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Temporal dynamics of the microbial community in an integrated fixed-film activated sludge system revealed by 16S rRNA MiSeq sequencing

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ABSTRACT

Illumina MiSeq high-throughput sequencing was used to reveal the temporal dynamic of microbial community structure and bacterial diversity in an integrated fixed-film activated sludge (IFAS) system over a seven-month period. Microbial community structure and bacterial diversity varied with different months in the system. The biofilm phase showed a more stable microbial community trait compared with the sludge phase with the fall in environmental temperature. Proteobacteria was the most predominant phylum in the IFAS system, followed by Bacteroidetes, Actinobacteria, Chloroflexi, Firmicutes, Planctomycetes and candidate division TM7. The most predominant ammonia oxidizing bacteria and nitrite oxidizing bacteria in the IFAS system were Nitrosomonas and Nitrospira, respectively. An evident shift in the abundance of nitrifying bacteria took place in the biofilm and sludge of the IFAS system during the sampling months. Principal coordinate analysis and a clustered heat map indicated that living conditions and temperature seemed to be the key factors in shaping the microbial community in the IFAS system.

Keywords: IFAS; Illumina MiSeq sequencing; Microbial community structure; Nitrifying bacteria; Temporal dynamics

1. Introduction

The integrated fixed-film activated sludge (IFAS) process is a modification of conventional activated sludge (CAS) process in wastewater treatment. Compared with the CAS process, the IFAS process has several distinct advantages, including a decreased sludge yield coefficient, better sludge settling characteristics, higher organic loadings and stable nitrogen removal at low temperature [1,2]. These advantages have resulted in widespread acceptance of the IFAS process as an upgrade for obsolete wastewater treatment plants (WWTPs) [3].

The current understanding of the IFAS process is largely obtained from the studies of wastewater treatment in laboratory-scale reactors fed by synthetic wastewater by monitoring of physicochemical parameters [1,4]. Nevertheless, it is well known that the bacterial community in bioreactors is the essential factor in determining the function, performance and stability of wastewater treatment [5]. The microorganisms are susceptible to the physiological stress encountered in the natural environment. Therefore, a better understanding of how community structure and bacterial diversity are affected by dynamic environmental conditions in IFAS WWTPs is indispensable to give us the guidance for design and operation of converted CAS WWTPs.

In previous studies, traditional molecular biology methods such as fluorescence in situ hybridization (FISH), denaturing gradient get electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) have been used to investigate the functional bacteria in the IFAS system [1,6,7].

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These conventional methods enable the detection of several significant bacterial community members in function but lack sufficient sequences to capture comprehensive and systematic information for the analysis of complete community structures. Furthermore, laboratory-scale reactors were utilized in these studies, which would be incapable of replacing the results about the real-world community in WWTPs. Other studies have already shown the differences in microbial community structure between WWTPs and bioreactors [8,9]. For some studies about IFAS WWTPs, they did not capture the temporal dynamic in the microbial community over time scales of months to years; thus, the temporal dynamic of bacterial community may be limited in WWTPs [10–12]. Wells et al. [13] explored bacterial community dynamics in a full-scale activated sludge WWTP over the course of one year and found that the bacterial community was more dynamic than expected. We might expect similar patterns to occur in the IFAS system.

Considering these factors, it is worthwhile and indispensable to thoroughly investigate the temporal dynamic of microbial community structure and bacterial diversity in an IFAS WWTP system by a method with high effectiveness and resolution. High-throughput sequencing can capture comprehensive and systematic information for analyzing the complete community structure at greater sequencing depths and has been applied to the screening of pathogens in environmental samples [14,15]. The primary objective of the study reported here was to reveal comprehensive and systematic information about the microbial community structure and bacterial diversity in the IFAS system. For this, Illumina MiSeq sequencing of bacterial 16S rRNA genes was used to investigate bacterial genes from activated sludge and biofilm samples in an IFAS WWTP system from summer to winter over the seven months covering the highest and lowest temperatures in a whole year.

