ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF NON STEROIDAL ANTI-INFLAMATORY DRUGS ON MICROBIAL KINETICS AND POPULATION

M.Sc. THESIS

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Environmental Biotechnology Programme

JANUARY 2020

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STEROİD YAPIDA OLMAYAN ANTİENFLAMATUVAR YAPIDAKİ İLAÇLARIN MİKROBİYAL KİNETİĞE VE POPULASYONA ETKİSİ

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To my homeland,

FOREWORD

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ABBREVIATIONS

DI : Distilled Water

LC-MS/MS : Liquid Chromatography Coupled with Tandem Mass Spectrometry

MP : Micropollutant

NH_4^+-N : Ammonium Nitrogen

NO_2^--N : Nitrite Nitrogen

NO_3^--N : Nitrate Nitrogen

NSAID : Non-Steroidal Anti-Inflammatory Drug

OTU : Operational Taxonomic Unit

OUR : Oxygen Uptake Rate

PCR : Polymerase Chain Reaction

sCOD : soluble Chemical Oxygen Demand

SRT : Sludge Retention Time

SPE : Solid Phase Extraction

TKN : Total Kjendal Nitrogen

TP : Total Phosphorus

TSS : Total Suspendend Solid

VSS : Volatile Suspended Solid

WWTP : Wastewater Treatment Plant

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EFFECT OF NON STEROIDAL ANTI-INFLAMATORY DRUGS ON MICROBIAL KINETICS AND POPULATION

SUMMARY

Recently, increasing attention has been paid to the contamination of the environment with pharmaceuticals because of their ubiquitous consumption and insufficient removal by the current conventional wastewater treatment plants, leading their accumulation in receiving aquatic bodies posing a potential adverse effect on the ecological system and human health. Biological wastewater treatment with activated sludge process exploits the activity of microorganisms for the reduction of the organic and inorganic matter and production of effluent that can be discharged safely into receiving waters. However, activated sludge microbial communities are not acclimated to different chemicals entering the wastewater, and perturbations in the activated sludge process may cause shifts in the microbial population and composition, and subsequently alter performance efficiency. One of the most frequently detected pharmaceuticals in the environment is the non-steroidal anti-inflammatory drugs (NSAIDs). This study investigated the effects of a mixture of six NSAIDs compounds on the treatability of a lab-scale aerobic sequencing batch reactor (SBR) as well as on the activated sludge microbial community structure, diversity, and kinetics. The SBR was operated at sludge retention time (SRT) of 5 days for 17 months, in which the first 2 months served as a control reactor and the rest included the addition of NSAIDs mixture (MP reactor). The performance of both reactors was monitored in terms of pH, suspended solid content of both sludge and effluent and the concentration of chemical oxygen demand (COD), ammonia, nitrate, and nitrite in the effluent. Moreover, the contribution of sludge adsorption and biodegradation to the removal of each drug in the NSAIDs mixture was evaluated by tracking their concentration in both the sludge phase and the effluent of MP reactor. Control culture feeding with the mixture of NSAIDs resulted in no change in the COD removal efficiency but nitrification process was enhanced. The results showed that the highest drug to be influenced by adsorption was Indomethacin while mefenamic acid was the lowest. However, adsorption effect

was negligible when compared with biodegradation that contributed to almost complete removal of ibuprofen (99.5%) and naproxen (98.6%) as well as moderate removal of indomethacin (88%), mefenamic acid (84%), ketoprofen (74%) and diclofenac (65%). Acute and chronic inhibitory effects of different concentrations of NSAIDs on activated sludge were tested with respirometry in the presence and absence of nitrification inhibitor. In general, NSAIDs caused no considerable variation in the oxygen uptake rate (OUR) profiles except for the highest tested concentration. The maximum OUR level was affected by the addition of 10 and 100 μg/L NSADs. Modeling of the acute OUR profiles indicated that amendment of activated sludge culture by 10 or 100 NSAIDs μg/L resulted in a decrease in the rate of the second hydrolysis process. Moreover, as a result of the addition of 100 μg/L all of the organic matter in the medium could not be oxidized and substrate binding was observed. A bacterial ribosomal RNA small subunit (16S) rRNA gene Illumina sequencing approach was used to characterize the diversity and structure of activated sludge bacterial community. Diversity indices suggested a considerable reduction in bacterial diversity by the addition of NSAIDs. Their presence also significantly enriched the phylum *Verrucomicrobia*. The species belonging to *Prosthecobacter* and *Paracoccus* predominated the MP reactor implying their ability to adapt well the new conditions and their potential capacity to degrade NSAIDs. A noticeable increase in aromatic hydrocarbon degradation functional group in the presence of NSAIDs was also detected. The findings revealed that pharmaceuticals even at the environmentally relevant concentrations that is at the microgram level have an impact on the kinetics, diversity and structure of activated sludge microbial community.

STEROİD YAPIDA OLMAYAN ANTİENFLAMATUVAR YAPIDAKİ İLAÇLARIN MİKROBİYAL KİNETİĞE VE POPULASYONA ETKİSİ

ÖZET

Farmasotik kimyasalların yaygın tüketimi ve mevcut geleneksel atık su arıtma tesislerinin bu kimyasalları giderimini hedeflememesi neticesindeki yetersiz arıtma nedeniyle özellikle sucul ortamlarda farmasötik kimyasallarla kirlenme konusunda yapılan çalışmalar daha önem kazanmaktadır. Yetersiz giderim alıcı su ortamlarında farmasötik birikimine sebep olarak ekolojik sistemler ve insan sağlığı üzerinde potansiyel olumsuz etki yaratmaktadır. Aktif çamur prosesleri biyolojik atıksu arıtımında, organik ve inorganik madde konsantrasyonunun azaltılması ve alıcı ortama güvenli bir şekilde deşarj edilebilecek kalitede çıkış sularının eldesi için mikroorganizmaların aktivitesinden yararlanır. Ancak, aktif çamur mikrobiyal komüniteleri atıksuya giren farklı kimyasallara karşı dirençli ve aklime değildir ve aktif çamur prosesindeki değişimler mikrobiyal popülasyon ve kompozisyonunda değişikliklere sebep olabilmektedir. Bu değişiklikler zamanla sistemin performans verimliliğini de etkileyebilmektedir. Atıksu arıtma tesislerinde en yoğun olarak tespit edilen farmasötiklerden biri steroidal yapıda olmayan anti-enflamatuar ilaçlardır (NSAID'ler). Bu çalışmada lab ölçekli ardışık kesikli aktif çamur reaktöründe (AKR) ölçümü hedeflenen antienflamatuvar yapıdaki ilaçların (diklofenak, ketoprofen, indometasin ve mefenamik asit, ibuprofen ve naproksen) karışımının aktif çamur sistemlerine beslenmesi neticesinde ortaya çıkabilecek değişiklikler incelenmiştir. Bu çalışma (i) seçilen NSAID'lerin aktif çamur sistemlerindeki aerobik arıtılabilirliği (ii) respirometrik analizler yardımı ile laboratuvar ortamında işletilen aktif çamur üzerindeki potansiyel akut ve kronik toksisiteleri, (iii) respirometrik sonuçların modellemesi neticesinde bu ilaçların aktif çamur kinetiği ve stokiyometrisi üzerindeki etkileri, (iv)ileri moleküler biyoteknoloji ve biyoinformatik araçlar kullanılarak elde edilen mikrobiyal komünitenin yapısında, çeşitliliğinde ve aktivitesindeki değişiklikler olmak üzere dört ana başlıktan oluşmaktadır. Laboratuvar ortamında işletilen ardışık kesikli reaktör çamur yaşı 5 gün olacak şekilde 17 ay süre ile

işletilmiştir. Reaktör işletiminde kullanılan aşı çamuru gerçek ölçekte işletilen İstanbul'da yer alan bir ileri biyolojik atıksu arıtma tesisinin havalandırma havuzundan alınmıştır. Reaktör işletimi iki kademede gerçekleştirilmiş olup; ilk iki ay reaktör kontrol reaktörü olarak işletilmiş ve mikrokirletici ilavesi yapılmadan işletilmiş, sonrasında ise antienflamatuvar yapıdaki ilaçların karışımının eklendiği reaktör (mikrokirletici reaktörü) işletilmiştir. Her iki reaktör işletimi adımında da reaktör verimi pH, askıda katı madde miktarı, çıkış suyundaki kimyasal oksijen ihtiyacı (KOİ), çıkış akımındaki askıda katı madde konsantrasyonu, amonyak, nitrat ve nitrit takip edilerek belirlenmiştir. Bunlara ek olarak, çamur adsorpsiyonu ve biyodegradasyonun karşılaştırılabilmesi amacıyla her bir ilacın çamurda ve reaktörlerin çıkış akımındaki konsantrasyonu takip edilmiştir.

Aktif çamurun çamur yaşı 5 gün olarak işletilen reaktörde antienflamatuar yapıdaki ilaçların karışımına aklime olması sonucunda organik maddenin biyodegradasyonu ve nitrifikasyon prosesi üzerinde uzun süreli bir inhibitör etki göstermediği gözlemlenmiştir. Reaktörde KOİ giderim verimi üzerinde bir değişiklik tespit edilmezken, nitrifikasyon veriminde mikrokirletici ilavesi yapılan reaktörde iyileşme olduğu gözlemlenmiştir. Çamur akımında yapılan ölçümler neticesinde, adsorpsiyonun en çok indometasinin kimyasalında, en az ise mefenamik asitte olduğu görülmektedir. Fakat çalışılan konsantrasyonlarda adsopsiyonun oldukça düşük seviyede olduğu ve bu kimyasalların su fazında yoğunlukla kaldığı görülmektedir. Sonuçta adsorpsiyon prosesi biyodegradasyon ile kıyaslandığında ihmal edilebilir seviyededir. Reaktörde ortalama olarak biyodegradasyon neticesinde %99.5 ibuprofen, %98.6 naproksen, %88 indometasin, %84 mefenamik asit, %74 ketoprofen, ve %65 diklofenak giderimi elde edilmiştir. NSAID'lerin farklı konsantrasyonlarının aktif çamur üzerindeki akut ve kronik inhibisyon etkisi nitrifikasyon inhibitörünün kullanıldığı ve kullanılmadığı respirometre testleri ile de belirlenmiştir. Genel olarak NSAID'ler test edilen en yüksek konsantrasyon haricinde, oksijen tüketim hızı (OTH) profilinde önemli değişikliğe sebep olmamışlardır. Mikrokirleticilerin en fazla etkisi kronik deneylerde 10 ve 100 µg/L NSAID ekleme yapılan setlerde OTH profillerinde gözlemlenmiştir. Akut OUR profillerinin modellemesi, aktif çamura 10 veya 100 µg/L NSAID eklenmesinin ikinci hidroliz hızının düşmesine sebep olduğunu göstermektedir. 100 µg/L'lik ekleme ile ortamdaki tüm organik madde oksitlenememiş ve yaklaşık %40 oranında KOİ ortamda giderilmeden kalmıştır.

Aktif çamur bakteri komünitesinin aktivite ve çeşitlilik karakterizasyonu için ribozomal RNA küçük altbirimi (16S) rRNA geni Illumina dizileme yöntemi kullanılmıştır. Çeşitlilik endeksleri açısından bakteri çeşitliliğinde NSAID eklenmesi ile önemli miktarda azalma olduğunu göstermektedir. Kontrol ve mikrokirletici ilavesi yapılan reaktörlerden alınınan çamur örneklerinin klon kütüphanelerinde gözlenebilen OTU sayıları 227 ve 171 olarak bulunmuştur. *Proteobacteria, Actinobacteria, Bacteroidetes* ve *Firmicutes* hem kontrol hem de mikrokirletici gruplarında bulunan dominant filumlardır. Mikrokirletici varlığı, *Verrucomicrobia* filumunu önemli ölçüde zenginleştirmiştir. *Prosthecobacter* ve *Paracoccus'*a ait türlerin mikrokirletici reaktörünü domine etmesi bu türlerin yeni koşullara uyum yeteneklerini ve NSAID'leri parçalama kapasitelerini işaret etmektedir. Bunun aksine NSAID'ler, *Paracoccus alkenifer* türlerinin %97'den fazlasının, *Nocardioides islandensis, Kineosphaera nakaumiensis, Niabella hirudinis,* ve *Leucobacter komagatae* türlerinin tamamının yok olmasına sebep olmuştur. Bu türlerin yok oluşu yeni koşullara uyum sağlayamadıklarını göstermektedir. Bu çalışma kapsamında aynı zamanda fonksiyonel grupların nasıl değişim gösterdiği de araştırılmıştır. Bu kapsamda aşı çamuru, kontrol reaktörü ve mikrokirletici reaktörlerinde türlerin dağılımı analiz edilmiştir. Kemoheterotroflar tüm örneklerin %96'sından fazlasını oluşturmaktadır. Kemoheterotroflar arasında kontrol ve mikrokirletici örneklerinde ham örneklere kıyasla daha fazla aerobik türler bulunmuştur. Aerobik türlerin bu numunelerde daha fazla olması önceki bölümlerde açıklanan baskın türlerle uyumluluk göstermektedir. Anaerobik türlerin kullanılan ham çamur (aşı çamuru) numunesinde baskın olduğu görülmüştür. Anaerobik türler tarafından gerçekleştirilen fumerat ve sülfat solunumuna ek olarak insan gastrointestinal yolunda bulunan farklı türler yalnızca ham numunede bulunmuştur. NSAID'lerin varlığında aynı zamanda aromatik hidrokarbon degradasyonundan sorumlu fonksiyonel bir grupta da kaydadeğer bir artış tespit edilmiştir. Sonuçlar göstermektedir ki bu çalışma kapsamında çevresel numunelerde gözlemlenen mikrogram seviyesindeki farmasötiklerin konsantrasyonları bile aktif çamur mikrobiyal komünitesinin kinetiği, çeşitliliği ve yapısı üzerinde etkili olmaktadır.

1. INTRODUCTION

Over the last decade, the detection of non-regulated chemicals at trace levels in various environmental compartments is increasingly becoming a worldwide concern due to their persistence in the environment, low biodegradability, and their bioaccumulative and toxic nature. In the literature, several terms are used to describe such chemicals including but not limited to; emerging micropollutants, contaminants of emerging concern and trace organic chemicals. All of them emphasize their adverse effects even they occurred at low concentration levels (ng/L - μg/L).

Pharmaceuticals are one of the major classes of micropollutants (MPs) besides others such as personal care products, endocrine disrupting compounds, disinfection by-products, antibiotics, and pesticides, all of which become a fundamental part of modern society. Even though micropollutants are grouped in different classes based on their properties, they all share posing important risks to both human health and ecosystem. The response of aquatic living organisms to both acute and chronic exposure to pharmaceuticals results in health impairment.

The types and the concentrations of pharmaceuticals reaching to WWTPs influent steams are mainly related to their consumption pattern. One of the frequently observed pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs) which are widely used without prescription. Their annual consumption has reached up to several hundred tons in developed countries (Daughton & Ternes, 1999). Moreover, a significant increase (131%) in the Turkish pharmaceutical markets has been recorded during the period between 2010 and 2018 (IEIS, 2019). Such statistics reflect their remarkable contribution to the level of pharmaceutical pollution in the receiving water sources.

The main direct route of MPs to water has been frequently identified as the central wastewater treatment plants (WWTPs) as they are not designed for this purpose. The concentration levels of non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, ketoprofen, naproxen, diclofenac, indomethacin, and mefenamic acid has been reviewed to lie between 0.001 and 4.11 μg/L (Ziylan & Ince, 2011). Regardless

of the continuous discharge of pharmaceuticals to the environment, there are still considerable gaps in the regulations that govern ecological risk assessment. Consequently, adopting a treatment approach for pharmaceutical elimination to protect the health of humans and ecosystems is a matter of great interest.

Biological treatment is an attractive option as it provides a low cost and low carbon footprint alternative in opposition to the existing physical and chemical technologies. However, inadequate and widely ranged pharmaceutical removal efficiencies (0 to 99%) were obtained so far from laboratory and pilot scale investigations evaluating biological treatment. For example, the removal efficiency of in full scale WWTPs has been reported to vary from <0 to 98% (Tran et al., 2018). Numerous variables such as operation condition (i.e., solid and hydraulic retention time) and system designs may cause such variations in the removal efficiencies (Stadler & Love, 2016). Moreover, several studies indicate that the absence or the low abundance of specific microbial phylotypes can also contribute to removal efficiencies variation (Kim et al., 2017).

In regard to this, more effort should be performed to identify the desirable conditions for MP degraders, the needed engineering strategies to favor the selection of microorganisms with the capacity to degrade pharmaceuticals and the operational conditions that lead to more predictable performance. Another important aspect related to introducing pharmaceuticals into WWTPs, is the physiological response of activated sludge microbial community; which is in other word is the toxicity posed by such complex chemicals to the microbial community. This response is greatly affected by the concentration of the introduced NSAIDs as well as the microorganisms in the incoming substrate. Most of the currently available examinations of microorganisms with a potential to biotransform NSAIDs are restricted solely to study the effect of a single individual compound on the microbial community without taking into consideration the possible synergistic and/or antagonistic effects of a mixture on NSAIDs. A limited number of studies have been performed in wastewater treatment systems in order to examine the effects of a mixture of NSAIDs at environmentally relevant concentrations on the diversity, composition and kinetics of the activated sludge microbial community. Therefore, expanding our knowledge about the diversity and structure of microbial community as well as to understand their response in the presence of the mixture of micropollutants is critical for proper functioning of biological wastewater treatment systems, and determining the shifts in the structure of

microbial community may elucidate the microbial communities responsible for the transformation of the target MPs.

1.1 Purpose of Thesis

This study aims to investigate the changes that can be induced by the presence of mixture of NSAIDs (diclofenac, ketoprofen, indomethacin, and mefenamic acid, ibuprofen, and naproxen) into lab-scale activated sludge sequencing batch reactor (SBR) over four main levels: (i) Aerobic treatability of the selected NSAIDs in SBR, (ii) Potential acute and chronic toxicity of the selected NSAIDs on activated sludge determined by respirometric analysis, (iii) Kinetic and stoichiometric coefficients and their variations in the presence of the selected NSAIDs by modeling respirometric results and (iv) Shift in microbial community structure, diversity, and functions employing advanced molecular biotechnology and bioinformatics tools.

2. LITERATURE REVIEW

2.1 Nonsteroidal Anti-İnflammatory Drugs (NSAIDs)

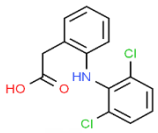
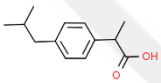
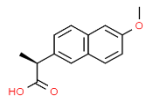
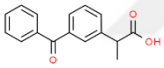
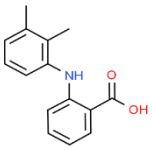
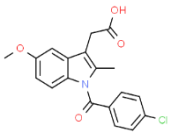
Pharmaceutically active compounds are one of the conspicuous classes of micropollutants which by one route or another, enter the environment as the parent compound or as pharmacologically active metabolites, where they may exert an undesirable biological effect. Approximately 3000 different substances are estimated to be used as pharmaceutical ingredients, including NSAIDs, painkillers, antibiotics, antidiabetics, beta-blockers, contraceptives, lipid regulators, antidepressants, and impotence drugs (Rodriguez-Narvaez et al., 2017). NSAIDs are an intermediary group of prescription and nonprescription drugs consisting of compounds having analgesics, antipyretic, and anti-inflammatory properties. Diclofenac, ibuprofen, naproxen, ketoprofen, mefenamic acid, and indomethacin are of the most commonly detected NSAID compounds in various water bodies and other environmental compartments (Feng et al., 2013).

The effect of pharmaceuticals on the health of animals and humans has been evaluated quite frequently; however, the real impact is still not clear. Nassef et al. (2010) demonstrated via laboratory and field scale investigations that the exposure of Japanese medaka fish to carbamazepine, an antiepileptic drug, or diclofenac causes a decrease in the performance of their feeding pattern and/or activity. The impact of pharmaceuticals can extend to threaten drinking water quality, yet their presence is thought not to exceed the danger-threshold and leave no impact on human health, for now at least. Other indirect effects of pharmaceutical (i.e. antibiotics) on human health may arise through the development of antibiotic-resistance genes (ARGs) and antibiotic-resistant bacteria (ARB), which negatively affect the effectiveness of antibiotic therapy (Tran et al., 2018).

The physicochemical properties (solubility, volatility, biodegradability, etc..) of the tested NSAIDs, listed in Table 2.1, may be helpful to understand their fate in WWTPs and these properties may reflect their level of persistence in different environmental compartments. For example, the level of hydrophobicity of the pharmaceuticals is

determined through their solubility and the octanol-water partition coefficient (K_{ow}). Evaluating these values is critical for predicting the sorption potential of the pollutant to sludge phase. Moreover, Henry-Law constant estimates contaminants' volatility and based on the value provided in Table 2.1, volatilization s is negligible for the tested drugs in the present study (Tiwari et al., 2017).

Table 2.1: Physical and chemical properties of the tested NSAIDs (Kim et al., 2019)

	Structure	Molecular Formula	Molecular Weight (g/m)	Vapor pressure (mmHg)	Solubility (mg/L)	pKa (20 ◦C)	log K_{ow}	Henry's constant (m^3/m)
Diclofenac		$C_{14}H_{10}C_{l2}NO_2$	296.1	$6.14.10^{-8}$	2.37	4.15	4.51	$4.73.10^{-12}$
Ibuprofen		$C_{13}H_{18}O_2$	206.28	$4.74.10^{-5}$	21	5.3	3.97	$1.5.10^{-7}$
Naproxen		$C_{14}H_{14}O_3$	230.26	$1.89.10^{-6}$	15.9	4.15	3.18	$3.39.10^{-10}$
Ketoprofen		$C_{16}H_{14}O_3$	254.28	$1.46.10^{-6}$	51	4.45	3.12	$2.1.10^{-11}$
Mefenamic acid		$C_{15}H_{15}NO_2$	241.28	$5.83.10^{-9}$	20	4.2	5.1	$1.7.10^{-8}$
Indomethacin		$C_{19}H_{16}ClNO_3$	357.8	$9.89.10^{-11}$	0.937	4.5	4.27	$3.13.10^{-14}$

Such compounds find their way to aquatic environments mainly by passing through WWTPs, originate from different sources. Pharmaceuticals and their metabolites, for example, enter wastewater through humans and animals' excretions. Other sources include direct discharge from hospitals, drug stores, and convenience stores where non-prescription medicines such as ibuprofen, naproxen and, aspirin are available. Many of these compounds are not biodegraded in conventional treatment systems, so they are commonly discharged with treated effluent into rivers, lakes, and estuaries.

Diclofenac, ibuprofen, acetylsalicylic acid, and ketoprofen were reported for the first time in sewage and river water early in 1996 (Daughton & Ternes, 1999). Other activities such as the fertilization of soil with sewage and the irrigation of soil with reclaimed wastewater allow micropollutants to contaminate soil and affect its inhabitants (Klatte et al., 2017). In addition, pharmaceuticals can reach the groundwater through leaching from municipal landfills of solid wastes. Peng et al. (2014) has reported ibuprofen and diclofenac among the most commonly detected anti-inflammatories in groundwater. The main route of pharmaceuticals into the environment are summarized in Figure 2.1.

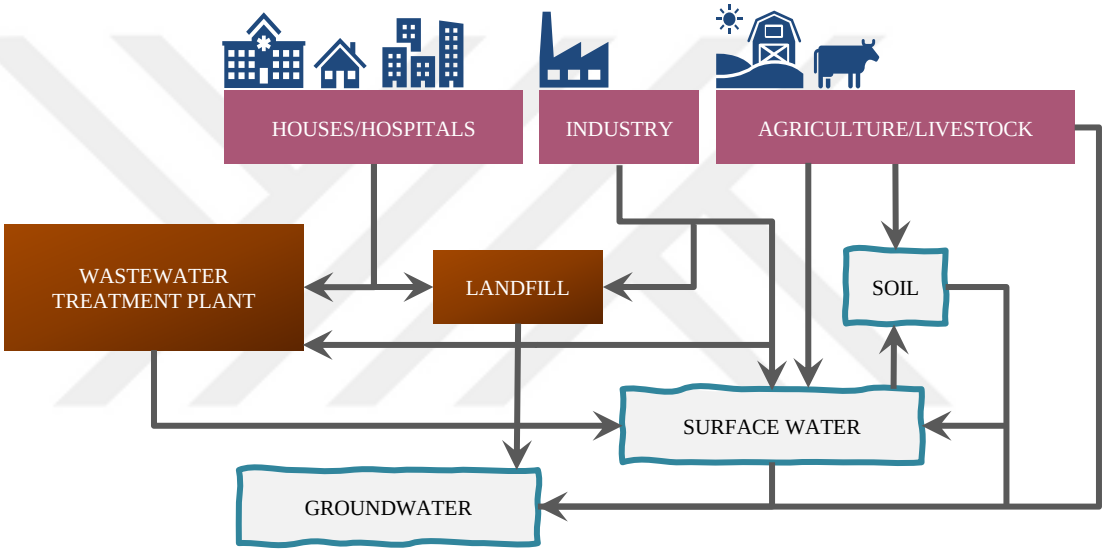


Figure 2.1 : Route of pharmaceuticals into the environment

Regarding environmental regulations, a list of 45 priority compounds was determined in The EU Directive 2013/39/EU (EC, 2013) in which the environmental quality standards to be followed in aquatic environments were included. At the same time, a watch list mechanism was created to monitor other substances with the potential to pose an environmental risk as a step before their addition to the priority list. By the decision number of 2015/495 (EU, 2015), ten additional chemicals were added to the watch list including the anti-inflammatory drug diclofenac. In United State, the U.S. Food and Drug Administration (FDA) has stipulated the assessments of veterinary pharmaceuticals since 1980 (Boxall et al., 2003), and by 1998 an environmental assessment report was required when the expected concentration of human pharmaceuticals in aquatic environment is equal to or greater than 1 µg/L (FDA, 1998).

In 2012 several pharmaceuticals (i.e., estradiol, erythromycin, quinolone) were included in the Drinking Water Contaminant Candidate List (EPA, 2016).

2.2 Occurrence and Removal Efficiencies of NSAIDS

World-wide investigations on the contamination of aquatic bodies by NSAIDs such as diclofenac, ibuprofen, ketoprofen, mefenamic acid, indomethacin, and naproxen have shown concentrations within ng-µg/L range signifying their occurrence at a high proportion in municipal sewage effluents. An overview of the total removal of selected NSAIDs in various municipal WWTPs in different regions of the world are presented in Table 2.2. The performance of conventional WWTPs for NSAIDs removal varies from very poor to complete breakdown. Although the mechanisms of breakdown are not clear, there is consensus on processes such as sorption, adsorption, sedimentation, and biotransformation which will be discussed in the next section. The variation in removal efficiencies in the influent seems to be associated with the consumption patterns. It is clearly seen in Table 2.2 the significant fluctuations in the concentrations of the selected NSAIDs in different geographical regions. For instance, the concentrations of diclofenac in both Europe and the US were considerably higher than in the Asian regions such as China where the population is equivalent to approximately 60% of the total world population. The large variation shown within different study areas can be explained by the differences in lifestyle, seasonal conditions, demography, and sampling techniques. The variation in removal efficiency, on the other hand, can be explained by variation in the unit operation design, climate and operational conditions such as hydraulic retention time and sludge retention time (Grandclement et al., 2017).

The wide range of diclofenac removal efficiencies (9-85%) based on the data collected in Table 2.2 can be due to the low biodegradability of the drug arising from the presence of –Cl and N–H functional groups that inhibit the rate of growth of sewage bacteria (Ziylan & Ince, 2011). Both ibuprofen and naproxen were found to achieve very high removal efficiencies with ranges of 72–99% and 85–97%, respectively. Ketoprofen was reported to be removed in conventional WWTPs in moderate efficiencies ranging between 31% and 85%. Fewer studies were available for mefenamic acid and indomethacin showing a wide variation in conventional WWTPs.

Table 2.2: Occurrence and removal efficiency of NSAIDs in various unit operations.

Drug	Influent (ng/L)	Effluent (ng/L)	Removal (%)	Unit operations	Country	Ref
Diclofenac	1660	430	74	Conventional WWTP	Spain	[1]
	0.8	0.2	75	Primary Settling, AS	Brazil	[2]
	445	69.2	84.4	Conventional WWTP	China	[3]
	2450	359	85	Conventional WWTP	Canada	[4]
	3020	2510	17	Conventional WWTP	Germany	[5]
	-	-	9-25	AS/N & phosphate removal	Finland	[6]
Ibuprofen	55975	1890	96.6	Sequencing batch reactor SBR	Malaysia	[7]
	2800	720	74	Conventional AS	Spain	[1]
	793	220	72	Conventional AS	Greece	[8]
	2500	16	99	Conventional WWTP	US	[9]
Naproxen	1180	190	84	Conventional WWTP	Spain	[1]
	7762	159	97	Conventional WWTP	China	[3]
	321	2.4	93	Conventional WWTP	Spain	[10]
	0.4	0.01	97	Primary Settling, AS	Brazil	[2]
Ketoprofen	5700	1620	71.5	Conventional WWTP	Italy	[11]
	250	37	85	Conventional WWTP	US	[12]
	133	20	85	Conventional WWTP	Spain	[10]
	286	183	37	Conventional WWTP	Korea	[13]
Mefenamic acid	-	-	55	AS/N & phosphate removal	Spain	[14]
	-	-	91	Primary Settling, AS	England	[15]
Indomethacin	449.4	61.4	86	Conventional WWTP	China	[3]
	-	-	40	AS/N & phosphate removal	Spain	[14]

[1] Fernandez-FontainaInes et al., 2013
[2] Stumpf et al., 1999
[3] Tran and Gin, 2017
[4] Metcalfe et al., 2003
[5] Hebereretal., 2002
[6] Lindqvist et al., 2005
[7] Subedi et al., 2015
[8] Papageorgiou et al., 2016
[9] Palmer et al., 2008
[10] Azzouz and Ballesteros, 2013
[11] Andreozzi et al., 2003
[12] Benotti et al., 2008
[13] Behera et al., 2011
[14] Jelic et al., 2011
[15] Jones et al., 2007

2.3 Removal mechanisms of micropollutants in activated sludge

WWTPs that adopt conventional activated sludge (CAS) have been proved to achieve a large assortment of removal efficiencies for different micropollutants. The main removal mechanisms that are used to govern their fate include biological degradation, sorption, hydrolysis, volatilization, and photolysis. Many laboratory works have been demonstrated that the major removal pathways of micropollutants are sorption and biodegradation while the rest are negligible for the majority of micropollutants (Verlicchi et al., 2012).

