Desalination and Water Treatment
www.deswater.com
doi: 10.5004/dwt.2020.26415

207 (2020) 205–212
December

The microbial mechanism of enhanced biological denitrification for advanced treatment of textile and dyeing wastewater based on iron

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Received 11 February 2020; Accepted 29 July 2020

ABSTRACT

After the advanced treatment of textile and dyeing wastewater, the concentration of total nitrogen (mainly nitrate) was still high while it lacked a biodegradable organic carbon source for further biological denitrification. Biological denitrification reactor with iron-based materials (both Fe and Fe/Cu) resulted in higher nitrogen removal efficiency (30% and 20%) compared to control (12%). However, the microbial mechanism was unknown. 16S rRNA genes sequencing and metagenomic sequencing were used to analyze the microbial community structure and functional genes in the process of iron-based materials enhanced biological denitrification of textile and dyeing wastewater. Microbial communities obtained from the two sequencing methods were quite different. The results of 16s rRNA genes sequencing indicated that both Fe and Fe/Cu influenced the microbial communities. Fe/Cu had a more profound effect on the microbial community and the diversity of the microbial community in the Fe/Cu reactor was relatively low. Metagenomic analysis showed that *Proteobacteria* was dominant in all samples (47%–80%). The biofilm formed on the surface of Fe and Fe/Cu enriched more functional microorganisms, which promoted denitrification of nitrate. The abundance of genes relating to nitrogen metabolism was higher in the samples on the surface of Fe and Fe/Cu, and therefore they had a stronger ability of nitrogen metabolism.

Keywords: Metagenomic analysis; Textile and dyeing wastewater; Biological denitrification; Iron-based materials

1. Introduction

Textile and dyeing is a water-intensive and highly polluting industry that accounts for about 80% of total wastewater emissions from the textile industry [1]. The content of organic pollutants in textile and dyeing wastewater is high, and its main components, dyes, and dyeing auxiliaries have potential toxicity and poor biodegradability, which may pose a threat to aquatic organisms [2]. Printing and dyeing textile wastewater and dye chemical wastewater contain a large number of refractory organics and toxic and harmful organics, such as polyvinyl alcohol, azo, nitrobenzene, and aniline compounds in dyes and raw materials [3]. Iron-based catalyst is a new type of advanced oxidation method, which can effectively produce hydroxyl ions [4]. For an advanced treatment process, the method of catalytic ozone does not need to be adjusted to a strong acid environment. In China, the wastewater standards that the wastewater can be directly discharged are as follows: chemical oxygen demand (COD) below 80 mg/L and total nitrogen (TN) below 15 mg/L. It was shown that the new technology of iron-based catalytic ozonation can effectively remove the organics in the biochemical effluent of textile and dyeing wastewater and reduce the COD concentration to below 80 mg/L, which met the corresponding discharge requirements [5].

However, after the advanced treatment, the TN (mainly nitrate, nitrate/TN > 95%) in the wastewater exceeds the standard and there is no easily degradable organic carbon

source for biological denitrification. Our previous study showed that the TN removal rate of dyeing wastewater was less than 10% after catalytic ozonation and biological denitrification. Zero-valent iron can be used as an electron donor for autotrophic denitrification, and the TN removal rates by Fe and Fe–Cu in continuous experiments were 30% and 20%, respectively, which were much higher than that of the control experiment (without iron-based materials) (12%) [5,6]. Both Fe and Fe–Cu contain zero-valent iron. The above results demonstrated that zero-valent iron can enhance biological denitrification. It was also found that the COD removal rate of the reactor was not affected, which showed that zero-valent iron did not affect the physiological activities of microorganisms, and a large number of iron-containing flocs in sludge even contribute to the adsorption and removal of organic matters.

Although the above results showed that zero-valent iron enhanced denitrification of the textile and dyeing wastewater after advanced treatment, the biological mechanism was still unknown. Therefore, high-throughput sequencing of 16S rRNA genes and metagenomic sequencing were used to reveal the changes of microbial communities and functional genes induced by the addition of Fe and Fe–Cu, and to elucidate the microbiological mechanism of zero-valent iron promoting biological denitrification.

