ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF SEASONAL REMOVAL EFFICIENCY OF DICLOFENAC IN A BIOLOGICAL WASTEWATER TREATMENT PLANT AND BIODEGRADABILITY POTENTIAL IN LAB SCALE ANAEROBIC REACTORS

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BİYOLOJİK ATIKSU ARITMA TESİSİNDE DİKLOFENAK'IN MEVSİMSEL GİDERİM VERİMİNİN VE LABORATUVAR ÖLÇEKLİ ANAEROBİK ARITILABİLİRLİĞİNİN İNCELENMESİ

YÜKSEK LİSANS TEZİ

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Sevgi SARI, a M.Sc. student of ITU Graduate School of Science, Engineering and Technology. Student ID 501111729, successfully defended the thesis entitled "Investigation of seasonal removal efficiency of diclofenac in a biological wastewater treatment plant and bidegradability potential in lab scale anaerobic reactors", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 5 May 2013
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To my family especially my mum and dad,

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ABBREVIATIONS

AD	**:** Anaerobic Digestion
ACN	**:** Acetonitrile
COD	**:** Chemical Oxygen Demand
DCF	**:** Diclofenac
DDD	**:** Defined Daily Dose
EC	**:** Effect Concentration
LC	**:** Lethal Concentration
LC-MS/MS	**:** Liquid Chromatography Coupled with Tandem Mass Spectrometry
LOEC	**:** Lowest Observed Effect Concentration
MeOH	**:** Methanol
NOEC	**:** No Observed Effect Concentration
NSAID	**:** Non-steroidal Anti-inflammatory Drug
ORP	**:** Oxidation Reduction Potential
TKN	**:** Total Kjehldal Nitrogen
TP	**:** Total Phosphorous
TS	**:** Total Solids
UPLC	**:** Ultra Performance Liquid Chromatograph
VFA	**:** Volatile Fatty Acid
VSS	**:** Volatile Suspended Solid
WWTP	**:** Wastewater Treatment Plant

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INVESTIGATION OF SEASONAL REMOVAL EFFICIENCY OF DICLOFENAC IN A BIOLOGICAL WASTEWATER TREATMENT PLANT AND BIODEGRADABILITY POTENTIAL IN LAB SCALE ANAEROBIC REACTORS

SUMMARY

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) that is used to treat the symptoms of pain and inflammation. Diclofenac and its metabolites excreted subsquently enter the ecosystem due to improper treatment in wastewater treatment plants and leads to toxic effects on the environmental ecology.

This research is conducted to understand the behavior of diclofenac in wastewater treatment plants as well as its bidegradability potential under anaerobic conditions.

To screen diclofenac behaviour in wastewater treatment plant, four sampling campaigns including summer, autumn, winter, and spring has been carried out in a biological wastewater treatment plant located in Istanbul. Diclofenac concentration in each units of wastewater treatment plant has been searched and removal efficiency in the wastewater treatment plant has been investigated. In addition, seasonal variations in diclofenac removal have been evaluated. Diclofenac concentrations in the plan influent have been measured as 846, 752, 1377, and 923 ng/L in summer, autumn, winter, and spring, respectively. In addition, diclofenac removal efficiencies have been found as 51%, 12%, 27%, and %18 in summer, autumn, winter, and spring, respectively. Operation efficiency activated sludge process has been correlated with diclofenac elimination, because diclofenac removal has been decreased with decreasing nitrogen removal.

The effect of diclofenac on anaerobic treatment has been studied by using anaerobic reactors operating in the presence of diclofenac for a long period of time.Batch experiments were conducted to understand the effect of initial diclofenac concentration, temperature, pH, and biomass concentration on its biodegradation potential. Parameters such as methane production, accumulation and consumption of

volatile fatty acids (VFAs), volatile suspended solids (VSS), total suspended solids (SS), and dissolved organic carbon (DOC) have been used to evaluate diclofenac removal efficiency as well as potential inhibition.

The behaviour of diclofenac in anaerobic bioreactors has been studied with the cultures developed using innoculum taken from different places. All reactors have been operated with 80 days sludge retention time and fed weekly with 300 and 15 mg/L glucose and yeast extract as substrate and 10 µg/L diclofenac dissolved in methanol. Four main reactors called first generation reactors inoculated with river sediment or anaerobic digestion sludge and kept at 22ºC in mesophilic condition in the dark. Diclofenac removal has been obtained between the range of 13 and 31% in these reactors. In addition, a reactor inoculated with anaerobic digester sludge has been operated at 35ºC in dark to examine temperature effect and 21% diclofenac removal has been obtained.

In order to understand the behaviour of diclofenac in anaerobic reactors, four anaerobic batch assays including different diclofenac concentrations, different temperature, different biomass concentration, and different carbon source have been performed.

To examine the toxic effect of diclofenac on anaerobic microorganisms five batch reactors with different diclofenac concentrations including 0, 10, 50, 200, and 1000 µg/L have been performed. 1000 µg/L of diclofenac concentrations has revealed very low inhibitory effects on methanogens that has shown itself by a decrease in methane production as well as in VSS concentrations**.**

Also, diclofenac removal efficiencies have examined in four different temperatures including 10ºC, 22 ºC, 35ºC, and 45ºC. No relation has been found between temperature and diclofenac removal efficiency.

Then, the effect of biomass concentration on diclofenac degradability assessed with 100, 75, 50, and 25 % dilution of biomass. Diclofenac removal efficiency has been increasing with an increasing initial biomass concentration at the first 3 days of the operation.

Finally, different carbon source assay has been carried out by using acetate as a carbon source where 25% diclofenac removal has been observed.

BİYOLOJİK ATIKSU ARITMA TESİSİNDE DİKLOFENAK'IN MEVSİMSEL GİDERİM VERİMİNİN VE LABORATUVAR ÖLÇEKLİ ANAEROBİK ARITILABİLİRLİĞİNİN İNCELENMESİ

ÖZET

Yüksek kullanımı ve biyolojik olarak zor ayrışabilen yapısı nedeniyle diklofenak, atıksu arıtma tesislerinin çıkış akımlarında, yüzeysel sularda, yer altı sularında ve hatta içme sularında rastlanan en önemli kirleticiler arasında yer almaktadır (Heberer, 2002). Diklofenak sentetik bir ilaç olarak steroid yapıya sahip olmayan antienflamatuar ilaçlar (NSAİDs) grubunda yer almaktadır. Hem insanlar tarafından hem de veterinerlik alanında enflamasyonu azaltmak ve ağrıyı dindirmek için artrit ve akut sakatlanmalarda kullanılmaktadır. Diklofenak bir çok ülkede yıllık onlarca ton mertebesinde kullanılmaktadır. Dünyada yıllık 940 ton kullanımıyla diklofenak, en çok tüketilen steroid yapıya sahip olmayan antienflamatuar ilaçlar arasında bulunmaktadır (Zhang ve diğ., 2008). Ayrıca ülkemizde 2009 yılında satılmış olan diklofenak miktarı yaklaşık 70 ton olarak belirlenmiştir (IMS Health, 2010).

Son yıllarda diklofenak maddesinin sucul ve diğer ortamlarda görülmesi ve bu ilacın sucul ortamlarda yaşayan canlılara olan olumsuz etkileri endişeleri arttırmıştır. Farmasötik maddelerin en toksik üyesi olarak düşünülen diklofenak, böbrek ve gastrointestinal dokular üzerinde zararlı etkilere sahiptir (Haap ve diğ., 2008). Kazara bu kimyasala maruz kalan Asya'daki akbaba populasyonu, yok olma tehlikesiyle karşı karşıya kalmıştır (EEA, 2010). Bu nedenle diklofenak bir çok araştırmacı tarafından çevresel bir tehdit olarak öne çıkarılmaktadır (Naidoo ve diğ., 2009; Oaks ve diğ., 2004).

Sucul ortamda diklofenak ekolojik toksisitesi nispeten düşük olmasına ve akut etkileri belirlenemeyen düzeylerde olmasına rağmen, sürekli bu ilaca maruz kalma durumunda canlılar üzerinde olumsuz etkileri gözlenmiştir.

Gökkuşağı alabalığı (*Oncorhynchus mykiss*) üzerinde yapılan kronik toksisite çalışmalarında, 28 gün boyunca 1 g/L diklofenağa maruz kalan alabalıklarda

karaciğer, böbrek ve solungaçlarda sitolojik değişikliklerin meydana geldiği gözlenmiştir. Ayrıca yapılan başka bir araştırmada 23 mg/L diklofenak konsantrasyonun alglerin çoğlamasını inhibisyona uğrattığı gözlenmiştir (Delorenzo ve diğ., 2008). Ayrıca 0.01-10 mg/L diklofenak konsantrasyonuna maruz kalan balıklarda yumurtadan çıkma döneminde gecikme ve yumurtadan çıkmada başarısızlık gözlenmiştir (Lee ve diğ., 2011).

Geleneksel evsel atıksu arıtma tesisleri organik maddeler, azot ve fosfor gibi nutrientlerin giderimini gerçekleştirmek amacıyla aktif çamur sistemleri ile projelendirilmiştir. Diklofenak konvansiyonel aktif çamur sistemleri ile yüksek oranda arıtılamadığından arıtma tesisi çıkış sularında ve alıcı ortamlarda ng/L ile µg/L seviyelerinde bulunmaktadır.

Bu çalışma Türkiye'de bulunan mevcut bir atıksu arıtma tesisinde üniteler bazında ve tesis bütününde diklofenak'ın mevsimsel giderimini incelemek, laboratuvar ölçekli yarı kesikli anaerobik reaktörlerde diklofenak'ın biyolojik arıtılabilirliğini araştırmak ve son olarak kesikli anaerobik testlerde çevresel ve işletme koşullarının (sıcaklık, diklofenak konsantrasyonu, biyokütle konsantrasyonu ve karbon kaynağı) diklofenak giderimine etkisini gözlemlemek amacıyla gerçekleştirilmiştir.

Diklofenak arıtılabilirliğini mevsimsel olarak incelemek amacıyla İstanbul'da bulunan 600000 m_3/gün kapasiteli ileri biyolojik atıksu arıtma tesisinden yaz, sonbahar, kış ve ilkbahar aylarında olmak üzere 4 defa numune alınmıştır. Tesis giriş akımında diklofenak konsantrasyonu yaz, ilkbahar, kış ve sonbahar aylarında sırasıyla 846, 752, 1377, 923 ng/L olarak ölçülmüştür. Tesis giriş ve çıkış akımlarından alınan 24 saatlik kompozit numunelerde yapılan diklofenak ölçümlerine göre yaz, ilkbahar, kış ve sonbahar aylarında diklofenak giderimi sırasıyla %51, 12, 27 ve 18 olarak bulunmuştur. Ayrıca karbon, azot ve fosfor gibi konvansiyonel parametreler incelenmiş ve aktif çamur sisteminin verimliliğinin diklofenak giderimini etkilediği de gözlemlenmiştir. Aktif çamur sisteminde azot arıtımındaki değişimler diklofenak giderimiyle benzer değişimler göstermiştir. Ünite bazında yapılan diklofenak ölçümleri neticesinde maksimum diklofenak giderimi anaerobik biyofosfor tankında %32 ile %40 arasında bulunmuştur.

Diklofenak'ın anaerobik koşullar altında biyolojik arıtılabilirliğini incelemek amacıyla laboratuvar koşullarında, yoğun kirliliğe maruz kalmış nehir yataklarından

alınan sedimentler ve atıksu arıtma tesislerinin anaerobik çürütücülerinden alınan çamurlar aşı olarak kullanılarak 2 L hacimli 5 adet anaerobik reaktör kurulmuştur. Reaktörler 80 gün çamur yaşında yarı kesikli olarak çalıştırılmıştır. Reaktörler glikoz ve diklofenak konsantrasyonu sırasıyla 300 mg/L ve 10 µg/L olacak şekilde beslenmiştir. 22ºC'de çalıştırılan reaktörlerde diklofenak giderimi %14 ila %31 arasında değişmiştir. Sediment etkisini azaltmak ve biyokütle aklimasyonunun diklofenak giderimine etkisini incelemek amacıyla reaktör içerisinden alınan çamurun aşı olarak kullanıldığı ikinci nesil yeni bir reaktör kurulmuş ve diklofenak giderimi bu reaktörde %35 olarak bulunmuştur.

Çevresel ve işletme koşullarının diklofenak giderimine etkisini incelemek amacıyla laboratuvar ortamında kesikli olarak beslenen 180 mL hacimli anaerobik reaktörler işletilmiştir. Sıcaklık, diklofenak konsantrasyonu, başlangıç biyokütle konsantrasyonu, ve farklı karbon kaynağı olmak üzere dört farklı set kurulmuştur.

Diklofenak giderimine sıcaklığın etkisini incelemek amacıyla 10, 20, 35 ve 45ºC olmak üzere dört farklı sıcaklıkta çalışılmıştır. Reaktörler kesikli olarak içerisinde 300 mg/L glikoz ve 50 µg/L diklofenak konsantrasyonu olacak şekide beslenmiştir. 10, 20, 35 ve 45ºC'deki reaktörlerde diklofenak giderimi sırasıyla %19, 19, 27 ve 22 olarak bulunmuştur.

Anaerobik reaktörlerde diklofenak konsantrasyonunun fermentativ mikroorganizmalar ve metanojenler üzerindeki inhibe edici etkisini incelemek amacıyla diklofenak konsatrasyonları 0, 10, 50, 200 ve 1000 µg/L olan beş farklı reaktör kurulmuştur. Reaktörler kesikli olarak içerisinde 300 mg/L glikoz olacak şekide 22ºC'de beslenmiştir. Ölçümler neticesinde diklofenak gideriminin diklofenak konsantrasyonunun artmasıyla arttığı gözlemlenmiştir. Ayrıca diklofenak konsantrasyonu 1000 µg/L olan reaktörde diğer reaktörlere göre biraz daha düşük metan üretimi gözlenmiştir.

Başlangıç biyokütle konsantrasyonunun diklofenak giderimi üzerinde etkisini incelemek amacıyla % 100, 75, 50 ve 25 biyokütle içeren dört farklı reaktör çalıştırılmıştır. Reaktörler kesikli olarak içerisinde 300 mg/L glikoz ve 50 µg/L diklofenak olacak şekide 22ºC'de beslenmiştir. İlk 3 günde %100 ve %75 biyokütle içeren reaktörlerde daha yüksek giderim gözlenmiş ama sonrasında bütün reaktörlerde birbirine yakın giderim verimleri gözlenmiştir. % 100, 75, 50 ve 25

biyokütle içeren reaktörlerde sırasıyla % 26, 23, 21 ve 21 diklofenak giderimi elde edilmiştir.

Farklı karbon kaynağı kullanımının diklofenak arıtımı üzerinde etkisini incelemek amacıyla karbon kaynağı olarak glikoz yerine asetatın kullanıldığı bir reaktör çalıştırılmıştır. Reaktör kesikli olarak içerisinde 600 mg/L asetat ve 50 µg/L diklofenak olacak şekide 22ºC'de beslenmiş ve bu deney neticesinde diklofenak giderimi %2 olarak elde edilmiştir.

Çevresel ve işletme koşullarının incelendiği kesikli testlerde elde edilen giderim verimleri karbon kaynağının reaktörde mevcut olduğu süre içinde elde edilmiştir. Karbon kaynağı reaktörde bittiğinde diklofenak konsantrasyonunda artış gözlenmiştir. Bu artışın nedeni diklofenak'ın öncelikle mikroorganizmalar tarafından ara bir ürüne çevrilmesi ve ortamdaki karbon kaynağı tükendiğinde tekrar diklofenak'a geri dönüşmesi olarak düşünülmektedir.

1. INTRODUCTION

Diclofenac has been increasing concern due to its increasing presence in environment with rising global consumption. Diclofenac is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAID) with annually 940 tonnes global consumption and used in the treatment of arthritis, ankylosing spondylitis, and acute muscle pain (Zhang et al., 2008; Barbieri et al., 2012).

Diclofenac has been identified as a problem for the water cycle because of its low removal rate during wastewater treatment processes (Huber et al., 2012). Recent studies report that the removal of diclofenac in wastewater treatment plants is often incomplete with treatment efficiencies ranging from less than 20–80% (Zhang et al., 2008). As a result, the diclofenac and its metabolites excreted subsequently enter to the ecosystem.

The threat of extinction in the Indian vultures due to accidental exposure to diclofenac in 2004 resulted in the designation of diclofenac as an environmental concern (EEA, 2010). The birds died because of renal failure after the consumption of livestock carcasses that consumed diclofenac (Oaks et al., 2004).

Although the concentration levels detected after wastewater treatment processes seem not to cause toxic effects on human health and in the aquatic environment, their continuous release into the aquatic environment may result in long-term chronic exposure (Garcia-Lor et al., 2012).

Potential toxic effects have been observed at environmentally relevant concentrations on aquatic organisms (Japanese medaka, rainbow trout, and brown trout) where it can bioaccumulate and change cellular reactions in liver, kidney and gills(Hong et al., 2007; Schwaiger et al., 2004; Hoeger et al., 2005).

The probability of biomagnification of diclofenac in the food chain ultimately in human requires the investigation of diclofenac treatment methods in wastewater treatment plants in addition to its toxicity survey.

The aim of this research is to investigate treatability of diclofenac in a biological treatment plant and in lab scale anaerobic reactors. Because diclofenac enters surface water via wastewater treatment plants, removal efficiency of diclofenac in existing wastewater treatment plant is significant subject to investigate. This research shows removal potential of diclofenac in existing WWTP in Turkey.

Study related to the anaerobic treatment of diclofenac is very limited in the literature. Result of this research sheds light on elimination potential of diclofenac by anaerobic treatment processes.

2. LITERATURE REVIEW

2.1 Diclofenac

During the last several years, pharmaceuticals that are diverse group of compounds designed to prevent, cure and treat disease have provoked increasing concern for presence of these ubiquitos, persistant and biologically active substances into water bodies (Ternes et al., 2007; Erickson, 2002; Heberer, 2002). In addition, pharmaceuticals are used in human as veterinary medicine to prevent illness; they are also used as growth promoters in livestock and fish farming as well as in agriculture.

Diclofenac is a nonsteroidal anti-inflamatuary drugs (NSAIDS) belongs to one of the most important groups of pharmaceuticals worldwide. Diclofenac is used primarily for the treatment of inflammation and pain caused by conditions such as rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. It is also effective in treating soft tissue inflammations due to tendinitis and bursitis, and treating dysmenorrhea (menstrual cramps).

Diclofenac is marketed under many brand names, such as Cataflam, Diclon, Flector patch, Voltarol, Voltaren, Oflam,etc with an estimated global annual consumption of 940 tonnes (Zhang et al., 2008). According to the number of prescribed defined daily doses (DDDs) for diclofenac (including combination preparates) amounted in 2006 in Netherland (CVZ, 2007), the emission to the environment would amount to approximately 6,8 tonnes/year assuming that all prescribed DDDs were consumed (DDD=100 mg, WHO, 2006) and annual consumption of diclofenac is also reported by another scientists as shown in Table 2.1.

The increasing use of diclofenac causes in problem for the water cycle due to its low removal rate during wastewater treatment processes and finally results in diclofenac occurance in wastewater treatment plant effluents and surface waters with concentrations ranging from ng/L to mg/L (Tixier et al., 2003; Andreozzi et al., 2003; Zorita et al., 2009). So diclofenac has started to be an environmental concern

due to the potential harmful effects on non-target organisms at environmentally relevant concentrations.

Table 2.1 : Annual consumption of diclofenac in different countries.

Annual Consumption [tonnes/year]	Country	Reference
17.4	Spain	Ortiz de Garcia et al., 2013
6.1	Austria	Calara et al., 2005
6.8	Netherlands	CVZ, 2007
5.9	Korea	Sim et al., 2010
3.9	Switzerland	Tauxe-Wuerce et al., 2005
1	Finland	Linquvist et al., 2005
328	China	Sui et al., 2010
7.5	Germany	Ternes et al., 2001
14.9	France	Metcalfe et al., 2004

The increasing use of diclofenac causes in problem for the water cycle due to its low removal rate during wastewater treatment processes and finally results in diclofenac occurence in wastewater treatment plant effluents and surface waters with concentrations ranging from ng/L to mg/L (Tixier et al., 2003; Andreozzi et al., 2003; Zorita et al., 2009). So diclofenac has started to be an environmental concern due to the potential harmful effects on non-target organisms at environmentally relevant concentrations.

2.1.1 Characteristics of diclofenac

Diclofenac, 2-[(2, 6-dichlorophenyl) amino] phenylacetic acid (Figure 2.1), a synthetic non-steroidal anti-inflammatory drug is known as a polar molecule being

relatively persisent in water (Bartels et al., 2007). The physico-chemical properties of diclofenac are given in Table 2.2.

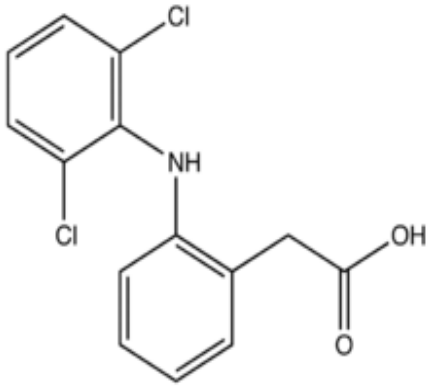


Figure 2.1 : Chemical structure of diclofenac.

Table 2.2 : Physico-chemical properties of diclofenac.

Properties	
CAS Number [1]	015307-86-5
Usage [2]	Analgesic, anti-inflammatory
Molecular Formula [1]	$C_{14}H_{11}Cl_2NO_2$
Molecular Weight [1]	296.16
Water Solubility [1]	2.37 mg/L at 25 ^oC
Log K_{ow} [3]	0.7- 4.5 (pH dependent)
Vapor Pressure [1]	6.14E-8 mm Hg
pK_a Dissociation Constant [1]	4.15
Henry's Law Constant [1]	4.73E-12 atm-m^3/mole at 25 ^oC (estimated)

[1] SRC The Physical Properties Database
[2] Zhang et al., 2008
[3] Ternes et al., 2006

The percentage of a compound that will be vaporized usually depends on Henry coefficient. Henry's law constant of diclofenac is relatively low (H < 3E-3) to consider the vaporization as a potential removal alternative.

K_{ow} value indicates the sorption potential of the organic compounds in terms of hydrophobicity or hydrophility. Organic contaminants with a strong hydrophobic character (log K_{ow} >4.5) were removed to a significant extent (approx. 85%), while hydrophilic compounds (log K_{ow} <3.5) were poorly removed (<20%) in wastewater

(Sui et al., 2010). Diclofenac with its K_{ow} value tend to remain in aqeous phase (Steven-Garson et al., 2011). Also compounds with low pK_a such as diclofenac are expected to be mainly in the aqeous phase (Jones et al., 2007).

2.1.2 Metabolites and pathways of diclofenac

Diclofenac is used as oral administration, dermal application, eye dropping and injection. Zhang et al. (2008) reported that oral application is the main form of administration and accounted for about 70% of the worldwide diclofenac sales.

In humans and mammals, a drug passes an initial activation reactions followed by conjugation reactions. After therapeutic use in humans and mammals, only 15% of the diclofenac is excreted as unchanged, the other part is converted to 3 and 4-hydroxy diclofenac. Then sulphate and glutathione conjugates, in fewer quantities are formed via conjugation reactions (Yu et al., 2005; Sarda et al., 2011). In addition, Zhang et al. (2008) reported diclofenac and its metabolites in urine with a percentage of 65 as shown in Figure 2.2.

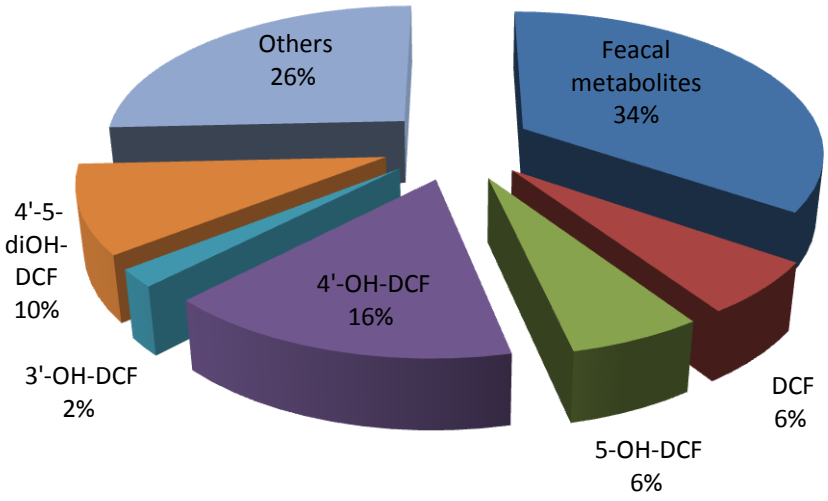


Figure 2.2 : Metabolites of diclofenac in feaces and urine (Zhang et al., 2008).

Parent compound and its metabolites are excreted into raw sewage and wastewater treatment systems. Diclofenac is mainly metabolized in humans to its hydroxylated or methoxylated derivatives and further conjugated, mostly to glucuronides. These metabolites contains 4'-OH-DCF, 3'-OH-DCF, 5-OH-DCF, and 4'-5-diOH-DCF, and 3'-OH-4'methoxy diclofenac (Stülten et al., 2008). However microbial metabolism on conjugates may cause separation of parent compound and its

conjugated biomolecule that results in re-release of the biologically active drug (Jelic et al., 2011).

Sewage treatment plant effluents are discharged to water bodies or reused for irrigation, and biosolids produced are reused in agriculture as soil amendment or disposed to landfill (Jelic et al., 2011). Thus, wastewater treatment plants without further diclofenac treatment are considered to be the primary pathway of pharmaceuticals to the environment (Figure 2.3).

