17α-Ethynylestradiol biodegradation in different river-based groundwater recharge modes with reclaimed water and degradation-associated community structure of bacteria and archaeb

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ARTICLE INFO

Article history:
Received 20 June 2016
Revised 1 November 2016
Accepted 6 December 2016
Available online 14 January 2017

Keywords:
EE2
Biodegradation
Groundwater recharge
Microbial community and diversity
Metabolic intermediates

ABSTRACT

This study investigated 17α-ethynylestradiol (EE2) biodegradation process and primary metabolic pathways associated with community structures of microorganism during groundwater recharge using reclaimed water. The attenuation rate is 1.58 times higher in wetting and drying alternative recharge (WDAR) than in continual recharge (CR). The primary biotransformation pathways of EE2 in WDAR system began with the oxidation of C-17 on ring D to form a ketone group, and D-ring was subsequently hydroxylated and cleaved. In the CR system, the metabolic pathway changed from the oxidation of C-17 on ring D to hydroxylation of C-4 on ring A, and ring A or B subsequently cleaved; this transition was related to DO, and the microbial community structure. Four hundred fifty four pyrosequencing of 16s rRNA genes indicated that the bacterial communities in the upper layer of the WDAR system were more diverse than those found in the bottom layer of the CR system; this result was reversed for archaeb. Unweighted UniFrac and taxonomic analyses were conducted to relate the change in bacterial community structure to the metabolic pathway. Microorganism community diversity and structure were related to the concentrations of dissolved oxygen, EE2 and its intermediates in the system. Five known bacterial classes and one known archaebal class, five major bacterial genera and one major archaebal genus might be involved in EE2 degradation. The findings of this study provide an understanding of EE2 biodegradation in groundwater recharge areas under different recharging modes and can facilitate the prediction of the fate of EE2 in underground aquifers.

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http://dx.doi.org/10.1016/j.jes.2016.11.022
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Introduction

Wastewater effluent and reclaimed water, which can contain estrogen compounds, are discharged into rivers; this discharge began as an approach to ameliorating serious water shortages that were drying rivers (Vulliet and Cren-Olivé, 2011; Li et al., 2013a, 2013b). In China, approximately $3.627 \times 10^8$ tons of wastewater effluent was discharged into the river in 2014, and nearly $7.5 \times 10^7$ tons of reclaimed water was naturally discharged through river utilization in Beijing (Ma et al., 2015a, 2015b). Using reclaimed water to implement artificial groundwater recharge through river utilization provides many advantages, such as storing reclaimed water for later usage, replenishing declining water tables and alleviating groundwater depletion (Wu et al., 2011; Li et al., 2014). When reclaimed water is discharged into the river, the water will naturally permeate the riverbed, recharging the groundwater. However, endocrine disrupting chemicals (EDCs) in reclaimed water are a potential threat to underground aquifers because EDCs accounted for 27% of priority organic compounds identified in groundwater recharging occurring in China (Li et al., 2014). Over the last two decades, China rated 17α-ethynylestradiol (EE2) as one of the highest priority organic compounds with an average concentration of 44.3 ng/L; EE2 was superseded by only nonylphenol (NP) and bisphenol A (BPA) (Li et al., 2014). Synthetic estrogen 17α-ethynylestradiol (EE2), the main ingredient in commonly used oral contraceptive pills, has been detected in sewage treatment effluents and surface waters at ng/L levels in many countries (Cicek et al., 2007; Dodgen et al., 2014). The occurrence of EE2 in rivers and groundwater has recently raised a serious public concern because EE2 has a high level of toxicity at low concentrations and is persistent. The harmful influence of EE2 on ecosystem systems and the ineffectiveness of general removal methods have driven many researchers to develop more effective microbial degradation techniques (Ren et al., 2007a). Most of this research has been focused on the identification of microorganisms capable of EE2 transformations, the metabolic pathways of a single microorganism, or the metabolic kinetics in wastewater treatment and sludge. Few studies have been conducted on the biodegradation and its metabolic pathway of EE2 during river-based groundwater recharge process with reclaimed water.

