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Evaluation of microbial diversity of activated sludge in a municipal wastewater treatment plant of northern China by high-throughput sequencing technology

Zhi-Bin Wang[a,b,1], Ming-Sheng Miao[b,1], Qiang Kong[a,*], Shou-Qing Ni[c]

[a]*College of Geography and Environment, Shandong Normal University, 88 Wenhua Donglu, Jinan 250014, Shandong, P.R. China, Tel. +86 531 86180745; Fax: +86 531 86180107; email: kongqiang0531@hotmail.com (Q. Kong)*
[b]*College of Life Science, Shandong Normal University, 88 Wenhua Donglu, Jinan 250014, Shandong, P.R. China*
[c]*School of Environmental Science and Engineering, Shandong University, Jinan 250000, Shandong, P.R. China*

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ABSTRACT

Samples collected in summer and winter were analyzed by high-throughput sequencing technology to reveal differences in the microbial community diversity of activated sludge at different temperatures in a municipal wastewater treatment plant of Northern China. A total of 36,438 validated reads were obtained from the samples, and the bacteria belonged to 25 phyla and 139 genera. The Shannon diversity index decreased from 5.367 in summer to 5.168 in winter. During summer, the dominant phylum was *Proteobacteria* (50.5%), while it was *Bacteroidetes* during winter (40.5%). At the genus level, *Chitinophaga* was most abundant during summer (8.7%), while *Haliscomenobacter* (2.8%) had the greatest abundance in winter. Almost all nitrogen and phosphorus removal-related functional microbes, including *Thiobacillus* (denitrifying bacteria), *Rhodocyclus* (phosphorus-accumulating bacteria), and *Nitrosomonadaceae* (ammonia-oxidizing bacteria), showed significantly higher abundance in summer than winter. Conversely, cryophilic microbial abundance was significantly higher in winter than summer.

Keywords: Microbial diversity; High-throughput sequencing; Activated sludge; Wastewater treatment plant

1. Introduction

Domestic wastewater treatment plants (WWTPs) play important roles in the treatment of urban sewage and wastewater recycling [1]. However, sewage treatment efficiency fluctuates seasonally. For example, the chemical oxygen demand (COD) removal rate was found to decrease as temperature declined from 30 to 20°C, as did nitrogen removal [2]. Conversely, the ammonia removal efficiency increased with temperature and was consistent with changes in nitrification activity [3].

Microbial structure is the fundamental factor affecting WWPT treatment effects [4,5]. Environmental factors such as temperature, light, and pH influence microbial growth; therefore, changes in these factors

*Corresponding author.

[1]The first two authors equally contributed.

lead to variations in microbial species and quantities [6,7]. However, no studies have systematically investigated the seasonal variations in microbial community composition and diversity in WWPTs to date.

Previous investigations of microbial community diversity were primarily based on 16S rRNA gene community fingerprinting, such as denaturing gradient gel electrophoresis, which only detects certain dominant microbial groups [8,9]. The advent of next-generation sequencing has led to systematic and comprehensive studies of microbial community [10]. In this study, differences in microbial community composition and diversity at various temperatures were investigated in a WWPT in Northern China by high-throughput sequencing of the 16S rRNA genes in the V4 hyper-variable region. The specific goal of this study was to provide information about the microbial population in different seasons.

2. Materials and methods

2.1. Study site and sample collection

In this study, the Jinan WWTP, which is an anaerobic–anoxic–oxic (A/A/O) treatment system located in northern China (N: 36˚40′, E: 117˚00′), was investigated. The average high and low air temperatures in the region during summer are 32 and 23˚C, respectively, while they are 5 and −4˚C, respectively, during winter. Activated sludge samples were collected from the same location (end of the aerobic basin) during summer and winter in 2014, when the respective working temperatures of the biochemical tank were 25 and 11˚C. The WWTP has a treatment capacity of 3×10^5 m^3 d^{-1} and serves a population of about 1,500,000. The wastewater treated in this plant is mostly domestic sewage with an average influent COD of 200 mg L^{-1} and an average suspended solids concentration (SS) of 150 mg L^{-1}. The wastewater composition and organic loading rate of the WWTP changed slowly with respect to seasonal changes [11].

