Bioresource Technology 211 (2016) 209–215

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Illumina MiSeq sequencing reveals long-term impacts of single-walled carbon nanotubes on microbial communities of wastewater treatment systems

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HIGHLIGHTS

- Long-term impacts of carbon nanotubes on microbial communities were investigated.
- Single-walled carbon nanotubes could have positive effects on removal of phenol.
- Microbial communities significantly shifted under phenol shock loading.
- Indigenous bacterial communities were responsible for phenol removal.

ARTICLE INFO

Article history:
Received 31 December 2015
Received in revised form 4 March 2016
Accepted 8 March 2016
Available online 11 March 2016

Keywords:
Single-walled carbon nanotubes
Phenol
Microbial community
Illumina MiSeq sequencing

ABSTRACT

In this study, phenol wastewater treatment systems treated with different concentrations of single-walled carbon nanotubes (SWCNTs) (0–3.5 g/L) were exposed to phenol and carbon nanotubes (CNTs) shock loadings to investigate the long-term impacts of SWCNTs on microbial communities. Phenol removal remained high efficiency (>98%) in SWCNTs-treated groups but decreased in non-treated group (85.1 ± 1.9%) when exposed to high concentration of phenol (500 mg/L). However, secondary dosing of SWCNTs in SWCNTs-treated groups would decrease the phenol removal efficiency. Illumina MiSeq sequencing revealed that the diversity, richness and structure of microbial communities were shifted under phenol shock loading, especially under high phenol concentration, but not under CNTs shock loading. In response to phenol and CNTs shock loadings, *Rudaea*, *Burkholderia*, *Sphingomonas*, *Acinetobacter*, *Methylocystis* and *Thauera* became dominant genera, which should be involved in phenol removal. These results suggested that a proper amount of SWCNTs might have positive effects on phenol wastewater treatment systems.

1. Introduction

Carbon nanotubes (CNTs), e.g. single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs), have been incorporated into diverse commercial products due to their unique chemical, physical and electrical properties (De Volder et al., 2013). The extensive manufacture and utilization of CNTs may lead to their release and accumulation in environment (soil, water, air and sediment), which raises a serious concern about their potential impacts on environmental and human health (Köhler et al., 2008; Petersen et al., 2011). Wastewater treatment plants are generally used to clean the sewage from home and businesses before safely released into environment, and the microbial communities of activated sludge from the biological process play a critical role in the removal of organic and inorganic pollutants (Hu et al., 2012; Ma et al., 2015a; Shu et al., 2015). Once the CNTs-containing sewage enters into wastewater treatment plants, which is almost inevitable, whether the CNTs would affect the microbial communities responsible for pollutants removal is worthy of attention (Petersen et al., 2011).

The impacts of CNTs on microbial communities of activated sludge are gradually being studied in recent years, but the roles of CNTs are still controversial. Hai et al. (2014) found that MWCNTs

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http://dx.doi.org/10.1016/j.biortech.2016.03.043
0960-8524/

had adverse effects on wastewater nutrient removal, and they would also inhibited the activities of key enzymes, such as ammonia monooxygenase, nitrite oxidoreductase, exopolyphosphatase and polyphosphate kinase. On the contrary, Zheng et al. (2014) found that neither the removal of nitrogen and phosphorus nor the activities of key enzymes were significantly affected by CNTs (including SWCNTs and MWCNTs). Furthermore, the toxicity of CNTs on microbial communities is suggested to be related with the concentration, physical properties (length and diameter) and types of functionalization (carboxylic and hydroxylic) (Goyal et al., 2010; Luongo and Zhang, 2010; Oleszczuk et al., 2011; Parise et al., 2014; Kerfahi et al., 2015). The floc architecture and original microbial community structure of activated sludge also have a profoundly influence on the behaviors and effects of CNTs (Petersen et al., 2011; Ma et al., 2015b). Thus, it is necessary to investigate the impacts of CNTs on activated sludge of wastewater treatment systems from different perspectives, which may provide a helpful guide for the safe use and disposal of nanomaterials. Recently, with the development of high throughput metagenomic technologies, it enables us to analyze the microbial community in a more comprehensive and informative way (Roh et al., 2010; Zhou et al., 2015), which can contribute to the research on ecological effects of CNTs.