2. Materials and methods

2.1. WWTP operation

The WWTP investigated in this experiment is a full-scale IFAS WWTP with a two-stage process (anoxic–aerobic) in Shanghai, China. The influent of the WWTP was mostly composed of domestic wastewater, and the treatment capacity of the WWTP was 10,000 m^3/d. Polyethylene carriers with a density of 0.95–0.98 kg/m^3 and a specific surface area of 500 m^2/m^3 were added into the aeration tank with a filling fraction of 30%. Detailed operational characteristics of the WWTP are presented in Table 1.

Table 1
Characteristics of the IFAS system used in this study and the operational data

IFAS system	WWTP[a]
Mixed liquor suspended solids (mg/L)	3,300 ± 200
Solids retention time (d)	15
Hydraulic retention time (h)	12
Temperature (°C)	10.5–29.5
Influent	
COD_{cr}	400 ± 80
NH_4^+	40 ± 10
TN	50 ± 10
Effluent	
COD_{cr}	35 ± 10
NH_4^+	7 ± 4
TN	15 ± 5

[a]WWTP: Wastewater treatment plant.

2.2. Sample collection and DNA extraction

According to different temperature phases, duplicate biomass samples from suspended sludge and biofilm were collected during stable operation condition of the IFAS WWTP in different months (August, September, November, December and February) covering the highest and lowest temperatures in a whole year. Besides, the duplicate samples in each phase were completely mixed for DNA extraction. The temperature of the IFAS reactor in August, September, November, December and February were 28.9°C ± 1.9°C, 26.2°C ± 2.4°C, 21.6°C ± 2.9°C, 16.3°C ± 2.5°C and 11.6°C ± 1.4°C, respectively. All samples were collected at the end of the aeration tank and preserved at −80°C before used. DNA of the suspended sludge and biofilm samples were extracted using the MoBio PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentrations and purities of the extracted DNA samples were analyzed by a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). The qualified DNA samples were stored at –80°C before used.

2.3. High-throughput Illumina MiSeq sequencing

A sequencing library was constructed using a MetaVx™ Library preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). Briefly, 5–50 ng DNA was used to generate amplicons separately covering the V3–V4 and V4–V5 variable regions of bacteria and Archaea 16S rRNA genes. GENEWIZ designed a panel of proprietary primers aimed at relatively conserved regions bordering the V3, V4 and V5 hypervariable regions of bacteria and Archaea 16S rRNA. The V3 and V4 regions were amplified using forward primers containing "CCTACGGRRBGCASCAGKVRVGAAT" and reverse primers containing "GGACTACNVGGGTWTCTAATCC". The V4 and V5 regions were amplified using forward primers containing "GTGYCAGCMGCCGCGGTAA" and reverse primers containing "CTTGTGCGGKCCCCCGYCAATTC". Sequencing was performed using a 2x250 paired-end configuration. Image analysis and base calling were conducted with MiSeq Control Software on the MiSeq instrument (Illumina, Inc., San Diego, CA, USA). Initial taxonomy analysis was carried out on the Illumina BaseSpace cloud computing platform. The sequence data have been deposited into the NCBI Sequence Read Archive.

2.4. Data analysis of sequencing

The 16S rRNA gene sequencing data was analyzed with QIIME software using default quality filters [16]. Briefly, demultiplexed sequences were clustered into operational taxonomic units (OTUs) at 97% similarity. Diversity analyses were performed by running a workflow on QIIME.

Depending on the sequencing results of the samples, bacterial diversity, richness, microbial community structure and main functional microorganism abundance were calculated.

3. Results and discussion

3.1. Microbial community diversity and richness in the IFAS systems

After trimming the adapters, barcodes and primers, and filtering out chimeras, a total of 557,288 effective sequences were generated totally from 10 samples at the same sequencing depth. The number of sequences was comparable with that of other studies [5]. The length range of effective sequences was between 400 and 480 bp, and the large number of sequences made it possible to estimate the microbial community diversity of the system.

