ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

BIODEGRADABILITY OF DICLOFENAC UNDER AEROBIC CONDITIONS

M.Sc. THESIS

Hülya CİVELEK

Department of Environmental Engineering

Environmenral Biotechnology Programme

Thesis Advisor: Assist. Prof. Dr. Gülsüm Emel ZENGİN BALCI

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<u>İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ</u>

AEROBİK ŞARTLARDA DİKLOFENAK'IN BİYOLOJİK ARITILABİLİRLİĞİNİN İNCELENMESİ

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Hülya Civelek, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 501121807, successfully defended the **thesis** entitled "**BIODEGRADABILITY OF DICLOFENAC UNDER AEROBIC CONDITIONS**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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To my family and nephews,

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ABBREVIATIONS

ACN : Acetonitrile
ASM1 : Activated Sludge Modelling No.1
b_H : Endogenous decay rate for X_H
COD : Chemical Oxygen Demand
C_{S1} : COD of iniitial biodegradable peptone mixture
DCF : Diclofenac
EC : Effect concentration
F/M : Food to microorganism ratio
k_{h1} : Maximum hydrolysis rate for S_{H1}
k_{h2} : Maximum hydrolysis rate for S_{H2}
K_{S1} : Half saturation constant for growth of X_H (for peptone mixture)
K_{S2} : Half saturation constant for growth of X_H (for methanol)
K_X : Hydrolysis half saturation constant for S_{H1}
K_{XX} : Hydrolysis half saturation constant for S_{H2}
LC : Letal Concentration
LC-MS/MS : Liquid Chromatography Coupled with Tandem Mass Spectrometry
LOEC : Lowest Observed Effect Concentration
MeOH : Methanol
MLSS : Mixed Luqiour Suspended Solids
MLVSS : Mixed Luqiour Volatile Suspended Solids
NOEC : No Observed Effect Concentration
NSAID : Non-steroidal Anti-inflammatory Drug
PHA : Polyhydroxyalkanoates
PHB : Polyhydroxybutyrate
S_{H1} : Readily hydrolizable substrate
S_{H11} : COD of initial readily hydrolizable peptone mixture
S_{H2} : Slowly hydrolizable substrate
S_{H21} : COD of initial slowly hydrolizable peptone mixture
S_S : Readily biodegradable substrate
S_{S1} : COD of initial readily biodegradable peptone mixture
S_{S2} : Initial methanol COD
SS : Suspended Solids
UPLC : Ultra Performance Liquid Chromatograph
VSS : Volatile Suspended Solid
WWTP : Wastewater Treatment Plant
X_H : Heterotrophic biomass
X_{H1} : Initial active heterotrophic biomass
X_S : Slowly biodegradable
X_T : Volatile suspended solids
Y_H : Yield coefficiend for X_H
$\hat{\mu}_{H1}$: Maximum specific growth rate for X_H (for peptone mixture)
$\hat{\mu}_{H2}$: Maximum specific growth rate for X_H (for methanol)

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BIODEGRADABILITY OF DICLIFENAC UNDER AEROBIC CONDITIONS

SUMMARY

Diclofenac, derives from its chemical name “2-(2,6-dichloranilino) phenylacetic acid” is a synthetic non-steroidal anti-inflammatory drug. Annual consumption of diclofenac worldwide is estimated as 940 tons. Increase in consumption of diclofenac causes several environmental problems. Because of its low removal rate, diclofenac is one of the most commonly detected anti-inflammatory in groundwater and surface water.

This study is conducted to understand the acute and chronic inhibition effect and biodegradability potential of diclofenac in different sludge ages under aerobic conditions.

To examine the effect of sludge age on biodegradability of diclofenac under aerobic conditions, two different sludge ages were selected as 10 days and 20 days considering literature review and real-scale systems. For each selected sludge age, control reactors were also operated. 10 μg/L diclofenac which dissolved in methanol was fed to diclofenac reactors and only methanol was fed to control reactors. Oxygen concentrations of the reactors were kept higher than 3 mg/L and reactors were throughly mixed.

10 days of sludge age reactors was operated for 160 days and suspended solids (SS), volatile suspended solids (VSS) and chemical oxygen demand (COD) parameters were measured weekly. According to SS, VSS and COD concentrations of 10 days of sludge age control and diclofenac acclimated reactors, diclofenac has no chronic inhibition effect on biomass and substrate degradation on 10 days of sludge age.

Similarly, 20 days of sludge age reactors were operated for 250 days and SS, VSS and COD parameters were measured weekly. According to SS, VSS and COD concentrations of reactors, diclofenac has a slight chronic inhibition effect on 20 days of sludge age on biomass and substrate degradation.

Samples were taken from 10 and 20 days of sludge age diclofenac reactors for diclofenac measurements. 22.5-34.3% diclofenac removal was observed in 20 days of sludge age reactor but no diclofenac removal was observed in 10 days of sludge age reactor. Diclofenac measurements were carried out also for sludge samples. It was observed that diclofenac cannot adsorb onto sludge.

Acute inhibition effect of diclofenac on acclimated and non-acclimated biomass was examined beside chronic inhibition effect. Respirometric experiments were carried out with different diclofenac concentrations.

According to respirometric sets which carried out with 10 days of sludge age control reactors’ biomass, only 1000 μg/L and 10,000 μg/L diclofenac had an acute inhibition effect on non-acclimated biomass. Similarly, 1000 μg/L diclofenac concentration had an acute inhibition effect on biomass that acclimated to diclofenac with 10 days of sludge age. In addition to this, acclimated and non-acclimated

biomass to diclofenac was compared with respirometric results. It was observed that long term exposure to diclofenac changes metabolic structure of biomass such as growth rate, hydrolysis, and storage mechanism.

According to respirometric sets which run with 20 days of sludge age control reactors' biomass, 10,000 μg/L diclofenac had an acute inhibition effect when 200 and 1000 μg/L diclofenac concentrations did not affect non-acclimated biomass. Likewise to non-acclimated biomass, 10,000 μg/L diclofenac concentration had an acute inhibition effect on biomass that acclimated to diclofenac in this sludge age. Similar to 10 days of sludge age, it was observed that long term exposure to diclofenac had an important effect on metabolic structure of biomass.

Modelling of OUR profiles of methanol addition (corresponds to 0 μg/L diclofenac concentration) to 20 days of sludge age control and diclofenac reactors was studied. Results showed that half saturation constant for growth of heterotrophic biomass, K_{S1}, was higher in control biomass than biomass that acclimated to diclofenac. Active biomass percentages were equal to each other. Acclimation to diclofenac did not effected hydrolysis rate. This situation shows that diclofenac had a chronic inhibition effect on biomass.

AEROBİK ŞARTLARDA DİKLOFENAK'IN ARITILABİLİRLİĞİNİN İNCELENMESİ

ÖZET

Hayvan ve insan tedavisinde kullanılan farmasotiklerin kullanımının her geçen gün artmasıyla birlikte doğaya salınımları da artmakta ve önem kazanmaktadır. En sık kullanılan farmasotiklerden biri olan, ağrı kesiciler olarak da bilinen anti-enflamatuarların kullanımı ise piyasada reçeteli ve reçetesiz olarak satışa sunulmakta ve dünyada yıllık birkaç tonu bulmaktadır. Gerek üretimi aşamasında, gerekse tüketildikten sonra doğaya yüksek miktarda salınımları gerçekleşen bu ilaçlar, acil eylem planı oluşturulması gereken kimyasallar olarak sınıflandırılmışlardır. Yüksek kullanım oranlarının yanı sıra sucul ortamlara kontrolsüz salınımları sadece alıcı ortamlardaki organizmalar için değil, aynı zamanda içme suları için de potansiyel risk oluşturmaktadır.

Steroid yapıya sahip olmayan bir anti-enflamatuar ilaç olan diklofenak da yüksek kullanım oranlarıyla dünyanın birçok ülkesinde en sık kullanılan steroid olmayan anti-enflamatuar ilaç olarak bilinir. Dünyada yıllık 940 ton tüketilen diklofenak'ın Türkiye'deki tüketim miktarıysa 2009 yılında 70 ton olarak belirlenmiştir.

Ayrıca diklofenak'ın anti-enflamatuarlar arasında en toksik üye olarak öne çıkması, diklofenak'ın birçok araştırmacı tarafından çevresel tehdit olarak ele alınmasına sebep olmuştur. Asya'da ineklere tedavi amaçlı verilen diklofenak, inekler öldükten sonra onların leşlerini yiyen akbabaların soylarını tükenme noktasına getirmiştir. Aynı zamanda diklofenak'ın sucul ortamlardaki toksisitesi de farklı çalışmalarda incelenmiş ve gözlenen en düşük etki konsantrasyonuna sahip olması sebebiyle en yüksek akut toksisite etkisine sahip steroid olmayan anti-enflamatuar olduğu bulunmuştur. Ayrıca yapılan çalışmalar diklofenak'ın farelerde, kuşlarda ve insanlarda ilk olarak böbreklerde zarara sebep olduğunu göstermiştir.

Diklofenak, biyolojik olarak zor parçalanabilen yapısı ve yüksek kullanımı sebebiyle atıksu arıtma tesislerinin giriş ve çıkış akımlarında, yeraltı sularında ve yüzeysel sularda en sık rastlanan anti-enflamatuardır. Yüzeysel sularda, arıtma tesislerinin giriş ve çıkış akımlarında diklofenak'a rastlama yüzdesi sırasıyla %96, %86 ve %93 olarak bulunmuştur. Diklofenak'ın yüzeysel sularda ve atıksu arıtma tesislerinin giriş konsantrasyonları incelendiğindeyse kullanım oranlarına bağlı olarak bölgesel ve mevsimsel olarak değiştiği görülmektedir. Yüzeysel sulardaki konsantrasyonu ise ng/L ile µg/L seviyeleri arasında olabilmektedir. Türkiye'de ise diklofenak konsantrasyonunun yüzeysel sularda 1.2 ng/L ile 45.7 ng/L seviyelerinde değişebildiği gözlemlenmiştir.

Diklofenak'ın atıksu arıtma tesislerindeki giderimleri incelendiğindeyse, çok çeşitli sonuçlarla karşılaşılmaktadır. Diklofenak'ın giderim verimi üzerinde hidrolik bekletme süresi, çamur yaşı gibi işletme parametrelerinin yanı sıra çevresel

faktörlerin ve sistemde çoğalan mikrobiyal çeşitliliğin de önemli etkilerinin olduğu bilinmektedir.

Diklofenak'ın aerobik koşullar altındaki biyolojik parçalanmasıyla ilgili literatürde oldukça değişken sonuçlarla karşılaşılmaktadır. Diklofenak bazı çalışmalarda biyolojik parçalanmaya dirençli olarak sınıflandırılırken, %93 mertebelerinde parçalanabildiği gözlenen çalışmalar da mevcuttur.

Bu çalışmada çamur yaşının aerobik koşullar altında diklofenak'ın biyolojik olarak parçalanabilirliği üzerindeki etkisi ve diklofenak'ın biyokütle üzerindeki akut ve kronik etkisi araştırılmıştır. Bu kapsamda literatürde yer alan çalışmalar ve gerçek ölçekli sistemler dikkate alınarak 10 gün ve 20 gün olmak üzere iki farklı çamur yaşı seçilmiş; çalışılan çamur yaşları için ayrıca kontrol reaktörleri kurulmuştur. 6 L çalışma hacmindeki laboratuvar ölçekli reaktörler aerobik şartlar altında 20^{o}C'de işletilmiştir. Reaktör içindeki diklofenak maddesinin fotolizini önlemek için reaktörler folyolarla kaplanmıştır. Reaktörler her gün 600 mg/L KOİ eşdeğerine sahip pepton solüsyonu, makro ve mikro nütrientleri içeren Çözelti A ve B ile beslenmiştir. Besleme sırasında diklofenak reaktörlerine metanol içerisinde çözünmüş olarak 10 µg/L diklofenak maddesi sürekli olarak beslenmiş; benzer koşulları sağlamak amacıyla kontrol reaktörlerine de aynı hacimde metanol ilavesi gerçekleştirilmiştir. Reaktörlerde pH değeri 6 ila 8 arasında olacak şekilde kontrol edilmiştir. Reaktörlerde aerobik koşullar laboratuvar merkezi havalandırma sistemine bağlı difüzörler ile sürekli olarak havalandırılarak sağlanmıştır. Sistemdeki oksijen konsantrasyonunun aerobik şartların sağlanması amacıyla 3 mg/L'nin üstünde tutulması amaçlanmıştır. Tam karışımı temin etmek için ise mekanik karıştırıcılar kullanılmıştır.

160 gün boyunca işletilen 10 gün çamur yaşındaki reaktörler denge haline geldikten sonra haftalık olarak AKM ve UAKM konsantrasyonları ile KOİ giderimleri incelenmiştir. Buna göre, diklofenak'ın KOİ giderimi ve biyokütle üzerinde kronik inhibisyon etkisinin olmadığı görülmüştür.

10 gün çamur yaşındaki diklofenak reaktöründen haftalık alınan diklofenak numunelerinin ölçümü sonucunda; ölçülen diklofenak konsantrasyonu, reaktöre beslenen diklofenak konsantrasyonuna yakın çıkmıştır. Diklofenak konsantrasyonunun çıkış değerinin giriş değerine yakın çıkması diklofenak'ın 10 gün çamur yaşında aerobik koşullar altında biyolojik olarak parçalanamadığını göstermiştir.

20 gün çamur yaşında işletilen kontrol ve diklofenak reaktörleri ise 250 gün boyunca işletilmiştir. Elde edilen sonuçlara göre diklofenak'ın biyokütle ve KOİ giderimi üzerinde az oranda kronik inhibisyon etkisinin olduğu görülmüştür. Çamur yaşının artmasıyla birlikte, diklofenak'ın biyokütle ve KOİ giderim verimi üzerinde etkisi artmıştır.

20 gün çamur yaşındaki diklofenak reaktöründen haftalık alınan diklofenak numunelerinin ölçümü sonucunda, diklofenak giderim veriminin %22.5-34.3 mertebelerinde olduğu tespit edilmiştir. Bu durum aerobik koşullarda çamur yaşının diklofenak giderimi üzerinde etkili olduğunu göstermektedir.

Diklofenak'ın işletilen reaktörlerin çamurlarındaki birikimini incelemek amacıyla 10 gün ve 20 gün çamur yaşında işletilen diklofenak reaktörlerinden haftalık çamur numuneleri alınmıştır. Diklofenak'ın her iki çamur yaşında işletilen reaktörlerin çamur numunelerinde de yaklaşık %2 oranında adsorblandığı tespit edilmiştir.

Diklofenak'ın çamurda tutunmadığı, daha çok çözünmüş formda olduğu gözlemlenmiştir.

Diklofenak'ın farklı çamur yaşlarındaki diklofenak'a aklime olmuş ve olmamış biyokütle üzerindeki potansiyel akut etkisini gözlemleyebilmek içinse respirometrik çalışmalar yürütülmüştür. Diklofenak maddesinin akut inhibisyonunu belirlemek için respirometre cihazında oksijen tüketim hızı (OTH) ölçülmüştür. Deneyler esnasında nitrifikasyon inhibitörü eklenerek nitrifikasyon aktivitesi engellenmiştir.

İlk olarak 10 gün çamur yaşındaki kontrol reaktöründen alınan biyokütleye 0, 200, 1000 ve 10,000 μg/L konsantrasyonlarında diklofenak eklenmiş ve diklofenak'ın akut etkisi incelenmiştir. Sonuçlarda 200 μg/L diklofenak konsantrasyonunun bir etkisi gözlenmezken, 1000 ve 10,000 μg/Ldiklofenak konsantrasyonlarında OTH profillerinin değiştiği görülmektedir. 10 gün çamur yaşında diklofenak'la beslenen reaktörden alınan biyokütleye ise 0, 10 ve 1000 μg/L konsantrasyonlarında diklofenak eklenmiştir. 0 ile 10 μg/L ve 1000 μg/L Diklofenak ilavesi yapılan deney setlerinin OTH profilleri karşılaştırıldığında diklofenak'ın akut inhibisyon etkisinin çok az olduğu ve biyokütlenin hidroliz hızını biraz düşürdüğü görülmektedir. Buna ilave olarak diklofenak reaktörü ve kontrol reaktörü ile yapılan respirometre sonuçları karşılaştırılmış ve diklofenak'ın uzun süreli maruziyet sonrasında biyokütle üzerinde büyüme hızı, hidroliz, depolama gibi metabolik özelliklerine önemli bir etkisinin olduğunu gözlemlenmiştir.

Diklofenak'ın 20 gün çamur yaşındaki biyokütleye akut inhibisyon etkisini gözlemlemek içinse hem kontrol hem de diklofenak reaktörlerinden alınan biyokütleyle respirometrik deneyler yürütülmüştür. 20 gün çamur yaşında işletilen kontrol reaktörüne ise 0, 200, 1000 ve 10,000 μg/L konsantrasyonlarında diklofenak eklenmiştir. 0, 200 ve 1000 μg/L diklofenak ilaveli respirometre setlerinin OUR profilleri birbirine yakınken, 10,000 μg/L konsantrasyonunda işletilen respirometre reaktörünün OTH profili diğerlerinden farklı çıkmış ve diklofenak'ın akut inhibisyon etkisi belirgin şekilde görülmüştür. 20 gün çamur yaşına sahip diklofenak reaktöründen alınan biyokütle ise respirometre deneyi sırasında 0, 10, 1000 ve 10,000 μg/L diklofenak konsantrasyonuna maruz bırakılmıştır. Kontrol reaktöründen elde edilen sonuçlara benzer şekilde, 10,000 μg/L diklofenak konsantrasyonu eklenen sette akut inhibisyonun etkisi belirginleşmiştir. 10 gün çamur yaşı çalışmalarına benzer olarak diklofenak reaktörü ve kontrol reaktörü ile yapılan respirometre sonuçları karşılaştırılmış ve yine aynı şekilde diklofenak'ın uzun süreli maruziyet sonrasında biyokütle üzerinde büyüme hızı, hidroliz, depolama gibi metabolik özelliklerine önemli bir etkisinin olduğunu gözlemlenmiştir.

Bunun yanı sıra 20 gün çamur yaşında işletilen kontrol ve diklofenak reaktörlerinden alınan biyokütleye yalnızca metanol ilavesiyle işletilen respirometre deneyinden elde edilen OTH profilleri modellenmiştir. Modelleme sonuçlarına göre biyokütle aktivitesinin her iki deneyde de %51 olduğu ve her iki biyokütlenin hidroliz hızlarında değişim olmadığı; heterotrofik yarı doygunluk katsayısı olan K_{S1} değerininse diklofenak'a aklime olan biyokütlede, kontrol reaktöründen alınan biyokütleye göre daha yüksek olduğu görülmüştür. Bu durum diklofenak'ın uzun süreli maruziyet sonrasında biyokütle üzerinde inhibisyon etkisinin olduğunu göstermektedir.

1. INTRODUCTION

Pharmaceuticals are chemicals consumed by human and animals for medical purpose. Extensive usage of pharmaceuticals has become a significant environmental problem because of their income to environment, their activeness or toxicity to biological systems (Mehinto, 2009). Pharmaceuticals can enter through environment with different ways such as inappropriate disposal to sewer line or discharging wastewater of pharmaceutical manufacturers (Boxall, 2004). Consumption of anti-inflammatories, which are one of the most commonly used pharmaceuticals, increased in few years and reached to several tons of weight (Ziylan and İnce, 2011).

Diclofenac is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAID) worldwide with annually 940 tons global consumption. Because of its high consumption rates and strength to biodegradability, it is the most commonly detected anti-inflammatory drug in influent and effluent of wastewater treatment plants (WWTPs) and even in surface waters (Heberer, 2002). The occurrence of diclofenac in influent, effluent of WWTPs and surface waters are 96%, 86% and 93%, respectively (Gros et al., 2010). Diclofenac concentrations in WWTPs and surface waters are changing between ng/L and μg/L with region and season according to their consumption rates. In Turkey, diclofenac concentration in surface waters is changing between 1.2-45.7 μg/L (Aydin et al., 2013).

Diclofenac is the most toxic NSAID (Mehinto, 2009). Because of its usage as veterinary pharmaceutical, millions of vultures which had eaten treated animals with diclofenac killed in Asia (EEA, 2010). Moreover, with lowest observed effect concentration (LOEC) often below 100 mg/l, diclofenac has been stated as the highest risks to aquatic species (Fent et al., 2006). The researches have also stated that the kidney is the main target to the damages of diclofenac in the other animals such as mice (Hickey et al., 2001), birds and humans (Oaks et al., 2004; Ng et al., 2006).

When removal of diclofenac in WWTPs is examined, it is faced with a wide variety of results. It is known that microbial diversity in the system, experimental design and environmental conditions are quiet important for diclofenac removal beside operational conditions like sludge age and hydraulic retention time (Suarez et al., 2010).

When biodegradability of diclofenac under aerobic conditions is investigated, there are remarkably various results about biological treatability of diclofenac in the literature. Some studies are classifying diclofenac as resistant to biodegradation (Joss et al., 2006; Quintana et al., 2005; Lahti and Oikari, 2011) when some studies states that up to 93% of diclofenac removal could be observed (Gröning et al., 2007; Kosjek et al., 2008; Yu et al., 2006).

The aim of this study is to investigate the acute and chronic inhibition effect of sludge age on biodegradability of diclofenac under aerobic conditions. After steady state conditions were achieved in the reactors, diclofenac removal in reactors was examined and removal efficiency was calculated. The biomass obtained from the reactors was used to perform respirometric tests to investigate acute and chronic inhibition effect of diclofenac on acclimated and non-acclimated biomass.

Study related to the aerobic treatment of diclofenac is very limited in the literature. And there is no study about respirometric evaluation of diclofenac. Result of this research sheds light on elimination potential of diclofenac under aerobic conditions and acute and chronic inhibition effect of diclofenac on biomass.

2. LITERATURE REVIEW

2.1 Pharmaceuticals

Pharmaceuticals are a group of chemical compounds consumed as human and animal medicines or vaccines. Pharmaceuticals can be classified in various groups besides their properties like mode of action, pharmaceutical activity, chemical properties, route of administration and therapeutic effectiveness. According to their therapeutic effectiveness, they can be classified as antipyretics, antibiotics, analgezycs, anti-inflammatories, and hormones.

Approximately 100,000 different pharmaceuticals are produced each year worldwide but the data about their consumption is scarce (Kümmerer, 2009). Pharmaceuticals can be obtained with and without prescription. Annual consumption of each pharmaceutical is variable among countries and changing from year to year. Most frequently used pharmaceutical consumption in England, Germany and Australia is recorded as hundreds of tons per year (Fent et al., 2006). In Germany, 836 tons acetylsalicylic acid, 622 tons paracetamol, 345 tons ibuprofen, 86 tons diclofenac which are non-steroidal anti-inflammatory drugs are sold in 2001 (Huchek et al., 2004).

Production and consumption of pharmaceuticals is increasing each year due to expansion of population and increasing income. According to World Health Organization’s (WHO) report (2004), production of medicines has grown four times faster than world’s income from 1985 to 1999 and they are mostly produced in the industrialized countries. For example, pharmaceutical consumption in Spain increased 264% from 1985 to 2000 (Bastida and Mossialos, 2000). Opioid analgesic consumption increased 47% in Israel between 2000 and 2008 (Ponizovsky et al., 2011). Studies for the 13-year period indicate that medicine consumption in Iran increased 28.38% annually (Kebriaeezadeh et al., 2013).

Calculations show that consumption of pharmaceuticals will increase demographically in future years (van der Aa et al., 2011). Amount of medicine that annually sold in Turkey is given in Figure 2.1.

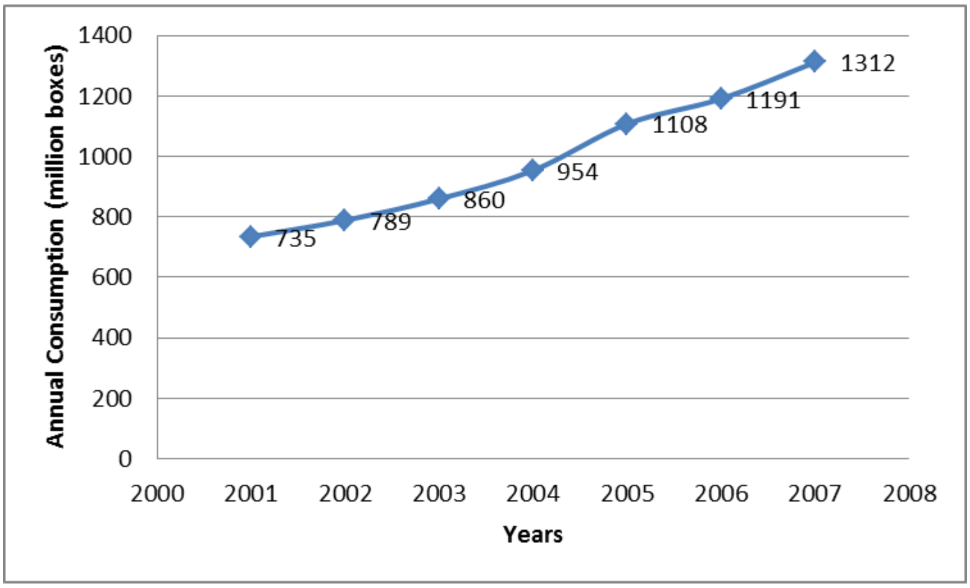


Figure 2.1 : Annual medicine consumption in Turkey.

