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Full Paper

Effect of a microbiota activator on accumulated ammonium and microbial community structure in a pilot-scale membrane bioreactor

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Yuya Sato,[1] Tomoyuki Hori,[1] Navarro R. Ronald,[1] Hiroshi Habe,[2,*] and Atsushi Ogata[1]

[1] *Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan*
[2] *Research Institute for Sustainable Chemistry, AIST, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan*

Microbiota activators (MAs) have been used to improve the reactor performances of biological wastewater treatment processes. In this study, to remove ammonium (NH_4^+) accumulated during the pre-operation of a pilot-scale membrane bioreactor (MBR) under high-organic-loading conditions, an MA was added to the MBR system and the resulting changes in reactor performances and microbial communities were monitored for 12 days. The NH_4^+ concentrations in the sludge and effluent decreased (from 427 to 246 mg/L in the sludge (days 1–9)), and mixed liquor suspended solid increased (from 6,793 to 11,283 mg/L (days 1–12)) after the addition of MA. High-throughput Illumina sequencing of 16S rRNA genes revealed that the microbial community structure changed along with the NH_4^+ removal resulting from the MA addition. In particular, the relative abundance of an *Acidovorax*-related operational taxonomic unit (OTU) increased significantly, accounting for approximately 50% of the total microbial population at day 11. In contrast, the ammonia-oxidizing bacteria and archaea showed low abundances (<0.05%), and no anaerobic ammonia oxidizers were detected. These results suggested that the *Acidovorax*-related OTU was mainly involved in the NH_4^+ removal in the MBR, probably due to its ammonia-assimilating metabolism.

Key Words: activated sludge; high-throughput sequencing; membrane bioreactor; microbial community structure; microbiota activator

Introduction

Membrane bioreactors (MBRs), which combine activated sludge treatment with membrane filtration technology, have gained increasing attention during the last two decades in the field of wastewater treatment. Compared with the conventional activated sludge method, MBRs have several advantages, including less production of excess sludge and smaller space requirements for the treatment, as the system can be operated with a much higher biomass concentration (Le-Clech, 2010). Maintaining metabolically active microbial communities in activated sludge is important for a stable reactor performance; however, the activity of microbial populations can be affected by the physicochemical parameters of the inlet wastewater. Operating MBRs with wastewater containing a high concentration of organic matter, hardly biodegradable matter, and/or toxic substances, often results in the deterioration of reactor performances (Dvorak et al., 2013). In such cases, microbiota activators (MAs), including nutrients, metal ions, surfactants, and adsorbent materials, are known to be effective in maintaining a stable reactor performance by balancing nutrient ratios and/or facilitating flocculation of the sludge (Jefferson et al., 2001; Li et al., 2012; Pala and Tokat, 2002). Nonetheless, our understanding of the response of microbial communities to the addition of MAs remains limited.

In our previous report, a pilot-scale MBR was operated with synthetic wastewater for three weeks, in which the inlet chemical oxygen demand (COD) increased from 450 to 900 mg COD/L (Sato, Y., unpubl.). It was observed that the COD removal rates were high (around 90%), even under high-organic-loading conditions, whereas the ammonium (NH_4^+) concentration increased significantly. The

*Corresponding author: Hiroshi Habe, Research Institute for Sustainable Chemistry, AIST, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan.
E-mail: hiroshi.habe@aist.go.jp

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release of high concentrations of NH_4^+ to the environment causes the eutrophication of rivers and lakes; thus, nitrogen removal is a major target for biological wastewater treatment (Terada et al., 2003).