There are different recharge modes which include wetting and drying alternative recharge (WDAR) and continual recharge (CR). In different recharge modes, recharge water might transform from aerobic to facultative anaerobic or anaerobic metabolisms with depth, which can influence the structure and diversity of the microbial community. Factors such as microbial diversity, microbial structure and dissolved oxygen (DO) can determine the biotransformation products and metabolic pathways of EE2 in CR and WDAR vadose zone soil (Zhang et al., 2012). Therefore, the biodegradation and ultimate fate of EE2 during artificial groundwater recharge is complicated. A few studies have explored the biodegradation metabolites of EE2 and the pathways of EE2 through the environment. The DO in reclaimed water was consumed very quickly as a result of microbial activity that occurred during the recharge process; however, the DO concentration is directly related to the recharge mode, which also determines the microbial diversity and structure. DO in aquifer medium can be recovered in the dry season in WDAR mode; while DO decreases with increasing depth in CR system. EE2 can be degraded by different microorganisms under anaerobic or aerobic conditions with different degradation mechanisms, but the biodegradation rate in aerobic conditions is much higher than in anaerobic conditions (Cajthaml et al., 2009; Aris et al., 2014). Li et al. (2013a, 2013b) reported that the degradation pathway of EE2 goes from EE2 to E1 to E3, but no further biodegradation pathway was proposed. The metabolic pathway of EE2 degradation has been reported for single strains, such as Sphingobacterium sp. JCR5, Rhodococcus zopfii, and Nitrosomonas europaea (Yoshimoto et al., 2004; Shi et al., 2002; Ren et al., 2007a; Aris et al., 2014). The EE2 biodegradation metabolite analysis showed that in the first step EE2 was oxidized to E1 with a subsequent ring cleavage (Cajthaml et al., 2009). EE2 at low μg/L levels in sludge and ng/L levels in wastewater could be co-metabolized with E1, E2, or E3; however, no other metabolites beside E1, E2, E3 and EE2 were detected, suggesting that total degradation and fission of aromatic rings occurred (Cajthaml et al., 2009; Witter et al., 2011; Rhee et al., 2014). There are no available reports about the relationship among the EE2 microbial degradation metabolic pathways and the shift of degradation-associated community structure of bacteria and archaea when the recharge conditions changed from aerobic to anaerobic. Therefore, knowledge of the biodegradation and associated microbial community structure can help us further predict the fate of EE2 in different river-based natural groundwater recharge modes that use reclaimed water.

In this study, a lab-scale percolation column was designed to further investigate the biodegradation of EE2 under different river-based natural aquifer recharge modes, and 454 pyrosequencing of 16s rRNA genes was used to analyze the shift of microbial community structure. The objective of this study is to investigate EE2 migration, biotransformation products and metabolic pathways in CR and WDAR vadose zone soil; to analyze the microbial community diversity and structure which is related to intermediate metabolites; and to understand the behavior and fate of EE2 associated with reclaimed water during natural recharge. This study will determine possible mechanisms on the shift in structures of bacterial communities in response to EE2 biodegradation pathway during river-based groundwater recharge.

1. Materials and methods

1.1. Vadose zone soil samples

Simulation columns were filled with representative soil vadose zone media from the middle of the Chaobai River (39°48′N, 116°21′E), which is a typical sandy clay from North China. Table 1 shows the physical and chemical properties of the representative soil vadose zone media. The cation-exchange capacity (CEC) was determined following the procedures defined by Chapman (1965). The total organic carbon (TOC) content was analyzed using a solid TOC analyzer (SCSH, TOC-VC-PH, Japan) (Gustafsson et al., 1997).
1.2. Raw water

The raw water used for lab-scale experiments was tertiary effluent from the Yinwenjichao Reclaimed Water Treatment Plant in the Shunyi District of Beijing. The main characteristics of the reclaimed water used for river-based groundwater recharge were: Chemical oxygen demand (COD) 50 mg/L, NH₄⁺ 1.0 mg/L, total nitrogen (TN) 6.7 mg/L, SO₄²⁻ 69 mg/L, and HCO₃⁻ 317 mg/L. The water sample for this study was collected and stored in dark for a maximum of 5 days. The concentration of 50 μg/L EE2 in reclaimed water was achieved by adding pure EE2. EE2 (99.9%) was obtained from Sigma-Aldrich (USA) and has a logKow of 4.1 and a water solubility (Sw) of 4.8 mg/L.

