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Responses of Microbial Communities to Single-Walled Carbon Nanotubes in Phenol Wastewater Treatment Systems

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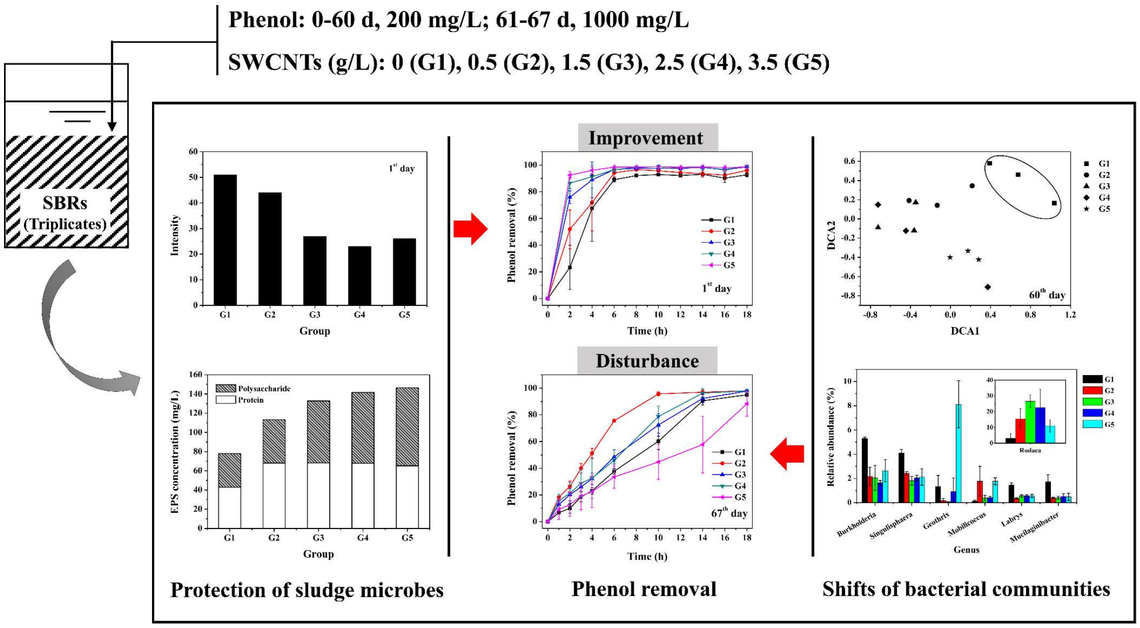

Phenol: 0-60 d, 200 mg/L; 61-67 d, 1000 mg/L
SWCNTs (g/L): 0 (G1), 0.5 (G2), 1.5 (G3), 2.5 (G4), 3.5 (G5)
SBRs
(Triplicates)
Improvement
Disturbance
Protection of sludge microbes
Phenol removal
Shifts of bacterial communities

ABSTRACT: The expanding use of single-walled carbon nanotubes (SWCNTs) raises environmental concerns. Wastewater treatment systems are potential recipients of SWCNTs containing influent, yet the impacts of SWCNTs on these systems are poorly documented. In this study, the microbial responses to SWCNTs in simulated phenol wastewater treatment systems were investigated. The phenol removal rates were improved in all SWCNTs-treated sequencing batch reactors during the first 20 days, but when facing higher phenol concentration (1000 mg/L) after 60 days, reactors with the highest concentration (3.5 g/L) of SWCNTs exhibited a notably decreased phenol removal capacity. Cell viability tests, scanning electron microscopy analysis and DNA leakage data suggested that SWCNTs protected microbes from inactivation possibly by producing more bound extracellular polymeric substances (EPS), which could create a protective barrier for the microbes. Illumina sequencing of 16S rRNA gene amplicons revealed that the bacterial diversity did not change significantly except for a minor reduction after the immediate addition of SWCNTs. Bacterial community structure significantly shifted after SWCNTs addition and did not recover afterwards. *Zoogloea* increased significantly upon SWCNTs shocking. At final stage, *Rudaea* and *Mobilicoccus* increased, while *Burkholderia*, *Singulisphaera*, *Labrys* and *Mucilaginibacter* decreased notably. The shifts of these dominant genera may be associated with altered sludge settling, aromatic degradation and EPS production. This study suggested that SWCNTs exerted protective rather than cytotoxic effects on sludge microbes of phenol wastewater treatment systems and they affected the bacterial community structure and diversity at test concentrations. These findings provide new insights into our understanding of the potential effects of SWCNTs on wastewater treatment processes.

■ INTRODUCTION

Carbon nanotubes (CNTs) are amongst the most promising engineering nanomaterials due to their unique physiochemical properties compared to bulk materials. They have been incorporated into a diverse array of commercial products such as pharmaceuticals, optical devices, cosmetics, electronics and antimicrobial coatings.[1,2] With the exponential increase in manufacturing and application of CNTs in nanotechnology, they will inevitably enter various environmental matrices.[3-6] However, the potential environmental risks are poorly understood and a comprehensive investigation is needed.

