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Bacterial community structures in MBRs treating municipal wastewater: Relationship between community stability and reactor performance

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ABSTRACT

Bacterial community structures in pilot-scale conventional membrane bioreactors (CMBRs) and hybrid MBRs (HMBRs) which were combined with pre-coagulation/sedimentation were analyzed by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescence in situ hybridization (FISH) techniques. The results were compared with the community structure in a full-scale activated sludge (AS) process treating the same municipal wastewater. The Dice index (Cs) of similarity analysis of DGGE banding patterns demonstrated that the microbial community in AS was more similar to those in CMBR1 and CMBR2 than HMBR1 and HMBR2. This suggested that influent wastewater composition had a larger impact on bacterial community structures. Long-term community structure changes in the HMBRs and CMBRs were monitored and analyzed over 240 days by Non-metric multidimensional scaling (NMDS) analysis of DGGE banding patterns. The NMDS analysis revealed that both HMBRs and CMBRs had marked changes in community structures during the first about 100 days. Thereafter the perpetual fluctuations of bacterial community structures were observed in both HMBRs and CMBRs, even though the stable MBR performances (the performance was measured as membrane permeability and removal of dissolved organic carbon, DOC) were achieved. These results suggest that not only the stability, but also the adequate dynamics ("flexibility") of the bacterial community structure are important for the stable performance of the MBRs treating complex municipal wastewater.

1. Introduction

Submerged membrane bioreactor (MBR) is increasingly becoming an important innovation in biological wastewater treatment, because they offer several advantages, e.g., high biodegradation efficiency, smaller footprint, and less sludge production (Fan et al., 2005; Howell et al., 2003). However, by their nature as membrane filters, membranes are prone to fouling as a result of interactions between the membrane and the mixed liquor. The membrane fouling significantly reduces

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the overall membrane performance, increases operating costs and shortens membrane life (Fan et al., 2005). The membrane fouling can be considerably suppressed by installing a coagulation/sedimentation process as pre-treatment for MBRs, since a large fraction of organic contaminants in raw municipal wastewater is associated with particles (>0.1 μm) (Itonaga and Watanabe, 2004).

MBRs have a longer solid retention times (SRT) and a lower F/M ratio as a consequence of complete biomass retention. This promotes endogenous decay (autolysis) of the retained biomass, which allows release of a large amount of dissolved organic matter (DOM). The DOM is in general slowly biodegradable and is thought to cause membrane fouling. Thus, the operating conditions of MBR (e.g., MLSS concentrations, HRT, SRT, and aeration rate) must be optimized to sufficiently reduce the DOM concentration, which consequently suppresses the membrane fouling and improves the permeate water quality. We therefore hypothesized that a complete food wed (i.e., microbial community) must be established for stable performance (i.e., degradation of DOM) of MBRs. However, to date, most of the related studies have focused mainly on treatment performance of MBRs and membrane fouling without considering the bacterial communities involved. Although a few researchers have investigated bacterial communities of MBRs (Luxmy et al., 2000; Stamper et al., 2003; Witzig et al., 2002), the information on structure, diversity, and stability of bacterial communities in MBRs treating domestic wastewater is still largely limited. This is partly because many previous studies have been performed with synthetic wastewater and relatively short operating times. Therefore, development of structure and diversity of bacterial community in MBRs treating municipal wastewater during a long-term operation has not been investigated yet using molecular-based approaches. Culture independent molecular techniques have been providing significant insights into bacterial communities in wastewater treatment processes in recent years (Wagner and Loy, 2002). A better understanding of structure and dynamics of bacterial community is essential to optimize the operating conditions of MBRs and to control membrane fouling.

The primary purpose of this research is, therefore, to monitor the bacterial communities of pilot-scale conventional membrane bioreactors (CMBRs) and conventional MBRs with pre-coagulation/sedimentation (hereafter called hybrid MBRs: HMBRs) treating municipal wastewater by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) and fluorescence in situ hybridization (FISH). The results were compared to that obtained from a conventional activated sludge (AS) process treating same municipal wastewater. Additionally, we discussed the relationship between the performance of the MBRs and the stability of bacterial communities during long-term operations.

2. Materials and methods

2.1. *Membrane bioreactors*

Fig. 1 shows a flow chart of pilot-scale MBRs used in this study. Four pilot-scale submerged MBRs were installed at a municipal wastewater treatment facility (Soseigawa treatment plant, Sapporo, Japan) receiving municipal wastewater from combined sewer system. All MBRs used in this study were equipped with 3 m^2 of hollow fiber microfiltration (MF) membranes (Mitsubishi Rayon Co., Ltd. Tokyo, Japan). Nominal pore size and material of the membrane were 0.4 μm and polyethylene, respectively. Two MBRs were directly fed with the primary clarifier effluent (hereafter defined as CMBRs) while other two were fed with the pre-treated wastewater by a jet mixed separator (JMS) for coagulation/sedimentation (hereafter defined as HMBRs) (Itonaga and Watanabe, 2004). The JMS with inclined tube settlers was used as a pre-coagulation/sedimentation unit (Watanabe et al., 1990, 1998). The treatment capacity and hydraulic retention time (HRT) in the JMS were set at 50 $m^3 day^{-1}$ and 1.5 h, respectively. An iron-based coagulant, poly-silicate iron (PSI) (Hasegawa et al., 1991), was used as a coagulant. A constant dosage of 10 mg-Fe l^{-1} was used throughout the experiments. Table 1 shows the characteristics of the raw wastewater and the operating conditions of the MBRs. Aeration was continuously carried