2.3.1 Sorption mechanisms of MPs

The extent of pharmaceutical sorption to sludge can be determined through the investigation of (i) the compound characteristics (i.e., lipophilicity and acidity), (ii) the environmental conditions (i.e., pH and redox potential), and (iii) the sludge properties which vary between different plants and with different operational conditions (i.e., sludge retention time, hydraulic retention time, supplied oxygen concentration). The removal of micropollutants through sorption occurs mainly via absorption in which the aliphatic and aromatic groups of micropollutants hydrophobically interact with the

lipid bilayers of biomass cell membrane and/or the fat molecules of the sludge. Another process in the removal of micropollutants is adsorption which referred to the electrostatic interactions between the positively charged micropollutant compounds and the negatively charged surface of biomass and sludge (Sipma et al., 2010).

One approach to estimate the sorption trend is the determination of solid-water distribution coefficient (K_d) value which represents the relative concentration of the chemical in the solid and in the aqueous phase at equilibrium conditions. Joss et al. (2005) noted that efficient sorption could be observed if K_d exceeds 0.3 $l\backslash g_{ss}$ while Ternes et al. (2004) considered sorption as a negligible removal mechanism if K_d is below 0.5 $l\backslash g_{ss}$. Considering this, the K_d values listed Table 2.3 indicate that sorption played a minor role in the removal of most of the pharmaceutical compounds. It is worth to note that the sorption behavior of both diclofenac and mefenamic acid are relatively higher compared to other pharmaceuticals.

2.3.2 Biodegradation mechanisms of MPs

Biological degradation, biodegradation, biotransformation are the terms generally used throughout the literature to refer to the transformation of chemical compounds from a complex, toxic form into simpler, less toxic products derived by the enzymatic activity of the microbial community. As previously stated, the major removal pathway of micropollutants has been reported as the biodegradation in WWTP. The efficiency of biodegradation is governed by the degree of micropollutants solubility in wastewater, the structure and functional group of the compounds and the operational parameters. Biodegradation rate was found to be higher in micropollutants including carboxyl groups, hydroxyl groups, and carbonyl groups within their structure, while the opposite was observed when ethers, halogens, aliphatic ethers, methyl groups and ring structures present (Bertelkamp et al., 2016). Studies reflected that the complex ring structure of aromatic compounds containing sulfate or halogen group exhibits a low rate of biodegradation when compared to long-chain aliphatic compounds (Schwarzenbach et al., 2005). This indicates that any alterations in the chemical structure of micropollutants may cause a change in their properties such as solubility and polarity and therefore affecting their fate in the environment. The biodegradation of micropollutants may result in either more hydrophobic or more hydrophilic metabolites. Those metabolites may be recalcitrant and may have a similar or higher level of toxicity (Kim et al., 2008). In terms of operational conditions, sludge retention

time (SRT) is one of the extensively studied factors that impact the biodegradation rate. It has been reported that the removal of pollutants at low SRT is governed by sorption while the higher values of SRT enhance biodegradation by allowing longer time of contact between the chemicals and the sludge (Byrns, 2001). There are two proposed mechanisms through which biodegradation of micropollutants may occur; namely metabolism and cometabolism. Several studies have found that autotrophs were able to contribute to micropollutants biodegradation solely via cometabolic reactions while heterotrophs utilize both cometabolism and/or metabolism pathways for the same purpose (Fischer & Majewsky, 2014).

2.3.2.1 Metabolism

Micropollutants biodegradation via metabolic pathways take place when microorganisms in activated sludge are able to utilize micropollutants as the sole carbon and/or energy source to support their microbial growth and to provide the necessary enzymes/cofactors for catabolism. For biomass to sustain under such conditions, the micropollutants should be available in sufficient concentrations and also be non-toxic to microbial growth. Previous investigations emphasize that hardly any micropollutants can be utilized by metabolic mechanisms as the only source of carbon and energy. In a study conducted by Quintana et al. (2005) to test the mechanism of biodegradation of five acidic pharmaceuticals in aerobic reactor inoculated with a seed sludge, it has been found that only ketoprofen has the potential for metabolic degradation while the removal of the rest, including naproxen and ibuprofen, was achieved cometabolically. Several other researchers reported microorganisms with the ability to use metabolism as a biodegradation pathway to remove micropollutants such as ibuprofen (Murdich & Hay, 2015), ketoprofen (Quintana et al., 2005), diclofenac (Rossmassler et al., 2019). The most commonly reported studies dealing with metabolic micropollutant removal have been conducted with heterotrophic organisms, however, as far as we know there are no reported autotrophic organisms with such metabolic capability.

2.3.2.2 Co-metabolism

Micropollutants usually occurred in WWTPs influent within a range of very low concentrations (μg/L – ng/L) with some reported to pose a potential risk of toxicity for activated sludge microbial community. In this manner, it is not very common for

micropollutants to be utilized as the sole substrate. Research on mechanisms of micropollutant elimination has suggested cometabolism as the main mechanism of micropollutant biodegradation in WWTPs. In the cometabolic reaction, microorganisms utilize micropollutants as a non-growth substrate in the presence of primary readily biodegradable substrates (i.e. glucose, peptone) nutrients (NH_4^+). Many attempts have been made aimed at studying the role played by ammonia-oxidizing bacteria (AOB) to improve micropollutants removal via cometabolic biodegradation. AOB, which utilizes ammonia (NH_4^+) as the primary substrate, induces monooxygenase (AMO) which is a non-specific enzyme known for its ability to breakdown aliphatic and aromatic chemical structure. Low concentrations of MPs detected in treatment plants reflected their biodegradation through cometabolism. Cometabolism has been demonstrated to be the key mechanism to explain the removal of estrogens under nitrifying conditions, through the enzyme ammonium monooxygenase (AMO) (Chen et al, 2019). In the study conducted by Bragança et al. (2016), a culture developed from a sample of soil obtained from an area located beside crude oil storage tanks and enriched with pentane for 2 years, was used to study the degradation level of a number of drugs. The results showed that the pentane enrichment culture could cometabolically transform ibuprofen, paracetamol, and naproxen but not diclofenac. Another studies examined pure cultures for their capacity to degrade pharmaceuticals in the presence of other easily biodegradable growth substrate such as glucose, acetate, tryptone, yeast extract, and peptone, will be discussed in the next section.

2.3.3 Mathematical modelling of MP removal

A growing number of studies in the literature has examined the mathematical models that predict the fate and transformation of micropollutants in both aquatic environment and WWTPs. A better understanding of such models would provide a promising tool for decision-makers to optimize the treatment processes. The reported micropollutants removal models include in most cases the three main removal mechanisms; volatilization, sorption and biological degradation considering both WWTPs operating conditions and physicochemical properties of the micropollutants. Volatilization has been considered in the models that deal with the fate of highly volatile micropollutants (i.e., fragrances) which usually determined by the value of Henry's law constant.

Table 2.3: Solid-water distribution coefficient (K_d) and biodegradation coefficient (K_{bio}) for the selected NSAIDs.

Compound	K_d (L /Kg_{ss})	K_{bio} (L/g_{ss}.d)	Bioreactor	Ref
Ibuprofen	30	29.28		Martínez-Alcalá et al., 2017
	170	50.16		Blair et al., 2015
	112	38.07	Continuous MBR	Fernandez-Fontaina et al., 2013
	24	6.03	Batch reactor	Fernandez-Fontaina et al., 2013
	240	2	CAS	Alvarino et al., 2014
		1.5–20		Suarez et al., 2010
		1.33–>3		Abegglen et al., 2009
		21-35		Joss et al., 2006
	200			Horsing et al. 2011
Naproxen	80	-		Martínez-Alcalá et al., 2017
	229	4.23	Continuous MBR	Fernandez-Fontaina et al., 2013
	47	0.5	Batch reactor	Fernandez-Fontaina et al., 2013
		0.2-9		Suarez et al., 2010
		0.08		Abegglen et al., 2009
		1.0-1.9		Joss et al., 2006
Ketoprofen	40	19.44		Alcala et al., 2017
		0.05	Aerobic	Xue et al., 2010
Diclofenac	110	31.44		Martínez-Alcalá et al., 2017
	78	0.1	Continuous MBR	Fernandez-Fontaina et al., 2013
	32	0.02	Batch reactor	Fernandez-Fontaina et al., 2013
		<0.02		Abegglen et al., 2009
		1.2		Suarez et al., 2010
		5		Plósz et al., 2012
Mefenamic Acid	513	2.02	WWTP	Jelic et al., 2011
	457	-	Primary sludge	Jones et al., 2002
Indomethacin		<0.3		Joss et al., 2006

However, the studies include modeling of pharmaceutical compounds, personal care products, and hormones mostly neglect volatilization as it does not significantly contribute to their elimination. In terms of sorption, there are three empirical models that are frequently used; the Langmuir model, the Freundlich model, and the linear model. The linear model has been found to show an acceptable fit when micropollutants occur at low concentrations (<1 μg/L). As previously mentioned, the biodegradation mechanism plays a major role in micropollutant removal and this section primarily focused on the models referred to biodegradation processes.

Generally, the basic equations that are used for biodegradation modeling (i.e. first order, pseudo-first-order, and Monod-type kinetics) are applied in the case of micropollutant biotransformation considering the requirement of additional parameters. Researchers modified these equations to meet their needs which makes it difficult to compare between different proposed models. In addition, some of the

proposed models were belts on the fact that biodegradation is a non-growing process while other models consider the growth and decay processes for micropollutants biodegradation (Henze et al., 1987). Most of the proposed models consider only the dissolved micropollutants to be available for biodegradation. However, there are other models that proposed the biodegradation for only sorbed and/or both dissolved and sorbed micropollutants (Pomiès et al., 2013). For simplification, some models exclude both parent compounds and the metabolites and consider only the specific micropollutants even though some metabolites might be more toxic and hence deviating away from the aim of modeling. Moreover, the specific biomass with the ability to degrade micropollutants directly is considered as a fraction among sludge in some models. The major limitation in assuming the metabolic biodegradation is that the over mentioned fraction of biomass is not measurable. The models that adopt the cometabolic biodegradation pathway has received much attention as it is considered as the main micropollutant removal mechanism. The early first cometabolic model considered only the cometabolic substrate and later on both primary and cometabolic substrate were included in the model. This is followed by the integration of other specific reactions, that take place and highly affect the fate of micropollutants, including the competition between the co-substrate or form the primary substrate and the inhibition caused by toxic metabolites (Peng et al., 2017).

2.4 Effective Microbial Communities for MP Removal

The majority of the researches, with the aim of understanding the role of biodegradation in micropollutant removal, has adopted the utilization of activated sludge microbial communities, derived from conventional WWTPs, to investigate removal efficiencies of these molecules as discussed in the previous section.

Up to date, little is known about the specific taxa that are responsible for the biotransformation of micropollutants. The identification of such strains is a promising tool in the field of bioremediation of pharmaceuticals-contaminated environments. The following part will summarize some of the identified microorganisms with the ability to biologically transform various types of pharmaceuticals.

2.4.1 Bacteria

Little effort has been made to isolate specific strains of bacteria from activated sludge and other environmental compartments samples with the capability to degrade micropollutants. Table 2.4 collects different bacteria that have been isolated and identified to have the potential to degrade ibuprofen, ketoprofen, naproxen, and diclofenac in. All the collected studies were conducted using laboratory-scale batch reactors.

In an investigation conducted by Marchlewicz et al. (2016), a Gram-positive bacterium, *Bacillus thuringiensis* B1, isolated from a soil sample, was found to own the capability to completely degrade 20 mg/L of ibuprofen within 6 days and 6 mg/L of naproxen within 35 days in the presence of 1 mg/L glucose as a main source of carbon. While ibuprofen exhibits the ability to metabolize 5 mg/L of ibuprofen within 2 days, naproxen has failed to provide an adequate carbon source for this strain. A more recent study examined the potential of the same species to degrade naproxen the presence of other growth substrates. The results showed that the addition of 1 g/L sodium benzoate has achieved a complete degradation of 6 mg/L on naproxen within 28 days (Górny et al., 2019). Complete degradation of ibuprofen and ketoprofen by nitrifying bacteria was achieved cometabolically at environmentally relevant concentrations (25–100 μg/L) (Dawas-Massalha et al., 2014). Degradation of ibuprofen by *Sphingomonas sp.* Ibu-2 was also reported to take place but in monosubstrate culture with ibuprofen as a sole carbon and energy source (Murdoch & Hay, 2005). Similarly, Ismail et al. (2016) studied ketoprofen degradation using a combination of microalgae and K2 bacterial consortium (*Raoultella ornithinolytica* B6, *Pseudomonas aeruginosa* JPP, *Pseudomonas sp.* P16, *Stenotrophomonas sp.* 5LF 19TDLC). The full biotransformation of ketoprofen by K2 consortium was accomplished within 2 days. Retardation of biodegradation was observed for higher concentrations of ketoprofen. *Raoultella ornithinolytica* B6, which is one of K2 consortium, was examined for diclofenac degradation capacity and within one month only 10% of the initial 6 mg/L diclofenac was removed (Domaradzka et al., 2016). Diclofenac is one of the most recalcitrant NSAIDs, with a removal efficiency ranged between 9-85% as indicated in Table 2.2. However, among the collected data, *Brevibacterium sp.* D4 (Bessa et al., 2017) and *Labrys portucalensis* F11 (Moreira et al., 2018) were found to achieve complete removal of diclofenac in the presence of acetate as a growth substrate in a lab-scale batch reactor.

Table 2.4: Biodegradation of NSAIDs in batch reactors using bacteria.

MP	Bacteria	MP Conc. (mg/L)	Removal effeciency (%)	Exp. Duration (d)	Mechanism	Ref
Ibuprofen	*Gordonia amicalis*	0.1	26	4.2	co-metabolism (1 g/L of yeast extract & tryptone)	[1]
	Acinetobacter bouvetii		12.8			
	Paracoccus aminophilus		16.2			
	Patulibacter americanus	0.1	35	4.2		
		0.25	50	13.5		
		1	28	13.5		
	Bacterial Consortium (Enterobacter hormaechei D15, Citrobacter youngae D16, Arthrobacter nicotianae S2, and Pseudomonas sp. S4)	3	23.08	2.0	Metabolism	[2]
			100		co-metabolism (50 mg/L glucose)	
	Patulibacter sp. I11	1	28	12.5	co-metabolism (M9 media + yeast extract and tryptone)	[3]
		0.25	50			
		0.05	62	3.8		
			92		co-metabolism (OD2-medium)	
	Nocardia sp. NRRL 5646	1000	100	5.0	co-metabolisn (glucose, yeast extract & soybean meal)	[4]
	Nitrifying Bacteria	0.025–0.1	100	1.0	cometabolism	[5]
	Bacillus thuringiensis B1	5	100	2.0	Metabolism	[6]
		20		6.0	co-metabolism (1 mg/L glucose)	
	Sphingomonas sp. Ibu-2	500	100	3.3	Metabolism	[7]
	Variovorax sp. Ibu-1	500	60	3.1	co-metabolism (0.2 % (v/v) LB; peptone & yeast extract)	[8]
	Sphingobium sp. IbD51	1	100	0.3	co-metabolism (positive for Casamino acid, Proteose, peptone, Tryptonem Whey protein, Yeast extract, Molasses)	[9]
Ketoprofen	*Methylobacterium populi*	0.1	12	4.2	co-metabolism (1 g/L of yeast extract & tryptone)	[1]
	Gordonia hydrophobica		22			
	Tsukamurella spumae		20.6			
	Paracoccus aminovorans		14.1			
	Rhodococcus qingshengii		13.6			
	Gordonia terrae		15.3			
	Rhodococcus zopfii		14.9			
	Bosea thiooxidans		13.4			
	Nitrifying Bacteria	0.025–0.1	100	6.3	cometabolism	[5]
	K2 bacterial consortium (Raoultella ornithinolytica B6, Pseudomonas aeruginosa JPP, Pseudomonas sp. P16, Stenotrophomonas sp. 5LF 19TDLC)	1000-5000	100	2.0	Metabolism	[10]

Table 2.4 (continued): Biodegradation of NSAIDs in batch reactors using bacteria.

MP	Bacteria	MP Conc. (mg/L)	Removal effeciency (%)	Exp. Duration (d)	Mechanism	Ref
Naproxen	*Planococcus sp.* S5	6	30	35.0	Metabolism	[11]
			75		co-metabolism (1 mg/L glucose)	
			86		co-metabolism (282.33 mg/L phenol)	
	Pseudomonas sp. CE21	0.1	22	3.0	co-metabolism (tryptone& yeast extract) + pharmaceutical mixture (Cefalexin, sulfamethoxazole, Caffeine, Salicylic acid, chloramphenicol, Naproxen, carbamazepine, Ibuprofen, Diclofenac)	[12]
	Bacillus thuringiensis B1	6	100	28.0	co-metabolism (1 g/L Sodium benzoate)	[13]
			<30	35.0	Metabolism	[6]
			>95	20.0	co-metabolism (1 mg/L glucose)	
	Stenotrophomonas maltophilia KB2	6	28	35.0	Metabolism	[14]
			40		co-metabolism (3 mM phenol)	
			78		co-metabolism (1 mg/L glucose)	
Diclofenac	*Enterobacter hormaechei D15*	10	52.8	2.0	Metabolism	[15]
			82	2.0	co-metabolism (50 mg/L glucose)	
	Brevibacterium sp. D4	10	61	15.0	metabolism	[16]
		10	99	30.0	co-metabolism (acetate)	
	Raoultella sp. DD4	6	10	28.0	metabolism	[17]
	Labrys portucalensis F11	5180	70	30.0	metabolism	[18]
		503.2	100	6.0	co-metabolism (acetate)	
	Klebsiella sp. KSC	70	high	3.0	metabolism	[19]

[1] Aissaoui et al., 2013
[2] Aissaoui et al., 2017
[3] Almeida et al., 2013
[4] Chen and Rosazza, 1994
[5] Dawas-Massalha et al., 2014
[6] Marchlewicz et al., 2016
[7] Murdoch and Hay, 2005
[8] Murdoch and Hay, 2015
[9] Zhou et al., 2013
[10] Ismail. et al., 2016
[11] Domaradzka et al., 2015
[12] lin et al., 2015
[13] Gorny et al. 2019
[14] Wojcieszynska et al., 2014
[15] Aissaoui et al., 2017
[16] Bessa et al., 2019
[17] Domaradzka et al., 2016
[18] Moreira et al. 2018
[19] Stylianou et al. 2018

2.4.2 Fungi

The employment of white-rot fungi cultures in the removal of pharmaceuticals is one of the promising biological options that has been extensively studied in recent decades. Such microorganisms can express several extracellular fungal oxidative enzymes, such as manganese peroxidase, lignin peroxidase, versatile peroxidase, or laccase (Wong, 2009), and intracellular enzymes, such as cytochrome P450 (Golan-Rozen et al., 2011) known by their ability to degrade a wide variety of xenobiotics. *Trametes versicolor*, *Phanerochaete chrysosporium*, *Myceliophthora thermophila*, *Pycnoporus sanguineus*, *Phanerochaete sordida* has shown to play important role in the removal of micropollutants. The ability of *T. versicolor* to secrete the four overmentioned extracellular enzymes might explain their potential in micropollutants degradation (Table 2.5). On the other hand, *P. chrysosporium* seems to have a good capacity for

the removal of micropollutants given that laccase and versatile peroxidase are absent from their enzymatic system (Hatakka, 1994). The results obtained by Rodarte-Morales et al. (2012) proved almost complete degradation of diclofenac, naproxen, and ibuprofen from both a stirred-tank reactor and a continuous fixed-bed reactor inoculated with free pellets of *P. chrysosporium*.

Table 2.5: Biodegradation of NSAIDs in different reactors/processes using fungi.

MP	Fungi	MP Conc. (mg/L)	Removal (%)	Exp. Time (d)	Reactor/Process	Mechanism	Ref
Ibuprofen	*Trametes versicolor*	0.013	100	1	fluidized bed batch reactor	co-metabolism (glucose) - Sterilized urban wastewater	[1]
		0.002				co-metabolism (glucose) - non-sterilized urban wastewater	
	T. versicolor	10	100	7	Batch	co-metabolism (glucose)	[2]
	Irpex lacteu						
	Anoderma lucidum						
	Phanerochaete chrysosporium		70				
	Phanerochaete chrysosporium	1	>93	50	Continuous stirred tank fermenter	co-metabolism (glucose) + mixture of pharmaceutical chemicals: (diclofenac, ibuprofen, naproxen, carbamazepine & Diazepam)	[3]
		1	100	100	Continuous fixed-bed reactor		
Ketoprofen	*T. versicolor*	0.00048	35	8	fluidized bed batch reactor	co-metabolism (glucose) - Sterilized urban wastewater	[1]
		0.00008	100	2		co-metabolism (glucose) - non-sterilized urban wastewater	
Naproxen	*T. versicolor*	0.036	100	1	fluidized bed batch reactor	co-metabolism (glucose) - Sterilized urban wastewater	[1]
	T. versicolor	10	100	0.25	Batch	co-metabolism (glucose)	[4]
		0.055	95	0.21			
	Phanerochaete chrysosporium	1	>90	50	Continuous stirred tank fermenter	co-metabolism (glucose) + mixture of pharmaceutical chemicals: (diclofenac, ibuprofen, naproxen, carbamazepine & Diazepam)	[3]
		1	100	100	Continuous fixed-bed reactor		
	Phanerochaete chrysosporium	20	81	7	Free growing batch system	Metabolism	[5]
			86			Cometabolism	
			88		Immobilized batch system	Metabolism	
			90			Cometabolism	
Diclofenac	*Phanerochaete chrysosporium*	1	>93	50	Continuous stirred tank fermenter	co-metabolism (glucose) + mixture of pharmaceutical chemicals: (diclofenac, ibuprofen, naproxen, carbamazepine & Diazepam)	[3]
		1	100	100	Continuous fixed-bed reactor		
	Phanerochaete sordida YK-624	30	100	6	Batch	Cometabolism	[6]
	T. versicolor	10	100	0.17	Batch	Metabolism	[4]
		0.045		0.08			
	T. versicolor	0.69	100	5	Batch	Metabolism	[7]
		0.35	55	2	MBR		

[1] Cruz-Morato et al. 2013
[2] Marco-Urrea et al. 2009
[3] Rodarte-Morales et al. 2012
[4] Marco-Urrea et al. 2010
[5] Li et al., 2015
[6] Hata et al., 2010
[7] Yang et al., 2013

2.4.3 Extracted enzymes

The removal of pharmaceuticals by enzymatic treatment is an attractive approach as it provides fewer chemicals, water, and energy consumption as well as less water production when compared to other chemically catalyzed bioprocesses. Laccase has been widely investigated for its degradation potential to a wide range of micropollutants. The study conducted by Margot et al., (2013) has proved that the extraction of laccase from the fungus *T. versicolor* would be more efficient as it can produce more than 20-times more laccase activity than the strains of the bacterial genus *Streptomyces* (*S. cyaneus, S. ipomoea, S. griseus, and S. psammoticus*) can do so with different carbon sources. Besides, laccase from *T. versicolor* showed higher activity and faster degradation kinetics under the conditions normally encountered in municipal wastewater (pH 7 and 10-25 °C). On the other hand, laccase in its purified or commercial form has presented a considerable removal efficiency for diclofenac and naproxen as collected in Table 2.6 (Kim and Nicell, 2006; Lloret et al., 2010; Tran et al., 2010) but not for ibuprofen (Tran et al., 2010). Moreover, it has been found that lignin peroxidase from *P. chrysosporium* can achieve complete degradation of diclofenac at pH 3.0-4.5 with 3-24 ppm H_2O_2 (Zhang and Geißen, 2010).

It is clearly seen from Table 2.6 that the degradation capacity of the extracted enzyme is affected by the presence and absence of redox mediators, which behave as electron carriers between the oxidizing enzyme and the target compounds. The influence of mediators varies depending on both micropollutant and mediator molecular structures (Kim & Nicell, 2006). For example, laccase firstly oxidizes the mediator which in turn transfers the electrons to the target substance. The most widely used redox mediators are 1-hydroxybenzotriazole, ABTS or violuric acid (Fabbrini et al., 2002).

2.5 Effect of MPs on Activated Sludge Microbial Community

The performance of a biological treatment system is critically related to the biological activity of activated sludge. It is essential to keep activated sludge away from any kind of shock loads so as not to harm their activity and disturb their performance. MPs are of interest as they can potentially affect, and harm activated sludge biomass. Their presence in WWTPs influents has been shown to partially inhibit activated sludge activity, negatively affect the rate of biological kinetics and obviously induce changes

Table 2.6: Biodegradation of NSAIDs in batch reactor using extracted enzymes.

MP	Enzyme	MP Conc. (mg/L)	Removal (%)	Exp. time (d)	Mechanism	Ref
Ibuprofen	crude laccase from *T. versicolor*	0.1	2	1	without 1-hydroxy benzotriazole mediator	[1]
			-*		with 1-hydroxy benzotriazole mediator	
		0.01	30	7	with ABTS mediators	[2]
Ketoprofen	crude laccase from *T. versicolor*	0.1	-*	1	without HBT mediator	[1]
			7		with HBT mediator	
		0.01	60	7	with ABTS mediators	[2]
Naproxen	Purified laccase from *T. versicolor*	20	>95%	1.25	co-metabolism (glucose) + 1-hydroxybenzotriazole mediator	[3]
		20	<10%	1.25	co-metabolism (glucose) - without mediator	
	commercial laccase from *Myceliophthora thermophila*	5	64	1	co-metabolism (50 mM sodium acetate) + 2 mM violuric acid mediator	[4]
	crude laccase from *T. versicolor*	0.1	-*	1	without 1-hydroxy benzotriazole mediator	[1]
			7		with 1-hydroxy benzotriazole mediator	
		0.01	60	7	with ABTS mediators	[2]
Diclofenac	commercial laccase from *Myceliophthora thermophila*	5	100	1	co-metabolism (50 mM sodium acetate) + 2 mM HBT mediator	[4]
	Crude lignin peroxidase from *Phanerochaete chrysosporium*	5	100	0.1	cometabolism (veratryl alcohol) at pH 3.0–4.5 and 3–24 ppm H_2O_2 + carbamazepine	[5]
	commercial laccase from *T. versicolor*	0.001	>90	0.833	metabolism + micropollutant mixture (diclofenac, mefenamic acid & bisphenol A)	[6]
	crude laccase from *T. versicolor*	0.1	72	1	without 1-hydroxy benzotriazole mediator	[1]
			95		with 1-hydroxy benzotriazole mediator	
		0.01	100	7	with ABTS mediators	[2]

* no removal
[1] Nguyen et al., 2013 [4] Lloret et al., 2010
[2] Tran et al., 2010 [5] Zhang and Geissen, 2010
[3] Marco-Urrea et al. 2010 [6] Margot et al., 2013

in the diversity and composition of activated sludge microbial community (Carballa et al., 2004; Papadimitriou et al., 2007).

2.5.1 Toxic and inhibitory effects of MPs on microbial activity

Wastewater treatment plants (WWTPs) frequently receive influent containing xenobiotic compounds that may pose an inhibition and/or have toxic potential to the biological community in activated sludge. The physicochemical properties of pharmaceuticals resemble to harmful xenobiotics in terms of having the ability to penetrate the membranes and being relatively persistent. Among the aspects considered while designing pharmaceuticals it was targeted to be persistent so as to maintain their chemical identity long enough to accomplish their therapeutic work. Toxicity tests have been widely applied to determine the inhibitory effect of these pharmaceuticals on the non-target organisms including activated sludge microorganism. Recent studies have revealed that the variation in the toxicity level of pharmaceuticals depend on their concentration, the target organism, exposure time, and the presence of other chemicals. Most of the conducted studies have mostly

focused on the short-term acute toxicity assessment even though pharmaceuticals are not expected to pose acute risks as they detected mostly at low concentration. Rather, pharmaceuticals will most likely pose chronic toxic effects due to their tendency to accumulate in the system as they continuously released at low concentrations. Another important issue to consider is that the pharmaceuticals reached to WWTPs influent are partially metabolized after human or veterinary use and in some cases metabolite concentrations may be more significant than the parent compounds. For example, the toxicity posed on algae by N4- acetylsulfapyridine, a metabolite of antibiotics, was found to be more effective than sulfapyridine, the parent compound. Furthermore, the co-presence of pharmaceuticals in WWTPs could promote synergistic or antagonistic interactions. Pharmaceuticals mixture referred as synergism when the toxicity of a mixture is greater than the sum of effects of the separate constituents and as antagonism on the contrary. This implies that in some cases the occurrence of single pharmaceuticals at low concentrations may not exert considerable toxic effect when acting individually while its presence as a part of a complex mixture can still pose a toxic risk. Synergetic toxicity effect was revealed by Ebele et al. (2016); whereby carbamazepine and clofibric acid showed much greater toxic effects to *Daphnia magna* than individual compounds at the same concentration. Quite the reverse, a binary mixture composed of ibuprofen and triclosan lead to clear antagonism for oxygen uptake rate and for enzymatic activity at low values of population affected fraction (Amariei et al., 2017).

2.5.1.1 Toxicity assessment in environmental samples

It is essential to screen incoming wastewater for toxicity to minimize the over mentioned adverse effects and to control the activated sludge system accordingly. There are several approaches mentioned in the literature used to track the impact of toxic chemicals on the biochemical activity of activated sludge. Most published aquatic toxicity data were measured based on the short-term acute toxic tests. Later, chronic aquatic toxicity tests have been implemented in the latest environmental risk assessment. In such tests, the target organisms are subjected to various concentrations of chemicals and monitored for long duration, or a considerable part of their lifetime. On the contrary to acute toxicity tests, which often based on only mortality to assess the impact, chronic tests usually consist of other impact measurements such as growth or reproduction.

The analysis applied by researchers to analyze the toxicity of emerging contaminants and other toxic chemicals in various environmental samples is summarized in Figure 2.2.

