BIODEGRADATION OF ESTROGENIC STEROIDAL HORMONES

A Dissertation

by

SANG HYUN KIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Robin L. Autenrieth
Committee Members,	Bill Batchelor
	Kung-Hui Chu
	Thomas J. McDonald
	Daren Harmel
Head of Department,	John Niedzwecki

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ABSTRACT

Biodegradation of Estrogenic Steroidal Hormones.

(August 2010)

Sang Hyun Kim, B.S., Chonnam National University;

M.Eng., Texas A&M University, College Station

Chair of Advisory Committee: Dr. Robin L. Autenrieth

Natural and synthetic estrogens are some of the most potent hormones detected in the environment. Agriculture fields often release higher concentrations of natural estrogens to the environment, but wastewater treatment plants (WWTPs) commonly release higher concentrations of synthetic estrogens. Estrogens can disrupt endocrine functions in wildlife and humans. Less attention has been paid to the fate and occurrence of estrogens in agricultural operations than WWTPs. Their fate is influenced by major mechanisms such as sorption and biodegradation. Sorption typically accounts for less than 10% of estrogen removal in WWTPs. However, biodegradation is a primary method for estrogen loss at high ammonia concentration in the agricultural and municipal operation. Less attention has been paid to the biodegradation kinetics of estrogens in the field application. Therefore, this dissertation focused on the occurrence of estrogens in agricultural fields and their biodegradation by a mixed culture and a pure culture. The estrogens in turkey litter amended fields might be biodegraded to some degree by turkey litter borne bacteria. The estrogen biodegradation by a mixed culture

showed different mechanisms for each estrogen. E1 and E2 were easily degraded as a carbon source of the mixed culture. E3 and EE2 were favorable for cometabolic degradation by AOB. EE2 was not readily biodegraded by the mixed culture due to a steric hindrance of enzyme expression and EE2 metabolism in the ethynylgroup of EE2. The cometabolic kinetics of individual estrogen was evaluated by using a pure culture. The cometabolism of estrogen was demonstrated by a reductant model. This model appropriately estimated the cometabolic kinetics of individual estrogens. In addition, the effect of antibiotics on the hormone degradation was investigated in Sequencing Batch Reactors (SBRs). No significant difference was detected for the removal efficiency of target compounds in the SBRs in presence or absence of antibiotics (oxytetracycline and chlortetracycline) during long sludge retention time (SRT). However, the effluent organic matter (EfOM) was less decomposed with the presence of antibiotics, especially causing less degradation of the humic-like substances in EfOM. The results indicated the flux of antibiotics to WWTPs did not affect hormone degradation, but reduced the decomposition of humic-like substance. Finally, the findings from the research provide insight into how biodegradation influences estrogen removal in agricultural fields and municipal WWTPs. The models developed in this research yielded valuable predictive values for engineered systems.

DEDICATION

This dissertation is dedicated to my father Jae-Hwa Kim, my mother Suk-Hee Kim, and my wife Jinsuk Lee.

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I would like to give my appreciation to my advisor, Dr. Robin L. Autenrieth. Without her support, advice, understanding and encouragement, I couldn't have completed any of this work.

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Finishing up this Ph.D. was one of the hardest things in my life. I'm proud of myself for being able to go through this tough time. Finally, my deepest gratitude goes to my wife, son, and daughter, Jin-suk Lee, Ju-yeon Kim and Ha-yun Kim for their love and support made it possible to complete my research work.

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CHAPTER I

INTRODUCTION

STATEMENT OF PURPOSE

The occurrence and fate of steroidal hormones and antibiotics has become a concern because of the endocrine disrupting effects and the growth and dispersion of antibiotic resistant bacteria, respectively (Hwang et al., 2001; Nash et al., 2004). The steroidal hormones exhibit high estrogenic potency compared to other endocrine disrupting chemicals (EDCs) and are often resistant to microbial degradation in ecosystems and engineered systems (Hoogenboom, 2001; Khanal., 2006). The risk of EDCs was listed on the EPA's endocrine disruptor screening program as a priority issue (EPA. 2009). Widespread use of antibiotics at confined animal feeding operations (CAPOs) results in their co-excretion with the steroidal hormones. Two significant sources of environmental exposure to the steroidal estrogens and antibiotics are non-point source runoff from agricultural operations associated with animal manures and litters and effluents from wastewater treatment plants (WWTPs). Agricultural operations are reported to contribute more to estrogenic contamination than municipal WWTPs (Colucci et al., 2001; Khanal et al., 2006). Exposure to the Antibiotics promotes antibiotic resistance in the environment. Understanding the fate of hormones and antibiotics in agricultural operations is necessary to initiate effective management practices and to prevent the exposures.

This dissertation follows the format of Chemosphere.

Characterizing the release, mobility, degradability and fate of steroidal estrogens in both natural and engineered systems for the purpose of identifying controlling parameters is the focus of this research. To understand the prevalence and mobility of the estrogens, bench and field studies were conducted to develop predictive models for the extent of release and rate of degradation. Monitoring microbial activity elucidated the degradation kinetics, extent of release, and antibiotic inhibition. The inhibitory effect of antibiotics on microbial activity is largely unknown in municipal operations. There is a critical need to understand and describe the dominant characteristics that govern the fate of estrogen in agricultural and municipal operations. This research addressed the fate and degradability of estrogen and antibiotic inhibition in municipal operations.

BACKGROUND

Chemical properties

Steroidal estrogens can be classified into two types of compounds. Natural estrogens include 17β-estradial (E2), estrone (E1), and estriol (E3). The synthetic hormone, 17α-ethynylestradiol (EE2), is primarily used for human contraception. Steroidal estrogens are characterized by three six-carbon rings (A, B, and C) attached to a fourth ring (D) with distinguishing functional groups, either –OH or C=O (Fig. 1.1.). The synthetic hormone (EE2) has an additional acetylene group on C17, on the fourth ring. This has triple bonding on D-ring. EE2 is the most persistent estrogen in many municipal operations, likely because of its extended molecular structure, which reduces enzyme activity. E1 has one – OH on the C3 position of estrogen skeleton; E2 has two –

OH groups on the C3 and C17 position; and, E3 has three –OH groups on C3, C17, and C19 position. The side chain functional groups of the different estrogens can be seen in Fig. 1.1. These subtle differences substantially alter the activity of these compounds.

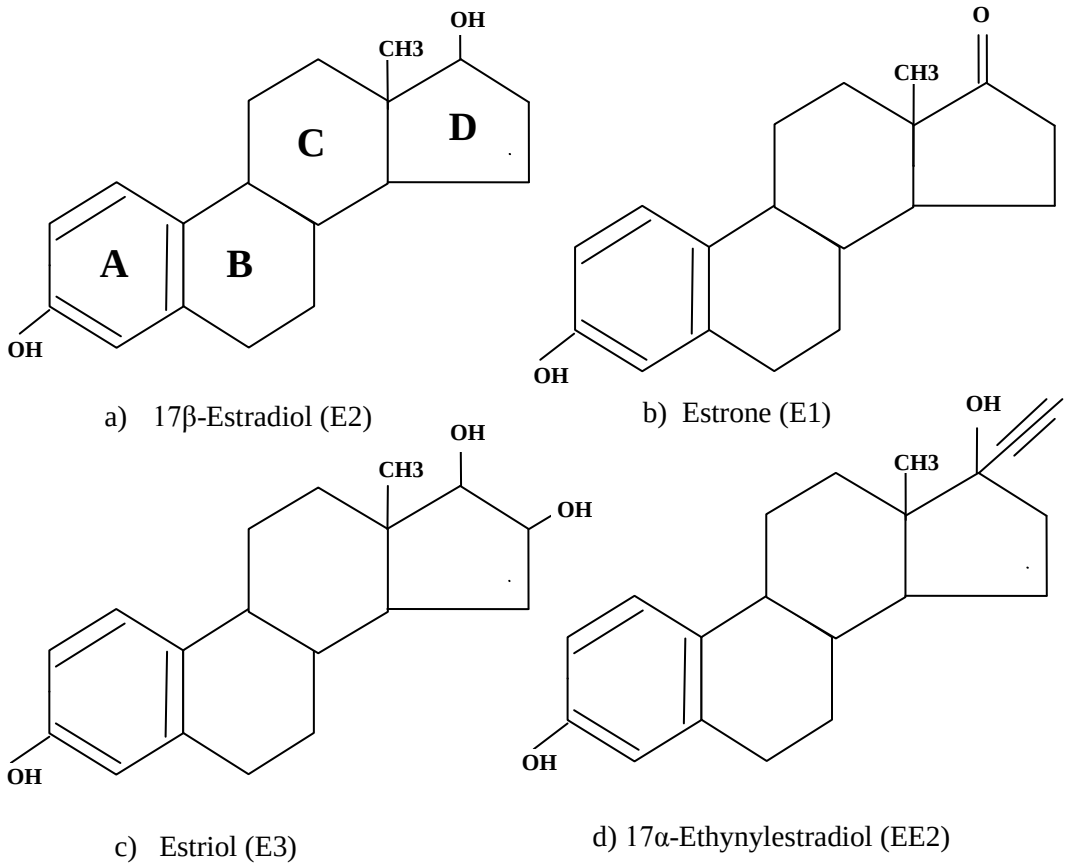


Fig. 1.1 Molecular structure of steroidal estrogens.

The physicochemical properties used to determine distribution and partitioning of hydrophobic organic contaminants (HOC) in the environment are applicable for steroidal hormones. The octanol-water partition coefficient (K_{ow}) is directly related to hydrophobicity and strongly correlated with the normalized organic carbon partition coefficient (K_{oc}) (Meylan and Howard, 1995). The water solubility of substrates (S_w) is empirically related to the rates of biodegradation of some HOCs. Therefore, K_{ow} and S_w should be considered important parameters governing the fate of hormones in agricultural fields and water systems. EE2 is the most hydrophobic compound among estrogenic hormones (Table 1.1). So, the sorption of EE2 is more favorable for its degradation than biodegradation in wastewater treatment plants (WWTPs).

Table 1.1 Select physicochemical properties of steroidal estrogens.

Estrogen	MW(g/mol)	S_w(mg/L)†	logK_{ow}‡	logK_{oc}(Sed)§
E1	270.4	13	3.43	3.40-3.81
E2	272.4	13	3.94	3.71-4.12
E3	288.4	13	2.81	
EE2	296.4	4.8	4.15	3.45-3.85

MW: Molecular weight,
†, S_w: Water solubility at 20 ^oC from Routledge et al. (1998).
‡, log K_{ow}: Octanol-Water partitioning coefficients from Lai et al. (2000).
§, log K_{oc} (Sed): Normalized organic carbon partitioning coefficients in sediment from Hanselman et al. (2003).

Physiological effects

Estrogen concentrations as low as 1-5 ng/L in water can adversely affect the reproductive systems of wildlife and humans by disrupting the normal function of their endocrine systems (Carlsen et al., 1995, Hoogenboom, 2001; Miller et al., 2007). E2 and E1 are metabolized to 2-hydroxyestrone and 16α-hydroxyestrone, respectively, at either the C2 or the C16 position (Zumoff, 1994; Eliassen et al., 2008). 2-hydroxyestrone contributes a modest anti-estrogenic effect as "the good estrogen." However, the 16α-hydroxyestrone and estriol (E3) are estrogen agonists (Zumoff, 1994). The hydroxylated E1 leads to tumor initiation in estrogen-sensitive tissue (Zumoff, 1994). This study reported that estrogen-related cancers including breast, ovary, uterus, and prostate, may be caused by increased 16α-hydroxylation of E2 or the ratio of 2-hyroxyestrone to 16α-hydroxyestrone (Elisassen et al., 2008). Human health risk from estrogenic hormones remains uncertain until estrogenic effects appear across the population.

The impact on wildlife exposed to estrogens has largely focused on aquatic organisms such as fish and turtles. The protein vitellogenin, whose production is stimulated upon exposure to estrogen in male and female organisms, serves as a good marker to indicate environmental exposure of estrogens in non-mammalian vertebrates. Several studies document that steroidal estrogens led to vitellogenin production at concentrations as low as 1 ng/L, some refer to this condition as the demasculinization of male fish (Irwin et al., 2001). The demasculization of fish can lead to an altered sex ratio within a population and sex reversals (Hutchinson et al., 1999; Lai et al., 2002). Physiological changes at three organizational levels in *Xenopus laevis* resulted from

exposure to E2 at 2.7 μg L^{-1} (Kloas et al., 1999). In the experimental lake area of north western Ontario, Canada, the population decrease of fathead minnow was caused by exposure to EE2 (Kidd et al., 2007).

Humans and other animals excrete steroidal estrogens through urine and feces, generating 25-100 μg per day depending on the phase of the menstrual cycle (Turan, 1995). Estrogens can be introduced to the environment through municipal and agricultural activities as both point and nonpoint sources. Estrogen concentration ranging 0.1 - 82 ng/L was observed in effluent from WWTPs (Table 1.2). The presence of the estrogens in the effluent of WWTPs is largely due to poor estrogen removal in activated sludge processes. For example, it was indicated that E1, E2, and EE2 can be removed up to 61%, 86%, and 85%, respectively, by the activated sludge process (Baronti et al., 2000). Another study reported that E1 and EE2 were removed up to 60% and 65%, respectively (Esperanza et al., 2004). Most WWTP effluents are discharged to surface waters and groundwater. The presence of these chemicals, at trace quantities, is known to have a negative impact on water quality.

Source of estrogens and antibiotics

Estrogens are released to the environment through human and animal urine and feces. Two significant sources of environmental exposure to steroid hormones are 1) non-point source runoff from agricultural lands with applied animal manures and litters; and 2) effluents from WWTPs (Ingerslev et al, 2003). The detection of estrogen in the effluent from WWTPs results from the endogenous excretion of hormones by humans. The excretion values for normal woman range from 50 to 450 μg per day via the urine

(Aheren and Briggs, 1989). The concentrations of estrogens in a range of various matrices from WWTPs are illustrated in Table 1.2.

Table 1.2 The detected concentration of estrogens in influent and effluent from WWTPs.

Estrogen	Sewage Influent (ng/L)†	Sewage effluent (ng/L)‡	Activated sludge (ng/g)§
E1	44-490	LOD-70	LOD-37
E2	11-180	LOD-64	LOD-49
E3	LOD-263	LOD-18	N.D
EE2	LOD-120	LOD-42	LOD-17

†, Busch et al., 2002; Sole et al., 2000; Baronti et al., 2000
‡ Ying et al., 2002
§, Ternes et al., 2002
LOD: Below limit of detection, N.D: no detection.

The 2002 US Census of Agriculture reports that there are approximately 400,000 animal feeding operations in the US (USDA, 2002) with the number of operations expected to increase. The poultry industry is the largest increased contributor among the primary contributors (swine, beef, dairy, and poultry). The U.S. poultry industry produced almost nine billion broilers with a total litter production of almost 13 billion kg (2008) (USDA, 2009). Growth in the poultry industry has lead to an increasing number of CAFOs (confined animal feed operations) that generate large quantities of animal wastes. One of the most concentrated poultry areas in the U.S. is the eastern shore of Maryland which produces 1.6 billion lbs of poultry litter for 600 million birds annually (USDA, 2002). Poultry litter is a good source of organic carbon and nutrients, but naturally occurring estrogenic steroids in the litter are a concern to environmental health (Nichols et al., 1997; Finlay-Moore et al., 2000; Shore et al., 2003; Raman et al., 2004).

The application of litter to soil is the primary means of disposal and utilization, providing nutrient sources to agricultural land (Moffitt, 1996; Kellogg et al., 2000). Poultry litter has been reported to contain up to 904 ng of E2 per gram of litter on a dry weight basis (Shore and Shemesh, 2003). E2 is naturally present in poultry litter. It is not legal to feed hormones to poultry in the U.S. The concentration of natural estrogens in poultry litter is dependent on gender, age, and type of bird. In total, poultry in the US produce approximately 160-760 ton/year of natural estrogens (Ingerslev et al., 2003). While the generated litter is valuable for soil amendment, the presence of hormones and antibiotics poses a concern if release in the watershed is not adequately controlled.

Agricultural runoff contributes to the deterioration of approximately 70% of impaired streams and rivers in the U.S. (USDA/USEPA, 1999). Table 1.3 shows the high concentrations of estrogens in various agricultural sources. Additionally, several studies of E2 and testosterone mobility in runoff from agricultural fields report measureable quantities of hormones (Nichols et al., 1997; Finlay-Moore et al., 2000). A farm in central Virginia (U.S.) with a 1.2-km^2 agricultural watershed, had runoff with E2 concentrations as high as 120 ng L^{-1}, that fed into a farm pond with E2 concentrations of 14 - 20 ng L^{-1} (Shore and Shemesh, 2003). In 0.8 ha fescue plots, E2 ranging from 305 - 820 ng L^{-1} was released into runoff following amendment with broiler litter (Finlay-Moore et al., 2000). The levels of estrogen in the runoffs from these various farms are high enough to cause harm to the health of fauna in the downstream ecosystems.

Table 1.3 Estrogen concentrations in runoff and lagoon from various sources at agricultural operation sites.

Source	Estrogen	Concentration (ng/L)	Reference
Poultry litter	E2	14-20	(Shore and Shemesh, 2003)
Poultry litter	E1	N.D.†	(Finlay-Moore et al., 2000)
	E2	20-2330	
Dairy manure slurry	E1	255-640	(Shore and Shemesh, 2003)
	E2	170-1,230	
Poultry litter	E2	1280	(Nicole et al., 1997)
	E1	N.D.	
Flushed dairy manure wastewater	E1	780	(Hanselman et al., 2004)
	E2	<1,310	
Dairy waste lagoon	E1	650	(Kolodziej et al., 2004)
	E2	650	
Diary manure	E1	N.D.	(Raman et al., 2001)
	E2	3300	

† N.D.: No detection

Antibiotics have been a great concern because of the development of antibiotic resistance in bacteria in water systems (Colborn et al., 1993, Levy, 1997). Annually, 50 million pounds of antibiotics are produced in the United States (FDA, 1999). The family of tetracycline antibiotics, such as chlortetracycline (CTC) and oxytetracycline (OTC), are of greatest environmental concern because they are highly consumed by humans and animal feeding operation (Col and O'Connor, 1987). Antibiotics have been detected at concentrations of 25-1000 μg/L in swine lagoons and 0.1 - 10 μg/L in effluent from wastewater treatment plants (Kolpin et al., 2002; Campagnolo et al., 2002; Karthikeyan and Bleam, 2003). Moreover, antibiotics delivered through these sources may cause long-term and irreversible change to the microorganism genome, subsequently reducing bacterial activity in activated sludge to degrade ammonia and organic matter (Halling-

Sørensen, 2001; Klavarioti et al., 2009; Prado et al., 2009).

Environmental fate

The fate of steroidal estrogens is governed by many physical, chemical, and biological processes, such as sorption, biodegradation, and photodegradation. These processes are complex and largely uncharacterized for natural or engineered systems. Moisture in agricultural soils favors estrogen biodegradation. Runoff and leaching from the agricultural soils is also favorable for estrogen biodegradation. To confirm biodegradation in agricultural soils, a dissipation study of estrogens in soils under aerobic conditions was undertaken by spiking the soil with ^{14}C-labeled estrogens to an initial concentration of 1-10 mg kg^{-1}. The results show that 91% of the total [^{14}C] was recovered during the experiment in natural soil: 6% of the total was trapped as $^{14}CO_2$; 12% was extracted from the soil; 73% was associated with natural organic matter (NOM) that was composed of 37% humic acid, 17% fulvic acids, and 19% humin (Fan et al., 2007). Twelve percent of the extracted [^{14}C] materials were transformed to E2 metabolites. This study suggests that sorption on NOM was mostly responsible for hormone loss in field soils. The sorbed estrogen may easily be transported by leaching or runoff to a downstream ecosystem, causing environmental problems to wildlife.

Sorption to various media, including soil, sediment, and colloids, affects the fate and transport of steroidal estrogens in the environment (Yamamoto et al., 2003). Sorption to soil can reduce the leaching potential to groundwater and reduce estrogen bioavailability to microorganisms (Xia et al., 2006). Sorption to sediment and colloids, such as clay and dissolved organic matter (DOM), can increase mobility through runoff

or leaching and enhance bioavailability to fixed bacteria and biofilms (Yamamoto et al., 2003; Das et al., 2004; Xia et al., 2006). Another study reports that estrogens were adsorbed on the sediment, colloids, and soil mineral depending on particle size and mineral type (Bonin and Simpson, 2007). Mineral influence on sorption increases when organic matter content in soil is low (<0.01%). However, when organic matter content in soil is high (6-8%), both the inorganic and organic composition affect sorption predictions (Gao et al., 1998). In addition, organic carbon content plays a role as a primary binding mechanism (Lee et al., 2003). The normalized organic carbon distribution coefficient (K_{oc}) is the distribution coefficient (K_d) normalized to total organic carbon content, suggesting the tendency of compounds to partition to organic matter. The log K_{oc} of estrogen for various colloids is listed in Table 1.4. The high log K_{oc} of estrogens promotes an affinity toward the organic matters. Therefore, log K_{oc} is useful in predicting the mobility of steroidal estrogens in agricultural and municipal operations. Higher log K_{oc} values correlated to less mobile organic chemicals while lower log K_{oc} correlated to more mobile organic chemicals, indicating that E2 is more mobile in soil-water systems than E1 (Zhou et al., 2007; Young and Borch, 2009). Additionally, NOM plays a role in estrogen sorption. As shown in Table 1.4, the order of sorption capacity on various organic materials for estrogen is as follows: Tannic acid>Humic acid>Fulvic acid> Polysaccharide (Table 1.4).

Table 1.4 Organic carbon normalized partition coefficients (K_{oc}) for the sorption of steroidal estrogens to various compounds.

Estrogen	Log K_{oc}	Type of sorbate	Organic Carbon	Reference
E2	3.5	River sediment	0.3 – 3.3 (%)	Lai et al. (2000)
	3.16-3.52	Soil	0.22-2.91 (%)	Lee et al. (2003)
	3.94	River colloids	3.0 mg/L	Zhou et al. (2007)
	4.94	River humic acid	100 %	Yamamoto et al. (2003)
	4.57	River Fulvic acid	100 %	
	2.76	Polysaccharide	100 %	
	5.28	Tannic acid	100 %	
	3.69	Soil	0.94-3.76 (%)	Hidebrand et al. (2006)
E1	3.69	River sediment	0.3 – 3.3 (%)	Lai et al. (2000)
	3.18-3.22	Soil	0.22-2.91 (%)	Lee et al. (2003)
	4.85	Stream colloids	2.3 mg/L	Zhou et al. (2007)
	4.83	River colloids	3.0 mg/L	
	4.67	River L'Aa colloids	2.9 mg/L	
	5.04	Seawater colloids	0.4 mg/L	
	4.64	Soil	0.94-3.76 (%)	Hidebrand et al. (2006)

Biodegradation is the primary removal mechanism for estrogens in natural and engineered systems. Initially, inactive conjugated estrogens (E2-3-glucuonide and E2-3-sulfate) are excreted to water systems (Ternes et al., 1999). The conjugated forms of estrogen can be easily transformed to the active unconjugated forms (E2) by bacterial enzymes (Ternes et al., 1999). The deconjugation occurs in thermodynamically irreversible reaction (Dray et al., 1972, Ternes et al., 1999). Estrogen biodegradation in municipal operations has been studied with activated sludge microorganisms, nitrifying bacteria, and other pure cultures. Estrogens can be degraded by ammonia oxidation

bacteria (AOB) or heterotrophic bacteria (Shi et al., 2004; Yoshimoto et al., 2004; Yu et al., 2007). *Rhodococcus zopfii* and *Rhodococcus equi* isolated from activated sludge degraded E1, E2, E3, and EE2 as their sole carbon and energy source under heterotrophic conditions with much better degradation capacity (Yoshimoto et al., 2004). Biodegradation of EE2 by nitrifying activated sludge was reported (Vader et al., 2000). A study also demonstrated that four estrogens were degraded by a pure culture of *Nitrosomonas europaea* (Shi et al., 2004). The results from both studies indicate that estrogen can also be degraded via cometabolism (Vader et al., 2000; Shi et al., 2004).

Photodegradation is a significant abiotic degradation mechanism for estrogen loss in natural water (Vialaton and Richard, 2002). Visible light, in the 200-700 nm range, is not good for saturated organic compounds, i.e., molecules with single bonds because visible light has low energy spectrum (Turro, 1991). However, unsaturated molecules, such as ring A on estrogens, can absorb visible light (Turro, 1991). In this manner, estrogens are photodegraded in natural ecosystems. In controlled experiments, to mimic advanced municipal treatment technology, estrogens were photodegraded up to 99% in less than 30 min under UV light (intensity-6mW/cm^2) by using a titanium dioxide (TiO_2) catalyst (Ohko et al., 2002). The efficiency of estrogen photodegradation is pH dependent, but not temperature dependent. Results suggest faster degradation of estrogens occur at pH 7+ (Liu et al., 2004; Kimura et al., 2004). Ideally, photodegradation plays a greater role in estrogen loss from agricultural fields when animal litters are applied because the urine is alkaline.

Degradation kinetic modeling

Degradation kinetics are important in modeling the persistence of steroidal estrogens in the agricultural and municipal WWTP operation. Data on estrogen biodegradation kinetics by cometabolic processes are limited. Cometabolic degradation of estrogen requires a reducing agent generated by primary substrate degradation and enzyme activity induced by ammonia oxidizing bacteria (AOB) (Stein et al., 1998). A kinetic model of estrogen cometabolic degradation was derived using four assumptions based on trihalomethanes (THM) cometabolic studies (Wahman et al 2006; Alvarez-Cohen and Speitel 2000; Arcangeli and Arvin 1997; Criddle 1993). The cometabolic degradation of estrogen could have a similar process as suggested by other cometabolic studies. The proposed metabolic pathway for cometabolism of estrogen by a nitrifier is illustrated in Fig. 1.2. Generally, the biological transformation of ammonia nitrogen (NH_3), which typically occurs in its ionic form (NH_4^+) in normal pH range, is oxidized to nitrate (NO_3^-) by nitrifying bacteria. AOB play a key role in the oxidation of NH_3 to NO_2^- as a first step. Ammonia is initially oxidized to hydroxylamine by ammonia monooxygenase (AMO) in *Nitrosomonas europaea (N. europaea)*, and the hydroxylamine is then further oxidized to NO_2^- with four electrons from hydroxylamine oxidoreductase (HAO) (Aziz et al., 1999; Ely et al., 1996). *N. europaea* is the most widely used AOB for cometabolism research of various organic compounds (Vannelli et al., 1990). Several studies indicate that estrogens can be biodegraded through cometabolic oxidation using *N. europaea* and mixed cultures of nitrifying activated sludge from WWTPs (Vader et al., 2000; Shi et al., 2004; Yi and Harper, 2007).

Microbial kinetic parameters for estrogen removal through cometabolism are not currently available.

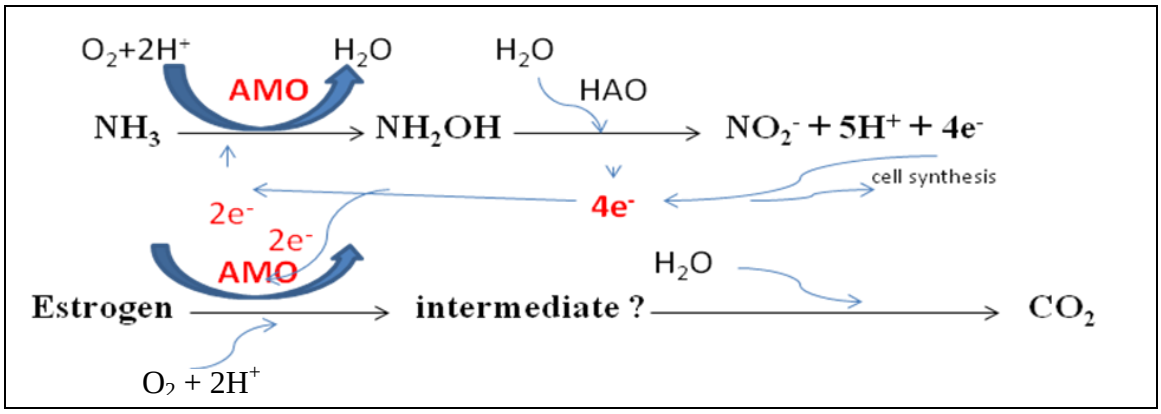


Fig.1.2. Proposed cometabolic pathway of estrogen using a nitrifier.

Effect of antibiotics

Antibiotics inhibit nitrification due to their potency against nitrifying bacteria (Halling-Sørensen, 2001). Different antibiotics can play a role in agricultural and municipal operations as inhibitors or stimulators of nitrification (Halling-Sørensen, 2001). The study reported inhibition levels of nitrification for 11 antibiotics by measuring EC_{50} values using a pour plate method. It was demonstrated that tetracycline, a broad spectrum antibiotic, inhibits the nitrification process, while sulfonamides stimulate nitrifiers. For activated sludge, the EC_{50} for chlortetracycline (CTC) and oxytetracycline (OTC) were 28 µg L^{-1} and 321 µg L^{-1}, respectively, by using a pour plate method, and 2 µg L^{-1} and 320 µg L^{-1} for *N. europaea* (Halling-Sørensen, 2001). Several

studies reported that estrogen degradation is linked to ammonia oxidation due to cometabolic degradation by AOB (Vader et al., 2000; Shi et al., 2004; Yi et al., 2006; Yi and Harper, 2007). This cometabolic degradation of estrogens was explained by an inhibition test using allythiourea, one of the AMO inhibitors in an *aerobic* batch test (Shi et al., 2004). It effectively inhibited nitrification and estrogen biodegradation, suggesting that hormone removal efficiency in WWTPs may be decreased by the presence of antibiotics. The high influx of antibiotics to WWTPs may reduce nitrification efficiencies by reducing enzymatic activity essential for cometabolism. The decreased AOB activity reduces estrogen removal in aquatic systems.

The inhibition of nitrification for various compounds has been determined by several standard methods. The extent of apparent nitrification inhibition by antibiotics depends on the assay. A study indicated that short-term (six hours) effects of antibiotics on nitrification might be less sensitive in standard methods (ISO 15522 and ISO 9509) than long-term test (3 days) (Backhaus et al., 1997). Alternatively, the long-term test using pure plate method with activated sludge and *N. europaea* was sensitive for most antibiotics compared to standard methods (Halling-Sørensen, 2001). The study failed to represent nitrification rates in batch tests. So, it was only possible to show the tendency of nitrification as either level of increased or decreased nitrification rate compare to a control, indicating that selective antibiotics including OTC and CTC are 10 times more potent with pure plate method than the standard method (Halling-Sørensen, 2001). Inhibition of antibiotics can strongly depend on the test duration and type of compounds (Kummerer, 2004). A long-term test via a sequential batch reactor (SBR) will be

effective for evaluating the inhibition of nitrification in activated sludge. Troubles arose with regard to the unknown length of the period in the inhibition test. Thus, the test duration was extended up to 14 days due to slow growth rate of pure culture nitrifiers in comparison to heterotrophic communities in activated sludge. Improved biodegradation of estrogens in nitrifying process can be related to increased sludge retention time (SRT) (Ternes et al., 1999; Holbrook et al., 2002; Anderson et al., 2003). Longer SRT for estrogen cometabolism yields more extensive biodegradation (Demes et al., 2005). Therefore, our study evaluated the effect of SRT on nitrification in the presence of antibiotics.

The need for water reuse and recycling of biologically treated sewage effluent (BTSE) is increasing as a strategy for conservation due to water scarcity and high rates of urbanization (Shon et al., 2006). The BTSE generally contains three classes of organic compounds, according to their sources, 1) natural organic matter (NOM), 2) synthetic organic compounds (SOC) and disinfection by-products (DBP), and 3) soluble microbial products (SMP) (Shon et al., 2006). The characteristic study of effluent organic matter (EfOM) or NOM in BTSE is of high concern because DOM serves as a precursor of disinfection byproducts (DBPs), which increases the amount of coagulants and oxidants in water treatment process, and causes major fouling problems on membrane surfaces in advanced processes (Singer, 1999; Lee et al., 2004; Sharp et al., 2004). However, little is known about the effect of antibiotics on neither the degradation characteristics of EfOM nor the degradation of xenobiotics in activated sludge processes of WWTPs. This research hypothesizes that these antibiotics will affect the rate of degradation of

activated sludge and other target bacteria in WWTPs, which will consequently cause incomplete removal of hormones by nitrification inhibition and less decomposition of organic matters in final effluents. Therefore, the objective of this study is to evaluate the inhibitory effects of selected antibiotics on degradation characteristics for ammonia, estrogen, and EfOM in SBR. Finally, the study will increase understanding of antibiotics' fate and transport in municipal and agricultural operations.