1.3. Lab-scale column recharge system

The recharge system includes two columns, which are schematically shown in Fig. 1. The two columns were filled with sandy clay. Each experiment was performed in triplicate. The columns were operated in CR mode and WDAR mode to simulate aquifer behavior during river-based groundwater recharge with reclaimed water. The flow rate was approximately 0.4–0.6 m/day. From the feed tank, the reclaimed water was pumped into the two aquifer treatment columns with the same flow rate. The two aquifer treatment columns were of 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 whole system covered with foil to avoid light. The lab-scale column recharge system sampled the water on day 5, 10, 30, 50, 70, and then approximately every fifty days according to the changes in water quality. The system was operated for 360 days. The wet and dry ratio of WDAR system was 4:1 (4 days recharge, 1 day dry without water). The room temperature was approximately (20 ± 5)°C. The columns were conditioned with CaCl₂ and then with reclaimed water with added EE2.

1.4. Instrumental and analytical methods

The water samples were pretreated by solid-phase extraction (Bravo et al., 2009; Zhou et al., 2010). The water sample was filtered with glass fiber filter (Whatmans, USA), and then concentrated into an Oasis HLB cartridge to extract EE2 and its metabolites. The cartridge was rinsed with methanol and ultra-pure water, and then the extraction was eluted with dichloromethane and acetone and cleaned up with a silica gel cartridge. Then, the extraction was concentrated by rotary evaporation with nitrogen. The concentrations of EE2 were

![Fig. 1 – Schematic diagram of the lab-scale column recharge system: (1) continuous recharge column (CR), (2) wet and dry alternating recharge column (WDAR), (3) feed tank, (4) peristaltic pump, (5) water sampling port, (6) piezometer, (7) supporting layer, (8) control valve, and (9) soil sampling port.](image-url)
determined by an Acquity UPLC-MS–MS system (Waters Corporation, USA) equipped with an Acquity UPLC BEH C18 column (100 mm × 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 volume ratio of mobile phase was a binary mixture of acetonitrile and ultra-pure water with gradient changing from 1:9 to 9:1 for 10 min. The parent ion (m/z), quantification ion (m/z), cone voltage (V), collision energy (eV) and dwell time (sec) were 295.1, 158.9, 37, 18 and 0.1. The metabolite identification was analyzed using the method described by Ren et al. (2007b) and Greca et al. (2008). The concentrations of EE2 metabolites were analyzed using an UPLC–MS–MS system (Waters, USA) and a GC-MS (Agilent 7890, Santa Clara, CA, USA). Mass spectrum was operated at the following conditions: ESI source, negative ion mode, 7890, Santa Clara, CA, USA. Mass spectrum was operated at 250°C.

1.5. Microbial community structure analysis using 454 pyrosequencing of 16s rRNA genes and FISH

For molecular analysis, soil samples (0.5 g, dry weight) from the CR and WDAR treatments were collected from depths of 15, 30, 55 and 90 cm. Soil Deoxyribonucleic acid (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–V8 regions from the extracted DNA. The sequence for the 5’ primer and 3’ primer comprised a 454 sequencing adaptor and 515F/806R, 50-GTGCACGCMGCCGCGGTAA-30/50-GGACTACHVGGGTWTCTAAT-30. Each Polymerase Chain Reaction (PCR) was performed in triplicate 30-μL reactions with 15 μL of the Phusion High-Fidelity PCR MasterMix (New England Biolabs, USA), 0.2 μmol/L of forward and reverse primers, and approximately 10 ng of template DNA. Reactions were cycled with an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 50°C for 30 sec, and elongation at 72°C for 30 sec. All dilutions were conducted using certified DNA-free PCR water. Pyrosequencing was performed at the Genome Center, Novogene, Beijing. Each amplicon sample was purified separately with a GeneJet Gel Extraction Kit (Thermo Scientific, USA) and quantified using an Agilent high sensitivity DNA assay on an Agilent Bioanalyzer 2100 system. Pyrosequencing was performed on a GC Junior System according to the manufacturer’s instruction (Roche Diagnostics Corp., Switzerland). The sequences were analyzed using the QIIME software package (Quantitative Insights Into Microbial Ecology), and in-house Perl scripts were used to analyze both α- and β-diversity metrics. Principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean were used to present the UniFrac distance metrics for comparing bacterial community diversity and structures in each soil sample. Data analysis involved analysis of variance with SPSS version 10.0 (SPSS Inc., Chicago, IL, USA). Bacterial classes and genera with p < 0.03 were considered EE2 degradation-associated bacteria.