2.2. DNA extraction, 16SrRNA amplification, and sequencing

Total DNA was extracted from 1.5 mL of sludge using a power soil DNA isolation kit (MO BIO Laboratories, Inc., Loker Ave West, Carlsbad, CA) according to the manufacturer's recommendations. The V4 region of the microbial 16S rRNA gene was amplified using the universal primer pair (forward primer: 5-AYTGGGYDTAAAGNG-3 and reverse primer: 5-TACNVGGGTATCTAATCC-3). The thermal profile started with a denaturation step of 5 min at 98˚C, followed by 27 cycles of denaturation (30 s at 98˚C), primer annealing (30 s), and extension (30 s at 72˚C), after which there was a final extension step of 72˚C for 5 min to ensure complete amplification. The amplified product was excised from 2% agarose gels and then purified using a SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, SK8131). V4 amplicons were sequenced using the pair-ended method by the Illumina Miseq sequencing platform at Personal Biotechnology Co., Ltd. (Shanghai, China).

2.3. Data processing and analysis

Alpha diversity was determined based on the Chao1, ACE, Simpson, and Shannon indices using the summary single command of the MOTHUR software [12]. All data were log transformed and then normalized as follows. The arithmetic mean of all transformed values was subtracted from each log-transformed measure, after which the difference was divided by the standard deviation of all log-transformed values for the given sample. After this procedure, the abundance profiles of all samples will exhibit a mean of 0 and a standard deviation of 1. The SPSS15.0 (IBM) software package was used for the general statistical analysis, as well as to conduct a chi-square test. The chi-square values were converted into *p*-values by excel (Microsoft Office) to indicate significance.

3. Results and discussion

3.1. Sequencing and classification

The V4 region of the 16S rRNA gene was PCR amplified from DNA samples extracted from activated sludge. PCR products of the V4 region were sequenced using the pair-ended method by Illumina Miseq. After quality control, a total of 36,438 validated sequence reads were obtained. These sequences were classified into different OTUs based on the identity level at 97%. Among the 36,438 sequences reads, a total of 1,421 OTUs were obtained, of which 1,318 and 373 were classified into the phylum and genus level, respectively. The OTU number reached a saturation value that indicated that samples selected in this study could reveal the WWTP microbial distribution ideally. Of the 1,421 OTUs, 1,056 were from summer samples, 961 from winter, and 596 were from both (Fig. 1).

3.2. Analysis of alpha diversity

Alpha diversity was estimated by the Chao, ACE, Simpson, and Shannon indices. As shown in Table 1, the Chao and ACE indices were higher in summer

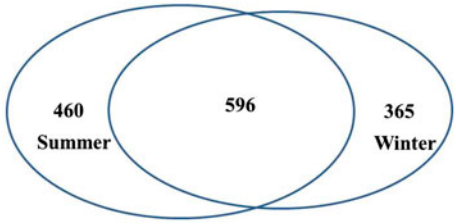

Fig. 1. Venn diagram of OTUs in summer and winter.

than in winter. However, this difference was not significant. Conversely, the Simpson's index was significantly ($p < 0.05$) lower in summer (0.012) than in winter (0.016), as was the Shannon's index (5.367 vs. 5.168 for summer and winter, respectively). Overall, these results showed that the diversity of the WWPT bacterial community was higher during summer than winter.

3.3. Analysis of community composition

Twenty-five phyla were detected in the activated sludge, five of which were found to comprise greater than 2% of the total populations during summer and winter. Therefore, the distribution of these organisms was compared for the summer and winter WWTP. The results revealed that *Proteobacteria* comprised 50.5 and 32.2% of the summer and winter population, respectively, while *Bacteroidetes* comprised 16.8 and 40.5%, *OD 1* 7.2 and 3.3%, *Verrucomicrobia* 6.9 and 4.1%, and *Chlorobi* 5.3 and 2.8% (Fig. 2(a)). *Proteobacteria* (50.5%) was the dominant phylum during summer which include many of the bacteria responsible for nitrogen fixation, including important ammonia-oxidizing bacteria such as *Betaproteobacteria* and *Gammaproteobacteria* [13,14]. *Betaproteobacteria* (32.3%) was the dominant class in summer, while *Bacteroidetes* (40.5%) was the dominant phylum during winter. *Saprospirae* (56%) was the dominant class of *Bacteroidetes*, followed by *Sphingobacteria* (21%). The proportions of the cryophilic microbes such as *Flavobacteria*, *Sphingobacteria* et al. in this study were increased in winter, while those cryophilic microbes were not reported having relationship to nitrogen removal [15]. All five

