

2011

Biodegradation of Bisphenol-A and 17B-Estradiol in Soil Mesocosms Under Alternating Aerobic/ Anoxic/Anaerobic Conditions

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Biodegradation of Bisphenol-A and 17 β -Estradiol in Soil Mesocosms Under
Alternating Aerobic/Anoxic/Anaerobic Conditions

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
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Date of Approval:
July 5, 2011

Keywords: EDCs, Water Reuse, Bioremediation, SPE, SPME

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DEDICATION

I would like to dedicate this dissertation
to my parents, who have always ultimately
supported and encouraged me

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my dissertation advisor, Dr. Jeffrey Cunningham for his guidance, support, and encouragement in completing Ph.D. research project. Thank you for giving me the freedom to pursue this project and for developing my research career as a 'scientist'. I will be forever grateful to you for your generosity, kindness, and wisdom over the years. I extend my appreciation to the members of my Ph.D. dissertation committee, Dr. Scott Campbell, Dr. Sarina Ergas, Dr. Valerie Harwood, Dr. Abdul Malik, and Dr. Daniel Yeh, who encouraged and led me to the right directions during my Ph.D. study. I would also like to thank Dr. Vinay Gupta, who served as an external committee chairperson for my dissertation defense.

This dissertation is based upon work supported by the state of Florida via the Sustainable Healthy Communities initiative at the University of South Florida (USF).

I would also like to thank Dr. Karoly Szekeres in College of Medicine Molecular Medicine, for his assistance; for handling down their new technical knowledge; for his assistance with Flow Cytometry analysis. I would also like to thank Timothy Ware, the Director of the Howard Curren Advanced WWTP, for his help to me in taking samples.

I would like to thank my entire fellows in the Lab., especially Hun-Young Wee, Joel Engleson, Shadab Anwar, Jung Kim, Mark Thomas, Jonathan Ticknor, and Claire Osborn, for their support and friendship. I would also like to thank Anh Do for his help and friendship and the time we have worked together for development to measure EDCs by GC/MS. I would also like to thank, Chungsik, Hyungtae, Huikyong, Museoung, Jein, Mansoo, Seungryong, Byungryong, my Korean friends in the USF. I am also grateful to Dr. Hyun Kim and Dr. Seokhun Kim, for their generosity, advice and encouragement.

Finally, a great thank you to my parents (Jung-gyu Kim and Jong-sook Choi) and my brother (Jinhyun Kim) for their tremendous love, support, and encouragement during my graduate student years. I could not have completed this long journey without them. All praise and glory goes to God the Father, Jesus the son and the Holy Spirit for their strength and sovereignty over this study.

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LIST OF ABBREVIATIONS

BAN	bromoacetonitrile
BPA	bisphenol-A
BSA	bovine serum albumin
BSTFA	N,O-bis-(trimethylsilyl) trifluoroacetamide
DOM	dissolved organic matter
E1	estrone
E2	17 β -estradiol
E3	estriol
EDCs	endocrine-disrupting compounds
EE2	17 α -ethinylestradiol (EE2)
GC/MS	gas chromatography (GC) with mass spectrometry (MS)
LC/MS	liquid chromatography with mass spectrometry
LLE	liquid-liquid extraction
MDL	method detection limit
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTBSTFA	N-methyl-N-(tert.-butyldimethyltrifluoroacetamide)
NOM	natural organic matter
NP	nonylphenol
NRB	nitrate-reducing bacteria
PFBBr	pentafluorobenzyl bromide
Redox	oxidation-reduction
SAT	soil-aquifer treatment
SPE	solid-phase extraction
SPME	solid-phase micro-extraction
SRB	sulfate-reducing bacteria
TMSI	N-tremethylsilyimidazole
WWTP	waste water treatment plant

ABSTRACT

Soil-aquifer treatment (SAT) has been proposed as a method for reusing treated municipal wastewater. SAT is characterized by alternating cycles of aerobic and anaerobic conditions in the subsurface, in response to alternating cycles of flooding and drainage of a surface impoundment. It is not yet known how these alternating redox conditions affect the removal of potentially harmful endocrine-disrupting compounds (EDCs) from treated effluent.

The overall objective of my doctoral research is to determine the fate of EDCs in alternating aerobic/anoxic/anaerobic conditions under simulated SAT conditions. To assess the fate of EDCs in simulated SAT conditions, I first had to develop appropriate analytical methods. Prior researchers have developed sophisticated analytical methods for measuring low concentrations of EDCs in water. However, it is not inherently clear which of these methods is preferable for analysis of any particular set of environmental samples. Therefore, in order to compare the analytical methods, solid-phase extraction (SPE) and solid-phase micro-extraction (SPME) were compared for the analysis of two EDCs, bisphenol-A (BPA) and 17 β -estradiol (E2), in water samples of water. Following extraction by SPE or SPME, the target EDCs were derivatized (silylated) and then analyzed by gas chromatography (GC) with mass spectrometry (MS). Also,

the performance of two candidate derivatization agents, N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), was compared. SPME is more convenient, is less labor-intensive, and allows for analysis of smaller sample volumes, but it is expensive because fibers need frequent replacement, and the range of linearity was limited. SPE has a lower material cost and allows for the analysis of a broader range of concentrations, but it is more labor-intensive and large sample volumes may be required. Therefore, the selection of which method is “best” depends upon the constraints (time, money, sample volume, acceptable detection limit) associated with any particular set of samples. The two derivatization agents performed equally when used in conjunction with SPE, but MSFTA yielded higher peak areas for headspace (on-fiber) derivatization during SPME.

To investigate how alternating redox conditions of SAT may affect the removal of harmful EDCs, a simulated SAT systems were constructed in 4-L reactors with 500 g of sediment (collected from a wetland) and 3 L of treated effluent from a municipal wastewater treatment plant; then BPA and E2 were spiked into reactors, two common EDCs often found in treated wastewater. Redox conditions in the mesocosms were controlled by switching the air between air (to induce aerobic conditions) and nitrogen (to induce anaerobic conditions); the length of the anoxic/anaerobic cycles was varied to determine how this affects biodegradation of the target EDCs. The mesocosm environment was supplemented with either nitrate or sulfate to serve as potential electron acceptors during the anoxic/anaerobic cycles. In addition to monitoring the

concentrations of the target EDCs in the mesocosms over time, I also monitored the concentration of dissolved oxygen in the water; the redox potential; the concentrations of nitrate, nitrite, and sulfate; and the concentration of bacteria in the water (estimated via flow cytometry).

BPA was biodegraded only during aerobic cycles, but E2 was biodegraded during both aerobic and anoxic/anaerobic cycles. Whenever the redox conditions in the system were switched, there was a temporary drop in the bacterial population, followed by a recovery of the population. When redox conditions were switched from anoxic/anaerobic to aerobic, biodegradation of the target EDCs commenced after a lag period during which no biodegradation was observed. The lag time for biodegradation in the aerobic cycle was longer when the anaerobic cycles were longer in duration. More biodegradation of E2 was observed under anoxic conditions than under anaerobic conditions.

SPE and SPME methods that included derivatization agent are useful method for detection and quantification of EDCs in water. I concluded that SAT is a viable technology to produce potable water from treated WWTP effluent, but the optimal length of flooding and drying cycles of SAT required removing the targeted contaminants during infiltration through the vadose zone.

CHAPTER 1

INTRODUCTION

1.1. Background and Motivation

Rapidly increasing demand for domestic water supply causes a limitation of fresh water, and the availability of potable water is a critical issue for many cities, especially in arid regions. As current water supplies begin to diminish and water costs increase, a sustainable supply of clean water that is reliable and inexpensive is required for both people and ecosystems. Water reuse technologies may be required to provide a sustainable supply of potable water, and soil-aquifer treatment (SAT) is a promising and successful technology for indirect reuse of tertiary treated municipal wastewater (Idelovitch and Michail 1984, Rice and Bouwer 1984). After SAT, effluents from wastewater treatment plants (WWTP) can be reused as potential indirect water sources. During SAT, treated wastewater is ponded in an unlined surface impoundment and is allowed to infiltrate through the vadose zone, thereby recharging a groundwater aquifer (Figure 1.1). The condition of vadose zone was changed between aerobic and anaerobic condition depend on the recharging process. Because impurities are removed during transport through the soil and the aquifer, low-quality wastewater can be purified to high-quality source water (Idelovitch and Michail 1984, Rice

and Bouwer 1984). Advantages of SAT are that it is cost effective and it can store treated wastewater in an aquifer for future use (Nema et al. 2001). However, if SAT is to be used as a means of potable reuse of wastewater, it must be determined whether harmful chemical and biological contaminants do not persist after treatment.

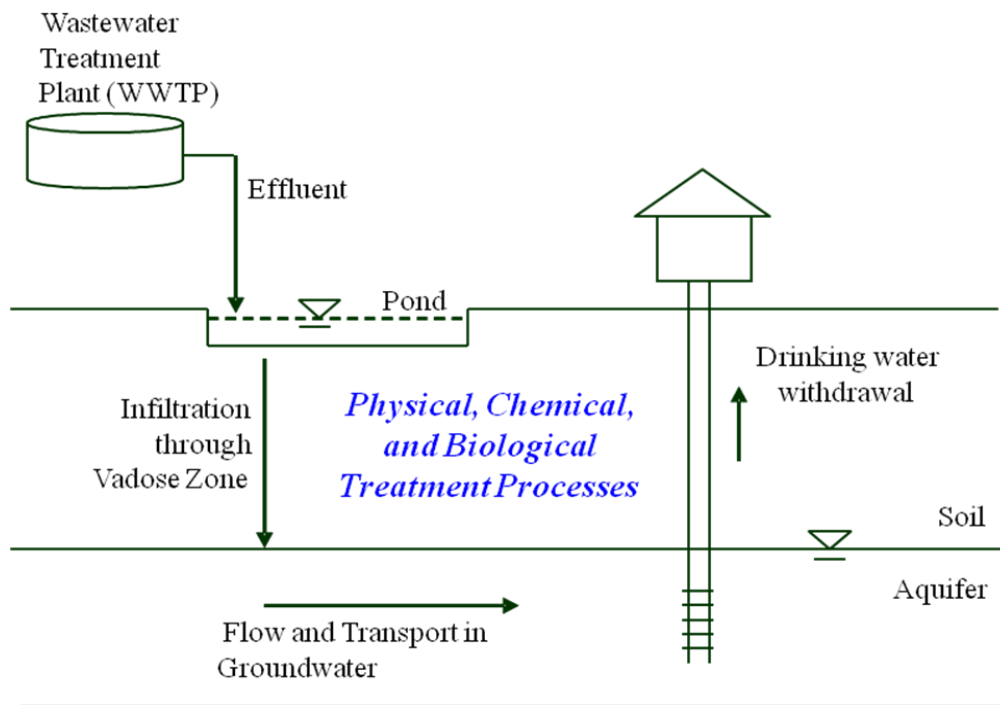


Figure 1.1. Soil-aquifer treatment (SAT) for indirect potable water re-use.

One of the most important challenges for water reuse is the presence of trace organic chemicals, especially endocrine-disrupting compounds (EDCs), in reclaimed water. EDCs present in the wastewater may threaten our potable supply if they are not sufficiently removed during SAT (Montgomery-Brown et al. 2003, Quanrud et al. 2004, Mansell et al. 2004a, Mansell et al. 2004b). The presence and fate of EDCs in the environment are now topics of worldwide concern. EDCs are environmental contaminants that interfere with the function of the endocrine system in wildlife and humans and can potentially cause severe health effects in humans (Colborn et al. 1993, Routledge et al. 1998, Iguchi et al. 2001, Silva et al. 2002). EDCs are commonly found in wastewater and may persist during SAT if operating conditions are not engineered properly (Quanrud et al. 2004, Mansell and Drewes 2004a). Classes of EDCs include phenols (e.g., bisphenol-A, bisphenol-F), hormone steroids (e.g., 17β -estradiol, estrone, estriol, testosterone, 17α -ethinyl estradiol, mestranol, diethylstilbestrol), alkylphenols and their metabolites (e.g., nonylphenol, octylphenol, nonylphenol ethoxylate, octylphenol ethoxylate, alkylphenoxy carboxylate, halogenated alkylphenols), and phytoestrogens (Nollet 2007). EDCs can be introduced into the environment in the effluent of WWTP (Halling-Sorensen et al. 1998, Daughton et al. 1999, Kolpin et al. 2002, Campbell et al. 2006).

Currently, the details of how SAT operates are not well known. Researchers have observed the improvements in water quality during SAT, but with little understanding of the mechanisms by which those improvements occur. Also, in the past, most researchers evaluated the performance and potential of

SAT based on the persistence of non-specific water quality indicators, such as dissolved organic carbon removal (Drewes et al. 2006, Rauch-Williams et al. 2006). The problem with this approach is that, even if the vast majority of dissolved organic carbon is removed during SAT, the small fraction that persists may be in the form of chemicals that are harmful even at low concentrations, such as EDCs.

The conditions of the vadose zone below the SAT pond typically changes between saturated and unsaturated conditions during alternating cycles of flooding and drying. This can cause alternating aerobic and anaerobic conditions of SAT, and can lead to cycles of varying oxidation and reduction conditions in the vadose zone (Amy et al. 1993, Greskowiak et al. 2005). Limited information is available in the literature about the degradation of EDCs under these types of conditions. Therefore, the success of SAT for potable reuse depends on improved understanding of biodegradation of EDCs during alternating aerobic/anoxic/anaerobic conditions.

1.2. Research Objectives

The *long-term objective of this research* is to contribute making SAT a viable technology for producing potable water from reclaimed water. The *overall objective of my doctoral research* is to determine the fate of EDCs under alternating aerobic/anoxic/anaerobic conditions using a simulated SAT systems. The *central hypothesis* is that biodegradation of EDCs during SAT exhibits a alternating cyclic behavior linked to the aerobic and anoxic/anaerobic cycles.

The *rationale* for this project is that determination of the fate of EDCs under alternating aerobic/anoxic/anaerobic conditions during SAT will allow for optimization of the removal mechanism and maximum efficiency of EDC removal during SAT. Therefore it will help to validate SAT as a technology for potable supply.

The overall objective of this project will be accomplished through achieving the following three specific goals.

1.2.1. Develop a Reliable Method for Detecting and Quantifying Target EDCs in Water Samples.

The working hypothesis of this goal is that target EDCs can be detected and quantified by solid phase extraction (SPE) and solid phase micro extraction (SPME) with derivatization, followed by gas chromatography and mass spectrometry (GC/MS).

1.2.2. Measure the Lag Time and the Biodegradation for Target EDCs under Alternating Aerobic/Anoxic Conditions.

The working hypothesis of this goal is that alternating between aerobic and anoxic conditions leads to a diauxic lag in the bacteria responsible for biodegradation of target EDCs. Therefore, longer anoxic cycles will lead to longer lag time and slower degradation of EDCs during the aerobic cycle under alternating aerobic/anoxic conditions.

1.2.3. Quantify the Biodegradation of EDCs under Different Anaerobic Terminal Electron Accepting Processes (TEAPs).

The working hypothesis of this goal is that different electron-accepting processes (e.g. reduction of nitrate or sulfate) lead to different biodegradation rates of EDCs during anaerobic cycles under alternating aerobic/anaerobic conditions. Furthermore, different electron-accepting processes during the anaerobic cycle will lead to different lag time duration and different rates of biodegradation of EDCs in the aerobic cycle during alternating aerobic/anaerobic conditions.

Accomplishment of these three research objectives will elucidate the linkage between biodegradation and alternating aerobic/anoxic/anaerobic conditions during SAT. This, in turn, will represent significant progress towards achievement of my long-term goals.

CHAPTER 2

LITERATURE REVIEW

2.1. Soil-Aquifer Treatment (SAT)

The subsurface environment can be used as a natural filtration treatment system, which is called soil-aquifer treatment (SAT). In SAT, treated effluent (or partially treated effluent) from a WWTP infiltrates through the vadose zone, also called the unsaturated zone, and reaches the groundwater (Rice et al. 1984, Idelovitch et al. 1984). SAT works as a natural bio filter that can remove odorous compounds, suspended solids, biodegradable materials, and endocrine-disrupting compounds from the wastewater (Routledge et al. 1998, Fox 2002, Asano et al. 2002, Mansell et al. 2004a, Mansell et al. 2004b, Conroy et al. 2005). In addition, heavy metals, nitrogen, and phosphorus can be removed by SAT systems (Lin et al. 2004, Cha et al. 2006).

2.1.1. Soil Materials for SAT

A soil with a high infiltration rate is required for SAT systems. If the infiltration rate is too low, then excessive basin areas are needed and high evaporation losses occur from the basins. In order to provide a high infiltration rate and good quality effluent after SAT, fine sand and/or loamy sand are

required for SAT systems. Fine suspended material in the effluent can cause the formation of a clogging layer on the bottom of the basin, and this causes a decrease in infiltration rate into the vadose zone.

2.1.2. Alternating Cycles of SAT

Because SAT is operated by alternating cycles of flooding and drying, the vadose zone below the wastewater pond typically undergoes cyclic changes between saturated and unsaturated conditions (Figure 2.1) and this causes alternating aerobic and anaerobic conditions (Greskowiak et al. 2005). In Step 1, saturated conditions beneath the pond are started and established, and the infiltration rate increases. During Step 2, saturated conditions prevail at the beginning. Unsaturated conditions start at the end of Step 2. During Step 3, a clogging layer occurs and the groundwater table declines. The infiltration rate decreased rapidly. Infiltration did not occur during step 4 and unsaturation condition prevailed. These changes result from the repeated formation of a clogging layer at the pond bottom. The formation of clogging layer causes a decrease in infiltration rate and air can penetrate into the unsaturated region. During the alternating cycles, the system experiences cycles of different oxidation and reduction conditions and reactions occurring in the soil (Greskowiak et al. 2005, Amy et al. 1993). Therefore we have to consider and investigate EDCs biodegradation processes under alternating aerobic and anaerobic conditions.

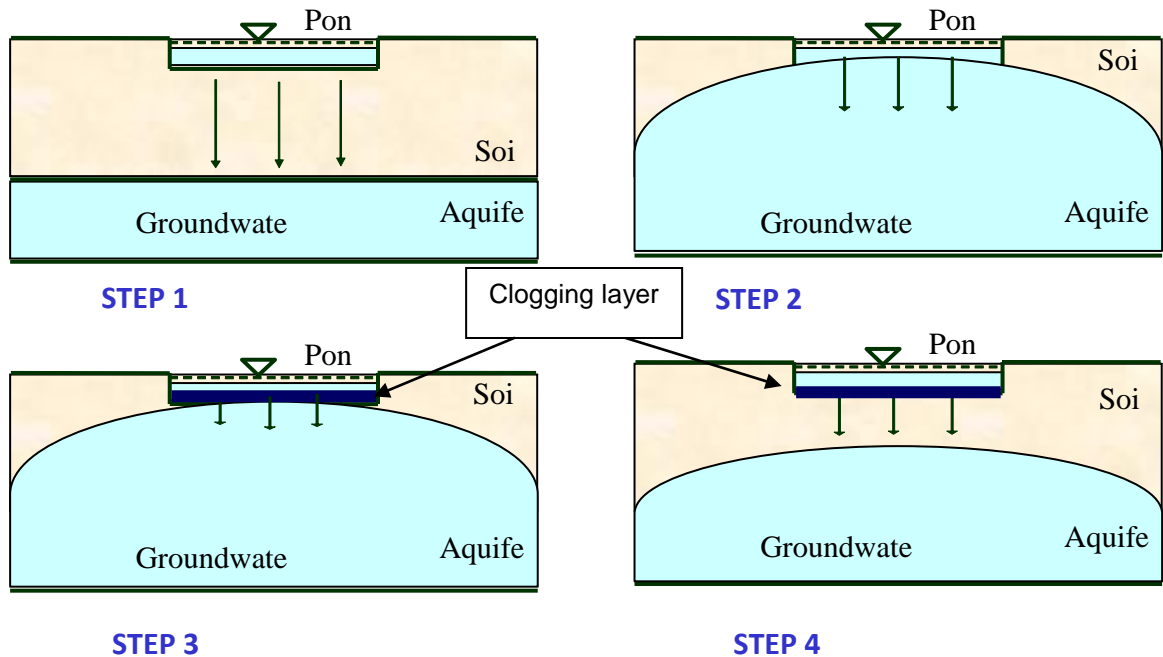


Figure 2.1. Infiltration process in SAT (adapted from Greskowiak et al. 2005)

2.1.3. Nitrification and Denitrification during SAT

Prior to SAT typically swage contains organic nitrogen at levels of 20 to 100 mg/L (as N) (Idelovitch et al. 2003, Miller et al. 2006). Nitrogen may be present as ammonium, nitrate, and/or organic nitrogen (Idelovitch et al. 2003, Miller et al. 2006). Nitrogen can be removed in a SAT system by controlling the hydraulic loading rates and flooding and drying periods of the basins (Leach and Enfield 1983). Flooding and drying periods cause aerobic and anaerobic conditions in the soil, which in turn may promote nitrification or denitrification (Greskowiak et al. 2005). Certain anaerobic bacteria present in the soil can reduce nitrate to free nitrogen gas. In one study, approximately 75% of the

nitrogen was removed by a SAT system (Idelovitch et al. 2003). Organic carbon is required for denitrifying bacteria as an energy source under anaerobic conditions (Starr et al. 2005). Currently, it is not known how denitrification in SAT depends upon the flooding and drying cycles.

2.2. Endocrine-Disrupting Compounds

Endocrine-disrupting compounds (EDCs) are a relatively new topic of worldwide concern. EDCs are environmental contaminants that interfere with the function of the endocrine system in wildlife and humans and can potentially cause severe health effects in humans (Colborn et al. 1993, Routledge et al. 1998, Iguchi et al. 2001, Silva et al. 2002). Steroid hormones detected in the environment can cause endocrine disrupting effects in aquatic wildlife (Jobling et al. 1998, Panter et al. 1998). Because EDCs exist ubiquitously in the environment but their concentrations are typically very low, it is difficult to understand their characteristics, sources, and effects on wildlife and humans (Colborn et al. 1993, Kuch et al. 2001, Braun et al. 2003).

2.2.1. Occurrence of EDCs

One reason why EDCs are so common in the environment is that treatment systems are not optimized for removing EDCs from wastewater. EDCs have been detected in wastewater treatment plant effluents, which can act as a source of EDCs to soil and surface water (Desbrow et al. 1998, Daughton et al. 1999, Kolpin et al. 2002, Campbell et al. 2006, Halling-Sorensen et al. 1998).

BPA is chemical that is widely used in the manufacture of phenolic resins and is released to environment (air, water, land, subsurface). Rudel et al. (1998) measured BPA levels of 0.1 - 1.7 µg/L in untreated septic system effluent and wastewater and 20 – 44 ng/L in drinking water wells. 17β-estradiol (E2), 17α-ethinyloestradiol (EE2), and estrone (E1) were detected in WWTP effluent at maximum concentrations of 12, 7.5, and 47 ng/L, respectively, in the Netherlands (Belfroid et al. 1999). E2, EE2, and E1 were detected in surface water at maximum concentrations of 93, 831, and 112 ng/L, respectively, in the U.S.A. (Kolpin et al. 2002). E2 has also been detected in ground water (Peterson et al. 2000). I focused on two particular EDCs, bisphenol-A (BPA) and 17β-estradiol (E2), because they are commonly found in wastewater effluents and receiving waters (Staples et al. 1998, Kolpin et al. 2002). BPA is a widely used monomer and an important compound, which is used in epoxy and polycarbonate plastic and flame retardants (Desvrow et al. 1998). E2 is a steroid estrogen hormone involved in high estrogenic activities (Desvrow et al. 1998, Hansen et al. 1998). EDCs have been detected, even though at trace concentration, in surface waters and river sediments around the world (Petrovic et al. 2004). Estradiol has been detected in the aquatic environment, mainly coming from municipal WWTP, agricultural production, and livestock farming (Ternes et al. 1999, Hanselman et al. 2003). The main sources of EDCs in nature originate from industrial, municipal, domestic, and animal farming activities. Negative potential effects of EDCs to animal and humans have caused an interest in removing EDCs from the environment.

2.2.2. Physical-Chemical Properties of EDCs

The chemicals that form the basis of this study are bisphenol-A (BPA) and E2. The physical-chemical properties of these EDCs are listed in Table 2.1 and molecular structures are shown in Figure 2.2. BPA (2,2-bis(4-hydroxyphenyl)propane; CAS Registry No. 80-05-7) is used most widely in the manufacture of phenolic resins (Staples et al. 1998). BPA has an acute toxicity to aquatic organisms in the range of 1 – 10 mg/L (Alexander et al. 1988). Under ambient conditions, BPA is a solid as crystals, prills, or flakes. Natural and synthetic estrogens, E1, E2 and EE2, have the strongest estrogenic effects. Due to a large amount of the estrogenicity in the municipal sewage treatment plant effluent, estrogens are often present in the aquatic environment (Anderson et al. 2003). EE2 and E2 are structurally similar and EE2 is used in oral contraceptives (Arcand-Hoy et al. 1998). E2 is commonly metabolized to E1 (Lee et al. 2003). EDCs generated in the manufacturing process are released during processing, handling, and transportation. BPA level is 0.1–1.7 mg/L in untreated septic- and wastewater and 20–44 ng/L in 2 of 28 drinking water wells (Rudel et al. 1998). EDCs can be naturally attenuated by subsurface of contaminated sites. Natural attenuation which low cost remediation is comprised by subsurface geology, hydrology, and microbial ecology. Intrinsic bioremediation in the subsurface is a potential remediation method of EDCs (Röling et al. 2002).

Table 2.1. Physical-chemical properties of EDCs

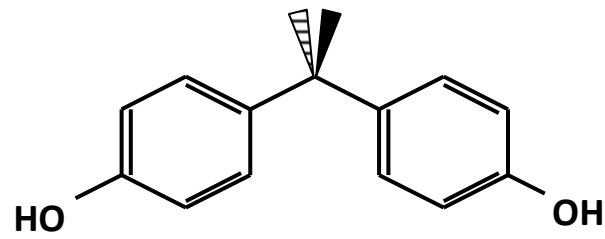
Compound name	Bisphenol-A	17 β -Estradiol	Estrone
M.W. (g/mol)	228.3	272.4	270.4
Water solubility (mg/L at 20°C)	120-300 [1]	3.9-13 5.4-13.3 [2]	0.8-12.4 [2] 6-13 [1]
LogK _{ow}	2.50-6.60 [1]	3.10-4.01 [1] 3.8-4.0 [2]	2.45-3.34 [1] 3.1-4.4 [2]
pKa	9.6-11.3 [1]	10.71 [1]	10.3-10.8 [1]
Vapor pressure (mm Hg)	5.3 X 10 ⁻⁸ [3]	3 X 10 ⁻⁸ [3]	3 X 10 ⁻⁸ [3]
Henry's law constant (atm·m ³ /mole at 25°C)	1 X 10 ⁻¹¹ [4]	3.64 X 10 ⁻¹¹ [4]	3.8 X 10 ⁻¹⁰ [4]
Formula	C ₁₅ H ₁₆ O ₂	C ₁₈ H ₂₄ O ₂	C ₁₈ H ₂₂ O ₂

[1] Campbell et al. 2006.

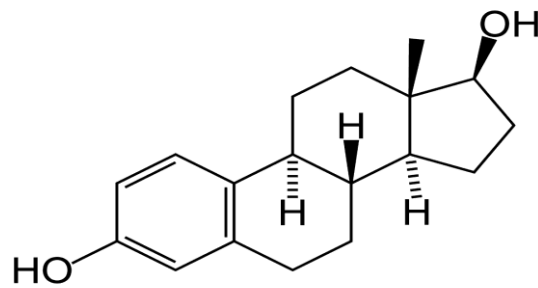
[2] Khanal et al. 2006.

[3] Sarmah et al. 2008.

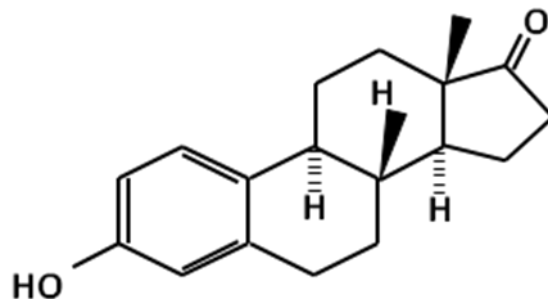
[4] Mes et al. 2005.



(a) bisphenol-A (BPA)



(b) 17β-estradiol (E2)



(c) estrone (E1)

Figure 2.2. Molecular structure of (a) bisphenol-A, (b) 17β-estradiol, and (c) estrone.

2.3. Fate of EDCs in the Environment

In these days, amount of man-made chemicals (EDCs, pharmaceutical materials, and personal care products) are increasing and it makes increasing the suspected EDCs amount which is discharged through WWTPs. The major EDC transformation processes in nature are photodegradation, sorption, and biodegradation. These processes are considered below.

2.3.1. Photodegradation

Recent research demonstrates that photodegradation is an important removal mechanism of EDCs from surface waters (Watanabe et al. 2003, Zhang et al. 2007). Photodegradation without biodegradation and sorption was the dominant removal mechanism in some batch reactor studies (Feng et al. 2005, Tsai et al. 2009). Phototransformation efficiency of EDCs depends on pH, concentration of Fe(III), and concentration of EDCs. The degradation efficiency of E1 varied from 14.2% to 98.4% under photo-Fenton system (Feng et al. 2005). The water matrix can affect the photodegradation of EDCs but the effects of natural organic matter (NOM) and dissolved organic carbon (DOC) on EDCs photodegradation are still under investigation (Neamtu et al. 2006). Photodegradation in SAT systems is restricted by lack of sunlight (Crump 2001).

2.3.2. Sorption

Sorption is defined as the uptake of a solute from a liquid phase to a solid phase. Sorption has an important function in the aquatic environment in the fate of EDCs. The transport of EDCs is retarded by sorption to soils and sediments (Lee et al. 2003, Yu et al. 2004, Casey et al. 2004, Das et al. 2004, Kim et al, 2007). Sorption can be the main removal mechanism of EDCs in the soil environment (Mansell et al, 2004a, b). There is little information about the sorptive nature of EDCs under natural conditions. Various environmental conditions, humic acid, black carbon, polyaromatic hydrocarbons and herbicides in soil can all affect the sorption coefficient and rates (Stevenson 1994, Schmidt et al. 2000, Xiao et al. 2004, Yu et al. 2006). More research is required to determine the effects of different functional groups and environmental conditions (i.e. ionic strength, surface complexation) on sorption of EDCs.