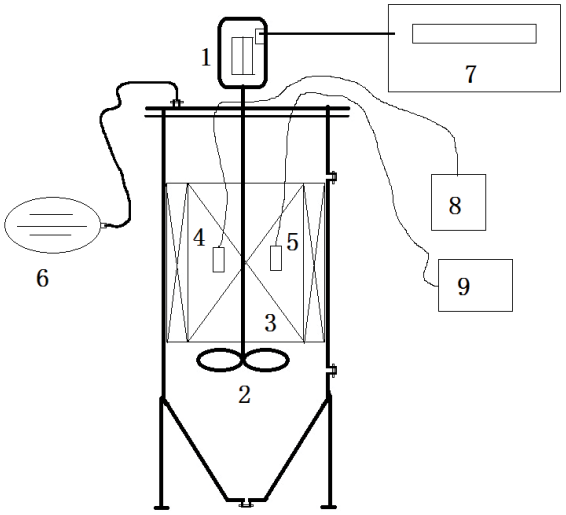

Fig. 1. SBR (sequencing batch reactor) reactor (C SBR, Fe SBR, and Fe–Cu SBR reactor). (1) Agitate motor, (2) stirrer blade, (3) ring packed bed, (4) pH/ORP probe, (5) DO (dissolved oxygen) probe, (6) gas sampling device, (7) programmable time controller, (8) pH/ORP measuring apparatus, and (9) DO measuring apparatus.

2. Materials and methods

2.1. Reactor operation

The effluent quality of textile and dyeing wastewater after the catalytic ozonation process was as follows: COD 71 mg/L, TN 58.7 mg/L, nitrate 58.3 mg/L, and pH 7.8. Three sequencing batch reactors (SBR) were set up (Fig. 1). One reactor was filled with iron (Fe SBR), one reactor was filled with Fe–Cu (Fe–Cu SBR), and the last one was used as a control without iron-based materials (C SBR). The influent was the effluent after advanced oxidation (COD/TN: 1/1.5). The water in and out of each operation cycle of three SBR reactors is 0.8 L, and the effective reaction volume was 4.8 L. All reactors were run continuously for 120 d. Detailed information about the operation of the reactors was described in Ma et al. [5,6].

2.2. DNA extraction

The fast DNA extraction kit (MP Biomedicals, USA) was used for DNA extraction, and DNA was extracted according to the operating instructions of the kit. The DNA was tested by 1% agarose gel electrophoresis and NanoDrop ND-1000 for quality control and concentration determination. DNA samples were stored in a refrigerator at –80°C.

2.3. 16S rRNA high-throughput sequencing

For 16S rRNA analyses, duplicate samples were collected from each reactor at steady-state (i.e., period with stable TN and nitrate removal rate with a daily variation lower than 10% for at least 5 d) at the end of reactor operation. The duplicate samples obtained from the sludge mixed liquor of the blank reactors were named as C1 and C2. The samples in the reactors with the addition of Fe and Fe–Cu were taken from the sludge mixed liquor (Fe1, Fe2, Fe–Cu 1, Fe–Cu 2) and sludge in static sedimentation (Fe–S1, Fe–S2, Fe–Cu–S1, and Fe–Cu–S2). Based on the high-throughput sequencing method, the PCR products in the V4 region of 16S rRNA were determined, and the results of microbial communities in each sample were analyzed. The PCR primer used was 338F/806R (ACTCCTACGGGAGGCAGCAG GGACTACHVGGGTWTCTAAT). The PCR products of different samples were added barcode sequences before the forward primers. The PCR products of different samples were evenly mixed and stored. The sequencing was performed on Illumina MiSeq platform (Illumina, USA). Clean reads were obtained by removing the unqualified sequence, less than 50 bp sequence, and PCR chimera. In order to unify the sequencing depth, the samples with the lowest sequencing depth in the same experiment were homogenized. The microbial diversity and community analysis were based on the previous study [7], The sequences were phylogenetically assigned to taxonomic classifications by RDP Classifier with a confidence threshold of 50% (http://rdp.cme.msu.edu/classifier/classifier.jsp). MOTHUR program (http://www.mothur.org/wiki/Mothur_manual) was used to set the distance limit to 0.03 and cluster the sequences into operable taxonomic units (OTU). The MOTHUR program also generates Shannon diversity index comparing the number of overlapping OTUs between samples and dendrogram based on Bray–Curtis similarity matrix.

2.4. Metagenomic sequencing and analysis

For metagenomic analysis, each of the duplicate samples in the corresponding sets were pooled together to minimize the potential variation. Replicates obtained from each reactor were named as C, Fe, Fe–Cu, Fe–S, and Fe–Cu–S. Genomic DNA was extracted by 1% agarose gel

electrophoresis. The extracted genomic DNA is fragmentated, and the size is about 300 bp (Covaris M220). Libraries with insert size of 180 bp were constructed according to the manufacturer's instructions (Illumina) for the samples. Sequencing was conducted using Illumina Hiseq 2000 platform by applying 101 bp paired-end strategy. The predicted gene sequences of all samples were clustered with CD-HIT software (http://www.bioinformatics.org/cd-hit/) (parameters: 95% identity, 90% coverage). The longest gene of each cluster was taken as the representative sequence to construct non-redundant gene set. Using SOAP aligner software (http://soap.genomics.org.cn/), the high-quality reads of each sample were compared with non-redundant gene sets (95% identity), and the abundance information of genes in corresponding samples was counted. BLASTP (version 2.2.28+) (http://blast.ncbi.nlm.nih.gov/blast.cgi) was used to compare the gene set with the NR database. The species annotation was obtained from the taxonomic information database corresponding to the NR database, and then the abundance of the species was calculated by the sum of gene abundances corresponding to the species, and the abundance of the species in each sample was calculated at the taxonomic levels of domain, kingdom, phylum, class, order, family, genus, and specifications so as to construct the abundance profile at the corresponding taxonomic level. The gene set sequence was compared with the database of eggNOG (evolutionary genealogy of genes: non-supervised original groups, http://eggnog.embl.de/), and the corresponding COG (clusters of orthologous groups of proteins) was obtained. In addition, the gene set sequence was compared with the gene database of KEGG. According to the comparison results, KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do) was used for function annotation.