To investigate the community diversity and richness of the IFAS process, the abundance-based coverage estimator (ACE) and Shannon index (Fig. 1) were calculated. The ACE index was in the range of 6,500–10,000 (a higher ACE value indicates higher community diversity) and the corresponding Shannon index value ranging from 6.7 to 10.2 (a higher Shannon value indicates higher community richness). The diversity and richness of the samples in our study were comparable with those of other WWTPs [15]. Obviously, from August to February, the community diversity and richness decreased every month for the sludge and biofilm as a whole. In the first three months, the community diversity and richness of the sludge and biofilm remained at a level higher than that of following months, and the sludge phase had a clear advantage in community diversity and richness than the biofilm phase. However, the biofilm phase displayed a dominant advantage in community diversity and richness compared with the sludge phase in December and especially in February. The dynamic of community characteristics may mainly be ascribed to the biological reaction tank temperature (Table 1), and a previous study showed that the drop in diversity during frigid months might be due to a drop in the growth rate of community members to the extent that they are washed out of the system [17]. The low temperature will severely reduce microorganism growth and prolong the generation cycle. In the IFAS system, the microorganism generation cycle of attached growth in biofilm could be decoupled from the influence of traditional sludge retention time (SRT) [18]. Therefore, compared with sludge phase, the biofilm phase possessed a remarkable advantage in community diversity and richness at low temperature. The Venn diagram of major OTUs based on DNA sequencing from samples in February and August were presented in Fig. 2. Both shared and total numbers of OTUs also supported this conclusion.

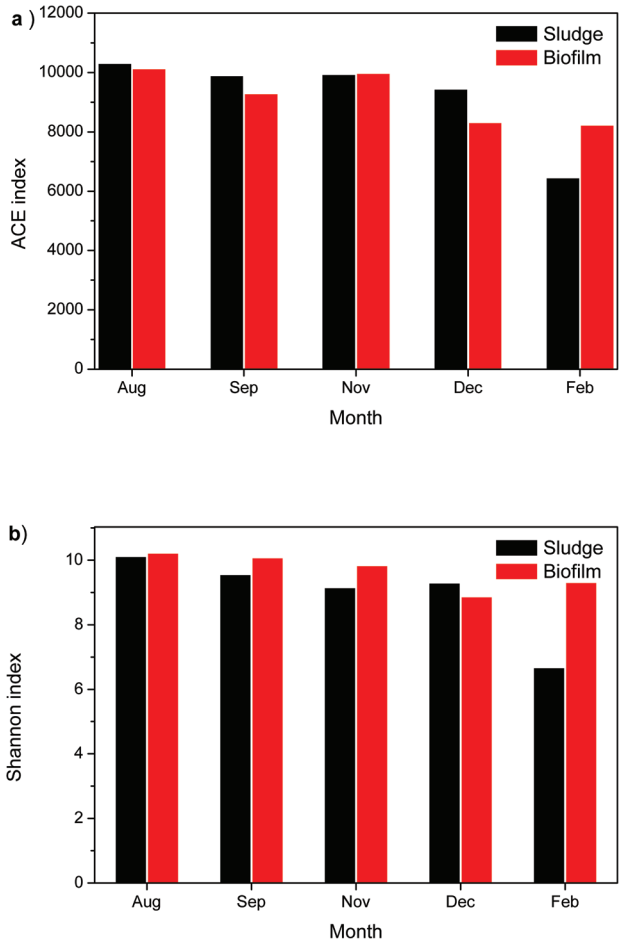

Fig. 1. Diversity and richness between sludge and biofilm in the IFAS system: (a) ACE index and (b) Shannon index.
Abbreviations: S – Sludge; B – Biofilm; Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

3.2. Microbial community structure in the IFAS systems

The RDP Classifier was used to assign selected effective bacterial sequences from 10 samples to different taxa levels from phylum to genus at 97% sequence identity. At the phylum level, nearly all of the sequences were assigned to bacteria, and only a few sequences (less than 0.1%) belonged

