Bacterial community evolution along full-scale municipal wastewater treatment processes

Lei Zhang, Yanan Cheng, Chang Qian and Wenxuan Lu

ABSTRACT

Sewage pollution is a major threat to public health because sewage is always accompanied by pathogens. Generally, wastewater treatment plants (WWTP) receive and treat sewage to control pathogenic risks and improve environmental health. This study investigated the changes in the bacterial community over the course of treatment by a WWTP. Illumina MiSeq high-throughput sequencing was performed to characterize the bacterial communities in the WWTP. This study found that potential pathogens in the WWTP, especially the genera *Arcobacter* and *Acinetobacter*, were greatly reduced. In addition, high chemical oxygen demand levels provided excessive growth substrates for the genera *Hyphomicrobium* and *Rhodoplanes*, the abundance of which could exceed autotrophic bacteria, increasing the ammonium removal. According to the network analysis, the bacterial assemblage was not randomly arranged in the WWTP, and various defined processes led to higher intra-phylum (such as *Proteobacteria*) coexistence than expected. Moreover, the metabolic functions of bacterial communities significantly improved in the WWTP compared with the influent. Together, the data in this study emphasize the need to understand the bacterial community of WWTPs better. When analyzing the risks of WWTP drainage systems to the environment and human health, these data should be considered.

Key words | bacterial diversity, Illumina MiSeq high-throughput sequencing, network analysis, PICRUSt analysis, wastewater treatment plant

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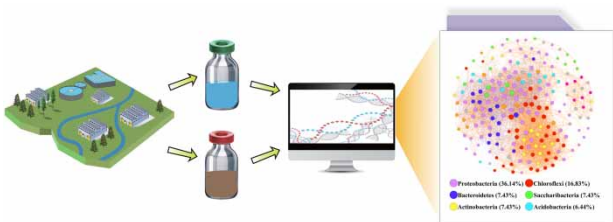
HIGHLIGHTS

- Heterotrophic nitrifiers have the potential to increase ammonium removal.
- The potential pathogens in the WWTP were greatly reduced.
- The bacterial assembly in wastewater treatment plants is nonrandom.
- The metabolic functions of the bacterial community improve in the WWTP compared with the influent.

GRAPHICAL ABSTRACT



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INTRODUCTION

The quality of fresh water has decreased significantly over the past few decades, posing a new threat to drinking water security (Palmateer *et al.* 1999; Rodell *et al.* 2018). One of the primary causes of water quality deterioration is sewage pollution, which is rich in both nutrients and microorganisms (Cai *et al.* 2014). Case studies have shown that groundwater in shallow rural wells is contaminated with high levels of human fecal bacteria and bacterial and viral pathogens (Ferguson *et al.* 2012). Approximately 5 billion people around the world live in areas with threatened drinking water (Vörösmarty *et al.* 2010). Therefore, water treatment is clearly a significant but complex global problem.

Normally, all wastewater is treated in a wastewater treatment plant (WWTP) and then discharged into the water environment. In order to assess the environmental health impact of wastewater from modern WWTPs, the microbiota can serve as an indicator of aquatic ecosystem health. This is because microorganisms are ubiquitous and widespread in aquatic ecosystems (Lawrence *et al.* 2005) and because the microbial community is the basis of the biogeochemical cycle (Azam 1998). Modern WWTPs can remove most bacteria (including coliforms) from sewage. Nevertheless, the intractable bacteria (particularly some pathogens) are mostly treated, because they threaten the quality of surface water (Marti *et al.* 2013; Kumaraswamy *et al.* 2014). Because of rapid air or water transmission and the high toxicity of bacterial pathogens, they will quickly cause disease and are harmful to human health (Cai & Zhang 2013; Ahmed *et al.* 2014; Gomi *et al.* 2015). Therefore, the key task of WWTPs is surveilling and monitoring the wastewater microbiome to record its removal efficiency.

Over many years, our group has performed a tremendous amount of research and development in microbial diversity in all types of habitats, resulting in great improvements (Shendure & Ji 2008; Kurilkina *et al.* 2016; Jiang *et al.* 2018). In many types of biological WWTP processes, the bacterial diversity has been revealed at high coverage and resolution through the high-throughput sequencing of 16S rRNA gene amplicons (Kwon *et al.* 2010; Zhang *et al.* 2012; Ibarbalz *et al.* 2013). These outstanding studies have described the relative abundance of taxa in a single bacteria, and we have contrasted diverse spatially distributed WWTP samples (e.g., unique and shared taxa) (Fang *et al.* 2018; Zhang *et al.* 2019). However, no studies have used sequencing data from multiple samples to probe latent interspecific inter-reactions. WWTPs with above average diversity (exceeding 700 genera (Zhang *et al.* 2012)) and high biomass (usually 2–10 g/L (Grady *et al.* 2011)) are a distinctive man-made microbial ecosystem, although it has long been known that dislodging organic and inorganic pollutants from wastewater (for example, nitrifying bacteria that convert ammonium to nitrite) (Ye *et al.* 2012) depends to a large extent on the coexistence of several groups of bacteria.

In recent years, the development of high-throughput molecular technologies has extended the bioinformatics data we can acquire by several orders of magnitude compared with traditional approaches, which offers the possibility of obtaining more comprehensive information (Kurilkina *et al.* 2016). Furthermore, Illumina MiSeq high-throughput sequencing has a lower cost per sequence than other platforms, enabling high-throughput microbial ecology at the greatest possible coverage to date (Caporaso *et al.* 2012). Therefore, in this study, we aimed to use Illumina MiSeq high-throughput sequencing to identify and monitor the wastewater microbiome and the pathogens present in the influent, effluent, wastewater samples from each treatment tank and activated sludge from a municipal WWTP. We also aimed to affirm the following assumptions: (i) the bacterial assembly in different activated sludge samples and different water samples are nonrandom in WWTPs; and (ii) the general situation of bacteria in influent water mirrors the human microbiome, and these bacteria can usefully be dislodged by WWTPs. Notably, a comprehensive introduction to the wastewater microbiome in different water samples and different activated sludge samples from a WWTP will be presented in an effort to comprehend the fate and environmental impact of WWTPs better. Furthermore, it is expected that by comparing the 16S rRNA gene sequencing results, the bacterial composition of influent wastewater in this study will provide a worthwhile reference

for other wastewater pollution investigations during similar seasons.

