/cm^2$ was calculated (Equation 2)

$$\frac{({}^{CFUs}/_{50\ ml})\times 1}{total\ area\ in\ cm^2} = {}^{CFUs}/_{cm^2} \quad \text{----------(2)}$$

DNA extraction, library preparation, sequencing and bioinformatics analysis

DNA was extracted from the biofilm-containing PBS solution by the CTAB method (Edwards *et al.*, 1991) with some modifications Amplicon libraries were prepared by using bacterial 16S rDNA's V3 to V4 hypervariable region-specific primers by following the Illumina 16S metagenomics library preparation guidelines (https://support.illumina.com/documents/ documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf) The sequences of primers with attached adapter sequences were as; forward primer (5'-TCG TCG GCA GCG TCA GAA AGA GAC AGC CTA CGG GNG GCW GCAG-3') and reverse primer (5'-GTC TCG TGG GCT CSGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC-3'). The amplicon libraries were sequenced in paired ends (300 x 2 bp) by using the MiSeq sequencer (Illumina Inc. USA).

The quality assessment of obtained raw reads were carried out with FastQC software (version 0.11.8) (Andrews, 2010). The filtration of low quality reads, denoising, merging followed by removal of chimeric sequences was carried in QIIME2 (Bolyen *et al*., 2019) environment using DADA2 (Callahan *et al*., 2016). The sequence similarity search of the filtered reads was carried using q2-feature-classifier plugin for taxonomic assignments of the reads against greengenes (McDonald *et al*., 2012) database (version 13.8) (McDonald *et al*., 2012). Rarefaction curves were built by core-metrics-phylogenetic command to a depth of 4000 sequences per sample with 10 iterations per depth. Observed Operational Taxonomic Units (OTUs), Shannon's diversity index, Simpson's diversity index, abundance-based coverage estimator (ACE) and Chao1 were calculated for the alpha diversity. For the beta diversity Bray-Curtis dissimilarity method emperor plots was used to generate principle coordinate analysis (PCoA).

Functional Predictions

For the metagenome functional predictions, PICRUSt (version 2) (Douglas *et al*., 2019) was used with default parameters to determine the possible pathways contributed by an OTU. The reads were placed into the reference tree in a single command place_seqs.py as command 1) aligned multiple sequences through HMMER, 2) placed most

likely sequences as the new tip of the tree through EPA-NG and 3) generated tree generation through GAPPA. The hsp.py command gave gene families count for each sequence by using castor. The hsp.py command also gave nearest-sequenced taxon index (NSTI) values for each OTU, however NSTI value greater than 2 were discarded. The PICRUSt 2 based functional predictions were analyzed to find KEGG ontologies (KO) involved in biofilm formation pathways, and their corresponding contributing bacterial taxa were filtered along with KO abundances. The resulting filtered files were analyzed in BURRITO (McNally *et al.*, 2018).

RESULTS

There has been an increase in the use of plastic pipes in DWDs and indoor installations because of their durability, ease of use and corrosion resistance. In this study, materials of five types of plastic pipes have been evaluated in terms of their potential of bacterial biofilm formation and diversity.

Physicochemical analysis of SW (inlet)

Physicochemical analysis of the SW water is given in table 1. All the parameters tested herein were under the permissible limits of WHO (WHO, 2011a) and US-EPA guidelines (Kumar and Puri, 2012). For instance, the pH was 8.2 ± 0.6 which confirms to the expected range of pH for drinking water. However, the turbidity level was 31 ± 1.02 way above the WHO recommended levels of 5 NTU. Two metals, lead 0.08 ± 0.02 ppm and chromium 0.23 ± 0.51 ppm, also exceeded the maximum desirable limits by small quantities as per WHO (lead ≤0.05 ppm), and US-EPA (chromium ≤0.05 ppm) guidelines (Kumar and Puri, 2012).

Heterotrophic Plate Count of biofilm bacteria

Heterotrophic plate count analysis showed the highest biofilm bacterial growth on ABS slides with 7.02×10^6 CFU/cm^2 and the least growth on PVC with 1.37×10^6 CFU/cm^2. After ABS, PC harbored the highest bacterial growth of 3.83×10^6 CFU/cm^2 followed by PP with 3.43×10^6 CFU/cm^2 and HDPE with 2.68×10^6 CFU/cm^2 (Table 2).

Amplicon sequencing and bacterial composition of biofilms

Collectively, more than 4.7 million reads were obtained from the MiSeq sequencing run. After removing the reads with low quality (average Phred quality score <20) score, more than 1 million good quality reads were obtained. The good quality reads were processed for DNA sequence similarity search against greengenes (McDonald *et al*., 2012) database for bacterial taxonomic assignments. Fig **2** shows bacterial abundances in biofilms formed on slides of different materials. Fig. **2** was generated by fixing the classification of bacterial taxonomy at level 1. The ABS pipe material had the highest bacterial relative abundance (27%) whereas the PVC was found with 18% bacterial relative abundance attached to it.

The taxonomic analysis at the phylum level showed difference in microbial communities in the SW (inlet) and the outlet water. However, biofilms, formed on different pipe materials, were relatively like each other in composition compared with the inlet and the outlet water. In the inlet water sample, *Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Planctomycetes, Verrucomicrobia, Spirochaetes, Chlorobi, Acidobacteria* were the most abundant phyla with contribution of 50.0, 14.9, 10.5, 8.0, 3.6, 2.0, 1.7, 0.9%, and 0.9%, respectively. The outlet water had *Proteobacteria, Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria, Chlorobi, NKB19, WWE1, Planctomycetes* as the most contributing phyla with 67.1, 21.8, 8.3, 1.4, 0.3, 0.1, 0.1, 0.1%, and 0.1%, respectively. In the biofilm samples, *Firmicutes, Bacteroidetes, Proteobacteria, Chlorobi, Actinobacteria, Chloroflexi, Planctomycetes, Nitrospirae, Cyanobacteria* were observed as top 9 bacterial phyla (Fig. **3**). These results suggested three phyla i.e. *Firmicutes, Bacteroidetes,* and *Proteobacteria* had the highest potential to form the biofilms in HDPE, PVC, PP, PC, and ABS pipe materials. Furthermore, the outlet water was observed as a mixture of both inlet water bacteriome and biofilm bacteriome, suggesting that bacteria were detaching from the biofilms and contaminating the water supply in the distribution system. Fig **3** shows top 10 most abundant phyla found in the inlet water, outlet water and biofilms.

