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Dynamics of the diversity and structure of the overall and nitrifying microbial community in activated sludge along gradient copper exposures

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Abstract Diversity and composition of the microbial community, especially the nitrifiers, are essential to the treatment efficiency of wastewater in activated sludge systems. Heavy metals commonly present in the wastewater influent such as Cu can alter the community structure of nitrifiers and lower their activity. However, the dynamics of microbial community along a gradient of metal exposure have largely been unexplored, partially due to the limitations in traditional molecular methods. This study explored the dynamics regarding the diversity and community structures of overall and nitrifying microbial communities in activated sludge under intermittent Cu gradient loadings using Illumina sequencing. We created a new local nitrifying bacterial database for sequence BLAST searches. High Cu loadings (>10.9 mg/L) impoverished microbial diversity and altered the microbial community. Overall, *Proteobacteria* was the predominant phylum in the activated sludge system, in which *Zoogloea*, *Thauera*, and *Dechloromonas* (genera within the *Rhodocyclaceae* family of the *Beta-proteobacteria* class) were the dominant genera in the presence of Cu. The abundance of unclassified bacteria at the phylum level increased substantially with increasing Cu loadings. *Nitrosomonas* and *Nitrospira* were the predominant nitrifiers. The nitrifying bacterial community changed through increasing abundance and shifting to Cu-tolerant species to reduce the toxic effects of Cu. Our local nitrifying bacterial database helped to improve the resolution of bacterial identification. Our results provide insights into the dynamics of microbial community in response to various metal concentrations in activated sludge systems and improve our understanding regarding the effect of metals on wastewater treatment efficiency.

Keywords Illumina sequencing · Activated sludge · Cu toxicity · Nitrifying bacteria · Microbial community

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Introduction

Activated sludge is a typical artificial microbial system widely used as a biological treatment process for wastewater. Activated sludge contains a huge amount and variety of microbes including autotrophic and heterotrophic bacteria, among which nitrifying bacteria play a central role in nitrogen removal through sequential oxidation of ammonia to nitrite and nitrate (Grady et al. 2011). These nitrifying bacteria are physiologically separated into ammonia- and nitrite-oxidizing bacteria (AOB and NOB), and functional species and activities of AOB and NOB have been studied extensively due to their essential contribution to the removal of nitrogen from wastewater (Fukushima et al. 2014; Purkhold et al. 2000; Ouyang et al. 2015). AOB and NOB grow slowly and are

sensitive to toxic stress caused by contaminants like heavy metal (Hu et al. 2003; You et al. 2009). The influent of biological treatment systems with activated sludge is often loaded with high concentrations of heavy metals, which could affect the community structure and activities of microbes and lower the treatment efficiency of wastewater (Ochoa Herrera et al. 2011). A few studies have demonstrated that structural complexity and diversity are correlated with the healthy functioning of AOB and NOB and the performance of activated sludge systems (Figuerola and Erijman 2010; Mertoglu et al. 2008).

Copper (Cu) is one of the most widely used heavy metals in manufacturing and commonly present in industrial wastewater. The reported concentrations of Cu in industrial wastewater ranged from 5 to 1000 mg/L (Sierra Alvarez et al. 2007; Stanković et al. 2009). Although trace amounts (microgram per liter) of Cu can serve as an important catalytic cofactor for microbial growth, high levels of Cu (e.g., greater than milligram per liter) is toxic to most organisms through increased production of highly reactive oxygen species, which cause lipid peroxidation, protein damage, and DNA cleavage (Dupont et al. 2011; Santos and Judd 2010). The inhibitory effects on bacterial activity in activated sludge systems have been observed at various concentrations of Cu (Cabrero et al. 1998; Jiang et al. 2013; Pamukoglu and Kargi 2007). However, previous studies have mainly focused on the impact of Cu on the efficiency of substrate removal in activated sludge, while information regarding shifts in the structure and diversity of the bacterial community in response to Cu loading is limited.

The change in the microbial community could be a result from the loading of harmful compounds. On the other side, the change could also be a strategy of the coexisting diverse bacterial populations to cope with the harmful conditions (Keshri et al. 2015). It has been reported that the macroscopical metabolism performance of activated sludge (e.g., substrate removal efficiency) was not affected by toxic heavy metals due to the enrichment of metal-tolerant microbial species (Demanou et al. 2006; Miao et al. 2015). The dynamic changes of the microbial community could be one of major factors determining the macroscopical metabolism performance of activated sludge. Thus, investigation of the dynamic changes of the overall microbial and nitrifying bacterial community would greatly aid in better understanding the effect of heavy metals on a mixed bacterial system like activated sludge. Over the past few decades, traditional molecular technologies based on the analysis of microbial 16S rRNA, including fluorescence in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphisms (T-RFLPs), have been utilized extensively to investigate microbial community structures in activated sludge (Kim et al. 2009; Munz et al. 2008; Yang et al. 2012). There have been some studies regarding changes in the nitrifying bacterial community under the stress of heavy metals. Mertoglu et al. (2008) applied DGGE techniques and found that the AOB population sharply shifted from *Nitrosomonas* sp. and *Nitrosococcus* sp. to *Nitrosospira* sp. under gradually increasing cadmium (Cd) loadings, while the NOB population had no obvious changes. Another study using T-RFLPs found that the dominant AOB group shifted from a *Nitrosomonas*-like cluster to an *N. oligotropha* lineage after 8 weeks in cultures exposed to less than 150 mg/L of nickel (Ni), while in cultures exposed to 150 mg/L of Ni, *Nitrosospira* became the dominant AOB group after 6 weeks (Yeung et al. 2013). In addition, a study using the FISH method revealed that *Beta-proteobacteria* abundance dramatically decreased in activated sludge under continuous Cu application, whereas *Gamma-* and *Alpha-proteobacteria* presented a relatively small change (Principi et al. 2006). Recently, an Illumina sequencing platform for 16S rRNA amplicon analysis, which can obtain thousands of sequences from multiple samples in parallel, has become a preeminent platform to explore the structure of microbial communities (Kwon et al. 2010). Many studies have shown that the Illumina sequencing technologies have sufficient sequencing depth and accuracy to cover complex bacterial communities (Degnan and Ochman 2012; Li et al. 2013; Ma et al. 2015a). The Illumina sequencing technologies can provide more insights into the dynamic changes of the microbial communities than these traditional molecular technologies mentioned above. However, to the best of our knowledge, the dynamics of the nitrifying community in response to Cu stress have rarely been detected through using the Illumina sequencing technologies.