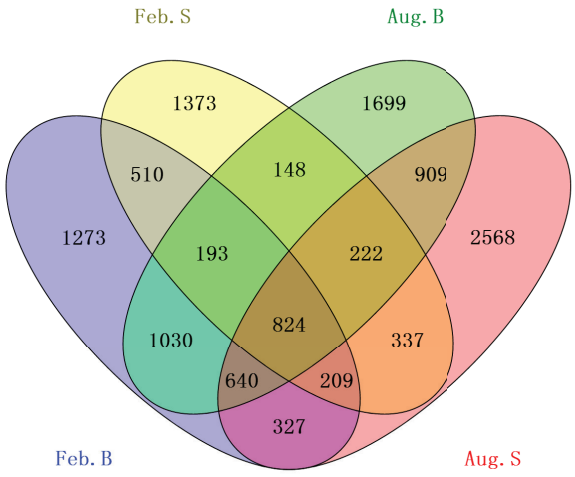

Fig. 2. Venn diagram of OTUs in sludge and biofilm in the IFAS system.
Abbreviations: S – Sludge; B – Biofilm; Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

to Archaea in total. The major phyla for each sample were shown in Fig. 3. Seventeen main identified phyla (more than 0.1%) were observed in the sludge and biofilm samples. The most predominant phylum in sludge during the detection period was Proteobacteria with median 39.7% sequences (minimum 29.8% in February and maximum 41.5% in August). This result was similar to previous analytical results of phylum taxonomy in various municipal WWTPs and wastewater treatment bioreactors [13–15]. The other major phyla in sludge were Bacteroidetes (median 23.2%, 11.5%–55.0%), Actinobacteria (median 10.8%, 1.6%–19.7%), Chloroflexi (median 10.9%, 0.6%–16.2%), Planctomycetes (median 2.7%, 1.6%–4.0%), candidate division TM7 (median 2.4%, 0.2%–4.2%) and Firmicutes (median 1.9%, 1.0%–4.0%), which were also widespread in other systems [19]. In keeping with the findings in the sludge samples, Proteobacteria was the most predominant phylum in biofilm with median 36.1% sequences (minimum 32.4% in December and maximum 41.7% in February). The major phyla in biofilm were Chloroflexi (median 22.0%, 3.8%–16.2%), Actinobacteria (median 10.2%, 4.4%–17.1%), Bacteroidetes (median 8.2%, each plant 2.5%–13.3%), Acidobacteria (median 6.3%, 3.0%–8.1%), Planctomycetes (median 5.8%, 5.2%–6.1%) and Chlorobi (median 4.6%, 2.2%–7.2%). Marked variation in microbial community compositions and dissimilarity between the sludge and biofilm were observed along with the seasonal change, even though the microorganisms living in the sludge and biofilm came from same system and the predominant phylum was identical. Firstly, the relative fluctuation in the abundance of the microbial population structure in biofilm manifested that the microbial community in the biofilm have higher stability than in sludge in terms of major phyla, especially in the low-temperature month. For example, the relative abundance of the most predominant phylum (Proteobacteria) in suspended sludge dramatically decreased from 50.1% in August to 29.8% in February that the coldest month in the sample period. However, this change in biofilm was inconspicuous with a variation of 34.5%–41.7% among Proteobacteria, let alone the change of Bacteroidetes. Secondly, the second-most predominant phyla in sludge and biofilm were Bacteroidetes (median 23.2%) and Chloroflexi (median 22.0%), respectively. As these two kinds of samples were collected from the same WWTP system, living conditions should be the most important factor affecting the microbial community structures and composition. According to another study [14], Bacteroidetes as a common phylum is widely spread in the CAS process. The phylum Chloroflexi is also abundantly present in most municipal WWTPs and plays a significant role in cell aggregation [20]. The isolated retrieved Chloroflexi from activated sludge have been proved to possess very slow growth rates, and a previous study showed that the operation of a membrane bioreactor (MBR) at longer SRT favors the slow-growing organisms [21]. Similar to the MBR system, the attached living biofilm in the present study could shelter organisms from the influence of SRT and favor the slow-growing organisms, which would provide Chloroflexi bacteria with ideal conditions for proliferation. Thirdly, the total relative abundance of Proteobacteria and Bacteroidetes reached 63.3% in August, which increased to 84.8% in February. This influence, however, was less apparent in biofilm. In short, these results seemed to suggest that temperature may greatly affect the community structure. The characteristics of community structure in the sludge and biofilm differed, and the biofilm had an advantage over sludge in community structure stability when facing shock caused by low temperatures.

