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Bacterial Community Shift during the Startup of a Full-Scale Oxidation Ditch Treating Sewage

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The oxidation ditch (OD) is one of the most widely used processes for treating municipal wastewater. However, the microbial communities in the OD systems have not been well characterized, and little information about the shift of bacterial community during the startup process of the OD systems is available. In this study, we investigated the bacterial community changes during the startup period (over 100 days) of a full-scale OD. The results showed that the bacterial community dramatically changed during the startup period. Similar to the activated sludge samples in other studies, Proteobacteria (accounting for 26.3%–48.4%) was the most dominant bacterial phylum in the OD system, but its relative abundance declined nearly 40% during the startup process. It was also found that Planctomycetes proliferated greatly (from 4.79% to 13.5%) and finally replaced Bacteroidetes as the second abundant phylum in the OD system. Specifically, some bacteria affiliated with genus *Flavobacterium* exhibited remarkable decreasing trends, whereas bacterial species belonging to the OD1 candidate division and Saprospiraceae family were found to increase during the startup process. Despite of the bacterial community shift, the organic matter, nitrogen, and phosphorus in the effluent were always in low concentrations, suggesting the functional redundancy of the bacterial community. Moreover, by comparing with the bacterial community in other municipal wastewater treatment bioreactors, some potentially novel bacterial species were found to be present in the OD system. Collectively, this study improved our understandings of the bacterial community structure and microbial ecology during the startup of a full-scale wastewater treatment bioreactor.

Keywords: Oxidation ditch, bacterial community, activated sludge, municipal wastewater treatment

Introduction

Oxidation ditch (OD) is a widely used process for simultaneously removing organic matter, nitrogen, and phosphorus from wastewater [1, 2]. Many studies have been conducted to investigate the performance of different OD systems affected by various parameters [1, 3, 4], including loading rate, temperature, hydraulic retention time, dissolved oxygen, operational mode, *etc*. As the main element of the OD system, the microbial community in activated sludge plays key roles for removing organic matter and nutrients. However, few studies have been conducted by to reveal the structure, metabolic functions, and ecology of the microbial community in the OD systems, which is partially due to the extremely high complexity of the microbial community in the activated sludge and the lack of powerful approaches to resolve the high complexity [5].

With the recent development of high-throughput sequencing technologies, the microbial community in various full-scale wastewater treatment bioreactors have been intensively studied [5–8]. Clear geographical differences were observed

among the activated sludge samples taken from municipal wastewater treatment plants (WWTPs) in Asia and North America [6]. Moreover, core and transiently abundant microorganisms were identified based on analyzing the microbial community in the activate sludge samples from multiple WWTPs [9]. The functional and taxonomic richness of the microbial communities in activated sludge were also investigated, and positive association was found between the functional and taxonomic richness [8]. However, few studies have been conducted to investigate the microbial community shift in the startup process of full-scale wastewater treatment bioreactors, and little is known about how the microbial community evolves over time in OD and other engineered wastewater treatment systems.

In this study, to understand the shift of bacterial community during the startup process of the OD system and to identify the relationship between the bacterial community and pollutant removal, we systematically investigated the changes of the microbial community in the startup process of a full-scale sewage treatment OD system based on high-throughput sequencing of the bacterial 16S rRNA gene. The performance of the OD systems was analyzed, and the microbial community shift and the possible reasons causing the shift were revealed. Bacterial species that significantly increased and decreased in relative abundance were identified. Besides this, we also compared the microbial community in the OD system with those in the activated sludge samples from other full-scale wastewater treatment bioreactors reported in previous studies and confirmed that most of the bacteria species are commonly present in different wastewater treatment bioreactors.

