SORPTION AND BIODEGRADATION OF PHARMACEUTICAL COMPOUNDS IN

BIOLOGICAL WASTEWATER TREATMENT PROCESS

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SORPTION AND BIODEGRADATION OF PHARMACEUTICAL COMPOUNDS IN BIOLOGICAL WASTEWATER TREATMENT PROCESS

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SORPTION AND BIODEGRADATION OF PHARMACEUTICAL COMPOUNDS IN BIOLOGICAL WASTEWATER TREATMENT PROCESS

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Taewoo Yi, son of JongWong Yi and MungSuk Lim, was born on Jan 12, 1972, in Boeun, Korea. He has one brother, Chanwoo Yi and one sister, Kyungkak Yi. After he completed elementary and high School in 1990, In March 1990 he entered Daejon University in Daejon, Korea and in February 1998 was awarded a Bachelor of Science degree in Environmental Engineering. He served as a military engineer in the Korean Army from April 1992 to June 1994. From September 1997, until July 1985, He attended the Graduate school in Biotechnology and Bioengineering at Korea University and then entered the Graduate studies at Iowa State University in September 2001 and received the degree of Master of Science (Civil and Environmental Engineering) in May 2003. In April 2007, he was awarded CH2M Hill Fellowship Award. Then, he came to Auburn University, Alabama and began work as a graduate student, in pursuit of the degree of Doctor of Philosophy in August 2003.

DISSERTATION ABSTRACT

SORPTION AND BIODEGRADATION OF PHARMACEUTICAL COMPOUNDS IN BIOLOGICAL WASTEWATER TREATMENT PROCESS

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This study was performed to investigate the removal mechanisms of pharmaceutical compounds (PhACs) in biological treatment processes. The removal efficiencies and byproducts of three model compounds including 17α-ethinylestradiol (EE2), Carbamazepine (CBZ), and Trimethoprim (TMP) were monitored in laboratory scale membrane bioreactor (MBR), sequencing batch reactor (SBR), and conventional bioreactor (CBR).

Laboratory scale bioreactors were used to investigate sorption and biodegradation of EE2. Results showed that the sludge taken from the MBR had partitioning coefficient (K_d) that was more than twice that of biomass derived from SBRs.

The MBR biomass had smaller particles and was more hydrophobic than the SBR biomass. Experiments with nitrifying sludge showed that sorption was more important when the initial ammonia concentration was 48 mg/L or less, but at higher initial

ammonia concentrations the role of biodegradation became more important. The ammonia monooxygenase (AMO) containing protein extract removed EE2 in batch tests.

The influence of biomass characteristics on K_d and sorption-hysteresis of EE2 using MBR and SBR was investigated under normal and nutrient deficiency condition at different SRT. Under normal growth condition, the biomass mean particle size had a dramatic effect on K_d and on sorption hysteresis index (HI). The EE2 partitioning coefficient and sorption hysteresis showed the considerable nonlinear relationship with the mean particle size. Visualization study confirmed this phenomenon. Although under nitrogen deficiency condition, K_d and HI had weak correlation with particle size, overall results showed that the magnitude of the K_d and sorption-hysteresis is affected by the particle size. This study also numerically explored the impacts of sorption hysteresis.

Batch experiments showed that ring A of EE2 is the site of electrophilic initiating reactions, including conjugation and hydroxylation. Ring A was also cleaved before any of the other rings are broken, which is likely because the Frontier Electron Density (FED) of the ring A carbon units is higher than those of rings B, C, or D. EE2 and NH_3 were degraded in the presence of an AMO containing protein extract, and the reaction stoichiometry was consistent with a conceptual model. Continuous tests showed a linear relationship between nitrification and EE2 removal in enriched nitrifying cultures.

Removal efficiencies of EE2, CBZ, and TMP were monitored in nitrifying sludge reactor and conventional bioreactor fed with toluene. EE2 was most efficiently removed in both reactors. The prediction tool combined with FED and degradation rules was applied to predict biodegradation reaction. Degradation reaction took place in the high FED region in three model compounds.

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I. INTRODUCTION

The presence of pharmaceutical compounds (PhACs) and their byproducts in water bodies may cause reproductive anomalies in invertebrates and possibly humans. Public awareness and concern has grown significantly over the past three decades and has brought this issue to the forefront in the water quality community. These concerns have been intensified even more over the past 15 years as improvements in analytical methods coupled with larger scale surveys revealed the broad range of apparently persistent PhACs that are present in the water cycle (Ternes *et al*., 1999a). Toxicologists are now working to quantify the risks associated with long term exposure, and governmental agencies are positioning themselves to eventually develop policies and perhaps regulations. Removing PhACs during biological wastewater treatment is probably important for preventing the proliferation of these chemicals in our environment. Accordingly, the wastewater treatment community has responded to these concerns with a great deal of applied research. Analytical methods are now available for low level detection of PhACs in wastewater, and it is now known that many PhACs are removed only partially in the activated sludge process (Ternes *et al*, 1999b). It is essential to understand the fate and behavior of pharmaceutical compounds discharged into a wastewater treatment processes. PhACs in the wastewater treatment plants are controlled by several important mechanisms including sorption/desorption, aerobic and anaerobic

biotransformation, abiotic-biodegradation, and volatilization. Sorption and biodegradation are main removal mechanisms in activated sludge processes.

It is well-known that operating conditions such as nutrient deficiency and solid retention time (SRT) affect biomass characteristics such as particle size and surface properties. Nitrogen deficiency causes the production of extracellular polymeric substances (EPS) (Ramirez-Castillo and Uriblarrea, 2004; Pratt *et al.*, 2007). The relationship between SRT and floc size has been investigated (Massé *et al.*, 2006; Liao *et al*., 2006; Liao *et al*., 2002) and it was believed that SRTs have an effect on floc size. Surface properties usually were represented by hydrophobicity. Hydrophobic sludge properties can enhance the removal of organic compounds, especially since organic pollutants have a relatively high octanol/water coefficient (Holthaus *et al.,* 2002). Properties of EPS could represent the surface properties of biomass. The biomass characteristics may have a significant effect on sorption. In addition, Particle size was considered as another main factor, because different size particle shows different sorbability to organic compounds. However, the relationship between sorption (or sorption-hysteresis) and surface properties or particle size have not been clearly investigated so far, although biomass characteristics have highly effect on sorption of pharmaceutical compounds.

PhAC biodegradation is likely due to cometabolic activity because PhACs are not present in high enough concentration to support substantial biomass growth. Much of the previous work on PhAC biodegradation has focused on the disappearance of parent compounds and/or the detection of daughter products. This data has been used to try to construct metabolic pathways, but these pathways are incomplete with missing

intermediates. Recently, Haiyan *et al.* (2006) proposed a new degradation pathway in which ethynyl group in EE2 was important. Previously, phenolic ring has been considered as the most reactive part. Construction of a complete metabolic pathway requires a prediction tool with which to predict and understand transformation. Frontier electron density (FED) has great promise for providing the organizing principle that is needed. Fukui pioneered much of the early work connecting FED to chemical reactivity in aromatic hydrocarbons. Wheland and Pauling (1935) successfully used FED to explain the reactivity of substituted aromatics. More recently, Ohura *et al*. (2005) showed that air-bore polycyclic aromatic hydrocarbons were abiotically chlorinated in positions that corresponded to high FED. Lee *et al.* (2001) used Fenton oxidation to remove polycyclic aromatic hydrocarbons, and they successfully used FED to predict the order of daughter product production. Although these previous attempts focused on abiotic reactions, they bolster the promise for predicting biological oxidations in the same way. Prior efforts to conduct predictions of biodegradation have generally focused on readily degradable substrates (e.g. glucose) that enter well-characterized metabolic pathways (e.g. glycolysis). FED presents the promise of predicting biodegradation on complex organics like PhACs; a contribution here will make a significant impact, most certainly stretching well beyond issues related to wastewater treatment.

Objectives and scope

The objectives of this research were (1) to evaluate the effect of hydrophobicity of biomass on EE2 sorption and biomass characteristics and to characterize the effect of particle size on partitioning coefficients and sorption-hysteresis for EE2 and potential

effects of sorption-hysteresis on effluent water quality, (2) to investigate the degradation mechanism of three model compounds in nitrifying sludge and conventional bioreactor fed with toluene, and (3) to develop a prediction tool for predicting biologically-mediated PhACs transformation in biological treatment processes. An improved understanding of the fundamentals of the biodegradation and sorption is essential to develop economical design of processes and to efficiently operate already installed wastewater treatment plants.

A specific objective of this paper is to determine the influence factors for sorption and desorption and the role of monooxygenase, especially ammonia monooxygenase and toluene monooxygenase, in degradation of model compounds.

The specific tasks accomplished during this research are as follows:

1) Conducted a comprehensive literature search pertinent to research carried out on the removal mechanisms.
2) Compared the capacity of sorption and sorption-hysteresis of the sludge from different types of reactor under various operating conditions.
3) Investigated degradation mechanisms and applied frontier electron density to predict biotransformation.

Organization

This dissertation is formatted in journal paper except chapter 1 and 7. For the purpose of accomplishing the research objectives, it was needed to distinguish four separate subtasks reflecting characteristics of sorption and sorption-hysteresis and degradation mechanisms in biological treatment processes. Each subtask was carried out

as an independent investigation presented in Chapters 3 through 6. This dissertation is presented in the following order:

Chapter 2 presents the currently investigated full-scale studies of sorption and biodegradation, cell properties effect on sorption, and interest enzymes which are well known in cometablic mechanism based on a review of the literature. Chapter 3 presents introductory experiments; sorption with MBR and SBRs and biodegradation using nitrifying sludge reactor and enzyme extracts. Chapter 4 describes the relationship between biomass properties under various operating conditions, including the SRT and nutrient limiting condition, and sorption (or sorption-hysteresis). Chapter 5 presents biotransformation mechanism of 17α-ethinylestradiol (EE2) in nitrifying sludge reactor. FED analyses was performed to predict biodegradation reaction and to investigate the applicability of FED to biological reaction.

Chapter 6 discusses prediction tool and removal of carbamazepine, trimethoprim, and 17α-ethinylestradiol in nitrifying sludge reactor and conventional bioreactor fed with toluene. Finally, Chapter 7 presents the summary and conclusions for all studies done and recommendations for future study.

II. LITERATURE REVIEW

Full-scale studies of sorption and biodegradation.

Numerous reports have explored the removal of various classes of pharmaceutical compounds (PhACs) at full scale, generally attempting to evaluate whether municipal wastewater treatment plants (WWTPs) are acting as persistent point sources for PhACs discharge to the environment. Ternes (1998) showed that the removal efficiencies ranged from 10 to 90% in wastewater treatment plants in Germany, and Ternes *et al*. (1999) showed that removal efficiencies for polar PhACs varied from 12 to 90% for WWTPs in Brazil. Gomez *et al*. (2006) conducted a one-year monitoring study at a sewage treatment plant in Spain, and they found that the removal efficiencies for 14 organic micro-pollutants varied from 20% (carbamazepine) to 99% (acetaminophen). Joss *et al.* (2006) showed that only 4 out of 35 compounds are 90% removed using state-of-the-art biological treatment systems, and 17 out of 35 are removed at less than 50% efficiency. These studies are in addition to others that present high removal efficiencies. Oppenheimer and Stephenson (2006) found that removal efficiencies for frequently detected PhACs were generally high (>80%), and another study by Jones *et al*. (2006) found that ibuprofen, paracetamol, salbutamol and mefenamic acid were removed at approx. 90% within a large sewage treatment plant in England. Overall, these efforts have shown that the removal efficiencies vary greatly.

That conclusion that PhAC removal in full-scale systems varies considerably is further supported by Lishman *et al*. (2006) who investigated the presence of selected acidic drugs, triclosan, polycyclic musks, and selected estrogens in WWTP influent and effluent at sites in Canada. They found that three analytes were never detected during the survey (clofibric acid, fenoprofen, fenofibrate) and two analytes were always removed at high efficiency for all treatment configurations (ibuprofen, naproxen, triclosan). Two analytes were removed at a low efficiencies (gemfibrozil, diclofenac), but better removals were observed for treatment configurations with higher solids retention times. Five polycyclic musks were surveyed; general conclusions could not be reached because of the small data set and because of numerous nonquantifiable results, but removal efficiencies generally were variable. E2 and E1 were both removed at high efficiency for all treatment systems. The removal efficiencies for different PhACs can vary significantly. Diclofenac removal efficiency is negative, suggesting that diclofenac may be deconjugated during the treatment process (Zwiener *et al*., 2003). Generally, these full-scale studies have not collected the type and amount of data necessary to organize mass balances for specific PhACs, so that a clear articulation of the relative roles of sorption and biodegradation in the full-scale process is generally unavailable. Some studies have complemented full-scale studies with batch experiments, so that the potential for sorption and/or biodegradation at full-scale can be assessed.

Removal efficiencies can vary as a function of the type of compound. Carballa *et al.* (2004) surveyed two cosmetic ingredients (galaxolide, tonalide), eight pharmaceuticals (carbamazepine, diazepam, diclofenac, ibuprofen, naproxen, roxithromycin, sulfamethoxazole and iopromide) and three hormones (estrone, 17β-

estradiol and 17α-ethinylestradiol) at municipal WWTPs in Spain. They found that the overall removal efficiencies ranged between 70–90% for the fragrances, 40–65% for the anti-inflammatories, approximately 65% for 17β-estradiol and 60% for sulfamethoxazole. However, the concentration of estrone increased along the treatment due to the partial oxidation of 17β-estradiol in the aeration tank. Nakada *et al.* (2006) measured a host of compounds, including six acidic analgesics or anti-inflammatories (aspirin, ibuprofen, naproxen, ketoprofen, fenoprofen, mefenamic acid), two phenolic antiseptics (thymol, triclosan), four amide pharmaceuticals (propyphenazone, crotamiton, carbamazepine, diethyltoluamide), three phenolic endocrine disrupting chemicals (nonylphenol, octylphenol, bisphenol A), and three natural estrogens (17β-estradiol, estrone, estriol) in 24 h composite samples of influents and secondary effluents from municipal WWTPs in Tokyo. They found that aspirin, ibuprofen, and thymol were removed efficiently during secondary treatment (>90% efficiency). They also found that amide-type pharmaceuticals, ketoprofen, and naproxen showed poor removal (<50% efficiency), probably because of their lower hydrophobicity (log K_{ow} <3). This study was also the first to report the presence of crotamiton (a topical treatment for scabies), and to show that it is persistent during secondary treatment. Overall, these results reinforce the conclusion that removal efficiencies vary for the various PhACs and suggest that chemical characteristics also may play an important role in determining the fate of each compound in biological wastewater treatment.

Removal efficiencies also can vary as a function of the sludge retention time (SRT). Oppenheimer and Stephenson (2006) studied the removal of 20 PhACs in full-scale and pilot scale WWTPs in the U.S, and they organized their data using a BIN

assignment system, which assigned each detected compound into a category related to the frequency of detection (i.e. infrequent, variable, and frequent) and into another category related to the removal efficiencies (excellent removal, moderate removal, poor removal). They found that half of the PhACs were frequently detected and were removed at less than 80% efficiency at a sludge retention time (SRT) of 5 days of less. Caffeine and ibuprofen were among 9 compounds that were both frequently detected and removed well for all the systems in the study. Galaxolide and musk ketone were also frequently detected but removed at 80% only when the SRT exceeded 25 days.

Membrane bioreactor systems (MBRs) have been evaluated as a possibly better technology for removing PhACs. MBRs use a suspended growth bioreactor, like in conventional activated sludge, but replaces gravity sedimentation with micro- or ultra-filtration. The MBR is an attractive treatment configuration because it eliminates the need for secondary clarification, which in turn allows the overall treatment process to be sited on a much smaller footprint. Kim *et al*. (2007) found that the MBR system was efficient for hormones (e.g., estriol, testosterone, androstenedione) and certain pharmaceuticals (e.g., acetaminophen, ibuprofen, and caffeine) with approximately 99% removal, but MBR treatment did not decrease the concentration of erythromycin, trimethoprim, naproxen, diclofenac, and carbamazepine. Oppenheimer and Stephenson (2006) used a limited data set to suggest that MBR provided no additional PhACs removal, when compared to similarly operated conventional systems. Kimura *et al*. (2005) found that MBRs exhibited much better removal regarding ketoprofen and naproxen, but with respect to the other compounds, comparable removal was observed between the MBRs and conventional systems. These data suggest that MBRs likely offer no inherent

advantage over conventional systems for removing PhACs, but because MBRs are operated at long solids retention times and at high mixed liquor suspended solids (MLSS) concentrations, those operational factors are likely the cause of any measured differences in PhAC removal efficiencies.

Finally, there remains a need to continue to conduct full-scale studies, with the goal of organizing accurate mass balance and fate data. To accomplish this, rigorous wastewater sampling methods must be employed. For example, these full scale studies collected data using time-weighted composite sampling using automatic samplers, equipped with sample storage in cooled compartments. This strategy allowed the reports to collect data that is likely to represent a reasonable estimate of the PhAC concentrations of interest, as well as the inherent variability; but this approach is not infallible. Many of the PhACs of interest are biodegradable, and may be transformed while the samples remain stored in the collection container. Still other compounds are highly hydrophobic and sorb strongly to biomass solids and colloidal materials that are also present in the original sample. In these cases, it is possible to underestimate the concentrations of interest, either because the solids are not properly re-suspended before sample analysis, or because of inadequate extraction techniques. Finally, time-weighted sampling collects a given wastewater volume at given time intervals, even if the wastewater flow is low. This means that time-weighted sampling may cause low-flow PhAC concentrations to be over-represented in the composite sample. For these reasons, future sampling campaigns should consider the use of flow-weighted sampling in combination with frequent grab sampling to minimize the error associated with sample collection. Each collected sample should also be mixed vigorously to resuspend settled material, and PhAC analysis should

be carried out on both the filtered and unfiltered samples. Improvements in sample collection methodology will strengthen the reliability of the data, which in turn will no doubt be the basis for future treatment plant optimization and regulatory action.

Sorption. In general, the partitioning of organic compounds from water onto activated sludge biomass is referred to as adsorption, although it may be more appropriate to refer to this as sorption because there may be some uncertainty as to whether the compound is on the surface (adsorption) or partitioning into another phase (absorption). When sorption is of interest, it is important to establish a relationship between what is on the surface and what is in the aqueous phase, a relationship generally referred to as a sorption isotherm. The term isotherm comes from the idea that the equilibrium is reached at a constant temperature to distinguish this type of partitioning from condensation. These relationships are determined experimentally and then the data is used to determine a partitioning coefficient, which is a measure for the affinity of a given compound for the activated sludge biomass.

Partitioning coefficients (K_d) have been determined in a number of studies to investigate PhACs sorption to activated sludge. Ternes *et al*. (2004) conducted a series of batch tests with primary and secondary sludge slurries to determine partitioning coefficients for a number of target PhACs. They found that the K_d values of pharmaceuticals ranged from <1 to 500 L kg−1, while that of the polycyclic musk fragrances AHTN and HHCB proved to be much higher and up to 5300 and 4900 L kg^{-1}, respectively. They also found significant differences between the K_d values obtained between primary sludge and secondary sludge; for acidic pharmaceuticals and musk

fragrances, the K_d values were higher when measured with primary sludge; the opposite was true with neutral pharmaceuticals, iopromide, and ethinyl estradiol.

Clara *et al*.(2004) found that the log(K_d) for steroid estrogens was 2.84 (2.64–2.97) and 2.84 (2.71–3.00) for E2 and EE2, respectively. In the work by Ternes *et al.* (2004) the log(K_d) for EE2 was determined to be 2.54 (2.49–2.58). Andersen *et al*.(2005) determined distribution coefficients (K_d) with activated sludge biomass for the steroid estrogens , estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in batch experiments, and they determined log K_d values for steroid estrogens of 2.6, 2.7, 2.8 respectively. When Andersen *et al*. (2005) corrected their log(K_d) values to account for the organic carbon content of the sludge, they found that the log(K_d) values were 3.16, 3.24, 3.32 respectively. These values were remarkably consistent with the sorption partitioning coefficients determined where soil is used as the sorbate (Holthaus *et al*., 2002; Bowman *et al*., 2003; Casey *et al*., 2003). Taken together, these partitioning coefficients enable practitioners to model PhAC sorption in activated sludge processes, and numerically evaluate the importance of sorption as a removal mechanism.

Sorption is not always an important removal mechanism. Ternes *et al*. (2004) found that, for compounds with the K_d values less than 500 L/Kg, only 20% of the target compound mass was associated with the sludge solids, which showed that the majority of the mass of the target compounds remained in solution. This result supported the idea that sorption is not an important removal mechanism for many pharmaceutical compounds. Yu *et al*. (2006) conducted aerobic batch biodegradation (using activated sludge as microbial inocula) experiments to evaluate the biodegradation behavior of 18 target PhACs at initial concentrations of 50, 10, and 1 μg/L. The target compounds included a

number of antiseptics, barbiturates, and anticonvulsants. Their sterile control studies showed no loss of target PhACs during the entire incubation period, and sorption to the biomass was found to be negligible for all testing conditions. Urase and Kikuta (2005) conducted a series batch experiment to examine the removal of three steroid estrogens (i.e. 17β-estradiol), two endocrine disruptors (i.e. bisphenol A), and 10 pharmaceutical substances by activated sludge. Many of the target PhACs in this study were hydrophilic, had lower water–sludge partition coefficients than the steroid estrogens, and remained in the aqueous phase, with only a small fraction partitioning to the activated sludge.

When sorption is important, there is a sorption/desorption cycle that should be investigated experimentally. In some cases, desorption fails to restore the full capacity of the sorbent, and when this happens, some of the sorption sites remain occupied. This is referred to as sorption hysteresis, and this has been reported for many organic compounds where either soil or sludge acts as the sorbent (Kim *et al*., (2005); Huang *et al*., 2003). Hysteresis has thus far received little attention where PhAC sorption to sludge is concerned. Recently, Kim *et al*. (2005) showed sorption hysteresis in the case of tetracycline sorption/desorption with activated sludge, but this is probably because tetracycline forms strong complexes with Ca (II) and other divalent cations known to be important for floc stability (Sobeck and Higgins, 2002; Martin, 1979). PhAC sorption hysteresis is a basic and relevant process that has not received great attention to date.

One cause of sorption hysteresis may be related to particle characteristics (e.g. size), and there is a need to study the possible fundamental connections. In general, activated sludge particles in conventional processes are typically 80-300 μm in diameter (Ng *et al*., 2005), and this structure typically consists of smaller microcolonies (approx 8-

15 μm) connected by exocellular polymeric and inorganic material, and with a few large flow channels that facilitate transport (Snidaro *et al*. 1997; Chu and Tay, 2005). Smaller activated sludge particles can be found in bioreactors like MBR (Ng and Hermanowicz, 2005) and smaller particles have less internal polymer, a higher number of cells per unit volume (Snidaro *et al*., 1997) and they do not have the large flow channels that facilitate transport.

Biodegradation. Biodegradation is likely due to cometabolic activity because PhACs are not present in high enough concentration to support substantial biomass growth. This means that PhAC transformation is most likely to occur during exponential growth stages and during active degradation of the primary substrates present in wastewater. The published reports of cometabolism of PhAC are currently limited. Most of the published reports that concern cometabolism focus on the removal of xenobiotics that are produced as a result of industrial and military activity (e.g. chlorinated solvents such as trichloroethylene, nitroaromatic compounds explosives, dyes, polyurethane foams). These compounds may be present in the environment at much higher concentrations than PhACs are, but many industrial pollutants and PhACs share some of the same structural features (i.e polyaromatic rings), so there may be common reaction mechanisms. It is also known that cometabolism is often an initiating reaction, producing intermediates that may be more biodegradable (and therefore would participate in the central metabolic pathways), or that may be suspectible to adsorption or polymerization reactions and rendered nonbioavailable (i.e. dead end product). Quintana *et al*. (2005) observed the cometabolic transformation of four acidic pharmaceuticals in laboratory-

scale experiments. Although cometabolism is likely when biodegradation is occurring, there is only limited information that clearly connects cometabolism with the removal of PhACs. One interesting example comes from Alexy *et al*. (2005) who found that each of 18 antibiotics was not biodegraded, but some partial biodegradation was observed when sodium acetate also was present. This suggests that when sodium acetate is available as a primary substrate, the antibiotics may be subject to cometabolism. Biodegradation may sometimes result in the formation of a stable byproduct. Haib and Kummerer (2006) found that diatrizoate (found in X-ray contrast media) was biodegraded aerobically to 3,5-diamino-2,4,6-triodobenzoic acid which was not further degraded by bacteria. Quintana *et al*. (2005) also found that biotransformation of ketoprofen and bezafibrate produced more stable metabolites.

A wide variety of mono and di-oxygenase enzymes can transform xenobiotics during exponential growth conditions (Schwarzenbach *et al*., 2005), but biotransformation of pollutants in the absence of bacterial growth also may occur as a result of enzymes previously produced by dead (non-viable) bacteria and as a result of extracellular enzymes excreted by viable bacteria (Madigan and Parker, 1997; Kragelund *et al*., 2005). Activated sludge communities are diverse and known to house a wide variety of nonspecific mono and di-oxygenase enzymes associated with both heterotrophic and autotrophic microorganism (Gessesse *et al*., 2003; Servos *et al*., 2004).

There is circumstantial evidence linking nitrifiers to a unique capability to biologically (perhaps cometabolically) transform steroid estrogens such as EE2. Surveys of municipal WWTPs indicated that nitrifying sludges remove EE2 more efficiently than those that do not nitrify (Servos *et al.,* 2004). Numerous experimental results further

supported this contention: Vader *et al*. (2000) degraded EE2 using nitrifying activated sludge, and they noted the presence of unidentified hydrophilic daughter products. Several groups (Shi *et al.,* 2004; Dytczak *et al.,* 2006) also biologically degraded EE2 using nitrifying mixed cultures. These combined results suggest that EE2 and NH_4 transformation rates are linked. A specific EE2 transformation mechanism may involve AMO, the key enzyme that catalyzes the conversion of ammonia to nitrite in nitrifying organisms. For example, AMO is also capable of co-metabolically oxidizing polycyclic aromatic rings (Chang *et al.,* 2003; Vannelli and Hooper, 1995). The active site of AMO is buried in the core of the protein, where four neighboring α-helices provide two histidine and four glutamic acids as iron ligands.(Zahn *et al*., 1996; Siegbahm *et al*., 1998). One face of the di-iron site contains a hydrophobic pocket, and may be well suited for organic substrates like EE2.

These results support the conclusion that nitrifying activated sludge cultures may play a role in biotransforming pharmaceuticals in biological WWTPs, but heterotrophic organisms also likely play a key role. Furthermore, these results show that nitrifiers may, at a minimum, provide for initial degradation of PhACs like EE2 into an intermediate that can then be degraded further by heterotrophic organisms. The work of Shi *et al*. (2004) also supports this idea. They conducted EE2 biodegradation experiments with a nitrifying pure culture and a nitrifying mixed culture. They detected daughter products in the pure culture experiments but not in the mixed culture experiments, perhaps because the heterotrophs completely degraded the daughter products produced by the nitrifiers. At this point, it is not clear if AMO is kinetically dominant in full-scale WWTPs among all enzymes that might be capable of transforming pharmaceuticals, especially if the

enzymes are present in fast-growing heterotrophic organisms. Proof that nitrifiers are responsible for transformation of steroids in full-scale systems has not been shown definitively. It may be that nitrifiers will cometabolically transform pharmaceuticals containing aromatic structures when they are present in low organic carbon, ammonium enriched environments through the AMO. However, heterotrophic cultures also may contribute to and, in fact, may predominate these biotransformations if the sewage also contains mono- and dioxygenase inducers that function in heterotrophic bacteria. Other scavenging, biodegradative mechanisms are likely to exist and function among the complex collection of heterotrophic bacteria present in the low organic carbon environments found during the nitrification phase of bioreactors. Questions related to the relative importance and the potentially synergistic interplay between nitrifiers and heterotrophs need to be further elucidated to clarify this issue.

Nyholm *et al*. (1996) suggested that biodegradation can be enhanced by operating at longer SRT. They operated laboratory-scale bioreactors over a range of SRT values (1-32 days) and sludge loadings (0.1 – 0.9 mgBOD5/mg MLSS/d), and they spiked five organic micropollutants (2,4-dichlorophenoxy acetic acid (2,4-D); 2,4,6-trichlorophenol (TCP); pentachlorophenol (PCP); 4-nitrophenol (4-NP) and lindane) into the influent. They found that adaptation was generally required, and that removal by biodegradation in successfully adapted systems were generally within a range of about 40 to about 95% except for 4-NP, which was degraded to concentration levels below the analytical detection limit. They found that PCP, TCP, and 2,4-D were degraded best at high sludge ages.

Biofilm experiments also have offered insight into the biodegradability of selected PhACs. For example, Boyd *et al*. (2005) investigated removal of naproxen and its chlorination products using a laboratory-scale biofilm bioreactor process. The bioreactor was a plug-flow bioreactor, and it used 31 m of polypropylene tubing as the support matrix for the biofilm. The bioreactor was fed a naproxen solution and then fed a solution at the same naproxen concentration following contact with free chlorine. Naproxen was not degraded biologically, and the naproxen solution containing products of chlorination caused biomass sloughing and discharge from the bioreactor. Zwiener and Frimmel (2003) investigated the biodegradation of three active compounds of pharmaceuticals (clofibric acid, ibuprofen, and diclofenac) in short-term tests with a miniaturized upflow biofilm bioreactor with an oxic/anoxic configuration. The biofilm reactor removed 85% of the applied dissolved organic carbon (DOC), but clofibric acid and diclofenac were not eliminated and were discharged at a level of approximately 95% of their initial concentration; they did find however that the elimination in the anoxic region of the biofilm reactor improved the removal efficiencies of clofibric acid and diclofenac to values between 60 and 80% of their initial concentration. Winkler *et al*. (2001) found that ibuprofen (as well as it's hydroxylated and carboxyated metabolites) was biodegraded in a river biofilm reactor, but clofibric acid was not.

Synthetic antibiotics, which do not appear to be readily biodegradable, deserve special attention. Ingerslev *et al.* (2001) studied the primary aerobic and anaerobic biodegradability of the antibiotics olaquindox (OLA), metronidazole (MET), tylosin (TYL) and oxytetracycline (OTC). They conducted batch experiments at intermediate concentrations (50–5000 μg/l) using shake flasks inoculated with C14-labeled antibiotic

compounds and mixed with sediment or activated sludge. They found that these compounds were slowly biodegradable during aerobic conditions, with half life values that were typically between 1-5 weeks. During anaerobic conditions the biodegradation rates were slower, with half life values of up to 12 weeks. Alexy *et al*. (2005) studied the biodegradability of 18 clinically important antibiotics, and in addition to finding that none of them were readily biodegradable, they also found that half of the antibiotics tested inhibited biological activity when present at ppb levels. A study by Kummerer *et al*. (2000) also revealed that none of the test antibiotic compounds (ciprofloxacin, ofloxacin, and metronidazole) were biodegraded, and that, in addition, the genotoxicity was not eliminated during batch experiments. Zhou *et al*. (2006) treated a high-strength pharmaceutical wastewater with a pilot-scale system composed of an anaerobic baffled reactor followed by a biofilm airlift suspension reactor. They found that ampicillin and aureomycin, with influent concentrations of 3.2 and 1.0 mg/L, respectively, could only be partially degraded, with overall removal efficiencies of less than 10% at steady state. These results imply that biodegradation is not likely to play a large role in determining the ultimate fate of synthetic antibiotics in conventional biological wastewater treatment systems.

Although a number of elucidating studies concerning biodegradation of PhACs have been conducted, research on the biodegradation of PhACs should continue, with particular attention to the identification of daughter products and the application of molecular methods to identify the important microorganisms and mechanisms. Currently, there are numerous examples in the literature reporting on the biodegradation of PhACs in biological treatment systems, but without any direct evidence of biotransformation (e.g.

metabolites). This is a weakness that currently exists in the literature, and it does not serve to clarify the dialogue concerning the fate of PhACs. There are also examples (Vader *et al*., 2000; Shi *et al*., 2004) of reports that show unidentified "daughter products" ; these reports will be strengthened with clear identification of metabolites, which can be readily accomplished by combining the latest tools in HPLC/MS/MS technology with well established methods such as thin layer chromatography and NMR.

The role of extracellular polymeric substances in sorption

The distribution of pollutants in water is highly dependent on the processes at the solid-liquid interface. Sorption phenomena can be explained by some interaction mechanism; hydrophobic interaction, van der Waals forces and electrostatic interaction between adsorbate molecules and the adsorbent. To understand the mechanism of sorption between PhACs and floc, investigators have also focused on the physicochemical properties of cell surface. The solid phase is represented by many different materials, mostly minerals but also metals and organics cells can sorb inorganic and organic solutes and particles. As sorption sites can serve: extracellular polymeric substances (EPS), cell walls, cell membranes and cell cytoplasm. These sites display different sorption preferences, capacities and properties.

EPS have been considered to be the most important sorption site as outmost component of cell. EPS as metabolic products are accumulated on the bacterial cell surface (Morgan *et al.,* 1990). They are composed of a variety of organic substances (Frølund *et al.,* 1996; Liao *et al*., 2001); carbohydrate, protein, humic substance, uronic acid and deoxyribonucleic acids (DNA). They are important from environmental and

engineering perspectives because they protected cells from phagocytosis in the environment and play a role in metal complexation. In addition, they affect microbial flocculation (Frølund *et al*., 1996; Rudd *et al*., 1984) and dewatering (Nielsen *et al*., 1996) in the activated sludge process.

EPS components have different roles, depending on the physical and chemical properties. Liao *et al.* (2000) found hydrophobicity was not influenced by the total EPS content of sludge. The change in the hydrophobicity of sludge floc was affected by variations in the protein, total carbohydrate, and DNA content in the EPS. Jorand *et al*. (1998) also found that the hydrophobic fraction of EPS was affected only by proteins and not carbohydrates. Their results showed protein in cell surface is very important as binding sites. Yi and Harper (2005) showed that membrane bioreactor (MBR) sludge is more hydrophobic than that of sequencing batch reactor (SBR) even when both are operated at the same SRT and the linear sorption coefficient (K_d) measured from MBR was twice that of SBR biomass. In addition, Danielsson *et al*. (1996) reported that sorption of organic compounds which have high log K_{ow} value (> 3.5) to activated sludge was dominant as removal mechanism. Thus, it is concluded that cell hydrophobicity can be affected by the change of EPS composition such as protein and sorption capacity depends on cell hydrophobicity. These results also reinforce the hydrophobic interaction between organic compound and biomass flocs are dominant.

Influence of sorption/desorption hysteresis on biodegradation

The mechanism of sorption-hysteresis is usually explained by irreversible chemical binding, sequestration of a solute into specific components of biomass, (or

entrapment of the solute into micro-porous structures), and time-dependent hysteresis that is a consequence of slow kinetics at given experimental conditions (Ravikovitch and Neimark, 2005).

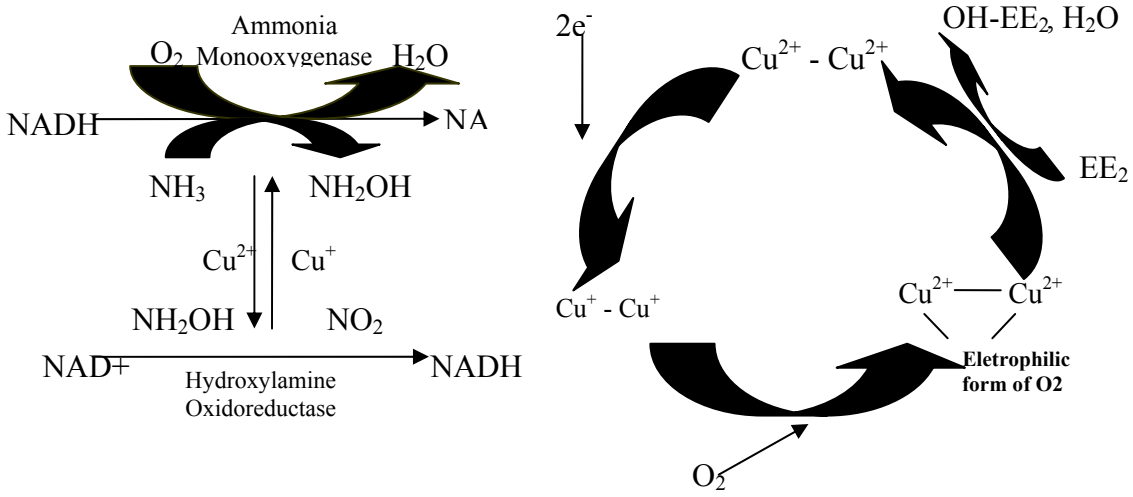
Sorption/sorption-hysteresis which influences intracellular degradation rates may be related to availability of the organic compounds to the degrading organisms. When organic compounds are bonded irreversibly or trapped in dead biomass or non-living organic particles, they are isolated from the degrading microorganisms and thus are protected from biodegradation. Ogram *et al*. (1985) reported that 2, 4-Dichlorophenoxy sorbed to organic particles was completely protected from biodegradation. The study of Stringfellow and Alvarez-chozen (1999) also showed that degradation rate of fluoranthene in the presence of non-degrading biomass was significantly reduced. These studies suggest that organic sequestration by inactive- or dead biomass and inorganic particles reduces the bioavailability of organic pollutant to degrading biomass.

Enzymes of interest

Microorganisms in our environment play a very important role in oxidizing or mineralizing many natural products and PhACs to carbon dioxide or to intermediate byproducts. Most of these compounds newly appear in the environment and microorganisms in nature do not have removal mechanisms or proper enzyme systems. Thus, many PhACs are known to be persistent in the environment because they are stable and thus resistant to enzyme catalyzed reactions. In cometabolic transformation of PhACs, monooxygenase enzymes play a very important role by initiating biodegradation. They catalyze the insertion of oxygen into a substrate, then reduce stability and, in most cases,

increase hydrophilicity. Many researchers start to have a great interest in their property to increase removal of recalcitrant PhACs and eventually prevent harmful effects of PhACs.

