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Effect of biochar on migration and biodegradation of 4-n-nonylphenol (NP) during river-based groundwater recharge with reclaimed water

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ABSTRACT

To investigate how biochar affects coupled migration-biodegradation of NP, a lab-scale column was set up to simulate the recharge process. The filler media of the two columns was mainly composed of silty clay (SC) and biochar amendment silty clay (BCSC). The results showed that the attenuation effect of NP in the BCSC system was 3.3% higher than that in the SC system. The attenuation rate constants of NP in the SC and BCSC systems were 0.20 and 0.24 m^{-1}, respectively, which followed first-order kinetics. The two turning points of NP concentration with depth in the leachate were 0.15 and 0.45 m as a result of fast sorption and biodegradation, respectively. The primary metabolites were not the only hydroxylated 4-nonyl-4-hydroxycyclohexa-2,5-dienone and 4-hydroxyphenyl-nonanoic-acid; detached branched alkyl moieties were also produced during the biotransformation process. The accumulation of NP in the soil was higher in BCSC soils than in the SC system. The bacterial community in the upper layer was more diverse than in the bottom layer, which was related to oxygen concentrations in the water-soil system. Five known bacterial classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteria*, and *Bacilli*) and 15 known bacterial genera (six major genera: *Methylobacillus*, *Azotobacterium*, *Sphingomonas*, *Pseudomonas*, *Bacillus*, and *Hyphomicrobium*) were considered to be NP-degradation-associated bacteria. The bacterial community diversity and percentage of NP-degradation-associated bacteria in the BCSC system were marginally greater than those in the SC system. The higher bacterial diversity and adsorption capacity in the BCSC system were able to mitigate the migration of NP into groundwater.

Keywords: Nonylphenol; Biochar; Adsorption; Biodegradation; Natural groundwater recharge; Microbial community

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1. Introduction

Nonylphenol (NP) is a xenobiotic compound used in the manufacture of antioxidants, lubricating oil additives, and the production of nonylphenol ethoxylate surfactants [1]. The major source of nonylphenol in the environment is the effluent from sewage treatment plants [2]. Most reclaimed water is discharged into nearby rivers and recharges the groundwater, which is a major pathway for the introduction of micropollutants to groundwater [3]. River-based groundwater recharge using reclaimed water is considered to be a promising method of alleviating groundwater depletion, especially in semi-arid and arid areas. In China, approximately 362.7×10^8 tons of reclaimed water was discharged into the river in 2014, and nearly 3.75×10^8 t of reclaimed water was recharged through river utilization in Beijing. Therefore, some non-readily biodegradable organic pollutants, such as endocrine disrupting chemicals (EDCs) and pharmaceuticals, might pose a potential threat to underground aquifers. EDCs accounted for 27% of priority organic compounds during groundwater recharge in China [4]. EDCs are difficult to remove from reclaimed water completely [5,6], meaning that some of them were introduced to groundwater, which poses risks to groundwater quality and humans. NP was the highest priority organic compound, with an average concentration of 947.79 ng/L, which even ranked above bisphenol A and ethinyl estradiol in surface water and groundwater [4]. In the Chaobai River, a large amount of reclaimed water and river water containing sewage effluent has infiltrated into the groundwater. Previous studies have shown that this accumulation of trace organic pollutants (e.g. EDCs, and PAHs) in underground aquifers influences normal hormone functions and ecosystems [3,4]. The concentration of NP in Chaobai River water and groundwater was 0.01–0.95 μg/L and 0.001–5.6 ng/L.

The concentrations of EDCs (except estriol (E3)) in groundwater were generally 1-2 orders of magnitude lower than those in river water because of sorption on soil, biodegradation by soil organisms, and oxidation by mineral materials [7,8]. Therefore, NP migration and biodegradation in the vadose zone aquifer media have caused great concern. Sorption and biodegradation/biotransformation were the two major dissipated pathways during the recharge process [3,9,10]. As sorption to soil and sediment is an underlying processes affecting migration, bioavailability and biodegradation, the sorption of NP has been widely studied in soil and sediment [3,10,11]. These studies reveal that soil organic matter (SOM) is the critical component affecting their sorption capacities. Biochar was produced by the straw incineration process, which does not fully carbonize and generates a product that is more like native SOM [12]. In many places in China, the soils and sediments have high biochar content due to stalk burning for several decades.