3. Results and discussion

3.1. High-throughput sequencing of 16S rRNA genes

3.1.1. Microbial diversity

The results of high-throughput sequencing of 16S rRNA genes after sampling from three reactors are shown in Table 1. The sequence numbers of the samples were in the range of 23,748–38,226, and the coverage index was above 0.988, indicating that the major OTUs were detected under the current sequencing depth. Shannon index reflected not only the species richness of microbial samples, but also the species evenness [8]. Fig. 2a showed the Shannon index values of all samples. It can be seen that the diversity of microbial communities in the sludge mixed liquor of the reactor with Fe was similar to that of the blank reactor, while the diversity of microbial communities enriched in the surfaces of Fe and Fe–Cu was slightly lower. The diversity of microbial community in the sludge mixed liquor of Fe/Cu reactor was significantly lower than that of the other samples, which might be due to the selective enrichment of some denitrification related microorganisms by the addition of Fe–Cu.

3.1.2. Microbial community structure

Fig. 3a shows the results of microbial community composition at phylum levels. It can be seen from the figure that the microbial community was mainly composed of *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Chlorofloxi*. At present, denitrifying bacteria reported in literature were distributed in more than 50 genera, mainly in *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and other phylum [9], which was consistent with the results of this study. These known denitrifying microorganisms can only produce part of the enzymes in the whole denitrification process, and cannot finish the whole denitrification process alone, and therefore the cooperation of a variety of denitrifying bacteria was necessary [10]. It can also be seen that *Proteobacteria* was dominant in all samples except for the samples Fe–Cu (Fe–Cu1 and Fe–Cu2). In the samples Fe–Cu (Fe–Cu1 and Fe–Cu2), *Firmicutes* were the dominant population. As mentioned above, the diversity (Shannon index) of Fe–Cu (Fe–Cu1 and Fe–Cu2) samples was the lowest, which might be related to the enrichment of *Firmicutes*. The abundance of *Chlorofeli* in Fe–S (Fe–S1 and Fe–S2) was significantly lower than that in Fe (Fe1 and Fe2), while the abundance of *Proteobacteria* in Fe–Cu–S (Fe–Cu–S1 and Fe–Cu–S2) was significantly higher than that in Fe–Cu (Fe–Cu1 and Fe–Cu2), which indicated that there was a great difference between the

Table 1
List of sample sequence information

Sample ID	Number of reads	Average sequence length (bp)	Minimum sequence length (bp)	Maximum sequence length (bp)
C1	36,241	441	299	452
C2	29,464	440	365	512
Fe1	32,292	438	386	495
Fe2	35,168	438	387	474
Fe–Cu1	35,572	442	409	461
Fe–Cu2	37,601	444	382	482
Fe–S1	38,226	444	387	482
Fe–S2	23,748	445	332	504
Fe–Cu–S1	36,311	446	396	486
Fe–Cu–S2	37,197	444	386	514

microbial community in the sludge mixed liquor and sludge in the reactor. The microorganisms in biofilm on the surfaces of Fe and Fe–Cu had longer residence time than that in the sludge mixed liquor, and therefore there were obvious differences of microbial community between sludge and the sludge mixed liquor. In addition, the microorganisms on the surfaces of Fe and Fe/Cu might be able to take advantage of the electrons released by Fe and Fe/Cu, and at the same time, they may also utilize organic matters. However, the microorganisms in the sludge mixed liquor might be more inclined to use organic matters present in the wastewater, which therefore led to the differences in the microbial community.