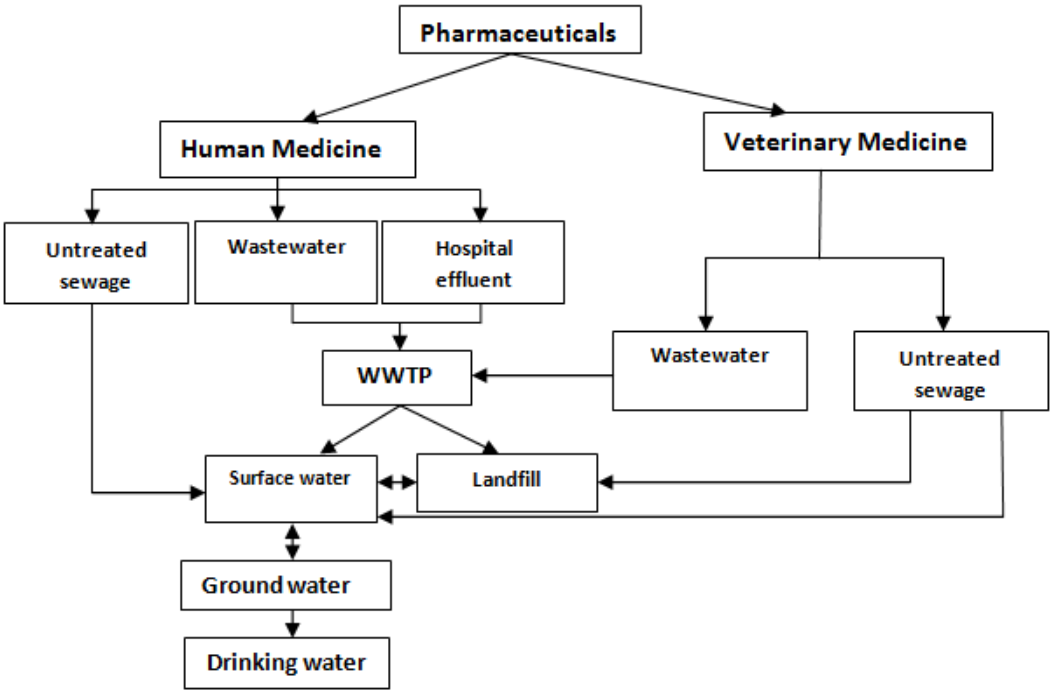


Figure 2.3 : Representative sources and fate of pharmaceuticals in the environment (Kummerer, 2011).

2.1.3 Diclofenac in surface waters

In the aquatic environment, diclofenac is one of the mostly detected at the highest frequency among the pharmaceuticals that are introduced into the environment through various ways during or after manufacturing or consumption. Especially discharge of wastewater from wwtps, agricultural irrigation using wastewater, improper disposal of expired pharmaceuticals, use of biosolids or animal excreta to amend agricultural soils and, in some cases areal deposition are the main routes of diclofenac to contaminate surface waters (Felix-Canedo et al., 2013). Diclofenac

concentrations in surface waters may vary between ng/L to µg/L as shown in Table 2.3.

Table 2.3 : Concentrations of diclofenac in surface waters in different countries.

Concentration [ng/L]	Region	Reference
74	Spain	Matamoros et al., 2013
1030	Germany	Heberer et al., 2002
4400	Pakistan	Scheurell et al., 2010
28-32	Mexico	Felix Conedo et al., 2013
1.2-45.7	Turkey	Aydın et al., 2013
195	UK	Thomas and Hilton, 2004
16	Korea	Choi et al., 2009

2.1.4 Toxicity of diclofenac

Diclofenac is considered as one of the most toxic type of anti-inflammatory drugs. Its harmful effects show itself by damaging renal and gastrointestinal tissue across several vertebrate taxa (Haap et al., 2008).Asian vulture populations face with near extinction due to the accidental exposure (Naidoo et al., 2009).

Almost tens millions of vultures killed in Asia because of diclofenac. Diclofenac causes acute renal failure and the vulture dies within a few days (Oaks et al., 2004). The birds became depressed at approximately 24 hour after exposure and finally succumbed after 36-48 hours (Swan et al., 2006). In the last 15 years, the population number of three species of vultures that eat ill live stock treated with diclofenac have decreased more than 97% and become endangered (EEA, 2010).

Ecotoxicity of diclofenac is relatively low and its acute effect also is undetectable at the concentration levels present in the environment. Diclofenac shows higher acute endpoints ranging from 47-67 mg/L that are much higher than environmentally relevant concentrations (Quinn et al., 2011). However combination of diclofenac with other drugs present in the water increases its toxic effects (Cleuvers, 2004).

Although environmentaly relevant concentrations seem not to cause acute toxic effects in the aquatic organisms, chronic effects of diclofenac on aquatic organisms is big concern.

According to Schwaiger et al. (2004), continuous exposure to environmentally relevant concentrations of diclofenac leads to impairment of the general health of fishes, inducing renal lesions and alterations of the gills. A decreasing trend in hatching success and delay in hatch are observed in fish that exposed to 0.001–10 mg/L of diclofenac for three months (Lee et al., 2011). On the other hand, lethality and teratogenicity were observed in zebra fish embryos after 96 h exposure to 480 ± 50 µg /L (LC_{50}/96 h) and 90 ± 20 µg /L of diclofenac (EC_{50}/96 h), respectively (Dietrich et al., 1998).

In long term toxic effect test in aquatic organisms, lowest observed effect concentration (LOEC) has found as 1 to 5 µg/L for rainbow trout (Schwaiger et al., 2004) while no observed effect concentration (NOEC) has been 0.5 µg/L for brown trout(Hoeger et al., 2005). Further, alterations in liver, kidney and gills even at 1 µg /L in rainbow trout have also been observed.

Also, 15 µg/L no observed effect concentration and 30 µg/L lowest observed effect concentration have been found for embryos and larvae of common carp (*Cyprinus carpio*) during a 30 days toxicity test by Islas-Flores et al. (2013).

2.1.5 Fate of diclofenac in the environment

Diclofenac is known as a polar molecule being relatively persistent in waters with low volatility. The chemical properties of the substance and the observed non-significant adsorption on suspended matter and sediments conclude that the bigger part of decrease of diclofenc concentration in surface waters cannot be described with adsorption processes. Diclofenac concentration can be decreased by photo-transformation when exposed to the natural sunlight (Bartel et al., 2007).

2.1.5.1 Phototransformation of diclofenac

Diclofenac is released in considerably high amounts to the aquatic environment including rivers, lakes, groundwaters and seas. Several studies have shown a rapid decomposition of diclofenac in surface waters when exposed to natural sunlight. Photo-transformation or photolysis of diclofenac is one of the main degradation pathway in surface waters (Schulze et al., 2010).

Bartels et al. (2007) observed that 16 days exposure to natural irradiation leads to decrease in diclofenac concentration. According to semi-natural laboratory tests and

in field experiment, it is found that sunlight stimulates the decomposition of diclofenac in surface waters. In summer, it is observed that diclofenac decomposition is increased up to 83 % in the surface layer of the water (0 to 5 cm) in Europe. However, the increase in water depth requires more time for decomposition of diclofenac. According to the same study, at 50 cm depth 96% of diclofenac was decomposed in two weeeks whereas at 100 cm, 2/3 of the initial diclofenac concentration remained.

Although photolysis was found to be the most important transformation pathway of diclofenac in surface waters leading to decomposition of up to 90% of the diclofenac within a few hours, photo-transformed diclofenac has been reported to be five times more toxic to green algae compared to the parent compound.

Schulze et al. (2010) reported that 2-[2-(chlorophenyl) amino] benzaldehyde (CPAB) formed during photo-transformation product has higher toxicity than diclofenac. The 50% effective concentration (EC_{50}) of CPAB and diclofenac was reported as 4.8 mg/L and 48.1 mg/L, respectively. So CPAB shows much higher toxicity than diclofenac due to the higher hydrophobicity of CPAB (log K_{ow} = 3,62) compared with diclofenac (log K_{ow} = 2,04) at pH 7,0.

2.1.5.2 Fate of diclofenac in soil and sediments

If the water that contains diclofenac is used to fertilize or irrigate crops, persistance and dissipation pathways of diclofenac in agricultural soil must be known. The major factors on diclofenac dissipation in soil are;

- Soil type,
- Temperature,
- Moisture,
- Presence or absence of biosolids.

Diclofenac is transported slowly in agricultural soil than the tracer in agricultural soil (Mersman et. al, 2002). Diclofenac showed significant retardation under experimental condition in the 0-5 cm soil sample by using wastewater from wwtp and 68 % of diclofenac removal was achieved (Chefetz et al., 2008).

In laboratory test by Al-Rajeb et al. (2010), diclofenac was rapidly mineralized without lag when added to soils varying widely in texture (sandy loam, loam, clay

loam). It is observed that diclofenac is readily biodegradable in agricultural soil with half-life less than 5 days.

Diclofenac is carboxylic acids with pK_a value of 4.16 and is negatively charged at pH of ambient water and sediment. Laboratory batch studies to characterize the sorption behavior of diclofenac in natural aquifer sediments show that sorption coefficients were generally quite low (Scheytt et al., 2005).

2.1.5.3 Bioaccumulation of diclofenac

Analysis on aquatic organisms showed accumulation of diclofenac in all organs related with the initial concentration of diclofenac (Schwaiger et al., 2004). For a concentration of 5 g/L on rainbow trout, renal lesions were evident as well as drug bioaccumulation was observed in the liver, kidneys, gills and muscle. Diclofenac bioconcentration factors were 10–2700 in the liver of fish and 5–1000 in the kidney, depending on the exposure concentrations (Schwaiger et al., 2004).

Also literature research reflects that bioaccumulation in biota or food webs are possible. The population of vultures significantly decreased right after the ingestion of diclofenac while scavenging on livestock treated with the drug (Naidoo et al., 2009).

2.1.6 Removal efficiency and behaviour of diclofenac in WWTPs

Due to their intrinsic biological activity that may cause adverse effects to aquatic and terrestrial ecosystems, particularly at chronic exposure, treatment of diclofenac has become emerging concern (Martin et al., 2012). At present, urban wastewaters are considered the most important source of diclofenac to aquatic environment, because wastewater containing diclofenac either unchanged or metabolites originated from different souces such as hospitals, veterinary clinics, households and even pharmaceuticals manufacturing facilities reach the wastewater treatment plants to discharge to receiving bodies after treatment processes.

Most of the treatment plants is operated with conventional wastewater treatment processes containing primary sedimentation and activated sludge system (either nitrification/denitrification or not) followed by final or secondary sedimentation. Because wastewater treatment plants have been designed to eliminate conventional pollutants such as organic matters, nutrients and solids, they are not capable of

removing diclofenac that joins wastewater via excretion or direct disposal of unused tablets or drops (Jones et al., 2005; Jelic et al., 2012; Suarez et al., 2008; Heberer, 2002).

Removal efficiencies can vary significantly from plant to plant and within a plant at different time periods (Vieno et al., 2007). Recent reports show that the concentration of diclofenac in the effluent of wastewater treatment plants reach to the level of μg/L as shown in Table 2.4.

Table 2.4 : Influent and effluent diclofenac concentration in WWTPs.

Influent [ng/L]	Effluent [ng/L]	Region	Reference
86-580	0-120	USA	Yu et al., 2013
1400	950	Switzerland	Sirbu et al., 2006
~500	~100	China	Sui et al., 2010
2800	1900	Germany	Quintana et al., 2005
131	24	Korea	Behera et al., 2011
1220	800	Greece	Samaras et al., 2013
905	780	Austria	Clara et al., 2005
720	530	Spain	Martin et al., 2012
1010	748	Canada	Lishman et al., 2006
58-376	25-182	Thailand	Tewari et al, 2013
2133	1617	Germany	Bernhard et al., 2006

Activated sludge processes are used in most of the wastewater treatment plants in order to mineralize the pollutant to water and carbon dioxide by microorganisms and degrade them into acceptable forms before discharging to the receiving bodies. In addition to these processes, pollutants can also be removed by stripping into air or by sorption onto sludge through the process. Some studies reveals that elimination of diclofenac in municipal wastewater treatment plants with activated sludge process is often incomplete with treatment efficiencies ranging between 9% and 62% as shown in Table 2.5.

Removal efficiencies of diclofenac in wastewater treatment plants are variable depending on the compound specific properties as well as factors related to the treatment processes factors such as the type of treatment process employed,

operational conditions of the treatment process (e.g., temperature, redox conditions, solids retention time and hydraulic retention time), and the climate conditions (e.g., temperature and sunlight intensity) (Castiglioni et al., 2006; Le-Minh et al., 2010).

Table 2.5: Removal efficiency of diclofenac in wwtps with activated sludge process.

Flow rate [m^3/day]	Removal [%]	Region	Treatment Process	Reference
650000	39	Greece	CAS	Samaras et al., 2013
350000	33	Thailand	CAS with A/O	Tewari et al, 2013
60000	27	Germany	-	Bernhard et al., 2006
62000	26	Spain	CAS	Martin et al., 2012
42000	41	Spain	CAS	Gracia-Lor et al., 2012
-	20-40	Switzerland	CAS with A/O	Jones et al., 2005
30000	21	Korea	CAS with A_2/O	Sim et al., 2011
-	9-25	Finland	-	Lindqvisk et al., 2005
40000	35	Italy	CAS	Verlichi et al., 2013
9300	0	Switzerland	CAS	Tauxe et al., 2005
27250	62	Spain	CAS	Matamoros et al., 2013

CAS: Conventional activated sludge process; A/O: continuous flow suspended growth process with anoxic and oxic stages; A_2/O: continuous-flow suspended-growth process with anaerobic, anoxic, and oxic stages.

Elimination of pharmaceuticals or its metabolites occurs in activated slugde processes at four mechanisms including biodegradation, sorption, air stripping and phototransformation (Zhang et al., 2008).

According to Poseidon (2006), the removal of diclofenac by air stripping is limited due to its low Henry coefficient (Table 2.1). Therefore, air stripping is omitted from the removal mechanism of diclofenac. Two processes are mainly responsible for the removal of pharmaceutical compounds: biodegradation and sorption onto sludge. Diclofenac can be transformed from the aqueous phase by biotransformation or by sorption to primary and secondary sludges (Le-Minh et al., 2010).

Diclofenac is classified under the poor biodegradable compounds with a 0.1 L/kg SS.day first order degradation rate constant (Joss et al., 2006). Lee et al. (2012) tested biodegradability of diclofenac by activated sludge and found no biodegradation through the 28 days without any significant change.

Similarly, the transformed percentage of diclofenac in a batch biodegradation study employing a similar methodology during 50 days incubation period resulted in 30% removal efficiency (Yu et al., 2006) and slow biodegradation was reported using other biological reactors by other researchers (Zwiener and Frimmel, 2003; González et al., 2006). Quintana et al. (2005) investigated the biodegradation of diclofenac by activated sludge. They found no transformation of diclofenac over 28 days incubation period, neither when diclofenac was the sole source of carbon, nor when it was dispersed in milk powder.

K_{ow} value shows hydrophobic or hydrophilic character of the compound to understant the tendency of compound to remain on aqeous phase or to sorp onto sludge. Organic contaminants hydrophilic compounds (log K_{ow} <3.5) were poorly removed (<20%) by sorption mechanism (Gasperi et al., 2010). The pharmaceuticals with low K_{ow} values and low pK_a values as diclofenac are mainly detected in wastewater instead of in sludge (Martin et al, 2012). However, diclofenac has partial biodegradability in activated sludge systems (Joss et al., 2006; Quintana et al., 2005)

The efficiency of diclofenac removal in the activated sludge is increased with high sludger retention time (SRT) that allows for the enrichment of slowly growing bacteria and formation of more diverse group of microorganisms capable of degrading wide range of pollutants. However, it is reported that diclofenac removal is not dependent with SRT ranging from 3 to over 30 days in a study conducted in several wastewater treatment plants (Lishman et al., 2006). In addition, Kreuzinger et al. (2005) did not found any correlation between diclofenac removal and SRT (up to 300 days).

2.2 General Information About Investigated Wastewater Treatment Plant

In order to investigate seasonal diclofenac removal efficiency, sampling campaigns have been carried out in a biological wastewater plant located in Istanbul. Wastewater treatment plant serves 2 400 000 people by operating with a 400 000 m^3/day advanced biological treatment capacity and a 600 000 m^3/day pre-treatment capacity.

Wastewater treatment plant collect the wastewater from some districts of the Istanbul through collectors, to convey such wastewater to the wastewater treatment plant,

where the wastewater is treated in advanced level by treatment processes such as coarse and fine screens, primary sedimentation, biological treatment, secondary sedimentation for wastewater; thickening, anaerobic digestion, dewatering and drying for sludge as shown in Figure 2.4.

Figure 2.4: Investigated advanced biological wastewater treatment plant.

2.2.1 Preliminary treatment

Wastewater reach to the plant first transferred through coarse screens with 50 mm spacing that is designed to protect the plant structure against large solid particles could create obstructions in the facility's units as well as negative affect for efficiency of treatment process. Wastewater pumped from the inlet pumping station passes through mechanically cleaned fine screens with 10 mm spacing.

Then wastewater enters to grit retaining chambers (6 chambers) each with 2 troughs. Grit chambers in the plant separate the grit and inorganic material up to 0.20 mm grain diameter by diffused air, while at the same time separate minerals and organic particles within the grit trap thus operational problems such grit sedimentation in aeration tanks and digesters, increased wear of equipment is prevented.

Wastewater from grit chambers follows through two primary sedimentation tanks which are large enough that settlable particles can settle and floating materials such as grease can rise to surface and be skimmed off thus both homogenous liquid capable of being treated biologically and settled sludge are obtained. Primary

sedimentation tanks generally equipped with mechanically driven scappres that continually drive the collected sludge towards a hopper in the base of the tank.

2.2.2 Biological treatment

Biological treatment is achieved by 5-stage Bardenpho system with 2 bio-phosphorous tanks, 2 oxic tanks where nitrification takes place and 2 anoxic tanks where denitrification occurs. Anoxic and oxic conditions are adjusted by diffusers in the tanks.

56% of wastewater fed into the aerated tank and wastewater come from primary sedimentation enters into 3 biological phosphorous tanks, which are operated in parallel. Anaerobic ambient conditions are met during the biological treatment.

Treatment system is based on the removal of carbon, nitrogen by flows through aerated (aerobic) and non-aerated (anoxic) tanks subsequently. Biological treatment system consists of 3 aeration tanks operating in parallel. The tanks are designed to ensure a continuous flow. 2-stage supply method is chosen due to improper carbon/nitrogen ratio. This way, the carbon source in the wastewater is distributed step by step. In this system, the SS density in the BioP Tank and first stage tanks is high, which optimizes the total volume of tanks. Activated sludge/wastewater mixture leaving the biological phosphorus unit enters the denitrification and nitrification tanks at the first section. At each stage, internal circulation is ensured by means of internal return pumps. Phosphorus, nitrogen and carbon are removed at the outlet of the biological unit.

Wastewater from aeration tanks are collected in distribution units and then enters to the final sedimentation tanks which are equipped with semi-rotary bridge scrappers operated continuously. Bottom scrappers convey the settled sludge to the sludge collecting zone while surface scrappers on the rotary bridges convey the scum on surface to the scum collecting chamber.

2.2.3 Sludge treatment

Primary sludge with 1083 m^3/day flow rate and excess sludge with 14 000 m^3/day flow rate flow through nine centrifugal sludge thickeners. With such centrifuges, the ratio of solid particles increase to 6 % and sludge are pumped to sludge digesters.

Sludge digesters consists of 6 cylinders made of reinforced concrete, each with a volume of 10 000 m^3. At such tanks, sludge stabilization is achieved under anaerobic conditions, resulting in a sludge volume decrease and biogas generation. Average detention period of the sludge in the digester is 16 days and average temperature is 35-37°C. The biogas is stored in two gas tanks. Further, the stabilized sludge is stored in the sludge storage tank for 1 hour and pumped to sludge dewatering unit.

Sludge is dewatered by centrifuges (in total 6 centrifuges) to increase suspended solid ratio from 6 % to 25%.

Sludge with 25 % suspended solid ratio is dried to become 90 % in drying unit after dewatering process.

3. MATERIALS AND METHODS

3.1 Sampling From Wastewater Treatment Plant

In this study, four sampling campaigns have been carried out in July 2012, November 2012, March 2013 and May 2013 to investigate diclofenac removal efficiency in different treatment units as well as to characterize seasonal changes in diclofenac concentrations. In the treatment plant, sampling of both mixed liquor and sludge samples were performed from 10 different sampling points as shown in Figure 3.1. Sampling types and dates in each season are shown in Table 3.1.

Table 3.1: Sampling dates and types.

Sampling Points	Summer Campaign July 2012	Autumn Campaign November 2012	Winter Campaign March 2013	Spring Campaign May 2013
Mixed Liquor				
Influent	Composite	Composite	Composite	Composite
Grit chamber	Grab	-	Grab	-
Primary clarifier	Grab	-	-*	-
Anaerobic tank	Grab	-	Grab	-
Anoxic tank	Grab	-	Grab	-
Oxic tank	Grab	-	Grab	-
Final clarifier	Grab	-	Grab	-
Effluent	Composite	Composite	Composite	Composite
Santrate	Grab	-	Grab	-
Sludge				
Primary sludge	Grab	-	-*	-
Secondary sludge	Grab	-	Grab	-
Digester sludge	Grab	-	Grab	-
Dried sludge	Grab	-	Grab	-

*Primary clarifier was not in operation in winter campaign.

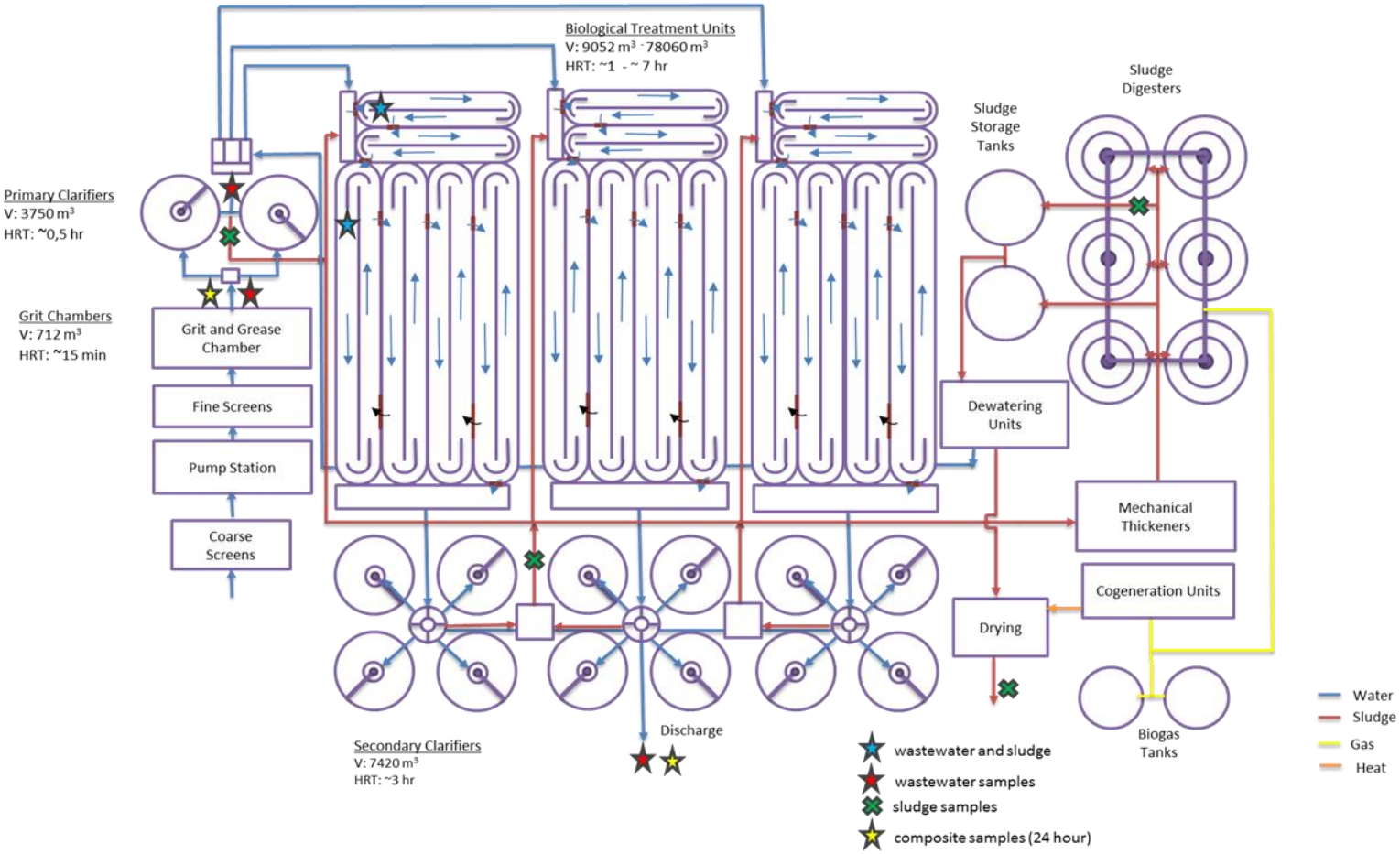


Figure 3.1: Sampling points in wastewater treatment plant.

In summer and winter campaigns, 24 hour composite samples from plant influent and effluent; grab samples from grit chamber effluent, primary clarifier effluent, anaerobic tank, anoxic tank, oxic tank, secondary clarifier effluent were taken as mixed liquor. In addition to this, grab sludge samples were taken from primary clarifiers, secondary clarifiers, anaerobic digesters, thickeners and drying unit. While 24-hour plant influent sample has been collected from effluent of grit chamber, 24-hour plant effluent sample has been collected from effluent of secondary clarifier.

In spring and autumn campaigns, samples were only collected form influent and effluent of wastewater treatment plant as 24-hour composite samples.

Samples were collected in fluorinated jerricans with a total volume of 10 L (Thermo Scientific) and transported to the laboratory in coolers. 1 L of samples have been preserved in amber bottles and acidified with 1 N hydrochloric acid (HCl) for chemical oxygen demand, total Kjeldahl nitrogen, total organic carbon and total phosphorous analysis while 1 L of samples has been reserved as unacidified form for other analyses (i.e., pH, total suspended solids, volatile suspended solids and ammonia).

Figure 3.2 : Storage of samples with teflon coated jerricans and amber glass bottles.

About 2 L of samples have been immediately centrifuged for 15 min at 10000 rpm in 500 mL teflon centrifuge tubes and then filtered using 0,22 µm filters (Millipore). Filtered wastewater has been kept in the amber glass bottles as shown in Figure 3.2.

All samples have been preserved in 4°C refrigerator. Sludge samples have been preserved at -20°C after centrifuge. Sludge samples have been dried under vacuum in freeze drier (Thermosavant) before diclofenac measurement.

As soon as samples have been taken, analysis of the samples has been started immediately. All analyses have been performed within one week.

3.2 Anaerobic Reactors Set Up

In order to investigate biodegradability potential in anaerobic conditions, semi-batch reactors have been performed in laboratory. Pressure resistant glass reactors with volumes changing between 2,5 L and 6 L have been filled with weighted or measured amount of inoculum. Then, three openings in the reactors were closed with teflon caps, teflon stoppers and valves (Figure 3.3).

