ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

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

M.Sc. THESIS

Burcu TEZCAN

Department of Environmental Engineering

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Thesis Advisor: Prof. Dr. Emine UBAY ÇOKGÖR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SENTETİK ÖSTROJEN 17 ALFA-ETİNİLESTRADİOL'UN NİTRİFİKASYON PROSESİ ÜZERİNE AKUT VE KRONİK ETKİSİ

YÜKSEK LİSANS TEZİ

Burcu TEZCAN
(501081803)

Çevre Mühendisliği Anabilim Dalı

Çevre Biyoteknolojisi Programı

Tez Danışmanı: Prof. Dr. Emine UBAY ÇOKGÖR

HAZİRAN 2012

Burcu Tezcan, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID **501081803,** successfully defended the **thesis** entitled "**ACUTE AND CHRONIC EFFECTS OF SYNTHETIC ESTROGEN 17 ALPHA-ETHINYLESTRADIOL ON BIOLOGICAL NITRIFICATION PROCESSES**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Prof. Dr. Emine UBAY ÇOKGÖR** .....
İstanbul Technical University

Jury Members : **Assoc. Prof. Dr. Elif PEHLİVANOĞLU MANTAŞ**

Assoc. Prof. Dr. Didem AKÇA GÜVEN
Fatih University

Date of Submission : 4 May 2012
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Dedicated to my family,

FOREWORD

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Burcu TEZCAN
(Environmental Engineer)

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ABBREVIATIONS

ASM	**:** Activated Sludge Modelling
COD	**:** Chemical Oxygen Demand
EDC	**:** Endocrine Disrupting Compound
EE2	**:** 17α Ethlylenestradiol
EPA	**:** Environment Protection Agency
ESI	**:** Electro Spray Ionisation
HPLC	**:** High Performance Liquid Chromatography
HRT	**:** Hydrolic Retention Time
IWA	**:** International Water Association
LOD	**:** Limit of Detection
MLSS	**:** Mixed Liquor Suspended Solids
MLVSS	**:** Mixed Liquor Volatile Suspended Solids
NAS	**:** Nitrifying Activated Sludge
OUR	**:** Oxygen Uptake Rate
PHA	**:** Polyhydroxyalkaonate
PHB	**:** Polyhydroxybutyrate
PHV	**:** Polyhydroxyvalerate
SKN	**:** Soluble Kjeldahl Nitrogen
SRT	**:** Sludge Retention Time
STP	**:** Sewage Treatment Plant
STW	**:** Sewage Treatment Work

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ACUTE AND CHRONIC EFFECTS OF SYNTHETIC ESTROGEN 17 ALPHA ETHINYLESTRADIOL ON BIOLOGICAL NITRIFICATION PROCESSES

SUMMARY

In this study, activated sludge taken from biological treatment plant in İstanbul, was used for the studying a selected hormon, namely, 17α-ethinylestradiol (EE2). Activated sludge was acclimated to synthetic peptone mixture having similar characteristics of domestic sewage. System was operated until biomass reached to steady state conditions at a sludge age of 10 days and hydraulic retention time of 1 day at 20 °C. After the acclimation of biomass to aimed operating conditions, the effect of EE2 to activated sludge was investigated under nitrifying conditions. Firstly, acute effects of EE2 was examined with respirometric analysis. Acute experiments representing the same conditions in respirometric tests were conducted in parallel for the only peptone mixture and together with 1 mg/L EE2 solution. After acute effects of EE2 was searched, the batch reactor was acclimated to EE2 during 40 days. Then, acclimation of EE2 with peptone mixture was performed and monitored through respirometric studies as well as conventional parameters. Batch experiments were conducted in 2 L reactors and they were started with biomass taken from batch reactor continuously operated at steady state. Each reactor was started with the necessary amount of acclimated biomass seeding alone to obtain endogenous oxygen uptake rate level of biomass. Changes in the oxygen uptake rate (OUR) of the system were monitored for 1 day. The volatile suspended solid concentrations were approximately 1200 mg VSS/L and influent COD conentrations 360 mg COD/L for all runs. Furthermore, EE2 analysis was performed with HPLC for solid and liquid phase, separately. Kinetic and stoichiometric coefficients were estimated via AQUASIM for optimum treatment conditions according to ASM 3. Therefore, it is getting more important to investigate the kinetic and stoichiometric coefficients for the systems treating the chemical.

SENTETİK ESTROJEN 17 ALFA ETİNİLESTRADİOL'UN NİTRİFİKASYON PROSESİ ÜZERİNE AKUT VE KRONİK ETKİSİ

ÖZET

Endokrin sistemini bozan kimyasallar, 1962'de biyolog Rachel Carson'un 'Silent Spring' adlı kitabında kimyasal maddelerin kuşlar üzerindeki zararlı etkileriyle ilgili incelemelerini yayınlamasıyla ortaya çıkmıştır (Carson, 2002). Finkelstein ve arkadaşları (1988), hazırladıkları makalede 50 yaşındaki bir cenaze hazırlayıcısı erkekte, tedrici libido kaybı (cinsel isteksizlik), testislerde küçülme ve sakal büyümesinde yavaşlama şikayetlerinin endokrin sistemini bozan kimyasallar ile ilişkili olduğunu belirlemiştir. Hasta serumunda bilinmeyen bir maddenin radyoaktif işaretlenmiş östrojen ile yer değiştirdiği ve maddenin kaynağının devamlı kullanılan mumya kremi olduğu saptanmıştır. Kremle karşılaşma kesilince şikayetler düzelmiştir. Colborn 1993'de tıbbi açıdan çevredeki kimyasal maddelerin endokrin sistem üzerine etkilerini vurgulamıştır. İnsan vücudunda ölçülebilen kimyasal maddelerin sayısı arttıkça ve bu kimyasalların endokrin sistem, üreme sistemi, immünolojik (tıbbın bağışıklık sistemi ile ilgilenen alt dalı) işlevler ve bu konunun en son noktasında yer alan karsinogenesis üzerine etkileriyle ilgili gözlem ve yayınlar çoğaldıkça; konu Birleşmiş Milletlerin, Dünya Sağlık Örgütü'nün ve hükümetlerin de gündemine girmiştir. 14 Mart 1997'de Birleşmiş Milletler Çevre ve Kalkınma Toplantısı'nda endokrin bozucular ele alınmıştır. Bu toplantıda insan vücudunda ölçülebilen en az 500 kimyasal maddenin taşındığı, bu maddelerin 1920'den önce insan kimyasının bir parçası olmadığını son 20 yılda doğan bebeklerin anne rahminde bu maddelerle karşılaştığını bildirmiştir. Başta Amerika Birleşik Devletleri olmak üzere pek çok ülke 'Çevre ve Doğal Kaynaklar Komiteleri' kurarak, endokrin bozucularla ilgili araştırmalar başlatmıştır.

Endokrin sisteminin normal fonksiyonunu engelleyen kimyasallar genel olarak EDC'ler olarak adlandırılır. Amerikan Çevre Koruma Teşkilatı (EPA) bir EDC'yi ve etki mekanizmalarını şöyle tanımlar (USEPA, 1997; USEPA, 1998): "organizmaya harici olarak alınan, doğal veya insan kaynaklı, bireysel veya popülasyon seviyelerinde geri dönüşümlü veya dönüşümsüz negatif etkiler yapan kimyasallardır. Vücudun normal hormonal sistemine etkileri: doğal hormonları taklit ederek normal sentezleri ve hormonal fonskiyonları engelleme, depolonan hormonları serbest bırakma, salgı, taşınım mekanizmalarını engelleme, bağlanma, ve doğal hormonları devre dışı bırakma."

Yeni, daha hassas ve daha düşük konsantrasyonları ölçebilen bilimsel metotların ve analitik ölçüm yöntemlerinin geliştirilmesiyle, halen mevzuatlarda olmayan ve önceki yıllarda tespit edilemeyen doğal sulardaki ve içme suyu kaynaklarındaki kimyasal kirleticiler son yıllarda özellikle de gelişmiş ülkelerde güncel hale gelmiştir. Özellikle EDC'lerin gündeme gelmesinin en büyük nedeni ürkütücü

sonuçlar ortaya koyan bazı bilimsel çalışmalardır. Vücuda alındığında doğal hormonları taklit edip üreme sistemini bozan EDC'lerin doğadaki birçok hayvan türlerinde (bazı balıklarda, kuşlarda, memelilerde, ve timsahlarda) cinsiyet bozuklukları, cinsiyetsiz doğumlar, sperm sayılarında azalmalar, erkek organizmalarda dişilik, dişi organizmalarda da erkeklik özelliklerini artırdığı tespit edilmiştir.

İnsanlardan ve hayvanlardan kaynaklı doğal östrojenler ve doğum kontrol hapları ile antidepresanlardan kaynaklı 17α-etinillestradiyol (EE2) olarak isimlendirilen sentetik östrojenler EDC olarak bilinmektedirler. Sentetik estrojen içeren ilaçlar, hastalar tarafından kullanıldıktan sonra bir kısmı metabolizma tarafından kullanılırken, kullanılmayan kısım idrar veya dışkı yoluyla dışarıya atılır. Aktif olmayan bağlı forma idrarla dışarı atılan EE2, lağım sularında bakterilerin etkisi ile aktif hale dönüşür. Lağım sularından arıtma tesisine ulaşan EE2, biyolojik olarak bozunmazsa yada arıtma tesisinde arıtım sırasında yok edilmezse içme sularına karışabilir. İlaç atıklarının sadece atıksularda değil sedimentlerde de biriktiği görülmüştür. Arıtma tesisi çıkış suları ve çamurları tarım alanlarında kullanılabilir. Bu kullanımlar sonucu bu atıklar toprağa, oradan da yeraltı sularına ulaşabilir. Ayrıca bitkiler üzerinde de birikim gözlenebilir. İngiltere'de bazı atıksu arıtma tesis çıkışlarında düşük seviyelerde de olsa 17β-estradiyol, estron, ve sentetik östrojen (EE2) bulunmuştur. Deşarjın olduğu nehir sularına maruz bırakılan alabalıkların üreme sistemlerinde olumsuzluklar tespit edilmiştir. EDC'lerin insanlarda da prostat ve meme kanseri artışlarına ve erkeklerde sperm sayısının azalmasına neden olabileceği varsayılmaktadır. Amerika Birleşik Devletleri'nde ise yapılan araştırmalar sonucunda, 57 tane ham içme suyu kaynağının 25 tanesinde, 56 tane arıtılmış içme suyunun 8 tanesinde, ve 15 tane arıtılmış atıksu çıkışının 11 tanesinde östrojenik aktivite bulunmuştur (Hemming ve ark., 2001). Ayrıca, yine ABD'de yapılan bir çalışmaya göre, atık su arıtma tesislerinden alınan numunelerde 5 ng/l östrojen tespit edilmiştir. Bu değer, bir göldeki balık populasyonunun büyük bir kısmını yok etmek için yeterlidir. Çünkü bu östrojen konsantrasyonu erkek balıkların kısırlaşması veya dişileşmesi için yeterlidir. Böylece bir ırmak veya göldeki balık yumurtalarının döllenebilme kabiliyeti yok olabilir. Canlıların iç salgı bezi sistemine tesir eden bu kimyevî maddelerin ortamdan uzaklaştırılması için her yüz metreküplük su için mevcut giderlere ilâveten 1-10 Euro finansmana ihtiyaç olduğu hesaplanmıştır. Atık su arıtma tesislerinde milyonlarca metreküp suyun temizlendiği göz önünde bulundurulursa, bu rakamın ne kadar büyük bir yekûn teşkil ettiği daha iyi anlaşılır.

Doğal östrojenik hormonlar ve özellikle sentetik steroid hormonlar biyolojik olarak çok güçlü bileşiklerdir. Yapılan araştırmalara göre, östrojenlerin çoğunun uygulanan atıksu arıtım tesislerinde tamamıyla giderilmediği, bu nedenle çıkış suyunda bulundukları belirtilmiştir. Çıkış suyunda bulunan bu kirleticilerin alıcı ortama deşarjıyla yüzey sularına, içme sularına ve yeraltı sularına bulaştığı belirlenmiştir. Sudan kirleticilerin giderimine etki eden en önemli faktör doğal (kil, sediment, mikroorganizmalar) veya ortama eklenen (aktif karbon, koagülant) katı partiküllerin birbirlerini etkileyerek fizikokimyasal (çökme, flotasyon) veya biyolojik proseslerle (biyodegradasyon) giderimidir. Gelişmiş ülkelerde bu maddelerin arıtılması amacıyla ileri arıtma teknolojileri kullanılmaktadır. Bunlar ozonlama, ultrafiltrasyon, ters osmoz gibi yüksek basınçlı membran sistemleri gibi fiziksel kimyasal prosesler ve adsorpsiyondur.

Korner ve ark. (2000)'nın Almanya'da yaptığı bir çalışmaya göre, atıksudaki östrojenik aktivitenin arıtma boyunca mikroorganizma üzerine adsorbe olmak yerine biyolojik olarak giderildiğini gözlemlemiştir. Bu çalışma sonucuna göre, %90 oranında giderilen östrojenik yükün, %3'ünden az bir kısmının çamura adsorbe olduğu sonucuna varılmıştır. Bir başka çalışmaya göre ise, yüksek katı madde konsantrasyonu sonucunda yüksek EE2 gideriminin sağlandığı sonucuna varılmıştır (Birkett ve Lester, 2003).pH ve tuzluluk konsantrasyonları da EE2 gideriminde etkilidir.

Nitrifikasyon sistemlerinde EE2 giderimi için önemli bir rol oynayan amonyağı okside eden bakteriler yüksek çamur yaşlarında çoğalırlar. Bu yüzden düşük çamur yaşı ile işletilen sistemlerde nitrifikasyon olmaz ve EE2 giderimi düşüktür.

Yüksek çamur yaşı gerektiren nitrifikasyon sistemlerinde EE2'nin giderilmesine yönelik bir çok araştırma yapılmıştır. Vader ve ark. (2000)'na göre, 50 µg/L EE2'nin nitrifikasyon sisteminde 6 gün içerisinde parçalanmaktadır. Shi ve ark. (2004) ise, sadece karbon gideren sistemde (inhibitörlü) ve nitrifikasyon sisteminde (inhibitörsüz) EE2'nin parçalanabilirliliğini izlemiş ve nitrifikasyon sisteminde EE2 parçalanma hızının sadece karbon gideren sisteme göre daha fazla olduğunu bulmuştur. Bu çalışmaların aksine, Gaulke ve arkadaşları (2008), nitrifikasyon sistemlerinde NH_4-N konsantrasyonun okside olduğunu ve bunun sonucunda sistemde NO_2-N konsantrasyonun yükseldiğini gözlemlemiştir. NO_2-N konsantrasyonunun yükselmesi ile pH düşmüş bu da EE2 giderimi için uygun koşulların sağlanmasına sebep olmuştur. Araştırmaya göre, sistemlere eklenen inhibitör EE2 giderimi için uygun koşullar yaratan NO_2-N oluşumunu engellemiş ve bu durum da yüksek seviyede EE2 giderimi sağlanamamıştır.

Bu çalışmada ise sentetik bir hormon olan 17α-etinilestradiolun (EE2) nitrifikasyon prosesi üzerine akut ve kronik etkileri, EE2'nin biyolojik olarak parçalanabilirliliği ve mikrooragnizmanın depolama özellikleri incelenmiştir. Bir arıtma tesisinden alınan aktif çamur, evsel atıksu karakteristiğine sahip olan pepton içerikli sentetik atıksuya aklime edilmiş ve sistem kararlı hale gelene kadar işletilmiştir. Çamur yaşı 10 gün olan sistemin hidrolik bekletme süresi 1 gündür. Sistem kararlı hale geldikten sonra, ilk olarak akut deneyler uygulanmış ve EE2'nin nitrifikasyon prosesi üzerine olan akut etkileri araştırılmıştır. Bu safhadaki deneyler, hacmi 2 L olan reaktörlerden birine sadece pepton karışımı, diğerine ise pepton karışımı ile birlikte 1 mg/L EE2 hormonu eklenmesi ile tamamlanmıştır. Akut deneylerin tamamlanması ile sisteme sentetik atıksu ile EE2 aklimasyonu birlikte uygulanmış ve konvansiyonel parametrelerle sistem izlenmiştir. Sisteme yapılan EE2 aklimasyonu ile birlikte, kronik deneylere geçilmiş ve 40 gün boyunca hacmi 2 L olan reaktörlerde oksijen tüketim hızındaki değişim izlenmiş ve EE2'nin nitrifikasyon prosesi üzerine olan kronik etkileri araştırılmıştır. Tüm deneyler için uçucu askıda katı madde miktarı 1200 mg UAKM/L iken giriş KOİ konsantrasyonu 360 mg KOİ/L'dir. Ayrıca, tüm bu deneylere ek olarak su ve katı fazda EE2 ölçümleri de yapılmıştır. Deneyler süresince mikroorganizmanın depolama kapasitesini tespit etmek için PHB ölçümleri yapılmıştır. Elde edilen verilere dayalı olarak, uygun arıtma şartlarını saptamak için ASM3'e göre kinetik ve stokiyometrik katsayılar AQUASIM programı yardımı ile hesaplanmıştır.

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1. INTRODUCTION

In recent decades there has been increasing concern over the potential for human and environmental health effects due to the presence of a wide range of organic contaminants in effluents of wastewater treatment plants which are often directly discharged to the environment. These include human derived chemicals, such as hormones, and other metabolic by-products. They also include various synthetic compounds used by humans as pharmaceuticals, personal care and household cleaning products.

Within the various pharmaceutical categories, particular attention is being focused on hormones. Hormonal activity has become a widely recognized mechanism of toxicity, and studies have shown that exposure to endocrine-modulating substances can impair reproductive function in adults of either sex, lead to irreversible abnormalities when administered during development, or in the most extreme cases cause cancer (Jobling et al., 1998). Example of these traits is the synthetic estrogen, 17α-ethinylestradiol (EE2), the main constituent in the contraceptive pill, which has been continually detected at the ng per liter level in sewage, ground and surface waters. These traits are amenable to biotransformation and bioconcentration and potentially may bioaccumulate ; as a consequence of this behaviour complex issues for environmental health arise.

Biological degradation of estrogens can occur during aerobic wastewater treatment (Johnson and Sumpter 2001). Investigations of removal of estrogens in wastewater treatment plants, as well as batch experiments with activated sludge, have demonstrated the potential for conversion of E2 to E1 and subsequent removal of E1 and, to a lesser extent, EE2, under aerobic conditions (Tanghe et al. 1998, Vader et al. 2000, Lee and Liu 2002, Andersen et al. 2003, Joss et al. 2004).

In this study, biological degradation of EE2 is investigated under nitrifying conditions. Moreover, acute and chronic effects will be searched and analyses done with respirometry will be modelled via AQUASIM and kinetic and stoichiometric coefficients will be estimated for optimum treatment conditions according to ASM 3.

1.1.Aim and Scope

Activated sludge taken from Paşaköy Wastewater Treatment Plant was used for the biodegradation studies of EE2, a synthetic hormon. Firstly, system was acclimated to Pepton synthetic wastewater having similar chracteristics of domestic sewage and acute affects of EE2 was evaluated. Then, the system was acclimated to EE2 with peptone mixture and chronic effects of EE2 was investigated during acclimation period. Acute and chronic effects of EE2 were monitored through respirometric studies. In parallel chemical oxygen demand, EE2 and some other conventional parameter analysis were measured. EE2 was determined via HPLC. Thereafter, same analyses were examined during acclimation period. The biodegradation mechanism was performed by using multi-component model.

2. LITERATURE REVIEW

2.1.Endocrine Disrupting Compounds (EDCs)

The existence of some specific micro-pollutants has become more concerning in the last decade. These pollutants are endocrine disrupting compounds (EDCs) – chemicals mimicking the action of desired hormones or acting on the proper endocrine systems. According to U.S. Environmental Protection Agency (EPA) the EDCs are exogenous agents that interface with synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction or behavior. Since the early 1990s some compounds released to the environment began to be recognized as pollutants of a new kind of mode of action. The first evidences of endocrine disruption in nature were observed in fishes and amphibians exposed to paper mills sewages. All xenobiotics present in the environment can be divided into two groups:

- Regulated pollutants
- Non-regulated pollutants.

Among these non-regulated xenobiotics, one can distinguish:

- Non-identified pollutants
- New emerging pollutants, as can be seen **Figure 2.1** (Kudlak and Namiesnik, 2008).

Many EDCs have now been reported as environmental contaminants. Natural and synthetic estrogens: estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are such endocrine disrupters and they display the strongest estrogenic effects however their concentrations are lower in the aquatic environment. The estrogenicity in the aquatic environment is largely due to their presence in the sewage treatment work effluent. Many of these steroids pass through wastewater treatment systems and are discharged continuously into the environment, mainly into surface waters. These compounds are amenable to biotransformation and bioconcentration and potentially

may bioaccumulate; as a consequence of this behavior complex issues for environmental health arise (Koh et. al., 2008).

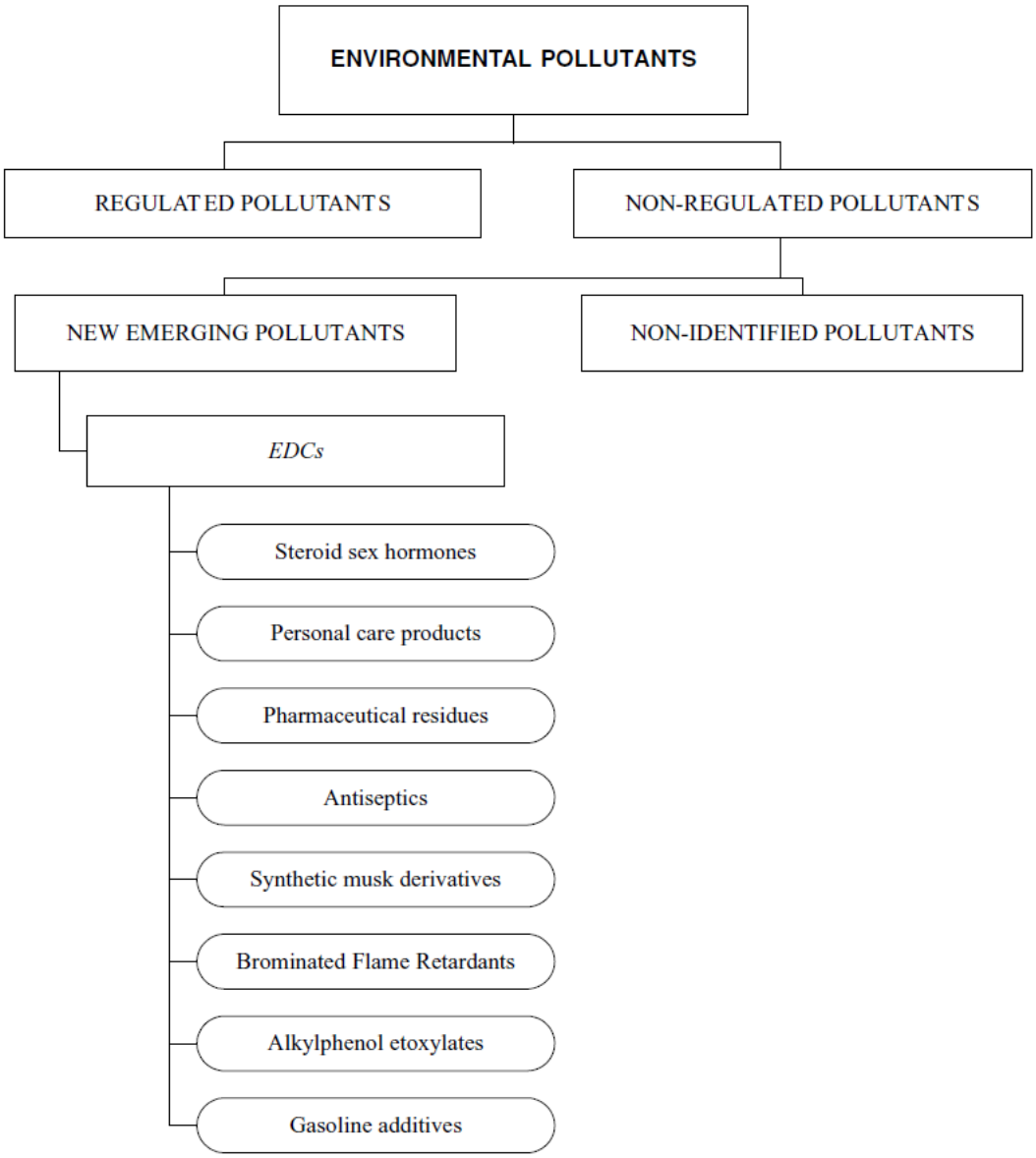


Figure 2.1: Classification of environmental pollutants due to their legal regulations (Kudlak and Namiesnik, 2008).

2.1.1. Definition of estrogens

Estrogens are defined as ‘’ any of a family of steroid hormones that regulate and sustain female sexual development and reproductive function’’. Like all steroids, estrogens share the same hydrocarbon ring nucleus as cholesterol, their parent compound. **Figure 2.2** illustrates this basic ring structure, consisting of three hexagonal rings, (A,B,C) and one pentagonal ring (D). Steroid estrogens are

characterized by their phenolic A-ring, which renders the 3-hydroxyl acidic and is essential for biological activity (Birkett and Lester, 2003).

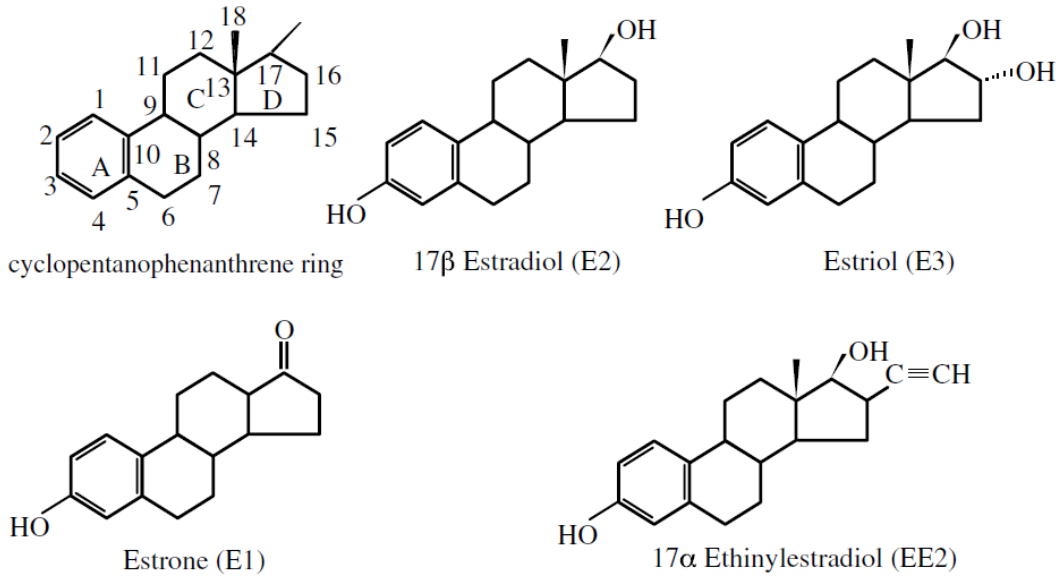


Figure 2.2: Structure of the steroid estrogens (Birkett and Lester, 2003).

The lipophilic cyclopentanophenanthrene nucleus shown in **Figure 2.2** is modified by the addition of hydrophilic groups to form different steroids. In the case of the three natural free estrogens, hydroxyl and carbonyl groups are added, while ethinyl groups are found in the structure of the synthetic free estrogen, 17β-ethinylestradiol, a component of the contraceptive pill. Substituent groups above the plane of the molecule are said to be in the ‘’β’’ position, whereas those situated under the plane of the molecule are said to be in the ‘’α’’ position. Log octanol/water coefficient (log K_{ow}) values for the free estrogens range from 2.81 and 4.15, and thus, it is evident that these compounds are lipophilic and are only sparingly soluble in water. When dissolved, these estrogens may be rapidly removed from the aqueous phase as a result of binding to suspended solids. Esterification with glucuronic or sulfuric acid, however, dramatically alters the physical-chemical properties of free steroid estrogens. Sulfate and glucuronide conjugates are far more hydrophilic than their unconjugated counterparts, although they are still soluble to some extent in organic solvents.

2.1.2. Sources of estrogens

The steroids estradiol, progesterone, and testosterone all produce growth effects in humans and animals. Because of this property, exogenous steroids have been used in meat-producing animals in the United States for almost 50 years. Several synthetic chemicals are also used as growth enhancers in cattle. A report by the Food and Agricultural Organization/Word Health Organization Joint Expert Committee on Food Additives (JECFA) found that levels of estradiol, estrone, progesterone, and testestorone in animal tissue were all significantly increased (at least twofold) in treated cattle compared with untreated herds. If the use of hormones increases steroid levels in edible tissues, then it is probable that there will be an increase in the steroid intake to humans from the consumption of such products. In Europe, the use of such steroids in meat production was banned in 1989. **Table 2.1** highlights the sources of steroid hormones, predominantly the estrogens (Birkett and Lester, 2003).

Table 2.1: Sources of Estrogens (Birkett and Lester, 2003).

Source	Steroid Hormone
Food - meat,fish,eggs,pork,dairy products	Estradiol, estrone, progesterone, testosterone
Sewage treatment work effluents	Estradiol, estrone, estriol, ethinyl estradiol
Sewage sludge	Estradiol, estrone, estriol, ethinyl estradiol
Oral contraception	ethinyl estradiol, mestranol
Hormone replacement therapy	Conjugated estrone, 17α and β-estradiol
Runoff	Estradiol, estrone
Agricultural waste	Estradiol, estrone

Detectable concentrations of steroid hormones (e.g., estradiol, estrone, progesterone, and testosterone) have also been found in fish, poultry, eggs, pork, cheese, milk, and milk products. However, in comparing the production of these hormones in adults and children with their daily intake, it is found that children, who have the lowest hormone production rate, show levels of pregosterone to be 20 times greater their intake, while estradiol and testosterone production levels are 1000 times higher. It is likely that no hormonal effects in humans can be expected from naturally occurring steroid compounds in food. However, use of synthetic EDCs and the presence of xenoestrogens in foodstuffs may be more problematic.

Within the steroid hormone group, it is perhaps the natural (estrone [E1], 17β-estradiol [E2], estriol [E3]) and synthetic (ethinylestradiol [EE2], mestranol) steroid estrogens that have received the most scientific attention. These compounds are the major contributors to the estrogenic activity observed in sewage effluent and the receiving water body. Estrogens serve as important point sources, especially in densely populated areas, **Figure 2.3** illustrates this pathway (Liu et. al., 2009). Their presence in the aquatic environment is attributed to their incomplete removal during the sewage treatment process. Although concentrations of steroid estrogens have been reported in the low ng/L levels, their estrogenic potency warrants cause for concern, as EE2 has been shown to induce vitellogenin (VTG) production (a female yolk protein) in male fish at 0.2 ng/L (Birkett and Lester, 2003).