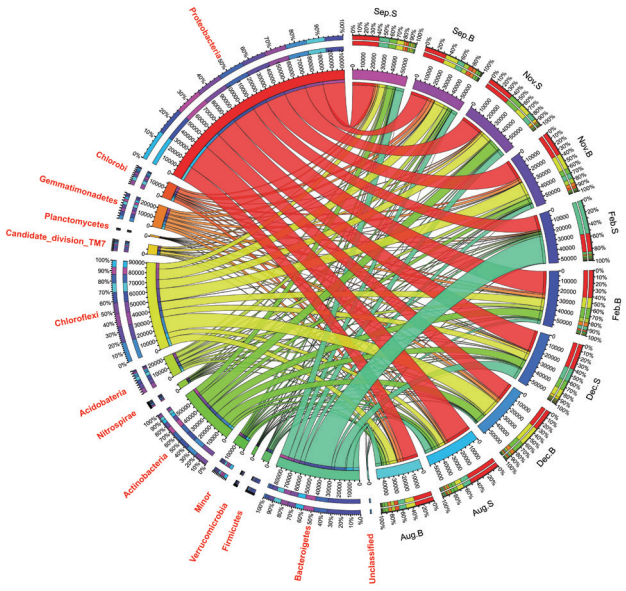

Fig. 3. Distribution of bacterial community at phylum level in sludge and biofilm. Minor phylum refers to maximum abundance less than 0.1% and circos graph showing the evolution of bacterial community was produced by the Circos version 0.7.
Abbreviations: S – Sludge; B – Biofilm; Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

3.3. Community and relative abundance of nitrifying microorganisms in the IFAS system

As is well known, nitrification is a significant step in nitrogen removal in wastewater treatment. During this process, ammonia is first oxidized aerobically to nitrite by ammonia oxidizing bacteria (AOB), and then the nitrite is subsequently converted by nitrite oxidizing bacteria (NOB) to nitrate [9]. Given the fastidious growth properties of nitrifying bacteria, nitrification failure frequently occurs due to low temperature in WWTPs [22]. The IFAS process has been shown to successfully enhance nitrification in a laggard CAS process with insufficient nitrification ability [1,3], even though the temporal dynamic of nitrifying bacteria populations and structure is still not completely clear. The sequencing results presented here suggested that the predominant AOB and NOB species were identical in different months. The AOB and NOB genera observed in this study were consistent with the previous publication [23]. The most dominant AOB species was Nitrosomonas, which was equally present in both the sludge and biofilm. Compared with other AOB such as Nitrosospira and Nitrosococcus, Nitrosomonas with a high growth rate is more adaptable to a changing environment and is often detected as the dominant AOB in WWTPs [24,25]. The most dominant NOB species was Nitrospira in sludge and biofilm in the studied system. Apart from the Nitrosomonas in the

AOB population, Nitrosococcus was also detected in the system. Nitrosospira and Nitrosococcus are the two typical AOB along with Nitrosomonas are present in activated sludge, and their occurrences have been reported in many studies [8]. Besides, no sequences were obtained for Nitrobacter in the present study, which is another typical NOB in wastewater treatment [26], suggesting that Nitrospira was the major NOB in the studied system.