Table 1. Physico-chemical parameters analyzed in the water supplied to the annular reactor (average ± standard deviation, n=2).

Parameters	Values
pH	8.2 ± 0.6
Temperature (°C)	27°C ± 0.4
DO (mg/L)	7 ± 0.22
EC (µS/cm)	605 ± 1.6
Turbidity (NTU)	31 ± 1.02
TDS (mg/L)	393 ± 1.24
Nitrate (NO_3^--N mg/l)	0.9 ± 0.1
NH3 (mg/l)	0.05 ± 0.01
F^- (mg/l)	0.3 ± 0.14
Chloride (mg/l)	19.4 ± 5
Alkalinity	100 ± 13
SO^{2-}_4 (mg/l)	23 ± 1
Hardness (mg/l)	121 ± 7
Nitrite (NO_2^--N ug/l)	4.5 ± 0.8
Phosphorous (mg/l)	2.2 ± 0.6
TOC (mg/l)	5 ± 2.3
Fe (mg/l)	0.1 ± 0.03
Pb (mg/l)	0.08 ± 0.02
Ni (mg/l)	0.03 ± 0.04
Cr^{3+} (mg/l)	0.22 ± 0.51
Free/residual chlorine	0.0 0.00

Table 2. Heterotrophic plate count in biofilms attached to plastic slides.

Pipe Material	HPC (CFU/cm^2)
ABS	7.02×10^6
PC	3.83×10^6
PP	3.43×10^6
HDPE	2.68×10^6
PVC	1.37×10^6

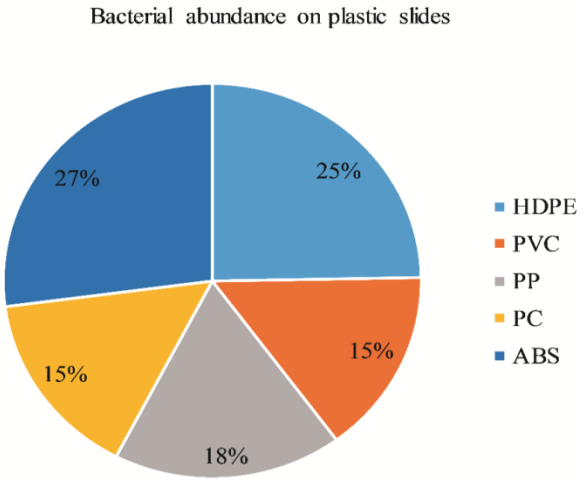

Fig. 2. Bacterial abundance in the biofilm samples extracted from 5 different pipe materials i.e. HDPE, PVC, PP, PC and ABS.

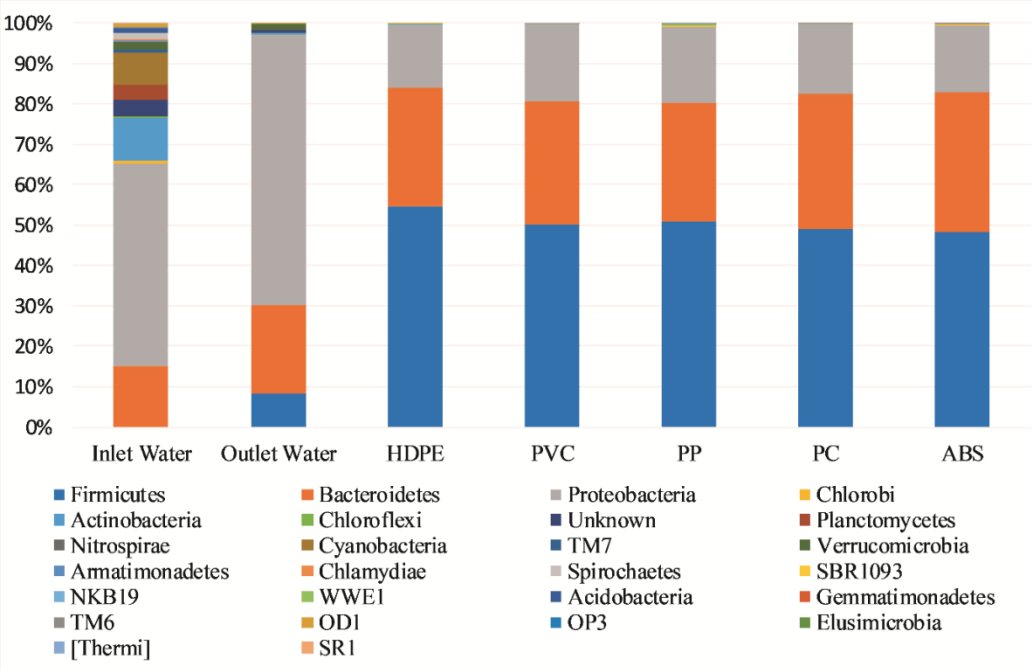

Fig. 3. Relative abundance at the phylum level in the biofilm samples extracted from the plastic slides.

At class level *Alphaproteobacteria* (28%) was the most abundant class followed by *Betaproteobacteria* (17%), *Saprospirae* (8%), *Actinobacteria* (7%) and *Synechococcophycideae* (5%) in the inlet water. In the outlet water, *Alphaproteobacteria* (40%), *Gammaproteobacteria* (19.6%), Bacteroidia (6.7%), *Betaproteobacteria* (6.5%) *Cytophagia* (4.7%), *Flavobacteriia* (3.7%) predominated (Fig. **4**). However, biofilms on each of the five pipe materials harbored class *Clostridia* (45 to 50%) predominantly. *Bacteroidia* (29 to 34%), *Gammaproteobacteria* (12 to 15%) were also found in abundance in biofilms of all the materials.

At genus level, the SW (inlet) water harbored *Planktophila* (4.5%), *Novosphingobium* (4.4%), *Denitromonas* (3.5%) and *Ilumatobacter* (3%) as the most abundant genera with >50% reads being unknown or unclassified. Likewise, the outlet sample comprised of unknown (27%), *Clostridium* (7.5%), *Aeromonas* (5.6%), *Bacteroides* (1.2%), and *Macellibacteroides* (1.2%) (Fig. **5**). Furthermore, it was noted that the *Clostridium* was the most abundant genus in the biofilms formed on all the five plastic materials. However, among the five pipe materials,

ABS had least percentage of *Clostridium*, whereas, HDPE had the highest percentage. Genus Bacteroides was the second highest abundant genus on plastic slides with ABS harboring the most and PVC harboring the least relative abundance of Bacteroidetes.

