ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ACUTE AND CHRONIC EFFECTS OF SYNTHETIC ESTROGEN 17 ALPHA-ETHINYLESTRADIOL ON BIOLOGICAL CARBON REMOVAL PROCESSES

M.Sc. THESIS

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Department of Environmental Engineering

Environmental Science And Engineering Programme

JUNE 2012

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17 ALFA-ETİNİLESTRADİOL SENTETİK ÖSTROJEN HORMONUNUN BİYOLOJİK KARBON GİDERİMİ PROSESLERİNDEKİ AKUT VE KRONİK ETKİLERİ

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To my family,

FOREWORD

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ABBREVIATIONS

AMO :Ammonia Monooxygenase
AOB :Ammonia Oxidizing Bacteria
AS :Activated Sludge
ASM :Activated Sludge Modelling
BPA :Bisphenol A
CAO :Chemical Advanced Oxidation
COD :Chemical Oxygen Demand
E1 :Estriol
E2 :Estradiol
E3 :Estrone
EDCs :Endocrine Disrupting Compounds
EE2 :17 Alpha-Ethinylestradiol
EPA :Environmental Protection Agency
EU :European Union
FDA :Food and Drug Administration
FQPA :Food Quality Protection Act
GC :Gas-chromatography
LC/MS :Liquid Chromatography–Mass Spectrometry
Log Kow :Octanol Water Partition Coefficient
MBR :Membrane Bioreactor
MS/MS :Tandem Mass Spectrometry
NF :Nanofiltration
NP :Nonylphenol
OUR :Oxygen Uptake Rate
PHA :Polyhydroxyalkanoate
PHB :Polyhydroxybutyrate
PHV :Polyhydroxyvalerate
PPCPs :Pharmaceuticals and Personal Care Products as Pollutants
RO :Reverse Osmosis
SBR :Sequencing Batch Reactor
SDWA :Safe Drinking Water Act
SPE :Solid-Phase Extraction
SRT :Sludge Retention Time
SS :Suspended Solids
STP :Sewage Treatment Plant
STW :Sewage Treatment Works
TKN :Total Kjeldahl Nitrogen
UPLC :Ultra Performance Liquid Chromatography
UV :Ultraviolet
VSS :Volatile Suspended Solids
WHO :World Health Organization
WWTP :Wastewater Treatment Plant

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SYMBOL LIST

$\mathbf{b_H}$	**:**Endogenous decay coefficient
$\mathbf{b_{STO}}$	**:**Respiration rate for X_{STO}
$\mathbf{f_{ES}}$	**:**Fraction of soluble inert products
$\mathbf{f_{EX}}$	**:**Fraction of inert particulate metabolic products
$\mathbf{k_h}$	**:**Maximum specific hydrolysis rate for S_H
$\mathbf{K_S}$	**:**Half saturation constant of substrate
$\mathbf{k_{STO}}$	**:**Maximum storage rate
$\mathbf{K_{STO}}$	**:**Half saturation coefficient of storage
$\mathbf{K_X}$	**:**Half saturation coefficient for S_H
$\mathbf{S_{ALK}}$	**:**Alkalinity
$\mathbf{S_H}$	**:**Rapidly hydrolysable COD
$\mathbf{S_I}$	**:**Soluble inert COD
$\mathbf{S_{ND}}$	**:**Soluble biodegradable organic nitrogen
$\mathbf{S_{NH}}$	**:**Ammonia concentration
$\mathbf{S_{NO}}$	**:**Nitrate nitrogen
$\mathbf{S_O}$	**:**Dissolved oxygen concentration
$\mathbf{S_P}$	**:**Soluble inert microbial products
$\mathbf{S_s}$	**:**Readily biodegradable substrate
$\mathbf{X_{B,A}}$	**:**Autotrophic biomass
$\mathbf{X_{B,H}}$	**:**Heterotrophic biomass
$\mathbf{X_H}$	**:**Heterotrophic biomass
$\mathbf{X_{STO}}$	**:**Internal storage products
$\mathbf{X_I}$	**:**Particulate inert COD
$\mathbf{X_{NB}}$	**:**Active mass nitrogen
$\mathbf{X_{ND}}$	**:**Biodegradable organic nitrogen
$\mathbf{X_{NI}}$	**:**Inert organic particulate matter
$\mathbf{X_{NP}}$	**:**Inert organic particulate products
$\mathbf{X_P}$	**:**Inert particulate product
$\mathbf{X_S}$	**:**Slowly biodegradable substrate
$\mathbf{X_{STO}}$	**:**Storage products
$\mathbf{Y_H}$	**:**Heterotrophic yield
$\mathbf{Y_{STO}}$	**:**Storage yield
$\mathbf{\mu_H}$	**:**Maximum growth rate for heterotrophs

ACUTE AND CHRONIC EFFECTS OF SYNTHETIC ESTROGEN 17 ALPHA- ETHINYLESTRADIOL ON BIOLOGICAL CARBON REMOVAL PROCESSES

SUMMARY

The existence and persistence of estrogenic chemicals in aquatic environments is a problem that may affect public and ecosystem wellness. Estrogenic compounds are known to cause endocrine disruption in wildlife and humans, including 17 alpha-ethinylestradiol, a widely used pharmaceutical. Ethinylestradiol (or 17 alpha-ethinylestradiol) is a synthetic hormone, which is a derivative of the natural hormone estradiol. Ethinylestradiol is an orally bio-active estrogen used in almost all modern formulations of combined oral contraceptive pills and is one of the most commonly used medications. Ethinylestradiol was the first orally active synthetic steroidal estrogen, synthesized in 1938 by Hans Herloff Inhoffen and Walter Hohlweg at Schering AG in Berlin. This compound enters the environment, primarily through discharges from wastewater treatment plants without being effectively degraded. Additionally, it is believed that some of the degradation products of ethinylestradiol formed during wastewater treatment have greater endocrine disrupting potential than the parent compound.

The goal of the research presented in this thesis was to assess acute and chronic effects of the selected compound 17 alpha-ethinylestradiol (EE2) and to further confirm its biodegradation. Activated sludge taken from a domestic wastewater treatment plant in Istanbul was acclimated to synthetic peptone mixture. A 12 liters of an aerobic batch reactor with a hydraulic retention time of 1 day and a sludge age of 10 days was installed then operated with the acclimated sludge. The system was fed with synthetic peptone mixture (600 mg COD/l) during five months. Some experiments were applied in order to ensure that the aerobic reactor procures steady state conditions.

In case the reactor was in equilibrium, activated sludge was subject to respirometric studies in order to determine acute effects. The behaviour of microorganisms through the experiment was monitored. Two concentrations of EE2 (1 mg/l – 5 mg/l) were used during acute experiments. Subsequent to acute experiments, the reactor was fed during 40 days with 17 alpha-ethinylestradiol solution, in company with peptone mixture, to determine chronic effects. During chronic period, 1 mg/l of EE2 was fed to the reactor. Every 5 days, activated sludge was subject to respirometric experiment to reveal the chronic effects of EE2. A nitrification inhibitor was used to prevent interference of nitrification.

Results of acute and chronic experiments were used to monitorize oxygen uptake rate (OUR) profile and to assess the inhibitory effects of EE2. OUR profiles were determined in the presence of inhibitor. Kinetic and stoichiometric coefficients were designated by using a multi-component model (ASM3).

During chronic experiments, hormone samples were taken to obtain information about the extent of EE2 biodegradation. Solid Phase Extraction (SPE) was conducted to observe the biodegradation of EE2. Also, EE2 concentration in the aqueous phase was analyzed. These were characterized by mass spectrometric methods as liquid chromatography tandem mass spectrometry (LC/MS/MS). According to chronic experiments, it was confirmed that 17 alpha-ethinylestradiol is degraded by heterotrophic microorganisms to some extent. It was observed that EE2 is not accumulated on solid phase (sludge) in contrast decreasing effluent concentrations of EE2 in the aqueous phase demonstrate that EE2 is degraded.

17 ALFA-ETİNİLESTRADİOL SENTETİK ÖSTROJEN HORMONUNUN BİYOLOJİK KARBON GİDERİMİ PROSESLERİNDEKİ AKUT VE KRONİK ETKİLERİ

ÖZET

Kararlı yapıdaki östrojenik kimyasalların su ortamında bulunması halk sağlığı ve ekosistemin yapısı açısından problem oluşturmaktadır. Endokrin sistemi bozan kimyasallar, son on yılda bilim insanlarını ciddi ölçüde kaygılandırmaya başlamıştır. Çünkü bu maddelerin hormonları taklit ettikleri, hormonsal etkileri engelledikleri veya arttırdıkları, hayvanların ve insanların üreme sistemlerinde ölümcül etkilere neden oldukları kaydedilmiştir.

Östrojenik bileşiklerin, özellikle ilaçlarda kullanılan 17 alfa-etinilestradiol hormonunun, hayvanlarda ve insanlarda endokrin bozucu etkisi olduğu bilinmektedir. Son yıllarda, balıkların üreme organlarında anomalilerinin arttığı ve interseks olgusuna çok daha sık rastlandığı rapor edilmektedir. Bu üreme bozukluklarının kaynağının östrojenik kirleticiler; örneğin nonylphenol, 17α-ethinylestradiol ve antiandrogenik pestisitler olduğu iddia edilmektedir. Östrojen ve androjenlerin; balıklarda cinsiyet belirlenmesinde, farklılaşmasında ve büyüme süreçlerinde çok önemli etkileri olduğu bilinmektedir.

Çevreye yayılan zehirlerin canlılara verdiği zararların başında, endokrin sistem (iç salgı bezleri) bozuklukları gelir. Bu bileşikler, canlılarda metabolizma sırasında üretilen endokrin sistemi hormonlarının tesirini maskeleyen veya onlar gibi davranarak fonksiyon gören, çevre ortamında (hava, gıda, su, toprak vs.) bulunan tabiî ve sentetik biyoaktif maddelerdir. Etinilestradiol (ya da 17 alfa-etinilestradiol, EE2) sentetik bir hormon olmakla birlikle doğal bir hormon olan estradiol hormonunun bir türevidir. Biyoaktif bir hormon olan ethinylestradiol tıp alanında takriben tüm modern formülasyonlarda, ağız yoluyla alınan ilaçlarda ve birçok ilaç tedavisinde kullanılmaktadır. Etinilestradiol, ilk olarak 1938 yılında Berlin'de Hans Herloff Inhoffen ve Walter Hohlwed tarafından sentezlenen sentetik steroid bir östrojen hormonudur. Bu hormon, tam olarak parçalanamadan atıksu arıtma tesislerinden çevreye ulaşan bir bileşiktir. Ayrıca, atıksu arıtımı sırasında bu bileşiğin bazı parçalanma ürünleri oluşmaktadır ve bu parçalanma ürünlerinin daha da fazla endokrin bozucu potansiyeli olduğuna inanılmaktadır.

Bu tez çalışmasında sunulan araştırmanın amacı, seçilen bir sentetik östrojen hormonunun (17 alfa-etinilestradiol, EE2) aktif çamur sistemlerine olan akut ve kronik etkilerini değerlendirmek ve biyolojik arıtılabilirliğini incelemektir. İstanbul sınırları içerisinde bir atıksu arıtma tesisinden alınan aktif çamur, sentetik pepton çözeltisine alıştırılmıştır. Bu sentetik atıksu ile laboratuar koşullarında 12 litre hacminde bir reaktör kurulmuştur. Kurulan reaktörün çamur yaşı 10 gün ve hidrolik bekletme süresi 1 gün olarak seçilmiştir. Reaktör içerisindeki tam karışım hava taşları ve mekanik bir karıştırıcı yardımıyla sağlanmıştır. Reaktör tam karışımlı, kesikli bir biyolojik reaktördür.

Reaktör alıştırma süreci boyunca, sistem her gün 600 mg KOİ/l organik yük olacak şekilde sentetik pepton çözeltisi ve bunun yanında mikro-makro nütrientler ile beslenmiştir. Alıştırma süreci, sıcaklık gibi laboratuar koşullarına bağlı olarak yaklaşık 5 ay sürmüştür.

5 ay boyunca biyolojik reaktörün denge koşullarına gelmesi beklenmiştir. Reaktör bu süreçte düzenli olarak alınan numune, ölçülen konvansiyonel parametre ve yapılan deneylerle izlenmiştir. Reaktör izleme süreci boyunca; sıcaklık, pH, askıda katı madde (AKM), uçucu askıda katı madde (UAKM) ve kimyasal oksijen ihtiyacı (KOİ) gibi konvansiyonel izleme parametrelerine bakılmıştır. İzleme süresince reaktör içerisindeki KOİ giderim verimi % 94 e ulaşmıştır. Reaktör içerisindeki UAKM miktarı 2000 mg/l, pH ise 7.0-7.5 mertebesinde tutulmuştur. Ayrıca, reaktör F/M oranı sürekli sabit olacak şekilde 0.30 alınmıştır.

Reaktörün denge koşullarını sağlaması ile birlikte ilk olarak bir kontrol deneyi ve ardından akut deneyler gerçekleştirilmiştir. Akut deneyler sırasında, 17 alfa-etinilestradiol östrojen hormonu iki farklı doz olarak uygulanmıştır. Bu dozlar 1 mg/l EE2 ve 5 mg/l EE2 dir. Farklı dozlarda EE2 verilen aktif çamur, respirometrik deneye tabi tutulmuştur. Akut deneyler boyunca respirometreye EE2 ile birlikte 360 mg KOİ/l olacak şekilde pepton çözeltisi de karbon kaynağı olarak eklenmiştir. Akut deneyler boyunca F/M oranı, reaktör F/M oranı ile aynı olacak şekilde (0.30) ayarlanmıştır. Deneyler, EE2 hormonun karbon giderimi üzerine olan akut etkisi ile ilgili olmasından dolayı nitrifikasyon inhibitörü kullanılmıştır. Akut deneyler sırasında belirli aralıklarla KOİ ve PHA numuneleri alınmış, AKM-UAKM deneyleri yapılmış, pH-sıcaklık takip edilmiştir. Ayrıca deney sonunda hormon numunesi de alınmış ancak EE2 hormonun aktif çamura anlık olarak beslenmesi sonucunda sıvı ya da katı fazda birikmiş hormon konsantrasyonuna rastlanmamıştır. Aynı zamanda, alınan PHA numunelerinde sisteme anlık EE2 verilmesi durumunda depolama etkisi görülmemiştir. Yapılan respirometre deneyleri sonunda, EE2 hormonunun akut etkisini gösteren oksijen tüketim hızı profili elde edilmiştir. Bu profile göre, farklı iki dozda uygulanan EE2 hormonunun yüksek dozda uygulanması durumunda maksimum oksijen tüketim hızında bir düşüş olduğu ve bunun sebebinin inhibisyon etkisi olduğu sonucuna varılmıştır.

Akut deneylerin ardından, pepton çözeltisine alıştırılmış aktif çamur 40 gün boyunca EE2 hormonu ve beraberinde sentetik pepton çözeltisi ile beslenmeye başlamıştır. Bu süreç, kronik periyot olarak adlandırılmıştır. Kronik periyot boyunca, reaktör her gün 1 mg/l olacak şekilde EE2 hormonu ve 600 mg KOİ/l olacak şekilde sentetik pepton çözeltisi ile beslenerek aktif çamur EE2 hormonuna alıştırılmıştır. Kronik periyot boyunca belirli günlerde reaktör içerisinden KOİ, PHA ve hormon numuneleri alınmış, düzenli olarak AKM-UAKM ve pH bakılarak reaktör izlenmiştir.

Reaktör pH sı 6.5 ile 8.0 arasında, UAKM değeri ise 2000 mg/l civarında tutulmuştur. KOİ giderim verimi ise % 95 olmuştur. Biyokütlenin depolama kapasitesine bakıldığında ise reaktöre beslenen toplam KOİ miktarının kronik periyot boyunca ancak % 6 sının biyokütle tarafından depolanabildiği sonucuna varılmıştır. Bu durumda depolama mekanizmasının sistem üzerinde önemli derecede bir etkisinin olmadığı söylenebilmektedir.

Kronik periyot boyunca her 5 günde bir respirometre deneyleri yapılarak aktif çamurun EE2 hormonuna verdiği tepki gözlenmiştir. Kronik deneyler boyunca respirometreye EE2 ile birlikte 360 mg KOİ/l olacak şekilde sentetik pepton çözeltisi de karbon kaynağı olarak eklenmiştir.

Kronik deneylerde F/M oranı, reaktör F/M oranı ile aynı olacak şekilde (0.30) ayarlanmıştır. Deneylerin, EE2 hormonun karbon giderimi üzerine olan kronik etkisi ile ilgili olmasından dolayı nitrifikasyon inhibitörü kullanılmıştır. Kronik deneyler sırasında belirli aralıklarla KOİ, PHA numuneleri alınmış, AKM-UAKM deneyleri yapılmış ve pH-sıcaklık takip edilmiştir. Her deney için oksijen tüketim hızı profilleri elde edilmiştir.

Deneyler sonucunda elde edilen oksijen tüketim hızı ve PHA depolama ürünü verileri, Aquasim olarak adlandırılan bir bilgisayar programı aracılığı ile modifiye edilmiş ASM3 modeline uygun olarak modellenmiştir.

Model kullanmanın amacı, ilgili stokiyometrik ve kinetik katsayıların belirlenmesidir. Bu tez çalışmasında kronik periyota ait kontrol, 1.gün ve 40.gün deneyleri modellenmiştir. Kronik kontrol ve 1.gün deneylerine ait kinetik katsayıların aynı olduğu ve değişmediği görülmüştür. Buna göre, kronik periyota ait ilk günler için EE2 hormonunun mikrobiyal kinetik üzerinde herhangi bir etkisinin olmadığı sonucuna varılmıştır. Oysa ki 40.gün deneyine ait model sonuçlarına bakıldığında, EE2 hormonun sistem üzerinde uyarıcı bir etkisinin olduğu açıkça görülmektedir. Bu etki sonucunda, artan enzim aktivitesi ile birlikte maksimum büyüme ve hidroliz hızlarında artış olduğu sonucuna varılmaktadır. Substratın az bir kısmı depolanmaya devam etmektedir. PHA depolama ürünü sonuçlarına bakıldığına, bu tip bir sistemde depolama mekanizmasının az miktarda etkili olduğu sonucuna varılmaktadır.

Kronik deneyler boyunca hormon numunesi de alınmıştır. Alınan hormon numunelerinden sıvı ve katı faz ölçümleri gerçekleştirilmiştir. Çamur numuneleri katı faz ekstraksiyonuna tabi tutulmuştur. Buradaki amaç; EE2 hormonunun katı fazdaki konsantrasyonlarının belirlenmesidir. Analiz sonuçlarından, EE2 hormonunun biyolojik arıtılabilirlik mertebesi belirlenmiştir.

Katı faz ekstraksiyonu sırasında katı fazda biriktiğine inanılan hidrofobik yapıdaki EE2 hormonunun sıvı faza geçirilerek kütle spektrometrik bir metot olan likit kromatografi tandem kütle spektrometrisi (LC-MS/MS) ile ölçülmesidir. Tüm numuneler Ultra Performans Likit Kromatografisi (UPLC) cihazı ile ölçülmüştür. Yapılan hormon ölçümlerinden elde edilen verilere göre, EE2 hormonunun hidrofobik yapısına rağmen çamur (katı faz) fazında önemli miktarda hormon konsantrasyonu görülmemiştir. EE2 konsantrasyonuna daha çok çıkış suyunda (sıvı faz) çözünmüş halde rastlanmıştır.

Karbon giderimi proseslerinde, heterotrof bakterilerin arıtma sürecinde görevli oldukları bilim dünyası tarafından ifade edilmiştir. Doğal hormonlara göre biyolojik olarak parçalanabilirliği zor olan sentetik östrojen hormonu EE2 nin, bilimsel araştırmalarda yapılan tür analizlerine göre bir grup heterotrof bakteri tarafından parçalanabildiği bilinmektedir.

Buna göre bu çalışma için, karbon gideren bir sistemde EE2 hormonunun katı fazda adsorbe olmak yerine karbon kaynağı yerine kullanılarak bir kısmının biyolojik olarak parçalandığı ve bir kısmının da sıvı fazda çözünmüş halde kaldığı görülmektedir. Sonuç olarak, karbon gideren biyolojik bir sisteme EE2 arıtılabilirlik verimi açısından bakıldığında yaklaşık % 84 giderim verimi olduğu sonucuna varılabilmektedir.

1. INTRODUCTION

There has been increasing concern over the past ten years regarding the occurrence and fate of low-level concentrations of pharmaceuticals, hormones, and other organic contaminants in the aquatic environment. Several studies have demonstrated some evidence that pharmaceutical substances are often not eliminated during wastewater treatment and additionally are not biodegraded in the environment. Within the various pharmaceutical categories, particular attention is being focused on hormones and endocrine disrupting substances. (O'Grady, 2007).

The aim of this study is to investigate acute and chronic effects of a synthetic estrogen 17 alpha-ethinylestradiol, referred to as EE2 and to confirm its biodegradability. Besides, respirometric method was applied. Results were used in modelling. A software, named Aquasim was used to model chronic effects of EE2 and kinetic coefficients were determined by using a multi-component model ASM3.

1.1 Purpose of Thesis

During this study, activated sludge taken from a biological treatment plant in Istanbul, was used to evaluate the biodegradability, acute and chronic effects of a selected endocrine disrupting compound named 17 alpha-ethinylestradiol (EE2). An aerobic batch reactor was installed and operated at a sludge age of 10 days. Activated sludge acclimated to peptone synthetic wastewater, having similar characteristics of domestic sewage was fed with peptone mixture. Respirometric studies were conducted to monitor the acclimation period and the behavior of EE2 to wastewater. In pursuit of respirometric experiments, some other parameters were also measured; such as COD, SS, VSS, pH and PHAs. Furthermore, EE2 concentrations in aqueous and solid phase were determined by using SPE (solid phase extraction) and analytical measurements by using LC/MS/MS analysis. Consequently, degradation efficiency for the synthetic estrogen EE2 was determined. Kinetic and stoichiometric coefficients for selected selected chronic experiments were also determined according to a multi-component model (ASM3) by using the Aquasim Software.

2. LITERATURE REVIEW

2.1 Endocrine disrupting compounds (EDCs)

In recent years, a group of xenobiotic compounds denominated endocrine disrupting compounds (EDCs) have been investigated due to their adverse effects in animals and humans inhibiting the normal action of the endocrine system (Cases et al., 2011).

According to Cargouet et al. (2004), endocrine disrupting compounds (EDCs) are a newly defined category of environmental contaminants that interfere with the function of the endocrine system.

The European Commission (1996) defined an EDC as an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine functions (Cases et al., 2011).

EDCs are a wide variety of both natural and man-made chemicals which typically exert effects, either directly or indirectly, through receptor mediated processes mimicking endogenous hormones by inhibiting the normal hormonal activities (Kumar and Mohan, 2011).

EDCs interfere with the endocrine system and may alter diverse physiological functions including reproduction and development in different species, including humans (Combalbert et al., 2010).

Due to the widespread presence in the environment and endocrine activity even at low concentrations e.g. as low as 0.1 ng/l of 17alpha-ethinylestradiol (EE2) that induces vitelogenesis in male rainbow trout. By mimicking natural hormones or disrupting signal pathways as endocrine disrupters, estrogens can stimulate the growth of human breast cancer cells or induce the expression of vitellogenin in fish; both mechanisms are used to prove estrogen activity in bioassays (Catjhmal et al., 2009).

Bolong et al. (2009) discussed the issues associated to EDCs to highlight the challenges in tackling the problems. The first issue is that of nonexistence of limiting regulations, especially for new compounds, by-products, pharmaceuticals and PPCPs as related to the water and wastewater treatment industry. None or very few precautions and monitoring were taken to ensure these unregulated or new compounds and by-products, specifically the micro-range pollutants, from being released to water sources. Yet some actions have been actively taken; for instance, by the European Commission that has developed strategies to deal urgently with endocrine disrupters. One of them is the amendment of the European Community on Risk Assessment and Directive on the classification of dangerous substances, whilst in the US no maximum limit of these substances in drinking or natural waters has been regulated.

However, the Food and Drug Administration (FDA) does require ecological testing and evaluation of pharmaceuticals when environmental concentration exceeds 1 μg/l. In 1996, the Food Quality Protection Act (FQPA) and amendments to the Safe Drinking Water Act (SDWA) have authorized the US Environmental Protection Agency (US EPA) to screen all chemicals and formulation on any potential endocrine activity in manufacturing or processing where drinking water and/or food supply line could be contaminated (Bolong et al., 2009).

This small sample of regulatory practices indicates that there is no coordinated ordinance that is accepted by the global community and nations.

Moreover, limits and regulation on pharmaceuticals and personal care products and new compounds have not yet specifically been made for water and wastewater treatment criteria.

The second issue of concern is that EDCs are comprised of an extensive and expanding spectrum of compounds. This is not surprising as the endocrine system has a complex function and involves a variety of compounds, as documented by various worldwide organisations including the World Health Organisation (WHO), the European Union (EU), US EPA, to name a few. These organizations have developed their own characterization lists and acceptable ranges of endocrine disrupters.

For example, the EDC priority list was first reported by the EU–Strategy for Endocrine Disrupters committee for 66 chemicals (including 60 that are considered likely to be exposed to humans). Then, a further 52 chemicals were identified and recently the total list included 564 chemicals. However, out of this total, 147 compounds are likely to be persistent in the environment (Bolong et al., 2009).

As the knowledge of endocrine disrupters increases, so does the list of chemicals that exhibits these endocrine disrupting properties. This means that more substances will be identified as endocrine disrupters as the number of chemicals increases. This will necessitate identification and removal of these compounds from the water system. Furthermore, removal of these compounds from the wastewater treatment process has not necessarily been effective due to their relatively low concentrations and the associated difficulty in analysis. The problem seems to be continuing and thus we need to upgrade the existing water and wastewater treatment system to cater to and solve these newly unregulated pollutants (Bolong et al., 2009).

Thirdly, these compounds are different in their form and mechanism of actions. Thus, the identification and evaluation of these compounds from the environmental matrixes have provided a unique challenge. This made the measurement and detection of EDCs difficult, for they sometimes include biological and instrumental methods. The accuracy of the determination methods is also still debated and progressively under research. In relation to measurement and detection of EDCs in water and wastewaters, some of the problems associated are listed. Detection of EDC compounds in water is at trace levels (μg/l or even ng/l); most analytical instruments are unable to directly detect compounds at these low levels.