RESEARCH OBJECTIVES

The overall goal of this research was to evaluate the biodegradability of selected estrogens in limited agricultural and municipal operations by determining their occurrence and mobility, the kinetics of microbial degradation, and the antibiotic inhibition of biological degradation. The biodegradability of estrogens was evaluated in representative agricultural and municipal samples, and compared to the performance of a pure culture of *N. europaea*. The specific objectives for each research question are as follows:

1. To develop an optimized detection method for estrogen in solid (turkey litter) and aqueous (runoff) matrices.
2. To measure biodegradation rate coefficients for individual estrogens (non-growth substrate) via cometabolism with an autotrophic pure culture (*N. europaea*).
3. To evaluate the microbial effects of turkey litter enrichment culture (TLEC) on estrogen biodegradation, using substrate utilization tests to discern key bacteria in biodegradation of estrogen on turkey litter amended fields.

4. To evaluate the inhibitory effects of selected antibiotics on hormone degradation in WWTPs.

CHAPTER II

GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC DETERMINATION OF STEROIDAL ESTROGEN IN TURKEY LITTER AMENDED FIELDS

OVERVIEW

Various analytical methods can detect trace levels of steroidal estrogens in animal litter and runoff generated from litter amended fields, but the complexity of the matrix challenges the accuracy and reproducibility of measurements. Agricultural studies commonly use enzyme immunoassay techniques for quantification of the steroidal estrogens due to the speed and simplicity of their use (no sample preparation). Our study evaluated derivatization protocols published previously as a sample preparation to determine the content of estrogens in field samples using gas chromatograph-mass spectrophotometer (GC-MS) with an effective extraction method. This sample preparation for a liquid and a solid matrix consists of sample preservation, filtration (liquid matrix only), ultrasonic solvent extraction (USE, solid matrix only), solid-phase extraction (SPE), aminopropil SPE as a cleanup step (solid matrix only), and optimized derivatization. This sample preparation procedure achieved improved efficacy to determine the concentration of steroidal estrogens in environmental samples. A derivatization protocol to form derivatives with sufficient efficacy used N, O-Bis trifluoroacetamide (BSTFA) as a silylation reagent, Trimethylchlorosilane (1% TMCS) as a catalyst, and pyridine as a solvent. Compared to other protocols, this method achieved better sensitivity immediately after 5 hours duration following a 30 minute

sand-bath at 70 ^{o}C. Moreover, recovery tests using isotopes indicated that the optimized method was suitable for determination of the steroidal estrogens in turkey litter and runoff from turkey litter amended fields with an average recovery of approximately 90% for liquid matrix and 50% for a solid matrix. As expected, only estrone (E1) and 17β-estradaiol (E2) were detected in the two matrices (turkey litter and runoff). The improved sample preparation protocol resulted in an optimized GC-MS analysis compared to previous sample preparation techniques. The study suggests that this sample preparation method is faster and simpler for determining the fate of estrogen using GC-MS in the turkey litter amended field study.

INTRODUCTION

Poultry litter is widely used for soil amendment both providing fertilizer and improving soil integrity to increase crop yield. The poultry litter contains natural quantities of steroidal estrogens depending on the type, age, and gender of birds. As a result of amending soil with the poultry litter, the steroidal estrogens can partition into runoff produced from poultry litter amended fields (Nichols et al., 1997; Nichols et al., 1998; Finlay-Moore et al., 2000; Shore and Shemesh, 2003). The occurrence of estrogens even at trace levels (part per trillion: ppt) in the aquatic environment can adversely affect the reproductive systems of wildlife and humans by disrupting the normal function of their endocrine system (Jobling et al., 1998; Miller et al., 2007). The number of published papers for quantification of estrogens in animal litter or manure is limited (Wenzel et al., 1998; Vethaak et al., 2002; Okkerman and Groshart, 2003).

Naturally occurring 17β-estradiol (E2) is readily transformed to estrone (E1) and estriol (E3) in soil and water systems. Relatively little is known of the fate of E1 or E3 compared to E2, even though it is likely that these metabolites would be detected on agricultural lands applied with poultry litter. Concern over the fate of E1 in the environment is due to its estrogenic activity, about one fifth that of E2, while that of E3 is much lower (Metcalfe et al., 2001; Conroy et al., 2007). E2 metabolites in agricultural field studies have rarely been reported. Colucci et al. (2001) suggest the need to monitor E1 in the agricultural non-point sources due to its high potency.

Various analytical techniques have been developed to monitor steroidal estrogens in various matrices including surface water, sediments, sludge, and soil (Belfroid et al., 1999; Lopez de Alda et al., 2000; Kolpin et al., 2002; Cargouet et al., 2004). However, analytical methods can be limited for an agricultural study of land litter application due to sample complexity (sample preparation is required), lower sample throughput, and interferences due to the presence of natural organic matter (NOM) (Johnson et al., 1999; Huang and Sedlak, 2001). Agricultural field studies evaluating the transport of steroidal estrogens generally use enzyme immunoassay assay (EIA) or the enzyme-linked immonosorbent assay (ELISA) methods due to their speed and simplicity of use (Finlay-Moore et al., 2000; Shore and Shemesh, 2003; Raman et al., 2004; Jenkins et al., 2009). However, these methods tend to overestimate the concentration of specific estrogens due to the tendency to form cross-linkages binding other contaminants on estrogen receptors resulting in overestimations (+63% and +49% in two studies) of the quantities of estrogens (Holbrook et al., 2002; Farré et al., 2007). A reliable method is needed for

accurate quantification of the estrogens in animal litter and runoff. Evaluation of the fate of estrogen in agricultural fields requires a chromatographic technique with superior separation and identification capabilities to quantify the low concentrations known to cause environmental impact (Hanselman et al., 2003; Kumar et al., 2006).

Gas chromatography-mass spectrometry (GC-MS) is widely used to determine the content of steroidal estrogens in environmental samples (Kolpin et al., 2002, Cargouet et al., 2004, Peng et al., 2008). Although it has less sensitivity and selectivity than GC-MS-MS or LC-MS/MS, it is significantly easier to use and more commonly available (Young and Borch, 2009). To increase GC separation efficacy, effective extraction and derivatization steps are essential steps in sample preparation. While there are many methods capable of identifying specific hormones from complex matrices, an effective sample preparation method is necessary for increasing the GC separation from exogenous natural organic matter, e.g. of poultry litter origin. To achieve high GC resolution, a derivatization step is needed to increase the volatility and thermal stability of target compounds for GC-MS analyses (Ding and Chiang, 2003). Various reagents, such as N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and N-methyl-N-trimethylsilyl trifluoroacetamide (MSTFA) are available for the derivatization. The various reagents can derivatize organic compounds containing multiple hydroxyl groups within their molecular structure for the detection of estrogens in complex matrices (Huang and Sedlak, 2001; Kolpin et al., 2002; Ingersleve et al., 2003; Raman et al., 2004). The reagents can convert an estrogen into trimethysilyl (TMS)-estrogen. Also, a catalyst such as Trimethychlorosilane (TMCS) can improve derivatization yield (Ding et

al., 2003). However, derivatization reagents can cause incomplete silylation or other transformations to form a single product (Ding et al., 2003; Zhang and Zuo, 2005). Shareef et al. reported that TMS-EE2 is partially converted to TMS-E1 when using BSTFA as TMS reagent (Shareef et al., 2006). Several studies have employed various final solvents, such as pyridine, acetonitrile, hexane and dichloromethane, for successful determination of E1 and EE2 derivatives by GC-MS (Zue et al., 2005; Zhang and Zuo, 2005; Shareef et al., 2006; Zhang et al., 2006). Zhang and Zuo indicated that the stability and efficiency of derivatization is dependent on the reagent, catalyst, final solvent, temperature, reaction time, and time after the reaction of derivatization (Zhang and Zuuo, 2005). Ideally, all hydroxyl groups on the estrogen's molecular structure should be replaced with TMS reagents to form TMS derivatives of estrogens for better quantification by GC-MS. However, it is uncertain how to achieve the ideal efficiency of the derivatization in sample preparation using complex matrices derived from turkey litter and runoff. Therefore, the objective of this study was to determine a suitable sample preparation method of turkey litter and runoff from an amended field for detection and quantification of steroidal estrogens using GC-MS. Derivatization efficacy was evaluated with the most relevant derivatization method among selected methods. The results from this study provide an analytical basis using GC-MS as a reliable way studying fate and behavior of steroidal estrogens in agricultural fields.

MATERIALS AND METHODS

Chemicals and sample collection

Synthetic standards for steroid estrogens, isotopes, derivatization reagents, and other solvents were purchased to evaluate peer reviewed protocols. E1, E2, E3, EE2 (17α-ethynylestradiol) and diethylestradiol (DES) were purchased from Sigma Aldrich (St. Louis, MO). Deuterated estrogens (d4-E2 and d4-E1) were purchased from CDN ISOTOPES (Quebec, Canada). Methanol, acetone, hexane, and diethyl ether were obtained from Sigma Aldrich. Dimethylformamide (DMF), BSTFA, and BSTFA + 1% TMCS were purchased from Supelco (Supelco Park, PA, USA). Pyridine was purchased from MC&B (Norwood, OH, USA). Turkey litter samples were obtained from the controlled field testing facility in Riesel, Texas. The collected samples were stored at -48°C before analysis. Runoff samples were collected from the fields (USDA, Riesel, TX) within 48 hours of a runoff event, and were acidified with concentrated HCl. The samples were then stored at -20 $^{\circ}$C until extraction.

Extraction method

An extraction method was chosen for revealing the most relevant chromatogram in GC-MS analysis among previously published papers. Using diethyl-ether (liquid extraction) or accelerated solvent extraction (ASE) suggested by Raman et al. (2004) and Chun et al. (2005) respectively was not suitable for determination of estrogen in field samples due to much interferences such as NOM in the raw samples, which resulted in unsatisfactory selectivity in the generated chromatograms (data not shown). Our study used a sample extraction method suggested by Lopez de Alda et al (2002). The

extraction method consisted of ultra sonic extraction (USE) and solid phase extraction (SPE). Removal of interference (NOM) in raw samples as a sample preparation step can achieve better selectivity of GC-MS. A cleanup step was included into the extraction method by using aminopropil (NH2) cartridge. Farré et al. demonstrated that addition of a cleanup step using an aminopropil (NH2) cartridge was more reliable than use of single SPE step for removal of the interferences (Farré et al., 2006). Based on each method suggested by Lopez de Alda et al. and Farré et al., a sequential extraction was used for determination of estrogens in the agricultural samples (Lopez de Alda et al., 2002, Farré et al., 2006). This method was composed of ultrasonic solvent extraction (USE), solid-phase extraction (SPE), and SPE for cleanup. The method was used for detection of estrogens in field samples with the optimized derivatization method prior to GC-MS analysis.

The turkey litter samples were pulverized, homogenized, and then sieved through a filter (2mm mesh size). A portion of turkey litter was added to a 40ml test tube. The samples were extracted with a mixture of methanol-acetone (1:1) while being ultrasonicated for 5 minutes. Sequential extractions were performed with 25 ml, 15 ml, and 15 ml of the mixed solvent. The liquid extracts were separated by centrifugation for 5 minutes, and then combined in 60 ml glass bottles. A total extract volume of 55 ml was concentrated to dryness by rotary evaporation, and then diluted by 2 ml of a mixture of acetone and methanol (1:1) and 18 ml of distilled deionized (DDI) water. The final volume of 20 ml was prepared for SPE using HLB cartridges from Waters Inc. (Milford, MA) in a 12 station vacuum manifold. The cartridges were conditioned with 3 ml of

diethyl ether, methanol, and water, respectively. The 20 ml of the extracts was loaded on the conditioned cartridges, and the cartridges were washed with 3 ml of DDI water, 40% methanol, and 10% NH_4OH. The cartridges were eluted with 6 ml of 10% methanol in diethyl ether. The eluates were concentrated to 200 µl using a gentle stream of nitrogen. The eluates were reconstituted with 1.8 ml hexane for further sample clean-up process. For the sample cleanup and enrichment, an aminopropyl cartridge was used to remove interferences from extracts. Sep-Pak plus NH2 cartridges from Waters Inc. (Milford, MA) were conditioned with 2ml hexane, and then 2 ml of the extracts were added to the conditioned cartridge. The cartridges were washed with 4 ml of 30% ethyl acetate in hexane and eluted with 4 ml of 50% ethyl acetate in acetone. For the water sample (runoff), the runoff sample was filtered with 0.45 µm glass filter using six stations manifold with vacuum pump. The filtered runoff samples were prepared for the SPE the same as described in sample preparation of turkey litters. 6 ml of diethyl ether as eluates was generated for runoff samples. Finally the extracts were reduced to dryness under a gentle stream of nitrogen gas at 60 $^{\circ}$C. The extracts were analyzed by GC-MS after derivatization.

Derivatization protocols

Four derivatization protocols used in previously published studies were evaluated to achieve high GC resolution for detection of estrogen in the agricultural field study. The protocols are listed in Table 2.1. Protocol A was formulated by using only BSTFA and DMF in the absence of a catalyst for derivatization at 20 ^{o}C overnight. The BSTFA and DMF are a derivatizing reagent and a final solvent used commonly in derivatization

(Raman et al., 2004). Protocols B was tested by using pyridine and a catalyst (1% of TMCS) at 70 ^{o}C sand-bath during 30 minutes. Also, protocols C and D were tested by using dichloromethane (DCM) and hexane, respectively, as the final solvents. Each protocol was evaluated with respect to a peak area based on the response of chromatogram by GC-MS. To evaluate sensitivity after the derivatization reaction, variable time durations subsequent to derivatization were tested, but only with the most suitable protocol for these four protocols to avoid heterogeneity of derivatization.

Table 2.1 Four derivatization protocols (Protocol A–D); BSTFA - N, O bis(trimethylsilyl) trifluoroacetamide; TMCS-Trimethychlorosilane; DCM-Dichloromethan; DMF-Dimethylformamide; N/A-not available.

	Silylation reagent	Catalyst	Solvent	Final solvent	Temperature & time	Reference
A	10.0 µl of BSTFA	N/A	90 µl of DMF	N/A	22 ^{o}C - 12 hrs	Raman et al. (2004)
B	49.5 µl of BSTFA	0.5 µl of 1% TMCS	50 µl of pyridine	N/A	70 ^{o}C - 30 min	Zhang an Zuo (2005)
C	50 µl of BSTFA	N/A	50 µl of pyridine	100 µl DCM	60 ^{o}C - 4 hrs	Thorpe et al. (2003)
D	49.5 µl of BSTFA	0.5 µl of 1% TMCS	50 µl of pyridine `	100 µl Hexane	70 ^{o}C - 30 min	Zhang et al. (2006)

Recovery test using matrix spikes

Three matrices (DDI water, effluent and turkey litter) were used for recovery test. Estrogen isotopes, such as deuterated estrogens (d4-E1 and d4-E2) were used for recovery tests in order to reduce matrix effects for turkey litters. The percent recovery of

the sample preparation method was calculated based on an extracted concentration of estrogen in the spiked samples.

Estrogen analysis

A THERMO TRACE GC Ultra^{TM} coupled with a mass selective detector and AS 3000 series autosampler was employed for all analyses. Samples were separated on a 30 m x 0.32 mm, 0.25 µm, DB-5 fused silica capillary column. The column temperature was programmed as follows: the initial temperature was 80 ^{o}C for 4 minutes, and increasing 200 ^{o}C at a rate of 20 ^{o}C per minute, then increasing to 300 ^{o}C at a rate of 8 ^{o}C per minute and holding at that temperature for 2.5 minutes. The total run time was 30 minutes. Ultra high purity helium with an inline Alltech oxygen trap was used as carrier gas. The carrier gas was set at 40 psi, column head pressure at 8 psi, injector temperature at 280 ^{o}C, and injection volume was 1.0 µl of the splitless mode. The interface temperature was held at 280 ^{o}C. Mass spectra were scanned from m/z 50-650 at a rate of 1.5 scan per second. The Electron impact ionization energy was 70 eV.

RESULTS AND DISCUSSION

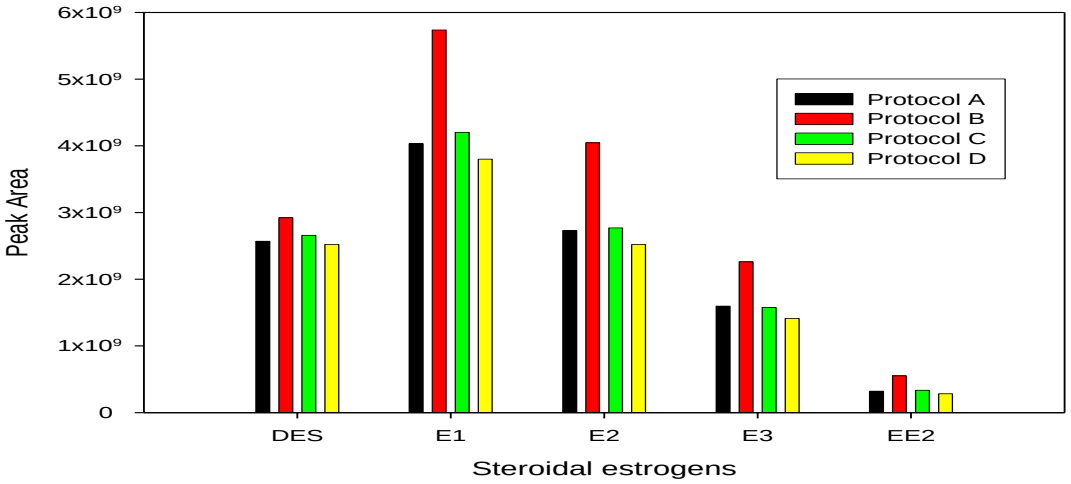


Fig. 2.1. Comparison of area abundance for the steroidal estrogens (DES, E1, E2, E3, and EE2) by four derivatization protocols published previously (Protocol A-D) in Table 2.1, spiked concentration of estrogens: 0.5 mg/L.

Derivatization is a key step in the analysis of steroidal estrogens using GC-MS. The four derivatization protocols were evaluated by comparing their chromatographic responses (peak area) of GC-MS in order to determine an optimized derivatization procedure (Table 2.1). Area abundance for the steroidal estrogens based on the four derivatization protocols was illustrated in Fig. 2.1. The results indicated that the use of pyridine as a final solvent achieved better sensitivity in derivatization by comparison of protocol B and other protocols (A, C, D). A study demonstrated that pyridine may facilitate the derivatization of steroidal estrogens with BSTFA and lead to a completely silylated product (Zhang and Zuo, 2005). It also indicates no favorable effects of other solvents (DCM and hexane) without improvement of the signal of derivatized estrogens

(Fig. 2.1.). The observation suggests that using pyridine with BSTFA+1%TMCS achieved better derivatization performance for estrogen detection in field samples.

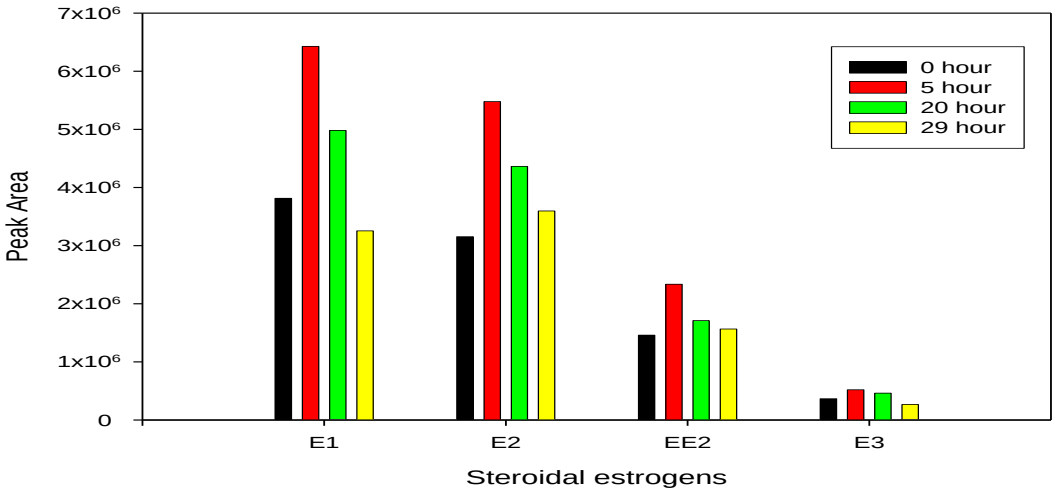


Fig. 2.2. Comparison of area abundance of steroidal estrogens for different durations (0, 5, 20, 29 hours) using protocol B at spiked concentrations of 0.5 mg/L.

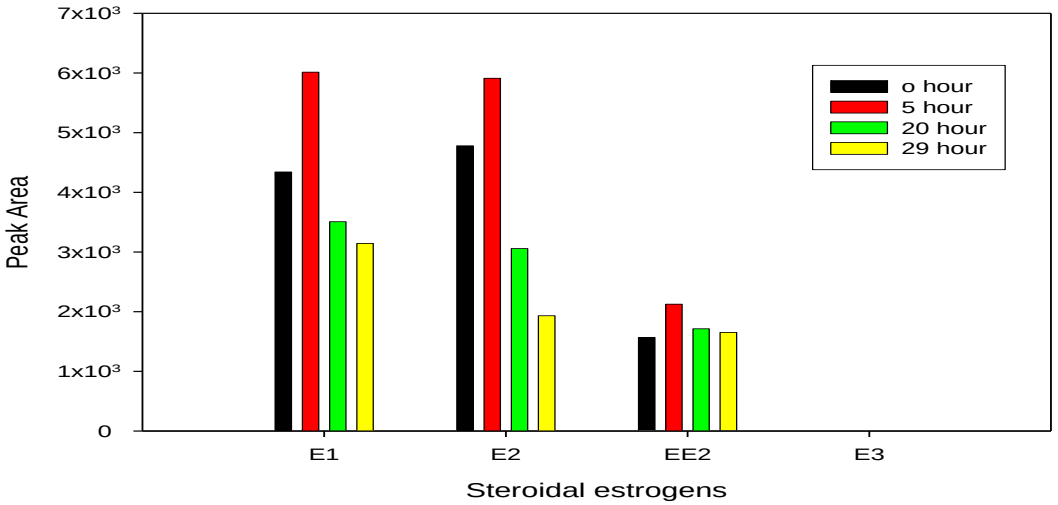
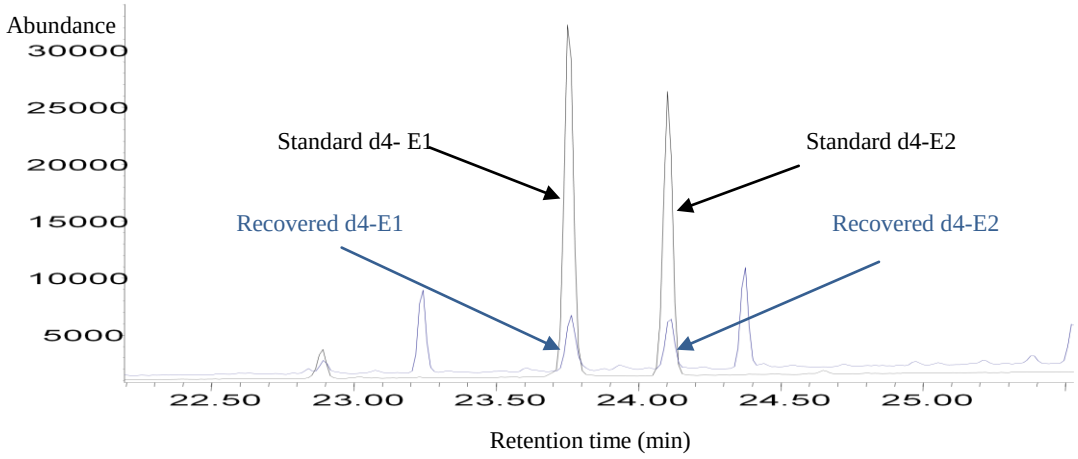


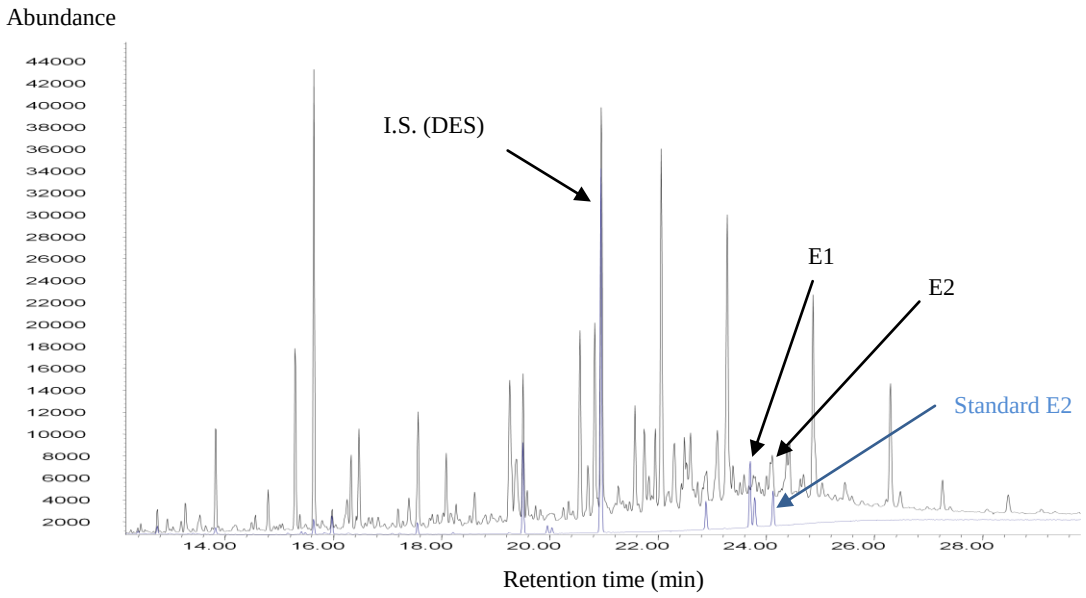
Fig. 2.3. Comparison of area abundance of steroidal estrogens for different durations (0, 5, 20, 29 hours) using protocol B at spiked concentrations of 0.1 mg/L.

The suitable reaction duration of the silylated derivatives of estrogens was determined for increasing durations for two concentrations of estrogens (0.1 and 0.5 mg L^{-1}). The area abundance of steroidal estrogens for different durations using the most suitable protocol (protocol B) was illustrated in Figs. 2.2.-2.3. It was observed that longer duration (more than 20 hours) became less satisfactory in derivatization of estrogens. Especially, it was interesting to observe that E1 and E2 were comparatively less stable than EE2 and E3 over the durations after derivatization. Using the area abundance as an indication of the quantity of the estrogenic steroids extracted, the 5 hour derivatization period resulted in higher areas abundance for all of the estrogenic steroids at both concentrations than shorter or longer duration after derivatization. Consequently, our study indicates that 5 hour duration after derivatization by protocol B achieved better derivatization efficacy when determining steroidal estrogens.



(a) Recovery test

Fig. 2.4. TIC chromatogram (selected ion monitoring) of steroidal estrogens; Recovery test by spiking isotopes (d4-E1, d4-E2) in turkey litter (a) – Blue: spiked sample, Black: standard sample, and turkey litter (b) – Blue: standard sample, Black: turkey litters, I.S.: internal standard.



(b) Turkey litter

Fig. 2.4. Continued.

Three distinct peaks generated in the total ion current (TIC) chromatogram were identified as DES, E2, and E1 in turkey litter (Fig. 2.4.). TIC chromatograms of a recovery test were illustrated in Fig. 2.4a. For the recovery test, deuterated estrogens (d4-E2 and d4-E1) were used as isotope. The standard chromatograms were well overlapped with recovered chromatograms at the same retention time in Fig. 2.4a. The TIC chromatogram yielded mass spectra of target estrogens using selected ion mode (SIM). The mass spectra were 346 m/z and 220 m/z for d4-E1, 320 m/z and 289 m/z for d4-E2 in the recovery test. TIC chromatogram of estrogen in turkey litter was exhibited in Fig. 2.3b. The two peaks were identified in the turkey litter as E1 and E2 appeared at 23.82 minutes and 24.23 minutes of retention time respectively (Fig. 2.4b.). The mass

spectra of target estrogens in turkey litter using selected ion mode (SIM) were 416 m/z and 285 m/z for E2, and 342 m/z and 257 m/z for E1 and 412 m/z and 397 m/z for DES as an internal standard.

Using the proposed extraction method and the optimized derivatization methodology, the method detection limit (MDL) was determined to be 5 µg kg^{-1} for E2, 10 µg kg^{-1} for E1, 20 µg kg^{-1} for EE2, and 50 µg kg^{-1} for E3 for solid matrix. The MDL for a liquid matrix was 5 ng L^{-1} of E2, 10 ng L^{-1} of E1, 50 ng L^{-1} of EE2, and 100 ng L^{-1} of E3. A percent recovery test of spiked estrogens on solid and liquid matrices was conducted by spiking deuterated estrogens (d4-E1 and d4-E2) into DDI water, effluent from WWTP, and turkey litter. The recovery of each estrogen was 115% of E2 and 106% of E1, 94% of EE2, and 92% of E3 for a liquid matrix using DDI water (Table 2.2). For the effluent, the recovery was 85% of E1, 59% of E2, 74% of EE2 and 84% of E3 (Table 2.2). The difference of recovery between DDI water and effluent is likely due to the presence of dissolved organic matter in the effluent. These interferences mask the selectivity and sensitivity in GC-MS analysis. In a solid matrix, the recovery was 48.9 ± 6.8% of E2 and 45.1 ± 12.2% of E1 in turkey litter. Relatively low recovery for solid matrix may be attributed from use of another SPE (NH2 cartridge) as a cleanup step. Farré et al. (2007) indicated that use of sequential extraction (with a cleanup step) was more reliable than use of single extraction (without a cleanup step) in estrogen analysis. Though, a percentage recovery using the sequential extraction was reduced by approximately 20%, comparing to use of single extraction (Farré et al. 2007). Further

testing of the sequential extraction is required to enhance recovery of estrogen in a solid matrix such as animal litter.

Table 2.2 The percent recovery of spiked estrogen and estrogen concentration in field samples (Turkey litter and runoff), DDI (Distilled deionized): n=3, Effluent after UV from WWTPs at College Station: n=2, turkey litter: n=2, N/D: not detected; N/A: not available.

Chemicals	Recovery (%)			Field samples	
	DDI water	Effluent	Turkey litter	Runoff ($ng\ L^{-1}$)	Turkey litter ($\mu g\ kg^{-1}$)
E1	129 - 135	75.3 - 87.6	36.4 - 53.7	2182	194.4
E2	115 - 135	44.5 - 69.1	44.1 - 53.7	481	249.9
EE2	96 - 113	54.7- 93.5	N/A	N/D	N/D
E3	78 - 134	80.2 - 87.1	N/A	N/D	N/D

Estrogen content of the field samples analyzed by the optimized method compared favorably with other studies published previously for detection of estrogens in poultry litter (Nichols et al., 1997; Nichols et al.; 1998; Finlay-Moore et al., 2000). In these studies, E2 content ranged from 33 to 904 $\mu g\ kg^{-1}$ in poultry litter, with our study showing that its content was 249.9 $\mu g\ kg^{-1}$ of E2, 194.4 $\mu g\ kg^{-1}$ of E1 (no detection of E3 and EE2) in turkey litter (Table 2.2). In a runoff matrix, Finlay-Moore et al. reported that the concentration of E2 ranged from 20 to 2530 $ng\ L^{-1}$ in runoff generated from broil litter amended fields at first storm by an immunoassay method (Finlay-Moore et

al., 2000). Using our optimized method, the concentrations of estrogen were 481 ng L^{-1} for E2 and 2182 ng L^{-1} for E1 in the runoff generated from fields amended with application quantities of 13.6 mega gram (10^6 gram) turkey litters per hectare in a pasture field (Table 2.2). The higher concentration of E1 in runoff may be a result of biodegradation by various bacteria activity in turkey litter after its field application. The transformation was demonstrated in a laboratory test using turkey litter enrichment culture (TLEC) in our other study. Finally, the sample preparation method, including the optimized derivation followed by GC-MS, made it possible to determine estrogen content in agricultural samples, providing a reliable quantification of estrogen for the fate of estrogen in turkey litter amended field.

