Removal of 17β-estradiol in a Biological Active Carbon Reactor with Acetic Acid and Humic Acid

Zhongtian Li[1*], Michael K. Stenstrom[1], Xu Li[2]

ABSTRACT: The objective of this study is to characterize the removal of 17*β*-estradiol (E2) and the microbial community of a biologically active carbon (BAC) reactor under acetic acid or humic acid as the primary carbon source. Influent E2 concentration was maintained at 20 g/L. Higher than 99% removal of E2 was achieved by the BAC reactor. The concentration of E2 increased from below detection limit (<5.8 ng/L) to 48 ± 8 ng/L after switching the primary carbon source from acetic acid to humic acid in the reactor influent. Meanwhile, effluent estrone concentration increased from 50 ± 15 to 55 ± 15 ng/L after the switch of primary carbon source in the reactor influent. 17*β*-estradiol degrading bacteria were isolated. Microbial community structures under different nutrient conditions were revealed by high throughput sequencing. The presence of readily biodegradable carbon source such as acetic acid benefited E2 removal in the BAC reactor. *Water Environ. Res.*, **89**, 871 (2017).

KEYWORDS: 17*β*-estradiol, estrone, bacterial community, biodegradation, carbon source.

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Introduction

17*β*-estradiol (E2), estrone (E1), and estriol (E3) are natural estrogens that can disrupt the function of the endocrine system at elevated levels. Surveys conducted in Europe, North America, and Asia show broad occurrences of E2 and E1 in surface water, groundwater, water resource recovery facility (WRRF) effluent, and drinking water (Batt et al., 2007; Benotti et al., 2009; Khanal et al., 2006; Labadie and Budzinski, 2005; Liu et al., 2015; Xu et al., 2012; Loos, 2010; Wang et al., 2012; Zhang et al., 2014). High concentrations of E2 at the micrograms per liter level were detected in agricultural lagoons (Hutchins et al., 2007). The presence of natural estrogens in water sources poses a potential threat to the health of aquatic species. Due to the broad occurrence and potential health concerns, the U.S. Environmental Protection Agency added E2, E1, and E3 to its Contaminant Candidate List 3 (U.S. EPA, 2009).

Bacterial strains capable of degrading natural and synthetic estrogens have been isolated from water and wastewater treatment systems (Iasur-Kruh et al., 2011; Kurisu et al., 2015; Larcher and Yargeau, 2013; Tan et al., 2013; Tan et al., 2015; Weber et al., 2005; Yu et al., 2007). For most E2 degrading bacterial isolates, E1 is the primary transformation product. Some E2 degrading bacterial isolates are capable of further degrading E1, such as members in the genera *Sphingomonas*, *Nitrosomonas*, *Rhodococcus*, and *Stenotrophomonas* (Ke et al., 2007; Kurisu et al., 2010; Li et al., 2012a; Shi et al., 2004; Yoshimoto et al., 2004; Yu et al., 2007). A review study showed that 76 identified E2 degrading bacterial strains belong to Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes (Yu et al., 2013).

Recent studies suggested that estrogen degrading bacteria were multiple substrate utilizers depending on broad expression of catabolic enzymes under low substrate concentration (Tan et al., 2013). Organic carbon profiling suggested that microbial organic products released during cell starvation could stimulate E1 degradation, but the specific mechanisms of the stimulation was not clear (Tan et al., 2015). Pure culture study isolated E1 degrading bacterial strain from activated sludge samples using starvation strategy, however the E1 biodegradation was not complete (Li et al., 2012a). Studies on linuron removal indicated that recalcitrant carbon source such as humic acid have positive effect on linuron degradation in suspended growth system (Horemans et al., 2013) but not in biofilm system (Egli, 2010). The previous study suggested that the impact of carbon source on estrogen degradation may be process-specific (Tan et al., 2013). There is a knowledge gap in understanding the impact of readily biodegradable carbon source and recalcitrant carbon source on estrogen biodegradation in biofilm system.

[1] Department of Civil and Environmental Engineering, University of California Los Angeles, Los Angeles, California.

[2] Department of Civil Engineering, University of Nebraska-Lincoln, Lincoln, Nebraska.

* Corresponding author: 5732 Boelter Hall, 420 Westwood Plaza, Los Angeles, CA 90095; telephone: (424) 256-5889; e-mail: zhongtian.li@ucla.edu. Current address: Centrisys Corporation, 9586 58th Place, Kenosha, WI 53144.

Table 1—The operating condition and performance of the BAC reactor[a].

Reactor operation and performance	Phase (Duration in days)			
	1 (1–178)	2 (178–240)	3 (240–350)	4 (350–735)
	Biomass sampling date			
	172	229	331	525
Influent				
Dissolved oxygen (mg/L)	8.0 ± 0.3	8.0 ± 0.3	8.0 ± 0.3	8.0 ± 0.3
Carbon source	Background[b]	Background	Background +acetic acid (5 mg-C/L)	Background +humic acid (5 mg-C/L)
Nitrogen source	Background	NH_4^+-N (3 mg-N/L)	NO_3^--N (4 mg-N/L)	NO_3^--N (4 mg-N/L)
E2 (μg/L)	20 ± 3	20 ± 2	20 ± 3	20 ± 3
EBCT (min)	30[g]	30	30[c]	30
Effluent				
TOC-C (mg/L)	0.4 ± 0.1	0.2 ± 0.1	0.8 ± 0.2	2.9 ± 0.2
NH_4^+-N (mg/L)	—[h]	0.13	—	—
NO_3^--N (mg/L)	—	—	3.3 ± 0.3	3.7 ± 0.3
NO_2^--N (mg/L)	—	—	0.07	0.06
E2 (ng/L)	224 ± 13	<MDL	<MDL	48 ± 8
E1 (ng/L)	<MDL	<MDL	50 ± 15	55 ± 15

[a] BAC reactor performance in the first 348 days has been published (Li et al., 2012b).
[b] Background TOC was between 0.4 and 0.8 mg-C/L, background NH_4^+-N, NO_3^--N, and NO_2^--N were below MDL.
[c] Multiple Empty Bed Contact Times (EBCTs) ranged between 48 and 4.8 minutes were tested; however, biomass samples were only collected when the EBCT equaled 30 minutes.
[d] Parameters not measured.

The objective of this study was to characterize the estrogen removal performance and the microbial community of a biologically active carbon (BAC) reactor under various influent compositions. Long-term performance (735 days) of the BAC reactor was assessed with respect to its ability to remove E2 and its primary degradation product E1 from synthetic surface water. Acetic acid and humic acid were separately added to the synthetic surface water to evaluate the impact of carbon sources on E2 and E1 removals. Bacterial strains capable of degrading E2 and/or E1 were isolated from the BAC reactor. The structures of the microbial communities were assessed using pyrosequencing. Results from this study suggest that a BAC process could effectively remove E2 and E1 in the synthetic drinking water. The presence of readily biodegradable carbon may benefit microbial estrogen removal in BAC process.