Figure was drawn according to medicine which having prescription (Turkish Union of Chambers and Commodity Exchanges, 2008). Medicine consumption increases with a 44% ratio from 2001 to 2007. Medicines consumed without prescription also forms a large amount.

2.1.1 Pharmaceuticals in the environment

About 100,000 chemicals are used by modern industrialized society and more than 3000 of these chemicals are pharmaceuticals (Jorgensen and Sorensen, 2000). Extensive usage of pharmaceuticals as human and veterinary medicines have become a significant environmental problem because of their activeness or toxicity to biological systems (Mehinto, 2009). According to Europan Environment Agency (2010), it becomes easier to understand better the potential eco-toxicity of many pharmaceuticals and mixtures of medicines that enter the environment with different ways, albeit in very small quantities.

Pharmaceuticals can enter to environment with several ways as shown in Figure 2.2. Entrance of pharmaceuticals to aquatic environment occurs with discharge of faeces and urine and directly discharge of expired drugs to sewer line. Because of their properties, pharmaceuticals are biologically active and remain unchanged during their passage through the human body. Unfortunately, a considerable amount of unused pharmaceutical products is discharged directly to sewer line (EEA, 2010). According to Kuspis and Krenzelok (1996), 35.4% of people flushed expired medicines down to toilet. Residuals of released medicines during manufacturing process and usage of antibacterials for the treatment of fish or shrimp are another reasons of contamination of pharmaceuticals to water bodies. Excrete of pasture animals that used veterinary medicines interfere to soil and water bodies. And also usage of these excrete as slurry causes soil contamination (Boxall, 2004). 95% of pharmaceuticals are not biodegradable. 15% of human pharmaceuticals are persistent in water and 50% remains in sediment. 50% of veterinary medicines are persistent in soil. (EEA, 2010).

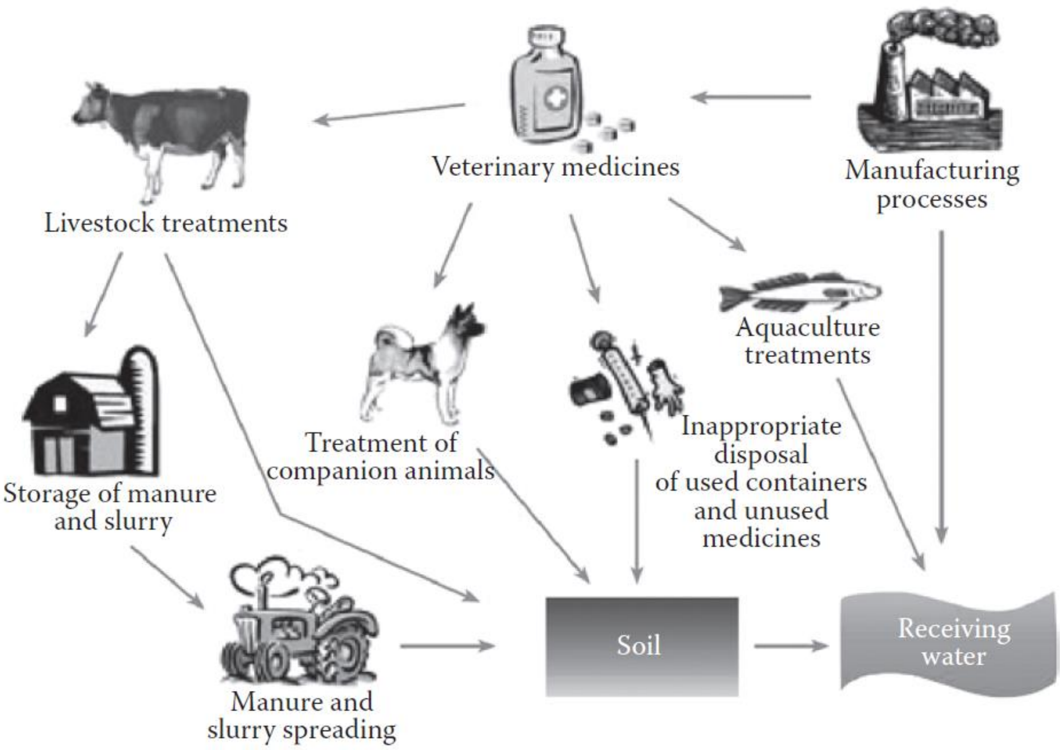


Figure 2.2 : Interference of pharmaceuticals to environment (Boxall, 2004, p.1111).

Richardson and Bowron (1985) have disclosed the importances of environmental impacts of pharmaceuticals firstly and number of studies are increased at the end of 1990s (Ternes 1998; Halling-Sorensen et al., 1998; Stumpf et al., 1999). Existing studies detected levels of hormones, steroids, antibiotics and parasiticides in soils, surface waters and groundwaters were very low, generally less then 1 μg/L. But there is no information about the mixture of veterinary and human medicines exist in soils and surface waters has an impact on beneficial organisms in the environment and on human health (Boxall, 2004).

2.2 Anti-Inflammatory Drugs in the Environment

Anti-inflammatory agents are medicines, which have effect on the reduction of edema, erythema, and resulting tissue damage associated with inflammatory conditions (Nickander et al., 1979). There are different types of anti-inflammatory drugs like phenylbutazone (as pyrazolones), indomethacin, sulindac, diclofenac, (as acetic acid derivatives), piroxicam (as oxicans), ibuprofen, naproxen, ketoprofen, fenoprofen (as propionic acid derivatives), mefenamic acid and tolfenamic acid (as fenemates) (Ziylan and İnce, 2011). Annual consumption of anti-inflammatory drugs increased in few years and reached to several tons of weight (Table 2.1).

Table 2.1 : Anti-inflammatory consumption.

Annual Consumption (tonnes/year)				**Country**	**Reference**
Ibuprofen	**Naproxen**	**Diclofenac**	**Ketoprofen**		
105	-	75	-	Germany, 1995	Ternes, 1998
344.9	-	85.8	-	Germany, 2001	Huschek et al., 2004
58.4	-	22.6	-	France, 2002-08	Laak et al., 2010
240	37.3	9.9	21.7	France, 2004	Besse et al., 2008
18	-	3.9	0.25	Switzerland, 2002	Tauxe-Wuersch, 2005
25	1.7	4.5	-	Switzerland, 2004	Fent et al., 2006
14.2	22.8	4.4	4.5	Austria, 1998	Tambosi et al., 2010
0.03	-	-	-	Denmark, 1997	Tambosi et al., 2010
162.2	35.1	26.1	-	England, 2000	Tambosi et al., 2010
1.9	-	-	-	Italy, 2001	Tambosi et al., 2010
276.1	42.6	32.3	-	Spain, 2003	Carballa et al., 2008

Concentrations of pharmaceuticals in water sources increase all over the world and become an important pollution source. Recent studies show that one of the most important pharmaceutical detected is non-steroidal anti-inflammatories (Diclofenac, ibuprofen, naproxen, ketolorac) (Gagné et al., 2006; Bormann et al., 2006; Zuccato et al., 2006; Khetan and Collins, 2007) and these chemicals are classified as pollutants that need to create an emergency action plan (Zuccato et al., 2006; Kim et al., 2007). Occurrence of anti-inflammatories in surface water, wastewater treatment plant (WWTP) influent and effluent are given in Table 2.2.

Table 2.2 : Country-wise occurrence of anti-inflammatories in surface water and influents/effluents of WWTPs (Ziylan and İnce, 2011, p. 28).

Drug	Surface Water (µg/L)	WWTP Influent (µg/L)	WWTP Effluent (µg/L)	Country
Diclofenac	0.020-0.150	1.4	0.95	Switzerlad
	n.d.-1.03	3.02	2.51	Germany
	-	0.105-4.11	0.035-1.95	France
	-	2.59	1.97	South Korea
	-	0.53-12.4	0.34	Spain[a]
	0.006	0.018	n.d.	China[b]
	0.02	3.1	1.5	Austria
Ibuprofen	0.010-0.400	-	0.100-1.5	Switzerland
	-	-	0.061-0.115	Romania
	-	12.4-14.6	n.d.	Spain[a]
	0.056	0.061	0.113	China[b]
NPX	0.022-0.107	-	-	USA
	0.010-0.400	-	0.100-3.5	Switzerland
	n.d.	0.732	0.261	Germany
	-	1.55	0.17	Spain[a]
	0.002	0.006	0.012	China[b]

(a)Gracia-Lor et al., 2011
(b)Huang et al., 2012

High consumption of pharmaceuticals and uncontrolled discharge to the aquatic environment is dangerous not only for organisms in the receiving body, but also for drinking water sources (Gagne et al., 2006). Pharmaceuticals are observed in wastewater and receiving environment very frequently due to their nonbiodegradable character and solubility. Their concentrations in different places varies with amount of production, releasing through urine and faeces, socioeconomic level, prices of pharmaceuticals, seasons and other demographic data (Santos et al., 2005; Sponberg and Witter, 2008; Daneshvar, 2010). Occurrence of anti-inflammatories in wastewater treatment plants and surface water is shown in Table 2.2. As given in Table, influent and effluent concentrations are varying among countries.

Concentrations of anti-inflammatories are in ng/L for surface waters but it is changing between ng/L to μg/L for WWTPs.

2.3 Diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) and functions as pain reliever. It is one of the most used pharmaceutical worldwide. Diclofenac is primarily used for the treatment of pain, tenderness, swelling and stillness resulting from osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, menstrual cramps or other pains. Diclofenac can be found as gel or cream that is used to treat actinic keratosis, a skin condition that may become cancerous if not treated.

Diclofenac can be found under many brand names, such as Dicloflam, Cataflam, Aclonac, Voltaren, Voltarol, Diclon, Zipsor, Flector patch, Cambia, Oflam, etc. 70% of the worldwide diclofenac sales in 2007 was for oral application (Zhang et al., 2008). Annual consumption of diclofenac and dose per capita worldwide are estimated as 940 tons and 618 mg, respectively (Zhang et al., 2008).

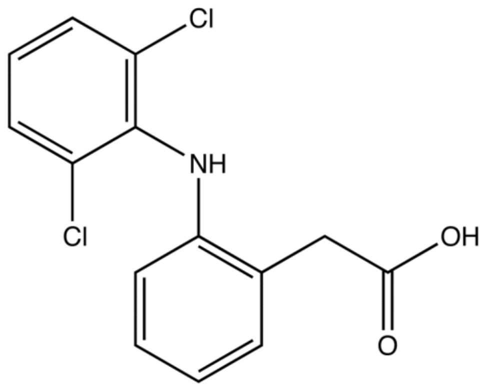
Increase in consumption of diclofenac causes several environmental problems. Because of its low removal rate, diclofenac is one of the most commonly detected anti-inflammatory in groundwater and surface water (Buser et al., 1998; Stumpf et al., 1999; Zhang et al., 2008; Heberer, 2002; Lin and Gan, 2011). Concentration of diclofenac in wastewater treatment plant effluents is ranging from ng/L to μg/L (Zorita et al., 2009; Henschel et al., 2003; Metcalfe et al., 2003).

2.3.1 General properties

Diclofenac, derives from its chemical name "2-(2,6-dichloranilino) phenylacetic acid" is a synthetic non-steroidal anti-inflammatory drug (Figure 2.3). It is a polar and relatively persistent molecule because of chloride in its structure (Bartels and Tümpling, 2007). Physico-chemical properties of diclofenac are given in Table 2.3.

Henry's law constant indicates the volatilization ability of compound. Henry's law constant is relatively low (< 3E-3) for diclofenac to consider removal of diclofenac with vaporization as a potential removal alternative.

K_{OW}, the octanol-water partition coefficient, indicates the sorption potential of the organic compounds in terms of hydrophobicity or hydrophility and generally used as



(a)

(b)

Figure 2.3 : (a) Chemical structure and (b) 3 dimensional structure of diclofenac.

Table 2.3 : Physico-chemical properties of diclofenac.

Properties	
CAS Number [1]	015307-86-5
Molecular formula [2]	$C_{14}H_{11}Cl_2NO_2$
Molecular weight (g/mol) [1]	296.16
Solubility in water (mg/L) (25ºC) [2]	2.37
pKa dissociation constant [2]	4.15
Vapor pressure (mmHg) [2]	6.14E-8
$logK_{ow}$ [2]	1.9 – 4.5 (pH dependent)
Henry's constant (atm.m3/mole at 25ºC) [2]	4.73E-12
K_{bio} (L/SS.d)	<0.1
EC_{50} Daphnia (mg/L) [2]	22-68
EC_{50} Algae (mg/L) [2]	72

1) Lindqvist et al., 2005
2) Ziylan and İnce, 2011

an indicator of the tendency of an organic compound to adsorb to soil. Organic contaminants having $logK_{OW}$ value higher than 4.5 (stong hydrophobic character) were removed approximately 85%, when $logK_{OW}$ value lower than 3 (hydrophilic character) were poorly adsorbed to particles (Sui et al., 2010). Diclofenac can remain both in aqeous and solid phase because of its pH-dependent $logK_{OW}$ value (Nieto et al., 2007; Matsuo et al., 2011). Also low pK_a value of diclofenac indicates its hydrophilicity (Ziylan and İnce, 2011).

Approximately 65% of consumed diclofenac is excreted through urine when the residue is defecated. Six metabolites of diclofenac have been identified in urine, however metabolites in faeces is not clear (Zhang et al., 2008). Diclofenac and its metabolites in urine is shown in Figure 2.4.

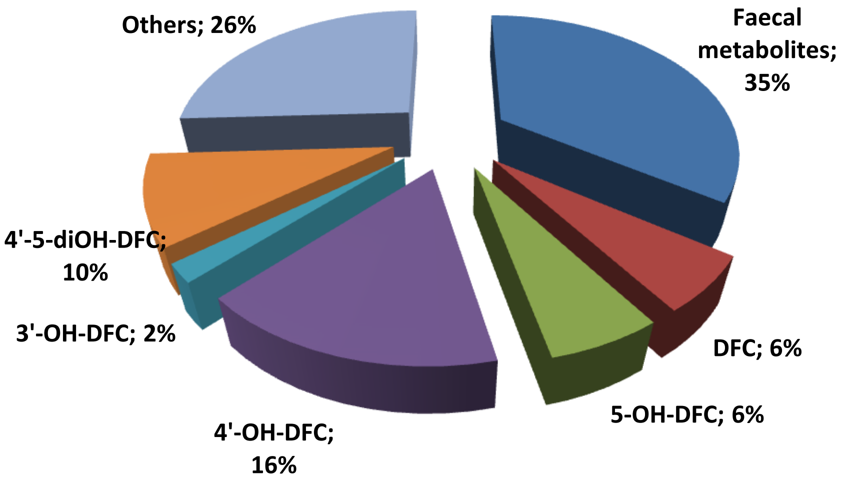


Figure 2.4 : Metabolites of diclofenac in faeces and urine (Zhang et al., 2008, p. 1153).

2.3.2 Diclofenac in the environment

Because of its high consumption rate and low biodegradability, diclofenac can be found in of wastewater treatment plants' effluent, surface waters, groundwater and even in drinking water (Ternes, 1998; Heberer et al., 2002; Rabiet et al., 2006; Comeau et al., 2008; Siemens et al., 2008; Wu et al., 2009; Lin and Gan, 2011). Diclofenac is the most commonly detected anti-inflammatory drug in effluent of wastewater treatment plants and high amount of diclofenac is releasing to the environment from wastewater treatment plants (Hilton and Thomas, 2003; Quintana et al., 2005). A study in Spain investigated the occurrence of diclofenac in influent, effluent of wastewater treatment plants and receiving bodies and detected as 92-96%, 86-100% and 93%, respectively (Gros et al., 2010; Jelic et al., 2011).

Occurence of diclofenac in drinking water have been reported in Germany for the first time and it was detected in groundwater and surface waters as 89 ng/L and 6 ng/L, respectively. (Heberer 2002). Daneshvar et al. (2010) observed diclofenac with 36 samples for surface waters and concentration was changing beween 25-170 ng/L. Diclofenac concentration in a lake in Switzerland was found as 1-7 ng/L (Buser et al., 1998). In Germany, 17 different wells that are used as drinking water supplies are investigated and diclofenac was observed with concentration up to 380 ng/L (Heberer et al., 1997). Diclofenac concentration is found up to 1-2 μg/L in rivers when directly discharge applies (Ternes et al., 1998). Concentrations of diclofenac in surface waters for different countries are given in Table 2.4. According Aydin et al. (2013), diclofenac concentration measured in the surface waters is quite low in Turkey compared to other contries. Consumption rates of diclofenac is one of the most important reason of this variance.

Table 2.4 : Diclofenac concentrations in surface waters for different countries.

Concentration [ng/L]	Region	Reference
1.2-45.7	Turkey	Aydın et al., 2013
4400	Pakistan	Scheurell et al., 2009
1030	Germany	Heberer et al., 2002
195	UK	Thomas and Hilton, 2004
74	Spain	Matamoros et al., 2013
28-32	Mexico	Felix Conedo et al., 2013
16	Korea	Choi et al., 2009

Diclofenac concentration was investigated in rivers, groundwater and wastewater treatment plant effluents by Maskaoui and Zhou (2010) and 37% of total diclofenac concentration was founded onto colloidal particles when the rest is soluble.

Vazquez-Roig et al. (2011) investigated the fate of pharmaceuticals in Mediterranean wetlands and diclofenac was found in all the examined surface water sources but it could not be seen due to photodegradation and decomposition to its metabolites.

2.3.3 Toxicity

Among anti-inflammatory drugs, diclofenac can be accepted as one of the most toxics. Detrimental effects of diclofenac occurs by harming renal and gastrointestinal tissue via vertebrate taxa. (Haap et al., 2008).

Because of accidental exposure, Asian vulture population is almost exposed to extinction. Near ten millions of vulture died due to use of diclofenac. After this event, it is seen that diclofenac results in acute renal failure and the vultures that are faced with this drug dies within few days. (Oaks et al., 2004). The birds firstly became depressed in the first 24 hours of exposure. After 36-48 hours, they succumbed. (Swan et al., 2006). The researches showed that, the population quantity of three species of vultures which had eaten animals treated with diclofenac disembarc more than 97%. (EEA, 2010).

According to Mehinto (2009), the studies that made related diclofenac useage has presented that diclofenac was the most toxic NSAID. On the other hand toxical effects in bacteria only appears when the concentration level is at least 1000 times higher than present in the environment.

Diclofenac's ecotoxicity is slightly low and its severe affect is imperceptible at the concentraion levels that appear in the environment. Acute endpoints of diclofenac range between 47-67 mg/L, which can be considered as very high value and this rate is higher than many other similar concentrations. (Quinn et al., 2011). On the other hand, mixture of diclofenac with other drugs in the water increase its toxic effects. (Cleuvers, 2004).

In the aquatic organisms, long term toxic damage of diclofenac are tested; and it is seen that lowest observed effect concentarion (LOEC) was observed as 1 to 5 μg/L for rainbow trout and no observed effect concentration (NOEC) was observed as 0.5 μg/L for brown trout (Hoeger et al., 2005; Schwaiger et al., 2004). Besides, distortion in liver, kidney and gills was detected as 1 μg /L in rainbow trout. In the 30 days toxicity test to embryos and larvae of Cyprus carpio, NOEC and LOEC were detected as 15 μg/L and 30 μg/L, respectively. (Islas-Flores et al. 2013).

Perpetual exposure of diclofenac causes deterioriation of fishes involving renal lesions and falsification of the gills. (Schwaiger et al. 2004). Letdown of hatching trend success and deferment in hatching were seen when fishes are exposed to 0.001 – 10 mg/L diclofenac for three months. (Lee et al., 2011).

2.3.4 Occurrence of diclofenac in wastewater treatment plants

Since chronic exposure of diclofenac may lead to negative effects to aquatic and terrestrial ecosystems, it is a serious concern to treat diclofenac (Martin et al., 2012).

Since hospitals, veterinary clinics, houses, and pharmatical manufacturing facilities discharge their wastewater including metabolites or unchanged diclofenac to sewer line, municipal wastewater can be accepted as a remarkable diclofenac source and danger to aquatic and terrestrial environment.

Occurrence and removal of diclofenac are variable among wastewater treatment plants. The occurrence of diclofenac in the influent and effluent of wastewater treatment plants and their removal efficiency is illustrated in Table 2.5.

Table 2.5 : Influent and effluent diclofenac concentration in WWTPs.

Influent [ng/L]	Effluent [ng/L]	Removal Efficiency (%)	References
188	147	21	Plosz et al., 2012
150	120	20	Xue et al., 2010
720	530	26	Martin et al., 2012
2590	1970	23.9	Han et al., 2006
283	175	37	Sui et al., 2011
110	90	18	Yu et al., 2006
730	120	84	Fatta-Kassinos et al., 2011
981	78	92	Zhou et al., 2009
3020	2510	17	Heberer et al., 2002
1500	900	40	Gomez et al., 2007

2.3.5 Removal of diclofenac

Generally, in the municipal wastewater treatment plants activated sludge processes are used. Diclofenac elimination in urban wastewater treatment by activated sludge process is generally pendant according to their treatment method and sludge retention time (SRT) (Vieno et al., 2007). Their treatment efficiencies range between 9% and 82% as shown in Table 2.6.

Operational parameters such as temperature, redox conditions, solids retention time and hydrolic retention time and climate related factors such as temperature, sunlight intensity effect the removal efficiencies of diclofenac in wastewater treatment plants. (Castiglioni et al., 2006; Le-Minh et al., 2010).

When diclofenac reaches the aquatic environment, various reactions, transformation, or translocation processes occur to reduce concentration of diclofenac in the water. Diclofenac is removed through biodegradation, bioaccumulation, biomagnification, chemical degradation, photodegradation, adsorption onto particles and sedimentation, infiltration in sediment and sorption, precipitation or volatilisation (Jiskra, 2008). Biodegradation, sorption, air stripping and phototransformation are the four mechanism of activated sludge processes in which elimination of pharmaceuticals and its metabolities occurs. (Zhang et al., 2008).

Table 2.6 : Removal efficiency of diclofenac in WWTPs process.

Treatment Method	SRT (days)	Removal Efficiency (%)	Reference
CAS	1.5	50	Santos et al., 2009
CAS	2	7.1	Clara et al., 2005
CAS	3	50	Radjenovic et al., 2007
CAS	5	50	Santos et al., 2007
CAS	20	13	Vieno et al., 2005
CAS	48	14	Clara et al., 2005
CAS	52	60	Clara et al., 2004
BNR	9.6	18	Yu et al., 2006
BNR	13-17	21	Sui et al., 2011
BNR	-	21	Sim et al., 2011
MBR	15	51	Kimura et al., 2007
MBR	37	23	Quintana et al., 2005
MBR	65	82	Kreuzinger et al., 2004

CAS: Conventional activated sludge process; BNR: Biological Nutrient Removal, MBR: Membrane Bioreactor

Because of its low Henry's coefficient, the removal of diclofenac through air stripping is limited. (Poseidon, 2006) (Table 2.3). Biodegradation and sorption onto sludge are two main processes for pharmaceutical compounds' removal. In the other words, through biotransformation and sorption to primary and secondary sludges, diclofenac can be transformed from the aqueous phase to sludge. (Le-Minh et al., 2010).

With a smaller than 0.1 L/kg SS.day first order degradation rate constant, diclofenac can be considered as a poor biodegradable compound (Joss et al., 2006). Lee et al. (2011) studied the biodegradability of diclofenac and observed that diclofenac concentration did not change during 28 days.

Yu et al. (2006) applied biodegradation test similar to Lee et al. (2011) and observed 30% of removal efficiency within 50 days. Some researchers that used other biological reactors reported 5% biodegradation rates (Zwiener and Frimmel, 2003). Diclofenac biodegradation was examined for 28 days and observed that, when diclofenac was the sole source of carbon or dispersed in milk powder, there was no transformation (Quintana et al. 2005).

K_{OW} value indicates the hydrophobic or hydrophilic character of the compound which shows the compound's tendency to remain on aqueous phase or to sorp onto sludge. According to Sui et al. (2010), organic contaminants of hydrophilic compounds (log K_{ow} <3.5) were defectively removed (<20%). It is more efficient to remove the pharmaceuticals with low K_{ow} and low pK_a values from the wastewater compare to sludge. (Martin et al, 2012). Nevertheless, some researchs have shown that diclofenac's biodegradability is partial in activated sludge systems. (Joss et al., 2006; Quintana et al., 2005)

In literature, it is generally stated that high sludge retention times (SRT) increases the efficiency of diclofenac's removal in the activated sludge systems which encourages the dominance of slow growing bacteria and microbial diversity. However, some researches in the various wastewater treatments have shown that, with 3 – 30 days ranging SRT, diclofenac removal is not dependent on SRT (Lishman et al., 2005). Besides, any correlation between diclofenac removal and SRT (up to 300 days) has not been found. (Kreuzinger et al. 2004). Beside to SRT, different operation conditions effect removal of diclofenac such as temperature, redox conditions, hydroulic retention time (Suarez et al., 2010).