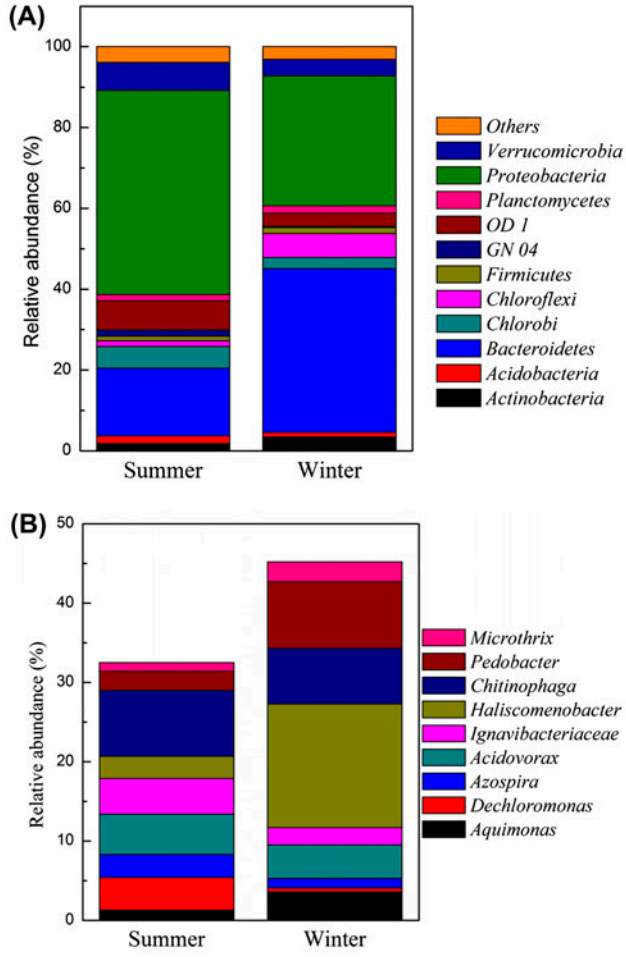

Fig. 2. Distribution of microbes in the WWTP during summer and winter.

phyla differed significantly ($p < 0.01$) between summer and winter, with *Proteobacteria*, *Verrucomicrobia*, *OD 1*, and *Chlorobi* being significantly higher in summer than winter and *Bacteroidetes* being higher during winter.

Additionally, 139 genera were detected, nine of which comprised greater than 2% of the total population. Of these nine genera, the proportions of *Aquimonas* (1.3% in summer vs. 3.6% in winter), *Haliscomenobacter* (2.8% in summer vs. 15.6% in winter), *Pedobacter* (2.4% in summer vs. 8.4% in winter),

Table 1
Analysis of microbial diversity

Group	Reads	Chao	ACE	Simpson	Shannon
Summer	18,814	1,218 ± 52	1,278 ± 55	0.012 ± 0.0004	5.367 ± 0.0250
Winter	17,624	1,152 ± 61	1,204 ± 55	0.016 ± 0.0006	5.168 ± 0.0280

Notes: Chao and ACE indicate richness, Shannon and Simpson indicate diversity.

Table 2
Functional microbes in the WWTP

Name of bacteria		Level	Summer read number	Winter read number	*p*-value
DNB	*Pseudomonas*	Genus	23	19	0.8620
	Paracoccus	Genus	5	4	0.8235
	Thiobacillus	Genus	359	6	[b][a]
	Bacillus	Genus	1	1	0.5431
PAO	*Pseudomonas*	Genus	23	19	0.8620
	Aeromonas	Genus	21	3	0.0008[a]
	Rhodocyclus	Genus	520	184	[b][a]
AOB	*Nitrosomonadaceae*	Family	457	151	[b][a]
NOB	*Nitrospirales*	Order	2	9	0.0187[a]
DNRA	*G. lovleyi*	Species	14	3	0.0172[a]
Anammox	*Planctomyces*	Genus	42	64	0.0132[a]

[a]Statistically significant at $p < 0.05$ and $p < 0.01$, respectively.
[b]Represent *p*-values less than 0.00005.

