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APPLIED AND MECHANISTIC STUDIES OF MICROBIAL 17BETA-ESTRADIOL DEGRADATION

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APPLIED AND MECHANISTIC STUDIES OF MICROBIAL 17BETA-ESTRADIOL DEGRADATION

By

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APPLIED AND MECHANISTIC STUDIES OF MICROBIAL 17BETA-ESTRADIOL DEGRADATION

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The presence of natural estrogens, a class of endocrine disrupting compounds, in water has caused increasing concerns over their adverse impacts on the health of aquatic eco-systems and human beings. In this study, adsorption characteristics of two natural estrogens, 17β-estradiol (E2) and estrone (E1), on granular activated carbon (GAC) were investigated in isotherm tests and in a GAC column. The GAC column was then converted to a biologically active carbon (BAC) column and the removal efficiency of E2 and its primary biodegradation intermediate E1 were monitored. During BAC operation, the impacts of various reactor operation parameters, such as the carbon (i.e., acetic acid vs. humic acid) and nitrogen sources (i.e., ammonium vs. nitrate) in reactor influent and empty bed contact times (i.e., 20 – 48 minutes), were systematically studied. Corresponding to each reactor operation, the structure of the bacterial community, particularly the E2 and E1 degrading bacterial populations, in the BAC reactor was monitored using the pyrosequencing technology. Finally, metabolic pathways involved in E2 biodegradation in an E1 degrading bacterial isolate, *Stenotrophomonas maltophilia* strain ZL1, were investigated using a quantitative proteomic approach.

The following conclusions were drawn from this study:

- The adsorption isotherm experiment showed that the F400 GAC had a higher adsorption capacity for E1 than for E2, and revealed mild competitive adsorption between E1 and E2.
- The GAC column cannot effectively remove E2 due to the long mass transfer zone. The microbial activities in the BAC column enhanced E2 removal. Highest removal of E2 and its primary degradation product E1 occurred when the BAC column was operated with long EBCTs and fed acetic acid as the energy source and ammonium as the nitrogen source.
- Microbial community analysis showed that E2 degrading bacteria were accumulated in the BAC reactor and E1 degrading bacteria were the most abundant when ammonium was used as the nitrogen source.
- Enzymes associated with fatty acid biosynthesis, protein biosynthesis and oxidative phosphorylation were up-regulated during the biodegradation of E2 and E1 in *Stenotrophomonas maltophilia* strain ZL1. An E2 assimilation pathway through aromatic amino acid biosynthesis was proposed.

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CHAPTER 1: INTRODUCTION

1.1 Background

Natural estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3) have been documented to cause disruption on the endocrine functioning of aquatic species at low ng/L level (Purdom 1994; Higashitani *et al.* 2003; Imai *et al.* 2005). Recent surveys revealed broad occurrence of low levels of E1and E2 in surface water in the U.S., Pan-European area, and Asia with concentrations up to 70 ng/L (Labadie and Budzinski 2005; Khanal *et al.* 2006; Kim *et al.* 2007; Benotti *et al.* 2009; Loos 2010). Due to increasing concerns of the adverse health effects of natural estrogens, US EPA recently added E1, E2, and E3 onto its Contaminant Candidate List 3 (EPA 1984).

E2 may be removed through microbial degradation in activated sludge and biofilm systems (Weber *et al.* 2005; Jiang *et al.* 2010). Identified E2 degrading bacteria are phylogenetically diverse, covering bacterial species belonging to *Alpha-*, *Beta-*, *Gamma-proteobacteria*, *Actinobacteria*, *Bacilli, and Flavobacteria* (Shi *et al.* 2004; Yu *et al.* 2007; Jiang *et al.* 2010). For most of the E2 degrading bacteria, E1 is the primary transformation product of E2 biodegradation (Weber *et al.* 2005; Yu *et al.* 2007). Among the E2 degrading bacteria, only a fraction of them were capably of degrading E1. Commonly detected E1 degrading bacteria are species from the genera *Sphingomonas*, *Nitrosomonas*, and *Rhodococcus* (Lee and Liu 2002; Shi *et al.* 2004; Yu *et al.* 2007). The frequent detection of phylogenetically diverse E2 degrading bacteria in engineered systems suggests that E2 degrading bacteria might be enriched in engineered water / wastewater treatment systems.

The E2 and other estrogen's degradation mechanisms have been studied through activate sludge and isolated bacterial cultures. Bench-scale studies showed that E2, E1, estriol (E3), and 17α-ethinylestradiol (EE2) could be degraded by nitrifying sludge and ammonia oxidizing bacteria. A recent study aiming to elucidate the function of ammonia oxidizing bacteria (AOB) for estrogen removal showed that ammonia monooxygenase (AMO) activity could accelerate the heterotrophic mineralization of EE2 by forming a hydroxyl group on the A ring of EE2 skeleton (Khunjar *et al.* 2011). Degradation studies based on *Sphingomonas* sp. growing with / without 3-chlorocatechol (a catechol dioxygenase inhibitor) showed dual pathways of E2 biodegradation, initiating both at the aromatic ring and saturated ring (Kurisu *et al.* 2010). Previous studies have shown that AMO and catechol dioxygenase might be responsible for the ring-cleavage on estrogen skeleton (Yi and Harper 2007; Yu *et al.* 2007; Kurisu *et al.* 2010; Khunjar *et al.* 2011). To date, the study of E2 degradation has primarily focused on the detection and identification of novel degradation intermediates and several oxygenases. However, the use of proteomic response has been lacked to investigate central metabolisms during E2 biodegradation.

Biologically active carbon (BAC) is biofilm-covered GAC. The dual mechanisms of adsorption and biodegradation make BAC reactor a potential treatment technology to remove low-level estrogens during drinking water treatment. An early study found that compared to GAC reactor, BAC reactor could better remove intermittent E2 spikes in water (Li *et al.* 2008). However, little effort has been directed on how well BAC reactor may remove estrogens that are constantly present in influent, or how various reactor operating conditions may affect BAC performance of E2 removal. Also, the impact of

reactor operating conditions on the bacterial community capable of E2 degradation has not been studied. In addition, the metabolism pathways involved in E2 biodegradation are largely unknown. These are critical questions in investigating the feasibility of using BAC systems to treat estrogen-contaminated drinking water.

1.2 Objective

The Objective of this study is to systematically investigate a biologically active carbon (BAC) reactor treating E2 contaminated drinking water and understand E2 biodegradation mechanisms. Three approaches were established to achieve the objective. First, E2 removal via abiotic adsorption was studied. Adsorption kinetics and isotherm experiments were developed to understand E2 adsorption capacity of Calgon Filtrasorb® 400 (F400) coal-based GAC. E2 removal through adsorption was also studied in a GAC column packed with the same F400 GAC. Second, the GAC column established in the first approach was modified to BAC reactor. E2 removal performances were evaluated under various reactor operation conditions, including different carbon and nitrogen sources and empty bed contact times (EBCTs). The bacterial community structure and estrogen degrading bacterial dynamics were monitored at different BAC reactor operation conditions. Third, a proteomic approach was established to investigate metabolism pathways in isolated E2 degrading bacterium.

1.3 Thesis Organization

This thesis includes five chapters, references, and appendices. Chapter 1 is the general introduction of the increasing concerns of E2 and other estrogens in natural and

engineered systems as well as research questions especially for estrogen removal in a BAC reactor. Chapter 2 describes E2 adsorption characteristics and its removal in GAC and BAC rectors. In chapter 3, the bacterial community structure and estrogen degrading bacteria dynamics in the established BAC reactor were reported. In chapter 4, proteomes of a *Stenotrophomonas maltophilia* strain during E2 biodegradation was investigated. A proposed E2 degradation pathway was established based on protein identification and pathway annotation. Chapter 5 provides the conclusions of the research and suggestions on future studies. Appendices include supplemental methods and data.

CHAPTER 2: REMOVING 17B-ESTRADIOL FROM DRINKING WATER IN A BIOLOGICALLY ACTIVE CARBON (BAC) REACTOR

2.1 Introduction

Endocrine disrupting compounds (EDCs), including natural estrogens, bring increasing concerns to the aquatic ecology and pose potential risk to human health. 17β-estradiol (E2) is one of the strongest natural estrogens based on yeast estrogenicity screening (YES) assay (Metcalfe *et al.* 2001; Rutishauser *et al.* 2004). It can cause adverse impacts on the reproduction system of male fish at a few tens of ng/L (Purdom 1994; Imai *et al.* 2005). Recent surveys in North America, Europe, and Asia detected E2 in surface water and ground water with concentrations up to 70 ng/L (Labadie and Budzinski 2005; Kim *et al.* 2007; Benotti *et al.* 2009; Loos 2010). In lagoons treating wastewater from concentrated animal feeding operations (CAFOs) the E2 concentration can be as high as 21 µg/L (Hutchins *et al.* 2007). Due to the increasing concerns over E2's adverse health impacts and its broad occurrence in surface water, US EPA recently added E2, along with two other natural estrogens estrone (E1) and estriol (E3), onto the EPA Contaminant Candidate List 3 (EPA 1984).

Estrogens in water may be removed using physicochemical treatment processes, such as membrane separation (Cartinella *et al.* 2006; Yoon *et al.* 2007; Benotti *et al.* 2009) and advanced oxidation (Maniero *et al.* 2008; Shappell *et al.* 2008; Hansen *et al.* 2010). Granular activated carbon (GAC) adsorption is also a viable option. The removal efficiencies of GAC on estrogens have been tested in laboratory-scale batch reactors and continuously flow filters, and surveyed in full-scale filtration plants (Zhang and Zhou 2005; Fukuhara *et al.* 2006; Benotti *et al.* 2009). Virgin GAC usually has high adsorption

capacities. However, the adsorption capacity of preloaded GAC is significantly reduced in the presence of natural organic matter (NOM) (Fukuhara *et al.* 2006).

Natural estrogens may also be removed directly from water through microbial degradation. Although biological estrogen removal has not been investigated in the context of drinking water treatment, studies on wastewater treatment showed that activated sludge appeared to be capable of degrading E2 (Holbrook *et al.* 2002; Kuster *et al.* 2010; Stanford and Weinberg 2010). Many E2 degrading bacteria can convert E2 to E1, but fail to further degrade E1, causing E1 to be a commonly detected E2 degradation intermediate (Yu *et al.* 2007). 16S rRNA gene analyses revealed that these estrogen degrading bacteria belong to multiple genera, e.g., *Sphingomonas* sp., *Aminobacter* sp., and *Rhodococcus* sp. (Fujii *et al.* 2002; Yoshimoto *et al.* 2004; Yu *et al.* 2007). One interesting finding is that many estrogen degrading bacteria species were isolated from nitrifying activated sludge, which led to the speculation that enzymes involved in nitrification may be responsible for estrogen degradation (Vader *et al.* 2000; Shi *et al.* 2004; Yi and Harper 2007).

Biologically active carbon (BAC) is a biofilm system that uses GAC as supporting medium. The dual mechanism of adsorption and biodegradation makes BAC filters a promising treatment technology to remove low-level estrogens during drinking water treatment. An early study showed that BAC filters had better performance of removing intermittently spiked E2 in water than did GAC filters (Li *et al.* 2008). However, little effort has been directed on how well BAC filters may remove estrogens that are constantly present in influent, or how various reactor operating conditions may

affect BAC performance of E2 removal. These are critical questions in investigating the feasibility of using BAC systems to treat estrogen-contaminated drinking water.

The objective of this study was to investigate the feasibility of the BAC process to remove E2 from drinking water and the impacts of various reactor operating conditions on estrogen removal efficiency. The reactor operating conditions include varying nutrient addition and adjusting the empty bed contact time (EBCT) by varying the flow rate. Particularly, the difference in removal efficiency of E2 between GAC and BAC was systematically studied. The operation of the laboratory-scale reactor system with an extended period provides useful information to evaluate the applicability of BAC to remove natural estrogens from drinking water in full-scale systems.

2.2 Materials and Methods

2.2.1 Chemicals

17β-estradiol, estrone, 17β-estradiol-16, 16, 17-d_3 (purity ≥98%), humic acid, and derivatization grade N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilaneacetic (TMCS) acid were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade anhydrous methanol, acetone, ethyl acetate, dimethyl formamide and phenanthrene were purchased from Fisher Scientific (Pittsburgh, PA).

2.2.2 Adsorption kinetics and isotherm experiments

Calgon Filtrasorb® 400 (F400) coal-based GAC was washed with Nanopure water 10 times to remove GAC fines prior to use. The dried GAC was passed through a 30 × 40 mesh. To prepare ground GAC, dried GAC was ground using an analytical mill and

sieved through a 200 × 350 mesh (Putz *et al.* 2005). Both GAC and ground GAC were dried at 105 ^{o}C for 24 h and stored in a sealed glass desiccator until use (Speitel and Digiano 1987).

Adsorption kinetics and isotherm experiments were conducted in pH 7.4 phosphate buffer saline (PBS) solution in amber glass bottles. Estrogen stock solutions in methanol were spiked into sterilized PBS to get desired, initial estrogen concentrations. The effect of methanol on estrogen adsorption was considered minimal. Five 1-mm diameter glass beads were added to each glass bottle to ensure thorough mixing. In adsorption kinetics experiments, ~6 mg of GAC or ground GAC was added to each bottle. Different equilibrium concentrations were achieved by varying the initial estrogen concentrations with constant activated carbon amount in each bottle. The bottles were tumbled at 80 rpm in dark. Adsorption isotherm experiments were conducted for E1 and E2 in both single- and dual-solute solutions. The amount of estrogen spiked to each 500-mL bottle ranged from 7.5 to 400 μg. For each sampling event, 100 mL water sample was centrifuged at 5,000 rpm for 15 min to remove activated carbon particles. 50 ml supernatant was collected and stored at -20 ^{o}C until analysis.

2.2.3 Reactor setup

A glass column with a 5 cm inner diameter and a 45 cm length was made by ACE Glass (Vineland, NJ) and packed with 270 g virgin F400 GAC resulting a 12.2 cm bed depth. Water treated by an in-house reverse osmosis (RO) system was used to prepare reactor influent. The RO water had a pH ranged between 6.9 and 7.2 and a total organic carbon (TOC) level fluctuated between 0.4 and 0.6 mg/L, and fed the reactor in a down-

flow mode. A 48 L glass bottle wrapped with aluminum foil was used to store water containing E2, while a 200 L polypropylene tank was used to store water containing other constituents in synthetic surface water. Since the BAC reactor had a constant carbon bed volume, different EBCTs were achieved by varying the flow rate (eq. 2.1). The influent composition is summarized in Table 2.1. NH_4Cl, $NaNO_3$, and CH_3COOH were added to stimulate the biomass growth while the left mineral salts were added to model typical surface water quality. The pH values of reactor influent and effluent were monitored throughout the operation periodically and were in range of 6.8 to 7.2. Teflon tubing wrapped with aluminum foil was used in connecting various reactor compartments to minimum algae growth.

Table 2.1 The composition of the influent to the reactor system

Chemical [a]	Concentration (mg/L)
E2[b]	0.02
Na_2HPO_4	142
KH_2PO_4	136
$NaHCO_3$	5.1
$MgSO_4.7H_2O$	200
$CaCl_2.2H_2O$	1
$FeSO_4.7H_2O$	1
EDTA	3
$ZnSO_4.7H_2O$	1
$MnCl_2.4H_2O$	0.3
H_3BO_3	3
$CoCl_2.6H_2O$	0.2
$CuCl_2.2H_2O$	0.1
$NiCl_2.6H_2O$	0.2
$Na_2MoO_4.2H_2O$	0.3
NH_4Cl-N[c]	3
$NaNO_3$-N[d]	4
CH_3COOH[d]	5

[a.] Day 178-Day 348, unless specified otherwise.
[b.] Day 0-Day 348
[c.] Day 178-Day 240
[d.] Day 240-Day 348

$$EBCT = \frac{Empty\ Bed\ Volume\ (cm^3)}{Flow\ Rate\ (\frac{cm^3}{min})} \quad (2.1)$$

2.2.4 Reactor operations

In the first 178 days (13,174 bed volumes, BVs), the GAC reactor was fed an influent containing 20 µg/L E2. Although no microbial seeds or external substrates were added to the influent, biofilm inevitably developed in the reactor towards the end of this operation period, which was detected by visual observation and verified by detecting E1, an intermediate of E2 biodegradation, in reactor effluent on Day 164 (12,602 BVs). Development of microbial activities in column tests without intentional seeding is not uncommon (Wang *et al.* 2007).

On Day 178, 3.0 mg/L NH4+-N along with other nutrients were added to reactor influent (Table 2.1). NH4+-N, dissolved oxygen (DO), and TOC were measured using a NitraVer® 5 kit (Hach, USA), an YSI 5000 Dissolved Oxygen Meter (YSI Incorporated, USA), and an Apollo 9000 TOC analyzer (Teledyne, USA), respectively. On Day 240, 3.0 mg/L NH_4^+-N was replaced by 4.0 mg/L NO_3^--N and 5.0 mg/L CH_3COOH as C was added to influent through a syringe pump to simulate the composition of natural surface water (Mitsch *et al.* 2005).

2.2.5 Estrogen measurements

An HP5890 series gas chromatography (GC) equipped with a DB-5 capillary column and coupled with an Agilent 5972 quadrapole Mass Spectrometer (MS) were used to measure E1, E2, and E3 in water. A published analytical method using BSTFA-TMCS derivatization was adapted and optimized for this study (Shareef *et al.* 2006). The

sampler preparation and analysis were conducted in the Waster Sciences Laboratory at the University of Nebraska-Lincoln. 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, USA). After 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^{o}C for 30 min. Derivatized samples were equilibrated to the room temperature and transferred to GC vials with 250 μL glass insert. 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 ng/L, 5.8 ng/L, and 11.4 ng/L, respectively, using a standard procedure (EPA 1984). The average of two measurement replicates was reported for each sample.

2.3 Results and Discussion

2.3.1 Adsorption kinetics and isotherm

Virgin GAC (30 × 40 mesh size) exhibited slow adsorption kinetics. In the kinetics experiments using GAC, the E2 concentration was still decreasing after 144 hours, indicating adsorption equilibrium had not been reached (Figure 2.1). In contrast, in the kinetic experiments using ground GAC (200 × 350 mesh size), approximate adsorption equilibrium was reached in less than 82 hours. Because the adsorption capacity of GAC can be approximated using the adsorption capacity of the ground GAC

of the same type (Putz *et al.* 2005), ground GAC was used in all subsequent isotherm experiments to assess the ultimate adsorption capacity of GAC F400 for estrogens.

This adsorption isotherm data can be well modeled by the Freundlich isotherm equations (Figure 2.2). The R^2 values of the Freundlich isotherm simulation ranged between 0.91 and 0.99 (Table 2.2). K_F describes adsorption capacity, while *1/n* denotes the abundance of high-energy adsorption sites on activated carbon surface (Yu *et al.* 2009). In the single-solute experiments in which either E1 or E2 was the sole adsorbate, the K_F value for E1 was 44% higher than that of E2, while the *1/n* values of the two compounds were very close (Table 2.2). E1 is the primary intermediate during the microbial degradation of E2 and is frequently detected in source water along with E2 (Benotti *et al.* 2009). Therefore, it is expected that E1 will coexist with E2 during drinking water treatment. In order to examine the adsorption behaviors of E1 and E2 in solutions where both compounds are present, a dual-solute experiment was conducted. Competitive adsorption was observed. Compared to their counterparts in the single-solute experiments, the K_F of E1 in the dual-solute experiment decreased by 52% and the K_F of E2 by 73%. The difference between the *1/n* values from single- and dual-solute experiments was not statistically significant for both compounds based on paired t-test.

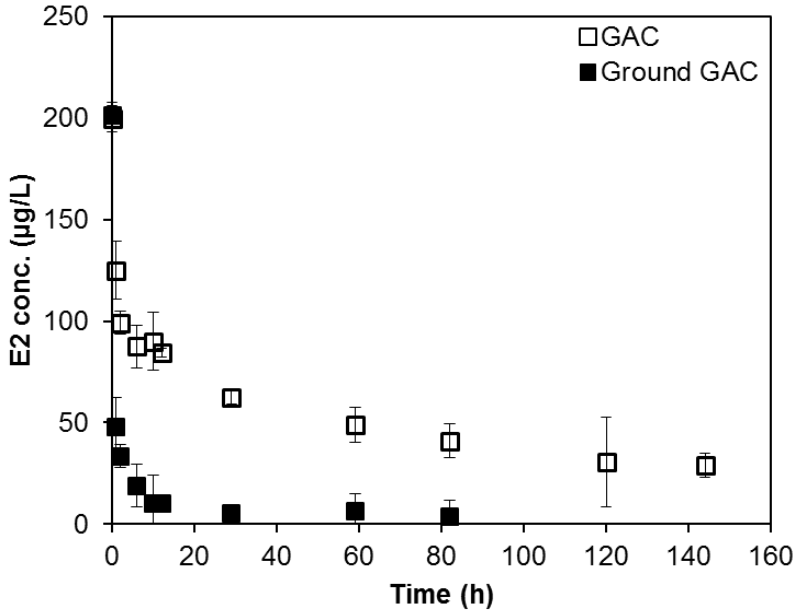

Figure 2.1 E2 adsorption kinetics using virgin GAC and virgin ground GAC.