Usually, extraction is used to concentrate the target compounds. However, this method has a limitation for the amount of contaminant subjected to the analysis that can be reduced. For instance, in the case of solid phase extraction, water samples are passed through a cartridge that is then dried by passing nitrogen or air (Bolong et al., 2009).

This is further followed by an elution process using a solvent. Such a series of processes of extraction can be detrimental for certain types of instrumental analysis. EDCs have a broad range of physiochemical characteristics; there is no standard or common method for EDC monitoring.

Each compound requires specific analysis by different techniques. Improved and advanced analytical and bioanalytical technologies that enable the detection of more xenobiotics at an even lower range of concentrations are required. The low level pollutants in complex matrices such as sludge and wastewater are difficult to analyze and could seriously affect their extraction and analysis. A highly sensitive measurement is essential. Thus, the development of a rapid, simple and low-cost procedure for detection of EDCs, specifically for their estrogenic activity in wastewater samples, is still a growing and interesting research area (Bolong et al., 2009).

2.1.1 Types and sources of EDCs

According to Bolong et al. (2009), EDCs comprise pharmaceuticals, personal care products, surfactants, various industrial additives and numerous chemicals purported to be endocrine disrupter.

EDCs include natural estrogens produced in humans and animals, such as estrone (E1), 17β-estradiol (E2), and estriol (E3); natural androgens such as testosterone (T), dihydrotestosterone (DHT), and androsterone (A); artificial synthetic estrogens or androgens used in medicine (e.g., birth-control drugs), such as ethinylestradiol (EE2), Norgestrel (N), and Trenbolone (Tr); phytoestrogens including isoflavonoides and coumestrol as well as other industrial compounds such as bisphenol A, nonylphenol. Such chemicals have been found existing in wastewater, surface waters, sediments, groundwater, and even drinking water (Liu et al., 2009; Sim et al., 2011).

Wastewater treatment plants, livestock farms, hospitals and pharmaceutical manufactures have been studied as a major source for EDCs and estrogens are discharged to sewer from human and animal sources in the conjugated form as sulphates or gluconarides. Especially, sewage and livestock wastewater are major pathways of estrogens in the aquatic environment (McAdam et al., 2010; Sim et al., 2011).

Wastewater treatment plants (WWTPs) receive a large spectrum of molecules from domestic and/or industrial waste, which are not totally eliminated during the treatment processes.

At the outlets of the WWTPs, a complex mixture of molecules including the partially eliminated wastewater molecules but also metabolites formed during treatment processes are finally discharged into the rivers. In this context, WWTP discharges are considered as a major source of estrogenic surface water pollution that may play a significant role in environmental contamination (Cargouet et al., 2004).

2.2 Estrogens

Estrogens are known to be a group of steroid hormones with high potential of endocrine disruption of organisms in the aquatic ecosystem (Sim et al., 2011).

2.2.1 Types and sources of estrogens

Most of emerging pollutants are generated from anthropogenic sources, while estrogens have both natural and synthetic sources. Natural estrogens (e.g., estrone, 17 β-estradiol and estriol) are produced in humans and animals, and synthetic compounds (e.g., 17 alpha-ethinylestradiol) are used in medicine (e.g., birth-control drugs) (Sim et al., 2011).

Estrogenic hormones are the most endocrine disrupting chemicals because the disrupting potency can be several thousand times higher than other chemicals such as nonylphenol. EDCs comprise pharmaceuticals, personal care products, surfactants, various industrial additives and numerous chemicals purported to be endocrine disrupter (Bolong et al., 2009).

The pollution of the aquatic environment by EDCs has become a major concern due to increasing evidences by which exposure to EDCs was linked to their reproductive and health effects on humans and other living things (Table 2.1). These are because the water resources always act as a sink for many types of pollution. Thus the aquatic environment (streams, rivers, marine and even groundwater) becomes susceptible to the effects of most contaminants.

EDCs enter the environment, specifically into the receiving waters, through a variety of pathways that can be categorised as point source (such as municipal sewage, industrial wastewaters, landfill) and nonpoint source (such as agricultural run-off, washoff from roadways, underground contamination) (Figure 2.1).

Most of the previous research concentrates on point source pollution, especially discharges of EDCs via sewage treatment. This is because one of the main sources of these contaminants comes from untreated wastewater and WWTP effluents. Most of current WWTPs are not designed to treat these types of substance and a high portion of emerging compounds and their metabolites can escape and enter the environment via sewage effluents. Thus, it is obvious that the development of more advanced technologies may be crucial to fulfill the requirements (Bolong et al., 2009).

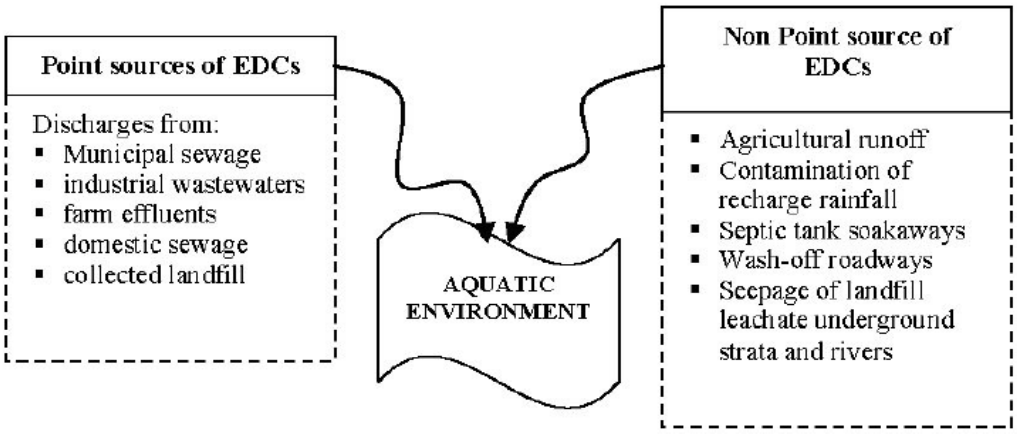

Figure 2.1 : Sources of EDCs in the environment, adapted from (Bolong et al., 2009).

Table 2.1 : Environmental effects of EDCs, adapted from (Bolong et al., 2009).

Endocrine disrupting compounds	**Effects**
Estrone and 17β-estradiol (steroidal estrogens) and 17α-ethinylestradiol (synthetic contraceptive) – contained in contraceptive pills	Cause feminization which observed for fish in sewage treatment. The discharge causes mimicking estrogen/hormone effect to non-target
Antibiotics (such as penicillin, sulfonamides, tetracylines)	Shown to cause resistance among bacterial pathogens that lead to altered microbial community structure in the nature and affect higher food chain
Phthalates – used as plasticizers in plastic, PVC baby toys, flooring	Exposure to high levels reported to cause miscarriage and pregnancy complication

2.2.2 Physicochemical properties of estrogens

All estrogens have very low vapor pressures indicating low volatility of these compounds.

Synthetic steroids have higher log Kow (octanol–water partition coefficient) values, 4.15 for EE2 and 4.67 for mestranol. In chemistry and the pharmaceutical sciences, a partition (P) or distribution coefficient (D) is the ratio of concentrations of a compound in the two phases of a mixture oftwo immiscible solvents at equilibrium. The terms "gas/liquid partition coefficient" and "air/water partition coefficient" are sometimes used for dimensionless forms of the Henry's law constant. Hence these coefficients are a measure of differential solubility of the compound between these two solvents. Normally one of the solvents chosen is water while the second is hydrophobic such as octanol. Hence both the partition and distribution coefficient are measures of how hydrophilic ("water loving") or hydrophobic ("water fearing") a chemical substance is (Url-2). Thus, from the physicochemical properties of steroids indicated above, it can be seen that estrogens are hydrophobic organic compounds of low volatility. It is expected that the sorption on soil or sediment will be a significant factor in reducing aqueous phase concentrations (Ying et al., 2002).

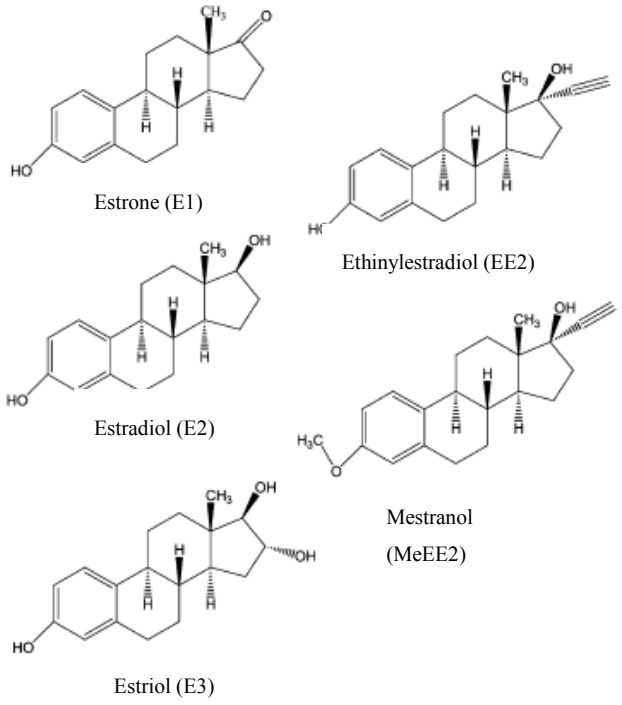

Figure 2.2 : Structures of hormone steroids, adapted from (Ying et al., 2002; Url-1).

Table 2.2 : Physicochemical properties of steroids, adapted from (Ying et al., 2002).

Chemical name	Molecular weight	Water solubility (mg/l at 25 ^{0}C)	log Kow
Estrone (E1)	270.4	1.30±0.08	3.43
17β-estradiol (E2)	272.4	1.51±0.04	3.94
Estriol (E3)	288.4	-	2.81
17α-ethinylestradiol (EE2)	296.4	9.20±0.09	4.15

2.2.3 Occurrence and fate of estrogens in the environment and sewage sludge

In the aquatic environment estrogens may be subject to biotransformation and bioconcentration leading to complex environmental health issues. Estrogens are discharged to sewer from human sources in the conjugated form as sulphates or gluconarides. Whilst significant reductions in their concentration occur within the sewage treatment works (STWs), secondary biological treatment of wastewater, as presently configured and operated, cannot afford adequate protection of the aquatic environment; consequently effluent discharges are major sources of these anthropogenic chemicals to the aquatic environment (Mc Adam et al., 2010; Ying et al., 2002).

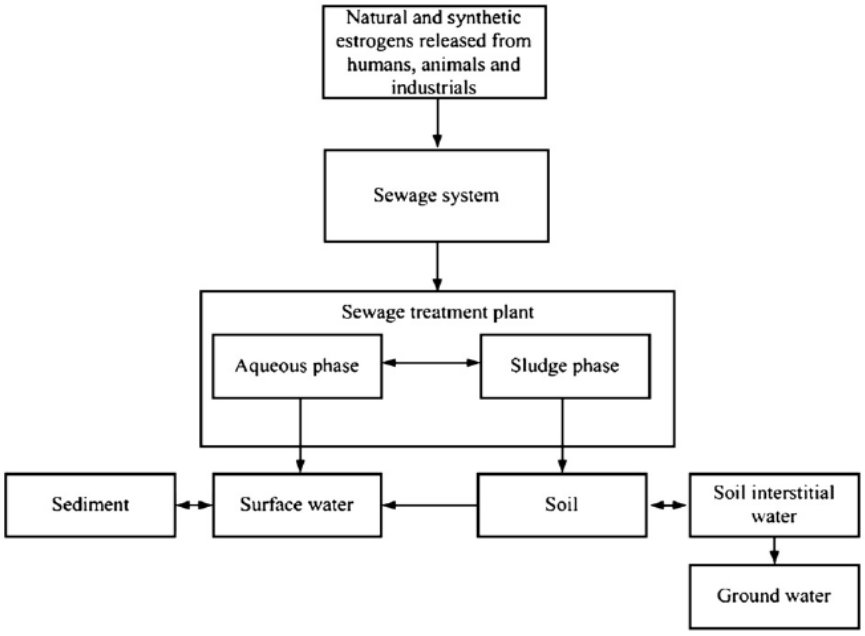

Figure 2.3 : Distribution of EDCs in the environment, adapted from (Liu et al., 2009).

Hormone steroids in the environment may affect not only wildlife and humans but also plants. Plants irrigated with sewage effluent, which contained hormone steroids, was observed to have elevated levels of phytoestrogens and the daily excretion of EE2 in the contraceptive pills was estimated as 35 μg/day (Ying et al., 2002).

Table 2.3 : Concentration of hormones in effluents of STPs, adapted from (Ying et al., 2002).

Location	Concentration (ng/l)			
	Estrone	17 β-Estradiol	Estriol	17 α-Ethinylestradiol (EE2)
Italy	2.5-82.1 (9.3)	0.44-3.3 (1.0)	0.43-18 (1.3)	<LOD-1.7 (0.45)
Netherlands	<0.4-47 (4.5)	<0.1-5.0 (<LOD)[b]	-	<0.2-7.5 (<LOD)
Germany	<LOD-70 (9)	<LOD-3 (<LOD)	-	<LOD-15 (1)
Canada	<LOD-48 (3)	<LOD – 64 (6)	-	<LOD-42 (9)
UK	1.4-76 (9.9)	2.7-48(6.9)	-	<LOD-7 (<LOD)
Japan	-	3.2-55 (14)[c] <LOD-43 (13)[d] 0.3-30 (14)[e]	-	-
USA	-	0.477-3.66 (0.9)	-	<LOD-0.759 (0.248)
Germany	<0.1-18 (1.5)	<0.15-5.2 (0.4)	-	<0.10-8.9 (0.7)

[a] Concentration range and median in parantheses.
[b] LOD=limit of detection.
[c] Summer sampling.
[d] Autumn sampling.
[e] Winter sampling.

The other major source of hormone steroids is livestock waste. Livestock such as sheep, cattle, pigs and poultry, as well as other animals, excrete hormone steroids. There are also some reports on the levels of estrogenic steroids in surface waters (Table 2.4). Recent studies have shown that disposal of animal manure to agricultural land could lead to movement of estrogenic steroids into surface and ground water (Ying et al., 2002).

Table 2.4 : Concentration of hormone steroids in surface waters, adapted from (Ying et al., 2002).

Location	Sample type	Concentration (ng/l)			
		Estrone	17 β-Estradiol	Estriol	17 α-Ethinylestradiol (EE2)
Japan	109 major rivers	-	<LOD-27 (2.1)[b,c,d] <LOD-24 (1.8)[b,e]	-	-
Germany	river water	0.10-4.1 (0.40)	0.15-3.6 (0.3)	-	0.10-5.1 (0.4)
Italy	Tiber river water	1.5	0.11	0.33	0.04
The Netherlands	Coastal/estuarine water and rivers (11 locations)	<0.1-3.4 (0.3)	<0.3-5.5 (<0.3)		<0.1-4.3 (<0.1)

[a] Concentration range and median in parantheses.
[b] Arithmetic mean (±standard deviation) in parentheses.
[c] LOD=limit of detection.
[d] Summer sampling.
[e] Autumn sampling.

2.3 Treatment alternatives of estrogens

2.3.1 Removal of estrogens by physicochemical treatment methods

The streoid estrogens are non-volatile with very low values of Henry's constant, so volatilisation is not a significant removal method for estrogens. Removal of organic compounds by sorption is dependent on the partitioning behaviour of the organic pollutant between the sludge or biofilm solids and the liquid phase. The partition coefficient is dependent on the organic content of the sludge and on the degree of hydrophobicity as measured by K_{ow} the octanol-water partition coefficient.

The steroid estrogens have log K_{ow} values of 3.43, 3.94 and 4.15 for E1, E2 and EE2 respectively, this makes them moderate to strongly adsorbable onto organic solids (Kanda and Churchley, 2008).

Some research results indicated that adsorption by activated carbon was effective for removing some estrogens. An activated carbon adsorption system is advantageous in terms of hydrophobic interactions in eliminating most organic compounds.

It has proven that sorption by powdered activated carbon and granular activated carbon was more efficient than coagulation, even in a hybrid system with nanofiltration membranes (Bolong et al., 2009).

Physicochemical treatment as a coagulation-flocculation process was generally found to be unable to remove EDCs. Chemical treatment such as coagulation, flocculation or lime softening shows ineffective removal for EDCs (Bolong et al., 2009).

In recent years, research on EDC removal by the membrane process has greatly increased. Studies have discovered that the rejection efficiency EDCs by membranes strongly depended on EDCs' physicochemical properties, such as molecular weight, K_{ow}, water solubility and so on (Liu et al., 2009).

Table 2.5 : Physicochemical properties of EDCs studied for membrane processes and their corresponding rejection, adapted from (Liu et al., 2009).

Compounds	Molecular weight (g/mol)	Water solubility (mg/l)	$\log K_{ow}$	Rejection (%)
Estrone (E1)	270.4	30	3.13	42-44
Estradiol (E2)	272.4	3.6	4.01	8-40
Estriol (E3)	288.4	441	2.45	38
17α-ethinylestradiol (EE2)	296.4	11	3.67	34-60

From Table 2.5, we see that EDCs rejection by the membrane processes has a very wide range, from 10 % to greater than 99.9 %. The reason for this is that apart from the EDCs' physicochemical properties, the rejection has a strong direct relationship with membrane types. In comparing membrane types, EDCs rejection rate by reverse osmosis is the highest, followed by nano-membrane types, then ultra-membranes, with the rejection of micro-membranes as the lowest (Liu et al., 2009).

2.3.2 Removal of estrogens by biological treatment methods

Conventional WWTPs are designed for the elimination of nutrients and solids; nevertheless, these treatment systems are only partially successful in removing estrogens from wastewater (Vega et al., 2010).

Biological treatments are efficient to remove estrogens from the dissolved phase, with removal rate around 90 % (Giraud et al., 2010).

Excreted estrogens are primarily removed from wastewater in an activated sludge system either by sorption or biodegradation as illustrated in Figure 2.4.

Although sorption occurs quickly, biodegradation is the primary removal means for estrogens in wastewater (Racz, 2010).

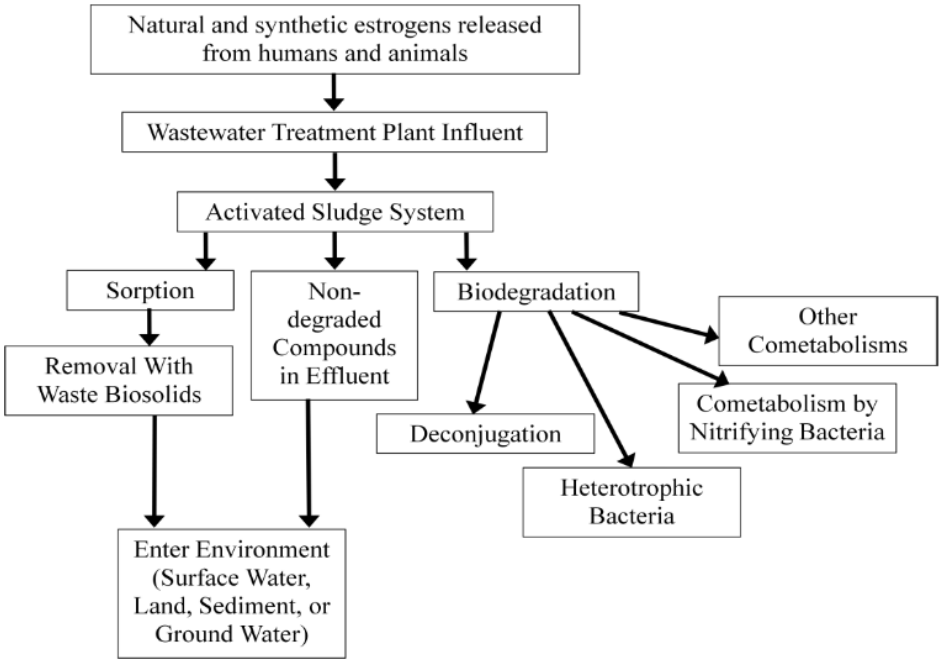

Figure 2.4 : Fate of estrogens in a wastewater treatment plant and in the environment, adapted from (Racz, 2010).

In activated sludge, E2 can be removed 44 % to 99.9 %, E3 can be removed 18 % to almost 100 %, E1 can be removed up to 98 %, and EE2 removal efficiencies vary from 34 % to almost 100 % (Racz, 2010).

Variations in wastewater treatment processes and operational conditions are generally regarded as the reason for fluctuations in removal efficiencies and effluent concentrations (Liu et al. 2009).

In particular, natural estrogens are poorly removed in highly loaded plants. Furthermore, plants with SRTs longer than 10 days tend to achieve better estrogen removal. Plants with good nitrification, which require long SRTs, also demonstrate better estrogen removal (Racz, 2010).

Sewage treatment system such as activated sludge and biological trickling filters can rapidly convert aqueous organic compounds into biomass that is then separated from the aqueous phase by settlement (clarifiers). Unfortunately, not all compounds such as steroid estrogens are completely broken down or converted to biomass (Bolong et al., 2009).

Additionally, although best available technology is adopted, biological treatment removes only a part of a wide range of emerging contaminants, particularly polar ones which are discharged via the final effluent. The limiting stage for removal was the transfer of substances from water phase to sludge phase (Bolong et al., 2009).

The preferred condition for the removal in the activated sludge was in the acidic conditions to ensure the transfer by adsorption of substances from water phase into sludge phase, and not by biodegradation. Similarly, less than 10 % of natural and synthetic estrogens are removed via biodegradation process, and although a considerable amount is adsorbed to the sludge, most of the compounds remain soluble in the effluent (Bolong et al., 2009).

On the other hand, it was observed that steroid estrogens were removed in the activated sludge, the degree of removal being consistent with their hydrophobicity and most removal involved adsorption to the organic-rich solid phase as it was not easily biodegraded. Biodegradation processes such as in the trickling filter case studied in Canada and Brazil were found incapable to remove estrogens due to their low SRT and HRT properties since this treatment method applies solid contact and attached growth process (Bolong et al., 2009).

Thus, suggestions were made toward biological treatment with longer HRT and SRT, which could increase the extent of the removal of the compounds. Similarly, it was pointed out that low effluent wastewater treatment plant concentration could be achieved at operating SRT higher than 10 days (Bolong et al., 2009).

Nitrification degree was also shown to affect biological treatment system and has potential on estrogens removal. This is an indication of an improve biological diversity and growth conditions which could increase biological transformation and thus lead to higher removal of the compounds (especially organics) (Bolong et al., 2009).

It was also found that sludges that failed to nitrify also significantly failed to degrade ethinylestradiol. It was examined sludge under both conditions (nitrifying and non-nitrifying) and found no degradation of ethinylestradiol at non-nitrifying environment, whereas at nitrifying conditions, ethinylestradiol was found to be oxidized to a more hydrophobic compound (Bolong et al., 2009).

The nitrification degree in biological treatment, however, depends on many factors such as pH, oxygen, temperature, etc., to ensure the growth of nitrifying bacteria (Bolong et al., 2009; Liu et al., 2009; McAdam et al., 2010).

As early as 1999, EDCs removal by activated sludge process was studied in Germany, Canada and Brazil. The removal efficiency for E1, E2 and EE2 was 83 %, 99.9 % and 78 %, respectively (Ternes et al., 1999).

In 2003, 20 wastewater treatment plants with no biological unit (chemical precipitation), activated sludge process and trickling filter were studied using in Sweden. Results denoted that the activated sludge process got the highest estrogenic removal, and trickling filters were better than chemical precipitation. The corresponding mean removal rates were 81 %, 28 % and 18 % (Svenson et al., 2003). The same tendency can be found in the references by Servos et al. (2005) and Johnson et al. (2007).

Andersen et al. (2003) investigated the fate of E1, E2 and EE2 at one German sewage treatment plant. They observed that an overall elimination efficiency of E1 and E2 was above 98 %, while EE2 elimination was slightly lower. About 90 % of E1 and E2 were found to be degraded in the activated sludge system while EE2 primarily was degraded only in the nitrifying tank.

Clara et al. (2005) found that SRT was a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove EDCs as well as other micro-pollutants, while Servos et al. (2005) found that SRT has no substantial relationship with EDCs removal, which was measured both by chemical analysis and bioassay.

In this case, the key role in transformation process could be attributed to ammonia oxidizing bacteria showing highest removal at high initial ammonia concentrations.

Table 2.6 : Estrogen concentrations in STP influent, adapted from (Auriol et al., 2006).

Sampling site	Influent concentrations (ng/l)			
	Estrone	17 β-Estradiol	Estriol	17 α-Ethinylestradiol (EE2)
Paris, France	9.6-17.6	11.1-17.4	11.4-15.2	4.9-7.1
England	1.8-4.1	<0.3	-	<LOD[a]
Germany	66	22.7	-	-
Italy	52	12	80	3
Roma, Italy	31	9.7	57	4.8
Barcelona, Spain	<2.5-115	<5.0-30.4	<0.25-70.7	<5.0
Japan	-	5	-	-

LOD: limit of detection; [a] 0.3 ng/l.

Table 2.7 : Estrogen concentrations in STP effluent, adapted from (Auriol et al., 2006).

Sampling site	Effluent concentrations (ng/l)			
	Estrone	17 β-Estradiol	Estriol	17 α-Ethinylestradiol (EE2)
Paris, France	6.2-7.2	4.5-8.6	5.0-7.3	2.7-4.5
Denmark	<2.0-11.0	<1.0-4.5	-	<1.0-5.2
Netherlands	<0.4-47	<0.6-12	-	<0.2-7.5
Sweden	5.8	1.1	-	4.5
England	1.4-76	2.7-48	-	<LOD[a]-4.3
Germany	9.0	<LOD[b]	-	1.0
Italy	3.0	1.4	20.4	0.6
Roma, Italy	24.0	4.0	11.7	1.4
Barcelona, Spain	<2.5-8.1	<5.0-14.5	<0.25-21.5	<5.0
Japan	2.5-34	0.3-2.5	-	-
Canada	3.0	6.0	-	9.0
California, USA	-	0.2-4.1	-	0.2-2.4

[a] 0.2 ng/l
[b] 1 ng/l

Even higher degradation efficiencies (tens mg/l of EE2) were recorded with Rhodococcus and Sphingobacterium sp. isolated from activated sludge.