and *Candidates microthrix* (1.1% in summer vs. 2.5% in winter) were lower in summer than in winter. Conversely, *Chitinophaga* (8.7 vs. 7%), *Ignnavibacteriaceae* (4.5 vs. 2.2%), *Dechloromonas* (4.1 vs. 0.5%), *Azospira* (2.9 vs. 1.2%), and *Acidovorax* (4.5 vs. 2.2%) showed higher abundance in summer than winter (Fig. 2(b)). Denitrifying bacteria (DNB) are another important type of microbiota in WWTP systems. Throbäck et al. found about 150 species of DNB belonging to 50 genera in typical WWTPs [16,17]. In the present study, we found that the dominant nitrifying bacteria in summer and winter were *Rhodospirillaceae* (35.7% in summer, 21.7% in winter) and *Pseudomonadaceae* (54.7% in summer and 73.9% in winter), respectively. *Pseudomonadaceae*, which belongs to *Betaproteobacteria*, are single-celled microbes with a cell size of 0.5–1.0 μm × 1.5–4 μm. *Pseudomonadaceae* are strict aerobes that use oxygen as the terminal electron acceptor under general conditions, although nitrate can be used as the terminal electron acceptor under conditions found in WWTPs. Additionally, the abundance of the 9 aforementioned genera differed significantly ($p < 0.01$) between summer and winter, with *Dechloromons*, *Azospira*, *Acidovorax*, *Ignavibacteriacea*, and *Chitinophaga* showing greater abundance in summer and *Aquimonas*, *Haliscomenobacter*, *Pedobacter*, and *Microthrix* having significantly higher abundance in winter.

3.4. Functional microbes in the WWTP

A total of 17,685 and 15,351 reads were obtained for summer and winter, respectively, and five important functional microbes were extracted DNB, phosphorus-accumulating bacteria (PAO), ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and anammox bacteria (Table 2).

DNB (*Pseudomonas*, *Paracoccus*, *Thiobacillus*, and *Bacillus* (*archaea*)) were detected in summer and winter [18]. The ratio of denitrifiers in summer was much higher than in winter, especially for *Thiobacillus*, which was the dominant denitrifier during summer. *Thiobacillus* is a class of chemoautotrophic microorganisms that uses CO_2 as a carbon source whose energy was obtained by oxidizing sulfide to elemental sulfur or sulfate, and nitrate could be an electron acceptor under anoxic or anaerobic conditions [19].

PAOs bacteria included *Pseudomonas*, *Arthobacter*, *Nocardia*, *Beyerinkia*, *Ozotobacter*, *Aromonas*, *Microlunatus*, and *Rhodocyclus* [18]. Only three genera were detected in summer and winter, which was similar to the distribution of denitrifiers. The ratio of PAOs was much higher in summer than in winter, with *Rhodocyclus* being the dominant genus.

The high-throughput sequencing test for AOB was limited at the family level. All of the AOB were assigned to *Nitrosomonadaceae* and divided into *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* [18]. The level of AOB in summer was significantly higher than in winter, with a *p*-value less than 0.01.

Few NOB were observed during summer and winter, even though the samples were collected from an aerobic basin. The relatively low concentration of NOB likely results in a low concentration of nitrate and therefore enhanced denitrifying dephosphatation [3,20].

Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are compting microbial nitrate-reduction process [21]. No successful DNRA enrichment culture has been reported. The enriched culture mainly consisted of *deltaproteobacteria*, closely related to *G. lovleyi*. *G. lovleyi* was detected both in summer

and winter, and the contribution status is shown in Table 2.

Anammox bacteria was also detected at the genus level during summer (42 reads) and winter (64 reads) indicating that the WWTP sludge can be used as a seed to enrich experimental systems with anammox bacteria [22].

4. Conclusion

The biodiversity of activated sludge in summer was higher than in winter, with Shannon index values of 5.4 and 5.2, respectively. The dominant phyla in summer and winter were *Proteobacteria* and *Bacteroidetes*, respectively, while *Chitinophaga* and *Haliscomenobacter* were the dominant genera. Cryophilic microbes showed significantly higher abundance in winter than in summer. Almost all nitrogen and phosphorus removal-related functional microbes were detected at significantly higher levels in summer than winter.

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