Fate and transport of endocrine disrupting chemicals in an aquifer

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FATE AND TRANSPORT OF ENDOCRINE DISRUPTING CHEMICALS IN AN AQUIFER

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Abstract

Endocrine disrupting chemicals (EDCs) are of growing concern in recent years, due to their harmful effects on human and wildlife development and reproduction. A wide variety of pollutants have been identified as potential EDCs. Estrogens (estrone, estradiol, estriol and ethylestradiol) are one of the most important groups of EDCs. They are selected as target compounds for this study because of their potential estrogenicity in the environment.

Soil Aquifer Treatment (SAT) is one of the principle technologies for water reuse. Many studies have shown that organic pollutants are significantly reduced during SAT. The reclaimed lands in Singapore have the potential to further polish treated wastewater. If this is the case, it is important to understand the behavior and transformation of selected contaminants which might be present in the water used for recharge.

The objectives of this study are to examine the behavior of the target compounds in the aquifer. A series of laboratory studies, including a batch study and column study has been carried out to achieve the objectives.

A sensitive LC-MS-MS method in conjunction with a single step solid phase extraction pretreatment method was developed for analyzing the estrogens. The single step solid phase extraction pre-treatment method showed good recovery for the ultrapure water when concentrated 1000 times and resulted in a method detection limit of ng/L levels for all the target compounds. However, when the method was applied to natural samples, the matrix effect was very significant and poor recovery was obtained. Therefore, for water with complex matrices a low concentration factor is suggested. In addition, a calibration standard prepared in the same matrix is also suggested for quantifying the water samples.

The sorption and degradation behaviour of estrogens were examined by batch experiments under different redox conditions. In addition, a set of columns packed with aquifer sand was used to simulate the SAT process and assess the attenuation behaviour

of these compounds under recharge conditions. The results indicate that the sorption ability of the estrogens were in the following order: $E3 < EE2 < E2 < E1$ which is inconsistent with their physicochemical properties. This may be due to the inadequate estimation of the K_{oc} values. The degradation study showed that E2 was the most readily degradable compound among these four target compounds. The rapid degradation of E2 was found in all the water matrices under aerobic conditions. Its primary metabolite, E1, was also found to rapidly degrade in all the matrices under aerobic conditions. Under anoxic conditions, E1 and E2 were inter-convertible. In contrast with E2, EE2 was more persistent. It was found to be biodegradable under both aerobic and anoxic conditions at relatively slow degradation rates. Chemical transformation could also be considered as one of the removal mechanisms for this compound.

Three strains of estrogen-degrading bacteria, namely LHJ1, LHJ3 and CYH, were isolated in this study from the aquifer system. LHJ1 and LHJ3 were found to belong to the genus *Acinetobacter* and *Agromyces* respectively, while CYH represents a novel species of the *Sphingobium* genus and was named *Sphingobium estrogenivorans* sp. nov. The three degraders showed the ability to degrade E2 under aerobic conditions. However, only CYH could degrade E1 and only LHJ3 could degrade E3. Under anoxic conditions, CYH and LHJ3 could only degrade E1 and E2 respectively, albeit the degradation rates were much slower than for aerobic conditions. None of the degraders could degrade E3 under anoxic conditions. An E3 degradation product was found and hypothesized to be 16-hydroxyl-estrone. With respect to the synthetic estrogen 17 α -ethinylestradiol (EE2), ground water samples from the aquifer site showed the ability to degrade EE2. However, the degrading microorganisms for this compound have not been isolated yet.

In summary, a sensitive LC-MS-MS method has been developed in this study. However, the solid phase extraction pretreatment step was not good enough to remove the interferences and good recoveries were not achieved for the natural samples. The fate of the target estrogens in aquifer materials was also investigated. Biodegradation was found to play an important role in their removal. In this study, three estrogen-degrading bacteria were isolated from the aquifer system and their estrogen-degradation ability has been

evaluated under different conditions. An E3 degradation product has been found as 16 α -hydroxyl-estrone in the degradation by LHJ3.

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LIST OF SYMBOLS / ABBREVIATIONS

ASR	Aquifer storage and recovery
AAS	Atomic absorption spectroscopy
$[M+H]^+$	protonated molecular ion
$[M-H]^-$	Deprotonated molecular ion
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
C ₁₈	Octadecyl
CAD	collision activated dissociation
CE	Collision energy
CF	concentration factor
CID	Collision-induced dissociation
CUR	Curtain gas
CWP	Clean Water Program
CXP	Collision cell exit potential
DP	declustering potentials
DCM	dichloromethane
DO	Dissolved oxygen
ELISA	Enzyme linked immunosorbent
E1	Estrone
E2	17 β -Estradi
E3	Estriolol
EDC	Endocrine disrupting chemical
EE2	17 α -ethinylestradiol
ESI	Electrospray ionization
FP	focusing potentials
GC	Gas chromatography
GCB	graphitized carbon black
GW	Groundwater

LIST OF SYMBOLS / ABBREVIATIONS

HPLC	High performance Liquid chromatography
HRMS	high-resolution mass spectrometry
ICP	Inductively coupled plasma
IDL	Instrument detection limits
K_m	half-saturation constant
K_{oc}	organic carbon normalized partition
K_{ow}	Octanol-water partition coefficient
K_f	sorption coefficients
LC	Liquid chromatography
LOD	Limit of detection
m/z	Mass to charge ratio
MDL	Method detection limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTBE	Methyl tertiary-butyl ether.
MW	molecular weight
NCBI	National Center for Biotechnology Information
NH_4AC	ammonium acetate
NH_4OH	ammonium solution
NI	Negative ionization
NTU	Nanyang Technological University
NW	Newater
ORP	Oxidation reduction potential
PBS	phosphate buffered saline
PCBs	Polychlorinated biphenyls
PI	Positive ionization
R^2	linear correlation coefficients
RP	reversed-phase

RO Reverse osmosis

LIST OF SYMBOLS / ABBREVIATIONS

RSD	Relative standard deviation
RW	Reservoir water
RDP II	Ribosomal Database Project II
SAT	Soil aquifer treatment
SE	Secondary effluent
S/N	signal to noise ratio
SIM	Selected-ion monitoring
SPE	Solid phase extraction
SRM	Selected-reaction monitoring
STPs	Sewage treatment plants
SW	surface runoff water
TEA	Triethylamine
TSA	Trypticase soy agar
TSB	Trypticase soy broth
TW	Tap water
TOC	Total organic carbon
UF	ultrafiltration effluent
V _{max}	maximum specific degradation rate

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Chapter 1 Introduction

1.1 Background

1.1.1 Endocrine Disrupting Chemicals

The European Union definition of an endocrine disrupting chemical (EDC) is “an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to change in endocrine function” (Bowman *et al.*, 2002; Fawell *et al.*, 2001; Lintelmann *et al.*, 2003; Lopez de Alda and Barcelo, 2000). EDCs constitute, therefore, a class of substances defined not by chemical nature but by biological effects (Lopez de Alda and Barcelo, 2001a). A wide variety of pollutants have been identified as potential EDCs, including organochlorine pesticides, polychlorinated biphenyls (PCBs), dioxins, alkylphenolic compounds, bisphenol A, natural and synthetic estrogens (Folmar *et al.*, 2002, Lopez de Alda and Barcelo, 2000).

Among the large range of endocrine disrupting chemicals, natural and synthetic estrogens are regarded as one of the most important groups due to their extremely low effective concentration and high estrogenic activity (Lopez de Alda and Barcelo, 2001b; Sole *et al.*, 2000). Jobling *et al.* (1998) indicated that natural and synthetic estrogens at concentrations of tens of nanogram per liter range were high enough to induce vitellogenin synthesis in male fish. Moreover, some research has shown that the estrogenic potentials of natural and synthetic estrogens are a few orders of magnitude higher than other types of EDCs, such as alkylphenols (Johnson *et al.*, 2000; Kuch and Ballschmiter, 2001; Lee and Liu, 2002; Sole *et al.*, 2000). Estrogens can cause endocrine-disrupting effects, such as decreased fertility, feminization and hermaphroditism of aquatic organisms, even at very low concentrations of around 1 ng/L (Kuster *et al.*, 2004). These compounds are also known to be human carcinogens and have been linked to the occurrence of breast cancer (Peterson *et al.*, 2000; NIEHS, 2004).

In view of their importance in the environment, natural estrogens (estrone, 17 β -estradiol, estriol) and the synthetic estrogen, 17 α -ethinylestradiol (EE2), were selected as target compounds for this study. Estradiol (E2) is one of the main female sexual hormones and the structural backbone for the engineering of some synthetic estrogens utilized in human hormone treatments (Kuster *et al.*, 2004). It is the most potent natural estrogen and can induce estrogenic response at concentrations of 1-10 ng/L (Routledge *et al.*, 1998). Although estrone (E1) is 2 to 5 times less potent than E2 (Johnson and Sumpter, 2001; Routledge *et al.*, 1998), it is frequently detected at concentrations greater than double that of E2 (Johnson and Sumpter, 2001). Estriol (E3) is considered as one of the major excretion products of E2 (Ying *et al.*, 2002a). The synthetic estrogen, EE2, is the major component in contraceptive pills (Desbrow *et al.*, 1998; Kuch and Ballschmiter, 2001), which has been reported to provoke feminization in some species of male wild fish at a concentration of 0.1 ng/L (Baronti *et al.*, 2000; Routledge *et al.*, 1998). The occurrences of these compounds have been frequently reported at only a few ng/L levels in water samples (Barnoti *et al.*, 2000; Belfroid *et al.*, 1999; Ternes *et al.*, 1999a). These compounds were found to be incompletely removed from sewage treatment plants (Baronti *et al.*, 2000; Beck *et al.*, 2005; Johnson and Sumpter, 2001; Kuster *et al.*, 2004; Lee and Liu, 2002). Therefore, their fate in the environment has become a particular concern in recent years.

1.1.2 Soil Aquifer Treatment

Advances in civilization coupled with rising population levels have resulted in an increasing need to treat and recycle available water resources (Mash *et al.*, 2003; Routledge *et al.*, 1998). Soil aquifer treatment (SAT) is one of the principle technologies for water reuse (Drewes *et al.*, 2003). During the SAT process, treated wastewater infiltrates into the ground from surface spreading basins, percolates through the vadose zone and eventually mixes with native ground water. Dissolved organic matter can be removed by a combination of biological, chemical and physical processes (biodegradation and adsorption being the most important) in the vadose zone and subsequently in the aquifer (saturated zone). This technology is currently being

practiced in the United States and it has been shown that the water quality can be improved significantly during SAT (Quanrud *et al.*, 1996a; Quanrud *et al.*, 1996b).

Singapore is considered a water-stressed country due to insufficient natural resources. About half of Singapore's freshwater daily consumption is currently provided by Malaysia, the other half comes from domestic reservoirs and storm water collection ponds (Segal, 2004). Moreover, its water demand has been increasing at a rate of about 4% over the past decade and will continue to rise in the coming decades. Therefore, one of the most important national strategies is to secure sufficient long term water resources in order to sustain growth. Recycling wastewater in the form of NEWwater and desalinating seawater has been recently introduced in Singapore. In addition, research is also being carried out on new water resources or storage mechanisms.

Due to the geologic nature of Singapore, groundwater has never presented itself as a potential candidate for water supply as there were no viable natural aquifers. However, over the years, Singapore has extended its land mass by reclaiming land from the sea. There is a huge reclaimed land area of over 2500 hectares at the eastern coastal area of Singapore. This original seabed is filled with graded gravel sand and can be considered as an unconfined coastal aquifer with the possibility to be used for groundwater storage. In addition, there is also a possibility of using treated wastewater to recharge for the further treatment, i.e. soil aquifer treatment (Ahmad *et al.*, 2001). If this is the case, it is imperative to understand the behavior and transformation of selected contaminants which might be present in the water used for recharge.

1.2 Objectives and Contributions

This study is aimed at determining the fate of estrogens (E1, E2, E3, and EE2) during soil aquifer treatment at an artificial tropical aquifer in Singapore. Consequently, the following questions need to be answered:

- Are the target compounds present in the potential injection water?
- What is the extent of sorption of these compounds onto aquifer sand?
- Are these compounds degradable in the aquifer?
- How does the environmental condition (redox conditions etc.) in the aquifer encourage or discourage the removal of contaminants?
- If the compounds are degradable, what are the mechanisms?
- What kind of microorganisms work on the biodegradation of these compounds?
- What are their biodegradation pathways and degradation products?

Therefore, the objectives of this study are to:

- Develop reliable method(s) to detect selected target compounds.
- Examine their occurrence in the potential injection water and ground water.
- Assess the removal mechanisms of target compounds in the coastal aquifer by studying their sorption and degradation behavior.
- Isolate and identify the microorganisms which can degrade the target compounds and identify the degradation products of the degradation by these microorganisms.

The methods developed in this study will be useful for detection of the target compounds in the aquatic environment. The information on the behavior of the target compounds contribute to a better understanding of the fate of estrogens in the environment and provide useful information for assessing the possibility of soil aquifer treatment in Singapore. This study also provides new resources for estrogen degrading bacteria.

1.3 Dissertation Overview

This thesis is presented in six chapters. Much of the material in Chapter three to chapter five has been prepared for publication in peer-reviewed technical journals. Chapter one gives a brief introduction to this study. Chapter two is a comprehensive literature

review on the topics related to this study including the source and occurrence of target compounds, the detection methods of the target compounds and sorption and degradation behavior of target compounds in the environment. Brief descriptions of Chapter 3 and Chapter 5 are presented below.

Chapter 3 describes the method development for detection of estrogens. The method development includes the development of a pretreatment method (i.e. solid phase extraction) and the LC-MS-MS method. Matrix effects are the main concern in the method development. In this chapter, different matrices were examined and the matrix effects are discussed.

In chapter 4, the background information of the site is given. This is followed by a description of the batch experiment to find out the sorption isotherms and degradation profiles. Column studies were used for simulating the SAT conditions to find out the attenuation behavior of the target compounds during the SAT process. The whole experiment was aimed at finding out the main removal mechanism during SAT.

Chapter 5 discusses the isolation and characterization of estrogen degrading bacteria. The bacteria were isolated from the SAT system consisting of potential recharged water, aquifer sand and ground water. The estrogen degrading ability and degradation kinetics were examined. In addition, the bacteria were identified and degradation products were investigated.

Chapter six summarizes the major finding of this study and outlines some recommendations for the future work.

Chapter 2 Literature Review

In the first section of this literature review, the occurrence of EDCs in the environment and their detection methods are reviewed. This is followed by a review of the physicochemical properties of the estrogens. Last but not least, the review will focus on their fate in the environment, including sorption and degradation behavior.

2.1 Source and Occurrence of Estrogens in the Environment

2.1.1 Source of Estrogens

The principal source of natural and synthetic estrogens (Figure 2.1) in the aquatic environment is due to excretion from humans and animals. The application of estrogens can be categorised into four main groups: contraception; management of the menopausal and postmenopausal syndrome (its widest use); physiological replacement therapy in deficiency states; and, treatment of prostate and breast cancers (Kuster *et al.*, 2004). The other application of estrogens are as growth promoters (Kuster *et al.*, 2004; Ying *et al.*, 2002a) and in developing single-sex populations of fish in aquaculture (Kuster *et al.*, 2004). Estrogens are mainly excreted from humans and animals as inactive conjugates of sulphuric and glucuronic acids. However, they dominate the water environment in the unconjugated form as the conjugates are usually cleaved during the sewage treatment process (D'Ascenzo *et al.*, 2003; Desbrow *et al.*, 1998; Snyder *et al.*, 1999; Williams *et al.*, 1999; Ying *et al.*, 2002a).

2.1.2 Levels in the Environment

Estrogens have been frequently detected in sewage effluents at varying concentrations in many countries. The typical range of the estrogens is in the range of 1-10 ng/L for estradiol and 5-20 ng/L for estrone, while ethinylestradiol usually is from

below detection limits to 1 ng/L (Baronti *et al.*, 2000; Christiansen *et al.*, 2002). Among the natural estrogens, E1 is the most frequently detected, possibly due to the fast conversion of E2 to E1 while in contact with activated sludge (Ternes *et al.*, 1999a). Although E3 is excreted in higher concentration from women than other estrogens (Baronti *et al.*, 2000; Christiansen *et al.*, 2002), it is detected at relatively lower concentrations in the effluents than other estrogens, in the range of <0.1-42 ng/L (Ternes *et al.*, 1999b).

Figure 2.1 Structures of estrogens (a) estrone, (b) estradiol, (c) estriol and (d) ethinylestradiol

Some studies have reported the presence of estrogens in surface waters (Baronti *et al.*, 2000; Belfroid *et al.*, 1999; Kuch and Ballschmiter, 2001). The concentrations in surface waters downstream of sewage treatment plants (STPs) were generally much lower than in corresponding effluents. This is presumably caused by the dilution in

rivers and streams (Ternes *et al.*, 1999b). In addition, biodegradation, adsorption or photolytic activities were also effective in reducing hormone levels (Barel-Cohen *et al.*, 2006; Petrovic *et al.*, 2004).

Although the occurrences of estrogens have been frequently reported in many countries, their presence in Singapore aquatic environments has not been reported yet. Gong *et al.* (2003) examined the sex hormone disruptor activity of surface and mid-depth seawater in the Singapore coast near industrial sites based on a human cell line expressing androgen receptors (ARs), and estrogen receptors (ER α - and E β -). Their results showed that the levels of hormone activity generated by the waters of Singapore may be high enough to affect hormone signaling in marine life.

2.2 Detection Methods

The analysis of these trace organic target compounds is very challenging because the environmental matrices are very complicated as well as the physiologically active concentration and the environmental concentration of these compounds are very low (Lopez de Alda and Barcelo, 2001a; Petrovic *et al.*, 2002). Therefore, very sensitive and selective sample preparation and analytical methodologies are required for their accurate determination. In general, sample preparation includes filtration, extraction, purification and evaporation (Lopez de Alda *et al.*, 2003; Snyder *et al.*, 2003). The extraction and purification processes are usually based on the application of solid-phase extraction (SPE) and to a lesser extent solid-phase microextraction (Lopez de Alda and Barcelo, 2001a; Petrovic *et al.*, 2004; Snyder *et al.*, 2003). The analytical determination of the target compounds has been dominated by chromatographic methods (GC and LC) coupled with sensitive and specific detection systems, such as MS, MS-MS or high-resolution MS (HRMS) (Petrovic *et al.*, 2002).

2.2.1 Solid Phase Extraction

2.2.1.1 Sorbent Selection

The most frequently used concentration and purification technique is solid-phase extraction (SPE) in which many solid phases (sorbents), eluent schemes and final solvents were used (Vanderford *et al.*, 2003). Octadecyl (C₁₈)-bond silica has been the adsorbent most widely used for estrogens extraction and purification (Kelly, 2000; Lopez de Alda and Barcelo, 2001a; Lopez de Alda *et al.*, 2003; Petrovic *et al.*, 2002; Zwiener and Frimmel, 2004b). Polymeric sorbents (Brossa *et al.*, 2004; Vanderford *et al.*, 2003) and graphitized carbon black (GCB) have also been employed ((Baronti *et al.*, 2000; Laganà *et al.*, 2000; Lagana *et al.*, 2001). The GCB material is presented as a fairly selective adsorbent that most of the co-extracted impurities, such as humic acids can be removed (Lopez de Alda *et al.*, 2003). Very good detection limit (0.008 ng/L for estrone, 0.03 ng/L for ethinylestradiol, 0.02 ng/L for estradiol and 0.02 ng/L for estriol) have been reported using SPE with GCB without clean-up procedure coupled with LC-ESI-MS detection (Baronti *et al.*, 2000). A highly crosslinked polymer (LiChrolut EN) was chosen by some researchers (Brossa *et al.*, 2005; Brossa *et al.*, 2004; Lopez de Alda and Barcelo, 2000). OASIS HLB cartridge has also been used in recent years (Benijts *et al.*, 2004; Komori *et al.*, 2004; Lagana *et al.*, 2004; Vanderford *et al.*, 2003). The OASIS is able to effectively retain both hydrophilic and hydrophobic compounds with high capacity by means of both van der Waals and H-donor-H-acceptor interaction (Lagana *et al.*, 2004). They were also effective for the isolation of all the analytes with recoveries ranging from 81% to 99%, relative standard deviation (R.S.D) below 9%.

In general, the desorption of trapped analytes is performed with methanol, acetone, dichloromethane, acetonitrile and their mixtures. Ethyl acetate-methanol (5:1, v/v) has been used for eluting the estrogen analytes from C18 cartridge (waters Sep-Pak C18) (Hu *et al.*, 2005; Ingrand *et al.*, 2003), while methyl tertiary-butyl ether (MTBE) has

been applied to 500 mg hydrophilic-lipophilic balance (HLB) cartridges (waters, Oasis) for elution.

2.2.1.2 Sample Loading Volume

The advantage of percolating a higher water volume is that the limit of detection (LOD) can be lower. On the other hand, the higher the water volume preconcentrated, the higher will be the effect of the water matrix and more interference will appear in the chromatogram. In addition, Clogging problems also severely limit the amount of sample that can be applied to a SPE cartridge. Therefore, the water volume preconcentrated is always a compromise between LOD and matrix effect (Lagana *et al.*, 2004). For the surface water and ground water, the concentration factor usually is 10^3 to 10^4 while for sewage influent and effluent the concentration factor usually is only 100 to 1000. However, Baronti *et al.* (2000) loaded a 4L river water sample, 0.15 L sewage treatment influent sample and 0.4 L sewage treatment plant effluent sample for SPE and finally concentrated the samples to 200 μ L. The concentration factors reached 80000 for river water, 750 for influent and 2000 for effluent samples.

