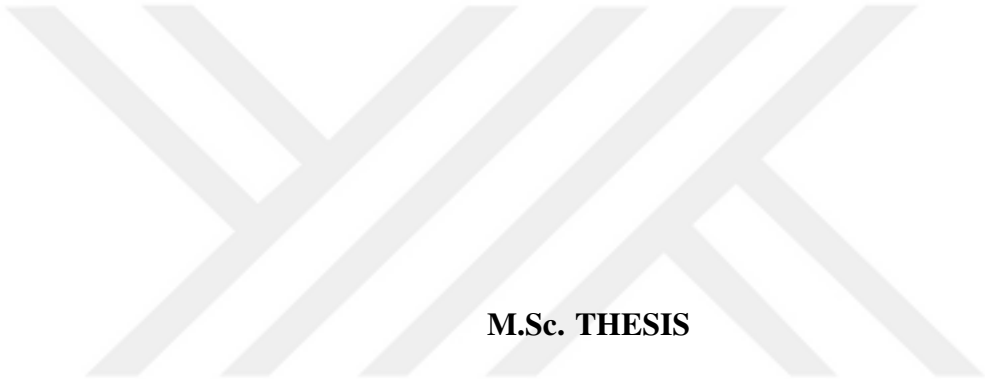


ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**EFFECT OF ANTI-INFLAMMATORY DRUG MICROPOLLUTANTS IN
HYBRID BIOFILM NITRIFICATION SYSTEMS ON MICROBIAL
COMMUNITY POPULATION**



M.Sc. THESIS

Roya DADKHAH

Department of Environmental Engineering

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JUNE 2025

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JUNE 2025

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**HİBRİT BİYOFİLM NİTRİFİKASYON SİSTEMİNDE
ANTIENFLAMATUVAR ÖZELLİKTEKİ MİKROKİRLETİCİLERİN
MİKROBİYAL POPÜLASYON ÜZERİNE ETKİSİ**

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To my beloved family and our Mother Nature,



FOREWORD

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June 2025

Roya DADKHAH

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ABBREVIATIONS

COD	: Chemical Oxygen Demand
SBR	: Sequencing Batch Reactor
MBBR	: Moving Bed Biofilm Reactor
sCOD	: Soluble Chemical Oxygen Demand
NO₂⁻	: Nitrite
NO₃⁻	: Nitrate
NH₄-N	: Ammonium Nitrogen
NSAIDs	: Non-Steroidal Anti-Inflammatory Drugs
CAS	: Conventional activated sludge system
MBR	: Membrane Bioreactor
SRT	: Sludge Retention Time
VSS	: Volatile Suspended Solids
TSS	: Total Suspended Solids
HRT	: Hydraulic Retention Time
AOPs	: Advanced Oxidation Processes
TCEP	: Tris(2-chloroethyl) Phosphate
TCPP	: Tris(1-chloro-2-propyl) Phosphate
BNR	: Biological Nutrient Removal
HybasTM	: Hybrid Attached Biofilm and Activated Sludge System
PE	: Polyethylene
IFAS	: Fixed-Film Activated Sludge
SSA	: Specific Surface Area
TN	: Total Nitrogen
AOB	: Ammonia-Oxidizing Bacteria
NOB	: Nitrite-Oxidizing Bacteria
WRRF	: Water Resource Recovery Facility
OLR	: Organic Loading Rate
OTUs	: Operational Taxonomic Units
ASA	: Acetylsalicylic Acid



SYMBOLS

T_C	: Total cycle time
T_F^*	: Duration of feeding phase
T_A	: Duration of aeration phase
T_S	: Duration of settling phase
T_D	: Duration of draw phase
T_I	: Duration of idle phase
V_0	: Initial volume
V_T	: Total volume



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EFFECT OF ANTI-INFLAMMATORY DRUG MICROPOLLUTANTS IN HYBRID BIOFILM NITRIFICATION SYSTEMS ON MICROBIAL COMMUNITY POPULATION

SUMMARY

Non-steroidal Anti-inflammatory Drugs (NSAIDs) are one of the most frequently used pharmaceuticals, as they are available over the counter worldwide. They are known among top 10 persistent pollutants with a significant environmental concern because of their incomplete degradation in wastewater treatment plants (WWTPs) and long-term toxic effects of NSAIDs even at trace concentrations on human health and ecosystem. Hybrid treatment technologies such as moving bed biofilm reactors (MBBRs) can be a promising alternative for conventional activated sludge system. Hybrid MBBR systems which, contain suspended and attached biomass, are capable of tolerating environmental fluctuations and have been reported to sustain effective removal activity in fluctuating organic loading conditions. There is no clear trend in literature about impact of organic loading rate (OLR) on removal efficiency of NSAIDs, and also their effect on microbial profile requires further investigation.

The objective of this study is to investigate the removal efficiency of targeted NSAIDs including diclofenac, ibuprofen, ketoprofen, naproxen, indomethacin, and mefenamic acid in hybrid systems, operated under low OLR condition, and to assess their impact on the structure and diversity of microbial communities.

For this purpose, laboratory scale sequencing batch reactors (SBRs) were operated as hybrid system by adding biofilm carriers to the reactors to achieve both suspended and attached biomass. The filling ratio for both reactors were 30%. Control and micropollutant (MP) reactors were operated under same operational conditions. The activated sludge was obtained from the aeration tank of an advanced biological WWTP in Istanbul. The chemical oxygen demand (COD) was 150 mg/L COD and the initial ammonium concentration was 60 mg/L. Sludge age was adjusted as 10 days. The micropollutant reactor were fed with six selected NSAIDs including diclofenac, ibuprofen, ketoprofen, naproxen, indomethacin, and mefenamic acid at environmentally relevant concentrations ranging from 1-10 µg/L. In order to track the performance of the reactor, COD, Ammonium Nitrogen ($\text{NH}_4\text{-N}$), Soluble Chemical Oxygen Demand (sCOD), Nitrite (NO_2^-), Nitrite (NO_3^-), Total Suspended Solid (TSS), Volatile Suspended Solid (VSS) analysis were conducted and they quantified. Sample collection was done after reaching steady state condition in the hybrid system to measure targeted NSAIDs concentrations.

Microbial analyses were started by DNA extraction from biomass. The V3–V4 region (widely used variable region for species identification) of the 16S ribosomal RNA gene were amplified by PCR. Then Next-generation sequencing (NGS) applied and finally the obtained sequencing went through bioinformatic analysis.

The results of effluent concentrations obtained to assess the hybrid reactor performance, showed a carbon removal of approximately 81% in hybrid control and 87% in hybrid MP reactor. The ammonium removal (NH_4^+) was measured as approximately 77% in hybrid control reactor and approximately 90% in presence of selected NSAIDs. Significant removal efficiencies of ibuprofen (100%), indomethacin (100%), naproxen (99%), mefenamic acid (98%), ketoprofen (96%) and diclofenac (69%) were achieved. The results showed that selected NSAIDs did not inhibit nitrification process and carbon removal, and also low OLR did not adversely affect the removal of selected NSAIDs.

The dominant phyla observed in the hybrid MP reactor were *Bacteroidota* (42.40%), *Proteobacteria* (35.13%) for suspended biomass and *Proteobacteria* (29.28%) and *Bacteroidota* (28.31%) for attached biomass.

At the genus level, in the hybrid MP reactor, the genera including uncultured genus belonging to the *Chitinophagaceae* family (10.08%), *Ferruginibacter* (7.82%), *Comamonas* (6.77%) dominated the suspended biomass, while *Fimbriimonadaceae* (8.73%) and *Parafilimonas* (6.46%) were seen as dominant genera in attached biomass.

Shannon index shows the diversity of microbial community. An increase in shannon index was observed in the attached biomass after addition of selected NSAIDs to the hybrid reactor, while diversity decreased in the suspended biomass of hybrid MP reactor, suggesting higher diversity in attached biomass under NSAIDs stress. Moreover, operational taxonomic units (OTUs) increased in the attached biomass after NSAIDs addition but a decrease was observed in the suspended biomass of hybrid MP reactor, implying that attached biomass had richer microbial profile than suspended biomass after NSAIDs addition. The case for the Pielou's evenness was opposite. This index decreased for both reactors after exposure to the selected NSAIDs. NSAID stress might contribute to the decrease of evenness in microbial community. Higher biodiversity and richness in attached biomass comparing to suspended biomass, might be attributed to an increase in microbial complexity which may enhance the coexistence of various functional bacterial in the biofilm as reported in a comparable study. The difference in biodiversity of hybrid control and hybrid MP reactors of suspended and attached biomass, might be due to the NSAIDs stress as seen in various studies.

Uncultured genus belonging to *Saprospiraceae* family and *Saccharimonadale* decreased significantly in both suspended and attached biomass of hybrid control reactor comparing to the inoculum sludge. On the other hand, a significant increase was detected for the suspended biomass of hybrid control reactor comparing to inoculum sludge in some genera such as *Niabella* and *Paracoccus*. For the attached biomass, significant increase was seen in *Paracoccus*, *Ottowia* and *Fimbrioglobus*. These observed significant changes were probably in response to the lab-scale experimental conditions, as well as selected operational conditions for this study such as low OLR.

uncultured genus belonging to *Chitinophagaceae* family, *Ferruginibacter* (belongs to *Chitinophagaceae* family) and *Comamonas* (belongs to *Comamonadaceae* family) that dominated suspended biomass of hybrid MP reactor, and also genus *Fimbriimonadaceae*, *Parafilimonas* (belongs to *Chitinophagaceae* family) and *Rickettsia*

which dominated the attached biomass of hybrid MP reactor, might contribute to the nitrification in hybrid system rather than common nitrifiers.

Families *Chitinophagaceae* and *Comamonadaceae* in similar studies, have been identified to be effective in nitrogen removal within MBBR.

Moreover, *Bacteroidota* and *Proteobacteria* have been reported to play an important role in nitrogen and MP removal.

Further research may be conducted by detailed qPCR and NGS with longer target gene region or alternative molecular techniques to more effectively investigate the relationship between microbial diversity, NSAID removal, and also its correlation with nitrification.





HİBRİT BİYOFİLM NİTRİFİKASYON SİSTEMİNDE ANTIENFLAMATUVAR ÖZELLİKTEKİ MİKROKİRLETİCİLERİN MİKROBİYAL POPÜLASYON ÜZERİNE ETKİSİ

ÖZET

Steroid olmayan antienflamatuar ilaçlar (NSAID'ler), en sık kullanılan farmasötikler arasında yer almaktadır. Bu maddeler çeşitli kaynaklardan sucul ortamlara ulaşmaktadır. Örneğin, hastanelerden kanalizasyon sistemlerine yapılan deşarjlar yoluyla doğrudan ya da insan ve hayvan dışkıları aracılığıyla dolaylı olarak sucul ortama girebilirler. NSAID'ler, yapılarında bulunan aromatik halka veya klor grubu nedeniyle ilk 10 kalıcı kirletici arasında yer almakta ve atıksu arıtma tesislerinde (AAT'ler) tam olarak parçalanmamaları ve düşük konsantrasyonlarda bile insan sağlığı ve ekosistem üzerinde uzun vadeli toksik etkileri nedeniyle önemli bir çevresel sorun oluşturmaktadır. Ayrıca, tarımsal ve endüstriyel faaliyetler nedeniyle su kaynaklarında artan azot bileşikler konsantrasyonu önemli çevresel problemlere yol açmaktadır. Kentsel atıksuların su ortamlarına deşarj edilmeden önce azot içeriğinden arındırılması çok önemlidir, çünkü bu durum doğal sularda ötrofikasyon riski ve sucul ekosistemler üzerindeki zararlı etkiler nedeniyle büyük önem taşımaktadır. Hareketli yatak biyofilm reaktörleri (MBBR'ler) gibi hibrit arıtma teknolojileri, geleneksel aktif çamur sistemlerine alternatif olarak umut verici olabilir. Askıda ve yüzeyde çoğalan biyokütle içeren hibrit MBBR sistemleri, heterotrofların çoğunlukla askıda büyümesini sağlarken, taşıyıcılar nitrifikasyon bakterilerinin gelişimini kolaylaştırır. Ayrıca, MBBR'ler çevresel dalgalanmalara karşı tolerans gösterebilir ve değişken organik yükleme koşullarında etkili arıtma faaliyetlerini sürdürebildikleri bildirilmiştir. Literatürde organik yükleme oranının (OLR) NSAID giderim verimliliği üzerindeki etkisi konusunda net bir eğilim bulunmamakta ve bu ilaçların mikrobiyal profil üzerindeki etkileri daha fazla araştırılmayı gerektirmektedir.

Bu çalışmanın amacı, düşük OLR koşullarında işletilen hibrit sistemlerde diklofenak, ibuprofen, ketoprofen, naproksen, indometasin ve mefenamik asit dahil olmak üzere seçilen NSAIDs'lerin giderim verimliliğini araştırmak ve mikrobiyal toplulukların yapı ve çeşitliliği üzerindeki etkilerini değerlendirmektir.

Bu amaçla, laboratuvar ölçekli ardışık kesikli reaktörler hibrit sistem olarak çalıştırılmış, reaktörlere biyofilm taşıyıcılar eklenerek askıda ve tutunmuş biyokütle elde edilmiştir. Her iki reaktör için doluluk oranı 30% olarak belirlenmiştir. Kontrol ve mikrokirletici reaktörleri aynı işletme koşullarında çalıştırılmıştır. Aktif çamur, İstanbul'da bulunan ileri biyolojik bir AAT'nin havalandırma tankından temin edilmiştir. Kimyasal oksijen ihtiyacı (KOİ) 150 µg/L ve başlangıç amonyum konsantrasyonu 60 mg/L olarak belirlenmiştir. Çamur yaşı 10 gün olarak ayarlanmıştır. Mikrokirletici reaktörü, çevresel olarak anlamlı konsantrasyonlarda (1–10 µg/L aralığında) seçilen altı NSAID bileşiği ile beslenmiştir.

Reaktör performansını izlemek amacıyla KOİ, Amonyum Azotu ($\text{NH}_4\text{-N}$), Çözünmüş Kimyasal Oksijen İhtiyacı (çKOİ), Nitrit (NO_2^-), Nitrat (NO_3^-), Toplam Askıda Katı Madde (AKM), Uçucu Askıda Katı Madde (UAKM) analizleri gerçekleştirilmiş ve miktarları belirlenmiştir. Hibrit sistemde kararlı duruma ulaşıldıktan sonra numune alımı yapılmış ve hedef NSAIDs konsantrasyonları ölçülmüştür. Mikrobiyal analizler, biyokütleden DNA ekstraksiyonu ile başlatılmıştır. Tür tayini için yaygın olarak kullanılan değişken bir bölge olan 16S ribosomal RNA geninin V3–V4 bölgesi PCR ile çoğaltılmıştır. Ardından yeni nesil dizileme (NGS) uygulanmış ve elde edilen diziler biyoenformatik analizden geçirilmiştir.

Hibrit reaktör performansını değerlendirmek amacıyla elde edilen çıkış konsantrasyonu sonuçları, hibrit kontrol reaktöründe yaklaşık 81% ve hibrit mikrokirletici reaktöründe yaklaşık 87% oranında karbon giderimi olduğunu göstermiştir. Amonyum giderimi (NH_4^+) hibrit kontrol reaktöründe yaklaşık 77%, seçilen NSAIDs'lerin varlığında ise yaklaşık 90% olarak ölçülmüştür. İbuprofen (100%), indometasin (100%), naproksen (99%), mefenamik asit (98%), ketoprofen (96%) ve diklofenak (69%) için anlamlı giderim verimliliği elde edilmiştir. Seçilen NSAID'ler, nitrifikasyon sürecini ve karbon giderimini inhibe etmemektedir. Ayrıca düşük OLR, seçilen NSAID'lerin giderimini olumsuz etkilememiştir.

Hibrit mikrokirletici reaktöründe gözlenen baskın filumlar askıda biyokütlerde 42.40% ile *Bacteroidota*, 35.13% ile *Proteobacteria*, tutunmuş biyokütlerde ise 29.28% ile *Proteobacteria* ve 28.31% ile *Bacteroidota* olarak belirlenmiştir.

Cins düzeyinde ise hibrit mikrokirletici reaktöründe askıda biyokütlerde baskın olan cinsler arasında *Chitinophagaceae* ailesine ait kültüre edilmemiş bir cins (10.08%), *Ferruginibacter* (7.82%) ve *Comamonas* (6.77%) yer alırken; tutunmuş biyokütlerde *Fimbriimonadaceae* (8.73%) ve *Parafilimonas* (6.46%) baskın olarak gözlemlenmiştir.

Mikrobiyal çeşitliliği gösteren Shannon indeksi, hibrit reaktöre seçilen NSAID'lerin eklenmesinden sonra tutunmuş biyokütlerde artış göstermiş, hibrit mikrokirletici reaktörünün askıda biyokütlesinde ise çeşitlilikte azalma gözlemlenmiştir. Bu durum, NSAID baskısı altında yüzeyde çoğalan, biyokütlerde daha yüksek çeşitliliğin olduğunu düşündürmektedir. Ayrıca, operasyonel taksonomik birimler (OTU) sayısı NSAID ilavesinden sonra, yüzeyde çoğalan biyokütlerde artarken, hibrit mikrokirletici reaktörünün askıda biyokütlesinde azalmıştır. Bu durum, yüzeyde çoğalan biyokütlerin NSAID ilavesi sonrası daha zengin bir mikrobiyal profile sahip olduğunu göstermektedir. Ancak Pielou's evenness indeksi için durum tersine olmuştur. Bu indeks, seçilen NSAID'lere maruz bırakıldıktan sonra her iki reaktörde de azalmıştır. NSAID baskısı, mikrobiyal topluluğun eşitliğindeki düşüşe katkıda bulunmuş olabilir. Yüzeyde çoğalan biyokütlerde askıda biyokütleye kıyasla daha yüksek biyoçeşitlilik ve zenginlik gözlemlenmiştir. Bu durum, biyofilm ortamında çeşitli fonksiyonel bakterilerin bir arada bulunmasını kolaylaştıran artan mikrobiyal karmaşıklığa bağlanabilir.

Hibrit kontrol ve hibrit mikrokirletici reaktörlerinde askıda ve yüzeyde çoğalan biyokütlerin biyoçeşitliliğindeki farklar, çeşitli çalışmalarda da görüldüğü gibi, NSAIDs baskısından kaynaklanmış olabilir.

İnokulum çamuruna kıyasla hibrit kontrol reaktörünün askıda ve tutunmuş biyokütlesinde *Saprospiraceae* ailesine ve *Saccharimonadales* takımına ait kültüre edilmemiş cinslerde önemli azalma görülmüştür. Öte yandan, inokulum çamuruna kıyasla hibrit kontrol reaktörünün askıda biyokütlesinde *Niabella* ve *Paracoccus* gibi bazı cinslerde önemli artış tespit edilmiştir. Yüzeyde çoğalan biyoküttelede ise *Paracoccus*, *Ottowia* ve *Fimbrioglobus* cinslerinde anlamlı bir artış gözlemlenmiştir. Bu önemli değişikliklerin, laboratuvar ölçekli deneysel koşullara ve bu çalışma için seçilen düşük OLR gibi işletme parametrelerine yanıt olarak ortaya çıktığı düşünülmektedir.

Hibrit mikrokirletici reaktörünün askıda biyokütlesinde baskın olan *Chitinophagaceae* ailesine ait kültürlenmemiş cins, aynı aileye ait *Ferruginibacter* ve *Comamonadaceae* ailesine ait *Comamonas* ile birlikte tutunmuş biyoküttelede baskın olan *Chitinophagaceae* ailesi ait *Parafilimonas* cins, *Rickettsia* ve *Fimbriimonadaceae*, hibrit sistemde yaygın nitrifiye edicilerden ziyade nitrifikasyona katkı sağlamış olabilir.

Benzer çalışmalarda, *Chitinophagaceae* ve *Comamonadaceae* ailelerinin MBBR sistemlerinde azot gideriminde etkili olduğu belirlenmiştir.

Ayrıca, *Bacteroidota* ve *Proteobacteria*, azot ve mikrokirletici gideriminde önemli rol oynayan filumlar olarak bildirilmiştir.

SILVA database, daha fazla takson tanımlayabildiği için bu çalışma için uygun bir veri tabanı olmuştur.

Mikrobiyal çeşitlilik, NSAID giderimi ve bunun nitrifikasyonla ilişkisini daha etkili bir şekilde incelemek amacıyla, daha uzun hedef gen bölgelerini içeren ayrıntılı qPCR ve NGS analizleri ya da alternatif moleküler teknikler kullanılabilir.



1. INTRODUCTION

Hybrid moving bed biofilm (MBBR) technology has been widely implemented for biological nutrient removal (BNR) processes, especially for nitrogen removal. The first full-scale MBBR plant was built in 1994 in Norway, designed for biological nitrogen removal (Rusten & Ødegaard, 2023). Hybrid MBBRs have emerged as a promising alternative of conventional activated sludge (CAS) due to their improved removal capacities. The hybrid MBBR supports the growth of microorganisms on moving carriers added to the aeration tank of CAS systems. This configuration enhances the concentration of biomass in the reactor without increasing the volume of the tank to improve the treatment efficiency (Godzieba et al., 2022; Tang et al., 2020). This hybrid configuration allows the coexistence of suspended biomass and attached biomass in one treatment step. The accumulation of nitrogen compounds in water bodies, caused by urbanization, agricultural and industrial activities, resulting in serious environmental issues such as eutrophication of natural waters. Consequently, effective nitrogen removal before discharge to environment is crucial (T. Liu et al., 2020; Phanwilai et al., 2020; Song et al., 2019). Conventional activated sludge systems are widely applied for this purpose, but they are unable to maintain sufficient nitrification performance under fluctuating conditions, especially low temperature and high nitrogen loads (Güneş et al., 2019a; Phanwilai et al., 2020). In contrast hybrid MBBRs, showed advantages over CAS systems for nitrogen removal by maintaining an favorable environment for nitrifiers to development in the system, resulting in effective ammonium conversion to nitrate even at fluctuating operational conditions such as high organic loading conditions and low temperatures. Hybrid systems could sustain higher sludge retention times effectively, thus enhances the growth of slow growing autotrophs. High proportion of the nitrifiers could accomplish full nitrification (Güneş et al., 2019b).

The configuration of hybrid MBBR technology improves the nutrient removal capacity of the system, especially by enhancing the nitrification, and enabling hybrid MBBR systems to be designed with smaller footprint comparing to the CAS systems (Tang et al., 2020).

Compared to the CAS system, hybrid MBBRs provides significant advantages. CAS systems have been commonly used in municipal wastewater treatment plants due to their ability to remove organic matter and nutrients; however, they are not specifically designed for the removal of pharmaceuticals or other emerging contaminants. Different studies reported poor removal efficiencies for pharmaceutical pollutants due to the low biodegradability of some commonly used drugs, such as diclofenac (Arola et al., 2017). Moreover, CAS system are sensitive to fluctuating loading conditions and show low nitrogen removal efficiency, especially in cold climate (Tang et al., 2020; Van Winckel, 2014). The increasing occurrence of nonsteroidal anti-inflammatory drugs (NSAIDs), as growing significant environmental contaminants in wastewater, along with their harmful effect on both human health and ecosystem, highlights the importance of developing innovative and effective treatment plants. Hybrid MBBR systems have shown improved efficiency in addressing this issue. For example, various studies reported significantly higher removal rates for low biodegradable pharmaceuticals such as diclofenac in the attached biomass of hybrid system comparing to CAS system (Falås et al., 2013). Furthermore, Wolff et al. (2021b), observed substantially higher overall transformation potential for several micropollutants (e.g., diclofenac) in attached biomass of three MBBR systems comparing to suspended sludge of the system. Therefore, Hybrid MBBR system showed to be an effective solution to remove NSAIDs.

Organic loading rate (OLR) is an important factor which considerably influence the removal efficiency of organic micropollutants such as NSAIDs in the WWTPs. Low OLR can promote the nitrification (Al-Sayed et al., 2023) and could be kept low for efficient nitrification in MBBR systems (Zellmer, 2024). In elevated OLRs, where more organic carbon is present, a competition between heterotrophic and autotrophic (nitrifying) organisms is expected (Al-Sayed et al., 2023). In some treatment plants low OLR may lead to contradictory results on organic micropollutants removal (Abtahi

et al., 2018; Kora et al., 2020). MBBR technology, which supports the attached biofilm formation, was reported to achieve effective treatment performance even in fluctuating organic loading conditions (Tang et al., 2020).

NSAIDS are pharmaceutically active compounds that are extensively used worldwide by human, as well as veterinary medicine, because of their analgesics (pain-relieving), antipyretic (fever-reducing), and anti-inflammatory properties. Most of them are available over-the-counter and can be sold without prescription (Green, 2001; Rastogi et al., 2021). Diclofenac, ibuprofen, naproxen, ketoprofen, mefenamic acid, and indomethacin are of the widely observed NSAID compounds in different water bodies. These compounds enter the environment from different pathways (Feng et al., 2013; Rastogi et al., 2021). Conventional treatment systems generally are not appropriate to remove most of these micro pollutants, they can discharge to the environment with treated effluent and threaten the human and animal life. In prolonged exposure, there is a risk of chronic toxic effect in organisms living in those environments even at low concentration of drugs because of their physicochemical characteristics. And also, there is a risk of accumulation of drugs or their metabolites in the food chain. Different studies claimed that, long term exposure could increase the risk of cancer in human (Zare et al., 2022). The removal of pharmaceuticals generally occurs with nitrification and carbon removal (Tang et al., 2020). There is a hypothesis that is partially supported by the literature, suggesting that nitrifying bacteria can improve the removal of certain micropollutants, such as diclofenac (Suarez et al., 2010; Torresi et al., 2017).

Microbial communities play a crucial role in the performance of wastewater treatment plants (WWTPs) due to their involvement in degradation of contaminants. Different factors such as operating conditions and also the composition of influent, particularly, the presence of emerging micropollutants such as mixture of NSAIDs in the WWTPs, can make a significant change in microbial community structure and diversity (Farkas et al., 2023; Y. K. Kim et al., 2019); therefore, evaluating only the chemical performance of hybrid systems does not provide a complete understanding of their overall performance in terms of microbial community dynamics. In this study, to assess the microbial community structure and diversity of biofilm and activated sludge in the hybrid reactor, 16s ribosomal RNA sequencing was applied, followed by statistical

analyses, as well as alpha diversity analysis. Alpha diversity quantifies the species diversity in an ecological community, focusing on richness and evenness of species (B.-R. Kim et al., 2017). Different indices are used to represent the alpha diversity, including observed OTUs (Operational Taxonomic Units), Chao 1, Shannon–Weaver Index (H), Pielou’s evenness, good coverage, Simpson diversity index, and Fisher diversity index. Shannon index reflect the microbial diversity and Chao1 index show the richness of microorganisms in the activated sludge (S. Liu et al., 2022). It is important to evaluate the diversity of the system because it gives us an overview for the stability of the system, as well as function of the community (Cook et al., 2006; Wu et al., 2019). Briones and Raskin (2003) stated that, system stability depends more on the different microbial community that are able to do the same job, than just diversity; which it helps the system to maintain stable functions. The effect of micropollutants on the microbial diversity and community structure is an interesting area for research. Several investigations reported higher diversity and richness in microbial community attached to the carriers (Ribera-Pi et al., 2020; Wolff et al., 2021b). Moreover, different studies have compared the microbial profile in activated sludge and biofilm but the results are not consistent; so, this study attempts to contribute to addressing this gap.

In this study, we aim to assess the performance of hybrid biofilm nitrification system to remove targeted NSAIDs mixture, including diclofenac, ibuprofen, ketoprofen, naproxen, indomethacin, and mefenamic acid under low organic loading condition, and also to investigate the changes in microbial community structure and to evaluate the biodiversity in both suspended and attached biomass and to compare the overall change in biodiversity of the hybrid system.

2. LITERATURE REVIEW

2.1 Micropollutants

Micropollutants (MPs), also known as emerging contaminants (ECs), comprise a wide range of substances such as pharmaceuticals, personal care products, and different bioactive chemicals. The concentrations of these contaminants change from micrograms to nanograms per liter and they have been identified in both urban wastewater and natural aquatic environments (C. Liang et al., 2021).

The rapid progress of global socio-economic advancement has led to the production of various new chemicals, significantly impacting the environment soon after their introduction. Chemical industry is responsible for producing more than 70000 diverse chemical products with a global sale value approximately \$5000 billion (Asthana, 2014). Some of these substances pose a substantial risk for human health and the environment after being introduced to the aquatic environment (Rogowska et al., 2020).

Regardless of significant advancement, many pollutants in the wastewater cannot be removed efficiently in conventional WWTPs. Analytic compounds, for instance endocrine-disrupting chemicals (EDCs) or pharmaceuticals have become of great interest because they were observed in the treated wastewaters which were ended up in receiving bodies like surface waters and lakes and rivers or, in some regions, sea waters. Therefore, these compounds and their transformation products and metabolites are of great concern to scientists and eventually end up in marine environments. Managing the environmental effect and potential health risks of these compounds is difficult because they are poorly understood (Rogowska et al., 2020).

In the last ten years, the European Union (EU) has increased its political attention on water quality by recognizing wastewater treatment plants (WWTPs) as significant sources of pollution (Corominas et al., 2013).

Some compounds are not strictly regulated by the law. For example, nowadays pharmaceuticals are one of the important groups which in the United States over 10,000 prescribed drugs and around 300 over-the-counter medications enter the environment through the process of manufacturing and usage (Dong et al., 2013). Due to the huge emission of them in the environment, they can be detected in wastewater. The second large group comprises unknown compounds. Moreover, the degradation and transformation of such chemicals may result in unknown structures which can be potentially more hazardous to the environment and the organisms than their parent compounds (Garnaga, 2012).

Physical and chemical properties and the affinity of micropollutants for the solid matter and water have a huge effect on the environmental consequences which results in a significant impact on their bioavailability. Moreover, the mobility of compounds and their ability to move up the food chain affects the living organisms, for example the substances can dissolve in water or can adhere to the solid particles and eventually accumulation in the tissue of marine organisms. Changes in the environmental conditions such as temperature, salinity, turbidity, or pH changes also influence the bioavailability of micropollutants. In this regard, the concentration of micropollutants in the living organism's tissue can also affect the rate of bioaccumulation. It can be in quantities like the level of micropollutants in the environment or even higher than those found in the environment (Garnaga, 2012).

Micropollutants have been increasing since industrialization began and are detected across diverse environmental matrices. They originate from diverse sources, including synthetic products like cosmetics, pharmaceuticals, steroid hormones, pesticides, surfactants, and persistent organic pollutants (POPs), etc. and they need precise monitoring to control their distribution and assess their variability over time. The physicochemical properties of These contaminants such as vapor pressure, solubility, and polarity govern their behavior, toxicity, and adequate treatment methods (Bunke et al., 2019; Luo et al., 2014a).

There are different pathways by which contaminants reach aquatic environments and pose serious threats to ecosystems and public health Gooty et al. (2022), dependent on

the origin and application of micropollutants. Therefore, scientists and stakeholders have been attempting to regulate the reuse of wastewater for irrigation (Rizzo et al., 2018). The primary routes that contaminants enter aquatic environments include runoff from agricultural or urban areas, industrial or municipal wastewater discharges, sludge accumulation, and accidental spills (Gooty et al., 2022).

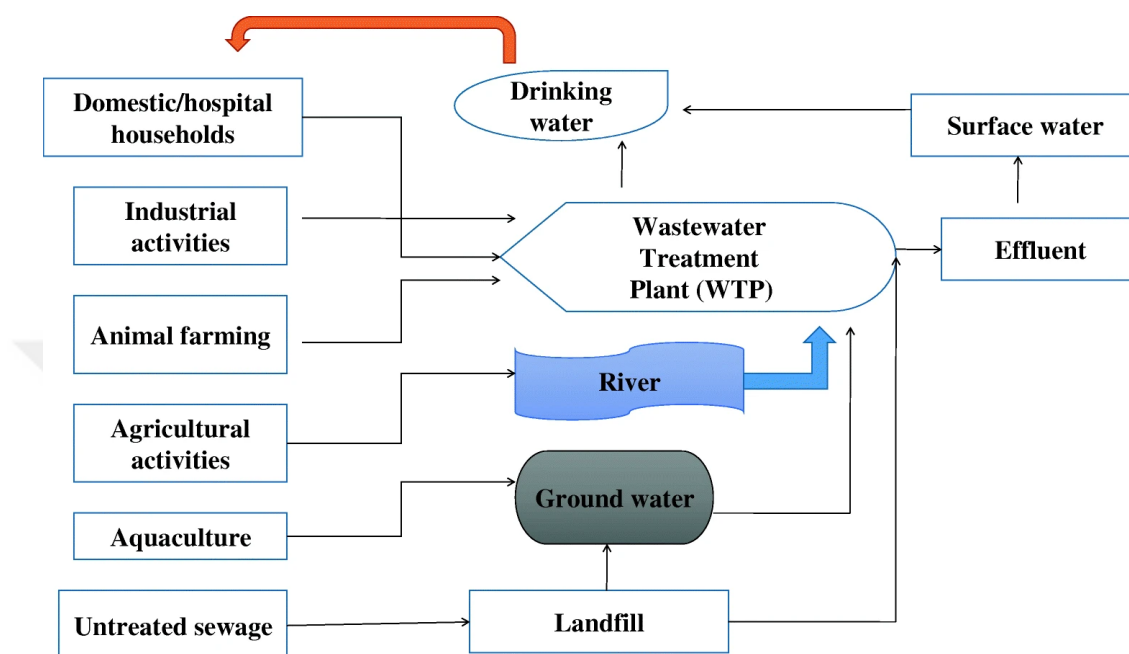


Figure 2.1: Representative sources and routes of micropollutants in the environment. Taken from Kanaujiya et al. (2019).