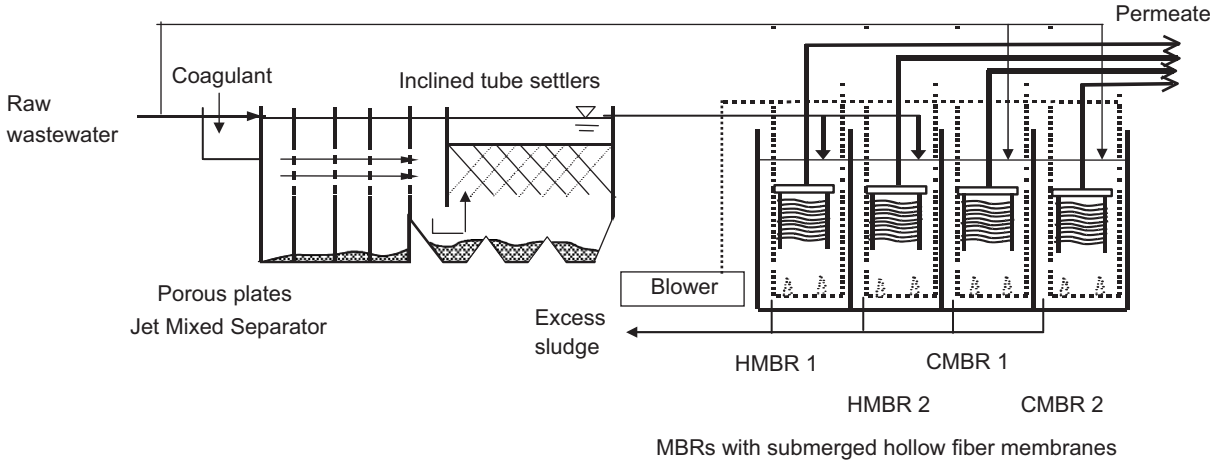

Fig. 1 – Schematic of the HMBRs and CMBRs used in this study.

Table 1 – Operating conditions and average influent water characteristics of the MBRs during the operation

	HMBR1	HMBR2	CMBR1	CMBR2
HRT (h)	4.5–6.0	3.6–4.5	4.5–6.0	3.6–6.0
Flux ($m\,day^{-1}$)	0.3–0.4	0.4–0.5	0.3–0.4	0.3–0.5
MLSS ($g\,l^{-1}$)	2–3	15–25	2–3	15–25
Influent water	JMS effluent		Primary clarifier effluent	
Temperature (°C)	17.8 (10.8–24.2)		17.8 (10.8–24.2)	
pH	6.8 (6.5–7.2)		7.7 (7.1–8.5)	
DO ($mg\,l^{-1}$)	1.9 (0.8–3.9)		1.7 (0.9–3.5)	
turbidity (NTU)	9.0 (1.7–26.0)		53.2 (33.3–89.1)	
TOC ($mg\,l^{-1}$)	16.7 (4.6–34.5)		35.3 (13.5–71.2)	
DOC ($mg\,l^{-1}$)	12.9 (4.6–29.7)		20.6 (10.1–40.3)	
T-N ($mg\,l^{-1}$)	20.7 (5.2–51.3)		29.7 (13.3–58.0)	
T-P ($mg\,l^{-1}$)	0.5 (0.1–1.1)		2.6 (0.5–6.5)	

Numbers in parentheses are minimum and maximum values.

out in all MBRs. Intermittent filtration (12-min filtration and 3-min pause) was also carried out. The MBRs were operated with the constant flow rate mode of filtration using suction pumps. Therefore, the required transmembrane pressure (TMP) increased as the operation period became longer or the membrane fouled. When the TMP increased significantly, membrane modules were taken out from the reactor and were cleaned physically or chemically. Physical membrane cleaning was carried out by spraying pressurized water on the membrane surface. Chemical membrane cleaning was carried out by submerging the membrane modules in a solution of sodium hypochloride (500 ppm) and then in a solution of hydrochloric acid (pH 2).

2.2. Analytical methods

The influent municipal wastewater and permeate were sampled 2–5 times per week and analyzed for total organic carbon (TOC), dissolved organic carbon (DOC), total nitrogen (T-N), total phosphorus (T-P), NH_4^+-N, NO_2^--N, NO_3^--N and turbidity. The concentrations of TOC and DOC were determined with the TOC analyzer (TOC-5000, Shimadzu, Kyoto, Japan). The concentrations of T-N and T-P were determined using an ultraviolet absorptiometry and the molybdenum blue absorptiometry, respectively. The concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were determined by ion chromatography (model DX-100; Nippon DIONEX, Osaka, Japan) equipped with an IonPac CS3 column for cations and AS4A column for anions (both Nippon DIONEX) after filtering with 0.45-μm-pore-size membrane filter, respectively. Concentration of mixed liquor suspended solids (MLSS) was measured according to the standard method (APHA-AWWA-WEF, 1998).

2.3. Bacterial community analysis

To monitor the changes in the bacterial community structures in the HMBRs (with low and high MLSS concentrations) and CMBRs (with low and high MLSS concentrations) during long-term operations, the mixed liquor samples were taken every month and analyzed by PCR-DGGE. To more quantitatively determine the bacterial community structures, grab samples of mixed liquor were taken from the HMBRs, the CMBRs, and the full-scale AS process on 78 days and analyzed by FISH and PCR-DGGE.