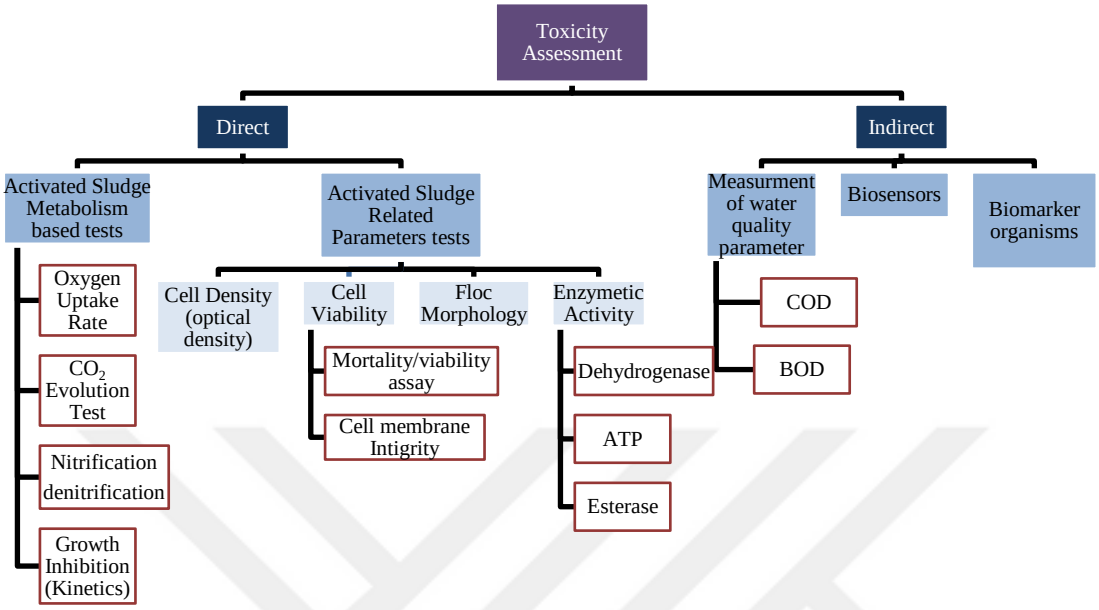


Figure 2.2 : Methods of toxicity assessment in environmental samples

For simplification, the methods are classified into direct and indirect toxicity assessments based on the target organism. The former referred to the assays using activated sludge as the target organism and to measure their response towards the applied toxicants, while the latest relied on other organisms or parameters. Among the direct assays, respirometric based methods has attracted considerable research effort in the past years. It involves oxygen uptake rate (OUR), the most widely applied test in aerobic processes and to less extent, CO_2 production method are used for toxicity assessment (Xiao et al., 2015). With the advancement in biological nutrient removal, toxicity assays specific to nitrification have been established as it has been proved to show more sensitivity towards toxicants. Such assays track the changes in ammonium consumption, nitrite consumption/accumulation and nitrate accumulation. Moreover, the level of inhibition can be measured directly by following up the deterioration of physiological functions and other community changes such as growth inhibition, cell density, cell viability (mortality and cell membrane integrity), floc morphology and the activity of several basic enzymes. In addition to the assays that target activated sludge directly, toxicity can be assessed using other indicator organisms (e.g., fish and invertebrate assays) termed as biomarkers in the field of ecotoxicology. Though this is

perceived as an indirect method as biomarkers are exogenous to the wastewater community, yet they can be used in the risk assessment of effluent wastewater on aquatic life. Another option for toxicity bioassays is biosensors which have been extensively researched and reviewed in the past decades to be used for the evaluation of toxicity in the aquatic environment and to some extent in biological WWTPs. Even though biosensor is thought to be a promising device to be used for on-line monitoring to provide an early warning for WWTP operators, the difference between the microbial community in reactors and the one in the biosensors is one of the main limitations.

The units that are used for influent and effluent toxicity expression varies throughout the literature, posing a challenge while comparing results between different sources. The most widely used way of expression is Effective Concentration 50 (EC_{50}). It is the dose that cause 50% production of the response. Yet other ways can be encountered such as using the raw data of growth inhibition without EC_{50} calculation, using the term Toxic Unit (TU, $1/EC_{50}$), and/or presenting effluent toxicity as a percentage of influent toxicity. Chemicals can be classified in terms of toxicity in several ways. The level of the toxicity classification defined by EC Directive (93/67/EEC) (European Commission, 1993) and Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (United Nation, 2011) is given in Table 2.7.

Table 2.7: Toxicity classification of chemicals by EC Directive and GHS.

EC Directive (93/67/EEC) (EC, 1993)		GHS (United Nation, 2011)	
µg/L	Risk Classification	µg/L	Risk Classification
EC_{50}<100	Extremely toxic	EC_{50}≤1000	Highly toxic
100< EC_{50}≤1,000	Very toxic	1000 < EC_{50} ≤10,000	Toxic
1,000< EC_{50}≤10,000	Toxic		
10,000< EC_{50}≤100,000	Harmful	10,000 < EC_{50} ≤100,000	Harmful
EC_{50}>100,000	Non-toxic	>100,000	Non-toxic

2.5.1.1.1 Respirometric methods

The most widely applied and direct method to evaluate the toxic effects of chemicals such as pharmaceuticals on the sludge activity, and thus the potential impact on its microbial community in aerobic processes is respirometric methods. Respiration rate can be quantified by different methods including the oxygen uptake rate (OUR) and CO_2 evolution rate.

OUR is the most frequently used method to evaluate the toxicity. This approach depends on the fact that the rate of oxygen consumed by activated sludge is depleted

in the presence of toxicants and thus the reduction on respirometric activity is a direct proof of toxicity (Tzoris & Hall, 2006). However, the test will not assess the overall toxicity as the observed inhibition is related only to the most common fast-grower microorganisms due to the high biodiversity and density of activated sludge microbial community (Guisasola et al., 2003).

Several respirometric based studies have been conducted to evaluate the acute and chronic toxic effect of MPs on non-acclimated and acclimated activated sludge samples, respectively. Acclimation refers to the exposition of the sample to the studied toxicants until steady state condition is achieved. Experiment on sulfamethoxazole and carbamazepine shows similar toxicity percentage for both compounds of around 17% at 1000 μg/L on acclimated activated sludge mixed culture, although the toxic effect posed by sulfamethoxazole was slightly higher than those obtained for carbamazepine in the studied range (100–1000 μg/L). Same study compared between the acute and chronic toxicity posed by those micropollutants together with caffein and found that higher values of toxicity and inhibition percentages were observed for the control as compared to the acclimated culture for all three pharmaceuticals. For instance, no toxicity was observed for both cultures in the presence of 200 μg/L caffeine. However, the toxicity of caffeine at 600 μg/L was increased from nondetectable to ca. 8% for acclimated and control culture, respectively. Similarly, the caffeine inhibition, which was negligible for the acclimated culture, took values up to 15% for the control culture (Vasiliadou et al., 2018). Furthermore, the results of the respirometric experiment, mentioned in previous section, indicated that ibuprofen and triclosan were respectively non-toxic and toxic to activated sludge previously acclimated to both compounds depending on the determined EC_{50} values. The presence of 0.32 ± 0.07 mg/L triclosan was found to cause 50% reduction of 125 mg/L activated sludge mixed liquor present in the test vessel while much higher concentration of ibuprofen (64 ± 13 mg/L) was required to cause the same inhibition for the same concentration of activated sludge. The toxic effect of both compounds was determined by the Combination Index-isobologram method. A clear antagonistic behavior was observed at lower values of affected population after which the mixture was recorded to behave additively and synergistically as the effect level increased (Amariei et al., 2017). Additionally, Garcia et al. (2014) utilized OUR-based method to measure the ecotoxicity of 26 pharmaceuticals on activated sludge acquired from the secondary treatment and reported EC_{50} value to express the extent of inhibition. The authors adopted the GHS

classification shown in Table 2.7 to classify the tested compounds. All of them were non-toxic except for 8 compounds. Only 1,4-benzoquinone (1.36 mg/L) and ciprofloxacin (9.55 mg/L) were evaluated as toxic. The rest including ibuprofen and naproxen were denoted as harmful chemicals with EC_{50} values of 21.00 and 32.82 mg/L respectively. The calculated EC_{50} value for ibuprofen in this study was less than the one mentioned in Amariei et al. (2017) by more than half. Liwarska-Bizukojcet al. (2018) also revealed that the inhibition caused by diclofenac to the respirometric activity of activated sludge acclimated to a mixture of three MPs (17α-Ethinylestradiol, diclofenac, and 4-nonylphenol) was the highest when compared to the other two compounds. This was associated with lowest removal degree of diclofenac from activated sludge system with a percentage of 58% while the removal level of 17α-Ethinylestradiol and 4-nonylphenol were 93 and 71%, respectively.

Moving to CO_2 evolution rate, this method is frequently used for the assessment of biodegradation potential of non-volatile compounds through the quantification of CO_2 evolution. It was previously known as modified Sturm tests and described by OECD 301-B. The design of the experiment involves an inhibition test that measure the biodegradability of the tested compound together with a readily biodegradable compound (i.e. sodium acetate) to act as a reference. The test provides the advantage of retaining the bacterial consortium away from any significant alterations by allowing the rapid usage of activated sludge. One of the experiments that employed this test was to determine the toxicity of tetracycline and tylosine antibiotics to activated sludge at different concentrations. The tests were conducted in 1 L batch reactor with sodium benzoate as referent, and the results were determined and found that the toxicity threshold to be in the range of 7 to 10 mg/L TOC for tetracycline and in the range of 4 to 5 mg/L TOC for tylosine (Prado et al., 2010). Another example in which a test based on CO_2-evolution principle was used to evaluate the toxicity of ibuprofen in WWTPs was by Davids et al. (2017). In their study they performed a batch incubation in OxiTop device, which is usually used for assessing volatile organic compounds biodegradability, for different ibuprofen concentration ranging between 50-5000 mg/L. The principle of OxiTop is based on the measurement of the reduction in bottles pressure due to the formation of CO_2 as a product of the aerobic degradation of the sample. In this experiment, the highest CO_2 evolution was detected in ibuprofen free set indicating the highest detected microbial activity (321 mg O_2/L BOD). The inhibiting effect, measured as biological oxygen demand (BOD), of ibuprofen

increased with increasing its concentration in which BOD was detected after 21 days incubation as 242 and 107 mg O_2/L in the bottles with 50 and 5000 mg/L of ibuprofen, respectively.

2.5.1.1.2 Enzymatic activity inhibition methods

The activity of several enzymes such as dehydrogenases, protease, phosphatases, glucosidases, esterase and hydrolytic enzymes are used to have an opinion on the biomass activity in biological treatment system. They believed to play a critical role in the initiation of the first rate-limiting hydrolytic reaction in the overall process of organic matter degradation and hence used widely for toxicity assessment. Dehydrogenase activity (DHA) assay has been applied very frequently for the purpose of MPs toxicity assessment. In an examination of the impact of diclofenac (DCF), 4-nonylphenol (4NP) and 17α-ethinylestradiol (EE2) on activated sludge the results of DHA test indicates that DCF and 4NP inhibit DHA at higher level than EE2 if they applied in a concentration ranged between 50-1000 μg/L while no inhibition was observed by EE2 and 4NP with concentrations lower than 50 μg/L (Liwarska-Bizukojct et al., 2018). Likewise, in acute enzymatic test, EE2 was found to cause a ~20% and 15% inhibition of the activity of dehydrogenase and hydrolytic enzyme respectively in activated sludge microorganisms at the highest tested concentrations with EC_{50} value to be greater than 100,000 μg/L after 30 minutes suggesting that EE2 is non-toxic based on EU Directive 93/67/EEC criteria.

The activity of esterase enzyme was also used to access the toxicity of pharmaceuticals on microbial community of activated sludge. Amariei et al. (2017) investigated how activated sludge are sensitive towards ibuprofen and triclosan by studying their effect on esterase activity. The obtained EC_{50} value for ibuprofen using 125 mg/L activated sludge was high enough (633 ± 63 mg/L) to classify it as non-toxic compound based on EU directives 93/67/EC. In contrast, triclosan was considered as a toxic compound as the maximum EC_{50} value obtained for a range of activated sludge concentrations (125–500 mg/L) did not exceed 5.5 mg/L.

2.5.2 Effect of MPs on microbial community structure and diversity

Activated sludge is composed of a mixture of microorganisms including protozoa, fungi, bacteria, archaea, and viruses with bacteria being the most dominant component (Wagner & Loy, 2002). The need to understand the key aspects of activated sludge

microbial community, including structure, diversity, and dynamics have been realized early as activated sludge is the most widely applied process in municipal WWTPs. However, the lack of robust techniques to explore highly complex systems was posed the largest obstacle to the advancement in this field. The traditional techniques depending mainly on microbial cultivation was not able to provide enough valuable information since most of the activated sludge microorganisms are unculturable. The utilization of the traditional PCR cloning approach was also limited due to its low coverage sequencing in comparison with the huge genetic diversity of activated sludge (Shendure & Ji, 2008). Until metagenomic sequencing (e.g. 454 pyrosequencing and Illumina sequencing technologies) has employed in this field, microbial community structure and composition of environmental samples have remained relatively little studied. This approach has simplified the way by which significant information about the taxa with low abundance can be acquired so to predict their function in activated sludge systems. Metagenomic sequencing has been employed on various environmental samples including drinking water (Chao et al., 2013), full-scale WWTPs (Ju et al., 2014; Wang et al., 2014; Zhang et al., 2012) and full-scale anaerobic digesters (Wong et al., 2013) to determine the microbial composition and diversity. The results of such studies proved the effectiveness of this tool to analyze a community structure with exceptionally high diversity.

The structure of pharmaceuticals and their metabolites is designed in a way that might cause a disturbance in the biological systems and likely pose an alteration in their microbial community structure. Since the commonly discussed pharmaceuticals are composed mainly of aromatic hydrocarbon, which is a structure widely detected among other wastewater constituents, their degradation pathways are expected to be somehow similar. This indicates minor structural shifts to take place on the microbial community, which is expected to become greater in the case of introducing pharmaceuticals at higher concentrations as in the hospital sewage (Kraigher et al., 2008).

Pharmaceuticals are designed in a way that disturb the biological systems and hence it is expected to pose an alteration in the microbial communities. Several studies in the literature has been focused on determining the effect posed by ibuprofen on the abundance and composition of activated sludge microbial population. Abdelrahman et al. (2018) examined how ibuprofen and tetracycline affect bacterial abundance and composition in activated sludge sample obtained from WWTP. A decrease in the

relative bacterial abundance, was observed in the three reactors to which the environmentally relevant low concentrations of ibuprofen (100 µg/L), tetracycline (50 µg/L), and combination of both (100 µg/L ibuprofen and 50 µg/L tetracycline) were added, compared to reference reactors where no pharmaceuticals were added. However, the addition of high and very high concentrations seems to trigger a recovery of the bacterial population as their relative abundance was not diminished considerably from the reference. The reduction in the bacterial diversity in activated sludge due to the exposition to low concentration (50 µg/L) of NSAIDs mixture (ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid) was also observed by (Kraigher et al., 2011; Kraigher et al., 2008), yet a larger structural divergence was detected in the reactors operated with 200 and 500 µg/L of pharmaceuticals. The results obtained by Jiang et al. (2017) on the other hands indicated a stimulation of bacterial diversity associated with the addition of lower concentrations to three SBRs reactor (5 µg/L diclofenac, 5 µg/L diclofenac +5 µg/L ibuprofen and 5 µg/L diclofenac+5 µg/L ibuprofen+5 µg/L naproxen). Similarly, a reduction in the microbial diversity was recorded in the presence of very high ibuprofen concentrations (500–1000 mg/L) with a recorded increase in the presence of 5000 mg/L (Davids et al., 2017). This indicates the lack of a known mode of action of NSAIDs against prokaryotes.

The impact of NSAIDs on the composition of activated sludge microbial communities is still poorly investigated. For example, Jiang et al. (2017) observed an increase in the abundance of *Actinobacteria* and *Bacteroidetes* together with a decrease in Proteobacteria as a response to the addition of a mixtures of ibuprofen, diclofenac, and naproxen (5 µg/L each) to a sequencing batch reactors (SBRs) fed with synthetic wastewater. Over the genus level, a reduction in *Micropruina* and *Nakamurella*, known for their ability to accumulate energy-storage chemicals under extreme conditions, was observed with the addition of NSAIDs. However, a reduction in the percentage of *Bacteroidetes* was observed when an ibuprofen-containing personal care product and pharmaceuticals mixture (50 µg/L each) was supplemented to a granular MBR system designed to treat acetate dominant wastewater (Wang et al., 2016). Likewise, in a study in which ibuprofen served as a sole source of carbon the results revealed a remarkable drop in the proportion of *Bacteroidetes*, *Planctomycetes*, and *Verrucomicrobia* (from 42.7 to 2.1%) coincide with a sharp increase in the relative abundance of *Alphaproteobacteria* and *Gammaproteobacteria* (from 29.1 to 80.8%) (Navrozidou et al., 2019).

The exposition of activated sludge microbial community to different concentrations of diclofenac ranged between 50-5000 μg/L in batch bioreactors, fed with glucose-based synthetic wastewater over two months, revealed no significant changes on their overall diversity and structure. Besides, an increase in the proportion of *Nitratireductor*, *Asticcacaulis*, and *Pseudacidovorax* genera was also recorded indicating they have a possible role in diclofenac biodegradation (Nguyen et al., 2019).

3. MATERIAL AND METHODS

3.1 Experimental Set-up

A laboratory-scale sequencing batch reactor (SBR), with a working volume of 8 L (total reactor volume 10 L) and a sludge retention time of 5 days, was operated in two consecutive phases. The first one is the control phase in which the reactor was acclimated solely to a synthetic organic substrate (peptone mixture) until steady state condition was achieved. This lasted 66 days after which the reactor started to be fed with peptone and micropollutant mixture (i.e., diclofenac, naproxen, ibuprofen, ketoprofen, indomethacin, and mefenamic acid). The reactor performance was monitored by conventional parameters such as suspended solids (SS), volatile suspended solids (VSS) and sCOD measurements. The acclimated biomass from both phases was used for respirometric experiments and microbial culture analyses. Long-term feeding with micropollutants was used for the investigation of (i) aerobic treatability potential of micropollutants especially for long time operational period, (ii) potential chronic toxicity to activated sludge and hence the aerobic treatment system posed by their presence and (iii) shift in microbial community structure and diversity caused by micropollutants.

3.2 Reactor Operation

The reactor was set up to be operated automatically employing timers. Throughout the operation, the reactors were maintained at constant room temperature (20 ± 2 °C) controlled by air conditioner, completely mixed using mechanical (IKA, Eurostar 40, Germany) and magnetic stirrers (VWR, VMS-C10, US), aerated at constant speed by the aid of high-power air pumps (RS Electrical-15000, China). Perforated Teflon pipes, specially designed to prevent the attachment of micropollutants, were placed in reactor's bottom. The feeding to and discharging from the reactor has been accomplished by peristaltic pumps (Seko, PR7, Türkiye; Antech, PER-A, Turkey). Both control and micropollutant reactors are illustrated in Figure 3.1.

Figure 3.1 : Control and micropollutant fed lab-scale SBRs

The seed sludge was obtained from the aeration unit of a municipal wastewater treatment plant located in Istanbul Province, Turkey. The sludge sample was first sieved with a stainless-steel sieve having 2 mm opening and later added to the reactor as seed sludge. The conventional characterization of the seed sludge is given in Table 3.1.

Table 3.1: The characterization of the raw sludge used to initiate reactors.

Parameter	Unit	Seed sludge
pH	-	7.8
TSS	mg/L	5470 ± 12
VSS	mg/L	3350 ± 31
Total COD	mg/L	4710 ± 20
TKN	mg/L	260 ± 10
NH_4^+-N	mg/L	56 ± 5
TP	mg/L	26 ± 6

The reactor was operated with 2 cycles daily. Each cycle was outlined to last 12 hours in which 630, 60, 20, and 10 minutes to be spent in reaction, settling, decanting and idle phases, respectively. Filling phase was taking place within the first 30 minutes of the reaction phase during which the reactor content was completely mixed and aerated. Maintaining the sludge age at 5 days was done by discharging 1600 mL excess sludge

once a day just before the end of reaction phase, which observed by the halt of aeration and stirring and the beginning of sludge settling. Effluent characterization was regularly carried out once a week using a sample that is obtained from the supernatant at the end of the settling phase. Directly after the decanting phase, where the rest of the supernatant is discharged leaving 4 L in the reactor, the feeding solution was pumped up to 8 L. Operating conditions and parameter of the reactor are given in Figure 3.2 and Table 3.2.

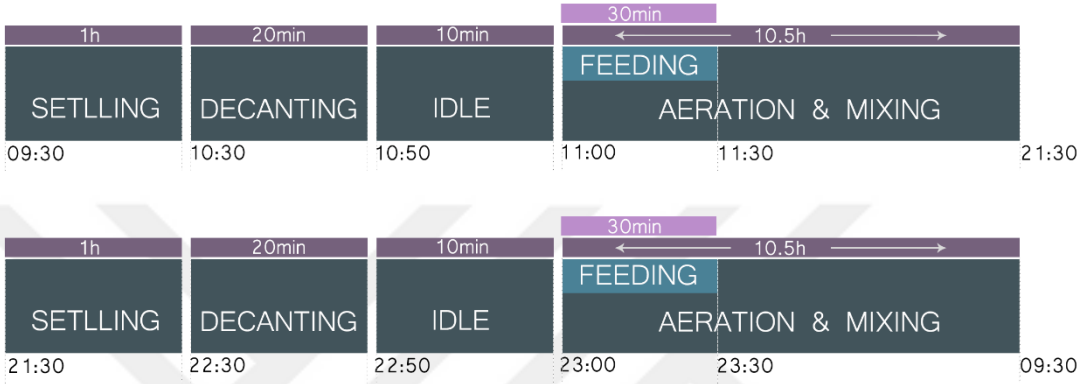


Figure 3.2 : Operational conditions of sequencing batch reactor

Table 3.2: Operating parameters of laboratory-scale sequential batch reactors.

Parameter	Symbol	Value	Unit
Total Cycle Time	T_C	12	hr
Feeding Phase	T_F	30	min
Aeration Phase	T_{AE}	630	min
Settle Phase	T_S	60	min
Draw Phase	T_D	20	min
Idle Phase	T_I	10	min
Initial Volume	V_0	4	L
Total Volume	V_T	8	L
V_0/V_T	-	0.50	-

Throughout the experiments the reactor was fed with a synthetic wastewater that provide a carbon source with concentration of approximately 400 mg COD/L per cycle and with essential macro- and micro- nutrients to supply the nutritional needs of the cells and to provide enough buffer capacity to the reactor so to keep pH between 6 and 8. The pH was adjusted if needed by the addition of 1 N H_2SO_4. Feeding solution constituents are listed in Table 3.3. Peptone mixture was used as an organic substrate owing to the shared biodegradation characteristics with domestic sewage (Insel et al., 2006). The amount to be added from the prepared peptone mixture stock solution to provide 400 mg COD/L was regularly determined based on the measured COD. The

macro- and micro- nutrient stock solution, designated as Solution A and Solution B respectively, were prepared freshly each week as two separate concentrated solution. The amount of Solution A and B were adjusted to supply 10 mL of solution for 1000 mg COD/L of carbon source. All the prepared stock solutions were stored in amber bottle to prevent photodecomposition and at +4^{o}C. Once steady states achieved in the control phase the reactor started to be fed by a mixture of NSAIDs (1 µg/L diclofenac, ketoprofen, indomethacin and Mefenamic Acid, and 10 µg/L for Ibuprofen and Naproxen) using concentrations reflecting real domestic wastewater together with the peptone mixture. Analytical standards of diclofenac, naproxen, ibuprofen, ketoprofen, indomethacin, and mefenamic acid were provided from Sigma-Aldrich (Steinheim, Germany). The stock solution (250 g / L for Ibuprofen and Naproxen; 25 g / L for Ketoprofen, Indomethacin, Mefenamic acid and Diclofenac) was prepared by dissolving in methanol, due to the low solubility of micropollutants in water, and stored in a glass amber bottle.

Table 3.3: Stock synthetic peptone mixture micropollutant mixture and inorganic nutrient solutions compositions.

Micropollutant Reactor Feeding							
Control Reactor Feeding							
Peptone Mixture	**(g/L)**	**Sol A**	**(g/L)**	**Sol B**	**(g/L)**	**Micropollutant Mixture**	**(g/L)**
Peptone	16	K_2HPO_4	320	$MgSO_4.7H_2O$	15	Ibuprofen	250
Beef Extract	11	KH_2PO_4	160	$FeSO_4.7H_2O$	0,5	Naproxen	250
Urea	3			$ZnSO_4.7H_2O$	0,5	Ketoprofen	25
NaCl	0,7			$MnSO_4.7H_2O$	0,41	Indomethacin	25
$CaCl_2.2H_2O$	0,4			$CaCl_2.2H_2O$	2,65	Mefenamic acid	25
						Diclofenac	25

3.3 Analytical Methods

3.3.1 Conventional characterization

The sample obtained from the wastewater treatment plant and the ones regularly obtained from the reactor's were subjected to several analysis to determine the concentration of conventional parameters as described in standard methods (APHA, 2005).

3.3.1.1 pH

The measurement of pH was performed with pH/milivolt meter (ThermoOrion) following the procedure indicated in the standard method of ISO 10523. (2008). The

calibration of pH meter was conducted daily where in use and the probe was rinsed and wiped with distilled water before, after and between each measurement. pH values were reported with two decimals from detection screen.

3.3.1.2 Total Suspended Solid (TSS) and Volatile Suspended Solid (VSS)

TSS and VSS were quantified using 1.2 mm Millipore AP40 glass fiber filters. Filters were first washed with three successive 20 mL distilled water, dried in an oven set to 104 ± 1ºC for 1 hour, placed into a desiccator until they have cooled to balance temperature and finally weighed as the filter tare mass in mg. To determine TSS, a volume of 5 mL and 100 mL was selected for the samples derived from mixed liquor and upper phase respectively, to pass through the prewashed filters, be dried in the oven at 104 ± 1ºC for 1 hour and be weighed after desiccated until constant weight. The difference in this weight and the tare weight represents TSS. In the case of VSS the same steps were followed except for burning of the filters with suspended solids residue in the ash furnace at 550 ±5 °C for 30 minutes. From the weight of the remaining solid, VSS values were calculated.

3.3.1.3 Chemical Oxygen Demand (COD)

COD measurements were carried out as described in ISO 6060. (1989). The sample obtained from wastewater treatment plant was used directly for TCOD determination while the ones obtained from reactor's upper phase were first passed through 0.45-μm filters to determine sCOD. In both cases, 2.5 mL of the sample, 1.5 mL of potassium dichromate digestion solution and 3.5 mL of sulfuric acid reagents were placed in the COD vials. The vials were then tightly capped, inverted several times and placed in COD digester at 150°C and refluxed for 2 hours. The contents of the COD vials, after cooling down, were transferred to a conical flask for titration with standardized 0.10M FAS using ferroin indicator which causes the solution to turn reddish-brown at the endpoint.

3.3.1.4 Ammonium nitrogen ($NH4^+$-N) measurement

The measurement of NH_4^+-N was done as described in APHA. (2005). After the addition of certain amount of upper phase sample, chosen to be 50 or 100 mL, to distillation flask, distilled water was used to reach a total volume of 500 mL, 2-3 glass silicate beads to achieve uniform boiling, 25 mL borate buffer solution and finally just

before assembling the distillation flask to distillation unit, 3 drops from 1 N NaOH solution were added to adjust pH. The distillation was then allowed to take place at a rate of 6 to 10 mL/min. At least 200 mL of the distillate was collected in a 250 mL Erlenmeyer flask containing 50 mL indicator boric acid solution. The amount of ammonia in the sample was then determined by titrating ammonia in the distillate with standard 0.02 N H_2SO_4 titrant until indicator turns to a pale lavender.

3.3.1.5 Total Kjeldahl Nitrogen (TKN)

TKN measurement in the raw wastewater sample was conducted according to (APHA, 2005). The volume of the sample to be used was decided to be 30 mL placed into a distillation flask and completed to 300 mL with distilled water. The flask was then placed into TKN digestion unit after the addition of a few boiling beads and 50 mL digestion solution to remove acid fumes. Due to boiling, a reduction in the volume of the content of the flask to about 20-25 mL and the appearance of copious white fumes were observed. Since then, the digestion continued for 30 minutes during which the turbid sample turned to be transparent and pale green. After cooling, the flask's content was diluted to 300 mL with distilled water and sodium hydroxide-thiosulfate reagent was added. The flask was then placed in the distillation unit and the distillate was collected in a 250 mL Erlenmeyer flask containing 50 mL indicator boric acid solution. The amount of ammonia in the sample was then determined by titrating ammonia in the distillate with standard 0.02 N H_2SO_4 titrant until indicator turns to a pale lavender.

3.3.1.6 Total Phosphorus (TP)

Firstly, the Micro-Kjeldahl flask, to which 5 mL of the raw wastewater sample, few glass silicates beads, 1 mL of concentrated sulfuric acid and 5 ml of nitric acid were added, was placed in the digestion unit. Heating was stopped once a reduction in sample volume to 1 mL was observed. After cooling, 20 mL distilled water, 1-2 drops phenolphthalein, 1 N sodium hydroxide were added until the color turned pink. The content of the flask was passed through a coarse filter and transferred to 100 mL volumetric flask to which 10 mL distilled water, 4 ml ammonium molybdate, and 0.5 mL tin chloride were added. After 10 min but before 12 minutes, the absorbance at 690 nm wavelength in 1 cm path-length cuvette was measured and used to calculate phosphate concentration.

3.3.2 Instrumental measurements

3.3.2.1 Microscopical visualization

Samples micrograph was demonstrated using an optical microscope, Olympus BX50 equipped with a Olympus U-ND6-2 video camera (Olympus, Japan). Gram staining was utilized to differentiate between filamentous and non-filamentous microorganisms. Gram stain are fundamental techniques in the examination of activated sludge (Jenkins et al., 2004).

3.3.2.2 Ion Chromatography (IC)

Measurement of NO_3^-, NO_2^- and PO_4^{3-} concentration was implemented utilizing ion chromatography (Dionex Corporation, Sunnyvale, CA, USA) with electrical conductivity detection system and an analytical column (AS14A). The samples obtained from reactor's upper phase were passed thorough 0.22-μm PVDF filters (Millipore) and kept at 4°C until the quantification.