Materials and Methods

BAC Reactor Operation and Biomass Sampling. Complete reactor operating condition (Day 0–735) is summarized in Table 1. Description of the BAC reactor is provided in the Supplementary Information (SI; found online). Mineral nutrients in the BAC reactor influent was reported in a previous study (Li et al., 2012b). In Phase 1 (Day 0–178), the reactor was operated as a granular activated carbon (GAC) filter with an influent made of synthetic surface water amended with 20 μg/L E2. The synthetic surface water was made using reverse osmosis (RO) water containing a background total organic carbon (TOC) concentration of 0.4 to 0.8 mg-C/L. Appropriate amount of minerals were added in the reactor influent (Li et al., 2012b). In Phase 2 (Day 178–240), 3.0 mg-N/L NH_4^+ was added to the reactor influent as a nitrogen source. In Phase 3 (Day 240–348), 5.0 mg-C/L acetic acid was added to the influent and the NH_4^+ was replaced with 4.0 mg-N/L NO_3^-. Reactor performance from Phase 1 to Phase 3 has been published elsewhere (Li et al., 2012b). In Phase 4 (Day 348–735), the acetic acid was replaced with 5.0 mg-C/L humic acid to simulate the natural organic matter (NOM) in surface water (Hyung and Kim, 2008). Biomass samples were collected at the quasi-steady state of each of the four reactor operation phases (i.e., Days 172, 229, 331, and 525). The quasi-steady states were evidenced by stable contaminant concentrations (<6% variation) in the effluent (Zhang et al., 2010). In each sampling event, 5 to 10 BAC granules were collected from the top layer of the BAC bed (Figure S1). Collected samples were transferred to sterile 2-mL RNase/DNase-free tubes and stored at −80 °C till analyses.

Isolation of E2 Degrading Bacteria from the BAC Reactor. Approximately 1 g wet weight of biomass was collected from BAC granules when the reactor showed steady E2 removal in Phase 3. Phase 3 was selected because the reactor had high biomass abundance and high E2 removal efficiency (>99.7%). The BAC granules were washed using sterilized phosphate buffer saline (PBS, pH = 7.2) twice before being transferred to a 200-mL E2-saturated nitrate mineral salts (NMS) medium (Chu and Alvarez-Cohen, 1996). The culture was grown aerobically for 24 hours while being stirred at 250 rpm to disperse the biofilm from the BAC surface. The culture was then transferred to fresh E2-saturated NMS medium (~3 mg/L of E2) at a 1:10 dilution ratio every 3 days. After the fourth transfer, the enrichment culture was diluted 10-fold in PBS and plated on R2A agar plates blended with ~3 mg/L E2. E2-R2A instead of E2-NMS agar was used because of slow growth on the latter (Yu et al., 2007). The E2-R2A agar plates were incubated at 30 °C for 3 days. Colonies with distinct morphologies were selected and regrown in liquid E2-saturated R2A media for 24 hours at 30 °C. Cells harvested were stored in 15% glycerol at −80 °C.

E2 Degradation Experiment by the Isolates. Each E2 degrading isolate was grown in 450-mL R2A medium for 24 hours at 30 °C. After incubation, 6 sets of 50-mL of cell culture was centrifuged (10 minutes, 8000*g* at 25 °C) separately for each isolate. The cell pellets were washed twice using PBS (pH = 7.2) before being transferred to 100-mL sterile E2-saturated NMS medium in a 500-mL flask. Then the culture was incubated on a shaking table (120 rpm) at 30 °C. A 70-mL solution from each sacrificial flask was centrifuged (10 minutes, 8000*g* at 4 °C) at 0, 4, 16, 48, 72, and 108 hour of incubation. A 50-mL supernatant was filtered (0.2 μm Teflon filter) and was collected in a 250-mL amber glass bottle. Water samples were stored at –20 °C until estrogen measurements.

DNA Extraction. Total DNA of E2 degrading isolates and BAC samples was extracted using a phenol-chloroform method (Chomczynski and Sacchi, 1987). For the biomass sample collected on Day 525, extra steps were taken after the phenol-chloroform extraction to remove the residual of humic acid, a polymerase chain reaction (PCR) inhibitor. Specifically, the DNA extract was further purified using an UltraClean Soil DNA Isolation Kit (MoBio, California) to remove the PCR inhibitor according to the purification steps in the manual. All DNA extracts were quantified using a NanoDrop 2000 (Thermo Scientific, Delaware), and their qualities were examined using gel electrophoresis. The DNA extracts were stored at –80 °C until use.

Phylogenetic Analyses of the E2 Degrading Isolates. The 16S rRNA genes of isolated estrogen degrading bacteria were amplified using PCR primers 27F (5′-AGRGTTT-GATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT-TACGACTT-3′) on a Mastercycler ep realplex (Eppendorf International, Hamburg, Germany) (Xia et al., 2010). Polymerase chain reaction conditions were available in the SI. The PCR products were purified and sequenced bidirectionally at Eurofins MWG Operon (Huntsville, Alabama). Sequence alignment using ClastalW and phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA) software were conducted according to a published protocol (Li et al., 2010a). Sequence alignment and phylogenetic tree construction were conducted according to a published protocol (Egli, 2010). The 16S rRNA gene sequences of the E2/E1 degrading isolates were deposited at the National Center for Biotechnology Information (NCBI) under accession numbers JX020950–JX020953.

Pyrosequencing of the 16S rRNA Gene in Biomass Samples. The V1 to V3 region of the 16S rRNA gene was amplified for pyrosequencing (Bibby et al., 2010; Martinez et al., 2009). Polymerase chain reaction was conducted at the Core for Applied Genomics and Ecology (CAGE) at the University of Nebraska–Lincoln (UNL) by following a published protocol by the facility (Martinez et al., 2009). The forward primer (A-8F) was 5′-GCCTCCCTCGCGCCATCAGAGAGTTT-GATCMTGGCTCAG-3′ with the A adapter sequence underlined. The reverse primer (B-518R) was 5′-GCCTTGCCAGCCCGCTCAG NNNNNNNN AT-TACCGCGGCTGCTGG-3′, with the B sequencing adapter underlined and an eight-base, sample-specific barcode marked as N (Martinez et al., 2009). Polymerase chain reaction conditions were available in the SI.

Prior to pyrosequencing, the quantity of the PCR products was measured using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Carlsbad, California) and the quality was assessed using an Agilent 2100 bioanalyzer (Santa Clara, California). Equal amounts of the bar-coded PCR products were mixed and loaded to a 454/Roche GS-FLX Titanium instrument (Roche, New Jersey). Pyrosequencing reads were filtered and trimmed (Martinez et al., 2009). Reads that met the following criteria were considered high quality: (a) longer than 200 base pairs, (b) perfect match with the forward primer and barcode sequences, (c) less than 2 interrupted and resumed signals from sequential flows, and (d) average quality score higher than 20.

