



# Metagenomic and quantitative insights into microbial communities and functional genes of nitrogen and iron cycling in twelve wastewater treatment systems



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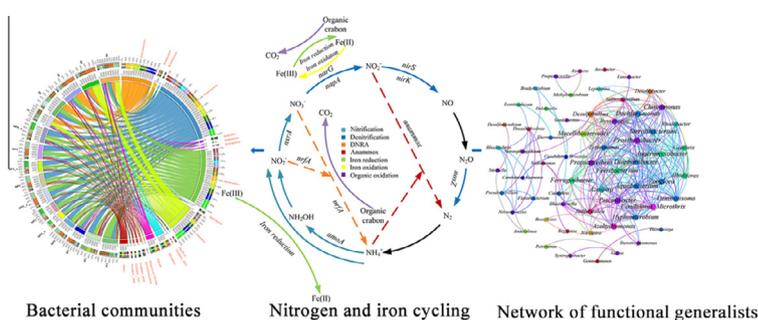
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## HIGHLIGHTS

- Bacterial communities and functional generalists were explored by MiSeq sequencing and qPCR.
- Anammox, *nrfA*, FeOB and FeRB genes had higher abundance in anammox bioreactors.
- Nitrification–anammox, denitrification–FeOB, and DNRA–FeRB showed co-occurrence patterns.
- Coupling of anammox, DNRA and FeRB were confirmed using correlation-based network analysis.
- Environmental factors have highly impacts on the N and Fe cycling-related bacterial communities.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 21 September 2015  
Received in revised form 10 January 2016  
Accepted 12 January 2016  
Available online 18 January 2016

### Keywords:

Co-occurrence patterns  
Functional gene group  
MiSeq sequencing  
Microbial community  
N and Fe cycling  
Network analysis

## ABSTRACT

To gain a better understanding of bacterial community structures, ecological inter-correlations and functional generalists of nitrogen- and iron-cycling-related bacteria in various wastewater treatment systems (WWTs), 16 samples collected from 3 industrial, 4 municipal and 5 anaerobic ammonium oxidation (anammox) WWTs were used to perform metagenomic analysis. A total of 9394 to 17,130 effective reads for 16 samples were obtained from the bacterial 16S rRNA V3–V4 regions using MiSeq sequencing. Taxonomic analysis revealed that *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and *Planctomycetes* were the dominant phyla in these samples. Furthermore, quantitative polymerase chain reaction (qPCR) was conducted and the results revealed that anammox, *nrfA*, FeOB (iron oxidizing bacteria) and FeRB (iron reducing bacteria) genes had higher abundance when *Candidatus Brocadia* was the dominant genera in anammox bioreactor. Finally, Spearman rank order coefficient correlation (SRCC) and redundancy discriminant analysis (RDA) analysis implicated that the groups of nitrification–anammox, denitrification–FeOB, and dissimilatory nitrate reduction to ammonium (DNRA)–FeRB showed positively

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correlations. Based on the co-occurrence pattern demonstrated by network analysis, the coupling of anammox, DNRA and FeRB was the noteworthy pathway for simultaneous removal of nitrogen and organic carbon. Overall, this work provides novel insights into the co-occurrence patterns and generalists of nitrogen and iron-cycling-related bacteria in wastewater treatment systems.

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## 1. Introduction

In recent decades, biological treatment processes has been widely applied for treating industrial and municipal wastewater due to their high removal efficiency, positive energy balance and low operational cost. However, the biological treatment processes is in a period of significant change motivated by two aspects. On one hand, it is necessary to renew some wastewater treatment systems (WWTSs) that were built in the last 30 years. On the other hand, the drive from the development of technological innovation toward more sustainable including energy recover and efficiency during mainstream wastewater treatment [1]. Moreover, increasingly effluent guidelines are being implemented across the world. However, the microbial influence on nutrients removal, recovery and function of microorganisms in mainstream biological approaches remains unclear. Therefore, to gain a better understanding of the microbial community and those functional genes of anaerobic or aerobic samples in WWTSs will not only be useful for illuminating the molecular mechanisms of nitrogen and organic matter removal, but also beneficial for promoting the maneuverability and stability of different WWTSs.

Previous studies [2,3] reported that nitrogen removal in WWTSs contains various biological processes, including anaerobic ammonium oxidation (anammox), nitrification, dissimilatory nitrate reduction to ammonium (DNRA), and denitrification. These nitrogen processes involved different 16S rRNAs and functional genes, namely anammox 16S rRNA, archaea ammonia monooxygenase (AOA-*amoA*), ammonia monooxygenase (AOB-*amoA*), nitrite oxidoreductase (*nxrA*), periplasmic nitrate reductase (*napA*) and membrane-bound nitrate reductase (*narG*), dissimilatory nitrate reductase (*nrfA*), copper-containing nitrite reductase (*nirK*), nitrite reductase (*nirS*), and nitrous oxide reductase (*nosZ*) [4,5]. At the same time, microbial iron cycling had been reported to play a key role in WWTSs for wastewater treatment. Microbial iron involves two processes, including iron-oxidizing (FeOB) and iron-reducing (FeRB). These two processes involved several bacterial 16S rRNAs genes, including FeOB (i.e. *Acidimicrobium* spp., *Ferrovum myxofaciens*) and FeRB (i.e. *Albidiferax ferrireducens*, *Geobacter* spp., and *Acidiphilium* spp.) [6,7]. Although the N (nitrogen) and Fe (iron) related microbial played the pivotal role in WWTSs, no attention has been paid to explore the bacterial interactions between the related microorganisms during N and Fe cycling. Moreover, the ecological linkages between bacterial community and operational parameters in different WWTSs are still unclear at present.

Currently, as the rapid development of high throughput sequencing technologies, MiSeq sequencing have been applied to explore the bacterial diversity and abundance of samples from WWTSs [8,9]. Nevertheless, the metagenomic and quantitative analysis for N and Fe cycling are few. Functional generalists, which were consisted of widely distributed bacterial genera affiliated with abundant phyla, were useful for maintaining the stability of the WWTSs. Their metabolic states have significant correlations with microbial communities and ecosystem functions. Last but not least, little is known about the co-occurrence associations among different bacteria and functional generalists in nitrogen and iron-cycling-related bacteria.

Given the above arguments, this study was performed with the following four objectives: (1) to explore the bacterial diversity and

their functional generalists in 16 sludge samples from different WWTSs; (2) to elucidate the community structures of anammox bacteria and iron-cycling-related bacteria and to quantitatively analyze the absolute abundance of nitrogen and iron-cycling-related bacteria; (3) to investigate the ecological linkages between operational parameters and related bacterial community; (4) to explore the co-occurrence patterns between bacterial communities and functional generalists using network analysis.

## 2. Methods

### 2.1. Description of wastewater treatment systems and sample collection

In this study, all anaerobic and anoxic sludge samples were collected from twelve WWTSs in Shaanxi and Kunming, China. Details of processes, treatment capacity, influents, effluents, and operational parameters of the 12 WWTSs were summarized in Table S1. Among these WWTSs, IP1, IP2, and IP3 were full-scale industrial WWTSs, MP1, MP2, MP3, and MP4 were full-scale municipal WWTSs. In addition, R1 was the pilot-scale WWTSs, and R2, R3, R4, and R5 were laboratory-scale WWTSs. These WWTSs differed mainly in their influents, effluents, and operational parameters. The anaerobic/anoxic/oxic (A/A/O) process was applied in MP1, MP2, and MP4 for treating municipal wastewater. MP2 was equipped with Intermittent Cycle Extended Aeration (ICEAS) plus Anaerobic Membrane Bioreactor (AMBR) for treating municipal wastewater. IP1 was operated with Upflow anaerobic sludge blanket process (UASB) plus Orbal oxidation ditch process for treating starch wastewater. UASB plus biological contact oxidation process was employed in IP2 for pulp and paper wastewater treatment. IP3 treated landfill leachate through the UASB plus A/A/O process for COD and nitrogen removal, and MBR was further used to enhance nitrogen removal. Additionally, R1, R2, R3, R4, and R5 were operated with anaerobic ammonium oxidation (anammox) process. R1 was controlled by continuous stirred tank reactor (CSTR) for nitrogen removal. R2, R3, R4, and R5 were equipped with sequencing batch reactor (SBR), SBR, UASB, and SBR, respectively.