3.3.2.3 Micropollutants quantification

3.3.2.3.1 Solid samples

Determination of the selected micropollutants concentration in the weekly obtained sludge samples, requires the extraction of micropollutants from solid to the liquid phase as a preparation step for measurement by LC-MS/MS. The optimization of applied to the sludge samples was based on the method described by Topuz et al. (2014). For each sludge sample taken from the reactor, the first step was lyophilization under high pressure using freeze-dryer. The dry sludge was then weighed and taken in the targeted amount. Isotope labeled standards (Diclofenac-d_4, ibuprofen-d_3, naproxen-d_3) were injected to the sample at 100 μg/L before starting the extraction process. Then, the dried sludge was extracted from the sludge phase to the solvent phase by ultrasonic sound waves in the ultrasonic extraction bath with the selected solvents. Based on the study of Topuz et al. (2014), 10 mL of methanol: acetone (1:1) mixture was added to the sludge sample and kept in the ultrasonic bath for 15 minutes. The sample was then taken into 50 mL teflon centrifuge tubes and the solvent phase was separated from the solid phase for 15 minutes at a speed of 9000 rpm using centrifugation. The use of teflon tubes in the centrifugation step was to prevent possible adherence of micropollutants to the wall of the centrifuge vessel. The

separated supernatant was filtered through 0.22 mm PVDF (Chromafil, Germany) syringe filters and placed into a beaker. The sludge phase remaining in the centrifuge tube was again subjected same steps starting from the ultrasonic bath and the solid phase was separated from the solvent phase in the same manner. The upper phase resulted from the second round was then combined with the upper phase obtained in the previous one. Using a rotary evaporator (Heidolp, Laborota 4000, Germany), the solvent was then evaporated under vacuum at 60°C. Then, the micropollutants remained in the flask was dissolved by the addition of 18 mL of ultrapure water and 2 mL of Methanol: Acetone mixture and the extraction from the sludge phase. After this stage, Solid Phase Extraction (SPE) treatment for liquid samples was applied and made ready for measurement by LC-MS/MS. The steps of the optimized method for measuring a solid phase contaminant with the LC-MS/MS instrument are shown in Figure 3.3.

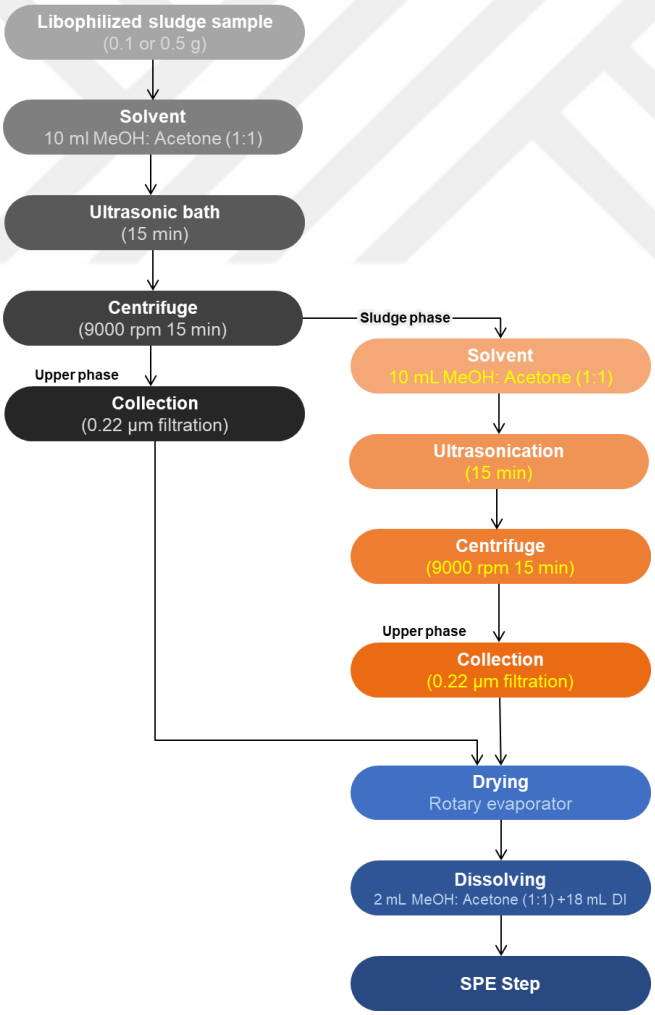


Figure 3.3 : General steps of MP extraction from solid to liquid phase

3.3.2.3.2 Liquid samples

Following up reactor's performance, in terms of micropollutant biodegradation, was accomplished through the measurement of micropollutants concentration in 200 mL samples obtained weekly from reactor's upper-phase. Samples were filtered through 0.2 μm membrane filters and stored in amber glass vials at +4 ° C until measurement. The quantification can be separated into two stages: sample purification and concentration by solid phase extraction (SPE) and micropollutants measurement by LC-MS/MS. All solvents and reagents used for SPE cartridge conditioning, elution, standard solution preparation, and LC-MS/MS measurements were HPLC grade and purchased from Sigma-Aldrich except LC-MS-grade water from Merck.

3.3.2.3.3 Solid Phase Extraction (SPE)

Solid phase extraction was applied as a pretreatment step to concentrate the samples and bring them to the concentration limits that can be measured with LC-MS/MS instrument and to clean the sample matrix from impurities. SPE was accomplished by the aid of a Neuberger (Mini Laboport Diaphragm Pump) vacuum pump and a vacuum system with 20 cartridge inserts (VocMaster, Biotage, Sweeden) to which Oa sis HLB branded cartridges (6 cc, 200 mg; Waters, Millford) were attached. The steps of SPE are depicted in Figure 3.4. Before SPE, isotope labeled diclofenac-d_4, ibuprofen-d_3, naproxen-d_3 were injected to the samples at 100 μg / L. In the first step of SPE, the cartridge was conditioned by the addition of 2 successive times 5 mL from each methanol and ultra-pure water, respectively. The sample was then passed through the conditioned cartridge by means of vacuum system at a flow rate of 5-6 mL/min. The cartridge was finally washed with 5 mL of ultra-pure water and dried under high vacuum for 1 hour. The collected micropollutants were then eluted from the cartridges by 3 successive additions of 2 mL methanol at a flow rate of about 2 mL/min. To remove methanol from the collected micropollutants, the samples were evaporated to dryness at 35°C, under 1.1 bar nitrogen stream using a TurboVap evaporator (TurboVap II, Caliper, Life Sciences, Hopkinton, MA, USA). By the addition of 1 mL of Methanol:DI (10:90) mixture the eluted micropollutants were collected, filtered through a 0.2 μm filter and transferred into 2 mL amber vial to be measured by LC-MS/MS.

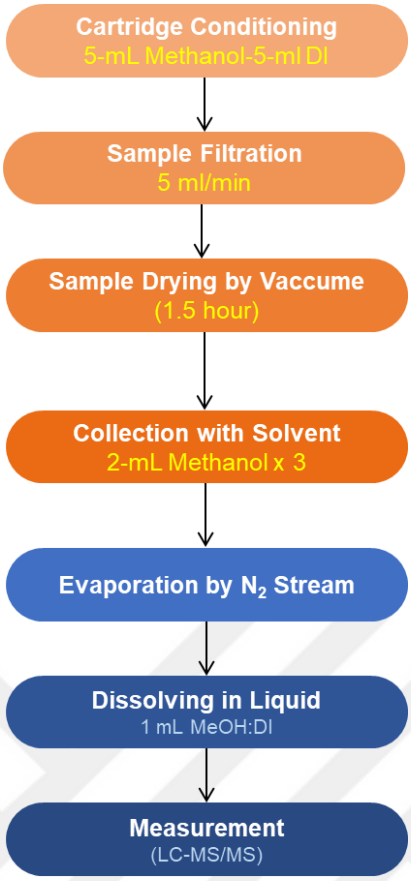


Figure 3.4 : General steps of solid phase extraction (SPE)

3.3.2.3.4 LC-MS/MS

An advanced analytical method; namely LC-MS/MS, that provide a precise measurement by avoiding the problems posed by the complexity of wastewater matrix, the polar structure of the micropollutants and their generally low molecular weight, has been selected to quantify micropollutants in this research. Micropollutants were measured by using Thermo Electron Cooperation TSQ Quantum Access triple quadruple mass spectrometer coupled with Accela Ultra Performance Liquid Chromatograph (UPLC) with a C18 column (Thermo, Hypersil Gold, aQ, 1.8 µm pore size) and an electrospray ionization interface.

3.4 Respirometric Analyses

Respirometric analyses were performed with samples taken from both micropollutant free control reactor and micropollutant containing reactor to determine both the acute and chronic effects of micropollutants on the biomass, respectively. In both cases, three trials at three different micropollutant final concentration (0, 1, 10 and 100 µg/L) were examined. As micropollutants stock solutions were dissolved in methanol, a

micropollutant-free trial, for both cases, was carried out by the addition of methanol equivalent to 60 mg/L COD to serve as a control. To determine the chronic effect on carbon removal and nitrification separately, additional three respirometric experiments, with micropollutant final concentration of 0, 10 and 100 µg/L, were carried out with the addition of nitrification inhibitor (Formula 2533TM-HACH) to prevent any possible interference induced by nitrification process. The starting conditions of the executed respirometric experiments are summarized in Table 3.4.

Table 3.4: Initial conditions of respirometric experiments.

	ExpID	Micropollutant Concentration (µg/L)	Feeding sCOD (mg/L)	IBU, NPX (µg/L)	DCF, KEP, MFA, IDM (µg/L)	Nitrification Inhibitor
Acute Effect	AE-0	0	200	-	-	-
	AE-1	1		1	0.1	-
	AE-10	10		10	1	-
	AE-100	100		100	10	-
Chronic effect	CE-0	0	200	-	-	-
	CE-1	1		1	0.1	-
	CE-10	10		10	1	-
	CE-100	100		100	10	-
	CE-I-0	0	200	-	-	+
	CE-I-10	10		10	1	+
	CE-I-100	100		100	10	+

Oxygen Uptake Rate (OUR) measurements were performed using Applitek RA-Combo-1000 continuous respirometer (Figure 3.5). The respirometric analysis system consists of a reactor vessel supplied with diffusers to provide a constant air flow and sampled continuously for OUR measurement. The samples are transferred to a closed vessel where the depletion of dissolved oxygen (DO) is monitored within a period of 30 seconds by the aid of oxygen probe. The connected computer is supplied with a data processor that reads the value of DO measurements, calculates the OUR values for each interval and comes up with a respirometric profile. In all experiments, the initial content of the aerated reactor vessel was composed of a calculated amount of biomass, nutrients (Sol A and Sol B), carbon source, micropollutants, nitrification inhibitor when needed, and completed to a total volume of 1600 mL with aerated tap water. However, in order to determine the internal respiration rate of the biomass, neither carbon source nor micropollutants were added until a steady endogenous

respiration level (b_H) has been reached. Experiments were terminated when the OUR profile decreased to b_H level. Samples were taken at certain intervals to monitor sCOD, nitrite, nitrate, and pH throughout the experiment.

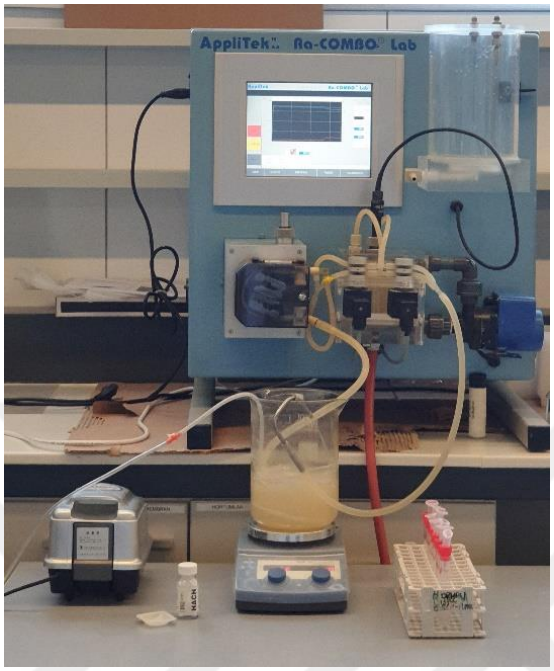


Figure 3.5 : Respirometric experiment set-up with respirometer

3.5 Modelling

Modelling of the OUR profiles obtained from the respirometric experiments was performed by AQUASIM simulation program (Reichert, 1998). A modified version of Activated Sludge Model No.1 (Henze et al., 2000) was used for kinetic interpretation of experimental batch OUR data. In accordance with the selected components, the model defined the kinetics of three microbial processes: growth on substrate; hydrolysis; and endogenous respiration of biomass. It is used to estimate the concentration of the readily biodegradable, rapidly hydrolysable, and slowly hydrolysable organic compounds (S_S, S_{H1} and S_{H2}), maximum specific growth rate ($\breve{\mu}_H$) half-saturation coefficient (K_S), maximum autotrophic growth rate ($\breve{\mu}_{NH}$ and $\breve{\mu}_{NO}$), rapid and slow hydrolysis rate coefficients (k_{H1} and k_{H2}) as explained by Insel et al. (2006). The process kinetics were evaluated as previously described (Insel et al. 2003).

3.6 Microbial Culture Analysis

3.6.1 Sampling and DNA extraction

Three samples were examined for their microbial community structure. The first one was sampled from the seed sludge at the same day of setting up the control reactor, while the second and the third samples were obtained at the end of both control and micropollutant reactors operation, respectively. Samples were taken directly from the completely mixed liquor during reactor operation, placed in 2 mL sterile tubes, and centrifuged at 12000 rpm (Beckman Coulter, microfuge 16, USA) for 10 minutes. After discarding the supernatant, the remaining pellets were stored at -80 °C until the time of extraction. Total DNA of the selected samples was extracted using NucleoSpin®Soil DNA extraction Kit (Macherey Nagel, Germany) following the manufacturer's protocol. The detailed steps are illustrated in Figure 3.6. The amount of extracted DNA and the corresponding quality were measured using a NanoDrop DNA/RNA-Concentration Measurement Spectrometer (ND-1000).

In the scope of optimizing DNA extraction method, it has been proved that the addition of SL2 and Enhancer SX provide the best DNA extraction performance. Besides, the obtained NanoDrop DNA concentration measurement indicates the need to add RNAzA (Macherey Nagel, Germany) to the DNA extraction procedure in order to minimize RNA contamination. RNAzA was added according to the manufacturer's instructions.

3.6.2 16S rRNA gene amplification

In order to determine the amplifiability of the extracted DNA, polymerase chain reaction (PCR) experiment, using the obtained DNA as a template, was performed. Amplification of the V4 region of the 16S rRNA gene was performed using primers bac515F (5′-GTGCCAAGCMGCCGCGGTAA-3′) and bac806R (5′-GTGCCAAGCMGCCGCGGTAA-3′) with TM PCR kit EP0402 (Thermo Scientific). PCR conditions included an initial denaturation stage of 3 min at 94 °C, followed by 28 cycles of 94 °C for 30 s, 40 °C for 53 s, 72 °C for 1 min, with a final elongation step at 72 °C for 5 min. All PCR reactions was performed in a total volume of 25 µL. PCR reactions contain 1 µL of each primer (Thermo Scientific 5U/µl), 2 µL 10 mM dNTP mixture (2.5 mM each) (Intron Biotechnology), 0.5 µL Taq DNA polymerase, 2.5 µL 10X taq buffer with KCl, 2.5 µL $MgCl_2$ (Thermo Scientific TM), and 1 µL

DNA template. The amplified DNA was then visualized by %1 agarose gel electrophoresis. For each run, 50 mL gel was prepared by dissolving 0.5 g agarose (Biomax Basic Agarose) in 1x TAE buffer to which 5 µL of SYBR® Safe DNA Stain was added.

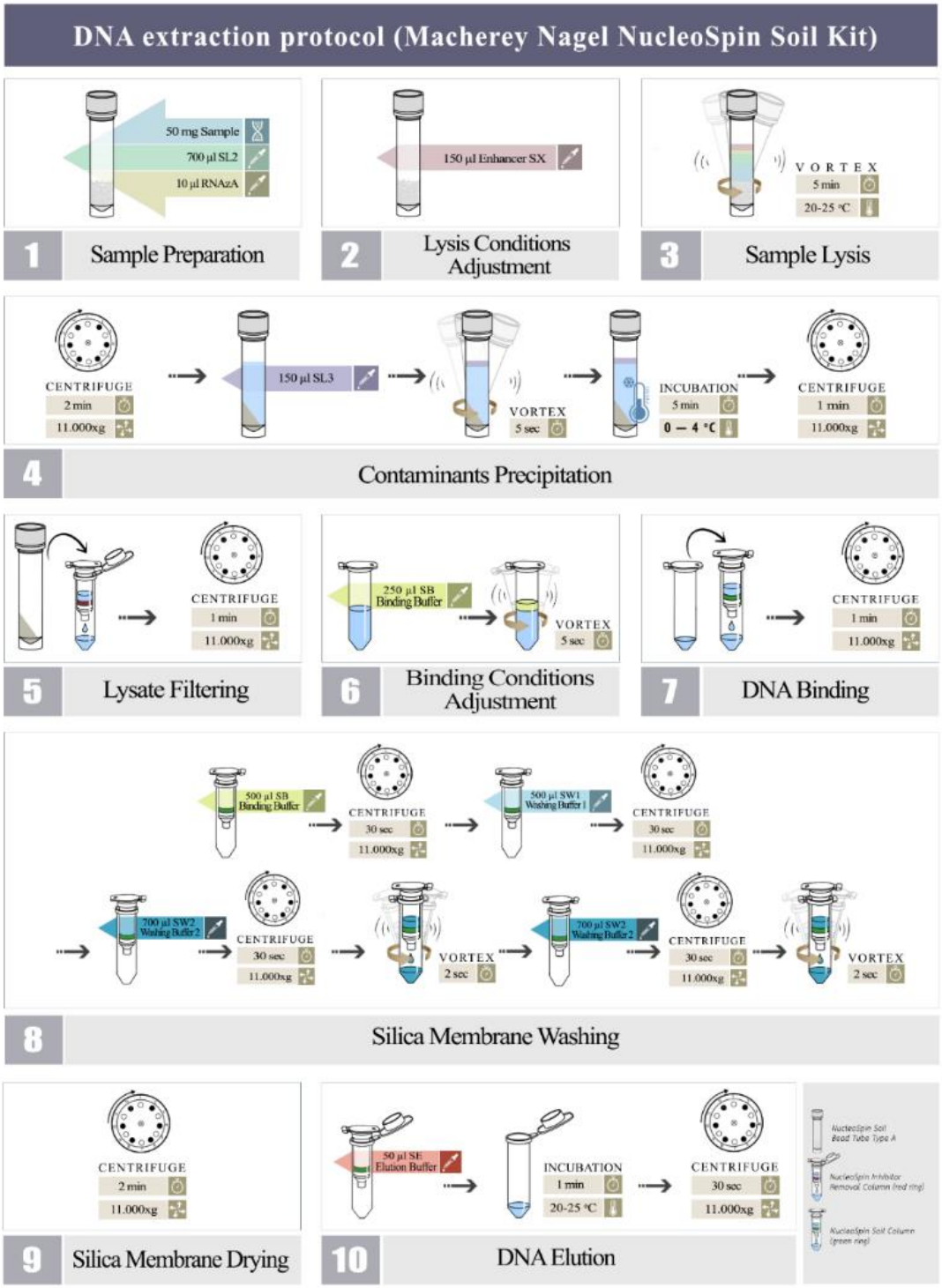


Figure 3.6 : DNA extraction protocol (NucleoSpin®Soil DNA extraction Kit)

3.6.3 Illumina sequencing

The PCR products were sent to BM Laboratuvar Sistemleri company (Ankara) to perform DNA sequencing and metagenomic analysis. They first performed a post-PCR purification using AMPure XP (Beckman Coulter) magnetic beads to get rid of any primer dimers and free primers from the 16S V3 and V4 amplicon. Then Nextera XT Index Kit was used to perform index PCR to attach dual indices and Illumina sequencing adapters. A second purification, using AMPure XP beads, was then applied to clean up the final library before quantification. DNA libraries are quantified using the Real Time PCR technique and then the normalization step takes place using magnetic beads. The normalized samples were then combined following pooling method. The prepared libraries were then loaded to MiSeq in which sequencing by synthesis method was adopted. A fluorescent pulse was observed and recorded for each added dNTP. The generated data after sequencing was converted to raw data for metagenomic analysis using bcl2fastq (v1.8.4).

3.6.4 Metagenomic analyses

The workflow of metagenomic analyses, performed by the same firm, starting from raw data is shown in Figure 3.7. Scythe (v0.994 BETA) and Sickle programs were used for trimming nonspecific adapter from the reading results. Readings shorter than 36 bases were removed for a clean data analyses. Bioinformatic analyse was performed to align the resulting reads with reference sequencings, classify them into several taxonomic levels: kingdom, phylum, class, order, family, and genus or species and determine the operational taxonomic units. The National Center for Biotechnology Information (NCBI) database was used to access biomedical and genomic information. Operational taxonomic units (OTUs) were defined based on 95% confidence level sequence similarity. Alpha diversity indices, including chao1 estimator of richness, observed OTUs, Shannon index, rarefaction analysis and Principal coordinate analysis (PCoA) were performed by QIIME. A 3D PCoA plot was generated using EMPEROR (Vazquez-Baeza et al. 2013). Heatmap was visualized in R (version 3.6.1) using Phylosec Packages. Cladogram was constructed by PhyloT (version 2019.1) based on the NCBI taxonomy and visualized by iTOL (version 5.4). The functions of 16S rRNA genes were annotated using Functional Annotation of Prokaryotic Taxa (FAPROTAX) (Louca et al., 2016). FAPROTAX includes software for converting taxonomic microbial community profiles (in the form of an OTU table) into putative functional

profiles, based on taxa identified in a sample. The taxa detected in this study, but their function characterization was not classified in FAPROTAX database were inserted into the database by us using the current literature on cultured strains.

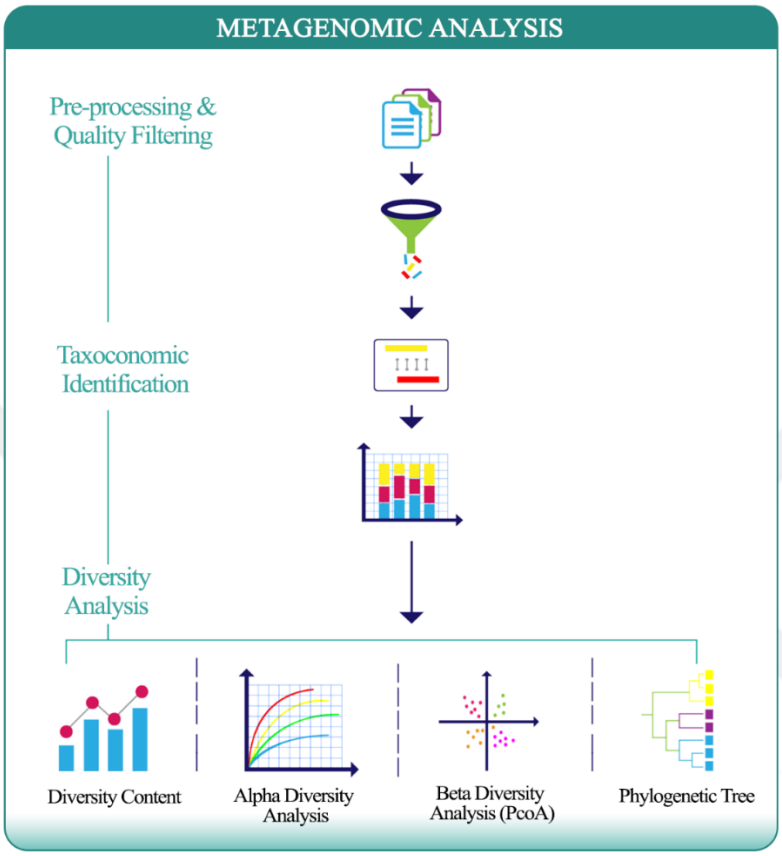


Figure 3.7 : Workflow of metagenomic analyses

4. RESULTS AND DISCUSSION

4.1 Evaluation of Reactors Performance

The reactor performance for control and micropollutant fed cultures was investigated via profiling their TSS/VSS (in both mixed liquor and upper phase), pH, effluent sCOD, and nitrogen species (NH_4^+, NO_3^-, NO_2^-) over the period of their operation (66 and 448 days, respectively).

4.1.1 Control reactor performance

The TSS and VSS profiles of the control reactor are illustrated in Figure 4.1. The average concentrations of TSS and VSS during the acclimation period were found to be 2830±165 mg TSS/L and 1840±30 mg VSS/L, respectively. After the acclimation was completed, identified by a period of 3 sludge ages (15 days), the average concentrations were measured as 2230±65 mg TSS/L and 1660±40 mg VSS/L, respectively. The VSS/TSS ratio was calculated as 0.74.

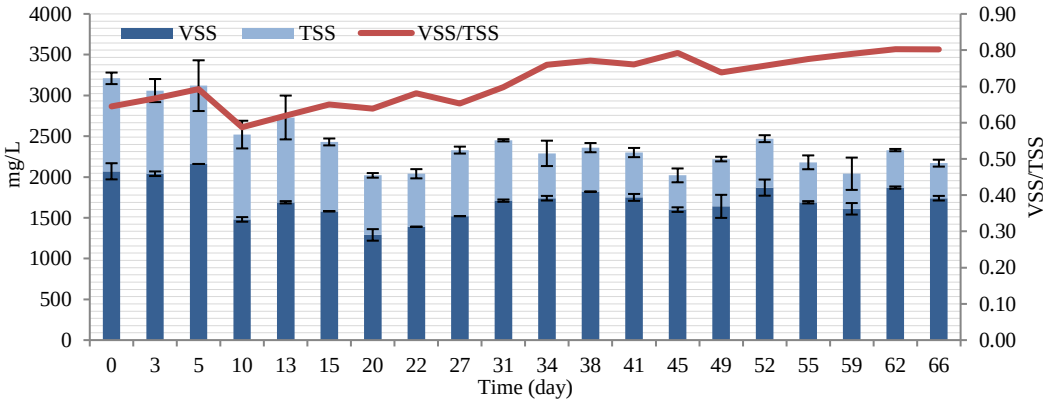


Figure 4.1 : TSS and VSS profile of control reactor

The change overtime in pH value was regularly monitored three times a week (Figure 4.2). The need to control pH was started shortly after acclimation period (20 days since installation), when the pH values were detected over the desired range. Such increase was expected as operating the reactors at short SRT (5 days) would hinder the nitrification from being effectively achieved. Short SRT prevents the slow growers autotrophic nitrifiers from maintaining long enough to oxidize the ammonium content

of the reactor leading to its accumulation. Controlling the pH was accomplished via the addition of 1 N sulfuric acid solution into the feeding mineral medium.

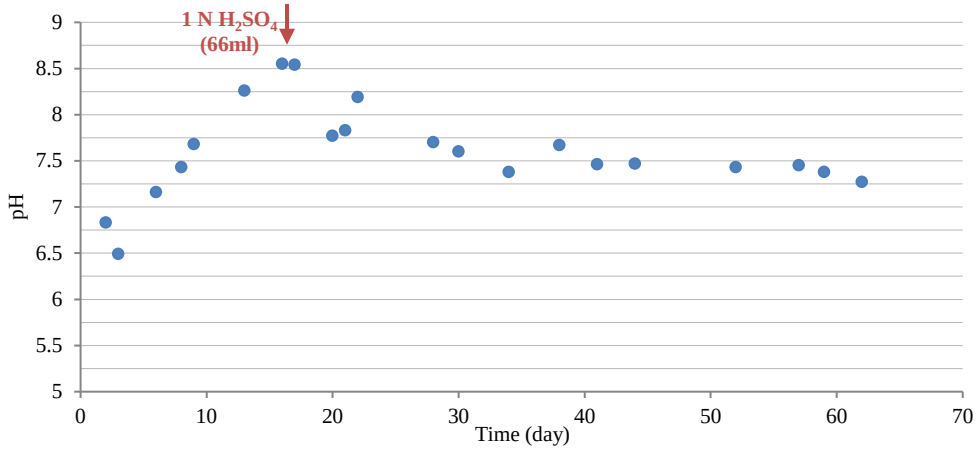


Figure 4.2 : pH profile of control reactor

The concentrations of sCOD in the reactor effluent, weekly measured in the upper phase layer after sludge settling, were depicted in Figure 4.3. The average concentration of sCOD in the control reactor output was found to be 40 mg/L. The effluent sCOD observed in the reactor is due to the accumulation of microbial inert products.

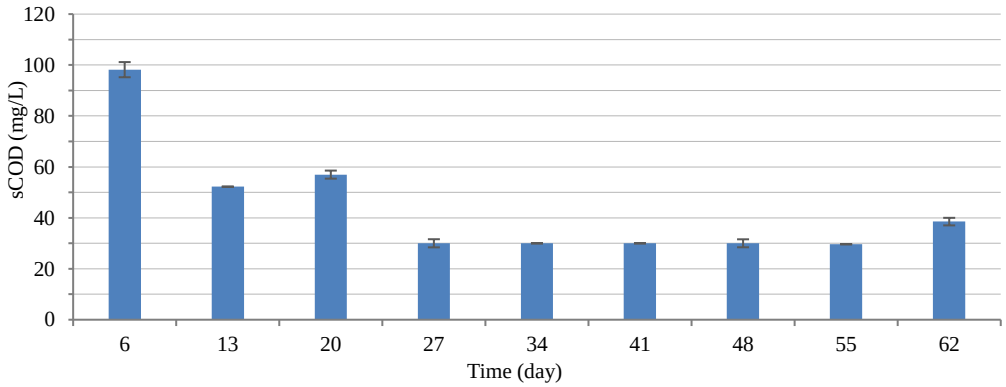


Figure 4.3 : sCOD profile of control reactor

The changes in the concentration of the three nitrogen species during the operation of control reactor is illustrated in Figure 4.4. After the acclimation period (between the days 20 and 66), the average concentration of ammonium nitrogen in the effluent was found to be 100 mg/L NH_4^+-N. There was no significant change in the nitrate concentrations during the operation of the control reactor (avg. 6 mg/L NO_3^--N), except for the early stages of acclimation in which a high nitrate concentration (approx. 55 mg/L NO_3^--N) was detected. The source of the utilized inoculum explains such

elevation since it was supplied from a wastewater treatment plant where full nitrification takes place. The impact of SRT on nitrification capacity has been extensively researched and the results showed insignificant nitrification capacity with low SRTs (up to 5 days). However, at the operated sludge age (5 days) nitrification process was not supported and nitrifying microorganisms were not maintained in the sludge.