The octanol-water partition coefficient (Kow) indicates contaminant mobility in the subsurface, which is frequently used to predict absorption of NP on solids [3]. NP is highly hydrophobic with a log Kow of 5.76, indicating high sorption potential, and thus, its concentration is mainly associated with SOM content in the soil environment [13,14]. Because of its strong affinity for biochar, the bioavailability and migration of NP may be decreased in biochar-amended soil [13]. A large number of studies have shown that biochar may enhance the sorption or sequestration of hydrophobic organic contaminants (HOCs) in soil [15,16]. Biochar has been used as a remediation option at contaminated sites to sequester HOCs in the bed sediment and to reduce their flux to the water column [17,18]. The NP concentration in groundwater is much lower in the Huanbei plain than in the Pearl Delta because the Huanbei plain soil exhibited a high content of biochar. The freely dissolved concentration of NP was directly correlated with the bioavailability and the biodegradability of NP. The freely dissolved NP can be decreased by biodegradation through the metabolism of microorganisms. The biodegradation of NP and microbial community diversity might be influenced by the toxicity of high concentrations of NP in the recharge water. However, biotoxicity might be reduced due to NP adsorption onto biochar. The biodegradation of NP includes hydroxylation, the removal of the alkyl chain from NP and aromatic ring cleavage [19,20]. Evidence of bacterial change in response to NP accumulation in the soil has been reported. Microbial community change during reclaimed water recharge was investigated using denaturing gradient gel electrophoresis (DGGE) and pyrosequencing-based high-throughput sequencing [21,22]. The intermediate products might be absorbed by the SOM, further affecting biodegradation. However, a report has not been made available about the effect of biochar on the migration and biodegradation of NP, nor on the changes to the microbial community structure and biotransformation products in response to NP biodegradation in the soils during groundwater recharge with reclaimed water.

In this study, a lab-scale percolation column was designed to simulate river-based aquifer recharge to further investigate the effect of biochar on the migration and biodegradation of NP. The objectives of this study were to investigate the effect of biochar on NP migration, biodegradation and fate in vadose zone

soil; to predict the effect of biochar on the removal efficiency and the degradation kinetics of NP in different recharge aquifer soil media; to analyze the effect of biochar on changes to the microbial community structure, core biodegradation bacteria of NP through 16S rRNA gene pyrosequencing, and intermediate biodegradation metabolites; and to propose an effective method to mitigate NP contamination associated with reclaimed water during natural recharge, thus supporting the development of control measures for NP during river-based natural groundwater recharge using reclaimed water.

2. Materials and methods

2.1. Chemicals

NP (99.9%) was obtained from Sigma–Aldrich (USA). NP has a log Kow of 5.76 and solubility (Sw) of 1.57 mg/L. A stock standard solution of NP was prepared in methanol. HPLC-grade methanol (MeOH) and acetonitrile were purchased from J.T. Baker (USA). All other chemical reagents ($CaCl_2$, NaN_3) were of analytical grade (Chengdu Best Reagent Co., Ltd, China).

2.2. Vadose zone soil samples

The Chaobai River vadose zone aquifer media were mainly composed of water-permeable gravel sand in the northern section, fine sand in the middle section, and less permeable silty clay (SC) in the southern section in Beijing of Huabei plain. Simulation column number 1 was filled with representative soil vadose zone media from the middle parts of the Chao River (N 39˚48′, E116˚21′), which are typical streambed in north China. Column 2 was filled with representative soil vadose zone media from the stalk burning area in the southern parts of the Chaobai River (N 39˚52′, E116˚22′).

The hydrogeological cross-sectional east-to-west view shows that the shallow stratigraphic number of 80 m increased from east to west as the lithology of the aquifer changed from fine sand to gradient gravel. Table 1 presents the physical and chemical properties of the two representative soil vadose zone media. The characteristics of the three representative soil vadose zone media were different. The cation exchange capacity (CEC) was determined following the procedures defined by Chapman [23]. The total organic carbon (TOC) and BC contents were analyzed using the method described by Gustafsson [24].

2.3. Raw water

The raw water used for lab-scale experiments was treated with municipal wastewater effluent from the YinWen JiChao Reclaimed Water Treatment plant in Beijing. The water treatment processes are mainly composed of ozone pre-oxidation, a membrane bioreactor, chemical phosphorus removal, disinfection, and constructed wetland. The main characteristics of the artificial recharge water were COD 50 mg/L, NH_4^+ 1.0 mg/L, TN 6.7 mg/L, SO_4^{2-} 69 mg/L, and HCO_3^- 317 mg/L. The water samples for this study were collected and stored in the dark for a maximum of five days. The concentration of NP was adjusted to 100 μg/L in reclaimed water by adding pure NP.

2.4. Lab-scale column recharge system

Lab-scale installations are schematically shown in Fig. 1. The recharge system includes four columns. Two columns were filled with SC, and the others were filled with SC with 20-year-old stalk burning biochar. Each experiment was performed in triplicates. The columns were operated in wetting and drying alternative recharge to simulate the aquifer behavior during river-based groundwater recharge with reclaimed water. The flow rate was approximately 0.5 m d^{-1}. From the feed tank, the reclaimed water was pumped into three aquifer treatment columns with the same flow rate. The aquifer treatment columns were the same size: 0.20 m in diameter and 1.50 m in height, with a packed bed height of 0.9 m and a supporting layer height of 0.15 m. The background leaching was conducted for 7 d. In the background leaching system,

Table 1
Physical and chemical properties of the two representative soil vadose zone media

Medium category	CEC (cmol kg^{-1})	TOC (%)	BC/TOC (%)	Clay mineral composition (%)	Clay content (%)	Bulk density (g cm^{-3})	Effective porosity (%)	Permeability coefficient (m d^{-1})
SC	0.10	0.63	13.8	33.65	32.38	2.14	0.12	1.05
BCSC	0.14	1.61	36.9	25.26	38.37	1.87	0.15	1.13

