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Identification and Quantification of the Bacterial Community on the Surface of Polymeric Membranes at Various Stages of Biofouling Using Fluorescence *In Situ* Hybridization

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The bacterial community on the surface of two polymeric membranes, polyvinylidene fluoride (PVDF) and polyethylene (PE), was investigated using fluorescence *in situ* hybridization. Three fouling states were defined during the study: low fouling (TMP < 10 kPa), fouled (TMP > 20 kPa), and extremely fouled (TMP > 40 kPa), and the changes in the microbial communities on the membrane surfaces were monitored with the progress of fouling. Results showed that the polyethylene membranes exhibited a faster fouling tendency compared to the polyvinylidene fluoride membranes. Although the same microbial groups were found on the PVDF and PE membrane samples, their community dominance exhibited different trends with the advent of fouling.

Keywords biofouling; fluorescence *in situ* hybridization; inclined plate membrane bioreactor; municipal wastewater; polymeric membranes

INTRODUCTION

A membrane separation bioreactor (MBR) combines the biological degradation capability of microorganisms and the separation ability of a membrane to treat various wastewaters (1,2). For municipal wastewater treatment, the use of MBRs has increased in recent years as it provides a compact system and it can reliably produce high effluent quality for water reuse or as pretreatment for more advanced processes such as nanofiltration or reverse osmosis. By their nature as filters, membranes are prone to fouling, which often constrains its use.

Fouling reduces the permeability of the membrane, and can lead to continuous flux decline and increased energy consumption (3,4). Severely fouled membranes necessitate cleaning by costly chemical reagents or replacement in order to continue operation. Understanding fouling and significantly controlling it could greatly improve membrane performance and reduce the maintenance and cleaning costs associated with fouling. Different aspects of membrane fouling have already been studied (3); however, the microbial communities that potentially contribute to this phenomenon have received limited attention. Some studies have been done with regard to the microbial communities in an MBR; however, the information on the structure and diversity of these communities treating domestic wastewater is still lacking. Witzig et al. (5) and Luxmy et al. (6) investigated the microbial communities of suspended solids in an MBR. Even more limited studies have been conducted focusing on the microbial communities on the membrane surface (7,8). The objective of this study is to identify and quantify the bacterial communities on the surface of two polymeric membranes during different stages of biofouling using fluorescence *in situ* hybridization (FISH).

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MATERIALS AND METHODS

Reactor Set-up

An inclined plate membrane bioreactor (iPMBR) was utilized for this study. The iPMBR was composed of two tanks in series, the upstream tank under anoxic condition and the downstream tank under aerobic condition. A schematic diagram of the system is shown in Fig. 1.

Feed water from the primary sedimentation basin of a Tokyo based sewage treatment plant first entered the system through the anoxic tank. The anoxic tank was constructed with several inclined plates installed within it. Liquor from the anoxic tank overflowed to the downstream aerobic tank via weir channels. A hollow-fiber, polyvinylidene fluoride (PVDF) membrane module (with surface area of 2.2 m^2 and pore size of 0.4 μm) was submerged inside the