Ammonia monooxygenase. The autotrophic ammonia oxidizing bacteria such as *Nitrosomonas* sp. are the most extensively studied (Hooper *et al*., 1984) and nowadays have been investigated intensively because of the capability of ammonia monooxygenase (AMO). Ammonia is converted to nitrite by oxygen-dependent reaction of AMO, which oxidizes ammonia to hydroxylamine consuming two electrons, and hydroxylamine oxidoreductase (HAO) oxidizes hydroxylamine to nitrite with obtaining four electrons. Two electrons are returned to AMO and provide energy for oxidations. The oxidization by AMO is called cometabolic reaction because the byproducts formed do not act as growth substrates for the ammonia-oxidizing bacteria. It is found that AMO can oxidize a wide variety of PhACs because of the nonspecificity of AMO, including aromatics, ethers (Hyman *et al*., 1994), *n*-alkanes (Hyman and Wood, 1983), *n*-alkenes (Hyman *et al*., 1988), and thioethers (Juliette *et al*., 1993). In addition, the substrate range of AMO is extended to polycyclic aromatic hydrocarbons (PAH) (Chang *et al.,* 2002) and the halogenated hydrocarbons (Rasche and Hyman, 1991). Some researchers (Shi *et al*., 2004; Vader *et al*., 2000) observed the biotransformation of estrogenic chemicals such as 17α-ethinylestradiol, estradiol, and estrone to hydrophilic daughter products in a highly enriched nitrifying culture. It is evident that ammonia oxidizing bacteria play an important role in natural bioremediation systems of recalcitrant chemicals. AMO is also induced by substrate (ammonia). It is found to increase AMO activity by at least 2-fold



Reaction: $1EE2+O_2+2NADH = 1Oxided\text{-}EE2+H_2O+2NAD^+$

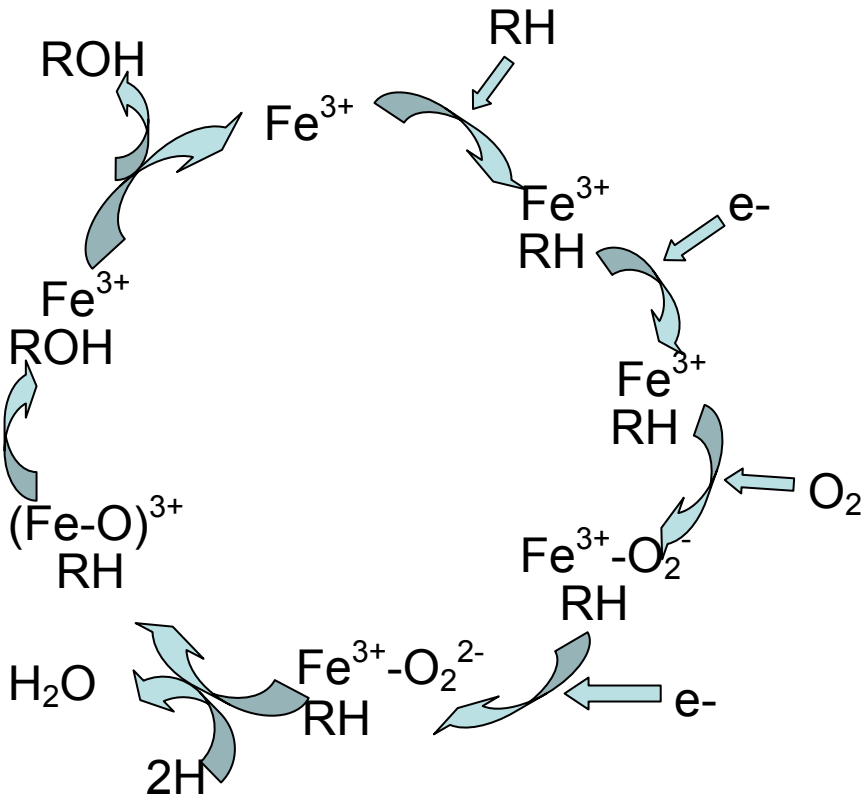
Figure 2.1 AMO degradation mechanism

when ammonia is present (Arciero *et al*., 1989). In surface water and wastewater, NH_3 is abundant and its concentration is relatively high, compared to possible inducers for other monooxygenase such as Cytochrome P450 or toluene monooxygenase.

Cytochrome P450s. Cytochrome P450 represents a huge family of enzymes and is found to catalyze the oxidation of a wide variety of chemicals, including many environmental pollutants such as PhACs and halogenated compounds. These enzymes are found in almost all living organisms. Especially, the P450s in drug industry was intensively investigated because they are deeply involved in the drug metabolism such as activation or inactivation and conversion of chemicals to reactive intermediates in the human body. It is known that the P450s can catalyze hydroxylation, epoxidation, sulfoxidation, or dealkylation reactions (Urlacher *et al.*, 2004) of organic chemicals and convert toxic chemicals into less toxic intermediates (usually more hydrophilic forms) that are more easily removable from the body.

In bacteria, P450s are involved in these catabolic reactions of pharmaceutical compounds and are very important in contaminant sites of toxic hydrocarbons.

There are three different induction systems; substrate induction, non substrate induction, and induction by environmental factors such as temperature and pH. It was found that *Pseudomonas* sp. and *Bacillus* sp. frequently observed in activated sludge can produce the enzymes called the cytochrome $P450_{cam}$ and $P450_{BM-3}$, respectively. $P450_{BM-3}$ and $P450_{cam}$ which are classified as substrate induction enzymes are induced by barbiturate and camphor, respectively. Although the roles of P450 were not fully or clearly investigated in biological treatment processes, when inducer is present in biological



Reaction: $RH+O_2+2NADH = ROH+H_2O+2NAD^+$

Figure 2.2 Reaction mechanism of Cytochrome P450 monooxygenase (Guengerich, 2007)

processes P450s may be considered a degrader for degradation of pharmaceutical compounds.

Toluene mono- and dioxygenase. It is known that toluene monooxygenases (TMOs) are able to hydroxylate toluene and a wide range of substrates including aromatic and phenolic compounds in metabolic pathways. TMOs initiate the oxidation of toluene by inserting oxygen into C-H bonds in toluene molecule, and enzymes have been classified according to the specificity in hydroxylation of a unique position C-H bond (Table 2.1), and the unique property of each TMO explains different removal efficiency for the same molecule.

Table 2.1 Toluene monooxygenase enzymes

Toluene monooxygenase	Microorganisms	References
Toluene 4-monooxygnease	*Pseudomonas mendocina*	Whited *et al.,* (1991)
Toluene 3-monooxygease	*Ralstonia pickettii*	Fishman *et al.*, (2004)
Toluene 2-monooxygenase	*Burkholderia cepacia*	Newman *et a*l., (1995)
Toluene 2,3-dioxygenasse	*Pseudomonas putida F1*	Jahng *et al.,* (1994)

For example, TMOs show different capability for the removal of trichloroethylene. *Pseudomonas putida* F1 (Toluene 2,3-dioxygenasse) and *Burkholderia cepacia* (Toluene 2-monooxygenase) are shown as the high TCE degrader, but *Pseudomonas mendocina* (Toluene 4-monooxygnease) is the poor degrader.

Frontier electron density

Frontier electron density (FED) profile on a molecule provides a useful means for the detailed characterization of donor-acceptor interactions (that is, highest occupied molecular orbital (HOMO) - lowest unoccupied molecular orbital (LUMO) interaction). In 1952, the FED was proposed by Fukui and his coworker and at the present time the HOMO-LUMO interactions became widely accepted by researchers, providing an important means in interpretation of the pathway of chemical reactions. This FED can be applied to electrophilic, nucleophilic, and radical positions reactions as follows:

1. For an electrophilic reaction, HOMO densities are normalized by the energy of the frontier molecular orbitals at ground state: $f\mathrm{r} = [2*\sum(C_{ri}\mathrm{HOMO})^2]$.

2. For an nucleophilic reaction, LUMO densities are normalized by the energy of the frontier molecular orbitals at ground state: $f\mathrm{r} = [2*\sum(C_{ri}\mathrm{LUMO})^2]$.

3. For a radical reaction, the sum of HOMO and LUMO densities are normalized by the energy of the frontier molecular orbitals at ground state: $f\mathrm{r} = [\sum(C_{ri}\mathrm{HOMO})^2 + \sum(C_{ri}\mathrm{HOMO})^2]$.

Where *r* is the number of carbon atoms in *i*: *2s, 2px, 2py*, and *2pz*. The highest *fr* value indicates the most reactive position, that most likely to be attacked by oxygen of enzyme.

The biochemical transformations of organic compounds in aerobic condition could be achieved by using enzymes carrying an electrophilic form of oxygen, which reacts with carbon atom in the highest *fr* positions of the organic compound. Especially in cometabolism, hydroxylation can be predicted by considering monooxygenase-

mediated reactions of organic compounds, regarding where the initial point of attack in molecule is located.

Fukui pioneered much of the early work connecting FED to chemical reactivity in aromatic hydrocarbons (Fukui, 1997). Wheland and Pauling (1935) successfully used FED to explain the reactivity of substituted aromatics. More recently, Ohura *et al.* (2005) showed that air-bore polycyclic aromatic hydrocarbons were abiotically chlorinated in positions that corresponded to high FED. Lee *et al*. (2001) used Fenton oxidation to remove polycyclic aromatic hydrocarbons, and they successfully used FED to predict the order of daughter product production. Although these previous attempts focused on abiotic reactions, they bolster the promise for predicting biological oxidations in the same way.

III. THE ROLE OF PARTICLE SIZE AND AMMONIUM OXIDATION IN REMOVAL OF 17A-ETHINYL ESTRADIOL IN BIOREACTORS

Abstract

Laboratory scale bioreactors were used to investigate sorption and biodegradation of 17α-ethinylestradiol (EE2). EE2 is among many emerging micropollutants that may cause endocrine disruption of aquatic organisms in the environment. Results showed that the sludge taken from the membrane bioreactor (MBR) had a sorption partitioning coefficient that was more than twice that of biomass derived from sequencing batch reactors (SBRs). The MBR biomass had smaller particles and was more hydrophobic than the SBR biomass. Experiments with nitrifying sludge showed that sorption was more important when the initial ammonia concentration was 48 mg/L or less, but at higher initial ammonia concentrations the role of biodegradation became more important. The ammonia monooxygenase enzyme extracted from a nitrifying mixed culture removed EE2 in batch experiments. These findings are the first that we are aware of to link biomass particle size, hydrophobicity, and sorption capacity. These results also support the notion that cometabolic biodegradation of EE2 can occur in nitrifying sludge.

Keywords: Pharmaceuticals, Sorption, Hysteresis, Bioreactors, Nitrogen, Wastewater

The purposes of this work were to evaluate the effect of hydrophobicity of biomass on EE2 sorption and biomass characteristics, and to investigate the degradation of EE2 in nitrifying sludges.

EE2 is more persistent than natural estrogens like estone (Ternes *et al.,* 1999a), but it can be removed in biological wastewater treatment processes (Ternes *et al.,* 1999b). Previous research showed that membrane bioreactors (MBRs) may be well suited to remove EE2 via sorption because of the hydrophobic properties of MBR sludge (Yi and Harper, 2005) and hydrophobic sludge properties can enhance the removal of EE2, especially since EE2 has a relatively high octanol/water coefficient (log K_{ow} for EE2 is 3.9, Holthaus *et al.,* 2002).

MBRs are also often operated to nitrify, and there is circumstantial evidence linking nitrifying sludge to a unique capability to biologically degrade EE2 (Servos *et al.,* 2004; Vader *et al.,* 2000). A specific EE2 degradation mechanism may involve ammonia monooxygenase (AMO), the key enzyme that catalyzes nitrification. The active site of AMO is buried in the core of the protein, and one face of the di-iron site contains a hydrophobic pocket, and may be well suited for organic substrates like EE2.

Methodology

Bioreactor configuration and operation. Fully-automated laboratory-scale bioreactors were operated, including a MBR, an anaerobic/aerobic SBR (AASBR), an aerobic SBR (ASBR), and a nitrifying continuous stirred-tank reactor (NCSTR). The operating details for the four bioreactors were presented previously (Yi and Harper, 2005). Each bioreactor was operated at a SRT of 20 days. The primary substrate in the

synthetic wastewater for the MBR and SBRs was acetate (360 mg as COD/L). For the NCSTR, the primary substrate was ammonia (140 mg/L as N).

Analytical methods. EE2 was detected by high pressure liquid chromatography (HPLC) (Hewlett-Packard, HP 1100) as explained previously (Yi and Harper, 2005). Surrogates samples were included in each batch of samples, and recovery always exceeded 90%. Total suspended solids (TSS), volatile suspended solids, and ammonia-N were analyzed according to Standard Methods (APHA 1992). The microbial adhesion to hydrocarbon test was used to measure sludge hydrophobicity (Guellil *et al.,* 1998). Particle size distribution was determined using a Horiba LA-920 laser scattering particle size distribution analyzer (Delta Analytical Instruments, North Huntington, PA). Extracellular polymeric carbohydrate and protein were determined as described by Ng and Hermanowicz (2005). Biomass was microscopically observed for filamentous microorganisms as described by Jenkins *et al* (1993).

Sorption Experiments. Sorption of EE2 onto the biomass was determined by adding EE2 at different concentrations ranging between 10 μg/L and 1000 μg/L. Time series sampling was done, beginning after 1 hour. Biomass inhibition was achieved with sodium azide at 0.2% w/v (Ning *et al.,* 1996). Soluble EE2 was measured directly by HPLC, as described above. Solid phase EE2 was determined by mass balance; this is possible because biodegradation was inhibited, EE2 did not associate with the walls of the glass bottles (as determined by controls), and EE2 has a low vapor pressure (1.7x10-6 Pa) so that evaporation was negligible.

Batch tests with nitrifying sludge. Batch tests with inhibited (with 0.2% sodium azide) and uninhibited NCSTR biomass were conducted. Biomass was washed and resuspended in fresh media with various initial ammonia concentrations ranging from 19 to 78 mg/L ammonia-N. The biomass was spiked with EE2 (1 μgEE2 per mg TSS) and then samples were collected over 24 hours for soluble EE2 analysis. The amount of biodegraded EE2 was calculated by taking the difference between the total EE2 removed from solution and the bound EE2 (which was calculated using experimentally-determined distribution coefficients and the measured soluble EE2 concentration).

Enzyme Experiments. AMO was extracted from NCSTR biomass as explained by Moir *et al*. 1996. The degradation of EE2 in the presence of the enzyme extract was conducted in 10mM Tris-HCl using eluted enzyme, 0.5mM NADH, 0.6 units diaphorase, 0.5mM duroquinone, and ammonia. A control test was also performed without the enzyme extract.

Results and Discussion

Sorption isotherms were developed using biomass from the MBR and SBRs. The linear isotherm model was used, $q=K_d \cdot C$, where q is the equilibrium biomass-associated EE2 (μg/g), K_d is the partitioning coefficient, and C is the equilibrium concentration of soluble EE2 (μg/L). The K_d measured for the MBR biomass was more than twice that measured for the biomass from either SBR (Table 3.1). The partitioning coefficients (K_d) were typically higher for the sludges that were more hydrophobic, and the MBR K_d and hydrophobicity values were statistically higher (at 99% confidence) than those of either

SBR, as determined by the one-tailed T test. This result supports the notion that sludge hydrophobicity affects the extent of EE2 sorption in bioreactors. MBRs appear to have a higher sorption capacity for EE2, and because MBRs remove TSS to very low levels, lower concentrations of EE2 can be expected from MBRs as compared to conventional bioreactors with gravity sedimentation.

Sludge hydrophobicity is also affected by filamentous organisms and extracellular polymeric substances (EPS) (Jenkins *et al.,* 1993), but the observed differences in this study can not be attributed to these factors because very few filaments were microscopically observed and the EPS contents of the three bioreactors were similar (typically 10-15% b. wt. extracellular polymeric carbohydrate, 13-18% b. wt. extracellular protein, for all bioreactors). Particle size is the key difference; the measured median particle diameter for the MBR sludge was 10 μm, compared to 120 μm for the ASBR and 123 μm for the AASBR. Because of the smaller floc in the MBR, the exposed surface area was greater for MBR biomass, which in turn likely contributed to the difference in the measured sludge hydrophobicity, because of the nature of the cell wall.

Figure 3.1 shows the effect of initial ammonia concentration on the removal of EE2 by sorption and biodegradation (the length of the error bars is twice the standard deviation). Sorption was most important when the initial ammonia concentration was 50 mg/L or less, but at higher initial ammonia concentrations the role of biodegradation became more important. These results appear reasonable because ammonia concentrations are known to regulate AMO activity (Sayavedra-soto *et al.,* 1996). At lower initial ammonia concentrations, there is less of the co-metabolic activity likely

responsible for EE2 degradation. As the initial ammonia concentration increases, respiration and cometabolism become more important.

Table 3.1 Statistical comparison of the EE2 sorption data

Parameter	**K_d**			**Hydrophobicity**		
	MBR	ASBR	AASBR	MBR	ASBR	AASBR
No. values	12	12	12	19	19	19
Average	0.45	0.22	0.3	68%	22%	35%
Std Dev	0.06	0.05	0.09	15%	6.80%	7.50%
Direct comparison						
Parameter	Kd			Hydrophobicity		
	MBR	MBR	ASBR	MBR	MBR	ASBR
	vs ASBR	vs AASBR	vs AASBR	vs ASBR	vs AASBR	vs AASBR
T value	10.5	4.7	2.4	13.7	82	9
Confidence	>99%	>99%	>97%	>99%	>99%	>99%

Figure 3.2 shows that EE2 was removed in the presence of the AMO enzyme, but not in the control. This was expected because AMO is capable of co-metabolically oxidizing polycyclic aromatic rings (Chang *et al.,* 2003, Vannelli and Hooper, 1995). The action of AMO enzyme can remove EE2, but the mode of action is not yet clear. AMO may catalyze degradation of EE2, or it may covalently interact with EE2, acting more as a reagent. Future work must identify daughter products to investigate the metabolic details.

Conclusions

The MBR sludge had K_d and hydrophobicity values that were significantly higher than those of SBR sludges. The MBR contained much smaller particles than SBRs, thus

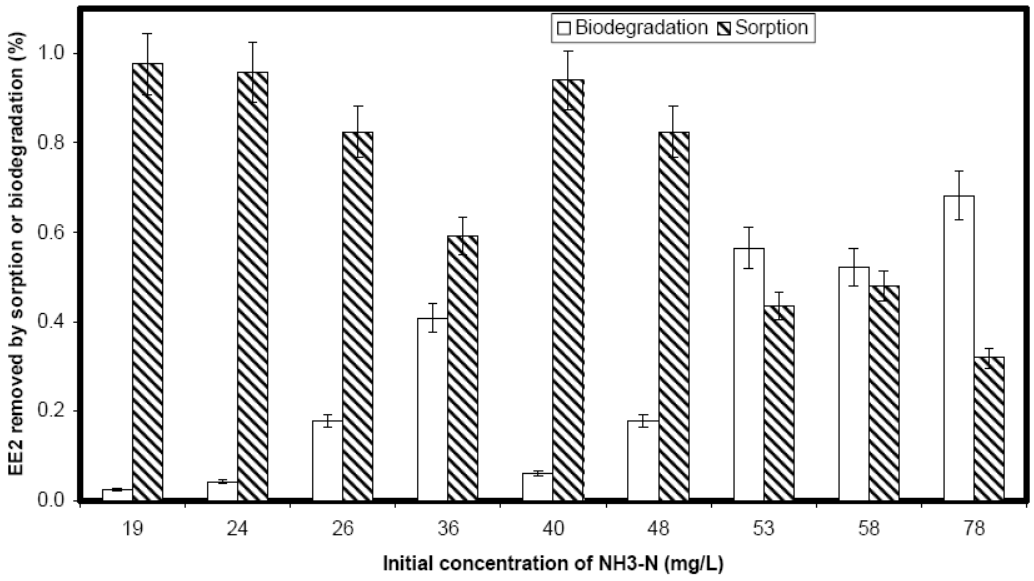


Figure 3.1 Effect of initial ammonia concentration on the sorption and biodegradation of EE2

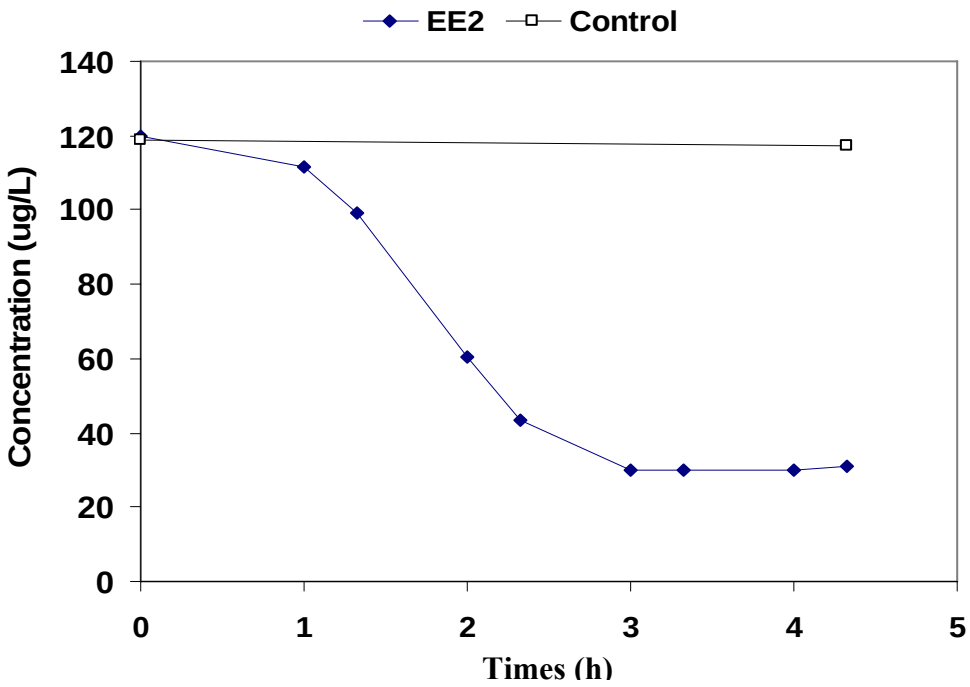


Figure 3.2 Degradation of EE2 with an AMO-containing extract

providing more exposed surface area for sorption. Ammonia affected the relative amounts of EE2 removed via sorption and biodegradation in nitrifying sludge. Sorption was most important when the initial ammonia concentration was 48 mg/L or less, but at higher ammonia concentrations biodegradation became more important. EE2 was removed in the presence of the AMO enzyme in batch tests; this further suggests that AMO possesses co-metabolic capability.

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IV. THE EFFECT OF BIOMASS CHARACTERISTICS ON THE PARTITIONING AND SORPTION HYSTERESIS OF 17*A*-ETHINYLESTRADIOL

Abstract

A membrane bioreactor (MBR) and a conventional bioreactor (CBR) were operated under various conditions to manipulate the biomass characteristics and evaluate the ensuing effects on the partitioning and sorption hysteresis of 17α-ethinylestradiol (EE2). When the biomass was grown without nitrogen limitation, the biomass mean particle size had a dramatic effect on the observed partitioning coefficient (K_d) and on sorption hysteresis index (HI). Visualization study confirmed this result. MBR K_d (0.33–0.57 L/g) values were equal to or larger than those of the CBR (0.25–0.33 L/g). Under nitrogen-deficient conditions, the correlations between the biomass particle size and K_d and HI were poor, likely because of extracellular polymeric substances. The K_d and HI were determined for initial EE2 concentrations between 100 and 1000μg/L. Changing the solid retention time (SRT) did not manipulate particle size, and the effects on K_d and HI were not dramatic. This study also numerically explored the impacts of sorption hysteresis on the removal of pharmaceutical compounds.

Keywords: Pharmaceuticals; Sorption; Hysteresis; Bioreactors; Nitrogen; Wastewater

The purposes of this work were to: (1) characterize the effect of particle size on partitioning coefficients and sorption-hysteresis for 17α-ethinylestradiol; (2) manipulate the biomass particle characteristics by changing the nutrient levels and operating solid retention time (SRT) and characterizing the ensuing effects on partitioning coefficients and sorption-hysteresis; and (3) investigate the potential effects of sorption-hysteresis on effluent water quality.

It is now known that removal efficiencies for the various classes of PhACs can vary greatly (Clara *et al.,* 2005; Joss *et al*., 2005). Carballa *et al.* (2004) found that the overall removal efficiencies ranged between 70% and 90% for the fragrances, 40–65% for the anti-inflammatories, around 65% for 17β-estradiol. Joss *et al.* (2006) showed that only 4 (ibuprofen, paracetamol, estradiol, estrone) out of 35 compounds are 90% removed using state-of-the-art biological treatment systems, and 17 out of 35 are removed at less than 50% efficiency.

Sorption occurs when PhACs associate with activated sludge biomass. The importance of sorption depends on the chemical characteristics of the parent compound; some PhACs are relatively hydrophilic and have low partitioning coefficients, while others are hydrophobic and will partition strongly onto the solid phase. When sorption is the primary removal mechanism, there is a sorption/desorption cycle that should be investigated experimentally. In some cases, desorption fails to restore the full capacity of the sorbent, and when this happens, some of the sorption sites remain occupied. This is referred to as sorption hysteresis, and this has been reported for many organic compounds where either soil or sludge acts as the sorbent (Kim *et al*., 2005; Conrad *et al.,* 2006; Huang *et al.,* 2003). Hysteresis has thus far received little attention where PhACs

sorption to sludge is concerned. Recently, Kim *et al*. (2005) showed sorption hysteresis in the case of tetracycline sorption/desorption with activated sludge, but this is probably because tetracycline forms strong complexes with Ca(II) and other divalent cations known to be important for floc stability (Martin, 1979; Sobeck and Higgins, 2002). PhAC sorption hysteresis is a basic and relevant process, and neglecting it may cause an overestimation of the long-term sorption capacity of the activated sludge.

One cause of sorption hysteresis may be related to particle characteristics (e.g. size), and there is a need to study the possible fundamental connections. The hypothesis of this work is that sorption hysteresis is more pronounced as the biomass particle size distribution shifts toward larger sizes. The rationale for this is smaller flocs are more dense and less permeable than larger floc (Snidaro *et al.,* 1997; Chu *et al.*, 2005) therefore allowing for much less intraparticle entrapment of PhACs. In general, activated sludge particles in conventional processes are typically 80-300μm in diameter (Metcalf and Eddy, 2003), and this structure typically consists of smaller microcolonies (approx. 8-15μm) connected by extracellular polymeric and inorganic material, and with a few large flow channels that facilitate transport (Snidaro *et al.,* 1997; Chu *et al.*, 2005). Smaller activated sludge particles can be found in bioreactors like membrane bioreactors (MBRs) (Yi *et al*., 2006; Ng and Hermanowicz, 2005), and smaller particles have less internal polymer, a higher number of cells per unit volume (Snidaro *et al.*, 1997) and they do not have the large flow channels that facilitate transport. The implications of this hypothesis may easily reach practitioners. Bioreactor particle size distributions can be affected in the design stage (e.g. MBRs will produce smaller particles than conventional

bioreactors (CBRs), Yi *et al*., 2006) or through operational adjustments (e.g. changing the mixing intensity or addition of non-ionic polymer).

Biomass particle characteristics may be affected by nutrient deficiency and the SRT. Nutrient deficiency is known to cause the production of extracellular polymeric substances (EPS), which can lead to viscous bulking (Jenkins *et al.*, 1993). Liao *et al.* (2006) recently showed that the particle size distribution for non-bulking sludge fit a log-normal distribution, but for bulking sludge, they observed a bi-modal distribution. SRT may also affect biomass particle size and structure. Massé *et al.* (2006) recently found that the floc size decreased as SRT increased. Liao *et al*. (2006) found that sludge flocs at low SRT were more irregular in shape and variable in size than those at higher SRT; this is probably because floc strength is greater at high SRT than at low SRT (Liao *et al.*, 2002). These previous results show that nutrient deficiency and SRT affect biomass characteristics, and they raise the possibility that these factors may be manipulated to affect particle size.

17α-Ethinylestradiol (EE2) is an ideal compound used to study sorption hysteresis in activated sludge. EE2 is strongly hydrophobic (log K_{ow} = 3.67), relative to other commonly detected PhACs, and it will therefore partition to activated sludge quite favorably. EE2 is also a synthetic steroid and the active ingredient in birth control pills; it is therefore of particular interest and concern because of the potential to cause endocrine disruption. Sorption hysteresis could affect EE2 bioavailability, which is important because EE2 can be biotransformed into estradiol and estrone which are more biodegradable.

Methodology

Experimental overview. Two laboratory-scale bioreactor systems were operated, a MBR and CBR, both operated in continuous flow mode. Since MBRs have smaller particles than CBRs, it is possible to investigate the effect of particle size by operating these two bioreactors in parallel. Both bioreactors were originally seeded with mixed liquor from the City of Auburn Southside Wastewater Treatment Facility. The experimental strategy was to harvest biomass from the bioreactors for use in a series of sorption/desorption batch tests. The data retrieved from the batch tests was used to determine sorption and desorption isotherms, and the slopes of these isotherms were used to determine the partitioning coefficients for sorption (K_d) and desorption (K_{ds}), respectively (an example is presented in Figure 4.1, which is discussed in the Results and Discussion section). The sorption hysteresis index (HI) was calculated as follows:

$$HI = \frac{K_{ds} - K_d}{K_d} T, C_r \qquad (1)$$

The subscript T (23 ^{o}C) and C_r (C_r level is 0.5) refer to specific conditions of constant temperature and residual solution phase concentration ratio, respectively. The partitioning coefficient determined from the sorption experiments is K_d, and the partitioning coefficient determined from the desorption experiments is K_{ds}.

The bioreactor operating mode was adjusted in two ways, in an attempt to manipulate the biomass particle characteristics and investigate the possible effects on sorption and desorption. First, three different SRTs were used, 5, 10, and 20 days. Second,

nitrogen-limiting conditions were imposed on the bioreactors by decreasing the influent ammonia–N concentration from 40.3 mg N/L (at normal growth conditions) to 8 mg N/L (at nitrogen-limiting conditions). After the bioreactor operating condition was changed, the bioreactors were operated for three SRTs before samples were taken for analysis.

Bioreactor operation. The MBR had a working volume of 60 L and was equipped with one, vertically mounted membrane module (pore size 0.08μm, physical size (0.55 m in total length), surface area ($0.5m^2$), courtesy of Vivendi/US Filter), completely submerged in a plexiglass vessel. The module was 56 cm in length, and had a 8 cm diameter. The height of the vessel was 91 cm, with 16 cm of freeboard, and a 76 cm water depth. The module was placed to allow a 10 cm clearance both from the vessel bottom and the water surface. The module was mounted in the middle of the vessel, and held in place by a plexiglass U-shaped support apparatus. The MBR SRT was varied (5, 10, and 20 days), and mixed liquor was withdrawn daily to control the SRT. Peristaltic pumps were used in order to control the influent and effluent flow. The airflow rate was maintained at 10 L/min to sustain a DO concentration of 2 mg/L and to provide mixing. The pressure drop across the membrane was monitored daily, and was typically 1.2 psi. The pH was controlled with an auto-pH meter (alpha pH 200 1/8-DIN pH/ORP Controller, EUTECH Instruments Pte Ltd, Singapore) and pH electrode (Thermo Orion Glass pH electrode, Orion Research, INC., Beverly, MA). The pH of each reactor was maintained in the range of 6.8–7.3 by the addition of 0.1 M HCL solution or 0.1 M NaOH solution. The volumes of acid and base were monitored daily and the addition of

new solution to the storage vials was recorded. The temperature was ambient (approx. 24 ^{o}C).

The CBR had a working volume of 4 L. The SRT was varied (5, 10, and 20 days) and sludge was manually wasted each day to control the SRT. Aerobic conditions were maintained by bubbling ambient air through a porous diffuser. Aerobic conditions were verified by measuring dissolved oxygen (YSI Model 57 Oxygen Meter with YSI Model 5793 Standard Membranes, YSI Incorporated, Yellow Springs, OH) and redox potential (Eutech Instruments, Model 200, Singapore). The pH was controlled with an auto-pH meter (WDP Series Dual Input pH/ORP Controller, Walchem Corporation, Holliston, MA) and pH electrode (WEL-PHF-NN electrode, Walchem Corporation, Holliston, MA) with a protective housing (Model 102606, Walchem Corporation, Holliston, MA). The pH of each reactor was maintained in the range of 6.8–7.3 by the addition of 0.1 M HCL solution or 0.1 M NaOH solution. The volumes of acid and base were monitored daily and the addition of new solution to the storage vials was recorded. There was no pH control during the settling or effluent withdrawal phases. The temperature was ambient (approx. 24^{o}C).

Synthetic wastewater. The synthetic wastewater used for the MBR and the CBR was the same. The organic substrate (acetic acid) and inorganic nutrients were added in separate feed streams. The composition of the synthetic feed was (on mg COD/L basis: acetate (360), casamino acids (20), yeast extract (<1)). The inorganic salts content was (as mg/L total influent concentration) KCl (210), $MgCl_2$–$6H_2O$ (394), $MgSO_4$–$7H_2O$ (26), $CaCl_2$ (80), H_3BO_3 (0.11), $ZnSO_4$–$7H_2O$ (0.0.50), KI (0.027), $CuSO_4$–$5H_2O$ (0.11),

$Co(NO_3)_2$–$6H_2O$ (0.135), $NaMoO_4$–$2H_2O$ (0.056), $MnSO_4$–H_2O (0.62), and $FeSO_4$–$7H_2O$ (0.55). The influent P concentration was supplied as NaH_2PO_4–$2H_2O$ and was always 8.0 mg P/L. The influent N was supplied as NH_4Cl and was always 40.3 mg N/L. The synthetic wastewater used for nitrogen-limiting conditions was the same, except that the influent nitrogen concentration was reduced to 8 mg N/L.

Experimental protocol for sorption and desorption. The EE2 sorption experiments were conducted using 200 mL biomass samples taken from the CBR and MBR. Sorption of EE2 onto the biomass was determined by adding EE2 into glass bottles at different concentrations ranging between 100 and 1000µg/L. To prevent biodegradation, sodium azide was added at 0.2% w/w; this concentration inhibited biodegradation (as confirmed with respirometry), did not cause cell lysis (as confirmed microscopically and with soluble carbohydrate measurements), and did not change the sludge hydrophobicity (as determined by the MATH Test protocol described by Guellil *et al*., 1998). Samples were mixed on an orbital shaker at 200 rpm at 23^{o}C, including additional control bottles without biomass to make sure that EE2 was not being lost to the glassware. Samples were taken after 1 h; preliminary kinetic tests indicated that equilibrium was reached in this time. Filtered supernatants (0.2-µm Teflon filter) were used for EE2 analysis. Biomass-bound EE2 concentrations were determined by mass balance. All sorption tests were done in triplicate. After the sorption experiment, desorption experiments were conducted as follows: the glass bottles were agitated in the dark at 200 rpm, 23^{o}C for 5 h then centrifuged for 20 min at 3000x*g*. Next, 100 mL of the supernatant was removed, and the supernatant was replaced with fresh background

solution, and the tubes were further agitated at 200 rpm, 23^{o}C for 1 h. Filtered supernatants (0.2-μm Teflon filter) were used for EE2 analysis. All desorption tests were done in triplicate.

Visualization of sorption-hystersis: A 250mL glass bottle with a working volume of 200mL was used for this test. Sorption of Diclofop-Methyl (DM) onto the biomass was determined by adding DM into glass bottles at the final concentration of 500ug/L. Sorption tests were conducted in an orbital shaker at 250rpm at 23^{o}C for 1day. Biomass inhibition was achieved using sodium azide (final concentration of 200mg/L). After sorption experiment, the glass bottles were centrifuged for 10 min at 2,000xg. Next, 100 mL of the supernatant were removed. The removed supernatant was replaced with fresh background solution (effluent) and the bottles were further agitated under the same conditions for 1day. After each step (sorption and desorption), biomass samples were taken and dried on the microscope slide, and then mount with Propidium Iodide was applied to slide. Accumulation of DM by biomass floc was visualized using confocal microscope. Setup of confocal microscope followed the study of Wolfaardt *et al*. (1995)

Analytical methods. EE2 was detected by HPLC (Hewlett-Packard, HP 1100). The system consisted of a degasser (G1322A), a Quaternary pump (G1311A), an ALS auto-sampler (G1313A), a Colcomp column oven (G1316A) and Variable Wavelength UV–VIS Detector (G1314A). A Hypersil ODS C18, (125x46mm,5 μm) column was used. HPLC operating conditions were as follows; UV detector wavelength, 197 nm and mobile phase, acetonitrile and water (40:60) with solvent delivered at a constant flow rate of 1 mL/min. The total runtime of the HPLC analysis was 10 min. Each batch of samples

included spiked surrogate samples, and recovery always exceeded 90%. Total suspended solids (TSS), volatile suspended solids, and ammonia–N were routinely analyzed according to Standard Methods (APHA, 1992). Particle size distribution and total specific surface area were determined on 15 mL samples from the MBR and CBR sludges utilizing a Horiba LA-920 laser scattering particle size distribution analyzer (Delta Analytical Instruments, North Huntington, PA). The measurement range for this instrument is 0.02–2000 microns. Extracellular polymeric substances (EPS) were extracted chemically, using formaldehyde and NaOH, as follows; 10 mL sludge was washed with DI water three times. Formaldehyde was added to the rinsed sludge and incubated for 1h at 4°C and then incubated for 3h with NaOH. The extracted EPS were harvested using centrifugation for 10 min at 6000xg, followed by membrane filter (0.2μm membrane) to remove solid materials. The extracted EPS was purified with a dialysis membrane of 3500 Da (Pierce, USA) for 24h. The extracted EPS was analyzed for total carbohydrates and protein. The carbohydrate and protein concentration in EPS were determined by the anthrone method (Gaudy, 1962) and Lowery method (Lowry *et al*., 1951), respectively.

Results and Discussion

EE2 partitioning. Figure 4.1 shows a typical sorption/desorption result for two different biomass floc suspensions. The suspension taken from the MBR had a mean particle size of 10μm, while that of the CBR had a mean particle size of 120μm. In this example, the sorption/desorption experiment yielded K_d and K_{ds} values of 0.47 and 0.56 L/g for the MBR biomass, and 0.32 and 0.61 L/g for the CBR biomass, respectively.

These K_d and K_{ds} values are the slopes of the respective isotherms. Using these values, the HI values for the MBR and CBR were 0.19 and 0.89, respectively. Results like this suggested that the particle size influenced the HI for EE2 sorption, and that the extent of the observed hysteresis (as reflected by the difference between the sorption and desorption lines) increased as the soluble EE2 concentration increases. As EE2 concentration increases, more intrafloc mass transfer and entrapment may occur.

Figure 4.2 shows the effect of the biomass mean particle size and specific surface area on the measured K_d values; the results from both the MBR and CBR sorption experiments are included. The mean particle size data points are shown with vertical bars that have a length equal to the standard deviation of the particle size distribution. K_d values increased as the mean particle size decreased and as the specific surface area increased. The observed trends were best approximated with a power law equation, which indicates that there was a scale invariant relationship between dependant and independent variables. In this case, the variable on the *x*-axis is the dependant variable, the partitioning coefficient, K_d, and the independent variables are the mean particle size and the specific surface area. Figure 4.2 indicates that the mean particle size and specific surface area can have a dramatic and nonlinear effect of the observed K_d. The reason for this nonlinear relationship may lie in the fact that a number of factors that affect the K_d also change as the biomass particle size changes (e.g. porosity, density, available surface area, the protein and carbohydrate content of the floc). These results suggest that the cumulative effect of the change in all of these factors produces the observed nonlinearity. Figure 4.2 also shows that the K_d values measured for the MBR were always equal to or greater than those measured for the CBR, because of the difference in the particle sizes.

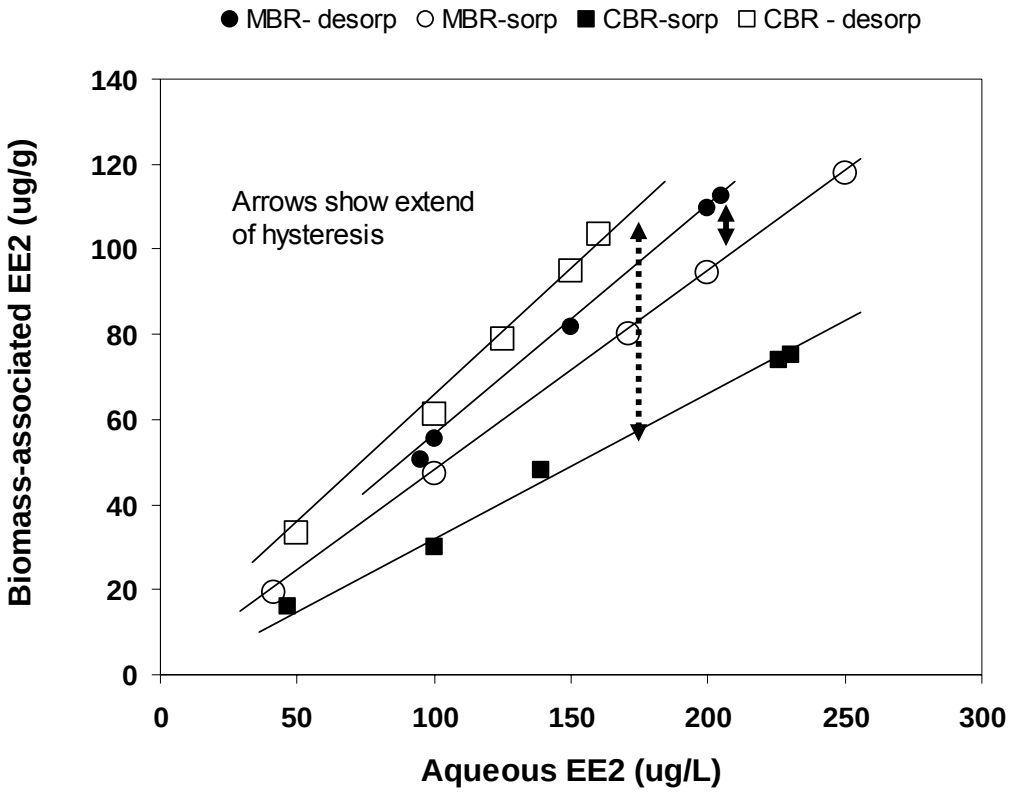


Figure 4.1 An example showing a set of sorption and desorption isotherms for MBR and CBR biomass.