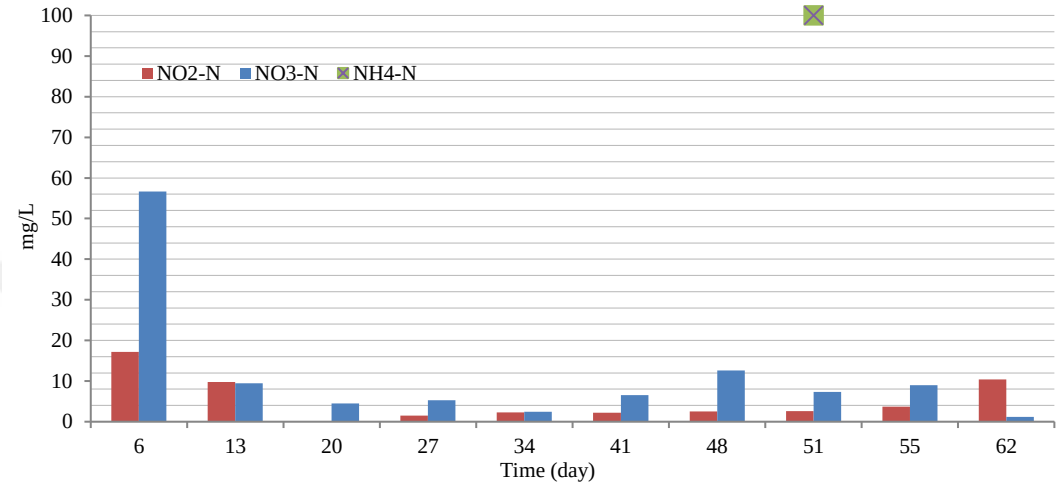


Figure 4.4 : Nitrate (NO_3^-), Nitrite (NO_2^-) and Ammonium (NH_4^+) profile of control reactor

4.1.2 Micropollutant reactor performance

TSS and VSS profiles covering both the acclimation and the steady state phases of the reactor to which micropollutants was added are shown in Figure 4.5. During the acclimation period the average values of TSS and VSS were 1900±40 mg TSS/L and 1305±30 mg VSS/L respectively, while in the steady states they were 1740±30 mg TSS/L and 1390±35 mg VSS/L respectively. The ratio of VSS to the TSS in the reactor were calculated as 0.75 and 0.8 during and after acclimation respectively. Generally, the concentrations of TSS and VSS were observed to be lower in the micropollutants reactor when compared to the control reactor while the VSS/TSS ratio exhibited an opposite trend.

The change in the pH was monitored regularly in samples taken three times a week in the micropollutants reactor as in the case of control reactor (Figure 4.6), however controlling pH with 1 N sulfuric acid solution was started since the initial setup of the reactor. The pH of the micropollutants reactor was controlled at an average value of 6.98.

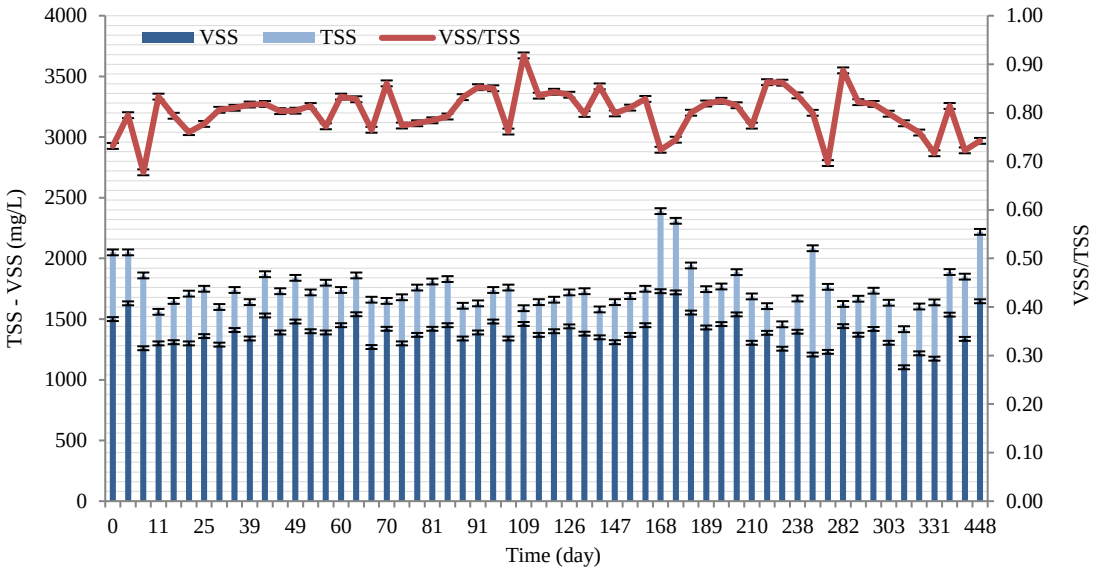


Figure 4.5 : Mixed liquor TSS and VSS profile of micropollutant reactor

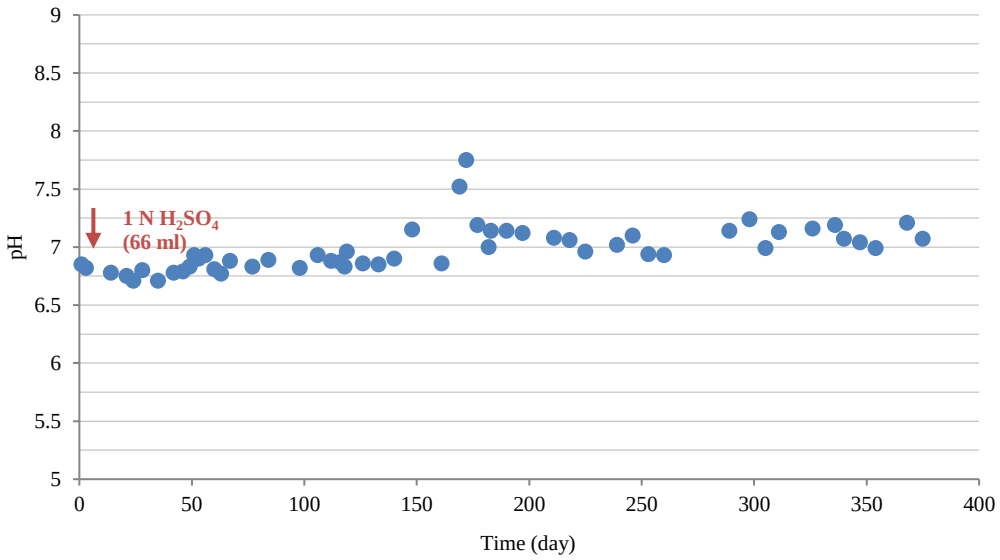


Figure 4.6 : pH profile of micropollutant reactor

The change in the concentrations of sCOD detected in the effluent of micropollutant reactor is given in Figure 4.7. The average sCOD concentration before and after acclimation was 55 mg/L and 35 mg/L, respectively. It is clearly seen that both control and micropollutant reactors exhibited approximately same level of sCOD concentration in their effluents, indicating no chronic inhibitory effect on the biodegradation of organic matter at SRT of 5 days. The detection limit for sCOD based on the applied method is 30 mgCOD/L. Therefore, the reported values as 30 mgCOD/L are the one which are equal to or lower than 30 mgCOD/L.

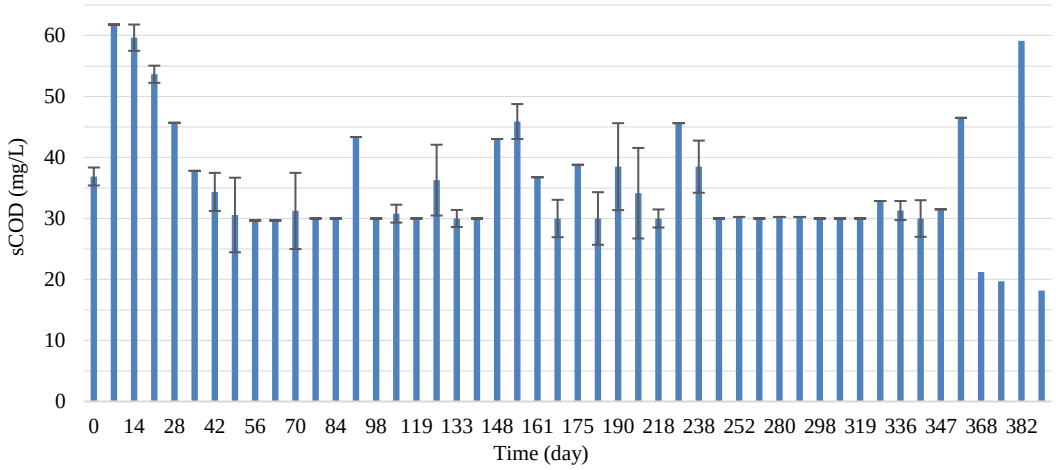


Figure 4.7 : Effluent sCOD profile of micropollutant reactor

The TSS and VSS concentrations measured in samples obtained in a weekly basis from the upper phase formed at the end of the cycle of the micropollutant reactor are illustrated in Figure 4.8. The TSS concentrations detected in the supernatant during the initial phase of the reactor were relatively high with an average value of 150 mg/L compared to 40 mg/L after acclimation period, reflecting a problem in the settling. The average VSS concentration during acclimation period was measured as 30 mg/L.

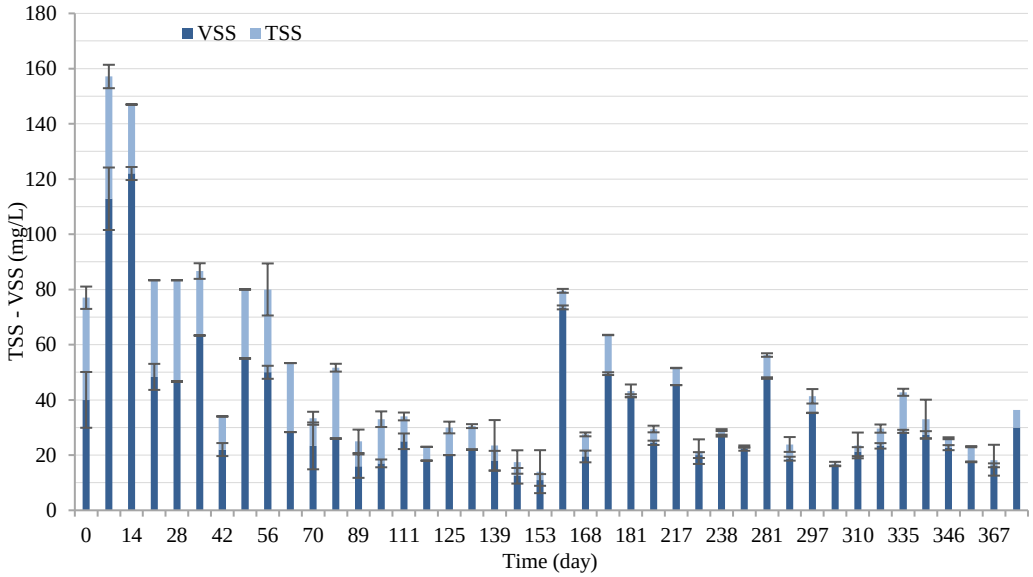


Figure 4.8 : Upper phase TSS and VSS profile of micropollutant reactor

Microscopic analyses were performed to examine the experienced precipitation problem since defining flock structures and density along with filamentous organisms is essential in the evaluation of sedimentation problems in activated sludge systems. It has been assumed that the cause of the problem was the reduction in the dissolved oxygen concentration, based on several previous experiments showing the negative impact posed by low dissolved oxygen concentration on the sludge settleability, leading to the proliferation of filamentous bacteria (Martins et al., 2003; Wilén & Balmér, 1999). The micrograph of the sample obtained during the initial period of the reactor indicates the high density of filamentous microorganisms and low density of flocks (Figure 4.9a) when compared to the micrograph after elimination of the problem (Figure 4.9b).

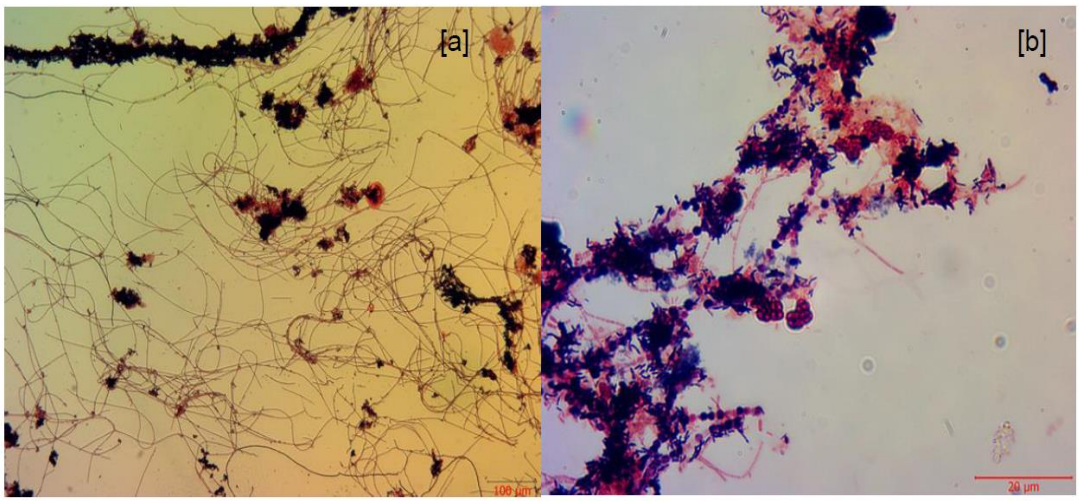


Figure 4.9 : Light microscopic micrographs of micropollutant reactor's activated sludge (a) At the beginning of acclimation (b) At steady state condition

The profiles of ammonia nitrogen, nitrite and nitrate nitrogen in the micropollutant reactor are shown in Figure 4.10. Ammonia and nitrate nitrogen concentrations were measured as an average of 60 mg /L and 31 mg/L, respectively in the outlet stream after the acclimation period. The observed decrease in ammonia nitrogen after the acclimation period is believed to be due to a partial nitrification taking place in the micropollutant reactor operated at SRT of 5 days. The acclimation of activated sludge to the micropollutants, i.e. ibuprofen, naproxen, ketoprofen, indomethacin, mefenamic acid and diclofenac, posed no chronic inhibitory effect on the nitrification process when compared with the results of control reactor. On the contrary, higher nitrification performance was observed in the micropollutant reactor than the control reactor.

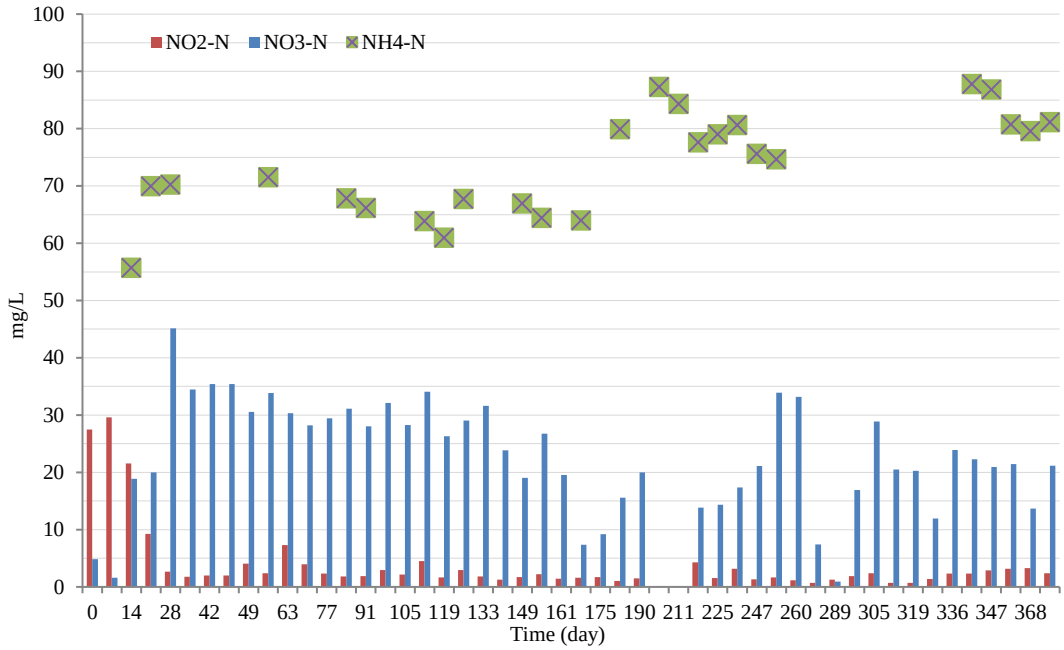


Figure 4.10 : Nitrate (NO_3^-), Nitrite (NO_2^-) and Ammonium (NH_4^+) profile of MP reactor

The operation of micropollutant reactor involved discarding half of the working volume (4L) that was later replaced by a feeding solution composed of the previously described synthetic feed in addition to NSAIDs mixture containing 10 µg/L Ibuprofen, 10 µg/L Naproxen, 1 µg/L Ketoprofen, 1 µg/L Indomethacin, 1 µg/L Mefenamic acid, and 1 µg/L Diclofenac. Therefore, the expected theoretical accumulation in the absence of any degradation process is calculated as 20 µg/L for Ibuprofen and Naproxen, and 2 µg/L for Ketoprofen, Indomethacin, Mefenamic acid and Diclofenac. The expected theoretical concentrations of micropollutants in the MP reactor are shown by the red line in Figure 4.11, Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15, and Figure 4.16. which also represent the concentration of diclofenac, ibuprofen, naproxen, ketoprofen, indomethacin, and mefenamic acid, respectively, in the liquid phase of the reactor. The obtained removal efficiencies reflect the extent by which biodegradation is contributed to the removal mechanisms.

The profile of diclofenac concentration throughout the operation of micropollutants is depicted in Figure 4.11. Its average concentration in the reactor effluent was found as 0.69 µg/L. Based on the theoretical accumulation value, the achieved diclofenac removal in the reactor was around 65%. Several studies in the literature concerned the performance of lab-scale reactor to remove diclofenac at different operating conditions. For example, Jiang et al. (2017) determined the removal efficiency of

diclofenac in SBRs operated at SRT of 20 days in which activated sludge was exposed to 5 µg/L diclofenac for 130 days. In this study removal efficiency of diclofenac was reported as 64.12%. Same study showed lower efficiencies when diclofenac was added together with other NSAIDs such as ibuprofen and/or naproxen. In the experiment conducted by Kruglova et al. (2016), no removal of diclofenac was observed within 24 hours from spiking 20 ± 10 µg/L of diclofenac into SBR containing activated sludge pre-acclimated 10 µg/L of diclofenac and operated at 12 days SRT. Such strong convergence result imply that higher sludge age may not lead to better performance. (Nguyen et al., 2019) found that activated sludge could remove <50% of 50 µg/L diclofenac in a batch-fed reactor operated at SRT of 10.5 days. The removal decreased significantly to below 15% when diclofenac concentrations increased to 500 and 5000 µg/L. The low diclofenac removal can be explained by the presence of chlorine atom in its structure, which contribute to its persistence (Joss et al., 2005) and it is in good agreement with the poor removal characteristics of diclofenac previously reported from WWTPs (Table 2.2). It is obvious from the variation of the reported value that influent concentration and SRT can influence the fate of diclofenac.

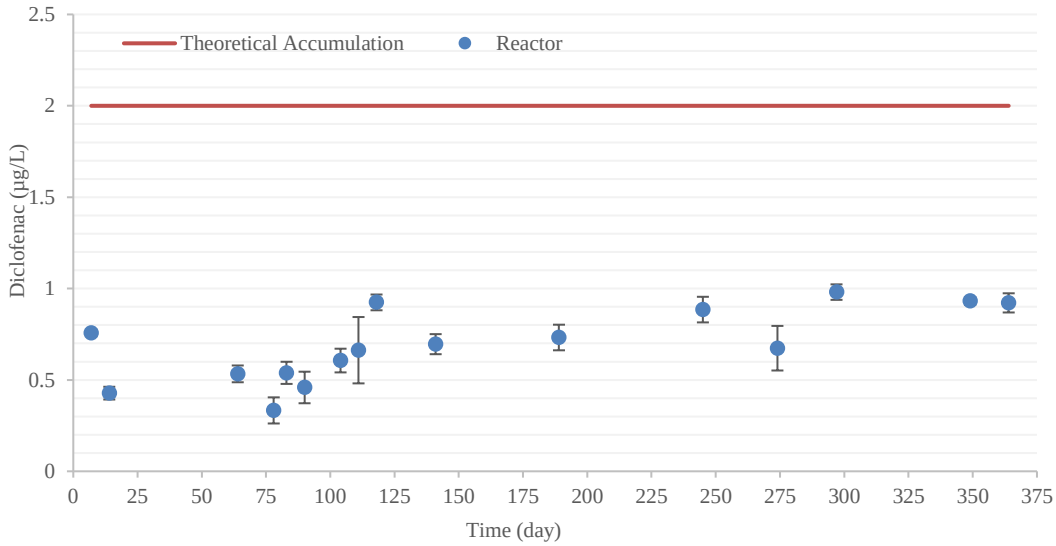


Figure 4.11 : Diclofenac profile of micropollutant reactor

The aerobic treatability of ibuprofen and naproxen was found to be much higher than other micropollutants (Figure 4.12 and Figure 4.13). Ibuprofen and Naproxen were measured in the reactor after acclimation in average values of 0.1 and 0.27 µg / L respectively, and their removal efficiencies according to the theoretical accumulation in the reactor were calculated as 99.5% and 98.6%, respectively. Likewise, high degree of degradation was recorded for both compounds in the literature in both lab-scale and

field-scale bioreactors. The results showed that ibuprofen was efficiently removed in the range of 63–90% in activated sludge SBR operated under a constant aeration rate at 1.0 L/min, 24 h HRT and various loadings of ibuprofen (1.71–5.1 mg/m^3 day). Jiang et al. (2017) has also determined the removal efficiency of ibuprofen in activated sludge when exposed to 5 µg/L ibuprofen and 5 µg/L diclofenac as 88.12±0.70% and with 5 µg/L naproxen as 85.64±1.01%. In the same study 79.96±0.37% of naproxen was removed when the activated sludge was subjected to a mixture of naproxen, ibuprofen and diclofenac (5 µg/L each). Moreover, naproxen showed the highest degree of degradation with 91.3 ± 2.9 % removal in SBRs operated at SRT of 10 days after 30 days from its addition with a concentration of 10 µM. A study performed by Kosjek et al. (2007) showed that steady-rate removal of NSAIDs including both ibuprofen and naproxen over a two-year monitoring period had been achieved. Elimination of 0.05 mg/L and 0.005 mg/L from each ibuprofen and naproxen in the continuous pilot WWTP operated at 0.5-2 days SRT was 90.8±13% - 91.5±6% and 93.6±8% - 86.6±11%, respectively.

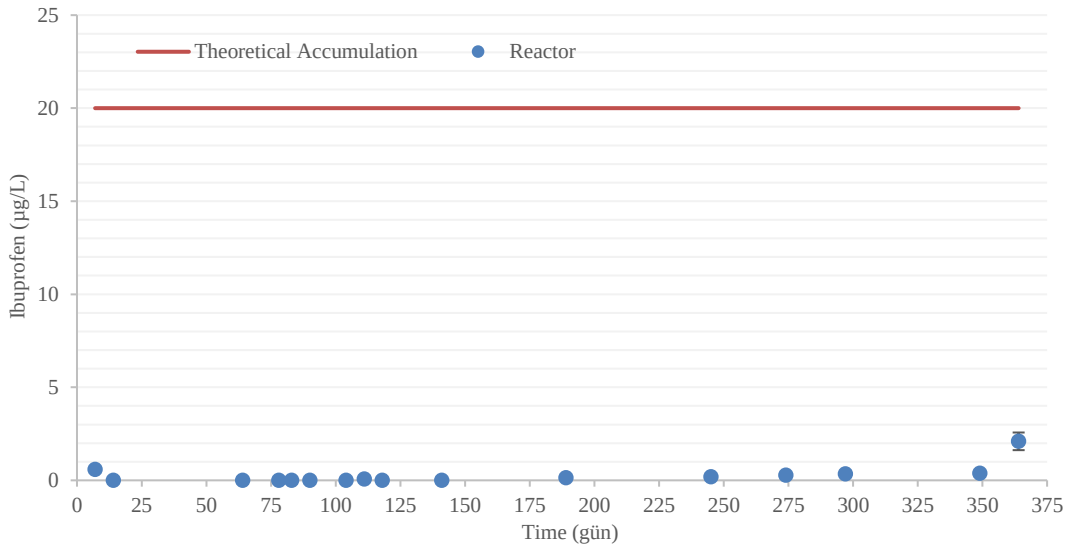


Figure 4.12 : Ibuprofen profile of micropollutant reactor

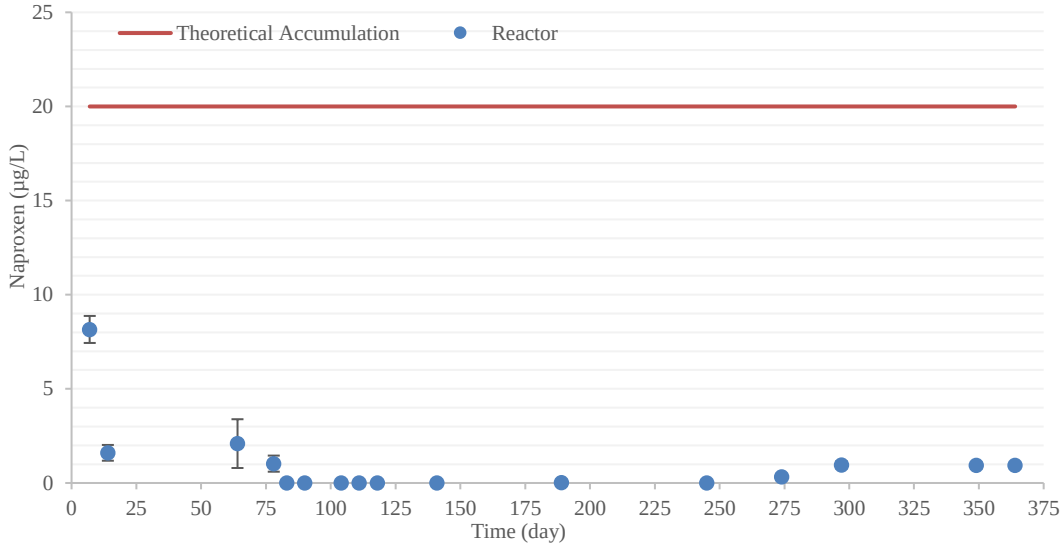


Figure 4.13 : Naproxen profile of micropollutant reactor

The variations in the result of ketoprofen and indomethacin reflected their susceptibility to operating problems encountered during the operation of the micropollutant fed reactor. Due to the experienced aeration problem, changes in their concentrations in the reactor effluent were observed (Figure 4.14 and Figure 4.15). For example, indomethacin was measured as 0.003 µg/L in the reactor in the post-acclimation period, however this was followed by an increase up to 1.02 µg/L coincided with the observed operational problems in the reactor. The average indomethacin and ketoprofen concentrations during 364 days of reactor operation were 0.24 µg/L and 0.53 µg/L, respectively. It is also important to note the quite low removal efficiency of ketoprofen during the first period of the reactor, which was fixed later after 50 days of operation. Beside ibuprofen and naproxen, Kosjek et al. (2007) determined that ketoprofen was eliminated from a pilot WWTP by 91.1±10% and 89.6±7% when ketoprofen was introduced at the concentrations of 0.05 mg/L and 0.005 mg/L, respectively. However, lower removal efficiencies of ketoprofen were observed by Abu Hasan et al. (2016). The results showed 21.9 ± 3.0% and 48.6 ± 5.0% removal in SBR operated at SRT of 10 days after 30 days from the addition of 0.1 and 10 µM ketoprofen, respectively. Removal efficiencies by conventional activated sludge and membrane bioreactor was reported as 23.4±22.3% and 46.6±23.2% for indomethacin and 51.5±22.9% and 91.9±6.55% for ketoprofen, respectively (Radjenovic et al., 2007).

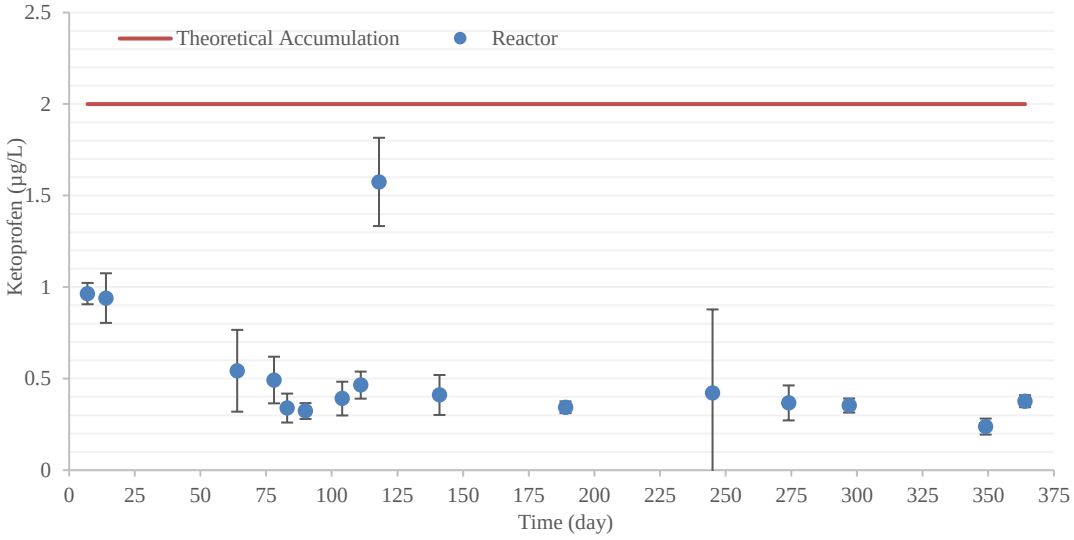


Figure 4.14 : Ketoprofen profile of micropollutant reactor

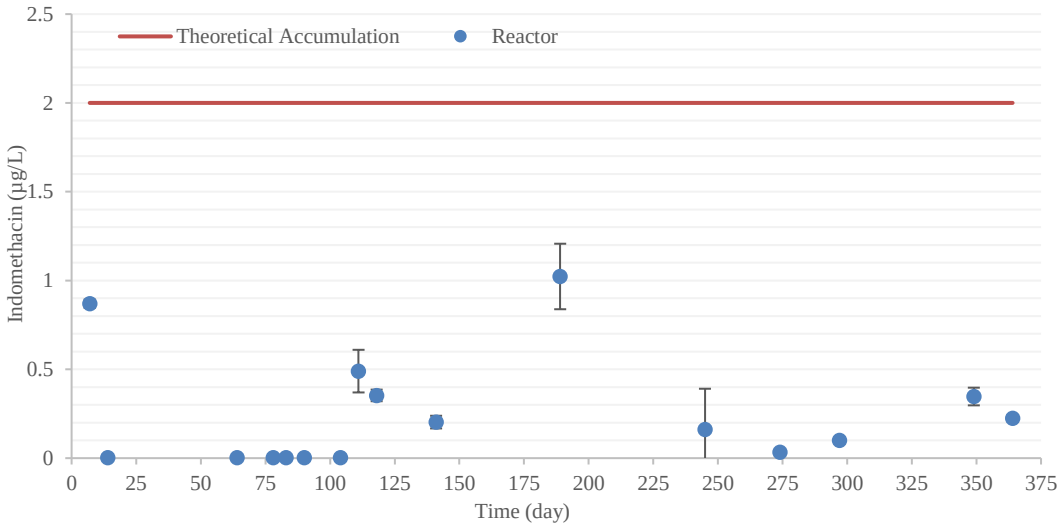


Figure 4.15 : Indomethacin profile of micropollutant reactor

Variations in the concentration of mefenamic acid measured in the laboratory-scale micropollutant reactor throughout its operation was also observed, with a maximum detected concentration of 0.60 µg/L and an average of 0.19 µg/L (Figure 4.16). The concentration of mefenamic acid in the effluent was 0.1 µg/L for the first 125 days which increased afterward up to 0.33 µg/L over the long-term operation. Moderate removal efficiency (30-80%) of mefenamic acid in SBR was reported by He et al. (2018). In full scale conventional activated sludge WWTPs, the achieved removal of mefenamic acid was reported as 29.4±32.3% (Radjenovic et al., 2007).