The sludge samples from MP1, MP2, MP3, and MP4 were collected from anaerobic and aerobic tank to compare the microbial abundance under different dissolved oxygen (DO) concentration. For others anaerobic sludge sample, they were taken from IP1, IP2, IP3, R1, R2, R3, R4, and R5. Taken together, 16 samples were obtained from 12 WWTSs. After the 16 sludge samples were collected, they were immediately fixed in 50% (v/v) ethanol aqueous solution and then stored in laboratory at  $-80^{\circ}\text{C}$  for DNA extraction.

### 2.2. DNA extraction, PCR amplification and Illumina MiSeq sequencing

For DNA extraction, 0.5 g wet sludge sample pellet was collected and DNA was extracted using the FastDNA<sup>®</sup> SPIN Kit for Soil (Mp Biomedicals, Illkirch, France) according to the manufacturer's protocol. Extracted genomic DNA concentrations were determined with Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA) and its quality was checked in 1.0% agarose gel electrophoresis. Microbial genomic DNA extraction was performed in triplicate for each sludge sample.

Before sequencing, genomic DNA were amplified by PCR using primer set 338F and 806R for the hypervariable regions V3–V4 of the bacterial 16S rRNA [10]. After amplification, the amplicons were pooled and purified with AxyPrep DNA Gel Extraction Kit (Axgen, USA) according to the instructions (Details are shown in Method S1 in the Supplementary data). Finally, the library was constructed and run on a MiSeq Illumina platform (Majorbio BioPharm Technology Co., Ltd, Shanghai, China). All the raw sequence data has been deposited to the NCBI SRA database (Accession number: SRR2297240).

### 2.3. Sequence processing and bioinformatics analysis

All the raw paired-end reads were merged using FLASH (<http://ccb.jhu.edu/software/FLASH/>), and then low quality reads, were trimmed off by Trimmomatic (<http://www.usadellab.org/cms/?page=trimmomatic>). After filtration, the remaining 16S rRNA sequences were cluster into operational taxonomic units (OTUs) by setting 97% similarity. Then, the taxonomic classification was performed using RDP classifier (<http://sourceforge.net/projects/rdp-classifier/>) via Silva SSU database (<http://www.arb-silva.de>) with a confidence threshold 70%. Furthermore, appropriate subsample depth was conducted to avoid unequal sampling depth biases during comparison of microbial diversity and to ensure adequate sample depth while retaining lowest sequences [11]. Subsequently, alpha and beta diversity were conducted with Mothur pipeline ([http://www.mothur.org/wiki/Main\\_Page](http://www.mothur.org/wiki/Main_Page)) (Method S2).

### 2.4. Quantitative real-time PCR

To obtain the quantitative distribution of bacteria 16S rRNA, anammox bacteria 16S rRNA, FeOB 16S rRNA, FeRB 16S rRNA and other functional genes (i.e. AOB-*amoA*, AOA-*amoA*, *nosZ*, *nirS*, *nirK*, *narG*, *napA*, and *nrfA*) in the 12 WWTSs, the copy numbers of these genes were quantified by Mastercycler ep realplex (Eppendorf, Hamburg, Germany) based on SYBR Green II method. All the primer pairs and protocols of qPCR were summarized (Method S3 and Table S2). The standard plasmids of these genes were prepared according to previous study [10]. In order to check reproducibility, each reaction was performed in triplicate.

### 2.5. Statistical and network analyses

Pearson correlation coefficients was applied to evaluate the significant correlations between above mentioned bacterial 16S rRNA and functional genes using SPSS Statistics 20 (IBM, USA) (<http://www-01.ibm.com/software/analytics/spss/>). The Spearman's rank correlation coefficient (SRCC) was performed to investigate the relationships between WWTSs operational parameters and abundance of these bacterial 16S rRNA and functional genes using SPSS Statistics 20. In order to further explore the correlations between above mentioned genes and environmental parameters, redundancy discriminant analysis (RDA) was used by R (<https://www.r-project.org/>) with “vegan” and “permute” packages in RStudio (<https://www.rstudio.com/>). Furthermore, the co-occurrence network analysis was performed in R environment with “vegan”, “igraph” and “Hmisc” packages. Then, network visualization was conducted on the Gephi platform (<https://gephi.org/>) (Method S4).

## 3. Results and discussion

### 3.1. Diversity of bacterial community composition and functional generalists

In this study, after filtering the low quality reads and trimming the chimeras, 9394–17,130 high quality reads were yielded for the

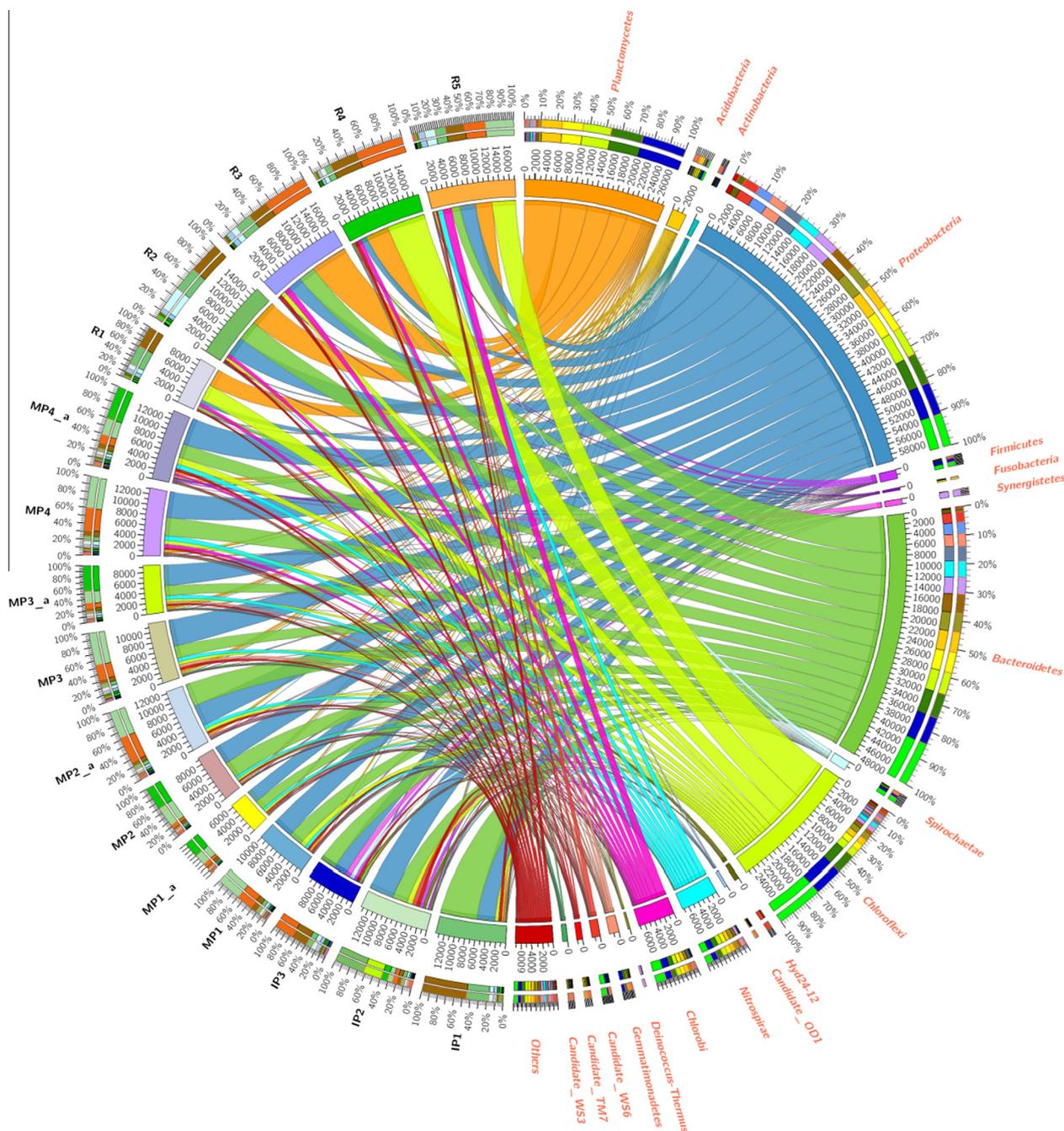
16 sludge sample by using MiSeq sequencing (Table S3). Based on the results of sequencing, OTUs were in the range of 110–748 for all 16 samples. Further analysis showed that 3, 304, 45 and 313 of 1352 OTUs were shared by samples from IP1–IP3, MP1–MP4, R1–R5, and MP1\_a–MP4\_a, respectively (Fig. S2). The result indicated that a high similarity of communities among municipal WWTSs and anammox WWTSs, respectively.