Cytotoxicity of CNTs to microbes has been demonstrated using pure microbial strains.[7-13] The underlying molecular mechanisms were proposed to be the synergistic impacts of cell membrane perturbation and oxidative stress. However, evidence has shown that CNT toxicity to pure microbial strains is a poor predictor of toxicity to microbial communities.[14,15] A number of studies have therefore investigated the effects of CNTs on microbial communities in aquatic and soil environments.[16-18] For instance, Chung et al. reported that high concentrations of CNTs significantly lowered biomass and some enzyme activities of microbial communities from an urban soil,[19,20] while Shrestha et al. suggested that soil respiration and extracellular enzyme activities were not significantly affected in a sandy loam soil even in the presence of extremely high concentrations of multi-walled carbon nanotubes (MWCNTs).[21] CNTs were also reported to affect microbial degradation of aromatic pollutants including 2,4-dichlorophenol and phenanthrene.[22,23] Therefore, it is necessary to investigate the responses of microbial communities to CNTs from various environments.

Wastewater treatment plants (WWTPs), as important receptors and the sink of waste streams, are amongst the most probable CNT recipients of industrial and domestic effluents.[24]

Moreover, CNTs are being tailored to work as catalyst supports, composite filters, adsorbents and antimicrobial agents in wastewater treatment processes, increasing the likelihood of contact with microbial communities in activated sludges.[2,25] Consequently, they may pose a risk to the microbial populations and their associated functions. In this respect, the effects of other nanoparticles (Cu-, Zn-, Ag-, Al_2O_3-, ZnO-, TiO_2-, SiO_2- nanoparticles), especially Ag-nanoparticles, have been extensively studied and most negatively affected waste removal efficacy.[26-28] Only a few studies have examined the effects of CNTs on wastewater microbial communities.[29-32] Luongo and Zhang found that MWCNTs inhibited the activated sludge respiration in a dose-dependent manner within three hours,[30] and that single-walled carbon nanotubes (SWCNTs) altered the structure of the bacterial communities in the sludge systems within five hours based on automated ribosomal intergenic spacer analysis (ARISA).[29] Very recently, two studies also revealed that CNTs affected bacterial community structure of activated sludge and its corresponding functions including methane production, nitrogen and phosphorus removal.[33,34] Notwithstanding, the ecological effects of CNTs on activated sludge system and microbial survival are far from clear.

Recently, reports have demonstrated that CNTs altered the composition and structure of microbial communities based on results from several culture-independent technologies including ARISA, PCR-denaturing gradient gel electrophoresis (DGGE), multiplex-terminal restriction fragment length polymorphism (M-TRFLP) and clone library analyses.[18,29,35] However, these methods only allowed identification of microbial populations at rather coarse taxonomic levels. Current advances in high-throughput sequencing techniques have not only increased sequencing depth at a lower cost, but also provided higher taxonomic resolution.[36-38] Especially, the Illumina MiSeq platform, which is able to achieve comparable or greater sequencing depth than related pyrosequencing, has been widely used to examine the phylogenetic/taxonomic diversity, composition and structure of microbial communities from a variety of environments.[21,39]

In this study, we aimed to: (1) explore the impacts of SWCNTs on pollutant removal efficiency in activated sludge systems, (2) investigate the microbial survival and cytotoxic mechanism, (3) monitor diversity and structure shifts of microbial communities, and (4) identify the dominant microorganisms susceptible to SWCNTs. To achieve these goals, sequencing batch reactors (SBRs) were constructed for treating phenol containing wastewater which were dosed with 0.5, 1.5, 2.5 and 3.5 g/L SWCNTs. Results demonstrated that SWCNTs played protective roles for sludge microbes, and in the meantime changed the structure of sludge bacterial communities.

■ MATERIALS AND METHODS

SWCNTs and Activated Sludge. Commercially available SWCNTs (>95%) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China) and were suspended in distilled water using ultrasonic treatment for 30 min to obtain a better dispersion. More details regarding the SWCNTs have been described previously.[40] Activated sludge was gathered from the secondary sedimentation tank of Chunliu River WWTP (Dalian, China).

Experimental Design. The reactors were 65 cm tall with an internal diameter of 8 cm and a working volume of 2.5 L. Fine air bubbles for aeration were supplied through an air pump at the reactor bottom with an airflow rate of 0.4 L/min. The synthetic wastewater consisted of 20 mg KH_2PO_4/L, 90 mg NH_4Cl/L, 10 mg NaCl/L, 12.5 mg $MgSO_4 \cdot 7H_2O$/L, 12 mg $CaCl_2$/L, 10 mg $FeSO_4 \cdot 7H_2O$/L, 785 mg glucose/L and 200 mg phenol/L. The SBRs (n=15) were seeded with activated sludge (2.57 g/L, dry weight at 105 ^{o}C), and domesticated with the synthetic wastewater. The SBRs were operated under identical conditions. Each cycle of SBR was operated for 24 h, including 2 h fill, 18 h aeration, 2 h settling, and 2 h decant. Effluent was discharged with a volumetric exchange ratio of 50%. After 15 days of domestication, the SBRs were divided into 5 groups, each receiving different concentrations of SWCNT (g/L): 0 (G1), 0.5 (G2), 1.5 (G3), 2.5 (G4) and 3.5 (G5). Each group contained three replicates. During the two month operation period, phenol concentrations of the influent and effluent were monitored daily, and the concentration of mixed liquor suspended solid (MLSS), the sludge volume after 0.5 h of settling (SV_{30}), pH and dissolved oxygen (DO) were measured every other day. Since the system was operated in good condition, and there was barely any sludge discharge during the whole process, the MLSS was relatively stable during the process (Figure S1), thus the sludge retention time was not considered herein. The influent phenol concentration was increased to 1000 mg/L on Day 61 to compare the robustness of the control reactors with the SWCNTs-treated ones. Phenol removal was monitored on Day 1, 20, 40, 60, 61 and 67 (the last day of operation).