Removal and therefore, the bioavailability of micropollutants in the environment is variable because of some factors such as: different environments (surface and groundwater, sediments, and wastewater treatment or water purification plants), their unique physicochemical properties (polarity, solubility, and vapor pressure) (Virikutyte & Varma, 2010). After being introduced into the ecosystem, these compounds depending on the type of molecule undergo several biotic and abiotic processes. Humification is a significant process where micropollutants are incorporated into humic macrostructures that finally transform into the sediment (Gooty et al., 2022).

Nonsteroidal anti-inflammatory drugs including, triclosan, sulfamethoxazole, and carbamazepine are the most frequent micropollutants in surface water. A study held in 2011 indicates that hospital effluents are the predominant source of high pollution concentration in Costa Rica (Gooty et al., 2022). Ibuprofen, ketoprofen, gemfibrozil,

and caffeine concentrations were measured as 36.8, 9.8, 17.0, and 1121.4 µg/L, respectively.

2.2 Pharmaceuticals

Pharmaceuticals are biologically active compounds. They play an important role in both human and animals. Herbal medicine such as plants crude extracts and shrubs are herbal medicine that are used for healing wounds, pain relief and also for treating different type of disease were the primary source of therapeutic in the beginning of 19th century (Tiwari et al., 2017a). The development of synthetic compounds like chloroform, aspirin became common after advancement in medical science, leading to the production of thousands of pharmaceuticals that are widely used today (Tiwari et al., 2017a). The daily intake of pharmaceuticals is increasing in private households. The occurrence of pharmaceuticals in wastewater has become well documented due to the improvement in some analytical high-resolution techniques for the screening of trace chemicals (Martínez-Alcalá et al., 2017; Sanganyado et al., 2017; Tran et al., 2018; Y. Yang et al., 2017).

A study showed a twofold increase in the daily use of antihypertensive, cholesterol-lowering, antidiabetic, and antidepressant drugs in OECD countries (Organization for Economic Cooperation and Development) from 2000 to 2013 (Indicators, 2015) (Tiwari et al., 2017a). By growing the consumption of pharmaceuticals, the concerns about their environmental impacts have raised. Many of these drugs end up in municipal wastewater treatment plants (WWTPs) through human's urine and feces because many of them are not fully metabolized in human body (Tiwari et al., 2017a).

Conventional wastewater treatment plants are basically designed for eliminating nitrogen, phosphorous and mostly biodegradable organic matter, therefore a significant concentration of non-biodegradable pharmaceuticals often can remain in the effluent of WWTPs (Tang et al., 2020). Different studies have identified the presence of various drugs, including metformin, atenolol, carbamazepine, clarithromycin, trimethoprim and anticancer drug tamoxifen and ciprofloxacin in river water and wastewater effluent

with concentrations in range of nanogram to microgram per liter (Ferrando-Climent et al., 2014; M. Kim, Guerra, Shah, Parsa, Alaei, & Smyth, 2014).

Distribution of sources and the socioeconomic conditions of the population influence the type and concentrations of these compounds in natural and artificial environments (Kumwimba & Meng, 2019). China is one of the largest producer and consumer of chemicals in the world along with US and Japan which use approximately 25000 tons of antibiotics per year (Bu et al., 2013; Hao et al., 2015). Over 20% of the global pharmaceutical production volume is by China (J.-L. Liu & Wong, 2013). Moreover, average antibiotic consumption is roughly 10 times more than United States (N. Wang et al., 2016).

Pharmaceutical pollutants enter the environment from different sources (Figure 2.2). In particular, the major contributor is hospitals because of usage of huge volume of water per bed which is approximately 400-1200 liters per day. Other important sources are pharmaceutical industries, direct discharge of agricultural runoff, livestock and aquaculture, urban and highway runoff and also using therapeutic compounds by researchers and improper exposure of drugs (Kumwimba & Meng, 2019; Tiwari et al., 2017a). Moreover, the wastewater coming from hospitals is not only contains pharmaceuticals, but also contains pathogens, drug conjugates, radioactive elements, other metabolites of pharmaceuticals and other chemicals that diminish the biodegradation process in wastewater treatment plants even if at diluted concentrations of pharmaceuticals (Pauwels & Verstraete, 2006). Continuous discharge of diclofenac in anoxic sludge treatment plants leads to a less gas production and also hinder the denitrification potential of microbial community (Ozdemir et al., 2015).

There is a considerable risk for the human health as well as aquatic ecosystem when face with these persistent compounds. Many short-term toxicity studies indicated that although, the low concentrations of pharmaceuticals in the environment do not have serious effect on aquatic environment, but long-term exposure to these compounds have long-term/chronic consequences (Tiwari et al., 2017a). According to a laboratory research, estrogen causes vitellogenesis in male *Oryzias latipes* (Japanese medaka), and high estrogen levels increase fish mortality (Jukosky et al., 2008). A well-known

example for the prolonged exposure to low concentration of pharmaceutical is the feminization of male fish because of estrogen in the water. Presence of dutasteride can decrease the fish fertility and also have effects on the reproductive functions of both male and female fish. A sharp drop in vulture populations in Asia is due to the veterinary drug diclofenac in their food that it is the reason of visceral gout, kidney failure, and finally death (Margiotta-Casaluci et al., 2013; Tiwari et al., 2017a).

Pharmaceuticals that have high values of octanol-water partition coefficients ($\log K_{ow}$) (a measure of hydrophobicity) are more likely to adsorb to sludge resulting in higher removal rates (Rogers, 1996). Other removal mechanisms such as volatilization and photodegradation are rarely operational due to the physical properties of these substances. Biodegradations of pharmaceuticals that plays an important role are influenced by various factors including their bioavailability, structure, redox potential, stereo chemical, pH and the chemical characteristics of sorbent and the sorbed molecules as well. The biodegradation of aromatic/long chain compounds are harder than short side chain/aliphatic compounds (Tiwari et al., 2017a). Presence of electron withdrawing/donating groups has effect on their removal in WWTPs (Wijekoon et al., 2013). Microbial diversity and abundance are influenced by sludge retention time (SRT) which has an impact, on the removal of pharmaceuticals by encouraging the growth of slow growing microbes that remove nitrogen efficiently over time. Hydraulic retention time (HRT) is important for removing pollutants with low sorption potential, and pH influences the degradation of ionizable compounds. Change in season and temperature affect the microbial activity like that higher removal rates are seen in warmer temperatures although some researches indicated that removal rates may not always be temperature dependent. Increasing STR is a possible strategy to address the seasonal fluctuations in removal efficiencies (Suarez et al., 2010).

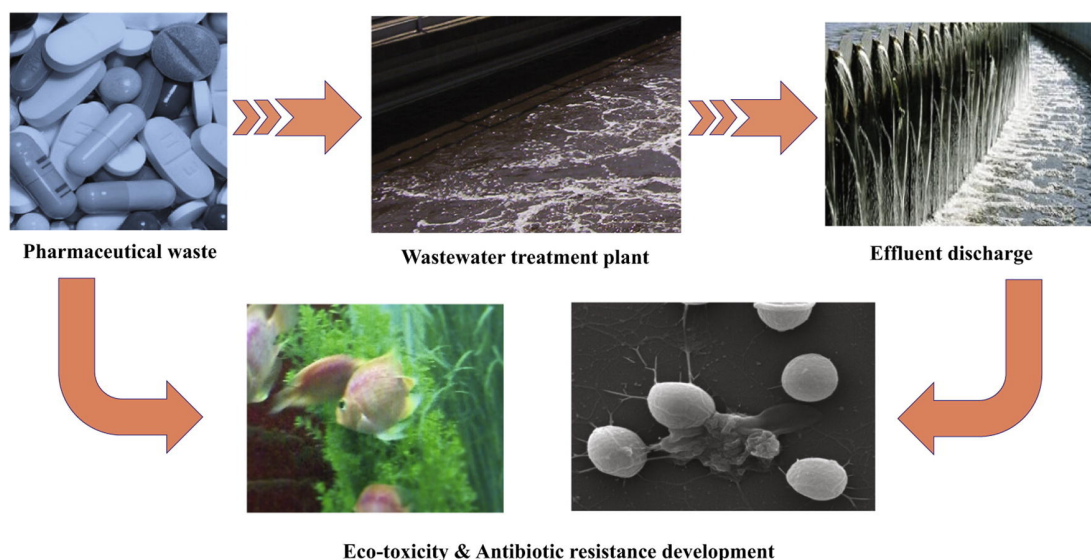


Figure 2.2: Source and pathways of pharmaceutical in the environment. Taken from Tiwari et al. (2017a).

2.3 Non-steroidal Anti-inflammatory Drugs (NSAIDs)

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs), which are among the top 10 persistent pollutants, have been identified as a significant environmental contaminant because of incomplete degradation in wastewater treatment plants (WWTPs) and also their physiological effects on human even at low concentrations over the past few years (Green, 2001; Rastogi et al., 2021).

Diclofenac (DCF), ibuprofen (IBF), and naproxen (NPX) are the most frequently identified NSAIDs in the environment (Izadi et al., 2020). According to the European Union classification, they are classified as high-priority pharmaceutical substances according to the persistence, occurrence, toxicity, etc. (De Voogt et al., 2009; Sousa et al., 2018).

It is estimated that about 3000 different substances are used as pharmaceutical ingredients such as NSAIDs, pain killers, antidiabetics, beta-blockers, antibiotics, impotence drugs and lipid regulators (Rodriguez-Narvaez et al., 2017). NSAIDs which have analgesics, antipyretic, and anti-inflammatory properties are considered as one of the most frequently used ones (pharmaceuticals) in both human and veterinary medicine because most of them can also be sold without a prescription.

The concentration levels of drugs differ between country to country and it is governed by some factors like population of the countries, drug consumption pattern, and also the effectiveness of treatment plants (Tijani et al., 2013). And also, there is a significant difference between conducted studies on monitoring of emerging contaminant in low and middle-income countries compare to high-income countries (Williams et al., 2019).

Diclofenac (DCF), ibuprofen (IBF), naproxen (NPX), ketoprofen (KTF), mefenamic acid (MFA), and indomethacin (IMC) are of the widely observed NSAID compounds in different water bodies and other environmental compartments (Feng et al., 2013; Rastogi et al., 2021). The solubility, biodegradability, volatility and other physicochemical properties of six commonly detected pharmaceuticals are shown in Table 2.1, may be useful to understand their fate in wastewater treatment plants and also their level of persistence in different environments. As an example, octanol-water partition (K_{ow}) and the solubility of pharmaceuticals define their level of hydrophobicity and it is important to predict the sorption potential of the pollutant to sludge phase. Furthermore, the Henry's Law constant is used to estimate the volatility of contaminants, and as it is shown in the Table 2.1.

Diclofenac, ibuprofen and naproxen have high-octanol partition coefficient ($\log K_{ow}$) (shows compound's hydrophobicity and potential for bioaccumulation) and low pKa values (Table 2.1). These drugs mitigate pain and inflammation through cyclooxygenase COX enzymes inhibition and blocking prostaglandin production which play crucial roles in standard biological functions and body regulation with pain relief and anti-inflammatory effects resulting from their suppression (Izadi et al., 2020).

Table 2.1: Physical and chemical properties of the common anti-inflammatory drugs.
Taken from Izadi et al. (2020).

NSAIDs	Molecular formula	Mass (g/mol)	pKa	Solubility (mg/L) (20°C)	LogK _{ow}	Henry's law constant (atmm ³ mol ⁻¹)	Environmental fate	Metabolic forms in water
Ibuprofen	C ₁₃ H ₁₈ O ₂	206.3	4.15	21	4.51	1.5×10^{-7}	Biodegradation, Sedimentation	2-[4-(2-Hydroxyl-2methylpropyl)phenyl] propionic acid, 2-[4-(2-carboxypropyl)phenyl] propionic acid, carboxy-hydratropic acid (carboxy-HA)
Naproxen	C ₁₄ H ₁₄ O ₃	230.3	4.15	144	3.18	3.4×10^{-10}	Biodegradation, Photodegradation	NPX, 6-O-desmethylated metabolite, DM-naproxen
Diclofenac	C ₁₄ H ₁₁ Cl ₂ NO ₂	296.2	4.91	2	4.51	3.13×10^{-14}	Partial biodegradation, photodegradation	4'-Hdroxy DCF, 4' 5'-dihydroxy DCF, 3'-hydroxy DCF, 5'-hydroxy DCF, 3' hydroxy-4'-methoxy DCF
Ketoprofen	C ₁₆ H ₁₄ O ₃	254.3	4.45	51	3.12	2.1×10^{-11}	Partial biodegradation, Photodegradation	2-(3-Benzoylphenyl)-propanoic acid, 2-[3-(3hydroxybenzoyl) phenyl]-propanoic acid, 2-[3-(4hydroxy benzoyl) phenyl]-propanoic acid, 2-[3 (hydroxy (phenyl)methyl)phenyl]-propanoic acid
Mefenamic acid	C ₁₅ H ₁₅ NO ₂	241.3	5.12	20	5.12	–	Partial biodegradation	Alkali-labile ester glucuronides
Indomethacin	C ₁₉ H ₁₆ ClNO ₄	357.8	4.5	9	4.51	4.7×10^{-12}	–	–

As mentioned in the previous section, NSAIDs along with other PPCPs find their way to environment through different pathways like, direct discharge from pharmaceutical industries or hospital wastewater treatment facilities, and leachate from landfills or solid waste treatment plants, topically at concentrations ranging from ng/L to $\mu\text{g/L}$ (Paíga et al., 2016). The continued and improper usage and discharge of NSAIDs resulting in accumulation in ecosystems, and consequently affects aquatic life and potentially disrupting human health (Rastogi et al., 2021).

NSAIDs are commonly found in both influent and effluent of treatment plants, with concentrations ranging from 0.1 to 10 $\mu\text{g/L}$. Ibuprofen concentration is high in wastewaters because of its frequent usage although it shows low excretion (5%) in compare to other NSAIDs like diclofenac with 6-39% rate. As the WWTPS cannot remove micropollutants efficiently, they would remain in treated effluent, resulting in contamination of the environment (Izadi et al., 2020).

Environmental factors like temperature, pH, nutrients and also inherent microbial species specifically influence the half-lives of NSAIDs in different types of water bodies (Rastogi et al., 2021). Different studies have conducted on persistency of NSAIDs which shows that NSAIDs are generally considered as persistent, though some shows varying results. For example, diclofenac and ibuprofen have half-life ranging from less than 30 days and 4.6-32 days, respectively (Bu et al., 2016); while another study reported a shorter half-life for the diclofenac under specific conditions in lake and raw river water and longer half-time (63.6 days) for ibuprofen in river waters and an even longer photolysis half-life of 413 days (Araujo et al., 2014; Yamamoto et al., 2009). For naproxen half-life varies from 9.6–18.5 days in lake and reservoir water and 27 days in rivers which effectively classifying it as persistent active pharmaceutical ingredient (Araujo et al., 2011), whereas the high biodegradability and low persistence of ketoprofen were based on its short life-time (hours rather than days) and its almost fully photodegradation in just 10 minutes in exposure to UV-B irradiation, as well as the fact that ketoprofen was not detected in many of the surface waters sources that were under the studies (Araujo et al., 2014; Bu et al., 2016; León et al., 2021). Moreover, naproxen and ibuprofen were reported to be more persistent in soil compared to water, while diclofenac's half-life in soil was in range of its half-life in water bodies (Lin & Gan, 2011). High concentrations of NSAIDs have been measured in certain rivers because of direct discharge of raw sewage, such as Beberibe River in Brazil, where diclofenac concentration reached as high as 193 $\mu\text{g/L}$ (Rastogi et al., 2021; Veras et al., 2019).

Although NSAIDs are present globally in surface waters, the Predicted Environmental Concentrations (PEC) are often lower than the Predicted No-Effect Concentrations (PNEC) resulting in PEC/PNEC ratio (risk quotient) smaller than 1 which means no significant environmental risk. PNEC values for diclofenac, ibuprofen, naproxen, and ketoprofen are 10, 7.1, 6.6, and 0.16 $\mu\text{g/L}$, respectively. However, the EU Watch List 2015 set a much lower PNEC value for diclofenac at 0.05 $\mu\text{g/L}$ (Commission, 2015; Rastogi et al., 2021).

2.3.1 Diclofenac (DCF)

Diclofenac ranks among the most commonly used nonsteroidal anti-inflammatory drugs (NSAIDs) which is recognized for its antipyretic and analgesic effects for treating different diseases, including cancer, neurodegenerative diseases and arthritis (Baranov et al., 2023). It is the first drug on the EU watch list of priority substances because of its significant uses, incomplete removal and also toxic effects (Nosek & Zhao, 2024). Diclofenac is an analgesic that appears as white crystal or crystalline powder and it is classified as non-steroidal derivative of benzene acetic acid (PubChem, 2023). It can be found in the environment because of its intensive use in the form of human waste (Baranov et al., 2023), but It is almost impossible to quantify the global consumption of diclofenac because of different reasons such as, sold through different trading names including: Voltaren Emulgel in Canada, Votalin in China, Volini in India, Diclo-Denk in Germany, Voltaren in Argentina, Australia, Belgium, Egypt, France, Germany, Israel, New Zealand, Norway, Portugal, Russia, South Africa, Sweden and Turkey (Lonappan et al., 2016). They are available as over-the-counter medicine and use for both human and veterinary applications. However, Y. Zhang et al. (2008) estimated the global consumption of diclofenac as 940 tons per year according to the data from Intercontinental Marketing Services (IMS) Health.

According to the International Union of Pure and Applied Chemistry (IUPAC), diclofenac is called 2-[2-(2,6-dichloroanilino) phenyl] acetic acid (PubChem, 2023). The annual consumption of diclofenac is 1443 ± 58 tons and it holds a market share nearly equal to the combined market share of the next three leading drugs (ibuprofen, mefenamic acid, and naproxen) (McGettigan & Henry, 2013). The molecular formula of diclofenac is $C_{14}H_{11}Cl_2NO_2$. The molecular weight and CAS number are 296.1 g/mol and 15307-86-5 respectively (PubChem, 2023). The terminal half-life of diclofenac, indicating that how fast the diclofenac itself is reduced in half in body, is about 2 hours while apparent half-life is extended to 25.8-33 hours which shows the longer time is needed for removing of both diclofenac and its metabolites in the body (PubChem, 2023). The melting point is measured to be 283–285°C. The

solubility of diclofenac in water at 25 °C is low, documented as 2.37 mg/L (PubChem, 2023). Toxicological and physico-chemical properties of diclofenac are demonstrated in Figure 2.1. Diclofenac's molecular configuration is illustrated in Figure 2.3. It tends to stay in the aqueous phase which makes it difficult to be removed from water and is due to its low K_{ow} and pK_a values (Izadi et al., 2020). The main mechanism of action of diclofenac is similar to all NSAIDs which works through the inhibition of the cyclooxygenase (COX) enzyme, result in suppressing the prostaglandin synthesis and contributes to its anti-inflammatory and pain-relieving properties (Warner et al., 1999). However, other mechanisms of action of NSAIDs associated with drug's membrane activity are also considered to play a role. Moreover, diclofenac has exhibited the capacity to chemically interact with the protective phospholipids of the gastrointestinal barrier, which might be responsible for gastrointestinal toxicity. The interactions of diclofenac with the plasma membrane are of interest because they can be helpful in explaining the therapeutic effect and side effects of diclofenac at molecular level (Baranov et al., 2023). For example, cardiovascular pathologies such as myocardial infarction, thrombosis and hypertension may rise as a result of change in lipid composition or membrane structure (Wallace, 2008).

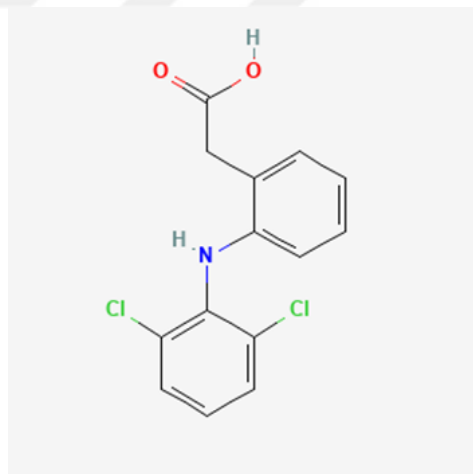


Figure 2.3: Chemical structure of Diclofenac (PubChem, 2023).

Studies on the toxicological effects of NSAIDs on living organisms is limited, while they are threatening for public health and non-target organisms as well. For example, for diclofenac some effect has been recorded such as bioaccumulation in the liver,

causing inflammatory changes in the esophagus and lower gastrointestinal tract (Izadi et al., 2020).

Toxicity of diclofenac was carried out word wile, demonstrating the effects of diclofenac on aquatic organisms (Lonappan et al., 2016). One of the first studies on toxicity of diclofenac were carried out by Ferrari et al. (2003), revealing relatively low toxicity at environmental concentrations on bacteria, algae, microcrustaceans and fish. On the other hand, later examinations showed potential environmental impacts on the environment.

According to risk assessment conducted by Hernando et al. (2006), the potential ecological risk of diclofenac in surface waters was considerable. Moreover, diclofenac showed harmful effects on aquatic organisms by using acute daphnia and algal tests (Cleuvers, 2004). The same study indicated that at environmental concentrations, adverse effects were minimal or negligible, although mixtures of pharmaceuticals could be highly toxic even at lower concentrations. In *Daphnia magna* species, acute concentrations (mg/L) of diclofenac led to high mortality rates. Studies showed that after 48 hours of exposure, diclofenac increased mortality, with EC₅₀ (half maximal effective concentration) values of 22.4 mg/L and 39.9 mg/L (Lonappan et al., 2016). According to Ferrari et al. (2003), *Ceriodaphnia dubia* species also showed mortality where an EC₅₀ value of 22.7 mg/L was documented. Conversely, Lee et al. (2011) reported EC₅₀ values three times higher than the same species tested for a period of 48-hour. Studies from Canada showed that diclofenac was of high concern even at low environmental concentration (10 – 100 ng/L) with threatening effects on the river biofilm community structure and function at much diluter concentrations of about 100 ng/L (Lonappan et al., 2016).

Diclofenac can accumulate in fish organs and muscles, and its bioconcentration factor (BCF), In the liver or bile of rainbow trout, differ considerably depending on exposure concentrations ranged from 12 to 2732 over a period of 28days at concentrations of 1–50 µg/L, 509 to 657 over a period of 20 days at concentrations of 0–25 µg/L, 291 to 1610 over a period of 10 days at concentrations of 1–50 µg/L, and 320 to 950 over a period of 10 days at concentrations of 1.7 µg/L (Nosek & Zhao, 2024).

According to different studies diclofenac has been found to cause ecotoxicological effects at low concentrations ($\mu\text{g/L}$) for example in certain fish, including endocrine-disrupting activities, also in muscles, crabs, daphnia and also river microbial biofilm (Nosek & Zhao, 2024).

The no observed effect concentration (NOEC) for diclofenac is between 0.5–7.3 $\mu\text{g/L}$. Although individual compounds may have minimal impact, the combined long-term (chronic) effects of multiple chemicals, such as metabolites, are possible. Diclofenac and its metabolites may interact with other contaminants in wastewater, potentially leading to new toxic compounds such as antibacterial metal complexes. This topic is explored in depth in literature (Nosek & Zhao, 2024).

Although pharmaceuticals are currently not subject to explicit regulation under the EU Water Framework Directive (WFD, 2000/60/EC) or the Environmental Quality Standards (EQS, 2008/105/EC), the European Commission introduced the first Watch List in 2015 (2015/4951/EC), which included diclofenac along with other major pharmaceuticals (e.g. estrogens and macrolide antibiotics), and required the chemicals to be monitored for between 12 months and four years, with updates every 24 months. In the 2018 update (2018/840/EC), diclofenac was removed from the list because of its low concentration in surface water which was reported across 25 European member states (median 0.027 to 0.047 $\mu\text{g/L}$), which was below the lower PNEC (predicted no-effect concentration) of 0.1 $\mu\text{g/L}$. Nevertheless, the concerns about the toxicological impact of diclofenac persisted, especially as more toxicity data became available on the combined effects of diclofenac (Maculewicz et al., 2022). As a result, the EQS for diclofenac was lowered from 0.10 $\mu\text{g/L}$ to 0.05 $\mu\text{g/L}$ in 2017 (Loos et al., 2018). Recently, Duarte et al. (2022) proposed a chronic PNEC of 0.01 $\mu\text{g/L}$ for diclofenac. In addition, the revised Watch List in 2020 (2020/1161/EC) included, for the first time, a metabolite of the pharmaceutical venlafaxine, O-desmethylvenlafaxine, which remained in the 2022 revision (2022/1307/EC), suggesting a real prospect that more pharmaceutical metabolites will be under closer scrutiny (Nosek & Zhao, 2024).

2.3.2 Ibuprofen (IBF)

Ibuprofen (IBU, 2-(4-Isobutylphenyl) propionic acid), is one of the most commonly prescribed active pharmaceuticals compound in the world and it is classified as non-steroidal anti-inflammatory drug (NSAIDs) (Ferrando-Climent et al., 2012; Jia et al., 2021a). Ibuprofen's molecular configuration is illustrated in Figure 2.4. The molecular formula of ibuprofen is $C_{13}H_{18}O_2$. The molecular weight and CAS number are 206.28 g/mol and 15687-27-1 respectively. Moreover, the melting point of ibuprofen measured to be 75-77.5 °C (PubChem, 2023). Toxicological and physico-chemical properties of ibuprofen are demonstrated in Figure 2.1. According to the International Union of Pure and Applied Chemistry (IUPAC), ibuprofen is called 2-[4-(2-methylpropyl) phenyl] propanoic acid (PubChem, 2023). Ibuprofen is the third most commonly used drug globally with a consumption of nearly 200 tons per year and it is often used to mitigate fever, pain and inflammation and also used for rheumatoid arthritis and osteoarthritis (Chopra & Kumar, 2020; Pietrini et al., 2015; Shaheen et al., 2022).

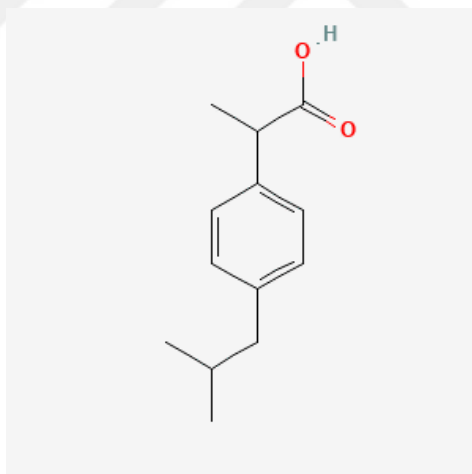


Figure 2.4: Chemical structure of ibuprofen (PubChem, 2023).

Human or animals can only metabolize 5% to 15% of the IBU and more than 85% will be excreted through feces and urine in both its original(pure) form and as different metabolites, some of the metabolites may be more toxic than the parent compound, finally they end up in wastewater treatment plants (WWTPs) (Osman et al., 2024).

Projections claim that by the year 2030, the population of the world will rise to 8.5 billion with a solid waste production of an overwhelming 2.59 billion tons. Therefore, a significant amount of ibuprofen has been measured in surface water, groundwater, and soil, contributing to a potential risk to the food chain and living organisms (Peng et al., 2023).

As a result, ibuprofen is commonly detected in the WWTPs effluent at concentrations ranging from 0 to 1673.0 µg/L, and also the concentrations in surface water and groundwater are 0–36.8 µg/L and 0–3.1 µg/L respectively, so that the environmental concern would rise due to the bioactive nature of ibuprofen (Jia et al., 2021b; Osman et al., 2024).

Although the removal efficiency of ibuprofen in WWTPs can archive up to 90%, its concentration in treated effluent varies significantly ranging from undetectable amounts to as high as 140 µg/L globally (Kermia et al., 2016).

Ibuprofen has functional groups such as carboxylic acids and benzene in its molecular structure so that leads to high mobility but limited solubility in water which allows it to remain in different environments (Osman et al., 2024).

As it is shown in Figure 2.5, ibuprofen could be accumulated in the aquatic environment as the result of improper waste release from hospitals, households, and pharmaceutical industries which leads to a risk of bioaccumulation and ecological harm after entering the food chain through organisms (Osman et al., 2024).

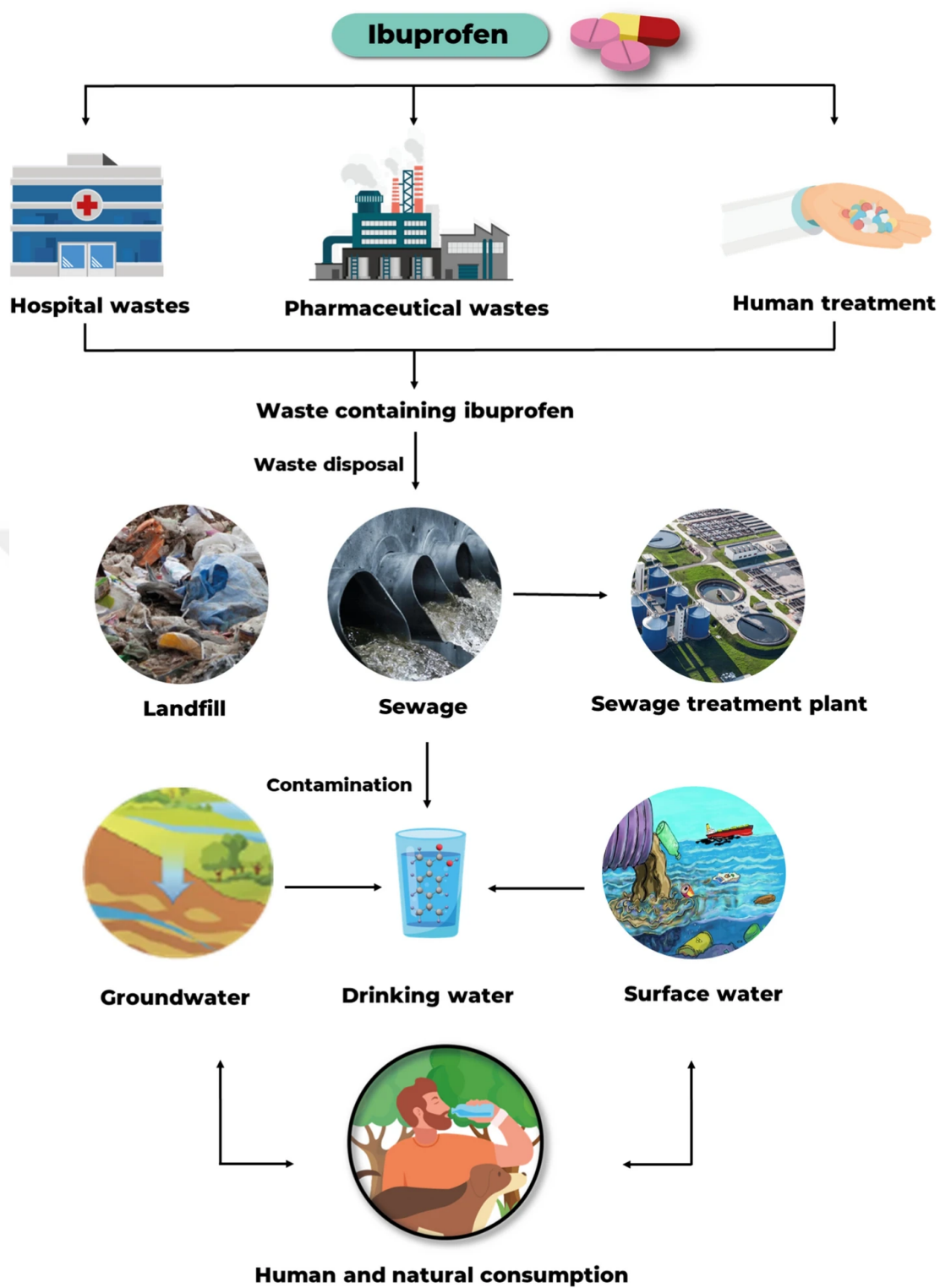


Figure 2.5: Fate of ibuprofen in the environment. Taken from Osman et al. (2024).