To better understand the nitrification characteristics of the IFAS system, the proportion of AOB and NOB in the sludge and biofilm were calculated, as shown in Fig. 4. Although, the system showed stable pollutant removal efficiency (Table 1), the proportion of AOB and NOB varied in the experimental months and displayed different results between the sludge and biofilm. The fluctuation in the proportion of AOB in biofilm was more gradual than that in sludge where the proportion of AOB drastically decreased from August to February with the fall in temperature (Fig. 4). In particular, the proportion of AOB in biofilm increased and surpassed that in sludge in February, whereas the result was just the opposite in the months before February. Similar to that of AOB, the proportion of NOB in biofilm in February was greater than that in sludge and was clearly predominant in the system. Trapani et al. [27] found that nitrification rate by biofilm doubled nitrification rate by activated sludge with operating temperature near 11.5°C in a pilot-scale IFAS reactor. The reason for this phenomenon is biofilm that had more abundant nitrifying bacteria than sludge in low temperature. Two distinctive living conditions enabled the shift in nitrifying bacteria between sludge and biofilm under different temperatures. The results clearly demonstrate that the nitrification characteristics of the IFAS system between sludge and biofilm were different over the whole year. The biofilm could better retain nitrifying bacteria compared with suspended sludge in cold months. Some studies of MBBR system demonstrated that nitrifying biofilm could quickly adapt low temperature and recover ammonia removal rate [28,29]. In a word, AOB and NOB were dominant in biofilm phase than sludge phase in the low-temperature period during which nitrifying bacteria can be severely affected by SRT and temperature, whereas this situation was the opposite in the warm-temperature period. This finding might explain the discrepancy between previous studies that Li et al. [30] observed a higher relative abundance of AOB and NOB in suspended sludge than in biofilm, but other researchers observed the opposite [1,7]. The shift of nitrifying organisms between sludge and biofilm exhibited a seasonal pattern and could be the paramount factor for the enhanced nitrification provided by the IFAS process in winter. We might surmise that there were two different growth stages in IFAS system: high-temperature period (August, September and November) and low-temperature period (December and February). Nitrification mainly took place in suspended sludge, which had a higher contribution to NH_4^+–N removal than biofilm at high temperature. And the abundance of nitrifying bacteria in suspended sludge was also higher than that in biofilm at the same time. However, nitrification is more dependent on biofilm than suspended sludge in the IFAS system at low temperature. In December and February, the ammonia oxidation rate of suspended sludge decreased, and the contribution of biofilm to ammonia removal increased. The abundance of nitrifying bacteria in biofilm was also higher than that in suspended sludge at the same time. The more robust ammonia removal rate at low temperatures by biofilm contributed to the relative stable removal of ammonia in the IFAS system. However, more studies of nitrification kinetics and contribution of sludge and biofilm to ammonia removal will be required to validate this hypothesis.

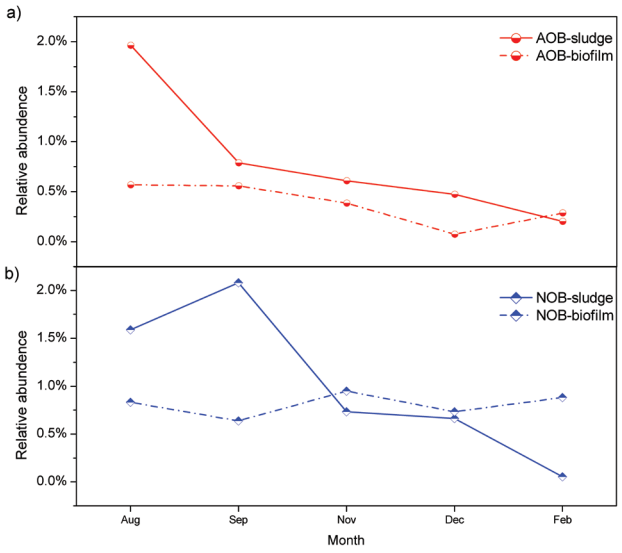

Fig. 4. Shift of nitrifying bacteria abundance in sludge and biofilm: (a) AOB and (b) NOB.
Abbreviations: Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

3.4. Comparison of microbial community dynamics in the IFAS system

To further expressly explore the temporal dynamic of the microbial community in the IFAS system, principal coordinate analysis (Fig. 5) was conducted and a clustered heat map (Fig. 6) was generated to investigate the similarities and dissimilarities of microbial community structure in the IFAS system. As demonstrated by the principal coordinate analysis (PCoA) and heat map, the microbial community in the system was divided into two distinctively different clusters, and the microbial community in biofilm was well separated from that in sludge. The result clearly showed that the microbial communities of biofilm were distinct from sludge in the IFAS system. The bacteria in biofilm formed one relatively tight group, whereas assemblages of bacteria in sludge seemed to be looser to some extent. Furthermore, seasonal change caused variation in the microbial community structure corresponding to the group divisions in the IFAS WWTP system. Interestingly, the PCoA plot showed that the samples obtained in December and February were separated from that in other months with high temperature, which were divided in two groups, suggesting the significant impact of temperature on assembling bacterial communities in the IFAS system. The result also suggested that organisms living in biofilm seemed to possess a more stable microbial community structure compared with that in sludge. This agreed with the hypothesis of two different growth stages in