In this study, MA (which contains minerals and organic nutrients) was added into the MBR, and we investigated its effects on reactor performance—including NH_4^+ removal—in a pilot-scale MBR. In addition, shifts in the microbial community structure in response to MA were analyzed. Because high-throughput sequencing has allowed for a comprehensive assessment of microbial communities in MBR systems (Gao et al., 2010, 2011; Ma et al., 2013), we employed the high-throughput Illumina MiSeq sequencing platform, in which millions of nucleotide sequences are determined in a single run (Bartram et al., 2011; Caporaso et al., 2012). Based on information on the changes in both the physicochemical parameters and fine-scale microbial community structures after MA addition, microbial populations potentially involved in NH_4^+ removal are discussed.

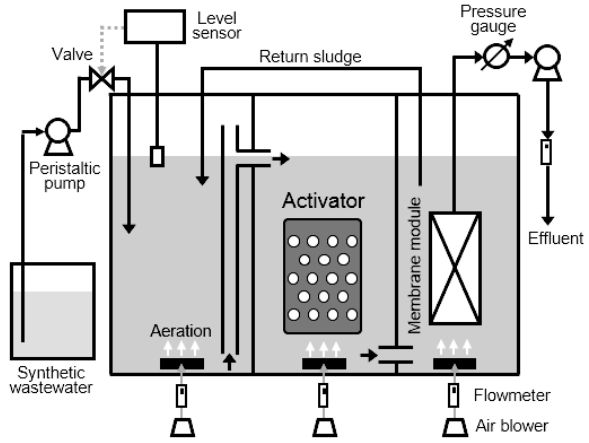


Fig. 1. Schematic configuration of a pilot-scale MBR.

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 continually aerated.

Materials and Methods

Experimental setup and operation of a pilot-scale MBR. A schematic configuration of a pilot-scale MBR used in this study is shown in Fig. 1. The MBR 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 30–35 L/min to maintain dissolved oxygen (DO) values between 0.11 and 1.06 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 a pause for 1 min, and the membrane surface was aerated continuously to reduce membrane fouling. The bioreactor was constantly fed with synthetic wastewater (see below) stored in a feed tank (20 L) at 4°C. The flow rates of both the input of synthetic wastewater and the output of the membrane-filtrated permeate were 115 L/day, and the hydraulic retention time (HRT) was adjusted to 2 days. During the experimental period, no sludge was withdrawn from the reactor (except for sampling). The return sludge flow rate was 115 L/day.

The activated sludge used in this study was obtained from a municipal wastewater treatment plant (Kinu aquastation, Ibaraki, Japan). Five kilograms of MA placed in a perforated cylindrical metal container, BRcnt-5E (CNT Corporation, Kagawa, Japan), were submerged in the second compartment of the MBR (Fig. 1), and kept in the reactor during the operation. The MA is composed of both porous pumice stones for microbial habitants and mixed organic-mineral pellets for biostimulation. The pellet contains 27.50–31.68% organic substances (unknown composition), 0.93–1.42% total nitrogen (TN, unknown composition), 50.83–52.00% SiO_2, 2.80–4.73% Fe, 0.19–0.71% CaO, 0.09–0.16% K_2O, 0.15–0.24% MgO, <0.14% P_2O_5, and others (10.16–16.17%). Before adding the activator to the bioreactor, the MBR was pre-operated for three weeks with 450 to 900 mg COD/L (Sato, Y., unpubl.). After addition of the additive, the MBR was further operated for 12 days. The substrate composition in the inlet (synthetic wastewater) used in this operation was as follows; 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). This COD value was 900 mg/L, which corresponds to 2,260 mg/L as the TOC value.

Analytical procedures. Mixed liquor suspended solid (MLSS), temperature, DO, pH, and transmembrane pressure (TMP) were monitored during operation. A 15-mL sample of activated sludge was taken nine times during the operation. After the solid constituent of the activated sludge was removed by centrifugation (15,300 × g, 15 min, 4°C), the resulting supernatant was further filtered using a cellulose acetate membrane (Φ, 0.20 μm, C020A025A; ADVANTEC, Tokyo, Japan). The amounts of TOC, COD, NH_4^+, NO_2^- and NO_3^- in the supernatant and treated effluent were analyzed as follows (Aoyagi et al., 2015b): TOC value was determined using a TOC analyzer (TOC-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^+, NO_2^-, and NO_3^- were determined using capillary electrophoresis (CE; Agilent, Santa Clara, CA, USA). All data were represented as the mean values from at least two different sampling points of the reactor: MLSS, temperature, DO, and pH were measured in all three compartments; TOC, NH_4^+, NO_2^-, and NO_3^- were measured in the first and second compartments of the reactor.