MATERIALS AND METHODS

Sampling site and sample collection

The sampling site was in Chuzhou city, China (Chuzhou Qingliu WWTP), which (including the tertiary treatment) aims to collect and treat 100,000 tons/day of domestic sewage from the primary urban area. This WWTP was under stable operation at the time of sampling. A_2/O biological tanks and ultraviolet ray disinfection processes are used in this WWTP, and a belt-type concentration dehydrator is used to treat sludge. After the sewage treatment is completed, the product is discharged into natural waters. The water quality of the effluent barely reaches the first-class A standard of the 'Discharge standard of pollutants for municipal wastewater treatment plant' (GB18918-2002).

Figure 1 shows the treatment processes of this WWTP and the corresponding sample names (the secondary sedimentation tank acts to separate the activated sludge from the sewage). This water sample had the same characteristics as the aerobic pond water sample, so it was ignored. All the samples were collected on the morning of 8 October 2018 (autumn). The sampling times were at the end of each treatment unit, and three samples were taken from each unit and mixed into one sample, except for the biological reaction tank. The temperature (T) and pH were measured immediately after collection. Inside the biological reaction tank is a mixture of sewage and activated sludge, so three samples measuring 1,000 ml each were collected from the bioreactor and placed on ice immediately after removal. The supernatant was collected from three samples and was used as the sample for each biological reaction cell. The sludge

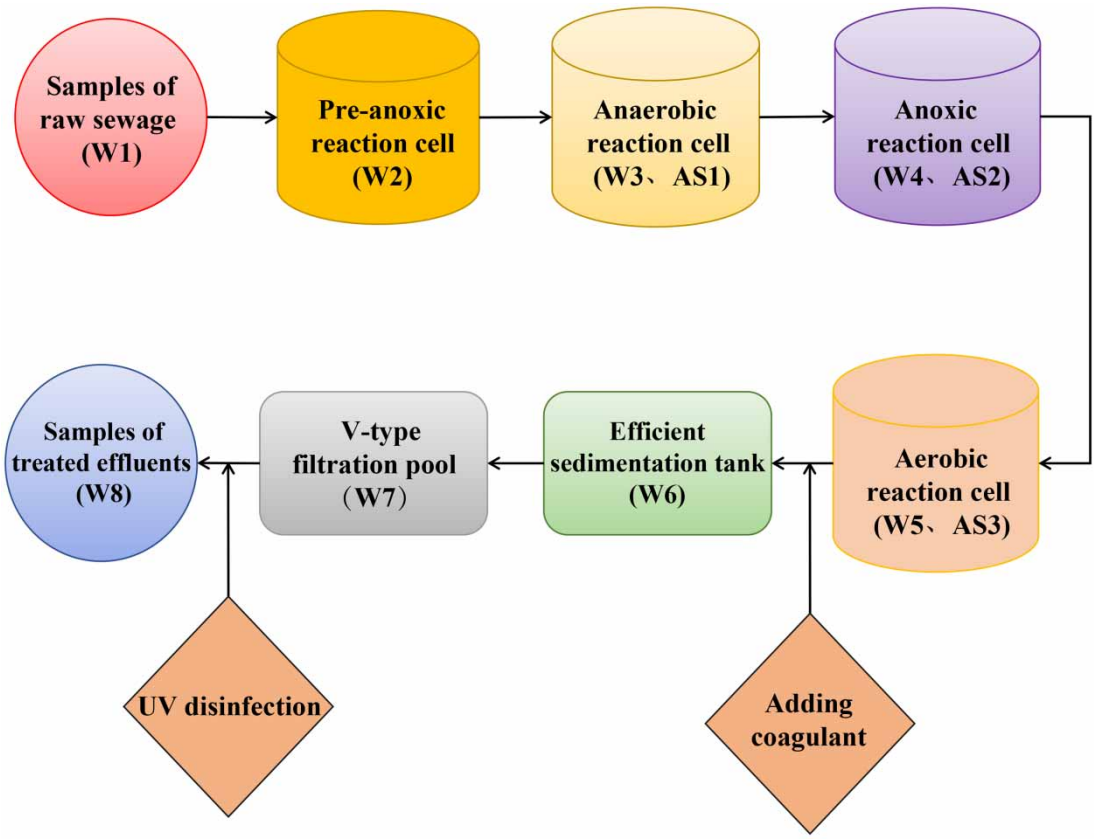

Figure 1 | Flow chart of the treatment processes of the Qingliu WWTP and the location of sampling sites.

(three samples were taken from each unit) was collected from the anaerobic tank (AS1), anoxic tank (AS2), and aerobic tank (AS3). Then, the wastewater and sludge were placed in sterile polyethylene bottles and transported to the laboratory on ice; the sludge samples were centrifuged immediately for 10 min at 6,000 rpm.

First, the water samples were applied to quantitative filter paper in the laboratory for routine filtration. To remove bulky suspended solids from the water sample, vacuum filtration was then performed using a 0.2 μm filter membrane. After the membrane was collected, a pair of scissors sterilized with 75% medical alcohol was used to cut the membrane, which was then placed inside a sterile centrifuge tube. The dewatered sludge was also placed in a sterile centrifuge tube. Lastly, the samples were stored in an ultralow temperature freezer at −20 °C until DNA extraction (Wang *et al.* 2012, 2016).

Determination of physical and chemical indices

A multiparameter water quality sonde (YSI 6600V2, USA) was used to measure the on-site water temperature (T) and pH. Chemical analyses (of the total nitrogen (TN), total phosphorus (TP), and ammonium nitrogen (NH_3-N)) of the water samples were conducted in the laboratory in accordance with standard methods (SEPA 2002). For the activated sludge samples, the NH_3-N, TP, and chemical oxygen demand (COD) were measured in the laboratory according to standard methods (Jin & Tu 1990).

DNA extraction and high-throughput 16S rRNA sequencing

The total DNA was extracted from eight water samples and three activated sludge samples from WWTPs using a DNA Isolation Kit (E.Z.N.A., Omega, Norcross, GA, USA) according to the manufacturer's instructions. Each sample was extracted in duplicate to minimize potential bias during DNA extraction. Duplicate DNA extracts (from three of the same samples) were then pooled to determine the DNA purity and yield using an ultraviolet spectrophotometer (Eppendorf, Germany). The DNA was stored at −20 °C until further processing (Keshri *et al.* 2018).