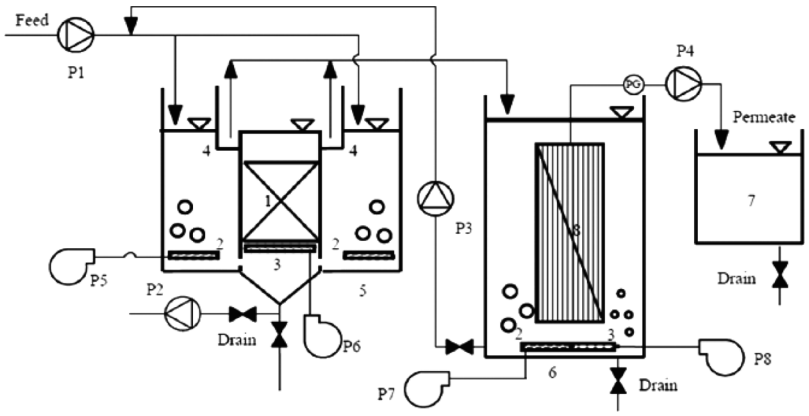

FIG. 1. Schematic diagram of the iPMBR. The notations on the image correspond to: P1 – Feed pump; P2 – Anoxic sludge pump; P3 – Internal recycle pump; P4 – Permeate pump; P5 and P7 – Air pumps for coarse bubble diffusers; P6 and P8 – Air pumps for fine bubble diffusers; 1 – Inclined plates; 2 – Coarse bubble diffusers; 3 – Fine bubble diffusers; 4 – Double-sided weir; 5 – Anoxic reactor; 6 – Aerobic reactor; 7 – Permeate tank and 8 – Membrane module.

aerobic tank. Beneath the membrane module, fine and coarse bubble diffusers were positioned to supply oxygen to the mixed liquor and to clean the surface of the membrane fibers by moving them and providing scouring action. The air flow rate was controlled by regulators attached to the pumps. A constant flux was maintained across the membrane module and filtration was carried out intermittently. Treated water was sucked through the membrane as a result of a partial vacuum created by a peristaltic pump. The transmembrane pressure (TMP) across the membrane was monitored by a digital vacuum gauge.

Operating Conditions, Polymeric Membranes and Auxiliary Tank

The iPMBR was operated under complete sludge retention for 458 days. No sludge was taken out from the system except for sampling. To investigate the microbial communities on the membrane surface, a batch experiment was conducted for 71 days using mini-membrane modules. Mini-membranes are smaller sized membrane modules that can be used for sampling purposes. For this study, two types of mini-membranes were used—polyvinylidene fluoride (PVDF) and polyethylene (PE). Each module of the former had a surface area of $0.07\,m^2$ and a pore size of $0.4\,\mu m$ while each module of the latter had a surface area of $0.03\,m^2$ and a pore size of $0.4\,\mu m$. Due to space limitations in the main aeration tank, another smaller tank was added to the system. This smaller, auxiliary tank was designed to accommodate several mini-membranes at a time. Diffusers were installed at the bottom of the tank to provide constant aeration to the mini-membrane modules. Mixed liquor from the main aeration tank was re-circulated to the smaller auxiliary tank by a peristaltic pump. MLSS as well as dissolved oxygen concentrations in the auxiliary tank were monitored to keep within range to that in the main aeration tank. Overflow from the auxiliary tank was returned to the main aeration tank through a weir channel at the side of the smaller tank. Each membrane was operated at a constant flux of $0.27\,m^3/m^2$-day.

The sampling times of the mini-membranes coincided with defined fouling phenomena occurring in the membrane to follow the progress of fouling. Three fouling states were identified based on the transmembrane pressure (TMP), which is often used as an indicator for fouling. In this study the following criteria were used: low fouling state, $TMP < 10\,kPa$; fouled state, $TMP > 20\,kPa$, and extremely fouled state, $TMP > 40\,kPa$. The samples were taken at different fouling states to follow the development of the biofouling in terms of changes in the microbial communities.

Sampling

Sludge samples from the tanks were taken for solid content analysis. The concentration of mixed liquor suspended solids (MLSS) were measured using standard methods (9). Dissolved oxygen (DO) in the tanks was determined using a DO-100 meter (Iijima Electronics Corporation, Japan). Sludge samples were also collected from the iPMBR for microbiological analysis. For membrane sampling, fibers from the PE and PVDF mini-membranes were cut, treated, and stored for further analysis.

