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Fine-scale monitoring of shifts in microbial community composition after high organic loading in a pilot-scale membrane bioreactor

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In biological wastewater treatment, municipal wastewater sometimes undergoes unexpected changes in physicochemical parameters, such as organic carbon concentration. The aim of this study was to understand how microbial communities in activated sludge in a membrane bioreactor (MBR) adapt to high organic loading and maintain their degradation ability during reactor operation. A pilot-scale MBR was operated for 19 days. On day 8, the concentration of organic matter in the synthetic wastewater increased from 450 to 900 mg chemical oxygen demand (COD)/L. Even under conditions of high organic loading, COD removal rates were high, ranging from 85.3 to 91.4%. High-throughput sequencing of 16S rRNA genes revealed that microbial communities changed drastically with increased organic loading. After day 8, *Aquabacterium*- and *Azospira*-related operational taxonomic units (OTUs) belonging to the class β-proteobacteria became dominant; this potentially enhanced the degradation of organic substances and decreased activated sludge microbial diversity. Due to the use of dissolved oxygen (DO) for degradation of organic substances, DO levels in the reactor decreased. This led to an increase in a subset of OTUs related to not only aerobic but also anaerobic bacteria, e.g., those in the class Clostridia. During this period, anaerobic microorganisms may have contributed to the degradation of organic substances to maintain MBR performance. On the other hand, high-throughput sequencing also made it possible to identify yet-to-be cultured or minor microorganisms affiliated with the candidate phylogenetic division SR1 and ammonia-oxidizing archaea in activated sludge.

[**Key words:** Membrane bioreactor; High-throughput sequencing; Microbial community structure; High organic loading; Activated sludge]

During the last two decades, much attention has been paid to membrane bioreactors (MBRs) in the field of wastewater treatment. MBRs combine activated sludge treatment and membrane filtration. Compared with conventional activated sludge systems, MBRs can be operated with much higher biomass concentration (1,2) and achieve greater efficiency with higher quality of effluent. Maintaining metabolically active microbial communities is required for stable reactor performance. However, municipal wastewater sometimes experiences huge and unexpected changes in its physicochemical parameters, such as pH, dissolved oxygen (DO), organic concentration, and salinity, which can affect microbial communities in MBRs. Because the assessment of reactor performance has been limited to measuring a few indices in the effluent, e.g., chemical oxygen demand (COD), it is difficult to understand, especially in large-scale MBRs, how microbial communities respond and acclimate to the compositional changes in wastewater during effective reactor operation. Nonetheless, understanding how reactor conditions affect the indigenous microbial communities is quite beneficial for maintaining stable reactor performance, as previously reported (3,4).

Most of the diverse microorganisms that coexist in activated sludge have not been isolated by traditional culture-dependent techniques. Hence, culture-independent molecular approaches are critical to uncover the microbial community structure. A number of methodologies have been developed on the basis of 16S rRNA genes, e.g., clone library analysis (5), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (6), terminal restriction fragment length polymorphism (T-RFLP) (7), and fluorescent *in situ* hybridization (FISH) (8). Up to now, microbial community dynamics in response to organic loads in MBR systems have been investigated by using such conventional methods (9). The conventional methods effectively detect major microorganisms, but their resolution is insufficient to comprehensively characterize microbial communities that include minor microorganisms. Recently, high-throughput pyrosequencing has been used to provide a more comprehensive assessment of microbial communities in MBR systems (10–12).

In this study, we operated a pilot-scale MBR under high organic loading conditions, for which the concentration of organic matter in the synthetic wastewater was increased from 450 to 900 mg COD/L during reactor operation. And then, we analyzed changes in microbial community structure in response to the organic loading by using the high-throughput Illumina MiSeq sequencing platform, in which millions of nucleotide sequences are determined in a single run (13,14). Compared to pyrosequencing, the Illumina MiSeq platform accomplishes a more sensitive and precise assessment of the microbial community dynamics. Based on all the information on changes in physicochemical parameters and fine-

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scale microbial community structure in the pilot-scale MBR, microbial adaptation to new environmental conditions with high concentrations of organic matter is discussed.