A soil sample from the top layer of CR was fixed in 4% paraformaldehyde for FISH (Soo et al., 2014). FISH was performed essentially as described by Daims et al. (2005). The oligonucleotide probes used for FISH were EUB 338 (GCTGCCTCCCGTAGGACT), BET 42 (GCCTTCCACTTGGTTT) and Thaumarchaeota 915 (GTGCTCCCCGCGCAATTCTC). The NON-EUB nonsense probe was used as a negative hybridization control (Wallner et al., 1993). Microscopic analysis was performed with an Axioskop epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

2. Results and discussion

2.1. EE2 biodegradation at different depths in two recharging modes

2.1.1. Spatial variation of EE2 migration in groundwater

The EE2 concentration in the leachate from different soil profiles showed high variance among the different depths in the WDAR and CR recharging systems (Fig. 2). While the value of DO, oxidation reduction potential (ORP), TOC and pH in the bottom leachate were stable at 0, −150 to −200, 6.8 and 7.7 mg/L after 70 days recharge. The EE2 concentrations gradually decreased with increasing depth, while the attenuation rates varied greatly in the two columns. The contents ranged from 0.23 to 1.12 μg/L in the WDAR effluent and from 0.41 to 4.23 μg/L in the CR effluent. The concentration of EE2 at different depths was lower in the WDAR system than in the CR system. The attenuation rate constants in the WDAR and CR systems were 0.098 and 0.062/m, respectively, which showed that EE2 could be effectively dissipated by the microorganism under wet and dry alternate condition. Many studies have revealed that EE2 is easily degraded by microorganisms under aerobic condition, while the anaerobic degradation rate was relatively low or no anaerobic degradation occurred (Cajthaml et al., 2009; Sarmah and Northcott, 2008). In the continuous recharge condition, the biodegradation changed rapidly from aerobic biodegradation to anaerobic because the DO in water was depleted quickly by microbes. However, the major degradation mechanism was aerobic biodegradation in WDAR system because the DO can be recovered in groundwater aquifers during the dry season. Therefore, the metabolic degradation pathway might be different in the WDAR system and the CR system.

2.1.2. EE2 biodegradation intermediates and the metabolic pathway in groundwater recharge

Based on the characteristics of biological catalysis and the results obtained from UPLC-MS–MS mass spectrum and GC–MS analysis, two major metabolic pathways of EE2 degradation by complex microbes are proposed in this paper (Fig. 4). According to the percentage of EE2 and intermediates under different recharge modes (Fig. 3), the major metabolic pathways were different in the two recharge systems. When the metabolic pathways transition from aerobic to anaerobic with depth, the biodegradation intermediates of EE2 were transforming. In the WDAR system, the percentage of EE2 decreased with the increasing depth, while the percentage of E1 and 7-hydroxy-1-hydroxymethyl-2-methyl-1,2,3,4,4a,9,10,10a-octahydroanthen-2-carboxylic acid increased first and then decreased. The percentage of E3 increased from 0% to 27.1% with depth; this accumulation shows that it is difficult to further biodegrade this metabolite. The accumulation of E3 showed that aromatic rings are difficult to
reduce and cleave (Al-Ansari et al., 2010). The detection of 2-hydroxy-2,4-diene-1,6-dioic acid may be a further metabolite. Therefore, the major biotransformation pathway of EE2 in the WDAR system is proposed as follows: EE2 → estrone(E1) → estriol(E3) → 7-hydroxy-1-hydroxymethyl-2-methyl-1,2,3,4,4a,9,10,10a-octahydropyrene-2-carboxylic acid → 2-hydroxy-2,4-diene-1,6-dioic acid. The biodegradation began with the oxidation of C-17 on ring D to form the ketone group, and then D-ring was hydroxylated and cleaved. Many studies have showed that the major metabolites were E1 and E3, but further biodegradation is different under different conditions (Li et al., 2013a, 2013b; Cajthaml et al., 2009; Ren et al., 2007a). However, the limitations of the analytical methods employed mean that some other further metabolites and may not be detected by UPLC–MS–MS analysis. Furthermore, it is possible that EE2 may be converted into a series of unknown intermediate metabolites.