2.4. Sample fixation

For FISH analysis, 4-ml mixed liquor samples were fixed for 3 h at 4 °C by adding 2 ml of 8% paraformaldehyde, which resulted in a final concentration of 4%. Subsequently, the samples were centrifuged at 10,000 × *g* for 8 min and washed three times with 2 ml of phosphate-buffered saline (10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2). After the fixation and washing steps, 5 μl of the samples were spotted on a gelatin-coated slide glass (Okabe et al., 1999).

2.5. Oligonucleotide probes and in situ hybridization

The 16S and 23S rRNA targeted oligonucleotide probes used in this study are shown in Table 2. The probes were labeled with fluorescein isothiocyanate (FITC), tetramethylrhodamine 5-isothiocyanate (TRITC), or the sulfoindocyanine dye Cy5 at the 5′ end. Dehydration and FISH were performed according to the procedure described by Amann (1995). The hybridization conditions used for FISH are also shown in Table 2. Simultaneous hybridizations with the probes requiring different stringency conditions were performed by using a successive hybridization procedure; hybridization with the probe requiring higher stringency was performed first, and then hybridization with the probe requiring lower stringency was performed. For determination of microbial compositions, the ratio of the area of bacterial cells stained with the group- or subgroup-specific probe to the area of all bacterial cells stained with the EUB338 mixed probes was determined after simultaneous in situ hybridization by using image analysis software provided by Zeiss (Okabe et al., 1999). The average was determined from at least 10 randomly chosen laser-scanning microscopy (LSM) projection images of each sample. The average number represents the relative abundance of each group- or subgroup-specific probe-hybridized cells in the EUB338 mixed probe-hybridized cells (most of the bacteria).

2.6. DNA extraction and PCR amplification

DNA was extracted from 200 μl of mixed liquor samples with a Fast DNA spin kit (Bio 101, Qbiogene Inc., Carlsbad, Calif.) as described in the manufacturer's instructions. 16S rRNA gene fragments were amplified from the extracted total DNA from the samples with Taq DNA polymerase (TaKaRa Bio Inc., Ohtsu, Japan) by using bacterial primer sets PRBA357F (5′-CCTACGGGAGGCAGCAG-3′) and PRUN518R (5′-GTATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). Primer 357F has an additional 40 nucleotide GC-rich sequences at its 5′ end. The GC-clamp has the following sequence: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′. The conditions used for the PCR were as follows: 5 min of initial denaturation at 94 °C and 30 cycles of 1 min at 92 °C, 1 min at annealing temperature, and 1 min at 72 °C. During

Table 2 – List and description of 16S and 23S rRNA targeted oligonucleotide probes used in this study

Probe	Target (position)[a]	Sequence (5′→3′)	Applied stringency (formamide conc.,%)	Reference
EUB338[b]	Domain bacteria 16S rRNA (338-355)	GCTGCCTCCCGTAGGAGT	0–50	Amann et al. (1990)
EUB338II[b]	Member of the phylum *Planctomycetales* 16S rRNA (338-355)	GCAGCCACCCGTAGGTGT	0–50	Daims et al. (1999)
EUB338III[b]	Members of the phylum *Verrucomicrobia* 16S rRNA (338-355)	GCTGCCACCCGTAGGTGT	0-50	Daims et al. (1999)
ALF1b	*Alphaproteobacteria* and some other bacteria 16S rRNA (19-35)	CGTTCGYTCTGAGCCAG	20	Manz et al. (1992)
Ntspa1026	*Nitrospira moscoviensis*, activated sludge clones A4 and A11 16S rRNA (1026–1043)	AGCACGCTGGTATTGCTA	20	Juretschko et al. (2002)
BET42a	*Betaproteobacteria* 23S rRNA (1027–1043)	GCCTTCCCACTTCGTTT	35	Manz et al. (1992)
GAM42a	*Gammaproteobacteria* 23S rRNA (1027–1043)	GCCTTCCCACATCGTTT	35	Manz et al. (1992)
SRB385[c]	Some sulfate-reducing bacteria of the *Deltaproteobacteria*, other *Deltaproteobacteria* and Grampositive bacteria	CGGCGTCGCTGCGTCAGG	35	Amann et al. (1990)
SRB 385Db[c]	Some sulfate-reducing bacteria of the *Deltaproteobacteria* and many non-sulfate-reducing bacteria	CGGCGTTGCTGCGTCAGG	35	Rabus et al. (1996)
GNSB941[d]	Phylum *Chloroflexi* (previously known as green nonsulfur bacteria)	AAACCACACGCTCCGCT	35	Gich et al. (2001)
CFX1223[d]	Phylum *Chloroflexi* (previously known as green nonsulfur bacteria)	CCATTGTAGCGTGTGTGTMG	35	Björnsson et al. (2002)
BAC303	*Bacteroidales*-group of the *Bacteroidetes* 16S rRNA (303-319)	CCAATGTGGGGGACCTT	0	Manz et al. (1996)
HGC69a	*Actinobacteria* (Gram-positive bacteria with high G+C content of DNA) 23S rRNA (1901–1918)	TATAGTTACCACCGCCGT	25	Roller et al. (1994)
LGC354a[e]	*Firmicutes* (gram-positive bacteria with low G+C content)	TGGAAGATCCCTACTGC	35	Meier et al. (1999)
LGC354b[e]	*Firmicutes* (gram-positive bacteria with low G+C content)	CGGAAGATTCCCTACTGC	35	Meier et al. (1999)
LGC354c[e]	*Firmicutes* (gram-positive bacteria with low G+C content)	CCGAAGATTCCCTACTGC	35	Meier et al. (1999)
CF319a/b	*Cytophaga-Flavobacteria* cluster 16S rRNA (319-336)	TGGTCCGTRTCTCAGTAC	35	Manz et al. (1996)