Materials and Methods

WWTP Description and Sample Collection

A municipal WWTP located in Nanjing, which treats approximately 100,000 m^3 of sewage per day by the three-tank OD treatment process (Fig. 1), was started up. Activated sludge from another WWTP in Nanjing with an anaerobic/anoxic/oxic process was used as the seeding sludge. During the startup process, human feces was added to increase the nutrients in the influent and maintain the growth of the biomass in the OD system. Twelve activated sludge samples from the middle tank were taken during the startup process in a time span of 122 days. Activated sludge samples were fixed on site by mixing with 100% ethanol at a volume ratio of 1:1, and then transported to our laboratory and stored at –20°C before DNA extraction. In addition, 1 L of influent and 1 L of effluent samples were taken for water quality analysis.

Water Quality and Biomass Analysis

Concentrations of chemical oxygen demand (COD), ammonium nitrogen, and total phosphorus of the influent and effluent samples from the OD system were determined to indicate treatment efficiency. The concentration of mixed liquor suspended solids (MLSS) was measured to reflect the changes of biomass in the OD system. All of these analyses were carried out according to the *Standard Methods for the Examination of Water and Wastewater* [10].

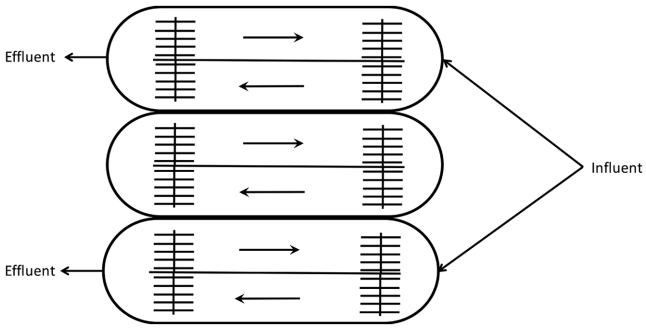

Fig. 1. Schematic diagram of the oxidation ditch.
The three tanks were operated in batch mode and served as both aeration sedimentation tanks during the wastewater treatment process.

DNA Extraction and PCR

The activated sludge samples were centrifuged at 4,000 rpm for 10 min at 4°C. About 200 mg of pellet was recovered for DNA extraction in duplicate with a FastDNA SPIN Kit for Soil (MP Biomedicals, USA). The DNA purity and concentration were determined with NanoDrop ND-2000 (NanoDrop Technologies, USA). The hypervariable V3-V4 region of the 16S rRNA gene was amplified from the DNA samples with the primer set V3F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and V4R (5′-TAC NVG GGT ATC TAA TCC-3′) [11]. The 10-nucleotide barcodes were incorporated between the 454 adaptor and the forward primers to distinguish reads from the data pool generated in a single pyrosequencing run. Each PCR solution (50 μl) contained 1× Pfx Amplification Buffer (Invitrogen, USA), 0.4 mM of dNTP, 2 mM of $MgSO_4$, 0.4 μM of each primer, 1 μl of template DNA and 2 U of Platinum Pfx DNA Polymerase (Invitrogen, USA). The PCR amplification was conducted in a thermal cycler under the following conditions: initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 sec, 62°C for 30 sec, and extension at 70°C for 45 sec, with a final elongation step at 70°C for 7 min. In order to minimize the impact of potential early-round PCR errors, amplicon libraries were prepared by combining three independent PCR products for each sample [12].

High-throughput Sequencing of 16S rRNA Gene and Sequencing Data Quality Filtration

The purified PCR products were qualified using Bioanalyzer 2100 (Agilent, USA) and then mixed accordingly to achieve an equal DNA mass in the final mixture, which was sequenced on a Roche 454 FLX Titanium platform (Roche, USA). Sequencing data were deposited into the Sequence Read Archieve under the

accession number PRJNA327654.