2.3.3 Ketoprofen (KTF)

Ketoprofen is also a commonly used NSAIDs. It is prescribed for the acute pain and chronic rheumatoid arthritis and it is known for the analgesic and antipyretic properties. According to the International Union of Pure and Applied Chemistry (IUPAC), ketoprofen is called 2-(3-benzoylphenyl) propanoic acid (PubChem, 2023). The molecular formula of ketoprofen is $C_{16}H_{14}O_3$. Molecular weight and CAS number are 254.28 g/mol and 22071-15-4 respectively (PubChem, 2023). Melting point is measured to be 94°C. And the solubility of ketoprofen in water at 22°C, documented as 51mg/L (PubChem, 2023). Toxicological and physico-chemical properties of ketoprofen are demonstrated in Table 2.1. And also, ketoprofen's molecular configuration is illustrated in Figure 2.6.

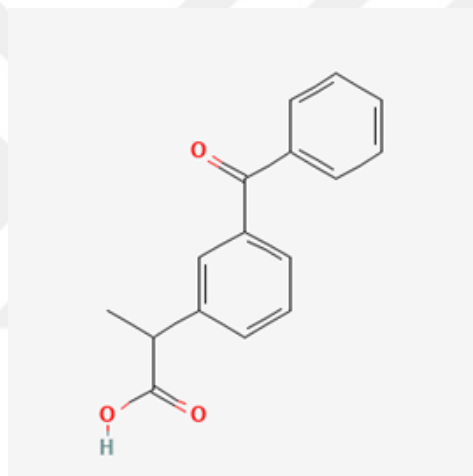


Figure 2.6: Chemical structure of ketoprofen (PubChem, 2023).

Ketoprofen has been frequently detected in surface water, groundwater, as well as drinking water; therefore, it is recognized as emerging environmental contaminant of concern. Low ketoprofen decomposition and its poor removal efficiency in conventional wastewater treatment plants result in accumulation of this drug in aquatic ecosystems (Tyumina et al., 2023). Ecotoxicological studies assess the short-term (acute) and long-term (chronic) toxic effects of substances. Acute toxicity is the adverse effects caused by one or several exposures in a short time that finally lead to the death. Chronic toxicity is important in evaluating the impact of pharmaceutical such as ketoprofen on living organisms. In contrast to acute toxicity, chronic toxicity focuses

long-term effects of continuous exposure contaminants (Parolini, 2020; Tyumina et al., 2023). According to the Global Harmonized System (GHS), ketoprofen is classified as hazardous to aquatic organisms with observing lethal dose (LD₅₀) and effective dose (ED₅₀) values in different species ($10 \text{ mg/L} < \text{LD}_{50} / \text{ED}_{50} \leq 100 \text{ mg/L}$) (United Nations, 2011).

Ketoprofen can bioaccumulate in aquatic vertebrates and invertebrates. Chronic toxicity is usually observed as sublethal effects such as cytotoxicity, which means cellular damage, genotoxicity, that is damage to genetic material, and also gene expression, metabolism, and hormone system disturbance (Świacka et al., 2021; Tyumina et al., 2023).

Bacterial biodegradation is an effective approach to remove ketoprofen. *Rhodococcus erythropolis* IEGM 746 is reported as a strain that can degrading approximately 40% of 100 mg/L KTP in 14 days (Bazhutin et al., 2022). Respirometry analysis revealed that *rhodococci* are very active in presence of ketoprofen, while atomic force microscopy showed changes in the shape and structure of *rhodococci* resulting from exposure to NSAIDs. Another study found that a gram-negative bacterium called *Raoultella* sp. KDF8 (isolated from composted waste) can degrade 60% of 1 g/L KTP in 96 hours at 28°C (Palyzová et al., 2018). Moreover, Ismail et al. (2016) reported that *Raoultella ornithinolytica* B6, *Pseudomonas aeruginosa* JPP, *Pseudomonas* sp., and P16 and *Stenotrophomonas* sp. as a group (all isolated from wastewater) could degrade ketoprofen as the sole carbon source. These bacteria could completely remove 5 µM of ketoprofen during 48 hours. during this process three by-products formed: (3-ethylphenyl)(phenyl)methanone, (3-hydroxyphenyl)(phenyl)methanone, and (3-hydroxyphenyl)(oxo)acetic acid that are less toxic than the parent compound. These results show that both single bacterial strains and mixed microbial groups can biodegrade pharmaceuticals such as ketoprofen. Since this process does not produce harmful byproducts, using bacteria for biodegradation is a promising and environmentally friendly approach to remove ketoprofen contamination.

2.3.4 Naproxen (NPX)

Naproxen is an analgesic that appears as white crystal or crystalline powder and it is classified as non-steroidal derivative of benzene acetic acid (PubChem, 2023). According to the International Union of Pure and Applied Chemistry (IUPAC), naproxen is called 2-[2-(2,6-dichloroanilino)phenyl]acetic acid (PubChem, 2023). The molecular formula of naproxen is $C_{14}H_{14}O_3$ (Figure 2.7). The molecular weight and CAS number are 230.26 g/mol and 22204-53-1 respectively (PubChem, 2023). Melting point is measured to be 152°C. Solubility of naproxen in water at 25°C is documented as 15.9 mg/L (PubChem, 2023). Toxicological and physico-chemical properties of naproxen are demonstrated in Figure 2.1.

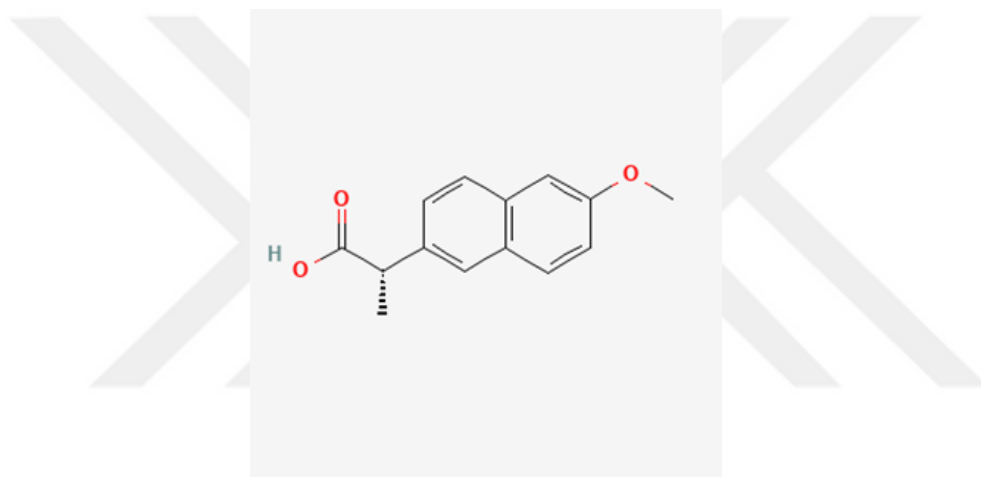


Figure 2.7: Chemical structure of Naproxen (PubChem, 2023).

Due to its incomplete degradation in certain wastewater treatment plants (WWTPs), naproxen has been detected in wastewater, surface water, groundwater, and even in drinking water. Naproxen enters the environment from different pathways. Naproxen can undergo metabolism in the body and resulting in two main products: O-desmethylnaproxen and naproxen glucuronide (Addison et al., 2000), therefore this drug can be found in the wastewater. Naproxen can be removed from almost complete to only 40% in WWTPs (Marco-Urrea et al., 2010). Different studies reported different concentrations of naproxen (ng/L to macrog/L) in the effluent of WWTPs (Marotta et al., 2013). Even low concentrations of Naproxen can increase the toxicity maybe because of the synergy effect with other contaminations. Naproxen can negatively

affect various organisms in the ecosystem directly or through toxicity of its metabolites which can be formed during physicochemical and biological processes (Jallouli et al., 2016; Rodríguez-Rodríguez et al., 2011). Some of these metabolites are more toxic than the parent compound such as photoderivatives of naproxen which toxic to aquatic organisms such as *Brachionus calyciflorus*, *Thamnocephalus platyurus*, *Vibrio fischeri*, *Ceriodaphnia dubia*, and *Daphnia magna* (DellaGreca et al., 2003; Jallouli et al., 2016). Most probably, it is because of easier cellular penetration after change in the structure that caused by photodegradation (Ma et al., 2014). Many studies confirmed the adverse effects of naproxen on aquatic invertebrates and vertebrates. Bioaccumulation of naproxen in bile of fishes was detected up to 1000 times higher than in samples of the lake. It might be due to the inhibition of the metabolizing enzyme activity (C. Xu et al., 2019). Moreover, environmentally concentrations of naproxen could have an effect on the mRNA expression which leads to gastrointestinal and renal effects in zebrafish (Ding et al., 2017). Furthermore, Grenni et al. (2014) indicated that, long-term exposure to naproxen has been associated with change in microbial community structure in river water, such as reduction in α -Proteobacteria, including ammonia-oxidizing bacteria, and an increase in β - and γ -Proteobacteria, which may play a role in naproxen degradation. Because of the potential risk of this drug for organisms in polluted environments, there is a crucial need to apply an effective method to remove this drug. According to the literature, the mixture of naproxen, diclofenac and ibuprofen can increase the biodiversity in the sequencing batch reactor (Jiang et al., 2017b). Same study reported an enrichment in the *Actinobacteria* and *Bacteroidetes*, while it was seen that *Micropruina* and *Nakamurella* decreased after addition of NSAIDs, a damage in the cell wall of the microorganisms was reported. Naproxen degradation under monosubstrate conditions was detected very low, whereas additional carbon source such as glucose, phenol, 4-hydroxybenzoic acid or 3,4-dihydroxybenzoic acid improved the degradation efficiency (Górny et al., 2019a; Wojcieszynska et al., 2014).

On the other hand, although the microbial degradation of NSAIDs has attracted increasing attention, there is limited information about the biodegradation of naproxen because of its chemical stability and complex aromatic structure. The most extensively

studied pathway for the bacterial degradation of naproxen has been investigated in *Bacillus thuringiensis* B1(2015b). Firstly, in this process, naproxen demethylated to form O-desmethylnaproxen, then converted to salicylate. Salicylate cleaved by salicylate 1,2-dioxygenase or hydroxylation to catechol or gentisate metabolites that are further processed by central metabolic pathways (Górny et al., 2019b).

Furthermore, transformation of naproxen with Fungal species such as *Trametes versicolor* showed high efficiency, almost 100%. It was reported that compared to crude laccase from *Trametes versicolor*, commercial laccase is more effective Tran et al. (2010) and ref125. Studies reported that monocultures are more efficient in naproxen degradation than mixed microbial communities (Wojcieszńska & Guzik, 2020).

2.3.5 Indomethacin (IMC)

According to the International Union of Pure and Applied Chemistry (IUPAC), indomethacin is called 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid (PubChem, 2023). The molecular formula of indomethacin is C₁₉H₁₆ClNO₄. Indomethacin's molecular configuration is illustrated in Figure 2.8. The molecular weight and CAS number are 357.8 g/mol and 53-86-1 respectively (PubChem, 2023). The melting point is measured to be 311 or 323.6°F (NTP, 1992). The solubility of indomethacin in water at 25°C, documented as 0.937 mg/L (PubChem, 2023). Toxicological and physico-chemical properties of indomethacin are demonstrated in Figure 2.1.

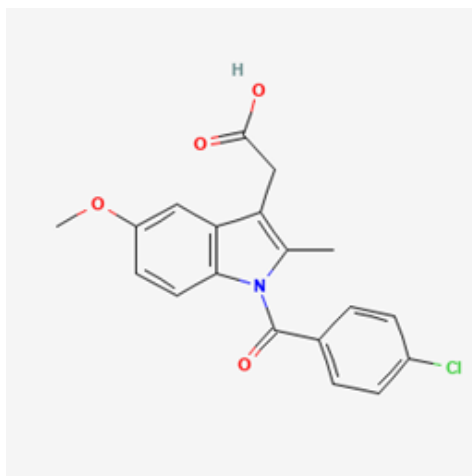


Figure 2.8: Chemical structure of Indomethacin (PubChem, 2023).

Indomethacin is a commonly used antipyretic, analgesic, and non-steroidal anti-inflammatory drug (NSAID), whose global consumption significantly increased following the COVID-19 pandemic. This rise in usage has led to heightened concentrations of the drug raising concerns for both human health and aquatic ecosystems. The primary concern stems from its environmental persistence and ecotoxicological impacts. Indomethacin is known to be poorly removed by conventional wastewater treatment processes, allowing it to enter aquatic environments through treated effluents. Reported environmental concentrations vary depending on geographic location (Jiménez et al., 2017).

Within the human body, Indomethacin undergoes extensive metabolism into inactive forms by processes such as demethylation, deacylation, and glucuronidation. These processing only handle about 10% of the dose (Munjal & Allam, 2025). The primary metabolites are O-desmethyl-indomethacin, O-deschlorobenzoyl-indomethacin and O-Desmethyl-N-deschlorobenzoyl-indomethacin, which are excreted with both urine and feces along with 10–20% of the unchanged parent compound (Gliszczyńska & Nowaczyk, 2021).

The transformation of indomethacin in water treatment can result in generation of degradation products, which may add extra ecological risks. It was reported that indomethacin can be removed when treated with sodium hypochlorite (NaClO) which is a disinfectant. Thirteen degradation products were identified. Seven of

these degradation products were not reported previously. Indomethacin degraded by degradation rate over 90% but only 33% of the degraded mass are identified as known byproducts, suggesting that some of the degradation products might be highly polar or volatile substances that conventional analysis is not able to detect them (Medici et al., 2025).

Concerns have been raised for chlorinated byproducts fate in the environment because they are resistance to biodegradation and photodegradation, and they might be more toxic than the parents' compound. Aldehyde is a degradation product that contains reactive functional groups which can form covalent bonds with biomolecules, which can cause cytotoxic and genotoxic effects (O'Brien et al., 2005). These results highlights the importance of evaluating potential environmental risks of the byproducts along with assessing the efficiency of pharmaceuticals like indomethacin that are removed during treatment.

2.3.6 Mefenamic acid (MFA)

Mefenamic Acid is called 2-[(2,3-dimethylphenyl)amino]benzoic acid (PubChem, 2023). Molecular formula of mefenamic acid is $C_{15}H_{15}NO_2$ (Figure 2.9). The molecular weight and CAS number are 241.29 g/mol and 61-68-7 respectively (PubChem, 2023). The terminal half-life of mefenamic acid, indicating that how fast the mefenamic acid itself is reduced in half in body, is about 2 hours while apparent half-life is extended to 25.8-33 hours which shows the longer time is needed for removing of both mefenamic acid and its metabolites in the body (PubChem, 2023). Melting point is measured to be 230-231°C. The solubility of mefenamic acid in water at 25°C is low, documented as approximately 4 mg/L (PubChem, 2023). Toxicological and physico-chemical properties of mefenamic acid are demonstrated in Figure 2.1.

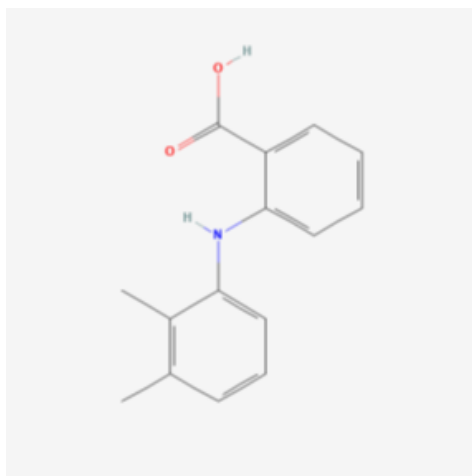


Figure 2.9: Chemical structure of Mefenamic Acid (PubChem, 2023).

Mefenamic acid is a derivative of diphenylamine, a pollutant class that is used in different applications and these days their environmental impact has become a concern. Accordingly, mefenamic acid has been classified as third-class priority pollutant in the European Union. Even prolonged exposure to low concentrations of this drug in surface water can result in health issues such as diarrhea and skin rash (P. Chen et al., 2016; Suwalsky et al., 2011). Mefenamic acid is an analgesic/anti-inflammatory pharmaceutical that is commonly present in the wastewater, and even drinking water. It can release to the environment by human excretion. It shows low biodegradability under conventional treatment system. Although pharmaceuticals such as mefenamic acid are designed to have an effect on specific metabolic pathway in humans' body, as well as domestic animals, they may also have unknown effects on metabolic systems of organisms that are not the targeted organisms like invertebrates (Velázquez & Nacheva, 2017).

Mefenamic acid is among the pharmaceuticals that can be degraded with the heterotrophic activity in bioreactors. Therefore, the role of microbial community in the biodegradation process of different pharmaceuticals should be studied more (Maeng et al., 2013; Majewsky et al., 2011; Tran et al., 2009).

Velázquez and Nacheva (2017) stated that, biodegradation was the main removal mechanism for mefenamic acid. Ammonium-nitrite-oxidizing and the aerobic heterotrophic consortium were able to biodegrade mefenamic acid, while nitrite-oxidizing

consortium showed the lowest removal efficiency. Same study reported sorption to biomass as a mechanism that contribute to mefenamic acid removal (approximately 6-14%).

2.4 The Need for Removal of NSAIDs From Wastewater

Pharmaceuticals find their way to aquatic environments. They can enter the wastewater from different sources like direct discharge of hospitals into sewer system or it can enter the wastewater with human and animal's excretions. And also, as conventional treatment systems are not appropriate to remove most of these micro pollutants, they can discharge with treated effluent and threaten the human and animal life. In long term exposure, there is a risk toxicity in organisms in those environments. and also, there is a risk of accumulation of drugs or their metabolites in the food chain. Moreover, some evidence claimed that in long term exposure, the risk of cancer would increase in human Figure 2.10 (Zare et al., 2022).



Figure 2.10: Routes and fate of discharged pharmaceuticals into aquatic environment. Taken from Zare et al. (2022).

2.5 Removal Mechanisms of NSAIDs in Wastewater Treatment Plants

Micropollutant removal in WWTPs is done by sorption or biodegradation process. Photodegradation and Volatilization (air stripping (Kumwimba & Meng, 2019)) have very little effect on pharmaceutical removal (M. Kim, Guerra, Shah, Parsa, Alaei, & Smyth, 2014). Biological decomposition (biodegradation) obtained many

advantages over conventional physicochemical methods because of its low cost and bacteria and fungi are responsible for biodegradation even at high concentrations of micropollutants. The efficiency of this process depends on nature of pollutants and different factors like pH and temperature (Kanauiya et al., 2019).

2.5.1 Sorption

The Sorption of pharmaceuticals refers to the attachment of micropollutants to the cell membrane of microorganisms or to lipid molecules in the sludge. Sorption involves two main steps: absorption and adsorption. Absorption happens due to hydrophobic interaction between aliphatic and aromatic group of a chemical and the lipophilic cell membrane of organisms, as well as the lipid components of sludge. Adsorption is because of the electrostatic forces of positively and negatively charged microbes and sludge (Kumwimba & Meng, 2019; Tiwari et al., 2017a). Efficiency of sorption is influenced by sludge adsorption coefficient (K_d), octanol-water coefficient (K_{ow}) and acid dissociation constant (Pka). physicochemical properties of several classes of pharmaceuticals showing that compounds with $\log K_{ow}$ more than 5 (high value) and high molecular weight are more likely to be sorbed onto sludge particles while compounds with $\log K_{ow}$ lower than 2.5 (low value) tend to remain in the aqueous phase and are less likely to be removed by sorption (Tiwari et al., 2017a). Most of the pharmaceuticals show insignificant sorption because of their low K_d values (Tiwari et al., 2017a). Based on a study it is indicated that compounds that have K_d lower than 500 LkgSS^{-1} will be removed less than 10% through sorption alone (Joss et al., 2004). Although sorption is important, it is typically a secondary mechanism for MPs removal and the dominant mechanism is biodegradation (Tiwari et al., 2017a).

2.5.2 Biodegradation

In biodegradation micro-organisms breakdown toxic and complex chemicals, with the help of enzymes that are secreted by micro-organisms, to simpler and less toxic products (Tiwari et al., 2017a; Y. Wang et al., 2017). The highest removal of organic MP in WWTP occurs with biodegradation (Tiwari et al., 2017a).

Martínez-Alcalá et al. (2017) investigated the percentage of biodegradation and sorption processes in a conventional activated sludge wastewater treatment plant located in Murcia, Spain. They discovered the importance of biodegradation (microbial degradation) for ibuprofen and ketoprofen with 99.7% and 54.3% contribution of the total removal for ibuprofen and also ketoprofen, respectively (Martínez-Alcalá et al., 2017). The solubility of pharmaceuticals in the wastewater affects the biodegradation efficiency of them, such that in case of hydrophobic compounds (low solubility), they tend to be retained in the sludge, which provides more time for microbial degradation; like that micropollutants are either consumed as a carbon source by microorganisms or degraded by catabolic microbial enzymes. On the other hand, hydrophilic micropollutants escape from wastewater treatment plants (WWTP) with effluent without biodegradation (Tiwari et al., 2017a).

Based on the functional and structural groups of compounds, the degradation rate and efficiency would differ in different compounds in both aerobic and anaerobic digestions. For example, low chlorinated compounds degrade faster in aerobic conditions, while polyhalogenated compounds degrade more slowly. Moreover, long-chain aliphatic compounds biodegrade more than aromatic compounds with halogen or sulfate groups in their ring structure (Tiwari et al., 2017a).

Operating parameters such as sludge retention time (SRT), pH, temperature, and also bioavailability and microbial community and complexity of micropollutants govern the rate of biodegradation (Tiwari et al., 2017a).

Byrns (2001) stated that a big portion of xenobiotics compounds would be removed by sludge discharge at low sludge retention time (SRT) by sorption. On the other hand, at high STRs, due to the longer contact time of sludge and microbial community, the removal rate of sludge waste decreases (Tiwari et al., 2017a). Also, another study indicated that the biodegradation of trace organic chemicals can be increased by higher mixed liquor suspended solids concentration that it occurs mostly in MBR with the high STR (Kumwimba & Meng, 2019). Biodegradation of MPs such as ketoprofen, ibuprofen, naproxen and Trimethoprim in aerobic and anaerobic conditions

is documented by many studies in the literature (M. Kim, Guerra, Shah, Parsa, Alaei, & Smyth, 2014; Tiwari et al., 2017a).

Biodegradation mechanisms can be classified as metabolic degradation or co-metabolic degradation (Tiwari et al., 2017b). They can be closely linked and substitutable (Kumwimba & Meng, 2019). In the metabolic pathways, micropollutants serve as the sole carbon and energy source to maintain the essential activities of degraders including growth and reproduction (Chandra et al., 2022; Fischer & Majewsky, 2014). On the other hand, in co-metabolic pathways, the MPs degradation occurs in the presence of another substrate which is catalyzed by nonspecific enzymes and serves as the primary energy source for microorganisms (Tiwari et al., 2017b). Many xenobiotics can be degraded by both metabolic and co-metabolic processes (Croom, 2012; Janke & Fritsche, 1985). Other studies have found that the biodegradation of trace organic chemicals is due to the co-metabolic activity of autotrophic bacteria in natural and engineered environments, while trace organic chemicals can be removed by co-metabolism and/or metabolism by heterotrophic bacteria (Kumwimba & Meng, 2019).

2.5.2.1 Metabolic degradation

The organisms present in the activated sludge systems need carbon and energy source to sustain their biomass and also to produce the necessary enzymes for redox reactions (Kumwimba & Meng, 2019). In the metabolic approach, they use micropollutants as their sole energy and carbon source (Kumwimba & Meng, 2019). The trace organic chemicals should be non-toxic for microbial growth processes and also, they should be present at sufficient concentrations to allow sustaining the biomass while simulating the production of required enzymes and co-factors which is necessary for degradation process (Tran et al., 2013). Different studies were conducted in this area, for example, Quintana et al. (2005) found that only ketoprofen exhibited metabolic biodegradation. On the other hand, naproxen, ibuprofen and bezafibrate were removed solely through co-metabolic processes. This experiment was conducted to study the microbial degradation of five acidic pharmaceuticals under aerobic conditions, while the sludge was used as inoculum. Likewise, another study mentioned that ketoprofen,

estradiol can be used by microorganisms as sole carbon/energy source (Kumwimba & Meng, 2019). Generally, the primary pathway for trace organic chemical removal is by co-metabolism such that NH_4^+ is used as the main substrate (energy source). One important metabolic limitation for microorganisms is inability of the enzymes to decompose certain trace organic chemicals (Kumwimba & Meng, 2019).

2.5.2.2 Co-metabolic degradation

Some of the MPs are toxic to organisms, therefore, microorganisms cannot utilize them as growth substrate, in this regard co-metabolic degradation can have advantageous over metabolic biodegradation, especially for trace organic chemicals as their low concentrations are not sufficient for supporting the growth (Kumwimba & Meng, 2019). Increasing the effectiveness of trace organic chemicals removal, in the presence of a growth substrate refers to the co-metabolic process. The nature and concentration of growth substrates, such as phenol and toluene, as well as the concentration of ammonium (NH_4^+), can notably influence the co-metabolism (Kumwimba & Meng, 2019; Z. Liu et al., 2013). Therefore, trace organic chemicals removal is basically linked to the removal of growth substrate, such as NH_4^+ . Higher elimination capacity of trace organic chemicals was seen with co-metabolism through ammonia oxidizing bacteria (AOB) (Y. Xu et al., 2016). Co-metabolism is executed by ammonia oxidizing bacteria (AOB) has been shown to be a principal process for elimination of trace organic chemicals. Another researcher stated that Ammonia monooxygenase enzyme produced by *Nitrosomonas europaea* (A type of AOB) can co-metabolize more than 35 halogenated compounds, while NH_4^+ was served as growth substrate and energy source (Kumwimba & Meng, 2019). Co-metabolic biodegradation has been used in both aerobic and anaerobic conditions to remove a wide range of chemicals in different ecosystems. Nowadays, aerobic co-metabolism considered as a better process than removal in anaerobic condition (Czajka & Londry, 2006). Moreover, both heterotrophs and autotrophs are responsible to degrade these compounds in engineered systems (Kumwimba & Meng, 2019). Compared to non-nitrifying waste water treatment facilities (WWTFs), the nitrifying WWTFs were more effective in biodegradation of most trace organic micropollutants. This is due to the co-metabolic biodegradation

that occurs by ammonia-oxidizing bacteria (AOB). Ammonia monooxygenase (AMO) has the potential to remove a big range of aliphatic and aromatic compounds by co-metabolic degradation (Kumwimba & Meng, 2019). According to a study on comparing single substrate degradation and co-metabolic process, co-metabolism was the major mechanism to remove ibuprofen, naproxen and bezafibrate whereas ketoprofen were removed partially as a sole carbon and energy source (Quintana et al., 2005). Primary removal mechanism for the antibiotic like norfloxacin, ciprofloxacin, tetracycline was sorption while for sulfamethoxazole and azithromycin degradation was the key removal mechanism; And some of the pharmaceutical pollutants were reported to have negligible removal in WWTPs, they are erythromycin, carbamazepine, sulfamethoxazole. This very low removal was explained by the fact that human metabolites were transformed back to their parental compounds (Tiwari et al., 2017a).

2.6 Treatment Processes of NSAIDs From Wastewater

Due to the prolonged harmful effects of NSAIDs on human health and ecosystem, the removal of these drugs has become a crucial concern globally, giving the need to meet regulatory standards. There are different treatment technologies to eliminate the NSAIDs from wastewater including biological treatment methods and also different advanced chemical processes. The major removal mechanisms are conventional activated sludge treatment (CAS), Membrane Bio-Reactor (MBR), attached growth MBR, using biological approaches. Other biological methods, such as constructed wetland, algae photobioreactor and stabilization ponds also play an important role for NSAIDs removal. Microbial communities have the responsibility of degrading NSAIDs in biological treatment systems, which can be further enhanced by combining membrane filtration and biological treatment to improve the removal efficiency (Barceló et al., n.d.; Tiwari et al., 2017b). Furthermore, ozonation, electrochemical oxidation, activated carbon adsorption, UV irradiation and advanced oxidation processes are advanced methods which are effective in degrading NSAIDs to less harmful products (Mussa et al., 2022).

There is not a single treatment process effective for all the MPs; meaning that for a specific MP, appropriate analytical methodologies should be done to evaluate the efficiency of the treatment plant in removing that particular MP (Barceló et al., n.d.).

2.6.1 Removal of NSAIDS with Conventional activated sludge systems

Conventional activated sludge (CAS) system is classified as one of the most effective and commonly used method for treating municipal wastewater which relies on the microbial activity (activated sludge) for nutrient removal and the oxidation of carbon-rich compounds (Kanaujiya et al., 2019; Xie et al., 2021). This process aims to remove the organic pollutants, suspended solids, and flocculated matter to meet the discharge standards by producing high quality effluent (Orhon, 2015). CAS systems combine industrial effluent with microbial culture in one aerated reactor before the next cycle begins (Kanaujiya et al., 2019). The main degradation mechanism in activated sludge processes is degradation or sorption through microbial flocs, with hydrophobic compounds being adsorbed due to their surface properties. Moreover, co-metabolism of various pollutants with the help of microbial consortium play a critical role in the effectiveness of activated sludge processes (Kanaujiya et al., 2019; Maeng et al., 2013).

Although, the sustainability of CAS systems is becoming a concern specially in terms of energy efficiency and operational reliability, they have economic advantages in terms of capital investment and operational costs in compare to chemical or thermal oxidation processes and etc. Process stability and cost-effectiveness of these systems are often affected because of some issues like settling problems (Van Winckel, 2014). To sustain the nitrifiers in the CAS systems and ensure effective nitrification, especially in colder climates where the growth rates of bacteria are reduced, a large reactor volume and extended sludge retention time (SRT) is required. Otherwise, it results in washing out of nitrifying bacteria, leading to a disruption in nitrogen removal, particularly in lower temperatures (Tang et al., 2020; Van Winckel, 2014).

While the CAS systems play a fundamental role in wastewater treatment, they need modifications such as integration of biofilm technology to overcome the energy efficiency and also operational limitation, ensuring compliance with the latest wastewater treatment standards (Güneş et al., 2019b; Sarioglu et al., 2017).

Conventional activated sludge (CAS) systems are not specifically designed for the removal of pharmaceuticals and other trace organic contaminants. For example, based on one different study, CAS systems showed poor efficiency in removing pharmaceuticals because of low biodegradability of certain drugs such as diclofenac and carbamazepine while they are considered as common pharmaceutical contaminants (Arola et al., 2017).

NSAIDs show different removal efficiencies in various studies in CAS, likely due to differences in biodegradability and sorption characteristics. For example, Salgado et al. (2012) reported low biodegradation rate, approximately below 25%, for diclofenac, while for ibuprofen and ketoprofen the biodegradation rate was reported above 75%. Behera et al. (2011) reported diclofenac removal efficiency of around 80% in Korean WWTPs, whereas it was 5% in Spanish conventional WWTPs.

Moreover, Martínez-Alcalá et al. (2017) reported low removal rates of below 20% for naproxen and carbamazepine, while diclofenac, ibuprofen showed removal rate between 20% and 90%; the higher values suggest that these compounds are greatly adsorbed onto sludge. The same study showed removal of approximately 50% for ketoprofen, however several studies (Verlicchi et al., 2012) reported ketoprofen removal rates ranging from 30 to 92%, depending on the operating sludge retention time.

2.6.2 Removal of NSAIDs with Membrane Bioreactor

Membrane bioreactor (MBR) technology integrates the conventional activated sludge treatment processes with the low-pressure membrane that act as a physical barrier to retain microorganisms. MBRs are more efficient technologies in micropollutant removal comparing to conventional activated sludge systems in some aspects including reduced sludge production, very low levels of suspended solids in permeate, effective elimination of viruses and pathogens and enhanced overall effluent quality (Tiwari et al., 2017a).

Sludge retention time (SRT) and hydraulic retention time (HRT) accounted as critical parameters that govern the removal efficiency in MBR systems (Judd, 2010). The long

STR in MBRs supports the growth of nitrifying bacteria which leads to a better removal rate of biodegradable micropollutants and also efficient nitrogen removal (Baek, 2004).

SRT is dependent on the organic load and F/M ratio for a particular wastewater that requires treatment (Kanauiya et al., 2019). The impact of SRT and HRT on micropollutant removal through MBR were studied by (Maeng et al., 2013). The finding showed high removal efficiencies for ibuprofen and acetaminophen at a low SRT (8 days). However, compounds such as carbamazepine and diclofenac that are categorized as hydrophilic compounds ($\log D_{pH} = 7.8 < 3.2$) indicated low removal rates under the same HRT. On the other hand, pH and temperature influenced the MP removal in MBR systems, like in other biological treatment processes (Kanauiya et al., 2019).

Membrane bioreactors (MBRs) typically function in two primary steps: side stream and submerge. In side stream arrangement, the membrane is set separate from the working bioreactor unit. An additional recirculation pump to circulate the feed from the bioreactor to the connected membrane module is needed in this setup plant. While these requirements make this process energy-intensive and high-cost process, the concept of submerged MBR system was taken into consideration. In the submerge configuration, the membrane module is kept inside the bioreactor which enables the feed/effluent to pass through while simultaneously treating or removing micropollutants. Micropollutant removal by MBRs include physical retention, air stripping, and biodegradation (Kanauiya et al., 2019; Sipma et al., 2010).

