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Insights into the bacterial species and communities of a full-scale anaerobic/anoxic/oxic wastewater treatment plant by using third-generation sequencing

Bin Ji,[1,*] Xuechun Zhang,[1] Shufei Zhang,[2] Hongjiao Song,[1] and Zehua Kong[3]

Department of Water and Wastewater Engineering, Wuhan University of Science and Technology, Wuhan 430065, China,[1] Guangdong Provincial Key Laboratory of Fishery Ecology and Environment, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, China,[2] and Department of Civil and Structural Engineering, The University of Sheffield, Sheffield S1 3JD, UK[3]

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For the first time, full-length 16S rRNA sequencing method was applied to disclose the bacterial species and communities of a full-scale wastewater treatment plant using an anaerobic/anoxic/oxic (A/A/O) process in Wuhan, China. The compositions of the bacteria at phylum and class levels in the activated sludge were similar to which revealed by Illumina Miseq sequencing. At genus and species levels, third-generation sequencing showed great merits and accuracy. Typical functional taxa classified to ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DB), anaerobic ammonium oxidation bacteria (ANAMMOXB) and polyphosphate-accumulating organisms (PAOs) were presented, which were *Nitrosomonas* (1.11%), *Nitrospira* (3.56%), *Pseudomonas* (3.88%), Planctomycetes (13.80%), Comamonadaceae (1.83%), respectively. *Pseudomonas* (3.88%) and *Nitrospira* (3.56%) were the most predominating two genera, mainly containing *Pseudomonas extremaustralis* (1.69%), *Nitrospira defluvii* (3.13%), respectively. Bacteria regarding to nitrogen and phosphorus removal at species level were put forward. The predicted functions proved that the A/A/O process was efficient regarding nitrogen and organics removal.

[**Key words:** PacBio's third-generation sequencing; Bacterial species; Anaerobic/anoxic/oxic process; Full-length 16S rRNA sequence; *Pseudomonas extremaustralis*; *Nitrospira defluvii*]

Activated sludge process has been wildly applied as a biological wastewater treatment technology more than one hundred years. Activated sludge is a complicated system consisted of bacteria, archaea, protozoan and viruses, in which bacteria plays the leading role (1). Among the methods of wastewater treatment, anaerobic/anoxic/oxic (A/A/O) process is one of the traditional activated sludge processes in domestic wastewater treatment, which combines biological nitrification and denitrification with biological phosphorus removal (2). The A/A/O process can be used for secondary wastewater treatment, tertiary wastewater treatment and reclaimed water reuse and it is still one of the main processes among the various Chinese wastewater treatment plants (WWTP). In the A/A/O process, nitrobacteria and denitrifying bacteria are mainly used to achieve nitrogen removal through two independent processes of aerobic nitrification and anoxic denitrification. Phosphorus is achieved through two steps of anaerobic phosphorus release and aerobic phosphorus uptake by polyphosphate-accumulating organisms. Under anaerobic conditions, anaerobic ammonia oxidation mainly utilizes anaerobic ammonia-oxidizing bacteria to convert nitrite into nitrogen to achieve denitrification. However, up to now, the functional bacteria at species level of activated sludge of A/A/O process were seldom reported deeply.

Recently, gene-sequencing technology has been consecutively innovated and the third-generation sequencing appears. The chain-termination sequencing method developed by Sanger and Coulson (3) and chemical sequencing method by Maxam and Gilbert (4) was called first-generation sequencing. The shortcomings of first-generation sequencing technology were high cost and slow speed, so it restricted its large-scale commercialization. The era of first generation sequencing quickly came to an end with the population of second-generation sequencing, including Illumina, 454 pyrosequencing and sequencing by oligonucleotide ligation and detection (5). Second-generation sequencing was a revolutionary change compared with first-generation sequencing, with superior technical performance and cost advantages. However, with the development and application of second-generation sequencing technology, some disadvantages are emerging. For example, the second generation sequencing reads a short length and brings difficulties in bioinformatics analysis such as sequence splicing, assembly, and annotation (6). Therefore, novel technologies like third generation sequencing are in development today. It has faster data reading speed, and PCR amplification was unneeded, which might offset these disadvantages of the second-generation sequencing in application (7).