For the bacterial hypervariable V3–V4 region of the 16S rRNA gene, the primer set of 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) was employed for polymerase chain reaction (PCR) amplification. The PCR product was detected by 1.5% agarose gel electrophoresis. All the samples were processed according to formal experimental conditions, and each sample was repeated three times. The PCR products were mixed and examined by 2% agarose gel electrophoresis; the bands were recovered using an AxyPrep DNA Gel Recovery Kit (Axygen Company), with Tris–HCl elution followed by 2% agarose electrophoresis and QuantiFluor™-ST Blue Fluorescence Quantitative System (Promega Company) detection. The genomic DNA was isolated from the membrane filters using a DNA Isolation Kit (E.Z.N.A., Omega, Norcross, GA, USA) according to the manufacturer's instructions. In accordance with the sequencing quantity requirements, the corresponding proportion mixing was performed, and the samples were denatured with sodium hydroxide to produce single-stranded DNA fragments. Using AMPure Beads, the PCR products were purified and then sequenced by Personal Biotechnology Co., Ltd, Shanghai, China. The sequences have been deposited in the NCBI Sequence Read Archive under accession number SRP241213.

Data quality filtration

Trimmomatic was used to demultiplex and filter the raw pyrosequencing data (Bolger *et al.* 2014). FLASH was applied to coalesce the overlapping reads into single long reads (Lie *et al.* 2014). We merged the paired-end 16S reads, trimmed primers and distal bases, and used USEARCH (version 7.0, http://drive5.com/uparse/) for quality filtering and removing singletons (Edgar 2010). To obtain species classification information corresponding to each OTU, the RDP Classifier (version 2.2, http://sourceforge.net/projects/rdp-classifier/) Bayesian algorithm (confidence threshold of 0.7) was applied, and 97% of the OTU representative sequences were classified at similar levels using the QIIME platform (http://qiime.org/scripts/assign_taxonomy.html) (Samarajeewa *et al.* 2015) for a bioinformatic analysis of the community composition in each sample.

Bioinformatic analysis

Based on the species abundance analysis, Mothur software (version v.1.30.1) was used to calculate the alpha diversity

index and to study the similarities and differences in the species composition of different samples. With respect to the taxonomic analysis, the community structure composition of different taxonomic levels (such as phylum and genus) could be acquired. Various network methods were used to analyze the data sets (Hartmann *et al.* 2015). The system was used as the source node, and the OTU was used as the target node to generate the network. The edge (i.e., the lines connecting the nodes) of the network corresponds to the association between a specific OTU and a specific system or the combination of systems. The functional and metabolic pathways of the bacteria were predicted with the PICRUSt software (Langille *et al.* 2013; Dong *et al.* 2018). To obtain different functional prediction information, a closed OTU table acquired from the QIIME was contrasted against the KEGG databases. The above bioinformatics analysis images were all drawn using the R language.

RESULTS

Physical and chemical indicator analysis

The environmental factors for the water samples from each treatment unit in the WWTP are shown in Supplementary material, Table S1. The Qingliu WWTP primarily treats domestic sewage from the Qingliu district of Chuzhou city; the influent effluent water quality is relatively stable, the removal efficiency of TN and NH_3-N is relatively high (TN removal, 88.9%; NH_3-N removal, 99.86%), and the removal efficiency of TP (TP removal, 68.9%) needs to be improved (Shichiji *et al.* 2013). The effluent water quality can reach the primary A standard in the 'Discharge standard of pollutants for municipal wastewater treatment plant'. Based on the change in the TN, NH_3-N, and TP contents, the removal of N and P during the sewage treatment process is basically performed during the biological treatment process, and the N and P removal effect is better. Based on the change in pH, it can be demonstrated that the pH in the water body following biological treatment decreased slightly, but the whole unit still exhibited weak alkalinity. Furthermore, the temperature reading indicates that the water temperature in each treatment unit was basically the same (22.6–23.0 °C), indicating that there is no special temperature requirement for each biological reaction tank during sewage treatment.

Analysis of bacterial community diversity

We performed microbial diversity analyses of eight water samples and three activated sludge samples from the Qingliu WWTP using high-throughput 16S rRNA sequencing technologies (Figure 2). To ensure the equality of data between samples, the indicated sequencing results were randomly selected to obtain a uniform amount of data. According to the minimum sample sequence number, a uniform amount of data was randomly extracted for each sample. For the 21,922 effective sequences, the OTU distribution was performed according to the standard of 0.97 similarities, yielding a total number of 1,519 OTUs. According to a sparse curve analysis, the slope of the curve gradually becomes gentle as the quantity of effective sequences increases (Figure 2(a)). This finding indicates that the number of sequencing samples accurately and reasonably reflects the microbial community structure and diversity.

This study used the alpha diversity index to analyze the richness and diversity of the bacterial communities in the samples. As shown in Supplementary material, Table S2, the coverage index of each sample was above 0.99, reflecting good community coverage. This result demonstrates that the sequencing findings reflect the bacterial community structure of each sample. After biological treatment, the number of OTUs in the water sample was significantly higher than that in the influent sample. The number of OTUs in the W4 sample from the anoxic tank was significantly lower than that from the other biological reaction tanks. The number of OTUs in the W4 sample was higher than only that in the W1 sample influent. The Simpson indices of the W1 samples were higher than those of the other samples, and the Shannon indices were lower than those in the other samples (Figure 2(b) and 2(c)). The Simpson and Shannon indices of the W1 and W4 samples were significantly different from those of the other wastewater samples, and activated sludge samples and the Ace and Chao indices of the W1 and W4 samples were significantly smaller than those of the other samples (Figure 2(d) and Supplementary material, Table S2).