According to different studies, MBR technology has advantages for treating hospital wastewaters; such that De Gusseme et al. (2009) reported high removal rates for 17 β -ethinylestradiol in nitrifier-enriched biomass as 99%. In another study, it was observed that higher removal of pharmaceuticals was achieved through higher nitrifying activity (Dawas-Massalha et al., 2014). There are many other examples in the literature which shows the higher efficiency of MBR to CAS systems. For instance, a decrease was observed in the concentrations of caffeine, acetaminophen, carbamazepine, gemfibrozil, and sulfamethoxazole after passing through the pilot MBR (Tiwari et al., 2017a). Kimura et al. (2005) stated same removal efficiencies like

CAS in the pharmaceuticals such as diclofenac, ibuprofen, and clofibrilic acid, while high degradation was reported in ketoprofen and naproxen through MBR.

Presence of aromatic ring or chlorine group in the structure of pharmaceuticals make them persistent and show low removal rates in both treatment plants but in general MBR system shows higher efficiency compared to CAS systems for removal of these kind of pollutants, especially for those who are not readily biodegradable (Tiwari et al., 2017a). Table 2.2 provides a comparison of the removal efficiency and removal mechanism of pharmaceuticals in CAS and MBR in different studies.

Briefly, MBRs are recognized as a promising and effective technology for removing micropollutants like NSAIDs. For instance, M.-L. Nguyen et al. (2022) reported an average removal percentage higher than 90% for ibuprofen compared to conventional methods. In recent decades, the use of MBRs has grown. Fouling is a major problem in MBR systems which can reduce the performance of the MBRs. Change in membrane configuration, modification of operating conditions and also using pretreatment techniques can be beneficial to overcome this issue (M.-L. Nguyen et al., 2022).

Table 2.2: Average removal efficiency of pharmaceutical and their removal mechanisms in conventional activated sludge system and MBR. Taken from Tiwari et al. (2017a).

Compound	%Removal MBR	%Removal CAS	Biodegradation%	Sorption
Ibuprofen	99	99	90–100	<5
Naproxen	95	94	55–85	<5
Diclofenac	32	50	5–45	<5
Ketoprofen	99	50	70	0
Mefenamic acid	63	36	55–58	<30
Indomethacin	50	–	40	<5

2.6.3 Removal of NSAIDs with advanced treatment processes

There are different advanced treatment methods like ozonation, activated carbon adsorption, UV irradiation, and advanced oxidation processes. There are a few studies on the benefits of upgraded wastewater treatment plants (WWTPs) with ozonation and activated carbon adsorption treatment processes in organic micropollutants. The most

commonly used advanced processes in organic micropollutants removal are activated carbon adsorption and advanced oxidation processes (Guillossou et al., 2019). The efficiency of the adsorption differs based on different factors including dose and type of the activated carbon, operational conditions and also the water's quality and composition. On average the removal rates generally are in range of 60% and 90% which are highly affected by different properties of the molecules like its charge, hydrophobicity and size (Guillossou et al., 2019).

According to a study conducted in 2019, they assessed the removal of 48 organic micropollutants in a WWTPs followed by an activated carbon pilot. The results indicated that although OMPs removal was not significant (<10%) for the MPs that were already well removed in the WWTP, it proved significant removal (>30%) for recalcitrant OMPs. Most of the targeted compounds achieved removal rates exceeding 50% and the average effluent concentration in the pilot were below 200 ng/L for 70% of the OMPs (Guillossou et al., 2019). In same study the only removal rate of citalopram reached over 80% as required by the Swiss regulations, while other compounds (carbamazepine, hydrochlorothiazide, irbesartan) did not meet the regulations indicating a need for higher dose of activated carbon or addition of an advanced treatment to reach the targeted 80% removal of Swiss regulations (Guillossou et al., 2019).

High removal of 8 OMPs including ibuprofen, ketoprofen, naproxen, acetaminophen, atenolol, citalopram, erythromycin, and trimethoprim were detected through combination of activated sludge systems with ozonation of granular activated carbon filters (Bourgin et al., 2018; Hollender et al., 2009). As a result, the mentioned compounds can be considered as easily eliminated in conventional WWTP and advanced treatment (Guillossou et al., 2019). X. Yang et al. (2011) reported a good removal of approximately 65% for diclofenac in WWTPs upgraded with activated carbon and also a better removal above 90% through ozonation.

Ozonation and advanced oxidation processes (AOPs) are efficient redox technologies that have some advantages comparing to conventional treatments including high degradation rates and non-selectivity that makes them effective against many

contaminants. Additionally, these technologies provide disinfection properties which is crucial for applications that involves direct human contact like household reuse (Luo et al., 2014b). Contaminants can be degraded through ozonation in two ways: directly and indirectly. Generally, ozone degrades the pollutants by formation of hydroxyl radicals ($\bullet\text{OH}$) that are stronger and less selective oxidizing agents. Some MPs such as naproxen and carbamazepine can be efficiently removed by both ozonation and AOPs. On the other hand, others like atrazine and meprobamate are only susceptible to $\bullet\text{OH}$. And also, some compounds such as tris(2-chloroethyl) phosphate (TCEP) and tris(1-chloro-2-propyl) phosphate (TCPP) exhibit resistance to both forms of oxidations (Luo et al., 2014b)

Based on different studies in the literature oxidation has been proved as an effective technique to decrease the MP load at full-scale WWTPs (Hollender et al., 2009). Moreover, it has been observed that generally all compounds can be removed efficiently from the biologically treated effluent with removal rates over 79% at the ozone doses of 15 mg/L (Hernández-Leal et al., 2011). Another study indicates that although some compounds, like bezafibrate, showed less removal efficiency (14%) at lower ozone dose of 5mg/L, this dose significantly reduced the concentration of some targeted MP (carbamazepine, diclofenac, indomethacin, sulpiride, trimethoprim) by more than 95% (Sui et al., 2010).

Gerrity et al. (2011) conducted a study during water reclamation on the application of $\text{O}_3/\text{H}_2\text{O}_2$ to remove different MPs (PPCPs and steroid hormones), indicated that most of the targeted contaminants were removed significantly by more than 90% removal efficiency, though some, like TCEP (tris(2-chloroethyl) phosphate), TCPP (tris(1-chloro-2-propyl) phosphate), atrazine, meprobamate and ibuprofen were more resistance with 13%, 26%, 69%, 80%, 83% removal efficiencies, respectively.

UV-based processes (UV and UV/ H_2O_2) have also showed high removal efficiencies (higher than 90%) for compounds such as ketoprofen, diclofenac and antipyrine. While it was ineffective to remove macrolides (24%–34%). Higher potential removal efficiencies improved up to 90% for 39 out of 42 compounds were observed when H_2O_2 were added (I. Kim et al., 2009). Moreover, according to De la Cruz et al.

(2012) an enhanced transformation of micropollutants were achieved (100%) through fenton/UV254 after 90 minutes.

While advanced treatment technologies like adsorption processes, advanced oxidation process, and membrane systems have demonstrated effective removal potential for MPs degradation, they have two main issues: One is about their high operational costs and the second one is forming of byproducts and concentrated residues. As a result, post-filtration is crucial for purification of treated water and also to reduce the residual byproducts (Luo et al., 2014b).

2.7 Removal of NSAIDs with Moving Bed Biofilm Reactors

Moving Bed Biofilm reactor (MBBR) was developed for the first time in Norway in the late 1980s and early 1990s (Santos et al., 2020). MBBR system has demonstrated success in biological treatment of both domestic and industrial wastewater with resistant properties (Leyva-Díaz et al., 2017; Tang et al., 2021). The Hybrid MBBR system is recognized as an innovative and mature technology that consists of two types of biomass: one is suspended biomass or activated sludge flocs, similar to conventional activated sludge systems, and another one is attached biofilm growing on the carriers. The combination of these two types of biomass in one reactor volume allows fast-growing bacteria, such as general heterotrophs responsible for carbon removal, and slow-growing bacteria, like autotrophs involved in nitrification, to develop on both suspended and attached biomass. This feature enables the system to maintain higher biomass and allows nitrification to occur at significantly shorter solid retention times compared to conventional activated sludge (CAS) systems, thereby increasing the nutrient removal capacity of existing plants or enabling the design of new plants with smaller footprints (Tang et al., 2020). Also, Falås et al. (2013) indicated higher overall removal efficiencies in hybrid system for some pharmaceuticals (e.g., Diclofenac) than suspended biomass or typical sludge of CAS.

Hybrid systems such as Moving Bed Biofilm (MBBR), Integrated Fixed Film Activated Sludge (IFAS), and Integrated Floating-Film Activated Sludge (IFFAS) are compact and simple to operate. They are easily adaptable for retrofitting in limited spaces and also, they showed low capital cost and reliable stability under

load variations. Therefore, MBBR systems considered as suitable system for reuse of treated wastewater. MBBR technology can be easily operated by adding carriers to the existing activated sludge systems without the need for extra construction. Also, they can tolerate high fluctuations in organic loadings (Gunes et al., 2023; Tang et al., 2020).

According to different studies in the literature, MBBRs have proven high efficiency in pharmaceutical removal from municipal wastewater effluent and also from hospital wastewater (Hapeshi et al., 2013; Ooi et al., 2018; Tang, Casas, et al., 2017; Tang, Ooi, et al., 2017; Tang et al., 2020).

In the pilot-scale Hybas™ system (a hybrid attached biofilm and activated sludge system) conducted by Tang et al. (2020), 14 out of 20 pharmaceuticals were removed by more than 50%, with ibuprofen showing a removal rate exceeding 99%.

2.7.1 Biofilm carriers

MBBR technology uses plastic carriers (media) to provide a surface area for growing microorganisms (biofilm) on them, facilitated by mechanical mixing or aeration which offers significant advantages, including a compact footprint. Also, these carriers, coated with biofilm, facilitate pollutant removal by mechanisms such as biodegradation, bioaccumulation, biomineralization, bioimmobilization and biosorption from the waste. Moreover, they have a density approximately equal to water's density which allows them to mix within the fluid (Radjenović et al., 2007).

K-series AnoxKaldnes were the first biocarrier applied in MBBR systems. These biocarriers are made of polyethylene, with a density of 0.95 g/cm^3 and their dimensions are different (di Biase, Alessandro and Kowalski, Maciej S and Devlin, Tanner R and Oleszkiewicz, Jan A, 2019). Although, different types of suspended carriers are available to be used in the MBBR systems including polyethylene (PE), polypropylene, polyurethane, ceramsite, diatomite, hydrogels, or poly vinyl alcohol gel, polyethylene are the most used ones because of their good performance in the MBBRs. Biocarriers can be launched by different companies under varying categories based on their size, structure (Radjenović et al., 2007).

In both Integrated Fixed-film Activated Sludge (IFAS) and Moving Bed Biofilm Reactor (MBBR) systems, carriers play a crucial role to support the biofilm. A study conducted on two full-scale municipal water resource recovery facility (WRRF), which implemented IFAS plants using AnoxKaldnes™ K1 (KC) (and polypropylene resin (RC) carriers, and two MBBR plants using KC and sponge carriers (SC) highlighted the importance of carrier design. The study recommended high specific surface area (SSA) carriers to increase the microbial growth and also retaining of slow-growing nitrifiers, although with a limited effect on denitrifies (Phanwilai et al., 2020).

Surface area is one of the important factors influencing the performance of carriers in wastewater treatment plants. For example, carriers with high surface area, like sponge carriers ($1094 \text{ m}^2/\text{m}^3$), showed a greater abundance of autotrophs (AOB and NOB) comparing to carriers with lower surface area (Phanwilai et al., 2020).

These findings were supported by result of Phanwilai et al. (2020), which reported high removal efficiencies for COD and BOD as 96%–98%. Moreover, removal for ammonium, total nitrogen ranging 72%-98% and 64%–77% respectively, with an expectation in one plant that was designed for lower total nitrogen removal in order to support local agricultural requirements (Phanwilai et al., 2020).

2.7.2 Filling ratio

Filling ratio represents an important operational factors in determination of MBBR system's efficiency. It is the ratio between the volume of supports (biocarriers) that occupied the reactor to the total volume of the reactor. The optimal range for this ratio is between 20% to 70% but it can be modified and adapted to the desired conditions (S. Chen et al., 2007).

According to Vaidhegi (2013) which conducted in a pilot-plant MBBR to treat mill wastewater, it was observed that the maximum degradation was achieved with a filling ratio of 50% and hydraulic retention time (HRT) of 8 hours, highlighting the crucial role of filling ratio on the organic removal because the removal depends on the biomass attached to the bioreactor.

A strong biomass attachment was observed in a study conducted by De Oliveira et al. (2014) within a MBBR pilot plant with 20 m³ capacity. An average HRT was 3.3 hours and the filling ratio was 10%. Moreover, in urban wastewater context, Calderón et al. (2012) observed more mature and also better colonized biofilm on the surface of carriers when the filling ratio were 50%.

2.8 Effect of Hybrid Systems on Nitrifying Activity

Higher amount of nitrogen compounds in water bodies which is due to the contamination from intensive urban, agricultural and industrial activities, resulting in significant environmental challenges (T. Liu et al., 2020). Eutrophication of natural waters is one the serious and significant environmental issues, so that nitrogen removal from municipal wastewaters prior to discharge to environment is crucial (Phanwilai et al., 2020; Song et al., 2019). As an example, in 2018, domestic and industrial ammonium emissions in China had already reached 1.4 million tons. Thus, nitrogen removal from wastewater is obligatory to meet the discharge standards But because of huge discharge of sludge from conventional activated sludge and short sludge ages in the activated sludge process, it is hard to meet efficient nitrification (T. Liu et al., 2020; Song et al., 2019).

Biological nitrogen removal involves two main processes, nitrification and denitrification. Nitrification is a biological process that converts ammonium (NH_4^+) into nitrite (NO_2^-) and then into nitrate (NO_3^-) under aerobic conditions (Phanwilai et al., 2020; Song et al., 2019).

According to Phanwilai et al. (2020) in a community that nitrification is carried out efficiently, the chemolithoautotrophic ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) are considered as dominant members. The denitrification process that is the second step, where NO_2^- and/or NO_3^- are reduced to nitrogen gas through intermediates such as nitric oxide (NO) and nitrous oxide (N_2O); this process is performed by heterotrophic bacteria (Y. M. Kim et al., 2013). Conventional biological nitrogen removal (BNR) through nitrification and denitrification is widely applied for this purpose. To keep the nitrifying bacteria in the reactor of conventional activated sludge systems, high reactor volumes are required to

have high sludge retention times (SRT). Otherwise, nitrifying bacteria may be washed out of the system, which is a failure in nitrogen removal, particularly during low temperatures in winter (Güneş et al., 2019b; Phanwilai et al., 2020).

According to different experiments, the high-rate activated sludge systems shows poor efficiency in nitrification due to the low STR (i.e., 0.5–2 days) which is below the minimum residence time that a cell needs for nitrifiers to grow at temperatures below 25 °C. Moving bed biofilm reactors (MBBR) significantly help to overcome growth limitations associated with sludge retention time (SRT) (Güneş et al., 2019b). Bio-carriers keep a high cell density to ensures effective nitrification (Chang et al., 2005; Manju et al., 2009). Thus, these systems allow slow-growing nitrifiers such as ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) to grow in the system which provides higher microbial retention times while are able to operate at high loading rates and also mitigates temperature sensitivity (Güneş et al., 2019b; Wijnffels et al., 1995).

Moreover, hybrid configurations such as MBBR facilitate simultaneous nitrification and denitrification (SND) by creating oxygen concentration gradients within biofilms, with AOB present in the oxygen-rich outer layers and denitrifying bacteria (DNB) in the anoxic inner layers (T. Liu et al., 2020; Phanwilai et al., 2020). This configuration allows hybrid systems to keep high nitrification efficiencies even under high loading rates and variable operational conditions.

Tang et al. (2020) hypothesized that higher nitrogen loads which is a result of intermittent feeding in this study, could lead to increasing the microbial population, most likely nitrifying bacteria which enhance the overall removal efficiency. Hybrid MBBR systems offers comparative advantages over conventional systems in terms of smaller reactor volume and lower operational expenditures (Casas et al., 2015; Güneş et al., 2019b; Song et al., 2019).

The increase in the biofilm thickness facilitates the development of an anoxic environment within biocarriers, which result in enhancing nitrification-denitrification (SND) and total nitrogen (TN) removal (Song et al., 2019).

2.9 Effect of Organic Loading Rate in Hybrid and CAS Systems on Removal of NSAIDs

Organic loading rate (OLR) can considerably affect the removal efficiency of organic micropollutants such as NSAIDs. It has been shown that MBBR technology, which supports biofilm formation on suspended plastic carriers, maintains high treatment efficiency even in the presence of fluctuating organic loading rate (OLR) conditions (Tang et al., 2020). In an experiment, Ødegaard (2006) stated that the configuration of the biocarriers should also rely on the operational conditions, such as OLR in terms of g COD/ m^2 , to maximize the removal efficiency of organic micropollutants (Madan et al., 2022). Additionally, the settleability of biomass leaving the reactor decreases with increasing the OLR, therefore, high-rate systems need to be equipped with enhanced settling systems like, coagulation or other separation techniques, to reduce the bio-media clogging (Al-Amshawee & Yunus, 2021; Ødegaard et al., 2000).

Although the role of OLR in organic micropollutant's removal is an interesting topic in biological systems, so far there are no clear trend in studies about the influence of OLR and the heterotrophic activity on biological kinetic constants (K_{biol}). As an example, Kora et al. (2020) observed that increase in the OLR enhances the removal efficiency of 3 out of 5 micropollutants in a methanogenic-aerobic moving bed biofilm reactor. In addition, Abtahi et al. (2018), which did an experiment on a moving bed biofilm reactor, observed that some OMPs achieved maximum removal efficiency at highest OLR tests, while the others showed the maximum removal at the lowest OLR. According to Carneiro et al. (2020), there is a negative impact of increasing OLRs in citalopram and sulfamethoxazole removal in anaerobic fix bed biofilm reactors. Also, Moya-Llamas et al. (2018) observed better removal of organic micropollutants at increased OLR in a upflow anaerobic sludge blanket (UASB) reactor integrated with a membrane bioreactor (MBR). In contrast, Gonzalez-Gil et al. (2018) found no correlation between OLR variations and the biotransformation of most organic micropollutants in methanogenic digesters. These findings highlight the complexity of removing organic micropollutants in hybrid MBBR systems and also show how important it is to optimize OLR in order to increase the removal efficiency of organic micropollutants.

Kennes-Veiga et al. (2021) evidenced that higher OLRs lead to an increase in the biotransformation rate of organic micropollutants along with the simultaneous removal of COD and organic micropollutants in activated sludge. This confirms that co-metabolism is the primary mechanism for organic micropollutants biotransformation under aerobic heterotrophic conditions. This increase in biotransformation rate was attributed to the increase in biotransformation kinetic constant (K_{biol}), showing that K_{biol} depends on the environmental conditions, as well as compound properties and also the intensity of metabolic activity. Moreover, the influence of heterotrophs on K_{biol} is different based on the compound. Same study concluded that, when sufficient HRT is provided, co-metabolic trend doesn't necessarily approach to a better biotransformation yield.

Besides, Al-Sayed et al. (2023) and Zellmer (2024) indicated that low OLR can promote the nitrification and should be kept low for efficient nitrification in MBBR systems. At higher OLRs, the increased concentrations of organic carbon may lead to a competition between heterotrophic and autotrophic (nitrifying) organisms (Al-Sayed et al., 2023).

On the other hand, low OLR may lead to contradictory results on organic micropollutants removal in some treatment plants (Abtahi et al., 2018; Kora et al., 2020). MBBR (moving bed biofilm reactor) technology, which supports the attached biofilm formation, was reported to achieve effective treatment performance even in fluctuating organic loading conditions (Tang et al., 2020).

2.10 NSAIDs Degradation by Microorganisms

Different studies have shown that biological transformation/biotransformation is the main removal mechanism of pharmaceuticals. Using advanced oxidation technologies to remove NSAIDs has been widely studied for all NSAIDs. However, these methods often lead to the formation of various potentially toxic byproducts, and their high energy consumption categorizes them as having a large ecological footprint. On the other hand, microbial degradation has been recognized as an effective and sustainable approach for addressing NSAID pollution (Ciftcioglu et al., 2021; Rastogi et al., 2021). Moreover, operational parameters such as solid retention time (SRT),

Hydraulic retention time (HRT), organic loading rate (OLR), and also supplied oxygen concentration effect on this process (Ciftcioglu et al., 2021).

Both bacteria and fungi have the ability to biodegrade NSAIDs. Different microbes or microbial consortia have demonstrated significant removal of different pharmaceuticals in the laboratory setup. The removal efficiencies vary depending on the type of pharmaceuticals and microorganisms involved (Rastogi et al., 2021).

Martínez-Alcalá et al. (2017) emphasized the importance of biodegradation for ketoprofen and ibuprofen in their study by reporting high contribution of microbial biodegradation in the total removal of ibuprofen and ketoprofen as 54.3% and 99.7% , respectively.

Lab scale studies reveal that fungi, algae, and bacteria are capable of efficiently degrade NSAIDs, but on a field scale of treatment (large-scale treatment), it is very challenging to maintain the pure culture. Several studies investigated the use of mixed microbial consortia to degrade individual and mixture of NSAIDs. Table 2.3 shows some studies that characterized the components of mixed consortia.

Table 2.3: Microbial degradation of NSAIDs by mixed cultures. Taken from Rastogi et al. (2021).

S. No	Microorganism Used	Initial Concentration	Removal %	Time Taken
1	<i>Gordonia amicalis</i> , <i>Acinetobacter bouvetii</i> , <i>Paracoccus aminophilus</i> and <i>Patulibacter americanus</i>	1000 µg/L or 500 µg/L ibuprofen	100%	2–6 h
	<i>Methylobacterium populi</i> , <i>Gordonia hydrophobica</i> , <i>Tsukamurella spumae</i> , <i>Paracoccus aminovorans</i> , <i>Rhodococcus qingshengii</i> , <i>Gordonia terrae</i> , <i>Rhodococcus zopfii</i> and <i>Bosea thiooxidans</i>	250 µg/L or 100 µg/L ketoprofen	100%	10-25 h
2	<i>Arthrobacter nicotianae</i> , <i>Pseudomonas sp.</i> , <i>Enterobacter hormaechei</i> and <i>Citrobacter youngae</i>	3 mg/L of each NSAIDs	23.08% for ibuprofen and 9.12% for diclofenac	48 h
		3 mg/L of each NSAIDs with supplementary feeding with glucose	100% for ibuprofen and 56% for diclofenac	48 h
3	K ₂ bacteria consortium (<i>Raoultella ornithinolytica</i> B6, <i>Pseudomonas aeruginosa</i> strain JPP, <i>Pseudomonas sp.</i> P16, <i>Stenotrophomonas sp.</i> 5LF 19TDLC)	1.27 g/L of ketoprofen under dark conditions	100%	48 h
	K ₂ bacteria consortium + microalga <i>Chlorella sp.</i> Iso 4	508.5 mg/L of ketoprofen under diurnal conditions	100%	10 days
4	Stable nitrifying enrichment culture	25–100 µg/L of ibuprofen	100%	24 h
		25–100 µg/L of ketoprofen	100%	~150 h
5	Pentane enrichment culture	2.9 mg/L of diclofenac, 2 mg/L ibuprofen and 2.3 mg/L naproxen respectively	0%, 100%, and 64% respectively	96 h

Many studies conducted on the degradation of NSAIDs by mixed cultures (Hasan et al., 2016; Martínez-Alcalá et al., 2017; L. N. Nguyen et al., 2019). For

example, a study conducted in 2019, reported that some different genera such as *Nitratireductor*, *Pseudacidovorax*, *Asticcacaulis*, and *Bacillus pseudomycoides*, *Rhodococcus ruber*, *Vibrio mediterranei* have demonstrated competitive advantages and also resistance to the toxicity of some NSAIDs including diclofenac, ibuprofen, and ketoprofen, implying their contribution to the biodegradation (L. N. Nguyen et al., 2019). Biodegradation pathways of NSAIDs in mixed cultures are not widely studied (Rastogi et al., 2021). In another study, biodegradation of ibuprofen, naproxen, diclofenac, and ketoprofen were studied considering them as sole carbon substrate while using activated sludge. They detected that only 20 mg/L of ketoprofen degraded through biphenyl pathway in 28 days, while all other compounds remained unchanged. Moreover, in the same study, it was reported that O-desmethylnaproxen together with 2-hydroxylated ibuprofen and 1-hydroxylated Ibuprofen were produced from demethylation of naproxen and hydroxylation of ibuprofen by mixed cultures. On the other hand, degradation of ketoprofen leads to the production of 3-(hydroxy-carboxymethyl) hydratopic acid and 3-(keto-carboxymethyl)-hydratopic acid through processes like dioxygenation, keto group reduction, ring-opening of catechol, hydrolysis, and the release of CO₂, water, and a CO group (Rastogi et al., 2021). Likewise, degradation of ketoprofen by means of K2 bacterial consortium yielded in tree specific metabolites as (3-ethylphenyl) (phenyl)methanone; (3-hydroxyphenyl) (phenyl)methanone, and (3-hydroxyphenyl) (oxo)acetic acid which were not detected in pure cultures (Rastogi et al., 2021).

Further researchers studied the use of algal-bacterial approach for the degradation of ibuprofen and naproxen in two photobioreactors (anoxic-aerobic and anaerobic-anoxic-aerobic). The dominant microalgae population in the photobioreactors were *Chlorella vulgaris*, *Phormidium sp.*, *Chlorella vulgaris*, *Pseudonabaena acicularis*, and *Scenedesmus acuminatus*, depending on the reactor phase (López-Serna et al., 2019).

Rutere et al. (2020) revealed that microbial degradation of ibuprofen in hyporheic zone sediments of a river yielded into 2 biotransformation products including 1-, 2-, 3-hydroxyibuprofen and carboxyibuprofen. Bacterial strains comprising 6 taxa of *Acidobacteria*, *Gemmatimonadetes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*

and *Latescibacteria* and two strains from genera *Pseudomonas* and *Novosphingobium* were identified as potentially contributing to degradation of ibuprofen. Another study observed that the population of *Actinobacteria* and *Bacteroidetes* were further enhanced in the presence of diclofenac and naproxen which is due to the adaption ability of bacteria to NSAIDs and eventually effectively degrade them (Jiang et al., 2017a).

Different environmental parameters including Illumination, nitrification, ammonium concentration and dissolved organic matter affect the mixed culture removal efficiencies. For example, the degradation rate of ketoprofen by K2 bacterial consortium elevated under dark conditions (Ismail et al., 2016). Another study observed a reduction in the biotransformation of ibuprofen at high ammonium concentrations (Dawas-Massalha et al., 2014). Moreover, in another research, results demonstrated that addition of dissolved organic matter improves the biotransformation of ibuprofen and naproxen, probably because of the increase in biomass production and microbial respiration which elevated the overall microbial activity that eventually leads to higher drug biodegradation capacity (He et al., 2018).

2.11 Effect of Micropollutant on Microbial Structure and Diversity

The influence of micropollutants on the microbial diversity and community structure has shown a great interest between many scientists. There are many scientific papers that have documented the impact of pharmaceuticals on the microbial population across different taxonomic groups. Except for the result shown in toxicity studies on individual organisms, the environmental impact of pharmaceuticals can extend far beyond these effects and potentially are able to substantially impact (disrupt) the entire microbial communities (Farkas et al., 2023).

According to a study conducted by Farkas et al. (2023), it was concluded that microorganisms have the ability to adapt and respond to the presence of emerging micropollutants. In their study, the microbial community's changes were evaluated in the presence of different concentrations of diclofenac (DCF), ibuprofen (IBU), and acetylsalicylic acid (ASA) through microcosm experiments. The taxonomic assessments showed that *Proteobacteria* were dominant in the absolute

controls and the representation of *Bacteroidetes* and *Patescibacteria* were significant. *Actinobacteria* were also abundant, while *Verrucomicrobia*, *Cyanobacteria*, and *Planctomycetes* were less represented. For archaea, *Nanoarchaeaeota* had a substantial abundance within the microbial community. *Thaumarchaeota* also were abundant but less in comparison to *Nanoarchaeota*. After three weeks of incubation, NSAIDs changed the microbial community's structure of treated samples, leading to an excessive presence of *Proteobacteria*. The bacteria showed higher resistance to ibuprofen / acetylsalicylic acid in compare to diclofenac like that a high reduction in the number of *Bacteroidetes* were observed in the samples treated with diclofenac while in IBU/ASA-treated samples, *Bacteroidetes* have remain abundant. Moreover, the number of *Actinobacteria* and *Patescibacteria* have decreased in all treated samples with NSAIDs. *Cyanobacteria* exhibited tolerance to IBU/ASA treated samples while for *Verrucomicrobia* and *Planctomycetes* the tolerance was observed in all treated samples. Therefore, it was concluded that generally these drugs led to a reduction in microbial diversity mostly in higher concentrations of NSAIDs (1000, 2000, and 6000 ppm) while increasing the overall cell count. Furthermore, this reduction in diversity indices of microbial community show a shift towards more resistant species (Farkas et al., 2023).

However, in another study, the Shannon-Wiener diversity index showed an increase in microbial diversity in the sequencing batch reactors (SBRs) that were subjected to environmentally relevant concentrations of diclofenac, ibuprofen and naproxen (individually and/or in mixture) (Jiang et al., 2017b). The increase in diversity was specifically due to *Actinobacteria* and *Bacteroidetes* enrichment which implies that both of them are capable of degrading NSAIDs and as a result they can adapt well with the presence of pharmaceuticals (Jiang et al., 2017b). The 16s rRNA gene sequencing in same study shows significant changes at phylum level of activated sludge: *Actinobacteria* and *Bacteroidetes* shows an increase, while a reduction was observed in *Proteobacteria* and *TM7* population, indicating that *Actinobacteria* shows adaptability whereas *Proteobacteria* imply less tolerant to the selected NSAIDs (Jiang et al., 2017b). The effect of micropollutants (MPs) on bacterial diversity at the genus level showed important shifts in microbial community structure. A

decrease in abundance of *Nakamurella* and *Micropruina* were observed. These are the key organisms in activated sludge systems. On the other hand, an increase in the population of some genera like *TM7_genera_incertae_sedis*, *Amaricoccus*, and *Haliscomenobacter* were documented, likely as a result of their ability to metabolize pharmaceuticals. *Zoogloea* also became more abundant, potentially playing a protective role against micropollutants (MPs). Furthermore, oxidative stress responses, like elevated superoxide dismutase (SOD) activity were observed, showing the complex effects of micropollutants on microbial ecosystems (Ballesteros Martin et al., 2011; Carr et al., 2011; Jiang et al., 2017b; X.-c. Wang et al., 2016).

D. Zhang et al. (2016a) mentioned the maximum Shannon-Wiener index in SBRs adding 5 µg/L of tetracycline and sulfamethoxazole. Also, Huang et al. (2015) observed an increase in the bacterial diversity after adding 300 µg/L of tetracycline, using two lab-scale anaerobic-anoxic-oxic systems. However, an increase in the concentration of pharmaceuticals to 10 µg/L suppress the bacterial diversity (D. Zhang et al., 2016a). Thus, these results suggested that the microbial adaptivity has its limits and future researches are needed to investigate the threshold value of pharmaceutical concentration (Jiang et al., 2017b).

Many studies focused on the short-term toxic effects of ibuprofen on activated sludge systems, while long-term effects are rare. Davids et al. (2017) reported that exposure to 50 mg/l ibuprofen can inhibit the activity of activated sludge. While even small amounts of ibuprofen (ng/L) can alter the reproduction rate of aquatic organisms (S. Liu et al., 2022). Therefore, there is possibility for inhibition of microorganisms due to the presence of ibuprofen and finally affect the stable operation of the activated sludge system. Many other examples can be found in the literature about the effects of ibuprofen exposure on activated sludge, such as altering the behavior of bacteria by reducing extracellular polymeric substances and microbial oxygen uptake (Amariei et al., 2017). Also, in another study a decrease in the community of *Nitrospira* and *Nitrosomonas* were observed with increase in ibuprofen concentration (G. Zhou et al., 2019).

Long-term changes in the microbial composition, diversity indices and also their interactions in activated sludge were studied extensively under the ibuprofen stress (S. Liu et al., 2022). Shannon and Simpson indexes reflect the microbial diversity and Chao1 and ACE indexes show the richness of microorganisms in the activated sludge. A decrease in both microbial diversity and structure were documented by a significant decrease in the Shannon index in control SBR after treating the SBR with 1 mg/L ibuprofen. Likewise, reduction in Ace and Chao1 were observed. Moreover, decrease in Operational Taxonomic Unit (OTU), which is related to the change of alpha diversity, was reported (S. Liu et al., 2022). In particular, ibuprofen decrease the microbial diversity through altering their structure, which result in a reduced population of nitrifying bacteria (*Nitrospira* and *Nitrosomonas*), while supporting the growth of denitrifiers (e.g., *Hyphomicrobium* and *Denitratisoma*). The mentioned changes show the selective pressure triggered by ibuprofen, which alters microbial communities by encouraging the growth of more resistance population to ibuprofen. Also, a reduction in the size and complexity of the molecular ecological network were conducted in the presence of ibuprofen, indicating a simplified (streamlined) microbial community structure with less keystone species and changed microbial interactions. For example, ibuprofen stress result in fewer taxa and links in the phylum *Proteobacteria*, which imply a reduction in the complexity of microbial network. On the other hand, an increase in the clustering coefficients were documented in the *Chloroflexi*, denoting a closer connection between microbial community (S. Liu et al., 2022).