One of the representatives of third generation sequencing was single molecular real-time technology, which improved considerably high accuracy by generating long reads spanning most repeat sequences (8). This technology had been widely used in medical science, pathologic studies and genetics. It was used in genomics research to assemble large genomes (9). It was also used in medical research to sequence of chromosomal base pairs of human body (10) and the character of hepatitis B virus quasi species (11).

* Corresponding author. Tel.: +86 27 68893426; fax: +86 27 68862793.
E-mail address: binji@wust.edu.cn (B. Ji).

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However, up to now, this method was rarely used in wastewater treatment systems to analyze microbial community of the activated sludge.

Therefore, for the first time, the full-length 16S rRNA sequencing was applied to research the functional bacteria of a full-sale A/A/O process WWTP at the species level in this study, which was in order to systematically investigate the bacterial community compositions and unravel functional characteristics in the activated sludge of A/A/O process. It was helpful to have a deep insight into the bacterial species and communities of a full-scale wastewater treatment plant, to understand the mechanism of pollutants removal in a comprehensive way.

MATERIALS AND METHODS

Sample collection The activated sludge sample (B1) was collected from three sites of the secondary clarifier, mixed uniformly, and then stored at −80°C from a secondary clarifier of a typical WWTP (30°35′19.9″N, 114°21′21.5″E) in Wuhan, China. The WWTP treats municipal wastewater by using modified A/A/O process with an anaerobic biological selection tank ahead. The quality of influent and effluent in June, 2017 was as follow: influent: COD, 78.91–136.37 mg L^{-1}; NH_4^+-N, 9.46–16.55 mg L^{-1}; TN, 13.45–19.12 mg L^{-1}; TP, 1.79–4.16 mg L^{-1}; effluent: COD, 15.35–19.12 mg L^{-1}; NH_4^+-N, 0.35–1.97 mg L^{-1}; TN, 7.46–9.87 mg L^{-1}; TP, 0.46–0.96 mg L^{-1}. NH_4^+-N was determined by Nessler's reagent spectrophotometry, TN by potassium persulfate oxidation ultraviolet spectrophotometry, TP by potassium persulfate digestion, molybdenum antimony spectrophotometry, and COD by potassium dichromate titration. The effluent quality accorded with the class I–B discharge standard of pollutants for municipal wastewater treatment plant (GB 18918-2002) in China.

DNA extraction and full length sequencing of 16S rRNA gene amplicon E.Z.N.A. soil DNA Isolation kit (Omega Biotek Inc., Norcross, GA, USA) was used for bacterial genomic DNA extraction following the manufacturer's instruction. The 16S rRNA genes (1500 bp) was amplified with a two-step PCR (25 μl) by using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′): initial denaturation for 120 s at 98°C, 25/10 cycles of 15 s denaturation at 98°C, 30 s annealing at 55°C and 30 s extension at 72°C, and final extension at 72°C for 5 min. After purification and quantification, PCR amplicons were pooled in equal amounts. PacBio Sequel platform (Pacific Biosciences, Menlo Park, CA, USA) was applied for single-molecule circular consensus sequencing of the nearly full-length 16S rRNA gene amplicon After seqencing, the raw sequences were rectified in the official workflow of PacBio through the PacBio SMRT Link portal (version 5.0.1.9585). To ensure the reliability and accuracy of the analysis results, software QIIME (Quantitative Insights Into Microbial Ecology, ver. 1.8.0) (12) was used to identify the interrogative sequence, and to check and remove chimera sequence. The accession number of the original gene sequences was SRR6318588 (Sequence Read Archive, http://www.ncbi.nlm.nih.gov/sra/).

Sequence Analysis and bioinformatics Sliding window approach was used to filter the low quality sequences. QIIME (ver. 1.8.0) (12) was applied to identify the interrogative sequence, invoking USEARCH (ver. 5.2.236) of QIIME to check and eliminate the chimera and interrogative sequence. After chimera detection, UCLUST of QIIME was used as a sequence alignment tool to normalize the sequence counts of each taxon and assign to operational taxonomic units (OTUs) at the levels of 97% similarities. Taxonomic binning of 16S rRNA reads was based on the National Center for Biotechnology Information gene databases by BLAST to acquire an OTU table. Rarefaction curve and the alpha diversity indices (Chao1, ACE, Shannon and Simpson) were determined based on the the OTU table in QIIME.. The high-quality and compact taxonomic and phylogenetic visualization of bacterial communities was produced by using GraPhlAn (Graphical Phylogenetic Analysis) (13). Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (14) was used to predict the functional content of the bacterial operational taxonomic units. Bacterial functional profiles were compared at Kyoto Encyclopedia of Genes and Genomes (KEGG) modules level 2.