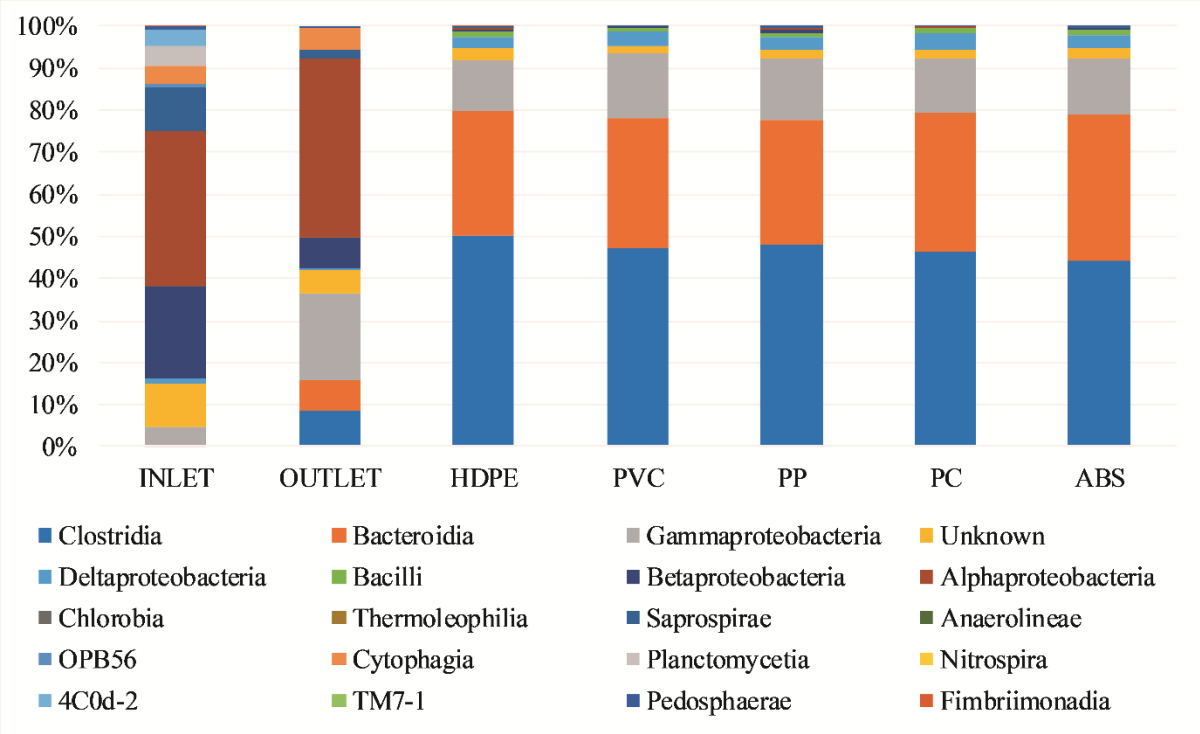

Fig. 4. Relative bacterial abundance at the class level showing relative abundances of 20 most abundant classes.

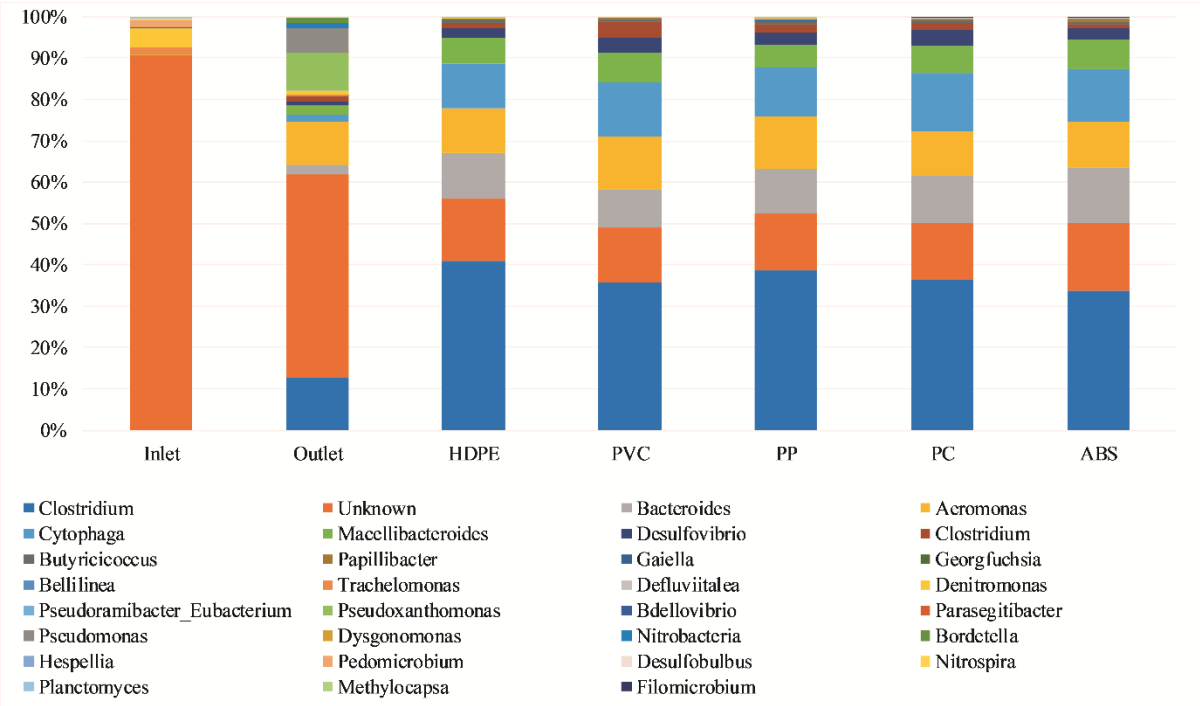

Fig. 5. Relative bacterial abundance of 30 most abundant genera in the Inlet, Outlet water and biofilm samples extracted from the plastic slides.