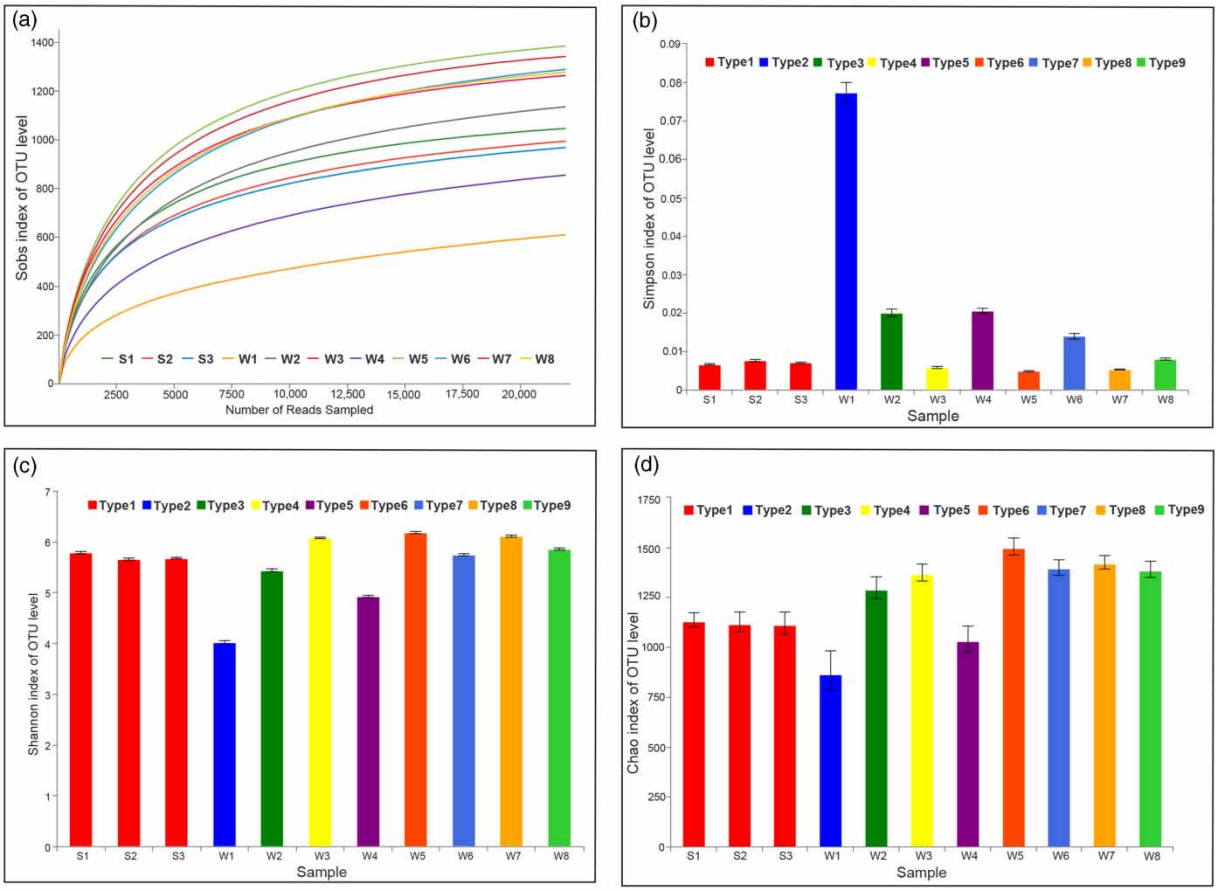

Figure 2 | Alpha diversity of eight water samples and three activated sludge samples of WWTP. (a) Observed OTUs, (b) Simpson's diversity, (c) Shannon's diversity, and (d) Chao richness.

Sample community composition analysis

Figure 3(a) shows the relative abundance of each bacterial phylum and the relationship with the sample. From the relative abundance of each bacterium in the sample, we observed that the phylum with the highest average abundance was *Proteobacteria* (average 35.61%). Other bacteria with relatively high average relative abundances were *Chloroflexi* (average 14.74%), *Saccharibacteria* (average 10.76%), *Actinobacteria* (average 7.70%), *Bacteroidetes* (average 6.38%), *Acidobacteria* (average 4.83%), *Firmicutes* (average 4.60%), *Parcubacteria* (average 4.57%), *Nitrospirae* (average 1.53%), *Planctomycetes* (average 1.17%), and *Gracilibacteria* (average 1.11%). In all the samples, the most abundant type was *Proteobacteria*, with a wide range of 65.25–17.63% in the wastewater samples and a relatively stable proportion of 32.99–22.93% in the activated sludge samples, similar to previous studies on wastewater (Cai *et al.* 2014) and activated sludge (Ma *et al.* 2016) that employed Illumina MiSeq sequencing. Interestingly, the most abundant bacterial phylum in the W4 sample was not *Proteobacteria* (relative abundance 17.63%), but rather *Saccharibacteria* (36.04%), which was relatively less abundant than *Proteobacteria* in the other samples. Notably, there was a significant difference between the bacterial community structure of the W1 samples and the other samples ($P < 0.05$). There was also a difference in the community structures between the samples in the bioreactor. This difference was primarily reflected in the relative abundance of individual bacteria. However, the bacterial community structures of the AS1, AS2, and AS3 samples were basically the same.

We next performed analyses of a single bacterium (Figure 3(b)). Although *Proteobacteria* was a dominant bacterial phylum in all the samples, there was a large difference in the *Proteobacteria* in each sample at the class level. The *Epsilonproteobacteria* continued to decrease as wastewater

