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Microbialdiversityandfunctionalrelationships

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PhD Thesis
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DTU Environment
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Preface

This thesis summarizes the results of a PhD project carried out at the Technical University of Denmark, Department of Environmental Engineering, and Veolia Water Technology AB, AnoxKaldnes (Lund, Sweden) from January 2014 to February 2017. My main supervisors were Associate Professor Benedek Gy. Plósz and Dr. Magnus Christensson, Senior Research Manager, AnoxKaldnes. Additionally, by 1 November, 2016, my former co-supervisor, Professor Barth F. Smets, was appointed as my main supervisor on behalf of DTU when Associate Professor Plósz has transferred to University of Bath, UK. My additional co-supervisor was Associate Professor Henrik R. Andersen. Project funding was provided by MERMAID, ITN-EU-FP7 funded by the People Programme (Marie Skłodowska-Curie Actions).

I **Elena Torresi**, S. Jane Fowler, Fabio Polesel, Kai Bester, Henrik R. Andersen, Barth F. Smets, Benedek Gy. Plósz, Magnus Christensson. Biofilm thickness influences biodiversity in nitrifying MBBRs – Implications on micropollutant removal. Environmental Science & Technology (2016), 50, 9279-9288.

II **Elena Torresi**, Fabio Polesel, Kai Bester, Stefan Trapp, Barth F. Smets, Magnus Christensson, Henrik R. Andersen, Benedek Gy. Plósz. Sorption and diffusion of micropollutants in nitrifying MBBRs- The influence of biofilm thickness. Submitted

III Fabio Polesel, **Elena Torresi**, Luca Loreggian, Monica Escolá Casas, Magnus Christensson, Kai Bester, Benedek Gy. Plósz. Elimination of pharmaceuticals in pre-denitrifying MBBR – Influence of organic substrate availability in single-stage and three-stage configurations. Submitted.

IV **Elena Torresi**, Arda Gulay, Fabio Polesel, Marlene M. Jensen, Magnus Christensson, Barth F. Smets, Benedek Gy. Plósz. Microbial composition and diversity of staged Moving Bed Biofilm Reactor for removal of micropollutants. Manuscript.

V **Elena Torresi**, Monica Escolá Casas , Fabio Polesel, Benedek Gy. Plósz, Magnus Christensson, Kai Bester. Impact of external carbon dose on the removal of micropollutants using methanol and ethanol in post-denitrifying Moving Bed Biofilm Reactors. Water Research (2017), 108, 95-105

This PhD study also contributed to international conferences with the following proceeding and conference papers:

- **Elena Torresi**, F. Jane Fowler, Fabio Polesel, Barth F. Smets, Henrik Rasmus Andersen, Magnus Christensson, Benedek Gy. Plósz. Microbial biodiversity enhances micropollutants biotransformation in Moving Bed Biofilm Reactors (MBBR) with controlled biofilm thickness. International Conference on Emerging Contaminants (EmCon), 2016, Sidney.
  Oral presentation awarded of *"Best Student Oral Presentation"*
- **Elena Torresi**, Fabio Polesel, Marlene M. Jensen, Monica Escola Casa, Barth F. Smets, Magnus Christensson, Kai Bester, Benedek Gy. Plósz. Enhancing the removal of pharmaceuticals in biological wastewater treatment: is MBBR the answer? International Poster at Conference on Emerging Contaminants (EmCon), 2016, Sidney.
  Poster presentation
- Fabio Polesel, **Elena Torresi,** Marlene M. Jensen, F. Jane Fowler, Monica Escola Casa, Barth F. Smets, Magnus Christensson, Kai Bester, Benedek Gy. Plósz. Does reactor staging influence microbial structure and functions in biofilm systems? The case of pre-denitrifying MBBRs. International Conference on Emerging Contaminants (EmCon), 2016, Sidney.
  Poster presentation
- Pedram Ramin, Andreas Libonati Brock, Fabio Polesel, **Elena Torresi**, Benedek Gy. Plósz. Improving the prediction of in-sewer transformation of illicit drug biomarkers by identifying a new modelling framework. International Conference on Emerging Contaminants (EmCon), 2016, Sidney
  Poster presentation
- **Elena Torresi**, Fabio Polesel, Henrik Rasmus Andersen, Barth F. Smets, Magnus Christensson, Benedek Gy. Plósz. Biodiversity positively associates with biofilm thickness in Moving Bed Biofilm Reactors (MBBRs) – implications on micropollutant removal and nitrification. MEWE, IWA Microbial Ecology in Water Engineering & Biofilms, 2016, Copenhagen.
  Oral presentation
- Fabio Polesel, **Elena Torresi,** Marlene M. Jensen, Monica Escola Casas, Kai Bester, Magnus Christensson, Barth F. Smets, Benedek Gy.

Plósz. The influence of reactor staging on microbial structure and functions in pre-denitrifying MBBRs. MEWE, IWA Microbial Ecology in Water Engineering & Biofilms, 2016, Copenhagen. Oral presentation by Fabio Polesel

- **Elena Torresi**, Fabio Polesel, Henrik Rasmus Andersen, Barth F. Smets, Magnus Christensson, Benedek Gy. Plósz. Can we enhance the biotransformation of pharmaceutical micropollutants by controlling biofilm thickness in MBBR? 18th International EWA Symposium, 2016, Munich.
  Oral presentation and Conference paper
- Fabio Polesel., **Elena Torresi**., Luca Loreggian, Monica Escolá Casas., Kai Bester, Benedek Gy. Plósz. Elimination of pharmaceuticals in single- and three-stage pre- denitrifying MBBR. Micropol & Ecohazard 2015, 9th IWA Specialist Conference on Assessment and Control of Micropollutants and Hazardous Substances in Water, 2015, Singapore. Oral presentation by Fabio Polesel.
- **Elena Torresi**, Henrik R. Andersen, Barth F. Smets, Benedek Gy. Plósz, Magnus Christensson. Influence of biofilm thickness on micropollutants removal in nitrifying MBBRs. Micropol & Ecohazard 2015, 9th IWA Specialist Conference on Assessment and Control of Micropollutants and Hazardous Substances in Water, 2015, Singapore.
  Oral presentation

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Summary

Numerous pollutants such as pharmaceuticals and personal care products are continuously released into municipal wastewater treatment plants (WWTP). Present at concentration of nano- to milligram per liter, they are defined as micropollutants. Micropollutants are only partially removed, possibly due to design and operational limitation of conventional WWTP. Eventually, micropollutant parent compounds and transformation products are discharged into receiving water bodies, possibly causing acute and chronic toxic effects on aquatic organisms even at very low concentrations. Therefore, research currently focuses on the enhancement of conventional WWTPs via physical-chemical and biological treatment processes.
Biofilm-based treatment processes, such as the Moving Bed Biofilm Reactor (MBBR), were shown to harbour bio-catalytic potential that can enhance the biotransformation of a number of micropollutants compared to conventional activated sludge. In MBBRs, biofilm grow on plastic carriers kept in suspension in the reactor basin via mechanical mixing or aeration, offering a suit of benefits, amongst all comparably small footprint. Despite few existing evidences in aerobic MBBR, an in-depth understanding of the fate of micropollutants in such systems under different operational conditions is still required.

In this context, this PhD thesis investigated different optimization strategies using MBBRs towards the removal of 23 commonly detected micropollutants (i.e., pharmaceuticals) in municipal wastewater. Specifically, I studied the impact of (i) biofilm thickness on the diffusion, sorption and biotransformation of the selected pharmaceuticals in nitrifying MBBR; and (ii) of organic carbon quality and availability on micropollutant biotransformation in anoxic pre- and post-denitrifying MBBRs. In both case, the influence of (i) and (ii) on the microbial activity (nitrification and denitrification) and microbial community composition and diversity were investigated. The existence of possible relationships between microbial diversity (analyzed via 16S rRNA amplicon sequencing) and biotransformation of micropollutants was evaluated to investigate which microbial processes and factors underlay the removal of micropollutants.
The PhD objectives were evaluated in long- and short-term experiments in three laboratory- scale MBBR systems for pre-denitrification (MBBR1), nitrification (MBBR2) and post-denitrification (MBBR3). Biokinetics of nitrification, denitrification and micropollutant biotransformation rate

constants (k_{Bio}, L g^{-1} d^{-1}) were estimated through batch experiments using Activated Sludge Models (ASMs) and ASM for Xenobiotics (ASM-X), respectively.

In the pre-denitrifying MBBR1 study, denitrification, biotransformation of micropollutants and microbial community were evaluated in three-stage (S) and single-stage (U) MBBR configurations. The three-stage configuration produced a prolonged exposure of the biofilm to a gradient of organic carbon loading and complexity, leading to a significant differentiation of denitrification and biotransformation kinetics in the three MBBR sub-reactors. The highest and lowest biotransformation kinetics were found in the first and the last stage, respectively (up to 4-fold decrease for selected compounds), suggesting a possible a correlation of micropollutant biotransformation with denitrification rates. The long term-operation with carbon availability and complexity gradient led to higher ($p<0.05$) biodiversity in the three-stage system, with a more diverse and even microbial community in the last stage. Specific taxa such as *Candidate division WS6* and *Deinococcales* were selected in S, possibly due to oligotrophic conditions occurring in the last reactor stage.

The influence of biofilm thickness was studied in nitrifying MBBR2 using newly developed Z-carriers that allow the control of defined biofilm thickness. The use of thinner biofilms (~ 50 µm), rather than thicker biofilms (>200 µm), had a positive effect on nitrification rates and on the biotransformation kinetics of a number of compound such as diclofenac (k_{Bio} up to 6 L g^{-1} d^{-1}) and the three sulfonamide antibiotics. However, the biotransformation of more than 60% of targeted compounds was enhanced in thicker biofilms, that exhibited higher ($p<0.05$) microbial diversity and were more even. Additionally, a biofilm model was developed and calibrated to evaluate sorption and diffusion of micropollutants in nitrifying biofilms. Sorption was significant only for eight out of the targeted compounds. All compounds removed by sorption were predicted to carry a net positive charge at the experimental pH, suggesting the importance of electrostatic interactions on sorption in biofilms. Sorption coefficients K_d (L g^{-1}) and effective diffusivity coefficients f increased with increasing biofilm thickness, suggesting reduced diffusion limitation and higher surface area accessibility in the thickest, least dense biofilm (~500 µm).

Two types of commonly dosed degradable carbon sources (methanol and ethanol) were investigated in two parallel post-denitrifying systems (MBBR3). The methanol-dosed MBBR exhibited in the enhancement of k_{Bio} (up to 2.5-fold) for a number of micropollutants (nine out 23) compared to

the ethanol-dosed MBBR, while for 10 compounds biokinetics were similar between the two reactors. The higher denitrification rates exhibited by the ethanol-dosed MBBR during batch experiments likely influenced the biotransformation of the sulfonamides antibiotics, in analogy with what observed in MBBR2. A strong cometabolic effect (i.e., an enhancement of micropollutant biotransformation in the presence of organic carbon) was observed for venlafaxine, carbamazepine, sulfamethoxazole and sulfamethizole. However, an increase in methanol or ethanol loading to the MBBRs during continuous-flow experiment did not influence the removal of the targeted micropollutants, most likely due to the short hydraulic residence time (2 hours) used in the study as well as in full-scale reactors.

Diversity-function relationships (assessed through Pearson correlation analyses) were tested by comparing diversity estimators against biomass-normalized biotransformation rates. A positive influence of biodiversity for most of the targeted compounds (~60%) was shown in MBBR2 study, while biotransformation of few compounds (diclofenac and sulfonamides) was positively associated to microbial activity (i.e., nitrification). Similarly, a positive association ($p<0.05$) with the specific denitrification rate was shown in MBBR1, while biotransformation of most of the detected pharmaceuticals in wastewater did not associate or negatively associated with biodiversity. The relationship between biodiversity and micropollutant biotransformation may depend on whether its biotransformation is catalysed by a narrow (i.e., performed by few species) or broad processes. It is likely that for highly redundant microbial processes (such as denitrification), micropollutant biotransformation may be catalysed by broadly distributed enzymes and pathways, and microbial diversity provides no benefit. Conversely, increasing biodiversity under nitrifying conditions may be necessary to increase the inclusion of microorganisms with specific functionality towards micropollutant biotransformation.

Overall, the biotransformation rates were significantly enhanced in MBBR3 compared to MBBR1 and MBBR2 for the majority of micropollutants (~60%) suggesting the positive impact of easily degradable carbon sources (such as methanol or ethanol) on micropollutant removal. Finally, the removal of compounds such propranolol atenolol, citalopram, venlafaxine (under post-denitrifying conditions) and diclofenac (under aerobic conditions) was improved compared to conventional activated sludge. It can be thus concluded that MBBRs can offer a suitable technology that can be optimized for the removal of micropollutants in municipal wastewaters under a range of operating conditions (nitrifying, pre- and post-denitrifying).

Dansk sammenfatning

Talrige miljøfremmede stoffer, såsom lægemidler og produkter til personlig pleje, udledes løbende til spildevandsrensningsanlæg. Stofferne er defineret som værende mikroforureninger, eftersom de findes i koncentrationer fra nano- til miligram per liter. Mikroforureninger fjernes kun delvist i de konventionelle spildevandsrensningsanlæg, hvilket kan skyldes designmæssige og operationelle begrænsninger. Mikroforureningerne og deres omdannelsesprodukter udledes herefter til vandmiljøet. Dette kan medføre akutte og kroniske giftpåvirkninger på vandlevende organismer selv ved meget lave koncentrationer. Som konsekvens heraf fokuseres på at forberede og udvikle de konventionelle biologiske rensningsprocesser i renseanlæggene ved hjælp af fysisk-kemiske og biologiske metoder.