Removal efficiency of estrogens during various sewage treatment processes were given in Table 2.8.

EE2 was found to be more resistant to bacterial biodegradation than natural estrogens, but its highly hydrophobic nature makes sorption a significant removal factor in WWTPs (Cajthaml et al., 2009).

Table 2.8 : Estrogens removal during various STPs treatment process, adapted from (Auriol et al., 2006).

Compound	Concentration		Removal efficiency (%)	Treatment process	Matrice type
	Influent	Effluent			
17 β-Estradiol	5.0 ng/l	<1ng/l	>80	1	Municipal waste landfill
	11.0 ng/l	1.6 ng/l	86	2	Municipal
	9.69 ng/l	4.0 ng/l	59	2	STP
	28.1 ng/l	1.2 ng/l	96	2	Domestic STP
	-	-	100	2	Domestic STP
					Municipal STP
Estrone	44.0 ng/l	17.0 ng/l	61	2	Municipal
	31.0 ng/l	24.0 ng/l	23	2	STP
	43.1 ng/l	12.3 ng/l	69	2	Domestic STP
	-	-	83	2	Domestic STP
					Municipal STP
Estriol	72.0 ng/l	2.3 ng/l	97	2	Municipal
	57.3 ng/l	11.71 ng/l	80	2	STP
	386ng/l	5.6 ng/l	99	2	Domestic STP
					Domestic STP
17 α-Ethinylestradiol	4.84 ng/l	1.40 ng/l -	71	2	Domestic STP
	-		78	2	Municipal STP

(1) Biodegradation/sedimentation + additional treatment with charcoal; (2) activated sludge

EE2 was found to be slowly decomposed by bacteria under anaerobic conditions. The dissipation time can exceed 1000 days and the degradation is attributed to sulfate, nitrate, and iron reducing conditions.

However, abiotic factors can also play an important role in the removal. Faster degradation can be also recorded for dissolved EE2 by seawater microbes were found to degrade EE2 after acclimation (Cajthaml et al., 2009).

Using also batch experiments, it was found that WWTP processes based on activated sludge are more effective in the EE2 removal; however, the results are strongly dependent on the operating parameters (e.g. temperature, SRT, redox potential etc.) (Cajthaml et al., 2009).

Another promising alternative for EE2 decomposition could be an application of ligninolytic fungi. A number of fungal strains were shown to degrade efficiently EE2 and other estrogens, and also a direct application of ligninolytic enzymes was proved to be successful in the EE2 degradation within a relatively short time (Cajthaml et al., 2009).

2.3.3 Removal of estrogens by chemical advanced oxidation

There are numerous studies on the removal of estrogens by using different chemical oxidants, known as chemical advanced oxidation (CAO). The main mechanism of CAO is the mineralization of pollutants in wastewater to CO_2 or the transfer of pollutants to some other metabolite products by some strong oxidizers through oxidation-reduction reactions (Liu et al., 2009).

The key point is the choice of oxidizer. The strength of redox potential can be ordered as $FeO_4^{2-} > O_3 > S_2O_4^{2-} > H_2O_2 > Cl_2 > ClO_2$. Some combinations such as UV/ O_3, UV/ H_2O_2, UV/Fenton are widely applied to the removal of estrogens to increase the removal effect. Results of removal of estrogens are summarized in Table 2.9 where most of the results are from laboratory research based on artificial sewage (Liu et al., 2009).

Chlorine is a good disinfectant, which is widely used in tap water or effluent of biological wastewater treatment process. Many byproducts were measured and possible degradation pathways were also proposed, but results evaluated on YES denoted the reaction by chlorination was incomplete. Especially for BPA, the estrogenic activity of the water solution was hardly decreased after chlorination. Compared to chlorination, O_3, UV/H_2O_2 and other combination methods yield more effective results. showed that the removal efficiencies of BPA, E2 and EE2 in aqueous solution were all above 90 % by UV/H_2O_2.

The high removal effectiveness of UV/H_2O_2 was further proven by studying the removal rates of both sole target chemicals and mixed target chemicals in water solutions by UV/H_2O_2 based on both chemical analysis and bioassays (Liu et al., 2009).

Table 2.9 : Research on estrogens removal by CAO, adapted from (Liu et al., 2009).

EDCs	Wastewater	CAO	Operational condition	Main conclusions
E2	Aqueous solution	NaClO	pH 7.5; T=25^{0}C	Estrogenic activity of aqueous solution decreased with the increase of chlorination time.
E1, E2, E3, EE2, NP, PR	Aqueous solution	NaClO	pH 3.5-12; T=20±2^{0}C	EDCs exhibited a pseudo-first order dependence on the EDCs concentration, their apparent second order rate constants suggest pH dependence, minimal at about pH = 5, maximal at pH between 8 and 10.
BPA, EE2, E2	Surface water or effluent of WWTPs	K_2FeO_4	pH=8;T=25°C	When concentration of Fe(VI) was above 1 mg/l and reacting for 30min, removal efficiency of spiked EDCs in surface water or effluent of WWTPs was over 99%.
EE2	Pure water and surface water	O_3 UV/H_2O_2	C_{O3}=0.1-2.0 mg/l	Removal effect differed greatly for different water solutions at the same reaction condition, the half-life time for lake water was 4min, however, for river water, the corresponding value was 75min.
EE2	Pure water	O_3	C_{O3}=0.5-20 mg/l	Removal efficiency of estrogenic activity could be 98.5% when O_3 adding concentration was 1.9 times of EE2 initial concentration.
E1, E2, EE2,	Effluent of WWTPs	O_3	Pilot-scale experiment. Q=2m^3/h HRT=8.4min LO_3=200 l/h	Removal efficiency of EDCs was over 90% when O_3 concentration was above 2 mg/l; SS in wastewater had no influence on removal efficiency of EDCs when SS was below 20 mg/l.
BPA, E2, EE2	Aqueous solution	UV; UV/H_2O_2	pH=6.8	Removal efficiency could be improved by increasing the strength of UV, and by adding of 15 mg/l H_2O_2, removal efficiency of EDCs could be increased from 20% to above 90%.

2.3.4 Removal of estrogens by advanced treatment methods

Advanced treatment options for removing EDCs include UV photolysis, ion-exchange and membrane filtration. Membrane filtration technology such as RO and NF has demonstrated itself as a promising alternative for eliminating micropollutants. Comparatively, the NF membrane is "looser" than RO. Therefore, RO will give almost complete removal but the higher energy consumption makes it more unfavorable (Bolong et al., 2009).

The transport during NF is produced by different mechanisms, namely convection, diffusion (sieving) and charge effects. Convection occurs due to the applied pressure difference over the membrane whereas diffusion mechanism happens due to concentration gradient across the membrane (Bolong et al., 2009).

The third mechanism that is the charge effects is due to electrostatic repulsion between a charged membrane and a charged organic compound. This mechanism has made NF an attracting removal technology specifically for micropollutants such as EDCs (Bolong et al., 2009).

Therefore, membrane processes such as NF may have a significant impact on EDC removal. Several key parameters for EDC properties in wastewater were identified to boost its removal through NF by properly controlling those key parameters. Additionally, a combination or a hybrid process (i.e., NF-activated carbon) will be able to enhance the removal performance of EDCs (Bolong et al., 2009).

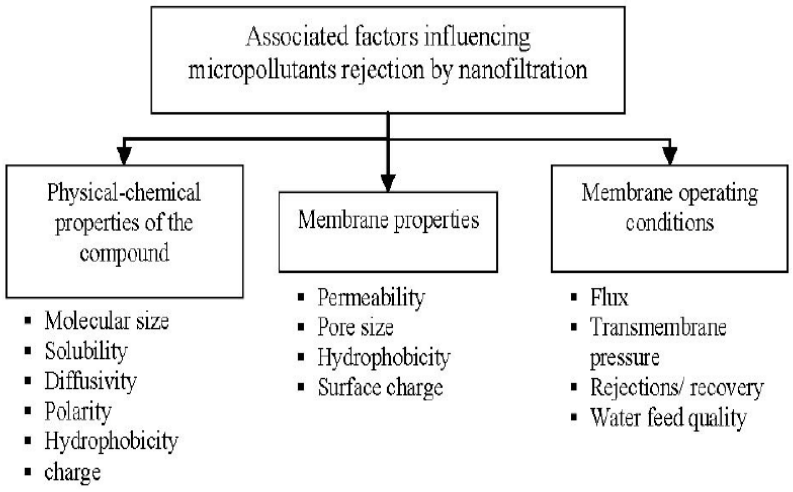

Figure 2.5 : Factors influencing rejection performance of NF membranes, adapted from (Bolong et al., 2009).

Unfortunately, most conventional wastewater treatment plants do not have treatment processes such as activated carbon, ozonation or membrane treatment, and therefore these emerging micro-pollutants are not removed but are easily released to the receiving natural waters (Bolong et al., 2009). Future wastewater treatments should be upgraded in order to cater to these fast-growing issues. Development towards a more compact and efficient treatment such as membrane technologies will have a stronger impact in the future (Bolong et al., 2009). Moreover, increased water consumption has triggered the consideration of wastewater reuse. This requires an effective and practical technology such as membrane separation processes having an advantage of purifying wastewater without using extensive chemicals (Bolong et al., 2009).

Table 2.10 : Removal of estrogens by advanced treatment processes, adapted from (Auriol et al., 2006).

Compound	Concentration	Removal (%)	Reaction time	Added dose
Ozonation	0.015µg/l[c]	>80	18 min	5 mg O_3/l
Estrone	9.7-28ng/l[d]	95	10 min	5 mg O_3/l
Estrone, 17β-estradiol	3.0-21 ng/l[d]			
Chlorination	50µg/l[e]	100	10 min	1.46 mg/l NaClO
17β-estradiol	10^{-7}M[e]	100[a]	36 h	
17β-estradiol	0.2 mmol/l[e]	100	5 min	1.5 mg/l Cl
17α-Ethinylestradiol				1mmol/l Cl
MnO_2	15 µg/l[e]	81.7	1.12 h	-
17α-Ethinylestradiol				
TiO_2	0.05-3µmol/l[e]	98	3.5h	-
17β-estradiol				
TiO_2 + UV	10^{-6}M[e]	99	30 min	1.0 g/l TiO_2 in suspension
17β-estradiol		100[b]	3 h	

[a] Complete removal of estrogenic activity.
[b] Decomposed completely into CO_2.
[c] Municipal STP effluent.
[d] Wastewater from secondary treatment.
[e] Synthetic water.

Consequently, more exploration of advanced wastewater treatment technologies that are able to eliminate these new unregulated micro-pollutants is highly desirable, not only to provide advantages to human, but also for the benefit of other living things. The question of whether these advanced technologies should be employed has become important, not only due to regulation requirements but also due to the sustainable development (Bolong et al., 2009).

2.4 Biodegradation of EE2 on activated sludge processes

Biodegradation is the primary removal mean for estrogens in wastewater. Biodegradation mechanisms include deconjugation, degradation as a carbon source for heterotrophic bacteria, cometabolism with nitrifying biomass, or other cometabolisms. Generally, the natural estrogens, E2 in particular, are quite readily biodegradable. However, EE2 is not nearly as easily biologically removed. The ethinylgroup of EE2 is assumed to sterically hinder enzyme expression, substrate-receptor binding, and EE2 metabolism. In addition, it is important to note that the bacterial communities in municipal wastewater treatment sludge have a much greater capacity to biodegrade estrogens than industrial wastewater treatment sludge, which can mineralize only 4 % of E2 (Racz, 2010).

2.4.1 Carbon removal systems

Various heterotrophic bacteria are capable of degrading estrogens. Achromobacter xylosoxidans and Ralstonia sp. as well as an unidentified gram negative bacterium, all isolated from activated sludge, directly used E2 as a growth substrate. A Sphingomonas bacterium, also isolated from activated sludge, was also able to use E2 as a sole carbon source and degrade it to nonestrogenic metabolites. While these bacteria were not able to degrade EE2, Rhodococcus zopfii, Rhodococcus equi, and Sphingobacterium sp. JCR5 isolated from activated sludge, as well as a fungus Fusarium proliferatum isolated from manure were able degrade the synthetic hormone as well as the natural hormones (Racz, 2010).

In addition, the denitrification process involving heterotrophic bacteria was reported that it significantly removes estrogens in wastewater (Racz, 2010).

It appears that degradation rates of estrogens increase with increasing initial concentrations, presumably as greater initial concentrations provide greater substrate for bacteria that metabolize these compounds (Racz, 2010).

Furthermore, the greater the concentration of total organic compounds, the less easily estrogens are degraded, possibly because bacteria preferentially utilize other organic compounds before utilizing estrogens (Racz, 2010).

2.4.2 Nitrification systems

Often, wastewater treatment plants remove ammonium ($NH4^{+}$), a common pollutant with a significant oxygen demand that can be toxic to aquatic macroorganisms. The nitrification process biologically removes ammonia from wastewater. Traditionally, the nitrification process begins with ammonia oxidizing bacteria (AOB), such as Nitrosomonas spp. and Nitrosospira, converting ammonia to NO_2^- (Racz, 2010).

Even though AOB require inorganic carbon for growth, AOB are quite diverse among wastewater treatment plants. AOB commonly coexist with heterotrophs in full-scale activated sludge systems. Temperature, solid retention time, high organic loads and high carbon-to-nitrogen (C/N) ratios, low dissolved oxygen concentrations and inhibitory compounds affect AOB metabolism. Several researchers have also studied the interaction between nitrifying and heterotrophic bacteria. Therefore, it is possible that there is some symbiotic relationship between heterotrophs and AOB although both of these feed on different carbon sources (Racz, 2010).

AOB oxidize ammonia to hydroxylamine via the ammonia monooxygenase (AMO) enzyme. AMO inserts oxygen into C-H bonds, catalyzing the hydroxylation of alkanes to produce primary and secondary alcohols. This hydroxylation is attributed as converting estrogens into hydrophilic products essentially devoid of estrogenic activity. Therefore, estrogen degradation in nitrifying biomass is typically attributed to cometabolism via AMO. The AMO enzyme is particularly important for EE2, the most recalcitrant estrogen. EE2 has been successfully degraded in nitrifying sludge where it could not be removed by heterotrophs alone (Racz, 2010).

Gusseme et al., 2009 suggested a possible competition between ammonia and estrogens, specifically EE2, for removal by the AMO enzyme as maximum EE2 removal rates occur when ammonia concentration is minimal.

AOB are likely responsible for the first degradation step of EE2, while heterotrophic bacteria in a mixed culture might remove the subsequent metabolites. However, nitrification could be less important for degrading E2 as it is suggested that heterotrophic bacteria are responsible for its degradation instead (Racz, 2010).

Interestingly, lab-scale nitrification bioreactors continued to degrade estrogens, specifically EE2. Also, nitrifying activated sludge does not require an adaptation period before degrading EE2 (Racz, 2010).

Estrogenic degradation is apparently improved at full-scale with extended SRT which promotes appropriate conditions for complete nitrification to proceed and maximises bacterial diversity. Consequently, the authors latterly hypothesise that at full-scale plants, when in the presence of low nitrite concentrations, heterotrophs may be principally responsible for the reduction of EE2 rather than autotrophic micro-organisms, or indeed, abiotic nitration (Racz, 2010).

Biodegradation of greater than 70 % can be achieved upon increasing SRT to values greater than 10 days and to more than 80 % once SRT increased over 20 days. Longer SRT in nitrification systems provides for enrichment of slow growing bacteria thus the establishment of more diverse biocoenosis and may explain the augmented total estrogen biodegradation observed at the nitrifying activated sludge plant operated with a relatively short HRT (Mc Adam et al., 2010).

2.4.3 Anaerobic systems

Muller et al. (2010) investigated the occurence of estrogens in urban sewage sludge and throughout a plant-scale anaerobic digestion process. It was concluded that the plant-scale anaerobic digestion showed low efficiency (<40 %) for removing estrogens and, regarding the final dewatering process, concentrations increased for E2 and EE2. Most authors found that the concentrations for natural estrogens E1, E2 increase, on the contrary decrease for EE2. Moreover, final sludge stabilization and dewatering by thermal-pressurized treatment tends to increase the estrogen content from anaerobic digestion, probably by enhancing their extractability.

Ivanov et al. (2010) studied the effect of the oxidant Fe(III) and facultative anaerobic iron-reducing bacteria on the anaerobic degradation of estrogens in reject water.

Reject water in a municipal wastewater treatment plant contains metabolic products of aerobic bacteria of aeration tank, as well as products of fermenting and denitrifying bacteria. Fe(III) is used as electron acceptor. EE2 remained resistant to anaerobic biodegradation by iron-reducing bacteria, while natural estrogens such as E2, E1 and E3 were removed by 92 %, 60 % and 27 % respectively (Ivanov et al., 2010).

2.4.4 Membrane bioreactors

Membrane bioreactor (MBR) technology was generally chosen to develop a community of autotrophic and nitrifying micro-organisms. Clouzot et al. (2010b) acclimated activated sludge (AS) in the MBR to a substrate specific to autotrophic biomass and resulted in an increase in nitrifying activity. Acclimated AS was used to successfully biodegrade EE2 (11 % increase in EE2 removal) and the overall removal of EE2 was determined to be 99 % (sorption+biodegradation). AS used directly from a WWTP without acclimation removed EE2 only through sorption (88 % removal of EE2). Therefore, higher nitrifying activity developed by acclimating AS allowed almost complete removal of EE2.

Nitrifying micro-organisms are autotrophic and grow more slowly than heterotrophic ones. Therefore, heterotrophic microorganisms typically outnumber nitrifying micro-organisms. The development of nitrifying micro-organisms during wastewater treatment can be improved with high sludge retention times. In a conventional activated sludge system, low settling abilities of sludge generally result in low SRT (15-20 days). However, with a membrane bioreactor, complete biomass retention allows control of a higher SRT. EE2 removal was predicted to be more efficient with acclimated, nitrifying activated sludge from a MBR (SRT=30 days) than with activated sludge from a conventional WWTP (SRT=11 days) (Clouzot et al., 2010b).

Clouzot et al. (2010a) also compared membrane bioreactor (MBR) technology with conventional activated sludge (CAS) systems. Contrary to previous studies on MBRs, continuous purification was combined with the determination of sorption and biodegradation parameters. In addition, EE2 removal was studied in two different MBR configurations.

Continuous purification with AS acclimated in the MBR resulted in the stabilization of the EE2 removal. In contrast, CAS resulted in EE2 accumulation in the permeate.

During batch kinetics, CAS was shown to remove EE2 only through sorption (87% removal of EE2) whereas AS acclimated to the MBR process developed biodegradation abilities towards the synthetic hormone (7% removal of EE2). Therefore, the membrane process combined with AS was shown to be essential to improve EE2 removal (with the autotrophic activity as the key factor) (Clouzot et al., 2010a).

2.5 Activated sludge modelling (ASM)

One of the most widespread biological wastewater treatment techniques is the activated sludge process. In this process, a bacterial biomass suspension is responsible for the removal of pollutants. Depending on the design and the specific application, an activated sludge wastewater treatment plant can achieve biological nitrogen removal and biological phosphorus removal, besides removal of organic carbon substances. The increased knowledge about the mechanisms of different biological processes taking place in an activated sludge plant was translated into dynamic models that were developed to describe the degradation processes in the activated sludge plant and modelling is an inherent part of the design of a wastewater treatment system (Henze et al., 2000).

2.5.1 ASM 1

In 1983, the International Association on Water Quality (IAWQ, formerly IAWPRC) formed a task group, which was to promote development, and facilitate the application of, practical models for design and operation of biological wastewater treatment systems.

The first goal was to review existing models and the second goal was to reach a consensus concerning the simplest mathematical model having the capability of realistically predicting the performance of single-sludge systems carrying out carbon oxidation, nitrification and denitrification. The final result was presented in 1987. Today the model is named Activated Sludge Model No.1, abbreviated ASM1 (Jeppsson, 1996).

ASM1 has a pioneering role on multi-component activated sludge modelling. ASM1 is basically structured on the assumption that active biomass is converted to a combination of particulate residual products and slowly biodegradable substrate, through death and lysis (Orhon et al., 2009).

The carbon material in ASM1 is divided into biodegradable COD, nonbiodegradable COD (inert material) and biomass (Figure 2.6). A soluble component is denoted S and a particulate component is denoted X. The biodegradable COD is further divided into readily biodegradable substrate (S_S) and slowly biodegradable substrate (X_S) (Jeppsson, 1996).

The readily biodegradable substrate is hypothesized to consist of simple soluble molecules that can be readily absorbed by the organisms and metabolized for energy and synthesis, whereas the slowly biodegradable substrate is assumed to be made up of particulate/colloidal/complex organic molecules that require enzymatic breakdown prior to absorption and utilization (Jeppsson, 1996).

A fraction of the slowly biodegradable substrate may actually be soluble although it is treated as a particulate material in the model. The non-biodegradable COD is divided into soluble (S_I) and particulate (X_I) material. Both are considered to be unaffected by the biological action in the system (Jeppsson, 1996).

The inert soluble material leaves the system by the secondary clarifier effluent, whereas the inert particulate material is enmeshed in the sludge mass and accumulates as inert VSS. The inert particulate material will be removed from the system by the removal of excess sludge and to some extent be present in the settler effluent as well. Moreover, the active biomass is divided into two types of organisms: heterotrophic biomass ($X_{B,H}$) and autotrophic biomass ($X_{B,A}$) (Jeppsson, 1996).

Finally, an extra state variable (X_P) for modelling the inert particulate products arising from biomass decay is included (Jeppsson, 1996).

In summary, the total COD balance of ASM1 is given by;

$$COD_{tot} = S_I + S_S + X_S + X_{B,H} + X_{B,A} + X_I + X_P \quad \textbf{(1.1)}$$

The nitrogenous material in the wastewater is divided according to Figure 2.7. Based on measurements of total Kjeldahl nitrogen (TKN), the nitrogen is divided into ammonia nitrogen (S_{NH}), organically bound nitrogen and active mass nitrogen, that is, a fraction of the biomass which is assumed to be nitrogen.

Similar to the division of the organic material, the organically bound nitrogen is divided into soluble and particulate fractions, which in turn may be biodegradable or non-biodegradable. It should be noted that only particulate biodegradable organic nitrogen (X_{ND}) and soluble biodegradable organic nitrogen (S_{ND}) are explicitly included in the model (Jeppsson, 1996).

The active mass nitrogen (X_{NB}) is included in the model only in the sense that decay of biomass will lead to a production of particulate biodegradable organic nitrogen (Jeppsson, 1996).

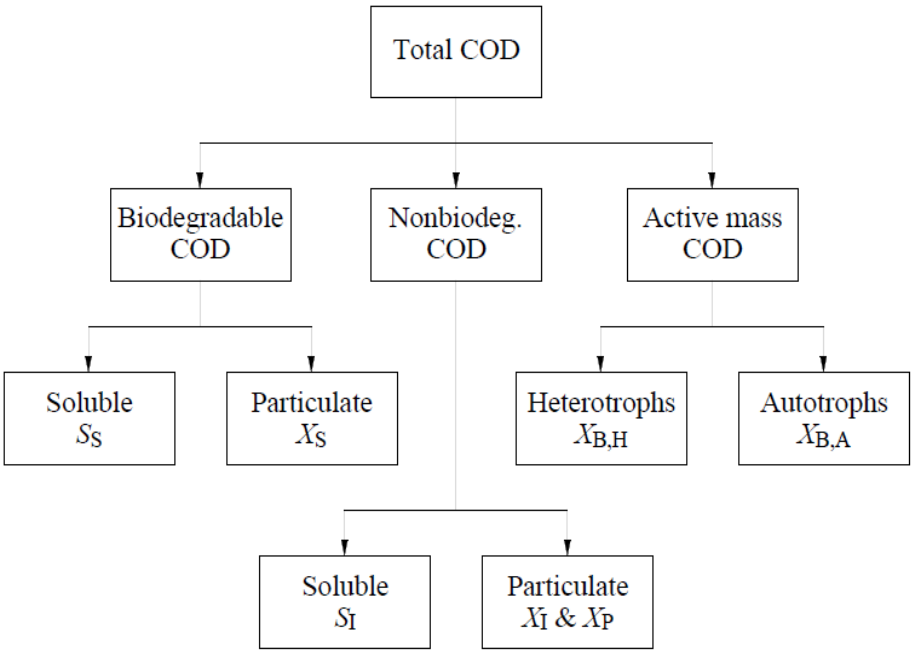

Figure 2.6 : COD Components in ASM1, adapted from (Jeppsson, 1996).

Organic nitrogen associated with the inert organic particulate products (X_{NP}) and the inert organic particulate matter (X_{NI}) can easily be calculated, although not described in the model matrix. Finally, the nitrification of ammonia to nitrate nitrogen (S_{NO}) is considered as a single step process. The last two components described in the ASM1 are the dissolved oxygen concentration (S_O), expressed as negative COD, and the alkalinity (S_{ALK}). The alkalinity does not affect any other processes in the model (Jeppsson, 1996) (Figure 2.7).

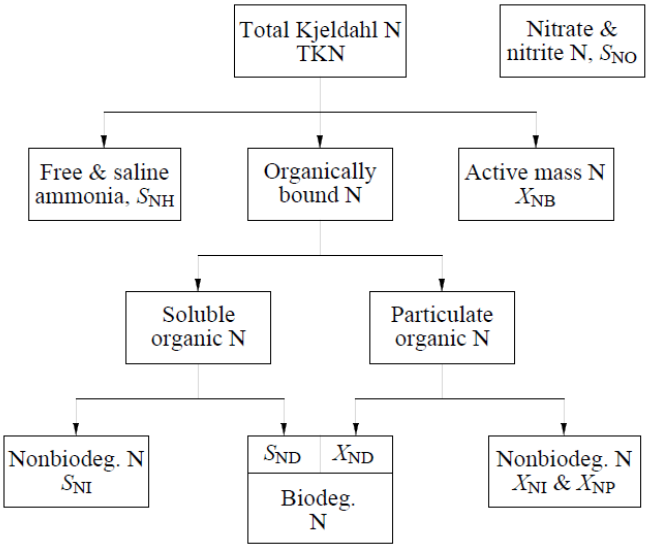

Figure 2.7 : Nitrogen components in ASM1, adapted from (Jeppsson, 1996).