In the CR system, the percentage of EE2 decreased with the increasing depth, but the decreasing rate was lower than the WDAR system, which is consistent with the results in Fig. 2. The percentage of E1 and E3 did not change significantly with the increasing depth, while the percentage of 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,4,17β-triol and 2-hydroxy-2,4-diene-1,6-dioic acid increased rapidly with depth compared to the WDAR system. The percentage of (2Z,4Z)-2-hydroxy-4-(3α-methyl-3,7-dioxododecylcarboxyl-6H-cyclopenta[a]naphthalene-6-yliene)but-2-enolic acid, 3,4-dihydroxy-9,10-secoandrosta-1,3,5,triene-9,17-dione and (2Z,4Z)-2-hydroxy-8-(7α-methyl-1,5dioxooctahydro-1H-inden-4-yl)6-oxo-octa-2,4-dienoic acid increased first and then decreased with depth. In the upper layer, E1, E3 and OH-EE2 were transformed from EE2 under aerobic conditions. However, in the middle and bottom layers, the biodegradation changed from aerobic degradation to anaerobic degradation as the DO became depleted; thus, the decay rate and metabolites of EE2 changed with increasing depth. The detected (2Z,4Z)-2-hydroxy-4-(3α-methyl-3,7-dioxododecylcarboxyl-6H-cyclopenta[a]naphthalene-6-yliene)but-2-enolic acid and 3,4-dihydroxy-9,10-secoandrosta-1,3,5-triene-9,17-dione might be further metabolites. Therefore, the major biotransformation pathway of EE2 in CR system is proposed as follows: (1) EE2 → 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,4,17β-triol → (2Z,4Z)-2-hydroxy-4-(3α-methyl-3,7-dioxododecylcarboxyl-6H-cyclopenta[a]naphthalene-6-yliene)but-2-enolic acid or 3,4-dihydroxy-9,10-secoandrosta-1,3,5-triene-9,17-dione → (2Z,4Z)-2-hydroxy-8-(7α-methyl-1,5dioxooctahydro-1H-inden-4-yl)6-oxo-octa-2,4-dienoic acid → 2-hydroxy-2,4-

![Fig. 2](image-url) Distribution of EE2 over time for depths of 0-90 cm in the two recharge systems. EE2: 17α-ethynylestradiol.

![Fig. 3](image-url) Percentage of EE2 and metabolites present under the two different recharge modes for the 360-day incubation.
diene-1,6-dioic acid; (2) the same biodegradation pathway proposed for the upper layer of the WADR system. The biodegradation began with the oxidation of C-17 on ring D and hydroxylation of C-4 on ring A, and then ring A or ring B cleaved. Many studies have shown that ring D and ring A are not the most favored sites for a biodegradation attack (Lee and Liu, 2002; Choudhary et al., 2004). The biotransformation product of EE2-OH as a metabolite by ammonia monooxygenase in Ammonia oxidizing bacteria (AOB) has been reported by Yi and Harper (2002). The hydroxyl group of steroidal estrogens could be cleaved on ring C and ring D by microbes (Zhou and Zhuang, 2002). In this study, the biotransformation changed from ring D to ring A as the recharge conditions were altered with depth.

2.2. Change of microbial community structure in the two recharging modes

2.2.1. Microbial diversity and structure

Differences in α-diversity metrics (i.e., Chao1 value, observed operational taxonomic units (OTUs), Shannon index, and phylogenetic diversity) were observed for the WDAR and the CR systems (Table 2). The bacterial community in the WDAR system was more diverse than in the CR system. The variation in soil microbial structure with depth changed, relative to the control soil, for both the WDAR and CR systems because DO consumption and accumulation of EE2 and its metabolites inhibited the growth of microorganisms. The microbial diversity decreased with depth in the CR system because the organic matter content decreased, and the microbial community transformed from aerobic to facultative anaerobic or anaerobic species. In the WDAR system, diversity decreased first and then increased, possibly because the concentration of EE2 and its metabolites decreased with increasing depth which associated shift in bacteria community structures (Wang et al., 2014; Aris et al., 2014). Thus, the microbial diversity was restored. Similar variations have been reported by Ma et al. (2015a, 2015b), who compared the microbial diversity of fine sand and silty clay recharge systems using the DGGE technology. The higher microbial diversity in the WDAR system was able to mitigate the migration of EE2. Read coverage was higher than 97% for all mixed soil samples in this study, indicating that the sequence library represented most species in eight samples.