2.3.6 Occurrence of diclofenac in sludge

Since pharmaceuticals can accumulate in sludge occurrence of pharmaceuticals in sludge should be examined. Because of its high K_{ow} value, diclofenac can accumulate onto biomass in wastewater treatment plants. But there is limited number of studies about diclofenac concentrations in sludge.

Waste sludge includes important amount of pharmaceuticals which can enter food chain and plants and animals can be exposed to them and can infiltrate into groundwater (Wild et al., 1994; Kreuzinger et al., 2004). Pharmaceutical concentration in sludges is an important parameter to identify its disposal and usage in agricultural application (Saleh et al., 2011).

Diclofenac was investigated in rivers, groundwater and wastewater treatment plant effluents by Maskaoui and Zhou (2010) and 37% of total diclofenac concentration was found in colloidal particles when the rest was soluble. This shows that diclofenac can be adsorbed onto solid phase. In Catalonia, Spain, 3 different anaerobic digestor sludges are investigated and it is found that diclofenac concentration in sludges is varied between 0.4-1 μg/L (Jelic et al., 2011). Another study conducted on biosolids detected diclofenac concentration as 0.18 mg/kg (Nieto et al., 2007). Diclofenac occurrence in waste sludges in Japan was found as 6-29 mg/kg (Matsuo et al., 2011). According to Plosz et al. (2012), diclofenac concentration in solid phase was found as 16%. In contradiction to these studies, Sari (2013) has found that there is no diclofenac accumulation in a waste activated sludge in Istanbul, Turkey.

2.4 Biological Treatability of Diclofenac

2.4.1 Aerobic treatability of diclofenac

In aerobic treatment of wastewater, organic pollutants are digested to carbondioxide and water and other end products by microorganisms. Although their different structure and low biodegradabilities, pharmaceuticals can be treated by aerobic treatment process beside physico-chemical treatment methods. In conventional activated sludge systems, efficiency of pharmaceutical removal depends on its physical and chemical properties like polarity, solubility in water (Ollers et al., 2001).

In the literature, there are remarkably various results about biological treatability of diclofenac under aerobic conditions. Some studies are classifying diclofenac as resistant to biodegradation (Joss et al., 2006; Quintana et al., 2005; Lahti and Oikari, 2011) when some studies states that up to 93% of diclofenac removal could be (Gröning et al., 2007; Kosjek et al., 2008; Yu et al., 2006). Source of microbial inoculum, experimental design and environmental factors are main reasons of this contrast (Lahti and Oikari, 2011). So diclofenac removal rate can vary between 0-90% in aerobic activated sludge systems of wastewater treatment plants (Joss et al., 2006).

Clara et al. (2005) worked on a lab-aerobic reactors to investigate pharmaceutical treatment efficiensies. 4 different sludge ages has been selected as 1, 5, 13 and 26 days. Diclofenac removal efficiency was observed below 20% in all reactors.

Suarez et al. (2010), studied biodegradability of 16 different pharmaceuticals (including diclofenac) with two lab-scale conventional activated sludge reactors. It is observed that biodegradability of diclofenac under aerobic conditions is 25%. It is observed that in adaptation period (corresponding to 170 days removal of diclofenac increased from 0% to 25%. At steady state, diclofenac removal reached to its maximum removal rate, around 74%. Biological transformation of diclofenac increased parallel to the increase of sludge concentration in the reactor. So, it has been suggested that microbial species growing in the reactor is much more effective than operating conditions like sludge age, temperature etc.

Zweiner and Frimmel (2003) examined biodegradability of diclofenac, clofibric acid and ibuprofen in short-term tests in a pilot sewage plant (including denitrification, carbon removal and sedimentation) and biofilm reactors (including oxic and anoxic parts). Experiments were conducted for 55 hours in pilot sewage plant and 48 hours in biofilm reactors. Diclofenac removal was <5% in pilot sewage plant and <4% in biofilm reactor.

Direct or cometabolic microbial transformation are the most important elimination processes especially for polar acidic pharmaceuticals. Microorganisms use pharmaceuticals to gain energy in direct metabolisation, when they are using more abundant and easily biodegradable compounds in cometabolic transformation (Lahti and Oikari, 2011). In the same study, they investigated contribution of organic readily degradable carbon to diclofenac removal. Two different experiments were carried out; in the first set diclofenac was sole carbon source and in the second setup sodium acetate was fed with diclofenac. Diclofenac removal was negligible in the first set whereas, sodium acetate addition increased the diclofenac removal up to 1-13% in the second set.

Quintana et al. (2005) operated two different aerobic systems to study the cometabolic effect on biotransformation of diclofenac. In the first setup, only 20 mg/L diclofenac was fed and 5 mg/L of diclofenac and 50 mg/L of powdered milk was fed to second set. No diclofenac removal observed in first set, but 60% of diclofenac removal occurred in second group as a proof of cometabolic degradation.

Similarly, biodegradability of diclofenac under aerobic and anoxic conditions is studied by Plosz et al. (2012). 130 mg/L COD was fed with diclofenac to a batch reactor. Diclofenac removal occurs only the presence of carbon source. Diclofenac removal occuring with cometabolic degradation.

2.4.2 Anoxic/Anaerobic treatability of diclofenac

Anaerobic treatment is generalley prefered for midium or high loaded wastewater types. Anaerobic treatment processes have various adventages compared to aerobic treatment processes like lower operation costs, less space requirements, less nutrient requirement and biogas production.

Anaerobic treatment microorganisms such as methanogenic archea can be adapted inhibitory effect of chemicals, such as pharmaceuticals (Fountoulakis et al., 2008). But because of strength of pharmaceuticals to biodegradation by anaerobic treatment microorganisms, anaerobic treatment methods can be unsufficient (Deegan et al., 2011). According to Drill et al. (2005), methanogenic archea are most sensitive microorganisms to inhibiton compared to orher anaerobic microorganisms and biogas production stops as a result of inhibition. Researches show that diclofenac causes a decrease on specific methane activity and production rate and it has proved with Anaerobic Digestion Model 1 (ADM1) (Fountoulakis et al., 2004).

There are limited number of studies about teratment of diclofenac under anoxic conditions. Generally anoxic treatment was applied together with anaerobic and/or aerobic treatment methods. Zwiener and Flimmel (2003) investigated that diclofenac removal in a anoxic-aerobic system is only 5%. According to Suarez et al. (2010) diclofenac is more strength under anoxic conditions compared to aerobic conditions.

2.5 Environmental Impact of Diclofenac

Pharmaceuticals can stay biologically active and interact with aquatic ecosystem. The researches had presented various and respectable data related toxic effects of pharmaceuticals. These studies can be used in the environmental risk assessments. (Henschel et al., 1997).

Due to frequent release of pharmaceuticals’s effect to aquatic populations, their development, reproduction, and survival ablities, it is important to examine their impact on the environment. As the mostly used pharmaceutical, NSAIDs are one of the most examined chemical in the environmental base. Diclofenac, as a NSAID, also examined by many researchers to understand its acute and chronic toxicity (Mehinto, 2009).

2.5.1 Acute toxicity

Toxicity tests have been played an important role in the environmental risk assessments. Invertebrates, bacteria, fish and algea are used for many standardised tests. (Crane et al.,2006) For instance, Farré et al. (2001) examined non-steroidal anti-inflammatory drugs acute toxicity on the luminescence of vibrio fischeri (bacteria) and stated that there was 50% reduction in bioluminescence (EC50= 30min) of 13.5 mg/l for diclofenac, besides, 19.2 mg/l for ibuprofen and ketoprofen and 35 mg/lfor naproxen. In another research it is also stated that Naproxen causes acute toxic effects in Ceriodahnia dubia (invertebrate species) with EC50 (24 hr) of 66.4 mg/l (Isidori et al., 2005).

Diclofenac, naproxen, ibuprofen and ketoprofen have been also examined by Jones et al. (2002) and Carlsson et al. (2006). According to results of these studies, diclofenac and ibuprofen have been found potantially dangeorus to the nature. Nevertheless, for naproxen and ketoprofen, there was not enough data to determine the risks for the environment.

Moreover, with lowest observed effect concentration often below 100 mg/l, diclofenac has been stated as the highest risks to aquatic species (Fent et al., 2006). The crustacean C. dubia, with LOEC (7 days) = 1 mg/1, was found as the most sensitive aquatic species to acute concentrations of diclofenac (Ferrari et al., 2003). For Lemnaminor (an aquatic plant), it was found that EC50 (7 days)= 7.5 mg/l by (Cleuvers, 2003). On the other hand, for rotifer Brachionus calyciflorus it was LOEC (48 hr) 25 mg/l (Ferrari et al., 2003).

Diclofenac’s acute effects on fish is barely konown. Rainbow trout has been examined for acute toxicity of diclofenac. And it is found that for cell toxicity EC50 (24 h) is 420 μM and for cytochrome P450 and enzymatic activity EC50 (24 hr) 63 μM (Laville et al. 2004). Medaka fish (Oryzias latipes) has been also examined in acute exposure. 1 μg/l and 8 mg/l diclofenac caused gene expression in the gills, liver, and intestine. Besides, significant increase of cyp1a, vitellogenin has been observed (Hong et al., 2007).

2.5.2 Chronic toxicity

In Pakistan and India, catastrophic decline of three vulture species because of toxicity of diclofenac has been stated in the former studies (Oaks et al., 2004). The vultures that eaten diclofenac-treated carrions; and eventualy, their metabolism failed to remove the drug. Even with LOEC= 0.007 mg/kg low concentration, diclofenac caused renal reverse in the vultures. Wherefrom, chronic toxicity of diclofenac in the environmental field has been searched.

According to Hallere et al. (2004), diclofenac has no effect in zebrafish's (Danio rerio) embryonic development but a slightly delay in hatching time (more than 1 and 2 mg/1 exposure).

In salmonid species, diclofenac's chronic effects have been observed. For instance, rainbow trout (Oncorhynchus mykiss) was tested 28 days by exposing diclofenac. Alterations in the morphology of the gills, renal lesions, bioconcentration of diclofenac in the liver, kidney, and muscless were reported. (Schwaiger et al., 2004). The liver of rainbow trout was the part with highest bioconcentration level. Also, in the liver, gills, and kidney, implicit subcellular harm has been observed. (Triebskorn et al., 2004).

Hoeger et al., (2005) reported that inflamnation of the gills and kidney of the brown trout (Salmo Trutta) was seen when 5 µg diclofenac/l was exposed for 21 days.

These studies has shown that diclofenac may cause renal functions in fish. The researches has also stated that the kidney is the main target to the damages of diclofenac in the other animals such as mice (Hickey et al., 2001), birds and humans (Oaks et al., 2004; Ng et al., 2006). Seitz and Boelsterli, (1998) and Hoeger et al., (2008) had examined toxicity of diclofenac in the small intestine.

2.6 Respirometric Evaluation of Pharmaceuticals

Oxygen utilization rate (OUR) is continuously monitored by respirometer throughout a controlled experiment in a batch reactor. OUR profile can be read for determination of stoichiometric and kinetic constants. OUR is known as a standard procedure for inhibition assesment. Respirometric analysis was used for evaluation of the inhibitory effect of heavy metals and verious compounds including pharmaceuticals (Pala-Özkök et al., 2011).

It is possible to investigate acute inhibition effect of a chemical on the microbial growth, storage of substrate and hydrolysis rate by using respirometric experiments instead of conventional methods. There is no study which is used respirometric methods to evaluate acute inhibition effect of diclofenac. In respirometric experiments which are carried out to investigate acute inhibition effect of xenobiotics, it is possible to observe kinetic and stoichiometric evalution of biomass.

In a study carried out on erythromycin antibiotic substance, it is observed that erythromycin has an acute inhibition effect on substrate utilization of biomass when erythromycin fed with peptone as substrate. Erythromycin have no effect on bacterial growth but it prevents storage of substrate and increases OUR rate of biomass (Pala-Ozkok et al., 2013a). Also in the respirometric experiments which are carried out with biomass acclimated to same antibiotic and having a lower sludge age, it has been found that the hydrolysis rate decreases. In another study which are run with antibiotic of sulfamethoxazole and peptone mixture and acetate as substrate, acute inhibition effect of sulfamethoxazole on biomass and effect of different substrate was investigated (Pala-Ozkok et al., 2013b). It has observed that sulfamethoxazole effects stoichiometry of biomass rather than kinetics when only peptone was fed to system. Also partial substrate utilization occured. When biomass fed with only acetate, acetate consumed completely but microbial growth kinetics was effected negatively. It has determined that sulfamethoxazole prevents storage of substrate on biomass and increases OUR rates.

In a study of Ubay-Cokgor et al. (2011), as relevant to acute inhibiton effect of acclimation to xenobiotics, it has observed that 2-6 dihydroxybenzoic acid, which is using in olive oil production to avoid decomposition of oils, has an acute inhibition effect on biomass when first exposure. When biomass acclimated to 2-6 dihydroxybenzoic acid, inhibition effect of chemical decreases. Olmez-Hanci et al., (2011) was studied on the H-acid (4-Amino-5-hydroxy-2,7-naphthalene disulfonic acid) and respirometric analyses was carried out. Experimental results showed that H-acid was not biodegradable even after prolonged acclimation periods. H-acid also has no inhibitory effect on biodegradation of synthetic sewage but accumulated as soluble inert COD in the treated wastewater. In another study which investigated the acute effect of a significant compound among polycyclic aromatic hydrocarbons, benzo[a]anthracene, it has observed that lower doses of benzo[a]anthracene had no effect on process kinetics and noticeable acute impact was only observed with the addition of 88 mg/L of benzo[a]anthracene (Başak et al., 2012).

2.7 Impact of Diclofenac on Nitrification and Nitrifiers Activity

Due to being the most sensitive microorganisms to inhibitory compounds, including pharmaceuticals, nitrifying bacteria are useful as biosensors to access toxicity contamination in wastewater treatment plants. The use of nitrifiers as biosensors against inhibitory substance enterance in aerobic environments is already well established in scientific literature, usually using nitrifying activated sludge. (Bressan et al., 2013)

There are different studies to assess the inhibition effect of diclofenac to nitrifier activity. Falas et al. (2012a) studied on the influence of SRT and nitrification capacity to removal of antiinflammatory substances. Diclofenac and clofibric acid showed no removal and for the nitrifying sludges, no dependency between the nitrification capacity and the pharmaceutical removal was observed. Similarly, Maeng et al., (2013) investigated the influences of solid retention time (SRT), nitrification, and microbial activity on the attenuation of pharmaceuticals and they observed that some acidic pharmaceuticals, like gemfibrozil, diclofenac, bezafibrate, and ketoprofen were partially degraded by nitrification. Same study illustrates that the removal of same pharmaceuticals was enhanced by ammonia oxydizing bacteria, suggesting the importance of nitrification in the degradation of pharmaceuticals.

When removal of diclofenac was assessed by batch experiments, with suspended biofilm carriers and activated sludge from several full-scale wastewater treatment plants, it was observed that biofilm carriers from full-scale nitrifying wastewater treatment plants demonstrated considerably higher removal rates per unit biomass of diclofenac compared to the sludges. (Falas et al., 2012b)

Nitrification activity in a small-scale reactors supplied with different concentrations of the pharmaceuticals including diclofenac were evaluated. It is observed that ammonia removal was not influenced by these pharmaceuticals and the removal efficiency was less than 60% for diclofenac. (Kraigher and Mandic-Mulec, 2011). In another study, biodegradation pharmaceuticals by enriched nitrifier cultures with ammonia oxidizing activity and it was obtained that the enriched nitrifier culture showed higher degradation of the target pharmaceuticals than the conventional activated sludge. The degradation efficiency of persistent pharmaceuticals such as diclofenac was increased with the increase in the ammonium concentration (Tran et al., 2009).

2.8 Activated Sludge Modelling No.1 (ASM1)

Different mathematical models have already been developed to identify the process kinetics of microorganisms in an activated sludge process during the aerobic degradation of both readily biodegradable and slowly biodegradable substrates. It is very important to understand the substrate removal mechanism for modelling of activated sludge due to explaining the process kinetics of substrate removal mechanisms in activated sludge processes. Improving the effluent quality of wastewater treatment plants are important to facilitate for design and operation of treatment plants.

Activated sludge model No. 1 (ASM1) has a pioneering role in activated sludge modelling. ASM1 involves COD fractionation for substrate and biomass components and assumes that the active bomass is converted to particulate residua products and slowly biodegradable substrate through death and lysis (Figure 2.5). ASM1 generally takes into consideration biomass, substrate and oxygen. Table 2.7 gives the part of ASM1 structure in the matrix format. (Orhon et al., 2009).

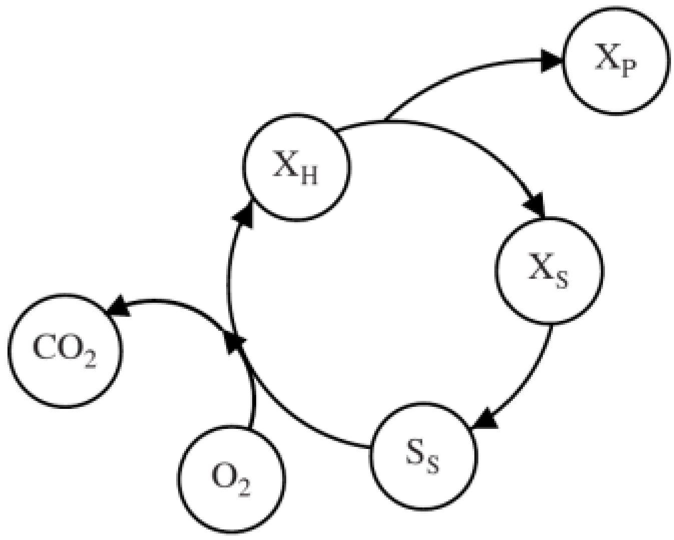


Figure 2.5 : Death regeneration process (Orhon and Artan, 1994, p.280).

Table 2.7 : Matrix presentation of ASM1 for organic carbon removal (Orhon et al., 2009, p.40).

Component→ / Process↓	S_S	X_S	X_H	X_P	S_0	Process Rate
Growth	$-\frac{1}{YH}$		1		$-\frac{(1-YH)}{YH}$	$\mu_{MAX}(\frac{Ss}{Ks+Ss})X_H$
Decay		$-f_{EX}$	-1	f_{EX}		$b_H X_H$
Hydrolysis	1	-1				$k_h(\frac{\frac{Xs}{XH}}{Kx+(\frac{Xs}{XH})})X_H$
Parameter	COD	COD	Cell COD	COD	O_2	-

As seen from the matrix representation of ASM1, model integrates two stoichiometric and six kinetic parameters to describe the utilization of wastewater by heterotrophic microorganisms. The biodegradable substrate in wastewater consists of two fractions: readily biodegradable (S_S) and slowly biodegradable (X_S) substrate. Readily biodegradable substrate is consist of simple molecules which are able to pass through the cell wall and immediately be used for microbial growth. Slowly biodegradable substrate (X_S), which consists of larger complex molecules, is adsorbed by biomass and firstly broken down by extracellular enzymes (hydrolysis) and then transferred through the cell wall and used for metabolism. The ASM1 can include three major processes: growth of heterotrophic biomass, endogenous decay and hydrolysis of slowly biodegradable organics.

The main stoichiometric parameters represented in ASM1 are the heterotrophic yield coefficient (Y_H) and fraction of particulate inert COD generated in biomass decay (f_{EX}).

3. MATERIALS AND METHODS

3.1 Seed Sludge

Laboratory scale fill and draw reactors were operated to examine the biodegradability of diclofenac under aerobic conditions. The biomass used for seed sludge was taken from an advanced biological wastewater treatment plant located in Istanbul, Turkey. Seed sludge for reactor setup was obtained from aeration tank of wastewater treatment plant.

Wastewater treatment plant has been constructed on an area of 507,000 m^2. Daily capacity of wastewater treatment plant is 500,000 m^3, which has a population equivalence of 2,500,000 people. Wastewater treatment plant that seed sludge is taken from is shown in Figure 3.1.

Figure 3.1 : Advanced biological wastewater treatment plant that seed sludge taken from.

Wastewater is treated in advanced level with the coarse and fine screens, grit chambers, biological treatment, secondary sedimentation, sand filtration, UV disinfection unit. Dewatering and drying steps are applied for sludge purification. Wastewater characterization of treatment plant is given in Table 3.1.

Table 3.1 : Influent and effluent characterization of wastewater treatment plant.

Parameters	Influent	Effluent
Suspended Solid (mg/L)	500	30-35
BOD (mg/L)	325	20-25
COD (mg/L)	600	125
TKN (mg/L)	70	10
TP (mg/L)	8	2-3

Seed sludge sample for reactor setup was taken from aeration tank. Characterization of seed sludge is given in Table 3.2. Diclofenac concentration of seed sludge was less than 5 ng/g.

Table 3.2 : Characterization of seed sludge.

pH	SS (mg/L)	VSS (mg/L)	Diclofenac (ng/g) *
6.92	7700±28	4950±42	< 5

*dry solid

3.2 Fill and Draw Reactors

Laboratory scale fill and draw reactors were operated to examine the effect of sludge age on biodegradability of diclofenac. Within this scope, two different sludge age were selected by considering literature review and real-scale systems. For each selected sludge age, control reactors were also operated.

14-liter graduated glass reactors were used to avoid adsorption of diclofenac with a working volume of 6 L. Reactors were packed with aluminum foil to prevent photodegradation of diclofenac as shown in Figure 3.2.

Air was supplied by installing airline from central aerial line of laboratory. Reactors were aerated by using airstones to obtain fine bubbles. Each reactor was aerated continually to ensure sufficient oxygen. It was aimed to keep the oxygen level of the reactors higher than 3 mg/L. Reactors were mixed with IKA RW 16 Basic mechanical mixers.

2 different sludge age were selected to compare the effect of sludge age on treatability of diclofenac and to observe the chronic effect of diclofenac. First set was operated with 10 days of sludge age (SRT) when second set was operated at 20 days

Figure 3.2 : Glass reactors which are covered with aluminum to prevent photodegradation of diclofenac.

of sludge age. Each set has 2 reactors including diclofenac reactor and control reactor. Diclofenac reactors were fed in daily basis with 10 μg/L of diclofenac solution (120 μL from 500 mg/L diclofenac stock solution each day) which dissolved in methanol. Same amount of methanol was added to control reactors in order to ensure same conditions with diclofenac reactors. First group reactors which has 10 days of sludge age were operated for 160 days. 20 days of sludge age reactors were operated for 250 days. Operation conditions of reactors are given in Table 3.3.

Table 3.3 : Operation conditions of lab-scale reactors.

Reactors	Sludge Age (Days)	Diclofenac Concentration (μg/L)	Operation Time (Days)
Diclofenac 10	10	0	160
Control 10	10	10	160
Diclofenac 20	20	0	250
Control 20	20	10	250

600 mL and 300 mL of sludge were wasted before daily feeding to obtain 10 and 20 days of sludge age, respectively. Volatile suspended solids concentrations of sludge age of 10 and 20 reactors were 2,000 mg/L and 3,000 mg/L, respectively. Food to microorganis (F/M) ratio of reactors were calculated as 0.3 and 0.2 mg COD/mg VSS for sludge age of 10 and 20 day reactors, respectively.

Reactors were operated with a working volume of 6 L. Reactors were kept in constant temperature room of laboratory at 20°C. All reactors were fed on daily basis with peptone solution, solution A, solution B and diclofenac or methanol. pH was controlled between 6-8.

Each reactor was fed with synthetic wastewater which prepared according to ISO 8192 standards. Synthetic wastewater was prepared with peptone solution, solution A and solution B. Peptone mixture was used as carbon source and Solution A and Solution B were added to systems to provide macro and micro nutrient requirement of the system, respectively. Organic and inorganic composition of synthetic wastewater is given in Table 3.4.

Table 3.4 : Synthetic wastewater solutions.

Solution	Stock (g/L)	Solution	Stock (g/L)	Solution	Stock (g/L)
Peptone Mixture		***Solution A***		***Solution B***	
Peptone	16	K_2HPO_4	320	$MgSO_4$•$7H_20$	15
Beef extract	11	KH_2PO_4	160	$FeSO_4$•$7H_20$	0.5
Urea	3			$ZnSO_4$•$7H_20$	0.5
NaCl	0.7			$MnSO_4$•$7H_20$	0.41
$CaCl_2$•$2H_20$	0.4			$CaCl_2$•$2H_20$	2.65
K_2HPO_4	2.8				
$MgSO_4$•$7H_20$	0.2				

Peptone mixture which has 30,000 mg/L of COD equivalent was kept under 4°C and consumed within a week to avoid deteroiration. Required amount of peptone was added to each reactor to provide 600 mg/L COD. Solution A and Solution B are added to systems to provide nutrient requirement of the system. 10 mL Solution A and Solution B were added for each 1000 mg COD. Feeding period was 24 hours for reactors. Diclofenac stock solution with a concentration of 500 mg/l which is dissolved into methanol was used as feeding solution for diclofenac reactors and kept under 4°C. 120 µL diclofenac solution was added to diclofenac reactors to keep diclofenac concentration in 10 µg/L. 120 µL methanol was added to control reactors to eliminate the effect of methanol for system.