2.3.3. Microbial Degradation of EDCs

Release of EDCs to nature over a long period has enabled certain bacteria to evolve pathways allowing them to use these compounds as an energy source. Steroid hormones can be used as the sole source of carbon and energy by certain bacteria. Under aerobic conditions, steroids can be degraded by aerobic bacteria, and a few degradation processes have been described (Kieslich 1985, Fujii et al. 2002, Fujii et al. 2003, Yoshimoto et al. 2004). During the aerobic biodegradation of steroids, molecular oxygen is used to form hydroxylated products from oxygenase-catalyzed reactions. During

denitrification in wastewater treatment or in sediments of lakes, anoxic conditions prevail, and oxygen has limited access to microbial activity. Less is known about biodegradation of steroids without molecular oxygen (Hylemon et al. 1999, Kniemeyer et al. 1999, Probian et al. 2003). The enterohepatic circulation in mammals by intestinal anaerobic bacteria is the most known transformation of steroids in anoxic habitats (Groh 1993). Under denitrifying condition, the mineralization of estradiol occurred, but more research is required to understand the responsible bacteria and oxidation products of estrone (Andersen et al. 2003, Joss et al. 2004). EDCs can be removed by microbial transformation. Organic compounds can be oxidized by microbes and the carbon was used as an energy source via direct metabolism. Alternatively, compounds can be transformed by microbial cometabolism (Benotti and Snyder. 2000). An oxidation and a corresponding accumulation of estradiol to estrone were reported and an accumulation of estrogens was suggested in anoxic environments (Czajka et al. 2006). BPA is generally rapidly biodegraded in surface waters, WWTPs, and biological waste treatment systems at greater than 96% efficiency (Staples et al. 1988) under aerobic conditions.

2.3.4. Biotransformation and Biodegradation Pathways for BPA and E2

Figure 2.3 shows the pathway for bacterial metabolism of BPA and Figure 2.4 shows the pathway of E2. Many bacteria, fungi, and algae that can degrade BPA and E2 have been identified from soils, river waters, and WWTP (Lobos et al. 1992, Spivack et al. 1994, Ike et al. 2000, Kang et al. 2002a,b, Kang et al.

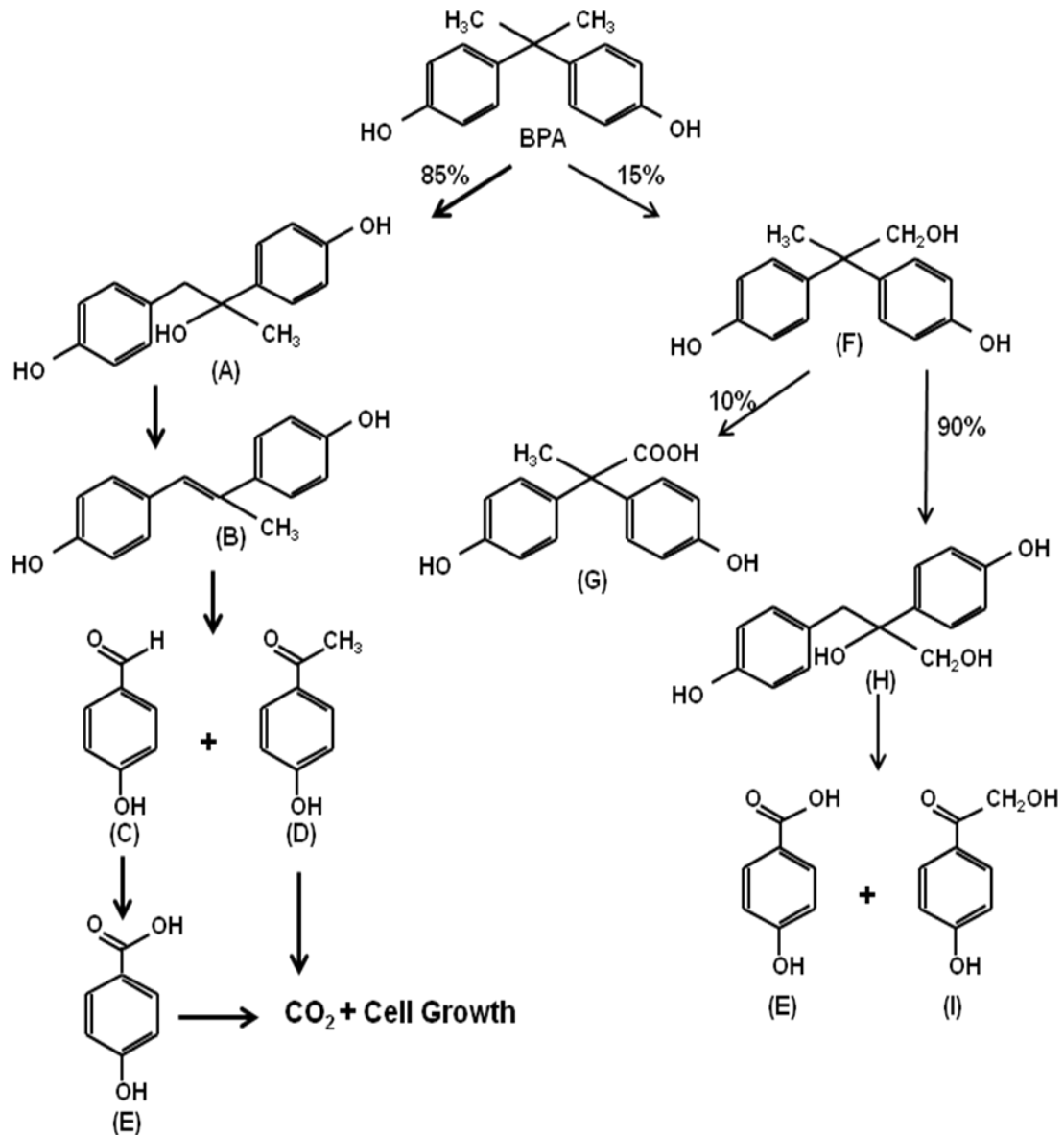


Figure 2.3. Proposed degradation pathway of BPA biodegradation by strain MV1 (adapted from Spivack et al. 1994).

- A: 1,2-bis(4-hydroxyphenyl)-2-propanol,
- B: 4,4'-dihydroxy- α -methylstilbene,
- C: 4-hydroxybenzaldehyde,
- D: 4-hydroxyacetophenone,
- E: 4-hydroxybenzoic acid,
- F: 2,2-bis(4-hydroxyphenyl)-1-propanol,
- G: 2,2-bis(4-hydroxyphenyl)propanoic acid,
- H: 2,3-bis(4-hydroxyphenyl)-1,2-propanediol,
- I: 4-hydroxyphenacyl

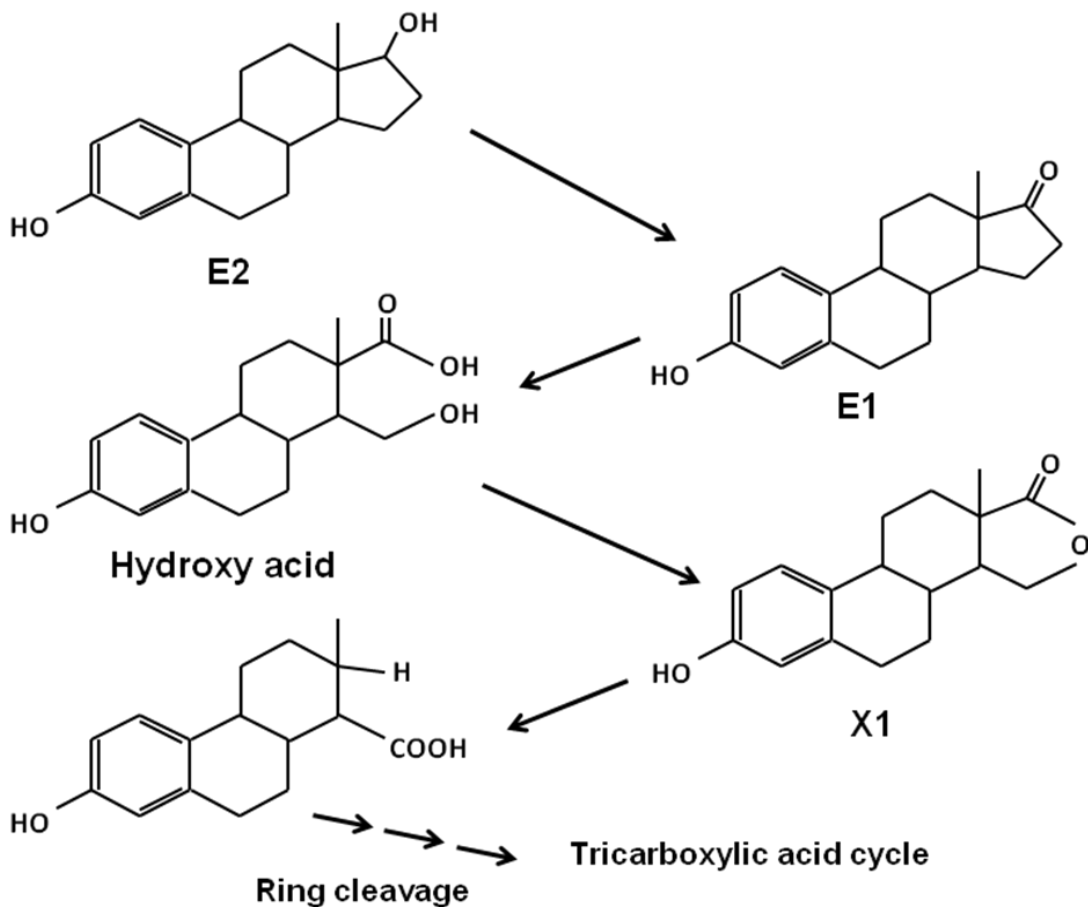


Figure 2.4. Proposed degradation pathway of estrone (adapted from Lee and Liu 2002).

2004, Sasaki et al. 2005). But Figure 2.3 and 2.4 are proposed degradation pathway of EDCs because the fate and degradation pathway of EDCs in environment are not yet fully understand. BPA metabolized routes by a gram-negative bacteria strain MV1 was found (Lobes et al. 1992, Spivack et al. 1994). MV1 is isolated from WWTP and MV1 uses BPA as the sole carbon and energy source. Figure 2.3 shows the major and minor pathways of BPA metabolism. Two primary metabolites (4-hydroxyacetophenone and 4-hydroxybenzoic acid) are produced from the major pathway and two primary metabolites (2,2-bis(4-hydroxyphenyl)-1-propanol and 2, 3- bis(4-hydroxyphenyl)-1, 2-propanediol) are produced from the minor pathway. Biotransformed BPA metabolites have no toxic and estrogenic effects of BPA: only 4-hydroxyacetophenone has a slight estrogenic activity compared with BPA (Ike et al. 2002). Figure 2.4 shows the pathways of E2 metabolism. Degradation of estradiol appeared to initiate at C-17 of ring D in E2, leading to the formation of a keto group at the same position (conversion of E2 to E1). In addition, E1 converse to X1 which was tentatively identified as a lactone. E2 biodegradation was not yet clear.

Tables 2.2 and 2.3 show the microorganisms capable of biodegrading or metabolizing BPA and E2. BPA was removed over 90% in a wastewater treatment process (Staples et al. 1998, Fürhacker et al. 2000) but BPA remaining in the effluent can be a source in the aquatic environment. A *Pseudomonas* species and a *Pseudomonas putida* strain showed high BPA biodegradability (about 90%). Moreover, *Streptomyces* sp. strain has high BPA biodegradability (>90%) (Kang et al. 2004). These bacteria which have high BPA biodegradability

may be useful for the bioremediation of the aquatic environment. E2, E1, estriol (E3), and EE2 were degraded by strains of *Rhodococcus* (Yoshimoto et al. 2004) and many microorganisms degrade the E2.

Table 2.2. Microorganisms capable of biodegrading or metabolizing BPA

Microorganisms	Strains	References
Bacteria	MV1 <i>Pseudomonas paucimobilis</i> FJ-4 <i>Pseudomonas sp.</i> <i>Pseudomonas putida</i> <i>Caragana chamlagu</i> <i>Streptomyces sp.</i> <i>Bacillus pumilus</i> 2CK <i>Bacillus pumilus</i> 21DK <i>Bacillus pumilus</i> 22DK <i>Sphingomonas sp.</i> AO1 <i>Achromobacter xylosoxidans</i> B-16	Lobos et al. 1992, Spivack et al. 1994 Ike et al. 2000 Kang et al. 2002a Kang et al. 2002a Chai et al. 2003 Kang et al. 2004 Yamanaka et al. 2005 Yamanaka et al. 2005 Yamanaka et al. 2005 Sasaki et al. 2005 Zhang et al. 2007
Fungi	<i>Pleurotus ostreatus</i> O-48 <i>Phanerochaete chrysosporium</i> ME-446 <i>Trametes versicolor</i> IFO-7043 <i>Trametes villosa</i> <i>Phanerochaete chrysosporium</i> ME-446 <i>Trametes versicolor</i> IFO-6482 <i>Aspergillus fumigatus</i> <i>Fusarium sporotrichioides</i> NFRI-1012 <i>Fusarium moniliforme</i> 2-2 <i>Aspergillus terreus</i> MT-13 <i>Emericella nidulans</i> MT-98 <i>Stereum hirsutum</i> <i>Heterobasidium insulare</i> <i>Irpex lacteus</i> <i>Trametes versicolor</i> <i>Irpex lacteus</i> 617/93 <i>Pleurotus ostreatus</i> 3004 CCBAS278 <i>Phlebia tremellosa</i>	Hirano et al. 2000 Tsutsumi et al. 2001 Tsutsumi et al. 2001 Fukuda et al. 2001, Uchida et al. 2001 Suzuki et al. 2003 Suzuki et al. 2003 Yim et al. 2003 Chai et al. 2005 Chai et al. 2005 Chai et al. 2005 Chai et al. 2005 Chai et al. 2005 Lee et al. 2005 Lee et al. 2005 Shin et al. 2007 Diano et al. 2007 Cajthaml et al. 2009 Cajthaml et al. 2009 Kum et al. 2009
Planktons	<i>Chlorella fusca</i> var. <i>vacuolata</i> <i>Nannochloropsis sp.</i> <i>Chlorella gracilis</i> <i>Stephanodiscus hantzschii</i> <i>Pavlova sp.</i>	Hirooka et al. 2003 Ishihara et al. 2003 Ishihara et al. 2003 Li et al. 2009 Shimoda et al. 2009

Table 2.3. Microorganisms capable of biodegrading or metabolizing E2

Microorganisms	Strains	References
Bacteria	<i>Novosphingobium</i>	Fujii et al. 2002
	<i>Nitrosomonas europaea</i>	Shi et al. 2004
	<i>Rhodococcus zopfii</i> Y50158	Yoshimoto et al. 2004
	<i>Rhodococcus equi</i> Y50155	Yoshimoto et al. 2004
	<i>Rhodococcus equi</i> Y50156	Yoshimoto et al. 2004
	<i>Rhodococcus equi</i> Y50157	Yoshimoto et al. 2004
	KC1	Yu et al. 2007
	KC2	Yu et al. 2007
	KC3	Yu et al. 2007
	KC4	Yu et al. 2007
	KC5	Yu et al. 2007
	KC6	Yu et al. 2007
	KC7	Yu et al. 2007
	KC8	Yu et al. 2007
	KC9	Yu et al. 2007
	KC10	Yu et al. 2007
	KC11	Yu et al. 2007
	KC12	Yu et al. 2007
	KC13	Yu et al. 2007
	KC14	Yu et al. 2007
	<i>Baculus pumilus</i> 1	Pauwels et al. 2008
	<i>Baculus pumilus</i> 2	Pauwels et al. 2008
	<i>Baculus pumilus</i> 3	Pauwels et al. 2008
	<i>Baculus pumilus</i> 7	Pauwels et al. 2008
	<i>Baculus pumilus</i> 8	Pauwels et al. 2008
	<i>Baculus pumilus</i> 10	Pauwels et al. 2008
Planktons	<i>Chlorella vulgaris</i>	Lai et al. 2002

2.4. Oxidation and Reduction

The removal of electrons from a compound (electron donor) is oxidation and the addition of electrons to a compound (electron acceptor) is reduction (DeLaune and Reddy, 2005). Transfer of electrons from one compound to another is a coupled reaction. During oxidation a compound is oxidized and its oxidation number is increased. During reduction a compound is reduced and its oxidation number is decreased. The redox potential of an environment is a measure of electrochemical potential or electron availability within soil, water, and marine systems. Wetland soils, usually anaerobic environments, are limited by electron acceptor and have a plentiful electron donor (DeLaune and Reddy, 2005). NH_4^+ , Fe^{2+} , Mn^{2+} , S^{2-} , CH_4 , and H_2 are reducing inorganic compounds. Aerobic condition soils are usually limited by electron donors and have an ample electron acceptor. O_2 , NO_3^- , MnO_2 , FeOOH , SO_4^{2-} , and HCO_3^- are oxidized inorganic compounds. Table 2.4 shows the redox potential range in soil and sediment, showing the microbial metabolism process and electron acceptor (DeLaune and Reddy, 2005). Redox potential is an identification method for recognizing whether an area is functioning as aerobic or anaerobic of the biogeochemical reactions in surface environments. The major removal mechanisms of EDCs are oxidation processes (Liu et al. 2009).

Table 2.4. Redox potential range in soil and sediment, showing the microbial metabolism process and electron acceptor.

Sediment condition	Anaerobic									Aerobic	
Redox condition	Highly reduced			Reduced		Moderately reduced				Oxidized	
Electron acceptor	CO ₂	SO ₄ ²⁻	Fe ³⁺	Mn ⁴⁺	NO ₃ ⁻	O ₂					
Microbial metabolism	Anaerobic		Facultative							Aerobic	
Oxidation reduction potential (mV)	-300	-200	-100	0	+100	+200	+300	+400	+500	+600	+700

Adopted from DeLaune and Reddy (2005)

2.5. Removal of EDCs during SAT

Multiple bench-scale studies and laboratory-scale soil column experiments have been performed to verify which mechanisms dominate the removal of the EDCs during SAT. Mansell et al. (2004a and 2004b) examined the fate and transport of EDCs during SAT and concluded that the dominating removal mechanism of EDCs during SAT is adsorption to soil. Additional removal mechanism by microorganism in redox condition (aerobic vs. anoxic) is required. For the purposes of this dissertation, I use the terms aerobic, anoxic and anaerobic according to the following definitions from Metcalf and Eddy (1991): “Aerobic processes are biological treatment processes that occur in the presence of oxygen; anaerobic processes are biological treatment processes that occur in the absence of oxygen; anoxic denitrification is the process by which nitrate nitrogen is converted biologically to nitrogen gas in the absence of oxygen” There are many studies on the fate of EDCs under aerobic and anaerobic

conditions. In this section, aerobic/anoxic/anaerobic biological degradation of EDCs will be described.

2.5.1. Aerobic Metabolism

There is a difference of BPA biodegradability between aerobic conditions and anaerobic conditions. BPA is able to degrade under aerobic condition (Staples et al. 1998, Ike et al. 2006, Zhao et al. 2008). BPA has been easily biodegraded under aerobic conditions in river water and spiked samples (>90%), but BPA degradation is difficult to find under anaerobic conditions (<10% for 10 days) (Kang and Kondo, 2002a). Ying et al. (2008) reported that BPA, E2, and EE2 were all degraded under aerobic conditions by groundwater microorganisms present in the aquifer.

2.5.2. Anoxic Denitrification

The EDCs removal in sludge from WWTP with nitrification/denitrification was investigated (Joss et al. 2004), and EDCs can be degraded under anoxic denitrification and EDCs were largely biodegraded in the denitrifying tanks in a municipal activated sludge system (Andersen et al. 2003).

2.5.3. Anaerobic Metabolism

Degradation of E2 under anaerobic condition was observed but degradation of BPA under anaerobic condition was not observed (Ike et al. 2006, Kang and Kondo 2002a, Ying and Kookana 2003). BPA was not biodegraded in

anaerobic slurry even after 3 months of incubation (Ronen et al. 2000). These results show that anaerobic bacteria have little capability for BPA biodegradation. Co-metabolic transformations of estrogens by the nitrifying bacterium *Nitrosomonas europaea* have been described (Veder et al. 2000, Shi et al. 2004), but the exact explanation of co-metabolism by microbial process is not yet fully understood (Wackett 1996). Under anaerobic conditions, removal of EDCs (E2, EE2, BPA, and 4-n-Nonylphenol (NP)) removal was attributed to the sulfate-, nitrate-, and iron-reducing conditions within the tested media; however, the overall degradation of the compounds was influenced by abiotic factors (Sarmah et al. 2008, Czajka and Londry 2006, Ying et al. 2008). Sulfate reducing bioremediation might not be applicable in every situation. Microbial activity and the intrinsic biodegradability are depending on the bioavailability of substrates, electron acceptors and nutrients, which are related to environmental conditions (Röling et al. 2002).

2.6. Diauxic Lag

During bioremediation, the microbes sometimes require a length of time to acclimate themselves to the environment (Crane and Novak, 2001). The length of acclimating time required for microbial inoculants in environment is the lag time of micro organism (Crane and Novak, 2001). When the enzyme is stored with a reversible inhibitor present, lag time is required for complete dissociation of the inhibitor. Bacteria generate their own enzymes, and there was lag time between enzyme application and results. Lag phase are also observed when

temperatures are not steady state condition (Copeland. 2000). Klečka et al. 2001 found that rapid biodegradation of BPA occurred after lag phases ranging from 2 to 4d. In biological systems, the amount of biomass and its growth are limited by the substrate in environment. Under physiological stress, endogenous processes effect to the amount of biomass (Lopez et al. 2006). During lag phase, the microbes are physiologically active and are adapting to the physiological stress, but the amount of microbes remains constant, and at the end of the lag phase, the bacteria start to divide (Madigan and Martinko. 2006). Diauxic lag is a lag period of little or no growth that occurs when bacteria switch electron donors or when they switch terminal electron acceptors during exposure to alternating aerobic and anoxic conditions (Monod 1949, Kodama et al. 1969). In some systems, the amount of dissolved oxygen in the oxic phase and the presence of nitrate during the oxic phase affect the length of diauxic lag (Liu et al. 1998, Gouw et al. 2001, Lisbon et al. 2002). The long aerobic condition cause the long diauxic lag after oxygen is removed, and the occurrence and length of diauxic lag is affected by the presence of nitrate and oxygen during the aerobic condition (Liu et al. 1998, Gouw et al. 2001, Lisbon et al. 2002). During alternating aerobic/anaerobic condition, diauxic lag might be an important factor to biodegradation of EDCs because the SAT leads to the cycles of different oxidation and reduction conditions. Therefore, investigation is required about the phenomenon of diauxic lag under alternating aerobic/anaerobic condition during SAT.

CHAPTER 3

DEVELOP A RELIABLE METHOD FOR DETECTING AND QUANTIFYING TARGET EDCS IN WATER SAMPLES

3.1. Introduction

The global concern over trace levels of EDCs in the environment has led to the development of sensitive analytical methods for detecting and quantifying EDCs in environmental samples (river water, ground water, and effluent from wastewater treatment plants) (Belfroid et al. 1999, Carpinteiro et al. 2004, Basheer et al. 2005). Due to the diversity of chemical properties of EDCs and the complexity of environmental matrices, quantification of EDCs in environmental samples at low concentrations is challenging. Numerous analytical methods have been developed to measure EDCs, most often by gas chromatography with mass spectrometry (GC/MS) or by liquid chromatography with mass spectrometry (LC/MS) (Castillo et al. 1997, Rudel et al. 1998, Mol et al. 2000, Huang et al. 2001, Ternes et al. 2001, Vanderford et al. 2003, Chang et al. 2005). Because the concentrations of EDCs are often very low ($\mu\text{g/L}$ or lower) in environmental samples, suitable methods for extracting and concentrating target EDCs must be applied prior to analysis by GC/MS. Extraction methods include solid-phase extraction (SPE), liquid-liquid extraction (LLE), and solid-phase

micro-extraction (SPME) (Arthur et al. 1992, Louch et al. 1992, Daughton et al. 1999, Mol et al. 2000, Kuch et al. 2001, Ternes et al. 2001, Lopez-Blanco et al. 2002, Braun et al. 2003). Furthermore, target EDCs that have highly polar functional groups need to be derivatized prior to GC/MS analysis to reduce peak tailing in the chromatogram and to improve the sensitivity, accuracy, and reproducibility of the analysis (Jeannot et al. 2002, Xiangli et al. 2006, Yang et al. 2006, Zhang et al. 2006, Moder et al. 2007, Pan et al. 2008).

Although the conventional methods (LLE and SPE) for the extraction and concentration of EDCs from environmental samples are effective, these methods require intensive labor as well as the use of expensive and potentially harmful organic solvents. Additionally, large sample volumes may be needed if the target contaminant concentration is low (ng/L) (López-Blanco et al. 2002, Chang et al. 2005, Zhang et al. 2006, Moder et al. 2007). In contrast, SPME does not require organic solvents or large sample volumes, but it frequently exhibits a higher detection limit ($\mu\text{g/L}$) (López-Blanco et al. 2002). This may raise concern because some EDCs are present at low concentrations in the environment. Therefore, it is unclear which method (SPE or SPME) is preferable depending on sample volume and target contaminant concentration.

Derivatization agents including N,O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), N-methyl-N-(tert.-butyldimethyltrifluoroacetamide) (MTBSTFA), and pentafluorobenzyl bromide (PFBBR) have been used with SPE (Mol et al. 2000, Jeannot et al. 2002, Zhang et al. 2006, Moder et al. 2007) and SPME (Basheer et al. 2004,

Carpinteiro et al. 2004, Basheer et al. 2005, Chang et al. 2005, Yang et al. 2006, Pan et al. 2008, Negreira et al. 2009, Viñas et al. 2009). Some investigations have compared derivatization agents and found BSTFA preferable to MSTFA or bromoacetonitrile (BAN) (Hsu et al. 2007, Szyrwińska et al. 2007). The comparison of derivatizing agent studies performed without a preceding extraction step such as SPE or SPME. Therefore, it is still unclear if one particular derivatization agent is preferable for use with SPE and/or SPME. Locicero et al. (2007) is found that MSTFA is more useful than the other derivatization agent (bovine serum albumin (BSA), N-Trimethylsilylimidazole (TMSI) and BSTFA). Szyrwińska et al. (2007) concluded that BSTFA is more useful than BAN. BSTFA and MSTFA are useful for confirming the presence of EDCs and its derivatization efficiency is almost the same (Basheer et al. 2005, Szyrwińska et al. 2007, Zhou et al. 2007, Sebők et al. 2008).

The purpose of this study was to compare analytical methods based on SPE and SPME with derivatization followed by GC/MS for detection and quantification of EDCs in water samples. We selected MSTFA and BSTFA over other possible derivatization agents in this study because these two have been observed to produce higher peak area than BAN, BSA, and TMSI. Derivatization efficiency of MSTFA and BSTFA is almost same and useful to EDCs. I focused on two particular EDCs, bisphenol-A (BPA) and 17 β -estradiol (E2), because they are commonly found in wastewater effluents and receiving waters (Staples et al. 1998, Kolpin et al. 2002). BPA is a widely used monomer and an important compound, which is used in epoxy and polycarbonate plastic and flame

retardants (Desvrow et al. 1998). E2 is a steroid estrogen hormone involved in high estrogenic activities (Desvrow et al. 1998, Hansen et al. 1998). The important contributions of this chapter are: (1) we determine which derivatization agent, MSTFA or BSTFA, is more effective in both SPE and SPME; and (2) we determine which extraction method (SPE or SPME) is preferable depending on operating factors such as the sample volume available and the concentration of the target analyte in the sample.

3.2. Materials and Methods

Solutions of known concentrations of BPA and E2 were created in purified water then those solutions were used to develop calibration curves for the analytical methods described below. This enabled us to determine such parameters as the method detection limit and the range of linearity of the calibration curves, thereby giving us a basis with which to compare the extraction methods and the derivatization agents.

3.2.1. Chemicals

Methanol (HPLC grade), BPA (purity grade > 99 %), E2 (purity grade > 99 %), 4-n-Nonylphenol (NP) (purity grade > 99.9), sodium chloride (NaCl, purity grade > 99.5 %), BSTFA with 1% trimethylchlorosilane (TMCS), and MSTFA with 1% TMCS were purchased from Aldrich (WI, USA).

3.2.2. *Aqueous Samples*

Primary stock standard solutions (1,000 mg/L) of each BPA and E2 were prepared in methanol by dissolving 0.100 g of analyte into 100 mL methanol. Stock solutions were stored at 4 °C in a refrigerator. Aqueous samples were prepared daily by dilution of the stock solutions into deionized water. The concentrations of the aqueous samples ranged from 1 ng/L to 100 µg/L for SPE, and from 30 ng/L to 1 mg/L for SPME. The aqueous samples were prepared from the primary stock solutions by diluting with deionized water, using sequential dilutions when necessary to obtain low concentrations. Methanol content in the aqueous samples was 0.1% or lower (by volume, before mixing) in all aqueous samples, and is therefore considered negligible. For SPME analyses, nonylphenol (NP) was spiked into the deionized water as an internal standard. The concentration of NP in all SPME samples was 10 µg/L. Samples were analyzed by SPE and SPME to compare the two methods.

3.2.3. *Solid-Phase Extraction (SPE)*

Here we describe the SPE method used to prepare a sample for analysis by GC/MS. The procedure is also shown in Figure 3.1. Oasis HLB glass cartridges (5 mL, 200 mg HLB) were purchased from Waters (Milford, MA) and placed on a vacuum manifold (SPE 24-port Vacuum manifold, purchased from Fisher). Figure 3.2 shows the extraction manifold (Vacuum Manifold) and pump. The cartridges were conditioned with 40 mL of deionized water and 25 mL of methanol, both of which were drawn through the cartridges under very low

vacuum to remove residual bonding agents. A known volume of aqueous sample was subsequently loaded onto the cartridge and flowed through under slight vacuum (flow rate = 60 mL/min). We tested different volumes of samples ranging from 10 mL to 4 L, and different EDC concentrations ranging from 1 ng/L to 100 µg/L. During the sample loading step, the target compounds are extracted from the aqueous samples onto the SPE cartridges. After loading, the cartridges

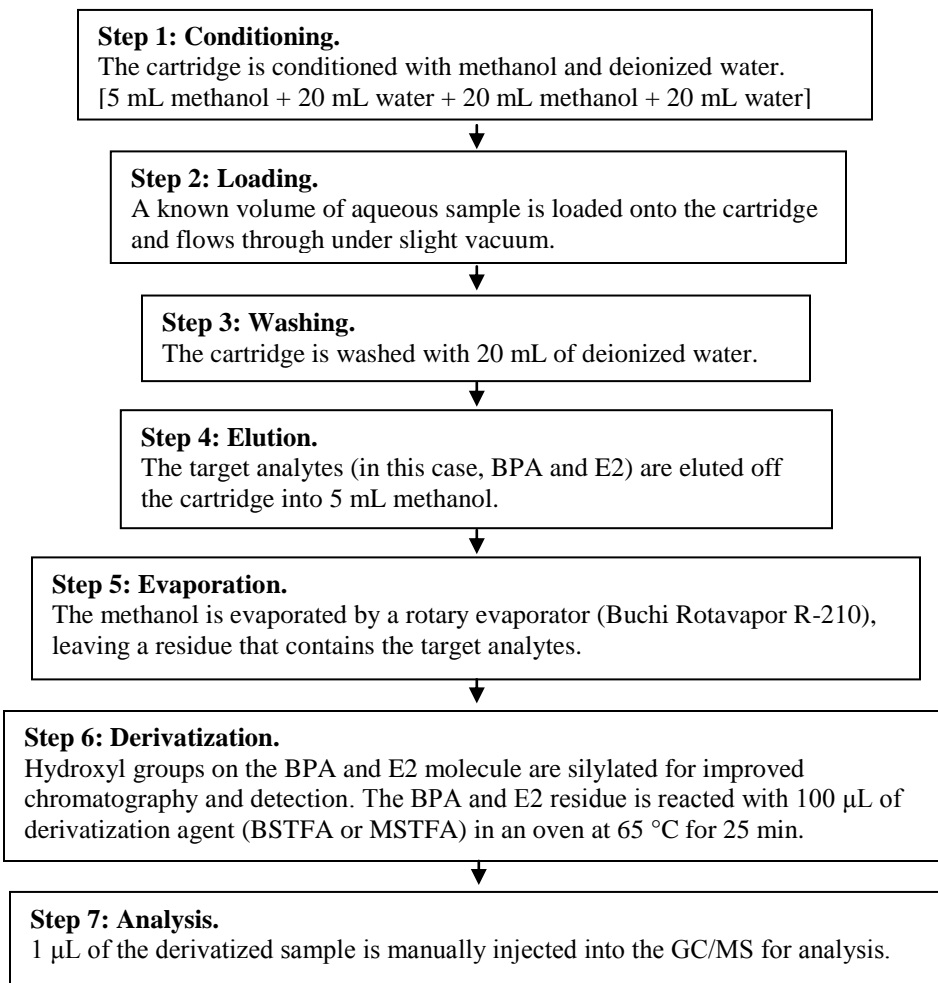


Figure 3.1. Solid-phase extraction (SPE) method: Aqueous samples are loaded onto an OASIS HLB glass cartridge, and then target analytes are extracted from the cartridge with methanol.