Fluorescence *In Situ* Hybridization

The procedures for fluorescence *in situ* hybridization (FISH) as described by Amann (10) were followed with some modifications. Fixation, immobilization on microscope slides, dehydration, *in situ* hybridization, and washing were undertaken. For the liquor samples, fixation was carried out as per protocol using 4% paraformaldehyde

TABLE 1
Oligonucleotide probes used

Probe	Sequence (5′–3′)	Specificity	FA[a] (%)	Target position[b]
EUB338[c]	GCTGCCTCCCGTAGGAGT	Eubacteria	0–50	16S rRNA, 338–355
EUB338II[c]	GCAGCCACCCGTAGGTGT	Planctomycetales	0–50	16S rRNA, 338–355
EUB338III[c]	GCTGCCACCCGTAGGTGT	Verrucomicrobiales	0–50	16S rRNA, 338–355
ALF968	GGTAAGGTTCTGCGCGTT	Alphaproteobacteria	20	16S rRNA, 968–985
BET42a[d]	GCCTTCCCACTTCGTTT	Betaproteobacteria	35	23S rRNA, 1027–1043
GAM42a[e]	GCCTTCCCACATCGTTT	Gammaproteobacteria	35	23S rRNA, 1027–1043
SRB385[f]	CGGCGTCGCTGCGTCAGG	Deltaproteobacteria[g]	35	16S, 385–402
SRB385Db[f]	CGGCGTTGCTGCGTCAGG	Deltaproteobacteria	35	16S, 385–402
GNSB941[h]	AAACCACACGCTCCGCT	Chloroflexi	35	16S, 941–957
CFX1223[h]	CCATTGTAGCGTGTGTGTMG	Chloroflexi	35	16S, 1223–1242
CF319a	TGGTCCGTGTCTCAGTAC	Bacteroidetes	35	16S rRNA, 319–336

[a]Formamide concentration used in the hybridization buffer.
[b]Position numbering based on the rRNA of Escherichia coli.
[c,f,h]Applied together in equal amounts.
[d]Applied together with an equal amount of the unlabelled probe GAM42a as a competitor to enhance specificity.
[e]Applied together with an equal amount of the unlabelled probe BET42a as a competitor to enhance specificity.
[g]Targeting sulfate reducing bacteria within the Deltaproteobacteria.

solution. For the membrane samples, the fibers with biofilms were cut into approximately 10-mm pieces using a sterile cutter knife. These cut pieces were then fixed with 4% paraformaldehyde solution. After fixation, the membrane samples were vortexed and sonicated at low power for 2 minutes to detach the microbial cells attached on the membrane. The samples were immobilized on gelatin-coated glass slides, air-dried and dehydrated by successive ethanol washes of 50%, 80%, and 98% concentration. The 16S and 23S rRNA-targeted oligonucleotide probes were obtained from Operon Biotechnologies, Inc., Tokyo, Japan. The probes were labeled with fluorescein isothiocyanate (FITC) or sulfoindocyanine dye (Cy3) at the 5' end. Unlabelled probes were also used to enhance the specificity of some probes. The group specific probes included ALF968, BET42a, GAM42a, SRB385/SRB385Db, GNSB941/CFX1223, and CF319a. The specific probes together with the EUB338 mixed probes (EUB338, EUB338II, and EUB338III) detecting almost all *Eubacteria* were applied simultaneously during the hybridization process. Additional information about the probes used are listed in Table 1. The hybridization buffer was prepared having the composition 0.9 M NaCl, 20 mM Tris-HCl at pH 7.2, 0.01% sodium dodecyl sulfate, and formamide at the concentrations appropriate for the probes used (20–35%). Hybridization was done at 46°C for 2–3 hours in an equilibrated sealed moisture tube. The final probe concentration applied was approximately 1 μM. Afterwards, a washing step was performed at 48°C for 20 minutes in 50 mL of pre-warmed washing solution (0.07 M to 0.2 M NaCl, 20 mM Tris-HCl at pH 7.2, 0.01% sodium dodecyl sulfate). Following hybridization and washing, the glass slides were air-dried. Anti-fading solution (Slow Fade Light, Molecular Probe, Eugene, OR) was applied before putting on the slide cover.