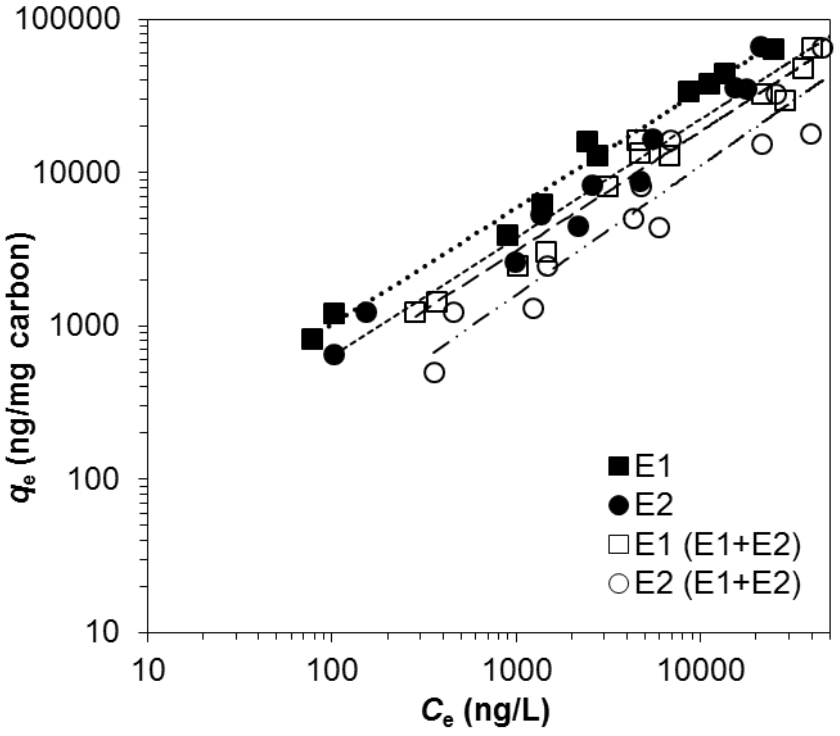

Figure 2.2 Adsorption isotherms for E1 and E2 in single- and dual-solute (i.e., E1+E2) solutions using ground GAC.

Table 2.2 Freundlich isotherm parameters in single-solute and mixed-solute solutions

Freundlich parameter	E1		E2	
	Single-solute	Dual-solute	Single-solute	Dual-solute
K_F (ng/mg)(L/ng)$^{1/n}$	29.84	14.22	18.06	4.78
$1/n$ (dimensionless)	0.77	0.78	0.77	0.84
R^2	0.99	0.97	0.97	0.91

The K_F values of E1 were higher than those of E2 in both the single- and dual-solute experiments, indicating a higher GAC adsorption capacity for E1. This finding is consistent with a previous study, which concluded that the adsorption of E1 onto activated carbon was 8% higher than that of E2 due to the higher hydrophobicity of E1 (Fukuhara *et al.* 2006). The results in Table 2.2 show that the adsorption capacity for E2 could significantly decrease in the presence of E1 (i.e., K_F dropped from 16.70 to 5.24). In order to evaluate E2 removal in continuous flow systems, a laboratory-scale GAC reactor was built and operated.

2.3.2 E2 removal in a GAC reactor

A continuous flow GAC reactor was built in the laboratory and operated abiotically. The influent E2 concentration was monitored periodically and appeared to be stable at 20±3 μg/L. E2 in the effluent was first observed above the MDL at only 1,819 BVs (Figure 2.3 middle panel, or 15 days). In the next 10,159 BVs (or 163 days), a series of EBCTs (48, 30, 20, 16, and 4.8 min) were tested by adjusting surface loading rate: 2.6, 4.1, 6.1, 7.6 and 25.5 L/m^2-min. After each change in EBCT, at least 396 BVs of water was passed through before an effluent water sample was collected and measured. Effluent E2 concentrations appeared to be responding to the EBCT changes. Their values

increased from 0.63 µg/L, to 2.2 µg/L, 2.72 µg/L, 4.38 µg/L and 6.48 µg/L, corresponding to the five EBCTs tested. Given that the F400 GAC has a relatively high adsorption capacity for E2 at the concentration range tested, the early occurrence of E2 in the effluent was likely due to a shallow mass transfer zone (MTZ) where leakage of the initial portion of the MTZ left the column relatively quickly. Previous research showed that the initial breakthrough of nonylphenol, a compound with a K_{ow} similar to E2, was significantly accelerated with a lowered external film diffusion coefficiency (Yu *et al.* 2009). It was believed that it was the low external film diffusion of E2 under the experimental condition that caused the early breakthrough of E2 in the effluent. Factors, such as the presence of natural organic matter in water sources could result in slow external film diffusion (Yu *et al.* 2009). Therefore, it is plausible to expect an early breakthrough in a full-scale GAC filter treating E2-contaminated surface water, which often contains natural organic matter. Given that an E2 concentration as low as 16 ng/L could cause negative ecological impacts, such as feminization in marine male fish (Imai *et al.* 2005), GAC adsorption alone might not be sufficient to keep effluent E2 level low at a safety level for an extended period of time.

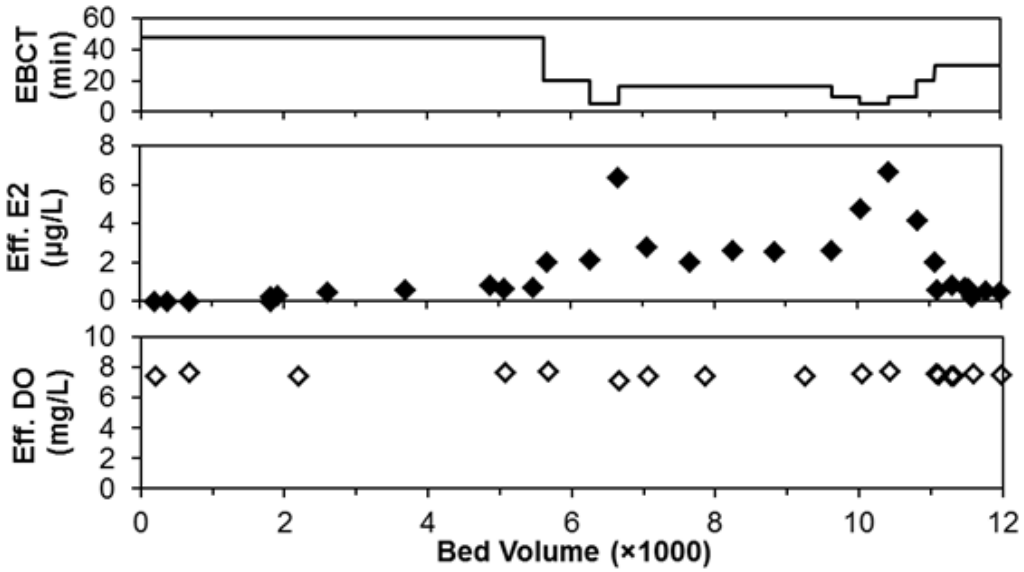

Figure 2.3 Effects of the EBCT on E2 removal in the GAC reactor.

2.3.3 Ammonium stimulated microbial E2 degradation

The addition of ammonium to the GAC reactor starting on Day 178 promoted microbial growth and stimulated microbial E2 degradation. Towards the end of the abiotic operation of the reactor, microbial activities developed inside the reactor, which was evidenced by the emergence of a frequently detected microbial degradation intermediate, E1, in reactor effluent. Since until then the reactor had not been intentionally seeded, the microbes in the reactor were likely introduced from the in-house RO water, which was used to make the influent, during the extended period of operation. In addition, TOC was detected at low levels in the in-house RO water and was likely used as a substrate by the microbes in the reactor towards the end of the 178 days of abiotic operation. Previous studies showed that ammonia oxidizing bacteria in pure and mixed cultures were capable of degrading E2. For example, ammonia oxidizing bacteria *Nitrosomonas europea* could biodegrade E2 and E1 (Shi *et al.* 2004). On day 178, 3.0

mg/L NH_4^+-N was pumped into the reactor influent to stimulate the growth of ammonia oxidizing bacteria.

Two distinct phases were observed in reactor performance after the GAC reactor was converted to a BAC reactor. In Phase 1 (Figure 2.4, Day 178 to 183 of the column run) effluent E1 concentration increased from below the method detection limit (MDL) to a peak value of 250 ng/L. Concurrently, effluent TOC decreased from 0.3 mg-C/L to 0.1 mg-C/L, effluent DO decreased from 7.8 mg/L to 3.7 mg/L, and effluent NH_4^+-N increased to 0.8 mg/L. In Phase 2 (Figure 2.4, Day 183 to Day 240), effluent E2 and E1 concentrations dropped to below their respective MDLs. In the meantime, NH_4^+-N gradually decreased back to below its MDL, and effluent DO and TOC stabilized at 3.5 mg/L and 0.2 mg/L, respectively.

The effluent profiles during the transition from GAC to BAC suggest some important characteristics of microbial communities capable of estrogen degradation, and have implications on the operation of full-scale engineered systems for estrogen removal. The water used to make the reactor influent, which was treated in an in-house RO system, did not contain any detectable levels of estrogen. The fact that bacteria originated from the in-house system accumulated in the BAC reactor and degraded estrogens suggests that bacteria in a system that has not been previously exposed to estrogens may process the enzymes related to estrogen degradation. In addition, the decrease of effluent E2 in Phase 1 suggests that E2 degrading bacteria were being enriched in the BAC reactor. The increase of effluent E1 in Phase 1 suggests a lack of E1 degrading bacteria in the BAC reactor upon the start of the BAC process. With respect to the overall microbial community, prior to ammonium addition, heterotrophic aerobes used the TOC in water as

an electron donor, but their activity levels were limited due to the lack of nutrients. After Day 178, the heterotrophic aerobes used NH_4^+-N as a nitrogen source and consumed more TOC (Figure 2.4).

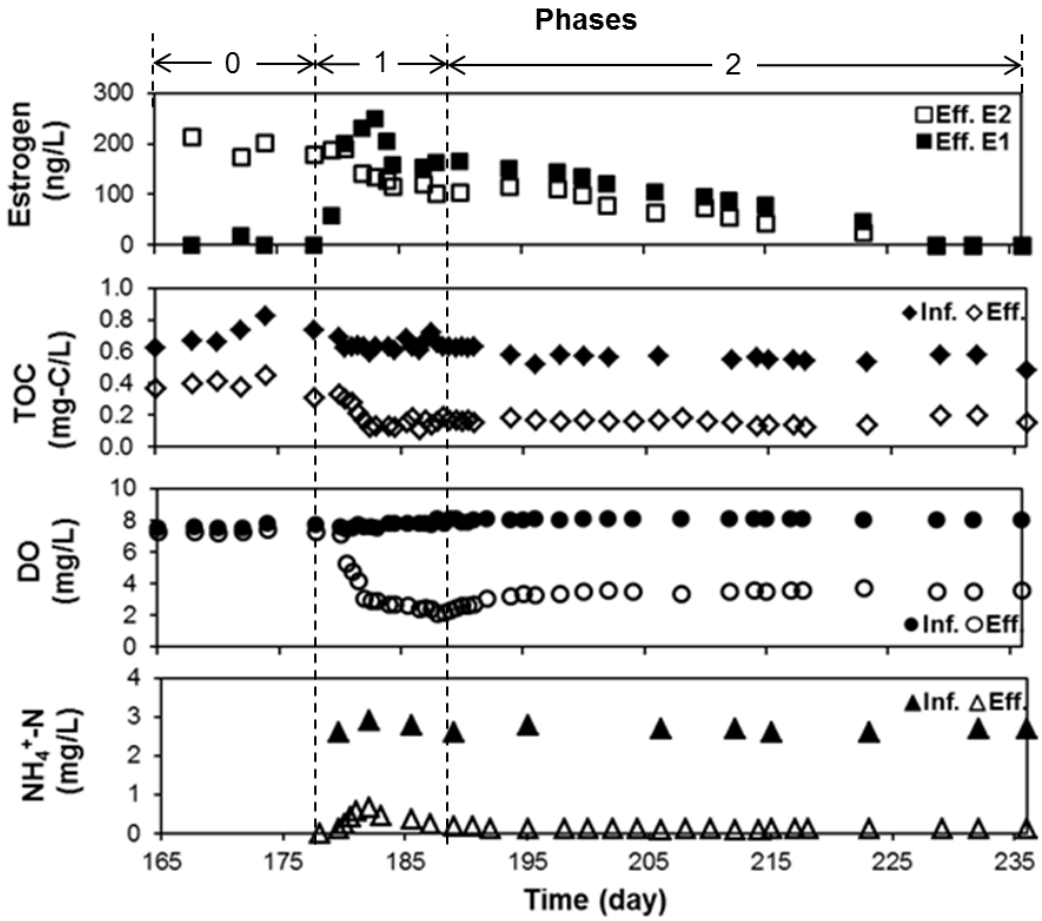

Figure 2.4 BAC reactor performance upon addition of NH_4^+-N into the reactor influent. 30 min EBCT was applied.

The decreases of effluent E1 and E2 in Phase 2 indicates that E2 and E1 degrading bacteria were gradually accumulated in the BAC reactor. The overlap of the effluent E1 and E2 profiles in Phase 2 suggests two possibilities: (1) bacteria capable of degrading both E2 and E1 accumulated in the reactor, or (2) separate E2 and E1

degrading bacteria accumulated simultaneously at the same rate. Results in the next section support the second possibility.

2.3.4 BAC operation with acetic acid and nitrate

To better simulate surface water composition, on Day 240 4.0 mg/L NO_3^--N was added to replace 3.0 mg/L NH_4^+-N as the nitrogen source and 5.0 mg-C/L CH_3COOH was added to reactor influent to simulate natural organic matters and (Figure 2.5). In Phase 3, the reactor was operated with a 30-min EBCT. The effluent E2 concentration was either close to or below the MDL, whereas E1 started to increase soon after the change in influent composition. Upon the change in influent composition, the BAC reactor consumed the majority of the influent TOC (primarily acetic acid) and DO. At the end of Phase 3 the effluent parameters appeared to reach quasi-steady state. In Phase 4 through 6, the EBCT was decreased from 40 to 25 and then to 20 minutes. Each time the EBCT was decreased (flow rated increased), the effluent DO increased and then recovered to near 2 mg/L. More biomass accumulation was also observed with a shorter EBCT during BAC operation. During these three phases, the effluent E2 concentration was not substantially impacted by the decreasing EBCTs, while the effluent E1 concentration increased with decreasing EBCTs. No pronounced differences were observed in the effluent DO or TOC profiles across the three phases. No shorter EBCT was tested due to reduced E1 degradation rate.

The results in Figure 2.5 have implications to estrogen removal in full-scale treatment systems. Nitrogen source and reactor operating conditions appeared to have substantial impact on the activities of E1 degrading bacteria. After the change in influent

composition, effluent E1 started to increase while effluent E2 was not significantly affected, suggesting that the two compounds were degraded by two separate microbial populations. Furthermore, the E1 degradation carried out by the E1 degrading bacteria in the BAC reactor seemed to rely on the presence of ammonium. Compared to the difference in response of E2 and E1 degradations to the change in influent composition (Phase 3), the differences in response of E1 and E2 degradations to decreasing EBCTs seemed more pronounced (Phases 4-6). Previous studies have suggested that E1 degradation is the limiting step during E2 biodegradation (Weber et al. 2005; Yu et al. 2007). The results reported in Figure 2.5 indicate that the presence of ammonium and sufficient contact time might be critical in achieving E1 degradation.

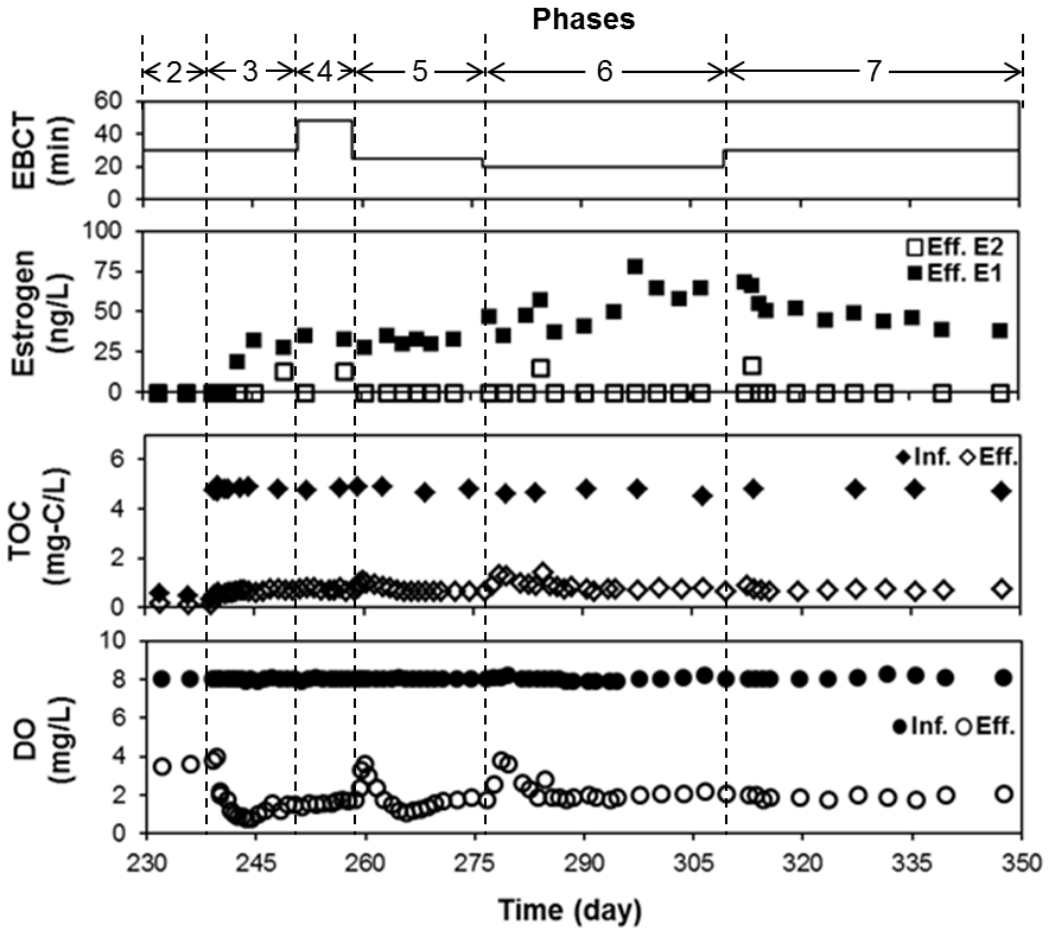

Figure 2.5 BAC reactor performance upon acetic acid supplement and ammonium starvation.

2.3.5 Comparison between GAC and BAC

By assuming no mass transfer limitations and no desorption of E2, the maximal usage of the total adsorption capacity of the activated carbon bed was only 1% with the 348 days of operation (Appendix A). Consistent with this fact, the effluent concentrations were relatively constant (±6%) for a given EBCT. Therefore, it can be assumed that the differences of reactor performance between GAC and BAC are primarily due to the biodegradation. The BAC showed a better E2 removal performance than GAC for all EBCTs. E2 removal data collected from GAC and BAC operations were plotted against EBCTs in Figure 2.6. The variance of estrogen concentrations at quasi-steady state of each operation phase was within 6% and no temporal trend was observed for a given EBCT. Complete E2 (C_e/C_i=0) removal was achieved in BAC with all EBCTs tested. As a comparison, the C_e/C_i ratio of E2 after GAC treatment increased from 0.04 to 0.32 as EBCT decreased from 48 to 4.8 min. In addition to microbial degradation in the BAC reactor, adsorption of estrogens to biofilm through hydrophobic interactions might also contribute to E2 removal from water. A study reported that estrogen adsorption to activated sludge can reach equilibrium within 10 minutes and the adsorption capacity was considered moderate at low estrogen concentration (Ren *et al.* 2007).