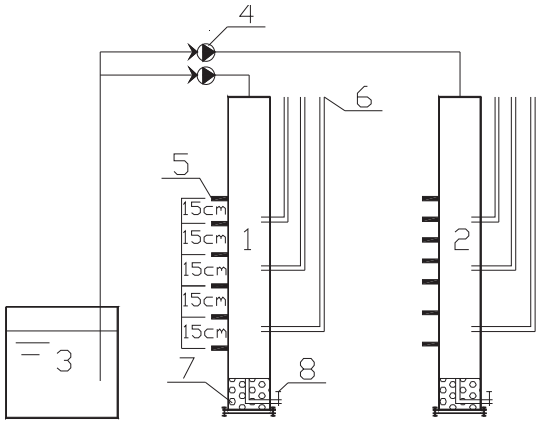

Fig. 1. Schematic diagram of the lab-scale column recharge system.
Notes: (1) SC aquifer treatment column (SC), (2) SC with stalk burning biochar (BCSC) aquifer treatment column, (3) feed tank, (4) peristaltic pump, (5) Sampling port, (6) piezometer, (7) Supporting layer, (8) Control valve.

samples were taken every day, while in the lab-scale column recharge system samples were taken every 15–80 d according to changes in water quality. The system operated for approximately one year. The room temperature was approximately 20 ± 5˚C. The columns were conditioned with $CaCl_2$ and then with reclaimed water without additional NP.

2.5. Instrumental analytical methods

The concentration of NP was determined by an Acquity UPLC-MS/MS system (Waters Corporation, USA) equipped with an Acquity UPLC BEH C18 column (100 × 2.1 mm, particle size 1.7 μm; Waters, USA) and a Quattro Premier XE tandem quadruple mass spectrometer (Waters, USA) equipped with an electrospray ionization source.

The metabolites were analyzed using the method described by Gabriel et al. and Różalska [19,20]. The metabolites' concentrations of NP were analyzed using UPLC-MS/MS system (Waters, USA) and GC-MS (Agilent 7890, Santa Clara, CA).

2.6. High-throughput sequencing

For molecular analysis, soil samples (0.5 g, dry weight) from the CR, WDAR, and control treatments were collected at depths of 10, 40, and 70 cm. Soil DNA samples were extracted using a Power soil DNA extraction kit (OMEGA, CA, USA). A broadly conserved primer set was used to amplify the 16S rRNA genes of the V4 region (515F/806R, 50-GTGCCAGCMGCCGCGGTAA-30/50-GGACTACHVGGGTWTCTAAT-30). The PCR primers were constructed as follows: forward primer = 454 Titanium Lib-l Primer A/5-base barcode/forward 16S primer and reverse primer = 454 Titanium Lib-l Primer B/reverse 16S primer [25,26]. All PCRs were performed in triplicate 30-μL reactions with 15 μL of the Phusion High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM forward and reverse primers, and approximately 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98˚C for 1 min, followed by 30 cycles of denaturation at 98˚C for 10 s, annealing at 50˚C for 30 s, and elongation at 72˚C for 30 s. All dilutions were conducted using certified DNA-free PCR water. The mixture PCR products were purified with a GeneJET Gel Extraction Kit (Thermo Scientific) and quantified using an Agilent high-sensitivity DNA assay on an Agilent Bioanalyzer2100 system. Paired-end reads from the original DNA fragments were merged using FLASH [27]. Paired-end reads were assigned to each sample according to the unique barcodes. The sequences were analyzed using the QIIME [28] software package (Quantitative Insights Into Microbial Ecology), and in-house Perl scripts were used to analyze alpha- (within samples) and beta- (among samples) diversity. Clustering of the index-coded samples was performed on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, USA). After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2000 platform, generating100-bp paired-end reads using the unweighted pair group method with arithmetic mean (UPGMA) clustering.

Analysis of variance was conducted with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Bacterial classes and genera with $p < 0.05$ were considered NP degradation-associated bacteria.

3. Results and discussion

3.1. Effect of biochar on spatial variation of NP migration in groundwater

The NP concentration in the leachate from different soil profiles showed a high variance among the different depths in SC and biochar amendment silty clay (BCSC) recharging systems (Fig. 2). The concentration of NP at different depths was lower in the BCSC system than in the SC system. The NP concentrations gradually decreased with increasing depth, while the attenuation rates varied greatly in the two columns. The contents ranged from 0.25 to 8.12 μg/L in the SC leachates and from 0.1 to 5.5 μg/L in the BCSC leachates. The results showed