To investigate the dynamics in diversity and structure of the overall and nitrifying microbial community in activated sludge under Cu exposures, we employed Illumina sequencing in this study. Five different intermittent Cu gradient loadings were introduced to the influent of a sequencing batch reactor (SBR). Activated sludge samples were drawn from the SBR at the end of Cu loading and recovery periods, and the abundances and composition of the microbial community were analyzed using Illumina sequencing. Furthermore, we created a local nitrifying bacterial database for local BLAST training of nitrifying bacterial strains to improve the resolution of nitrifying bacteria identification. Our study is among the first to characterize the community dynamics of the overall microbes and nitrifying bacteria along a Cu exposure gradient in activated sludge systems.

Materials and methods

Synthetic domestic wastewater and activated sludge

Synthetic wastewater with 500 mg/L chemical oxidation demand (COD) and 75 mg/L NH_4^+-N was prepared by dissolving the following chemicals in ultrapure water (in mg/L):

glucose (480), NH_4Cl (288), KH_2PO_4 (30), $NaHCO_3$ (580), $MgSO_4\ 7H_2O$ (20), $FeSO_4\ 7H_2O$ (2.5), $ZnSO_4\ 7H_2O$ (0.25), $CaCl_2\ 2H_2O$ (10), $CoCl_2\ 6H_2O$ (0.05 μg/L), and MoO_3 (1.50 μg/L). The influent pH was measured at 7.5 ± 0.2. A Cu stock solution of 50 g/L was prepared by dissolving $CuCl_2$ in an acidic solution (containing 0.37 mg/L HCl) to stabilize the Cu ions in solution. Activated sludge was collected from an aerobic tank of a local sewage treatment plant, which employed an A^2/O treatment system. The collected activated sludge was screened through a 2-mm sieve to remove coarse particles and was cultivated in an SBR with a total effective volume of 5 L. A schematic of the SBR can be found in a previous paper (Ouyang et al. 2015).

Experimental design

The SBR was operated sequentially in 6-h cycles: influent filling (15 min), aeration (240 min), settling (95 min), and effluent withdrawal (10 min). During each cycle, 2.5 L of wastewater was treated, resulting in an average hydraulic retention time of 12 h. The mixed liquid suspended solids (MLSS) were kept at approximately 5000 mg/L by periodically discharging excess sludge from the reactor, with a solid retention time of 30 days. The temperature in the reactor was controlled at 24 ± 1 °C using a water bath layer. The dissolved oxygen concentration was maintained at 2.00–4.00 mg/L by aeration.

After a long acclimation period in the SBR, the concentrations of COD, ammonia, nitrite, and nitrate in the effluent were stable at 17.8 ± 2.5, 0.2 ± 0.1, 0.07 ± 0.01, and 36.6 ± 0.3 mg/L, respectively. Then, the experiment with intermittent Cu gradient loadings was started. The SBR was operated in five continuous periods, each of which included 8 running cycles with Cu loadings and 24 (or 46) subsequent running cycles without Cu loadings (i.e., the recovery periods). Because Cu precipitation was observed in the storage tank of influent, a coarse filtration system with a sand core filter plate (3–4 μm) was fixed at the end of the influent collecting tube. During the five loading periods, 10, 20, 30, 40, and 50 mg/L were added into the storage tank of influent. However, measured soluble concentrations of Cu in the influent were only 5.5, 9.2, 10.9, 13.5, and 28.2 mg/L; thus, they were used as the actual dosages of Cu into the SBR. The concentrations of ammonia, nitrate, and COD in the effluent were measured every two cycles. For the microbial population analysis, an aliquot of 10.0 mL of activated sludge solution was withdrawn during each settling process at cycles 0 (as the control), 8, 32, 40, 64, 72, 96, 104, 128, 136, and 183. These samples were numbered S_0, S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_8, S_9, and S_{10}, respectively.

DNA extraction, PCR amplification, and dual-index Illumina sequencing

The activated sludge sample of 10.0 mL was centrifuged at 4000 rpm for 10 min at 4 °C. After centrifugation, 200 mg of the pellet of each sample was collected in duplicate for DNA extraction using the FastDNA® SPIN Kit for Soil (Q-Biogene, CA, USA). The duplicate DNA extracts were then merged. Then, the DNA was examined for yield, purity and suitability for PCR by both electrophoresis and spectrometry (NanoDrop-1000; Thermo Scientific, MA, USA).

The hypervariable V4 region (~240 bp) of the bacterial 16S rRNA gene was amplified with 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Three replicates of the 100-mL PCR reaction solutions were prepared for each sample using MightyAmp polymerase (TaKaRa, Bio, Japan) according to the instructions. The PCR was performed in an i-Cycler (BioRad, CA, USA) under the following conditions: initial denaturation at 95 °C for 2 min; 30 cycles at 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final extension at 72 °C for 10 min. Then, the triplicate PCR products were mixed and purified with the PCR quick-spin PCR Product Purification Kit (iNtRON Biotechnology, Bio, Korea). After the PCR products were quantified using a NanoDrop-1000 spectrometer (Thermo Scientific, MA, USA), the PCR products were mixed to obtain an equal concentration of DNA fragments for each sample. These samples were sent to Majorbio Institute (Shanghai, China) for Illumina high-throughput sequencing on a MiSeq platform (Illumina, CA, USA).