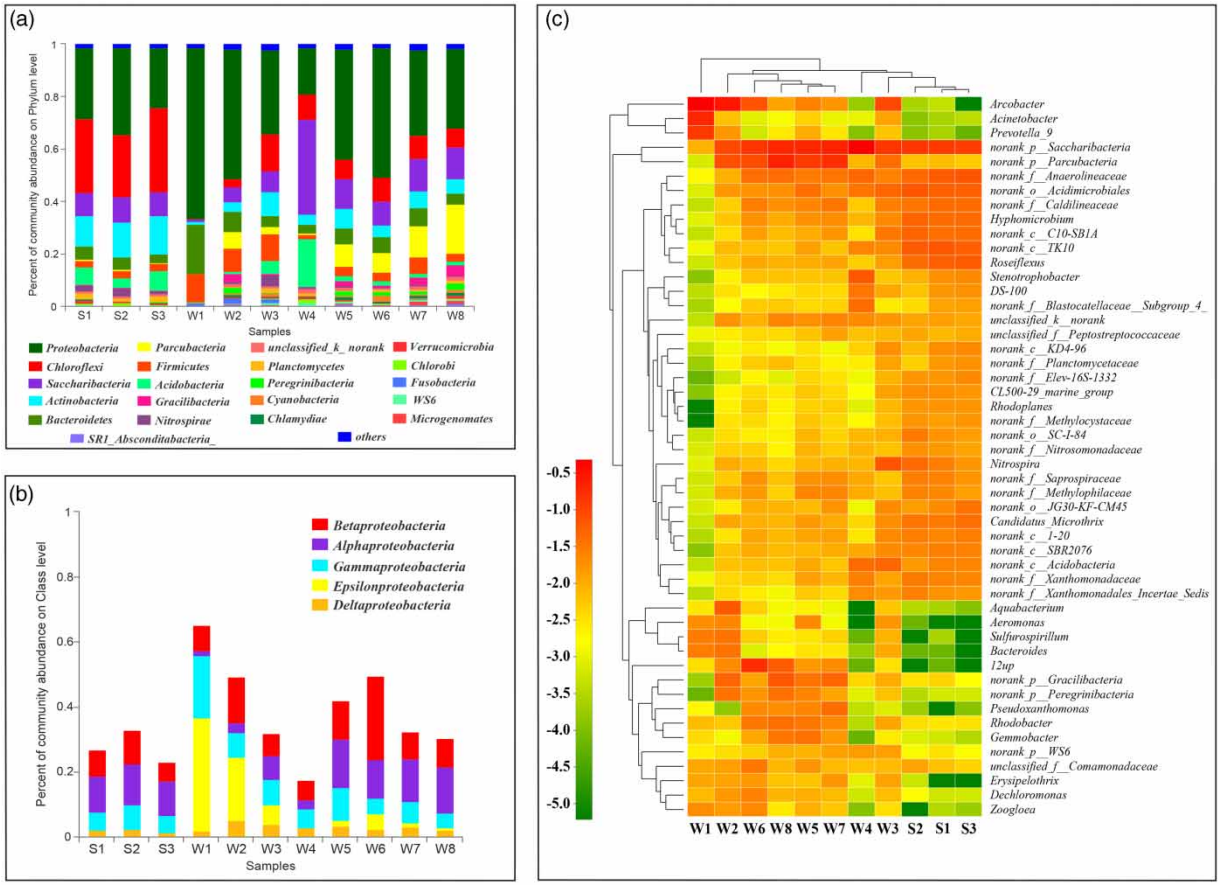

Figure 3 | Microbial community composition of (a) bacterial phyla (>0.1%), (b) class level for Proteobacteria, and (c) classified genera (top 50).

treatment progressed. The trend between *Alphaproteobacteria* and *Epsilonproteobacteria* was just the opposite; the bacterial abundance in the samples increased, but the amplitude was smaller than that observed for *Epsilonproteobacteria*. The abundance of *Gammaproteobacteria* declined slightly, while the relative abundance of *Betaproteobacteria* remained basically unchanged. However, the abundance of *Proteobacteria* in the bacterial community structure of the activated sludge samples (AS1, AS2, and AS3) was largely the same.

As shown in Figure 3(c), the relative abundances of the bacteria in all the samples from highest to lowest were unclassified *Saccharibacteria* (average 11.14%), *Arcobacter* (average 5.72%), unclassified *Parcubacteria* (average 3.96%), unclassified *Anaerolineaceae* (average 2.49%), unclassified *Acidimicrobiales* (average 2.25%), unclassified *TK10* (average 1.72%), *12up* (average 1.59%), and *Acinetobacter* (average 1.40%). Additional species with relatively low average abundances were not listed. The comparison of the bacterial community structures from each sample shows that the relative abundance of some bacteria (such as the genera *Acinetobacter*, *Prevotella 9*, *Cloacibacterium*, and *Macellibacteroides*) in the water samples after treatment decreased; in some cases, it was close to 0 (Figure 4). There were also cases in which the abundance of some bacterial genera (such as unclassified *Saccharibacteria*, unclassified *Anaerolineaceae*, and *Pseudoxanthomonas*) increased after handling. Additionally, some genera that were not found in the influent samples were found in the treated samples. For example, in the W1 sample, the relative abundance of *Arcobacter* was 32.5%, and while the relative abundance decreased to 1.5% after the bioreactor treatment, the bacterial abundance was only 0.73% in the effluent. *Acinetobacter* had a relative abundance of 2% in the W1 sample, while after prehypoxic and anaerobic treatments, the relative abundance of *Acinetobacter* in the water was reduced to 0.

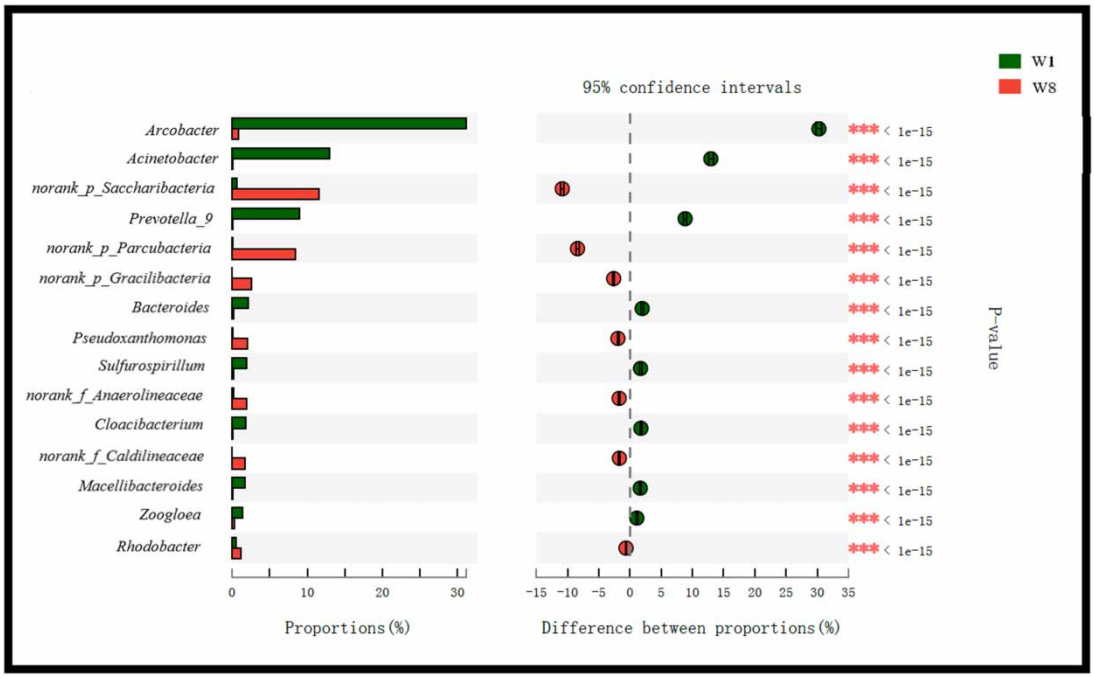

Figure 4 | Microbial community evolutions and core genera between W1 and W8 of the WWTP.