The substrate removal and the oxygen consumption can be given as (Orhon et al., 2009):

$$-\frac{1}{Y_H} \quad \textbf{(1.2)}$$

and

$$-\frac{(1-Y_H)}{Y_H} \quad \textbf{(1.3)}$$

The effect of each model component is conveniently modelled with a Monod-type saturation function:

$$\frac{dX_H}{dt} = \mu_H \frac{S_S}{K_S + S_S} X_H \quad \textbf{(1.4)}$$

Where the maximum heterotrophic growth rate, μ_H; the half saturation coefficient for heterotrophic biomass, K_S.

Hydrolysis is assumed to be the rate limiting step for the utilization of the slowly biodegradable COD:

$$\frac{dX_S}{dt} = -k_h \frac{\frac{X_S}{X_H}}{K_X + \frac{X_S}{X_H}} X_H \quad \textbf{(1.5)}$$

The endogenous decay coefficient, b_H is defined as a function of the active heterotrophic biomass concentration:

$$\frac{dX_H}{dt} = -b_H X_H \quad \textbf{(1.6)}$$

Generation rate of particulate inert products are given as follows:

$$\frac{dX_P}{dt} = f_{EX}\frac{dX_H}{dt} \quad \textbf{(1.7)}$$

Where f_{EX} is a fraction of the active biomass does not udergo any further reaction and accumulates in the activated sludge as particulate residual metabolic products, X_P.

Activated Sludge Model No.1 is extended and Activated Sludge Model No.2 (ASM2) is developped. ASM2 presents a concept for dynamic simulation of combined biological processes for chemical oxygen demand (COD), nitrogen and phosphorus removal (Henze et al., 2000).

The strong movement towards effluent criteria for both nitrogen and phosphorus has created a need for a tool to model biological phosphorus removal processes. ASM2 is introduced as a further developmentof ASM1; introduces phosphorus accumulating organisms (PAO) and allows us to simulate the behaviour of biological nutrient removal activated sludge systems. ASM2 has many limitations because it is based on information from municipal wastewater treatment processes and ASM2 is more complex, includes many more components (Henze et al., 2000).

In 1995, the Activated Sludge Model no. 2 was published. This model included nitrogen removal and biological phosphorus removal. In 1994, when the ASM2 was finished, the role of denitrification in relation to biological phosphorus removal was still unclear, so it was decided not to include that element. However, the development in research was fast, and denitrifying PAOs (phosphorus-accumulating organisms) were needed for simulation of many results from research and practice. Because of this, the ASM2 model was expanded in 1999 into the ASM2d model, where denitrifying PAOs were included (Henze et al., 2000). However, this thesis does not deal with ASM2 because phosphorus was not analyzed during laboratory studies. ASM3 was used to model respirometric analysis during this thesis.

Table 2.11 : Matrix representation of ASM1 for organic carbon removal, adapted from (Orhon et al., 2009).

Component →	1	2	3	4	5	6	7	8	9	Process rate
Process ↓	S_I	X_I	S_S	X_S	X_H	X_P	S_O	S_{NH}	S_{ALK}	$ML^{-3}T^{-1}$
1 Growth			$-\frac{1}{Y_H}$		1		$-\frac{(1-Y_H)}{Y_H}$	$-i_{XN}$	$-\frac{i_{XN}}{14}$	$\mu_H \frac{S_S}{K_S+S_S} X_H$
2 Decay				$1-f_{EX}$	-1	f_{EX}				$b_H X_H$
3 Hydrolysis			1	-1						$k_h \frac{\frac{X_S}{X_H}}{K_X+\frac{X_S}{X_H}} X_H$
Parameter, ML^{-3}	COD	COD	COD	COD	CellCOD	COD	O_2	NH_3-N	Alkalinity – Molar units	

2.5.2 ASM 3

Models generally presume that microbial growth is the only biochemical mechanism for growth; substrate is directly converted into biomass which is subsequently used for endogenous respiration. It is now strongly argued that this mechanistic approach may not always be accurate, especially in the case where substrate is not continuously available in the reactor either temporally or spatially (Orhon et al., 2009).

In fact, some biological treatment systems like SBR, operate with an intermittent wastewater feeding which creates a feast phase followed by a famine phase after depletion of all available external substrate and these phases follow one another during the cyclic SBR operation (Orhon et al., 2009).

A similar set of conditions may also be sustained in a continuous flow biological reactor where the biodegradable COD in the wastewater is rapidly utilized and consumed in the inital section of the reactor so that the remaining volume of the reactor basically functions for endogenous respiration (Orhon et al., 2009).

Experimental findings indicate that under these operating conditions biomass tends to accumulate internal storage polymers which are subsequently utilized for growth when external substrate is no longer available (Orhon et al., 2009).

A new modelling approach, recently introduced as Activated Sludge Model No.3 (ASM3) assumes that storage is the sole initial biochemical mechanism for the utilization readily biodegradable COD also uses the concept of COD fractionation for substrate and biomass with two major differences compared with previous models (Orhon et al., 2009).

The major difference between the ASM1 and ASM3 models is that the latter introduces the concept of storage-mediated growth of heterotrophic organisms, assuming that all readily biodegradable substrate (S_S) is first taken up and stored into an internal cell polymer component (X_{STO}) which is then used for growth (Iacopozzi et al., 2007).

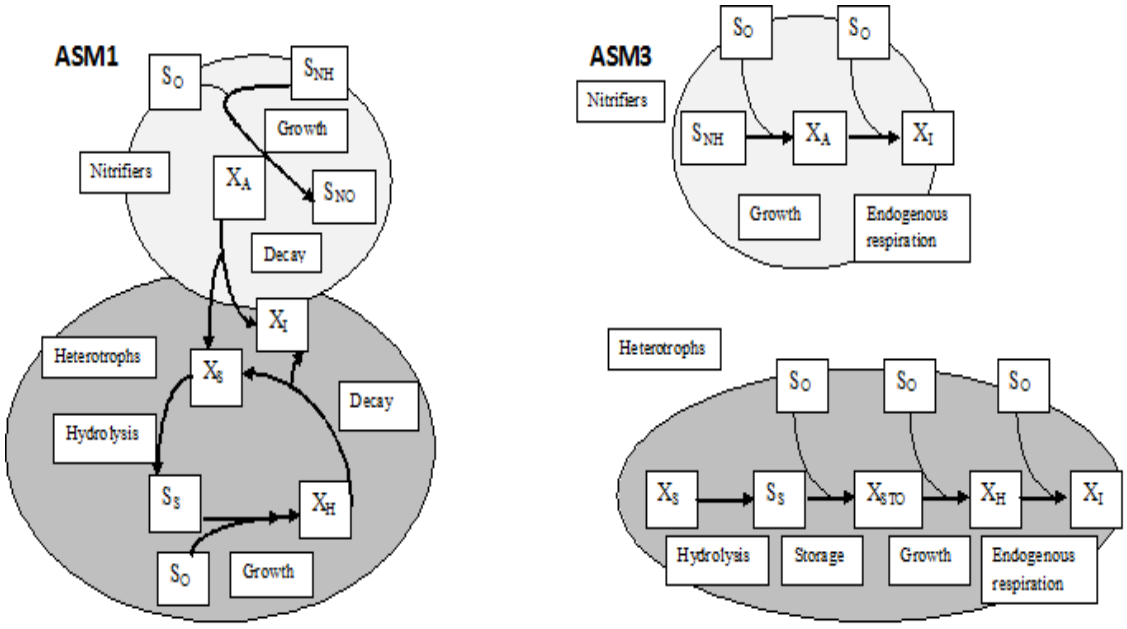

Figure 2.8 : Flow of COD in ASM1 and ASM3, adapted from (Henze et al., 2000).

ASM3 models provide a common base for the simulation of nitrogen removing activated sludge systems for chemical oxygen demand as well as organic carbon based characterization of wastewater and biomass (Henze et al., 2000).

ASM3 model covers both S_S and S_H (S_S+S_H) defined in ASM1 and endogenous decay models. Second, ASM3 assumes, as previously mentioned, that all biodegradable COD is initally converted into internal storage products and growth occurs only at the expense of stored polymers (Orhon et al., 2009)

Storage is generally observed as a faster process compared with growth and it is characterized in terms of a kinetic coefficient, k_{STO} defining the maximum storage rate. The concentration of storage, X_{STO} is inevitably incorporated into the model structure as a particulate model component, an additional biomass fraction (Orhon et al., 2009)

The conversion of S_S into X_{STO} is defined by means of the storage yield coefficient, Y_{STO}. ASM3 also accounts for the generation of soluble inert microbial products, S_P. Matrix representation of ASM3 structure was given in Table 2.12 (Orhon et al., 2009).

Table 2.12 : Matrix representation of ASM3 modified for the generation of microbial products, adapted from (Orhon et al., 2009).

Component →	1	2	3	4	5	6	7	8	Process rate
Process ↓	S_I	X_I	S_S	X_H	X_P	S_P	X_{STO}	S_O	$ML^{-3}T^{-1}$
Storage of readily biodegradable substrate			-1				Y_{STO}	$-(1-Y_{STO})$	$k_{STO}\frac{S_S}{K_S+S_S}X_H$
Growth on storage product				1			$-\frac{1}{Y_H}$	$-\frac{1-Y_H}{Y_H}$	$\mu_H\frac{\frac{X_{STO}}{X_H}}{K_{STO}+\frac{X_{STO}}{X_H}}X_H$
Endogenous respiration			1	-1	f_{EX}	f_{ES}		$-(1-f_E)$	$b_H X_H$
Respiration of storage product							-1	-1	$b_{STO}X_{STO}$
Parameter, ML^{-3}	COD	COD	COD	CellCOD	COD	COD	COD	O_2	

3. MATERIALS AND METHODS

3.1 Reactor Operation

Experimental part of this study was started with the setup of an aerobic batch reactor. Activated sludge was taken from a domestic wastewater treatment plant, in İstanbul. Complete mixing in the reactor was provided by adequate aeration and mechanical mixing. Activated sludge was acclimated by feeding peptone mixture having 600 mg COD/l. The reactor was operated with a working volume of 12 liters. The operating sludge age of the reactor was 10 days and the hydraulic retention time was 1 day. The volume of wasted sludge from the complete mix reactor was 1.2 liters per day. The temperature of the reactor was kept constant at 20^0C. The peptone mixture was prepared according to (ISO 8192) method. Macro and micronutrients were also added to the reactor in order to support microbial growth and to maintain pH at neutral levels. The ingredients of peptone mixture and nutrient solutions were given in Table 3.1. The operated system was given in Figure 3.1.

Any respirometric experiments were conducted until the system had reached steady state conditions. After the acclimation period, fate and effect of 17 alpha-ethinylestradiol to activated sludge were investigated. Respirometric analysis were performed in order to specify these effects. A nitrification inhibitor (Formula 2533 TM, Hach Company) was used to prevent interference of nitrification. COD concentration of the peptone mixture was 36 g COD/l (Table 3.1).

Table 3.1 : Ingredients of Peptone mixture, Solution A and Solution B, adapted from (ISO 8192).

Compound	Feed Concentration (g/l)
Peptone Mixture	
Peptone	16
Meat Extract	11
Urea	3

Table 3.1 (continued) : Ingredients of Peptone mixture, Solution A and Solution B, adapted from (ISO 8192).

NaCl	0.7
$CaCl_2.2H_2O$	0.4
$MgSO_4.7H_2O$	0.2
K_2HPO_4	2.8
Solution A	
K_2HPO_4	320
KH_2PO_4	160
Solution B	
$MgSO_4.7H_2O$	15
$FeSO_4.7H_2O$	0.5
$ZnSO_4.7H_2O$	0.5
$MnSO_4.7H_2O$	0.41
$CaCl_2.2H_2O$	2.65

Figure 3.1 : Aerobic batch reactors.

3.2 Analytical Procedure

Conventional parameters such as SS, VSS, COD and pH analysis were performed in order to monitor and to control reactor operation. The criteria for reaching steady state conditions was monitored by measuring VSS concentration and COD. pH was an important parameter during the operation and experiments. It was kept in the range of 6.5-7.5 which is suitable for biological activity. pH measurements were performed by a 520Aplus pH meter. Samples taken for COD measurements, were filtred through 0.45 µm membrane filters (Whatman, Mainstone, UK) for the separation of the bacterial cells from the liquid. COD samples were preserved with H_2SO_4. COD measurements were performed according to the ISO 6060 method (ISO 6060, 1986). In order to determine the storage properties of the activated sludge Polyhydroxyalkanoate (PHA) samples were taken during experiments and preserved at -20^0C refrigerator. SS, VSS, COD, pH and PHAs parameters were defined according to the Standards Methods (1998). Respirometric tests were performed with AppliTek Ra-Combo respirometer for evaluation and modelling purposes. To enlighten the treatment mechanism and the effect of EE2 on activated sludge systems, EE2 concentrations were measured. Selected EE2 concentrations are higher than that of in domestic wastewaters therefore the analytical method for the measurement of EE2 was chosen as Ultra Performance Liquid Chromatography (UPLC)/Tandem Mass Spectrometry (MS/MS). The average SS and VSS concentrations were 2477 mg/l and 1916 mg/l respectively.

3.3 Experimental Procedure

3.3.1 Respirometric analysis

Oxygen uptake rate (OUR) profiles should be comprehended for the evaluation of substrate removal mechanisms. All the tests were run at steady state conditions. The respirometric tests were first conducted for the control. Then, respirometric experiments were continued with relevant acclimated biomass seeded with the test substance, carbon source and mineral nutrients. At the beginning of each respirometric test, acclimated biomass was seeded with only mineral nutrients to obtain endogenous oxygen uptake rate level of biomass.

Samples having desired S_o/X_o ratios were added to the respirometer reactor with 2 liters of volume and the OUR data was monitored. Experimental studies were conducted by using AppliTek Ra-Combo respirometer. Experiments were conducted by using activated sludge operated at the sludge age of 10 days. A nitrification inhibitor (Formula 2533 TM, Hach Company) was used to prevent interference of nitrification. The same conditions in respirometric tests were provided in other reactors of 2 liters of volume in parallel with the respirometer. The average VSS concentrations of the reactor were between 1700 and 2000 mg/l during the experiments. The summary of respirometric studies were given in Table 3.2 and in Table 3.3. The monitored data for experimental sets was detailed in Table 3.4.

3.3.2 PHA analysis

Polyhydroxyalkanoates (PHAs) are one of the mostly observed storage polymer in activated sludge. The commonly known part of PHAs is polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV). PHAs samples were taken into 2x10 ml centrifuge tubes containing 2 drops of formaldehyde to prevent the biological activity. Then the samples were centrifuged and the liquid phase was discarded. 4.5-5 ml of phosphate buffer was added and the samples were centrifuged again. Collected sludge pellets were freeze-dried (ThermoSavant, ModuloD Freeze dryer) for at least 48 hours at -50^0C. Freeze-dried pellets were crushed, 20-30 mg of sludge pellets and the same amount of caproic acid were weighed then the amount was recorded. The weighed amount was added into glass tubes with PTFE lined screw caps (Schott GL18 Max 200^0C). Freeze-dried biomass are subjected to extraction, hydrolization and esterification in a mixture of hydrochloric acid, 1-propanol and dichloroethane at 100^0C for 2 hours. 50 µl of internal standard was added to each sample. Benzoic acid was used as an internal standard throughout the procedure. After boiling, samples were cooled down. 3 ml of distilled water was added to the samples then the samples were shaked vigorously for 10 minutes by vortex. Then they were centrifuged for 5 minutes at 2500-3000 rpm. Waited for phase separation for a few minutes. The resulting organic phase was extracted with water to remove free acids. Around 1 ml of the lower organic phase was taken and then transferred into vials. Glasswool and sodium sulfate were put into blue pipette tips. 1 ml of organic phase was filtered from sodium sulfate column. Samples then were measured with Gas-chromatography (GC).

Table 3.2 : Acute experiment conditions conducted (SRT: 10 days).

Set	Substrate Type	
	Peptone mixture	**EE2**
Set 1	360 mg COD/l	-
Set 2	360 mg COD/l	1 mg/l
Set 3	360 mg COD/l	1 mg/l

Table 3.3 : Chronic experiment conditions conducted (SRT: 10 days).

Set	**Substrate Type**		**Acclimation Period (day)**
	Peptone mixture	**EE2**	
Set 4	360 mg COD/l	-	Control
Set 5	360 mg COD/l	1 mg/l	1
Set 6	360 mg COD/l	1 mg/l	5
Set 7	360 mg COD/l	1 mg/l	10
Set 7.1	360 mg COD/l	-	10
Set 8	360 mg COD/l	1 mg/l	15
Set 9	360 mg COD/l	1 mg/l	20
Set 10	360 mg COD/l	1 mg/l	25
Set 11	360 mg COD/l	1 mg/l	30
Set 11.1	360 mg COD/l	-	30
Set 12	360 mg COD/l	1 mg/l	40
Set 12.1	360 mg COD/l	-	40

Table 3.4 : Monitored data for experimental sets.

Time (min)	pH	SS/VSS	COD filtered	PHA	EE2
-10		X	X	X	X
10	X		X	X	
20			X	X	
30			X	X	
60	X		X	X	
90			X	X	
120			X	X	
150	X		X	X	
200			X	X	
250			X	X	
300			X	X	
360	X		X	X	
420			X	X	
1440	X	X	X	X	X

3.3.3 EE2 extraction method

Wastewater and sludge samples were collected during experiments in order to measure EE2 concentration in aqueous phase and solid phase. 250 ml of wastewater was taken from the mixed liquor. 10 drops of was added to the sample in order to prevent the biological activity. 250 ml wastewater sample was divided into 5x50 ml falcon tubes then they were centrifuged at 7000 rpm during 5 minutes. Supernatants were filtered through 0.22 µm membrane filters (Whatman, Mainstone, UK) then stored at + 4^0C for aqueous phase analysis. Sludge samples were preserved at - 20^0C for SPE (solid phase extraction).

Supernatants were diluted in the ratio of 1:100. Conventionally conditioned SPE cartridges were used to filter supernatants. 3 ml of distilled water was passed through the SPE cartridge before vacuum drying. SPE cartridges were dried during 1 hour using a vacuum manifold system connected to a vacuum pump.

Finally, samples were collected in a glass tube by passing 8 ml acetoneitrile through the SPE cartridge. The rest was concentrated to dryness in a gentle N_2 flow (Figure 3.2). The residue was redissolved in 0.5 ml methanol, filtered through 0.22 µm membrane filter and then collected in glass vials for UPLC measurement.

Figure 3.2 : Turbo vaporizer.

Sludge samples were first freeze-dried for at least 48 hours at -50^0C. 0.5 g of crushed sludge sample was weighed and transferred to a 50 ml beaker. 20 ml of acetone – methanol (in the ratio of 1:1) mixture was added in to the sludge sample. Then samples were sonicated with 50 % power for 3 minutes. Bandelin Sonopuls Ultrasonicator was used for ultrasonic extraction. Then the mixture was centrifuged at 4000 rpm during 5 minutes. Supernatant was separated and the solid phase was subjected to the same procedure at least 3 times.

For each time, supernatant was filtered through 0.22 µm membrane filter and collected in a 50 ml beaker. At the end of the first cycle, 20 µl of 134 C labelled EE2 was injected to the solid phase (40 µl of 134 C labelled EE2 was also injected to the aqueous phase). Collected aliquots were concentrated by rotary evaporator for SPE (Figure 3.3). The rest was solubilized in 2 ml acetone – methanol (in the ratio of 1:1) mixture and 18 ml distilled water. Prior to use, SPE cartridges were conditioned conventionally.

In order to condition SPE cartridges; 5 ml acetonitrile, 5 ml methanol and 5 ml distilled water were used respectively. Then concentrated samples were filtered through 0.22 µm membrane filter and passed through conditioned SPE cartridges. SPE cartridges were washed with 3 ml of distilled water before vacuum drying. SPE cartridges were dried during 1 hour using a vacuum manifold system connected to a vacuum pump.

Figure 3.3 : Rotary evaporator.

Finally, samples were collected in a glass tube by passing 8 ml acetoneitrile through the SPE cartridge. The rest was concentrated to dryness in a gentle N_2 flow at 25^0C under 30 bar pressure (Figure 3.2). The residues were redissolved in 1.0 ml methanol, filtered through 0.22 µm membrane filter and then transferred to glass vials for UPLC measurement. All LC-MS/MS analysis were performed using Thermo Accela UPLC and Thermo Quantum tandem MS. In order to eliminate matrix effects, internal standard was used. Isotope diluton method was used to quantify EE2. Regression coefficient of the calibration curves were always above 99 %. Relative standard deviation for the measurements were below 20 %.

4. RESULTS AND DISCUSSION

The peptone mixture reactor was operated at SRT of 10 days at steady state for a period of approximately 5 months. The monitoring results of biomass were represented in Figure 4.1.

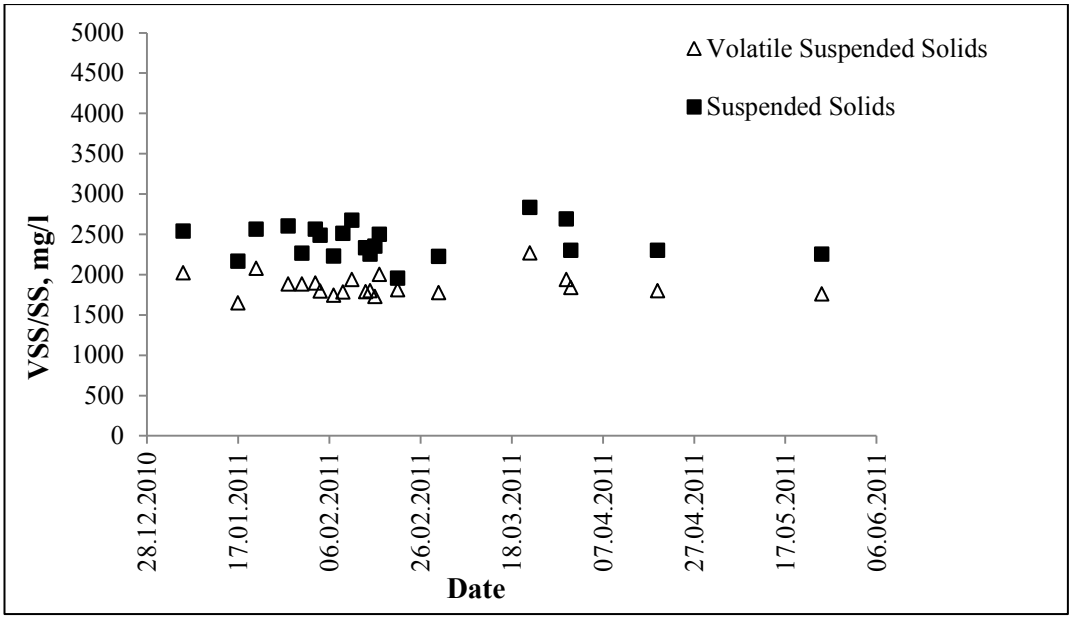

Figure 4.1 : Monitoring results of biomass of the peptone mixture reactor.

The peptone mixture reactor characteristics at steady state conditions were given in Table 4.1. pH of the reactor was maintained at neutral level.

Table 4.1 : The peptone mixture reactor characteristics at steady state conditions.

Substrate Type	S_o/X_o (mg COD/ mg VSS)	SS (mg/l)	VSS (mg/l)	VSS/SS ratio	COD_{inf} (mg/l)	COD_{eff} (mg/l)	Removal Efficiency (%)
Peptone mixture	0.30	2500±200	2000±140	0.80	600±100	33±10	95±3

4.1 Acute Experiments

Two experiments were performed to evaluate the acute effect of EE2 on acclimated sludge. Two concentrations of EE2; 1 mg/l and 5 mg/l of EE2 were used during acute experiments. A control experiment was also performed before these two experiments.

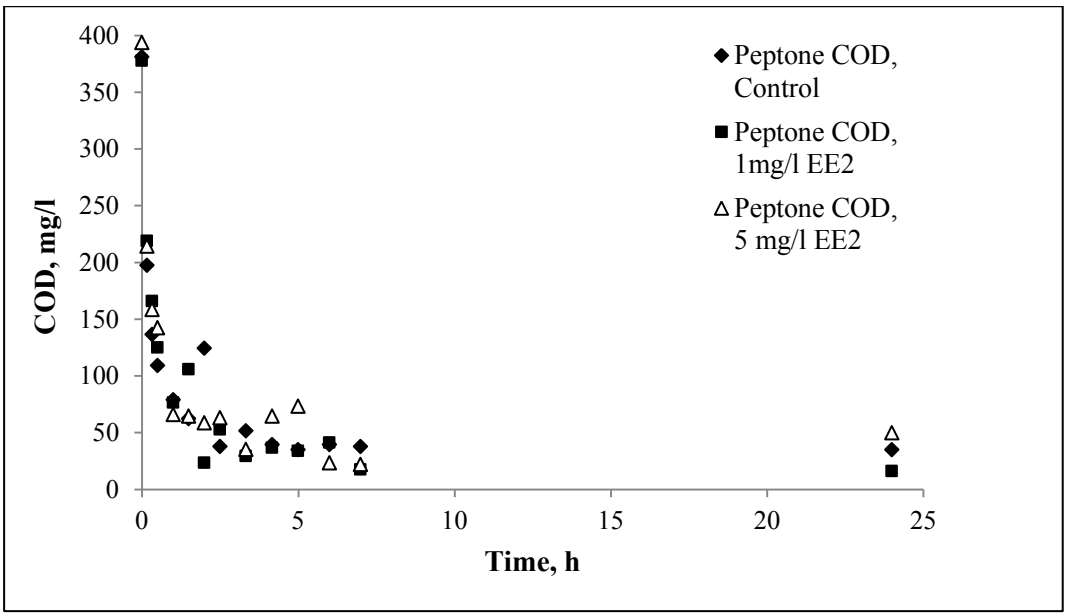

Figure 4.2 : COD concentrations versus time (Set 1-Set 2-Set 3).