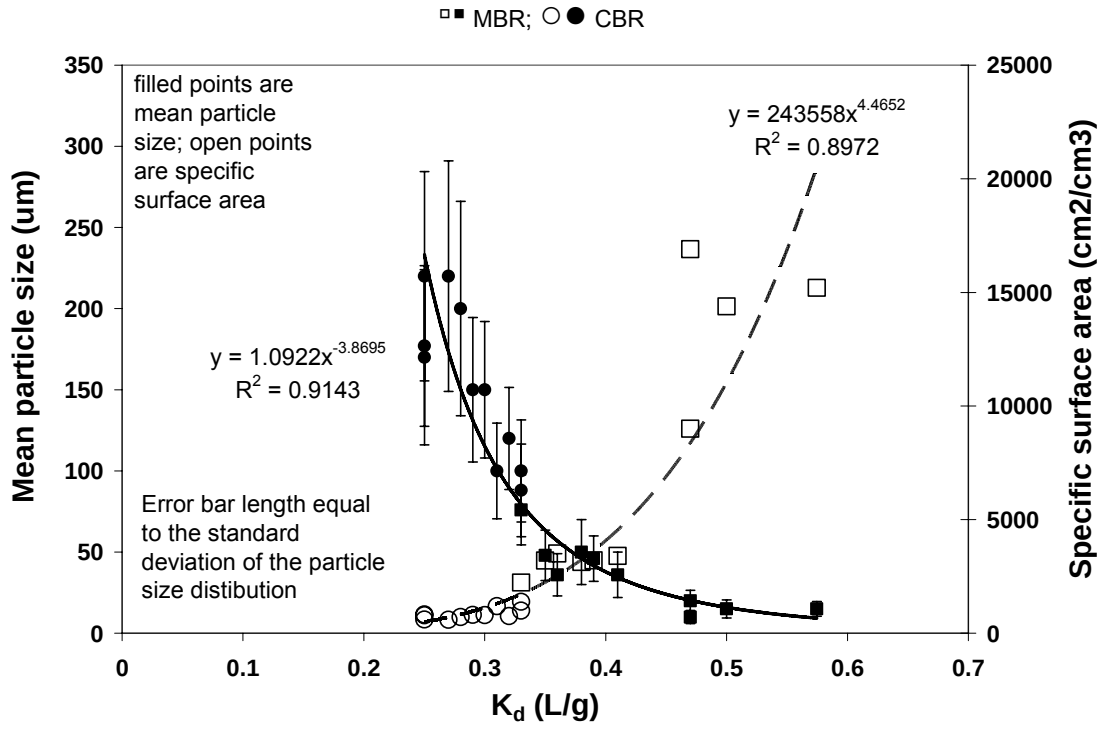


Figure 4.2 The effect of mean particle size and specific surface area on K_d: normal growth conditions.

The CBR K_d values ranged between 0.25 and 0.33 L/g, while those of the MBR were between 0.33 and 0.57 L/g. This means that the EE2 sorption capacity of MBR sludge was up to twice that of CBR sludge. This result corroborates previous findings which show that MBR K_d values for EE2 are higher than those of CBR sludges (Yi *et al*., 2006). Figure 4.3 shows the effect of the biomass mean particle size and specific surface area on the measured K_d values in the case where the biomass was grown under nitrogen-limiting conditions. The relationships between measured K_d values and the particle size and specific surface area were not approximated well by the power law (or by any other linear or nonlinear model). There are weak trends showing that K_d increased as mean particle size decreased and as specific surface area increased, but the data scatter and poor curve fit show that nitrogen-limiting conditions significantly interfere with the trends otherwise present under normal growth conditions. Figure 4.3 also shows that the CBR K_d values sometimes exceeded those of the MBR, another trend different from that observed in Figure 4.2. Under nitrogen-limiting conditions, the slime production was noticeable and the EPS-associated carbohydrate levels were significant (Table 4.1). EPS is known to alter the surface characteristics of biomass (Liao *et al*., 2001) and is therefore a plausible cause for the poor correlations.

Hysteresis index. There were good correlations between the HI and both the mean biomass particle size and specific surface area (Figure 4.4). The MBR HI values ranged from 0.15 to 0.67, while those of the CBR were from 0.65 to 0.92, so the HI was nearly always higher for CBR biomass than for MBR biomass. The power law was the best model with which to approximate the data, which again showed the nonlinear effect

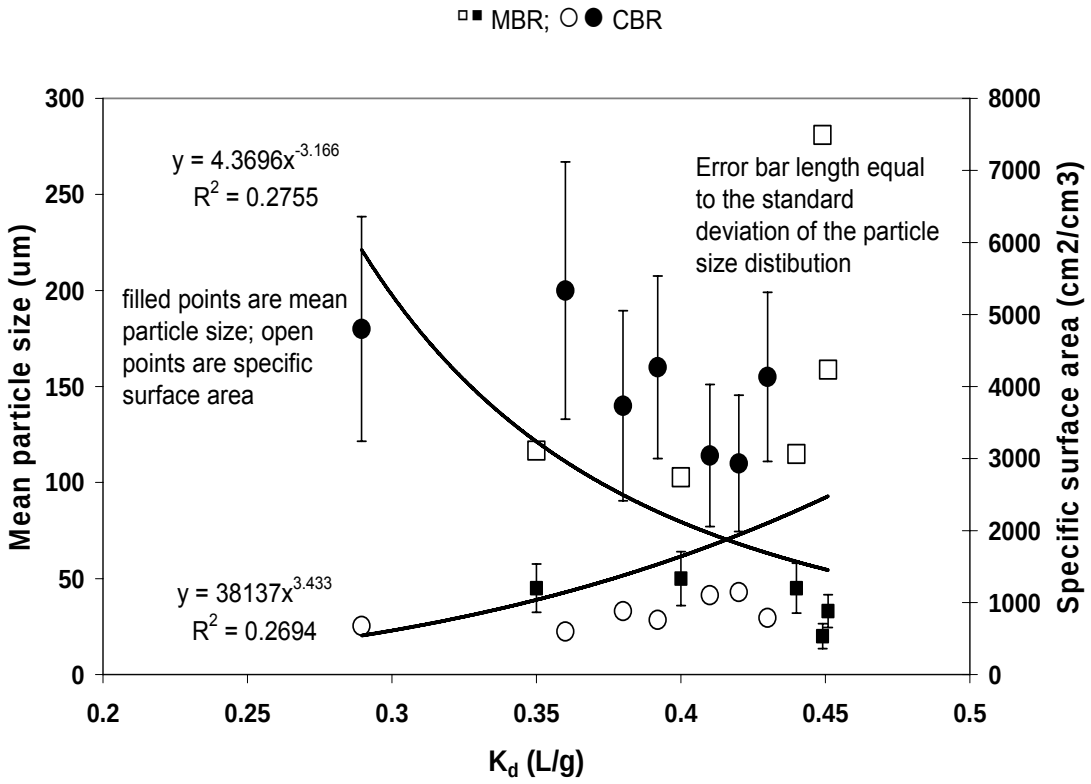


Figure 4.3 The effect of mean particle size and specific surface area on K_d: nitrogen-limited growth.

Table 4.1 The effect of nitrogen-limitation on exocelluar polymer production

Bioreactor	SRT	Extracellular protein/carbohydrate (average of three measurements)	
		Normal	Nitrogen-limiting conditions
MBR	5	9.3/23	9.1/[a]
	10	16/18	20/28
	20	7.5/24	7.8/44
CBR	5	6.6/17	13/23
	10	13/14	16/24
	20	6.4/29	7.3/37

The production of extracellular polymer substances was significant.
[a] Data not available.

that biomass particle size has on HI values. For example, as the mean particle size increased from 50 to 150μm, the HI values take a dramatic increase from approximately 0.50 to 0.80; this is particularly interesting given that the 50-150μm range is the particle size range that represents the transition from those particle sizes typically found in MBRs to those commonly found in more conventional activated sludge systems. Selection of MBRs in the design of biological wastewater treatment systems appears to offer the advantage of lower sorption hysteresis of EE2, and this result may also apply to other micropollutants with similar chemical characteristics.

This result shows that the biomass particle size can have a dramatic effect on the entrapment of EE2 within activated sludge floc, which in turn may affect the ultimate fate of EE2. Figure 4.5 shows the relationship between the HI and the mean biomass particle size and specific surface area, when the biomass was initially grown under nitrogen-limiting conditions. The correlations were not as good as those derived when the biomass was grown under normal conditions, but the larger particles tended to have higher HI values. Under nitrogen limited conditions, the MBR HI values ranged from 0.32 to 0.6, while those of the CBR were between 0.53 and 0.9, so that the MBR biomass tended to have lower HI values. The overall range of HI values measured for the MBR and CBR were similar under normal growth and nitrogen-limited conditions.

The effect of SRT. Figure 4.6 shows the effect of SRT and nitrogen limitation on the measured K_d. When the biomass was grown under normal growth conditions the average MBR K_d generally decreased with the increase of SRT. When the biomass was grown under nitrogen-limiting conditions, the average MBR K_d values were relatively stable, 0.45 L/g at a 5 day SRT, 0.45 L/g at a 10 day SRT, and 0.35 L/g at a 20 day SRT. Under normal conditions the average CBR K_d values were 0.47 L/g at a 5 day SRT, 0.64 L/g at a 10 day SRT, and 0.25 L/g at a 20 day SRT. The average CBR K_d value measured at a 10 day SRT was surprising, given that previous results have shown that MBR K_d values are higher than those of comparably operated CBRs (Yi *et al*., 2006). The CBR K_d values measured during nitrogen-limiting conditions were lower than those measured during normal growth conditions, except for when the SRT was 20 days. Overall, different K_d values were observed at different SRTs, but these results did not

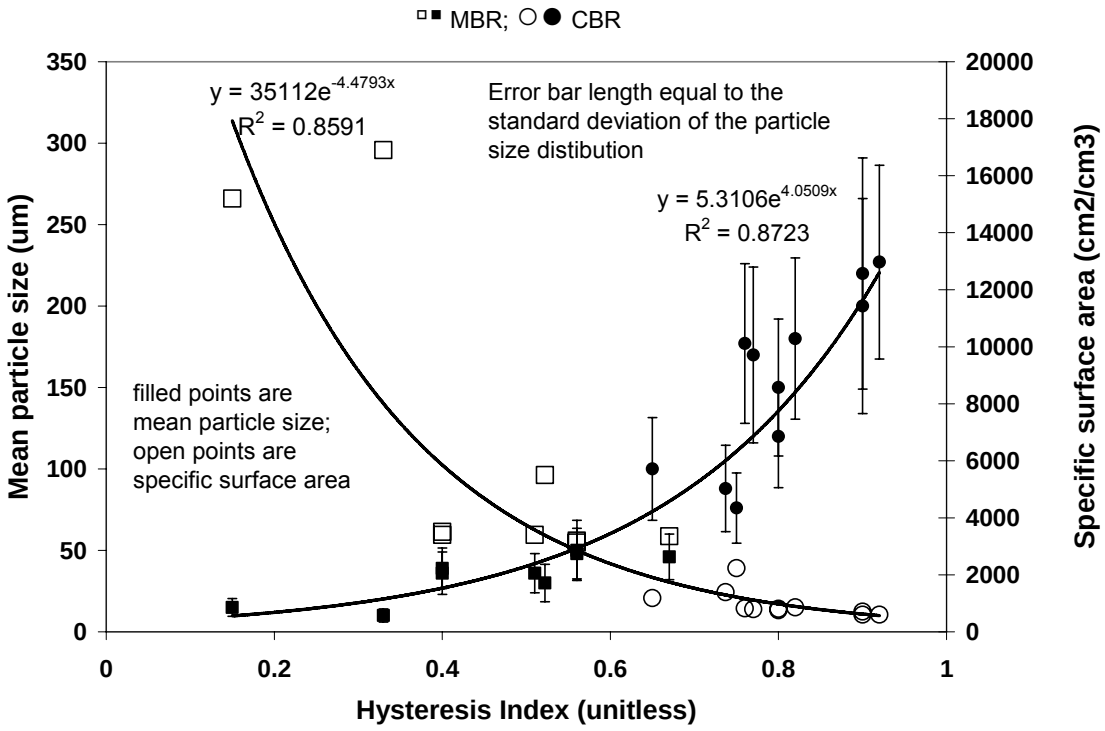


Figure 4.4 The effect of mean particle size and surface area on HI: normal growth.

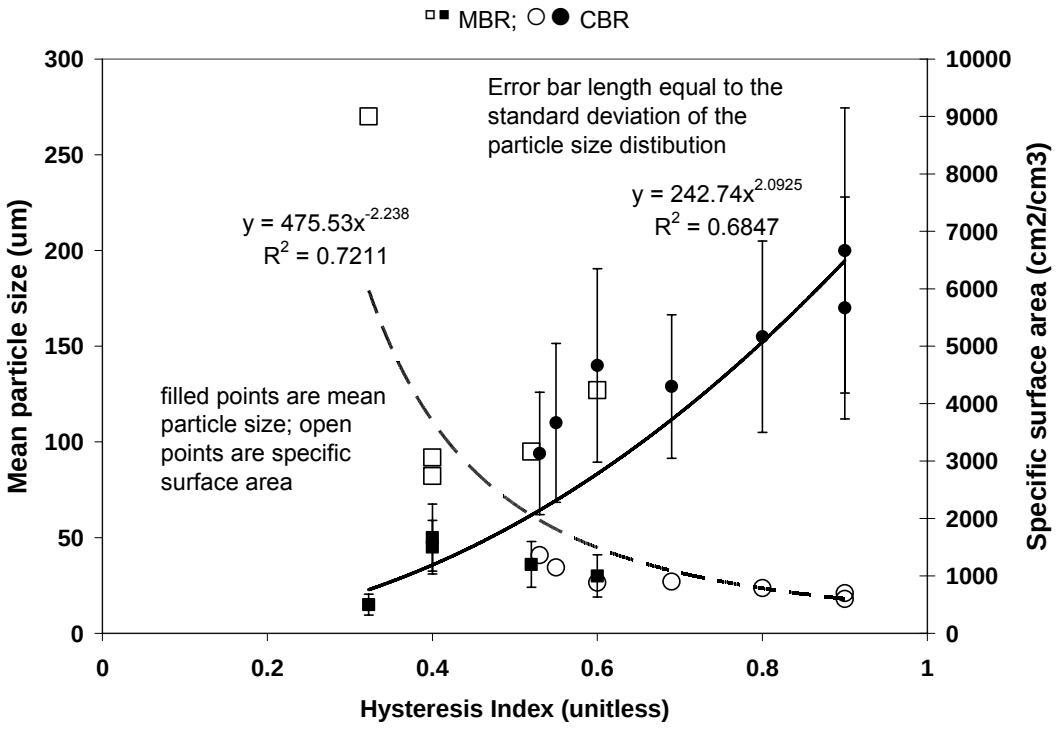


Figure 4.5 The effect of mean particle size and surface area on HI: nitrogen-limited growth.

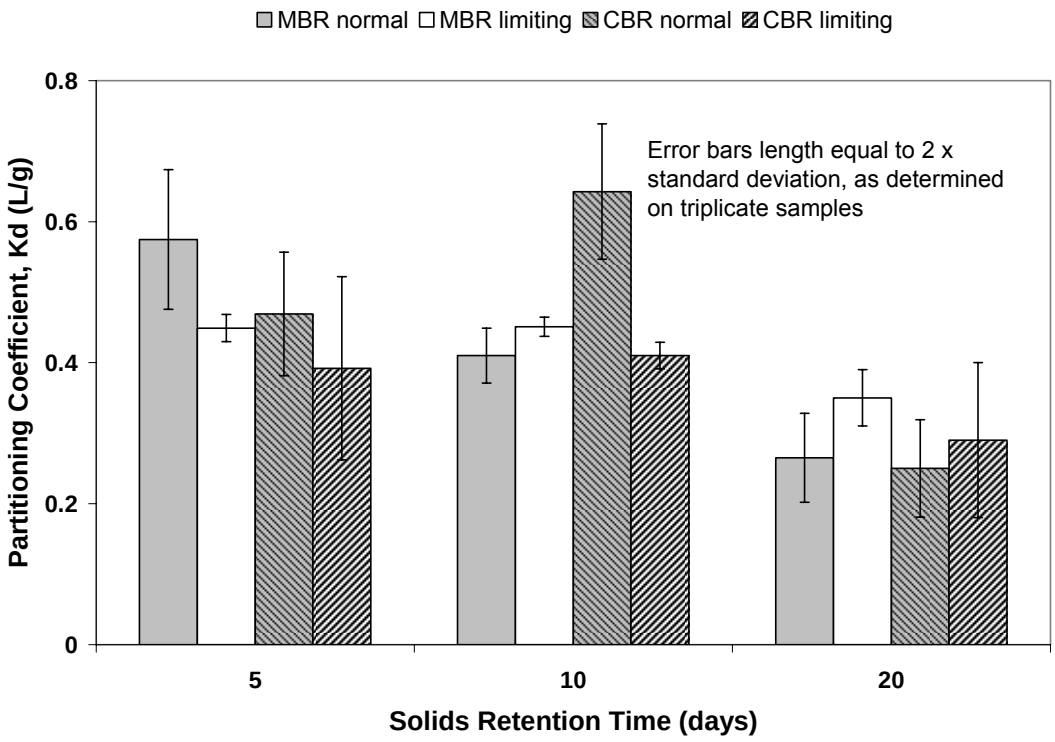


Figure 4.6 The effect of solids retention time on K_d

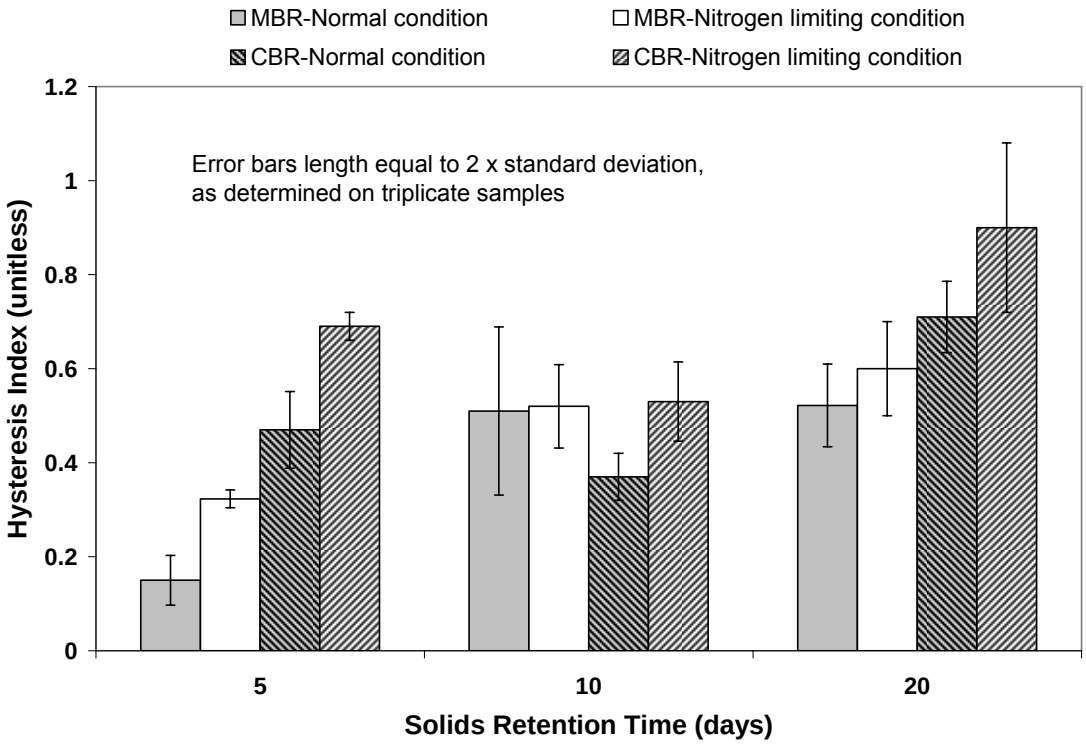


Figure 4.7 The effect of sludge retention time on the hysteresis index.

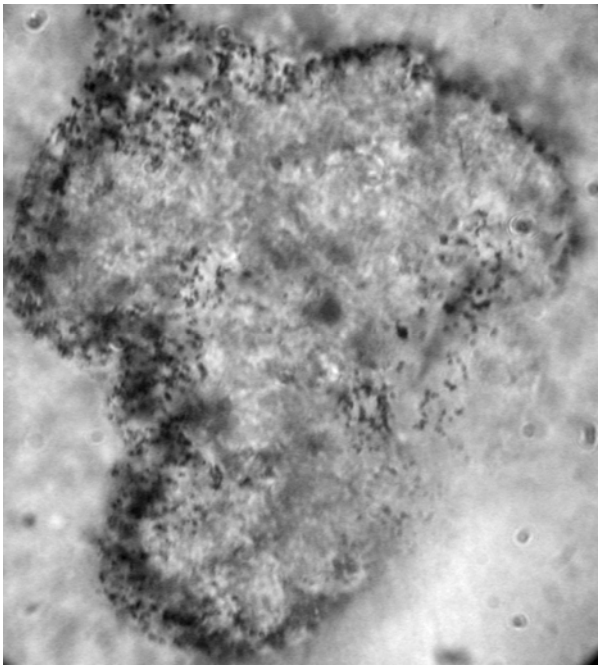
Table 4.2 The effect of SRT of the means of the particle size distribution

Bioreactor	SRT	Mean of the particle size distribution	
		Average	Standard deviation
MBR	5	18	14
	10	36	16
	20	30	14
CBR	5	129	46
	10	94	54
	20	170	44

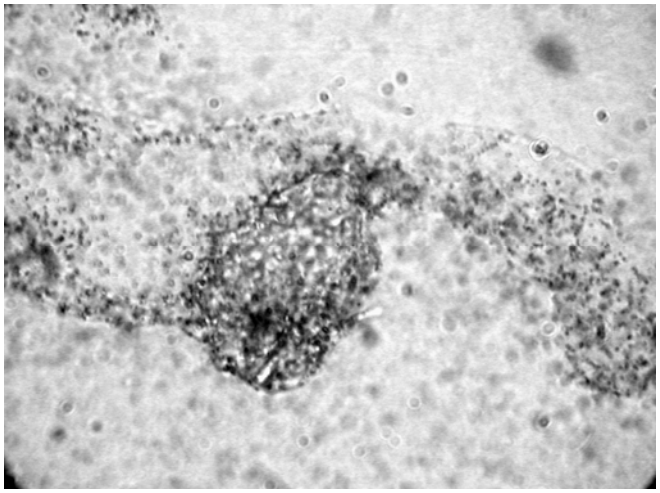
produce clear and generally applicable relationships that applied to both the MBR and CBR biomass. Under non-nitrogen-limiting conditions, the CBR K_d values did not show clear relationships with SRT, and the K_d value determined at a 10 day SRT was surprisingly high. MBR K_d values decreased as the SRT increased; this might be because, as the SRT increases, the MBR may have been retaining more colloidal material that had different surface properties than the activated sludge biomass. Under nitrogen-limiting conditions, there were also no clear relationships that can be applied to both the MBR and CBR biomass. Nitrogen-limitation is known to induce EPS production, but how this affects the structural and chemical properties that bear directly on K_d is still not well understood. Future research should endeavor to fill this knowledge gap. Figure 4.7 shows the effect of SRT and nitrogen limitation on the HI. At a 5 day SRT, the average HI values were lower for the MBR, and the nitrogen-limiting conditions increased the HI for

both bioreactors. At a 10 day SRT, the average HI for the bioreactors were more similar, except that the CBR had a lower than expected HI under normal growth conditions. At a 20 day SRT, the MBR again maintained a lower HI than the CBR, and the nitrogen limitation caused a significant change in the HI for the CBR but not the MBR. Overall, the HI appeared to increase gradually with SRT for both bioreactors, perhaps because of a general effect that SRT has on the structure of activated sludge floc. There is recent evidence that shows that, as the SRT is increased, the floc surface becomes covered with tightly bound EPS (Liao *et al*., 2002; Li and Yang, 2006); this tightly bound polymer may be trapping of compounds like EE2. SRT proved to be a less effective means of directly manipulating the biomass characteristics (Table 4.2). The mean particle size of the MBR was unaffected by SRT, and the CBR mean particle size was not affected in a systematic way. Therefore, it follows that the effects of SRT on the K_d and the HI, although present, were not as dramatic. When the SRT was 5 or 20 days, nitrogen limitation increased the HI values; this result implies that it is *possible* to cause increased accumulation of EE2 in activated sludge floc during nitrogen-limiting conditions.

Visualization of sorption/desorption Hysteresis in sludge flocs. The fluorescence intensity observed in the study was typically sustained for 1 day using the sludge from MBR and CBR, after which no further accumulation was observed. Figure 4.9 shows the confocal microscopic images from the sludge flocs of SBR and MBR. The red staining highlighted the position of bacteria and the green staining represented diclofop-methyl (DM) accumulation. After 1day of sorption, DM was completely diffused into floc regardless of the floc size, and even distributed. After desorption,

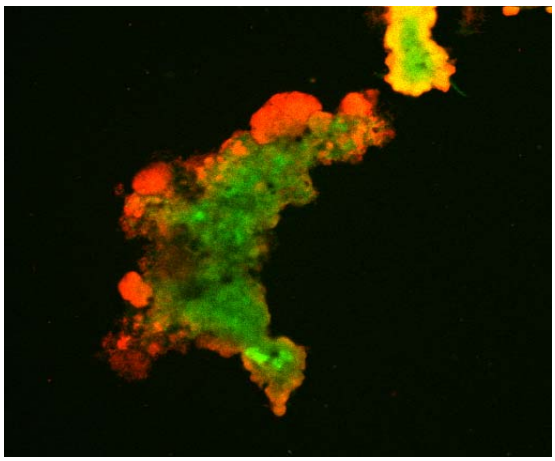


(a) SBR particle

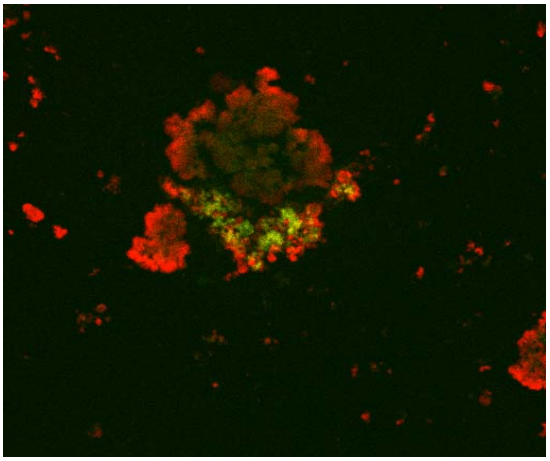


(b) MBR particle

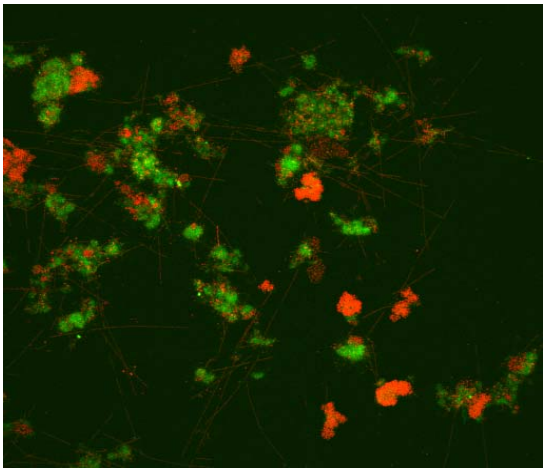
Figure 4.8 Visualization of Sorption using Miroautoradiograpy



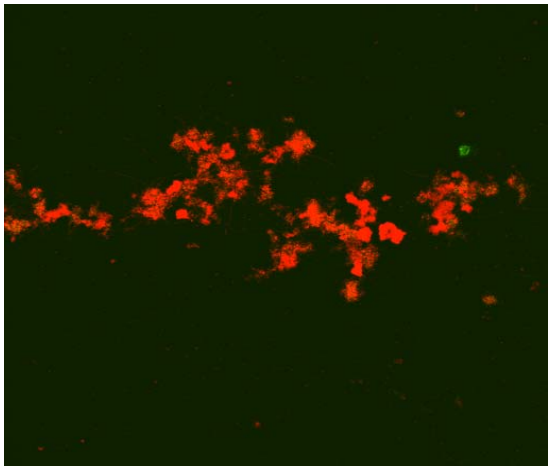
(a) After sorption in SBR particles



(b) After desorption in SBR particles



(a) After sorption in MBR particles



(b) After desorption in MBR particles

Figure 4.9 Visualization of Hysteresis using confocal microscope; Biomass (red) and green (Diclofop-methly)

intensity of DM in different size of flocs was different, that is, the bigger flocs still have DM inside. In smaller floc, most of DM was released and not observed (Figure 4.8). This experiment clearly showed that previous results that particle size affects desorption processes. The bigger particles are, the bigger HI values. Another sorption test using micro autoradiography showed how sorption is affected by particle size. Cell surface was absolutely shown as a good sorption site (Figure 4.9).

The connection between the HI and the loss of sorption capacity. When sorption hysteresis occurs, the removal capacity of the biomass is reduced because some of the surface sites are unavailable. This reduction in the removal capacity cannot be ignored, because neglecting it may cause an overestimation of the sorption capacity of the activated sludge. Before the operating implications of sorption hysteresis can be quantitatively explored, the HI and the loss of sorption capacity must be connected. The HI is a measure of the potential for PhAC entrapment, but the actual loss of sorption capacity occurs when the biomass, having been previously exposed to a higher soluble PhAC concentration, is exposed to a lower PhAC concentration, and then again to a higher PhAC concentration. Figure 4.10 illustrates an example, which is formulated using data collected in this study. Consider point #1, which corresponds to a biomass-associated PhAC concentration of 76μg/g(q1) and suspended in wastewater with a soluble PhAC concentration of 230μg/L(c1). When this biomass is exposed to a lower PhAC concentration (e.g.100μg/L($c2$), located at point #2 in Figure 4.10), hysteresis may occur; so that the amount of the PhAC associated with the biomass will be 64μg/g($q2'$) located at point #$2'$, instead of 32μg/g($q2$) located at point #2. The quantity $q2'$-$q2$

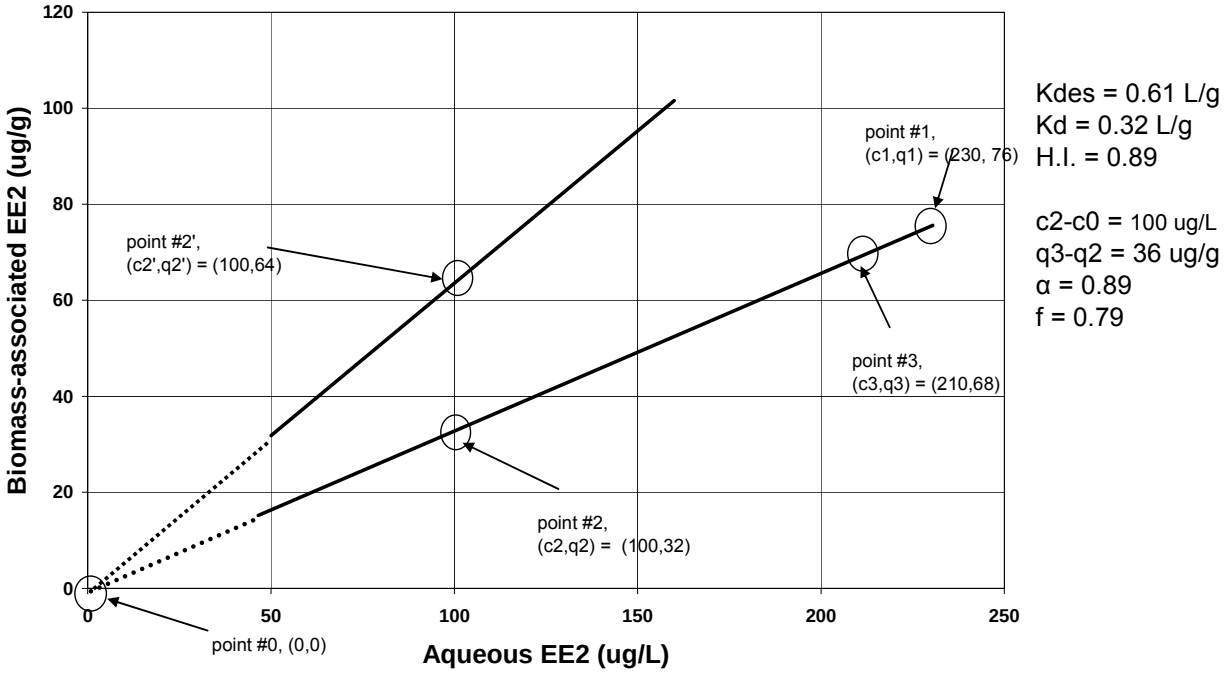


Figure 4.10 Typical sorption and desorption isotherms for CBR biomass: an example illustrating the loss of sorption capacity and the determination of α.

represents the PhAC amount that remains associated with biomass, possibly entrapped within the floc. The loss of sorption capacity is realized when an "upshift" occurs, or in other words, when the biomass is now exposed to a higher PhAC concentration, $c3$ (210μg/L) at point #3, where the biomass can only remove $q3\text{-}q2'$ (as opposed to $q3\text{-}q2$). In this case, the fractional loss of sorption capacity (f) is

$$\frac{(q2'-q2)}{q3-q2} \tag{2}$$

Therefore, f is a function of $q2'\text{-}q2$, which can be quantitatively related to the HI. The geometries of the sorption and desorption lines can be exploited to show

$$f = HI \cdot K_d \cdot \frac{(c2-c0)}{(q3-q2)} \tag{3}$$

or

$$f=\text{HI}*\alpha, \tag{4}$$

where

$$\alpha = K_d \cdot \frac{(c2-c0)}{(q3-q2)} \tag{5}$$

The α term is therefore dependent on four factors: (1) the slope of the sorption line, which determines K_d, (2) the relationship between the sorption and desorption lines, which determines the $c2\text{-}c0$ term, (3) the magnitude of the "upshift", which determines the $q3\text{-}q2$ term, and (4) the magnitude of $c2\text{-}c0$, which depends on the location of point #2. The

example shown in Figure 4.10 produced α=0.89, and with HI=0.89, this produced f=0.79. In general, f is proportional to HI, and their respective values are normally on the same order of magnitude, but the exact relationship between f and HI depends on the relationship between the sorption and desorption lines and the nature of the transient phenomena.

Operating implications. The operating implications of sorption hysteresis depend on the configuration of the treatment plant in question. To quantify the operating implications for a single CSTR, the following schematic is considered (Figure 4.11).