In previous study, five groups of sequencing batch reactors (SBRs) dosing with 0, 0.5, 1.5, 2.5 and 3.5 g/L SWCNTs, respectively, were constructed for the treatment of phenol wastewater (Qu et al., 2015). The results showed that SWCNTs exerted protective effects on sludge microbes, but they would also alter the structure and diversity of microbial community. Herein, the SBRs continued to run under phenol and CNTs shock loading conditions, and the long-term impacts of SWCNTs on the microbial communities of phenol wastewater treatment systems were assessed with the aid of Illumina MiSeq sequencing technology. The present study should provide insights into the understanding of SWCNTs nanotechnology.

2. Methods

2.1. Chemicals and activated sludge

SWCNTs (>95%) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China), and the characteristics of the SWCNTs have been described previously (Shen et al., 2013; Qu et al., 2015). All other chemicals were of analytical grade. Activated sludge was collected from Chunliu River Wastewater Treatment Plant (Dalian, China).

2.2. Experimental setup

Five groups of SBRs were constructed previously (Qu et al., 2015), and each group was performed in triplicate under identical conditions (Fig. S1). The synthetic wastewater consisted of 20 mg/L KH_2PO_4, 90 mg/L NH_4Cl, 10 mg/L NaCl, 12.5 mg/L $MgSO_4{\cdot}7H_2O$, 12 mg/L $CaCl_2$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 785 mg/L glucose and 200–500 mg/L phenol. SWCNTs were added directly to each SBR at the beginning of operation with different concentrations (g/L): 0 (G1), 0.5 (G2), 1.5 (G3), 2.5 (G4) and 3.5 (G5). No additional SWCNTs were supplemented during the operation unless otherwise indicated. Each cycle of SBR was operated for 24 h, including 2 h filling, 18 h reacting, 2 h settling, and 2 h decanting. To simulate phenol shock loading, the SBRs were operated for 40 days with the concentrations of phenol in influent increasing from 200 mg/L (0–20 days) to 500 mg/L (20–40 days). Samples were collected at Day 0, 20 and 40 for Illumina MiSeq sequencing analysis. Afterwards (41st day after phenol shock), extra SWCNTs (2.5 g/L) was added into G4 to investigate the effects of CNTs shock loading on the performances of wastewater treatment systems and microbial communities. The SBRs were operated with the synthetic wastewater containing 500 mg/L phenol for another 20 days, and samples were taken at Day 0, 1 and 20 (designated as Day C0, C1 and C20) for sequencing analysis. During the operation process, the concentrations of phenol in influent and effluent were monitored daily, and the concentrations of mixed liquor suspended solid (MLSS), the sludge volume after 0.5 h of settling (SV_{30}) and the pH of effluent were measured at certain days.

2.3. Analytical methods

To determine the concentrations of phenol, the effluent samples were centrifuged at 10,000*g* for 10 min to remove suspended sludge, and the supernatants were analyze using a JASCO V-560 UV–vis spectrophotometer (Japan). MLSS and SV_{30} were measured using the standard methods, and the pH of effluents was determined by a Mettler-Toledo S30 pH meter (Switzerland).

2.4. DNA extraction, PCR and Illumina MiSeq sequencing

The genomic DNA was extracted from the activated sludge samples using the protocol reported by Purkhold et al. (2000), and the V4 region of 16S rRNA gene was amplified using the primer set 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). PCR amplification was carried out based on the methods described previously (Qu et al., 2015; Shi et al., 2015; Zhang et al., 2015), and the resulting PCR amplicons were used for sequencing on Illumina MiSeq platform at the Institute for Environmental Genomics, University of Oklahoma.

2.5. Data analysis

After sequencing, the data processing was conducted as described previously (Ma et al., 2015a; Qu et al., 2015; Zhang et al., 2015). Operational taxonomic units (OTUs) were generated using CD-HIT method, and the taxonomic assignment of OTUs were performed by RDP classifier. The Shannon diversity index (H), Pielou's evenness index (J), species richness estimator of Chao1