Municipal wastewater is one of the main routes of emission into the environment for most important EDCs like natural and synthetic estrogens via urine, as well as ingredients of personal care products and detergent via grey (washing) water, phytoestrogens from food processing and consumption and ubiquitous chemicals used in different industrial products getting in contact with water (Siegrist, 2005)

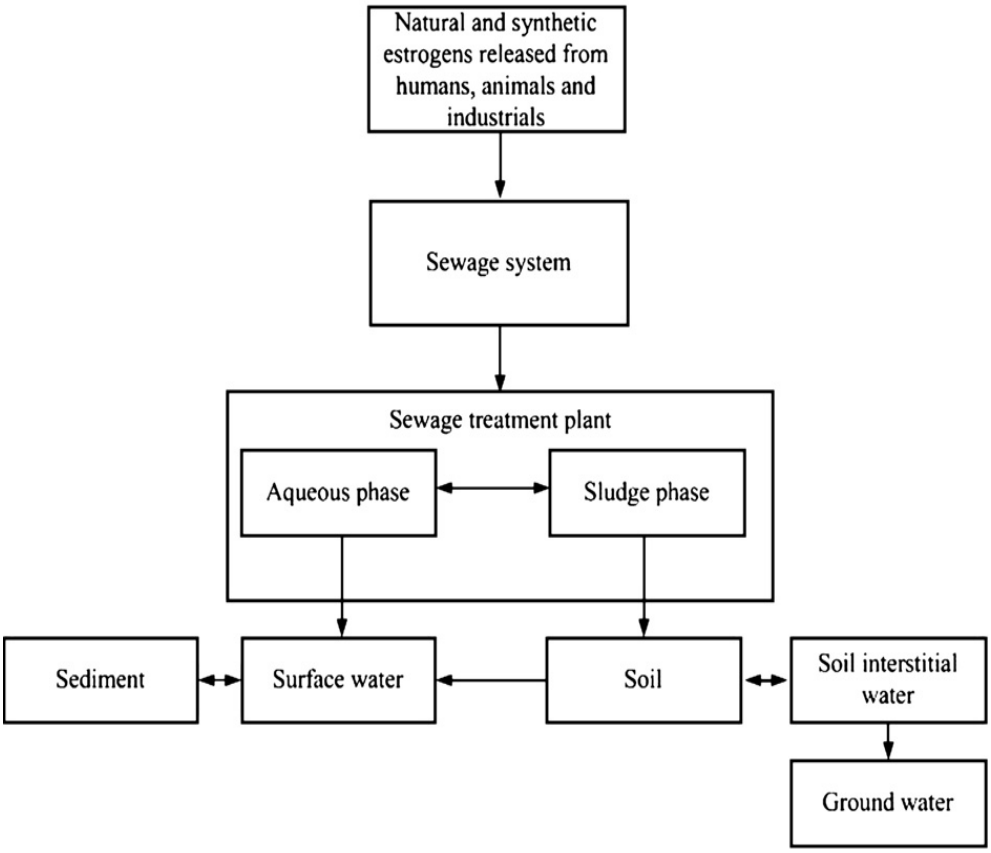


Figure 2.3: EDCs distribution in the environment (Liu et. Al., 2009)

The presence of steroid estrogens in Sewage Treatment Work (STW) effluent arises from mammalian excretion, in particular females of reproductive age and those who are pregnant. Women excrete 10 to 100 μg of estrogen per day depending on the phase of their cycle, and pregnant women can excrete up to 30 mg per day. The major excretion product is estriol, with daily excretion rates for women at a maximum of 64 μg per day. The excretion rate of estrone and 17β-estradiol is 3 to 20 μg and 0.5 to 5 μg per day, respectively. The majority of these estrogens are excreted from the human body within urine in a biologically inactive,conjugated form (predominantly as glucuronides and sulfates). However, because free estrogens have been observed in STW effluent, this implies that deconjugation has occurred at some stage during or prior to sewage treatment. Moreover, the amount of natural and synthetic estrogens entering the STW is unlikely to decrease due to the origin and use. Steroid estrogens have also been detected in sewage sludge. Concentrations of estradiol, estrone, and estriol in sewage sludge from 14 different STWs in the United States range from 0.01 to 0.08 ng/L, while mean concentrations of ethinyl estradiol in raw and treated sewage were 1.21 ng/L and 0.81 ng/L, respectively. The application of such sludge to agricultural land is likely to increase the estrogen content from other sources, such as runoff (Birkett and Lester, 2003).

The main uses of synthetic estrogens are in oral contraception and hormone replacement therapy. In 1990, of the 58 million women in the United States who actively practiced contraception, 10 million used oral contraception. These contraceptives contain a combination of estrogen and progestin, with the active ingredient being ethinyl estradiol (EE2), or sometimes mestranol. Typical concentrations of EE2 range from 20 to 50 μg, with 35 μg the most commonly prescribed. It is estimated that of the 40 million menopausal American women, 5 to 13 million are using hormone replacement therapy. The active ingredients in hormone replacement therapy drugs are the conjugated equine estrogens, as well as conjugated estrone and 17α and 17β-estradiol, with a typical daily dose of conjugated estrogens of 0.625 mg. Arcand-Hoy et al. (1998) produced an estimated introduction concentration (EIC) of EE2 into the aquatic environment, based on the amount of pharmaceuticals sold in the United States. The EIC for EE2 was found to be 2.16 ng/L (Birkett and Lester, 2003).

2.1.3. Effects of estrogens

Estrogens disrupt normal hormonal functions and cause unsafe results to humans and wildlife, such as decreased fertility and feminization, hermaphroditism, even at concentration levels as low as pg-ng/L water (O'Grady *et. al.*, 2007).

Many scientific groups worldwide have stated the hypothesis of an association between increased estrogens in the environment and the adverse trends in reproductive health, and the prevalence of cancer in endocrine sensitive tissues. For humans, a causal relation has not been firmly established. However, for fish there are direct correlations with the discharge of STP effluents in surface water and the feminisation of male fish and early life exposure can affect sex ratio by increasing the female phenotype. Concentrations as low as 0.5 ng/l of EE2 leads to an induction of vitellogenin, a protein responsible for the formation of oocytes, in male trout after 10 days exposure. Beside the direct effects on the aquatic environment, there is a suspected bioaccumulation via the food chain for all three compounds. There is even an effect observed on the plant alfalfa; irrigation with wastewater cause to an elevated level of phytoestrogens. Adding E1 and E2 to irrigation water in a concentration range of 5-500 ng/l did increase growth, while higher concentrations in the range 50-500 µg/l inhibited growth (O'Grady *et. al.*, 2007).

2.1.4. Determination of estrogens by chemical techniques

Estrogens occurring in wastewaters or WWTP effluents have been reported in several research papers in concentration levels of µg/l. Since these substances represent a potential danger for human health and because of their environmental levels, it is important to monitor in water. Therefore, robust and reliable analytical methods are required which will allow estimating the presence of such compounds in water samples (Rodriguezi *et al.*, 2011).

A variety of chromatographic methods, with a range of detection techniques (e.g. GC-MS, LC-MS and GC- or LC-MS-MS), have been employed in environmental monitoring of EDCs (Kuch et al. 2000; Mol et al. 2000; Heemken et al. 2001; Lopez de Alda et al. 2001; Promberger et al. 2001; Xiao et al. 2001; Brossa et al. 2003; Ding et al. 2003; Liu et al. 2004; Quintana et al. 2004; Garcia et al. 2005; Labadie et al. 2005). The choice of analytical method for determination of selected organic compounds in domestic wastewater streams can depend on a number of factors

including the physicochemical properties of the analytes as well as the nature of sample (e.g. water, soil, sediments) and the effect of matrices. The cost of analyses is another important factor in choosing suitable analytical methods (Shareef et. Al., 2008).

2.1.4.1.Gas chromatography-mass spectrometry (GC-MS)

GC-MS has been a popular method of analysis for the trace levels determinations of a range of organic compounds in environmental samples for many decades. The use of GC-MS allows characterization of unknown
compounds as well as their naturally occurring metabolites and breakdown products. However, the technique is only suitable for analysis of thermally stable, volatile or semivolatile organic compounds (Evershed 1993; Halket et al. 2003). Therefore, highly polar compounds are often derivatised to eliminate polar groups by blocking polar functionalities such as hydroxyl, carboxylic or amino groups (Halket et al. 2003).

Many EDCs and PPCPs including the xenoestrogens OP, NP, mono- and diethoxylates of NP (NP_1EO and NP_2EO), BPA, the natural hormones E1, E2, E3, and the synthetic hormone EE2, and musk fragrances in environmental samples have been determined by GC-MS. Until recently GC-MS has also been used widely in the residue analyses of a range of pharmaceuticals. However, sample preparation can often involve tedious steps such as derivatisation of acidic compounds (Shareef et. al., 2008).

2.1.4.2.Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Recent advances in liquid chromatography tandem mass spectrometry (LC-MS/MS) technologies have enabled a greater enhancement in the rapid detection of polar organic contaminants in the environment (e.g. polar pharmaceuticals).

For instance, Pozo et al. (2006) recently reported improved detection limits (e.g. 0.4-4.3 ng/L in water) for a range of human antibiotics using online SPE coupled to LCMS/MS. Recently, LC-MS/MS with electro spray ionisation (ESI) methods which achieved significantly improved sensitivities for antibiotics in surface water: 8-10 ng/L, wastewater influents: 13-18 ng/L and wastewater effluents: 8-15 ng/L have also been developed (Cha et al. 2006).

2.1.4.3.Suitability of analytical methods

While both GC-MS and LC-MS methods are commonly used in the trace analyses of emerging contaminants, there are some limitations associated with these methods. For example, due to the very low levels (ng/L) of typical emerging compounds that occur in the environment, clean-up and pre-concentration of water samples using solid phase extraction (SPE) technique are necessary for quantitative determination of the target analytes.

LC-MS/MS in ESI mode has become the most commonly used method for determination of pharmaceuticals. Detection limits of 5-20 ng/L are typically reported with good recoveries from STP effluent samples (Miao et al. 2002; Pedrouzo et al. 2007).

Some GC-MS analyses reported of pharmaceuticals have also appeared in the recent literature. For example, Zhang et al. (2007) recently used GC-MS and quantified many PPCPs including clofibric acid, ibuprofen, acetaminophen, caffeine, naproxen, TCS, BPA, carbamazepene, E1 and E2 in river water samples.

GC-MS in electron impact (EI) ionisation mode has been reportedly the most widely used technique in the analysis of the polycyclic musks for a number of reasons. High selectivity and sensitivity can be achieved through the characteristic EI spectra of the musks which show several characteristic mass fragments. HPLC methods have also been recently used to analyse the musk compound HHCB in aqueous samples. Detection limits of fluorescence and ultraviolet (UV) detection were 5 μg/L and 1.5 mg/L, respectively (Rimkus 1999).

2.1.5. Estrogen levels in the environment

2.1.5.1. Wastewater

Estrogenic steroids have been detected in influents and effluents of sewage treatment plants in different countries. Average concentrations of estrogenic steroids (E3, E2, E1 and EE2) in influents of six Italian activated sludge STPs were 80, 12, 52 and 3 ng/l, respectively. However, E3 was rarely reported to occur in such a high concentration (80 ng/l). E3 was not detected in most of the influents studied. In the raw sewage of the Brazilian STPs, estrogenic steroids E2, E1 and EE2 were detected with average concentrations of 21, 40 and 6 ng/l, respectively. Estrogen levels were lower with average concentrations of 15, 27 and 1.4 ng/l for E2, E1 and EE2,

respectively. Estrogenic steroids were detected in three Dutch STPs with concentrations ranging from < LOD to 48 ng/l for E2, from 11 to 140 ng/l for E1 and from < 0.2 to 8.8 ng/l for EE2. The concentrations of E2 in influents of Japanese STPs ranged from 30 to 90 ng/l in autumn and from 20 to 94 ng/l in summer (Ying et al., 2002).

The concentrations of estrogenic steroids in the effluents ranged from below detection limit (LOD) to 64 ng/l for E2, from < LOD to 82 ng/l for E1, from 0.43 to 18 ng/l for E3 and from < LOD to 42 ng/l for EE2 (**Table 2.2**). From the table, it can be seen that E2 was present at higher concentrations in the effluents from STPs in Canada, UK and Japan than those from other countries. E2 was detected in Japanese STP effluent samples with concentrations ranging from 3.2 to 55 ng/l in summer and from 2.8 to 30 ng/l in autumn. The average concentrations in the effluents were 18 and 12 ng/l, respectively. Nasu et al. (2000) also measured estrogenic steroids in effluents of Japanese STPs with similar concentration ranges. In British STPs, the concentrations of E1 in the effluents varied widely from 1.4 to 76 ng/l, while E2 concentrations lie in a similar range to that of Japanese STPs. However, EE2 was only found in 7 of 21 effluent samples from domestic STPs in UK, with concentrations ranging from < LOD to 7 ng/l. In Canadian STPs, E1 and E2 were determined with maximum concentrations of 48 and 64 ng/l, respectively. EE2 was detected in 9 of 10 effluent samples with a maximum concentration of 42 ng/l. In comparison, the concentrations of E2 in the effluents from German, Italian, Dutch, Swedish and American STPs were lower, ranging from < LOD to 5.2 ng/l. However, Spengler et al. (2001) recently reported a maximum concentration of 15 ng/l for E2 in effluents of STPs in SE Germany, and they also detected mestranol with a maximum concentration of 2.7 ng/l. The levels of estrone in the effluents from different countries are quite comparable. Estriol (E3) was only reported in Italian STP influents and effluents (Ying et. al.,2002).

2.1.5.2.Surface water

There are some reports on the levels of estrogenic steroids in surface waters . Tabata et al. (2001) conducted an extensive survey of estrogenic steroids in 109 Japanese rivers and found E2 in 222 of 256 samples in summer with a mean concentration of 2.1 ng/l and in 189 of 261 samples in autumn with a mean concentration of 1.8 ng/l (**Table 2.3**).

Table 2.2: Concentration of hormones in effluents of sewage treatment plants (STPs) (Ying et al., 2002)

Location	17β-Estradiol (ng/L)	Estrone (ng/L)	17α-Ethinylestradiol (ng/L)	References
Italy	0.44-3.3	2.5-82.1 (9.3)	<LOD-1.7 (0.45)	Baronti et. al. (2000)
Netherlands	<0.1-5.0 (<LOD)[a]	<0.4-47 (4.5)	<0.2-7.5 (<LOD)	Belfroid et. al. (1999)
Germany	<LOD-3 (LOD)	<LOD-70 (9)	<LOD-15 (1)	Ternes et. al. (1999a)
Canada	<LOD-64 (6)	<LOD-48 (3)	<LOD-42 (9)	Ternes et. al. (1999a)
UK	2.7-48 (6.9)	1.4-76 (9.9)	<LOD-7 (<LOD)	Desbrow et. al. (1998)
USA	0.477-3.66 (0.9)	-	<LOD-0.759 (0.248)	Snyder et. al. (1999)
Japan	3.2-55 (14)[b] <LOD-43 (13)[c] 0.3-30 (14)[d]	-	-	Nasu et. al. (2000)

[a]LOD = Limit of Detection
[b]Summer sampling
[c]Autumn sampling
[d]Winter sampling

Table 2.3: Concentration of hormones in surface water (Ying et al., 2002).

Location	17β-Estradiol (ng/L)	Estrone (ng/L)	17α-Ethinylestradiol (ng/L)	References
Japan	<LOD[a]-27 (2.1)[b] <LOD-24 (1.8)[c]	-	-	Tabata et. al. (2001)
Germany	0.15-3.6 (0.3)	0.10-4.1 (0.40)	0.10-5.1 (0.4)	Kuch and Ballschmiter (2001)
Italy	0.11	1.5	0.04	Baronti et. al. (2000)
The Netherlands	0.3-5.5 (<0.3)	<0.1-3.4 (0.3)	<0.1-4.3 (<0.1)	Belfroid et. al. (1999)

[a]LOD = Limit of Detection
[b]Summer sampling
[c]Autumn sampling

The measurements in Germany resemble the situation in the Netherlands. Estrogenic steroids were also detected in some drinking water samples from southern Germany with an average concentration of 0.4, 0.7 and 0.35 ng/l, respectively. E3 was found in

Tiber river water in Italy with a concentration of 0.33 ng/l, while E2 and E1 were 0.11 and 1.5 ng/l in the river water, respectively (Ying et al., 2002). Estrone (E1) was detected in 7 of 11 Dutch coastal/estuarine and freshwater samples with a median concentration of 0.3 ng/l, while E2 and EE2 were only detected in 4 and 3 of 11 samples, with most of the concentrations below the quantification limit of < 1 ng/l.

2.1.5.3.Ground water

Recent studies have shown that disposal of animal manure to agricultural land could lead to movement of estrogenic steroids into surface and ground water. E2 has been found mobile and detected in runoff from manured land. Nichols et al. (1998) determined an average E2 concentration of 3500 ng/l in the runoff from a pastural land applied with 5 Mg/ha of manure (poultry litter). Ground water has been reported to be contaminated with E2. Shore et al. (1995) believed that a constant E2 concentration of about 5 ng/l in spring waters was caused by infiltration of E2 through the soil profile to the ground water. Peterson et al. (2001) measured E2 concentrations ranging from 6 to 66 ng/l in mantled karst aquifers in northwest Arkansas. The observed E2 concentration trends imitated the changes in stage over the recharge event. The contamination was associated with poultry litter and cattle manure waste applied on the area (Ying et al., 2002).

2.1.6. Estrogens in sewage treatment process

A conventional STW is typically a three stage process consisting of preliminary treatment, primary sedimentation and secondary treatment. Wastewater treatment begins at the head of the works with preliminary treatment, typically inlet screens. However steroid biodegradation and biotransformation have been found to occur before the STW within the sewage system. This arises from the presence of bacterial slime which accumulates on the walls leading often to anaerobic biodegradation. In large catchment areas, the retention time of the sewage system can be significant allowing a high degree of transformation and degradation. Conventional wastewater treatment provides the best model to study the mechanisms through which natural estrogens are attenuated in nature and in engineered system. It is generally belived that transformation and biodegradation are the two main processes for estrogen removal from wastewater, with some disputing that adsorption can play a significant role in estrogen removal (Koh et. al., 2008).

2.1.6.1.Preliminary treatment

Initial raw sewage screening occurs at the preliminary treatment at the head of the works where large floating objects, grit and dense inorganic solids are removed. A small amount of organic material is removed from the screens. Little or no removal of organic micropollutants and steroid hormones is observed at this stage (Koh et. al., 2008).

2.1.6.2.Primary sedimentation

The raw sewage then passes into the primary sedimentation tanks where the most significant mechanism is adsorption onto solids, which under the influence of gravity settle to form primary sludge. The degree of pollutant removal is largely dependant on suspended solids removal, which is controlled by settling characteristics of the particles (their density, size and ability to flocculate); the retention time in the tank; and the surface loading. The retention time in the tank is referred to as the sludge retention time (SRT) or sludge age and is a measure of the time bacterial cells remain in the system. It is the total amount of sludge in the system divided by the total rate of sludge leaving the system and is typically 4 to 9 days. Removal od organic compounds can be affected by temperature and the solids content of the effluent is higher during winter months when the temperature is low. Fats, oils, and greases adsorb significant amount of hydrophobic compounds, including many EDCs, and are removed from the surface of the tank and added to the sludge prior to sludge treatment. **Figure 2.4** indicates some of the possible removal mechanisms for EDCs during primary sedimentation. Primary sedimentation is used in the majority of STW; in some cases flocculants are added to aid flocculation. Coagulants, such as aluminum and ferric salts have been used to remove organic matter, although their use is often deemed impractical due to the high costs. Where oxidation ditches are employed for wastewater treatment there is no primary sedimentation, and removal of EDCs probably conforms to the mechanisms identified or postulated for activated sludge treatment (Birkett and Lester, 2003).

2.1.6.3.Secondary treatment

It has been shown that secondary biological treatment is the key process behind the ability of some STWs to remove most, if not all, estrogenic activity. Transformation and biodegradation are thought to play a significant role in the hormone removal

since some of the microorganisms present in biological STWs posseses the potential to utilize steroid estrogens amongst other micropollutants as carbon sources for metabolism.

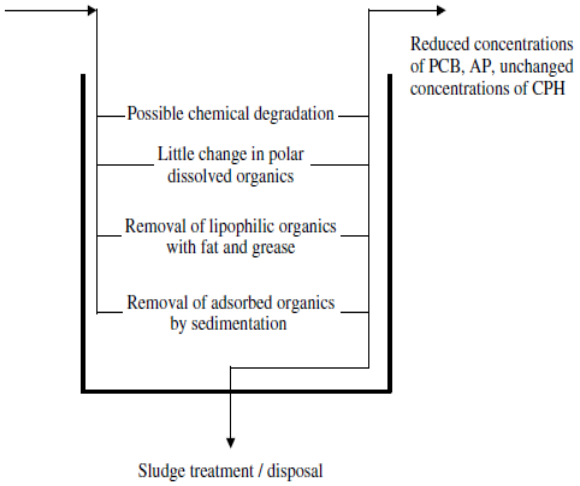


Figure 2.4: Removal mechanisms during primary sedimentation for EDC removal (Birkett and Lester, 2003).

The most rapid and complete degradation of pollutants present in the STWs is brought about under aerobic conditions through catabolic pathways. Correspondingly, anaerobic, nitrifying-denitrifying and methanogenic bacteria that carry out the detoxification of effluent contaminants have been discovered from various STWs (Koh et al., 2008).

The population dynamics of such microorganisms depend greatly on the type of process i.e. trickling filter, activated sludge process, membrane bioreactors and enhanced biological process. This section focuses on the impact of biological treatment design on EDC removal, since that is the key component of a conventional STW for estrogen removal. A recent study in England showed particularly dramatic benefits of adding a biological step. Simply adding a short secondary treatment stage of fine bubble aeration to a domestic STW that previously had only primary settlement produced a sudden and sustained reversal in feminization trends in downstream fish (Koh et. al., 2008).

Removal of Estrogens Using Trickling Filters

Trickling filters are generally less effective than activated sludge systems in eliminating estrogenic activity. Less than one third of the total estrogenicity was

removed in a trickling filter system. In a study to map the distribution of natural estrogens from 18 Canadian municipal treatment plants, researchers found poor estrogen removals at plants that incorporated trickling filter. Elsewhere, elevated levels of E1, E2 and EE2 were found in effluent from a trickling filter, over two sampling periods. This supports the work of Turan et al. (1995) who found that estrogens, particularly synthetic compounds, are stable enough to withstand the sewage treatment process. Conversely, a more extensive treatment train with two stage trickling filter process and two stage post sedimentation was able to achieve removals of 58% for E1 and 82% for E2 which was comparable to an activated sludge process (Koh, et. al., 2008).

Removal of Estrogens Using Activated Sludge Process

Conventional activated sludge process is commonly used to treat domestic and industrial wastewater mainly to remove organic compounds in STW influent. Batch studies have indicated that E1 and EE2 will not be completely removed in activated sludge in the present process. Ternes et al. (1999) found low elimination efficiencies for E1 and EE2 (<%10) but approximately two thirds of E2 was eliminated in the STWs. This was in agreement with Komori et al. (2004) who observed a 45% reduction in E1 which was considerably less than the reduction found for E2 and E3. The persistence of EE2 under aerobic conditions and rapid degradation of E1 and E2 were found also in laboratory experiments using STW sludge. In two plot scale municipal wastewater treatment plants, removal efficiencies for E1 and EE2 were 60% and 65% respectively with elimination of more than 94% for E2 entering the aeration tank.

In activated sludge systems, SRT seems to be an especially important factor in EDC removal. Several researchers have noted improved removal with increased SRT. A retention time of at least 10 to 12.5 days has been suggested as the period required for the growth of organisms that decompose estrogens.

Removal of Estrogens Using Membrane Bioreactors

In existing STWs where it may not be possible to adequately increase the SRT because of expense or site constraints, membrane bioreactors (MBR) could offer advantages of more flexibility to operate at higher SRTs in a smaller footprint. MBR technology is often considered a promising development in wastewater treatment

which integrates biological degradation of waste products with membrane filtration. These treatment systems are effective in removing organic and inorganic compounds as well as biological contaminants from wastewater. Steroids removal rates of greater than 90% were achieved in membrane bioreactors with nitrification and denitrification (SRT of 12-15 days).

Biological degradation has been cited as the important factor in the removal of estrogens and other endocrine disrupters in membrane bioreactor. This is in agreement with Ivashechkin et al. who operated conventional activated sludge and MBR pilot units in parallel, operating both for denitrification at two different SRTs (12 and 25 days), and applying the same influent wastewater and sludge loading rate to each system. The authors did not find an appreciable difference in removal of EE2 between the two systems and determined that estrogen removal was due primarily to biodegradation; removed estrogens were not sorbed onto sludge particles, nor were they retained in the membrane material or the membrane biofilm. Weber et al. report that E2 turnover rates to E1 did not differ greatly between conventional and membrane activated sludge. Furthermore, no degradation was observed for the persistent EE2 in both sludges. While microfiltration membranes themselves will not provide an enhanced degree of estrogens removal, it has been suggested that estrogens adsorption to particulate matter that is retained by the membrane would reduce estrogens concentration in the effluent. Some researchers have found that microfiltration membranes are able to display some retention of smaller particles or colloidal material onto which estrogens may adsorb (Koh et al., 2008).

Removal of Estrogens Using Biological Nutrient Removal Plants (BNR)

Of the biological treatment plants, BNR employed for nutrient removal such as nitrogen and phosphorous exhibits significant overall estrogen removals. To achieve biological phosphorus removal, an anaerobic zone between the activated sludge and influent wastewater before aerobic degradation is necessary. Biological nitrogen removal involves nitrifying and denitrifying reactions.

Nitrification relies on the metabolic activities of a group of chemoautotrophic bacteria essentially utilizing ammonia nitrogen, S_{NH}, and oxidizing it to nitrate nitrogen, S_{NO} for energy.

An activated sludge system for nitrification and denitrification including sludge recirculation has been observed to eliminate appreciably natural and synthetic estrogens. Andersan et al. (2003) found that to a large extent, the natural estrogens were degraded biologically in the denitrifying and aerated nitrifying tanks of the activated sludge system, whereas EE2 was only degraded in the nitrifying tank.

Some workers found that nitrifying plants exhibited greater removals than those without nitrifying capabilities. The presence of aerobic, anoxic, anaerobic zones in the BNR allows for most of the removal processes i.e. anaerobic biodegradation, adsorption, anoxic biodegradation and aerobic biodegradation to occur. A high sludge age is usually required to achieve nitrification and nutrient removal because the autotrophic bacteria involved grow very slowly (Koh et. al., 2008).

2.1.7. Behavior of estrogens in sewage treatment works

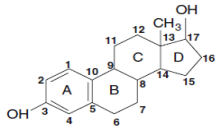
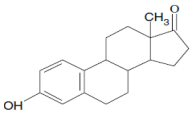
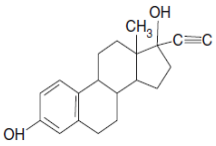
The behavior of estrogens in STWs is dependent on their physicochemical properties (Table **2.4**). Aqueous solubility (mg/L), organic carbon/water partition coefficients (K_{oc}), and K_{ow} influence the partitioning and sorption of a compound during treatment. The H_c is their volatilization potential and is also an indicator of the likelihood of evaporation during treatment.

2.1.7.1.Adsorption

Sewage sludge is a complex mixture of fats, proteins, amino acids, sugars, carbohydrates, lignin, celluloses, humic material and fatty acids. In secondary sludge, the large amounts of live and dead microorganisms provide a large surface area. Estrogens preferentially adsorb onto these suspended particulates because of their hydrophobic properties. The K_{ow} values often correlate with the degree of association between an organic compound and the solid phase. K_{ow} is the concentration ratio at equilibrium of an organic compound partitioned between an organic liquid and water and is one of the quantitative physical properties that correlates best with biological activity. It can be used as a measure of lipophilicity and is therefore used to predict sorption onto solids (Birkett and Lester, 2003). Log K_{ow} values increase with increasing lipophilicity and correlate inversely with solubility. Large K_{ow} values are characteristic of large hydrophobic molecules that tend to associate with solid organic matter, while smaller hydrophilic molecules have low log K_{ow}. It is better indication of the extent of adsorption by microorganisms than solubility. Below a log

K_{ow} of approximately 4, the removal of estrogens during primary treatment is dominated by advection of the dissolved compounds.

Table 2.4: Physicochemical properties of steroids (Birkett and Lester, 2003).

Name	17β-Estradiol (E2)	Estrone (E1)	17α-Ethinylestradiol (EE2)
Structure			
Formula	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_2$	$C_{20}H_{24}O_2$
Molecular weight (g)	272.39	270.37	296.40
Aqueous solubility (mg/l)	12,96	12,42	4.83
Log K_{OW}	3.94	3.43	4.15
Size (nm)	0.398	0.396	
H_c (atm m^3/mol)	6.3×10^{-7}	6.2×10^{-7}	3.8×10^{-7}

A log K_{ow} of less than 2.5 demonstrates a low sorption potential, and a log K_{ow} greater than 4 shows a high sorption potential. At a log K_{ow} greater than 4, the major removal process is sorption to the settled sludge and with that associated with suspended matter. The organic carbon content of the solid phase and the significance of the polarity and composition of orgaic matter have been highlighted as major surface variables influencing sorption for the majority of organic compounds (Birkett and Lester, 2003).

The K_{oc} is also an important parameter when considering adsorption. K_{oc} is the ratio between the concentration of the organic compound on organic carbon (mg/g) and its concentration in water (mg/l) at equilibrium; it can be estimated from log K_{ow} values or solubility. The likelihood that a compound will sorb to organic matter, such as sewage sludge, can be assessed using log K_{oc}. Generally compounds with a high log K_{oc} will tend to adsorb onto sewage sludge, while those with lower values will tend to remain in the aqueous phase (Birkett and Lester, 2003).

Increasing the SRT reduces the amount of sludge wastage from the activated sludge system. This results in a decrease in compound concentrations in the final effluent because of increased sorption and biodegradation. At low SRTs, the high mass flows of wasted sludge and the wash-out of slow growing specific degraders result in the

majority of estrogens being removed in the sorbed phase. Removal of estrogens in settled sludge from primary sedimentation varies as a function of the partitioning behavior to sludge through log K_{ow}, a compound's removal can be influenced by changes in sedimentation efficiency. A reduction in sedimentation efficiency would result for example from an increase in influent flow rate.

Sorption onto inorganic and biological solids is an important removal mechanism because adsorption onto cellular material is often the first stage in biological degradation of these compounds. However, compounds that are strongly bonded onto inorganic particles are less available for degradation and volatilization. Organic compounds are adsorbed onto raw wastewater solids during primary treatment and onto biological sludges during secondary treatment (Birkett and Lester, 2003).

2.1.7.2. Biological degradation and transformations

Due to the endocrine disrupting potency of natural and synthetic estrogens, there has been an increasing interest in biodegradation of these estrogens by using microorganisms.

Biological degradation may occur as a result of intra- and extracellular enzymes. The hydrolysis process, for example, involves regulation of extracellular enzyme synthesis in the cells. It takes place by enzymes secreted by the cells before the substrate can be taken up by the microorganisms and be metabolized. Chemical properties of a compound influence their entry into microbial cells, which is a prerequisite for the induction of intracellular enzymes. Reactions that involve extracellular enzymes are important for large volume molecules and possibly molecules with large molecular masses. These enzymes are excreted from cells into solution or are released when aged cells undergo lysis in low growth conditions (Birkett and Lester, 2003).

Unlike naturally occuring compounds, anthropogenic compounds tend to be relatively resistant to biodegradation. This is partly due to the fact that microorganisms lack the necessary enzymes required for transformation, so a longer acclimation period may be required. However, the biodegradation of anthropogenic compounds can be facilitated by co-metabolism. Co-metabolic transformations are the transformations of a compound by metabolic reactions that do not contribute carbon or energy to the biological growth of the organism. The organism uses co-substrates to support its growth (Birkett and Lester, 2003). The importance of

biotransformation increases with SRT and increasing log K_{ow} is their influence on biosorption. Biotransformation rates increase to a maximum at log K_{ow} 3 to 3.5 (e.g., estrone has a log K_{ow} of 3.43) and then decline rapidly as sorption to sludge dominates the removal mechanisms for more hydrophobic compounds. Biotransformation has more influence on compounds with log K_{ow} in the range 1.5 to 4, such as estradiol with log K_{ow} 3.94. K_{ow} values also play a role in determining bioconcentration in the food chain. Compounds that are easily metabolized or are polar tend to have lower K_{ow} values and do not easily enter the food chain. Lipophilic compounds with high K_{ow} values however do accumulate (Birkett and Lester, 2003).

Role of Nitrifying Bacteria in Biodegradation

Longer SRTs allow for the establishment of slow-growing nitrifying bacteria (i.e., ammonia oxidizing bacteria and nitrite-oxidizing bacteria). Several studies evaluated whether nitrifying bacteria improve the biodegradation of estrogens.

Lee et al. (2002) showed that E2 of 200 μg/l has been almost quantitatively oxidized to E1 by sewage bacteria under both aerobic and anaerobic conditions. Michael et al. (2001) and Colucci et al. (2001) reported that E2, E1 and EE2 were rapidly biodegraded in agricultural soils. Ying et al. investigated degradations of E2 and EE2 using aquifer materials, or a marine sediment and seawater under aerobic condition, indicating that E2 of 1 μg/g in the sediment was degraded quickly with a half life of 2 or 4.4 d, while EE2 was degraded much more slowly with a half life of 81 or >20 d. Jurgens et al. (2002) showed that microorganisms in the river water sample were capable of transforming E2 to E1, and that E1 was then degraded at similar rates, but EE2 was much more resistant. Regarding EE2, Vader et al. (2000) demonstrated that nitrifying activated sludge (NAS) could degrade EE2 at an initial concentration of 50 μg/l within 6 d; a result allowing us to surmise that NAS may degrade natural estrogens including E1, E2, E3 (Shi et al., 2004).