Additionally, the Shannon, Chao1, and ACE estimators of 8 samples from 4 municipal WWTSs were varied by 1.25–2.50 times higher than that of samples from industrial and anammox WWTSs. These results revealed that municipal WWTSs samples had higher diversity of bacterial 16S rRNA than samples from industrial and anammox WWTSs, which was consistent with other studies on different WWTSs [9]. The rarefaction curves of 16 samples were also drawn. Results showed that the rarefaction curves of MP1–MP4 and MP1\_a–MP4\_a did not reach plateau (Fig. S1), revealing clearly that rare species continued to emerge after 7000 sequences with MiSeq sequencing. Furthermore, rarefaction curves of IP1–IP3 and R1–R5 showed that the diversity of microbial communities in those samples can be covered by the sequence libraries, while samples from municipal WWTSs had higher diversity of microbial communities than industrial and anammox WWTSs.

All effective sequences for each sample were further assigned to the corresponding taxonomy levels (from phylum to genus). As shown in Fig. 1, *Proteobacteria* was the most abundant phylum in 16 samples, accounting for 7.4–46.4% of total effective sequences. The other dominant phyla included *Bacteroidetes* (1.4–54.7%), *Chloroflexi* (2.6–49.4%), and *Planctomycetes* (0–44.4%), which was similar to the result of bacterial communities in municipal WWTSs [9], suggesting *Proteobacteria* and *Bacteroidetes* were the most dominant phyla. Additionally, previous studies [12,13] have reported that *Proteobacteria*, *Chloroflexi* and *Planctomycetes* were the significant phyla in nitrification–anammox system. As shown in Fig. 1, it clearly showed that *Planctomycetes* (i.e. *Candidatus\_Brocadia*) and *Chloroflexi* (i.e. *Anaerolinea*) were more abundant in anammox WWTSs (R1–R5) than that of samples from IP1–IP3 and MP1–MP4. Therefore, it can be concluded that *Planctomycetes* and *Chloroflexi* has made major contribution to anammox processes. Within *Proteobacteria*,  $\alpha$ -*Proteobacteria* (0.0–4.2%),  $\beta$ -*Proteobacteria* (0.1–27.5%),  $\gamma$ -*Proteobacteria* (1.0–10.8%),  $\delta$ -*Proteobacteria* (0.0–26.5%) and  $\epsilon$ -*Proteobacteria* (0–1.4%) appeared in more than 10 samples (Fig. S3a). Additionally, it also found that  $\alpha$ -*Proteobacteria*,  $\beta$ -*Proteobacteria*,  $\gamma$ -*Proteobacteria*, and  $\delta$ -*Proteobacteria* had been shared by 5 anammox WWTSs. Hence, it could be concluded that these four *Proteobacteria* classes may play a major role in N and Fe-cycle for the removal of nitrogen. Results from the order and family level showed that *Brocadia*, *Anaerolineaceae*, *Rhodocyclaceae*, et al. were more abundant and shared by 5 anammox WWTSs (R1–R5) (Fig. S3b–c), suggesting that these orders and families have made great contributions to nitrogen removal.

Among the 386 assigned genera, 154 were most abundant, accounting for 28.4–69.9% of classified sequences (Fig. S3d), of which only 27 were commonly shared by more than 2 sludge samples from IP1–IP3, and 96 were shared by more than 4 sludge samples from MP1–MP4 and MP1\_a–MP4\_a. Additionally, 19 out of 154 genera appeared in R1–R5. These results implicated that these shared genera in different WWTSs were considered to be core genera and played key roles in wastewater treatment.

Given the major roles of some groups of functional bacteria in maintaining the stability of the different WWTSs, there are reasonable to believe that these functional groups should be cosmopolitan different generalists widely distributed in WWTSs. Here, the top 61 dominant genera in 16 samples were selected and analyzed their function using heatmap (Fig. 2). Results showed that 40.4% of sludge generalists were identified as 16 functional groups, including 3 anammox bacteria, 3 AOB, 6 denitrifier, 3 FeOB, 7 FeRB, and



**Fig. 1.** Distribution of phyla in the 16 sludge samples based on the taxonomy annotation via SILVA SSU database using RDP classifier. The thickness of each ribbon represents the abundance of each taxon. The absolute tick above the inner segment and relative tick above the outer segment stand for the reads abundances and relative abundance of each taxon, respectively. Others refer to those unclassified reads. The data were visualized using Circos (Version 0.67, <http://circos.ca/>).

11 other functional groups, indicating the popularization of a core set of functional groups in all 16 samples distributed in industrial, municipal and anammox WWTSs.

Based on UniFrac analysis, results showed that bacterial communities in 16 samples could be clustered into five groups: (1) Group I was sludge samples from IP1; (2) Group II contained two samples from IP2 and IP3; (3) Group III involved 8 samples from all municipal WWTSs; (4) Group IV was the samples from R4; (5) Group V contained 4 samples, including R1, R2, R3, and R5 (Fig. S4a). PCoA analysis found the maximum variation was 36.34% (PC1) and 23.82% (PC2) (Fig. S4b–c). These results indicated that the slight variances were present in industrial WWTSs due to their different influents, although there had some similarities

between Group I and Group II. In addition, it can be found that 8 samples from Group III tended to clustering together and showed highly similarity of bacterial communities. Furthermore, Group IV and Group V showed highly discrepancy due to their different anammox bacteria.