CONCLUSION

A method appropriate for sample preparation for the study of occurrence and mobility of steroidal estrogens derived from turkey litter amended fields was developed. The method using USE, two SPEs, and optimized derivatization based on published papers proved to be relevant in determination of estrogens in field samples. The derivatization protocol using BSTFA+1% TMCS and only pyridine as a final solvent, and 5 hour duration after its reaction (30 minutes at 70 ^{o}C sand-bath) achieved better efficacy for the analysis of estrogens with an average recovery of 90% in the liquid matrix and approximately 50% in the solid matrix. Moreover, this method was successfully applied to the analysis of estrogenic hormones in runoff and turkey litter from turkey litter amended fields using GC-MS. However, an even more robust and

reliable sequential method in sample preparation is needed for the quantification of estrogens in the turkey litter with higher recovery.

CHAPTER III

DEGRADATION KINETICS OF STEROIDAL ESTROGEN VIA COMETABOLIC REACTIONS

OVERVIEW

Steroidal estrogens are environmentally significant because of their high estrogenic potency, toxicity, and persistence. Microbial degradation is considered to be a major mechanism of estrogen removal from the environment. Biodegradation kinetics of individual parent estrogen compounds by pure and mixed cultures are available, but not for the cometabolic reaction of estrogen as a non-growth substrate. Biodegradation kinetics of estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), estriol (E3) and 17α-estradiol (17α-E2) were evaluated in the cometabolic system using *N. europaea*. Four models to represent the cometabolism of estrogen were proposed. Normalized residual sum of square (NRSS) analysis revealed that the reductant model is the most appropriate method to yield biokinetic parameters, (k_{NH3}, $k_{s,NH3}$, $k_{estrogen}$) from individual estrogen experiments. The model assumes that estrogen degradation requires both a limited reductant generated from ammonia oxidation and no competition with ammonia or its oxidative product (NO_3^- or NO_2^-). The model predicted cometabolic degradation of estrogens in high ammonia concentrations. The estimated estrogen degradation rate constants were comparable to some previously reported values. Ninety-five percent confidence limits were applied for individual estrogen rate constants to account for culture variability. The ratio difference between two rate constants ($k_{estrogen}/k_{NH3}$) resulted

from differing cometabolic activity and enzyme activity for each estrogen. For EE2, the higher ratio that EE2 is degraded faster cometabolically than other estrogens. The kinetic coefficients determined by estrogen cometabolism help explain the limited removal of estrogen by ammonia oxidation bacteria (AOB) cometabolism of the engineered and agricultural systems.

INTRODUCTION

The presence of hormones in the environment can potentially have an adverse effect on humans and ecosystem even at surprisingly low concentrations (ng/L). Steroidal estrogens are of are more potency, compared to other endocrine disrupting chemicals (EDCs) (Hanselman et al., 2003). Removal of the steroidal estrogens in WWTPs is strongly associated with the sorption and biodegradation in the activated sludge systems. Over the past 10 years, considerable research has been performed on the fate of estrogen in wastewater treatment plants (WWTPs) (Ternes et al., 1999; Johnson and Sumpter, 2001; Anderson et al., 2003). Reported results suggest that biodegradation is the primary mechanism of estrogen removal in WWTP activated sludge systems (Anderson et al., 2005).

Estrogen biodegradation has been studied extensively with various microbial cultures, including activated sludge, enrichment cultures, and pure cultures (Vader et al., 2000; Shi et al., 2004; Yoshimoto et al., 2004; Yu et al., 2007). Yoshimoto et al. isolated four strains capable of heterotrophically biodegrading 17β-estradiol (E2), estrone (E1), estriol (E3), and 17α-ethynylestradiol (EE2), using these estrogens as the sole carbon

and energy source. In their study, *Rhodococcus zopfii* and *Rhodococcus equi* were isolated from activated sludge at WWTPs, with a degradation efficiency of 70 - 96% in 6 hours (Yoshimoto et al., 2004). Vader et al. (2000) report that biodegradation of 17α-ethynylestradiol (EE2) was performed by nitrifying activated sludge cometabolically. The degradation of four estrogens (E1, E2, E3, and EE2) with a nitrifier, *N. europaea* was reported by Shi et al (2004). In 2007, Yi and Harper showed that the nitrification rate is significantly (R^2=0.9432) related to EE2 biotransformation. The recent study by de Gusseme suggests that AOB contributes to EE2 biodegradation in WWTPs (de Gusseme et al., 2008). Low biodegradation rates of AOB bacterial activity may be explained by reductant depletion, competition with key enzymes, or formation of toxic intermediates in the cometabolic reaction (Aziz et al., 1999; Alvarez-Cohen et al., 2000).

N. europaea is the most commonly reported organism used to evaluate the cometabolism of hormones. Shi et al. (2004) demonstrated that *N. europaea* can cometabolize the steroid estrogens at rate ranging from 0.0384 mg/L day to 0.0528 mg/L day ssuming pseudo-first order kinetics. The cometabolism of estrogens by *N. europaea* is proposed to be in two steps. Generally, the nitrifying bacteria transform ammonia (NH_3) or ammonium (NH_4^+) to nitrate (NO_3^-). AOB plays a key role in the oxidation of NH_3 to NO_2^- as the first step of estrogen degradation by cometabolism. Ammonia is initially oxidized to hydroxylamine by ammonia monooxygenase (AMO) from *N. europaea.* In the second step, the hydroxylamine is further oxidized to NO_2^- by hydroxylamine oxidoreductase (HAO) generating four electrons. The estrogens are biodegraded through the cometabolic reaction with the electrons (as a reductant) and

AMO (as an enzyme). The kinetic models for estrogen cometabolism were evaluated based on the assumptions that estrogens might be cometabolized by two limiting reactants without any competition between them (Wahman et al. 2006). Also, this kinetic study revealed the underlying mechanism of the estrogen biodegradation by cometabolism. The objective of our study is to determine the biodegradability of estrogen using a represented model based on fitting of experimental results.

MATERIALS AND METHODS

Chemicals and chemical analysis

Steroidal estrogens E1, E2, E3, EE2, 17α-E2, and diethylstilbestrol (DES) with high purity (+95%) (Sigma Chemicals Co., St Louis, MO) were used. BSTFA+1% TMCS (Supelco, Supelco Park, PA) and pyridine (MC&B, Norwood, OH) were used for derivatization. The protein assay was performed with the kits with Bovine Serum Albumin (BSA) standard (Bio-Rad, Hercules, CA). For ammonia analysis, High Range Test ‘N Tube Nitrogen-Ammonia reagents set for 0.4 to 50 mg/l NH_3-N were used (HACH CO., Loveland, CO).

A HP 5890 Series II gas chromatographic-mass spectrometry (GC-MS) coupled with a HP mass selective detector was used to quantify the steroidal estrogens. Samples were separated on a 30 m x 0.32 mm, 0.25 µm, DB-5 fused silica capillary column. The column temperature was programmed as follows: the initial temperature was 80 ^{o}C for 4 min and increase to 200 ^{o}C at 20 ^{o}C/min, and then it was increased to 300 ^{o}C at 8 ^{o}C/min and held for 2.5 min. The total run time was 30 min. Ultra high purity helium with an

inline Alltech oxygen trap was used as carrier gas. The carrier gas was set at 40 psi, column head pressure at 8 psi, injector temperature was maintained at 280 ^{o}C, and the injection volume was 1.0 µl in the splitless mode. The interface temperature was held at 280 ^{o}C. Mass spectra were scanned from m/z 50-650 at a rate of 1.5 scan/sec. The electron impact ionization energy was 70 eV.

Cell growth

A pure culture of *N. europaea* (ATCC 19718) was obtained from American Type Culture Collection (ATCC). The culture was grown in 2-L Erlenmeyer flasks with aluminum foils on 1-L of media including 50 mM $(NH_4)_2SO_4$, 43mM KH_2PO_4, 0.73 mM $MgSO_4$, 0.2 mM $CaCl_2$, 0.01 mM $FeSO_4$, 0.017 mM EDTA, 0.007 mM $CuSO_4$, 4.4 mM NaH_2PO_4, and 0.04% (wt/vol) Na_2CO_3. Approximately 10% of the culture was used as an inoculum for cell transfer every 10 days. The flasks were placed on rotary shakers in a dark room at 30 ^{o}C. The suspension in the flasks should be turbid in 3 or 5 days after cell transfer and before the kinetic experiment.

Batch kinetic assay

For kinetic experiments, pre-grown *N. europaea* organisms were harvested from Erlenmeyer flasks by centrifugation, washing, centrifugation, and resuspension in fresh buffer medium (8 mM phosphate and 10 mM carbonate, pH 8). The initial concentration of substrate was adjusted with 100 ml of growth medium after evaporation of 250 µl of 2 g/L stock solution in 300-ml amber bottles to obtain approximately 0.5 mg/L of each estrogen. The required volume of the suspension of concentrated cells was added to each reactor to obtain an OD_{600} of 0.4. The biomass concentration was quantified at the

beginning of each test by a protein assay using BSA as a standard. 5-ml samples from each reactor were added to 20-ml amber vials containing 5 ml solvent (diethyl ether) for extraction at predetermined sampling times. The amber vials were placed on a rotary shaker for overnight to allow complete transfer of the estrogens into the diethyl ether phase. The extracts were derivatized by using BSTFA + 1% TMCS and pyridine. The derivatized extracts were analyzed using GC-MS. For ammonia-nitrogen analysis, 1.0 ml samples from each reactor were filtered with 0.2 μm membrane filters to 10-ml centrifuge tube, and then were measured with HACH High Range nitrogen-ammonia reagent kits and an UV-visible spectrophotometer (Agilent 8453) according to HACH colorimetric method 10023 (Salicylate method). pH was measured using an pH electrode (HACH SENSION) for each sampling at predetermined time.

Kinetic modeling approach

The kinetic model for microbial degradation of ammonia was developed based on the Monod equation. The model (eq. 3.1) assumes that the estrogens do not compete with ammonia.

$$-\frac{dC_{TOTNH3}}{dt} = \frac{k_{TOTNH3} C_{TOTNH3} \alpha_1}{K_{S_{NH3-N}} + C_{TOTNH3} \alpha_1} X \qquad (3.1)$$

where,

C_{TOTNH3} is the concentration of total ammonia in the liquid phase (mol/L),

k_{TOTNH3} is the ammonia maximum specific rate of degradation,

$C_{estrogen}$ is estrogen concentration,

$k_{estrogen}$ is a pseudo first-rate constant of estrogen,

$K_{S_{NH3-N}}$ is the ammonia half saturation constant,

X is the biomass concentration (mg-BSA/L), and

α_1 is the NH_3-N fraction to $TOTNH_3$.

Using estrogen degradation models based on a first-order kinetic, substrate competition, reduction, and the combination of competition-reduction, four forms of the model resulted and is expressed in equations 3.2–3.5. Model 1(Equation 3.2) assumes first-order kinetics, that estrogens and ammonia do not compete with each other, and estrogen is the limiting reactant.

$$-\frac{dC_{estrogen}}{dt} = k_{estrogen}C_{estrogen}X \tag{3.2}$$

For estrogen degradation based on substrate competition kinetics, ammonia must compete with estrogen and estrogen is the limiting reactant (Equation 3.3).

$$-\frac{dC_{estrogen}}{dt} = \frac{k_{estrogen}C_{estrogen}}{1+\frac{C_{TOTNH_3}\alpha_1}{K_{S_{NH3-N}}}}\ X \tag{3.3}$$

Assuming that ammonia does not compete with estrogen and there are two limiting reactants (estrogen and reductant), a reductant model results (Equation 3.4)

$$-\frac{dC_{estrogen}}{dt} = \frac{k_{estrogen}C_{estrogen}}{1+\frac{K_{S_{NH3-N}}}{C_{TOTNH_3}\alpha_1}}\ X \tag{3.4}$$

Combining the competition and the reductant models and assuming ammonia competes with estrogen, two limiting reactants (estrogen and reductant) are represented in the model (Equation 3.5).

$$-\frac{dC_{estrogen}}{dt} = \frac{k_{estrogen}C_{estrogen}}{\left(1+\frac{C_{TOTNH_3}\alpha_1}{K_{S_{NH3-N}}}\right)\left(\frac{K_{S_{NH3-N}}}{C_{TOTNH_3}\alpha_1}+1\right)}X \tag{3.5}$$

The four models were evaluated by comparing the sum of square error (SSE) values to determine kinetic coefficients for the best-fit simulation among them. A small SSE value indicates a tight fit between the model and the data.

$$SSE = \sum_{i=1}^{n} (C_i^{obs} - C_i^{pred})^2 \quad (3.6)$$

Estimation of kinetic coefficients

If the initial substrate concentrations are higher than substrate affinity constant (K_s) or if the ratio of initial substrate concentration to biomass (S_0/X_0) as an extant condition is low in the experiment, the biokinetic coefficients can be measured independently (Ellies et al., 1996, Grady et al., 1996). The use of nonlinear regression techniques is widely accepted (Smith et al., 1996) as an accurate method of analysis (Leatherbarrow, 1990). For the kinetic coefficients of ammonia degradation as a first step, a kinetic model was developed where ammonia (NH_3) is the only species that binds to the active site of AMO (Suzuki et al., 1974). Ammonia concentrations in the models were calculated by using both the results of the ionization fraction and experimentally measured totals of ammonia ($TOTNH_3$-N), including ammonia (NH_3-N) and ammonium (NH_4^+-N). The experimental data obtained from the each set of experiments was used to estimate the Monod parameters by fitting the Monod-typed saturation kinetic equation (Eq. 3.1). An alternative means of estimating the Monod parameters is by solving the integrated form of the Monod equation in computer code or simple spreadsheet (Smith et al., 1996, 1998). The numerical integration of the differential equations has been commonly achieved through a fourth-order Runge-Kutta numerical approximation (Eq. 3.2). The approximated value was fit to the experimental data by minimizing the sum of

square errors (SSE) between the model predicted values and the experimental values. This value was based on an iterative search by the Solver spreadsheet function (Microsoft Excel 2007). To estimate the kinetic coefficients for estrogens degradation as a second step, the four aforementioned kinetic models were employed based on their major assumption.

The assumptions adopted in this study were based on a proposed model for cometabolic degradation of various halogenated compounds using *N. europaea* by several researchers (Smith et al., 1996, Aziz et al., 1999, Wahman et al., 2006). The models were evaluated by fitting them to data on estrogen depletion. Ammonia half saturation constants ($k_{s,NH3}$), estimated from the biokinetic experiments of ammonia degradation, were used for determination of the estrogens degradation rate constants ($k_{estrogen}$). Finally, the estrogen rate constants and the initial concentrations of each estrogen were determined using non-linear regression. The kinetic models used in this study and their major assumptions used are provided in a previous section. The nonlinear regression analysis yielded estimates of the estrogen rate constant ($k_{estrogen}$), the ammonia maximum specific rate of degradation (k_{TOTNH3}), and the ammonia half-saturation constant ($k_{s,NH3}$), as well as the initial concentration (C_0) for ammonia and each estrogen. The uncertainty in the fitting parameters was determined by a method suggested by Smith et al. (1998).

RESULTS AND DISCUSSION

The time dependent substrate depletion data was generated for individual

estrogens of E1, E2, E3, EE2, 17α-E2, and corresponding total ammonia nitrogen for each estrogen (Figs. 3.1-3.5). Each experiment was repeated two times and the duplicated data was treated as an independent data set. The kinetic models were fitted to the experimental data measured from the biokinetic experiment to generate biokinetic parameters such as the estrogen rate constant ($k_{estrogen}$), the maximum specific degradation rate (k_{TOTNH3}), and the half saturation constant ($k_{s,NH3}$), as well as, the initial concentration (C_0) of ammonia and individual estrogen. The kinetic models were generated with the replicated experimental data (Figs 3.1-3.5). The 95% confidential intervals of the model determined with the method as described by Smith et al. (1998) are illustrated in Fig. 3.6. Each estrogen was evaluated using normalized residual sum of square (NRSS) values that were approximated by non-linear regression (Table 3.1).

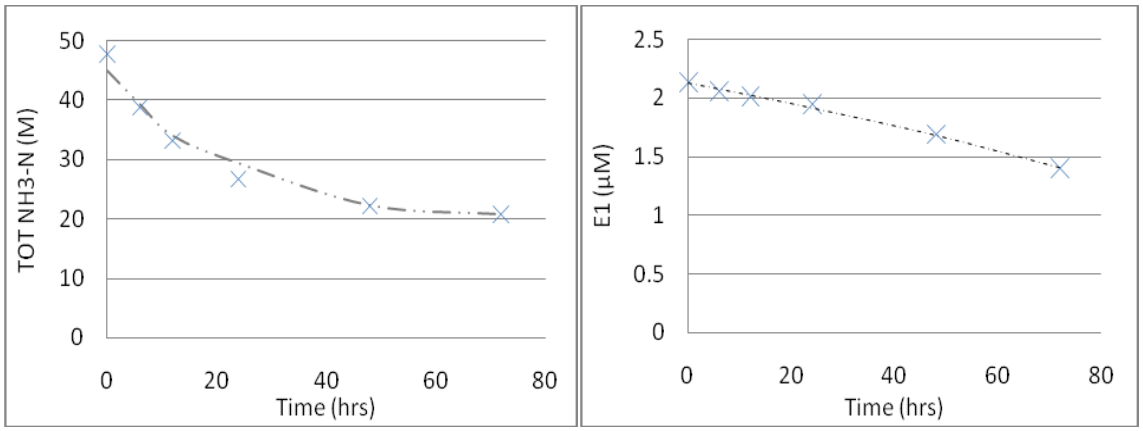


Fig. 3.1. Kinetics of ammonia (saturation model) & estrone (E1) degradation; Open symbols denote experimental observations and dashed lines represented the reductant model.

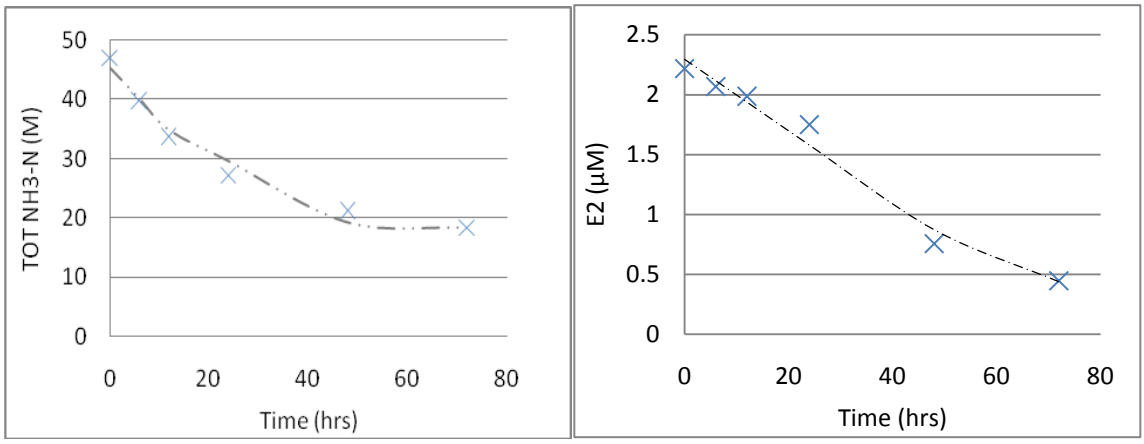


Fig. 3.2. Kinetics of ammonia (saturation model) & 17β-estradiol (E2) degradation; Open symbols denote experimental observations and dashed lines represented the reductant model.

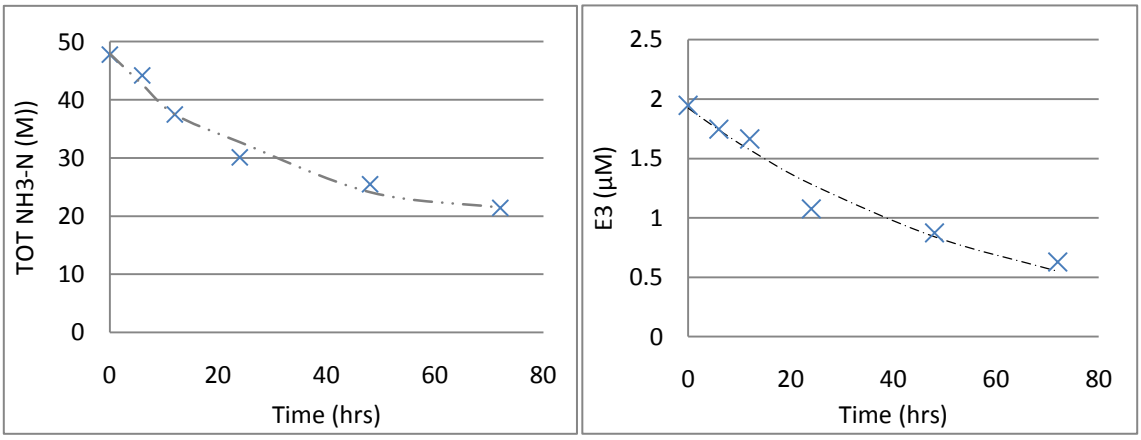


Fig. 3.3. Kinetics of ammonia (saturation model) & estriol (E3) degradation; Open symbols denote experimental observations and dashed lines represented the reductant model.

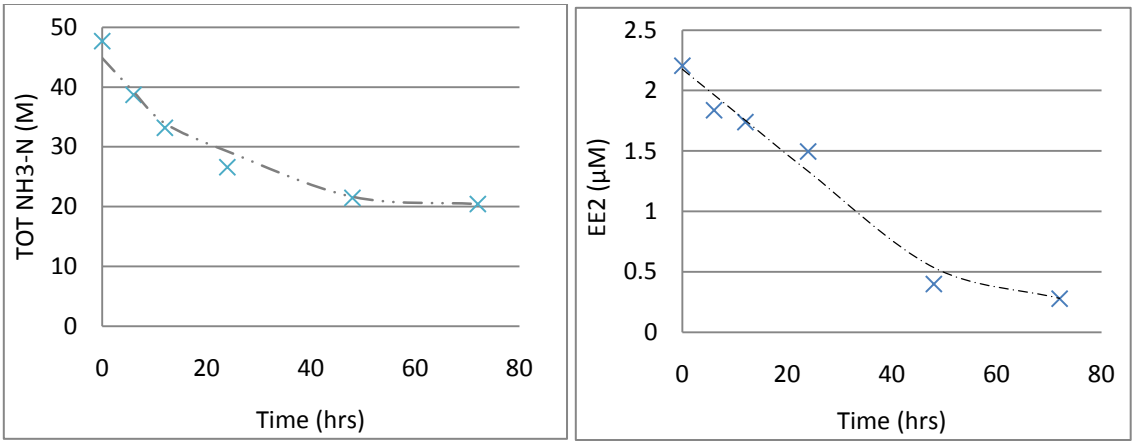


Fig. 3.4. Kinetics of ammonia (saturation model) & 17α-ethynylestradiol (EE2) degradation; Open symbols denote experimental observations and dashed lines represented the reductant model.

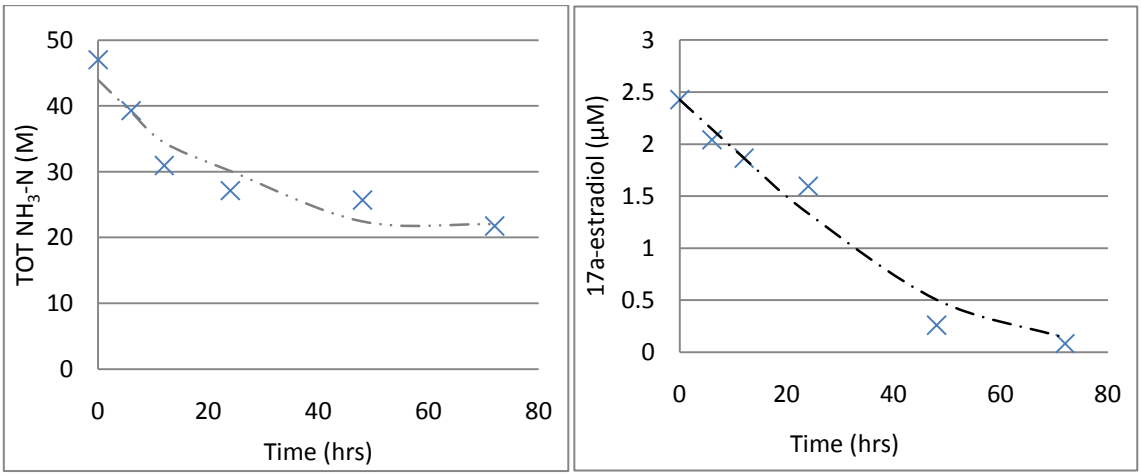


Fig. 3.5. Kinetics of ammonia (saturation model) & 17α-estradiol (17α-E2) degradation; Open symbols denote experimental observations and dashed lines represented the reductant model.

Table 3.1 NRSS (Normalized residual sum of squares) of five estrogens, bold numbers indicate the best fit model with an observed value.

Models	NRSS (10^{-3})				
	E1	E2	E3	EE2	17α-E2
1st order	0.48	19.7	1.08	30.4	28.1
Competition	0.42	17.6	2.53	24	24.9
Reductant	**0.38**	**13.5**	**0.45**	**13**	**3**
Combined	0.39	17.9	1.65	35	23.9

The NRSS approach revealed that all estrogens were best fit with the reductant model as determined by the lowest values (Table 3.1)., indicating that this model is the most relevant to represent the estrogen cometabolism in batch experiments. The fit of the ammonia and estrogen concentrations generated by the reductant model of E1, E2, E3, EE2, and 17α-E2 are illustrated in Fig. 3.1. through 3.5. The ammonia biodegradation of each experiment was limited at approximately 20 M of ammonia concentration because other toxic intermediates (NO_3^--N or NO_2^--N) would be generated (Figs. 3.1- 3.5). The degradation of some estrogens (E3, EE2, 17α-E2) was limited from 42 hours because reducing power was not enough from 42 hours or the toxic intermediates were generated (Figs. 3.1-3.5). E1 was least degraded among other estrogens. The estrogen kinetic coefficients ($k_{estrogen}$) estimated using the reductant model generated values higher than typically reported for nitrifiers. The Monod half saturation coefficient ($K_{S_{NH3-N}}$) for ammonia degradation ranged from 17 - 41 mg L^{-1} NH_3-N (Table 3.2). The estrogen rate constants ($k_{estrogen}$) ranged from $3.2x10^{-3}$ to $7.7x10^{-3}$ (L/mg BSA-day) which is a fairly small range of values. The narrow range of values

reported by Shi et al. (2004) (3.8 - $5.2x10^{-2}$ mg/L-day) are approximately 10 times. This difference is attributed to the ammonia rate constants in the reductant model because their study assumed a zero-order reaction model. The reductant model provided better prediction of estrogen rate constants for cometabolic kinetic study.

Table 3.2 Kinetic coefficients of estrogens estimated by a reductant model and the kinetic coefficient ratio (Estrogen/Total ammonia)

Chemicals	X	k_{TOTNH3}	$k_{estrogen}$	$k_{estrogen}/k_{TOTNH3}$
	(mg BSA/L)	(mg $TOTNH_3$/mg BSA-day)	(L/mg BSA-day)	(L/mg $TOTNH_3$)
E1	68.80 ±7.63	11.53±0.45	3.17E-3±0.003	2.75E-04
E2	48.50±11.80	13.21±1.09	7.71E-3±0.005	5.83E-04
EE2	89.20±15.98	7.34±0.24	6.77E-3±0.0001	9.22E-04
E3	52.90±10.47	15.88±0.57	4.13E-3±0.004	2.60E-04
17α-E2	69.90 ±1.63	9.82±0.87	5.18E-3±0.002	5.28E-04

The estrogen degradation rate ($k_{estrogen}$) varied in proportion to the ammonia degradation rate (k_{TOTNH3}) (Table 3.2). Taking the ratio of estrogen to ammonia rate constants, the cometabolic level or enzyme activity of each estrogen can be evaluated. The ratio of k_{EE2}/k_{TOTNH3} was highest among the estrogens, indicating that EE2 degradation is favored by cometabolism. AOB has been reported to be effective for EE2 removal in WWTPs, suggesting that EE2/NH_4^+ cometabolism under high nitrification activity could be mediated by AMO (Ren et al., 2007). Estrogen degradation was found to be preferred by some microorganisms in activated sludge (AOB or Heterotrophs), implying that EE2 degradation can be favored by AOB while E3 degradation is favored by heterotrophic bacteria (Ren et al., 2009). These authors proposed that nitrifiers

initially degrade EE2 to other metabolites that are subsequently removed by heterotrophic bacteria which supports the theory that removal of EE2 is due to cometabolism than that of other estrogens.

Fitting of the kinetic models examined to the experimental data resulted in different degradation rate constants due to the cometabolic assumptions. That the constants are different suggests that the mechanisms of estrogen degradation in the presence of high ammonia concentrations emphasize the importance of AOB in estrogen removal in WWTPs. The degradation of all estrogens (E1, E2, EE2, E3 and 17α-E2) were governed by both a reductant (electrons) and no competition by either ammonia or its oxidative metabolites (NO_2^- or NO_3^-). The availability of reducing agents from the oxidation of ammonia (a growth substrate) limits the rate and extent of cometabolic degradation of estrogen (a non-growth substrate). However, whether ammonia or its oxidative metabolites compete with each estrogen as non-growth substrates is not clear. The model-based diagnostics were an aid to understanding the behavior of estrogen cometabolism in the presence of high ammonia concentration.

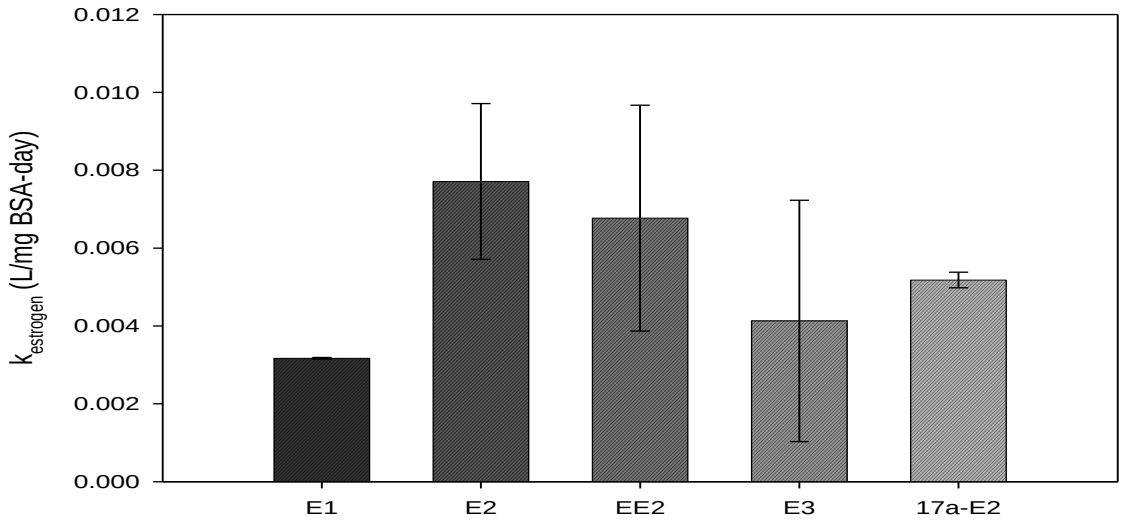


Fig. 3.6. Estrogen kinetics 95% confidential limits for batch kinetic experiments with *N. europaea.*

Estimation of 95% confidential limit for the estrogen rate constant was determined by the method described by Smith et al. (1998). The kinetic parameters fit to the reductant model of estrogen were the initial estrogen concentration (C_0) and the rate constant ($k_{estrogen}$). The 95% confidential limit for two kinetic parameters is illustrated as the value of $k_{estrogen}$ plus two standard deviations (2σ) and the value minus two standard deviations (Fig. 3.6.).

Table 3.3 Initial estrogen removal performance prediction based on *N. europaea* kinetic parameter under various sources, WW: Wastewater.