DNA preparation and PCR amplification. The activated sludge samples from the second compartment of the MBR were washed once with a 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-beat-

ing twice, phenol-chloroform extraction, and ethanol precipitation (Hori et al., 2015; Noll et al., 2005). RNA was digested with RNase (Type II-A; Sigma-Aldrich, St. Louis, MO, USA). Purified DNA was quantified using NanoDrop Lite (Thermo Fisher Scientific, Waltham, MA, USA), and was used as template for PCR amplification with a high-fidelity DNA polymerase (Q5; NEB, Ipswich, MA, USA). The V4 region of 16S rRNA genes was amplified using the universal primers 515F and 806R (Caporaso et al., 2012). Both primers were modified to contain an Illumina adapter region, and the reverse primer contained a 12-bp barcode for multiplex sequencing (Bartram et al., 2011). The thermal conditions of PCR were as follows: initial denaturation at 98°C for 90 s followed by 35–40 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 at 72°C for 2 min.

High-throughput Illumina sequencing of 16S rRNA gene amplicons. High-throughput sequencing was performed as described previously (Aoyagi et al., 2015a). Briefly, the PCR products were purified using an AMPure XP kit (Beckman Coulter, Brea, CA, USA). The resulting 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 Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The DNA concentration was determined spectrophotometrically using 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 performed according to a previous report (Itoh et al., 2014). Contaminating PhiX sequences in the Illumina sequence libraries were detected using a homology search against the Greengenes database (DeSantis et al., 2006) using the Burrows-Wheeler Aligner, version 4.0.5 (Li and Durbin, 2009). The PhiX sequences were then removed from the library by self-written scripts. The paired-end sequences were joined using a fastq-join tool in the ea-utils software package, version 1.1.2-301 (Aronesty, 2013). The joined sequences with Q scores of ≥30 were collected using the software package QIIME, version 1.7.0 (Caporaso et al., 2010), and aligned using the program Mothur, version 1.31.2 (Schloss et al., 2009), after which the chimeric sequences were detected and excluded from the library. The sequences in each library were characterized phylogenetically using the QIIME software package (Schloss et al., 2009). The α-diversity indices (i.e., Chao1, Shannon, and Simpson reciprocal) were calculated using the QIIME software package. The closest relative of the OTUs was determined based on the results of BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparisons of their 16S rRNA sequences with those in the DDBJ nucleotide sequence database (http://www.ddbj.nig.ac.jp/). The raw sequence data in this study were deposited in the MG-RAST database (http://metagenomics.anl.gov/) as a "Microbial dynamics in response to microbiota activator in MBR 2015" project under the IDs: 4631878.3–4631886.3.

