Activated sludge microbial community responses to single-walled carbon nanotubes: community structure does matter

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ABSTRACT

The ecological effects of carbon nanotubes (CNTs) have been a worldwide research focus due to their extensive release and accumulation in environment. Activated sludge acting as an important gathering place will inevitably encounter and interact with CNTs, while the microbial responses have been rarely investigated. Herein, the activated sludges from six wastewater treatment plants were acclimated and treated with single-walled carbon nanotubes (SWCNTs) under identical conditions. Illumina high-throughput sequencing was applied to in-depth analyze microbial changes and results showed SWCNTs differently perturbed the alpha diversity of the six groups (one increase, two decrease, three no change). Furthermore, the microbial community structures were shifted, and specific bacterial performance in each group was different. Since the environmental and operational factors were identical in each group, it could be concluded that microbial responses to SWCNTs were highly depended on the original community structures.

Key words | activated sludge, diversity, high-throughput sequencing, microbial community, single-walled carbon nanotubes

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INTRODUCTION

Carbon nanotubes (CNTs) have been extensively used in diverse industry fields due to their unique physico-chemical properties (Baughman *et al.* 2002). Consequently, more and more CNTs are released into environmental matrices. CNTs have been proven to be toxic to pure culture strains through oxidative stress and membrane perturbation (Kang *et al.* 2007, 2009; Rodrigues & Elimelech 2010). In the meantime, the discharge and accumulation of CNTs in soil and aquatic environments would also impact indigenous microbial community structures and activities (Chung *et al.* 2011; Petersen *et al.* 2011; Khodakovskaya *et al.* 2013); thus study of the ecological effect of CNTs is being put on the agenda nowadays. For example, Jin *et al.* (2013) found that high concentration of CNTs significantly decreased enzyme activities and biomass in an urban soil, and Rodrigues *et al.* (2012) pointed out that single-walled carbon nanotubes (SWCNTs) could negatively affect the fungi and bacteria associated with carbon and phosphorus biogeochemical cycling. Nevertheless, studies addressing CNT ecological effects are limited and sparse information is available.

Previous research has shown that the antimicrobial activity of CNTs is closely associated with their physico-chemical properties such as sizes, aspect ratios, functional modifications and electronic structures (Kang *et al.* 2008; 2009; Tong *et al.* 2012). Furthermore, the environmental variables such as natural organic matter, ionic strength and CNT aggregation state in the environment highly determine the toxicity level (Kang *et al.* 2009; Petersen *et al.* 2011). The diverse microbial communities also complicate CNT cytotoxicity in the real environmental scenario. Microbes in the microbial communities will interact with each other (i.e. commensalism, competition, predation, synergism), which in turn affects the microbial ecological behavior (Brenner *et al.* 2008). Therefore, it can be speculated that a microorganism may display distinct responses to CNT addition if in different microbial communities. However, the answer to whether the microbial community structure of a certain biotope will affect CNT ecological effects remains unknown.

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Activated sludge is acting as one of the most important receptors for CNT release as increasing CNT-related products are directly or indirectly released into the sewages (Goyal *et al.* 2010; Neale *et al.* 2013). Yet the impacts of CNTs on sludge community are poorly understood and more experimental attempts should be made to fill the knowledge gap, aiming to predict and assess CNT ecological effects. Among the limited reports, most studies were performed using traditional DNA fingerprint techniques such as denaturing gradient gel electrophoresis and terminal-restriction fragment length polymorphism analyses (Nyberg *et al.* 2008; Rodrigues & Elimelech 2010); thus the overall response of microbes (i.e. diversity and abundance change) has been greatly ignored due to technical low-resolution restrictions. High-throughput sequencing (HTS) has been widely used in various biological studies recently, and can provide massive amounts of information on community analysis (Deng *et al.* 2012; Shrestha *et al.* 2013). But as far as we know, few studies have explored the CNT and sludge community interactions using HTS technologies.

Herein, the responses of microbial communities from six different activated sludges treated with identical operations to SWCNTs were investigated using Illumina HTS for the first time, aiming to explore the specific microbial behavior towards SWCNTs in different community systems.