Analytical Methods. Since activated sludge used in this study was gathered from a municipal WWTPs and aerated immediately which was transparent and colorless after centrifugation, the concentration of phenol was measured directly using a UV-vis spectrophotometer (V-560, JASCO, Japan). MLSS and SV_{30} were determined according to standard methods. The pH and DO were measured using a pH meter (S20, Mettler-Toledo, Switzerland) and a DO meter (FLX310, Flow Electronic, China), respectively. Scanning electron microscopy (SEM) images of the SWCNTs and activated sludges were recorded using a Field Emission Scanning Electron Microscope (KYKY-1000B, KYKY Technology, China). For the detection of live/dead cells, the 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Beyotime, China) and propidium iodide (PI, Beyotime, China) staining assays were performed according to the manufacturer's instructions. The efflux DNA was determined by fluorescence spectroscopy (Hitachi, Japan) using DAPI as the fluorescent dye (excitation 364, emission 454 nm) after filtration through a 0.22 μm membrane. The bound extracellular polymeric substance (EPS) of activated sludge was extracted using ethylenediaminetetraacetic acid. Concentrations of protein and carbohydrate were measured using the Lowry and anthrone methods, respectively.[41]

DNA Extraction, PCR Amplification and Sequencing. Activated sludge samples were collected at Day 1, 20, 40, 60 and 67 before sludge settling, and the genomic DNA was extracted using a protocol based on Purkhold et al.[42,43] DNA concentration was measured with Pico Green assays using a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Germany). For high-throughput sequencing, the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V4 region of the 16S rRNA gene.[44] PCR was conducted in a 25 μL mixture containing 0.1 μL AccuPrime High Fidelity Taq Polymerase (Invitrogen, USA), 2.5 μL of 10×AccuPrime PCR buffer II, 1 μL of each primer (10 μM), 1 μL template DNA and 19.4 μL nuclease-free water under the following conditions: 94 °C for 1 min; 35 cycles of 94 °C for 20 s, 53 °C for 25 s, and 68 °C for 45 s; and a final extension at 68 °C for 10 min. Each sample was amplified in triplicate. PCR products were pooled, purified through QIAquick Gel Extraction Kit (Qiagen), and quantified by Pico Green analysis. The 16S rRNA high-throughput sequencing was conducted on Illumina MiSeq platform at the Institute for Environmental Genomics, University of Oklahoma.

Sequencing data analysis of 16S rRNA gene amplicons. After sequencing, PhiX sequences were removed and primers were trimmed (mismatch 1.5), and the paired-end reads were joined using the Flash program (phredOffset 33, standard deviation of fragment lengths 20).[45] Sequences containing ambiguous reads (N) and reads shorter than 240 bp were removed.[46] The resulting sequences were screened for Chimeras using UCHIME.[47] Operational taxonomic units (OTUs) were categorized using CD-HIT at a 97% sequence similarity threshold,[48] and the taxonomic assignment of OTUs was performed by RDP classifier with 50% confidence.[49] The above processes were performed through a pipeline (http://zhoulab5.rccc.ou.edu/) (not published). Detrended correspondence analysis (DCA) and correlation tests were calculated using R v2.15.1 (http://www.r-project.org/). Three nonparametric tests, including multiple response permutation procedure (MRPP), permutational multivariate analysis of variance (Adonis), and analysis of similarity (ANOSIM) were performed to test dissimilarity among treatment groups based on bray-cutis distance index (http://ieg.ou.edu/).

■ RESULTS AND DISCUSSION

Characterization of SWCNTs. Cytotoxicity of CNTs was closely related to the physicochemical properties. The pristine SWCNTs used here were 5-16 μm in length, and less than 2 nm in diameter, with a surface area of 500-700 m^2/g. SEM and TEM micrographs were obtained to confirm the manufacturer's description.[40] The impact of low metal impurities (carbon content > 95%) was not considered since it showed insignificant antibacterial activity on soil and activated sludge communities.[15,29,35] With the increasing manufacturing and application of CNTs, more and more CNTs will inevitably enter WWTPs via CNT production facility's release, manufacturing and disposal of CNT containing products.[4,5,50,51] CNTs released from composites such as sports equipment, tires, electronics, etc. have been reported elsewhere.[51] CNTs may agglomerate and accumulate in activated sludge due to their high hydrophobic and biodegradation-resistant characteristics.[5,34] In previous reports, low concentrations of CNTs have been proven to exert no or little impact on microbial and enzyme activities of soils and activated sludge.[19,33,35] Our preliminary experiments also showed that the microbial communities were not changed with 0.1 g/L SWCNT, while a noticeable shift was observed in 2.0 g/L SWCNT group using PCR-DGGE analysis (data not shown). Therefore, the concentrations of SWCNTs used in the present study were set at a relative high level from 0.5 to 3.5 g/L.