Network analysis

The WWTP bacterial network was composed of 202 nodes (OTUs) and 3,961 edges (39.218 edges per node on average) (Figure 5). The diameter was six edges, and the average path length was 2.474 edges. The modular index (the value >0.4 indicates that the network has a modular structure) was 0.822, and the clustering coefficient was 0.657. In the

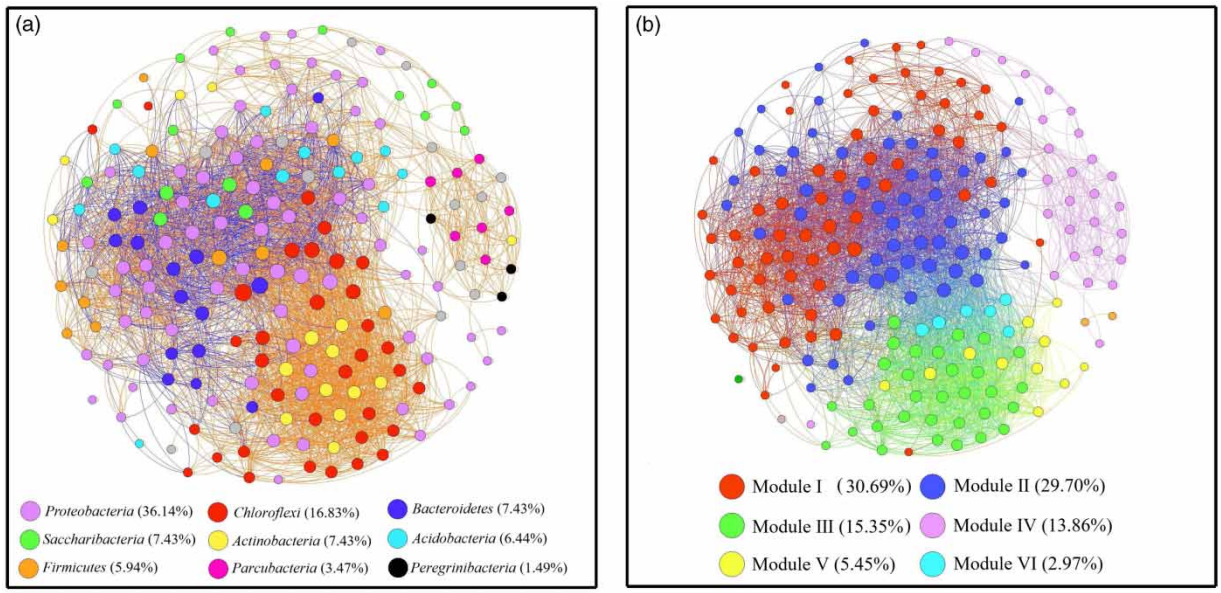

Figure 5 | Network of co-occurring bacterial OTU based on correlation analysis ($P < 0.05$). The size of the node is proportional to the genera abundance. (a) Co-occurring network colored by phylum. Edge color represents positive (orange) and negative (blue) correlations. (b) Co-occurring network colored by the modularity class. Please refer to the online version of this paper to see this figure in color: http://dx.doi.org/10.2166/wh.2020.092.

network, the nodes were primarily allocated to nine bacterial phyla. Among them, six types of phyla (including *Proteobacteria*, *Actinobacteria*, *Saccharibacteria*, *Bacteroidetes*, *Acidobacteria*, and *Chloroflexi*) occupied 81.7% of all the nodes and were widespread. All the nodes were divided into six primary modules, when the distribution of nodes was modularized. The nodes in module I were primarily *Proteobacteria*; the nodes in module II were primarily *Chloroflexi* and *Acidobacteria*; the nodes in module III were primarily *Actinobacteria* and *Chloroflexi*; the nodes in module IV were primarily *Saccharibacteria* and *Parcubacteria*; the nodes in module V were primarily *Proteobacteria* and *Actinobacteria*; and the nodes in module VI were primarily *Actinobacteria*.

16S rRNA function prediction

As shown in Figure 6, the relative abundances of the bacterial functional category in all the samples from highest to lowest were metabolism (71.4%), genetic information processing (17.1%), environmental information processing (8.6%), and cellular processes (2.9%). Additional bacterial functional categories with relatively low average abundances were not listed. To compare the differences among the various samples better, the top 35 bacterial potential functions in different groups were further selected (Figure 6 and Supplementary material, Table S3). The potential bacterial functions of each sample showed that 25 important metabolism pathways were found, such as the biosynthesis of amino acids, carbon metabolism, purine metabolism, pyruvate metabolism, and oxidative phosphorylation. The primary pathways in environmental information processing were ABC transporters, the two-component system, and the bacterial secretion system. Aminoacyl-tRNA biosynthesis and ribosome and quorum sensing were the primary pathways in genetic information processing and cellular processes, respectively.

DISCUSSION

The OTU diversity for the bacterial community in the Qingliu WWTP samples is shown in Figure 2 and Supplementary material, Table S2. The Simpson's diversity index in the bacterial community of WWTP samples was found to be significantly different ($P < 0.05$). The Simpson index showed the highest influent content. The most likely explanation is that in a hyperoxic environment, some bacteria are unable to grow or die. Therefore, the treatment process in the WWTP limits the Simpson index. In addition, the bacterial community richness in the water changed slightly during coagulation and sedimentation, which explains why the physicochemical treatment method used in the sewage treatment plant has less of an effect on the bacterial community richness. Moreover, the richness and evenness of microorganisms in the sludge were found to be higher than those in the influent community, which showed no difference compared with previous studies (Tong *et al.* 2019).

In this study (Figure 3), many bacteria (such as the genera *Acinetobacter*, *Bacteroides*, and *Prevotella 9*) were found in the human intestine, surface water, and soil. This result indicates that these bacteria could originate from the soil and human microbiome, and they could help shape the characteristics of the bacteria in WWTPs. Compared with the samples from other WWTPs, the differences in the geographical location and wastewater characteristics of WWTPs may lead to differences in the bacterial composition (Ma *et al.* 2013; Do *et al.* 2019). Some of the top 50 genera, such as *Arcobacter*, *Acinetobacter*, and *Erysipelothrix*, are the most important genera for human health. This finding suggests that one of the potential sources of bacterial pathogens released into the environment may be wastewater from WWTPs. The genus *Acinetobacter* is known as an opportunistic human pathogen (Visca *et al.* 2011), and it has not only clinical importance but also difficult to treat. A relationship between these bacteria and pneumonia, meningitis, wound infection, and urinary tract infection has been reported (Dijkshoorn *et al.* 2007). The genus *Erysipelothrix* is also a pathogen, and although the incidence of human infection may decrease due to the technological progress of the animal industry, this pathogen will still be present in a specific environment (Wang *et al.* 2010). The genus *Arcobacter* has also been shown to be associated with human diseases (Vandenberg *et al.* 2004; Ferreira *et al.* 2014; Figueras *et al.* 2014). *Arcobacter butzleri* is a pathogen that causes abdominal colic and persistent watery diarrhea (Arguello *et al.* 2015). It can survive in aquatic environments, so it may pose a threat to human health downstream of the