Det er blevet påvist, at biofilmsbaserede behandlingsteknologier, såsom "Moving-Bed" biofilm reaktoren (MBBR), besidder et større biokatalytiske potentiale, sammenlignet med den konventionelle aktiveret slam-proces. Det større potentiale i MBBR kan forbedre biotransformationen af visse mikroforureninger. I MBBR dannes biofilmen på plastbærere, som holdes suspenderet i vandsøjlen ved mekanisk omrøring eller beluftning. Dette medfører bl.a. den fordel, at reaktoren er meget kompakt og har et lille fodaftryk. Selv om der allerede foreligger bevis på fjernelse af mikroforureninger i MBBR, mangler der stadigvæk en dybdegående forståelse af mikroforureningers skæbne i reaktorsystemet under forskellige driftsforhold.I dette ph.d.-projekt undersøgte jeg 23 udvalgte lægemidlers skæbne i MBBR under forskellige driftsforhold. Dette blev gjort med sigte på at optimere reaktordesignet og driftsforholdene, således at både konventionelle forureninger og mikroforureninger fjernes effektivt. Fokus var særligt på: (i) hvilken indvirkning biofilmtykkelsen havde på diffusion, sorption og biotransformation af lægemidlerne i nitrificerende MBBR; og (ii) hvilken effekt tilgængeligheden af organisk kulstof havde på biotransformationen af lægemidlerne i anoksiske præ- og post-denitrificerende MBBR. Derudover blev mulige sammenhænge mellem den mikrobielle diversitet og biotransformation af lægemidlerne undersøgt for at afdække, hvilke mikrobielle processer og faktorer der er årsag til deres omdannelse.

Studiets formål blev evalueret i både lang- og kortsigtede eksperimenter i tre MBBR systemer i laboratorieskala med tre forskellige driftsforhold. De tre reaktorer var henholdsvis præ-denitrificerende (MBBR1), nitrificerende (MBBR2) og post-denitrificerende (MBBR3). De biokinetiske omdannelses-

rater for nitrifikation og denitrifikation (den primære metabolisme) samt biotransformationsraten (k_{bio}, L g^{-1} d^{-1}) blev estimereret ved at kombinere batchforsøg med følgende velkendte modeller: Activated Sludge Model (ASM) og Activated Sludge Modelling framewrok for Xenobiotics (ASM-X).

Forskelle i denitrifikation, biotransformation af lægemidler og den mikrobielle sammensætning mellem et tre-trins reaktorsystem (S) og enkelt-trin (U) blev undersøgt i forsøget med den præ-denitrificerende reaktor (MBBR1). S-konfigurationen medførte, at biofilmen i systemet blev eksponeret for en gradient af både mængden af organisk kulstof samt dets kompleksitet. Dette resulterede i en betydelig differentiering af dentrifikation- og biotransformationskinetikken i de tre trin. I det første trin fandtes den højeste kinetik, i det sidste trin den laveste (op til 60% forskel). Dette tyder på, at der eksisterer en korrelation mellem biotransformation af mikroforureninger og denitrifikationspotentialet. Den længerevarende eksponering til denne gradient medførte en højere mikrobiel diversitet i S-konfigurationen sammenlignet med U-konfigurationen ($p < 0.05$). I det sidste trin fandtes således et mere mangfoldigt og mere lige mikrobielt samfund. Specifikke taksonomiske grupper såsom *Candidate division WS6* og *Deinococcales* blev selekteret i S-systemet, hvilket muligvis kan skyldes de oligotrofe forhold, der forekommer i det sidste trin. Effekten af biofilmens tykkelse blev undersøgt i den nitrificerende reaktor (MBBR2) ved anvendelse af de nyudviklede *Z-carriers*, der gør det muligt at kontrollere biofilmens tykkelse. Anvendelse af tyndere biofilm (~50µm) snarere end tykkere (< 200 µm) havde en positiv effekt på nitrifikationsraten og biotransformationskinetikken for en række af lægemidlerne, bl.a. diclofenac (k_{bio} op til 6 L g^{-1} d^{-1}) og tre sulfonamidantibiotika. For 60% af de undersøgte stoffer blev biotransformationen forøget i de tykkere biofilm, som havde en højere ($p < 0.05$) og mere lige mikrobiel diversitet.

En model til beskrivelse af biofilmen blev udviklet og kalibreret, således at mikroforureningernes diffusion og sorption i den nitrificerende biofilm kunne estimeres. Sorption var kun signifikant for otte af de 23 stoffer. Disse otte stoffer havde alle en positiv nettoladning ved den anvendte pH, hvilket tyder på, at elektrostatiske interaktioner spiller en vigtig rolle i forbindelse med sorptionen. De estimerede sorptionskoefficienter, K_d (L g^{-1}), var proportionale med biofilmtykkelsen. Det skyldes sandsynligvis den øgede porøsitet og det øgede overfladeareal forbundet med den tykkere biofilm. Den estimerede effektive diffusivitet, *f*, steg også med biofilmtykkelsen, hvilket indikerer en begrænsning af diffusion ved højere biofilmdensitet.

Effekten af letnedbrydelige kulstofforbindelser (methanol og ethanol) blev undersøgt i to parallelle post-dentrificerende systemer (MBBR3). Doseringen

af methanol resulterede i en forøgelse af k_{bio} (ca. en fordobling) for ni af stofferne sammenlignet med doseringen af ethanol. For 10 af stofferne var kinetikken sammenlignelig. Den højere denitrificeringsrate i batchforsøget med det ethanol-doseret system havde sandsynligvis en effekt på biotransformationen af sulfonamidantibiotika.
En stærk co-metabolisk effekt blev observeret i MBBR3-forsøget (dvs. biotransformationen af mikroforureningerne blev forhøjet ved tilsætning af organisk kulstof), hvorfor en co-metabolisk model blev anvendt til at estimere de biokinetiske parametre. En øget kulstoftilsætning i de to reaktorer under anvendelse af kontinuert flow havde ikke en positiv effekt på biotransformationen. Dette kan skyldes den korte hydrauliske opholdstid (2 timer).
Diversitet-funktionsrelationer blev undersøgt ved at sammenligne diversitetsestimatorer mod biomasse-normaliseret biotransformationsrater (vurderet ved at anvende Pearson korrelationsanalyser). Biodiversiteten havde en positiv indflydelse på størstedelen af stofferne (60%) i MBBR2-forsøget, mens biotransformationen af diclofenac og sulfonamider var positivt korreleret med den mikrobielle aktivitet (dvs. nitrifikation). Ligeledes fandtes flere positive korrelationer ($p < 0.05$) med den specifikke dentrifikationsrate i MBBR1-forsøget. Dog korrelerede biotransformationen for størstedelen af lægemidler enten negativt med biodiversiteten eller slet ikke. Forholdet mellem biodiversitet og biotransformation af mikroforureninger kan afhænge af, hvorvidt biotransformationen katalyseres af en specifik proces (dvs. kun udført af få arter) eller af generelle processer. Det er sandsynligt, at biotransformationen for meget redundante mikrobielle processer (såsom dentrifikation) kan katalyseres af de hyppigt forekommende enzymer og reaktionsveje, hvorved en øget mikrobiel diversitet ikke medfører fordele. I modsætning hertil er de situationer (bl.a. under nitrificerende og aerobe heterotrofe betingelser), hvor en øget mikrobiel diversitet er nødvendige for, at mikroorganismer med en specifik funktionalitet i forhold til biotransformation findes i biofilmen.
Samlet set blev biotransformationsrater markant forbedret i MBBR3-forsøget for mere end 60% af stofferne sammenlignet med biotransformationsraterne observeret i MBBR1- og MBBR2-forsøgene. Dette tyder på, at tilstedeværelsen at letnedbrydelige kulstofforbindelser har en positiv indvirkning på nedbrydning af mikroforureninger. Endelig blev fjernelse af stoffer såsom propranolol, atenolol, citalopram, venlafaxine (under post-dentrificerende betingelser) og diclofenac (under aerobe betingelser) forbedret sammenlignet med den konventionelle aktiveret slam-proces. Dette viser, at MBBR-teknologien er velegnet til fjernelse af mikroforureninger i spildevand.

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1. Introduction

1.1. Background and motivations

Water is valuable resource and is the foundation of all living organisms and crucial for human activities, from the domestic use to agriculture and industry purposes. Entering in an era of water scarcity (Postel, 2000), water recycling and reuse can provide tremendous benefits to the environment and to the society, by decreasing fresh water uptake and use reclaimed water for landscape irrigation, to recharge groundwater aquifers or for drinking. However, several contaminants of emerging concern such as micropollutants can compromise the reliability of reclaimed water, by contaminating vital aquatic compartments such as surface water, groundwater and eventually drinking water (Barbosa et al., 2016).

Micropollutants consists of man-made chemicals which are found in the environment at trace level (from ng L^{-1} to µg L^{-1}) and mostly include organic compounds such as pharmaceuticals, illicit drugs, personal care products, pesticides, herbicides and surfactants. Several are the sources that contribute to their presence in environmental compartments, consisting mainly in (i) human consumption (e.g., of pharmaceuticals and illicit drugs), (ii) manufacturing (e.g., pharmaceuticals industry) and (iii) veterinary and agricultural use (e.g., of antibiotics and pesticides/herbicides). Micropollutants may be directly released into the environment as for example by the use of manure or biocides application on soil. However, through administration to human and animals, as well as manufacturing, micropollutants eventually reach municipal wastewater treatment plants (WWTPs). On the one hand, municipal WWTPs represents the major source of the emission of micropollutants in the environment (Kolpin and Meyer, 2002; Ternes, 1998), on the other hand they represents also the last defence against their spread.

Humans excrete a significant fraction of prescribed pharmaceutical dose, both as unchanged chemical (parent compound) as well as metabolites. Pharmaceuticals have been extensively detected in surface water, groundwater and drinking water (Barbosa et al., 2016; Benner et al., 2013; Focazio et al., 2008) and they have been associated with detrimental impacts on aquatic life even at very low concentrations (Jobling et al., 1998; Painter et al., 2009). The possible effects range from acute and chronic toxicity on aquatic organisms (e.g., zooplankton or algae) (Flaherty and Dodson, 2005;

Yang et al., 2008), feminization of male fishes (Brodin et al., 2014; Jobling et al., 1998) to the spread of antibiotic resistance genes (Daughton and Ternes, 1999; Sharma et al., 2016).

Although no legal discharge limit for micropollutants is currently defined in Europe, some regulations have been published in the last decades (Barbosa et al., 2016). Following the Directive 2000/60/EC (first mark in the European policy, Directive 2000), 33 priority substances/group of substances were ratified with the respective environmental standards in the Directive 2008/105/EC (Directive 2008). Four years ago, the Directive 2013/39/EU recommended the monitoring and the treatment options for 45 substances, rectifying the first watch list of 10 substances/group of substances for European Union monitoring. The watch list included the anti-inflammatory diclofenac and the synthetic hormone 17-alpha-ethinylestradiol -EE2, a natural hormone (17-beta-estradiol—E2) and three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), along with some pesticides (i.e., Barbosa et al., 2016).

To comply with Environmental Quality Standards (EQS) of European directives, advanced processes such as activated carbon (Solisio et al., 2001; Stoquart et al., 2016) or advanced oxidation with ozone and chlorine (Benner et al., 2013; Knopp et al., 2016) have been used to treat effluent wastewater. However, the economic costs associated with ozone treatment (Knopp et al., 2016) and the possibility of the formation of persistent oxidation products with equal or greater toxicity than the parent chemical (Benner et al., 2013) underpin the need to optimize secondary processes and thus biological wastewater treatment.

Recent studies have proposed biofilm systems, e.g., Moving Bed Biofilm Reactor (MBBR), as a promising alternative to conventional activated sludge systems (CAS) with respect to the attenuation of micropollutants via biological treatment (Escolà Casas et al., 2015; Falås et al., 2016, 2012; Hapeshi et al., 2013). In MBBR, biofilm grows on plastic carriers that are continuously mixed and retained in the reactor. The enhanced removal of several micropollutants in MBBR compared to CAS have been hypothesized to be related to longer biomass retention, as well as the capability to enrich the microbial community of “specialists” able to improve the removal of a number of micropollutants (Falås et al., 2012). Despite these preliminary studies, we still lack a mechanistic understanding of the fate of micropollutants in MBBR as well as of operational strategies to optimize

their performance towards removal of micropollutants. Furthermore, understanding the ecology and the physiology of microorganisms in biofilm systems and their possible link to the biotransformation of micropollutants is necessary to optimize biological processes and to make them competitive over alternative treatment technologies (i.e., ozonation and activated carbon).

1.2. Aim of the thesis

The overall aim of this research project was to investigate the capability of the MBBR technology for removal of micropollutants, with the intention to improve the overall performance at different stages of existing wastewater treatment plant (Figure 1.1). Notably, Figure 1.1 represents a schematic and simplified configuration of a conventional WWTP, where only the processes that were the focus of this study were reported. The work is divided in three core aims as listed below.