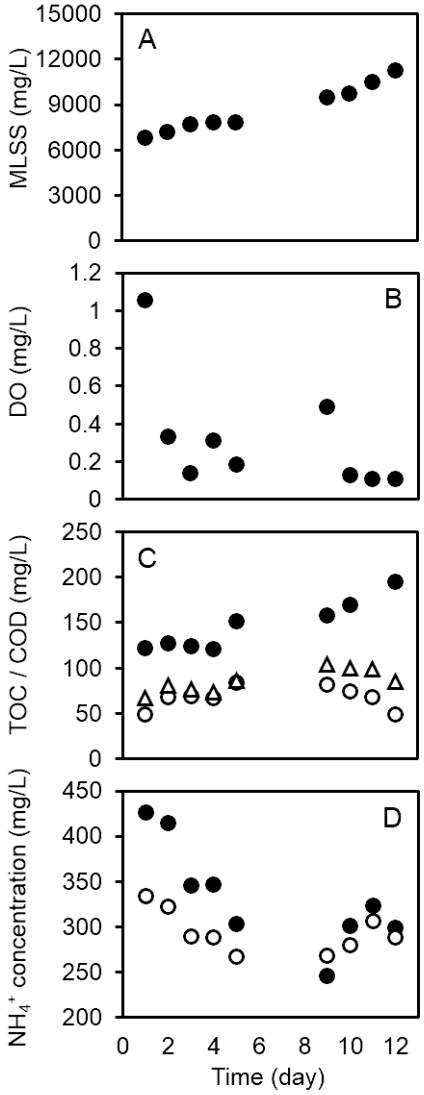


Fig. 2. Changes in physicochemical parameters of the sludge and effluent during the operation.

(A) MLSS in the sludge. (B) DO in the sludge. (C) Closed circle, TOC in the sludge; open circle, TOC in the effluent; open triangle, COD in the effluent. (D) Closed circle, NH_4^+ in the sludge; open circle, NH_4^+ in the effluent. The samples for days 6–9 were not analyzed.

Results and Discussion

Reactor performance

In this study, a pilot-scale MBR (Fig. 1) was operated for 12 days after a three-week pre-operation under high-organic-loading conditions (Sato, Y., unpubl.) at constant flow rates for the input and output (115 L/day). On day 1 (i.e., immediately after MA was placed into the MBR), concentrations of MLSS, DO, TOC value, and NH_4^+ concentrations in the sludge were 6,793 mg/L, 1.06 mg/L, 122 mg/L, and 427 mg/L, respectively. Time courses of major physicochemical parameters during the 12-day operation are summarized in Fig. 2. The MLSS value gradually increased from 6,793 to 11,283 mg/L. The DO value drasti-

cally decreased at day 2, probably because oxygen was highly consumed by microorganisms stimulated by the MA. After day 2, DO was maintained lower than 0.50 mg/L during the operation, indicating that the sludge was under microaerobic conditions. The TOC value in the sludge gradually increased to 195 mg/L at day 12; however, both TOC and COD in the effluent stayed at lower levels throughout the operation (TOC, 49–83 mg/L; COD, 67–104 mg/L). Based on the TOC and COD values, high removal rates of organic matter ranging from 88.4% to 92.6% were obtained throughout the operation. Notably, the concentration of NH_4^+ in the sludge decreased after the addition of MA, suggesting that biological consumption of NH_4^+ was stimulated by the MA. Neither NO_2^- nor NO_3^- were detected throughout the operation. The TMPs varied between –6 and 27 kPa, and coincided with the change in effluent-flow rates from 67 to 90 mL/min throughout the operation. Based on the values of the physicochemical parameters shown in Fig. 2, the reactor performance of the MBR system was stable, especially in terms of the removal of COD and NH_4^+.

Population dynamics of microbial communities in the MBR

The microbial population dynamics in response to the addition of MA was evaluated using high-throughput Illumina sequencing of 16S rRNA genes from activated sludge samples. The total number of sequences characterized for nine sludge samples was around 0.65 million, corresponding to an average of 71,953 sequences per library. Alpha-diversity indices (i.e., Chao1, Shannon, and Simpson reciprocal) obtained from the Illumina sequencing are summarized in Fig. 3. All three α-diversity indices increased during the first 3–5 days, indicating that the microbial community was diversified by the addition of MA, probably because some dissolved nutrients and minerals from the activator supported the growth of microorganisms present in small populations. After that, the diversity gradually decreased.