Sequence processing

The retrieved reads were processed using MOTHUR 1.32.1 software according to a published method (Schloss et al. 2011) and an online MiSeq standard operating procedure (MiSeq SOP) (http://www.mothur.org/wiki/MiSeq_SOP). In brief, contigs between read pairs were assembled as described in the MiSeq SOP. A total of 1,024,894 contig reads (called as sequence below) were obtained. The primers and barcodes were removed, and sequences with ambiguous base pairs, more than two mismatches in the primer sequence, more than one mismatch in the barcode sequence, more than eight homopolymers, or less than 250 base pairs were filtered out. The sequences were aligned using the SILVA reference database (http://www.mothur.org/wiki/Silva_reference_alignment) according to the MiSeq SOP, and those sequences that did not align to the correct region were culled. The ends of the sequences were trimmed so that the sequences all started and ended at the same alignment coordinates. After identifying the unique sequences and their frequency in each sample, a preclustering algorithm was

utilized to further denoise sequences within each sample. The resulting sequences were screened for chimeras using UCHIME. We then used a naive Bayesian classifier to classify each sequence against the Ribosomal Database Project 16S rRNA gene training set (version 9) (RDP-II for short) that was customized to include rRNA gene sequences from mitochondria and Eukaryota. We required an 80 % pseudo bootstrap confidence score. Those sequences that were either classified to the level of kingdom or classified as Archaea, Eukaryota, chloroplasts, or mitochondria were culled. This processing resulted in 744,084 sequences. The sequences were clustered into operational taxonomy units (OTUs) at a similarity cutoff of 97 % and further assigned to phylotypes from the phylum to the genus level using the classify.otu command according to the MiSeq SOP. The richness estimator rarefaction curves and diversity estimator Shannon index were calculated for each sample. A *t* test was used to test the significant difference of the rarefaction curves or Shannon index between the individual groups (S_1-S_{10}) and the control group (S_0). A weighted UniFrac principal coordinate analysis (PCoA) was performed using MOTHUR 1.32.1 to compare the phylogenetic distances of the microbial communities. The raw reads have been deposited into the NCBI Sequence Read Archive (Accession Number: SRP061818).

Database for nitrifying bacteria

In the Illumina sequencing data analysis, a microbe is typically identified by comparing a detected 16S rRNA sequence with sequences from a professional nucleic database, such as the SILVA reference database, NCBI database, or RDP-II. These databases contain hundreds of thousands of sequences distributed in a wide range of taxa and are extensively used for overall environmental microbial sequence BLAST searches. However, for profiling specific sub-dominant functional groups, analyses using these databases are often of low resolution. Guo and Zhang (2012) reported that creating a special database for some of the sub-dominant functional groups was helpful in identifying bacterial species. In this study, a local nitrifying bacterial database was created to improve the resolution for identification. The reference sequences of this database included 16S rRNA gene sequences (>1000 bp) of nitrifying bacteria in NCBI, including species of *Nitrosococcus*, *Nitrosomonas*, *Nitrosospira*, *Nitrosolobus*, *Nitrosovibrio*, *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina*. Sequences identified from all matrices, such as sludge, freshwater, and soil, were also included in the database. To avoid repeat hits, redundant sequences were filtered out (at a 97 % similarity cutoff at the V4 region), and only one reference sequence for each type (or subtype) was kept in the database.

The tags of each activated sludge sample were locally BLASTed against the local nitrifying bacteria database. The sequence processing method used was derived from a previous study (Guo and Zhang 2012). In brief, the e-value was set at 1×10^{-60}, under which the lowest similarity between reference sequences and the query tags is about 80 %. For each hit tag, only one of the closest reference sequences was listed. Then, the results both having a similarity of less than 97 % and having more than three uncovered bases (i.e., query coverage is less than 99 % for 253 bp pyrotags) were filtered. Then, the sequences were ready for statistics. Heat maps displaying the abundance of the nitrifying bacterial species were generated in Matlab2013.

Analytical methods

COD and concentrations of ammonia nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) of the effluent were determined according to standard methods (APHA, AWWA, WEF, 2005). The MLSSs of the SBR were monitored regularly as an indicator of reactor performance using standard methods (APHA, AWWA, WEF, 2005). The pH of the influent and effluent were measured using a pH meter (Hach, CO, USA).

Results

Effects of Cu on the overall bacterial metabolic activity in the SBR

COD has been used as an index of the metabolic activity of heterotrophic bacteria (Moussa et al. 2005). The COD value in the effluent did not change when the intermittent Cu loading was increased from 5.5 to 13.5 mg/L (Fig. 1a), indicating that Cu loadings up to 13.5 mg/L did not inhibit the metabolic activity of the heterotrophic bacteria in this system. When the Cu loading concentration was increased to 28.2 mg/L, substantial inhibition of COD degradation was observed, indicating a severe toxic impact on the metabolic activity of the heterotrophic bacteria.

The ammonia oxidizing community was more sensitive to Cu toxicity than the heterotrophic bacteria. As shown in Fig. 1b, the Cu loadings at 9.2 and 10.9 mg/L caused short-term, slight inhibitory effects on nitrification, while Cu loadings of 13.5 and 28.2 mg/L caused significant inhibitory effects on nitrification. In cycle 152, ammonia oxidation almost ceased and then slowly recovered in the following cycles. Interestingly, the inhibitory effects on nitrification did not happen during the Cu loading periods; instead, they occurred in the subsequent recovery periods.