3. MATERIAL AND METHOD

This thesis is a part of TUBITAK Research Project No. 117Y087. This study focused on evaluating the microbial population dynamics in hybrid biofilm nitrification systems used for biological removal of NSAIDs.

Detailed information on the experimental set-up, reactor operation and analysis conducted to evaluate reactor performance and results can be found in in MSc thesis of Uçan (2024).

3.1 Experimental Set-up

Laboratory-scale sequencing batch reactors (SBR) were operated to study the biodegradability of micropollutants in hybrid systems (moving bed biofilm reactors, MBBR). Hybrid systems combine suspended growth-activated sludge processes with attached growth biofilm processes, allowing for simultaneous growth. Hybrid systems allow higher biomass concentrations to be retained in the reactor, resulting in increased organic matter removal and nitrification efficiency. In this context, laboratory-scale reactors were modified by adding carriers to reactors to ensure the retention of both suspended biomass and biofilm in the system. The sludge age was determined as 10 days. Control hybrid reactor operated for 150 days, while the operation period for micropollutant hybrid reactor was 215 days with a constant SRT of 10 days for both control reactors and micropollutant-added reactors.

3.2 Control Hybrid SBR Experimental Set-up

The control hybrid SBRs were set up using laboratory-scale cylindrical glass reactors with a working volume of 6 L (total reactor volume of 10 L). In order to have a stable temperature during the operation of the reactors, the SBRs were kept in a temperature-controlled room with a constant temperature of $20\pm 2^{\circ}\text{C}$. SBRs were stirred with magnetic (VWR, VMS-C10, USA) and mechanical (IKA, Eurostar

40, Germany) equipment and aerated with constant air flow. The attachment of micropollutants was prevented by using specially designed perforated Teflon hoses. High-power air pumps (RS Electrical-15000, China) connected to timers were used to supply the air at a constant rate. The feeding and discharge of the reactors were done with peristaltic pumps (Seko, PR7, Turkey; Antech, PER-A, Turkey) connected to timers. The carriers were from the Aquaflex brand (Bioaqua, 2025), and the reactors were operated with a filling ratio of 30%.



Figure 3.1: Carriers added to the reactors.

The activated sludge was obtained from the aeration tank of an advanced biological wastewater treatment plant located in Istanbul. Lab-scale SBRs were fed with synthetic wastewater prepared in accordance with the composition of domestic wastewater (Table 3.1). SBRs were fed twice a day with a peptone mixture having a chemical oxygen demand (COD) of 150 mg/L. The reactors were additionally supplied with Solution A and Solution B to satisfy the inorganic nutrient requirements of the microorganisms. The initial ammonium concentration of the SBRs were set as 60 mg/L.

Table 3.1: Composition of the Synthetic Wastewater.

Pepton Mixture	Stok (g/L)	Solution A	Stok (g/L)	Solution B	Stok (g/L)
Peptone	16	K ₂ HPO ₄	320	MgSO ₄ ·7H ₂ O	15
Beef Extract	11	KH ₂ PO ₄	160	FeSO ₄ ·7H ₂ O	0.5
Urea	3			ZnSO ₄ ·7H ₂ O	0.5
NaCl	0.7			MnSO ₄ ·7H ₂ O	0.41
CaCl ₂ ·2H ₂ O	0.4			CaCl ₂ ·2H ₂ O	2.65

Sludge age of 10 days was adjusted by the daily discharge of the constant volume of 600 mL mixed liquor as the waste sludge from the reactor. The pH was kept between

6 and 8 through regular monitoring. A cycle time of 12 hours were regulated for the operation of the reactors. Operating conditions and parameters of the SBRs are depicted in Figure 3.2 and Table 3.2.

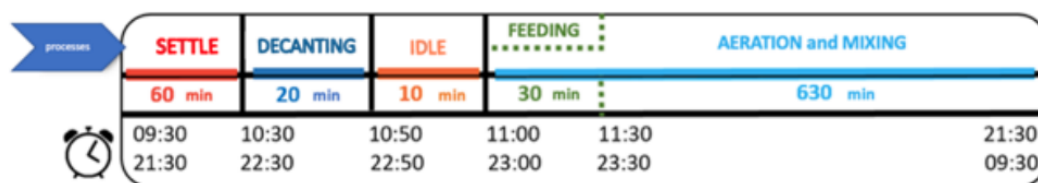


Figure 3.2: Operating conditions of reactors in laboratory-scale sequential batch hybrid reactors (Reference project).

Table 3.2: Operating Parameters of Laboratory-Scale SBRs.

Parameter	Symbol	Value	Unit
Total cycle time	T_C	12	h
Duration of feeding phase*	T_F^*	30	min
Duration of aeration phase	T_A	630	min
Duration of settling phase	T_S	60	min
Duration of draw phase	T_D	20	min
Duration of idle phase	T_I	10	min
Initial volume	V_0	3	L
Total volume	V_T	6	K
V_0/V_F	-	0.50	-

*First 30 minutes of aeration phase.

3.3 Hybrid Reactors Fed with Micropollutants

Hybrid reactors with micropollutant addition, have the same operational conditions of control hybrid reactors. An aluminum foil covered up the reactors and stock feed solution to avoid photolysis of micropollutants (Figure 3.3).

The micropollutant concentrations introduced to the reactors were determined as 10 µg/L for Ibuprofen and Naproxen, and 1 µg/L for Ketoprofen, Indomethacin, Mefenamic acid, and Diclofenac.

Total Suspended Solid (TSS), Volatile Suspended Solid (VSS), Chemical Oxygen Demand (COD), Soluble Chemical Oxygen Demand (sCOD), Nitrite (NO_2^-), Nitrate (NO_3^-), Ammonium Nitrogen ($\text{NH}_4\text{-N}$) analyses were conducted on the samples taken from a fully mixture before feeding once every seven days to track the performance

of the reactors. Samples from the micropollutant reactors that reached steady state were taken once a week before feeding in order to measure the concentration of micropollutants. In all reactors, a single microbial population analysis was performed at steady state condition.



Figure 3.3: Hybrid reactor with micropollutant addition and feed solution bottle.

3.4 Measurement of Conventional Parameters

COD was determined based on the ISO 6060 standard. Measurement of TSS, VSS, and $\text{NH}_4\text{-N}$ was carried out in accordance with the Standard Methods (APHA). The samples were kept at $+4^\circ\text{C}$ until measurement. NO_2^- and NO_3^- analyses were conducted by Ion Chromatography, which was equipped with a conductivity detector and an analytical column (AS14A). The pH was measured with an OrionA211 pH meter based on the ISO 10523 Standard (2008).

3.5 Micropollutant Measurement

A Thermo brand ultra-performance liquid chromatograph combined with a triple quadrupole mass spectrometer (LC-MS/MS) was applied for measuring the micropollutants. A detailed description for this section of the study can be found in Topuz et al. (2014).

3.6.2 Polymerase chain reaction (PCR) analysis

Species identification was conducted by amplification of V3 and V4 regions of the 16S ribosomal RNA gene. V3 and V4 regions are considered as the most frequently used variable region for species identification. They were amplified through forward (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3') and reverse (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC3') primer sequences (300 base pair region). The components of the PCR mixture are provided in Table 3.3.

Table 3.3: PCR Mixture Contents.

	volume
Microbial DNA (5 ng/μL)	2.5μL
Forward Primer 1 μM	5μL
Reverse Primer 1 μM	5μL
2X KAPA HotStart PCR Mix	12.5μL
Total	25μL

The PCR conditions were as follows: 3 minutes at 95°C, 8 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C, followed by a final extension of 5 minutes at 72°C.

PCR products were purified using 20 μL of AMPure XP (Beckman Coulter) magnetic beads to eliminate primer dimers and free primers. Samples containing magnetic beads were washed twice with 200 μL of 80% ethanol, then the purified PCR products were kept in 50 μL of 10 mM pH 8.5 Tris solution. After the purification process, an index PCR was conducted. In this step, specific base sequences, functioning as “index barcodes” to differentiate between several samples during next-generation sequencing and also enable the processing of multiple samples in a single run, were introduced to the PCR products. Moreover, to attach the PCR products to the specific oligo attachment sites in the next-generation sequencing device, the “adapter” sequences were added. During this process, which is carried out through the Nextera XT index kit, two index barcode regions were linked to each sample. The purified

PCR products were stored at +4°C. The purified PCR products were further sent to BMLabosis (Ankara) via a cold chain (packaged in a special box with dry ice) for the characterization of the microbial community via DNA sequencing analysis.

The bioinformatics analysis was carried out for the taxonomic classification of the resulting sequence data, identification of operational taxonomic units (OTU), alignment to reference sequences, and grouping based on species similarities (Bolyen et al., 2019). Reads shorter than 36 bases were removed from the data analysis. SILVA 138 SSU database was used for microbiome analyses. OTUs were determined at a 97% confidence level with sequence similarity.

The analysis of collected sequences initially focused on the phylum level. Variations at the phylum level under varying operating conditions were observed. A more detailed and closer analysis of these changes was done at the species level. To achieve this, the number of species observed at different sequencing depths was assessed and rarefaction curves were generated to show changes in richness across various samples. Furthermore, non-parametric statistical indicators were conducted to conclude the population analysis of reactors operated under various conditions. To examine the changes in richness in the samples, the Chao1 parameter was used. While Pielou's evenness parameter was used to indicate if the population exhibited a dominant structure, the Shannon diversity index was applied to assess and compare species diversity. Goods coverage was used to estimate the representative percentage of species in the samples.



4. RESULTS AND DISCUSSION

4.1 System Performance of Control and Micropollutant Reactors

Effluent COD, ammonium (NH_4^+), Nitrite (NO_2^-) and Nitrate (NO_3^-) and also VSS/SS ratio and PH were measured to evaluate the performance of control and micropollutant hybrid (MP) reactors. Initial organic load was 150 mg/L COD and initial ammonium concentration was 60 mg N/L. Control hybrid reactor operated for 150 days, while the operation period for micropollutant hybrid reactor was 215 days with a constant SRT of 10 days for both reactors. The detailed explanation of system performance of control and MP hybrid reactors were explained in MSc thesis of Uçan (2024).

4.1.1 Control hybrid reactor

After acclimation period, the average values of the system performance were measured for the control hybrid reactor. SS and VSS concentrations in control hybrid reactor were measured as 1050 ± 52 mg/L and 798 ± 39 mg/L, respectively, with a VSS/SS ratio of 0.77 and pH of 6.6 ± 0.95 .

4.1.2 Micropollutant hybrid reactor

In the hybrid micropollutant reactor, inoculum sludge fed with micropollutants including ibuprofen (10 $\mu\text{g/L}$), diclofenac (1 $\mu\text{g/L}$), indomethacin (2 $\mu\text{g/L}$), naproxen (10 $\mu\text{g/L}$), mefenamic acid (1 $\mu\text{g/L}$), and ketoprofen (1 $\mu\text{g/L}$). For MP reactor, SS and VSS concentrations were measured as 884 ± 44 mg/L and 779 ± 38 mg/L, respectively, with a VSS/SS ratio of 0.88 and pH of 6.6 ± 0.9 . Lower concentrations of SS, VSS were observed in the hybrid MP reactor.

Furthermore, Table 4.1 compares the performance of hybrid control and hybrid MP sequencing batch reactors (SBRs) at sludge retention time of 10 days by illustrating effluent COD, ammonium ($\text{NH}_4\text{-N}$), Nitrite (NO_2^-) and Nitrate (NO_3^-) as 29 ± 1.1

mg/L, 14±0.15 mg/L, 3.3 mg/L and 44.7 mg/L, respectively for the control reactor. These effluents, except nitrate (NO_3^-) was lower in the effluent of hybrid MP reactor than hybrid control reactor. The concentrations of effluent COD, ammonium, nitrite and nitrate were measured as 20±1.6 mg/L, 6±0.1 mg/L, <0.1 mg/L and 45.7 mg/L, respectively in the MP hybrid reactor. Briefly, the concentrations of COD and ammonium reduced significantly.

Table 4.1: SBR performance of the control hybrid and MP hybrid reactors

Parameters	SRT10 Control Hybrid Reactor	SRT10 MP Hybrid Reactor
Effluent COD (mg/L)	29±1.1	20±1.6
Avg. $\text{NH}_4\text{-N}$ (mg/L)	14±0.15	6±0.1
Avg. $\text{NO}_2^- \text{-N}$ (mg/L)	3.3	<0.1
Avg. $\text{NO}_3^- \text{-N}$ (mg/L)	44.7	45.7

The effluent concentrations and removal efficiencies were measured for six selected NSAIDs, illustrated in Table 4.2. A complete removal was measured for the ibuprofen (100%) and indomethacin (100%). Other NSAIDs including naproxen (99%), mefenamic acid (98%), and ketoprofen (96%) showed significantly high removal, except for diclofenac (69%). Comparing to other Pharmaceutically Active Compounds (PhACs) which are more readily biodegradable, diclofenac is one of the most recalcitrant ones (dos Santos et al., 2022).

Table 4.2: Treatment efficiency of selected NSAIDs

NSAID	Theoretical Influent ($\mu\text{g/L}$)	Effluent ($\mu\text{g/L}$)	Removal Efficiency (%)
Ibuprofen	20	0.03	100
Diclofenac	2	0.61	69
Indomethacin	2	0.01	100
Naproxen	20	0.24	99
Mefenamic acid	2	0.03	98
Ketoprofen	2	0.09	96

4.2 Effect of Selected NSAIDs and Low Organic Loading on Microbial Community in Hybrid System Based on SILVA Database

This study mainly focused on the effect of six selected non-steroidal anti-inflammatory drug (NSAID), as micropollutants, on the different microbial community structure in hybrid biofilm systems evaluated using sequencing batch reactors (SBRs) with

biofilm carriers at SRT of 10 days. DNA extraction and 16S rRNA gene sequencing were done and then the data were processed. The microbial community's relative abundance is depicted in the following figures at phylum level, genus level and species level. The V3-V4 variable region was selected for taxonomic analyses as V3-V4 has a good balance of taxonomy coverage and resolution for short-read sequencing analysis. SILVA database was used for taxonomic classification of operational taxonomic units (OTUs).

4.2.1 Effect of selected NSAIDs at phylum level

Relative abundance of microbial phyla in the inoculum sludge, suspended biomass in hybrid control reactor, and suspended biomass in hybrid MP reactor is illustrated in Figure 4.1 Only the phyla with frequency above 1% were included.

The most dominant phylum is *Bacteroidota*, with a relative abundance that gradually increased from 30.68% in the inoculum sludge to 31.62% in the control hybrid reactor, reaching its maximum at 42.40% in the hybrid MP reactor. *Bacteroidota* are heterotrophic microorganisms capable of degrading high molecular weight organic compounds, such as petroleum hydrocarbons, and they contribute to denitrification (Eshamuddin et al., 2024; Gargouri et al., 2014).

The higher percentage of *Bacteroidota* in the presence of selected NSAIDs can be supported by Jiang et al. (2017b), who found that *Bacteroidota* abundance increased in reactor by increasing the concentration of the mixtures of selected NSAIDs of diclofenac, ibuprofen and naproxen. This finding can also be corroborated by the results of Drury et al. (2013), which reported an increase in the *Bacteroidota* at urban locations with higher concentrations of complex organic compounds.

The second most abundant phylum is *Proteobacteria*. It was observed that its relative abundance increases from 16.24% in the inoculum sludge to 29.00% in the hybrid control reactor up to 35.13% in the hybrid MP reactor. This phylum is recognized as the most dominant phylum in marine and soil environments (von Mering et al., 2007). *Proteobacteria* dominate biological wastewater treatment systems as they are able to remove organic matter and nutrients (Vasiliadou et al., 2018). Moreover, they

are among one of the most common phyla contributing to degrade different pollutants including hydrocarbons, pharmaceuticals and etc. (Farkas et al., 2023).

Planctomycetota and *Patescibacteria* make a significant contribution to the microbial composition in the inoculum sludge (15.01% and 14.02%, respectively) but its abundance declined in both hybrid control and hybrid MP reactors. The reduction in the *Patescibacteria* abundance can be supported by (Farkas et al., 2023) which observed a reduction in number of this phylum in all NSAIDs-treated microcosms (250 mL, clean, sterile glass bottles- a small controlled system) (with different dosage of diclofenac, ibuprofen, and acetylsalicylic acid. de Bento Flores, Carlos Eduardo (2013), reported that, in addition to acetaminophen and caffeine, diclofenac was the only studied NSAID that adversely affect *Planctomycetes* (*Planctomycetota*) growth which leads to a complete loss of cell viability. *Planctomycetota*, contributes to the anaerobic ammonia oxidation (anammox), carbon and nitrogen cycles and transformation of inorganic sulphur (Eshamuddin et al., 2024).

A significant reduction was observed in *Actinobacteriota* from 11.18% in hybrid control reactor to 2.58% in the presence of selected NSAIDs in suspended biomass of hybrid MP reactor almost same as its relative abundance in the inoculum sludge as 2.83%. The reduction in the *Actinobacteriota* abundance can be supported by Farkas et al. (2023) which reported a reduction in number of this phylum in all NSAIDs-treated microcosms (with different dosage of diclofenac, ibuprofen, and acetylsalicylic acid). On the other hand, same study stated that similar to *Bacteroidota* and *Planctomycetes*, *Actinobacteriota* were unable to utilize diclofenac but comparing to the control it was reported that despite the reduction in abundance, this phylum remained relatively high suggesting an adaption to the selected NSAIDs in the study (Farkas et al., 2023).

Briefly, the first four dominant phyla in the inoculum sludge are *Bacteroidota* (30.68%), *Proteobacteria* (16.24%), *Planctomycetota* (15.01%), and *Patescibacteria* (14.02%) with less abundance of Chloroflexi, Verrucomicrobiota, *Acidobacteriota*, *Actinobacteriota*, *Myxococcota*, *Firmicutes*, and *Nitrospirota*, respectively. Contrary to our finding, in another study, conducted on hybrid anoxic/oxic (AO) and anoxic/oxic

membrane bioreactor (AO-MBR), *Chloroflexi* was observed as the most crucial bacterial phylum in nitrite oxidation and denitrification in activated sludge Qin et al. (2018), contributing to production of the filamentous scaffolding of flocs, carbohydrate degradation, and sludge settleability (Nierychlo et al., 2019; Speirs et al., 2019). *Verrucomicrobiota*, contributes to the degradation of polysaccharide, while *Chloroflexi* could degrade the nitrogen and phosphorus and also involve in the removal of macromolecular organic matter (Eshamuddin et al., 2024).

In the control hybrid reactor also *Bacteroidota* (31.62%) and *Proteobacteria* (20.00%) emerged as dominant taxa. After these two dominant taxa, *Actinobacteriota* (11.18%) and *Planctomycetota* (8.42%) were observed as phyla with high relative abundance. Other taxa such as *Patescibacteria*, *Acidobacteriota*, *Verrucomicrobiota*, and *Firmicutes* are less abundant, respectively.

Following treatment with a mixture of six selected NSAIDs, the results demonstrated a high relative abundance of *Bacteroidota* (42.40%) and *Proteobacteria* (35.13%) same as control hybrid reactor and inoculum sludge, while the relative abundance of *Patescibacteria* (5.69%), *Cyanobacteria* (4.58%), and *Planctomycetota* (4.39%) is less. Furthermore, the representation of *Actinobacteriota*, *Verrucomicrobiota*, *Abditibacteriota*, and *Firmicutes* is detected in low values after addition of selected NSAIDs.

Despite the results of this study, Farkas et al. (2023), reported that, *Verrucomicrobiota* was observed as one of the dominant phyla in diclofenac-treated microcosms, along with *Proteobacteria* and *Bacteroidetes*. Navrozidou et al. (2019) documented that, *Verrucomicrobiota* were high in the immobilized biomass of a bioreactor after addition of commercial ibuprofen, while they decreased when only pure ibuprofen was added. Interestingly, *Cyanobacteria* could be recovered in the presence of selected NSAIDs in the suspended biomass of hybrid reactor. It aligns with previous study Farkas et al. (2023), confirming that *Cyanobacteria* have shown tolerance to Ibuprofen (IBU)/ acetylsalicylic acid (ASA) treatment in microcosms.

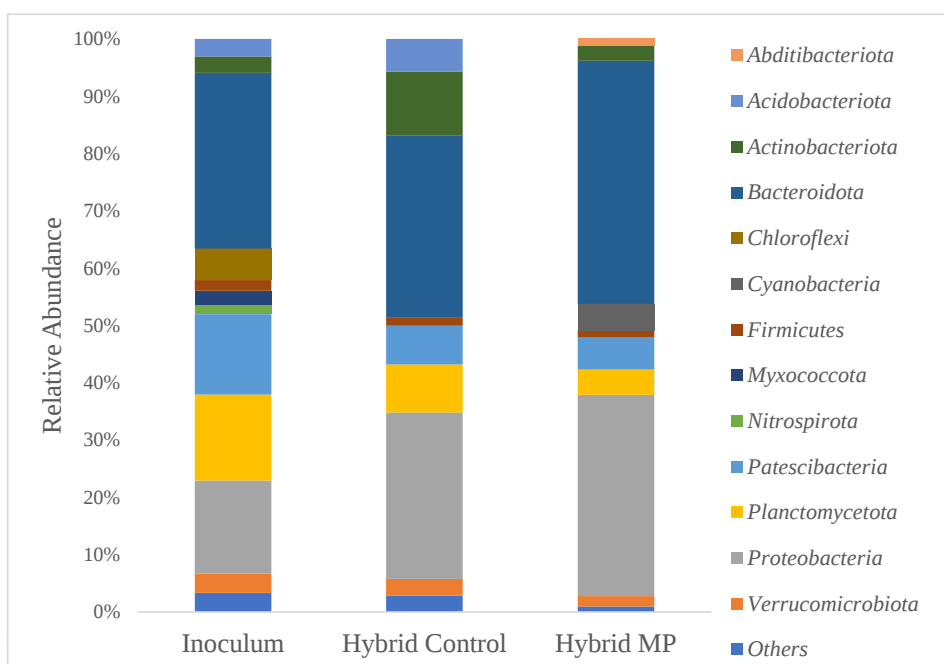


Figure 4.1: Comparison of Relative Abundance at the Phylum Level for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, and Suspended biomass – Hybrid MP Reactor (SILVA Database).

The relative abundance of microbial phyla in attached biomass of hybrid control reactor and attached biomass of hybrid MP reactor is shown in Figure 4.2, including the data only with a frequency more than 1%.

The dominant phyla in the attached biomass of hybrid control and hybrid MP reactor were detected for *Proteobacteria* with the relative abundance of 39.65% and 29.28%, respectively. Decrease of *Proteobacteria* in the attached biomass of hybrid MP reactor indicated that this phylum may not adapt well in the presence of NSAIDs (Jiang et al., 2017b).

The second most abundant phylum in the attached biomass of hybrid control reactor is *Bacteroidota* (18.10%), proliferating significantly in the presence of selected NSAIDs within attached biomass (28.31%).

Another abundant phylum in attached biomass of hybrid control reactor and attached biomass of hybrid MP Reactor is *Actinobacteriota* with relative abundance of 16.16% and 9.00%, respectively. Even though, *Actinobacteriota* abundance decreased, their abundance still remained relatively high in the presence of selected NSAIDs which

might be due to the adaption of this phylum to the selected NSAIDs (Farkas et al., 2023).

Even though, *Planctomycetota* shows lower presence after addition of selected NSAIDs (5.37%), its abundance was high in hybrid control reactor (10.40%).

Notably, *Armatimonadota* and *Acidobacteriota* might have increased in the presence of selected NSAIDs (8.85% and 6.11%, respectively), probably due to its contribution to the degradation of selected NSAIDs.

Other phylum, including *Patescibacteria* had relatively high abundance in the attached biomass of control reactor (4.55%) and could able to tolerate the addition of selected NSAIDs (4.59%).

In other words, *Proteobacteria* (29.28%) and *Bacteroidota* (28.31%) were recorded as dominant phyla in the attached biomass after addition of selected NSAIDs. The presence of *Actinobacteriota* (9.00%) and *Armatimonadota* (8.85%) were less, whereas in the attached biomass in hybrid control reactor *Proteobacteria* (39.65%), *Bacteroidota* (18.10%), *Actinobacteriota* (16.16%), and *Planctomycetota* (10.40%) were identified as four most abundant phyla.

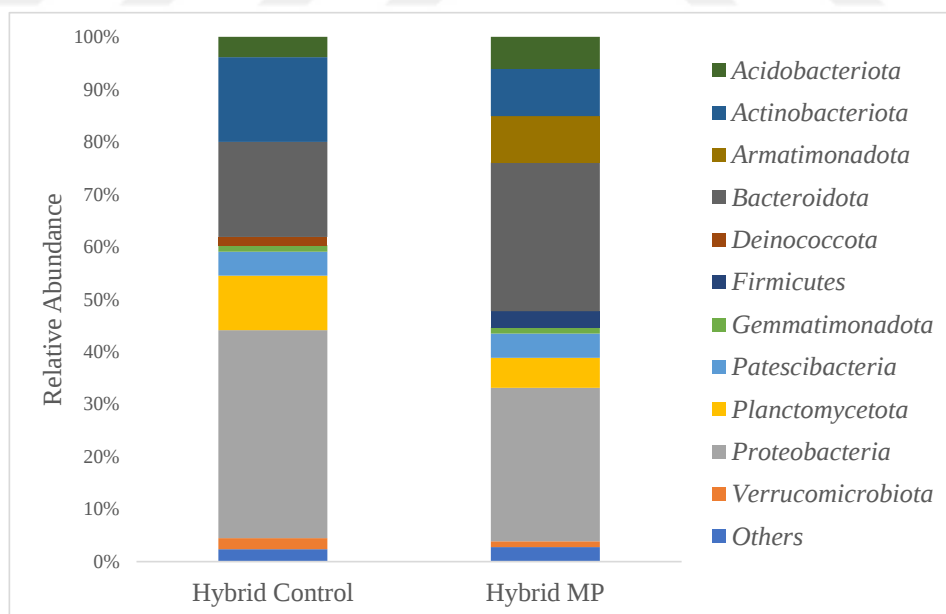


Figure 4.2: Comparison of Relative Abundance at the Phylum Level for Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database)

Figure 4.3 was illustrated to compare the microbial communities in the suspended and attached biomass of hybrid control reactor, displaying only the phyla with relative abundance of above 1%.

The four most dominant phyla in the suspended and attached biomass of hybrid control reactor are almost same, differing in their abundance.

Proteobacteria is notably high in the attached biomass of hybrid control reactor showing as 39.56%. The same trend was observed in the suspended biomass of hybrid control reactor for *Proteobacteria* with the difference that the abundance of this phylum is less in suspended biomass but still it is high as 29.00%.

Although, *Bacteroidota* showed less frequently (18.10%) in the attached biomass of hybrid control reactor, still it counts as the second most abundant phylum in attached biomass. In the suspended biomass of hybrid control reactor, *Bacteroidota* is the most abundant phyla showing as 31.62% frequency which is relatively high.

Previous studies reported *Proteobacteria* and *Bacteroidetes* as the dominant phyla in the WWTPs (Madigan et al., 2008). These phyla acted significantly in the degradation of organic matter and nutrients (Zeng et al., 2022). *Bacteroidetes* (like *Actinobacteria*) might be able to play a crucial role in nitrogen removal (M. Wang et al., 2019).

The third most abundant phylum in both suspended and attached biomass of hybrid control reactor is *Actinobacteriota*, showing more tendency to proliferate in attached biomass comparing to the suspended biomass. The relative abundance of this phyla is 11.18% and 16.16% in suspended and attached biomass of hybrid control reactor, respectively.

Planctomycetota also exhibited high frequency in both suspended and attached biomass of hybrid control reactor and reported as fourth most abundant phylum in both biomass, showing high abundance as 8.42% in suspended and 10.40% in attached biomass. Other detected phyla had lower frequency as shown in the relative abundance graph (Figure 4.3). For example, the relative abundance of *Patescibacteria* and *Acidobacteriota* were observed approximately in lower concentrations, while they are slightly richer in the suspended biomass than attached biomass. Other phyla such as

Gemmatimonadota and *Deinococcota* were observed only in the attached biomass, while *Firmicutes* was observed in suspended biomass of hybrid control reactor.

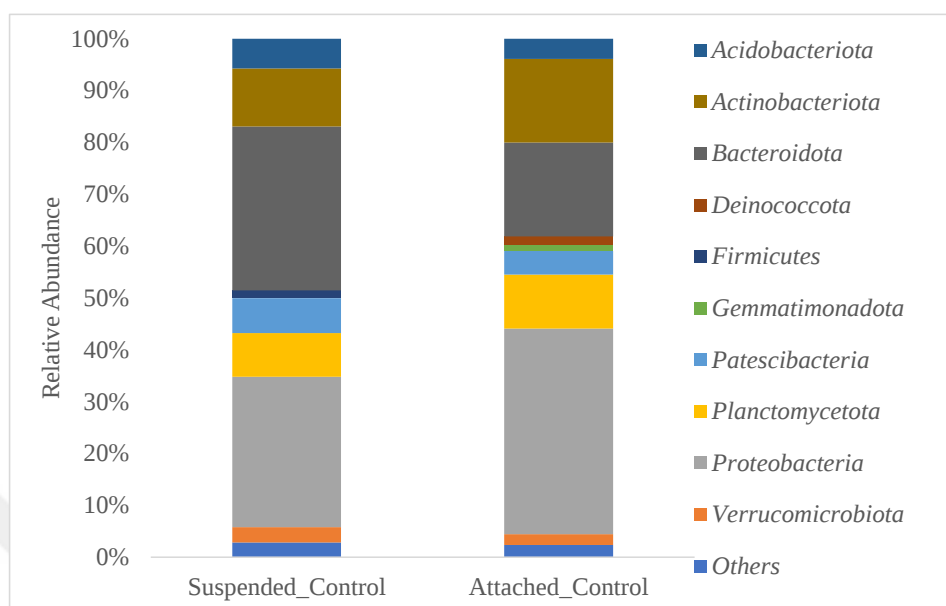


Figure 4.3: Comparison of Relative Abundance at the Phylum Level for Suspended biomass – Hybrid Control Reactor, and Attached biomass – Hybrid Control Reactor (SILVA Database).

The relative abundance of microbial phyla in suspended and attached biomass of hybrid MP reactor is shown in the Figure 4.4. Phyla with a relative abundance exceeding 1% were considered for inclusion.

Bacteroidota and *Proteobacteria* play a crucial role in both suspended and attached biomass after exposure of the reactor to the selected NSAID.

In the attached biomass of hybrid MP reactor some phyla including *Proteobacteria* (29.28%), *Bacteroidota* (28.31%), *Actinobacteriota* (9.00%) and *Armatimonadota* (8.85%) dominated the attached biomass while *Acidobacteriota* (6.11%), *Planctomycetota* (5.73%), *Patescibacteria* (4.59%), *Firmicutes* (3.05%) represented lower but still notable abundances.

In the suspended biomass, same as attached biomass, *Bacteroidota* (42.40%) and *Proteobacteria* (35.13%) showed significantly high relative abundances and dominated the microbial community but the difference is that, based on higher observed relative

abundancies in suspended MP biomass, these phyla have more tendency to grow in suspended biomass than attached biomass in the presence of selected NSAIDs. As illustrated in the Figure 4.4, *Patescibacteria* and *Planctomycetota* showed up in almost close relative abundances in both attached and suspended biomass.

In addition, some phyla such as *Cyanobacteria* and *Armatimonadota*, are detected exclusively in either suspended or attached biomass. For example, *Cyanobacteria* proliferated in the presence of selected NSAIDs only in the suspended biomass. while, *Armatimonadota* was observed only in the attached biomass of hybrid MP reactor.

Various studies on the MBBR systems, indicated *Proteobacteria* as the dominant bacteria in both attached and suspended biomass in MBBR systems (L. Cao et al., 2022; Li et al., 2019; Wolff et al., 2021a). And *Bacteroidetes*, were detected as the second most dominant phylum on the carriers (C. Fu et al., 2017; Ribera-Pi et al., 2020; Zhu et al., 2023).

Wolff et al. (2021a), reported *Bacteroidetes* with higher relative abundances in the suspended biomass than attached biomass. On the other hand, T. Liu et al. (2017b), which conducted on improving partial nitrification in MBBRs, reported *Bacteroidetes* and then *Proteobacteria* as the dominant phyla in the Mpd_biofilm (The biofilm that attached to the modified carriers).