2.2.1.3 Purification

Sample purification has been performed in most of the studies for the elimination of co-eluted compounds which might disturb the final determination (Beck *et al.*, 2005; Hu *et al.*, 2005; Ingrand *et al.*, 2003; Komori *et al.*, 2004; Lagana *et al.*, 2004; Zuehlke *et al.*, 2005). This has been achieved by different methods, including washing the cartridge with solvent and water (Lagana *et al.*, 2004; Vanderford *et al.*, 2003; Zuehlke *et al.*, 2005), liquid-liquid extraction and purification with florisil cartridges purification (Hu *et al.*, 2005; Ingrand *et al.*, 2003), silica gel column (Beck *et al.*, 2005; Zuehlke *et al.*, 2005), sep-pak, NH_2 column (Beck *et al.*, 2005; Komori *et al.*, 2004), size-exclusion chromatography (Schlüsener and Bester, 2005) or their combination (Ingrand *et al.*, 2003).

2.2.2 Analytical Methods

Generally, GC-MS and GC-MS-MS have been more commonly employed for environmental analysis of these compounds. However, due to the poor volatility of estrogens, derivatization steps are required to improve the sensitivity of subsequent analysis by GC. Hence, sensitivity is often compromised due to loss of sample during the additional manipulation (Lopez de Alda and Barcelo, 2001a; Petrovic *et al.*, 2002; Vanderford *et al.*, 2003; Richardson, 2006).

Liquid chromatography-mass spectrometry (LC-MS) became more popular in the last few years due to the sensitivity, ruggedness and ease of use given by atmospheric pressure ionization (API) interfaces, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI) combined with the new generation of MS equipment (Lopez de Alda and Barcelo, 2001a; Lopez de Alda and Barcelo, 2001c; Richardson, 2006; Tichardson and Ternes, 2005).

The limit of detection (LOD) by the instruments for LC-MS, GC-MS-MS and LC-MS-MS are as follow: LC-MS (LOD 200 pg/μl) > GC-MS-MS (LOD 20 pg/μl) > LC-MS-MS (LOD 5 pg/μl) (Petrovic *et al.*, 2004). The LC with tandem mass spectrometric detection (LC-MS-MS) promises both high sensitivity and selectivity for the unambiguous identification and quantification of environmental contaminants at trace-level concentrations regardless of matrix interferences (Croley *et al.*, 2000; Zuehlke *et al.*, 2005). Therefore, LC-MS-MS is not only widespread, but also a routine method for determination of estrogens in environmental samples (Beck *et al.*, 2005; Petrovic *et al.*, 2002). Therefore, this review focuses on LC-MS-MS.

2.2.2.1 LC Separation

Prior to the MS detection, C18 columns have been commonly used for the separation of estrogens by LC (Beck *et al.*, 2005; Benijts *et al.*, 2004; Brossa *et al.*, 2004; Ingrand *et al.*, 2003; Komori *et al.*, 2004; Rodriguez-Mozaz *et al.*, 2004a). Hu *et*

al. (2005) used Capcell Pak C18 column, Xterra MS Phenyl column, and UG 120 Capcell Phenyl column.

As mobile phases, water-acetonitrile has been frequently used with gradient elution. Methanol-water has also been used as mobile phases (Beck *et al.*, 2005; Gomes *et al.*, 2005; Schlüsener and Bester, 2005). Hu *et al.* (2005) found that using methanol as an organic phase produced a higher response in comparison to using acetonitrile. Schlüsener and Bester (2005) also found that the use of pure methanol instead of pure acetonitrile as the organic phase gave ionization ratios of two to three times higher in ESI mode for the estrogens. However, Ingrand *et al.* (2003) concluded that the best overall results were achieved with water-acetonitrile eluents. Benijts *et al.* (2002) also came to the same conclusion. This difference in response may be the result of the lower viscosity of the water/acetonitrile mixture compared with that of the water/methanol mixture, which may improve the fast formation of small droplets shrinking rapidly to the size required for ion evaporation (Benijts *et al.*, 2002).

Different concentrations of ammonium acetate (Beck *et al.*, 2005; Croley *et al.*, 2000) or ammonium solution (Baronti *et al.*, 2000), formic acid (D'Ascenzo *et al.*, 2003), acetic acid (Brossa *et al.*, 2005; Brossa *et al.*, 2004), and triethylamine (TEA) (Isobe *et al.*, 2003; Johnson *et al.*, 2000) have been added in the mobile phase as modifier to improve the sensitivity. Methanolic acetate (Baronti *et al.*, 2000), triethylamine (Johnson *et al.*, 2000) and ammonium solution (Komori *et al.*, 2004) have also been added in by post-column to enhance ionization efficiency.

However, some researchers claim that the addition of buffers, such as ammonium acetate or ammonium hydroxide, and ammonium formate at varying concentrations to the mobile phase caused a decrease in the responses of the analytes due to lower ionization ratios (Schlüsener and Bester, 2005; Zuehlke *et al.*, 2005). In addition, Benijts *et al.* (2002) who studied in detail the influence of different mobile phase compositions on the ionization efficiency of an ionspray interface concluded that a

mixture of water and acetonitrile, without additional bases or buffer is the best choice for optimal ionization of estrogens. The use of mobile additives, was also investigated by Benijts *et al.* (2002) and it was found that none of these volatile bases improved the estradiol signal. The effects of mobile-phase additives on ionization efficiency were also evaluated by Lopez de Alda and Barcelo (2000). They found that modification of the acetonitrile-water mobile phase with methanol in various proportions, acetic acid (0.5%), or triethylamine (5 mM), did not significantly improve the MS signal. Schlüsener and Bester (2005) also found a decrease in the response of analytes when adding a post-column of methanolic ammonia solution. This finding is consistent with the study by Benijts *et al.* (2002) but is in contrast with those expressed by Baronti *et al.*, (2000), Johnson *et al.* (2000) and Isobe *et al.* (2003). Baronti *et al.* (2000) and Johnson *et al.* (2000) found that the post column addition of a basic agent, such as methanolic ammonia and TEA, serves to promote deprotonation of weakly acidic estrogens, thus resulting in a drastic increase in the response of the ESI-MS system. Isobe *et al.* (2003) also found that TEA resulted in an increase in the sensitivity of LC-ESI(-)-MS-MS although they used the same model of mass spectrometer (API 2000) as Schlüsener and Bester (2005).

2.2.2.2 Mass Spectrometry

Triple-quadrupole mass spectrometers are most widely used for sensitive and selective quantification of target compounds that show specific mass transitions in multiple reaction monitoring mode (MRM) (Ingerslev *et al.*, 2003; Zwiener and Frimmel, 2004a). In general, MRM operation is performed at fixed mass to charge ratio (m/z) values of the quadrupoles (Q)1 and 3, whereas quadrupole 2 (Q2) serves as the collision cell. Therefore, the instrument monitors not only the precursor ion but also the fragmented product ions. Hence, far better limits of detection can be achieved compared to the single-quadrupole LC-MS. In this study, the detection of estrogens was carried out using LC-MS-MS. Therefore, this review shall focus on the LC-MS and LC-MS-MS methods.

Electrospray (ESI) and atmospheric-pressure chemical ionization (APCI) are the HPLC-MS interfaces currently most widely used in the environmental analysis of organic pollutants (Lopez de Alda and Barcelo, 2001a). ESI in negative ion mode has been widely used for the determination of estrogens in water samples because of its observed better sensitivity compared to the APCI interface (Brossa *et al.*, 2004; Gomes *et al.*, 2003; Ingrand *et al.*, 2003; Lagana *et al.*, 2004; Lopez de Alda and Barcelo, 2001a). However, Laganà *et al.* (2000) have reported the use of LC/APCI(PI)-MS/MS for analysis of estrogens in water with sensitivities comparable to those achieved in many cases by LC/ESI(NI)-MS/MS where the detection limit of 0.5 ng/L for E2 and EE2, and 1 ng/L for E1 and E3 was reached. Schlüsener and Bester (2005) compared the analysis of steroid hormones by using APCI and ESI. They found that APCI has an advantage on reduction of matrix effects. Therefore, the APCI mode should be preferred to ESI even though less sensitivity is obtained in standard solutions. On the other hand, many researchers have reported that ESI in negative mode provides better overall sensitivity for steroids of interest than APCI (Baronti *et al.*, 2000; Ingrand *et al.*, 2003; Lagana *et al.*, 2004; Lopez de Alda and Barcelo, 2000). For example, Lopez de Alda and Barcelo (2000) have reported that in term of sensitivity, the ESI interface has been shown to provide limits of detection about one order of magnitude better than those achieved with the APCI interface. Ingrand *et al.* (2003) also concluded that the sensitivities in APCI⁺ and ESI⁻ were in the same range, but the S/N ratios were greater with ESI⁻, leading to a lower LOD.

The base peak selected for quantitation of estrogens in the selected-ion monitoring (SIM) mode, or as precursor for collisionally induced dissociation (CID) in MRM mode corresponds to the $[M+H-H_2O]^+$ ion for E2, E3, EE2, and $[M+H]^+$ for E1 when using the APCI interface in positive mode (Laganà *et al.*, 2000; Lopez de Alda and Barcelo, 2001c). Figure 2.2 shows MS/MS spectra and hypothetical chemical structures of precursor and product ions used in the transitions selected for the analysis.

The $[M-H]^-$ was observed as the base peak in the mass spectrum of estrogens for ESI in negative mode, regardless of the instrument used. The CID spectrum for estradiol showed losses consistent with ring cleavage (i. e. losses of $C_5H_{12}O$, $C_8H_{14}O$ and $C_8H_{16}O$) to give major product ions at m/z 183, 145, and 143 respectively. The major product ions for $[M-H]^-$ ion from estriol (m/z 287) are m/z 171, 145, and 143 upon excitation. These ions relate to losses of $C_6H_{12}O_2$ and $C_8H_{14}O_2$ and $C_8H_{16}O_2$ respectively, from the estroidal ring system. The $[M-H]^-$ ion from estrone at m/z 269 gives major product ions at m/z 145 and 143 (loss of $C_8H_{12}O$ and $C_8H_{14}O$) (Croley *et al.*, 2000; Lopez de Alda *et al.*, 2003; Petrovic *et al.*, 2002) and the $[M-H]^-$ ion from EE2 gives five major fragment ions at m/z 199, 183, 159, 145, 143 (Figure 2.3) (Croley *et al.*, 2000; Díaz-Cruz *et al.*, 2003; Petrovic *et al.*, 2002). The observation of m/z 145 in daughter ion mass spectrum for each of the four estrogens is consistent with the stability of the phenol ring in the system. Thus it can serve as an indicator for the target estrogens (Croley *et al.*, 2000).

In the analyses of estrogens by LC-ESI-MS/MS, usually two different MRM transitions should be monitored for each compound: the first one is the more abundant for quantification, and the second one for confirmation. When using LC-MS-MS for the identification of an analyte in aqueous samples, the following criteria should be emphasized (derived from guidelines in recent literatures) (Laganà *et al.*, 2000; Lopez de Alda and Barcelo, 2001a; Petrovic *et al.*, 2002; Rodriguez-Mozaz *et al.*, 2004b; Zwiener and Frimmel, 2004b):

1. LC retention times of the analytes should be within 2% of the retention time for a reference standard of the same compound analyzed under the same conditions.
2. The ion abundance ratios, relative to the most abundant ion, are within $\pm 20\%$ of the ion ratios obtained for the standards.

Figure 2.2 MS/MS spectra and hypothetical structures of precursors and products ions by using APCI positive ion mode for the analysis of estrogens (Langanà et al., 2000)

Figure 2.3 Negative ion ESI-MS/MS spectra of the analytes at a concentration of 5 pg/ μ l and their fragmentation pathway (insets). (a) E2 at 45V CID, (b) E3 at 45 V CID and (d) EE2 at 40 V DIC (Croley *et al.*, 2000)

2.2.2.3 Matrix Effect

The major problem when using quantitative HPLC/MS/MS is the unexpected matrix effects. The matrix effect defined as the effect of co-eluting residual matrix components on the ionization of the target analyte, typically results in either signal suppression or enhancement (Benijts *et al.*, 2004). Moreover, interfering matrix components can affect the reproducibility and accuracy of the developed procedure, leading to compromising or erroneous results (Beck *et al.*, 2005; Benijts *et al.*, 2004; King *et al.*, 2000). The mechanism of the matrix effect in LC-MS is still not fully resolved. It is assumed that solid analyte precipitation or co-precipitation with other non-volatile matrix components causes gas phase ion suppression in APCI (Benijts *et al.*, 2004; King *et al.*, 2000). However, in ESI, competition between matrix components and analytes for access to the droplet surface for gas phase emission has been suggested as a possible cause of matrix effects (Benijts *et al.*, 2004). King *et al.* (2000) concluded that it is most likely that ionization suppression is the result of high concentrations of nonvolatile materials present in the spray with the analytes. The nonvolatile solute causing ionization suppression can be any chemical structure. Salts such as sulfates and phosphates are well known to cause ionization suppression. Any mechanism that might decrease the production rate of small droplets, and ultimately gas phase analyte ions, could participate in ionization suppression (King *et al.*, 2000). The analysis of estrogens run in ESI negative mode showed much more matrix effect than that ran in APCI mode (Schlüsener and Bester, 2005; Vanderford *et al.*, 2003). Schlüsener and Bester (2005) have reported that the matrix effects in APCI are 3-10 times less pronounced than that in ESI mode.

Different strategies are established to compensate these matrix effects which include (Schlüsener and Bester, 2005):

1. Matrix calibration: This is an internal standard calibration in the presence of uncontaminated matrix to avoid matrix effects. This matrix is produced by

the same sample preparation which is used for the analysis of the samples.

This matrix is added to the calibration standards.

2. Standard addition: The sample is divided into several sub-samples. As a result, a calibration curve is generated for each sample.
3. Isotope dilution: Quantification with isotopically labeled internal standards. These standards have the same chemical nature, and co-elute with the respective analyte and the matrix. Thus same effect occurs to the internal standard as well as to the analyte.

These three options help to compensate for the matrix effects, but none of these options reduce these effects. A more fundamental approach would be to remove the disturbing matrix components prior to the ionization process (Reemtsma, 2001; Reemtsma, 2003). Two strategies are available:

1. Improved sample clean-up. This can be achieved by using a more selective sorbent or elution procedure in SPE. Alternatively, the clean-up procedure in SPE can also help in removing the matrix (Reemtsma, 2001).
2. Improved chromatography. Interfering matrix components may be separated from a target analyte by improving the chromatographic separation. However, this may not be possible if a complex matrix is present and a variety of target analytes with differing physico-chemical properties has to be quantified (Reemtsma, 2001).

2.3 Physicochemical Properties

The physicochemical properties of compounds affect their distribution and fate in the environment. These properties include the chemical structure, water solubility, vapor pressure and water partitioning coefficient (Miyamoto and Klein, 1998).

The structure of estrogens (E1, E2, E3, and EE2) is shown in Figure 2.1. All of them contain an aromatic A-ring as a distinctive part of their teracyclic molecular frame work and all of them possess hydroxyl groups at the C-3 position (Figure 2.3) (Hanselman *et al.*, 2003). Key structural differences arise in the D-ring structure owing to the type and stereochemical arrangement of functional groups at the C-16 and C-17 positions. Estrone (E1) has a carbonyl group at C17, which exhibits electrostatic potential. Therefore, estrone is a good electron acceptor but poor electron donor, i.e. has oxidant characteristics (Schafer *et al.*, 2003). 17 β -Estradiol (E2) has a hydroxyl group at C-17 position that projects upward from the molecule (β configuration). Estriol (E3) features hydroxyl groups at both possess the C-16 and C-17 position and thus, has four epimers (Hanselman *et al.*, 2003). Ethinylestradiol process both hydroxyl group and ethinyl group in C-17 position.

The physicochemical properties are summarized in Table 2.1. As shown, these compounds have very low water solubility, especially for the synthetic estrogens (EE2). They have very low vapor pressures, ranging form 2.3×10^{-10} to 6.7×10^{-15} , indicating low volatility of these compounds. They are weak acids with pKa value around 10. This indicates that they will dissociate under very basic conditions (pH>8.7) forming an organic anion (Lee *et al.*, 2003). The logK_{ow} for estrogens ranges from 2.81 to 4.15, which indicates that their behavior is similar to that of moderately hydrophobic contaminants (Sedlak *et al.*, 2000).

Table 2.1 Physicochemical properties of estrogens

Compounds	Chemical name	Formula (MW) ^e	Water Solubility (mg/l)	Vapour pressure (mm Hg) ^a	pKa ^f	Log K _{ow} ^a
Estrone (E1)	3-hydroxyestra-1,3,5(10) - trien-17-one	C ₁₈ H ₂₂ O ₂ (270.37)	13 ^a , 30 ^b	2.3*10 ⁻¹⁰	10.5-10.7	3.43 ^{c,a}
17β-Estradiol (E2)	1,3,5(10)-estratriene-,17β-diol	C ₁₈ H ₂₄ O ₂ (272.3)	13 ^a	2.3*10 ⁻¹⁰	10.3-10.8	3.94 ^{e,a} , 4.4 ^d
Estriol (E3)	1,3,5(10)-estratriene-3,16α,17β-triol	C ₁₈ H ₂₄ O ₃ (288.39)	13 ^a , 32 ^c	6.7*10 ⁻¹⁵	10.4	2.81 ^{e,a}
17α-ethynyl-estradiol (EE2)	(17α)-19-norpregna-1,3,5-(10)trine-20-yne-3,17-diol	C ₂₀ H ₂₄ O ₂ (296.4)	4.8 ^a	7.5*10 ⁻¹⁰	10.21 ^g	4.15 ^{e,a}

MW : molecular weight. K_{ow}: octanol-water partition coefficient

Source: ^a (Lai *et al.*, 2000); ^b (Schafer *et al.*, 2003); ^c (Lee *et al.*, 2003) solubility from a control tablet comprising estriol and α-cyclodextrin.; ^d (Sedlak *et al.*, 2000), ^e (Lai *et al.*, 2002a). ^f (Hanselman *et al.*, 2003); ^g (Yu *et al.*, 2004).

2.4 Fate in the Environment

EDCs enter the aquatic environment via the effluents from sewage treatment and wastewater treatment plants. Their fates in the environment are determined by various processes and factors such as sorption, degradation, evaporation etc. Among these processes, sorption and degradation (including biodegradation and abiotic transformation) are of great importance. Therefore, they are the main focus in this study.

2.4.1 Sorption

Sorption will significantly affect the persistence of the compounds in the environment as sorption to the solid phase (sediment, soil etc), will reduce the degradation rate of the compounds. This is because that the chemicals are easier to be attacked by bacteria when they are dissolved in the water phase(Andersen *et al.*, 2005).

Therefore, the stronger a compound adsorbs to the solid phase the longer it will persist in the environment (Bowman *et al.*, 2003).

The distribution and partitioning of estrogenic compounds in the environment are the result of physical, chemical and electrostatic interactions between the solid surface and the compounds (Carlson, 1999; Li and Lee, 1999; Ying *et al.*, 2002a). The mechanisms to sorption is dependent on their chemical properties (water solubility, charge distribution, polarity, and molecular size) and the available soil domains (cation-exchange sites and soil organic matter) (Carlson, 1999; Li and Lee, 1999).

The pK_a value of estrogens indicates that these compounds are usually uncharged in the pH range of most natural waters and wastewaters (Nghiem *et al.*, 2002). Therefore, hydrophobic partitioning could be the dominate sorption mechanism. According to physicochemical properties, the synthetic estrogen, EE2, with a higher K_{ow} than that of natural estrogens, is more readily removed from the water phase than natural estrogens. Among the four steroids, E3 should have the weakest sorption to the solid phase.

In addition to physicochemical properties, different environmental conditions also affect the behavior of estrogens sorption. Some studies show that estrogens have high correlation between sorption and organic carbon, where the sorption of estrogens increased with increasing TOC content of sediment, albeit the presence of organic carbon was not a prerequisite for the sorption (Casey *et al.*, 2003; Lai *et al.*, 2000; Lee *et al.*, 2003; Ying *et al.*, 2003; Ying *et al.*, 2002a). These phenomena are consistent with hydrophobic theory. Casey *et al.* (2003) reported a high correlation between E2 sorption and soil surface area and cation exchange capacity. Lai *et al.* (2000) found that the amount of estrogens sorbed is influenced by sorbent binding site availability and competition for binding sites. The estrogens will compete for sorption sites, both between estrogens and with other hydrophobic chemicals. Yu *et al.* (2004) found that the sorption affinities of these chemicals were dependent upon specific sorbent-sorbate systems and the concentration. At high equilibrium concentration (C_e) ($=0.5 S_w$, where

S_w is the solubility limit), E1, E2 and EE2 have very similar $\log K_{oc}$ (where K_{oc} is the organic matter partition coefficient) values (i.e. 3.14 – 3.49). As C_e Values decreased the $\log K_{oc}$ values increased. This is because sorbing molecules at lower concentration ranges may preferentially interact with sorption sites having greater affinities or site energies, exhibiting greater sorption capacities.