The generated raw reads of activated sludge samples were processed by using the Pyrosequencing Pipeline Initial Process [13] of the Ribosomal Database Project (RDP): (i) to assign the reads into different samples according to the barcodes, (ii) to trim off the adapters, barcodes, and primers using the default parameters, and (iii) to remove reads containing more than one ambiguous base (N) and reads shorter than 150 bp. Subsequently, the sequencing data were denoised using the pre.cluster command in the Mothur software [14] to remove the sequences containing errors. Then, PCR chimeras were filtered out using the chimera.slayer command in Mothur. Furthermore, the archaeal sequences were filtered out after the archeal and bacterial sequences of all samples were identified using RDP Classifier (ver. 2.6) [15]. In order to fairly compare all the samples at the same sequencing depth, 5,820 sequences in each sample were randomly selected for the subsequent bioinformatics analysis (Table S1).

Bacterial Community Analysis

After quality filtration, Quantitative Insights Into Microbial Ecology (QIIME) [16] was used for de novo operational taxonomic unit (OTU) clustering with the uclust method at 94% (genus level) and 97% (species level) identity. The representative sequence of each OTU was assigned to specific taxonomy with RDP Classifier [15]. The alpha-diversity indices (*i.e.*, Chao 1 estimator, abundance-based coverage estimator (ACE), and Shannon estimator) were also calculated by using QIIME. The representative sequences of the OTUs were compared with 16S rRNA gene sequences of activated sludge published in previous studies [6, 9] with the BLAST program.

Statistical Analysis

Distances between microbial communities in different activated sludge samples were calculated using the weighted UniFrac beta diversity metric via QIIME. Principal coordinates analysis (PCoA) was used to visualize the pairwise UniFrac distances among different samples.

Results and Discussion

Performance of the OD System during Startup

In this study we investigated the startup process of a full-scale sewage treatment OD system in a newly build municipal WWTP, which has a treatment capacity of about 20,000 m^3/day. The three tanks of the OD system were operated in sequential mode and served as both aeration tank and sedimentation tank. Activated sludge from another full-scale WWTP with an anaerobic/anoxic/oxic process was transferred to this OD system and used as seeding sludge. During the startup process, both operation parameters and the microbial community were monitored. As shown in Fig. 2, the COD concentration in the influent