### 3.2. Phylogenetic-based anammox bacteria, FeOB and FeRB diversity analysis

To explore the community composition of anammox bacteria, the 16S rRNA gene sequences were retrieved from MiSeq PE libraries. The anammox bacteria NJ tree revealed that OTU 539 (8.8%), OTU 929 (2.3%), and OTU 919 (0.06%) were affiliated to

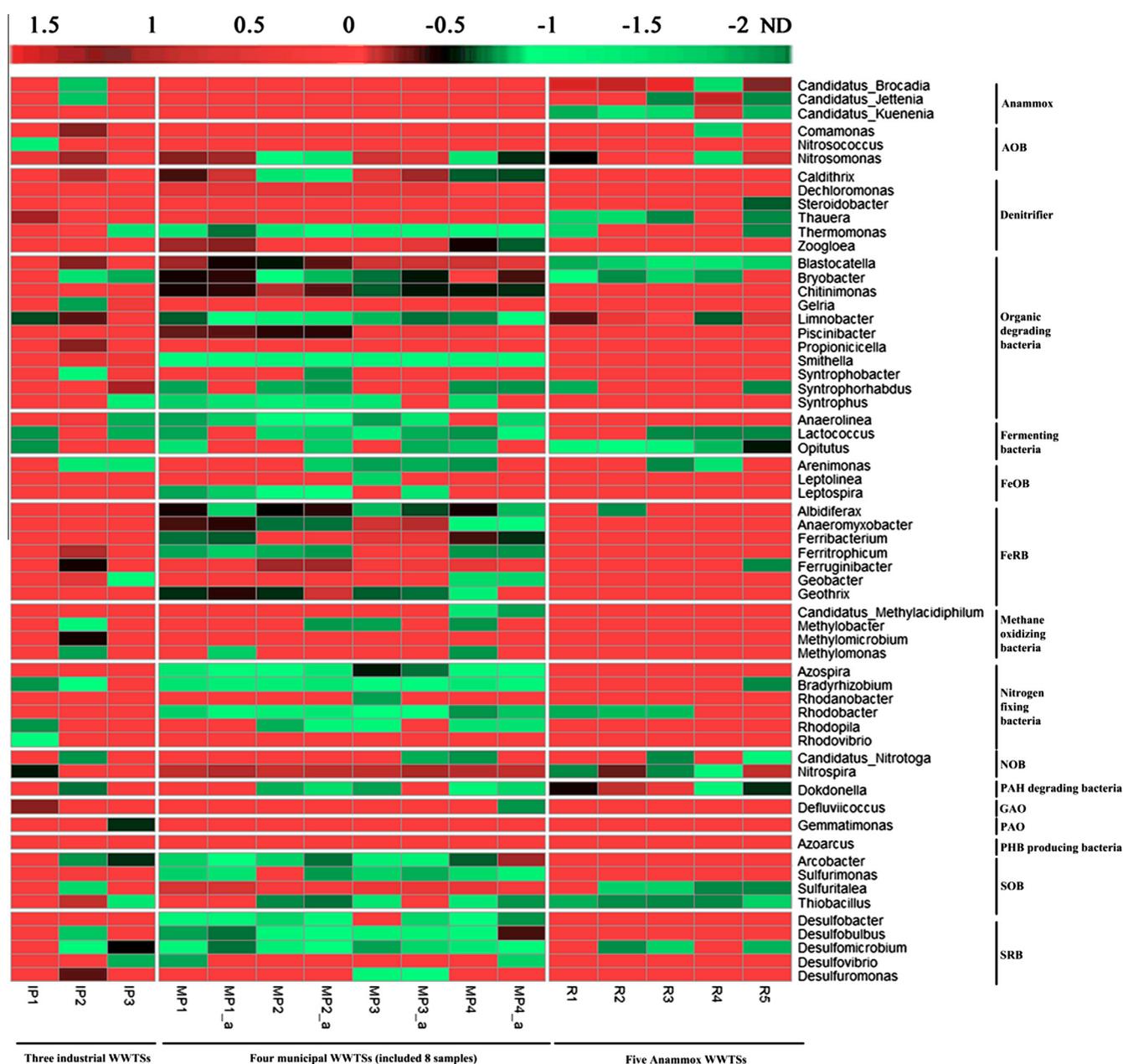


Fig. 2. The relative abundance (log scale) of total 61 functional genera in the 16 sludge samples.

the genera of *Candidatus Brocadia*, *Candidatus Kuenenia*, and *Candidatus Jettenia*, respectively (Fig. S5a). The percentage of *Candidatus Brocadia* in R1–R3, and R5 were in the range of 17.25%–40.9%, which were higher than sample from R4 and IP2. However, compared with *Brocadia*, *Kuenenia*, and *Jettenia* were the most dominant species in R4. One possibility is that the seeding sludge in these four anammox WWTs, including R1, R2, R3, and R5 were incubated with active sludge from municipal WWTs. For anammox WWTs R4, the seeding sludge in this reactor was inoculated with anaerobic granule sludge from industrial WWTs. Another possibility is that the sequences of *Candidatus Brocadia* in municipal WWTs were more than that of industrial WWTs. Moreover the sequences of *Candidatus Kuenenia* and *Candidatus Jettenia* in industrial WWTs were more than that of municipal WWTs. Therefore, it can be conclude that *Brocadia* might be the dominant anammox species in municipal WWTs, while *Kuenenia* showed higher diversity in industrial WWTs, which was consistent with previous studies [14,15].

The NJ phylogenetic tree of FeOB based on OTU taxa showed that FeOB OTUs fell into two phyla: *Proteobacteria* and *Spirochaetae* (Fig. S5b). It was found that OTU 60 took only 0.04% of all *Proteobacteria* sequences, which as mainly distributed in IP2, IP3, MP3, R3, and R4. The result indicated that sample from IP3 had more abundant than other industrial, municipal and anammox WWTs. Furthermore, OTU 820 and OTU 1200 accounted for 1.1% and 0.44% of *Spirochaetae* sequences (Fig. S5b). These results revealed that FeOB had more abundant in anoxic niche, which was accordant with previous studies [7]. In this study, 7, 4 of 11 *Proteobacteria* OTUs were affiliated to *Geobacter* and *Aeromonas* cluster, respectively, as well as 4 OTUs and 3 OTUs belonged to *Bacteroides* and *Geothrix* cluster, respectively. Results of OTU taxa showed that OTU 11, OTU837, OTU1099, and OTU 1188 had more abundant in IP2 than other samples from municipal and anammox WWTs. This finding was consistent with previous studies [16], indicating that high chemical oxygen demand (COD) in IP2 could enhance the abundance of iron reducing populations.

### 3.3. Quantification of 16S rRNA and functional genes

qPCR was further applied to validate the absolute abundance of 16S rRNA and functional genes. As shown in Fig. 3(a), the gene copies of anammox in R1–R5, were nearly one to five order of magnitude higher than those of samples in IP1–IP3, MP1–MP4 and MP1\_a–MP4\_a. Among the three industrial WWTSs, the AOB *amoA* gene abundance was varied between  $4.44 \times 10^6$  and  $5.79 \times 10^7$  copies  $g^{-1}$  wet sludge, and it were nearly 1–3 order of magnitude higher than AOA *amoA* and *nrxA* genes. For samples from municipal WWTSs, AOA *amoA* gene abundance in MP1, MP1\_a, MP3, MP3\_a, and MP4\_a were ranged from  $3.88 \times 10^2$  to  $1.03 \times 10^6$  copies  $g^{-1}$  wet sludge, and it were nearly 1–2 order of magnitude higher than that of samples in MP2, MP2\_a, and MP4. In MP1, MP2, MP3 and MP4\_a, the *nrxA* gene copies were in the same order of magnitude, and were nearly 1–2 orders of magnitude higher than MP1\_a, MP2\_a, MP3\_a, and MP4. For five samples from anammox reactors, AOB *amoA* gene copies were greater than AOA *amoA* and *nrxA* genes copies (Fig. 3b).