MATERIALS AND METHODS

SWCNTs and activated sludges

SWCNTs (>95%) were purchased from Shenzhen Nanotech Port Co., Ltd (Shenzhen, China) and detailed information regarding the SWCNTs has been described previously (Shen *et al.* 2013). The SWCNT length was 5–15 μm with diameter less than 2 nm. The carbon content of SWCNTs was above 95% and the effects of metal impurities in SWCNTs were not investigated since it had been proven that they had insignificant impacts on activated sludge communities (Goyal *et al.* 2010; Tong *et al.* 2012). SWCNTs were suspended in the distilled water by ultrasonic treatment for 30 min to obtain better dispersion. The activated sludges were gathered from the secondary sedimentation tank of six different Chinese coking wastewater treatment plants in Zhangjiagang, Taiyuan, Shanghai, Jinan, Xingtai and Tangshan, designated as G1, G2, G3, G4, G5 and G6, respectively.

Experimental design

To eliminate the residual substances in original sludges, sludge samples were no-effluent aerated for 24 h. Activated sludge was acclimatized in 250 mL simulated sequencing batch reactors (SBRs) using synthetic wastewater which 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 $FeSO_4{\cdot}7H_2O$ and 1,200 mg/L glucose (C:N:P = 100:5:1). Each cycle of SBRs was operated for 24 h. After acclimation for dozens of cycles to reach steady state (chemical oxygen demand removal rates above 95%), each sludge was divided and transferred into three parallel SBRs. In the meantime, 3.5 g/L SWCNT was added into each parallel SBR to mimic CNT shock loading. All the operation conditions for each sample were identical with the same influent. The activated sludge samples before and after SWCNT addition (24 h) were taken and stored at −80 °C until use (Figure S1, available online at http://www.iwaponline.com/wst/071/095.pdf).

DNA extraction, amplification and sequencing

DNA extraction followed the method of Purkhold *et al.* (2000). The primers 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′) were used to amplify the V4 region of bacterial and archaeal 16S rRNA genes (Bates *et al.* 2010). Polymerase chain reaction (PCR) was conducted using AccuPrime High Fidelity *Taq* Polymerase (Invitrogen, Carlsbad, CA, USA) in triplicate. PCR products were pooled, purified through a QIAquick Gel Extraction Kit (Qiagen), and quantified by Pico Green analysis. The 16S rRNA HTS was conducted on Illumina MiSeq platform in IEG, USA (http://ieg.ou.edu/).

Sequencing data analysis

The raw sequences were cleaned using Flash, Mothur and UCHIME programs through the pipeline (http://zhoulab5.rccc.ou.edu/). Operational taxonomic units (OTUs) were generated using CD-HIT method at 97% sequence similarity threshold, and the taxonomic assignment of OTUs was performed by RDP classifier with 50% confidence. Alpha diversity calculation and detrended correspondence analysis (DCA) were carried out by R software v2.15.1. Hierarchical clustering analysis was performed using Cluster and visualized using Treeview (Ma *et al.* 2015).

RESULTS AND DISCUSSION

Overview of the sequencing data

After removing chapters, primers, low quality sequences and chimeras, the sequence number for each sample was normalized to 15,000. The resulting sequences were sorted by CD-HIT and 200–337 OTUs were generated. Sequences for the three parallel sludges in each group were similar and clustered by hierarchical clustering analysis (Figure S2, available online at http://www.iwaponline.com/wst/071/095.pdf), suggesting Illumina sequencing was credible and reproducible. Rarefaction curves of each sludge group (Figure 1(a)) did not reach the plateau, indicating that there were still some species undetermined.