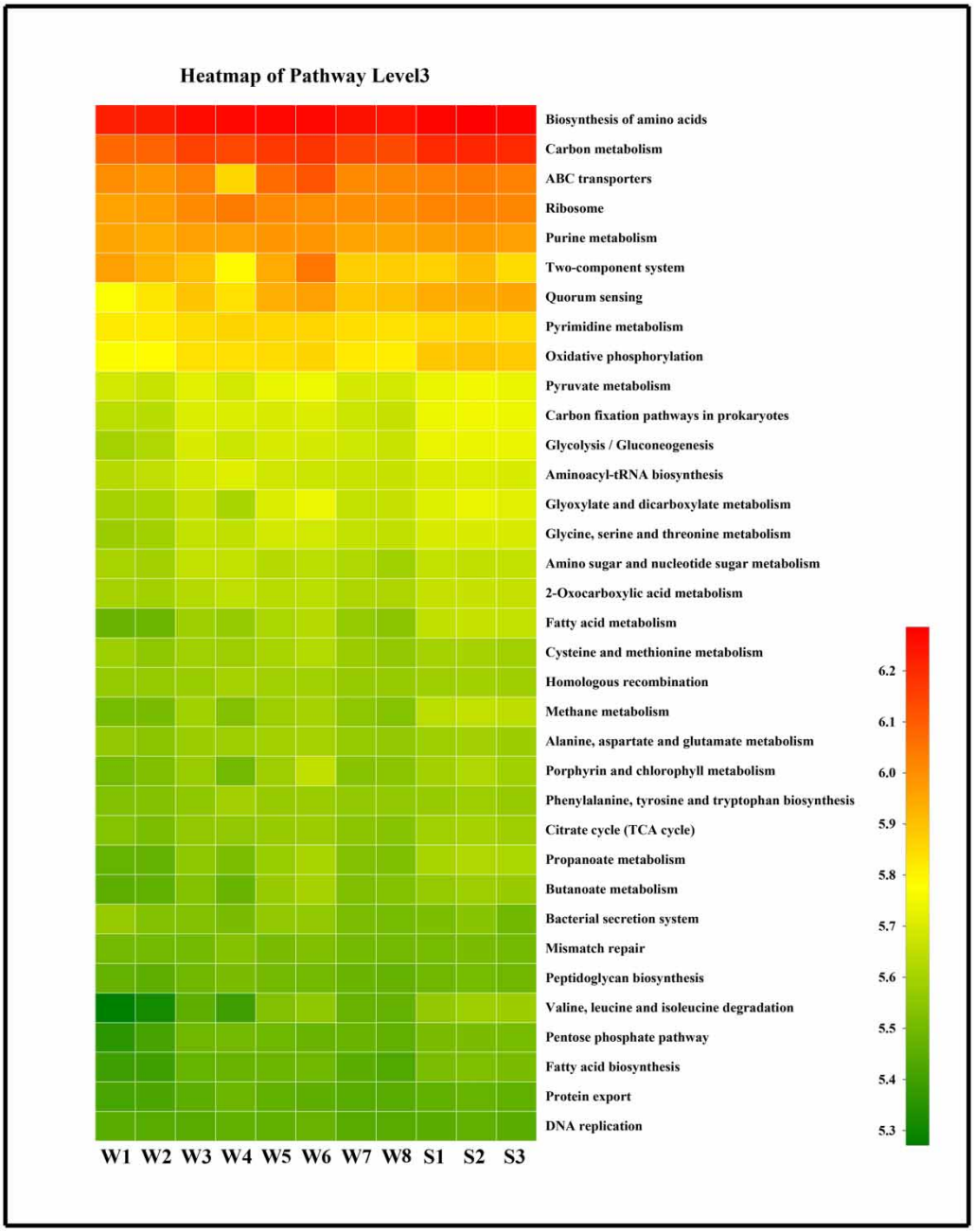

Figure 6 | Relative abundance of various predicted functions of microbial communities in the water bacteria and activated sludge bacteria in the WWTP using PICRUSt grouped into level 3 functional categories.

release site or when used to irrigate vegetables (Rubino *et al.* 2011). The genus *Bacteroides* was found in the WWTP, with an average abundance of 0.49%. Although it is an indicator of fecal pollution, *Bacteroides* is not the most common genus in wastewater (Xu *et al.* 2003). In our work, the analysis of the 16S rRNA gene sequence confirmed the existence of bacterial genera rather than species. However, identifications at the genus level do not reveal the presence of pathogenic species. Therefore, further research is needed to identify the pathogens.

As shown in Figure 4, the abundance of some bacterial pathogens (such as the genera *Arcobacter*, *Bacteroides*, *Acinetobacter*, and *Prevotella 9*) in the effluent was significantly lower than that in the influent ($P < 0.01$). These results show that the WWTP can effectively remove most bacterial pathogens. The secondary wastewater treatment systems (W2, W3, W4, and W5) of WWTPs effectively dislodged the genus *Arcobacter* (32.5%), with an average abundance of 6.01% in the secondary treatment process and an average abundance of 3.29% in the tertiary treatment process (W6 and W7). Similarly, after the secondary treatment, the abundance of the genera *Bacteroides* and *Acinetobacter* decreased by 1.8–13.4%. These results showed that the secondary treatment process can remove a large number of potential bacteria and pathogens, while the tertiary treatment process can further reduce these bacteria. This reduction is primarily due to the influence of ultraviolet rays on pathogens. UV irradiation is used to an effective and competitive advance disinfection process, and it has become the most commonly used alternative to chlorination, with a comparable and often more effective disinfection efficiency against viruses and bacteria (Krzeminski *et al.* 2017; Ignatev & Tuhkanen 2019).

As a result of the high concentration of ammonia and the serious environmental factors from coking wastewater, nitrification failure occurs often (Thomsen *et al.* 2007). Nitrification is usually performed in two consecutive steps, with the conversion of autotrophic ammonia oxidation to nitrite by ammonia oxidizing bacteria (AOB) and autotrophic nitrite oxidation to nitrate by the nitrite oxidation bacterium (NOB), which are the two important types of nitrifiers (Siripong & Rittmann 2007; Zhang *et al.* 2011; Kim 2013). In this study, *Nitrospira* was the NOB in the WWTP, and this result is similar to previous research, in which this genus was the primary NOB in the study WWTP system (Xu *et al.* 2018).