The mass balance for a given PhAC is as follows:

$$QC_i = QC_o + Q_{was} \cdot X_{was} \cdot C_{sludge} + Q_{prim} \cdot X_{prim} \cdot C_{prim} + k_{biol} \cdot C_0 \cdot V \cdot X_{sludge} \qquad (6)$$

where Q is the wastewater flow (volume/time); C_i the influent PhAC concentration (mass/volume); C_0 the effluent PhAC concentration (mass/volume); Q_{was} the waste activated sludge flow (volume/time); X_{was} the waste activated sludge concentration (mass/volume); C_{sludge} the PhAC concentration in sludge (g/g); Q_{prim} the primary sludge flow (volume/time); X_{prim} the primary sludge concentration (mass/volume); C_{prim} the PhAC concentration in primary sludge (mass/volume); k_{biol} the rate of co-metabolic PhAC biotransformation resulting in CO_2 production (volume per mass per time); X_{sludge} the mixed liquor suspended solids concentration (mass/volume); and V the bioreactor volume (volume). In Eq. (6), the pharmaceutical concentrations in the waste activated sludge and in the mixed liquor are assumed to be the same (C_{sludge}). Also, loss of PhACs

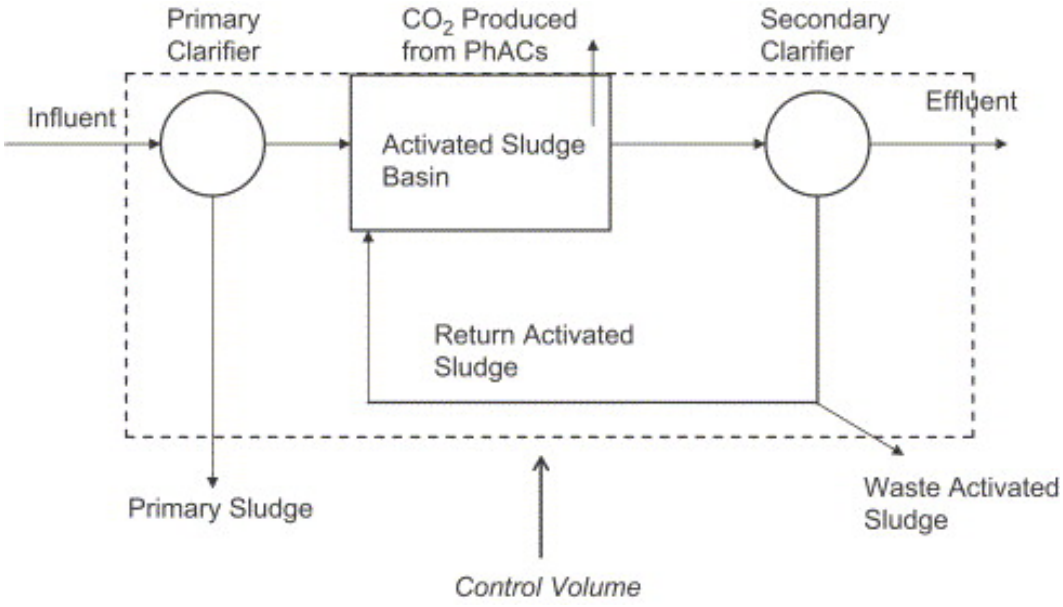


Figure 4.11 Continuous flow (CSTR) activated sludge wastewater treatment plant schematic.

due to volatization is neglected. The next step is to connect the aqueous and biomass phase PhAC concentration using the appropriate partitioning coefficient

$$C_{sludge} = K_d \cdot C_0 \qquad (7)$$

$$C_{prim} = K_d^{'} \cdot C_i \qquad (8)$$

where K_d and K_d' represent the partitioning coefficients associated with the mixed liquor and the primary solids, respectively. Finally, sorption equilibrium is assumed and Eq. (6) is rearranged and then normalized with respect to the influent concentration to produce the following:

$$\frac{C_0}{C_i} = \frac{Q - Q_{prim} \cdot X_{prim} \cdot K_d^{'}}{Q + Q_{was} \cdot X_{was} \cdot K_d + k_{biol} \cdot X_{sludge} \cdot V} \qquad (9)$$

The K_d term can now be replaced with (1-f) K_d,

$$\frac{C_0}{C_i} = \frac{Q - Q_{prim} \cdot X_{prim} \cdot K_d^{'}}{Q + Q_{was} \cdot X_{was} \cdot (1-f) K_d + k_{biol} \cdot X_{sludge} \cdot V} \qquad (10)$$

The f term is introduced to represent the fraction of the adsorptive capacity lost due to hysteresis, and theoretically it can hold values between 0 and 1. When f is equal to 0, the full sorption capacity is available, and no sorption hysteresis is observed; very high values of f indicate that a larger fraction of the sorption capacity has been lost. An expression similar to Eq. (10) can be derived for a system of CSTRs operating in series. Joss *et al*. (2006) recently presented the appropriate expression, which accounted for

recycle streams and is fundamentally based on the same mass balance presented in Figure 4.11:

$$\frac{C_0}{C_i} = \frac{1 - K_d^{'} \cdot X_{prim}}{1 + (1-f) K_d \cdot X_{sludge}} \times \frac{1}{(1+R)\xi + (1 + (1-f) K_d SP) / (1 + (1-f) K_d \cdot X_{ss})} \qquad (11)$$

Where

$$\xi = \left[1 + \frac{k_{biol} \cdot X_{ss} \cdot \Theta_{hrt}}{\left[(1+R)(1 + (1-f) K_d X_{ss}) \cdot n \right]^b} \right] \qquad (12)$$

SP is the specific sludge production (mass/volume); R the recycle rate (unitless); Θ_{hrt} the hydraulic retention time (time); and n the number of reactors in series. Figure 4.12 presents the quantitative effect of sorption hysteresis. The relative normalized effluent quality is shown on the y-axis, and it is the ratio of the normalized effluent quality (at a given value of f) divided by the normalized effluent quality when f is zero:

$$\varepsilon = \frac{(C_0 / C_i)_{fi}}{(C_0 C_i)_{f=0}} \qquad (13)$$

The relative normalized effluent quality is calculated for four cases; cases 1–3 are for a single CSTR, and for the case when biotransformation is important (#1), when biotransformation is negligible and sorption is favorable K_d=2L/G (#2), and when biotransformation is negligible and sorption is very strong K_d=10L/G (#3). Eq. (10) is used for the first three cases. Eq. (11) is used for the fourth case, which is when three CSTRs in series are considered, and both sorption (K_d=0.5) and biotransformation play a

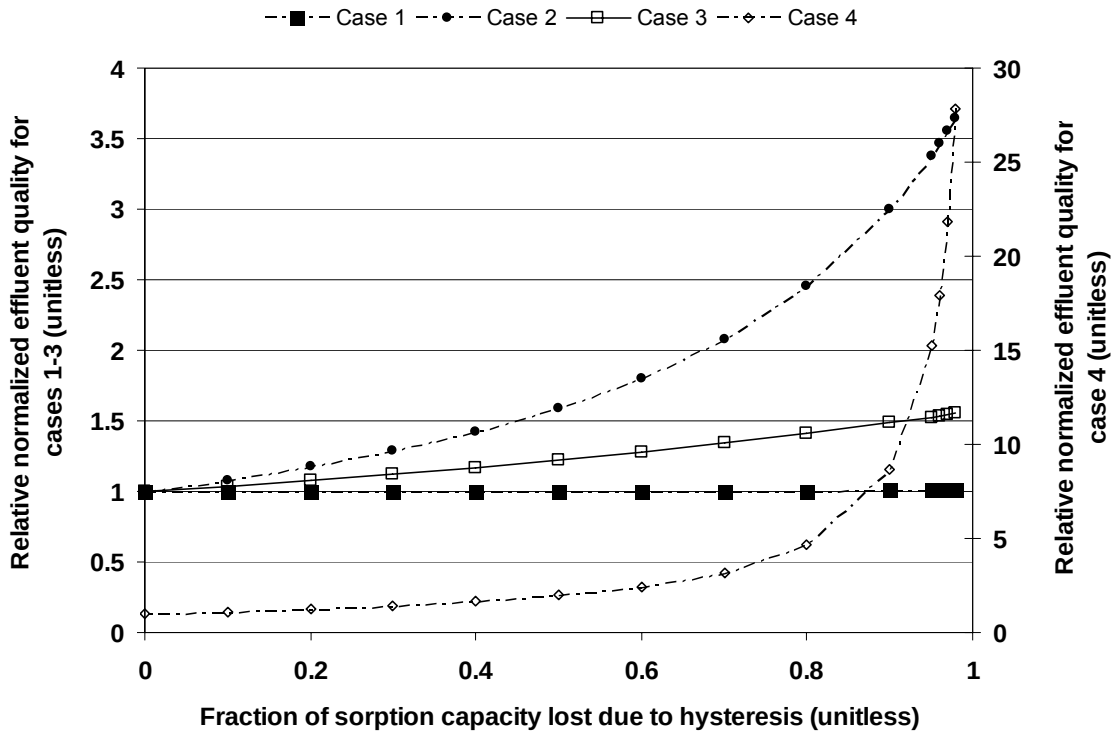


Figure 4.12 The effect of sorption hysteresis on relative normalized effluent quality.

role. For case 1, sorption hysteresis has a negligible effect on ε, which is to be expected since biotransformation (and not sorption) is the principle removal mechanism. For the second case, the effect of sorption hysteresis is moderate, and it is only at very high values of *f* that the relative normalized effluent quality begins to approach 1.5. For case 3, sorption hysteresis causes a more dramatic increase in the relative normalized effluent quality, which is greater or equal to 1.8 when *f* is 0.6 or greater. These first three cases are in sharp contrast to case 4. When *f* is 0.5, ε is 2, and as *f* increases, more dramatic increases in normalized effluent quality occur. Values of *f* greater than 0.9 cause the effluent quality to deteriorate by a factor of at least 9. Thus, in some bioreactor configurations and for chemicals like EE2, severe cases of sorption hysteresis can cause the effluent concentrations to increase dramatically. The results of the current work show that severe levels of sorption hysteresis are possible with larger biomass particle sizes typically found in CBR systems. For example, the CBR HI values observed during this study were between 0.53 and 0.92; this translates into *f* values between 0.47 and 0.82 (when α=0.89). This loss of sorption capacity would result in ε values between 2 and 5 for a series of three CSTRs (case #4). MBRs alleviate this problem by producing smaller biomass particles, less amenable to sorption hysteresis.

Conclusions

The conclusions from this study are as follows:

• Biomass characteristics can have an important effect of sorption of EE2 to activated sludge biomass. The mean particle size and specific surface area can have a dramatic and

nonlinear effect of the observed EE2 partitioning coefficient and on sorption hysteresis. As the mean particle size decreases, more EE2 partitions to activated sludge biomass and less sorption hysteresis is observed. The partitioning coefficient and the HI both correlated well with mean particle size, and the relationships were best approximated by a power law expression. MBR sludge generally has higher EE2 partitioning coefficients and smaller HI values than CBR sludge.

• When the biomass was grown under nitrogen-limited conditions, the observed partitioning coefficients and HI values did not correlate as well with biomass size.

• Changing the SRT did not prove to be an effective means of directly manipulating the biomass characteristics, or the measured EE2 partitioning coefficients or HI values.

• Visualization study proved the effect of particle size on sorption hysteresis.

• Severe levels of sorption hysteresis may potentially lead to significant increases in the effluent concentrations of PhACs, depending on the process configuration and the relative roles of sorption and biodegradation. When sorption is the most important removal mechanism, severe levels of sorption hysteresis can cause a significant increase in the effluent concentration of PhACs in a single CSTR system. When both biodegradation and sorption are important removal mechanisms for three CSTRs in series, severe levels of sorption hysteresis can cause the relative effluent concentrations of PhACs to increase by at least a factor of 9.

Acknowledgements

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V. THE LINK BETWEEN NITRIFICATION AND BIOTRANSFORMATION OF 17A-ETHINYLESTRADIOL

Abstract

Biological treatment processes are probably important for preventing the proliferation of steroidal compounds in the environment, and a growing number of reports suggest that nitrification may play a role in removing these chemicals from wastewater. The link between nitrification and biotransformation of 17α-ethinylestradiol (EE2) was investigated using enriched cultures of autotrophic ammonia-oxidizers. Batch experiments showed that ring A of EE2 is the site of electrophilic initiating reactions, including conjugation and hydroxylation. Ring A was also cleaved before any of the other rings are broken, which is likely because the frontier electron density of the ring A carbon units is higher than those of rings B, C, or D. EE2 and NH_3 were degraded in the presence of an ammonium monooxygenase (AMO) containing protein extract, and the reaction stoichiometry was consistent with a conceptual model involving a binuclear copper site located at the AMO active site. Continuous tests showed a linear relationship between nitrification and EE2 removal in enriched nitrifying cultures. Taken together, these results support the notion that EE2 biotransformation can be cometabolically mediated under operating conditions that allow for enrichment of nitrifiers.

Keywords: Frontier electron density, Nitrifying bacteria, 17a-EthinylEstradiol, Carbamazepine, Trimethorprim

Previous research (Shi *et al*., 2004; Vader *et al*., 2005; Kim *et al.,* 2005; Yi *et al.,* 2006; Dytczak *et al.,* 2006) has provided useful information about the removal of EE2 in nitrifying cultures, but there remain important and unanswered three questions. The first unanswered question concerns the reaction mechanisms (e.g. ring cleavage, hydroxylation) which are at work when EE2 is initially biotransformed. Most previous experiments were not conducted in the manner that allowed for the identification of metabolites, leaving the nature of the chemical reactions unclear. There has also been little effort dedicated toward the development of a better fundamental and conceptual understanding of EE2/NH_3 cometabolism. This need raises the second unanswered question, which concerns identifying conceptual model can be used to understand the stoichiometry of EE2/NH_3 cometabolism. There is also not enough information in the literature to analytically characterize the relationship between nitrification and EE2 biotransformation, which are presumably linked cometabolically. The third question concerns the relationship between EE2 and NH3 biotransformation rates. The purposes of this study are to answer these questions using laboratory-scale experimentation.

Recent applied research has demonstrated that micropollutant removal efficiencies depend on a number of factors including the chemical characteristics of the compound(s) (e.g. log K_{ow}), the operating solids retention time, and the biomass particle characteristics (Lishman *et al*., 2006;Clara *et al.,* 2005; Yi *et al*., 2006; Oppenhiemer and Stephenson, 2006). One class of micropollutants that has attracted considerable attention

in the literature is the steroidal compounds, including natural estrogens such as 17β-estradiol (E2) and estrone (E1), and the synthetic steroid 17α ethinylestradiol (EE2). These compounds tend to adsorb strongly onto activated sludge particles, and much of the previous work has determined equilibrium partitioning coefficients (K_d). The values available in literature generally show good agreement. Clara *et al*. (2004) found that the "log(K_d)" for steroid estrogens was 2.84 (2.64–2.97) and 2.84 (2.71–3.00) for E2 and EE2, respectively (Clara *et al*., 2004). In the work by Ternes *et al*. (2004), the "log(K_d)" for EE2 was determined to be 2.54 (2.49–2.58). Yi *et al*. (2006) found that the "log(K_d)" for EE2 was 2.7 for membrane bioreactor sludge and 2.3 when the sludge was taken from a sequencing batch reactor. Andersen *et al.* (2005) determined distribution coefficients (K_d) with activated sludge biomass for the steroid estrogens , E1, E2 and EE2 in batch experiments, and they determined "log(K_d)" values for steroid estrogens of 2.6, 2.7, 2.8 respectively (Andersen *et al.,* 2005). Taken together, these partitioning coefficients enable practitioners to model sorption in activated sludge processes, and numerically evaluate the importance of sorption as a removal mechanism.

Biotransformation of steroidal compounds is an area where the consensus is still developing. Biotransformation is likely due to cometabolic activity because steroidal compounds (like other micropollutants) are not present in high enough concentration to support substantial biomass growth. Clear evidence of cometabolism is still needed, but progress is being made, as there is a growing body of reports suggesting that EE2 can be biotransformed in enriched autotrophic nitrifying cultures. Vader *et al.* (2000) degraded EE2 using nitrifying activated sludge, and they noted the presence of unidentified hydrophilic daughter products. Yi *et al*. (2006), Shi *et al*. (2004), and Dytczak *et al.*

(2006) also biologically degraded EE2 using nitrifying mixed cultures, and in each case, the simultaneous disappearance of EE2 and ammonia was reported. Cometabolic activity requires a catalyst, and Yi *et al.,* (2006) investigated the possibility that ammonium monooxygenase (AMO) can mediate EE2/NH_3 cometabolism. Their data suggested that AMO can remove EE2 and ammonia simultaneously, but their results were in conflict with others that suggested that AMO may be inhibited by acetylene (an analogue of the C17 EE2 function group) (Teissier and Torre, 2002; Bollmann and Conrad, 1997; Hyman and Arp, 1990); since EE2 contains an acetylene group (at C17), the possibility of EE2 inhibiting AMO must be considered.

Methodology

Experimental overview. A nitrifying completely-mixed stirred tank reactor (NCSTR) with sludge recycle was operated to cultivate an enriched nitrifying microbial community. This bioreactor was originally seeded with mixed liquor from the H.C. Morgan Water Pollution Control Facility in Auburn, Alabama. The experimental strategy was to use the waste activated sludge for a series of batch experiments. The batch experiments involved extracting the AMO enzyme from the biomass and incubating the protein extract with EE2, E2 and NH_3. These experiments were done to investigate reaction stoichiometry and to determine whether the EE2 acetylene group at the C17 inhibited nitrification. These batch tests were done three times, and samples were collected and analyzed in triplicate. Another series of batch tests involved incubating whole cells with EE2, NH_3, and sometimes allylthiourea to confirm the link between nitrification and EE2 removal. After the batch tests were completed, the NCSTR was

used for continuous experiments in which EE2 was included in the influent and the effluent was collected for metabolite detection. During the continuous experiments, the influent $NH4^+$ concentration was varied in order to change the nitrification rate and to evaluate the ensuing effects on the rate of EE2 biodegradation. Finally, computational experiments were carried out in order to determine with electron density of the EE2 compound, and to investigate whether the electron density of EE2 is related to the sites where the initial biotransformation steps take place.

Nitrifying bioreactor. The NCSTR (with sludge recycle) was operated at a HRT of 0.75 d and an SRT of 20 d. Peristaltic pumps were used to control the flow of influent and effluent, and the pH was controlled between 7.5 and 8.5. The SRT was maintained by manual wasting of solids directly from the bioreactor, and in most cases the waste sludge was used for analytical purposes. The influent feed consisted of the following (as mg/L total influent concentration): $(NH_4)_2SO_4$ (660), $MgSO_4$ (40), KH_2PO_4 (83.3), $CaCl_2$ (34), $CuSO_4$ (0.2), $NaHCO_3$ (1500), $FeCl_3$ (0.4). The influent EE2 concentration was 300 ug/L and influent ammonium concentrations were between 100 – 400 mg/L as NH_4^+-N. Ammonia was used as the primary substrate to select for an autotrophic microbial community, and fluorescence in situ hybridization (FISH) was done at each operating condition to confirm the structure of the bioreactor population. FISH tests were performed using the hybridization and washing buffers provided by Vermicon AG (Munich, Germany) and as described by Tarre and Green (2004). The hybridized samples were analysed with a BioRad Laser Scanning Confocal Microscope, and the results confirmed the presence of autotrophic nitrifiers (Figure 5.1).

Standard analytical methods. EE2 and E2 were detected by HPLC (Hewlett-Packard, HP 1100). The system consisted of a degasser (G1322A), a quaternary pump (G1311A), an ALS auto-sampler (G1313A), a colcomp column oven (G1316A) and variable wavelength UV-VIS detector (G1314A). A Hypersil ODS C18, (125x46 mm, 5um) column was used. HPLC operating conditions were as follows; UV detector wavelength, 197nm and mobile phase, acetonitrile and water (40:60) with solvent delivered at a constant flow rate of 1mL/min. The total runtime of the HPLC analysis was 10min. Total suspended solids (TSS), volatile suspended solids (VSS), and ammonia-N were analyzed according to Standard Methods (APHA 1992). NADH was measured colorimetrically as described by Hage and Hartmans, (1999).

Thin layer chromatography. The effluent from nitrifying sludge reactor was collected and metabolites were extracted using solid phase extraction (SPE). Extracted samples were dried in Savant Speed Vac System (GMI, USA). Solid samples were dissolved in methanol. Chromatography was conducted using TLC plates of Silica Gel (Whatman, no. 4745-010, Florham Park, NJ) with a solvent system of hexane/ethyl acetate (3:1 v/v). The metabolites on the TLC plate were visualized by exposure to iodine vapors (see Figure 5.2).

Column chromatography. SPE was carried out as described above and metabolites were eluted using methanol. The eluted samples were mixed with fresh silica gel (Whatman 4745-010, Kent, UK) and then dried in vacuum evaporator. A glass column was packed with silica gel. Silica gel containing dried metabolites was loaded on

the top of the silica gel column. The column was eluted with solvent mixture of hexane/ethyl acetate (3:1 v/v) by gravity and the eluting solvent was collected at the bottom of the silica gel column in each 50mL fraction. Total volume of eluting solvent was 1L. Each fraction of solvent was dried in vacuum evaporator and then sample was dissolved in acetone-D6 for NMR tests.

Nuclear magnetic resonance spectroscopy (NMR). The 1H NMR of purified metabolites was obtained using Bruker 400 MHz model (Bruker-Oxford Imaging Comp, Oxford, UK). The samples were dissolved in acetone-D6 (Aldrich chemical, no. 29621-0, Milwaukee, WI). 600 ul samples were placed in a new NMR tube. Figure 5.3 shows an example of an NMR spectrum that was observed.

AMO extraction and activity. AMO enzyme extraction was explained previously (Yi and Harper, 2006; Schwarzenbach *et al*., 2003). Briefly, biomass from the NCSTR was harvested by centrifugation at 5000xg at 4ºC for 30min and resuspended in 10mM Tri-HCl (pH 8.0). The resuspended pellet was sonicated for 10min at 40% amplitude in an ice bath using Fisher scientific Sonic Dismembrator (model 550, maximum power of 500W at a frequency of 20 kHz). The particulate fraction of the sonicated product was separated by centrifugation at 5000xg and the pellet was reconstituted in 10mM Tris-HCl (pH 8.0) supplemented with 1% dodecyl-β-D-maltoside. The resuspended pellet, which contained the membrane-bound AMO was investigated in 10mM Tris-HCl using eluted enzyme, electron donors (0.5mM NADH, 0.6 units diaphorase and 0.5mM duroquinone), and ammonia. Control tests were always performed

in the same way without the enzyme extract; these controls confirmed the absence of abiotic transformation of EE2. Protein was incubated for 1h, and then separated by centrifugation at 5000xg at 4ºC for 1hour. Chromatography was performed on the supernatants and the resuspended pellet in a glass column (1cm x 10cm) packed with a DEAE Sepharose CL6B (weak anion exchange resin). The column was eluted with 140mM NaCl in 10mM Tris-HCl (pH 9.0) + 0.02% dodecyl- β-D-maltoside. The removal of EE2 or E2 was investigated in 10mM Tris-HCl using eluted enzyme, electron donors (0.5mM NADH, 0.6 units diaphorase and 0.5mM duroquinone), and ammonia. Control tests were always performed in the same way without the enzyme extract; these controls confirmed the absence of abiotic transformation of EE2 or E2.

FED analysis. Frontier electron density (FED) values were obtained by using Gaussian 03 program on the supercomputer in Alabama supercomputer center. Optimization of the EE2 structure was carried out with STO-3G basis set at level of Unrestricted Hartree-Fock (UHF). Based on optimized structure, highest occupied molecular orbital (HOMO) were calculated using the same method and basis set. The frontier electron density fr can be calculated as

$$f\mathrm{r} = [2^{*}\Sigma\,(C_{\mathrm{ri}}\mathrm{HOMO})^{2}]$$

Where r is the number of carbon atoms in i: 2s, 2px, 2py, and 2pz.

Results and Discussion

Electrophilic initiating reactions and Ring A cleavage. EE2 is polycyclic with a single aromatic ring A, and it includes polycyclic rings B, C, and D; ring D carries a

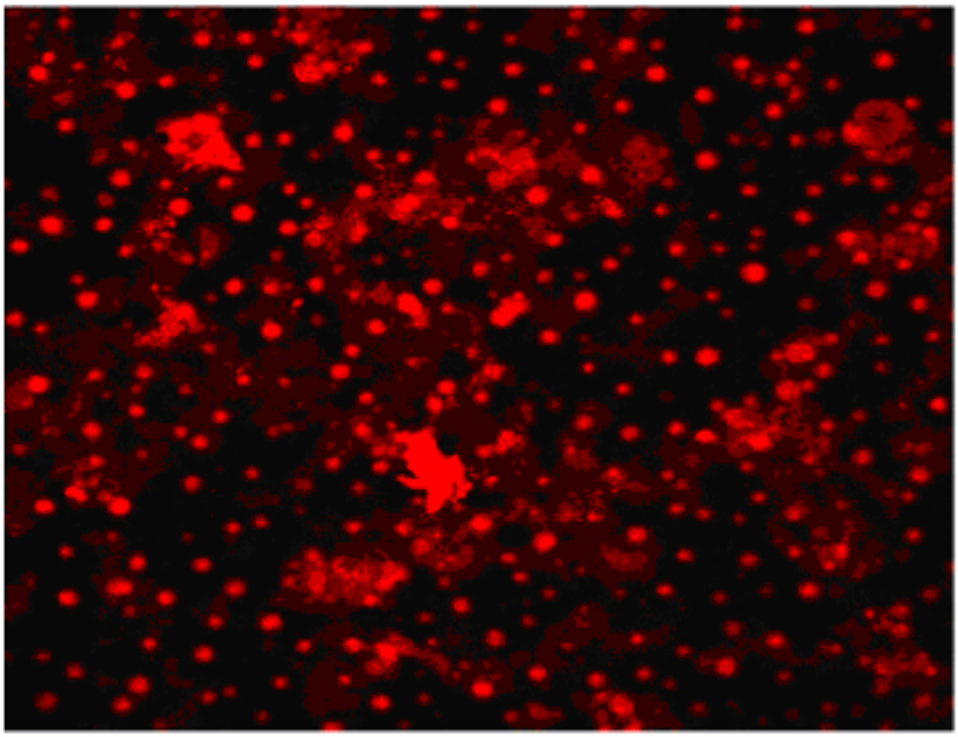


Figure 5.1 Fluorescent *in-situ* hybridization of *Nitrosomonas* sp., *Nitrosococcus* sp. and *Nitrosospira* sp. cells from nitrifying sludge reactor. Cell hybridization was done using Nitri-VIT(Vermicon AG)

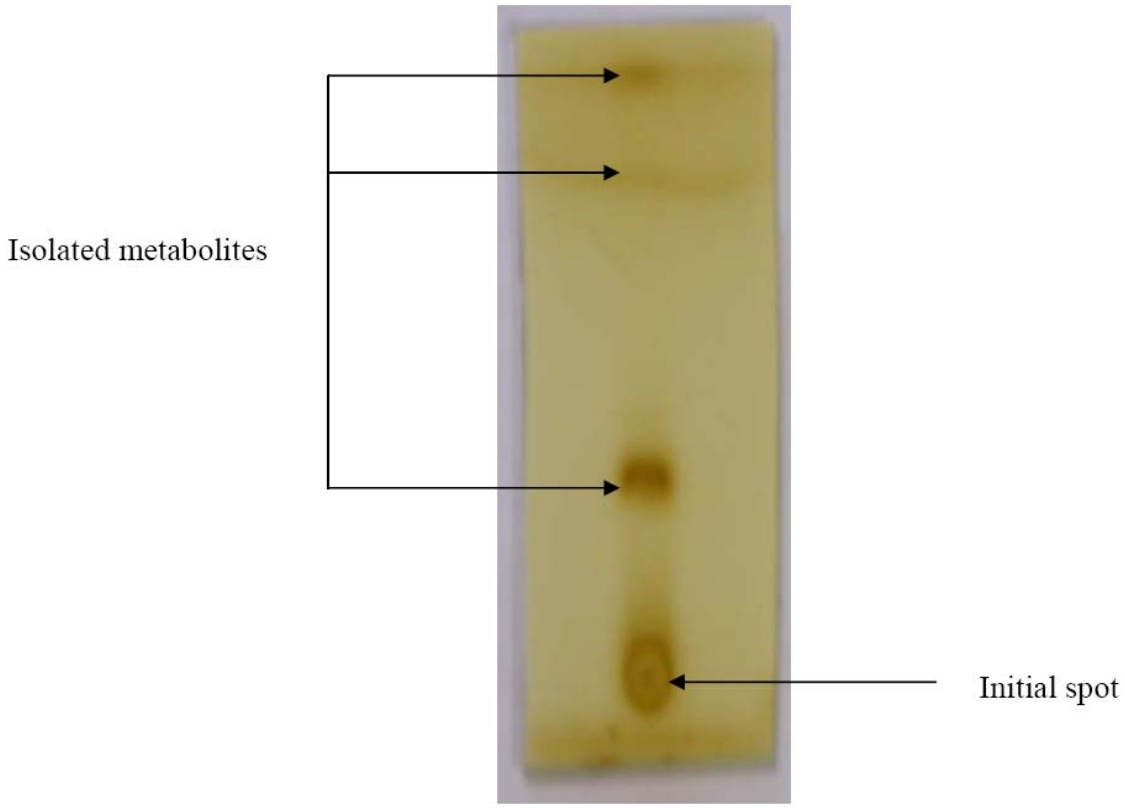


Figure 5.2 Typical Thin Layer Chromatography Plate

hydroxyl group and acetylene group at C17. The electron density associated with ring A is significantly higher than in other rings of the compound (Figure 5.4). The pi electrons associated with this ring are sterically unhindered (i.e. accessible to attacking reagents because of their location in the circular clouds above and below the plane of ring A). This leaves ring A vulnerable to electrophilic substitutions that may serve as initiating reactions. Therefore, we expected to identify daughter products that showed electrophilic substitution, and we further expected to identify daughter products that demonstrated ring A cleavage. Using NMR, three primary daughter products were identified: ETDC (3-ethynyl-3a,6,7-trimethyl-2,3,3a,4,5,5a,8,9,9a,9b-decahydro-1H-cyclopenta[a]naphthalen-3-ol), EE2-OH, and EE2-SO4 (Table 5.1). The first, ETDC, shows that ring A was removed, which was expected since the electron density around the EE2 ring A was relatively high. The second, EE2-OH, is hydroxylated at the C-2, and EE2-SO_4 is conjugated at C3, and C3 and C2 are also high FED carbon units. These results show that the high FED regions of the EE2 structure are involved in initiating reactions. These results also show ring A cleavage can occur before modification of ring B or C; this latter finding is different from what was found by Haiyan *et al.* (2006). They used a Sphingobacterium sp. JCR5 to degrade EE2, and based on the daughter products they detected, they proposed that EE2 is initially oxidized to E1, and that the pathway continues with ring opening oxidation reactions on ring B, leaving ring A initially intact. These current results offer another view of these initiating reactions by whole cells because they show that ring A cleavage can occur before ring B is broken.

Acetylene group inhibition. Previous results have shown that acetylene (an analogue of EE2) irreversibly inactivates AMO (Teissier and Torre, 2002; Bollmann and Conrad, 1997; Hyman and Arp, 1990); since EE2 contains an acetylene group (at C17), the possibility of EE2 inhibiting AMO must be considered. EE2 and E2 were both incubated in the presence of an AMO-containing extract and with NH_4^+. Figure 5.5 shows that the rate of ammonia removal for both incubations is similar. When EE2 is present, the ammonia removal efficiency is 90% and with E2 the ammonia removal efficiency is 85%. The EE2 removal efficiency was 70% and that of E2 was 63%. Since the only difference between EE2 and E2 is the C-17 acetylene group of EE2, and the action of AMO does not appear to be inhibited by the C-17 acetylene group. This result makes sense in light of the previously shown evidence (from Table 5.1) that initial transformations occur at ring A of the EE2 structure and not at C17. Whole cell incubations with E2 and EE2 also did not show any inhibition of NH_3 removal rates (Figure 5.9). This result shows that the C-17 group does not affect AMO activity or nitrification.

Cometabolic reaction. Figure 5.6 is a conceptual picture showing a detailed example of how EE2 and nitrification may be connected. AMO converts NH_3 to NH_2OH in the presence of oxygen. This step requires reducing power that is regenerated as NH_2OH is oxidized to NO_2- by hydroxylamine oxidoreductase. Electrons then enter a catalytic cycle involving a binuclear copper site located at the AMO active site. Oxygen reacts to convert the Cu(I) to Cu(II), but the oxygen remains bound as an electrophilic radical. This oxygenated form of the enzyme then reacts with organic substrates to

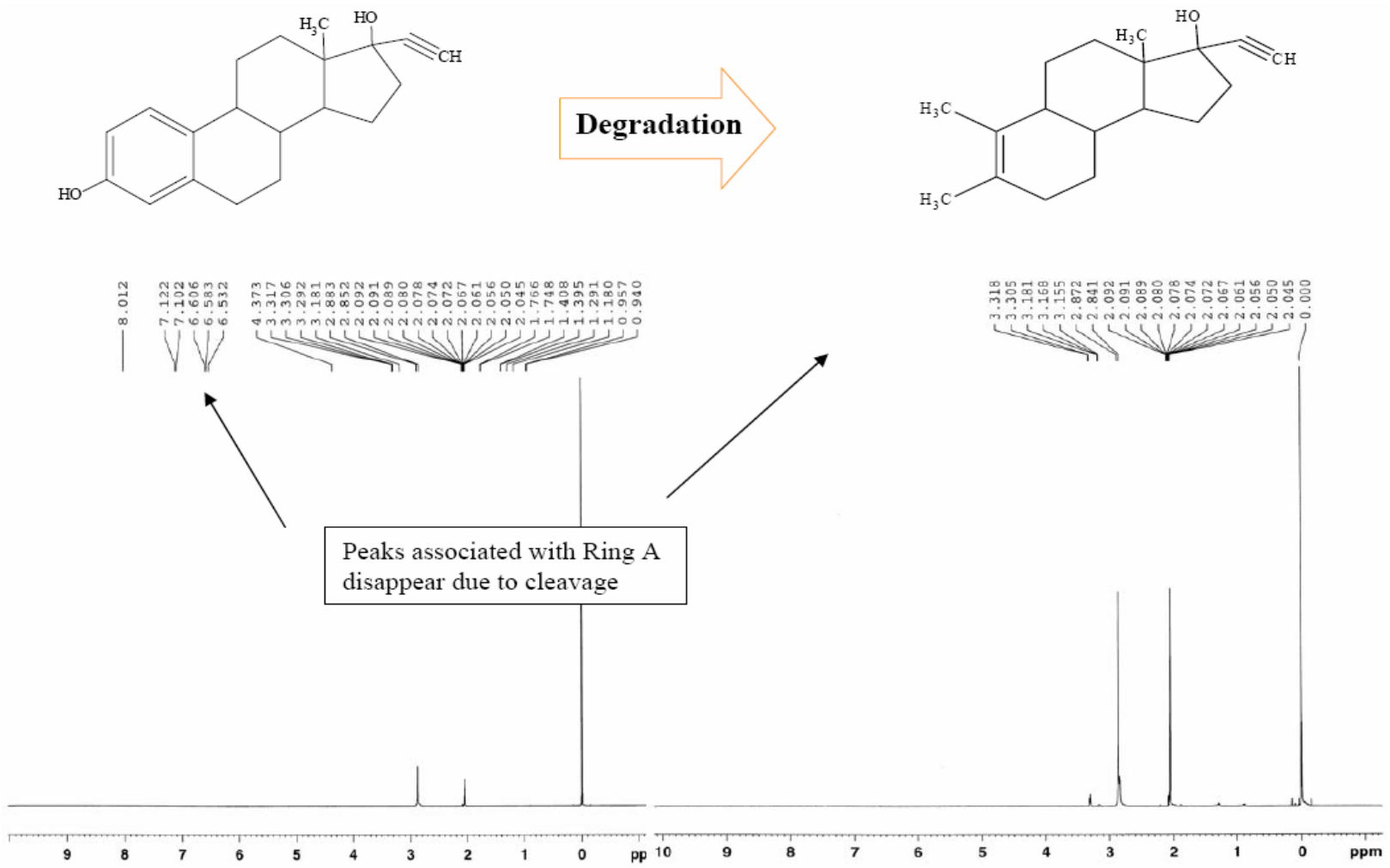
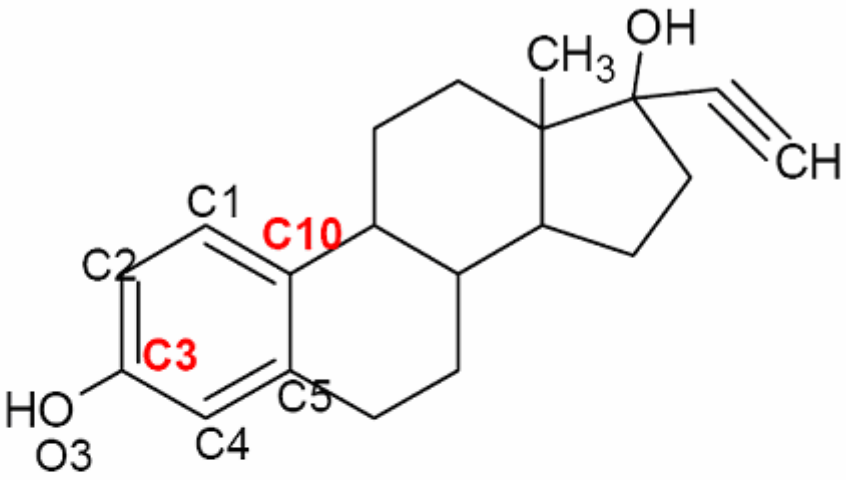


Figure 5.3 Example of an observed NMR spectrum: This shows that Ring A of EthinylEstradiol was cleaved



Electron density values for 17α-ethinylestradiol	
Atom No.	Electron density, *fr*
C3	**0.26**
C4	0.10
C5	0.09
C1	0.09
C2	0.15
C10	**0.38**
O3	0.18
All other electron density values < 0.01.	

Figure 5.4 17α-Ethinylestradiol structure with electron density shown for the carbon units with the highest FED values

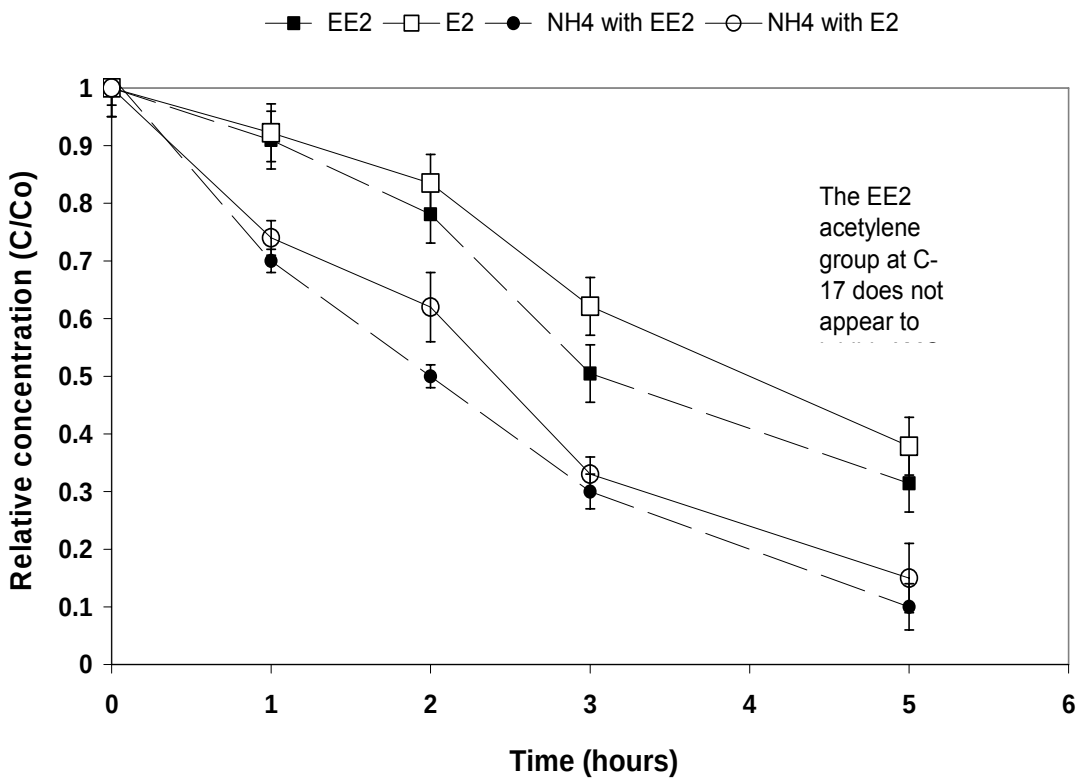
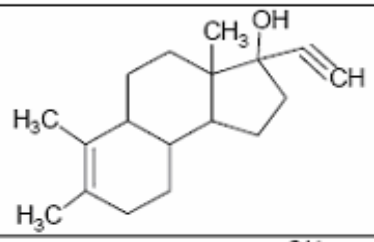
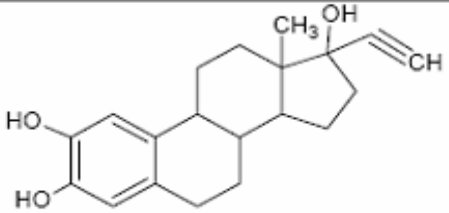
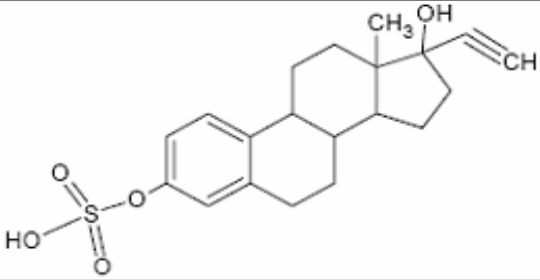


Figure 5.5 EE2 and E2 removal in the presence of an AMO-containing extract

produce the Cu(II) form. This conceptual model is based on monooxygenase activity, but dioxygenase enzymes must be considered also, and for two reasons in particular. First, dioxygenase enzymes are capable of mediating cometabolic biotransformation of polyaromatic compounds (Schwarzenbach *et al*., 2003). Second, the microorganisms in this study were harvested from an *enriched* (not pure) culture of nitrifiers, so it is possible that heterotrophic activity may be present, which would increase the possibility that dioxygenase activity may be occurring. Fortunately, cometabolic dioxygenase-mediated biotransformation of EE2 can be distinguished from monooxygenase-mediated activity because the NADH/EE2 molar ratio of the former is 1:1 (as opposed to 2:1). Therefore, in order to investigate whether the biotransformation of EE2 was monoxygenase or dioxygenase mediated, the ratio of EE2/NADH removed was determined by incubating EE2 and NADH in the presence of an AMO-containing enzyme extract. The molar ratio of NADH/EE2 determined during the incubation was 2.2, which is consistent with the action of monooxygenase-mediated biotransformation. This result demonstrates the potential for monooxygenase-mediated EE2 biotransformation *in vitro* and it also provides a conceptual model which could be useful for the design of future experimental efforts.