As shown in Fig. 3(c), *napA* involved in dissimilatory N reduction was more abundant than *narG* and *nrfA* in most samples except IP2 and IP3. Additionally, compared with other 15 samples, the absolute abundance of *nrfA* genes in IP2 had a maximum gene copies. Notably, *nrfA* and anammox gene were more abundant in IP2, indicating that these two genes might have a mutual contribution to nitrogen removal in IP2. *nirS* and *nosZ* involved in denitrification were more abundant in industrial and municipal WWTSs (Fig. 3d). Moreover, the gene copies of *nirK* in five anammox WWTSs were nearly 1–2 orders of magnitude higher than *nosZ* gene, while those two genes copies were nearly 2–3 orders of magnitude lower than *nirS* gene.

As shown in Fig. 3(e), the gene copies of *Acidimicrobium* and *Ferroplasma* 16S rRNA gene involved in FeOB group varied widely, and *Ferroplasma* 16S rRNA gene were more abundant. For FeRB groups, including *Albidiferax* 16S rRNA, *Geobacter* 16S rRNA, and *Acidiphilium* 16S rRNA genes, varied markedly in all 16 samples. In general, *Albidiferax* 16S rRNA were not detected in MP1, MP1\_a, MP4, MP4\_a, and R4, the absolute abundance of *Geobacter* 16S rRNA were nearly 1–6 and 2–4 orders of magnitude higher than *Albidiferax* 16S rRNA and *Acidiphilium* 16S rRNA genes, respectively (Fig. 3f).

Based on the qPCR results, the AOB/AOA ratios in three industrial WWTSs samples, eight municipal WWTSs samples, and five anammox reactors ranged from 0.8823–4235.2573, 0.0003–12.3830, and 4.1661–25.3650, respectively. Moreover, One-way ANOVA results indicated that the AOB *amoA* gene copies had significantly influence on AOA *amoA* gene copies ( $p < 0.05$ ) in the five anammox reactors. The results were accordant with previous studies [17,18]. However, it was lower than other studies [19]. It was found that AOB *amoA* genes outnumbered AOA *amoA* genes in most of samples in this study. Interestingly, the (AOB + AOA)/anammox ratios in industrial WWTSs, anammox reactors ranged from 0.011–0.2255, and 0.0038–0.0348. It was indicated that AOA and AOB coexisted with anammox bacteria may play a pivotal role in nitrogen removal from high ammonia concentration wastewater. The anammox/*nrfA* ratios in five anammox reactors varied from  $5.45 \times 10^3$ – $3.43 \times 10^6$ . In addition, anammox gene had significantly effects on *nrfA* gene in five anammox WWTSs ( $p < 0.05$ ) and the conversion of DNRA was mainly regulated by *nrfA* gene. Thus, DNRA may play a significant role in anammox reactor for nitrogen removal. Additionally, the FeOB/FeRB ratios in industrial WWTSs, municipal WWTSs, and anammox reactors ranged from 0.2098–11.8093, 0.5815–4.6851, and 0.0014–6.2750, respectively. Based on the above analyses, the nitrogen related gene ratios varied markedly, suggesting that not only linkages between environmental factors and abundance of anammox, nitrification, dissimilatory N reduction, denitrification, FeOB and FeRB genes

should be further investigated, but relationships between nitrogen and iron cycling functional genes remains unclear and requires further research.

### 3.4. Ecological associations between the bacterial 16s rRNA and functional genes

As displayed in Table 1, the pairs of (AOA + AOB)–*nrxA*, (AOA + AOB)–*nrfA*, (AOA + AOB)–*nosZ*, *nrxA*–*nrfA*, *nrxA*–*nosZ*, *nrfA*–*nosZ*, (*narG* + *napA*)–(*nirK* + *nirS*), (*narG* + *napA*)–FeOB, (*nirK* + *nirS*)–FeOB, *nrxA*–FeRB, *nrfA*–FeRB, *nosZ*–FeRB, and FeOB/FeRB showed significant positive correlation. Their Pearson correlation coefficients ( $r$ ) exceeded 0.504 ( $p < 0.05$ ).

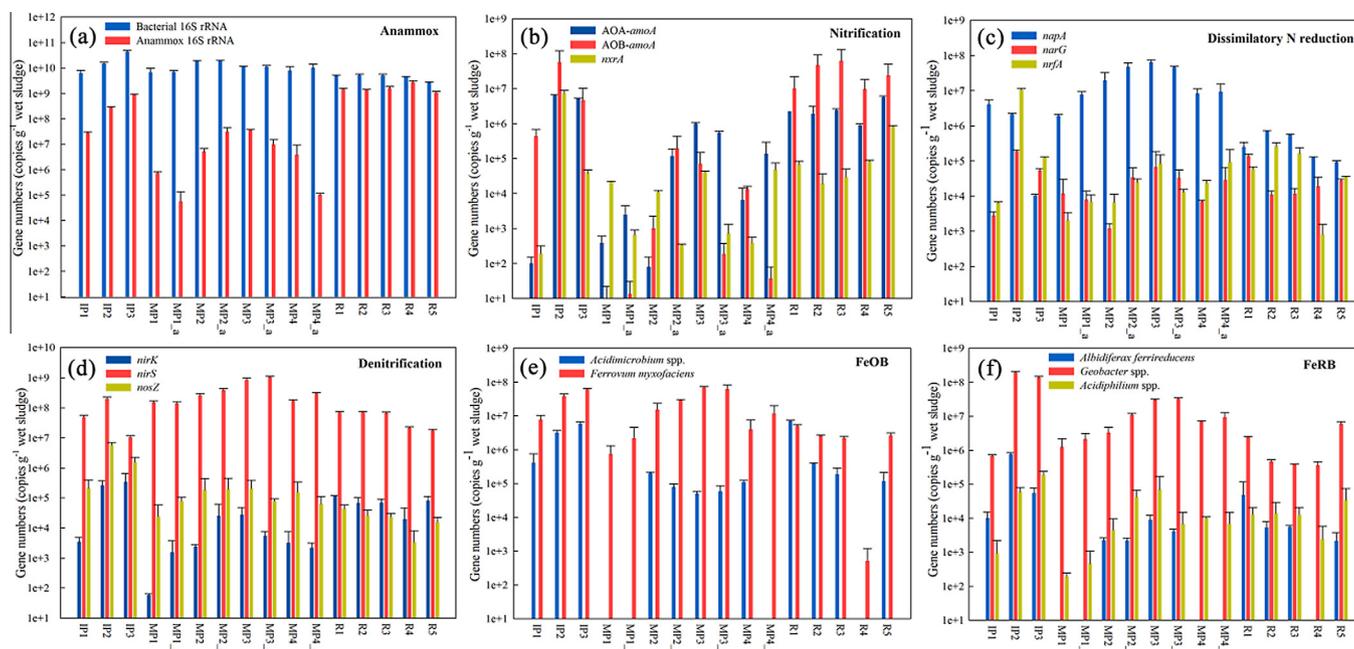
In the ammonia oxidation step, the conversion of  $NH_4^+$ -N to hydroxylamine or  $NO_2^-$ -N, is catalyzed by AOB and AOA–*amoA* genes [3]. The  $NH_4^+$ -N was consumed by AOA and AOB and they provided substrate  $NO_2^-$ -N for NOB and other microorganisms. In the second step, conversion of  $NO_2^-$ -N to  $NO_3^-$ -N is catalyzed by *nrxA* gene. It consumed  $NO_2^-$ -N and provided  $NO_3^-$ -N for other nitrogen removal pathway. Thus, the results indicated that the pair of (AOA + AOB)–*nrxA* showed a significantly protocoeoperation (Table 1).

The *nrfA* gene is the key gene for the conversion of  $NO_2^-$ -N to  $NH_4^+$ -N [4]. It is clearly indicated that AOA, AOB, and *nrxA* provided the substrate  $NO_2^-$ -N for *nrfA* gene under anoxic condition. The *nosZ* gene, encoding the  $N_2O$  reductase, is the key gene of conversion from  $N_2O$  to NO or  $N_2$ , which is often used as a marker for the final denitrification step.