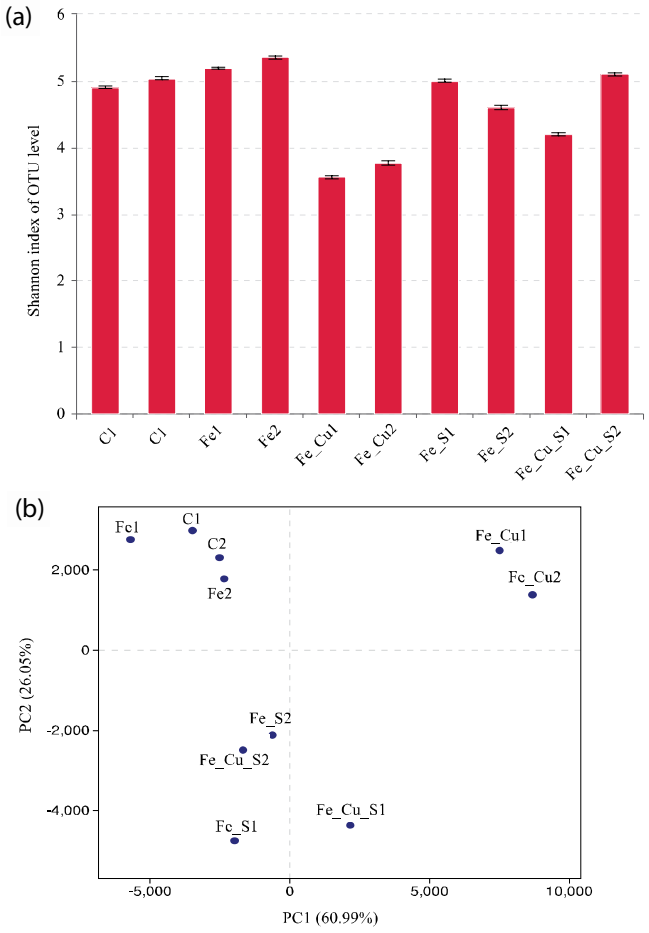

Fig. 2. (a) Shannon index of all samples and (b) PCA (Bray–Curtis distance) analysis of all the samples based on microbial community composition at the genus level.

Fig. 3b shows the microbial communities of all samples at the genus level. The results showed that the microbial community structure of samples C (C1 and C2) and Fe (Fe1 and Fe2) were similar, but the microbial community structure of samples Fe–Cu (Fe–Cu1 and Fe–Cu2) was quite different. The above results showed that the microbial communities in the reactors were significantly affected by the different types of electron donor. When Fe was added, the microbial community in the sludge mixed liquor had no significant change compared to the control reactor, while Fe/Cu resulted in the significant change of the microbial community in the sludge mixed liquor. In this study, duplicate samples were taken during the steady-state as biological repeat, and the two samples of a biological repeat were basically clustered together, which also reflected that the microbial community in the reactor was relatively stable when sampling. Compared with other samples, *Bacillus* was significantly enriched in Fe/Cu reactor (40%), which also explained the large difference in the microbial community between Fe/Cu reactor and the other reactor. It was reported that most microorganisms belonging to *Bacillus* can achieve denitrification for both nitrate and nitrite [11]. In addition, it has been reported that microorganisms belonging to *Bacillus* can reduce iron [12]. Therefore, the enrichment of *Bacillus* might be related to the addition of Fe–Cu and the reduction of nitrate by using iron as an electron donor. *Gallionellaceae_unclassified* has been enriched in Fe reactor and Fe–Cu reactor (>10%). It has been reported that microorganisms belonging to *Gallionellaceae* can realize autotrophic respiration and denitrification by using divalent iron [13]. In this study, it was found that *Gallionellaceae_unclassified* had high relative abundances only in the reactor with Fe and Fe/Cu added, while it was not detected in the sludge mixed liquor. Therefore, *Gallionellaceae_unclassified* was likely to participate in the reduction of iron to achieve autotrophic denitrification, but it cannot use organic matters in the sludge mixed liquor to achieve heterotrophic denitrification. *Arenimonas* was enriched on the reactor with Fe and Fe/Cu added (2%–5%). Previous studies have shown that *Arenimonas* could achieve autotrophic denitrification [13]. *Pseudomonas* was relatively abundant in the reactor and

Table 2
Metagenomic sequencing and analysis results

Sample-ID	C	Fe	Fe–Cu	Fe–S	Fe–Cu–S
Number of high quality sequences	45,148,010	41,087,113	40,439,341	43,200,973	46,412,656
Contig numbers	105,563	137,959	94,089	89,367	100,052
Maximum Contigs (bp)	87,606	56,905	234,959	96,059	181,190
Minimum Contigs (bp)	500	500	500	500	500
ORF	181,166	221,375	160,550	160,872	190,444
Average length of ORF (bp)	588	553	564	581	614
Maximum ORF (bp)	11,045	8,699	10,293	13,275	12,268
Minimum ORF (bp)	100	100	100	100	100