Shi et al. (2004) show that EE2 can be degraded by certain microorganisms. Nitrifying activated sludge (NAS) containing a lot of nitrifying bacteria is used to oxidize ammonia to nitrite and nitrate. *Nitrosomonas europaea* in nitrogen removal systems, an obligate chemolithotrophic ammonia – oxidizing bacterium, is usually responsible for the oxidation of ammonia to nitrite, deriving its energy for growth exclusively from the oxidation of ammonia to nitrite. It is also known to be capable of oxidizing various hydrocarbon compounds such as methane, methanol, phenol,

and benzene, as well as halogenated hydrocarbons. In the presence of the ammonia oxidation inhibitor, ammonia oxidized bacteria in NAS did not grow. At an initial estrogen concentration of 0.2 mg/l, the degradation rate constants were 0.004 h^{-1} for E1, 0.32 h^{-1} for E2, and 0.0085 h^{-1} for EE2 with the inhibitor, whereas the degradation rate constants were 0.036 h^{-1} for E1, 0.60 h^{-1} for E2, and 0.059 h^{-1} for EE2 without the inhibitor. These results suggested that ammonia oxidation bacteria together with microorganisms in NAS degraded the estrogens (Shi et. al., 2004).

Studies by Yi and Harper (2007), Khunjar et al. (2007), and others have focused on the mechanisms of estrogen removal during nitrification. Possible mechanisms include sorption of estrogens to solids and biotransformation within the treatment facility, especially in the presence of nitrifying activated sludges (Khunjar et al., 2007). Ammonia oxidizing bacteria have monoxygenase enzymes for ammonia oxidation and these enzymes have been shown previously to be nonspecific and able to accomplish cometabolic degradation of recalcitrant organics. Cometabolic degradation is a reasonable hypothesis for estrogen degradation because this compound is present at low ng/L concentrations that are below those expected to support microbial growth on that compound alone (EPA, 2009).

One goal of the Yi and Harper (2007) study was to establish whether biotransformation of EE2 is due to cometabolic activity. They conducted batch experiments using enriched cultures of autotrophic ammonia oxiders. Their study and others (Vader et al., 2000, Shi et al., 2004, as reported in Yi and Harper, 2007) showed a strong relationship between nitrification and EE2 removal in enriched nitrifying cultures. Based on batch tests with and without a nitrifying bacteria inhibitor, they concluded that EE2 biotransformation can be cometabolically mediated in bioreactors that are enriched for autotrophic nitrifiers. However, Yi and Harper (2007) noted that the heterotrophic microorganisms, if present in activated sludge processes, may also be responsible for some micropollutant biotransformations. Further work is needed in this area as these tests did not identify the EE2 degradation product to confirm cometabolic degradation and the role of heterotrophs was not accounted for in some tests (EPA, 2009).

The focus of a Khunjar et al. (2007) study was to identify the role of ammonia oxidizing bacteria compared to heterotrophic bacteria in the biotransformation of EE2. They used pure cultures of ammonia oxidizing *Nitrosomonas europaea* and

heterotrophic cultures that were enriched with monooxygenase and dioxygenase enzyme systems. Nitrifying activated sludge mixed liquors were taken from two WWTPs to seed the cultures. EE2 concentrations were 10 - 15 µg/L. The results of their study showed significant sorption of EE2 to the predominantly heterotrophic culture but none to the *N. europaea* culture. In addition, biotransformation of EE2 was significant in the *N. europaea* culture. They observed three major EE2 metabolites at different phases of *N. europaea* culture growth that suggest differential action on each byproduct by the nitrifying bacteria; however, additional work is needed to identify these byproducts. The authors also noted that additional research is needed with continuous flow cultivated *N. europaea* to determine whether these metabolites are likely to be present in nitrifying activated sludge. Also, *N. europaea* was not significantly inhibited at EE2 concentrations at or below 10 µg EE2/L, suggesting that ammonia oxidation may not be significantly impacted by concentrations of EE2 that may be typical of those found in the environment (EPA, 2009).

2.1.7.3.Volatilization

Volatilization is the transfer of a compound from the aqueous phase to the atmosphere from the surface of open tanks such as clarifiers. However, the majority of losses occur through air stripping in aeration vessels. A proportion may be lost during sludge treatment at the dewatering or thickening stage, particularly if the sludge is aerated or agitated. The activated sludge stage of treatment aeration allows air stripping to occur. Low molecular mass, nonpolar compounds with low aqueous solubilities, and low vapor pressures are known to be transferred to the atmosphere during aeration in wastewater treatment. However, due to the static nature of sedimentation process, losses by volatilization are small. A peak removal rate is seen for compounds with a log K_{ow} of around 2. At less than 2, increased water solubility inhibits volatilization. H_c can be used to predict losses by volatilization. Generally, compounds with an H_c greater than 10^{-3} mol^{-1} m^{-3} can be removed by volatilization (Birkett and Lester, 2003).

2.1.8. Fate of estrogens in sewage treatment works

Steroid removal can be influenced by HRTs and SRTs in STWs as demonstrated in **Table 2.5**. Other factors include the time it takes to reach STW, the nutrient status,

type of treatment and the activity and stability of resident biota, and the use of secondary treatment processes.

Research has shown that the majority of steroid estrogens enter the STW in their conjugated form. Natural estrogens, i.e., estrone (E1), 17β-estradiol (E2) and estriol (E3), are excreted by humans and animals through their urine principally as inactive polar conjugates such as glucuronides and sulphates. The synthetic estrogen, 17α-ethynylestradiol (EE2), is a key ingredient in oral contraceptives and is mainly eliminated as conjugates in urine. Many of these conjugates of natural and synthetic estrogens are cleaved to free estrogens through microbial processes before or during sewage treatment. Ascenza et al. have investigated the concentrations of both free and conjugated estrogens in the six sludge treatment plants (STPs) in Roman indicating that E3-16-glucuronide, E2-3-glucuronide, and E2-3-sulfate surviving in the sewer system were completely removed by the STP treatment, while E1, E2, E3, E1-3-glucuronide, E1-3-sulfate and E3-3-sulfate were detected in the effluents. In addition, *Escherichia coli* synthesize large amounts of the enzyme β-glucuronidase, which deconjugates the steroids in the gut. These bacteria are also present in the STW, so it is expected that they will deconjugate steroids during treatment.

Ternes et al.(1999) looked at the behavior and occurrence of estrogens in aerobic batch experiments with activated sludge. 17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide were cleaved in contact with the sludge to form 17β-estradiol E2. E2 was shown to oxidize to E1 on contact with the activated sludge, but no further degradation occurred and estrone was the final product. The concentration of E2 was immediately reduced. After 1 to 3 hours, 95% had been removed and the level of E1 had risen by 95%. The synthetic hormone mestranol was quickly degraded and EE2 was produced in small amounts; EE2 was then only slightly reduced demonstrating its persistence.

Table 2.5: Removal Rates of Steroid Estrogens at Varying Retention Times

	13 – 15 °C		18 – 19 °C	
	E1 % loss	E2% loss	E1 % loss	E2% loss
18-hour SRT 6-day SRT	64	92	75	-
18-hour SRT 11 day SRT	94	98	>98	-
26-hour SRT 20 day SRT	66	>75	98	>94

Baronti et al. (2000) reported average E1, E2, E3 and EE2 removals of 61%, 86%, 95% and 85% respectively, in Rome activated sludge treatment plants. The efficiency for estrone is lowest, and at 4 out of 30 sites it exhibited an increase in concentration from influent to effluent. This can partly be explained by the biological oxidation of E2 to E1.

Removal rates of EE2 in NAS were at a maximum for the first 2 days with a degradation rate of 1 $\mu g\ g^{-1}$ Dry Weather h^{-1}. After this period, the degradation rate slowed. This was most likely due to the affinity of microorganisms for EE2 at lower concentrations or a decrease in the activity of the nongrowing cells. The low concentration may result in the bacteria being in a starved condition (senescent cells) in the phase between death and the breakdown of the osmotic regulatory system (in moribund state). The oxidation of EE2 was confirmed by the formation of hydrophilic organic compounds that were not identified. When hydrazine was added as an external electron donor to provide unlimited reducing energy, degradation of EE2 was slightly higher than without hydrazine addition. This demonstrated that EE2 degradation is mediated by monooxygenase activity. The capacity to transform EE2 by the nitrifying sludge may become a function of the reducing energy.

A study of mass balances of estrogens in STW in Germany demonstrated that most of the estrogenic activity in the wastewater was biodegraded during treatment rather than adsorbed onto suspended solids. There was a 90% reduction in estrogenic load, and less than 3% of the estrogenic activity was found in the sludge. Radiolabelled E2 was used in a study of estrogen fate in STW. At low concentrations the majority of the radiolabelled E2 remained in the liquid phase.

In another study, suspended solids content was an important factor. A higher suspended solids content resulted in a higher removal of estrogens, while an increase in influent estrogen concentration caused a decrease in removal. Salinity and pH also affect the removal. pH changes influence the amount and the type of bonding involved in sorption; a higher sorption rate was observed at neutral pH and at high salinity. Competition for binding sites effects estrogen removal, the addition of estrogen-valerate with a very high Kow (6.41) reduced the amount of estrogen removed, as was also shown by Lai et al. (2000) during a study of Japanese night soil treatment processes, the highest amount of estrogenic activity was found to be in the sludge from the sludge tank, while the effluent contained very much lower

concentrations. E2 accounted for 16% of the estrogenic activity, and the estrogenic activity in the aqueous phase decreased by 1000 times during biological treatment. The estrogens were considered to be accumulating in the sludge and passed through the biological treatment.

In municipal biosolids, 84% of estradiol and 85% of estrone were mineralized in 24 hours, compared to less than 4% in industrial biosolids. No correlation between BOD and suspended solids removal with mineralization was observed, although temperature was seen to have an effect. At different temperatures no significant differences in first order rate constants were seen for EE2. However, E2 was significantly different, and even at cold temperatures, it was rapidly removed by biosolids.

2.2. Activated Sludge Modeling

2.2.1. The need for models

WWTPs are complex systems that depend on numerous biological, chemical, and physical processes to achieve effluent goals. Because of the complex behavior of the processes and the variability in wastewater characteristics, biological populations, and plant design, it is not always possible to predict how changing any one variable will affect the effluent quality. Plant designs that work for one influent wastewater and climate may not perform well in different conditions. Pilot scale or full scale trials can help to determine the effect of various parameters, but costs and time to cover all possibilities may be prohibitive. Therefore, models fill an important need by enabling simulation of a process and estimating the impact that changing parameters will have on the treatment effectiveness.

Models can be used for a number of purposes including the design of new WWTPs, the design of retrofits or upgrades to existing plants, determining how changes in operations may affect effluent concentrations of permitted contaminants, determining how plants will respond to changes in influent quality or flow, and for training operators. Not all models can achieve all of these purposes, so models should be selected with the desired use in mind (EPA, 2009).

Models can be used for determining how plants will respond to changes in influent quality or flow and design of refrofits or upgrades to existing plants. Moreover, it can

be used for determining how changes in operation may effect effluent concentration of permitted contaminants.

2.2.2. Overview of models

Model evaluation is now considered as an integral component of biological systems, as it yields numerical information about related process kinetics and this way, it provides a meaningful correlation between results derived from different experimental sets (Katipoğlu Yazan et al., 2012).

Models are sets of equations, generally based on theory and grounded in empirical data, that represent a wastewater treatment process. Each unit process is represented by its own model. Model equations for processes such as clarification and settling are well known and fairly simple. Modeling biological wastewater processes such as activated sludge, however, is much more complicated. The primary set of models for activated sludge processes has been compiled by the International Water Association (IWA) (formerly IAWQ and IAWPRC). The first model was developed in 1986 and was called the activated sludge model (ASM). Later known as Activated Sludge Model No.1 (ASM 1), this model was able to model the biological oxidation of carbon, nitrification, and denitrification (EPA, 2009).

Although the ASM model gained widespread use among both academia and industry, it had limitations. In order to improve the model, IWA developed other ASM models; ASM 2 and ASM 3. The ASM 3 models were intended to deal with limitations such as the independence of the ASM 1 model of temperature and carbon source (**Table 2.6**).

Table 2.6: Activated Sludge Models (WERF 2003, Gernaney et al. 2004)

Model Name	Wastewater Treatment Unit	Reference
ASM 1	Carbon oxidation, nitrification, denitrification	Henze et al. 1987
ASM 2	Carbon oxidation, nitrification, denitrification, enhanced biological phosphorus removal, fermentation, chemical phosphorus removal	Henze et al. 1995
ASM 3	Carbon oxidation, nitrification, denitrification	Henze et al. 1999

The model has some processes that it enables calculation of oxygen consumption, ammonia, and nitrate in tanks of treatment plants and in effluents. In addition, mixed liquor suspended solids, solid retention time and sludge production can also be assessed by using this type of models (Jördening et al., 2005).

In the multicomponent model, COD is selected as the most suitable parameter for defining the carbon sources as it provides a link between electron equivalents in the organic substrate, biomass, and oxygen utilized. Organic carbon removal can be modeled by using ASM 1 and ASM 3, which involve different processes.

2.2.2.1. ASM 1

In ASM 1, COD is divided into fractions based on its solubility, biodegradability, biodegradation rate, and viability for biomass. The main COD fractions are defined as soluble (S), and particulate (X) COD. They are further divided into non-biodegradable fraction and biodegradable fraction. The non-biodegradable fraction is biologically inert and passes through an activated sludge system in an unchanged form. The inert soluble organic matter (S_I) leaves the system at the same concentration as it enters. Inert particulate matter (X_I) in the influent wastewater is removed with particulate organic matter produced via decay processes by sludge wastage (Orhon et al., 1994).

Growth, decay and hydrolysis are basic processes which are involved in ASM 1. The basic relationship is given in **Figure 2.5**. According to the model, carbon removal is slightly coupled with nitrification. Heteretrophs utilize organic matter directly or after hydrolysis process whereas autotrophic bacteria utilize ammonia for their growth. Decay of organisms results in particulate matter formation which in turn can be utilized for growth following hydrolysis.

The biodegradable matter is divided into soluble readily biodegradable, (S_S) and slowly biodegradable substrate (X_S). Some of the X_S is assumed soluble. S_S is assumed to be simple organic matter that is utilized by heterotrophic organisms for growth. On the other hand, regeneration of slowly biodegradable particulate matter on nonviable biomass is observed in death-regeneration model while the rest of it is converted to inert particulate product (X_P). On the contrary, X_S consists of relatively complex molecules that require enzymatic breakdown prior to utilization to S_S such as hydrolysis. Heterotrophic biomass X_H and autotrophic biomass X_A are generated by growth on S_S or by growth on ammonia nitrogen S_{NH}. The biomass is lost via the decay process and converted to some other particulate components (Orhon et al., 1994).

In endogenous decay model, S_S is utilized in only growth process. In addition, generation of inert particulate products is linked to the active biomass decay, which a fraction of biomass (f_{EX}) turns into inert particulate products, X_P. These products do not go any further reaction and accumulate in the system until they are removed by sludge wastage.

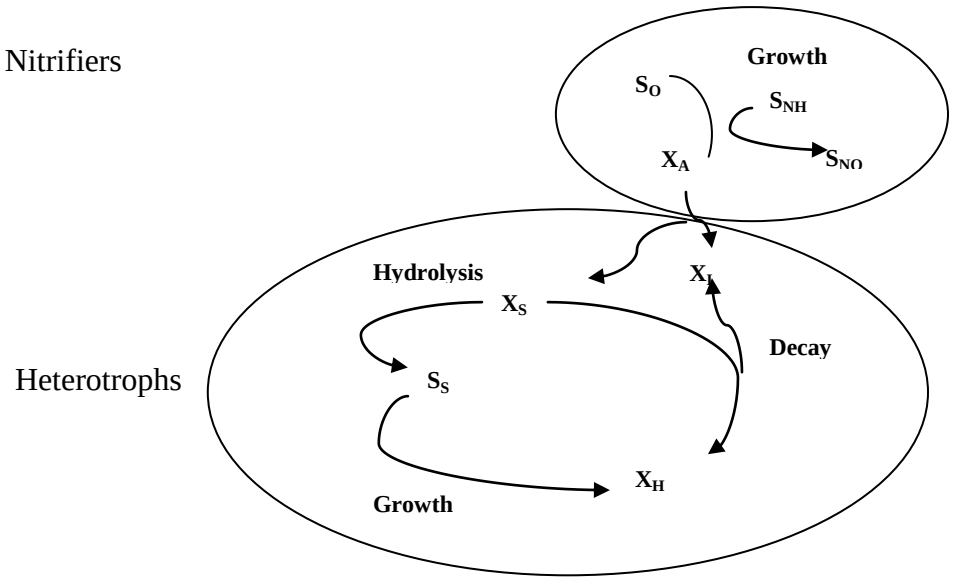


Figure 2.5: Processes for Heterotrophic and Nitrifying Bacteria in ASM 1 (Jördening et al., 2005)

On the other hand, soluble inert product formation is assumed through decay of a fraction of biomass (f_{ES}) (Orhon et al., 1994).

The decrease of biomass can be given as (McKinney, 1962):

$$\frac{dX}{dt} = \frac{dX_H}{dt} + \frac{dX_p}{dt} \quad \textbf{(1.1)}$$

b_H is defined as the endogenous decay coefficient. The change in active biomass is expressed as :

$$\frac{dX_H}{dt} = -b_H X_H \quad \textbf{(1.2)}$$

Generation rate of particulate inert products are given as follows:

$$\frac{dX_p}{dt} = f_{EX}\frac{dX_H}{dt} \quad \textbf{(1.3)}$$

When the maximum growth rate of heteretrophs and half saturation constant of substrate are defined as μH and, KS respectively, biodegradation rate of SS which is directly used in growth is given as follows:

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

The decay associated soluble inert product formation rate can be given as follows:

$$\frac{dS_p}{dt} = f_{ES} b_H X_H \quad \textbf{(1.5)}$$

Where K_X and k_h are maximum specific hydrolysis rate and half saturation coefficient for hydrolysis of slowly biodegradable substrate, hydrolysis of this fraction to S_S is given as:

$$\frac{dX_S}{dt} = k_h \frac{X_S/X_H}{(K_X + X_S/X_H)} X_H \quad \textbf{(1.6)}$$

Matrix representation of basic relationships between process components of endogenous model is given in **Table 2.7**. The S_I and X_I components are not included in the matrix since they do not go through biochemical processes.

Table 2.7: Simplified Matrix Representation of ASM1 Involving Endogenous Decay

Component → / Process ↓	1 S_S	2 X_S	3 X_H	4 X_P	5 S_P	6 S_0	Process Rate $ML^{-3}T^{-1}$
Growth	$-\frac{1}{Y_H}$		1			$-\frac{(1-Y_H)}{Y_H}$	$\mu_H \frac{S_S}{(K_S+S_S)} X_H$
Hydrolysis	1	-1					$k_h \frac{X_S/X_H}{(K_X+X_S/X_H)} X_H$
Decay			-1	f_{EX}	f_{ES}	$-(1-f_{EX}-f_{ES})$	$b_H X_H$
Parameter, ML^{-3}	COD	COD	Cell COD	COD	COD	O_2	

2.2.2.2.ASM 3

ASM 3 is one of the multi component models involving carbon and nitrogen removal with additional substrate storage process to ASM 1.

ASM3 is one of the first models to address the storage phenomenon. To keep the modeling exercise simple (Gujer et al., 1999), it assumed that all readily biodegradable substrate (S_S) is first stored as internal storage products before it is used for growth during the famine phase. The basic process that are involved in ASM 3 is shown in **Figure 2.6**. The substrate flow is given as storage, growth, and maintenance.

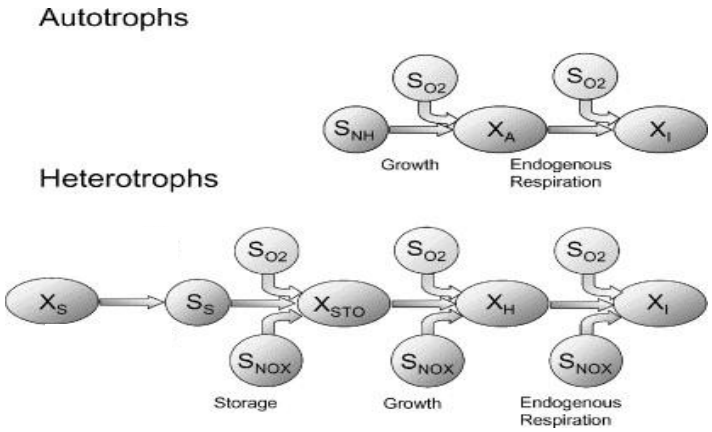


Figure 2.6: Process for Heterotophic Organisms in ASM 3.

According to metabolic model of storage compounds, PHB is taken by microorganisms and it is converted into acetyl-CoA. Acetyl-CoA is used for biosynthesis, as energy source and in the storing processes. Famine conditions forces biomass to hydrolyze acetyl-CoA. On the other hand, glucose is taken up and used for the production of Glucose-6-phosphate is also an intermediate in catabolic reactions. When the external substrate is over, glycogen is used for the synthesis of Glucose-6-phosphate. Aerobic storage of readily biodegradable substrate is the main process that differs from ASM 1. Under transient loading, heterotrophic bacteria can store organic matter, S_S in the form of polyhydroxyalkanoate (PHA). S_S is first stored in the biomass and converted to internal storage polymers in an energy requiring process. The storage products X_{STO} are used in aerobic heterotrophic growth process when there is not external substrate in the environment. In aerobic endogenous respiration process, all forms of biomass loss are involved including decay,

endogenous respiration. The respiration process of storage products is the other main process that is similar to endogenous respiration in ASM 1 that emphasize both storage products and biomass decay. In ASM 3, conversion of X_S to S_S is also involved. In ASM 3 substrate consumption rate is given as follows. k_{STO} is maximum rate of storage [M COD.(M Cell COD.T)$^{-1}$].

$$\frac{dS_S}{dt} = k_{STO} \frac{S_S}{K_S + S_S} X_H \quad \textbf{(2.1)}$$

The storage product formation rate is given in equation 2.1 where Y_{STO} is storage yield [M COD.(M COD.T)$^{-1}$]. Y_{STO} reflects the stoichiometric amount of substrate converted into storage products followed by utilization for growth.

$$\frac{dX_{STO}}{dt} = Y_{STO} k_{STO} \frac{S_S}{K_S + S_S} X_H \quad \textbf{(2.2)}$$

Growth of biomass under both feast and famine conditions is described depended on storage polymer concentration and half saturation constant of storage respectively X_{STO} [M COD L^{-3}], and K_{STO} [M COD.(M COD^{-1})].

$$\frac{dX_H}{dt} = \mu_H \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} X_H \quad \textbf{(2.3)}$$

Decay rate of storage products is given depended on heterotrophic yield.

$$\frac{dX_{STO}}{dt} = \frac{\mu_H}{Y_H} \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} X_H \quad \textbf{(2.4)}$$

The process of endogenous decay is given as a function of endogenous decay rate of heterotrophs, b_H and X_H :

$$\frac{dX_H}{dt} = b_H X_H \quad \textbf{(2.5)}$$

Respiration of storage products is a function of endogenous respiration rate of storage products, b_{STO} and X_{STO} [T^{-1}].

$$\frac{dX_{STO}}{dt} = b_{STO} X_{STO} \quad \textbf{(2.6)}$$

The simplified matrix representation of ASM 3 is given in **Table 2.8**.

According to Katipoğlu-Yazan et al. (2012), the model adopted for the study was structured to include model components and processes for: organic carbon removal; ammonia release, and nitrification. Accordingly, the first-unit of the model, which constitutes the backbone of all similar activated sludge models, defined the organic substrate and the heterotrophic biomass. 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, SH_1 and SH_2 with markedly different hydrolysis rates and a small readily biodegradable COD (SS) fraction (Insel et al., 2003; Orhon et al., 2009). Consequently, major metabolic activities of the heterotrophic active biomass, X_H were defined as growth on S_S; hydrolysis of SH_1 and SH_2 ; and endogenous decay of X_H. Dissolved oxygen, S_O, was included as the significant model component in the OUR experiments. Generation of particulate residual microbial products, X_P, should also be accounted for, as X_P needs to be considered for nitrogen mass balance in the system. Studies also reported that substrate storage as polyhydroxyalkanoates (PHAs) occurred as an auxiliary biochemical process during the biodegradation of the peptone mixture in batch systems (Katipoglu et al., 2010; Orhon et al., 2009). In fact, since experiments were carried out in batch systems involving initial/pulse substrate feeding, they were likely to induce substrate storage, which makes it necessary to also account for storage. Therefore, the sub-model was designed as a simultaneous growth-storage model, successfully implemented in many similar studies (Cokgor et al., 2011; Krishna and van Loosdrecht; 1999), also including storage products, X_{STO}, as model component and two new biochemical processes, namely storage and growth on stored PHAs. The second unit of the model mainly addressed the ammonia release mechanism as the major objective of the study. Its mechanistic structure was primarily based on the assumption that soluble organic nitrogen, S_{ND} was a fraction of the readily and slowly biodegradable COD, SH_1 and SH_2.

$$S_{ND} = i_{SNH1} + i_{SNH2} \cdot S_{H2} \qquad \textbf{(2.7)}$$

where, i_{NSH1} and i_{NSH2} are the nitrogen fractions of SH_1 and SH_2 (mg N/mg COD), respectively. Consequently, generation of simpler (soluble) organic nitrogen, S_{ND}, could conveniently be defined in terms of hydrolysis:

$$\frac{dS_{ND}}{dt} = i_{NSH1} k_{h1} \frac{S_{H1}/X_H}{K_X + S_{H1}/X_H} X_H + i_{SNH2} k_{h2} \frac{S_{H2}/X_H}{K_{XX} + S_{H2}/X_H} X_H \qquad \textbf{(2.8)}$$

Table 2.8: Simplified matrix representation of ASM 3 for organic carbon removal

Component	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**Process Rate $ML^{-3}T^{-1}$**
Process	**S_0O_2**	**S_S**	**S_I**	**X_S**	**X_I**	**X_H**	**X_{STO}**	
Hydrolysis		$(1-f_{SI})$	$-f_S$					$k_h \frac{X_S/X_H}{\left(K_X + X_S/X_H\right)} X_H$
Storage of S_S	-(1-Y_{STO})	-1						$k_{STO} \frac{S_S}{K_{STO}+S_S} X_H$
Growth on X_{STO}	$-\frac{(1-Y_H)}{Y_H}$					1	Y_{STO}	
Endogenous Respiration	$-(1-f_I)$				1	-1	$-\frac{1}{Y_H}$	$b_H X_H$
Respiration of X_{STO}	-1			f_I	-1		-1	$b_{STO} X_{STO}$
Parameter, ML^{-3}	O_2	COD	COD	COD	COD	Cell COD	COD	

Release of ammonia, S_{NH}; was attributed to the breakdown of S_{ND} as suggested in similar activated sludge models (Lu et al., 2001; Wang et al., 2007):

$$\frac{dS_{NH}}{dt} = -\frac{dS_{ND}}{dt} = k_a S_{ND} X_H \quad \textbf{(2.9)}$$

where, ka is the ammonification coefficient (m^3/g COD day). Accordingly, this sub-model added S_{ND} and S_{NH} together with two processes, i.e. ammonification and hydrolysis modified with the i_{NSH1} and i_{NSH2} coefficients, to the main model structure. The third sub-model concerning nitrification obviously requires concentrations of ammonia, S_{NH}, and nitrate, S_{NO3}, with alkalinity and dissolved oxygen, S_O. The complete description of the overall model is outlined in the usual matrix format in **Table 2.9** and **Table 2.10**.

Table 2.9: Kinetic equations of modified ASM 3 model structure (Katipoğlu-Yazan et al., 2012).

Process	Rate Equation
Growth of X_H	$\mu_H \frac{S_S}{K_S + S_S} \frac{S_O}{K_{OH} + S_O} X_H$
Growth of X_A	$\mu_A \frac{S_{NH}}{K_{NH} + S_{NH}} \frac{S_O}{K_{OH} + S_O} X_A$
Growth on X_{STO} by X_H	$\mu_{STO} \frac{X_{STO}/X_H}{K_{STO} + X_{STO}/X_H} \frac{S_O}{K_{OH} + S_O} X_H$
Decay of X_H	$b_H X_H$
Decay of X_A	$b_A X_A$
Hydrolysis of S_{NH1}	$k_{h1} \frac{S_{H1}/X_H}{K_X + S_{H1}/X_H} X_H$
Hydrolysis of S_{NH2}	$k_{h2} \frac{S_{H2}/X_H}{K_{XX} + S_{H2}/X_H} X_H$
Storage of PHA by X_H	$k_{STO} \frac{S_S}{K_S + S_S} X_H$
Ammonification of S_{ND}	$k_A S_{ND} X_H$

Table 2.10: Matrix representation of modified ASM 3 model structure (Katipoğlu-Yazan et al., 2012).

Process	**S_{O2}**	**S_S**	**S_P**	**S_{NH}**	**S_{ND}**	**S_{NOX}**	**S_{H1}**	**S_{H2}**	**X_H**	**X_A**	**X_{STO}**	**X_P**	**Alk**
Growth of X_H	$\frac{(1-Y_H)}{Y_H}$	$-\frac{1}{Y_H}$		$-i_{NBM}$					1				$-\frac{i_{NBM}}{14}$
Growth of X_A	$-\frac{(4{,}57-Y_A)}{Y_A}$			$-\frac{1}{Y_A}-i_{NBM}$		$\frac{1}{Y_A}$				1			$-\frac{i_{NBM}}{14}-\frac{2}{14Y_A}$
Growth on X_{STO} by X_H	$\frac{(1-Y_H)}{Y_H}$			$-i_{NBM}$					1		$-\frac{1}{Y_H}$		$-\frac{i_{NBM}}{14}$
Decay of X_H	$-(1-f_{ES}-f_{EX})$		f_{ES}	$i_{NBM}-i_{NXP}f_{EX}-i_{NSP}f_{ES}$					-1			f_{EX}	$\frac{i_{NBM}-i_{NXP}f_{EX}-i_{NSP}f_{ES}}{14}$
Decay of X_A	$(1-f_{ES}-f_{EX})$		f_{ES}	$i_{NBM}-i_{NXP}f_{EX}-i_{NSP}f_{ES}$						1		f_{EX}	$\frac{i_{NBM}-i_{NXP}f_{EX}-i_{NSP}f_{ES}}{14}$
Hydrolysis of S_{NH1}		1			$-i_{SNH1}$								
Hydrolysis of S_{NH2}		1			$-i_{SNH2}$		1						
Storage of PHA by X_H	$(1-Y_{STO})$	-1						1			Y_{STO}		
Ammonification of S_{ND}				1	-1								$\frac{1}{14}$
COD (g COD)	-1	1	1			-4,57	1	1	1	1	1	1	-
N (g N)	-	-	i_{NSP}	1	1	1	i_{SNH1}	i_{SNH2}	i_{NBM}	i_{NBM}	-	i_{NXP}	-
Ionic Charge				$\frac{1}{14}$		$\frac{1}{14}$							-1

3. MATERIALS AND METHODS

3.1.Reactor Operation

Activated sludge taken from Paşaköy Wastewater Treatment Plant was used as inoculum. The activated sludge was fed with OECD as a complex organic carbon source (**Table 3.1**) with the concentration of 600 mg COD/l. Batch reactor was used which had a working volume of 12 L. Micro and macronutrients also added to the reactor in sufficient quantities for biological growth. The reactor was operated at a sludge ages of 10 days and hydraulic retention time of 1 day at 20 °C. In addition, dissolved oxygen concentration in the reactor was kept as 3 mg/L. The system was operated until biomass reached to steady state conditions. After the acclimation period, acute and chronic effects of 17α- ethinylestradiol (EE2) to activated sludge were investigated by performing the respirometric tests.

Table 3.1: Composition of OECD nutrient solution (ISO 8192, 1999).

Compound	Feed Concentration (g/l)
Peptone	16
Meat Extract	11
Urea	3
NaCl	0,7
$CaCl_2.2H_2O$	0,4
$MgSO_4.7H_2O$	0,2
K_2HPO_4	2,8

3.2.Conventional Parameters

COD was measured according to procedure defined by ISO 6060 (ISO 6060, 1986). For determination of soluble COD, samples were filtered through 0,45 μm membrane filters and preserved with H_2SO_4. MLSS and MLVSS analysis were performed by using the procedure defined in Standard Methods (1998). The Millipore AP40 glass fiber filters were used for MLSS and MLVSS measurements. pH measurements were performed by a 520Aplus pH meter. Nitrite and nitrate nitrogen were determined by Ion Chromatography (DIONEX ICS – 1500 model). On the other hand, soluble TKN

and ammonia nitrogen analysis were performed as defined in the Standard Methods (1998).

3.3.Respirometric Analysis

Respirometric tests were conducted to generate the OUR profiles of the acclimated biomass. OUR measurements were performed with an Applitek RA-Combo-1000 continuous respirometer.

Respirometric tests were performed in twelve parallel batch reactors. One of the batch reactors is served as a control reactor. Firstly, the acute impact of the EE2 was investigated. Reactor was started with a 1 mg/L dose of EE2. Detailed information about the OUR batch experiments are given in **Table 3.2**.