Nitrification and EE2 biotransformation. The aforementioned whole cell cultures were used as a resource for further evaluating links between nitrification and EE2 biotransformation. One of the objectives of this work is to characterize the relationship between nitrification rate and EE2 biotransformation rate. Figure 5.7 shows the relationship between the measured NH_3 removal rate and the measured EE2

Table 5.1. EE2 biotransformation byproducts detected by NMR

Name	Structure	Reaction mechanism
ETDC (3-ethynyl-3a,6,7-trimethyl-2,3,3a,4,5,5a,8,9,9a,9b-decahydro-1*H*-cyclopenta[*a*]naphthalen-3-ol) (IUPAC)		Ring A cleavage
OH-EE2		Hydroxylation
Sulfate-EE2		Conjugation

biotransformation rate. Current data, as well as data taken from Shi *et al.* (2004) and Vader *et al.* (2000) is shown.

The continuous experiments showed a linear relationship between nitrification and EE2 biodegradation rates over the range of NH_3 and EE2 biotransformation rates tested. The EE2 biotransformation rate increased from 1.1 to 4.1 μmol EE2/g VSS/h, while the NH_3 biotransformation rate increased from 0.3 to 3.1 mmol NH_3/VSS/h. The current data agree well with the results of Shi *et al.* (2004), who measured an EE2 biotransformation rate of 1.5 μmol/g VSS/h at a nitrification rate of 0.1 mmol/g VSS/h. The EE2 biotransformation rates observed by Vader *et al.* (2000) where less than those reported by Shi *et al*. (2004) and by the current work, but the Vader *et al.* (2000) work

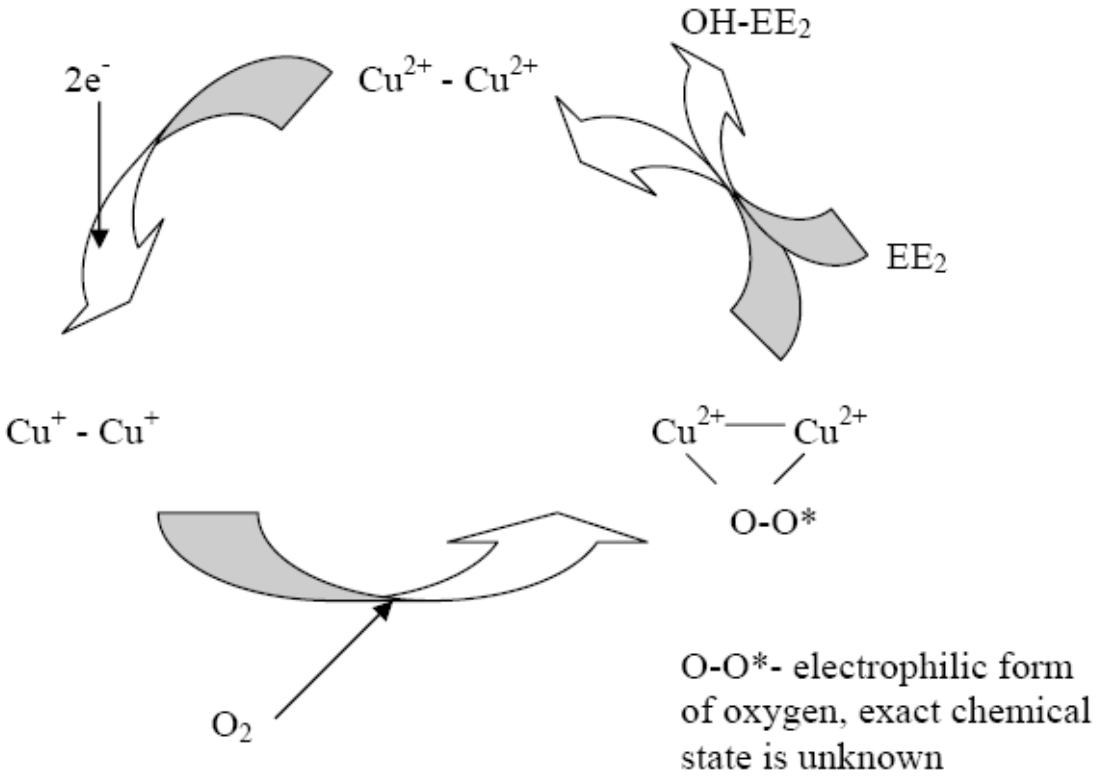


Figure 5.6 Conceptual model for AMO role in cometabolic transformation: Catalytic reaction cycle involving a binuclear copper site

showed a trend that is consistent with the current data. These data taken together strongly show a linear link between nitrification and EE2 removal in enriched nitrifying cultures, and therefore supports the notion that EE2 biotransformation can be cometabolically mediated in bioreactors that are enriched for autotrophic nitrifiers. Results from the whole cell experiments provide evidence for AMO involvement in EE2 biotransformation. The appearance of EE2-OH as a metabolite (from Table 5.1) is consistent with monooxygenase activity, and the correlation between the EE2 and NH3 biotransformation rate (from Figure 5.7) are both strongly suggestive. Also, nitrifying whole cells were incubated with NH4+-N and EE2, both with and without allylthiourea (a nitrification inhibitor). Nitrification and EE2 removal were observed without the inhibitor, but in the presence of the inhibitor, EE2 was not removed and nitrification did not occur (Figure 5.9). This result also supports the idea that AMO is involved because allylthiourea inhibits nitrification by reacting with AMO (Bedard and Knowles, 1989). There are also other reports presenting similar evidence for AMO oxidation of organic compounds (Wahman and Speitel, 2005). Taken together these observations offer a considerable body of evidence.

The current and previous results make it tempting to assert that autotrophic cultures are keys to controlling the passage of micro pollutants through full scale biological wastewater treatment plants, but the role of heterotrophic organisms must be considered. There are many fast-growing heterotrophic microorganisms in the activated sludge processes that have a variety of mono- and dioxygenase enzymes; these cultures may contribute to or even dominate micro pollutant biotransformations. The current results show that nitrifiers can initially degrade EE2 into intermediates; these compounds

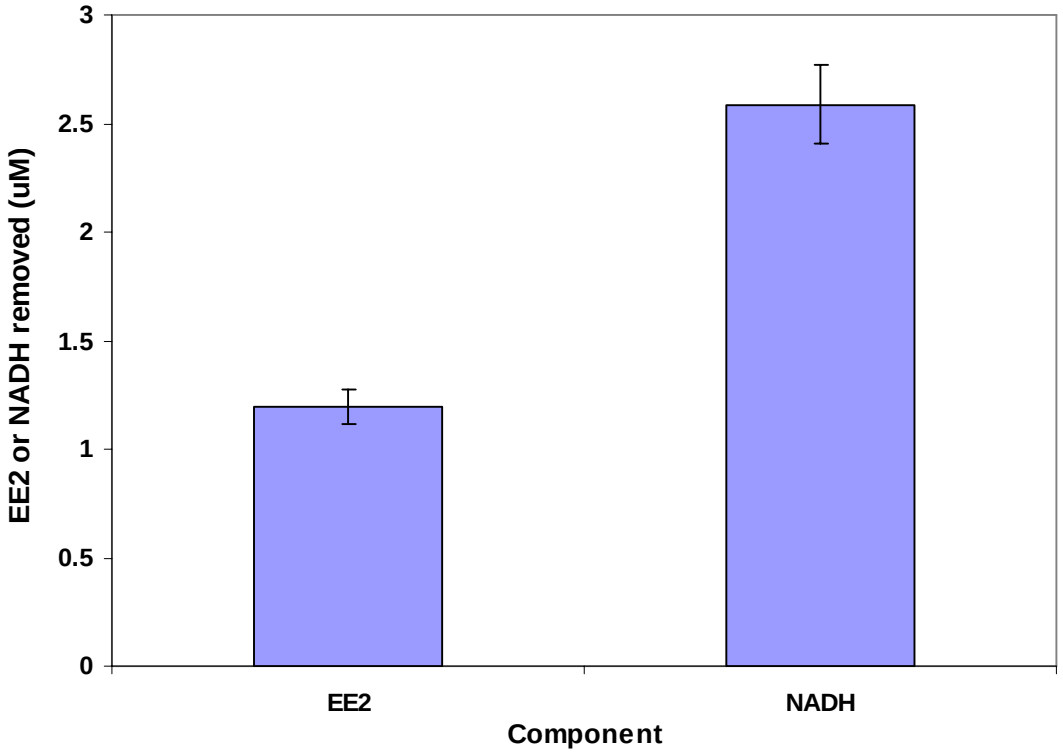


Figure 5.7 Stoichiometry of EE2 and NADH removal

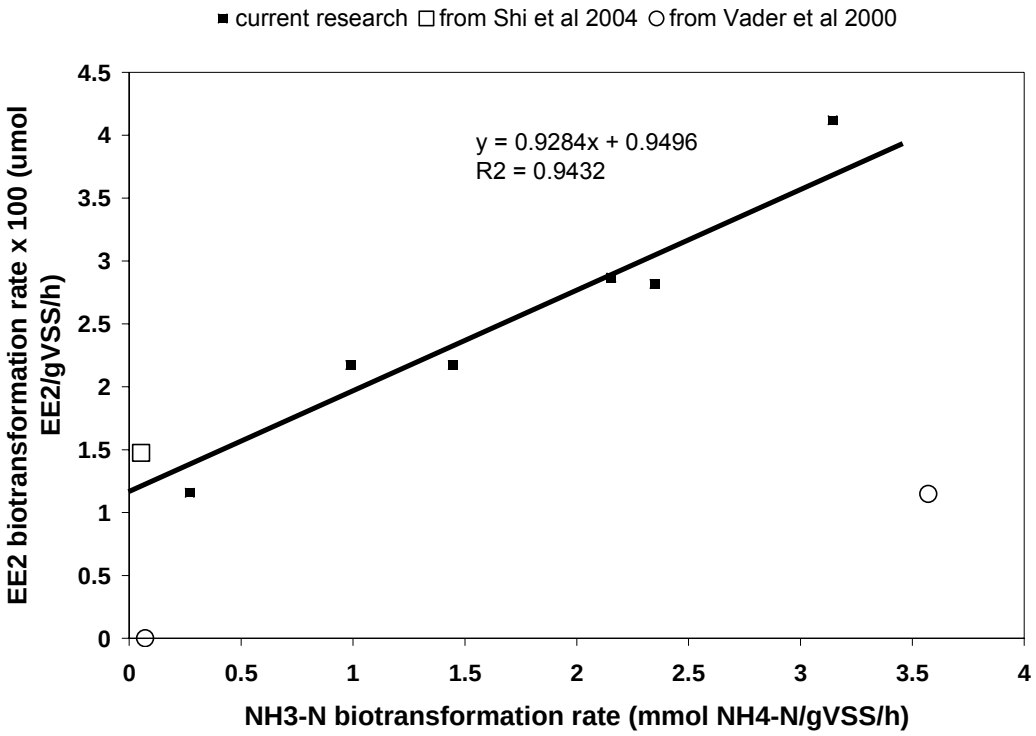


Figure 5.8 Relationship between NH_3-N and EE2 biotransformation rate.

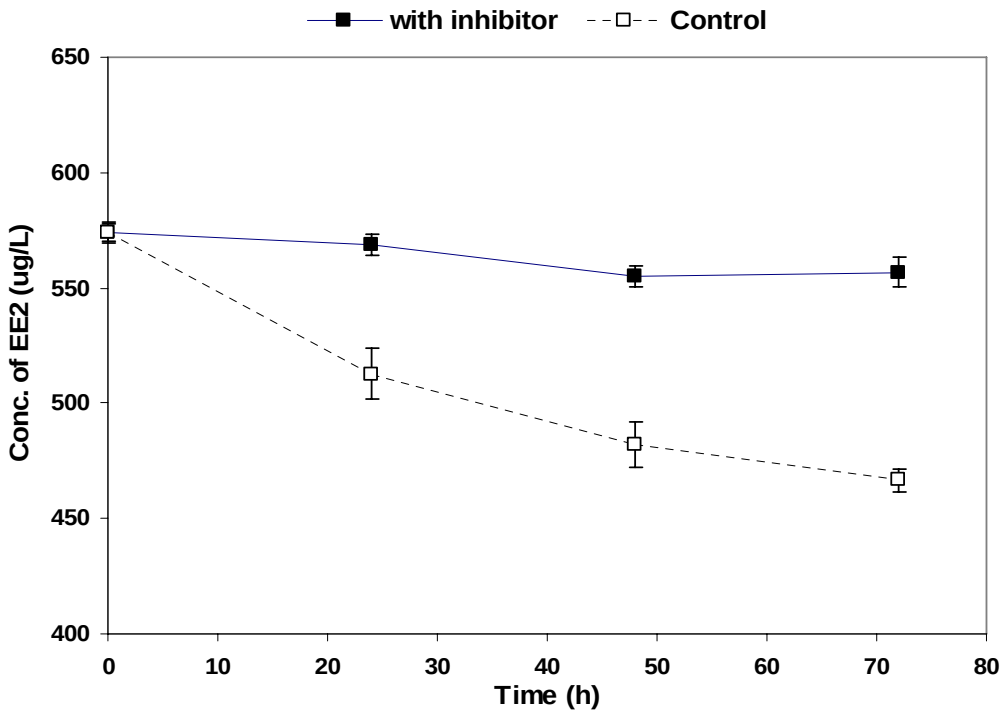


Figure 5.9 Degradation of EE2 with inhibitor (allylthiourea)

may serve as a substrate for heterotrophic organisms. The work of Shi *et al. (*2004) also supports this idea. They conducted EE2 biodegradation experiments with a nitrifying pure culture and a mixed culture of nitrifiers and heterotrophs. They detected daughter products in the pure culture experiments but not in the mixed culture experiments, perhaps because the heterotrophs completely degraded the daughter products. It is not clear that autotrophic nitrifiers can play a significant role in transforming micropollutants in systems that support significant heterotrophic populations. Extracellular enzymes and other scavenging, biodegradative mechanisms are also present in bioreactors. The relative importance of nitrifiers and heterotrophs remains open for future investigations.

Conclusions

- FED profiles of EE2 showed that ring A is the most reactive region in EE2 molecule. Batch experiments using whole cell taken from nitrifying sludge reactor showed that ring A of EE2 is the site of electrophilic initiating reactions, including conjugation and hydroxylation. Ring A was also cleaved before any of the other rings are broken, which is likely because the frontier electron density of the ring A carbon units is higher than those of rings B, C, or D.
- EE2 and NH_3 were degraded in the presence of AMO containing protein extract, and the reaction stoichiometry was consistent with a conceptual model
- Continuous experiments showed a linear relationship between nitrification and EE2 removal in enriched nitrifying cultures. These results support the notion that EE2 biotransformation can be cometabolically mediated under operating conditions that allow for enrichment of nitrifiers.

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VI. DEGRADATION OF 17A-ETHINYLESTRADIOL, CARBAMAZEPINE, AND TRIMETHOPRIM IN NITRIFYING SLUDGE REACTOR AND CONVENTIONAL ACTIVATED SLUDGE FED WITH TOLUENE

Abstract

Prediction of biological metabolic pathway is important for understanding the fate of pharmaceutical compounds (PhACs) in biological processes. A metabolic pathway prediction tool was developed and predictive accuracy was assessed using two bioreactors fed with monooxygenase inducers; ammonia and toluene. The removal of 17α-EthinylEstradiol (EE2), Carbamazepine (CBZ), and Trimethoprim (TMP) were monitored and byproducts were detected and identified. Removal efficiencies of EE2 were relatively higher in both reactors. Removal efficiencies were 60 % and 40% in nitrifying sludge reactor and conventional bioreactor fed with toluene, respectively. And TMP and CBZ were not significantly removed in both reactors. Byproducts detected in continuous experiments clearly showed that biotransformation took place in the high frontier electron density region of EE2 and TMP. Although byproduct of carbamazepine (CBZ) was not detected, previous study showed biotransformation of CBZ was well correlated with FED profile. However, we could not prove whether or not the full degradation pathway developed by prediction tool was accurate due to not having enough substantial byproducts data in this research and previous studies.

Keywords: Frontier electron density, Degradation rules, Degradation pathway, monooxygenase enzyme

The purposes of this study were to develop and prove the prediction tool and the capability of FED analysis in the biological systems. In this study, we tried to give a more accurate and logical way to predict degradation pathway by applying theoretical based approach (frontier electron density analysis) and degradation rules developed based on previously established metabolic pathways.

The recent studies conducted by few researchers (Shi *et al.,* 2006; Yi and Harper, 2007; Kim *et al*., 2005; Batt *et al*., 2006) have shown that some pharmaceuticals undergo a transformation in activated sludge treatment processes in the presence of nitrifying bacteria. It is well known that monooxygenase enzymes such as Cytochrome P450, ammonia monooxygenase, or toluene monooxygenase which are the highly non-specific enzyme are capable of co-metabolizing a variety of compounds. Shi *et al.* (2004) and Yoshimoto *et al.* (2004) reported the appearance of 17α-ethinylestradiol (EE2) degradation products and the study of Kim *et al.* (2006) suggested carbamazepine (CBZ) and trimethoprim (TMP) degradation in enriched nitrifying sludge reactor were observed. Yi and Harper (2007) and Haiyan *et al.* (2007) investigated the degradation mechanism and proposed the pathway of EE2, although each study suggested different degradation mechanism. Therefore, it is believed that some bacteria in presence of inducers are capable of producing monooxygenase enzyme and oxidizing pharmaceutical chemicals. However, more effort is needed to reveal degradation mechanisms. To experimentally develop a complete degradation pathway in most cases is generally very difficult because

byproducts are quickly removed and concentration of byproducts is usually under detection limit. Several degradation prediction tools (Pathway prediction systems, METEOR, MetabolExpert, and META) have been introduced for xenobiotic metabolism (Hou *et al*., 2004; Darvas, 1988; Greene, 1999; Klopman and Tu, 1997; Klopman *et al.,* 1995; Long, 2002). However, these prediction tools for microbial metabolism of pharmaceutical compounds need huge database collected from previously established pathways. This system is not able to theoretically explain the degradation pathway because empirical data are used and pathway is predicted based on functional group. Thus, if a compound has several different functional groups, many different pathways are proposed by prediction system.

To simply and logically predict the first reaction in cometabolism and whole metabolic pathway, the detail of how enzyme affects the chemically different transformation of pollutants and how microorganisms deal with oxidated or hydroxylated products at the first step are really of interest to study the fate of pharmaceuticals.

Methodology

Experimental overview. Two different types of bioreactor were operated; a nitrifying sludge reactor (NSR) fed with NH_3-N and conventional bioreactor (CBR) fed with toluene. These bioreactors were originally seeded with mixed liquor from the H.C. Morgan Water Pollution Control Facility in Auburn, Alabama. These two reactors were operated continuously and EE2, CBZ, and TMP were fed to each reactor to investigate and prove the proposed degradation pathway which was developed by the combination tool of FED theory and degradation rules.

Reactor configuration and operation. NSR and CBR were fed with ammonia and toluene as an induer and substrate, respectively. Both reactors were operated at a HRT of 2 day and an SRT of 20 day. The flow of influent and effluent was controlled by peristaltic pumps, and the pH was controlled between 7.5 and 8.5. The SRT was controlled by manually wasting of mixed liquor directly from the bioreactor. The composition of the NSR influent feed (as mg/L total influent concentration) consisted of $(NH_4)_2SO_4$ (660), $MgSO_4$ (40), KH_2PO_4 (83.3), $CaCl_2$ (34), $CuSO_4$ (0.2), $NaHCO_3$ (1500), $FeCl_3$ (0.4). The influent concentration of model compounds (EE2, CBZ, and TMP) was fixed at 300 μg/L. The primary substrate in the synthetic wastewater for the CBR was acetate (360 mg as COD/L) and influent ammonium concentration for NSR was 100mg/L as NH_3-N. The operating details for the bioreactors were presented previously (Yi and Harper, 2007 and Yi and Harper, 2005).

Standard analytical methods. EE2, CBZ and TMP were detected by HPLC (Hewlett-Packard, HP 1100). The system consisted of a degasser (G1322A), a quaternary pump (G1311A), an ALS auto-sampler (G1313A), a colcomp column oven (G1316A) and variable wavelength UV-VIS detector (G1314A). A Hypersil ODS C18, (125x46 mm, 5um) column was used. HPLC operating conditions were as follows; UV detector wavelength, 197nm, mobile phase, acetonitrile and water (40:60 for EE2 and CBZ; 10:90 for TMP) with solvent delivered at a constant flow rate of 1.5mL/min. The total runtime of the HPLC analysis was 30min. Total suspended solids (TSS), volatile suspended solids (VSS), and ammonia-N were analyzed according to Standard Methods (APHA 1992).

Column chromatography. Solid phase extraction (SPE) was carried out using C18 disc and then metabolites were eluted using methanol. The eluted samples were mixed with fresh silica gel (Whatman 4745-010, Kent, UK) and then dried in vacuum evaporator. A glass column was packed with fresh silica gel. Silica gel containing dried metabolites was loaded on the top of the silica gel column. The column was eluted with solvent mixture of hexane/ethyl acetate (3:1 v/v for EE2 and 1:1 v/v for CBZ and TMP) by gravity and the eluting solvent was collected at the bottom of the silica gel column in each 50mL fraction. Total volume of eluting solvent was 2L. Each fraction of solvent was dried in vacuum evaporator and then sample was dissolved in methanol-D4 for nuclear magnetic resonance spectroscopy (NMR) tests.

Nuclear magnetic resonance spectroscopy. After column chromatography, the 1H NMR of purified metabolites was obtained using Bruker 400 MHz model (Bruker-Oxford Imaging Comp, Oxford, UK). The samples were dissolved in Methanol-D4 (Aldrich chemical, no. 29621-0, Milwaukee, WI). 0.6 ml samples were placed in a clean NMR tube. An example of an NMR spectrum was observed.

FED analysis. Frontier electron density (FED) analyses were performed to determine highest electron density site in a compound, using Gaussian 03 program on the supercomputer in Alabama supercomputer center. Structure optimization of the model compounds was conducted with 6-31G(d) basis set at level of Unrestricted Hartree-Fock (UHF). After structure optimization, FEDs of carbon atoms in the model compounds

were calculated in the same method and basis set. The frontier electron density fr can be calculated as

$$f\mathrm{r} = [2*\Sigma\,(C_{ri}\mathrm{HOMO})^{2}]$$

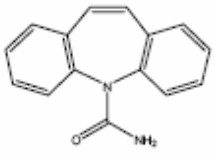
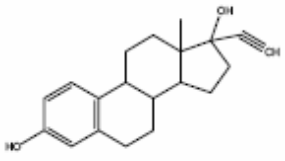
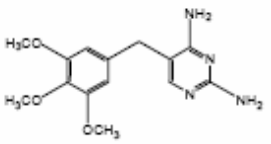
Where r is the number of carbon atoms in i: 2s, 2px, 2py, and 2pz.

Degradation rules. FED values were used to determine the location of the most reactive part in the model compounds. To determine what happens at the reactive position, we invoke five *degradation rules* (Figure 6.1) as follows (Kamath and Vaidyanathan, 1990; Hay and Focht, 1998; Nosova *et al*., 1997; Steffan *et al*., 1997; Casellas *et al*., 1997; Dean-Ross *et. al.,* 2001; Brzostowicz *et. al*., 2005; Nakazawa and Hayashi, 1978; Olsen *et. al.,* 1994);

Rule 1 - Enzyme attacks carbon atom at the highest f_r value position.

Rule 2 - The phenol ring is cleaved after oxidized to catechol. The oxygenolytic cleavage

Table 6.1 Structures and Properties of model compounds

Chemical	Carbamazepine	17α-Ethinylestradiol	Trimethoprim
Structure			
Log K_{OW}	2.45 (63)	3.9 (63)	0.91 (44)
Log K_d (pH 7)	2.67^{+}	4.52^{+}	0.28^{+}
Use	Anti-seizure drug	Contraceptive/ Hormone Replacement	Antibiotic

of the phenol ring occurs via *Ortho*- or *meta*-cleavage. *Ortho*- or *meta*-cleavage was determined by f_r value of adjacent carbon atom which has hydroxylated carbon with highest f_r value. Ring cleavage takes place between hydroxylated carbon with highest f_r value and carbon with higher f_r out of two adjacent carbons.

Rule 3 - The cyclohexane and cyclopentane ring are open after oxidized to cyclohexanone and cyclopentanone, respectively. Ring cleavage of cylcohexanone and cyclopentanone are determined by the same rule with phenol ring cleavage.

Rule 4 - After ring opening, carbon chains are degraded to hydroxyl-, ketone, and carboxylic acid and then de-carboxylation step is followed.

Rule 5 –Ring cleavage of heterocyclic ring occur as shown in Figure 6.1 and hydrolytic de-amination and dehalogenation are followed.

Rule 6 - Although a carbon is in the highest f_r value position, if not applicable to degradation rules (rule 1 to 5), metabolic reaction occurs at the a carbon atom which has second highest f_r value.

Results and Discussion

Degradation of model compound in nitrifying sludge reactor and activated sludge reactor fed with toluene. CBZ, EE2, and TMP were separately fed into both reactors. The influent concentrations of NH_3-N for NSR and toluene for CBR were fixed at 100 mg/L and 520 mg/L, respectively. After a few days, NH_3-N and toluene concentration in each reactor was stabilized at 5mg/L (± 1.7mg/L) and 3.5 mg/L (± 1mg/L), respectively. To determine the volatilized amount of toluene, control test was performed and toluene concentration was stabilized at 5mg/L.

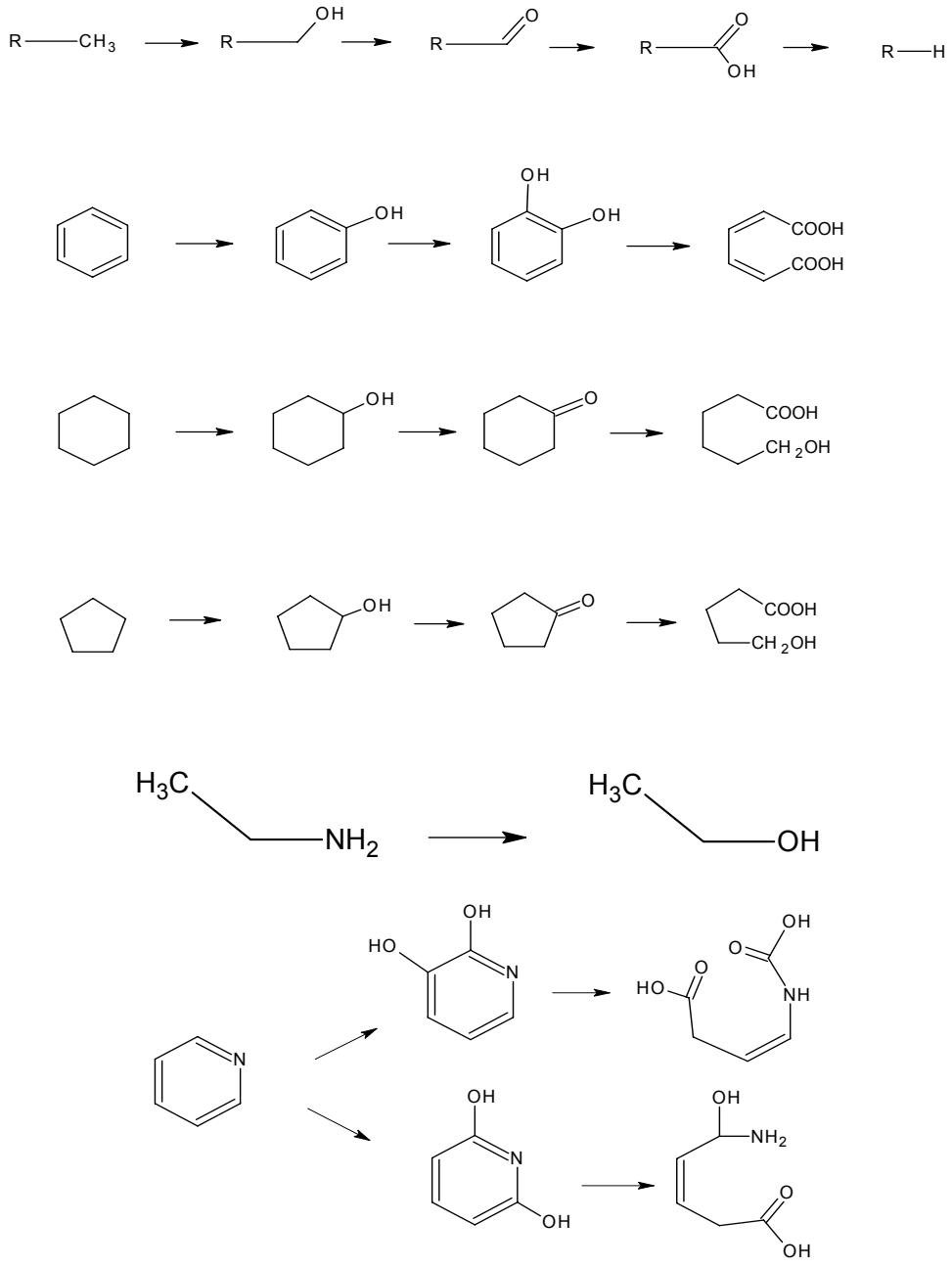


Figure 6.1 Degradation rules

EE2 was most easily degraded in both reactors and removal efficiencies were 60% and 40% in NSR and CBR, respectively (Figure 6.2), and disappearance of EE2 was not observed in control tests. Previous studies (Vader *et al*., 2000; Shi *et al*., 2004; Yi and Harper, 2006) showed that enriched bacterial culture or monoculture of nitrifiers can remove or initiate degradation of EE2. The current data agree well with previous finding. TMP and CBZ were highly persistent in both reactors. Removal efficiencies of CBZ and TMP were 10 % and 5 % in NSR, respectively and close to zero in CBR. Batt *et al.* (2006) achieved 70 % removal of TMP in fresh activated sludge fed with NH_3-N as substrate. However, Khunjar *et al.* (2007) observed no removal of TMP and CBZ in the monoculture of nitrifying bacteria and concluded the various removal efficiencies of TMP were attributed to other heterotrophic bacteria which can not produce monooxygenase. Biomass composition may explain differences in the observed CBZ removal efficiencies. Batt *et al*. (2006) used fresh activated sludge, which is presumably diverse in terms of microbial community composition. The sludges used in the current study and by Khunjar *et al*. (2007) were taken from the bioreactor which was operated on synthetic wastewater, and the community composition was not likely to maintain a comparable level of microbial diversity. A diverse microbial community is more likely to have a broader pool of enzymes capable of transforming micro-pollutants.

Removal efficiencies of three model compounds observed in both reactors were in the following order: EE2 > CBZ > TMP. This order was well correlated with log K_{ow} values order (Table 6.1). Danielsson *et al*. (1996) reported that biotransformation rates of organic compounds increase with increase of log K_{ow} value and reach the maximum at around log K_{ow} 3.5, and then fall into a decline. It is note worthy to mention sorption is

often the first step of biotransformation and increase of log K_{ow} has the influence on sorption.

Theoretical studies and detected byproducts. Molecular orbital (MO) calculations were carried out to calculate FED for EE2, CBZ, and TMP. FED profiles show that which region or position in a molecule is most reactive. However, it does not tell how reaction will go or what will happen at the high FED position. We developed the prediction tool based on FED theory and degradation rules. FED indicates reactive part or position in a molecule and degradation rules designate what happens in this region or position. The prediction tool was applied to each degradation step. Degradation pathways of EE2, CBZ, and TMP were theoretically developed. All intermediates were derived on the basis of combination of FED and degradation rules. If the highest FED position was not applicable in degradation rules, the degradation rules were applied to the next highest FED position. The brief pathway is shown in Figure 6.6. Although steric hiderance effect is important in chemical reactions, it was not applied in degradation rules, because the framework needed for this is still missing.

EE2: These FEDs analysis are found to be high at phenolic group in EE2, especially at the atom No. 13, followed by atom No. 16, 15, and 17 (Figure 6.3). It is determined that the first reaction takes place at atom No. 13 and then oxidation of Atom No. 15 takes place next (Figure 6.6). However, byproduct **1** clearly shows that first reaction occurred at atom No. 15. It is expected that atom No. 15, 16 or 17 may be more vulnerable than atom No. 13 because atom No. 13 associated with the ring structure can

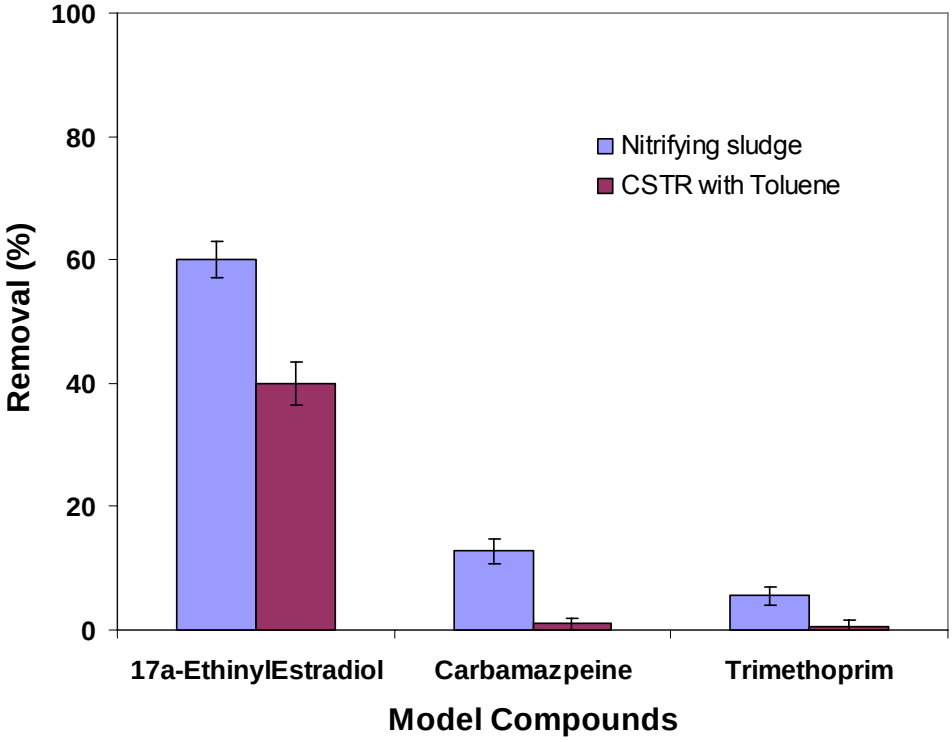


Figure 6.2 Removal Efficiency of model compounds

be sterically hindered. Figure 6.6 shows that after the hydroxylation of atom No. 15 ring A cleavage occurs. Byproduct **2** also demonstrates that ring A cleavage take place ahead of ring cleavage of B, C, D, and the elimination of ethinyl group. However, Haiyan *et al.* (2006) proposed the oxidation of EE2 was initialized at ethinyl group and then ring B cleavage took place earlier than ring A. Yi and Harper (2007) investigated EE2 and estradiol (E2) removal rates in enzyme extract to estimate the inhibition of AMO by

Table 6.2 Byproducts of 17α-EthinylEstradiol identified

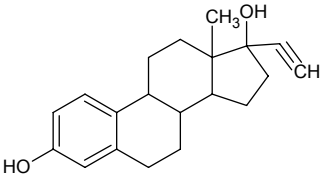
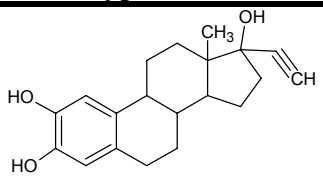
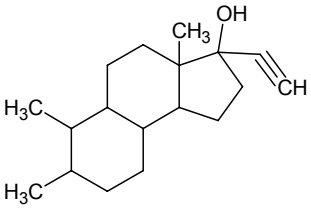
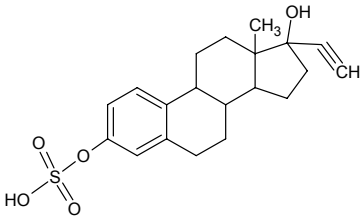
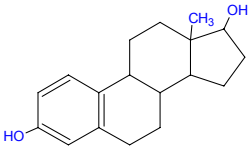
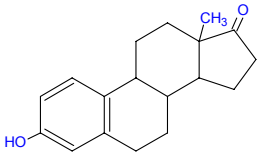
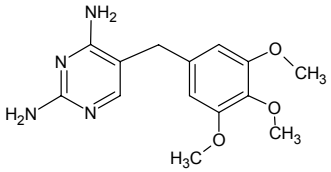
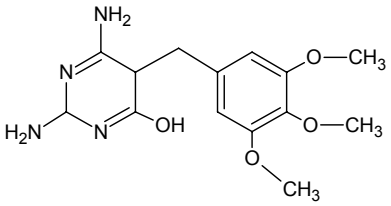
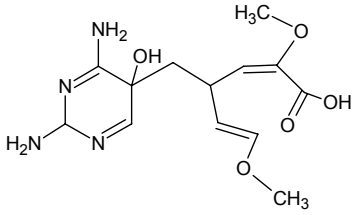
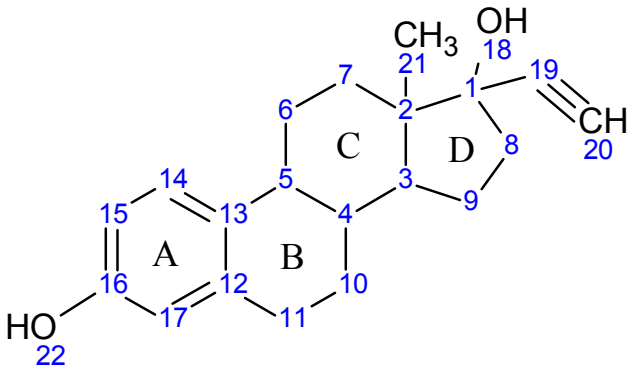
Parent compounds	Byproducts	Byproduct No.	Reference
 17a-EthinylEstradiol (EE2)		1	Yi and Harper (2007)
		2	Yi and Harper (2007)
		3	Yi and Harper (2007)
		4	Hayian *et al.* (2007)
		5	Hayian *et al.* (2007)

Table 6.3 Byproducts of Trimethoprim identified

Parent compounds	Byproducts	Byproduct No.	Reference
 Trimethiprim (TMP)		6	This Study
		7	This Study

ethinyl group in EE2 molecule because only structural difference between EE2 and E2 is ethinyl group of EE2. In their study, they found that biotransformation rates of both compounds were very similar. It is indicative that there was no or very little effect by ethynyl group on biotransformation and Shi *et al.* (2006) also found no significant difference of biotransformation rates among the four different estrogens including EE2 and E2 using *Nitrosomonas europaea*. Brzozowski *et al.* (1997) reported that phenol group in Estradiol (E2) has a significant effect on interaction with human estrogen receptor (hER). It is determined that FED profile of E2 is very similar with EE2 (data not shown). The study of Brzozowski *et al*. (1997) well agrees with FED profile of E2 and it indicates that phenol group plays an important role in the interaction between EE2 (or E2) and an enzyme. Therefore, it is expected the complex between enzyme and EE2 is associated with phenol group and it is highly possible that first hydroxylation occurs in phenol group.