Variation in the diversity of the overall bacterial community

A total of 7274 OTUs were detected in the samples. At a similarity cutoff of 97 %, approximately 500 to 2500 of the

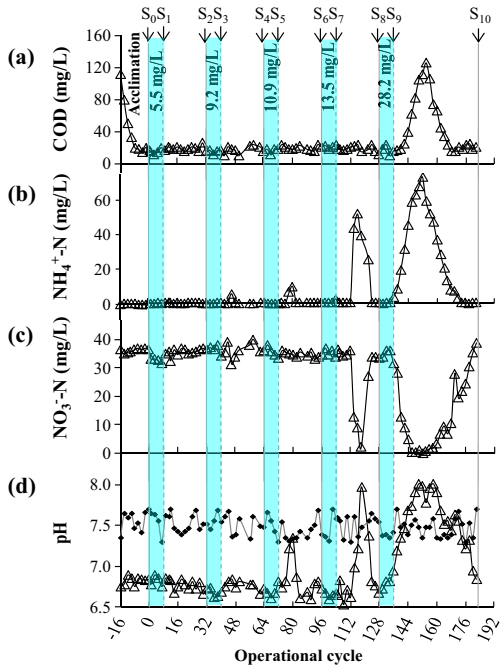

Fig. 1 SBR effluent COD (**a**), NH_4^+-N (**b**), NO_3^--N (**c**), and pH (**d**) under intermittent Cu gradient loadings. *Diamonds* indicate pH of the influent

OTUs of the overall bacterial community were identified among S_0–S_{10}, and rarefaction curves were generated. A steeper slope of the rarefaction curve indicates higher species richness. As shown in Fig. 2a, the OTUs of S_1 (2427) and S_2 (2485) were richer than that of S_0 (2264). However, the *t* test of the rarefaction curves suggested that the differences of S_0 from S_1 ($p > 0.05$) and S_2 ($p > 0.05$) were not significant. This indicates that the loading of Cu at 5.5 mg/L might slightly enhance the richness of the overall bacterial community. For S_3–S_{10}, the richness exhibited a significantly decreasing trend with increasing Cu loadings (*t* tests showed that the differences of S_0 from S_3–S_{10} were very significant with $p < 0.01$).

Since traditional rarefaction curves only presented species richness, we used Shannon rarefaction curves, which were calculated based on both richness and distribution uniformity of bacterial species (Allen et al. 2009), to further examine changes in diversity. As shown in Fig. 2b, all Shannon curves achieved their plateaus, suggesting that the sequencing data was large enough to retain most of the information for the microbial community in each sample. The plateau values of S_1 were significantly lower than that of S_0 (*t* test: $p < 0.01$) suggesting that the diversity of the overall bacterial community in S_1 significantly decreased after facing the Cu loading for the first time. However, the decrease in diversity seemed to be tentative. The plateau values of S_2–S_4 were close to or a little higher than that of S_0 and *t* tests showed that differences of S_2–S_4 from S_0 were not significant (*p* values all above 0.05). It indicates that the overall bacterial community might adapt to the presence of Cu after the loading of 5.5 mg/L Cu, leading to the increases of diversity of the overall bacterial community in the 5.5 mg/L Cu recovery periods and 9.2 mg/L Cu loading and subsequent recovery periods. Higher Cu concentrations significantly reduced the diversity of the overall bacterial community in S_5–S_{10} (*t* test: $p < 0.01$). For S_1–S_4, variations of species diversity in the Shannon curves were different from those in the rarefaction curves. This suggests the importance of accounting for distribution uniformity in calculating the rarefaction curves. For example, the loading of 5.5 mg/L Cu could increase the richness of bacterial species (leading to a steeper slope of S_1 than S_0 in the rarefaction curves) but could simultaneously decrease the distribution uniformity of bacterial species. If the contribution of the decrease in distribution uniformity exceeds the contribution of the increase in richness, it would lead to a lower plateau value of S_1 than S_0 in the Shannon rarefaction curves.

Shifts in the overall bacterial community structure and bacterial phylogenetic identification

The similarity of the samples with each other is shown in a dendrogram (Fig. 3a). The microbial communities in the 11 samples could be grouped into four distinct clusters: Cluster I contained samples S_0 and S_1; Cluster II contained samples S_2, S_3, S_4, and S_5; Cluster III contained samples S_6, S_7, S_8, and S_9; and Cluster IV contained S_{10}. This is consistent with the results of the weighted UniFrac PCoA analysis, which depicted the dynamic change in the microbial community structure along with the Cu loadings (Fig. 3b).

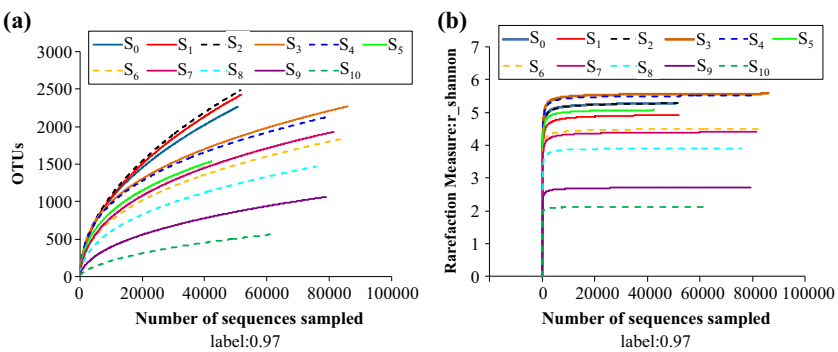

Fig. 2 **a** Rarefaction curves of OTUs defined by 3 % sequence variations in samples S_0-S_{10}; **b** Shannon rarefaction curves defined by 3 % sequence variations in samples S_0-S_{10}

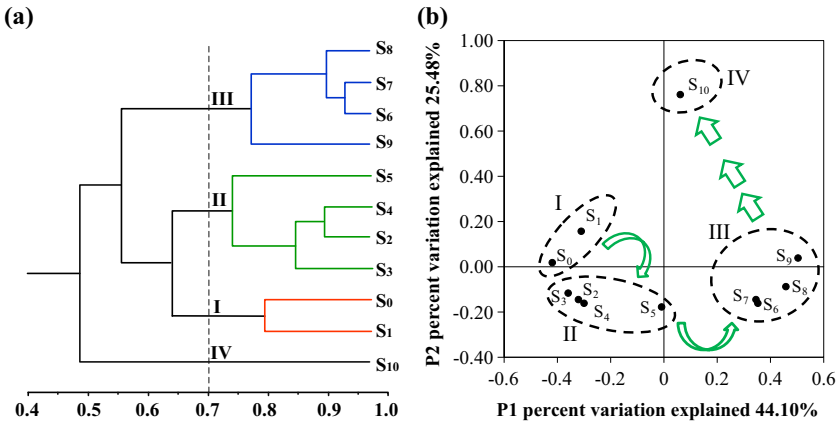

Fig. 3 Dendrogram (**a**) and PCoA analysis (**b**) of samples S_0–S_{10} based on weighted UniFrac algorithm