Supernatant of the reactors were removed till 1.5 and 2 L level after biomass settled down for 10 day and 20 day sludge age reactors, respectively. Since a small amount of supernatant had been left in the reactor to prevent biomass escaping during removing the supernatant. Diclofenac concentration increased each day. So, theoretical diclofenac concentration of the reactors increased to 13.3 μg/L and 15 μg/L for sludge age 10 days and sludge age 20 days reactors, respectively.

Reactors were monitored weekly through measurements of pH, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), soluble chemical oxygen demand (sCOD) and diclofenac. After obtaining samples, measurements were carried out immediately.

After maintaining steady state conditions, respirometric experiments were performed to examine possible acute effect of diclofenac on aerobic biological treatment. Respirometric analyses were done with biomass taken from reactors.

3.3 Respirometric Experiments

Respirometric measurements were carried out with different concentrations of diclofenac to determine potential microbial acute toxicity of diclofenac. Experiments were run with predetermined amount of activated sludge that taken from both control and diclofenac-feeded reactors. Oxygen utilization rate (OUR) had been measured in respirometer apparatus during experiments to establish acute toxicity of diclofenac.

Applitek RA-Combo-1000 Respirometer were used for experiments (Figure 3.3). Respirometer used in the experiments involved a continuous flow-through measurement using the dissolved oxygen (DO) concentration in the liquid phase. Oxygen utilization rate (OUR) of activated sludge is calculated by using DO concentration. In principle, the activated sludge is continuously transferred from respiration vessel to respirometer reactor with a peristaltic pump. After passing through 0.75 L inlet volume of respiration vessel, where the DO level at the inlet and outlet is measured by a probe. Activated sludge continuously recirculated. The respirometer reactor was continuously aerated with airstones. Nitrification inhibitor (Formula 2533TM-HACH) was added to prevent any possible interference induced by nitrification.

Respirometric experiments were run with biomass that taken from fill and draw reactors continuously operated at steady state conditions. Sample that taken from reactors was settled and supernatant was removed and only settled biomass was used

Figure 3.3 : Applitek RA-Combo-1000 Respirometer.

for respirometric experiment. Solution A, solution B, nitrification inhibitor and tap water was added. Respirometric experiment was run without substrate till endogenous respiration level (b_H) becomes steady. When b_H level became steady, calculated peptone mixture which corresponding to 300 mg/L of COD and decided amount of diclofenac which dissolved into methanol were added to reactor and reactor volume was completed to 2 L. Biomass was adjusted to an initial food to microorganism (F/M) ratio approximately same with the F/M level in the fill and draw reactor. The performance of the reactors was monitored through measurements of COD, SS, VSS and PHA measurements beside oxygen uptake rate (OUR) measurements.

15 sets of respirometric experiments were done for 4 reactors. List of respirometer sets are given in Table 3.5.

All reactor's sludge was also run in respirometric experiment with only methanol (no diclofenac addition) to see the effect of methanol on biomass. 40 μL methanol was added for these control set groups. To be able to add the same volume of diclofenac-methanol solution, 500 mg/L, 10,000 mg/L, 50,000 mg/L and 200,000 mg/L diclofenac stock solutions were prepared. 40 μL diclofenac-methanol solution was added from 500 mg/L for 10 μg/L respirometric sets. Similarly, 40 μL diclofenac-methanol stock solution was added from 10,000 mg/L and 50,000 mg/L to 200 μg/L

Table 3.5 : Respirometric experiments.

	10 Control Reactor	10 Diclofenac Reactor	20 Control Reactor	20 Diclofenac Reactor
Diclofenac Concentration	0 μg/L	0 μg/L	0 μg/L	0 μg/L
	200 μg/L	10 μg/L	200 μg/L	10 μg/L
	1000 μg/L	1000 μg/L	1000 μg/L	1000 μg/L
	10,000 μg/L		10,000 μg/L	10,000 μg/L

and 1000 μg/L respirometric sets, respectively. Because of solubility of diclofenac into methanol, it is impossible to prepare 500,000 mg/L diclofenac-methanol solution. So, 200,000 mg/L dilofenac stock solution was prepared for 10,000 μg/L respirometer sets and 100 μL diclofenac-methanol solution was added to respirometer sets.COD equivalence of 40 μL and 100 μL methanol are 17 mg/L and 42 mg/L, respectively. So, 17 mg/L and 42 mg/L COD is added when respirometer data was modelling.

During respirometric analyses, PHA samples were taken to study the storage mechanism of system.

3.4 Analyses

3.4.1 pH

SM 4500-H^+ B Method was used for pH measurements. pH was measured using pH/milivoltmeter (Thermo Orion SA 520). pH-meter was calibrated in each measurement. Probe was washed with distilled water and dried for each measurement. pH of biomass was controlled each day and controlled between 6-8.

3.4.2 Mixed liquor suspended solid (MLSS)

SM 2540 D Method was used for MLSS studies. Glass fiber filter papers were washed with distilled water and then burned in furnace before MLSS analyses. Washed filter papers are stored in desiccator to avoid moisture grabbing.

Filter papers were weighed before filtering of samples. Samples which taken from reactors were rinsed and 5 mL sample was filtered with the help of vacuum. Tweezers was used for transportation of filter papers from vacuum. Filtered samples were dried in oven under 105^0C at least 1 hour. After drying process, samples were cooled in the desiccator to remove moisture inside. Samples were weighed again. Weight difference between filtered filter paper and empty filter paper gives suspended solid amount as milligram in 5 mL. All samples were studied duplicate.

3.4.3 Mixed liquor volatile suspended solid (VSS)

SM 2540 C E Method was used for MLVSS esperiments. After determining the final weight in the total suspended solids analysis, filter papers were placed into furnace and burned at 550^{0}C for 30 minutes. Samples were cooled in the desiccator and weighed. Weight difference between burned filter paper and dried filter paper gives volatile suspended solid amount as milligram in 5 mL. All samples were studied duplicate.

3.4.4 Chemical oxygen demand

ISO 6060 Method was used for COD measurements. Samples were filtered with 0.45 μm PVDF filters (Chromafil) and 2.5 ml sample was taken into glass tubes. Samples should be diluted, if required as procedure is applicable to COD values between 40 and 400 mg/L. 1.5 mL of 0.01667 M standard potassium dichromate digestion solution was added to each sample. 3.5 mL of sulfuric acid reagent was added as cataliyst to each tube. Tubes were mixed several times and closed with TFE-lined screw caps. Tubes were placed into block digester preheated to 150ºC and waited for 2 hours. Samples were taken from heater and cooled down to room temperature.

Digested sample were taken into an Erlenmeyer flask and titrated with 0.1 M standard ferrous ammonium sulfate titrant (FAS).

3.4.5 Polyhydroxy butiric acid (PHB) measurement

2-3 drops of formaldehyde were added in 20 mL samples taken from reactor to prevent biological activity of sludge. Samples were centrifuged (Hettich, Universal 320) at 5000 rpm for 5 minutes and liqud phase was discharged. 10 mL phosphate buffer which prepared with 0.58 g K_2HPO_4 and 0.23 g KH_2PO_4 in 1 L was added to each sample. Samples were shaked and centrifuged at 5000 rpm for 5 minutes. Upper phase was discharged and sludge samples were kept in freezer at -20 ^{o}C. Sludge samples were dried in freeze-dryer (Thermo Savant, Modulyo D-230) shown in Figure 3.4 under vacuum at -45 ^{o}C for 2 days. 20-30 mg dry sludge was weighed and placed into glass tubes with TFE-lined screw caps.

Figure 3.4 : Freeze dryer and centrifuge.

For the extraction of PHB from the sludge, 50 μL internal standard (20 mg benzoic acid in 1 mL 1-propanol), 1.5 mL acid mixture (37% HCl and 1-propanol; 1:4 v/v) and 1.5 mL 1-2 dichloro ethane was added to each sample and boiled in block heater preheated to 100 ^{o}C for 2 hours. Samples were shaked every 15 minutes. Samples were cooled down for 2 hours and distilled water was added to each sample and vortexed for 10 minutes. Then centrifged at 3000 rpm for 5 minutes to separate organic and inorganic phase. Organic phase was taken with a Pasteur pipette and placed into vials after passing through sodium sulphate and glass wool. Samples were measured in gas chromatograph (Agilent, 6890N).

3.4.6 Diclofenac measurements

Diclofenac measurements can be classified into 2 groups as aqueous phase and solid phase.

3.4.6.1 Diclofenac measurement in aqueous phase

Diclofenac measurement in aqueous phase includes three steps; solid phase extraction (SPE), evaporation and measuring in LC-MS/MS. In solid phase extraction diclofenac was captured in cartridges by passing through it and then solved with methanol and acetonitrile. In second step, solvents of diclofenac were evaporated. Samples were prepared by solving with solvents and placed into vials inn third step.

Vacuum manifold with 20 cartridges ports (VocMaster, Biotage) was used to place cartridges (Oasis HLB; Waters). Cartridges were conditioned before solid phase extraction with 10 mL acetonitrile (Merck), 5 mL methanol (Merck) and 5 mL distilled water (Merck), respectively.

Samples were filtered with 0.22 µm PVDF filters (Chromafil) to prepare for diclofenac analyses. Theoretical diclofenac concentration in reactors were 13.3 µg/L and 15 µg/L for sludge age 10 days and 20 days reactors, respectively. So, 5 mL sample was sufficient to obtain diclofenac concentration from LC-MS/MS. d_4-diclofenac (isotopically labelled diclofenac) was added to each sample as internal standard for measurement in LC-MS/MS and placed into ultrasonic bath (Intersonic, Min 12) to provide transition. Samples were passed through cartridges with a flowrate of 3.5 mL/minutes without waiting right after conditioning period. 5 mL distilled water was added to wash cartridges after sample loading. Wet cartridges were dried at least 60 minutes under vacuum (Figure 3.5). Diclofenac was captured into cartridges with this method. 2 mL acetonitrile and 2 mL methanol was passed through cartridges to dissolve and samples was collected in a glass tubes.

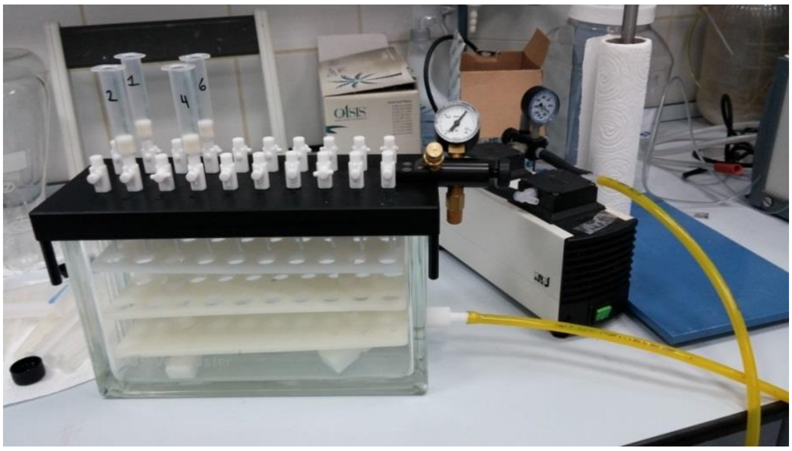


Figure 3.5 : SPE of samples.

Subsequently, solvents was evaporated in Turbovap II (Caliper Life Sciences, USA) at 35^{o}C and 10 bar N_2 flow (Figure 3.6).

Diclofenac remained in Turbovap tubes was collected with 1 mL mixture of MeOH-water (10:90 v/v) and placed into amber vials to be measured in Thermo Electron Cooperation TSQ Quantum Access triple quadruple mass spectrometer coupled with Accela Ultra Performance Liquid Chromatograph (UPLC) after filtering with 0.22 µm filters.

Figure 3.6 : Turbovap II and LC-MS.

Schematic diagram of diclofenac measurement in queous phase is given in Figure 3.7.

3.4.6.2 Diclofenac measurement in solid phase

Sludge samples which centrifuged and stored at -20 ^{o}C were dried in freeze dreyer (Thermo Savant, Modulyod-230) . 0.1 g dry solid was taken into teflon tubes and d_4-diclofenac and 10 mL acetone-methanol mixture (1:1, v/v) were added to samples. Samples were placed into ultrasonic bath for 10 minutes to provide transition of diclofenac from sludge to solvent mixture.

Samples were centrifuged at 9000 rpm for 15 minutes to separate solid and liquid phase after ultrasonication. Supernatant was placed into another tube. And 10 mL methanol-acetone mixture was added to solid phase again and same procedure was applied.

Both first and second supernatant was collected together and evaporated into evaporator at 45 ^{o}C (Heidolph, Laborota 4000). Dried samples were dissolved into 2 mL of acetone-methanol mixture and 18 mL distilled water. The procedure that applied to liquid phase for diclofenac measurement was applied to samples.

Schematic diagram of diclofenac measurement in queous phase is given in Figure 3.8.

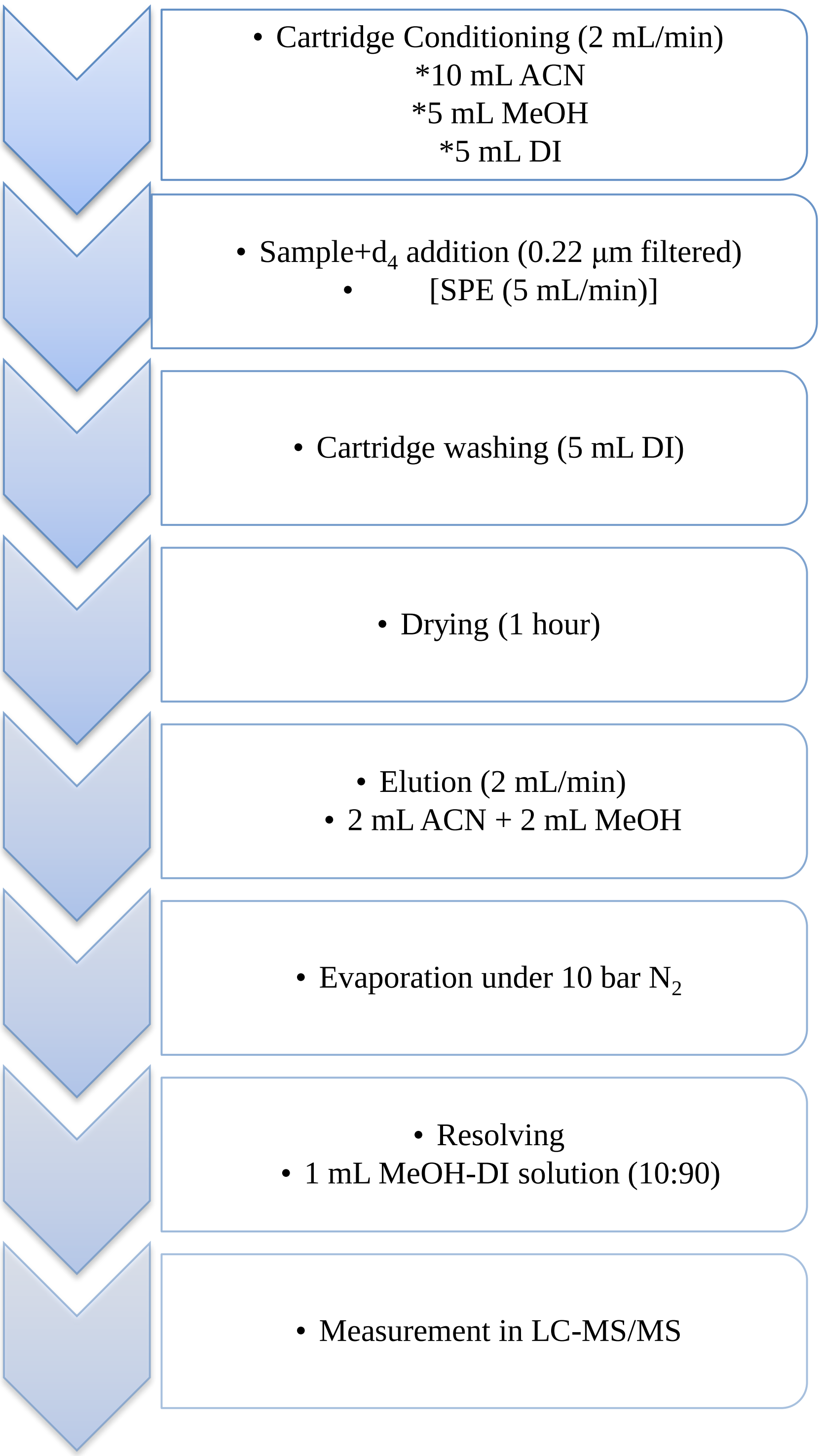


Figure 3.7 : Schematic diagram of diclofenac measurement in aqueous phase.

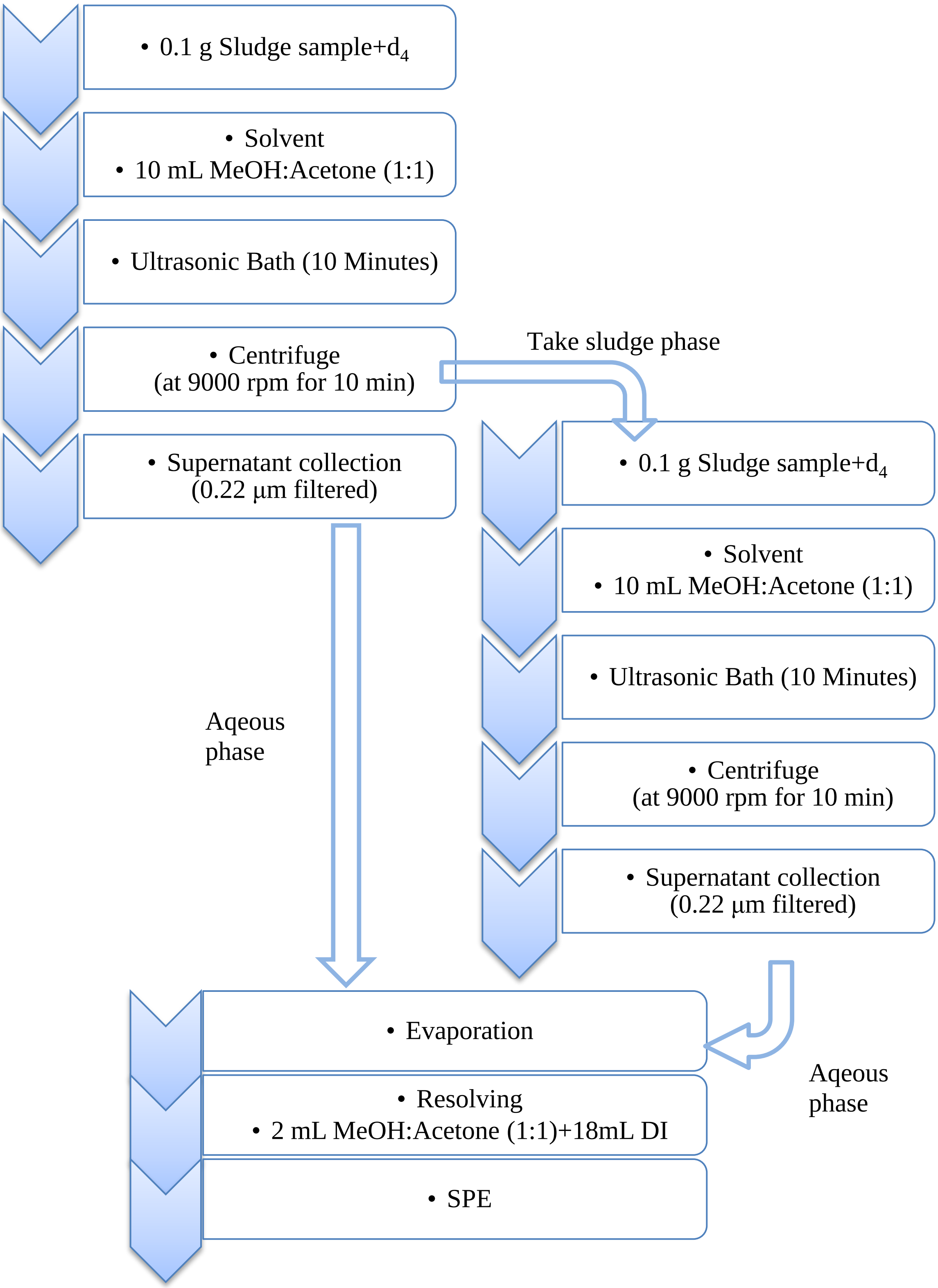


Figure 3.8 : Schematic diagram of diclofenac measurement from solid phase.

4. RESULTS AND DISCUSSION

4.1 Reactor Operations

Four laboratory-scale reactors (two test and two control) with selected sludge ages were operated in parallel to compare the effect of sludge age on biodegradability of diclofenac under aerobic conditions and to observe the chronic effect of diclofenac to aerobic treatment systems. First set was operated at a sludge age of 10 days and in the second set sludge age was selected as 20 days. Studies in the literature and real-scale systems have been taken into account when sludge age was selecting. Each set has 2 reactors including diclofenac and control reactor. Diclofenac reactors were fed with 10 μg/L diclofenac solution which dissolved in methanol. Same amount of methanol was added to control groups in order to ensure same conditions with diclofenac reactors.

4.1.1 Operation at a sludge age of 10 days

Two reactors were operated to investigate the biological treatability of diclofenac under aerobic conditions at a sludge age of 10 days for 160 days. Diclofenac concentration of seed sludge was less than 5 ng/L. Control group was fed with peptone, solution A, solution B and methanol and diclofenac group was fed with peptone, solution A, solution B and diclofenac.

Systems were fed with 600 mg/L COD equivalent peptone solution. Reactors were acclimated with diclofenac and reached to steady state where suspended solids and volatile suspended solids amount of reactors and theroretical diclofenac concentrations of reactors stabilized. SS and VSS concentrations of control and diclofenac acclimated reactors during operation time are given in Figure 4.1.

Control reactors were started with initial SS and VSS value of 4150±155 and 2780±113 mg/L, respectively. During acclimation period, SS and VSS concentrations stabilized and at the end of the acclimation period less fluctuation was observed. SS concentration was 3506±526 mg/L when VSS concentration was

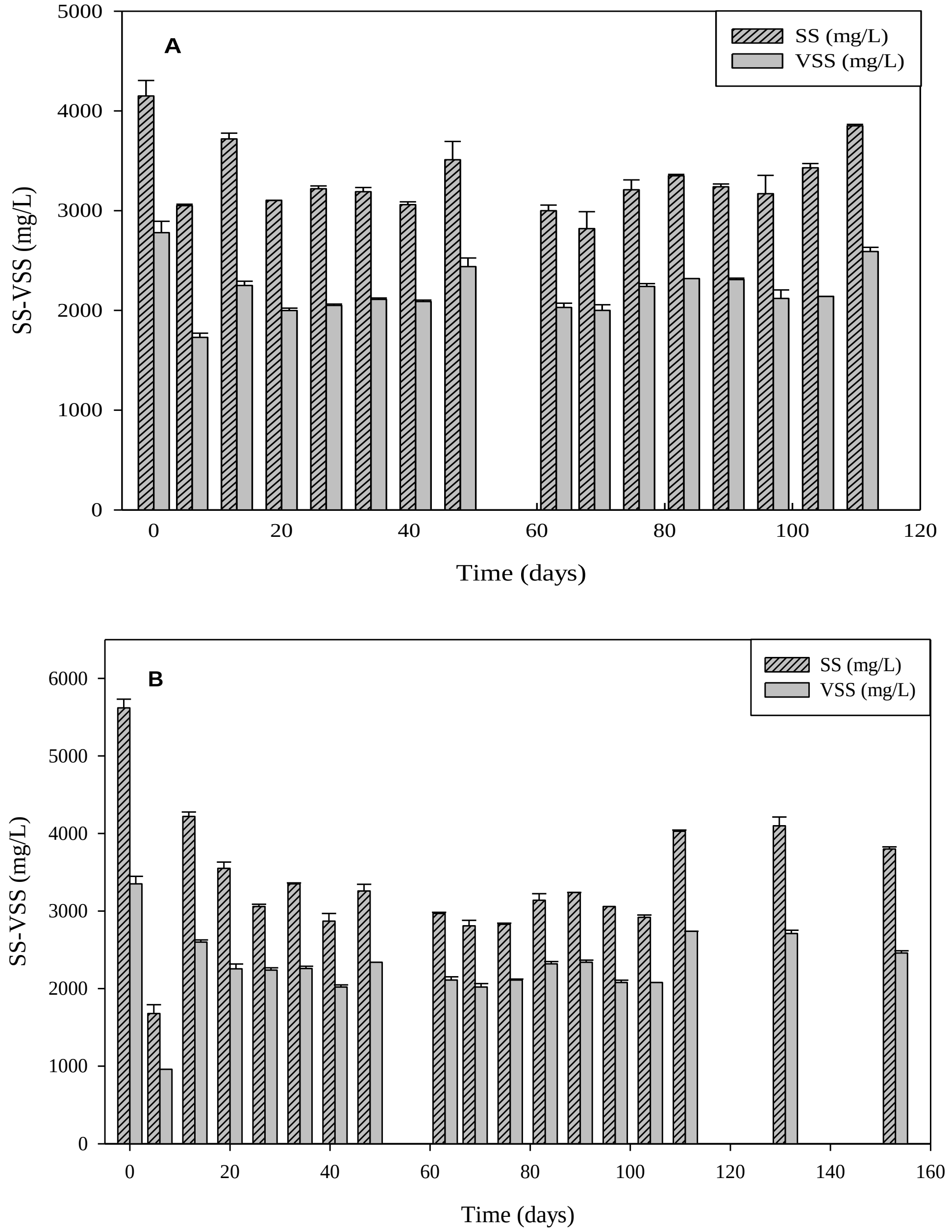


Figure 4.1 : SS and VSS profiles of 10 days sludge age A) Control reactor, B) Diclofenac acclimated reactor.