RESULTS AND DISCUSSION

Bacteria community composition and diversity Different sequencing technologies for microbial community showed clear platform-specific benefits. The average sequence length of the third generation sequencing was 1500 bp, and 6797 effective sequence reads were yielded out by PacBio sequencing. After excluded the low-quality reads, sequences were used for microbial community analyses, which was compared to our previous Illumina MiSeq sequencing (Illumina, San Diego, CA, USA) as depicted in Table 1 (15). Although the number of sequence generated by second-generation sequencing was seven times as it generated by third generation sequencing, the third-generation sequencing generated more OTUs. This was owing to the deeper sequencing depth and longer read lengths span, which could better represent the sample thus enabling new insights into sequence diversity as indicated by Chao1, ACE, Shannon, and Simpson values in Table 1. The high Coverage estimators (> 0.996) could convey that the collected gene sequences could well represent the microbial community. As shown in Fig. 1A, the long rarefaction curve indicated that the sequencing depth was good. And the smooth rarefaction curve indicated that the recovered sequences well represented the diversity of the bacterial communities of the activated sludge sample B1.

TABLE 1. Community richness and diversity of sample B1.

Technology	Sequences	OTUs	Chao1[a]	ACE[a]	Shannon[b]	Simpson[b]
Illumina MiSeq	49453	1582	2191.17	2226.00	8.79	0.99
Pacific Biosciences	6797	2585	2585.00	2586.26	10.96	1.00

[a] Community richness.
[b] Community diversity.

Overall taxonomic bacterial structures at phylum and class levels The relative abundances of the taxon assignments for the bacterial communities of the activated sludge of the WWTP are shown at phylum (Fig. 1B) and class levels (Fig. 1C). The bacterial community was dominated by phylum Proteobacteria (38.32%), followed by Planctomycetes (13.80%), Chloroflexi (13.38%), Bacteroidetes (10.03%), Firmicutes (7.81%), Actinobacteria (3.79%), Nitrospirae (3.64%), and Acidobacteria (2.18%). At class level, the bacterial community of the activated sludge consisted of Planctomycetia (13.02%), Betaproteobacteria (12.86%), Gammaproteobacteria (10.21%), Alphaproteobacteria (8.57%), Anaerolineae (8.36%) Deltaproteobacteria (4.91 %), Clostridia (4.02 %), Nitrospira (3.61 %), Bacilli (3.52 %), Acidimicrobiia (1.68 %), Acidimicrobiia (1.53%), Bacteroidia (1.51%), Epsilonproteobacteria (1.50%) Flavobacteriia (1.42%), Sphingobacteriia (1.42%), Ktedonobacteria (1.21%) and Verrucomicrobiae (1.18%), based on the identified total operational taxonomic units. In general, the compositions of the bacteria at phylum and class levels in the activated sludge were in accordance with the microbial community of influent in the same WWTP by using 454 pyrosequencing (16) and the bacterial community of the activated sludge in the same WWTP by using Miseq sequencing (16).

It could be concluded that the most predominant phyla Proteobacteria was the one of the main reason for organics, nitrogen and phosphorus removal. Betaproteobacteria was the second abundant class, which was believed to be the most important source component of polyphosphate-accumulating organisms (PAOs), such as *Accumulibacter* and Comamonadaceae (17). The third abundant class Gammaproteobacteria was proved to be closely related to denitrification as heterotrophic bacteria. Moreover, it could be deduced that anaerobic ammonium oxidation (ANAMMOX) might happen in the A/A/O process as the ANAMMOX process was conducted by Planctomycetes (18), which was the second abundant phylum in the wastewater treatment system.