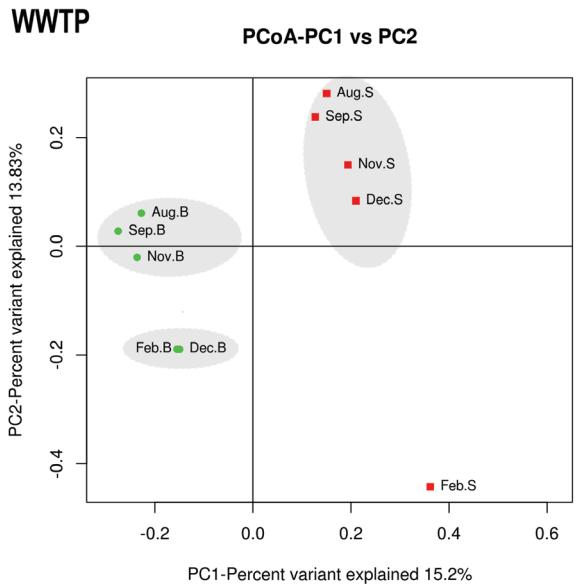

Fig. 5. Principal coordinates analysis (PCoA) based on the OUT profiles retrieved from sludge (red) and biofilm (green).
Abbreviations: S – Sludge; B – Biofilm; Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

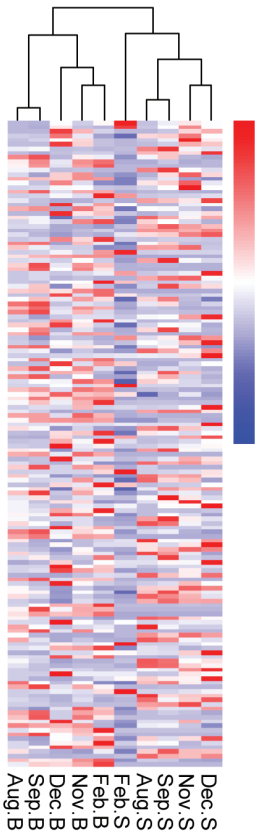

Fig. 6. Heat map analysis of sludge and biofilm in the IFAS system. From blue to red means the increment of abundance.
Abbreviations: S – Sludge; B – Biofilm; Aug – August; Sep – September; Nov – November; Dec – December; and Feb – February.

the IFAS system and the nitrification characteristics of sludge and biofilm. In a word, biofilm showed a distinctly different microbial community trait compared with sludge, and living conditions and temperature appeared to be the key factors in shaping the microbial community in the IFAS system.

4. Conclusion

Microbial community structure and bacterial diversity were varied in different months in the studied IFAS system. Biofilm showed a more stable microbial community trait compared with that in the sludge with the fall in temperature. Proteobacteria was the most predominant phyla in the IFAS system, followed by Bacteroidetes, Actinobacteria, Chloroflexi, Firmicutes, Planctomycetes and candidate division TM7. The most predominant AOB and NOB in the IFAS system were Nitrosomonas and Nitrospira, respectively. The shift in nitrifying organisms between the sludge and biofilm exhibited a seasonal pattern and could be the paramount factor for enhanced nitrification of the IFAS process in winter. The influence of temperature on microorganisms in diverse living conditions seemed to be different, and the microorganisms in suspended sludge were more susceptible to the temperature fluctuation than that in biofilm, which may be easier to retain microorganisms. The presence of biofilm would mitigate the effect of temperature fluctuation on IFAS microbial communities. The enhanced understanding of IFAS microbial communities provided by this study will be helpful in developing efficient strategies to guide the design and operation of converted CAS WWTPs.

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