that the concentration of NP varied greatly at different depths during the 360-d recharge period and that biochar reduced the migration of NP into groundwater. The NP concentrations in the leachates were much lower than those in recharge water due to sorption on soil and biodegradation by soil micro-organisms [3,29,30]. At the beginning of the recharge stage, the adsorption was mainly the removal mechanism due to the weak microbial degradation capacity. The adsorption capacity of NP onto soil and sediment had a good correlation with TOC and biochar content ($p < 0.01$) [9,31]. The content of biochar in the BCSC system was 1% higher than that of the SC system which might explain the weak migration capacity of NP in the BCSC system. The fast sorption rate constants (k_1) and slow sorption rate constants (k_2) ranged from 2.40 to 4.12 h^{-1} and 0.40 to 0.47 h^{-1} for SC and BCSC soil samples, respectively (Fig. 3). The fast adsorption rate in the BCSC system showed that NP could adsorb onto soil in a short period of time and prevent the migration of NP from upper layer to bottom groundwater, which played an important role in preventing NP from migrating to groundwater at the beginning of the recharge stage. The fast sorption compartment fraction (f_1) and the slow sorption compartment fraction (f_1) for SC and BCSC soil sample were 0.82 and 0.18, and 0.89 and 0.11, respectively. On one hand, the fast sorption compartment gradually decreased after the initial two had leveled off at the end of the sorption experiments with the fraction. On the other hand, the weight of the slow sorption compartment progressively increased, and ultimately approached a stable stage. However, the slow sorption undoubtedly played a major role from the subsequent 3 to 24-h water retention time and the 360 d recharge time. Therefore, the order of the NP attenuation effect was BCSC > SC, which showed the relatively fast and strong sorption characteristics of BCSC soil. With the increased recharge time, biodegradation became the predominant mechanism because the adsorption ability reduced and the microbial community structure gradually stabilized. In the later period of recharge, the concentration of NP in the BCSC column was lower than in the SC column, showing that the BCSC system can improve biological degradation capacity because micro-organisms were capable of adhering to or forming biofilms on biochar [32].

3.2. Effect of biochar on the attenuation of NP

The fitting curves of NP attenuation rate constants in SC and BCSC columns using origin 8.0 are shown in Fig. 4. The results showed that the attenuation effect of NP was in the order of BCSC > SC, which was consistent with the NP concentration in the leachate (Fig. 2). Therefore, biochar reduced the migration risk of NP to groundwater. The average NP concentration at different depths is represented by C_i, and the influent concentration is C_0. The attenuation rate constants of NP in the SC and BCSC systems were 0.20 and 0.24 m^{-1}, respectively, which were obtained by fitting curves to the concentration as a function of depth. The decay rate of NP was 1.2 times higher in BCSC than in SC system, which showed that high biochar content of the soil medium aquifer could be effectively trapped NP contamination in groundwater recharge process. The results showed that the attenuation of NP in the two recharge modes was in the order of BCSC > SC and followed first-order kinetics. The concentration at different depths can be calculated using the following formulae for the different recharge modes:

$$C_{i,SC} = C_0 e^{0.20 h_i}$$

$$C_{i,BCA} = C_0 e^{0.24 h_i} \quad (1)$$

where h_i is the soil depth below the soil surface; C_i is the NP concentration at soil depth h_i, and C_0 is the NP concentration of raw recharge water.

Adsorption plays an important role in the fate of NP in soil-water systems, which accounts for the fate of NP during the recharge process. Many studies have revealed that NP is easily adsorbed onto soil particles because of the higher hydrophobicity of NP, which was proposed to account for its stronger affinity with sorption sites [20,33]. The residual NP at different depths of BCSC were 1.5–2.2 times higher than those in the SC system, which indicated that NP migrated slower in the BCSC column than that in the SC column due to adsorption (Figs. 2 and 5). The average removal rates of NP in the leachate of SC and BCSC systems were 97.5 and 99.7%, while the proportions of the soil adsorption section were 18.1 and 31.7%, which indicated that the biodegradation effect in SC system was stronger than that in the BCSC system. The residual NP amounts indicated that the biodegradation was the main decay mechanism and biochar strengthens the adsorption process. There is a negative correlation between biological availability of NP and absorption efficiencies [13]. During the degradation process, free NP and desorbed NP from soil were bioavailable. Due to the high adsorption capacity and low desorption capacity of NP onto BCSC soil, which resulted in the reduction of bioavailable and biodegraded amounts of NP.

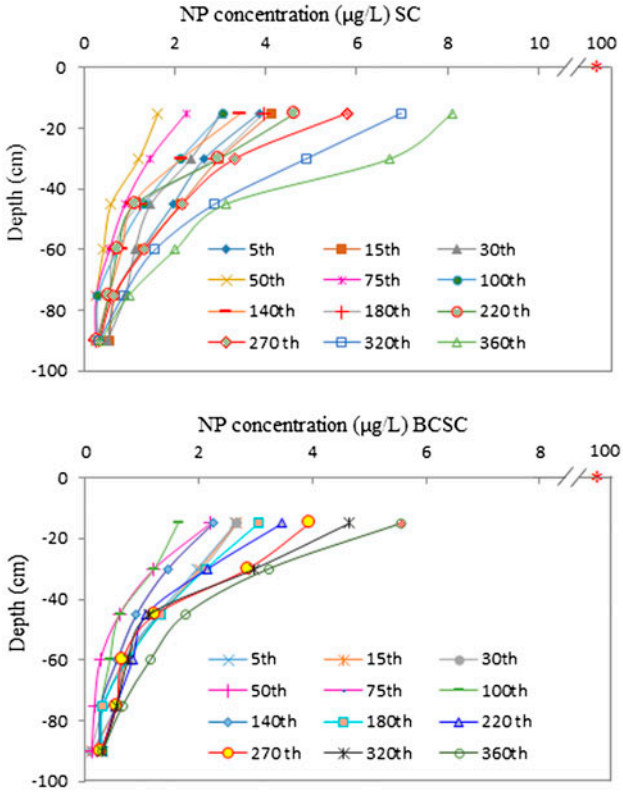

Fig. 2. Distribution of 4-n-nonylphenolover time at depths of 0–90 cm in the two recharge systems.