A deep insight into the bacterial communities at genus and species levels The top 20 genera and species revealed by PacBio sequencing in the A/A/O WWTP are presented in Table 2. At the genus level, *Pseudomonas* (3.88%) and *Nitrospira* (3.56%) were the most predominating two genera. *Pseudomonas* was always related to denitrification, aerobic denitrification (19) and denitrifying phosphorus removal (20). *Nitrospira* was able to finish ammonia

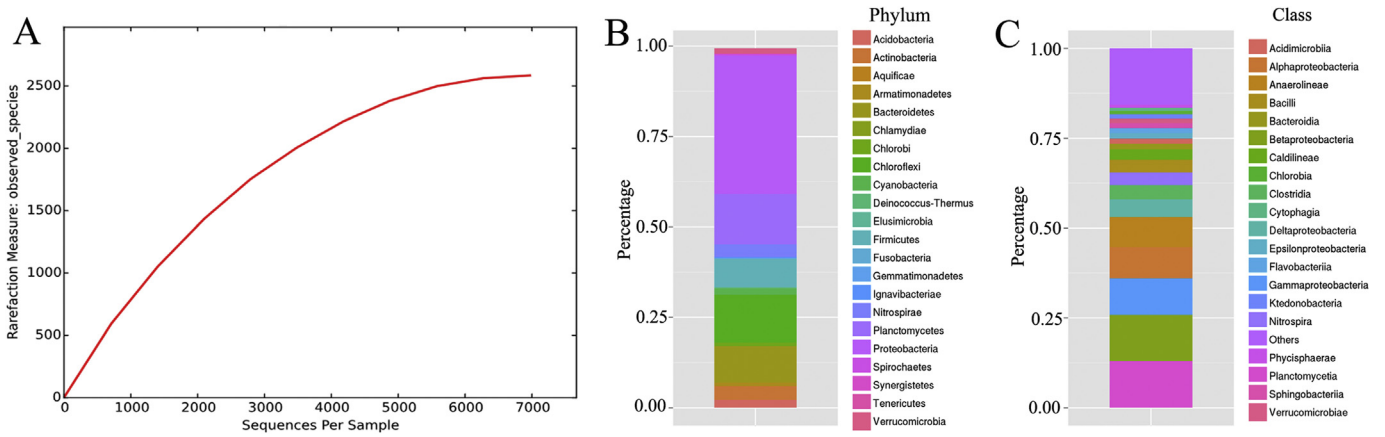

FIG. 1. (A) Rarefaction curve. The smooth curve indicates that the sequencing depth is effective to reflect the bacterial diversity by full-length 16S rRNA sequencing. (B) Bacterial components at phylum level. (C) Bacterial components at class level.

oxidation, nitrite oxidation and complete nitrification (21). So, the dominant position of the two genera was the reflection of the function of nitrogen and phosphorus removal for A/A/O process. It drew our attention that some genera, such as *Zavarzinella* (2.61%) and *Pelolinea* (2.32%), had been seldom reported in wastewater treatment systems, which need further research.

The general components of the activated sludge were quite different from our previous report on the bacterial community of the same sample of the WWTP by secondary Miseq sequencing. Illumina's Miseq sequencing indicated that the relative abundance of *Nitrospira* was only 2.88% and few *Pseudomonas* ($< 0.1\%$) existed in the sludge (15). While the abundance of *Nitrospira* and *Pseudomonas* were 3.88% and 3.56% as revealed by more acute third-generation sequencing. The most predominant genera were *Nitrospira and Planctomyces* from the identified OTUs by Miseq platform (15). We concluded tentatively that the whole length of 16S rRNA sequencing tended to be much more reliable than the Miseq sequencing targeting the V4–V5 hypervariable regions of 16S rRNA.