In a recent study by Atasoy et al. (2023), the influent used for three different bioreactors, including MBBR, were from a secondary clarifier that was spiked with a mix of 16 MPs (2 µg/L of each MP), reported high abundance for *Proteobacteria*, *Cyanobacteria*, and *Actinobacteria*, respectively, in the attached biomass of MBBR. In same way, different studies supported the significantly high relative abundance of *Proteobacteria* in MBBRs which were fed with the wastewaters that were fed with MPs (D. Liang et al., 2022; Song et al., 2020; Wolff et al., 2021a; X. Zhang et al., 2020).

It is worth noting that, *Actinobacteriota* could be enriched significantly in the attached biomass, while its presence in the suspended biomass after addition of selected NSAIDs was approximately 70% lower. In a study conducted by Y. Zhang et al. (2016), a significant increase was observed for *Actinobacteria* in ibuprofen-enriched

planted beds in compare to the control. Although, the focus of this study is on six selected NSAIDs, it is worth mentioning another study that assessed the continuous exposure of activated sludge to erythromycin for three days resulting in a preferential growth and dominance of *Actinobacteriota*, with increasing the abundance from 22% to 48% (Pala-Ozkok et al., 2014). Furthermore, *Actinobacteria* is able to degrade diclofenac into different products by using cytochrome P450 enzyme (Prior et al., 2010). *Actinobacteria* same as *Bacteroidetes* may contribute to the nitrogen transformation as well (M. Wang et al., 2019).

An enrichment in the relative abundance of *Actinobacteria* and *Bacteroidetes* in a MBBR system was stated by D. Liang et al. (2022), after the addition of different antibiotics to the system. Adversely, *Actinobacteria* that was counted as the third most abundant phylum in a study conducted by X. Zhang et al. (2020), showed a decline in the presence of higher concentrations of MPs. The same study reported an increase for the *Bacteroidetes*, especially in the MBBR that were exposed to *carbamazepine*, indicating that *Bacteroidetes* could tolerate this MP.

Song et al. (2020) which conducted on a sponge-based aerobic nitrifying moving bed biofilm reactor, reported *Actinobacteria* as the second most dominant phylum, indicating that pharmaceuticals, 1 mg/L sulfadiazine, 2 mg/L ibuprofen and 5 mg/L carbamazepine, stimulate the proliferation of this phylum, suggesting their ability to tolerate the toxicity of such products.

Furthermore, in a study conducted by X. Zhou et al. (2022), *Proteobacteria* and *Actinobacteriota* were observed as dominant phyla in the post-anoxic biofilms and oxic biofilms, respectively and some *Proteobacteria* have an important role in the denitrification (Zhao et al., 2019). Moreover, *Actinobacteriota* noted to be able to remove organic matter (X. Zhou et al., 2022). In the same study, *Acidobacteriota*, *Bacteroidota*, and *Chloroflexi* enriched in the pre-anoxic biofilm. *Actinobacteriota* and nitrogen removal in wastewater treatment are linked (Kristensen et al., 2021), on the other hand *Bacteroidota* are recognized for their role in the hydrolysis of macromolecules in anaerobic or anoxic conditions. Also, *Chloroflexi* have been linked to the degradation of organics (Zhao et al., 2019).

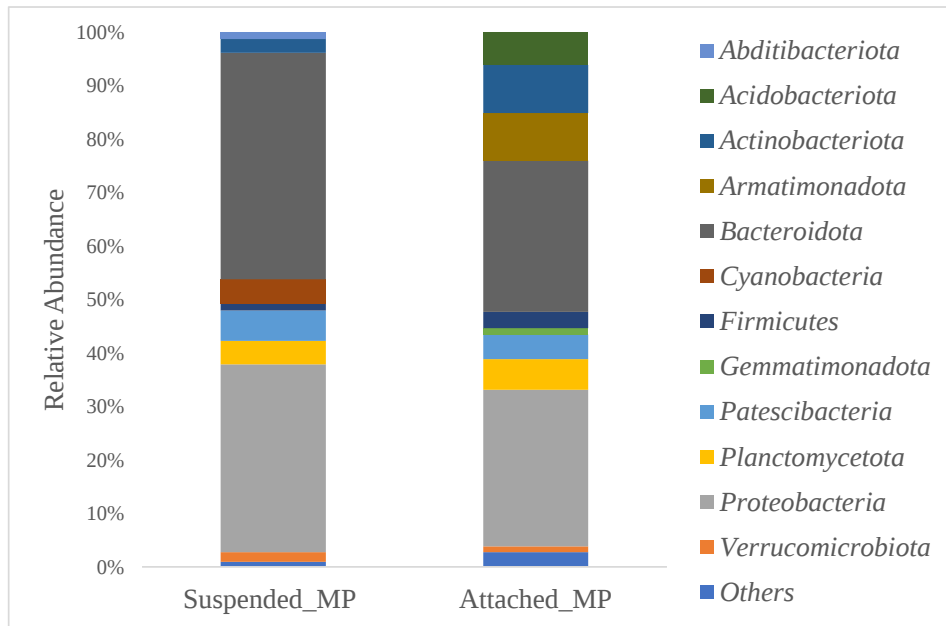


Figure 4.4: Comparison of Relative Abundance at the Phylum Level for Suspended biomass – Hybrid MP Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database).

Figure 4.5 illustrates the overall shift in community composition at the phylum level in inoculum sludge, suspended biomass in hybrid control reactor and hybrid MP reactor and suspended biomass in hybrid control reactor and hybrid MP reactor incorporating only the relative abundances above 1%.

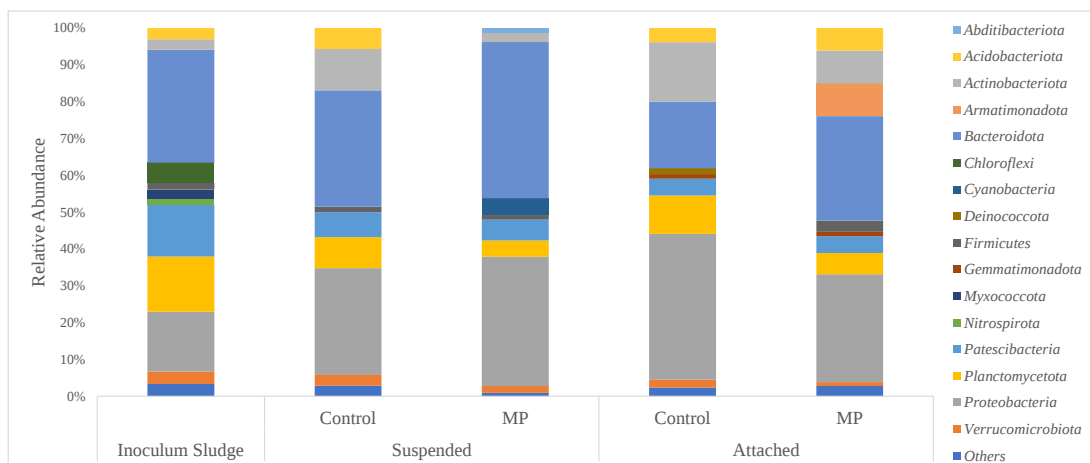


Figure 4.5: Comparison of Relative Abundance at the Phylum Level for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, Suspended biomass – Hybrid MP Reactor, Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database).

4.2.2 Effect of selected NSAIDs at genus level

The heatmap of bacterial abundance for community composition variations at the genus level are represented in the Figure 4.6 for the inoculum sludge, suspended biomass of hybrid control reactor, suspended biomass of hybrid MP reactor, as well as attached biomass of hybrid control reactor, and attached biomass of hybrid MP reactor, taking into account for the only taxa greater than 3%.

At the genus level for the data above 1%, thirteen genera were characterized as “uncultured”. Therefore, in this study we name them based on the lowest available taxonomic level.

According to the results, the operational conditions within the SBR and also addition of MPs, the uncultured genus belonging to the *Saprospiraceae* family (8.77%) and *Saccharimonadales* genus (7.31%) were observed as dominant genera in the inoculum sludge. These two genera decreased drastically in the hybrid control reactor. Also, *SJA-28* genus (3.81%), uncultured genus belonging to the *Rubinisphaeraceae* family (3.77%), uncultured genus belonging to the *Planctomycetales* order (3.60%) had relatively high abundance in the inoculum sludge, while decreased or disappeared in the suspended biomass of hybrid control reactor.

The four most dominant genera in the suspended biomass of hybrid MP reactor after addition of selected NSAIDs are uncultured genus belonging to the *Chitinophagaceae* family (10.08%), *Ferruginibacter* (7.82%), *Comamonas* (6.77%), and *Rhodanobacter* (6.23%), while these taxa had very low frequency in the suspended biomass of hybrid control reactor ranging from 0.01% to 1.16%. It was reported that *Comamonas* genus (belongs to the *Burkholderiales* order) had the ability to oxidize ammonia to nitrite (Q. Chen & Ni, 2011; T. Zhang et al., 2012).

Other genera including *Parafilimonas* (6.00%), *Flavobacterium* (5.90%), *TM7a* (5.34%), uncultured genus belonging to the *Microscillaceae* family (4.60%), *Obscuribacteraceae* (4.57%), and *NS9_marine_group* (4.55%) proliferated to a

considerable amount in the suspended biomass of hybrid MP reactor in the presence of selected NSAIDs comparing to their low abundance in the hybrid control reactor.

Ferruginibacter, *Flavobacterium* were reported among the dominant genera in only preanoxic and in preanoxic /postanoxic compartments of the biofilm system in a novel two-stage anoxic/oxic MBBR and may have a role in denitrification (X. Zhou et al., 2022).

Although, *Niabella* (9.50%), *Mucilaginibacter* (6.21%), uncultured genus belonging to the *Intrasporangiaceae* family (5.87%), *Paracoccus* (5.38%) were detected as dominant genera in suspended biomass of hybrid control reactor, these taxa could not tolerate the addition of selected NSAIDs.

In the attached biomass of hybrid control reactor, three most dominant detected genera are *Paracoccus* (10.34%), *Nakamurella* (7.45%), and *Ottowia* (6.03%). Also, other genera including *Fimbriiglobus* (4.20%), uncultured genus belonging to the *Intrasporangiaceae* family (4.15%), and *Knoellia* (3.90%) showed relatively high abundance in the attached biomass of hybrid control reactor, while their abundance decreased in the attached biomass in presence of selected NSAIDs. According to a study, *Nakamurellaceae* family (belongs to the *Nakamurella* genus) was detected among the abundant families in the initial biofilm before starting the experiment (Q. Liu et al., 2023). Moreover, a decrease was observed in the relative abundance of *Nakamurella* in the SBR with mixture of diclofenac, ibuprofen and naproxen (Jiang et al., 2017b).

Fimbriimonadaceae (8.73%), *Parafilimonas* (6.46%) and *Rickettsia* (5.55%) genera seems to be enriched in the attached biomass of hybrid MP reactor following the addition of selected NSAIDs to the hybrid control reactor, showing very low abundance for these bacteria in attached biomass of hybrid control reactor.

Niabella (3.56%), uncultured genus belonging to the *Blastocatellaceae* family (3.30%) are other genera that slightly increased in the attached biomass in the presence of selected NSAIDs comparing to the control reactor. Moreover, *Saccharimonadales* genus (3.08%) showed a slight reduction in exposure to the selected NSAIDs comparing to the control reactor.

Some of the mentioned dominant genera in the suspended biomass (control and MP reactor) and attached biomass of MP reactor including *Niabella*, uncultured genus of *Chitinophagaceae* family, *Ferruginibacter*, *Parafilimonas*, *Niabella* belonging to the *Chitinophagaceae* family. Even though, Farkas et al. (2023), reported *Chitinophagaceae* family as one of the most abundant families among *Bacteroidetes* in NSAID-treated microcosms, there is no previous report on their role for degrading NSAIDs. Du et al. (2016) revealed the capability of *Chitinophagaceae* bacterium for degradation of 2-Methylisoborneol (a common detected odor causing compound in drinking water source).

Ottowia is the third dominant genus in attached biomass of control reactor, belonging to the *Comamonadaceae* family. Petrilli et al. (2024) reported *Comamonadaceae* and *Chitinophagaceae* family among the microbial community accumulated in the biofilms, suggested a role for them in nitrification. Moreover, the same study stated that *Paracoccus* (the most dominant genus in attached biomass of control reactor in this study), along with *Ferruginibacter* and *Comamonas* as the most prevalent genera in biofilm (Petrilli et al., 2024).

Although J. Cao et al. (2021) reported that *Lactococcus* was widely present in all investigated WWTPs; in this study, its abundance was relatively low and only slightly increased (1.34%) in the attached biomass of the hybrid MP reactor. *Haliangium* and *Sulfuritalea* decreased gradually in the suspended biomass and disappeared in the presence of selected NSAIDs. The same study stated that the functional genera such as *Haliangium* and *Sulfuritalea* showing high abundance in domestic WWTP. In the same study, *Nitrospira* was observed in high abundance in the municipal WWTP (J. Cao et al., 2021). While in this study *Nitrospira*, which had a relative abundance of 1.70% in the inoculum sludge, gradually decreased in the suspended biomass of both hybrid control reactor and the hybrid MP reactor.

The relative abundance of genus *Sphingomonas*, increased in the suspended biomass of hybrid MP reactor (2.99%), after addition of selected NSAIDs. This observation is consistence with previous findings that *Sphingomonas* species were reported to

degrade salicylate and ibuprofen effectively (Jouanneau et al., 2007; Murdoch & Hay, 2015).

In this study, the genera *Acinetobacter* and *Pseudomonas*, both belonging to the phylum *Proteobacteria* and class *Gammaproteobacteria*, showed an increasing pattern in response to the addition of selected NSAIDs. *Pseudomonas* demonstrated an increase in the suspended biomass of the hybrid MP reactor (2.09%), while *Acinetobacter* exhibited a decrease in the suspended biomass (2.90%) and a slight increase in the attached biomass (1.38%) in the MP reactor comparing to their control reactor.

Acinetobacter showed a higher tendency for surviving in the suspended biomass compared to attached biomass, with relative abundances of 2.90% and 1.38%, respectively. Similarly, *Pseudomonas* proliferated in a noticeable amount in the suspended biomass, reaching a relative abundance of 2.09%, while its abundance was below 0.05% in the inoculum sludge and suspended biomass of hybrid control reactor. Conversely, a previous study, identified *Pseudomonas* and *Acinetobacter* as two most abundant bacterial genera of *Gammaproteobacteria* class in pharmaceutical-polluted environments (Farkas et al., 2023). A similar observation was made in another study on the biodegradation of paroxetine and bezafibrate, they reported these two genera among the highest number within the *Gammaproteobacteria* class (Fernandes et al., 2020). Moreover, another study confirmed the ability of *Pseudomonas* to degradation of aromatic compounds (Seo et al., 2009).

As mentioned previously, *Paracoccus* was detected as the fourth most abundant genus in the suspended biomass of hybrid control reactor but there was a notable drop in the abundance of this genus in the suspended biomass of hybrid MP reactor following the addition of selected NSAIDs to the hybrid control reactor, decreasing from 5.38% in the suspended biomass of hybrid control reactor to 0.40% in the hybrid MP reactor. Same trend was observed in the attached biomass; however, in the attached biomass of the hybrid control reactor, *Paracoccus* was the most dominant genus, with a relative abundance of 10.34%. This genus was unable to tolerate exposure to the selected NSAIDs, resulting in a reduction in its abundance to 2.18%. Moreover,

Prosthecobacter was observed almost in low relative abundance in the hybrid control reactor of both suspended and attached biomass (1.87% and 1.04% respectively), this genus even decreased more after addition of selected NSAIDs to the hybrid control reactor. These findings contrast with a previous study conducted by Salih (2020) which reported *Prosthecobacter* (*Verrucomicrobia*) and *Paracoccus* (*Proteobacteria*) as the most abundant genera in the reactor fed with NSAIDs mixture.

Comamonas that was detected as the third dominant genus in the suspended biomass of hybrid MP reactor and *Ottowia* which was also third dominant genus in attached biomass of control reactor, both belonging to the family *Comamonadaceae*. The genera of this family were reported among the dominant genera in suspended biomass in a study conducted for biological nitrogen removal by a novel two-stage anoxic/oxic MBBR but the function of these genera was stated as unknown (X. Zhou et al., 2022).

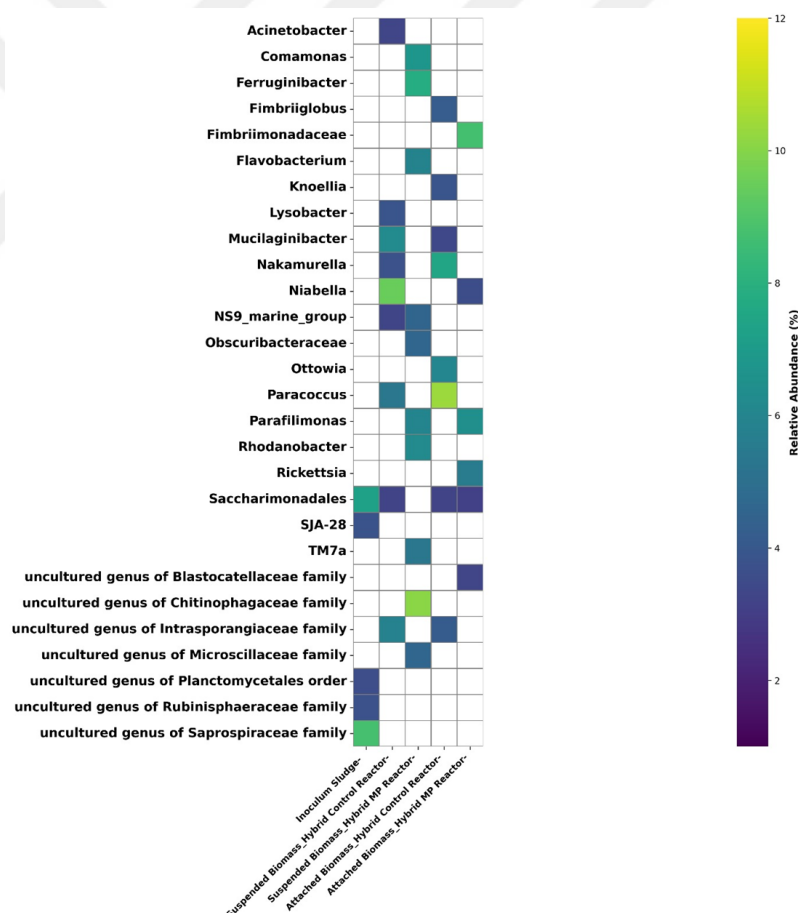


Figure 4.6: Microbial profile at genus level in Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, Suspended biomass – Hybrid MP Reactor, Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database) (above 3%).

4.2.3 Effect of selected NSAIDs at species level

Changes in community composition at the species-level in inoculum sludge, suspended and attached biomass of hybrid control reactor and suspended and attached biomass of hybrid MP reactor including only the taxa with relative abundance greater than 1% are shown in the Figure 4.7 and Figure 4.8. An overall representation is shown in Figure 4.9 for the taxa with a relative abundance above 3% because the number of taxa in species level was too large to display comprehensively.

High-Throughput Sequencing (next generation sequencing (NGS)) evidenced that uncultured species belonging to the *Saprospiraceae* family (8.77%) and *Saccharimonadales* sp. (7.31%) were two most abundant species observed in the inoculum sludge. Among the observed microbial communities in suspended biomass of hybrid control reactor *Niabella* sp. (9.50%), *Mucilaginibacter* sp. (6.21%), uncultured species belonging to the *Intrasporangiaceae* family (5.87%), and *Paracoccus* sp. (5.38%) were four most abundant species. While after introducing the selected NSAIDs into the hybrid MP reactor, uncultured species belonging to the *Chitinophagaceae* family (10.08%), *Ferruginibacter* sp. (7.82%), *Comamonas* sp. (6.77%), *Rhodanobacter* sp. (6.23%), *Parafilimonas* sp. (6.00%) and *Flavobacterium* sp. (*Flavobacteriaceae* family, *Flavobacterium* genus) (5.90%) became the six most abundant species in suspended biomass of hybrid MP reactor (Figure 4.7). *Flavobacterium* sp. have the ability to degrade 17 β -estradiol to estrone (Leiviskä & Risteelä, 2022). The genus *Flavobacterium* showed strong floc-forming properties, and it has been reported as one of the dominant genera in both biofilm and activated sludge in MBR process (Jo et al., 2016).

Furthermore, in attached biomass of hybrid control reactor, *Paracoccus* sp. (10.34%), *Nakamurella* sp. (7.45%) and *Ottowia* sp. (6.03%) were observed as dominant species, whereas *Fimbriiglobus* sp. (4.20%), uncultured species belonging to the *Intrasporangiaceae* family (4.15%), *Knoellia* sp. (3.90%), and *Mucilaginibacter* sp. (3.37%) were less abundant (Figure 4.8).

According to Leiviskä and Risteelä (2022), the relative abundance of *Rhodobacteraceae sp.*, mainly *Paracoccus sp.*, increased during August to November in both MBR and CAS effluent, suggesting the ability of this species in nitrogen removal at that time.

Following the addition of selected NSAIDs to the hybrid MP reactor *Fimbriimonadaceae sp.* (8.73%), *Parafilimonas sp.* (6.46%), and *Rickettsia sp.* (5.55%) were become dominant in the attached biomass of hybrid MP reactor. In contrast, *Niabella sp.* (3.56%), uncultured species of *Blastocatellaceae* family (3.30%), and *Saccharimonadales sp.* (3.08%) proliferate less in the presence of selected NSAIDs (Figure 4.8).



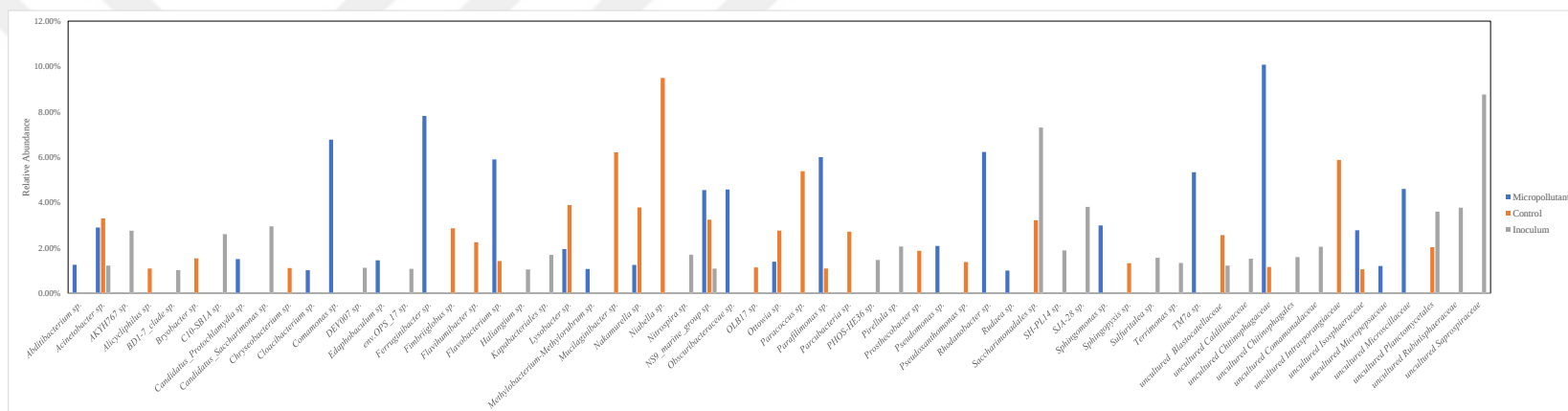


Figure 4.7: Comparison of Relative Abundance at the Species Level for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, and Suspended biomass – Hybrid MP Reactor (SILVA Database) (above 1%).

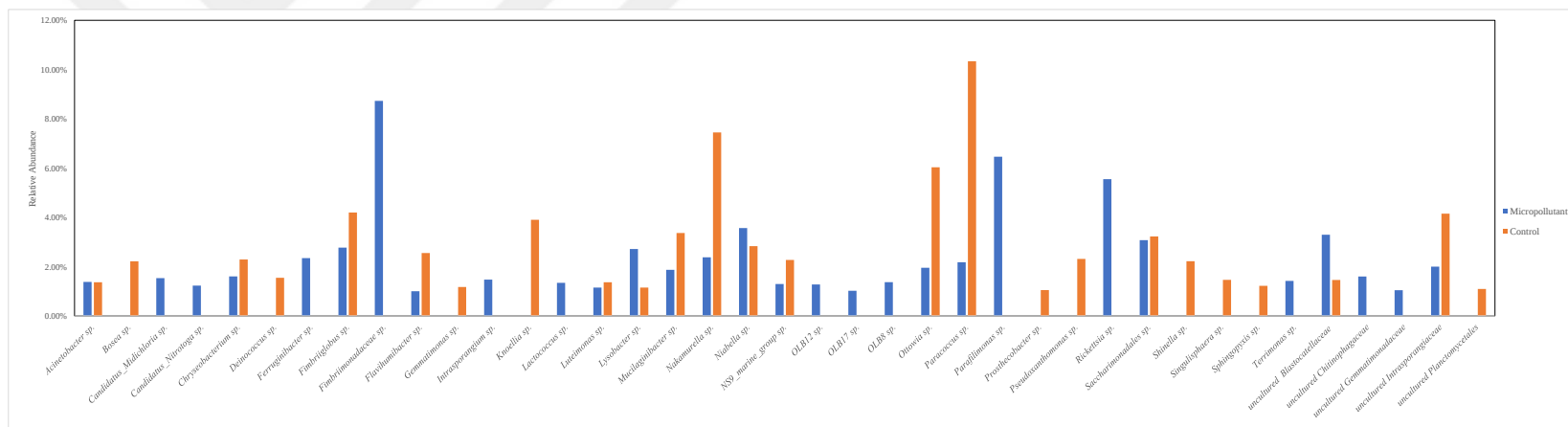


Figure 4.8: Comparison of Relative Abundance at the Species Level for Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database) (above 1%).

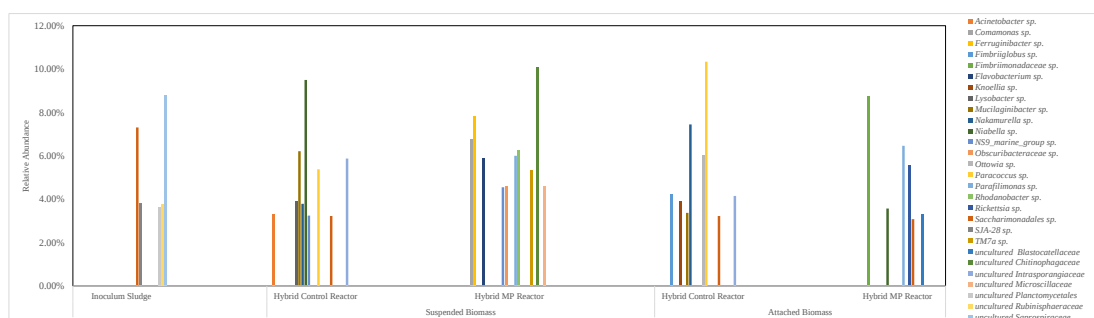


Figure 4.9: Comparison of Relative Abundance at the Species Level for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, Suspended biomass – Hybrid MP Reactor, Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor (SILVA Database) (above 3%).

The Microbial community composition for the phyla with a relative abundance above 1% illustrated in the form of sunburst chart for five samples. The sunburst chart shows taxa in different taxonomic levels including Class, Order, Family, Genus and Species within each phylum. The microbial profile of inoculum sludge, suspended biomass of hybrid control reactor, suspended biomass of hybrid MP reactor and also attached biomass of hybrid control reactor and attached biomass of hybrid MP reactor displayed in Figures 4.10, 4.11, 4.12, 4.13, and 4.14, respectively.

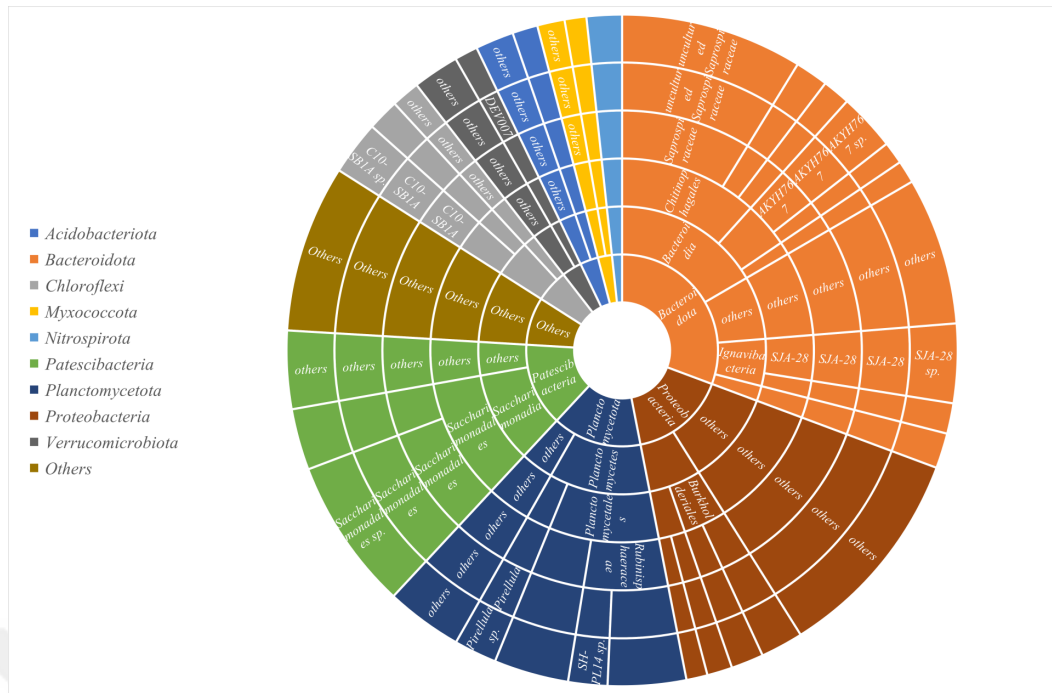


Figure 4.10: Microbial community composition of the inoculum sludge for the phyla with a relative abundance above 1% and Class, Order, Family, Genus and Species level within each phylum (SILVA database).

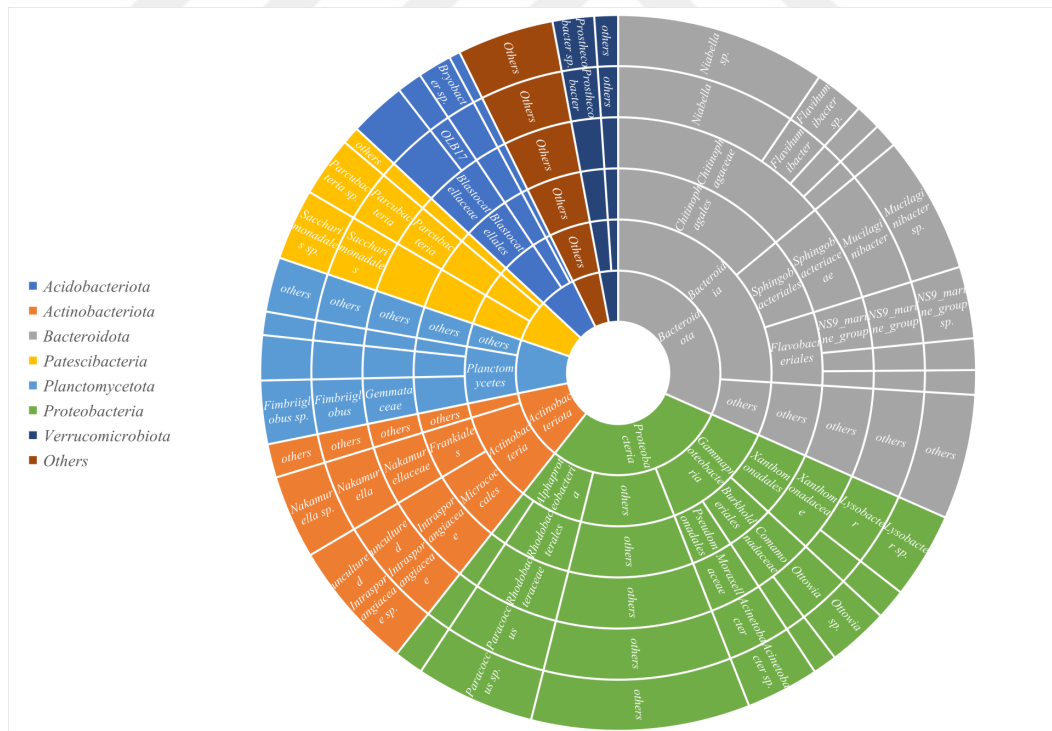


Figure 4.11: Microbial community composition of the suspended biomass of hybrid control reactor for the phyla with a relative abundance above 1% and Class, Order, Family, Genus and Species level within each phylum (SILVA database).