Figure 3.2. Extraction manifold (vacuum manifold) and pump

were washed with 20 mL of deionized water, and then dried for 5 min under vacuum in order to remove the excess of water remaining on the cartridge. The adsorbed analytes were eluted from the cartridges into 10 mL vials with 5 mL methanol at a flow rate of 5 mL/min.

Due to the presence of polar functional groups in BPA and E2, which can give rise to poor chromatographic peaks, derivatization was necessary. The methanol eluent collected from SPE was evaporated with a rotary evaporator (Buchi Rotavapor R-210). The dry residues were derivatized either by BSTFA with 1% TMCS or by MSTFA with 1% TMCS. For either agent, 100 μ L of

derivatization reagent was added into each reaction vial. Then, the vials were closed and placed in an oven at 65 °C for 25 min. Once the derivatization was completed, 1 µL of the reaction mixture was injected into the GC/MS system in 30 min to avoid reaction inversion.

3.2.4. *Solid-Phase Micro-Extraction (SPME)*

SPME procedures were performed with a CombPAL auto-sampler (CTC Analytics) using a polyacrylate (PA) fiber of 85 µm thickness. The procedure is shown in Figure 3.3 and SPME fiber is shown in Figure 3.4. The PA fibers were purchased from Supelco. The PA fiber has higher extraction capacities for phenols, anilines, amides, and many drugs and pesticides. In addition, PA fiber has more efficient and linear range of response over a wide range of phenols concentration in water samples than polydimethylsiloxane (PDMS) fiber (Endo et al. 2011). Each fiber was conditioned in the injector of the GC for 90 min at 280 °C before its first use, as described in Supelco's conditioning instructions. Conditioning was followed by blank analysis to determine the conditioning quality. For sample analysis, 10 mL of aqueous sample was placed into a vial with 1.75 g NaCl. Vials were sealed with Teflon-coated silicone septa held by open-top screw caps. SPME extraction was performed by piercing the septum of the sample vial with the autosampler needle and immersing the PA fiber into the aqueous sample. Extraction was performed at 45 °C, controlled by the CombiPAL auto-sampler. During extraction, the samples were continuously agitated with an agitating block at about 400 rpm for duration of 50 min, which we

had previously determined was sufficient time to reach equilibrium. After extraction, the fiber was transferred into the headspace derivatization vials. The headspace derivatization vial contained 1 mL of derivatization reagent and was maintained at 70 °C with a heating block. The SPME needle pierced the septum and the fiber was exposed to the headspace. This allows the EDCs absorbed on the fiber to be derivatized with either BSTFA (1% TMCS) or MSTFA (1% TMCS) vapor rising from the bottom of the vial. During derivatization, the vial was continuously agitated with an agitating block at about 250 rpm. After 5 min of derivatization, the SPME fiber was withdrawn from the derivatization vial and inserted into the GC injection port to perform thermal desorption. The temperature of the injection port was 280 °C, and the desorption time was 3 min.

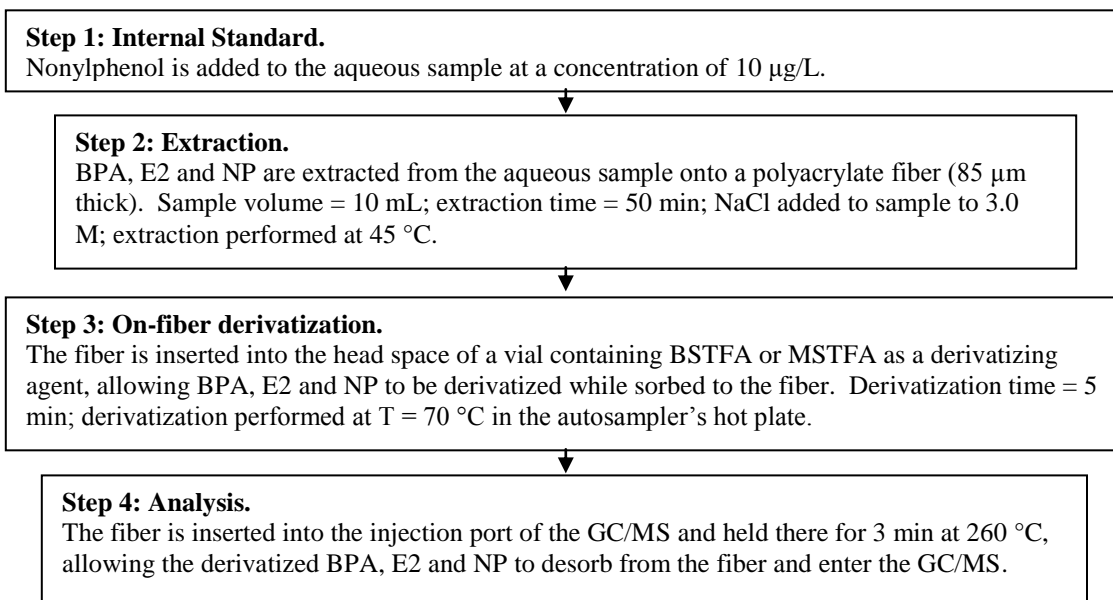
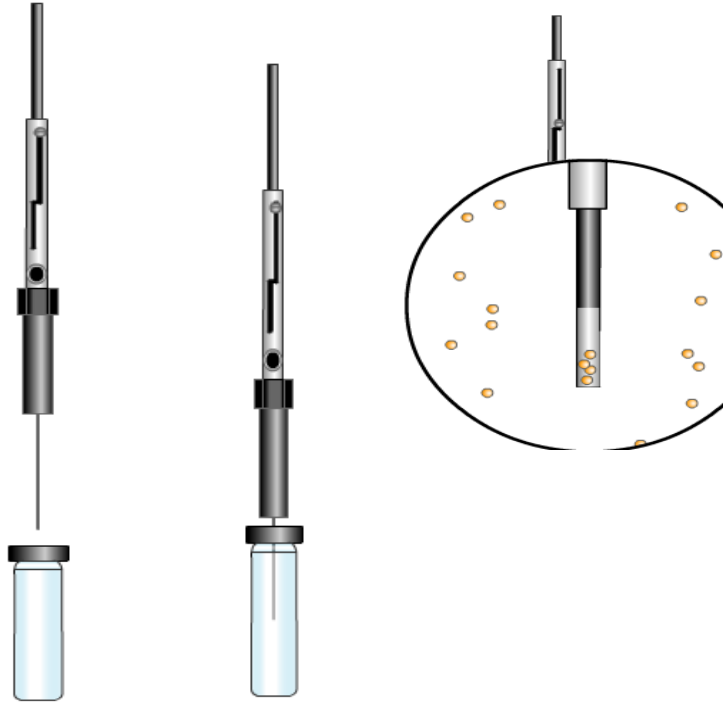


Figure 3.3. Solid-phase micro extraction (SPME) method: Target analytes in aqueous samples are extracted and concentrated onto a fiber. The procedure is fully automated on the Combi-PAL auto-sampler.



http://www.sigmaaldrich.com/Brands/Supelco_Home/Spotlights/SPME_central.html#spmeanim

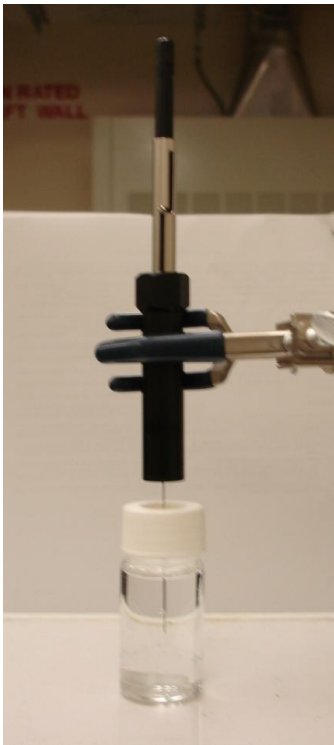


Figure 3.4 SPME fiber

3.2.5. GC/MS Instrumentation and Operating Conditions

Analyses were carried out on a Varian CP-3800 gas chromatograph directly connected to a Saturn 2000 ion-trap mass spectrometer (Varian). GC/MS is shown in Figure 3.5. A HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film, 5% phenyl-dimethylsiloxane phase, Agilent) was used for chromatography. Helium (99.9995% purity) was used as carrier gas at a constant flow rate of 1.0 mL/min. The injection port temperature was 280 °C with splitless mode. The GC oven temperature program was as follows: hold for 1 min at 80 °C, increase at 15 °C/min to 240 °C, hold for 1 min, increase at 10 °C/min to 280 °C, and hold for 5 min. Data acquisition was performed in full scan mode measuring from m/z 69 to 614. The transfer line temperature of the GC/MS was set at 170 °C, and the manifold temperature was set at 160 °C. The electron emission current of GC/MS was 10 μA (70 eV), multiplier voltage was 1500 V, and automatic gain control (AGC) target was 20,000.

BPA, E2, and NP were quantified by the area of the peak corresponding to a particular fragment on the MS. We refer to these fragments as the diagnostic ions for each compound. The m/z ratios for the diagnostic quantitative ions are 357 for BPA, 416 for E2, and 179 for NP. These m/z ratios correspond to major peaks in the mass spectra of the derivatized (silylated) compounds.

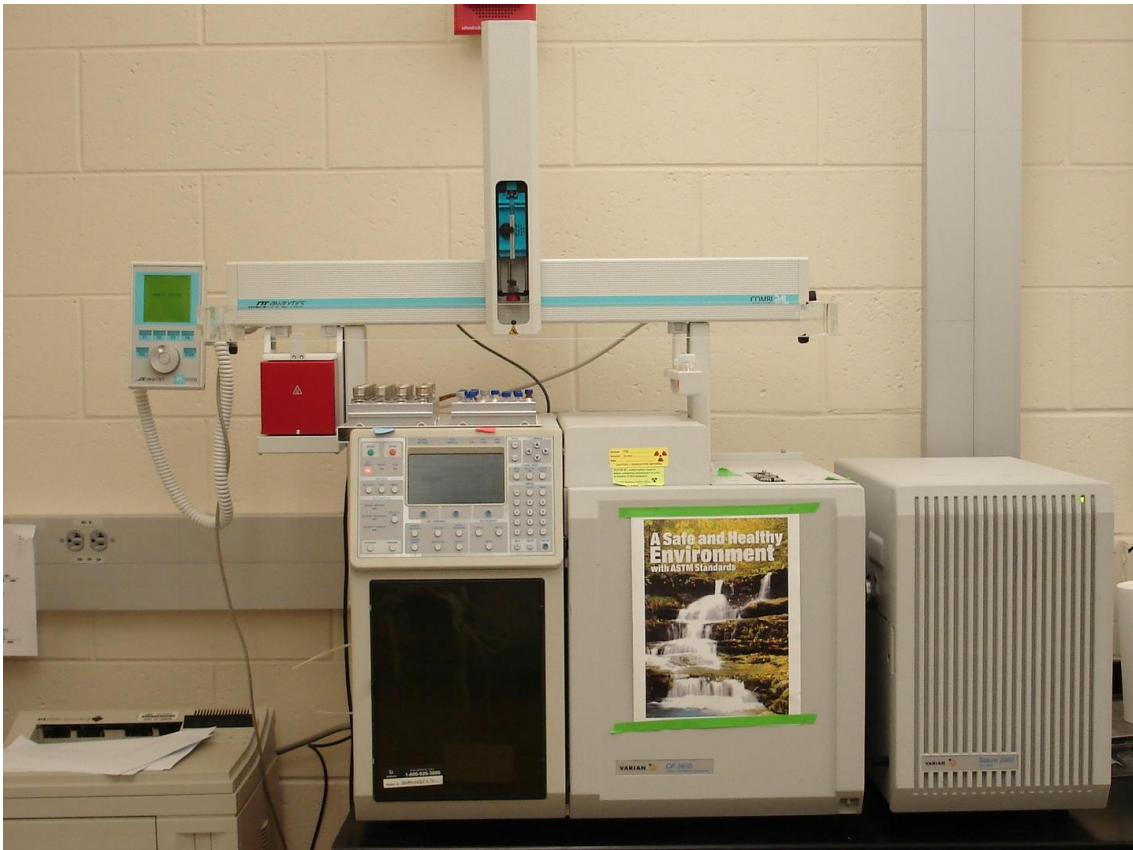


Figure 3.5. Gas Chromatography Mass Spectrometry (GC/MS)
[Varian CP-3800 GC, Saturn 2000 GC/MS, Combi PAL auto sampler]

3.2.6. Method Detection Limit for SPME

Seven replicate samples of concentration 30 ng/L were analyzed by SPME to determine the method detection limit (MDL) based on USEPA procedure 40 CFR, part 136. With this method, the MDL is calculated as the standard deviation of replicate analyses times the student's t value for the 99% confidence level with $n-1$ degrees of freedom (Ripp, 1996). The procedure was conducted for both BPA and E2 at concentrations of 30 ng/L.

3.3. Results

The retention times and mass spectra of the target EDCs (BPA and E2) and the internal standard (NP) were recorded. Retention times are shown in Table 3.1 for both the derivatized and non-derivatized forms of the chemicals. Retention times increased by about 0.1 min for most silylated compounds compared to the non-derivatized compounds.

Table 3.1. Retention time (RT) and mass spectrometric data for endocrine disruptors and their silylated derivatives

Compound	Not derivatized		Derivatized	
	RT (min)	Diagnostic ion	RT (min)	Diagnostic ion
4-Nonylphenol	10.03	220	10.10	179
Bisphenol-A	12.65	213	12.78	357
17 β -Estradiol	16.31	272	16.45	416

López-Blanco et al. (2002) and Braun et al. (2003) compare the SPE and SPME concluded that both extraction methods are good for EDCs extraction because a dilute aqueous sample can become a more concentrated sample. Jeannot et al. (2002) and Zhang et al. (2006) studied SPE method with BSTFA derivatization agent. Chang et al. (2005) studied SPME method with BSTFA derivatization agent. They concluded that derivatization is useful step for effective detection by GC/MS. Because highly polar compounds do not give sharp chromatographic peaks in GC, it is difficult to get a good chromatographic peak. The derivatization reduces the polarity of compounds and thus produces sharp peaks helping better detection. Yang et al. (2006) investigated the optimized condition for SPE and SPME with derivatization. Szyrwińska et al. (2007) compare different kind of derivatization agent and concluded that BSTFA is more useful than BAN. The comparison test is required for selection of which extraction method (SPE and SPME) and which derivatization agent (BSTFA and MSTFA) is “better” to detect and quantify the EDCs in water samples.

3.3.1. SPE: Calibration Curves

The calibration curves of EDCs extracted by SPE are presented in Figures 3.6 and 3.7 as measured peak area versus injected EDC mass. The injected EDC mass is calculated as the volume, V , of sample loaded onto the SPE cartridge (ranging from 20 mL to 4000 mL) times the concentration, C , of target EDC in the sample (ranging from 0.001 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$).

For BPA (Figure 3.6), the calibration curve was generated from samples that met three criteria: the concentration C_{BPA} was between 0.010 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$; the sample volume V was between 20 mL and 4000 mL; and the BPA mass loaded ($M = V \cdot C_{\text{BPA}}$) was between 30 ng and 5000 ng. The third criterion implies, for instance, that for samples where we used a volume $V = 100$ mL, the calibration curve includes all results for which $0.30 \mu\text{g/L} \leq C_{\text{BPA}} \leq 50 \mu\text{g/L}$, but not for samples outside this concentration range. As can be seen from Figure 3.6, the measured peak area is linear with respect to the BPA mass injected for samples meeting the three necessary criteria.

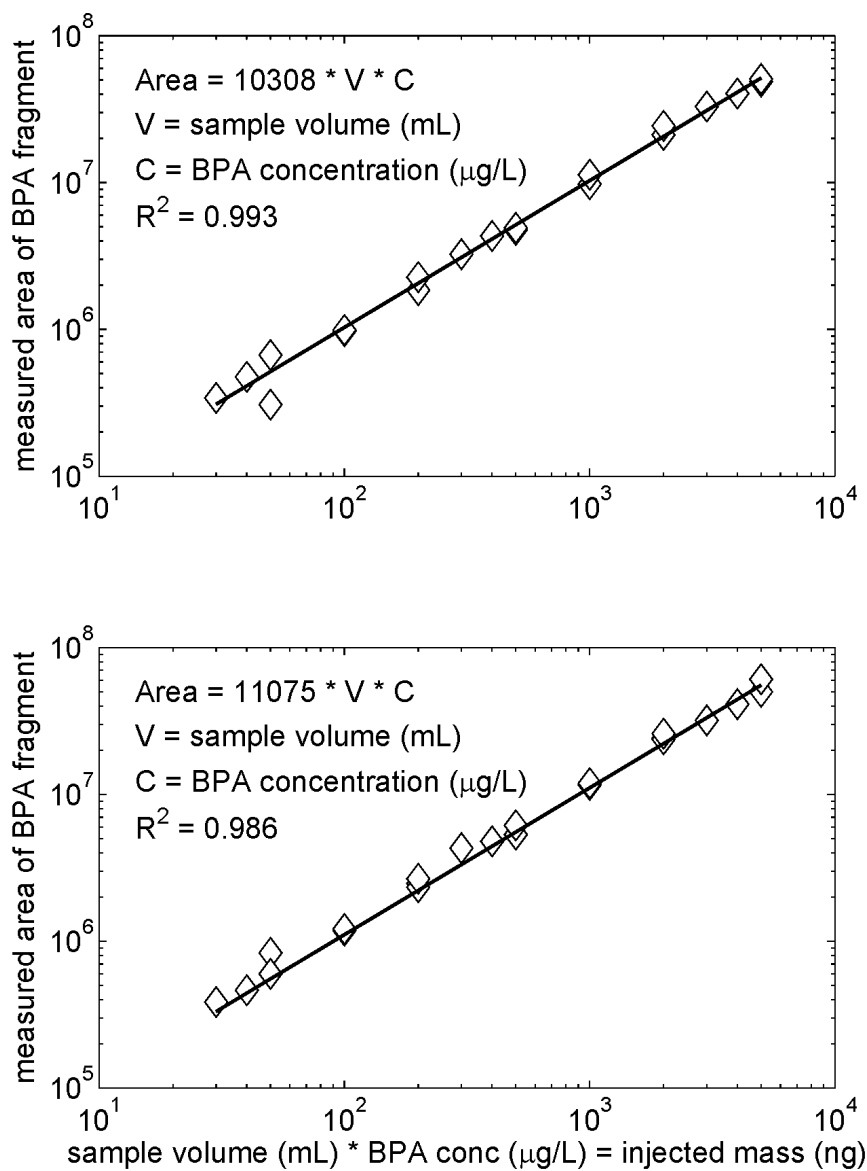


Figure 3.6. Calibration curves for BPA samples extracted by SPE. Top panel: derivatized with BSTFA. Bottom panel: derivatized with MSTFA.

For E2 (Figure 3.7), similar behavior was observed, but the range of linearity was even greater for E2 than it was for BPA. For E2, the calibration curves were generated from samples which met the following three criteria: the concentration C_{E2} was between 0.010–100 $\mu\text{g/L}$; the sample volume V was between 20–4000 mL; and the E2 mass loaded ($M = V \cdot C_{E2}$) was between 20 ng and 20,000 ng. The third criterion implies, for instance, that a sample volume of $V = 500$ mL could be used to quantify concentrations in the range $0.040 \mu\text{g/L} \leq C_{E2} \leq 40 \mu\text{g/L}$. As can be seen from Figure 3.7, the measured peak areas were linear (log scale) with respect to the E2 mass injected for samples meeting these criteria.

For both BPA and E2, we did test several samples that did not meet one of the requisite criteria (e.g., samples of concentration $C < 10$ ng/L, or samples for which $V \cdot C$ is not in the specified range). These samples generally did not follow the same linear behavior, and are not included in Figures 3.6 and 3.7. Hence, there is some limitation on the range of linearity for the SPE method; if the concentration is too low or too high, the measured peak area is not likely to fall on the calibration curves provided. However, this limitation is not severe; simply by choosing the sample volume appropriately, the SPE method may be applied to samples of BPA or E2 in the concentration range 10 ng/L to 100 $\mu\text{g/L}$, a range of four orders of magnitude. We found that $C = 10$ ng/L is a practical lower limit of quantification for the SPE method for both BPA and E2.

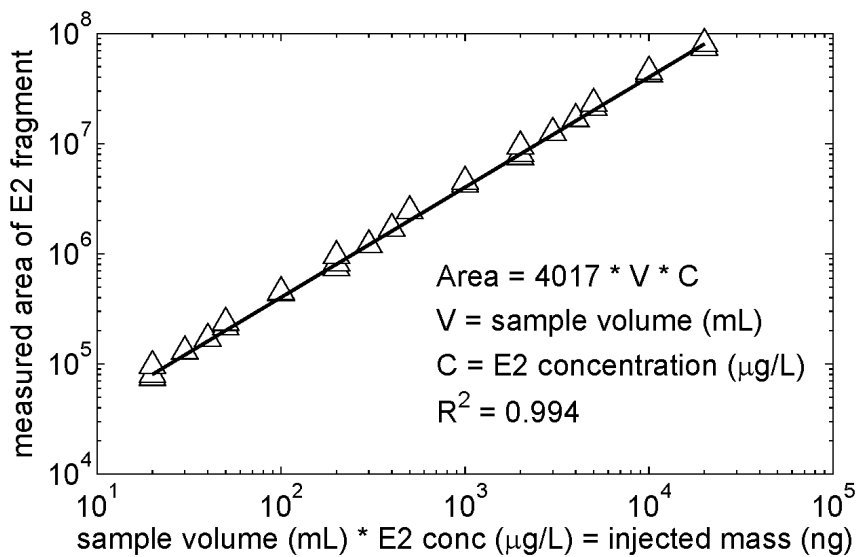
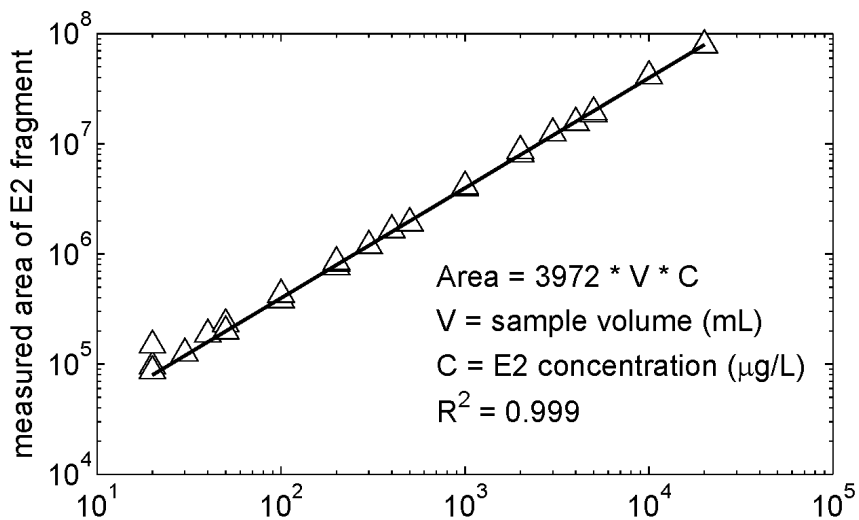


Figure 3.7. Calibration curves for E2 samples analyzed by SPE. Top panel: derivatized with BSTFA. Bottom panel: derivatized with MSTFA.

3.3.2. SPME: Calibration Curves

The calibration curves of EDCs extracted by SPME are presented in Figures 3.8 and 3.9 as ratio of peak areas versus aqueous EDC concentration. The ratio of peak areas is the quotient of the measured area of the diagnostic ion for the target EDC (either BPA or E2) divided by the measured area of the diagnostic ion for the internal standard (NP). We found that the performance of the SPME fibers changes over time, and therefore it is not acceptable to use only the peak area of the BPA or E2 fragment; the fragment area must be normalized by that of the internal standard to account for the transient behavior of the SPME fibers (and for other sample-to-sample variability of the automated SPME procedure).

Figure 3.8 shows that the calibration curves are linear with respect to EDC concentration in the high concentration range (50–1000 $\mu\text{g/L}$). However, at lower concentration ranges, we consistently found that the ratio of peak areas was not linear with respect to the EDC concentration. Figure 3.9 is presented on logarithmic axes, and it is observed that the calibration curves in the lower concentration range (0.030–30 $\mu\text{g/L}$) are log-linear but not linear. In general, it is expected that SPME should produce a linear response factor, and if deviation from linearity is observed, it is more likely to be in the high concentration range (i.e., if the SPME fiber becomes saturated) rather than in the low concentration range (Tuduri et al. 2003). Hence, the behavior observed here is unexpected. However, we consistently observed this deviation from linearity in the low

concentration range, for both BPA and E2, and with both MSTFA and BSTFA derivatization agents.

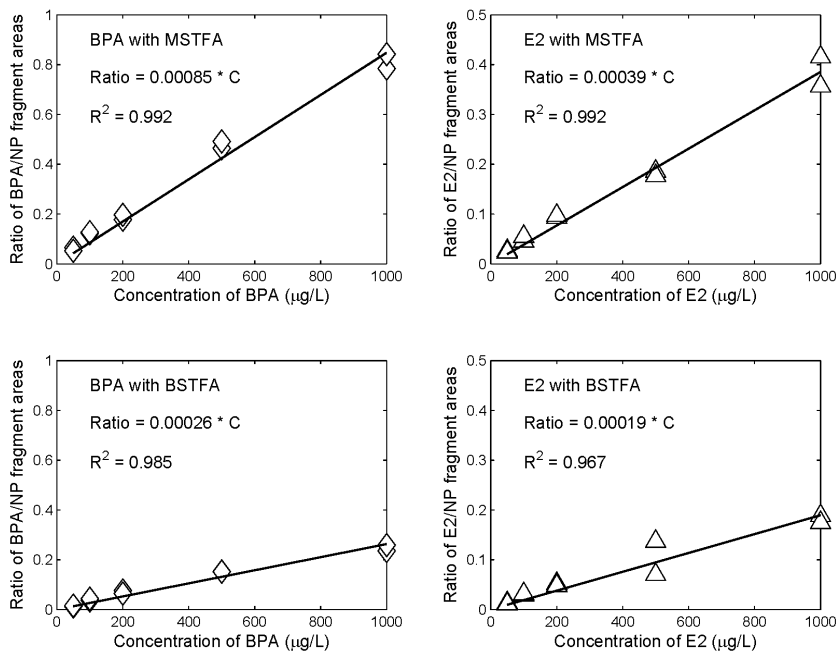


Figure 3.8. Calibration curves for BPA and E2 extracted by SPME, high concentration range (50–1,000 $\mu\text{g/L}$).

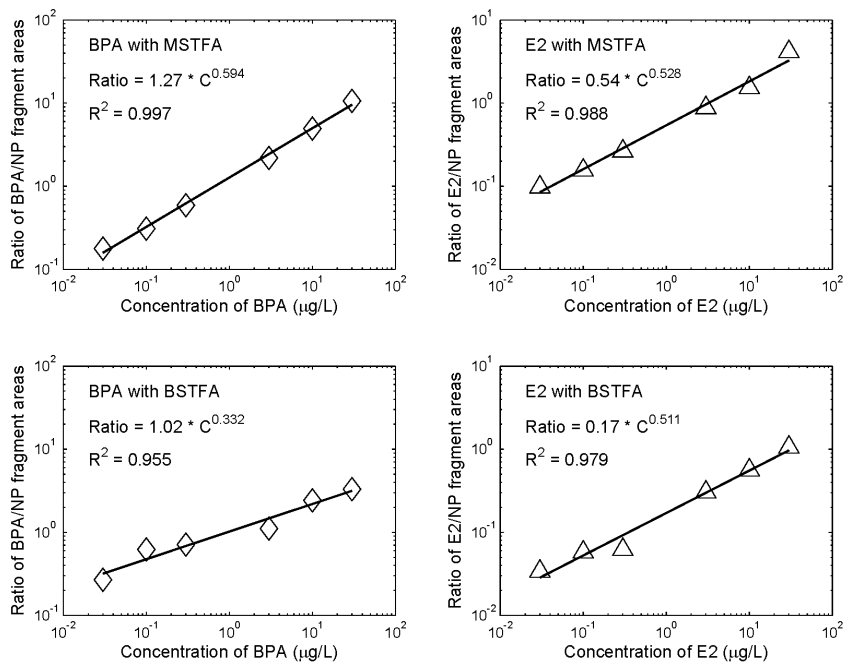


Figure 3.9. Calibration curves for BPA and E2 extracted by SPME, low concentration range (0.030–30 μg/L).

For analysis using SPME, we observed that the calibration curves depend on the individual SPME fiber employed, and hence a calibration curve developed with one fiber would not be applicable to analyses performed with a different fiber. For instance, in comparing Figures 3.8 and 3.9, it can be seen that the ratio of BPA/NP areas is often higher in the low concentration range (Fig. 3.9) than in the high concentration range (Fig. 3.8). This is because the SPME fiber was changed between analyses of the different concentration ranges. Hence, only data collected with the same SPME fiber may be compared to each other. For the experimental procedure employed in this study, we observed that a single SPME fiber can be used for somewhere between 20 and 30 samples before it must be changed.

3.3.3. SPME: Method Detection Limits and Performance

Replicate analysis of BPA and E2 samples at concentration 30 ng/L allowed us to determine the method detection limit (MDL) of the SPME procedure. For both target analytes, the MDL was found to be 10 ng/L when MSTFA was used, and 15 ng/L when BSTFA was used.