Sources	Δ $TOTNH_3$ (mg N/L)	Estrogen Percent Removal (%)				
		E1	E2	EE2	E3	17α-E2
Drinking Water	4	0.1	0.2	0.4	0.1	0.2
Municipal Wastewater	30	0.8	1.7	2.7	0.8	1.6
Animal Wastewater	300	7.9	16.0	24.1	7.5	14.7
High con. Wastewater	900	21.9	40.8	56.3	20.9	37.9

The ratio of $k_{estrogen}/k_{TOTNH3}$ can be used as a first approximation to the expected performance of the various biofilter processes for various sources such as drinking water, municipal wastewater and animal wastewater (Equation 3.7) (Wahman et al., 2005 and 2006). The normalized estrogen effluent concentration ($\frac{C_{estrogen}(t)}{C_{estrogen}(0)}$), or estrogen fractional removal, is independent of the influent estrogen and $TOTNH_3$ concentration (Equation 3.7). Also for a given $TOTNH_3$ removal, the ratio is dependent on the estrogen rate constant ($k_{estrogen}$).

$$\frac{C_{estrogen}(t)}{C_{estrogen}(0)} = e^{-\Delta TOTNH_3 \frac{k_{estrogen}}{k_{TOTNH3}}} \quad (3.7)$$

Equation 3.7 can be used to approximate the maximum expected removal of each estrogen as a function of total-ammonia removal ($\Delta TOTNH_3$). ΔTOTNH3 is a function of time. Using average values of the kinetic coefficients from Table 3.3, initial performance predictions are made with likely maximum TOTNH3 removal for various sources. The results of this analysis are summarized in Table 3.3 with removals ranging from 0.1-56.3%, depending on water sources. Drinking water containing the low ammonia concentration is less effective for estrogen removal by cometabolism.

However, the estrogen removal performance in real facilities seems to be increased due to kinetics high enough for in-situ application by the mixed culture of nitrifiers. Animal wastewater containing the high ammonia concentration (approximately 300 - 2500 mg N/L for swine wastewater) is expected to effectively treat estrogens.

CONCLUSION

Kinetic coefficients of estrogen degradation were successfully determined using *N. europaea* to generate data for a reductant model. The implications are that estrogen cometabolism is, under the experimental conditions of this study, governed by a mechanism that requires both a limited reductant produced from ammonia oxidation and no competition with ammonia or its oxidative intermediates. Further, the removal of estrogen increased at greater ammonia concentrations because the rate of estrogen degradation depends on both the concentration of the estrogen and ammonia. The reductant model also predicted no estrogen degradation in the absence of ammonia. The kinetic coefficients of estrogen cometabolism normalized by kinetic coefficients of ammonia degradation suggest a further approach for an engineered application. The kinetic experiments imply that AOB likely grown in various water treatment facilities and agricultural fields could play a role to degrade estrogen to some extent by themselves depending on ammonia removal. The results suggest that the ratio of $k_{estrogen}/k_{NH3}$ should be considered in designing effective bioremedial strategies. The role of nitrifiers and predicting the biodegradability of individual estrogen through cometabolism are some of the key results from this study.

CHAPTER IV

THE EFFECT OF MICROBIAL ACTIVITY ON STEROIDAL ESTROGEN REMOVAL USING TURKEY LITTER ENRICHMENT CULTURE (TLEC)

OVERVIEW

The effect of aerobic microbial activity on steroidal estrogen removal was evaluated for a turkey litter enrichment culture (TLEC). Favorable bacteria in the TLEC for the biodegradation of steroidal estrogens in aerobic batch test were identified using a substrate utilization test (SUT). Based on materials balance calculations of the amount of naturally occurring hormone applied via litter amendment of fields and measured runoff calculations, it was indicated that indigenous microorganisms have the potential to degrade the steroidal estrogens found on agricultural fields amended with turkey litter. The aerobic degradation of estrogens followed pseudo-first order kinetics. The rate constants for estrogen biodegradation were calculated: 0.050 hr^{-1} for E1, 0.031 hr^{-1} for E2, 0.027 hr^{-1} for E3, and 0.012 hr^{-1} for EE2. The results indicate that the synthetic estrogen, EE2, is more resistant to aerobic biodegradation than the natural estrogens by the TLEC using nitrogen and carbon substrates. Moreover, SUT data indicate that the biodegradation of E1 and E2 occurs through co-metabolism by ammonia oxidizing bacteria (AOB). By contrast, the biodegradation of E3 and EE2 by TLEC was largely due to the heterotrophic bacteria in the consortia. The results for only the natural estrogens (E1, E2, and E3) are in agreement with previous results for municipal operations. This study indicates that the TLEC is composed of estrogen degraders that

can impact the fate of estrogen in turkey litter amended fields. The microbial activity of the turkey litter biota for estrogen degradation is primarily attributed to the composition of the microbial population. The evidence suggests that the performance of indigenous microorganisms can be encouraged by managing environmental factors (nutrients and organic carbon) or a composting process prior to application may be effective in reducing the concentrations of steroidal estrogens in turkey litter and thereby eliminating the environmental consequences of these releases to waterways.

INTRODUCTION

Steroidal estrogens can potentially impact ecosystems and engineered systems due to their higher estrogenic potency than other endocrine disrupting chemicals (EDCs) (Jobling et al., 1998; Metcalfe et al., 2001). In particular, exposure can influence sex ratios and reproductive systems of wildlife by disrupting the normal function of their endocrine systems even at trace concentrations in the environment (Jobling et al., 1998; Miller et al., 2007). Steroidal estrogens have been detected at trace levels in runoff (12 – 1256 ng E2/L) from poultry litter amended fields and effluent (0.1 - 30 ng E2/L) from wastewater treatment plants (WWTPs) (Hewitt and Servos, 2001; Raman et al., 2001; Young and Borch, 2009). Efforts to trace the fate of these chemicals in the environment has revealed that natural and synthetic estrogens can be biodegraded and this process is considered a significant parameter governing the fate of estrogens in ecosystems (Donova, 2007; Young and Borch, 2009). However, relatively little is known about the biodegradation of steroidal estrogens introduced to

agricultural fields when amended with animal litter. Agricultural runoff can constitute a significant fraction of estrogens released to surface and groundwater even when compared to municipal sources (Colucci et al., 2001).

Turkey litter is a good source of nutrients and organic carbon, but co-occurring steroidal estrogens in the litter can become an environmental concern. The US Census of Agriculture reported that the U.S. poultry industry produced almost 8.9 billion broilers with a total litter production of almost 14 billion kg (2008) (USDA, 2009) Growth in the poultry industry has led to an increasing number of confined animal feeding operations (CAFO) that generate large quantities of animal waste (Shore and Shemesh, 2003). Application of litter to the land is not the only means of disposal or utilization, but also it is desirable because the litter provides nutrients that increase crop yield (Ingersleve et al., 2003). All animals release some level of hormones in their urine and poultry litter contains 17β-estradiol (E2) ranging from 126 to 904 ng g-1 on a dry weight basis depending on the gender, age, and type of bird (Nichols et al., 1997). The quantities of steroidal estrogens in U.S. poultry are estimated to ranging from 160 tons/year up to 750 tons/year (Finlay-Moore et al., 2000). The reported concentration of steroidal estrogens detected in runoff ranges from 14 to 1256 ng/L, depending on the size of plots, precipitation rate, litter rate, and land use in fields applied with animal litter (Raman et al., 2004).

Biodegradation of steroidal estrogens can significantly reduce their concentrations, particularly in agricultural fields. A composting process, a form of biodegradation, as an on-farming animal litter management practice may provide

effective removal of the steroidal estrogens (Hakk et al., 2005). Composting is a controlled aeration process that enhances the performance of microorganisms capable of decomposing organic materials in poultry litter (de Bertoldi et al., 1998). Many studies report that the biodegradation of estrogens in the composting or animal litter amended fields is governed by the microbial activity at the application sites (Raman et al., 2004; Hakk et al., 2005). Hakk et al. show that 80% of E2 can be removed in 139 days of composting (Hakk et al., 2005). However, most available data has been reported from estrogen degrading cultures isolated from activated sludge in wastewater treatment plants (WWTPs) (Fujii et al., 2002; Yoshimoto et al., 2004; Yu et al., 2007). A source of much debate is whether the key bacteria responsible for estrogen biodegradation are the heterotrophic bacteria or the ammonia oxidizing bacteria (AOB) (Shi et al., 2004; Yi and Harper, 2007; Yu et al., 2007; Gaulke et al., 2008). Although some contend that heterotrophic bacteria dominate for estrogen biodegradation, others argue that estrogen biodegradation is coupled to nitrification with cometabolic degradation using AOB or nitrifiers (Vader et al., 2000; Shi et al., 2004; Yi and Harper, 2007; Gusseme et al, 2009). Some authors suggest that each estrogen is degraded by specific cultures based on dominant populations in activate sludge, indicating that the heterotrophic bacteria degrade E1, E2, and EE2; and the AOB degrade E3 (Ren et al., 2007)

Turkey litter may harbor estrogen degrading cultures which in turn seed amended fields with capable estrogen degrading cultures. Yang et al. indicate that the enrichment cultures obtained from manure-borne bacteria demonstrated a hormone

biodegradation performance depended on the organic carbon content and field temperature (Ren et al., 2007; Yang et al., 2010). Based on results reported from similar studies, estrogen biodegradation by the TLEC was evaluated by aerobic substrate utilization tests (SUT) conducted in batch reactors. Four substrates were evaluated to determine those bacteria responsible for estrogen biodegradation under simulated agricultural operation using turkey litter amendments. The extent of TLEC microbial degradation of selected hormones will provide guidance to improve our understanding of the fate of steroidal estrogen in agriculture fields and impacted ecosystems. The objectives of this study are 1) to determine if the steroidal estrogens are aerobically degraded by enrichment cultures from turkey litter (TLEC) and if so, 2) determine the type of bacteria (AOB or heterotroph) responsible for the degradation if they are being degraded. The objective of this study will be achieved by conducting substrate depletion kinetics and substrate utilization tests.

MATERIALS AND METHODS

Culturing techniques

An enrichment culture was developed using turkey litter (TLEC) following the protocol described by Herman and Mills. (2003). One Ziploc bag (2L) of turkey litter was collected from USDA-ARS, Riesel, TX, (2007, September) and preserved at -50 ^{o}C until culturing. The turkey litter was ground with 200 µm of mesh for use as inoculums. The buffered mineral-salts (BMS) solution was prepared for cell culturing using the following compounds: 5.5 g of KH_2PO_4, 10 g of Na_2HPO_4, 2 g of $(NH_4)_2$

HPO_4, 1.5 g of $(NH_4)H_2PO_4$, 15 mg of $CaCl_2$, 200 mg of $MgSO_4 \cdot 7H_2O$, 0.6 mg of $Fe_2(SO_4)_3$, 0.2 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, and 0.2 mg of $MnSO_4 \cdot H_2O$. The pH of the BMS solution was adjusted to 7.0 with KOH. Stock solutions of steroidal estrogens were prepared in acetone. Stock dissolved organic carbon (DOC) was prepared with acetate and glucose in a 1:1 molar ratio in the BMS solution for a final concentration of 100 g L^{-1}. Duplicate 25 ml of BMS solution containing 1.0 g L^{-1} DOC were each added to two sterilized 125-ml Erlenmeyer flasks. Then, 1 g of turkey litter was added as the inoculum followed by spiking 50 µl of the stock solution of estrogen to obtain a final concentration of 1.0 µg estrogens L^{-1}. The two Erlenmeyer flasks were stored in a dark incubator at approximately 30 ^oC under aerobic conditions. The culture suspensions were centrifuged at 8500 g for 15 minutes. The supernatant was decanted from the centrifuge bottles. The remaining cells were washed three times by centrifuging after resuspending them in 25 ml of distilled deionized (DDI) water. Then, 1 ml of the microbial suspension was transferred to the two new 125-ml Erlenmeyer flasks including the BMS-Estrogens-DOC growth medium prepared as described. The culture enrichment process was repeated four times. The nutrient solution for AOB growth was composed of several chemicals to encourage biomass growth and was developed based on the work of Hyman (1994) with nitrifying bacteria (Hyman et al., 1994). Compounds for the growth medium for nitrifiers are detailed in Table 4.1.

Table 4.1. Growth media composition for nitrifiers.

Chemicals	$(NH_4)_2 \cdot SO_4$	KH_2PO_4	$MgSO_4 \cdot 7H_2O$	$CaCl_2 2H_2O$	$FeSO_4$	$CuSO_4 \cdot 5H_2O$	NaH_2PO_4	Na_2CO_3
Concentration (mM)	25	43	0.7	0.18	0.01	0.53	3.89	3.77

Biodegradation experiments

Aerobic biodegradation experiments were conducted in batch reactors for the following compounds: estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2). The experiments were conducted in 100 ml amber serum bottles (reactors) at 22 ^oC lab condition. Reactors containing 45 ml of BMS solution and 5 ml of a DOC stock solution were prepared and autoclaved. 50 µl of the stock solution of estrogens was spiked into each treatment flask to yield 1.0 mg of estrogen L^{-1} in the reactors. The TLEC was centrifuged and then resuspended in 25 ml of DDI water for use as inoculums. Each treatment flask was inoculated with 250 µl of the cell suspension to obtain an absorbance A600 of 0.4 in each reactor. Four flasks of each treatment for each estrogen were spiked with 250 µl of DDI water as a control. Each flask was sampled by taking 5 ml of the suspension at predetermined sampling times. The sampling vials were a 16 ml screw cap tube fitted with Teflon coated caps containing 5 ml of acetone. Reactors containing only the aqueous estrogen mixture solution without any culture suspension represent controls for the experiment. The

sample vials were placed on a rotary shaker for 10 hours to allow complete partitioning of the steroidal estrogens into the acetone phase. After 10 hours, the estrogens were extracted from the acetone phase. Following this, 1 ml of the solution from the acetone was transferred to GC vials and 10 µl of internal standard added. The extract was completely evaporated and derivatized with BSTFA + 1% TMCS and pyridine at sand-bath 70 ^{o}C for 30 minutes. Gas chromatography-mass spectrometry (GC-MS) analysis was used to quantify the estrogen concentrations.

Substrate utilization test (SUT)

Preliminary microbial performance was evaluated using four substrates. The four different substrates used to evaluate estrogen removal behavior of heterotrophic bacteria and AOBs indigenous to the TLEC included combinations of glucose and ammonia (Table 4.2). To encourage both heterotrophic bacteria and AOBs, reactors consisting of both glucose and NH_4Cl were initiated. To separate the dual substrate performance, reactors composed of glucose only, only NH_4Cl, and no substrate were initiated. As expected, both heterotrophs and AOBs were active in the presence of both glucose and NH_4Cl. Only heterotrophs were active with glucose substrates; only AOBs were active with NH_4Cl, and no activity occurred in the absence of any substrate.

Table 4.2.
Substrate influence on estimated heterotrophic and AOB microbial activity in the presence or absence of either glucose or ammonia substrates.

Substrate	Estimated microbial degradation	
	Heterotroph	AOB
Glucose, NH_4Cl	Positive	Positive
Glucose	Positive	Negative
NH_4Cl	Negative	Positive
No addition	Negative	Negative

Analytical methods

A THERMO TRACE GC UltraTM coupled with a mass selective detector and AS 3000 series autosampler was used to quantify the hormones. Samples were separated on a 30 m x 0.32 mm, 0.25 µm, DB-5 fused silica capillary column. The column temperature was programmed as follows: the initial temperature was 80 ^{o}C for 4 minutes and was increased to 200 ^{o}C at 20 ^{o}C/min, then increased to 300 ^{o}C at 8 ^{o}C/min, and finally held for 2.5 minutes. The total run time was 30 minutes. Ultra high purity helium with an inline Alltech oxygen trap was used as carrier gas. The carrier gas was set at 40 psi, column head pressure at 8 psi, injector temperature was maintained at 280 ^{o}C, and the injection volume was 1.0 µl in the splitless mode. The interface temperature was held at 280 ^{o}C. Mass spectra were scanned from m/z 50-650 at a rate of 1.5 scan/s. The electron impact ionization energy was 70 eV.

RESULTS AND DISCUSSION

Biodegradation tests were conducted for the individual steroidal estrogens of E1, E2, E3, and EE2 using the TLEC under aerobic condition. The log normalized concentrations of estrogens are illustrated in Fig. 4.1. TLEC was found to significantly degrade steroidal estrogens under aerobic conditions. In the presence of nitrogen and additional carbon source, the TLEC degraded the estrogens within 118 hr (Fig. 4.1.).

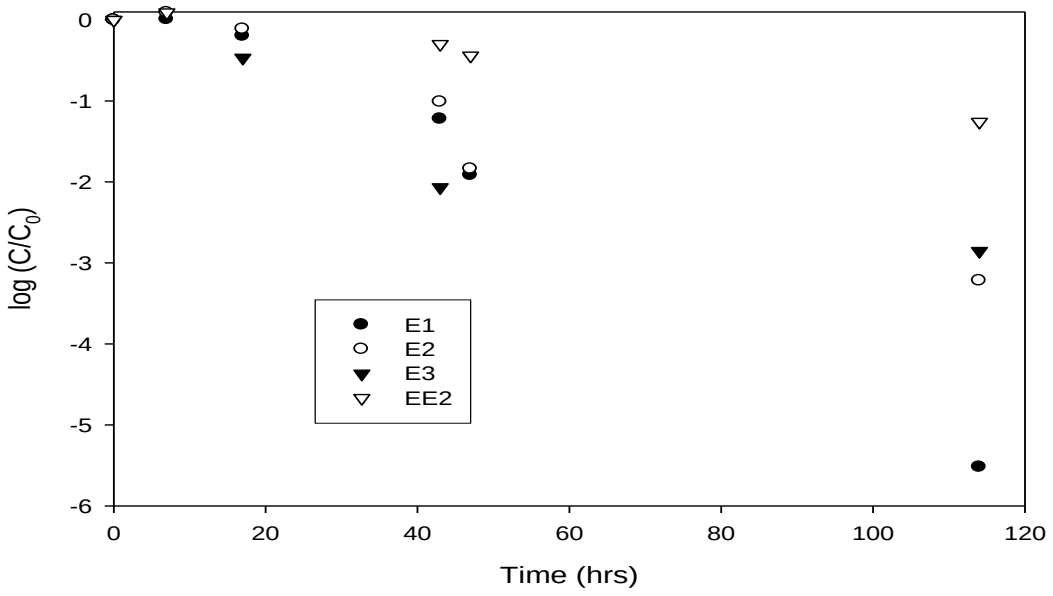


Fig. 4.1. Aerobic degradation of steroidal estrogens by the TLEC for initial concentration (C_0) as follows: E1-3.61 µM; E2-3.24 µM; E3-2.76 µM; EE2-2.94 µM. Symbols are defined as: closed circle-E1, open circle-E2, open reverse triangle-EE2, closed reverse triangle-E3.

Table 4.3. The rate constant of steroidal estrogen biodegradation and their removal efficiency for a given time.

Estrogens	E1	E2	E3	EE2
Degradation rate constant (hr^{-1})	0.050	0.031	0.027	0.012
Removal efficiency (%)	85.3	84.6	93.6	35.7

Although the experiments were conducted in batch reactors, a pseudo-first-order reaction rate was estimated using data from the linear portion of the degradation curve. Others have reported that estrogen removal can be described by a pseudo-first-order reaction in municipal operations (Layton et la., 2000; Li et al., 2005; Khanal et al., 2006). Simple first-order reaction equation was used to generate a pseudo first-order rate from a slope of the straight line. The generated estrogen biodegradation rate constants were 0.05 hr^{-1}, 0.031 hr^{-1}, 0.027 hr^{-1}, and 0.012 hr^{-1} for E1, E2, E3, and EE2, respectively (Fig. 4.1.). The rate constant (0.031 hr^{-1} for E2) was comparable to previously reported pseudo first-order rate constant for E2 degradation (Yang et al., 2010). The study reported that the E2 degradation rate was 0.025 hr^{-1} (22 ^{o}C) using pre-enriched culture of swine manure-borne bacteria (Yang et al., 2010). The degradation of natural estrogens appeared to be faster than a synthetic estrogen. Specifically, the removal efficiency (35.65%) of EE2 was shown to be 2-3 times less than those (84.62 - 93.61%) of the natural estrogens tested at a given time (Table 4.3). This observation indicates that EE2 degradation was more resistant than natural estrogen degradation by the TLEC in aerobic batch test (Table 4.3). These results also indicate that EE2 may be

more persistent in turkey litter amended fields than the other natural estrogens. Similar results were observed for the persistency of EE2 in WWTPs due to steric hindrance of enzyme expression and EE2 metabolism in ethynylgroup of EE2 (Miller et al., 2001; Anderson et al., 2003; Pauwels et al. 2008; Skotnicka-Pitak et al., 2009). Poultry, including turkey, are not feed EE2 for any growth in the U.S. The relatively low EE2 degradation rate constant by the TLEC can also be attributed from a lack of bacterial adaptation to EE2 which had never been exposed in turkey litter.

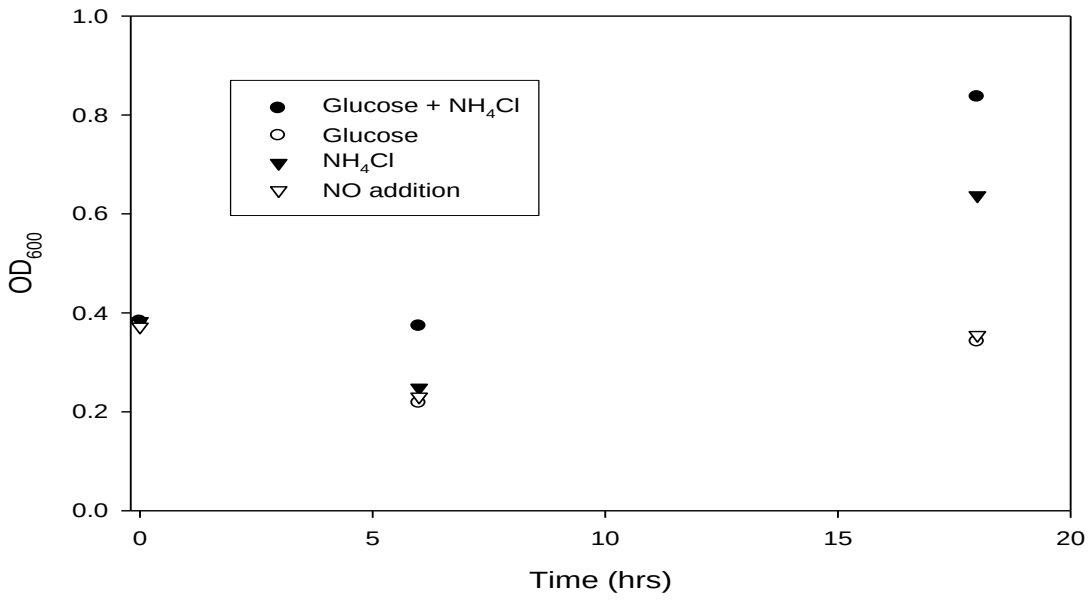


Fig. 4.2. Microbial growth (OD_{600}) using four substrate combinations: Glucose+NH_4Cl (closed circle); Glucose only (closed reverse triangle); NH_4Cl only (open circle); No glucose and NH_4Cl substrates (open reverse triangle).

To evaluate the microbial growth of the TLEC in the presence or absence of glucose and ammonia, optical density (OD_{600}) was measured. It was also observed (Fig. 4.2.) that in the presence of glucose and NH_4Cl, microbial growth was the highest indicating that the enrichment culture was the most prolific. When only NH_4Cl was provided, microbial growth marginally lagged that of the combination. Without any substrate, no growth was observed, nor was any growth observed using only glucose. AOB demonstrated growth dominance over the heterotrophs.

The implication of these substrate growth responses is that the AOB members of the TLEC appear responsible for steroidal estrogen biodegradation. Biodegradation of the steroidal estrogens is likely associated with AOB co-metabolism. Theoretically, the proposed cometabolic transformation of estrogens is based on cometabolism of other halogenated compounds (Aziz et al., 1999, Alvarez-Cohen et al., 2001). This requires that ammonia is first oxidized to hydroxylamine by AMO induced from nitrifiers. Then, the hydroxylamine is oxidized to nitrite, generating five hydrogen ions and four electrons as a reductant. Two of the electrons are consumed for cell synthesis; the other two electrons are recycled to ammonia oxidation or co-metabolism of estrogen. However, the co-metabolism of estrogen could result in depletion of the electrons for the nitrifiers and would require enough ammonia to provide a source of electrons for sustained cometabolic degradation.

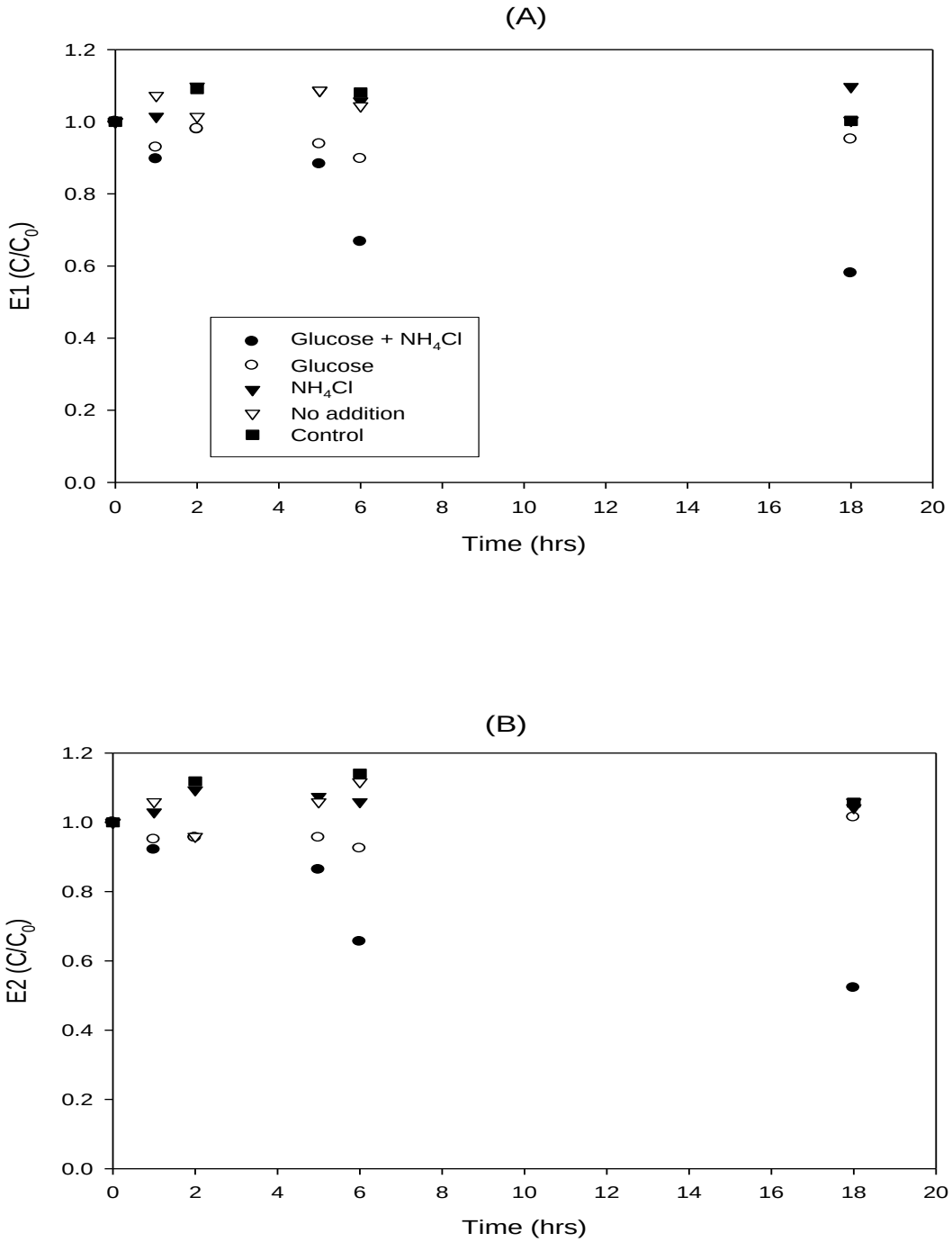


Fig. 4.3. Aerobic degradation of steroidal estrogen by the TLEC for four substrate conditions: Glucose+NH_4Cl (closed circle); Glucose only (closed reverse triangle); NH_4Cl only (open circle); No glucose and NH_4Cl substrates (open reverse triangle); abiotic control (closed rectangular). Initial concentrations were: E1-3.70 µM; E2-3.30 µM; E3-2.43 µM; EE2-3.04 µM.

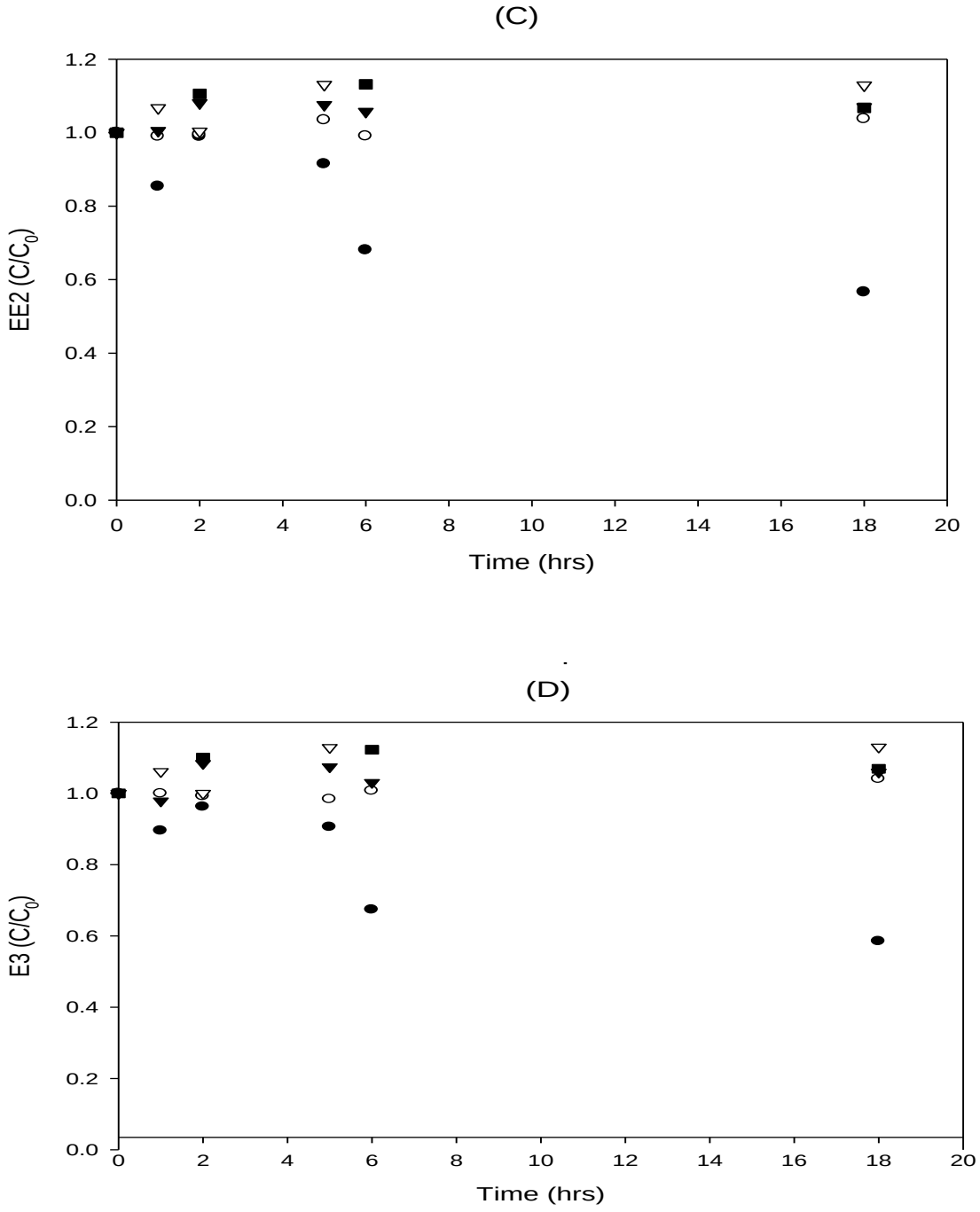


Fig. 4.3. Continued.