Each reactor has been flushed with pure N_2 gas under 1-2 psi for 30 minutes to provide anaerobic conditions by replacing oxygen gas with nitrogen. After flushing, each reactor has filled with anaerobic media to the desired level to provide 42-52 % empty headspace in the reactors. Then decided amount of substrate (glucose and yeast extract) and diclofenac were added to reactor. Reactors have been operated in the dark and mixing has been achieved with magnetic stirrers.

Figure 3.3 : Configuration of anaerobic reactors.

3.2.1 Anaerobic media

10 L of pressure resistant glass bottle (Simax, Czech Republic) has been used for media preparation. The silicone cap with two pipes mounted on the glass bottle for feeding and flushing purposes has been autoclaved under high pressure and temperature to sterilize materials for avoiding microbial activity. Chemicals used in the media preparation that are essential for microbial growth are shown in Table 3.2.

Table 3.2 : Constituents of anaerobic media.

Component	Concentration
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
NH_4Cl	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
Trace Metal Stock Solution	1 mL/L
Resazurin Stock Solution	2 mL/L
Vitamin Stock Solution	0.2 mL/L
$NaHCO_3$	1.2 g/L
$Na_2S \cdot 9H_2O$	0.5 g/L

To prepare metal stock solution, chemicals shown in Table 3.3 have been weighted and added to 1 L of volumetric flask and completed to 1 L with deionized water (DI). Then it has been transferred to amber glass bottle and preserved at $4^{\circ}C$ in refrigerator.

Table 3.3 : Constituents of trace metal stock solution.

Component	Concentration, g/L
$ZnCl_2$	0.5
$MnCl_2 \cdot 4H_2O$	0.3
H_3BO_3	3
$CoCl_2 \cdot 6H_2O$	2
$CuCl_2 \cdot 2H_2O$	0.1
$NiSO_4 \cdot 6H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.3

In order to prepare resazurin stock solution, 100 mg resazurin has been weighted and transferred to 100 mL of volumetric flask and completed with deionized water to abtain 1 g/L solution (Table 3.4). Prepared solution has been transferred to amber glass bottle and preserved at 4 $^{\circ}$C in refrigerator.

Table 3.4 : Constituents of resazurin stock solution.

Component	**Concentration**
Resazurin	1 g/L

To prepare vitamin stock solution, chemicals shown in Table 3.5 have been weighted and added to 1 L volumetric flask. After transferred to amber glass bottle, it has been preserved at 4^{o}C in the refrigerator.

Table 3.5 : Constituents of vitamin stock solution.

Component	**Concentration, g/L**
Biotin	0.2
Folic Acid	0.2
Pyridoxine Hydrochloride	1
Riboflavin	0.5
Thiamine	0.5
Nicotinic Acid	0.5
Pantothenic Acid	0.5
Vitamin B_{12}	0.01
p-Aminobenzoic Acid	0.5
Thioctic Acid	0.5

8 L glass bottle graduated with 2 L intervals have been filled with distilled water to 6 L and chemicals have been added into bottles for 8 L (7.2 g K_2HPO_4; 4 g KH_2PO_4, 4 g NH_4Cl; 0.8 g $CaCl_2 \cdot 2H_2O$; 1.6 g $MgCl_2 \cdot 4H_2O$; 0.8 g $FeCl_2 \cdot 6H_2O$; 8 mL of trace metal stock solution and 16 mL of resazurin stock solution).

Prepared solution has been completed to exceed (~ 200 mL) 8 L taking into account the losses during the evaporation. Solution has been heated with continuous mixing until it boiled (Figure 3.4). After ten minutes boiling, cap with pipes has been sealed and vanes have been placed into pipes to provide impermeability of gas. Oxygen gas has been replaced with nitrogen gas while the media is still hot by flushing the media

bottle for 30 minutes with pure N_2 gas. Flushing also facilitated the cooling of the media.

$Na_2S \cdot 9H_2O$ (4 g) have been injected to media to provide reduced condition. After media has been cool down, 9.6 g $NaHCO_3$ and 1.6 mL of vitamin stock solution has been added into media bottle by transferring with a syringe under anaerobic conditions.

Figure 3.4 : Preparation of anaerobic media.

3.2.2 Anaerobic culture development

3.2.2.1 Culture developed at 22ºC

Reactor A.I, B.I, C.I, D.I, and E.I have inoculated with 80 g Alibeyköy river sediment (Eyup, Istanbul), 200 mL of anaerobic digestion sludge from an advanced biological wastewater treatment plant-1, 80 g of Kağıthane river sediment (Sisli, Istanbul), 80 g of America river sediment (Bayou d'Inde, LA, USA) and 750 mL of anaerobic digestion sludge from a wastewater treatment plant-2, respectively. Glass reactors with a total volume of 3650 mL have been flushed with nitrogen gas under 1-2 psi pressure after decided amount of sludge was placed into the reactor. Then, reactors have been filled with anaerobic media until 2 L as shown in Figure 3.5.

These five main reactors have been the first generation anaerobic reactors due to direct inoculation with sediment or anaerobic digestion sludge.

Figure 3.5: First generation anaerobic reactors at 22 °C.

Reactor A.I, B.I, C.I, D.I, and E.I have been inoculated at 22°C in dark with 80 day SRT. To provide 80 day of SRT 350 mL of sludge has been drawn off from each reactor and then reactors recompleted up to 2 L with anaerobic media every 14 days.

Reactors have been fed weekly with glucose and yeast extract to provide 300 mg/L and 15 mg/L concentrations in the reactors, respectively. Reactor A.I, B.I, C.I, and D.I have been amended with diclofenac in methanol weekly to provide 10 µg/L diclofenac concentrations (40 µL from 500 mg/L diclofenac stock solution in methanol). Diclofenac stock solution has been prepared into methanol du to diclofenac's low solubility. Diclofenac is dissolved in a solvent such as methanol tointroduce the culture. Reactor E.I has been operated as control reactor without diclofenac addition. However, 40 µL of methanol has been added to Reactor E.I weekly to compensate extra carbon coming from diclofenac stock solution in methanol in other reactors.

In order to eliminate the sediment that may be originated from the innoculum, another reactor as second generation (Reactor A.II) have been installed using inoculum from Reactor A.I. 100 mL of inoculums have been placed into reactors and closed with teflon caps and teflon stoppers. After 30 minutes flushing with nitrogen gas, reactor filled with media until 2 L (Figure 3.6). The reactors were

operated at 22ºC in the dark with the amendment of 1 µg/L diclofenac, 200 mg/L glucose, and 15 mg/L yeast extract. In order to maintain 80 day SRT, 350 ml of sludge have been replaced with anaerobic media every 14 days.

Figure 3.6 : Reactor A.II and A.III operated at 22°C

A third generation (Reactor A.III) culture was also initiated with the innoculation of 270 ml sludge from Reactor A.II as a third generation anaerobic reactors. After flushing, it has been filled with anaerobic media until 4 L (Figure 3.6). It was operated at 22ºC in the dark with 1 µg/L diclofenac and 200 mg/L glucose and 15 mg/L yeast extract concentration. In order to maintain 80 day SRT, 700 ml of sludge was replaced with anaerobic media every 14 days. Reactor A.III which is the third generation mixed culture has been used as a sediment-free culture source for all batch assays.

3.2.2.2 Culture developed at 35ºC

100 mL culture inoculum taken from anaerobic digestion sludge of a wastewater treatment plant-2 has been placed into reactor to build Reactor F. Reactor bottle with a total volume of 2420 mL have been sealed with teflon caps, teflon stoppers and valves and flushed with nitrogen gas under 1-2 psi.

After flushing, reactor was filled with anaerobic media until 1.4 L. Reactor F has been performed at 35 °C in continuously stirred incubator (Thermoforma, orbital shaker) (Figure 3.7). It has been operated in the dark with 80 day SRT. Every 14

days, 245 mL of sludge has been discharged from reactor and refilled up to 1.4 L with anaerobic media to sustain 80 day SRT. Reactor F have been fed weekly with glucose, yeast extract and diclofenac to provide 300 mg/L, 15 mg/L and 10 µg/L diclofenac concentrations in the reactor.

Figure 3.7 : Reactor F.I in incubator at 35 ^{o}C.

All reactors have been mixed mechanically everyday and gas production, gas composition, pH, volatile fatty acids (VFA) concentration, and dissolved organic carbon concentration have been followed to observe microbial activity in each reactor summarized in Table 3.6.

Table 3.6: Summary of anaerobic reactors.

Reactor	Generation number	Inoculum	Diclofenac Concentration [µg/L]	Temperature [^{o}C]
A.I	1st	Alibeyköy river sediment	10	22
B.I	1st	Anaerobic digestion sludge of a WWTP-1	10	22
C.I	1st	Kağıthane river sediment	10	22
D.I	1st	America river Sediment	10	22
E.I	1st	Anaerobic digestion sludge of a WWTP-2	-	22
F.I	1st	Anaerobic digestion sludge of a WWTP-2	10	35
A.II	2nd	Anaerobic culture from A.I	1	22
A.III	3rd	Anaerobic culture from A.II	1	22

3.3 Anaerobic Batch Assays Set Up

Pressure resistant glass serum bottles with a total volume of 245 mL have been sealed with teflon lined stoppers. Serum bottles have been flushed for 20 min at 1-2 psi with N_2 gas to provide anaerobic conditions by replacing oxygen gas with nitrogen. Then, a total of 180 mL culture and anaerobic media has been transferred anaerobically with 60 mL syringes and decided amount of substrate (glucose and yeast extract or acetate) and diclofenac was added.

3.3.1 Different initial diclofenac concentration assay

Batch assay has been conducted to investigate the effect of diclofenac concentration on anaerobic microorganisms and methanogenic activity. Six culture series have been prepared in 245 mL of serum bottles with different diclofenac concentrations including 0 µg /L (Control 1 and 2), 10 µg /L (Set 1a), 50 µg /L (Set 1b) , 200 µg /L (Set 1c), and 1 mg/L (Set 1d), respectively (Figure 3.8).

Figure 3.8 : Anaerobic reactors with different diclofenac concentrations.

Serum bottles have been sealed tightly with teflon lined stoppers (Wheaton, USA) and anaerobic conditions have been provided by flushing bottles for 20 minutes with nitrogen gas at 1-2 psi. Serum bottles have been filled with appropriate amount of anaerobic media and culture from Reactor A.II and Reactor E.I indicated in Table 3.7 by syringe anaerobically.

Table 3.7 : Matrix of anaerobic batch assay with different initial diclofenac concentrations.

	Reactor E.I Culture	Reactor A.II Culture	Anaerobic Media	Glucose + YE[1]	MeOH	Diclofenac in MeOH Stock I.A[2]	Diclofenac in MeOH Stock I.B[3]	Diclofenac in MeOH Stock I.C[4]	Diclofenac in MeOH Stock I.D[5]
Control 1	60 mL	-	120 mL	270 µL	100 µL	-	-	-	-
Control 2	60 mL	60 mL	60 mL	270 µL	100 µL	-	-	-	-
Set 1a (10 µg/L diclofenac)	60 mL	60 mL	60 mL	270 µL	-	100 µL	-	-	-
Set 1b (50 µg/L diclofenac)	60 mL	60 mL	60 mL	270 µL	-	-	100 µL	-	-
Set 1c (200 µg/L diclofenac)	60 mL	60 mL	60 mL	270 µL	-	-	-	100 µL	-
Set 1d (1 mg/L diclofenac)	60 mL	60 mL	60 mL	270 µL	-	-	-	-	100 µL

[1] 200 + 10 g/L glucose and YE; [2] 18 mg/L diclofenac in MeOH; [3] 90 mg/L diclofenac in MeOH; [4] 360 mg/L in MeOH; [5] 1.8 g/L diclofenac in MeOH.

Two serum bottles have been prepared for each culture series. One of them has been used for liquid samping whereas the other one has been used for gas sampling. All serum bottles have been kept at 22ºC in dark and shaken manually once a day.

Diclofenac concentrations for each culture series have been provided by adding same amount of stock solutions in methanol (100 µL) that prepared separately for each culture series. Because methanol coming from the stock solution is a carbon source for microorganisms, same amount of methanol has been also added to each reactor. Due Control 1 and Control 2 culture series have been fed without diclofenac addition, 100 µL methanol has also been injected into them.

Each serum bottles have been amended with glucose and yeast extract to sustain 300 and 15 mg/L concentrations, respectively. Thus, 1000 mg/L COD has been introduced to serum bottles by adding methanol and glucose.

Gas production and composition, diclofenac, VFA, DOC, pH and ORP parameters have been measured to investigate microbial activity.

3.3.2 Different temperature assay

Batch assay has been conducted to investigate temperature effect on the biodegradation of diclofenac in anaerobic condition. Four culture series with their control have been prepared in 245 mL of serum bottles in different temperatures including 10°C (Set 2a), 20 °C (Set 2b), 35°C (Set 2c), and 45°C (Set 2d).

Serum bottles have been sealed tightly with teflon stoppers and flushed for 20 min at 1-2 psi with N_2 gas to provide anaerobic conditions by replacing oxygen gas with nitrogen. Then, a total of 180 mL culture and anaerobic media has been transferred to serum bottles anaerobically with 60 mL syringes (Table 3.8).

Each culture series have contained one control that has consisted of 180 mL of anaerobic media to observe the degradation potential of diclofenac in abiotic conditions at four different temperature. To eliminate microbial contamination, sterilized syringes and needles have been used for preparation and sampling of abiotic controls.

Table 3.8 : Experimental setup to assess the temperature effect on diclofenac degradation under anaerobic conditons.

	Raector E.I Culture	Reactor A.III Culture	Anaerobic Media	Glucose + YE[1]	Diclofenac in MeOH Stock [2]	Serum Bottles
Set 2a (10 °C)	40 mL	70 mL	70 mL	275 µL	100 µL	245.8 mL (x5)
Set 2a Control (10 °C)	-	-	180 mL	275 µL	100 µL	245.8 mL (x1)
Set 2b (20 °C)	40 mL	70 mL	70 mL	275 µL	100 µL	245.8 mL (x5)
Set 2b Control (20 °C)	-	-	180 mL	275 µL	100 µL	245.8 mL (x1)
Set 2c (35 °C)	40 mL	70 mL	70 mL	275 µL	100 µL	245.8 mL (x5)
Set 2c Control (35 °C)	-	-	180 mL	275 µL	100 µL	245.8 mL (x1)
Set 2d (45 °C)	40 mL	70 mL	70 mL	275 µL	100 µL	245.8 mL (x5)
Set 2d Control (45 °C)	-	-	180 mL	275 µL	100 µL	245.8 mL (x1)

[1] 200 + 10 g/L glucose and YE; [2] 90 mg/L diclofenac in MeOH.

5 serum bottles have been prepared for each culture series. One of them has been used for gas sampling, two of them have been used for liquid sampling of first feeding cycle, the others have been used for liquid sampling of second feeding cycle.

Before the addition of glucose, yeast extract and diclofenac, all culture series have been incubated for the acclimation at their respective temperature for one day.

100 µL of diclofenac stock solution in methanol have been injected to all serum bottles except controls to sustain 50 µg/L concentrations. Controls have been amended with 100 µL of methanol to balance the substrate in terms of methanol. All serum bottles amended with glucose and yeast extract to provide 300 mg/L and 15

mg/L concentration, respectively. Thus, 1000 mg/L COD has been introduced to serum bottles by adding methanol and glucose.

10^{o}C (Set 2a) , 20 ^{o}C (Set 2b), 35^{o}C (Set 2c) and 45^{o}C have been performed at 10^{o}C refrigerator, room temperature, 35^{o}C incubator (Thermoforma, orbital shaker) and, 45^{o}C incubator (Memmert) in dark as shown in Figure 3.9. All serum bottles have been shaken manually or mechanically once a day.

Figure 3.9 : Anaerobic batch assay reactors with different temperatures.

Gas composition and production, diclofenac, VFA, DOC, pH and ORP parameters have been measured to investigate microbial activity at different temperatures.

3.3.3 Different biomass concentration assay

A batch assay has been conducted to investigate the effect of biomass concentrations on the biodegradation of diclofenac under anaerobic condition. Different biomass concentrations including 100 % (Set 3a), 75 % (Set 3b), 50 % (Set 3c) and 25 % (Set 3d) have been performed into 245 mL of serum bottles with same diclofenac concentration.

Serum bottles have been sealed tightly with teflon stoppers, then anaerobic conditions have been provided by flushing serum bottles with nitrogen gas at 1-2 psi. Anaerobic media, culture from Reactor A.II and Reactor E.I have been transferred to serum bottles to provide decided amount of biomass as shown in Table 3.9.

Table 3.9 : Experimental set up to assess the biomass concentration effects on diclofenac biodegradation under anaerobic conditions.

	Reactor E.I Culture	Reactor A.II Culture	Anaerobic Media	Glucose + YE[1]	Diclofenac in MeOH Stock [2]	Serum Bottles
Set 3a (100 % of biomass)	90 mL	90 mL	0 mL	275 µL	100 µL	245.8 mL (x3)
Set 3b (75 % of biomass)	67.5 mL	67.5 mL	45 mL	275 µL	100 µL	245.8 mL (x3)
Set 3c (50 % of biomass)	45 mL	45 mL	90 mL	275 µL	100 µL	245.8 mL (x3)
Set 3d (25 % of biomass)	22.5 mL	22.5 mL	135 mL	275 µL	100 µL	245.8 mL (x3)

[1] 200 + 10 g/L glucose and YE; [2] 90 mg/L diclofenac in MeOH..

Three serum bottles have been prepared for each culture series. Two of them have been used for liquid samping whereas the other one has been used for gas sampling. All serum bottles have been kept at 22ºC in dark and shaken manually once a day.

All culture series have been amended with glucose, yeast extract, and methanol to provide 300 mg/L, 15 mg/L and 50 µg/L concentrations, respectively. Thus, 1000 mg/L COD has been introduced to serum bottles by adding methanol and glucose.

3.3.4 Different carbon source assay

In this batch assay, acetate was used instead of glucose to examine the effect of a different carbon source on diclofenac biodegradation.

Serum bottles have been sealed tightly with teflon stoppers and flushed for 20 min at 1-2 psi with N_2 gas to provide anaerobic conditions by replacing oxygen gas with nitrogen. 60 mL of culture from Reactor A.II, 60 mL of culture from Reactor E.I, and 60 mL of anaerobic media have been transferred to serum bottles anaerobically with 60 mL syringe.

3 serum bottles have been prepared during the experiment. One of them has been used for gas sampling, the others one have been used for liquid sampling for consecutive two feeding cycles.

Diclofenac in methanol and acetate have been injected to each serum bottles to provide 50 µg/L and 600 mg/L concentrations, respectively. Thus, 1000 mg/L COD has been introduced to serum bottles by adding methanol and acetate.

All culture series have been kept at 22ºC in dark and shaken manually once a day. Gas composition and production, diclofenac, VFA, DOC, pH and ORP parameters have been followed.

3.4 Analyses

3.4.1 Analytical methods

3.4.1.1 pH

pH was conducted using pH/milivolt meter (ThermoOrion) as shown in Figure 3.10 with calibration for each measurement. Probe was celeaned and dried for each sample then inserted into the sample. Data was recorded after ready sign was seen.

3.4.1.2 Oxidation reduction potential (ORP)

ORP was conducted using pH/milivolt meter (Thermo Orion) by replacing pH probe with ORP probe (Orion). The meter and electrode output periodically calibrated using an ORP reference solution (Light solution: 1 M ferrous ammonia sulphate, 0,1 M ferric ammonium sulphate, and 1 M sulfuric acid). The difference between the meter ORP reading and the theoretical value of reference solution (455 mV at 25ºC) has been taken into account in all measurements. Probe was cleaned and dried for each sample then inserted to sample that was taken immediately before the measurement. Electrode was fitted tightly in the bottle filled with sample. Data was recorded after ready sign was seen.

3.4.1.3 Total gas production

Gas measurement has been conducted by using monometer (Lutran, PM-9107) that performs measurement between 0 and 7000 mbar (Figure 3.11). Meaurement in mbar unit has been converted to mL unit by using Equation 3.1.

$$P\ (mL) = \frac{P\ (mbar) * V_h \times V^0 \times 10^6}{R \times T} \qquad (3.1)$$

V_h: Head space volume in the reactor (L),

V^0: Molar volume of gas at relevant temperature (K),

R : Ideal gas constant (L.atm/mol.K)

T: Temperature (K)

Figure 3.10 : Manometer.

3.4.1.4 Gas chromatography

Flame ionization detection

Volatile fatty acids concentrations including acetic, propionic, iso-butyric, butyric, iso-valeric, valeric have been measured by using gas chromatography (Agilent Technologies, 6890N) equipped with a flame ionization (FID) detector and capillary column (DB-FFAP 125-3232).

For the VFA analysis, the temperature of the injection port and detector have been 230°C and 250°C, respectively. The sample has been injected with splitless injection The oven temperatue have reached 100°C in first 5 minutes and then 160°C; it has been kept at this temperature for 5 minutes and fixed at 230°C in 3 minutes. Column has been operated with helium as the carrier gas at a constant flow rate of 4 mL/min

In VFA analyses, 1.6 mL of sample filtrated from 0.22 μm membrane filter has been transferred to 2 ml HPLC vial and then 20 μL phosphoric acid (10 N) has been added. VFAs in the vials have been quantified in gas chromatoraphy (GC).

Thermal Conductivity Detection

Gas chromatography (Agilent Technologies, 785CA) has been equipped with two columns and two thermal conductivity detectors have been used to measure gas composition. Methane, nitrogen has been separated with CPT530 column (Agilent Technologies) and carbon dioxide has been separated with HP-PLOT/Q column (Agilent Technologies) column. Both columns have been operated with helium as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector has been maintained at 150ºC, and the detector temperature has been set at 150ºC. All gas analyses have been performed by injecting 100 μL gas sampe while detectors' temperature was set to 150^{o}C.

3.4.1.5 Total Kjeldahl nitrogen (TKN)

Total Kjeldahl nitrogen experiment has been occured in three steps; digestion included decompisition of organic nitrogen to ammonia, distillation includedcollection of condensed nitrogen in boric acid and titration.

Sample volumes decided according to Table 3.10 was placed into 800 mL Kjehldal flasks and completed to 300 ml with distilled water. Kjeldahl flasks have been placed into digestion part of TKN set (Gerhardt, Germany) that designed to remove acid steams after 50 mL digestion solution and a few boiling chips have been added into it. Boiling has proceeded until approximately 30 minutes after white fumes have been observed to obtain 25 – 30 mL light green solution in Kjeldahl flask.

After cooling, light green solution has been diluted to 300 mL distilled water and 50 mL neutralization solution has been added into flasks. Subsequently, erlenmayers have filled with 50 mL boric and Kjeldahl flasks have been placed into distillation part of TKN set and nitrogen in the Kjeldahl flasks has been distilled into boric acids in the erlenmayers. Then samples in the erlenmayers have been titrated with 0.02 N H_2SO_4 solution until light purple colour has been obtained after 2 drops of mixed indicator is added.

Table 3.10 : Samples volumes for TKN according to Standard Methods.

Organic-N in Samples [mg/L]	Sample Volume [mL]
0-1	500
1-10	250
10-20	100
20-50	50
50-100	25

3.4.1.6 Ammonia

Ammonia nitrogen experiment has been occured in two steps; distillation included collection of condensed nitrogen in boric acid and titration.

Sample volumes decided according to Table 3.11 have been placed into 800 mL Kjeldahl flasks and completed to 500 ml with distilled water. Kjeldahl flasks have been placed into distillation part of TKN set after 25 mL borate buffer and a few boiling chips have been added into it.

Table 3.11 : Sample volumes for ammonia according to Standard Methods.

Ammonia-N in Samples [mg/L]	Sample Volume [mL]
5-10	500
10-20	100
20-50	50
50-100	25

Subsequently, erlenmeyers have filled with 50 mL boric acid solution ang Kjehldal flasks have been placed into distillation part of TKN set and nitrogen in the Kjehldal flasks have been distilled into boric acid in the erlens. Then samples in the erlens have been titrated with 0.02 N H_2SO_4 solution until light purple colour has been obtained after 2 drops of mixed indicator is added.

3.4.1.7 Total phosphorous

Micro-Kjeldahl flasks have been filled with 5 mL of samples. 1 mL of concentrated sulfuric acid and 5 mL of concentrated nitric acid have been added into flasks with a few boiling chips and Kjeldahl flasks have placed into digestion part of TKN set. Boiling has been proceeded until 1ml sample remaining in the flasks. After cooling, 25 mL of distiled water has been added into flasks. Concentrated sulphuric acid and 1 N sodium hydroxide have been used to adjust pH. Sample in the flask has been tranferred to 100 mL volumetric flask by filtering and completed to 100 mL with distilled water. 4 ml of ammonium molybdate and 0.5 mL of tin chloride have been added into volumetric flask. Samples have been transferred to cell and measured in spectrophotometer at 690 nm wavelength between 10 and 12 minutes after tin chloride has been added.

3.4.1.8 Chemical oxygen demand

COD measurements have been performed as described in the ISO 6060 Method.. COD tubes filled with 2.5 ml of samples have been put in 1,5 mL of potassium dichromate digestion solution. 3.5 mL of sulphuric acid reagents have been also added to tubes as catalyst. COD tubes have been sealed tightly and placed into COD digester at 150^{o}C for 2 hours.

After 2 hour digestion and cooling to the room temperatures, samples have been transferred to conical flask. Samples have been titrated with ferrous ammonium sulfate until reddish colours have been observed after 2 drops of ferroin indicator has been added.

3.4.1 General methods

3.4.1.1 Diclofenac measurement in aqueous phase

Diclofenac in aqueous phase has been measured in three steps; solid phase extraction that diclofenac has been extracted with solvent from cartridge diclofenac captured in, evaporation that only diclofenac has been obtained by evaporation of extracting solvent, and measurement in LC-MS/MS after dissolved diclofenac with methanol has been filled into a vial.

Vacuum manifold with 20 sampling ports (VocMaster, Biotage) used to place Oasis HLB cartridges (6 cc, 200 mg; Waters, Millford) for solid phase extraction as shown in Figure 3.11. Sample volumes introduced to the cartridges have been decided according to estimated diclofenac concentrations existed in sample. 100 ml and 1 – 10 mL samples filtered from 0.22 µm PVDF filter (Chromafil) have been used for WWTPs samples and anaerobic reactors' samples, respectively.

Figure 3.11 : SPE of reactor samples.