I. Operational strategies of MBBR for removal of micropollutants

(i) Influence of biofilm thickness on nitrification and biotransformation of selected micropollutants in nitrifying MBBR (**Paper I**)

(ii) Influence of a three-stage MBBR configuration vs single-stage configuration – and thus tiered organic substrate availability – on the denitrification and on the biotransformation of micropollutants under pre-denitrifying conditions (**Paper III**)

(iii) Impact of type and availability of additional carbon sources (methanol and ethanol) on the biotransformation of micropollutants in post-denitrifying MBBR (**Paper V**)

II. Fate processes of micropollutants in MBBR

(i) Quantify the relative contribution of biotic and abiotic transformation of micropollutants in biofilm systems (**Paper I-V**)

(ii) Quantify sorption and diffusive transport of micropollutants in biofilms at different biofilm thicknesses (**Paper II**)

(iii) Develop and calibrate a model to separately describe diffusion and sorption of micropollutants onto biofilm at different biofilm thicknesses (**Paper II**)

III. Relationship between microbial structure and function

(i) Evaluate relationships between microbial density and diversity with biotransformation of micropollutants (**Paper I, IV**). The understanding of these relationships will contribute to the possibility of managing microbial communities in wastewater engineering to improve the overall performance.

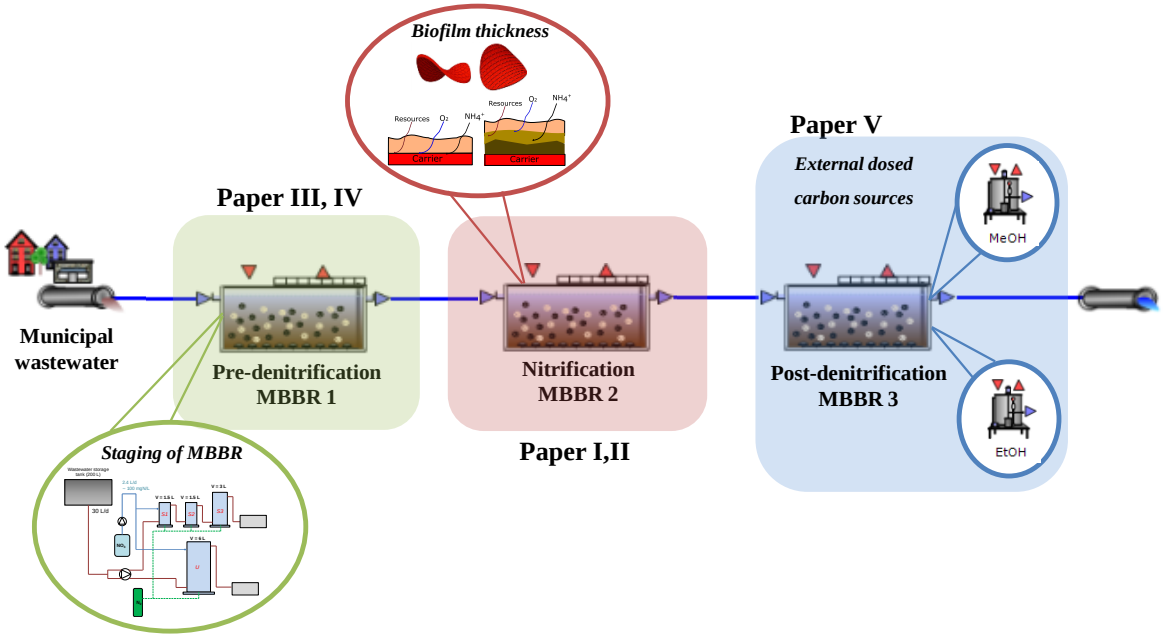


Figure 1.1. Schematic representation of the research conducted in this thesis. The study performed on MBBR1 (pre-denitrifying MBBR) is focus of Paper III and IV; the nitrifying MBBR2 is the focus of Paper I and II and post-denitrifying MBBR3 of Paper V.

1.3. Scope and overview of the thesis

The PhD research was developed by mainly working in laboratory-scale MBBR systems working under three different conditions: (i) anoxic pre-denitrification (MBBR1); (ii) aerobic nitrification (MBBR2) and (iii) anoxic post-denitrification (MBBR3) (Table 1.2). The result of this work was then summarized in five manuscripts: **Paper III** and **IV** for the anoxic pre-denitrification MBBR (i), **Paper I** and **II** for the nitrifying MBBR (ii) and

Paper V for the post-denitrifying MBBR (iii) (Fig. 1.1). For all the three MBBR systems, the impact of the different operational strategies on the microbial functionality (i.e., carbon and nitrogen removal, micropollutant biotransformation) and microbial structure (composition and diversity) was evaluated. Subsequently, microbial structure and function relationships were assessed in all the three studies to elucidate how different operational strategies impact the microbial diversity and its association with the microbial functionality.

Chapter 1 presents an overview of the biological removal in WWTP and introduces the biofilm technology used in this work (i.e., MBBR) as well as the biofilm properties which characterize biofilm systems.

Chapter 2 focuses on the fate of micropollutants and the processes which lead to their removal in WWTP. The models used to identify micropollutants biokinetics are also introduced.

Chapter 3 gives an overview of the major factors influencing the removal of micropollutants, giving focus to those parameters and operational conditions studied in this work. Chapter 3 also identify the state-of-the art of biological treatment for micropollutants.

Chapter 4, 5, 6 summarize the main findings of the three studies with focus on biotransformation and sorption of micropollutants in nitrifying MBBR (Chapter 4 and 5, respectively) and in pre- and post-denitrifying MBBR (Chapter 6). In Chapter 4 and 6, the impact of the operational parameters on the microbial diversity is also reported.

Chapter 7 elucidates the relationship between microbial structure and functionality in the three studies.

Chapter 8 summarizes the three studies with respect on the biotransformation of micropollutants in order to give possible application and engineering significance to the work.

1.4. Overview of methods

Throughout the PhD research similar approach in terms of material and methods was used to investigate the aims of this thesis in the three MBBR systems (Fig. 1.1). The methods mainly consisted of (i) continuous-flow operation of the MBBR; (ii) batch experiments; (iii) biokinetics assessment;

and (iv) characterization of the microbial community. Statistical analyses were used to investigate relationships between microbial structure and functionality.

1.1.1 Continuous-flow operation of the systems

In this research work, continuous-flow operation of MBBR mainly consisted of the use of laboratory scale glass reactors (of operating volume from 1.5 to 3 L) fed with municipal wastewater from local municipal WWTPs (generally collected semi-weekly and stored in cooling tanks at 4 ºC). By using real wastewater, continuous-flow operation of the three systems was highly influenced by the variation of the wastewater characteristics. The use of real wastewater rather than synthetic wastewater (containing a limited range of growth substrates as compared to real wastewater) can avoid change in enzyme capability and thus underestimation of biotransformation kinetics (Polesel et al., 2016). Continuous-flow operation was performed without spiking of reference substances but only using native micropollutants concentration at trace level in wastewater. Furthermore, biomass adaptation to micropollutants has been observed not to enhance microbial activity in removal of micropollutants (Alidina et al., 2014a), making the spiking of reference substances redundant during continuous-operation. In this work, continuous-flow operation of MBBR systems was required to both (i) establish biofilms on new carriers (**Paper I**) and (ii) reach a stable microbial activity after implementing new operational strategies (i.e., from single to three stages in **Paper III-IV** and different carbon source dosing in **Paper V**).

1.1.2 Batch experiment

Batch experiments with minimum 24 h duration were used to estimate biokinetics of primary metabolism (removal of conventional pollutants such as carbon and nitrogen) and micro-pollutants removal. Batch experiments have previously been shown to simulate reasonably well biotransformation kinetics observed in full-scale WWTPs (Kern et al., 2010).

Generally, batch experiments were performed after reaching stable performance during continuous-flow operation in terms of conventional pollutants (carbon, ammonium and nitrate). MBBR systems were disconnected from continuous-flow and batches were performed in separately glass-reactors with wastewater spiked with reference pharmaceuticals (at concentration of 1 ± 0.5 µg L^{-1}) **(Paper I, II, V**). For the study under pre-anoxic conditions **(Paper III, Paper IV**) only micropollutants found in pre-clarified wastewater were studied. In all the batch experiments, electron donor was dosed at condi-

tions similar to continuous-flow operation and ensuring excess of electron acceptors (oxygen and nitrate for **Paper I** and **Paper III-IV**, respectively).

Twenty-three environmentally relevant micropollutants were selected for this study (Table 1.1). The targeted pharmaceuticals were grouped in six categories according to their use: (i) beta-blocker; (ii) X-ray contrast media; (iii) sulfonamides antibiotics; (iv) macrolide antibiotics; (v) anti-inflammatory; (vi) antiepileptic/ antidepressants. HPLC-MS/MS via direct injection was used to quantify micropollutants (Escolà Casas et al., 2015).

Table 1.1. The 23 pharmaceuticals targeted in this study.

	Compound	**Abbreviation**
Sulfonamide antibiotics and combination product	Acetyl-sulfadiazine	Ac-SDZ
	Sulfadiazine	SDZ
	Sulfamethizole	SMZ
	Sulfamethoxazole	SMX
	Trimethoprim	TMP
Beta-blockers	Atenolol	ATN
	Metoprolol	MET
	Propranolol	PRO
	Sotalol	SOT
Macrolide antibiotics	Clarithromycin	CLA
	Erythromycin	ERY
	Roxithromycin	ROX
Anti-inflammatory	Diclofenac	DCF
	Phenazone	PHE
	Ibuprofen	IBU
Antiepileptic/ antidepressants	Carbamazepine	CBZ
	Citalopram	CIT
	Venlafaxine	VFX
X-ray contrast media	Diatrizoic acid	DIA
	Iohexol	IOH
	Iomeprol	IOM
	Iopamidol	IOP
	Iopromide	IOPR

1.1.3 Biokinetics for primary metabolism and micropollutants removal

Primary metabolism under nitrifying and denitrifying condition was measured under batch experiment as removal rate of ammonium and nitrogen species (Sözen et al., 1998), respectively, normalized to the biomass concentration in the reactor. In addition, biokinetics of nitrification were estimated by developing a one-dimensional (1D) two-step nitrification biofilm model (**Paper I**). A two-step activated sludge model (ASM, adapted from Pan et al., (2015)) for denitrification was also developed to estimate biokinetics of denitrification (**Paper V**). Activated Sludge Model for Xenobiotics ASM-X (Plósz et

al., 2010, 2012) was used in this work to estimate biotransformation kinetics (describe in 3.1.2).

1.1.4 Microbial characterization

Microbial composition was investigated by (i) quantitative polymerase chain reaction (qPCR) to measure microbial density in nitrifying and denitrifying biofilms; and (ii) by next generation Illumina sequencing of the bacterial 16S rRNA gene. Previous studies observed the benefit of using a combination of molecular based-tools, given the divergence that can arise by using universal primers (used in pyrosequencing) compare to family- and genus-specific primers (used in qPCR) (Pellicer-Nàcher et al., 2014).

In this research, the results from the microbial characterization were used for different purposes. Abundance of nitrifying guilds obtained by qPCR was included in the 1D two-step nitrification biofilm model (**Paper I**). Denitrifying functional gene abundances in the pre-denitrifying MBBR study were compared with microbial functionality under different operational conditions. Alpha-diversity measured by 16S rRNA amplicon sequences (at 97% of similarity for **Paper I** and **V**, 93%, 95%, 97% and 99% for **Paper IV**) was linked to microbial functionality in MBBR1 and 2 study (Chapter 8).

1.1.5 Statistics

Statistical analyses were used to identify significance between estimated parameters and to evaluate relationships between results from qPCR analysis and 16S rRNA gene sequences to microbial activity. The statistical methods that were used in this thesis comprise:

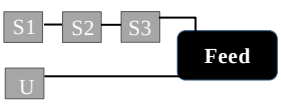
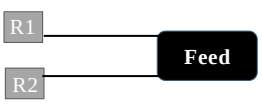
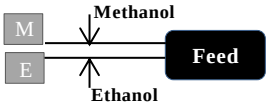
- Parametric tests such as Pearson correlation analysis to test association between variables. In this case, Pearson correlation coefficient or Pearson´s r provide a measure of the linear dependence between the two variables. Pearson´s r ranges between +1 and -1, where 1 determine the positive linear correlation, 0 no correlation and -1 the negative linear correlation (Baltosser and Zar, 1996).
- Nonparametric tests such as Spearman correlation analysis to test association between variables. As a nonparametric test, Spearman correlation makes no assumption on the underlying distribution of the values and it is based on ranks. The Spearman correlation coefficient, rs, has the same range as Pearson´s r (Baltosser and Zar, 1996).
- Linear and nonlinear regression analysis to find the best-fit model to the measured data. The goodness of the fit of the regression is quanti-

fied by the coefficient of determination r^2 or R^2 for linear and nonlinear regressions respectively.

- One-way analysis of variance (ANOVA) with non-parametric test (Kruskal-Wallis test) to compare means of the samples and to evaluate significance of difference between parameters.

According to Baltosser and Zar (1996), the Spearman correlation analysis should be used when the size of the population (N, measurements) is higher than 10 (N>10). Although recommended, in this study it was not always possible to perform Spearman correlation analysis (due to N<10), and instead parametric tests were used. However, normality tests (Wilk-Shapiro) were previously performed (when possible) to evaluate the correctness of using the linear distribution assumption of parametric-test.