3.3. Effect of biochar on NP Biodegradation

The biodegradation intermediate products of NP in the leachate from different soil profiles showed a high variance among the different depths in two recharging columns during the 360-d incubation (Fig. 6). The

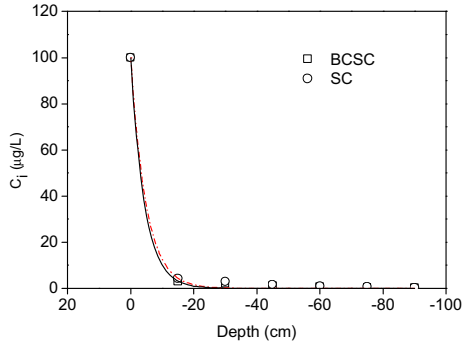

Fig. 4. Fitting curve of 4-n-nonylphenol attenuation rate constants in the two recharge systems.

percentage change of NP and metabolites have the same trends with different depths in SC and BCSC systems. The two turning points of NP percentage in the leachate were 0.15 and 0.45 m. The first turning point is due to the fast adsorption, which leads to a significant reduction in the concentration of NP. The second turning point is due to the biotransformation, which is correlated with the functional micro-organism (Fig. 8). NP could be decreased by biodegradation in both aerobic and anaerobic environments [14].

The average concentration of NP and the main metabolites was 5.21 μg/L in the SC system, which was 2.68 μg/L higher than in the BCSC system during the recharge period. This result indicated that the migration in the BCSC system was relatively poor compared with that in the SC system. The percentage of NP was 7.5% in the SC leachates and 5.1% in the BCSC leachates, and it varied greatly at different depths. The results showed that biochar can reduce the impact of NP on groundwater. Under the

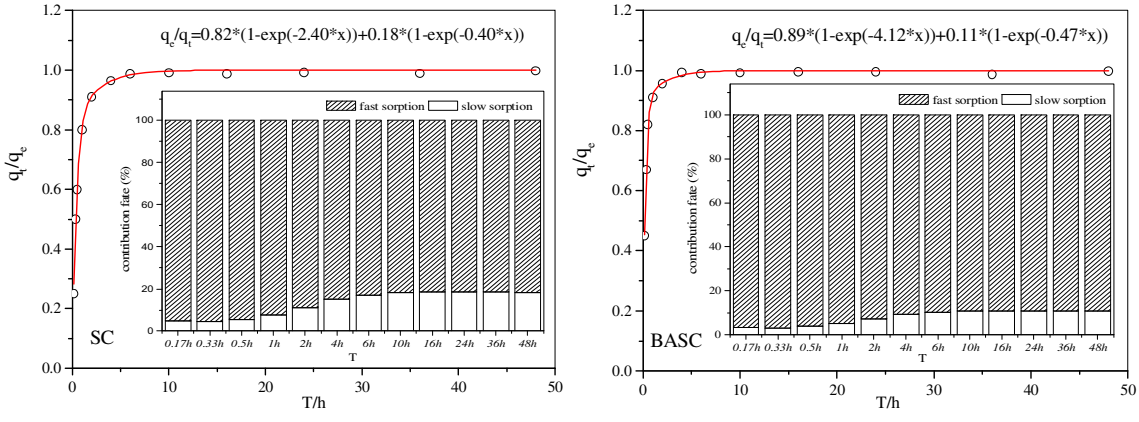

Fig. 3. The kinetics fitting results of SC and BCSC samples by the two-compartment first-order model (solid line). The embedded plots exhibited the contributions of the fast sorption and slow sorption to the total sorption capacities at the different time intervals.

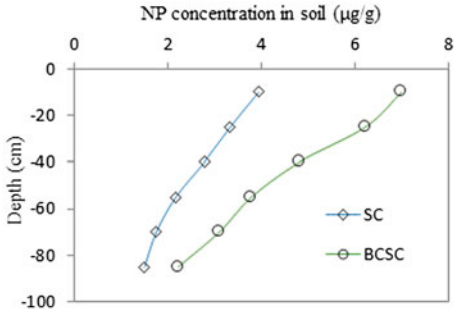

Fig. 5. Concentrations of NP in recharge soils along the depth.