Initially cartridges have been conditioned with 5 + 5 mL of acetonitrile (Merck), 5 mL of methanol (Merck) and 5 mL of distilled water (DI) (Merck). Then decided amount of samples have been introduced to the cartridges at flowrate of 3.5 mL/minutes after d_4-diclofenac (isotopically labelled diclofenac) has been added to samples as internal standards for measurement in LC-MS/MS. After sample loading, cartridges have been washed with 5 mL of water and dried under vacuum for 60 min, and then have been eluted with 2 mL of acetonitrile and 2 mL of methanol as shown in Figure 3.12.

Subsequently, extracts have been evaporated at 35 $^{\circ}$C, under 10 bar N_2 flow in Turbovap II (Caliper Life Sciences, USA) as shown in Figure 3.13.

Diclofenac remained in the Turbovap tubes has been reconstructed with 1 mL MeOH–water (10:90, v/v) and placed into vial to be measured in LC-MS/MS after filtering with 0.22 µm filter. Samples have been analyzed using a Thermo Electron Cooperation TSQ Quantum Access triple quadruple mass spectrometer coupled with Accela Ultra Performance Liquid Chromatograph (UPLC).

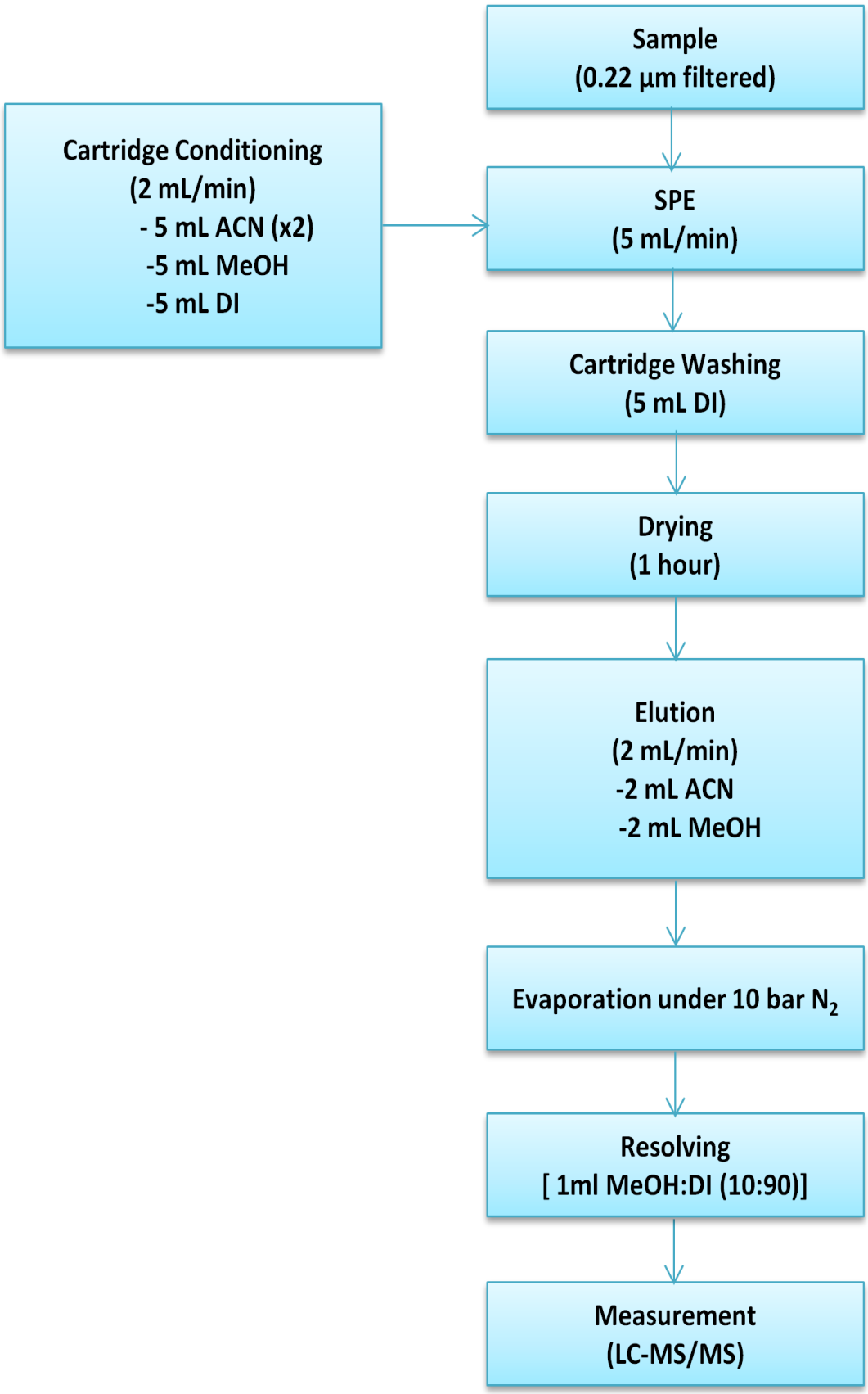


Figure 3.12 : Schematic diagram of diclofenac measurement in aqueous phase.

Figure 3.13 : Evaporation of samples in Turbovap.

3.4.1.2 Diclofenac measurement in sludge phase

0.1 g of sludge samples dried at freeze dry (ThermoSavant, Modulyo D) under vacuum and have been weighted and placed into teflon centrifuge tubes. 10 mL of methanol-acetone (1:1, v/v) has been added into sludges after injection of d_4-diclofenac. To provide transition of diclofenac from sludge to solvent 10 minutes ultrasonic bath (Intersonik, MIN12) has been applied (Figure 3.14).

Soon after, supernatants have been separated after centrifuging at 9000 rpm for 15 minutes. Then 10 mL methanol- acetone has been added again into teflon tubes and ultrasonic bath and centrifugation have been applied with the similar conditions as shown in Figure 3.15.

Figure 3.14 : Ultrasonic bath.

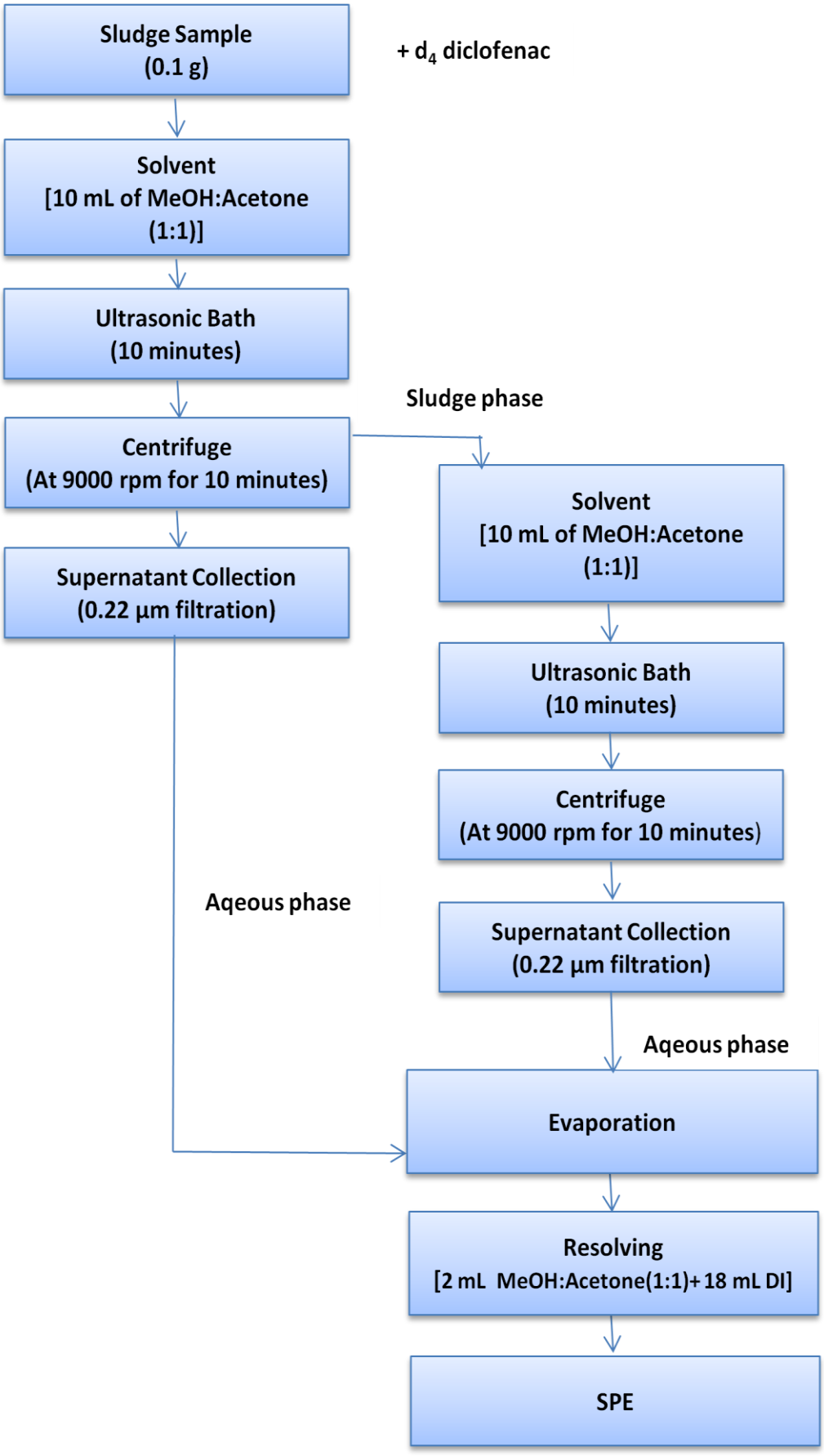


Figure 3.15 : Schematic diagram of diclofenac measurement in sludge phase.

First and second supernatants have been collected and placed into evaporator tubes and evaporated to dryness at 45 $^{\circ}$C at evaporator (Heidolph, Laborota 4000) and dissolved with a mixture of 2 mL methanol-acetone and 18 ml water. Subsequently, solid phase extraction has been applied without d_4-diclofenac addition.

4. RESULTS AND DISCUSSIONS

4.1 Characterization and Diclofenac Removal in wwtp

4.1.1 Summer Campaign

Sampling for summer campaign has been carried out in dry weather conditions in July 2012. In total 12 samples including 24 hours composite influent and effluent samples have been collected from different units of wastewater treatment plant. Conventional characterization of the samples has been performed by monitoring the parameters such as COD, TKN, NH_3, TP, etc. in all samples and showed in Table 4.1.

The pH throughout the units has been varied between 7.0 and 7.9 without further change. COD concentration of plant influent has been measured as 865 mg/L in 24 hours composite sample. This value has been decreased to 26.9 mg/L in plant effluent by achieving 96 % COD removal in the plant. The ratio between COD and soluble COD has been found as 24 % at the influent of the plant. According to measurement in 24 hour composite samples, soluble COD has decreased from 205 mg/L to 25 mg/L with 88% of removal yield.

TOC and DOC measurement have been also performed in the samples. The ratio between TOC and DOC has been found as 30 % at the influent of the plant. According to measurement in 24 hour composite samples from influent and effluent, 94% and 86% of removal yield have been obtained for TOC and DOC, respectively.

TKN and NH_3 concentration in the 24 hours influent sample have been measured as 73 and 36 mg/L, respectively. Throughout the plant, 92% of TKN removal has been observed while TKN concentration has decreased to 5.94 mg/L for 24 hours composite sample taken from the effluent of the plant. Also, 95% of NH_3 removal has been observed while NH_3 concentration has decreased to 2.24 mg/L that has been measured in 24 hours effluent sample.

Table 4.1 : Conventional characterization results of WWTP in summer campaign (July 2012).

Sample	pH	TSS (mg/L)	VSS (mg/L)	TOC (mg/L)	DOC (mg/L)	COD (mg/L)	sCOD (mg/L)	TKN (mg/L)	NH_3-N (mg/L)	TP (mg/L)
Influent[a]	7.5	630 ± 5	380 ± 4.7	240	72	865 ± 6	205.7 ± 5	73.92	36.96	10.99
Grit Chamber[b]	7.8	510 ± 30	350 ± 4.7	220 ± 4	53	425± 12	155.1 ± 5	87.47	47.60	9.65
Primary Clarifier[b]	7.9	180 ± 20	155 ± 20	102.18	29	360 ± 56	170 ± 5	62.44	36.40	7.88
Anaerobic Tank[b]	7.1	10920 ± 50	6110 ± 40	2750 ± 258	595	7520 ± 111.9	25 ± 5	477.40	28.00	40.74
Anoxic Tank[b]	7.2	8660 ± 250	4900 ± 225	1968 ± 362	517	4510 ± 111.9	20.6 ± 6.7	395.92	22.40	35.80
Aerobic Tank[b]	7.7	8780 ± 30	4980 ± 60	2047 ± 223	496	4895 ± 111.9	20.6 ± 5	118.16	14.00	24.69
Final Clarifier[b]	7.7	4	2	16,48	10	39.6 ± 2.2	19.0 ± 4.8	3.78	0.98	5.65
Effluent[a]	7.9	10	9	14.72	9.7	30 ± 4.5	25.3 ± 6.7	5.94	2.24	3.28
Primary Sludge[b]	7.7	57400 ± 495	24000 ± 141	13402 ± 1940	-	33554 ± 859	-	1668.80	140.00	397.53
Secondary Sludge[b]	7.0	3680 ± 42	2125 ± 7	1205.40	-	3560 ± 559.5	-	256.48	33.60	170.37
Digestion sludge[b]	7.4	37575 ± 459	14625 ± 813	9180 ± 2992	-	10760 ± 895	-	1936.48	974.40	646.91
Santrate[b]	7.6	10630 ± 42	5580 ± 57	3324	106	7120 ± 223.8	234.2 ± 4.5	977.76	627.20	383.95

[a] 24 hours composite samples; [b] Grab samples.

In addition, TKN and NH_3 concentration in grab samples of grit chamber have been 87 and 48 mg/L, respectively. Activated sludge process has achieved almost complete removal of TKN and NH_3 providing removal efficiency of 94% and 97%, respectively that has resulted in 3.78 mg/L of TKN and 0.98 mg/L of NH_3 in final clarifier effluent.

According to measurements in 24 hours composite samples from influent and effluent of the treatment plant, a decrease in the total phosphorous concentration was observed from 10.99 mg/L to 3.28 mg/L with 70% of removal efficiency.

Diclofenac concentration in 24 hours composite influent sample has been measured as 846.39±57.65 ng/L as shown in Figure 4.1. Higher concentration has been observed such 1058, 64±86 ng/L in grit chamber effluent that collected as grab samples. The reason of this has been variations in pollution load during the 24 hours in composite sample. 51 % of diclofenac removal efficiency has been observed while diclofenac concentration has decreased to 412.90±43.21 ng/L in the plant effluent.

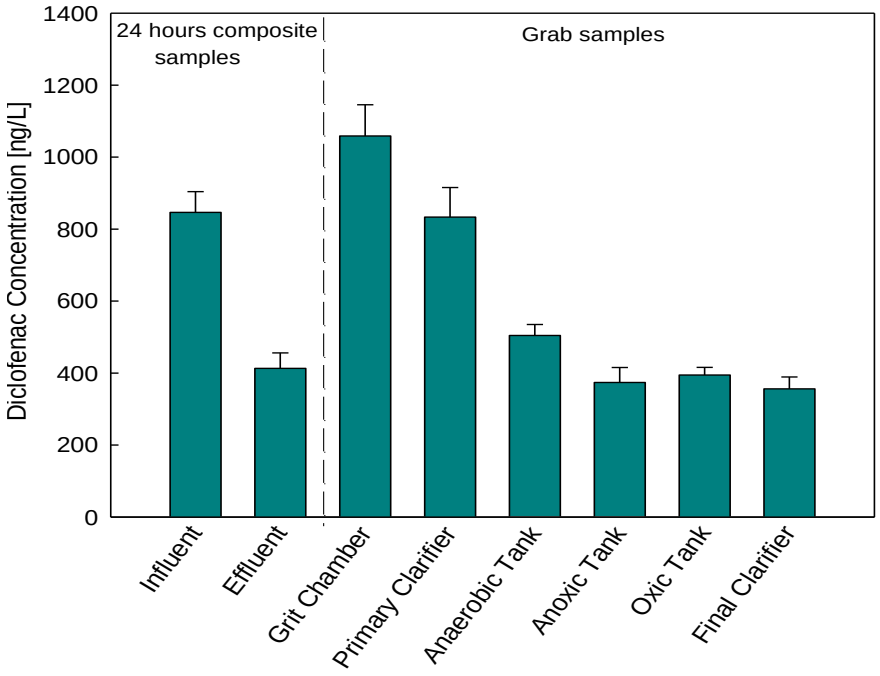


Figure 4.1 : Diclofenac concentration in each unit of WWTP in summer campaign (July, 2012).

Diclofenac removal efficiency has been investigated for primary treatment and secondary (biological) treatment by using grab samples. It is observed that 21% of diclofenac removal has been achieved in primary treatment. Similar to this value, maximum removal efficiency in primary treatment has been found by Behera et al. (2011) as maximum 28%. Due to low K_{ow}, diclofenac has insignificant adsorption potantial to particles removed in primary treatment. Most of the removal efficiency (approximately 45%) has been observed in secondary treatment. Similar to our findings, it is reported that 28–53% of diclofenac, has been removed by secondary treatment in the wastewater treatment plants (Sui et al., 2010).

Diclofenac measurement has been also performed in the sludge phase of each sample, but negligible amount has been detected as 36.87±9.81 ng/g as shown in Table 4.2. Diclofenac has not been detected in the sludge samples where diclofenac has beem measured. Similar to our study, in a study performed in Japan they have found the diclofenac concentration in the sludge samples in the range of 6-29 ng/g (Matsou et al., 2011). It has been reported by several authors that removal of diclofenac from wastewater has been achieved by biodegradation instead of sorption onto sludge (Nakata et al., 2006; Jones et al., 2005; Martin et al., 2012).

Table 4.2 : Diclofenac concentrations in sludge samples.

Sampling point	**Diclofenac Concentration [ng/g]***
Primary Sludge	< 5
Anaerobic Tank	< 5
Anoksik Tank	< 5
Aerobic Tank	36.87±9.81
Secondary Sludge	< 5
Anaerobic Digester Sludge	< 5
Santrate	< 5
Dried Sludge	< 5

*Dried weight basis.

4.1.2 Autumn Campaign

Sampling for the characterization study in autumn has been carried out in November 2012. 24 hours influent and effluent composite samples collected from the effluent of grit chamber and final clarifier, respectively. Conventional parameters such as COD, TKN, NH_3, TP, etc. have been measured in all samples and showed in Table 4.3.

450 mg/L of COD has decreased to 110 mg/L in plant effluent by achieving 76% COD elimination in the overall processes. The ratio between total COD and soluble COD has been found as 61% at the influent of the plant. 89% of soluble COD removal has been observed by decreasing 275 mg/L in plant influent to 30 mg/L in plant effluent.

Table 4.3 : Conventional caharacterization results of WWTP in autumn campaign (November 2012).

Parameter	Unit	Influent	Effluent	Removal
pH	-	8.20	7.82	-
COD	mg/L	450±60	110±30	76
sCOD	mg/L	275±5	30±5	89
TOC	mg/L	310±124	47±6	84
DOC	mg/L	95±0.01	26.3±5.6	94
TSS	mg/L	350±5	20±5	-
VSS	mg/L	250±5	15±5	-
TKN	mg/L	84.5±0.24	46	45
NH_3-N	mg/L	53.03±0.24	40.6±0.3	24
TP	mg/L	84±1.8	2.2±0.2	74

TKN and NH_3 concentration in the influent sample have been measured as 84.5 and 53.03 mg/L. Throughout the plant, 45% of TKN removal has been observed while TKN concentration has decreased to 46 mg/L that has been measured in 24 hours effluent sample. 23% of NH_3 removal has been observed while NH_3 concentration has decreased to 40.60 mg/L which has been measured in 24 hours effluent composite sample. Also 73% removal has been achieved for total phosphorus by a decrease from 8.37 mg/L to 2.22 mg/L.

Diclofenac concentration in plant influent sample has been measured as 856.92±62 ng/L. 36% diclofenac removal efficiency has been observed while diclofenac concentration has decreased to 545.55±33 ng/L in the plant effluent. Removal of diclofenac has been reported as within the range of 40% in wwtps depending on the configuration and operation condition of WWTPs as well as wastewater characteristics (Zhang et al., 2008).

4.1.3 Winter Campaign

Sampling has been carried out in March due to continuous precipitation in winter season. A total of ten samples including 24 hours influent and effluent composite samples have been collected. Conventional parameters such as COD, TKN, NH_3, TP, etc. have been measured in all samples and showed in Table 4.4.

The pH have been observed between 7.0 and 8.0 in all samples taken from ten units of the plant. 638 mg/L of COD have been measured in 24 hour composite sample of plant influent. It has decreased to 79 mg/L in plant effluent by achieving 88% COD elimination in the plant. The ratio of soluble COD to total COD has been found as 20% at the influent of the plant. 81% of sCOD removal has been observed by decreasing 128 mg/L in plant influent to 24 mg/L in plant effluent.

TOC and DOC measurements have been also performed to investigate carbon removal. According to measurement in 24 hour composite samples of effluent and effluent, 60% of diclofenac removal yield have been obtained for TOC. TKN concentration in the plant influent have been measured as 83 mg/L. Throughout the plant, 24% of TKN removal has been observed while TKN concentration has decreased 63 mg/L that has been measured in the plant effluent.

In addition, TKN and NH_3 concentration in grab samples of grit chamber have been 83 and 44 mg/L, respectively. Activated sludge process has achieved 29% and 25% of TKN and NH_3 elimination by reaching 54 mg/L of TKN and 47 mg/L of NH_3 in final clarifier effluent concentrations.

According to measurement in 24 hours composite samples of influent and effluent, total phosphorous concentration has decreased from 7.57 mg/L to 3.49 mg/L with 55% removal efficiency. In activated sludge process 83% of removal efficiency has achieved for phosphorous which has been decreased from 7.6 mg/L in primary clarifier effluent to 1.41 mg/L in final clarifier effluent.

Table 4.4 : Conventional characterization results of WWTP in winter campaign (March 2013).

Numune Adı	pH	TSS (mg/L)	VSS (mg/L)	TOC (mg/L)	DOC (mg/L)	COD (mg/L)	sCOD (mg/L)	TKN (mg/L)	NH_3-N (mg/L)	TP (mg/L)
Influent[a]	7.5	673±9	358±7	80±13	47.68	638±0	128±2	83.08±0.6	44.63	7.57±0.1
Grit Chamber[b]	8.0	536±5	331±12	93±9	58±10	786±21	134±4	89.55±16	63.09	7.6±0.1
Anaerobic Tank[b]	7.0	11416±24	7833±0	591	134±8	9371±0	43±2	759.40±107	53.51	120±22
Anoxic Tank[b]	7.3	5363±5	3626±9	565	143.8±40	3942±736	37±4	390.73±56	50.22	72.5±.2.6
Aerobic Tank [b]	7.2	5133±47	3483±71	422	85±13	3867±210	15±2	419.24±11	49.16	69.6±1.1
Final Clarifier[b]	7.5	33±4	26±0.7	30±0.1	20±2.2	56±4	13±0	54.04±4	47.30	1.41
Effluent[a]	7.5	59±4	44±0.7	32±0.6	22±0.03	79±2	24±2	63.80±4	50.30	3.49±0.1
Secondary Sludge[b]	7.0	12025±177	8200±71	450±140	389±11	7719±0	-	961.38±17	64.97	190.1±8.7
Digestion sludge[b]	7.4	32800±141	16550±71	1163±18	230±45	24049±1259	321±2	2616±125	1134.84	539.5±75

[a]24 hours composite samples; [b] Grab samples.

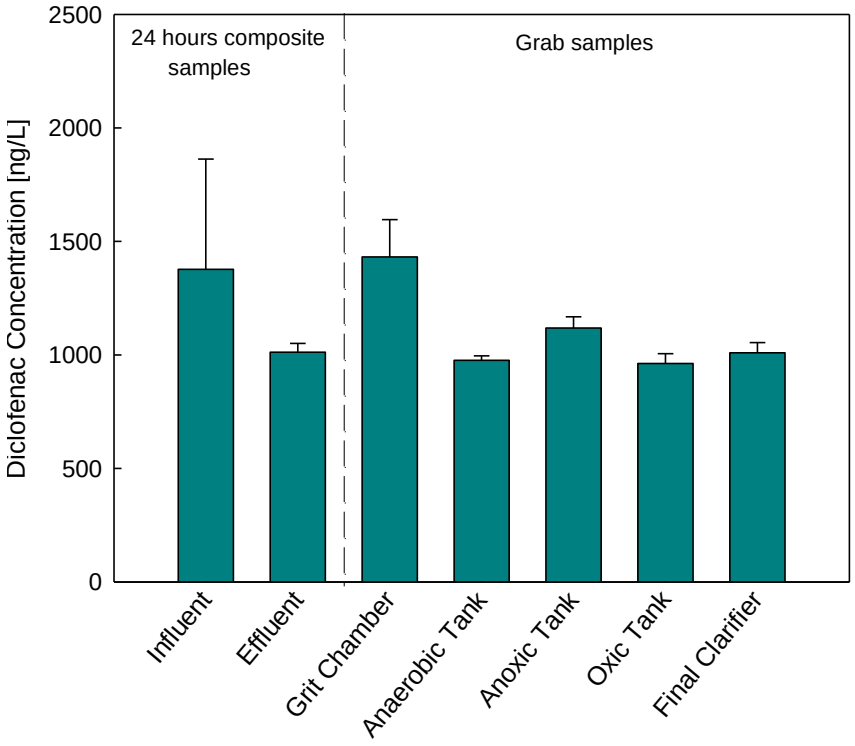


Figure 4.2 : Diclofenac concentration in each unit of WWTP in winter campaign (March, 2013).

In the influent of the wastewater treatment plant based on the 24 hours composite sample, the diclofenac concentration have been detected as 1376±486 ng/L as shown in Figure 4.2. Higher concentration has been observed (i.e., 1431±164 ng/L) in grit chamber effluent that has been collected as grab samples. The reason of this has been the variations in pollution load during the day.

27% of diclofenac removal efficiency has been observed while diclofenac concentration has decreased to 1012±39 µg/L in the plant effluent. It is reported that diclofenac was not completely removed during wastewater treatment process with 20-30% of removal efficiency (Suarez et al., 2008). During secondary treatment, 30% of removal yield has been obtained from measurements conducted using grab samples. According to a research, 28–53% of diclofenac was removed by secondary treatment in the conventional wastewater treatment plant (Tauxe-Wuershe et al., 2005).