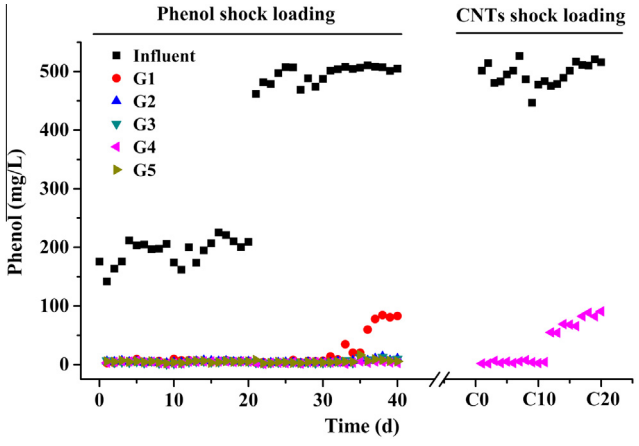

Fig. 1. Phenol removal performances of the SBRs under phenol and CNTs shock loading conditions. SBRs were treated with different concentrations of SWCNTs (g/L): 0 (G1), 0.5 (G2), 1.5 (G3), 2.5 (G4) and 3.5 (G5). Each group was performed in triplicate. For phenol shock loading, the concentrations of phenol in influent was increased from 200 mg/L (0–20 days) to 500 mg/L (20–40 days). For CNTs shock loading, 2.5 g/L SWCNTs was additionally added into G4 and the concentrations of phenol in influent were maintained at 500 mg/L. C0, C1 and C20 represent the sampling time of Day 0, Day 1 and Day 20, respectively, under CNTs shock loading conditions.

Table 1
Alpha diversity of activated sludge samples at each sampling time.

Index	Phenol shock loading: Day 0					Phenol shock loading: Day 20				
	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
Shannon	4.692	4.122	4.167	4.417	4.380	3.948	4.517	4.594	4.308	4.688
Evenness	0.648	0.595	0.590	0.630	0.623	0.564	0.637	0.651	0.612	0.660
Chao1	2080	2010	2027	2151	2119	1937	2412	2154	2204	2231
OTU	1467	1027	1166	1113	1131	1099	1208	1164	1143	1216
Index	Phenol shock loading: Day 40					CNTs shock loading				
	G1	G2	G3	G4	G5	Day C0	Day C1		Day C20	
Shannon	3.947	4.184	4.131	4.092	4.366	3.991	4.166		4.066	
Evenness	0.569	0.602	0.589	0.592	0.618	0.571	0.592		0.579	
Chao1	1874	1878	2035	1889	2173	2182	2192		1924	
OTU	1042	1048	1106	1006	1175	1083	1132		1115	

and rarefaction curves were generated using Mothur program. The statistics analyses, including detrended correspondence analysis (DCA), multiple-response permutation procedure (MRPP), permutational multivariate analysis of variance (Adonis), analysis of similarity (ANOSIM) and Pearson correlation test, were performed using R environment (version 3.2.1; http://www.r-project.org/). Heat map was generated using HemI (Heatmap Illustrator, version 1.0) (Deng et al., 2014).

3. Results and discussion

3.1. Phenol removal performance of SBRs systems

The 15 SBRs were operated for more than two months, and the MLSS, SV_{30} and effluent pH had no significant changes (Fig. S2), indicating the systems were stable during the operation. Fig. 1 depicts the long-term phenol removal performances of the SBRs. When phenol concentration was around 200 mg/L, each of the SBRs exhibited excellent phenol removal efficiencies (>98%), which was in accordance with the previous study (Qu et al., 2015). When phenol concentration increased to 500 mg/L, the SBRs treated with different concentrations of SWCNTs maintained high efficiencies of phenol removal (>98%), while the phenol removal efficiency of G1 declined from 98.8% to 84.0% over the long-term operation. These results suggested the positive influences of SWCNTs on phenol wastewater treatment systems under phenol shock loading conditions over the long-term operation.

To further investigate the effects of CNTs shock loading on wastewater treatment efficiency of SBRs, G4 was chosen and inoculated with an additional dose of 2.5 g/L SWCNTs. During the first 10 days of operation, the phenol removal efficiencies had no significant changes and remained around 99%. However, after 20 days of exposure, the phenol removal efficiencies significantly decreased (<85%). The results indicated that secondary high dosing of CNTs would negatively affect reactor performance.

Although SWCNTs could be used as the efficient absorbents for the removal of aromatics, SWCNTs could not improve the absorption of phenol when added into the activated sludge since there were no differences among the autoclaved sludge systems with or without SWCNTs (Qu et al., 2015). Thus, the indigenous bacteria should play the most significant roles in the removal of phenol.