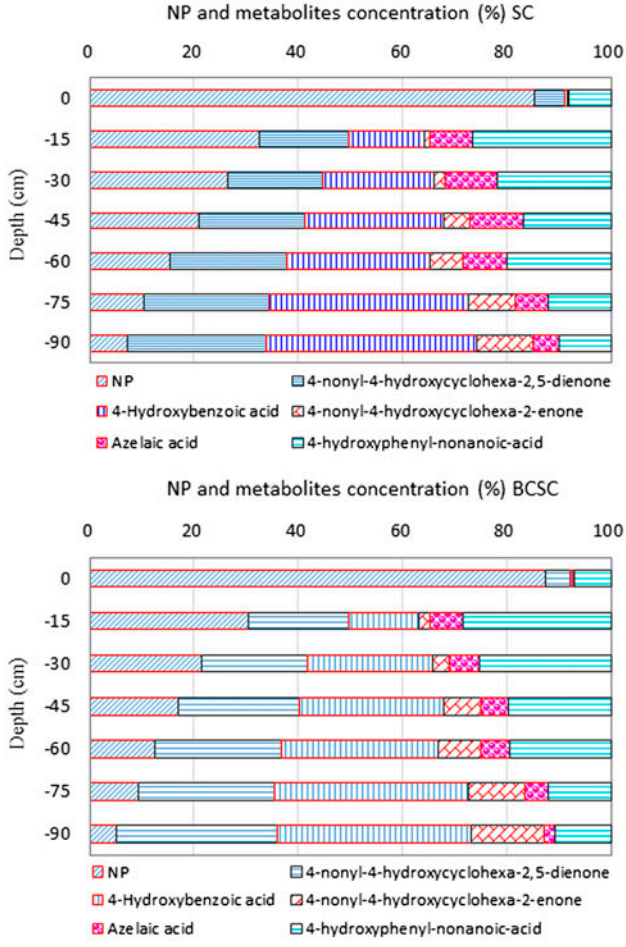

Fig. 6. NP and its metabolites' profile under different recharge mode incubation for 360 d.

condition of recharge state transition from aerobic to anaerobic with depth, biodegradation intermediates from NP to 4-nonyl-4-hydroxycyclohexa-2,5-dienone and 4-hydroxyphenyl-nonanoic-acid were found in both SC and BCSC systems, but the percentage of 4-nonyl-4-hydroxycyclohexa-2,5-dienone increased with depth. The percentage of 4-nonyl-4-hydroxycyclohexa-2-enone increased from 1.89 to 13.59% with depth, showing that it is difficult for further biodegradation because of accumulation. The accumulation of 4-nonyl-4-hydroxycyclohexa-2,5-dienone and 4-nonyl-4-hydroxycyclohexa-2-enone showed that the aromatic ring is difficult to reduce and cleave. The detected 4-hydroxybenzoic acid and azelaic acid might be the further metabolites of 4-hydroxyphenyl-nonanoic-acid. However, the percentage of azelaic acid was much lower than that of 4-hydroxybenzoic acid and it showed that the cleavage of aromatic ring was difficult and the straight chain hydrocarbons were more likely to be utilized by the micro-organisms. These results agree with those of [19,20]. The cleavage of aromatic radicals (112 kcal/mol) require more energy than the rupture of C–C or C–OH bonds (25–35 kcal/mol) between branch and aromatic radicals, which implied the hydrogen reduction reaction mainly forming 4-hydroxybenzoic acid products [34]. We proposed the two main metabolic pathways of NP. In the first, the linear carbon carboxylated and subsequently alkyl chain removal from the aromatic ring was followed by the aromatic ring cleavage. In the second, the hydroxylated aromatic ring at the ipso position formed 4-nonyl-4-hydroxycyclohexa-2,5-dienone and then reduced to form4-nonyl-4-hydroxycyclohexa-2-enone. The ipso-hydroxylation pathway was responsible for the removal of the alkyl chain from NP by *Sphingomonas* strains [35,36]. However, because of the limitations of the analytical methods employed, some other further metabolites and 4-nonyl-4-hydroxycyclohexa-2,5-dienone and 4-nonyl-4-hydroxycyclohexa-2-enonedegradation byproducts may not be detected by the GC-MS analysis. Furthermore, it is possible that this OH-enone might be converted into a series of unknown intermediate metabolites. However, very few studies have been reported about the effect of biochar on aerobic and anaerobic degradation of NP [13,14]. The NP concentration was higher in the SC system than in the BCSC system, especially in the beginning of the recharge process, because the high adsorption capacity of biochar decreased the concentration and toxicity of NP, and the relatively high dissolved oxygen (DO) concentration resulted in more aerobic microbial activity in the upper layer. The oxygen content and contaminant bioavailability were of major importance in NP degradation in soil [2], which influenced the half-life of NP. Some studies indicated that biochar application promotes HOCs mineralization in the presence of abundant micro-organisms [37,38]. NP might be oxidized in the upper layer by aerobic micro-organism, then be subjected to reductive

cleavage by anaerobic micro-organisms, forming low-carbon hydrocarbon isomers in the bottom layer. Research has revealed that the mechanism of aerobic biodegradation of NP involves hydroxylation, detach branched alkyl, and ring opening [2,14,19,20]. The concentration and percentage of NP decreased with depth, especially in BCSC system, suggesting that biochar reduced the risk of groundwater contamination by NP during the recharge process.