3.4. Discussion

3.4.1. Selection of Derivatization Agent (MSTFA or BSTFA)

When using SPE to extract the target analytes from aqueous solution, either MSTFA or BSTFA may be used. Examination of Figures 3.6 and 3.7 shows that the calibration curves are nearly identical for the two derivatization

agents. For analysis of BPA (Fig. 3.6), the slopes of the calibration curves differ by only about 7% for the two derivatization agents. For analysis of E2 (Fig. 3.7), the slopes differ by only about 1%.

However, for on-fiber derivatization during the SPME extraction, MSTFA was consistently found to result in larger peak areas for the diagnostic ions as compared to BSTFA. This suggests that MSTFA would probably result in more reliable analysis (higher signal-to-noise ratios) of target EDCs in the low concentration range. Furthermore, MSTFA produced higher BPA/NP and E2/NP ratios than BSTFA, as can be seen from both Figure 3.8 and Figure 3.9; however, a higher ratio of peak areas does not necessarily mean a “better” analysis. If, for instance, nonylphenol had been the target analyte and 17 β -estradiol had been the internal standard, then MSTFA would likely have resulted in lower NP/E2 peak ratios, but would still probably be preferable to BSTFA because the magnitude of all measured peak areas is larger. The larger peak areas produced with MSTFA may be a result of MSTFA having a higher vapor pressure than BSTFA (Shareef et al. 2006, cf. Donike 1969), and therefore being present at a higher concentration in the head space during the on-fiber derivatization step of the SPME analysis. However, we were not able to find measured values of vapor pressure of MSTFA and BSTFA to support this hypothesis; hence, the reason for the higher peak areas obtained with MSTFA is still uncertain.

3.4.2. Selection of Extraction Method (SPE or SPME)

The selection of which extraction method is “better” depends on a number of factors, such as time, money, the amount of sample volume available, and the expected concentration range of the samples to be analyzed.

When either time or sample volume is a limiting factor, SPME may be preferable to SPE. The SPME method is much less labor-intensive because it can be automated by the CombiPAL auto-sampler. The SPE method, however, requires multiple steps that must be performed by hand. Also, although SPE was successful with sample volumes as low as 20 mL, the effective detection limit increases as the sample volume decreases (because the calibration curves are linear only if $V \cdot C$ satisfies a minimum criterion). With a sample volume of 20 mL, our SPE procedure is applicable to BPA concentrations down to 1.5 $\mu\text{g/L}$ and E2 concentrations down to 1.0 $\mu\text{g/L}$. In contrast, the SPME method requires a sample volume of only 10 mL and had a method detection limit of 0.010 $\mu\text{g/L}$ for BPA and 0.015 $\mu\text{g/L}$ for E2.

However, there are also conditions under which the SPE method may be preferable to SPME. The SPME method has a higher materials cost because SPME fibers are relatively expensive and can only be used for approximately 20–30 analyses (based on the conditions of our method), some of which must include calibration standards, because the instrument must be re-calibrated each time the fiber is changed. Hence, each sample analyzed by SPME is costly. In contrast, SPE cartridges are relatively inexpensive, and we found that a cartridge may be used multiple times (six times were tested) without loss of performance.

Also, the SPE calibration curves were linear over several orders of magnitude, whereas the SPME calibration curves were linear only in the high concentration range (50 µg/L – 1,000 µg/L) and were log-linear at lower concentration ranges. Therefore, if a number of samples must be analyzed which might consist of widely varying concentrations, SPE is probably preferable to SPME because of its much wider range of linearity.

In terms of detection limits, we found that about 10 ng/L was a practical lower limit of analysis for either method. With SPME, the method detection limit was determined to be 10 ng/L for BPA and 15 ng/L for E2. With SPE, the calibration curve was found to be linear with respect to concentration only if the concentration was 10 ng/L or higher. Hence, neither method offered a significant advantage in terms of detection limit, as long as sufficient sample volume is available for the SPE method.

3.5. Conclusions

The purpose of this study was to compare analytical methods based on SPE and SPME with derivatization followed by GC/MS for detection and quantification of EDCs in water samples. Two particular EDCs, bisphenol-A (BPA) and 17β-estradiol (E2) was focused on this chapter. The important contributions of this paper are: (1) I determined which derivatization agent, MSTFA or BSTFA, is more effective in both SPE and SPME; and (2) I determined which extraction method (SPE or SPME) is preferable depending on

operating factors such as the sample volume available and the concentration of the target analyte in the sample.

With regard to derivatizing agent, either MSTFA or BSTFA may be used when SPE (HLB cartridge) is the extraction method. Calibration curves were nearly identical for the two derivatization agents. For on-fiber derivatization during the SPME extraction, MSTFA was consistently found to result in larger peak areas for the diagnostic ions as compared to BSTFA. This suggests that MSTFA would probably result in more reliable analysis (higher signal-to-noise ratios) of target EDCs in the low concentration range. I suspect that the larger peak areas produced with MSTFA may be a result of MSTFA having a higher vapor pressure than BSTFA, and therefore being present at a higher concentration in the head space during the on-fiber derivatization step of the SPME (PA fiber) analysis. With regard to extraction method, the selection of which method is “better” depends on a number of factors, such as time, money, the amount of sample volume available, and the expected concentration range of the samples to be analyzed. When either time or sample volume is a limiting factor, SPME may be preferable to SPE, because the SPME procedure can be automated on the CombiPA auto-sampler, and because the SPME method allows a low detection limit with only 10 mL of sample. The SPE method is labor-intensive and requires large sample volumes to achieve low detection limits. There are also conditions under which the SPE method may be preferable to SPME. The SPE method has a significantly lower materials cost, despite the need for an extraction solvent like methanol, because SPME fibers are relatively

expensive and can be used for only a limited number of samples. Also, the SPE calibration curves were linear over several orders of magnitude, so if a number of samples must be analyzed which might consist of widely varying concentrations, SPE is probably preferable to SPME.

Of course a number of factors have not been considered in this study. For instance, my conclusions are based on calibration curves generated by spiking the target analytes into purified water; therefore I have not considered matrix effects which might be important in samples collected from a natural environment or from a wastewater treatment plant. Nevertheless, the comparison provided here can offer useful guidance to scientists and engineers who are trying to develop and select a useful analytical procedure for endocrine-disrupting compounds in aqueous samples at the ng/L to $\mu\text{g/L}$ range.

CHAPTER 4

BIODEGRADATION OF TARGET EDCs UNDER ALTERNATING AEROBIC AND ANAEROBIC CONDITIONS

4.1. Introduction

During alternating aerobic/anaerobic conditions, diauxic lag might be an important factor for biodegradation of EDCs. However, limited information is available in the literature about the degradation of EDCs under these types of conditions. Therefore, the purpose of this chapter is to determine the fate of EDCs during alternating redox conditions. The lag time and the phenomenon of diauxic lag were investigated for target EDCs under alternating aerobic/anaerobic conditions for efficient EDC removal. The objectives of this chapter are to quantitatively distinguish between sorption-based and biodegradation-based removals, measure the lag-phase of aerobic biodegradation depending on the period of alternating cycles between aerobic and anaerobic conditions in simulated SAT systems, and investigate the rate of biodegradation for target EDCs under alternating aerobic/anaerobic conditions.

4.2. Materials and Methods

4.2.1. Chemicals

SYTOX Green nucleic acid stain was purchased from Molecular Probes (OR USA). Sodium azide (NaN_3) and sodium nitrate (NaNO_3) were purchased from Aldrich (WI USA). Methanol (HPLC grade), BPA (purity grade > 99 %), E2 (purity grade > 99 %), 4-n-Nonylphenol (NP) (purity grade > 99.9 %), sodium chloride (NaCl , purity grade > 99.5 %), and MSTFA with 1% TMCS were purchased from Aldrich (WI, USA).

4.2.2. Soil and Wastewater

Tertiary-treated effluent (final effluent) was obtained from Howard F Curren Advanced Waste Water Treatment Plant (WWTP) in Tampa, FL. Aquifer soil was obtained from a constructed Wetland Wastewater Treatment System in Lakeland, FL. Characterization of effluent from WWTP is shown in Table 4.1. The plant has a design capacity of 96 million gallons per day and the effluent water is discharged to Hillsborough Bay or used as reclaimed water for cooling and irrigation. Soil samples were collected from the top 10 cm, 50 cm, and 100 cm of the pond bed and placed in glass jars. Upon arrival back to the laboratory, the jars were kept under refrigeration. Collected soil was mixed in the laboratory.

Table 4.1. The quality of effluent in Howard F. Curren Advanced WWTP

	2009 Annual Average	Permit Requirement	Percent Removal
Flow	54.2 MGD		
BOD ₅	1.5 mg/L	5.0 mg/L	99.1
TSS	0.5 mg/L	5.0 mg/L	99.7
Total Nitrogen	2.33 mg/L	3.0 mg/L	92.4

Source: Howard F. Curren Advanced WWTP

http://www.tampagov.net/dept_wastewater/information_resources/Advanced_Wastewater_Treatment_Plant/facts_of_interest.asp

4.2.3. Batch Mesocosm Reactors

Simulated SAT systems were set up in 4 L reactors with 3 L effluent from WWTP and 500 g aquifer soil placed in each reactor. BPA and E2 were spiked to simulated SAT system at an initial concentration of 1,000 µg/L. This concentration is higher than could be observed in most WWTP effluent streams. However, for this research, I chose to use a high EDCs concentration in order to prove the clear variation of EDCs during alternating conditions. If the initial EDCs concentration were too low then won't be able to observe removal of up to 99%. In general, batch simulated SAT mesocosms were run under alternating aerobic/anaerobic conditions. The simulated SAT systems were operated in a dark environment (the reactor was wrapped in aluminum foil) at room temperature (20 °C). Schematic diagram of a simulated SAT reactor is shown in Figure 4.1 and the photograph of a simulated SAT reactor is shown in Figure 4.2.

Because the SAT system is alternating between aerobic and anaerobic condition, simulated SAT systems were established by purging the system with

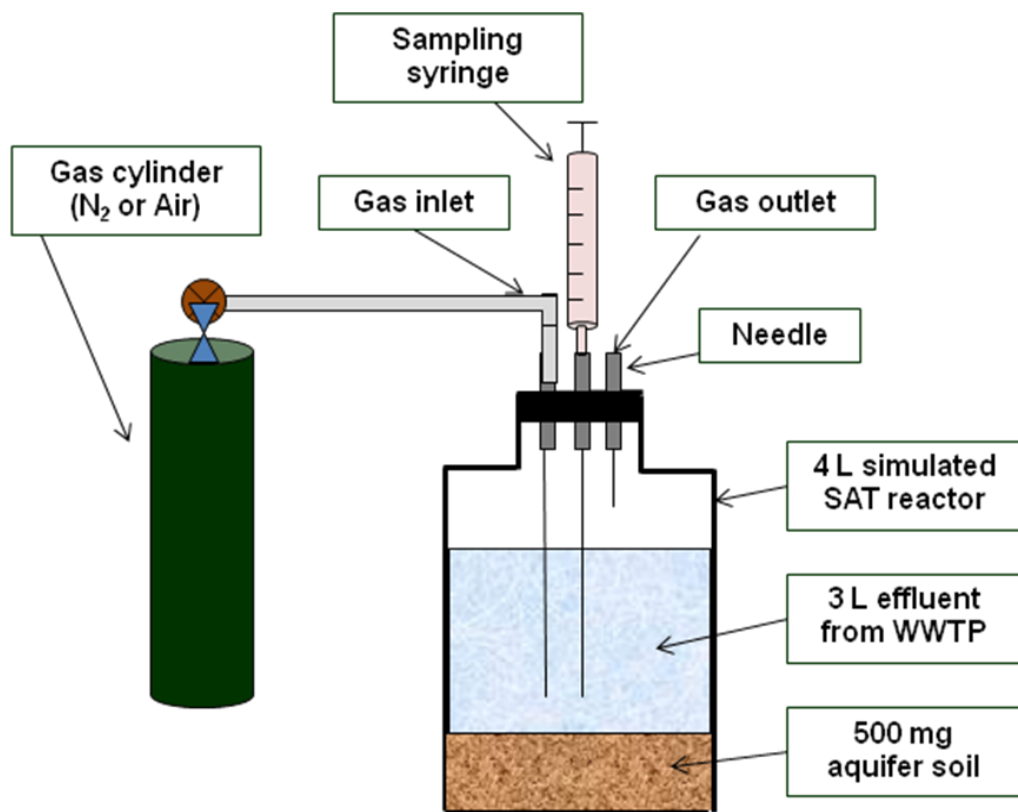


Figure 4.1. The schematic diagram of simulated SAT reactor.



Figure 4.2. The picture of simulated SAT reactor.

either air (21% O₂) or N₂ gas (O₂ free) in order to make the alternating aerobic and anaerobic conditions. Aerobic environment was established by air gas passing through the simulated SAT system, and then it was switched to anaerobic environment by N₂ gas passing through the system. The gas tank was connected with the simulated SAT reactor by fluoropolymer tubing and a syringe. The simulated SAT system was capped by silicone sealing in order to control the aerobic/anaerobic condition, and the air gas and N₂ gas were connected through this cap by syringe. Aerobic environment and anaerobic environment were manually switched by reconnecting the fluoropolymer tube and syringe. In addition, a sampling syringe was connected through the cap.

In order to make nitrate-reducing condition during anaerobic cycles, nitrate was spiked to simulated SAT system at an initial concentration of 1,000 mg/L. This concentration is higher than could be observed in most WWTP effluent streams. However, for this research, I chose to use a high EDCs concentration in order to prove the clear variation of nitrate during alternating conditions and won't be able to observe removal of up to 99%. Batch mesocosm reactors were used for lab experiments, and different periods of alternating conditions were tested. Nine simulated SAT batch reactors were prepared (Table 4.2 shows the condition of reactor; test was based on the triplicate); three control tests for sorption test without microbiological activity (Reactor C1, C2, and C3; same condition), and the other six test for comparison of different anaerobic duration (two days; reactor NS1, NS2, and NS3; same condition, and four days; reactor NL1, NL2, and NL3; same condition). Control test was studied to distinguish

between sorption to the soil and biodegradation during simulated SAT system. In order to know only sorption amount to the soil without microbiological activities, 1,000 mg/L NaN_3 was used for soil sterilization in control test (Kao et al. 2004, Zhang et al. 2009).

Table 4.2. The condition of nine simulated SAT batch reactors

Reactor #	Condition of Reactor		Duration (day)	
C1	Control for sorption test		20	
C2	Control for sorption test		20	
C3	Control for sorption test		20	
	Alternating system	During anaerobic	Aerobic-Anaerobic	
NS1	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
NS2	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
NS3	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
	Alternating system	During anaerobic	Aerobic-Anaerobic	
NL1	3 aerobic/ 4anoxic	Anoxic denitrification	31	3-4-3-4-3-4-3-4-3
NL2	3 aerobic/ 4anoxic	Anoxic denitrification	31	3-4-3-4-3-4-3-4-3
NL3	3 aerobic/ 4anoxic	Anoxic denitrification	31	3-4-3-4-3-4-3-4-3

4.2.4. Sampling and Analysis

On the first day of each aerobic and anaerobic condition, six samples were taken (volume of sample is 10 mL, sampling time is 1, 3, 6, 10, 16, and 24 hr). Then, three samples of each condition were taken in each day thereafter (the frequency of sampling is 1 per 8 hour). BPA and E2 were extracted from sample by SPME and analyzed by GC/MS (as described in Chapter 3). When the concentration of EDCs was below 100 $\mu\text{g/L}$ (90 % removal), EDCs was re-spiked (100 $\mu\text{g/L}$ additional).

Nitrate and nitrite were analyzed by Metrohm Ion Chromatography (881 Compact IC pro and 863 Compact Autosampler), using a Metrosep A Supp 7-250 (250 mm x 4 mm) analytical column, the eluent was sodium carbonate (3.6 mmol/L), and the flow rate was 0.7 ml/min.

The microorganism population density was estimated by flow cytometry (BD FACSCanto II Analyzer, High-throughput flow cytometer). SYTOX Green Nucleic Acid Stain was used to dye the bacteria. 1 mL aqueous sample was mixed with 1 mL 4% paraformaldehyde and stay 5 min. It was centrifuged and discard the supernatant. Put the stain (5 μ M, 300 μ L) into precipitation and resuspension. Put 200 μ L sample into 96 tray and detect the microbes by flow cytometry. It is a direct enumeration method (Gunasekera et al. 2000, Chen et al. 2001). Microorganisms stained with SYTOX Green Nucleic Acid Stain yield bright and stable fluorescent signals that could be detected by flow cytometry.

Dissolved oxygen concentration monitored by YSI DO200 DO meter, and the redox state was checked by pH100 redox potential meter.

4.3. Results and Discussion

4.3.1. Control Test

BPA and E2 (1,000 μ g/L) were spiked into reactors for control test with no microbiological activity (1 g/L NaN_3). EDCs concentration in the aqueous phase is shown in Figure 4.3. Because there is no microbiological reaction, sorption is the main removal mechanism in reactor. BPA and E2 were rapidly decreased in first-day of reaction by sorption to soil, and they were maintained until the end of

the reaction. The equilibrium concentration of BPA is 110 $\mu\text{g/L}$ and E2 is 210 $\mu\text{g/L}$ in aqueous phase. In real application of SAT, sorption is probably not a viable removal mechanism over long periods of time, because eventually the soil equilibrate with the percolating water. Therefore biodegradation must be sustained in order to have sustained removal of EDCs form infiltrating water.

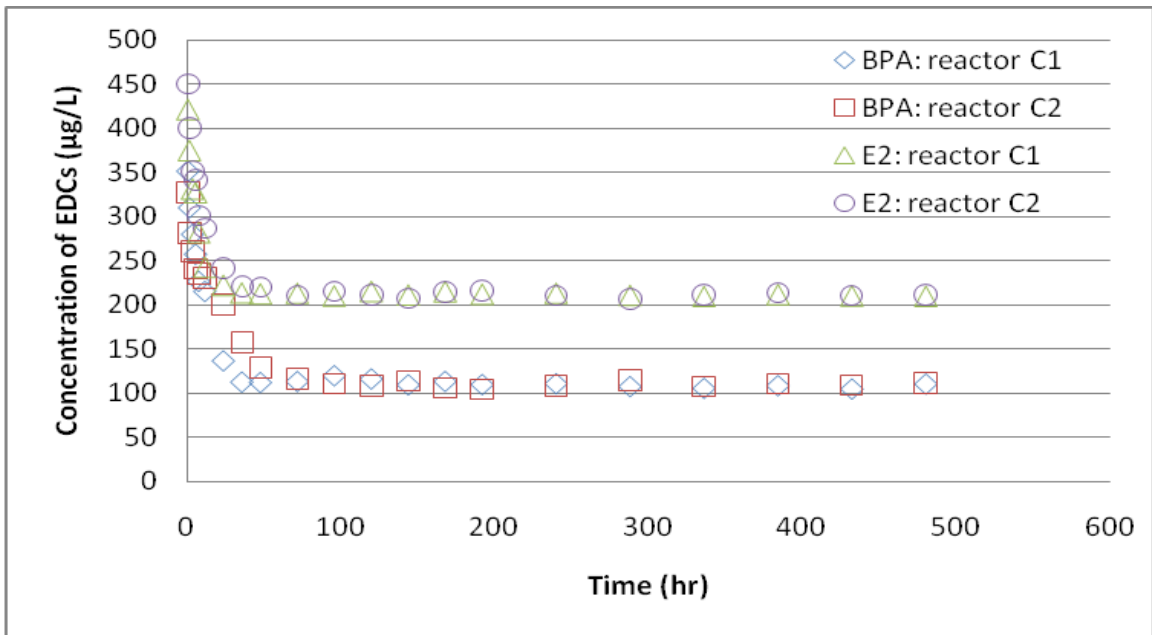


Figure 4.3. Concentration of EDCs as a function of time in reactors without microbiological reaction.

4.3.2. Dissolved Oxygen

Dissolved oxygen concentration in alternating between aerobic and anaerobic condition is shown in Figure 4.4 and 4.5. Because simulated SAT system was established by passing either air (21% O₂) or N₂ gas (O₂ free) in order to make the alternating aerobic and anaerobic conditions, during aerobic condition the dissolved oxygen level is between 7.5 mg/L and 7.9 mg/L (oxygen concentration in saturated condition at room temperature: 9.09 mg/L at 20 °C, and 8.26 mg/L at 25 °C), and during anaerobic condition the dissolved oxygen level is between 0.3 mg/L and 0.5 mg/L. Aerobic environment was established and continued by air gas passing through the simulated SAT system, and then it was switched to anaerobic environment by N₂ gas passing through the system. As seen in Figure 4.4 and 4.5, the concentration of dissolved oxygen dropped very quickly when the reactor was switched from air to N₂, and rose very quickly when the reactor was switched from N₂ to air.

Oxygen dissolves to the water by absorption from air or by photosynthesis. The aerobic bacteria and plants consume the dissolved oxygen in aquatic environment. The aerobic and anaerobic/anoxic conditions of aquatic environment depend on the rates of dissolution and consumption. During aerobic condition, aerobic microbes use organic matter and dissolved oxygen, and aerobic microbes produce additional cells, partially oxidized organic compounds, and carbon dioxide. During absence of dissolved oxygen, anaerobic microbes perform the fermentative metabolism in order to produce the energy for growth.

The aerobic condition is that when the microorganism(s) used O_2 as the terminal electron acceptor, and the anaerobic/anoxic condition is that when the microorganism(s) carry out the fermentation without terminal electron acceptors or uses chemicals other than oxygen (nitrate, sulfate, iron, etc.) as terminal electron acceptors.

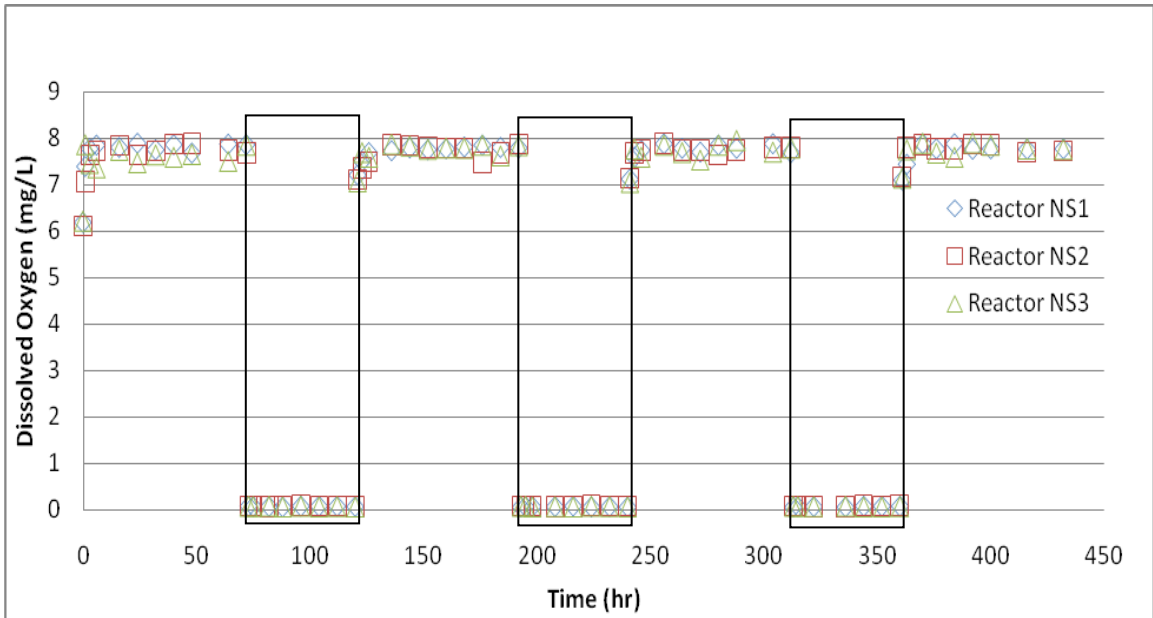


Figure 4.4. Concentration of dissolved oxygen as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

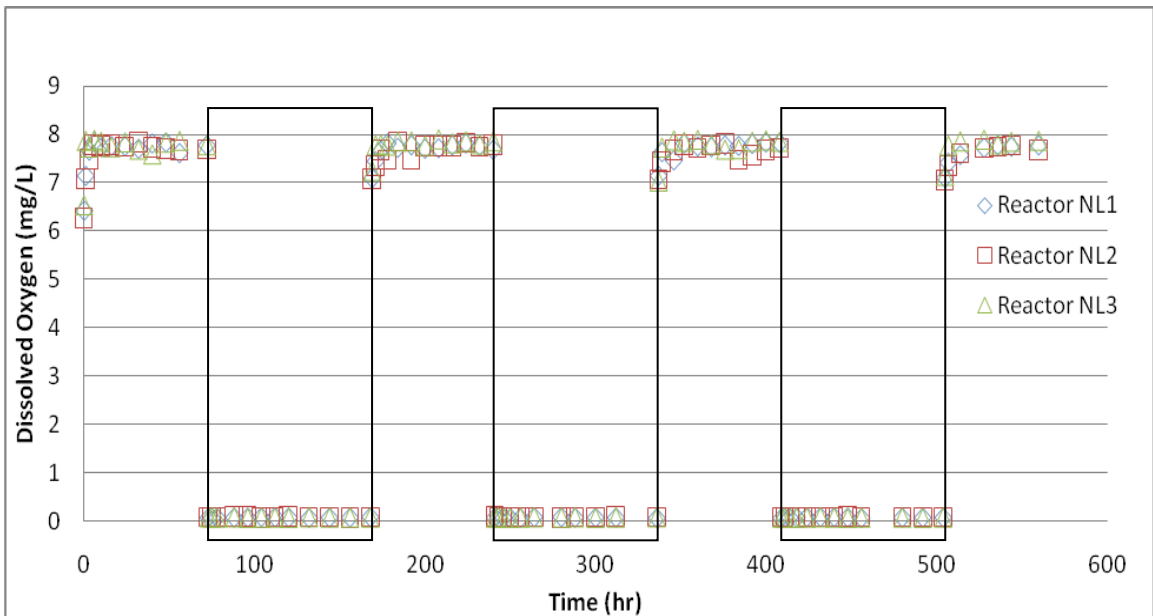


Figure 4.5. Concentration of dissolved oxygen as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

4.3.3. Oxidation-Reduction (Redox) Potential Level

The redox potential of the water in the reactor responded rapidly to changes in the aeration status of the water. Figure 4.6 and Figure 4.7 shows the redox potential level in each 2 days and 4 days anaerobic period. The maximum redox potential under aerobic conditions for all treatments was approximately +240 mV. The redox potential rapidly decreased right after air was switched by N₂ gas. The redox potential level was fallen down to approximately -100 mV and remained at this value throughout anaerobic condition. On the contrary to this, the redox potential rapidly increased right after N₂ gas was switched to air. For the aerobic condition the redox potential level increased rapidly to approximately + 240 mV and remained constant for the entire aerobic period (3 days).

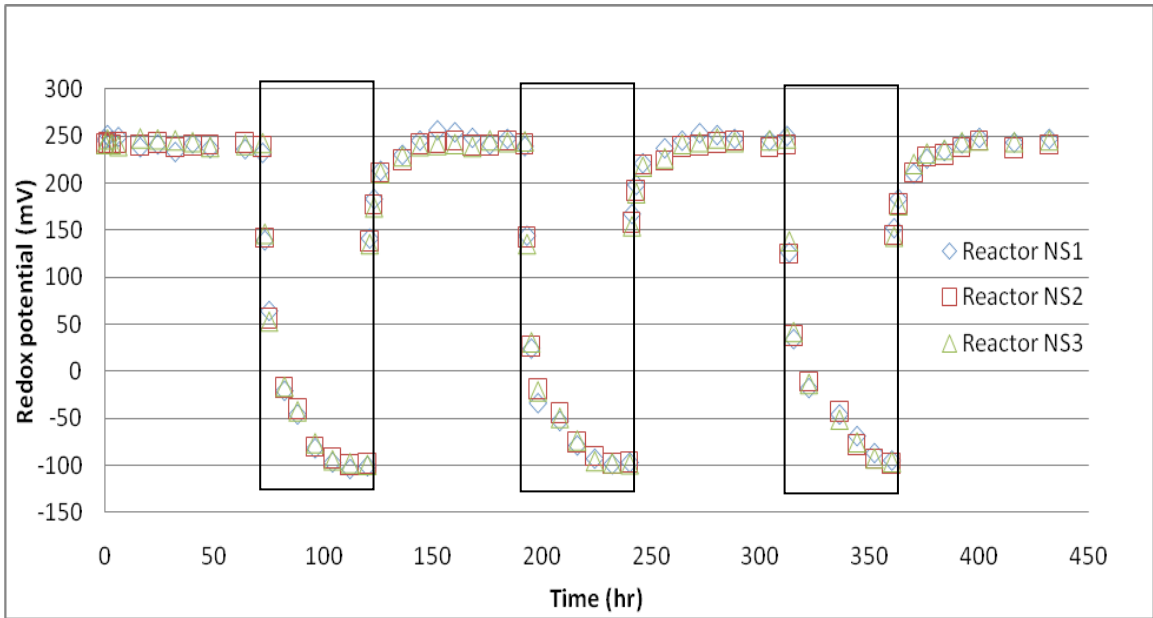


Figure 4.6. Redox potential as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

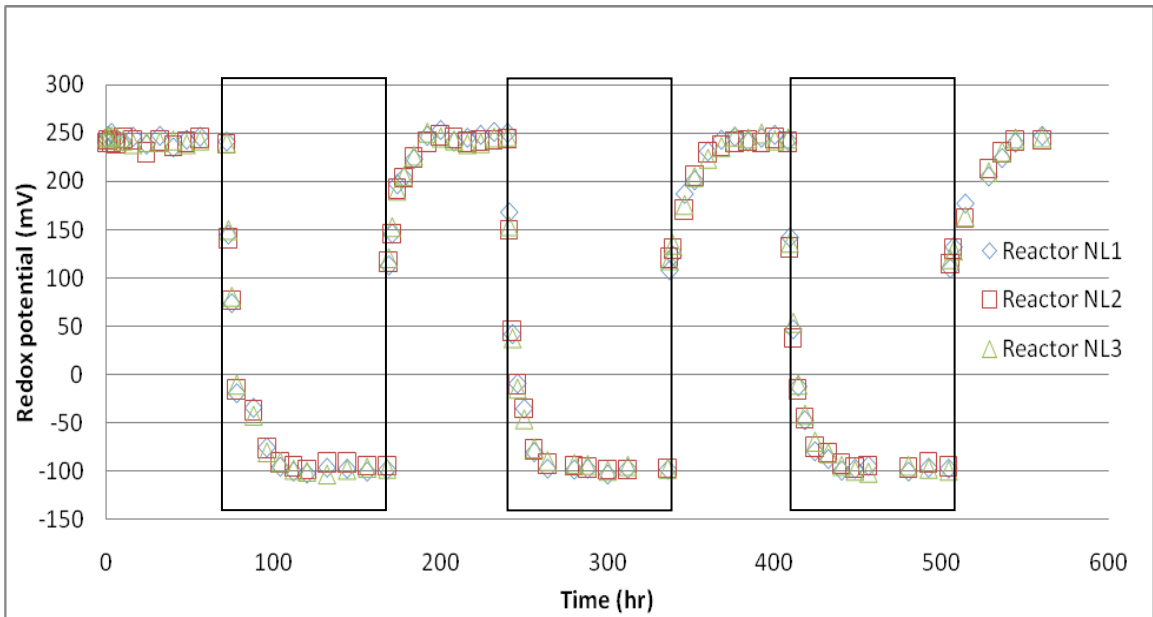


Figure 4.7. Redox potential as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

4.3.4. Concentration of Electron Acceptor

Nitrate and nitrite concentration in simulated SAT system are shown in Figure 4.8 and 4.9. In aerobic cycles, the data shows that nitrate increases slightly and nitrite decreases slightly. The total amount of nitrate and nitrite is constant. Because nitrification occurred during aerobic cycles, NO_2^- is converted to NO_3^- both 2-days and 4-days cycles.