Bacterial Community Analysis. Black Box Chimera Check (B2C2) was used to check chimeric sequences (Gontcharova et al., 2010). Sequencing de-noising was completed using QIIME by UNL CAGE (Reeder and Knight, 2010). The pyrosequencing data were then processed using the Ribosomal Database Project (RDP) Pyrosequencing Pipeline available online (http://pyro.cme.msu.edu/). Taxonomic classification was conducted using the RDP Classifier 2.6 (Wang et al., 2007a). High-quality pyrosequencing reads were aligned using Infernal, a sequence alignment software (Nawrocki et al., 2009). Shannon Index and Chao1 estimator were calculated based on 97% sequence similarity using the programs available within the RDP Pyrosequencing Pipeline.

Differences between microbial communities based on phylogenetic information were compared using unweighted pair group method with arithmetic mean (UPGMA) method and principal coordinate analysis (PCoA) (Hwang et al., 2009; Lozupone and Knight, 2005). Ribosomal Database Project Classifier 2.6 was used to generate "allrank" file and then to pull out 16S rRNA gene sequences of interested bacterial genera in pyrosequencing datasets (Wang et al., 2007a). Library Compare functionality was applied to compare the 16S rRNA gene sequences of isolated E1/E2 degrading bacteria with16S rRNA sequences in pyrosequencing datasets that belong to the same taxonomy based on Naïve Bayesian Classifier (Wang et al., 2007a). Pyrosequencing reads were deposited at NCBI Sequence Read Archive under accession numbers SRS345215 and SRS345219-SRS345221.

Estrogen Measurements. Estrogens were quantified in the UNL Water Sciences Laboratory. An HP5890 series gas chromatography (GC) equipped with a DB-5 capillary column and coupled with an Agilent 5972 quadrapole mass spectrometer (MS) was used to measure E1, E2, and E3 in water. A published protocol using BSTFA-TMCS derivatization was adapted and optimized for this study (Shareef et al., 2006). Essentially, water samples were spiked with 20 μL of 10 mg/L 17β-estradiol-16, 16, 17-d_3, and 20 μL of 10 mg/L phenanthrene as internal standards, and concentrated using a C-18 solid phase extraction (SPE) cartridge (Waters, Milford, Massachusetts, USA). After being eluted from the SPE cartridges by ethyl acetate, extracts were blown dry by nitrogen gas, reconstituted, and derivatized with 50 μL dimethyl formamide and 50 μL BSTFA+1% TMCS at 75

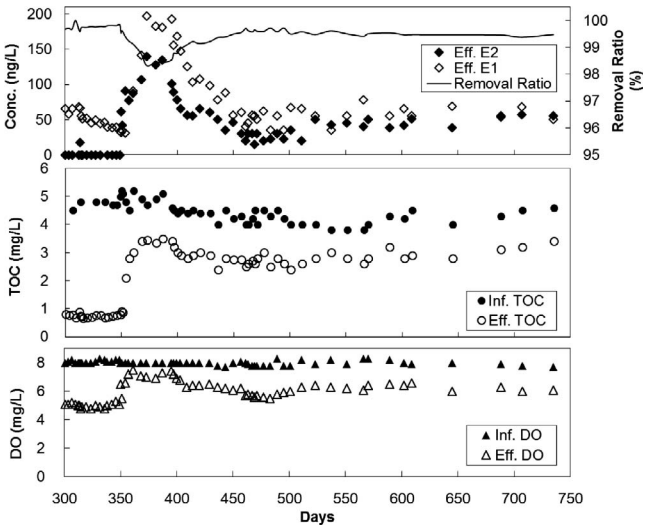

Figure 1—Response of BAC reactor performance when additional carbon source was switched from acetic acid to humic acid on Day 350. BAC reactor was operated at a constant EBCT of 30 minutes from Day 300 to Day 735. Removal ratio is the calculated based on detectable estrogens in reactor influent and effluent.

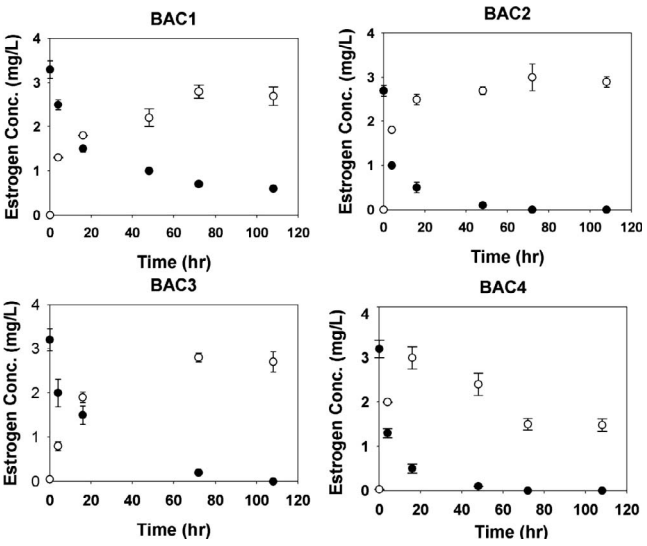

Figure 2—Estrogen degradation profiles of four E2 degrading isolates (BAC1–BAC4). Solid and open circles represent E2 (•) and E1 (○) concentration, respectively. Error bars are standard deviation of duplicate analyses.

°C for 30 minutes. Derivatized samples were equilibrated to the room temperature and transferred to GC vials with 250-μL glass inserts. All samples were analyzed within 36 hours after derivatization. A five-point standard curve was included in each GC-MS run. The method detection limit (MDL) for E1, E2, and E3 was determined to be 14.4, 5.8, and 11.4 ng/L, respectively, by following a standard procedure (U.S. EPA, 1984).

Results

Biologically Active Carbon Reactor Performance. Three major changes in operating conditions divided reactor operation into four phases in the 735 days of reactor operation (Materials and Methods and Table 1). The first 348 days of BAC reactor operation was reported previously (Leu et al., 2012). The previous data showed that BAC reactor had higher E2 removal efficiency and stability compared with GAC reactor at empty bed contact time (EBCT) from 4.8 to 48 minutes with acetic acid as the primary carbon source. In this study, the effects of switching the primary carbon source from acetic acid to humic acid on E2 and E1 removals were investigated from Day 349 to Day 735 at 30 minutes' EBCT.

As shown in Figure 1, the effluent E2 concentration increased from below detection limit to 135 ng/L, and the effluent E1 concentration increased from 44 to 197 ng/L after switching the primary carbon source from 5 mg-C/L acetic acid to 5 mg-C/L humic acid on Day 354. Starting on Day 377, effluent E2 and E1 concentrations gradually decreased and stabilized at 50 ± 15 ng/L and 55 ± 15 ng/L. The presence of humic acid in the influent decreased the E2 removal efficiency, although the E2 removal ratio was higher than 99%.