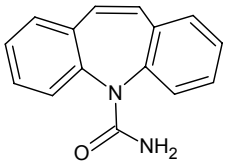
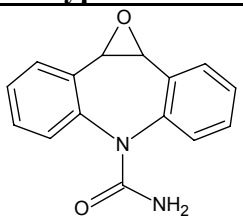
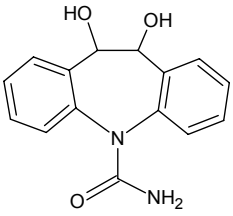
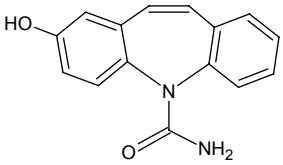
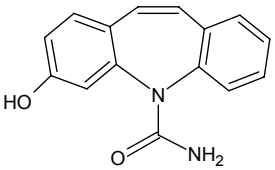
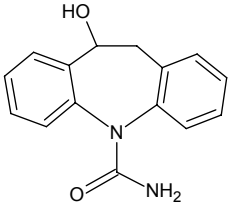


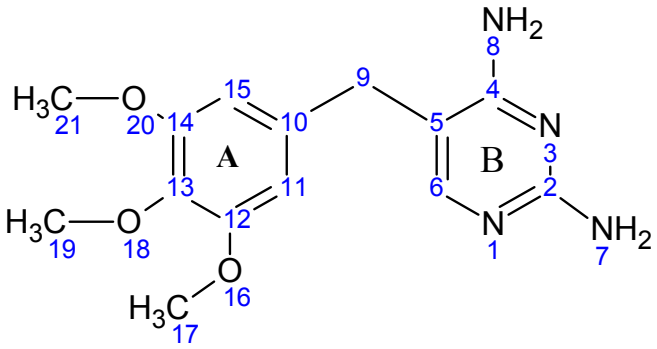
Atom No.	2FEDhomo
1	0.000371005
2	0.000553011
3	0.002074564
4	0.015643466
5	0.023331228
6	0.066403301
7	0.018288454
8	2.08166E-05
9	0.000467225
10	0.003232121
11	0.00569899
12	0.089349825
13	**0.377681475**
14	0.085472475
15	**0.149679794**
16	**0.26059811**
17	**0.103886755**
18	6.66368E-05
19	0.000355099
20	0.000391955
21	0.000214882
22	**0.182670974**

Figure 6.3 Frontier Electron Density profile for 17α-EthinylEstradiol

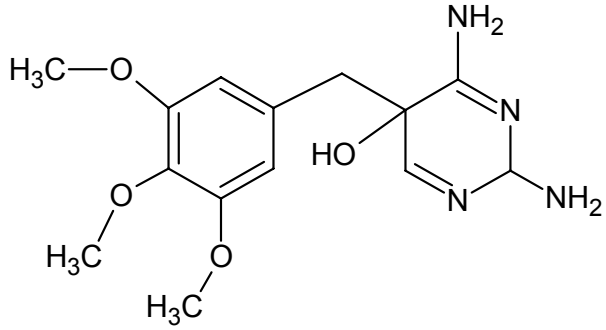
Byproduct **3** also shows conjugation occurred at high FED region. After the cleavage of ring A, the next degradation occurs in where the carbon chain generated by phenol cleavage was degraded to short chain. The predicted pathway shows that the elimination of ethinyl group takes place next, followed by the cleavage of ring B, C, and D (Figure 6.6).

Table 6.4 Byproducts of Carbamazepine identified

Parent compounds	Byproducts	Byproduct No.	Reference
 Carbamazepine (CBZ)		8	Miao *et al.* (2005), Masubuchi *et al.*,(2001) By Cytochrom P450
		9	Miao *et al.* (2005)
		10	Miao *et al.* (2005)
		11	Miao *et al.* (2005)
		12	Miao *et al.* (2005)



(a)



OH-TMP

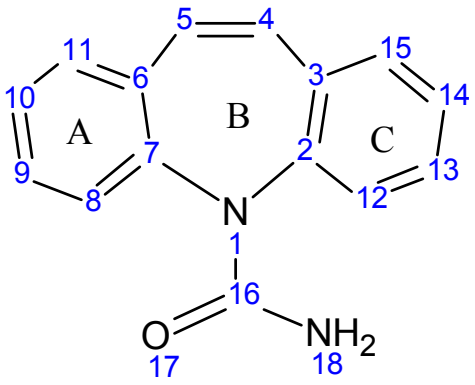
(b)

Atom No.	(a)	(b)
1	**0.24741766**	0.00449815
2	**0.08417355**	0.00104102
3	0.027575708	0.00187971
4	**0.055900837**	0.00261573
5	**0.49185134**	0.0538014
6	**0.051353883**	0.00381706
7	**0.248716172**	0.00137362
8	**0.109231687**	0.00035407
9	0.017677641	0.01452419
10	0.0030356	**0.28086663**
11	0.007253743	0.03636656
12	0.01931808	**0.14960749**
13	0.002774946	**0.35524935**
14	0.009524638	**0.05285957**
15	0.020068421	**0.12729231**
16	0.005108807	0.04885564
17	0.001546981	0.00973651
18	0.000195218	**0.09631899**
19	0.000608386	0.02665585
20	0.002096012	0.01628797
21	0.000958108	0.00313194

Figure 6.4 Frontier Electron Density profile for (a) Trimethoprim (b) OH-TMP

Trimethoprim: FED profile of TMP is shown in Figure 6.3. High FED region is located in Ring B and the highest FED position is atom No. 5 and atom No. 6 is the second highest FED position. Accordingly, we expected that first reaction occurs by adding oxygen into atom No. 5. However, byproduct **6** shows first hydroxylation reaction took place at atom No. 6. This result indicated that first hydroxylation reaction may be affected by steric hindrance effect. High FED profile is significantly changed after the first hydroxylation reaction (Figure 6.4). It causes the ring A cleavage ahead of the cleavage of ring B. Byproduct **7** supported this prediction.

Carbamazepine: It is determined that ring B is the most reactive part. The FED associated with atom No. 4 is significantly higher than that of other atoms of the compounds and atom No. 5, 6, and 9 are also high FED positions (Figure 6.5). It is predicted that first hydroxylation reaction occurs in atom No. 4, followed by atom No. 5 and then ring cleavage take place at ring B. Ring cleavage A and C follows ring B (Figure 6.6). Although byproducts were not detected in this study, Miao *et al.* (2005) reported the metabolic byproducts of CBZ detected in wastewater treatment plants. And these byproducts agree well with FED profile of CBZ. Byproduct **8**, **9**, **11**, and **12** reported by Mia *et al.* (2005) shows that hydroxylation reaction of CBZ took place at high FED position. Most dominant metabolites are associated with the highest or second highest FED positions (atom No. 4 and 5). Masubuchi *et al.* (2001) also proposed 2 and 3-Hydroxy CBZ and 10, 11-Epoxide (byproduct **8**) as CBZ metabolites using cytochrome P450.



Atom No.	2FEDhomo
1	0.028598102
2	0.053538417
3	0.086247973
4	**0.193466787**
5	**0.154936677**
6	**0.142871562**
7	0.060326586
8	0.041501186
9	**0.142826907**
10	0.015609155
11	0.074303102
12	0.035776825
13	0.092644276
14	0.006538912
15	0.057707472
16	0.011709527
17	0.002420261
18	0.002528383

Figure 6.5 Frontier Electron Density profile for Carbamazepine.

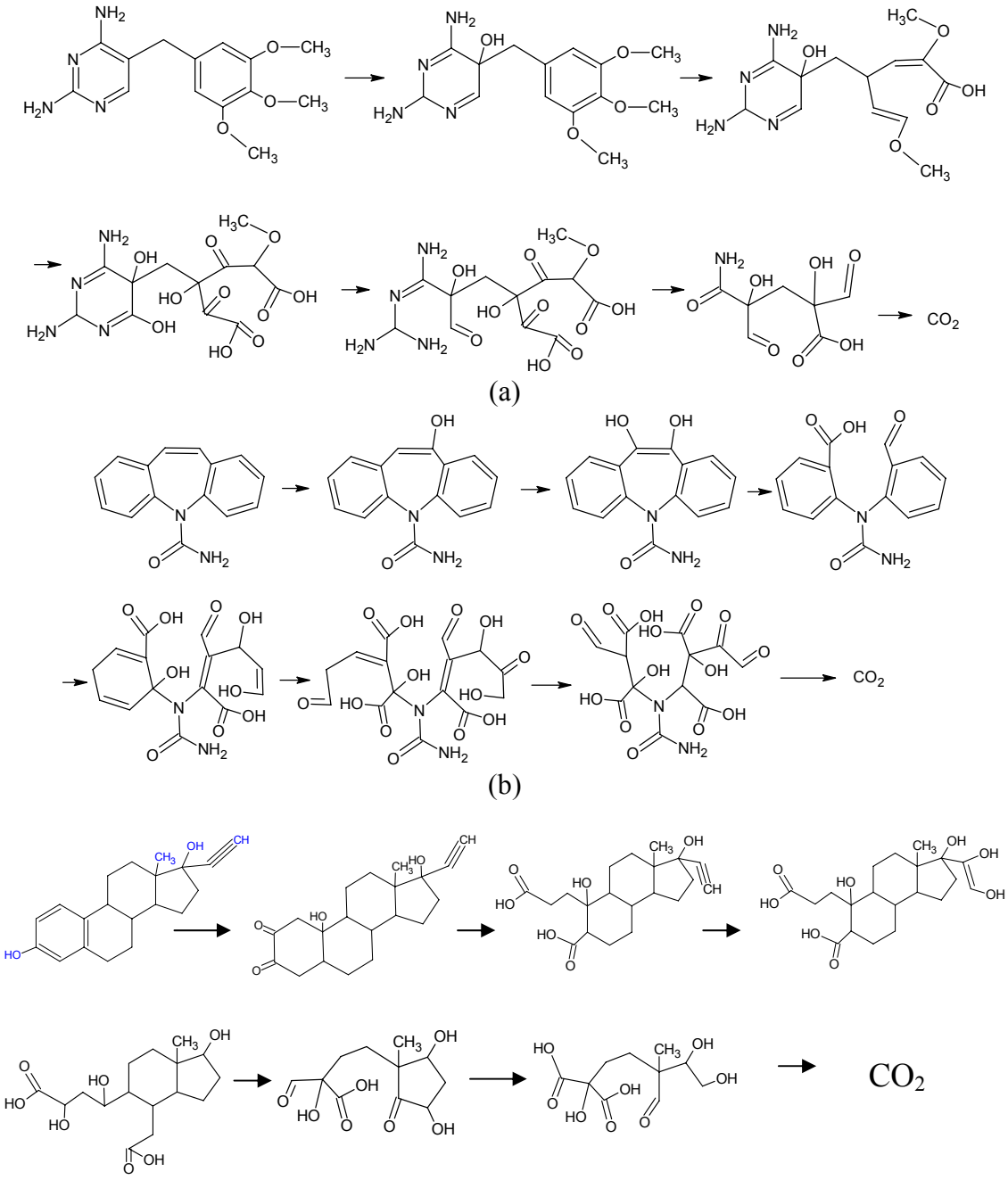


Figure 6.6 Metabolic pathways of model compounds predicted by prediction tool; (a) Trimethoprim, (b) Carbamazepine, and (c) 17α-EthinylEstradiol

Conclusions

- Degradation of EE2, TMP, and CBZ was investigated using two reactors fed with ammonia and toluene. Results indicated that EE2 among three model compounds was most efficiently removed in both reactors. The removal efficiencies of EE2 were 60% and 40% in nitrifying sludge reactor and conventional bioreactor fed with toluene respectively. However, CBZ and TMP were not removed significantly in both reactors.
- FED theory was applied to predict biodegradation reaction. Byproducts detected in this research and reported in previous studies clearly shows that biotransformation reaction takes place in high FED position or region. However, we could not prove if the prediction tool well predict degradation pathway of pharmaceutical compounds through full pathways due to not having enough substantial data of byproducts in this and previous studies.

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VII. CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Summary and Conclusions

Pharmaceutical compounds have been detected in relevant amounts in surface and wastewater. Biological treatment process plays an important role in preventing the negative effects from pharmaceutical compounds in the environment. The improved understanding of fundamental of removal mechanisms of these compounds in biological system is essential to increase removal efficiency. Three different types of bioreactor were continuously operated: membrane bioreactor (MBR), sequencing batch reactor (SBR), and conventional reactor (CBR).

Sorption is one of main mechanisms responsible for removal of pharmaceutical compounds from wastewater. Biomass characteristics such as hydrophobicity and particle size were manipulated by operating MBR and CBR under various conditions (nitrogen limitation and SRTs) and monitored to determine the effects on K_d and sorption-hysteresis of EE2. MBR always had smaller particle than that of SBR, and MBR K_d was equal to or larger than that of SBR. SRT change does not manipulate particle size, and the effects on K_d and HI were not dramatic. The biomass particle size had a dramatic effect on the observed K_d under normal conditions. K_d values of MBR and CBR were 0.33-0.57 and 0.25-0.33, respectively. Under nitrogen-limiting conditions, the correlations between the biomass particle size and K_d and HI were weak. It was found that the hysteresis index was greater for biomass suspensions with larger particle sizes.

This means that the selection of a MBR may potentially result in the less entrapment of EE2 with activated sludge biomass. These results showed that the magnitude of the partitioning coefficient and sorption-Hysteresis depended on the particle size characteristics (i.e. specific surface area). Visualization study showed that after desorption, in bigger particles from SBR, large amounts of substrate remained inside particles. The result clearly confirms particle size has an effect on sorption-hysteresis. This study also numerically explored the impacts of sorption hysteresis on the removal of pharmaceutical compounds.

Cometabolic reaction is important in degradation of pharmaceutical compounds. An enriched nitrifying sludge reactor was prepared and tested to estimate the role of nitrifying bacteria in degradation of EE2. Frontier electron density (FED) analysis was carried out to determine the highest FED region or position for electrophilic reaction using Gaussian 03 program. FED analysis shows that ring A of EE2 is the highest FED region and it is predicted that ring A is cleaved before rings B, C, or D was cleaved and ethinyl group was eliminated. Byproducts suggested that oxidation reaction and ring cleavage occurred at ring A of EE2 and Batch experiments using nitrifying sludge indicated that disappearance of EE2 was attributed to the presence of an ammonium monooxygenase (AMO) containing enzyme extract and EE2 degradation was improved with increase of removal of NH_3, simultaneously. There are good linear relationship between nitrification and disappearance of EE2. The reaction stoichiometry between a binuclear copper site located at the AMO active site of ammonia monooxygenase enzyme and EE2 was consistent with a proposed conceptual model. Removal mechanism proved in these experiments suggested EE2 biotransformation may be cometabolically mediated

enzyme reaction mainly involving nitrifying bacteria.

Two reactors that have different inducer conditions were tested to investigate the removal of EE2, TMP and CBZ in different monooxygenase condition. Removal efficiencies of the model compounds which have different chemical properties were monitored in each reactor. Removal efficiency results showed that EE2 was most efficiently removed in the nitrifying sludge reactor and conventional stirred tank reactor fed with toluene. Removal efficiencies of EE2 were 60% and 40%, respectively. CBZ and TMP were not removed significantly in both reactors. FED was applied to predict biodegradation reaction. Results clearly showed that degradation reaction took place in the high frontier electron density region. The full degradation pathway was developed using frontier electron density theory and degradation rules. It was determined that the first reaction was well matched with experimental results. However, we could not prove if the prediction tool predict degradation pathway of pharmaceutical compounds well due to not having enough substantial data of the byproducts in this research and references.

Suggestions for Future Work

This study raises two questions; (1) why the different structure of chemicals are removed at different removal efficiencies and (2) how the microbial community structure affects removal of pharmaceutical compounds. The complex between enzyme and organic compounds should be investigated because favorable complex shows higher removal of organic compounds. In cometabolism, the first reaction is very important because usually the first reaction is rate-limiting step. The study of a complex between monooxygenase enzyme and a organic compound will be more informative and can

determine why the various compounds which have different structures have different removal efficiencies even if in same condition. Docking Simulation between target monooxygenase enzymes and pharmaceutical compounds can be very nice tool to answer this question.

The microbial structural diversity should be investigated using molecular techniques such as denaturing gradient gel electrophoresis (DGGE) or terminal-restriction fragment length polymorphism (T-RFLP). In this and previous studies, it is believed that microbial community structure of other heterotrophic bacteria as well as monooxygenase production bacteria such as ammonia monooxygenase is important for degradation of pharmaceutical compounds.

In addition, more degradation byproducts should be detected to prove the capability of the prediction tool.

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APPENDICES

Appendix A

An example of Gaussian Code for Structure optimization and FED calculation

%chk=Trimethoprim
 # opt uhf/6-31g(d)

Trimethoprim

0 1

C							
C	1	B1					
C	2	B2	1	A1			
C	3	B3	2	A2	1	D1	
C	4	B4	3	A3	2	D2	
C	1	B5	2	A4	3	D3	
H	2	B6	1	A5	6	D4	
H	4	B7	3	A6	2	D5	
C	3	B8	2	A7	1	D6	
C	9	B9	3	A8	2	D7	
C	3	B10	2	A9	1	D8	
H	11	B11	3	A10	2	D9	
H	11	B12	3	A11	2	D10	
N	9	B13	3	A12	2	D11	
N	10	B14	9	A13	3	D12	
O	5	B15	4	A14	3	D13	
O	6	B16	1	A15	2	D14	
O	1	B17	2	A16	3	D15	
C	16	B18	5	A17	4	D16	
H	19	B19	16	A18	5	D17	
H	19	B20	16	A19	5	D18	
H	19	B21	16	A20	5	D19	
C	18	B22	1	A21	2	D20	
H	23	B23	18	A22	1	D21	
H	23	B24	18	A23	1	D22	
H	23	B25	18	A24	1	D23	
C	15	B26	10	A25	9	D24	
C	17	B27	6	A26	1	D25	
H	28	B28	17	A27	6	D26	
H	28	B29	17	A28	6	D27	
H	28	B30	17	A29	6	D28	
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H	32	B32	14	A31	9	D30	
N	10	B33	9	A32	3	D31	
H	34	B34	10	A33	9	D32	
H	34	B35	10	A34	9	D33	
O	27	B36	15	A35	10	D34	
H	37	B37	27	A36	15	D35	

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B2	1.40140226
B3	1.40140141
B4	1.40140135
B5	1.40140152
B6	1.07000086
B7	1.07000356
B8	2.51481727
B9	1.39494235
B10	1.54000157
B11	1.06999988
B12	1.07000097
B13	2.35905254
B14	1.33786616
B15	1.43000187
B16	1.43000087
B17	1.42999959
B18	1.43000244

B19	1.07000386
B20	1.06999779
B21	1.07000481
B22	1.43000099
B23	1.06999711
B24	1.07000035
B25	1.06999867
B26	1.34423457
B27	1.43000467
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A8	147.80491235
A9	119.99979053
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A11	109.47122415
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A15	119.99987921
A16	120.00014364
A17	109.47121850
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A23	109.47118265
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Trimethoprim

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C	17	B27	6	A26	1	D25	
H	28	B28	17	A27	6	D26	
H	28	B29	17	A28	6	D27	

H	28	B30	17	A29	6	D28
C	14	B31	9	A30	3	D29
H	32	B32	14	A31	9	D30
N	10	B33	9	A32	3	D31
H	34	B34	10	A33	9	D32
H	34	B35	10	A34	9	D33
O	27	B36	15	A35	10	D34
H	37	B37	27	A36	15	D35

B1	1.40140033
B2	1.40140226
B3	1.40140141
B4	1.40140135
B5	1.40140152
B6	1.07000086
B7	1.07000356
B8	2.51481727
B9	1.39494235
B10	1.54000157
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B21	1.07000481
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B23	1.06999711
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B25	1.06999867
B26	1.34423457
B27	1.43000467
B28	1.07000012
B29	1.07000164
B30	1.07000036
B31	1.33786909
B32	1.06999955
B33	1.47000000
B34	1.00000000
B35	1.00000000
B36	1.43000000
B37	0.96000000
A1	120.00011735
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A16	120.00014364
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A22	109.47117925
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A25	120.74844672
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A35	119.46609692
A36	109.50000006
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D2	0.00000000
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D11	87.79703810
D12	147.19310163
D13	180.00000000
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D23	60.00150599
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D25	-89.99822483
D26	-180.00000000
D27	-59.99899057
D28	60.00110472
D29	18.98143950
D30	180.00000000
D31	-32.80550585
D32	132.60073377
D33	-107.39925144
D34	-180.00000000
D35	-66.93506356

Appendix B

Routine Operational Data

Table B.1 Synthetic influent feed

Feed	Stock Solution	Influent Solution
Carbon Feed		
$CH_3COONa{\cdot}3H_2O$	21240 mg/L	425 mg/L
Casamino Acids	1500 mg/L	30 mg/L
Nutrient Feed		
KCl	1600 mg/L	118.4 mg/L
$MgCl_2{\cdot}\ 6H_2O$	3000 mg/L	222 mg/L
$MgSO_4{\cdot}\ H_2O$	200 mg/L	14.8 mg/L
$CaCl_2$	620 mg/L	45.9 mg/L
Yeast Extract	112.5 mg/L	83 mg/L
10% HCl	3.25 mg/L	0.24 mg/L
Trace element solution	2.75 mg/L	0.2 mg/L
$FeSO_4$ solution	2 mg/L	0.15 mg/L
NH_4Cl	1548 mg/L	114.5 mg/L
$NaH_2PO_4{\cdot}2H_2O$	186 mg/L	13.8 mg/L
Trace Elements Solution		
H_3BO_3	300	0.061 mg/L
$ZnSO_4{\cdot}7H_2O$	1500	0.305 mg/L
KI	75	0.015 mg/L
$CuSO_4{\cdot}\ 5H_2O$	300	0.061 mg/L
$Co(NO_3)_2{\cdot}\ 6H_2O$	367.1	0.075 mg/L
$Na_2MoO_4{\cdot}\ 2H_2O$	150	0.031 mg/L
$MnSO_4{\cdot}H_2O$	1700	0.342 mg/L
$FeSO_4$ Solution		
$FeSO_4{\cdot}\ 7H_2O$	2054 mg/L	0.304 mg/L
Note: 500mL of influent solution consists of 10mL of carbon feed, 37mL nutrient feed, 453mL deionized water		

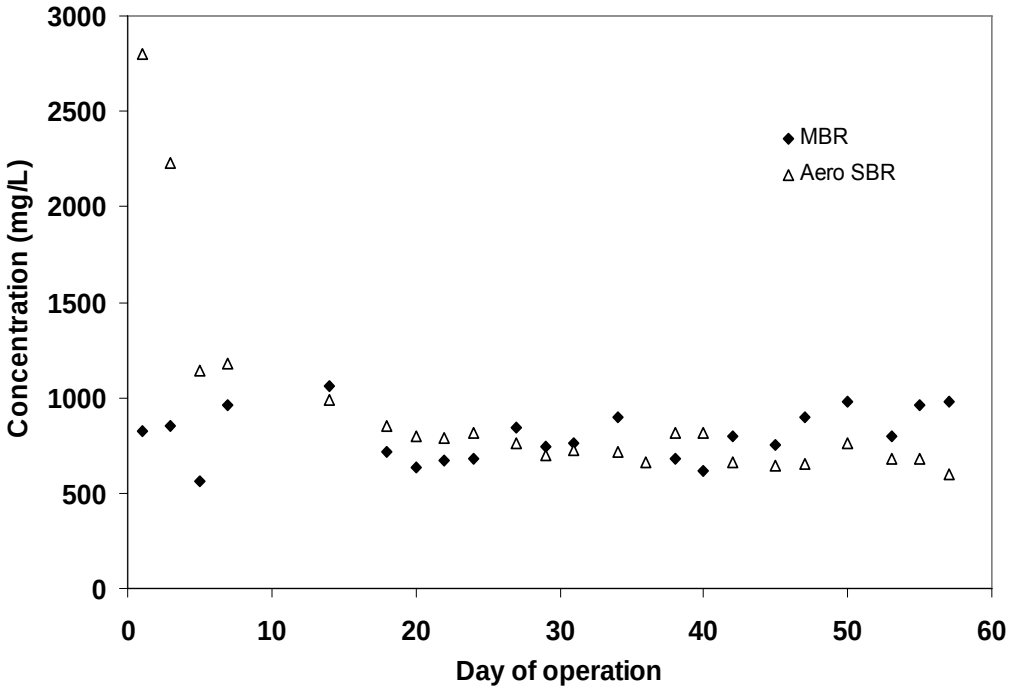


Figure B.1 MLSS concentration for SBR and MBR

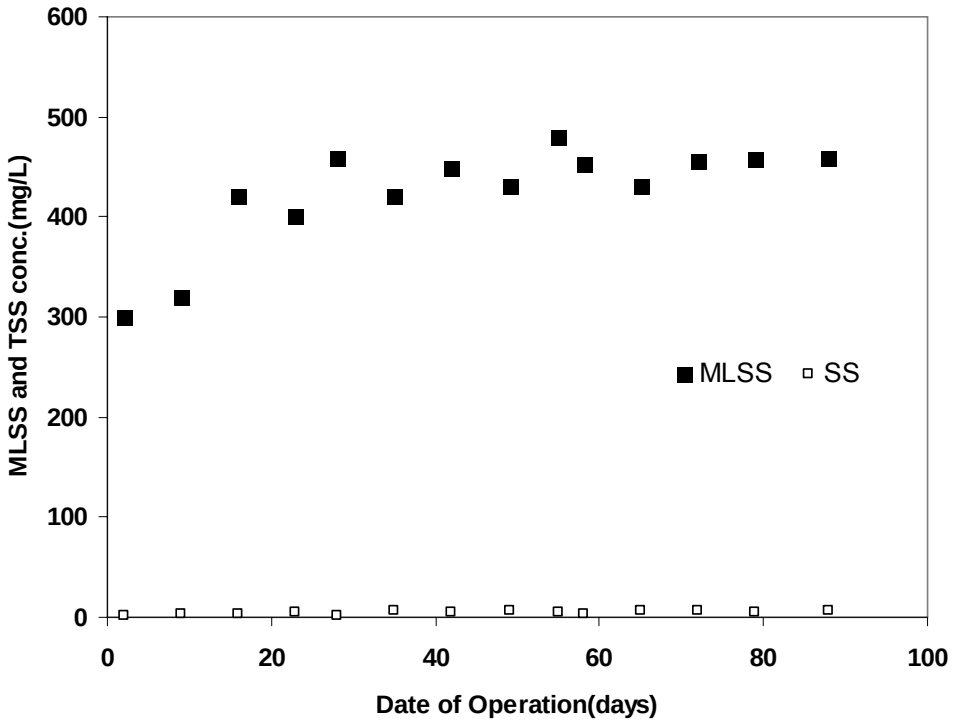


Figure B.2 MLSS and Effluent TSS concentration for Nitrifying sludge reactor

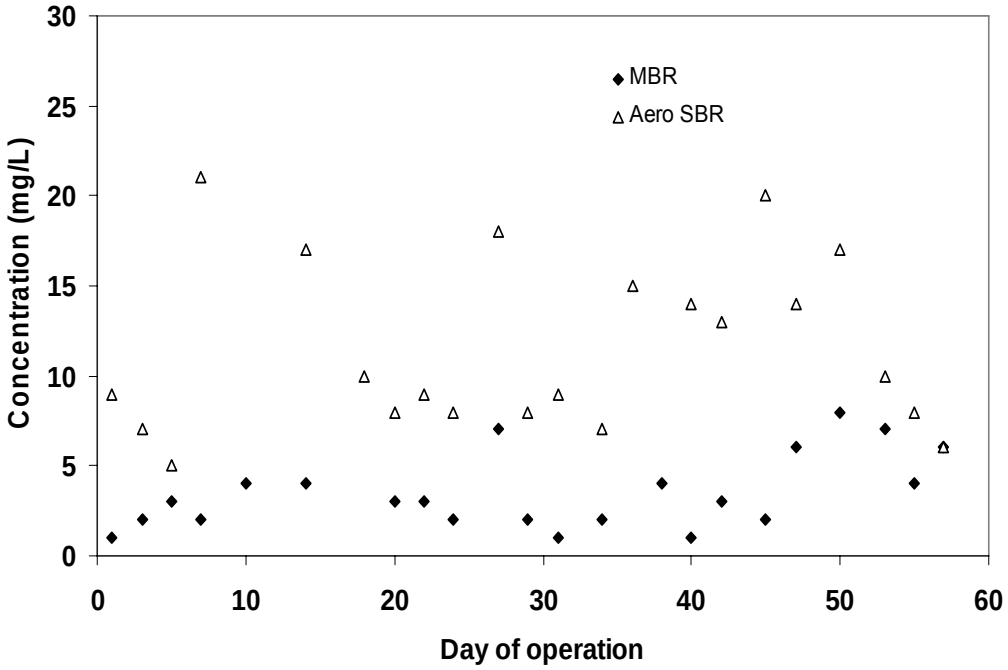


Figure B.3 Effluent TSS concentration for SBR and MBR

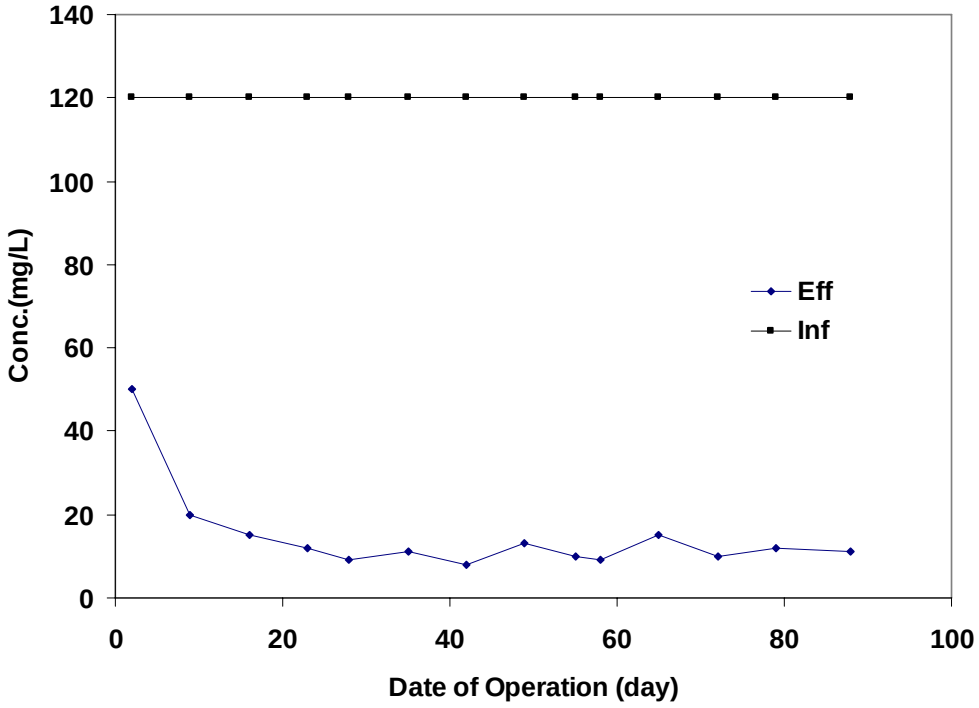


Figure B.4 NH_3-N Concentration in Influent and Effluent

Appendix C

Reactors and reactor configuration

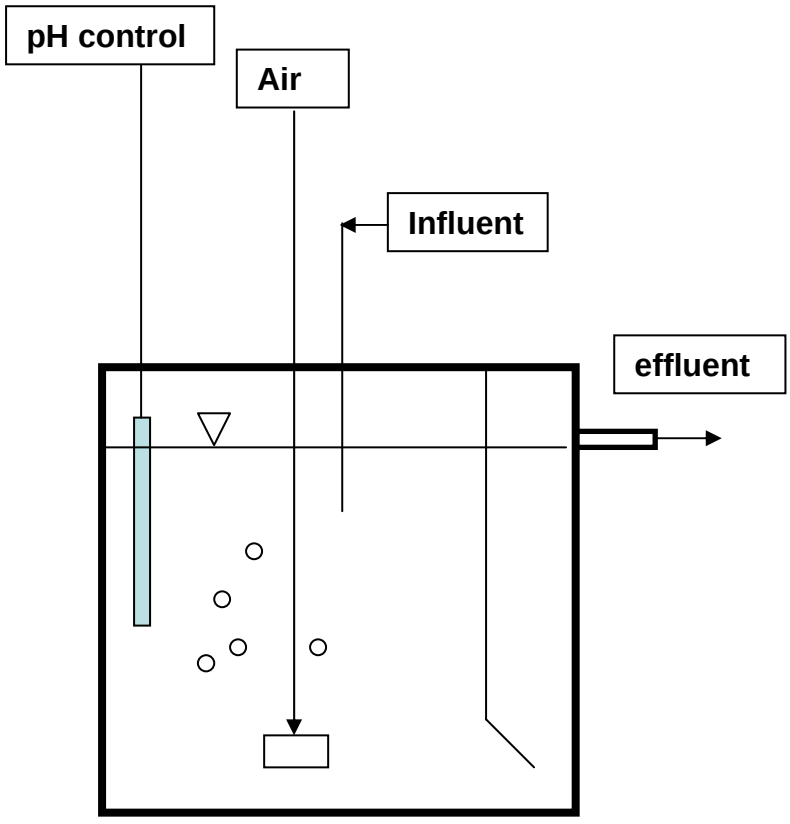


Figure C.1 CSTR fed with toluene

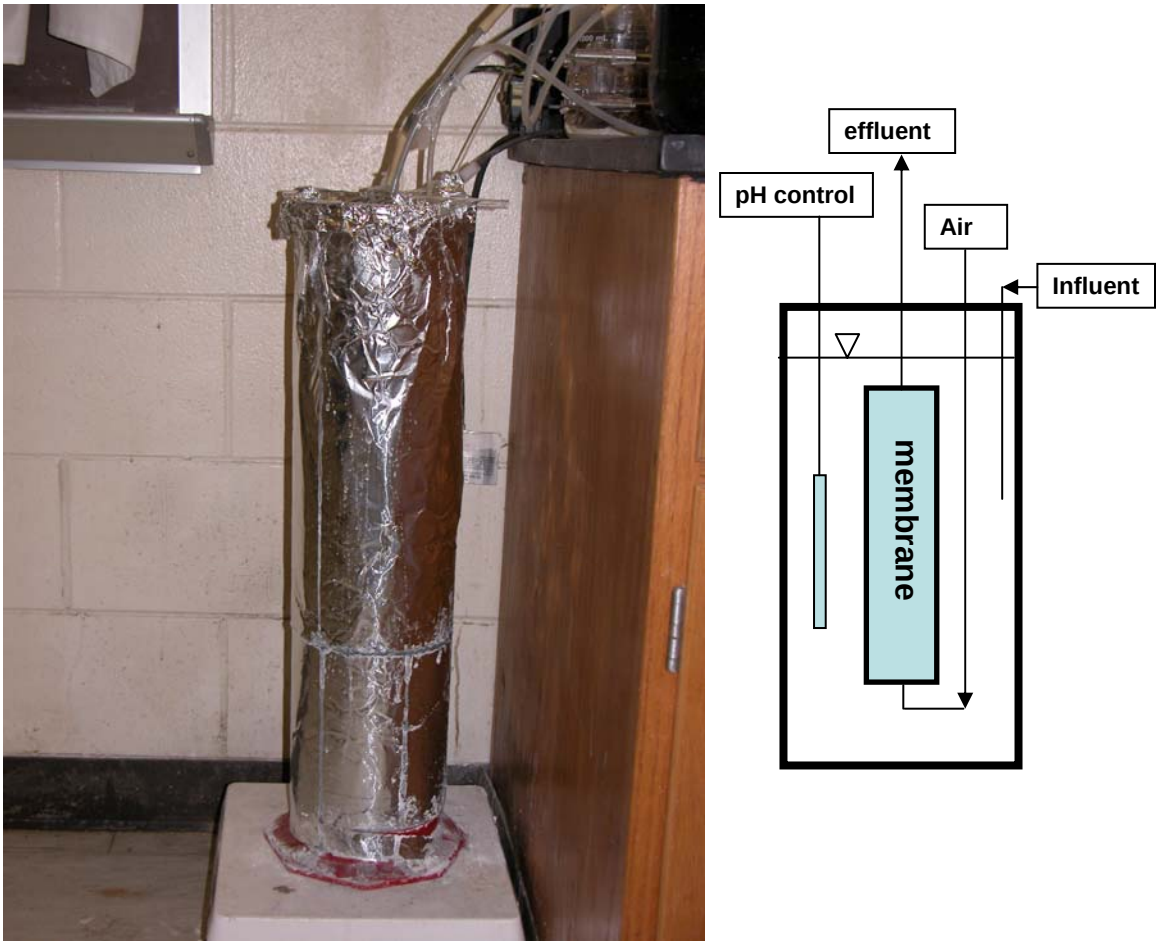


Figure C.2 Nitrifying membrane bioreactor

Figure C.3 Membrane bioreactor

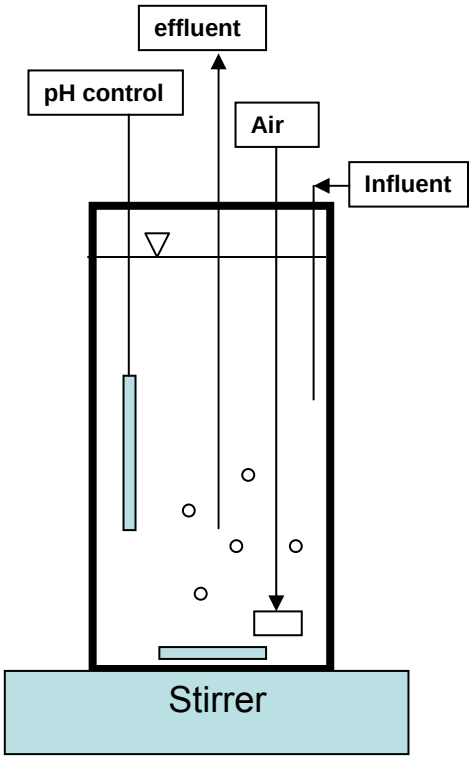


Figure C.4 Sequencing bioreactor

Appendix D

Degradation experiment data

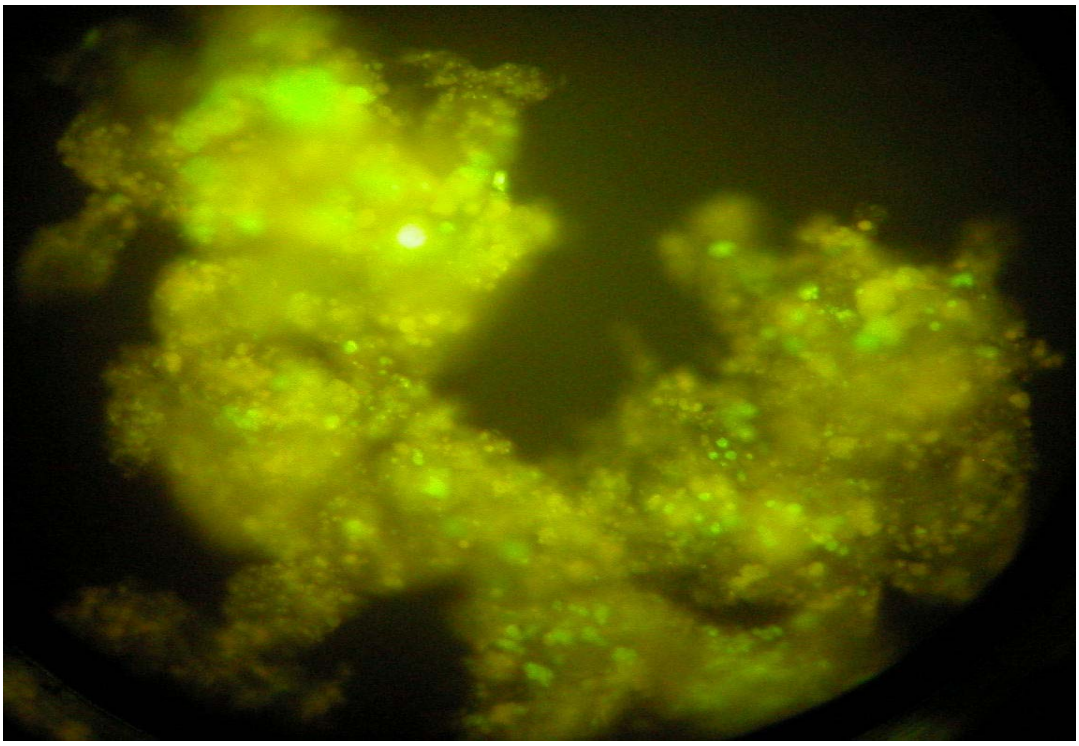


Figure D.1 Nitrobacter sp. FISH image of nitrifying sludge using vermicon kit

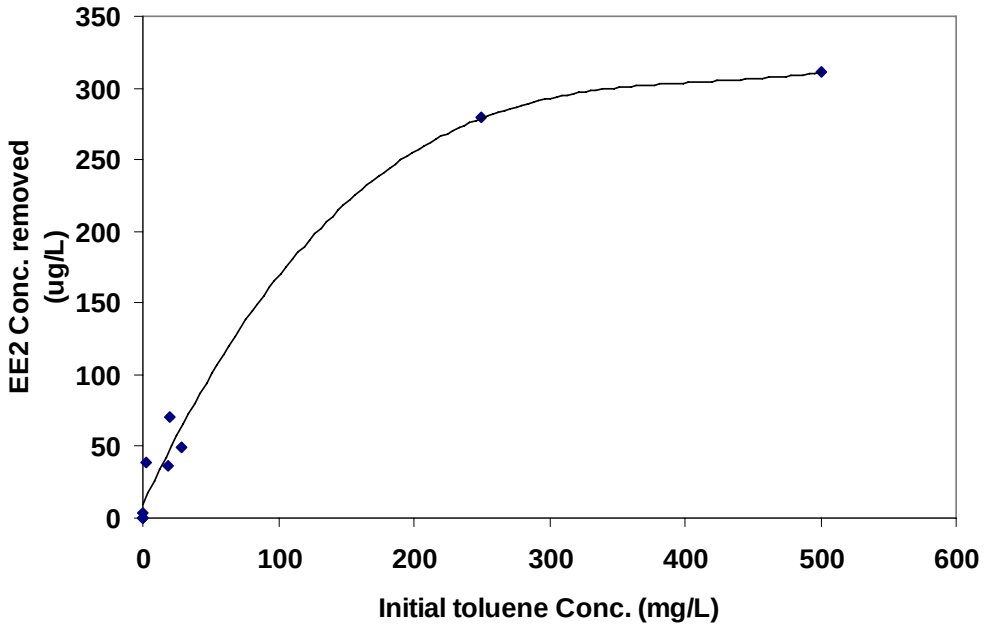


Figure D.2 EE2 removed at different initial toluene concentration in batch tests

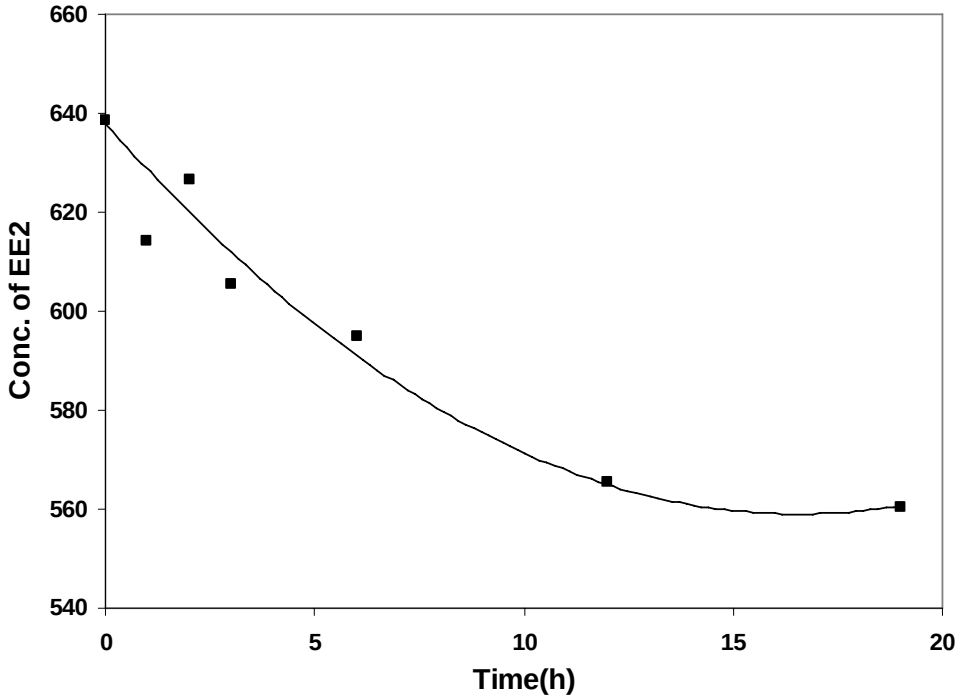


Figure D.3 Degradation of EE2 with Toluene in batch tests (intial concentration of Toluene = 50 mg/L)