The class-level phylogenetic analyses of Illumina sequence data clarified the changes in microbial community composition in response to the addition of MA (Fig. 4). Microorganisms belonging to the α-, β-, γ-proteobacteria, Sphingobacteriia, and Flavobacteriia were highly abundant throughout the operation, as is commonly found in activated sludge systems (Hu et al., 2012). At day 1, the dominant sludge microorganisms were affiliated within α-, β-, γ-, δ-proteobacteria, Sphingobacteriia, and Flavobacteriia, with relative abundances of 9.3%, 13.6%, 39.4%, 5.6%, 9.3%, and 14.0%, respectively. In contrast, at the end of the operation, the relative abundances of γ- and δ-proteobacteria decreased (4.5% and 0.19%, respectively), whereas those of β-proteobacteria, Clostridia, and Bacteroidia increased (53.4%, 3.4%, and 7.5%, respectively). This increase in the relative abundance of β-proteobacteria suggested that some species belonging to this taxonomic group were largely stimulated by the addition of MA. The increase in relative abundance of anaerobic bacteria such as Clostridia and Bacteroidia could be explained in relation to the microaerobic conditions in the MBR (Fig. 2B).

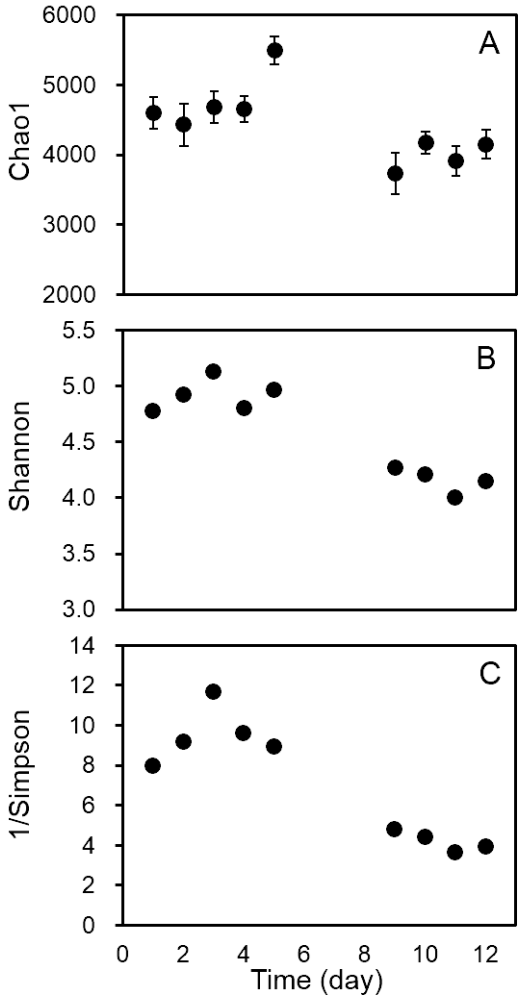


Fig. 3. Alpha diversity indices calculated based on the obtained sequence data.

(A) Chao1, (B) Shannon, and (C) Simpson reciprocal. Each index was calculated based on an equal amount of sequences (33,585) sub-sampled 10 times from original libraries (mean ± SD). For each index, higher values represent more diverse microbial communities. The samples for days 6–9 were not analyzed.

OTUs increased in response to the addition of MA

Figure 5 shows changes in the relative abundance of the 10 most abundant bacterial OTUs at day 12. OTU 1 belonging to β-proteobacteria was exclusively dominant throughout the operation. Its abundance reached 52.2% at day 11, which corresponded to 95% of the total β-proteobacterial population. This OTU was 100% identical in the 16S rRNA gene sequence to *Acidovorax ebreus* (GI: 444304167), a facultative anaerobic bacterium, belonging to the family Comamonadaceae (Carlson et al., 2013). The *A. ebreus* genome harbors a complete set of genes for denitrification and three different terminal oxidases including high oxygen affinity types (i.e., cbb_3 and cytochrome *d* oxidases) (Byrne-Bailey et al., 2010). This suggests that these respiratory enzymes allowed this bacterium to survive under microaerobic and anaerobic environments, and most likely emerged during the operation of the MBR (Fig. 2B).