[a] *Escherichia coli* numbering.
[b,c,d,e] These probes were applied in combination, respectively.

the reaction cycle, the annealing temperature was decreased by 1 °C from 65 to 56 °C every second cycle in the first 20 cycles and in the last 10 cycles the annealing temperature was 55 °C. A hot-start PCR program was used for all amplifications to minimize nonspecific amplification (Muyzer et al., 1993). Final extension was carried out for 4 min at 72 °C. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

2.7. DGGE

The PCR-amplified DNA fragments were separated on polyacrylamide gels (8%, 37.5:1 acrylamide-bisacrylamide) in 0.5 × TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM Na_2EDTA, pH 7.4) using a denaturing gradient ranging from 30% to 60% (100% denaturant contains 7 M urea and 40% (vol/vol) formamide). The amplicons were purified with Wizard PCR preps (Promega), and then aliquots (2 μl) of purified amplicons were quantified densitometrically. For DGGE, 100 ng of purified amplicons was used. DGGE was performed by using a D-Code system (Bio-Rad Laboratories, Inc., Tokyo Japan) Electrophoresis was initially at 60 °C for 20 min at 100 V, and thereafter for 5 h at 200 V. Following the electrophoresis, the gel was soaked for 15 min in SYBR Gold (Molecular Probes; Eugene, OR, USA) and then visualized with a UV transilluminator (302 nm).

2.8. Numerical analysis of DGGE banding patterns

Dice index (Cs) of similarity was used to evaluate the similarity of bacterial community among bioreactors (LaPara et al., 2002). DGGE fingerprints were manually scored by the presence or absence of co-migrating bands without consideration of the band intensity. This was done at least three times to ensure constant results. Pair wise community similarity was quantified using the Dice index of similarity, $Cs = 2j/(a+b)$, where j is the number of common bands between samples A and B; a and b are the total number of bands in samples A and B, respectively. This index ranges from 0 (no common band) to 1.0 (identical band patterns).

Shannon diversity index (H′) was introduced to analyze the bacterial community diversity (species richness) (Eichner et al., 1999). In the Shannon diversity index, in contrast to the Dice index of similarity, the band intensity should be considered. Here, each band is considered as a single species and the band intensity as the species abundance (Eichner et al., 1999). This index was calculated using the following equation: $H' = -(n_i/N)(\log n_i/N)$, where n_i/N is the proportion of community that is made up by species i (brightness of the band i/total brightness of all bands in the lane). The Shannon diversity index was influenced by both the species number and species abundance. Populations with more species and even distribution of individuals have higher diversity than other populations with either fewer species or disproportioned populations of each species.

Equitability index (EI) was introduced to analyze the species distribution during the period of operation (Stamper et al., 2003). This index was calculated using the following equation: $EI = H'/\log n$, where, n is the total number of species in the sample (Stamper et al., 2003). In contrast to the Shannon diversity index, the Equitability index have a maximum value of 1, which means completely even species distribution (e.g. 10 species, each at 10% abundance).

Nonmetric multidimensional scaling (NMDS) was introduced to visualize the changes in bacterial community during the period of operation. The DGGE banding patterns were converted to a binary matrix to make the data accessible to statistical analysis. The presence or absence of DGGE bands at the same position was used to generate a binary data set. From this binary matrix a distance matrix was calculated using SPSS 11.5J (SPSS Inc, Tokyo, Japan) to construct a map that shows the community structure at a particular point in time (e.g., the bacterial community of one day) as one dotted point, and by connecting consecutive points, relative changes in the community over the time can be visualized and interpreted. The data are presented in a Euclidean plane such that highly similar measurements are plotted close together. The dimensions (axes) in the map have no special significance and can be rotated without influencing the relative distance between the points. NMDS has proven to be useful as a tool for analysis of DGGE data (Van Hannen et al., 1999).

3. Results and discussion

3.1. MBR performance

HMBR1 and CMBR1 were operated for about 120 days, whereas HMBR2 and CMBR2 were operated for about 240 days. Fig. 2 shows the changes in MLSS concentration of each MBR during the operation. The MLSS concentrations in the HMBR2 and CMBR2 were not controlled until 148 days and thereafter were maintained around 15 $g\,l^{-1}$. Table 3 summarizes average water quality of permeate in all the MBRs. DOC concentrations in all MBRs were below 5.0 $mg\,l^{-1}$, in which biodegradable DOC was almost zero. T-P concentrations in the HMBRs were lower than those in the CMRBs because of pre-coagulation/sedimentation process. On the other hand, nitrification was deficient in the HMBRs because alkalinity was consumed by pre-coagulation/sedimentation process, resulting in low pH (< 5.5). Fig. 3 shows changes in TMP in each MBR during the operation. Since physical washing did not restore membrane permeability in the CMBR1, chemical washing was carried out more frequently (Fig. 3A). This is probably because the DOC concentration in

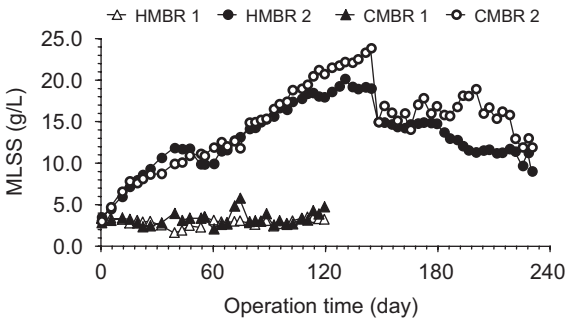

Fig. 2 – Changes in MLSS concentrations in HMBRs and CMBRs during the operation. The HMBR1 and CMBR1 were operated for only 120 days.