3.2. Shifts in microbial communities of SBRs

To investigate the microbial community shifts in response to phenol and CNTs shock loading, Illumina MiSeq sequencing analysis was performed. After removing low-quality reads, at least 35,700 effective sequences were obtained and normalized for each sample. The sequences were then grouped into OTUs at a clustering threshold of 0.97 using CD-HIT method, and the Chao1 estimator was calculated using Mothur program. It was found that the microbial richness (OTUs and Chao1) slightly increased from Day 0 to Day 20, except G1, but then significantly decreased at Day 40 (ANOVA, $P < 0.05$) (Table 1). Likewise, both Shannon (H) and evenness (J) indices showed the similar variation as the richness indices (Table 1). These results indicated that high concentrations of phenol would reduce the α-diversity of microbial community due to the toxicity effects of phenol on microorganism. When facing CNTs shock loading, the microbial richness and diversity had no significant changes upon either short-term (1 day) or long-term (20 days) exposure ($P > 0.10$) (Table 1).

To assess the overall variation of microbial communities in response to phenol and CNTs shock loading, DCA analysis was performed. As shown in Fig. 2, the communities underwent a clear

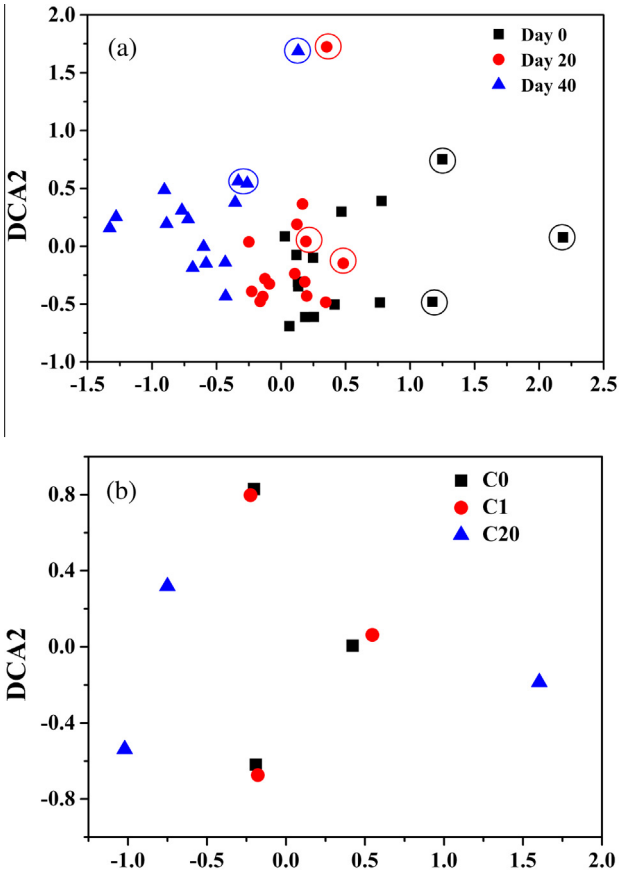

Fig. 2. DCA analysis of microbial communities under phenol (a) and CNTs (b) shock loading conditions. The samples in circle represent the microbial communities of G1 at different sampling time. C0, C1 and C20 represent the sampling time of Day 0, Day 1 and Day 20, respectively, under CNTs shock loading conditions.

shift from Day 0 to Day 40 with the concentrations of phenol in influent increasing from 200 to 500 mg/L. The results of three dissimilarity tests, i.e. Adonis, ANOSIM and MRPP, showed that the community structures at Day 0, 20 and 40 were significantly different from each other ($P < 0.05$) (Table S1). However, the CNTs shock loading had no significant effects on microbial communities as observed from DCA plots and dissimilarity tests ($P > 0.10$) (Fig. 2 and Table S1).