Bowman *et al.* (2002) concluded that solids concentration (SC) had a major influence on the sorption of E1 and E2 which increased with an increase in SC. This is the so-called colloidal effect. The true partition coefficients on colloids are two orders of magnitude greater than those on sediment particles. Surfactants were also found to enhance the solubility of these compounds. The effect of salinity also has been investigated in the study by Bowman *et al.* (2002) where an increase in partition coefficient (K_p) for estrone was observed with increased salinity. This can be explained by the decrease in aqueous solubility due to the presence of salts, resulting in the compound being more attracted to particles. Lai *et al.* (2000) also found similar results for the relationship between salinity and sorption of estrogens.

The sorption behavior of estrogens during activated sludge treatment, in soil and sediment has been widely studied. Andersen *et al.* (2005) concluded that for a typical sewage treatment plant (STP), the removal of steroid estrogens with excess sludge was estimated to be only 1.5-1.8% of the total loading if an equilibrium condition exists. Sorption is therefore not important for the fate of steroid estrogens in STPs compared to biodegradation. Sorption of steroid estrogens to sediments was evaluated in several recent studies (Bowman *et al.*, 2002; Casey *et al.*, 2003; Lai *et al.*, 2000; Yu *et al.*, 2004) and the results appear to be contradictory. Lai *et al.* (2000) reported an initial rapid sorption (4.0-9.4 $\mu\text{g/g/h}$) between 0 and 0.5 hour, and attained apparent equilibrium within 1 hour for the sorption of six steroid estrogens to estuary sediments. The Freundlich isotherm for each estrogen was nonlinear with sorption coefficients $\log K_f$. Holthaus *et al.* (2002) studied the potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. They found that 80 to 90% of

binding to bed sediments was complete within 1 day, but that an equilibrium had not been reached after 2 days. The Freundlich isotherm was a linear isotherm in this study. Yu *et al.* (2003) reported sorption of E1, E2 and EE2 by seven soil and sediment samples at both equilibrium and rate-limiting conditions. Their study suggests that, at sub-micrograms per liter levels, these estrogenic chemicals may exhibit even slower rates and greater capacities of sorption by soils and sediments than those at their experimental concentration. The sorption of E2 and EE2 in an aquifer material with low organic carbon content (0.5%) was studied by Ying *et al.* (2003). The sorption was nonlinear with sorption exponents, $1/n$, of 0.40 for E2 and 24.2 for EE2. Most of these studies revealed that estrogens are moderately mobile. Therefore, in comparison to other more hydrophobic compounds, these compounds would be expected to remain mainly in the aqueous phase and have a strong tendency for bioaccumulation (Baronti *et al.*, 2000; Bowman *et al.*, 2002; Ying and Kookana, 2005; Ying *et al.*, 2003).

2.4.2 Degradation

2.4.2.1 Degradation in the Environment

Biodegradation has been reported to play a major role in the removal of these compounds from aquatic environments. Table 2.2 summaries some of the studies on the biodegradation of estrogens in different environments. The rapid degradation of natural estrogens under aerobic conditions were found in activated sludge (Lee and Liu, 2002; Ternes *et al.*, 1999a), river water (Jurgens *et al.*, 2002), marine sediment (Colucci *et al.*, 2001; Ying and Kookana, 2003), agriculture soil (Ying and Kookana, 2005) and aquifer material (Ying *et al.*, 2003). Based on first order kinetics, the half-lives of estradiol and estrone ranged from 0.2 to 9 days and from 0.1 to 11 days respectively in river water (Jurgens *et al.*, 2002), 2 days in soil for estradiol (Ying and Kookana, 2005), 4.4 days in marine sediment (Ying and Kookana, 2003) and 2 days in aquifer material (Ying *et al.*, 2003). Shi *et al.* (2004a) studied the biodegradation of estrogens using activated sludge and night soil composting microorganism. Their result showed that among the three natural estrogens, E2 was found to be the most easily degraded. Lucas

and Jones (2006) studied the biodegradation of 17 β -estradiol in grassland soils amended with animal wastes. The results indicated that the rate of hormone mineralization was strongly influenced by both soil type and matrix in which the hormone was added to the soil. A 2-10 days lag phase was observed in this study for the degradation of E2 involving sheep urine. It was hypothesized that this lag phase occurs as a result of the time taken for the soil microbial community to adapt to the urine. This adaptation may be due to metabolic changes caused by the alteration in chemical environment or induction of enzymes and membrane transporters required for hormone biodegradation (Lucas and Jones, 2006).

Table 2.2 Summaries of literature on estrogen degradation in different environments.

Material	Target compounds	Degradation behavior	references
Activated sludge	E2 and its metabolites	E2 and its metabolites were not persistent and could be degraded by sewage bacteria under both aerobic and anaerobic conditions.	Lee and Liu, 2002
Activated sludge	E2, EE2 and 17 β -estradiol-17-glucuronide	E2 was oxidized to estrone and further eliminated in the batch experiments. Estrone were not further eliminated. Little or no EE2 transformation over 20h.	Ternes et al., 1999a
River water	E1 and E2	Half-lives of estradiol and estrone ranged from 0.2 to 9days and 0.1 to 11 days respectively. EE2 degradation was much slower than that of E2 under the same incubation conditions.	Jurgens et al., 2002
Marine sediment	EE2	<ul style="list-style-type: none"> • EE2 could be readily biodegraded in agricultural soil. The degradation pathways involced the transformation of EE2 by hydroxylases in the aerated soils.	Colucci et al., 2001
Marine sediment	E2 and EE2	<ul style="list-style-type: none"> • Half-lives in marine sediment for estradiol is 4.4 days. • Degradation of natural estrones in anaerobic conditions appeared to much slower than that under aerobic 	Ying and Kookana 2003

		conditions. EE2 degradation half-lives are >20 days.	
Agriculture soil	E1, E2, E3, EE2	<ul style="list-style-type: none"> • Half-lives in soil for estradiol is 2 days. • Half life of of E2 degradation in anaerobic soils was 24 days. • EE2 degradation in soil is comparable to that of E2 under the same conditions with half-life of 4.5 days. 	Ying and Kookana, 2005
Aquifer material	E2 and EE2	<p>Half-lives in aquifer material is 2 days under aerobic conditions.</p> <p>Estimated half-life of E2 under anaerobic conditions is 107 days.</p> <p>EE2 degradation concentration decreased from 1 to 0.62 µg/g in aquifer material within 70 days.</p>	Ying et al., 2003
Activated sludge and night soil composting microorganism.	E1, E2, E3	First-order kinetics with half-lives of 2.2 days for E1, 0.6 days for E2, 1.5 days for E3.	Shi et al., 2004
Grassland soils amended with animal wastes	E2 and EE2	<ul style="list-style-type: none"> • The rate of hormone mineralization was strongly influenced by both soil type and matrix . • Anaerobic degradation half-life of the hormones (E1 and E2) in soils ranged from 5 to 25 days. • A 2-10 days lag phase was observed for the degradation of E2 in urine. • EE2 were not degradable under anaerobic conditions. 	Lucas and Jones, 2006
Aquifer material supplemented with sewage effluent	E2 and EE2	<ul style="list-style-type: none"> • E2 showed rapid biodegradation with DT50 (time for 50% loss) of ~2 days under aerobic conditions. • E2 degraded slowly in both aquifer media under anaerobic conditions. • EE2 was found to be resistant to biodegradation and remained almost nchanged within 70 days under anaerobic conditions. 	Ying et al., 2004

The degradation of natural estrogens in anaerobic conditions appeared to be much slower than that under aerobic conditions. Approximately 50% E2 was degraded after 70 days anaerobic incubation in marine sediments (Ying and Kookana, 2003). Ying and Kookana (2005) also found slow E2 degradation in anaerobic soils with an estimated half-life of 24 days. In aquifer material, only 40% of E2 was lost over 70 days with an estimated half-life of 107 days (Ying *et al.*, 2003). In activated sludge, E2 was more persistent in anaerobic conditions than under aerobic conditions but still biodegradable. After 7 days incubation, about 50% of the spiked E2 was removed. Czajka and Londry (2006) studied the anaerobic biotransformation of estrogens using lake water and sediments under methanogenic, sulfate-, iron-, and nitrate-reducing conditions. E2 was found to be transformed to E1 under all four anaerobic conditions. The initial 14 day loss was fastest with iron followed by sulfate, carbon dioxide and slowest with nitrate. However, the loss of E2 was accompanied by an accumulation of E1. Upon further incubation under iron-reducing conditions, the E2 concentration actually increased and then decreased again as it was re-oxidized back to E1. Except for E2, there have been no reports on the degradation of the other two natural estrogens (E3 and E1) under anaerobic conditions.

In contrast with natural estrogens, EE2 appeared to be much more resistant to biodegradation under aerobic conditions. Czajka and Londry (2006) explained that the ethynyl group in the 17-position of the synthetic EE2 blocks the potential formation of ketone (as observed for the natural estrogen E2) and sterically hinders access to the hydroxyl group in the 17-position, making this compound more recalcitrant than its natural counterpart. Ternes *et al.* (1999a) witnessed little or no EE2 transformation over 20 h, using an activated sludge batch test system. Ying *et al.* (2003) reported that EE2 concentration decreased from 1 to 0.62 µg/g in aquifer material within 70 days. EE2 degradation in marine sediments was also much slower than that of E2, with half-lives of >20 days (Ying and Kookana, 2003). In English river water, the aerobic biodegradation of EE2 was much slower than that of E2 under the same incubation conditions (Jurgens *et al.*, 2002).

However, Colucci *et al.* (2001) reported that EE2 could be readily biodegraded in agricultural soils. The removal in aerobic nonsterile soil was initiated without a lag phase and the dissipation kinetics were generally first order indicating that microbial transformation did not require an adaptive phase and that there was no proliferation of the biodegrading organisms. The speculated degradation pathways involved the transformation of EE2 by hydroxylases in the aerated soils (Colucci *et al.*, 2001). Vader *et al.* (2000) also found good EE2 removal with nitrifying activated sludge. In addition, Ying and Kookana (2005) reported EE2 degradation in soil with half-lives of 4.5 days, which is comparable to E2 degradation under the same condition. On the other hand, no EE2 degradation has been reported under anaerobic conditions.

2.4.2.2 Estrogen Degrading Microorganisms

As biodegradation plays an important role in the removal of estrogens, attention has recently focused on the isolation of estrogen degrading microorganisms from environmental sample. The first E2 degrading bacteria (*novosphingobium tardaugens* sp. nov.) was isolated from activated sludge (Fujii *et al.*, 2002; Fujii *et al.*, 2003). This microorganism is a gram-negative, oval-shaped bacterium. The strain was found to degrade E1, E2 and E3, but not EE2. About 40% of E1 was degraded in 20 days, while E3 was degraded almost completely within 10 days. E2 was degraded by this strain to compounds with very low molecular mass (for instance, CO₂) or simple organic acid and no toxic or accumulative metabolites of E2 were produced from the degradation pathway (Fujii *et al.*, 2003).

Although EE2 was found to be more recalcitrant in the environment than the natural estrogens, an EE2 degrading microorganism has been isolated from cowshed samples by Shi *et al.* (2002). *Fusarium proliferatum* strain is a fungus which removed 97% of EE2 at an initial concentration of 25 mg/l, with a first order rate constant of 0.6 d⁻¹. Unknown products of EE2 degradation, which may be more polar compounds that

have a phenolic group, remained in the culture medium. In addition, the highest EE2 biodegradation rate was obtained at pH 7.2 (Shi *et al.*, 2002).

Yoshimoto *et al.* (2004) isolated four strains of *Rhodococcus* which have the ability to degrade four estrogens (E1, E2, E3 and EE2). *R. zopfii* Y5018 and *R. equi* Y 50155, Y 50156 and Y 50157 were isolated from the activated sludge of wastewater treatment plants. These four strains began degrading E2, E1, E3 and EE2 (100 mg/l) immediately after starting culture. E2 and E1 were degraded completely in 24 hours, and E3 and EE2 were degraded by about 80% of their concentrations in 24 hours (Yoshimoto *et al.*, 2004). *R. zopfii* Y50158 showed particularly strong degrading activities with all four estrogens completely degraded in 24 hours. Strain Y5018 selectively degraded E2 during jar fermentation, even when glucose was used as a readily utilizable carbon source in the culture medium. The experiment also suggested that these strains degrade E2 into substances without estrogenic activity.

2.4.2.3 Degradation Mechanisms

The biodegradation of E2 appeared to initiate at the hydroxyl group at C-17 (ring D) of the molecule, leading to the formation of the major metabolite, E1. The formation of E1 in the degradation of E2 was found in many studies under aerobic conditions (Jurgens *et al.*, 2002; Lee and Liu, 2002; Lucas and Jones, 2006; Shi *et al.*, 2004b; Ternes *et al.*, 1999a; Ying and Kookana, 2003; Ying and Kookana, 2005), anaerobic conditions (Czajka and Londry, 2006; Lee and Liu, 2002; Ying and Kookana, 2005) and even in autoclaved soil samples (Colucci *et al.* (2001a). E2 was found to be finally mineralized to CO₂ (Jurgens *et al.*, 2002).

Lee and Liu (2002) studied the degradation of E2 and its metabolites by sewage bacteria and reported several E2 metabolites (Figure 2.4). During the very early stage (1-5 hours) of E2 degradation by the culture, a metabolite X1 was observed. This

compound is a labile metabolite and was identified as a lactone produced from the oxidation of E1(Lee and Liu, 2002).

Lactones are easily formed by elimination of water molecules from γ -hydroxy acids, It was further speculated that the precursor of X1 was an unstable hydroxyl acid produced by the further oxidation of E1 and the cleavage of ring D. The metabolic pathway of E2 was proposed by Lee and Liu (2002) (Figure 2.5).

Figure 2.4 Chemical structure of E2 and some metabolites.

Coombe *et al.* (1966) investigated the degradation mechanisms of estrone by *Nocardia sp.* isolated from soil. In this study, three degradative products were formed and characterized as 3ad-H-4a-[3'-propanoic acid]Q-[Z-ketopropyl]-7a/3-methyl-1-indanone (Ia), 3ao(-H-4a-[3'-propanoic acid]-5P-[4'-but-3-enoic acid]-7a/3-methyl-1-indanone(IIa), and Z-carboxy-7afl-methyl-7-keto-9aoc-H-indano-[5,4f]-5aa, 10, 10a,B,

11-estrahydroquinoline (IIIa). The presented mechanism (Figure 2.6) indicated that the degradation of the steroid skeleton appears to involve the cleavage of the A ring prior to the B ring. The cleavage of the A ring was also found in the degradation of estradiol in English rivers where radiolabeled E2 at the 4th position of the A ring was used (Jurgens *et al.*, 2002).

Figure 2.5 Proposed metabolic pathway of E2 by sewage bacteria (Lee and Liu, 2002).

Lai *et al.* (2002b) studied the biotransformation and bioconcentration of steroid estrogens by the algae *Chlorella vulgaris*, using batch-shaking experiments with incubation for 48 h in the light or dark. Estradiol and estrone were inter-convertible in both light and dark conditions. However, this biotransformation showed a preference for estrone. In the light, 50% estradiol was further metabolized to an unknown product.

Monooxygenases can carry out reactions by inserting oxygen into C-H bonds. The activity of nitrifying activated sludge probably results in hydroxylation, converting EE2 into hydrophilic products. These hydroxylated degradation products have no estrogenic activity. The EE2 degradation by nitrifying microorganisms is a cometabolic process (Vader *et al.*, 2000).

As mentioned earlier, Czajka and Londry (2006) studied the anaerobic biotransformation of estrogens. In this study, E2 was transformed to E1 under four anaerobic conditions. The oxidation of E2 to E1 was not inhibited by E1. Under some conditions, reversible inter-conversion of E2 and E1 was observed, and the final steady state concentration of E2 depended on the electron-accepting conditions present but was independent on the total amount of estrogens added. E3 was found as a metabolite accumulating in the system; however, this could not be confirmed. One metabolite was conclusively identified as 17 α -estradiol. The authors concluded that under all the anaerobic conditions tested, the observed transformations of E2 would only partially reduce the estrogenicity of the water, as E2 was mostly converted to estrone or to 17 α -estradiol.

2.5 Summary of Literature Review

Estrogens are regarded as one of the most important groups of endocrine disrupting chemicals due to their extremely low effective concentration and high estrogenic activity. They enter the aquatic environment mainly via sewage treatment plants. These compounds have been widely detected in sewage effluent, surface waters, and ground waters at ng/l levels.

Due to the complexity of environmental matrices and typically extremely low concentrations of the estrogens, the analysis of these compounds is very challenging. Thus, very sensitive and selective sample preparation and analytical methodologies are required for their accurate determination. In order to reach very low detection limits, solid-phase extraction (SPE) has been widely used for concentrating and purifying the

sample. In addition, LC-MS and LC-MS-MS have become more popular in the last few years for the detection of these compounds. However, the matrix effect is still a major problem encountered in the detection of these compounds.

With respect to the fate of these compounds in the environment, many studies have been carried out on sewage treatment plant processes, surface water and sediments. Some studies have been done on aquifer materials (Ying *et al.*, 2003), and the soil aquifer treatment (SAT) process (Mansell and Drewes, 2004). Biodegradation has been reported as the main mechanism for the removal of these compounds. However, their fate in the environment is still not totally understood. In addition, the fate of the target compounds is dependent on specific environmental conditions. In this study, the fate of the target compounds will be explored in a tropical aquifer.

E2 was found to be the most rapidly degradable compound among the four target estrogens. Aerobic conditions have been reported as a favorable condition for the degradation of these compounds. E1 is the key degradation intermediate in the degradation of E2 and was found in many studies under both aerobic and anaerobic conditions. However, further degradation of E1 is still unknown. E2 was also found to be finally mineralized to CO₂. In contrast with E2, EE2 was found to be more recalcitrant in the environment. Most of the studies found that EE2 degraded much more slowly than natural estrogen under aerobic conditions, while no reports have been found for EE2 degradation under anaerobic conditions. In the case of E3, little work has been carried out and information on the degradation of this compound is still lacking.

Chapter 3 Method Development

3.1 Introduction

A sensitive and reliable method is the prerequisite for studying the fate of trace contaminants in the environment. High performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (LC-MS-MS) in conjunction with a single step solid phase extraction pretreatment method was developed in this study. The method was applied to different water matrices to improve its performance. The matrix effect was the major problem encountered in this study. Lower recoveries of target compounds have been reported in the presence of natural waters (Bonfiglio, 1999; Ikononou, 1991). The presence of a matrix which results from co-eluting residual non-volatile matrix components non-volatile can affect the ionization efficiency of target analytes and lead to erroneous results (Vanderford, 2003). It can also affect the reproducibility and accuracy of the developed method while compromising the sensitivity of the method when different matrices are present. Hence, evaluation of the matrix effect is also a main task in this study and is discussed in this chapter.

3.2 Introduction to LC-MS-MS

The LC-MS-MS system used in this study is a high performance liquid chromatography (HPLC) (Shimadzu, Singapore) system coupled to API 3000 tandem mass spectrometry (AB Applied Biosystems, Singapore) (Figure 3.1).

The HPLC system consists of two pumps (LC-10ADVP), a degasser (DGU-14AM), a column with column oven (C70-10ASVP) and an autosampler (SIL-10ADVP). The inorganic mobile phase (A) and organic mobile phase (B) are distributed by pump A and pump B respectively. Mobile phases pass through the degasser for removing air bubbles and are then mixed with samples, which are introduced by the autosampler. The mixture passes through the column and different analytes are separated. The separation involving a mobile phase of constant composition (irrespective of the

number of components it contains) is termed isocratic elution, while that in which the composition of the mobile phase is changed by time is termed gradient elution (Adrey, 2003). The mobile phase contents and compositions need to be chosen for providing adequate separation.

Figure 3.1 Schematic of the LC-MS-MS system (API 3000)

The MS system used in the study is API 3000 triple quadrupole mass spectrometry. It can be connected to an electrospray ionization (ESI) or an atmospheric-pressure chemical ionization (APCI) ion source. In ESI, the eluate from LC system is passed through a capillary at atmospheric pressure, which is maintained at high voltage. The liquid stream breaks up with the formation of highly charged droplets which are desolvated as they pass through the atmospheric-pressure region of the source towards a counter electrode. Desolvation is assisted by a stream of nitrogen gas (curtain gas), being continually passed into the spraying region. The analyte ions are obtained from

these droplets. In positive mode, the analytes are protonated and form protonated molecular ions ($[M+H]^+$), while in negative mode, deprotonation occurs and deprotonated ($[M-H]^-$) ions form. The electrospray interface enables mass spectra to be obtained from highly polar and ionic compounds (Adrey, 2003; Benijts *et al.*, 2002).

APCI shares many common features with ESI. The differences between the techniques are the methods used for droplet generation and the mechanism of subsequent ion formation. The analytes in solution from HPLC eluate is introduced into a pneumatic nebulizer and converted to a thick fog. The droplets are displaced by gas flow through a heater which vaporizes the mobile phase and the sample. After desolvation, they are carried along a corona discharge where ionization occurs. the primary ions (N_2^+ and O_2^+) interact with vaporized solvent molecules and form solvent ions. The solvent ions then ionize the analyte by proton transfer. Under the positive mode, the ions are formed by protonation, while under the negative mode the ions are formed by deprotonation. The sentence was rewrote in the current version.