Effects of SWCNTs on Reactor Performances. Acute and chronic influences of SWCNTs on the phenol wastewater treatments were mimicked over a course of two months' operation. Phenol was almost completely removed after one cycle of operation in all reactors (Figure 1). On the first 20 days, phenol removal rates were SWCNT-dose-dependent with the order of G5>G4>G3>G2>G1 (Figure S2). On Day 1, the removal rate reached 96.1 ± 2.4% within two hours in G5, while it took eight hours for G1 to reach a similar removal rate, indicating a positive influence of SWCNTs on phenol removal in this system. After a 40-day operation, all groups reached similar phenol removal rates (Figure S2). Therefore, despite the potential toxicity, SWCNTs appeared to exhibit positive effects on sludge microbial communities, especially during the early stages of operation. SWCNTs have been widely used as adsorbents for heavy metals and aromatics removal by virtue of their unique properties.[52-54] Results from this study also indicated a dose-dependent relationship between phenol removal and SWCNTs, and 3.5 g/L SWCNTs could adsorb 58.9% phenol (initial concentration 180 mg/L) within 6 hours (Table S1). Thus, we initially speculated the elevated phenol removal rates on Day 1 and 20 were attributed to phenol adsorption by SWCNTs. However, when using autoclaved sludge systems, phenol adsorption did not improve in SWCNTs-treated groups (Table S1). It was previously proven that carbon-based nanomaterials could adsorb free EPS and improve EPS production, which could form a protective barrier for the microbes.[12,31,34] Therefore, we determined the bound protein and polysaccharide concentrations of the five groups (Figure S3), which revealed higher bound EPS concentrations in SWCNTs-treated SBRs compared to the control group. Altogether, our results suggested that the potential protective mechanism of SWCNTs possibly resulted from the higher bound EPS production.

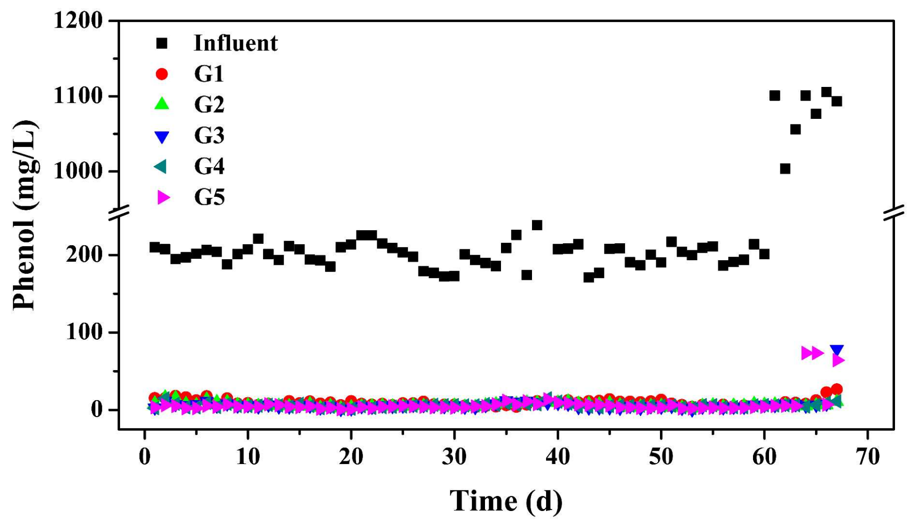

Figure 1. Phenol removal performances of each group during the 67-day operation process. Each group was performed in triplicate with different concentrations of SWCNTs (g/L): 0 (G1), 0.5 (G2), 1.5 (G3), 2.5 (G4) and 3.5 (G5).

The phenol concentration in the influent was increased to 1000 mg/L on Day 61 to investigate the robustness of the constructed systems. Over 94% of the phenol was removed within 14 hours in all groups with slightly lower removal rate in G5 (Figure S2). On Day 67, all five groups showed different removal performances. G1 had a similar removal rate compared to Day 61; G2, G3 and G4 showed higher removal rates, in which 96% phenol was removed within 10 hours; G5 had a considerably lower removal rate, requiring over 18 hours to achieve 88% phenol removal (Figure S2). Therefore, different concentrations of SWCNTs had different effects on wastewater treatment systems. At low concentrations, they improved treatment performance. Once past a threshold, i.e. 3.5 g/L in the present study, they could result in a negative impact after a longtime interaction.