Table 3.2: Characteristics of acut batch experiments

Runs	Substrate Type	S_0/X_0 (mg COD/mg VSS)
Run 1.1	Peptone mixture	0.3
Run 1.2	Peptone mixture + EE2	0.3

Then, chronic impact of the EE2 was studied. Each reactor was started with the necessary amount of acclimated biomass seeding alone to obtain endogenous oxygen uptake rate level of biomass. Both peptone mixture having 360 mg COD/L and EE2 solution (1mg/l) was added to the reactor to obtain desired S_0/X_0 ratio and the OUR data was monitored. Also, 50 mg/L NH_4Cl alone or together with the 1 mg/L EE2 solution was added to two of the batch reactors. Experimental studies are conducted by using activated sludge operated at the sludge age of 10 days. The summary of Chronic respirometric studies is given in and **Table 3.3**.

During the experiments pH, SS and VSS, COD, NO_3-N, NO_2-N, NH_3-N,soluble TKN, PHA and EE2 analysis were conducted in parallel to respirometric tests. The monitored data for experiment was detailed in **Table 3.4**.

3.4.PHA Analysis

Polyhydroxyalkanoates (PHAs) are linear polyesters produced in nature by bacterial fermentation of sugar or lipids. They are produced by the bacteria to store carbon and energy.

Table 3.3: Characteristics of chronic batch experiments

Runs	Substrate Type	Acclimation Period (day)
Run 2.1	Peptone mixture + EE2	1.
Run 2.2	Peptone mixture + EE2	5.
Run 2.3	Peptone mixture + EE2	15.
Run 2.4	Peptone mixture + EE2	20.
Run 2.5	Peptone mixture + EE2	25.
Run 2.6	Peptone mixture + EE2	30.
Run 2.7	Peptone mixture + EE2	40.
Run 2.8	Peptone mixture	40.
Run 2.9	NH_4Cl	40.
Run 2.10	NH_4Cl + EE2	40.

Table 3.4: Monitored Data for Experimental Runs

Time (min)	pH	SS/VSS	COD Filtered	NO_3-N	NO_2-N	NH_3-N	TKN Filtered	PHA	EE2
-10	X	X	X	X	X	X	X	X	X
10			X	X	X	X		X	
20	X		X	X	X	X		X	
30	X		X	X	X	X		X	
60			X	X	X	X		X	
90			X	X	X	X		X	
120	X		X	X	X	X		X	
150			X	X	X	X		X	
200	X		X	X	X	X		X	
250			X	X	X	X		X	
300	X		X	X	X	X		X	
360	X		X	X	X	X		X	
420			X	X	X	X		X	
1440	X	X	X	X	X	X	X	X	X

The commonly known PHA components are polyhydroxybutyrate (PHB), polyhydroxyvalerate (PHV) and 3-hydroxy-2-methylvalerate (3H2MV). Once the population has reached a substantial level, the nutrient composition is changed to force the microorganisms to synthesize PHA. The yield of PHA obtained from the intracellular inclusions can be as high as 80% of the organism's dry weight.

For the analysis, 8 ml samples taken into tubes from mixed liquor. 2 ml of sodium hypochlorite (NaOCl) was added to each tubes. The samples centrifuged with SED

Model 5X Centrifuge and the liquid phase discarded. 5 ml phosphate buffer added and then the sample were centrifuged again before vortex. Collected sludge pellets in a sample tube were freezed at -20°C. The pellets freeze dried at least 48 hours at -50°C with ThermoSavant, ModulyoD Freeze dryer. Then, freeze dried sludge pellets crushed and 20 – 30 mg with a microgram balance weighed and recorded exact amount. The weighed amount of sample in glass tubes with PTFE lined screw caps (Schott GL18 Max 200°C) putted. 3 standards each 2-3 mg for each series of 15 samples were prepared. Internal standard, acid mixture and dichloro ethane were added each samples. The samples and standards boiled for 2 hours at 100°C and shaked every 15 minutes. After cooling down, 3 ml distilled water added and shaked vigorously for 10 min by vortex. The samples centrifuged for 5 min. at 3000 rpm and waited for phase separation for a few minutes. Around 1 ml from the lower organic phase taken. Glass fiber putted in blue pipet tips and sodium sulfate added for the 1 ml of organic phase filtered.

The samples were analyzed by Agilent 6890N gas chromatograph.3-hydroxybutyricacid-co-3-hydroxyvaleric acid, PHV and caproic acid sodium salt was used as standards for PHB, PHV and 3H2MV measurements, respectively. COD equivalents of the measured components were calculated according to conversion ratios of 1.38 mg COD/mg PHB, 1.63 mg COD/mg PHV, and 1.82 mg COD/mg 3H2MV.

3.5.EE2 Analysis

The reliable methods were optimized to determine EE2 using solid-phase extraction (SPE) for sample preparation and Ultra Performance Liquid Chromatography (UPLC)/Tandem Mass Spectrometry (MS/MS) for analysis.

3.5.1. Extraction of EE2

3.5.1.1. Sample preparation

250 ml samples taken into tubes from mixed liquor and formaldehyde added a few drops each tubes for inhibited the biological activity. The samples centrifuged during 5 min. at 7000 rpm. Liquid phase separated from solid phase that were freezed at -20°C. Then solid phase freeze-dried for at least 48 hours at - 50°C and samples crushed for the weighing. 0.5 g of crushed samples were transferred to 50 ml glass

flasks. In addition, supernatant was filtered through 0.22 μm membrane filters (Whatman, Mainstone, UK).

3.5.1.2.Ultrasonic extraction

20 ml of methanol:acetone mixture (1:1) added the glass flasks and sonicated with %50 of power at 3 min. by used ultrasonicator which was used for extraction. The mixture centrifuged during 5 min. at 4000 rpm. and aliquot separated from solid phase. While aliquot was filtered through 0.22 μm membrane filters, 14 C labelled EE2 were injected into solid phase. This cycle was assayed for two more times and aliquots were collected. Finally, aliquot evaporated at 65 °C with rotary evaporator. Residual phase was dissolved with 18 ml distilled water and 2 ml methanol: acetone mixture (1:1).

3.5.1.3.Solid phase extraction

Prior to use SPE cartridges were conditioned using 5 ml of acetonitrile, 5 ml of methanole and 5 ml of distilled water. Then, the sample of 20 ml was filtered through 0.22 μm membrane filters and transferred to the SPE cartridge at a flow rate of about 5-6 ml/min using a vacuum manifold system connected to a vacuum pump. SPE cartridges washed with 3 ml of distilled water and drying during one hour. 8 ml of acetonitrile passed through each of SPE cartridges. Elution solvent was evaporated to dryness ml by a gentle nitrogen stream and the residues were dissolved in 1 ml of methanol. In the end, 1 ml of sample filtered through 0.22 μm membrane filters and concentration of EE2 of solid phase was measured by using LC-MS/MS analysis.

For EE2 measurement at liquid phase, 10 ml of filtered sample was passed through conditioned SPE cartridges and applied only solid phase exraction procedure.

Recovery of EE2 for 120 μg/g is 120%.

3.5.2. Measurement of EE2 concentrations by HPLC

All LC-MS/MS analyses were performed using Thermo Accela and Thermo Quantum tandem MS. The column dimensions was 50x4 mm and separation was achieved by gradient elution in acetonitrile: water: Amonium hydroxide in water (70:10:20) with a pressure of 400 bar and flow rate of 400 μL/min.

Internal standard was used for the elemination of matrix effects and isotope dilution method was used for the quantification of EE2 by using 13 C EE2 both in the

standards and samples. Regression coefficient of the calibration curves were always above 99%. Relative standard deviation for the measurements were always below 20%.

4. RESULTS AND DISCUSSION

Batch reactor was designed to have a volume of 12 L. It was started using the seed sludge taken from the aeration tank of a domestic wastewater treatment plant and fed with the peptone mixture which have 600 mg COD/L. Average pH of the system was kept 7.2 at 24°C. The reactor characteristics at steady state conditions was given in **Table 4.1**. The batch reactor operated at SRT of 10 days at steady state condition in a period of 10 months. The average reactor analysis results represented for effluent soluble COD and final MLSS - MLVSS in **Figure 4.1** and **Figure 4.2**, respectively.

Table 4.1: The Batch Reactor Characteristics at Steady State

Substrate Type	**S_0/X_0**	**SS**	**VSS**	**VSS/SS**	**COD_{inf}**	**COD_{eff}**	**Removal Efficiency**
	mgCOD/ mgVSS	mg/L	mg/L	mgVSS/ mgSS	mg/L	mg/L	%
Peptone mixture	0.3	2500±141	2000±237	0,8	600	33±5	94

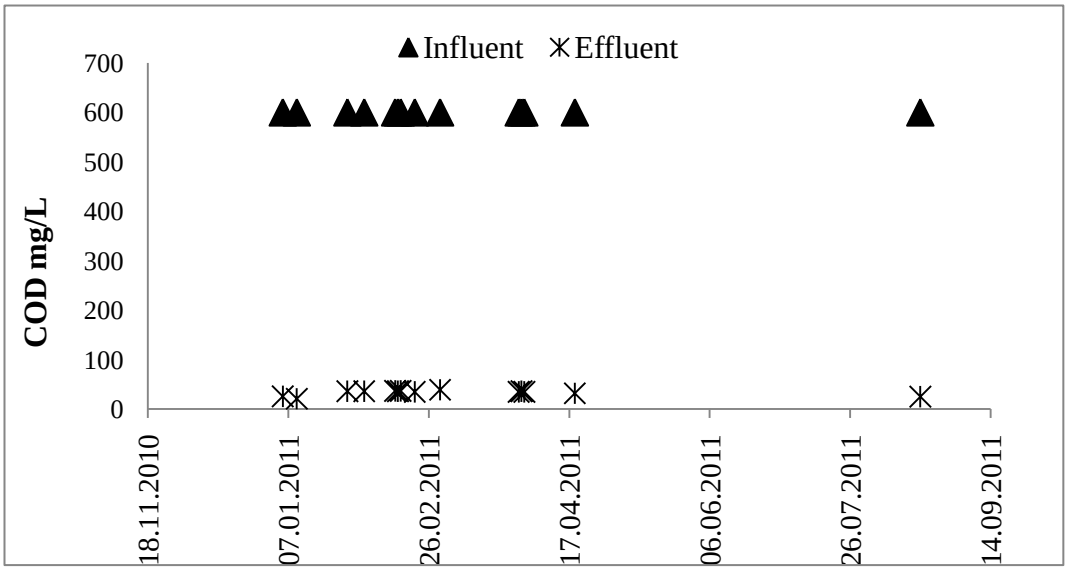


Figure 4.1: Final effluent COD results of acclimated pepton mixture system.

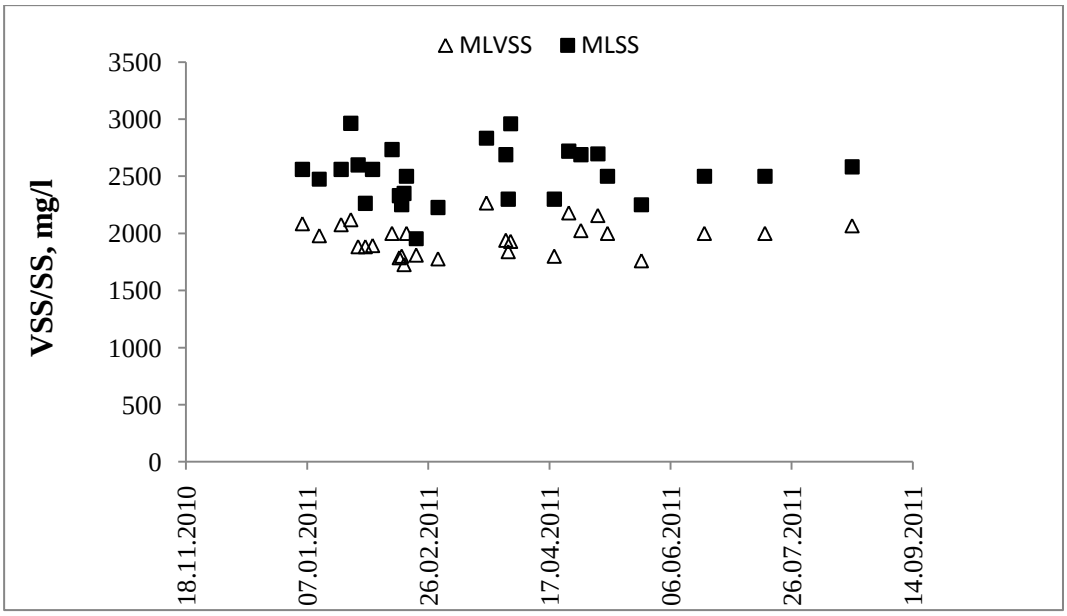


Figure 4.2: Final MLSS-MLVSS results of acclimated peptone mixture system.

4.1. Acute Results

After reaching the steady state conditions, respirometric tests were performed to figure out the acute effects of EE2. Acute experiments representing the same conditions in respirometric tests were conducted in parallel for the only peptone mixture and 1 mg/L EE2 solution with peptone mixture, separately. The volatile suspended solid concentrations were approximately 1200 mg VSS/L and influent COD concentrations 360 mg/L for all runs.

After conducting the respirometric analysis for pepton mixture only by keeping the loading ratio 0.3 mg COD/ mg VSS, 1 mg/L EE2 solution was added to system with the peptone mixture. Moreover, effluent NH_3-N concentration was measured and found under the 5 mg/L.

4.1.1. 360 mg COD/L pepton mixture (control)

In the first set, control, 360 mg COD/L peptone mixture was added to the system. The OUR profile is illustrated in **Figure 4.3**. As seen from the figure the maximum oxygen uptake rate was reached at nearly 100 mg/L/h when 360 mg COD/L peptone mixture added to the system.

The respirometric analysis for the non-acclimated system, the control analysis was performed by feeding the system only with the peptone mixture by keeping the loading ratio 0.3 mg COD/mg VSS.

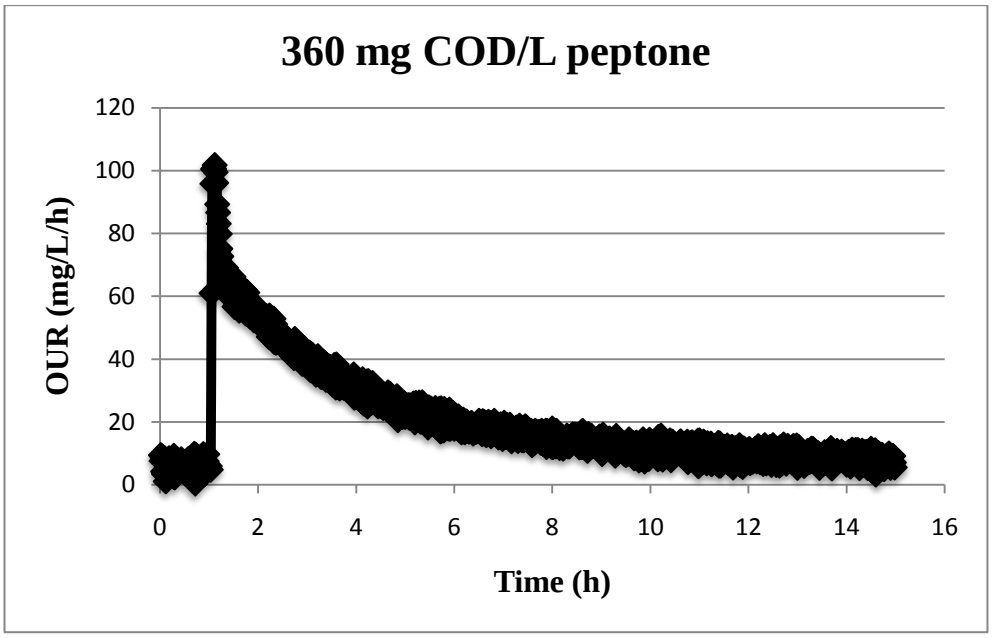


Figure 4.3: OUR data versus time (Acute Run 1.1).

Parallel to the respirometer analysis, COD, NO_X and pH was monitored. The soluble organic matter removal, NO_X concentration and pH are illustrated in the **Figure 4.4**, **Figure 4.5** and **Figure 4.6**, respectively. In addition, PHA analysis results for control set of system are illustrated in **Figure 4.7**.

As seen from the **Figure 4.4**, most of the organic matter given to the system is removed nearly 24 hours, in the control set of system. Removal efficiency of the control system is 92,5%.

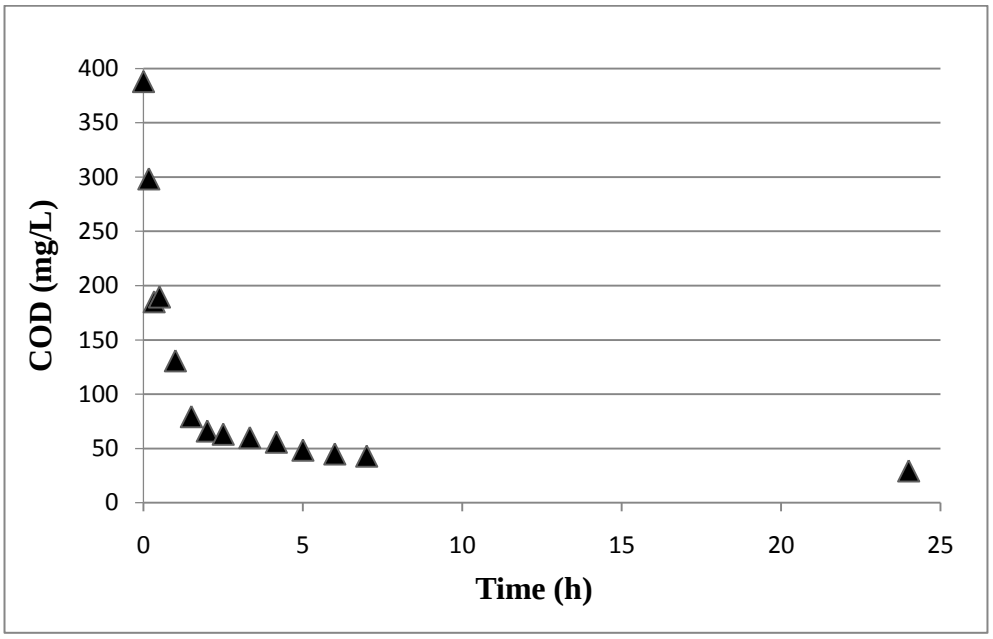


Figure 4.4: COD concentration versus time (Acute Run 1.1).

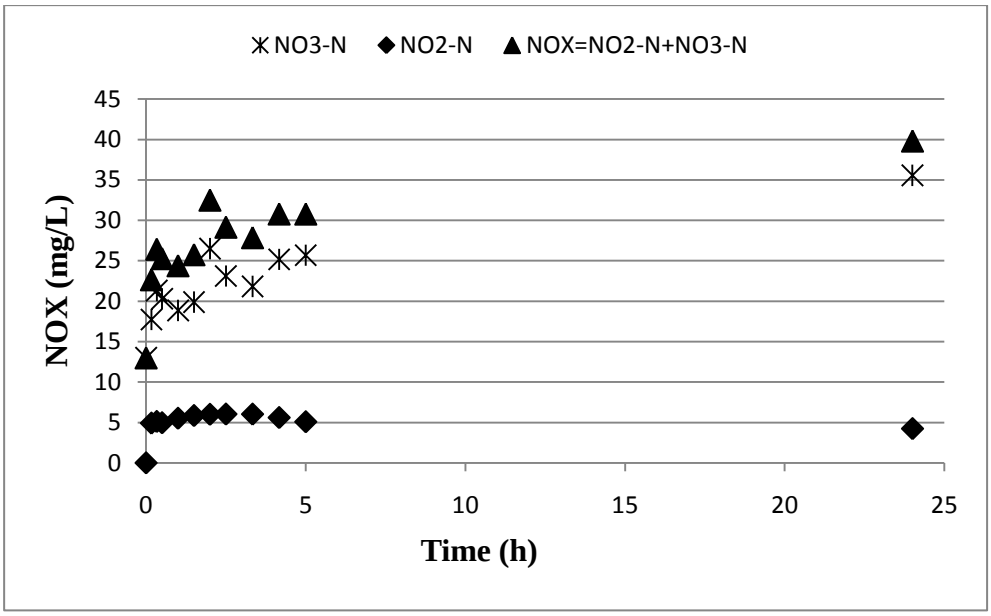


Figure 4.5: NO_X versus time (Acute Run 1.1).

According to Katipoğlu-Yazan (2012), soluble kjeldahl nitrogen (SKN) concentration of the 455 mg COD/L peptone mixture is 56 mg N/L. In this study, 44,3 mg/L S@KN is added to the system by feeding 360 mg COD/L peptone mixture. Hereby, influent total nitrogen concentration is 62.3 mg N/L (**Figure 4.5**).

According to **Figure 4.6**, average pH of the system is kept approximately 7.2 during the experiment.

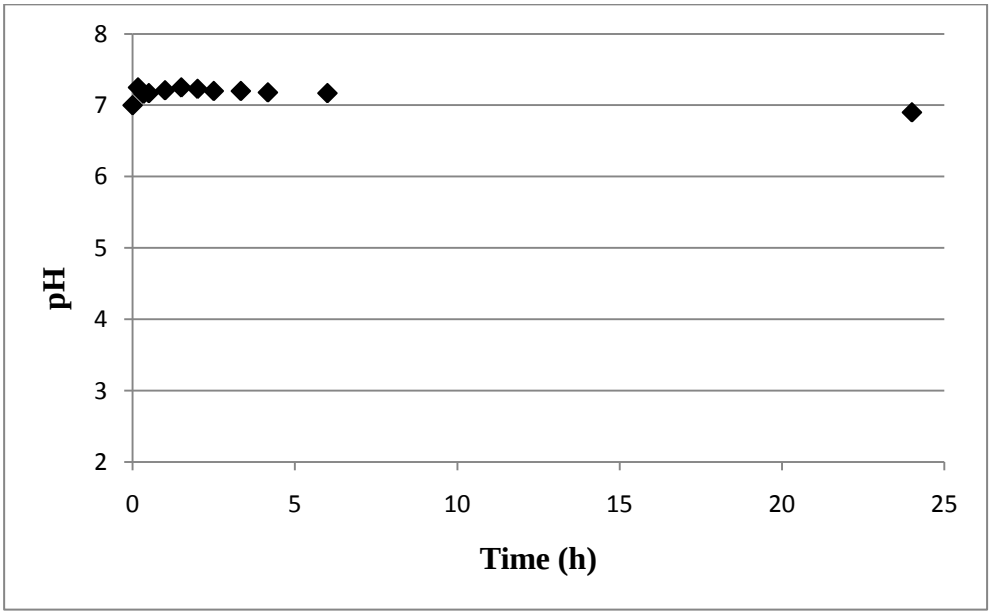


Figure 4.6: pH versus Time (Acute Run 1.1).

Storage of PHA was also observed which is about 15 mg COD/L before the addition of peptone mixture. PHA storage increased to 34 mg COD/L during the experiment and decreased to nearly 4 mg COD/L. (**Figure 4.7**).

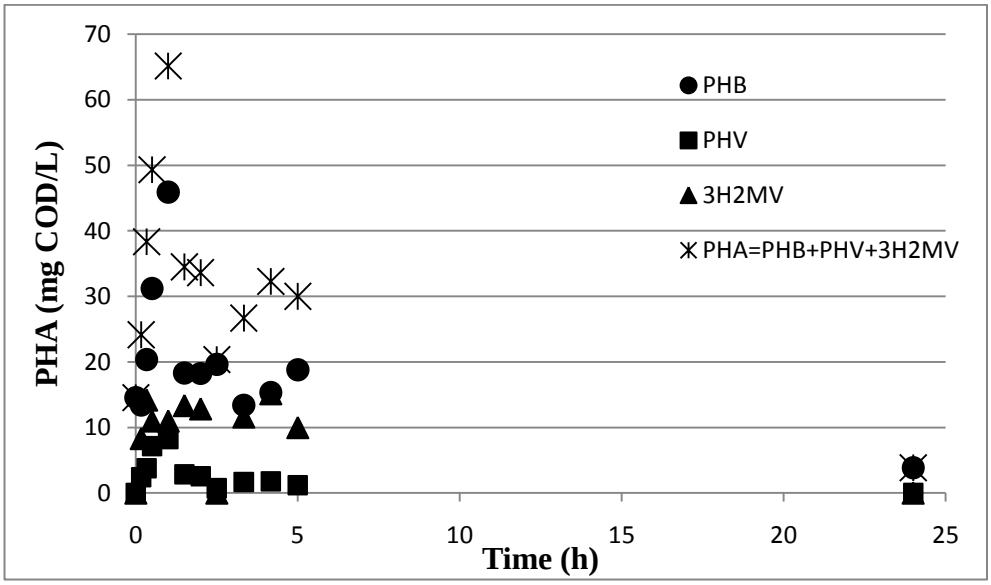


Figure 4.7: PHA versus Time (Acute Set 1.1).

4.1.2. 360 mg COD/L peptone mixture + 1 mg/L EE2 solution

After the control set was performed, same steps were followed for the acute effect of 1 mg/L EE2 solution. The OUR profile of the acute analysis of second run is given in **Figure 4.8**. When 360 mg COD/L peptone mixture added to system with 1mg/L EE2 solution, maximum OUR data is 100 mg/L/h same with OUR data of control set.

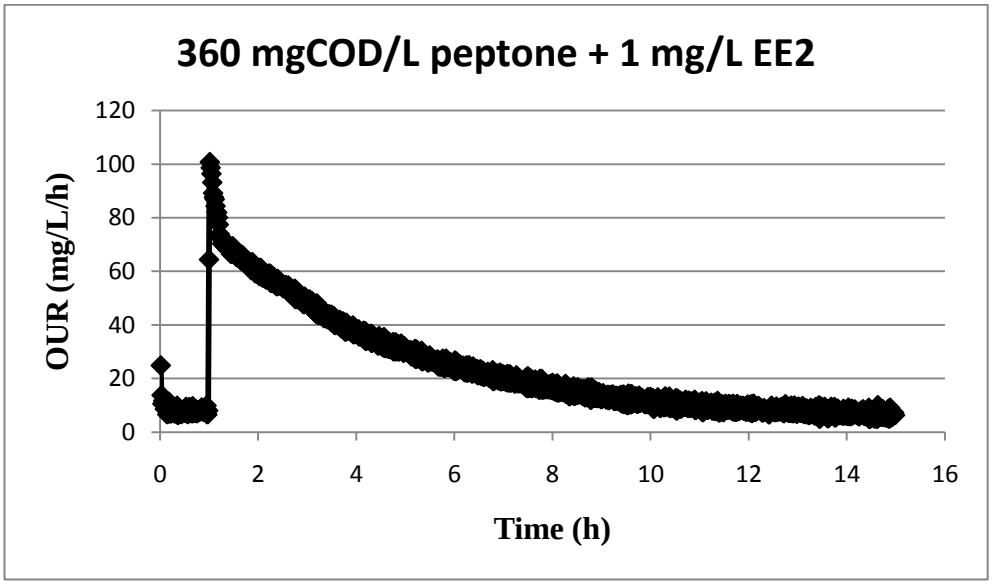


Figure 4.8: OUR Data versus Time (Acute Run 1.2)

The change of COD and NO_X concentrations of the experiment are illustrated in **Figure 4.9** and **Figure 4.10**, respectively.

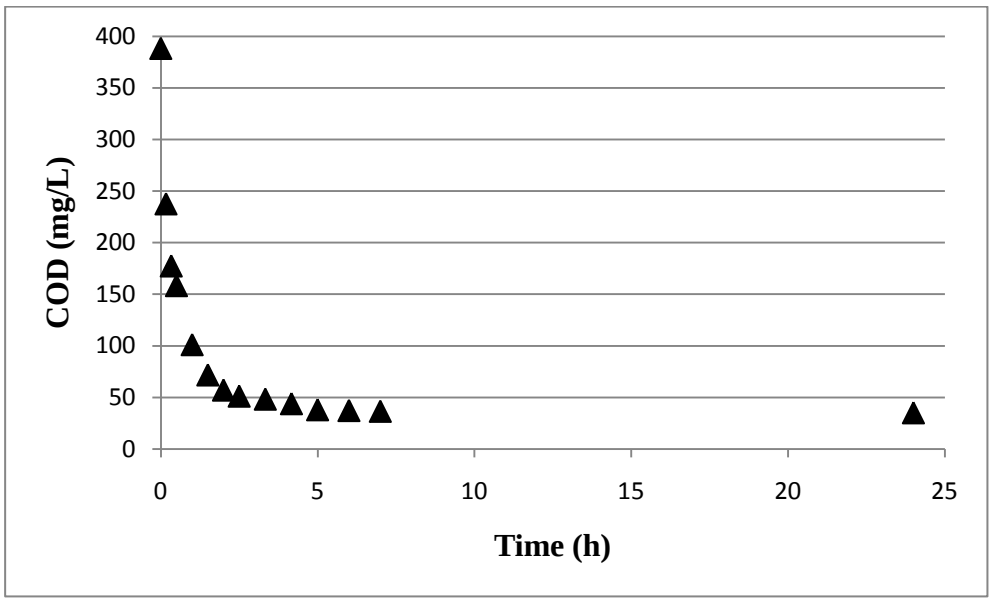


Figure 4.9: COD concentration versus Time (Acute Run 1.2)

As seen from the **Figure 4.9**, when 360 mg COD/L peptone mixture added to the system with 1 mg/L EE2 solution, COD concentration measured as nearly 35 mg COD/L at the end of the experiment. Removal efficiency of the acute experiment is 91 % less than control set.

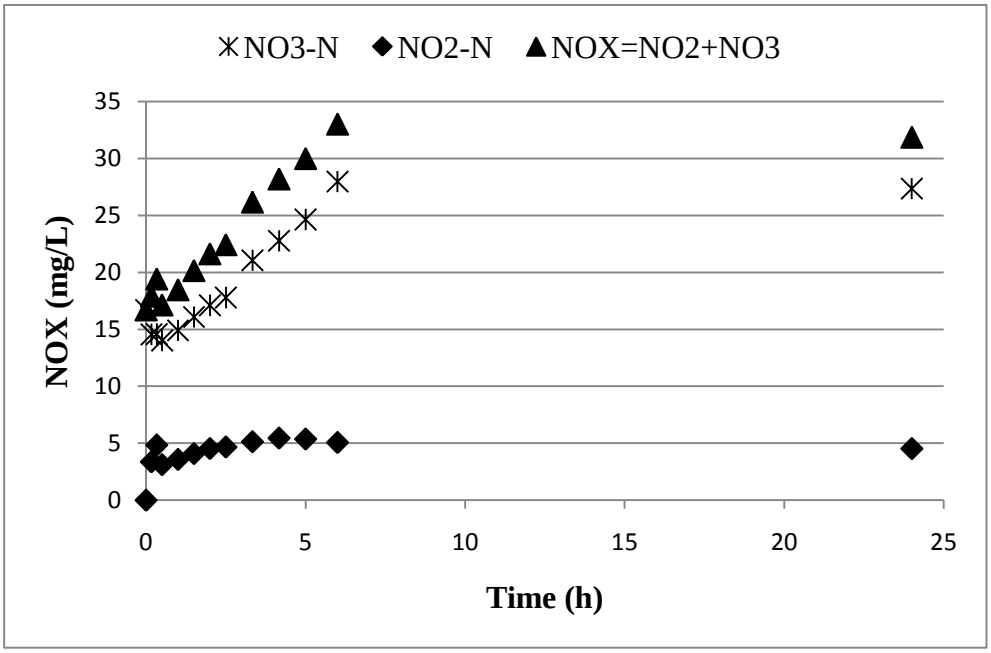


Figure 4.10: NO_X versus Time (Acute Run 1.2)

4.2.Chronic Results

After acute effects of EE2 was searched, the batch reactor was acclimated to EE2. System is set to 2000 mg VSS/L and the feeding amount is adjusted to 600 mg COD/L peptone mixture with 1 mg/L EE2 solution. MLSS and MLVSS concentration of the system was watched during chronic experiments. According to **Figure 4.11**, avarage MLSS and MLVSS concentrations are approximately 2540 ± 478 mg/L and 2000 ± 255 mg/L, respectively. Moreover, effluent NH_3-N concentration was measured and found under the 5 mg/L.

Moreover, effluent organic matter content of the system was watched and the results are demonstrated in **Figure 4.12**. As seen from the figure, influent COD amount is 600 mg COD/L for all days and average effluent COD concentration of the system is 32 ± 11 mg COD/L during 40 days.

NO_X concentrations and pH values of the system was monitored during chronic experimets (**Figure 4.13** and **Figure 4.14**). Avarage pH of the system is kept 7.2 during 40 days.

Storage of the PHA of the system were also observed during 40 days. Results are illustrated in **Figure 4.15**. Avarage PHA generation of the system is 37 mg COD/L, corresponding to around 6% of the initially available substrate.