Figure 3.2 Triple quadrupole system

The triple quadrupole system consists of three sets of quadrupole rods in series (Figure 3.2). Only monitored precursor ions from the ion source can pass through the first set of rods (Q1). The second set of rods (Q2) act as a collision cell, where fragmentation of ions transmitted by the first set of quadrupole rods is carried out by collision-induced dissociation (CID) gas, and as a device for focusing any product ions into the third set of quadrupole rods. The third quadrupole rods (Q3) allow the transmission of product ions (produced in Q2) with certain m/z ratio.

3.3 Materials and Methods

3.3.1 Chemical and Reagents

All chemicals used for experiments were of analytical grade or better. Estrone (E1) (min. 97%) were purchased from Fluka Chemika (Steinheim, Germany) while 17β -estradiol (E2) (min. 97%) was purchased from Lancaster Synthesis (Morecambe, England). Estriol (E3) (min. 98%) was purchased from Aldrich Chem, Co. (WI, USA), 17α -ethynylestradiol (EE2) (min. 98%) from Sigma (Steinheim, Germany). Internal standards, i.e. isotopically labeled estrone (E1-d4) (min. 98% D), was obtained from CDN Isotopes (Quebec, Canada). Sodium sulfate (anhydrous GR for analysis), ammonia solution (25%) and ammonium acetate (ACS, GR for analysis), together with the solvents, methylene chloride, acetonitrile and methanol (all HPLC grade) were all obtained from Merck kGaA (Darmstadt, Germany). Sulphuric acid (min. 98%) was purchased from LAB-SCAN Anal. Sci. (Bangkok, Thailand) while formic acid (min. 90%) was purchased from APS Chemicals Limited (NSW, Australia). Sodium thiosulphate was purchased from Ajax Chemicals (NSW, Australia). Ultrapure water was used for all experiments.

Direct infusion MS experiments were performed using a Harvard 11 syringe pump (Merk, Poole, UK) with a Hamilton 500 μ l gas tight syringe (Supelco, Poole, UK). LC-

MS-MS system is as described above (section 3.2). ESI ion source was used in this study.

3.3.2 Preparation of Standards

Primary stock solutions of E1, E2, E3, EE2 were prepared individually at a concentration of 1 g/L in methanol or acetonitrile. A standard solution was then prepared by spiking 10 μ L of each primary stock solution in a 10 mL volumetric flask and diluting to volume with methanol to produce a 1 mg/L of each target compounds. A 1 mg/L standard for the mixture of target compounds was also prepared. An internal standard solution with 1 g/L of E1-d4 was prepared in methanol and diluted 1000 times to give a concentration of 1 mg/L. The 1 mg/L internal standard solution was further diluted to produce working solutions of lower concentrations. All solutions were stored at 4°C in 10 mL amber glass bottles with Teflon lined closure caps and warmed to room temperature before use.

3.3.3 MS Optimization

ESI was operated in negative mode. Initial mass data acquisition was via direct infusion of 1 mg/L standard of each target compound at 5.0 μ L/min to the MSMS system. The optimization of precursor ion was done by acquiring the Q1 scan over a range of 50 to 500 mass to charge ratio (m/z) for a duration of 5 min with a dwell time of 2 s. The pre-Q2 voltages DP and FP, collision energy (CE) and collision cell exit potential (CXP) were automatically optimized for maximum sensitivity by the machine using the quantitation optimization function of the Analyst software (version 1.4.1) (Applied Biosystems Asia Pte. Ltd.). The most sensitive fragment ion was also optimized when using quantitation optimization.

3.3.4 LC optimization

Chromatographic separation was performed with a Phenomenex Luna 3 μ m C18(2) column, 2.0 x 75 mm (Phenomenex, Torrance, CA, USA). A 100 μ g/L mixture standard of the target compounds was used to optimize the LC conditions. Methanol

and water were used as the mobile phase A and mobile phase B. Isocratic elution was performed, ranging from 10% B to 90% B for the determination of the retention time of target compounds. Base on the isocratic study, a gradient program program was designed to optimize performance.

Methanol and acetonitrile were selected as the organic mobile phase and pure water, 10 mM ammonium acetate, and 0.1% formic acid were selected as the inorganic mobile phase. Six combinations of mobile phase, namely methanol and water, acetonitrile and water, methanol and 10% formic acid, acetonitrile and 0.1% formic acid, methanol and ammonium acetate in water, acetonitrile and ammonium acetate in water were tried as mobile phase A and B.

3.3.5 Solid phase Extraction

The overall schematic procedure is presented in Figure 3.3. Solid phase extraction (SPE) was performed with Alltech extract-clean C-18 columns (1000 mg/8 mL) (Alltech, Deerfield, IL, USA). The cartridges were sequentially conditioned with 4 mL acetonitrile, 4 mL methanol and 4 mL of acidified water (pH 2). The water samples were then passed through the cartridges at a flow-rate of 5 mL/min. After loading the water samples, cartridges were washed three times with 2 mL wash solution (2% acetic acid in 20% methanol aqueous) followed by three times with 2 mL acidified water. The cartridges were then dried by vacuum for 30 min and then eluted with 3×2 mL acetonitrile. The extracts were then evaporated to dryness in a vacuum evaporator (Concentrator 5301, Eppendorf, Hamburg, Germany) and reconstituted to a final volume of 1 mL with acetonitrile and water (v/v, 1:1) containing 10 µg/L E1-d4 as internal standard.

3.3.6 Water Survey and Evaluation of Matrix Effect

3.3.6.1 Sample Collection and Preservation

Various types of water were collected to evaluate the performance of the developed methods (Table 3.1). Surface runoff waters were collected from different locations in Marina Basin, Newater was collected from Seletar and Kranji Newater treatment plants and reservoir water was obtained from Kranji reservoir. Tap waters were collected from the NTU laboratory. In addition, four samples were collected after different treatment stages from the Bedok Newater treatment plant (i.e. secondary effluent (SE), ultrafiltration effluent (UF), reverse osmosis (RO), and Newater (NW)). All water samples were collected in 1 L kapclean precleaned amber bottles with Teflon-lined caps (Qorpak, Bridgeville, PA). All water samples were filtered with 934-AH glass microfiber filter paper of 1.5 μm pore size (Whatman, NJ, USA) followed by Nylon 66 membranes of 0.45 μm pore size (Supelco, PA, USA). The filtrates were then acidified with 1:3 v/v sulphuric acid/ultrapure water to pH2 and stored at 4 $^{\circ}\text{C}$ until extraction. Samples were extracted within 14 days of collection. Prior to extraction, the water sample was warmed to room temperature and measured to required volume.

Figure 3.3 Solid phase extraction procedures

Table 3.1 Sampling surveys

Sample date	Type of water	Source	Concentration factor
July, 2004	Surface runoff (2 locations)	Marina Basin	1000
July, 2004	Surface runoff just after raining (4 locations)		1000
July, 2004	NWwater	Seletar plant	1000
July, 2004	NWwater	Kranji plant	1000
July, 2004	Tap water	NTU lab	1000
July, 2004	Reservoir water	Kranji Reservoir	1000
August, 2004	Secondary effluent	Bedok NEwater treatment plant	100
August, 2004	Ultrafiltered water		100
August, 2004	Reverse osmosis water		250
August, 2004	Newater		250

All the samples were triplicates. Same amount of ultra pure water underwent the same process as water sample to serve as blank. In addition, water sample spiked with the mixture of target compounds were process as same as the water same to evaluate the method recovery.

3.3.6.2 Evaluation of Matrix Effect on recovery and detection limit

The matrix effect on the recovery was conducted by loading different volumes of SE. 10 mL, 50 mL, 100 mL and 500 mL of SE was spiked with 50 µl of 1 ppm mix standard. The samples were concentrated according to the SPE protocol. All samples were concentrated and reconstituted to a final volume of 1 mL methanol containing 10 µg/L E1-d4 as internal standard. The SE was choosen based on its complex matrix. All the samples were triplicates. The recovery of the method were calculated as follow:

$$R = \frac{C_{final}}{(C_{spike} - C_{blank})}$$

where R is recovery; C_{final} is the final concentration we obtained; C_{spike} is the target compounds' concentration which we spiked into the sample; C_{blank} is the target compounds' concentration in the sample without spiking with standard.

Martix effect on the detection limit was evaluated by comparing the detection limit of LC-MS/MS for target compounds spiked into Kranji reservoir water concentrated with SPE at different concentration factors. 1, 10, 25, 50, 250 and 500 mL of Kranji reservoir water samples were concentrated according to the SPE protocol except for the 1 mL sample. As the 1 mL water sample was too small a volume to be concentrated with the available SPE cartridge, liquid-liquid extraction with 1 mL dichloromethane was used instead (detailed procedure see appendix 1). All samples were concentrated and reconstituted to a final volume of 1 mL with methanol and acetonitrile (50/50 v/v) and spiked with low concentrations of target compounds enough to produce a chromatographic peak with three to ten times for the signal to noise (S/N) ratio, and 10 $\mu\text{g/L}$ of E1-d4 as internal standard. As the target compounds were spiked after extraction, the difference observed in MS/MS response could thus be attributed to the effect of sample matrix on ionization efficiency only (Tsukamoto et al., 2004). The method detection limit (MDL) was then determined by consecutively injecting the samples 5 times and multiplying the standard deviation of the replicate measurements by the appropriate Student T value, and multiplying that value by the reciprocal of the concentration factor and recovery of the sample.

3.4 Results and Discussion

3.4.1 LC-MS-MS Optimization

3.4.1.1 MS-MS Optimization

The Q1 scan of the four target compounds is shown in Figure 3.4. All the target compounds have their most intensive precursor ion in deprotonated ($[\text{M-H}]^-$) ion form. The optimized MS conditions are summarized in Table 3.2. Two sensitive product ions were selected for each target compound to avoid false readings. However, only one product ion was selected for the internal standard (E1-d4). The declustering potentials (DP) and focusing potentials (FP) are dependent on the Q1 mass and collision energy (CE) and cell exit potential (CXP) are the parameters of fragmentation energy, which is related to the product ion mass.

Figure 3.4 Q1 scan for the four target compounds (a) E1, (b) E2, (c) E3, and (d) EE2.

3.4.1.2 Optimization of the LC Conditions

The gradient program was optimized as follows: (A) 70%, (B) 30% for initial, (A) 30% and (B) 70% at 1 min and hold for 1 min; (A) 5% and (B) 95% at 2.3 min and hold for 2.5 min; (A) 70% and (B) 30% at 5 min and hold for 1.5 min. Methanol and pure water without any modifier gave the best S/N for the detection. However, when methanol was used as the organic phase, E1 and E2 could not be separated. Moreover, E2 was converted to E1 during the ionization process (Figure 3.5). Therefore, E1 has to be separated from E2 to avoid false reading of E1. Thus, we used a mixture of methanol and acetonitrile (50/50 v/v) as the organic phase for the separation of estrogens, for which a better sensitivity was given than using pure acetonitrile.

Table 3.2 Optimized MS conditions for estrogens

Compounds	Parent ion (m/z)	Product ion (m/z)	DP	FP	CE	CXP
E1	269	145	-76	-250	-52	-13
		143			-66	-9
E2	271	183	-96	-260	-52	-11
		145			-58	-9
E3	287	171	-71	-320	-46	-17
		145			-54	-3
EE2	295	143	-41	-200	-60	-11
		159			-44	-7
E1-d4	273	145	-76	-300	-42	-5

DP: Declustering potential; FP: focusing potential; CE: collision energy; CXP: collision cell exit potential

3.4.1.3 Other Conditions

The column was kept at 40 °C in order to obtain a sharp peak. Total flow rate for pump A and pump B was set at 0.25 ml/min and the injection volume was 10 µl. The conditions for the mass spectrometer were as follows: Nebulizer gas 3; iron source temperature 450 °C; curtain gas (CUR) 9.00; ion spray voltage -400V; Collision activated dissociation gas (CAD):9.00. The resolution used for Q1 and Q3 was low and unit respectively and the dwell time for each MRM was 150 msec.

Figure 3.5 MRM chromatography of (a) E1 (269/145) and (b) E2 (271/145) using mobile phase (A) H₂O and (B) methanol/ acetonitrile (50/50, v/v).

3.4.1.4 Method Evaluation

The method performance was studied by valuating the linearity, repeatability, accuracy, precision and detection limit. The calibration curves for the determination of analytes were obtained by performing a linear regression analysis on the standard solution using the ratio of standard area to internal standard area. Calibration curves were obtained ranging from 1, 10, 25, 50 to 100 µg/L of target compound. The linearity of each analyte was high and all r^2 -values were higher than 0.99 (Table 3.3). The instrument detection limits (IDL) were estimated at a signal-to-noise (S/N) ratio of 3 (Table 3.3). The IDL could reach 0.1 pg as injected amounts. This indicates that the method is very sensitive and comparable with other published methods (Beck *et al.*,

2005, Brossa *et al.*, 2004, D'Ascenzo *et al.*, 2003, Isobe *et al.*, 2003, Johnson *et al.*, 2000). The reproducibility of this method was tested by running 1 ppb mix of standard solution for 10 times. The relative standard deviation (RSD) ranged from 4.7 to 9.2 (Table 3.3), which is less than 10. Thus this method is reliable (Isobe *et al.*, 2003).

Table 3.3 Multiple reaction monitoring mode (MRM), linear correlation coefficients (R^2), instrument detection limit (IDL) and relative standard deviation (RSD) for the optimized LC-MS-MS method

Compounds	MRM	R^2	IDL		RSD
			pg	ng/L	
E1	269/145	0.9991	0.1	10	4.76
	269/143	0.9992	0.5	50	7.17
E2	271/183	0.9980	0.1	10	7.07
	271/145	0.9993	0.5	50	9.32
E3	287/171	0.9996	0.5	50	8.03
	287/145	0.9993	1	100	7.84
EE2	295/143	0.9999	1	100	6.19
	295/159	0.9986	1	100	6.02
E1-d4	273/145	-	1	100	3.32

3.4.1.5 Application in various environmental sample

As there have been many reported cases of single step SPE being successfully applied to environmental water sample preparation for LC/MS or LC/MS/MS analysis, a single step SPE which was previously developed for GCMS was further optimized for LC-MS/MS with spiked ultrapure water. The performance of the method is summarized in Table 3.4. The recoveries for all the target compounds are between 98% to 109% and the detection limit was ng/L level.

Table 3.4 Limits of detection (LOD) and recoveries of target compounds in ultra pure water. Calculated recoveries of 10 ng of each of the selected compounds spiked into ultrapure water prior to extraction, relative to internal standards. Relative standard deviations (RSD) based on triplicate samples.

	E1	E2	E3	EE2
LOD (ng/L)	0.9	1.1	4.5	4.9
Recovery	103	98	109	102
RSD	4.2	3.8	5.7	15

However, when the optimized method was applied to different type of water sample (Table 3.1) at a concentration factor of 1000, low recoveries of 40% for all target compounds were obtained for tap water and no peaks were observed in the chromatogram of RW samples as shown in Table 3.5.

Table 3.5 Recoveries of target compounds in various types of water. SE: secondary effluent; UF: ultrafiltration water; RO: reverse osmosis water; NW: new water; TW: Tap water; RW: reservoir water; SW: surface runoff water; CF: concentration factor, RSD: relative standard deviations (RSD)

Water type	E1		E2		E3		EE2		CF
	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD	Recovery (%)	RSD	
SE	102	7.07	149.5	12.0	156	5.7	330	24.0	100
UF	69	15.6	137	29.7	77	25.4	192	62.2	100
RO	0.5	0.7	0	-	0.5	0.7	0.5	0.7	250
NW	0	-	0	-	0	-	0.5	0.7	250
TW	44.7	50.2	26.9	25.5	33.1	32.6	28.7	19.0	1000
SW	0	-	0	-	0	-	0	-	1000
RW	0	-	0	-	0	-	0	-	1000

It was suspected that the cause of poor recovery could be due to the co-extracted materials in the water, as signals of most of the target compounds including

the internal standard were completely suppressed by the background noise, as compared to target compounds spiked into ultrapure water, which showed clear sharp peaks (Fig. 3.6 A). The extraction ion chromatogram of internal standard (E1-d4) showed a raised level of noise in the region where the peak was completely suppressed (Fig. 3.6 C). As a result, it was not possible to use the internal standardization method of quantitation for the reservoir water. However, this high noise level phenomenon together with the reduction in signal intensity was not observed for the E1-d4 peak in the tap water sample (Fig. 3.6 B). The concentration factors were subsequently lowered to a factor of 250 for seawater and the cleaner water samples from Bedok Newater Plant (i.e. RO and Newater) while a factor of 100 was used for samples with complex matrices, i.e. the secondary effluent and ultrafiltered water (SE and UF) which still had a slight brownish appearance. As for secondary effluent, it was observed that even at a concentration factor of only 100, the matrix was not sufficiently removed and target compounds such as E2, E3, and EE2 showed signal enhancement. UF water also showed signal enhancement for E2 and EE2 but suppression of the other compounds. The water types which showed close to zero or no recovery were, surprisingly, either from very clean matrices (tap water and Newater) or very complex matrices (reservoir water).

In the case of TW and Newater, these samples had previously undergone chlorination, it was suspected that they contained relatively high levels of free chlorine which consumed the spiked target analytes. The removal of free chlorine was thus essential as chlorine is known as a strong oxidizing agent and is likely to modify the chemical and biological nature of water, most notably its organic characteristics. 80 mg of sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, granular) was added per liter of sample to remove the residual chlorine present (APHA 6410B, pg 13710). The recovery of each compound before and after adding sodium thiosulfate is illustrated in Figure 3.7.

Figure 3.6 Chromatograms of target compounds spiked into (A) ultrapure water (B) tap water and (C) reservoir water (top panel). The corresponding extracted ion chromatogram for internal standard (E1-d4) is shown in the lower panel.

Figure 3.7 Effect of sodium thiosulfate on the recovery of analytes in tap water
□ not adding sodium thiosulfate, ▨ add in sodium thiosulfate.

Without sodium thiosulfate, E2 and E3 were not recovered while the recovery of EE2 was 532 %. The unrealistically high EE2 recovery may be due to the single enhancement by the matrix. The detail mechanism is not clear. The addition of sodium

thiosulfate increased the recoveries of the selected compounds where the recoveries of E1, E2, EE2 ranged from 70 – 124 %. In the case of E3, relatively low recovery was observed even though the recovery was increased from 0 to 50.5 %. Similar results were also obtained when isolute ENV and LiChrolut EN solid phase extraction cartridges were used to concentrate 1000 mL of distilled water. The recovery of E3 was about 37.7 % and 39.4 % respectively (Lopez de Alda and Barcelo, 2000).

3.4.1.6 Matrix effect study

The concentration factor has been reported to have significant effect on the recovery and detection limit (Langanà et al., 2004). High concentration factors have the advantage in increasing the detection limit. However, the higher the water volume preconcentrated, the higher will be the effect of the water matrix and more interference will appear in the chromatogram. Thus, the water volume preconcentrated is always a compromise between the LOD and matrix effect (Langanà *et al.*, 2004). Therefore, a balance must be achieved between the concentration factor and the detection limit. In this study we evaluated the effect of concentration factor on the recovery by loading different volumes of SE spiked with 50 µl of a 1 ppm mix standard to the SPE cartridge and optimizing the SPE process. As shown in Table 3.6, the recoveries for all analytes fell within 80-120%, except for E3 in secondary effluent concentrated 10, 50 and 100 times. The recovery for E3 was above 120 when concentrated beyond 50 times and increased with increasing concentration factor. As the calibration standards used in this study was prepared in methanol, it was clear that the concentrated SE matrices enhanced the E3 signals. However, when the concentration factor increased to 500, the recovery for E1 was reduced slightly (from around 100% to around 90%); but the E2 and EE2 signals were completely suppressed and the recovery for EE2 was increased to more than 500%. It is thus important that for samples with complex matrices, the SPE concentration factor is kept low (factor of 10 or below) or the standard addition method of spiking standard compounds into the water could be used to quantify the water samples with complex matrices. New standards were prepared in the sample matrix and

also ultrapure water by adding 1, 10, 25, 50 and 100 μL of stock standard solution to 100-1000 mL of water sample. This technique compensates for a sample constituent that enhances or suppresses the analyte signal, thus producing a different slope from that of the calibration standards which gave recoveries of 80-120%. This quantitation method was used to quantify the samples collected in August and the site background study.

Table 3.6 Recovery of target analytes spiked into secondary effluent (SE) at different concentration factor.

Analytes	MRM	Recovery at different concentration factor (%)			
		10	50	100	500
E1	269/143	98.3	103	99	85
	269/145	104	101	100	94
E2	271/183	101	103	105	-
	271/145	104	105	110	-
E3	287/171	115	134	138	514
	287/145	120	138	144	780
EE2	295/143	103	98	98	-
	295/159	103	97	98	-

The matrix effect on the detection limit were evaluated by spiking the target compounds to the water samples which has been concentrated by SPE with different concentrate factors. The method detection limits of different concentration factor for reservoir samples are shown in Table 3.7. The result showed that method detection limits did not decrease linearly with the increase in concentration factor as expected. This is due to the higher the water sample concentrated, the higher the effect of water matrix. The optimum concentration factor is thus set at 50, giving the best MDL of 0.001-0.003 $\mu\text{g/L}$ for E1 and 0.01-0.07 $\mu\text{g/L}$ for E2, E3, EE2 for Kranji Reservoir water.