Table 3 – Average effluent water characteristics of the MBRs during an experimental period

	HMBR 1	HMBR 2	CMBR 1	CMBR 2
Turbidity (NTU)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
DOC ($mg\,l^{-1}$)	4.6±2.6	3.2±2.2	5.0±1.4	4.2±1.9
E260 ($1\,cm^{-1}$)	0.08±0.03	0.07±0.04	0.11 ±0.02	0.10±0.03
T-P ($mg\,l^{-1}$)	0.03±0.02	0.03±0.04	0.44 ±0.36	0.68 ±0.46
T-N ($mg\,l^{-1}$)	16.9±4.6	16.5±4.9	18.8±5.3	18.4±7.6
NH_4^+-N ($mg\,l^{-1}$)	3.1 ±3.4	1.8±1.5	0.7±0.9	0.6±1.5
NO_2^--N ($mg\,l^{-1}$)	1.5±3.0	0.1 ±1.1	0.9±0.8	0.1±0.3
NO_3^--N ($mg\,l^{-1}$)	11.2±4.6	14.1 ±3.8	14.7±3.5	14.9±4.9
Alkalinity ($mg\,l^{-1}$)	8.8±15.8	4.0±10.4	20.7±6.2	27.0±11.2
pH	5.5±0.9	4.8±0.8	6.7±0.3	6.5±0.4

Numbers are means±SD.

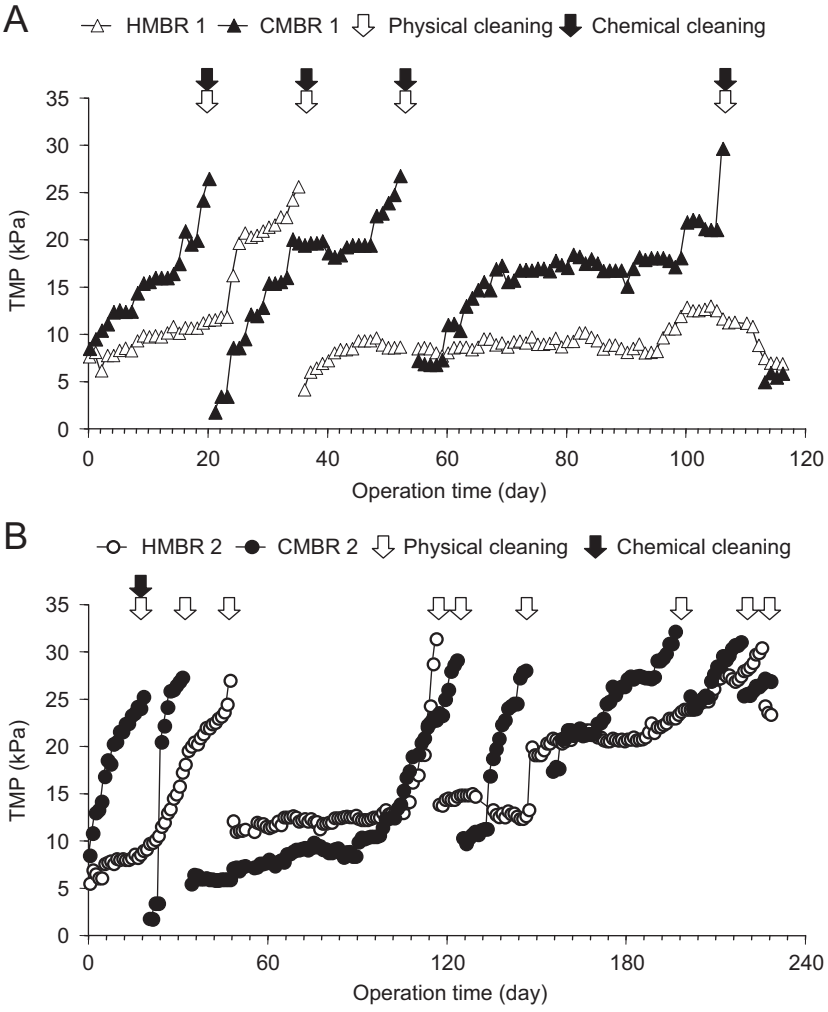

Fig. 3 – Changes in transmembrane pressure (TMP) in the HMBRs and CMBRs during the operation. (A) HMBR1 and CMBR1 operated with low MLSS concentrations, (B) HMBR2 and CMBR2 operated with high MLSS concentrations.

the CMBR1 was about two times higher than those in the other MBRs during the entire operation (Fig. 4A). This result indicated that the biological degradation of DOC could not occur sufficiently due to insufficient amount of biomass and higher load of organic matter including particulates (Table 1), resulting in the higher F/M ratio than the other MBRs. The DOC concentration in the CMBR2 was also high until day 100 (Fig. 4B) because the MLSS concentration did not accumulate enough (Fig. 2) and higher load of organic matter including particulates (Table 1). These results suggest that the pre-treatment of feed

wastewater was one of the crucial measures to prevent membrane fouling in the MBR processes when MLSS concentration was low. Thereafter the DOC concentration decreased to the level similar to that in the HMBR2. The TMP increased rapidly in the CMBR2 during the first 40 days, which resulted in more frequent physical washing (Fig. 3B). This is partly because that the mixed liquor viscosity in the CMBR2 gradually increased with increasing the MLSS concentration, whereas that in the HMBR2 remained low during the entire operation. Excessive mixed liquor viscosity has a positive impact on membrane fouling (Itonaga and Watanabe, 2004). These demonstrated that the overall performances of the HMBRs, in terms of permeate water quality and membrane permeability, were better than those of the CMBRs.