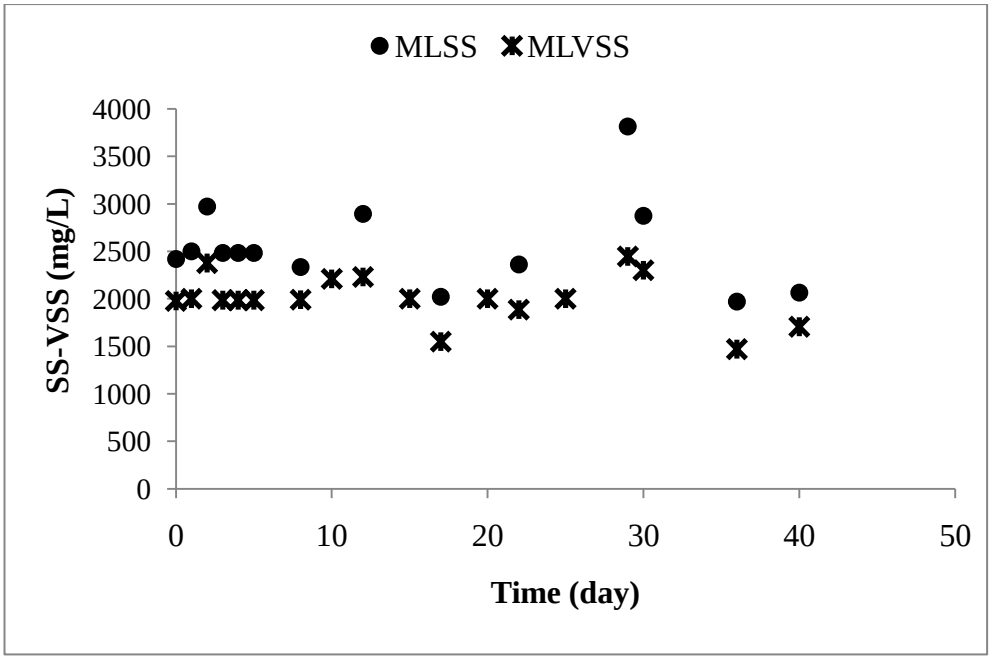


Figure 4.11: MLSS-MLVSS versus Time.

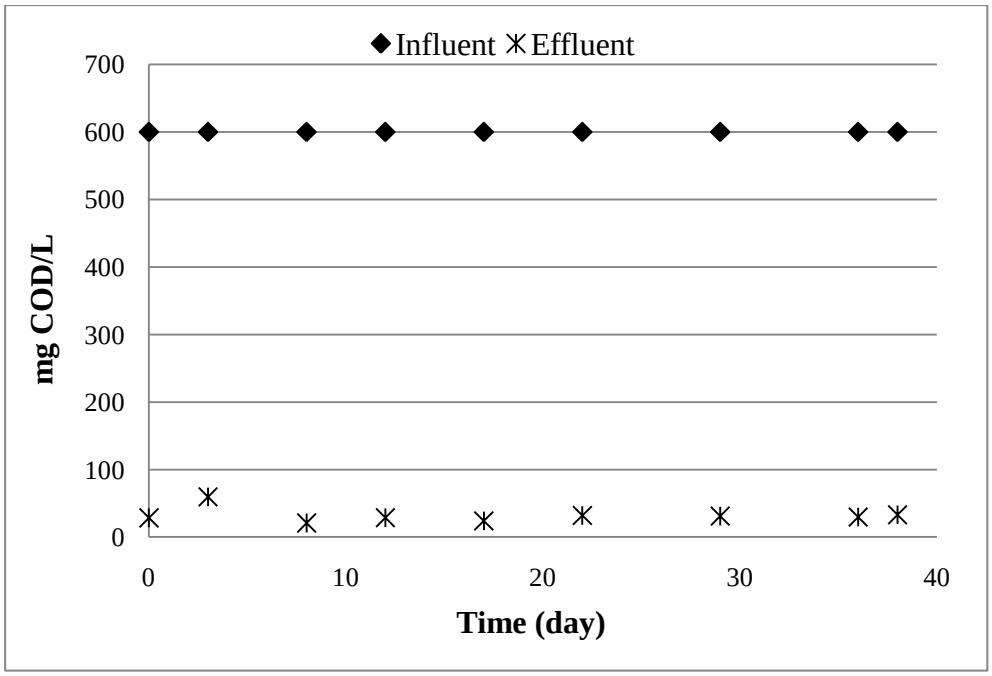


Figure 4.12: COD influent and effluent versus Time.

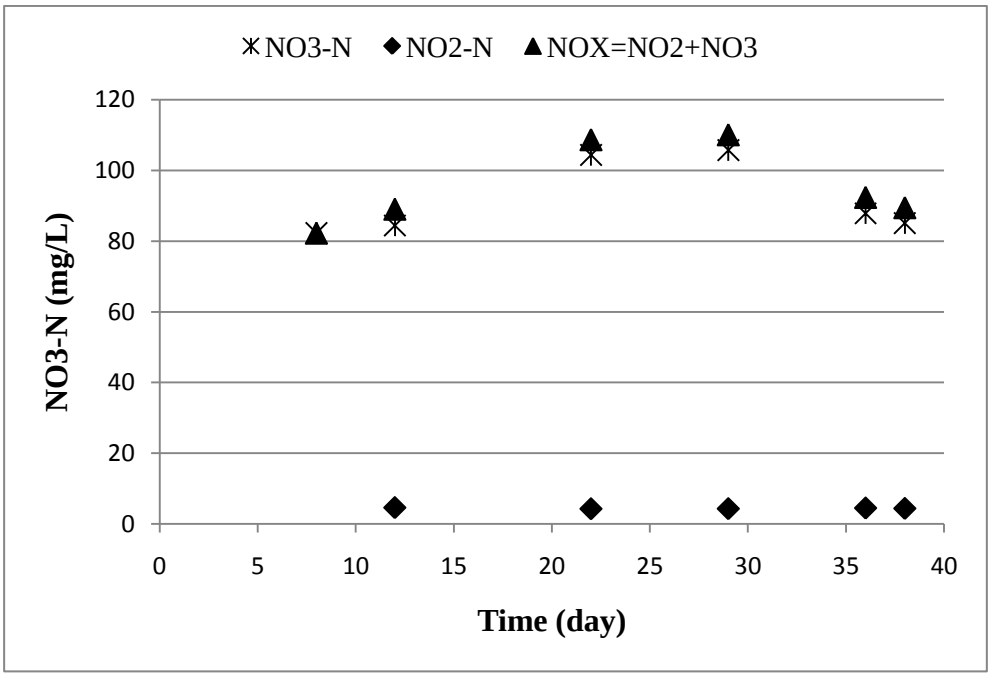


Figure 4.13: NO_X versus Time.

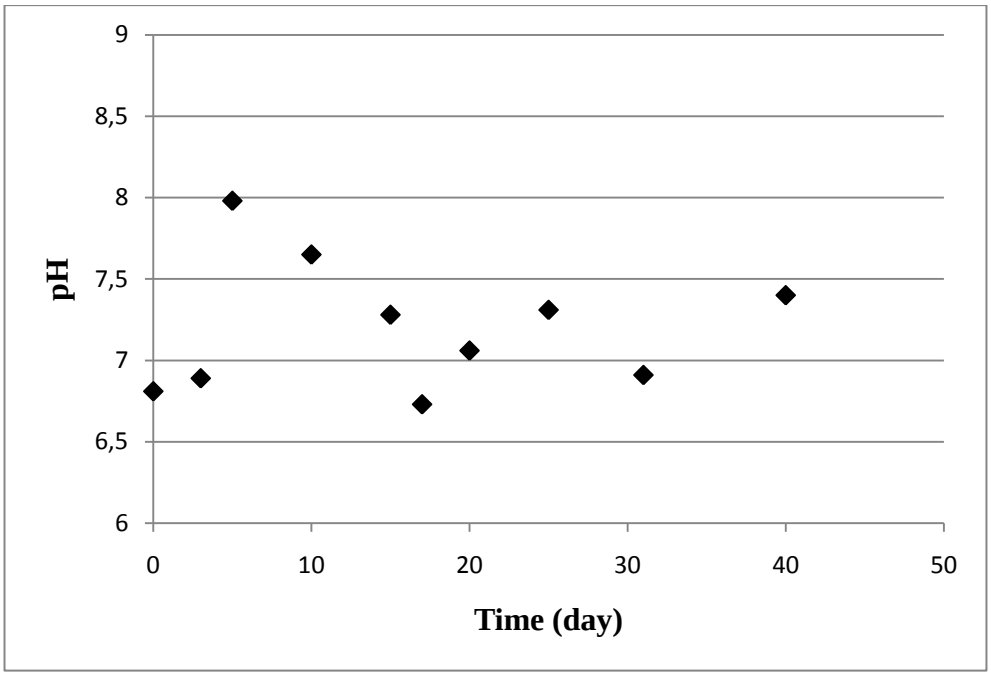


Figure 4.14: pH versus Time.

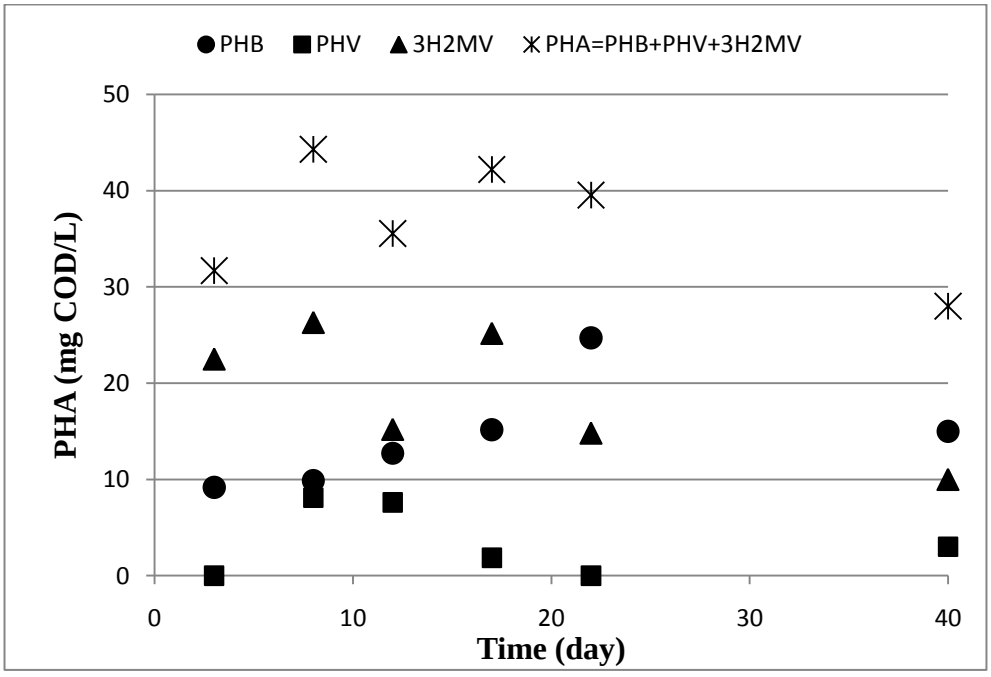


Figure 4.15: PHA versus Time.

The EE2 concentration of the system was observed during 40 day acclimation period via HPLC both solid phase and liquid phase and the results are shown in **Figure 4.16** and **Figure 4.17**. Avarage EE2 concentration of the reactor is 3.8 µg/g at solid phase. At liquid phase, EE2 concentration of the system is decreased during 40 days.

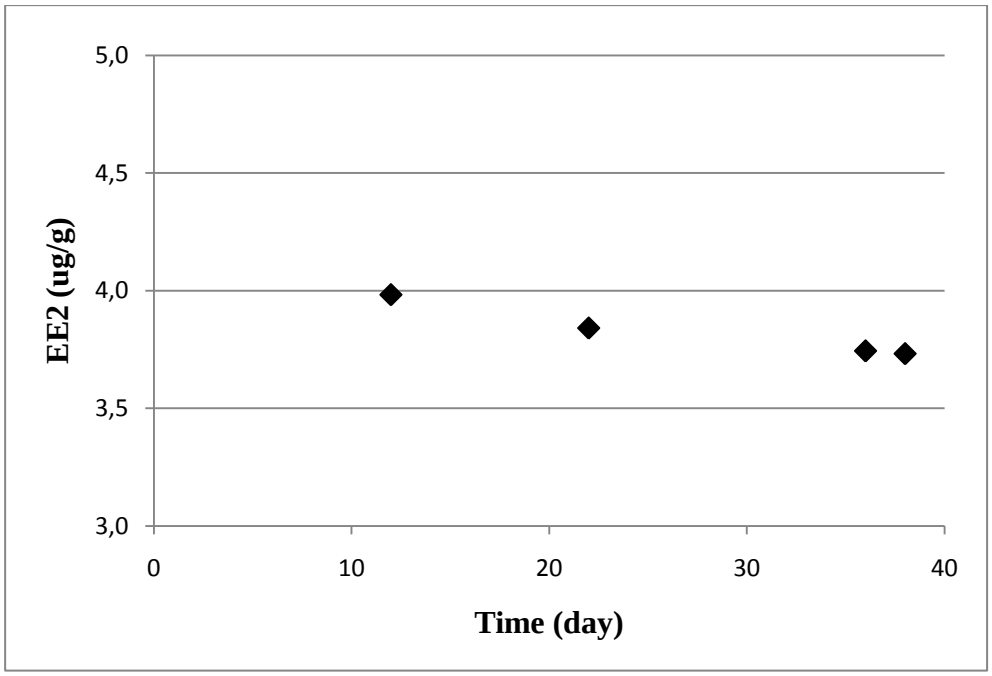


Figure 4.16: EE2 versus Time (solid phase).

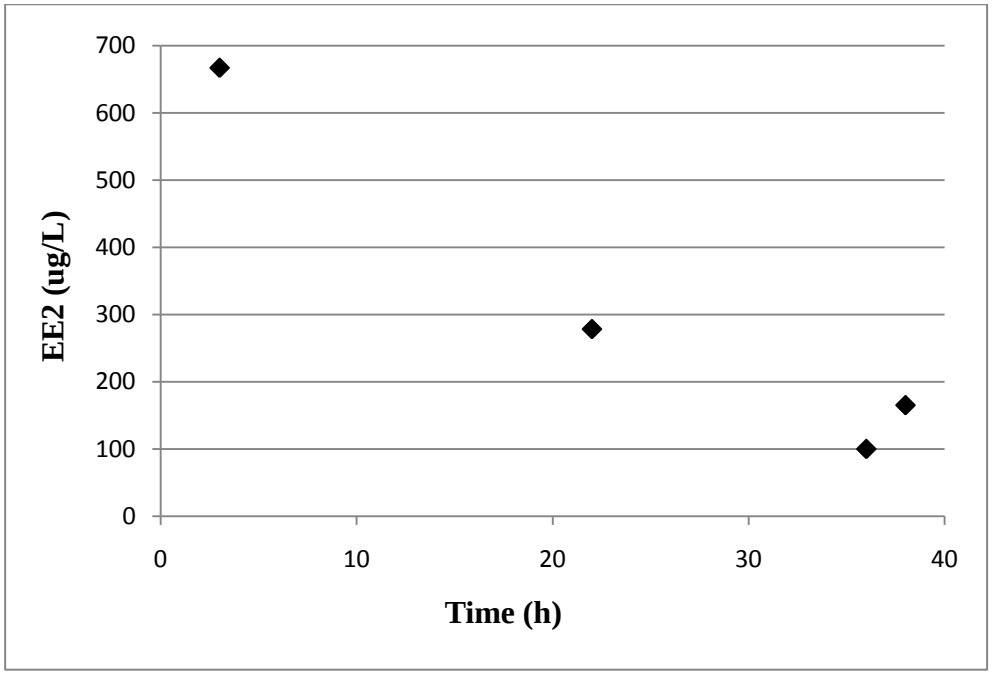


Figure 4.17: EE2 versus Time (liquid phase).

4.2.1. 1 st Day 360 mg COD/L peptone + 1mg/L EE2

In the first day, when the system was fed with 360 mg COD/L peptone mixture with 1 mg/L EE2 solution, the OUR profile was determined as shown **Figure 4.18**. During the experiment the samples taken for the analysis of organic matter, NO_X and pH.

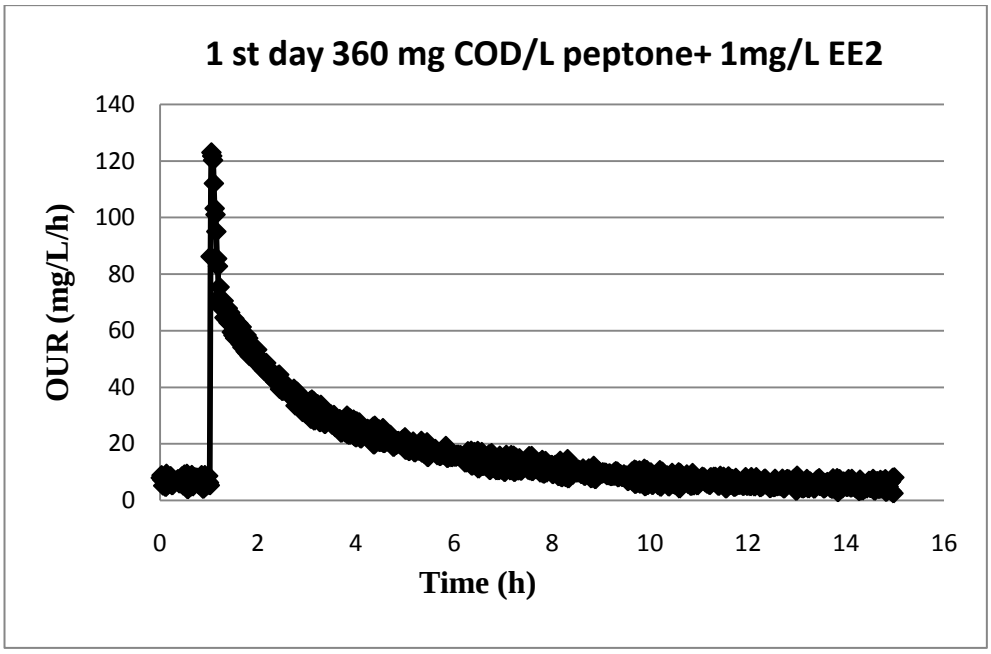


Figure 4.18: OUR Data versus Time (Chronic Run 2.1).

The organic matter removal is shown in **Figure 4.19**. As seen from the figure, COD concentration is decrease during experiment and removal efficiency of 1st day is calculated 95% higher than control set.

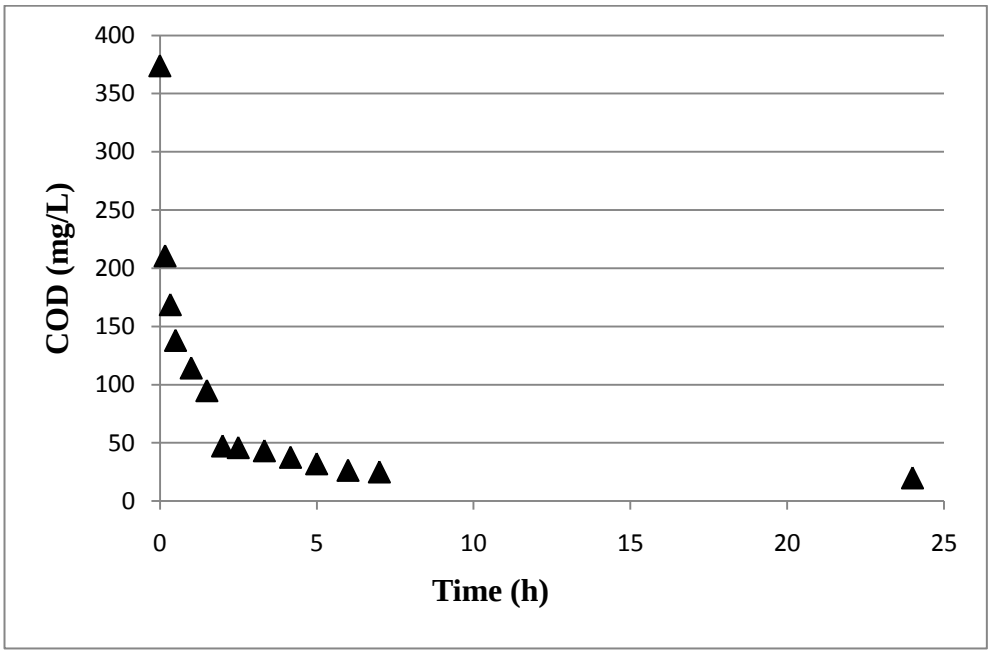


Figure 4.19: COD versus Time (Chronic Run 2.1).

As seen from the **Figure 4.20**, NO_X concentration of the system is increased during the experiment and total influent nitrogen concentration estimated 59,4 mg/L. pH of the system is decreased from 7.24 to 6.75 during the experiment (**Figure 4.21**).

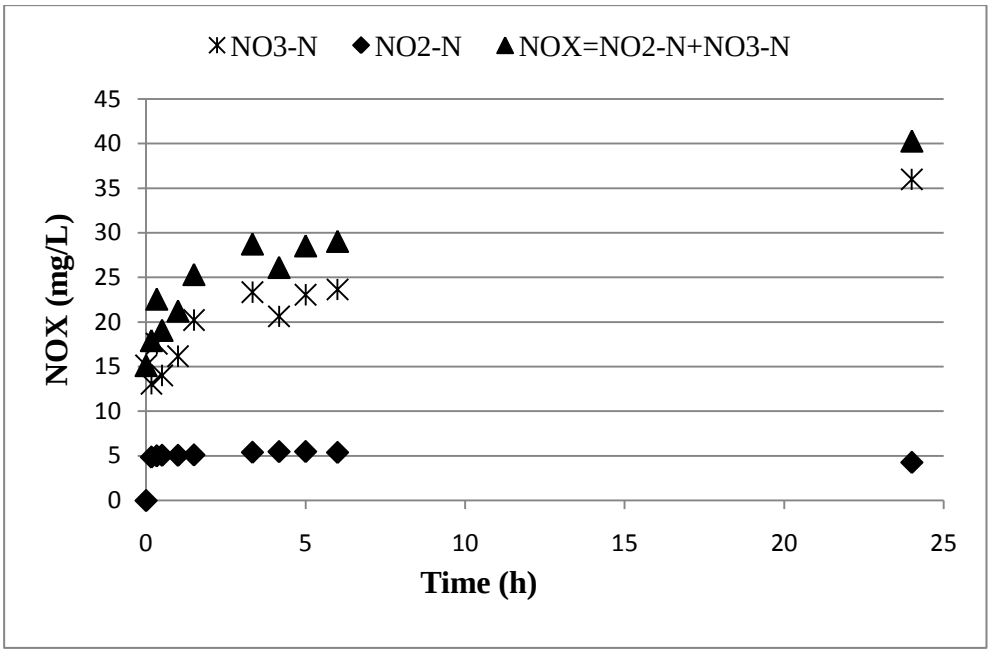


Figure 4.20: NO_X versus Time (Chronic Run 2.1).

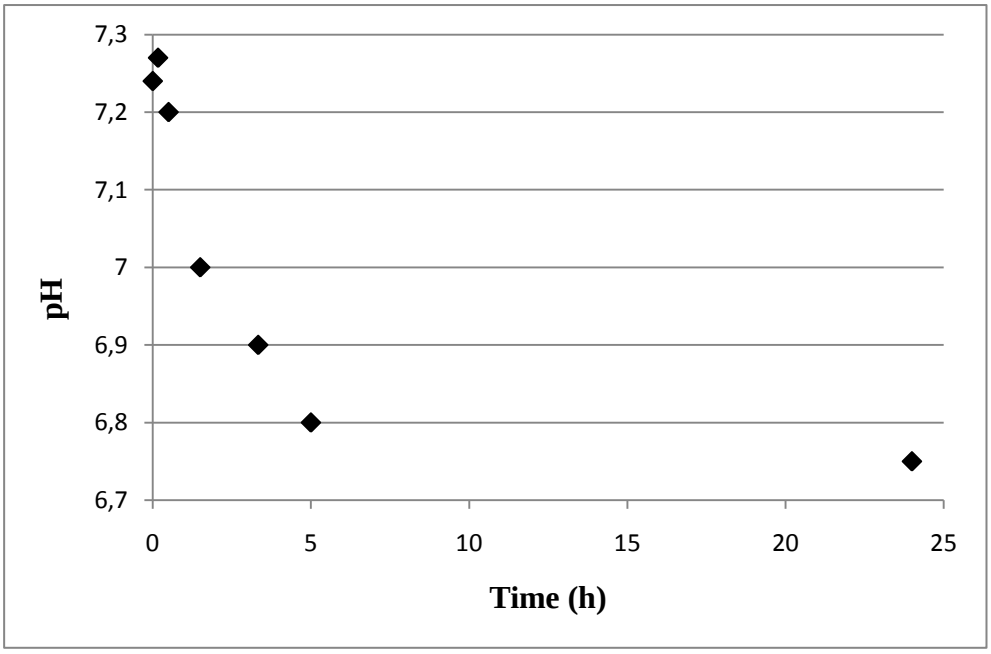


Figure 4.21: pH versus Time (Chronic Run 2.1).

4.2.2. 5 th Day 360 mg COD/L peptone + 1mg/L EE2

5^{th} day of the operation respirometric analysis was experimeted and the OUR profile is determined as illustrated in **Figure 4.22**. The system was fed with 360 mg COD/L with 1 mg/L EE2 solution and the maximum OUR is reached 120 mg/L/h as same as the first day data. During the experiment the samples taken for the analysis of organic matter, NO_X, pH, PHA and EE2.

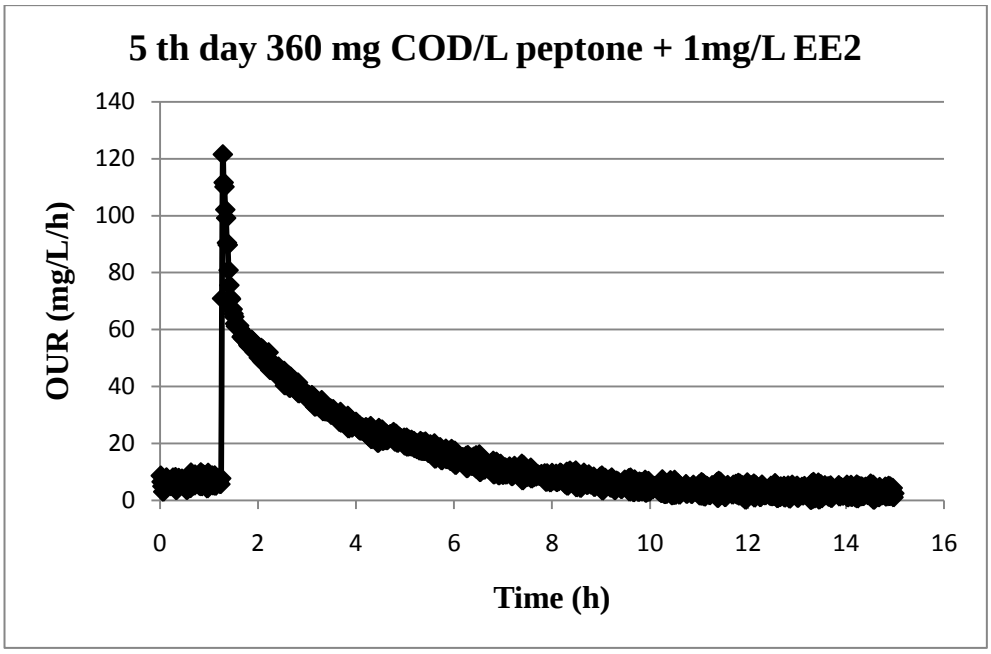


Figure 4.22: OUR Data versus Time (Chronic Run 2.2).

The COD change was in run 2.2 is like in **Figure 4.23** and decreased to 20 mg COD/L from 382 mg COD/L at the end of the day. The removal ratio is 95 % on the fifth day of acclimation as same as the first day data.

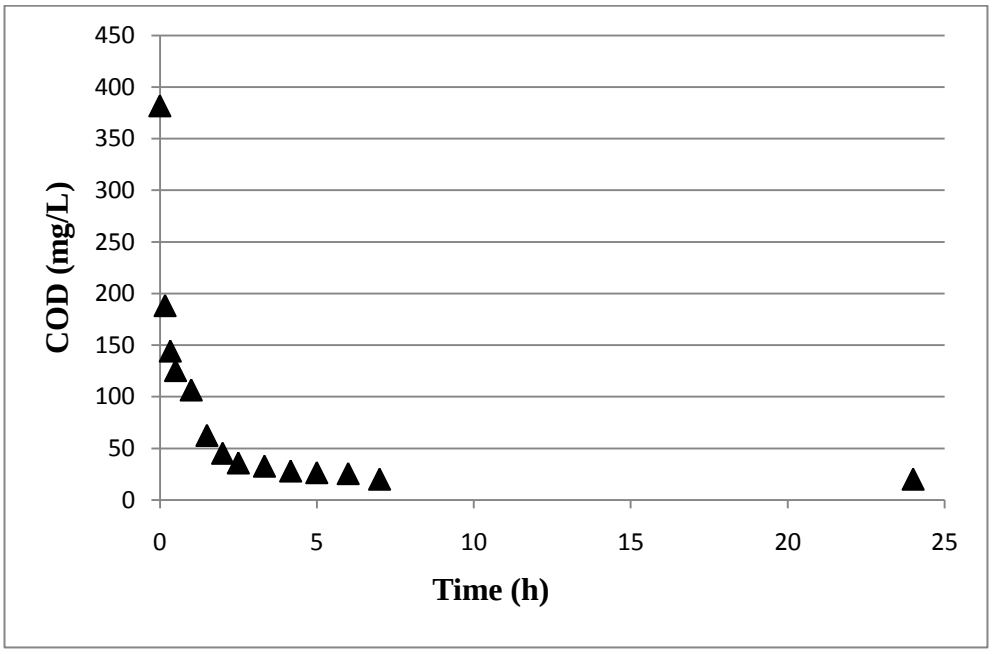


Figure 4.23: COD versus Time (Chronic Run 2.2).

Moreover, NO_X concentration graph shown in **Figure 4.24**. Total influent nitrogen concentration is calculated 59,4 mg/L. pH of system was about 7 during a one day period with a slight increase (**Figure 4.25**).

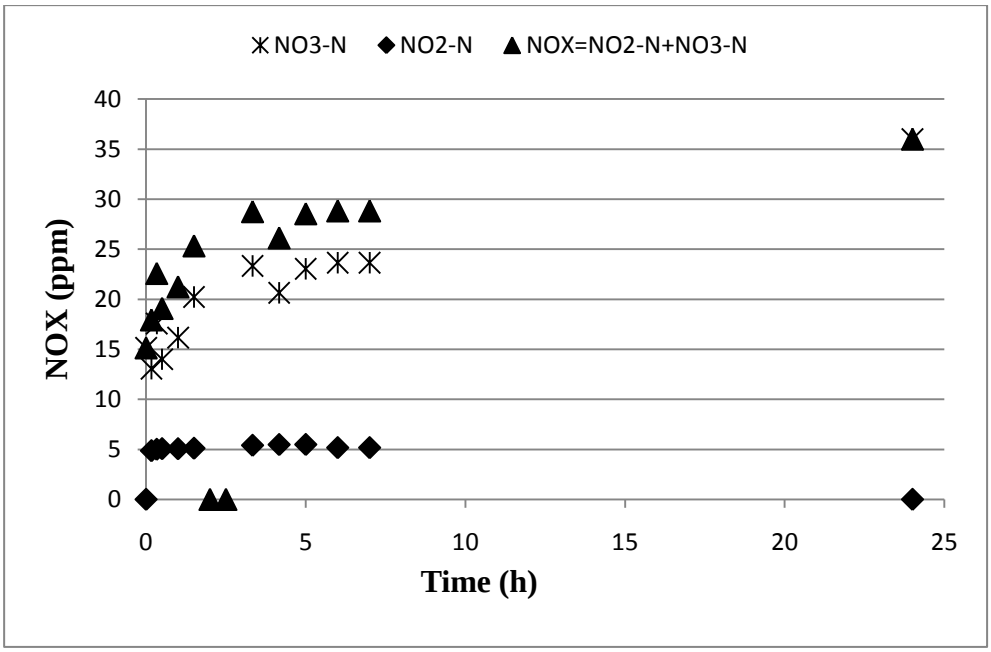


Figure 4.24: NO_X versus Time (Chronic Run 2.2).