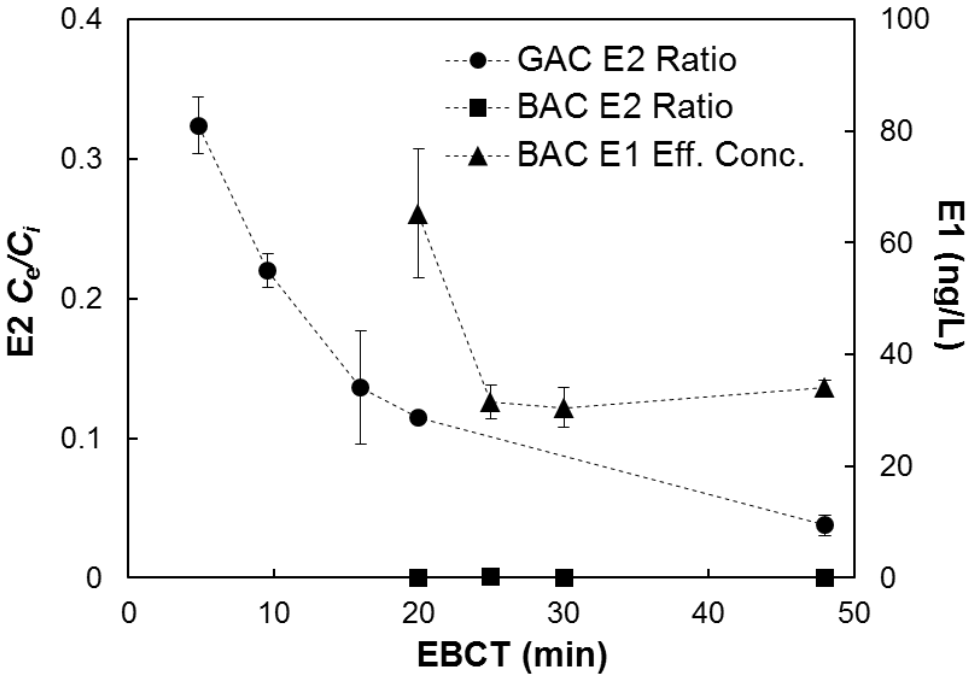

Figure 2.6 Estrogen removal efficacies of GAC and BAC (Phases 3-7) as a function of EBCT. C_e and C_i represent the effluent and influent E2 concentrations, respectively.

While E2 was completed removed, E1 appeared in effluent during BAC operation. Complete E1 removal was only achieved when NH_4^+-N was added as the nitrogen source. After NH_4^+ was replaced by NO_3^--N, effluent E1 stabilized between 35 to 40 ng/L when EBCTs were set at between 25 and 48 minutes. Because the estrogenic potency of E1 was only 14% of the estrogenic potency of E2 (Metcalfe *et al.* 2001), the overall estrogenicity of the water decreased. This study indicated that further optimizations of BAC rectors to remove estrogens should be focused on promoting the abundance and activities of E1 degrading bacteria.

CHAPTER 3: BACTERIAL COMMUNITY DYNAMICS IN A BIOLOGICALLY ACTIVE CARBON (BAC) REACTOR TREATING 17B-ESTRADIOL CONTAMINATED DRINKING WATER

3.1 Introduction

17β-estradiol (E2), estrone (E1), and estriol (E3) are natural estrogens and may cause disruption in the function of the endocrine. E2 has the highest estrogenic potency among natural estrogens, followed by E1 and E3 (Metcalfe *et al.* 2001; Aerni *et al.* 2004; Rutishauser *et al.* 2004; Khanal *et al.* 2006). Surveys conducted worldwide have showed limited occurrence of E2 and E1 in surface water, ground water, and drinking water (Labadie and Budzinski 2005; Kim *et al.* 2007; Loos 2010). In addition, E2 has been detected at μg/L level was observed in agricultural lagoons receiving wastes from animal feeding operations (Hutchins *et al.* 2007). The presence of natural estrogens in water sources poses a potential threat to the health of aquatic species and human being. Due to the occurrence and potential health concerns, US EPA added E2, E1, and E3 to its Contaminant Candidate List 3 (EPA 1984).

Bacterial isolates capable of degrading E2 are phylogenetically diverse. E2 degrading bacteria isolated from activated sludge systems include species in *Alpha-*, *Beta-*, *Gamma-proteobacteria, Actinobacteria, Bacilli, and Flavobacteria* (Shi *et al.* 2004; Yu *et al.* 2007; Jiang *et al.* 2010; Iasur-Kruh *et al.* 2011; Minz *et al.* 2011). The frequent detection and the phylogenetically diverse nature of E2 degrading bacteria suggests that enzymes responsible for biotransforming E2 to E1 might be widely distributed among bacteria common in natural and engineered systems. For most of the E2 degrading bacterial isolates, E1 is the primary transformation product of E2

biodegradation (Weber *et al.* 2005; Yu *et al.* 2007). A smaller number of E2 degrading bacterial species are capable of further degrading E1. These E1 degrading bacteria belong to the genera *Sphingomonas*, *Nitrosomonas*, and *Rhodococcus* (Lee and Liu 2002; Shi *et al.* 2004; Yu *et al.* 2007).

Microbial populations capable of degrading estrogens have been quantified using a range of molecular techniques for various systems. A method called real-time terminal restriction fragment length polymorphism was used to quantify E2 and E1 degrading bacterial populations by detecting distinctive terminal restriction fragment (T-RF) patterns in activated sludge samples separately enriched with E2 and E1 (Yu *et al.* 2005). In a recent study, denaturing gradient gel electrophoresis (DGGE) was used to study the microbial community in a wetland that received E2 as the sole carbon source. Sequencing results of DGGE bands showed that *Bacteroidetes*, *Novosphingobium* strain JEM-1, and *Hydrogenophaga intermedia* dominated the microbial population at the end of E2 enrichment (Iasur-Kruh *et al.* 2011). In another study, microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) was used to visualize and quantify E1 degrading bacteria in activated sludge floc. It was estimated that approximately 1~2 % of the microbial population was capable of assimilating E1, and they belonged to *Beta*- and *Gamma-proteobacteria* (Zang *et al.* 2008). These studies confirm the findings from culture-based studies that estrogen degrading bacteria are phylogenetically diverse, and suggest that E1 degrading bacteria may have low abundance in mixed microbial community. Therefore, molecular method that is capable providing high sequencing resolution is best suited for microbial populations degrading E2 and E1.

Pyrosequencing, a parallel sequencing technique, relies on emulsion polymerase chain reactions and sequences DNA based on a "sequencing by synthesis" principle (Margulies *et al.* 2005). Compared to the Sanger sequencing method that has been used in the construction of traditional clone libraries, pyroseqeuncing could achieve an approximately 100-fold increase in throughput and thus more suitable for identifying bacteria with low abundance in microbial community studies (Jones *et al.* 2009). The pyrosequencing technique has been used to study microbial communities in drinking water distribution systems, denitrifying reactors, and microbial electrolysis cells (Hong *et al.* 2010; Lee *et al.* 2010; Zhang *et al.* 2010). The high sequencing resolution granted by pyrosequencing makes the technique ideal for quantifying E2 and E1 degrading bacteria in environmental samples.

A biologically active carbon (BAC) reactor was established by encouraging biofilm growth on the surface of granular activated carbon (GAC), and operated to remove E2 from surface water. The performance of the BAC reactor, with respect to removing E2 and its degradation intermediates, was evaluated under different reactor operating conditions (e.g., nutrient conditions, empty bed contact times, and natural organic matter). Results showed that the adsorption of E2 onto the GAC granule could reduce E2 from 20 µg/L in the influent to ~200 ng/L in the effluent, and the microbial activities were able to further lower effluent E2 concentration from ~200 ng/L to below the detection limit (i.e.,5.8 ng/L). In this study, we investigated the bacterial community, particularly the estrogen degrading populations in the BAC reactor. The objective of this study is to investigate the dynamics of the E2 and E1 degrading populations in the BAC reactor under various reactor operating conditions. The reactor operations that benefit the

growth of estrogen degrading bacteria in mixed communities may benefit the design and operation of biological treatment systems to remove estrogens in water.

3.2 Materials and Methods

3.2.1 BAC reactor operation and biomass sampling

Reactor operating condition is summarized in Table 3.1. In Phase 0 (Day 0 – 178), the reactor was operated abiotically as a GAC filter with an influent made of reverse osmosis (RO) water amended with 20 μg/L E2. The RO water was treated using an in-house system, and contained a background total organic carbon (TOC) concentration of 0.4 mg-C/L, which happened to be within the typical range found in US drinking water treatment plant effluents (Lechevallier *et al.* 1991). On Day 178, chemicals were added to the influent to promote microbial growth and to convert the GAC filter to a BAC reactor. In Phase 1-2 (Day 178 – 240), 3.0 mg/L NH_4^+-N was added to the reactor influent as a nitrogen source. In Phase 3-7 (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/L NO_3^--N. In Phase 8 (Day 348 – 613), the acetic acid was replaced with 5.0 mg-C/L humic acid to better simulate the natural organic matter (NOM) in surface water (Hyung and Kim 2008). The estrogen concentrations were quantified using a modified GC-MS method (Shareef *et al.* 2006).

Biomass samples were collected on Days 172 (Phase 0), 229 (Phase 2), 331 (Phase 7), and 525 (Phase8), one representing each of the four phases at their perspective quasi-steady state. The quasi-steady conditions were evidenced by stable contaminant concentrations in the effluent (Zhang *et al.* 2010). In each biofilm sampling event, about 5 ~10 BAC granules were collected from the top layer of the BAC bed through a

specially designed sampling port using a sterilized inoculation loop. A schematic of the biofilm sampling location could be found in the Appendix B. Collected samples were transferred to sterile 2-ml centrifuge tubes free of RNase and DNase and stored in -80℃ freezer till DNA extraction.

Table 3.1 The operating condition and reactor performance of the BAC reactor[a]

Reactor Operation and Performance	Phase (Duration in days)			
	0 (1-178)	2 (183-240)	7 (310-348)	8 (348-613)
	Biomass Sampling Day			
	172	229	331	525
Influent				
Dissolved Oxygen (mg/L)	7.8±0.4	8.2±0.2	8.1±0.3	8.2±0.2
Carbon Source	Background[b]	Background	Background +Acetic acid[c]	Background +Humic acid[d]
Nitrogen Source	—[h]	NH_4^+-N[e]	NO_3^--N[f]	NO_3^--N[f]
17β-Estrodial (μg/L)	20	20	20	20
Effluent				
TOC-C (mg/L)	0.4	0.2	0.7	3.2
NH_4^+-N (mg/L)	—	0.12	—	—
NO_3^--N (mg/L)	—	—	3.8	3.2
NO_2^--N (mg/L)	—	—	0.07	0.06
17β-Estrodial (ng/L)	230	Below MDL	Below MDL	50
Estrone (ng/L)	Below MDL	Below MDL	44	55
EBCT (min)	30	30	30[g]	30

[a] BAC reactor performance in the first 348 days is also reported in another study.
[b] Background TOC was below 0.8 mg-C/L.
[c] Acetic acid concentration was 4~5 mg-C/L.
[d] Humic acid concentration was 3.5~5 mg-C/L.
[e] NH_4^+ concentration was 3 mg-N/L.
[f] NO_3^- concentration was 4 mg-N/L.
[g] Multiple EBCTs ranged between 48 min and 4.8 min were tested, and the biomass sample was collected when the EBCT equaled 30 min.
[h] Parameters not measured.

3.2.2 Isolation of E2 degrading bacteria from the BAC reactor

Five BAC granules were collected from the reactor when it showed steady E2 removal in Phase 7. Phase 7 was selected because of the reactor had more biomass in this Phase than the other Phases. The BAC granules were washed using sterilized phosphate buffer saline (PBS) solution (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 on the BAC surface. The culture was then transferred to fresh E2-saturated NMS medium at a 1:10 dilution ratio every 3 days (Yu *et al.* 2007). After the 4th transfer, the enrichment culture was diluted 10 folds in PBS and plated on R2A agar plates blended with ~ 3mg/L E2 (Yu *et al.* 2007). The E2-R2A agar plates were incubated at 30 ℃ for 3 days. Colonies with distinct morphologies were selected and re-grown in liquid R2A media containing ~ 3mg/L E2 for 24 hr at 30℃. Cells were harvested using centrifuge (10,000 × g, 15 min, 4℃) and stored in 15% glycerol at -80℃.

3.2.3 E2 Degradation experiment by the isolates

Each isolated E2 degrading bacterial strain was grown in a 250-mL R2A medium for 24 hours at 30℃. 70-mL of each cell culture was centrifuged (8,000 g, 15 min at 4 ℃) and washed using PBS (pH=7.2) twice before being transferred to 100 ml sterile E2-saturated NMS medium (~3mg/L). The culture was incubated on a shaking table (120 rpm) at 30℃. At various times, 70 ml solution was filtered (0.2 μm Teflon® filter) and stored in an amber glass bottle at -20 ℃ for estrogen measurements. Sterile E2-NMS medium without cells were used as controls.

3.2.4 DNA extraction

Total DNA of E2 degrading isolates and BAC samples was extracted using a phenol-chloroform method (Appendix C). For the BAC sample collected from Phase 8, extra steps were taken after the phenol-chloroform extraction to remove humic acid, a PCR inhibitor, in the BAC sample. The DNA from phenol-chloroform extraction was purified using the UltraClean® Soil DNA Isolation Kit (MoBio, CA, USA) according to the purification step in the manual: 100 µL of DNA in Tris-EDTA buffer was loaded to a Spin Filter from the kit and centrifuged at 10,000 × g for 1 min at 4℃ to remove humic acid and other impurities. Detailed steps of purification were provided in Appendix C. All DNA extracts were quantified using a NanoDrop 2000 (Thermo Scientific, DE), and their qualities were examined using gel electrophoresis. The DNA extracts were stored at-80℃ till use.

3.2.5 Phylogenetic analyses of the E2 degrading isolates

The DNA extracts of the E2 degrading isolates were amplified using PCR with a forward primer 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and a reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Xia *et al.* 2010). Each 25-µL reaction volume contained 1.0 µL of 50 ng/µL DNA template working solution, 1 µL of each primer (25 pmol/µL), 0.1 µL high fidelity Ex-Taq polymerase (5 units/µL, Takara Bio Inc., Madison, WI), 2 µL dNTP (2.5 mM each), 2.5 µL buffer (10×, $MgCl_2$ free), 2 µL $MgCl_2$ (25 mM), and 15.4 µL PCR grade water (Sigma-Aldrich, MO, USA). The PCR reactions were carried out on a Mastercycler® ep Realplex (Eppendorf International, Hamburg, Germany): 2 min initial denaturation at 95 ℃ and 30 cycles of 2 min at 95℃,

45 sec at 58℃, 2 min at 72℃, and a final extension for 10 min at 72℃. PCR products were examined using 2.0% agarose gel electrophoresis. DNA purification and sequencing was completed by Eurofins MWG Operon (Huntsville, AL).

3.2.6 Pyrosequencing of the 16S rRNA gene of the biomass samples

The V1 to V3 region of the 16S rRNA gene was selected for PCR amplification for pyrosequencing (Yoshimoto *et al.* 2004; Bibby *et al.* 2010). The forward primer (A-8F) contained an A sequencing adapter (5'-GCCTCCCTCGCGCCATCAGAGAGTTTGATCMTGGCTCAG -3') with the A adapter sequence underlined. The reverse primer (B-518R) contains a B sequencing adapter with an eight-base, sample-specific barcode (5'-GCCTTGCCAGCCCGCTCAG NNNNNNNNATTACCGCGGCTGCTGG-3') with the B adapter underlined and a sample specific barcode marked as N (Yoshimoto *et al.* 2004). A 50-µL PCR reaction contained 1.5 µL DNA template (50~100 ng/µL), 1 µL of each primer (25 pmol/µL), 0.3 µL high fidelity *Ex-Taq* polymerase (5 units/µL), 4 µL dNTP (2.5 mM each), 5 µL buffer (10×, $MgCl_2$ free), 4 µL $MgCl_2$ (25 mM), and 33.2 µL PCR grade water (Sigma-Aldrich, MO, USA). The PCR reaction was carried out in a Mastercycler® ep realplex (Eppendorf International, Hamburg, Germany) with the following PCR conditions: (1) an initial denaturation at 95 ℃ for 5 min; (2) 30 cycles of denaturing at 95 ℃ for 45 sec, annealing at 55 ℃ for 45 sec, and extension at 72 ℃ for 120 sec in each cycle; and (3) a final extension at 72 ℃ for 10 min.

The quantity of the PCR products was measured using a Quant-iT PicoGreen double-stranded DNA assay (Invitrogen, Carlsbad, CA), and the quality was assessed

using an Agilent 2100 bioanalyzer (Santa Clara, CA) at the Core for Applied Genomics and Ecology at the University of Nebraska-Lincoln. Equal amounts of the PCR products were mixed and loaded to a 454/Roche GS-FLX Titanium instrument (Roche, NJ) (Martinez *et al.* 2009). Pyrosequencing reads were filtered, trimmed and annotated following a published protocol (Benson *et al.* 2010). Briefly, reads that meet the following criteria were considered acceptable: (1) Length between 200 and 500 nucleotides; (2) perfect match with the forward primer and barcode sequences, (3) less than 2 interrupted and resumed signals from sequential flows, and (4) average quality score larger than 20.

3.2.7 Bacterial community analysis

The pyrosequencing data were processed using the RDP Pyrosequencing Pipeline (http://pyro.cme.msu.edu/). Taxonomic classification was conducted using the Ribosomal Database Project (RDP) CLASSIFIER (Wang *et al.* 2007). Acceptable pyrosequencing reads were aligned using Infernal, a secondary multiple sequence alignment software (Fahrbach *et al.* 2006; Skotnicka-Pitak *et al.* 2008). Number of operational taxonomic units (OTUs), rarefaction curve, Shannon Index, and Chao1 estimator were calculated at the 95 % sequence similarity level using the programs available within the RDP Pyrosequencing Pipeline. Principle component analysis (PCA) based on pyrosequencing reads was conducted using a customized software pipeline that could generate the input files for UniFrac.

3.3 Results

3.3.1 BAC reactor performance at the times of sampling

Changes in reactor operation had profound impacts on reactor performance. Three major changes in operating conditions divided reactor operation into four Phases (Table 3.1 and Figure 3.1). Towards the end of Phase 0, effluent E2 concentration stabilized at ~230 ng/L, while E1 was occasionally detected in the effluent at concentrations merely above the method detection limit (MDL, Figure 3.1). Soon after Phase 1 started, effluent E2 concentration started to decrease, and dropped to below MDL at the end of Phase 2. In the meantime, effluent E1 concentration increased to ~250 ng/L on Day 183 and after that gradually decreased to below MDL in 40 days. One characteristic that marked Phase 7 is the complete removal of E2 and the accumulation of E1 at ~50 ng/L in the effluent. During Phase 8, the removal rate of the BAC reactor for both E2 and E1 were decreased in the first 51 days, and then gradually recovered. Effluent E2 and E1 concentrations were both stabilized at about 65 ± 15 ng/L after about 150 days operation into Phase 8.

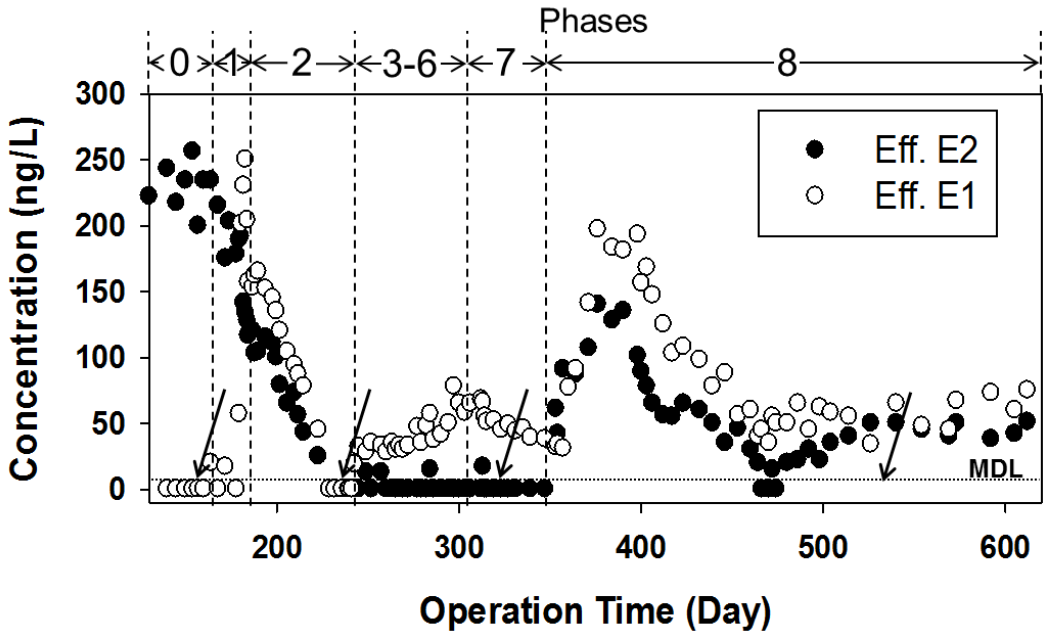

Figure 3.1 Effluent E2 and E1 concentrations of the BAC reactor. The arrows indicated the biomass sampling events on Day 172, Day 229, Day 331, and Day 525.

3.3.2 Degradation profiles of isolated E2 degrading bacteria

Knowledge about the taxonomic classification of estrogen degrading bacteria is critical to the study of estrogen degrading populations in mixed microbial communities. Taxonomic analyses revealed that the four bacterial strains isolated from the BAC reactor, ZLB1, ZLB2, ZLB3 and ZLB4, 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. During the degradation experiment, strains ZLB1, ZLB2, and ZLB3 converted E2 to E1 in a stoichiometric manner, but could not further degrade E1. In contrast, strain ZLB4 completely converted E2 to E1 in the first 48 hours and then partially degraded the E1 accumulated till the degradation ceased at 72 hour (Figure 3.2). These four isolates, along with the estrogen degrading bacterial genera reported in the literature, are listed in Table 3.2. Most of the bacterial strains listed in Table 3.2 could only transform E2 to E1, and only a few could further degrade E1.