3.4. Effect of biochar on the change of microbial community structure

The microbial communities of different recharge soils were analyzed by high-throughput sequencing when all reactors were operated for 360 d. In the environment, NP can be decreased by biodegradation through the action of micro-organisms under aerobic or anaerobic conditions [14]. The seven soil samples came from the upper, middle, and bottom layer of the SC and BCSC systems and were named SC1, 3, 5; BCSC1, 3, 5, and control, respectively. In total, the produced 16S rDNA gene sequences ranged from 36,894 to 51,006 for seven different experimental samples through high-throughput sequencing analysis. The number of biological classification groups in the seven samples is presented in Table 2. The microbial community composition at class and genus levels is shown in Figs. 7 and 8, respectively. The result showed that the bacterial biodiversity was very rich in the two recharge systems. The variety of different soil microbial structures with depths had some differences compared to the control soil because the DO consumption and the accumulation of NP and its metabolites inhibited the growth of micro-organisms, especially aerobic micro-organisms in the upper layer. The micro-organism amount in the middle and bottom layer decreased because the organic matter content decreased and the microbial community transited from aerobic to facultative or anaerobic species. A Shannon index expressed the diversity between the microbial communities of the reactors [39]. The bacterial community in the upper layer was more diverse than in the bottom layer, as shown by the Shannon index values (Table 2). The Shannon index value of the recharge systems was lower than that of the control, which showed that bacterial diversity might be affected by the toxic organic compounds in reclaimed water. The Shannon index value of the BCSC system was marginally greater than that of the SC system in the upper layer, demonstrating a higher capability of toxicity resistance in the BCSC system compared with the SC system. The lower bacterial diversity and weak adsorption capacity in SC system could have led to the migration of NP to groundwater.

Furthermore, the accumulation of NP facilitated the dominance of some bacterial groups. The major known bacterial classes were *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteriia*, and *Bacilli* (66–84%) and the major known bacterial genera were *Sphingomonas*, *Pseudomonas*, *Methylobacillus*, *Hyphomicrobium*, *Azotobacterium*, and *Bacillus* (4.0–65.0%). To identify the bacteria associated with degradation of NP, proportions of each class and genus found in the samples were used to compute the Pearson correlation coefficient, with NP as the remaining percentage in the degradation experiments. Five known bacterial classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteriia*, and *Bacilli*) were identified that have negative correlation with NP remaining percentage (increasing with NP degradation). Similarly, 15 known bacterial genera (six major genera: *Methylobacillus*, *Azotobacterium*, *Sphingomonas*, *Pseudomonas*, *Bacillus*, and *Hyphomicrobium*) that have negative correlations with NP remaining concentration were identified. Three archaeal genera (*Methylotenera*, *Bellilinea*, and *Planomicrobium*) that have positive correlations with NP remaining percentage were identified.

Table 2
Number of sequence, operational taxonomic units, classification, and diversity indexes

Samples	No. of sequences	OTUs	Phylum	Class	Order	Family	Genus	Shannon index	Chao1
SC1	49,198	3,017	45	112	181	224	275	8.7	3,620.6
SC3	36,894	1909	36	106	162	204	268	5.7	2,085.9
SC5	42,422	3,117	43	117	181	237	323	8.5	3,068.8
BCSC1	47,089	2,953	43	128	182	212	252	8.9	3,393.2
BCSC3	56,874	2,701	51	136	201	234	293	8.1	2,626.8
BCSC5	49,782	1898	44	121	176	227	280	5.6	1,865.3
Control	51,006	2,737	36	108	176	200	205	8.8	3,135.6

Note: OUT = operational taxonomic unites.

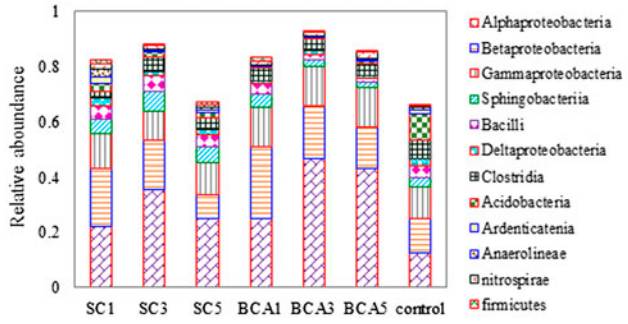

Fig. 7. Bacterial community composition (known bacterial classes) in seven recharge soil samples.

To obtain more details regarding the bacteria associated with degradation of NP at different depths and system, the distribution of bacterial genera with sequence frequencies negatively correlated with NP remaining percentage was analyzed. Five bacteria classes (*Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteriia*, and *Bacilli*) were common at different depths, while the percentage changed in different systems. These results are similar to Wang et al. [40], who reported that *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidetes* were the largest bacterial groups and micro-organisms from bacterial genera *Brevundimonas*, *Flavobacterium*, *Lysobacter*, and *Rhodobacter* might be involved in NP degradation in river sediment. Many NP-degrading micro-organisms in water and soil ecosystems from diverse bacterial genera have been reported, such as *Rhizobium*, *Sphingobium*, *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* [41–43].