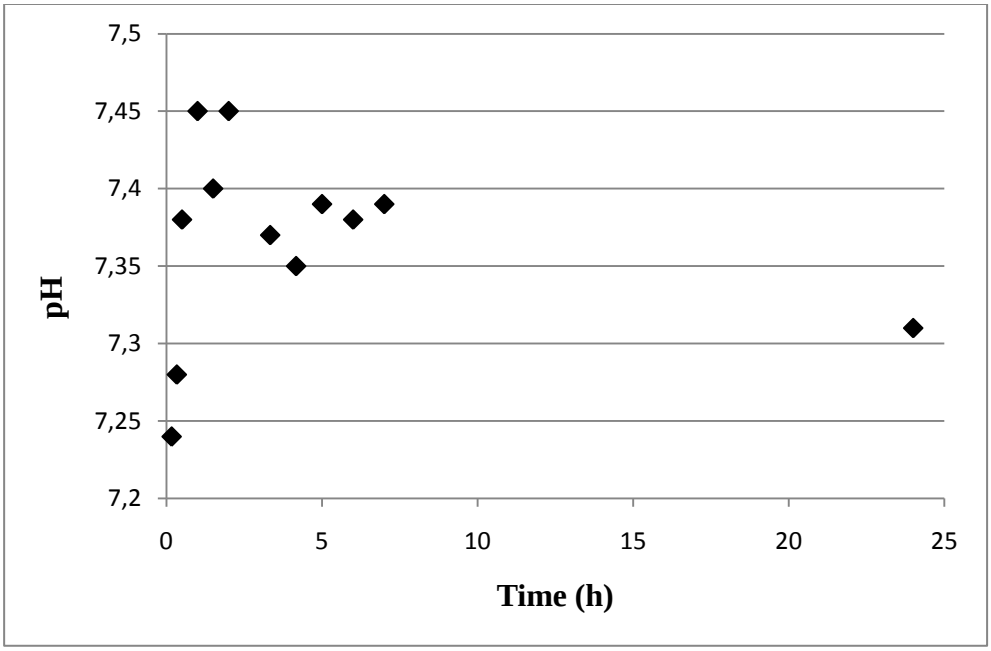


Figure 4.25: pH versus Time (Chronic Run 2.2).

Storage of PHA were also observed which is about 17 mg COD/L before the addition of peptone mixture and EE2 solution. PHA storage increased to 60 mg COD/L during the experiment and decreased to its initial concentration (**Figure 4.26**).

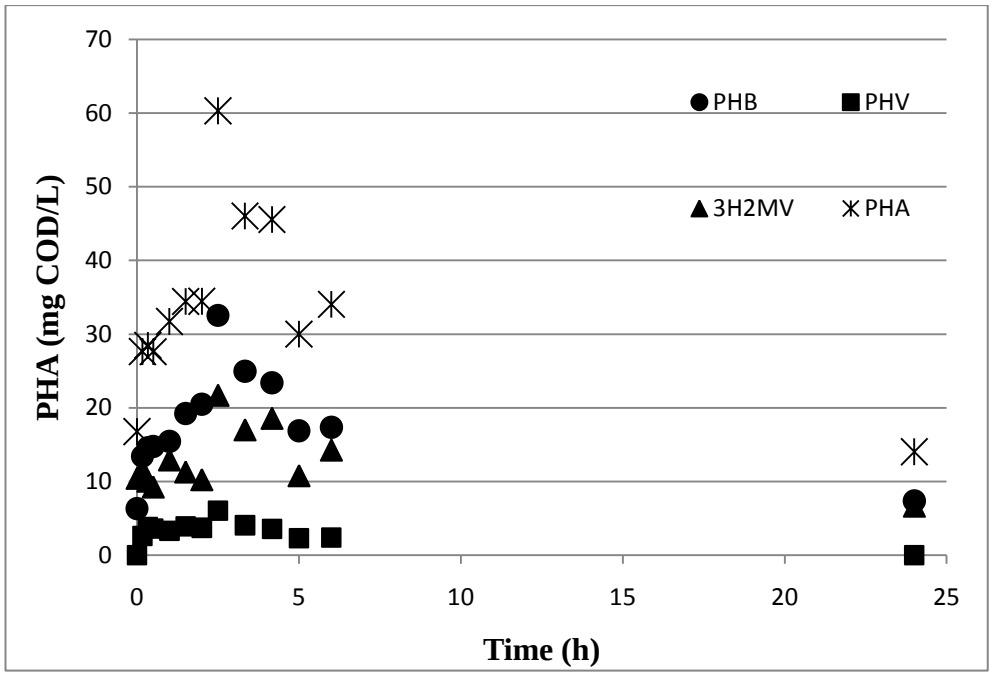


Figure 4.26: PHA versus Time (Chronic Run 2.2).

4.2.3. 15 th Day 360 mg COD/L peptone + 1mg/L EE2

In 15th day, the OUR profile observed when 360 mg COD/L peptone and 1 mg/L EE2 solution were added to the system is seen **Figure 4.27**. The maximum OUR is reached nearly 85 mg/L/h less than 5th day.

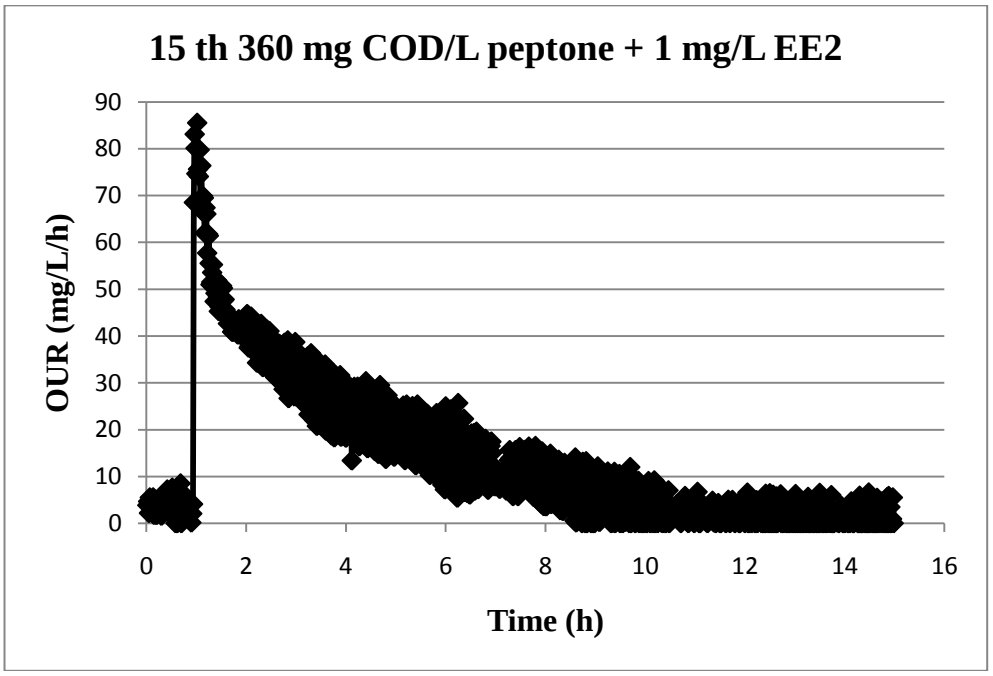


Figure 4.27: OUR Data versus Time (Chronic Run 2.3).

The change in organic matter content of 15th day is shown in **Figure 4.28**. COD concentration is decreased to about 22 mg COD/L from 380 mg COD/L during experiment. The removal ratio is 94 % lower than the 5th day.

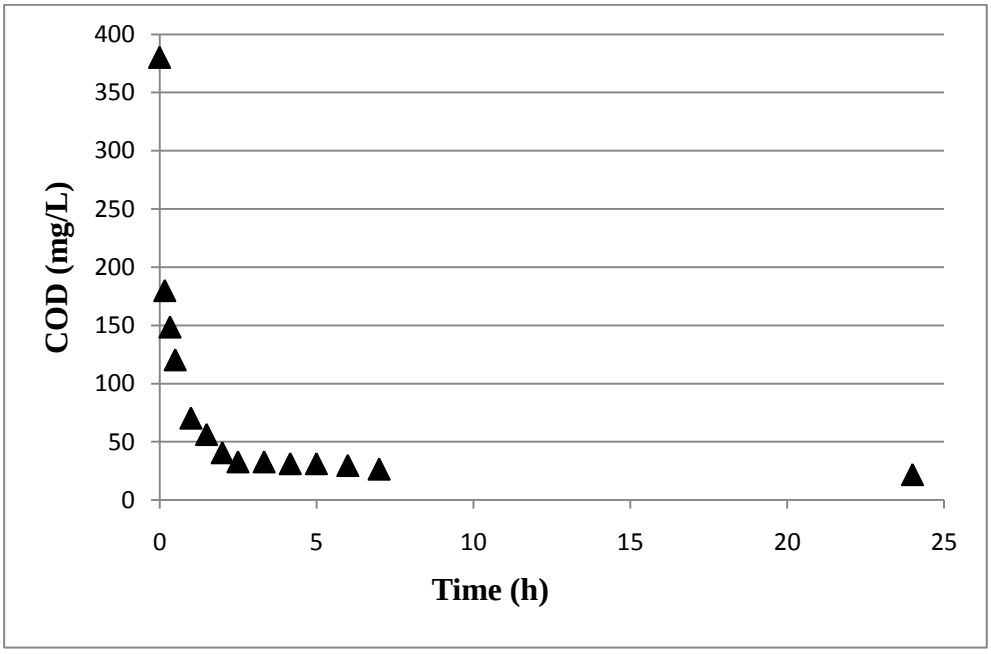


Figure 4.28: COD versus Time (Chronic Run 2.3).

As seen from the Figure **4.29**, NO_X concentration increased to 42 mg/L during experiment and influent total nitrogen concentration is calculated 64,8 mg/L.

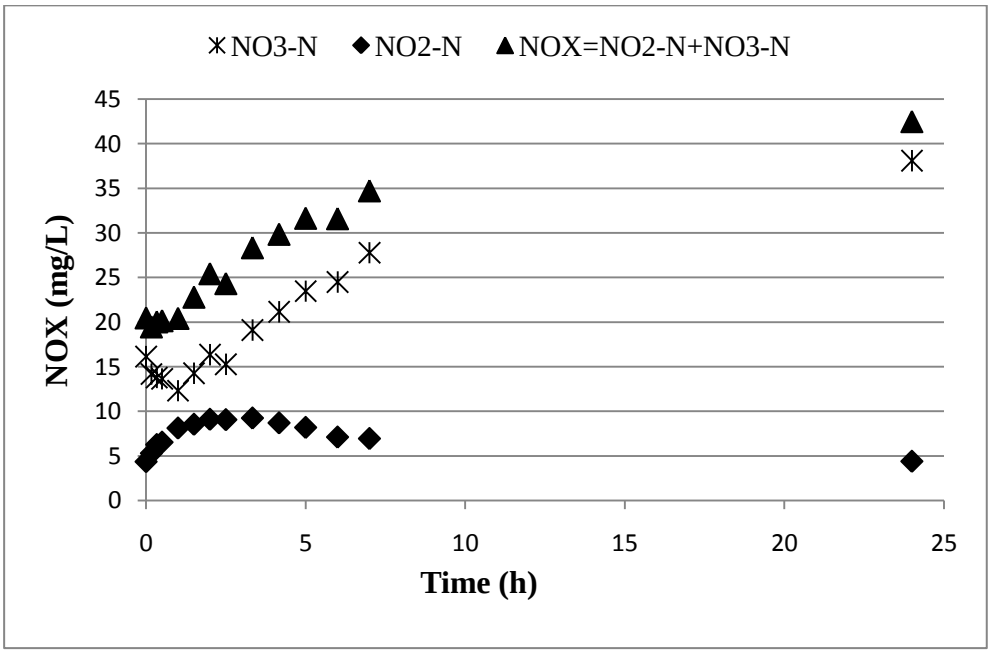


Figure 4.29: NO_X versus Time (Chronic Run 2.3).

Storage of PHA were also observed which is about 13 mg COD/L before the addition of peptone mixture and EE2 solution. PHA storage increased to 35 mg COD/L during the experiment and decreased to its initial concentration (**Figure 4.30**).

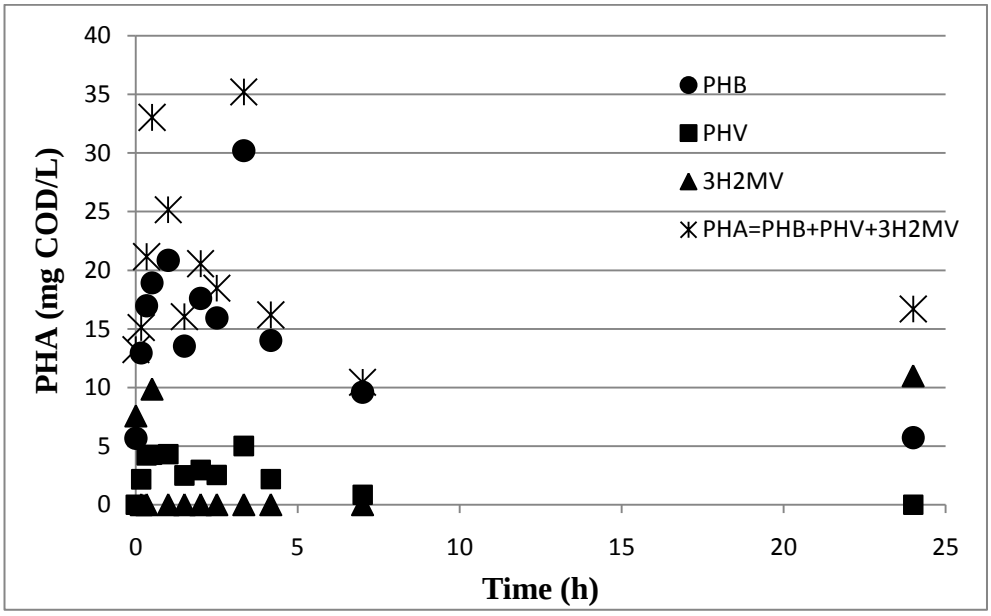


Figure 4.30: PHA versus Time (Chronic Run 2.3).

4.2.4. 20 th Day 360 mg COD/L peptone + 1mg/L EE2

Run 4 representing twentieth day of 1 mg/L EE2 solution addition was conducted with 360 mg COD/L peptone mixture. The OUR profile is given in **Figure 4.31**. The maximum OUR is reached nearly 120 mg/L/h higher than 15th day because of higher VSS concentration of the system.

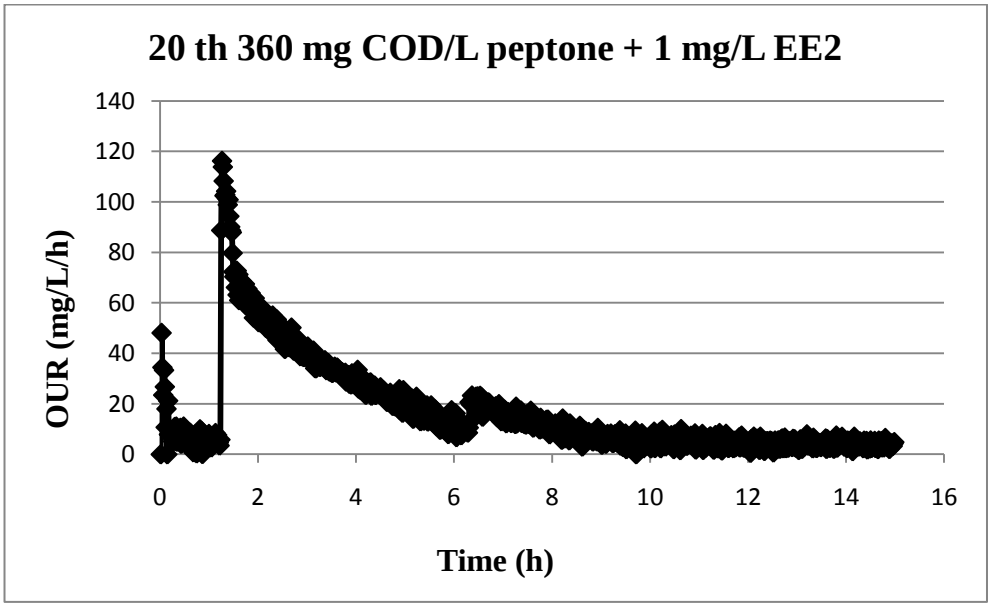


Figure 4.31: OUR Data versus Time (Chronic Run 2.4).

According to **Figure 4.32**, COD concentration is decreased to 33 from 382 in the course of time. The removal ratio of 20th day is higher than the 15th day.

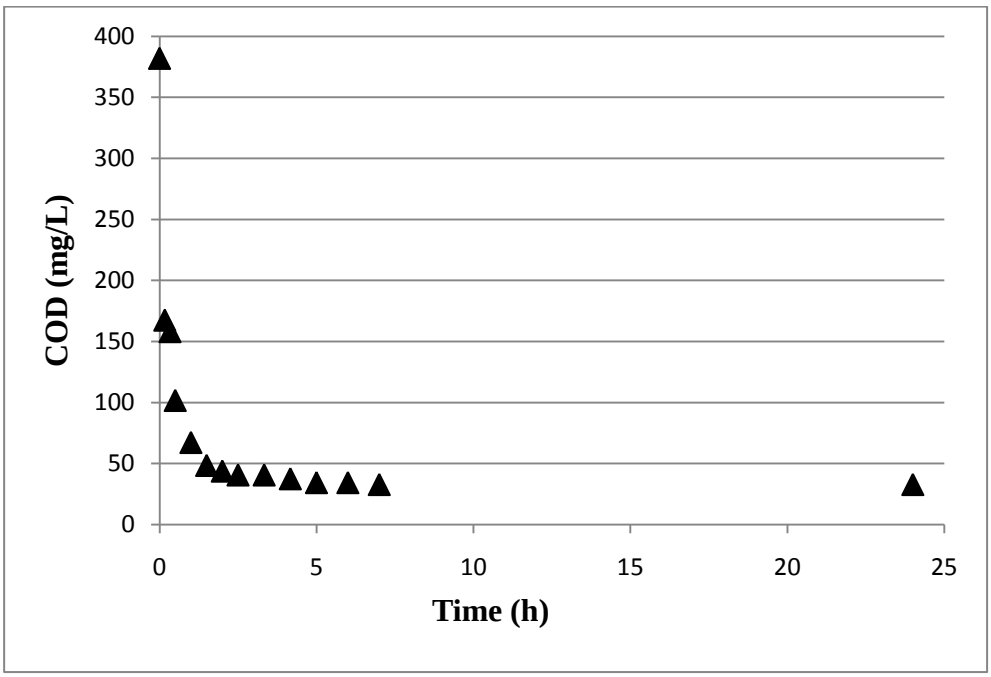


Figure 4.32: COD versus Time (Chronic Run 2.4).

As seen from the **Figure 4.33**, NO_X concentration increased to 43 mg/L during experiment and influent total nitrogen concentration is calculated 65 mg/L.

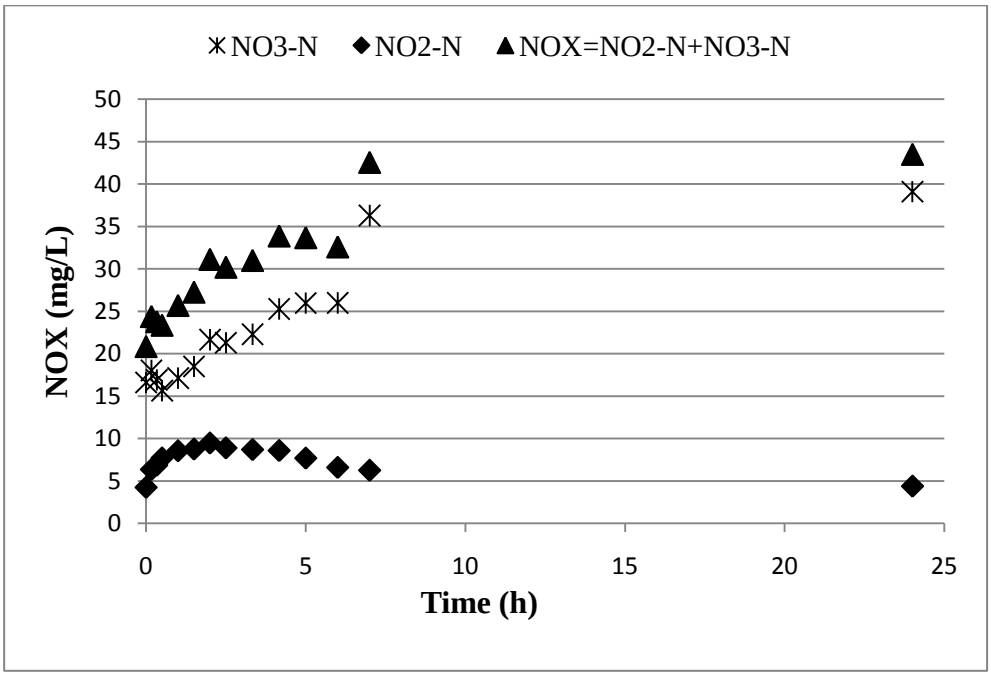


Figure 4.33: NO_X versus Time (Chronic Run 2.4).

4.2.5. 25 th Day 360 mg COD/L peptone + 1mg/L EE2

In the 25th day, respirometric analyses were done with peptone mixture and EE2 solution together. The OUR profile of the experiment of the system was fed with 360 mg COD/L peptone mixture and 1 mg/L EE2 solution is seen in **Figure 4.34**.

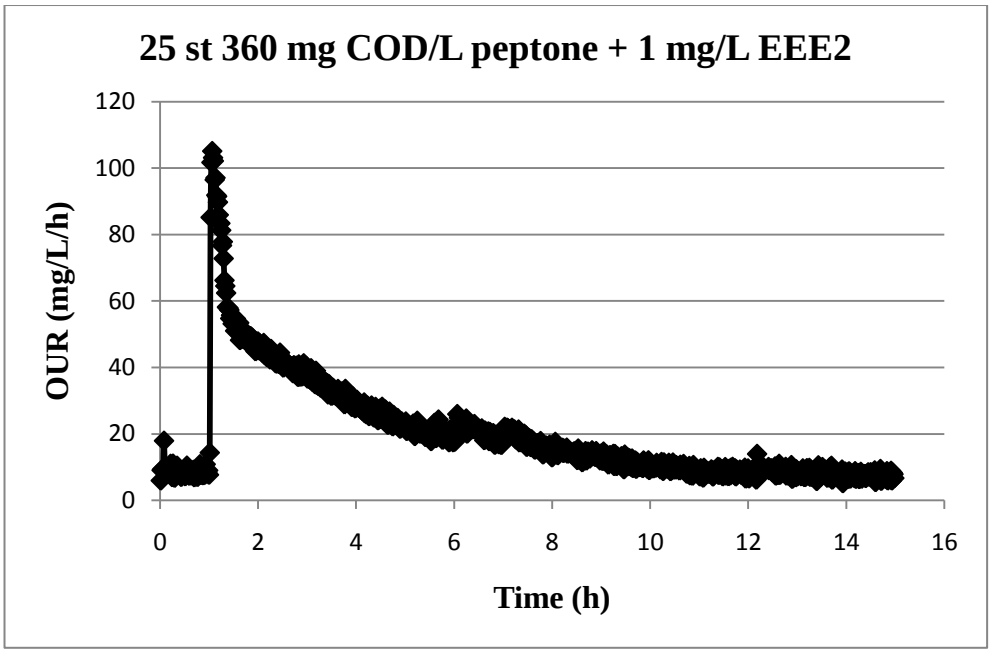


Figure 4.34: OUR Data versus Time (Chronic Run 2.5).

The COD change of experiment is illustrated in **Figure 4.35**. COD concentration is decreased to 27 from 389 in the course of time and the removal ratio is 93% .

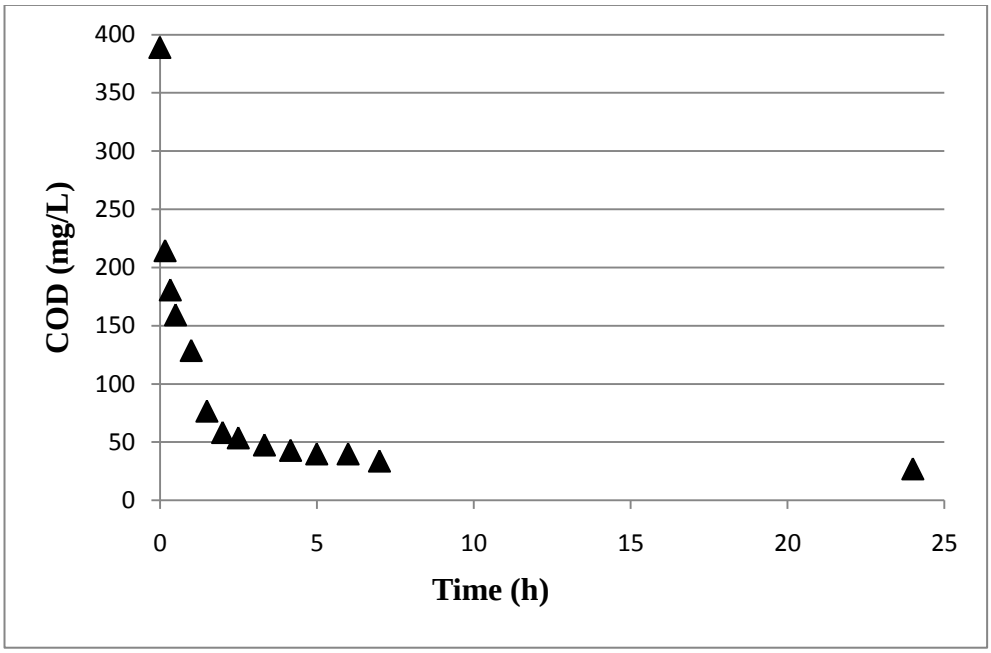


Figure 4.35: COD versus Time (Chronic Run 2.5).

According to the Figure **4.36**, NO_X concentration increased to 44 mg/L during experiment and the influent total nitrogen concentration is calculated 59 mg/L.

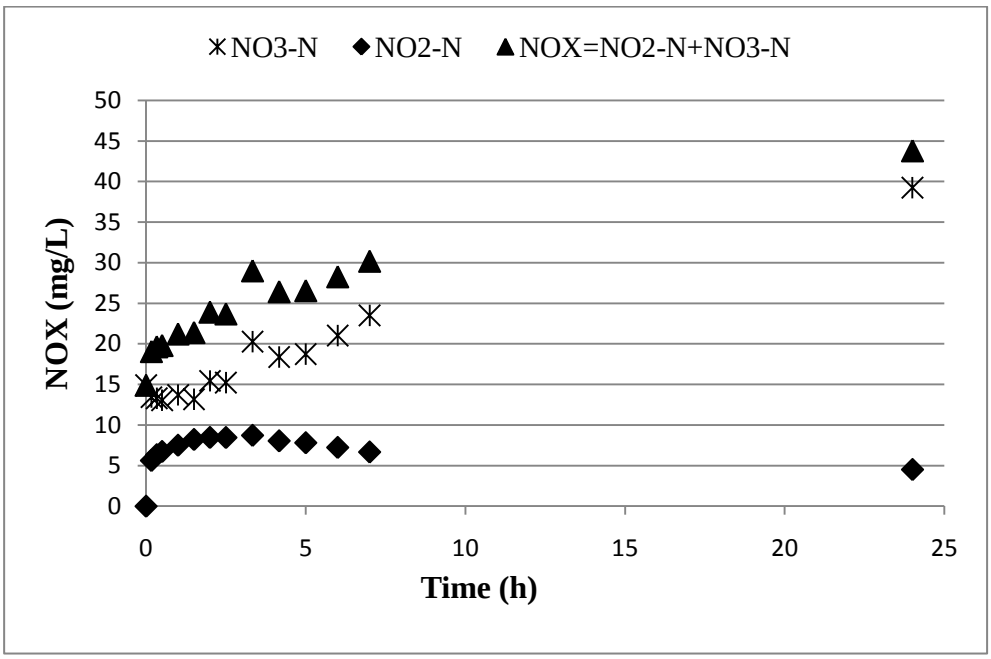


Figure 4.36: NO_X versus Time (Chronic Run 2.5).

4.2.6. 30 th Day 360 mg COD/L peptone + 1mg/L EE2

30^{th} day of the operation respirometric analysis was experimented and the OUR profile is determined as illustrated in **Figure 4.37**.

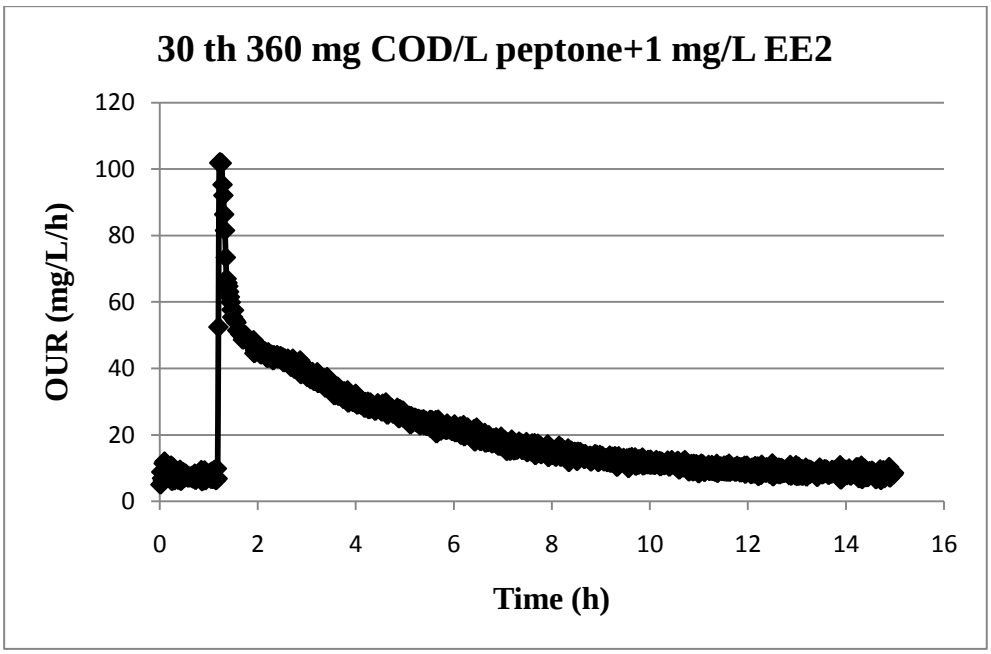


Figure 4.37: OUR Data versus Time (Chronic Run 2.6).

The initial COD value, 376 mg COD/L is decreased to nearly 26 mg COD/L at the end of the experiment and the removal ratio is 93% (**Figure 4.38**).

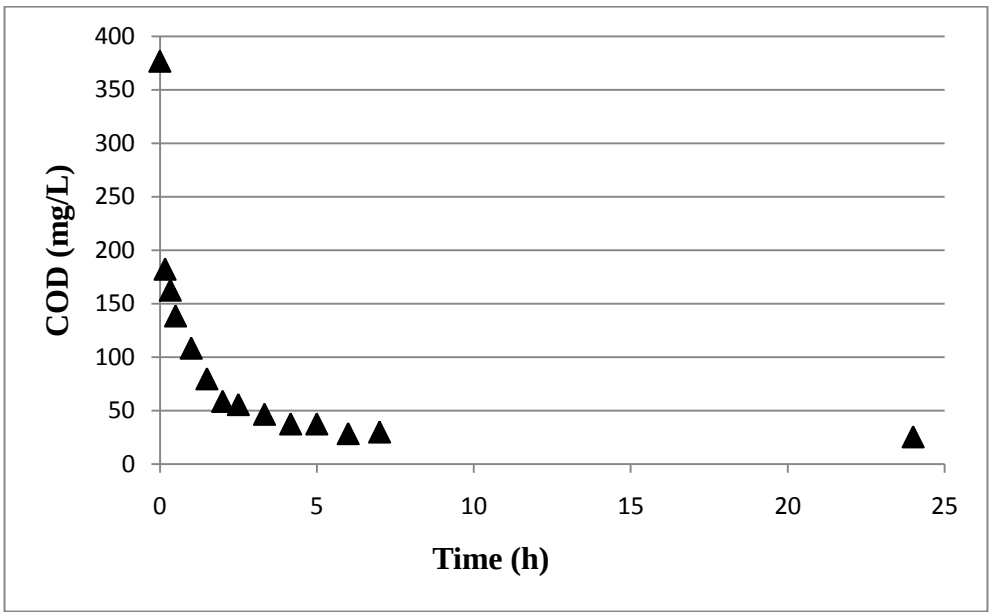


Figure 4.38: COD versus Time (Chronic Run 2.6).

According to the **Figure 4.39**, NO_X concentration increased to 39 mg/L during experiment and the influent total nitrogen concentration is calculated 61 mg/L.