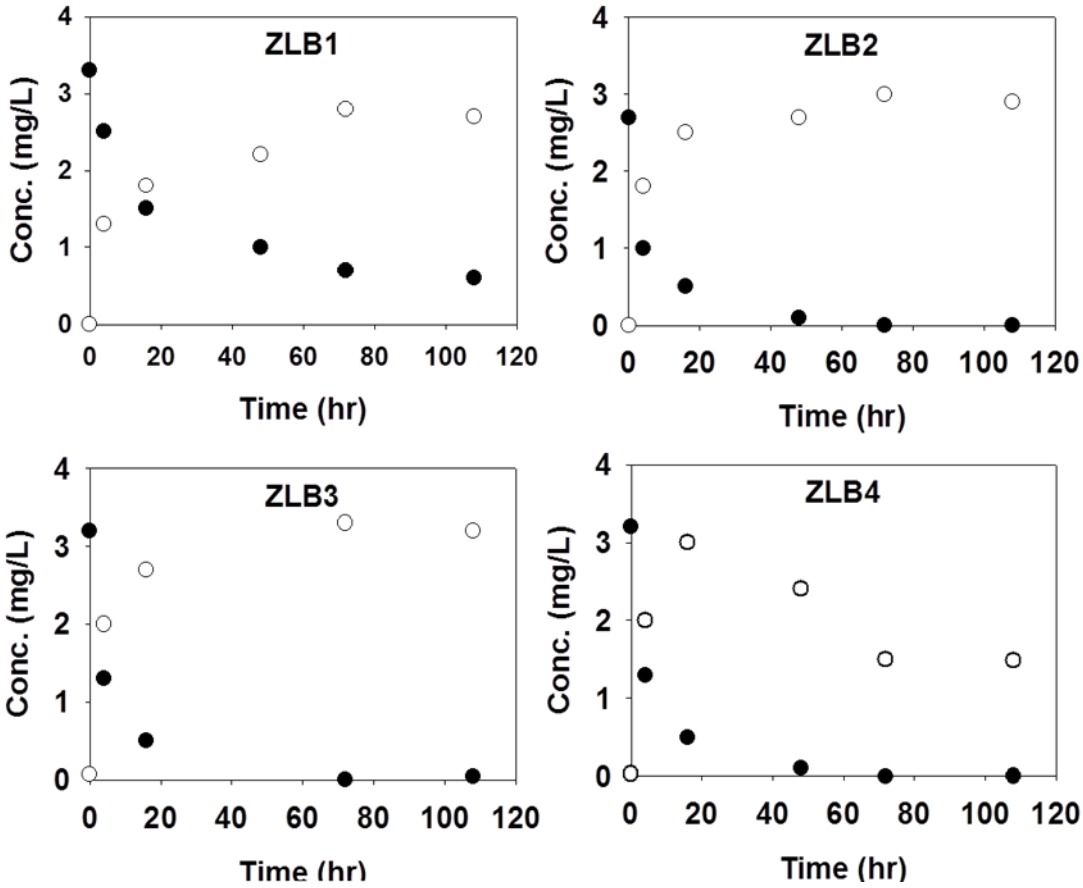

Figure 3.2 Estrogen degradation profiles of four E2 degrading isolates. Solid circle indicates E2 and empty circle indicates E1. Average values of duplicate measurements are reported.

Table 3.2 Isolated E2 and E1 degrading bacteria

Class	Genus	Sources	References
Alpha-Proteobacteria	*Aminobacter*	activated sludge	(Yu *et al.* 2007)
	Brevundimonas	activated sludge	(Yu *et al.* 2007; Muller *et al.* 2010)
	Sphingomonas[a]	activated sludge, wetland	(Fujii *et al.* 2002; Lee and Liu 2002; Fujii *et al.* 2003; Shi *et al.* 2004; Yu *et al.* 2007; Kurisu *et al.* 2010; Iasur-Kruh *et al.* 2011)
Beta-Proteobacteria	*Achromobacter*	activated sludge, wetland	(Weber *et al.* 2005; Iasur-Kruh *et al.* 2011)
	Denitratisoma	activated sludge	(Fahrbach *et al.* 2006)
	Nitrosomonas[a]	activated sludge	(Shi *et al.* 2004; Skotnicka-Pitak *et al.* 2008)
	Ralstonia	wetland	(Weber *et al.* 2005)
	Zoogloea	wetland	This study
Gamma-Proteobacteria	*Acinetobacter*	compost, activated sludge	(Pauwels *et al.* 2008), this study
Actinobacteria	*Escherichia*	activated sludge	(Yu *et al.* 2007)
	Stenotrophomonas[a]	activated sludge	This study
	Microbacterium	activated sludge	(Yu *et al.* 2007)
	Nocardioides	activated sludge	(Yu *et al.* 2007)
	Rhodococcus[a]	activated sludge, soil	(Yoshimoto *et al.* 2004; Yu *et al.* 2007; Kurisu *et al.* 2010)
Bacilli	*Bacillus*	activated sludge	(Jiang *et al.* 2010)
	Staphylococcus	wetland	(Iasur-Kruh *et al.* 2011)
Flavobacteria	*Flavobacterium*	activated sludge	(Yu *et al.* 2007)

[a] Genera containing strains that have been reported with E1degrading capability.

3.3.3 Diversity, richness, and similarity of the bacterial communities

A total of 69,651 acceptable high quality pyrosequencing reads were obtained from the four biomass samples. Sequences sharing >95% similarity were defined as an operational taxonomic unit (OUT). Despite that each sample included more than 10,000 pyrosequencing reads, none of the rarefaction curves completely leveled off, suggesting that additional sequences would introduce new OTUs (Figure 3.3). The diversity and the richness of the bacterial communities in the four samples were assessed using the Shannon Index and Chao1 estimator, respectively (Table 3.3). The Shannon diversity index assesses both the richness and the evenness of a microbial community. According to the Shannon Index, the microbial community during Phase 2 had the highest level of diversity, while the microbial community during Phase 8 had the lowest level of diversity. The Chao1 index estimates the ultimate number of OTUs in a microbial community. The Chao1 index was the highest during Phase 2, and then decreased in Phases 7 and 8, a trend similar to the Shannon index. In addition to the assessments on richness and diversity, the bacterial communities in the four biomass samples were also evaluated for their overall similarities using principle component analyses. The four samples were best explained by the PC1 and PC2 axes extracted using the software UniFrac (Figure 3.4). The bacterial communities from Day 172 and Day 229 were grouped, while the bacterial communities from Day 331 and Day 525 were distinctively distanced from each other and from those of Day 172 and Day 229.

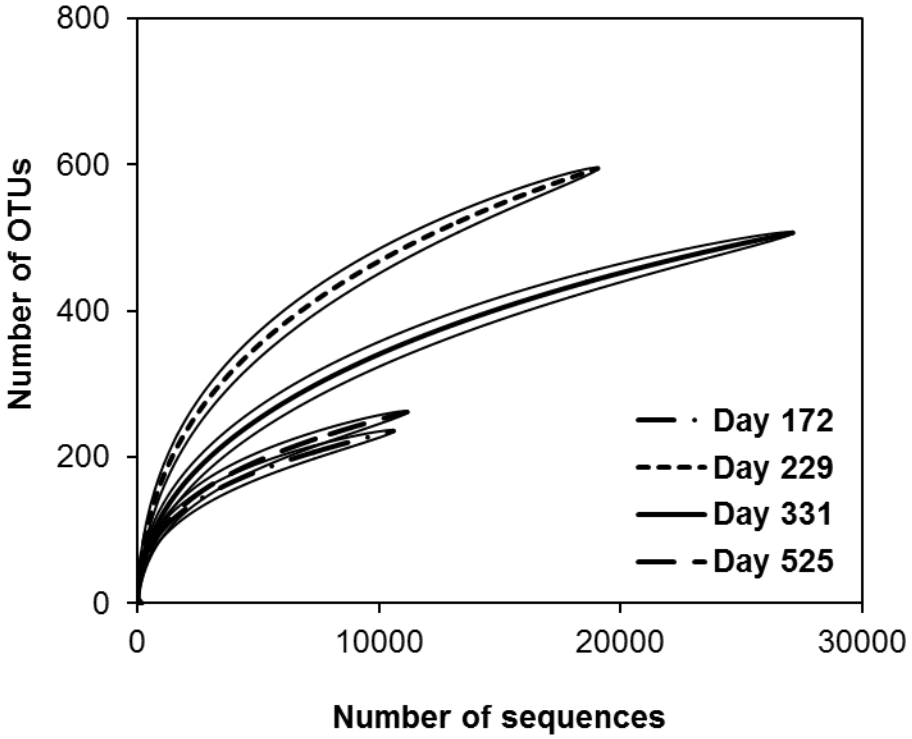

Figure 3.3 Rarefaction curves of the biomass samples collected from the four reactor operating phases. Rarefaction curves were constructed with 95% confidence intervals.

Table 3.3 Bacterial community diversity indices

Operation Phase	Sampling Date	OTUs	Shannon Index	Chao1
1	172	236	3.57	375
2	229	595	4.12	817
3	331	513	3.54	507
4	525	262	3.03	262

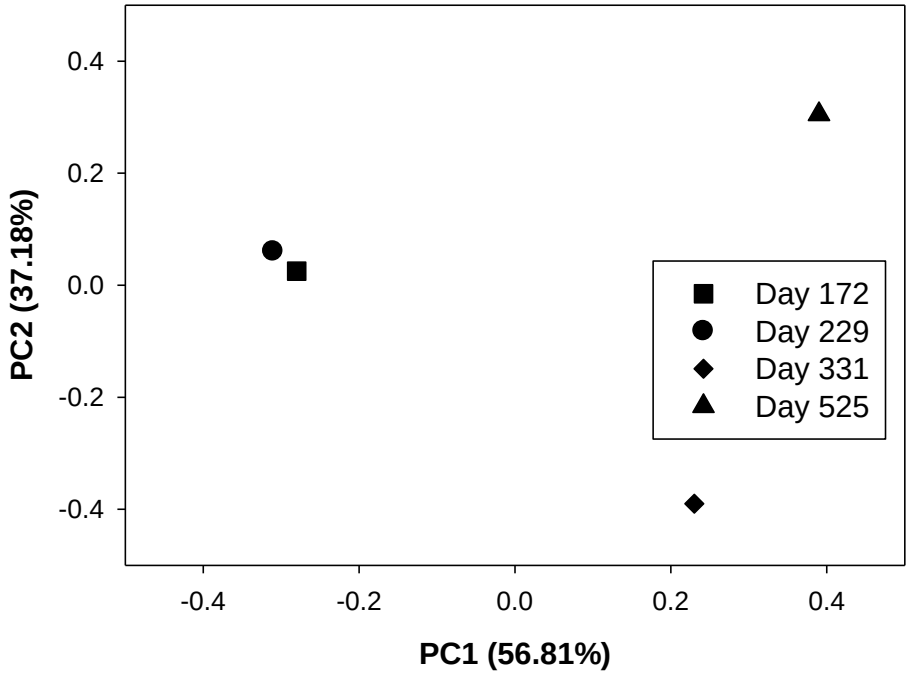

Figure 3.4 Statistical comparison of the bacterial communities in biomass samples collected from the four Phases (Day 172: Phase 0, Day 229, Phase 2; Day 331: Phase 7; Day 525: Phase 8).

3.3.4 Bacterial community dynamics in the BAC reactor

Biomass samples collected on Days 172, 229, 331, and 525 had sequences that belonged to 13, 11, 13, and 10 classes, respectively (Figure 3.5). Four classes, *Betaproteobacteria*, *Alphaproteobacteria*, *Sphingobacteria*, and *Gammaproteobacteria*, appeared in all four samples and together constituted 80.4%, 84.1%, 96.2%, and 98.5% of the bacterial communities on the four sampling dates. *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.

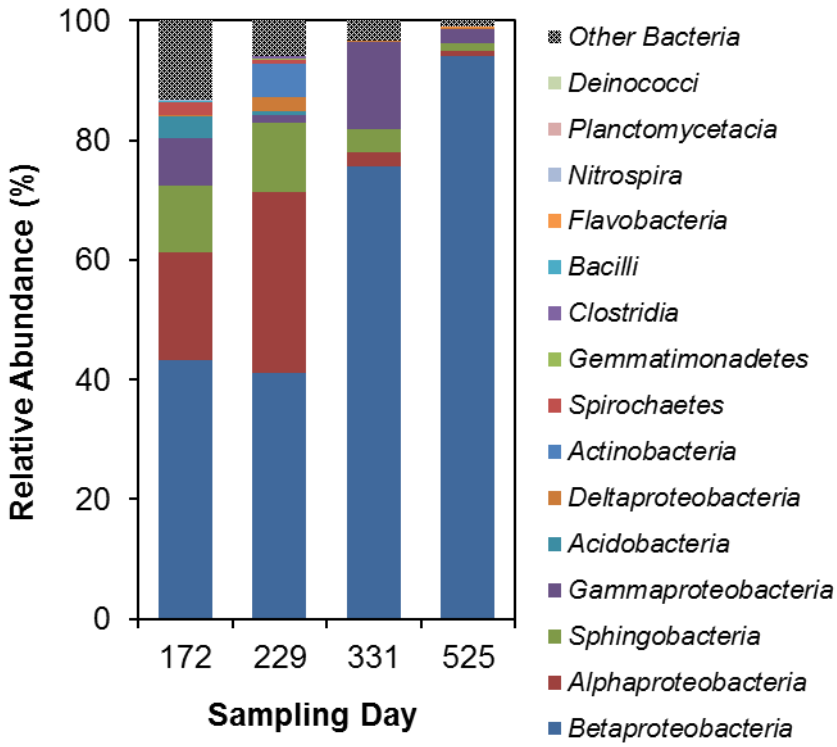

Figure 3.5 Taxonomic breakdown at the class level for the bacterial communities in the four biomass samples. The relative abundance of each class was calculated as the number of sequences affiliated with that class divided by the total number of sequences in that sample.

The microbial community within the BAC reactor was also analyzed at the genus level. According to Table 2, six known E2 and/or E1 degrading bacterial genera were detected in one or more of the four biomass samples (Figure 3.6). The relative abundance of *Zoogloea* sp., which could biotransform E2 to E1 based on degradation profile of Strain ZLB3 in the current study (Figure 3.2), was enriched 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. In contrast, the relative abundance of *Ralstonia* was changed from 7.7% on Day 172 to 0.35% on Day 229, to 1.4% on Day 331, and to 1.1% on Day 525. Three bacteria genera capable of degrading E1, *Rhodococcus*, *Sphingomonas*, and

Stenotrophomonas, were detected in the biomass samples. The relative abundances of *Sphingomonas* and *Rhodococcus* were the highest on Day 229, and were undetected in samples collected on Days 331 and 525. In contrast, *Stenotrophomonas* gradually accumulated in the BAC reactor, accounting for 0.3% of the bacterial population on Day 331 to 2.0% on Day 525. It is noted that complete E2 and E1 degradations were only achieved at the end of Phase 2 (Figure 3.1), when *Sphingomonas* and *Rhodococcus* were most abundant.

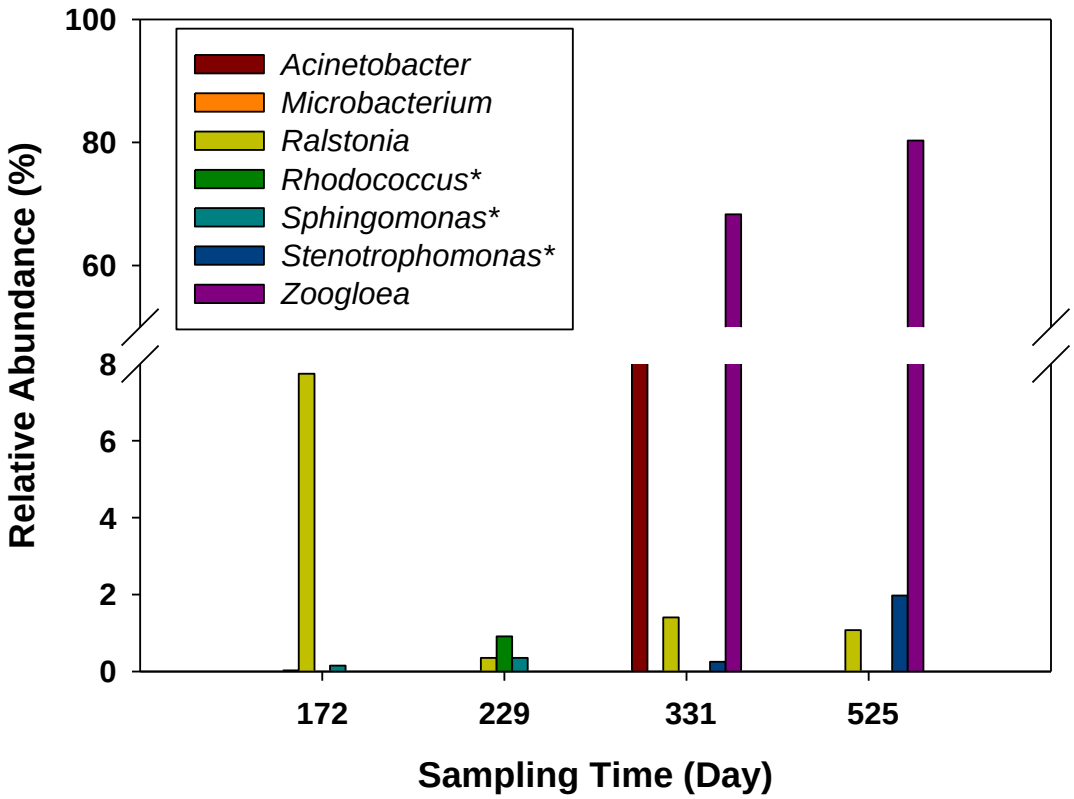

Figure 3.6 Dynamics of identified E2 degrading bacterial genera in the BAC reactor. Bacteria capable of degrading both E2 and E1 were marked by asterisk in the legend.

3.5 Discussion

This study reported the microbial community structure, with a particular focus on the E2 and E2 degrading bacterial populations, of a BAC reactor operated to treat E2-contaminated water. Although pyrosequencing provided unprecedented sequencing resolution, the rarefaction curves indicate that the bacterial communities were still not fully sequenced. This observation is not uncommon for microbial communities in bioreactor systems, such as membrane-biofilm reactor, biogas fermenter, and anaerobic digester (Zhang *et al.* 2009; Zhang *et al.* 2010; Jaenicke *et al.* 2011). On the other hand, the pyrosequencing data were able to detect bacterial populations with very low abundance. Given that E2 concentration in contaminated acquatic environment is usually orders of magnitude lower than the background NOM (as simulated in this study), the resolution granted by pyrosequencing analyses is necessary to detect the quantitatively insignificant but functionally important bacterial populations (i.e., bacterial populations capable of degrading E2 and its degradation intermediates).

The BAC reactor in this study was not intentionally seeded. It is believed that the microbes in the BAC reactor were mostly originated from in-house treated RO water that was used to make the reactor influent. To some extent, this suggests common occurrence of E2 degrading bacteria in the environment, which is consistent with the observation that E2 degrading bacteria was widespread in engineered systems (Shi *et al.* 2004; Yoshimoto *et al.* 2004; Yu *et al.* 2007; Kurisu *et al.* 2010; Iasur-Kruh *et al.* 2011). In this study, *Beta-* and *Gamma-proteobacteria* together accounted for 51.3%, 42.4%, 90.0%, and 98.5% of total the bacterial community on Days 172, 229, 331, and 525, respectively (Figure 3.5). Zang and co-workers found *Beta-* and *Gamma-proteobacteria* were

involved in the initial stage of E1 biodegradation (Zang *et al.* 2008). The authors also reported that the E1 degrading bacterial population, which included members from the *Beta-* and *Gamma-proteobacteria* as well as some other bacteria, accounted for 1~2% of the bacterial population in the activated sludge samples tested. This finding and the finding from this study suggest that certain bacterial species within the *Beta-* and *Gamma-proteobacteria* may be capable of carrying out ring cleavage on the estrogen skeleton and facilitating further degradation.

The predominant E2 degrading populations changed during the course of the reactor operation. *Ralstonia* was the most abundant E2 degrading bacteria in Phase 0, while *Zoogloea* was the most abundance E2 degrader in Phase 8 (Figure 3.6). Ad egradation experiment showed that *Zoogloea* strain ZLB3 could rapidly biotransform E2 to E1 (Figure 3.2). *Zoogloea* is frequently detected in water / wastewater treatment systems wherever biofilm exists, as they are good slime former (Larsen *et al.* 2008; Shao *et al.* 2009). For example, *Zoogloea* sp. made up 17.9 % to 52.0 % of the total bacterial community in a pilot scale BAC reactor system for biological drinking water treatment (Li *et al.* 2010). The common existence of *Zoogloea* in biofilm and its ability to biotransform E2 suggest that water/wastewater treatment systems containing biofilm have a good probability of reducing the total estrogenic activity by converting E2 to E1 (Metcalfe *et al.* 2001; Yu *et al.* 2007). Interestingly, despite high abundance of *Zoogloea* sp. in Phase 8, effluent E2 concentration stabilized at 50 ng/L. One possible reason is that only certain strains (e.g., ZLB3) of the *Zoogloea* population in the BAC biofilm (Figure 6) were actually able to biotransform E2. The 16S rRNA gene is not a reliable

biomarker to deduce the activity of a microbial population. However, currently no other biomarkers are available for estrogen degrading bacteria.