Table 1.2. Overview of the experiments performed in the thesis

	MBBR 1	**MBBR 2**	**MBBR 3**
Conditions	Pre-denitrification (anoxic)	Nitrification (aerobic)	Post-denitrification (anoxic)
Configuration			
Carriers	K1	Z-carriers	K1
Conditions tested	Three-stages (S) vs single stage (U)	Different biofilm thicknesses	Two different carbon sources
Continuous-flow operation	3 MBBRs in series (S) 1 MBBR (U)	MBBR with Z200-Z500 carriers (R1) MBBR with Z50 (R2)	MBBR with methanol (M) MBBR with ethanol (E)
Batch experiments	**How**: Separated batch experiments for each MBBR (4 batches) **When**: ~at 3 and 15 months after start-up	**How**: Separated batch experiments for each Z-carrier (5 batches) **When**: ~at 5 and 9 months after start-up	**How**: Separated batch experiments for the 2 MBBRs (5 batches) **When**: ~at 3.5 months after start-up
Feed	Pre-clarified wastewater and addition of NO_3-N	Effluent wastewater and addition of NH_4-N	Nitrified wastewater and addition of methanol or ethanol
Hydraulic residence time	~8.9 h	~2 h	~2 h
Volume	6 L for each system	3 L for R1, 1.5 L for R2	1 L for each MBBR

2. Biofilms for biological wastewater treatment

Municipal WWTPs typically include at least a primary and a secondary biological treatment process. While in the primary treatment, particulate pollutants are removed via screening and sedimentation, in the secondary treatment, conventional pollutants are removed by biological processes to reach the required quality.

In biological treatment, microorganism can convert primary pollutants into harmless metabolites such as carbon dioxide, water and nitrogen gas and into new microbial biomass. Separation methods, such as settling or filtration are then used to separate biomass and particulate matters from wastewater requirement for effluent wastewater.

2.1. Biological processes

Generally, biological treatment in WWTP includes (i) the removal of organic matter (expressed as chemical or biochemical oxygen demand, COD and BOD, respectively) through aerobic and/or anoxic conversion by heterotrophic bacteria; and (ii) the removal of nitrogen under aerobic and anoxic conditions carried out by autotrophic and heterotrophic bacteria (processes referred to as nitrification and denitrification, respectively); (iii) the removal of phosphorus via biological treatment through a combination of anaerobic and aerobic tanks (e.g., enhanced biological phosphorus removal, EBPR).

Nitrification

During nitrification, ammonium (NH_4^+) is oxidized by nitrifying bacteria to nitrate (NO_3^-), through two sequential microbial oxidation processes (nitritation and nitratation), during which the valence state of nitrogen pass from -3 (NH_4^+) to +5 (NO_3^-) (Eq. 2.1) (Barnes and Bliss, 1983).

$$NH_4^+ + 1.382O_2 + 1.982HCO_3^- \rightarrow 0.018C_5H_7NO_2 + 0.982NO_2^- + 1.891H_2CO_3 + 1.036H_2O \quad (2.1)$$

In nitritation, ammonium is first oxidized into hydroxyl-amine (NH_2OH) catalysed by the enzyme ammonia monooxygenase (AMO). This is followed by NH_2OH oxidation to nitrite (NO_2^-) catalysed by the hydroxylamine oxidoreductase (HAO). Aerobic ammonia oxidizing bacteria (AOB) and archea

(AOA) are responsible for nitritation. AOB, as chemi-autotrophs (i.e., use of inorganic carbon for biomass synthesis and ammonium oxidation for energy) are slow-growing bacteria, with growth rates of ca. 2 d^{-1}. AOB found in wastewater are mainly affiliated to the subphylum of beta-proteobacteria, *Nitrosomonas* and *Nitrosospira* genera (Purkhold et al., 2000). The gene encoding ammonia monooxygenase subunit A (*amoA*) has been widely used as phylogenetic marker to quantify ammonia oxidizers in different environmental compartments (Klotz and Stein, 2008). Additionally, many *amoA* gene containing bacteria have been reported capable of oxidizing ammonia as a cometabolic process (Bock and Wagner, 2013).
In nitratation, nitrite is oxidized to nitrate in the presence of oxygen by nitrite oxidizing bacteria (NOB), catalysed by the nitrite oxidoreductase (NXR) as in the reaction (Eq. 2.2) (Barnes and Bliss, 1983):

$$NO_2^- + 0.0025NH_4^+ + 0.4875O_2 + 0.01H_2CO_3 + 0.01HCO_3^- \rightarrow 0.0025C_5H_7NO_2 + NO_3^- + 0.0075H_2O \quad (2.2)$$

NOB presents slower growth rates (~1.4 d^{-1}) and higher diversity than AOB. *Nitrobacter* genus of the *Alphaproteobacteria* and *Nitrospira* genera (of the *Nitrospira* phylum) account for the majority of NOB found in WWTP (Daims et al., 2006, Sorokin et al., 2012). NOB can be both quantified by 16S rRNA gene and functional genes, with *nxrA* proposed for *Nitrobacter* and *nxrB* proposed for *Nitrospira* coverage (Pester et al., 2014).

In WWTP, nitrification process is usually performed either in a single-sludge configuration (where ammonium and carbon are removed in a single tank) or in a two-stage configuration (where the first stage remove mainly carbonaceous and the second ammonium).

Denitrification

Denitrification is the sequential reduction of nitrate (Eq. 2.3) or nitrite (Eq. 2.4) (used as electron acceptor) to dinitrogen gas in the absence of free oxygen (anoxic), carried out by heterotrophic bacteria, which oxidizes biodegradable organic carbon for energy source (electron donor).

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2 + HCO_3^- + 1.68H_2O + 0.47N_2 \quad (2.3)$$

$$NO_2^- + 0.67CH_3OH + 0.53H_2CO_3 \rightarrow 0.04C_5H_7NO_2 + HCO_3^- + 1.23H_2O + 0.48N_2 \quad (2.4)$$

Complete denitrification is the combination of 4 energy-generating processes, catalysed by 4 different enzymes and that is reduction of: (i) nitrate to nitrite by nitrate reductase (Nar); (ii) nitrite to nitric oxide by nitric oxide reductase (Nor); (iii) nitric oxide to nitrous oxide (Nor) and (iv) nitrous oxide to nitrogen (Nos). Denitrification is a capability wide spread in both bacteria and archea, leading to a relative high diversity and species richness in WWTP (Lu et al., 2014). Denitrifier diversity (mainly influenced by the type of carbon source) lies between many phyla, although they are mostly identified as alpha-and beta- *Proteobacteria*. The functional genes *nirS* and *nirK*, have been mostly used to identify denitrifying bacteria (Braker et al., 2000; Kandeler et al., 2006).

In WWTP, denitrification is usually performed in four main configurations: (1) pre-denitrification, (2) post-denitrification, (3) simultaneous nitrification/denitrification. In this thesis, only configurations 1 and 2 were studied.

Pre-denitrification. In the WWTP, a pre-denitrification configuration consists of stage before the aerobic tank, where nitrate (produced in aerobic nitrification process) is recirculated in the pre-denitrification tank. The native carbon in the influent wastewater of the WWTP is used, and thus, the rate of denitrification is affected by the influent biodegradable COD, biomass concentration and temperature (Tchobanoglous and Burton, 1991). The pre-denitrification configuration is the most common configuration for denitrification due to no need for external carbon addition and the production of alkalinity before nitrification.

Post-denitrification with an external carbon source. Prior to the late 1970s or early 1980s, the main approach to remove the nitrogen (as nitrate) was to add a process after the nitrification stage in WWTP that needed to meet nitrogen requirement (Tchobanoglous and Burton, 1991). Because of the low amount of carbon left in the nitrified effluent, an external carbon source must be added to supply energy for the heterotrophic biomass to perform denitrification (Tchobanoglous and Burton, 1991). Commonly used external carbon sources are methanol, ethanol (Gavazza et al., 2004; Mokhayeri et al., 2009). Additional carbon required for nitrate removal (C-to-N ratio) is one of key design factors to be considered.

There are several technologies for biological wastewater treatment that can be categorized mainly in (i) suspended growth systems (activated sludge) and (ii) attached growth systems (biofilms systems)
In suspended growth systems, the microorganisms grow in flocs, which are retained in the systems by separation from the wastewater generally with settling, and subsequently recirculated in the system.
In the attached growth system, microorganisms grow on different support material as biofilm and they are retained in the system either by its own density or with outlet sieves. Attached growth systems can then be differentiated in fixed-film (e.g., trickling filter) or suspended-film, where the biofilm (attached on supported material) is then suspended in the bioreactor. Activated sludge systems may require relatively low maintenance but also a large volume (e.g., for the growth of slow growing bacteria), as well as a good control to ensure separation (settleability) of the biomass. In comparison, attached growth systems (fixed-film) can be more compact and less sensitive to varying environmental condition, but could be sensitive to the incoming suspended solids, leading to clogging of the support material (e.g. trickling filters).
In response to this, attached-growth systems with suspended biofilms can combine advantages of both fixed-biofilms and activated-sludge, and they will be described in the following section.

2.2. Moving Bed Biofilm Reactor

The MBBR technology is a biofilm-based technology, where the biofilm growths on plastic carriers which are then suspended in the reactor by mechanical mixing (in case of anoxic/anaerobic conditions) or aeration. (Ødegaard, 2006) (Fig. 2.1). The continuing mixing of the carriers in the reactor prevents carriers from clogging and enhances the mass transfer of substrate into the biofilm, improving treatment capacity (Ødegaard, 1999). The carriers are contained in the reactor by retention sieves over the reactor outlet, ensuring that biomass is retained independent of the flux of the wastewater (McQuarrie and Boltz, 2011). Due to this feature, HRT and SRT are decoupled, allowing operation of the system at shorter HRT than activated sludge, while biomass is retained in the system at long SRT. Thus, the long sludge age of the biofilms developed on carriers allow the growth of slow growing bacteria (e.g. nitrifying), making MBBR more compact and competitive to activated sludge systems for several processes (e.g., nitrification).

Furthermore, MBBR is particular advantageous when upgrading and retrofitting existing WWTP (as for example by introducing carriers in activated sludge reactors) to meet future and increasing effluent requirement.
The carriers are typically extruded or molded from virgin high-density polyethylene or from recycled high-density polyethylene (McQuarrie and Boltz, 2011). An important feature of the carriers in MBBR is the **carrier surface area** that in this study was defined as the exposed (or projected) biofilm area (EBA), as the "*area of biofilm exposed to the bulk liquid in the process*". In conventional MBBR carriers, which are generally cylinder-shaped with voids, the surface area mainly consists of the inner carriers surfaces (Ødegaard et al., 1994). The number of carriers (expressed in volume) in the operating volume of reactors further define filling ratio.

A wide range of plastic carriers were developed over the time for different treatment purposes, differing in surface area, shape and specific design (Ødegaard et al., 1994). In this work, two types of carriers from AnoxKaldnes (Veolia) were used (Fig. 2.2). AnoxKaldnes K1 carrier has same feature of the original MBBR carrier developed in Norway (Rusten et al., 1992). The Z-carriers are newly designed carriers (Piculell et al., 2016), which allow controlling biofilm thickness. While biofilm grows in the inside of voids in K1, in the Z-carrier the biofilm is retained on the carrier surface due to a grid covering the entire carriers.

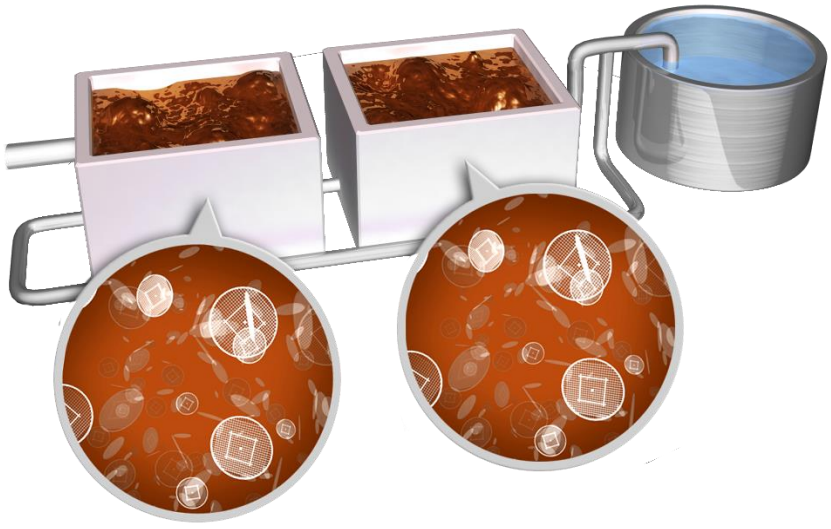


Figure 2.1. Schematic representation of MBBR systems containing polyethylene carriers in suspension.

The height of the grid can determine the maximum biofilm thickness, since the excess biofilm growing above the grid height will be scraped off by the abrasion from the other carriers in suspension during continuous MBBR operation. The saddle shape of the Z-carriers enhances mixing and oxygen transfer (Piculell et al., 2016). Five different types of Z-carriers were developed with 50, 200, 300. 400 and 500 µm grid wall (Z50-Z500 carriers).

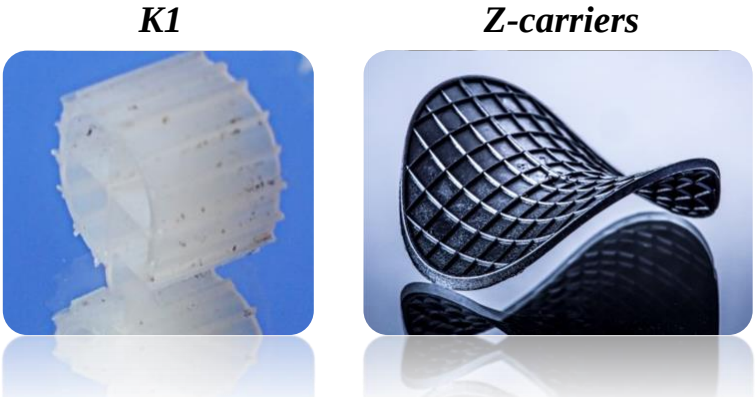


Figure 2.2. AnoxKaldnes K1 carriers and Z-carriers used in this study.