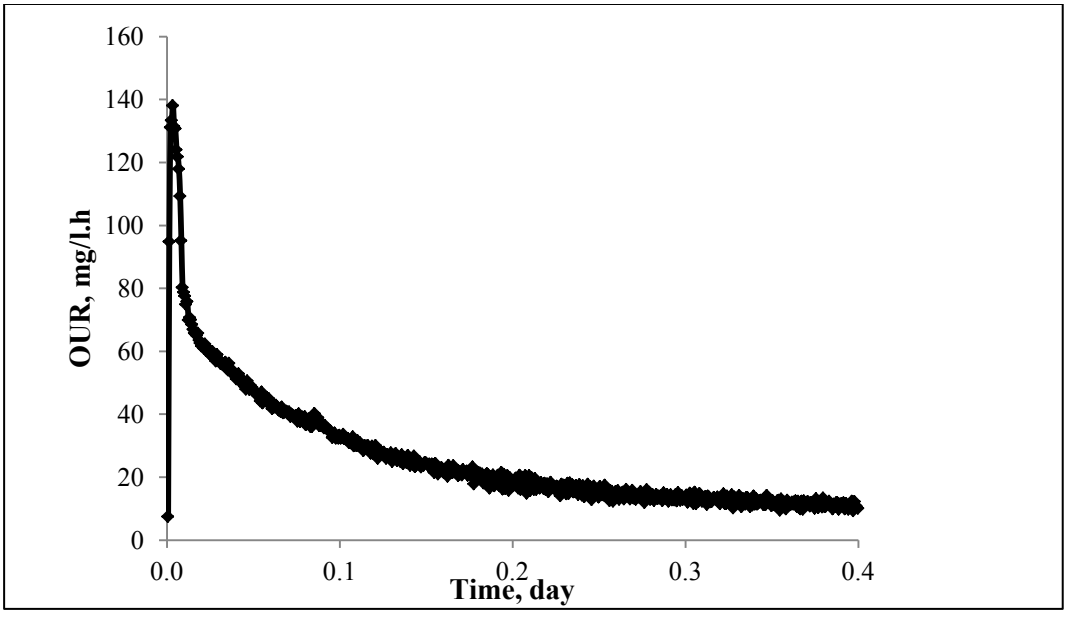

Figure 4.3 : OUR profile of Set 1.

Addition of EE2 with peptone mixture resulted in a decrease of maximum oxygen uptake rate from 138 mg/l.h to 48 mg/l.h according to Set 3 (Figure 4.5).

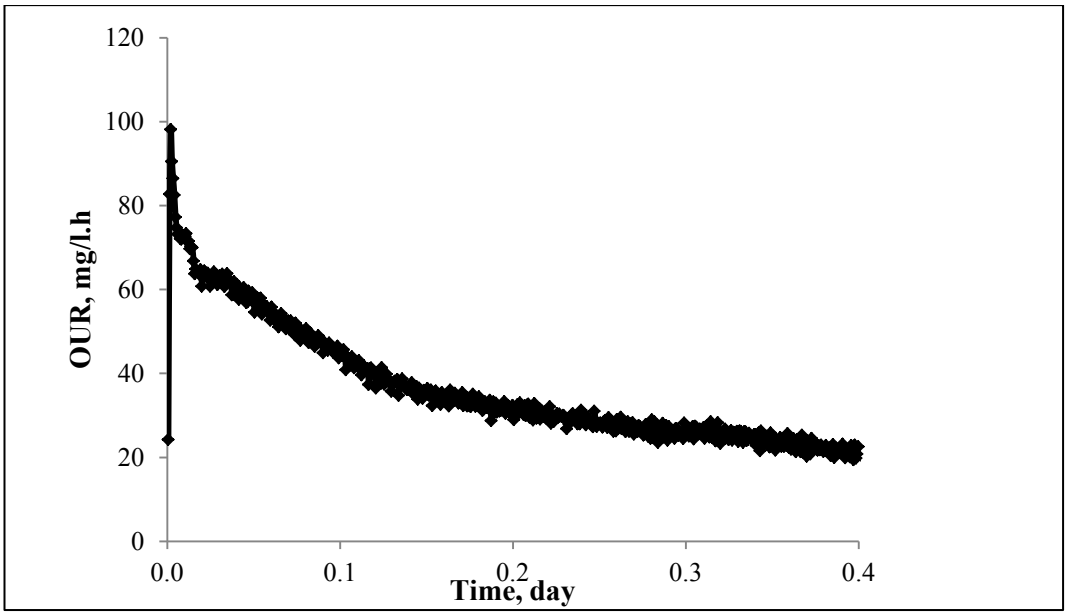

Figure 4.4 : OUR profile of Set 2.

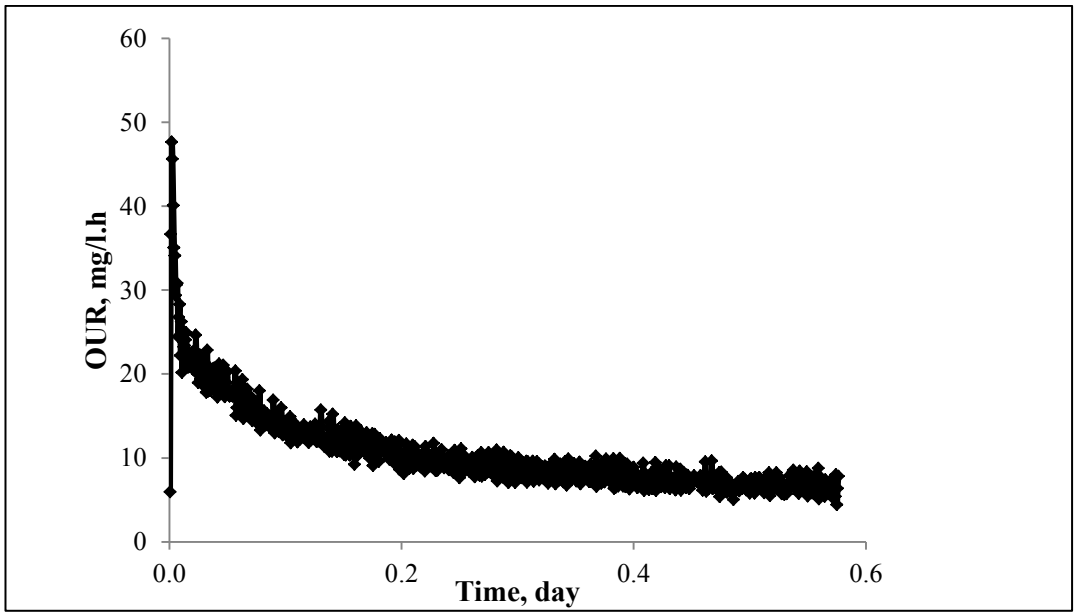

Figure 4.5 : OUR profile of Set 3.

The results indicate that EE2 had an inhibitory effect of 65 % on peptone mixture acclimated activated sludge. However, the trend of the OUR profile remained the same (Figure 4.3-4.5). It can be concluded that the acute addition of different dosage of EE2 affects the maximum oxygen uptake rate leading to an inhibition effect.

EE2 removal and PHA storage did not observed. pH of the system was at neutral level during the experiments. COD was removed from 360 mg/l to 17 mg/l. However the trend of peptone COD degradation did not change, it remained the same (Figure 4.2).

4.2 Chronic Experiments

Chronic period took 40 days. During chronic period, the reactor was fed everyday with the peptone mixture having 600 mg COD/l and 1 mg/l of 17 alpha-ethinylestradiol solution. The reactor was monitorized during 40 days. Figure 4.6-4.8 illustrate the data obtained throughout the chronic period.

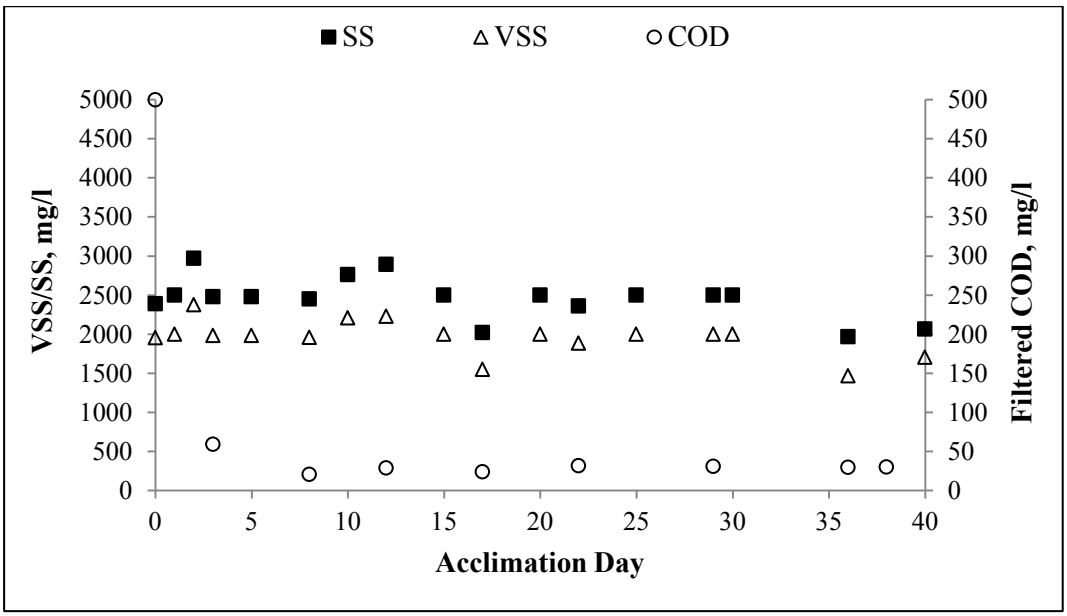

Figure 4.6 : Monitoring results of the chronic period.

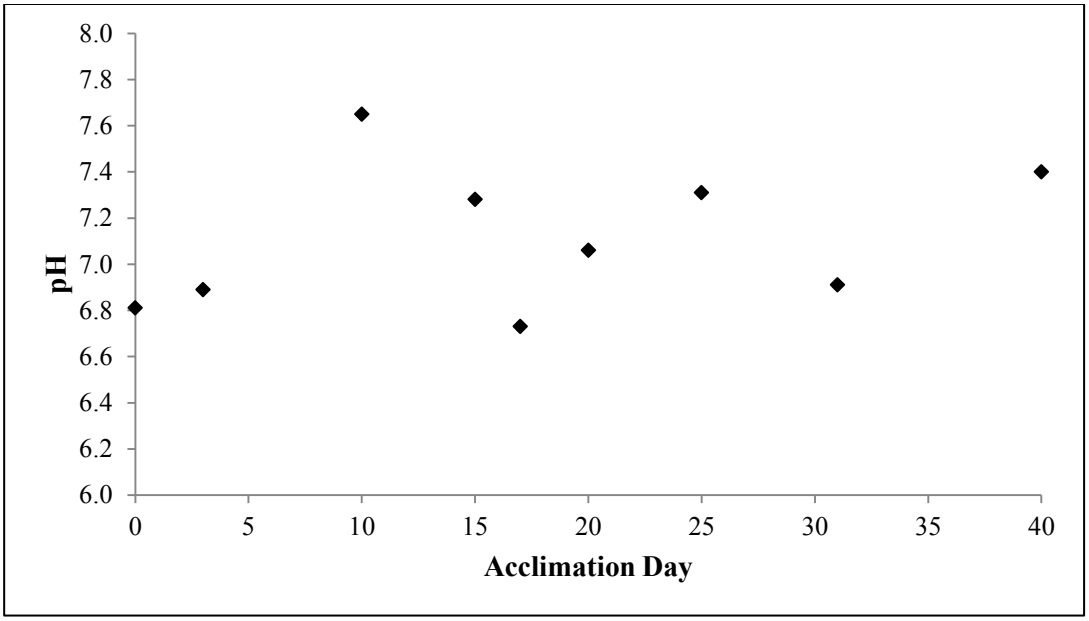

Figure 4.7 : pH versus time.

Conventional parameters such as; COD, SS, VSS and pH were measured during 40 days for the reactor. COD removal efficiency was measured 95 % and the average pH was 7.2. The average VSS of the reactor during 40 days was measured 1960±200 mg/l.

Figure 4.8 illustrate the storage capacity of biomass during chronic period. It was observed that the average PHAs storage amount in the reactor was about 37 mg COD/l till twentyfifth day. The reactor, having an inital feed of 600 mg/l total COD, it can be concluded that the biomass can only store 6 % of total COD approximately. Storage compound analysis supported that the system stored small amount of PHAs and the storage mechanism is not an important factor in this system.

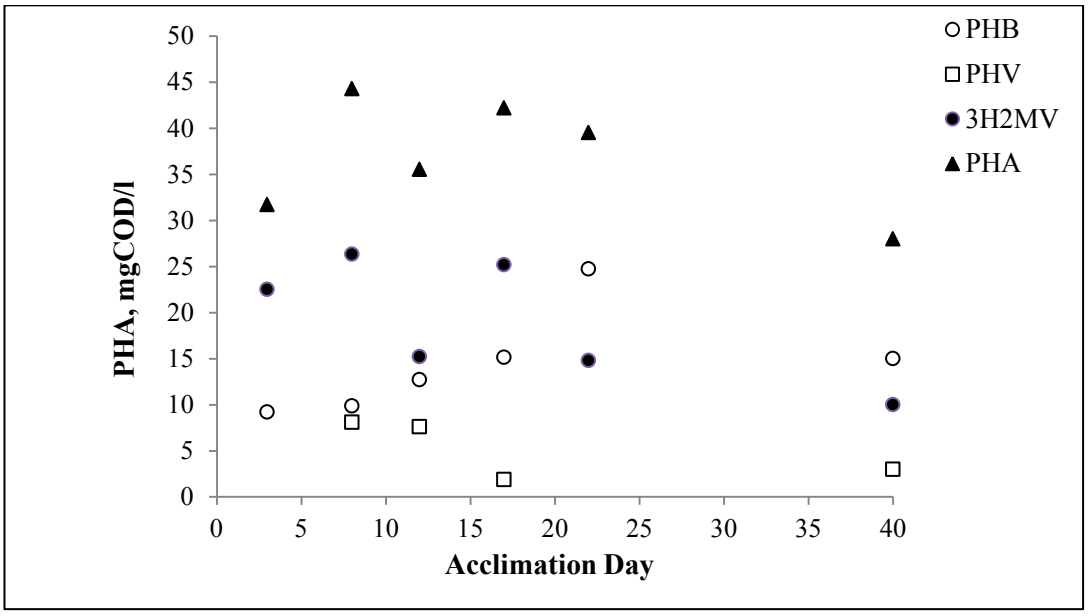

Figure 4.8 : PHA versus time.

Table 4.2 : EE2 amounts in aqueous and solid phase (Reactor).

Day	Aqueous Phase (EE2, µg/l)	Solid Phase (EE2, µg/g)
0	1000	-
3	667	-
12	83.7	4.0
22	278.5	3.8
36	100.3	3.7
38	165.4	3.7

Table 4.2 shows aqueous and solid phase concentrations of EE2 in the operated reactor. The average solid phase concentration of EE2 was 3.8 µg/g.

The recovery during EE2 measurements was 120 % for 100 µg/g. The results indicate that EE2 was not accumulated on the solid phase but it was found in soluble form in the aqueous phase as cited in Racz et al. (2012). Figure 4.9 illustrate that EE2 effluent concentrations in the reactor decrease during chronic period. The initial feeding concentration of EE2 to the reactor was 1000 µg/l (1 mg/l). The aqueous phase concentration of EE2 resulted in a decrease of 1000 µg/l to 165 µg/l. This result indicates that the removal efficiency of EE2 was 84 %.

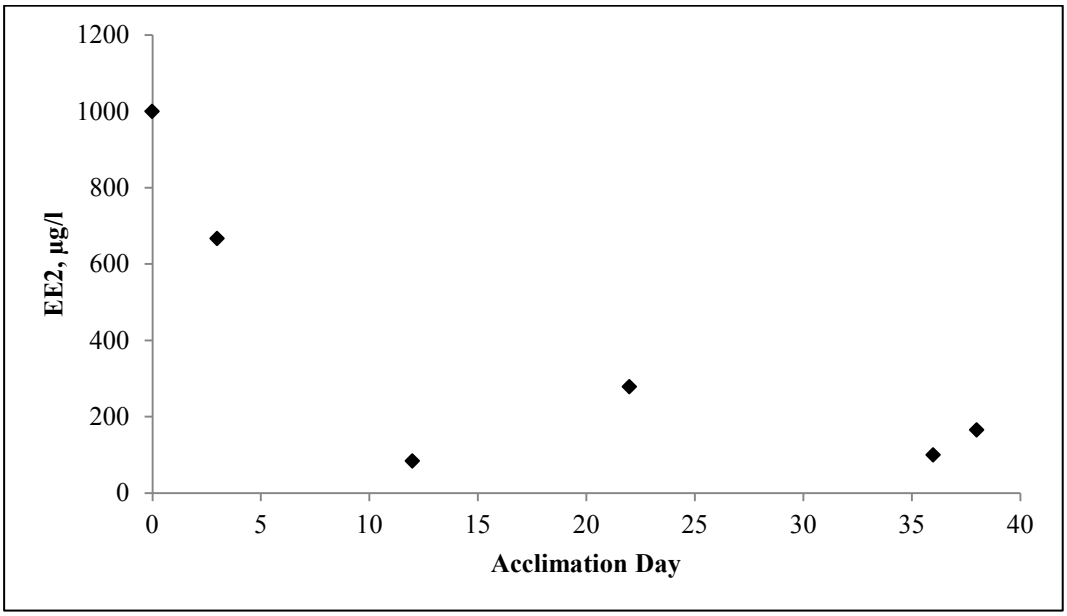

Figure 4.9 : EE2 effluent concentrations versus time (Reactor).

Notwithstanding there were noticeable EE2 removals that could be attributed to nitrifiers, heterotrophs are active for EE2 removal. Heterotrophic bacteria in the activated sludge processes have a variety of enzymes that can dominate micropollutant biotransformations and they can readily degrade EE2 by using it as a carbon source. This suggests that the differences in microbial populations may affect abilities of bioreactors to degrade estrogens. These explanations support the observations of this thesis study that not only can nitrifiers degrade EE2 but heterotrophs can also significantly contribute to EE2 transformation (Racz et al., 2012).

Twelve respirometric experiments were performed to monitor the chronic period and the behavior of EE2 to wastewater. 360 mg COD/l synthetic peptone mixture and 1 mg/l EE2 solution were fed to the respirometer during each experiment. S_o/X_o ratio kept the same (0.30) approximately for each experiment.

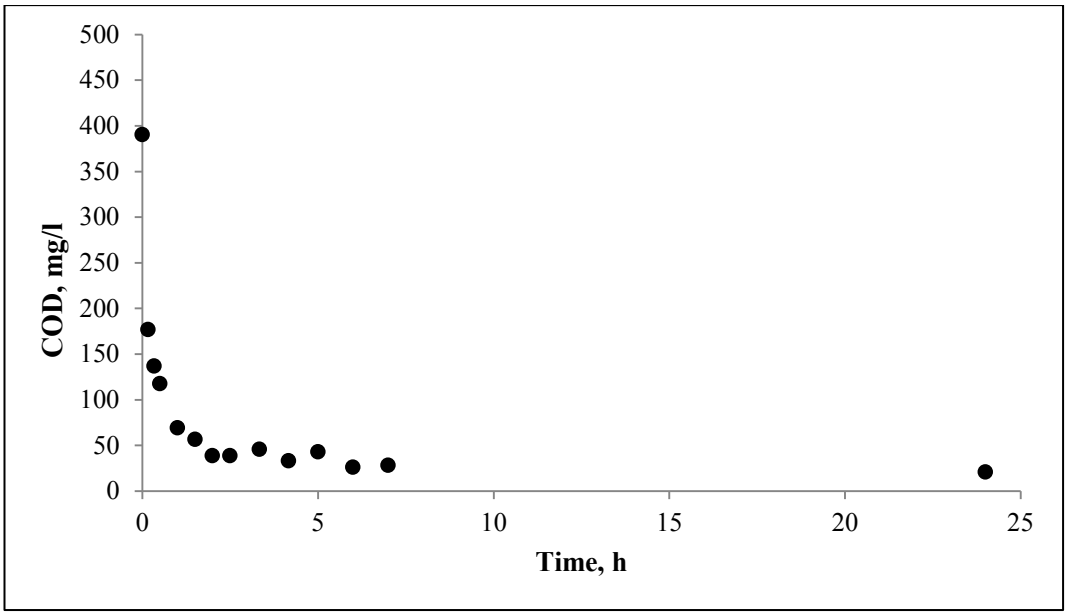

Figure 4.10 : Filtered COD concentration versus time (Set 4).

A control experiment was conducted before EE2 feeding (before first day) to the reactor (Set 4).

Peptone mixture alone was fed to the respirometer for the control experiment as a carbon source. Figure 4.10-4.13 illustrate the data obtained from the control experiment (Set 4).

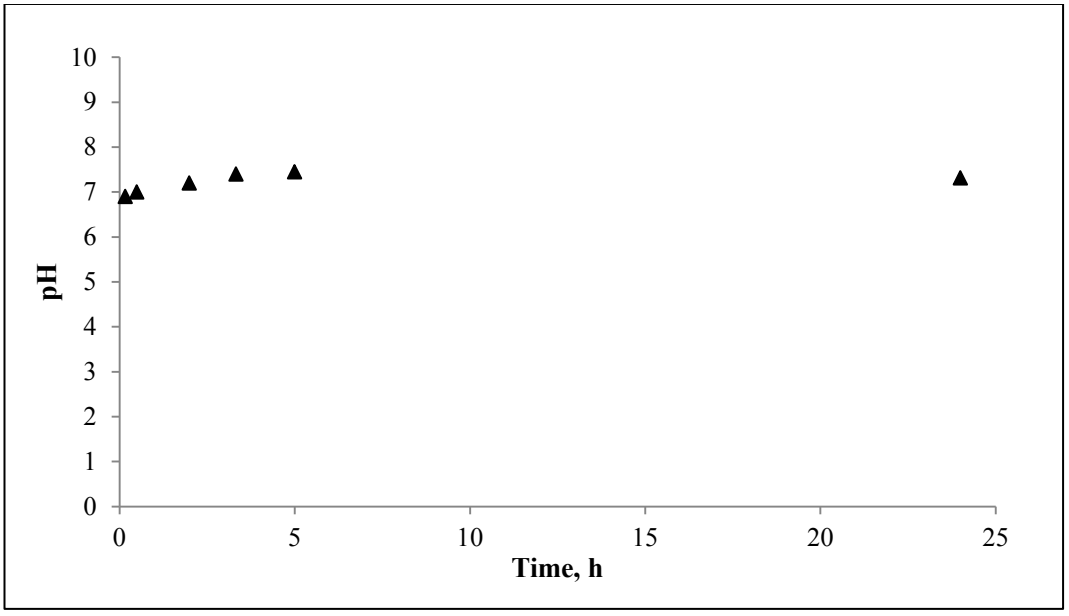

Figure 4.11 : pH versus time (Set 4).

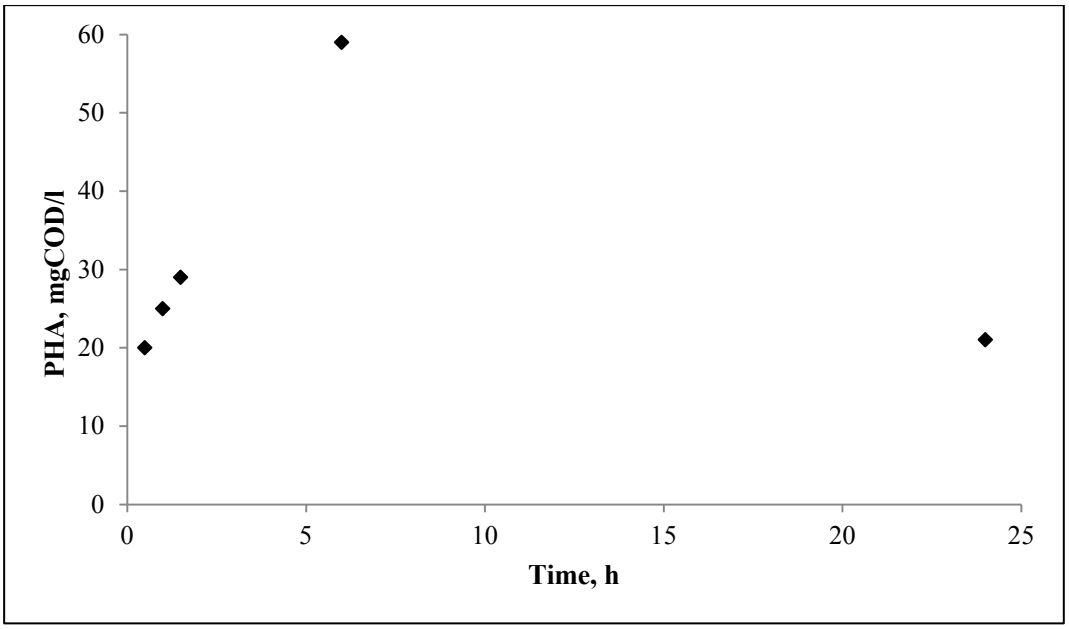

Figure 4.12 : PHA versus time (Set 4).

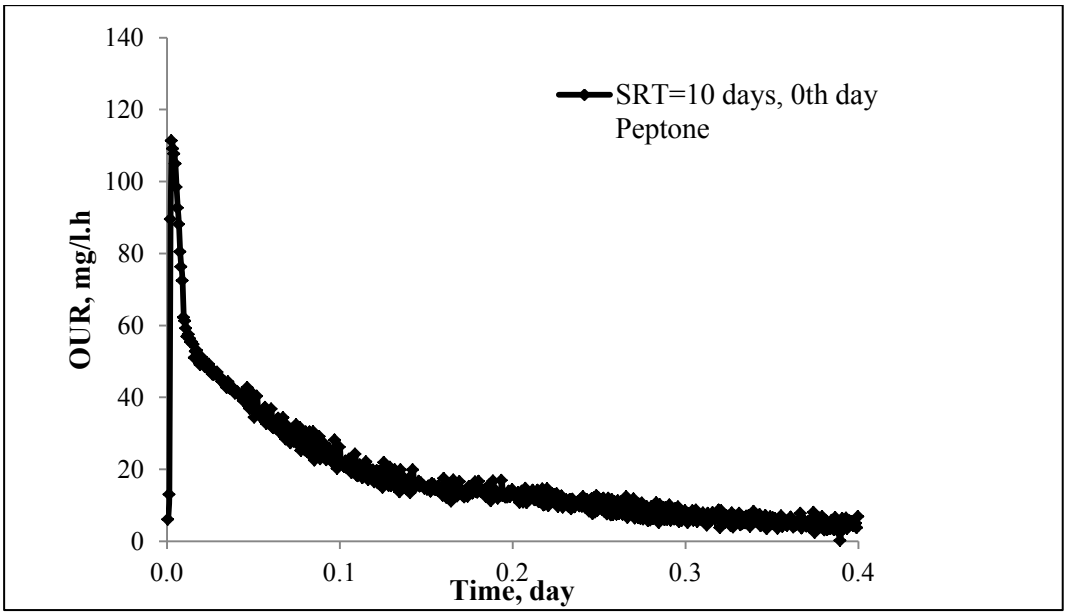

Figure 4.13 : OUR profile of Set 4.