3.3. Microbial community structure analysis

To identify taxonomic diversity of microbial communities from these 15 SBRs, RDP identifier was employed to assign the sequence tags to different taxonomic levels (from phylum to genus) at 50% threshold. At the phylum level, a total of 30 phyla were detected and the majority belonged to 10 phyla, which covered over 97% of the effective sequence tags. *Proteobacteria* was the most abundant phylum in all samples, accounting for 58.1–81.4% of total sequences (Fig. 3a). Other dominant phyla included *Bacteroidetes* (5.3–15.7%), *Acidobacteria* (3.1–9.7%) and *Planctomycetes* (1.9–6.7%). Notably, the abundances of *Proteobacteria* in G1 were significantly higher ($P < 0.05$) than those in other four groups under phenol shock loading conditions (at Day 20 and Day 40). While under CNTs shock loading conditions, the abundances of *Proteobacteria* had almost no changes after 1 day of operation ($P > 0.10$) but significantly decreased after 20 days ($P < 0.05$) (Fig. 3a). Besides, the abundances of *Acidobacteria* and *Planctomycetes* in G1 were seemed to be lower than those in SWCNTs-treated groups.

Within *Proteobacteria*, *Gammaproteobacteria* (11.0–36.8%), *Alphaproteobacteria* (13.2–36.1%) and *Bataproteobacteria* (9.8–27.7%) were more abundant in all samples (Fig. 3b). The abundances of *Gammaproteobacteria* showed similar patterns as *Proteobacteria* under phenol and CNTs shock loading conditions, which decreased after long-term exposure to SWCNTs (Fig. 3b). These results indicated that SWCNTs might have adverse effects on *Proteobacteria* under long-term exposure, especially for *Gammaproteobacteria*. On the contrary, previous study by Hai et al. (2014) indicated that MWCNTs would increase the abundances of *Proteobacteria* in activated sludge, especially for *Alphaproteobacteria* and *Gammaproteobacteria*.

Among the 527 assigned genera, the 10 most abundant genera in each treatment at different sampling time were used for heat map analysis, and a total of 17 genera were selected for comparison