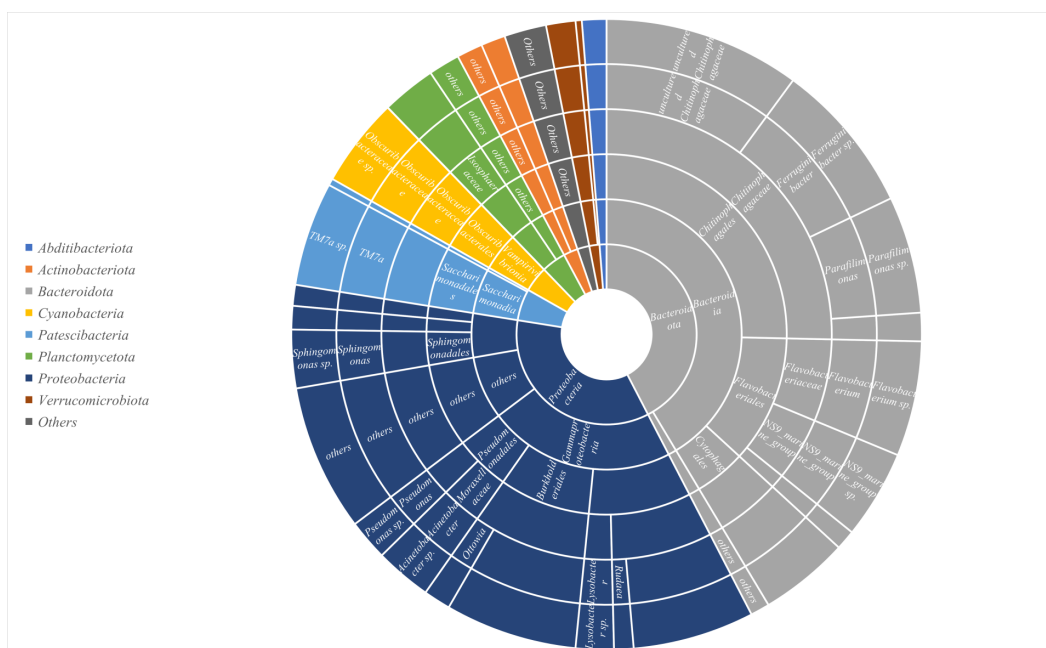


Figure 4.12: Microbial community composition of the suspended biomass of hybrid MP reactor for the phyla with a relative abundance above 1% and Class, Order, Family, Genus and Species level within each phylum (SILVA database).

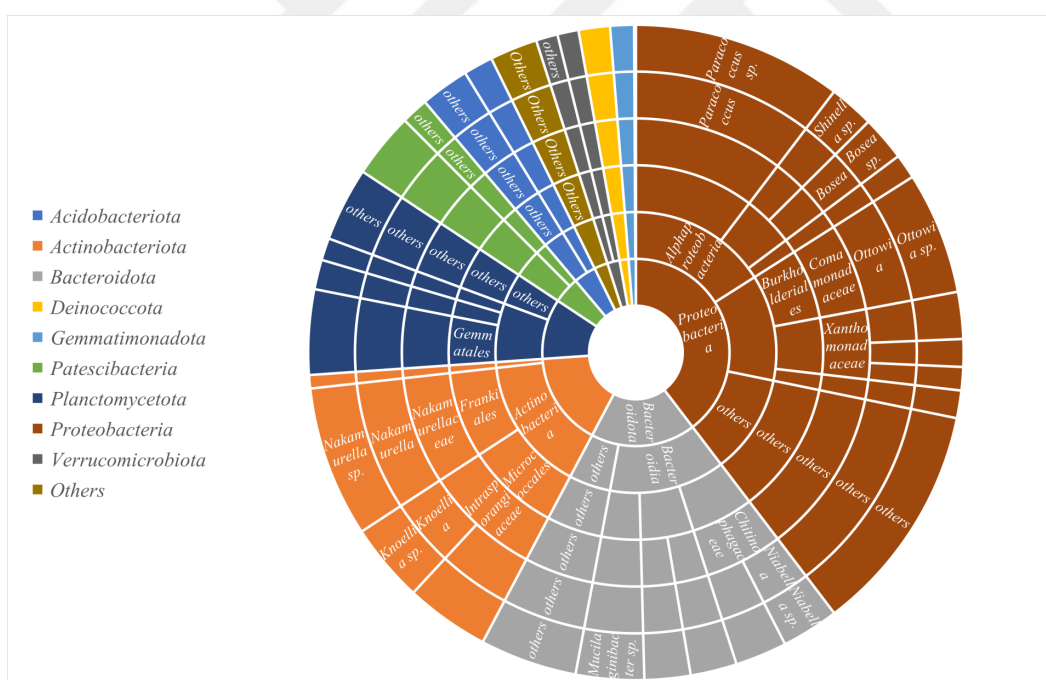


Figure 4.13: Microbial community composition of the attached biomass of hybrid control reactor for the phyla with a relative abundance above 1% and Class, Order, Family, Genus and Species level within each phylum (SILVA database).

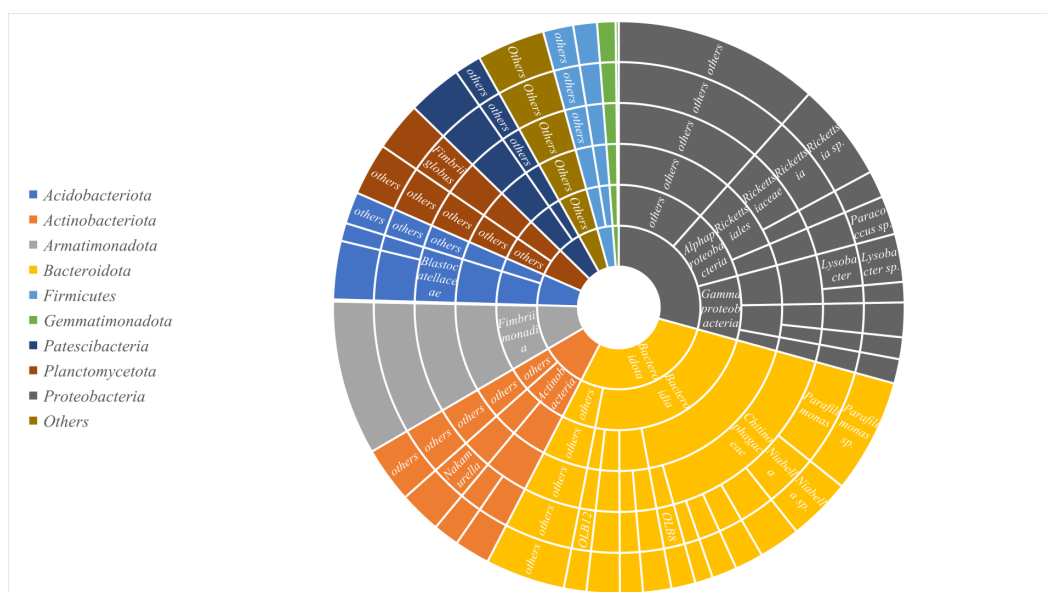


Figure 4.14: Microbial community composition of the attached biomass of hybrid MP reactor for the phyla with a relative abundance above 1% and Class, Order, Family, Genus and Species level within each phylum (SILVA database).

4.3 Statistical Analysis of the Effect of Selected NSAIDs on Microbial Community in Hybrid System

Five independent samples obtained from inoculum sludge and two hybrid reactors (control and micropollutant reactors). A total of 312247 Illumina reads, with an average length of 300 bp and an acceptable average quality score, were derived from inoculum sludge with 55276 reads, suspended biomass of hybrid control reactor with 77084 reads, suspended biomass of hybrid MP reactor with 63297 reads, attached biomass of hybrid control reactor with 53502 reads, and attached biomass of hybrid MP reactor with 63088 reads. Diversity analysis were conducted by alpha diversity. Alpha diversity shows the diversity within a sample (B.-R. Kim et al., 2017).

4.3.1 Alpha diversity analysis

The key properties of a bacterial community in a specific environment are the number of different species that is present and also the relative abundance of those species, which define bacterial diversity. To compare the bacterial diversity in different microbial samples, different bioinformatic tools have been introduced (B.-R. Kim et al., 2017). Alpha diversity quantifies the species diversity in an ecological

community, focusing on richness and evenness of species. As mentioned earlier, the term richness represents the number of distinct taxa or number of taxonomic groups, while evenness shows the relative abundance distribution of the group. Therefore, diversity increases when richness and evenness increase (B.-R. Kim et al., 2017).

As long as, Alpha diversity indices may change in a community due to various factors, it is common to apply alpha diversity analysis from amplicon sequencing data (16S rRNA gene) as the starting point in microbial ecology to reveal and compare the community differences between different environments (Willis, 2019). Different indices are used to represent the alpha diversity, including Observed OTUs (Operational Taxonomic Units), Chao 1, Shannon–Weaver Index (H), Pielou’s evenness, good coverage, Simpson diversity index, and Fisher diversity index. The description of five indices that are applied in this study are represented in Table 4.3. Moreover, The Singletons are the OTUs that appear only once in a given sample and used in richness estimators (Chao, 1984). In order to estimate undetected species richness, the Chao1 use the Singletons (the species observed once-rare taxa) and doubletons (the species observed twice) (Chao, 1984).

Table 4.3: Description and References for Common Alpha Diversity Indices

Diversity Indices/Parameters	Target	Description	Reference
Observed OTUs	Species richness	Counts the number of distinct OTUs (operational taxonomic units-taxa) observed in a sample	(B.-R. Kim et al., 2017)
Chao1	Estimat species richness	Estimates number of rare taxa missed from under-sampling. (Predicts total species richness by considering rare taxa)	(Chao, 1984)
Shannon Index (H)	Richness and evenness	Accounts for both the number of species and their relative abundance distribution. Calculates richness and diversity using a natural logarithm	(B.-R. Kim et al., 2017)
Pielou’s Evenness	Evenness	Measures how evenly individuals are distributed among species (ranges from 0 to 1)	(Pielou, 1966)
Good’s Coverage	Sampling completeness (Sequencing depth completeness)	Estimates the percent of an entire species that is represented in a sample (values close to 1 = sufficient)	(Good, 1953)

Table 4.4 and Figure 4.15 illustrates the alpha diversity indices calculated for five samples: inoculum sludge, suspended biomass of hybrid control, suspended biomass

of hybrid micropollutant (MP) reactor, and Attached biomass of hybrid control reactor, and attached biomass of hybrid MP reactor.

Among all samples, the inoculum sludge represented the highest number of OTUs (492), implying the highest microbial community richness. Also, Shannon index was measured as (8.02), representing that the inoculum sludge has the most complexity and diverse microbial community. The number of singletons (193), Chao1 (675.45), and Pielou's evenness (0.897) are recorded as the highest as well, showing richness with an even distribution of individual taxa (B.-R. Kim et al., 2017).

This finding is in accordance with another study which reported the highest and lowest diversity for inoculum and suspended biomass, respectively (T. Liu et al., 2017a). Also, same study, measured the highest richness for attached biofilm.

Comparing to the inoculum sludge, for the suspended biomass of hybrid control reactor, the diversity decreased notably by calculating the Shannon index (6.299). Other alpha diversity indices also reduced remarkably such as OTUs (216) and Chao1 (311), suggesting a loss in the richness of microbial community after acclimation. Although, the biodiversity reduced, still it was higher compared to after exposure to the selected NSAIDs in the suspended biomass of hybrid MP reactor. Moreover, In the control reactor, the number of OTUs represented by a single read is 76 which had a positive correlation with Chao 1 index. Pielou's evenness calculated sufficiently high (0.81), indicating a stable and evenly distributed microbial community without the stress of selected NSAIDs. The suspended biomass of hybrid MP reactor exhibited the lowest microbial biodiversity among all samples and also lower comparing to its control reactor, showing Shannon index as 5.628, reflecting the negative influence of selected NSAIDs on the microbial community in the suspended biomass. Other indices including significantly lower OTU number (154), the lowest Singletons (49), and Chao 1 (197.556) among all samples, demonstrating the lowest richness. Moreover, the addition of selected NSAIDs reduced the evenness (0.774) in suspended biomass, implying that the microbial community structure of the suspended biomass was less evenly distributed (few taxa became dominant) under pharmaceutical stress. This finding is in accordance a study conducted by D. Zhang et al. (2016b), showed a

significant decrease in the microbial diversity in wetland microcosm (a small-scale wetland in order to mimic the constructed wetlands) in the presence of ibuprofen (250 µg/L), comparing to the control. Moreover, Farkas et al. (2023) studied the impact of NSAIDs (diclofenac, ibuprofen, and acetylsalicylic acid in range of 200-600 ppm) on microbial community, reported a decrease in the microbial community, while observed higher cell counts after addition of selected pharmaceuticals. In contrast, an increase in the bacterial diversity was observed sequencing batch reactors after addition of diclofenac, ibuprofen and naproxen in various concentrations in the range of 1 to 5 µg/L, indicated that environmental concentrations of NSAIDs could stimulate the biodiversity (Jiang et al., 2017b).

The attached biomass of hybrid control reactor had almost higher microbial diversity and the microbial community is more evenly distributed comparing to the suspended biomass of the same reactor by observing of the alpha diversity indices. The control attached biomass showed 223 number of distinct OTUs, 6.446 for Shannon index, and 0.826 for Pielou's evenness. Also, the attached biomass of hybrid control reactor showed lower number of rare species (singletons) (58) and subsequently Chao 1 (264.325). After exposure the control reactor to the selected NSAIDs, the attached biomass of MP reactor, maintained higher microbial diversity comparing to the suspended biomass of hybrid MP reactor. It showed increased values for observed OTUs (308), Shannon index (6.656), and Chao1 richness (454.3). Even though, richness or the number of different taxa increased in the presence of selected NSAIDs in the attached biomass of hybrid MP reactor, Pielou's evenness decreased slightly to 0.805 and became less even distributed comparing to its control reactor but still is higher comparing to the suspended biomass. Wolff et al. (2021c) compared the carrier-attached biomass and suspended biomass in three hybrid MBBR, reported higher values for species richness and Shannon index in the carrier-attached biomass. Moreover, same study reported averaged biodiversity indices in three investigated systems, showed elevated values for attached biomass comparing to suspended biomass, suggesting high biodiversity for the microbial community attached to the carriers (Wolff et al., 2021c). This finding is consistent with results from Gao et al. (2014) and Silva et al. (2010) which evaluated the biodiversity in biocake and aerobic

mixed liquor and also in CAS and membrane bioreactors. This difference in the microbial community dynamics might be because of the occupation of metabolic and territorial niches on carrier-attached biofilm; It can be because of the long sludge retention time, low food-to-microorganisms (F/M) ratio or due to the different substrate and redox gradients within the biofilm (Choi et al., 2007; De Wever et al., 2007; Wolff et al., 2021c).

Overall, compared to the suspended biomass, the attached biomass could tolerate better presence of selected NSAIDs and remained more diverse, as evidenced by higher observed OTUs values, Shannon index, Pielou's evenness, and Chao1; reflecting higher richness, evenness, and overall microbial diversity. Moreover, the selected NSAIDs had negative effect on the microbial community structure and diversity in the suspended biomass, showing a decrease in Shannon index from control reactor of the suspended biomass to the MP reactor of the suspended biomass, while the microbial community could proliferate and remain divers in the attached biomass in the stress of selected NSAIDs; Shannon index increased comparing to its control in attached biomass. Same trend was observed in the Chao1 (and Singletons) but as presented in Figure 4.15, the Chao 1, an estimator of species richness, increased significantly in the attached biomass, suggesting a substantial rise in the number of rare taxa that is in accordance with the formulation of Chao1 index (B.-R. Kim et al., 2017).

In MBBR systems, the differences in the environmental conditions for suspended and attached biomass, could have a considerable effect on the microbial community structure and diversity (Yuan et al., 2023). For instance, according to the Fowler et al. (2023), the microbial community reported for biofilm, is more biodiverse than the microbial community that forms suspended biomass. Moreover, various articles reported higher diversity and richness in attached biofilm comparing to suspended biomass, as well (B. Fu et al., 2010; Ribera-Pi et al., 2020; Wolff et al., 2021b).

Additionally, other studies, support the finding of this study on the number of observed OTUs for different attached and suspended biomass. for example, B. Fu et al. (2010) and Mazioti and Vyrides (2024), reported higher observed OTUs in the biofilm comparing to the suspended biomass, indicating that attached biofilm in the system,

has increased the microbial community complexity. Therefore, it allows different functional bacteria to coexist (Eshamuddin et al., 2024).

The good's coverage values ranging between the lowest value of 0.9035 in inoculum sludge and highest value of 0.9755 in suspended biomass of hybrid MP reactor.



Table 4.4: Diversity indices for inoculum sludge, Suspended biomass — hybrid control/MP reactor, and Attached biomass — hybrid control/MP reactor of different samples (depth=2000, iter=10)

Description	Sample	Observed Species (OTUs)	Singletons	Chao1	Shannon	Pielou's evenness	Goods coverage
10 d - control	Suspended	216	76	311	6.299361941	0.812308617	0.962
	Carrier	223	58	264.325	6.446434252	0.82637059	0.97
10 d - MP	Suspended	154	49	197.555556	5.627503554	0.774413419	0.9755
	Carrier	308	133	454.3	6.656211365	0.805175183	0.9335
inoculum sludge		492	193	675.445446	8.019548629	0.896788999	0.9035

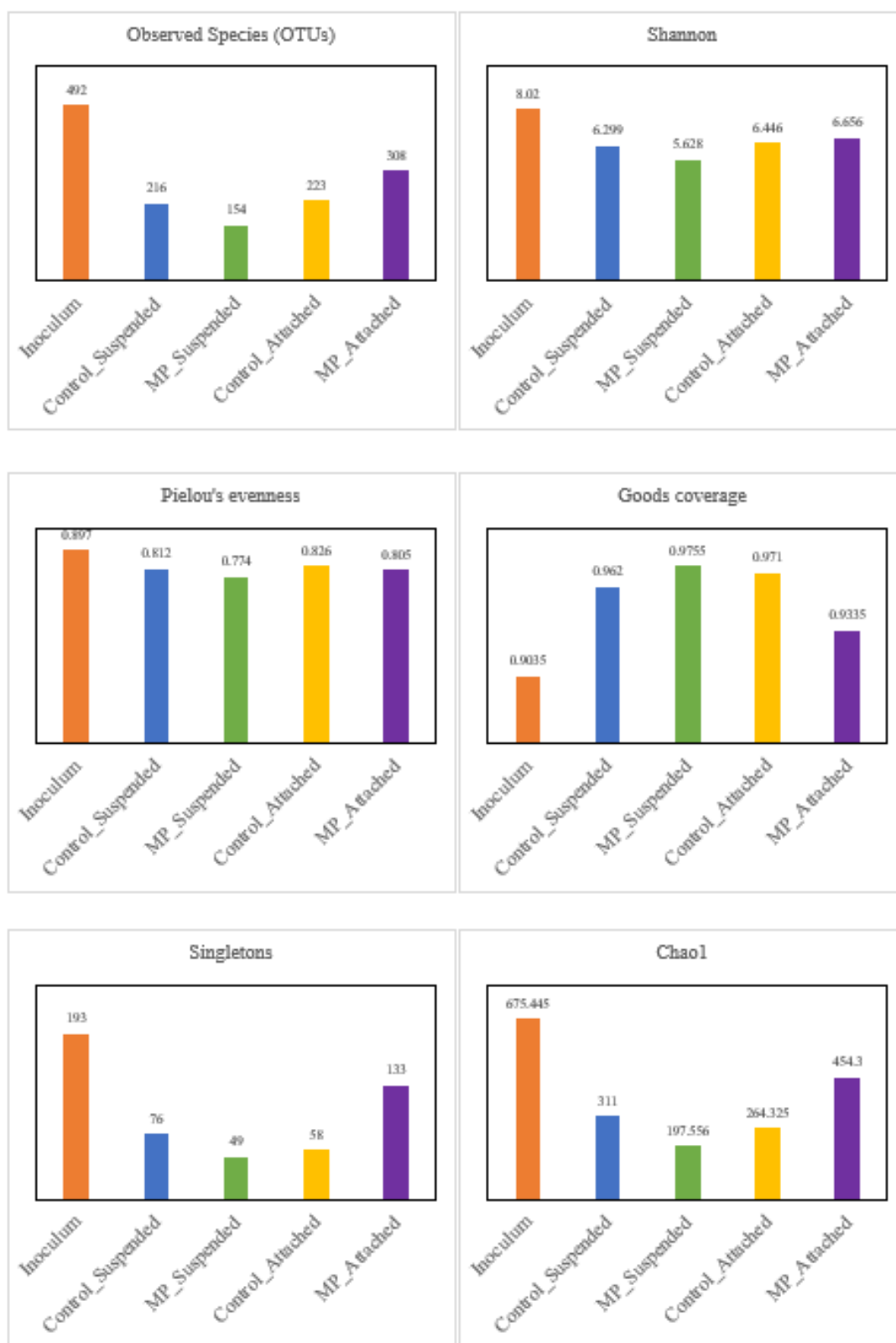


Figure 4.15: Representation of diversity indices including Observed Species (OTUs), Singletons, Chao1, Shannon, Pielou's evenness, and Good's coverage for inoculum sludge, Suspended biomass — hybrid control/MP reactor, and Attached biomass — hybrid control/MP reactor.

4.3.2 Rarefaction curves

Rarefaction curves measure the number of observed OTUs at a specific sequencing depth and are used to compare the species richness between communities that are samples unequally. In other words, rarefaction helps us to compare the samples even if they are in different size (B.-R. Kim et al., 2017). In this study rarefaction analysis was performed for observed OTUs in ten sequencing depth, illustrated in Figures 4.16, 4.17, 4.18, 4.19, 4.20 and 4.21, respectively. A steeper slope was observed in the inoculum sludge comparing to other samples. When analyzing rarefaction curves, they showed that presence of NSAIDs leads to a decrease in richness in suspended biomass but increase in the attached biomass.

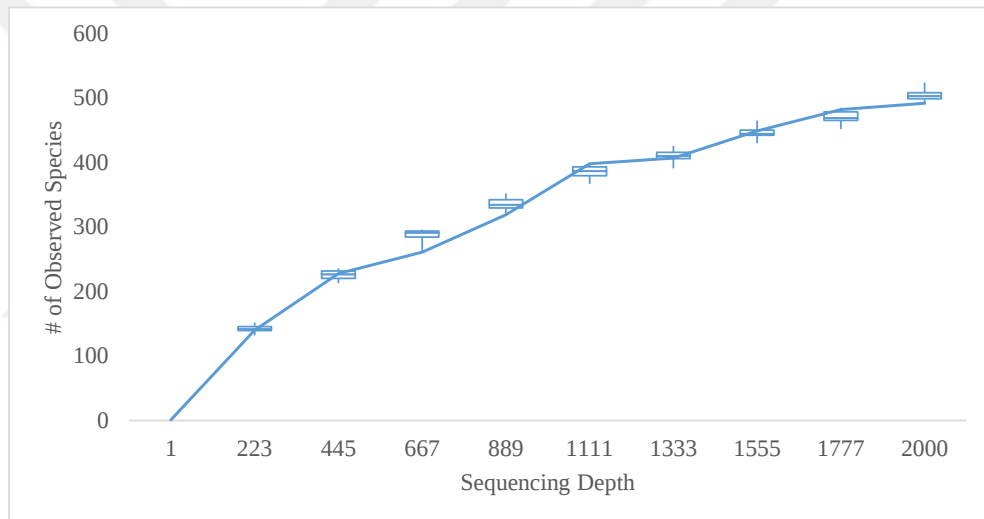


Figure 4.16: Rarefaction curve illustrated for inoculum sludge.

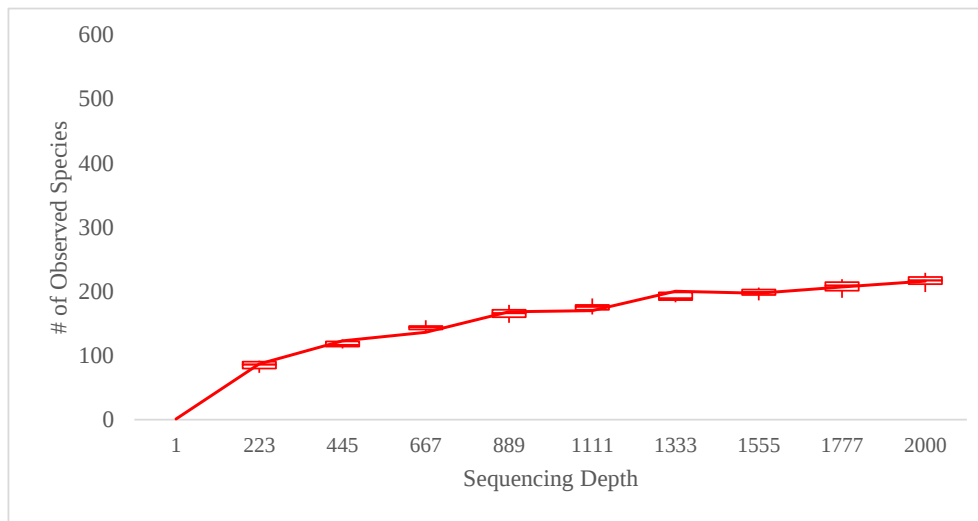


Figure 4.17: Rarefaction curve illustrated for suspended biomass — hybrid control reactor.

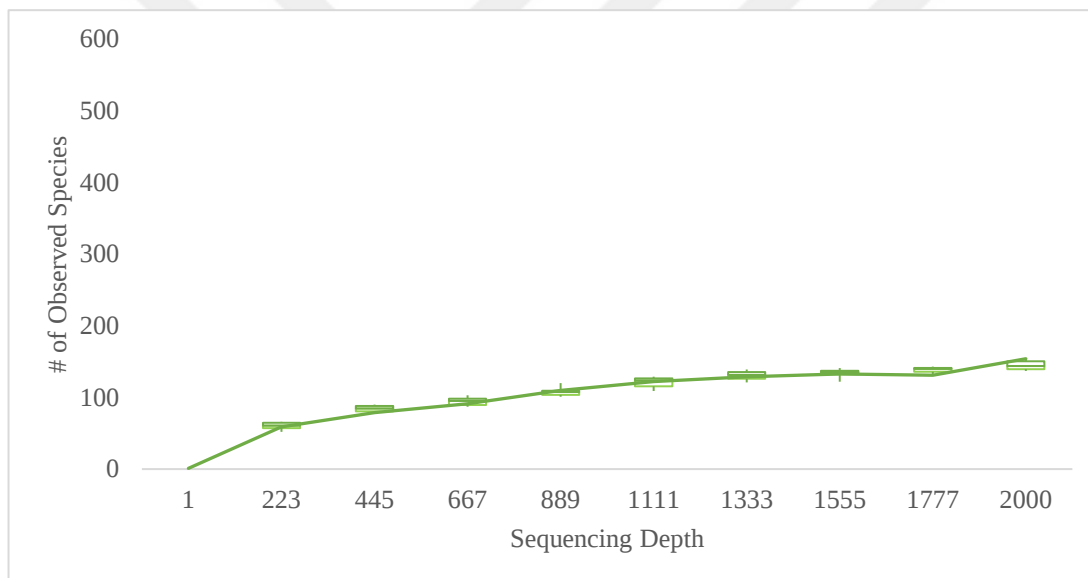


Figure 4.18: Rarefaction curve illustrated for suspended biomass — hybrid MP reactor.

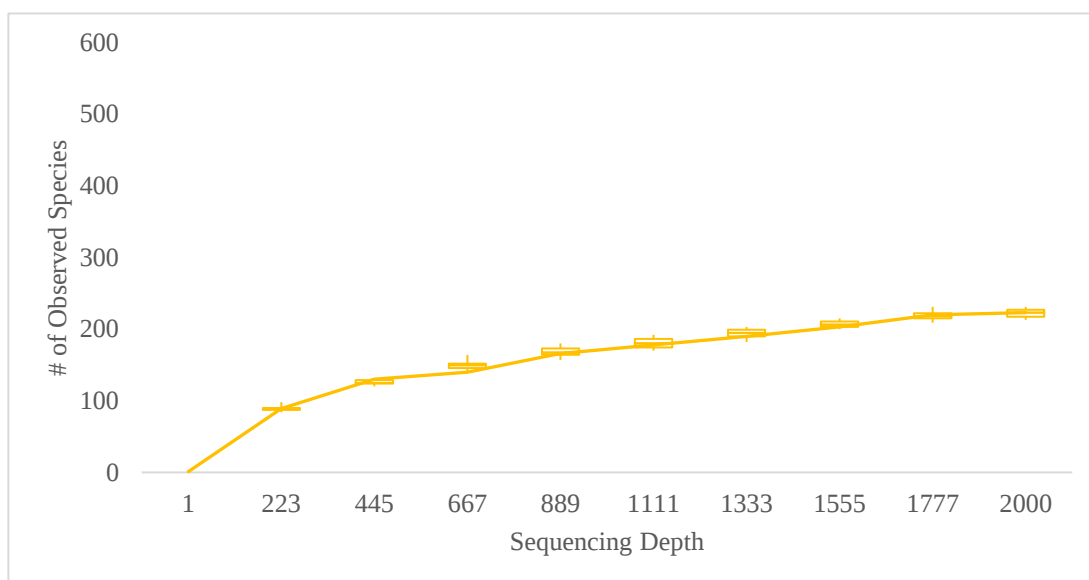


Figure 4.19: Rarefaction curve illustrated for attached biomass — hybrid control reactor.

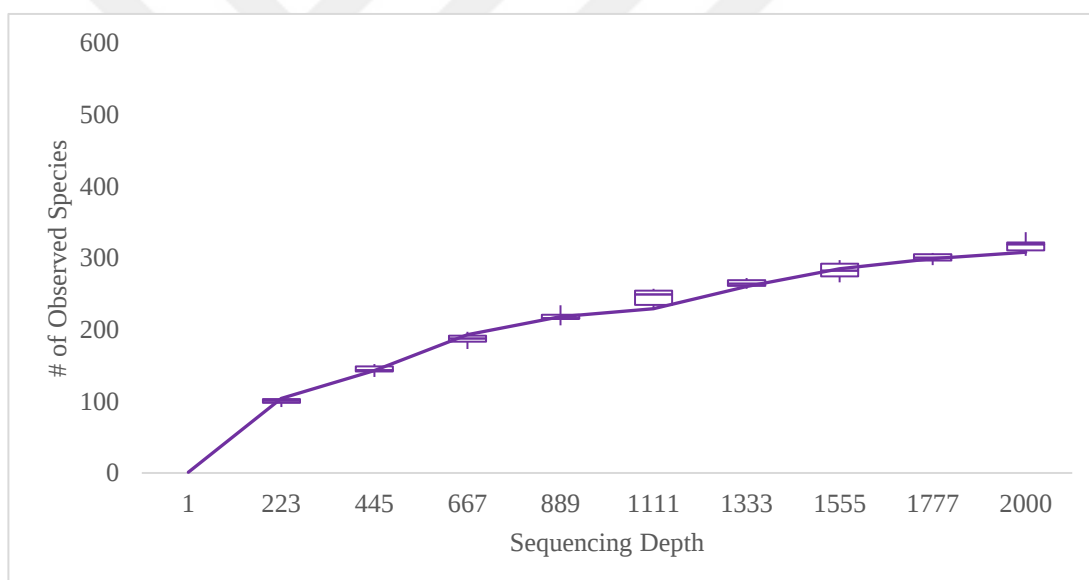


Figure 4.20: Rarefaction curve illustrated for attached biomass — hybrid MP reactor.

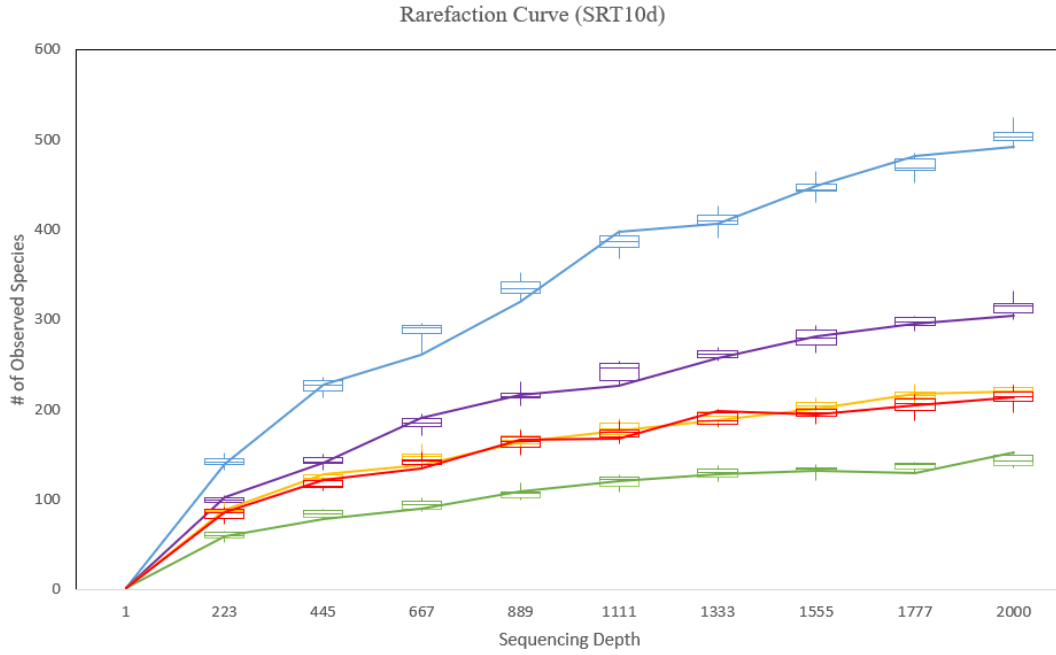


Figure 4.21: Rarefaction curve illustrated for inoculum sludge, suspended biomass — hybrid control/MP reactor, attached biomass — hybrid control/MP.