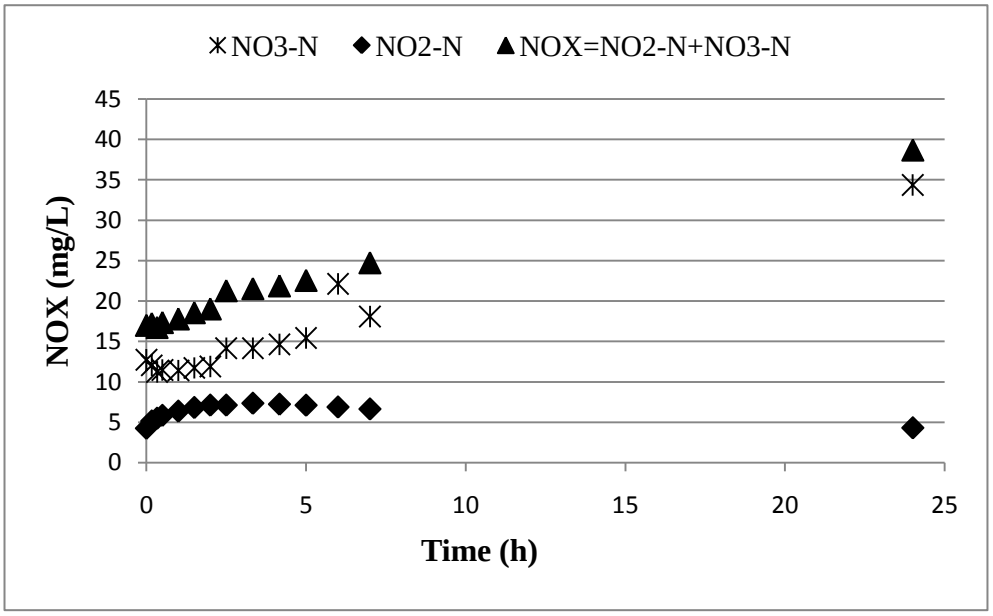


Figure 4.39: NO_X versus Time (Chronic Run 2.6).

4.2.7. 40 th Day 360 mg COD/L peptone + 1mg/L EE2

In 40th day, respirometric analysis were done with peptone mixture and EE2 solution together and only peptone mixture seperately. The OUR profile of the experiment of

the system fed with 360 mg COD/L peptone mixture and 1 mg/L EE2 solution is seen in **Figure 4.40**.

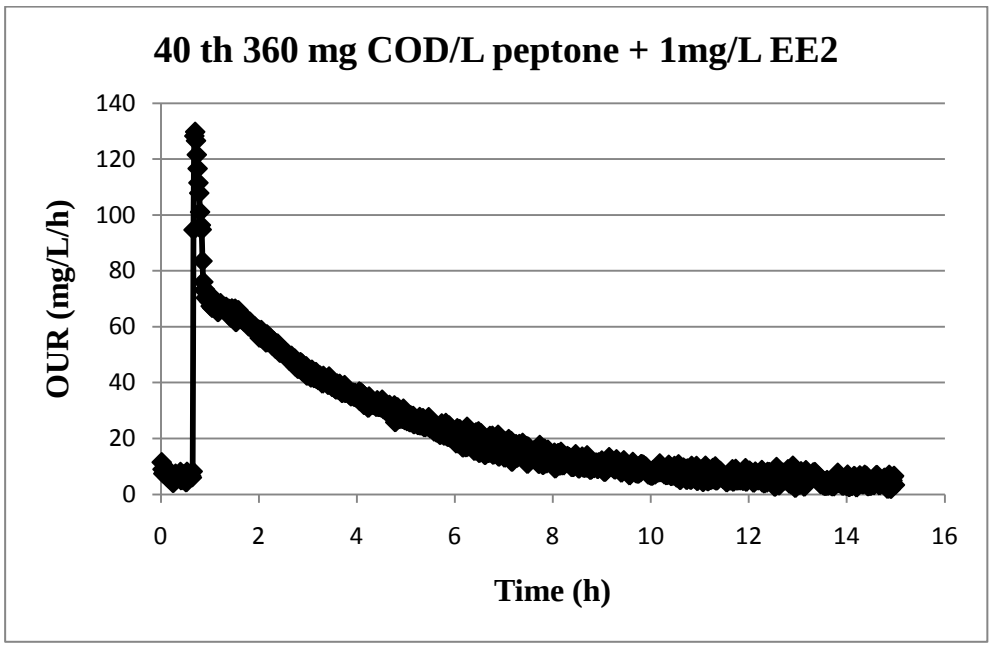


Figure 4.40: OUR Data versus Time (Chronic Run 2.7).

The COD change of experiment is illustrated in **Figure 4.41**. Concentration of COD is decreased from 394 mg COD/L to 22 mg COD/L and the removal ratio is 94%.

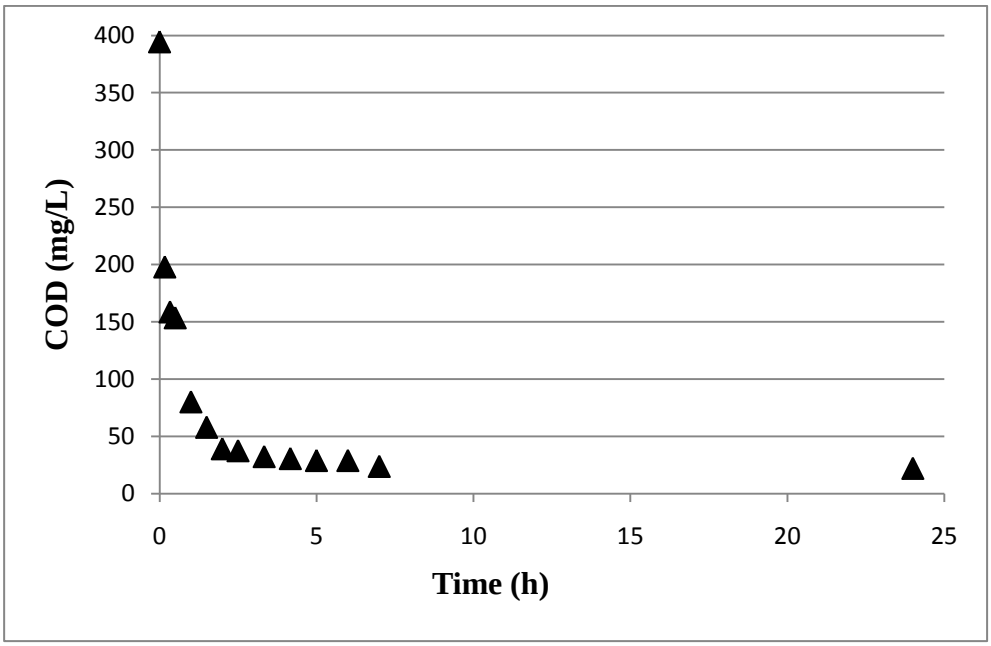


Figure 4.41: COD versus Time (Chronic Run 2.7).

As seen from the **Figure 4.42**, NO_X concentration increased to 61 mg/L during experiment and influent total nitrogen concentration is calculated 67 mg/L.

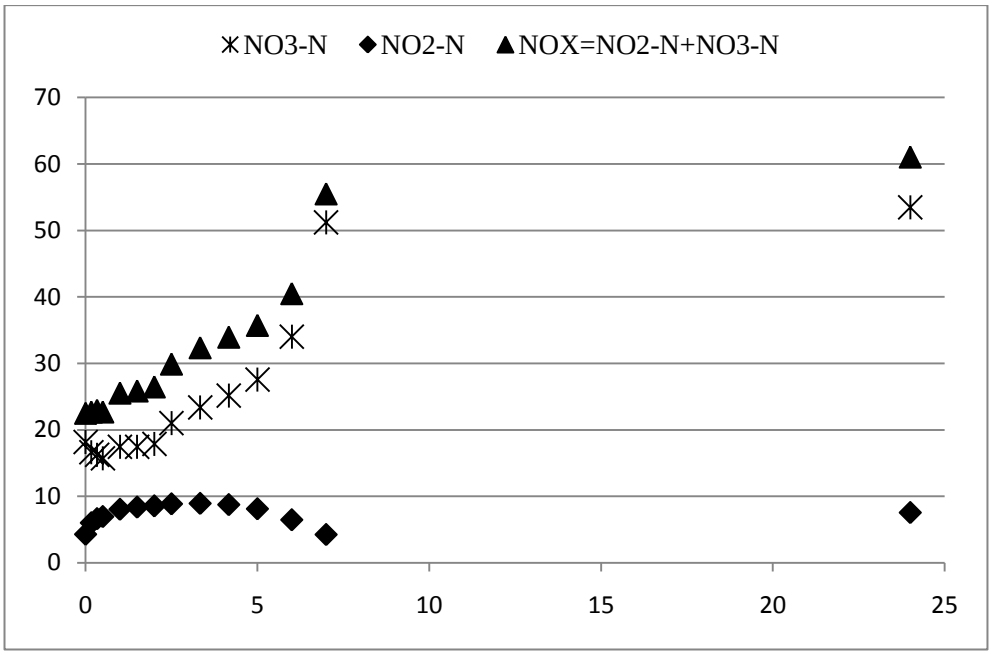


Figure 4.42: NO_X versus Time (Chronic Run 2.7).

Storage of PHA were also observed which is about 15 mg COD/L before the addition of peptone mixture and EE2 solution. PHA storage increased to 30 mg COD/L during the experiment and decreased to 6 mg COD/L (**Figure 4.43**).

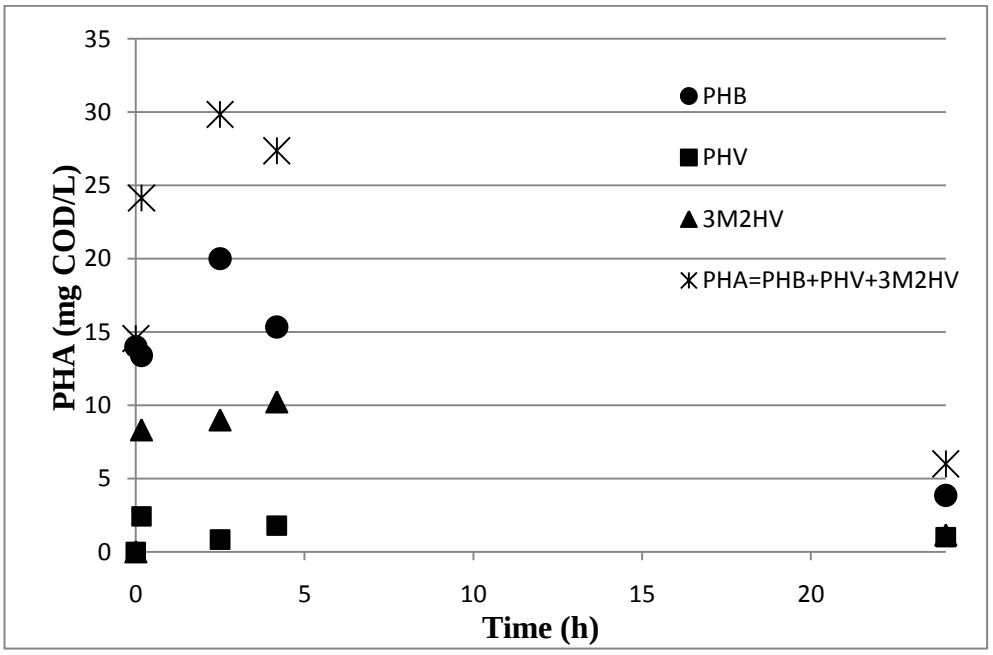


Figure 4.43: PHA versus Time (Chronic Run 2.7).

The OUR profile observed when only 360 mg COD/L peptone mixture was added to the system is seen in **Figure 4.44**. The maximum OUR data is increased by comparison with the Run 2.7.

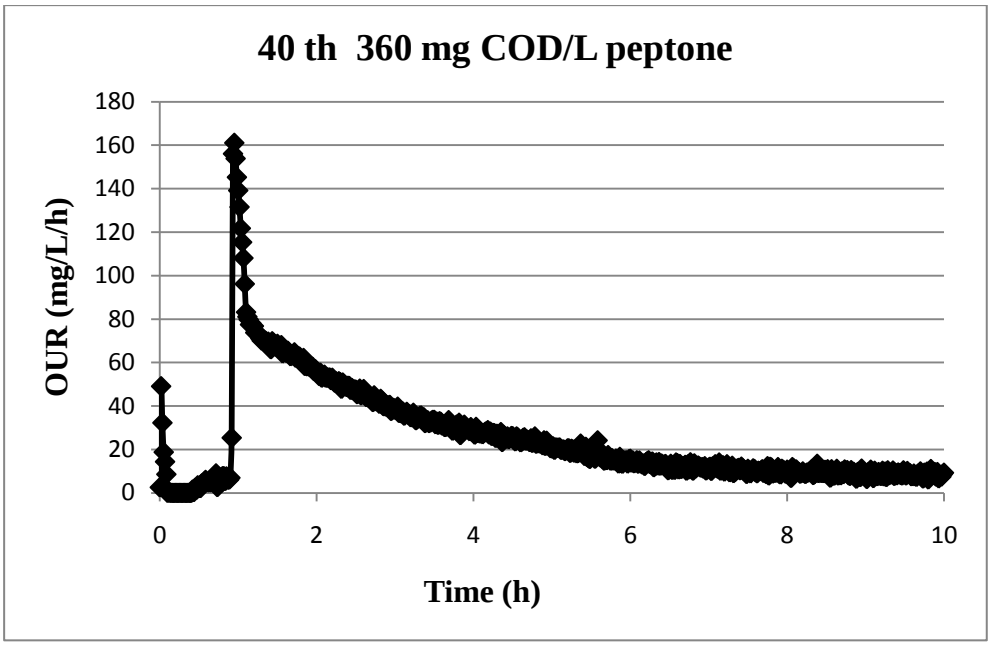


Figure 4.44: OUR Data versus Time (Chronic Run 2.8).

It is seen that there is a slight change in the effluent COD concentration without any EE2 solution. The effluent COD concentration is decreased to 32 mg COD/L from 393 mg COD/L. While removal ratio is 94% with EE2, it decrease to 92% without EE2 (**Figure 4.45**).

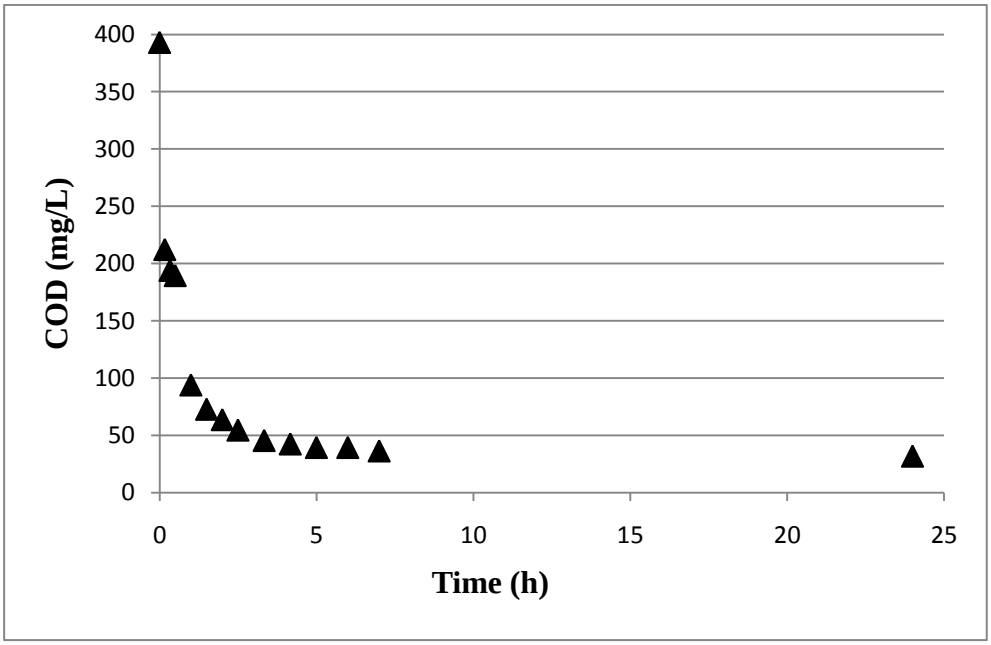


Figure 4.45: COD versus Time (Chronic Run 2.8).

As seen from the **Figure 4.46**, NO_X concentration increased to 42 mg/L during experiment and influent total nitrogen concentration is calculated 67 mg/L.

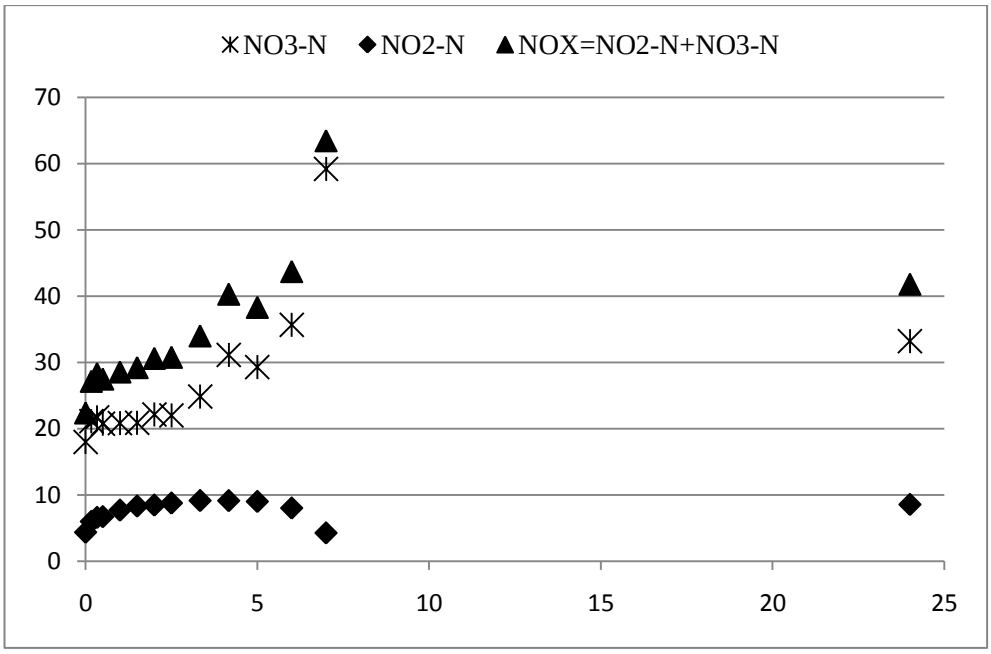


Figure 4.46: NO_X versus Time (Chronic Run 2.8).

4.2.8. 40 th day 50 mg/L NH_4Cl

The OUR profile observed when only 50 mg/L NH_4Cl was added to the system is seen in **Figure 4.47**. During the experiment the samples taken for the analysis of TKN, NO_X, pH, NH_3 and EE2. Effluent TKN concentration is measured 3.8 mg/L and ammonia concentration found under the 5 mg/L. NO_X concentration of the run 2.10 is given in **Figure 4.48**.

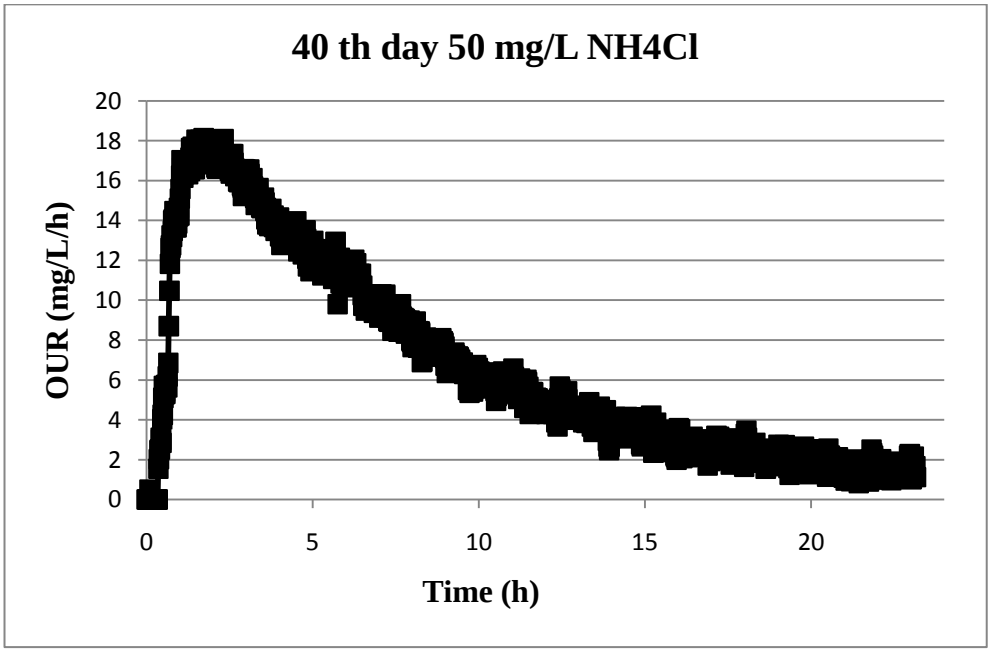


Figure 4.47: OUR Data versus Time (Chronic Run 2.9).

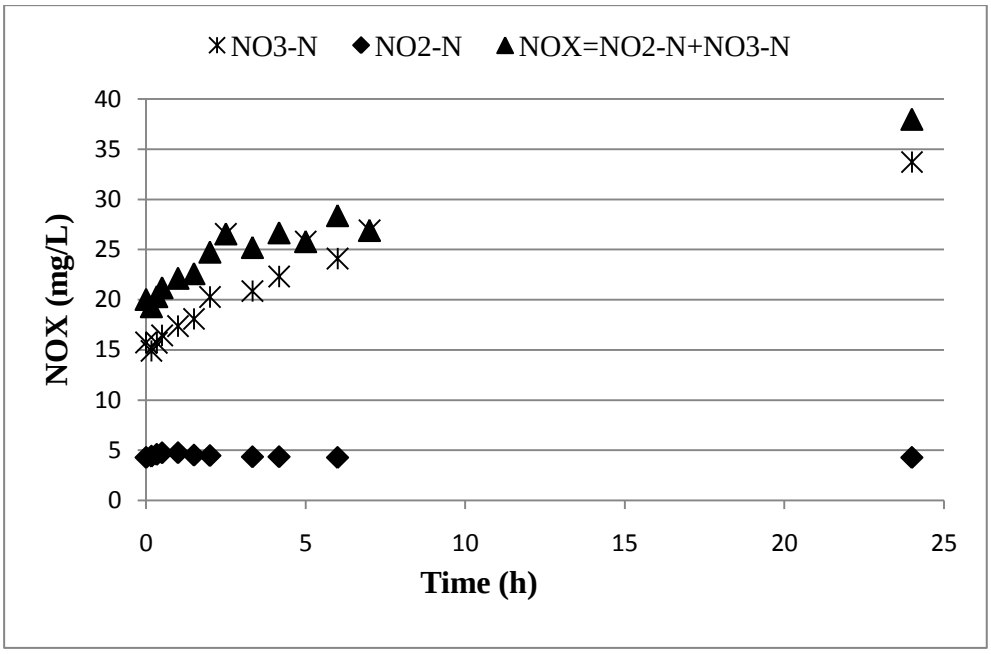


Figure 4.48: NO_X versus Time (Chronic Run 2.9).

The OUR profile observed when 50 mg/L NH_4Cl with 1 mg/L EE2 solution were added to the system is seen in **Figure 4.49**. During the experiment the samples taken for the analysis of soluble TKN, NO_X, pH, NH_3 and EE2. Effluent TKN concentration is measured 3.9 mg/L and ammonia concentration found under the 5 mg/L. NO_X concentration of the run 2.10 is given in **Figure 4.50**.

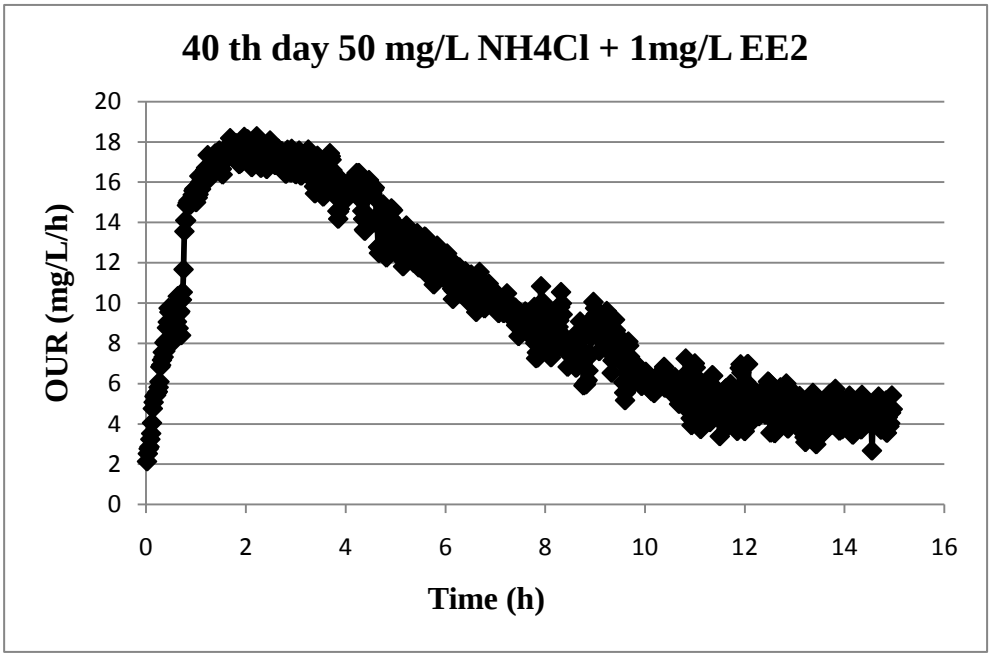


Figure 4.49: OUR Data versus Time (Chronic Run 2.10).

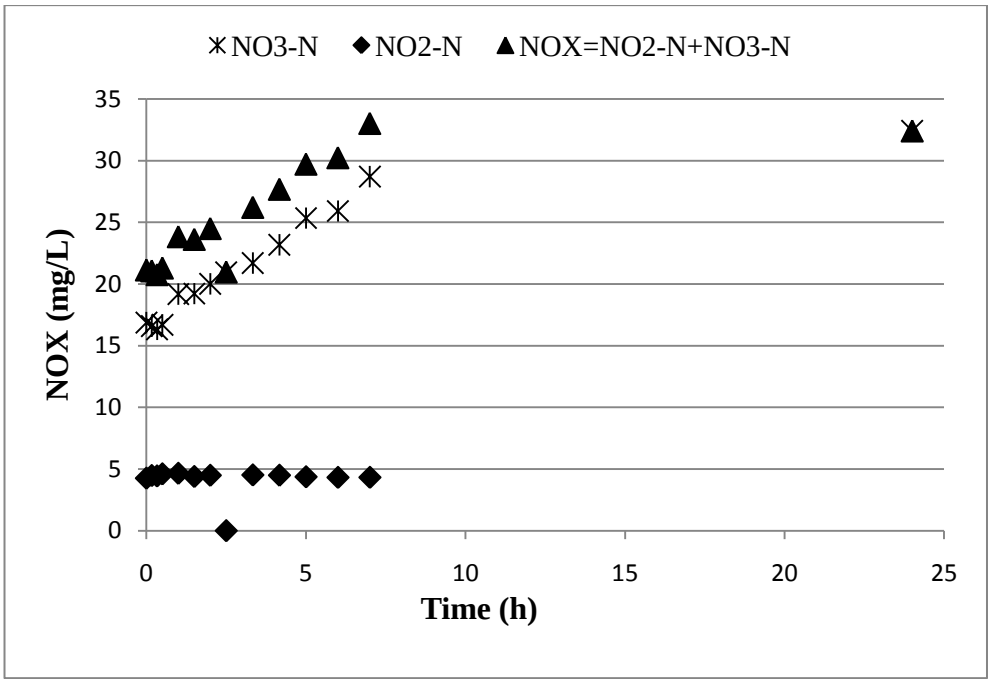


Figure 4.50: NO_X versus Time (Chronic Run 2.10).

4.3.EE2 Results

EE2 results of the chronic experiments for solid and liquid phase illustrated in **Table 4.2** and **Table 4.3**, respectively. 1 mg/L EE2 solution with 360 mg/L peptone mixture is added to the system. According to **Table 4.2**, effluent EE2 concentration of the liquid phase is decreased in time. Removal ratio of EE2 is found 96% at the end of experiment. However, for solid phase, effluent EE2 concentration is almost stable for all days. Avarage effluent EE2 concentration of the system is found 4.2 µg/g during 40 days.

Table 4.2: EE2 Results for solid phase

Day	Pepton+EE2 (µg/g)	
	Influent	Effluent
5	1300	4,0
10	1200	4,4
15	1100	4,2
20	1080	4,4
25	1080	3,7
30	1106	4,7
40	1080	4,3

Table 4.3: EE2 Results for liquid phase

Day	Pepton+EE2 (µg/L)		Only Peptone (µg/L)		NH_4Cl (µg/L)	
	Influent	Effluent	Influent	Effluent	Influent	Effluent
5	1300	247	-	-	-	-
15	1100	81,7	-	-	-	-
40	1080	40,6	80	31	-	-
40	-	-	-	-	1099	83

4.4.Comparison of NOX concentrations

According to **Figure 4.51**, addition of EE2 at the system is increased the NO_X concentration. Also, when mass balance of the system is measured, ıt is observed to increased NO_X concentration. Mass balance of the system is calculated according to following equations and results are given in **Table 4.4**:

$$TN_{inf} = NO_{X,inf} + TKN_{inf} \quad \textbf{(3.1)}$$

$$NO_X = NO_{X,eff} - NO_{X,inf} \quad \textbf{(3.2)}$$

$$N_X = C_S \cdot \frac{Y_H}{1+k_d\theta_x} \frac{g\ N}{g\ VSS} \quad \textbf{(3.3)}$$

$$TKN_{rem} = NO_X + N_X \quad \textbf{(3.4)}$$

$$SKN_{eff} = TKN_{peptone} - TKN_{rem} \quad \textbf{(3.5)}$$

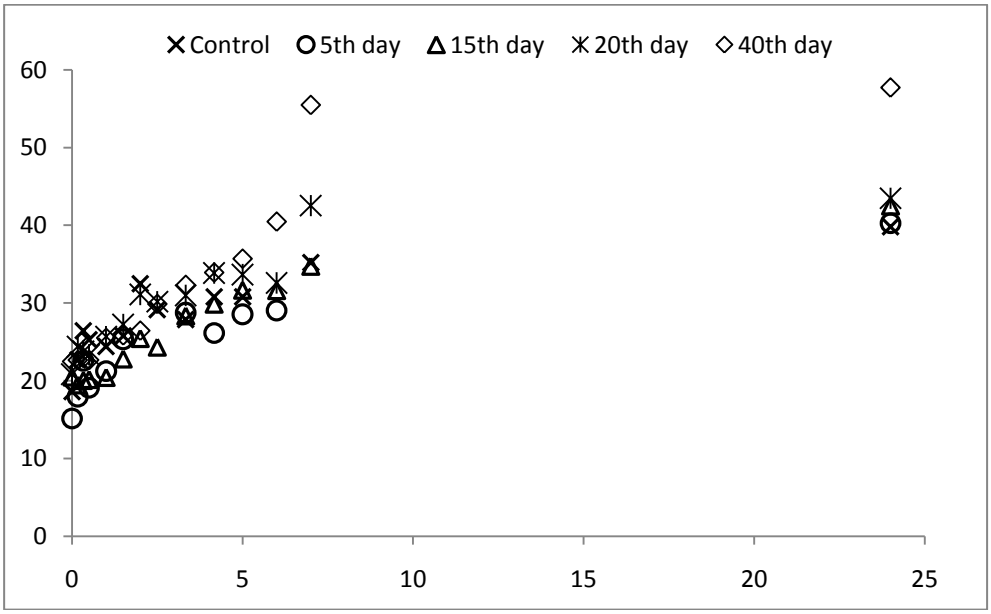


Figure 4.51: Comparison of NOX concentrations

Table 4.4: Comparision of Control and 40th day experiments

Parameter	Experiments	
	Control	40 th day
$SKN_{Peptone}$	44.30	44.30
TN_{Inf}	62.30	67.00
NO_X	21.81	33.01
N_X	10.70	10.70
SKN_{Rem}	32.51	43.71
SKN_{Eff}	11.79	0.59

According to this results, addition of EE2 cause hydrolysis of organic nitrogen fraction of the system during experiments.