Bacterial diversity and richness

Alpha Diversity

For the alpha diversity OTUs richness, Shannon diversity index, Simpson diversity index, ACE, Chao 1 Estimator were calculated in QIIME2 (Bolyen *et al*., 2019) environment. The inlet water had the highest alpha diversity among the samples with 793 OTUs (richness), a Shannon diversity index of 7.35 (richness and evenness), a Simpson diversity index of 0.98, ACE of 820 and a Chao 1 Estimator of 818.45. Whereas among biofilms, ABS had the highest alpha diversity with Shannon index of 5.27 followed by the HDPE (Shannon 5.25), PP (Shannon 5.22), PC (Shannon 5.13) and PVC (Shannon 5.08) (Table 3). The alpha rarefaction curve at a sequencing depth of 4000 showed the index had leveled out and reached a plateau at approximately a sequencing depth of 4000 (Fig **6**).

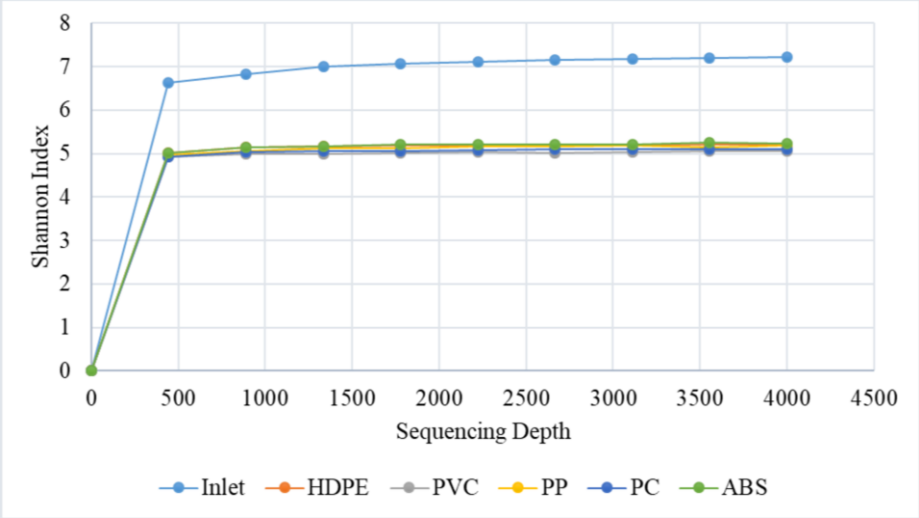

Fig. 6. Alpha rarefaction curve (Shannon's diversity index) plotted as a function of the sequencing depth.

Table 3. Summary of the diversity and evenness indices for bulk water and biofilm samples extracted from the plastic slides.

Sample	OTUs	Shannon	Simpson	ACE	Chao 1
Inlet	793	7.35	0.98	820	818.45
HDPE	234	5.25	0.96	255.25	255
PVC	172	5.08	0.95	179	179
PP	228	5.22	0.95	243.25	243
PC	181	5.13	0.95	188	188
ABS	251	5.27	0.95	278	278

The low alpha diversity of the biofilm samples relative to the inlet sample could be due to less bacterial species had the potential for biofilm formation. It is noteworthy that both Shannon and Simpson's reciprocal indices also account for evenness within a sample, the results showed that there is a high evenness among the species.

Beta Diversity

The PCoA plot (Fig **7A**, **B** and **C**) showed biofilm samples plotted near to each other compared to inlet and outlet samples suggesting inlet sample compositionally was different than the biofilm samples (Fig **7 A-C**), however, the outlet sample had a composite of both inlet and biofilm bacteriome. Furthermore, among biofilm samples HDPE, PP and ABS biofilms were compositionally more like each other, whereas, PVC and PC were like one another. The scatter plots [Fig **7 D-F**] show the correlation between inlet and outlet water, inlet and biofilm samples, and outlet and biofilm sample. There was 49.7% correlation between the inlet and the outlet water samples R^2=0.497 (Fig **7D**). Whereas, inlet and biofilm samples of different pipe materials showed very less correlation of R^2=0.084 (Fig **7E**). Same pattern of less correlation was also observed in the outlet and biofilm samples as 17.8% R^2=0.178 (Fig **7F**), however, it was slightly higher than inlet water, suggesting that the outlet water was a composite of bacteriome of both the inlet water and biofilm bacteriome.