On the contrary to this, nitrite increases slightly and nitrate decreases slightly in anoxic cycles. The total amount of nitrate and nitrite decreases. Because denitrification occurred during anoxic cycles, NO_3^- is converted to NO_2^- and finally it transferred to N_2 both 2-days and 4-days cycles. Nitrate was respiked at the beginning of 3rd aerobic cycles in 2-days, and at the beginning of 2nd and 3rd anoxic cycles in 4-days.

The result shows that more denitrification occurred during anoxic cycles in 4-day cycles than 2-day cycles. Nitrate was re-spiked once for reactors with 2-day cycles and re-spiked twice for reactors with 4-day cycles. The nitrate concentration decreases quickly in 4-day anoxic cycles. It looks like greater extent of denitrification with 4-day cycles causes more conversion to N_2 .

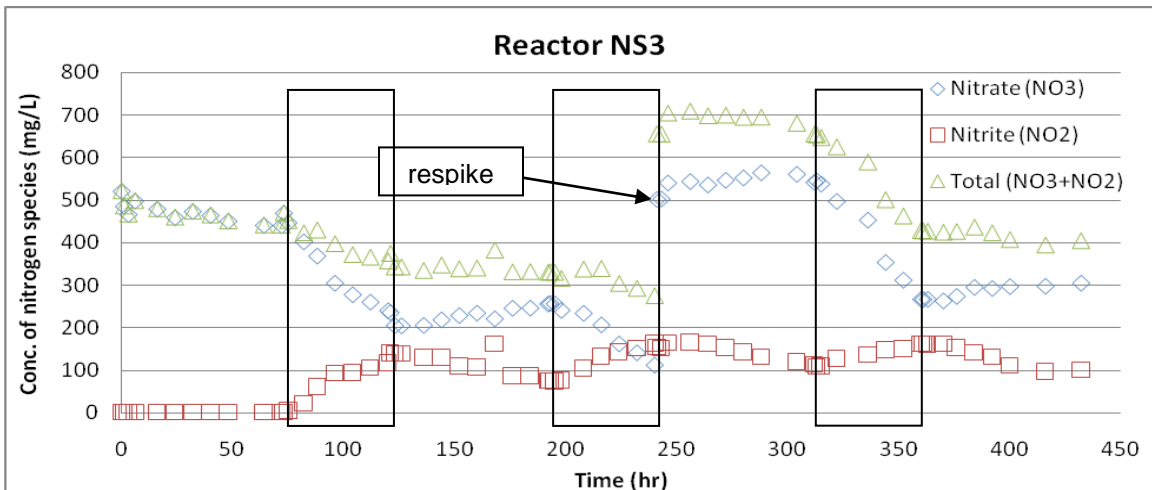
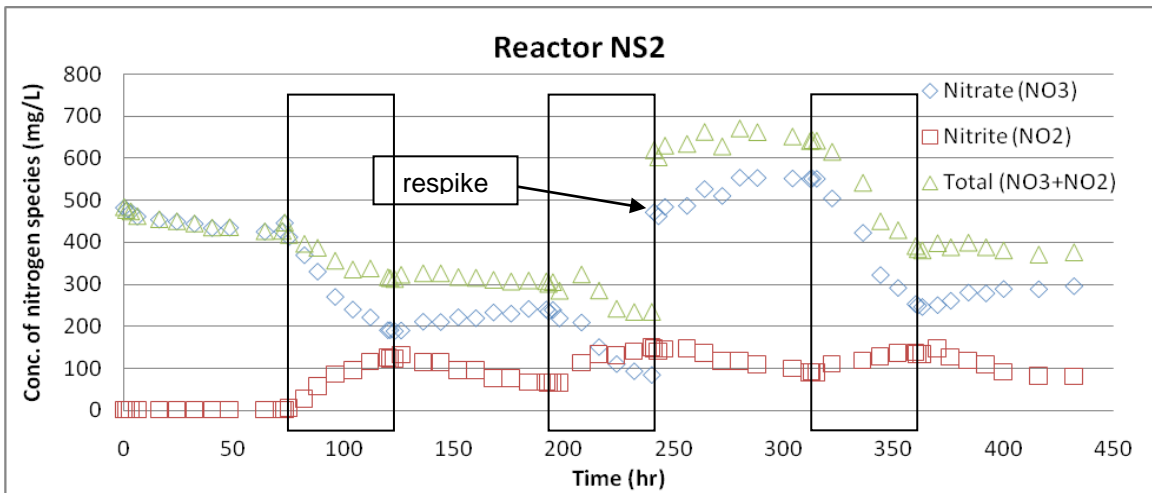
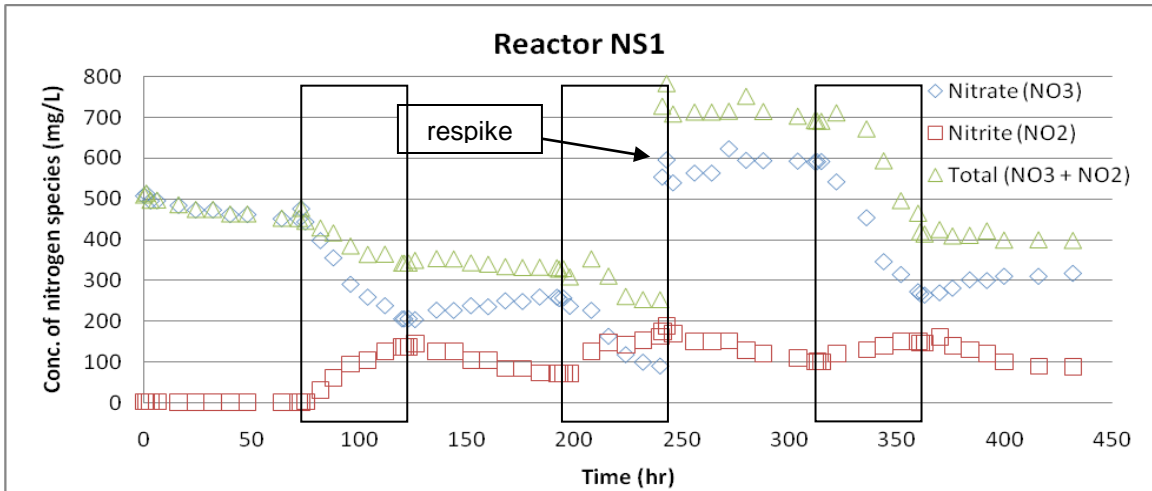


Figure 4.8. Concentration of nitrate and nitrite as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

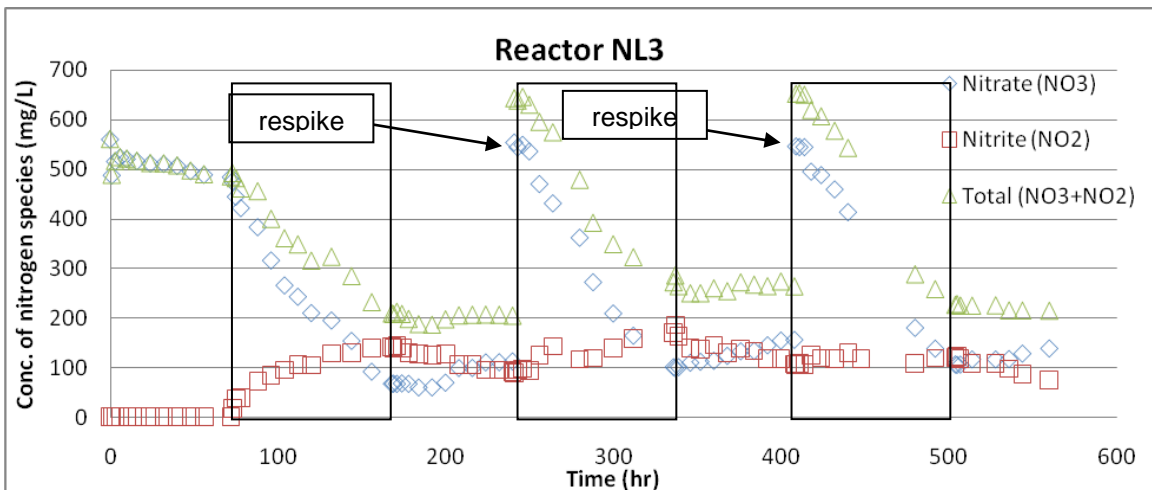
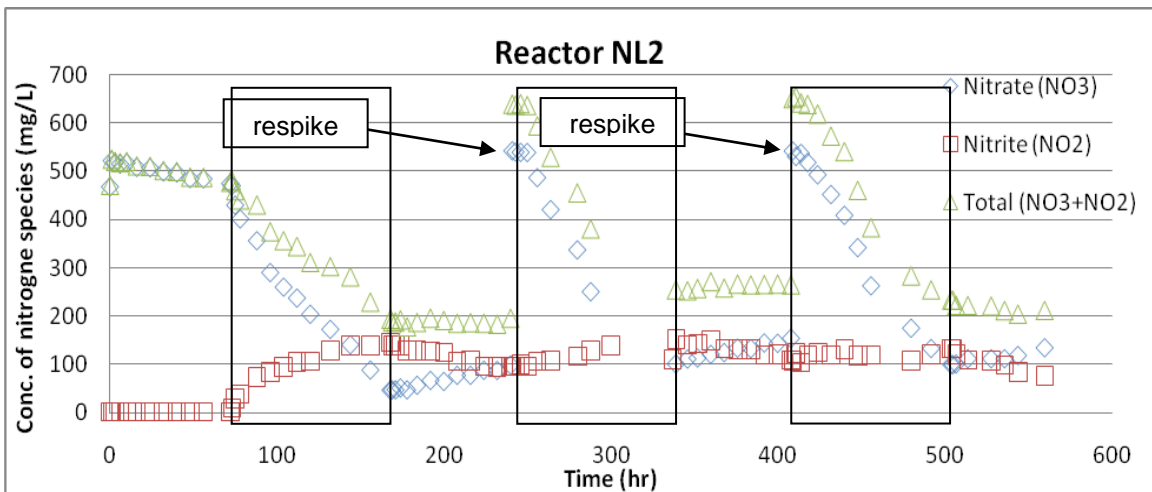
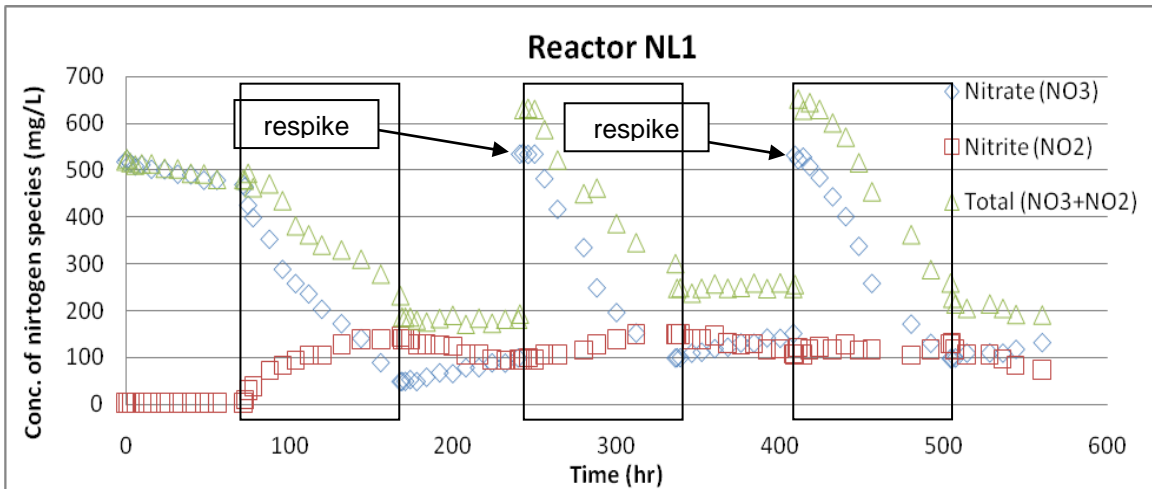
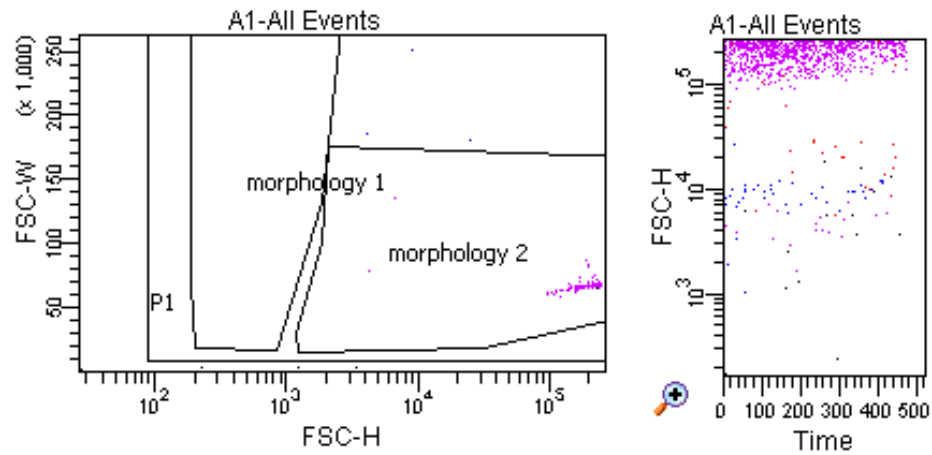


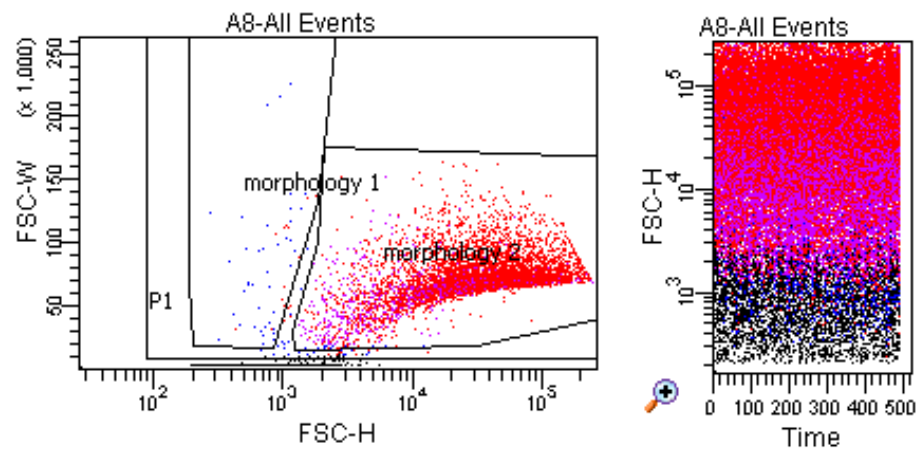
Figure 4.9. Concentration of nitrate and nitrite as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

4.3.5. Quantification of Biomass

The individual cells or bacteria were measured with flow cytometry. Bacteria densities were quantified by measuring the fluorescence from cells stained. The bacteria were stained by SYTOX Green dye. Figure 4.10 shows the number of bacteria in control sample and alternating aerobic/anoxic sample. In control sample the number of bacteria is below detection limit. Figures 4.11 and 4.12 show the densities of microorganism during test. When the condition was changed from aerobic to anoxic, and from anoxic to aerobic conditions, the microbes require a length of time to acclimate themselves to the environment (Crane and Novak, 2001) which is lag time. Exposure of aerobic microbes to anoxic environments and exposure of anoxic microbes to aerobic environments caused physiological stress. When the condition was changed, the number of microbes decreased then increased after a lag time. The lag time in aerobic cycles after longer anoxic condition (4 days) is longer than after short anoxic condition (2 days). The population of microbes generally increased over time in reactors NS1, NS2, NS3, and overall trend is increasing. The population of microbes goes up and down like 'sine waves' in reactors NL1, NL2, NL3, and is not increasing overall.



(a)



(b)

Figure 4.10. The number of bacteria in (a) control sample without microbiological activity, and (b) alternating aerobic/anoxic sample with microbiological activity.

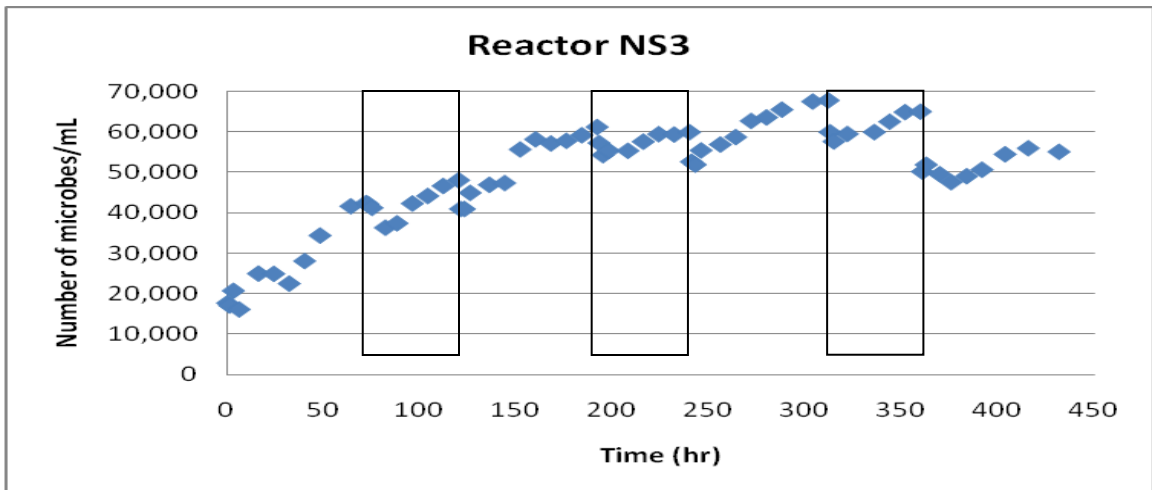
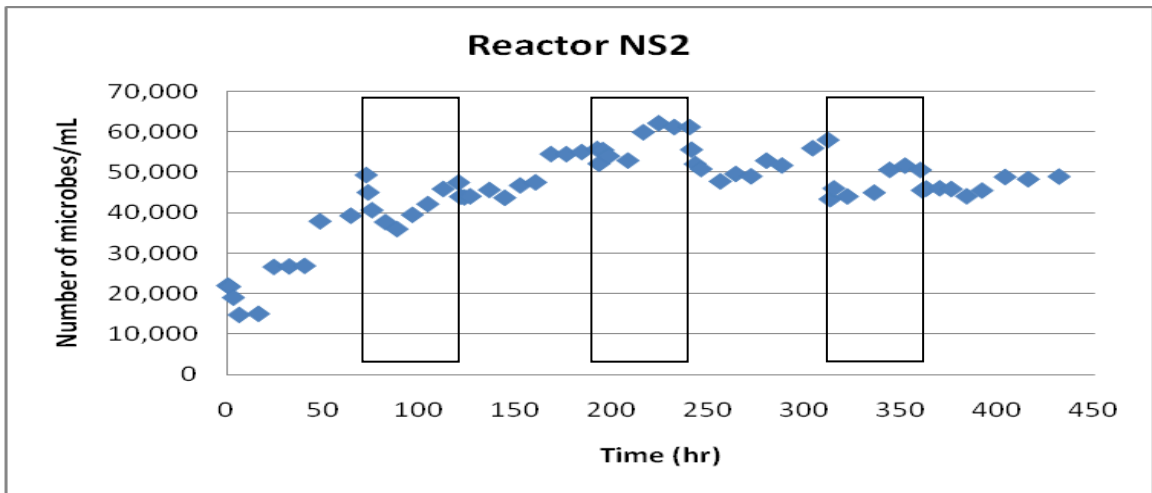
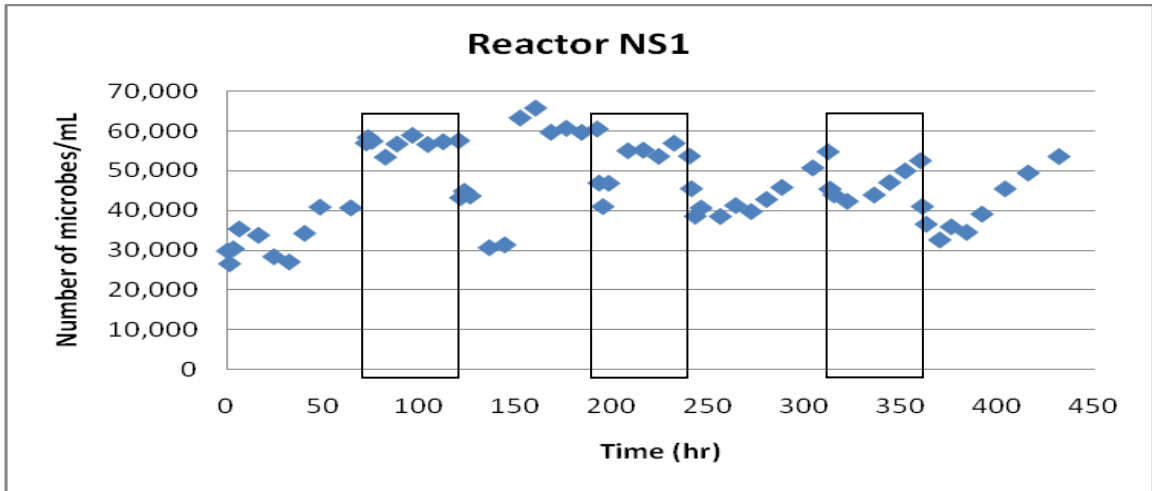


Figure 4.11. The number of microbes by Flow Cytometry as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

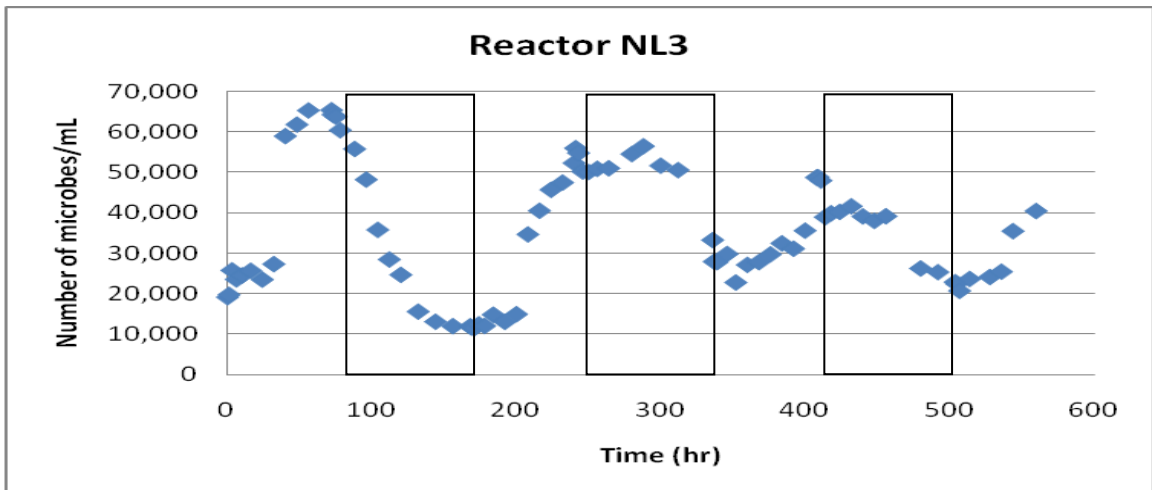
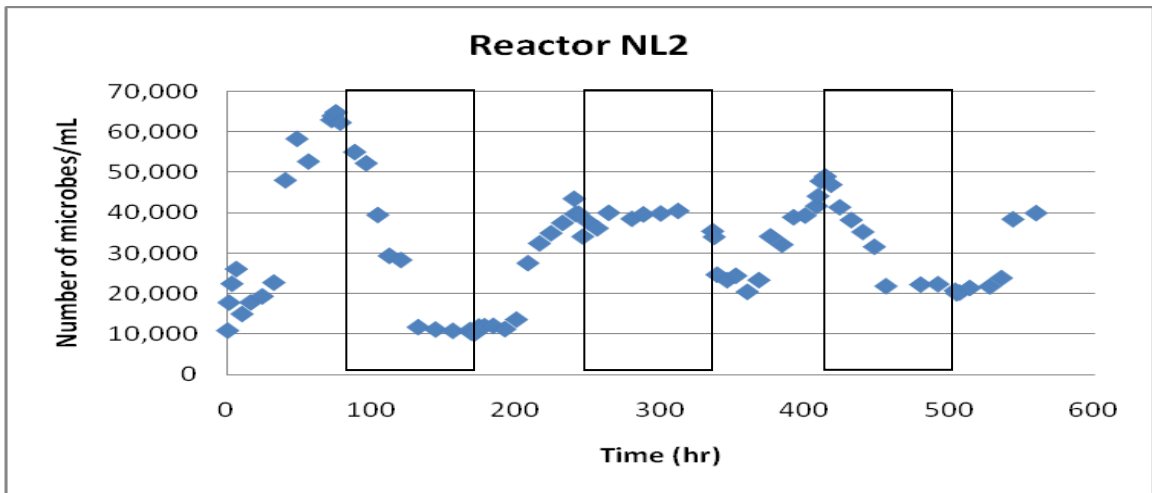
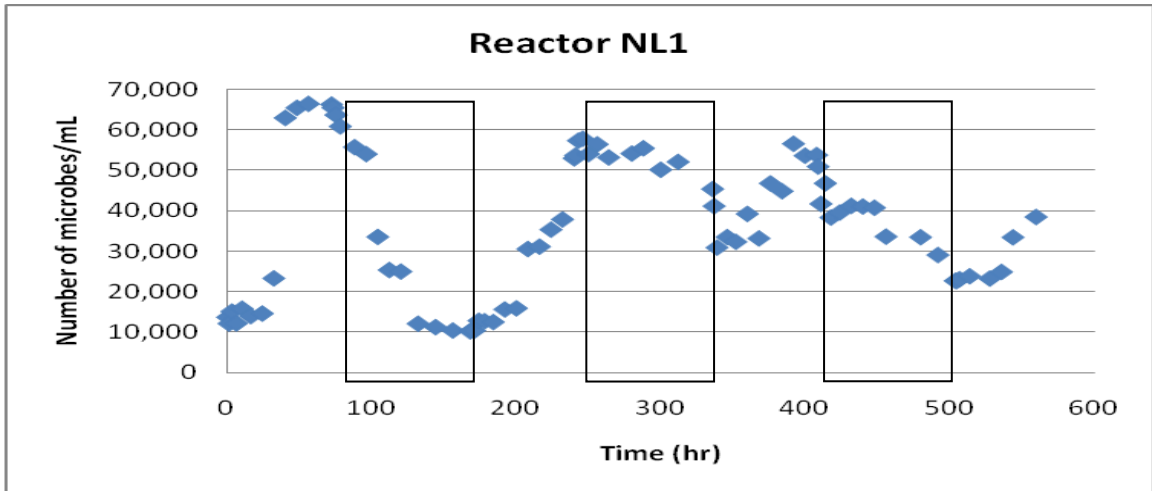


Figure 4.12. The number of microbes by Flow Cytometry as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

4.3.6. Concentration of EDCs

This study investigated the degradation of EDCs and lag time of microorganism in different conditions (alternating aerobic and anoxic condition) under simulated SAT systems. The change from aerobic to anoxic and from anoxic to aerobic conditions causes the most significant changes, including the oxidation of EDCs, reduction of electron acceptor, and microbiological activity. Nitrate as an electron acceptor was added into simulated SAT reactors in order to create nitrate reducing conditions. So, nitrate reducing conditions were dominant during anoxic conditions (Figure 4.8 and 4.9). This study demonstrates the different EDCs degradation in different aerobic or anoxic conditions, and the different lag time in aerobic condition after different anoxic period.

The concentration of EDCs in different anoxic period tests (2 days and 4 days) are shown in Figure 4.13 and 4.15 respectively. EDCs were respiked at the beginning of 3rd aerobic cycles in 2-days, and at the beginning of 2nd and 3rd anoxic cycles in 4-days. Biodegradation and sorption of EDCs occurred at the same time in the initial aerobic period. After 48 hr, biodegradation of EDCs was the main removal phenomenon because sorption equilibrates within 48 hr (Figure 4.3). E2 degradability is slower under anoxic conditions than aerobic conditions and BPA is not degrading in anoxic conditions. A small amount of biotransformation of E2 to E1 is observed during reaction. E1 concentration is showed in Figure 4.14 (2-day) and 4.16 (4-day). The trend of degradation is strongly linked to the redox conditions. EDCs oxidation was dependent upon oxygen reduction in aerobic condition and nitrate reduction in anoxic condition.

E2 was degraded in both aerobic and anoxic conditions, and BPA was degraded only during aerobic condition.

Overall, EDCs degradation has similar trend in different alternation period both 2 days and 4 days systems. However, the results show the different lag time in different alternation period both 2 days and 4 days systems. There are longer lag time in aerobic condition right after longer anoxic period. Figure 4.17 shows the results of lag time. When the condition was changed from aerobic to anoxic, and from anoxic to aerobic conditions, the microbes require a length of time to acclimate themselves to the environment (Crane and Novak, 2001) which is lag time. Exposure of aerobic microbes to anoxic environments and exposure of anoxic microbes to aerobic environments caused physiological stress. Because microbes have physiological stress, biodegradation of EDCs were decreased during lag time. Lag time was decided by when concentration decreases less than 5 %. The longer lag time (34-42 hr) is showed in 4-day cycle and shorter lag time (18-26 hr) is showed in 2-day cycle.