4.1.4 Spring Campaign

Spring sampling has been carried out in May 2013. 24 hours composite samples have been collected from wwtp influent and effluent. Conventional parameters such as COD, TKN, NH_3, TP, etc. have been measured in two samples and showed in Table 4.5.

758 mg/L of COD has decreased to 53.6 mg/L in plant effluent by achieving 93% COD elimination in the overall processes.The ratio between COD and soluble COD has been found as 23% at the influent of the plant. 77% of TOC removal has been observed by decreasing 230 mg/L in plant influent to 53.6 mg/L in plant effluent.

Table 4.5 : Conventional characterization results of wwtp in spring campaign (May 2013).

Parameter	Unit	Influent	Effluent*	Removal
pH	-	7.66	7.91	-
COD	mg/L	758±32	53.6±4.2	93
sCOD	mg/L	174±6.3	44.6±8.4	74
TOC	mg/L	230±18	88.9±5.8	77
DOC	mg/L	143±3.01	53.6±4.2	38
TSS	mg/L	408±11	119 ± 45	-
VSS	mg/L	248±45	86±31.3	-
TKN	mg/L	97.6±1.6	47.6±0.17	51
NH_3-N	mg/L	55.98±0	36.25±0.29	35
TP	mg/L	9.22±0.68	1.9±0.069	79

*30 minutes settling has been applied in laboratory conditions.

TKN and NH_3 concentration in the influent sample have been measured as 97.6 and 55.98 mg/L. Throughout the plant, 51% of TKN removal has been observed while TKN concentration has decreased to 47.6 mg/L that has been measured 24 hours

effluent sample. Also, 35% of NH_3 removal has been observed while NH_3 concentration has decreased to 36.25 mg/L that has been measured 24 hours effluent sample. Also, 79% phosphorous removal has been obtained.

Diclofenac concentration in plant influent sample has been measured as 923±31 ng/L. The removal of diclofenac has been observed as 18 % with 756±9 ng/L diclofenac concentrations in the plant influent and effluent. Lower treatment efficiencies has been found in literature even Tauxe-Wuerch et al. (2005) has pointed also no elimination in wastewater treatment plants in Switzerland.

Higher diclofenac concentration has been measured in winter as shown in Figure 4.3. It has been related to higher consumptions of drugs during the cold period of the year. Inflow loads during winter time have been reported two times higher than loads during summer (McArdell et al., 2003).

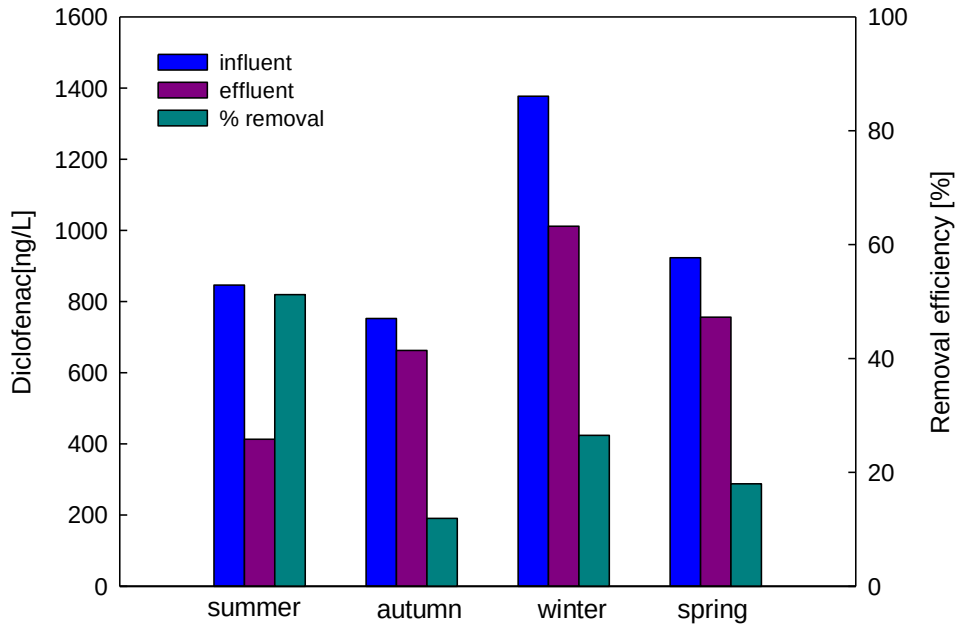


Figure 4.3 : Diclofenac removal efficiency and concentrations in influent and effluent.

Maximum diclofenac removal yield has been observed in summer season as shown in Figure 4.3. In summer season, conventional parameters such as COD, TKN, NH_3, and TOC have shown better removal efficiency with 96, 92, 94, and 90% removal with respect to other seasons. The seasonal differences of pharmaceuticals concentration in the treated effluents have seemed to reflect the performance of

treatment processes. These compounds have been more effectively removed in warmer temperature of summer in the presence of abundant sunlight (Yu et al., 2013).

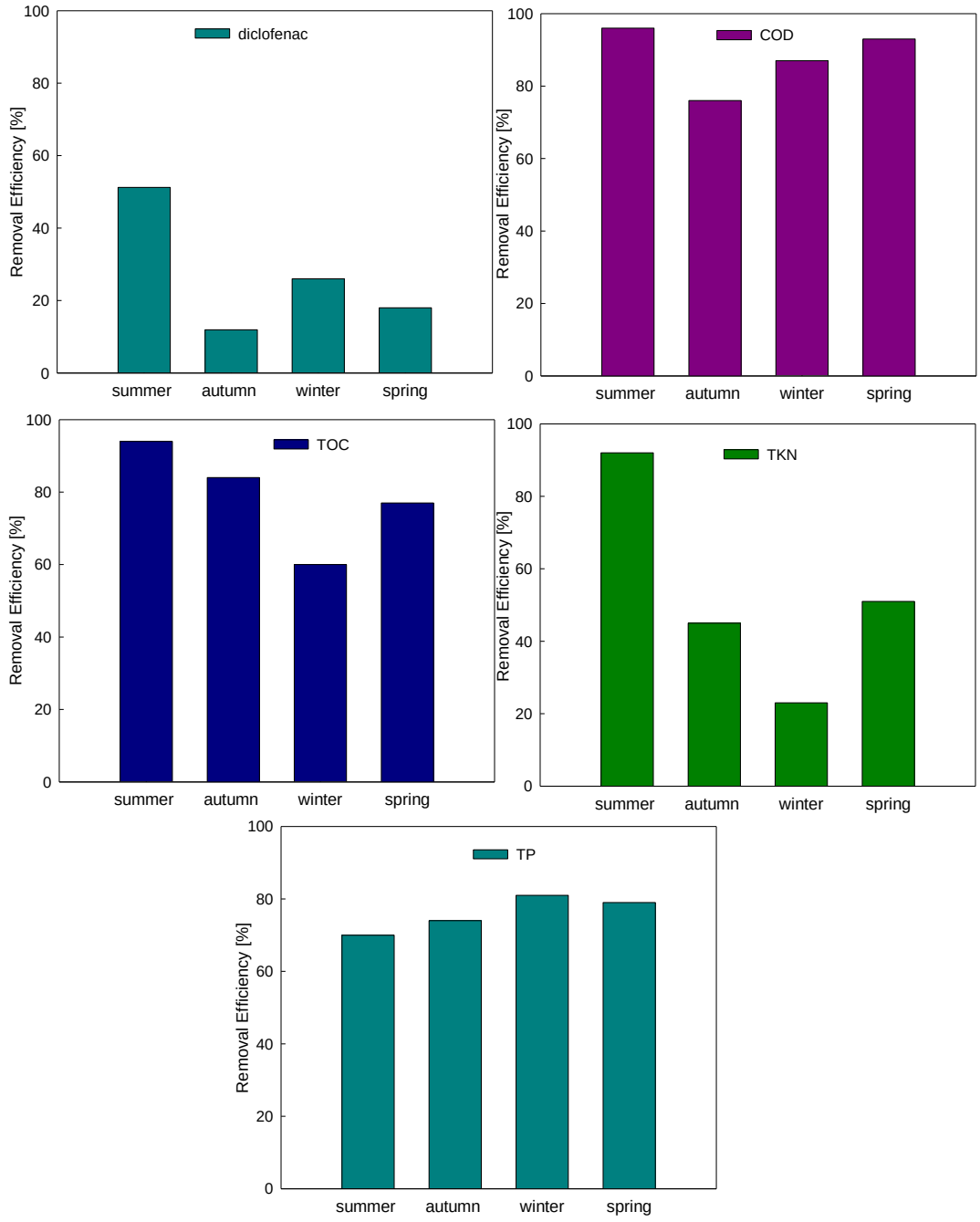


Figure 4.4: Removal efficiencies of diclofenac, COD, TOC, TKN, and TP.

Diclofenac removal efficiency has shown similar trend with COD during a year. Higher removal efficiency for diclofenac has been observed in summer while lower elimination has been observed in autumn. Also, COD has shown declining trend in

autumn. Positive correlations have been found by Santos et al. (2009) through the association of these compounds to the dissolved organic matter present in wastewater which is commonly characterized by BOD and COD values.

Activated sludge efficiency has been related with diclofenac removal efficiency because most part of the diclofenac elimination has been achieved in secondary treatment processes. By secondary treatment, 45% of diclofenac removal has been achieved in summer wheraes 28% of diclofenac removal has been obtained in winter.

TKN and NH_3 removal efficiencies that indicates activated sludge process' performace have been higher in summer resulting in higher diclofenac elimination with respect to other seasons. Diclofenac removal efficiency has decreased in other seasons with decrease in TKN and NH_3 removal. Lower diclofenac elimination has been obtained in autumn where lowest TKN and NH_3 efficiencies have been found with respect to other seasons.

4.2 Anaerobic Culture Development

4.2.1 Cultures developed at 22ºC

Culture series were developed under fermentative-methanogenic conditions to investigate the effect of diclofenac on fermentation, methanogenesis and diclofenac biodegradation. First generation anaerobic batch reactors including Reactor A.I, B.I, C.I, and D.I reactors have been operated for a long time with same diclofenac (10 µg/L) and substrate concentration (300 mg/L glucose and 15 mg/L yeast extract). Inoculum characteristics are shown in Table 4.6.

Table 4.6 : Characteristics of sludges and river sediments used as inoculums.

Inoculum	pH	TS [%]	VS [%]	TOC [mg/g]*
Alibeyköy river sediment	7.35	63.71 ± 0.60	4.58 ± 0.26	18.36
AD sludge of WWTP-1	7.39	6.78	3.85	11.30
Kağıthane river sediment	7.22	51.60 ± 1.99	5.63 ± 0.00	22.04
America river sediment	6.15	41.99	35.05	48.72

*dry matter.

During the operation the pH has been remained in the neutral range (6.0-7.0), and the ORP has been observed nearly in the range of -200 and -330 mV in all reactors (Table 4.7).

Table 4.7 : pH and ORP values of Reactor A.I, B.I, C.I, and D.I.

Reactor	pH	ORP [mV]
A.I	7.19 ± 0,30	-210
B.I	7.22 ± 0,30	-330
C.I	7.31 ± 0,28	-215
D.I	7.09 ± 0,38	-253

Reactors A.I, B.I, C.I and D.I have been fed weekly with 350 mg/L COD consisted of glucose and methanol. Theoretically 329, 338, 333, and 348 mL of biogas production have been expected by Reactor A.I, B.I, C.I, and D.I with methane percentage of 73, 71, 72, and 69 that has been measured in GC (Table 4.8).

While lower biogas production have been observed in all of the reactors at the beginning of the acclimation period due to acclimation and development of microorganisms in the new conditions, biogas production has been increased in the following feeding cycles.

Table 4.8 : Biogas composition and theoretical gas production of Reactor A.I, B.I, C.I, and D.I.

Reactor	CH_4 [%]	CO_2 [%]	Theoretical Biogas Production [mL]
A.I	73	27	329
B.I	71	29	338
C.I	72	28	333

Biogas production profiles of reactors shows that, Reactor A.I, B.I, and C.I have been performed better biogas production trends with respect to Reactor D.I (Figure 4.5).

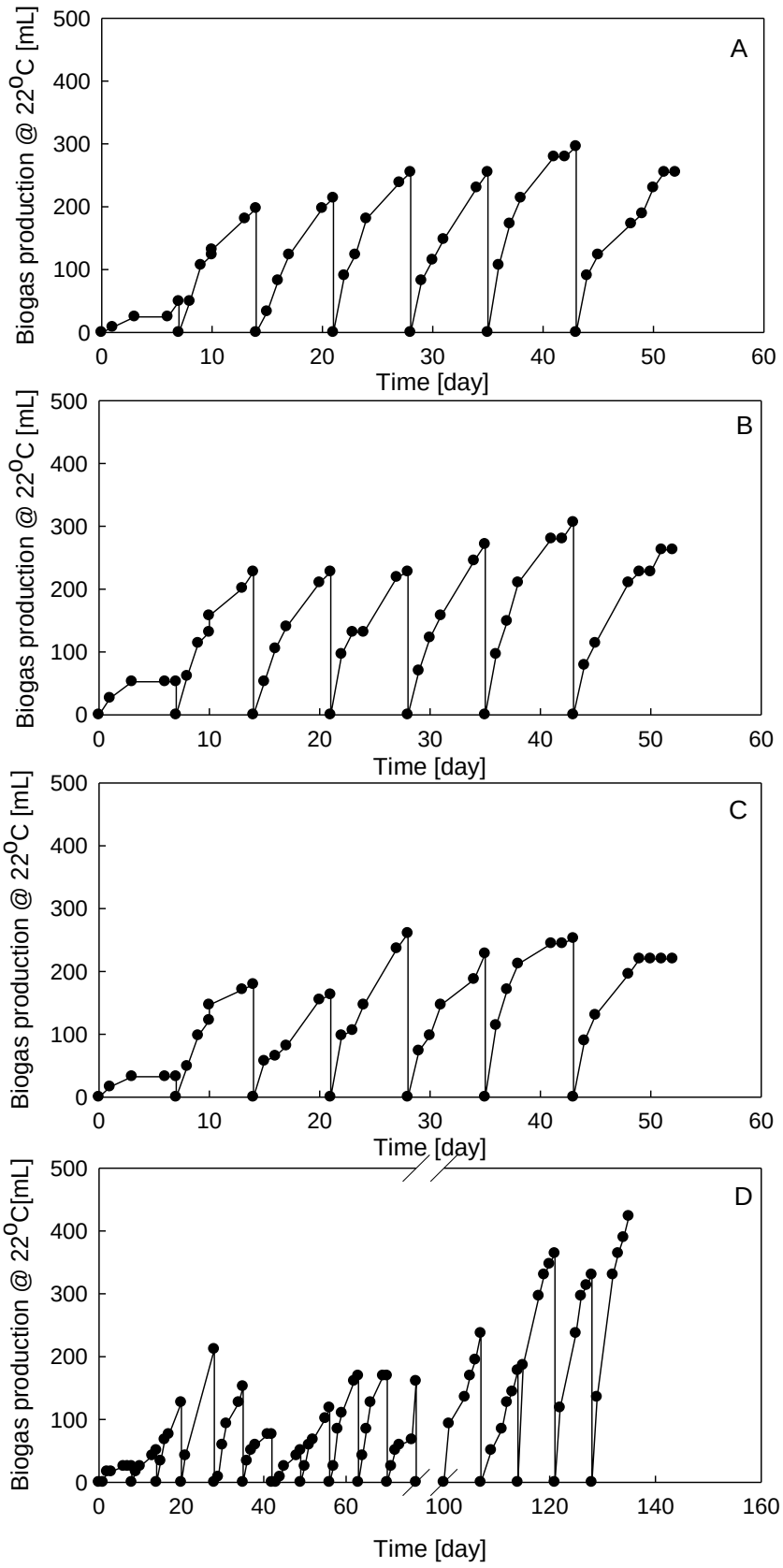


Figure 4.5 : Biogas production of Reactor A.I (A), B.I (B), C.I (C), and D.I (D).

While Reactor A.I, B.I, and C.I have shown increasing biogas production performance along the feeding period, Reactor D.I has not revealed stable biogas production performance despite 160 days long operation time.

Reactor A.I, B.I, and C.I have not reach their theoretical biogas concentration and produced 265, 267 and 240 mL of biogas, respectively. Diclofenac may be the cause of depletion in biogas production with a loss of 19, 21, 28% in Reactor A.I, B.I, and C.I, respectively.

Biogas production of Reactor D.I has been varied for every feeding cycle. pH of the reactor have tended to decresase but it has been adjusted to 7 with the addition of $NaHCO_3$ (0.5 g/L).

Reactor A.I and C.I have been examined in terms of volatile fatty acids that were converted from substrate by fermentative microorganisms for one feeding cycle. As shown in the Figure 4.6, volatile fatty acids including acetic acid and propionic acid have been both produced and then consumed by microorganisms in the reactors and participated in biogas formation.

Reactor A.I , B.I, and C.I have been fed weekly with diclofenac by providing 10 µg/L diclofenac concentration in each feeding cycle during 52 days. During 52-day operation period, averagely 20, 21, and 36.8 % of diclofenac removal have been achieved in Reactor A.I , B.I, and C.I, respectively.

The reason why high elimination has been observed in Reactor C.I can been the high particulate matter content which comes from the inoculum,where diclofenac can be adsorbed. It also explained the decrease in diclofenac removal after two weeks in this reactor.

After the day 52, the feeding with both carbon source and diclofenac in Reactor A.I , B.I, and C.I have stopped. Although the reactor has not been fed with additional carbon source between the day 52 and 121, diclofenac being a potential carbon source has not biodegraded in the reactor. Although, no carbon source has been present in the reactor except than diclofenac, diclofenac removal was not observed.

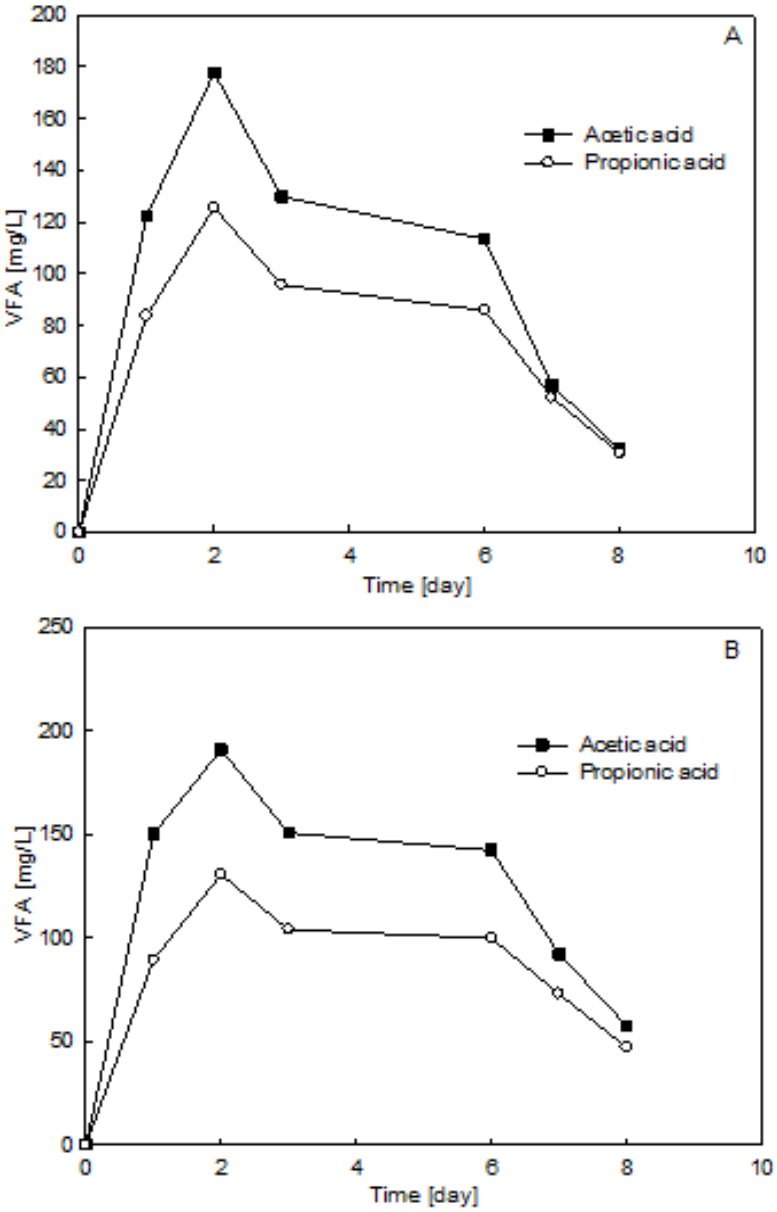


Figure 4.6 : Volatile fatty acids formation in the Reactor A.I (A) and C.I (B).

Reactor D.I has been fed weekly with diclofenac to provide 10 µg/L diclofenac concentration. A decrease in diclofenac concentration have been observed after five feeding cycle but due to the lack of proper biogas performance or methanogenic activity, the removal rate of diclofenac in the Rector D.I has been very low averagely 12%.

At the end of the operation diclofenac elimination has been achieved as 31, 20, 30 and 13 % in Reactor A.I, B.I, C.I, and D.I, respectively (Figure 4.7).

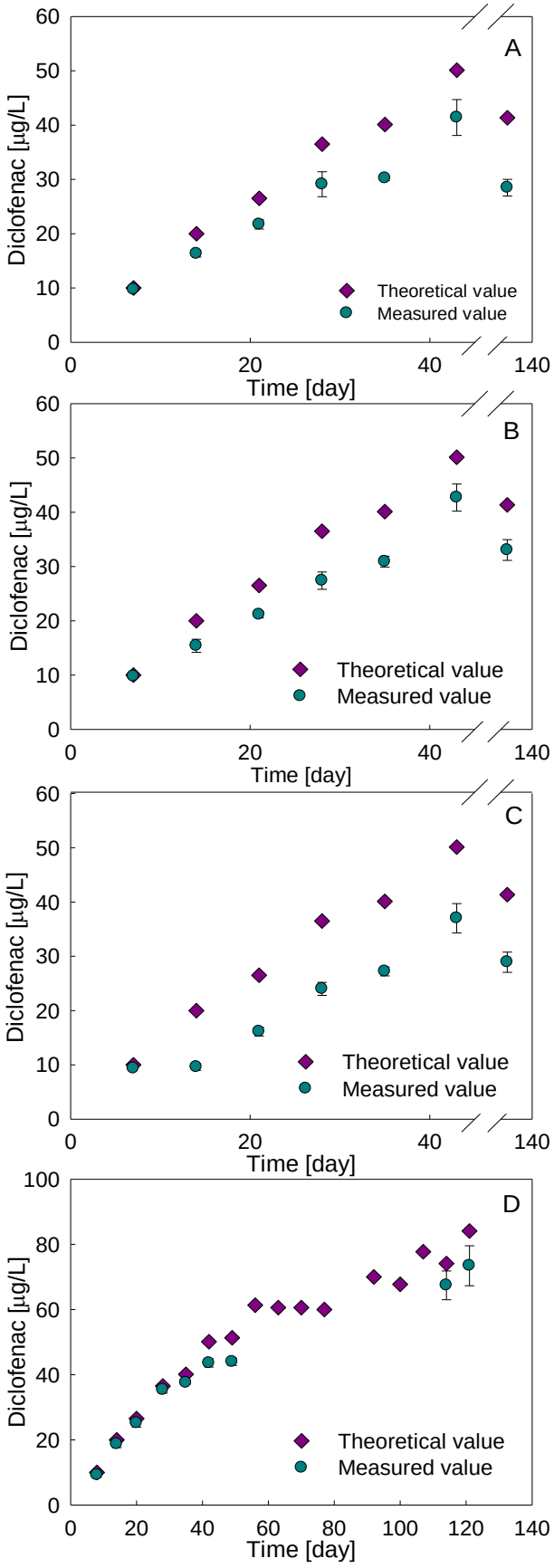


Figure 4.7 : Theoretical and measured diclofenac concentrations in Reactor A.I (A), B.I (B), C.I (C), and D.I (D).

Average diclofenac removal has been observed as 24% in the anaerobic reactors performed properly. It is shown that adequate diclofenac removal has not been obtained in first generation anaerobic reactors such that diclofenac exhibit some resistance to anaerobic degradation. It is reported that no significant removal of diclofenac has been observed in upflow anaerobic sludge blanket (UASB) feeding with black water at 25ºC (de Graaff et al., 2011). However, diclofenac removal has been achieved as 26 % at 35ºC (Lahti and Oikari, 2011).

Diclofenac removal by sorption onto sludge has been inadequate in anaerobic reactors. However, study on secondary treatment of WWtp has showed that diclofenac has been shown to persist in the aqueous phase of process. Diclofenac removal has been achived by biodegradation as 24% and by sorption onto sludge as 7% (Khan and Ongerth, 2004).

Sorption potential of diclofenac have been investigated on the sludge for the Reactor A.I, B.I, and C.I. 176, 964 and 55 ng diclofenac have been detected in per g of sludge in Reactor A.I, B.I, and C.I (Table 4.9). Maximum sorption potential with 6% have been observed in Reactor B.I inoculated with anaerobic sludge of wwtp. Diclofenac has been existed in aqeous phase with low affinity to sorb to anaerobic sludge and low biodegradation rate in first generation anaerobic reactors.

Table 4.9 : Fractions of diclofenac in Reactor A.I, B.I, and C.I.

Reactor	Biodegradation [%]	Sorption onto sludge [%]	Aqeous phase [%]
A.I	22.67	2.04	75.29
B.I	16.61	6.34	77.05
C.I	31.20	0.98	67.82

In order to eliminate sediment interruption, after 50 days incubation period a culture transfer was performed by diluting 100 mL of the Reactor A.I in anaerobic culture media (Reactor A.II). Reactor A.II inoculated as second generation reactor and have been fed during 150 days at 22ºC. During the operation the pH has been remained between 6.8 and 7.9 and the ORP has been observed nearly -231 mV.

350 mg/L of COD have been introduced into the reactor by addition of glucose and methanol.

Until the fifth feeding cycle, low amount of biogas production hasbeen observed in Reactor A.II. as shown in Figure 4.8 because Reactor A.II has been inoculated with less amount of sludge, and the growth and acclimation of biomass has taken relatively long time. Biogas production has been increased after acclimation but not reached to the theoretical biogas production more likely due to presence of diclofenac.