The resulting OTUs were aligned by RDP classifier with 50% confidence. The primers of sequencing were designed for the V4 region of 16S rRNA gene amplicons, and nearly all the sequences (99.9%) were assigned to bacteria (Bates *et al.* 2010). Thus, the following analysis was concentrated on bacterial compositions and shifts. As shown in Figure 1(b), there were only 1.03% sequences not classified and the overwhelming majority of the sequences belonged to phylum *Proteobacteria* (93.28%), followed by *Acidobacteria* (1.32%), *Bacteroidetes* (1.22%) and *Cyanobacteria/Chloroplast* (1.02%). Bacterial community structures before and after SWCNT shocking in each group were similar, indicating SWCNTs did not result in community shifts at phylum level. The bacterial community compositions differed at low taxonomic levels. For example, the average percentages of the major three classes, *Betaproteobacteria, Gammaproteobacteria* and *Alphaproteobacteria*, were 56.82%, 28.95% and 14.21%, respectively, which were similar to the sludge microbial structures reported previously (Zhang *et al.* 2012). However, they occupied different proportions in each group (Figure 1(b)). Simultaneously, the proportions of these classes were also different before and after SWCNT addition, especially in G1 and G3.

Shifts of alpha diversity upon SWCNT dosing

The microbial diversity changes upon CNT addition have been rarely documented. In the present study, the alpha diversity of each sludge sample before and after SWCNT shocking is shown in Figure 2 and Table S1 (available online at http://www.iwaponline.com/wst/071/095.pdf). The higher Shannon index value, the higher alpha diversity. There were no changes for G2, G5 and G6, while G1 and G4 underwent significant reduction in alpha diversity with Shannon index values shifting from 2.75 and 2.65 to 1.59–1.80 and 2.46–2.55, respectively. For G3, an opposite trend was exhibited with Shannon index increasing from 2.54 to 3.07–3.14. In the meantime, the OTU numbers and Simpson index values altered consistently with that of Shannon index values. Thus it could be concluded that the alpha diversities of different microbial communities responded distinctly upon SWCNT addition. The environmental and operational conditions for each group were identical in the present study to eliminate interferences of other influence factors, proving initial microbial community structure was an important determinant affecting microbial diversity changes.

Shifts of bacterial structure upon SWCNT dosing

Clearly from the DCA plot (Figure 3), all sludge communities were shifted after SWCNT loading except for G2, implying that SWCNTs exerted an exogenous stress to indigenous microbes. The community changes at phylum level are shown in Figure 1(b). It was notable that bacteria displayed different variation in different groups. For example,

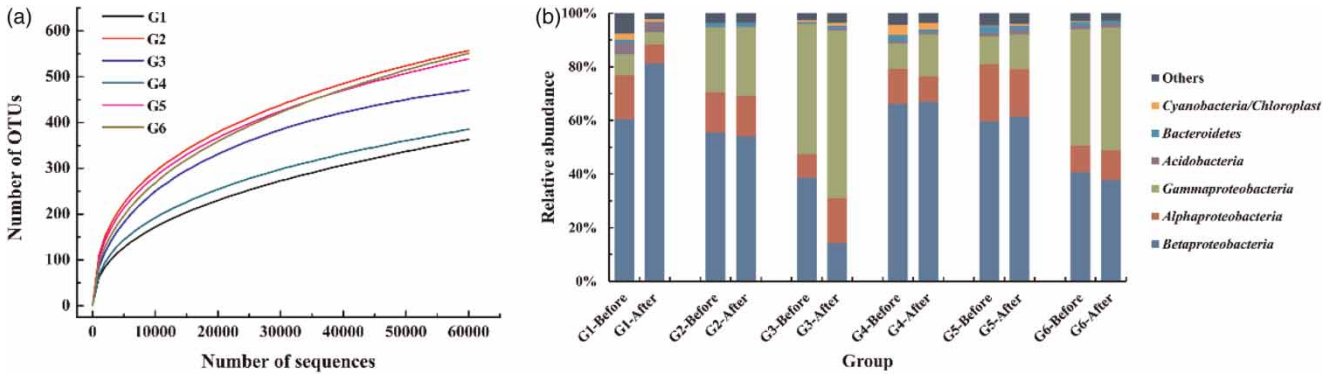

Figure 1 | Rarefaction curves of the sequencing results (a) and percentages of the major phyla (average sequence percentage >1%) in each group before and after SWCNT shocking (b).