2.3. Biofilm characteristics

Biofilm differs greatly from activated sludge for their governing transport mechanisms and their microbial composition.

Dissolved chemicals present in the bulk are transported into biofilms via advection and ***molecular diffusion***, where the latter is generally considered as the dominant mechanism (Zhang and Bishop, 1994a). In biofilms, diffusivity of substrates is reduced as compared to free aqueous media (Wanner and Reichert, 1996) due to increased path length needed for diffusive transport through the biofilm thickness. This reduction is described by the *effective diffusivity coefficient f*, also defined as 'tortuosity' (Zhang and Bishop, 1994b), which can be expressed according to Eq. 2.6:

$$D_{bf,i} = f \cdot D_{W,i} \qquad (2.6)$$

where $D_{bf,i}$ and $D_{w,i}$ (m^2 d^{-1}) is the effective diffusivity of the chemical compound (i) within biofilm and water (respectively) and where f (-) is always lower than 1.

Due to diffusion and metabolic activities in biofilms, ***concentration gradients*** of different substrates will lead to stratification throughout the biofilm (Stewart and Franklin, 2008). The oxygen penetration in the

nitrifying depth will depend on the microbial activity, the bulk liquid concentration, reactor hydrodynamics (as mixing, aeration) and ***biofilm thickness***. At increasing biofilm thickness, higher mass transfer limitation and concentration gradients will lead to stronger stratification. Similar gradients will be observed for other substrates, while metabolites (as e.g. nitrate during nitrification) will have reverse concentration profiles (Stewart and Franklin, 2008). The thickness of the boundary layer (i.e., stagnant layer of liquid surrounding the biofilm dependent on the bulk liquid turbulence) can negatively influence the mass transfer of substrates into biofilms (Boltz et al., 2011).
Diffusion limitation is often seen as drawback of biofilms technologies (as for example in nitrifying biofilms by limiting the diffusion of oxygen and thus nitrification in the biofilm). However, diffusion limitation also enables a higher microbial stratification (Stewart and Franklin, 2008), the co-existence of several functional groups and possibly the increase of the ***biodiversity*** of the biofilm.

Substrate availability strongly regulates the microbial competitions in biofilms. Microbial growth kinetics in relation to substrate availability is often described by Monod kinetics (Eq. 2.7), where μ (d^{-1}) define the specific growth rate, μ_{max} is the maximum specific growth rate, S the substrate concentration and K_S (g m^{-3}) is the substrate half saturation constant:

$$\mu = \mu_{\max} \frac{S}{S + K_S} \qquad (2.7)$$

Two types of ecological strategies can be identified in terms of microbial kinetics, which determine the ***r-K selection*** (Andrews, 1986). The r-strategists have high μ_{max} but low K_S, while K-strategists have the opposite characteristics and typically higher yield than the r-strategists (Andrews, 1986).
Hence, the r-strategists dominate nutrient-rich habitats and K-strategists would thrive under oligotrophic conditions.
Finally, ***biofilm density*** (ρ) and ***porosity*** (ε) are also key parameters to understand biofilm dynamics and functionality. These properties can vary considerably depending on e.g., reactor configuration, substrate availability, shear conditions. A gradient of biofilm porosity can be created through the biofilm thickness (Zhang and Bishop, 1994c), possibly influencing substrate availability and thus system performance.

3. Fate of micropollutants in wastewater treatment

Bioavailability of micropollutants (as dissolved concentration accessible to the microbial cells) is a prerequisite for microbial transformation. Processes such as sorption to biomass and retransformation of metabolites to parent compound (Schwarzenbach et al., 2003) can affect the bioavailability of the compounds. Furthermore, release from fecal matter (Göbel et al., 2007) or from hydrolyzed particulate (Delgadillo-Mirquez et al., 2011) (colloidal and dissolved organic matter) have been observed to increase dissolved concentration of micropollutants in wastewater. In this Chapter, the main processes influencing the fate of micropollutants in WWTP will be described:

3.1. Fate processes

3.1.1. Sorption and desorption

Solid-liquid partioning of organic chemicals is a physico-chemical process involving two reverse mechanisms: sorption from aqueous to solid phase and desorption from solid to aqueous phase (Joss et al., 2006b). Solid-liquid partioning to biomass is considered in equilibrium when the rate of sorption equals the rate of desorption.

At equilibrium, the micropollutant concentration sorbed onto biomass ($C_{S,eq}$, µg L^{-1}) is proportional to the dissolved concentration ($C_{L,eq}$, µg L^{-1}) and the solid-liquid distribution coefficient K_d (L g-1) can be defined as (Eq. 3.1) (Joss et al., 2006b):

$$K_d = \frac{C_{S,eq}}{C_{L,eq} X} \qquad (3.1)$$

The initial measured concentration ($C_{L,0}$, µg L^{-1}) and the measured final concentration at equilibrium ($C_{L,eq}$, µg L^{-1}) can be used to determine $C_{S,eq}$ (Eq. 3.2)

$$C_{S,eq} = C_{L,0} - C_{L,eq} \qquad (3.2)$$

with the assumption of negligible initial sorbed concentration at the onset of the sorption experiment (i.e., $C_{SL,0} = 0$).

Eq. 3.2 represents a simplified (linear) case of the empirical Freundlich equation (Freundlich, 1909).

Sorption/desorption equilibrium has been previously assumed to be reached instantaneously, defining partitioning as a non-rate-limiting process during wastewater treatment. Experimental observations confirmed this assumption in activated sludge, showing that equilibrium can be reached within 0.5–1 h (Hörsing et al., 2011; Wendell O. Khunjar and Love, 2011; Ternes et al., 2004a). However molecular diffusion trough biofilms pores may have a major role in determining partitioning kinetics in biofilms. Thus, when considering biofilm systems, biofilm porosity, along with sorption, can also influence the decrease of micropollutant concentrations in the bulk liquid. Eq. 3.2 can be revised by considering the relative contribution of bulk aqueous volume and biofilm pore volume (Eq. 3.3):

$$K_d = \frac{C_{L,0} - C_{L,eq}}{C_{L,eq} X_{biomass}} \frac{V_{bulk}}{V_{bulk} + V_{PW}} \tag{3.3}$$

where V_{bulk} (L) denotes the volume of the bulk liquid and V_{PW} (L) the volume of the pore water in the biofilm matrix, not accounting for water inside the cells. Biofilm characteristics (density, porosity) have been accordingly found to influence diffusion of a number of organic and inorganic chemical species in biofilm (Zhang and Bishop, 1994b).

Sorption of organic chemicals has been mostly associated with their chemical properties, involving different mechanisms such as: (i) hydrophobicity of the chemical or van der Waals interactions and/or (ii) electrostatic interaction between ionized chemicals and biomass. Hydrophobicity is usually described by the octanol-water partition coefficient, K_{OW}, or the species-dependent K_{OW} estimated at experimental pH, D_{OW} (Schwarzenbach et al., 2003).

The pH and the ionic strength of the solution can also highly influence the activity of the ionizable micropollutants, which can be found in the solution in non-dissociated or dissociated form depending on the acid dissociation constant, pK_a. This process is called **ionization**, and it is instantaneous process in aqueous solution where the neutral and the ionized species (anionic, cationic and zwitterionic) are in equilibrium. Given the negative charged surface of biomass, ionization is likely to influence sorption of organic chemicals in WWTP, leading e.g. to the limited sorption potential of negatively charged compounds (Franco et al., 2009; Vasudevan et al., 2009).

Sorption process may involve other processes such as cation exchange, surface complexation and hydrogen binding (Hyland et al., 2012; Trapp et al.,

2010) and may also associated with multiple interactions (MacKay and Vasudevan, 2012).

Finally, when using biological reactor and carriers, abiotic removal via sorption of organic chemicals can also occur on bioreactor walls (e.g, plastic or glass) and on plastic carriers.

3.1.2. Biotransformation

Biotransformation describes the microbially-mediated transformation of micropollutants (parent compound) to other chemical products (defined as transformation products, TP, and/or metabolites). Biodegradation is a term that often substitutes biotransformation, although implies the complete mineralization of the micropollutants to inorganic carbon, nitrogen and water. Biodegradation rarely occurs in WWTP, leading to an accumulation of several transformation products (e.g., Boix et al., 2016; Helbling et al., 2010). TP of organic chemicals produced by oxidative processes typically are more polar and consequently more mobile and less toxic than the parent compounds (Boxall et al., 2004). However, several evidences exist on TP that exhibit a similar or even more toxic action than the parent compound (Boxall et al., 2004), leading to the introduction of TP identification for environmental risk assessment (Escher and Fenner, 2011).

Biotransformation of organic chemicals can be described by Monod-model (Eq 2.7). However, pharmaceuticals and human metabolites are found at low concentration in wastewater, from ng L^{-1} to µg L^{-1}. When the substrate concentration (S) is significant lower that the half-saturation coefficient, and thus the biomass activity increases linearly with S, Eq. 3.4 can written as the pseudo-first order kinetic model (Joss et al., 2006) :

$$\frac{dC_L}{dt} = \frac{-k_{Bio}}{(1+K_d X)} C_L X \qquad (3.4)$$

where k_{Bio} (L gX^{-1} d^{-1}) defines the biotransformation rate constant, C_L (µg L^{-1}) the concentration of micropollutant and X the biomass concentration (g L^{-1}), and K_d accounts for the relative contribution of sorption and desorption during biotransformation. The term “pseudo” refers to the dependence of the

relation to X, which is assumed constant when considering negligible biomass growth during batch experiments (Joss et al., 2006b).
The biomass concentration X can be expressed as different unit measures, in terms gram of total suspended solids (gTSS; e.g., Joss et al., 2006a,b), volatile suspended solids (gVSS, e.,g., Fernandez-Fontaina et al., 2014) or active biomass (gCOD, e.g. Sathyamoorthy et al., (2013). In this study, the attached biofilm was measured as total attached solids (gTAS), by the difference in weight of 3 or higher dried carriers (105 °C for >24 h) before and after biofilm removal (using 2 M H_2SO_4 with subsequent brushing) (Falås et al., 2012).
Furthermore, Eq. 3.4 assumes that biotransformation of micropollutants takes place exclusively in the aqueous phase, considering C_L as the only biologically active phase (Schwarzenbach et al., 2003) as commonly assumed (Pomiès et al., 2013). Models including biotransformation of the only sorbed concentration or biotransformation of both aqueous and sorbed fraction were also used to describe biotransformation of micropollutants (Pomiès et al., 2013). Furthermore, the aqueous concentration can include both freely dissolved fraction and the fraction sorbed into dissolved and colloidal material (Delgadillo-Mirquez et al., 2011).

Biotransformation of a number of micropollutants was described as a metabolic process and thus carried out by specific degraders (Pomiès et al., 2013). This assumption implies that the biomass can grow and sustain with the only presence of the micropollutants (Siegrist et al., 1989; Lindblom et al., 2009). However, micropollutants present in concentration of ng to µg L^{-1} generally do not support biomass growth and energy requirements (Alexander, 1985, Rittmann, 1992). Thus, a co-substrate (such as readily organic carbon or ammonium) is necessary and it is defined as the growth substrate (or primary substrate). With this terminology, the micropollutant is defined as secondary substrate and its biotransformation as **cometabolism** (Rittmann, 1992). The obligate presence of the growth substrate is needed to produce the enzymes (non-specific enzymes) for the biotransformation of the micropollutants (Dalton et al., 1982). Cometabolic biotransformation comes from the lack of specific enzymes catalysing defined transformation processes (Dalton et al., 1982) and it is often described as a ‘fortuitous’ biotransformation (Fischer and Majewsky, 2014) catalysed by broad spectrum enzymes in autotrophic and heterotrophic communities (Wendell O Khunjar and Love, 2011; Tran et al., 2013).

A number of models were developed to describe cometabolism of micropollutants and the beneficial effect of the primary substrate on the cometabolic biotransformation (Alvarez-Cohen and Speitel, 2001; Delgadillo-Mirquez et al., 2011; Fernandez-Fontaina et al., 2014; Liu et al., 2015; Plósz et al., 2010), that were inspired by the original reductant model developed by Criddle, (1993). The **cometabolic-biotransformation model** (by Plósz et al. (2012)) used in this study also derives from the reductant model (Eq. 3.5), assuming pseudo-first order with respect to micropollutants biotransformation:

$$\frac{dC_L}{dt} = -\frac{\left(q_{Bio}\frac{S}{S+K_S}+k_{Bio}\right)}{(1+K_d X)}C_L X \qquad (3.5)$$

where q_{Bio} (L gX^{-1} d^{-1}) defines the cometabolic-biotransformation rate constant in the presence of the primary substrate (S) and half saturation constant of S. The model was developed in mixed activated sludge culture by Plósz et al. (2012) for the case when the consumption of reductive forces by means of micropollutants is insignificant in the primary metabolic mass balance; it was also used to predict micropollutant removal in nitrifying biomass (Sathyamoorthy et al., 2013).

Finally, in this study, the effect of diffusion into biofilm on the removal of pharmaceuticals from bulk aqueous phase was lumped in the biotransformation rate constants, as previously considered by (Escolà Casas et al., 2015; Falås et al., 2013, 2012; Hapeshi et al., 2013), to allow comparison to literature. However, a biofilm model to estimate micropollutants removal kinetics was previously tested in a study in rotating biological contactor (Vasiliadou et al., 2014).