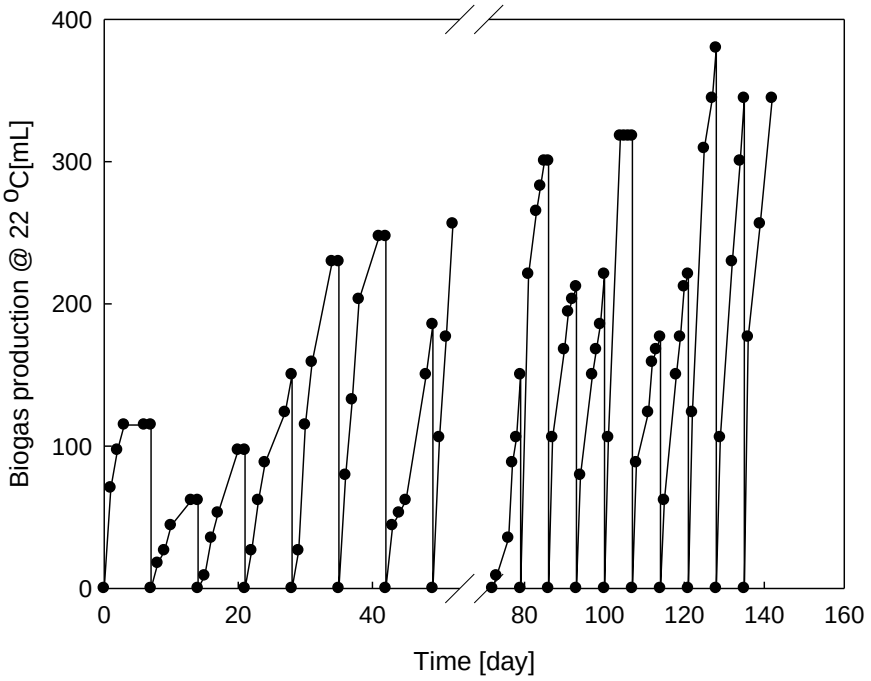


Figure 4.8 : Biogas production performance of Reactor A.II.

Reactor A.II has been amended with 1 µg/L diclofenac. During initial 56 days semi-continuous feeding period, increasing diclofenac elimination has been observed. Approximately 35% of diclofenac removal has been achieved in Reactor A.II.

While Reactor A.I hasbeen performed with averagely 24% of diclofenac removal, Reactor A.II has been performed with 35%.

Higher aqueous phase diclofenac elimination has been observed in Alibeyköy A.II reactor that has been set up using the first generation reactor sludge as inoculum (Figure 4.9). It has been understood that biomass acclimation favored biodegradation of diclofenac. Similar to our findings, Carballa et al. (2007) have been reported the advantage of acclimation on diclofenac removal.

At the day 49, 720 mL of the culture have been taken from Albeyköy A.II as inoculum in order to setup batch assays and the reactor has been filled with fresh media and fed again. The higher decrease in the measured diclofenac concentration has been a result of this transfer for batch reactors setup.

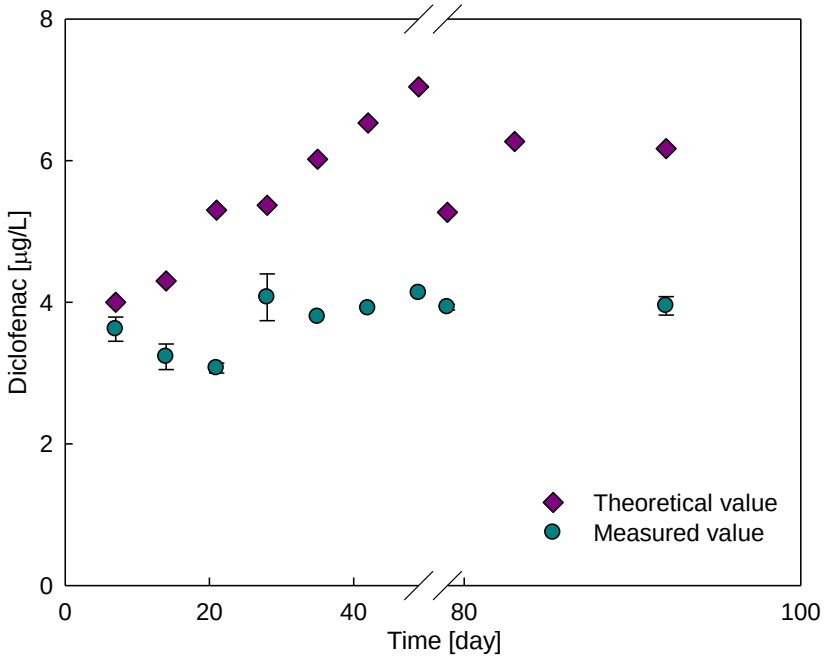


Figure 4.9 : Theoretical and measured diclofenac concentrations in Reactor A.II.

4.2.1 Culture developed at 35ºC

Reactor F.I has been operated at 35ºC as a first generation anaerobic reactor. It has been fed weekly with glucose and methanol to provide 343 mg/L COD concentration in the reactor.

Because, lower biogas productions have been obtained in first feeding cycle, additional 50 mL inoculums have been transferred to Reactor F.I at 10th day. After that, an increase in the biogas production was observed.

Reactor F.I has been revealed continuous biogas production performance with 57 % methane portion (Figure 4.10).

Reactor F.I has been fed with diclofenac weekly with 10 µg/L diclofenac concentration and kept at 35ºC in the dark. At the beginning of the culture development diclofenac removal was not achieved. However, after 70 days

acclimation period partial diclofenac degradation was observed in the reactor. In the reactor, maximum diclofenac removal efficiency has been 21% averagely. (Figure 4.11).

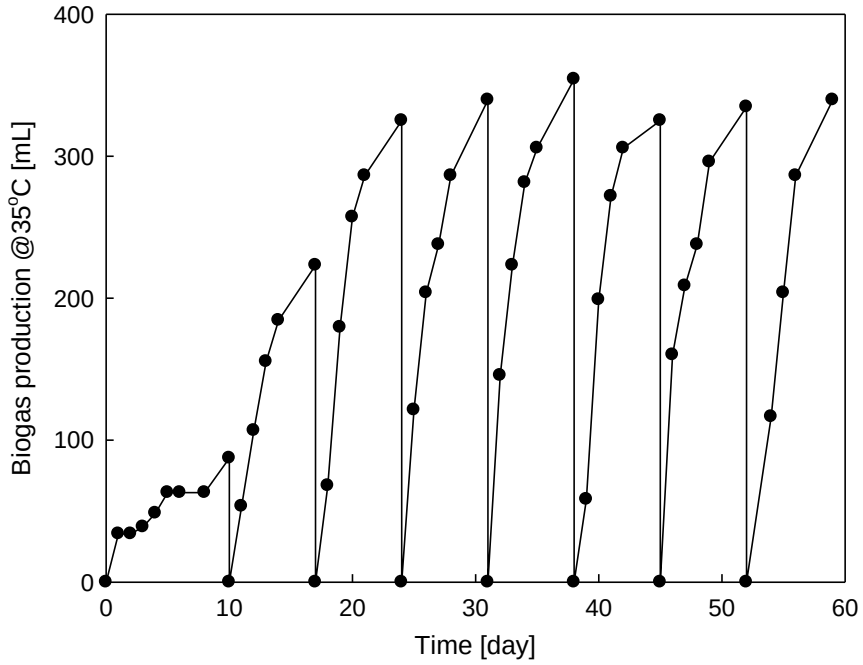


Figure 4.10 : Biogas production in Reactor F.I.

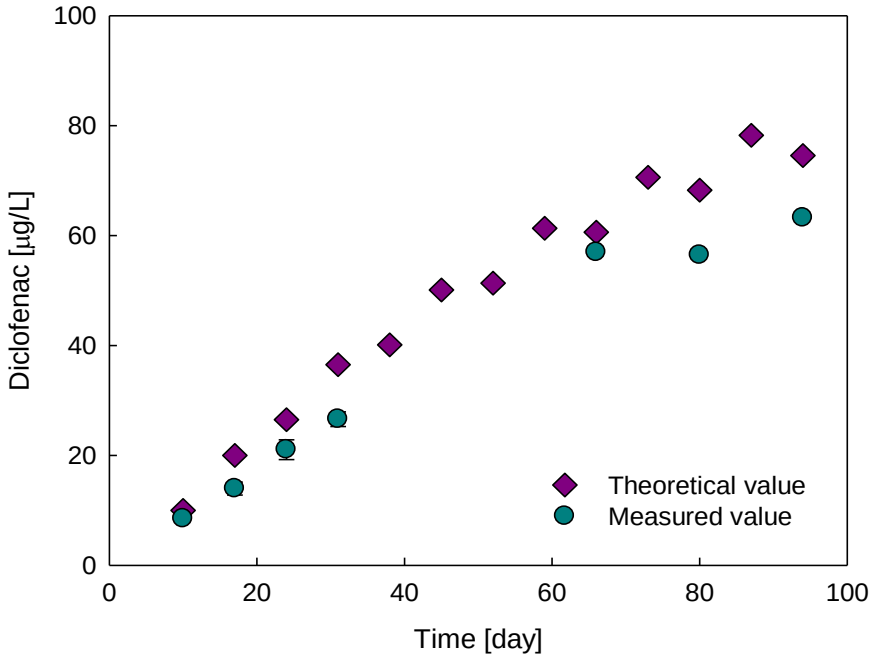


Figure 4.11 : Theoretical and measured diclofenac concentrations in Reactor F.I.

Similar to our result, Lahti and Oikari (2011) has been reported 26 % of diclofenac elimination during the experiments with anaerobically digested sludge at 35ºC. Removal of diclofenac has also found as 60±18% in mesophilic digestion at 37ºC (Carballa et al., 2007).

Reactor F.I operated at 35ºC has not shown distinctive increase in removal of diclofenac as 21 % in comparison to reactors operated at 22ºC including A.I, B.I, C.I, and D.I with 20, 21, 36, and 12%.

4.3 Anaerobic Batch Assays

4.3.1 Different diclofenac concentrations

Four different concentrations including 0 (Control 1 and Control 2), 10, 50, 200 and 1000 µg/L have been introduced to the developed fermentative-methanogenic culture to investigate the effect of diclofenac on fermentation, methanogenesis and diclofenac biodegradation. The cultures were monitored for 45 days incubation period.

pH has been remained in the range of 7.2-8.0 and ORP has been measured in the range of -190 mV- -305 mV during the incubation for all culture series.

Glucose and methanol were used as a carbon source, so 1000 mg/L COD resulting from both glucose and methanol has been introduced into the all culture series. Theoretical methane productions according to the COD introduced have been calculated as 62 mL in serum bottles (180 mL liquid volume). Also diclofenac has been a carbon source resulted in 1.8 mg/L COD in culture series with 1000 µg/L. It has been negligible value in terms of methane production (approximately 0.11 mL).

Control 1 which have been inoculated with only culture from Reactor A.II have been produced 62 mL of methane as shown in Figure 4.12. However, other culture series that inoculated with culture from Reactor A.II and E.I have been exceeded this value. The reason of excess methane production compared to the theoretical value has been the additional COD coming from culture of Reactor E.I. Approximately 100 mg/L of COD has been transferred to culture series with culture from Reactor E.I.

Despite no significant change in methane production has been seen in lower concentrations such as 10, 50 and 200 µg/L, approximately 8 mL of decrease has been observed in the culture amended with 1000 µg/L diclofenac (Figure 4.12).

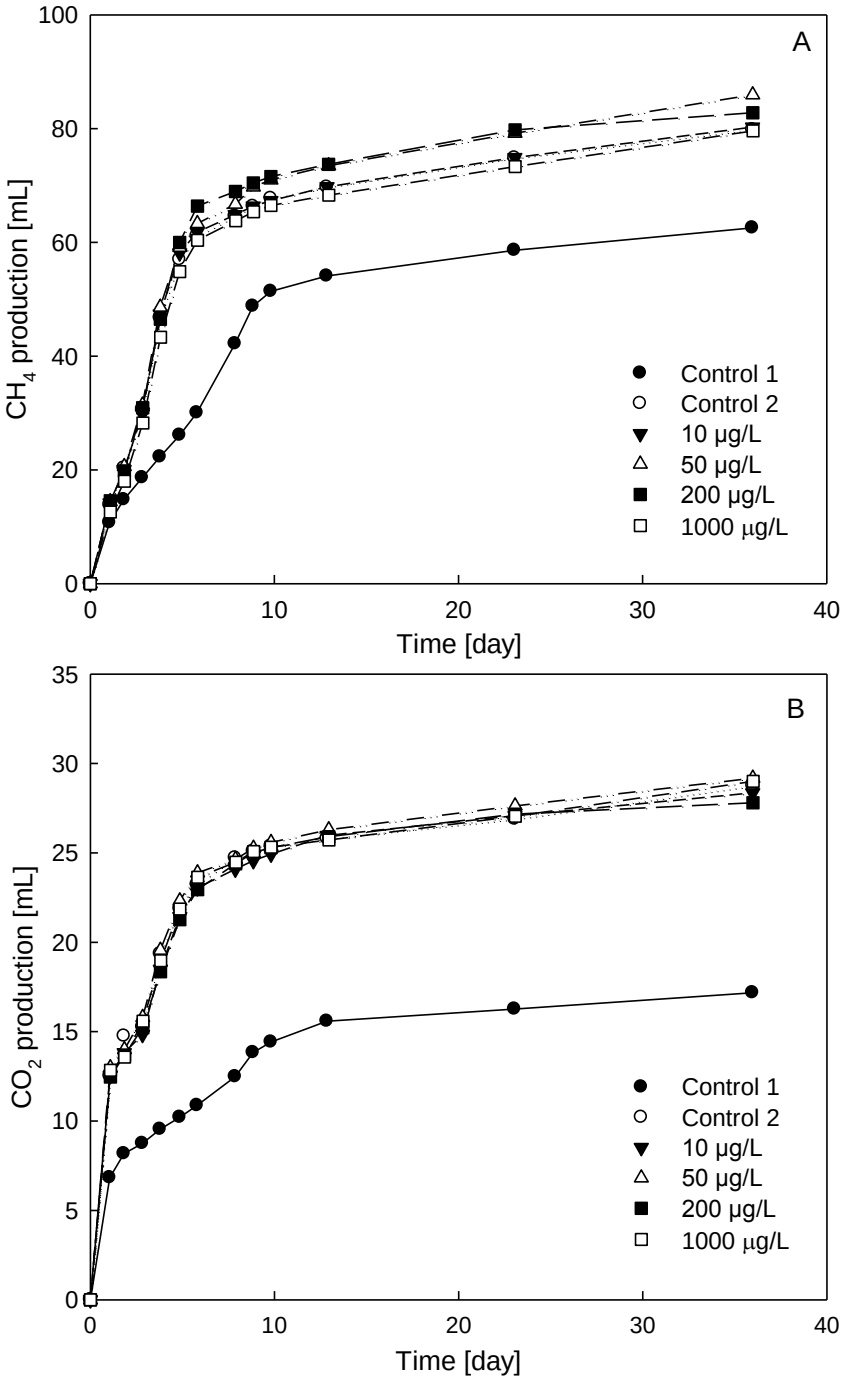


Figure 4.12 : Methane (A) and carbondioxide (B) production in batch test with different diclofenac concentrations.

It has also found that diclofenac caused severe inhibition on methanogenic activity at high concentrations according to a research where concentrations ranging from 0 up to 400 mg/L have been tested (Fountoulakis et al., 2004). Additionally, the concentration of VSS decreased at the end of 30 days incubation period.

Carbon dioxide profiles have showed that diclofenac concentration has not effect carbondioxide production. All culture series except Control 1 have been produced averagely 28-29 mL of carbon dioxide as shown in Figure 4.12.

Volatile suspended solids (VSS) concentrations have been decreased through the operation period in all culture series except Control 1, which has not been amended with diclofenac as shown in Figure 4.13.

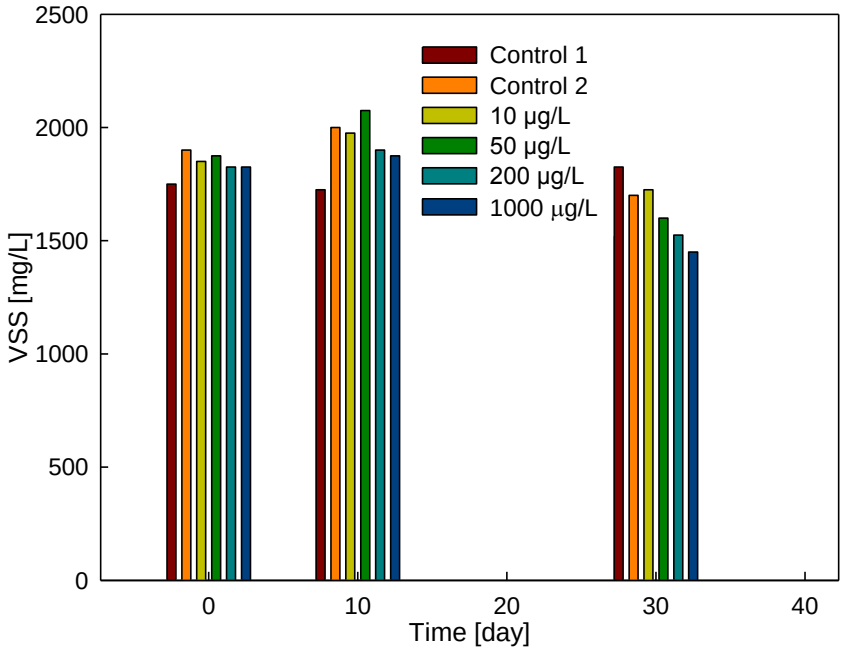


Figure 4.13 : Suspended solids behaviour in batch anaerobic test of different diclofenac concentrations.

COD introduced into culture series in the form of glucose have converted into the VFAs. Acetic acid, propionic acid, and isobutyric acid have been produced in all culture series.

Due to unconsumed VFAs coming from culture of Reactor E.I, VFAs concentration in Control 2, 10 µg/L, 50 µg/L, 200 µg/L, and 1 mg/L of culture series have not been zero at the time 0 as shown in Figure 4.14. Initial VFA concentration was determined

as 20 mg/L in all of the reactors except Control 1 that was not amended with the culture from Reactor E.I.

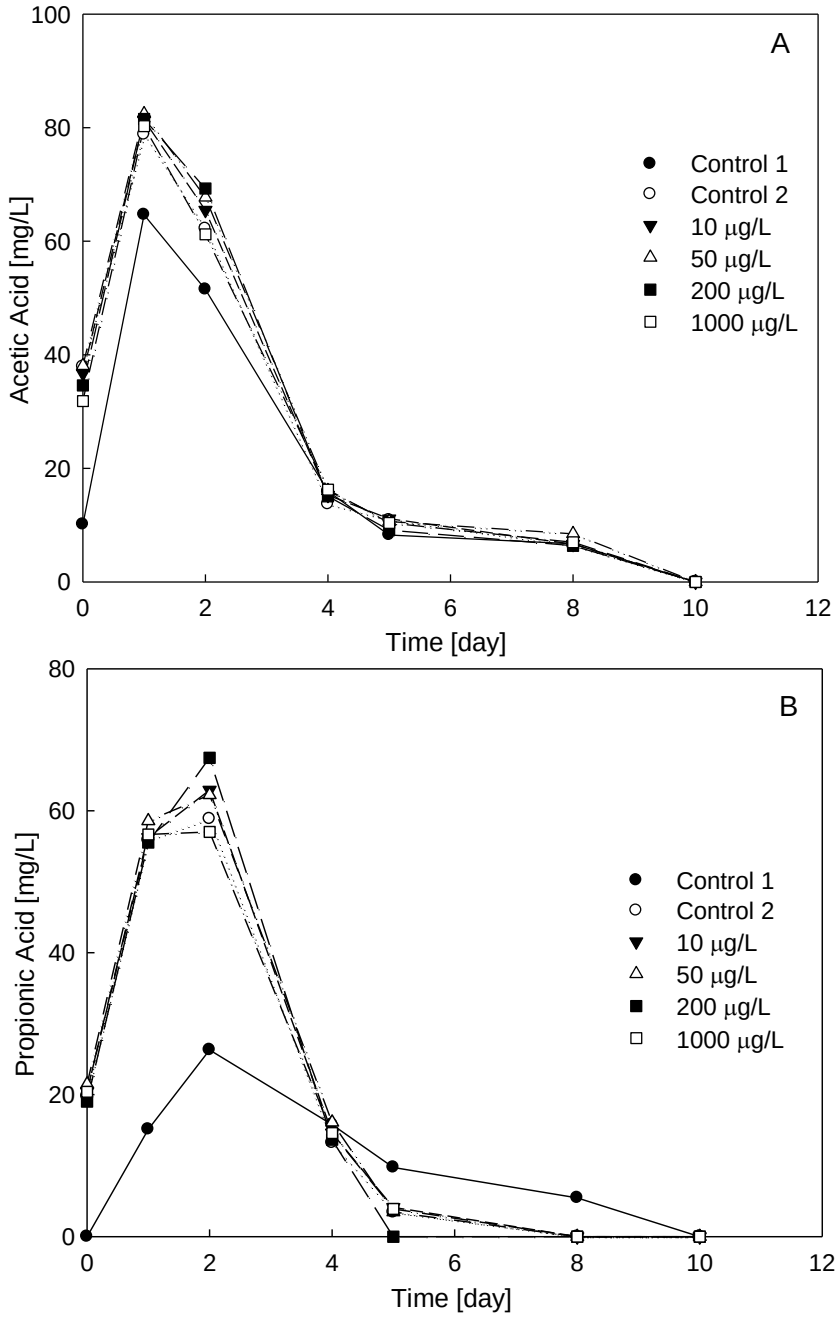
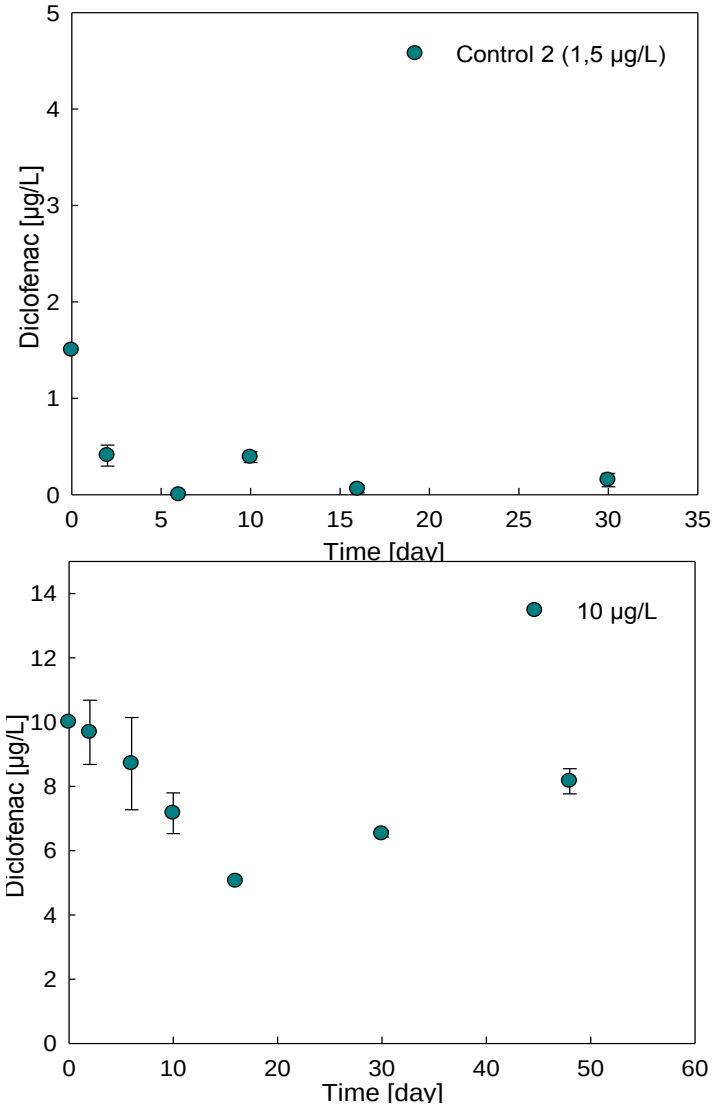


Figure 4.14 : Acetic (A) and propionic acid (B) concentration in anaerobic batch test with different diclofenac concentrations.

VFAs in all of the reactors have been consumed completely at the end of 15 days incubation period as shown in Figure 4.14. The VFAs production and consumption profiles were very similar in all of the reactors indication no interference of diclofenac on fermentative microorganisms.

As shown in the Figure 4.15, elimination of diclofenac has been achived between 18 and 54 %, and higher removal efficiencies has been observed in higher concentrations of diclofenac. Moreover, a quantitative relationship has been recognized between diclofenac removal and VFAs. In 15 day period of batch set in other words when VFAs have been existed in the reactor, diclofenac removal process has been occurred and in the absence of VFAs diclofenac degradation has been stopped.



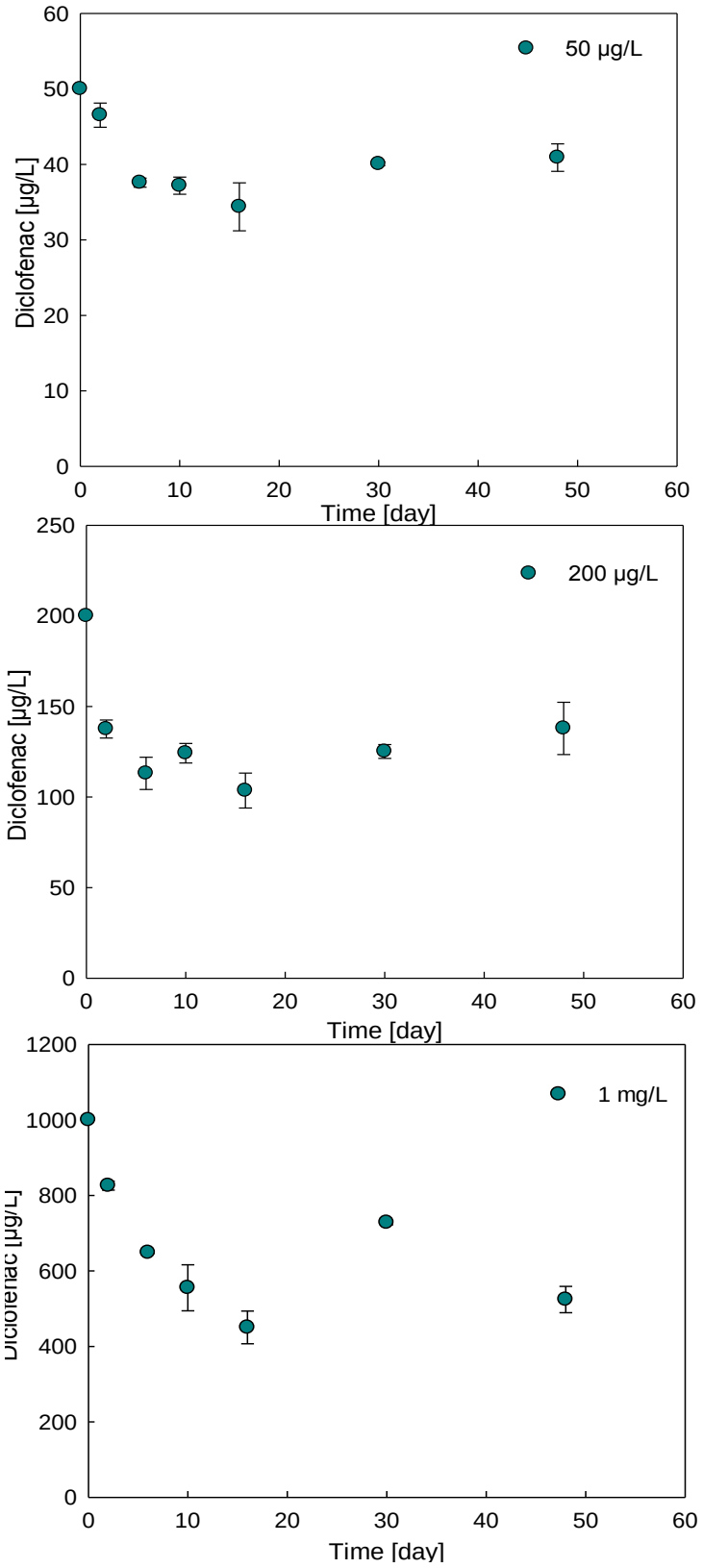


Figure 4.15 : Diclofenac concentrations in anaerobic batch reactors with different diclofenac concentrations.