Thus, three nitrification functional genes could provide the substrate  $NO_2^-$ -N and  $NO_3^-$ -N for *nosZ* gene. It was demonstrated that (AOA + AOB)–*nrfA*, (AOA + AOB)–*nosZ*, *nrxA*–*nrfA*, *nrxA*–*nosZ* showed mutually beneficial relationship (Table 1). It reported that the accumulation of  $NO_2^-$ -N in WWTSs have badly impacts on microorganisms [2], while the *nrfA* and *nosZ* genes could consume the  $NO_2^-$ -N. Obviously, co-existence of these two genes were beneficial to eliminate the toxic effects because of the  $NO_2^-$ -N accumulation. Furthermore, DNRA and denitrifying bacteria are chemolithotrophic and both of them have capacity to utilized organic carbon as the source of electron donor. Thus, COD can be removed together in the WWTSs by those two heterotrophic bacteria. In this respect, *nrfA* and *nosZ* showed a collaboration relationship (Table 1). For *narG* and *napA* genes, which catalyze the conversions of  $NO_3^-$ -N to  $NO_2^-$ -N. The *nirK* and *nirS* genes were the key genes for converting  $NO_2^-$ -N to NO [5]. Apparently, *narG* and *napA* genes consumed  $NO_3^-$ -N and provided substrate  $NO_2^-$ -N for *nirK* and *nirS* genes. Thus, (*narG* + *napA*)–(*nirK* + *nirS*) showed a symbiosis relationship.

The *Acidimicrobium* spp. and *F. myxofaciens* [6] are the two mainly groups involved in microbial iron oxidation process. The electrons from the conversion of  $NO_3^-$ -N to  $NO_2^-$ -N could be utilized by microbial iron oxidation. Likewise, the electrons and substrates NO from the conversion of  $NO_2^-$ -N to NO could be provided for microbial ferrous oxidation. Therefore, (*narG* + *napA*)–FeOB and (*nirK* + *nirS*)–FeOB showed beneficial cooperation, indicating nitrate, nitrite and NO reduction couple dissimilatory ferrous oxidation (NAFO) may make a significant contribution to nitrogen removal in nitrogen and iron cycling [20,21].

The three groups *A. ferroreducens*, *Geobacter* spp., and *Acidiphilium* spp. [6], are mainly bacteria associated with microbial iron reduction process. Given the DO was consumed by *nrxA* gene, it could result in a suitable oxygen level for FeRB bacteria. Previous studies [22] reported that anammox can be coupled to ferric iron (Fe(III)) reduction (Feammox) to produce  $NO_2^-$  or  $N_2$ . Obviously, *nrfA* gene consumed  $NO_2^-$  and provided  $NH_4^+$  for growth of FeRB. In addition, *nrfA* gene, *nosZ* gene and FeRB have the capacity to oxidize and utilize the organic matter. Therefore, these four pairs of



**Fig. 3.** Quantitative analysis of nitrogen and iron-cycling-related genes in the 16 sludge samples. Error bars represent standard deviation calculated from three independent experiments.

**Table 1**

Pearson correlation coefficients between 16S rRNAs and functional genes (Pearson correlation coefficients values with  $p < 0.01$  and  $p < 0.05$  are shown in blue,  $n = 16$ ).

	Anammox	AOA + AOB	<i>nxrA</i>	<i>narG + napA</i>	<i>nrfA</i>	<i>nirK + nirS</i>	<i>nosZ</i>	FeOB	FeRB
Anammox	1.0000								
AOA + AOB	0.4531	1.0000							
<i>nxrA</i>	-0.0749	0.572*	1.0000						
<i>narG + napA</i>	-0.4646	-0.4010	-0.1663	1.0000					
<i>nrfA</i>	-0.0921	0.568*	0.993**	-0.1479	1.0000				
<i>nirK + nirS</i>	-0.4712	-0.3218	-0.0607	0.892**	-0.0397	1.0000			
<i>nosZ</i>	-0.1078	0.504*	0.956**	-0.1540	0.967**	-0.0674	1.0000		
FeOB	-0.2966	-0.1232	0.1950	0.643**	0.2182	0.672**	0.3561	1.0000	
FeRB	-0.0994	0.3750	0.785**	-0.0667	0.798**	0.0221	0.918**	0.606*	1.0000

\* Correlation is significant at the 0.05 level (2-tailed).

\*\* Correlation is significant at the 0.01 level (2-tailed).

*nxrA*–FeRB, *nrfA*–FeRB, *nosZ*–FeRB, and FeOB–FeRB could not only benefit the nitrogen removal, but also be beneficial to take full advantage of residual ferrous and ferric salt yielded in the WWTSs.

### 3.5. Linkages between operational parameters and microbial communities

To obtain the variability related to the operational parameters, SRCC and RDA analysis were further applied to investigate the linkages between operational parameters and bacterial communities. Results of SRCC (Table S4) showed that anammox gene abundance had positive correlation with effluent pH, influent  $\text{NH}_4^+\text{-N}$ , effluent  $\text{NH}_4^+\text{-N}$ , influent  $\text{NO}_2^-\text{-N}$ , temperature, and  $\text{NO}_2^-\text{-N}$  removal rate, while had negative correlation with COD removal rate, indicating that anammox bacteria was chemoautotrophic and pH was the key factor in the anammox process as well as temperature [23,24]. AOA *amoA* and AOB *amoA* genes abundance were positively correlated with effluent pH, influent  $\text{NO}_2^-\text{-N}$ , temperature,  $\text{NO}_2^-\text{-N}$  removal rate, influent and effluent  $\text{NH}_4^+\text{-N}$ , while were negatively correlated with  $\text{NH}_4^+\text{-N}$  removal rate (Table S4), showing that high ammonia concentration was in favor of AOA and AOB [18]. *nxrA* gene abundance was positively correlated with effluent

pH, temperature, and  $\text{NO}_2^-\text{-N}$  removal rate, while was negatively correlated with COD removal rate and  $\text{NH}_4^+\text{-N}$  removal rate (Table S4), showing that pH and temperature played key roles in the conversion of nitrite oxidation. In addition, *napA* and *narG* genes abundance had negative correlation with influent  $\text{NO}_2^-\text{-N}$ , temperature,  $\text{NO}_2^-\text{-N}$  removal rate, influent and effluent  $\text{NH}_4^+\text{-N}$ , while had positive correlation with COD removal rate. This result showed that high concentration of ammonia and nitrite could result in accumulation of free ammonia (FA) and free nitrous acid (FNA), which had adverse effects on denitrification step. *nirK* and *nirS* genes were also negatively correlated to influent  $\text{NO}_2^-\text{-N}$ , temperature,  $\text{NO}_2^-\text{-N}$  removal rate, influent and effluent  $\text{NH}_4^+\text{-N}$  as well as MLSS (Table S4), indicating more FA and FNA accumulated, more denitrification step inhibited. In this study, *nosZ* gene showed positive correlation with influent COD and COD removal, and had negative correlation with influent and effluent pH,  $\text{NO}_2^-\text{-N}$ , showing that organic matter was favorable for the growth of denitrifying bacteria. Moreover, it also indicated that the denitrifying bacteria had higher tolerance to the pH and FNA stresses. The group of FeOB and FeRB had positive correlation with influent COD and COD removal, while had negative correlation with effluent  $\text{NO}_2^-\text{-N}$ , showing that organic matters can be oxidized in the conversion of iron-oxidizing and iron-