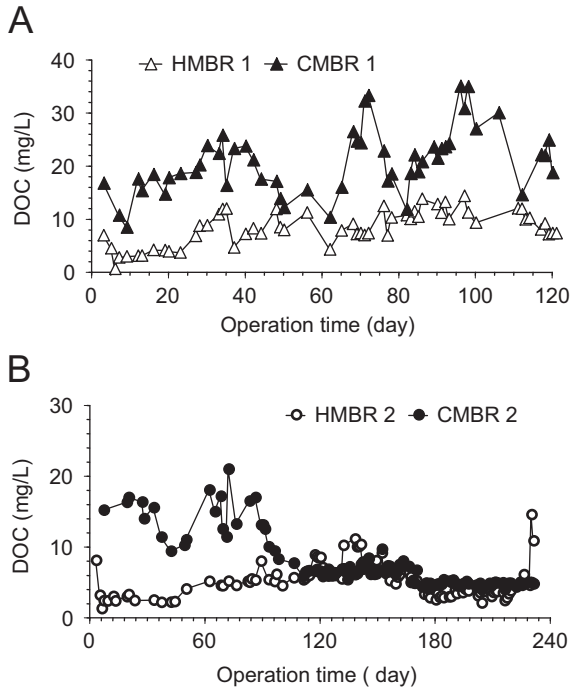

Fig. 4 – Changes in mixed liquor DOC concentrations (mg l^{-1}) in the HMBRs and CMBRs throughout the operational period. (A) HMBR1 and CMBR1 operated with low MLSS concentrations, (B) HMBR2 and CMBR2 operated with high MLSS concentrations.

3.2. *Bacterial community analysis*

The similarity of PCR-DGGE banding patterns among bacterial communities in the AS process and four MBRs taken on days 30, 78, 90 and 120 were quantified using the Dice index of similarity (Cs) (Table 4). The bacterial community structures in HMBRs were different from those in the CMBRs and AS process (Cs < 0.6). The bacterial communities in the CMBRs showed 0.57 to 0.73 similarity to that in the AS process, indicating moderately similar bacterial communities. The bacterial communities in CMBR 1 and CMBR 2 were similar to each other (Cs = 0.64–0.79). These results indicated that the pre-coagulation and sedimentation process had a larger impact on bacterial community structures than MLSS concentration did, because the influent water quality of the HMBRs (e.g., turbidity, TOC, DOC, T-P and pH) were considerably different from that of the CMBRs and the AS process (Table 1). In particular, the pH of the effluent was lower in the HMBRs than in the CMBRs (Table 3), which probably led to the different microbial communities in the HMBRs. In addition,

Table 4 – Dice indexes (Cs) comparing the similarity of PCR-DGGE fingerprints from the AS process, HMBRs, and CMBRs on days 30, 78, 90 and 120

		Similarity (Dice index, Cs)				
		AS	HMBR 1	HMBR 2	CMBR 1	CMBR 2
Day 30	AS					
	HMBR 1	0.49				
	HM BR 2	0.44	0.60			
	CMBR 1	0.71	0.57	0.50		
	CMBR 2	0.65	0.58	0.54	0.75	
Day 78	AS					
	HMBR 1	0.38				
	HM BR 2	0.45	0.64			
	CMBR 1	0.71	0.59	0.47		
	CMBR 2	0.69	0.53	0.52	0.79	
Day 90	AS					
	HMBR 1	0.42				
	HM BR 2	0.40	0.56			
	CMBR 1	0.73	0.47	0.40		
	CMBR 2	0.71	0.44	0.49	0.64	
Day 120	AS					
	HMBR 1	0.48				
	HM BR 2	0.37	0.48			
	CMBR 1	0.65	0.46	0.33		
	CMBR 2	0.57	0.32	0.34	0.69	

most of the incoming microorganisms could be removed by the pre-coagulation/sedimentation, which also significantly influenced the bacterial community structures in the HMBRs.

PCR-DGGE is an effective method that enables analysis of many samples simultaneously. However, it has some inherent biases and limitations such as DNA extraction efficiency, preferential PCR amplification (LaPara et al., 2002), co-migration of several PCR fragments (Rölleke et al., 1996), and so on. This suggests the importance of combining different analysis. We, therefore, performed FISH to quantitatively determine the bacterial community structures in four MBRs and the conventional AS process.

3.3. *Detectability of cells by FISH*

The *bacteria*-specific EUB338 mixed probes (an equimolar mixture of EUB338, EUB338II and EUB338III) were used to assess the overall physiological state of the bacterial communities in four MBRs and in the AS process. In situ probing with the EUB338 mixed probes hybridized less than 60% of all DAPI-stained bacteria in the HMBR2 and CMBR2, both of which were operated with high MLSS concentrations ($>15\,g\,l^{-1}$). On the other hand, the HMBR1 and CMBR1 operating with low MLSS concentrations ($2–3\,g\,l^{-1}$) showed that approximately 80% of DAPI-stained bacteria were hybridized with the EUB338 mixed probes. The AS biomass ($MLSS = 2\,g\,l^{-1}$) revealed that more than 95% cells were detectable with the EUB338 mixed probes. These results are in agreement with the previous study showing that 40–50% of all DAPI-stained cells were detectable with *Bacteria*-specific EUB338 mixed probes in MBR and 80% in the conventional AS (Witzig et al., 2002).