In most of the reactors an increase in the diclofenac concentration has been observed right after the complete degradation of VFAs in the system. This observation can be explained by the metabolism or/and transformation products converted back to the parent compound in the lack of VFA in other words substrate.

Acidic polar pharmaceuticals have been removed mostly by cometabolic transformation (Quintana et al., 2005). Diclofenac has been used as cometabolic substrate in the presence of readily biodegradable substrate such as glucose, acetate etc. that microorganisms has gained energy from. Cometabolic substrate transformation capacity of microorganism has been related with the energy yield and the reductive force regeneration potential of microorganisms, derived from substrates (Chang and Alvarez-Cohen, 1995). In the presence of readily biodegradable substrate, cometabolic biotransformation of chlorinated solvents that diclofenac molecule have had in its structure can be occurred.

No reports have been present about cometabolic transformation of diclofenac in anaerobic treatment system. However, Lahti and Oikari (2011) have been studied on aerobic degradation of diclofenac in the presence and absence of readily biodegradable substrate as acetate. The removal efficiencies of diclofenac have been observed in the range of 1-13% in the presence of acetate. In comparison to that, the concentration of diclofenac without acetate has been remained unchanged.

4.3.2 Different temperatures

Experiments with four culture series with different temperature have been conducted to observe temperature effect on diclofenac degradation at 10, 22, 35, and 45ºC. Culture series have been incubated for 70 days. The cultures have been fed twice during the acclimation period to supply sufficient electron donor. The second feeding has been performed at the 34th day of the incubation.

During the first feeding period pH and ORP values of culture series have varied in the range of 7.0-8.5 and -120 mV – -350 mV while they have varied in the range of 6.9-8.0 and -100 mV--290 mV during the second feeding, respectively.

For each feeding, 1000 mg/L COD have been added to culture series in the form of glucose and methanol from stock solution. According to COD value, theoretical methane production have been calculated as 62 mL.

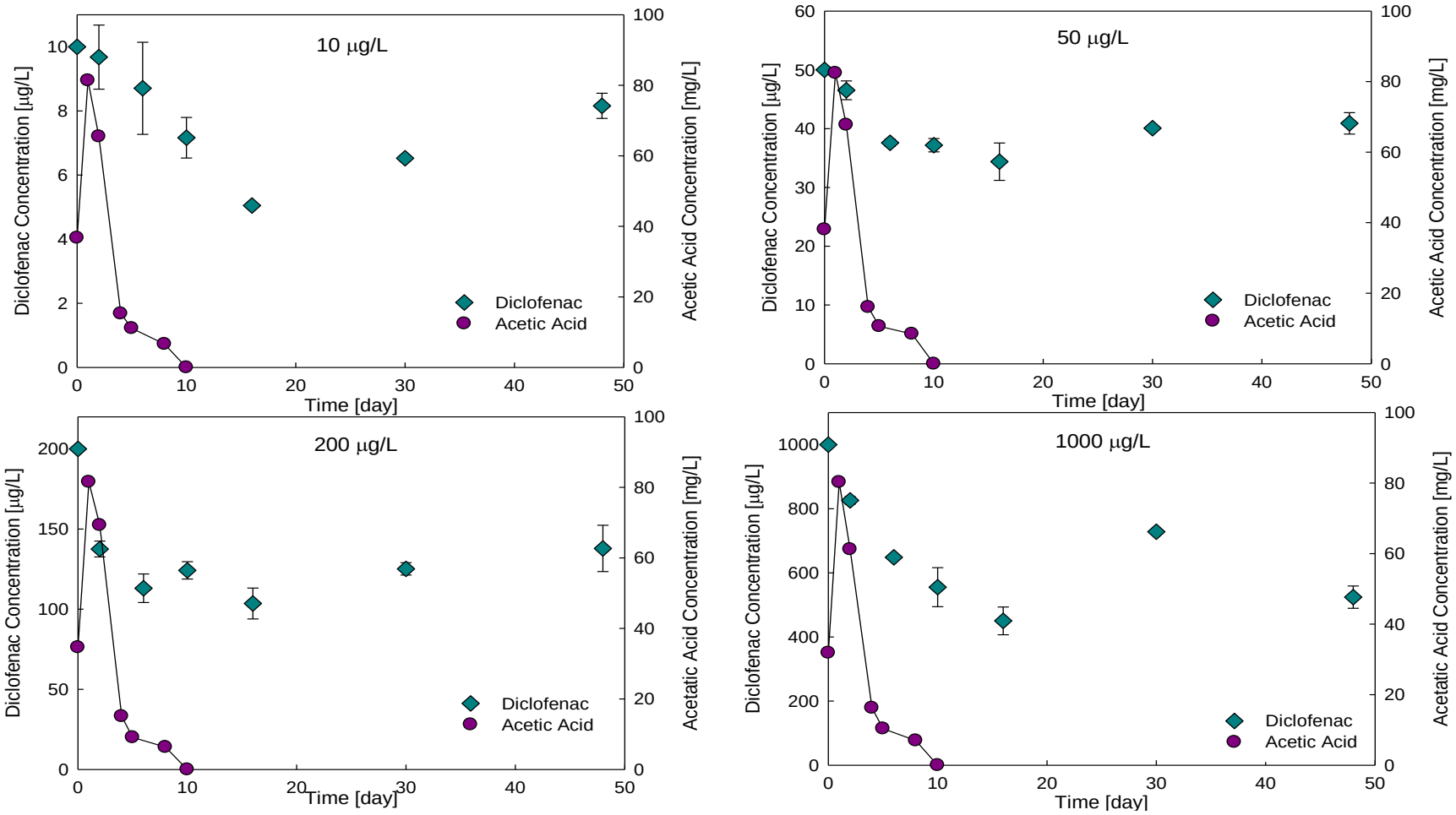


Figure 4.16 : Diclofenac and VFA concentrations in culture series with different diclofenac concentrations

However, culture series except 10ºC have produced more than theoretical value. Because, culture from Reactor E.I have provide approximately 200 mg/L COD to culture series. This excessive 200 mg/L of COD has showed theoretically itself as 13 mL of methane gas.

For each feeding, culture series at 10ºC have lower methane production with 15 mL as shown in Figure 4.17 because temperature sensitive methanogenic bacteria have not performed at this temperature.

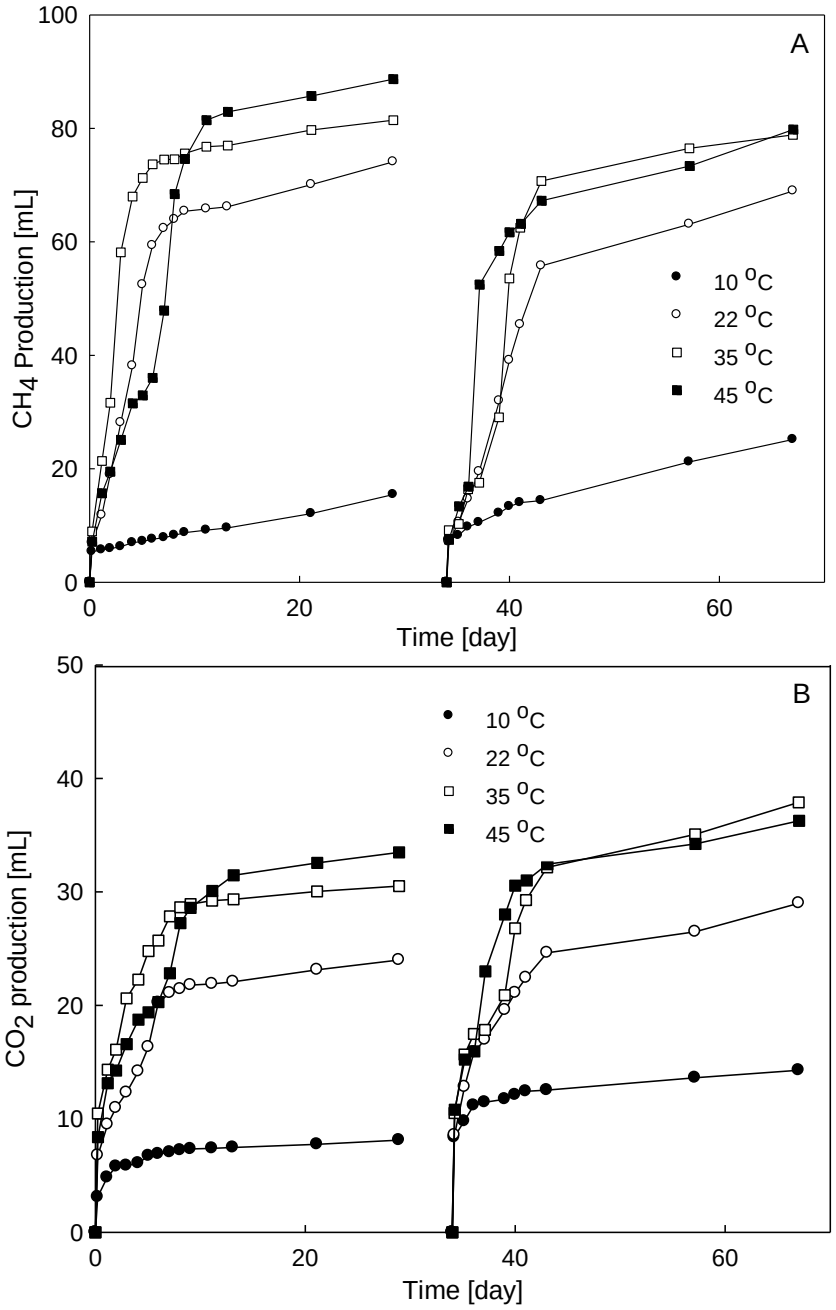


Figure 4.17 : Methane (A) and carbondioxide (B) production in batch test with different temperatures.

Methane production profiles have showed that rate of methanogenesis has increased with increasing temperature. For first feeding, methane production profile has also showed that methane production was increased with increasing temperature. For second feeding, culture series at 35 and 45ºC has shown higher methane production with respect to 22ºC because methanogenic activity has been increased with increased temperature and long acclimation period.

Carbon dioxide production had same behavior with methane production. Culture series at 10ºC have shown the less production with 8 and 14 mL for the first and the second feeding due to lower methanogenic activity at this temperature. For other culture series, carbon dioxide production has been increased with increasing temperature as shown in Figure 4.17.

1000 mg/L of COD introduced into culture series in the form of glucose and methanol have converted to VFAs. Acetic acid and propionic acid have been produced in culture series.

VFA produced by acetogens have not consumed by methanogens and accumulated in culture at 10ºC as shown in Figure 4.18.

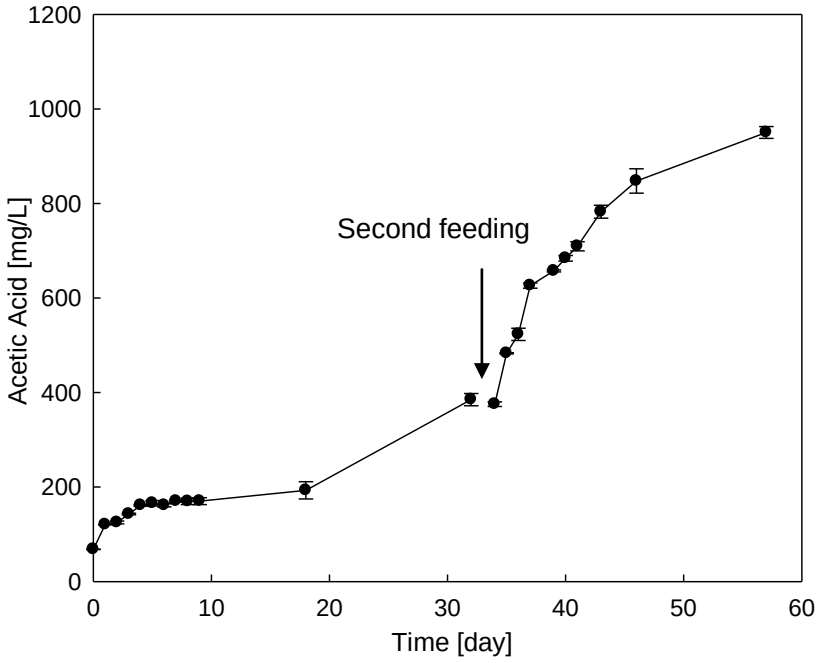


Figure 4.18 : Acetic acid concentration at 10ºC.

Due to the carbon coming from culture of Reactor E.I, VFA concentration in each culture series at different temperatures have not been zero at the time 0. VFA consumption rate has been increased with increased temperature.

VFA levels at time 0 have not been same in all temperatures because initial one day incubation has been performed to acclimate biomass to relevant temperature before substrate addition where some of the VFAs has been consumed.

For the cultures incubated at 35ºC and 45ºC, at first feeding cycle the type of VFA which have been mainly produced and accumulated was propionic acid whereas in the second feeding it has been acetic acid as shown in Figure 4.19.

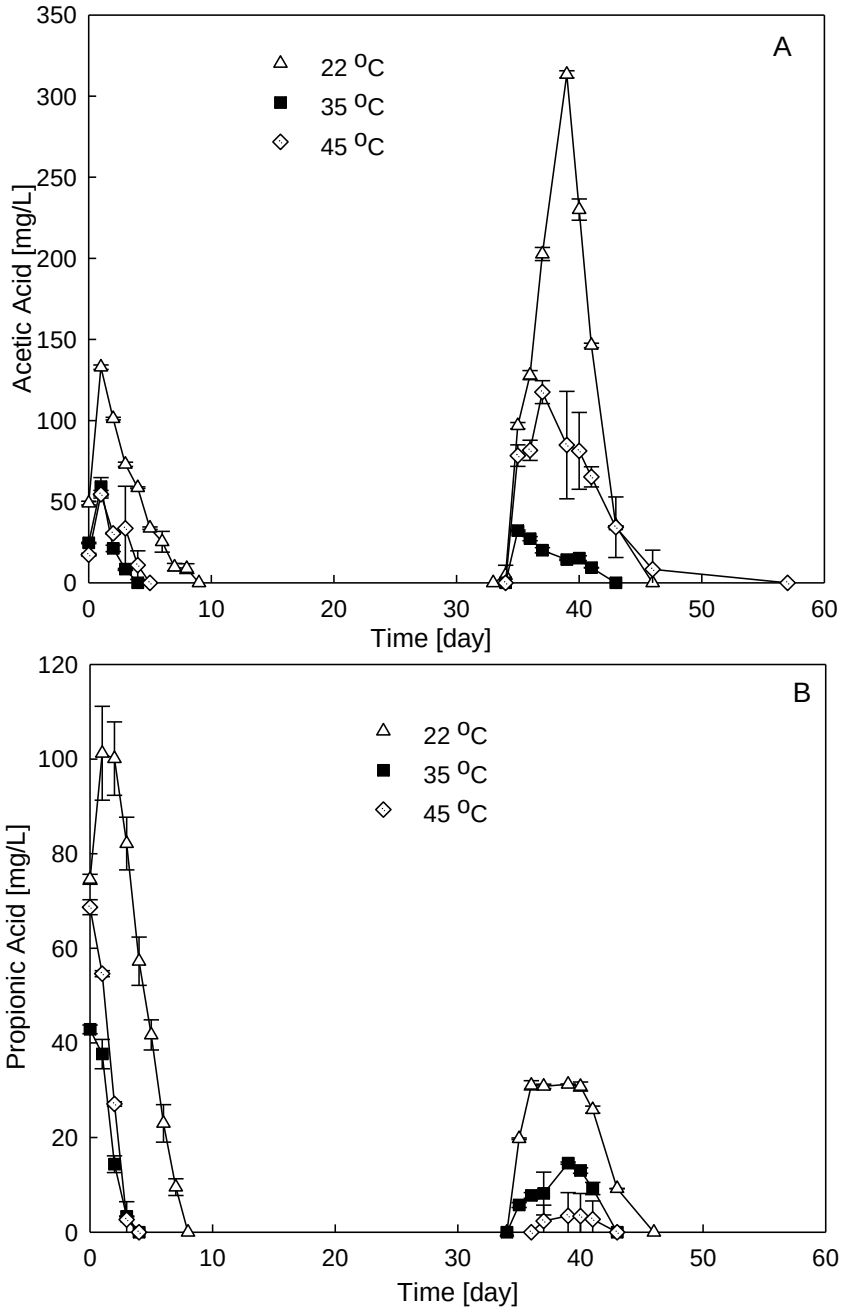


Figure 4.19 : Acetic acid (A) and propionic acid (B) concentration in anaerobic batch test with different temperatures.

In the first feeding cycle VSS concentration has been tend to increase until 15 days. At the day 32, depletion on VSS concentration has been observed due to endogenous respiration in the absence of carbon source. However, VSS concentration has showed an increasing trend throughout the second feeding as shown in Figure 4.20.

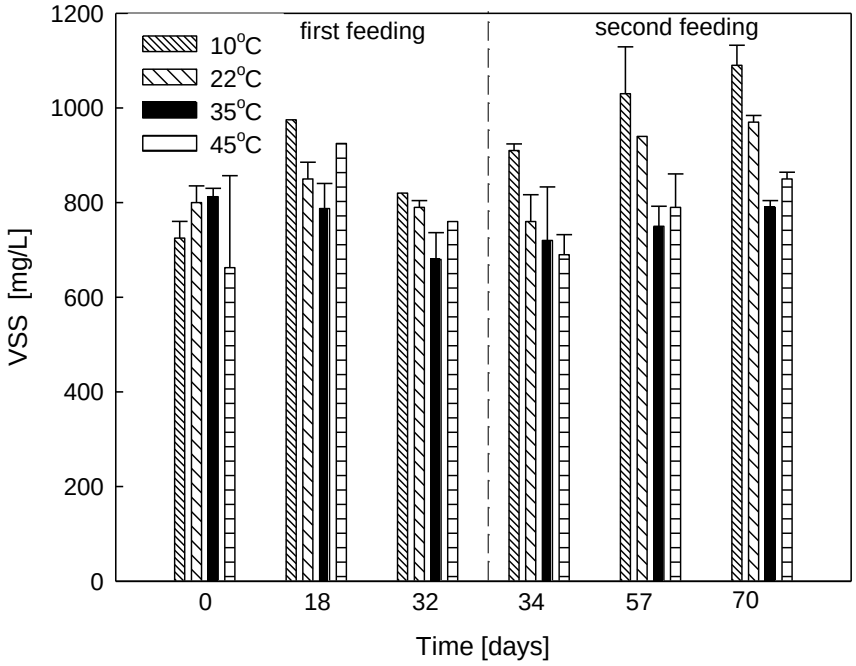


Figure 4.20 : VSS concentration in anaerobic batch test with different temperature.

For the first feeding cycle, diclofenac removal efficiency has not changed considerably with temperature as shown in Figure 4.19. Diclofenac removal efficiency has been obtained as 19%, 19%, 27%, and 22 % at 10, 22, 35, and 45ºC, respectively.

Carballa et al. (2007 and 2008) have been studied on temperature effect on elimination of diclofenac with mesophilic and thermophilic anaerobic digester at 37ºC and 45ºC and with 20 day and 10 day of SRT, respectively. They observed that elimination of diclofenac has been occurred after sludge adaptation and acclimation result in presence of more active microbial population. Removal of diclofenac has been increased 60±18 and 73±9% after sludge adaptation at 37ºC and 45ºC, respectively. However, no significance influences of temperature has been observed. It has also been reported that diclofenac removal efficiency in WWTP has been varied between 9 and 41% without any significant change with temperature (Oosterhuis et al., 2013).

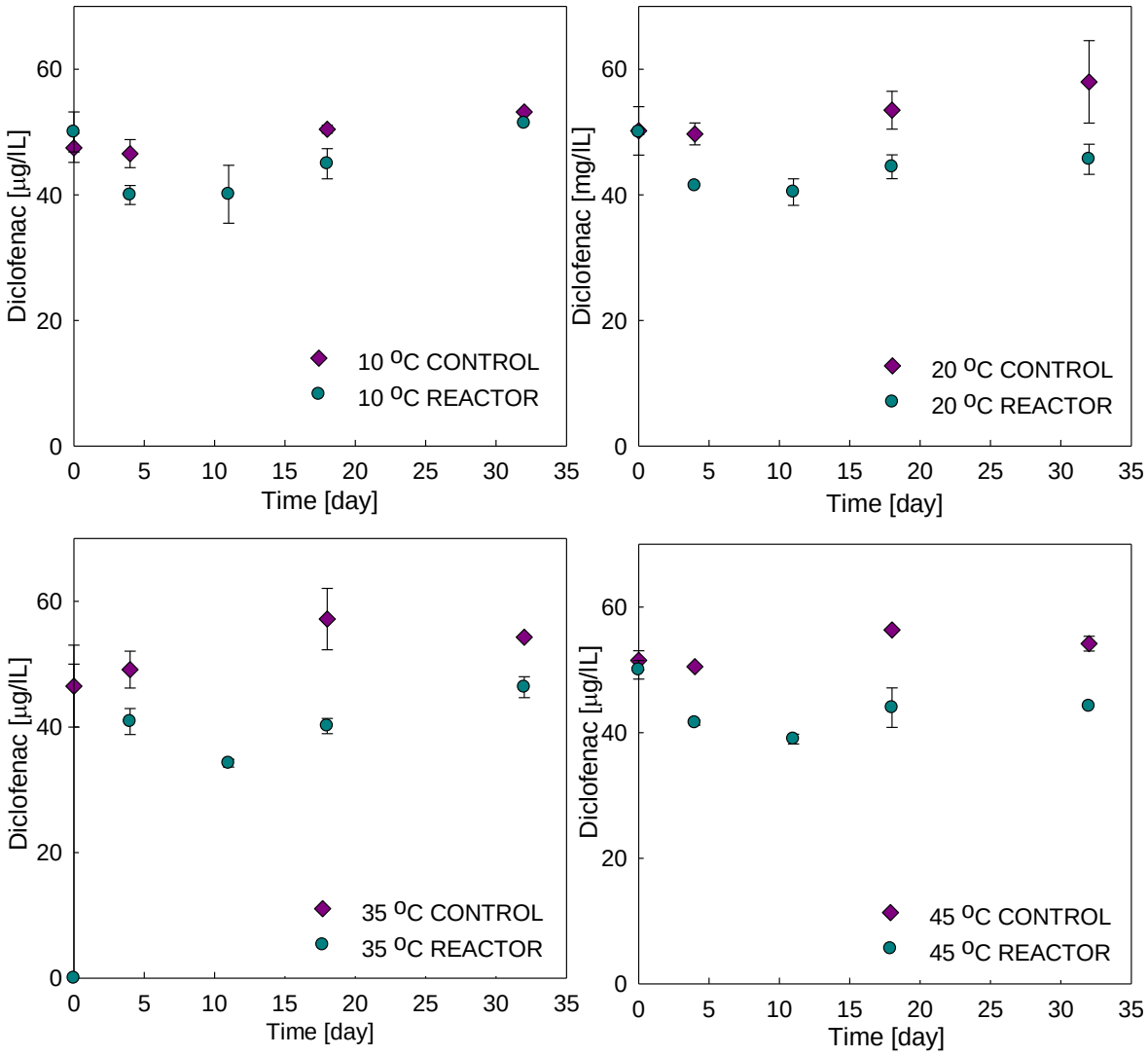


Figure 4.21 : Diclofenac concentrations in batch assay with different temperature for first feeding.

However, after second feeding, diclofenac elimination has not been observed in significant extend as shown in Figure 4.22. In the reactors, fluctuations and increasing trend in diclofenac concentration have been observed with negative removal. The negative removal can be explained by the formation of the unmeasured by products of bacterial metabolism which has been converted back to parent compound of diclofenac.

It has been also observed that diclofenac removal in control of each culture series at relevant temperature has shown similar trend with no depletion around 50 µg/L. It has been understood that diclofenac elimination achieved in culture series has been achieved by biodegradation without photodegradation and abiotic degradation.