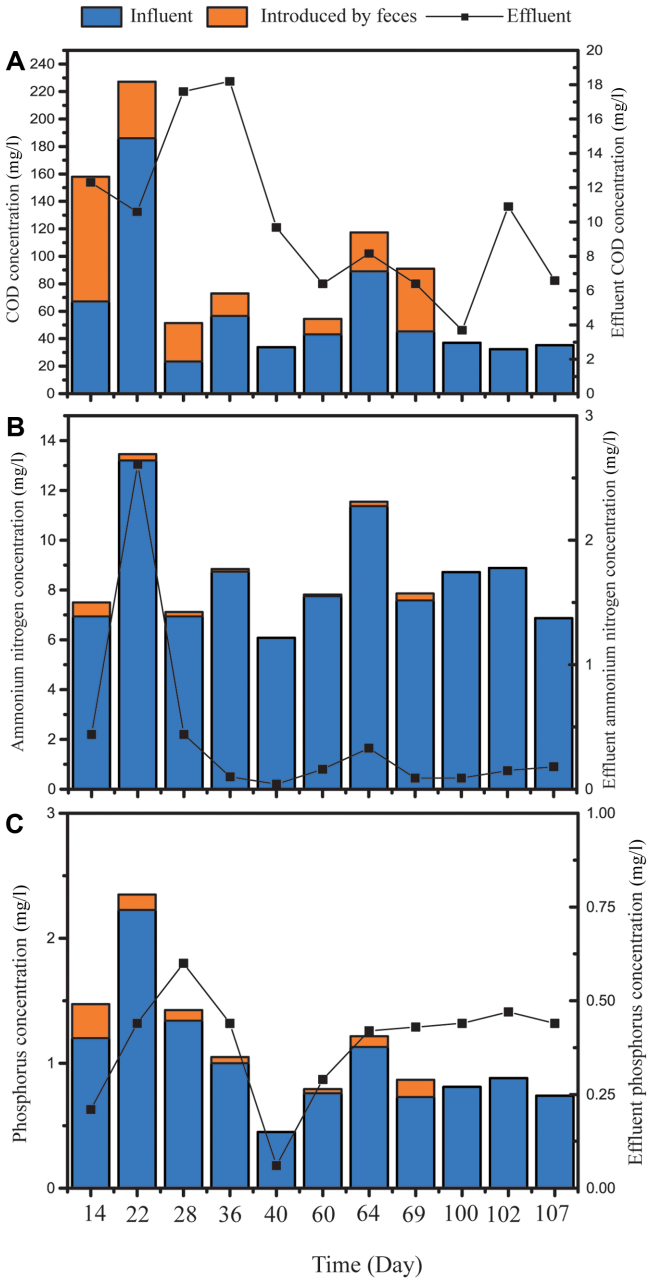

Fig. 2. COD (**A**), ammonium nitrogen (**B**), and phosphorus (**C**) concentrations in the influent and effluent samples taken on different days from the OD system.

The orange color bars in each panel represent the parts of pollutants (COD, ammonium nitrogen, or phosphorus) introduced by adding fecal matter to the influent.

(average value 59.0 mg/l) was lower than those of the common municipal WWTPs [6, 17]. This is because the WWTP was newly built and some pipelines transporting sewage were not well constructed; therefore, some rain

water or drinking water might enter the sewage pipeline. Owing to the low COD concentration in the influent, the biomass concentration in the OD system was also at a relatively low level. The MLSS in the first 20 days was about 1,000 mg/l. This is much lower than the values (around 3,000–5,000 mg/l) in other OD systems [18, 19]. To maintain the biomass concentration for the possible increase of organic loading rate, certain amounts of human feces were added to the OD system periodically. As shown in Figs. 2A–2C, after adding human feces, the COD concentration in the influent could be increased about 25.30–135.30 mg/l. Moreover, ammonium nitrogen and phosphorus concentrations were brought up by 0.07–0.56 mg/l and 0.03–0.27 mg/l, respectively.

By adding human feces, the biomass (MLSS concentration) in the OD system could generally be maintained at about 1,500 mg/l after day 30. It was also found that the COD concentrations in the effluent ranged from 12.3 to 18.2 mg/l before day 36, while after day 40, it decreased to 6.4–9.6 mg/l. This suggested that the removal efficiency was obviously improved after the increase of biomass. On the other hand, the ammonia nitrogen and total phosphorus concentrations were always in a low level (<1 mg/l) except on day 22 (ammonia nitrogen concentration > 2 mg/l), indicating that the biomass in the OD system has sufficient capacity to remove the ammonium nitrogen and phosphorus.

Based on the performance of the OD system and the effluent quality, the startup process was quite successful. Even though the quality of influent fluctuated, both the organic matter and nutrients could be reduced to a very low level. Despite of the good performance, it is of great interest to investigate the organisms in the activated sludge that are responsible for the removal of organic matter and nutrients and to examine whether the microbial community shifted during the startup process.

Taxonomic Complexity of the Bacterial Community

By using 16S rRNA gene high-throughput sequencing, we analyzed the bacteria community in 12 activated sludge samples taken from the OD system during the startup process in more than 100 days. Alpha diversity analysis results (Fig. 3) showed that the Shannon index increased slightly, suggesting that the bacterial richness and evenness changed slightly during the startup process.