The detectability of bacterial cells by FISH is dependent on their ribosomal content and consequently on their physiological state (Amann et al., 1995). It could be an advantage to describe the composition of the more active and hence ecologically relevant part of the community instead of the total existent population to evaluate the biodegradation capacity of the MBRs. The concentrations of active biomass in both HMBR2 and CMBR2 were much higher than those in the AS, HMBR1 and CMBR1 due to the higher MLSS concentrations. This was probably the reason that the average DOC concentrations in the HMBR2 and CMBR2 were slightly lower than those in the HMBR1 and CMBR1 during the operation (Table 3). It was also speculated that bacteria with low ribosomal contents still actively participate in degradation of organic matters (Witzig et al., 2002), even though F/M ratios in HMBR2 and CMBR2 were very low. This clearly indicates that CMBRs and HMBRs have high viable-cell concentrations and, thereby, high volumetric treatabilities.

3.4. *Bacterial community structure analysed by FISH*

FISH analysis was performed to quantitatively characterize the overall composition of the bacterial populations in the AS process, HMBRs and CMBRs (Table 5). The mixed liquor samples were taken on day 78 (the same samples with PCR-DGGE analysis). The *Alpha*-, *Beta*-, and *Gammaproteobacteria* dominated the microbial community in the AS process and both MBRs. These results are consistent with data obtained from municipal wastewater treatment plants (Daims et al., 1999; Wagner et al., 1993; Witzig et al., 2002). These *proteobacteria* could be responsible for removal of various organic matters from municipal wastewater (Wagner et al., 1993).

Interestingly, filamentous bacteria such as *Actinobacteria* and *Chloroflexi* were found in large amounts in the CMBR2 and AS process (Table 5). In particularly, the abundance of *Chloroflexi* in the CMBR2 was significant, and its fluorescence intensity was also very high. In contrast, they were absent in the CMBR1 and HMBR1 operating with low MLSS concentrations. This is the first time that filamentous *Chloroflexi* was reported to be one of dominant members in MBRs. The phylum *Chloroflexi* contains a number of diverse environmental clones retrieved from various wastewater treatment plants with only a few cultured representatives. Thus, their

Table 5 – Bacterial community structures of the AS process, HMBRs and CMBRs on day 78, which were determined by FISH

	Relative abundance (%)				
	AS	HMBR 1	HMBR 2	CMBR 1	CMBR 2
Alphaproteobacteria	18±16	13±16	12±10	15±7	16±15
Nitrospira	2±3	0	1±3	1±1	1±1
Betaproteobacteria	25±22	21±22	16±18	47±2	23±13
Gammaproteobacteria	10±11	9±12	9±12	12±4	9±11
Deltaproteobacteria	5±4	2±4	4±3	6±6	10±5
Chloroflexi	12±13	0	6±5	0	19±21
Bacteroidetes	1±2	0	3±3	1±2	1±1
Actinobacteria	14±14	8±10	3±4	1±2	7±5
Firmicutes	3±4	0	4±10	4±5	5±7
Other bacteria[a]	10	47	42	13	9

All the values (means ± SD) are presented as percentage of group- and subgroup-specific probe hybridized bacterial cells against total bacterial cells hybridized with EUB338 mixed probes.

[a] The segment "other bacteria" refers to the bacteria detectable with EUB338 mixed probes, but not with any other group or subgroup specific probes used in this study.

roles in wastewater treatment plants have not been well studied in relation to the ecophysiology of *Chloroflexi* bacteria.

More than 40% of bacterial cells in the HMBRs could not be identified by any of the probes used, which targeted bacteria frequently reported in AS systems. This is partly due to limitation of the coverage and specificity of the probes used and the lower fluorescence signal intensity of cells in the HMBRs.

3.5. MBR performance and changes in bacterial community structure

The DGGE fingerprints of all four MBRs were analyzed using Shannon diversity index (H') and Equitability index (EI). The four MBRs did not show significant differences in their diversity and species distribution ($H' = 1.3$–1.6 and EI = 0.96–1.0). Comparing with other studies, the bacterial communities in our MBRs were very diverse. For example, the Shannon diversity index (H') in the MBR treating gray water of the US Navy ship was in average 0.82 during 100-day operation (Stamper et al., 2003), approximately half of our community diversity. This is probably attributed to the simpler composition of the gray water feed (10% galley water and 90% laundry water) than the municipal wastewater used in this study. Species or phenotypes need to be functionally redundant to some extent for stable treatment of complex wastewater whose compositions are constantly fluctuating. In this case, the biodiversity acts as insurance for MBR functions against temporal changes in environmental factors. The biodiversity is positively related to ecosystem stability (i.e., resistance to disturbance and rate of recovery after disturbance) (Reinthaler et al., 2005; Saikaly et al., 2005). Therefore, the high bacterial diversity in the MBRs could allow better and stable water quality of permeates.

3.6. NMDS analysis of DGGE banding patterns

To understand the relationship between the bacterial community succession and the reactor performance (performance was measured as membrane permeability and removal of DOC), changes in bacterial community structure in the HMBRs and CMBRs were monitored and analyzed by NMDS analysis of DGGE banding patterns (Fig. 5). In the NMDS plots, the distance between the points reflects the similarity of the DGGE profiles at a given time. Similar community structures are closer together. Hence, the degrees of the bacterial community changes could be related to the MBR performance.