The hierarchical clustering based on the unweighted UniFrac metric shows significant differences in bacterial community structure for different depths of the two recharge systems (Fig. 5). This observation indicates that the DO concentration, the content of EE2 and its metabolites in different depths and the mode of recharge led to the development of different bacterial communities in the recharge bioreactor. Different clusters were also observed for samples obtained over a range of depths from both recharge systems, probably due to the natural variation of bacteria.

Clustering analysis obtained from the UniFrac analysis was further examined by PCoA (Fig. 6). Along PC1 vector, similar bacterial communities were observed for the upper layers, especially for the WDAR system. All CR system soil samples from different depths were clearly distinguishable from those of the control and the WDAR samples along the PC1 vector (Fig. 4). In contrast, EE2 increase modified microbial communities in the recharge system. Additionally, natural variation of the microbial communities also occurred at the different depths, as indicated by different PCoA plots of mixed soil samples taken over a range of depths from both the WDAR and the CR recharge systems along the PC2 vector.

2.2.2. Microbial community structure in different depths

Taxonomic analysis revealed the variation in the microbial community structure in response to the different recharge modes and depths (Fig. 7 and Table 2). The microbial biodiversity was rich in the two recharge systems, while, compared to the control, the variety of the different soil microbial structure decreased because the DO consumption and the accumulation of EE2 and its metabolites inhibited the growth of microorganisms, especially the aerobic microorganisms in the upper layer. The microorganism abundance and diversity decreased with depth in the CR system because the organic matter content decreased, and the microbial community transitioned from aerobic to facultative anaerobic or anaerobic species. The abundance of microorganisms decreased in the middle and then increased in the bottom layer of the WDAR system, which showed that the microbial community rebounded with the decrease of EE2. DO might also be an important factor. The Shannon index value of the WDAR system was marginally greater than that of the CR system, demonstrating a higher capability of toxicity resistance in the WDAR system compared to the CR system.
to the silty clay (SC) system. As a result, the biological treatment of the two recharge modes adversely affected the biodegradation metabolic intermediates. The lower microbial diversity in the CR system could have led to the migration of EE2 to the groundwater.

The EE2 increased the abundance of several bacterial phyla, including proteobacteria, Firmicutes, and Nitrospirae, which might be EE2 associated degrading bacteria. These bacteria were also found to be effective in the microbial degradation of EE2 compounds (Roh and Chu, 2010; Yu et al., 2011). Further analysis at the class level showed the growth of some bacterial classes, such as β-proteobacteria, Clostridia, α-proteobacteria, Acidobacteria-6, and δ-proteobacteria. Their percentages were higher in both the CR and the WDAR systems than in the control soil after the EE2 appeared in the reclaimed water. The percentage of increase was different between the WDAR and CR systems. Furthermore, the accumulation of EE2 facilitated the dominance of some microbial

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**Fig. 4** – Proposed metabolic pathway of EE2 biodegradation during groundwater recharge (A, B, C, and D represent the position of different rings).

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**Fig. 5** – Hierarchical clustering based on the unweighted UniFrac metric.
groups. Proteobacteria was the most abundant phylum in both recharge systems, and the phylum Proteobacteria contained mainly the class β-proteobacteria, α-proteobacteria, δ-proteobacteria, and γ-proteobacteria. The percentage of each of these classes varied with depth in each recharge system. In this study, the percentage of β-proteobacteria (bacterial) and Thaumarchaeota (Archaea) was higher than in the control, especially for the bottom layer, which suggests that they might play an important role in EE2 degradation for different recharge systems. The presence of β-proteobacteria and Thaumarchaeota cells in the bottom layer of the CR system was examined using a FISH with probe. Fig. 8 shows that the β-proteobacteria mix-probe-fluorescing cells and Thaumarchaeota mix-probe-fluorescing cells contributed more than 20.8% and 2.5% of EUBmix-stained cells, respectively. This abundance was comparable to or higher than the abundance found in soil samples from the Chaobai River

Fig. 6 – Principal coordinate analysis (PCoA) based on the unweighted UniFrac metric.

Fig. 7 – Microbial community structure in the eight recharge soil samples at the class and genus levels.
recharge aquifer. β-Proteobacteria cells were clustered into densely packed microcolonies, while Thaumarchaeota appeared as singly, loosely dispersed cells.