Fluorescent images were recorded with an epifluorescence microscope (BX51, Olympus, Japan) equipped with a charge-coupled device (CCD) camera (DP70, Olympus, Japan). Quantification was carried out using a Leica Qwin image analysis software V2.3 (Leica Microsystems, Wetzlar, Germany). The software detects the area occupied by probe hybridized cells. For each sample, twenty different microscopic fields were randomly chosen and for each field, the ratio of the area of those cells labeled by the specific probe to the area of all bacterial cells stained by an EUB probe mix (EUB338, EUB338II, and EUB338III) was determined. The average and standard deviations of these ratios were calculated.

RESULTS AND DISCUSSION

Mini-Membrane Experiment

During the batch experiment using mini-membranes, six modules (3 PE and 3 PVDF) were immersed in the auxiliary tank receiving recycled mixed liquor from the main aerobic tank. The experiment was run for 71 days. Each membrane was operated at a constant flux of $0.27\,m^3/m^2$-day. The change in TMP for all the mini-membranes was monitored. Additionally, conditions at the auxiliary tank (MLSS and DO) were also checked to ensure that they were comparable to those in the aerobic tank. Figure 2 shows the MLSS and DO concentrations in the aerobic and auxiliary tanks. MLSS and DO

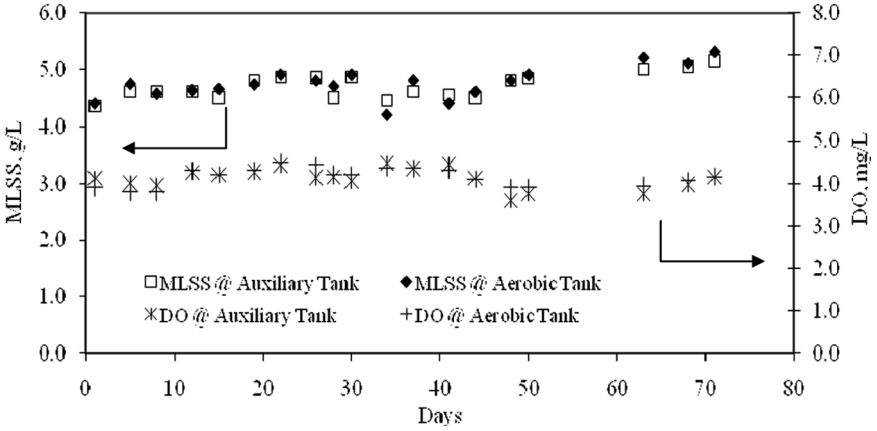

FIG. 2. MLSS and DO concentrations at the aerobic and auxiliary tanks. Comparable MLSS and DO values were measured at both tanks.

concentrations did not vary significantly between the tanks. The MLSS in the aerobic tank during the 71 days of operation varied from 4.2 to 5.3 g/L. On average the MLSS was about 4.8 g/L. Dissolved oxygen on the other hand varied from 3.8 to 4.5 mg/L, averaging 4.1 mg/L. In the auxiliary tank the MLSS ranged from 4.4 to 5.2 g/L with an average of 4.7 g/L. Dissolved oxygen ranged from 3.6 to 4.5 mg/L, averaging 4.1 mg/L.

The change in the transmembrane pressure across each mini-membrane was detected using a digital vacuum gauge. For this experiment, three fouling states based on the transmembrane pressure were identified. The following criteria were used: low fouling state, TMP < 10 kPa; fouled state, TMP > 20 kPa and extremely fouled state, TMP > 40 kPa. The TMP measurements for each membrane were recorded to check whether any of the TMP criterion was reached. Once a mini-membrane registered a TMP within the specified range, it was taken out of the tank for sampling. No physical cleaning was conducted for any of the membranes. They remained within the auxiliary tank until another TMP criterion was reached. Filtration was not interrupted also for the remaining modules. Two sets of 3 PE and 3 PVDF mini-membranes were used. For each set of membranes, one was sampled at the low fouling state, one at the fouled state, and one at the extremely fouled state. Membrane sampling was conducted whenever a TMP criterion was met. Liquor samples were also taken simultaneously from other parts of the reactor. Figure 3 shows the changes in TMP across the PE and PVDF mini-membranes.