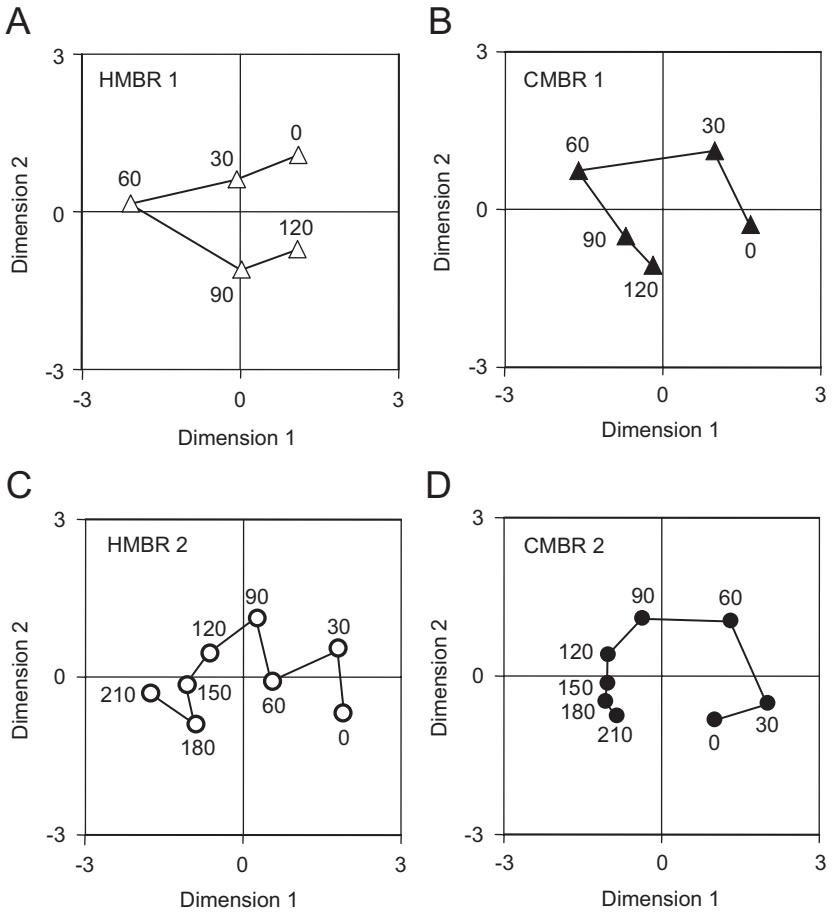

Fig. 5 – Nonmetric Multidimentional Scaling (NMDS) map of the bacterial communities, showing the community changes during the operation. The numbers indicate the days when the samples were taken.

The NMDS plots showed a general trend that relatively large changes in bacterial communities occurred in the first 100 days of operation, and thereafter, small community changes were constantly observed in all MBRs (Fig. 5). It seems that the community changes in the MBRs operated with low MLSS concentrations (HMBR1 and CMBR1) were more dynamic than those in the MBRs with high MLSS concentrations (HMBR2 and CMBR2), which was probably due to constant removal of biomass (Fig. 2). The initial large changes in bacterial community could be attributed to adaptation of the seeded AS to new MBR conditions where the F/M ratio was low and the SRT (sludge retention time) was long. In addition, the production of the slowly biodegradable DOM from decay of the retained biomass in the MBR could be much higher than in the conventional AS process. Thus, the seeded AS might need a relatively long adaptation period.

After about first 100 days, small perpetual fluctuations of bacterial community structures were observed in both CMBR2 and HMBR2, even though the water quality (e.g., DOC) of permeate became stable (Fig. 4). These results indicate that stable communities (i.e., climax communities) were not established during the entire operating period in this study. This was partly due to temporal fluctuation of environmental parameters (i.e. temperature, influent wastewater characteristics, and so on) and/or interactions between community members (e.g., predation). In addition, biomass decay would become significant in the HMBR2 and CMBR2, resulting in zero net biomass production. Under these conditions, a stable bacterial community was hard to be achieved. Compared to the CMBR2, the HMBR 2 showed an irregular change in the community structure after 180 days, which was probably due to the effect of $NaHCO_3$ addition ($40\,mg\,l^{-1}$ as $CaCO_3$) to improve nitrification efficiency.

These results suggest that not only the stability, but also the adequate dynamics of the bacterial community (flexibility to adapt in response to changes in environments) are important factors for the stable performance of the CMBR and the HMBR. The similar relationship can be found in the literature (Ayala-del-Río et al., 2004; Fernández et al., 1999). For example, a study performed with a functionally stable methanogenic reactor revealed that microbial communities could be very dynamic, suggesting that stable function is not always correlated with stable community structure (Fernández et al., 1999).

However, the presence of numerically minor but important populations (e.g., populations responsible for degradation of DOM) in the MBRs may not be revealed at 16S rDNA level analysed in this study. The changes in these minor populations may have a profound impact on the functional stability of the MBRs. Thus, further researches are needed to understand the relationship between the bacterial community structure and reactor function by analyzing genes encoding specific function that represents specific bacterial community.

4. Conclusions

Analysis of DGGE of PCR-amplified 16S rRNA genes and FISH indicated that the bacterial communities of the HMBRs were different from those of the AS process and CMBRs, while the bacterial communities in the CMBRs were closer to that in the AS process. The microbial community structures were strongly determined by the influent water quality and microbial composition in these systems. In addition, the changes in the bacterial community structures in the HMBRs and CMBRs were monitored by NMDS analysis of DGGE banding patterns and related to the performances of the HMBR and CMBR. The results revealed that adequate dynamics of the bacterial community or flexibility to adapt to changes in environments are important for the stable performance of both the CMBR and HMBR.

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