The unique depth of sequencing of the third-generation PacBio sequencing gave us a possibility to have a glance at the specific bacterial community of the activated sludge. The results in Table 2 indicate the typical bacterial species in the wastewater treatment system. The mixotrophic *Nitrospira defluvii* owned the key enzyme nitrite oxidoreductase, and was significant in nitrogen cycle along with Planctomycetes (such as *Gemmata obscuriglobus*) by comparative genomic analyses (22). *Pseudomonas extremaustralis* could degrade alkane under a microaerophilic atmosphere (23) and produce polyhydroxybutyrate (PHB) (24). The specific mechanisms of COD, nitrogen and phosphorus degradation stayed unclear as most of the varied species lacked deep research, especially when these species formed complex bacterial communities in the wastewater treatment system. However, we could find some functional taxa regarding to nitrogen and phosphorus removal, which were classified to ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DB) and PAOs. *Nitrosomonas* (1.11%) and *Nitrospira* (3.56%) were representatives of AOB and NOB, respectively. The typical bacteria belonging to DB were *Pseudomonas* (3.88%), *Denitratisoma* (0.63%), *Azoarcus* (0.56%), *Rhodobacteraceae* (0.53%), *Novosphingobium* (0.37%), *Bacillus* (0.34%), *Zoogloea* (0.20%), *Rhodocyclaceae* (0.20%), *Thauera* (0.17%), *Azospira* (0.14%), *Acidovorax* (0.14%), *Nitratireductor* (0.06%), *Arcobacter* (0.06%) and *Flavobacterium* (0.03%). PAOs related bacteria were much fewer than the reported A/A/O process (25) according to the typical reported *Accumulibacter* (0.21%) and *Tetrasphaera* (0.09%). But the novel PAOs, Comamonadaceae (1.83%) were much more abundant relatively, which suggests that large amounts of PAOs with richness were in the WWTP as an enhanced biological phosphorus removal technology.

At the species level, the relationship and mechanism between nutrients removal and bacterial community are put forward in

TABLE 2. Top 20 genera and species revealed by PacBio sequencing in the A/A/O WWTP.

Genera	Relative abundances (%)	Species	Relative abundances (%)
Pseudomonas	3.88	*Nitrospira defluvii*	3.13
Nitrospira	3.56	*Zavarzinella formosa*	2.61
Zavarzinella	2.61	*Pelolinea submarina*	2.32
Pelolinea	2.32	*Bellilinea caldifistulae*	2.28
Bellilinea	2.28	*Pirellula staleyi*	1.89
Pirellula	1.89	*Pseudomonas extremaustralis*	1.69
Caldilinea	1.83	*Caldilinea aerophila*	1.58
Gimesia	1.49	*Gimesia maris*	1.49
Enterococcus	1.46	*Portibacter lacus*	1.43
Portibacter	1.43	*Gemmata obscuriglobus*	1.35
Gemmata	1.35	*Thermogutta hypogea*	1.22
Thermosporothrix	1.20	*Thermosporothrix hazakensis*	1.20
Nitrosomonas	1.11	*Litorilinea aerophila*	1.09
Terrimonas	1.09	*Levilinea saccharolytica*	0.92
Litorilinea	1.09	*Longilinea arvoryzae*	0.86
Dechloromonas	1.03	*Fimbriimonas ginsengisoli*	0.82
Levilinea	0.92	*Planctopirus limnophila*	0.77
Longilinea	0.86	*Piscinibacter aquaticus*	0.77
Fimbriimonas	0.82	*Dechloromonas hortensis*	0.77
Lewinella	0.82	*Thermanaerothrix daxensis*	0.75