In this study, the unforeseen low percentage of NOB and almost no AOB seemingly conflict with successful nitrification. Nevertheless, a similar phenomenon was also found in previous studies. In a large-scale WWTP in Hong Kong, only 0.05% AOB and 1.01% NOB were found (Ye *et al.* 2011). Almost no AOB was detected in oil refinery WWTPs with high nitrification efficiency (Figuerola & Erijman 2007). In this study, potential heterotrophic nitrifiers (such as the genera *Hyphomicrobium* and *Rhodoplanes*) grew excessively due to the presence of organic carbon sources and high concentrations of COD. They may be more abundant than autotrophic bacteria and have a greater effect on removing ammonium. However, the nitrification mechanism and the specific function of heterotrophic bacteria require further study. The process management is of great significance for obtaining a thorough understanding of these functional nitrifying species.

The degradation of some organic pollutants and the transformation of inorganic nutrients in wastewater usually involve many steps, which benefit from cooperation between different bacterial groups but simultaneously suffer from competition among bacteria that use the same substrate. Thus, it can be expected that a nonrandom symbiosis mode of intertaxon variation occurs. The results show that the bacterial assembly of these species may not be driven by random factors, such as mutualism and competition, but they may be more driven by certainty factors (Ju *et al.* 2014; Ju & Zhang 2015; Rui *et al.* 2015). Previous studies have shown that different habitats of the human microbiome (Faust *et al.* 2012) and the WWTP (Ju *et al.* 2014) also affect the network structure. In each type of the WWTP, specific and core species probably play different roles. In the WWTP of this study, core species usually participate in basic electron transfer processes, such as denitrification (genera *Hyphomicrobium* and *Rhodoplanes*) (Sperl & Hoare 1971; Hiraishi & Ueda 1994) and sulfate reduction (genus *Desulfovibrio*) (Lovley & Phillips 1992). In addition, the core species that can degrade organic carbon are widespread in the WWTP, for example, aromatic species (such as the genus *Sphingomonas*) (White *et al.* 1996). In particular, the WWTPs have more specific OTUs (Operational Taxonomic Units), which can withstand the pressure of highly inhibited bacteria (Kümmerer *et al.* 2000). The special OTUs of the WWTP, which are labeled as the genus *Prevotella 9*, are bacteria that have been found to be resistant to a variety of antibiotics (Falagas & Siakavellas 2000). The specific resistant species may change from rare species to dominant species under antibiotic stress (Li *et al.* 2013; Huang *et al.* 2014).

In addition, the modular structure was obvious: the nodes were divided into six primary modules (Figure 5), and the nodes in different modules performed different functions (Newman 2006). In module I of the network, some bacteria were related to electron transfer. For example, the sulfur-oxidizing bacteria in the genus *Arcobacter* (Sievert

et al. 2008), iron-reducing bacteria in the genus *Sulfurospirillum* (Sikorski *et al.* 2010), and iron-oxidizing bacteria in the genus *Aquabacterium* (Straub *et al.* 2004) were found, and they are related to electron transfer. Biological electron transfer plays a significant role in the microbial degradation of organic matter (Stams *et al.* 2006). For example, the genera *Hydrogenophaga* and *Rhodobacter* in module I are primarily related to the degradation of organic contaminants (Contzen *et al.* 2000; Merugu *et al.* 2014). In module V of the network, some bacteria may be involved in biogeochemical C and N cycles. For example, the nitrite-oxidizing function of bacteria in the genus *Nitrospira* has been well studied. In module V of the network, other bacteria are also involved in C and N cycling, including the families Arenicellaceae, Caldilineaceae, Methylocystaceae, Saprospiraceae, and Rhodobacteraceae (Justice *et al.* 2014). In general, the network analysis enabled us to conclude that the bacterial assemblage in the WWTP is not random.

The bacterial communities in the water environment play an important role in the degradation of nutrients during cycling and organic matter (Newton *et al.* 2011). Accordingly, the metabolic function of the bacterial community is an important factor that affects the stability of the WWTPs. Some studies have used 16S rRNA function prediction to analyze the functional differences between bacterial communities, and good results have been obtained (Wong *et al.* 2016; Zhang *et al.* 2016). In this study, the major functional genes in different samples were metabolic-related functions, which accounted for the largest proportion (71.4%, Figure 6), indicating that metabolism plays an extremely important role in WWTP processing, which is consistent with the previous research results (Yang *et al.* 2011). Moreover, the results showed that the metabolic functions of the bacterial community in the WWTP increased significantly after treatment. In addition, amino acid metabolism and carbohydrate metabolism accounted for a higher proportion of the secondary functional layer related to metabolism in this study (Supplementary material, Table S3). Studies show that amino acid metabolism occurs primarily through deamination, transamination, combined deamination, and decarboxylation. Through decomposition into α-keto acids, amines, and carbon dioxide, amino acid metabolism is closely related to the nitrogen cycle (Kuypers *et al.* 2018). In terms of carbohydrate metabolism, some studies have shown that *Firmicutes* may play a very important role in carbohydrate metabolism (Ibrahim & Anishetty 2012). According to the results of animal intestinal flora research, some *Bacteroidetes* have many genes that encode glycoside hydrolase and polysaccharide lyase in the genome, and these encoded enzymes can promote the degradation of polysaccharides (El Kaoutari *et al.* 2013). Therefore, changes in the bacterial communities in WWTPs are of great interest for maintaining the stability of the water environment.

CONCLUSIONS

In the present study, the high-throughput sequencing of the 16S rRNA gene provides a comprehensive insight into the microbial community structure and opportunistic pathogen distribution pattern in the WWTP. In addition, the nitrifying bacterial community structure in the system was limited to one genus. We found that in WWTPs, high concentrations of COD and organic carbon sources provide excess growth substrates for potential heterotrophic nitrifiers, such as the genera *Hyphomicrobium* and *Rhodoplanes*, which may outperform autotrophic bacteria and have a greater influence on ammonia removal. The results showed that most of the potential pathogens can be eliminated in the WWTP. The network analysis reveals that the bacterial assembly in different activated sludge samples and different water samples is nonrandom in the WWTP. Furthermore, the PICRUSt analysis shows that the metabolic functions of bacterial communities significantly improved in the WWTPs compared with the influent. These findings provided insights into the relationship between the microbial community and the performance of WWTPs.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT

All relevant data are available from an online repository or repositories (SRP241213).

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