Degradation Profiles of E2 Degrading Isolates. Four isolated bacterial strains were isolated from the BAC reactor and were selected for E2 degradation experiment. Taxonomic analyses revealed that BAC1, BAC2, BAC3, and BAC4 shared 99 to 100% similarity in the 16S rRNA gene with *Acinetobacter* sp. DR1 (GenBank accession number CP002080), *Acinetobacter* sp. PRGB15 (EF195345), *Zoogloea* sp. A5 (DQ342276), and *Stenotrophomonas maltophilia* R551 (NC_011071), respectively. Two patterns of E2 degradation were observed (Figure 2). Strains BAC1, BAC2, and BAC3 converted E2 to E1 in a stoichiometric manner, but could not further degrade E1. In contrast, strain BAC4 completely converted E2 to E1 in the first 48 hours and then partially degraded the accumulated E1 till the degradation ceased at hour 72. Bacterial growth was observed only in BAC4 culture during E2 degradation experiment.

Diversity, Richness, and Differences of the Bacterial Communities. A total of 69 651 high-quality pyrosequencing reads were obtained from the four biomass samples. Sequences sharing >97% similarity were defined as an operational taxonomic unit (OTU). Rarefaction curves showed satisfactory sequencing coverage (Figure S2). The diversity and the richness of the bacterial communities in the four samples were assessed using the Shannon Index and Chao1 estimator, respectively (Table 2). The Shannon diversity index, which assesses both the richness and the evenness of a microbial community, indicates that the microbial community during Phase 2 had the highest level of diversity and the microbial community during Phase 4 had the lowest. The Chao1 index, which estimates the ultimate number of OTUs in a microbial community, reached the highest in Phase 2 and then decreased in Phase 3 and 4, a trend similar to the Shannon index. The PCoA analysis of 16S rRNA gene sequences showed that bacterial communities on Days 172 and

Table 2—Bacterial community diversity and richness indices.

Operation phase	Sampling date	OTUs	Shannon index	Chao1
1	172	455	4.13	815
2	229	1263	4.8	2178
3	331	1054	4.14	1868
4	525	576	3.89	991

229 were clustered together, and bacterial communities on Days 331 and 525 were clustered distantly based on PC1 (56.81%) and PC2 (37.18%) (Figure 3). The PCoA analysis based on PC1 and PC2 indicated that primary carbon source may be the dominating environmental factor affecting bacterial communities in the BAC reactor.

Bacterial Community Dynamics in the BAC Reactor at the Class Level. Biomass samples collected on Days 172, 229, 331, and 525 had sequences that belonged to 11, 11, 12, and 9 bacterial classes, respectively (Figure 4a). Seven bacterial classes were detected in all samples: Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Sphingobacteria, Acidobacteria, and Actinobacteria (Figure 4b). Together, sequences belonging to these seven classes constituted 84.1, 92.8, 96.6, and 98.7% of the bacterial communities on the four sampling dates (Table S1). Betaproteobacteria was the most predominant bacterial class in all samples, and its relative abundance in the BAC reactor increased from 43.3% of the bacterial community on Day 172 to 93.8% on Day 525.

Bacterial Community Dynamics in the BAC Reactor at the Genus Level. Bacterial genera *Acinetobacter* sp., *Zoogloea* sp., and *Stenotrophomonas* sp. were investigated in detail because they might contain E2/E1 degrading bacterial strains since isolates from the BAC reactor belonging to the three genera showed E2/E1 degrading capacity (Figure 2). As shown in

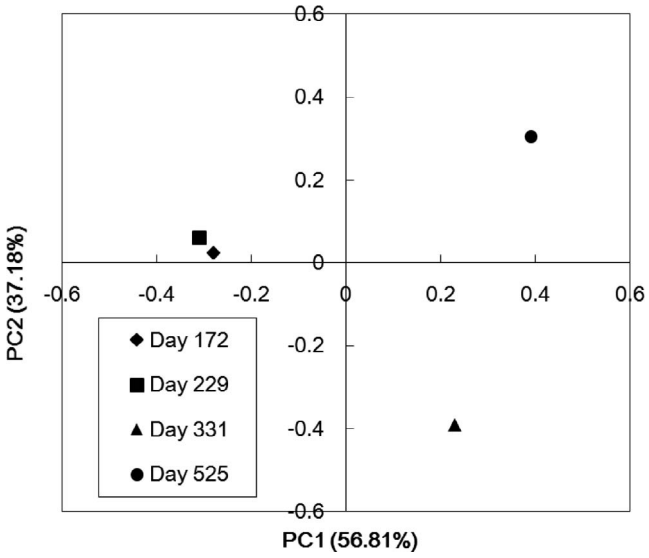

Figure 3—PCoA analysis of the bacterial communities collected on Days 172, 229, 331, and 525.

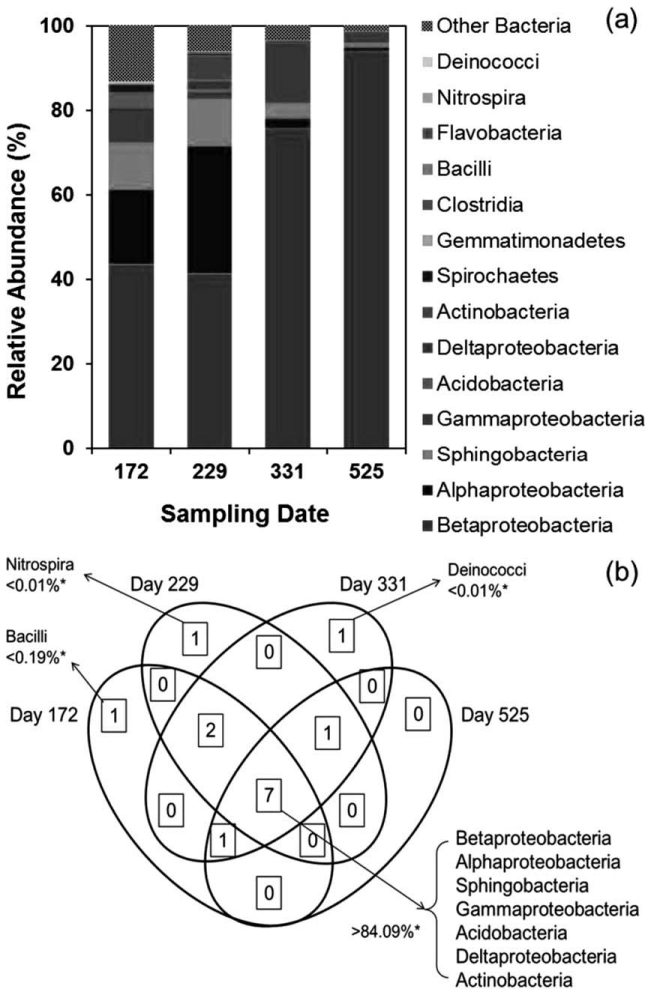

Figure 4—Taxonomic analyses of the bacterial communities in the four biomass samples at the phylum level. (a) Relative abundance of identified bacterial phyla. (b) Overlapping of identified bacterial phyla. Numbers in the Venn diagram represent the number of bacterial phylum, while the percentages with asterisks represent the relative abundance of the corresponding bacterial phylum. The relative abundance of each phylum was calculated as the number of sequences affiliated with that phylum divided by the total number of sequences in that sample.