MATERIALS AND METHODS

Experimental setup and operation of a pilot-scale MBR The pilot-scale MBR used in this study was composed of three compartments, the operating volumes of which were 92, 80.5, and 57.5 L (from left to right, in Fig. 1), respectively, and the sludge was stirred by continuous aeration. Air was provided through an air diffuser set in each compartment with a flow rate of 22.5–35 L/min to maintain DO values between 0.15 and 1.75 mg/L. A pilot-scale M-fine flat membrane module (Awa Paper Mfg, Co., Tokushima, Japan) made of polyacrylonitrile was submerged in the reactor during operation. The effective surface area of the membrane module was 0.24 m^2 with a pore size of 0.07 μm. The membrane module was operated with a cycle of permeate extraction for 9 min and pause for 1 min, and the membrane surface was aerated continuously to reduce membrane fouling. The bioreactor was constantly fed with the synthetic wastewater (see below) stored in a feed tank (20 L) at 4°C. The flow rates of both input of synthetic wastewater and output of membrane-filtrated permeate were 115 L/day, and hydraulic retention time (HRT) was adjusted to 2 days.

The reactor was started up with an inoculum of activated sludge obtained from a municipal wastewater treatment plant (Kinu aqua-station, Ibaraki, Japan). During the whole experimental period, no sludge was withdrawn from the reactor except for sampling. The return sludge flow rate was 115 L/day.

Substrate composition The substrate concentration in the influent was initially adjusted to 450 mg COD/L, and on day 8 of operation of the MBR system, the substrate concentration was increased to 900 mg COD/L. The composition of the synthetic wastewater used on days 1–7 was as follows; CH_3COONa (2.65 g/L), NH_4Cl (0.376 g/L), KH_2PO_4 (0.109 g/L), peptone (0.706 g/L), $FeCl_3 \cdot 6H_2O$ (0.782 mg/L), $CaCl_2$ (1.56 mg/L), $MgSO_4$ (1.56 mg/L), KCl (1.56 mg/L), and NaCl (1.56 mg/L). On days 8–19, the composition of the synthetic wastewater used was twice that at days 1–7: i.e., CH_3COONa (5.30 g/L), NH_4Cl (0.751 g/L), KH_2PO_4 (0.217 g/L), peptone (1.41 g/L), $FeCl_3 \cdot 6H_2O$ (1.57 mg/L), $CaCl_2$ (3.13 mg/L), $MgSO_4$ (3.13 mg/L), KCl (3.13 mg/L), and NaCl (3.13 mg/L). The COD values 450 mg/L and 900 mg/L correspond to the total organic carbon (TOC) values 1130 mg/L and 2260 mg/L, respectively.

Analytical procedures Mixed liquor suspended solid (MLSS), temperature, DO, pH, and transmembrane pressure (TMP) were monitored during operation of the reactor. A 15-mL sample of the activated sludge was taken 5 times per week. The solid constituent of the activated sludge was removed by centrifugation (15,300 ×*g*, 15 min, 4°C), and the resulting supernatant was further filtered by using a cellulose acetate membrane (*Φ*, 0.20 μm, C020A025A; Advantec, Tokyo, Japan). The amounts of TOC, total nitrogen (TN), COD, NH_4^+, and NO_3^- in the supernatant and treated effluent were analyzed as follows: TOC and TN values were determined by using a TOC-TN analyzer (TOC-L/TNM-L; Shimadzu, Kyoto, Japan); COD was measured with a COD analyzer (DR2800 and DRB200; Hach, Loveland, CO, USA) using appropriate kits (TNT820 or TNT821; Hach); concentrations of NH_4^+ and NO_3^- were determined by capillary electrophoresis (CE; Agilent, Santa Clara, CA, USA). All data are represented as the mean values from at least two different sampling points of the reactor: MLSS, temperature, DO, pH were measured in all three compartment; TOC, TN, NH_4^+ and NO_3^- were mean values of first and second compartment of the reactor.

DNA preparation and PCR amplification The collected activated sludge from first and second compartments of the MBR was washed once with 50 mM sodium phosphate buffer (pH 7.0) and stored at −20°C as a pellet until use. DNA was extracted from 50 mg of activated sludge pellet according to a direct lysis protocol that includes bead-beating twice, phenol–chloroform extraction, and ethanol precipitation steps (15). RNA was digested with RNase (Type II-A; Sigma–Aldrich, St. Louis, MO, USA). The purified DNA was quantified by using NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA), and was used as the template for PCR amplification with a high-fidelity DNA polymerase (Q5; NEB, Ipswich, MA, USA). In this study, the V4 region of 16S rRNA gene was targeted for the PCR because many studies on the microbial communities have been performed using this region, and a large amount of such sequences has been accumulated in databases. The V4 region of 16S rRNA genes was amplified by using the universal primers 515F and 806R (14). Both primers were modified to contain an Illumina adapter region, and the reverse primer contained a 12-bp barcode for the multiplex sequencing (13). The thermal conditions of PCR were as follows: initial denaturation at 98°C for 90 s, 30–35 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s; and a final extension step at 72°C for 2 min.