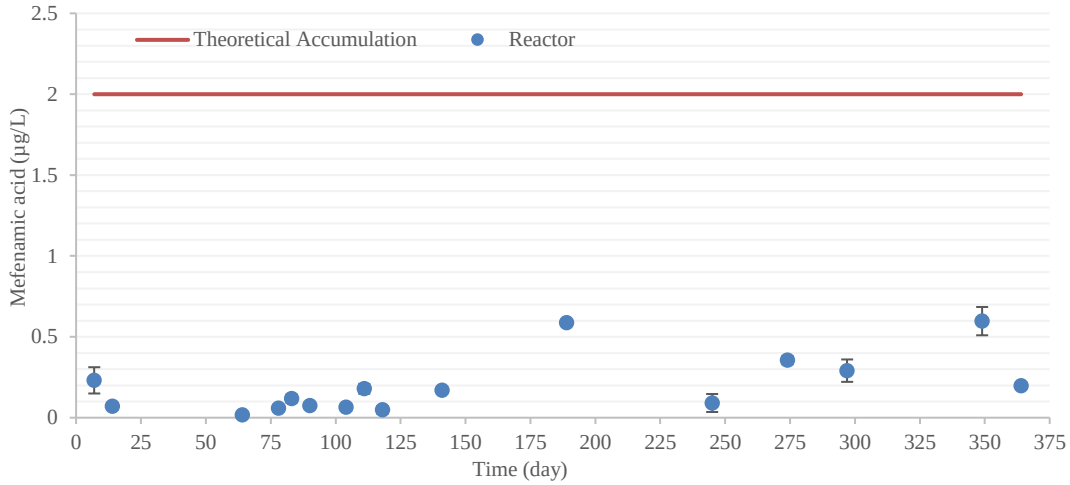


Figure 4.16 : Mefenamic acid profile of micropollutant reactor

The concentration of micropollutants in sludge samples was measured to determine whether sorption played a crucial role in their removal from the reactor or not. The calculation of the sorbed fraction of micropollutant to the sludge was performed considering both the average TSS of MP reactor in the steady state (1740 mgTSS/L) and the measured concentrations of micropollutants in the sludge phase (Table 4.1). The amount of diclofenac, ibuprofen, naproxen, indomethacin and mefenamic acid, that sorbed to the sludge were calculated as 0.86, 0.90, 1.7, 2.1, and 0.40 µg, respectively. It is important to consider the accumulated amount of micropollutants due to the continuous feeding while reading these amounts. For example, the amounts to the daily added amount of micropollutant. For example, 1.97 µg of indomethacin constitutes about 25% of the 8 µg Indomethacin fed to the reactor. The amount of mefenamic acid is only 5% compared to 8 µg mefenamic acid given to the reactor at each feed. However, mefenamic acid exhibits a high ability of binding to solids (Jones et al., 2006).

Table 4.1: Micropollutants measurements in sludge phase.

Micropollutant	Concentration (ng/g)	Sorption Percentage (%)
Diclofenac	62	10.8
Ibuprofen	65	1.1
Naproxen	125	2.2
Indomethacin	148	25.8
Mefenamic Acid	29	5.0

4.2 Evaluation of In-cycle Performance

In order to determine the reactor performances in both control and micropollutant reactors, in-cycle monitoring was performed. The change of COD, pH, NO_3^--N and NO_2^--N parameters in the reactors in 12-hour cycle (between 11:00 and 23:00 o'clock) was measured in samples taken directly from the reactors.

4.2.1 In-cycle performance of control reactor

The sCOD profile observed in the control reactor is shown in Figure 4.17. The concentration of sCOD in the sample taken from the reactor just before the addition of feeding solution into the was measured as 70 ± 20 mg/L. It is clearly seen in Figure 4.17 that 150 minutes was required for the system to recover the same concentration after feeding addition. In other word, the 400 mg/L COD added to the system within the feeding solution required approximately 150 minutes to be eliminated.

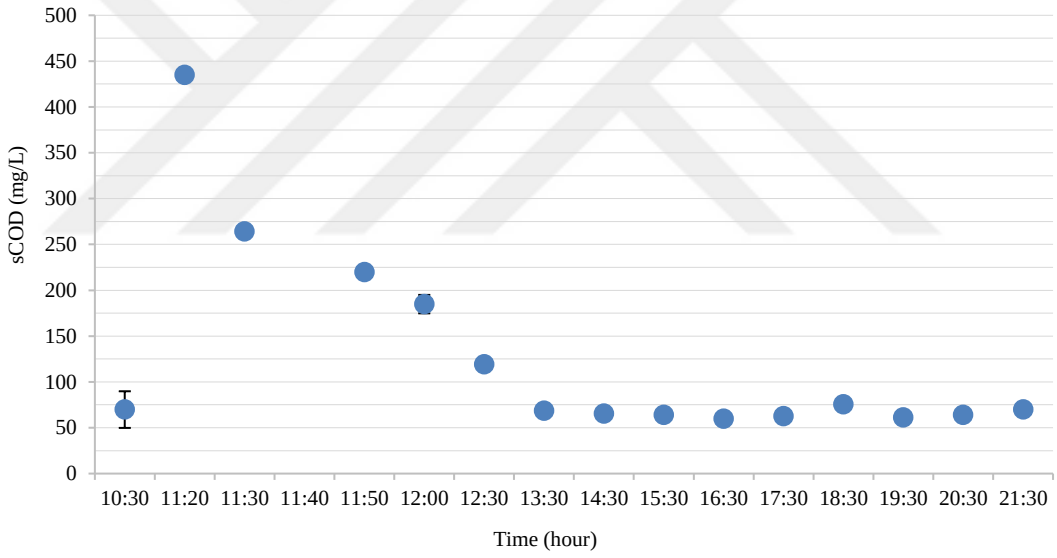


Figure 4.17 : In cycle change of sCOD in control reactor

The COD proportion corresponding to the soluble microbial products formed in the reactor was calculated as 8%. The calculation of this percentage assumes that the measured 70 mg/L reflects the sCOD in a volume of 4L. Therefore, the sCOD, measured at the end of the previous cycle after treatment completion, is determined to be 35 mg/L, considering that the total volume reached 8L after feeding in the new cycle. The concentration of sCOD in the system was calculated as 435 mg/L since feeding solution that has been added at the beginning of the cycle contains 400 mg/L. Given that the residual COD at the end of the cycle (35 mg/L) is proportional to the

initial COD at its starting point (400 mg/L), the accumulation of the soluble microbial products in the system can be calculated.

The examination of the reactor in terms of nitrogen parameters revealed that only partial nitrification was accomplished, and that nitrification process was not fully developed. While nitrate-nitrogen did not demonstrate a significant change in the system, the partial nitrification has led to 11.7 mgNO_2^--N /L increase in nitrite nitrogen (Figure 4.18). Moreover, there was no recorded change in the on-line pH values related with nitrogen parameters.

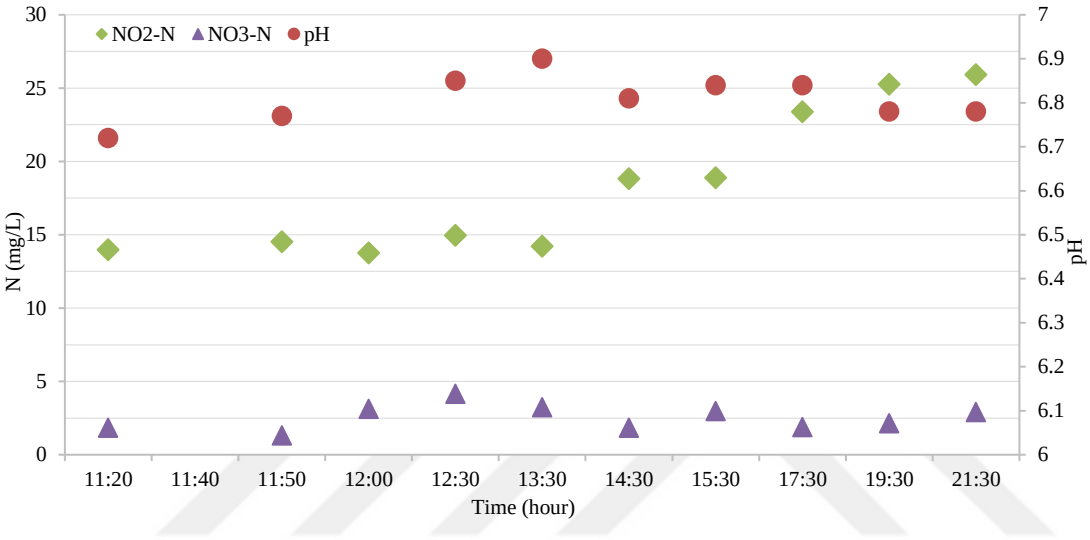


Figure 4.18 : In cycle change of pH, nitrate and nitrite nitrogen in control reactor

4.2.2 In-cycle performance of micropollutant reactor

The change of sCOD observed in micro-pollutant reactors over one cycle period (12-hour) is given in Figure 4.19. Before the feeding of the micropollutant reactor, the sCOD concentration was detected as 40 ± 2 mg/L. Within 150 minutes after feeding, the sCOD was measured as 55 ± 2 mg/L. Comparing to the control reactor, the result indicates no significant effect on organic matter biodegradation potential to be posed by micropollutants.

The soluble microbial product accumulated in the micropollutant reactor was calculated as 5% which is lower than the rate of accumulation in control reactor.

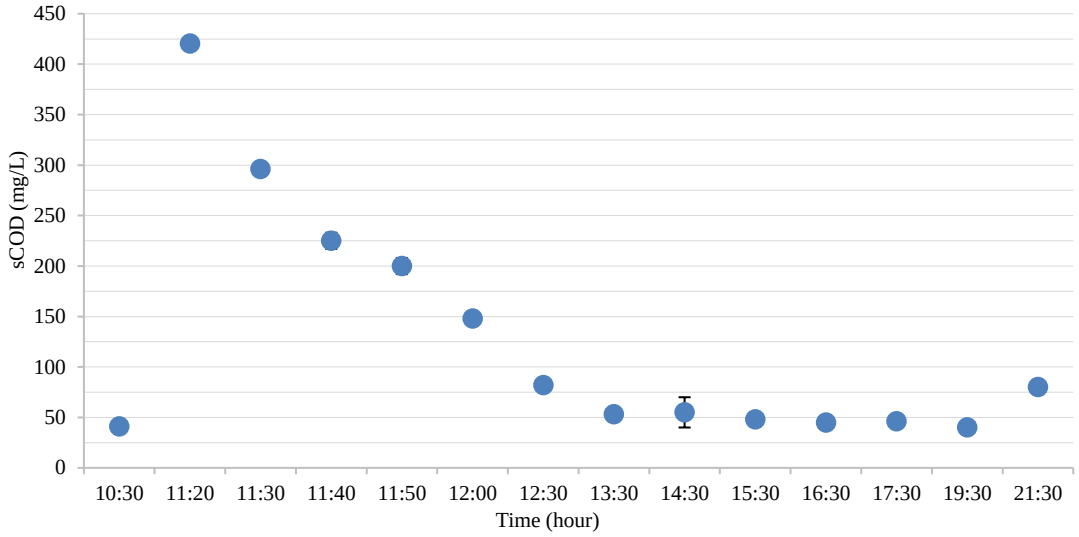


Figure 4.19 : In cycle change of sCOD in micropollutant reactor

Similarly, the on-line changes in nitrogen parameter indicated that nitrification was not achieved completely in the micropollutant reactor, yet there was a significant difference compared to the control reactor (Figure 4.20). In the micropollutant reactor, nitrite nitrogen was measured as 1.56 NO_2^--N/L at the end of the cycle; and nitrate nitrogen reached 19.1 NO_3^--N/L.

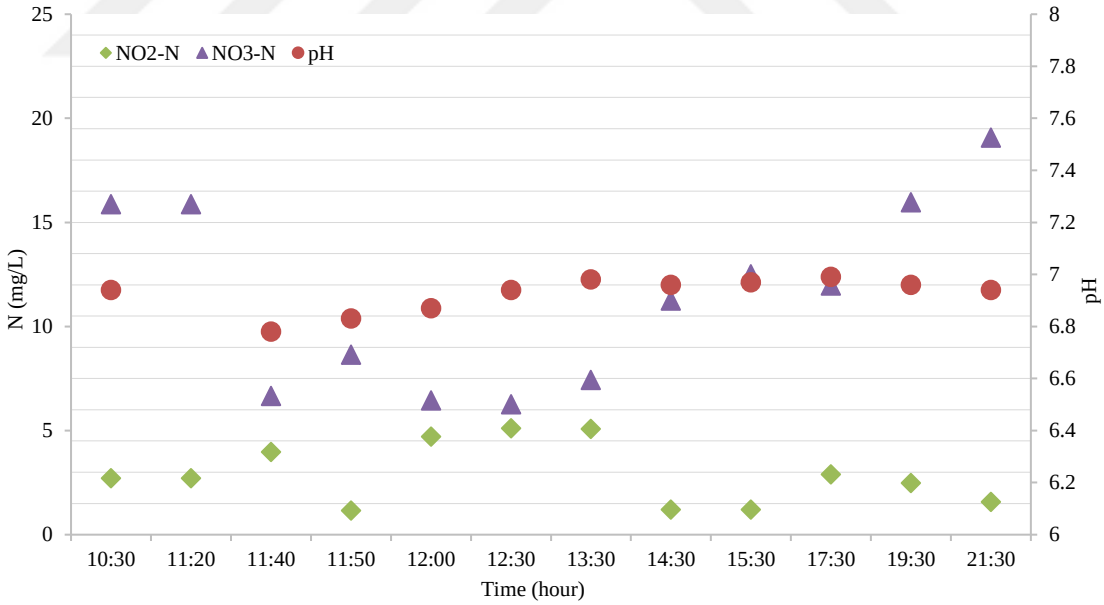


Figure 4.20 : In cycle change of pH, nitrate and nitrite nitrogen in MP reactor

4.3 Acute and Chronic Toxicity of Micropollutants on Activated Sludge

4.3.1 Acute effect evaluation

Activated sludge samples taken from the control reactor after acclimation, which operated without the addition of MPs, were subjected to respirometric analyses to evaluate their response (acute effect) when exposed to MPs for the first time. A comparison between the effect posed by different concentrations of MPs is an important approach to understand the resulting changes on the level of sludge activity (Figure 4.21).

The major findings of the executed respirometric experiments are summarized in Table 4.2. The first 4 experiments (i.e., AE-0, AE-1, AE-10, and AE-100) will be covered in this section and their related OUR and sCOD profiles are represented in Figure 4.22.

Table 4.2: Summary of MPs acute and chronic effects analysis results.

ExpID	Initial TSS (mg/L)	Initial VSS (mg/L)	F/M	b_H levels (mg/L/h)	max. OUR (mg/L/h)	Initial COD (mg/L)	Final COD (mg/L)	Duration (min)
AE-0	1080	850	0.24	11	126	284	69	460
AE-1	1050	820	0.24	10.4	129	225	56	480
AE-10	1080	860	0.24	9	120.5	219	82	420
AE-100	1070	790	0.24	13.6	126.8	227	89	433
CE-0	685	540	0.37	6	98	346	30	370
CE-1	670	535	0.37	6.5	108	340	<30	370
CE-10	640	525	0.38	6.2	81	338	<30	330
CE-100	640	335	0.6	4.8	87	320	30	330
CE-I-0	700	565	0.35	7.4	110	326	<30	330
CE-I-10	745	370	0.54	7.5	111	339	60	330
CE-I-100	700	445	0.45	5.6	110	385	40	330

The detected b_H levels in all sets were in the range between 9 and 13.6 mg/L/h indicating the active status of the biomass. Generally, the results of all experiment sets showed that the oxygen consumption rates of biomass started to decrease to b_H level with the decrease of substrate concentration. During all experiments, pH level was detected to be between 7.25 and 8. The maximum OURs, detected immediately after feeding, were consistent with and without MP addition indicating no influence on the maximum specific growth rate. For AE-0 and AE-1, initial COD concentration decreased from 284 and 225 mg/L to 69 and 56 mg/L within 450 minutes, respectively. Considering the results of the in-cycle monitoring of the control reactor, the majority of feeding substrate was removed within 150 minutes, retaining only 31 mg/L COD at

the end of the cycle (Figure 4.17). As peptone mixture, known for its high biodegradability, was used as the substrate, it can be concluded that the remaining 31 mg/L COD represents the soluble microbial product fraction. Unlike to the control reactor, the respirometric results revealed that 69 and 56 mg/L COD have retained at the end of the AE-0 and AE-1 experiment sets, respectively. These amounts represent the sum of both the unused substrate and the soluble microbial product (Figure 4.22a & b). The remaining COD at the end of AE-10 (82 mg/L) and AE-100 (89 mg/L) experiments were higher than the residual CODs in AE-0 and AE-1, implying that besides the unused substrate and the soluble microbial products, a portion of substrate was adsorbed to the sludge as a consequence of the addition of higher concentration of MPs. By looking at all OUR profiles depicted in Figure 4.21, it is clearly seen that both AE-0 and AE-1 coincide with each other and that AE-10 shares a high degree of similarity with them as well. The most significant difference was observed in the case of AE-100 where the experiment involved the addition of 100 µg/L MPs indicating the significance of adsorption mechanism and therefore affecting the rate of secondary hydrolysis.

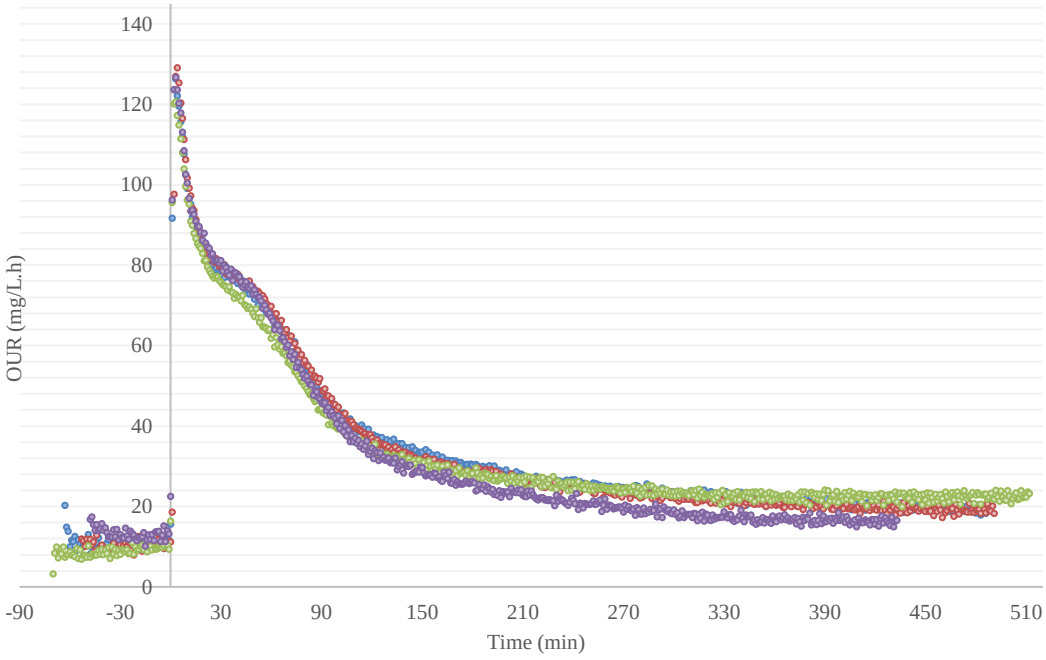


Figure 4.21 : OUR profiles obtained from control reactor respirometric experiment with 0 (●), 1 (●), 10 (●), and 100 (●) µg/L micropollutants addition (acute effect)

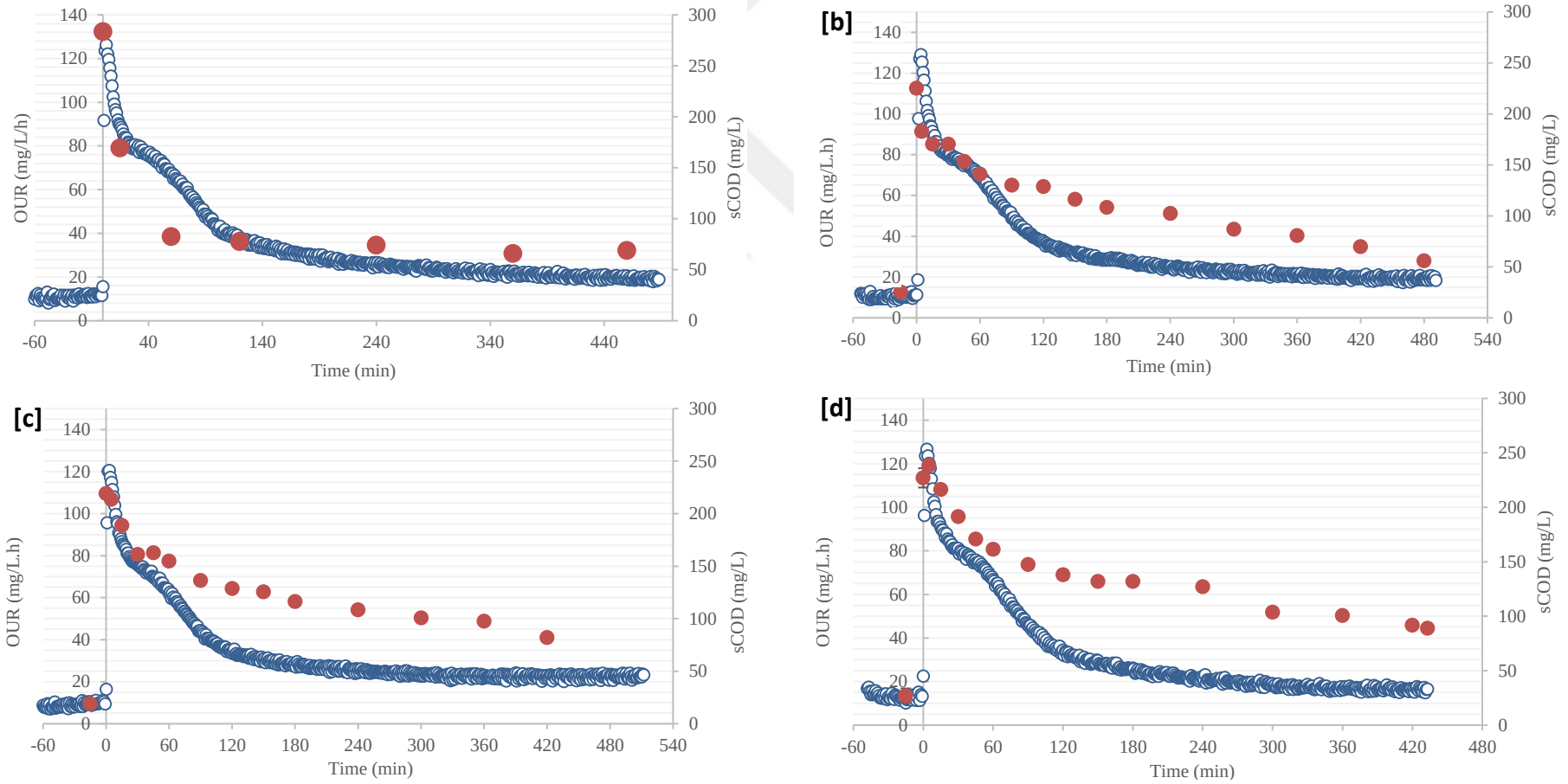


Figure 4.22 : OUR (○)and sCOD (●)profiles obtained from control reactor respirometric experiments (acute effect) in which MP was added at concentration of (a) 0 µg/L [AE-0] (b) 1 µg/L [AE-1] (c) 10 µg/L [AE-10] and (d) 100 µg/L [AE-100]

4.3.2 Chronic effect evaluation

The chronic effect of different concentrations of micropollutants was determined using activated sludge samples acclimated to micropollutant which obtained from the reactor operated with the addition of micropollutants after its stabilization. The determination of chronic effect was carried out in two different respirometric experiment sets, i.e. in the presence or absence of nitrification inhibitor, to allow the evaluation of carbon removal and nitrification processes together and separately. As shown in Table 4.2, the first set; i.e. CE-0, CE-1, CE-10, and CE-100 were executed in the absence of nitrification inhibitor while the second set, i.e. CEI-0 CEI-10 CEI-100, in its presence.

4.3.2.1 Experiments without nitrification inhibitor

All the data obtained from respirometric experiments aimed to determine the chronic toxicity of micropollutants on acclimated activated sludge obtained from the main micropollutant reactor are comparatively represented in Figure 4.23. The profiles of OUR, sCOD, nitrogen and pH of the experiments involving no addition of nitrification inhibitor are represented in Figure 4.24 and Figure 4.25. The detected b_H levels in all sets were in the range between 4.8 and 6.5 mg/L/h indicating the active status of the biomass. Generally, the result of all experiment sets showed that the oxygen consumption rates of biomass started to decrease to bH level with the decrease of substrate concentration. During all experiments, it was observed that the pH increases from 6.7 to 7.45.

The amount of COD remained at the end of these experiments (<30 mg/L) was found to be lower than the ones observed in acute experiments set. Considering the results of the in-cycle monitoring of the MP reactor, the majority of the feeding substrate was removed within 150 minutes, retaining only 50 mg/L COD at the end of the cycle (Figure 4.19). This means that the low fractions of COD observed at the end of these respirometric experiments are merely soluble microbial products (Figure 4.24 and Figure 4.25). The results of MP reactor in-cycle monitoring also revealed that after 210 minutes full nitrification was achieved and that 12 hours were needed for the formation of only 20 mg/L nitrate (Figure 4.20). In similar way, the results of respirometric experiments revealed that approximately 15 mg/L ammonium nitrogen in the peptone mixture was converted to a very low amount of nitrate measured as 1.84, 1.46, 4.8, and 6.8 mg/L after 330 minutes since the beginning of CE-0, CE-1,

CE-10, and CE-100, respectively (Figure 4.24 and Figure 4.25). It is worth to mention that the addition of 100 µg/L, unlike all other sets, cause the substrate (COD) removal rate to be lower than the other sets.

In view of all the respirograms depicted in Figure 4.23, it is apparent that both CE-0 and CE-1 exhibited similarity in the first hours with very close OUR values, yet, some differences appeared at the latest hours. Similarly, the initial values of OUR obtained by addition of 10 µg/L and 100 µg/L micropollutants (CE-10 and CE-100) were similar and found to be lower than the initial OUR values in the control and experiments with 1 µg/L (CE-0 and CE-1). By evaluating all OUR and sCOD profiles together, these differences indicate substrate binding to the sludge, especially in the case of CE-100, and also influencing the rate of secondary hydrolysis.