Table 3, *Acinetobacter* sp. was only detectable on Day 331 at relative abundance of 13.5% when acetic acid served as the primary carbon source. The relative abundance of *Zoogloea* sp. increased in the BAC reactor from nondetectable on Days 172 and 229 to 68.3% on Day 331 and further to 80.3% on Day 525. The relative abundance of *Stenotrophomonas* sp. increased in the BAC reactor, accounting for 0.3% of the bacterial population on Day 331 to 2.0% on Day 525. To further investigate the 16S rRNA gene sequences of interested bacterial genera in pyrosequencing datasets, sequences of *Acinetobacter* sp., *Zoogloea* sp., and *Stenotrophomonas* sp. were pulled out from pyrosequencing datasets based on classification by RDP

Table 3—Relative abundance (%) of interested bacterial genera in BAC biomass samples[1].

	Sampling date			
	182	229	331	525
Acinetobactoer sp.	NA	NA	13.5 (7.3) [1]	NA
Zoogloea sp.	NA	NA	68.3 (45.8)	80.3 (45.0)
Stenotrophomonas sp.	NA	NA	0.3 (0.2)	2.0 (1.2)

[1] Values in parentheses indicated the percentage of 16S rRNA gene sequences in pyrosequencing datasets that share 100% similarity compared with 16S rRNA gene sequences of isolated E2/E1 degrading bacteria based on Naïve Bayesian assignment.

Classifier 2.6 (Wang et al., 2007a). The percentage of 16S rRNA gene sequences in pyrosequencing datasets that shared 100% similarity with 16S rRNA gene sequences of isolated E2/E1 degrading bacteria based on naïve Bayesian assignment ranged from 54.0% to 67.1% (Table 3).

Discussion

Switching from acetic acid to humic acid in the BAC reactor influent initially deteriorated E2 and E1 removals. Effluent E2 and E1 concentrations increased from below detection limit to 135 and 181 ng/L from Day 354 to Day 377 (Figure 1). The inhibitory effect of humic acid on E2 biodegradation was observed in previous study (Lee et al., 2011). The study showed that the kinetics of E2 and E1 degradation was negatively impacted with increasing humic acid concentrations. The authors attributed the decrease of estrogen degradation in the presence of humic acid to decreased cell densities and enzyme activities. In this study, effluent TOC concentration increased from 0.7 ± 0.1 to 2.9 ± 0.3 mg/L (Figure 1). The increased effluent TOC concentration indicated that recalcitrant portion of humic acid could not be utilized by microorganisms under tested conditions. Because less TOC was consumed and humic acid was less biodegradable than acetic acid, the maintained cell density in the BAC reactor was expected to decrease.

After Day 377, effluent E2 and E1 concentrations in reactor effluent plateaued, decreased, and stabilized at 50 ± 15 and 55 ± 15 ng/L, respectively. This observation illustrated differential effect of humic acid on E2 and E1 removal. Unlike E1, which stabilized at similar concentrations with either acetic acid or humic acid as primary background carbon source, E2 concentration was elevated from undetectable to 50 ± 15 ng/L after the BAC reactor restabilized with humic acid as background carbon source. Event with extended reactor operation from Day 377 to Day 735, no further improvement of E2 removal was observed. Data from this study showed that the presence of readily biodegradable carbon source may promote the biotransformation from E2 to E1. Comparably, only a slight increase of E1 concentration (from 50 ± 15 to 55 ± 15 ng/L) was observed when the primary carbon source was changed from acetic acid to humic acid. This finding was in agreement with what Tan et al. (2015) observed in biodegradation of E1 under different organic matter quality that dissolved organic carbon were not causative factors for E1 degradation. It was suggested that microbiologically derived organic matter may be associated with E1 degradation (Tan et al., 2015). This study's findings, together with previous study, indicated that although biological process could remove the majority of natural estrogens in the water treatment, other physiochemical means (e.g., ultraviolet light—advanced oxidization process) may be needed to further reduce residual estrogens in water (Cédat, et al., 2016).

The BAC reactor in this study was not intentionally seeded. It is believed that the microbes in the BAC reactor mostly originated from the in-house treated water that was used to make the reactor influent. Pyrosequencing analysis showed that 7 out of 14 detected bacterial classes were shared under different operation conditions (Figure 4b). This finding suggested that microbes occurred in the reactor before adding carbon source and nutrients were already phylogenetically diverse. The development of microbial activity without seeding was previously observed in a GAC reactor (Velten et al., 2011; Wang et al., 2007b). The naturally developed E2 biodegradation is reasonable because the broad occurrence of diverse E2 degrading bacteria has been observed in the aquatic environment (Yu et al., 2013).

To validate the existence of E2/E1 degrading bacteria in the BAC reactor, bacterial strains were isolated and tested for their E2/E1 degradation capacities. The isolated bacterial strain BAC1 (*Acinetobacter* sp. DR1), BAC2 (*Acinetobacter* sp. PRGB15), and BAC3 (*Zoogloea* sp. A5) could biotransform E2 to E1 (Figure 2). The biotransformation from E2 to E1 are well-observed in previous studies, including isolates from *Aminobacter* sp., *Brevundimonas* sp., *Achromobacter* sp., *Denitratisoma* sp., *Escherichia* sp., *Microbacterium* sp., *Nocardioides* sp., *Bacillusm* sp., *Staphylococcus* sp., *Sphingomonas* sp., and *Flavobacterium* sp. (Jiang et al., 2010; Li et al., 2012a; Muller et al., 2010; Weber et al., 2005; Yu et al., 2007; Yu et al., 2013). Being good slime formers, *Zoogloea* sp. are detected in water/wastewater treatment systems where biofilms exist (Larsen et al., 2008; Li et al., 2010b; Shao et al., 2009) and in estrogen-degrading consortia (Yu et al., 2005).

An E1 degrading bacterium, BAC 4 (*S. maltophilia* R551), was isolated from the BAC reactor. The E1 biodegradation by *S. maltophilia* was investigated by a proteomic approach (Li et al., 2012a). Estrone was proposed to undergo ring-cleavage and form 3-(4-hydroxyphenyl)-2-hydroxyprop-2-enoic acid, which was further biotransformed to 4-hydroxyphenylpyruvate, and eventually converted to tyrosine. The ring-cleavage characteristic of *S. maltophilia* has been well-documented (Boonchan et al., 2000; Juhasz et al., 2000; Urszula et al., 2009). *S. maltophilia* isolated from activated sludge could biodegrade several aromatic hydrocarbons, such as benzoate, catechol, 4-hydroxybenzoic acid, protocatechuic acid, and vanillic acid, by various inducible dioxygenases (Urszula et al., 2009). The occurrence of *S. maltophilia* in both biological water and wastewater treatment processes and its ring-cleavage capacity suggested its importance in estrogen biodegradation in aquatic environment.