PE mini-membranes labeled PE-A, PE-B and PE-C, and PVDF mini-membranes labeled PVDF-A, PVDF-B, and PVDF-C were submerged in the tank. During the initial days of operation, the membranes were monitored for the low fouling state, TMP < 10 kPa. The first PE membrane and first PVDF membrane to meet this criterion were taken out for sampling. After 12 days of operation, the TMP for PE-B reached 9 kPa while the rest remained below 7 kPa. At this time, PE-B had met the criterion of the low fouled state and it was taken out for sampling. The rest of the membranes remained in the tank for further monitoring. On Day 22, PVDF-A reached a TMP of 9 kPa and it was sampled. For the next several days, the remaining membranes were monitored for those reaching TMP > 20 kPa. On Day 28, PE-A showed a significant jump in TMP from a previous measurement of 16 kPa to 23 kPa. PE-A was removed from the tank and sampled. On Day 37, PVDF-C showed an increase in TMP (from 16 kPa to 25 kPa) and was taken out for sampling. The two membranes left in the tank were monitored for TMP > 40 kPa. It took 41 days for PE-C to reach 44 kPa and 71 days for PVDF-B to reach 42 kPa. When TMP > 40 kPa was reached the membranes were taken out for sampling. Every time membrane sampling was conducted, liquor samples from various parts of the system were also collected. Based on

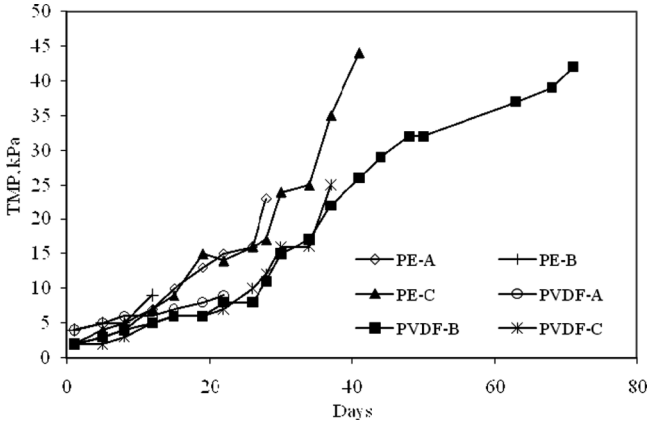

FIG. 3. TMP variation across the PE and PVDF mini-membranes. Two sets of 3 PE and 3 PVDF membranes were monitored and sampled during low fouling state (TMP < 10 kPa), fouled state (TMP > 20 kPa), and extremely fouled state (TMP > 40 kPa). Membranes were sampled at the end of each curve above.

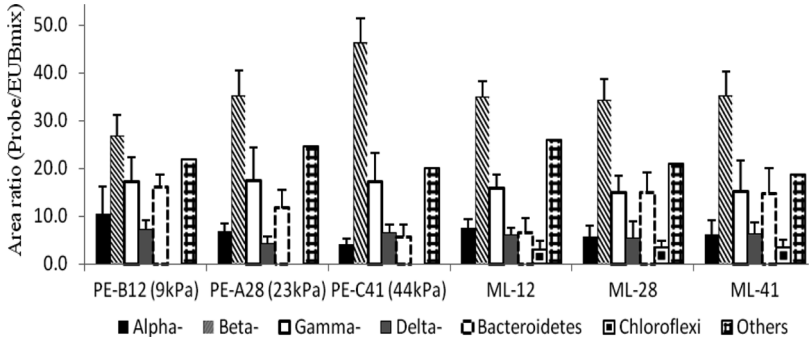

FIG. 4. Relative abundance of groups in the PE biofilm and mixed liquor samples taken on Days 12, 28 and 41.

Fig. 3, the PE membranes showed a faster tendency for fouling compared to the PVDF membranes. At each TMP criterion, the PE membranes were always the first to become fouled.