After the control experiment, the peptone mixture and EE2 were started to be fed together with the peptone mixture acclimated sludge reactor during 40 days.

During 40 days, the sludge age of the reactor was kept 10 days and the hydraulic retention time was kept 1 day. Set 5 represents the experiment of first time addition of EE2 to the reactor.

The results obtained from Set 5 are illustrated in Figure 4.14-4.16. As regards to COD removal, the degradation efficiency did not change with respect to the control experiment (Figure 4.14).

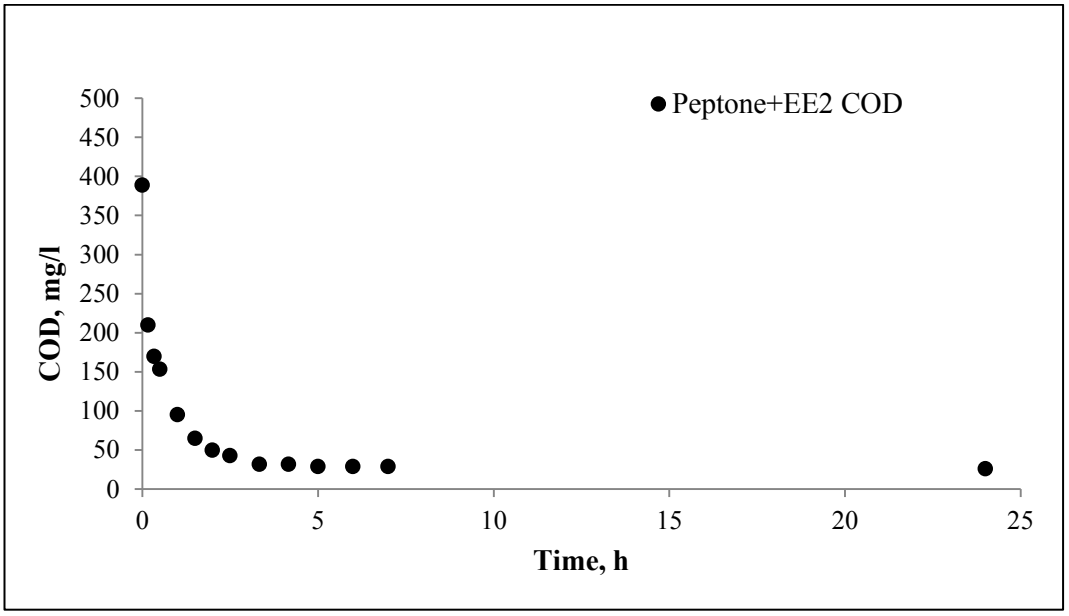

Figure 4.14: Filtered COD concentrations versus time (Set 5).

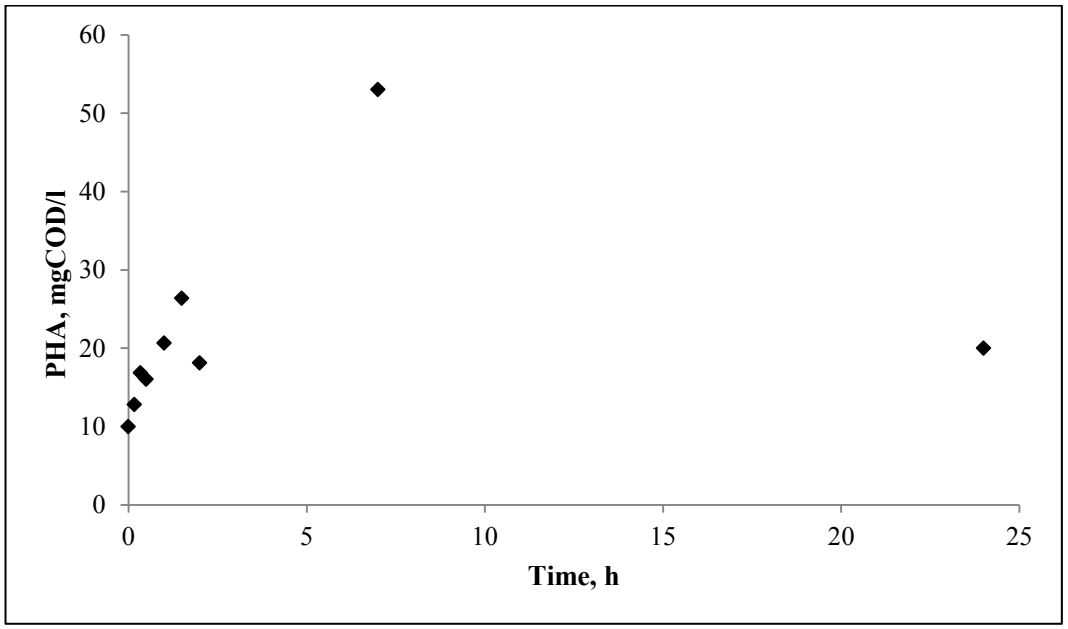

Figure 4.15 : PHA versus time (Set 5).

PHAs concentrations were also observed during the experiment. Initial PHAs concentration was 10 mg COD/l and it increased to 53 mg COD/l then decreased to its initial concentration at the end of the experiment. pH was at neutral level during Set 5.

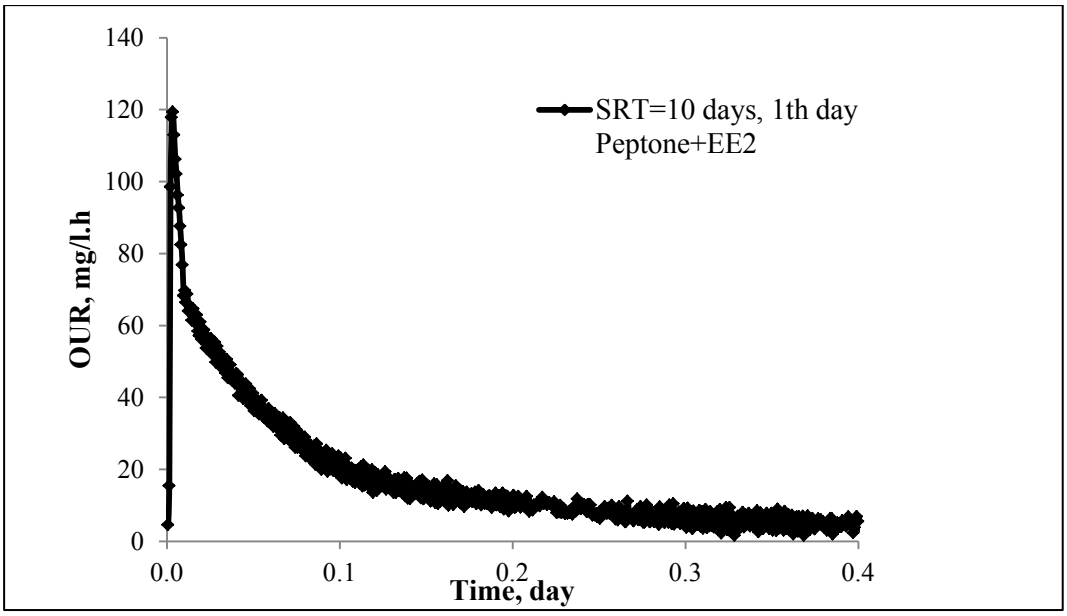

Figure 4.16 : OUR profile of Set 5.

Addition of EE2 with peptone mixture resulted in an increase of maximum oxygen uptake rate from 111 mg/l.h to only 119 mg/l.h. This increase is not very high. The trend of the OUR profile remained the same by comparison with the control experiment (Figure 4.16).

Set 6 represents fifth day of EE2 addition to the peptone mixture acclimated sludge reactor. The results obtained from Set 6 are illustrated in Figure 4.17-4.18.

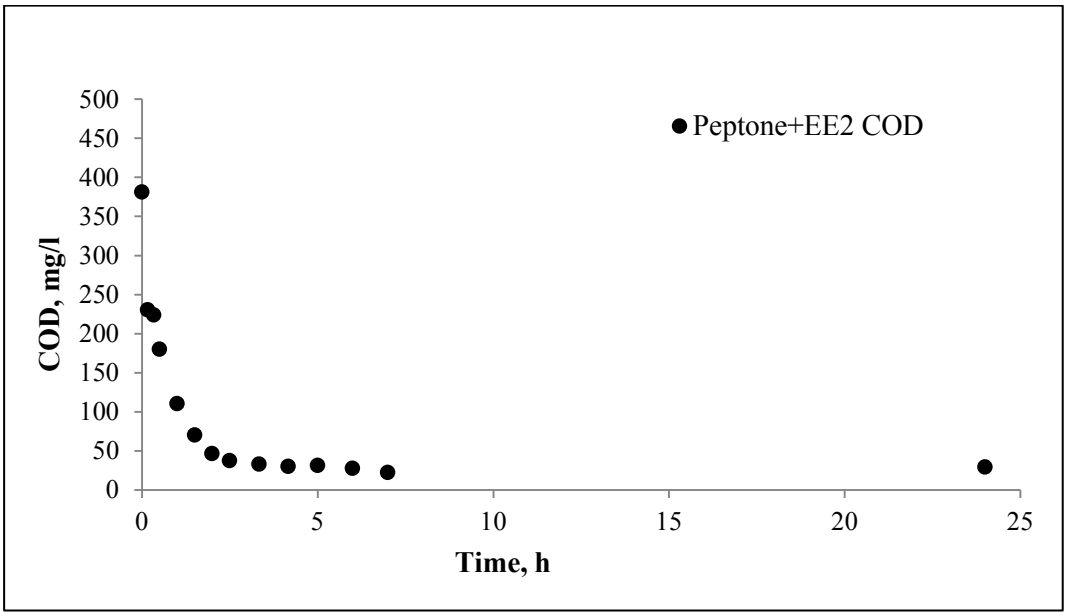

Figure 4.17 : Filtered COD concentrations versus time (Set 6).

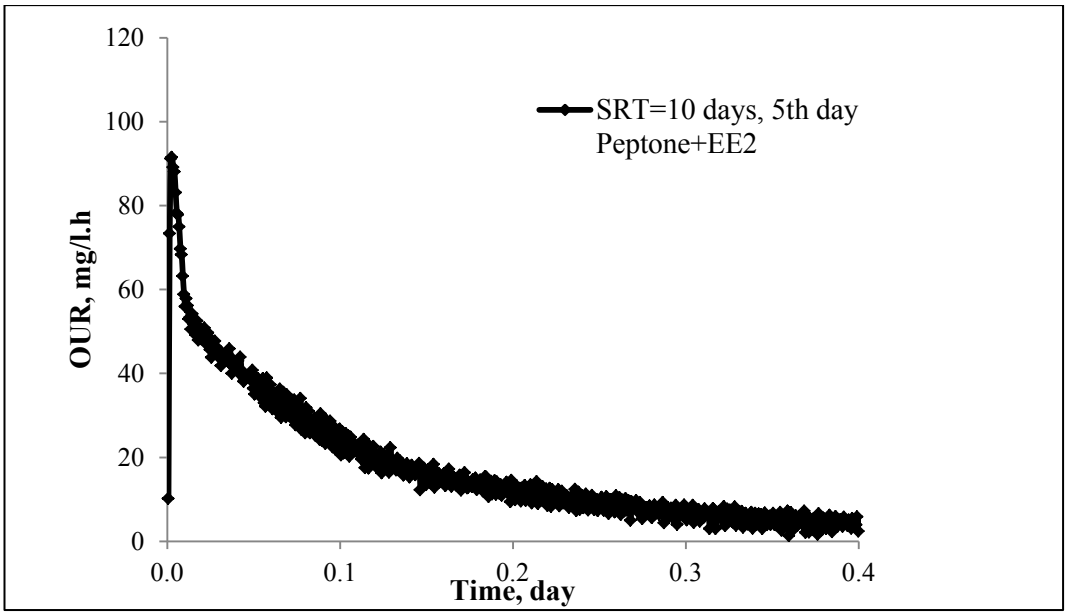

Figure 4.18 : OUR profile of Set 6.

Oxygen uptake rate decreased to 92 mg/l.h on the fifth day of chronic period. However, the trend of the OUR profile remained the same (Figure 4.18).

As regards to COD removal, the degradation efficiency had not a big change with respect to the control and first day experiment. The removal efficiency was about 90 % (Figure 4.17). The average pH was about 7.5 during the fifth day experiment. Also, the effluent aqueous phase concentration of EE2 was measured. It was determined as 169 µg/l.

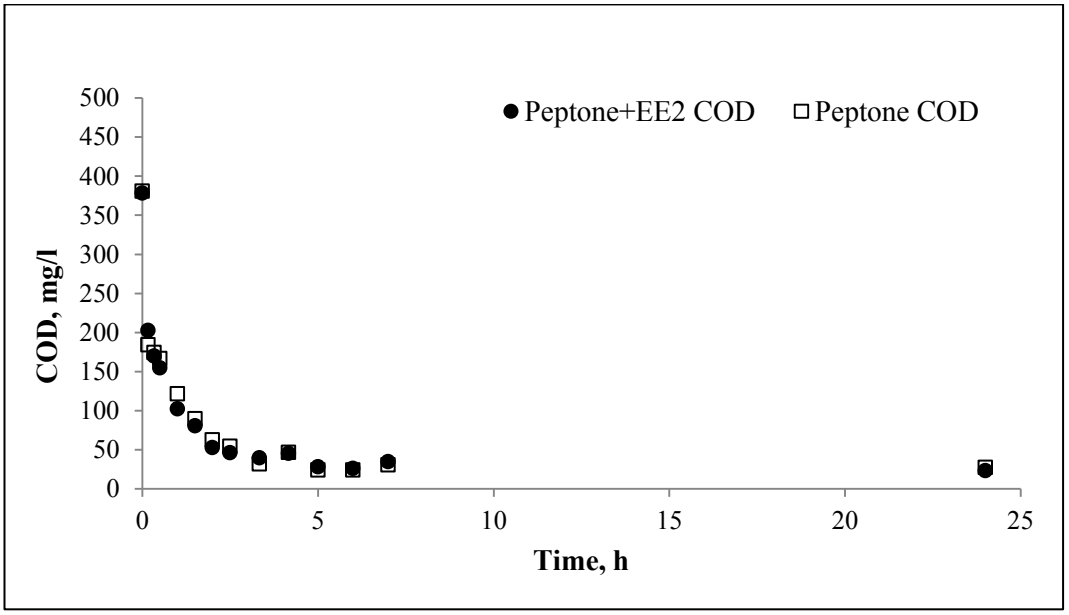

Figure 4.19 : Filtered COD concentrations versus time (Set 7 – Set 7.1).

Set 7 and Set 7.1 represents tenth day of EE2 addition to the peptone mixture acclimated sludge reactor. The results obtained from Set 7 and Set 7.1 are illustrated in Figure 4.19-4.25.

On the tenth day of EE2 addition, two experiments were conducted in parallel by the addition of the peptone mixture and EE2 together (Set 7) and the peptone mixture alone (Set 7.1) to the respirometer as carbon source (Figure 4.20, 4.21).

As regards to COD removal, the degradation efficiency did not change. The trend of COD degradation for the two experiments did not change (Figure 4.19).

The average pH was about 7.2 during tenth day experiments.

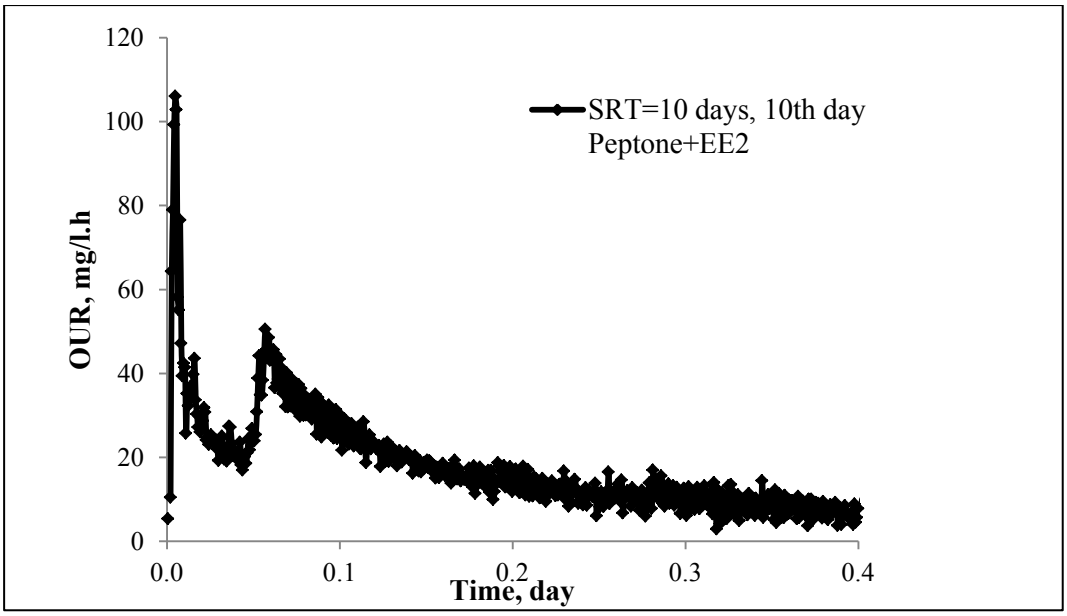

Figure 4.20 : OUR profile of Set 7.

Oxygen uptake rate decreased to 106 mg/l.h on the tenth day of chronic period (Figure 4.22). However, it increased to 129 mg/l.h when the peptone mixture was added alone as carbon source (Figure 4.21).

The result indicates that EE2 can prevent the oxygen uptake of biomass related with the maximum heterotrophic growth rate and the active heterotrophic biomass.

The solid phase concentration of EE2 was measured at the end of Set 7. It was measured as 5 µg/g. Due to its hydrophobic structure as indicated in the literature, EE2 is not accumulated on sludge.

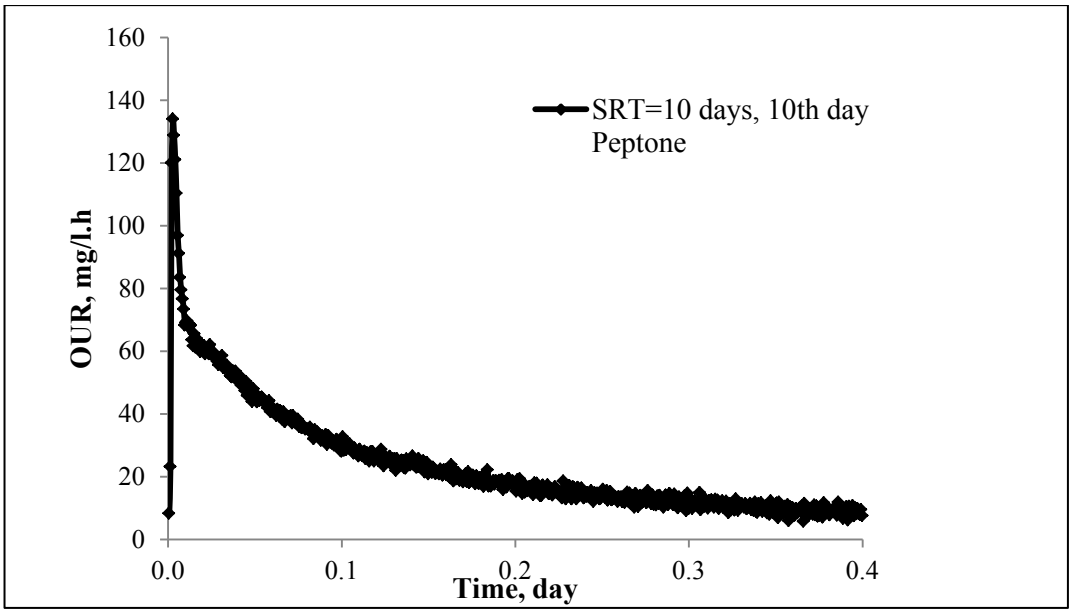

Figure 4.21 : OUR profile of Set 7.1.

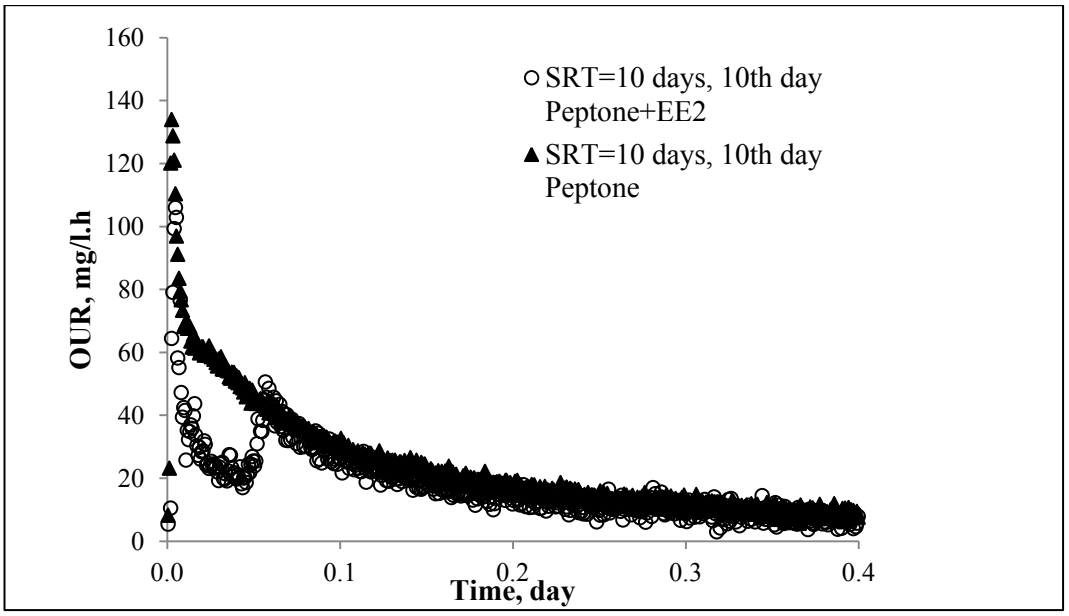

Figure 4.22 : OUR profile comparison of Set 7 and Set 7.1.

Set 8 represents fifteenth day of EE2 addition to the peptone mixture acclimated sludge reactor.

The results obtained from Set 8 are illustrated in Figure 4.23 and in Figure 4.24.

Figure 4.23 shows the COD degradation efficiency of Set 8. As regards to COD removal for Set 8, the degradation efficiency did not change. The removal efficiency was about 94 % (Figure 4.23). The trend of COD degradation did not change when compared to other experiments.

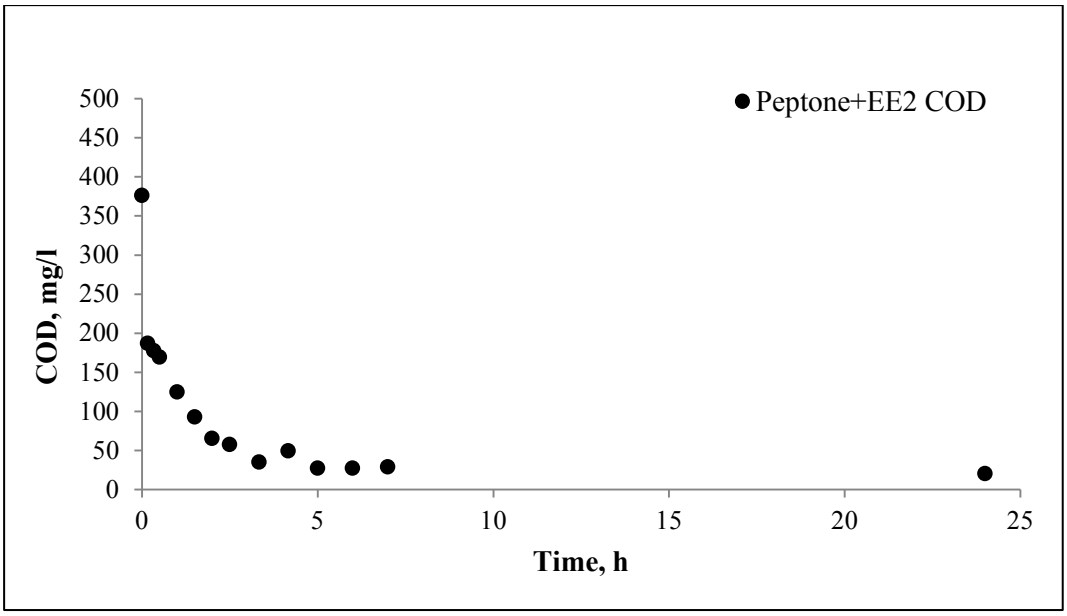

Figure 4.23 : Filtered COD concentrations versus time (Set 8).

Oxygen uptake rate increased to 119 mg/l.h as it was on the first day of EE2 addition. The trend of the OUR profile remained the same (Figure 4.24). The average pH was about 7.3 during the fifteenth day experiment. On the fifteenth day, the effluent aqueous phase concentration and the solid phase concentration of EE2 were measured. The aqueous phase concentration was 252 µg/l however the solid phase concentration was only 3.7 µg/g.