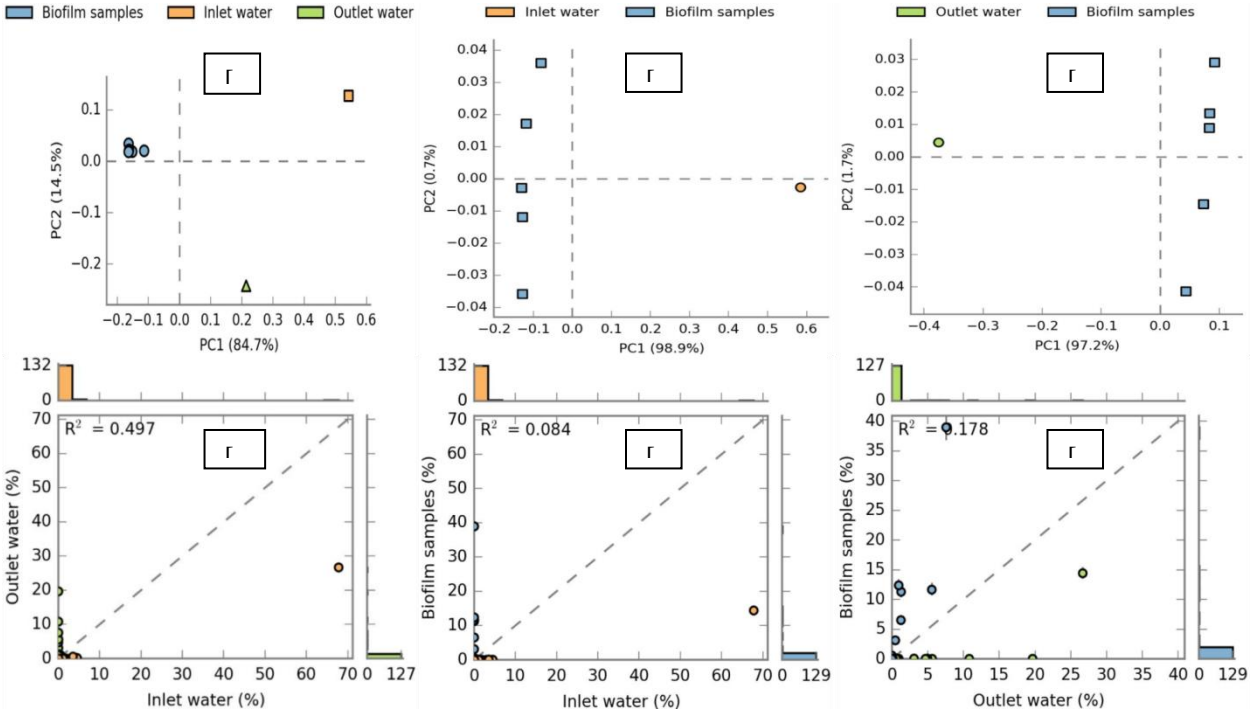

Fig. 7. PCoA plots [A-C] showing correlation in Inlet water, outlet water and biofilm samples [A], Inlet and biofilms [B] and Outlet water and biofilms [C]. Whereas scatter plots [D-E], showing correlation between Inlet and Out water samples [D], Inlet water and Biofilms samples [E], Outlet water and Biofilm samples [F].

Functional Predictions

The functional predictions based on 16S rDNA through PICRUSt version 2 predicted 6961 gene ontologies in KEGG database which were contributing to 418 KEGG pathways. From 6961 KEGG gene ontologies, 124 gene ontologies were predicted to be involved in biofilm formation. Those 124 predicted genes contributed to six level-1 KEGG pathways including cell process, environmental information processing, genetic information processing, human diseases, metabolism, and organism systems. Moreover, at level 3 pathway classification 27 KEGG pathways were biofilm contributing pathways. Those twenty-seven level-3 pathways most abundantly were predicted to be contributed, at phylum level, by *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* phylum as 34.75%, 33.39%, and 27.04% (Fig. **8**), respectively. Proteobacteria were predicted to be involved in overall 27 biofilms related KEGG pathways at level 3, in which transcription factors was the most contributed function by *Proteobacteria* (99.89%), however, overall abundance of transcription factors in all samples was 1.07%. *Firmicutes* also contributed to all 27 found KEGG biofilm related pathways, whereas, it most abundantly (57.38%) contributed to peptidases, however, overall, in samples 0.43% peptidases were predicted. Phylum *Bacteroidetes* contributed to 26 KEGG pathways whereas, they most abundantly (47.11%) contributed to multiple KEGG pathways with same percentage including ferroptosis, exosomes, peroxisomes, fatty acid biosynthesis, fatty acid degradation, lipid biosynthesis proteins, adipocytokine signaling pathway, PPAR signaling pathway, and thermogenesis. Average attribution from the total for these pathways was 0.24% for all pathways.

While analyzing the KEGG pathways at level 3 of the filtered taxonomies it was found that quorum sensing was the most abundant function in all samples (36.26%) and was most abundantly contributed by *Proteobacteria* (56.23%), followed by the phylum *Firmicutes* (32.81%) and *Bacteroidetes* (7.63%) (Fig. **8**).

At genus level *Clostridium*, *Aeromonas* were the two most abundant genera contributing to biofilm pathways with 17.67%, and 14.52% relative abundances, respectively. *Clostridium* most abundantly contributed for peptidases (32.74%), *Aeromonas* to prokaryotic defense system (91.99%), while other two genera (data not shown) which were also abundant in biofilms formation including *Macellibacteroidetes* which most abundantly contributed to phenazine biosynthesis (13.05%), and *Elstera* was mostly attributed to contribute for transporters (6.76%) (Fig. **9**). While most abundant contribution of function by bacterial genera was quorum sensing, mostly contributed by Aeromonas (29.19%) (Fig. **9**). These results suggested that various bacterial genera could have contributed for the biofilms formation through various level 3 pathways.