Table 3.7 Method detection limit (MDL) for Kranji Reservoir water spiked with target compounds and concentrated with SPE at different concentration factors

Compounds	MDL (ng/L)					
	1×	10×	50×	100×	250×	500×
E1	60	40	3	4	3	10
E2	680	160	10	10	30	40
E3	490	130	10	10	30	70
EE2	730	160	30	10	60	120

1×: liquid-liquid extraction was used. Others (10×; 50×; 100×; 250× and 500×) SPE were used.

3.5 Conclusions

A sensitive LC-MS-MS method in conjunction with a single step solid phase extraction pretreatment method was developed in this study. The instrument detection limit of the LC-MS-MS method could reach 0.1 pg (absolute injected amounts). The linearity of each target compound was higher than 0.99 at concentrations ranging from 1 to 100 µg/L. The single step solid phase extraction pre-treatment method showed good recovery for the ultrapure water when concentrated 1000 times, which result in a method detection limit of ng/L level. However, when the method was applied to natural water samples, poor recoveries were observed even for the clean water matrices. The poor recoveries for the clean water matrices were due to the presence of free chlorine which oxidized the spiked target analytes. Thus, sodium thiosulfate was added to remove the free chlorine and the recoveries of the target compounds were improved. With respect to water with complex matrices, the method detection limit did not decrease linearly with the increase in concentration factor, as the sample matrix was also concentrated. Therefore, for water with complex matrix low concentration factor is suggested. In addition, a calibration standard prepared in the same matrix as the sample is also suggested for quantifying the water samples.

Chapter 4 Fate of estrogens during Soil Aquifer Treatment (SAT)

4.1 Introduction

By 2002, roughly 30 km² of land, located close to Changi International Airport has been reclaimed from the sea with potential for aquifer storage and recovery (ASR) (www.ntu.edu.sg/cwp/index.html). The reclaimed area comprises three layers: the upper layer consists of very loose silty fine to medium sand, 0 to 13 m thick (Ngonidzashe, 2003). The middle layer consists of medium dense silty fine sand, 0 to 13 m thick, and the lower layer consists of very loose silty fine to medium sand, 0 to 8 m thick (Ngonidzashe, 2003). A study launched as the Clean Water Program (CWP) was initiated in 2002. The objectives of the programme were to determine the water quality and hydrologic suitability of ASR in this reclaimed land. Therefore, physical, chemical and biological transformations during the ASR process were studied. To achieve the objectives, a test site (450m×500m) was allocated for the study. Four sampling wells were installed in four corners of the test site which measured 500m × 450m (Figure 4.1).

In this Chapter, the fate of target compounds in the field and under the simulated field conditions were investigated. The fate of the target compounds is dependent on specific environmental conditions and hence a background study was first conducted to characterize the site conditions. The characterization of target compounds in groundwater of the test site was also carried out prior to recharge of ultra-filtered secondary effluent water (UF) to the test site. As sorption and degradation were considered to be the major removal mechanisms, batch experiments were also conducted to determine the sorption and biodegradation behavior of target estrogens in the presence of sand and groundwater/UF water (v/v, 1/1), to simulate the conditions during soil aquifer treatment (SAT). In addition, laboratory scale column studies were carried out to determine the rate and mechanisms of removal in the presence of aquifer sand.

Figure 4.1 Changi site location (a) and sampling wells location C1~ C4 (b).

4.2 Background Study

The background study is aimed at understanding the environmental baseline conditions of the aquifer site, and the water quality for a potential input of recharge water from a water reclamation plant, namely secondary effluent (SE), ultrafiltered secondary effluent (UF), and reverse osmosis effluent (RO) from a Newater plant.

4.2.1 Materials and Methods

4.2.1.1 Sand Characteristics

Surface sand was collected from the Changi reclaimed land for this study. The top 30 cm of soil was scraped off before collection of surface sand samples (duplicate). The site sand analysis was carried out by an external laboratory (Setsco services Pte. Ltd).

4.2.1.2 Water Sample Collection and Pretreatment for Analysis of Water Quality

There were five rounds (January, April, July and October of 2003 and February of 2004) of analysis for background characterization of the water at the Changi reclamation site. Aquifer waters were taken from four corners of the test site (Figure 4.1). General water quality parameters were measured *in situ* by a YSI 556 MPS probe (Yellow Springs Instrument Co., Inc., USA). Samples for target estrogens compounds and total organic carbon analysis were collected in 1 L pre-cleaned amber glass bottles. Samples for nutrients and inorganic analysis were collected in 1 L polyethylene bottles. Samples for metals and total hardness analysis were collected in 500 mL glass bottles. After collection, all samples were kept in an icebox whilst transported to the laboratory at Nanyang Technological University (NTU). As soon as the samples arrived in the laboratory, they were filtered through 1.5 µm (GF/C) prior to filtration through 0.45 µm cellulose ester filter papers. Samples for trace organics, namely the target estrogens compounds, and metals analysis were preserved with 1:3 H₂SO₄ (v/v) and concentrated

HNO₃ to pH 2-2.5 and pH below 2 respectively. All preserved samples were kept cool at 4 °C until analysis.

In addition, further background characterization for the target estrogen compounds at the Changi site was carried out on 18 May 2005, just prior to recharge of UF for the test site. Samples were taken along the path of groundwater flow, according to the direction of an injected fluoresce tracer. All water samples were collected using pre-cleaned 40 mL amber glass bottles with Teflon-lined caps and stored in an ice-box during transportation. Sodium thiosulphate was added to a final concentration of 80 mg/L to remove the free chlorine followed by addition of concentrated sulphuric acid/ultrapure water (v/v 1:3) to adjust the pH to 2. Samples were then stored at 4 °C until extraction, typically within 7 days of collection. Prior to extraction, the water samples were warmed to room temperature.

The potential injection water SE and UF were taken from a Newater plant in Singapore in Aug 2003 and July 2004 and for analyzing the target compounds.

4.2.1.3 Analytical Methods

Total organic carbon (TOC) was measured by a total organic carbon analyzer (TOC-VCSH, Shimadzu, Singapore). Inorganic parameters (nitrate, sulfate) were measured by a Hach DR 2400 spectrophotometer and metals were detected by inductively coupled plasma (ICP) spectrometry (Optima 2000DV).

Samples taken in 2003 and 2004 for analysis of the target estrogen compounds were concentrated 1000 times with SPE as described in section 3.3.5.2, while the samples taken in 2005 from the Changi site were concentrated ten times by means of liquid-liquid extraction prior to analysis by liquid chromatography tandem mass spectrometry (LC-MS-MS). Dichloromethane (DCM) was added as the extraction

solvent followed by evaporation in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany) to dryness and reconstituted with 50/50 acetonitrile/water spiked with internal standards. After centrifuging for 3 min at 10,000 rpm, samples were transferred into inserts fitting into 2 mL HPLC amber vials. A ten times concentrate was made by extracting 1 mL of sample and reconstituting to a final volume of 100 μ L. Deuterated estrone (E1-d4) was used as the internal standard.

Calibration standards were prepared in the same matrix and went through the same procedure as samples at concentrations ranging from 1, 10, 30, 50 to 100 μ g/L of target compounds and 10 μ g/L of internal standards. From the results of the initial background study, a sample which had undetectable levels of any of the target compounds was chosen as the standard matrix.

Recovery was evaluated by spiking 10 μ L of 100 μ g/L standard solution in the same sample and concentrated 10 times as with the samples. A method blank was also performed using ultrapure water and processed in the same way as a sample for assessing contamination during the sample processing.

The conditions for LC-MS-MS were optimized as described in section 3.3.3 and 3.3.4 and summarized as follow: 10 μ L extract were injected onto a Luna 2.0 $\times$ 75 mm column, C18 reversed phase column (Phenomenex, UK) at 40°C and eluted with a flow rate of 0.25 μ L/min using a gradient with water as phase A and acetonitrile and methanol (v/v, 1:1) as phase B. The gradient was as follows: (A) 70%, (B) 30% for initial, (A) 30% and (B) 70% at 1 min and hold for 1 min; (A) 5% and (B) 95% at 2.3 min and hold for 2.5 min; (A) 70% and (B) 30% at 5 min and hold for 1.5 min. The turbo ion spray source was operated in negative-ion mode with ion spray voltage at -4kV and the probe temperature was maintained at 450 °C. Nitrogen gas was used as the nebulizer (NEB), curtain (CUR) and for collision activated dissociation (CAD). The setting for these parameters were: NEB: 10, CUR: 12, CAD: 6.

4.2.2 Results and discussion

4.2.2.1 Characterization of Site Conditions

Table 4.1 and 4.2 summarize the characteristics of aquifer sand, aquifer water, SE and UF. Detail of the sand characteristics and general water quality results are shown in Appendix 2 and Appendix 3, respectively. In general, the aquifer was weakly basic with a pH of around 8. Temperature for the aquifer water was approximately 30 °C. The oxidation reduction potential was around -200 mV, which indicates a highly reducing environment.

For aerobic biodegradation, free oxygen is needed to serve as the terminal electron acceptor. Under anaerobic conditions, inorganic compounds, such as nitrate, sulfate, can be used as terminal electron acceptors. Therefore, nitrate, sulfate, and dissolved oxygen are important inorganic parameters to monitor. The result indicates that the aquifer water has high sulfate (900 mg/L) and nitrate (0.65 mg/L) content, compared with an Australian aquifer, which has 250 mg/L SO₄ and 0.02 mg/L NO₃-N (Ying *et al.*, 2003). Dissolved oxygen in the aquifer water is also very low, general below 1 mg/L, therefore, the aquifer conditions can be consider as anoxic.

The results for the sand characterization indicate that the site sand has a low organic carbon content of around 0.3%. It contains a very high concentration of iron (3085 mg/kg), which can act as an electron acceptor in the biodegradation process if conditions are favorable. The sand is weakly basic with a pH of 7.9 and has a cation exchange capacity of 2.7 meq/100g.

4.2.2.2 Occurrence of Target Compounds

All the target compounds were not detected in aquifer water and the potential recharged water, SE and UF in the background study in 2003 and 2004. This may be

due to the extremely low concentration of the target compound in the water and the poor recovery for the SPE methods as discussed in section 3.4.1.2.

For the sample taken in 2005, the recovery study shows that 10 times concentrated by LLE gave good recovery for all the target compounds, i.e. 92%-108% for E1, 83%-118% for E2, 82%-114% for E3 and 81%-115 for EE2. E1 was detected in 13 out of 48 background samples at concentration ranging from 7 ng/L to 25 ng/L which is higher than the concentration reported in other groundwater studies (Bursch *et al.*, 2004) but within the range of reported concentration in the surface water (Kolpin *et al.*, 2002). However, the other three estrogens E2, E3 and EE2 were not detected in the background samples.

Although only E1 was detected in the background samples, the potential adverse impacts by all the target compounds during the SAT process and the long term ASR will still exist as these compounds may potentially exist in the recharge water and adsorb or accumulate in the system. Therefore, the fate of all the target estrogens in this tropical aquifer environment is addressed.

Table 4.1 Characteristic of aquifer sand used in this study

pH saturated paste	Total carbon (%)	Organic carbon (%)	Volatile matter (%)	Nitrogen (%)	Iron ppm	Phosphorus ppm
7.9	1.6	0.29	0.65	<0.01	3085	67

Table 4.2 Characteristic of groundwater and potential recharge water used in this study

Parameter	groundwater	Ultrafiltered secondary effluent	secondary
pH	7.9	7.3	7.35
Total organic carbon (mg/L)	2.9	7.7	6.7
Dissolved inorganic carbon (mg/L)	-	6.6	12.3
Oxidation Reduction Potential (mV)	-181	273	-
Conductivity (mS/cm)	3.02	0.73	0.71
NO ₃	0.52	5.79	7.50
SO ₄	900	55.4	83.16
Fe	0.002	-	-
Dissolve oxygen (mg/L)	0.35	-	-

4.3 Sorption and Degradation

In this study, we aim to investigate the removal efficiency and mechanism of the target compounds at the Changi site. Batch studies were carried out to investigate the sorption and biodegradation of these compounds under conditions of the Changi site. A series of column studies were also conducted to simulate the percolation of treated municipal effluent through sand from a study site on the reclaimed land.

4.3.1 Materials and Methods

4.3.1.1 Aquifer Materials

The aquifer materials used in this study were surface sand collected from the Changi site. Sand was first air dried and sieved through a 2 mm sieve before use. UF and groundwater (GW) (v/v, 1/1) were chosen as the aquatic matrix to simulate the SAT recharge condition after UF was injected into aquifer sand. The UF recharge water was collected from a Newater plant and refrigerated prior to use. The physiochemical properties of aquifer materials are described in Table 4.1.

4.3.1.2 Sterilization of Materials

All the water, sand and bottles used in this study were sterilized. UF and GW were passed through 0.2 μm membrane for removal of bacteria. Sand was sterilized at 180 °C for three days. All the bottles, pipette tips, measuring cylinders and conical flasks used in this study were autoclaved at 121 °C for 15 minutes.

4.3.1.3 Sorption Kinetics

Sorption kinetics were conducted to determine the sorption equilibrium time of the target compounds. 10 g of sterilized sand was added to 30 mL of the filtered mixture of GW/UF (v/v 1:1) containing 100 $\mu\text{g/L}$ each of the target compounds. Controls without sand but with the same concentration of target compounds were prepared in the same way as samples. Samples were shaken with an overhead shaker during the experiment period and the concentration of target compounds were monitored at 0h, 2h, 4h, 8h, and 24 h.

4.3.1.4 Sorption Isotherm

Samples and controls for this study were prepared as in the sorption kinetics study (section 4.3.1.3). Five concentrations were used in this test (i.e. 5, 10, 25, 40 and 50 $\mu\text{g/L}$). Based on the kinetic study, samples were taken at the equilibrium time. The initial concentration and the equilibrium concentrations of each analyte were measured.

The relationship between the equilibrium concentration in solution and the total amount adsorbed on a solid phase can be described by fitting the sorption data into the Freundlich isotherm equation.

$$S = K_f \cdot C_e^{1/n} \quad (1)$$

where

S- is the concentration of a chemical adsorbed by the solid phase [$\mu\text{g /kg}$]

K_f -is the Freundlich sorption coefficient

C_e -is the equilibrium solution concentration [$\mu\text{g /L}$] and

$1/n$ - is the linearity parameter

The sorption data were fitted to the logarithmic transform of the Freundlich equation.

$$\log S = \log K_f + \frac{1}{n} \log C_e \quad (2)$$

When the value of n is unity, we have the simple linear isotherm

$$S = K_d \cdot C_e \quad (3)$$

where K_d is the sorption coefficient [kg/L]

Many studies have shown that in the absence of strong ionic interactions, the sorption of chemicals closely depends on the organic content in the soil/sediment. By normalizing sorption from a total soil basis to an organic carbon basis, the organic carbon sorption is defined as follows:

$$K_{oc} = \frac{K_d}{f_{oc}} \quad (4)$$

where f_{oc} is the % organic carbon (Ying et al., 2003)

4.3.1.5 Degradation Study

Batch experiments were carried out to study the degradation behaviour of target estrogens compounds in a recharge aquifer environment under both aerobic and anoxic conditions. Aquifer sand (20 g) and 60 mL UF/GW blend (v/v 1:1) were spiked with 100 µg/L each of the target analytes. All experiments were performed in duplicate, including abiotic controls and blanks. The soil to water ratio was the same as in the sorption study. Bottles and flasks used in the experiment were sterilized by autoclaving.

Aerobic microcosms were constructed in 250 mL sterilized conical flasks, placed in an amber plastic bag, maintained at 30 °C, and shaken at 150 round/min. The anoxic samples were prepared in 40 mL amber glass bottles placed in an anaerobic chamber. The UF and GW blend was purged with nitrogen gas for 1 hr followed by spiking the target compounds. All samples were kept in the anaerobic chamber. Abiotic controls were constructed with 20 g of sterilized sand, UF/GW blend filtered through 0.2 µm membrane followed by the addition of 2.0 mM sodium azide.

All operations were performed in the bio-safety hood to prevent bacterial contamination. Duplicates of blanks without spiked target compounds were also prepared for the experiment to determine initial concentrations of the target compounds. Microcosms were sampled weekly and analyzed for the target compounds.

4.3.1.6 Analytical Methods

0.6 mL samples were taken from each flask. After centrifuging for 15 min at 10,000 rpm, 0.5 mL supernatant sample was transferred into a 2 mL centrifuge tube. The samples were then extracted by liquid-liquid extraction (LLE) with dichloromethane and subsequently analyzed by LC-MS/MS. Details of the LC-MS-MS method were described in section 4.2.1.4. The limits of quantitation are 1 ng/L for E1, 5 ng/L for E2, 10 ng/L for E3.

4.3.2 Results and Discussion

4.3.2.1 Sorption Kinetics

The sorption kinetics of four target compounds is shown in Figure 4.2. Rapid sorption was observed in five hours and sorption equilibrium was achieved between 5 and 24 hours, consistent with other studies (Casey *et al.*, 2005; Holthaus *et al.*, 2002; Lai *et al.*, 2000; Yu *et al.*, 2004). Around 60% of E1 and E2 were adsorbed onto the aquifer sand when equilibrium was reached while only around 40% of E3 and EE2 were adsorbed.

Figure 4. 2 Sorption kinetics of estrogens (○) (a) E1, (b) E2, (c) E3 and (d) EE2 and corresponding control (□), measurements were made in duplicate.

4.3.2.2 Sorption Isotherm and K_{ow}

The sorption distribution coefficient is defined as the ratio of the concentration of the compound bound to the sediment and the concentration in the solution following equilibration. The concentration bound to the sediment was measured indirectly as the difference between the concentration at the beginning and the concentration in the solution after equilibration. Controls gave recoveries between 90%-110% for all analytes, when kept shaken for 1 day. The sorption isotherms of the four target compounds are given in Figure 4.3.

Fig 4.3 Sorption isotherms for the target compounds on Changi aquifer material (a) E1, (b) E2, (c) E3 and (d) EE2.

The experimental data were subjected to regression analysis using a logarithmic transform of the Freundlich model equation (2). The values of the Freundlich sorption coefficients (K_f) and the linearity parameter ($1/n$), as well as correlation coefficients (R^2) are given in Table 4.3.

Table 4.3 Sorption coefficients of the target estrogens compounds on Changi aquifer sand ($f_{oc} = 0.3\%$, sand (97.1%), clay and silt (2.9%)). The solution matrix was a mixture of groundwater and ultrafiltered water at a 1:1 ratio.

Analytes	K_f	$1/n$	R^2	K_{oc}	K_{oc} from Lit.
E1	4.25	0.94	0.97	1614.3	4882 ^b , 2961 ^c ,
E2	3.45	1.02	0.99	1210.6	4360 ^a , 3300 ^b , 3714 ^c , 909-2174 ^d ,
E3	0.65	1.11	0.99	347.63	1944 ^b , 1404 ^c ,
EE2	1.937	1.05	0.99	791.57	4840 ^a , 4770 ^b , 5433 ^c , 1926-1000 ^d ,

^aLimestone sediment from ASR well, South Australia (~150m), groundwater (300m) Sand (83%), clay (3.1%), silt (1.1%), f_{oc} (0.5%) (Ying *et al.*, 2003)

^bsediment from the Blackwater estuary, U.K., river water, sand (1.6%), clay (25%), silt (74%) (Lai *et al.*, 2000).

^cfour representative soils from agricultural land in south Australia. Clay (10%-25%), Silt (2%-20%), sand (27%-85%), TOC (0.85%-2.9) (Ying and Kookana 2005). The data showed here are the mean of these four soils.

Bed sediments in some English rivers. Clay (3%-9%), Silt (12%-77%), Sand (14%-85%), TOC (0.1%-7%) (Holthaus *et al.*, 2002).

The data shows that E3 has the lowest K_f value (0.65), followed by EE2 (1.94), E2 (3.45), and E1 (4.25). As the linearity parameter ($1/n$) ranged from 0.87 to 1.11 (i.e. relatively close to 1), the sorption of the target compounds was simplified by the linear isotherm, equation (3). As most analytes were primarily sorbed by soil organic matter, the corresponding organic carbon normalized partition coefficient (K_{oc}) values were estimated using Eq. (4). The organic carbon normalized K_{oc} (in L/kg) values varied from 348 (E3), 792 (EE2), 1211 (E2) to 1614 (E1). These indicate that the relative strength of sorptive affinities among the estrogens follows an order of $E3 < EE2 < E2 < E1$.

The synthetic estrogen, EE2, has been reported has the strongest adsorption among the four estrogens, as it is considered to be more hydrophobic than other steroid estrogens (Johnson and Sumpter 2001; Ying and Kookana 2005; Ying *et al.*, 2003). However, in this study, EE2 showed lower sorption ability than E1 and E2 while E3 has the lowest sorption ability. These results are inconsistent with their physicochemical properties (Table 2.1) and hence, hydrophobic partitioning may not be the dominant sorption mechanism. This may due to the low organic carbon content (OC) for the aquifer sand used in this study. Casey *et al.* (2005) reported that the contribution of nonhydrophobic processes in the sorption process increased as OC decreased. Yu *et al.* (2004) gave an explanation for nonhydrophobic sorption processes. They suggested that the phenolic group of estrogens can interact with humic acid or mineral surfaces via hydrogen bonding or covalent bonding. The phenolic function at C-3 and the hydroxyl function at C-17 tend to react with carboxylic function groups of humic materials associated with soils and sediments. In addition, the rapid sorption of these compounds showed that adsorption was a more likely sorption mechanism for these compounds. Therefore, the Freundlich model could have caused an inadequate estimation of the K_{oc} values.