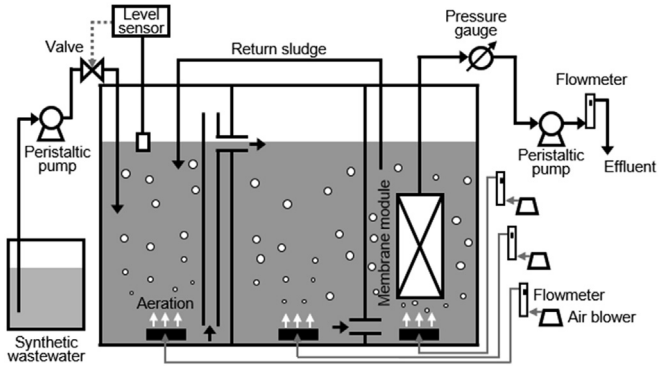

FIG. 1. Schematic of pilot-scale membrane bioreactor (MBR). The total volume of the pilot-scale MBR was 230 L. The flow rates of influent synthetic wastewater, return sludge, and effluent were 115 L/day (HRT = 2 days). The activated sludge was continuously aerated.

High-throughput Illumina sequencing of 16S rRNA gene amplicons High-throughput sequencing was carried out as described previously (16). Briefly, the PCR products were first purified by using an AMPure XP kit (Beckman Coulter, Brea, CA, USA). The resultant DNA solution was subjected to agarose-gel electrophoresis and the target DNA band was excised. Recovery of DNA in the gel slice was performed with a QIAquick gel extraction kit (Qiagen, Venlo, Netherlands). The DNA concentration was determined spectrophotometrically with a Quant-iT PicoGreen dsDNA reagent and kit (Life Technologies, Carlsbad, CA, USA). An appropriate amount of the 16S rRNA gene segments and an internal control (PhiX Control V3; Illumina, San Diego, CA, USA) were subjected to paired-end sequencing with a 300-cycle MiSeq reagent kit (Illumina) and a MiSeq sequencer (Illumina). Removal of PhiX, low-quality (Phred value score [Q], <30) and chimeric sequences, and assembly of the paired-end sequences were carried out according to a previous report (17). Contaminating PhiX sequences in the Illumina sequence libraries were detected by means of a homology search against the Greengenes database (18) with the use of Burrows–Wheeler Aligner, version 4.0.5 (19). Then the PhiX sequences were removed from the library by self-written scripts. The paired-end sequences were joined by a fastq-join tool in the ea-utils software package version 1.1.2–301 (20). The joined sequences with Q scores of ≥30 were collected by using the software package QIIME, version 1.7.0 (21), and aligned by using the program Mothur, version 1.31.2 (22), and then the chimeric sequences were detected and excluded from the library. The sequences in each library were characterized phylogenetically by using the QIIME software package (21). The α-diversity indices (i.e., Chao1, Shannon, and Simpson reciprocal) and the weighted UniFrac distances for principal coordinate analysis (PCoA) were calculated by using the QIIME software package (23). The closest relative of the operational taxonomic units (OTUs) was determined based on the results of BLAST search comparisons of their 16S rRNA gene sequences with those in the DDBJ nucleotide sequence database (http://www.ddbj.nig.ac.jp/). The hypothetical populations of the OTUs were calculated by multiplying relative abundance (%) by the MLSS value (mg/L). The raw sequence data in this study were deposited in the MG-RAST database (http://metagenomics.anl.gov/) as a "Microbial dynamics under high organic loading in MBR 2015" project under the IDs 4618790.3–4618819.3.

RESULTS

Reactor performance A pilot-scale MBR was constructed (Fig. 1) and operated for 19 days with constant input and output flow rates. On day 8, the concentration of organic matter in the synthetic wastewater (i.e., influent) doubled from 450 to 900 mg COD/L; this allowed us to analyze the response of microbial communities to high organic loading. Major physicochemical parameters of MBR performance are summarized in Table 1. As the hydraulic retention time (HRT; the average length of time a soluble compound remains in the reactor) was set to 2 days, the turnover of concentrated synthetic wastewater required at least a 6-day operational period (24). Therefore, the operational period was divided into three organic loading phases: (i) low organic loading (days 1–7); (ii) transition state (days 8–14); and (iii) high organic loading (days 15–19). Compared with earlier phases, the high organic loading phase was associated with higher concentrations of MLSS, NH_4^+, and TOC and lower concentrations of DO and NO_3^- (Table 1) in the activated sludge. This implied that reactor conditions became more anaerobic under conditions of high organic loading. Nonetheless, high removal rates of organic compounds (85.3–91.4%) were achieved during high organic loading. The values for TMP and effluent flux remained steady, indicating that membrane fouling did not occur during