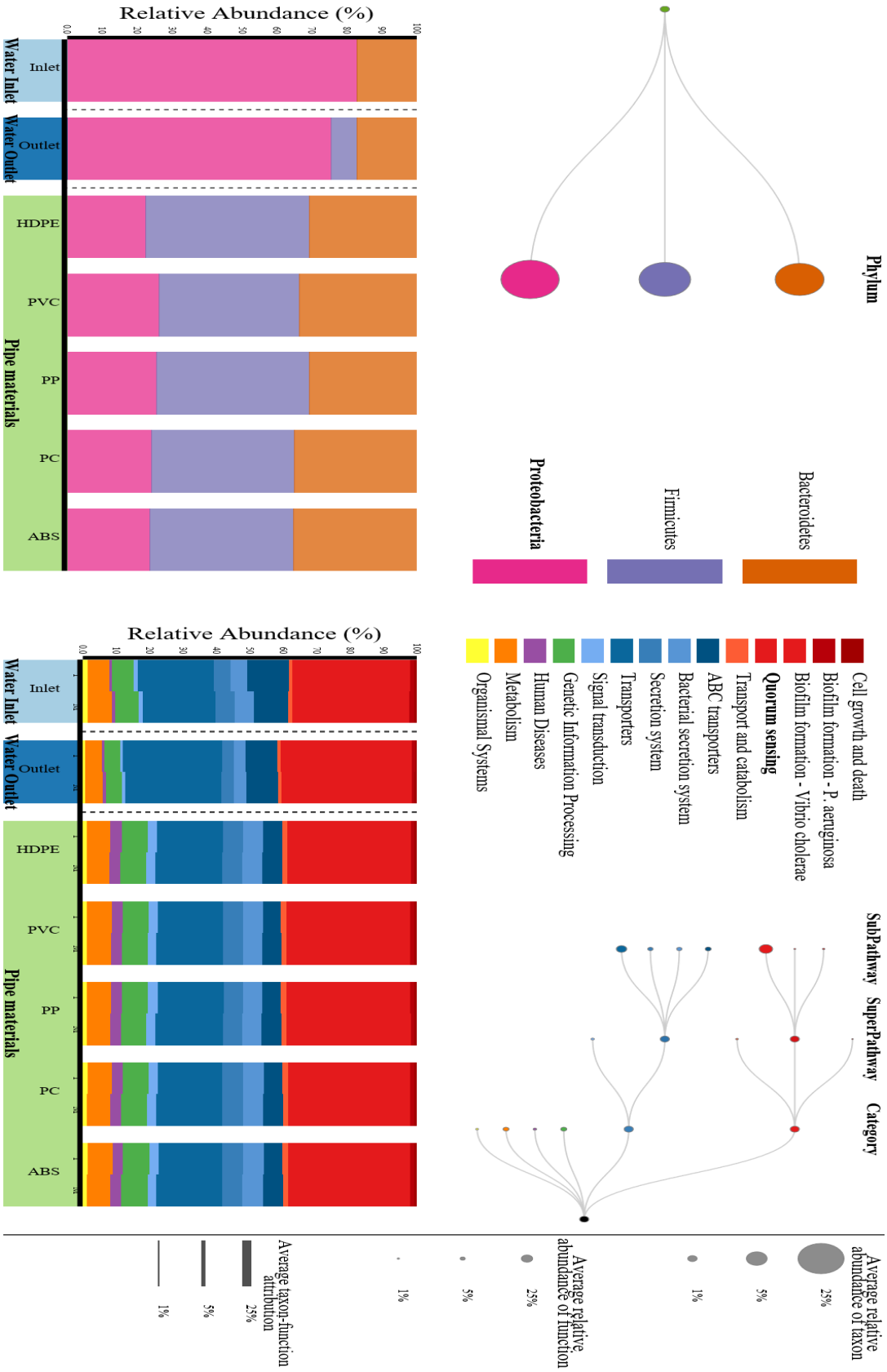

Fig. 8. Biofilm related pathways contributed by 3 most abundant bacterial phyla. Top left are bacterial phyla contributing to biofilm pathways, top right is KEGG pathways, in which 2 most abundant pathways biofilms formation pathways are extended at level 3, left down are relative abundances of bacterial phyla, whereas, left down is the relative abundances of KEGG pathways. Font bolded phylum and level 3 pathway quorum sensing were highly contributing phyla and highly attributed pathway for biofilm formation, respectively.

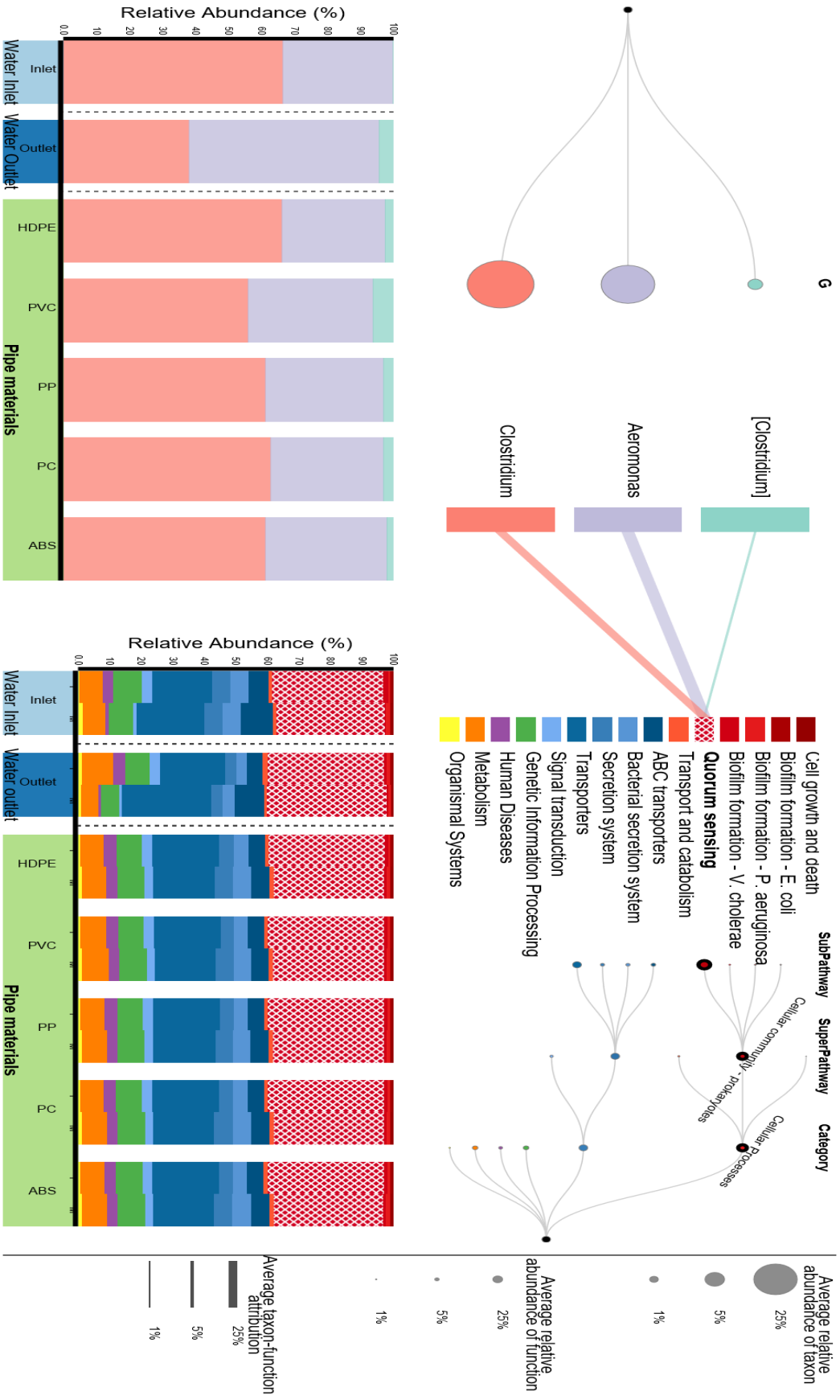

Fig. 9. Biofilm related pathways contributed by most abundant bacterial genera. Top left are bacterial genera contributing to biofilm pathways, top right is KEGG pathways, in which 2 most abundant pathways are extended at level 3, left down are relative abundances of bacterial genera, whereas, left down is the relative abundances of KEGG pathways. Font bolded level 3 pathway quorum sensing was highly attributed pathway for biofilm formation mostly contributed by *Aeromonas* genus.