Taxonomic assignment results (Fig. 4) showed that 12 abundant phyla (>1% in at least one sample) appeared in these samples. Most of these phyla were also observed in activated sludge samples of the previous studies [5, 6, 20]. Proteobacteria and Bacteroidetes were the two most

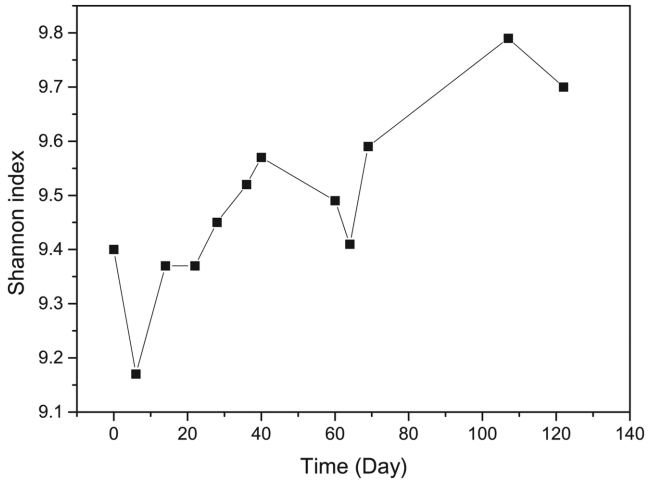

Fig. 3. Shannon index of bacterial diversity in different samples taken from the OD system during the startup process.

dominant bacterial phyla in the seeding sludge (Day 0), which accounted for 43.0% and 12.3%, respectively. This is consistent with most of the activated sludge samples in previous studies [6, 20]. The number of genus level OTUs (singletons were excluded) increased gradually from 528 on Day 0 to 658 on Day 122, which agreed with the Shannon index and indicated that the taxonomic complexity of the bacterial community increased during the startup process.

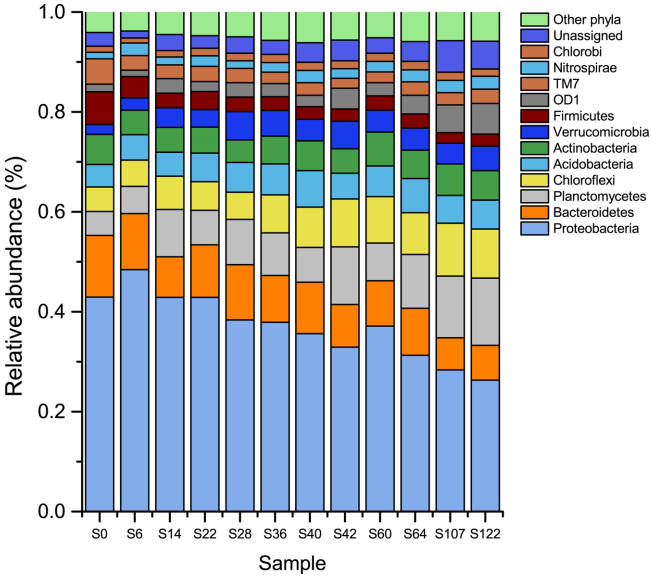

Fig. 4. Relative abundance of bacteria phyla in different samples taken from the OD system.
Only abundant phyla are shown in this figure; phyla in low relative abundances (<1% in all samples) were combined into "Other phyla."

Bacterial Community Shift in the OD System during Startup

As shown in Fig. 4, Planctomycetes proliferated greatly (from 4.79% on day 0 to 13.5% on day 122) during the startup process and replaced Bacteroidetes as the second phylum from day 64. Planctomycetes was widely reported to be present in activated sludge in previous studies [6, 20, 21]. However, compared with the results of this study, the abundance of this phylum in those previous studies was relatively lower (<5%). Chouari *et al*. [22] reported that diverse species of Planctomycetes could grow in activated sludge in under aerobic and anoxic conditions, which is similar to the conditions in the OD system of this study. Although this phylum was widely found, the detailed functions of this phylum in activate sludge were not completely understood. It is known that this phylum contains some bacteria species responsible for anaerobic ammonia oxidation (anammox) [23]. Chamchoi and Nitisoravut [24] successfully cultivated an anammox consortium from activated sludge in lab-scale sequencing batch reactors in 4 months. However, these anammox species may only represent a small fraction of the Planctomycetes phylum. Further studies are needed to investigate the functions of this phylum in the activated sludge, especially in the bioreactor that contains high abundance of this phylum, like the OD system in this study.