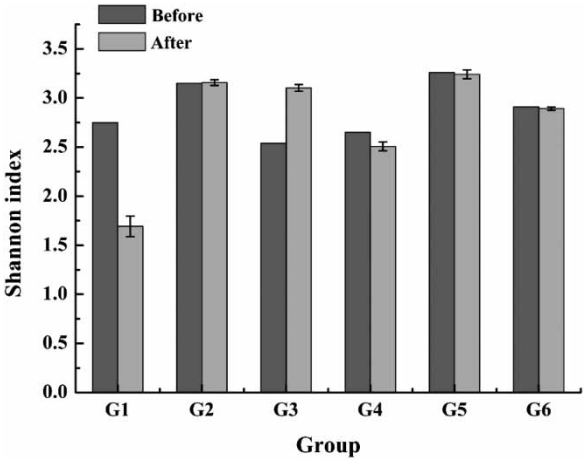

Figure 2 | Shannon index values of the six groups before and after SWCNT shocking.

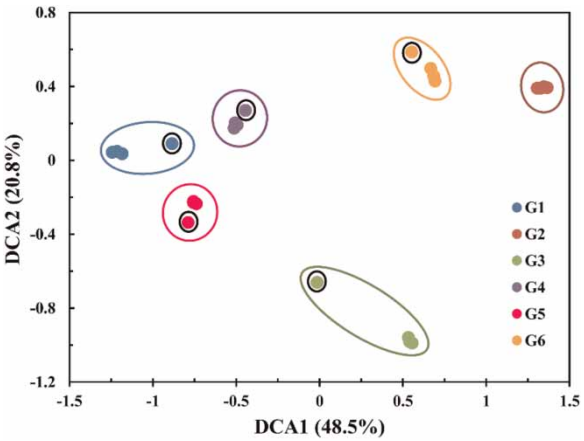

Figure 3 | DCA plot of the six activated sludges before and after SWCNT shocking (the dot in black circle represents the community before SWCNT shocking).

Betaproteobacteria increased noticeably in G1, but decreased in G3 and kept unchanged in other groups. The composition and dynamic of the bacterial community were further analyzed at genus level as shown in Figure S3 (available online at http://www.iwaponline.com/wst/071/095.pdf), and the top 10 genera were *Burkholderia* (20.62%), *Ralstonia* (8.26%), *Dyella* (6.86%), *Thiobacillus* (5.36%), *Caulobacter* (4.35%), *Pseudomonas* (3.12%), *Azoarcus* (3.07%) *Rhodoplanes* (1.37%), GP4 (1.22%) and *Sphingomonas* (0.88%). The community structures were different in each group, and the shifts of these genera were also distinct in different groups. Taking *Burkholderia*, for example, the relative abundance increased in G1 and G5, but decreased in G3 and G6. Among the six groups, most of the bacteria did not shift significantly in G2, suggesting the community shift was not an inevitable result of CNT shocking. This was consistent with the previous study that the sludge microbial community was impregnable upon carbon-based nanomaterial fullerene dosing using denaturing gradient gel electrophoresis analysis (Nyberg *et al.* 2008).

More and more emerging research pointed out that CNT addition would result in microbial community shifts. For instance, Shrestha *et al.* (2013) observed that some potential pollutant degraders such as *Rhodococcus*, *Cellulomonas*, *Nocardioides* and *Pseudomonas* increased in the presence of 10,000 mg/kg multi-walled CNTs by pyrosequencing analysis. Khodakovskaya *et al.* (2013) found that *Bacteroidetes* and *Firmicutes* increased while *Proteobacteria* and *Verrucomicrobia* decreased in the CNT-treated soil used to grow tomato plants grown soil. Jin *et al.* (2013) also found that SWCNT addition negatively affected the biomass of major microbial groups and might alter community composition. Our study result further proved that CNTs could change the microbial structures to some extent. The shifting structure might result in the perturbation of community ecological functions such as aromatic pollutant degradation and nutrient cycling (Yin & Zhang 2008; Luongo & Zhang 2010; Rodrigues *et al.* 2012; Zhou *et al.* 2013). A certain type of bacteria behaved differently in the six similar systems, suggesting the ecological effects of CNTs were more complicated than expected. It also further confirmed that the pure-culture antibacterial study conclusion could not be generalized to community research (Kang *et al.* 2009).