To determine cell survival rates upon addition of SWCNTs, cell viability tests (DAPI/PI staining) was performed at each sampling time (Figure2, Figure S4). The percentage of inactivated cells in the control group was similar to G2 and was much higher than those in G3, G4 and G5. This phenomenon was consistent throughout the whole operation. Therefore, the addition of SWCNTs at relatively high concentrations apparently lowered phenol toxicity to cells. DNA leakage data also revealed a higher cell-free DNA intensity in G1, confirming the lower cell membrane damage rates in the SWCNT groups (Figure S5). The results further confirmed the positive influences of SWCNTs on the vitality of the microbial communities, which were also consistent with the EPS data. Three major cytotoxic mechanisms of CNTs have been recognized in previous studies, i.e. generation of oxidative stress, release of certain impurities and physical perturbation.[33,34,55] The reduced cell death and high purity of SWCNTs in the present study implied oxidative stress and metal purities should not be significant. SEM images showed a prevalence of aggregates of SWCNTs, largely reducing their direct contact with microbial cells (Figure S6). Only a few morphologically changed cells intertwined with SWCNTs, indicating limited physical toxicity of SWCNTs.[15,29] Meanwhile, the bound EPS concentrations in SWCNTs-treated groups were higher than those of the control group, further reducing the possibility of SWCNT physical toxicity. Therefore, the cytotoxicity of SWCNTs on sludge microbial communities was very limited and negligible. However, our results also suggested that G5 displayed a decreased phenol removal capability after phenol shock on Day 61, contrary to the results derived from microscopic observations. The distinct phenol degrading capability could be primarily, if not completely, due to community shifts resulting from SWCNTs addition.

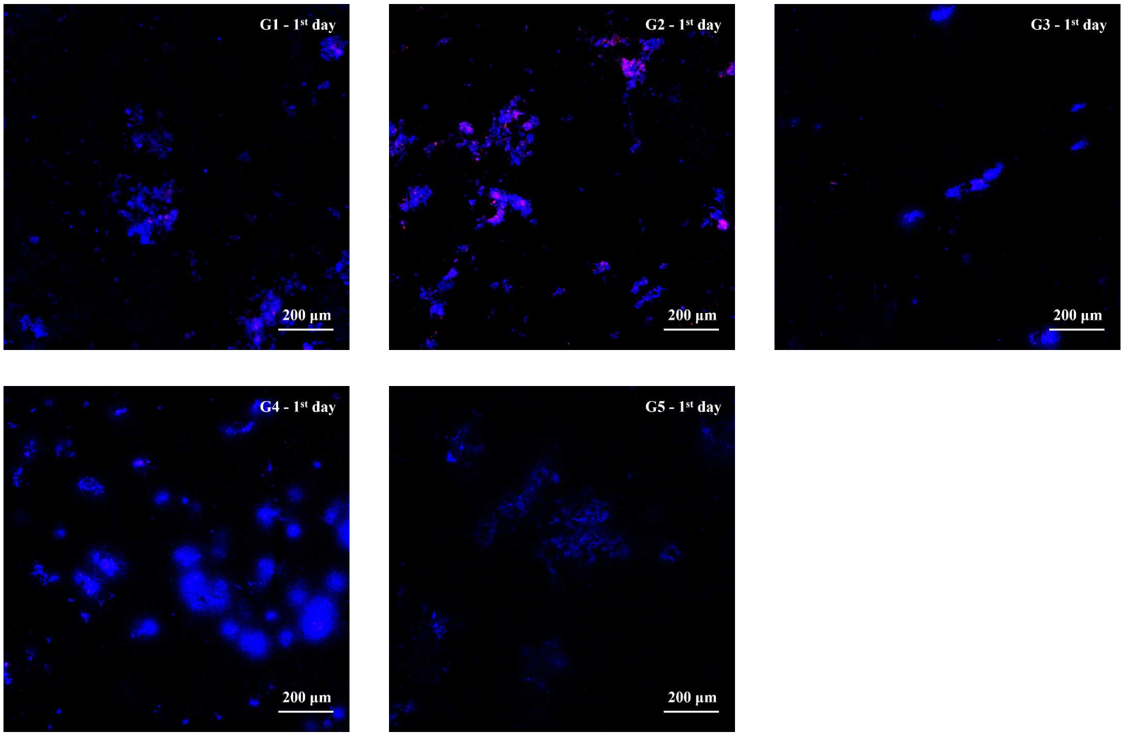

Figure 2. DAPI/PI staining results of each group on Day 1. Blue parts represent the active cells stained with DAPI and red parts represent the inactivated cells stained with PI.

Shifts of Bacterial Community Diversity and Structure. The sludge samples from Day 1, 20, 40, 60 and 67 were sequenced using the Illumina Miseq platform. After removing low quality sequences and chimeras, the sequence number of each sample was rarefied to 15131, resulting in 226-579 OTUs at the clustering threshold of 0.97. Our results showed that 99.9% of the sequences belonged to bacteria. SWCNTs addition significantly reduced Shannon diversity and evenness (ANOVA P=0.027 and 0.025, respectively) on the first day (Table 1), while the richness (OTU and Chao1) did not change (P>0.05). Thereafter, the Shannon indices, OTU richness and evenness were similar among all groups (P>0.05), suggesting a shock loading effect upon SWCNTs addition, followed by a gradual recovery of bacterial diversity. Our results were in accordance with the report by Khodakovskaya et al. that CNTs did not affect soil bacterial diversity after a nine-week influence,[39] while Hai et al. reported that continuous addition of 20 mg/L MWCNTs significantly decreased bacterial diversity,[33] indicating the influence of CNTs on microbial diversity was inconclusive.