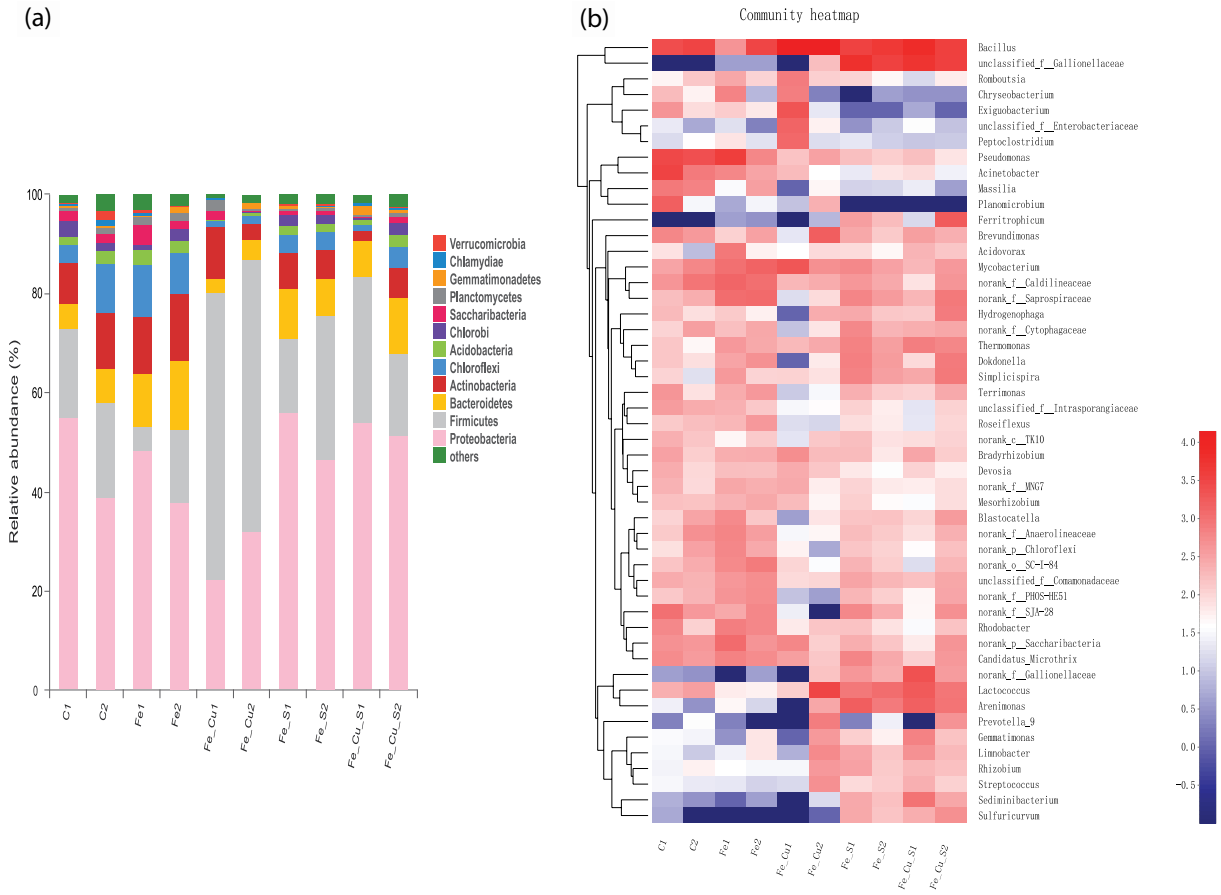

Fig. 3. (a) Relative abundance of 12 kinds of predominant bacterial phylum type detected in all samples and (b) heatmap showing the relative abundance of 50 kinds of predominant bacteria genus detected in all samples.

Fe reactor, which could realize heterotrophic denitrification [14]. Therefore, its existence in this study was mainly related to the use of organics to achieve denitrification. The above results further showed that the microbial community in the sludge mixed liquor samples (Fe1, Fe2, Fe–Cu1, and Fe–Cu2) was significantly changed by adding Fe or Fe/Cu. However, these microorganisms with denitrification function were enriched in sludge samples (Fe–S1, Fe–S2, Fe–Cu–S1, and Fe–Cu–S2), but not in the sludge mixed liquor samples.

The PCA results are shown in Fig. 2b, which are consistent with Heatmap cluster results. The microbial community structure in the sludge mixed liquor of Fe and control reactor was similar, and Fe/Cu had a greater impact on the microbial community structure in the sludge mixed liquor. Principal components 1 and 2 of PCA analysis can explain 60.99% and 26.05% of microbial community differences, respectively. The above results showed that the addition of Fe and Fe/Cu had a greater impact on the microbial community in the reactors.

3.2. Metagenomic sequencing

3.2.1. Overview of the metagenomic sequences

Based on the high-throughput sequencing of 16S rRNA gene, the metagenome of microbial samples was further sequenced (Table 2). After quality control, the high-quality sequence numbers of all the samples were between 40,439,341 and 46,412,656. The number of contigs larger than 500 bp was between 89,367 and 137,959 after the assembly of the sequences, and the longest contigs were up to 234,959 bp. The number of contigs obtained in this study was more than that of activated sludge samples (54,114–71,760) reported in the literature [15], mainly due to the higher sequencing depth in the present study. An open reading frame (ORF) was used to predict the contigs in the assembled results. The number of ORF was between 160,550 and 221,375, and the average length of ORF was 553–614 bp.