4.5. Comparison of the Carbon Removal and Nitrification System

Comparision of OUR profiles of carbon removal and nitrification system for control experiment will be given in **Table 4.4** and **Figure 4.51**. Two different runs of batch experiments were carried out to evaluate the biodegradation of EE2. Nitrification was prohibited by means of selected inhibitor (TCMP) in the Carbon Removal experiments, and in the Nitrification system, it was allowed to proceed. In the carbon removal systems, the batch reactor system was used to monitor the OUR profile, only reflecting reactions associated with the heterotrophic activity. In the nitrification system included a parallel batch reactor to obtain OUR profile, reflecting reactions together with the heterotrophic and autotrotrophic activity.
The resulting OUR profile of nitrification yielded, as expected, a longer oxygen consumption due to nitrification simultaneously occured with the biodegradation of the peptone mixture. For example, ın the control set, the area of the OUR curve above the endogenous respiration level, calculated as 169 mg O_2/L for nitrification removal system and 105 mg O_2/L for carbon removal system.

Run 1.1, Run 2.7, Run 2.9 and Run 2.10 were modelled according to modified ASM 3 model (Katipoğlu-Yazan et. al., 2012) and the model parameters were estimated for those sets. The model with the OUR profiles, nitrogen profiles of the system, storage profiles of the system were shown for control set (Run 1.1) and 40 th day (Run 2.7) in **Figure 4.53**, **Figure 4.54**, **Figure 4.55** and **Figure 4.56**, **Figure 4.57** and **Figure 4.58**, respectively.

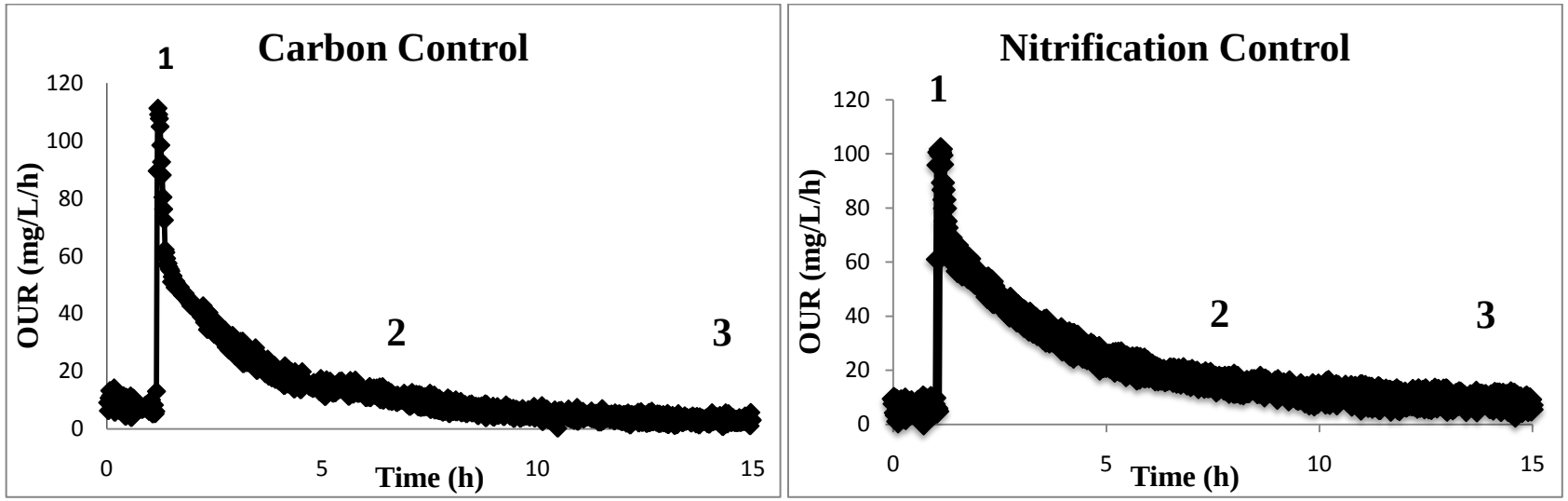


Figure 4.52: Comparison of carbon removal and nitrification system

Table 4.5: Results of chronic experiments

Day	Consuming Area (mg/L/h)		Nitrate parameter at point of [2] (mg/L)	NO_X (mg/L)
	Nitrification	Carbon Removal		
0 (Control)	169	105	14.00	26.81
1	165	105	14.86	25.14
5	165	108	13.7	26.14
15	157	105	12.67	22
30	153	105	10	21.7
40	242	105	30	38.5

The model and the OUR profile for Run 2.9 (only NH_4Cl) and Run 2.10 (NH_4Cl + EE2) were given in **Figure 4.59** and **Figure 4.60**, respectively. The estimated model parameters were illustrated in **Table 4.4** and **Table 4.5**.

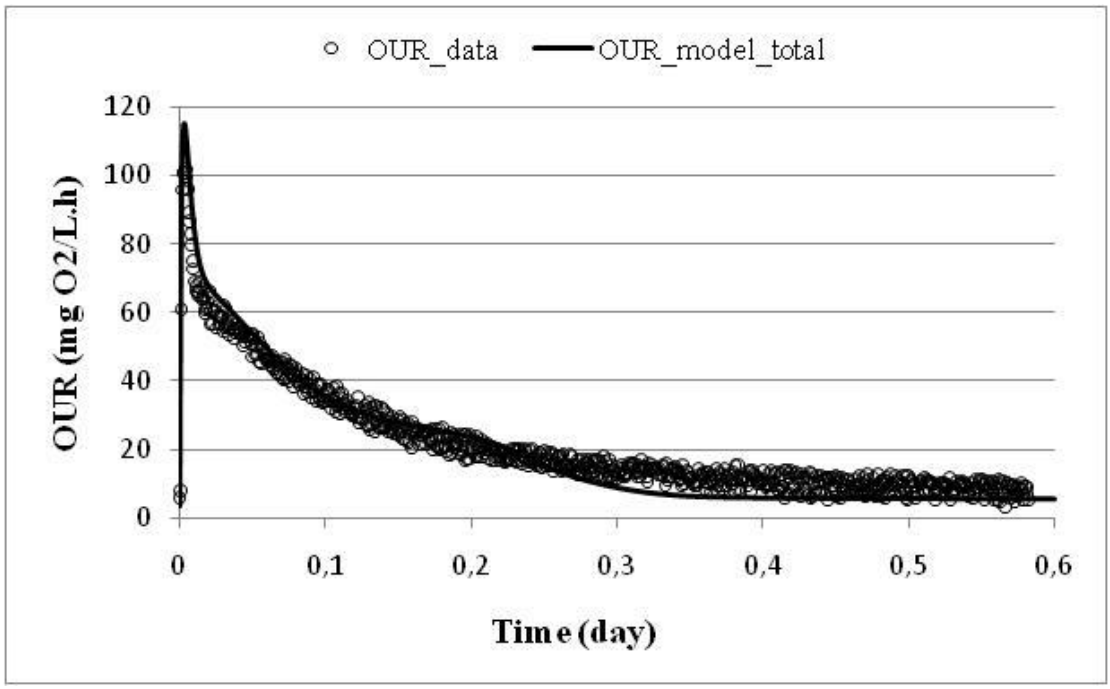


Figure 4.53: Modified ASM 3 simulation of OUR data of the control set (Run 1.1).

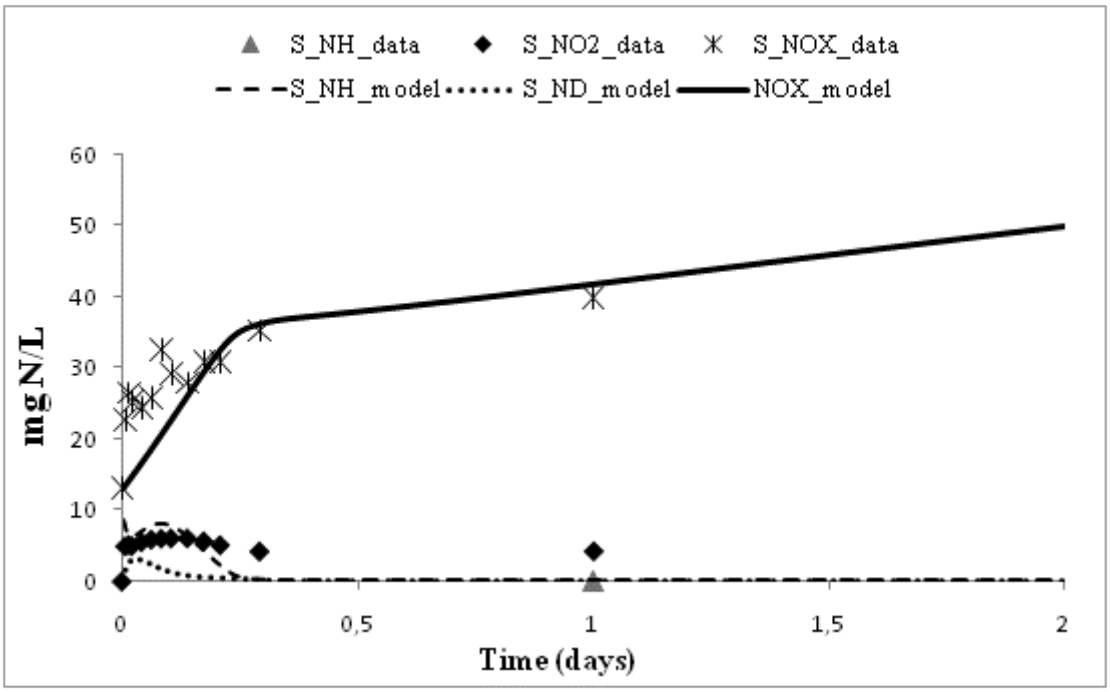


Figure 4.54: Modified ASM 3 simulation of nitrogen data of the control set (Run 1.1).

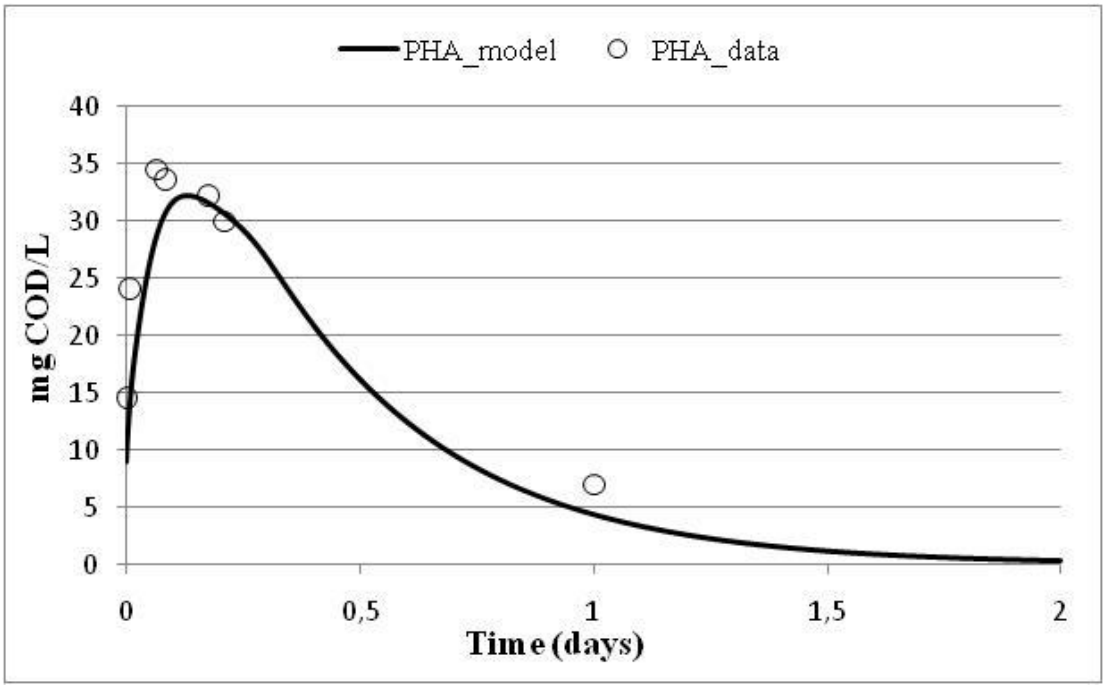


Figure 4.55: Modified ASM 3 simulation of PHA data of the control set (Run 1.1).

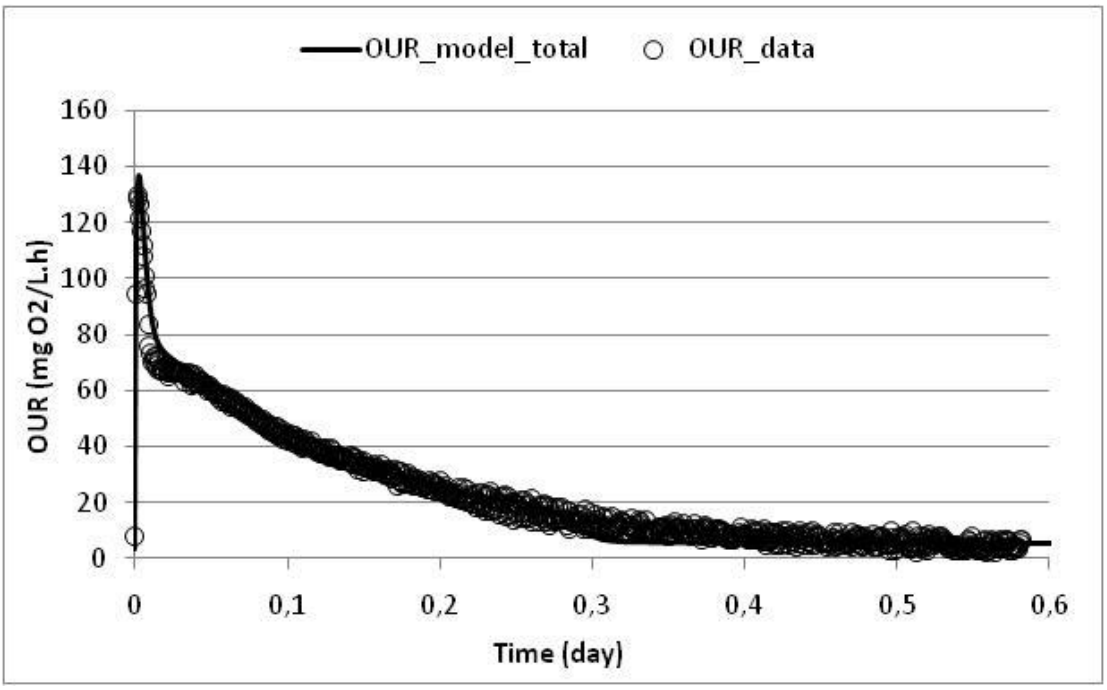


Figure 4.56: Modified ASM 3 simulation of OUR data of the 40 th day (Run 2.7).

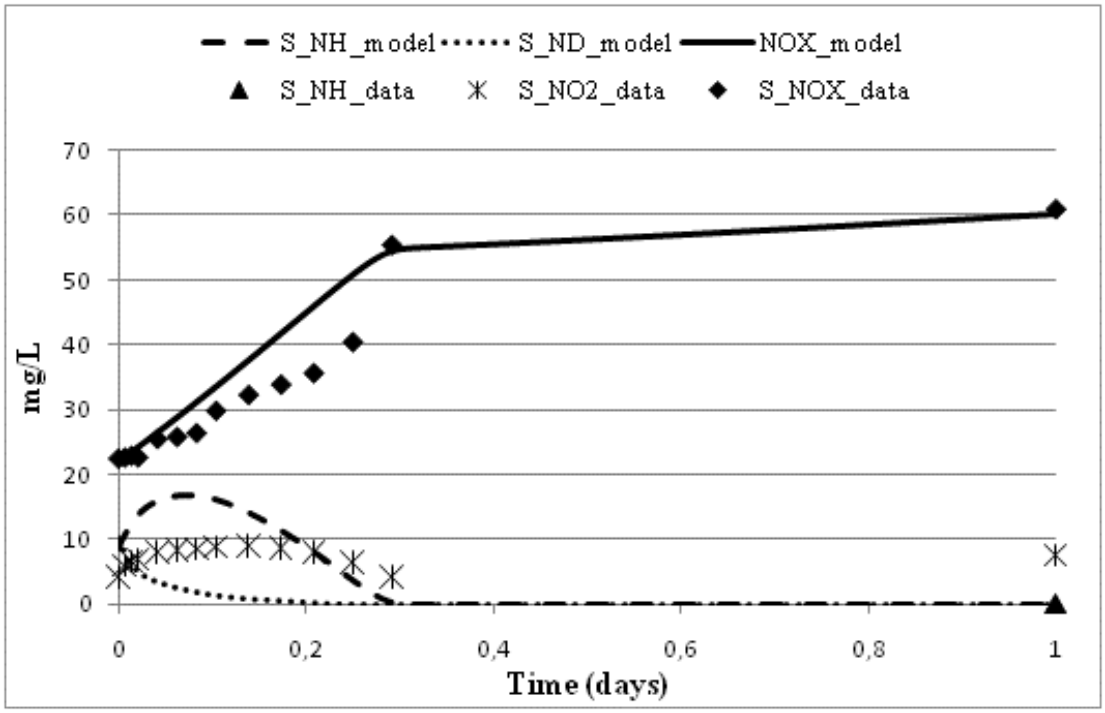


Figure 4.57: Modified ASM 3 simulation of nitrogen data of the 40 th day (Run 2.7).

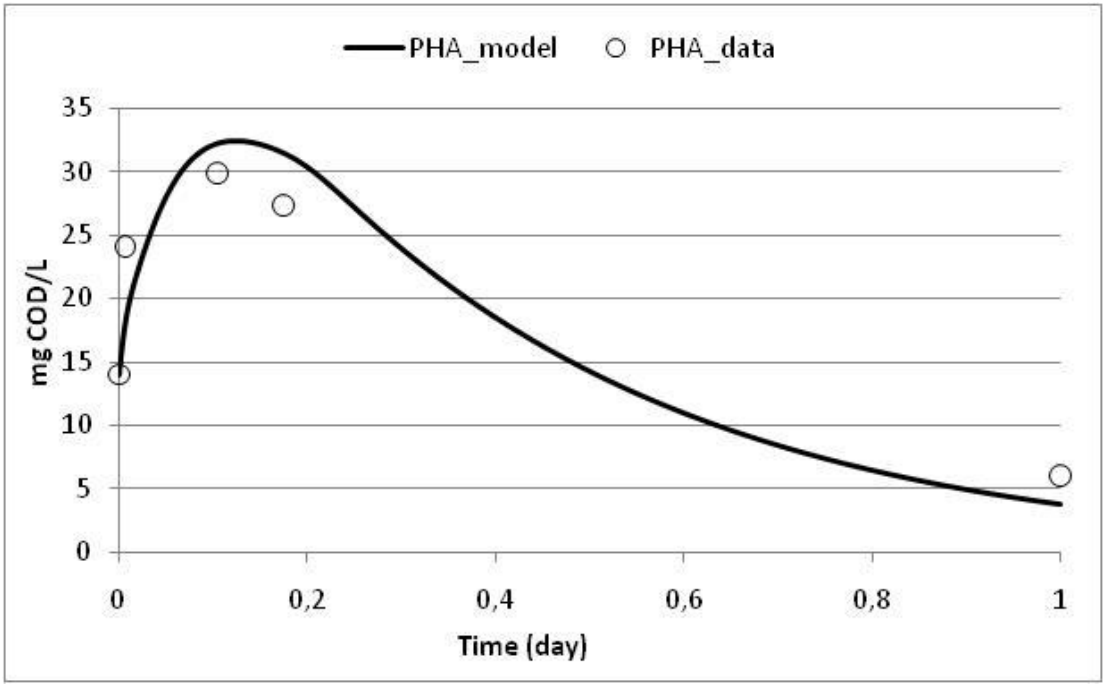


Figure 4.58: Modified ASM 3 simulation of PHA data of the 40 th day (Run 2.7).

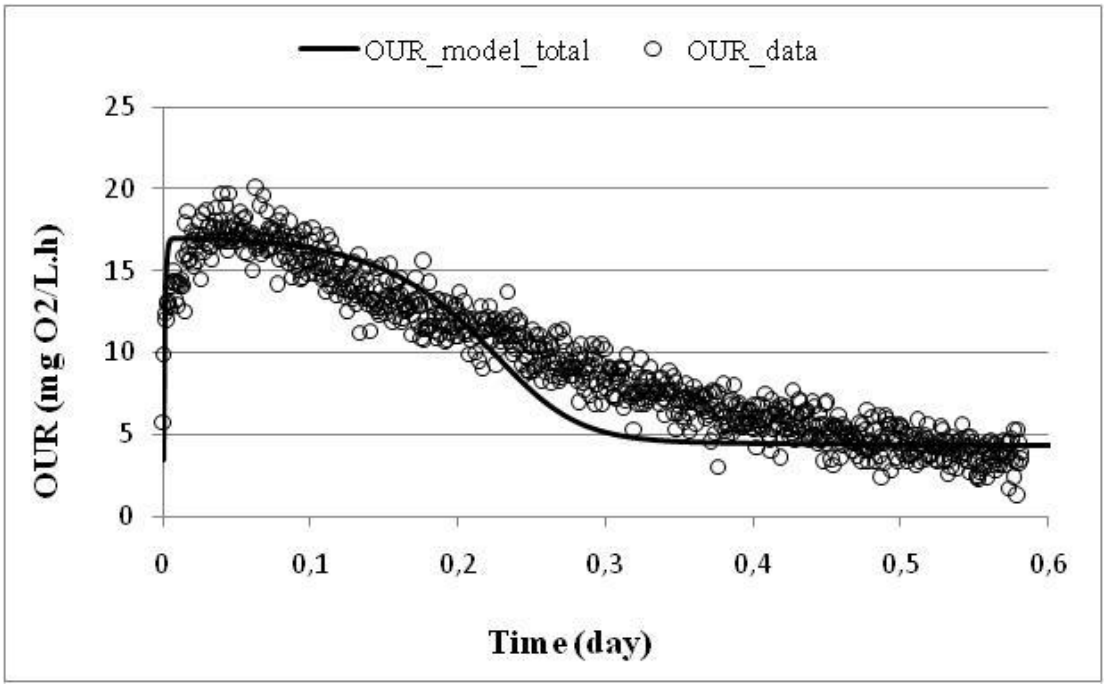


Figure 4.59: Modified ASM 3 simulation of OUR data of addition of NH_4Cl (Run 2.10).

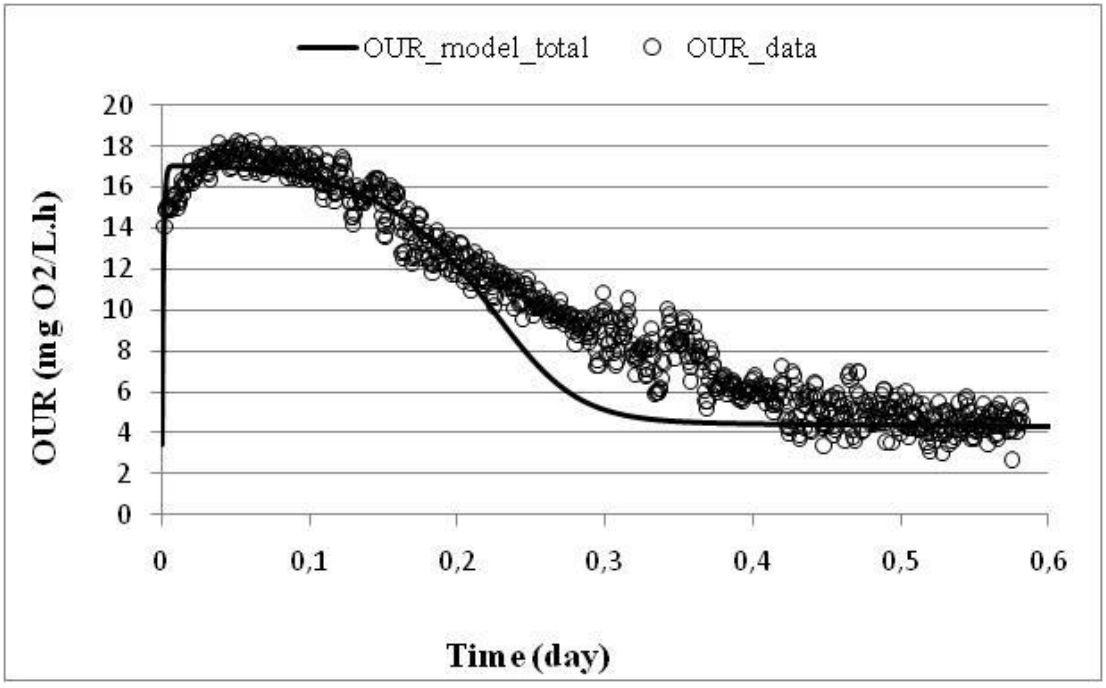


Figure 4.60: Modified ASM 3 simulation of OUR data of addition of NH_4Cl+EE2 (Run 2.11)

Table 4.6: Results of model calibration for the Run 1.1, Run 2.7, Run 2.10 and Run 2.11 (Model parameters)

Model Parameters		Unit	Run 1.1	Run 2.7	Run 2.10	Run 2.11
Maximum growth rate for X_H	μ_H	1/day	6.8	8	-	-
Half saturation constant for growth of X_H	K_S	mg COD/L	24	24	-	-
Maximum growth rate for X_A	μ_A	1/day	0.8	1.0	1.0	1.0
Half saturation constant for growth of X_A	K_{NH}	mg N/L	0.9	0.9	3.0	3.0
Endogenous decay rate for X_H	b_H	1/day	0.1	0.1	0.1	0.1
Endogenous decay rate for X_A	b_A	1/day	0.1	0.1	0.1	0.1
Half saturation coefficient of oxygen for X_H	K_{OH}	mg O_2/L	0.2	0.2	0.2	0.2
Half saturation coefficient of oxygen for X_A	K_{OA}	mg O_2/L	0.4	0.4	0.4	0.4
Maximum hydrolysis rate for S_{H1}	k_{h1}	1/day	6.3	6.3	-	-
Hydrolysis half saturation constant for S_{H1}	K_X	g COD/g COD	0.20	0.20	-	-
Maximum hydrolysis rate for S_{H2}	k_{h2}	1/day	0.5	0.5	-	-
Hydrolysis half saturation constant for S_{H2}	K_{XX}	g COD/g COD	0.01	0.01	-	-
Maximum storage rate of X_{STO} by X_H	k_{STO}	1/day	1.60	1.60	-	-
Half saturation constant for growth of X_H on X_{STO}	K_{STO}	mg COD/L	0.50	0.50	-	-
Maximum growth rate on X_{STO} for X_H	μ_{STO}	1/day	0.8	0.8	-	-
Ammonification rate of S_{ND}	k_a	m3/g COD day	0.08	0.08	0.08	0.08
Yield coefficient for X_H	Y_H	g COD/g COD	0.60	0.60	-	-
Yield coefficient for X_A	Y_A	g COD /g N	0.24	0.24	0.24	0.24
Storage yield of X_{STO}	Y_{STO}	g COD/g COD	0.80	0.80	-	-
Fraction of nitrogen in biomass	i_{NBM}	g N/g COD	0.085	0.085	0.085	0.085
Fraction of nitrogen in S_{H1} and S_{H2}	i_{NSH1},i_{NSH2}	g N/g COD	0.09	0.09	0.09	0.09
Fraction of nitrogen in particulate products from biomass	i_{NXP}	g N/g COD	0.03	0.03	0.03	0.03
Fraction of nitrogen in soluble products from biomass	i_{NSP}	g N/g COD	0.04	0.04	0.04	0.04
Fraction of biomass converted to S_P	f_{ES}	-	0.05	0.05	0.05	0.05
Fraction of biomass converted to X_P	f_{EX}	-	0.15	0.15	0.15	0.15

Table 4.7: Results of model calibration for the Run 1.1, Run 2.7, Run 2.10 and Run 2.11 (State Variables)

State Variables		Unit	Run 1.1	Run 2.7	Run 2.10	Run 2.11
Initial active heterotrophic biomass	X_{H1}	mg COD/L	1000	1000	1000	1000
Initial active autotrophic biomass	X_{A1}	mg COD/L	30	30	30	30
Initial amount of PHA	X_{STO1}	mg COD/L	14	14	9	9
Initial biodegradable peptone mixture COD	C_{S1}	mg COD/L	360	360	-	-
Initial readily biodegradable peptone mixture COD	S_{S1}	mg COD/L	33	33	-	-
Initial readily hydrolizable peptone mixture COD	S_{H11}	mg COD/L	202	202	-	-
Initial slowly hydrolizable peptone mixture COD	S_{H21}	mg COD/L	125	125	-	-
Initial ammonia nitrogen	S_{NH1}	mg N/L	8.70	8.70	20.00	20.00
Initial soluble organic nitrogen	S_{ND1}	mg N/L	0	0	0	0
Initial oxidized nitrogen	S_{NOX1}	mg N/L	18.00	22.49	20.40	21.13

Model calibration of modified ASM 3 providing best fit with the experimental data yielded a value of 6.8 d^{-1} for the maximum heterotrophic growth rate of Run 1.1, Run 2.10 and Run 2.11, μ_H for 40 th day fed with peptone and EE2 is higher than other runs. The maximum growth rate for autotrophs is 1 d^{-1} for Run 2.7, Run 2.10 and Run 2.11, but 0.8 d^{-1} for control set and the corresponding half saturation coefficient, K_{NH} of 0.9 mg N/L for all runs. Henze et al. (1987) recommended a default , μ_A value of 0.8 d^{-1} for domestic sewage. It can be commented that the addition of EE2 is increased the growth of heterotrophic and autotrophic microorganisms.

5. CONCLUSION AND RECOMMENDATIONS

The purpose of this study is evaluating the biodegradation of EE2 under nitrifiying conditions. An activated sludge system with biomass acclimated to a synthetic substrate mixture was investigated for acute and chronic effects of EE2 solution. The experiments conducted parallel to the respirometer, organic matter removal efficiency, nitrogen concentration, PHA and EE2 amounts were monitored.

The batch tests were started with COD level of 360 mg/L; the initial biomass level was adjusted to maintain the same food to microorganism ratio (S_0/X_0) of 0.3 mg COD/mg VSS. The resulting OUR profile yielded, as expected, larger oxygen consuption by comparision with carbon removal system, due to the nitrification simultaneously occuring with the biodegradation of the peptone mixture.

Model calibration yielded a close fit with the experimental OUR data for the set of stoichiometric and kinetic coefficients. It also defined the relevant COD fractionation associated with the peptone mixture, which indeed included three components with different biodegradation characteristics, namely: a small readily biodegradable COD fraction , S_S, of 33 mg/L (9.2%) together with a readily hydrolysable COD, S_{H1}, of 202 mg/L (56%) and a slowly hydrolysable COD, S_{H2}, of 125 mg/L (34.8%), in accordance with the shape of the OUR curve. Optimum calibration was obtained with a heterotrophic yield, Y_H, value of 0.60 mg cell COD/mg COD and an endogenous decay coefficient, b_H, of 0.1 day^{-1}.

According to modified ASM 3, the control run and final day analyses were modelled. And it is seen that the addition of EE2 is increased the growth of heterotrophic and autotrophic microorganisms. The maximum growth rate (μ_A) for control experiment was 0.8 d^{-1} and a corresponding half saturation coefficient, K_{NH} of 0.9 mg N/L. For 40 th day experiment, μ_A was 1.0 d^{-1} and a corresponding K_{NH} value of 0.9 mg N/L. Henze et al. (1987) recommended a default μ_A value of 0.8 d^{-1} for domestic sewage. Moreover, with the addition of EE2 cause hydrolysis of organic nitrogen fraction of the system during experiments.

During 40 days, 1 mg/L EE2 with 360 mg COD/L was added to the system and EE2 concentration of the system was monitored at liquid and solid phase. For liquid phase removal ratio of the EE2 was found 96% at the end of the experiment. However, for solid phase, effluent EE2 concentration is almost stable for all days (4.2 μg/g).

According to Run 2.10 modelled with modified ASM 3, the OUR profile approximately similar to the one using the NH_4Cl with EE2 at the same sludge age of 10 days. At this experiment, pH of the system decreased in time, therefore, model calibration didn't yield a close fit with the experimental OUR data of Run 2.10 and Run 2.11.

Reliable modelling of substrate utilization also requires consideration of intracellular storage: It is well known that in biological reactors (such as batch reactors in this study) sustaining sequential feast (abundance of external substrate) and famine (absence of external substrate) conditions, a fraction of available substrate is likely to be diverted to storage (Krishna and Loodsdrecht, 1999). In this context, generation of the storage products (PHAs) were also monitored, parallel to the OUR profiles. It should be mentioned that PHA assessment involved separate measurements of all relevant components, i.e. PHB, PHV and 3H2MV. The results of this study indicated a slight PHA generation (8-10%) of the initially available substrate.

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CURRICULUM VITAE

Name Surname: Burcu Tezcan

Place and Date of Birth: İstanbul, 21.01.1986

E-Mail: brctzcn@hotmail.com

B.Sc.: Trakya University