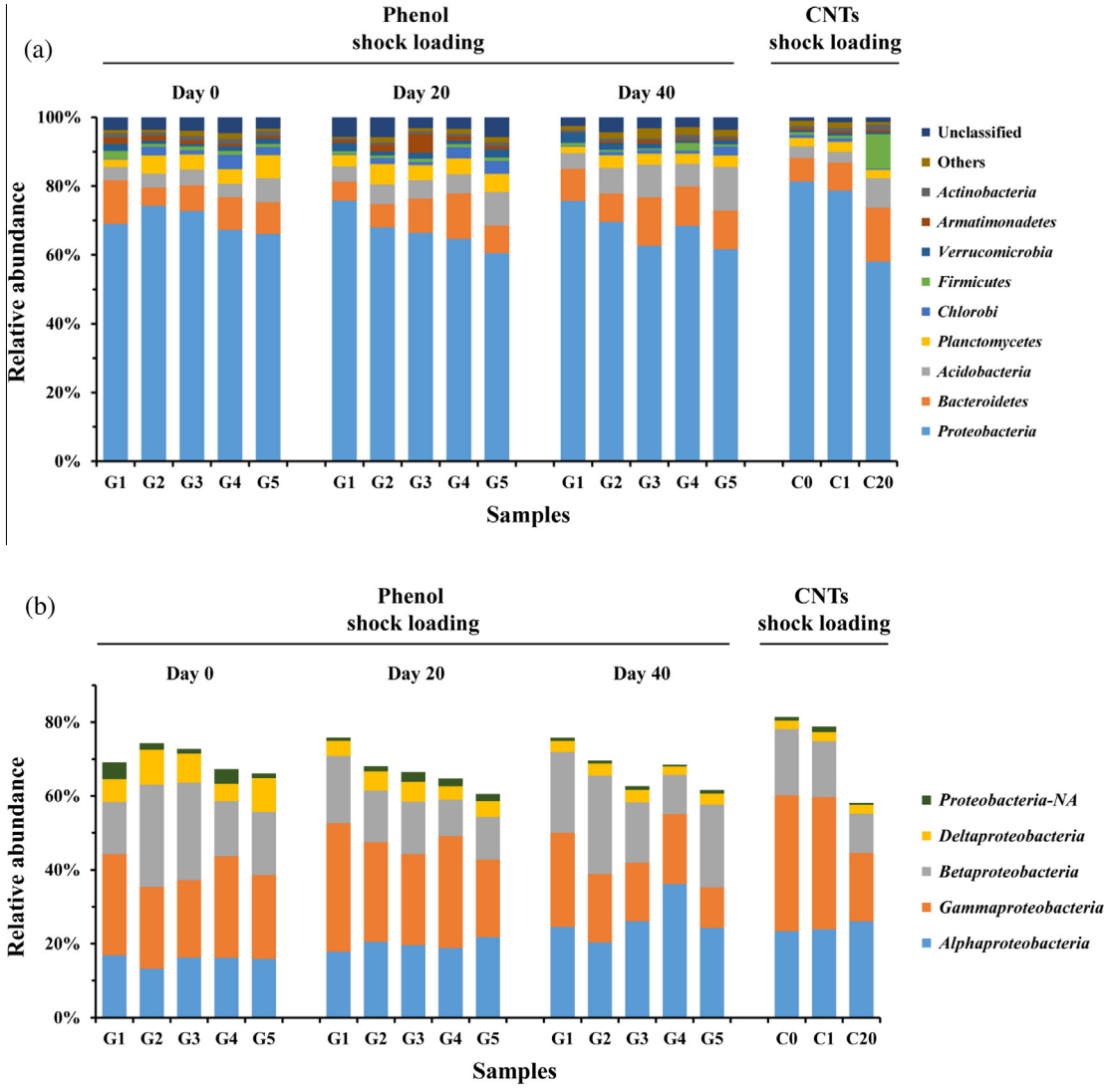

Fig. 3. Relative abundance of phyla (a) and Proteobacterial class (b) in the SBRs. The relative abundance was displayed in terms of percentage in total effective sequences in each sample from the triplicated systems. C0, C1 and C20 represent the sampling time of Day 0, Day 1 and Day 20, respectively, under CNTs shock loading conditions.