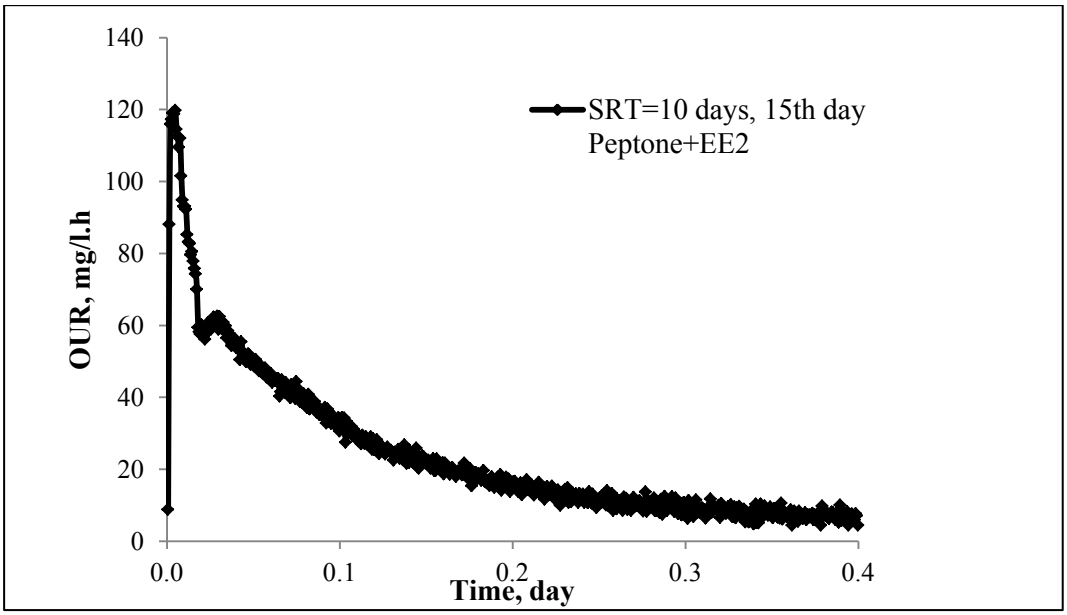

Figure 4.24 : OUR profile of Set 8.

Set 10 represents twentyfifth day of EE2 addition to the peptone mixture acclimated sludge reactor. The results obtained from Set 10 are illustrated in Figure 4.25, 4.26.

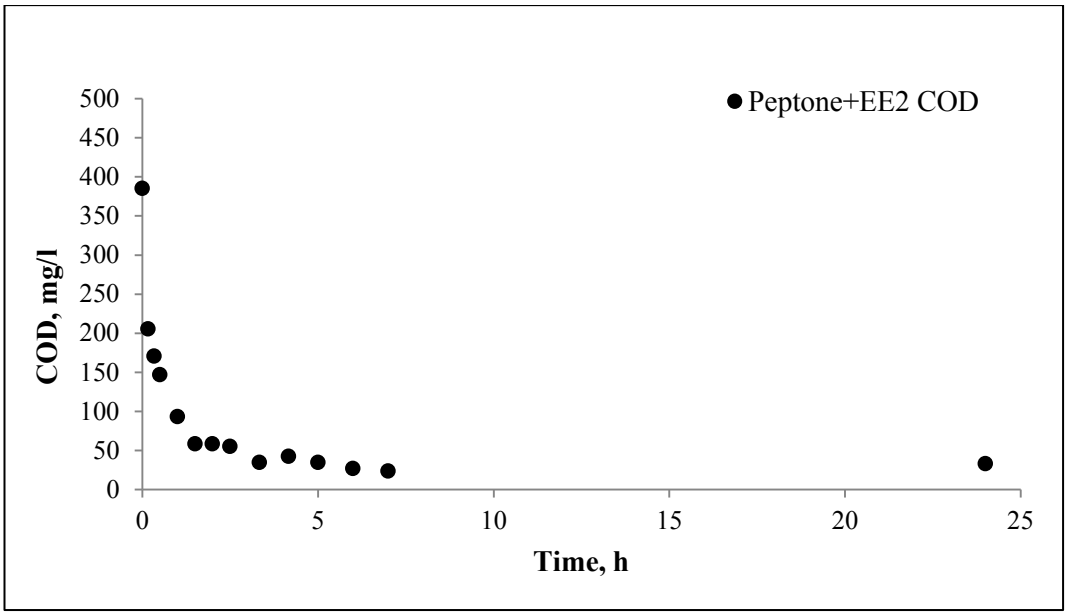

Figure 4.25 : Filtered COD concentrations versus time (Set 10).

As regards to COD removal, the degradation efficiency did not change. The trend of COD degradation also did not change and the removal efficiency was 90 % (Figure 4.25). pH was at neutral level.

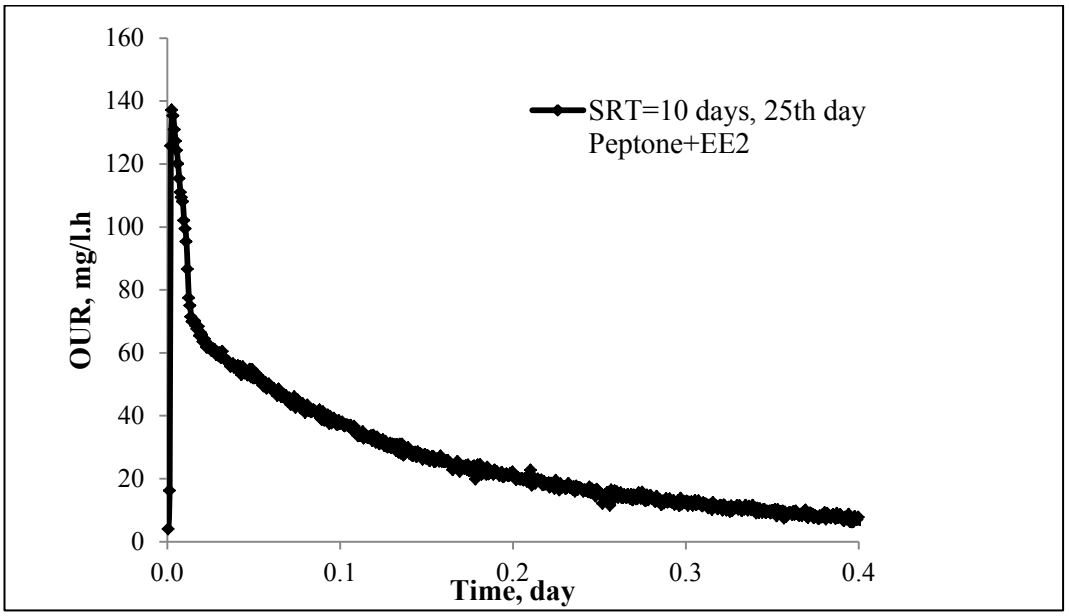

Figure 4.26 : OUR profile of Set 10.

On the twentyfifth day of the chronic period, the maximum oxygen uptake rate decreases and it was measured as 137 mg/l.h. The trend of the OUR profile did not change (Figure 4.26).

The solid phase concentration of EE2 was measured at the end of Set 10. It was measured as 3.9 µg/g indicating that EE2 is not accumulated on sludge.

Set 11 and Set 11.1 represents thirtieth day of EE2 addition to the peptone mixture acclimated sludge reactor.

The results obtained from Set 11 and Set 11.1 are illustrated in Figure 4.27-4.30.

On the thirtieth day of EE2 addition, two experiments were conducted in parallel by the addition of the peptone mixture and EE2 together and the peptone mixture alone to the respirometer.

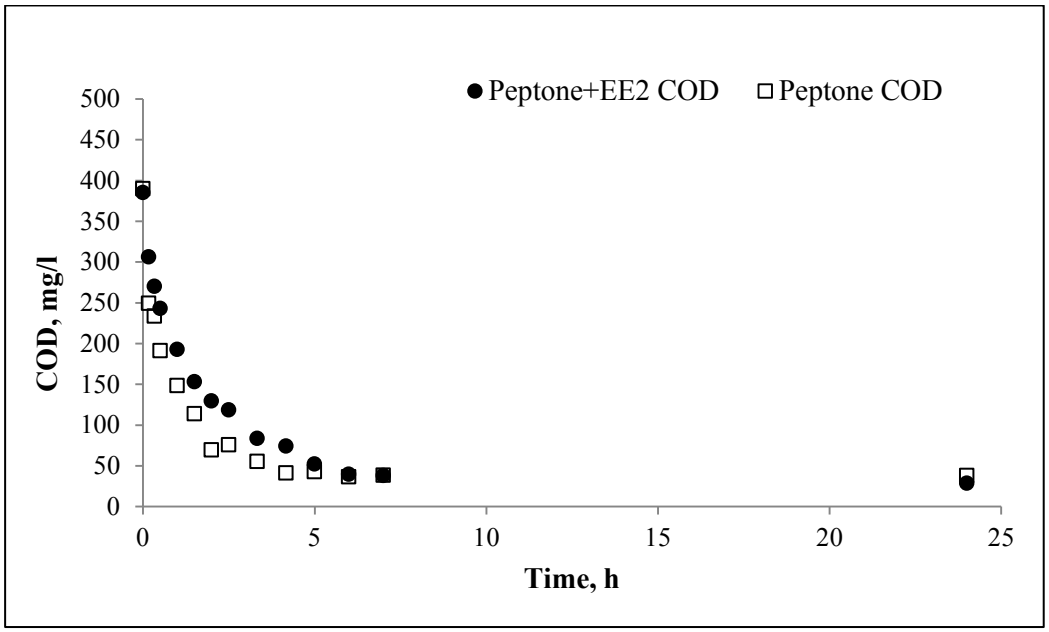

Figure 4.27 : Filtered COD concentrations and pH values versus time (Set 11-Set 11.1).

As regards to COD removal, the degradation efficiency did not change. The trend of COD degradation for the two experiments did not change (Figure 4.27).

The average pH was at neutral level during thirtieth day experiments.

Maximum oxygen uptake rate was measured as 136 mg/l.h when the peptone mixture and EE2 were fed together to the respirometer as carbon source (Figure 4.28).

However, it was measured as 150 mg/l.h when the peptone mixture was added alone as carbon source (Figure 4.29).

The results indicate that EE2 can prevent the oxygen uptake of biomass to some extent (Figure 4.30).

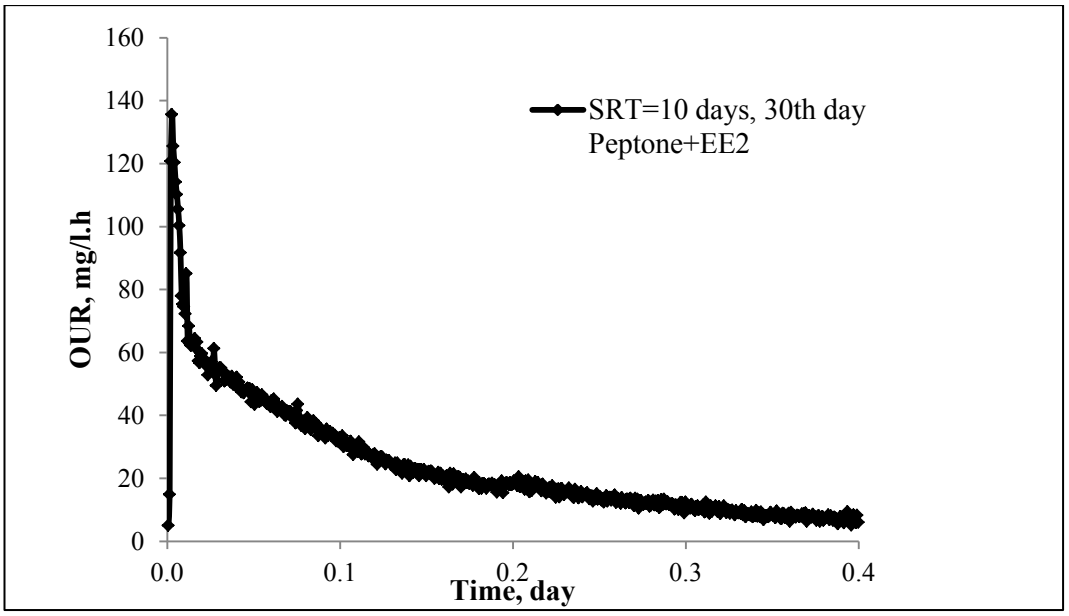

Figure 4.28 : OUR profile of Set 11.

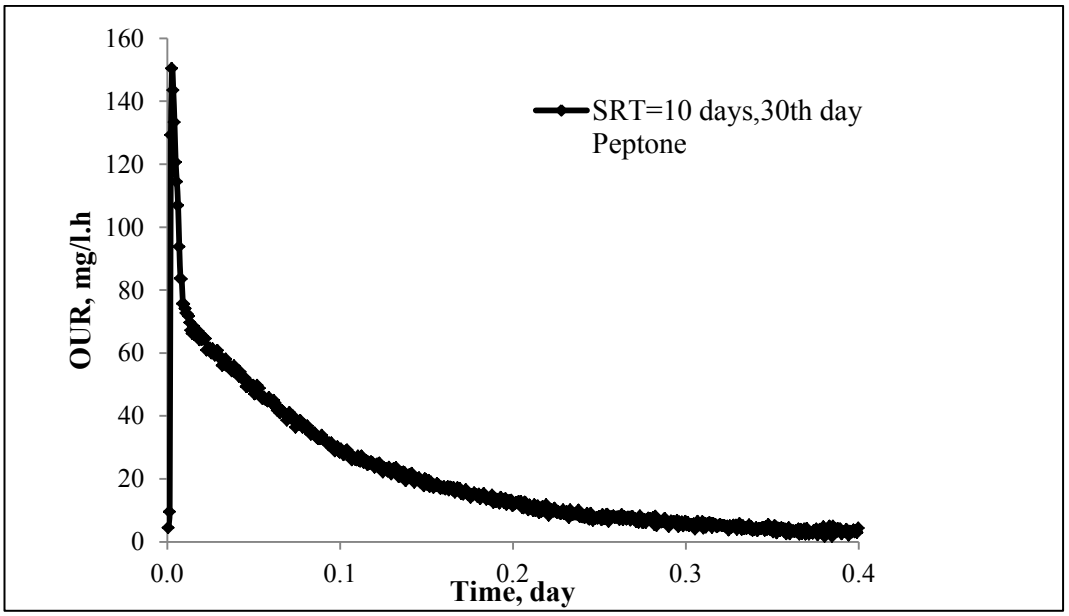

Figure 4.29 : OUR profile of Set 11.1.

The solid phase concentration of EE2 was measured at the end of Set 11. It was measured as 3.8 µg/g indicating that EE2 is not accumulated on sludge.

Figure 4.30 shows OUR profile comparison of Set 11 and Set 11.1. In this figure, it is clearly seen that when peptone was added as sole carbon source, the system gives higher maximum oxygen uptake rate level.

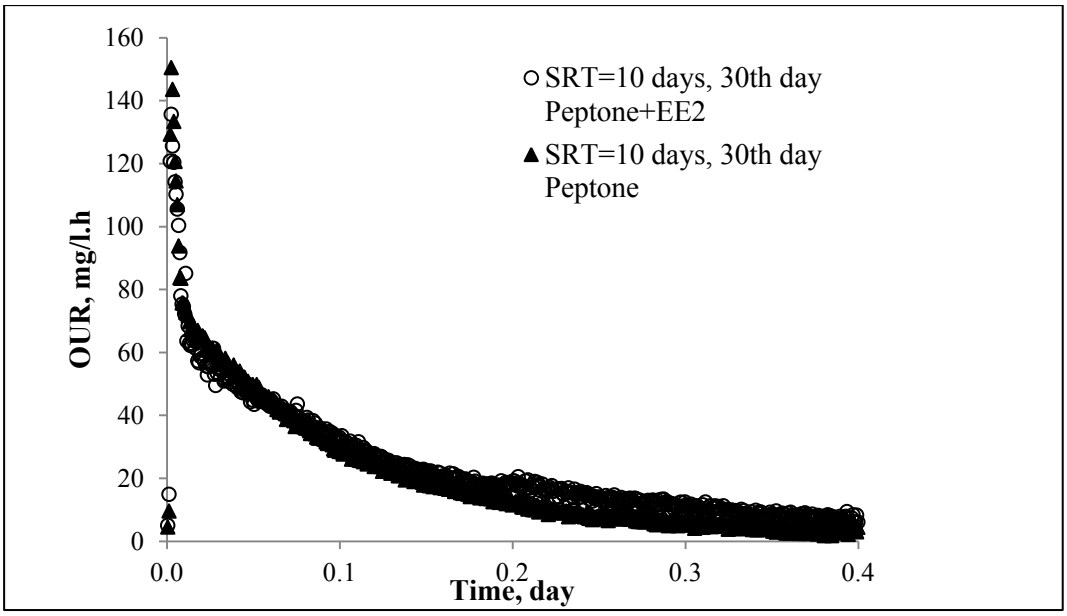

Figure 4.30 : OUR profile comparison of Set 11 and Set 11.1.

Set 12 and Set 12.1 represents fortieth day of EE2 addition to the peptone mixture acclimated sludge reactor. The results obtained from Set 12 and Set 12.1 are illustrated in Figure 4.31-4.35.

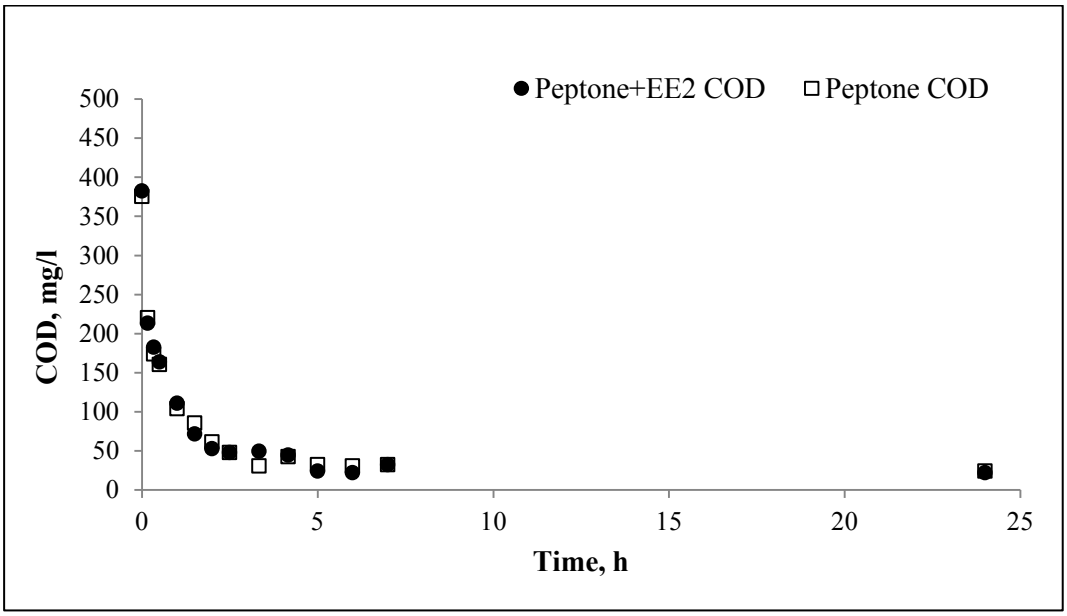

Figure 4.31 : Filtered COD concentrations and pH values versus time (Set 12-Set 12.1).

On the fortieth day of EE2 addition, two experiments again were conducted in parallel by the addition of the peptone mixture and EE2 together and the peptone mixture alone to the respirometer.

As regards to COD removal, the degradation efficiency did not change. The trend of COD degradation for the two experiments did not change (Figure 4.31). pH was at neutral level during experiments.

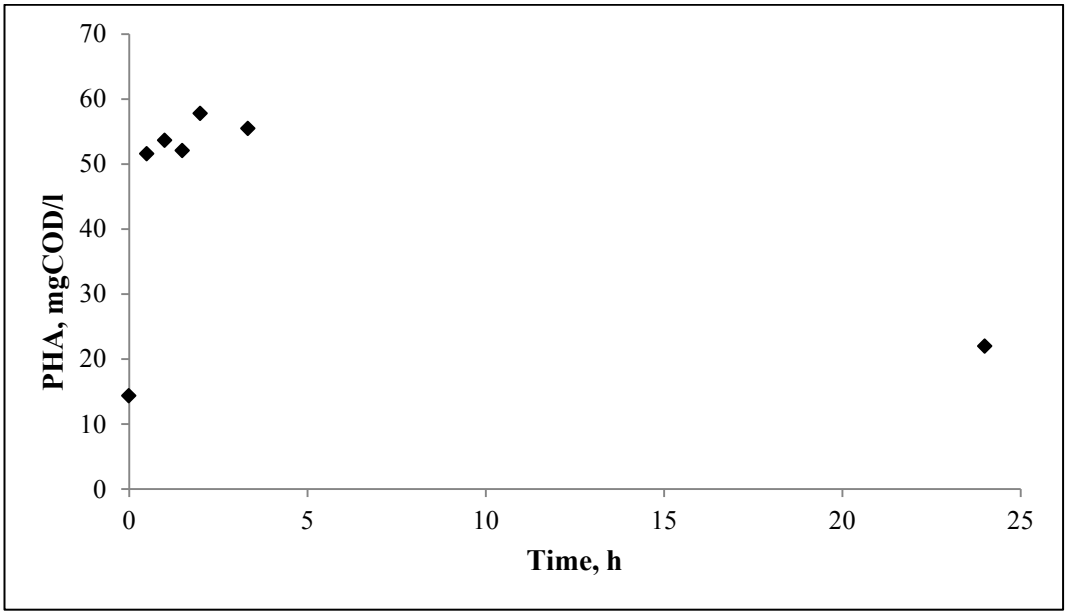

Figure 4.32 : PHA concentrations versus time (Set 12).

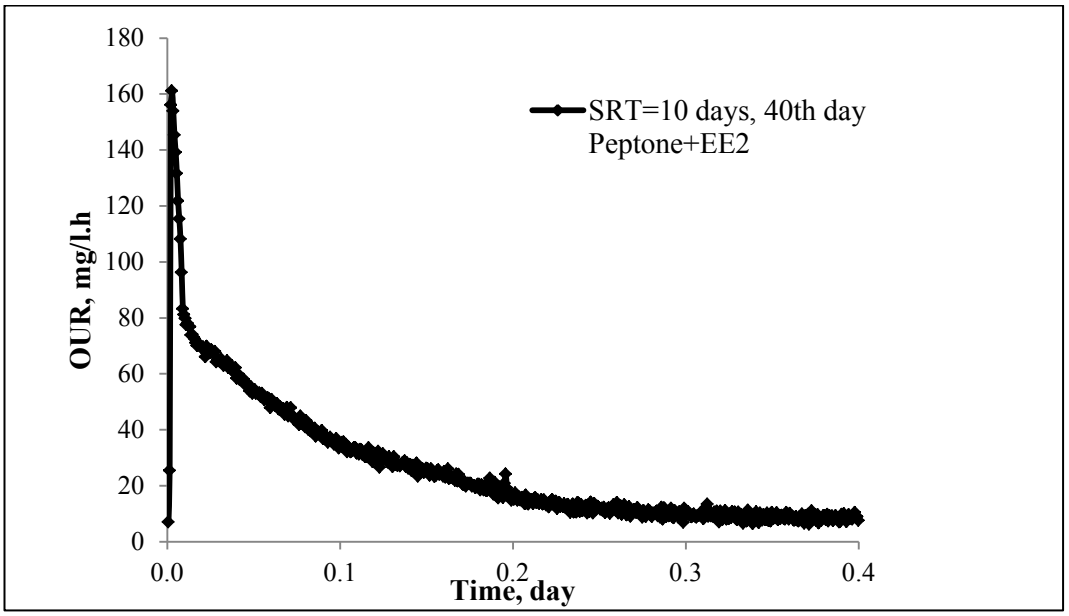

Figure 4.33 : OUR profile of Set 12.

Maximum oxygen uptake rate was measured as 161 mg/l.h when the peptone mixture and EE2 were fed together to the respirometer as carbon source (Figure 4.33). However, it was measured as 150 mg/l.h when the peptone mixture was added alone as carbon source (Figure 4.34).

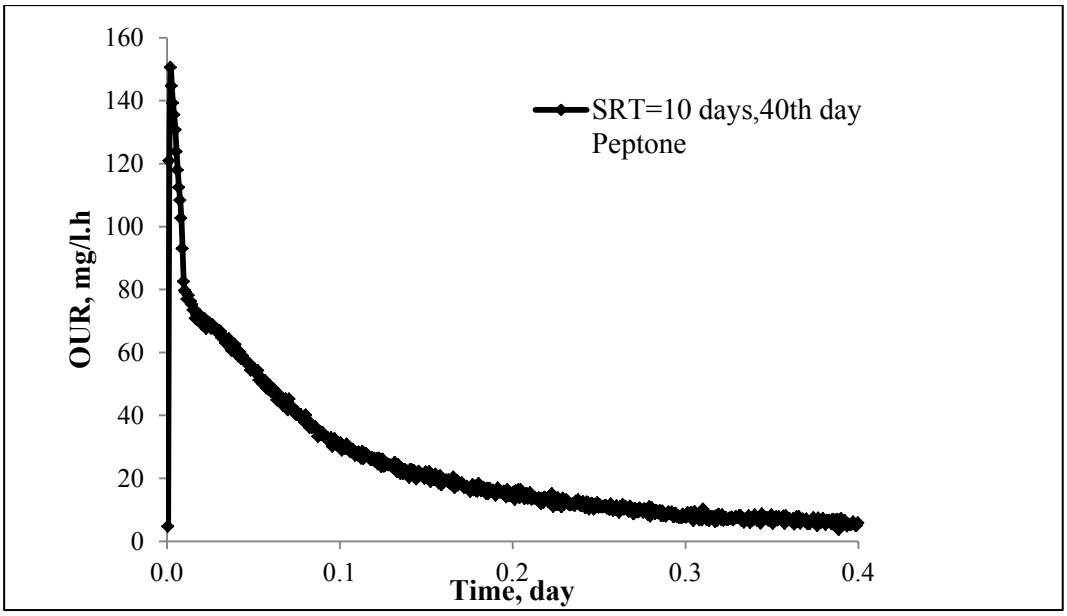

Figure 4.34 : OUR profile of Set 12.1.

PHAs concentrations were also observed during the experiment. The initial PHAs concentration for Set 12 was 14 mg COD/l (Figure 4.32). PHAs concentration increased to 58 mg COD/l then decreased to its initial concentration at the end of the experiment. It was observed that the storage process continued during the chronic period from first day till fortieth day with the acclimation of activated sludge to EE2. However storage compound analysis supported that the system stored small amount of PHAs and used available substrate for their growth resulting in high maximum growth and hydrolysis rates.