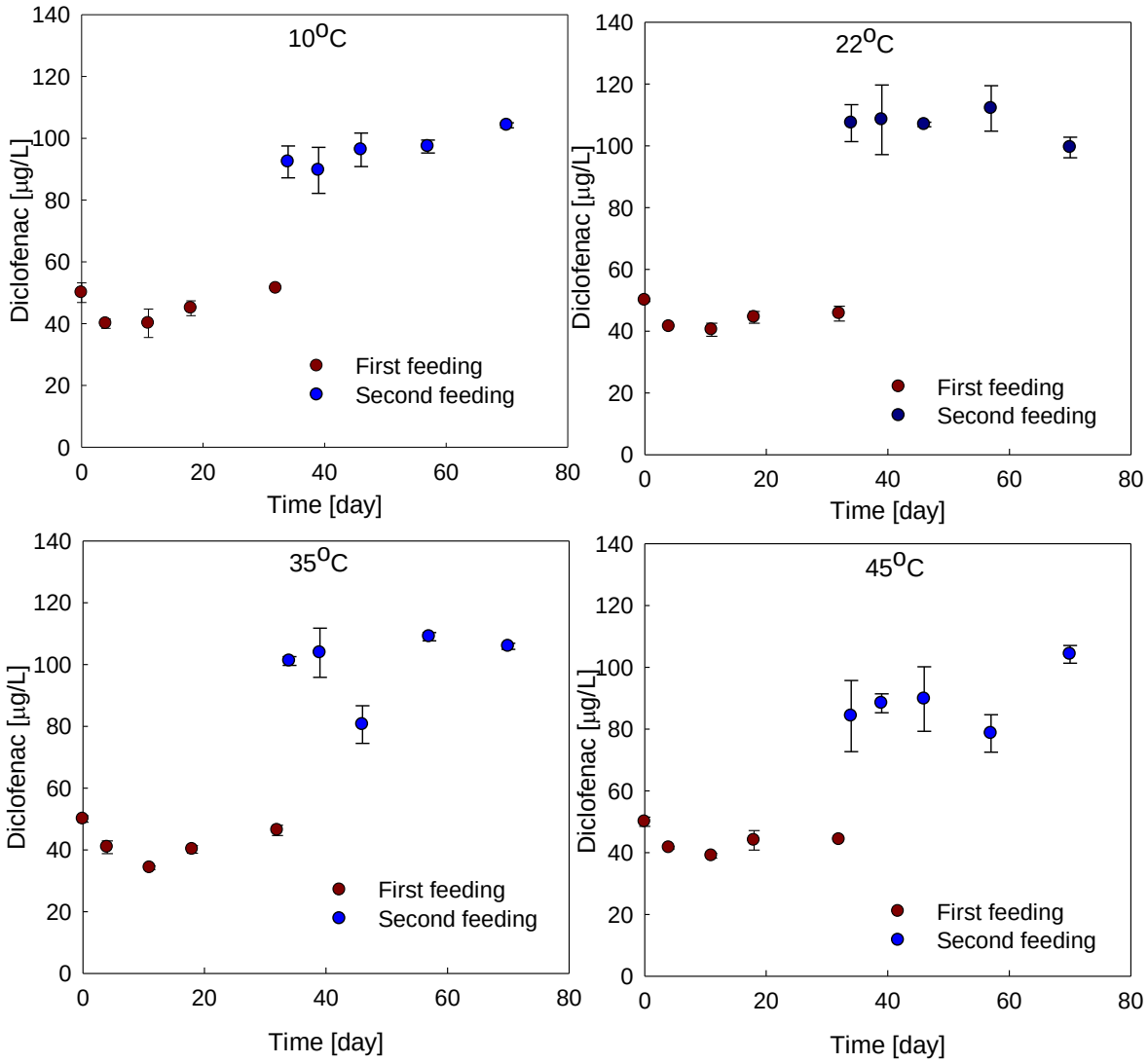


Figure 4.22 : Diclofenac concentrations in batch assay with different temperature for first and second feeding,

4.3.3 Different biomass concentration

Four culture series with different initial biomass concentration including 25, 50, 75, and 100 % have been conducted to observe effect of initial biomass concentration on diclofenac degradation. Culture series have been operated in total 33 days.

During the operation period pH and ORP values of culture series have varied in the range of 6.6-7.2 and -117 mV – -222 mV during the operation period.

1000 mg/L COD have been added to culture series in the form of glucose and methanol from stock solution. According to COD value, theoretical methane gas productions have been calculated as 62 mL. However, culture series have produced more than theoretical value. Because, culture from Reactor E.I have provide approximately 130, 94, 73, and 47 mg/L COD

to culture series with 100, 75, 50, and 25 % of biomass, respectively. This excessive COD have showed theoretically itself as 8, 6, 5, and 3 mL of methane gas as shown in Figure 4.23.

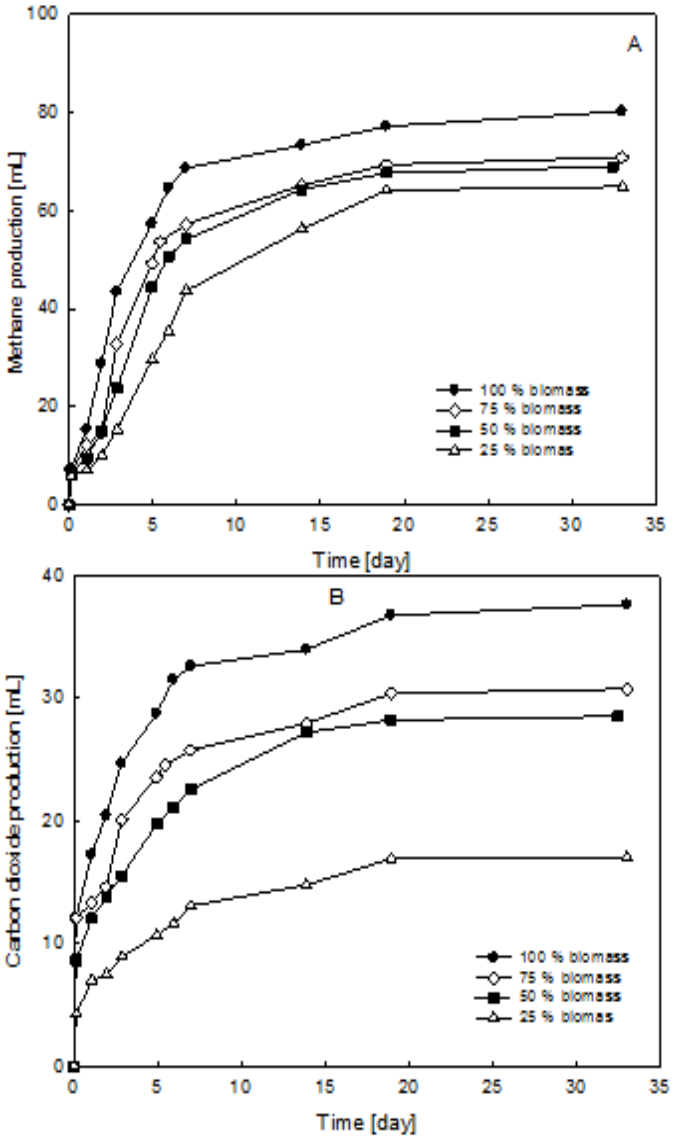


Figure 4.23 : Methane (A) and carbondioxide (B) production in batch test with different biomass concentrations.

Methane production profiles have shown that rate of methanogenesis has increased with increased initial biomass concentrations. Methane content has been increased with time and reached steady state condition. At the end of the operation methane content has been measured in GC as 70, 71, 72, and 72 % culture series with 100, 75, 50, and 25 % of biomass, respectively.

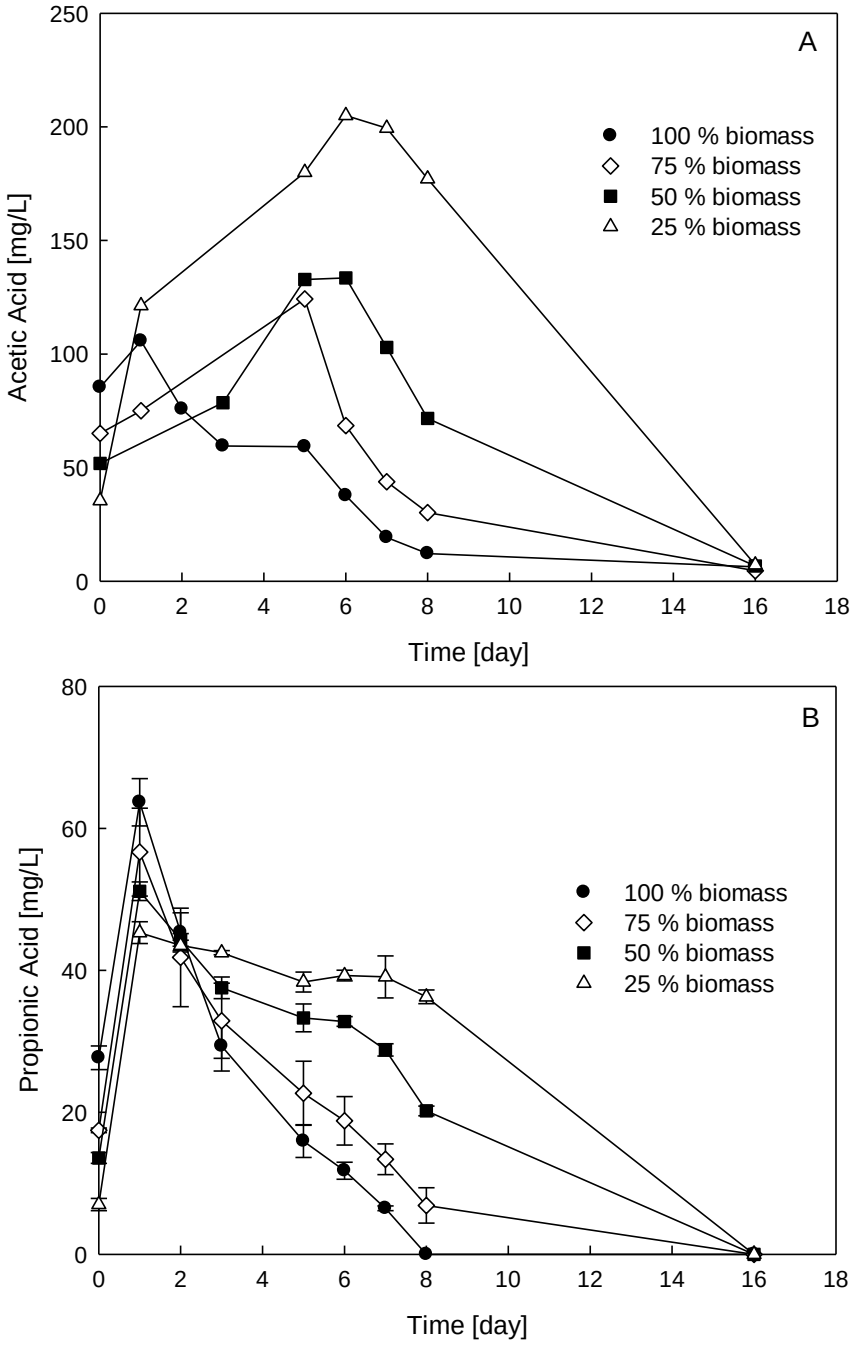


Figure 4.24 : Acetic acid (A) and propionic acid (B) concentration in anaerobic batch test with different biomass concentrations.

Carbon dioxide production has had same behavior with methane production. It has been increased with increasing initial biomass concentration as shown in Figure 4.23**.**

Due to carbon coming from culture of Reactor E.I, VFA concentration in each culture series at different biomass concentrations have not been zero at the time 0. VFA consumption rate has been increased with increased initial concentrations as shown in Figure 4.24.

100, 75, 50 and 25% of biomass have been planned to add four culture series. However, the measurements have shown 100, 89, 59, and 36% of biomass at the day of 0.

VSS concentration has been decreased at the end 33 days of incubation period due to the endogenous decay.

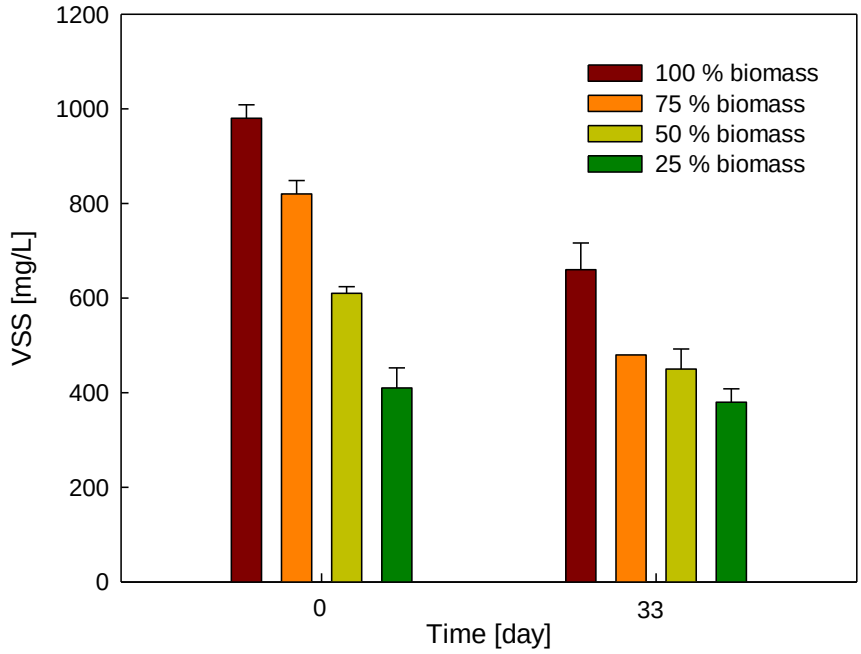


Figure 4.25 : Suspended solids behaviour in batch anaerobic test of different biomass concentrations

Until the day 16, diclofenac removal efficiency has been obtained as 26, 23, 21, and 21% in the culture series of 100, 75, 50, and 25 % biomass, respectively. After the day 16, diclofenac concentration has tended to increase in lack of VFA as shown in Figure 4.26. The reason of this has been explained by metabolism or transformation products formed by bacteria throughout the processes have been converted back to parent compound (Jelic et al., 2011).

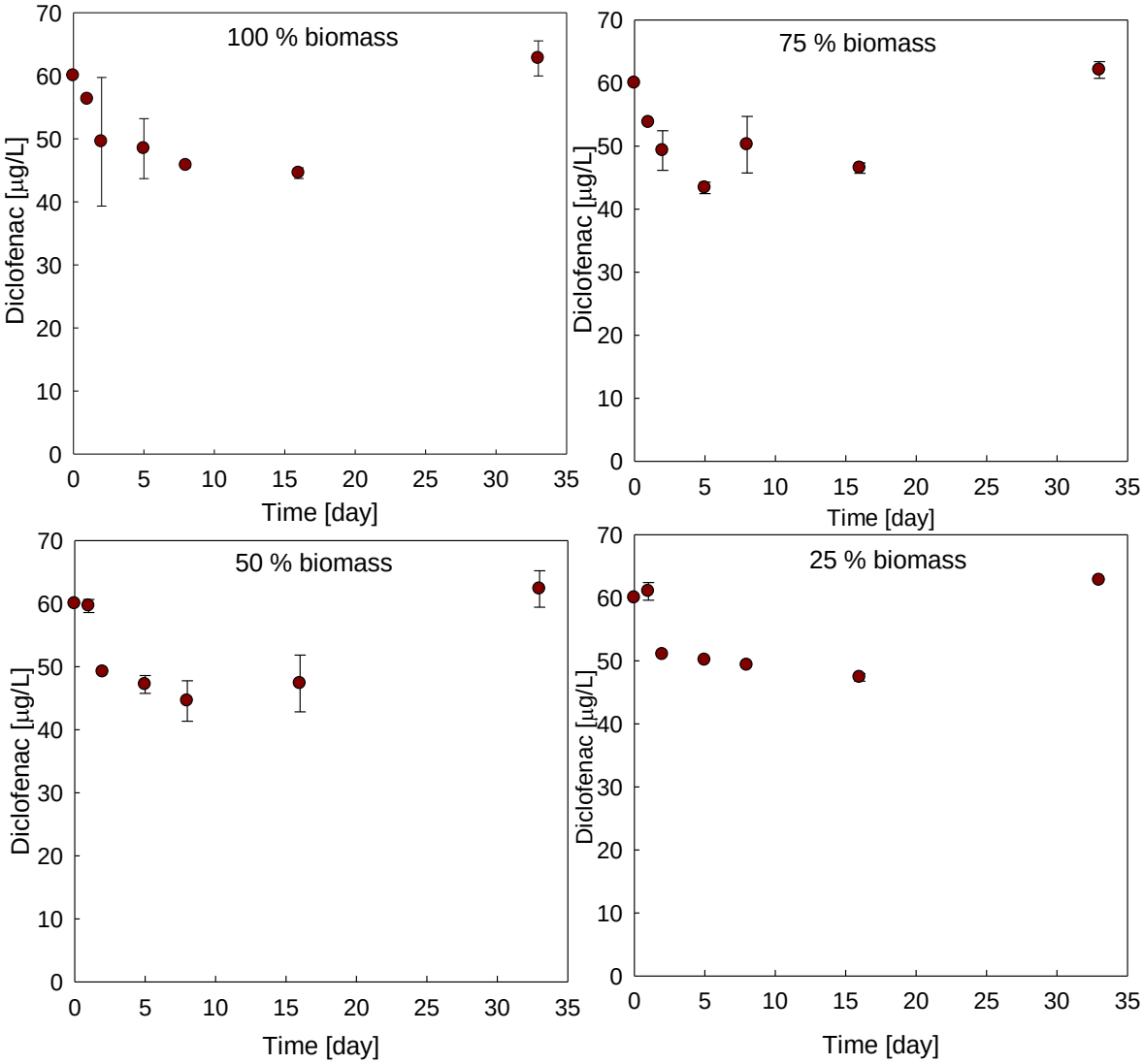


Figure 4.26: Diclofenac concentrations in batch assay with different biomass concentrations.

4.3.4 Different carbon source

In order to investigate effect of different carbon source on diclofenac removal efficiency, culture series was fed with acetate as electron donor.

During the first operation period pH and ORP values of culture series have varied in the range of 6.96-7.13 and -167 mV – -233 mV, respectively.

For first feeding period, 1248 mg/L COD have been added to culture series in the form of sodium acetate and methanol from stock solution. According to COD value, theoretical methane production have been calculated as 79 mL for first feeding. However, reactor has produced more than theoretical value. Because, culture from Reactor E.I have provided extra COD to reactor. For second feeding, the reactor has fed with only sodium acetate and 578

mg/L COD have been introduced to reactor. According to COD value, theoretical methane production have been calculated as 36 mL for second feeding

Culture series with acetate feeding has shown higher methane production rate and consumed the COD in shorter time compared to the culture series with glucose (Figure 4.27). At the end of the operation culture series with acetate feeding has reached 80 % of methane content in the biogas. It has been higher than the value measured in culture series fed with glucose.

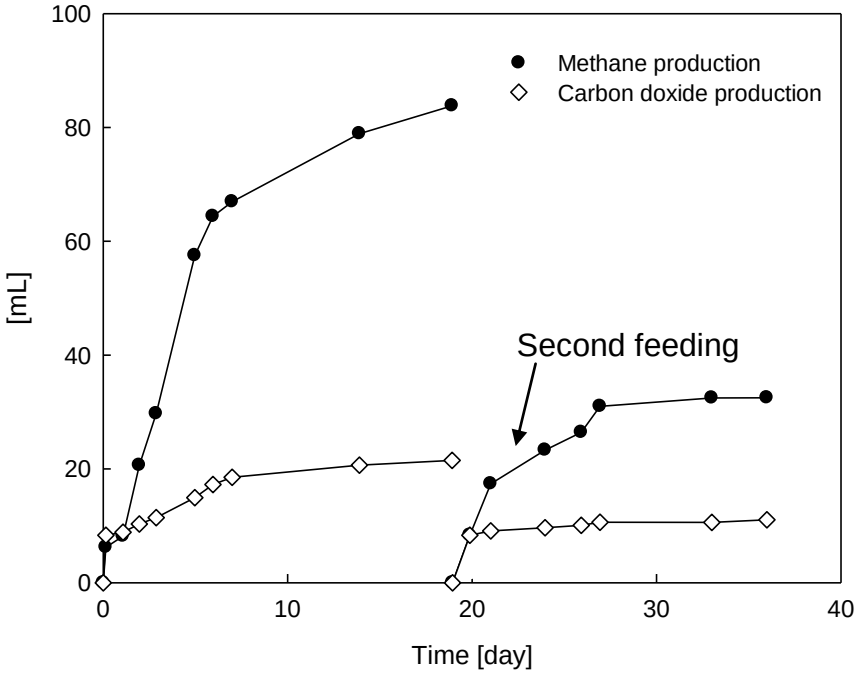


Figure 4.27 : Methane and carbon dioxide production in acetate fed culture series.

Due to carbon coming from culture of Reactor E.I, VFA concentration have not been zero at the time 0. Near the day 8, acetate has been consumed completely in the system as shown in Figure 4.28.

Diclofenac removal efficiency has observed 25% at the day 8 as shown in Figure 4.29. After that, diclofenac concentration has been increased with decreasing acetate concentration. Culture series fed with glucose have performed better diclofenac elimination with respect to culture series fed with acetate.

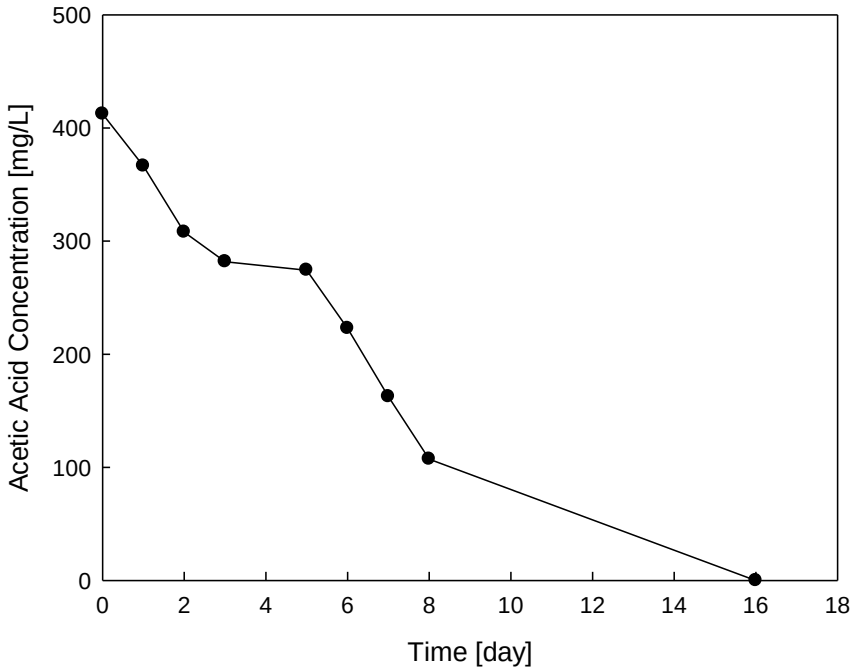


Figure 4.28 : Acetic acid concentrations in culture series with acetate feeding.

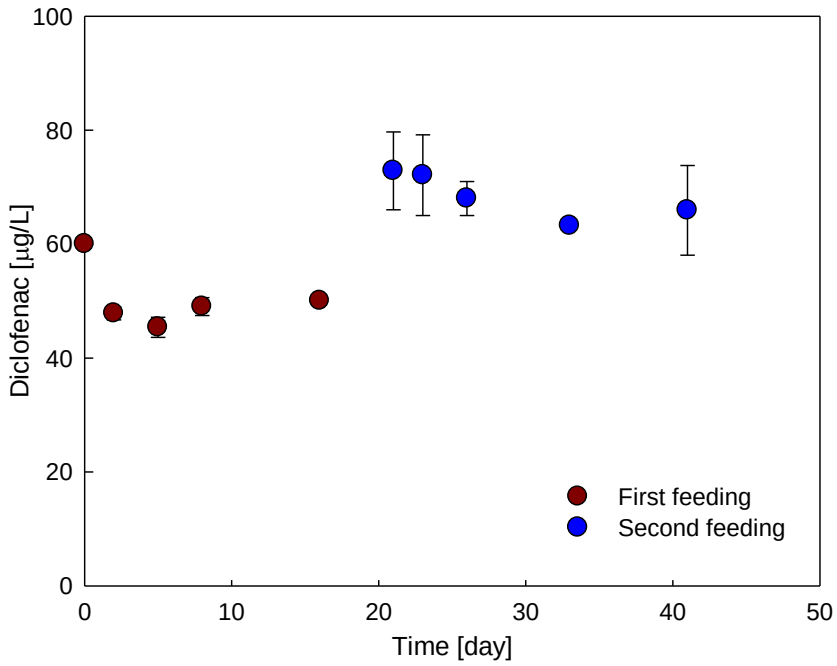


Figure 4.29 : Diclofenac concentration in culture series with acetate feeding.

5. CONCLUSION AND RECOMMENDATIONS

According to sampling campaigns carried out in WWTP in 4 seasons of the year, maximum diclofenac concentration of 1376 µg/L has been detected in winter due to higher consumtion of diclofenac with increasing illnesses in colder months. Maximum removal efficiency of diclofenac has been found as 51% in summer with increasing ambient temperature. Diclofenac removal profile has shown similar trend with nitrogen removal profile. Both has shown same increasing and decreasing pattern throughout the year. It is observed that 45 and 27 % of diclofenac removal have been achieved by secondary treatment in summer and winter, respectively. Operation efficiency of secondary treatment as activated sludge process with A_2O has been correlated with diclofenac elimination, because diclofenac removal has been decreased with decreasing TKN, NH_3, and TP removal.

Laboratory experiments have shown that removal of diclofenac has not completely achieved under anaerobic conditions. Diclofenac has shown significant resistance to biodegradation. It has also been tend to remain in aqeous phase instead of sorption onto the sludge. Maximum 2 % of diclofenac has remained in sludge phase whereas most of them have been occurred in aqueous phase. Anaerobic reactors operated at 22ºC as first generation reactor has been performed with 13 - 31 % diclofenac removal. Due biomass adaptation has been enchanced biodegradation of diclofenac, second generation anaerobic reactors at 22ºC has been achieved better removal efficiency with respect to first generation reactors. Also, 21 % of diclofenac elimination has been obtained in anaerobic reactor operated at 35ºC.

Among 10, 50, 200, and 1000 µg/L of diclofenac concentrations applied in batch assays, 1000 µg/L of diclofenac concentrations has revealed very low inhibitory effects on methanogens that has shown itself decreasing in methane production and VSS concentrations. Diclofenac concentration has been tend to increase right after the complete consumption of VFAs.

Even at different temperatures, diclofenac removal was not achieved under abiotic conditions reflecting microbial activity importance on its degradation. Diclofenac removal has been observed in the range of 19-27%. No relation has been found between temperature and diclofenac removal efficiency. In addition, diclofenac removal efficiency has tended to

increase with an increasing initial biomass concentration at the first 3 days of the operation then reactors have been shown similar removal efficiencies in the presence of substrate.

Studies have shown that diclofenac has not been completely removed inconventional biological treatment processes used by municipal wwtps. Conventional WWTPs needs to be upgraded with tertiary treatment process to treat this kind of emerging micropollutants.

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