The three E1 degrading bacterial genera, *Sphingomonas*, *Stenotrophomonas*, and *Rhodococcus* (Figure 3.6), constituted less than 2% of the bacterial communities, which is comparable to the relative abundance of E1 degrading bacteria in sewage sludge (Zang *et al.* 2008). According to the literature, E1 degrading species that belong to *Sphingomonas* could completely remove E2 and its degradation intermediate E1 within three days, and *R. zopfii* Y 50158 could completely remove E1, E2, E3, and ethinylestradiol (EE2) in 24 hours (Yoshimoto *et al.* 2004). *Sphingomonas* and *Rhodococcus* often co-occur at sites contaminated with polycyclic aromatic hydrocarbons (PAHs) (Hernandez-Raquet *et al.* 2006; Cebron *et al.* 2008; Li *et al.* 2009), and both have PAH-ring hydroxylating dioxygenase, an enzyme involved in the initial step of the ring cleavage of PAH (Cebron *et al.* 2008). Therefore, given that dioxygenase activities might be responsible for the biodegradation of estrogen intermediates such as 4-hydroxy-estradiol and 4-hydroxy-ethinylestradiol (Kurisu *et al.* 2010; Khunjar *et al.* 2011), it was not surprising that the BAC reactor completely removed E2 and E1 when these two genera were abundant (i.e., Phase 2, Table 3.1 and Figure 3.1). In contrast, *Stenotrophomonas* has never been reported as an estrogen degrading bacteria. E1 degrading strain that belong to *Stenotrophomonas* could not degrade E1 to below 1.6 mg/L (ZLB4 in Figure 3.2), suggesting that the strain might have a minimum substrate concentration for E1 degradation. Maybe this explained why an effluent E1 concentration between 19 and 65 ng/L was observed when *Stenotrophomonas* was the only E1 degrader in the reactor (i.e., Phase 3 and 4).

Previously identified ammonia oxidizing bacteria were not detected at an appreciate amount in the BAC reactor. Previous studies reported that ammonia oxidizing bacteria, in pure culture and in nitrifying activated sludge, could biotransform E2 and degrade E1 (Shi *et al.* 2004). Among the all four biomass samples analyzed in this study, only one nitrite oxidizing bacterial genus, *Nitrospira*, was detected at 0.01% of the bacterial population in Phase 2. This finding suggests estrogen degradation in the BAC reactor was mainly contributed by heterotrophic bacteria. Another interesting finding comes from the comparison of the reactor performance between Phase 7 and Phase 8. Compared to Phase 8 when humic acid was used the primary energy and carbon source, a higher efficiency of microbial estrogen degradation was observed in Phase 7 when the more readily biodegrable acetic acid was used. The presence and absence of *Acinetobacter* might be responsible for this difference (Figure 3.6). The less-than-ideal removal efficiency in Phase 8 is consistent with the fact that both E2 and E1 have been detected at appreciable amount in natural surface water.

CHAPTER 4: PROTEOMIC ANALYSIS OF 17B-ESTRADIOL DEGRADING BACTERIUM *STENOTROPHOMONAS MALTOPHILIA*

4.1 Introduction

17β-estradiol (E2) and its primary microbial degradation product, estrone (E1), have been detected in a few samples at surface water, groundwater, and wastewater effluent in North America, Europe, and Asia (Baronti *et al.* 2000; Andersen *et al.* 2003; Joss *et al.* 2004; Khanal *et al.* 2006; Kim *et al.* 2007; Benotti *et al.* 2009; Loos 2010). E2, a strong estrogenic compound, can cause adverse impacts on the reproductive systems of male fish at tens of ng/L (Purdom 1994; Imai *et al.* 2005). Due to the increasing concerns over the broad occurrence in source water and the potential health impact on human beings, US EPA recently added E2 and E1 to its Contaminant Candidate List 3 (EPA 1984).

E2 degrading bacteria have been isolated from a variety of environmental systems, such as activated sludge, compost, and constructed wetland (Weber *et al.* 2005; Yu *et al.* 2007; Pauwels *et al.* 2008; Iasur-Kruh *et al.* 2011). Identified E2 degrading bacteria are phylogenetically diverse, including species from *Alpha-*, *Beta-*, *Gama-proteobacteria*, *Actinobacteria*, *Bacilli*, and *Flavobacteria* (Shi *et al.* 2004; Yu *et al.* 2007; Jiang *et al.* 2010; Iasur-Kruh *et al.* 2011). The majority of the identified E2 degrading bacteria cannot degrade E1 and thus often cause accumulation of this compound as the primary microbial degradation intermediate (Yu *et al.* 2007; Iasur-Kruh *et al.* 2011). A few E2 degrading bacterial strains, which belong to the genera of *Sphingomonas*, *Nitrosomonas*, and *Rhodococcus*, are able to degrade E1 (Shi *et al.* 2004; Yu *et al.* 2007; Iasur-Kruh *et al.* 2011). It was speculated that these bacterial strains degrade E1 by utilizing it as a sole

carbon and/or energy source. However, the proposed degradation mechanism has never been demonstrated at the molecular level.

The mechanisms of microbial degradation of natural and synthetic estrogens have been studied in pure and mixed cultures. Monooxygenase was believed to be responsible for the initial steps in estrogen degradation. Ammonia oxidization bacteria (AOB) in pure cultures and in nitrifying activated sludge exhibited degradation capacity of E2, E1, estriol (E3), and 17α-ethinylestradiol (EE2). A study demonstrated that a protein extract containing ammonia monooxygenase (AMO) could attack the A ring (the aromatic ring) of EE2 and form hydroxyl EE2 (OH-EE2) as a degradation intermediate before the modification on ring B and C could occur (Yi and Harper 2007). Dioxygenase was also thought to be involved in estrogen biodegradation, because it could mediate ring cleavageof polyaromatic compounds (Schwarzenbach 2003). A *Sphingomonas* species could initiate E2 degrading with ring cleavage on the aromatic ring of the compound, a process possibly catalyzed by dioxygenase. When a catechol dioxygenase inhibitor was added, the same species altered the degradation pathway by initiating ring cleavage at the saturated ring (Kurisu *et al.* 2010). Another recent study showed that AOB could biotransform EE2 to intermediates like 4-hydroxy-EE2 (4-OH-EE2) but could not further mineralized them. In comparison, heterotrophic bacteria could mineralize EE2. The study also showed that in a reactor containing both AOB and heterotrophs, by modifying the A ring of EE2, the ammonia monooxygenase of AOB facilitated the mineralization of EE2 which was carried out by the dioxygenase of heterotrophs through meta-cleavage (Khunjar *et al.* 2011). To date, studies on the mechanisms of microbial degradation of natural and synthetic estrogens primarily relied on the detection and identification of

degradation intermediates under various conditions. A complementary approach is to study the enzymatic systems in estrogen degrading bacteria and link degradation products profiles with enzyme expression profiles.

As a "post-genomic" science, proteomics directly addresses the level of gene products present in a given cell state (Ong and Mann 2005). Quantitative proteomics typically includes liquid chromatography (LC) separation, tandem mass spectrometry (MS/MS) detection, and bioinformatics that identify proteins based on MS spectra (Aebersold and Mann 2003; Schneider and Riedel 2010). The resolution on protein expression levels offered by quantitative proteomics grants the technology great potential in studying microbial processes under environmental conditions. For example, proteomics revealed metabolisms, e.g., Pi transformations, polyhydroxyalkanoate (PHA) cycling in *Accumulibacter phosphatis* were specific to enhanced biological phosphorus removal (Wilmes *et al.* 2008). The proteomic approach was also used to monitor differentially expressed proteins associated with energy generation and a shift of iron reduction to sulfate reduction at uranium bioremediation sites (Callister *et al.* 2010). A comparative proteomic study showed that reductive dehalogenases were preferentially expressed in *Dehalococcoides* during anaerobic reductive dehalogenation (Morris *et al.* 2007). Quantitative proteomics could also confirm the validity of biomarkers in vinyl chloride remediation (Chuang *et al.* 2010).

The successful applications of quantitative proteomics in these studies suggest that it could be a powerful tool in studying enzymes involved in the biodegradation natural estrogens. Elucidating key enzymes in biodegradation could contribute to completing the microbial estrogen degradation pathways and suggest candidate

biomarkers to monitor estrogen degradation capacity of a microbial community, such activated sludge in wastewater treatment. This study reports the global protein expression profile of *Stenotrophomonas* strain ZL1 during E2 biodegradation. Two distinctive degradation phases were identified and studied coupled with differential protein expression profiles. A proposed E2 degradation pathway was established based on protein identification and pathway annotation.

4.2 Materials and Methods

4.2.1 Isolating estrogen degrading bacteria

Two batch reactors were established in 1-L amber glass bottles with 700 mL of E2- or E1-saturated acetone-free nitrate mineral salts medium (NMS) (Chu and Alvarez-Cohen 1996). Activated sludge from a local municipal wastewater treatment plant was used to seed the reactors. The operating conditions of the batch reactors were adapted from a published paper (Yu *et al.* 2007). In brief, the two reactors were aerated with 0.2-μm filtered air at a flow rate of 100 mL/min and operated at room temperature. 100-mL of well-mixed reactor content was replaced with fresh E2- or E1-saturated NMS medium every 7 days. After 78 days of enrichment, estrogen degrading bacteria were isolated using R2A plates amended with ~3 mg/L E2 or E1. A screening experiment was conducted to evaluate the capability of each strain to degrade estrogens (Supplementary Information). Among all five isolates, the strain *Stenotrophomonas maltophilia* ZL1 was selected for further analyses due to its ability to degrade both E2 and E1.

4.2.2 DNA extraction, sequencing and phylogenetic tree construction

Total DNA was extracted from the bacterial isolates using a phenol-chloroform method (Appendix C) and its quality was examined using 2% agarose gel electrophoresis. The 16S rRNA gene was amplified using PCR primers 27F (5'-AGRGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') on a Mastercycler® ep realplex (Eppendorf International, Hamburg, Germany) (Xia *et al.* 2010). *TaKaRa Ex Taq*™ kit was used for PCR amplification (TAKARA Bio Inc., Shiga, Japan). A 25-μL PCR reaction volume contained: 50 ng of DNA template, 1 pmol/μL of each primer, 0.1 μL high fidelity *Ex-Taq* polymerase (5 units/ μL), 0.2 mM each dNTP, 2.5 μL 10×, $MgCl_2$-free buffer, 2 mM $MgCl_2$ and 15.4 μL water (Sigma-Aldrich, MO, USA). The PCR condition included 2 min denaturing at 95℃ followed by 30 cycles of 2 min denaturing at 95℃, 45 sec of annealing at 58℃, and 2 min extension at 72℃, and a final extension for 10 min at 72℃. PCR products were purified and sequenced bi-directionally at Eurofins MWG Operon (Huntsville, AL). Sequence alignment and phylogenetic tree construction were following a published protocol (Li *et al.* 2010).

4.2.3 E2 degradation profile of *S. maltophilia* ZL1

S. maltophilia ZL1 was grown in R2A medium for 24 hours (Yu *et al.* 2007) before 5-mL culture was transferred to one of the eighteen flasks, six per set, containing 100 mL sterile R2A medium and grew for another 10 hours. Cells in two sets of the flasks were washed with phosphate buffer saline (PBS, pH=7.2) twice and then transferred to 12 new flasks each containing 100 mL of E2-saturated NMS medium. Cells in the third set of the flasks were processed in the same manner, except that they

were autoclaved prior to the transfer. A fourth set of six flasks containing only sterile E2-saturated NMS medium were also prepared as negative controls. All flasks were incubated at 30ºC on a shaking table set at 120 rpm. At 0, 4, 16, 36, 68 and 104 hours after the experiment started, cell samples were harvested (centrifuging at 10,000×g for 5 min at 4 ℃) and underwent downstream sample preparation for proteomic analysis. 70 mL filtrate (0.2 μm Teflon® filter) of reactor content from each reactor was stored at -20 ℃ in an amber glass bottle for estrogen measurements.

4.2.4 Sample preparation for proteomic analysis

Sample preparation and in-solution trypsin digestion for the proteomic analysis were done as described in (Nandakumar *et al.* 2011). Briefly, the harvested cells were re-suspended in 1mL 50 mM ammonium bicarbonate containing 8 M urea and 1.5 mM phenylmethylsulfonyl fluoride (PMSF). The cells were lysed in a bead-beater (Manufacturer) for 2.5 min. Cell lysates were centrifuged (16,000×g, 10 min at 4℃) to remove cell debris and glass beads. The bacterial proteins in the supernatant were precipitated using acetone and re-suspended in 100mM ammonium bicarbonate containing 6M urea. The proteins were further reduced with 10 mM dithiothreitol, alkylated with 40 mM iodoacetamide, digested with sequencing-grade trypsin (Roche, USA) at 1:50 trypsin to protein ratio at 37 ℃ overnight. Protein samples were desalted and concentrated using solid phase extraction (PepClean C-18 spin column, Pierce, USA), vacuum-dried, and stored at -80 ℃ till analysis.

4.2.5 2D nano LC-MS/MS analysis for proteomics

Quantitative proteomics was performed on an Ultimate 3000 Dionex MDLC system (Dionex Corporation, CA) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer (Thermofinnigan, USA) (Nandakumar *et al.* 2011). The first dimensional separation was performed on a SCX column (Polysulfoethyl, 1 mm I.D. × 15 cm, 5 µm, 300A, Dionex). 25 µL sample was loaded onto the first dimension SCX column and eluted using a salt gradient of 0–600 mM. Selected fractions based on the UV absorbance of the eluted peptides were subjected to second dimension analysis. The second dimension separation incorporated an on-line sample pre-concentration and desalting using a monolithic C18 trap column (Pep Map, 300 µm I.D. × 5 mm, 5 µm, 100A, Dionex). The desalted peptides was then eluted and separated on a C18 Pep Map column (75 µm I.D. × 15 cm, 3 µm, 100 A) applying an acetonitrile (ACN) gradient (ACN plus 0.1% formic acid, 90 min gradient) at a flow rate of 300 nL/min. The LCQ was operated in data dependent mode with 4 MS/MS spectra for every full scan, 5 microscan averaged for full scans and ms/ms scans, 3 m/z isolation width for ms/ms isolations and 35% collision energy for collision induced dissociation. Dynamic exclusion was enabled with exclusion duration of 1 min.

4.2.6 Protein functional category classification and pathway mapping

Identified proteins were classified into functional groups using annotation based on Clusters of Orthologous Groups (COG) using NCBI COGnitor tool ((Tatusov *et al.* 2000; Tatusov *et al.* 2001). In order to group identified proteins into known metabolisms, all identified protein sequences were also mapped into KEGG metabolic pathway using KEGG Automatic Annotation Server (KAAS) (Moriya *et al.* 2007).

4.2.7 Gas chromatography (GC)-mass spectrometry (MS) for estrogen quantification

E2 and its biodegradation intermediates, E1 and E3, were quantified using GC-MS protocol with solid phase extraction (SPE) and modified derivatization (Shareef *et al.* 2006). Briefly, 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 1 gm SPE cartridge (C-18, Waters, USA). Ethyl acetate was used to elute adsorbed E2 and its biodegradation intermediates. Eluted samples were blown dry, reconstituted, and derivatized using 50 µL dimethyl formamide and 50 µL BSTFA+1% TMCS at 75^{o}C for 30 min. Derivatized samples were transferred to GC vials with 250 µL insert. Derivatized samples were analyzed within 36 hours. The method detection limits (MDL) for E1, E2, E3 were determined to be 14.4 ng/L, 5.8 ng/L, and 11.4 ng/L, respectively, by following a standard procedure (EPA 1984).

4.3 Results

4.3.1 Selection of isolated E2 degrading bacterium for proteomic analysis

Five E2 degrading bacteria were isolated from the enrichment experiment. Taxonomic analyses based on the 16S rRNA gene revealed that the five isolates belonged to the genera *Stenotrophomonas* (strain ZL1), *Alcaligenes* (ZL2), *Microbacterium* (ZL3), and Rhodococcus (ZL4 and ZL5) (Figure 4.1). To select a bacterial strain for proteomic analysis, the following criteria were used: capability of estrogen degradation, availability of genome information, and availability of annotated metabolism pathways. A preliminary degradation experiments using end-point measurements was conducted on the isolates to test their abilities to degrade E2 an E1 (Appendix D). Results showed that

among the five isolates the *Stenotrophomonas* strain ZL1. and *Rhodococcus* strains ZL4 and ZL5 could degrade both E2 and E1. In addition, strain ZL1 shared a >99% similarity in the 16S rRNA gene with *Stenotrophomonas maltophilia* R551, whose complete genome has been sequenced (Johnson *et al.* 2008). In comparison, *Rhodococcus* strains (ZL4 and ZL5) did not have closely related strains with genome information available. In addition, the metabolism pathways of *S. maltophilia* R551 were annotated by the KEGG Orthology. Therefore, the *S. maltophilia* strain ZL1 was selected for further analyses in this study. The 16S rRNA gene sequences were deposited in NCBI under accession numbers JN085951-JN085955.

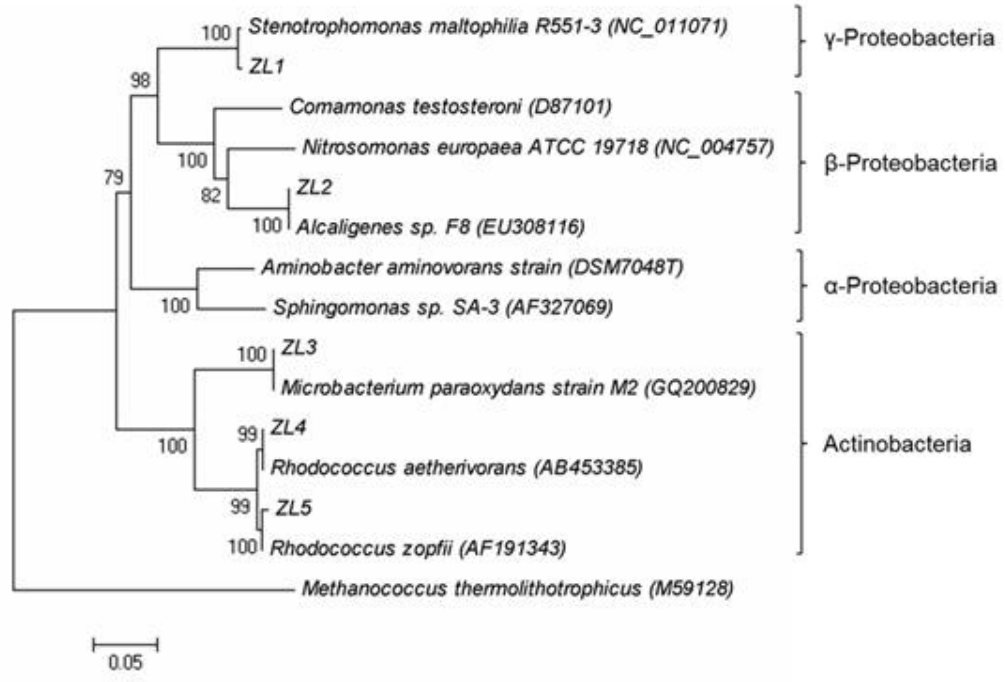

Figure 4.1 Phylogenetic tree for the estrogen degrading bacteria isolated from the enrichment experiment. Construction of the phylogenetic tree was based on the neighbor-joining method with 1000 bootstrap replications. An archaeal strain *Methanococcus thermolithotrophicus* was used as an out-group for the phylogenetic tree.

4.3.2 E2 degradation by *S. maltophilia* strain ZL1

E2 degradation by strain ZL1 showed two distinctive phases (Figure 4.2). In Phase 1 (0 – 16 hr), E2 concentration dropped from 3.3mg/L to a very low level, while E1 concentration increased from below detected to about 3.5 mg/L, suggesting that E2 was biotransformed to E1. Optical density (OD) reading indicated that there was no significant cell growth of strain ZL1 during Phase 1. In Phase 2 (16 – 36 hr), E2 concentration remained low, while E1 concentration dropped to 1.9 mg/L. OD reading increased by approximate 0.2 unit. In Phase 3 (36 – 104 hr), both E2 and E1 concentrations remained stable, while the optical density reading dropped about 0.1 unit towards the end of the phase. At the end of the experiment, E2 was nearly 100% degraded and the about 50% of the E1 formed from E2 biotransformation was degraded by strain ZL1. The control set without biomass (Control 1) and the control set with autoclaved ZL1 (Control 2) showed no significant decrease in E2 concentration and no E1 accumulation. Based on the E2/E1 and OD profiles, biomass samples were collected at 0, 4, 16, and 36 hour for proteomic analyses.