The substrate utilization test (SUT) was conducted to identify favorable bacteria for the biodegradation of steroidal estrogens in aerobic batch tests. Autoclaved culture was used as a control to elucidate the potential of abiotic degradation and sorption. The depletion concentration for individual estrogens over time was illustrated in Fig. 4.3. However, some control samples (abiotic control and no addition) exceeded slightly initial concentration of each estrogen during 1-6 hours in batch tests. Though, the presence of nitrogen and carbon sources favored estrogen biodegradation in the TLEC inoculated cultures (Fig. 4.3.). In the presence of only glucose or only NH_4Cl, no estrogen biodegradation was observed. Abiotic controls showed no change in estrogen concentration. The observation is attributed with growth-related degradation because using both glucose and NH_4Cl achieved higher bacterial growth using OD_{600} (Fig. 4.2). These results from Fig. 4.3 were not shown to relevantly identify bacteria which are favorable for estrogen biodegradation. For further identification of the favorable bacteria for estrogen biodegradation in TLEC, specific AOB growth medium as suggested by Hyman et al. (1996) was used for estrogen biodegradation in aerobic batch test.

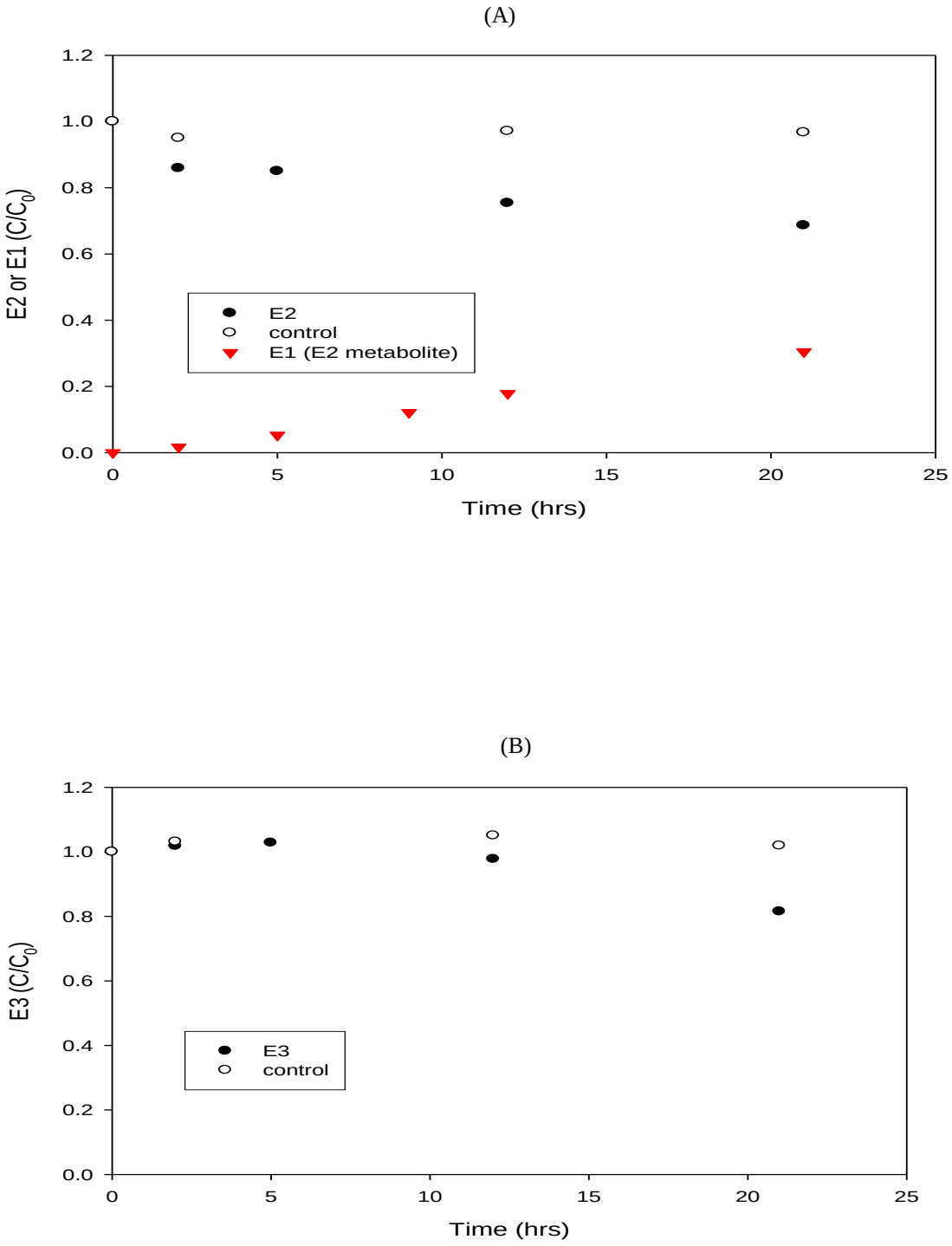
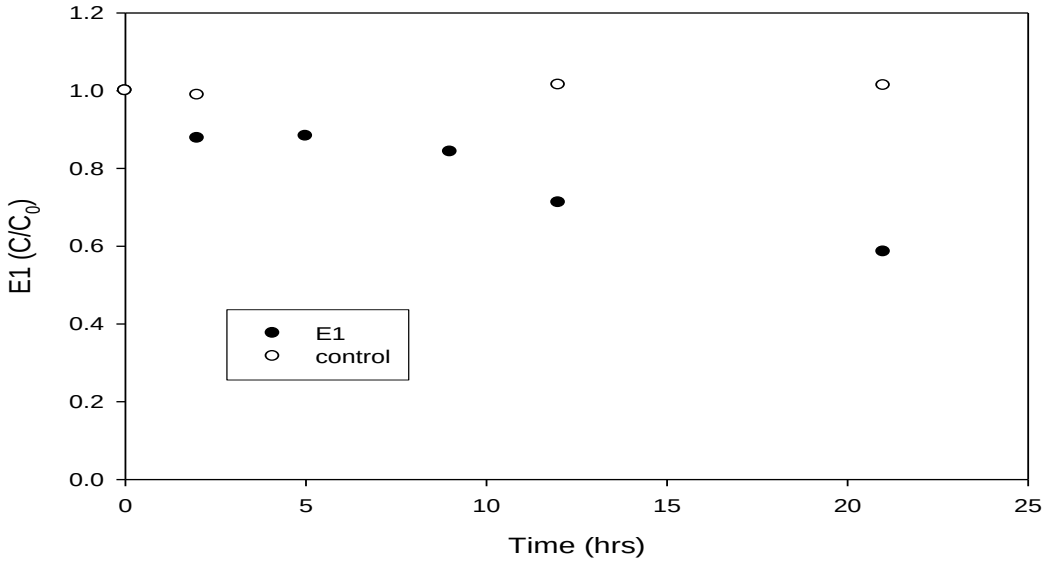


Fig. 4.4. Aerobic degradation of steroidal estrogens by the TLEC for AOB growth medium, Initial concentrations were: E1-2.57 µM; E2-2.42 µM; E3-2.05 µM; EE2-2.71 µM; autoclaved culture as a control (open circle); estrogen (closed circle).

(C)



(D)

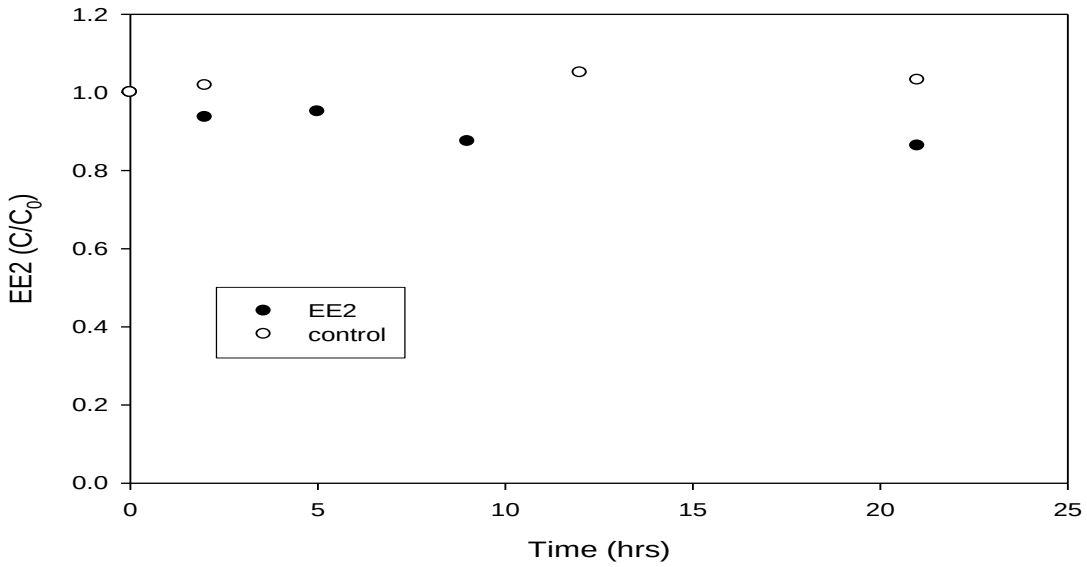


Fig. 4.4. Continued.

Table 4.4 Removal efficiency of individual estrogens by the TLEC during 21 hours in aerobic batch test under AOB growth medium; C_0: initial molar concentration of estrogens, C: final molar concentration of estrogens after 21 hours.

	Steroidal estrogens				
	E2		E1	E3	EE2
	E2	E1 (as *E2-M.)			
C_0 (µM)	8.88	0.00	9.51	7.11	9.14
C (µM)	6.09	2.74	5.58	5.79	7.89
Removal (or generation) efficiency (%)	31.4	(30.9)	41.3	18.5	13.6

*E2-M.: E2 Metabolite in E2 biodegradation test

Biodegradation of all steroidal estrogens tested occurred to some degree when the TLEC was cultured in the AOB medium. A proportional stoichiometric generation of E1 from E2 was observed (Fig. 4.4A.) and E1 was readily degraded. The extent of degradation during 20 hours of incubation was interpreted using a percent removal approach. The degradation of E2, resulted in the stoichiometric conversion (30.9%) of E1 (Fig. 4.4A., Table 4.4). This transformation indicates that TLEC may be composed of a dominant AOB culture responsible for E2 biodegradation. Presumably the E1 generated from E2 would also be degraded as was demonstrated with the 41.3% degradation of pure E1 spiked culture (Fig. 4.4B.). In contrast, the removal efficiency of E3 and EE2 was 18.5% and 13.6% respectively (Table 4.4). The minimal degradation of both E3 and EE2 indicates that they are persistent under the test conditions with ammonia only and no carbon sources provided (Fig. 4.4C. and 4.4D.). On the other hand, this observation implies that cometabolic degradation of E3 and EE2 by AOB in the consortia might be less likely in turkey litter amended fields. The activity of the TLEC on estrogen indicates that the biodegradation of E1 and E2 may be more

associated with heterotrophic bacteria than with AOB in turkey litter amended field. By contrast, the biodegradation of E3 and EE2 appears to be governed by heterotrophic bacteria in estrogen biodegradation by the TLEC.

Results from this study show that the fate of steroidal estrogen in agricultural lands amended with turkey litter appears to be governed by the observed bacterial degradation in turkey litter if agricultural soil would be incubated with the turkey litter after it is to be spread to soil. Ren et al. (2007) demonstrated that heterotrophic bacteria cultured from nitrifying activated sludge are favorable for E3 biodegradation, and AOBs preferably degrade E1, E2, and EE2 in activated sludge from WWTPs. Our observations are consistent with these results in that E1, E2, E3, and EE2 were degraded by a consortium isolated from turkey litter and cultured under nitrifying conditions.

CONCLUSION

Microorganisms indigenous to turkey litter demonstrated the potential to degrade the steroidal estrogens that may be deposited on the agricultural fields when amended with turkey litter. Biodegradation rate constants for individual estrogens calculated using pseudo-first order kinetics revealed that E1, E2, and E3 are fairly degradable under favorable conditions. However, the synthetic estrogen, EE2, appears to be more resistant to biodegradation than natural estrogens which indicate that it could be more persistent in the environment. Further, heterotrophic metabolism was shown to be favorable for E3 and EE2 biodegradation. By contrast, the biodegradation of E1and E2 is favored by AOB in TLEC. These results indicate the potential to reduce endocrine hormone levels

with proper management of fields that enhance the performance of capable microorganisms.

CHAPTER V

THE EFFECTS OF SELECTED ANTIBIOTICS ON DEGRADATION: STEROIDAL HORMONE AND EFFLUENT ORGANIC MATTER (EFOM)

OVERVIEW

The influx of antibiotics to wastewater treatment plants (WWTPs) can disrupt key bacterial cycles/processes critical to its process efficiency. The effect of selected antibiotics on microbial degradation at an environmentally relevant concentration of antibiotics (10 μg L^{-1}) in a sequential batch reactor (SBR) was evaluated. Ammonium-nitrogen, nitrate-nitrogen, TOC and hormone concentrations were monitored in the SBRs treated with hormone only and hormone plus antibiotics at solids retention time (SRT) of 14 days. No significant difference was observed for removal efficiency of all target compounds in the two SBR units. However, EE2 degradation was affected by the SBR cycle length (analogous to HRT), suggesting that the HRT should be considered as a key operating parameter for complete removal of all hormones in WWTPs. Concomitantly, the inhibitory effect of antibiotics on effluent organic matter (EfOM) was characterized using 3D-EEM and $SUVA_{254}$. The inhibition was quantified using FRI analysis. As a result, the decomposition of humic-like substance from dissolved organic matter (DOM) in reactors with antibiotics was shown to be inhibited up to 7% in one region by less humification. If the fraction of DOM was not degraded because the antibiotics prevented degradation, the DOM would be available to sorb the hormones. Environmentally relevant concentrations of antibiotics in wastewaters did not alter the

degradation of hormones. However, the antibiotics did reduce the decomposition of EfOM which has implications for the reuse and recycling of effluents from WWTPs.

INTRODUCTION

Hormones and antibiotics in the environment have generated concern because of their developmental effects on wildlife and humans, as well as, development of antibiotic resistance of bacteria in water systems (Colborn et al., 1993, Levy, 1997). In the U.S., annually approximately 3 million pounds of antibiotics are prescribed for human consumption and prophylactic use of 25 million pounds for animals (Egger et al., 2002, Null et al., 2005). The tetracycline group of drugs, including chlortetracycline (CTC) and oxytetracycline (OTC), is second most widely used for humane therapy and animal feeding operations (Col and O'Connor, 1987). Antibiotics have been detected at concentrations ranging from 25 to 1000 μg L^{-1} in swine lagoons and 0.1 to 10 μg L^{-1} in effluent from wastewater treatment plants (WWTPs) (Campagnolo et al., 2002; Karthikeyan and Bleam, 2003; Kim et al., 2005). Moreover, point and nonpoint sources of antibiotics in the environment may cause long-term and irreversible changes to the microorganism genome, rendering them incapable of degrading ammonia, organic matter and other target contaminants (Hernando et al., 2006).

Antibiotics are linked with nitrification because of the potential to disrupt nitrifiers/bacterial cycles (Halling-Sørensen, 2001; Costanzo et al., 2005). Antibiotics may play a role in agricultural and municipal operations as an inhibitor or a stimulator of

nitrification (Halling-Sørensen, 2001). Nitrification inhibition studies caused by 11 antibiotics demonstrated that the tetracycline group, a broad spectrum antibiotic, inhibits the nitrification process, while sulfonamides stimulate it (Halling-Sørensen, 2001). EC_{50} values for CTC and OTC were 2 μg L^{-1} and 320 μg L^{-1} for *N. europaea,* respectively (Halling-Sørensen, 2001). Several studies report that estrogen degradation is linked to ammonia oxidation due to cometabolic degradation by ammonia oxidation bacteria (AOB) (Vader et al., 2000; Shi et al., 2004; Yi et al., 2006; Yi and Harper, 2007). The cometabolic degradation of estrogens effectively inhibited nitrification and estrogen biodegradation, suggesting that hormone removal efficiency may decrease in the presence of antibiotics (Shi et al., 2004). The high influx of antibiotics via wastewaters to WWTPs may result in less nitrification which will result in lower removal rates for hormones and higher discharge concentrations causing exposures to aquatic systems. The presence of antibiotics in WWTPs may inhibit cometabolic degradation of hormones by reducing bacterial activity of heterotroph or AOB in the activated sludge.

Nitrification inhibition by antibacterial compounds can be determined using several standardized methods. The extent of apparent nitrification inhibition by antibiotics depends on the assay. The short-term (six hours) effect of antibiotics on nitrification is not as well detected by the less sensitive standard methods (ISO 15522 and ISO 9509) than in long-term test (three days) (Backhaus et al., 1997). Long-term tests using pure plate methods with an activated sludge and *N. europaea* was especially sensitive for most antibiotics compared to the standard methods (Halling-Sørensen,

2001). Inhibition of antibiotics can strongly depend on the test duration and type of compounds (Kummerer, 2004). The long-term test is effective for evaluating the nitrification inhibition using activated sludge in a sequential batch reactor (SBR) in the presence of antibiotics. The test duration was extended up to 15 days due to the slow growth rate of nitrifiers compared to heterotrophs in the activated sludge. Several studies report that improved biodegradation of estrogens in the nitrifying process is related to an increase in SRT (Ternes et al., 1999; Holbrook et al., 2002; Anderson et al., 2003). Longer SRT is more efficient for estrogen biodegradation (Demes et al., 2005).

Reuse and recycling of biologically treated sewage effluent (BTSE) is foreseen as a necessary strategy for water conservation due to scarcity and increased urbanization (Shon et al., 2006). BTSE is composed of three general constituents according to their sources, including 1) natural organic matter (NOM), 2) synthetic organic compound (SOC) and disinfection by-product (DBP), and 3) soluble microbial product (SMP) (Shon et al., 2006). Characterizing the effluent organic matter (EfOM) or NOM in BTSE is vital because DOM serves as a precursor of disinfection byproducts (DBPs), increases the amount of coagulants and oxidants needed in water treatment process, and causes major fouling problems on membrane surfaces in advanced processes (Singer, 1999; Lee et al., 2004; Sharp et al., 2004). However, little is known about the effect of antibiotics on EfOM or xenobiotics degradation in the activated sludge processes in WWTPs.

The hypothesis for this study is that antibiotics adversely affect the capacity of activity sludge or specific target bacteria in WWTPs, consequently causing incomplete removal of hormones by nitrification inhibition or less decomposition of organic matters

in final effluents. The main objective of this study is to evaluate the inhibitory effect of selected antibiotics (CTC and OTC) on degradation for selected hormones (testosterone, progesterone, 17α-ethynleatradoil, estrone and 17β-estradiol) and DOM, and identify the inhibited fraction through characterization of EfOM in SBR. This will allow us to understand the exposure of the antibiotics to municipal or agricultural operations.

MATERIALS AND METHODS

Experimental design

The inhibitory effect of selective antibiotics was evaluated using two sequencing batch reactors (SBR) to compare the effect of the hormone only (unit H) and hormone plus antibiotics (unit H+A) (Fig. 5.1.). The effluent collected daily from primary clarifier of the Clark County Water Reclamation Facility (CCWRD) in Las Vegas, NV was used as influent to the SBR. Each reactor received enough stock solution of hormones and antibiotics (10 mg L^{-1}) to attain a final concentration of 1 μg L^{-1} of each hormone (testosterone, progesterone, E2, E1, EE2) and 10 μg L^{-1} of antibiotics plus 1 μg L^{-1} of hormone in two SBRs (Unit H and Unit H+A), respectively. Activated sludge from the CCWRD was used to maintain a mixed liquid suspended solid (MLSS) of approximately 2 g L^{-1} in both SBRs. Dissolved oxygen (DO) concentration in the SBRs was maintained between 6 to 8 mg L^{-1} by pumping air into the reactors. Decanted samples taken from SBR were preserved with 0.1 % sodium azide, transferred to the Southern Nevada Water Authority (SNWA) lab where they were kept at 4 ^{o}C until samples were extracted. The

SBR system consisted of 5-L flasks as reactors, two feeding tanks as a primary clarifier, and an aeration unit. Aeration was supplied by air-pump and air stone diffuser. The SBR was operated in 24 hour cycles including five sequential steps: fill, react, settle, draw, and idle. Each cycle consisted of 1 hour fill, 19.5 hours aeration, 2 hours sedimentation, 1 hour draw of the supernatant and 0.5 hour idle (Fig. 5.1).

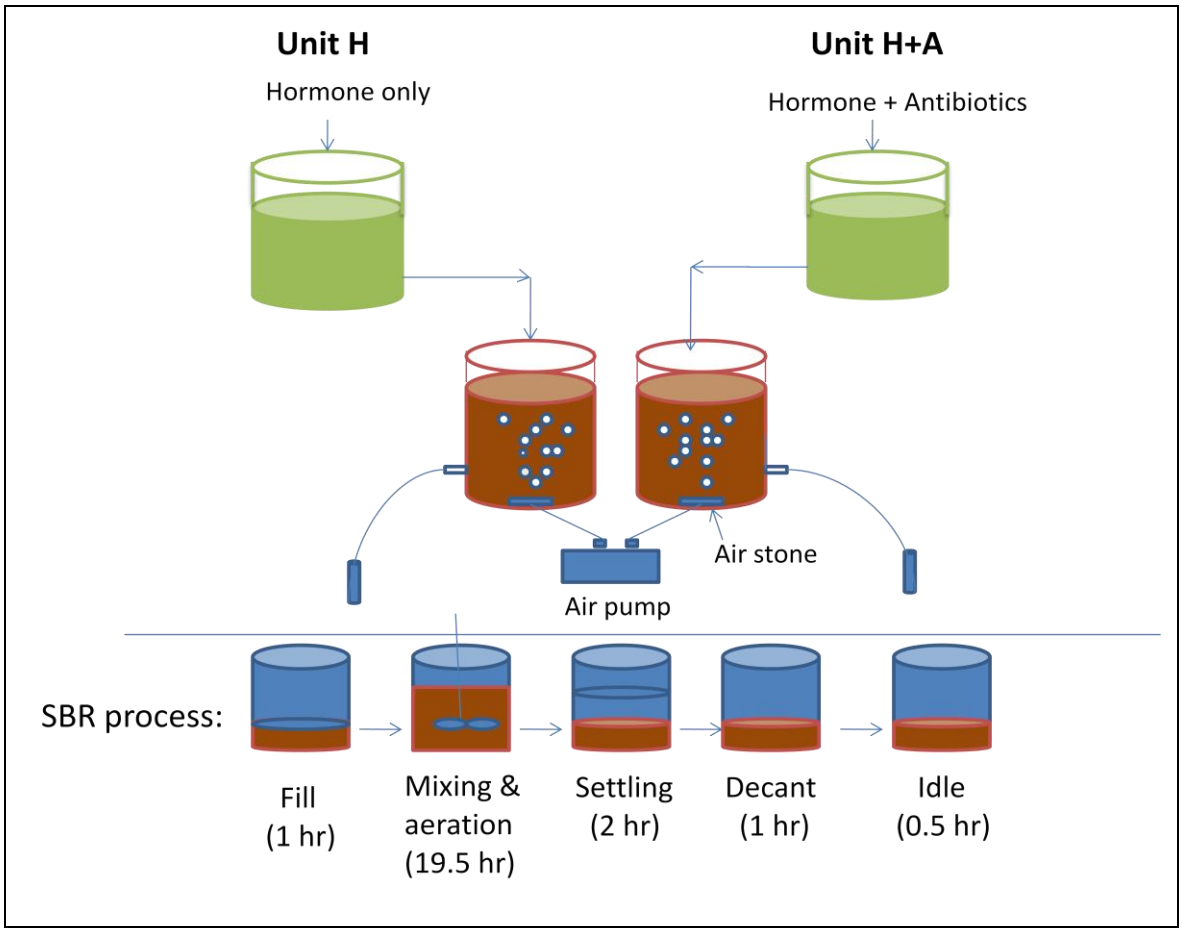


Fig. 5.1. SBR scheme; unit H: hormone only; unit H+A: hormones plus antibiotics; SBR cycle: 1 hour fill, 19.5 hours aeration, 2 hours sedimentation, 1 hour draw of the supernatant and 0.5 hour idle; dissolved oxygen (DO) concentration: 6-8 mg/L.

Chemicals

The hormones (17β-estradiol: E2, estrone: E1, 17α-ethynylestradiol: EE2, testosterone: T, and progesterone: P) and antibiotics (Tetracycline and oxytetracycline) used in this study were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated estrogen (estradiol-d4) (Cambridge Isotope Laboratories, Andover, MA) was used for hormone analysis. All standard solutions of nutrients were obtained from Hach Company (Loveland, CO). All stock solutions were prepared in methanol. Each chemical used was ACS certified.

Analytical method

Samples were collected in 1L ultra clean, pre-silanized, amber glass bottles (Eagle-picher, Miami, OK) as suggested by Ahrer et al. (2000), to prevent compounds from binding to the glass surface of the collection bottles. Sample bottles were kept on ice during transport to the laboratory, where they were immediately preserved by adding 0.1% sodium azide and stored at 4 ^{o}C until extraction. Prior to extraction, the samples (influent and effluent) were filtered through 90 mm GF/F filters (Whatman, England). Ammonium-nitrogen and nitrate-nitrogen were measured using the Hach Salicylate method 10031 (DR/2000, Hach). TOC was measured by a standard method (SM5310B) using Shimadzu Model TOC 5050A. Mixed liquid suspended solid (MLSS) was measured using a portable MLSS analyzer (InsiteIG Model 3150) after compared with a standard method for better calibration.

A rapid on-line solid phase extraction (SPE) and LC/MS/MS technique was used for hormone analysis. The extraction and analysis of 100 mL of treated water was performed using an on-line solid phase extraction and liquid chromatography with

tandem mass spectrometry (SPE-LC-MS/MS). An automated solid phase extractor (Spark Holland) and a 4000 QTRAP (Applied Biosystems, Foster City, CA) mass spectrometer were used. Oasis HLB (Waters CO., Milford, MA) cartridges were used for SPE. Separation was performed on a C18 column (Phenomenex) and with a mobile phase consisting of 5 mM ammonium acetate in DI water:methanol gradient. All samples were analyzed using positive electrospray ionization (ESI) for EE2, E1, E2 and testosterone, atmospheric chemical ionization (APCI) for progesterone with tandem mass spectrometry, or multiple reaction monitoring (MRM). Two MS/MS transitions were used to quantify and confirm each compound. Quantitation was performed using isotope dilution.

Three-dimensional, excitation-emission matrix spectroscopy (EEM) was measured using a PTI fluorometer (Birmingham, NJ, USA). The parameters of fluorescence EEM were as follows: the range of excitation from 220 to 460 nm at 5 nm increments, the range of emission from 280 to 580 nm at 4-nm increments, 2 nm bandwidth and 0.1s integration time. The samples were analyzed in duplicate. The intensity of 3D-EEM spectra was normalized by the intensity of blank Raman water line (350 nm excitation and 397 nm emission) using a analytical tool coded by Matlab (E. Dickenson, 2009).

The inhibitory effect of antibiotics on EfOM degradation for each region can be quantified. The fluorescent spectra for each region were quantified using the fluorescence regional integral (FRI) analysis as described by Chen et al. (2003):

$$\phi_i = \int_{ex}\int_{em} I(\lambda_{ex}\lambda_{em})d\lambda_{ex}\lambda_{em} \quad (5.1)$$

$$\phi_{i,n} = MF_i\phi_i \quad (5.2)$$

$$\phi_{T,n} = \sum_{i=1}^{6} \phi_{i,n} \quad (5.3)$$

$$P_{i,n} = \frac{\phi_{i,n}}{\phi_{T,n}} \times 100\% \quad (5.4)$$

where,

ϕ_i is the volume beneath region "i" of the EEM,

$d\lambda_{ex}$ is the excitation wavelength interval,

$d\lambda_{em}$ is the emission wavelength interval,

MF_i is multiplication factor for each region,

$\phi_{i,n}$, $\phi_{T,n}$ are normalized excitation-emission area volumes, and

$P_{i,n}$ is percent distribution of volumetric fluorescence among six regions.

The percent removal inhibition from equation 6.4 is described by:

$$I(\%) = \frac{B - A}{B} \times 100 \quad (5.5)$$

where,

I (%) is the % removal inhibition of DOM in a specific region,

B is % removal of DOM in a specific region in SBR (unit H+A),

A is % removal of DOM in a specific region in SBR (unit H) as control.

RESULTS AND DISCUSSION

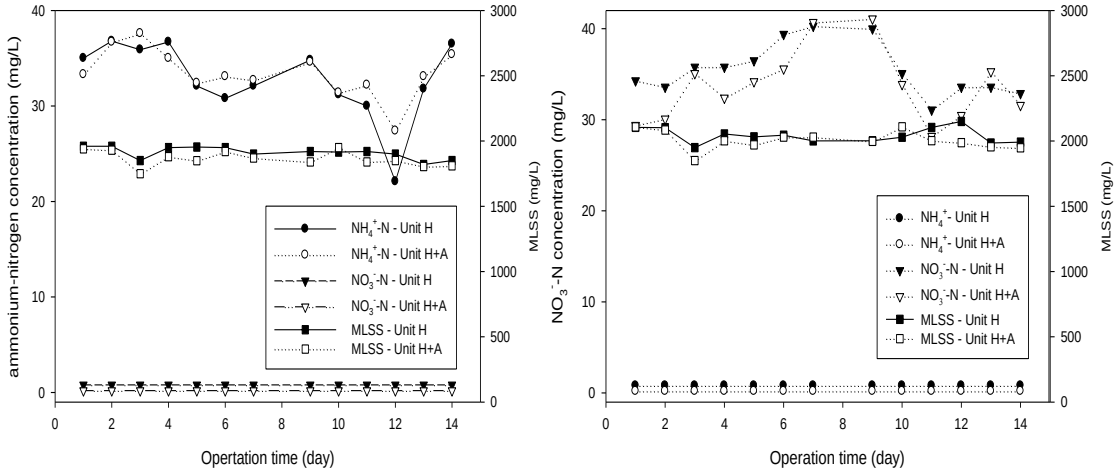
The effect of selected antibiotics on microbial performance in an activated sludge system was evaluated by monitoring ammonium-nitrogen and TOC. Selected hormones (testosterone, progesterone, estrone, 17β-estradiol, and 17α-ethynylestradiol) were also monitored over two weeks with different SBR cycle lengths. Dissolved organic matter (DOM) was characterized. Primary clarifier effluent from CCWRD was used to provide a more realistic simulation of conditions for WWTP microbial community response. Two SBRs were operated to evaluate the microbial performance influenced by antibiotics. Unit H, hormone only, was used as a control representing the absence of antibiotics in the SBR. Unit H+A, hormone plus antibiotic, was spiked with antibiotics in the SBR.

Table 5.1 Two scenarios to indentify target bacteria responsible for inhibition of antibiotics in activated sludge, AOB: ammonia oxidizing bacteria.

Inhibition as a percent removal			Target bacteria on hormone biodegradation
TOC	NH_4^+-N	Hormones	
Yes	No	Yes	Heterotrophs
No	Yes	Yes	AOB

Target bacteria inhibited by the presence of antibiotics in the SBR were identified by monitoring the concentrations of ammonium-nitrogen (NH_4^+-N) and total organic carbon (TOC) (Table 5.1). Two possible scenarios to identify bacteria inhibited by antibiotics are illustrated in Table 5.1. If the percent removal of TOC and hormones

in the SBR was decreased due to reduced microbial activity caused by the antibiotics, the biodegradation of the hormones is assumed to be governed by heterotrophs in the activated sludge (Table 5.1). On the contrary, if a percent removal of NH_4^+-N and hormones is decreased, the biodegradation of the hormones is assumed to be governed by AOBs.



(a) Ammonium-nitrogen (b) Nitrate-nitrogen

Fig. 5.2. Ammonium-nitrogen, nitrate-nitrogen and MLSS concentration of influent and effluent streams from Unit H and Unit H+A; open symbol: Unit H+A, closed symbol: Unit H, circle: ammonium-nitrogen, reverse triangles: nitrate-nitrogen, squares: mixed liquid suspended solid (MLSS); SRT: 14 days.