3.1.3. Retransformation

The term retransformation indicates the increase of concentration of parent compounds in wastewater as results of different processes (Polesel et al., 2016). Microbial **deconjugation of human metabolites** to parent forms is the major process leading to an increase of parent compound in wastewater and identifies the cleavage of the moiety of conjugated metabolites. Human

metabolites of pharmaceuticals can derive by functionalization reactions (e.g, oxidation, hydroxylation, known as Phase I) and conjugation reactions (e.g., glucoronation, sulphatation, acetylation, known as Phase II) in human body, although the latter have been observed also to occur exclusively (Polesel et al., 2016). Notably, human conjugates may represent up to 22% of the excreted form of pharmaceutical (Testa et al., 2012), suggesting the possible impact of this process in WWTP. Microbial deconjugation of human metabolites to parent compound have been hypothesized or observed e.g., for diclofenac (Plósz et al., 2012; Vieno and Sillanpää, 2014) and for sulfamethoxazole from N4-acetyl-SMX (Göbel et al., 2007; Joss et al., 2006). In case of deconjugation of human metabolites, a retransformation-biotransformation model describes the variation of aqueous concentration of the parent compounds (C_L) and a term is added in Eq. 3.6.

$$\frac{dC_L}{dt} = \frac{-k_{Bio}}{(1+K_d X)} C_L X + k_{dec} C_{CJ} X \tag{3.6}$$

where C_{CJ} accounts for the fraction of micropollutant present as conjugate and k_{Dec} (L g^{-1} d^{-1}) define the retransformation rate constant. Negligible sorption can be considered for the fraction C_{CJ} due to its potentially high hydrophobicity (Göbel et al., 2005; Plósz et al., 2010a).

3.1.4. Other processes

A number of other processes can influence the removal of micropollutants in wastewater. Volatilization and stripping (e.g., induced by aeration) can be considered negligible in this study due to the extremely low volatility of the pharmaceuticals (Trapp and Harland, 1995) and as observed in several studied (e.g., (Carballa et al., 2005; Li and Zhang, 2010). Similarly, photolysis and chemical hydrolysis processes are generally considered insignificant in the removal of pharmaceuticals, due to e.g., the high turbidity of the wastewater (hindering photolysis) (Li and Zhang, 2010).

3.2. Factors influencing the removal of micropollutants

3.2.1 Redox conditions

As WWTP normally operate under a combination of aerobic, anoxic and anaerobic conditions, removal of micropollutants under different redox condition is an emerging body of research (e.g., Falås et al., 2016; Stadler et al., 2015; Suarez et al., 2010; Joss et al., 2004a). Despite this, most of the studies focused on the aerobic removal of micropollutants (reviewed by e.g., Grandclement et al., 2017), while few evidences are available under anoxic conditions and even fewer in biofilm systems. However, while compounds such as diclofenac, metoprolol, erythromycin and roxithromycin were found to be transformed mainly under aerobic conditions in activated sludge using synthetic wastewater (Suarez et al., 2010) and in hybrid biofilm-systems (Falås et al., 2013), some of the investigated chemicals had similar (e.g., atenolol, clarithromycin) or higher (i.e., levetiracetam) removal under anoxic conditions than under aerobic conditions. Hence, in this thesis, a more detail investigation of removal of micropollutants under anoxic conditions was carried out (**Paper III, V**) to fill this gap.

Microbial biotransformation pathway under aerobic conditions has been intensively studied (e.g. Dagley, 1971; Hayaishi, 1994), identifying hydroxylation (i.e., incorporation of one of two oxygen molecules into the compounds) by oxygenase enzymes (mono- or di-oxygenase) as the key metabolism, favouring the subsequently biotransformation of the micropollutants by other enzymes. In anaerobic metabolism, the aromatic ring is reduced (commonly by the bezoly-CoA reductase) rather than oxidized and central intermediates (e.g., benzoyl-CoA) are formed for breaking the aromaticity and cleaving the ring (Freiburg and Fuchs, 1997). Following, hydroxylation of carbon atoms can be the primary mechanism in certain low redox environments, for ring cleavage and further transformation of the aromatic ring (Pitter and Chudoba, 1990). Several and contrasting results can be found regarding the redox conditions which enhance a specific micropollutant rather than another. Compounds such as atenolol were suggested to be ubiquitously biotransformed under aerobic and anaerobic conditions (Stadler et al., 2015), while venlafaxine and diatrizoate removal enhanced under anaerobic conditions (Falås et al., 2016). In Chapter 8.2, a detailed review of the removal of the 23 targeted compounds under different redox condition in this study will be provided.

3.2.2 Primary substrate

As previously reported, primary substrates (such as organic carbon and nitrogen) play an important role in the removal of micropollutants, supporting biomass growth and energy flow. Generally, primary substrates were shown to enhance the biotransformation of micropollutants in wastewater (Mazioti et al., 2015; Plósz et al., 2012; Su et al., 2015; Tran et al., 2013, 2009). On the other hand, competitive inhibition between primary substrate and specific micropollutants has been observed (e.g., Alvarez-Cohen and Speitel, 2001; Plósz et al., 2010a), as results of inhibition to the nonspecific enzyme active site (Criddle, 1993).

Particular interest in research was shown with respect to ammonium as primary substrate. A positive link between **nitrification** under ammonium non-limiting conditions and biotransformation of several micropollutants has been reported (Kassotaki et al., 2016; Khunjar et al., 2011; Sathyamoorthy et al., 2013; Tran et al., 2009). They suggested that removal of specific micropollutants occur as a cometabolic process carried out by AOB via the broad capability of the enzyme ammonia monooxygenase AMO (Arp et al., 2002). This was also tested through inhibition of AMO using e.g., allythiourea (ATU), which, in turn, can lead to substantially lower removal rate of several micropollutants during inhibition (Khunjar et al., 2011; Shi et al., 2004; Tran et al., 2009). On the contrary, Helbling (2012) statistically evaluated a positive relation with ammonium removal, but not with AMO, suggesting that other enzymes (e.g., hydroxylamine oxidoreductase) could be responsible for the removal of a number of micropollutants. However, high concentration of ammonium was also shown to re-press cometabolic transformation of specific pharmaceuticals by AOB (Fernandez-Fontaina et al., 2012).
In this work, correlation between biotransformation of 23 targeted micropollutants, nitrification rates and *amoA* abundance was investigated in nitrifying biofilms of different biofilm thicknesses (**Paper I**).

Fewer evidences on the important of organic carbon source on the removal of micropollutants are reported in literature for wastewater systems, limited to addition of humic acid to MBBR to enhance micropollutant removal (Tang et al., 2017) or to managed aquifer recharge systems (Alidina et al., 2014b). In this thesis, we investigated the importance of carbon availability under two different operation conditions: in pre-denitrifying MBBR, creating a gradient of carbon availability through staging (**Paper III**) and by using two different

additional carbon sources in post-denitrifying MBBR and evaluating them at different concentrations (**Paper V**).

3.2.3 Operational parameters

The relevance of the parameter **solid retention time (SRT)** on removal of micropollutants have been widely investigated thought lab- and full-scale experiments (Clara et al., 2005a, 2005b; Kreuzinger et al., 1998; Plósz et al., 2012) and discussed in several reviews (Grandcl Ement et al., 2017; Polesel et al., 2016; Pomiès et al., 2013). The SRT indicates the mean residence time of microorganisms in the reactor, and it is related to the growth rate of microorganisms. The positive relationship between micropollutants removal and SRT is generally attributed to the higher microbial diversity at longer SRT with positive effect on micropollutant biotransformation (Falås et al., 2016).

With respect to biofilm systems and in specific MBBR, since the carriers are retained in the bioreactor, SRT depends on the biofilm detachment (Henze et al., 2008). Assuming the biofilm thickness at steady state, the detachment rate of biomass can be considered equal to biofilm growth, making it possible to obtain extremely long SRT in biofilms compared to activated sludge systems when the organic loading is low. However, due to shear forces, MBBR will also give rise to suspended biomass and that can potentially influence the performance of the reactor (Mašić and Eberl, 2014)

Thus, the enhancement of micropollutants biotransformation in biofilm systems have been mainly associated to the longer SRT—a factor that can lead to higher microbial diversity (as previously observed in laboratory- (Xia et al., 2012) and full-scale (Vuono et al., 2014) activated sludge system) or the enrichment of nitrifiers (Ternes et al., 2004b).

The **hydraulic residence time** (HRT), corresponding to the mean residence time of the liquid phase in the reactor, has also been positively observed to influence the removal of several micropollutants in activated sludge (Maurer et al., 2007; Petrie et al., 2014) and MBBR (Mazioti et al., 2015). The contact time between biomass and aqueous concentration of micropollutant can be beneficial for the general slow removal rates estimated for micropollutants. However, extended HRT may not be feasible at full-scale treatment plant due to the increase of investment costs (e.g., large reactor volumes).

Other parameters, such as temperature and pH have been investigated. Temperature can positively affect microbial activity, but inconclusive results have been reported so far (Tran et al., 2013). pH can influence the physiology

of the microbes but above all the solubility of micropollutants, and the ionization of organic chemicals and thus the sorption onto biomass (Tran et al., 2013).

3.2.4 Microbial diversity

The term biodiversity includes the taxonomic and functional aspects of microbial organisms, with species richness (the number of taxa, also defined as OTU, operational taxonomic unit) and evenness (the relative abundance of species) as two of the major descriptors of microbial diversity (Wittebolle et al., 2009). Taxonomic diversity is generally measured with the Shannon index which couples microbial richness and evenness (Shannon and Weaver, 1949). Microbial richness is generally expressed by the taxonomic indices Chao and ACE (Chao, 1987; Chao et al., 1993), while evenness use Hill´s number (Hill, 1973).

The relationship between microbial diversity and microbial functionality is an area of considerable debate and research, but generally a positive relationship between the two is postulated and – sometimes – experimentally confirmed (Cardinale, 2011; Cardinale et al., 2012). A positive relationship between diversity (or richness) would emerge (i) if the microbial community consists of a number of strains which exploit unique niches (ii) if facilitative interactions (i.e., complementarity effects) would occur (Cardinale, 2011; Cardinale et al., 2012; Loreau et al., 2001). With the emerging of next generation sequencing tools, microbial ecologists have also begun to investigate diversity-function relationships (Johnson et al., 2015a) as well as the ecological consequences of disturbances on ecosystem function. In previous studies involving in 10 different full-scale WWTP, positive relationships between microbial diversity (and in particular microbial richness) and biotransformation rates of several, but not all, micropollutants were observed at full scale (Johnson et al., 2015a), as well as in laboratory-scale experiments under different DO concentration (Stadler and Love, 2016). Additionally, functional diversity (based on the phenotypes inferred from taxonomic descriptors and based on mRNA sequencing) and taxonomic diversity (based on 16S rRNA amplicon sequencing) associated positively with the rate constants of specific micropollutants (Johnson et al., 2015a). Furthermore, functional and taxonomic richness was also found to correlate with each other, confirming that communities with more taxa likely harbour more functional traits (Johnson et al., 2015b).

4. Removal of micropollutants in nitrifying MBBR

During nitrification, molecular diffusion of substrates from the bulk liquid into the biofilm (in particular oxygen as the limiting-recourse) and microbial activities create substrate gradients in the biofilm. At increasing **biofilm thickness**, greater concentration gradients and stratification of metabolic processes throughout the biofilm can possibly lead to a more heterogeneous and biodiverse biofilm (due to competition on limiting resources, 2.3). The increase of microbial diversity can likely have a positive impact on the microbial activities and in specific on micropollutants biotransformation. These hypotheses were investigated in **Paper I**, using Z-carriers (2.2), which were able to develop controlled biofilms of different thicknesses.

Two parallel MBBR systems (R1, R2) containing Z-carriers, where operated in continuous-flow operation for more than 300 days under nitrifying conditions (dissolved oxygen concentration, DO, of ~4.5 mg L^{-1}, effluent wastewater from municipal WWTP spiked with ammonium concentration 50 mgN L^{-1}, phosphorus =0.5 mgP L^{-1}) (Fig. 4.1). R1 (3 L operating volume) contained a mix of Z200, Z300, Z400 and Z500 carriers, while R2 (1.5 L) only Z50. The nitrifying biofilms were enriched on Z50, Z200, Z300, Z400 and Z500 carriers under similar conditions, resulting in biofilm with thickness between 50 and 500 µm, respectively. Two batch experiments were performed after reaching stable ammonium removal (1.88 ± 0.22 gN d^{-1} m^{-2} and 2.38 ± 0.47 gN d^{-1} m^{-2} for R1 and R2, respectively) for each Z-carrier in batch 1 and for Z50, Z200 and Z500 biofilm in batch 2 by spiking with the 23 targeted micropollutants described in 1.1.2. A separate batch experiment was also performed to assess the abiotic removal of targeted compounds. The experiment was divided in two parts: (i) without plastic carriers and using only filtered effluent wastewater to assess abiotic transformation and sorption onto glass walls and (ii) with new carriers to investigate sorption onto plastic carriers.

4.1. Influence of biofilm thickness on nitrification and micropollutants

Nitrification was described as (i) ammonia uptake rate per gram of biomass, r_{NH4_B}, or per carrier surface area, r_{NH4_S} (gN d^{-1} g^{-1} and gN d^{-1} m^{-2} respectively), assessed through linear regression of NH_4^+−N concentration

during batch experiment; and (ii) by estimating kinetic parameters such as maximum specific growth rate for AOB and NOB, $\mu_{max,AOB}$ and $\mu_{max,NOB}$, with a 1D two-step nitrification biofilm model that included growth and decay of AOB and NOB. Data obtained through qPCR by targeting 16S rRNA for AOB and NOB were used in the model as initial conditions for the biomass.