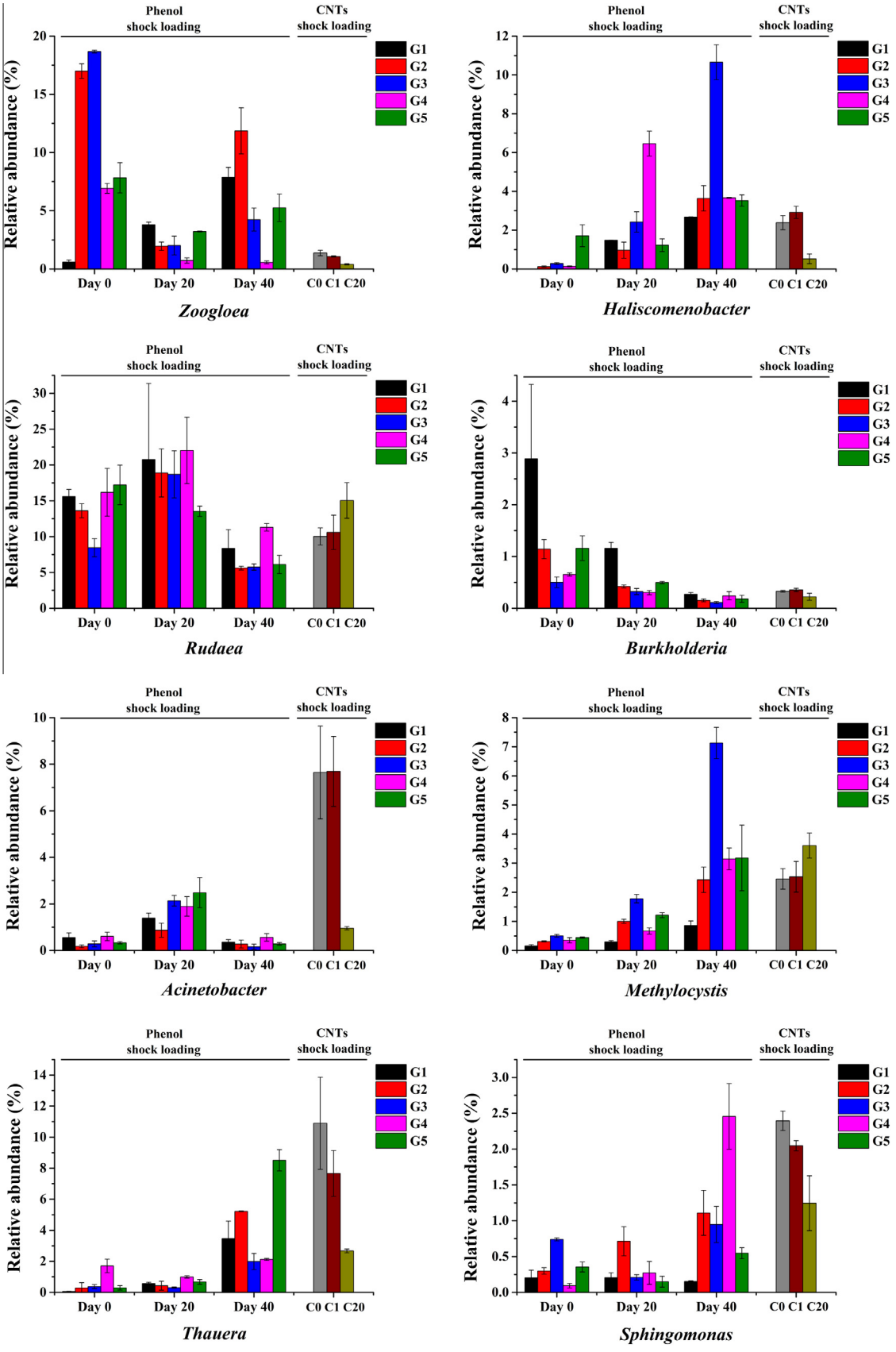

Fig. 4. Relative abundance of the dominant genera in the SBRs. The relative abundance was displayed in terms of percentage in total effective sequences in each sample from the triplicated systems. C0, C1 and C20 represent the sampling time of Day 0, Day 1 and Day 20, respectively, under CNTs shock loading conditions.

(Fig. S3). Among the dominant genera, *Zoogloea* and *Haliscomenobacter* could be related to sludge settling properties, while *Rudaea*, *Burkholderia*, *Acinetobacter*, *Methylocystis*, *Thauera* and *Sphingomonas* could play important roles in phenol degradation.

Zoogloea and *Haliscomenobacter* were widely spread in activated sludge, which have been regarded as floc-forming and filamentous bacteria, respectively (Guo and Zhang, 2012; Zhao et al., 2013). *Zoogloea* played a key role in the flocculation of activated sludge, while *Haliscomenobacter* could be related to sludge bulking and foaming. On Day 0, the relative abundances of *Zoogloea* in G2-G4 were higher than that in G1 (Fig. 4), suggesting that SWCNTs might have a positive effect on sludge settling ability, which was in accordance with the results of SV_{30} measurement (Fig. S2). Under phenol shock loading conditions, the abundances of *Zoogloea* in SWCNTs-treated groups decreased firstly and then increased when phenol concentration was raised, while the abundances of *Haliscomenobacter* increased in each treatment. Under CNTs shock loading conditions, the long-term exposure would significantly decrease the abundance of *Haliscomenobacter*. The results indicated that SWCNTs might significantly affect the settling ability of activated sludge (Yin et al., 2009; Hai et al., 2014; Qu et al., 2015).

Rudaea was detected in all treatments with considerably high abundance (5.6–22.0%) (Fig. 4). The abundance of *Rudaea* decreased when the concentration of phenol was 500 mg/L, but it would be improved after the long-term operation under CNTs shock loading conditions. Previous studies have indicated that genus *Rudaea* was detected in soil and activated sludge contaminated with aromatic compounds, such as biphenyl, naphthalene and phenol (Uhlik et al., 2012; Qu et al., 2015). Thus, *Rudaea* could have a considerable potential in bioremediation of aromatic pollutants (such as phenol).

Burkholderia and *Acinetobacter* showed high versatility in genetic diversity and metabolic capacity, which enabled them to degrade various natural and xenobiotic pollutants, such as toluene, phenols and polychlorobiphenyls (Mahenthiralingam et al., 2005; Al-Khalid and El-Naas, 2012). The relative abundance of *Burkholderia* decreased during the long-term operation, while the relative abundance of *Acinetobacter* had a slight increase under low concentration of phenol (200 mg/L), but then decreased after long-term exposure to either high phenol concentration or CNTs shock loading (Fig. 4).