Phylogenetic analysis of the sequences revealed 20 major bacterial phyla in the 11 activated sludge samples (Fig. 4a). The absolute majority of the sequences belonged to *Proteobacteria*, which accounted for 23.65–56.59 % of the total reads in each bacterial community. Other major bacterial phyla included *Acidobacteria* (0.42–18.08 %, averaging 3.71 %), *Actinobacteria* (0.51–2.44 %, averaging 1.72 %), *Bacteroidetes* (2.55–20.43 %, averaging 11.24 %), *Planctomycetes* (0.14–7.84 %, averaging 3.53 %), and *Nitrospira* (0.06–3.78 %, averaging 0.93 %) as well as a certain amount of unclassified sequences at the phylum level (11.66–60.66 %, averaging 26.32 %). The degradation efficiencies of COD and ammonia were close to 100 % in S_0, in which the dominant bacterial phyla included *Acidobacteria* (8.69 %), *Bacteroidetes* (13.73 %), *Planctomycetes* (4.66 %), *Proteobacteria* (46.54 %), and *Nitrospira* (3.76 %) as well as unclassified sequences at the phylum level (11.66 %). This microbial composition had a high capacity for nutrient removal. The increase in Cu loadings led to an increase in the abundance of unclassified bacteria, which rose to 59 % when the Cu loading concentration was 28.2 mg/L.

At the phylum level, *Proteobacteria* were the most abundant. The overall abundance of *Proteobacteria* remained in the range of 46.54–56.59 % among S_0–S_8 (except for S_1, in which it was 39.84 %), while there was a substantial decrease in S_9 and S_{10}. There were five subclasses of *Proteobacteria*, i.e., *Alpha-*, *Beta-*, *Gamma-*, *Delta-proteobacteria*, and the *Unclassified*, detected in all of the samples (Fig. 4b). The abundance of *Alpha-proteobacteria* did not change until the Cu loading was increased to 28.2 mg/L. The *Beta-proteobacteria* were the most abundant among the five subclasses, and their proportion increased with increasing Cu loadings. The abundance of *Gamma-proteobacteria* exhibited a general decreasing trend with increasing Cu loadings in S_0–S_8, but it increased abruptly in S_{10}. The abundance of *Delta-proteobacteria* varied greatly among the samples and was extremely low in S_{10}. The abundance of the *Unclassified* had no clear trend and was also extremely low in S_{10}.

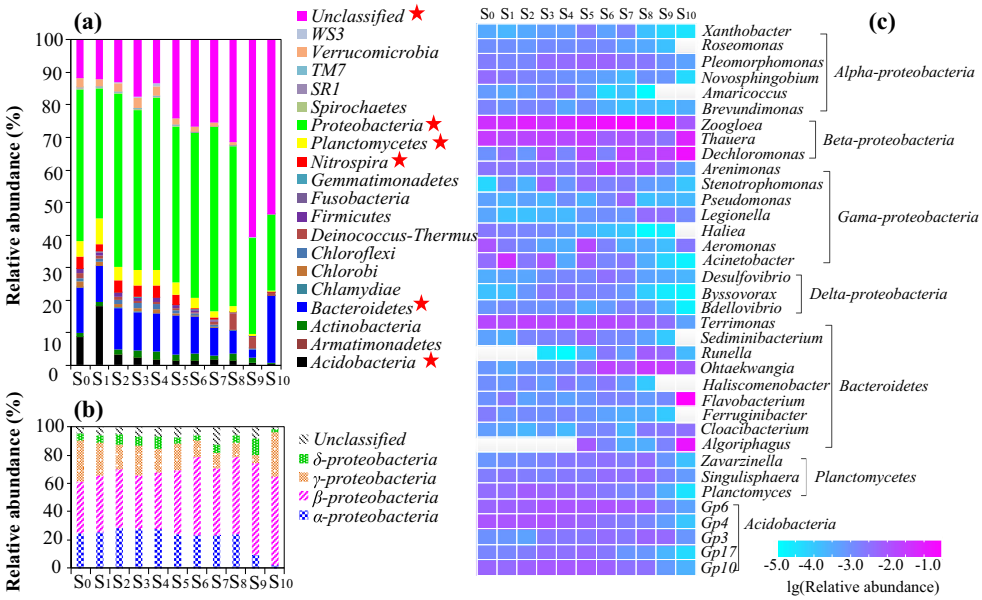

Fig. 4 **a** Taxonomic classification of bacterial DNA sequences from communities of the samples (S_0–S_{10}) at the phylum level; **b** class-level distribution of *Proteobacteria*; **c** abundance of the major bacterial genera (>0.1 % in any sample) in the samples (S_0–S_{10}). Each row is a genus with one reference sequence and each column represents an activated sludge sample. The numbers of the tags were logarithmized and then shown as *colored blocks*. The *white blocks* are undetected groups. *Red stars* represent major bacterial phyla

We also analyzed the abundances of the major genera present in S_0–S_{10}. As shown in Fig. 4c, among the genera, *Zoogloea*, *Thauera*, and *Dechloromonas* (genera within the *Rhodocyclaceae* family of the *Beta-proteobacteria* class) had relatively high abundances during the Cu loadings, but their responses to Cu stress were different. *Zoogloea* had a very high tolerance to Cu, as its abundance increased from 3.89 % in S_0 to 19.98 % in S_9 then decreased in S_{10}. While *Thauera* and *Dechloromonas* were not as abundant as *Zoogloea* in S_0–S_9, and they became the predominant genera in S_{10} (6.71 % for *Thauera* and 16.31 % for *Dechloromonas*).