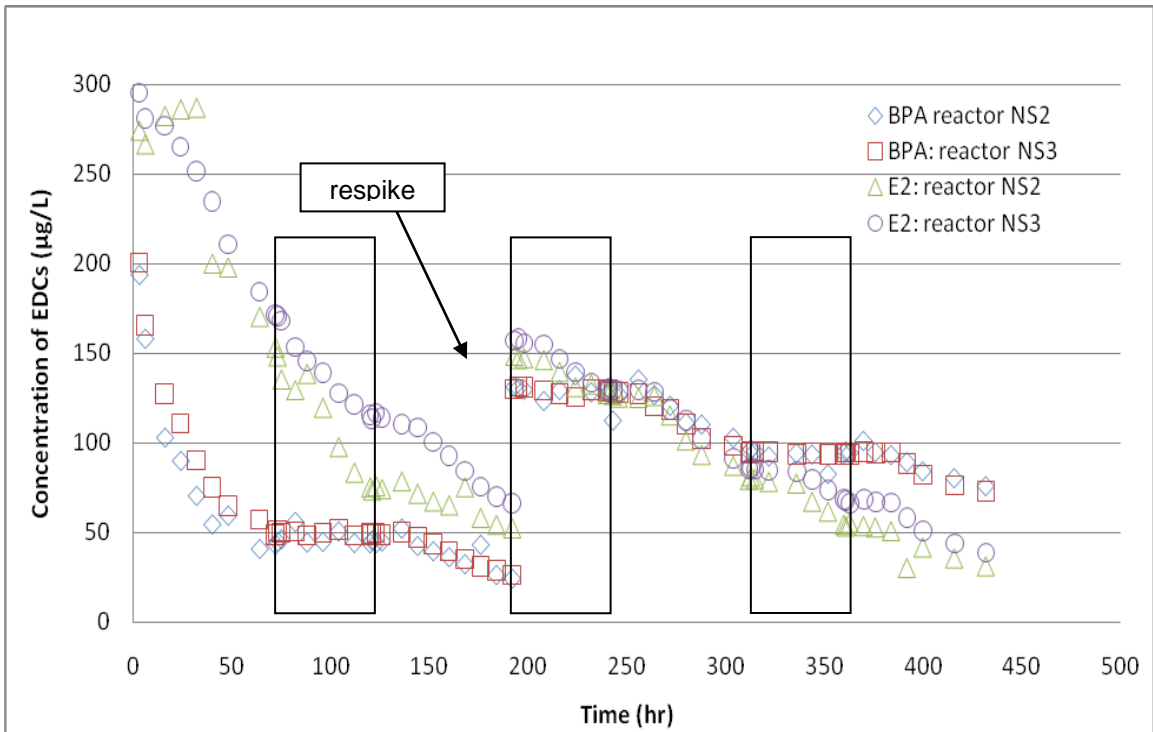


Figure 4.13. Concentration of EDCs as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

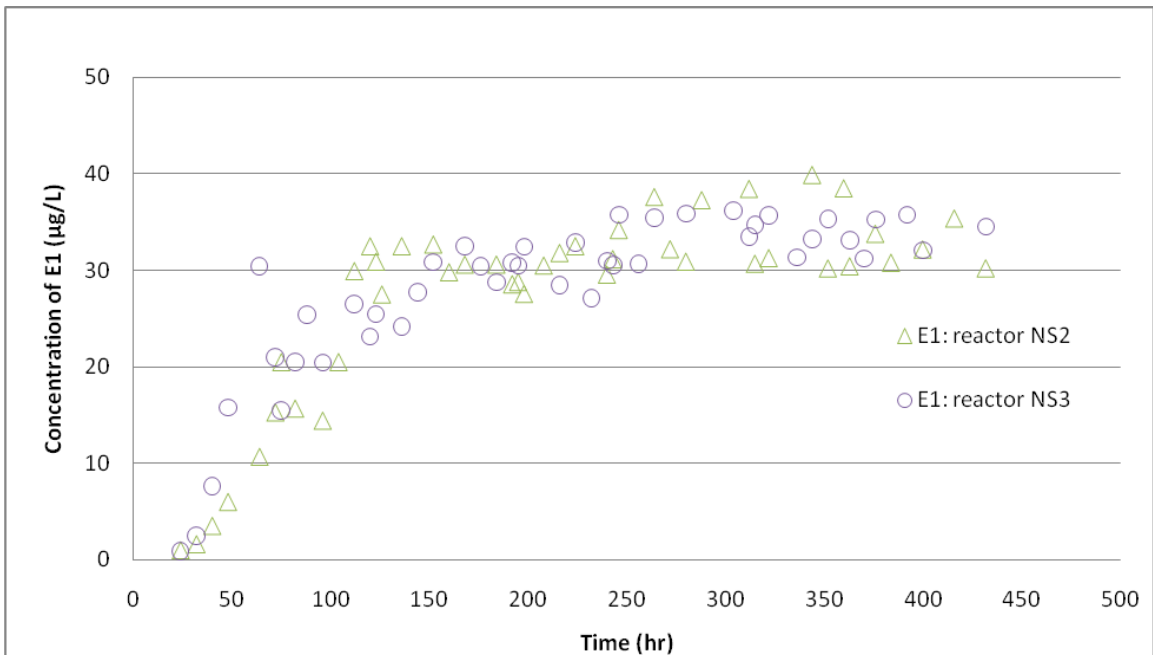


Figure 4.14. Concentration of E1 as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles.

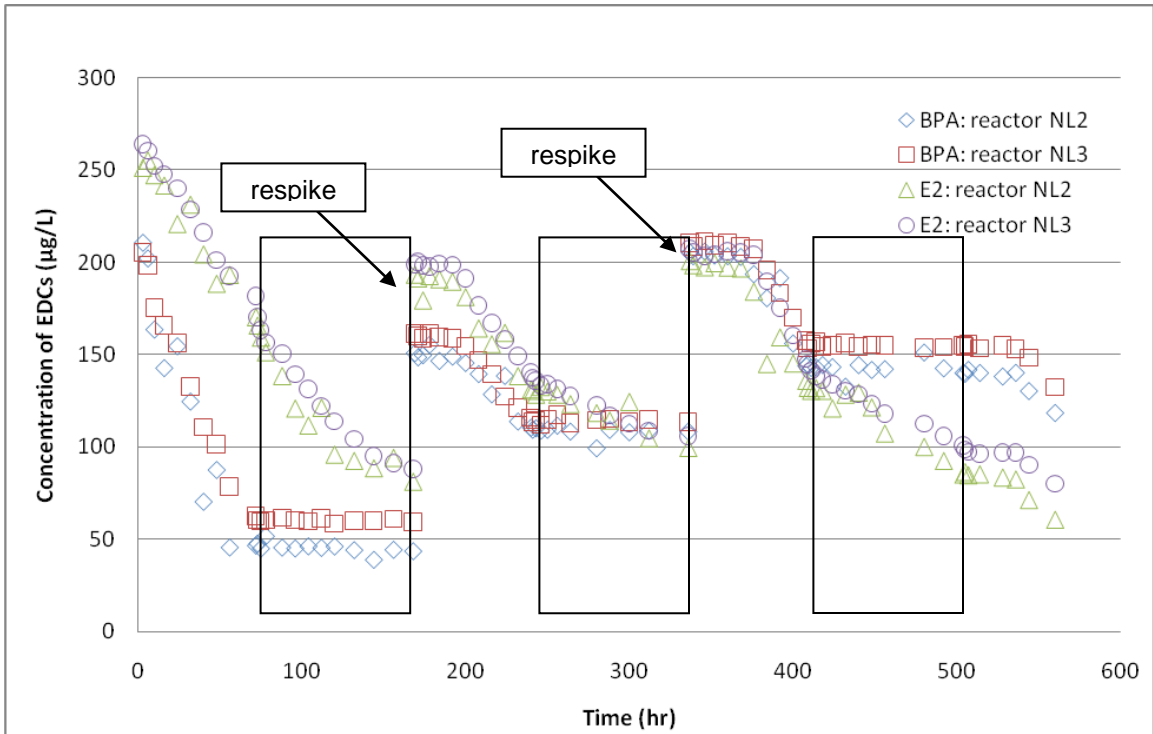


Figure 4.15. Concentration of EDCs as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

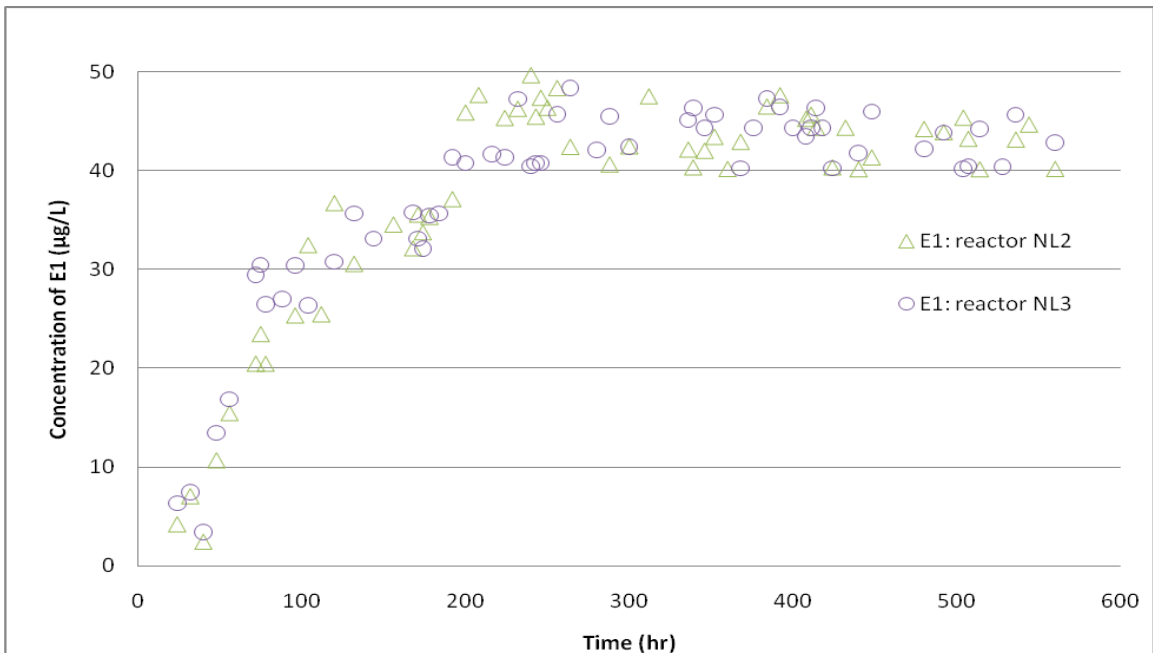
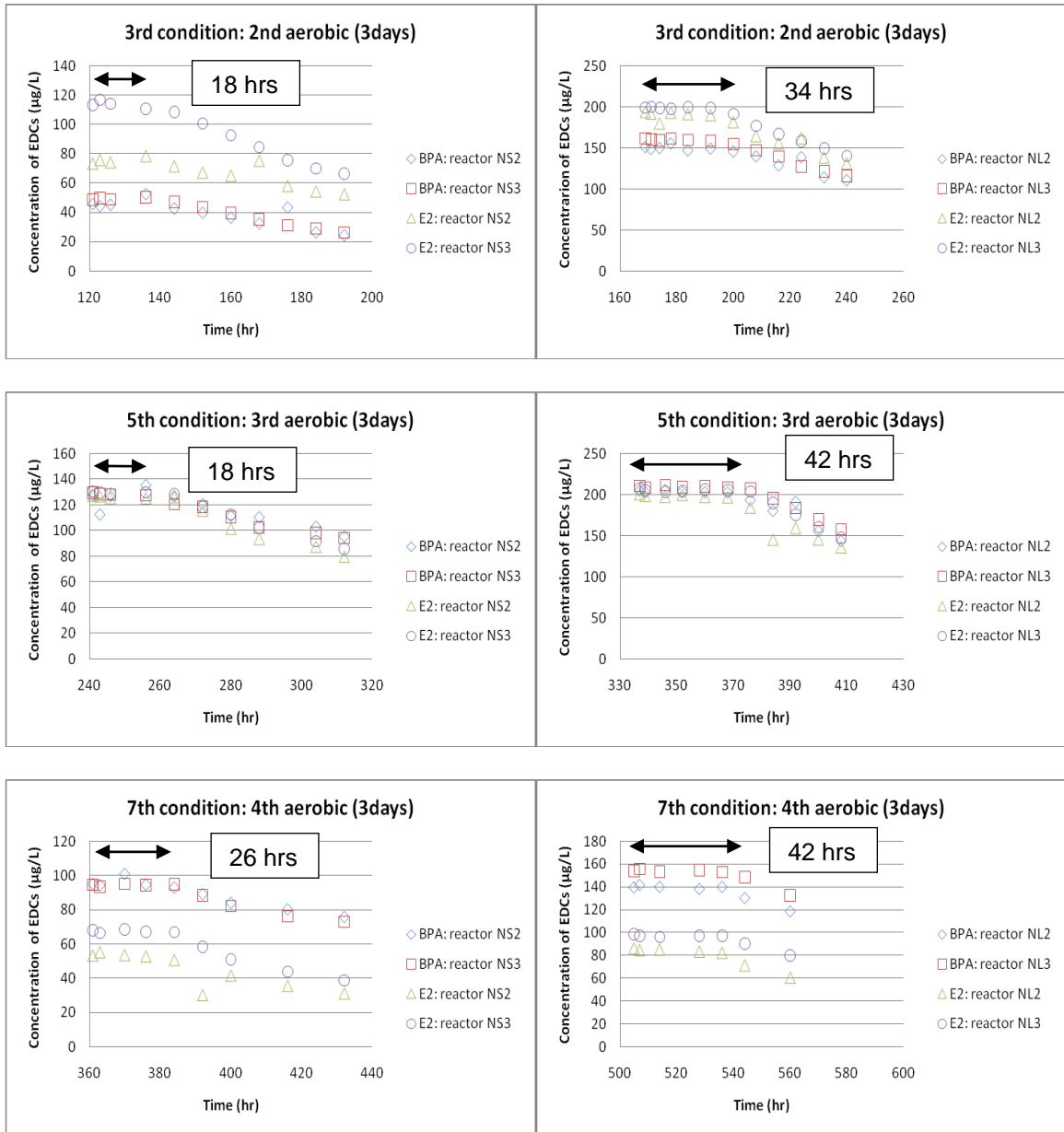


Figure 4.16. Concentration of E1 as a function of time in reactors with a 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles.



(a) aerobic condition in 2-day

(b) aerobic condition in 4-day

Figure 4.17. Concentration of EDCs as a function of time in reactors with a 2-day and 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (a) aerobic condition in 2-day (left side), (b) aerobic condition in 4-day (right side).

4.3.7. Mass Balance of EDCs

Mass balance of EDCs is checked before and after the reaction. Figure 4.18 shows the mass balance of EDCs in sorption test (reactor C1 and C2), 2-day cycles (reactor NS2 and NS3), and 4-day cycles (reactor NL2 and NL3). Figure 4.17 compares the spiked and final amount of EDCs. Spiked amount of BPA and E2 is 3,000 µg for reactor C1 and C2, 3,300 µg for reactor NS2 and NS3 (respiked once), and 3,600 µg for reactor NL2 and NL3 (respiked twice). After finishing the reaction, aqueous and soil samples were collected and analyzed by GC/MS. 5 samples of aqueous phase and soil were tested, and the average results are shown in Figure 4.18. In statistical t-test, the t value is equal to or less than 0.05. Average results, standard deviation, and sampling mass of EDCs are shown in Table 4.3. During test I collected 89 samples for 2-day cycles (reactor NS2 and NS3), and 107 samples for 4-day cycles (reactor NL2 and NL3). Each sample was 10 ml and contained BPA and E2. Therefore a significant mass of the EDCs were removed from reactors during sampling and must be taken into account in the mass balance. EDCs in aqueous phase were extracted by SPME, and in soil were extracted by methanol. 2 g soil samples was collected and dried at room temperature (20 °C, 24 hr), and 10 ml methanol was inserted into soil and shaken for 24 hrs in a 40 ml glass vial. Methanol was separated by centrifuge and evaporated by gentle nitrogen gas. EDCs were derivatized by MSTFA and detected by GC/MS. Final EDCs amount is shown in Figure 4.18. In reactor C1 and C2, where biodegradation was suppressed by NaN₃, the average recovery of BPA was 96.8 % and the average recovery of E2

was 98.3 %. In reactor NS2 and NS3, average recoveries were 70.1 % and 61.1 % for BPA and E2 respectively. In reactor NL2 and NL3, average recoveries were 72.5 % and 63.1 %. This shows that biodegradation was minimal in the sorption control tests and was similar in reactors with 2-day and 4-day anaerobic cycles. E2 is more biodegradable than BPA.

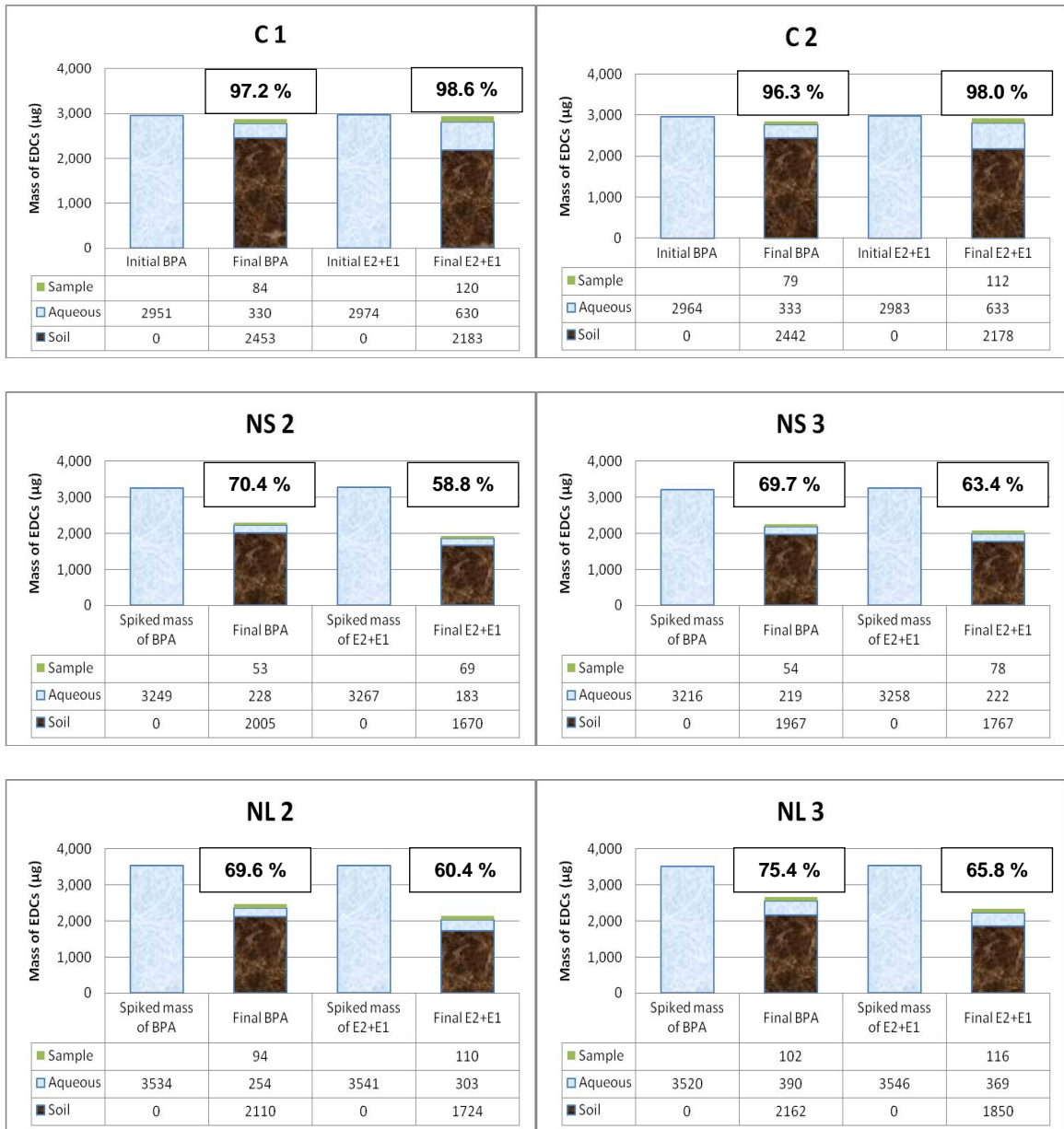


Figure 4.18. Mass balance of EDCs in aqueous and soil with a control test, 2-day and 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles.

Table 4.3. Mass balance of EDCs in aqueous and soil with a control test, 2-day and 4-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. Average data from 5 samples and standard deviation.

C1	Initial BPA		Final BPA		Initial E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			84				120	
Aqueous	2951	39	330	8	2974	27	630	12
Soil			2453	29			2183	29
Total	2951	39	2867	23	2974	27	2933	21

C2	Initial BPA		Final BPA		Initial E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			79				112	
Aqueous	2964	25	333	6	2983	17	633	12
Soil			2442	20			2178	35
Total	2964	25	2854	20	2983	17	2923	25

NS2	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			53				69	
Aqueous	3249	52	228	6	3267	45	183	9
Soil			2005	26			1670	28
Total	3249	52	2286	23	3267	45	1922	34

NS3	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			54				78	
Aqueous	3216	26	219	7	3258	32	222	9
Soil			1967	21			1767	26
Total	3216	26	2240	22	3258	32	2067	34

NL2	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			94				110	
Aqueous	3534	23	254	11	3541	20	303	9
Soil			2110	35			1724	19
Total	3534	23	2458	44	3541	20	2137	24

NL3	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			102				116	
Aqueous	3520	21	390	15	3546	18	369	12
Soil			2162	15			1850	17
Total	3520	21	2654	21	3546	18	2335	14

4.3.8. Sorption vs Biodegradation

Figure 4.3 shows the results of sorption test, because there is no microbiological activity. Initial concentration of EDCs is 1,000 $\mu\text{g/L}$, and final concentration is 210 $\mu\text{g/L}$ for E2 and 110 $\mu\text{g/L}$ for BPA after 24 hr. The concentration of EDCs does not change after 24 hr in control test for sorption test, but the concentration of EDCs does change in 2-day and 4-day cycles test. In the beginning part of reaction (0 -24 hr), sorption is the main EDCs removal mechanism, but biodegradation occurs, so the EDCs decrease continually (Figure 4.13 and 4.15). E1 (bio-transformed from E2) is observed in reactor NS2, NS3, NL2, and NL3, but it is not observed in reactor C1 and C2. Because the concentration of BPA is 110 $\mu\text{g/L}$ and E2 is 210 $\mu\text{g/L}$ in aqueous phase of control test after 24 hr, the difference concentration of EDCs between abiotic test (reactor C1 and C2) and biotic test (reactor NS2, NS3, NL2, and NL3) is due to biodegradation.

4.4. Conclusions

Simulated SAT reactor system has been constructed and demonstrates desired behavior during alternating conditions. The redox condition had linkage with alternating aerobic/anaerobic condition. Nitrification occurred during aerobic condition and denitrification occurred during anaerobic condition. During alternating aerobic/anaerobic condition, after longer anaerobic condition, the adaptation to aerobic condition from anaerobic is dampened. Biodegradation was differentiated from sorption and the biodegradation was investigated depending on the duration of alternating aerobic/anaerobic condition. BPA and E2 can biodegrade during aerobic condition but only E2 can biodegrade during anaerobic condition. This study quantifies the removal of BPA and E2 by the process of biodegradation; and most significantly, I am the first to measure how the lag time of microbiology during SAT depends upon the transient redox conditions (alternating aerobic/anaerobic conditions), which are controlled by the system operation. After longer anaerobic condition, longer lag time occurred in aerobic condition right after longer anaerobic condition. Since the simulated SAT system in this phase are more realistic condition about alternating aerobic/anaerobic condition, the results from this phase provide a better understanding to real SAT system. The results from this study give good information to determine an optimized SAT operation time (no longer 4-day anaerobic condition in aquifer under the SAT pond) in order to get high removal efficiency of targeted material.

CHAPTER 5

**QUANTIFY THE BIODEGRADATION OF EDCS UNDER DIFFERENT
ANAEROBIC TERMINAL ELECTRON ACCEPTING PROCESSES**

5.1. Introduction

Denitrification or anoxic denitrification is a standard biological process for the remediation of nitrogenous compounds from wastewater. During denitrification, NO_3^- acts as an electron acceptor and reduced to N_2 gas. Nitrate-reducing bacteria (NRB) or denitrifiers involved in the denitrification process and NRB is usually facultative bacteria. The facultative bacteria can survive in aerobic and/or anaerobic respiration. Sulfate-reducing bacteria (SRB) which is anaerobic bacteria use the sulfate as an electron acceptor. Organic matter can be degraded by SRB in the presence of sulfate in an anaerobic environment. Hydrogen sulfide (H_2S) gas was produced in anaerobic condition when sulfate acts as an electron acceptor. Most SRB is obligate anaerobes. Oxygen is the most favorable electron acceptor and the next best electron acceptor is NO_3^- in anaerobic environment. The electron acceptor affinity of microorganism is the following order: $\text{O}_2 > \text{NO}_3^- > \text{MnO}_2 > \text{FeOOH} > \text{SO}_4^{2-} > \text{CO}_2$ (Kiene 1991).

EDCs biodegradation study in aerobic and anaerobic condition was performed in respectively (Wackett 1996, Ronen et al. 2000, Veder et al. 2000,

Kang and Kondo 2002a, Shi et al. 2004). However, limited information is available in the literature about the degradation of EDCs under different types of electron acceptor. Therefore, it was required that electron acceptor study in EDCs remediation during alternating condition between aerobic and anaerobic condition.

The objective of this study is to compare the different electron acceptors of anaerobic microbiology and quantify the biodegradation of EDCs under different electron acceptors during alternating cycles between aerobic and anaerobic in simulated SAT system. The working hypothesis of this goal is that different electron-accepting processes (nitrate or sulfate) lead to different duration of lag time and different biodegradability of EDCs during anaerobic cycles under alternating aerobic/anaerobic conditions. Simulated SAT in batch microcosms under alternating aerobic/anaerobic conditions was done, and the reduction of nitrate and sulfate conditions in anaerobic condition was compared.

5.2. Materials and Methods

5.2.1. Chemicals

SYTOX Green nucleic acid stain was purchased from Molecular Probes (OR USA). Sodium sulfate (Na_2SO_4) was purchased from Aldrich (WI USA). Methanol (HPLC grade), BPA (purity grade > 99 %), E2 (purity grade > 99 %), 4-n-Nonylphenol (NP) (purity grade > 99.9), sodium chloride (NaCl, purity grade > 99.5 %), and MSTFA with 1% TMCS were purchased from Aldrich (WI, USA).

5.2.2. *Soil and Wastewater*

Tertiary-treated effluent (final effluent) was obtained from Howard F Curren Advanced Waste Water Treatment Plant (WWTP) in Tampa, FL. Aquifer soil was obtained from a constructed Wetland Wastewater Treatment System in Lakeland, FL. Characterization of effluent from WWTP is shown in Table 4.1. The plant has a design capacity of 96 million gallons per day and the effluent water is discharged to Hillsborough Bay or used as reclaimed water for cooling and irrigation. Soil samples were collected from the top 10 cm, 50 cm, and 100 cm of the pond bed and placed in glass jars. Upon arrival back to the laboratory, the jars were kept under refrigeration. Collected soil was mixed in the laboratory.

5.2.3. *Batch Mesocosm Reactors*

This study compared and measured the biodegradability of EDCs under different oxidation-reduction conditions. In this study, simulated SAT reactor as described in Chapter 4 was used. Simulated SAT systems were set up in 4 L reactors with 3 L effluent from WWTP and 500 g aquifer soil placed in each reactor. BPA and E2 were spiked to simulated SAT system at an initial concentration of 1,000 $\mu\text{g/L}$. This concentration is higher than could be observed in most WWTP effluent streams. However, for this research, I chose to use a high EDCs concentration in order to prove the clear variation of EDCs during alternating conditions. If the initial EDCs concentration were too low then won't be able to observe removal of up to 99%. In general, batch simulated SAT mesocosms were run under alternating aerobic/anaerobic conditions. The

simulated SAT systems were operated in a dark environment (the reactor was wrapped in aluminum foil) at room temperature (°C). Schematic diagram of simulated SAT reactor is shown in Figure 4.1 and the photograph of a simulated SAT reactor is shown in Figure 4.2.

Because the SAT system is alternating between aerobic and anaerobic condition, simulated SAT systems were established by passing either air (21% O₂) or N₂ gas (O₂ free) in order to make the alternating aerobic and anaerobic conditions. Aerobic environment was established by air gas passing through the simulated SAT system, and then it was switched to anaerobic environment by N₂ gas passing through the system. The gas tank was connected with the simulated SAT reactor by fluoropolymer tubing and a syringe. The simulated SAT system was capped by silicone sealing in order to control the aerobic/anaerobic condition, and the air gas and N₂ gas were connected through this cap by syringe. Aerobic environment and anaerobic environment were manually switched by reconnecting the fluoropolymer tube and syringe. In addition, sampling syringe was connected through the cap.

In order to make anoxic or anaerobic condition during anaerobic cycles, nitrate or sulfate was spiked to simulated SAT system at an initial concentration of 1,000 mg/L. This concentration is higher than could be observed in most WWTP effluent streams. However, for this research, I chose to use a high concentration in order to prove the clear variation of nitrate or sulfate during alternating conditions and won't be able to observe removal of up to 99%. When I put the nitrate or sulfate into the reactor respectively, nitrate condition refers to

anoxic, and sulfate condition refers to anaerobic condition. Batch mesocosm reactors were used for lab experiments, and different electron acceptors were tested. Nine simulated SAT batch reactors were prepared (Table 5.1 shows the condition of reactor; test was based on the triplicate); three control tests for sorption test without microbiological activity (Reactor C1,C2, and C3; same condition), and the other six test for comparison of different electron acceptors (nitrate; reactor NS1, NS2, and NS3; same condition, sulfate; reactor S1, S2, and S3; same condition). Test was based on the triplicate. Control test was studied to distinguish between sorption to the soil and biodegradation during simulated SAT system. In order to know only sorption amount to the soil without microbiological activities, 1,000 mg/L NaN_3 was used for soil sterilization in control test (Kao et al. 2004, Zhang et al. 2009).

Table 5.1. The condition of nine simulated SAT batch reactors

Reactor #	Condition of Reactor		Duration (day)	
C1	Control for sorption test		20	
C2	Control for sorption test		20	
C3	Control for sorption test		20	
	Alternating system	During anaerobic		Aerobic-Anaerobic
NS1	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
NS2	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
NS3	3 aerobic/ 2anoxic	Anoxic denitrification	23	3-2-3-2-3-2-3-2-3
	Alternating system	During anaerobic		Aerobic-Anaerobic
S1	3 aerobic/ 2anoxic	Sulfate present	23	3-2-3-2-3-2-3-2-3
S2	3 aerobic/ 2anoxic	Sulfate present	23	3-2-3-2-3-2-3-2-3
S3	3 aerobic/ 2anoxic	Sulfate present	23	3-2-3-2-3-2-3-2-3

5.2.4. Sampling and Analysis

On the first day of each aerobic and anaerobic condition, six samples were taken (volume of sample is 10 mL, sampling time is 1, 3, 6, 10, 16, and 24 hr). Then, three samples of each condition were taken each day thereafter (the frequency of sampling is 1 per 8 hour). BPA and E2 were extracted from samples by SPME and analyzed by GC/MS (as described in Chapter 3). When the concentration of EDCs was below 100 µg/L (90 % removal), EDCs were re-spiked (100 µg/L additional).