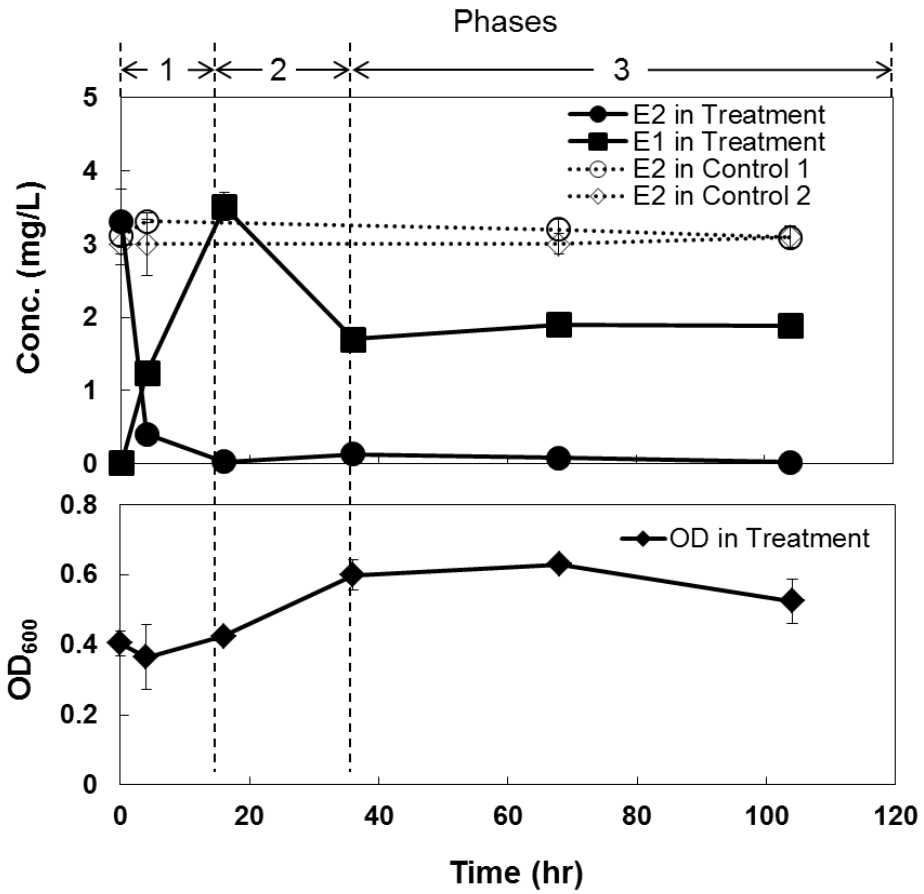

Figure 4.2 Concentration profiles of E2 and E1 in a degradation experiment by strain ZL1 (top), and the optical density at 600 nm (OD_{600}) of strain ZL1 in an E2-saturated NMS medium. The bars indicate the ranges of measurements from duplicate experimental sets. Control 1 was an E2-saturated NMS medium containing no cells, and Control 2 was an E2-saturated NMS medium containing autoclaved strain ZL1.

4.3.3 Protein identification

All MS/MS spectra were searched against *Stenotrophomonas maltophilia* R551 genome database using MASCOT (Version 2.2 Matrix Science, UK) with the following parameters: enzyme (trypsin); missed cleavages (2); mass (monoisotropic); fixed modification (carbamidomethyl (C)); peptide tolerance (1.5 Da); MS/MS fragment ion tolerance (1 Da). Probability assessment of peptide assignments and protein identifications were accomplished using Scaffold (Proteome Software Inc., Portland,

OR). Only peptides with ⩾90% probability were considered in this study. Criteria for protein identification included detection of at least two unique peptides in a protein and a protein probability score of ⩾90%. Relative quantitation of the proteins was developed using a label-free method of spectral counting with normalized spectral counts (Liu *et al.* 2004). A total of 263 proteins were identified with at least two unique peptides. 1.5 folds increased or 0.75 folds decreased abundance combined with a P-value ⩽0.05 (Fisher's exact test) was used to select for proteins that were differentially expressed.

4.3.4 Global functional distribution of *S. maltophilia* ZL1 proteome during E2 degradation

The proteins identified in the *S. maltophilia* ZL1 samples collected from the four time points were classified into 19 functional categories using COGnitor (Figure 4.3). Quantitatively, proteins in three functional categories were most abundant in *S. maltophilia* ZL1: energy production and conversion (C), translation, ribosomal structure and biogenesis (J), and post translational modification, protein turnover and chaperones (O). The functional distribution of the identified proteins did not show significant temporal changes during the course of E2 degradation. The number of proteins in two of the aforementioned functional categories, C and J, and in the category inorganic ion transport and metabolism (P) showed a general trend of decrease during the time course tested. The proteins with functions in carbohydrate transport and metabolism (G), DNA replication, recombination and repair (L), and cell envelope biogenesis (M) became increasingly abundant after strain ZL1 was exposed to E2. Notably, the number of

proteins in the functional category of secondary metabolites biosynthesis, transport and catabolism (Q) decreased 85% during 16 hr and 36 hr.

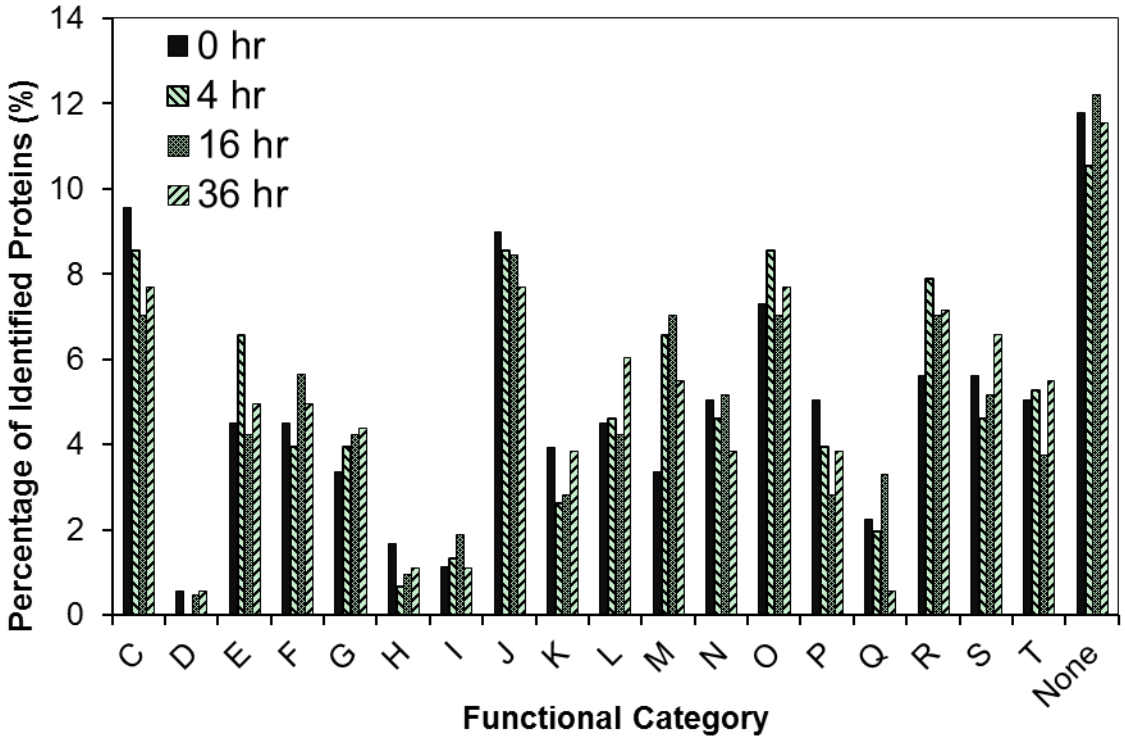

Figure 4.3 Functional category distribution of proteins in strain ZL1 during E2 biodegradation. COG categories are as follows: C: energy production and conversion; D: cell division and chromosome partitioning; E: amino acid transport and metabolism; F: nucleotide transport and metabolism; G: carbohydrate transport and metabolism; H: coenzyme metabolism; I: lipid metabolism; J: translation, ribosomal structure and biogenesis, K: transcription; L: DNA replication, recombination and repair; M: cell envelope biogenesis, outer membrane; N: cell motility and secretion; O: post translational modification, protein turnover, chaperones; P: inorganic ion transport and metabolism; Q: secondary metabolites biosynthesis, transport and catabolism; R: general function prediction only; S: function unknown; T: signal transduction mechanisms; and None: no related COGs.

4.3.5 E2 and E1 in catabolic and anabolic processes

Glycolysis, tricarboxylic acid (TCA) cycle, oxidative phosphorylation, amino acid metabolism, and lipid metabolism were selected for detailed analyses (Table 4.1),

because the expression profiles of the enzymes involved in these processes exhibited interesting correlation with the E2 and E1 degradation profiles shown in Figure 4.1. The time course covered by the four time points (i.e., 0, 4, 16, and 36 hr) was divided into two phases (i.e., Phases 1 and 2, as marked in Figure 4.2). A protein was considered up-regulated when the expression level increased by 1.5 folds, and was considered down-regulated when the expression level dropped by 25 percent (Habicht *et al.* 2011). The connections of the proteins involved in glycolysis, TCA cycle, oxidative phosphorylation, and biosyntheses of proteins and lipid metabolism are illustrated in Figure 4.4.

Glycolysis. During Phase 1 (0-16 hr) the expression level of pyruvate kinase decreased considerably, suggesting decreased glycolysis activities (Figure 4.4). This was deemed reasonable, because at 0 hr the strain ZL1 was transferred from a R2A medium in which glucose and soluble starch were ingredients to an acetone-free NMS medium to which E2 was added as the only energy and carbon source. During Phase 2 (16-36 hr), pyruvate kinase and pyruvate dehydrogenase were up-regulated, although the increase in expression of the former enzyme was not significant. Two enzymes, phosphoglycerate kinase and dihydrolipoyl transacetylase, significantly decreased in this phase.

Oxidative phosphorylation and TCA cycle. ATP synthase, an integral membrane protein complex, derives energy from proton motive force and transfers the energy to adenosine diphosphate (ADP) molecules to form adenosine triphosphate (ATP) molecules. The expression levels of the alpha, beta, and epsilon subunits of F_1F_0 ATP synthase varied during the biodegradation experiment. During Phase 1 (0-16 hr), all three ATP synthase subunits were first down-regulated and then up-regulated. Specifically and noticeably, between 4 and 16 hr, the expressions of the alpha and beta

subunits were up-regulated by 3.0 and 2.6 folds, respectively, and the epsilon subunit changed from non-detectable to detectable. During Phase 2 (16-36 hr), the three subunits behaved differently from one another. The expression pattern of ATP synthase coincided with the expression level of the malate dehydrogenase in the TAC cycle (Table 4.1 and mdh in Figure 4.4). The coincidence is likely due to the fact that the NADPH molecules generated by malate dehydrogenase could be used to feed the electron transport chain, which could generate proton motive force to feed ATP synthase (Figure 4.4). Finally, the high level of spectra counts matching ATP synthase at 36 hr suggests high energy demands of *S. maltophilia* ZL1 during E1 degradation and cell growth.

Amino acid metabolism. Amino acid metabolism was important to cell vitality and growth. Aspartate-semialdehyde dehydrogenase, an enzyme catalyzing an initial step in synthesizing amino acids lysine, methionine, leucine and isoleucine from aspartate, was up-regulated by 6 folds at the end of the Phase 1 and further increased 1.8 folds at the end of Phase 2. In addition, aromatic-amino-acid transaminase, a protein capable of converting 4-hydroxyphenylpyruvate to tyrosine, was up-regulated throughout the two phases. It is not surprising that the enzymes related to amino acid biosynthesis were up-regulated while biomass growth occurred in Phase 2 (Table 4.1 and mdh in Figure 4.4).

Table 4.1 Differential expression of proteins in selected metabolic processes. Proteins with normalized MS spectral counts 25% lower than the reference time are highlighted in green and proteins with normalized spectral counts 50% higher are highlighted in red. For Phase 1, time zero is the reference point. For Phase 2, 16 hr is the reference point

Classification	Accession No.	Gene	Protein name	Normalized spec. count			
				Phase1			Phase2
				0	4	16	36
Glycolysis	YP_002029595	*fbaB*	fructose-bisphosphate aldolase	12	25	19	17
	YP_002029598	*pgk*	phosphoglycerate kinase	7	0	6	3
	YP_002027840	*eno*	phosphopyruvate hydratase	11	19	16	13
	YP_002029596	*pyk*	pyruvate kinase	17	14	5	6
	YP_002030129	*pdhB*	pyruvate dehydrogenase	0	0	1	3
	YP_002030131	*pdhC*	dihydrolipoyl transacetylase	12	0	24	14
TCA cycle	YP_002029635	*gltA*	citrate synthase	8	9	16	10
	YP_002028226	*acnB*	aconitate hydratase	2	2	0	0
	YP_002029545	*sucD*	succinyl-CoA synthetase alpha subunit	13	19	20	20
	YP_002029038	*fumA*	fumarate hydratase	6	0	0	1
	YP_002027178	*mdh*	malate dehydrogenase	64	19	50	37
Oxidative Phosphorylation	YP_002029895	*atpA*	F_1F_0 ATP synthase subunit alpha	17	6	18	16
	YP_002029893	*atpD*	F_1F_0 ATP synthase subunit beta	21	17	45	33
	YP_002029892	*atpC*	F_1F_0 ATP synthase subunit epsilon	2	0	4	8
Amino Acid Metabolism	YP_002029237	*asd*	aspartate-semialdehyde dehydrogenase	1	0	6	11
	YP_002026409	*tyrB*	aromatic-amino-acid transaminase	3	3	8	12
	YP_002029395	*purA*	adenylosuccinate synthase	3	6	7	3
	YP_002029010	*purB*	adenylosuccinate lyase	0	2	2	0
Lipid Metabolism	YP_002027260	*fabG*	3-oxoacyl-[acyl-carrier-protein] reductase	6	2	1	8
	YP_002030404	—	glutathione peroxidase	0	2	2	3

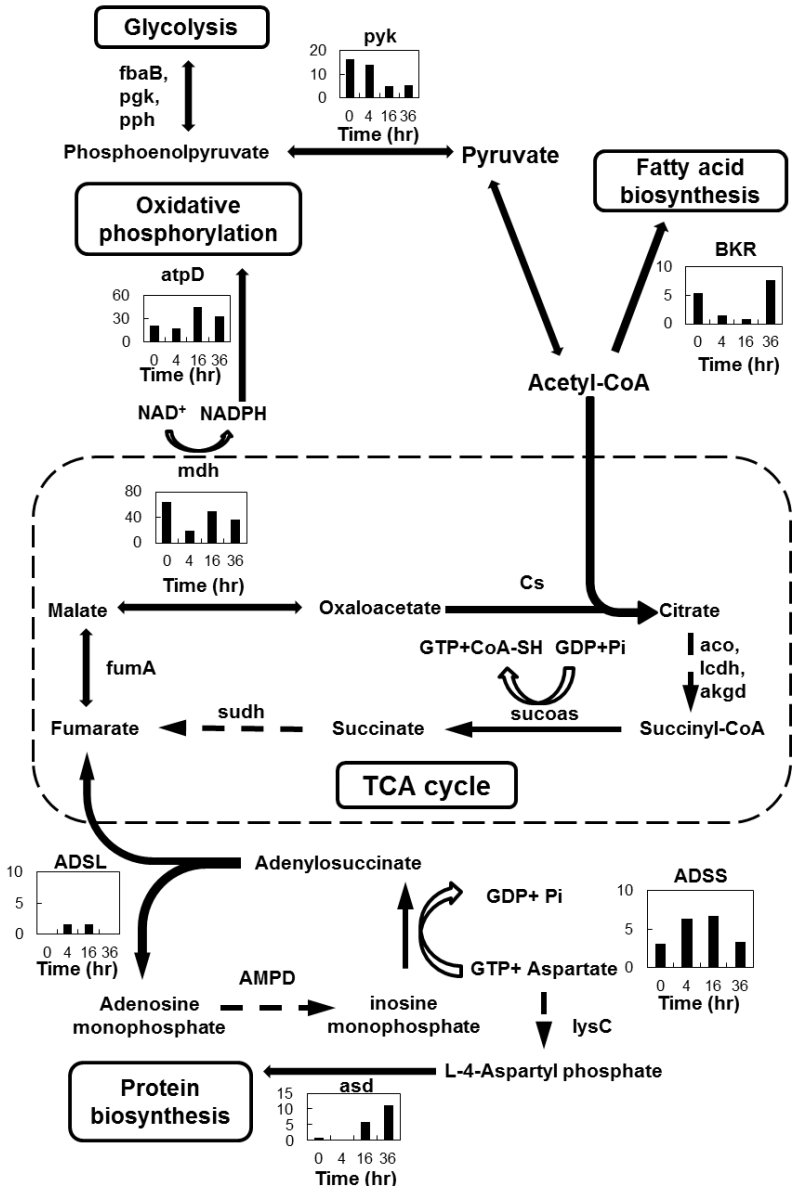

Figure 4.4 Connections among glycolysis, oxidative phosphorylation, fatty acid biosynthesis, protein biosynthesis, and TCA during 17β-estradiol biodegradation, as well as proteins involved and their expression levels. Y-axies in the column plots are normalized spectral counts of selected protein. fbaB, fructose-bisphosphate aldolase; pgk, phosphoglycerate kinase; pph, ; pyk phosphoglycerate kinase; BKR, 3-oxoacyl-[acyl-carrier-protein] reductase; atpD, F1F0 ATP synthase subunit beta; mdh, malate dehydrogenase; Cs, citrate synthase; aco, aconitase; Icdh, isocitrate dehydrogenase; akgd, alpha ketoglutarate dehydrogenase; sucoas, succinyl-CoA synthetase; sudh, succinyl-CoA dehydrogenase; fum, fumarase; ADSL, adenylosuccinate lyase; ADSS, adenylosuccinate synthase; AMPD, adenosine monophosphate deaminase; lysC, aspartate kinase; asd, aspartate-semialdehyde dehydrogenase. Solid arrows indicate detected pathways, while dashed arrows indicate pathways coded by *S. maltophilia* R551-1 genome but not detected.

Lipid metabolism. 3-oxoacyl-[acyl-carrier protein] reductase (BKR) was down-regulated by 6 folds during Phase 1 and was then up-regulated by 8 folds during Phase 2. Lipid biosynthesis typically involves four recurring steps, each of which adds 2 carbons to fatty acids per cycle. BKR is an enzyme involved in the fourth recurring step of lipid biosynthesis, and responsible for reducing (3R)-3-hydroxyacyl-[acyl-carrier-protein] to (3R)-3-hydroxybutanoyl-[acyl-carrier-protein], two lipids with different numbers of carbon (Magnuson *et al.* 1993). In addition, glutathione peroxidase, an enzyme responsible for reducing lipid hydroperoxides and reducing free hydrogen peroxide (H_2O_2) to water (Mills 1957), was gradually up-regulated during the biodegradation experiment. It was worth of noting that the up-regulation of glutathione peroxidase occurred simultaneously with the elevated electron transport chain activity, a process that could produce hydrogen peroxide (González-Flecha and Demple 1995).

4.4 Discussion

The global proteome responses of *S. maltophilia* strain ZL1 during E2 degradation was investigated using functional category distribution and pathway annotation. The pathway analyses, including catabolism and anabolism, showed that E1, the primary degradation product of E2, was utilized by the strain as both carbon and energy sources for cell growth. The E2 degradation pattern of ZL1 was similar to that of an *Amonobacter* strain named KC7 (Yu *et al.* 2007). Compared to the strain KC7, *S. maltophilia* ZL1 converted E2 to E1 at a faster pace and showed a more pronounced biomass growth. In this study, we compared the proteomic profiles within both Phase 1 (i.e., 0-16 hr), and Phase 2 (16-32 hr). The majority of identified proteins were shared

throughout the degradation experiment, with only a few unique proteins at each time point (Appendix E). A recent study on EE2 removal in lab-scale chemostat reactors simulating wastewater treatment reported that basal catechol oxygenase activity in heterotrophs was sufficient for aerobic mineralization of the aromatic ring in EE2 (Khunjar *et al.* 2011). Those findings, together with the largely shared proteome profiles at different degradation stage in this study, suggested that proteins involved in other metabolism pathways could also involve in E2 biodegradation.

In the E2-NMS medium, *S. maltophilia* strain ZL1 experienced a lag phase in Phase 1 and underwent exponential growth in Phase 2. The OD_{600} profile in Figure 4.2 coincides with the expression profile of aspartate-semialdehyde dehydrogenase (asd in Figure 4.4), which catalyzes one of initial steps of the biosynthesis of amino acids and cell-wall components in bacteria (Cox *et al.* 2005). In addition, adenylosuccinate synthase (purA) and adenylosuccinate lyase (purB), which connect aspartate to the TCA cycle by incorporating aspartate to adenylosuccinate (Figure 4.4), were up-regulated during Phase 1 and down-regulated in Phase 2 (Figure 4.1). We speculate that the E1 and/or its degradation products were channeled into the TCA cycle and used for amino acid biosynthesis through aspartate. Interestingly, both E1 degradation and biomass growth ceased after 36 hours.