Contrary to the Planctomycetes phylum, the relative abundance of the Proteobacteria phylum decreased from 43.0% to 26.3% in the startup process, and the abundance of Bacteroidetes decreased from 12.3% to 6.0%. Overall, from Fig. 4, it can be seen that the bacterial community shifted clearly during the startup process at phylum level. Furthermore, the microbial community was also analyzed by clustering the sequences into genus-level OTUs. Fig. 5 shows the relative abundances of the 33 most dominant (relative abundance >1% in any sample) OTUs in these activated samples. These 33 OTUs belonged to 10 phyla, and 12 OTUs were affiliated with Proteobacteria. It was found that two OTUs affiliated with the *Flavobacterium* genus exhibited remarkable decreasing trends from Day 0 to Day 122. Their abundance declined to almost zero from 1–1.5%. A previous study [6] showed that *Flavobacterium*

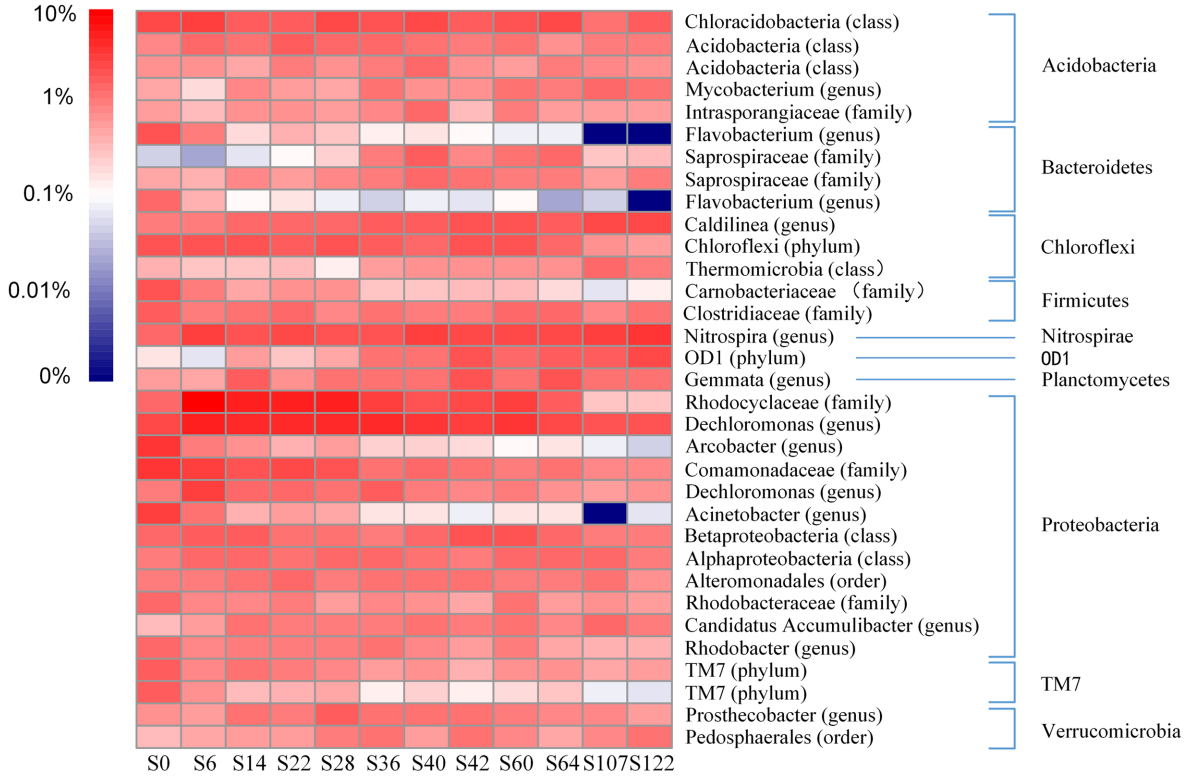

Fig. 5. Heatmap showing the relative abundances of the main genus-level OTUs in the activated sludge samples taken from the OD system.