Nitrate, nitrite and sulfate were analyzed by Metrohm Ion Chromatography (881 Compact IC pro and 863 Compact Autosampler), using a Metrosep A Supp 7-250 (250 mm x 4 mm) analytical column, the eluent was sodium carbonate (3.6 mmol/L), and the flow rate was 0.7 ml/min.

The microbial population was estimated by the flow cytometry (BD FACSCanto II Analyzer, High-throughput flow cytometer). SYTOX Green Nucleic Acid Stain was used to dye the bacteria. 1 mL aqueous sample was mixed with 1 mL 4% paraformaldehyde and stay 5 min. It was centrifuged and discard the supernatant. Put the stain (5 µM, 300 µL) into precipitation and resuspension. Put 200 µL sample into 96 tray and detect the microbes by flow cytometry. It is a direct enumeration method (Gunasekera et al. 2000, Chen et al. 2001). Microorganism stained with SYTOX Green Nucleic Acid Stain yield bright and stable fluorescent signals that could be detected by flow cytometry.

Dissolved oxygen concentrations monitored by a YSI DO200 DO meter, and the redox state was checked by pH100 redox potential meter.

5.3. Results and Discussion

5.3.1. Dissolved Oxygen

Dissolved oxygen concentration in alternating between aerobic and anaerobic condition is shown in Figure 5.1 and 5.2. Although different (nitrate and sulfate) reducing condition was compared under anaerobic condition, dissolved oxygen concentration is similar with Chapter 4. In both reducing condition, during aerobic condition the dissolved oxygen level is between 7.5 mg/L and 7.9 mg/L (oxygen concentration in saturated condition at room temperature: 9.09 mg/L at 20 °C, and 8.26 mg/L at 25 °C), and during anaerobic condition the dissolved oxygen level is between 0.3 mg/L and 0.5 mg/L, because simulated SAT system was established by passing either air (21% O₂) or N₂ gas (O₂ free) in order to make the alternating aerobic and anaerobic conditions. Aerobic environment was established and continued by air gas passing through the simulated SAT system, and then it was switched to anaerobic environment by N₂ gas passing through the system. As seen in Figure 5.1 and 5.2, the concentration of dissolved oxygen dropped very quickly when the reactor was switched from air to N₂, and rose very quickly when the reactor was switched from N₂ to air.

The aerobic condition is that when the microorganism(s) used O₂ as the terminal electron acceptor, and the anaerobic/anoxic condition is that when the microorganism(s) carry out the fermentation without terminal electron acceptors or uses the chemicals (nitrate, sulfate, iron, etc.) as terminal electron acceptors.

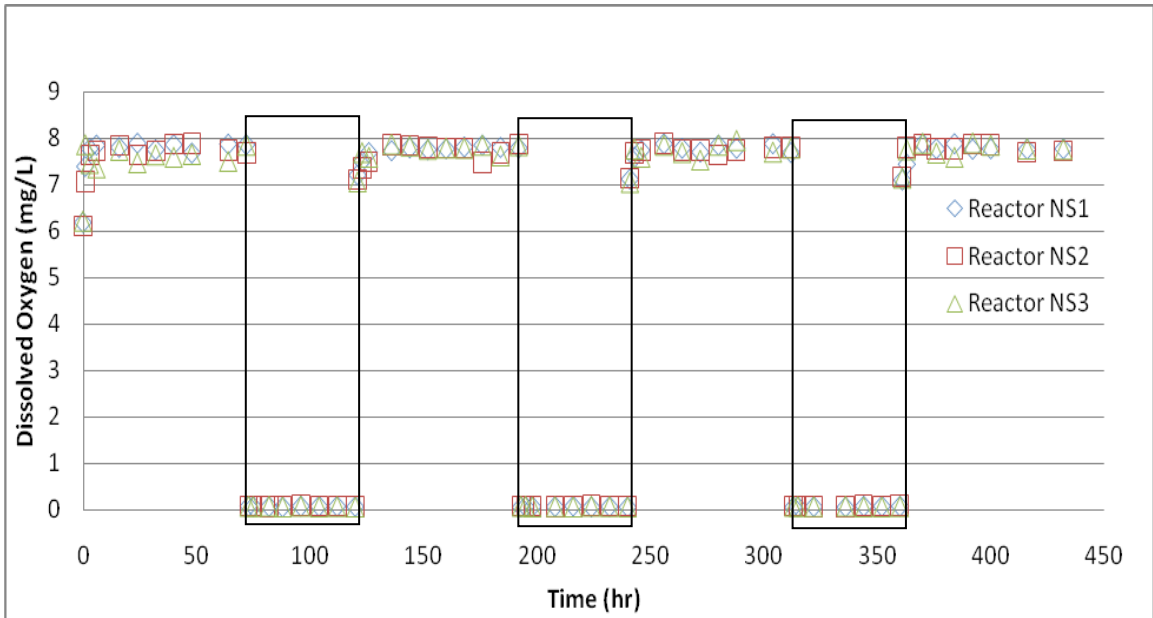


Figure 5.1. Concentration of dissolved oxygen as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

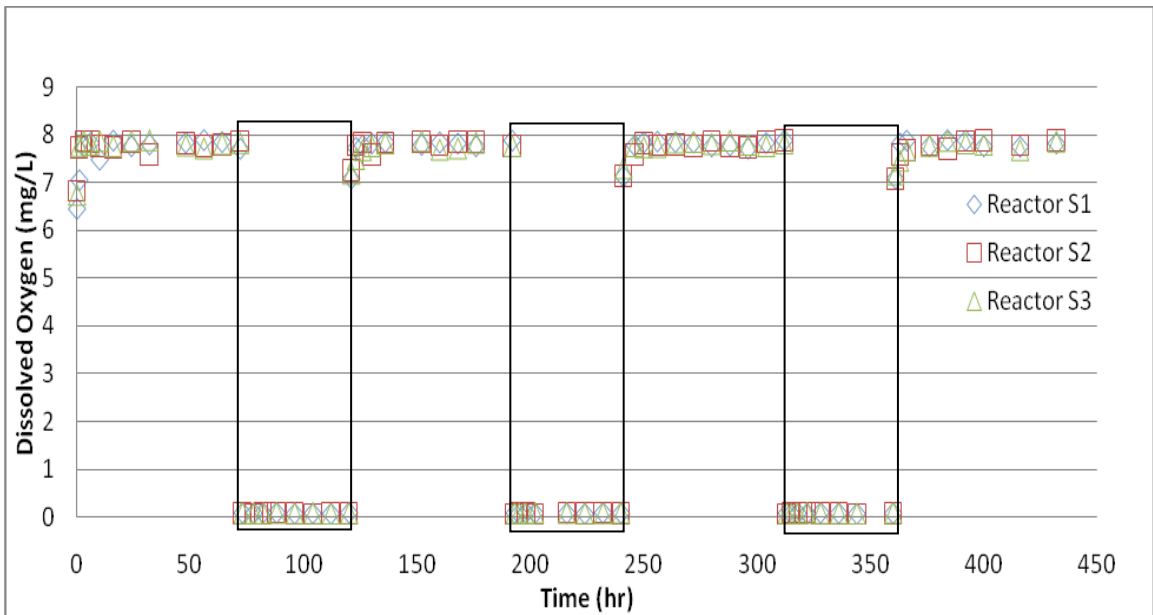


Figure 5.2. Concentration of dissolved oxygen as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

5.3.2. Oxidation-Reduction (Redox) Potential Level

The redox potential of the water in the reactor responded rapidly to changes in the aeration status of the water. Figure 5.3 and 5.4 shows the redox potential level in each nitrate and sulfate added condition. The maximum redox potential under aerobic conditions for all treatment was approximately +240 mV. The redox potential was rapidly decreased right after air was switched by N₂ gas. The redox potential level was fallen down to approximately -100 mV in anoxic condition and -150 mV in anaerobic condition, and remained at this value throughout anaerobic condition. On the contrary to this, the redox potential was rapidly increased right after N₂ gas was switched by air. For the aerobic condition the redox potential level increased rapidly to approximately + 240 mV and remained constant for the entire aerobic period (3 days).

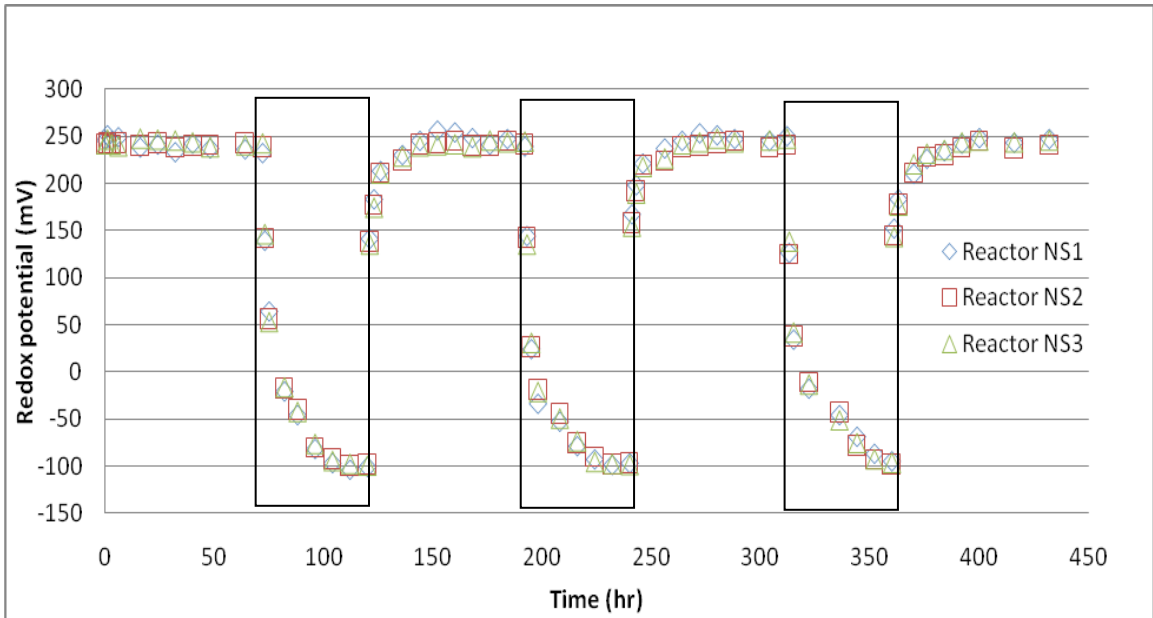


Figure 5.3. Redox potential as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

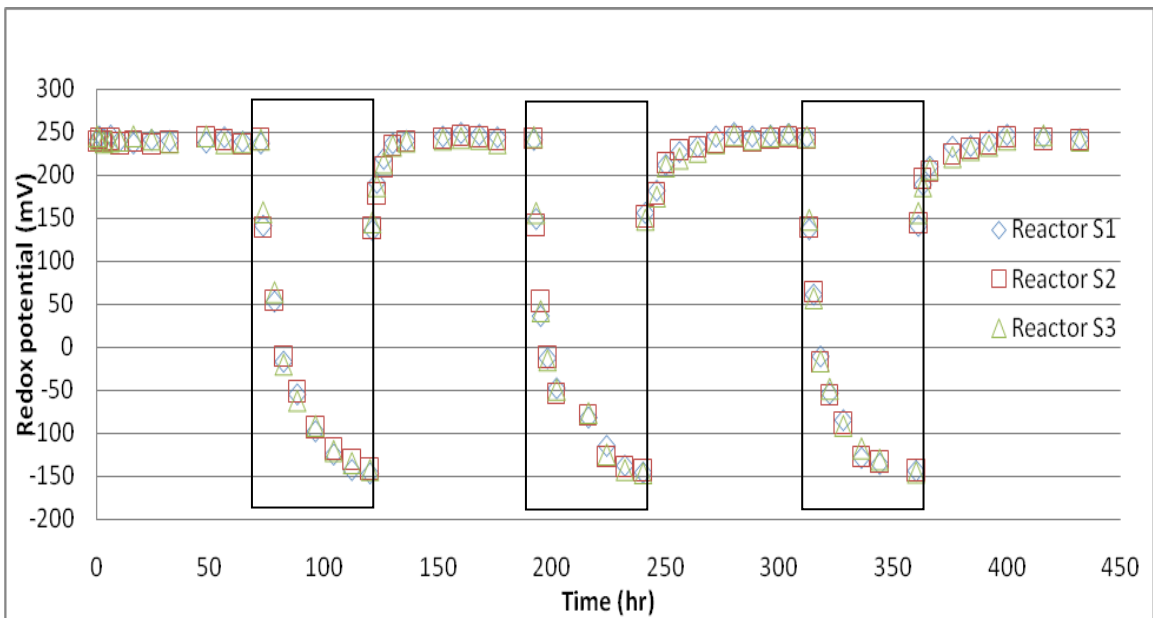


Figure 5.4. Redox potential as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

5.3.3. Concentration of Electron Acceptor

Different electron acceptors were tested and the results and the behavior are showed in Figure 5.5 and 5.6. Nitrate and sulfate was selected because these elements play a major role in the redox chemistry. As described in Chapter 4, the nitrification and denitrification phenomenon is observed in Figure 5.5. Even though sulfate reduction was observed in anaerobic conditions, the level was relatively low. The role of nitrate as an electron acceptor is effective in alternating aerobic and anaerobic systems, but the sulfate is not effective as an electron acceptor.

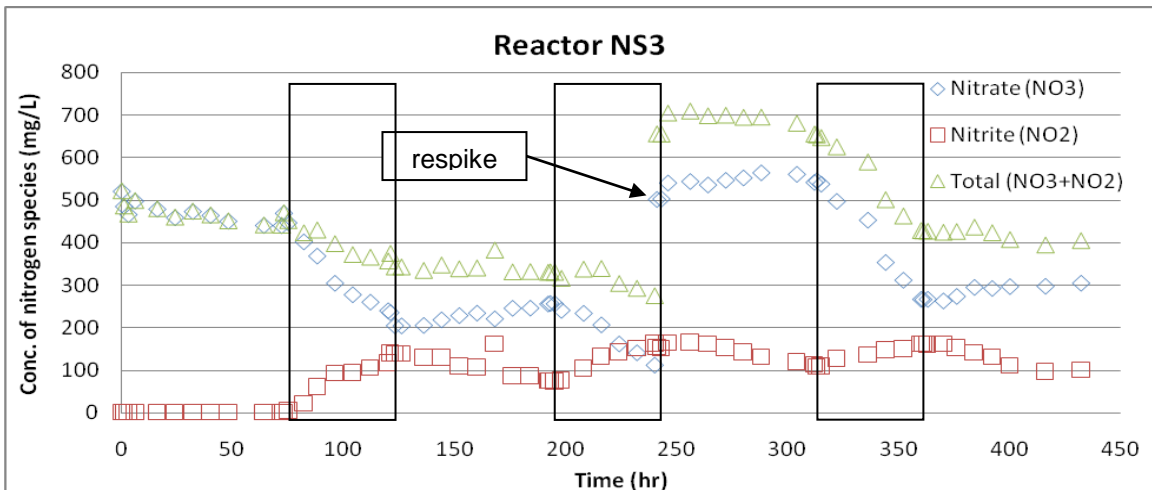
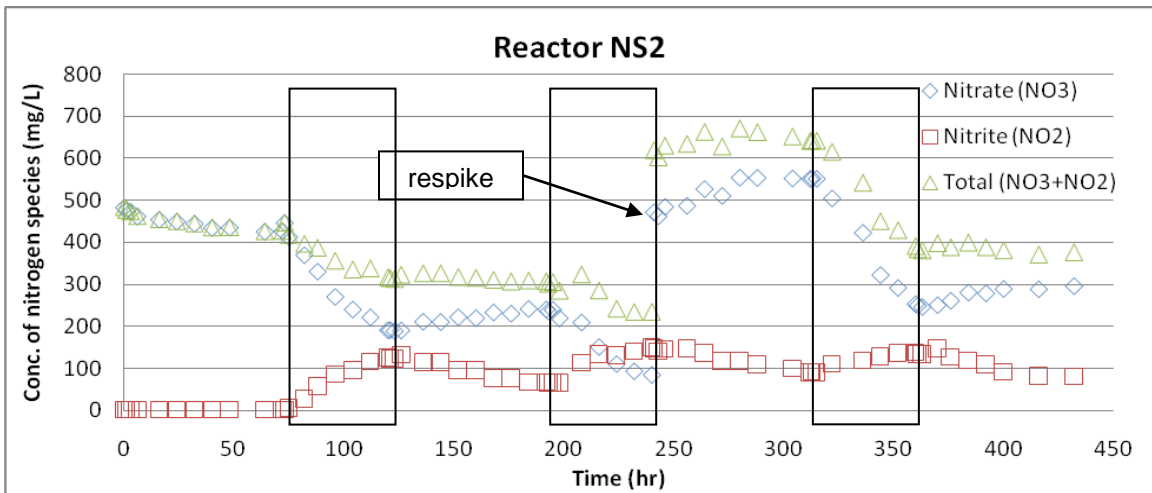
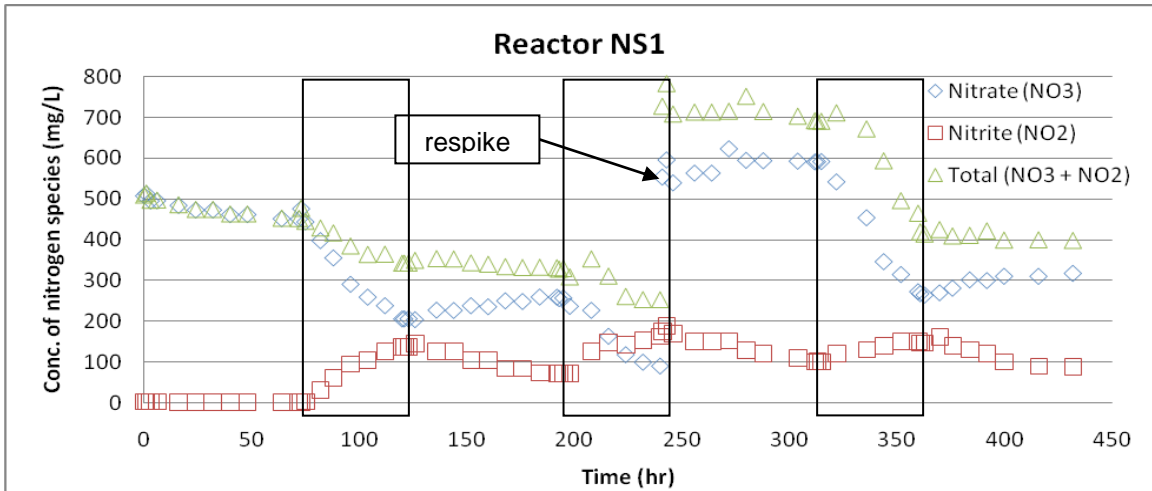


Figure 5.5. Concentration of nitrate and nitrite as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

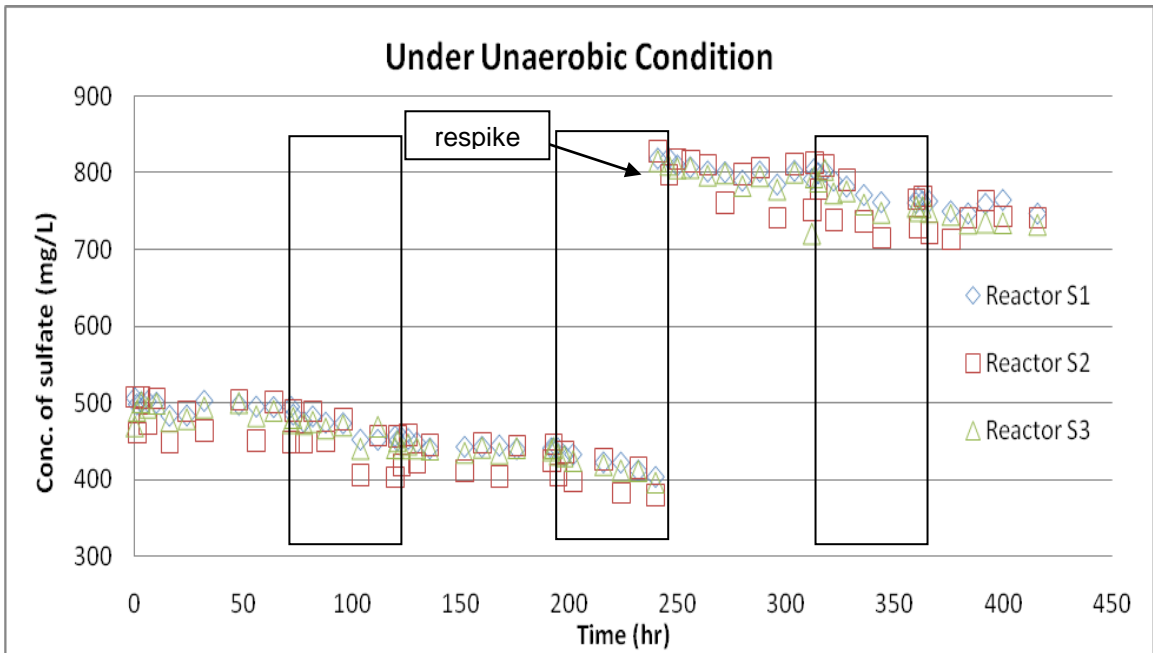


Figure 5.6. Concentration of sulfate as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

5.3.4. Quantification of Biomass

The individual cells or bacteria were measured with flow cytometry. Figures 5.7 and 5.8 show the number of microorganism during test. Bacteria number was quantified by measuring the fluorescence from cells stained. The bacteria were stained by SYTOX Green dye. The number of bacteria was compared with different electron acceptor (nitrate and sulfate) conditions, and the result shows that bacteria are at greater concentrations in nitrate reducing condition compared to sulfate present condition. When the condition was changed from aerobic to anaerobic, and from anaerobic to aerobic conditions, the microbes required some length of time to acclimate themselves to the environment (Crane and Novak, 2001) which is lag time. Exposure of aerobic microbes to anaerobic environments and exposure of anaerobic microbes to aerobic environments caused physiological stress. When the condition was changed, the concentration of microbes initially decreased, and increased after a lag period. The lag time in aerobic conditions followings sulfate present conditions was longer than after nitrate reducing condition.

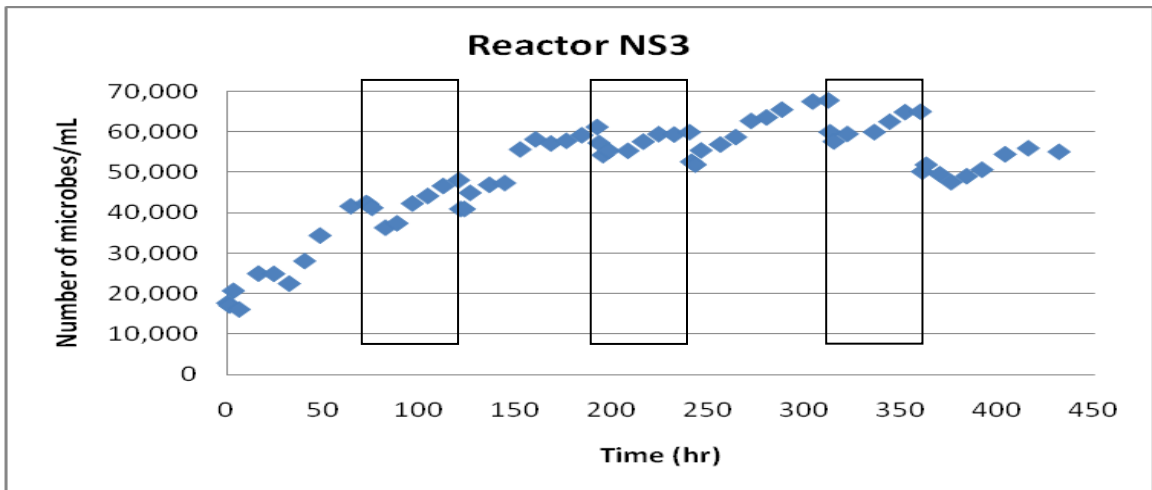
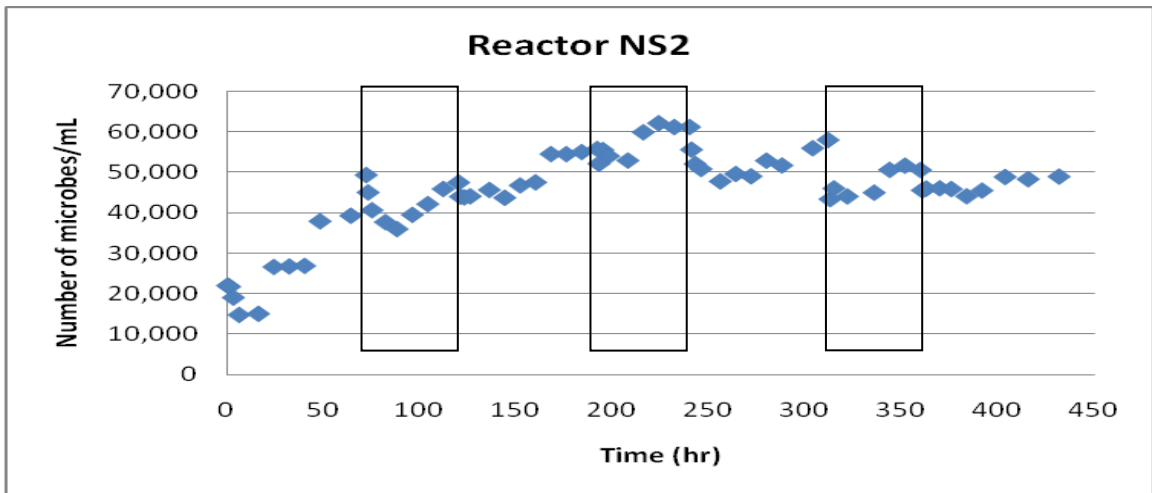
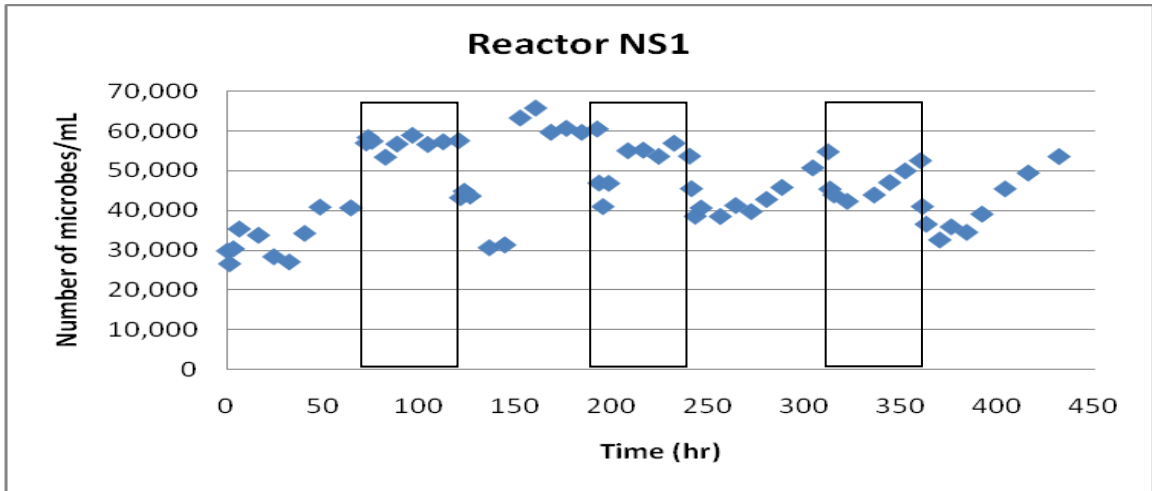


Figure 5.7. The number of microbes by Flow Cytometry as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

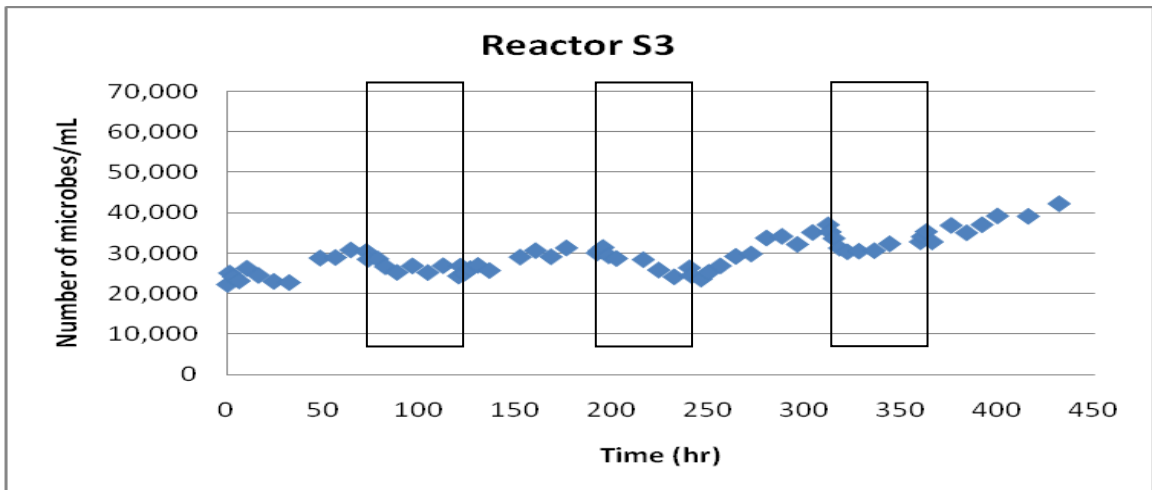
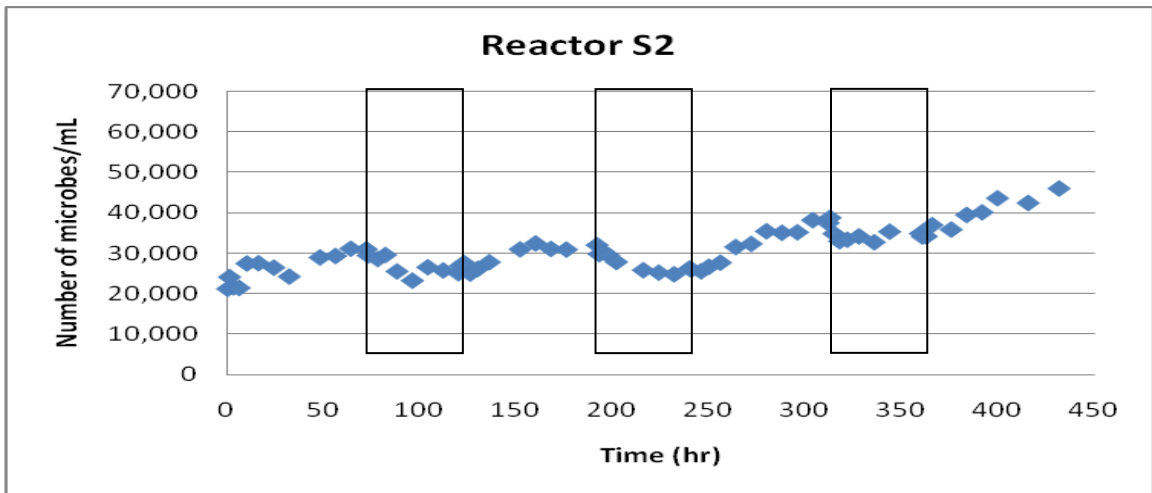
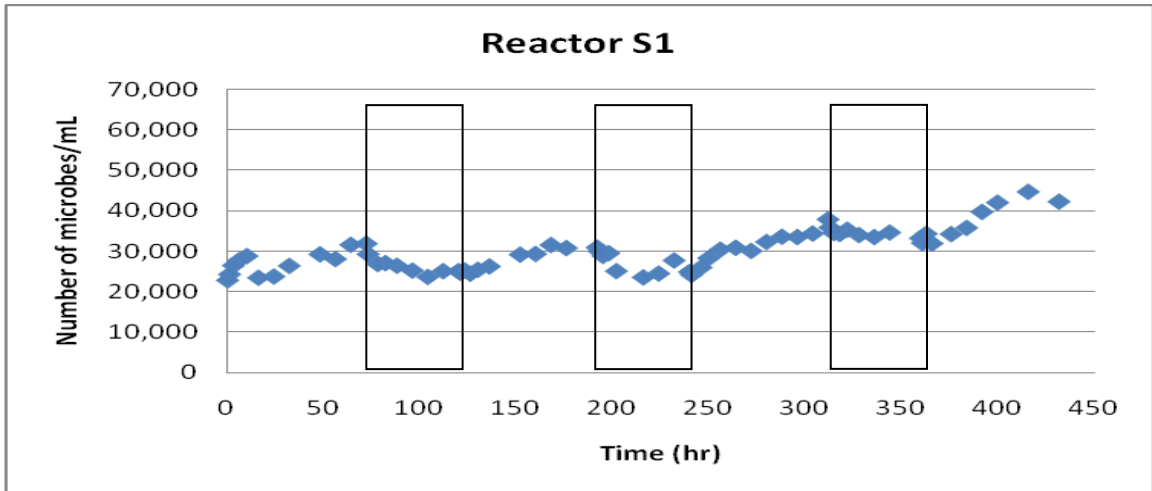


Figure 5.8. The number of microbes by Flow Cytometry as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

5.3.5. Concentration of EDCs

This study investigated the degradation of EDCs in the presence of different electron acceptors (nitrate and sulfate) under simulated SAT systems. The electron acceptor change causes the microbiological activity in simulated SAT systems. Nitrate and sulfate as an electron acceptor was added into separate simulated SAT reactors in order to create nitrate and sulfate reducing conditions respectively. EDCs oxidation was dependent upon nitrate reduction or sulfate reduction and the biodegradation of EDCs was compared. This study demonstrates the different EDCs degradation with different electron acceptor. The results of EDCs concentration in different electron accepting tests (nitrate and sulfate) are shown in Figure 5.9 and 5.11 respectively. Biodegradation and sorption of EDCs occurred at the same time in the initial aerobic period. After 48 hr, biodegradation of EDCs was the main removal phenomenon because sorption equilibrates within 48 hr (Figure 4.3). Biotransformation of E2 to E1 was observed over the course of the experiment. E1 concentration is shown in Figure 5.10 (nitrate) and 5.12 (sulfate). E2 degradation was slower under anaerobic conditions than aerobic conditions and BPA was not degraded under anaerobic conditions in nitrate reducing and sulfate present condition. In sulfate present condition, EDCs degradation is much slower than nitrate reducing condition. E2 degradation amount is higher under nitrate reducing condition than sulfate present condition. Overall, EDCs degradation has different trend in different electron accepting systems. E2 is more biodegradable than BPA, and EDCs biodegradation is much higher with nitrate reducing condition than sulfate present

condition during simulated SAT system. Moreover, the results show the different lag time in different electron accepting systems. There are longer lag time in aerobic condition right after the anaerobic condition within sulfate present condition than nitrate reducing condition. Figure 5.13 shows the results of lag time. When the condition was changed from aerobic to anaerobic, and from anaerobic to aerobic conditions, the microbes require a length of time to acclimate themselves to the environment (Crane and Novak, 2001) which is lag time. Exposure of aerobic microbes to anaerobic environments and exposure of anaerobic microbes to aerobic environments caused physiological stress. Because microbes have physiological stress, biodegradation of EDCs were decreased during lag time. Lag time was decided by when concentration decreases less than 5 %. The longer lag time (34-42 hr) is showed in sulfate reducing condition and shorter lag time (18-26 hr) is showed in nitrate reducing condition.