Bacterial Community Identification and Quantification Using FISH

Fluorescence *in situ* hybridization was applied to the liquor and membrane biofilm samples obtained from the iPMBR. However, this section will focus mostly on the results obtained from the membrane biofilm samples. The following sample naming convention was used: i.e., PE-B12 – PE membrane B sampled on day 12, PVDF-A22 – PVDF membrane A sampled on day 22, ML-12 – Mixed liquor sampled on day 12, etc.

The PE membranes exhibited a faster fouling tendency compared to the PVDF membranes as shown in Fig. 3. They were sampled on Days 12, 28, and 41 corresponding to the low fouling, fouled, and extremely fouled states respectively. Figure 4 shows the relative proportions of the major groups detected in the PE biofilm sample as well as the mixed liquor, which was sampled at around the same time as the membrane.

The bars in Fig. 4 are clustered according to sample (PE-B12, ML-12, etc.). Beside the PE sample names, the transmembrane pressure at the time of sampling are also indicated. Each column shows the degree of detection of the bacterial groups (Alpha-, Beta-, Gamma-, etc.).

In the case of the PE biofilm sample, the advent of fouling was indicated by the increase in TMP from 9, 23 to 41 kPa. *Betaproteobacteria* is seen to predominate the community increasing in detection from 27% to 47% (Fig. 5). *Bacteroidetes*, on the other hand, showed a decrease in numbers with increase in TMP (from 16% to 5%). *Gammaproteobacteria* remained relatively stable whatever the TMP. *Chloroflexi* was not detected in the membrane samples and was not included in the figure. In the mixed liquor, the percentages of *Betaproteobacteria* and *Gammaproteobacteria* remained relatively stable at ~35% and ~17%, respectively. *Bacteroidetes* increased in the mixed liquor from 7 to 15%. While undetected in the membrane biofilm samples, *Chloroflexi* was identified in the mixed liquor although at low levels (~3%).

A study by Miura et al. (8) reported a similar dominance of the *Betaproteobacteria* when they analyzed biofilm samples taken from an MBR treating municipal wastewater. They observed this predominance, however, when aeration was increased in the MBR. At a higher aeration rate, *Betaproteobacteria* became the most dominant phylogenetic group in the biofilms. They could detect the group accounting for as much as 70% of EUB stained cells. Even after the membrane has been chemically cleaned, *Betaproteobacteria* became the dominant group on the biofilm after some time. They attributed the dominance of the *Betaproteobacteria* to their ability to overcome the shear stress provided by higher aeration and continue thriving on the membrane surface.

In this study, *Betaproteobacteria* was already dominant during the low fouled state and the group persisted throughout the operation. No physical cleaning was conducted during the experiment and the operating conditions were not changed in the middle of the operation. The group's dominance until the extremely fouled state could primarily be accounted to its continued growth in the biofilm. Once attached on the membrane, they could adapt and thrive to the conditions surrounding the membrane.

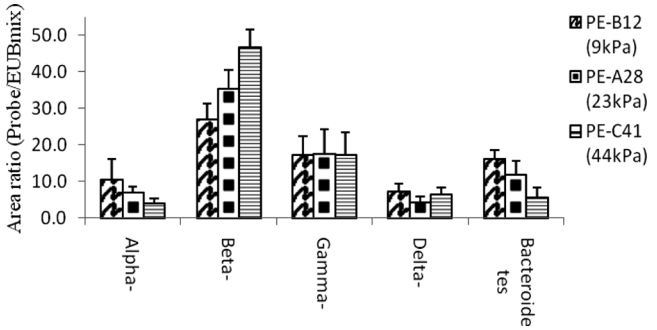

FIG. 5. Relative abundance of groups in the PE membrane biofilm samples taken on Days 12, 28 and 41.