Methylocystis, *Thauera* and *Sphingomonas* all increased in relative abundances as the phenol concentration increased, especially in SWCNTs-treated groups when phenol concentration was raised to 500 mg/L (Fig. 4). However, the CNTs shock loading would also decrease the abundances of *Thauera* and *Sphingomonas*. Previous studies have shown that *Methylocystis* could be used for the degradation of chlorinated aromatic compounds (e.g. chlorobenzene) and trichloroethylene (McDonald et al., 1997; Jechorek et al., 2003), while *Thauera* was an important population in wastewater treatment plants (Liu et al., 2006; Ma et al., 2015a), which had a versatile capacity to degrade a wide spectrum of aromatic compounds, such as phenol, indole and toluene (Shinoda et al., 2004; Mao et al., 2010). *Sphingomonas* possessed extraordinary metabolic versatility, which could be used to degrade a wide range of recalcitrant natural and anthropogenic compounds, such as biphenyl(s), naphthalene(s), phenols and pesticides (Stolz, 2009). Therefore, *Methylocystis*, *Thauera* and *Sphingomonas* could be more responsible for the efficient removal of phenol during the operation.

3.4. Effects of SWCNTs on microbial community

In previous studies, CNTs, in most cases, exhibited adverse or no significant effects on microbial communities (Hai et al., 2014; Zheng et al., 2014; Kerfahi et al., 2015). In contrast, the present study suggested that SWCNTs could have positive influences on phenol wastewater treatment systems. The effects of SWCNTs on microbial community could be observed in three aspects. Firstly, SWCNTs could change the richness and diversity of microbial community. It was noticed that the microbial richness (OTU and Chao1) and diversity (Shannon and evenness) of SWCNTs-treated groups (G2-G5) were a little higher than those of non-treated group (G1) at Day 20 and Day 40 (Table 1), suggesting that SWCNTs might have protective effects against phenol. Previous studies indicated that SWCNTs could induce the microbial community to produce more extracellular polymeric substance, which might protect the microbes from the toxicity of phenol (Yin et al., 2009; Qu et al., 2015), thus leading to the high level of richness and diversity in SWCNTs-treated groups.

Secondly, SWCNTs could alter the composition and structure of microbial community. DCA plots showed that the communities of G1 clustered separately from other groups treated with different concentrations of SWCNTs (Fig. 2), which was further confirmed by dissimilarity tests (Table S1), suggesting the addition of SWCNTs would affect the assembly of microbial communities. In previous studies, although CNTs (SWNCTs and MWCNTs) might have positive or adverse effects on wastewater treatment efficiency, they could alter the diversity and structure of microbial communities in activated sludge (Goyal et al., 2010; Hai et al., 2014; Ma et al., 2015b; Qu et al., 2015).

Thirdly, SWCNTs could affect the taxa and function of microbial community. To discern the impact of SWCNTs on microbial community composition, Pearson correlation between the abundance of different taxa and the concentration of SWCNTs was examined. The results indicated that more taxa at the class, family and genus level showed positive relationships rather than negative, especially after the long-term exposure to SWCNTs (Table S2). Among the dominant genera, *Acinetobacter*, *Methylocystis*, *Thauera* and *Sphingomonas* were reported to be capable of degrading various xenobiotic pollutants (Al-Khalid and El-Naas, 2012; Jechorek et al., 2003; Mao et al., 2010; Stolz, 2009). Thus, the higher abundances of these genera in SWCNTs-treated groups than the non-treated group might contribute to the better efficiency of phenol removal. Although the diversity and structure of microbial community had no significant changes under CNTs shock loading condition, the dominant genera *Acinetobacter*, *Thauera* and *Sphingomonas* decreased notably after the long-term exposure, which might cause the fluctuations in phenol removal.

4. Conclusions

The long-term impacts of SWCNTs on microbial communities of wastewater treatment systems were determined. SWCNTs might have positive effects on phenol removal, but secondary high dosing of SWCNTs would also have adverse effects on systems in long-term operation. Phenol shock loading, rather than CNTs shock loading, would significantly affect the diversity, richness and structure of microbial communities. The dominant genera *Rudaea*, *Burkholderia*, *Acinetobacter*, *Methylocystis*, *Thauera* and *Sphingomonas* should be more responsible for the high efficiency of phenol removal. This study should provide important information for comprehensive analysis of the environmental effects of CNTs.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 21176040 and 51508068), the Program for New Century Excellent Talents in University (No. NCET-13-0077), the Fundamental Research Funds for the Central Universities (No. DUT14YQ107), and the Open Project of State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of

Technology (No. ESK201529). The authors would like to thank Jizhong Zhou and Yujia Qin at the Institute for Environmental Genomics, University of Oklahoma for Illumina MiSeq sequencing technical help.

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.biortech.2016.03.043.

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