The K_{oc} values of estrogens for the Changi aquifer sand were generally lower than those reported in other similar studies (Table 4.3). Presumably the low sorption observed in this study was due to the different compositions of the aquifer materials.

4.3.2.3 Degradation Study

Rapid degradation of natural estrogens was found under aerobic condition (Figure 4.4). The half-lives are defined as the time that the concentration of target compounds is half of the initial concentration and they were 1.8 days, 2.3 days, and 15 days respectively. The degradation is comparable to the degradation observed in the other studies. For example, Shi *et al.* (2004a) studied the biodegradation of estrogens using activated sludge and night soil composting microorganism where the decrease in

estrogen concentrations obeyed first-order kinetics with half-lives of 2.2 days for E1, 0.6 for E2, 1.5 days for E3.

Figure 4.4 Degradation of estrogens under aerobic (□) , anoxic (○) , and abiotic (Δ) conditions for (a) E1, (b) E2, (c) E3 and (d) EE2

The half-life of E2 degradation in an Australian aquifer was 2 days (Ying *et al.*, 2003). E3 is reported to have a half-life of 23.1 hours for an initial concentration of 1000 $\mu\text{g/L}$ in nitrifying activated sludge (Shi *et al.*, 2004a) and a half-life of 37.8 hours and 61.6 hours respectively at initial concentrations of 20 to 25 mg/L in activated sludge collected from Korea and night soil-composting microorganisms (Shi *et al.*, 2004b). The present study showed E3 was almost completely degraded within 32 days under aerobic conditions (Figure 4.4 c).

The primary metabolite, E1, was formed in the degradation of E2 (Figure 4.5) which is consistent with other studies (Jurgens *et al.*, 2002; Lee and Liu, 2002; Lucas and Jones 2006; Shi *et al.*, 2004b; Ternes *et al.*, 1999b; Ying and Kookana 2003; Ying and Kookana 2005). The E1 concentration reached a maximum (50% of the initial E2 concentration) on the third day and subsequently degraded to below the detection limit together with E2 after one day.

Figure 4.5 E2 degradation under aerobic conditions E2 (□), E1 (○).

Under anoxic conditions, E1 and E2 also showed degradation ability. However, the degradation of these compounds was considerably slower than under aerobic conditions. E1 and E2 were inter-convertible and reached steady state around 15 days (Figure 4.6). At the steady state, $6 \mu\text{g/L} \pm 0.35$ E1 and $37 \mu\text{g/L} \pm 0.31$ E2 remained after the degradation of E1 at an initial concentration of $100 \mu\text{g/L}$. On the other hand, in the degradation of E2 (initial concentration of $100 \mu\text{g/L}$), $40 \mu\text{g/L} \pm 0.71$ E2 and $21 \mu\text{g/L} \pm 0.07$ E1 remained in the system after steady state was reached. The inter-conversion phenomenon between E2 and E1 was found in the study by Czajka and Londry (2006) who studied the anaerobic transformation of estrogens under methanogenic, sulfate, iron, and nitrate reducing conditions. They reported that the

final steady state concentration of E2 depended on the electron-accepting condition but was independent of the total amount of estrogens added.

Figure 4.6 E1 (○) and E2 (□) degradation under anoxic conditions (a) E1 degradation, (b) E2 degradation.

A black precipitate was found in the anoxic degradation of E1 and E2 and is likely due to the formation of iron sulfide on the sand surface. The sulfate content in the ground water is relatively high at 900 mg/L (Table 4.1). Therefore, sulfate reducing conditions probably resulted in the loss of E1 and E2. Similar observations were also found in other aquifer studies (Ying *et al.*, 2004).

In contrast to natural estrogens, the synthetic estrogen, EE2, showed slow degradation under aerobic conditions (Figure 4.4d); only 60% of the initial EE2 was removed after 40 days incubation. EE2 has been reported principally for its persistence in the environment. Its dissipation time in river systems has been reported to range from 20 to 40 days (Jurgens *et al.*, 2002). In aquifer material, its half-life was estimated around 81 days (Ying *et al.*, 2003). EE2 also has been found to slowly degrade under anoxic conditions. Its degradation behavior was similar to that under abiotic conditions, where around 40% of EE2 was removed after 40 days incubation. Therefore, the removal mechanism may be due to chemical transformation. The chemical transformation of EE2 has been found in many studies (Liu *et al.*, 2003; Moriyama *et al.*, 2004; Rudder *et al.*, 2004).

4.4 Column Study

While the above studies are useful to study kinetics under controlled conditions, they are limited because they apply to small scales. In order to better simulate the SAT conditions on a larger scale, a column study was conducted. E2 was selected to represent a natural estrogen and was studied together with the synthetic estrogen, EE2. A set of batch experiments was conducted to examine the abiotic transformation and sorption ability of the target compounds as it was difficult to sterilize the columns for this purpose.

4.4.1 Materials and Methods

4.4.1.1 Batch Experiments

The set up for abiotic batch experiments is similar to that in the degradation study reported in section 4.3.2.3. However, in this batch experiment, E2 and EE2 mixture were added into UF water alone which is consistent with the feed water for the column study. The preparation of aerobic, anoxic, and abiotic samples was the same as the

degradation study (section 4.3.2.3). Another set of abiotic controls without sand was performed to investigate the sorption of these compounds in the UF and sand system. The experimental setup is shown in Table 4.4.

Table 4.4. Setup of microcosms for the batch experiment of target compounds in UF

conditions	Vol. of UF water (ml)	Aquifer sand (g)	Target compounds concentration (µg/L)	Sterilization	Aeration
Aerobic	100	10	100	No	Yes
Anoxic	100	10	100	No	No
Abiotic with sand	100	10	100	Yes	Yes
Abiotic w/o sand	100	0	100	Yes	Yes

4.4.1.2 Column Study

Laboratory-scale columns with eight 1.0 meter long and 0.1 meter diameter columns were packed with sand as shown in Figure 4.7. For sampling purposes, a series of side ports with Teflon[®] lined septa was installed at multiple depths (25, 50, 75 cm) of the column while a three way valve was installed at the bottom end (100 cm). In order to minimize the loss of target compounds due to sorption, all wetted components were either Teflon[®] tubing or stainless steel 303. The transparent parts of the set-up were wrapped with aluminium foil to prevent algae growth and photodegradation.

Two similar sets of eight columns were operated in series in a flow through regime at room temperature ($20\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$). The residence time of each column was determined via a tracer test with chloride used as the conservative tracer. With a porosity of 0.44 and average flow rate of 0.55 mL/min, the average residence time for each set of columns was about 35 days. Each of the eight columns was fed with UF water augmented with mixture of 100 ppb E2 and EE2. For each set of columns, feed that was sufficient for 24 hours of continuous operation was prepared daily and warmed to ambient temperature prior to recharge. The spiked recharged effluent was continuously mixed by a magnetic stirrer before entering the columns.

Prior to add in the target compounds, the columns were saturated with UF for one month and then the target compounds were added into the feed. The concentrations of the target compounds in the column effluents were monitored.

A glass syringe was used to collect 3 mL of sample from sampling port 1 to port 3 (Figure 4.7) when the plume of contaminants travelled to the sampling port (0.0325 pore volumes to each sampling port). When the plume reached the 4th port of each column, samples were collected in 40 mL amber glass vials through the three-way valves. First 5 mL was withdrawn and discarded, then a 3 mL sample was collected. All samples were acidified to pH 2 using sulphuric acid and kept in the dark at 4°C until analysed.

Figure 4.7 Schematic diagram of the laboratory SAT columns (~ 35 days retention time)

4.4.2 Results and Discussion

4.4.2.1 Batch Experiments

The degradation profiles of E2 and EE2 are shown in Figure 4.8. The loss of target compounds in the abiotic control without sand is considered as chemical transformation while the loss of target compounds in the abiotic control with sand minus the loss in the abiotic sample without sand is considered as sorption. The result indicates that E2 was degraded quickly under aerobic conditions. Half-lives of E2 degradation under aerobic conditions were estimated to be around 1.6 to 2 days which is comparable to that in the UF and GW mixture (section 4.3.2.3). Formation of E1 was

also observed, but E1 was also quickly degraded under aerobic conditions (Figure 4.9a). Maximum E1 recovery was detected after 3 days of incubation, and the maximum E1 recovery was around 15-20% of the initial E2 concentrations. This indicates that E1 did not stoichiometrically accumulate but degraded together with a slower rate than E2.

Figure 4.8 E2 (a) and EE2 (b) degradation in UF under aerobic (□), anoxic (○), abiotic (Δ) and abiotic without sand control (▲) conditions.

Under anoxic conditions, E2 was degraded at much slower rates and reached a steady state concentration (around 40% (w:w) of the initial E2 concentration) after 50 days incubation. E1 was observed to accumulate under anoxic conditions, with a

maximum E1 recovery of approximately 28% (w:w) of the initial E2 concentrations (Figure 4.9 a). This may be due to the inter-conversion of E1 and E2 and reached steady state as mentioned in section 4.3.2.3. In addition, the result in UF is consistent with that in the UF and GW mixture, where at steady state, around 40% of E2 and 20% of E1 remained. A black precipitate was also found under anoxic conditions similar to the degradation study (section 4.3.2.3).

Figure 4.9 E2 degradation under (a) aerobic and (b) anoxic conditions (○) E2, (□) E1.

E2 was observed to be persistent under abiotic conditions. When aquifer sand and bacteria were absent, the E2 concentrations remained unchanged; while in the abiotic controls with sand, E2 concentrations were reduced to 40% of initial levels

within the first 10 days and remained unchanged thereafter. This indicates that sorption of E2 onto the aquifer sand also contributes to the removal of this compound. In both abiotic microcosms, no E1 was detected.

In contrast with E2, EE2 was degraded at similar rates in both aerobic and anoxic microcosms. Based on first order kinetics, the degradation rate constants for EE2 were 0.025 and 0.028 day⁻¹ respectively under aerobic and anoxic conditions. Compared with biotic conditions, EE2 was more persistent in the absence of bacteria. However, there was still approximately 30% disappearance of EE2 due to abiotic transformation after 27 days of incubation (Figure 4.8 b). By comparing the EE2 concentrations in the UF abiotic microcosms with and without sand, it can be shown that sorption is insignificant.

4.4.2.2 Column Study

Based on the batch study, it can be seen that E2 is a readily degradable compound. For the column study, E2 was reduced to below the detection limit (0.1 µg/L) in the first 0.25 meters infiltration (0.0325 PV). The E2 primary metabolite, E1 was found within 1 meter infiltration in the first round but was not detected thereafter (Figure 4.10a). This indicates that E1 is also a readily degradable compound which is consistent with the batch study. In addition, acclimatization of the aquifer sand microbial community appeared to be one of the factors causing the increased removal for E1.

Figure 4.10 Percentage removal in concentration as a function of pore volume (a) E1 and (b) EE2 in the UF column system (1st round (○): 80 days after start up; 2nd round (□): 103 days after start up and 3rd round (Δ): 119 days after start up.

EE2 exhibited more persistence than the natural hormone, E2. A bell-shaped curve was obtained for all three rounds of measurement. There was a sharp drop in concentration in the first few metres of infiltration, followed by a gradual increase in

columns 2-5 and finally a decrease in the later columns (Fig. 4.10b). No firm conclusions can be made. Sorption/desorption and retardation may be a possible explanation for the removal trend observed. A desorption experiment, however, was not conducted in this study.

4.5 Conclusions

In this study, the sorption and degradation behaviour of estrogens were examined by batch experiments under different redox conditions for a UF/GW mixture. In addition, a set of columns packed with aquifer sand were used to simulate the SAT process and assess the attenuation behaviour of these compounds under UF recharge conditions. Another set of batch experiments was conducted to serve as a control for the column study. The conclusions are summarized as follows:

1. The sorption abilities of the estrogens were in the following order: $E3 < EE2 < E2 < E1$. This is inconsistent with their physicochemical properties and hence, hydrophobic partitioning may not be the dominant sorption mechanism in this study. In addition, it may be due to the inadequate estimation of K_{oc} value by using Freundlich model.
2. The K_{oc} values of estrogens in this study were significantly lower than those reported in other similar studies. This is due to the different compositions of the aquifer materials.
3. E2 was the most readily degradable compound among these target compounds. The rapid degradation of E2 was found in all the water matrices under aerobic conditions. Its metabolite, E1 was also found to rapidly degrade in all the matrices under aerobic conditions. Under anoxic conditions, E1 and E2 were

inter-convertible and reached steady state after 50 days incubation in UF and after 20 days incubation in GW/UF (v/v 1:1). E2 exhibited sorption ability to the aquifer sand in the presence of UF. In the column study, E2 was removed rapidly and could not be detected after 0.25 m infiltration in the column. Its degradation metabolite, E1, could be detected only within 1 meter infiltration in the UF column.

4. EE2 was found to be more persistent than the natural estrogen, E2. It was found to be biodegradable under both aerobic and anoxic conditions. Chemical transformation could also be considered as one of the removal mechanisms. In the column study, a bell shaped curve was obtained for the removal of EE2. The mechanism is not clear but sorption and desorption may be the reason for this removal trend.
5. Acclimatization of the aquifer sand appears to be an important factor in enhancing the removal of target compounds. This is important for the practice of SAT as the target compounds have a greater potential to be removed if there is sufficient long term storage.
6. Degradation plays a more important role than sorption in the removal of target compounds especially for the readily degradable compound E2.

Chapter 5 Isolation and Characterization of Estrogen-Degrading Bacteria

5.1 Introduction

Significant attenuation of target compounds was found in the Changi aquifer material in the fate study (chapter 4). In addition, soil aquifer materials as well as reclaimed waters are often nutrient poor and limited in sustaining microorganism growth and survival which might reduce the microbial degradability of EDCs. Therefore, it is important to study microorganisms which are capable of surviving and degrading EDCs under such difficult conditions.

The aim of this study was to isolate, characterize, and evaluate estrogen-degrading bacterial strain from Changi site sand and potential recharge water, ultrafiltered effluent (UF). Three estrogen-degrading bacteria, LHJ1, LHJ3 and CYH, were isolated. Based on gram-strain morphology and 16s rRNA sequence homology, LHJ1 and LHJ3 belong to the genus *Acinetobacter* and *Agromyces* respectively; CYH represents a novel species within the *Sphingobium* genus. Data from this study extends our current knowledge on the estrogen-degrading bacteria surviving in tropical aquifer environments. In addition, this will aid in the development of bioremediation schemes for estrogen-containing reclamation waters.

5.2 Materials and Methods

5.2.1 Enrichment and Isolation of Estrogen-degrading Bacteria

Sand was collected from the surface of the Changi reclaimed land site and ground water from the underlying artificial aquifer where rain water had displaced seawater. UF

biologically treated effluent water was obtained from the NEWater treatment plant in Singapore. The characteristics of aquifer sand, groundwater and UF are shown in Table 4.1. 250 mL conical flasks with 50 mL of liquid minimal medium (MM) (Table 5.1) (Shi *et al.*, 2002) were inoculated with 2 mL water sample or 2 g of sand. Each MM with sample was supplemented with 1 mg/L of each of the estrogens as the enrichment substrate. No other carbon sources were added to the medium and thus, estrogen was considered as the sole carbon source. The samples were covered by aluminium foil and then incubated in a shaker at 150 rpm at 30°C in the dark. The estrogen concentrations were monitored daily. When significant degradation of the target compound was found, the microcosm was enriched by transferring 10% of the sample to a new medium. The samples were also serially diluted by phosphate buffered saline (PBS, pH 7.2), plated onto Bacto trypticase soy agar (TSA), inverted and incubated at 30°C for up to 2 days. Visible colonies with distinct morphology were picked and transferred to fresh plates until pure. CYH was isolated as a degrader of E1 whereas LHJ3 and LHJ1 were isolated as E2 degraders.

Table 5.1: Composition of minimal medium for the enrichment

Composition	Concentration (g/L)
K ₂ HPO ₄	1.0
NH ₄ NO ₃	1.0
NaCl	0.1
MgSO ₄ .7H ₂ O	0.2
Fe(NO ₃) ₃	0.01
CaCl ₂	0.1

5.2.2 Identification of Isolated Degradors

5.2.2.1 Phenotypic Characterization 16 s Ribosomal DNA (rDNA) Sequences

Cells of the isolates were observed with light microscopy (Olympus BX-FLA-3 epifluorescence microscope, Japan). Gram stain was performed. The isolates were smeared onto a glass slide using an inoculator and then emulsified with droplets of water. The slide was then heat fixed under a naked flame and primary stain (crystal violet) was added for 1 min. The slide was then washed with alcohol before secondary stain (safranin pink) was added for 30 seconds. The slide was then washed with water and viewed under microscopy (Smibert and Krieg 1994).

The strains were identified by determining their 16S ribosomal DNA (rDNA) sequences. Direct lysis PCR method was used to amplify 16S rDNA as described by Zhuang et al. (2002). Forward primer Eubac27F and reverse Universal primer 1492R1 were used for amplification of the nearly full-length 16s rDNA gene (Lane 1991). A single colony from the isolated growth was aseptically collected with a sterile pipette tip and smeared onto the bottom of a 0.5 ml PCR tube and 94 μ l of PCR buffer (added with $MgCl_2$) was added. The PCR tube was heated at 98 °C for 30 min in a thermal cycler (Mastercycler, Eppendorf) to enable cell lysis to occur after which a mix of eubacterial primers 27F and 1492R, dNTP and Taq polymerase was added into the PCR tube to initiate PCR. Thermal cycling which consists of 30 cycles of 1.5 min at 94 °C, 15 min at 62 °C, and 2 min at 72 °C was carried out with the exception of the last step of the last cycle which was continued for 10 min, followed by cooling at 4 °C. The PCR products were purified by Wizard SV Gel and PCR clean up system (Promega, USA) following the manufacturer's instructions. The 16S rRNA sequences of the isolates was obtained in both directions for the isolates using the ABI PRISM BigDye Terminator Cycle Sequencing ready-reaction kit (version 3.0) and the ABI model 310A DNA sequencer (Applied Biosystem, Foster City, CA, USA). Partial sequences were compiled and aligned using BioEdit software and analyzed with BLAST.

5.2.2.2 Further Identification for Strain CYH

Based on the full sequence results, it was shown that CYH has high potential to be a new species. Identification analysis was therefore conducted for this strain. Enzyme profiles and carbon substrate characteristics were determined using the API ZYM and API 20E assays according to the manufacturer's instructions (BioMérieux, France).

A final data set of 16S rRNA sequence with 1275 bases was obtained from homologous sites at which the sequence of strain CYH was aligned unambiguously with reference sequences of *Sphingomonas/Sphingobium* and other close relatives from the Ribosomal Database Project II (RDP II) (Cole *et al.*, 2005) and GenBank (Benson *et al.*, 2005). Distance and maximum likelihood analyses were performed on the final data set with PHYLIP (Felsenstein 1989) and fastDNAm1 (Smibert and Krieg 1994). A maximum likelihood phylogenetic tree with bootstrap values was constructed as previously reported (Zhuang *et al.*, 2002).

5.2.3 Investigation of Estrogens-degrading Ability

The estrogen-degrading abilities of the isolated strains were investigated under aerobic and anoxic conditions. The degrader cultures were prepared by inoculating one colony of the degrader in 10 mL of trypticase soy broth (TSB) overnight with shaking at 30 °C. Prior to inoculating the overnight cultures to the medium, the cultures were washed with PBS five times to wash out the TSB. After washing, the cells were suspended in 10 mL of phosphate buffered saline (PBS). One mL of this washed overnight cultures were inoculated separately in 50 mL of MM with 500 µg/L mixed estrogens (mixture of E1, E2, E3 and EE2). All the flasks and bottles used in this experiment were autoclaved before use. The liquid media and PBS were passed through 0.2 µm filter for sterilization.

Anoxic experiments were as follows: prior to incubating the overnight culture, the MM containing estrogens was purged with nitrogen gas for two hours to remove the

dissolved oxygen and passed through a 0.2 μm filter in an anaerobic incubation chamber to prevent contacting with oxygen. Dissolved oxygen (measured using an oxygen electrode) was approximately 0.1 mg/L after purging. A consortium of the three isolates was also used as the inoculum in another 50 mL of liquid minimal medium. An autoclaved mixture of the three isolates was used as inoculum for the negative control and to test for biological adsorption of estrogens. Aerobic cultures were prepared in 250 mL conical flasks to ensure sufficient aeration and placed in amber plastic bags to prevent photodegradation. Flasks were shaken at 150 rpm at 30°C for a period of 15 days. Anoxic samples were prepared in 50 mL amber glass bottles with limited headspace and kept in an anaerobic chamber for 40 days.