To evaluate the effects of antibiotics on AOBs in the activated sludge, ammonium-nitrogen and nitrate-nitrogen were monitored in the influent and effluent streams of the SBRs (Fig. 5.2.). The average influent concentrations of ammonium-nitrogen and nitrate-nitrogen in unit H were 32.8 mg L^{-1} and below detection limit (BDL) (<0.2 mg L^{-1}), respectively. Average effluent concentrations of ammonium-

nitrogen and nitrate-nitrogen from unit H were below detection limit (<0.5 mg L^{-1}) and 35.3 mg L^{-1}, respectively. MLSS concentration in the SBR remained relatively constant at 2.0 g L^{-1} for both SBRs throughout all experiments. Ammonium was completely oxidized to nitrate. In unit H+A, the influent concentration of average ammonium-nitrogen and nitrate-nitrogen from the SBR was 33.4 mg L^{-1} and below detection limit, respectively. The average effluent concentration of ammonium-nitrogen and nitrate-nitrogen was below the detection limit and 33.9 mg L^{-1}, respectively. The effluent data from unit H+A suggests that there was no significant inhibitory effect of antibiotics on nitrification in the SBR, indicating that the antibiotics (CTC and OTC) did not affect the nitrifying bacteria containing about 20% AOB population in the activated sludge in spite of their broad spectrum of activity. This may be explained by low populations of *N. europaea* in the AOB community due to their slower growth rate compared to that of heterotrophic bacteria commonly found in sludge. A study by Dytchzak et al. (2007) quantified AOB and NOB populations for different types of treatment (aerobic and alternating aerobic/anoxic regimes) using fluorescent *in situ* hybridization (FISH) analysis. *N. europaea* in constituted only 3.8% of the total AOB population in the aerobic treatment which is comparable to our treatment design. But the proportion of *N. europaea* in the alternating treatment was 21.1% (Dytchzak et al., 2007). Based on our results and those of Dytchzak et al. (2007), the inhibitory effect of antibiotics on nitrification appear to be dependent on treatment types which affect the population of specific nitrifiers in activated sludge.

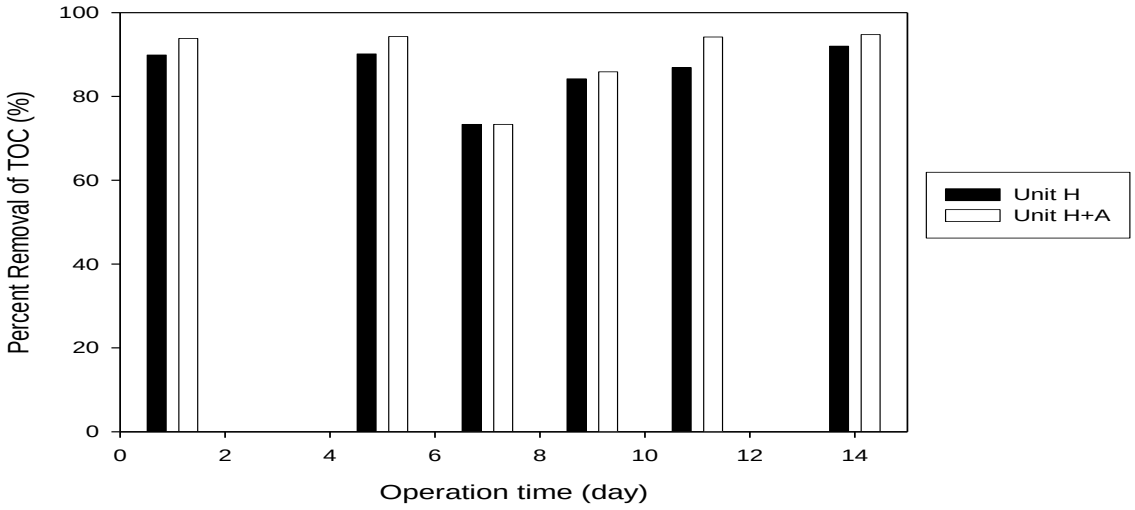


Fig. 5.3. TOC percent removal of influent and effluent streams from Unit H and Unit H+A; SRT: 14 days.

To evaluate the effects of antibiotics on heterotrophic bacteria in the activated sludge, the TOC concentrations of the effluent and influent streams of SBRs were monitored. The TOC percent removal was calculated by multiplying 100 for removed concentration of TOC divided by influent concentration of TOC. The average percent removals of TOC from the SBRs were 86.1±6.2 % for unit H and 89.4±7.8 % for unit H+A (Fig. 5.3.). A significant inhibition of percent removal by antibiotics was not observed in the SBR (Unit H+A). This result indicates that the antibiotics do not have significant inhibitory effect on the activity of the heterotrophic bacteria in the activated sludge. This operation showed stable and good removal for TOC and ammonium-nitrogen throughout all experiments.

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Table 5.2 Hormone removal efficiency in a primary clarifier after equilibrium mixing (20 minutes) after spiking some amount of hormones to yield 1000 ng L^{-1} of theoretical influent concentrations of hormones.

Hormone	Primary clarifier (ng L^{-1})		Removal efficiency (%)	
	Unit H	Unit H+A	Unit H	Unit H+A
Testosterone	465±136	466±105	53.5±14	53.4±10
Progesterone	462±104	452±99	53.8±10	54.8±10
EE2	735±117	791±84	26.5±12	20.9±8
E1	917±77	1030±121	8.3±8	0.0±5
E2	426±125	437±117	57.4±12	56.3±12

The theoretical concentration of hormones in the effluent from the primary clarifier may be substituted for an influent concentration in the SBRs before introduction to a primary clarifier. The concentration of hormones from the SBRs was monitored after equilibrium mixing (20 minutes) (Table 5.2). The concentration detected from the SBRs may be replaced to the concentration of hormones in the primary clarifier. In all cases, approximately 50% of the initial concentration of some hormones (testosterone, progesterone, and E2) was lost prior to introduction into the SBRs (Table 5.2). A higher concentration of E1 (lower loss) was observed compared to other hormones. Biotransformation of E2 to E1 occurs easily by some bacteria of suspended solids in the effluent. Esperanza et al. (2007) demonstrated that 48% and 51% of the influent E1 and E2, respectively, were found in the effluent of the primary clarifier. The fraction of estrogenic hormones degraded is in partial agreement with our data; 49% of influent EE2 was degraded versus 24% degraded in our study. The difference in the faction of EE2 measured is attributed to the differences in the influent characteristics between

experiments tested. Irreversible adsorption to clay or other organic matters and abiotic degradation is cited as the sink for the hormones (Esperanza et al, 2007). In our study, the loss is explained by the presence of suspended solids (approximately 150 mg L^{-1}) containing bacteria and clay particles in the primary clarifier effluent.

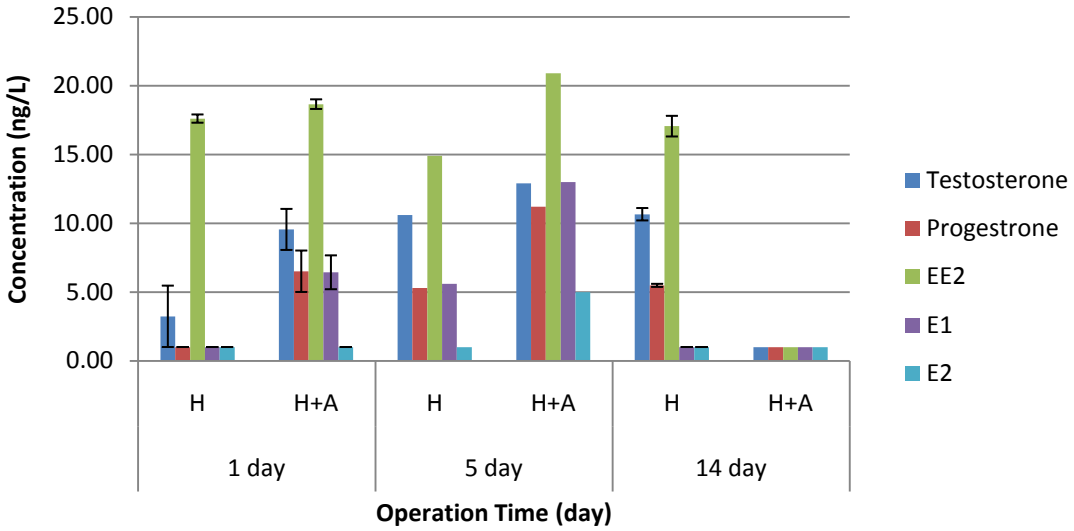

Fig. 5.4. Hormone concentration from effluent for 14 days in unit H and unit H+A; DO concentration: 6-8 mg/L; SRT: 14 days; Temperature: 22 ^{o}C.

Table 5.3 Mean hormone removal efficiency for SRT 14 day in Unit H and Unit H+A; a cycle length of SBR-24 hours; SRT 14 days; DO: 6-8 mg/L; Temperature: 22 ^{o}C.

Hormone	Primary clarifier (ng L^{-1})		Effluent (ng L^{-1})		Removal efficiency (%)	
	Unit H	Unit H+A	Unit H	Unit H+A	Unit H	Unit H+A
Testosterone	465±136	466±105	8.2±4.3	7.8±6.1	98.1±0.2	97.9±1.0
Progesterone	462±104	452±99	3.9±2.5	6.2±5.1	98.8±0.2	98.2±0.8
EE2	735±117	791±84	16.5±1.4	13.5±10.9	97.7±0.6	98.1±1.2
E1	917±77	1030±121	2.5±2.7	6.8±6.0	99.4±0.1	99.2±0.5
E2	426±125	437±117	BDL	BDL	98.7±0.4	98.8±0.4

BDL: below detection limit.

To evaluate the effect of the antibiotics on hormone degradation, the concentration of several hormones were monitored in effluent streams of the SBRs. Hormone concentrations over three sampling times (1, 5, and 14 day) when the system was operated at the SRT of 14 days reveal an overall hormone removal efficiency of greater than 97% (Table 5.3). The hormone removal efficiency from unit H was not significantly different from that of unit H+A, indicating that hormone degradation was not inhibited by the presence of antibiotics at an environmentally relevant concentration. Based on the measured values of ammonia and TOC, there are no antibiotic induced inhibitory effects on hormone degradation after 14 days and there is minimal relationship to a shift in specific bacterial populations (AOB or heterotrophs). Hormone degradation is known to be positively correlated with SRT (Ternes et al., 1999; Holbrook et al, 2002; Andersen et al., 2003). A more diverse and specific microbial culture develops at a higher SRT, especially nitrifiers (Andersen et al., 2003; Saino et al., 2004; Koh et al., 2008). Although the evidence of a relationship between SRT and

hormone removal has been reported, this relationship is statistically weak (Johnson et al., 2005; Servos et al., 2005). Additionally, the AOB population was not related to SRT in a batch test using the activated sludge with a molecular method (Noh et al., 2009). According to the results for unit H (Fig. 5.4), the removal of EE2 of all the hormones is less correlated with SRT in the activated sludge system.

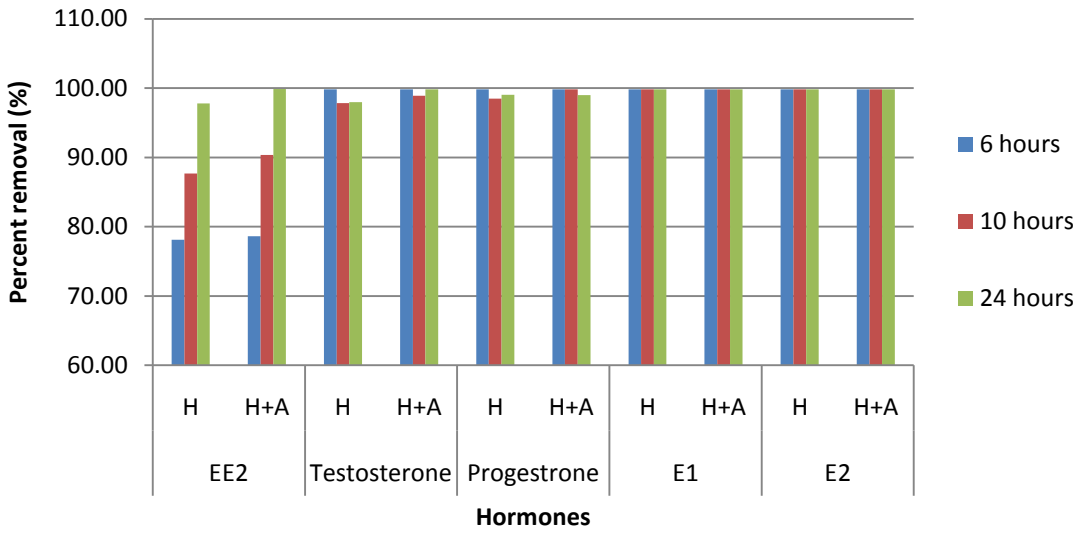

Fig. 5.5. Hormone removal efficiency for various cycle lengths (hour) of SBR in Unit H+A and Unit H, a cycle length of SBR-24 hours; DO (mg/L): 6-8; Temperature: 22 ^{o}C.

The SBR cycle length (analogous to hydraulic retention time-HRT) can improve hormone removal efficiency (Suarez et al., 2008). The effect of various cycle lengths (6, 10, 24 hours) on hormone removal evaluated in the SBR demonstrated that the removal efficiency (average 98%) of all hormones was not affected by cycle lengths except for

17á-ethynylestradiol (EE2) (Fig. 5.5.). EE2 was removed up to 78% and 88% in unit H, and 78% and 90% in unit H+A at 6 and 10 hours of cycle length, respectively (Fig. 5.5.). The results also revealed that variance in cycle lengths did not affect the inhibition of hormone degradation in the presence of antibiotics. EE2, a synthetic hormone, is the most persistent among all the hormones detected in WWTPS because of the steric hindrance of enzyme expression and EE2 metabolism in ethynylgroup of EE2 (Miller et al., 2001, Anderson et al., 2003, Pauwels et al., 2008, Skotnicka-Pitak et al., 2009). The estrogenic potency of EE2 is approximately 10^7 times higher than that of nonylphenol (NP) as measured by yeast estrogen screen (YES) assay (Folmar et al., 2002). The removal efficiency of EE2 is largely dependent on SBR cycle length, suggesting that an optimized cycle period is a key operating parameter for complete removal of EE2 in WWTPs.

Table 5.4 SUVA of DOM from two effluents without (Unit H) and with antibiotics (Unit H+A).

Effluent	UVA_{254} (1 cm^{-1})	TOC(mg L^{-1})	SUVA(L mg^{-1}•m^{-1})
Unit H	0.31	6.0	5.2
Unit H+A	0.38	6.3	6.0

An increase in bacterially-produced dissolved organic matter (DOM) would facilitate sorption of tetracycline on the DOM (Sun et al., 2007). The abundance of aromatic rings in humic substance would favor the sorption of hormones (Sun et al,

2007). Characterization of DOM in the two systems could clarify the questions regarding the sorption of hormones on humic substance in the DOM. Specific ultraviolet adsorption (SUVA) is used to identify humic content (>4.0 L mg^{-1}•m^{-1}) and non-humic content (<2.0 L mg^{-1}•m^{-1}) in DOM (Edzwald et al., 1990). It is often used as an index of EfOM aromaticity in the humic fraction. SUVA is calculated by dividing the UVA_{254} value by TOC. The SUVA calculated for the suspension in the reactor without antibiotics is 5.2 and with antibiotics is 6.0 L mg^{-1}•m^{-1} (Table 5.4). This difference is enough to indicate that there is a higher (13%) aromaticity of humic content than in the presence of antibiotics in SBR. The difference of intensity in region V (humic like substance region) of the EEM spectra would occur if this region was less degraded due to microbial inhibition caused by the antibiotics.

(a) Unit H (Influent) **(b) Unit H+A (Influent)**

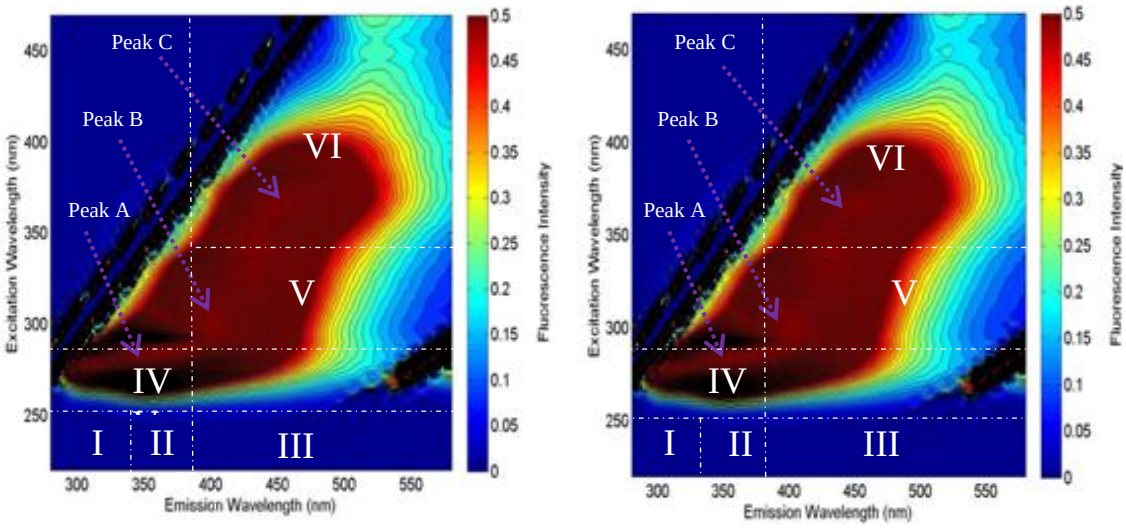

Fig. 5.6. 3D-EEM fluorescent spectra of DOM sample from influent and effluent in unit H and unit H+A of SBR at a final cycle of 14 day SRT.

(c) Unit H (Effluent) **(d) Unit H+A (Effluent)**

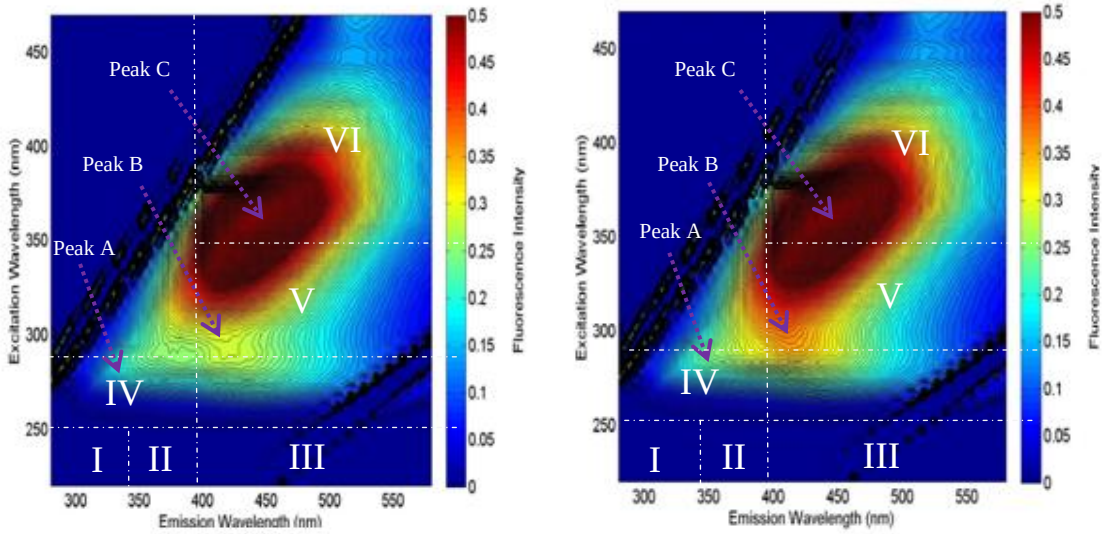

Fig. 5.6. Continued.

Three-dimensional excitation emission matrix fluorescence spectroscopy (3D-EEMS) was used to evaluate the effect of antibiotics on the degradation characteristics of EfOM. Six regions of DOM from influent and effluent samples were divided by fluorescent spectra using 3D-EEM with previous peer-reviewed references (Fig. 5.6.) (Stedmon et al., 2003; Nam et al., 2008; Wang et al., 2009). Three peaks were observed with the fluorescent contours. Region I was in the range of λ_{ex} less than 250nm and λ_{em} less than 330nm for aromatic protein like substances. Region II was in the range of λ_{ex} less than 250nm and λ_{em} between 330nm and 430nm for aromatic protein like substances (II). Region III was in the range of λ_{ex} less than 250nm and λ_{em} more than 330nm for Fulvic acid-like substances. Region IV was in the range of λ_{ex} between 250nm and

282nm, and λ_{ex} less than 380nm for soluble microbial by-product-like substance (SMBP). Region V was in the range of λ_{ex} between 282 nm and 340nm, and λ_{em} more than 380nm for humic acid-like substance. Region VI was in the range of λ_{ex} more than 340nm and λ_{em} more than 380nm for humic acid-like substance (II). Three peaks (A-C) were observed at the three EEMs ($\lambda_{ex}/\lambda_{em}$=280nm/335nm), ($\lambda_{ex}/\lambda_{em}$=305nm/404nm), and ($\lambda_{ex}/\lambda_{em}$=360nm/452nm) respectively. The fluorescent spectra from both effluents were partly blue shifted for all regions divided (Fig. 5.6.). Specifically, region V from the SBR with antibiotics was relatively red shifted compared to that of the SBR without antibiotics. A blue shift of fluorescent spectra can be attributed to the decomposition of condensed aromatic moieties and the break-up of the molecular into smaller molecules (Chen et al., 2003; Wang et al., 2009). The increase detected in region V (humic like substance) when antibiotics are present may be due to less decomposition of humic substance, suggesting that humic like substances in region V are inhibited by the two selected antibiotics.

Table 5.5 Percent removal of DOM in each region from influent and effluent samples, % inhibition for DOM removal of each region between Unit H and Unit H+A.

region	% Removal		Inhibition (%)
	SBR(H)	SBR(H+A)	
I	100	100	0
II	100	100	0
III	100	100	0
IV	87	87	0
V	57	53	7
VI	30	30	0

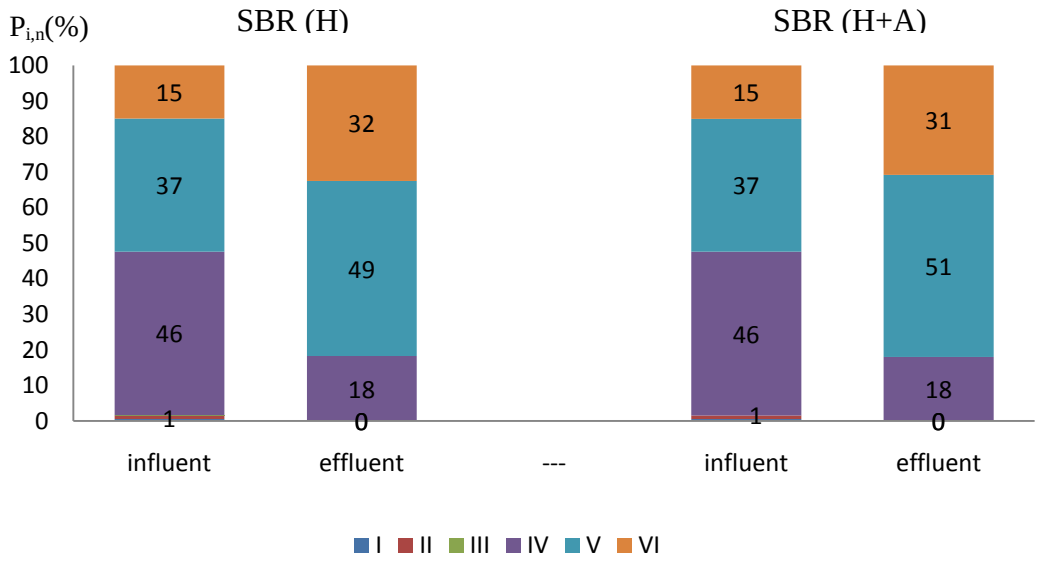

Fig. 5.7. Percent distribution of FRI (Fluorescence Regional Integration) in DOM from influent and effluent samples from Unit H and Unit H+A.

The percent distribution of DOM FRI from influent and effluent samples was determined using Equations 5.1 through 5.4. It is a reasonable assumption that the influent organic matter fraction consisted primarily of three substances: soluble microbial product (SMP) (46%), humic-like substance (53%), and protein-like substance (1%). Further, the EfOM fraction had less soluble microbial product (18%), more humic-like substance (81%), and less protein-like substances (0%) (Fig. 5.7.). Most of the SMP and protein-like substances found in the influent organic matter were degraded by the activated sludge. In contrast, humic-like substances were persistent compared to the SMP in EfOM. Additionally, humic-like substances of EfOM in the effluent samples of

SBR (H+A) was more persistent than that of SBR (H), indicating that less decomposition (or humification) of EfOM in SBR (H+A) is attributed to the presence of the antibiotics in the activated sludge system. The small difference of percent distribution in humic substance region (V) between SBR (H) and SBR (H+A) may be attributed from small dose (10 μg L^{-1}) of antibiotics in the comparative experiment. The percent inhibition of DOM removal of each region using FRI analysis was calculated. Seven percent of the inhibition was measured for humic-like substance removal when in the presence of the antibiotics in the activated sludge systems (Table 5.5). Moreover, the characteristics of the EfOM provide an evidence for the inhibitory effect of antibiotics on hormone degradation. Less humification due to the presence of antibiotics, results in better degradation of hormones later in the operational period (14 day) (Fig. 5.4.). This may be due to sorption of hormones onto 7% of the humic-like substance following on less decomposition of DOM. The humic substances played a role in the sorption of hormones, suggesting that approximately 15 to 50% of the steroidal estrogens are bound in typical natural water (Yamamoto et al., 2003; Holbrook et al., 2004). Although the hormone degradation in the SBRs was not significantly affected by the presence of antibiotics with environmental relevant concentration, some fraction of EfOM was less decomposed due to the presence of the antibiotics. Therefore, the influx of antibiotics to WWTPs cannot cause inhibitory effect on hormone degradation, but reduction of humic substance degradation in EfOM can be detected at environmental relevant concentration. Further study is needed to evaluate the inhibition for hormones and EfOM using high dose or different kind of antibiotics.

CONCLUSION

The effect of antibiotics on the degradation of hormones and EfOM in SBRs using primary clarifier effluent containing environmental relevant concentration (10 μg L^{-1}) of antibiotics revealed significant effects in the effects of antibiotics and surprising shifts in the degradation of organic matter. The degradation of ammonium-nitrogen, TOC, and hormones was not significantly altered by the antibiotics when the SBR was operated with the SRT of 14 days. However, variation in hormone concentration from effluents was observed over the operational period. This may be due to comparative sorption and alteration of cell surface charge by the toxicity induced from antibiotic exposure. EE2 degradation was dependent on cycle lengths of SBR. The cycle length of SBR should be considered as a key operational parameter for complete EE2 removal. Humic-like substance of EfOM was less decomposed up to approximately 7% in the presence of the antibiotics in SBR. Sorption of hormones onto the humic-like substance is proposed as the cause for shifts in hormone removal due to the presence of antibiotics.

CHAPTER VI

CONCLUSIONS

SUMMARY

Growing concerns over estrogens (natural and synthetic) and antibiotics in the environment have been linked to farming operations where cattle, poultry, and hogs are raised. Bacteria that are resistant to available antibiotics are increasing which poses human health concerns when people become infected. Hormones are only fed to cattle in the U.S., but all animals excrete natural hormones. Low level concentrations of hormones and antibiotics are impacting the quality of waterways and pose human and ecological health concerns. Environmental transport of these two groups of chemicals are linked via their common sources and co-occur nutrients whose loading to receiving waters has been well documented. Ideally methods that would reduce environmental exposure to all of these potential contaminants simultaneously would be the most cost effective approach for all in the stakeholder community.

Antibiotics are an important group of pharmaceuticals in both human and veterinary medicine. Of the 50 million pounds of antibiotics produced annually, an estimated 70 % are being used in confined animal feeding operations (CAFOs) not only to treat or prevent diseases, but also as growth promoters (Mellon, et al., 2001). Nationwide, an estimated 13.5 million pounds of the total antibiotics used are excreted annually by grazing animals on farmlands through urine and feces and/or using animal manure as fertilizers (Halling-Sørensen et al., 1998). Most of these antibiotics are mobile

in the environment and because of their hydrophilicity, they can contact surface water as a result of runoff or groundwater by leaching (Ingerslev et al., 2001).

Chapter II focused to optimize the sample preparation method to determine the content of steroid estrogens in field samples (runoff or turkey litter) using GC-MS, compared to previous published protocols. The sample preparation method is composed of sample preservation, filtration (liquid sample only), ultra sonic extraction (solid sample only), solid phase extraction and derivatization. Derivatization is an important step to increase thermal stability of free estrogen before GC-MS analysis. It is suggested that the best derivatization protocol is to use BSTFA as a derivatization reagent, 1% TMCS as a catalyst and pyridine as solvent. The mixture should be reacted in 30 minutes 70^{o}C at sand-bath. This method achieved better sensitivity if the derivatized sample would be analyzed by GC-MS at approximately 5 hour-duration after the 30 minute reaction in sand-bath. The method effectively applied to analysis of estrogen in agricultural field samples.

Chapter III showed that kinetic coefficients for estrogen degradation were successfully determined using the pure culture *N. europaea* through the reductant model providing the best fit of the data. The implications of this model are that estrogen cometabolism may be governed by a mechanism that requires both a limited reductant produced from ammonia oxidation and no competition with ammonia or its oxidative intermediates. The kinetic experiments revealed that the removal of estrogen increases at greater ammonia concentrations because the rate of estrogen degradation depends on both the concentration of the estrogen and ammonia. The reductant model also predicts

no estrogen degradation in the absence of ammonia. The kinetic coefficients of estrogen cometabolism normalized by kinetic coefficients of ammonia degradation provided further approach for various sources as an engineered application. The kinetic experiments imply that AOB likely grown in various water treatment facilities and agricultural fields could degrade estrogen to some extent by themselves depending on ammonia removal. The results suggested that the ratio of $k_{estrogen}/k_{NH3}$ should be considered in designing effective bioremedial strategies. This study helps understand the role of nitrifiers in various water treatment facilities, predicting biodegradability of individual estrogen through cometabolism.

Chapter IV demonstrated that Turkey litter enrichment culture (TLEC) has a potential to biologically degrade the steroidal estrogens in the agricultural fields amended with turkey litter. Biodegradation rate constants of individual estrogen were calculated using pseudo-first order kinetics: 0.050 hr^{-1} for E1, 0.031 hr^{-1} for E2, 0.027 hr^{-1} for E3, and 0.012 hr^{-1} for EE2. The observation from the data implies that a synthetic estrogen, EE2 appears to be more persistent to biodegradation than natural estrogens if agricultural soil would be incubated with the turkey litter after it is to be spread to soil. Also, heterotrophic bacteria favor for E3 and EE2 biodegradation in TLEC. By contrast, the biodegradation of E1and E2 is favorable by AOB in TLEC. This study provides understanding of biodegradation of estrogens governing their fate and occurrence in turkey litter amended fields.

Chapter V evaluated the effect of antibiotics on degradation of hormone and EfOM in SBR using actual primary clarifier effluent containing environmental relevant

concentration (10 μg L^{-1}) of antibiotics. The degradation of ammonium-nitrogen, TOC, and hormones was not significantly disturbed by the antibiotics when the SBR was operated with the SRT of 14 days. However, variation in hormone concentration from effluents was observed over an operational period. This may be because comparative sorption and alteration of cell surface charge by antibiotics. Additionally, EE2 degradation was dependent on a cycle length of SBR. A cycle length of SBR should be considered as a key operational parameter for a complete EE2 removal. Humic-like substance of EfOM was less decomposed up to approximately 7% in the presence of the antibiotics in SBR. It is implied that the fraction may contribute to the sorption of hormone onto humic-like substance.

REFERENCES

Aheren, G.W., Briggs, R., 1989. The relevance of the presence of certain synthetic steroids in the aquatic environment. J. Pharm. Pharmacol. 41, 735-736.

Alvarez-Cohen, L., Speitel Jr., G.E., 2001. Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation. 12, 2, 105-126.

Anderson, H., Siegrist, H., Halling-Sorensen, B., Ternes, T.A., 2003. Fate of estrogens in a municipal sewage treatment plant. Environ. Sci. Technol. 37, 4021-4026.

Anderson, H., Siegrist, H., Halling-Sorensen, B., Ternes, T.A., 2005. Assessment of the importance of sorption for steroid estrogens removal during activated sludge treatment. Chemosphere. 61, 1, 139-146.

Aziz, C.E., Georgiou, G., Speitel G.E., 1999. Cometabolism of chlorinated solvents and binary chlorinated solvent mixtures using *M. trichosporium* OB3b PP358, Biotechnol. Bioeng. 65, 1, 100–107.