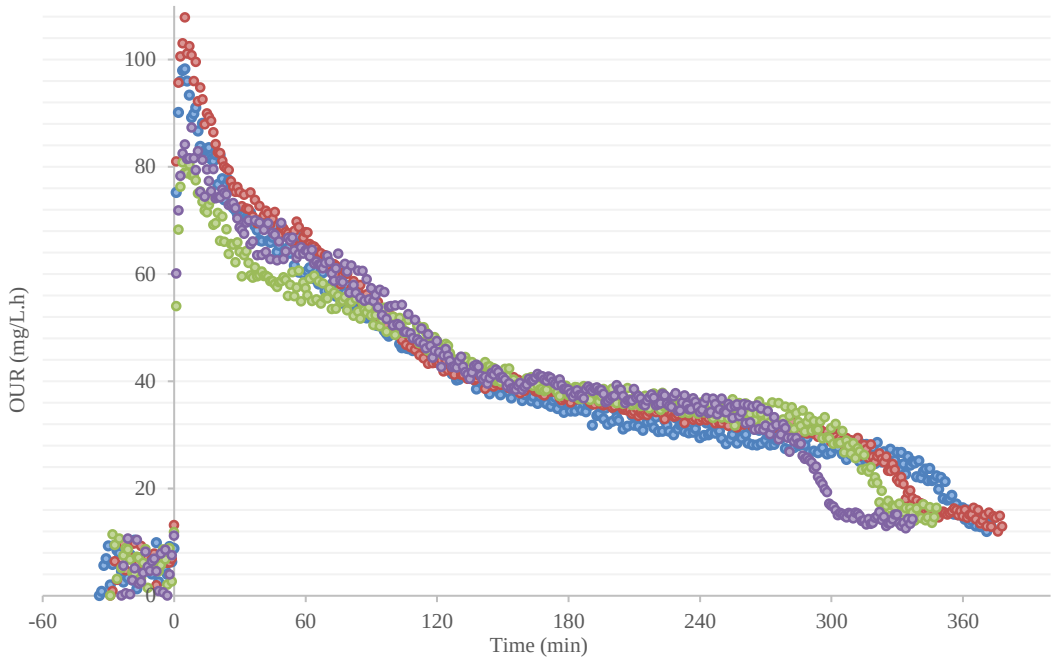


Figure 4.23 : OUR profiles obtained from MP reactor respirometric experiment with 0 (●), 1 (●), 10 (●), and 100 (●)µg/L micropollutants addition (chronic effect)

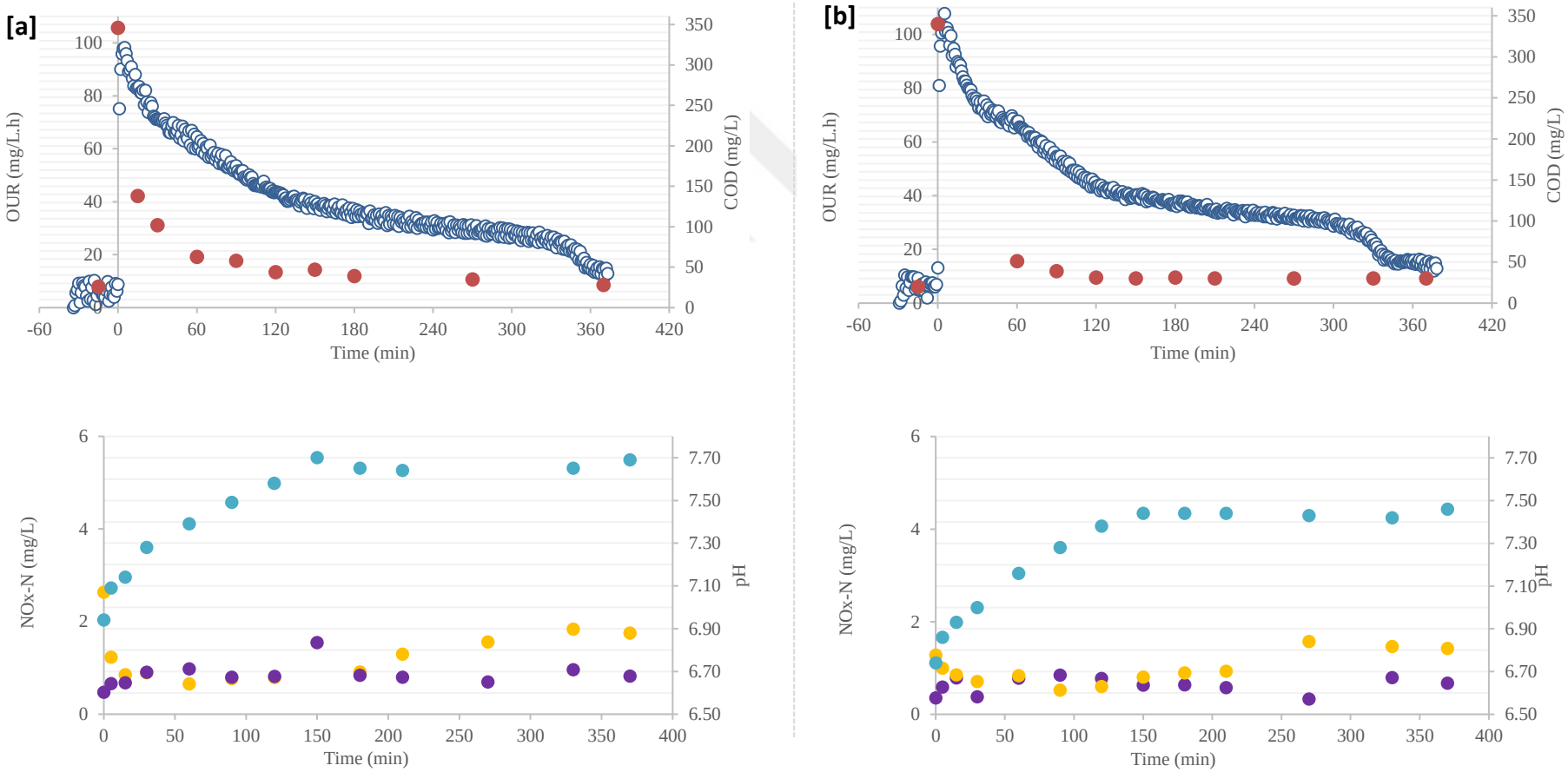


Figure 4.24 : OUR (○), sCOD (●), NO_2-N (●), NO_3-N (●), and pH (●) profiles obtained from MP reactor respirometric experiments (chronic effect) in which MP was added at concentration (a) 0 µg/L [CE-0] and (b) 1 µg/L [CE-1] – without nitrification inhibitor

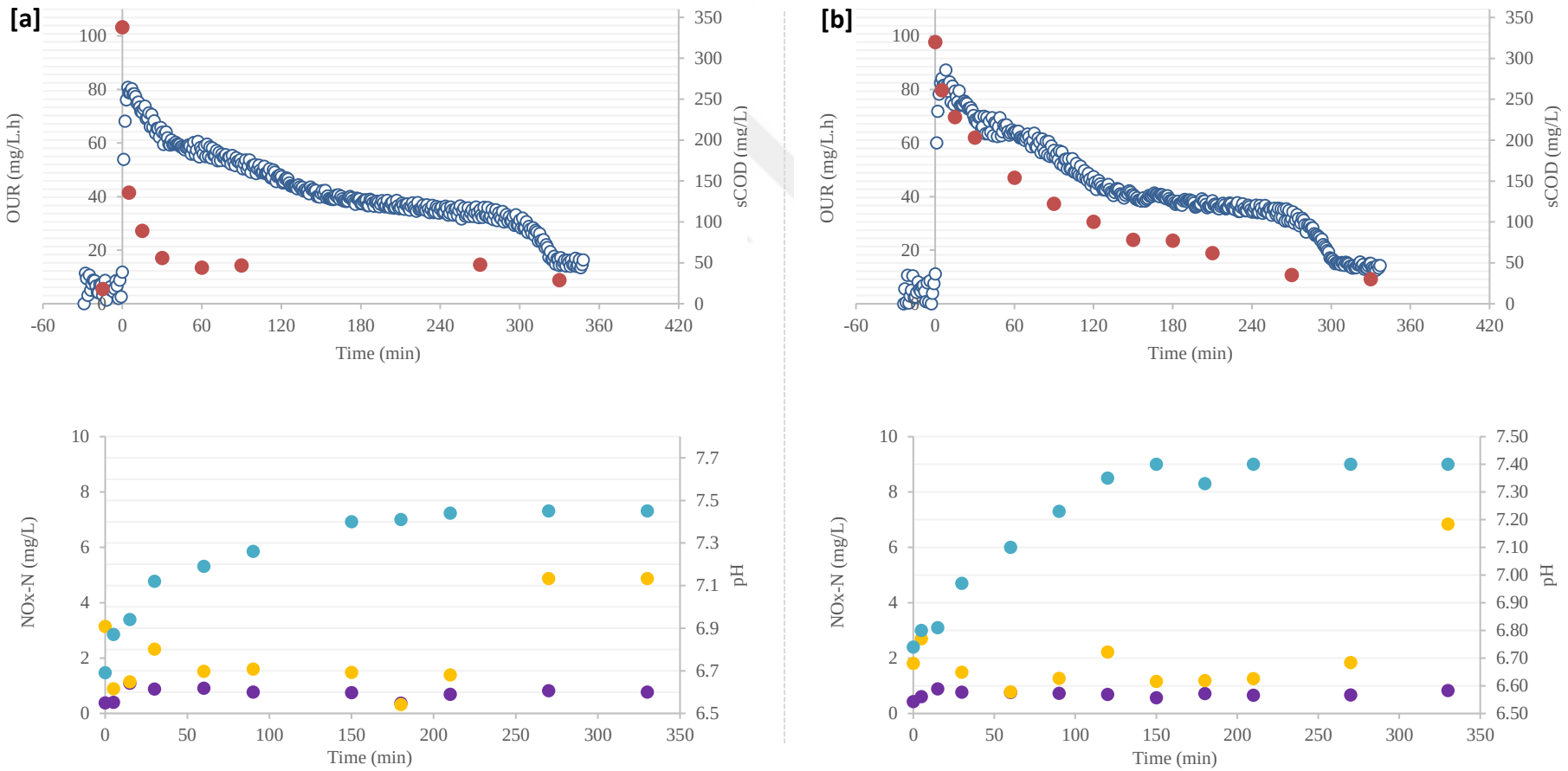


Figure 4.25 : OUR (○), sCOD (●), NO_2-N (●), NO_3-N (●), and pH (●) profiles obtained from MP reactor respirometric experiments (chronic effect) in which MP was added at concentration (a) 10 µg/L [CE-10] and (b) 100 µg/L [CE-100] – without nitrification inhibitor

4.3.2.2 Experiments with Nitrification Inhibitor

All the OUR profiles obtained from chronic toxicity respirometric experiments to which nitrification inhibitor has been added are illustrated in Figure 4.26. The OUR and sCOD profiles of the CEI-0, CEI-10, and CEI-100 experiments are represented separately in Figure 4.27. These experiments allow the evaluation of the chronic effect of micropollutants on carbon removal and nitrification separately. During all experiments, it was observed that the pH increases from about 6.5 to 7.4. Moreover, no nitrite or nitrate formation was observed, indicating that the nitrification inhibitor was effective. The detected b_H levels ensure biomass activity and the rate of biomass oxygen consumption decreased to b_H level with the decrease of substrate concentration. The amount of COD remained at the end of these experiments (330 minutes) were decreased to below the detection limit (<30 mg/L) in the case of CEI-0 while the values were slightly higher in the case of CEI-10 (60 mg/L) and CEI-100 (40 mg/L). Considering the interpretation of Figure 4.19, remained COD values at the end of these respirometric experiments referred to the soluble microbial product accumulation at the end of the respirometric assay.

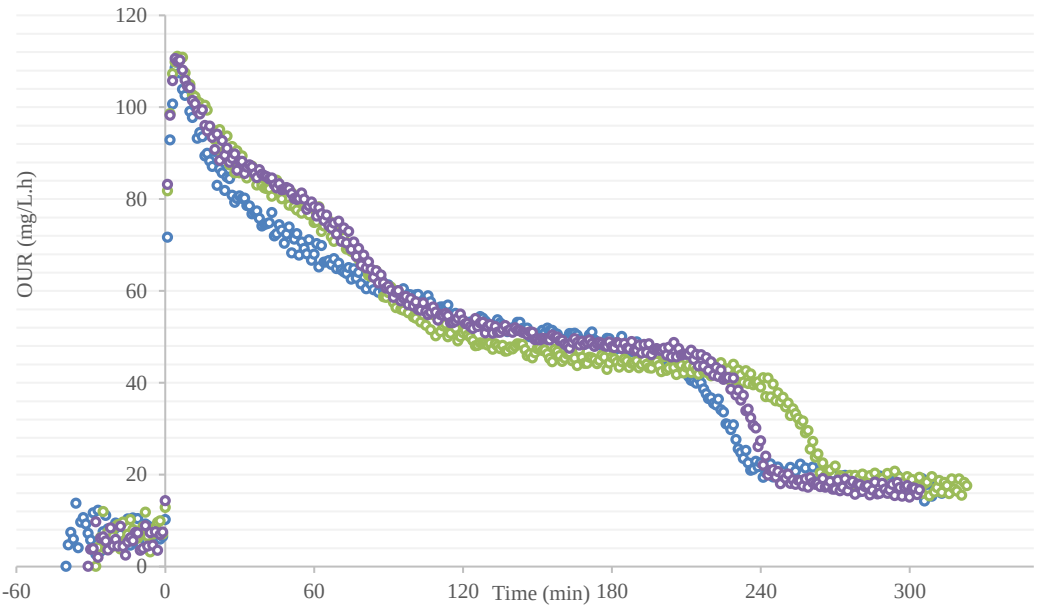


Figure 4.26 : OUR profiles obtained from MP reactor respirometric experiment with 0 (●), 1 (●), 10 (●), and 100 (●)µg/L micropollutants addition (chronic effect) – with nitrification inhibitor

Very close OUR values were observed in CEI-0 and CEI-10 in the first hours, yet, some differences appeared at later hours. Similarly, the initial values of CEI-10 and CEI-100 OUR profiles were similar and found to be higher than the initial OUR values in the control experiment (CEI-0).

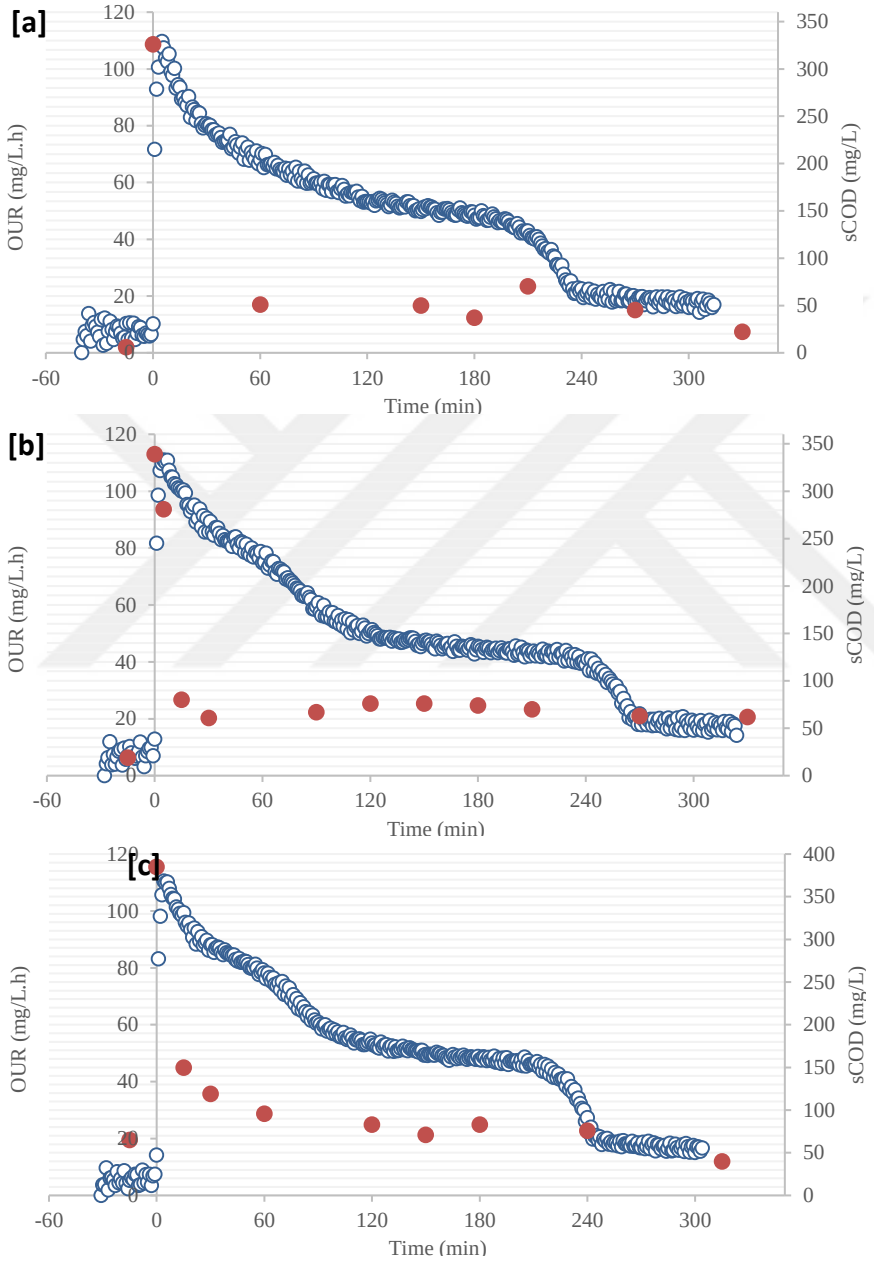


Figure 4.27 : OUR (○) and sCOD (●) profiles obtained from MP reactor respirometric experiments (chronic effect) in which MP was added at concentration of (a) 0 µg/L [CEI-0] (b) 10 µg/L [CEI-10] and (c) 100 µg/L [CEI-100]-With Nitrification inhibitor

4.4 Acute Effects of Micropollutants on Activated Sludge Kinetics

4.4.1 Acute effect modeling

The OUR profiles obtained from respirometric analysis to determine the acute effect at different micropollutant concentrations (0, 1, 10 and 100 µg/L), were used together with ASM1 modeling approach to estimate the concentrations of easily biodegradable, rapidly and slowly hydrolyzable organic substrates (S_S, S_{H1}, and S_{H2}, respectively) as well as the maximum heterotrophic specific growth rate ($\hat{\mu}_H$), half-saturation constant (K_S), maximum autotrophic growth rates ($\hat{\mu}_{NH}$ and $\hat{\mu}_{NO}$), rapid and slow hydrolysis rates (k_{h1} and k_{h2}). The obtained model parameters and initial concentrations are summarized in Table 4.3. A high level of compatibility was achieved between the applied models and the data obtained from OUR profile (Figure 4.28 and Figure 4.29)

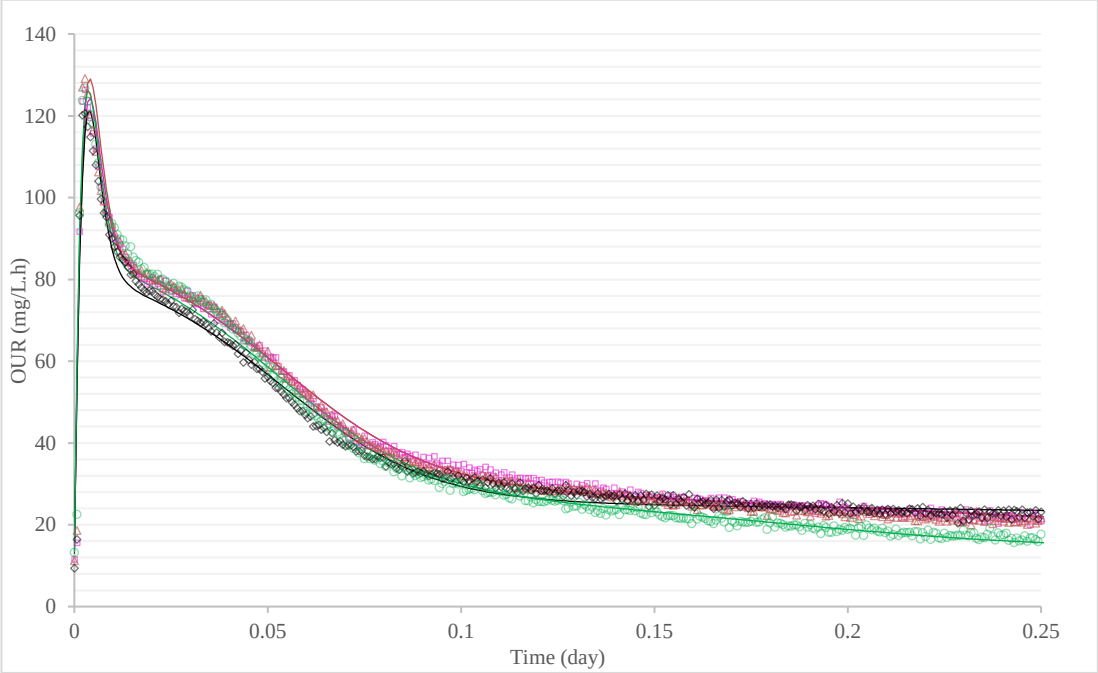


Figure 4.28 : Acute effect modeling, 0 (), 1 (), 10 (), and 100 () µg/L

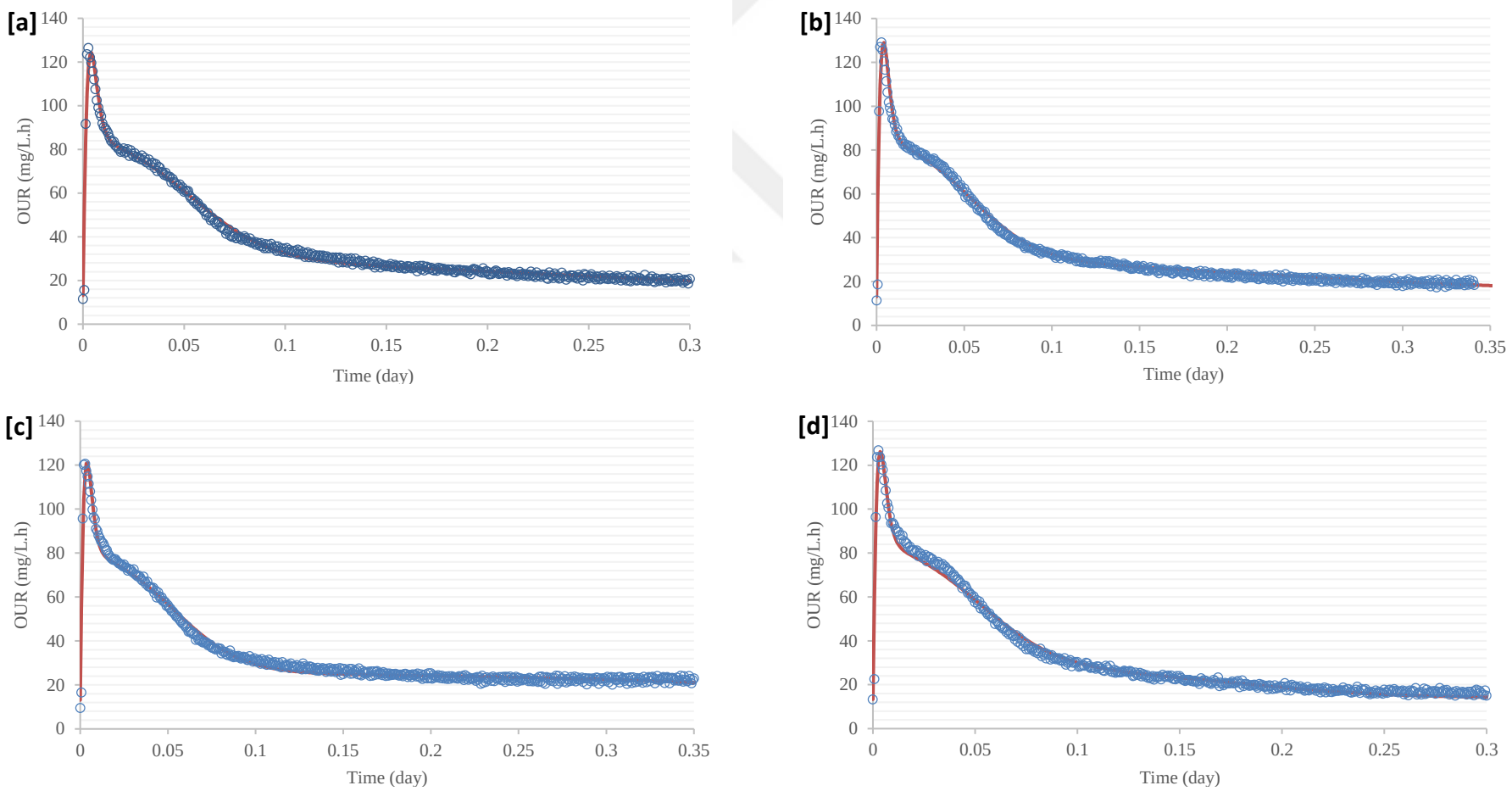


Figure 4.29 : Acute effect modeling (—) and observed data (o) for (a) 0, (b) 1, (c)10 and (d) 100 µg/L

Modeling results indicate that the addition of 1 μg/L cause no significant effect on carbon removal kinetics with reference to the control where no micropollutant was added. However, increasing the added micropollutant concentration to 10 μg/L decelerates the rate of the second hydrolysis, indicated by lower k_{H2} value. On the other hand, by the addition of 100 μg/L micropollutants, not all of the organic matter in the medium could be oxidized and about 40% of COD remained in the reactor as a result of the substrate binding.

Table 4.3: Kinetic and stoichiometric coefficients obtained from modeling.

Model Parameters	Symbol	Unit	NSAIDs Concentration (μg/L)			
			0	**1**	**10**	**100**
Maximum specific growth rate	$\hat{\mu}_H$	1/day	10	10	10	10
Half saturation coefficient of S_S	K_S	mg COD/L	22	22	22	22
Rapid hydrolysis coefficient of S_{H1}	k_{h1}	1/day	4.9	4.9	4.9	4.9
Half saturation coefficient for rapid hydrolysis of S_{H1}	K_X	g COD/gCOD	0.09	0.09	0.09	0.09
Slow hydrolysis coefficient of S_{H2}	k_{h2}	1/day	1.2	1.2	0.63	0.84
Half saturation coefficient for slow hydrolysis of S_{H2}	K_{XX}	g COD/g COD	0.091	0.091	0.025	0.025
Endogenous decay coefficient	b_H	1/day	0.4	0.4	0.4	0.4
Total biomass	X_T	mg VSS/L	850	820	860	790
Total biomass	X_T	mg COD/L	1207	1164	1221	1122
Initial heterotrophic active biomass	X_{HI}	mg COD/L	983	983	983	1000
Initial Concentrations						
Total Biodegradable COD	C_S	mg COD/L	405	405	405	405
Rapidly Biodegradable COD	S_S	mg COD/L	18	18	18	18
Rapidly hydrolyzable COD	S_{H1}	mg COD/L	180	180	180	180
Slowly hydrolyzable COD	S_{H2}	mg COD/L	207	207	207	125
Y_H: 0.58 g COD/g COD						

4.5 Evaluation of the Selected NSAIDs Effect on Microbial Community

4.5.1 Effect of selected NSAIDs on microbial community diversity

A total of 350,571 Illumina reads with average length of 300 bp and acceptable average quality score were derived from three independent samples obtained from raw seed sludge (80214 reads), control reactor sludge (161307 reads), and micropollutant containing reactor sludge (109050 reads). Diversity analysis were performed at two levels; within a sample (alpha diversity) and between samples (beta diversity). Biodiversity is essential to enable and facilitate particular ecosystem functions and thus biodiversity indices are considered important variables to describe microbial communities (Hernandez-Raquet et al., 2013). Alpha diversity indices are shown in Table 4.4.

Richness is expressed as the number of observed OTUs. Analysis of the 16S rRNA genes revealed that 128, 227, and 171 distinct OTUs were found in the raw, control

Table 4.4: Diversity indices for raw, control and MP samples

Sample	Observed OTU	Single	Chao1	Shannon	Pielou's evenness	Goods coverage
Raw	128	1	128.0	6.21	0.89	1.00
Control	227	45	257.8	6.48	0.83	0.98
Mp	171	30	188.7	6.04	0.82	0.99

and MP clone libraries, respectively. In addition, richness was estimated by the abundance-based coverage estimator (ACE), which is a nonparametric richness estimator based on distribution of abundant (>10) and rare (≤10) OTUs, and the richness estimator Chao1, which is a nonparametric richness estimator based on distribution of singletons and doubletons. The three indices indicate that addition of micropollutants induces a reduction in the microbial community richness. In contrast to the species richness, the Shannon-Weiner's index (H) takes the number of species as well as their relative abundances into account and was calculated for every sample. The values of H represent high bacterial diversity, which was 3.17 (raw sludge), 3.74 (control), and 3.16 (micropollutant).

Many studies have calculated Shannon diversity index in aquatic systems and values typically range from 0.5 to 5, with values of 0.5–2.0 implying low diversity and values of 2.0–5.0 indicating high diversity (Garrido et al., 2014). Considering the fact that H of control was significantly larger than raw sludge and MP reactor, it could be inferred that the OTU in the control community was distributed more evenly than those in the raw sludge and MP-enriched reactor. Likewise, introducing ibuprofen-enriched wastewater at a concentration of 250 μg/L into wetland mesocosms resulted in a significant reduction in microbial diversity (3.19-3.40) when compared to diversity to the control beds (4.19-5.60). Moreover, results obtained by Kraigher et al. (2008) suggested that the presence of selected pharmaceuticals in wastewaters at concentrations of both 5 and 50 mg/L causes microbial diversity to decrease. In contrast to the obtained results, Jiang et al. (2017) showed that environmental concentration of NSAIDs (5 μg/L) could stimulate the bacterial diversity. Similarly, Zhang et al. (2016) observed that the maximum Shannon-Wiener index occurred in SBRs with the addition of 5 μg/L tetracycline and sulfamethoxazole. Huang et al. (2018) used two lab-scale anaerobic and anoxic-oxic systems containing 300 μg/L of tetracycline to investigate bacterial diversity, showing that microbial diversity increased after tetracycline addition.

Rarefaction analysis (Figure 4.30) was performed for observed OTUs to determine if samples were sequenced deeply enough to capture the presence of minority species. Deep sequencing refers to sequencing the required genomic region numerous times so that the detection of rare microbes covering as little as 1% of the original sample will be possible. Samples with low sequence depth consider only the presence of few dominant species resulting in lower species diversity.

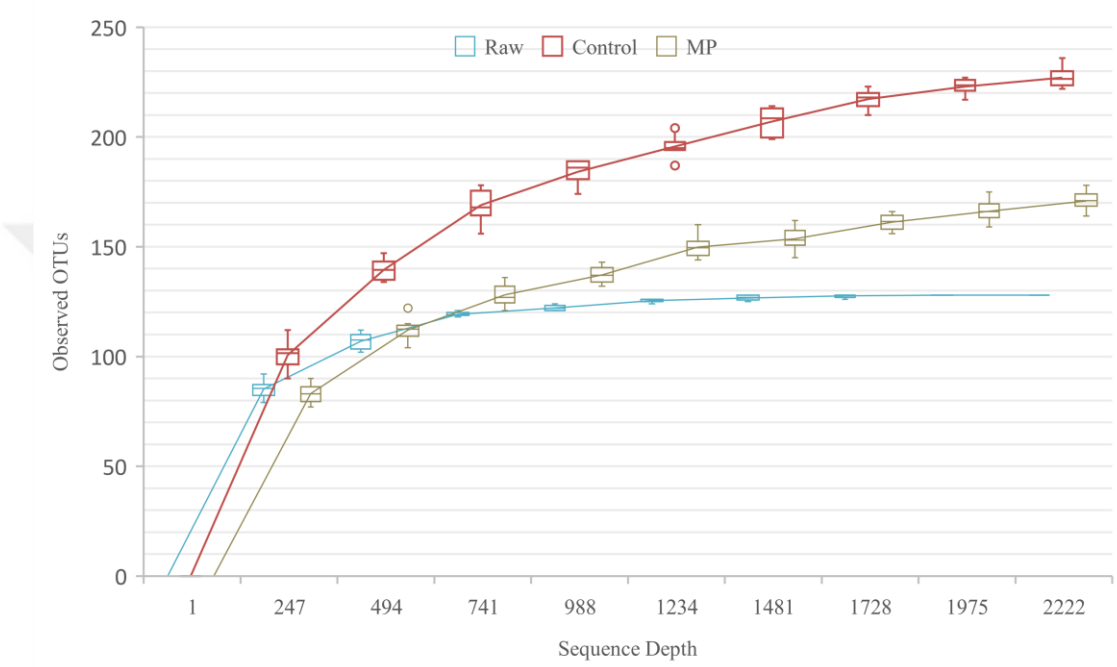


Figure 4.30 : Rarefaction Curve for observed OTUs

Beta diversity is illustrated as PCoA in Figure 4.31. PCoA visualizes the dissimilarity matrix between the three samples, such that samples that were more similar were closer in space than samples that were more divergent. It is clearly seen that the ellipses related to the samples obtained from lab-scale reactor are closer to each other than the sample obtained from raw sludge.