DISCUSSION

During the last few decades, the understanding of biofilms has changed from the slime-entrapped innocuous aggregates of microorganisms to convoluted structures providing shelter to a variety of microbes ranging from bacteria and viruses to protozoa (Szewzyk *et al.*, 2000). It has been agreed upon that biofilms play a detrimental role in the quality of drinking water. Being able to thrive in challenging environments and resist disinfectant agents, biofilms are likely to act as hosts to opportunistic pathogen organisms (Lehtola *et al*., 2007). Biofilms could also change in color of the water (Husband *et al.*, 2008), taste and odor (Szewzyk *et al*., 2000) of water along with causing corrosion, scale formation and obstruction in pipe-flows (McNeiill and Edwards, 2001). Hence, owing to the biofilm significance, in this study its bacterial structure in biofilms was analyzed using both culture dependent and culture independent methods in different pipe materials. Furthermore, the correlations between biofilms of pipes of different material with inlet and outlet and between inlet and outlet water samples were evaluated.

All physicochemical parameters were analyzed in replicates and once in a week. Analysis of physicochemical parameters of the inlet water showed that all the parameters were under the permissible limits of the WHO (WHO, 2011a), EPA and USEPA guidelines (Kumar and Puri, 2012), except for turbidity, lead and chromium which were slightly higher than the recommended limits. The increase in lead and chromium metals is harmful for the human health. Excessive lead intake accumulates in the body and causes anemia and reproductive disorders. Chromium changes the water color to yellowish, and its high intake was reported to increase cancer mortality rate (Wang *et al.*, 2017). The increase in lead and chromium in water were also reported in 2009, in Karachi, Pakistan. The possible source of lead and chromium was untreated water discharge, directly into the Indus river, without proper treatment by the towns, industries and agriculture farms (Nadeem-ul-Haq *et al.*, 2009).

The reactor was operated with SW for 2 weeks such that the biofilms could acquire representation of all the bacteria of SW. After two weeks, the bacterial inoculum which was prepared from the SW was inoculated in the reactor. The inoculum was made by adding 100ml SW in 900 ml TSB media and incubated at 37^{o}C for 24 h for the bacterial growth. After 24 h, this media containing high abundance bacterial growth was used as inoculum for the AR and followed by 12 h operation with same SW from Mehran University treatment plant. The main purpose for media grown bacterial inoculum in the AR was to shorten the duration of the biofilms formation and let the bacteria to form biofilms effectively. Moreover, by using same SW as culture source only those bacteria were supposed to grow which were present in the SW. The 12 h post inoculum addition, the SW was continuously passed through to let bacteria form biofilms on the surfaces of the slides, to simulate the real shear stress of the drinking water distribution system, and to remove the TSB media from the reactor slowly such that bacteria could form biofilms at given 12 hours' time. The inoculum of bacteria has grown enough biofilm which could be processed for further analysis.

The comparison of 16S rDNA-based amplicon sequencing and HPC showed consistent results for the PVC and ABS pipe materials. Both PVC and ABS were found to be the least and most prone to biofilm growth, respectively. However, sequencing results differed from the results of HPC in sample HDPE as in HPC, HDPE was found to be the 2nd least susceptible material to biofilm formation. However, in sequencing results it harbored the most bacteria after ABS. Likewise, in sequencing analysis, PC was the 2nd least prone to biofilm formation among the five plastic materials, but HPC analysis showed it to be the most prone to biofilm formation after ABS. These difference in the number of bacteria attached to different materials agrees with the already published research finding that surface properties play a significant role in the attachment rate of microorganisms. For instance, Chang *et al* reported that materials with greater surface roughness (i.e. galvanized steel and cast iron) support greater biofilm formation than smooth materials (i.e. PVC) (Chang *et al*., 2003). Generally, rough and hydrophobic surfaces strengthen and accelerate the microbial attachment due to the diminished shear effects and greater surface area (Donlan, 2002). Moreover, these differences can be attributed to unclassified bacteria, since their culture conditions are unknown so there are number of bacteria which still need to be classified (Burtscher *et al.*, 2009). These finding were also reported in which greater than 65% genera were found unclassified in pyrosequencing (Chao *et al.*, 2015).

In similarity search against greengenes data base (version 13.8) in QIIME2 environment, phylum *Proteobacteria* was found most abundant in Inlet and Outlet sample. Its presence in such a high relative abundance in drinking water distribution systems is extensively reported. For instance, Hou et al evaluated the spatiotemporal changes in bacterial community in a drinking water distribution system and found *Proteobacteria* as a dominant phylum (Hou *et al*., 2018). Moreover, same trend were also observed in a findings while assesing the abundance of bacterial community in the multi-step filtration water treatment plant (Lautenschlager *et al*., 2014). Likewise, Phylum *Bacteroidetes*, which was the second most abundant phylum in inlet and outlet water sample, and *Actinobacteria*, which was abundant in inlet sample, are well known fresh water inhabitants too (Eichler *et al*., 2006; Li *et al*., 2010). While presence of *Cyanobacteria* was also reported in many studies in comparatively low

percentages (Douterelo *et al*., 2016). An upsurge in the abundance of *Cyanobacteria* has been observed extensively in the surface waters due to the increase in nutirent concentration (He *et al*., 2016; Szlag *et al.*, 2015; WHO, 2011b). They produce cyanotoxins with can cause gastroenteritis, neurotoxicity and inflammation of eyes, skin and respiratory tract. Their presence is also marked with taste and odor problems and generate consumer complaints (WHO, 2011b). Other phyla, including *Planctomycetes, Verrucomicrobia, Spirochaetes, Chlorobi, Acidobacteria, Chlamydiae, Gemmatimonadetes, Chloroflexi, Nitrospirae*, *Firmicutes*, etc., were also present in the inlet water sample. These bacterial phyla present in this study exhibit striking resemblance with the community found in the study conducted in 2015 in which NGS was used to investigate microbial community in biofilms (Chao *et al*., 2015).

Only three phyla were abundant in the biofilms attached to the plastic slides installed in the bioreactor. Those were *Firmicutes, Bacteroidetes* and *Proteobacteria* in descending order. The high abundance of Firmicutes in the outlet water sample suggests that detached microbes from the plastic slides were entering the outlet water sample. Furthermore, high abundance of *Bacteroidetes* and *Proteobacteria* on plastic slides, agrees with a study (Rożej *et al*., 2015). In this study, they compared the biofilm potential of three plastic materials including PVC, HDPE and PEX and found these two phyla in the biofilm samples taken from all the three plastic materials. Although, Firmicutes was the most abundant phylum in the biofilm samples in our study, irrespective of the type of material, however, no Firmicutes were present in the biofilms in the Rożej study (Rożej *et al*., 2015). This might be due the different characteristics of SW or difference in the method employed for the characterization of microbial diversity as Rozej et al used PCR-DGGE based characterization, while 16S rDNA based taxonomic characterization was carried in this study. Moreover, Firmicutes are reported in the drinking water distribution systems (Eichler *et al*., 2006; Li *et al*., 2010).