Backhaus, T., Froehner, K., Altenburger, R., Grimme, L.H., 1997. Toxicity testing with *Vibrio fischeri*: a comparison between the long term (24 h) and the short term (30 min) bioassay, Chemosphere. 35, 2925–2938.

Baronti, C.,Curini, R., D'Ascenzo, G., Di Corcia, A, Gentili, A., Samperi, R., 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. 34, 5059-5066.

Batta, A.L., Kim, S., Aga, D.S., 2004. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere. 68, 3, 428-435.

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schafer, A.J., Rijs, G.B.J., Wegener, J., Cofino, W.P., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Tot. Environ. 225, 1-2, 101-108.

Bonin, J.L., Simpson, M.J., 2007. Sorption of steroid estrogens to soil and soil constituents in single- and multi-sorbate systems. Environ. Toxicol. Chem. 26, 12, 2604-2610.

Busch, K., Hegemann, W., Spengler, P., Metzger, J.W., 2002. Influence of different process techniques in sewage plants to eliminate selected endocrine disrupters, IWA, Melbouren, e21435.

Campagnolo, E.R., Johnson, K.R., Karpati, A., Rubin, C.S., Kolpin, D.W., Meyer, M.T., Esteban, J.E., Currier, R.W., Smith, K., Thu, K.M, McGeehin, M., 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299, 89-95.

Cargouet, M., Perdiz, D., Mousatassim-Souali, A., Tamisier-Karolak, S., Levi, Y., 2004. Assessment of river contamination by estrogenic compounds in Paris area (France). Sci. Tot. Environ. 324, 1-3, 55-66.

Carlsen, E., Giwercman, A., Keiding, N., Skakkebaek, N.E., 1995. Declining semen quality and increasing incidence of testicular cancer: is there a common cause?. Environ. Health Perspect. 103, 137-139

Chiara, B., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., Samperi, R., 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. 34, 24, 5059-5066.

Chun, S., Lee, J., Geyer, R., White, D.C., 2005. Comparison of three extraction methods for 17β-estradiol in sand, bentonite, and organic-rich silt loam, J. Environ. Sci. Heal. B. 731-740.

Col, N.F., O'Connor, R.W., 1987. Estimating worldwide current antibiotic usage: report of task force I. review of infectious disease. 9, 232-243.

Colborn, T., vom Saal, F.S., Soto, A.M., 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ. Health. Perspective. 101, 378-384.

Colucci, M.S., Bork, H., Topp, E., 2001. Persistence of estrogenic hormones in agricultural soils I 17β-estradiol and estrone. J. Environ. Qual. 30, 2070-2076.

Conroy, O., Saez, A.E., Quanrud, D., Ela, W., Arnold, R.G., 2007. Changes in estrogen/anti-estrogen activities in ponded secondary effluent. Sci. Total. Environ. 382, 311-323.

Crommentuijn, T., Kalf, D.F., Polder, M.D., Posthumus, R., van de Plassche, E.J., 1997. Mazimum permissible concentrations and negligible concentrations for

pesticides. National Institute of Public Health and the Environment. Report no. 601501002. 174pp.

Crommentuijn, T., Kalf, D.F., Polder, M.D., Posthumus, R., van de Plassche, E.J., 2000. Maximum permissible and negligible concentrations for some organic substances and pesticides. Journal of Environmental Mangement. 58, 297-312.

Das, B.S., Lee, L.S., Rao, P.S.C., Hultgren., R.P., 2004. Sorption and degradation of steroid hormones in soils during transport: column studies and model evaluation. Environ. Sci. Technol. 38, 1460-1470.

de Bertoldi, M., Citterio, B., Civilini, M., 1988. Composting management: a new process control through O2 feedback. Waste Management Research. 239-259.

de Gusseme, B., Pycke, B., Hennebel, T., Marcoen, A., Vlaeminck, S.E., Noppe, H., Boon N., Verstraete, W., 2009. Biological removal of 17α-ethinylestradiol by a nitrifier enrichment culture in a membrane bioreactor Water Res. 43, 2493–2503.

de Mes, T., Zeeman, G., Lettinga, G., 2005. Occurrence and fate of estrone, 17β-estradiol, ethynylestradiol in STPs for domestic wastewater. Review in Environ. Sci. Biotech. 4, 275-311.

Dickenson, E., **2009**. personal communication.

Ding, W. H., Chiang, C. C., 2003. Derivatization procedures for the detection of estrogenic cheimcals by GC/MS. Rapid Communication in Mass Spectrometry. 17, 56-63.

Donova, M.V., 2007. Transformation of steroids by actinobacteria: A review. Applied Biochemistry Microbiology. 43, 1, 1-14.

Dray, J., Dray, F., Ullmann, A., Tillier, F., 1972. Hydrolysis of urinary steroid conjugates with Escherichia-coli beta-glucuronidase. Ann. Inst. Pasteur. 123, 853-857.

Eliassen, A.H., Missmer, S.A., Tworoger, S.S., Hankinson, S.E., 2008. Circulating 2-hydroxy and 16-a hydroxyl estrogen levels and risk of breast cancer among postmenopausal women. Cancer Epidemiol Biomarkers Prev. 17, 8, 2029-2035.

Ellis, T.G., Barbeau, D.S., Smets, B.F., Grady, C.P.L., 1996. Respirometric technique for determination of extant kinetic parameters describing biodegradation. Water Environ. Res. 68, 917-926.

EPA, 2009. Drinking Water Contaminant Candidate List and Regulatory Determination, Washington, DC.

Esperanza, N., Suidan, M.T., Nishimura, F., Wang, Z-M., Sorial, G.A., 2004. Determination of sex hormones and nonylphenol ethoxylates in the aqueous matrixes of two pilot-scale municipal wastewater treatment plants. Environ. Sci. Technol. 38, (11), 3028-3035.

Esperanza, M., Suidan, M.T., Marfil-Vega, R., Gonzalez, C., Sorial, G.A., McCauley, P., Brenner, R., 2007. Fate of sex hormones in two pilot-scale municipal wastewater treatment plants: conventional treatment, Chemosphere. 66, 1535–1544.

Farré, M., Brix, R., Kuster, M., Rubio, F., Goda, Y., de Alda, M.J.L., Barcelo, D., 2006. Evaluation of commercial immunoassays for the detection of estrogens in water

by comparison with high-performance liquid chromatography tandem mass spectrometry HPLC-MS/MS (QaQ). Anal Bioanal Chem. 385, 1001-1011.

Farré, M., Kuster, M., Brix, R., Rubio, F., de Alda, M.J.L., Barcelo, D., 2007. Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography–tandem mass spectrometry, and ultra performance liquid chromatography–quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. J. Chromatogr. A. 166–175.

Finlay-Moore, O., Hartel, P.G., Cabrera, M.L., 2000. 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. 29, 1604-1611.

Fujii, K., Kikuchi, S., Satomi, M., Ushio-Sata, N., Morita, N., 2002. Degradation of 17β-estradiol by a gram-negative bacterium isolated from activated sludge in a sewage treatment plant in Tokyo, Japan. Appl. Environ. Microbiol. 68, 2057-2060.

Gao, J.P., Maguhn, J., Spitzauer, P., Kettrup, A., 1998. Sorption of pesticides in the sediment of the Teufelsweiher Pond (Southern Germany). I: equilibrium assessments, effect of organic carbon content and pH. Water Research. 32, 5, 1662-1672.

Gaulke, L.S., Strand, S.E., Kalhorn, T.F., Stensel, H.D., 2008. 17α-ethinylestradiol transforamtion via abiotic nitration in the presence of ammonia oxidizing bacteria. Environ. Sci. Technol. 42, 7622-7627.

Grady, C.P.L., Smets, B.F., Barbeau, D.S., 1996. Variability in kinetic parameter estimate: a review of possible causes and a proposed terminology. Wat. Res. 30, 742-748.

Hakk, H., Millner, P., Larsen, G., 2005. Decrease in water-soluble 17β-estradiol and testosterone in composted poultry manure with time. J. Environ. Qual. 34, 943–950.

Halling-Sørensen, B., Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Lutzhoft, H.C.H., Jorgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substances in the environment- A review. Chemosphere. 36, 2, 357-393.

Halling-Sørensen, B., 2001. Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. Arch. Environ. Contam. Toxicol. 40, 451-460.

Hanselman, T. A., Graetz, D. A., Wilkie A. C., 2003. Manure-borne estrogens as potential environmental contaminants; a review. Environ. Sci.Technol. 37, (24), 5471-5478.

Hanselman, T. A., Graetz, D.A., Wilkie, A.C., Szabo, N.J., Diaz, C.S., 2006. Determination of steroidal estrogens in flushed dairy manure wastewater by gas chromatography-mass spectrometry. J. Envion. Qual. 35, 695-700.

Her, N., Amy, G., Sohn, H-R., von Gunten, U., 2008. UV absorbance ratio index with size exclusion chromatography (URI-SEC) as an NOM property indicator. J. Water Supply: Research and Technology-AQUA. 57, 1, 35-44

Herman, J.S., Mills, A.J., 2003. Biological and hydrogenological interactions affect the persistence of 17β-estradiol in an agricultural watershed. Geobiology. 1, 141-151.

Hewitt, M., Servos, M., 2001. An overview of substances present in Canadian aquatic environments associated with endocrine disruption. Water Qual. Res. J. Can. 36, 319–330.

Holbrook, R.D, Love, N.G., Grizzard, T.J., Novak, J.T., 2002. Estrogen receptor agonist behavior during wastewater and biosolids treatment processes: a mass balance analysis, Environ. Sci. Technol. 4533-4539.

Hoogenboom, L.A.P., 2001. Estrogenic activity of estradiol and its metabolites in the ER-CALUX assay with human T47D breast cells. APMIS. 109, 101-107.

Huang, C.H., Sedlak, D.L., 2001. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. Environ. Toxicol. Chem. 20, 1, 133-139.

Hutchinson, T.H., Pounds, N.A., Hempel, M., Williams, T.D., 1999. Impact of natural and synthetic steroids on the survival, development, and reproduction of marine copepods (*Tisbe battagliai*). Sci. Total Environ. 233, 167-179.

Hwang, C.H., Renew, J.E., Smeby, K.L., Pinkstone, K., Sedlak, D.L., 2001. Assessment of potential antibiotics contaminants in water and preliminary occurrence analysis. Water Res. 30-40.

Hyman, M.R., Kim, C.Y., Arp, D.J., 1990. Inhibition of Ammonia Monooxygenase in *Nitrosomonas europaea* by Carbon Disulfide. Journal of Bacteriology. 172, (9), 4775-4782.

Hyman, M.R., Page C.L., Arp, D.J., 1994. Oxidation of methyl fluoride and dimethyl ether by ammonia monooxygenase in *Nitrosomonas europaea*, Appl. Environ. Microbiol. 60, 3033–3035.

Ingerslev, F., Vaclavik, E., Halling-sørensen, B., 2003. Pharmaceuticals and personal care products: a source of endorcrine disruption in the environment. Pure Appl. Chem. *75*, 1881-1893.

Irwin, L.K., Gray, S.L., Oberdorster, E., 2001. Vitellogenin induction in painted turtle, *Chrysemys picta*, as a biomarker of exposure to environmental levels of estradiol. Aquat Toxicol. 55, 49-60.

Jenkins, M.B., Endale, D.M., Schomberg, H.H., Hartel, P., Cabrera, M., 2009. 17β-estradoil and testosterone in drainage and runoff from poultry litter applications to tilled and no-till crop land under irrigation. J. Environ. Manage. 90, 2659-2664.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. Environ. Sci. Technol. 32:2498–2506.

Johnson, A.C., William, R.J., Ulahannan, T., 1999. Comment on "identification of estrogenic chemicals fractionation and *in vitro* biological screening." Environ. Sci. Technol. 33, 369-370.

Johnson, C.A., Sumpter, P.J., 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. Environ. Sci. Technol. 35, 24, 4697-4703.

Karthikeyan, F.G., Bleam, W.F., 2003. DNR Project 163 Final Project Report, Wisconsin Department of Natural Resources, Wisconsin Department of Agriculture, Trade and Consummer Protection: Wisconsin, http://www.dnr.state.wi.us/org/water/dwg/gw/research/reports/169.pdf.

Kellogg, R.L., Lander, C.H., Moffitt, D.C., Gollehon, N., 2000. Manure nutrients relative to the capacity of cropland and pastureland to assimilate nutrients: Spatial and temporal trends for the U.S. U.S. Department of Agriculture, Natural Resources Conservation Service. 140, 18-157.

Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S., van Leeuwen, J., 2006. Fate, transport, biodegradation of natural estrogens in the environment and engineered systems. Environ. Sci. Technol. 40, 6537-6547.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. U.S.A. 104, 21, 8897-8901.

Kim, M.H., Hao, O.J., 1999. Cometabolic degradation of chlorophenols by *Acinetobacter* species. Water Research. 33, 2, 562-574.

Kim, S., Eichhorn, P., Jensen J.N., Weber, A.S., Aga, D.S., 2005. Removal of antibiotics in wastewater. Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process, Environ. Sci. Technol. 39, 5816-5823.

Klavarioti, M., Mantzavinos, D., Kassinos , D., 2009. Removal of residual pharmaceuticals from aqueous systems by advanced oxidation processes. Environ Int. 35, 402-417.

Kloas, W., Lutz, I., Einspanier, R., 1999. Amphibians as a model to study endocrine disruptors: II. Estrogenic acitivity of environmental chemicals *in vitro* and *in vivo.* Sci. Total Environ. 225, 1-2, 59-68.

Koh, Y.K.K, Chiu, T.Y., Boobis, A.R., Carmell, E., Scrimshaw, M.D., Lester, J.N., 2008. Treatment and removal strategies for estrogens from wastewater. Environ. Technol. 29, 3, 245-267.

Koh, Y.K.K, Chiu, T.Y., Boobis, A.R. Scrimshaw, M.D., Bagnall, J.P., Soares, A., Pollard, S., Cartmell, E., Lester, J.N., 2009. Influence of operating parameter on the biodegradation of steroid estrogens and nonylphenolic compounds during biological wastewater treatment processes. Environ. Sci. Technol. 43, 17, 6646-6654.

Kolodziej, E.P., Harter, T., Sedlak, D.L., 2004. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environ. Sci. Technol. 38, 6377-6384.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other wastewater contaminants in US streams, 1999-2000. Environ. Sci. Technol. 36, 1202-1211.

Körner, W., Bolz, U., Süßmuth, W., Hiller, G., Schuller, W., Hanf, V., Hagenmaier, H., 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. Chemosphere. 40, 1131-1142.

Kumar, K., Gupta, S., Singh, A., Gupta, S., Chander, Y., Hundal, Y., Cox, A., Granato, T., 2006. Estrogens and estrogenic activity in swine manure. ASA-CSSA-SSSA International Annual Meetings. 2005MN102B, November 12-16, 2006.

Kummerer, K., 2004. Resistance in the environment. J. Antimicro. Chemo. 54, 2, 311-320.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D., and Lester, J.N., 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environ. Sci. Technol. 34, 3890-3894.

Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. Crit. Rev. Toxicol. 32, 113-132.

Layton, A.C., Gregory, B.W., Seward, J.R., Shultz, T.W, Sayler, G.S., 2000. Mineralization of steriodal hormones by biosolids in wastewater treatment systems in Tenessee U.S.A. Environ. Sci. Technol. 34, 3925-3931.

Leatherbarrow, R.J., 1990. Using linear and non-linear regression to fit bio-chemical data. Trends Biochem Sci. 15, 455-458.

Lee, L.S., Strock, T.J., Sarmah, A.K., Rao, P.S.C., 2003. Sorption and dissipation of testosterone, estrogens, and their primary transformation products in soils and sediment. Environ. Sci. Technol. 37, 18, 4098-4105.

Lee, N., Amy, G., Croué, J., Buisson, H., 2004. Identification and understanding of fouling in low-pressure membrane (MF/UF) filtration by natural organic matter (NOM), Water Res. 38, 4511–4523.

Lei, B., Huang, S., Zhou, Y., Wang, D., and Wang, Z., 2009. Levels of six estrogens in water and sediment from three rivers in Tianjin area, China. Chemosphere. 76, 36-42.

Levy, S.B., 1997. Antibiotic resistance: an ecological imbalance. In *Antibiotic Resistance: Origins, Evolution, Selection and Spread*, (Chadwick, D. & Goode, J., Eds), Wiley, Chichester (Ciba Foundation Symposium 207). 1–14.

Li, F., Yuasa, A., Obara, A., Mathews, A.P., 2005. Aerobic batch degradation of 17β-estradiol (E2) by activated sludge: effects of spiking E2 concentration, MLVSS and temperature, Water Res. 39, 2065–2075.

Lopez de Alda, M.J., Barcelo, D., 2000. Determination of steroid sex hormones and related synthetic compounds considered as endocrine disrupters in water by liquid chromatography-diode array detection-mass spectrometry. J. Chromatography A. 892, 1-2, 391-406.

Lopez de Alda, M.J., Gil, A., Paz, E., Barcelo, D., 2002. Occurrence and analysis of estrogens and progestogens in river sediments by liquid chromatography-electrospray-mass spectrometry. Analyst. 127, 1299-1304.

Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koeng, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals

detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 297-308.

Meylan, W.M., Howard, P.H., 1995. Atom/fragment contribution method for estimating octanol-water partition coefficients. J. Pharm. Sci. 84, 1, 83-92.

Miller, D.H., Jenson, K.M., Villeneuve, D.L., Kahl, M.D., Makynen, E.A., Durhan, E.J., Ankley, G.T., 2007. Linkage of biochemical responses to population-level effects: a case study with vitellogenin in the fathead minnow. Environ Toxicol Chem. 26, 3, 521-527.

Moffitt, L.A., 1996. Nutrient use in cropland agriculture (commercial fertilizer and manure): Nitrogen and phosphorus. Working Paper. 14. USDA, Government Printing Office, Washington, DC., USA.

Nichols, D.J., Daniel, T.C., Moore, P.A., Jr. Edwards, D.R., Pote, D.H., 1997. Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. J. Environ. Qual. 26, 1002-1006.

Nichols, D.J., Daniel, T.C., Edwards, D.R., Moore, P.A., Pote, D.H., 1998. Use of grass filter strips to reduce 17β-estradiol in runoff from fescue-applied poultry litter. J. Soil Water Conserv. 53, 74-77.

Okkerman, P.C., Groshart, C.P., 2003. Chemical study on estrogens. Report no. 2001.028. National Institute for Coastal and Marine Management (RIKZ), The Hague, The Netherlands.

Pauwels, B., Noppe, H., de Brabander, H., Verstraete, W., 2008. Comparison of steroid hormone concentrations in domestic and hospital wastewater treatment plants. J. Envir. Engrg. 134, 11, 933-936.

Peacock, A.D., Mullen, M.D., Ringelberg, D.B., Tyler, D.D., Hedrick, D.B., Gale, P.M., White, D.C., 2001. Soil microbial community responses to dairy manure or ammonia nitrate applications, Soil Biol. Biochem. 33, 1011-1019.

Peng, X., Yu, Y., Tang, C., Tan, J., Huang, Q., Wang, Z., 2008. Occurrence of steroid estrogens, endocrine-disrupting phenols, and acid pharmaceutical residuals in urban riverine water of the Pearl River Delta, South China. Science of the Total Environment. 397, 1-3, 158-166.

Petrovic, M., Gonzalez, S., Barcelo, D., 2003. Analysis and removal of emerging contaminants in wastewater and drinking water. Trends Anal. Chem. 22, 685-696.

Prado, N., Renault, E., Ochoa, J., Amrane, A., 2009. Development and validation of a rapid method for the determination of tetracycline in activated sludge by SPE clean-up and HPLC–UV detection. Environ. Technol. 30. 469–476.

Raman, D.R., Layton, A.C., Moody, L.B., Easter, J.P., Sayler, G.S., Burns, R.T., Mullen, M.D., 2001. Degradation of estrogens in dairy waste solids: effect of acidification and temperature. Trans. ASAE, 44, 1881−1888.

Raman, D.R., Williams, E.L., Layton, A.C., Burns, R.T., Easter, J. P., Daugherty, A.S., Mullen, M.D., Sayler, G.S., 2004. Estrogen content of dairy and swine wastes. Environ. Sci. Technol. 38, 3567-3573.

Ren, Y., Nakano, K., Nomura, M., Chiba, N., Nishimura, O., 2007. Effects of bacterial activity on estrogen removal in nitrifying activated sludge. Water Res. 41, 3089-3096.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., Sumpter, J.P., 1998. Identification of estrogenic chemicals in STW effluent: 2. *In vivo* responses in trout and roach. Environ. Sci. Technol. 32, 1559-1565.

Saino, H., Jamagato, H., Nakajima, H., Shigemura, H., Suzuki, Y., 2004. Removal of endocrine disrupting chemicals in wastewater by SRT control. J. Japan. Soc. Water Environ. 27, 61-68.

Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S.A., Ternes, T.A., 2004. Distribution of estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. Science of the Total Environment. 336, 1-3, 155-170.

Shareef, A., Angove, M.J., Wells, J.D., 2006. Optimization of silylation using N-methyl-N-(trimethylsilyl)-trifluoroacetamide, N,O-bis-(trimethylsilyl)-trifluoroacetamide and N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide for the determination of the estrogens estrone and 17α-ethinylestradiol by gas chromatography–mass spectrometry J. Chromatogr. 1108, 121-128.

Sharp, E.L., Parsons S.A., Jefferson, B., 2004. The effects of changing NOM composition and characteristics on coagulation performance, optimization and control, *Water Sci. Technol. Water Supply*. 4, 4, 95–102.

Shi, J., Fujisawa, S., Nakai, S., Hosomi, M., 2004. Biodegradation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. Water Res. 38, 9, 2322-2329.

Shon, H.K., Vigneswaran, S., Kim, S., Cho, J., Ngo, H.H., 2006. Fouling of ultrafiltration membrane by effluent organic matter: A detailed characterization using different organic fractions in wastewater. J. Mem. Sci. 278, 232-238

Shore, L.S., Correll, D.L., Chakraborty, P.K., 1995. Fertilization of fields with chicken manure is a source of estrogens in small streams. Animal waste and the land-water interface. 155-162. *ln* K. Steele (ed.) Lewis Publ., Boca Raton, FL.

Shore, L.S., Shemesh, M., 2003. Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. *75*, 1859-1871.

Singer, P.C., 1999. Humic substances as precursors for potentially harmful disinfection by products, Water Sci. Technol. 40, 9, 25–30.

Skotnicka-Pitak, J., Khunjar, W.O., Love, N.G., Aga, D.S., 2009. Characterization of metabolites formed during 17a-ethynylestradiol by nitrosomonas europaea in batch and continuous flow bioreators. Environ. Sci. Technol. 43, 10, 3549-3555.

Smith, L.H., Kitanidis, P.K., McCarty, P.L., 1996. Numerical modeling and uncertainties in rate coefficients for methane utilization and TCE cometabolism by a methane-oxidizing mixed culture. Biotechnology and Bioengineering. 53, 320-331.

Smith, L.H., Kitanidis, P.K., McCarty, P.L., 1998. Spreadsheet method for evaluation of biochemical reation rate coefficients and their uncertainties by weighted

nonlinear least-squares analysis of the intergrated Monod equation. Appl. Environ. Microbiol. 64, 2004-2050.

Sole, M., de Alda, M.J.L., Casillo, M., Porte, C., Ladegaad-Pedersen, K., Barcelo, D., 2000. Estrogenicity determination in sewage treatment plants and surface waters from the Catalonian area (NE Spain). Environ. Sci. Technol. 34, 5076-5083.

Sone, K., Hinago, M., Kitayama, A., Morokuma, J., Ueno, N., Watanabe, H., Iquchi, T., 2004. Effects of 17β-estradiol, nonylphenol, and bisphenol-A on developing Xenopus laevis embryos. Gen comp endocrinol. 138, 3, 228-236.

Stedmon, C.A., Markager, S., Bro, R., 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. Mar. Chem. 82, 239-254.

Suzuki, I., Dular, U., Kwok, S.C., 1974. Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. Journal of Bacteriol. 120, (1), 556-558.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigation in Germany, Canada and Brazil. The Science of the Total Environment. 225, 1-2, 81-90.

Ternes, T.A., Anderson, H.R., Gilberg, D., Bonerz, M., 2002. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal. Chem. 74, 3498-3504.

Thies, K.L., Champlin, F.R., 1989. Compositional factors influencing cell surface hydrophobicity of *Pasteurella multocida variants.* Current Microbiology. 18, 385-390.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal oestrogens in fish. Environ. Sci. Technol. 1142-1149.

Turan, A., 1995. Excretion of natural and synthetic estrogens and their metabolites: occurrence and behavior in water. In Endocrinically Active Chemicals in the Environment. Vol. UBA Texte 3/96. Umweltbundesamt, Berlin.

Turro, N.J., 1991. Modern Molecular Photochemistry. University Science Books: Sausalito, CA.

U. S. Department of Agriculture (USDA), 2002. 2002 Census of Agriculture. National Agricuture Statistics Services. Washington, DC.

U.S. Department of Agriculture (USDA), 2009. Poultry – Production and Value: 2008 summary, Natural Agricultural Statistics Service. Washington, DC.

USDA & USEPA., 1999. Unified National Strategy for Animal Feeding Operations, USDA and USEPA, Washington, DC.

Vader, J.S., van Ginkel, C.G., Sperling, F.M.G.M., de Jong, J., de Boer, W., de Graaf, J.S., van der Most, M., Stokman P.G.W., 2000. Degradation of ethinyl estradiol by nitrifying activated sludge. Chemosphere. 41, 8, 1239-1243.

Vethaak A.D., Rijs G.B.j., Schrap S.M., Ruiter, H., Gerritsen, A., Lahr, J., 2002. Estrogens and xeno-estrogens in the aquatic environment of The Netherlands.

report no 2002.001. Institute of Inland Water Management and Waste Water Management and Water Treatment (RIZA) and the Dutch Institute for Coastal and Marine Management (RIKZ), Lelystad, The Netherlands.

Vialaton, D., Richard, C., 2002. Phototransformation of aromatic pollutants in solar light: photolysis versus photosensitized reactions under natural water conditions. Aquatic Science. 64, 2, 207-215.

Wahman, D.G., Henry, A.E., Katz, L.E., Speitel Jr., G.E., 2005. Cometabolism of trihalomethanes by *Nitrosomonas europaea*, Appl. Environ. Microbiol. 71, 12, 7980–7986.

Wahman, D.G., Henry, A.E., Katz, L.E., Speitel Jr., G.E., 2006. Cometabolism of trihalomethanes by mixed culture nitrifiers. Water Res. 40, 3349-3358.

Wang, L., Wu, F., Zhang, R., Li, W., Liao, H., 2009. Characterization of dissolved organic matter fractions from lake Hongfeng, southwest China plateau. J. Environ. Sci. 21, 5, 581-588

Wenzel, A., Kuchler, T., Henschel, K-P., Schnaak, W., Diedrich, M, Muller, J., 1998. Konzentrationen ostrogen wirkender Substanzen in Umweltmedien. Forschungsbericht 216 02 011/11: German Federal Environmental Agency, Berlin.

Xia, X.H., Yu, H., Yang, Z.F., Huang, G.H., 2006. Biodegradation of polycyclic aromatic hydrocarbons in the natural waters of the Yellow River: effects of high sediment content on biodegradation. Chemosphere. 65, 3, 457-466.

Yang, Y-Y., Borch, T., Young, R., Goodridge, L.D., Davis, J.G., 2010. Degradation kinetics of testosterone by manure-boren bacrteria: influence of temperature, pH, glucose amendmetns, and dissolved oxygen. J. Environ. Qual. 39, 1153-1160.

Yamamoto, H., Liljestrand, H.M., Shimizu, Y., Morita, M., 2003. Effects of physical-chemcial characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates. Environ. Sci. Technol. 37, 12, 2646-2657.

Yi, T., Harper, Jr. W.F., Holbrook R.D., 2006. The role of particle size and nitrification in removal of 17α-ethinylestradiol in bioreactors. ASCE J Environ Eng. 132, 11, 1527–1529.

Yi, T., Harper, Jr. W.F., 2007. The link between nitrification and biotransformation of 17α-ethinylestradiol. Envion. Sci. Technol. 41, 4311-4316.

Ying, G-G., Kookana, R.S., Ru, Y-J., 2002. Occurrence and fate of hormone steroids in the environment. Environmental International. 28, 6, 545-551.

Yong, W.F., Whitehouse, P., Johnson, I., Sorokin, N., 2002. Proposed predicted-no-effect-concentration (PNECs) for natural and synthetic steroid oestrogens in surface waters. WRc-NSF Report No EA5098. 1-172.

Yoshimoto, T., Nagai, F., Fujimoto, J., Watanabe, K., Mizukoshi, H., Makino, T., Kimura, K., Saino, H., Sawada, H., Omura, H., 2004. Degradation of estrogens by *Rhodococcus zopfii* and *Rhodococcus equi* isolates from activated sludge in wastewater treatment plants. Appl. Environ. Microbiol. 70, 9, 5283-5289.

Young, R.B., Borch, T., 2009. Source, presence, analysis, and fate of steroid sex hormones in freshwater ecosystems – A review. In: Aquatic Ecosystem Research

Trends. Nairne, G. H., Ed. Nova Science Publisher, Inc.: Hauppauge, New York, Chapter 4. 103-164.

Yu, C.P., Roh, H., Chu, K.H., 2007. 17β-Estradiol-degrading bacteria isolated from activated sludge. Environ. Sci. Technol. 41, 486-492.

Yutaka, T., Akihiro, T., Haruhiko, F., Kaneshi, T., 2003. Livestock wastes as a source of estrogens and their effects on wildlife of Manko tidal flat, Okinawa. Mar. Pollut. Bull. 47, 143-147.

Zhang, K., Zuo, Y., 2005. Pitfalls and solution for simultaneous determination of estrone and 17a-ethynylestradiol by gas chromatography-mass spectrometry after derivatization with N, O-bis (trimethylsilyl)trifluoroacetamide Anal. Chim. Acta 554, 190-196.

Zhang, Z.L., Hibberd, A., Zhou, J.L., 2006. Optimization of derivatization for the analysis of estrogenic compounds in water by soild-phase-extraction gas chromatography-mass spectrometry Anal. Chim. Acta. 577, 52-61.

Zhou, J.L., Liu, R., Wilding, A., Hibberd, A., 2007. Sorption of selected endocrine disrupting chemicals to different aquatic colloids. Environ. Sci. Technol. 41, 1, 206-213.

Zumoff, B., 1994. Hormonal profiles in women with breast cancer. Obstetrics and Gynecology Clinics of North America. 21, 751-772.