Each row represents one OTU, and all OTUs were grouped by phylum. The lowest assigned taxonomy of each OTU was shown on the right side of the figure.

was dominant in the activated sludge samples taken from WWTPs in North America, with abundance levels ranging from 1.83% to 7.44%; however, its relative abundances were lower than 1% in the samples from mainland China and Singapore. This indicated the possible geographical difference of this genus. The present study suggested that this genus may not be abundant in the activated sludge of the OD system, even with the relatively high abundance in the seeding sludge. It has been reported that *Flavobacterium* species are usually distributed in soil and freshwater habitats [25]. The species in this genus could also be isolated from diseased fish, domestic drain conduits, and potable dental lines [26]. Recently, Jo *et al*. [27] reported that it could be abundantly present in full-scale membrane bioreactors. These results suggested that this genus probably prefers biofilm environments and may contribute to biofilmformation. The decrease of this genus was probably because the complete mixing condition in the OD system, which was not favorable for the attached growth of this kind of bacteria.

Besides this, *Arcobacter* and *Acinetobacter*, which both belong to Proteobacteria, also declined to a very low level (about 0.4%) at the end of the study period. Both genera contain human pathogenic species and were widely found in human feces [28, 29]. These two genera may come from the fecal matter added in the influent, and after the feces addition was stopped, they were gradually eliminated to the system. Moreover, it was found that one OTU affiliated to TM7 also decreased clearly in the startup process, but the reason for this decrease was not clear. In addition, an OTU belonging to OD1 and an OTU belonging to Saprospiraceae family increased during the startup process. Moreover, some other taxa, like OTUs in the Acidobacteria phylum and *Verrucomicrobia* genus, remained almost unchanged during the startup process, suggesting that these bacteria could well adapt themselves to the environment of the OD system.

To further examine the shift of the microbial community during the startup process, PCoA based on UniFrac distances was used to investigate the microbial community similarity among different activated sludge samples. Fig. 6 shows the microbial community shift among the startup period. Samples taken on day 0 and day 6 were clearly separated, suggesting that the condition of the OD system may change the microbial community in the seeding sludge. After fecal matter was added to the OD system, the microbial community shifted further from day 14 to day 64, and then the microbial community continued to evolve although feces addition was discontinued. These results

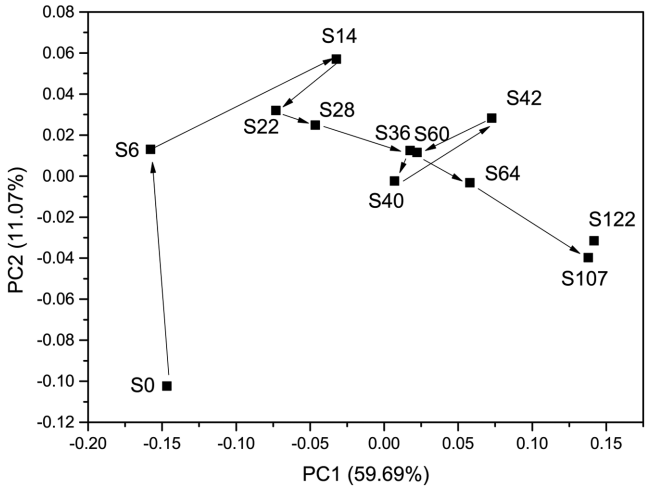

Fig. 6. Principal coordinates analysis plot showing the microbial community shift during the startup process of the oxidation ditch.