The effects of BAC reactor operating conditions on bacterial communities were investigated. Statistical parameters, for example, OTU, Shannon index, and Chao 1, showed that the microbial communities were most diverse on Day 229, followed

by Days 331, 525, and 172 (Table 2). Microbial community diversity decreased when the primary carbon source was changed from acetic acid to humic acid (Phase 3 vs Phase 4, Table 2). A similar observation was reported that glucose in feed yielded higher microbial diversities in reactors than did complex sugars (Pholchan et al., 2010), suggesting that the presence of readily biodegradable substrates could increase microbial diversity and yield more stable reactor performance. Principal coordinate analysis indicated that carbon source may be the dominate driver of bacterial community succession. It must be noted that samples collected in the BAC reactor were from the top layer of BAC bed. Changes of bacterial community richness and evenness with bed depth were observed in BAC system (Boon et al., 2011). In this study, focuses were put on the top layer of the BAC bed. The top layer of the BAC bed usually contained the highest biomass density (Han et al., 2013; Liao et al., 2013) in down-flow BAC.

The metabolism pathways of estrogens may be significantly affected by the presence of primary carbon sources and nutrients that are at concentration several folds of magnitude higher. Four E2 biodegradation pathways with different initiating steps by aerobic bacteria have been proposed and summarized (Kurisu et al., 2010; Lee and Liu 2002; Nakai et al., 2011; Yu et al., 2013). The current knowledge indicated that enzymes responsible for E2 biodegradation may be depended on growth media and environmental conditions, which is a challenge for selecting suitable functional genes as quantifiable biomarkers. Currently, 16S rRNA genes of isolated E2 degrading bacteria seem to be the best available biomarkers to screen possible estrogen degrading community. More studies are needed to illuminate the dominate biodegradation pathways for E2 under different carbon sources.

Conclusions

(1) Switching of primary carbon source from acetic acid to humic acid in the BAC reactor influent increased the effluent E2 concentration from below detection limit (<5.8 ng/L) to 48 ± 8 ng/L. Higher than 99% removal ratio of E2 was maintained regardless of the type of primary carbon source. The presence of readily biodegradable carbon source was beneficial for E2 removal in the BAC reactor.

(2) Effluent E1 concentration was increased from 50 ± 15 to 55 ± 15 ng/L when the primary carbon source was switched from acetic acid to humic acid. This observation suggested that the removal of E1 in the BAC reactor was not significantly inhibited by the presence of humic acid.

(3) The BAC reactor was not intentionally seeded. The isolation of E2 and E1 degrading bacteria from the BAC reactor suggested that naturally occurring biofilm in activated carbon filter was likely contributed to estrogen removals in drinking water treatment process.

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Supplemental Information

Supplementary data associated with this article can be found in the online version.

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References

Batt, A. L.; Kim, S.; Aga, D. S. (2007) Comparison of the Occurrence of Antibiotics in Four Full-Scale Wastewater Treatment Plants with Varying Designs and Operations. *Chemosphere*, **68** (3), 428–435.

Benotti, M. J.; Trenholm, R. A.; Vanderford, B. J.; Holady, J. C.; Stanford, B. D.; Snyder, S. A. (2009) Pharmaceuticals and Endocrine Disrupting Compounds in US Drinking Water. *Environ. Sci. Technol.*, **43** (3), 597–603.

Bibby, K.; Viau, E.; Peccia, J. (2010) Pyrosequencing of the 16S rRNA Gene to Reveal Bacterial Pathogen Diversity in Biosolids. *Water Res.*, **44** (14), 4252–4260.

Boon, N.; Pycke, B. F. G.; Marzorati, M.; Hammes, F. (2011) Nutrient Gradients in a Granular Activated Carbon Biofilter Drives Bacterial Community Organization and Dynamics. *Water Res.*, **45** (19), 6355–6361.

Boonchan, S.; Britz, M. L.; Stanley, G. A. (2000) Degradation and Mineralization of High-Molecular-Weight Polycyclic Aromatic Hydrocarbons by Defined Fungal-Bacterial Cocultures. *Appl. Environ. Microbiol.*, **66** (3), 1007–1019.

Cédat, B.; de Brauer, C.; Métivier, H.; Dumont, N.; Tutundjan, R. (2016) Are UV Photolysis and UV/H2O2 Process Efficient to Treat Estrogens in Waters? Chemical and Biological Assessment at Pilot Scale. *Water Res.*, **100**, 357–366.

Chomczynski, P.; Sacchi, N. (1987) Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction. *Anal. Biochem.*, **162** (1), 156–159.

Chu, K. H.; Alvarez-Cohen, L. (1996) Trichloroethylene Degradation by Methane-Oxidizing Cultures Grown with Various Nitrogen Sources. *Water Environ. Res.*, **68**, 76–82.

Egli, T. (2010) How to Live at Very Low Substrate Concentration. *Water Res.*, **44** (17), 4826–4837.

Gontcharova, V.; Youn, E.; Wolcott, R. D.; Hollister, E. B.; Gentry, T. J.; Dowd, S. E. (2010) Black Box Chimera Check (B2C2): A Windows-Based Software for Batch Depletion of Chimeras from Bacterial 16S rRNA Gene Datasets. *Open Microbiol. J.*, **4**, 47–52.

Han, L.; Liu, W.; Chen, M.; Zhang, M.; Liu, S.; Sun, R.; Fei, X. (2013) Comparison of NOM Removal and Microbial Properties in Up-Flow/Down-Flow BAC Filter. *Water Res.*, **47** (14), 4861–4868.

Horemans, B.; Vandermaesen, J.; Vanhaecke, L.; Smolders, E.; Springael, D. (2013) *Variovorax* sp.-Mediated Biodegradation of the Phenyl Urea Herbicide Linuron at Micropollutant Concentrations and Effects of Natural Dissolved Organic Matter as Supplementary Carbon Source. *Appl. Microbiol. Biotechnol.*, **97** (22), 9837–9846.

Hutchins, S. R.; White, M. V.; Hudson, F. M.; Fine, D. D. (2007) Analysis of Lagoon Samples from Different Concentrated Animal Feeding Operations for Estrogens and Estrogen Conjugates. *Environ. Sci. Technol.*, **41** (3), 738–744.

Hwang, C. C.; Wu, W. M.; Gentry, T. J.; Carley, J.; Corbin, G. A.; Carroll, S. L.; Watson, D. B.; Jardine, P. M.; Zhou, J. Z.; Criddle, C. S.; Fields, M. W. (2009) Bacterial Community Succession During in Situ Uranium Bioremediation: Spatial Similarities Along Controlled Flow Paths. *ISME J.*, **3** (1), 137–137.

Hyung, H.; Kim, J. H. (2008) Natural Organic Matter (NOM) Adsorption to Multi-Walled Carbon Nanotubes: Effect of NOM Characteristics and Water Quality Parameters. *Environ. Sci. Technol.*, **42** (12), 4416–4421.