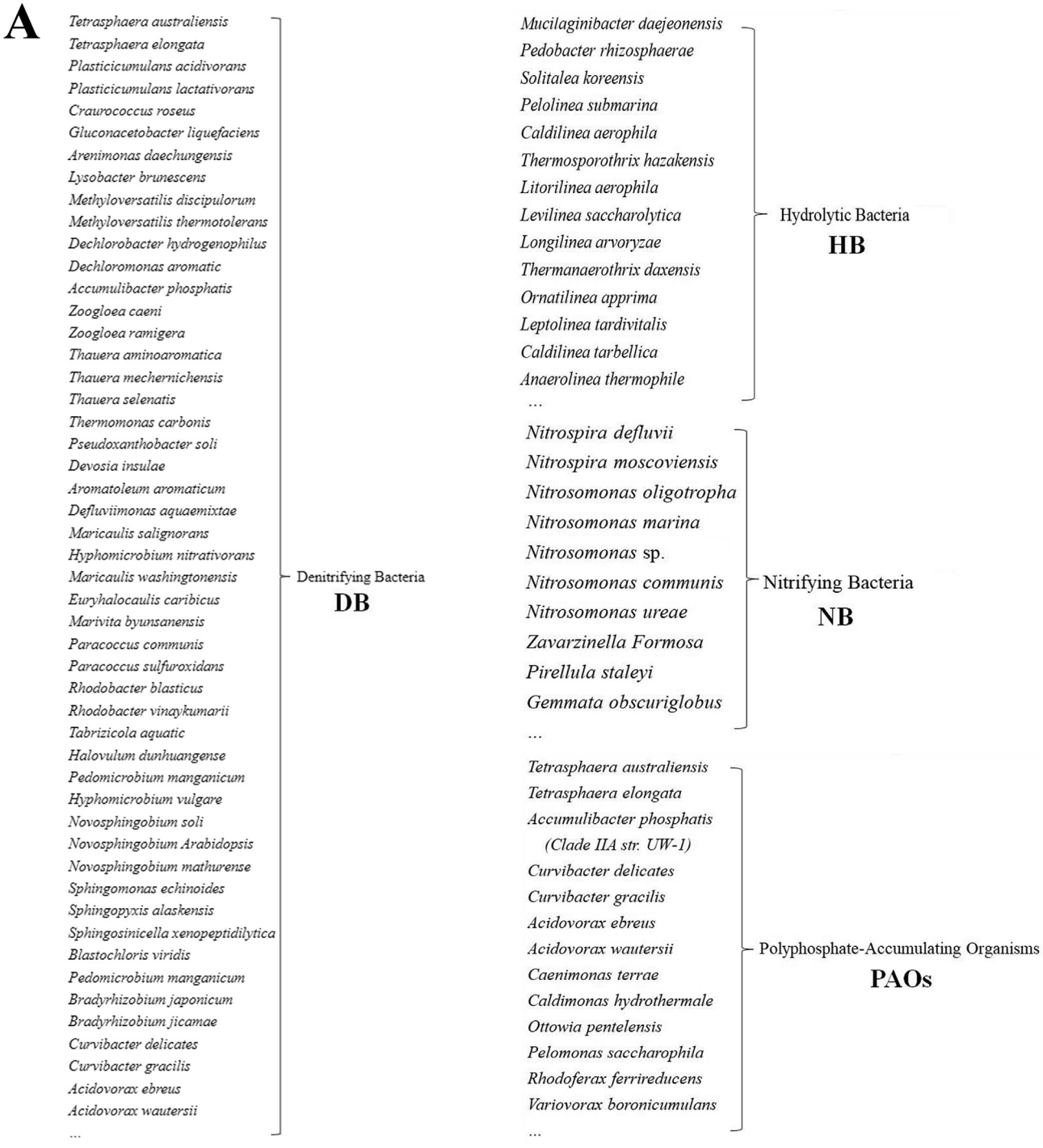

FIG. 2. (A) Specific species of the functional bacteria for potential nitrogen and phosphorus removal. (B) Proposed detailed process of nitrogen and phosphorus degradation by the functional bacteria.

Fig. 2. Results revealed that not only the reported *Tetrasphaera australiensis*, *Tetrasphaera elongate* and *Accumulibacter phosphatis* Clade IIA str. UW-1 exited in the biological wastewater treatment system, but also the bacteria of Comamonadaceae were vital as potential PAOs, including *Curvibacter delicates*, *Curvibacter gracilis*, *Acidovorax ebreus*, *Acidovorax wautersii*, *Caenimonas terrae*, *Caldimonas hydrothermale*, *Ottowia pentelensis*, *Pelomonas saccharophila*, *Rhodoferax ferrireducens* and *Variovorax boronicumulans*. The organic substrate was hydrolyzed to generate some volatile fatted acids and amino acids by hydrolytic bacteria (HB) under an anaerobic atmosphere. Partial of the VAF were used by PAOs to release phosphorus. And the PAOs could absorb phosphorus excessively to achieve phosphorus enrichment from wastewater to the cells of PAOs in the activated sludge (26). For nitrogen removal, the ammonia released by amino acids was transformed to nitrate or nitrite by Nitrifying Bacteria (NB), including AOB and NOB. The most abundant DB could realize the reduction of nitrate and nitrite. Overall, the specific species of the functional bacteria for nutrients removal revealed by third-generation sequencing help us gain an insight into the final scale of the bacterial component of the wastewater treatment system accurately.