3.2.2. Microbial community compositions

The composition of the microorganisms based on metagenomic sequencing is shown in Fig. 4. *Proteobacteria* was shown to be dominant bacteria in all samples (47%–80%), which was not exactly consistent with the high-throughput sequencing of 16s rRNA genes analysis. In high-throughput sequencing of 16s rRNA genes analysis, although *Proteobacteria* was also the dominant bacteria in most samples, the relative abundance of *Proteobacteria* in the reactor with Fe/Cu added was only about 30%, which was significantly lower than that of the control reactor and Fe

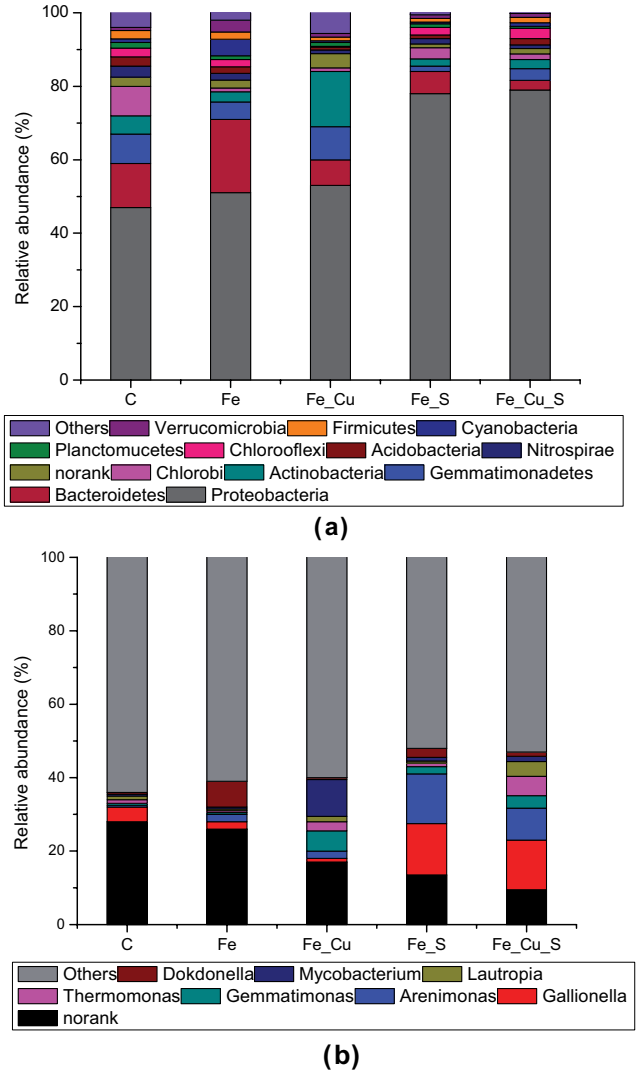

Fig. 4. Bacterial community structures at phylum level (a) and genus level (b) obtained via the sequencing analysis of metagenomics.

reactor (about 40%). However, the relative abundance of *Proteobacteria* in Fe/Cu reactor was slightly higher than that in the control and Fe reactor by metagenomic analysis. In addition, *Firmicutes* was also accounted for a large proportion (5%–60%) of all microbial samples in the high-throughput sequencing of 16s rRNA genes analysis. However, the results of the metagenomic analysis suggested that the relative abundance in all samples was less than 5%. The above results showed that the results of high-throughput sequencing of 16s rRNA genes analysis were quite different from those of metagenomic analysis. The reason for the differences might be that PCR amplification was involved in high-throughput sequencing of 16s rRNA genes analysis, and there were base mismatch and different primer specificity in the PCR amplification process, which might lead to the difference of analysis results [16]. In contrast, PCR was not involved in the metagenomic sequencing, and therefore the errors introduced by PCR were avoided. Moreover, metagenomic sequencing can identify microorganisms to the species level or even the strain level. Metagenome sequencing involves randomly breaking the microbial genome and assembling small fragments into longer sequences. Metagenome is one of the most advanced technical methods for studying microbial communities. Although there may be some technical errors, there is currently no more precise technology to avoid errors. Therefore, the results of microbial community analysis based on metagenomic sequencing might be more able to reflect the real community structure.