suggested that the microbial community in seeding sludge evolved during the startup of the OD system, and this process could be affected by adding fecal matter. Similar results were also observed in the lab-scale membrane bioreactors [30], which showed that the acclimated microbial communities could evolve over time in engineered systems even though the operational parameters were left unchanged. So far, although few studies have been conducted on the microbial community shift in the startup process of bioreactors, the microbial community shift is widely found in the operation process of various reactors, and the reason for the shift could be due to changes of dissolved oxygen, temperature, or other operational parameters [31–33]. In many cases, like this study, the microbial community shift has little effect on the performance of the bioreactors, indicating the functional redundancy of the microbial community in the bioreactors. However, monitoring the microbial community is still of great importance, since many operation problems (*e.g.*, effluent deterioration or sludge bulking) could be caused owing to the specific bacterial species decrease or overgrowth.

Comparison of the Bacterial Community in the OD System with That in Other Bioreactors

Furthermore, we also compared the dominant bacterial species (top 100 most abundant OTUs) (Table S2) observed in this study with those in the previous studies [6, 9] by using the BLAST program. The results in Fig. 7 show that most (97%) of the investigated OTUs have relatively high identity (>94%) with sequences in the previous studies,

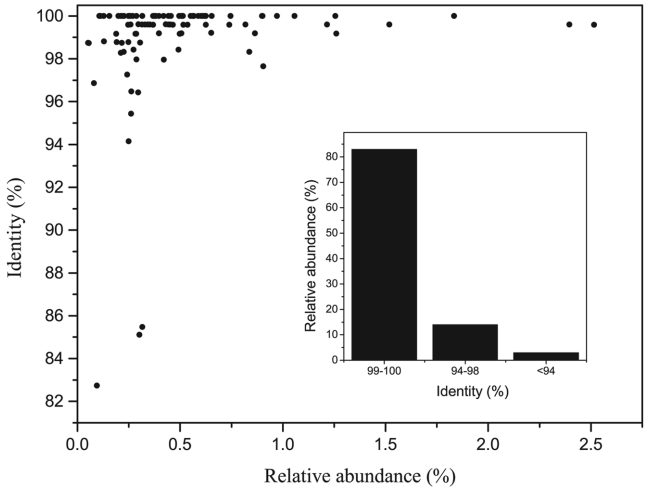

Fig. 7. Similarity of the top 100 abundant OTUs with the microbial species in the activated sludge samples of previous studies [6, 9].
The bar plot in the subpanel shows the proportions of the OTUs with different similarities.

suggesting that the activated sludge in different bioreactors treating sewage contain similar microbial communities. This confirmed the core community concept of activated sludge [9]. However, it was also found that three OTUs had relatively lower identities (82–85%) with the sequences in previous studies. One of the OTUs was assigned to candidate division TM7, and the other two could not be assigned to known phyla. All three were in relatively low abundance in the activated samples (<0.3%). These sequences could probably indicate that some novel bacteria species were present in the OD system of this study, and also could possibly be the sequencing noise that could not be eliminated. Further studies based on isolation or other molecular methods are needed to further investigate this issue.

In conclusion, an obvious microbial community shift was observed in the strartup process of a full-scale OD system treating municipal wastewater in this study. The relative abundance of Proteobacteria declined nearly 40% during the startup period, whereas Planctomycetes increased greatly (from 4.79% to 13.5%) during this period and replaced Bacteroidetes as the second abundant phylum in the OD system. It was found that distinct bacterial communities were formed in the OD system, and some potentially novel bacterial species appeared in the OD system according to the comparison with the bacterial community in other municipal wastewater treatment bioreactors. The fecal matter addition could be a helpful approach for the startup of the OD system and could affect the microbial community structure. Despite of the microbial community shift, the performance of the OD system was general stable, and the organic matter, nitrogen, and phosphorus in the effluent met the discharge standards, suggesting the functional redundancy of the microbial community. Future studies focusing on the functional redundancy of the microbial community in the activated sludge may provide us with more helpful information on improving the operation stability and solving the process problems for OD and other engineered wastewater treatment bioreactors.

Acknowledgments

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