Compared with S_0, the abundance of *Acidobacteria* exhibited a notable increase of 18.08 % in S_1 and then significantly decreased with increasing Cu loadings (Fig. 4a). Similar to *Acidobacteria*, *Planctomycetes* increased in S_1 and then decreased throughout the whole experiment (Fig. 4a). *Bacteroidetes* seemed to be resistant to the impact of Cu at relatively low Cu concentrations. The abundance of *Bacteroidetes* did not significantly change among S_0–S_6, then decreased in S_7–S_9, and became very high in S_{10} (20.42 %). As shown in Fig. 4c, *Flavobacterium* (a genus within the *Flavobacteriaceae* family of the *Bacteroidetes* class) maintained a constant level of abundance in S_0–S_9 and then increased substantially in S_{10}. *Algoriphagus* (a genus within the *Cyclobacteriaceae* family of the *Bacteroidetes* class) was not observed in S_0–S_4 but appeared in S_5–S_{10}, accounting for 12.72 % of the total community when Cu reached 28.2 mg/L. The increases in *Flavobacterium* and *Algoriphagus* might contribute to the increased abundance of *Bacteroidetes* in S_{10}. The abundance of *Nitrospira*, which plays a central role in the oxidation of nitrite, varied to a limited extent among S_0–S_5 but almost dropped to zero in S_6–S_{10}.

Shifts in the nitrifying bacterial community

The newly created local nitrifying bacterial database was used in identification of nitrifying bacteria and then the identified results were compared with their taxonomy assignments referring to RDP-II. As shown in Table 1, there were 29 OTUs identified as nitrifying bacteria at genus at a similarity cutoff of 97 % using the two databases. The local nitrifying bacterial database, which was extracted from NCBI, had a higher resolution than the RDP-II reference database. According to the local nitrifying bacterial database, the 29 OTUs were identified as AOB or NOB at the genus level: the detected AOB belonged to *Beta-proteobacteria*, including the genera *Nitrosospira*, *Nitrosomonas*, and *Nitrosovibrio*; and the detected NOB belonged to *Nitrobacter* and *Nitrospira*. According to RDP-II reference database, only 15 OTUs among the 29 OTUs were identified as AOB or NOB at the genus level: the detected AOB included *Nitrosospira* and *Nitrosomonas*; and the detected NOB only included *Nitrospira*. Additionally, the application of the local nitrifying bacterial database saved the search space.

Fig. 5 shows the relative abundances of nitrifying bacterial OTUs in Table 1. The abundances of nitrifying bacteria (0.23–4.19 %) identified according to our local nitrifying bacterial database were higher than those (0.15–2.83 %) identified according to the RDP-II reference database. The abundances of nitrifying bacteria identified using the two databases exhibited similar profiles along the Cu loading gradient. Loadings of 5.5–10.9 mg/L seemed to promote the overall abundances of nitrifying bacteria in S_1–S_5, while the loading of 28.2 mg/L reduced the overall abundance of nitrifiers.

A profile showing the shifts in nitrifying bacterial communities in S_0–S_{10} was created for OTUs with more than 60 sequences in Table 1, according to our local nitrifying bacterial database at a similarity cutoff value of 97 % (Fig. 6). The AOB mainly included five groups (i.e., 5 OTUs): OTU91, OTU275, OTU381, OTU431, and OTU555. Although OTU91, OTU275, OTU381, and OTU555 were all identified as genera of *Nitrosomonas*, their adaptations to Cu toxicity were different. OTU91 was the predominant AOB group and had a high tolerance to Cu, while OTU275, OTU381, and OTU555 had weaker adaptation to Cu toxicity than OTU91. OTU431, which belonged to *Nitrosovibrio*, maintained a relatively constant abundance in S_0–S_7. The NOB included six OTUs: OTU20, OTU40, OTU105, OTU174, OTU293, and OTU766. OTU20 and OTU40, both identified as *Nitrospira*, were two predominant NOB species and had a high tolerance to Cu. OTU20 had a high abundance in S_1–S_7, whereas OTU40 had a high abundance in S_2–S_9. OTU105, OTU174, and OTU766 were all identified as *Nitrobacter*. The abundances of OTU105 and OTU174 reached their maxima in S_3. Cu seemed to greatly inhibit OTU766, of which the abundance substantially decreased with the presence of Cu in S_1–S_{10}. OTU293, identified as a *Nitrospira*, had a relatively low abundance in S_0–S_2 and then became highly abundant in S_3–S_9.

Discussion

To further understand the impact of Cu on microbial community, we analyzed the correlations between the microbial community structure and the efficiency of the activated sludge system.

Effect of Cu on dynamic changes of the overall bacterial community

In this study, it was found that Cu loadings reduced the diversity of the overall bacterial community, shown both by the rarefaction curves and the Shannon rarefaction curves. McCann (2000) demonstrated that diversity was positively

Table 1 All identified nitrifying bacterial OTUs according to the new local nitrifying bacterial database and the RDP-II reference database