The influent and effluent aqueous phase concentrations of EE2 were measured for Set 12. The influent aqueous phase concentration was 142 µg/l however the effluent aqueous phase concentration was measured as 40.7 µg/l.

For Set 12.1, the effluent aqueous phase concentration was measured as 47.3 µg/l. This result indicates that biomass can degrade approximately the same amount of EE2 in case of EE2 and the peptone mixture were added together to the respirometer or the peptone mixture alone whenever the sludge is acclimated to EE2.

The degradation efficiency of EE2 in the aqeuous phase at the end of fortieth days experiment was observed as 71 %.

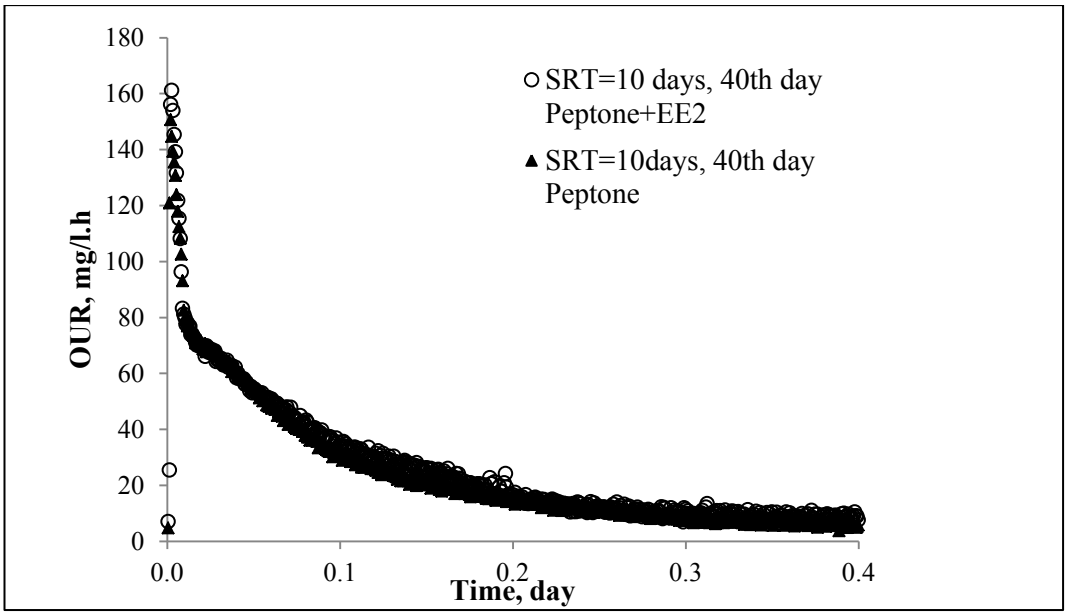

Figure 4.35 : OUR profile comparison of Set 12 and Set 12.1.

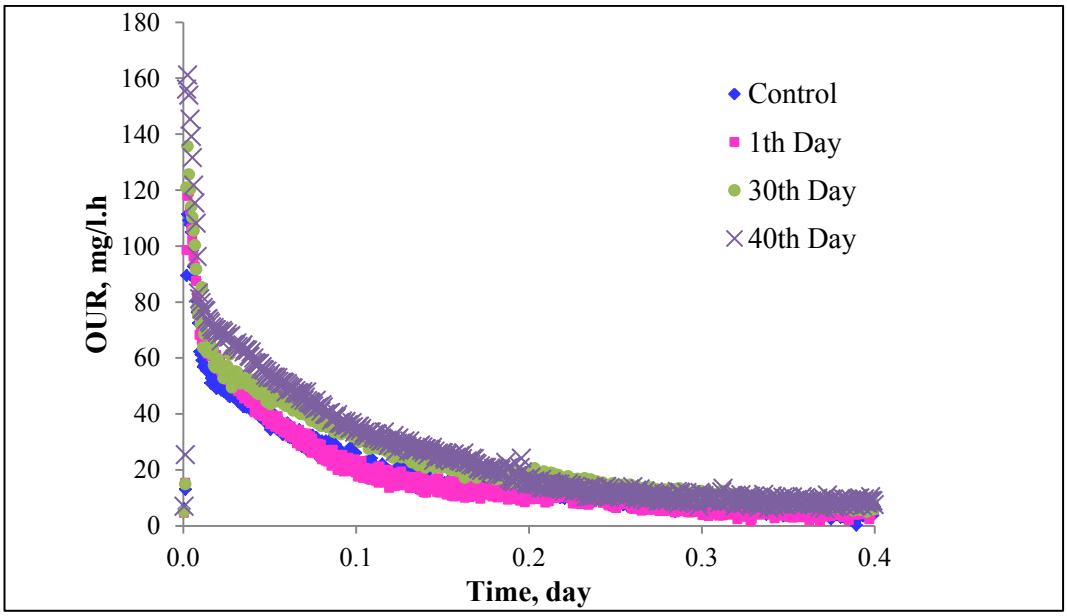

Figure 4.36 : OUR profile comparison of the chronic period.

During the chronic period, the trend of OUR profiles remained approximately the same by comparison to the control experiment (Figure 4.36).

As a result, it is clearly seen that the sludge is acclimated to EE2 at the end of the chronic period giving more elevated maximum oxygen uptake rate level (Figure 4.36).

4.3 Modelling Results

In this thesis, the Aquasim Software was used to model selected chronic experiments; Set 4, Set 5 and Set 12 in order to determine related kinetic and stoichiometric coefficients. A multi-component model referred to as modified Activated Sludge Model No.3 (ASM3) was used in modelling studies.

Modelling results of Set 4, Set 5 and Set 12 according to ASM3, were illustrated with experimental data in Figure 4.37-4.42.

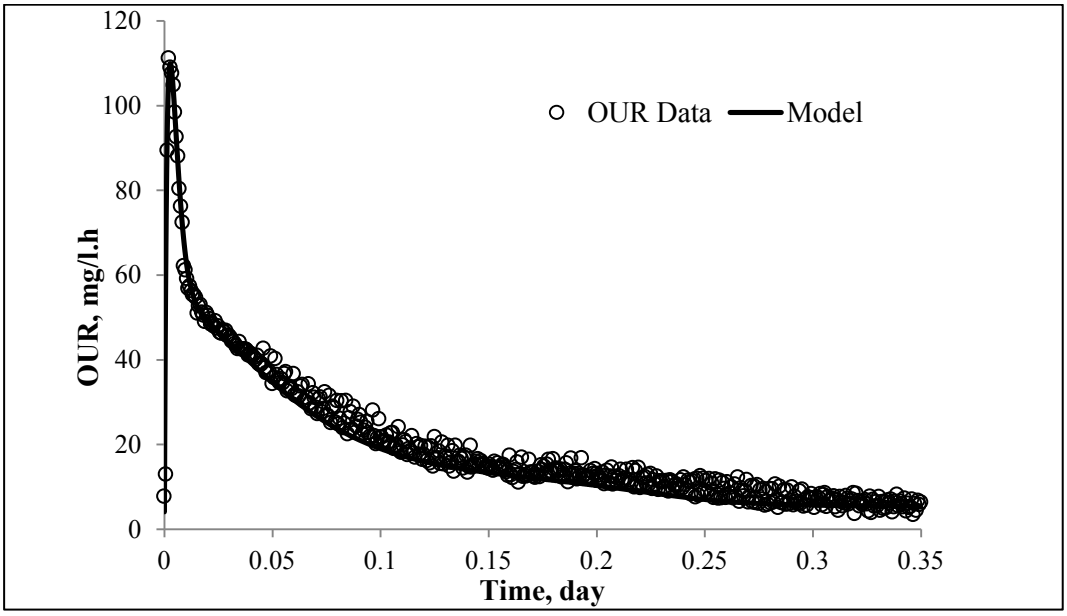

Figure 4.37 : Model simulation of OUR data for Set 4 (ASM3).

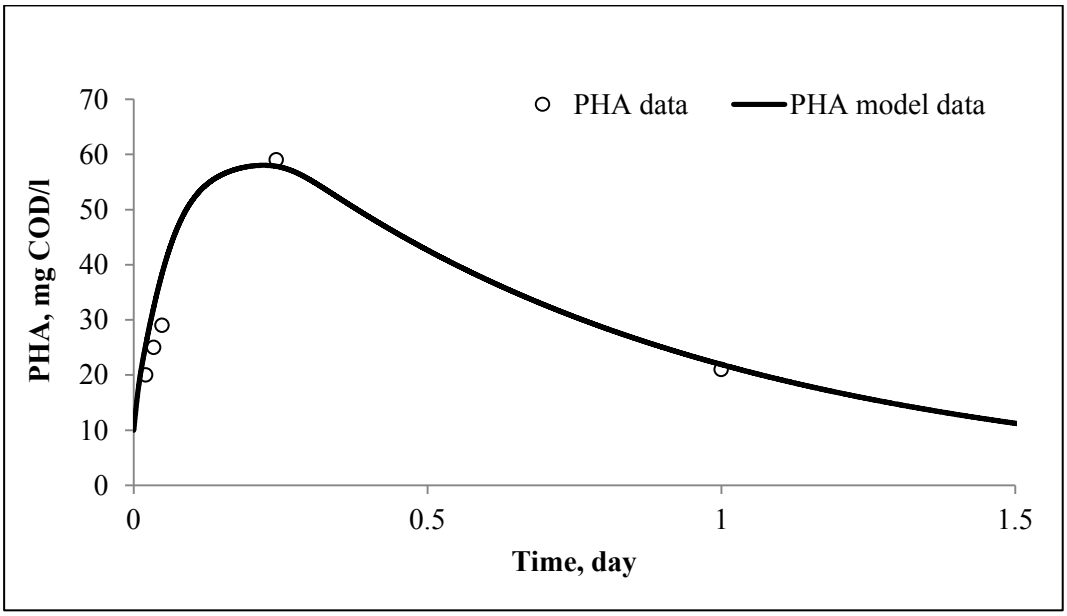

Figure 4.38 : Model simulation of PHA data for Set 4 (ASM3).

Necessary kinetic information derived from the experimental results of Set 4 and Set 5 were given in Table 4.3. Modelling simulation results of ASM3 for Set 4 and Set 5 indicate that the maximum heterotrophic growth rate, μ_H is 6.8 day^{-1}, half saturation constant for growth, K_s is 24 mg COD/l and endogenous decay rate, b_H is 0.1 day^{-1}. Simulation estimations used for Set 4 and Set 5 are findable in the study of Katipoglu et al. (2012).

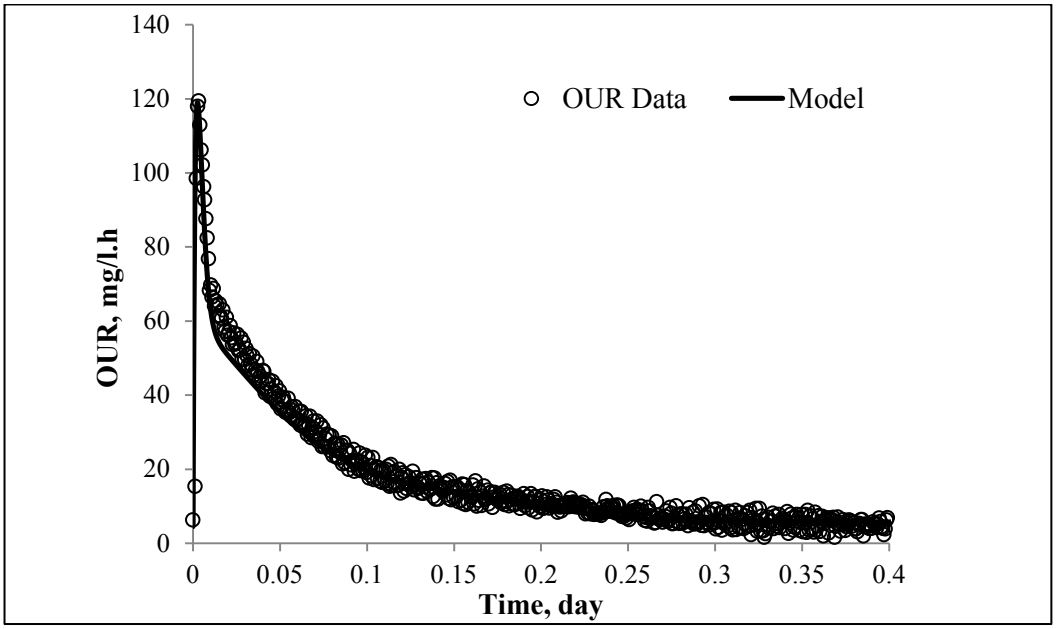

Figure 4.39 : Model simulation of OUR data for Set 5 (ASM3).

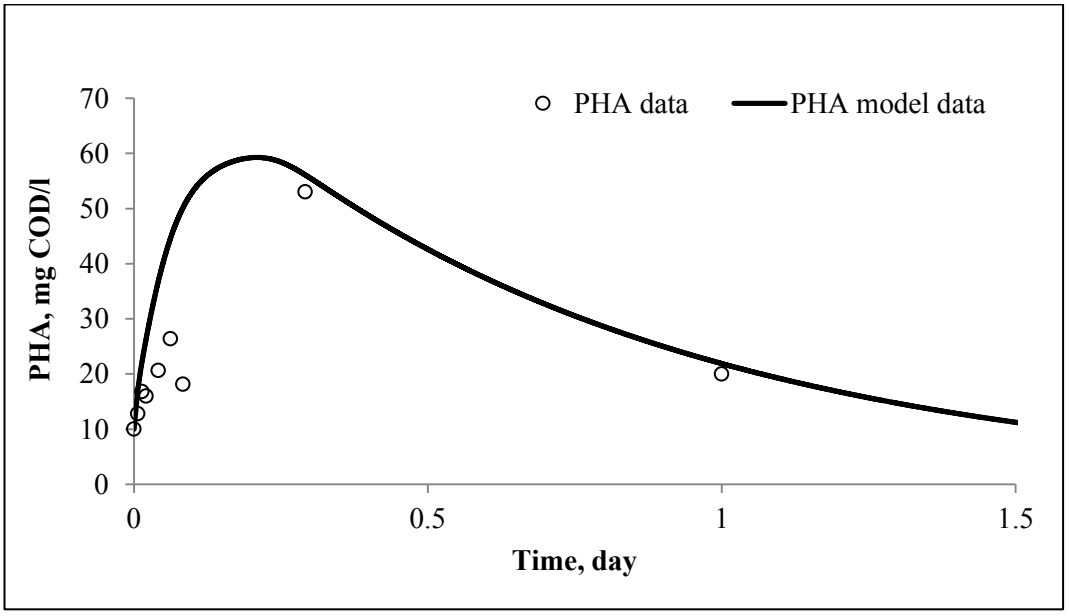

Figure 4.40 : Model simulation of PHA data for Set 5 (ASM3).

Previous evaluations indicated that the peptone mixture was quite similar to domestic sewage in terms of COD fractionation, mainly involving two slowly biodegradable COD components S_H and X_s with different hydrolysis rate and a small readily biodegradable COD (S_s) fraction (Katipoglu et al., 2012).

The estimated kinetic parameters for Set 4 and Set 5 were similar to reported values for domestic sewage by Sözen et al. (1998).

Generally, the maximum heterotrophic growth rate range is reported as 0.6-3.75 day^{-1} for domestic sewage (Orhon and Artan, 1994) whereas a much wider μ_H range of 1.5-7.0 day^{-1} and 20 mg COD/l of K_s are depicted by Sözen et al. (1998) and Orhon et al. (2009).

As a result, all estimated kinetic parameters for Set 4 and Set 5 are same (Table 4.3) indicating that EE2 addition has no effect on microbial kinetics.

Necessary kinetic information derived from the experimental results of Set 12 was also given in Table 4.3.

Modelling simulation results of ASM3 for Set 12 indicate that the maximum heterotrophic growth rate, μ_H is 9.0 day^{-1}, the half saturation constant for growth, K_s is 24 mg COD/l and the endogenous decay rate, b_H is 0.1 day^{-1}

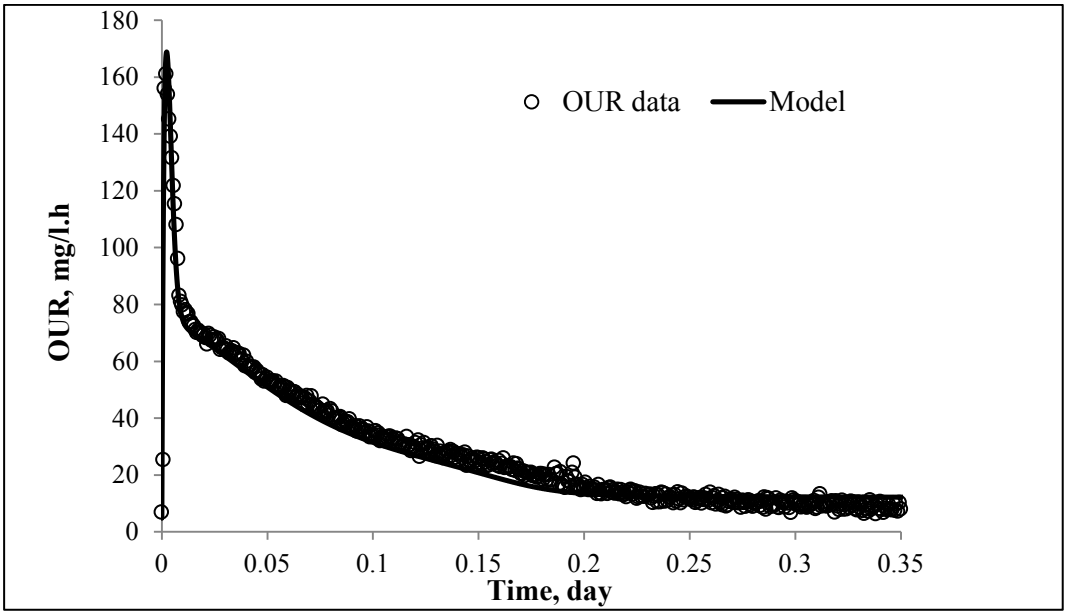

Figure 4.41 : Model simulation of OUR data for Set 12 (ASM3).

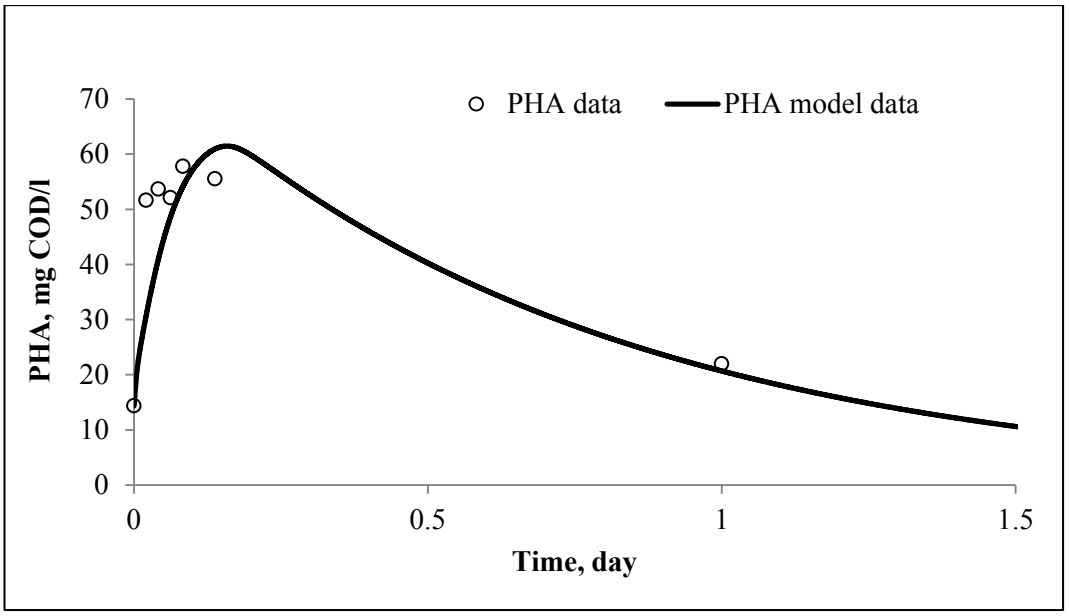

Figure 4.42 : Model simulation of PHA data for Set 12 (ASM3).

Based on model simulations of Set 12, the maximum heterotrophic growth rate and the maximum hydrolysis rate for slowly hydrolizable products increased when compared to the parameters estimated for Set 4 and Set 5.

The maximum hydrolysis rate for slowly hydrolizable products, X_s increased from 0.5 to 0.72 day^{-1} and the maximum heterotrophic growth rate, μ_H increased from 6.8 to 9.0 day^{-1}.

It was observed that the storage process continued during the chronic period from first day till fortieth day with the acclimation of activated sludge to EE2 (Figure 4.38, Figure 4.40, Figure 4.42). For the storage process; μ_{STO}, Y_{STO}, K_{STO}, k_{STO} remained the same for Set 4, Set 5 and Set 12.

Increasing hydrolysis rate during fortieth days may result in available substrate for both growth and storage process. As cited in Kavarova-Kovar and Egli (1998), it has been frequently pointed out that for growth of a microbial strain, the steady-state extracellular concentration of the growth-controlling substrate and the content of the enzymes involved in transport and catabolism of this substrate influence each other.

As a result, increasing maximum growth rate and hydrolysis rate for Set 12 indicate that transport enzymes are affected by EE2 involving in more substrate utilization. μ_H level is a function of r-RNA level of the cells stimulating protein synthesis mechanism leading to an increase in the maximum growth rate for Set 12.

Table 4.3 : Results of model calibration for Set 4, Set 5 and Set 12.

Model Parameters		**Unit**	**Set 4 (Control)**	**Set 5 (Day 1)**	**Set 12 (Day 40)**
Maximum growth rate for X_H	$\hat{\mu}_H$	1/day	6.8	6.8	9.0
Half saturation constant for growth of X_H	K_S	mg COD/l	24	24	24
Endogenous decay rate for X_H	b_H	1/day	0.10	0.10	0.10
Half saturation coefficient of oxygen for X_H	K_{OH}	mg O_2/l	0.01	0.01	0.01
Maximum hydrolysis rate for S_H	k_{hs}	1/day	6.30	6.30	6.30
Hydrolysis half saturation constant for S_{H1}	K_X	g COD/g COD	0.20	0.20	0.20
Maximum hydrolysis rate for X_S	k_{hx}	1/day	0.50	0.50	0.72
Hydrolysis half saturation constant for X_{S1}	K_{XX}	g COD/g COD	0.01	0.01	0.01
Maximum storage rate of X_{STO} by X_H	k_{STO}	1/day	1.60	1.60	1.60
Half saturation constant for growth of X_H on X_{STO}	K_{STO}	mg COD/l	0.50	0.50	0.50
Maximum growth rate on X_{STO} for X_H	$\hat{\mu}_{STO}$	1/day	0.80	0.80	0.80
Yield coefficient for X_H	Y_H	g COD/g COD	0.60	0.60	0.60
Storage yield of X_{STO}	Y_{STO}	g COD/g COD	0.80	0.80	0.80
Fraction of biomass converted to to S_P	f_{ES}	-	0.05	0.05	0.05
Fraction of biomass converted to X_P	f_{EX}	-	0.15	0.15	0.15
State variables					
Total biomass	X_H	mg VSS/l	1130	1240	1330
Initial active heterotrophic biomass	X_{H1}	mg COD/l	1100	1215	1300
Activity		%	69	69	69
Initial PHA	X_{STO1}	mg COD/l	10	10	14
Initial biodegradable peptone mixture COD	C_{S1}	mg COD/l	360	360	360
Initial readily biodegradable peptone mixture COD	S_{S1}	mg COD/l	34	34	34
Initial readily hydrolizable peptone mixture COD	S_{H1}	mg COD/l	202	202	202
Initial slowly hydrolizable peptone mixture COD	X_{S1}	mg COD/l	125	125	125

5. CONCLUSION

17 alpha-ethinylestradiol (EE2) is a synthetic estrogen hormone having hydrophobic structure and low volatility. Estrogens are primarily removed from wastewater via sorption, biodegradation or both. It is reported in the literature that natural estrogens are more biodegradable than the synthetic estrogen EE2 and EE2 is adsorbable to the solid phase due to its hydrophobicity. In contrast, experiments conducted in this thesis study demonstrate that EE2 is biodegradable by heterotrophic microorganisms to some extent and heterotrophs can readily degrade EE2 by using it as carbon source.

Acute and chronic respirometric experiments conducted during this thesis study showed a useful information about the inhibitory effect of EE2 and the acclimation period of EE2 to the peptone mixture acclimated activated sludge. It can be concluded that acute addition of different dosage of EE2 affects the initial oxygen uptake rate level. Also when it comes to sewage treatment plant's influent EE2 concentrations, which are in ng/l levels, an inhibition in the system is not expected.

Chronic experiments conducted show that until twentyfifth days the maximum oxygen uptake rate and the storage compound amount were not changed significantly. The acclimation of the system to EE2 was observed on the twentyfifth day of acclimation period. The inactivation impact was suppressed and the mixed culture became acclimated to EE2. After the twentyfifth day, the maximum oxygen uptake rate datas decreased very slowly and are close to each other.The shape of OUR profiles are similar to each other during chronic experiments. EE2 concentration is very small on the solid phase in contrast EE2 is found in soluble form in the aqueous phase indicating that EE2 is degraded.

Model simulation estimations proved that kinetic parameters for the microbial culture does not change during the control and the first day experiment. This indicates that EE2 addition has no effect on microbial kinetics.

For the fortieth day experiment, model simulation results showed that the maximum heterotrophic growth rate and the maximum hydrolysis rate for slowly hydrolizable products increased due to the stimulated enzyme activity.

Storage compound analysis supported that the system stored small amount of PHAs and used available substrate for their growth resulting in high maximum growth and hydrolysis rates.

In conclusion, acute addition of EE2 has an inhibitory impact by suppressing the microbial system but the acclimation of EE2 is possible in the presence of available substrate as peptone mixture which may be a result of cometabolism. EE2 removal is also possible from the aqueous phase as a consequence of biodegradation mechanism by heterotrophic microorganisms. EE2 also stimulates the system leading to an increase in the enzyme activity and so does the growth rate.

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