Although biotransformation of E1 has been observed in multiple studies (Weber *et al.* 2005; Yu *et al.* 2007), the initiating ring cleavage location on the estrogen skeleton is uncertain. Based on the detection of a putative intermediate that contained a keto group on the D ring of E1, it was proposed that the ring cleavage of E1 by sewage bacteria was initiated at that ring (Lee and Liu 2002). Another study using an enriched

ammonia oxidizing bacterial culture suggested that the aromatic A ring on EE2 was more vulnerable because the electron density associated with the A ring was significantly higher than the other rings (Yi and Harper 2007). A third study that used a *Sphingomonas* culture reported that before ring cleavage E2 might be converted to intermediates 4-hydroxyestrone (4-OH-E1) and 4-hydroxyestradiol (4-OH-E2), or intermediates keto-estradiol (keto-E2) and keto-estrone (keto-E1, Figure 4.5) (Kurisu *et al.* 2010). The authors proposed that ring cleavage could happen either at the aromatic A ring of 4-OH-E2 and 4-OH-E1, or at the saturated ring of keto-E2 and keto-E1. The intermediate compound VI (i.e., 3-(4-hydroxyphenyl)-2-hydroxyprop-2-enoic acid) can be isomerized to compound X (i.e., 4-hydroxyphenylpyruvate) by phenylpyruvate tautomerase, an enzyme encoded by the A. maltophilia genome. Compound X could be converted to tyrosine by aromatic-amino-acid transaminase, which was detected in our proteomic analysis and was up-regulated throughout the two phases (Table 4.1). Therefore, we propose that E2 was primarily biotransformed to E1 by *S. maltophilia* strain ZL1; then E1 and it degradation intermediates were utilized in aromatic amino acid biosynthesis (Figure 4.5). This proposed pathway is partially supported by the results from a study using radiochromatograms, which showed that radio-labeled carbon on the estradiol structure in EE2 was incorporated into the biomass (Yi *et al.* 2011). It is worth noting that although in the original paper E2 was directly converted to keto-E1 (Kurisu *et al.* 2010), it's possible that E2 was converted to E1 first and then to keto-E1 before the tyrosine is formed (personal communication with Dr. Kurisu).

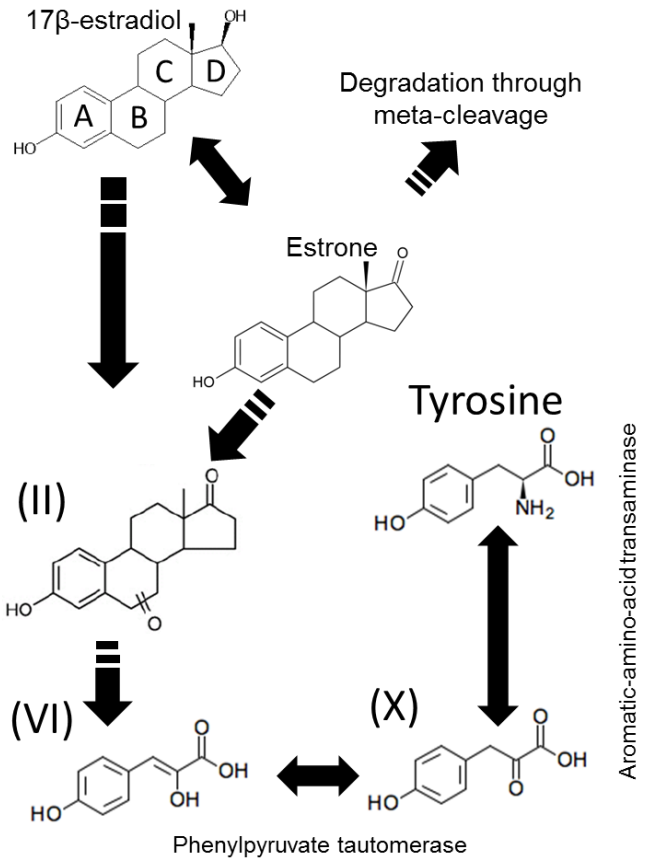

Figure 4.5 Proposed 17β-estradiol biodegradation pathway suggested by the proteomic analyses on *S. maltophilia* ZL1. Compound II: keto-E1; compound VI: 3-(4-hydroxyphenyl)-2-hydroxyprop-2-enoic acid; compound X: 4-hydroxyphenylpyruvate. Compound numbers were adopted from the original paper (Kurisu et al). Solid arrows indicate evidenced reactions, while dashed arrows indicate proposed reactions.

Although this is the first study to report *S. maltophilia* as an estrogen degrader, biodegradation of aromatic hydrocarbon by *S. maltophilia* has been reported. *S. maltophilia* strain KB2 isolated from activated sludge could degrade various monocyclic aromatic hydrocarbon, e.g., benzoate, catechol, 4-hydroxybenzoic acid, protocatechuic acid and vanillic acid (Urszula *et al.* 2009). The ability of an *S. maltophilia* strain (VUN10,003) to degrade high molecular weight polycyclic aromatic hydrocarbons (PAHs), e.g., benzo[*a*]pyrene, dibenz[*a,h*]anthracene, and coronene, was also reported

(Juhasz *et al.* 2000). *S. maltophilia* strain VUN 10,010 isolated from soil showed increased degradation capacity for high molecular weight PAHs when incubated with fungus (Boonchan *et al.* 2000). Several dioxygenases are believed to be involved in aromatic biodegradation in *S. maltophilia* cultures. Protocatechuate 3,4-dioxygenase was extracted from a system where 3,4-dihydroxybenzoate was a growth substrate and nitrophenols were co-metabolites (Wojcieszynska *et al.* 2011). Different types of dioxygenases could be induced by different growth substrates. For example, catechol 1,2-dioxygenase was induced when benzoate and catechol were carbon sources, while protocatechuate 3,4-dioxygenase was induced after incubation with 4-hydroxybenoic acid, vanillic acid and protocatechuic acid (Urszula *et al.* 2009). Neither catechol 1,2-dioxygenase nor protocatechuate 3,4-dioxygenase were not detected in our proteomic analyses, which might be due to the ability of the method to detect proteins with low abundance (Garbis *et al.* 2005; Ray *et al.* 2011). This limitation might be particularly relevant, because estrogens have low solubility in water and are usually present at low concentrations (Khunjar *et al.* 2011). Targeted proteomics by selected reaction monitoring mass spectrometry provides a higher resolution, and thus allows for detection of low abundant proteins (Garbis *et al.* 2005; Doerr 2011; Elschenbroich and Kislinger 2011). Target proteomics could be used in future studies of E2 biodegradation in pure or mixed cultures, by using the characteristic enzymes suggested in this study and/or oxygenases (e.g., monooxygenases and dioxygenases) as potential biomarkers for microbial E2 degradation.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

This thesis investigated the applied and mechanistic aspects of microbial E2 degradation. The adsorption characteristics of E2 onto GAC were evaluated through kinetics, isotherm, and column experiments. The impacts of exogeneous energy source (i.e. acetic acid and humic acid), nitrogen sources (i.e. ammonium and nitrate) and EBCTs on E2 removal were systematically investigated for the BAC reactor. The structure of the bacterial community inside the BAC reactor was monitored using pyrosequencing. Metabolism pathways involved in E2 biodegradation in *S. maltophilia* was investigated. The following conclusions may be drawn from this study:

- The adsorption isotherm experiment showed that the F400 GAC had a higher adsorption capacity for E1 than for E2, and revealed competitive adsorption between E1 and E2.
- Early leakage of E2 occurred to the GAC reactor, likely due to the development of a shallow mass transfer zone in the GAC column. Effluent E2 decreased with increased EBCTs, however, no complete E2 removal was observed even with the longest EBCT tested (i.e., 48 minutes).
- Near complete removal of E2 was achieved at the quasi-steady states during Phase 2 when ammonium served as the nitrogen source and possibly the energy source in the BAC reactor and during Phase 7 when acetic acid and nitrate served as energy and nitrogen source, respectively. Trace amount of E2 (65 ± 15 ng/L) was detected in the effluent with humic acid was the exogenous energy source.

This study suggested that substrate and nutrient conditions were crucial to achieve satisfactory E2 removal in a bioreactor.

- The BAC reactor exhibited high E1 removal efficiency when ammonium was added as the nitrogen source, and correspondingly, *Sphingomonas* and *Rhodococcus* were the primary E1 degrading populations.
- 2D LC-MS/MS based proteomic approach was successfully applied to investigate metabolism pathways of *S. maltophilia*. Pathways involved in fatty acid biosynthesis, protein biosynthesis, and oxidative phosphorylation were up-regulated during E2 biodegradation. This study provided direct evidence that E2 and its degradation products could be used as sole carbon and energy source in *S. maltophilia*.
- E2 and its degradation products may be used as substrates for aromatic amino acid biosynthesis. Aromatic-amino-acid transaminase, an enzyme converting 4-hydroxyphenylpyruvate to tyrosine, could be a potential biomarker of microbial E2 degradation.

5.2 Recommendations for Future Research

This study investigated several critical questions related to microbial E2 degradation. Potential directions for future research include

- When humic acid was the sole energy source in the influent, the BAC reactor exhibited poor removal efficiencies of E2 and E1. Competitive adsorption between NOM and estrogens has been reported (Fukuhara *et al.* 2006). The GAC adsorption of E2 in presence of humic acid needs to be evaluated.

- This study confirms the finding of several published works that the presence of ammonium was beneficial to E2 degradation in mixed cultures. In order to elucidate the mechanisms behind this phenomenon, the pure culture of *S. maltophilia* will be studied for its protein expression characteristics when ammonium or nitrate is the sole nitrogen source.
- Proteomic approach utilized in this study uncovered potential metabolism pathways and biomarkers for E2 biodegradation. However, several interested enzymes, including nonspecific monooxygenase and dioxygenase were not detected. More sensitive proteomic approach, e.g., targeted proteomics by selected reaction monitoring mass spectrometry, would be useful to detected low abundant but functionally important enzymes expressed in estrogen degrading bacteria.

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Appendix A. Theoretical Usage of E2 Adsorption Capacity of the GAC/BAC Reactor

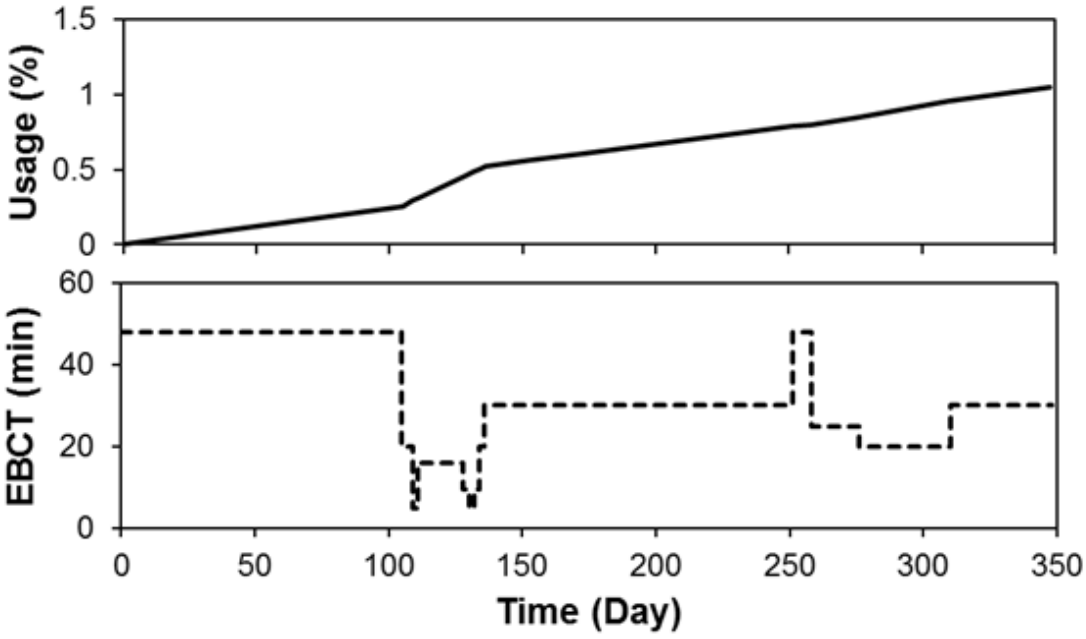


Figure A-1. Adsorption capacity usage of the activated carbon bed of the first 348 days, assuming no mass transfer limitations, no desorption of E2, and no biodegradation.

Appendix B. Schematic of the BAC Reactor Configuration

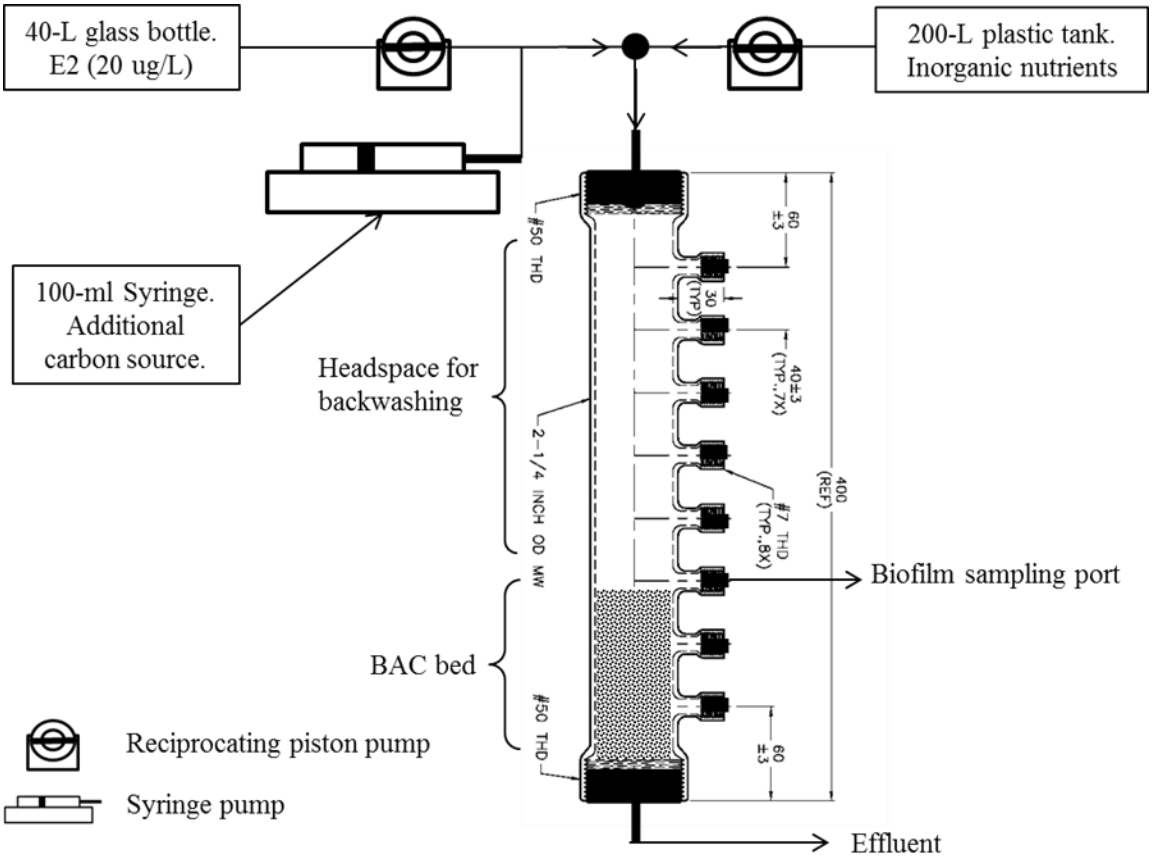


Figure B-1. BAC reactor configuration.

Appendix C. DNA isolation From Bacterial Cells

1. Into 2.2 ml screw cap, conical vials place:

   100-300 mg cells (wet weight) and 0.5 mL TE buffer

2. Suspend cells (if not already suspended), and then add:

   0.5 g baked zirconium beads (0.1 mm diameter)

   50 µL 20% SDS

3. Fill tube with 1 mL phenol:chloroform:isoamyl alcohol (25:24:1) and vortex.
4. Incubate for 30 seconds at 60 ℃.
5. Beat 2 minutes on bead beater.
6. Incubate 10 min at 60 ℃.
7. Beat another 2 minutes.
8. Centrifuge at 12,500 ×g for 10 min to pellet beads.
9. Remove 500 µL of aqueous phase containing nucleic acids and transfer aqueous phase to a 2.0 mL conical tube.
10. Rinse beads with 250 µL TE buffer, beat 2 minutes, centrifuge, and pool this aqueous with previous phase.
11. Add equal volumes (EV) phenol:chloroform:isoamyl alcohol, 25:24:1 and mix thoroughly by repeated inversions.
12. Incubate on ice for 10 minutes.
13. Centrifuge at 14,000 × g for 5 minutes.
14. Transfer aqueous phase to a new centrifuge tube.
15. Repeat steps 11-14.
16. Add EV chloroform and mix thoroughly by repeated inversions.
17. Centrifuge at 14,000 × g for 5 minutes.
18. Transfer aqueous phase to a new centrifuge tube.

19. Repeat steps 16-18.
20. Precipitate nucleic acid from final aqueous phase by adding 0.5 volumes 7.5 M NH_4Ac and 0.6 volumes isopropanol. Mix gently.
21. Keep at -20 ℃ overnight.
22. Centrifuge 30 minutes at 12,000 × g.
23. Decant carefully.
24. Wash precipitate with 1 mL 80% ethanol.
25. Decant carefully.
26. Air dry 5-10 minutes.
27. Re-suspend sample in 100 µL TE buffer* and then pipette 50 µL to a second tube.
28. Store stock solution at -80 ℃

* For biomass samples in presence of humic acid, the 100 µL DNA extract in TE buffer is directed to DNA purification steps of UltraClean® Soil DNA Isolation Kit (MoBio, CA, USA). The solution numbers in the manual of the UltraClean® Soil DNA Isolation Kit was adopted in this protocol:

29. Shake to mix Solution S3 before use. Add 1.3 mL of Solution 3 to the 100 µL DNA extract in TE buffer and vortex for 5 seconds. Solution 3 helps to bind DNA on silica membrane on the spin filter.
30. Load about 700 µL mixed solution in step 29 to a spin filter and centrifuge at 10,000 × g for 1 minute.
31. Discard the flow through, add the remaining mixed solution in step 29 to the spin filter, and centrifuge at 10,000 × g for 1 minute. Repeat until all supernatant has passed through the spin filter.

32. Add 300 µL of Solution S4 and centrifuge for 30 seconds at 10,000 × g. Solution 4 is an ethanol based wash solution used to clean the DNA that is bound to the silica membrane in the spin filter. Solution 4 removes residues of salt, humic acid, and other contaminants while allowing DNA to stay bound to the silica membrane.
33. Discard the flow through from the 2 mL collection tube.
34. Centrifuge again at 10,000 × g for 1 minute.
35. Carefully place spin filter in a new clean centrifuge tube. Avoid splashing any Solution S4 onto the spin filter.
36. Add 50 µL of Solution S5 to the center of the white filter membrane. Solution S5 is to release desired DNA on the silica membrane.
37. Centrifuge at 10,000 × g for 30 seconds.
38. Discard the spin filter, store the DNA solution at -80 ℃.