Shifts of major OTUs upon SWCNT dosing

The shifts of bacterial community at OTU level were analyzed and are shown in Figure 4. There were 11 major OTUs with average sequence proportion above 1% in total. The specific OTU proportion and corresponding taxonomical classification are shown in Table S2 (available online at http://www.iwaponline.com/wst/071/095.pdf). Nearly all major OTUs were differently impacted among the six groups. For example, OTU_11 increased by 74.28%, 23.11%, 51.88% in G1, G4 and G5, respectively, while it decreased by 66.79%, 666.4% and 164.29% in G2, G3 and G6, respectively. OTU_169 and OTU_157 remained almost unchanged in G1 to G5, but significantly increased in G6. OTU_46, which was aligned to genus *Ralstonia*, increased in almost all groups (unaffected in G1). *Ralstonia* was reported to be able to mineralize aromatics, produce the polyhydroxyalkanoate and resist heavy metals (Mergeay *et al.* 2003; Pohlmann *et al.* 2006), and the increase of this genus in the present study might imply it was were more tolerant to SWCNTs, which requires further verification.

Since the bacteria behaved differently among the groups, attempts to look for the potential biomarkers

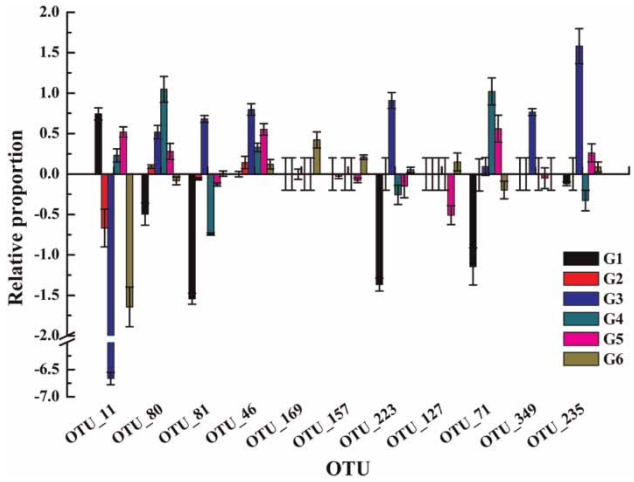

Figure 4 | Shifts of the major OTUs in each group (the relative proportion was calculated as follows: $(x_1–x_2)/(x_2)$ for increasing bacteria while $(x_1–x_2)/(x_1)$ for decreasing bacteria, where x_1 and x_2 were the OTU sequence numbers after and before SWCNT shocking, respectively).

sensitive to or indicative of SWCNTs ended in failure. We also constructed a phenol wastewater–SWCNT interaction system, and results indicated that the impacts of SWCNTs on microbes were of a temporal nature. The microbial responses would be affected by various environmental variables and microbial interactions. Meanwhile, CNTs could also exhibit different environmental behaviors such as aggregation, adsorption and biodegradation (Petersen *et al.* 2011); thus we speculated that there might not be a universal biomarker in response to CNTs.

CONCLUSIONS

In summary, six different activated sludges were domesticated and operated under identical conditions to explore the microbial responses to SWCNTs shocking by Illumina HTS. Results showed that the alpha diversities of the sludge communities changed diversely with one increase, two decrease, and the remaining three of no changes. The microbial community structures of each group shifted based on DCA analysis. However, the bacterial behavior was different among the groups and the attempts to seek for the biomarkers in response of SWCNTs were not successful. It could be concluded that the microbial responses to SWCNTs were microbial community structure-dependent, implicating that studies on ecological effects of CNTs should be studied case by case.

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