Table 1. Alpha-diversity of all the groups at each sampling time

	Day 1					Day 20				
Group	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
Shannon index	3.85	3.61	3.56	3.48	3.48	2.66	2.60	2.54	2.50	2.81
Evenness	0.61	0.57	0.56	0.56	0.56	0.49	0.46	0.44	0.43	0.48
Chao1	861	868	926	857	824	401	484	526	595	630
OTU	533	543	550	533	522	237	300	325	334	364
	Day 40					**Day 60**				
Group	G1	G2	G3	G4	G5	G1	G2	G3	G4	G5
Shannon index	3.37	3.69	4.00	3.87	3.98	3.79	3.46	3.41	3.49	3.92
Evenness	0.57	0.61	0.65	0.63	0.66	0.65	0.65	0.59	0.57	0.59
Chao1	553	655	742	695	665	502	540	654	596	663
OTU	379	405	474	482	418	340	348	387	369	435
	Day 67									
Group	G1	G2	G3	G4	G5					
Shannon index	3.10	3.00	2.84	2.87	3.16					
Evenness	0.64	0.61	0.59	0.59	0.65					
Chao1	578	559	565	567	579					
OTU	340	345	324	349	368					

Pearson correlations between taxa abundances and CNT concentrations were assessed to estimate the impact of SWCNTs on taxonomic groups (Table S2). On Day 1, more taxa were positively affected than negative ones at phylum, class, genus and OTU levels, whereas the opposite trend was observed at all other sampling time. For example, the majority of significantly impacted OTUs (78/106) were of lower abundances on Day 1, yet much smaller fractions of those were reduced on other sampling days (32/155, 27/93, 31/98, 55/126 for day 20, 40, 60 and 67, respectively). Therefore, the short-term exposure to SWCNTs repressed the growth of the majority of bacterial populations, while continuous interaction exhibited positive effects.

DCA analysis showed that the G1 samples at all sampling time separated from the other groups on a two-axes plot (Figure 3). Hierarchical clustering analysis also showed that the triplicate samples of G1 clustered together and separated from the samples of all other groups (Figure S7). Dissimilarity analyses by Adonis, ANOSIM and MRPP confirmed that G1 was significantly distinct from the other samples (data not shown, P<0.01) at all time points. The changes in bacterial communities, especially the dominant populations, may lead to disturbed system functions.