Sample preparation was carried out in a biosafety cabinet for the aerobic samples and in an anaerobic chamber (Sheldon Manufacturing, Inc., Cornelius, Oregon) for the anoxic samples. The anaerobic chamber was filled with mixture of N_2 , H_2 , CO_2 (v/v/v 90:5:5). All experiments were performed in duplicates. During the cultivation period, the estrogen concentrations were monitored by liquid chromatography (Shimadzu, Kyoto, Japan) equipped with a triple quadrupole tandem mass spectrometer (API 3000 Applied Biosystems Foster City, CA) (LC-MS/MS) as described in section 4.2.1.4 following liquid-liquid extraction using dichloromethane (1:1 v/v).

5.2.4 Degradation Kinetics Study

The influence of the concentration on the aerobic degradation rate was evaluated by varying the initial substrate concentrations from 50 $\mu\text{g/L}$ to 2000 $\mu\text{g/L}$. This concentration range was selected to approximate recharge conditions as much as possible. It is expected that at these concentrations, growth is not significant (Berg and Nyholm 1996) and therefore cell growth was not monitored. Kinetic studies focused on CYH degrading E1 and E2, LHJ degrading E2 and E3 under aerobic conditions as well as CYH degrading E1 under anoxic conditions.

A predetermined volume of overnight culture solution was added to 50 mL of MM containing one of the degradable estrogens substrates to obtain a final cell concentration of approximately 10^7 cells/mL. The culture flasks were placed in amber plastic bags and incubated on the shaker at 150 rpm at 30°C. Anaerobic cultures were kept in an anaerobic chamber. The concentration of estrogens and the initial concentration of cells were monitored by LC-MS/MS (as described in section 4.2.1.4) and flow cytometry (see below) respectively. All experiments were performed in duplicates. Culture flasks were autoclaved before use and all the sample preparation and sampling were performed under aseptic conditions.

5.2.5 Enumeration of Microbes

The initial cell concentrations in the degradation kinetic study were counted by flow cytometry. 100 μ L of the overnight culture was diluted in 1 mL PBS. The samples were stained with 10 μ L of the nucleic acid-binding cyanine dye, SYBR Green[®] I, followed by incubation for 15 min in the dark at room temperature. Flow cytometric analysis was conducted with a FACSCalibur flow cytometer (Becton, Dickinson, San Jose, CA) equipped with an air cooled 15 mW argon ion laser, emitting at a fixed wavelength of 488 nm. Cells that were stained by SYBR Green[®] I fluoresce green upon excitation by a blue laser (Xie Shuang, personal communication).

5.2.6 Identification of Degradation Products

For identification of degradation products, initial concentrations were increased to 1 mg/L. LHJ3 cultures degrading E3 were collected daily; CYH cultures degrading E1 were collected at 0 hour, 2 hours, 4 hours, and daily thereafter. The LC gradient for sample analysis was as above with the following modification: Initially 10% (B), increase to 50% (B) in 10 min and to 90% (B) in 18 min, hold 90% (B) until 24 min and return to 10% (B) at 25 min and stop after 30 min. MS/MS data was acquired for 30 min in 815 cycles, each

cycle consisting of a Q1 scan from 50 to 350 m/z , followed by a product-ion scan of the most intensive ions in the Q1 scan.

5.3 Results

5.3.1 Isolation, Characterization and Identification of Isolates

From microcosms constructed with aquifer sand and UF, three phenotypically distinct (based on colony morphology) strains of bacteria capable of growing on E1 and E2 were isolated. The three strains were denoted as strain LHJ1, LHJ3 and CYH and were assumed to originate from the sand rather from the UF because ultrafiltration is expected to have removed bacterial cells.

Strain CYH was obtained from a yellow color colony, which appeared after 2 days on TSA plates inoculated with enrichment culture supernatant. It grew relatively faster than other estrogen-degrading bacteria isolated from this study. Strain CYH is a rod-shape, gram negative, catalase positive, oxidase positive bacterium. Cells were between 0.5 and 3 μm in length and were 0.2-0.4 μm in diameter when grown in TSB medium at 30°C (Figure 5.1). Cells were nonmotile and flagella were not observed. LHJ1 was a gram-negative coccoid bacterium and LHJ3 was a gram-positive long rod shaped bacterium (Figure 5.1).

The 16s rDNA results were input to the BLAST algorithm from the National Center for Biotechnology Information, USA (NCBI) and known sequences in the GenBank databases for searching the similarities between the sequences of the isolated degraders and other species. The BLAST result is shown in Table 5.2.

Table 5.2 BLAST result for the isolates: CYH, LHJ1 and LHJ3

degrader	Most closet sequence	Access No.	Similarity	Base Pair
CYH	<i>Sphingomonas amiense</i>	SPP16SRD6	95%	1444
LHJ1	<i>Acinetobacter sp. AU783</i>	AY847284.1	98%	1427
LHJ3	<i>Agromyces sp.auranticcus</i>	AF389342.1	98%	1386

5.3.1.1 Phylogenetic Analysis of CYH

Base on the Blast analysis CYH was most similar to *Sphingobium amiense*, with a 16S rRNA sequence identity of 95.7%. Therefore, CYH could potentially be a new species of this genus. The sequence of the strain CYH has been deposit to GenBank with asscession number of DQ855413. The 16S rRNA gene sequence of strain CYH was almost identical to a newly isolated, unidentified *Sphingomonas* sp. str. JEM-14 (AB219361). Phylogenetic analysis based on 1275 unambiguous bases placed strain CYH with other members of the genus *Sphingomonas/Sphingobium*. A maximum likelihood tree generated by fastDNAm1 is shown in Figure 5.2. Strain CYH clustered closely with *S. chlorophenolica* ATCC 33790(T), *S. yanoikuyae* IFO 15102(T), *S. suberifaciens* IFO 15211, *S. herbicidovorans* str. MBIC3166, and *S. amiense* YT. A distance and identity matrix was calculated (Appendix 4) and showed that the sequence of strains CYH/JEM-14 was most similar to *S. amiense* with the sequence identity of 95.7%. The sequence identities between strain CYH and other representative species ranged from 95.5 to 92.4%. Based on taxonomic and 16S rRNA data, strain CYH was named *Sphingobium estrogenivorans* sp. nov.

5.3.1.2 Physiological Characteristics for CYH

The physicological characteristics for CYH was tested by ABI ZYM and API 20E assays. The following characteristics are positive for strain CYH: alkaline phosphatase, esterase, esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase,

trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, lysine decarboxylase, acetoin production and gelatinase.

The following characteristics are negative for strain CYH: lipase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, arginine dihydrolase, ornithine decarboxylase, citrate utilization, H_2S production, urease, tryptophane deaminase, indole production, glucose, mannitol, inositol sorbitol rhamnose sucrose melibiose amygdalin, arabinose and nitrate respiration.

Figure 5.1 Phase contrast microscopic pictures of the strain (a) CYH (bar 2 μ m), (b) HJ 3 (bar 4 μ m) and (c) LHJ1 (bar 1 μ m).

Fig. 5.2 16S rRNA phylogenetic tree for *Sphingobium estrogenivorans* sp. nov. str. CYH. The numbers at the branch nodes are bootstrap values based on 100 resampling for maximum likelihood. Only bootstrap values greater than 60% are shown. The sequence for strain CYH has been deposited in the GenBank database under accession no. DQ 855413. The GenBank accession numbers of the other sequences used in the phylogenetic analysis are shown after each species name. Scale bar Nucleotide divergence of 1%.

5.3.2 Estrogen-degrading Ability and kinetic study

The three isolates were examined individually and as a consortium for their ability to degrade E1, E2, E3 and EE2, which were fed as a mixture. Result obtained under aerobic conditions are shown in Figure 5.3. The consortium transformed E1, E2 and E3 within 2 days whereas EE2 was stable in the consortium and in individual cultures (Figure 5.3d). CYH and LHJ3 transformed E2 rapidly (<2days) but in LHJ1 it took 15 days. E3 was degraded by the consortium and by LHJ3 only. E1 was transformed rapidly by the consortium and CYH but was formed in LHJ1 and LHJ3. In LHJ3 E1 remained stable at approximately 140% suggesting the E1 was a stable metabolite. In LHJ1, however, the appearance of E1 appears to have been transient with its concentration first increasing to 200% before declining, suggesting further transformation. All four substrates were stable in the control sample.

The formation of E1 as a transient metabolite under aerobic conditions was investigated for CYH by feeding E2 as the sole substrate (Figure 5.4). The transformation of E1 was rapid relative to its formation with its maximum concentration never exceeding approximately 20% of the initial concentration.

Under anoxic conditions (Figure 5.5), E2 was completely degraded by LHJ3 and to approximately 80% and 25% respectively, by CYH and the consortium. It is not clear whether the apparent stability after the initial drop in concentration is because some trace nutrients are lacking. None of the degraders degraded E3 and EE2 under anoxic conditions. Interestingly, degradation of EE2 was observed when minimal medium was inoculated aerobically with groundwater. Figure 5.6 shows the removal of EE2 in groundwater enrichments, which took 40 days at an initial concentration of 1200 µg/L.

Figure 5.3 Aerobic degradation profiles of (a) estrone (E1), (b) 17 β -estradiol (E2), (c) estriol (E3) and (d) 17 α -ethinylestradiol by CYH (Δ), LHJ1 ($\circ$), LHJ3 ($\square$), consortium ($\bullet$) and negative control (autoclaved cells) (*).

Lag phases were only observed for the degradation of E1 by CYH and E3 by LHJ3 under aerobic conditions at high concentrations (above 500 $\mu\text{g/L}$ E1 and above 2000 $\mu\text{g/L}$ E3 for LHJ3) (Figure 5.7a and Figure 5.7b). Under aerobic conditions, the relationship between the different initial substrate concentrations and the initial degradation rates was linear ($R^2 > 0.9$) (Figure 5.8a). The degradation of E1 and E2 by CYH was much faster than the degradation of E2 and E3 by LHJ3 for the same initial concentration, while the degradation of E1 and E2 had similar degradation rates by the strain CYH. In contrast to

the linear relation, the degradation of E1 by CYH under anoxic conditions fitted well to the Michaelis-Menten equation (Figure 5.8 b) with a specific degradation rate (V_{max}) of 18.9 $\mu\text{g/L-h}$ and a half-saturation constant (K_m) of 106 $\mu\text{g/L-h}$.

Figure 5.4 E2 ($\square$) degradation by the strain CYH under aerobic condition [E1 ($\circ$) metabolite was formed].

Figure 5.5 Anoxic degradation profiles under conditions (a) estrone (E1), (b) 17 β -estradiol (E2), (c) estriol (E3) and (d) 17 α -ethinylestradiol (EE2) by CYH (Δ), LHJ1 ($\circ$), LHJ3 ($\square$), consortium ($\bullet$) and negative control (autoclaved cells (*)).

Figure 5.6 Degradation of EE2 in ground water enrichment sample under aerobic conditions: first round enrichment sample—○— where 2 mL of groundwater is inoculated into the MM containing 1 mg/L EE2; second round enrichment sample —□— where 5 mL of the first round sample is inoculated into the new MM containing 1 mg/L EE2.

Figure 5.7 Aerobic degradation of E1 by the strain CYH (a) and E2 by the strain LHJ3 (b) under different initial concentration.

Figure 5.8 degradation kinetic under aerobic condition (a) and anoxic conditions (b): (□) E1 by CYH, (■) E2 by CYH, (▲) E2 by LHJ3 and (Δ) E3 by LHJ3.

5.3.3 Degradation Products

In aerobic cultures of LHJ3 fed with E3 we detected a transient transformation product with an apparent molecular weight of 286 ($[M-H]^- = 285$) (Figure 5.9). The metabolite eluted at a retention time of 10.4 minutes and showed major fragments at m/z 213, 145, and 171 (Figure 5.10). It appeared on the first day, accumulated for three days, and then decreased until it disappeared on day 6.

Figure 5.9 Q1 spectrum for the E3 metabolite (3 days sample) for $[M-H]^-$ ions at a retention time of 10.4 minutes.

This product was confirmed as 16 α -hydroxyestrone (Figure 5.11), by injecting a standard and comparing the mass spectrum and retention time. This compound was previously identified as a metabolites of E2 degradation by sewage bacteria (Lee and Liu 2002) and of E1 by intestinal bacteria *Streptococcus faecalis* and *Bacteroides fragilis*

(Järvenpää *et al.*,1980). In addition, *Staphylococcus aureus* has been found to reduce 16 α -hydroxyestrone to E3 under anaerobic conditions (Jävenpää *et al.*, 1980).

Figure 5.10 Production-ion spectra of the E3 metabolite (3 days sample) for [M-H]⁻ ion m/z 285.

5.4 Discussion

5.4.1 Estrogen-degrading Bacteria

This study demonstrates the presence of estrogen degrading bacteria in marine sand used to reclaim land, consistent with observations by others that such bacteria are wide spread in the environment. Several estrogen-degrading microorganisms have been isolated and characterized in recent years. A novel Gram-negative *Novosphingobium* species was identified and its degradability of E2 was characterized in detail (Fujii *et al.*, 2002). In

addition, two Gram-positive *Rhodococcus* species, *R. zopfii* and *R. equi*, were reported to completely and rapidly degrade 100 mg L⁻¹ of E1, E2, E3 and EE2 (Yoshimoto *et al.*, 2004). This is the only published data where pure microbial cultures were shown to degrade all four estrogens. Other microorganisms reported to degrade estrogens include a fungal species (Shi *et al.*, 2002) as well as several mixed cultures (Shi *et al.*, 2004b, Yu *et al.*, 2005). A more recent study by Yu *et al.* (2006) characterized 14 phylogenetically diverse 17 β -degrading bacteria from sewage sludge. As a matter of fact, almost all of these isolates were obtained from enrichment cultures using activated sludge as an initial inoculum source. It is well known that activated sludge has typically a high degree of microbial diversity, which may enhance the likelihood of selecting for specific bacterial strains with the desired catabolic capability. However, strain CYH presented in this study was isolated from a tropical reclaimed aquifer site for possible water reclamation purposes. With respect to this site, the aquifer groundwater as well as recharge water contained very limited nutrients (Total organic carbon 4 mg/L; NO₃ 0.65 mg/L; P 0.67 mg/L in ground water) which might not efficiently support the growth of microorganisms and microbial activity as compared to normal activated sludge systems (Chua *et al.*, 2006). The fact that isolates can survive in such harsh conditions reveals that this bacteria has strong compatibility and the capability to adapt and thrive in oligotrophic aquifer environments. Since isolates are indigenous bacterium capable of degrading estrogens, it can potentially be used in *in situ* or *ex situ* bioremediation schemes to treat estrogens containing reclaimed and/or recharge waters. Furthermore, the presence of naturally occurring estrogens-degrading bacteria at the aquifer site shows a strong potential that residual estrogens in recharge water could be successfully removed during the soil aquifer treatment (SAT) process.

One of the isolates, *Acinetobacter* spp., has been found to be widespread in water and soil (Abdel-EI-Hallem, 2003). It has been reported that several strains of *Acinetobacter* can degrade various xenobiotic pollutants, such as phenol, toluene, and benzoates (Abdel-EI-Hallem 2003), This report demonstrates its ability to degrade estrogens. However, the application of the other isolate, *Agromyces* spp., in the environment is limited.

Figure 5.11 MRM chromatograms of 1 day sample for the degradation of E3 by LHJ3 under aerobic conditions.

Phylogenetic analysis placed the other isolate, strain CYH, in the *Sphingobium* genus, a recent classification from genus *Sphingomonas*. The old *Sphingomonas* genus included more than 40 species which carried relatively diverse physiological and phylogentic characterizations. As a result, the genus *Sphingomonas* was proposed to be subdivided into four genera including *Sphingomonas*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi et al., 2001). The sphingomonads are widely distributed in nature and have been isolated from many different aqueous and terrestrial habitats, as well as from plant root systems (Balkwill et al., 2003). It is interesting that sphingomonads have been reported to have the capability to degrade a broad range of xenobiotic contaminants. *S. TTNP3* and *S. coacae* S-3^T were able to assimilate chlorinated phenols (Corvini et al.,

2004, Fujii et al., 2001). *S. xenophaga* has been reported to assimilate several aromatic and chloroaromatic compounds (Stolz *et al.*, 2000) and a *Novosphingobium* sp., as mentioned earlier, isolated from activated sludge has been found to degrade E2 (Fujii et al., 2002, Fujii et al., 2003). The ubiquitous distribution and broad xenobiotic compounds utilization make the sphingomonads an attractive microbial agent to be applied to bioremediation processes.

5.4.2 Degradation of Estrogens

The results of the present study are consistent with previous observations that E2 is highly susceptible to biodegradation with the transformation to E1 being the first step. The kinetic data shows that the degradation rate of E1 by CYH is faster than those observed for nitrifying activated sludge (Shi *et al.*, 2004a). Although the degradation of E3 by LHJ3 was the slowest among the degradation of estrogens by these three isolates, its degradation ability is comparable to the study by Shi *et al.* (2004a). Considering that the expected aerobic residence times in the ground are relatively short (7 days) it is important that this compound is degraded rapidly.

Under aerobic conditions, the degradation rates of the estrogens increased with the initial concentration (Figure 5.7). It is interesting to note that the degradation of E3 did not occur below a threshold initial concentration ($\sim 80\mu\text{g/L}$) (Figure 5.9) in contrast to the degradation of E1 and E2 by CYH and the degradation of E2 by LHJ3. The fact that E3 was not degraded below a minimum concentration is interesting and could be of practical significance because in effluent and under recharge conditions, concentrations are likely much lower. Therefore it may not be possible to extrapolate these results directly to environmental conditions where concentrations are typically many orders of magnitude lower. On the other hand, under anoxic conditions, the degradation kinetic of E1 by CYH well obey the Michaelis-Menten model. This indicates that the degradation mechanisms were different under aerobic and anoxic conditions. Hence, different enzymes may be involved in the degradation under aerobic and anoxic conditions. That lag phase was

observed only under aerobic conditions also confirms the different mechanisms under the two conditions.

The fact that E2 was degraded under anoxic conditions is consistent with other studies. For example, slow E2 degradation was found in an aquifer material from Australia with a half-life of 107 days (Ying *et al.*, 2003); E2 was also degraded after 70 days of anaerobic incubation in marine sediments which is much slower than that under aerobic conditions (Ying and Kookana 2003). However, to date, there have been no reports on the degradation of E1 under anoxic conditions, which could be an important characteristic from a practical point of view. At actual aquifer site in this study, the groundwater is not strictly anaerobic but contains approximately 1.0 mg/L dissolved oxygen.

It is noted that the performance for degradation of E1 and E2 by a consortium of these three degraders was better than the individual degrader under aerobic conditions, but was worse under anoxic conditions. The reason is not clear. It is also noted that under anoxic conditions, the degradation of E2 by the consortium was not complete within 30 days but the degradation of E1 was completed within 14 days. This indicates that E1 was more readily degraded by the degraders than E2.

In the case of EE2, degradation was found in the enrichment sample but not by the isolated degraders under aerobic conditions. Therefore, the removal of EE2 may be attributed to other bacteria from the groundwater. These bacteria have not been isolated by our current isolation method possibly because it is non-culturable.

5.5 Conclusions

These three isolated degraders showed the ability to degrade E2 under aerobic conditions. However, only CYH could degrade E1 and only LHJ3 could degrade E3. Under anoxic conditions, CYH and LHJ3 could only degrade E1 and E2 respectively but

the degradation rates were much slower than for aerobic conditions. None of the degraders could degrade E3 under anoxic condition. Under aerobic condition, an E3 degradation product was found to be 16-hydroxyl-estrone. With respect to the synthetic estrogen, 17 α -ethinylestradiol (EE2), ground water samples from the aquifer site showed the ability to degrade EE2. However, the degrading microorganisms for this compound have not been isolated yet.

The rapid degradation of E1 and E2 under aerobic conditions indicates that these compounds have high potential to be removed during the SAT process. The Changi aquifer site is generally anoxic where the average dissolved oxygen concentration of ground water is around 1 mg/L. E1 and E2 can also be removed under anoxic conditions, albeit the rate is much slower. Therefore, the majority of E1 and E2 could be removed during the infiltration process and further attenuation of E1 and E2 can be expected to happen in the aquifer during the storage stage. Although slow degradation of E3 and EE2 was observed under aerobic conditions, they tend to be more persistent under anoxic conditions as found in the aquifer.

Chapter 6 Conclusions and Recommendations

This doctoral dissertation is a study on the fate of estrogens in a tropical sandy aquifer in Singapore. The study includes three parts: (1) development of analytical methods for estrogens; (2) analysis of the sorption and degradation behavior in the sandy aquifer and (3) isolation of the estrogen-degrading bacteria from the aquifer recharge system and characterization of the bacteria in terms of identifying the bacteria and evaluation of the degradation ability for estrogens. In this chapter, the results which we have obtained in this thesis are summarized and future research issues are discussed.

6.1 Conclusions

In this study, a sensitive LC-MS-MS method in conjunction with a single step solid phase extraction pretreatment method was developed. The instrument detection limit of the LC-MS-MS method could reach 0.1 pg (absolute injected amount). The linearity of each target compounds was higher than 0.99, at concentrations ranging from 1 to 100 µg/L. The single step solid phase extraction pre-treatment method showed good recovery for ultrapure water when the sample was concentrated 1000 times, which resulted in the method detection limit of ng/L level. However, when the method was applied to natural water samples poor recoveries were observed even for the cleaner water matrices (i.e. tap water and Newater). The poor recoveries for the clean water matrices were due to the presence of free chlorine which oxidized the spiked target analytes. Thus, sodium thiosulphate was added to remove the free chlorine and the recoveries of the target compounds were improved. With respect to water with complex matrices, the method detection limit did not decrease linearly with the increase in concentration factor as the sample matrix was also concentrated. Therefore, for water with complex matrices, low concentration factors are suggested. In addition, a calibration standard prepared in the same matrix as the sample is also suggested for quantifying the water samples.