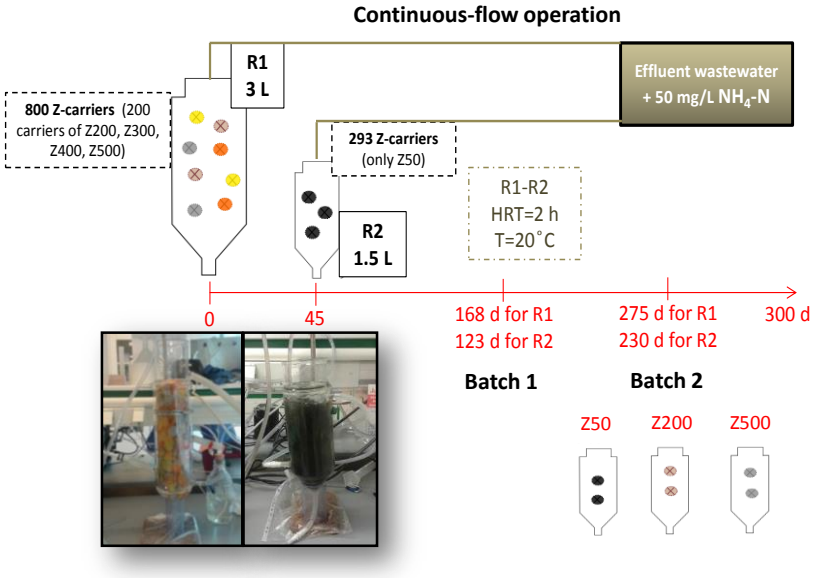


Figure 4.1. Schematic representation and pictures of the continuous-flow operation of the two nitrifying MBBRs; batch experiments performed over long time operation.

In batch experiment 2, the thinnest biofilm (50 µm) presented higher ($p<0.05$) r_{NH4_B} compared to the other biofilm thickness, and similar between Z200 and Z500 (Fig. 4.2). The estimated $\mu_{max,AOB}$ presented similar trend as the nitrification rates, suggesting that biofilm thickness beyond 200 µm may not result in any significant increase in nitrification activity. Furthermore, considering the results from the biofilm model, along with the affinity constant for ammonium, K_{NH4_AOB} (gN m^{-3}) that was found higher in the thinnest biofilm (see supplementary information of **Paper I**), r- and K-selection theory may have selected for different nitrifying communities, with r-strategist and thus fast-growing organisms enriching thinnest biofilm unlike thickest biofilm.

Most of the targeted micropollutants were removed according to pseudo-first order equation during the batch experiments (Eq. 3.4). Only diclofenac presented an increase of concentration at the beginning of the experiment, most likely due to retransformation from its human metabolites such as sulphate and glucuronide conjugates (Stierlin et al., 1979) (possibly present

in the feed wastewater). The abiotic batch experiment revealed a negligible contribution of abiotic degradation (<10%).

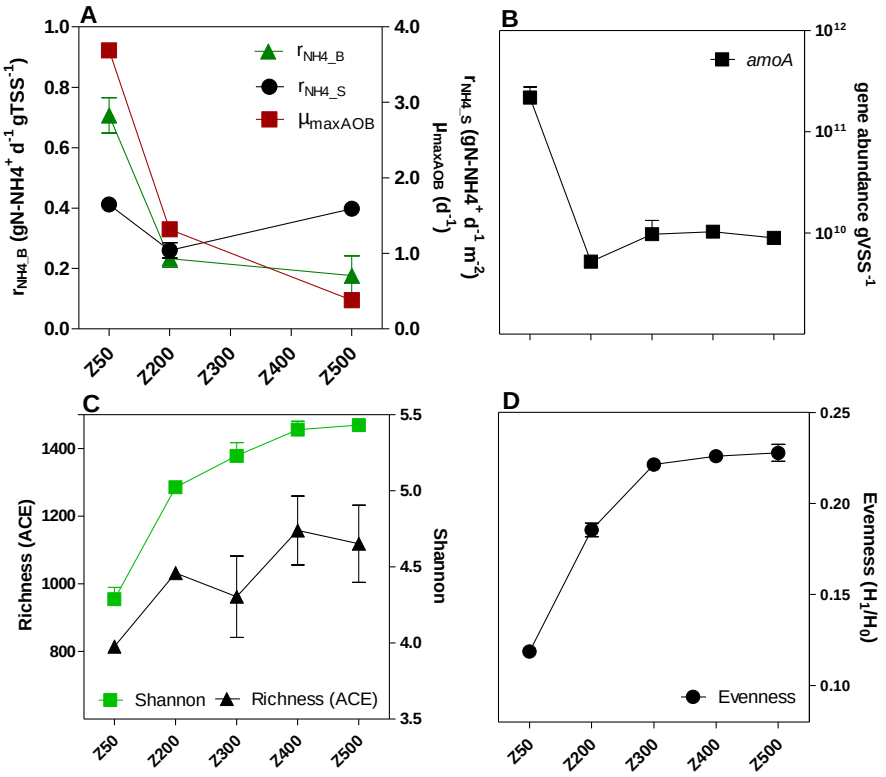


Figure 4.2. Nitrification rates (r_{NH4_B}, r_{NH4_S}) and specific growth rates of AOB (μ_{maxAOB}) for Z50, Z200 and Z500 (A); amoA gene abundance (B); extrapolated taxonomic richness (ACE), Shannon biodiversity (C) and evenness indices (D) estimated for the 5 Z-carriers (x-axis). Errors bars show standard deviation.

Correlation analyses were then performed between the estimated biotransformation rates k_{Bio} and biofilm thickness (Fig. 4.3). The results from Pearson´s correlation analysis (1.1.5) were grouped in three different categories, whether it was observed: (i) positive correlations ($r \geq 0.9$), (ii) low correlations ($-0.2 < r < 0.2$) and (iii) negative correlations ($r < 0$, and in specific $r = -0.9$) between k_{Bio} and biofilm thickness. Most of the targeted compounds (~ 60%) were part of the first category. Notably, it was observed that (i) the group X-ray contrast media (and venlafaxine) could not be biotransformed to any extent in the thinner biofilms, (ii) while the sulfonamide antibiotics and diclofenac presented biotransformation up to 5 times higher in the thinner biofilms compared to the thickest one.

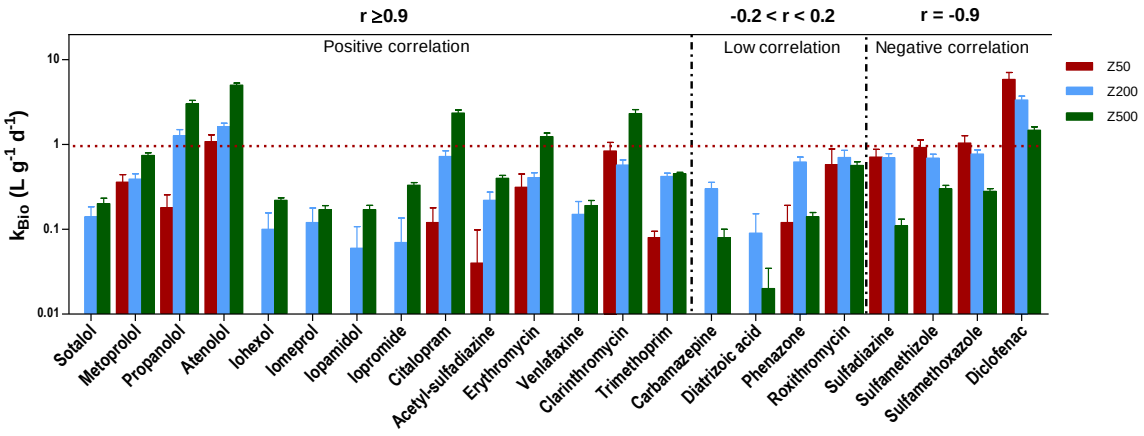


Figure 4.3. Biotransformation rates (k_{Bio}) estimated for 22 micropollutants for 3 Z-carriers. The fast removal of ibuprofen prevented the estimation of k_{Bio}. Pearson's coefficient r was used to measure the correlation between k_{Bio} and biofilm thickness.

Taken together, we concluded that although thin biofilms (~50µm) can achieve higher nitrification and increase the removal of some specific compounds, biofilm technologies based on thicker biofilms (at least 500 µm) could possibly enhance the removal of a major number of micropollutants.

4.2. Biodiversity and microbial density at different biofilm thicknesses

The control of biofilm thickness by using Z-carrier had likely influenced the microbial composition of the nitrifying biofilms. Estimated indices of biodiversity (Shannon), richness (ACE) and evenness increased with biofilm thickness (Fig. 4.2 C and D). Values of Shannon and evenness indices were significantly lower in Z50 compared to the other biofilm thicknesses. This suggested that after 200 µm, biofilm thickness does not significantly influence biodiversity (and in specific evenness) as observed for the microbial activities such as nitrification. qPCR data also revealed higher density of AOB, NOB and *amoA* abundance in the thinnest per gram of biomass, supporting the finding of higher nitrification rates observed during batch experiments.

5. Sorption and diffusion of micropollutants in nitrifying biofilms

In wastewater systems, micropollutants can sorb onto raw biomass, reaching equilibrium during sewer transport, and during secondary treatment. Subsequently removal of sorbed micropollutants occurs via primary and secondary sedimentation and with subsequent wastage of a fraction of activated sludge. Understanding the sorption of micropollutant in WWTP is important to understand their fate, and to predict for example a sudden desorption and release of micropollutants in the wastewater due to changes in wastewater conditions (e.g., pH).

Despite several investigations on sorption of organic micropollutants in activated sludge (Hörsing et al., 2011; Hyland et al., 2012; Stevens-Garmon et al., 2011; Ternes et al., 2004a), there is a lack a systematic study on sorption of trace organic chemicals in biofilm systems to understand the fate of micropollutants in biofilms. Furthermore, while in activated sludge, equilibrium time is considered sufficiently fast to take into account mass transfer limitation (Joss et al., 2004; Wang and Grady, 1995) (Barret et al., 2011), molecular diffusion may have a major role in determining partitioning kinetics in biofilms. As described in 2.3, diffusivity of organic chemicals in biofilm is reduced compared to bulk liquid and this reduction is described by the *effective diffusivity coefficient f*. While a number of studies, determined *f* for oxygen, phenol, sodium chloride and sodium nitrate (e.g., Fan et al., 1990; Horn and Morgenroth, 2006; Zhang and Bishop, 1994a), no evidence exists currently for micropollutant diffusion in biofilms.

In **Paper II**, diffusive transport and sorption of micropollutants in biofilms were investigated by using the two-consecutive-step approach: (i) calculation of the coefficient K_d by means of batch experiments (section 3.1.1); (ii) calibration of the diffusion-sorption model against experimental data and estimation of the effective diffusivity coefficient *f*.

Nitrifying biofilms of different thicknesses enriched on Z-carriers (of 50, 200 and 500 µm on Z50, Z200, Z500 carriers) described in Chapter 4 were used. First, batch experiments (in 200 ml glass beakers) were performed separately for each type of Z-carrier to experimentally determine K_d for the 23 spiked micropollutants dissolved in effluent wastewater. Biomass activity was inhibited during batch experiments by spiking of sodium azide and ATU in the wastewater feed that was sparged with nitrogen gas (Fig. 5.1).

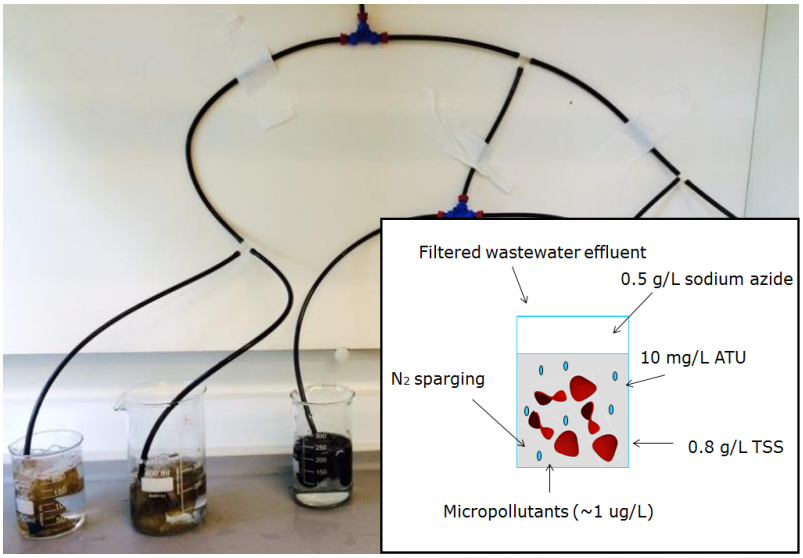
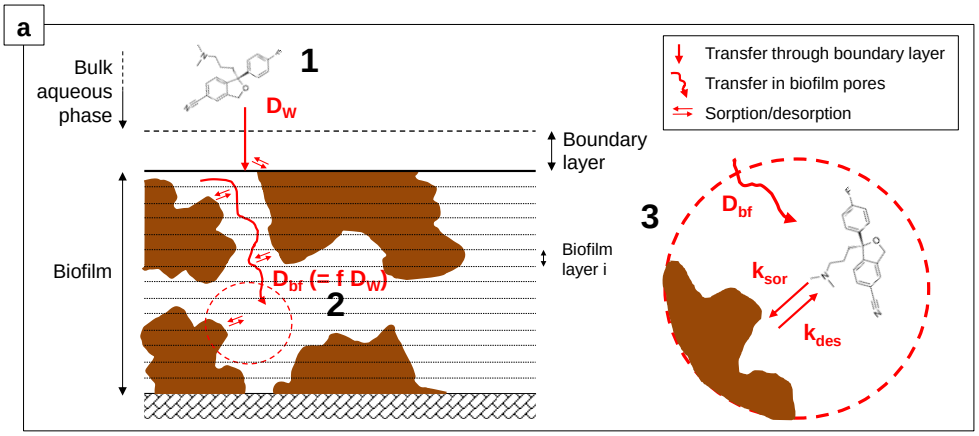


Figure 5.1. Pictures of the batch experiments containing Z50, Z200 and Z500 together with a schematic representation of the batch experiment and chemicals used for biomass inhibition.