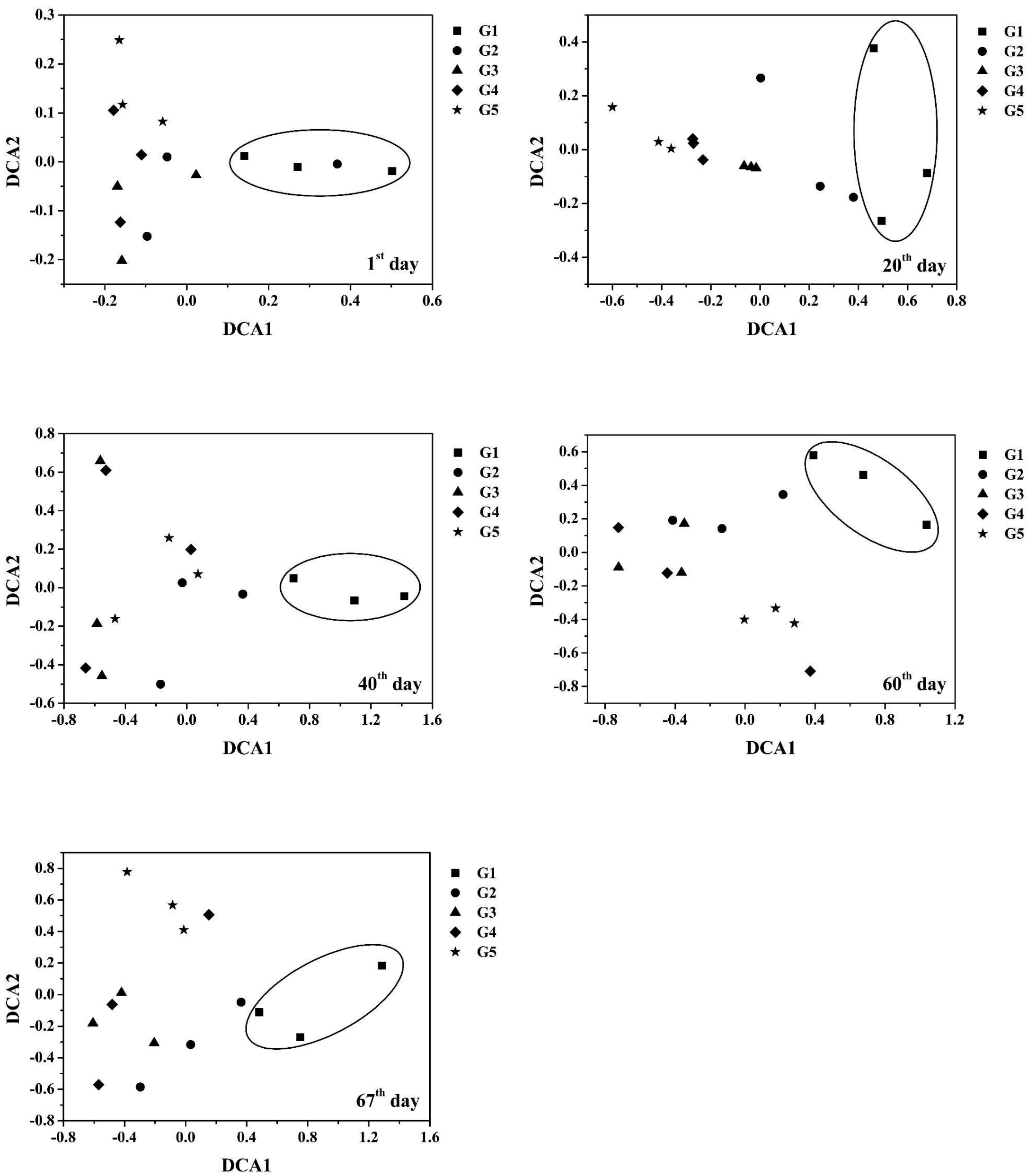

Figure 3. DCA plots of all samples showing the relationships of microbial community structures among different groups at each sampling time (Day 1, 20, 40, 60 and 67). Symbols represent the samples from different groups. A distinct cluster can be defined from the samples of G1 based on the DCA ordination, which suggests the differences in microbial community structures between G1 and other four groups.

Susceptible Genera in Response to SWCNTs. The influences of SWCNTs on dominant genera (relative abundances over 1%) were further assessed at the initial (Day 1) and final (Day 60) stages of SWCNTs addition. On Day 1, there were 11 dominant genera in all groups, among which only *Zoogloea* exhibited different abundances among groups (ANOVA, P<0.05) and it did not survive at final stage (Figure S8). The SV_{30} values in our experiment were in the order of G1 (72.8% in average)>G2 (62.4%)>G3≈G4≈G5 (50.3%) in the first 10 days, which indicated that the addition of SWCNTs improved sludge settling ability (Figure S9). Yin et al. also reported SWCNTs addition improved sludge settling ability by 5.7-10.8% within five hours.[31] Species of *Zoogloea* (*Zoogloea ramigera*) were widely spread in activated sludge, and have been regarded as the key populations responsible for the flocculation of activated sludges.[37,56,57] Therefore, the increased *Zoogloea* upon SWCNTs addition in this study might have positively influenced the sludge settling ability.

There were 19 dominant genera on Day 60 (Figure S10), and 7 of them showed significant shifts (Figure 4). *Rudaea*, the most predominant population at final stage, remarkably increased from 3.17% in G1 to 10.90-26.55% in G2-G5 (Figure 4). The relative abundance of this genus on Day 67 (3.69% in G1, 11.47-26.48% in others) was similar to that of Day 60 (Figure S11). *Rudaea* has been identified in long-term contaminated soils with biphenyl, benzoate and naphthalene, as well as in a petroleum refinery wastewater treatment plant, thus it is of considerable potential for aromatics biodegradation.[58,59] Meanwhile, it is also known for cellulose degradation and was detected as the predominate and sensitive genus susceptible to metal nanoparticles, especially nano-TiO_2.[60,61] In our study, the increase of *Rudaea* in SWCNTs-treated groups might lead to improved aromatic (such as phenol) degradation capacity.

The abundances of *Burkholderia*, *Singulisphaera*, *Mucilaginibacter* and *Labrys* in SWCNTs-treated groups were notably lower than that in G1. Members of *Burkholderia* were known to play important roles in bioremediation of recalcitrant xenobiotics, as well as polyphosphate uptake and accumulation in activated sludge systems.[62-64] Type strains of *Labrys* were reported to be capable of degrading fluorobenzene, chlorobenzene and various pharmaceuticals.[65,66] Thus, the changes of these two bacteria might cause the fluctuation of xenobiotic removal performance. *Singulisphaera* was a newly established genus belonging to the order *Planctomycetales* with biopolymers-degrading ability.[67] Certain species from *Mucilaginibacter* including *Mucilaginibacter gracilis* and *Mucilaginibacter paludis* were also proficient in degrading various biopolymers (pectin, xylan, laminarin, etc.).[68] Therefore, the higher abundances of *Singulisphaera* and *Mucilaginibacter* in G1 might result in relatively more EPS degradation, which may explain the higher EPS concentration in SWCNTs-treated groups. *Mobilicoccus* was another increased genus in response to SWCNTs. It has so far only been isolated from fish intestinal tracts and its ecological role in WWTPs kept unknown.[69] *Geothrix* was an anaerobic Fe(III)-reducing bacterium which usually existed in hydrocarbon-contaminated matrix.[70] The roles of this genus also needed further investigation.