However, in 16S rDNA based functional predictions through PICRUSt, *Proteobacteria* was most abundantly contributing to the biofilm pathways, followed by *Firmicutes* and *Bacteroidetes*. To our best knowledge this is the first-time functional predictions based on 16S rDNA through PICRUSt. PICRUSt results at phylum level as well as at genus level suggested that quorum sensing was most probable function of the bacteria to form the biofilms on the pipe material.

Class *Clostridia* belongs to the phylum *Firmicutes* which is also the most abundant phylum in the biofilms formed on plastic slides. *Bacteroidia, Gammaproteobacteria* were also found in abundance in biofilms from all the materials. Species belonging to class *Bacteroidia* are known to be the part of mammalians' gut microbiota. Genus *Bacteroide* belonging to Class *Bacteroidia* is well-known as host specific fecal indicator because of its ability to be specific to the digestive environment of host animal. The probability of its growth in outside environment is very low and considered an authentic indicator of recent pollution (Ibekwe *et al.*, 2016). Alpha and beta proteobacteria were found abundant in inlet water and outlet water but *Gammaproteobacteria* was found in high percentage in biofilm material. Previous researches reported higher abundances of alpha, beta and gamma proteobacteria, in bacterial communities of DWDS as well as in biofilm (Mathieu *et al.*, 2009; Tokajian *et al.*, 2005). However, in this study we only found gamma proteobacteria to be in abundance in biofilms. *Planktophila* is an Actinobacterium found in abundance in fresh aquatic systems. It was never considered numerically important taxa in fresh water systems in the past due to the use of culture-dependent methods of characterization, however, in the past two decades, it has been identified as one of the most abundant bacterioplankton as a result of the employment of cultivation-independent techniques (Jezbera *et al.*, 2018).

Genus *Clostridium*, belonging to phylum Firmicutes, was observed as most abundant genus in biofilms of all pipe materials. Which suggested that this genus might had the higher potential for biofilm formation on different pipe materials. In 16S rDNA based functional predictions the pathways which were associated with biofilm were mostly contributed by *Clostridium*, which suggested that potentially *Clostridium* contributed in biofilm formation (Fig 9). The most abundant pathway predicted for *Clostridium* was peptidases. Peptidases break the proteins into peptides and amino acids. Two main function related to biofilm and peptidases were reported as 1) numerous peptidases were suggested to provide amino acid to biofilm cells (Poquet *et al*., 2018), 2*) Clostridium difficile* reported for example, to modulate its adhesion with surface while secreting Pro-Pro endopeptidase PPEP-1, a type of peptidases, to cleaving collagen binding protein (Hensbergen *et al*., 2015). This is also consistent to other studies as *Clostridium* species have been found to be involved in the biofilm formation in varying environments (Bouttier *et al.*, 2014). These species produce spores which help them persist in adverse conditions and many of them are well known pathogens. The presence of *Clostridium perfringens* in the intestines of humans and animals makes it an important indicator of fecal contamination in the water (UK Standards for Microbiology-Clostridium Species, 2016). It has also been associated with gas gangrene in humans and animals (Shrestha and Mcclane, 2015). Hence, its presence in the drinking water of MUET is a matter of concern and it might indicate fecal contamination in the SW. Furthermore, ingestion of water contaminated with species related to genus *Aeromonas* could cause gastroenteritis infections. They may also the cause wound infections if wound get exposed to the contaminated water (Parker and

Shaw, 2011). Moreover, *Aeromonas* was predicted as the second most abundant genus which could have contributed to the biofilm formation in functional predictions through PICRUSt. Whereas *Aeromonas* most likely had contributed to quorum sensing. The biofilm formation by *Aeromonas hydrophila* through quorum sensing was also reported in another study on stainless steel (Lynch *et al*., 2002).

CONCLUSION

This study indicated the difference in the number and diversity of bacteria in the biofilms scraped from five different plastic pipe materials namely, HDPE, PC, PVC, PP and ABS. ABS plastic was found to be the most prone to the bacterial colonization in terms of biofilm formation, as it harbored the maximum number of bacteria (HPC counts) and 16S amplicon sequencing and had the high diversity index. On the other hand, PVC showed least potential for the biofilm formation. Hence, in terms of suitability of plastic material to be used for water supply system within buildings, considering the biofilm formation affinity, which is the potential cause of water quality deterioration, should be employed. This study showed that the five types of plastic materials possessed affinity for biofilm formation in the descending order as ABS>HDPE>PP>PC>PVC. Our findings suggest that PVC pipes are the most suitable for DWDS and ABS being the least. Moreover, high abundance of *Clostridium spp.* in biofilm samples might have contributed mostly to biofilm formation as it was seen in PICRUSt analysis. The most abundant function was quorum sensing pathway, which was mostly contributed by *Aeromonas* genus. Additionally, the bacteriome in the inlet water, the biofilms, and the outlet water show that biofilms formed on the pipes of different materials release bacteria in the drinking water.

RECOMMENDATIONS

Forgoing in view, we suggest that the concerned authorities should make stringent measures to implement the standards of WHO and/or EPA Pakistan guideline to operate the Mehran University treatment plant to start its water-treatment operation. Moreover, the least potential of PCV for supporting biofilms formation may be replicated in-real DWDS setup with same drinking water shear stress, pH, temperature, and water flow time/contact time in order to get conclusive findings regarding better plastic material to be used for the DWDSs and inside the buildings.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CONTRIBUTIONS

Junaid Ahmed Kori helped in library preparation, did data analysis, and manuscript writeup. Huma Tariq carried the lab experiments, analyzed the data, and wrote the manuscript Muhammad Raffae Vistro helped in lab experiments, in data analysis and in manuscript write-up. Rasool Bux Mahar designed and supervised the project. Ishtiaq Ahmad Khan and Muhammad Shakeel helped in sequencing. Ramesh Goel co-supervised the project.

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