2189±447 mg/L during acclimation period (first two sludge age). After acclimation period, SS and VSS concentration of control reactor was 3254±263 and 2168±140 mg/L, respectively. VSS/SS ratio was calculated as 0.666 after acclimation period.

Diclofenac reactors were initiated with SS and VSS concentration of 5620±113 and 3350±99 mg/L, respectively. SS concentration was 3767±1637 mg/L when VSS concentration was 2291±998 mg/L during acclimation period (first two sludge age).

After acclimation period, SS and VSS concentration of acclimated diclofenac reactor was measured as 3246±432 and 2273±234 mg/L, respectively. VSS/SS ratio was calculated as 0.7 after acclimation period. It was observed that SS and VSS concentrations of diclofenac-acclimated reactor and control reactor were nearly equal to each other.

Samples for sCOD were taken from reactors immediately before feeding of reactors, sCOD were measured immediately after sampling.

Soluble chemical oxygen demand amount of 10 days sludge age reactors (control and diclofenac-acclimated) are given in Figure 4.2.

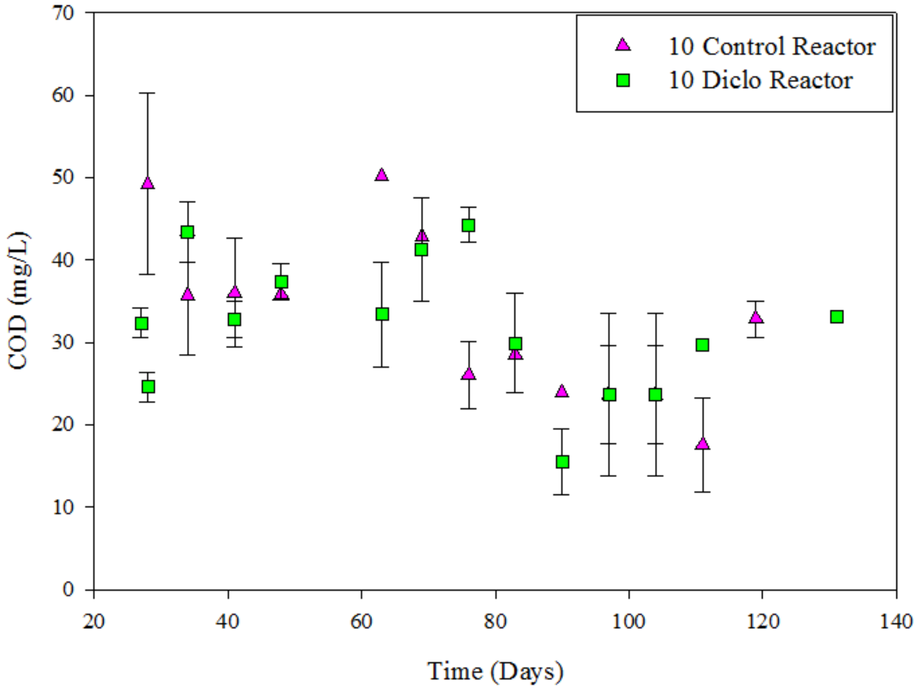


Figure 4.2 : Soluble COD profile of 10 days sludge age reactors.

sCOD concentration of 10 days sludge age control reactor during acclimation period was decreased from 600 mg/L to 35±18 mg/L. 20 days later (first two sludge age) fluctuations of sCOD profile became stable and average sCOD concentration was found as 33±10 mg/L.

For diclofenac reactor, average sCOD value for first two sludge age (20 days) was found as 25±5 mg/L during acclimation period. After acclimation period, sCOD value was measured as 32±8 mg/L.

Soluble COD value for both control and diclofenac reactors were same during reactor operation. So, it is observed that diclofenac have no chronic effect on the removal of organic matter.

Diclofenac concentration in domestic wastewater can change between 0.11-3 μg/L as shown in Table 2.6 (Yu et al., 2006; Heberer et al., 2002). 10 μg/L diclofenac concentration was selected for reactor operation. This concentration was selected in order to observe the change in diclofenac concentration more easily.

Diclofenac sample was taken from diclofenac-acclimated reactor weekly after acclimation period to examine biodegradability of diclofenac with a sludge age of 10 days. Samples were taken from reactor right before feeding. Reactor was fed with 10 μg/L diclofenac but since a small amount of supernatant had been left in the reactor to prevent biomass escaping during removing the supernatant. Diclofenac concentration increased each day. So, theoretical diclofenac concentration of 10 days of sludge age reactor was 13.3 μg/L. Diclofenac concentration of reactor and theoretical diclofenac concentration into reactor are given in Figure 4.3.

As shown in Figure 4.3, diclofenac concentration of 10 days sludge age diclofenac-acclimated reactor was nearly same with theoretical value. Diclofenac concentration was 13.4±1.7 μg/L, averagely. Results show that diclofenac could not be degraded under aerobic conditions with this sludge age.

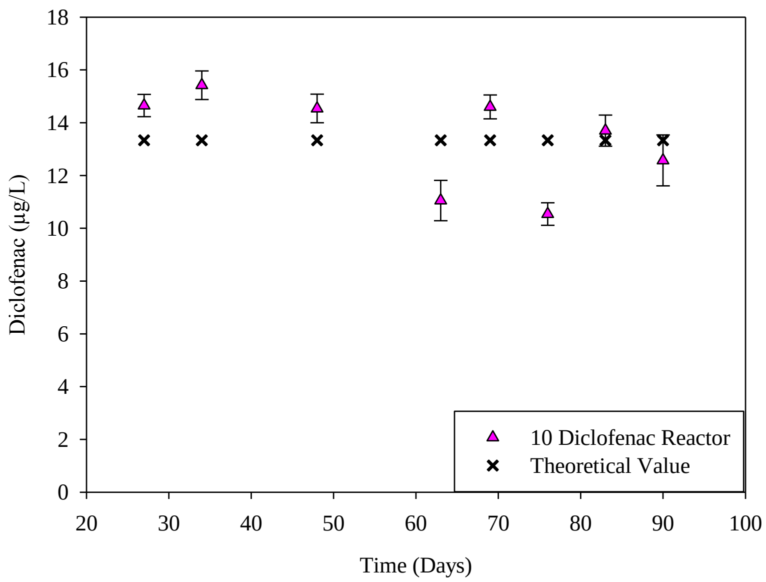


Figure 4.3 : Theoretical and actual diclofenac concentration of 10 days sludge age diclofenac-acclimated reactor.

There are limited number of studies about biodegradation of diclofenac in lab-scale reactors under aerobic conditions and results are quite different from each other. In general, it is observed that diclofenac can not be degraded biologically and it has a strength to biodegradation (Joss et al., 2006; Quintana et al., 2005; Lahti and Oikari, 2011; Zwiener and Frimmel, 2003; Suarez, 2010; Clara et al., 2005). Ternes and Joss (2006) studied with 35 different pharmaceuticals with different sludge ages. It is observed that in low sludge ages (<4 days) pharmaceuticals can not be removed, but in higher sludge ages, like 10-15 days, they can be degraded with a certain rate. In this study, biodegradation constant of diclofenac was found as 0.01 L/g SS.d and this value is considerable low when compared to other pharmaceuticals, like ibuprofen and paracetomol.

Accumulation of diclofenac onto sludge was also investigated in this study. Sludge samples were taken from reactor for seven weeks and diclofenac measurement in solid phase was measured. Two different samples were taken every week; first one is just before feeding (24 hours later than last feeding) and the second one is just after feeding. Diclofenac measurements in sludge were done after acclimation period of reactor. Results of diclofenac measurement in solid phase for sludge age 10 days are given as percentage in Table 4.1.

Table 4.1 : Percentage of diclofenac that adsorbed onto sludge of 10 days diclofenac-acclimated reactor.

Time (Days)	Diclofenac percentage in sludge before feeding (%)	Diclofenac percentage in sludge after feeding (%)
27	1.2	1.1
48	3.0	1.8
63	-	1.5
69	1.0	1.0
76	2.3	1.6
83	1.2	1.3
90	1.1	1.0

Percentages given in Table 4.1 correspond to the ratio of the diclofenac amount adsorbed onto sludge to the diclofenac amount present in the reactor. Average of 1.6% of total diclofenac present in the reactor was adsorbed onto sludge according to values that obtained from first column (before feeding values). The samples that taken immediately after feeding of reactors shows that 1.3% of total diclofenac adsorbed to sludge.

Percentages show that accumulation of diclofenac onto sludge is negligible for 10 days sludge age under aerobic conditions.

4.1.2 Operation at a sludge age of 20 days

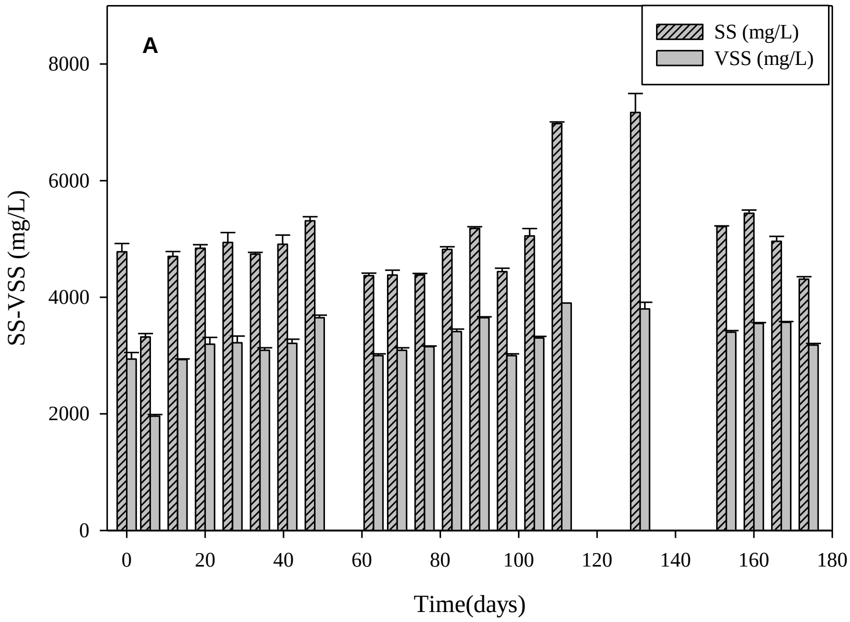
Two reactors were operated to investigate biological treatability of diclofenac under aerobic conditions. Within this scope two reactors at a sludge age of 20 days were operated for 250 days. Diclofenac concentration of seed sludge was less than 5 ng/L. Control group was fed with peptone, solution A, solution B and methanol and diclofenac group was fed with peptone, solution A, solution B and diclofenac. Environmental factors were kept same for both 10 days and 20 days sludge age reactors.

Systems were fed with 600 mg/L COD equivalent peptone solution. Reactors were acclimated with diclofenac and reached to steady state where suspended solids and volatile suspended solids amount of reactors and theroretical diclofenac concentrations of reactors stabilized. SS and VSS concentrations of control and diclofenac acclimated reactors during operation are given in Figure 4.4.

Initial SS and VSS value of control reactor were 4780$\pm$141 and 2940$\pm$113 mg/L, respectively. During acclimation period, SS and VSS concentration of control reactor stabilized and at the end of the acclimation period less fluctuations were observed. SS concentration was 4604$\pm$572 mg/L when VSS concentration was 2935$\pm$447 mg/L during first two sludge age (40 days). After acclimation period, SS and VSS concentration of control reactor was 5143$\pm$907 and 3403$\pm$293 mg/L, respectively. VSS/SS ratio was calculated as 0.662 after acclimation period.

Diclofenac reactor was initiated with SS and VSS concentration of 4610$\pm$71 and 2800$\pm$28 mg/L, respectively. SS concentration was 4594$\pm$431 mg/L when VSS concentration was 2873$\pm$323 mg/L during first two sludge age period. After acclimation period, SS and VSS concentrations of acclimated diclofenac reactor was measured as 4734$\pm$864 and 3216$\pm$378 mg/L, respectively. VSS/SS ratio was calculated as 0.679 after acclimation period.

SS concentration of diclofenac-acclimated reactor was approximately 400 mg/L less than control reactor's. Similarly, VSS concentration of control reactor was approximately 200 mg/L higher than diclofenac-acclimated reactor. This situation could be a reason of slight chronic inhibition effect of diclofenac on biomass in this sludge age. There was not such an effect on sludge age of 10 days reactors.



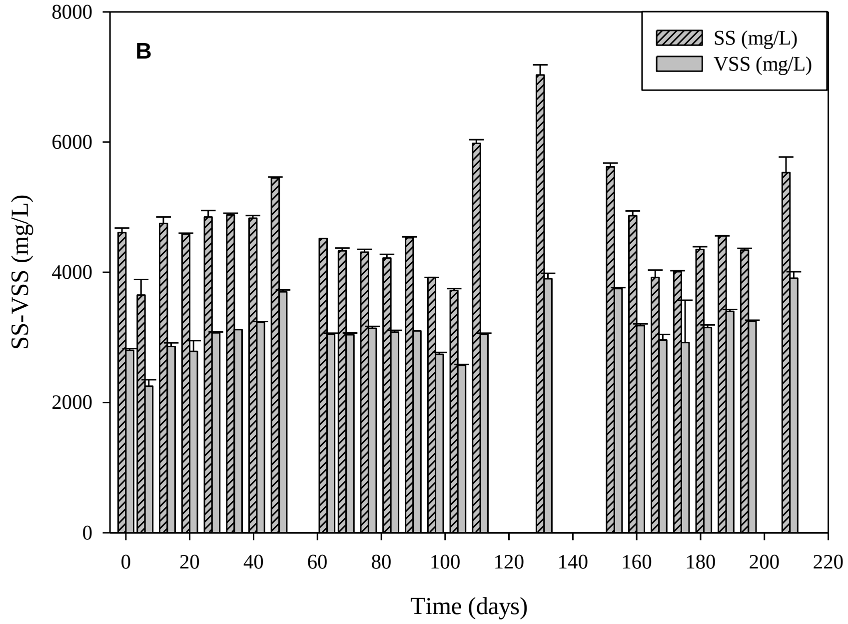


Figure 4.4 : SS and VSS profiles of 20 days sludge age A) Control reactor, B) Diclofenac acclimated reactor.

Soluble COD of 20 days of sludge age control and diclofenac-acclimated reactors are given in Figure 4.5.

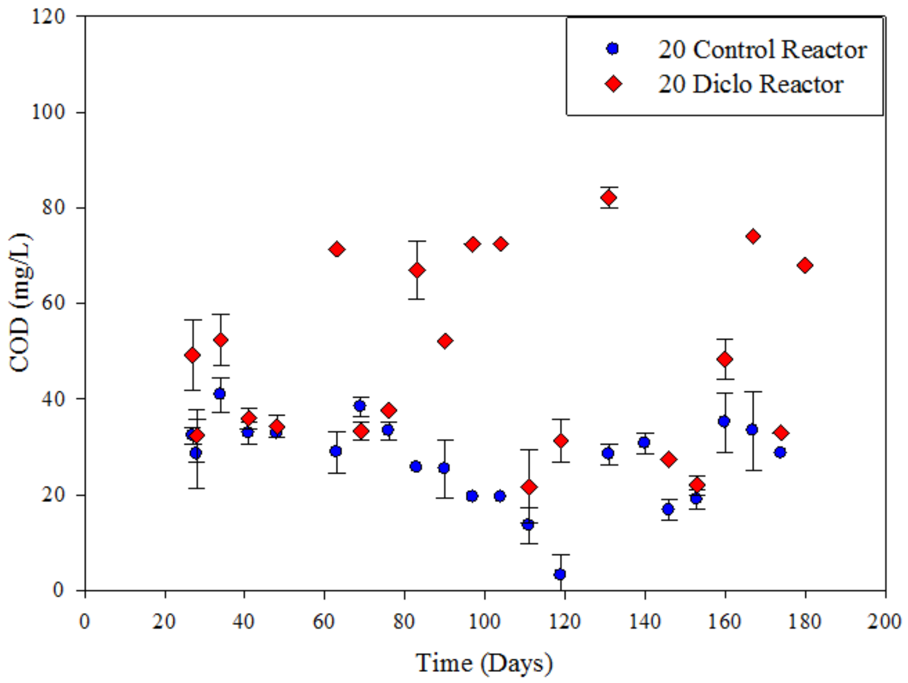


Figure 4.5 : Soluble COD profile of 20 days sludge age reactors.

sCOD concentration of 20 days of sludge age control reactor during acclimation period was decreased from 600 mg/L to 41±38 mg/L. 40 days later (equal to first two sludge age) fluctuations of sCOD profile became stable and average sCOD concentration was 25±10 mg/L.

For 20 days of sludge ages diclofenac reactor, average sCOD value for 40 days (equal to first two sludge age) was found as 63±44 mg/L during acclimation period. After acclimation period, sCOD value was measured as 53±18 mg/L.

sCOD concentration of diclofenac-acclimated reactor and control reactors were measured every week during reactor operation. It was found that biodegradation of organic matter in control reactor of 20 days of sludge age is more effective than diclofenac-acclimated reactor. There was approximately 28 mg/L difference between control and diclofenac-acclimated reactor’s COD values. Same situation was observed in SS and VSS concentrations of reactors. This may be attributed to chronic inhibition effect of diclofenac on biomass in this sludge age. There was no effect of diclofenac on VSS and COD for sludge age of 10 days reactors. It could be said that inhibition effect of diclofenac on biomass is changing with sludge age of reactors. But it should be emphasized that the change in sludge age from 10 days to 20 days showed only a small difference in COD and SS/VSS concentrations. So a slight chronic effect of diclefenac on sludge age of 20 days is observed within 250 days of operation.

Diclofenac sample was taken from 20 days of sludge age diclofenac-acclimated reactor weekly after acclimation period to investigate biodegradability of diclofenac. Reactor was fed with 10 μg/L diclofenac but theoretical diclofenac value into the reactor increased to 15 μg/L with time. Diclofenac concentration of reactor and theoretical diclofenac concentration in the reactor are given in Figure 4.6.

As shown in Figure 4.6, diclofenac concentration of 20 days of sludge age diclofenac-acclimated reactor was nearly same with theoretical value for first 50 days. Diclofenac concentration was found as 15±1.8 μg/L till first 50 days. This period corresponds to approximately two sludge age (acclimation period) of diclofenac reactor. After first 50 days of reactor, average diclofenac concentration decreased to 10.5±0.6 μg/L.

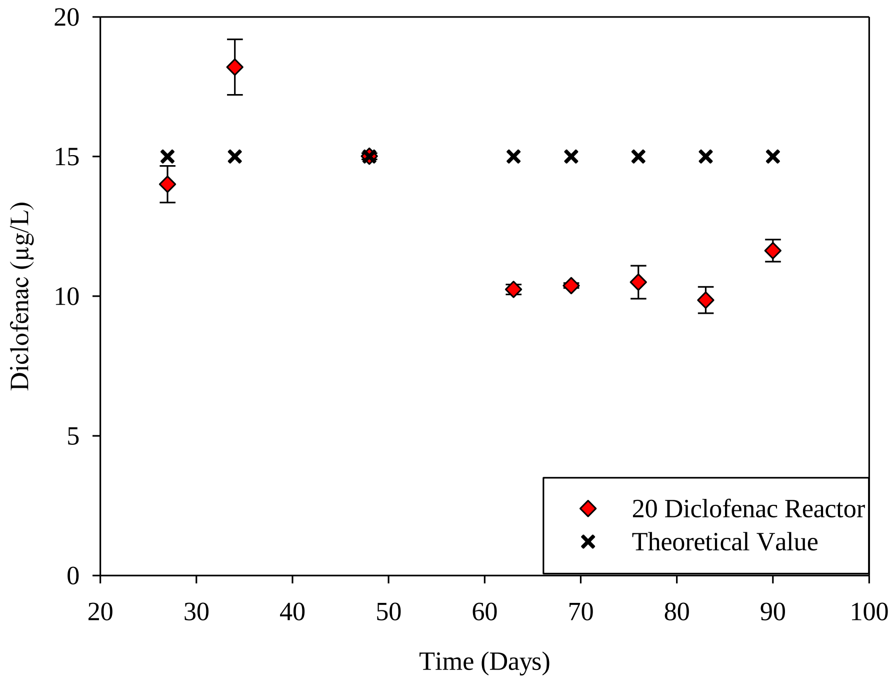


Figure 4.6 : Theoretical and actual diclofenac concentration of 20 days sludge age diclofenac-acclimated reactor.

According to data obtained, it could be said that biodegradability of diclofenac under aerobic conditions can only occur with high SRT values. There was no removal with 10 days of sludge age. But 22.5-34.3% diclofenac removal was occurred in 20 days sludge age reactor.

It is seen that acclimation of biomass to diclofenac is also important for biodegradation. During acclimation period of biomass there was no decrease on diclofenac concentration. After acclimation period, diclofenac concentration in the reactor was decreased from 15 μg/L to approximately 10 μg/L. Average diclofenac removal was observed around 30% after acclimation period.

There are different results about biodegradation of diclofenac in lab-scale reactors under aerobic conditions. According to Lahti and Oikari (2011), main reasons of these contrasts are due to the source of microbial inoculum, experimental design and environmental factors. Suarez et al. (2010) studied on biodegradation of diclofenac and it was observed that biodegradation of diclofenac occured just after the growth of specific bacteria. Beside these factors, operational parameters, like sludge age, are effective as well as microbial culture. For example, Kimura et al. (2007) found that biodegradation of diclofenac was more effective in an MBR operating with an SRT value of 65 days with respect to a CAS operating with an SRT value of average 7 days.

For measurement of diclofenac in solid phase, sludge samples were taken from 20 days sludge age reactor for seven weeks. Two different samples were taken every week; first one is just before feeding (24 hours later than last feeding) and the second one is just after feeding. Diclofenac measurements in sludge were done during and after acclimation period of reactor. Results of diclofenac measurement in solid phase are given as percentage in Table 4.2.

Percentages given in table correspond to the ratio of the diclofenac amount adsorbed onto sludge to the diclofenac amount present in the reactor. Average of 1.7% of total diclofenac present in the reactor was adsorbed onto sludge according to values that obtained from first column (just before feeding values). The samples taken immediately after feeding of reactors shows that 2.1% of total diclofenac adsorbed to sludge.

Table 4.2 : Percentage of diclofenac that adsorbed onto sludge of 20 days diclofenac-acclimated reactor.

Time (Days)	Diclofenac percentage in sludge before feeding (%)	Diclofenac percentage in sludge after feeding (%)
27	-	1.8
48	2.5	3.3
63	1.5	2.0
69	1.3	2.3
76	1.3	1.8
83	1.5	1.6
90	1.8	1.9

Diclofenac has been existed in aqeous phase with low affinity to sorb to aerobic sludge. Similar results were obtained from 10 days of sludge age reactors. It is observed that there is no relationship between sludge age and adsorption of diclofenac on sludge. Scheytt et al. (2005) also obtained similar results. Laboratory batch studies to characterize the accumulation of diclofenac in natural aquifer sediments showed that sorption coefficients were quite low compared to other pharmaceuticals (Scheytt et al., 2005).

4.2 Respirometric Analyses

Respirometric analyses were done to investigate the acute toxicity of diclofenac on diclofenac-acclimated biomass and non-acclimated biomass. Different concentrations of diclofenac were fed to respirometer reactor and effect of diclofenac onto biomass was observed. Biomass was taken from both control and diclofenac-acclimated reactors. Analyses were run with Applitek RA-Combo-1000 Respirometer. Respirometer measured Oxygen Utilization Rate (OUR) of reactor during experiments. Unit of OUR is mg/L/hr.

15 sets of respirometric analyses were done with 0, 10, 200, 1000 and 10,000 μg/L diclofenac concentrations. Diclofenac was added to respirometer reactor as dissolved in methanol. 4 different stock solutions were prepared for respirometric experiments. 500 mg/L, 10,000 mg/L, 50,000 mg/L and 200,000 mg/L diclofenac stock solutions

was prepared for 10, 200, 1000 and 10,000 μg/L diclofenac concentrations, respectively.