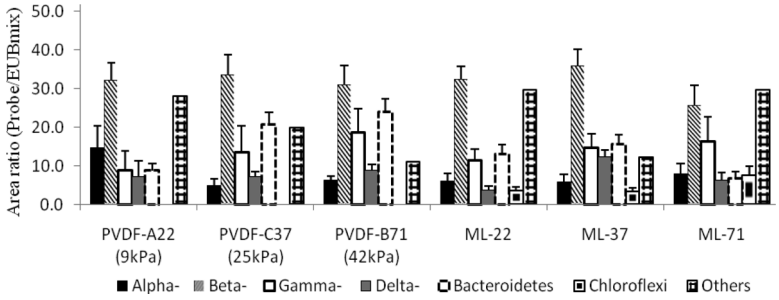

FIG. 6. Relative abundance of groups in the PVDF membrane biofilm and mixed liquor samples taken on Days 22, 37, and 71.

Their high presence in the biofilm suggests that they probably contributed significantly to fouling in this study.

The PVDF membranes were sampled on Days 22 (TMP = 9 kPa), 37 (TMP = 25 kPa), and 71 (TMP = 42 kPa) for the low fouling, fouled, and extremely fouled states respectively (Fig. 6). For the PVDF biofilm, *Betaproteobacteria* distribution in the community seemed stable with the advent of fouling (average values around ~32%). The detection of *Bacteroidetes*, on the other hand, increased with the rise in TMP (from 9% to 24%). The same trend was observed with *Gammaproteobacteria* (increasing from 9 to 19%) Fig. 7. *Chloroflexi* was not detected in the PVDF biofilm samples similar to the PE biofilm. The observation about *Chloroflexi* is supported by the results of the PCR-cloning-sequencing experiment (data not shown). No clone close to *Chloroflexi* was derived from the PVDF membrane biofilm sample. However, the rest of the samples had one or two clones affiliated with *Chloroflexi.* In the mixed liquor, the distribution of *Betaproteobacteria* was fluctuating with time but generally had the highest proportion compared to others (between 26% to 36%). The detection of *Gammaproteobacteria* in the mixed liquor increased as fouling progressed on the membrane. *Chloroflexi* was also identified in the mixed liquor albeit at low levels (from 3 to 8%).

Unlike in the PE samples, the proportion of *Betaproteobacteria* in the PVDF biofilm community remained relatively stable with the progress of fouling. *Bacteroidetes* and *Gammaproteobacteria,* however, were observed to be increasing as fouling progressed. In the PVDF membranes, these groups probably contributed significantly to fouling.

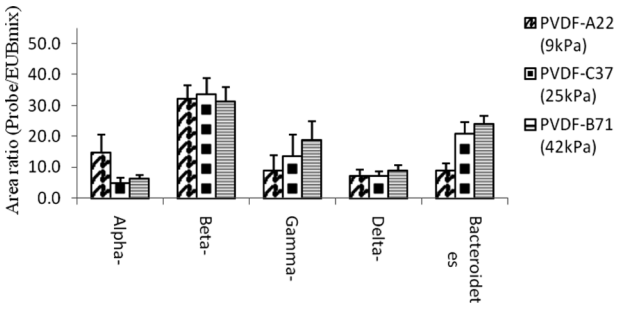

FIG. 7. Relative abundance of groups in the PVDF membrane biofilm samples taken at Days 22, 37, and 71.

CONCLUSIONS

During the study, the PE membranes exhibited a faster fouling tendency compared to the PVDF membranes. Comparing the two membranes, although the same groups were found in the samples, their dominance exhibited different trends with the advent of fouling. For the PE membrane biofilm, *Betaproteobacteria* increased in detection while *Bacteroidetes* decreased with increase in TMP. *Gammaproteobacteria* remained relatively stable whatever the TMP. For the PVDF membrane biofilm, *Betaproteobacteria* remained stable with the advent of fouling while the detection of *Bacteroidetes* and *Gammaproteobacteria* increased with the rise in TMP. Both types of membranes were put in the same tank receiving the same mixed liquor; however, they showed some difference in microbial community. Possibly, membrane material and configuration had some effect on how the microbial community dominance evolved.

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