OTU	Total sequence number	Local nitrifying bacterial database in this study		Accession no.	RDP-II reference database	
		Closest genus or species	Similarity (%)		Closest genus (families)	Similarity (%)
20	6068	*Nitrospira*	100	HQ424545	*Nitrospira* (*Nitrospiraceae*)	100
40	3517	*Nitrospira*	97.6	AB113596	*Nitrospira* (*Nitrospiraceae*)	100
91	2284	*Nitrosomonas*	98.5	JQ936545	*Unclassified* (*Unclassified*)	100
105	954	*Nitrobacter*	100	HM486336	*Devosia* (*Hyphomicrobiaceae*)	93
174	379	*Nitrobacter*	100	HM486336	*Unclassified* (*Unclassified*)	97
275	369	*Nitrosomonas*	99.6	JQ936534	*Unclassified* (*Unclassified*)	100
293	719	*Nitrospira*	100	GU229885	*Nitrospira* (*Nitrospiraceae*)	100
381	232	*Nitrosomonas*	99.2	AB500061	*Unclassified* (*Nitrospiraceae*)	100
431	62	*Nitrosovibrio*	98.4	AM773598	*Unclassified* (*Unclassified*)	100
555	68	*Nitrosomonas*	100	AY138525	*Unclassified* (*Unclassified*)	100
766	88	*Nitrobacter*	100	KC172240	*Unclassified* (*Enterobacteriaceae*)	95
1092	16	*Nitrosomonas*	100	EU127375	*Unclassified* (*Nitrosomonadaceae*)	88
1500	13	*Nitrosomonas*	98.42	AJ224410	*Nitrosomonas* (*Nitrosomonadaceae*)	100
2654	3	*Nitrosomonas*	98.0	AB500058	*Unclassified* (*Unclassified*)	100
2773	7	*Nitrospira*	100	EF073284	*Nitrospira* (*Nitrospiraceae*)	100
2774	12	*Nitrospira*	100	FN394313	*Nitrospira* (*Nitrospiraceae*)	100
3394	1	*Nitrosomonas*	99.2	JN648262	*Nitrosomonas* (*Nitrosomonadaceae*)	100
3404	1	*Nitrosomonas*	98.8	AB500061	*Unclassified* (*Nitrosomonadaceae*)	100
3405	1	*Nitrosospira*	99.6	AJ275876	*Nitrosospira* (*Nitrosomonadaceae*)	100
3406	1	*Nitrosomonas*	100	NR_104815	*Nitrosomonas* (*Nitrosomonadaceae*)	100
3696	1	*Nitrospira*	99.6	HQ424545	*Nitrospira* (*Nitrospiraceae*)	100
3697	2	*Nitrospira*	99.2	JX493374	*Nitrospira* (*Nitrospiraceae*)	100
3698	1	*Nitrospira*	95.9	AB113596	*Nitrospira* (*Nitrospiraceae*)	100
3699	1	*Nitrospira*	96.4	HQ424545	*Nitrospira* (*Nitrospiraceae*)	100
3700	3	*Nitrospira*	95.7	AB113596	*Nitrospira* (*Nitrospiraceae*)	100
3701	4	*Nitrospira*	100	AB500062	*Nitrospira* (*Nitrospiraceae*)	100
4692	1	*Nitrosospira*	97.2	GU472967	*Unclassified* (*Unclassified*)	100
7078	2	*Nitrospira*	99.6	FN394313	*Unclassified* (*Unclassified*)	100
7199	1	*Nitrospira*	98.8	DQ414434	*Unclassified* (*Unclassified*)	100

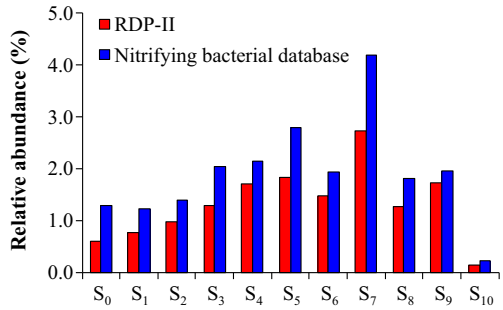

Fig. 5 Relative abundances of nitrifying bacteria at the genus level in the samples (S_0–S_{10}) based on the RDP-II reference database and the local nitrifying bacterial database. The abundances are the percentages of the tags to the total pyrotags at a cutoff value of 97 %

related to environmental stability, which could resist external interferences. Therefore, a decrease in diversity may indicate that the structure and functions of the microbiological system in activated sludge become vulnerable to toxicants and other negative shocks.

In addition to the reduction in microbial diversity, the overall microbial community structure gradually shifted over the Cu loading gradient. As shown in Fig. 3b, the Cu loading of 5.5 mg/L (S_1) might only change the microbial community structure slightly. This change in the microbial community continued even after the termination of the 5.5 mg/L Cu loading (S_2). Consequently, S_2 was grouped in Cluster II together with S_3, S_4, and S_5, which corresponded to the 9.2 mg/L Cu loading cycles, the recovery cycles, and the 10.9 mg/L Cu loading cycles, respectively. Because Cluster I and II were still in a monophyletic lineage (Fig. 3a), we propose that the Cu loadings of 5.5–10.9 mg/L, which had no impact on COD degradation and a short-term, slight metabolic inhibition on nitrification, caused a moderate change in the overall microbial structure. A significant change in the microbial community became recognizable in S_6, the recovery cycles after the 10.9 mg/L Cu loading. S_{10}, being Cluster IV alone, was very distant from the other samples, suggesting that the microbial

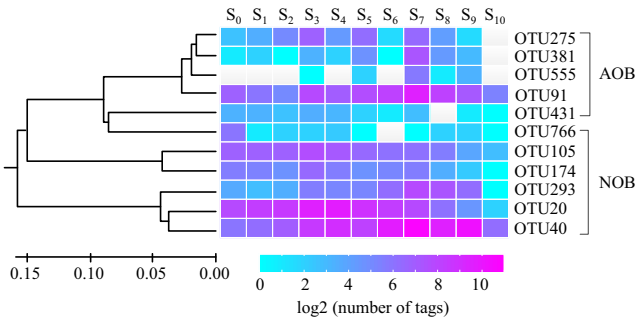

Fig. 6 A heat map showing the profile of the nitrifying bacteria communities with more than 60 sequences in the samples (S_0–S_{10}). Each row is a nitrifying group with one reference sequence and each column represents an activated sludge sample. The clustering among rows is inferred using the UPGMA method and drawn on Mega 5.0 software. The numbers of tags were logarithmized and shown as *colored blocks*. The *white blocks* are undetected groups

communities drastically diverged in the recovery phase after the 28.2 mg/L Cu loading.

Among the overall bacteria, *Proteobacteria* was the dominant phylum, which is consistent with other studies that applied high throughput sequencing to investigating the microbial community structure in activated sludge. For example, Kwon et al. (2010) demonstrated that *Beta-proteobacteria* and *Gamma-proteobacteria* were the top two classes in phylum *Proteobacteria*, whereas Ye and Zhang (2013) found that *Alpha-proteobacteria* was the top class in the phylum of *Proteobacteria*. In this study, *Alpha-proteobacteria*, *Beta-proteobacteria* and *Gamma-proteobacteria* were the top three classes in activated sludge without Cu loadings. As Cu was loaded in the influent, the abundance of *Beta-proteobacteria* increased. Among the genera of *Beta-proteobacteria*, *Zoogloea* was found to have the highest tolerance to Cu. Previous studies demonstrated that *Zoogloea* was common in sewage plants and was successfully used for Cu adsorption (Chen et al. 2014; Norberg and Persson 1984; Sağ and Kutsal 1995). In addition, we found that *Thauera* and *Dechloromnas* were resistant to Cu toxicity and maintained high abundances under the highest Cu exposure (e.g., 28.2 mg/L), which significantly reduced the abundances of other bacteria. *Thauera* and *Dechloromnas* are two frequently detected genera of the *Beta-proteobacteria* under heavy metal exposures since they possess orthologous Cu resistance proteins (Chihomvu et al. 2015; Martins et al. 2010; Wu et al. 2013).