40 μL methanol was added for respirometer experiments which are running with 0 μg/L diclofenac concentration. 40 μL methanol or diclofenac-methanol solution was added to respirometer reactors for all concentrations except 10,000 μg/L. 100 μL diclofenac solution was added to these sets from 200,000 μg/L diclofenac stock solution.

4.2.1 Respirometric analyses carried with 10 days of sludge age reactors

Acute effect of diclofenac on acclimated and non-acclimated biomass which was run with 10 days of sludge age was investigated. 4 sets of respirometer measurements (with 0 μg/L, 200 μg/L, 1000 μg/L and 10,000 μg/L diclofenac concentrations) were run for control reactor and 3 sets (with 0, 10 and1000 μg/L diclofenac concentrations) were run for diclofenac reactor. Nitrification inhibitor was added to all sets to avoid nitrification.

4.2.1.1 Control reactor

Respirometer experiments were run to investigate the acute toxicity of diclofenac on biomass taken from control reactor which has 10 days sludge age and did not acclimate to diclofenac. Respirometer experiment graphs of 10 days of sludge age control reactor are given in Figure 4.7, 4.8, 4.9, 4.10.

Biomass that taken from 10 days of sludge age control reactor was run with 0 μg/L diclofenac concentration (Figure 4.7). Nitrification inhibitor eliminated the nitrifiers activity in the reactor. System was run for half an hour to obtain endogenous decay level of microorganisms. b_H which is the abbrevation of endogenous decay level, was obtained from graph as 9.3 mg/L/hr. 40 μL methanol and 300 mg/L COD equivalence of peptone was added to respirometer reactor and OUR versus time and COD versus time profiles are given in Figure 4.7. COD equivalence of 40 μL methanol corresponds to 17 mg/L. So, there is an additive COD source which effects OUR profile. 349 mg/L COD concentration which added to respirometer reactor at t=0 minute is decreased to 50±0 mg/L COD value at the end of the 150 minutes. At time t=0 minute, OUR value was read as 107 mg/L/hr. OUR level decreases to b_H level after 300 minutes because of substrate depletion in the reactor. pH of reactor during respirometer experiment was measured as 6.9±0.4. SS and VSS value was found at the beginning of experiment as 3210±42 and 1520±0 mg/L, respectively.

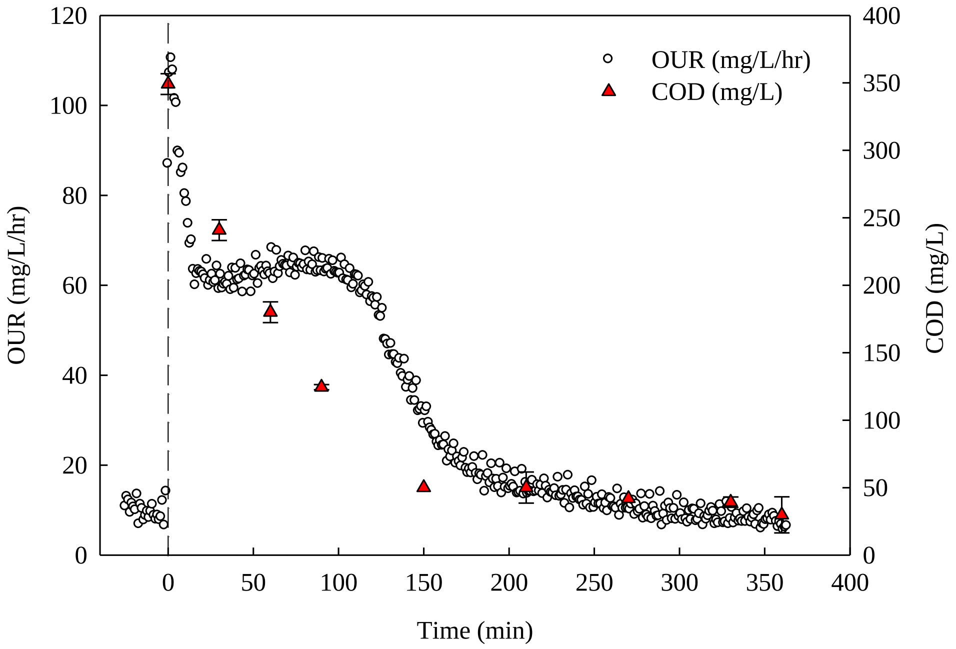


Figure 4.7 : OUR and COD profile of respirometer that run with 0 µg/L diclofenac and biomass of 10 days sludge age control reactor.

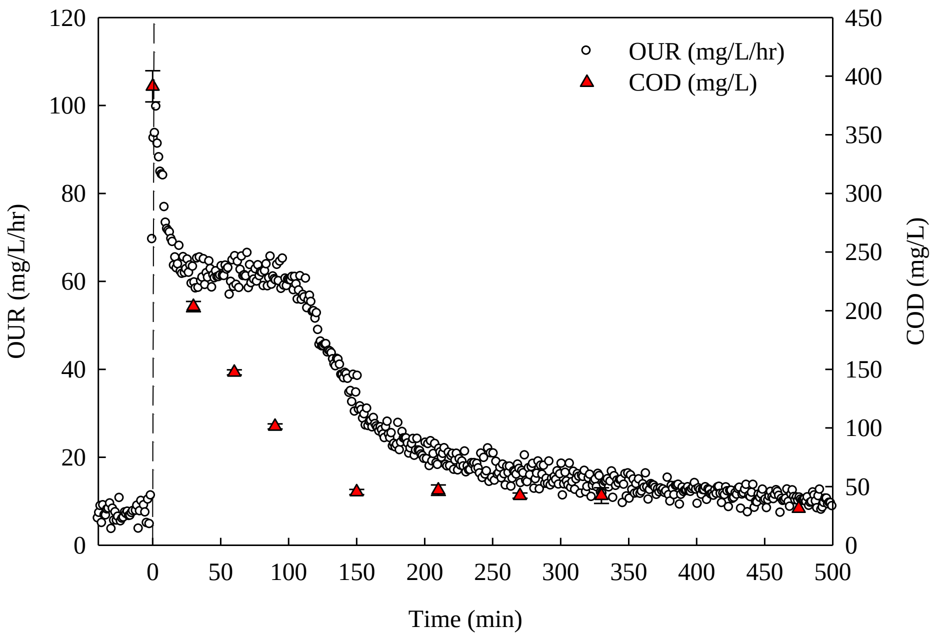


Figure 4.8 : OUR and COD profile of respirometer that run with 200 µg/L diclofenac and biomass of 10 days sludge age control reactor.

Respirometric graph of 200 µg/L diclofenac addition to 10 days sludge age reactor is given in Figure 4.8. b_H level during first 40 miutes was found as 6.9 mg/L/hr. 40 µL diclofenac from 10,000 mg/L diclofenac stock solution was added to respirometer reactor together with peptone. OUR level jumped up to 100 mg/L/hr when peptone and diclofenac added. 390 mg/L COD value at t=0 minute decreases to 45±2 mg/L COD. COD values were close to 0 µg/L diclofenac concentration's COD value. OUR level decreases to b_H level 400 minutes later. pH of biomass that taken from reactor was measured ad 7.6. SS and VSS values were found at the beginning of experiment as 2810±14 and 1525±14 mg/L, respectively.

Biomass that taken from 10 days of sludge age control reactor was run with 1000 µg/L diclofenac concentration (Figure 4.9) System was run for half an hour to obtain endogenous decay level of microorganisms. b_H level was found as 13.5 mg/L/hr. 40 µL diclofenac from 50,000 mg/L diclofenac stock solution was added to respirometer reactor together with peptone and OUR profile obtained. 325 mg/L COD concentration which added to respirometer reactor at t=0 minute is decreased to 28±6.6 mg/L COD value at the end of the 150 minutes. At time t=0 minute, OUR value was read as 134 mg/L/hr. pH of reactor during respirometer experiment was

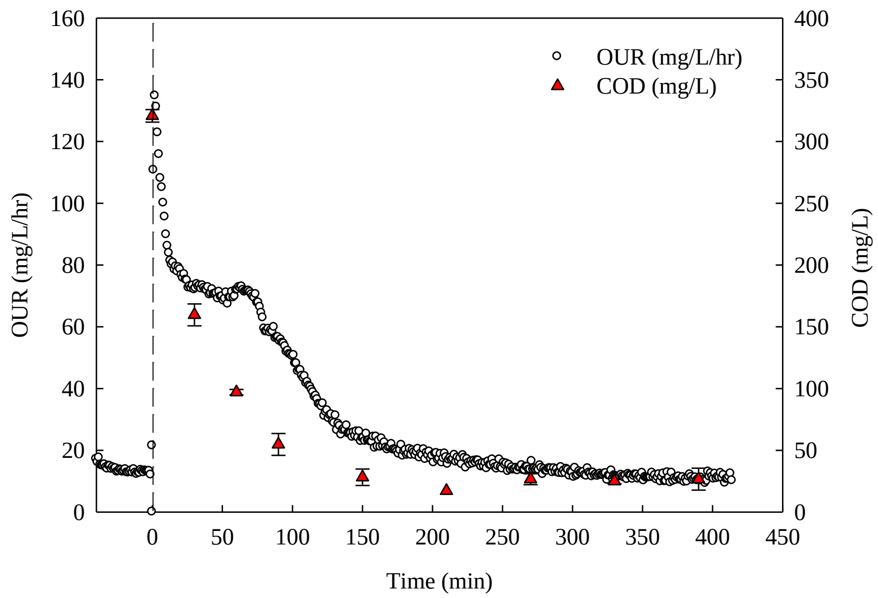


Figure 4.9 : OUR and COD profile of respirometer that run with 1000 µg/L diclofenac and biomass of 10 days sludge age control reactor.

measured as 7.95. SS and VSS values were found at the beginning of the experiment as 4110±155 and 1820±56 mg/L, respectively.

OUR profile of the respirometer that run with 10.000 μg/L is given in Figure 4.10. Highest concentration which added to control reactor was selected as 10,000 μg/L for 10 days of sludge age control reactor. 100 μL diclofenac was added from 200,000 μg/L diclofenac stock solution to reactor to obtain this diclofenac concentration. System was run for a while to obtain endogenous decay level of microorganisms. b_H level was obtained as 7.8 mg/L/hr. After addition of peptone and diclofenac-methanol solution, OUR was increased to 113 mg/L/hr. COD equivalence of 100 μL methanol corresponds to 43 mg/L and that value makes profile different than previous. At the end of the 150th minute, COD value was measured as 85±6.6 mg/L. In this set, in contrast to lower diclofenac concentration sets, COD value decreased to 50 mg/L barely after 400 minutes. It is observed that different OUR and COD profiles was obtained when compared to other concentrations. pH of reactor at the beginning of respirometer experiment was measured as 7.6. SS and VSS value was found at the beginning of experiment as 3600±56 and 1650±14 mg/L, respectively.

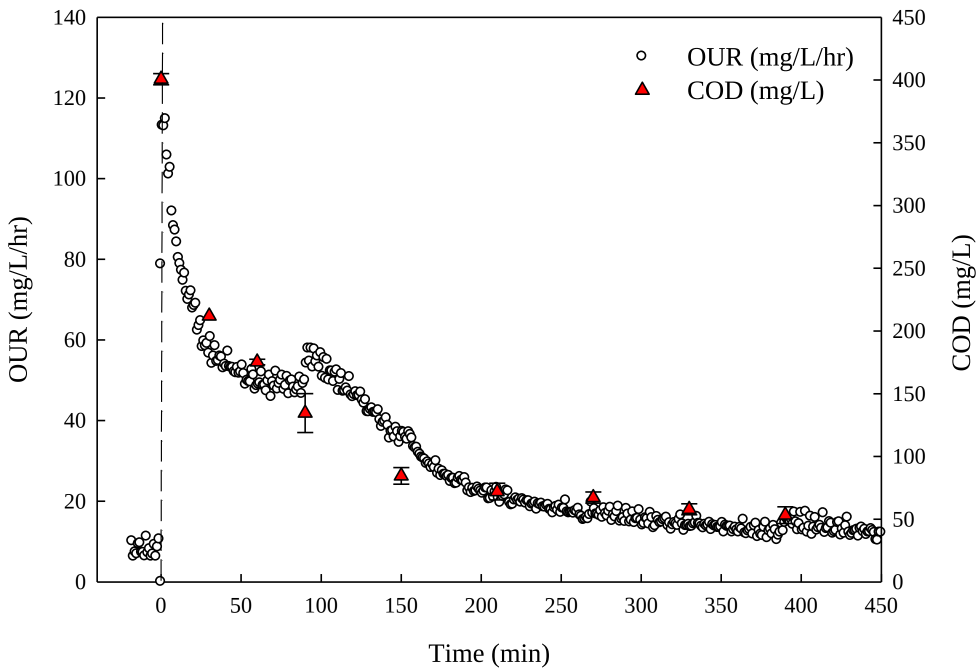


Figure 4.10 : OUR and COD profile of respirometer that run with 10,000 μg/L diclofenac and biomass of 10 days sludge age control reactor.

All data obtained from 10 days of sludge age control reactor's respirometer sets are given in Figure 4.11 to be comperable. When OUR profiles of 0 µg/L and 200 µg/L sets are compared, no difference was observed. So, 200 µg/L diclofenac did not have a significant effect on oxygen utilization rate of biomass. Moreover, OUR profiles completely changed when diclofenac concentration increased. COD amount which added to system in all sets were equal to each other, except 10,000 µg/L diclofenac. An additional 25 mg/L COD was coming from methanol in this set. Area calculated under the curves that showed respirometric data of different concentrations were equal to each other and it showed that substrate were completely degraded by microorganisms. It is observed that OUR values start to decrease to b_H level when substrate degraded in all sets. b_H levels were changing between 7 and 13 for all sets and it is a proof of activeness of biomass. OUR value of all sets except 10,000 µg/L decreased to b_H levels just after than 300 minutes. But it did not decrease to b_H level till 500 minutes in 10,000 µg/L diclofenac concentration set.

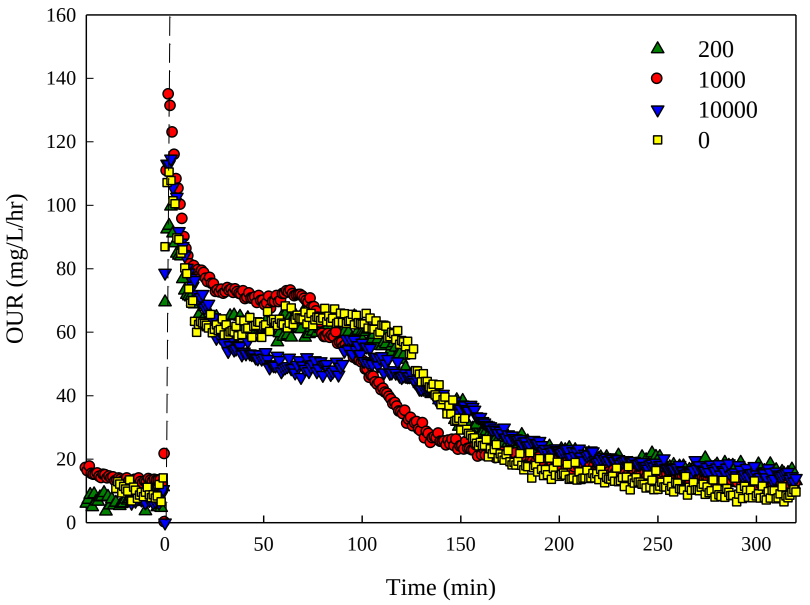


Figure 4.11 : All respirometric data obtained from sludge age 10 days control reactor's biomass.

4.2.1.2 Diclofenac reactor

Respirometric experiments were run to investigate the acute effect of diclofenac on an acclimated biomass obtained from the 10 days of sludge age reactor. 3 sets of respirometer experiments were carried out at this stage. Respirometer experiment graphs of 10 days sludge age diclofenac-acclimated reactor are given in Figure 4.12, 4.13, 4.14. Nitrification inhibitor was added to all sets to avoid nitrification activity in the respirometer reactor.

Respirograph of diclofenac-aclimated biomass obtained from 10 days of sludge age reactor with no diclofenac addition is given in Figure 4.12. b_H level was obtained from graph as 9.9 mg/L/hr. 40 µL methanol and peptone was added to respirometer reactor. 150 minutes later, COD value decreased from 400 mg/L COD to 25±2 mg/L. At time t=0 minute, OUR value was read as 198 mg/L/hr. OUR level decreased to b_H level after 250 minutes because of substrate depletion in the reactor. pH of reactor during respirometer experiment was measured as 7.7. SS and VSS values were found at the beginning of experiment as 3400±0 and 1700±14 mg/L, respectively.

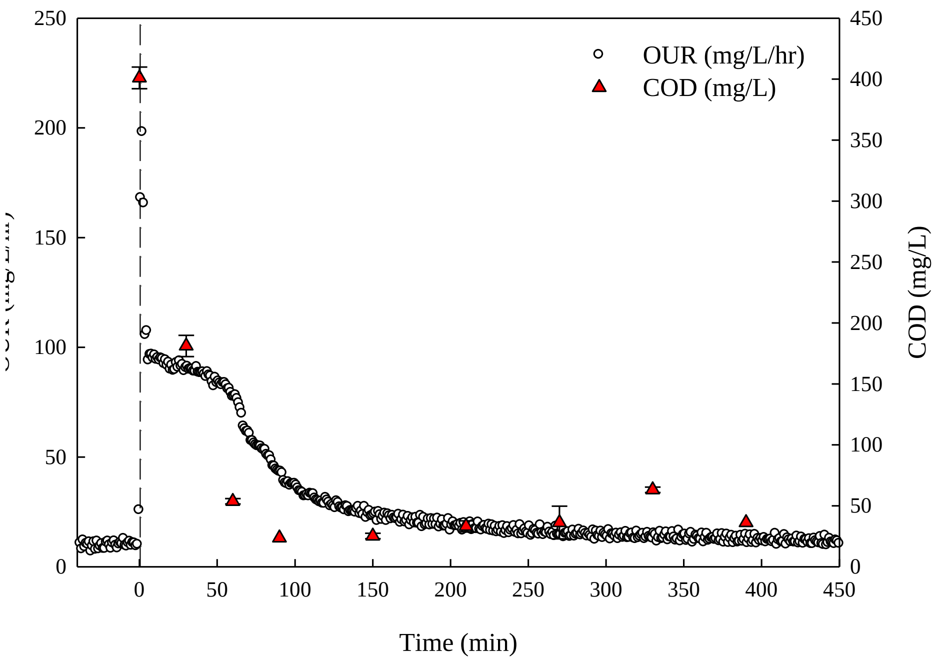


Figure 4.12 : OUR and COD profile of respirometer that run with 0 µg/L diclofenac and biomass of 10 days sludge age diclofenac-acclimated reactor.

10 µg/L diclofenac was added to respirometer reactor (Figure 4.13) to be able to see the similar conditions with the main reactor. It was observed that COD concentration after 150th minute decreased to 32±11 mg/L COD. OUR value increased to 163 mg/L/hr right after peptone addition and decreased to b_H level 250 minutes later. b_H level was observed as 6.5 mg/L/hr. SS and VSS concentrations in the respirometer reactor was measured as 2180±28 mg/L and 1460±56 mg/L, respectively.

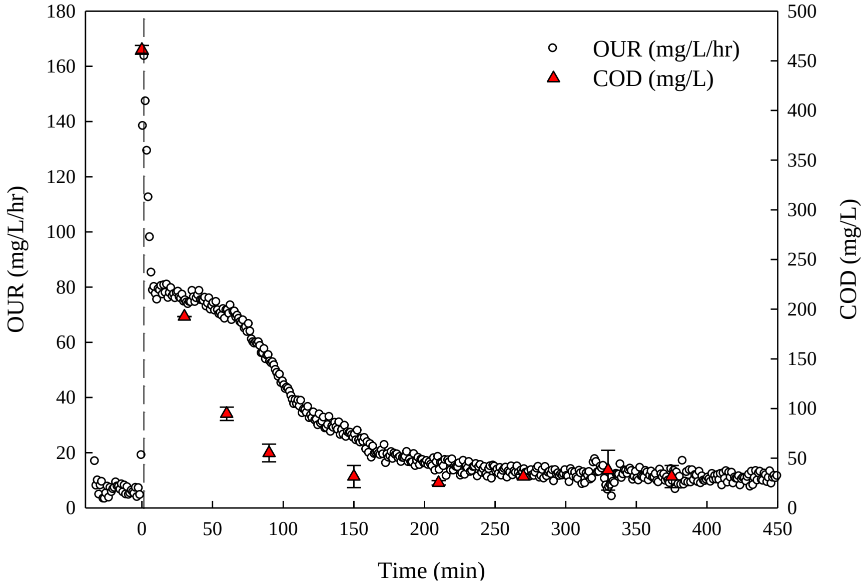


Figure 4.13 : OUR and COD profile of respirometer that run with 10 μg/L diclofenac and biomass of 10 days sludge age diclofenac-acclimated reactor.

Figure 4.14 shows the respirometer set which was carried out with 1000 μg/L diclofenac concentration with diclofenac acclimated reactor of 10 days sludge age. It was seen that COD value decreased from 345 mg/L to 20±0 mg/L level. OUR level jumped to 156 mg/L/hr and than decreased to b_H level after 300th minutes. b_H level was found as 7.6 mg/L/hr. SS concentration in the respirometer reactor was

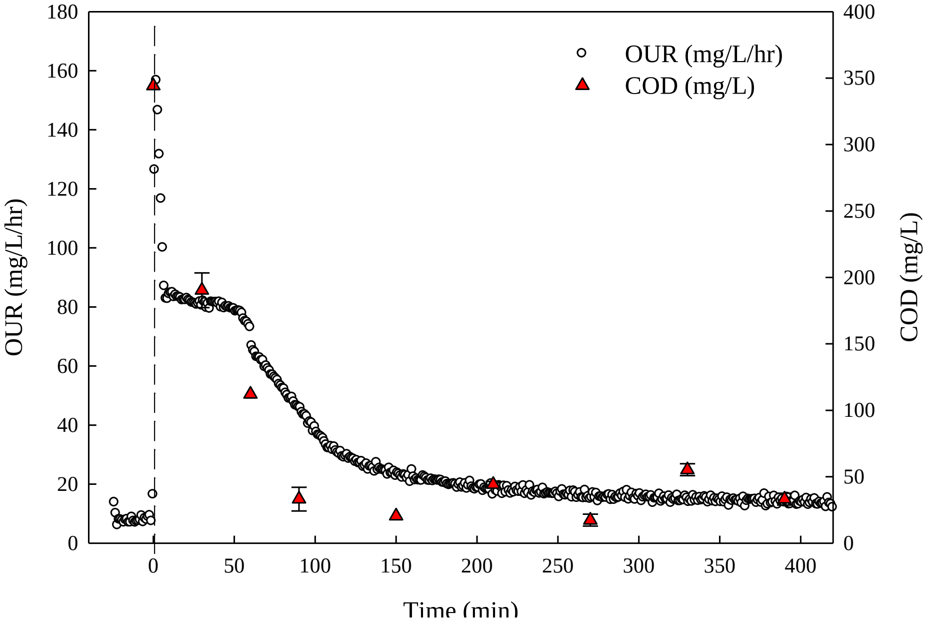


Figure 4.14 : OUR and COD profile of respirometer that run with 1000 μg/L diclofenac and biomass of 10 days sludge age diclofenac-acclimated reactor.

measured as 3050±212 mg/L when VSS concentration was measured as 1400±85 mg/L.

All respirometric experiments which carried out with 10 days of sludge age diclofenac-acclimated reactor are given in Figure 4.15 to be comperable. When OUR profiles of first two concentrations (0 μg/L and 10 μg/L) and 1000 μg/L diclofenac concentrations are compared, it is seen that acute inhibition effect of diclofenac is very low and it slightly decreases the hydrolysis rate of biomass.

When diclofenac acclimated reactor is compared with control reactor, it is obtained that biomass that acclimated to diclofenac can tolerate higher concentration of diclofenac compared to control reactor biomass. Diclofenac has a higher acute inhibitory effect on non-acclimated biomass.

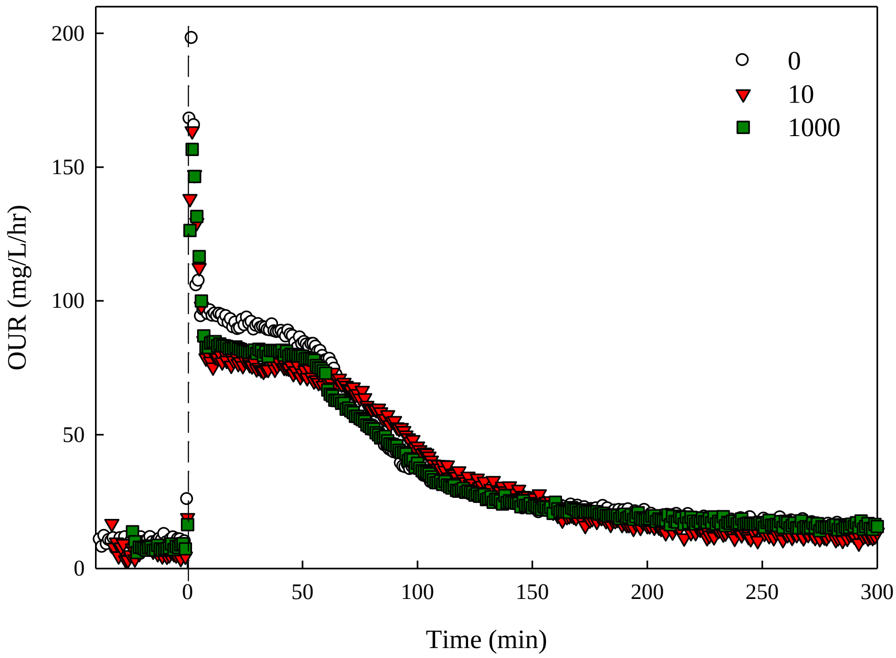


Figure 4.15 : All respirometric data obtained from sludge age 10 days diclofenac-acclimated reactor's biomass.