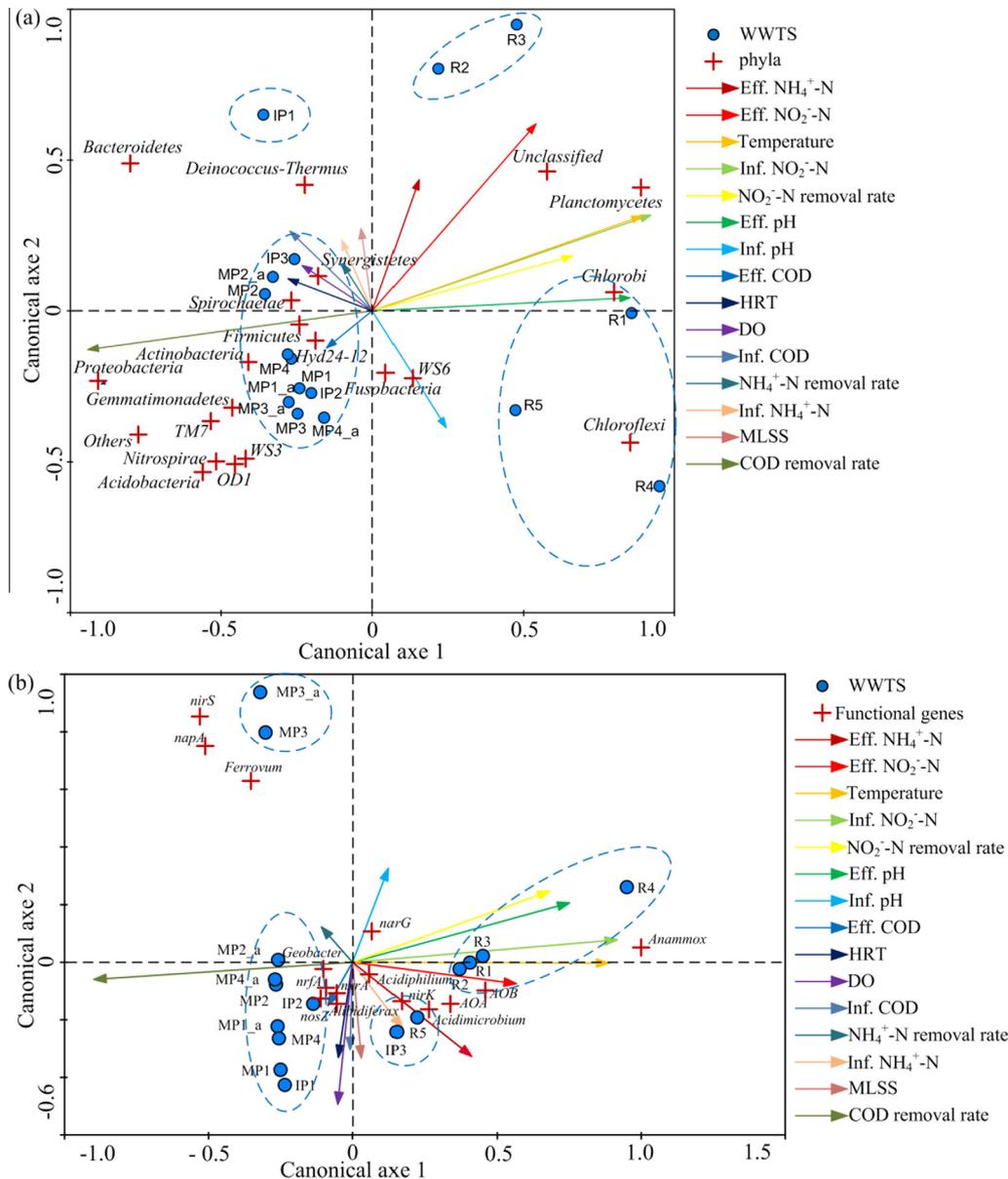


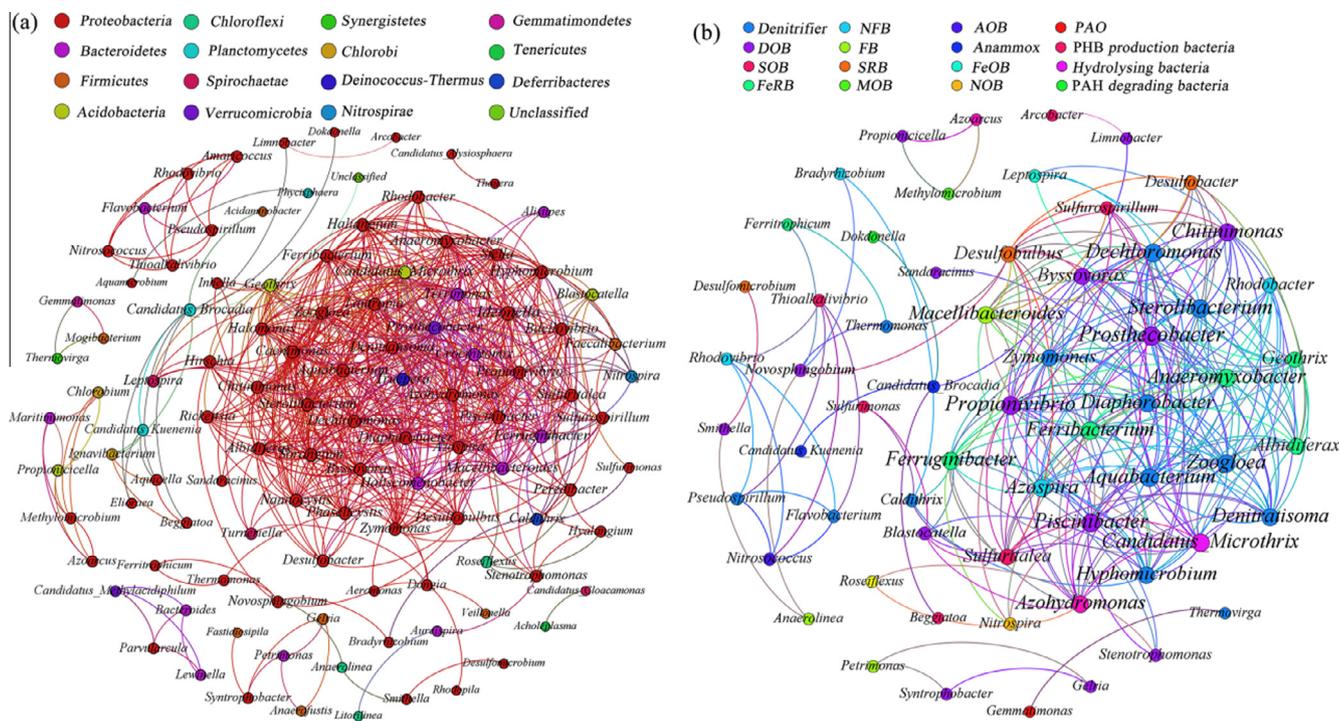
Fig. 4. Redundancy analysis (RDA) diagram illustrating (a) the relationship between different phyla with environmental factors in all samples; (b) the relationship between different functional genes with environmental factors in all samples.

reducing, which was consistent with previous studies [25]. Additionally, the activity of FeRB could be severely affected by  $\text{NO}_2^-\text{-N}$  due to the  $\text{NO}_2^-\text{-N}$  inhibited electrons transport to Fe(II) [26].