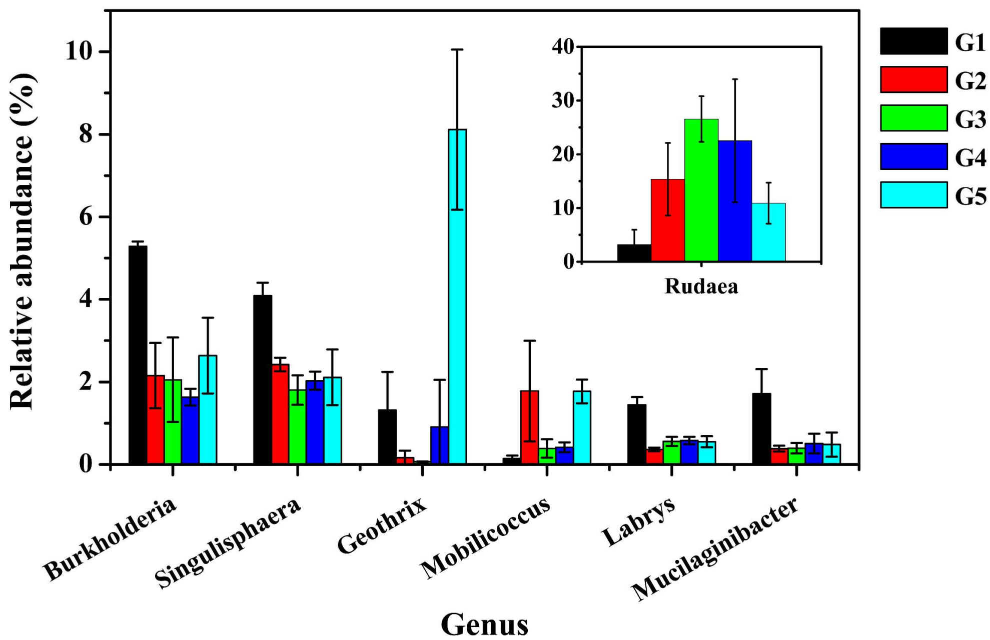

Figure 4. Relative abundances of significantly shifted genera in SWCNTs-treated groups compared with control group on Day 60. ANOVA analysis was adopted and the 7 genera had $P<0.05$.

Rhodanobacter was another predominant genus with relative abundance of over 10%. Although it showed no significant differences among G1 to G4 (ANOVA, $P>0.05$), it was significantly lower in G5 (7.54%) compared with G1 (16.73%) at final stage (Figure S11). *Rhodanobacter* was reported to be capable of aromatics degradation and denitrification.[71,72] The decrease in abundance of this genus might negatively affect aromatic degradation and nitrogen removal performance in G5.

Most of the previous studies only searched the shifted microbes upon SWCNTs addition at one time point, yet the long-time and termly detection of community changes could bring us more useful insights. Our results revealed similar responses of *Rudaea*, *Burkholderia*, *Geothrix*, *Mobilicoccus* and *Labrys* at any sampling time, indicating that they were more susceptible to

SWCNTs in phenol wastewater treatment systems (Figure S11). However, SWCNTs exhibited varied or converse effects on some other taxa including *Rhodanobacter*, *Singulisphaer* and *Mucilaginibacter* at different time points. It suggested the impacts of SWCNTs were of temporal relations. Under *in situ* conditions, more complicated surroundings will induce disparate impacts on the community members. Therefore, the ecological effects of SWCNTs should be investigated case by case.

It was previously reported that high concentrations of SWCNTs could significantly reduce urban soil enzyme activity and affect 2,4-dichlorophenol mineralization, possibly by inhibiting the activity of soil endogenous microorganisms, whereas low concentrations of SWCNTs showed no or little influence on the microbes.[19,22] Based on our study, 3.5 g/L seemed to be the threshold for SWCNT toxicity, above which the performance of the SBRs could be unstable upon high phenol influent shock. For controllable engineered system-bioreactors, the performance and stability correlated with functional redundancy.[73-75] Hence, the specific contributions and interactions of the community members, and particularly the impacts of SWCNTs on related functional genes need further investigation to obtain an in-depth understanding of the underlying mechanisms of the SWCNT ecological effects. Since SWCNTs can significantly alter the microbial community structure, both improving and inhibiting wastewater treatment system performance, application of CNTs to wastewater treatment systems should be carefully considered for balancing both positive and negative effects.

Implications. Although SWCNTs were shown to be toxic to bacteria elsewhere, this study showed that addition of SWCNTs to phenol wastewater treatment systems could reduce cytotoxicity and increase phenol removal rates. However, the performance of bioreactors receiving a 3.5 g/L dose of SWCNT was significantly lowered upon loading with high concentrations of phenol, suggesting SWCNTs also posed a potential threat to the treatment systems. SWCNTs addition significantly altered the composition and structure of indigenous activated sludge microbial communities. *Zoogloea*, *Rudaea*, *Mobilicoccus*, *Burkholderia*, *Singulisphaera*, *Labrys* and *Mucilaginibacter* were significantly shifted which might result in microbial community function fluctuations. The high-throughput sequencing technology has proven to be a feasible method for detecting subtle microbial changes resulting from CNT contamination in realistic environmental matrixes, which will contribute to promoting understanding of SWCNT nanoecotoxicology.

■ ASSOCIATED CONTENT

Supporting Information

This file contains Figures S1-S11 and Tables S1-S2. The information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest

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