Phylogenetic visualization and related metabolic functions Fig. 3 shows the phylogenetic tree at all levels of classification resulting from the 16S rRNA sequencing profile of the activated sludge of the WWTP by using the emerging visualization tool of GraPhlAn, which provides a fast way to find dominant microbial groups from complex community data. The classification units are distinguished by different colors, and their abundance distribution was reflected by the size of the nodes. The dominant bacteria at phylum and class levels revealed by Fig. 3 are in accordance with the above description. Three most dominant families are outstanding from Fig. 3, which are Planctomycetaceae, Anaerolineaceae and Pseudomonadaceae, suggesting that anaerobia played an indispensable role in pollutants removal.

The predicted functions of operational taxonomic units based on KEGG modules level 2 is presented in Fig. 4. The abscissa of Fig. 4 is the second level functional group of KEGG, and the ordinate is the relative abundance of each functional group in the sample. It indicated that much more bacteria involved in substance metabolism than energy metabolism. Amino acid metabolism, carbohydrate metabolism, energy metabolism, cofactors and vitamins metabolism, and lipid metabolism were the top five functions of bacteria in the activated sludge, which was similar to our previous study by using Miseq sequencing (15). It proved that the A/A/O process was efficient with regard to nitrogen and COD removal.

By targeting the full length of 16S rRNA, PacBio's third-generation sequencing presented us more acute recognitions of the bacterial components of the full-scale WWTP, especially at species level, than Illumina's second-generation sequencing. Some functional taxa regarding to nitrogen and phosphorus removal were disclosed, including AOB, NOB, DB, anaerobic ammonium