Iasur-Kruh, L.; Hadar, Y.; Minz, D. (2011) Estradiol-Degrading Bacterium—Isolation, Bioaugmentation and Integration into Mature Biofilm. *Appl. Environ. Microbiol.*, **77** (11), 3734–3740.

Jiang, L. Y.; Yang, J.; Chen, J. M. (2010) Isolation and Characteristics of 17 beta-estradiol-Degrading *Bacillus* spp. Strains from Activated Sludge. *Biodegradation*, **21** (5), 729–736.

Juhasz, A. L.; Stanley, G. A.; Britz, M. L. (2000) Microbial Degradation and Detoxification of High Molecular Weight Polycyclic Aromatic Hydrocarbons by Stenotrophomonas maltophilia Strain VUN 10,003. *Lett. Appl. Microbiol.*, **30** (5), 396–401.

Ke, J. X.; Zhuang, W. Q.; Gin, K. Y. H.; Reinhard, M.; Hoon, L. T.; Tay, J. H. (2007) Characterization of Estrogen-Degrading Bacteria Isolated from an Artificial Sandy Aquifer with Ultrafiltered Secondary Effluent as the Medium. *Appl. Microbiol. Biotechnol.*, **75** (5), 1163–1171.

Khanal, S. K.; Xie, B.; Thompson, M. L.; Sung, S. W.; Ong, S. K.; Van Leeuwen, J. (2006) Fate, Transport, and Biodegradation of Natural Estrogens in the Environment and Engineered Systems. *Environ. Sci. Technol.*, **40** (21), 6537–6546.

Kurisu, F.; Ogura, M.; Saitoh, S.; Yamazoe, A.; Yagi, O. (2010) Degradation of Natural Estrogen and Identification of the Metabolites Produced by Soil Isolates of Rhodococcus sp and Sphingomonas sp. *J. Biosci. Bioeng.*, **109** (6), 576–582.

Kurisu, F.; Zang, K.; Kasuga, I.; Furumai, H.; Yagi, O. (2015) Identification of Estrone-Degrading Betaproteobacteria in Activated Sludge by Micro-autoradiography Fluorescent In Situ Hybridization. *Lett. Appl. Microbiol.*, **61** (1), 28–35.

Labadie, P.; Budzinski, H. (2005) Determination of Steroidal Hormone Profiles Along the Jalle d'Eysines River (near Bordeaux, France). *Environ. Sci. Technol.*, **39** (14), 5113–5120.

Larcher, S.; Yargeau, V. (2013) Biodegradation of 17α-ethinylestradiol by Heterotrophic Bacteria. *Environ. Pollut.*, **173**, 17–22.

Larsen, P.; Nielsen, J. L.; Otzenj, D.; Nielsen, P. H. (2008) Amyloid-like Adhesins Produced by Floc-Forming and Filamentous Bacteria in Activated Sludge. *Appl. Environ. Microbiol.*, **74** (5), 1517–1526.

Lee, H. B.; Liu, D. (2002) Degradation of 17 beta-estradiol and Its Metabolites by Sewage Bacteria. *Water, Air, Soil Pollut.*, **134** (1-4), 353–368.

Lee, J. H.; Zhou, J. L.; Kim, S. D. (2011) Effects of Biodegradation and Sorption by Humic Acid on the Estrogenicity of 17 beta-estradiol. *Chemosphere*, **85** (8), 1383–1389.

Leu, S. -Y.; Chan, L.; Stenstrom, M. K. (2012) Toward Long Solids Retention Time of Activated Sludge Processes: Benefits in Energy Saving, Effluent Quality, and Stability. *Water Environ. Res.*, 84, 42–53.

Li, X.; Upadhyaya, G.; Yuen, W.; Brown, J.; Morgenroth, E.; Raskin, L. (2010a) Changes in the Structure and Function of Microbial Communities in Drinking Water Treatment Bioreactors upon Addition of Phosphorus. *Appl. Environ. Microbiol.*, **76** (22), 7473–7481.

Li, X.; Upadhyaya, G.; Yuen, W.; Brown, J.; Morgenroth, E.; Raskin, L. (2010b) Changes in the Structure and Function of Microbial Communities in Drinking Water Treatment Bioreactors Upon Addition of Phosphorus. *Appl. Environ. Microbiol.*, **76** (22), 7473–7481.

Li, Z. T.; Dvorak, B.; Li, X. (2012b) Removing 17 beta-estradiol from Drinking Water in a Biologically Active Carbon (BAC) Reactor Modified from a Granular Activated Carbon (GAC) Reactor. *Water Res.*, **46** (9), 2828–2836.

Li, Z.; Nandakumar, R.; Madayiputhiya, N.; Li, X. (2012a) Proteomic Analysis of 17beta-Estradiol Degradation by Stenotrophomonas maltophilia. *Environ. Sci. Technol.*, **46** (11), 5947–5955.

Liao, X. B.; Chen, C.; Wang, Z.; Wan, R.; Chang, C. H.; Zhang, X. J.; Xie, S. G. (2013) Changes of Biomass and Bacterial Communities in Biological Activated Carbon Filters for Drinking Water Treatment. *Process Biochem.*, **48** (2), 312–316.

Liu, Z. H.; Lu, G. N.; Yin, H.; Dang, Z.; Rittmann, B. (2015) Removal of Natural Estrogens and Their Conjugates in Municipal Wastewater Treatment Plants: A Critical Review. *Environ. Sci. Technol.*, **49** (9), 5288–5300.

Loos, R. (2010) Pan-European Survey on the Occurance of Selected Polar Organic Persistent Pollutants in Ground Water. *Water Res.*, **44**, 4115.

Lozupone, C.; Knight, R. (2005) UniFrac: A New Phylogenetic Method for Comparing Microbial Communities. *Appl. Environ. Microbiol.*, **71** (12), 8228–8235.

Martinez, I.; Wallace, G.; Zhang, C. M.; Legge, R.; Benson, A. K.; Carr, T. P.; Moriyama, E. N.; Walter, J. (2009) Diet-Induced Metabolic Improvements in a Hamster Model of Hypercholesterolemia Are Strongly Linked to Alterations of the Gut Microbiota. *Appl. Environ. Microbiol.*, **75** (12), 4175–4184.

Muller, M.; Patureau, D.; Godon, J. J.; Delgenes, J. P.; Hernandez-Raquet, G. (2010) Molecular and Kinetic Characterization of Mixed Cultures Degrading Natural and Synthetic Estrogens. *Appl. Microbiol. Biotechnol.*, **85** (3), 691–701.

Nakai, S.; Yamamura, A.; Tanaka, S.; Shi, J.; Nishikawa, M.; Nakashimada, Y.; Hosomi, M. (2011) Pathway of 17β-estradiol Degradation by Nitrosomonas europaea and Reduction in 17β-estradiol-derived Estrogenic Activity. *Environ. Chem. Lett.*, **9** (1), 1–6.