Due to the increase in relative abundance, *Acidovorax*-

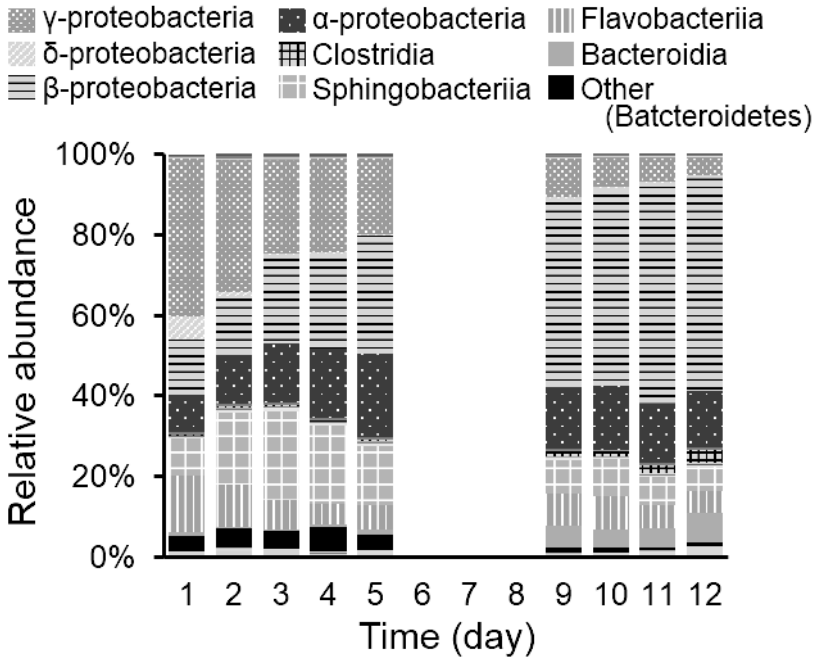
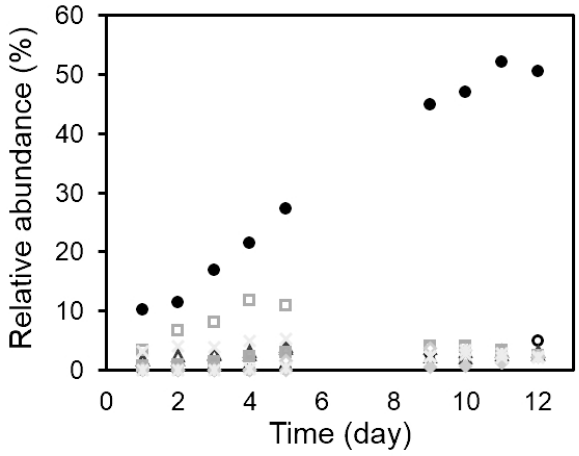


Fig. 4. Structural changes in microbial communities characterized at the class level.

Microbial community structure in activated sludge samples was analyzed using high-throughput sequencing of 16S rRNA genes. Relative abundances of the sequences at the class level are shown. The samples for days 6–9 were not analyzed.



OTU	Related species	Identity (%)
1	*Acidovorax ebreus*	100
2	Bacterium B1C2-8 (Bacteroidetes)	99
3	*Rhodobacter* sp.	100
4	*Rhodobacter* sp.	99
5	*Brevundimonas terrae*	100
6	*Ferruginibacter* sp.	96
7	*Fusibacter* sp.	100
8	*Fluviicola taffensis*	92
9	*Paracoccus* sp.	99
10	*Solitalea koreensis*	87

Fig. 5. Changes in relative abundance of the bacterial operational taxonomic units (OTUs).