Respirometeric experiments were carried out also to investigate the chronic effect of diclofenac on biomass that exposed to diclofenac for a long-term period. OUR profiles of respirometric sets which run with 0 μg/L diclofenac additon (only methanol and peptone addition) to diclofenac acclimated and control reactor's biomass are given in Figure 4.16. SS and VSS concentrations of respirometer reactors at the beginning of experiments were 3210±42 mg SS/L and 1520±0 mg

VSS/L for control reactor; 3400±0 mg SS/L and 1700±0 mg VSS/L, respectively. Diclofenac acclimated biomass made an higher jump than control biomass after substrate addition. The main reason of this situation is the initial VSS difference of reactors. It is observed that diclofenac has an important effect on biomass' metabolic structure such as growth rate, hydrolysis and storage after a long term exposure.

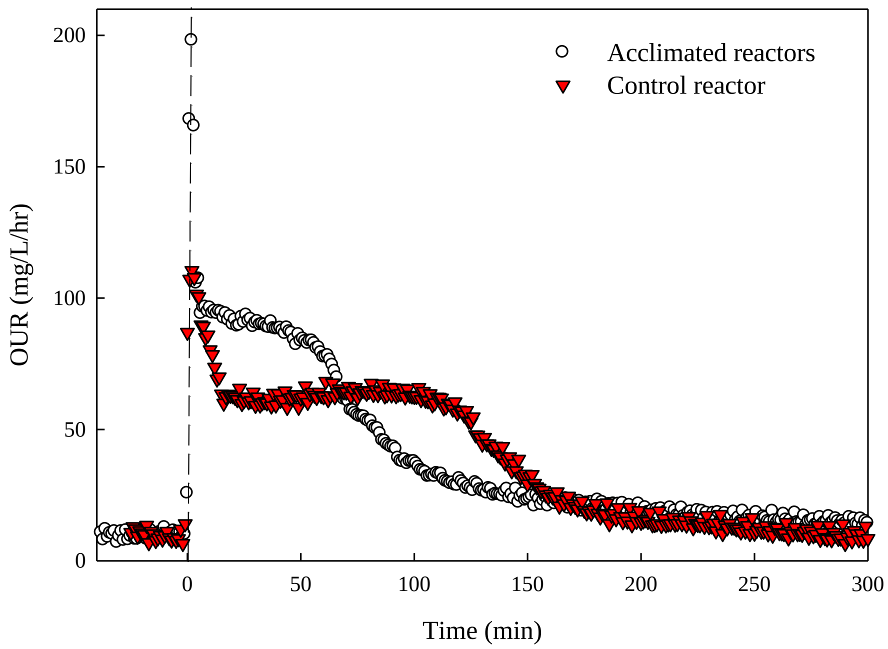


Figure 4.16 : All respirometric data obtained from sludge age 10 days diclofenac-acclimated reactor's biomass.

4.2.2 Respirometric analyses carried with 20 days of sludge age reactors

Acute effect of diclofenac on biomass which was run with 20 days of sludge age was investigated. 4 sets of respirometer analyses (with 0 µg/L, 200 µg/L, 1000 µg/L and 10,000 µg/L diclofenac concentrations) were run with control reactor and 4 sets of respirometer analyses (with 0 µg/L, 10 µg/L 1000 µg/L and 10,000 µg/L diclofenac concentrations) were run for diclofenac reactor.

4.2.2.1 Control reactor

Respirometer experiments were run to investigate acute toxicity of diclofenac on biomass which was obtained from 20 days of sludge age reactor and did not acclimate to diclofenac. Respirographs of 20 days sludge age control reactor are given in Figure 4.17, 4.18, 4.19 and 4.20. Nitrification inhibitor was added to all sets to eliminate nitrification activity into respirometer reactor.

Biomass that taken from 20 days of sludge age control reactor was run with 0 µg/L diclofenac concentration (Figure 4.17) Methanol was added instead of diclofenac-methanol solution to obtain same conditions with other respirometer sets. System was run for half an hour to obtain endogenous decay level of microorganisms. b_H was obtained from graph as 11.3 mg/L/hr. 40 µL methanol peptone was added to respirometer reactor after determining of b_H level.Same as 10 days of sludge age reactors, there was an additive COD source because of methanol addition which effected OUR profile. 376 mg/L COD concentration which added to respirometer reactor at t=0 minute was decreased to 31±2 mg/L COD value end of the 150 minutes. At time t=0 minute, OUR value was read as 145 mg/L/hr. OUR level decreased to b_H level after 250 minutes because of substrate depletion in the reactor. SS and VSS value was found at the beginning of experiment as 2520±0 and 1930±42 mg/L, respectively.

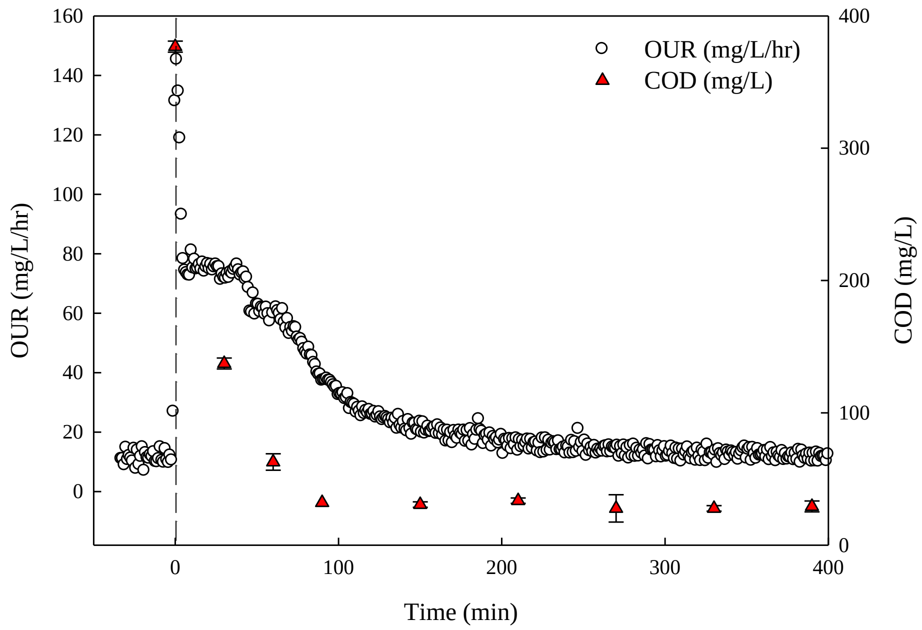


Figure 4.17 : OUR and COD profile of respirometer that run with 0 µg/L diclofenac and biomass of 20 days sludge age control reactor.

Respirometric graph of 200 µg/L diclofenac addition to 20 days sludge age reactor is given in Figure 4.18. b_H level during first one hour was found as 14.3 mg/L/hr. 40 µL diclofenac from 10,000 mg/L diclofenac stock solution was added to respirometer

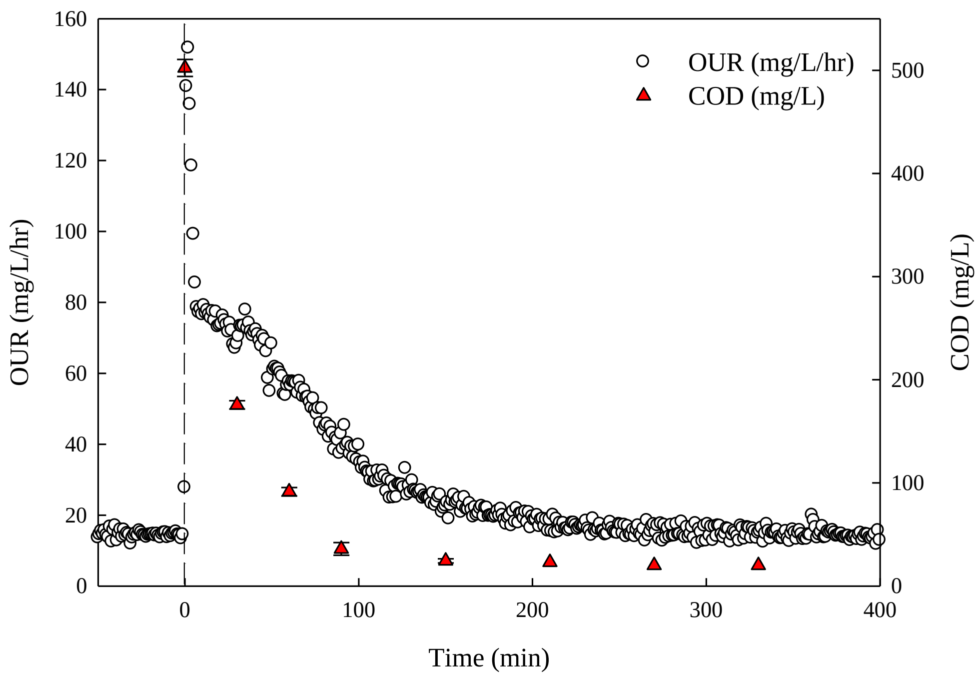


Figure 4.18 : OUR and COD profile of respirometer that run with 200 μg/L diclofenac and biomass of 20 days sludge age control reactor.

reactor together with peptone. OUR level jumped up to 151 mg/L/hr when peptone and diclofenac added. 500 mg/L COD value at t=0 minute decreased to 24±2 mg/L COD. OUR profile and also COD values were relatively close to 0 μg/L diclofenac concentration's COD value. It was seen that acute effect of 200 μg/L diclofenac on biomass was quite low. OUR level decreased to b_H level 250 minutes later. SS and VSS value were found at the beginning of experiment as 2440±28 and 1840±28 mg/L, respectively.

20 days of sludge age control reactor biomass was run with 1000 μg/L diclofenac concentration (Figure 4.19). System was run for a while to obtain endogenous decay level of microorganisms. b_H level was found as 15.7 mg/L/hr. 40 μL diclofenac from 50,000 mg/L diclofenac stock solution was added to respirometer reactor together with peptone and OUR profile obtained. 522 mg/L COD concentration which added to respirometer reactor at t=0 minute was decreased to 53.7±0 mg/L COD value at the end of the 150 minutes. At time t=0 minute, OUR value was read as 137 mg/L/hr. SS and VSS value was found at the beginning of experiment as 2850±42 and 2160±28 mg/L, respectively.

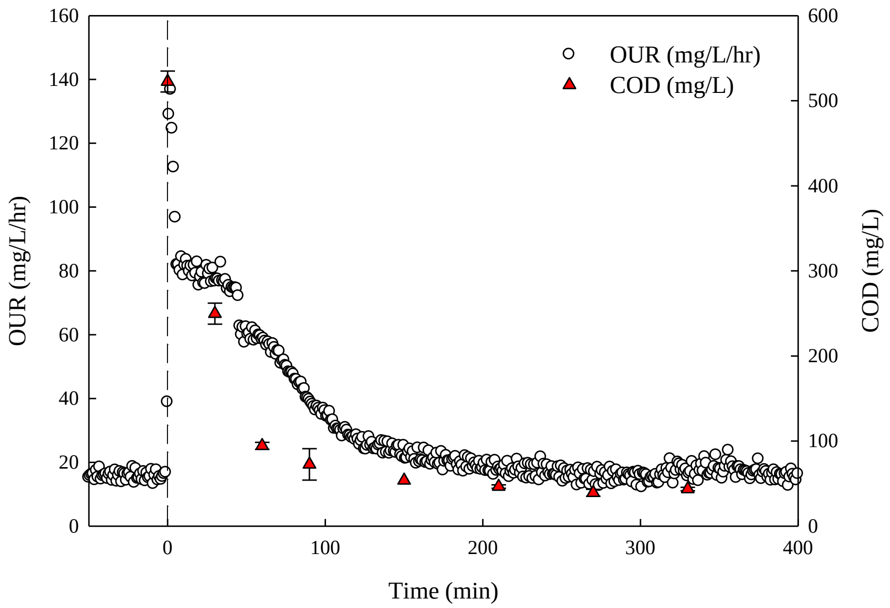


Figure 4.19 : OUR and COD profile of respirometer that run with 1000 µg/L diclofenac and biomass of 20 days sludge age control reactor.

OUR profile of respirometer that run with 10.000 µg/L is given in Figure 4.20. Highest concentration to investigat the acute toxicity of 20 days of sludge age control reactor was selected as 10,000 µg/L for 20 days sludge age control reactor. 100 µL diclofenac was added from 200,000 µg/L diclofenac stock solution to reactor to obtain this diclofenac concentration. b_H level was obtained as 13.4 mg/L/hr. After addition of peptone and diclofenac, OUR was increased to 88 mg/L/hr. COD equivalence of 100 µL methanol corresponds to 42 mg/L and that value makes profile different than previous. OUR value decreased to b_H level 450 minutes later than substrate addition. At the end of the 150th minute, COD value was measured as 53±0 mg/L. In this set, in contrast to 10 days sludge age reactor's, COD was utilized in 150 minutes. This shows that acute inhibition effect of diclofenac on 10 days sludge age biomass is much higher than 20 days's. Inhibitory effect of diclofenac decreased with the increase in the sludge age. It was observed that different OUR and COD profiles was obtained when compared to other concentrations SS and VSS value was found at the beginning of experiment as 2310±42 and 1840±28 mg/L, respectively.

All data obtained from 20 days of sludge age control reactor's respirometer sets are given in Figure 4.21 to be comperable. OUR profiles of 0 µg/L, 200 µg/L and 1000 µg/L were quite similar. So, 200 µg/L and 1000 µg/L diclofenac concentrations did

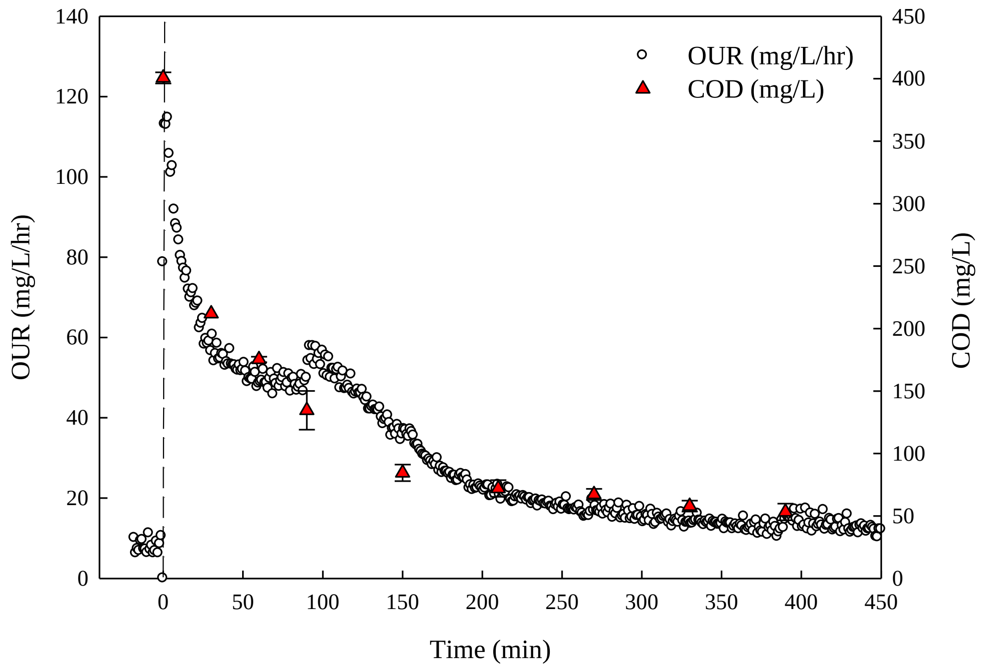


Figure 4.20 : OUR and COD profile of respirometer that run with 10,000 μg/L diclofenac and biomass of 20 days sludge age control reactor..

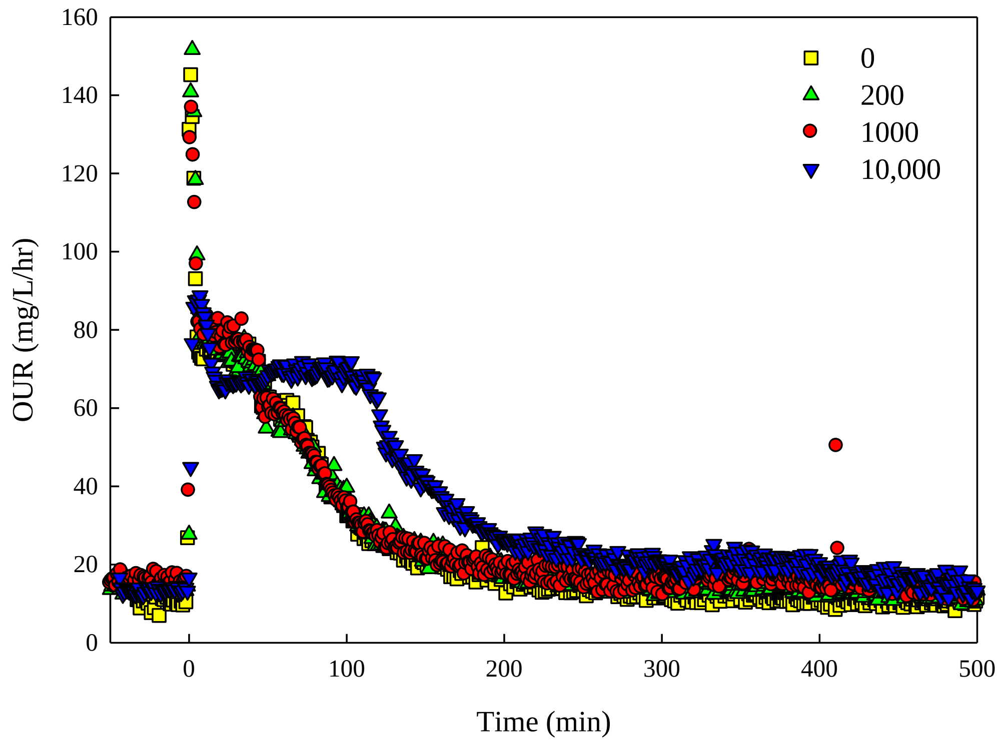


Figure 4.21 : All respirometric data obtained from sludge age 20 days control reactor's biomass.

not have a significant effect on oxygen utilization rate of biomass. Moreover, OUR profiles were completely changed when diclofenac concentration increased to 10,000 μg/L. COD amount which added to system in all sets were equal to each other, except 10,000 μg/L diclofenac concentration. An additional 25 mg/L COD was coming from methanol in this set. Area under the curve of all of respirographes are equal to each other except 10,000 μg/L. That showed the COD amount that added to each sets were same and these substrates are degraded by microorganisms in all sets. It was observed that OUR values started to decrease to b_H level when substrate was degraded. OUR value of all sets except 10,000 μg/L decreased to b_H levels just after than 300 minutes. But it did not decrease to b_H level till 500 minutes in 10,000 μg/L diclofenac concentration set.

4.2.2.2 Diclofenac reactor

Respirometric experiments were run to investigate the acute effect diclofenac on an acclimated biomass obtained from 20 days of sludge age reactor. 4 sets of respirometer experiments with a concentration of 0 μg/L, 10 μg/L, 1000 μg/L and 10,000 μg/L were carried out at this stage. Respirometer experiment graphs of 20 days sludge age diclofenac-acclimated reactor are given in Figure 4.22, 4.23, 4.24 and 4.25. Nitrification inhibitor was added to all sets to avoid nitrification activity into respirometer reactor.

Respirograph of diclofenac-aclimated biomass obtained from 20 days of sludge age reactor with no diclofenac addition is given in Figure 4.22. b_H level was obtained from graph as 8.2 mg/L/hr. 40 μL methanol and peptone was added to respirometer reactor. 150 minutes later, COD value decreased from 378 mg/L COD to 32±2 mg/L. At time t=0 minute, OUR value was read as 126 mg/L/hr. SS and VSS value was found at the beginning of experiment as 2720±56 and 2080±56 mg/L, respectively.

10 μg/L diclofenac was added to respirometer reactor to be able to see the similar conditions with the main reactor. It was observed that COD concentration after 150th minute decreased to 20±8 mg/L COD. OUR value increased to 126 mg/L/hr after peptone addition and decreased to b_H level 300 minutes later. b_H level was observed as 6.7 mg/L/hr. Initial SS and VSS concentrations were measured as 2020±28 mg/L and 1750±42 mg/L, respectively.

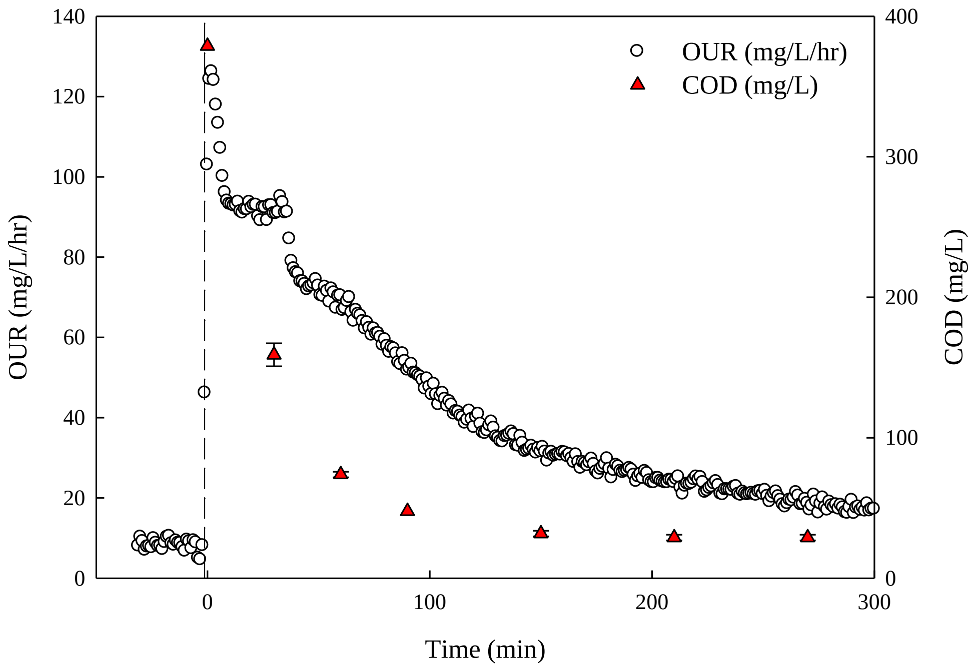


Figure 4.22 : OUR and COD profile of respirometer that run with 0 μg/L diclofenac and biomass of 20 days sludge age diclofenac-acclimated reactor.

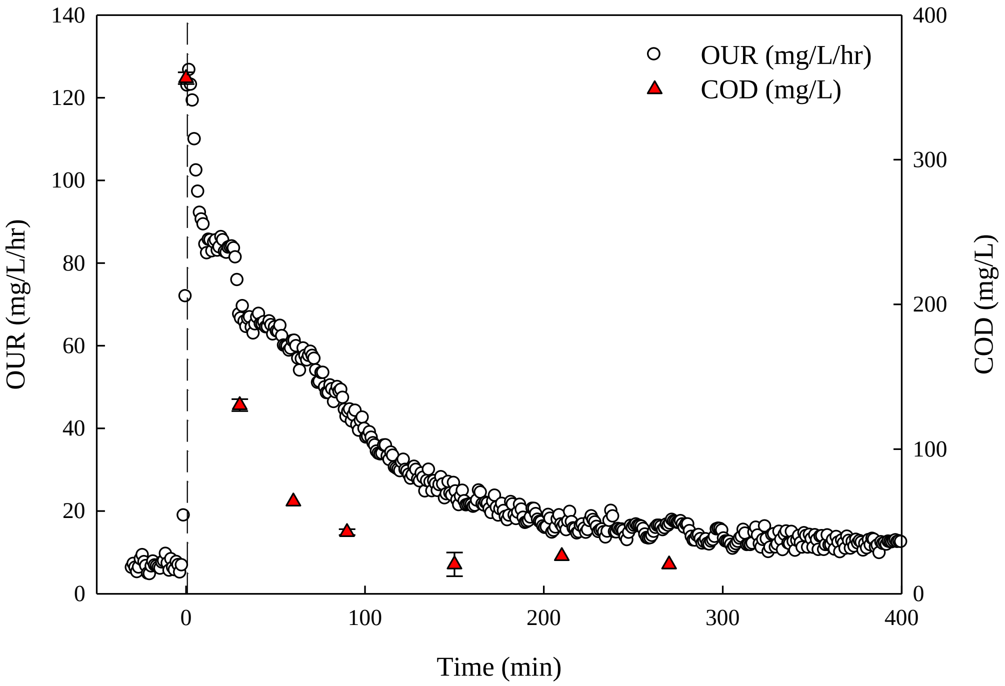


Figure 4.23 : OUR and COD profile of respirometer that run with 10 μg/L diclofenac and biomass of 20 days sludge age diclofenac-acclimated reactor.