4.4 Comparison of SILVA and NCBI Database Results

An overall comparison for the microbial community in SILVA and NCBI database is illustrated in Figure 4.22 and Figure 4.23.

Figure 4.22 shows the bacterial phyla, filtered for data above 1% and Figure 4.23 depicts the bacterial community at genus level, filtered for data above 5%.

Attention should be drawn to the phyla *Pseudomonadota*, *Actinomycetota* and *Bacillota* in the NCBI database which are proposed names for *Proteobacteria*, *Actinobacteriota* and *Firmicutes*, respectively (Panda et al., 2022). In Figure 4.22, the names of the phyla are summarized according to the original data set but in the following paragraph, common names for both database is selected for comparison.

At the phylum level in both SILVA and NCBI databases, the phyla *Bacteroidota* and *Proteobacteria* exhibited dominance in five samples including, inoculum sludge, suspended biomass of hybrid control and hybrid MP reactors and also attached biomass of hybrid control and hybrid MP reactors (Figure 4.22).

Moreover, in SILVA database, *Actinobacteriota* detected in high abundance in the suspended biomass of hybrid control reactor, while decreased significantly in the reactor fed with selected NSAIDs. Also, this phylum dominated the microbial community in the attached biomass of both hybrid control and hybrid MP reactors in the NCBI database (Figure 4.22).

Furthermore, in the NCBI database it was detected that *Acidobacteriota* showed elevated abundance (Figure 4.22).

In the SILVA database, the two phyla comprising *Patescibacteria* and *Planctomycetota* were dominant in the inoculum sludge and also had high relative abundance in the suspended biomass of hybrid control reactor while decreased in the MP reactor. conversely, the *Patescibacteria* phylum were not reported in the NCBI database and *Planctomycetota* had below 1% abundance. Before the reactor was fed with selected NSAIDs, *Planctomycetota* abundance were higher in attached biomass than suspended biomass (Figure 4.22).

Briefly, at the phylum level, both databases identified *Bacteroidota*, *Proteobacteria*, and *Actinobacteriota* as dominant phyla in the samples. In contrast to the NCBI results, SILVA also identified *Patescibacteria* and *Planctomycetota* as two of the dominant phyla in almost all samples.

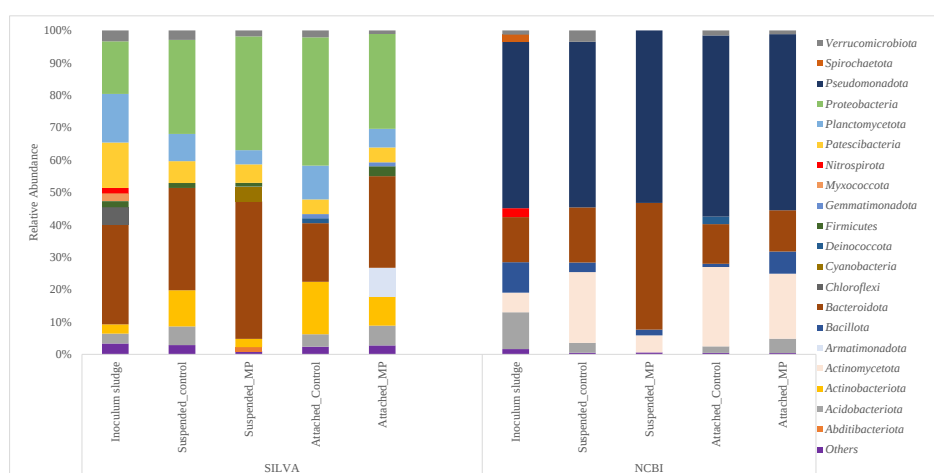


Figure 4.22: Comparison of SILVA and NCBI Database Results at Phylum Level for the data above 1% for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, Suspended biomass – Hybrid MP Reactor, Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor.

The genera depicted in Figure 4.23 all are almost the dominant genera. The genera including, *Paracoccus*, *Nakamurella*, *Ottowia*, *Comamonas*, *Ferruginibacter* and *Paraflimonas* detected as the common genera in both SILVA and NCBI databases. The remaining available genera in Figure 4.23 are seen in either SILVA or NCBI database.

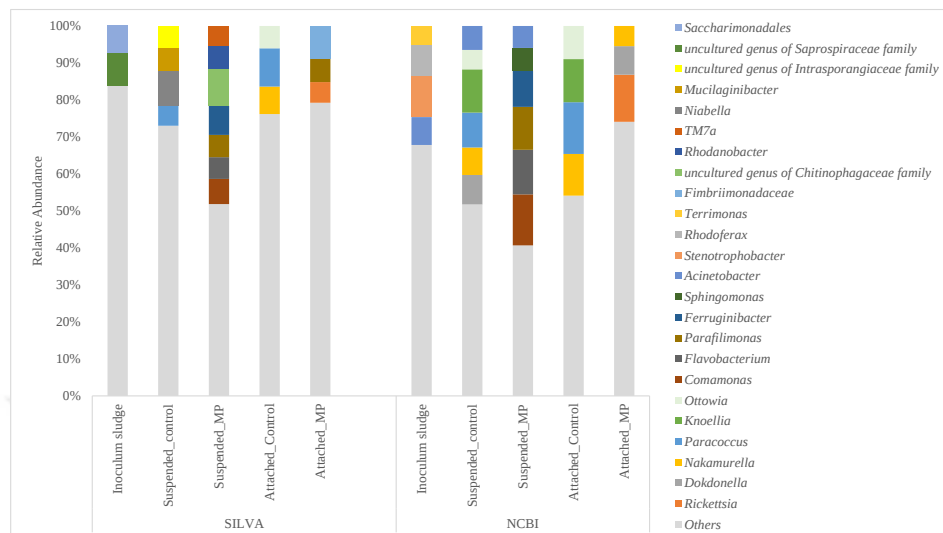


Figure 4.23: Comparison of SILVA and NCBI Database Results at Genus Level for data above 5% for Inoculum Sludge, Suspended biomass – Hybrid Control Reactor, Suspended biomass – Hybrid MP Reactor, Attached biomass – Hybrid Control Reactor, and Attached biomass – Hybrid MP Reactor.

OTU (Operational Taxonomic Unit) is commonly used in microbiome studies where organisms with similar DNA sequences are grouped together. The OTU numbers in five samples including inoculum sludge, suspended biomass of hybrid control reactor, suspended biomass of hybrid MP reactor and attached biomass of hybrid control reactor and attached biomass of hybrid MP reactor illustrated in Table 4.5. The number of OTUs obtained in SILVA database was higher than number of OTUs in NCBI database for all five samples. In SILVA database higher microbial sequencing were identified; therefore, we selected SILVA reference database for further analysis.

4.5 Microbial Taxa that Change Significantly with the Addition of NSAIDs in Hybrid Systems Operated at Low Organic Loading

In this section, the microbial profile is evaluated based on the significant increase and/or decrease in relative abundance in hybrid systems operated under low organic loading. First, significant changes were assessed in the hybrid control reactor

Table 4.5: Comparison of OTU numbers obtained at different taxonomic levels based on SILVA and NCBI reference databases.

		SILVA (OTUs)	NCBI (OTUs)
Suspended biomass	Control	196	171
	MP	171	122
Attached biomass	Control	177	155
	MP	326	285
Inoculum sludge		364	196

compared to the inoculum sludge at both phylum level and genus level. Subsequently, these changes were evaluated after the addition of selected NSAIDs and compared to corresponding control reactor, again at both the phylum and genus level. The evaluation was done for the data above 1%.

4.5.1 At phylum level

Planctomycetota, *Patescibacteria* and *Chloroflexi* which were reported as the dominant phyla in the inoculum sludge, significantly reduced in the suspended biomass of hybrid control reactor, most probably due to low OLR selected for this study compared to influent COD concentration of domestic WWTP as well as lab-scale experimental conditions. The reduction was 44%, 52% and 97% for *Planctomycetota*, *Patescibacteria* and *Chloroflexi* respectively (Table 4.6).

Conversely, in the suspended biomass *Acidobacteriota*, *Actinobacteriota* and *Proteobacteria* increased significantly in the hybrid control reactor. *Acidobacteriota* and *Proteobacteria* increased approximately 80% in the control reactor, while the increase in the frequency of *Actinobacteriota* was about fourfold of its abundance in the inoculum sludge (Table 4.7).

Table 4.6: Significant decrease of microbial community in suspended biomass at phylum level.

Phylum	Inoculum	Control
<i>Planctomycetota</i>	15.01%	8.42%
<i>Patescibacteria</i>	14.02%	6.74%
<i>Chloroflexi</i>	5.50%	0.19%

Table 4.7: Significant increase of microbial community in suspended biomass at phylum level.

Phylum	Inoculum	Control
<i>Acidobacteriota</i>	3.07%	5.74%
<i>Actinobacteriota</i>	2.83%	11.18%
<i>Proteobacteria</i>	16.24%	29.00%

Same as suspended biomass, in the attached biomass, *Patescibacteria* and *Planctomycetota* decreased significantly by 68% and 31%, respectively. *Bacteroidota* which was observed as the first dominant phylum in the inoculum sludge, decreased by 41% in the attached biomass of hybrid control reactor in response to selected operational conditions for this study such as low OLR and lab-scale experimental conditions (Table 4.8).

On the other hand, *Actinobacteriota* showed approximately a fivefold increase in the attached biomass of hybrid control reactor. The observed increase in *Proteobacteria* was also notable, with more than twofold rise in the control reactor (Table 4.9).

Therefore, it is worth mentioning that significant changes in the relative abundance of *Patescibacteria*, *Planctomycetota*, *Actinobacteriota* and *Proteobacteria* in both suspended and attached biomass of hybrid control reactor were observed.

Table 4.8: Significant decrease of microbial community in attached biomass at phylum level.

Phylum	Inoculum	Control
<i>Bacteroidota</i>	30.68%	18.10%
<i>Patescibacteria</i>	14.02%	4.55%
<i>Planctomycetota</i>	15.01%	10.40%

Table 4.9: Significant increase of microbial community in attached biomass at phylum level.

Phylum	Inoculum	Control
<i>Actinobacteriota</i>	2.83%	16.16%
<i>Proteobacteria</i>	16.24%	39.65%

The addition of selected NSAIDs caused significant changes in microbial composition of both suspended and attached biomass of hybrid MP reactor, compared to

corresponding hybrid control reactor. For example, *Actinobacteriota*, *Acidobacteriota* were significantly suppressed in the suspended biomass of hybrid MP reactor compared to the its hybrid control reactor, with reductions of approximately 77% and 98%, respectively (Table 4.10). On the other hand, in the attached biomass, a notable reduction, nearly by half, was observed in the hybrid MP reactor for phyla including *Actinobacteriota* and *Planctomycetota* (Table 4.11). These phyla might be sensitive to the mixture of selected NSAIDs, under low OLR conditions.

Table 4.10: Significant decrease in suspended biomass at phylum level after long-term exposure to NSAIDs.

Phylum	Control	MP
<i>Actinobacteriota</i>	11.18%	2.58%
<i>Acidobacteriota</i>	5.74%	0.14%

Table 4.11: Significant decrease in attached biomass at phylum level after long-term exposure to NSAIDs.

Phylum	Control	MP
<i>Actinobacteriota</i>	16.16%	9.00%
<i>Planctomycetota</i>	10.40%	5.73%

Moreover, three phyla proliferated significantly in the presence of a mixture of selected NSAIDs under low OLR conditions. *Cyanobacteria*, which had an abundance of less than 1% in suspended biomass of hybrid control reactor, increased significantly to 4.58% in suspended biomass of hybrid MP reactor (Table 4.12). Similarly, *Bacteroidota* and *Armatimonadota* in the attached biomass showed a notable increase in the hybrid MP reactor compared to the corresponding control reactor (Table 4.13). It can be said that, these phyla might contribute to the degradation of selected NSAIDs.

Table 4.12: Significant increase in suspended biomass at phylum level after long-term exposure to NSAIDs.

Phylum	Control	MP
<i>Cyanobacteria</i>	0.01%	4.58%

Table 4.13: Significant increase in attached biomass at phylum level after long-term exposure to NSAIDs.

Phylum	Control	MP
<i>Bacteroidota</i>	18.10%	28.31%
<i>Armatimonadota</i>	0.19%	8.85%

4.5.2 At genus level

The two most abundant genera in the inoculum sludge were uncultured genus belonging to *Saprospiraceae* family and *Saccharimonadales* genus. These two genera drastically decreased in suspended biomass of hybrid control reactor (96% and 56%, respectively), under selected operational conditions like low OLR in this study, and also lab-scale experimental conditions (Table 4.14).

As summarized in the Table 4.15, seven genera showed significant increase in the suspended biomass of hybrid control reactor. The genera including *Niabella*, *Paracoccus*, *Lysobacter*, *Acinetobacter*, *NS9_marine_group*, *Fimbriiglobus* and *Ottowia* increased significantly and became dominant in the suspended biomass of the hybrid control reactor under low OLR conditions and also lab-scale experimental conditions (Table 4.15).

Table 4.14: Significant decrease of microbial community in suspended biomass at genus level.

Genus	Inoculum	Control
uncultured <i>Saprospiraceae</i>	8.77%	0.31%
<i>Saccharimonadales</i>	7.31%	3.22%

Table 4.15: Significant increase of microbial community in suspended biomass at genus level.

Genus	Inoculum	Control
<i>Niabella</i>	0.22%	9.50%
<i>Paracoccus</i>	0.13%	5.38%
<i>Lysobacter</i>	0.01%	3.89%
<i>Acinetobacter</i>	1.22%	3.30%
<i>NS9_marine_group</i>	1.09%	3.24%
<i>Fimbriiglobus</i>	0.07%	2.86%
<i>Ottowia</i>	0.55%	2.76%

Various genera such as *SJA28*, uncultured genus belonging to *Rubinisphaeraceae* family, *Candidatus_Saccharimonas*, *C10SB1A*, uncultured genus belonging to *Comamonadaceae* family, *Kapabacteriales*, uncultured genus belonging to *Chitinophagales* order, *PHOS-HE36*, *DEV007*, *BD1-7_clade* and *Obscuribacteraceae* showed a complete disappearance in suspended biomass of hybrid control reactor, indicating a potential intolerance to low OLR and lab-scale experimental conditions (Table 4.16).

Table 4.16: Disappearance of microbial community in suspended biomass at genus level.

Genus	Inoculum	Control
<i>SJA-28</i>	3.81%	0.00%
uncultured <i>Rubinisphaeraceae</i>	3.77%	0.00%
<i>Candidatus_Saccharimonas</i>	2.95%	0.00%
<i>C10-SB1A</i>	2.60%	0.00%
uncultured <i>Comamonadaceae</i>	2.05%	0.00%
<i>Kapabacteriales</i>	1.69%	0.00%
uncultured <i>Chitinophagales</i>	1.59%	0.00%
<i>PHOS-HE36</i>	1.47%	0.00%
<i>DEV007</i>	1.12%	0.00%
<i>BD1-7_clade</i>	1.02%	0.00%
<i>Obscuribacteraceae</i>	0.03%	0.00%

In the attached biomass of the hybrid control reactor, similar to suspended biomass, genera including uncultured genus belonging to *Saprospiraceae* family and *Saccharimonadales* were detected as taxa which showed significant decrease in control reactor (Table 4.17).

Furthermore, *Paracoccus*, *Ottowia*, and *Fimbriiglobus* showed a significant increase in the attached biomass of hybrid control reactor, similar to suspended biomass, with a higher tendency of these genera to grow on carriers and become dominant in the attached biomass of hybrid control reactor (Table 4.18).

Table 4.17: Significant decrease of microbial community in attached biomass at genus level.

Genus	Inoculum	Control
uncultured <i>Saprospiraceae</i>	8.77%	0.23%
<i>Saccharimonadales</i>	7.31%	3.22%

Table 4.18: Significant increase of microbial community in attached biomass at genus level.

Genus	Inoculum	Control
<i>Paracoccus</i>	0.13%	10.34%
<i>Ottowia</i>	0.55%	6.03%
<i>Fimbriiglobus</i>	0.07%	4.20%

Table 4.19 presents the genera that were initially present in the inoculum sludge but completely disappeared in the attached biomass of hybrid control reactor, suggesting a possible inability to tolerate the low OLR and lab-scale experimental condition. For instance, genera including *BD1-7_clade*, *C10-SB1A*, *Candidatus_Saccharimonas*, *DEV007*, *PHOS-HE36*, *SJA-28*, *env.OPS_17*, *Sulfuritalea* and also uncultured genus belonging to *Chitinophagales* order and uncultured genus belonging to *Rubinisphaeraceae* family completely disappeared in attached biomass of hybrid control reactor.

Table 4.19: Disappearance of microbial community in attached biomass at genus level.

Genus	Inoculum	Control
<i>BD1-7_clade</i>	1.02%	0.00%
<i>C10-SB1A</i>	2.60%	0.00%
<i>Candidatus_Saccharimonas</i>	2.95%	0.00%
<i>DEV007</i>	1.12%	0.00%
<i>env.OPS_17</i>	1.08%	0.00%
<i>PHOS-HE36</i>	1.47%	0.00%
<i>SJA-28</i>	3.81%	0.00%
<i>Sulfuritalea</i>	1.56%	0.00%
uncultured <i>Chitinophagales</i>	1.59%	0.00%
uncultured <i>Rubinisphaeraceae</i>	3.77%	0.00%

The introduction of selected NSAIDs resulted in significant changes in the microbial profile at the genus level in both suspended and attached biomass of hybrid MP reactor, compared to corresponding hybrid control reactor. Table 4.20 represents the genera that were significantly suppressed in the suspended biomass after addition of selected NSAIDs under low OLR conditions, including *Niabella*, *Mucilaginibacter*, uncultured genus belonging to *Intrasporangiaceae* family *Paracoccus*. Likewise, Table 4.21 illustrates the genera that showed notable suppression in attached biomass of hybrid

MP reactor for genera including *Paracoccus*, *Nakamurella* and *Ottowia*. These genera might be sensitive to the mixture of selected NSAIDs and may not tolerate the toxic effects of NSAIDs under low OLR conditions.

Table 4.20: Significant decrease in suspended biomass at genus level after long-term exposure to NSAIDs.

Genus	Control	MP
<i>Niabella</i>	9.50%	0.02%
<i>Mucilaginibacter</i>	6.21%	0.08%
<i>uncultured Intrasporangiaceae</i>	5.87%	0.39%
<i>Paracoccus</i>	5.38%	0.40%

Table 4.21: Significant decrease in attached biomass at genus level after long-term exposure to NSAIDs.

Genus	Control	MP
<i>Paracoccus</i>	10.34%	2.18%
<i>Nakamurella</i>	7.45%	2.38%
<i>Ottowia</i>	6.03%	1.95%

The combined effect of NSAIDs-induced stress and low OLR conditions may have contributed to a significant increase in some specific genera in suspended and attached biomass of hybrid MP reactor. For example, uncultured genus belonging to Chitinophagaceae family, *Ferruginibacter*, *Comamonas* and *Rhodanobacter* showed significant elevated abundance in suspended biomass of hybrid MP reactor (Table 4.22), while *Fimbriimonadaceae*, *Parafilimonas* (belongs to *Chitinophagaceae* family) and *Rickettsia* dominated the attached biomass in the presence of selected NSAIDs. These genera may contribute to NSAIDs removal, as their abundance increased significantly in the presence of selected NSAIDs, suggesting a potential tolerance to NSAIDs stress (Table 4.23).

Table 4.22: Significant increase in suspended biomass at genus level after long-term exposure to NSAIDs.

Genus	Control	MP
<i>uncultured Chitinophagaceae</i>	1.16%	10.08%
<i>Ferruginibacter</i>	0.95%	7.82%
<i>Comamonas</i>	0.03%	6.77%
<i>Rhodanobacter</i>	0.01%	6.23%

Table 4.23: Significant increase in attached biomass at genus level after long-term exposure to NSAIDs.

Genus	Control	MP
<i>Fimbriimonadaceae</i>	0.02%	8.73%
<i>Parafilemonas</i>	0.15%	6.46%
<i>Rickettsia</i>	0.02%	5.55%

4.6 Effect of Low Organic Loading on Nitrifying Profile in Hybrid Systems

At a constant ammonium loading, decrease in COD loading rates result in less availability of carbon source for the heterotrophic bacteria. The heterotrophic population decreased, while the autotrophic nitrifying bacteria may become dominant in the mixed culture (B. Fu et al., 2010). Moreover, the observed relation between C/N ratio and nitrifiers indicated higher relative abundance of nitrifying bacteria at lower C/N ratio (B. Fu et al., 2010). In other words, in the biological wastewater treatment systems, the heterotrophic/nitrifying bacteria population ratio is influenced by the important factor of COD/N ratio (Lindemann & Wiesmann, 2000). In a study conducted by Shi et al. (2023), it was reported that, the high C/N ratio (calculated as 20) was unfavorable for the competitive growth of nitrifiers.

As mentioned in section 4.2.2, the dominant genera observed in the suspended biomass of hybrid MP are uncultured genus belonging to *Chitinophagaceae* family, *Ferruginibacter*, *Comamonas*, *Rhodanobacter*, and *Parafilemonas* genera, respectively. In same trend the dominant genera in attached biomass after exposure to the NSAIDs are *Fimbriimonadaceae*, *Parafilemonas*, *Rickettsia* and *Niabella*.

Interestingly, the most dominant uncultured genus in suspended biomass of hybrid MP reactor and also *Ferruginibacter* and *Parafilemonas* genera from suspended biomass and *Parafilemonas* and *Niabella* from attached biomass belong to the *Chitinophagaceae* family. Moreover, the two genera *Comamonas* and *Ottowia*, detected in high abundances (third dominance in samples) in suspended biomass of MP reactor and attached biomass of control reactor, respectively. These two genera belong to the *Comamonadaceae* family. Additionally, *Sphingomonadaceae* family detected with a relative abundance of 3.08% in suspended biomass of hybrid MP reactor, while in the attached biomass the frequency was seen as 1.14%.

According to a study conducted by Petrilli et al. (2024) on ammonia removal in moving bed biofilms, *Chitinophagaceae*, *Comamonadaceae* and *Sphingomonadaceae* families were reported as the microbial community accumulated in biofilms, contributing to the high removal rate of ammonium, suggesting a critical role for them in nitrification process.

Rosenberg et al. (2014), reported that *Comamonadaceae* family could be detected in freshwater, groundwater, soil, and activated sludge. Most *Comamonadaceae* family are aerobic and heterotrophic bacteria and some of them could contribute to the denitrification (Rosenberg et al., 2014). Also, according to Khan et al. (2002), the members of this family, tend to dominate under poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-acclimated denitrifying conditions, utilizing organic compounds as their electron donor (Morais et al., 2016).

Chitinophagaceae family includes many heterotrophic ammonia-oxidizing bacteria (AOB) that use organic matter as their energy source (Petrilli et al., 2024). This family was recorded among the most represented families in a study done on removing nitrogen in a pilot-scale reactor (Z. Wang et al., 2020).

Likewise, another study done by Garcia et al. (2022), reported the presence of *Comamonadaceae* and *Sphingomonadaceae* families in the bench-scale MBBR bioreactors. Most of the mentioned detected families in the MBBR systems include many heterotrophic nitrifying bacteria, specifically indicating *Comamonadaceae* with the ability of simultaneous nitrification/denitrification (Janka et al., 2022). The same study, reported *Ferruginibacter*, *Comamonas*, and *Paracoccus* as the most prevalent genera in biofilm (Petrilli et al., 2024). In this study *Paracoccus* genus were also observed in abundance in the suspended and attached biomass of hybrid control reactor, reducing significantly in the presence of selected NSAIDs.

It is worth noting that, in this study *Nitrosomonas* and *Nitrospira* as common ammonia oxidizing bacterial (AOB) and *Nitrospira* and *Nitrobacter* as common nitrite oxidizing bacteria (NOB), were detected in very low relative abundances. Same situation observed by some of other studies as well (Petrilli et al., 2024).

In this study, the initial ammonium concentration (60 N mg/L) decreased significantly in both hybrid control and hybrid MP reactor, reported the ammonium concentrations in the effluent of hybrid control and hybrid MP reactor as 14 ± 0.15 and 6 ± 0.1 mg N/L, respectively. These results show approximately 76.67% and 90.00% removal efficiency for ammonium in the hybrid control and hybrid MP reactor, respectively. At the same time, the concentration of nitrate increased to 44.7 mg/L in hybrid control reactor and 45.7 mg/L in hybrid MP reactor, suggesting that nitrification proceeded. According to these findings, most probably the hybrid system supported (proliferated) other genera which might contribute to nitrification rather than the common nitrifiers.



5. CONCLUSIONS AND RECOMMENDATIONS

The objective of this study was to investigate the effect of low organic loading rate (OLR) in hybrid nitrification systems on removal of six selected non-steroidal anti-inflammatory drugs (NSAIDs) including diclofenac, ibuprofen, ketoprofen, naproxen, indomethacin, and mefenamic acid, as well as assessing its impact on microbial population structure and diversity.

Based on the analyzed data, the study leads to the following key findings:

- Mixture of selected NSAIDs did not chronically inhibit biological degradation of organic matter in the hybrid system, as almost similar concentrations of COD were observed in the effluents of both acclimated control and micropollutant (MP) reactors. COD removal efficiencies calculated as approximately 81% and 87% in the hybrid control reactor and hybrid MP reactor, respectively.
- Similarly, mixture of selected NSAIDs did not inhibit nitrification process, as evidenced by comparable effluent ammonium concentrations in both hybrid control reactor and hybrid MP reactor. It was observed that, the biomass of hybrid system enhanced nitrification performance in hybrid MP reactor than in hybrid control reactor. Nitrification efficiencies calculated as approximately 77% and 90% for hybrid control and hybrid MP reactors, respectively. In parallel, the effluent concentration of nitrate in both hybrid reactors, reflecting an active nitrification in the hybrid system. It can be said that, low OLR conditions promote nitrification as evidenced in literature.
- Low OLR did not negatively affect the removal of targeted NSAIDs, showing high to complete removal for all selected NSAIDs as follows: ibuprofen (100%), indomethacin (100%), naproxen (99%), mefenamic acid (98%), ketoprofen (96%) and diclofenac (69%). Lower removal efficiency of diclofenac was predictable

as literature well documented the limited biodegradability of diclofenac due to its complex structure.

- Conventional characterization alone is not enough for a complete understanding of hybrid system performance; understanding microbial community dynamics is also important. For this reason, in this study high-throughput 16S rRNA gene sequencing was conducted, followed by statistical and alpha diversity analyzes to evaluate the structure and diversity of microbial communities in the hybrid system.
- The three most dominant species in the suspended biomass of hybrid MP reactor are uncultured species belonging to *Chitinophagaceae* family, *Ferruginibacter* and *Comamonas*, while *Fimbriimonadaceae*, *Parafilimonas* and *Rickettsia* dominated the attached biomass of hybrid MP reactor. To our knowledge, the role of these species in nitrification or NSAIDs removal has not yet been studied in literature. Investigating their specific function would require further work beyond the scope of this thesis, but it remains an open question and a valuable direction for future research. However, according to the result of this study, it may be concluded that these species could tolerate the toxic effects of NSAIDs and low OLR conditions, and may contribute to the degradation of selected NSAIDs as well as to nitrification.
- On the other hand, the addition of selected NSAIDs caused significant changes in microbial composition of both suspended and attached biomass of hybrid MP reactor, compared to corresponding hybrid control reactor. For example, genera including, *Niabella*, *Mucilaginibacter*, uncultured genus belonging to *Intrasporangiaceae* family *Paracoccus* were suppressed significantly in the suspended biomass of hybrid MP reactor under the addition of mixture of selected NSAIDs, as well as low OLR conditions. Similarly, in the attached biomass notable suppression was observed in hybrid MP reactor for genera including *Paracoccus*, *Nakamurella* and *Ottowia*. It can be inferred that these genera might be sensitive to the mixture of selected NSAIDs under low OLR conditions.

Moreover, the combined effect of NSAIDs-induced stress and low OLR condition may contribute to a significant increase of following genera in the suspended and attached biomass of hybrid MP reactor, compared to hybrid control

reactor. Uncultured genus belonging to *Chitinophagaceae* family, *Ferruginibacter*, *Comamonas* and *Rhodanobacter* in suspended biomass, while *Fimbriimonadaceae*, *Parafilimonas* (belongs to *Chitinophagaceae* family) and *Rickettsia* dominated the attached biomass. These taxa might contribute to nitrification in the hybrid system rather than common nitrifiers.

- Families *Chitinophagaceae* and *Comamonadaceae* became dominant in the hybrid MP reactor and have been reported in similar studies to be effective and play an important role in nitrification in MBBRs.
- Addition of selected NSAIDs leads to an enrichment of two phyla in suspended biomass including *Bacteroidota* and *Proteobacteria*. And also, *Bacteroidota* enriched in attached biomass of hybrid MP reactor. Both phyla were reported to be effective contributors in nitrogen and NSAIDs removal.
- The microbial diversity varied in suspended biomass and attached biomass in response to the stress caused by selected NSAIDs addition and the low OLR condition. Evaluating the diversity of the system is important because it provides insight into stability and function potential of the microbial community of the system. Alpha diversity quantifies the species diversity in an ecological community, focusing on richness and evenness of species. Shannon index reflect the microbial diversity and Chao1 index and number of OTUs show the richness of microorganisms in the activated sludge. Based on the Shannon index, chao1 and rarefaction curves generated based on OTU numbers for all samples, biodiversity and richness were observed to decrease in both suspended and attached biomass of hybrid reactor compared to the inoculum sludge as expected due to the differences in lab-scale and full-scale conditions. Moreover, biodiversity and richness decreased in suspended biomass of hybrid MP reactor compared to the corresponding control reactor. In contrast, an increase in biodiversity and richness was observed in the attached biomass of the hybrid MP reactor relative to its control. NSAIDs stress might be responsible for this change in biodiversity as detected in various similar studies. Moreover, attached biomass might supports the coexistence of different functional bacteria in the biofilm, as reported in a similar

study. Generally, higher diversity in the attached biomass might contribute to a more stable community and improved system performance, as reported in similar studies.

- Higher biodiversity provides functional redundancy, meaning that multiple species perform similar roles. This enables the system to tolerate stress and fluctuations. Therefore, it can be said that higher biodiversity in the attached biomass might maintain system stability as reported in similar studies.
- The decrease in diversity (richness and evenness) in the suspended biomass of the hybrid MP reactor could be the result of selective dominance by certain taxa that may resist chronic NSAID exposure and potentially contribute to NSAID removal.
- Evenness decreased in both attached and suspended biomass of the hybrid MP reactor compared to hybrid control reactor. This reduction might be due to NSAIDs stress, as well as low OLR which could have promoted the dominance of a few resilient species.
- In the attached biomass of hybrid MP reactor, although evenness decreased, a considerable increase in richness may have compensated for the decline, allowing overall diversity to remain high. Therefore, selective dominance could be possible for attached biomass as well. This may lead to dominance of certain species that resist chronic NSAID exposure and potentially contribute to NSAID removal in the attached biomass.

Briefly, the results clearly supported that selected NSAIDs did not have any chronic inhibition on nitrification and carbon removal. And also, low OLR did not adversely affect the removal of selected NSAIDs. Therefore, a good nitrification performance as well as high NSAIDs removal were achieved in hybrid system. The hybrid system, operated at low OLR, showed advantages over conventional WWTPs for nitrogen removal, most likely by maintaining a favorable environment for nitrifiers, resulting in effective ammonium conversion to nitrate. Some taxa different other than common nitrifiers might have contributed to the nitrification in hybrid system. It seems that the stability of the hybrid reactor increased due to the higher diversity observed in

the attached biomass. There is a need for use of real time-qPCR that would help us to study the functional genes of microbial communities to obtain a better view from the metabolic activity of microbial community. Moreover, further studies using detailed qPCR and next generation sequencing (NGS) with longer target gene regions or alternative molecular techniques can be conducted for a better investigation of relationship between microbial diversity and NSAIDs removal and also its correlation with nitrification. Finally, these approaches could be implemented in full-scale WWTPs to evaluate the behavior of microbial community under real operational conditions.





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- G.E. Zengin, **R. Dadkhah**, D. Soylu, I. Pala-Ozkok, D. Güven, G. Özyıldız, B. Uçan, G. Insel, E. Çokgör (2025). Response of microbial interactions in hybrid biofilm system with low organic loading to micropollutant removal. 12th International water association Symposium on Waste Management Problems in AGRO-Industries, 2025, (Upcoming oral presentation).