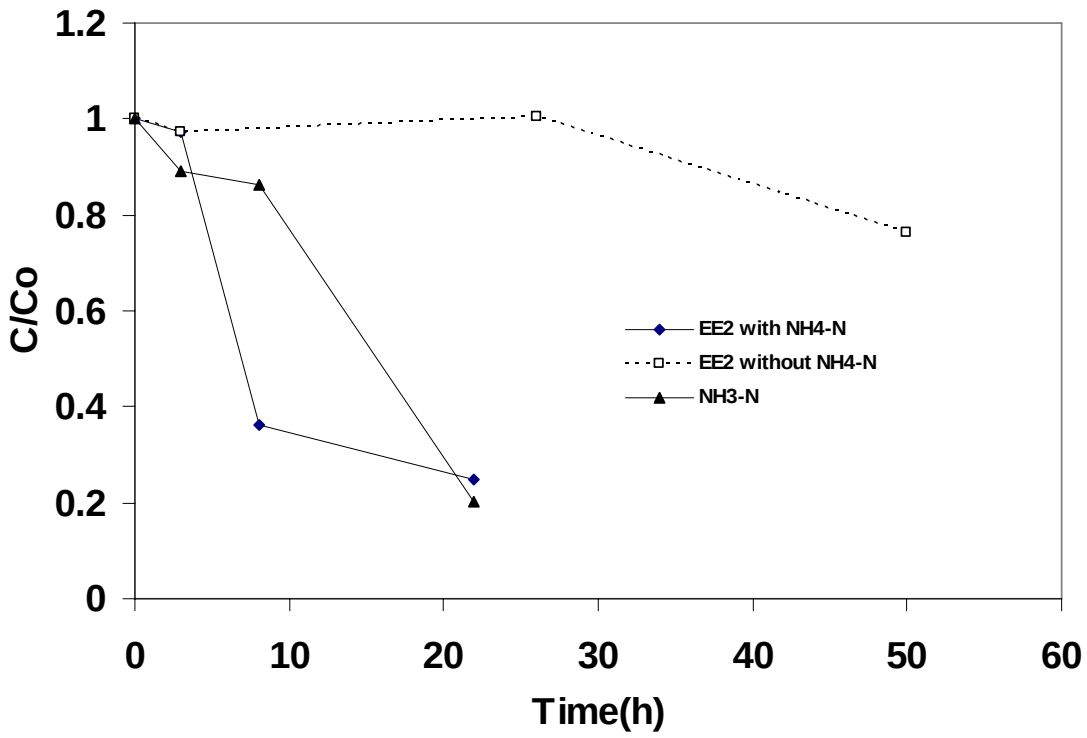
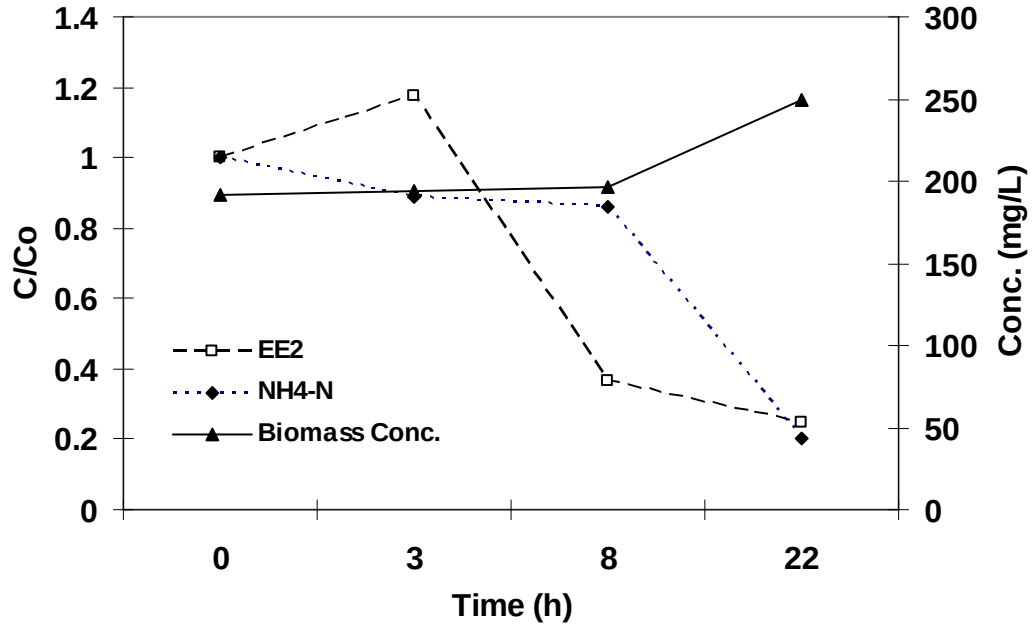


Figure D.4 Degradation of EE2 with whole cell



Figrue D.5 Degradation Tests using nitrifying sludge (initial concentration of EE2 = 100ug/L, NH_3-N = 30mg/L)

Appendix E

Sorption experiment data

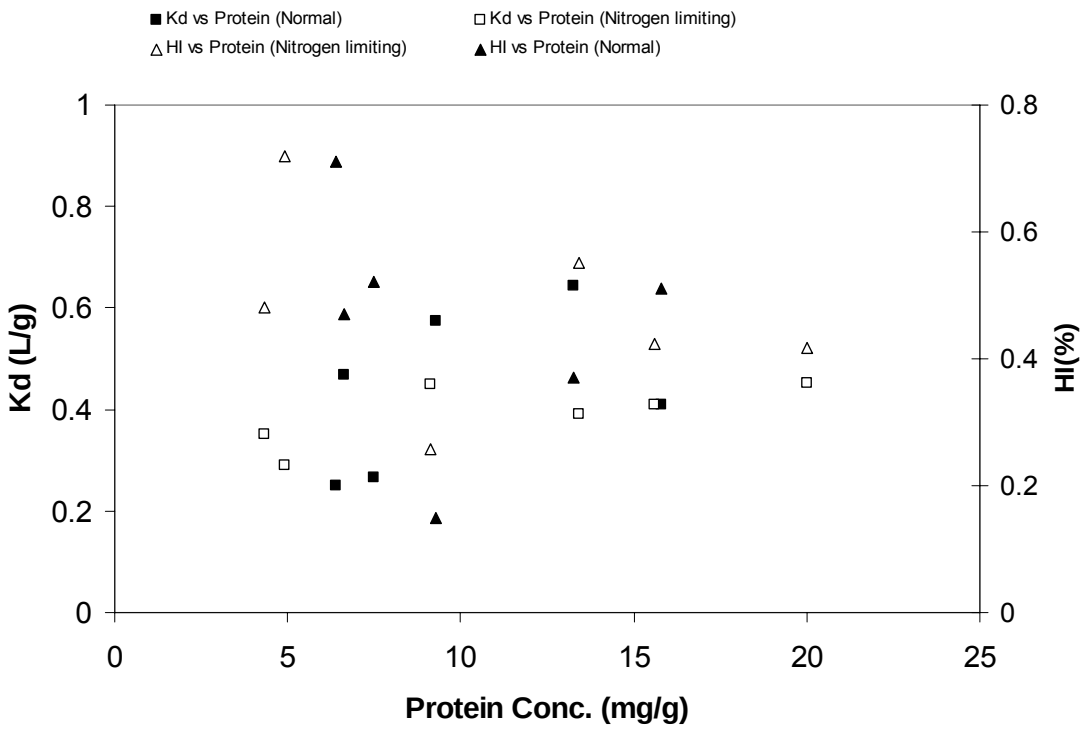


Figure E.1 Relationship between protein conc. of EPS and Partitioning coefficient (K_d)

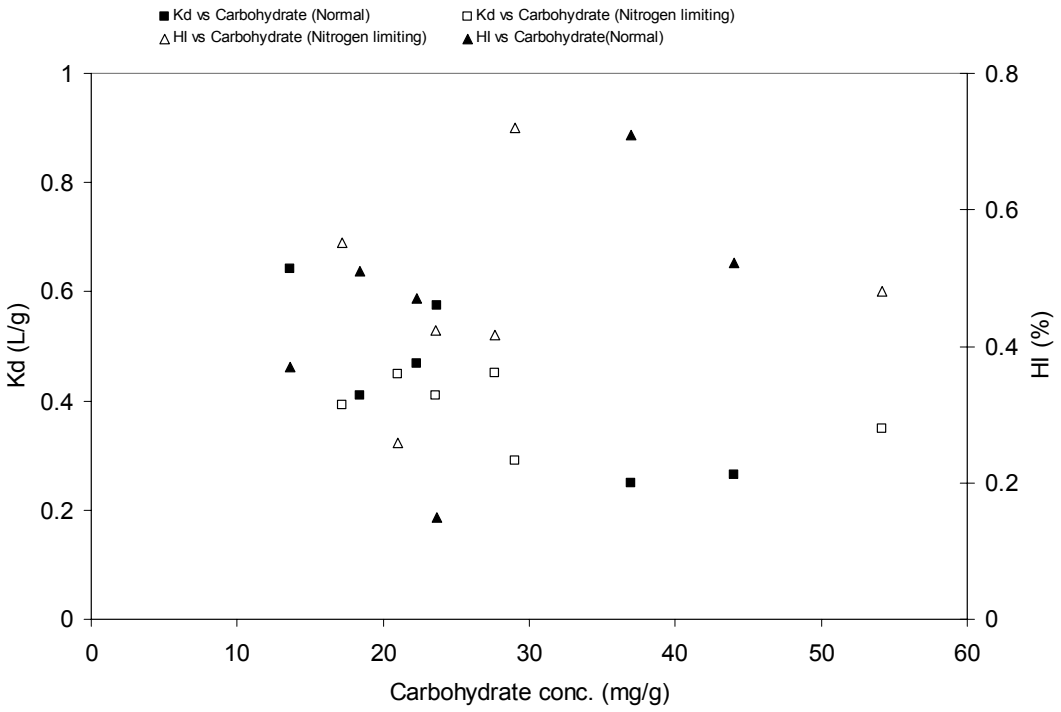


Figure E.2 The relationship between Carbohydrate conc. of EPS and partitioning coefficient (K_d)

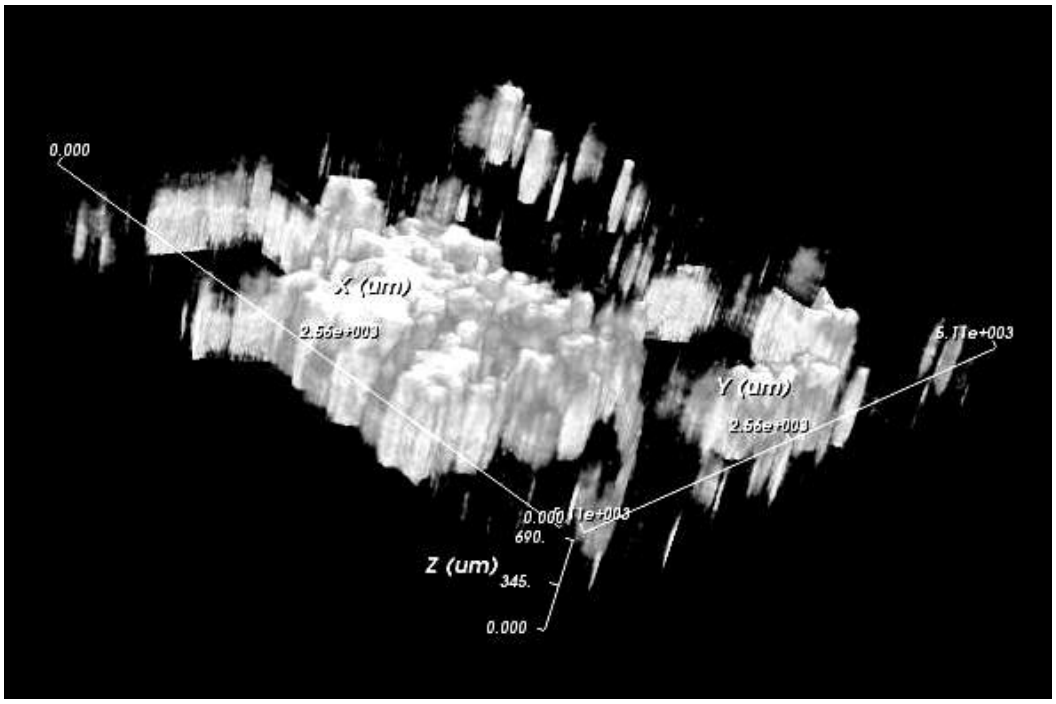
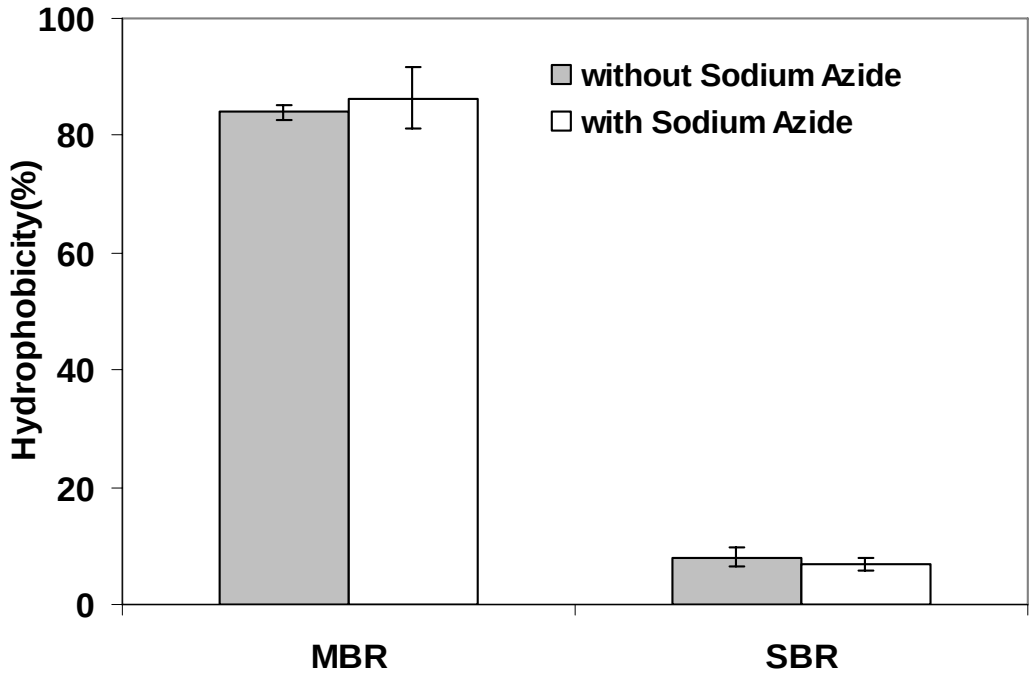


Figure E.3 Floc 3D Structure using confocal microscope



Figrue E.4 Hydrophobicity change with/without sodium azide (control test)

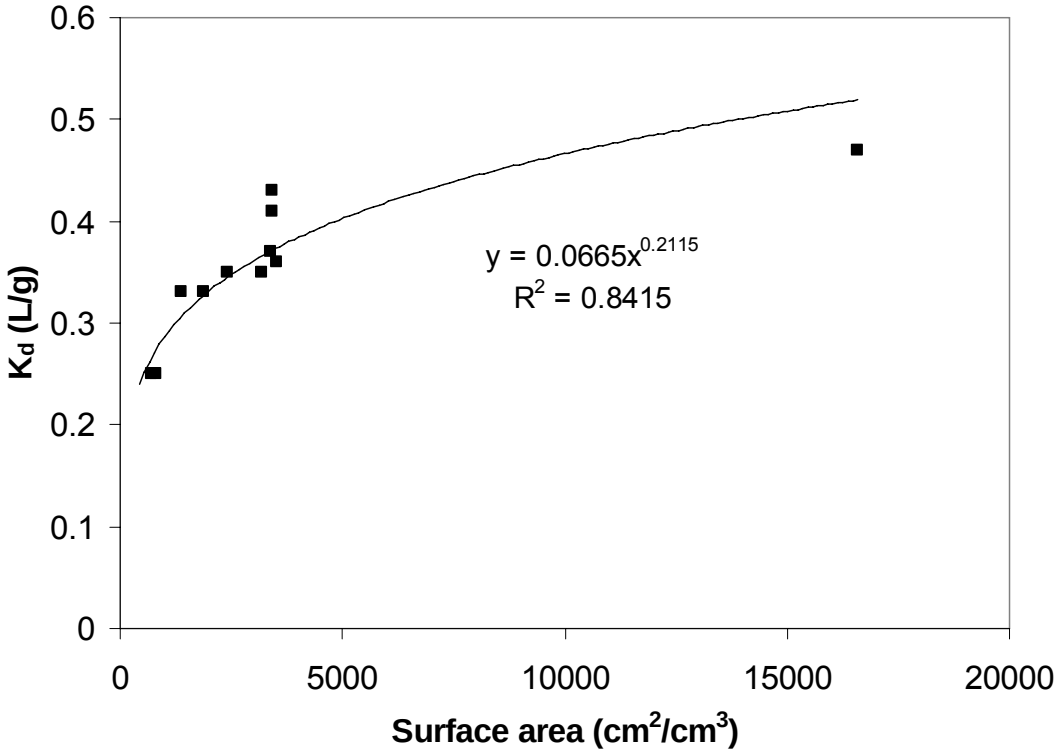


Figure E.5 Partitioning coefficienty (K_d) vs. Surface area of floc

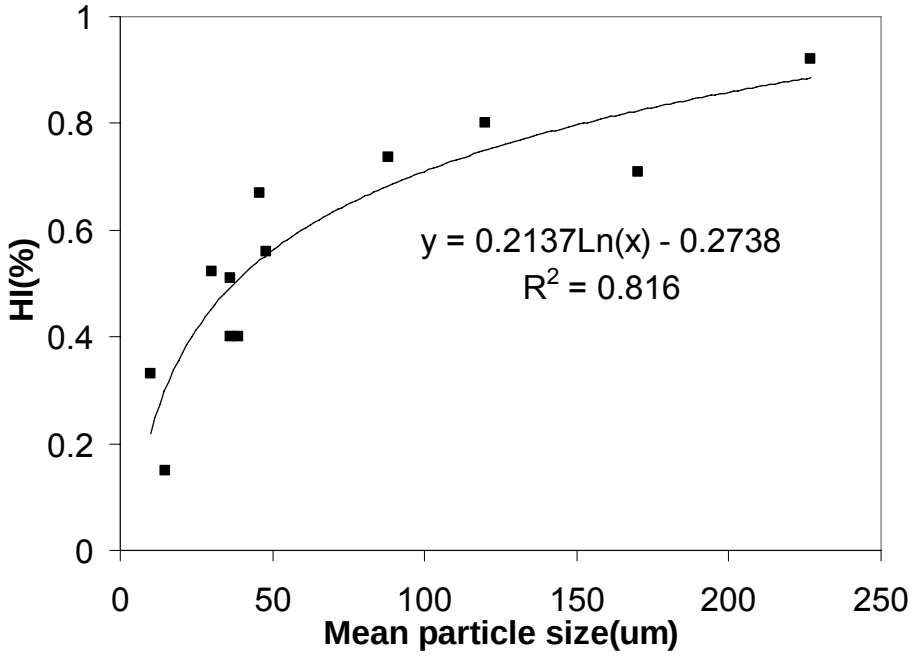


Figure E.6 Hysteresis Index (HI) vs. Mean particle size of floc

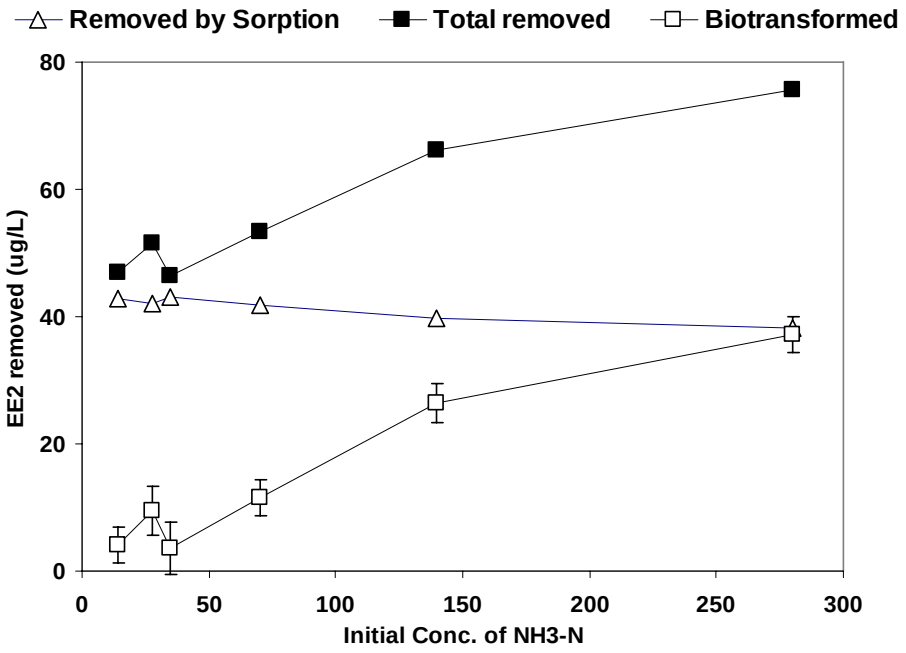


Figure E.7 EE2 romoved in batch test at different concentration of NH_3-N

Appendix F

TLC plates for continuous reactor

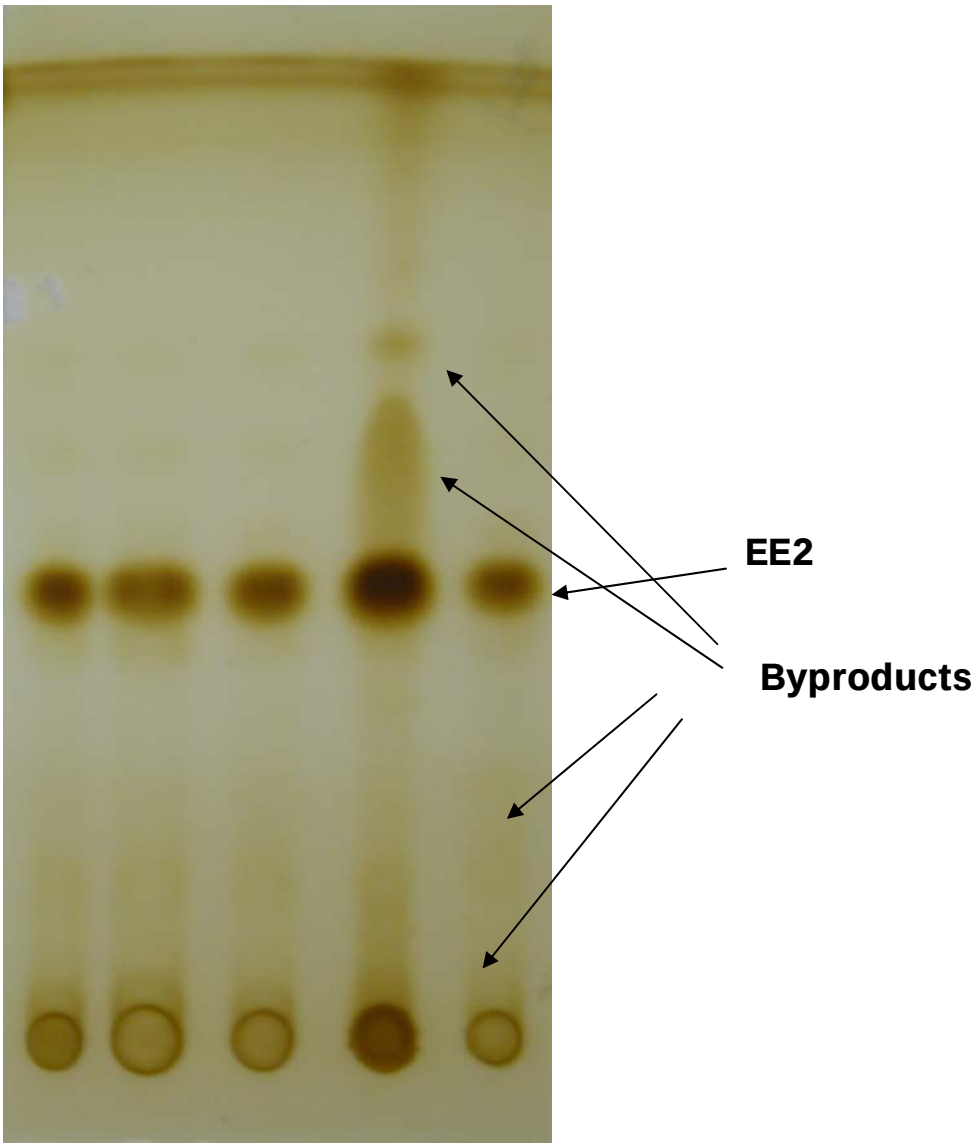


Figure F.1 TLC plate for EE2 in Nitrifying sludge reactor

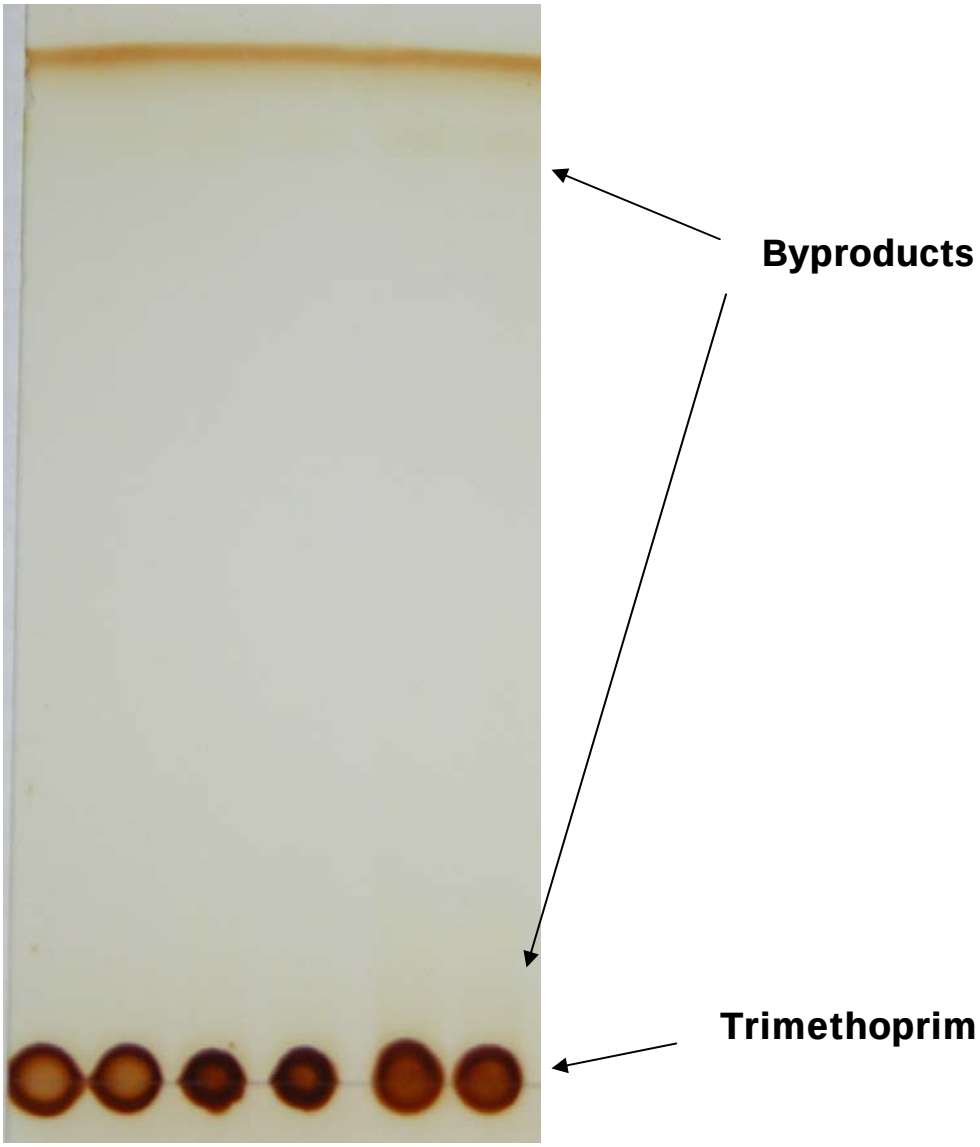


Figure F.2 TLC plate for Trimethoprim in Nitrifying sludge reactor

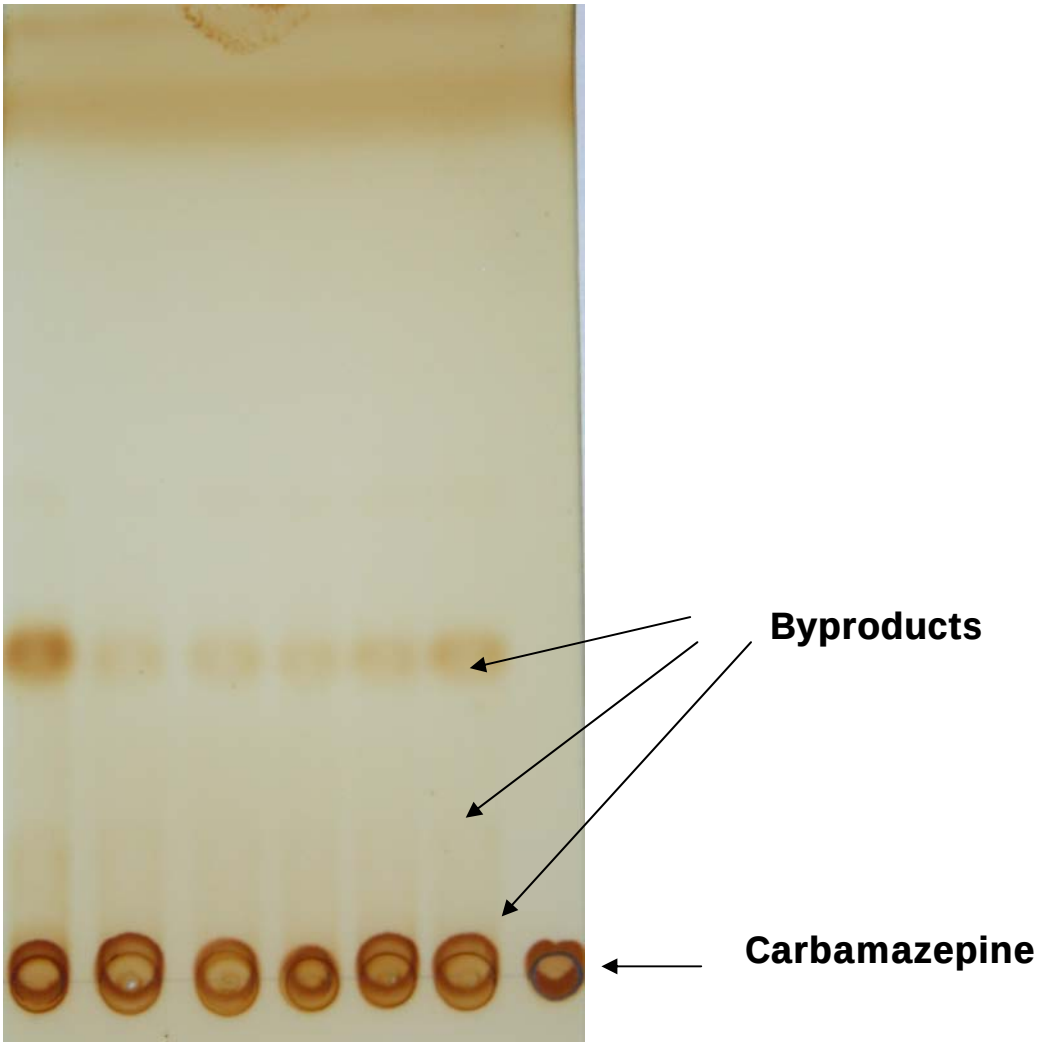


Figure F.3 TLC plate for Carbamazepin in Nitrifying sludge reactor

Figure F.4 TLC plate for EE2 in CSTR fed with toluene

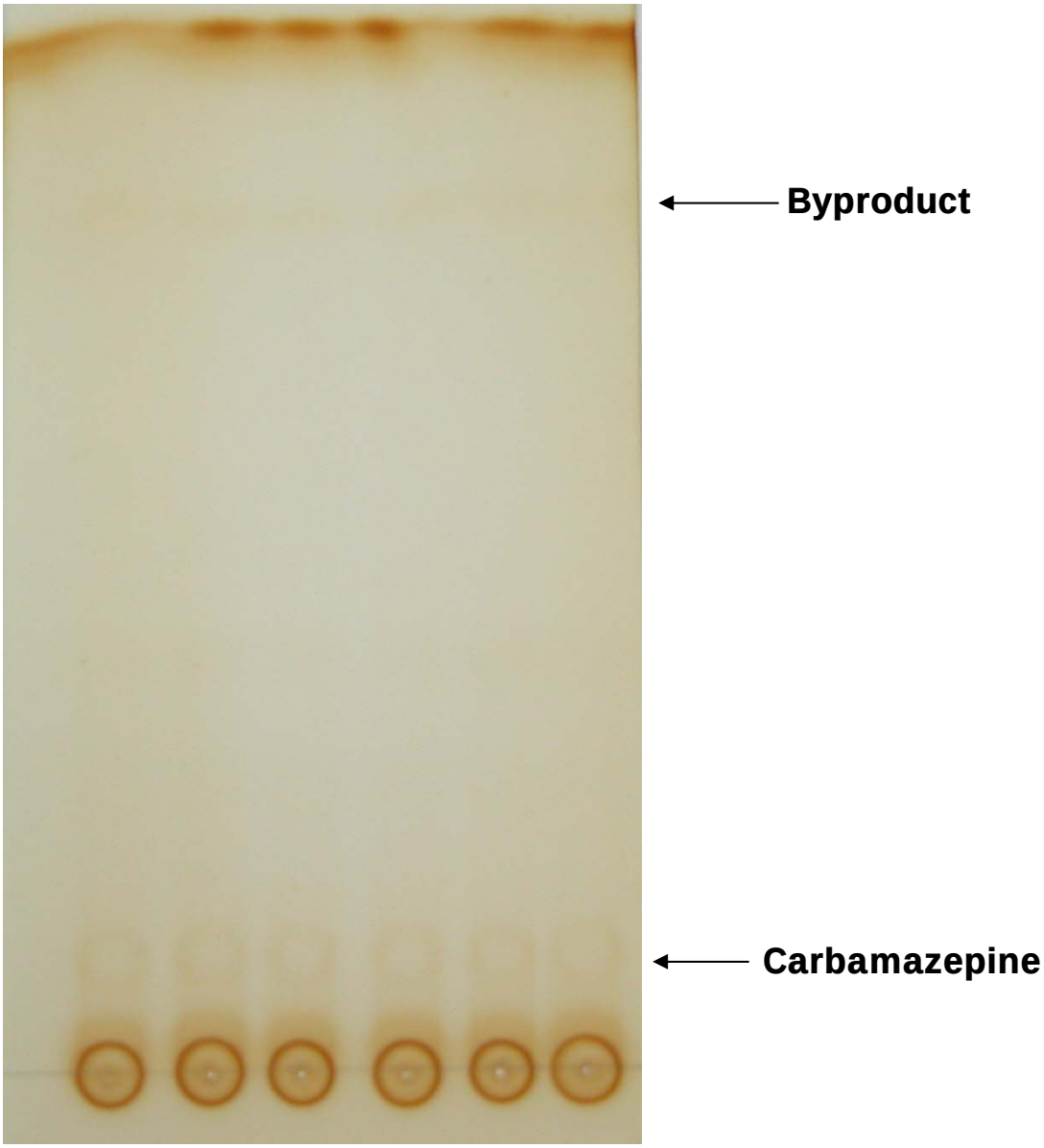


Figure F.5 TLC plate for Carbamazepine in CSTR fed with toluene

Figure F.6 TLC plate for Trimethoprim in CSTR fed with toluene

Appendix G

Full degradation pathways predicted using FED and degradation rules and 1H NMR data of byproducts