Figure 4.24 shows the respirometer set which carried out with 1000 µg/L diclofenac concentration with diclofenac acclimated reactor of 20 days sludge age reactor. It was seen that COD value decreased to 31±0 mg/L level. OUR level jumped to 106 mg/L/hr and than decreased to b_H level after 300th minutes. b_H level was found as 7.0 mg/L/hr. SS concentration in the respirometer reactor was measured as 1910±70 mg/L when VSS concentration was measured as 1590±70 mg/L.

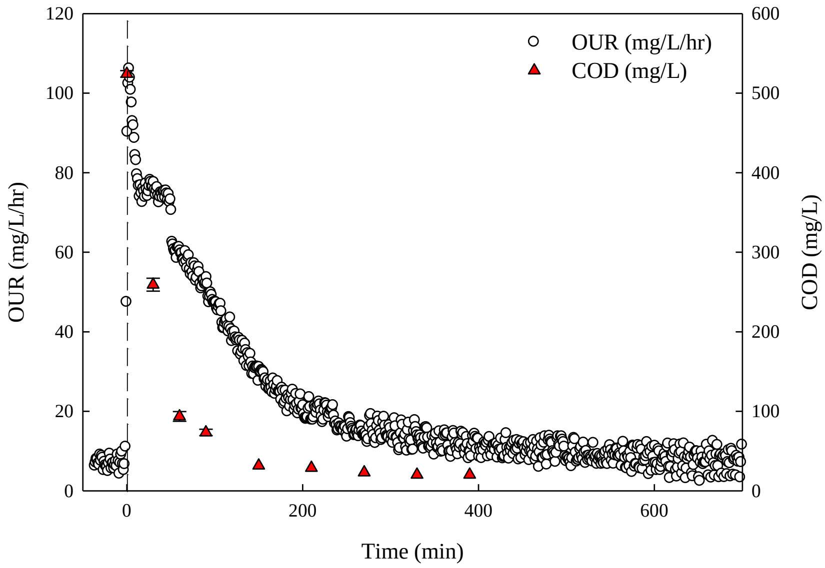


Figure 4.24 : OUR and COD profile of respirometer that run with 1000 µg/L diclofenac and biomass of 20 days sludge age diclofenac-acclimated reactor.

As different from 10 days of sludge age diclofenac acclimated reactors, an additional set of 10,000 µg/L diclofenac concentration was carried out with 20 days of sludge age because sludge age 20 days is more tolerant to the inhibition effect of diclofenac than 10 days sludge age. OUR and COD curve of this set is given in Figure 4.25. b_H level of this set was found as 6.5 mg/L/hr. OUR value spiked to 76 mg/L/hr after substrate addition and than decreased to b_H level after 350 minutes. COD value decreased under 50 mg/L barely after 250 minutes. Initial SS concentration in the respirometer reactor was measured as 1620±56 mg/L when VSS concentration was measured as 1300±28 mg/L.

All respirometric experiments which carried out with 20 days sludge age diclofenac-acclimated reactor are given in Figure 4.26 to be comperable. OUR profiles of first three concentrations 0 µg/L, 10 µg/L and 1000 µg/L were quite similar. But in 10,000 µg/L concentration, decrease of hydrolysis rate and acute inhibition effect of

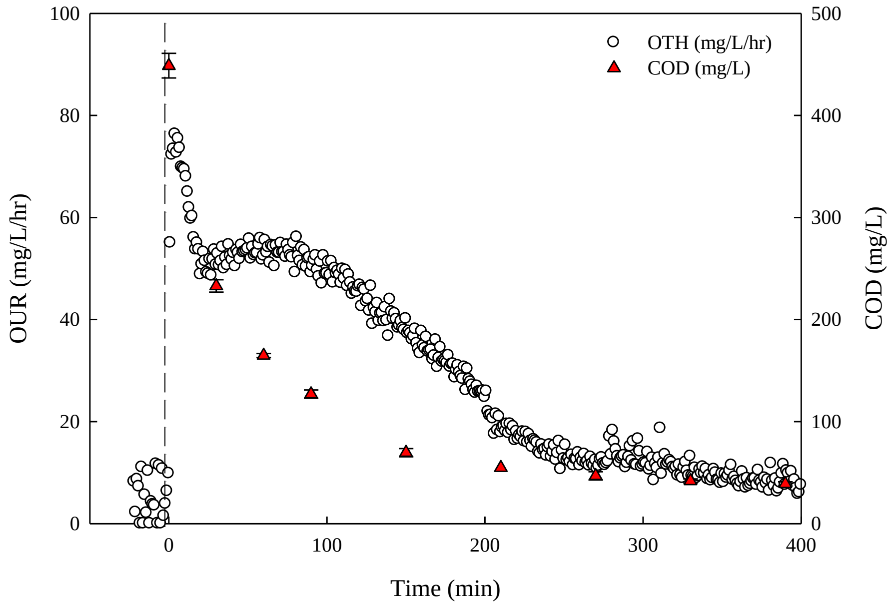


Figure 4.25 : OUR and COD profile of respirometer that run with 10,000 µg/L diclofenac and biomass of 20 days sludge age diclofenac-acclimated reactor.

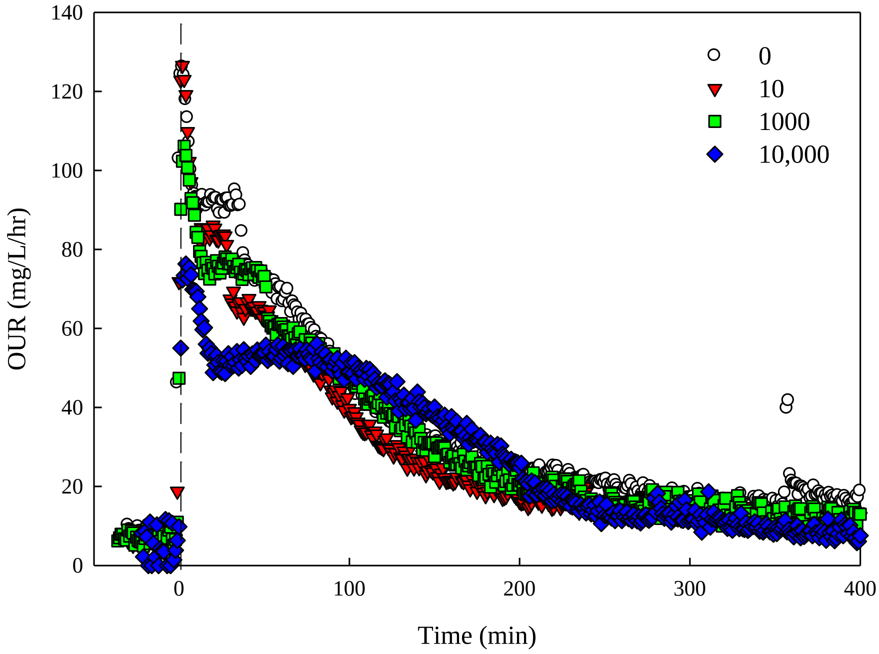


Figure 4.26 : All respirometric data obtained from sludge age 20 days diclofenac-acclimated reactor's biomass.

diclofenac is seen clearly. Due to additive methanol, curve of 10,000 µg/L set has a different way than others. Highest jump of OUR value is observed in 0 and 10 µg/L concentrations because of higher VSS concentrations.

When diclofenac acclimated reactor is compared with control reactor, it is observed that biomass that acclimated to diclofenac can tolerate higher concentration of diclofenac compared to control reactor biomass. Diclofenac has a higher acute inhibitory effect on non-acclimated biomass.

Respirometeric experiments were carried out also to investigate the chronic effect of diclofenac on biomass that exposed to diclofenac for a long-term period. OUR profiles of respirometric sets which run with 0 μg/L diclofenac additon (only methanol and peptone addition) to diclofenac acclimated and control reactor's biomass are given in Figure 4.27. SS and VSS concentrations of respirometer reactors at the beginning of experiments were 2520±0 mg SS/L and 1930±42 mg VSS/L for control reactor; 2720±56 mg SS/L and 2080±56 mg VSS/L, respectively. Same as 10 days of sludge age biomass, diclofenac had also an important effect on 20 days sludge age biomass' metabolic structure such as growth rate, hydrolysis and storage after a long term exposure. But these effects were more apparent in 10 days of sludge age.

It is possible to investigate acute inhibition effect of a chemical on the microbial growth, storage of substrate and hydrolysis rate by using respirometric experiments instead of conventional methods. There is no study which is used respirometric methods to evaluate acute inhibition effect of diclofenac. In respirometric experiments which are carried out to investigate acute inhibition effect of xenobiotics, it is possible to observe kinetic and stoichiometric evalution of biomass.

In a study carried out on erythromycin antibiotic substance, it was observed that erythromycin had an acute inhibition effect on substrate utilization and substrate storage of biomass when erythromycin fed with peptone. Erythromycin did not have an effect on bacterial growth (Pala-Ozkok et al., 2013a). In the same study, it was also found that hydrolysis rate decreased on the biomass having lower sludge age. In another study which studied by Pala-Ozkok et al. (2013b), respirometric experiments were carried out with acclimated biomass and antibiotic of sulfamethoxazole and peptone and acetate was fed to system seperately as substrate to identify acute inhibition effect of sulfamethoxazole on biomass. It was observed that effect of sulfamethoxazole differed depending on the substrate. For peptone, partial substrate utilization was observed but for acetate, a slightly inhibition on microbial growth with full substrate utilization was observed.

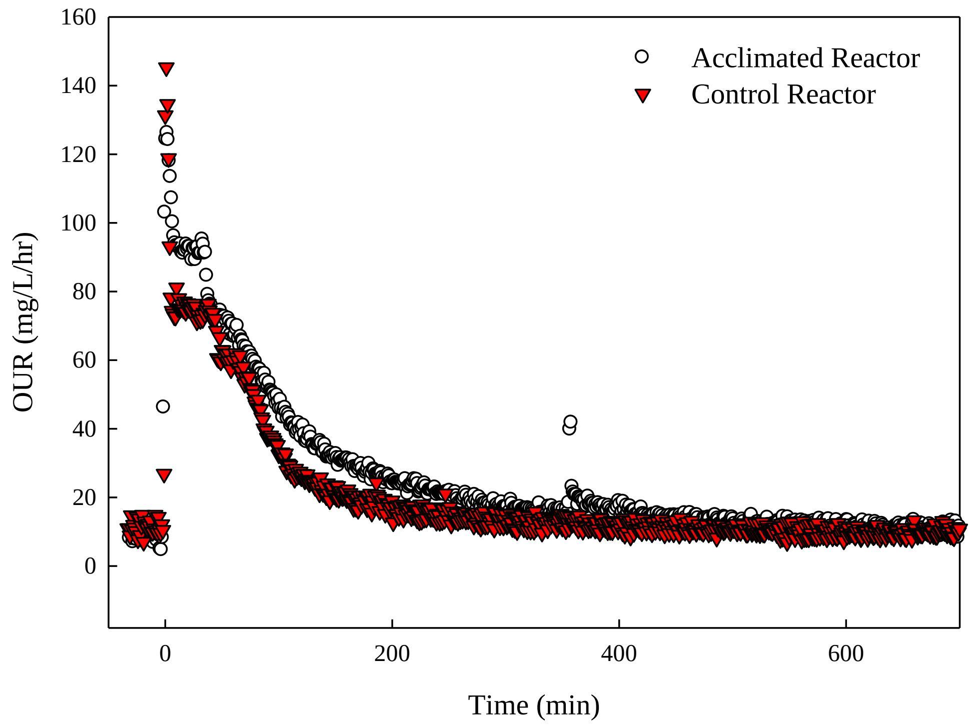


Figure 4.27 : All respirometric data obtained from sludge age 20 days diclofenac-acclimated reactor's biomass.

In a study of Ubay-Cokgor et al. (2011), as relevant to the acute inhibiton effect of biomass which was acclimated and non-acclimated to 2-6 dihydroxybenzoic acid, it was observed that 2-6 dihydroxybenzoic acid had an acute inhibition effect on non-acclimated biomass. When biomass acclimated to same xenobiotic, inhibition effect of chemical decreased. Olmez-Hanci et al., (2011) studied the effect of H-acid (4-Amino-5-hydroxy-2,7-naphthalene disulfonic acid) and respirometric analyses were carried out. Experimental results showed that H-acid was not biodegradable even after prolonged acclimation periods. H-acid also had no inhibitory effect on biodegradation of synthetic sewage but accumulated as soluble inert COD in the treated wastewater. In another study which investigated the acute effect of benzo[a]anthracene, a significant compound among polycyclic aromatic hydrocarbons, it was observed that lower concentrations had no effect on process kinetics but noticeable acute impact was observed with the addition of 88 mg/L of benzo[a]anthracene (Başak et al., 2012).

4.3 Modelling Results

Modelling is now regarded as a useful instrument for understanding substrate utilization and kinetic and stoichiometric changes in biomass.

Model simulations of 20 days of sludge age control and diclofenac reactors which were run with only methanol and peptone addition was studied. Activated Sludge Model-1 (ASM-1) was used in this study due to no PHA storage occurred. Kinetic and stoichiometric parameters were estimated using AQUASIM simulation program.

Model simulation results of OUR profile of 20 days of sludge age control reactor which carried out with only methanol addition is given in Figure 4.28. 40 μL methanol was added to respirometer reactor and total volume of reactor was 2 L. Nitrification inhibitor was added to system.

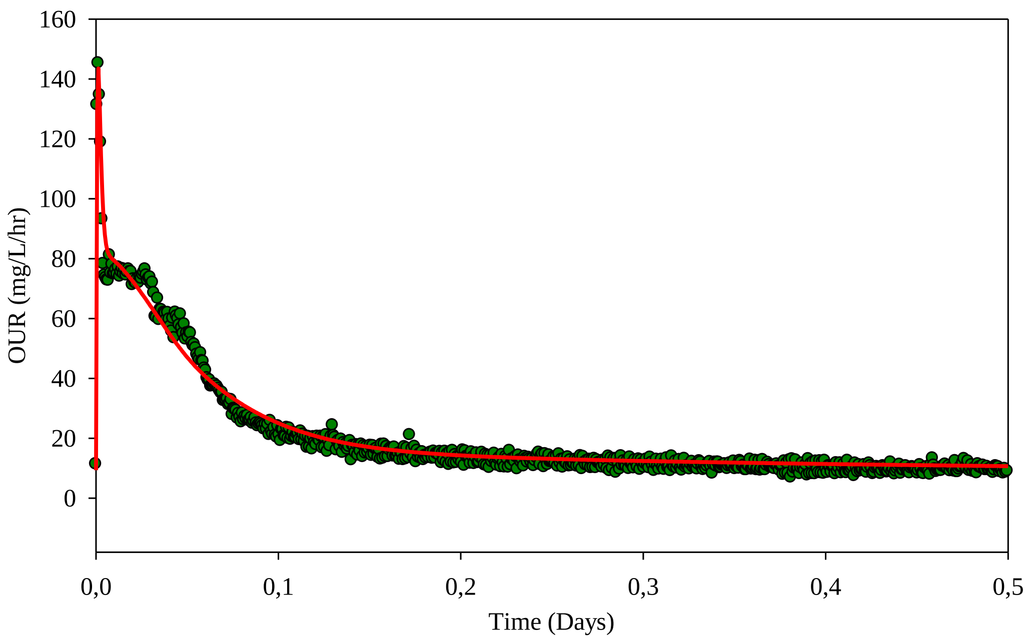


Figure 4.28 : Model simulation of OUR profile for 20 days of sludge age control reactor with 40 μL methanol addition.

20 days of sludge age diclofenac acclimated biomass was run with 40 μL methanol in respirometer reactor. Model simulation results of OUR profile is given in Figure 4.29.

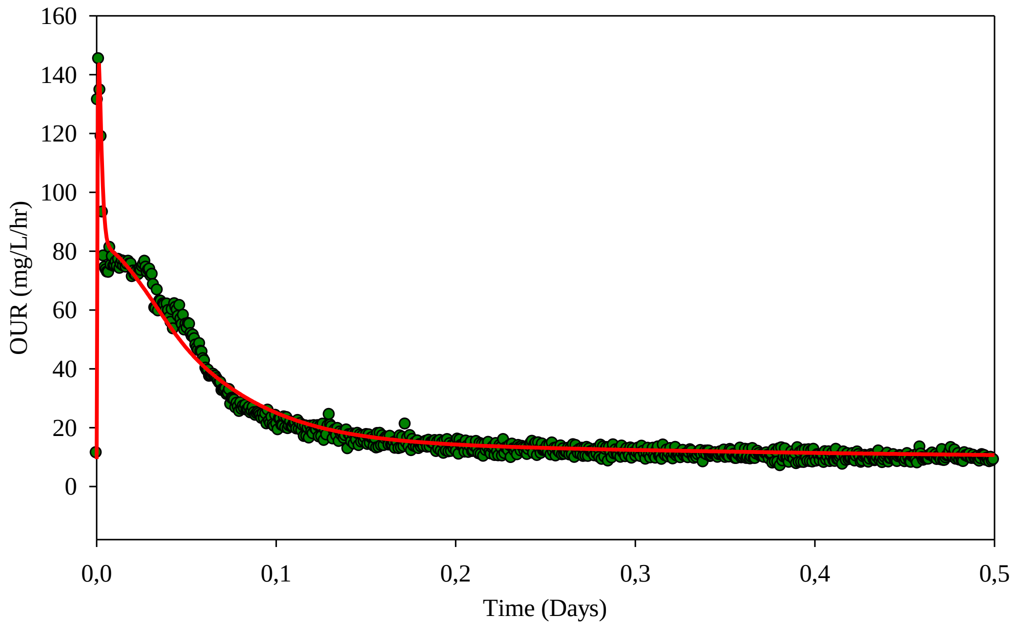


Figure 4.29 : Model simulation of OUR profile for 20 days of sludge age diclofenac reactor with 40 µL methanol addition.

Model simulation results based on ASM-1 of modelled OUR profiles are given in Table 4.3.

According to calibration results obtained from OUR profiles which are given in Table 4.3, all values are same except half saturation constant for growth of heterotrophic biomass, K_{S1}. This value for control reactor's biomass, which is acclimated to methanol during 160 days, was found as 15 mg COD/L. K_{S1} value increased to 30 mg COD/L for diclofenac-acclimated biomass.

Increment of K_{S1} value from 15 mg COD/L to 30 mg COD/L shows that diclofenac had a chronic inhbition effect on 20 days of sludge age biomass. But diclofenac did not have a chronic inhibition effect on total activity of biomass, biomass activity percentage for both two experiment was found as 51%.

Similarly to this result, Pala-Ozkok et al. (2013a) studied the acute impact effect of erythromycin on biomass and they found that K_S value increased with increment of erythromycin concentration. In another study observing the effect of different substrates on acute effect of sulfamethoxazole (Pala-Ozkok et al., 2013b), likewise previous study, half saturation constant increased and activity decreased with increment of sulfamethoxazole concentration when acetate was using as substrate.

Table 4.3 : Calibration results of the methanol addition to 20 days of sludge age control and diclofenac reactor biomass.

State Variables		**Unit**	**Control**	**Diclofenac**
Peptone Mixture				
Maximum specific growth rate for X_H	$\hat{\mu}_{H1}$	1/day	7	7
Half saturation constant for growth of X_H	K_{S1}	mg COD/L	15	30
Maximum hydrolysis rate for S_{H1}	k_{h1}	1/day	6	6
Hydrolysis half saturation constant for S_{H1}	K_X	g COD/g COD	0.22	0.22
Maximum hydrolysis rate for S_{H2}	k_{h2}	1/day	0.93	0.93
Hydrolysis half saturation constant for S_{H2}	K_{XX}	g COD/g COD	0.22	0.22
Endogenous decay rate for X_H	b_H	1/day	0.2	0.2
Methanol				
Maximum specific growth rate for X_H	$\hat{\mu}_{H2}$	1/day	0.28	0.28
Half saturation constant for growth of X_H	K_{S2}	mg COD/L	6	6
Yield coefficient for X_H	Y_H	g COD/g COD	0.58	0.58
Volatile suspended solids	X_T	mg /L	1930	2080
Volatile suspended solids	X_T	mg COD/L	2740	2953
Total activity	-	%	51	51
Initial active heterotrophic biomass	X_{H1}	mg COD/L	1400	1500
Initial biodegradable peptone mixture COD	C_{S1}	mg COD/L	300	430
Initial readily biodegradable peptone mixture COD	S_{S1}	mg COD/L	12	16
Initial readily hydrolizable peptone mixture COD	S_{H11}	mg COD/L	181	260
Initial slowly hydrolizable peptone mixture COD	S_{H21}	mg COD/L	155	103
Initial methanol COD	S_{S2}	mg COD/L	16	16

5. CONCLUSION AND RECOMMENDATION

The objective of this study was to investigate biodegradability of diclofenac under aerobic conditions and its acute and chronic effect on biomass with different sludge age. Therefore, this research focused on the fate and effect of diclofenac, which is a non-steroidal anti-inflammatory (NSAID) drug. The chronic inhibitory effect of diclofenac on the biomass concentration, substrate utilization rate and biodegradability assays were investigated under aerobic conditions with different sludge ages.

Laboratory scale fill and draw reactors were operated to examine the effect of sludge age on biodegradability of diclofenac and two different sludge age were selected within this scope. According to laboratory scale reactor operations, it was observed that removal of diclofenac changed with sludge retention time and also acclimation of biomass to diclofenac. No diclofenac removal was observed in 10 days of sludge age when 30% diclofenac removal achieved right after acclimation period of 20 days of sludge age. Beside this, a slight chronic inhibition effect of diclofenac on biomass concentration and substrate utilization rate was observed only for 20 days of sludge age. Maximum 2% of diclofenac has adsorbed onto sludge when most of them stayed in aqueous phase. It has tendency to remain in aqueous phase instead of sorption onto the solid phase.

Respirometric measurements were carried out with different concentrations of diclofenac to determine potential microbial acute toxicity of diclofenac. According to respirometric sets which carried out with 10 days of sludge age control reactor biomass, it was seen that 200 μg/L diclofenac concentration did not have an acute inhibition on biomass. Acute inhibition effect of diclofenac on non-acclimated biomass was observed with 1000 μg/L of dclofenac concentration. Similarly, 1000 μg/L diclofenac concentration had an acute inhibition effect on biomass that acclimated to 10 μg/L diclofenac with 10 days of sludge age. In addition to this, acclimated and non-acclimated biomass was compared according to respirographs. It was observed that long term exposure to diclofenac changes metabolic structure of biomass such as growth rate, hydrolysis, storage mechanism.

According to respirometric sets which run with 20 days of sludge age control reactors' biomass, 10,000 μg/L diclofenac had an acute inhibition effect when 200 and 1000 μg/L diclofenac concentrations did not affect non-acclimated biomass. Likewise to non-acclimated biomass, 10,000 μg/L diclofenac concentration had an acute inhibition effect on acclimated biomass to diclofenac in this sludge age. Similar to 10 days of sludge age, it was observed that long term exposure to diclofenac had an important effect on metabolic structure of biomass.

Consequently, higher SRT values and acclimation of biomass to diclofenac have a positive effect on biodegradability of diclofenac. In addition, acute inhibition effect of diclofenac decreased with increment of SRT. At the same time, chronic inhibition effect of diclofenac increased with an increase in SRT.

According to modelling results, it was seen that half saturation constant for growth of heterotrophic biomass (K_{S1}) of 20 days of sludge age control reactor was lower than diclofenac-acclimated biomass which had the same sludge age. This was the indicator of acute inhibition effect of diclofenac on biomass at 20 days sludge age.

Finally, it is recommended that future studies on diclofenac should include biodegradation of diclofenac and its toxic inhibition effects on biomass. Tertiary treatment methods should be applied beside aerobic treatment for removal of diclofenac. Studies on the microbial groups and species that are responsible for biodegradation of diclofenac have not been done yet. Different configurations should be applied beside aerated systems. And also treatment mechanism and byproducts of diclofenac should be examined to understand the diclofenac removal mechanism.

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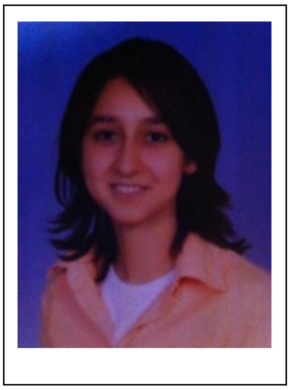

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