Appendix D. Preliminary E2 Degradation Experiment of Isolated E2 Degrading Bacteria

Table D-1. E2 degradation capacity of isolated E2 degrading bacteria in preliminary degradation experiment

Bacteria	Initial E2 conc. measured (mg/L)	Final E2 conc. measured (mg/L)	Initial E1 conc. measured (mg/L)	Final E1 conc. measured (mg/L)
ZLB1	3.30	0.27	0.00	0.51
ZLB2	3.05	0.38	0.00	2.60
ZLB3	3.11	0.52	0.00	2.45
ZLB4	3.15	0.32	0.00	0.63
ZLB5	3.24	0.41	0.00	0.67

Appendix E. Comparison of Identified Proteins

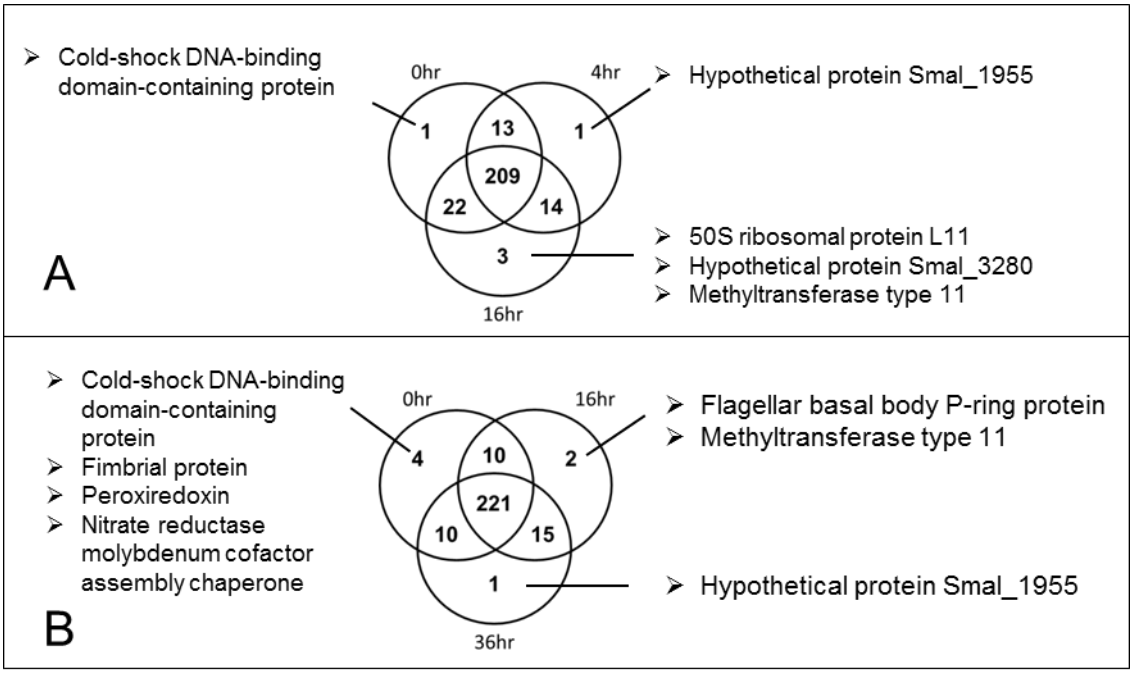


Figure E-1. Venn diagram comparing expressed protein profiles among the three time points in Phase 1 (0, 4 and 16 hr) (A) and expressed protein profiles amount the two times points in Phase (16 and 36 hr) and time zero. Unique proteins were listed for each sampling event.

Appendix F. Protocol of Sample Analysis on Gas Chromatography (GC) – Mass Spectrometry (MS)

1. Sample collection and preservation

(1) Items needed

250 ml amber bottle, Amber bottle cap with Teflon® Liner, syringe needle, 4 degree refrigerator (lab 348), -25 degree freezer (lab 348).

(2) Operation

- Before sample collection, stop influent pump and wait for 1 min until no effluent comes out of the effluent tubing; weigh and record the weight of each amber bottle, and make a label on the outside wall indicating water volume of 50.0 ml.
- Insert clean syringe needle into the desired sample port septa and then start influent pump. Drop the first 15~20 ml water sample comes out of the syringe needle; after that stop the influent pump again, and fix the amber bottle on iron support with bottle lip just below the end of syringe needle.
- Start the influent pump to begin sample collection until the water level reaches the label of 50.0 ml. Stop the influent pump and take off the amber bottle from the iron support.
- Note: The sample volume can be calculated by weighing the sample bottle, using 1g/ml as water density.
- Sample is preserved in the 4 °C refrigerator if it would be analyzed in 24 hours; otherwise, sample is preserved in the -25 °C degree freezer.

2. Solid-phase extraction

(1) Items needed

Vacuum manifold, SPE vacuum manifold (Sigma-Aldrich), SPE manifold cover (Sigma-Aldrich), PTFE transportation lines, disposable PTFE needle (Sigma-Aldrich), 6 ml disposable glass tube (Fisher Scientific), 3 ml disposable glass tube (Fisher Scientific), C18 SPE cartridge (Waters), 105 °C oven (Fisher Scientific).

(2) Operation

- Transportation lines are washed first by 10 ml MiliQ water, then by 5 ml methanol. Filter (0.45 μm) should be used if total suspended solid (TSS) is high in water sample.
- C18 SPE cartridge is conditioned with 5.0 ml of hexane, 5.0 ml of ethyl acetate, 5.0 ml of methanol, and 5.0 ml of MiliQ water [1]. When conditioning, the cartridge should not be dried. If dried, the cartridge should be reconditioned.
- 100 μL 17beta-estradiol-d3 (10 ng/μL) and 100 μL phenanthrene (10 ng/μL) are spiked into water sample bottle. After spiking, the sample bottles are shaken several times to homogenize water sample.

  Note: Phenanthrene is used only to test derivatization efficiency.
- The transportation lines are inserted to each sample bottle, make sure the end of transportation line is reached the bottom of sample bottle; the flow rate is controlled at about 3 ml/min [2].

  Note: The flow rate may vary, but to make sure the liquid is trickled down.

- After the water sample going through SPE, the cartridge is dried by air and is connected to SPE manifold.
- 3.0 ml ethyl acetate-methanol mixture (4:1 V/V) is used to elude the adsorbed steroids in C18 SPE cartridge [3]; the flow rate is controlled at about 0.5 ml/min.
- Note: The flow rate here cannot be accurately controlled; however, the elution should be at least 5 min to get good recovery.
- The extracts are collected by 6 ml disposable glass tube, and then the organic phase is concentrated to about 0.5 ml by gentle dry nitrogen gas applied through the SPE manifold cover;
- Reaction vials are fired at 350 C for 4 h, and washed with acetone. After that, the reaction vials are preserved in 105 °C oven until use [2]. The concentrated organic elutes are transferred to each reaction vial, which is cooled down to room temperature in desiccators. Then 50 µL BSTFA+ 1% TMCS and 50 µL dimethyl formamide are added [4]. The Teflon cap is screwed onto reaction vial firmly. After that, the vial is votexed for 30 s to sufficiently mix the liquid.

Note: The 50 µL BSPFA+1% TMCS is taken out by 50 µL syringe. The syringe should be washed by acetone, and then dried completely in oven to prevent moisture go into BSTFA bottle.

Note: The BSTFA is very sensitive to moisture. To prevent contamination and degradation of BSTFA, Para film is used to seal the septa on the BSFTA bottle.

Note: Pyridine and dimethyl formamide are both catalysts for BSTFA; however, for the specific GC-MS5972, pyridine may be a reason jeopardizes the resolution with unknown mechanism.)

- The reaction vials can be washed by acetone directly, bypassing firing at 350 C.
- The reaction vials are heated at 75 C for 30 min [4]. After 30 min derivatization, the reaction vials are taken out of the hot plate, and are cooled down to room temperature.
- The liquid in each reaction vial is transferred to GC vials. After that, the vial is sealed, votexed for 30 s, and ready for analysis.

3. Sample analysis on GC-MS

(1) Preparation

- Check washing solutions

The washing solution bottles are on the auto-sampler. Check the wash bottle 1 and bottle 2 to make sure the solutions are at least 2/3 full. If not, fill ethyl acetate for bottle 1 and methanol for bottle 2.

Note: Insufficient wash solutions may cause injection problem, such as syringe damage.

(2) Open GC-MS program

Double click the icons “5972”, “GC-MS Instrument #2”, and “Data Analysis #1”.

(3) Check leaking problem

In the program “5972”, select “view” in the title bar. In the “view” menu, click “Manual Tune”. In the window of “Manual Tune”, select “AdjPram” and put cursor on “PFTBA”, then select “Closed”. At the right bottom of “Manual Tune”, click “Scan” and watch the spectra (typically 2~3 min): the abundance of H2O (18) should be at about 4000~7000 or below, and the nitrogen peak (on the right side of H2O peak) should below the H2O peak. After checking, click “MSoff” and then click “Ok”. In the title bar, select “View”, click “Instrument Control”, select “OK” to go back to instrument control window.

Note: If any suspicious problem was observed, analysis should be stopped to prevent possible MS damage. Consult with professional staff in the Water Sciences Lab as soon as possible.

(4) Sample layout

- Open sequence program

  In the window “5972 MSTop”, select “Sequence” in the title bar. In “Sequence” menu, click “Edit sequence”. The sequence program is then opened.

- Layout the sequence table

  For calibration vials, select “calibration” as property, input method name, file name, and sample name. For sample vials, select “sample” as property, input method name, file name, and sample name.

- Save sequence

  In the “Sequence” tag, select “Save” to save edited sequence.

(5) Method establishment

- Set parameters

In the window of "Instrument Control", five icons are listed horizontally, representing for syringe, injection port, column, oven, and MS detector. Lots of parameters can be set in "Instrument Control", and some of the parameters are universally used for different methods. Therefore, only key parameters should be changed in method establishment.

Syringe: Click the "Syringe Icon", in the window, select "Syringe Vol." and input the total volume (10 ul) of the syringe that is used; select "Injection Vol." and input the injection volume (1 ul) of the method.

Injection port: Click the "Injection Icon", in the window, select "Pressure" and input 7.5 psi; select "Tem." and input 280 C.

Column: Nothing is needed to change in "Column Icon" except changing to another kind of column.

Oven: The operating interface is quite straight forward. "Initial tem" is the initial oven temperature; "Initial Time" is the time period for initial temperature. The optimized oven condition is: the oven temperature is programmed at 80 C for 2 min, ramps at 15 C/min to 250 C, then ramps at 10C/min to 280 C, and then ramps at 15 C/min to 300 C and maintains at this temperature for 10 min.

MS detector: In "Full Scan" model, no ion information is needed; in "SIM" model, the monitored ions are: phenanthrene (188), 17beta-estradiol-d3 (419, 328, 403), estrone (342, 257, 218), 17beta-estradiol (416, 326, 401), estriol (311, 345, 504)

- Save established method

After method establishment, click “saves” in the “Method” tag to save the method with a unique file name.

(6) Method selection

In the “Method” tag, first select “load” to load desired method that is already established; after that select “save” to save the method with an unique file name (For example, ES072109 indicated the method was used on July 21, 2009).

Note: To prevent overwrite existing method, build a new method file for each analysis is recommended.

(7) Start analysis

- Final check before start.

  Check the “Method” and “Sequence” to be of the right file names to prevent accidentally overwriting. Check the wash solutions to make sure they are at least 2/3 full. Check vials location to make sure each vial is on the right sample hole.

- Start analysis

  In the window “5972 MSTop”, click “sequence”. In the menu, click “Run sequence”. In the window “Run sequence”, input the file location (make sure to prevent unwanted overwriting). Click “run” to start analysis.

- Watch the first analysis

  It is recommended to watch the first analysis in person. Pay attention on the following aspects:

  If the auto-sampler wash the syringe needle 3 times using wash solution 1 and 3 times using wash solution 2;

If "Run" is showed on the window "5972";

If the chromatography is similar with previous one (not applicable for the first analysis).

(8) Quantification

- Set ions information

The program cannot provide quantification results at the first run (usually in "Full Scan" model) because the program do not know which peak should be quantified. To get quantification results, compound information is needed. In "Data Analysis #1", clicks "Calibrate" tag and then selects "Edit Compounds". Input Compound Name, Retention Time, and target ions in corresponding blank (the window is straight forward to find correct blank). The target ions for target compounds are: phenanthrene (188), 17beta-estradiol-d3 (419, 328, 403), estrone (342, 257, 218), 17beta-estradiol (416, 326, 401), estriol (311, 345, 504).

References

1. Zuo, Y. G.; Zhang, K.; Lin, Y. J., Microwave-accelerated derivatization for the simultaneous gas chromatographic-mass spectrometric analysis of natural and synthetic estrogenic steroids. Journal of Chromatography A 2007, 1148, (2), 211-218.

2. Fine, D. D.; Breidenbach, G. P.; Price, T. L.; Hutchins, S. R., Quantitation of estrogens in ground water and swine lagoon samples using solid-phase extraction, pentafluorobenzyl/trimethylsilyl derivatizations and gas chromatography-negative ion chemical ionization tandem mass spectrometry. Journal of Chromatography A 2003, 1017, (1-2), 167-185.

3. Zuo, Y. G.; Zhang, K.; Deng, Y. W., Occurrence and photochemical degradation of 17 alpha-ethinylestradiol in Acushnet River Estuary. Chemosphere 2006, 63, (9), 1583-1590.

4. Shareef, A.; Angove, M. J.; Wells, J. D., Optimization of silylation using N-methyl-N-(trimethylsilyl)-trifluoroacetamide, N,O-bis-(trimethyl.silyl)-trifluoroacetamide

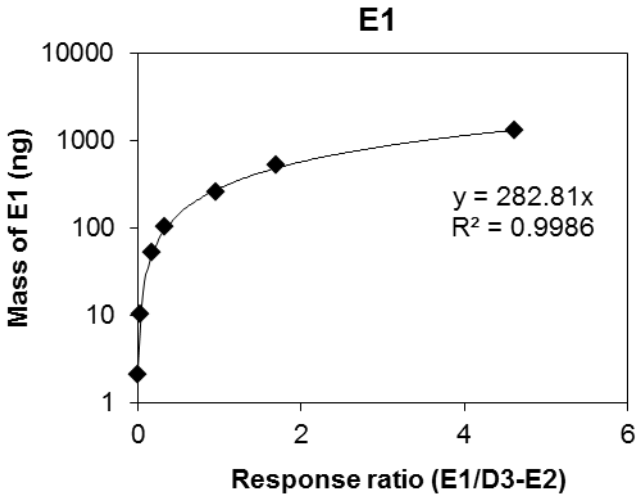
and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide for the determination of the estrogens estrone and 17 alpha-ethinylestradiol by gas chromatography-mass spectrometry. Journal of Chromatography A 2006, 1108, (1), 121-128.

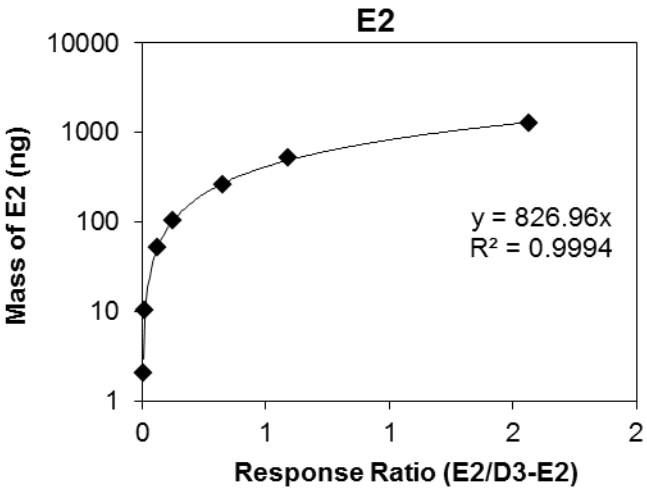
Appendix G. Standard Curves and Method Detection Limits of Selected Estrogens

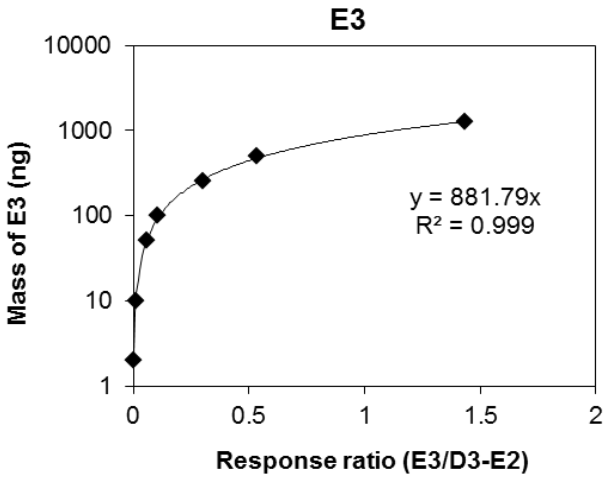
1. Standard Curves for E1, E2, and E3

Table G-1. Calibration data for E1, E2, and E3

	Calibration Series	Response				Response Ratio					
									Mass (ng)	Mass (ng)	Mass (ng)
Mass of target chemicals (ng)	Mass of internal standard (ng)	E1	E2	E3	D3-E2	E1	E2	E3	E1	E2	E3
1250	1000	755286	255806	234669	163525	4.6188	1.5643	1.4351	1293.75	1286.25	1256.25
500	1000	252680	88110	79650	148962	1.6963	0.5915	0.5347	517.5	514.5	502.5
250	1000	116870	39843	36806	122659	0.9528	0.3248	0.3001	258.75	257.25	251.25
100	1000	43374	16043	14048	130544	0.3323	0.1229	0.1076	103.5	102.9	100.5
50	1000	22125	7859	7490	130401	0.1697	0.0603	0.0574	51.75	51.45	50.25
10	1000	4955	1806	1625	146062	0.0339	0.0124	0.0111	10.35	10.29	10.05
2	1000	1148	479	360	133252	0.0086	0.0036	0.0027	2.07	2.058	2.01







2. Method Detection Limit (MDL)

The determination of method detection limit (MDL) was followed by the Code of Federal Regulations, U.S. Environmental Protection Agency, 40 CFP Part 136 [1].

Table G-2. Raw data for E1, E2, E3 MDLs estimation

	Response				Ratio of Responses		
No.	E1	E2	E3	D3-E2	E1/D3-E2	E2/D3-E2	E3/D3-E2
1	1554	740	509	143082	0.0109	0.0052	0.0036
2	1467	707	556	138555	0.0106	0.0051	0.0040
3	1362	658	486	128967	0.0106	0.0051	0.0038
4	1481	716	538	135697	0.0109	0.0053	0.0040
5	1322	711	513	125836	0.0105	0.0057	0.0041
6	1727	735	591	155053	0.0111	0.0047	0.0038
7	1741	836	625	155120	0.0112	0.0054	0.0040
8	1719	790	630	152467	0.0113	0.0052	0.0041

Table G-3. Statistical data for E1, E2, E3 MDLs estimation

Xi			**Xi^2**			**S^2**		
E1	E2	E3	E1	E2	E3	E2	E1	E3
3.07	4.28	3.14	9.43	18.29	9.84	0.0076	0.0465	0.0287
2.99	4.22	3.54	8.97	17.81	12.52		S	
2.99	4.22	3.32	8.92	17.80	11.04	E2	E1	E3
3.09	4.36	3.50	9.53	19.04	12.22	0.0874	0.2156	0.1694
2.97	4.67	3.59	8.83	21.83	12.92	t(7,0.99)=	3.3554	
3.15	3.92	3.36	9.92	15.37	11.30			
3.17	4.46	3.55	10.08	19.86	12.62	**MDL as mass**		
3.19	4.28	3.64	10.17	18.36	13.28	E2	E1	E3
SUM			**SUM**			ng	ng	ng
24.62303	34.41346	27.64666	75.84021	148.3612	95.74304	0.2934	0.7235	0.5683

Reference

1. EPA, U. (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 CFP Part 136: 554.

APPENDIX H. EFFLUENT E2 AND E1 DATA FOR GAC / BAC REACTOR

Table H-1. Effluent E2 and E1concentrations during GAC reactor operation

Bed Volume	Effluent E2 (ng/L)	Effluent E1 (ng/L)
198	0	0
258	0	0
318	0	0
382	0	0
678	0	0
1817	223	0
1820	235	0
1909	278	0
2189	431	0
2606	450	0
3689	599	0
4879	811	0
5077	659	0
5077	661	0
5474	700	0
5672	1987	0
6267	2102	0
6664	6387	0
7060	2786	0
7655	2032	0
8250	2621	0
8845	2564	0
9639	2602	0
10035	4734	0
10432	6645	0
10829	4168	0
11072	1986	0
11102	550	0
11323	797	0
11494	675	0
11542	623	0
11594	234	0
11786	536	0
11978	426	0
12266	200	0
12410	234	0
12122	256	0
12602	234	20
12792	215	0
12934	175	17

Table H-2. Effluent E2 and E1concentrations during BAC reactor operation

Time (Day)	Effluent E2 (ng/L)	Effluent E1 (ng/L)
180	189	57
181	191	201
182	141	230
183	134	250
184	128	204
185	116	157
187	120	153
188	103	162
190	104	165
194	115	152
198	111	145
200	100	135
202	79	120
206	65	104
210	73	94
212	56	87
215	43	78
223	25	45
229	0	0
232	0	0
236	0	0
240	0	0
240	0	0
242	0	0
243	0	19
245	0	32
249	13	28
252	0	35
258	13	33
260	0	28
264	0	35
266	0	30
268	0	33
270	0	30
273	0	33
278	0	47
280	0	35
283	0	48
285	15	57
287	0	37

291	0	41
295	0	50
298	0	78
301	0	65
304	0	58
307	0	65
313	0	68
314	17	66
315	0	55
316	0	51
320	0	52
324	0	45
328	0	49
332	0	44
336	0	46
340	0	39
348	0	38
354	61	32
355	42	34
358	91	31
361	77	77
365	87	91
372	107	141
377	140	197
385	128	183
391	135	181
399	101	193
401	89	156
404	78	168
407	65	147
413	56	125
418	55	103
424	65	108
433	60	98
440	50	78
447	35	88
454	46	56
461	30	60
465	20	40
467	0	45
471	0	35
473	15	55

475	0	50
481	20	50
487	22	65
493	30	45
499	22	62
505	35	58
515	40	55
527	50	34
541	50	65
555	45	48
570	40	45
574	50	67
593	38	73
606	42	60
613	51	75