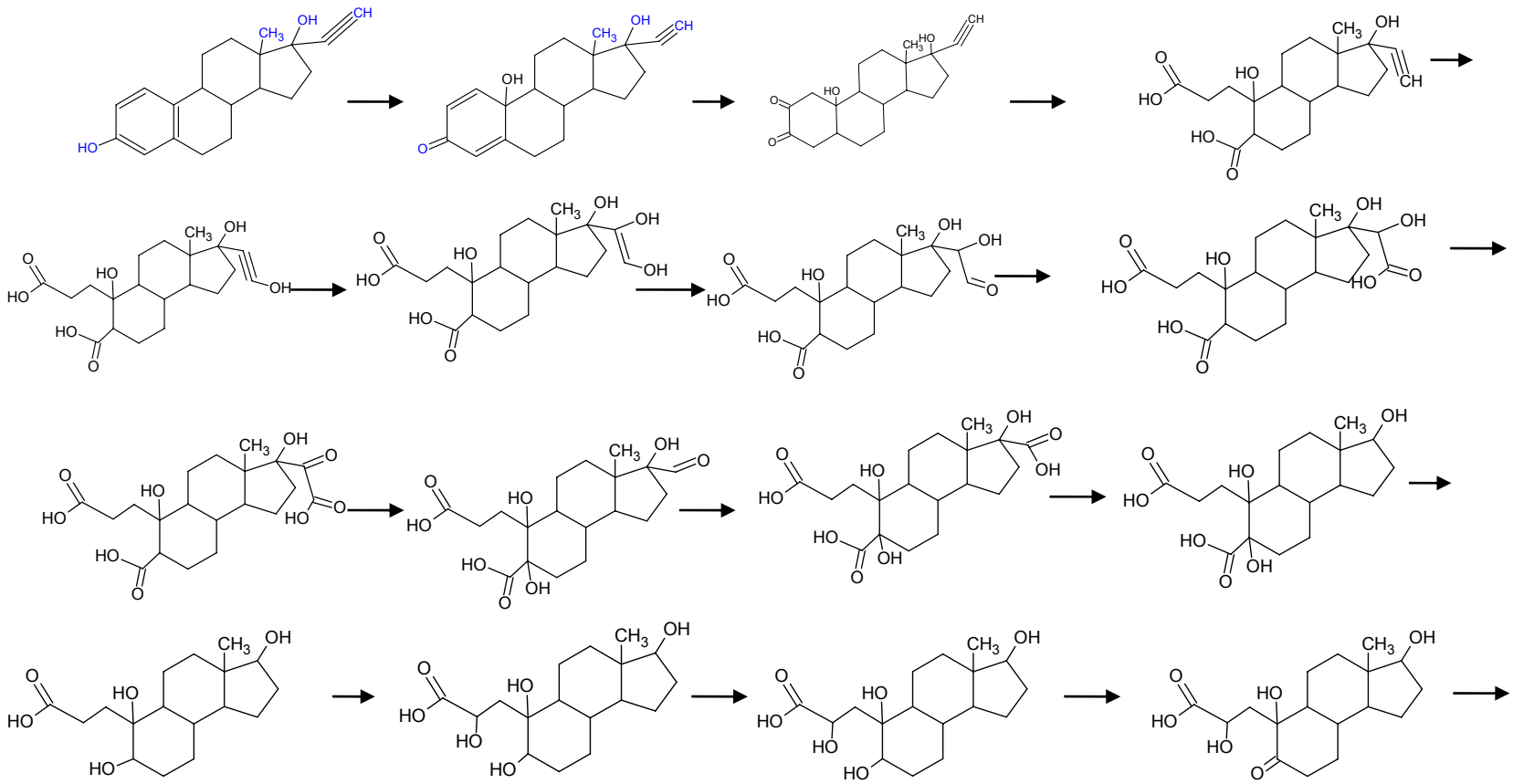


Figure G.1 EE2 degradation (full) pathway using MO theory and degradation rules

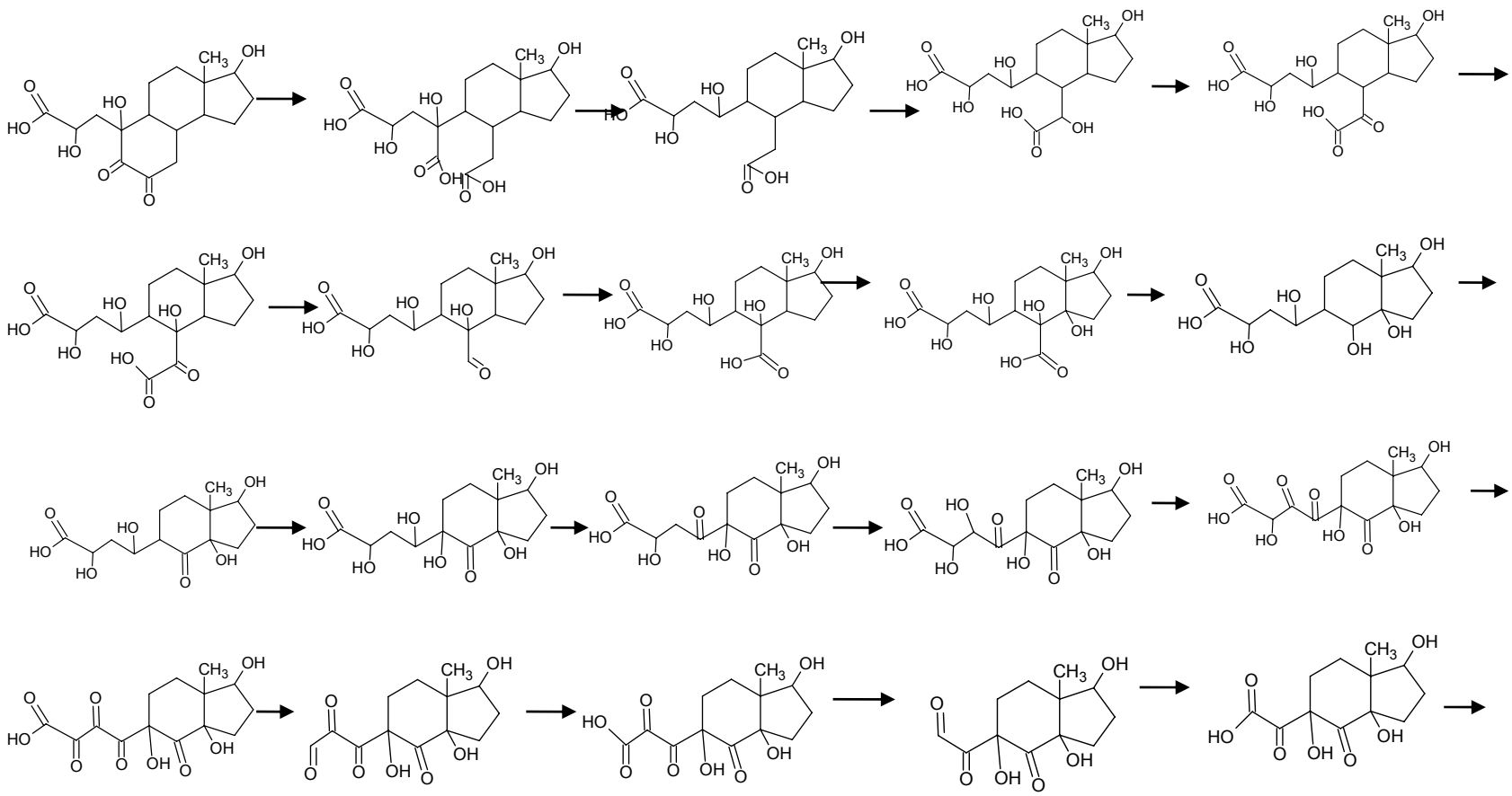


Figure G.1 EE2 degradation (full) pathway using MO theory and degradation rules (continued)

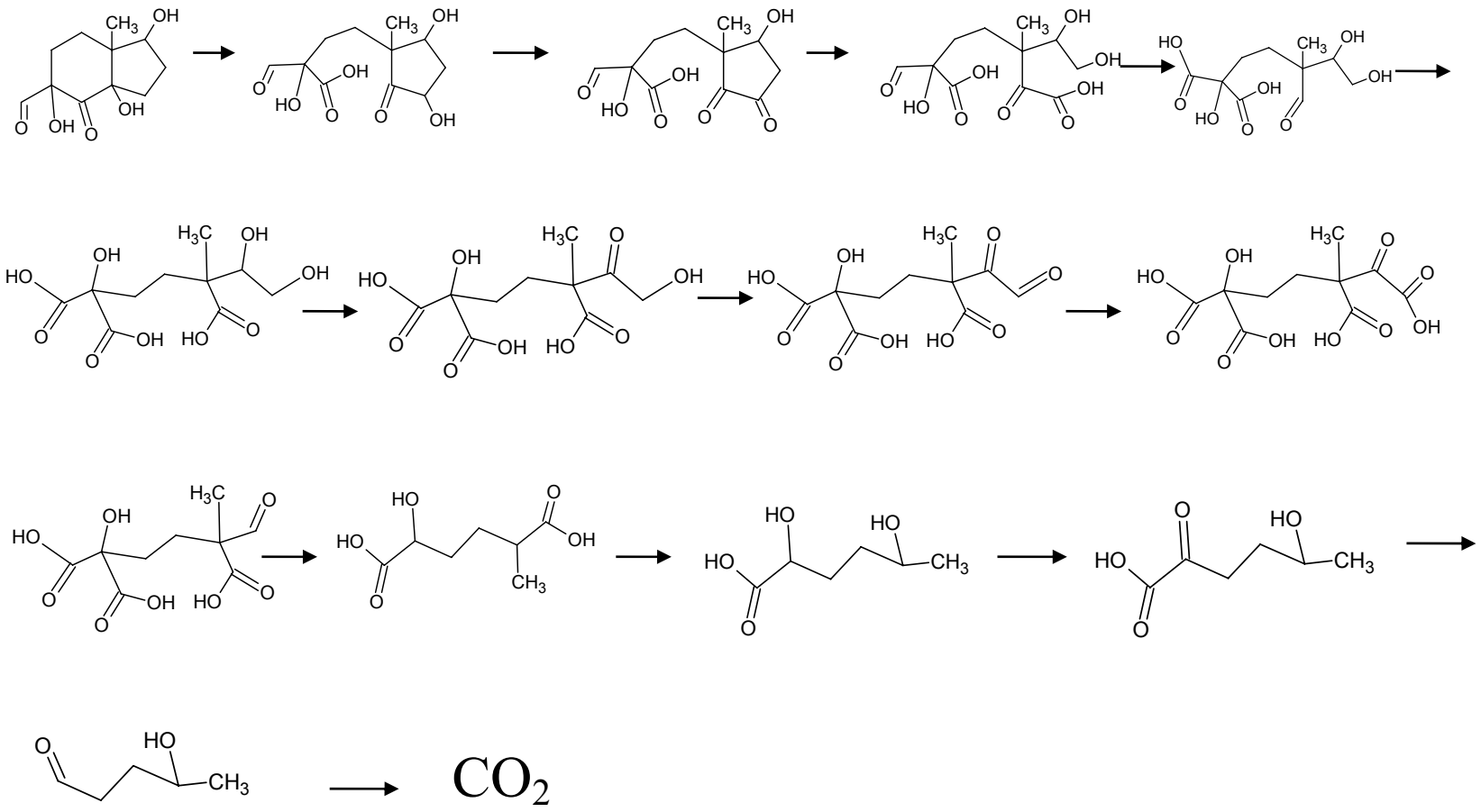


Figure G.1 EE2 degradation (full) pathway using MO theory and degradation rules (continued)

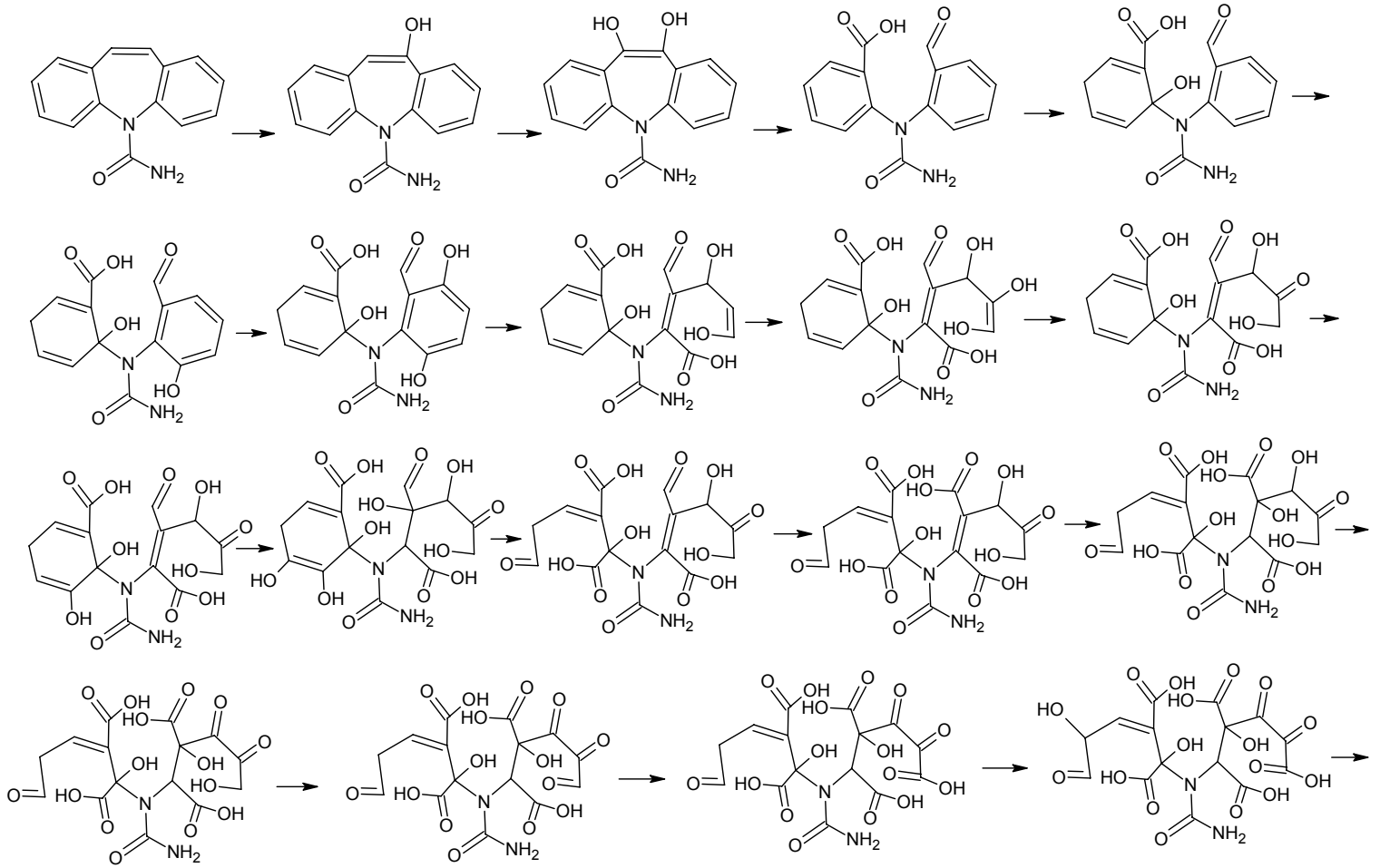


Figure G.2 Carbamazepine degradation (full) pathway using MO theory and degradation rules

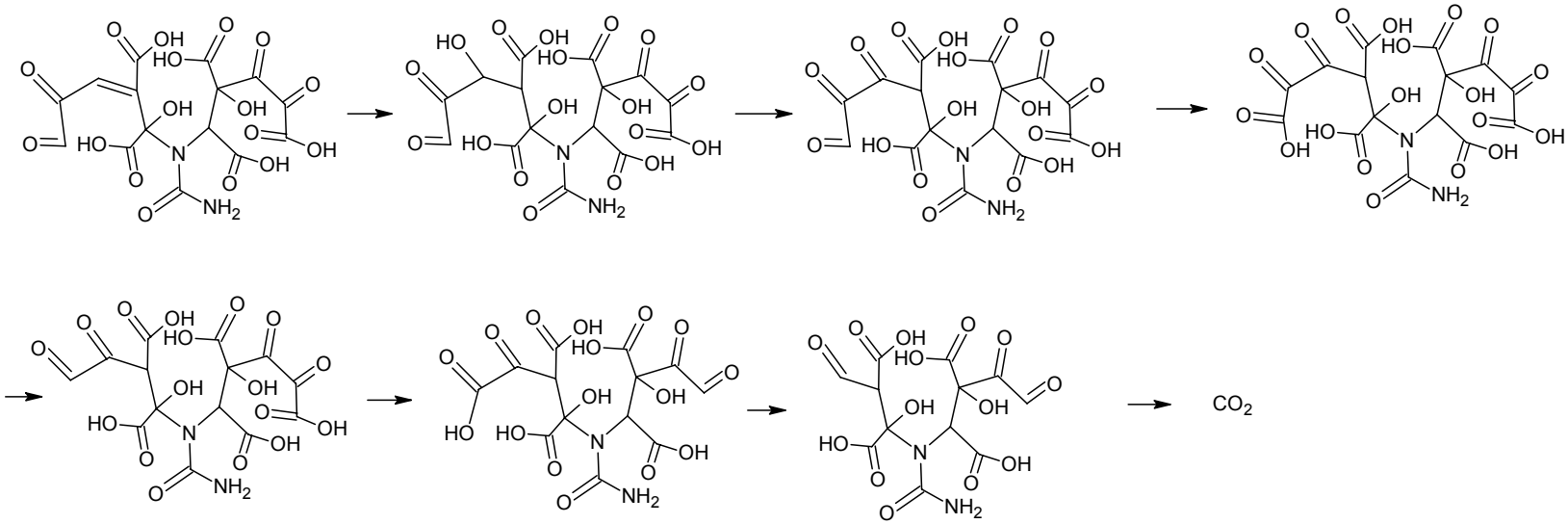


Figure G.2 Carbamazepine degradation (full) pathway using MO theory and degradation rules (continued)

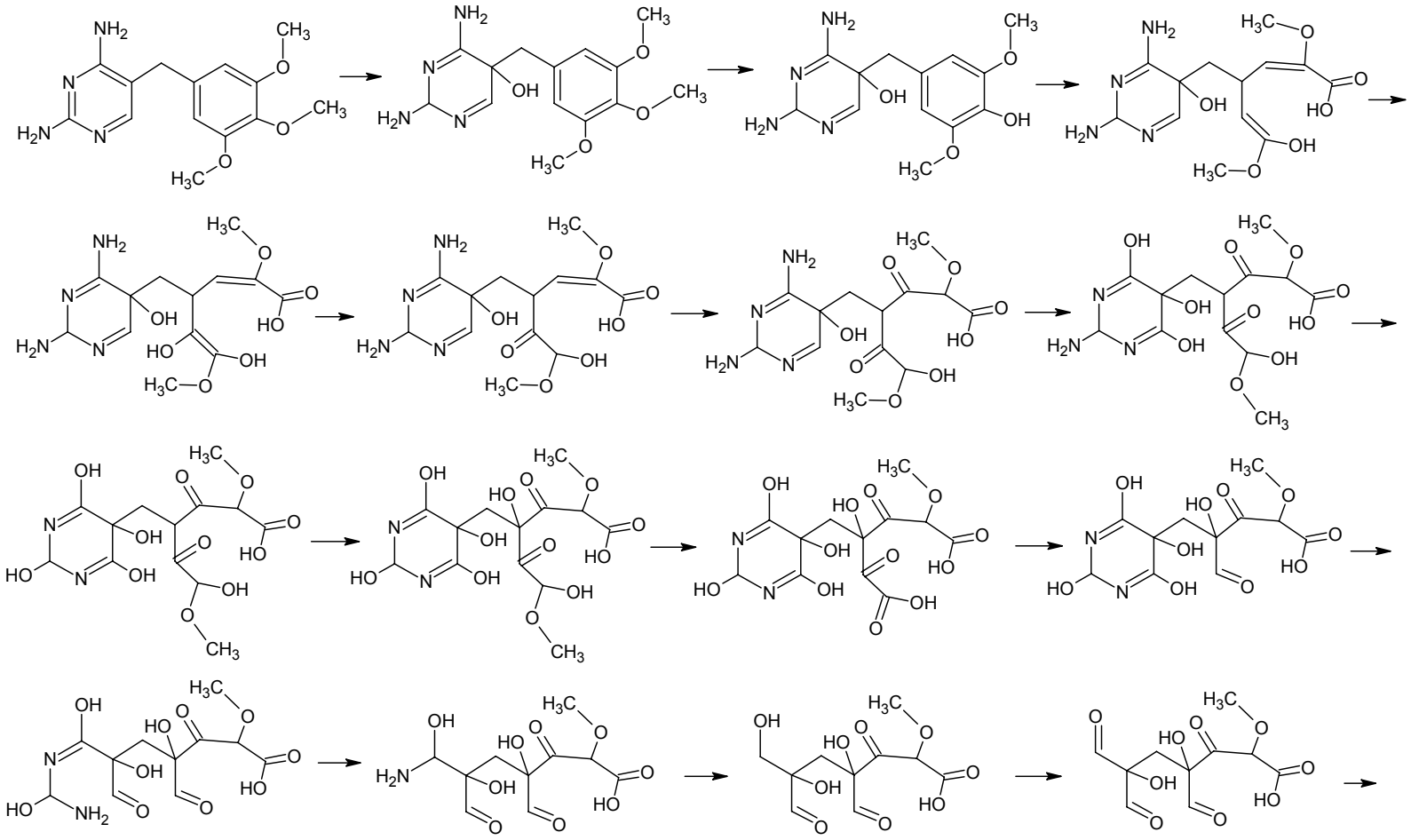


Figure G.3 Trimethoprim degradation (full) pathway using MO theory and degradation rules

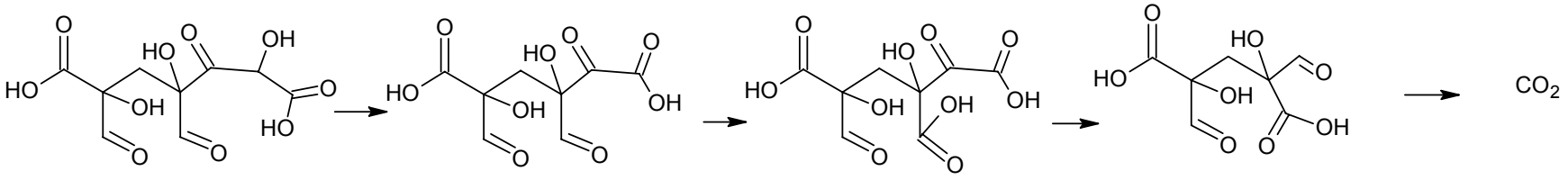
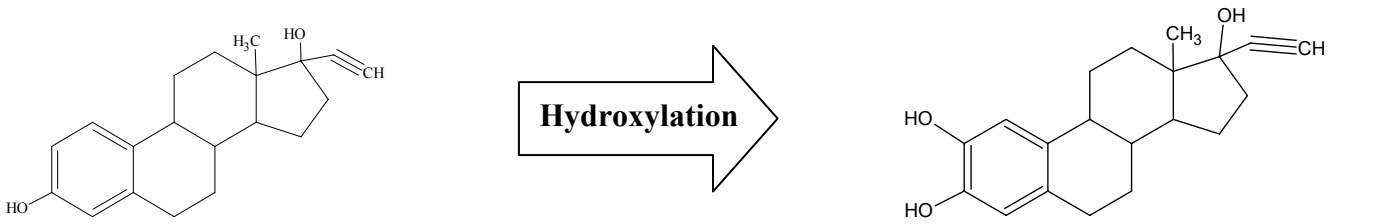


Figure G.3 Trimethoprim degradation (full) pathway using MO theory and degradation rules (continued)



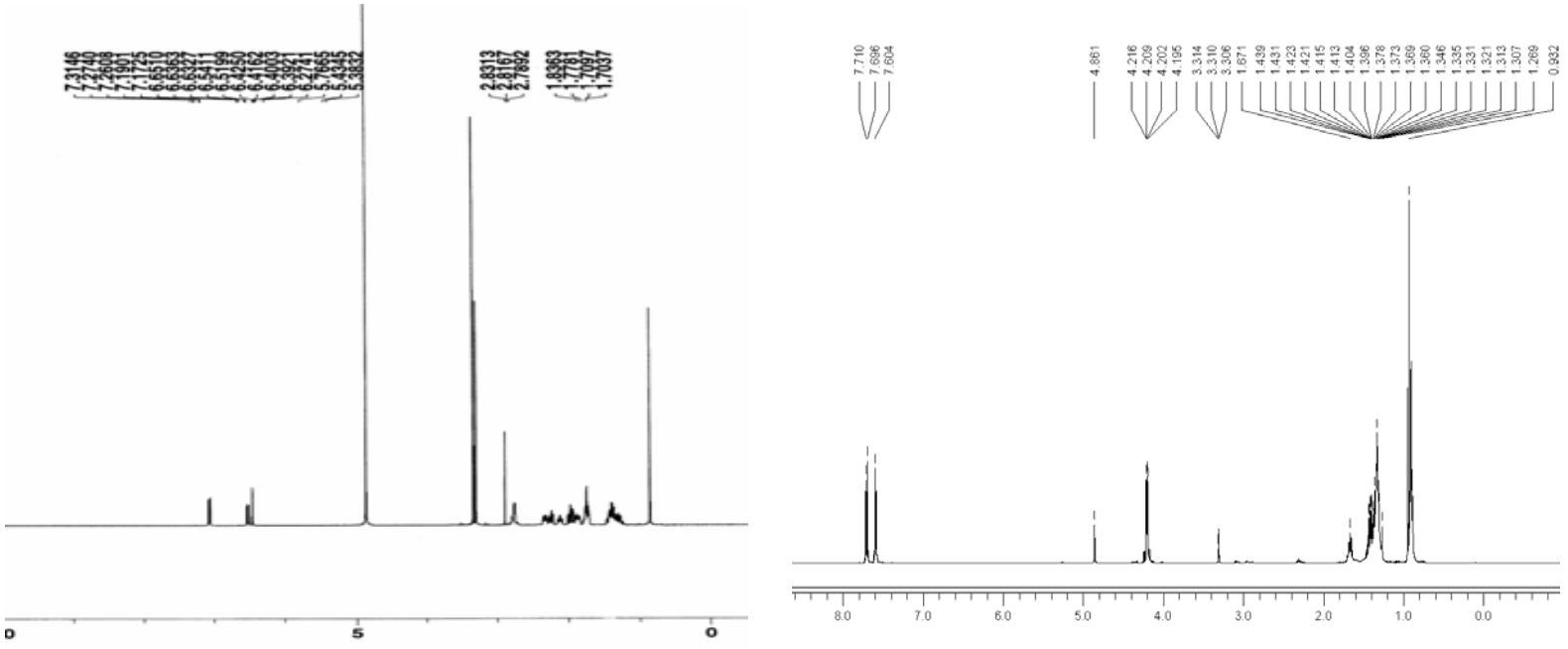


Figure G.4 The observed NMR spectrum: This shows that hydroxylation of EthinylEstradiol

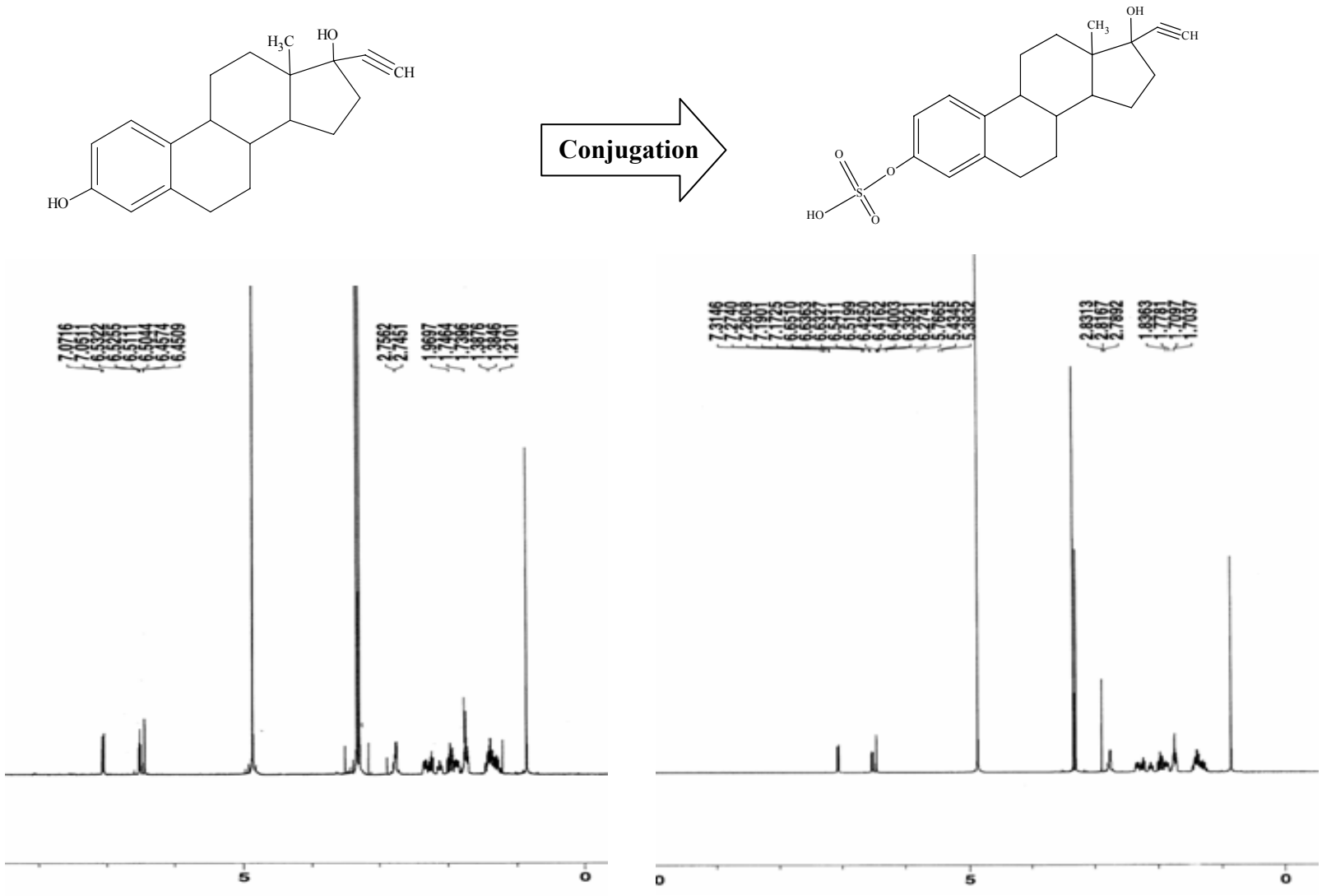


Figure G.5 The observed NMR spectrum: This shows that conjugation of EthinylEstradiol

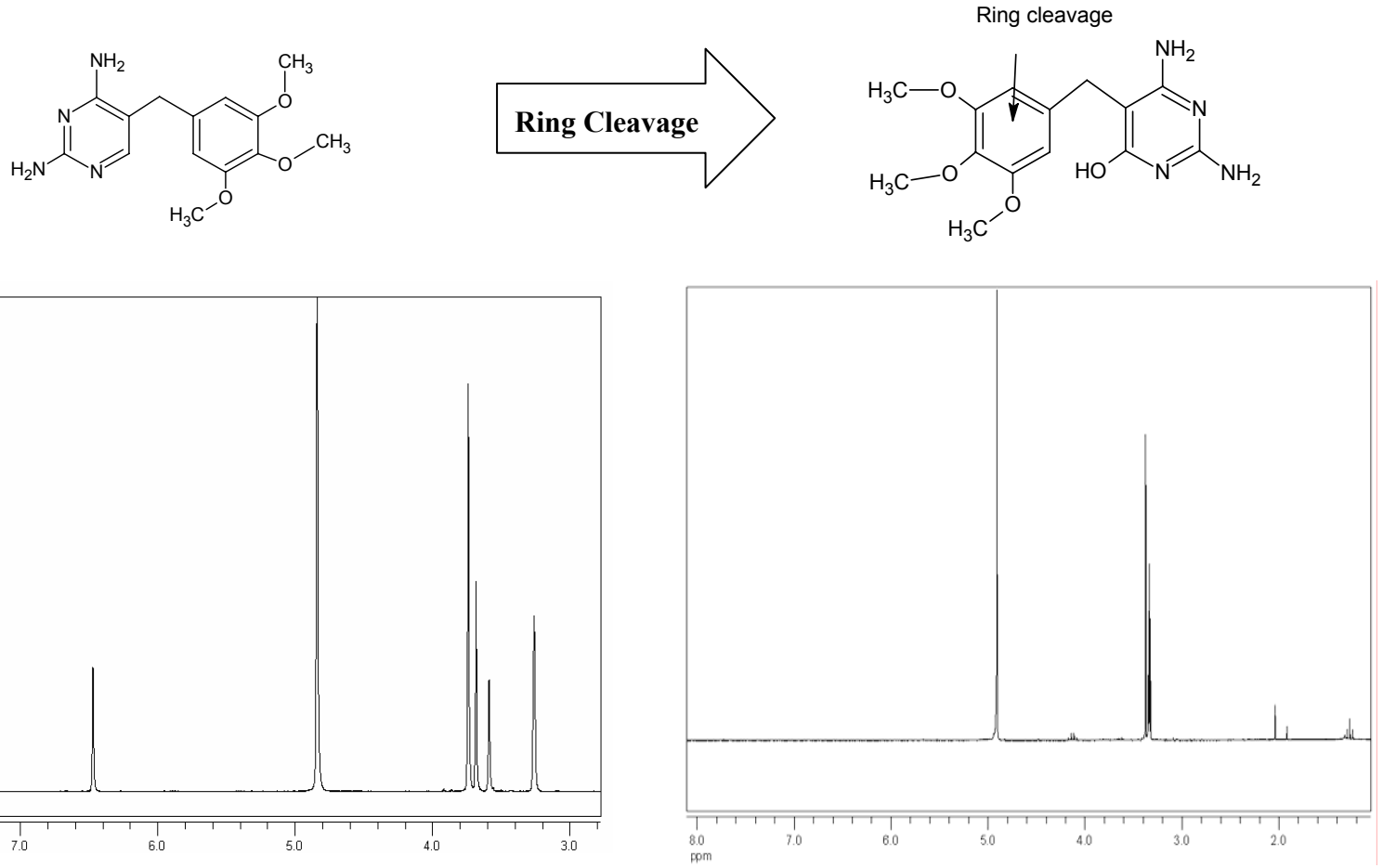
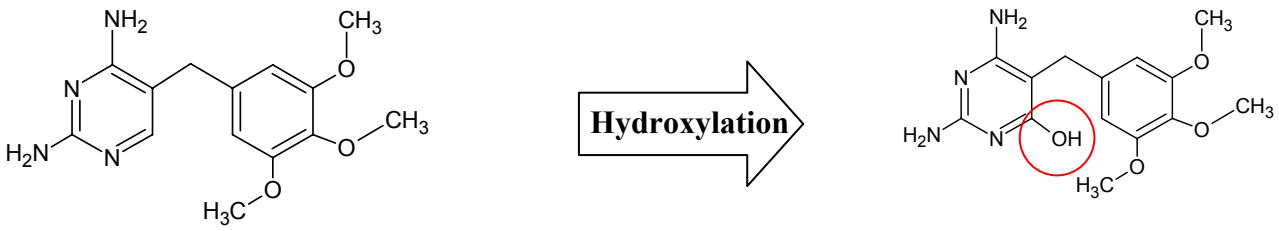


Figure G.6 1H NMR spectrum for Trimethoprim byproduct; ring cleavage



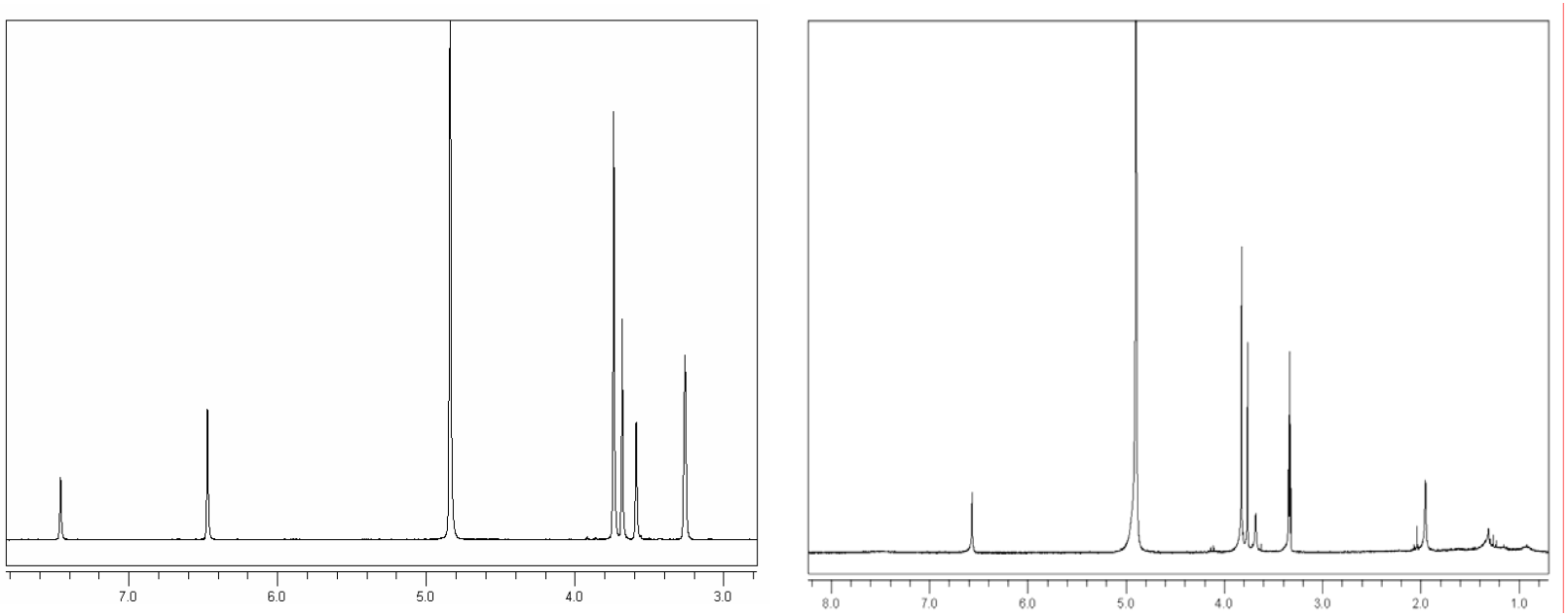


Figure G.7 1H NMR spectrum for byproduct of Trimethoprim; Hydroxylation