It can be seen from Fig. 4a that compared with the control reactor, *Bacteroidetes* in Fe was enriched, while *Actinobacteria* in Fe–Cu was enriched. The above results presented that the addition of Fe and Fe/Cu changed the microbial community structure in the sludge mixed liquor, while Fe and Fe–Cu had different effects on the microbial communities. *Proteobacteria* were enriched (about 80%) on Fe–S and Fe–Cu–S, which was significantly higher than the other samples (<60%). This may be due to the biofilm formed on the surface of Fe and Fe/Cu increasing the residence time of microorganisms, which significantly changed the microbial community compared to the sludge mixed liquor. The microbial community structure at the genus level is presented in Fig. 4b, from which it can be seen that *Gallionell*a and *Arenimonas* were obviously enriched in Fe–S and Fe–Cu–S samples and both of them can realize denitrification by autotrophic respiration [13,17]. This result was consistent with the high-throughput sequencing of 16s rRNA genes analysis, and the autotrophic microorganisms on the surface of Fe and Fe/Cu were further enriched for denitrification. This result presented the microbial mechanism of Fe action, mainly because the formation of a biofilm on its surface is conducive to denitrification. Compared with C sample, *Dokdonella* in Fe was enriched, while *Mycobacterium* in Fe–Cu was enriched. However, it has not been reported that these two kinds of microorganisms can realize autotrophic denitrification in the literature at present. The reason for the enrichment of these two kinds of microorganisms in Fe and Fe–Cu samples was not clear at present.

3.2.3. Functional gene composition

In order to further understand the function of the microbial community, COG annotation was conducted on the sequences. Different samples had different proportions of annotation, and they were 18.5% for C, 16.5% for Fe, 13.6% for Fe–Cu, 26.4% for Fe–S, and 31.2% for Fe–Cu–S, respectively (Fig. 5). The results presented that the samples obtained from sludge were better annotated. The COG analysis results are shown in Fig. 5. It can be seen that the COG function classification of the five samples were relatively close. Most of the functional genes were concentrated in energy production and conversion, amino acid transport and metabolism, and replication. Although there was no Fe in the control reactor, the proportion of the sequences belonging to organic transport and metabolism in the microbial samples was not much different from that in other samples, which indicated that the control reactor might also have the potential ability to metabolize iron. However, because there were no Fe and Fe/Cu in the reactor, these genes might not be expressed. Further, KEGG

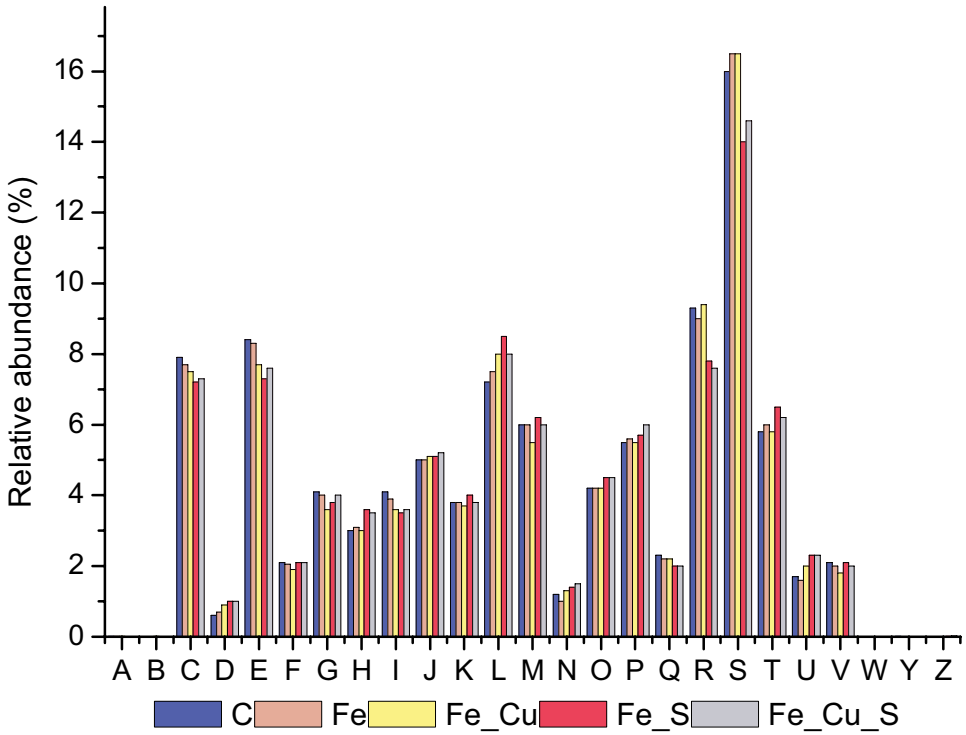

Fig. 5. Relative abundance of COG functional classes in all samples. [A] RNA processing and modification, [B] chromatin structure and dynamics, [C] energy production and conversion, [D] cell cycle control, cell division, and chromosome partitioning, [E] amino acid transport and metabolism, [F] nucleotide transport and metabolism, [G] carbohydrate transport and metabolism, [H] coenzyme transport and metabolism, [I] lipid transport and metabolism, [J] translation, ribosomal structure, and biogenesis, [K] transcription, [L] replication, recombination, and repair, [M] cell wall/membrane/envelope biogenesis, [N] cell motility, [O] post-translational modification, protein turnover, and chaperon functions, [P] inorganic ion transport and metabolism, [Q] secondary metabolites biosynthesis, transport, and catabolism, [R] general function prediction only, [S] function unknown, [T] signal transduction mechanisms, [U] intracellular trafficking, secretion, and vesicular transport, [V] defense mechanisms, [W] extracellular structures, [Y] nuclear structure, and [Z] cytoskeleton.