The predominance of β-proteobacteria could be further attribute to the orders Methylophilales, Burkholderiales and MND1, which were richer in the CR system than in the WDAR system. Rhizobiales and Sphingomonadales were main contributors to the abundance of α-proteobacteria, which were richer in the WDAR system than in the CR system. Clostridiales and RB41 were main contributors to the abundance of Clostridia, which were rich in the CR system. Many results have reported α-proteobacteria, β-proteobacteria and Clostridiales to be effective in the biodegradation of estrogenic compounds (Fujii et al., 2002; Aris et al., 2014). In addition, the order Nitrososphaerales in Thaumarchaeota of Archaea was also more abundant in the CR system than in the WDAR system. Many studies reported that this type of ammonia oxidizing archaea can participate in co-metabolic transformations EE2 (Yi and Harper, 2007; Ren et al., 2007a).

Detailed analysis at the genus level identified the dominant genera of EE2-tolerant or EE2-biodegradation microorganism in the recharge system (Fig. 7). The major known bacterial genera *Hyphomicrobi um*, *Bacteroides*, *Methylversatilis*, *Nitrospira*, *Methylotenera*, and *Escherichia* and one major archaea genus *Candidatus Nitrososphaera* became more abundant in the two recharge systems. In contrast, *Parabacteroides* decreased compared to the control. The relative abundance of most bacterial genera decreased with the increasing depth, while the archaea genus *Candidatus Nitrososphaera* experienced the opposite trend. Some genera were more dominant in the CR system, such as *Oscillospira* and *Clostridium*, while *Kaistobacter* and *Sphingomonas* were more dominant in the WDAR system. *Sphingomonas*, EE2-degrading bacterium, can oxidize EE2 to E1 (Ren et al., 2007a) and might be related to the ring A metabolic pathway in the WDAR system. Larcher and Yargeau (2013) and Ribeiro et al. (2010) reported that *Hyphomicrobi um* and *Rhodococcus* could remove EE2 from wastewater. *Nitrospira* was able to decompose EE2 and EE2-OH as a co-metabolic transformation product (Gaulke et al., 2008). Microorganisms from a total of twenty-one bacteria genera and five main genera *Sphingobium*, *Hyphomicrobi um*, *Bacteroides*, *Methylversatilis* and *Bellilinea* may have been linked to the removal of a variety of ring compounds. Bacteroides and *Methylversatilis* were also able to utilize E2 and EE2 (Liu et al., 2008). The composition of bacteria and archaea may affect the biodegradation of EE2. The EE2-degrading microbial community can be used as imbedding immobilized microorganisms in circulating percolation well to improve the biodegradation capacity during river-based groundwater recharge in Chaobai River.

Fig. 8 – Composite fluorescence in situ hybridization (FISH) micrographs of the two highest classes of Achaea and bacteria in the bottom recharge soil of CR. CR: continual recharge.

3. Conclusions

The attenuation rate of EE2 was higher in the WDAR system than in the CR system, which indicated that the microbial metabolism was different under aerobic and anaerobic conditions. The main metabolic pathway in the WDAR system first oxygenized a carbon on ring A. In the CR system, the metabolic pathway changed from oxidation on A ring to hydroxylation on D ring with increasing depth, which was related to the DO content and the microbial community structure. Bacterial community diversity and structure analysis using 454 pyrosequencing of 16s rRNA genes revealed that the microbial diversity decreased with depth in the CR system, while the diversity in the WDAR system decreased at first and later increased. β-Proteobacteria, Clostridia, α-proteobacteria, Acidobacteria-6, δ-proteobacteria, Chloridobacteria and γ-proteobacteria were the predominant classes. *Candidatus*, *Nitrososphaera*, *Hyphomicrobi um*, *Bacteroides*, *Methylversatilis*, *Nitrospira*, *Methylotenera*, *Treponema* and *Escherichia* were the major genera in the recharge soil, which might influence the biodegradation and metabolic pathways of EE2 in different recharge systems. FISH analysis revealed that the top class of bacteria and archaea, β-proteobacteria and Thaumarchaeota, appeared as different
dispersed cells. These results suggest that both the recharge mode and changes in the bacterial composition can affect the biodegradation and fate of EE2. This also provides insight into the bacterial community structure and diversity involved in EE2 biodegradation during reclaimed water recharge. The findings of this study may be useful for predicting the environmental fate and ecological risks of EE2 in natural environments, especially when soils and groundwater act as receptors.

Acknowledgments
This work was supported by Beijing Municipal Science and Technology Commission (No. D161100000216002) and the National Natural Science Foundation of China (No. 51678052).

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