Concerning the variation of bacteria at phylum level, the principal component 1 (PC1) explained 70.9% of the variation of species-environment relation, the PC1 and PC2 together explained 86.8% (Fig. 4a). It can be found that Group II and Group III had higher bacterial diversity than Group I, Group IV and Group V. These results were accordant with taxonomic distribution of the bacterial communities (Fig. 1). RDA analysis revealed that the phyla of *Planctomycetes* was clearly related to effluent  $\text{NH}_4^+\text{-N}$ , temperature,  $\text{NO}_2^-\text{-N}$  removal rate, influent  $\text{NO}_2^-\text{-N}$  and effluent  $\text{NO}_2^-\text{-N}$ . Phylum of *Chloroflexi* was related to influent and effluent pH. Phylum of *Proteobacteria* was strongly related to effluent COD and COD removal rate. Phylum of *Bacteroidetes* was related to HRT, DO, influent COD, influent  $\text{NH}_4^+\text{-N}$  and  $\text{NH}_4^+\text{-N}$  removal rate. Based on these results, the phylum of *Planctomycetes* made a great contribution to ammo-

nia and nitrite removal in the anammox process. Furthermore, *Proteobacteria* and *Bacteroidetes* were the primary phyla in the removal of organic matters and ammonia.

The results of RDA analysis for all bacterial 16S rRNA and functional genes were shown in Fig. 4(b). It demonstrated that the PC1 represented 90.9% of variance, the PC1 and PC2 together explained 99.4% of variance. In addition to SRCC results, RDA analysis showed that anammox had strongly related with effluent pH, influent  $\text{NO}_2^-\text{-N}$ , and  $\text{NO}_2^-\text{-N}$  removal rate. AOA, AOB, *nirK*, *Acidiphilium*, and *Acidimicrobium* genes were clearly related to influent  $\text{NH}_4^+\text{-N}$ , effluent  $\text{NO}_2^-\text{-N}$  and  $\text{NH}_4^+\text{-N}$ . *nrxA*, *nosZ* and *Alibifera* genes were related to HRT, DO and influent COD. *Geobacter* and *nrfA* genes had positive correlation with effluent COD and COD removal rate. *Ferrovum*, *napA* and *nirS* showed positive correlation with COD and  $\text{NH}_4^+\text{-N}$  removal rate. These results were accordant with the SRCC results, which further revealed that *F. myxofaciens*, *A. ferrireducens*, and *Geobacter* spp. were accountable for COD removal



**Fig. 5.** Networks analysis of co-occurring bacterial and functional generalists in 16 sludge samples. A connection stands for a strong (Spearman's  $p > 0.8$ ) and significant ( $p$ -value  $< 0.01$ ) correlation. (a) Co-occurring network colored by phylum. (b) Co-occurring network colored by phylum functional group. For each panel, the size of each node is proportional to the number of connections.

in the anammox reactor under low C/N ratio wastewater. Moreover, results found that AOA was related to influent and effluent COD (Fig. 4b), indicating that AOA might have had mixotrophic nature.

Interestingly, AOA *amoA* and AOB *amoA* genes had significant positive correlation with anammox 16S rRNA genes (Fig. 4b). The result was consistent with other studies [3], which reported that AOA and AOB couple anammox were responsible for nitrogen loss in the marine oxygen minimum zones (OMZs). In addition, it can be found that *nrfA* and anammox genes had also positive correlations with *nosZ* and *nirK* genes. The result demonstrated that co-existence anammox, DNRA and denitrification were responsible for nitrogen removal in WWTSs. FeOB 16S rRNA had clearly related to denitrification genes, indicating that NAFO could be used as a valuable biological process for nitrogen removal [27]. *Acidiphilium* spp. had strongly positively correlation with anammox, indicating Feammox could fuel nitrogen loss in ecosystems, which was consistent with other studies [22]. Furthermore, results revealed that *Geobacter* spp. was strongly related to *nrfA* gene, and had also positive correlation with COD removal (Fig. 4b). This result showed that organic matter not only can be used as an electron donor by DNRA bacteria to remove nitrate [28], but also were oxidized by *Geobacter* spp. in conversion of Fe (III) reduction [25]. Although the linkages between operational parameters and microbial communities were investigated, further work is required to insight into the relationships between Fe-metabolizing, DNRA bacteria and anammox bacteria.

### 3.6. Network analysis of microbial co-occurrence patterns

The co-occurrence patterns among genera were investigated using network inference based on Spearman's coefficient  $> 0.8$  and  $p$  value  $< 0.01$  [29]. As shown in Fig. 5(a), the positive network of genus has 155 nodes and 713 edges. Based on the phylum level, the entire network was parsed into 16 phyla, with 16 of 155 total

vertices occupied by the six major phyla. These nodes, which were densely connected in each phyla, were defined as the "hub" following previous study [30]. The results from the Fig. 5(a) showed that the phyla of *Lautropia*, *Terrimonas*, *Faecalibacterium*, *Candidatus\_Microthrix*, *Roseiflexus*, and *Candidatus\_Brocadia* were the hub of *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria*, *Chloroflexi*, and *Planctomycetes*, respectively. One possible explanation for these hubs and related co-occurring genera in each phyla is that they shared the same habitat preferences in different environments and might be included in certain microbial taxa. Another reason is that these hubs could be used as representatives of genera and acted as the indicators to explore their abundance and co-occurring genera in different WTSSs.

Furthermore, all genera were selected and then were explored in co-occurrence analysis according to functional group. As shown in Fig. 5(b), network of functional group has 91 nodes and 277 edges. Results of co-occurrence patterns revealed that 16 functional group were speculated. And, anammox bacteria, FeOB, and FeRB accounted for 7.69%, 3.3%, and 3.3% of functional group, respectively. Additionally, *Candidatus\_Brocadia* was the hub of anammox group. The denitrifier group was consisted of *Aquamicrobium*, *Dechloromonas*, and *Sterolibacterium*. The FeRB group involved the genera of *Ferribacterium* and *Anaeromyxobacter*. The phyla of *Leptospira* was found to be the host of FeOB group. For the entire positive network of functional group, anammox bacteria (i.e. *Candidatus\_Brocadia*) were positively correlated with organic degrading bacteria (i.e. *Limnobacter*) (Fig. 5b). The result indicated that anammox bacteria could be oxidized some organic, which were accordant with other research [31]. The phyla of *Leptospira* in the FeOB group had positive correlation with denitrifier (i.e. *Dechloromonas* and *Steroidobacter*), indicating that ferrous oxidizing process could couple denitrification for nitrogen removal, which was consisted with previously study [27]. The FeRB group (i.e. *Geothrix* and *Albidiferax*) was found to have synergetic relationships with denitrifier and organic degrading bacteria. The result

indicated that the co-existence of iron reducing bacteria coupled denitrification and organic degrading could be beneficial for the simultaneous removal of nitrogen and organic matter in the N and Fe cycling.

Overall, as mentioned above, these findings had highly consistency with previous studies and indicated that the network analysis provide further insights into the discrepancy for the different microbial taxa and functional group, as well as their co-occurrence patterns in complex WWTSs.

#### 4. Conclusion

Results of MiSeq sequencing showed that *Bacteroidetes*, *Chloroflexi*, *Proteobacteria*, and *Planctomycetes* were the dominant phyla in 16 samples. qPCR results found that the absolute abundance of anammox, *nrfA*, FeOB and FeRB genes were higher than other functional genes in anammox bioreactors when *Candidatus Brocadia* was the dominant anammox bacteria. Moreover, SRCC and RDA analyses revealed that four functional gene pairs, including (AOA + AOB)–anammox, (*narG* + *napA*)–FeOB, (*nirK* + *nirS*)–FeOB, and *nrfA*–FeRB had positive correlations with each other. Finally, network analysis implicated that the coexistence of anammox, DNRA and FeRB could be useful for simultaneous nitrogen and organic carbon removal in mainstream wastewater treatment processes.

#### Acknowledgement

This study was financially supported by the Science and Technology Innovative Program of Shaanxi Province (2011KTZB03-03-01). The authors are grateful to Hong Yue for her assistance in equipment supply and network analysis.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.cej.2016.01.024>.

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