gene database was used to annotate the sample sequence. The metabolic pathway of nitrogen is shown in Fig. 6. The red box indicated the annotated genes. The same genes were annotated in the five samples, indicating that the five microbial samples had relatively complete metabolic pathways of nitrogen. The sequence proportions of metabolic pathways of nitrogen were 0.21% for C, 0.18% for Fe, 0.16% for Fe–Cu, 0.31% for Fe–S, and 0.36% for Fe–Cu–S, respectively, which showed that microorganisms on sludge had a stronger ability to metabolize nitrogen. It can also be seen from the above results that the sequence proportion of the annotated metabolic pathway to nitrogen in the sludge mixed liquor after adding Fe or Fe/Cu was slightly lower than that in the control reactor. Therefore, the above results further indicated that the biofilm formed on the surface of Fe and Fe/Cu could enrich more functional microorganisms, which promoted denitrification of nitrate.

In general, this study deeply reveals the microbiological mechanism of Fe and Fe/Cu, which is mainly related to the biofilm formed on their surface. This finding emphasized the impact of biofilms formed on the surface of exogenous additives on the microbial community structure. Moreover, the importance of iron-based materials in the denitrification process was highlighted at the genetic level. Subsequent research can focus on optimizing the denitrification process parameters to enhance the enrichment of dominant microorganisms and further improve performance. Another research focus is on further characterization and improvement of iron-based materials. Simultaneous optimization of process parameters and exogenous materials is also applicable to the biological denitrification of other types of wastewater.

4. Conclusions

The current research presented Fe or Fe/Cu bimetallic could change the microbial community structure. Fe and Fe/Cu could selectively enrich denitrification-related microorganisms and reduce the diversity of the microbial community. COG annotation of the sequence revealed that the microbial community in the reactor with added Fe and Fe–Cu can get more functional annotations. Analysis of metagenomics data using the KEGG database showed that genes on the nitrogen metabolism pathway were significantly up-regulated after adding Fe and Fe/Cu. This study explained that the reactor with Fe and Fe/Cu added had stronger denitrification ability at the genetic level.

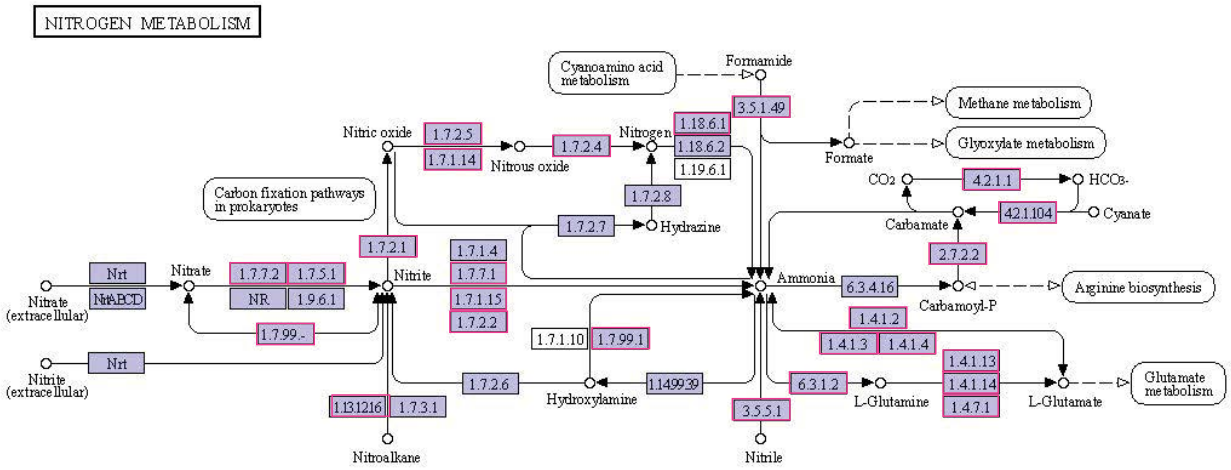

Fig. 6. KEGG nitrogen metabolism pathway. Gene present in all samples is labeled in red.

Acknowledgment

This study was funded by Fuxiao Youth Talent Program from Shanghai Municipal Engineering Design Institute (Group) Co., Ltd.

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