4.5.2 Effect of selected NSAIDs on microbial community at taxonomic levels

The composition of microbial community at different taxonomical level in both control and micropollutant samples is illustrated in Figure 4.33 and Figure 4.34, respectively. The comparison at taxonomical levels can give a general overview about the shit in microbial community structure as a result of certain trigger. Micropollutants have been demonstrated that their presence, alone or in combination could inhibit microbial metabolism, and cause the changes in the performance and microbial community of

water treatment systems (Harb et al., 2016; Kraigher et al., 2008; Sheng et al., 2018). For example, Kraigher et al. (2008) demonstrated that the concentration of TrOCs (at 5 µg/ L) (e.g., ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid) had a strong effect on the community structure and resulted in a shift in bacterial community.

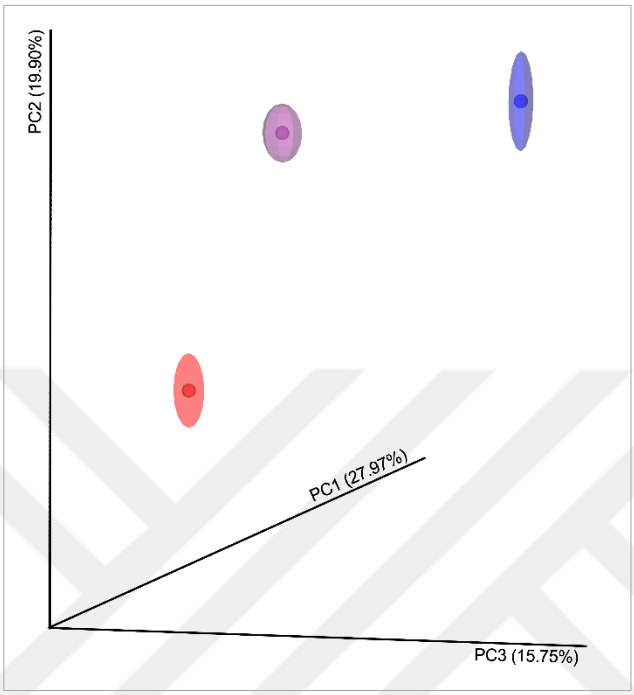


Figure 4.31 : Beta diversity of raw, control and MP samples

4.5.2.1 Effect of selected NSAIDs on microbial community at phylum level

The changes in activated sludge microbial community at phylum level is shown in Figure 4.32. Nine bacterial phyla were recovered from the samples. In terms of average abundance, it is clearly seen that *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicute* were the four most abundant phyla in the three samples, and they are frequently observed in activated sludge (Nguyen et al., 2019; Zhang et al., 2012). *Proteobacteria* usually predominate in domestic sewage sludges, corresponding to 30-65% of total sequences, presenting wide diversity and metabolic capacity, and acting in important environmental functions, such as the C, N, and S cycles. Their abundance in the raw sludge sample were 29%, 15%, 17% and 20%, respectively. Compared to raw sludge, it was observed that, under laboratory conditions, species belonging to *Firmicute* and *Acidobacteria* decreased by more than 95% while the ones belonging to *Proteobacteria* and *Actinobacteria* exhibited an increase of about 38% and 50%, respectively. It is also worth to note that both *Ignavibacteriae* and *Fusobacteria* phyla were detected in raw sludge sample, even at low abundance, and totally disappeared

under laboratory conditions. Same observation for the same phyla was recorded by (Navrozidou et al., 2019) who studied the effect of feeding an immobilized cell bioreactor with commercial ibuprofen tablets and pure ibuprofen on bacterial community structure. The changes induced by the addition of NSAIDs mixture at phylum level can be clearly visible for *Verrucomicrobia*, which was only 3% in the control sample and increased up to 20% after the addition of NSAIDs mixture. Similarly, an increase in *Verrucomicrobia* was recorded when an immobilized cell bioreactor was fed with wastewater containing commercial ibuprofen tablets (Navrozidou et al., 2019). Such increase suggests that the species belonging to *Verrucomicrobia* could adapt well with the presence of selected NSAIDs.

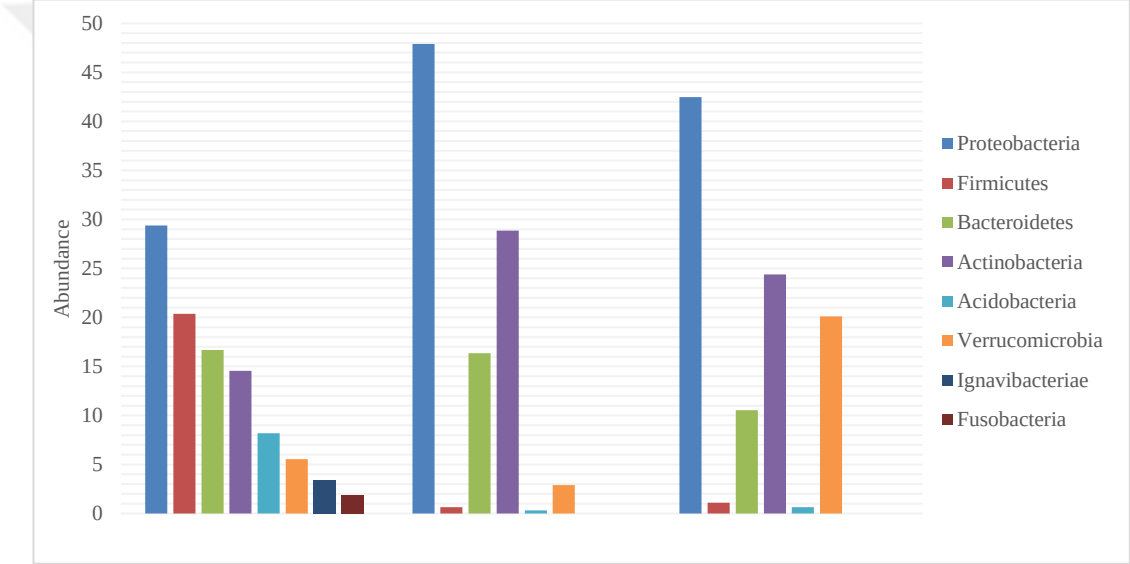


Figure 4.32: Changes at phylum level in raw sludge, control and MP samples

Figure 4.33: Microbial population in control reactor

Figure 4.34: Microbial population in MP reactor

4.5.2.2 Effect of selected NSAIDs on microbial community at genus level

The heatmap of bacterial abundance at the genus level is shown in Figure 4.35. It is quite clear that the conditions in the lab-scale reactors could not sustain the growth of species belonging to various genera which were detected in raw sludge samples with a relatively high abundance. For example, *Trichococcus flocculiformis*, *Romboutsia timonensis*, *Ruminococcus faecis*, *Streptococcus salivarius* and others were totally disappeared due to their obligatory and/or facultatively anaerobic nature (Scheff et al., 1984, Ricaboni et al., 2016, Kim et al., 2011, Kaci et al., 2014). *T. flocculiformis* is macrophallic or facultatively anaerobic and in opposite to the result obtained in this study, the same species was detected as the highest abundance and exhibited an increase of about 80% by the addition of exogenous organic micropollutants in feed wastewater of a membrane bioreactor system (Harb et al., 2016). In the reactor receiving NSAIDs mixture, the most abundant genera were *Prosthecobacter* (*Verrucomicrobia*) and *Paracoccus* (*Proteobacteria*), which accounted for up to 17 and 16% of the total read abundance, respectively. In a study conducted by Rossmassler et al. (2019), *Prosthecobacter* sp. were found to be highly abundant in a reactor inoculated with activated sludge pre-acclimated to a mixture of pharmaceuticals and personal care product including ibuprofen and diclofenac and fed with phenol as a carbon source. Moreover, *Paracoccus sp* are characterized as oxidase positive microorganisms (Baker et al., 1998). Oxidase enzymes have great potential as biocatalysts for micropollutant and organic waste breakdown. Two of such class of enzymes are the oxygenase Cytochromes P450 (CYPs or Cyt P450), a highly efficient group of monooxygenases responsible for the destruction of drugs and toxins in organisms, and the laccases (EC 1.10.3.2), a class of copper-containing oxidase enzymes used by microorganisms to break down lignin). Both enzymes have been shown to efficiently degrade a vast array of organic micropollutants in pure enzyme assays (Singhal and Perez-Garcia, 2016). Additionally, it has been reported that *Paracoccus sp.* are able to conduct heterotrophic nitrification at high nitrogen load in laboratory-scale aerobic granules (Cydzik-Kwiatkowska, 2015). Several studies in the literature provide evidence that nitrifying activity significantly contributes to the biotransformation of a number of micropollutants.

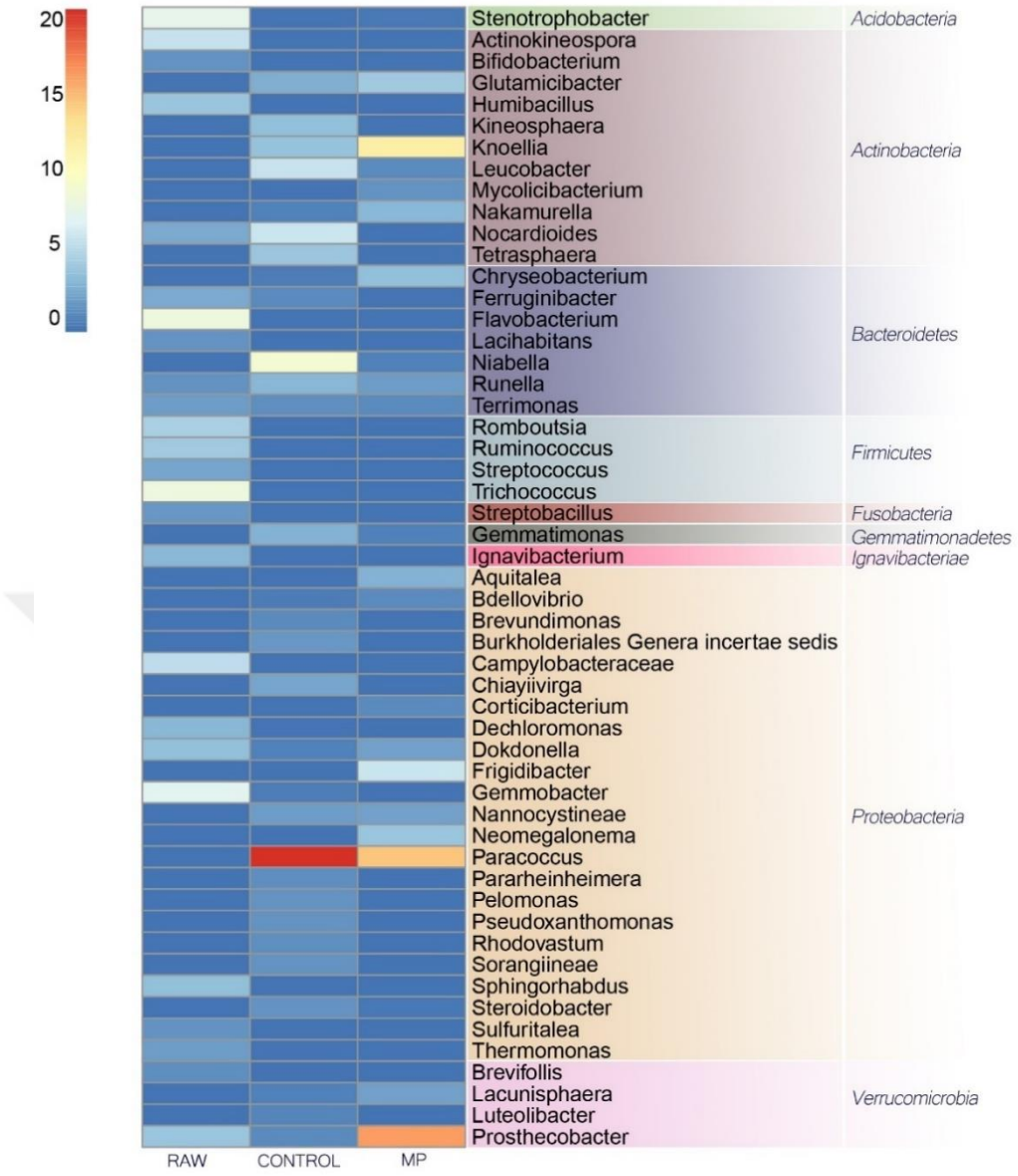


Figure 4.35: Heatmap of genera occurring in raw, control and MP samples

4.5.2.3 Effect of selected NSAIDs on microbial community at species level

To focus on the most highly enriched taxa, Figure 4.36 illustrates a taxonomic cladogram reporting the most abundant 53 detected species in the three analyzed samples. Color of squares beside species names represents different samples. The cladogram shows the taxonomic relatedness of different taxa and their distribution within the samples. The two variables between samples thought to cause shifts in microbial structure are the addition of micropollutants and the acclimation under lab conditions. It is obvious that certain species disappeared and/or appeared responding to the addition of micropollutants, and other disappeared from the raw sludge as a result of acclimation under lab conditions. Five of the highest abundant species that

found to disappear or significantly decrease, and the ones appear or significantly increase by the addition of micropollutants are shown in Table 4.5 and Table 4.6, respectively. Table 4.7 presents the species disappeared from raw sludge under lab conditions.

Table 4.5: Species disappeared or significantly decreased by the addition of MPs

Species	Raw (%)	Control (%)	MP (%)
Paracoccus alkenifer	0	9.2	0.22
Nocardioides islandensis	0	6.5	0
Kineosphaera nakaumiensis	0	3.9	0
Niabella hirudinis	0	3.5	0
Leucobacter komagatae	0	3.1	0

Table 4.6: Species appeared or significantly increased by the addition of MPs

Species	Raw (%)	Control (%)	MP (%)
Knoellia aerolata	0.0	4.1	12.7
Paracoccus mangrove	0.0	1.4	11.1
Prosthecobacter vanneervenii	4.2	1.1	17.1
Paracoccus cavernae	0.0	0.6	4.3
Frigidibacter albus	0.0	0.0	6.4

Table 4.7: Species disappeared under lab conditions

Species	Raw (%)	Control (%)	MP (%)
Stenotrophobacter terrae	8.2	0.0	0.3
Gemmobacter lanyuensis	7.7	0.0	0.0
Romboutsia timonensis	4.8	0.0	0.0
Ruminococcus faecis	4.5	0.0	0.0
Ignavibacterium album	3.4	0.0	0.0

Micropollutants caused the disappearance of more than 97% of the species *Paracoccus alkenifer*, while *Nocardioides islandensis*, *Kineosphaera nakaumiensis*, *Niabella hirudinis*, and *Leucobacter komagatae* were totally disappeared indicating their failure to sustain in the new condition. All the species in Table 4.7 were characterized as obligatory anaerobes, a thing that explains their disappearance in control and MP reactors. The species with highest relative abundance in raw sample was *Stenotrophobacter terrae*. Although it was characterized as aerobic chemoheterotroph, it disappeared totally under lab conditions as it lack the ability to utilize pepton as a growth substrate (Pascual et al., 2015).

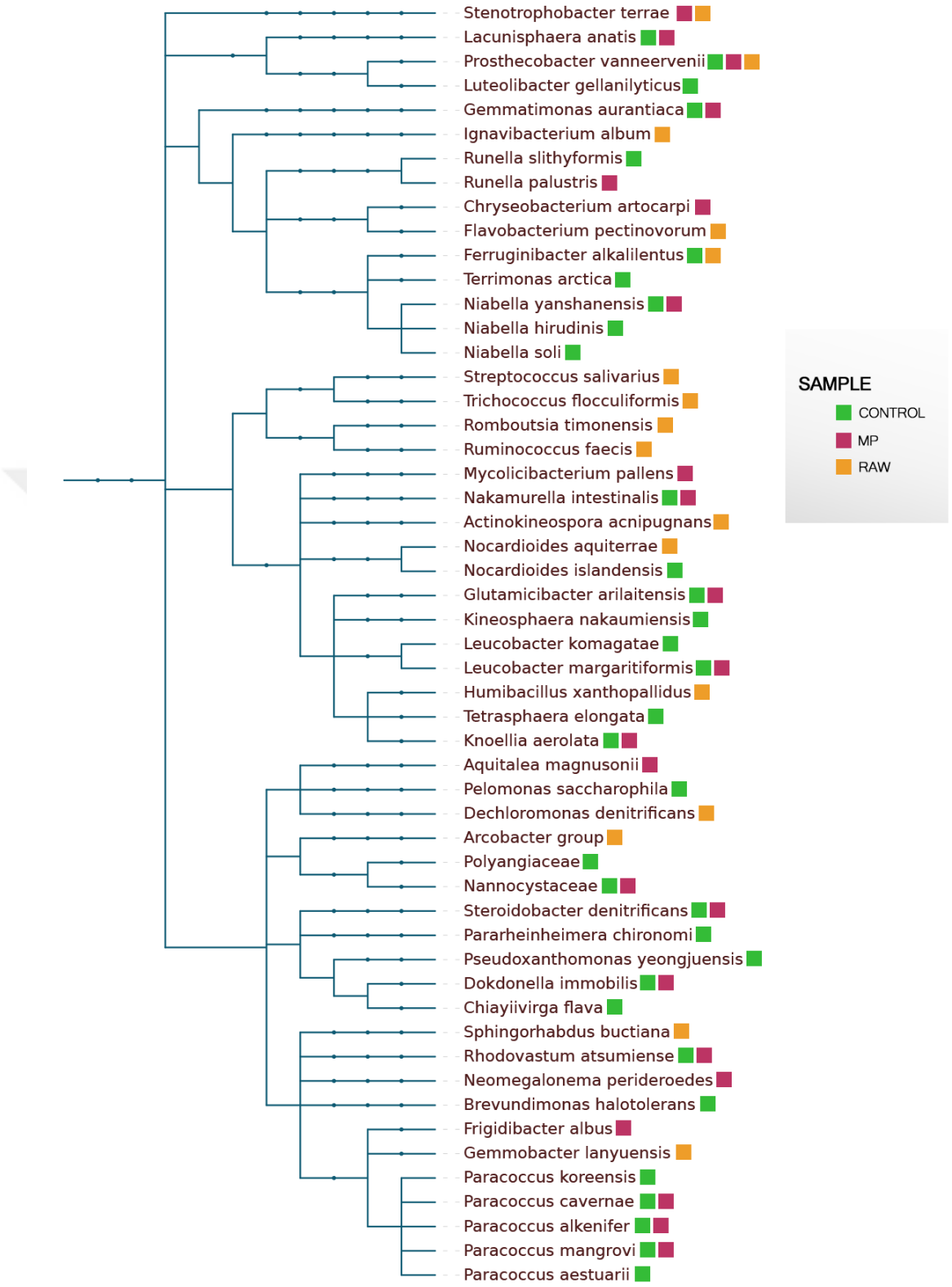


Figure 4.36: Cladogram of high relative abundance species

4.5.3 Effect of pharmaceuticals on functional traits distribution

Classification of the detected bacteria into functional groups and tracing their changes between different samples were performed based on FAPROTAX database that maps prokaryotic clades (genera or species) to establish metabolic or other ecologically relevant functions, using the current literature on cultured strains. Chemoheterotrophs constituted more than 96% in all samples. Other main functional groups and subgroups observed in the samples are described in Table 4.8 and Figure 4.37.

Table 4.8: Main functional groups and subgroups in FAPROTAX

Main Function Groups	Categories
Chemoheterotroph	Aerobic Chemoheterotroph
	Fermentation
	Aromatic Compound Degradation
	Cellulolysis
Nitrogen Denitrification	Nitrate Denitrification
	Nitrite Denitrification
	Nitrous Oxide Denitrification
Nitrate Reduction	
Nitrification	
Nitrogen Fixation	
Ureolysis	
Methanol Oxidation	
Respiration of sulfur compounds	
Fumarate Respiration	
Hydrogen Oxidation	
Human Gut	

Within chemoheterotrophs, aerobic species were found more in control and micropollutant fed reactor samples when compared to raw sample which is in convenient with the species with high relative abundance present as described in previous sections. The opposite trend can be observed in the case of fermentation group, in which anaerobic species are more dominant in raw sludge sample. In addition to fumarate and sulfate respiration, performed by anaerobic species, there are other species that reside in human gastrointestinal tract appeared only in raw sample. Over 99% of the bacteria in the gut are anaerobes. Since the lab-scale reactor operated under aerobic conditions those function groups disappeared or significantly decreased in control and MP samples.

There is a noticeable increase in aromatic hydrocarbon degradation in MP reactor. This was expected since the selected NSAIDs have aromatic structures. The occurrence of

hydrogen and methanol oxidation in the samples obtained in the lab can be related to the presence of species belonging to *Paracoccus* genus. As described previously, *Paracoccus* genus has the highest relative abundance in both control and MP samples. Most *Paracoccus* strains have been isolated from wastewater treatment plants or bioremediation sites of contaminated soils due to their degradative capabilities. Paracoccus has a versatile degrading capacity. Besides being chemoorganotrophs, most *Paracoccus* species can reduce nitrate to N, under anaerobic conditions. *Paracoccus* strains can also grow methylotrophically with simple methyl compounds such as methanol or methylamine. Also, facultative chemolithotrophic growth with hydrogen has been described for several strains of *Paracoccus* (Bergey et al., 1984).

There are two main functional groups related to nitrogen appeared in high abundance in all the three samples. An average of 35% of the detected species shown to perform nitrate reduction. According to the description of this functional group in FAPROTAX, the species belong to this group can carry out both assimilatory and dissimilatory nitrate reduction. Assimilatory nitrate reduction refers to the uptake of nitrate, its reduction to ammonium, and its incorporation into biomass while dissimilatory nitrate reduction to ammonia, a process distinct from denitrification, generally thought to be mediated by anaerobic, or facultatively anaerobic bacteria, using NO_3^- as a terminal electron acceptor in respiration (Mohan & Cole, 2007). Nitrogen denitrification on the other hand, constitutes 16, 27, and 19% of row, control and MP reactors respectively, ends in production of N_2O and/or N_2 gas, which can then be lost from the system to the atmosphere (O'Neil & Capone, 2008). It is widely known that facultative anaerobic bacteria perform denitrification as a type of respiration and reduce nitrate in the absence of oxygen. However, various genera of microorganisms describe the use of nitrate (NO_3^-) as oxidizing agents under an aerobic atmosphere known as aerobic denitrifiers. The periplasmic nitrate reductase is vital for aerobic denitrifiers and its presence may be the proof of aerobic denitrification. Phylogenetic analysis revealed that aerobic denitrifiers mainly belong to α-, β- and γ-Proteobacteria. Aerobic denitrifiers tend to work efficiently at 25 ~ 37°C and pH 7 ~ 8, when dissolved oxygen concentration is 3 ~ 5 mg/L and C/N load ratio is 5 ~ 10 (Ji et al., 2015).

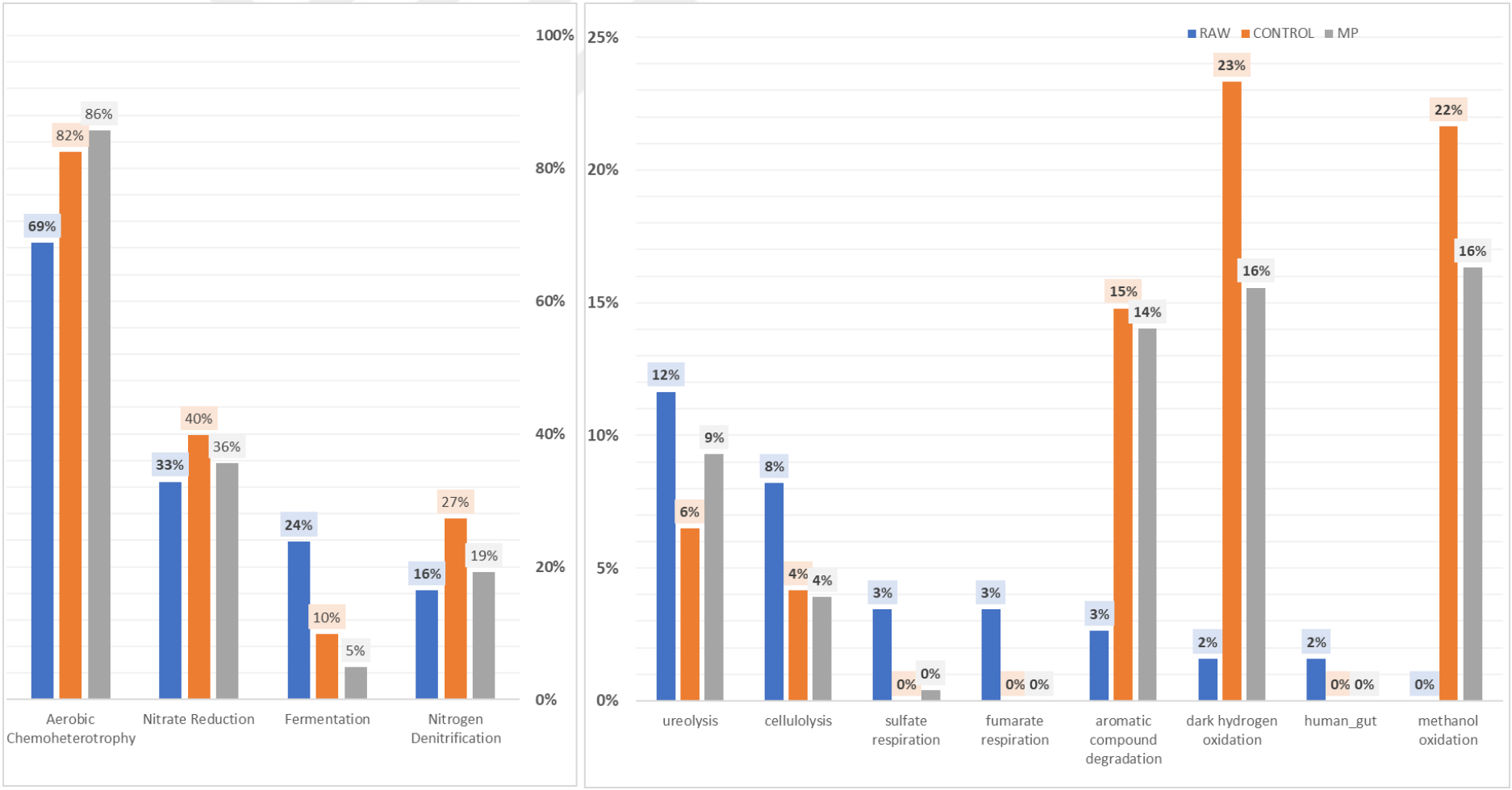


Figure 4.37: Function distribution of species presents in raw, control and MP samples

5. CONCLUSIONS AND RECOMMENDATIONS

Since understanding the behavior of activated sludge microbial communities under new conditions developed by the addition of chemicals with a potential risk (i.e. NSAIDs) would provide insights for their biological degradation in wastewater treatment systems, the overall goal of this research was to determine the impact of NSAIDs on the bacterial population of activated sludge. The key findings of this work can be summarized as follow:

- Acclimation of activated sludge to the NSAIDs mixture, i.e. ibuprofen, naproxen, ketoprofen, indomethacin, mefenamic acid and diclofenac under SRT of 5 days, posed no long-term inhibitory effect on both the biodegradation of organic matter and the nitrification process. While no change in the COD removal efficiency was observed, the performance of nitrification process was enhanced in the presence of micropollutant.
- NSAIDs at environmentally relevant concentrations were effectively removed in SBR operated at SRT of 5 days particularly for ibuprofen and naproxen in which almost full removal was observed. The contribution of adsorption to the selected NSAIDs removal mechanism was found negligible when compared with biodegradation.
- Evaluation of toxicity and inhibition in both non-acclimated control culture (acute) and micropollutant acclimated cultures (chronic) under the given operational conditions, revealed no considerable inhibition in terms of OUR profiles. However, long adaptation of mixed cultures to 10-100 μg/L NSAIDs seems to negatively impact the maximum specific growth rate. In addition, based on the modeling results, the kinetics of non-acclimated biomass was affected by NSAIDs, in which the rate of the second hydrolysis was slow down and not all of the organic matter in the medium could be oxidized when the added concentration was 100 μg/L.
- Long adaptation of mixed cultures to NSAIDs compounds significantly reduce the diversity of microbial community.
- Massive DNA sequencing analysis of the activated sludge revealed a substantial enrichment of *Verrucomicrobia* phylum in culture acclimated to NSAIDs. *Proteobacteria* and

Actinobacteria in both NSAIDs dominate both in acclimated and non-acclimated culture. However, a great alteration of the bacterial composition at the genus level was detected. The species belonging to *Prosthecobacter* and *Paracoccus* predominated the acclimated culture implying their ability to adapt well to the new conditions and their potential capacity to degrade NSAIDs

- A noticeable increase in aromatic hydrocarbon degradation functional group in the presence of NSAIDs was detected.

The findings of this study showed that bioreactor's operation at SRT 5 days with a constant presence of pharmaceuticals altered the diversity of microbial culture. Therefore, it is recommended to investigate the effect of other operational parameters such as SRT. Moreover, deeper studies to observe nitrification process effect on NSAIDs removal efficiency are necessary. More insights should be performed to understand nitrifying organisms on the micropollutant removal potential.

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- B. Ciftcioglu, E. Demirkaya, **E. Saleh**, D. Soylu, G. Ozyildiz, G.E. Zengin, D. Guven, I. Pala-Ozkok, E. Cokgor, D. Okutman Tas (2019). Acute effect of micropollutants on an activated sludge system with high sludge retention time operated as a sequencing batch reactor. 10th IWA International Symposium on waste management problems in agro industries, AGRO, 2019, 19-21 June, Rhodes, Greece.
- B. Ciftcioglu, E. Demirkaya, **E. Saleh**, D. Soylu, G. Ozyildiz, G.E. Zengin, D. Guven, E. Topuz, I. Pala-Ozkok, G. Insel, E. Cokgor, D. Okutman Tas (2019). Insights into the acute effect of anti-inflammatory drugs on activated sludge systems with high solids retention time. Environmental Technology (Accepted for publication)