APPENDIX I

1. **α (ammonia fraction of TOT ammonia) derivation**

$$\alpha = \frac{1}{1+10^{(pK_a - pH - s)}} = \frac{[NH_3]}{[TOTNH_3]}$$

$$pK_{a,T} = -\log K_{a,T} = 0.09018 + \frac{2729.92}{T+273.16}$$

$$pH = -\log\{H^+\}$$

$$s = log_{10}\gamma_{NH+} = -AZ^2\left[\frac{I^{1/2}}{1+I^{1/2}} - 0.2I\right]$$

$$A = 1.82483 \times 10^6[E(T+273.16)]\text{^} - 1.5$$

$$E = 87.74 - 0.4008T + 9.398 \times 10^{-4}T^2 - 1.41 \times 10^{-6}T^3$$

$$\mathrm{I} = \frac{1}{2}\sum \mathrm{C_i}\,\mathrm{Z_i^2}$$

2. **Ammonia degradation (ammonia saturation model based on Monod equation)**

$$\frac{dS_{TOTNH3}}{dT} = -\frac{q_{max}XS_{NH3-N}}{K_{s_{NH3-N}}+S_{NH3-N}} = \frac{q_{max}XS_{TOTNH3}\,\alpha}{K_{s_{NH3-N}}+S_{TOTNH3}\alpha}$$

3. **Estrogen degradation**

3.1 First order model

3.1.1 Assumption

- Ammonia does not compete with estrogens
- Estrogens do not compete with the ammonia
- Reductant is not a limiting reactant.

$$\frac{dS_{estrogen}}{dt} = -\frac{k_{estrogen}X\,S_{estrogen}}{K_{s_{estorgen}} + S_{estrogen}}$$

$$if\ Simplifying\ (only\ when K_{s_{estrogen}} \gg S_{estrogen})$$

$$\frac{dS_{TOTNH3}}{dT} = -k_{estrogen}\,XS_{estrogen}$$

3.2 Competition model

3.2.1 Assumptions

-Ammonia compete with estrogens

-Estrogen do not compete with ammonia

-Reductant is not a limiting reactant

$$\frac{dS_{estrogen}}{dt} = -\frac{k_{estrogen} X\, S_{estrogen}}{K_{S_{estorgen}} + S_{estrogen} + \frac{K_{S_{estrogen}}}{K_{S_{NH3-N}}} S_{NH3-N}}$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{K_{S_{estorgen}} + S_{estrogen} + \frac{K_{S_{estrogen}}}{K_{S_{NH3-N}}} S_{TOTNH3}\alpha}$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{K_{S_{estorgen}}\left(1 + \frac{S_{estrogen}}{K_{S_{estrogen}}} + \frac{S_{TOTNH3}\alpha}{K_{S_{NH3-N}}}\right)}$$

$$if\ Simplifying\ (only\ when K_{S_{estrogen}} \gg S_{estrogen})$$

$$\frac{dS_{estrogen}}{dt} = \frac{k_{estrogen} X\, S_{estrogen}}{1 + \frac{S_{TOTNH3}\alpha}{K_{S_{NH3-N}}}}$$

3.3 Reductant model

3.3.1 Assumptions

-Ammonia does not compete with estrogens

-Estrogens do not compete with ammonia

-Two limiting reactants (estrogen and reductant)

$$\frac{dS_{estrogen}}{dt}$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{K_{s_{estorgen}} + S_{estrogen}} \left(\frac{S_{NH3-N}}{K_{s_{NH3-N}} + S_{NH3-N}} \right)$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{K_{s_{estorgen}} + S_{estrogen}} \left(\frac{S_{TOTNH3}\alpha}{K_{s_{NH3-N}} + S_{TOTNH3}\alpha} \right)$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{K_{s_{estorgen}} + S_{estrogen}} \left(\frac{1}{\frac{K_{s_{NH3-N}}}{S_{TOTNH3}\alpha} + 1} \right)$$

$$if\ Simplifying\ (only\ when K_{s_{estrogen}} \gg S_{estrogen})$$

$$\frac{dS_{estrogen}}{dt} = \frac{k_{estrogen} X\, S_{estrogen}}{1 + \frac{K_{s_{NH3-N}}}{S_{TOTNH3}\alpha}}$$

3.4 Combined model

3.4.1 Assumption

-Ammonia compete with estrogens

-Estrogens do not compete with ammonia

-Two limiting reactants (Estrogens and Reductant)

$$\frac{dS_{estrogen}}{dt} = -\frac{k_{estrogen} X\, S_{estrogen}}{\left(1 + \frac{S_{NH3-N}}{K_{s_{NH3-N}}}\right)} \left(\frac{1}{\frac{K_{s_{NH3-N}}}{S_{NH3-N}} + 1} \right)$$

$$= -\frac{k_{estrogen} X\, S_{estrogen}}{\left(1 + \frac{S_{TOTNH3}\alpha}{K_{s_{NH3-N}}}\right)} \left(\frac{1}{\frac{K_{s_{NH3-N}}}{S_{TOTNH3}\alpha} + 1} \right)$$

$$if\ Simplifying\ (only\ when K_{s_{estrogen}} \gg S_{estrogen}$$

$$\frac{dS_{estrogen}}{dt} = \frac{k_{estrogen} X\, S_{estrogen}}{\left(1 + \frac{S_{TOTNH3}\alpha}{K_{s_{NH3-N}}}\right)\left(\frac{K_{s_{NH3-N}}}{S_{TOTNH3}\alpha} + 1\right)}$$

4. $\frac{k_{estrogen}}{k_{TOTNH3}}$ - Batch Reactor Derivation to represent a plug flow reactor with hydraulic residence time (t).

Assumptions: 1. Batch reactor, 2. Ammonia does not compete with estrogens

3. Estrogens do not compete with ammonia. 4. Two limiting reactants

(Estrogen and Reductant)

Ammonia degradation - $\frac{dS_{TOTNH3}}{dT} = -\frac{q_{max} X S_{NH3-N}}{K_{s_{NH3-N}} + S_{NH3-N}}$

Estrogen degradation - $\frac{dS_{estrogen}}{dt} = -k_{estrogen} X S_{estrogen}\left(\frac{S_{NH3-N}}{K_{s_{NH3-N}} + S_{NH3-N}}\right)$

Divide ammonia degradation by estrogen degradation and simplify

$$\frac{\frac{dS_{TOTNH3}}{dT}}{\frac{dS_{estrogen}}{dt}} = \frac{-\frac{q_{max} X S_{NH3-N}}{K_{s_{NH3-N}} + S_{NH3-N}}}{-k_{estrogen} X S_{estrogen}\left(\frac{S_{NH3-N}}{K_{s_{NH3-N}} + S_{NH3-N}}\right)}$$

$$\frac{dS_{TOTNH3}}{dS_{estrogen}} = \frac{k_{TOTNH3}}{k_{estrogen} S_{estrogen}}$$

Separating variables and integrating

$$\int_{S_{TOTNH3}(0)}^{S_{TOTNH3}(t)} dS_{TOTNH3} = \frac{k_{TOTNH3}}{k_{estrogen}} \int_{S_{estrogen}(0)}^{S_{estrogen}(t)} \frac{dS_{estrogen}}{S_{estrogen}}$$

$$S_{TOTNH3}(t) - S_{TOTNH3}(0) = \frac{k_{TOTNH3}}{k_{estrogen}} \left(lnS_{estrogen}(t) - lnS_{estrogen}(0)\right)$$

$$-\Delta TOTNH_3 = \frac{k_{TOTNH3}}{k_{estrogen}} \left(lnS_{estrogen}(t) - lnS_{estrogen}(0)\right)$$

$$-\Delta TOTNH_3 = \frac{k_{TOTNH3}}{k_{estrogen}} ln \frac{S_{estrogen}(t)}{S_{estrogen}(0)}$$

$$-\Delta TOTNH_3 \frac{k_{estrogen}}{k_{TOTNH3}} = ln \frac{S_{estrogen}(t)}{S_{estrogen}(0)}$$

$$\frac{S_{estrogen}(t)}{S_{estrogen}(0)} = e^{-\Delta TOTNH_3 \frac{k_{estrogen}}{k_{TOTNH3}}}$$

APPENDIX II

17α-estrogen (17α-E2)

		OD600		0.4000							obs-1st									
											0.4701									
	mg Es/mg protein/hr	qmax		0.000			qmax/Ks	-0.0022												
	mg/L	Ks		0.123																
	mg BSA/L	X		79.1000																
		mg/L	uM	mg/L	uM															
TOTNH3	Time	Cobs	Cobs	Cpred	Cpred	dt	dC/dt	K1	Ci + K1/2	K2	Ci+K2/2	K3	Ci+K3	K4	2K2	2K3	K	error	Error Sq	STD DEV
780.8452	0	0.66105	2.427119	0.6618	2.4298	6	0.0125	0.0749	0.6992	-0.1094	0.6071	-0.1069	0.5548	-0.1053	-0.2188	-0.2139	-0.0772	-0.0007	0.0000	0.0712
676.7549	6	0.556108	2.041814	0.5846	2.1465	6	0.0117	0.0701	0.6196	-0.1073	0.5310	-0.1044	0.4802	-0.1024	-0.2146	-0.2088	-0.0760	-0.0285	0.0008	0.0426
583.8376	12	0.508038	1.865317	0.5086	1.8676	12	0.0108	0.1295	0.5734	-0.2118	0.4028	-0.1970	0.3116	-0.1844	-0.4236	-0.3941	-0.1454	-0.0006	0.0000	0.0059
511.526	24	0.434885	1.596729	0.3632	1.3336	24	0.0095	0.2279	0.4772	-0.4090	0.1587	-0.2897	0.0735	-0.1923	-0.8180	-0.5795	-0.2270	0.0717	0.0051	0.0114
425.6617	48	0.070639	0.25936	0.1363	0.5003	24	0.0061	0.1475	0.2100	-0.3244	-0.0259	0.1372	0.2734	-0.3548	-0.6487	0.2744	-0.0969	-0.0656	0.0043	0.0007
409.2815	72	0.022665	0.083216	0.0393	0.1444	24	0.0028	0.0664	0.0725	-0.1907	-0.0560	0.4294	0.4688	-0.4075	-0.3814	0.8589	0.0227	-0.0167	0.0003	0.0007
																			0.0105313	

Estriol (E3)

		OD600		0.4000							obs-1st									
											0.4701									
mg Es/mg prot		qmax		-0.033			qmax/Ks	-0.00065												
mg/L		Ks		50.009																
mg BSA/L		X		38.4810			0.381													
					38.8620															
		mg/L		mg/L																
TOTNH3	Time	Cobs		Cpred		dt	dC/dt	K1	Ci + K1/2	K2	Ci+K2/2	K3	Ci+K3	K4	2K2	2K3	K	error	Error Sq	STD DEV
808.8756	0	0.561595	1.947413	0.5552	1.9251	6	0.0137	0.0825	0.5964	-0.0888	0.5108	-0.0762	0.4790	-0.0715	-0.1776	-0.1523	-0.0532	0.0064	0.0000	0.0712
719.541	6	0.503781	1.746935	0.5020	1.7408	6	0.0124	0.0746	0.5393	-0.0804	0.4618	-0.0689	0.4331	-0.0647	-0.1608	-0.1379	-0.0481	0.0018	0.0000	0.0426
634.5638	12	0.479967	1.664355	0.4539	1.5740	12	0.0113	0.1350	0.5214	-0.1555	0.3762	-0.1125	0.3414	-0.1022	-0.3110	-0.2250	-0.0838	0.0261	0.0007	0.0059
556.3836	24	0.310096	1.075304	0.3701	1.2832	24	0.0092	0.2205	0.4803	-0.2867	0.2267	-0.1360	0.2341	-0.1404	-0.5734	-0.2720	-0.1275	-0.0600	0.0036	0.0114
420.1658	48	0.252053	0.87403	0.2425	0.8410	24	0.0060	0.1448	0.3149	-0.1886	0.1482	-0.0891	0.1535	-0.0922	-0.3771	-0.1781	-0.0838	0.0095	0.0001	0.0007
456.8808	72	0.181434	0.629151	0.1587	0.5504	24	0.0040	0.0949	0.2062	-0.1238	0.0969	-0.0583	0.1005	-0.0604	-0.2475	-0.1165	-0.0549	0.0227	0.0005	0.0007
																			0.0049251	

17α-Ethynylestradiol (EE2)

		OD600		0.4000							obs-1st								
											0.4701								
mg Es/mg prote		qmax		0.000			qmax/Ks	-0.00658											
	mg/L	Ks		0.027															
	mg BSA/L	X		89.2840		dX	0.884												
		mg/L		mg/L															
TOTNH3	Time	Cobs		Cpred		dt	dC/dt	K1	Ci + K1/2	K2	Ci+K2/2	K3	Ci+K3	K4	2K2	2K3	K	error	Error Sq	STD DEV
822.6856	0	0.648541	2.187985	0.6731	2.2708	6	0.0106	0.0638	0.7050	-0.0922	0.6270	-0.0918	0.5813	-0.0915	-0.1845	-0.1836	-0.0660	-0.0246	0.0006	0.0712
716.6761	6	0.59664	2.012887	0.6071	2.0483	6	0.0101	0.0607	0.6375	-0.0919	0.5612	-0.0914	0.5158	-0.0910	-0.1837	-0.1827	-0.0661	-0.0105	0.0001	0.0426
619.2883	12	0.541529	1.826959	0.5410	1.8252	12	0.0095	0.1146	0.5983	-0.1832	0.4494	-0.1806	0.3604	-0.1781	-0.3665	-0.3613	-0.1319	0.0005	0.0000	0.0059
537.1211	24	0.46124	1.556086	0.4091	1.3803	24	0.0089	0.2132	0.5157	-0.3640	0.2271	-0.3422	0.0669	-0.2725	-0.7279	-0.6844	-0.2453	0.0521	0.0027	0.0114
419.7312	48	0.127577	0.430406	0.1639	0.5529	24	0.0072	0.1737	0.2507	-0.3457	-0.0090	0.1882	0.3520	-0.3557	-0.6913	0.3763	-0.0828	-0.0363	0.0013	0.0007
440.1424	72	0.080828	0.272688	0.0810	0.2734	24	0.0064	0.1534	0.1578	-0.3268	-0.0824	-0.5720	-0.4910	-0.4056	-0.6536	-1.1440	-0.3416	-0.0002	0.0000	0.0007
																			0.0047468	

Estrone (E1)

		OD600		0.4000							obs-1st								
											0.4701								
mg Es/mg prote		qmax		0.000			qmax/Ks	0.000117											
	mg/L	Ks		-0.224															
	mg BSA/L	X		80.3960			0.00017134												
					0.7960		0.00017807												
		mg/L		mg/L															
TOTNH3	Time	Cobs		Cpred		dt	dC/dt	K1	Ci + K1/2	K2	Ci+K2/2	K3	Ci+K3	K4	2K2	2K3	K	error	Error Sq	STD DEV
804.5355	0	0.577245	2.13502	0.5741	2.1235	6	0.0034	0.0207	0.5845	-0.0206	0.5639	-0.0211	0.5531	-0.0213	-0.0412	-0.0421	-0.0140	0.0031	0.0000	0.0712
694.3220	6	0.555765	2.055571	0.5501	2.0718	6	0.0035	0.0210	0.5706	-0.0209	0.5497	-0.0214	0.5387	-0.0217	-0.0418	-0.0428	-0.0142	-0.0044	0.0000	0.0426
599.4313	12	0.544193	2.012773	0.5459	2.0192	12	0.0036	0.0427	0.5673	-0.0420	0.5249	-0.0443	0.5016	-0.0459	-0.0839	-0.0886	-0.0293	-0.0017	0.0000	0.0059
533.7617	24	0.526365	1.946831	0.5166	1.9109	24	0.0037	0.0889	0.5611	-0.0845	0.4744	-0.0962	0.4204	-0.1088	-0.1690	-0.1925	-0.0636	0.0097	0.0001	0.0114
467.1703	48	0.457094	1.690626	0.4531	1.6758	24	0.0041	0.0996	0.5029	-0.0916	0.4073	-0.1129	0.3402	-0.1490	-0.1832	-0.2259	-0.0764	0.0040	0.0000	0.0007
436.4352	72	0.37887	1.4013	0.3767	1.3932	24	0.0052	0.1242	0.4388	-0.1038	0.3248	-0.1641	0.2126	0.9173	-0.2076	-0.3281	0.0843	0.0022	0.0000	0.0007
																			0.0001471	

$$r_{nitrogen} = \frac{k_{1,nitrogen} X S_{nitrogen}}{1 + \frac{K_{NH3-N}}{S_{TOTNH3}} + \frac{K_{2,nitrogen}}{S_{TOTNH3}}}$$

17β-estradiol (E2)

		OD600		0.4000							obs-1st									
											0.4701									
mg Es/mg prot		qmax		0.000			qmax/Ks	-0.02239												
mg/L		Ks		0.016																
mg BSA/L		X		32.1180			31.8													
					0.3180															
		mg/L		mg/L																
TOTN-t3	Time	Cobs		Cpred		dt	dC/dt	K1	Ci + K1/2	K2	Ci+K2/2	K3	Ci+K3	K4	2K2	2K3	K	error	Error Sq	STD DEV
822.2558	0	0.604015	2.217464	0.6250	2.2946	6	0.0083	0.0498	0.6499	-0.0686	0.5907	-0.0685	0.5566	-0.0684	-0.1373	-0.1369	-0.0488	-0.0210	0.0004	0.0712
740.5964	6	0.56357	2.068984	0.5762	2.1155	6	0.0080	0.0483	0.6004	-0.0685	0.5420	-0.0683	0.5079	-0.0682	-0.1370	-0.1366	-0.0489	-0.0127	0.0002	0.0426
658.7612	12	0.541085	1.986435	0.5273	1.9359	12	0.0077	0.0929	0.5738	-0.1368	0.4589	-0.1359	0.3914	-0.1351	-0.2736	-0.2718	-0.0979	0.0138	0.0002	0.0059
576.7453	24	0.476554	1.749527	0.4294	1.5763	24	0.0074	0.1766	0.5177	-0.2728	0.2930	-0.2666	0.1628	-0.2558	-0.5456	-0.5332	-0.1930	0.0472	0.0022	0.0114
413.0109	48	0.206295	0.757353	0.2364	0.8678	24	0.0063	0.1510	0.3119	-0.2674	0.1027	-0.2429	-0.0065	0.1849	-0.5349	-0.4857	-0.1141	-0.0301	0.0009	0.0007
408.4143	72	0.121845	0.447319	0.1223	0.4489	24	0.0059	0.1421	0.1933	-0.2595	-0.0075	0.2391	0.3614	-0.2693	-0.5191	0.4782	-0.0280	-0.0004	0.0000	0.0007
																			0.0039220	

APPENDIX III

- **Ammonia-nitrogen, nitrate-nitrogen, MLSS concentration from influent and effluent samples in Both SBRs (Unit H and Unit H+A)**

Influent

Time (day)	NH4+-N (mg/L)	NH4+-N (mg/L)	NO3--N (mg/L)	NO3--N (mg/L)	MLSS (mg/L)	MLSS (mg/L)
	Unit H	Unit H+A	Unit H	Unit H+A	Unit H	Unit H+A
1	35	33.3	BD	BD	1960	1940
2	36.8	36.7	BD	BD	1960	1930
3	35.9	37.6	BD	BD	1850	1750
4	36.7	35	BD	BD	1950	1880
5	32.1	32.4	BD	BD	1954	1849
6	30.8	33.1	BD	BD	1950	1920
7	32.1	32.7	BD	BD	1900	1870
9	34.8	34.6	BD	BD	1920	1840
10	31.2	31.4	BD	BD	1915	1950
11	30	32.2	BD	BD	1920	1840
12	22.1	27.4	BD	BD	1900	1850
13	31.8	33.1	BD	BD	1820	1805
14	36.5	35.4	BD	BD	1850	1810
Average	32.8	33.4	BD	BD	1911.5	1864.2
STDEV	3.9	2.5	BD	BD	44.6	56.8

Effluent

	Unit H	Unit H+A	Unit H	Unit H+A	Unit H	Unit H+A
1	BD	BD	34.3	29.4	2105	2108
2	BD	BD	33.6	30.1	2102	2084
3	BD	BD	35.8	35.1	1950	1850
4	BD	BD	35.8	32.4	2054	2002
5	BD	BD	36.5	34.2	2032	1970
6	BD	BD	39.4	35.6	2045	2030
7	BD	BD	40.3	40.7	2001	2030
9	BD	BD	40	41.1	2002	1998
10	BD	BD	35.1	33.9	2029	2109
11	BD	BD	31.1	28.2	2104	2003
12	BD	BD	33.6	30.5	2150	1987
13	BD	BD	33.6	35.3	1985	1956
14	BD	BD	32.9	31.6	1992	1948

- **Hormone, TOC, TN concentration from effluent concentration in Both SBRs (From SNWA)**

Sample ID		09070346-001	09070346-002	09070396-001	09070396-002	09070396-003	09070396-004
Date Collected		8/3/2009 11:30	8/3/2009 11:30	8/4/2009 11:30	8/4/2009 11:30	8/4/2009 11:30	8/4/2009 11:30
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H dup.	Effluent H+A	Effluent H+A dup.
Tap Location		Day 1	Day 1	Day 2	Day 2	Day 2	Day 2
Total Nitrogen	mg/L	36	37	35	35	30	31
Total Organic Carbon	mg/L	72	110	7.3	6.8	6.8	6.7
Testosterone	ng/L	308	346	BDL	5.5	8.5	11
Progesterone - APCI	ng/L	350	350	BDL	BDL	7.6	5.4
Ethynylestradiol	ng/L	607	698	18	17	19	18
Estrone	ng/L	835	1160	BDL	BDL	7.3	5.6
Estradiol	ng/L	282	303	BDL	BDL	BDL	BDL

Sample ID		09070405-001	09070405-002	09070406-001	09070406-002
Date Collected		8/7/2009 11:00	8/7/2009 11:00	8/8/2009 11:30	8/8/2009 11:30
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H+A
Tap Location		Day 5	Day 5	Day 6	Day 6
Total Nitrogen	mg/L	35	36	38	35
Total Organic Carbon	mg/L	70	110	6.9	6.3
Testosterone	ng/L	537	535	11	13
Progesterone - APCI	ng/L	479	460	5.3	11
Ethynylestradiol	ng/L	835	815	15	21
Estrone	ng/L	928	920	5.6	13
Estradiol	ng/L	500	492	BDL	BDL

Sample ID		09080260-001	09080260-002	09080260-003	09080260-004
Date Collected		8/9/2009 0:00	8/9/2009 0:00	8/10/2009 15:03	8/10/2009 15:03
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H+A
Tap Location		Day 7	Day 7	Day 8	Day 8
Total Nitrogen	mg/L	34	30	41	39
Total Organic Carbon	mg/L	24	24	6.4	6.4
Testosterone	ng/L	NA	NA	NA	NA
Progesterone - APCI	ng/L	NA	NA	NA	NA
Ethynylestradiol	ng/L	NA	NA	NA	NA
Estrone	ng/L	NA	NA	NA	NA
Estradiol	ng/L	NA	NA	NA	NA

Sample ID		09080260-005	09080260-006	09080260-007	09080260-008
Date Collected		8/11/2009 12:00	8/11/2009 12:00	8/11/2009 12:00	8/11/2009 12:00
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H+A
Tap Location		Day 9	Day 9	Day 9	Day 9
Total Nitrogen	mg/L	37	36	40	39
Total Organic Carbon	mg/L	43	46	6.8	6.5
Testosterone	ng/L	NA	NA	NA	NA
Progesterone - APCI	ng/L	NA	NA	NA	NA
Ethynylestradiol	ng/L	NA	NA	NA	NA
Estrone	ng/L	NA	NA	NA	NA
Estradiol	ng/L	NA	NA	NA	NA

Sample ID		09080090-001	09080090-002	09080090-003	09080090-004	09080091-001	09080091-002
Date Collected		8/6/2009 0:00	8/6/2009 0:00	8/12/2009 9:57	8/12/2009 9:57	8/7/2009 0:00	8/7/2009 0:00
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H+A	Effluent H	Effluent H+A
Tap Location		Day 10	Day 10	Day 10	Day 10	Day 11	Day 11
Total Nitrogen	mg/L	38	37	40	41	38	35
Total Organic Carbon	mg/L	48	120	6.3	7.0	6.3	6.1
Testosterone	ng/L	517	404	NA	NA	BDL	BDL
Progesterone - APCI	ng/L	460	398	NA	NA	BDL	BDL
Ethynylestradiol	ng/L	850	745	NA	NA	BDL	BDL
Estrone	ng/L	1556	1100	NA	NA	BDL	BDL
Estradiol	ng/L	310	311	NA	NA	BDL	BDL

Sample ID		09080092-001	09080092-002	09080093-001	09080093-002	09080093-003	09080093-004
Date Collected		8/10/2009 0:00	8/10/2009 0:00	8/17/2009 11:00	8/17/2009 11:00	8/17/2009 11:00	8/17/2009 11:00
Sub Location		Influent H	Influent H+A	Effluent H	Effluent H dup.	Effluent H+A	Effluent H+A dup.
Tap Location		Day 14	Day 14	Day 15, 24 hour	Day 15, 24 hour	Day 15, 24 hour	Day 15, 24 hour
Total Nitrogen	mg/L	48	46	34		32	
Total Organic Carbon	mg/L	75	120	6.0		6.3	
Testosterone	ng/L	549	518	11	10	BDL	BDL
Progesterone - APCI	ng/L	556	547	5.4	5.6	BDL	BDL
Ethynylestradiol	ng/L	763	861	16	18	BDL	BDL
Estrone	ng/L	987	1010	BDL	BDL	BDL	BDL
Estradiol	ng/L	496	517	BDL	BDL	BDL	BDL

- **Matlab code (3D-EEM analysis for EfOM samples from two SBRs) from Eric Dickenson in SNWA.**

```
% This code was adopt from Eric (Colorado School of Mines). Mei modified it
% for SNWS R&D group.
% Areas in Purple Color need to be changed with the proper information before
% running the program.

% Purpose of this code is to correct and plot an EEM without sample UV correction
files. Corrected EEMs and
% figures are saved to set folders.
% read in excel EEM (check the format of the file)

%INPUT

% change directories and file names to read in uncorrected sample eem.
% Uncorrected eem data must be in the Sheet 'Uncorrected Data')
% define ifile.

A_full = xlsread('V:\R & D\LK\3D-Flurorescence\SRT\sample\012.xls', 'Uncorrected
Data');
ifile = '012';

% read in blank eem
BLANK_full = xlsread('V:\R & D\LK\3D-Flurorescence\SRT\sample\DI Blank.xls',
'Uncorrected Data');

%type in the integrated raman number from daily instrument test
%raman area average based on 070507 raman scatter
raman_area = 2023526;

% change directories and file names to read in instrument correction files
% (specific to fluor instrument 2 or 3)
MC = xlsread('V:\R & D\LK\3D-Flurorescence\SRT\Emcorr.xls', 'Emcorr');
XC = xlsread('V:\R & D\LK\3D-Flurorescence\SRT\Excorr.xls', 'Excorr');

% OPTIONAL UV Correction.  This requires an individual UV correction file
% for each sample.  This is NOT really necessary for samples with
% UVA@254 < .1/cm
%UC = xlsread('C:\Documents and Settings\xinm\My Documents\MATLAB\Mei
Files\Water Quality Project\March 2008\CO Aurora Fin 1\Corrections_UV_CO Aurora
Fin 1.xls', 'Correction');
```

```
% Input intensity maximum for plot scale
max = 0.5; % Figure EEM intensity scale

%CUT EEM MATRIX AND DEFINE WAVELENGTH INDICES

% Define size of A_full
size_A_full = size(A_full);
[row1,col1]= size(A_full);

% define wavelength indices
% (this may be diff't if you use diff't wavelengths)
ex = A_full(1, 2:col1);
em = A_full(2:row1, 1);

% chop wavelength (Row,Col), rename interior matrix, "A_raw"
A_raw = A_full;
A_raw(:,1) = [];
A_raw(1,:) = [];

% assign variable names for the number of wavelength indices for the
% non-interpolated (condensed) matrix and display (should be 76,51)
[num_em, num_ex] = size(A_raw);


% assign variable names for the starting and ending wavelength and display
% to check wavelength ranges
ex_start = ex(1);
ex_end = ex(num_ex);
em_start = em(1);
em_end = em(num_em);


%CUT BLANK MATRIX
% assign variable name to cut matrix (BLANK) and define size.

BLANK = BLANK_full(:,2:col1);
BLANK = BLANK(2:row1,:);
size_BLANK = size(BLANK);


%SUBTRACT BLANK FROM EEM
```

```
% create new matrix with zeros "A_sub"
A_sub=zeros(num_em,num_ex);

%subtract each value in matrix (A_raw) by the corresponding value in BLANK
%to get corrected matrix (A_sub).

for i=1:num_ex
   for j=1:num_em
      A_sub(j,i)=A_raw(j,i)-BLANK(j,i);
   end
end


%INTERPOLATION

% define x,y, and z of matrix
x = ex;
y = em;
z = A_sub;

% assign the intervals to interpolate to
[xi, yi] = meshgrid(ex_start:2.5:ex_end, em_start:1:em_end);


% interpolate z from x,y to xi,yi, rename interpolated matrix: "A_int",
A_int = interp2(x, y, z, xi, yi, 'spline');

%Put zeros in for deadzones:
%Num_xi = find(xi==ex_end);
 %      Num_yi = find(yi==em_end);

% assign variables to size of interpolated matrix and DISPLAY
[num_em, num_ex] = size(A_int);
num_em;
num_ex;

%Put zeros in for deadzones:
%Num_xi = find(xi==ex_end);
 %      Num_yi = find(yi==em_end);

      for i=1:num_ex
            for j=1:num_em
                  if xi(i)>yi(j)
```

```
                A_int(j,i) = 0;
              else A_int(j,i) = A_int(j,i);
               end
            end
    end

    k = find(A_int<0);
    A_int(k)=0;

%A_int(1:1,41:44)=0;
%A_int(1:11,45:48)=0;
%A_int(1:21,49:52)=0;
%A_int(1:31,53:56)=0;
%A_int(1:41,57:60)=0;
%A_int(1:51,61:64)=0;
%A_int(1:61,65:68)=0;
%A_int(1:71,69:72)=0;
%A_int(1:81,73:76)=0;
%A_int(1:91,77:80)=0;
%A_int(1:101,81:84)=0;
%A_int(1:111,85:85)=0;



% X and M INSTRUMENT CORRECTIONS

%convert correction files to diagonal matrices for matrix multiplication
X=diag(XC);
M=diag(MC);

% the matrix (A_int) is multiplied by the diagonal x-correct file (X).
% the result is transposed before multiplied by the diagonal mcorrect file (M).
% the result is transposed back to create the X-, M- corrected matrix (A_XM).
A_XM=[[A_int*X]'*M]';



%OPTIONAL INTERFILTER CORRECTION (FOR UV ABSORBANCE)

% create new matrix with zeros "A_UV"
A_UV=zeros(num_em,num_ex);

%divide each value in matrix (A_XM) by the corresponding value in the UV
```

```
%correction matrix (UC) to get UV corrected matrix (A_UV).

% for i=1:num_ex
%    for j=1:num_em
%        A_UV(j,i)=A_XM(j,i)/UC(j,i);
%    end
% end


%NORMALIZE MATRIX BY RAMAN AREA
A_final = A_XM/raman_area;     %IF NO UV CORRECTION
%A_final = A_UV/raman_area;    %IF USING UV CORRECTION

xi(1,:)
yi(:,1);
A_final_new = zeros(num_em+1,num_ex+1);
A_final_new(2:num_em+1,2:num_ex+1) = A_final;
A_final_new(2:num_em+1,1)=yi(:,1);
A_final_new(1,2:num_ex+1)=xi(1,:);

%EXPORT NORMALIZED / CORRECTED MATRIX
% export data in ascii format

pathname = 'Final Matrix';
for i=1:length(A_final_new)
   pathname(length(pathname) + 1) = A_final_new(i);
end

%append .xls
%If pathname is a vector of characters, you can manipulate it by the index.
%You want to add 4 characters to the next spot after the end of the
%pathname.
pathnamelength = length(pathname);
pathname(pathnamelength + 1: pathnamelength + 4) = '.xls';

save(pathname, 'A_final_new', '-ascii', '-double', '-tabs');


%PLOT NORMALIZED / CORRECTED MATRIX

%Set wavelength range and increments for plot
ex1 = ex(1):2.5:ex(length(ex));
em1 = em(1):1:em(length(em));
```

```
%transpose normalized / corrected matrix (A_final) for plotting
Aplot=A_final';

% Create graph ...
figure;

% now draw the graph with 30 contour lines or more if needed
contourf(em1,ex1,Aplot,100);

%Label x and y axes
xlabel('Emission Wavelength (nm)','fontsize',14);
ylabel('Excitation Wavelength (nm)','fontsize',14);
title(ifile, 'fontsize',14);

%set handle
handle = gca;
set(handle,'fontsize', 14);

% set color scheme
colormap(jet);

% set min and max intensity values
caxis([0 max]);

% prevent auto-setting of caxis by changing caxis to manual control
caxis('manual');

%Format colorbar legend
H = colorbar('vert');
set(H,'fontsize',14);
text(648,290,'Fluorescence Intensity','fontsize',14,'rotation',90);%change from
'603'to'648'

%saves current object, this won't work if you close the figure first.
%this command saves the current object only

pathname = 'Figure ';

for i=1:length(ifile)
   pathname(length(pathname) + 1) = ifile(i);
```

```
end
pathnamelength = length(pathname);
%pathname(pathnamelength + 1: pathnamelength + 4) = '.jpeg';
saveas(gcf, pathname, 'jpeg');
saveas(gcf, pathname, 'fig');
```

VITA

Name:	Sang Hyun Kim
Address:	Environmental and Water Resource Engineering Division Department of Civil Engineering WREC Room 212F Texas A&M University College Station, Texas, 77843-3136
Email Address:	ludwig114@hotmail.com
Education:	B.S., Mineral and Energy Resource Engineering, 2000 Chonnam National University M.Eng., Civil Engineering, 2004 Texas A&M University Ph.D., Civil Engineering, 2010 Texas A&M University