The sorption and degradation behaviour of estrogens were examined by batch experiments under different redox conditions for a UF/GW mixture. In addition, a set of columns packed with aquifer sand was used to simulate the SAT process and assess the attenuation behaviour of these compounds under UF recharge conditions. Another set of batch experiments using UF was conducted to serve as control for the column study.

The results from the sorption study indicate that the sorption ability of the estrogens were in the following order: $E3 < EE2 < E2 < E1$. This is inconsistent with their physicochemical properties and hence, hydrophobic partitioning may not be the dominant sorption mechanism. In addition, it may be due to the inadequate estimation of K_{oc} value by using Freundlich model. The K_{oc} values of estrogens in this study were significantly lower than those reported in other similar studies. This is due to the different compositions of the aquifer materials, especially the low organic content of the aquifer material used in this study ($f_{oc}=0.3\%$).

The degradation study showed that E2 was the most readily degradable compound among these target compounds. The rapid degradation of E2 was found in all the water matrices under aerobic conditions. Its metabolite, E1 was also found to rapidly degrade in all the matrices under aerobic conditions. Under anoxic conditions, E1 and E2 were inter-convertible and reached steady state after 50 days incubation in UF and after 20 days incubation in GW/UF (v/v 1:1). E2 exhibited sorption ability to the aquifer sand in the presence of UF. In the column study, E2 was removed rapidly and could not be detected after 0.25 m infiltration in the column. Its degradation metabolite, E1, could be detected only within 1 meter infiltration in the UF column. In contrast with E2, EE2 was more persistent. It was found to be biodegradable under both aerobic and anoxic conditions. Chemical transformation could also be considered as one of the removal mechanisms. In the column study, a bell shaped curve was obtained for the removal of

EE2. The mechanism is not clear but sorption and desorption may be the reason for this removal trend.

The column results revealed that acclimatization of the aquifer sand appears to be an important factor in enhancing the removal of target compounds. This is important to the practice of SAT as the target compounds have potential to be removed if there is sufficient long term storage. In addition, degradation plays a more important role than sorption in the removal of target compounds especially for the readily degradable compound, E2.

Three strains of estrogen-degrading bacteria, namely LHJ1, LHJ3 and CYH, were isolated from the aquifer sand/ ultra-filtered recharge water system in this study. Based on gram-stain morphology and 16S rRNA sequence homology, LHJ1 and LHJ3 were found to belong to the genus *Acinetobacter* and *Agromyces* respectively, while phylogenetic analysis revealed that strain CYH was most similar to *Sphingobium amiense*, with a 16S rRNA sequence identity of 95.7%. Based on taxonomic and 16S rRNA data, strain CYH was named *Sphingobium estrogenivorans* sp. nov. These three degraders showed the ability to degrade E2 under aerobic conditions. However, only CYH could degrade E1 and only LHJ3 could degrade E3. Under anoxic conditions, CYH and LHJ3 could only degrade E1 and E2 respectively but the degradation rates were much slower than for aerobic conditions. None of the degraders could degrade E3 under anoxic conditions. Under aerobic conditions, an E3 degradation product, 16-hydroxyl-estrone, was found. With respect to the synthetic estrogen, 17 α -ethinylestradiol (EE2), ground water samples from the aquifer site showed the ability to degrade EE2. However, the degrading microorganisms for this compound have not been isolated yet.

6.2 Recommendations

The SPE method developed in this study cannot efficiently remove the interferences from complex matrices. Further optimization can be done by using different cartridges for concentrating the samples. The newly developed polymer based C18 cartridge can be used for sample concentrate and clean up. According to literature, Oasis HLB C18 column and GCB cartridge may give better performance (Lagana *et al.*, 2004; Baronti *et al.*, 2000). A sequential clean up step could be used for further eliminating the interference from the sample, such as using a silica column or Florisil cartridges for further removing the interferences. On the other hand, considering the time consuming and labour intensive method of SPE, on-line SPE system can be considered which has been reported to result in improved repeatability and accuracy and speed (López de Alda *et al.*, 2003). In addition, solid phase microextraction (SPME) could also be considered as an alternative pretreatment method. It is characterized by high precision, reproducibility, good linearity, fast, simple and solvent free operation (Braun *et al.*, 2003). This method is usually coupled with gas chromatography, but a successful analysis of estrogens in environmental water with in-tube solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry has been developed by Mitani *et al.* (2005), with a detection limit of 2.7 ng/L for E1, 7.4 ng/L for E2, 11.7 ng/L for E3 and 10.5 ng/L for EE2. Therefore, the SPME method coupled with LC-MS-MS can be explored.

The strain CYH represents a novel species of *Sphigobium* genus which is able to degrade E1 and E2 rapidly under aerobic as well as degrade E1 under anoxic conditions. The experiment results indicate that the degradation mechanisms are different under aerobic and anoxic conditions. In addition, under anoxic conditions, the degradation may undergo an aerobic degradation pathway followed by anaerobic degradation pathways. Therefore, there is a need to understand the relationship between the oxygen content and the degradation behavior of E1. This information may be useful to predict the degradation behaviour in actual site conditions, as our site condition is microaerophilic. In addition, *Sphigobium* was divided from genus *Shpingomonas*, which has been reported to have the capability to degrade a broad range of xenobiotic

contaminants (Corvini et al., 2004, Fujii et al., 2001 Stolz et al., 2000). Therefore, the degradation ability of the strain, CYH, for other endocrine disrupting chemicals could be further investigated.

The detailed degradation pathways were not investigated in this study due to the limitation of triple-quadrupole mass spectrometer which has low sensitivity in the identification of metabolites by the full-scan mode (Kostianinen *et al.*, 2003). Therefore, other analytical methods could be used. For example, ion-trap MS with its MSⁿ (multi-stage mass spectral scans) capability is highly efficient in the structural analysis of metabolites (Kostianine *et al.*, 2003). In addition, in order to assess the environmental impact of the degradation of target compounds, bioassays could be used to examine the estrogenic activities of the degradation products formed from these target compounds.

Finally, the experiments conducted in this thesis were done under laboratory controlled conditions. Care should be taken when extrapolating these results to real environmental conditions. At the test site, a screening study for the target compounds before and after recharging the UF water was conducted. However, due to the very low concentration of target compounds in the groundwater and UF water (only E1 was detected in the ground ranging from 7 ng/L to 25 ng/L) we were unable to find a removal trend for the target compounds in the field study. Therefore, a more sensitive detection method may need to be used in future studies or the target compounds could be spiked into the site to investigate the removal of such compounds in the real environment.

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Appendix 1: liquid-liquid extraction procedure:

1. Measure 1 mL water sample in 2 mL centrifuge tube.
2. Add in 1 mL dichloromethane in the tube.
3. Vortex the tube for 3 minutes.
4. Discard the aqueous layer.
5. Evaporate the remaining organic to dryness by vacuum centrifuge.
6. reconstitute the sample with reconstitute solution.

Appendix 2

Changi site sand characteristics

Sample	A	B	Average
Dry loss, %	0.18	0.18	0.18
Nitrogen, %	<0.01	<0.01	<0.01
Total carbon, %	1.66	1.61	1.64
Carbonate C, %	1.39	1.31	1.35
Organic C, %	0.27	0.30	0.29
Volatile matter, %	0.64	0.66	0.65
Iron, ppm	3880	3730	3805
Phosphorus, ppm	76	58	67
Volatile solids at 550°C, %	0.64	0.66	0.65
Volatile solids at 750°C, %	5.60	5.27	5.44
Cation exchange capacity, CEC, meq/100g	2.7	-	2.7
pH, saturated paste	7.9	-	7.9

Appendix 3

water quality of Changi site aquifer water

1. general water quality

Location	Parameters	Sampling date (day/month/year)				
		20/01/2003	10/04/2003	04/07/2003	09/10/2003	12/02/2004
AQ1	pH	-	7.57	9.11	7.14	7.66
	Temperature (° C)	-	30.16	30.58	-	30
	Conductivity (mS/cm)	-	12.916	3.684	9.868	1.43
	Salinity (ppt)	-	7.37	1.92	5.50	-
	Total suspended sloid (mg/L)	3.6	14.2	11.6	9.5	2.15
	Oxidation Reduction Potential (mV)	-	-174	-218	-179	-214
AQ2	pH	7.55	7.83	8.54	-	7.96
	Temperature (° C)	30.62	30.00	30.76	-	30.00
	Conductivity (mS/cm)	3.290	12.900	8.034	-	2.12
	Salinity (ppt)	1.70	7.36	4.41	n.s.	-
	Total suspended sloid (mg/L)	6.2	14.8	10.5	-	1.65
	Oxidation Reduction Potential (mV)	-	-179	-236	-	-212
AQ3	pH	7.69	7.65	8.48	8.10	7.55
	Temperature (° C)	29.73	30.06	30.25	n.s.	30
	Conductivity (mS/cm)	12.670	11.960	6.087	n.s.	3.09
	Salinity (ppt)	7.22	6.75	3.28	-	-

	Total suspended sloid (mg/L)	4.0	30.0	16.5	6.0	5.0
	Oxidation Reduction Potential (mV)	-	-182	-192	-107	-221
AQ4	pH	7.56	7.36	8.12	7.92	7.57
	Temperature (° C)	29.99	30.11	30.79	30.29	29
	Conductivity (mS/cm)	3.223	21.330	14.490	3.137	1.14
	Salinity (ppt)	1.67	12.72	8.34	1.59	-
	Total suspended sloid (mg/L)	5.2	62.1	5.5	5.0	5.05
	Oxidation Reduction Potential (mV)	-	-155	-178	-75	-203

AQ: aquifer water and location in parentheses (see Figure 4.1); -: not available; n.s. not stable reading.

2. Inorganic parameters in aquifer

Location	Parameters	Sampling date (day/month/year)				
		20/01/2003	10/04/2003	04/07/2003	09/10/2003	12/02/2004
AQ1	Nitrate (mg/L)	0.5	0.3	0.35	0.55	<0.10
	Sulfate (mg/L)	340	332.5	500	434	205
	Dissolved oxygen (mg/L)	-	0.02	0.08	1.35	0.31
AQ2	Nitrate (mg/L)	0.4	0.7	0.65	-	<0.1
	Sulfate (mg/L)	430	925	575	-	431
	Dissolved oxygen (mg/L)	-	0.03	0.06	-	0.35
AQ3	Nitrate (mg/L)	0.5	1.0	0.95	0.5	0.35
	Sulfate (mg/L)	460	687.5	550	424	-
	Dissolved oxygen (mg/L)	-	0.02	0.05	0.10	0.32
AQ4	Nitrate (mg/L)	0.6	0.2	0.65	0.3	0.35
	Sulfate (mg/L)	380	537.5	1013	344	206
	Dissolved oxygen (mg/L)	-	0.03	0.27	0.21	0.97

AQ: aquifer water and location in parentheses (see Figure 4.1); -: not available

3. Metals and total organic carbon

Location	Parameters	Sampling date (day/month/year)				
		20/01/2003	10/04/2003	04/07/2003	09/10/2003	12/02/2004
AQ1	Fe (III) (mg/L)	0.0282	0.0148	0.0256	0.0231	0.0310
	Mn (IV) (mg/L)	0.1117	0.1570	0.2961	0.1142	0.0449
	Total organic carbon (mg/L)	-	2.325	2.089	1.982	1.162
AQ2	Fe (III) (mg/L)	0.0270	<DL	0.0401	-	0.0227
	Mn (IV) (mg/L)	0.1060	1.8300	0.3417	-	0.3246
	Total organic carbon (mg/L)	-	3.822	3.868	-	2.116
AQ3	Fe (III) (mg/L)	0.0247	0.0087	0.0252	0.0181	0.0271
	Mn (IV) (mg/L)	0.1723	0.4439	0.2095	0.1280	0.2623
	Total organic carbon (mg/L)	-	4.484	4.918	3.284	3.170
AQ4	Fe (III) (mg/L)	0.0265	0.0121	0.0255	0.0208	0.0310
	Mn (IV) (mg/L)	0.0427	0.2396	0.3999	0.0734	0.2623
	Total organic carbon (mg/L)	-	2.946	3.804	2.069	1.045

AQ: aquifer water and location in parentheses (see Figure 4.1); -: not available; <DL below detection limit.

Appendix 4

levels of 16S rDNA similarity for the members of the genus *Sphingomonas*.

strain	% rDNA similarity
<i>Sphingobium estrogenivorans</i> sp. CYH	100
<i>Sphingomonas</i> sp. JEM-14	99.8
Uncultured soil bacterium Clone749-2	97.4 97.5
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	95.2 95.3 95.3
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	94.8 94.9 94.9 96.3
<i>Sphingomonas aquatilis</i> KCTC 2882.	95.0 95.2 94.5 93.3 93.3
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	94.6 94.8 94.3 93.1 92.7 97.3
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	93.7 93.9 93.9 95.6 98.8 93.7 93.1
Uncultured soil bacterium Clone749-2	94.5 94.7 94.2 92.8 92.8 96.8 98.8 93.4
<i>Sphingomonas</i> sp. JEM-14	95.5 95.7 95.5 94.0 94.0 97.9 96.6 94.3 96.7
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	95.4 95.6 95.8 93.9 93.7 97.7 96.5 94.0 96.6 99.3
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	95.4 95.6 95.7 93.7 93.7 97.9 96.3 94.0 96.4 99.3 99.8
<i>Sphingobium herbicidovorans</i>	94.8 94.9 94.8 96.2 97.9 93.1 92.6 97.0 92.5 93.6 93.6 93.4
<i>Novosphingobium aromaticivorans</i> IFO 16084	92.5 92.6 92.5 92.5 91.6 94.8 93.0 92.2 93.5 94.3 94.8 94.8 91.8
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	92.4 92.5 91.9 92.7 91.3 93.2 92.3 91.8 92.8 93.1 93.7 93.6 91.2 97.8
<i>Sphingomonas xenophaga</i>	93.7 93.8 94.8 95.2 95.3 94.1 93.7 95.3 93.7 94.7 94.4 94.4 95.2 93.1 92.8
<i>Sphingobium amiense</i> YT	95.7 95.8 95.7 97.5 97.4 94.3 93.6 96.9 93.7 94.6 94.3 94.3 96.9 93.0 92.9 96.7
<i>Sphingomaonas aurantiaca</i> MA101b	94.3 94.4 94.0 93.0 92.9 96.2 94.9 92.9 94.6 96.1 96.5 96.5 92.8 94.8 93.7 93.2 93.6
<i>Sphingomonas cloacae</i> JCM 10874	93.6 93.7 94.2 95.4 96.3 94.8 93.6 96.6 93.1 94.5 94.3 94.5 96.5 92.7 91.6 95.0 96.3 93.9
<i>Sphingobium francense</i>	93.5 93.7 93.7 95.3 98.6 94.0 93.1 99.5 93.3 94.0 93.7 93.7 96.7 92.5 91.7 95.3 96.8 92.7 96.3
<i>Sphingomonas melonis</i> DAPP-PG224	95.3 95.4 95.3 93.9 93.1 97.4 96.4 93.5 96.7 97.9 98.6 98.6 93.3 94.8 93.7 94.2 94.1 96.7 94.6 93.3
<i>Sphingomonas phyllosphaerae</i> FA2	93.9 94.1 93.8 93.0 93.1 97.9 96.6 93.5 96.5 96.7 96.7 96.6 93.1 93.4 92.3 93.5 93.6 95.0 93.9 93.7 96.8
<i>Sphingomonas roseiflava</i> MK314	94.6 94.8 94.2 93.1 92.9 96.2 98.3 92.7 98.6 96.0 95.9 95.8 92.8 92.8 92.3 93.6 93.7 94.4 92.9 92.6 96.5 96.0
<i>Sphingomonas suberifaciens</i> IFO 15211	94.6 94.8 94.9 94.8 94.1 94.4 93.6 94.3 93.7 95.1 95.7 95.5 94.6 94.3 93.7 95.4 94.7 94.0 94.9 94.1 95.5 94.0 93.1
<i>Sphingomonas aerolata</i>	94.8 94.9 94.5 93.1 93.3 95.8 94.5 93.0 94.6 96.2 96.6 96.6 93.1 95.2 94.3 93.3 93.8 99.1 93.5 92.8 96.7 94.6 94.3 94.1
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	92.0 92.2 92.8 91.5 91.4 92.7 92.1 91.2 92.2 92.5 92.3 92.2 90.9 93.4 93.1 92.4 92.5 92.1 91.2 91.5 91.7 92.3 91.7 92.1 92.8
<i>Erythrobacter longus</i> DSM 6697	91.0 91.2 91.6 90.3 89.7 91.5 90.9 89.4 91.0 91.0 91.1 91.0 89.6 92.6 92.1 90.3 90.6 91.0 89.5 89.7 90.5 91.2 90.4 90.7 91.5 97.1
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	90.7 90.9 91.3 90.1 91.0 92.4 91.9 91.2 92.0 92.7 92.7 92.8 91.5 91.7 91.5 91.2 91.2 92.0 91.2 91.5 92.5 92.0 91.7 92.4 92.5 91.8 90.8
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	76.8 77.0 77.5 77.0 77.5 78.2 78.0 77.5 77.9 78.2 78.2 78.1 77.6 78.5 78.8 77.5 77.3 77.4 77.6 77.6 78.1 78.8 77.8 78.3 77.4 78.3 77.6 79.6 100
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingobium herbicidovorans</i>	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Sphingomaonas aurantiaca</i> MA101b	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium francense</i>	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas aerolata</i>	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084.	
<i>Novosphingobium capsulata</i> ATCC 14666 (T).	
<i>Sphingobium herbicidovorans</i>	
<i>Sphingomonas pruni</i> IFO 15498 (T).	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T).	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingobium herbicidovorans</i>	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Sphingomaonas aurantiaca</i> MA101b	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium francense</i>	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas aerolata</i>	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084.	
<i>Novosphingobium capsulata</i> ATCC 14666 (T).	
<i>Sphingobium herbicidovorans</i>	
<i>Sphingomonas pruni</i> IFO 15498 (T).	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T).	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingobium herbicidovorans</i>	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Sphingomaonas aurantiaca</i> MA101b	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium francense</i>	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas aerolata</i>	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084.	
<i>Novosphingobium capsulata</i> ATCC 14666 (T).	
<i>Sphingobium herbicidovorans</i>	
<i>Sphingomonas pruni</i> IFO 15498 (T).	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T).	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingobium herbicidovorans</i>	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Sphingomaonas aurantiaca</i> MA101b	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium francense</i>	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas aerolata</i>	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084.	
<i>Novosphingobium capsulata</i> ATCC 14666 (T).	
<i>Sphingobium herbicidovorans</i>	
<i>Sphingomonas pruni</i> IFO 15498 (T).	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T).	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15498 (T)	
<i>Sphingobium herbicidovorans</i>	
<i>Novosphingobium aromaticivorans</i> IFO 16084	
<i>Novosphingobium capsulata</i> ATCC 14666 (T)	
<i>Sphingomonas xenophaga</i>	
<i>Sphingobium amiense</i> YT	
<i>Sphingomaonas aurantiaca</i> MA101b	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium francense</i>	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas aerolata</i>	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Rhodanobacter lindaneticus</i> str. RP5557 LMG 18385 (T)	
<i>Sandaracinobacter sibiricus</i> str. RB16-17 (T)	
<i>Erythrobacter longus</i> DSM 6697	
<i>Erythrobacter litoralis</i> str T4 DSM 8509 (T)	
<i>Sphingomonas suberifaciens</i> IFO 15211	
<i>Sphingomonas roseiflava</i> MK314	
<i>Sphingomonas phyllosphaerae</i> FA2	
<i>Sphingomonas melonis</i> DAPP-PG224	
<i>Sphingobium francense</i>	
<i>Sphingomonas cloacae</i> JCM 10874	
<i>Sphingobium amiense</i> YT	
<i>Novosphingobium aromaticivorans</i> IFO 16084.	
<i>Novosphingobium capsulata</i> ATCC 14666 (T).	
<i>Sphingobium herbicidovorans</i>	
<i>Sphingomonas pruni</i> IFO 15498 (T).	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T).	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T).	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium estrogenivorans</i> sp. CYH	
<i>Sphingomonas</i> sp. JEM-14	
Uncultured soil bacterium Clone749-2	
<i>Sphingobium yanoikuyae</i> GIFU 9882 (T)	
<i>Sphingobium chlorophenolica</i> ATCC 33790 (T)	
<i>Sphingomonas aquatilis</i> KCTC 2882.	
<i>Sphingomonas sanguinis</i> IFO 13937 (T).	
<i>Sphingobium japonicum</i>	
<i>Sphingomonas parapaucimobilis</i> JCM 7510 (T)	
<i>Sphingomonas asaccharolytica</i> IFO 10564.	
<i>Sphingomonas mali</i> str. Y-347 IFO 15500 (T)	
<i>Sphingomonas pruni</i> str. Y-250 IFO 15	