Nawrocki, E. P.; Kolbe, D. L.; Eddy, S. R. (2009) Infernal 1.0: Inference of RNA Alignments. *Bioinformatics*, **25** (10), 1335–1337.

Pholchan, M. K.; Baptista, J. D.; Davenport, R. J.; Curtis, T. P. (2010) Systematic Study of the Effect of Operating Variables on Reactor Performance and Microbial Diversity in Laboratory-Scale Activated Sludge Reactors. *Water Res.*,**44** (5), 1341–1352.

Reeder, J.; Knight, R. (2010) Rapidly Denoising Pyrosequencing Amplicon Reads by Exploiting Rank-Abundance Distributions. *Nat. Methods*, **7** (9), 668–669.

Shao, Y. Q.; Chung, B. S.; Lee, S. S.; Park, W.; Lee, S. S.; Jeon, C. O. (2009) Zoogloea caeni sp nov.; A Floc-Forming Bacterium Isolated from Activated Sludge. *Int. J. Syst. Evol. Microbiol.*, **59**, 526–530.

Shareef, A.; Angove, M. J.; Wells, J. D. (2006) Optimization of Silylation using N-methyl-N- (trimethylsilyl)-trifluoroacetamide, N,O-bis- (trimethyl. silyl)-trifluoroacetamide and N- (tert-butyldimethylsilyl)-N-methyltrifluoroacetamide for the Determination of the Estrogens Estrone and 17 alpha-ethinylestradiol by Gas Chromatography-Mass Spectrometry.*J. Chromatogr. A*,**1108** (1), 121–128.

Shi, J.; Fujisawa, S.; Nakai, S.; Hosomi, M. (2004) Biodegradation of Natural and Synthetic Estrogens by Nitrifying Activated Sludge and Ammonia-Oxidizing Bacterium Nitrosomonas europaea. *Water Res.*, **38** (9), 2323–2330.

Tan, D. T.; Arnold, W. A.; Novak, P. J. (2013) Impact of Organic Carbon on the Biodegradation of Estrone in Mixed Culture Systems. *Environ. Sci. Technol.*, **47** (21), 12359–12365.

Tan, D. T.; Temme, H. R.; Arnold, W. A.; Novak, P. J. (2015) Estrone Degradation: Does Organic Matter (Quality), Matter? *Environ. Sci. Technol.*, **49** (1), 498–503.

Urszula, G.; Izabela, G.; Danuta, W.; Sylwia, L. (2009) Isolation and Characterization of a Novel Strain of Stenotrophomonas Maltophilia Possessing Various Dioxygenases for Monocyclic Hydrocarbon Degradation. *Braz. J. Microbiol.*, **40** (2), 285–291.

U.S. EPA (1984) *Definition and Procedure for the Determination of the Method Detection Limit, Revision 1. 11. U. S.*; Environmental Protection Agency, Code of Federal Regulations, 40 CFR Part 136, 554.

U. S. EPA (2009) Drinking Water Contaminant Candidate List 3. http://www.epa.gov/fedrgstr/EPA-WATER/2009/October/Day-08/w24287.htm (accessed Oct 1, 2016).

Velten, S.; Boller, M.; Koster, O.; Helbing, J.; Weilenmann, H. U.; Hammes, F. (2011) Development of Biomass in a Drinking Water Granular Active Carbon (GAC) Filter. *Water Res.*, **45** (19), 6347–6354.

Wang, H. X.; Ho, L.; Lewis, D. M.; Brookes, J. D.; Newcombe, G. (2007b) Discriminating and Assessing Adsorption and Biodegradation Removal Mechanisms During Granular Activated Carbon Filtration of Microcystin Toxins. *Water Res.*, **41** (18), 4262–4270.

Wang, L.; Ying, G. G.; Chen, F.; Zhang, L. J.; Zhao, J. L.; Lai, H. J.; Chen, Z. F.; Tao, R. (2012) Monitoring of Selected Estrogenic Compounds and Estrogenic Activity in Surface Water and Sediment of the Yellow River in China Using Combined Chemical and Biological Tools. *Environ. Pollut.*, **165**, 241–249.

Wang, Q.; Garrity, G. M.; Tiedje, J. M.; Cole, J. R. (2007a) Naive Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.*, **73** (16), 5261–5267.

Weber, S.; Leuschner, P.; Kampfer, P.; Dott, W.; Hollender, J. (2005) Degradation of Estradiol and Ethinyl Estradiol by Activated Sludge and by a Defined Mixed Culture. *Appl. Microbiol. Biotechnol.*, **67** (1), 106–112.

Xia, S. Q.; Duan, L. A.; Song, Y. H.; Li, J. X.; Piceno, Y. M.; Andersen, G. L.; Alvarez-Cohen, L.; Moreno-Andrade, I.; Huang, C. L.; Hermanowicz, S. W. (2010) Bacterial Community Structure in Geographically Distributed Biological Wastewater Treatment Reactors. *Environ. Sci. Technol.*, **44** (19), 7391–7396.

Xu, N.; Xu, Y. F.; Xu, S.; Li, J.; Tao, H. C. (2012) Removal of Estrogens in Municipal Wastewater Treatment Plants: A Chinese Perspective. *Environ. Pollut.*, **165**, 215–224.

Yoshimoto, T.; Nagai, F.; Fujimoto, J.; Watanabe, K.; Mizukoshi, H.; Makino, T.; Kimura, K.; Saino, H.; Sawada, H.; Omura, H. (2004) Degradation of Estrogens by Rhodococcus zopfii and Rhodococcus equi Isolates from Activated Sludge in Wastewater Treatment Plants. *Appl. Environ. Microbiol.*, **70** (9), 5283–5289.

Yu, C. -P.; Deeb, R. A.; Chu, K. -H. (2013) Microbial Degradation of Steroidal Estrogens. *Chemosphere,* **91** (9), 1225–1235.

Yu, C. P.; Ahuja, R.; Sayler, G.; Chu, K. H. (2005) Quantitative Molecular Assay for Fingerprinting Microbial Communities of Wastewater and Estrogen-Degrading Consortia. *Appl. Environ. Microbiol.*, **71** (3), 1433–1444.

Yu, C. P.; Roh, H.; Chu, K. H. (2007) 17 beta-estradiol-degrading Bacteria Isolated from Activated Sludge. *Environ. Sci. Technol.*, **41** (2), 486–492.

Zhang, H. S.; Ziv-El, M.; Rittmann, B. E.; Krajmalnik-Brown, R. (2010) Effect of Dechlorination and Sulfate Reduction on the Microbial Community Structure in Denitrifying Membrane-Biofilm Reactors. *Environ. Sci. Technol.*, **44** (13), 5159–5164.

Zhang, H.; Shi, J. H.; Liu, X. W.; Zhan, X. M.; Chen, Q. C. (2014) Occurrence and Removal of Free Estrogens, Conjugated Estrogens, and Bisphenol A in Manure Treatment Facilities in East China. *Water Res.*, **58**, 248–257.