In this study, the percentage of *Alphaproteobacteria* was higher than that of the control, especially for the bottom layer, which suggests that *Alphaproteobacteria* might play an important role in NP degradation in recharge systems. The enhanced abundance of the phylum *Proteobacteria* in the BCSC recharge system was mainly contributed by the classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*. The predominance of *Alphaproteobacteria* could be further attributed to the order Rhizobiales, followed by *Sphingomonadales*. Similar results have been reported by Luo et al. [44]. At the genus level, three bacterial genera (*Azotobacterium*, *Pseudomonas*, and *Methylobacillus*) were rich in all depth settings. The total percentage of these three bacterial genera was higher in the SCSC system than in the SC system, which might account for the efficient degradation in BCSC column. The genera *Pseudomonas* and *Hyphomicrobium* became more abundant in the middle and bottom layers in SCSC system. The percentage of *Sphingomonas* decreased with depth, especially for SC system, which could account for the concentration change of NP with depth. The percentage of *Hyphomicrobium* decreased with depth in the SC system, while the percentage increased with depth in the BCSC system. These bacteria may represent the core bacteria in degradation of NP. *Sphingomonas*, a group of strictly aerobic bacteria, existed only in the top and middle layer, which might be directly correlated with the aerobic degradation of NP. Gabriel et al. reported the biodegradation and its metabolic pathway of 4-NP by *Shpinggomonas xenophaga* Bayram [20,45]. The *Methylobacillus* and *Pseudomonas*, a genus of gram-negative bacteria existed in each layer, are known for degradation of a variety of environmental pollutants, such as petroleum hydrocarbons, NP, and chlorpyrifos [45]. It is reported that the enrichment of *Sphingobium* as well as *Methylobacillus* and *Xanthomonas* might have specific roles in NP biodegradation [46]. It is reported that *Bacilli* and *Azotobacterium* in mixed populations can enhance the bioavailability of aromatic hydrocarbons [47]. *Bacilli* were predominant in the middle layer of SC and bottom layer of BCSC, which might be related to the rapid hydroxylated of NP in this layer for both columns. *Hyphomicrobium* was rich in the middle and bottom layers, especially in the BCSC system, which might be related

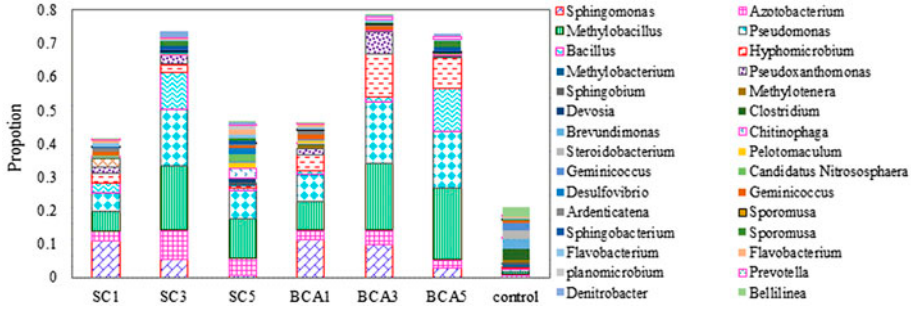

Fig. 8. Bacterial community composition (known bacterial genera) in seven recharge soil samples.

to completely carbonized NP. It is reported that *Hyphomicrobium* spp. are methylotrophic bacteria that typically use C-1 carbon sources, such as methanol, but can grow on variety of complex carbons [48]. The composition of bacteria may affect the biodegradation of NP.

4. Conclusions

The total attenuation amount in the leachate and the accumulation in the soil of the NP was in the order of BCSC > SC; this was the same order with the biodegradation intermediate products concentration of NP, which showed that the migration and microbial metabolism of NP was affected by biochar. The attenuation rate constants of NP in the SC and BCSC systems were 0.20 and 0.24 m^{-1}, respectively, which follow the principle of first-order kinetics. The detected intermediate products showed that the metabolic pathways might include carboxylation and hydroxylation followed by alkyl chain removal and aromatic ring cleavage. The bacterial community diversity decreased from the upper layer to the bottom layer in the BCSC soils and increased after an initial decrease in the SC system. *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Sphingobacteriia*, and *Bacilli* were predominant in recharge soil, which might influence the biodegradation and biotransformation of NP in recharge systems. A variety of biomass from different genera might have links with the hydroxylation, carboxylation, and mineralization of NP in the recharge process. Six major genera, *Methylobacillus*, *Azotobacterium*, *Sphingomonas*, *Pseudomonas*, *Bacillus*, and *Hyphomicrobium*, were considered NP degradation-associated bacteria (their sequence frequency negatively correlated with NP remaining concentration). These results suggest that biochar could improve the adsorption capacity and bacterial diversity in a BCSC system, and prevent the migration of NP into groundwater. The findings of this study provide a potential pathway to prevent groundwater pollution during natural groundwater recharge with reclaimed water, especially when soils and groundwater are their receptors.

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