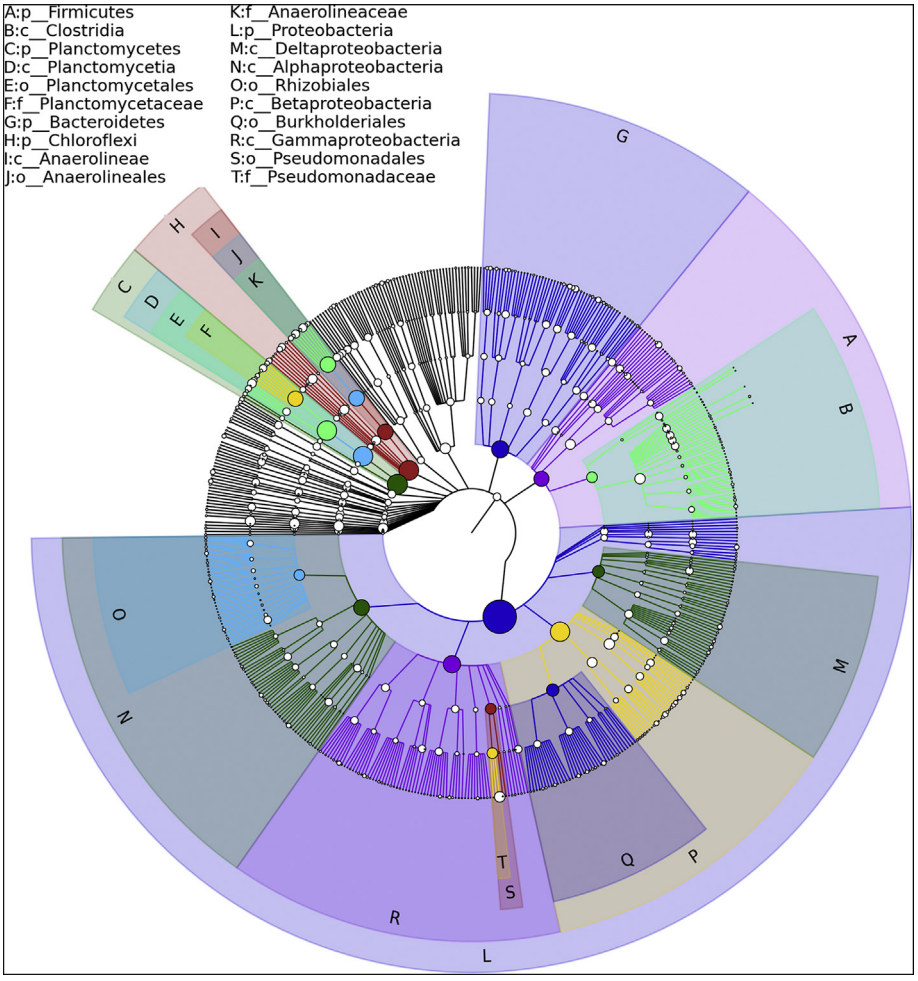

FIG. 3. Phylogenetic tree at all levels of classification (from phylum to species) based on GraPhlAn. The node size represents the mean relative abundance of each classification. Relative abundance of the top 20 classification units are identified by letter A–T in the graph. The color of the letter is the same as the corresponding node. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

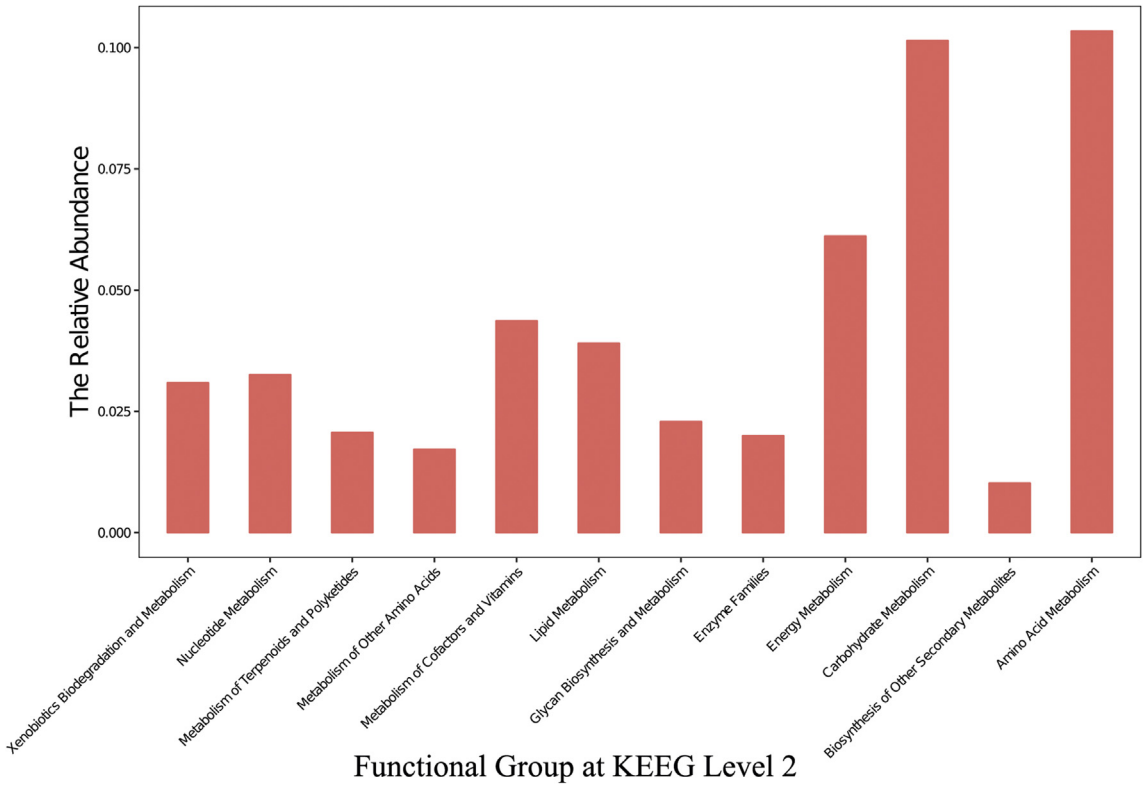

FIG. 4. Predicted functions of OTU based on KEGG modules level 2.

oxidation bacteria and PAOs. *Pseudomonas* and *Nitrospira* were the most predominating two genera. Genera like *Zavarzinella* and *Pelolinea* need further research. The reported A/A/O process was efficient for nitrogen and COD removal. Comamonadaceae was proved to be the main kind of PAOs responsible for biological phosphorus removal.

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