Time courses of relative abundances of the 10 most predominant bacterial OTUs during a 12-day operation are shown. The OTUs are indicated by symbols as shown in the table on the right. The closest relative species and their identity obtained by the BLAST search are provided. The samples for days 6–9 were not analyzed.

related OTU was believed to be largely involved in the consumption of NH_4^+ in the MBR. The fact that the NH_4^+ concentration began to increase at days 9–10 (Fig. 2D), accompanied with the growth of OTU 1 reaching the stationary phase (Fig. 5), supports the contribution of this OTU to NH_4^+ removal. Biological ammonia consumption is classified into assimilation and dissimilation, and most microorganisms are capable of assimilating ammonia *via* the biosynthesis of amino acids. The genes encoding glutamine synthetase and glutamate dehydrogenase (Accessions: WP_015912807.1 and WP_015913052.1; EC numbers: 6.3.1.2 and 1.4.1.4), which catalyze the ammonia-assimilation reactions, were found in the *A. ebreus* genome.

In contrast, the potential contribution of other microorganisms to dissimilatory NH_4^+ consumption was investigated using sequencing data. The dissimilatory ammonia-metabolisms are divided into aerobic and anaerobic types;

namely, nitrification and anammox (ANaerobic AMMonia OXidation) (Ge et al., 2015; Hatzenpichler, 2012). The nitrification proceeds in the following two steps: (i) ammonia oxidation to nitrite, which is the first and rate-limiting reaction, catalyzed by ammonia-oxidizing bacteria (AOB) and archaea (AOA); (ii) nitrite oxidation to nitrate catalyzed by nitrite-oxidizing bacteria (NOB). Although both AOB and AOA could be detected in the MBR, their relative abundances were quite low (<0.05%) (Fig. S1) and their populations did not change regardless of the NH_4^+ concentration. In addition, NOB was not detected in this study. These results suggested that the contribution of nitrification by AOB and AOA to NH_4^+ consumption was quite small. This assumption was also supported by the absence of nitrite, the product of aerobic ammonia oxidation. On the other hand, none of the five anammox genera reported so far (i.e., *Kuenenia*, *Brocadia*, *Anammoxoglobus*, *Jettenia*, and *Scalindua*) (Kartal et al., 2013) were detected in this study, suggesting that the NH_4^+ consumption by anammox bacteria was minor. Based on these results, ammonia assimilation by the *Acidovorax*-related OTU, rather than dissimilation, largely contributed to NH_4^+ removal from the MBR. To verify the impact of the *Acidovorax*-related OTU, isolation and further characterization of this OTU will be required.

Conclusions

To evaluate the effect of an MA on the accumulated NH_4^+ and microbial community structure, we investigated the reactor performances and fine-scale dynamics of microbial communities in the pilot-scale MBR using a combination of physicochemical analyses and high-throughput sequencing of 16S rRNA genes. After the addition of MA, the NH_4^+ concentration in both the sludge and effluent precipitously decreased, indicating that the application of MA was effective in decreasing the NH_4^+ concentration. During the same period, the relative abundance of *Acidovorax*-related OTU increased, accounting for approximately 50% of the total microbial population. Meanwhile, the relative abundances of AOB and AOA remained lower than 0.05% throughout the operation. Neither NOB nor anammox bacteria were detected in the MBR. These results strongly suggested that the decrease in the NH_4^+ concentration mainly stemmed from the assimilatory metabolism of the *Acidovorax*-related OTU; thus, enhancing the growth of the OTU may be effective for the removal of ammonia from municipal wastewater.

Acknowledgments

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Supplementary Materials

Fig. S1. Structural changes in ammonia-oxidizing bacteria (AOB) and archaea (AOA) communities during the MBR operation.

The histograms indicate the relative abundances of the OTUs. For each OTU, the closest species determined by the BLAST analysis are shown on the right. The samples for day 6–9 were not analyzed.

Supplementary figure is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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