The abundance of *Acidobacteria* and *Planctomycetes* at the phylum level exhibited similar trends along the Cu exposure gradient. *Acidobacteria* contain many genera that contribute to COD degradation, while increased Cu stress reduced the abundance of all *Acidobacteria* at the genus level in this study (Fig. 4c). *Planctomycetes* has been reported to have great metabolic versatility (Chouari et al. 2003), and the *Planctomycetes* group also includes chemoorganotrophs, autotrophs, and phototrophs (Fuerst 1995; Miskin et al. 1999). In this study, one of the major genera of *Planctomycetes* was *Planctomyces* (Fig. 4c), which included aerobic chemoheterotrophs and could play an important role in COD degradation.

Our results suggest that *Bacteroidetes* were more tolerant to Cu toxicity than *Acidobacteria* and *Planctomycetes*. Ma et al. (2015b) observed a distinct increase in *Bacteroidetes* in activated sludge at the end of continual loadings of Ag^+ (gradually increased from 0.1 to 20.0 mg/L). However, they did not investigate the shift in *Bacteroidetes* abundance along the Ag^+ loading process. Another study reported random variations in *Bacteroidetes* abundance in response to an increase in Cr (VI) stress using Illumina sequencing datasets (Miao et al. 2015). It seems that the response of *Bacteroidetes* to heavy metals varies with experimental conditions and metal species.

In addition to the impact of Cu, there may be competitive/inhibitory interactions among coexisting bacteria within the

bacterial community. For example, nitrification generally leads to a low pH in the reactor, since it is a process of alkalinity consumption. Our results indicate that inhibition of nitrification was accompanied by a corresponding increase in the pH value of the effluent (Fig. 1d). Among the detected microbes, *Acidobacteria* are known to be very sensitive to pH (Ward et al. 2009). Therefore, a significant increase in pH could aggravate the inhibition on *Acidobacteria*.

Effects of Cu on the nitrifying bacterial community

When exposed to Cu, the nitrifying bacterial community changed both in abundance and species. During Cu-loading cycles of 5.5–9.2 mg/L Cu (S_1 and S_3) and the corresponding recovery periods (S_2 and S_4), the total abundances of the nitrifying bacteria kept increasing with running cycles, and the structure of the nitrifying community shifted. Meanwhile, nitrification appeared to be only slightly inhibited during S_1–S_4 (Fig. 1b). This suggests that nitrifying bacterial community changed its structure to adapt to the toxic effect caused by Cu. It has been reported that the shifts in nitrifying communities from the non-tolerant to the tolerant species can reduce the toxic effects of heavy metals (Demanou et al. 2006). The loading concentrations of 5.5 and 9.2 mg/L of Cu may place a selective pressure on nitrifying communities and result in an enrichment of certain Cu-tolerant AOB and NOB, such as OTU 275, OTU 91, OTU20, and OTU40. Increases in the abundance of the nitrifying bacteria were still observed in the 10.9 and 13.5 mg/L Cu-loading cycles (S_5 and S_7). However, in S_6, S_8, and S_{10} (the three recovery periods after loadings of 10.9, 13.5, and 28.2 Cu mg/L, respectively), abundances of the nitrifying bacteria notably decreased. This decrease may explain the inhibition on nitrification efficiency in these recovery cycles (Fig. 1b).

Various AOB (or NOB) groups had different favorable levels of Cu. A moderate level of Cu could reduce certain nitrifying bacteria while simultaneously facilitating the proliferation of other nitrifying bacteria, leading to a phylogenetic change in the nitrifying bacterial community. However, a Cu loading of 28.2 mg/L exceeded the Cu tolerance threshold of the majority of the nitrifying bacteria. *Nitrosomonas* was found to be the main AOB group in this study. This is consistent with past studies showing *Nitrosomonas* as one of the most widely found AOB groups in activated sludge systems and resistant to the changing environment (Bai et al. 2012). However, some researchers found that the *Nitrosospira* group was one of the main AOB group and seemed to be more tolerant to heavy metal toxicity than the *Nitrosomonas* group (Ma et al. 2015a). Therefore, AOB composition in wastewater treatment systems should be evaluated by case. The majority of the detected NOB groups belonged to *Nitrospira*, which could make a significant contribution to the nitrite oxidizing process in our activated sludge system. *Nitrobacter* was the second major NOB groups in our study, yet it seemed to have a weaker adaptation to Cu toxicity than *Nitrospira*.

The present study used a high-throughput sequencing approach to elucidate the effect of Cu on the diversity and community structure of the overall bacteria and nitrifiers in activated sludge. It was found that *Zoogloea*, *Thauera*, and *Dechloromonas* (within the *Rhodocyclaceae* family of the *Beta-proteobacteria* class) were the dominant genera in the presence of Cu. In addition, we found that *Nitrosomonas* and *Nitrospira* were the predominant nitrifiers, and different nitrifiers had various responses to the Cu toxicity. The nitrifying bacterial community changed through increasing the absolute abundance and shifting from the non-tolerant to the tolerant species as a way to reduce the toxic effects of Cu. The local nitrifying bacterial database we developed helped to improve the resolution of bacterial identification. Our results provide insights into the dynamics of the overall and nitrifying bacteria community under the stress of various metal concentrations and improve our understanding regarding the effect of metals on wastewater treatment efficiency.

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Compliance with ethical standards

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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