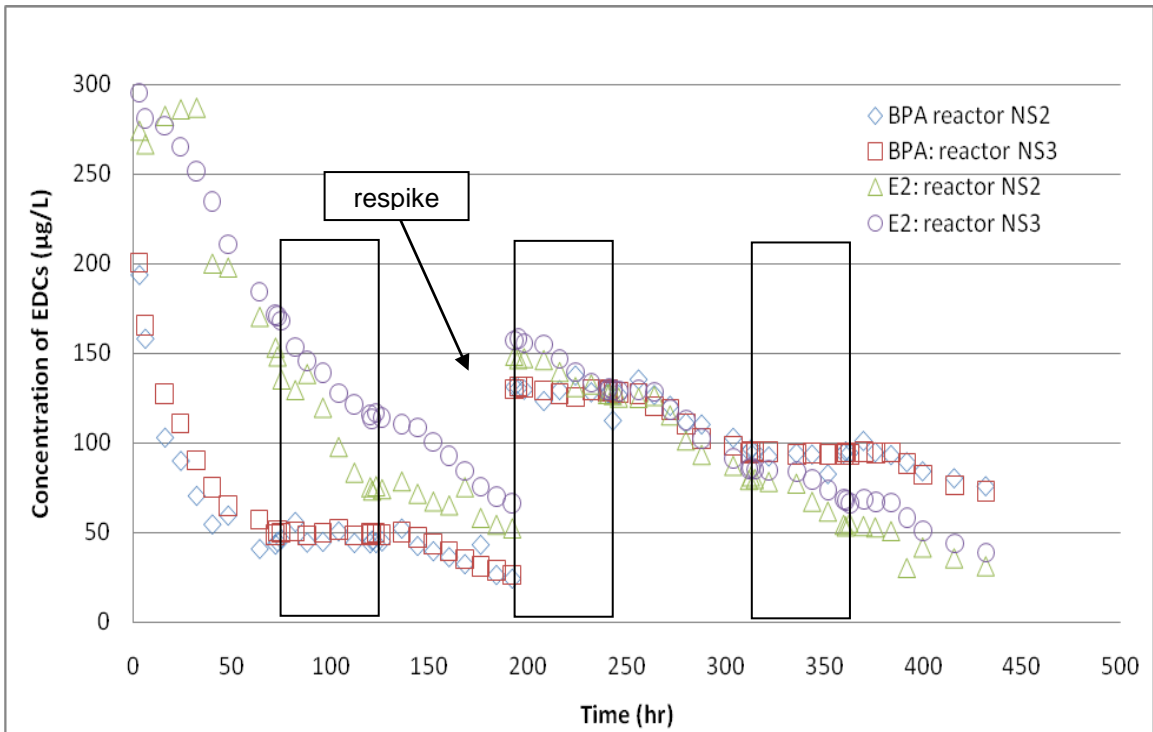


Figure 5.9. Concentration of EDCs as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

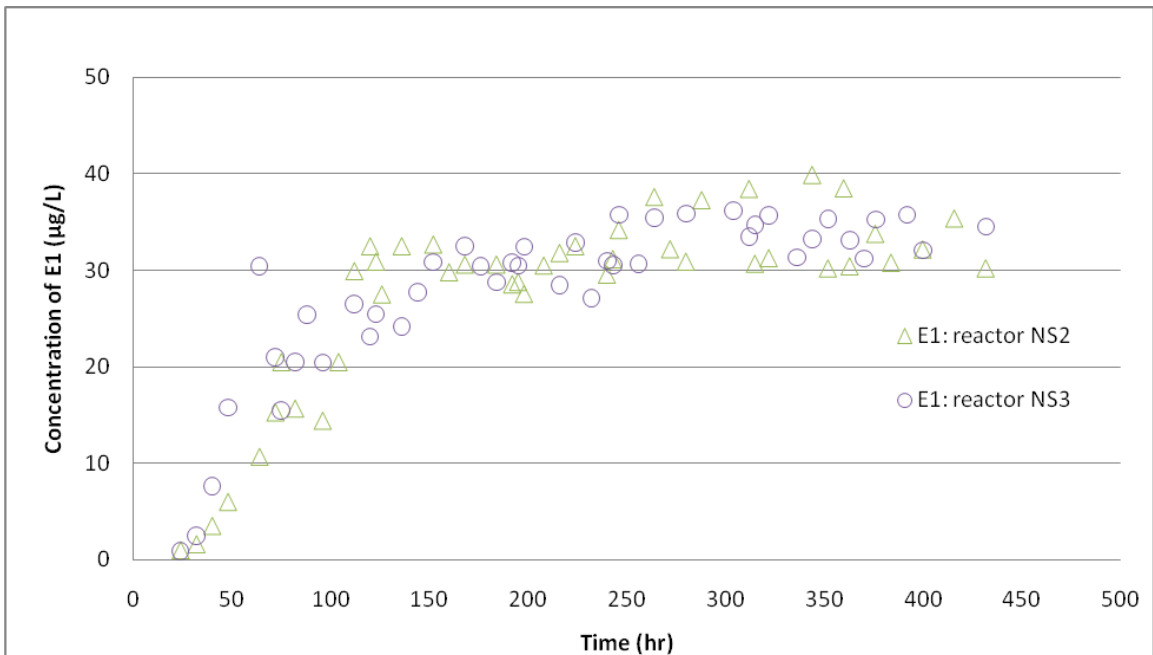


Figure 5.10. Concentration of E1 as a function of time in reactors with a 2-day anaerobic cycle. Nitrate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

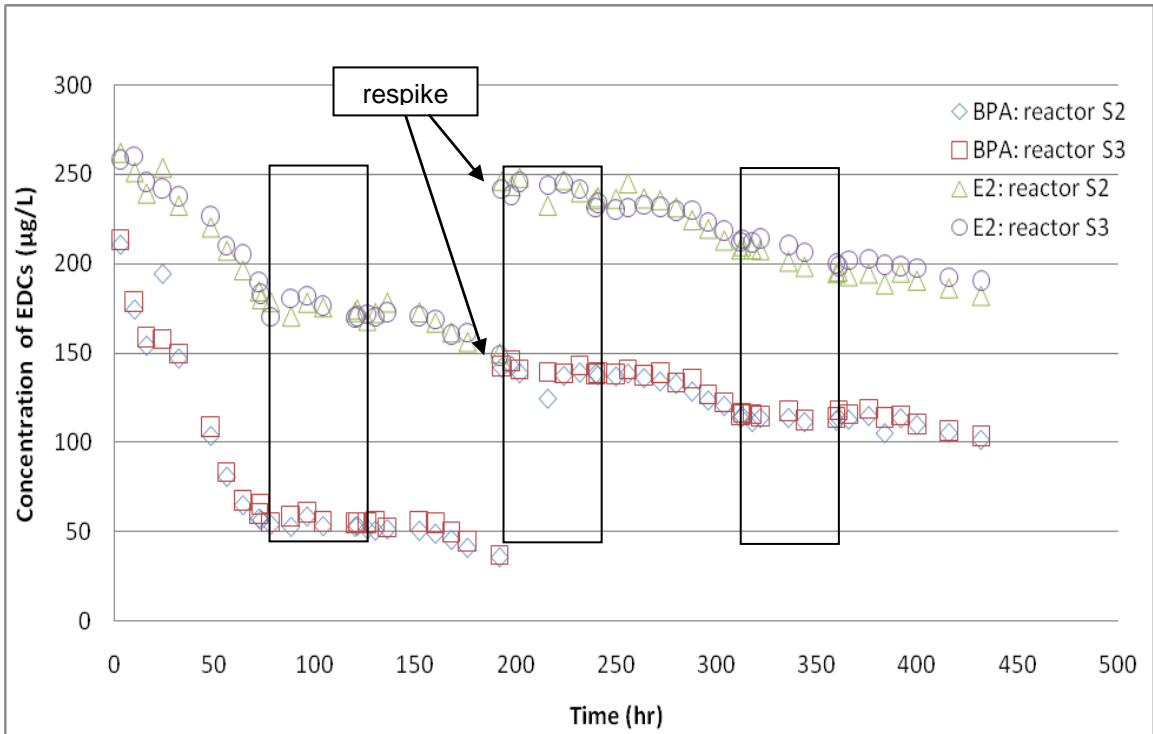


Figure 5.11. Concentration of EDCs as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)

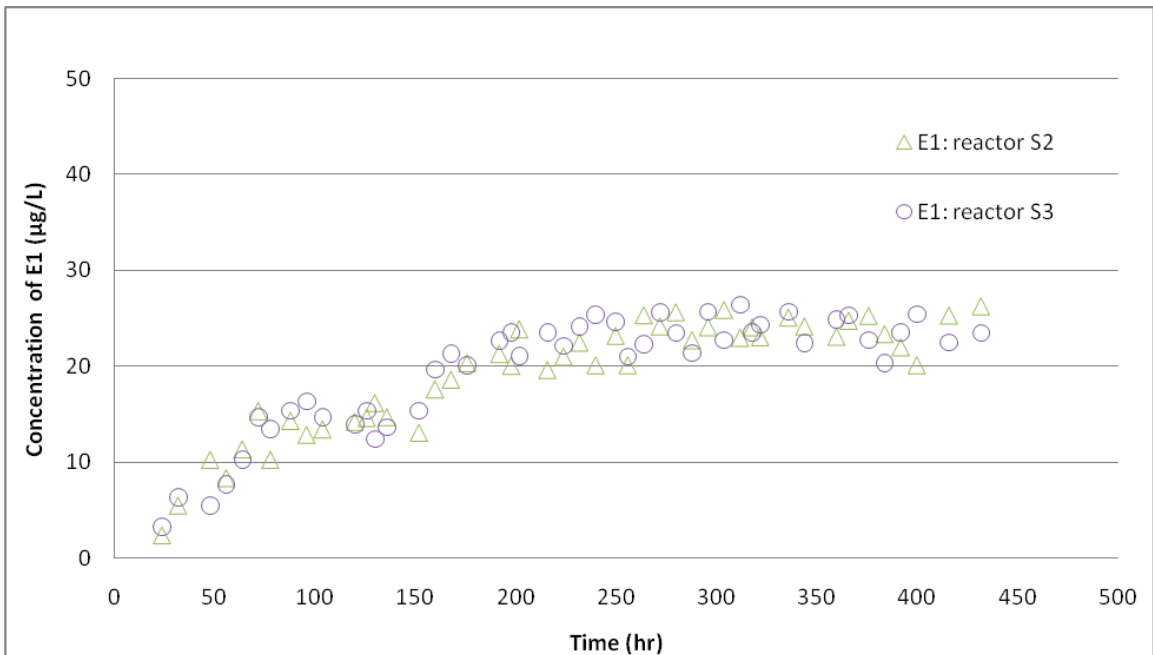
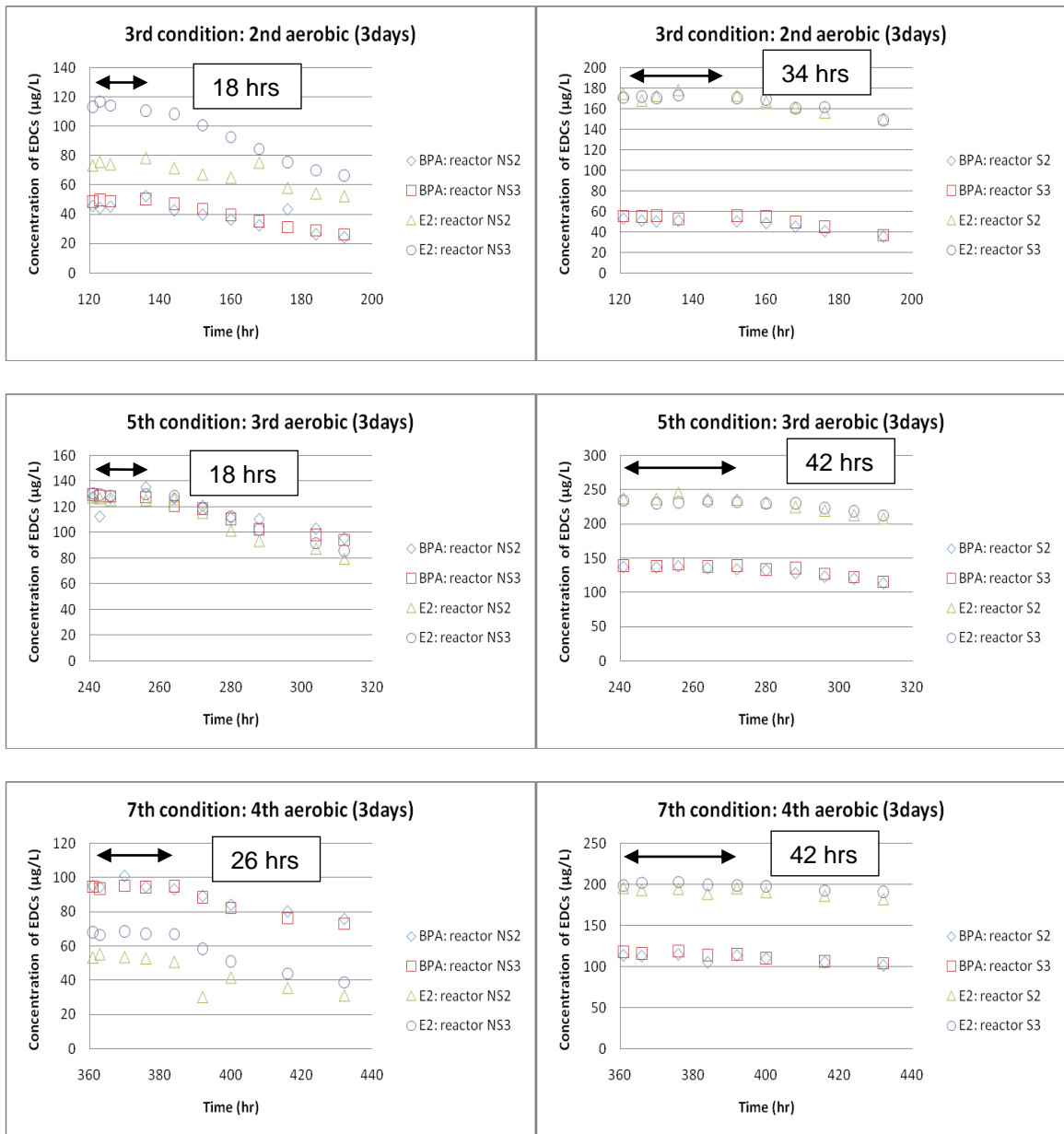


Figure 5.12. Concentration of E1 as a function of time in reactors with a 2-day anaerobic cycle. Sulfate was provided as an electron acceptor for anaerobic cycles. (Rectangular boxes indicate anaerobic periods)



(a) aerobic condition with nitrate

(b) aerobic condition with sulfate

Figure 5.13. Concentration of EDCs as a function of time in reactors with a 2-day anaerobic cycle. Nitrate and sulfate was provided as an electron acceptor for anaerobic cycles. (a) nitrate as a electron acceptor (left side), (b) sulfate as a electron acceptor (right side).

5.3.6. Mass Balance of EDCs

Mass balance of EDCs is checked before and after the reaction. Figure 5.14 shows the mass balance of EDCs in sorption test (reactor C1 and C2), nitrate reducing condition (reactor NS2 and NS3), and sulfate present condition (reactor S2 and S3). Figure 5.14 compares the spiked and final amount of EDCs. Spiked amount of BPA and E2 is 3,000 µg for reactor C1 and C2, 3,300 µg for reactor NS2, NS3, S2, and S3 (respike once). After finishing the reaction, aqueous and soil samples were collected and analyzed by GC/MS. 5 samples of aqueous phase and soil were tested, and the average results are shown in Figure 5.14. In statistical t-test, the t value is equal to or less than 0.05. Average results, standard deviation, and sampling mass of EDCs are shown in Table 5.2. During test I collected 89 samples for 2-day cycles (reactor NS2 and NS3), and 89 samples for 2-day cycles (reactor S2 and S3). Each sample was 10 ml and contained BPA and E2. Therefore a significant mass of the EDCs were removed from reactors during sampling and must be taken into account in the mass balance. EDCs in aqueous phase were extracted by SPME, and in soil were extracted by methanol. 2 g soil samples were collected and dried at room temperature (20 °C, 24 hr), and 10 mL methanol was inserted into soil and shaken for 24 hrs in a 40 ml glass vial. Methanol was separated by centrifuge and evaporated by gentle nitrogen gas. It was derivatized by MSTFA and detected by GC/MS. Final EDCs amount is shown in Figure 5.14. In reactor C1 and C2, where biodegradation was suppressed by NaN_3 , the average recovery of BPA was 96.8 % and the average recovery of E2 was 98.3 %. In reactor NS2

and NS3, average recoveries were 70.1 % and 61.1 % for BPA and E2 respectively. In reactor S2 and S3, average recoveries were 82.6 % and 86.9 %. This shows that biodegradation was minimal in the sorption control tests and biodegradation amount of EDCs in nitrate reducing conditions (reactor NS2 and NS3) is higher than sulfate present conditions (reactor S2 and S3).

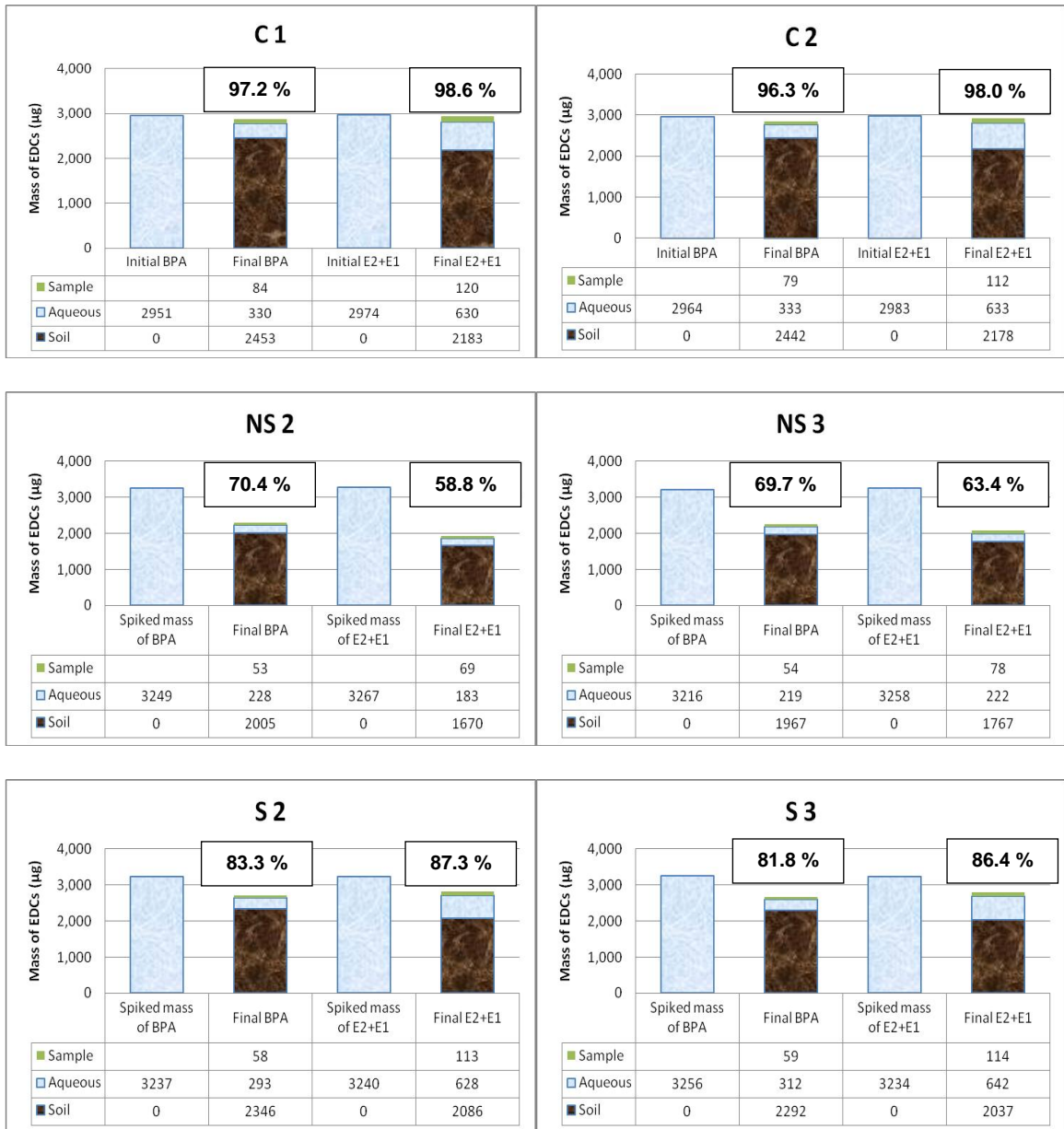


Figure 5.14. Mass balance of EDCs in aqueous and soil with a control test and 2-day anaerobic cycles. Nitrate and sulfate was provided as an electron acceptor for anaerobic cycles.

Table 5.2. Mass balance of EDCs in aqueous and soil with a control test, 2-day and 4-day anaerobic cycle. Nitrate and sulfate was provided as an electron acceptor for anaerobic cycles. Average data from 5 samples and standard deviation.

C1	Initial BPA		Final BPA		Initial E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			84				120	
Aqueous	2951	39	330	8	2974	27	630	12
Soil			2453	29			2183	29
Total	2951	39	2867	23	2974	27	2933	21

C2	Initial BPA		Final BPA		Initial E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			79				112	
Aqueous	2964	25	333	6	2983	17	633	12
Soil			2442	20			2178	35
Total	2964	25	2854	20	2983	17	2923	25

NS2	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			53				69	
Aqueous	3249	52	228	6	3267	45	183	9
Soil			2005	26			1670	28
Total	3249	52	2286	23	3267	45	1922	34

NS3	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			54				78	
Aqueous	3216	26	219	7	3258	32	222	9
Soil			1967	21			1767	26
Total	3216	26	2240	22	3258	32	2067	34

S2	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			58				113	
Aqueous	3237	17	293	13	3240	13	628	12
Soil			2346	16			2086	23
Total	3237	17	2697	29	3240	13	2827	34

S3	Spiked mass of BPA		Final BPA		Spiked mass of E2+E1		Final E2+E1	
	Average	S.D.	Average	S.D.	Average	S.D.	Average	S.D.
Sample			59				114	
Aqueous	3256	15	312	10	3234	14	642	11
Soil			2292	12			2037	15
Total	3256	15	2663	21	3234	14	2793	26

5.3.7. Sorption vs Biodegradation

Figure 4.3 shows the results of sorption test, because there is no microbiological activity. Initial concentration of EDCs is 1,000 $\mu\text{g/L}$, and final concentration is 210 $\mu\text{g/L}$ for E2 and 110 $\mu\text{g/L}$ for BPA after 24 hr. The concentration of EDCs is not change after 24 hr in control test for sorption test, but the concentration of EDCs is change in nitrate reducing and sulfate present cycles test. In the beginning part of reaction (0 -24 hr), sorption is the main EDCs removal mechanism but the biodegradation is exist, so the EDCs is decreasing continually (Figure 5.9 and 5.11). E1 (bio-transformed from E2) is observed in reactor NS2, NS3, S2, and S3, but it is not observed in reactor C1 and C2. Because the concentration of BPA is 110 $\mu\text{g/L}$ and E2 is 210 $\mu\text{g/L}$ in aqueous phase of control test after 24 hr, the difference concentration of EDCs between abiotic test (reactor C1 and C2) and biotic test (reactor NS2, NS3, S2, and S3) is biodegradation.

5.4. Conclusions

In this Chapter the simulated SAT reactor with different electron acceptor (nitrate and sulfate) and compare the lag time and EDCs bioremediation. The important innovation is that the biodegradability is differentiated depend on different electron acceptor. Nitrate and sulfate as the electron acceptor of anaerobic condition affects the biodegradation of EDCs during anaerobic condition in alternating system under simulated SAT reactor. And it affect the duration of lag time and different biodegradability of EDCs in the aerobic cycle during alternating aerobic/anaerobic conditions. In nitrate reducing condition, the EDCs biodegradability is much higher than sulfate present condition. The lag time is longer in sulfate present condition than nitrate reducing condition.

CHAPTER 6

CONCLUSIONS

The important innovation in this study is the linkage of the observed EDCs degradation to the prevailing redox conditions in the simulated SAT system. This project will help SAT to become a viable means of providing a sustainable and low-cost supply of clean water around the world.

Important conclusions from this work include: (1) The comparison between SPE and SPME provided useful guidance to scientists and engineers who are trying to develop and select a useful analytical procedure for EDCs in aqueous samples at the ng/L to $\mu\text{g/L}$ range; (2) A broader range of concentrations with large sample volumes can be analyzed by SPE, and it has a lower material cost, but more labor is required; (3) Small sample volume and limited range of concentration can be analyzed by SPME, and it is expensive due to frequent replacement of fiber, but less labor-intensive; (4) MSTFA yield higher peak areas than BSTFA for headspace (on-fiber) derivatization during SPME; (5) E2 is biodegraded during aerobic, anoxic and anaerobic cycles, but BPA is biodegraded only during aerobic cycles; (6) The lag period is observed whenever the redox condition in the systems is switched and there is no biodegradation of EDCs during lag time; (7) The long anaerobic cycle (4-day) cause long lag time

for biodegradation in the aerobic cycle. So, anaerobic cycles should not last longer than 4 days because longer anaerobic condition cause the longer lag time and lower EDCs biodegradability; (8) Nitrate reducing condition is more suitable than sulfate present condition in EDCs biodegradation in alternating SAT system between aerobic and anaerobic condition.

I expect this research to have an *impact at the national and international level*, for several reasons. First, interest in water reuse is increasing rapidly, both in the US and abroad (Metcalf and Eddy 2007). Second, I am, to the best of my knowledge, the first researcher to consider biodegradation of EDCs under alternating aerobic/anaerobic conditions. These alternating cycles are likely to control biodegradation of EDCs not only during SAT, but also during other low-cost water-treatment strategies such as riverbank filtration, which is widely applied in Europe. Third, the results of this work have important practical implications for the management of SAT systems. For instance, I am the first to demonstrate how the operating parameters (e.g., length of flooding and drying cycles) affect the quality of the re-used water. Thus, as a result of this project, we may be able to determine the optimal length of flooding and drying cycles required to ensure that the targeted contaminants are removed during percolation through the vadose zone. Hence, I anticipate that this project will have a significant impact on our *ability to provide a sustainable water supply* at low cost.

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