Secondly, the diffusion-sorption model (schematized in Fig. 5.2) was calibrated using the experimental data and the calculated K_d for each chemicals. Three consecutive steps can be considered for partioning of micropollutants (Joss et al., 2004; Weber, 1973): (1) diffusion of dissolved micropollutants from bulk aqueous phase, through the boundary layer, into the biofilm matrix (described by diffusivity of the chemical in water, $D_{w,i}$; (2) diffusion of dissolved micropollutants through biofilm pores (described by the diffusivity of the chemical in biofilms (Fig 5.2); (3) sorption to biofilm solid matrix (biofilm is assumed to be a porous media). Sorption/desorption kinetics were described using first-order rate equations (see matrix in Fig. 5.2). When sorption is assumed (i) as an instantaneous process, by attributing an arbitrary high value to the desorption rate k_{des} (Plósz et al., 2012), and (i) linear (due to low concentration of micropollutants), diffusion within the biofilm is the rate-limiting steps for solid-liquid partitioning and f can be estimated as identifiable parameter.



b

Assumptions

(i) Same diffusivity in bulk liquid and boundary layer

(ii) Biofilm is a homogeneous porous medium

(iii) Sorption to biofilm solid matrix is instantaneous, i.e. sorption kinetics limited by diffusion though boundary layer and biofilm pores

(iv) Sorption is assumed linear (K_d)

c

Process	Component C_W	Component C_S	Process rate
Desorption	1	-1	$k_{des}C_S$
Sorption	-1	1	$k_{des}K_dC_WX$

Figure 5.2. Conceptual model for diffusion and sorption of micropollutants into biofilms, including (a) a graphical description of the biofilm as porous medium, with discretization in finite completely mixed layers, and of the consecutive steps required for partitioning onto biofilm solids (processes 1–3, see text); (b) the assumptions considered in the model; and (c) the process matrix describing sorption and desorption kinetics (X=$X_{biomass}$).

5.1. Sorption coefficients at different biofilm thicknesses

As described in section 3.1.1., sorption coefficient in biofilms was calculated as according to Eq. 3.3, accounting for biofilm porosity. Additionally, $K_{d,4h}$ and $K_{d,eq}$ were assessed respectively (i) by considering the last measurement (t=4 h) as dissolved concentration at equilibrium ($C_{L,eq}$ = $C_{L,4h}$) in agreement with previous studies on activated sludge where equilibrium was assumed to be reached within 0.5 h (Göbel et al., 2005; Hörsing et al., 2011; Ternes et al., 2004a); and (ii) with $C_{L,eq}$ as the asymptotic aqueous concentration estimated by fitting measured concentration profiles with a first-order decay equation (see SI **Paper II**).

Sorption was considered significant when a concentration drop ($C_{L,0}$ – $C_{L,4h}$)/$C_{L,0}$ higher than 10% was observed (Hörsing et al., 2011), thus not considering analytical uncertainty.

Out of the 23 targeted substances, sorption was significant only for eight micropollutants, namely atenolol, metoprolol, propranolol, citalopram, venlafaxine, erythromycin, clarithromycin and roxithromycin. These compounds were all positively charged (>90% cationic fraction) compounds

at the experimental pH 7.5. Higher sorption potential of positively charged compounds compared to negatively charged or neutral compounds was previously observed in activated sludge solids (Stevens-Garmon et al., 2011; Polesel et al., 2015) and soil (Franco and Trapp, 2008).
Furthermore, we compared $K_{d,eq}$ and $K_{d,4h}$ (L g^{-1}) for each chemical at different biofilm thicknesses by calculating the relative deviations Δ (%) of the two coefficients (Table 5.1). For most of the compounds, relative deviations for Z50 and Z200 were on average around 10%, with the exception of atenolol (>50%). Conversely, the relative deviations Δ (%) between the two coefficients in Z500 biofilms were higher than 30% (up to 80% for atenolol), suggesting that while the assumption of equilibrium reached within 4 h seems overall reasonable for Z50 and Z200, it may not be valid for Z500. This hypothesis was also investigate with the diffusion-sorption model (5.2).

Table 5.1. Sorption coefficients calculated using the asymptotic equilibrium concentration ($K_{d,eq}$, L g^{-1}) and the last measured aqueous concentration (t=4 h) during batch experiments ($K_{d,4h}$, L g^{-1}) for eight of the 23 spiked chemical compounds. The parameter Δ defines the relative deviation between the two K_d values, indicating as well deviation from partitioning equilibrium.

	Z50			Z200			Z500		
	$K_{d,eq}$ (Lg^{-1})	$K_{d,4h}$ (Lg^{-1})	Δ %	$K_{d,eq}$ (Lg^{-1})	$K_{d,4h}$ (Lg^{-1})	Δ %	$K_{d,eq}$ (Lg^{-1})	$K_{d,4h}$ (Lg^{-1})	Δ %
Atenolol	1.12	0.26	77	1.10	0.68	38	4.72	0.95	80
Metoprolol	0.08	0.08	3	0.19	0.16	15	0.27	0.15	44
Propranolol	0.50	0.54	-9	1.69	1.67	1	1.90	1.92	-1
Clarithromycin	0.42	0.34	20	0.41	0.39	4	10.90	5.63	48
Erythromycin	0.33	0.34	-3	0.19	0.20	-4	10.68	6.13	43
Roxithromycin	~0.0	0.00	/	0.85	1.05	-24	10.80	3.92	64
Citalopram	0.47	0.46	1	0.66	0.61	8	2.46	2.06	16
Venlafaxine	~0.0	0.00	/	0.12	0.09	25	0.14	0.12	16

Among the compound that presented significant sorption, macrolides presented the highest sorption potential (especially in Z500 biofilm). K_d for macrolides was shown to present high variability for soil (Uhrich et al., 2014), with estimated values >8 L g^{-1}, and for humic acids (Sibley and Pedersen, 2008), with estimated values >20 L g^{-1}.

When comparing $K_{d,eq}$ at different biofilm thicknesses, we observed an increase of sorption potential with biofilm thickness, with $K_{d,eq}$ for Z500 from 4-fold higher for most of the compounds to 30-fold higher for the macrolides antibiotics. Biofilm properties such as porosity and density were measured and calculated (Tchobanoglous and Burton, 2003) revealing increasing porosity, ε, with biofilm thickness (76, 91 and 93% for Z50, Z200 and Z500 biofilm, respectively) and decreasing values of biofilm density in wet biofilm, ρ (up to 16 kg m^{-3} for the thinnest biofilm). Although, further investigation is required, porosity could have likely influence the sorption potential in the three biofilms by affecting the available surface area inside the biofilm as previously observed (Wendell O. Khunjar and Love, 2011; Yi and Harper, 2007).

Finally, according to Ternes et al., (2004a), removal by sorption for compounds with K_d < 0.5 L g^{-1} can be a minor removal pathway in the overall mass balance of wastewater treatment. Although, this observation refers to activated sludge with different biomass production and properties than biofilms, our results suggests that by using thinner biofilms (up to 200 µm), sorption can be considered minor for most of the compounds (with the exception of atenolol and propranolol), while relevant for most of the encountered compounds (with exception of metoprolol and venlafaxine) at higher biofilm thickness (500 µm).

5.2. Simulation results and effective diffusivity estimation

Measured and simulated C_L in bulk, along with simulated data of C_L in the biofilm pored liquid of three selected micropollutants are shown in Fig. 5.3. The model predicted reasonably well the removal of the targeted compounds ($R^2 > 0.9$). At equilibrium condition, simulated C_L in bulk and in the biofilm should converge. This condition was satisfied for most of the compounds in Z50 and Z200 within 4h experimental time (as for the case of propranolol, Fig. 5.3a), but not in the case of the thickest biofilm Z500 (e.g., for clarithromycin, Fig 5.3c). Possibly, due to their large molecular volume, for macrolide antibiotics, simulation results suggested a time for partitioning equilibrium of approximately 10 days which is in good agreement with values

(days, months and year) reported in another study (Delle Site, 2001).

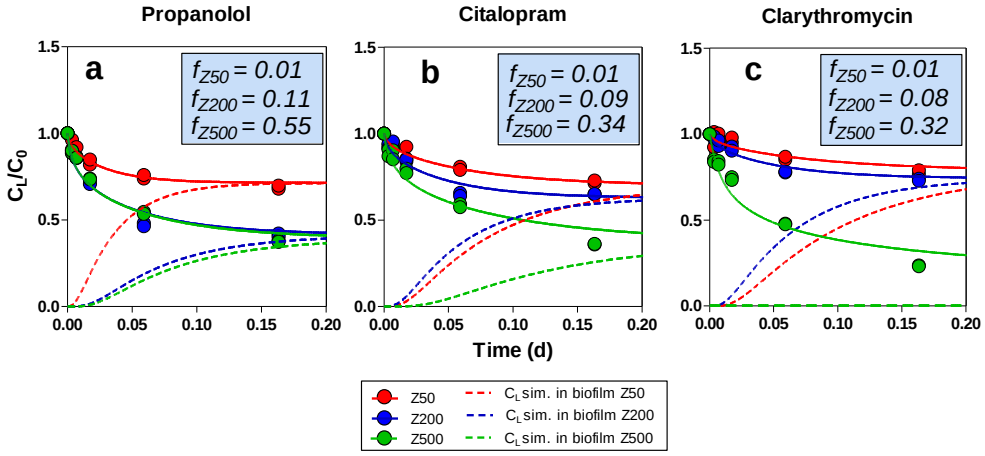


Figure 5.3. Measured (technical replicates, in circles) and simulated (continuous line) aqueous concentrations) C_L in bulk aqueous phase (normalized over initial aqueous concentration $C_{L,0}$ and simulated concentrations in biofilm pores liquid (dashed lines) of six selected chemicals compounds during batch experiments with Z50, Z200 and Z500 biofilms. Simulated C_L in biofilm is the concentration at the bottom layer of the biofilm.

Despite the widely held assumption of sorption equilibrium reached in shorter period of time (i.e. minutes to few hours) in activated sludge (e.g., Hörsing et al., 2011; Pomiès et al., 2013), results from this study suggest that sorption equilibrium in biofilms may not be reached in the short time frame of batch experiment considered previous in literature, e.g., from 4 h (Hörsing et al., 2011) up to 74 h (Hyland et al., 2012). Thus, to reduce uncertainties in sorption experiments, sorption coefficient should be calculated by using the asymptotic aqueous concentration value obtained using e.g., the first-order decay equation. By calibrating the sorption diffusion model, effective diffusivity coefficient f (-) for the targeted compounds and at different biofilm thickness were estimated (Fig. 5.3 for selected micropollutants and in **Paper II**). For most of the compounds, f decreased with biofilm density and thus increased with biofilm thickness and porosity, suggesting a possible diffusion limitation in thinner biofilms due to higher density. Decreasing values of f with increasing biofilm density were previously observed in studies in biofilms for solutes with lower molecular weight (< 100 mol^{-1}) and with high solubility (e.g., O_2, sodium chloride, sodium nitrate) (e.g., Guimerà et al., 2016; Horn and Morgenroth, 2006; Zhang and Bishop, 1994a).

6. Removal of micropollutants in denitrifying MBBR

6.1. Pre-denitrifying MBBR

A number of technologies and reactor design optimization have been investigated for enhancing the biological removal of micropollutants. Among these, staging of biological reactors has been hypothesized to optimize the removal of conventional pollutants in activated sludge (Plósz, 2007; Scuras et al., 2001) and denitrifying biofilm reactors (Plósz et al., 2010b) based on reaction kinetics principles.

By staging pre-denitrifying treatment systems, the biomass is likely exposed to a decreasing gradient of the influent organic carbon loading and composition, from higher concentration of easily organic carbon in the first stage to more recalcitrant substrates in the last stage. Long operation to such conditions can lead to a biomass adaptation to the different fractions of organic carbon, leading to improved utilization kinetics compared to a single/unstaged configuration (Plósz et al., 2010b). However, while previous studies focused on the removal of conventional pollutants, further research is needed to study the influence of staging of biological systems on the removal of micropollutants (focus of **Paper III**), also on composition and biodiversity of the microbial community developed under long-term operation to such conditions (focus of **Paper IV**).

To do so, two laboratory-scale MBBRs (containing K1 carriers), i.e., single stage (U) and three-stages (S=S1+S2+S3) were operated under pre-denitrifying conditions for more than 400 days (Fig. 6.1). The biofilm inoculum was collected from a full-scale post-denitrification MBBR (Sjölunda WWTP, Sweden), where methanol is continuously dosed. During continuous-flow operation, the two systems were operated in parallel under similar conditions and thus (i) by feeding pre-clarified municipal wastewater spiked with excess of nitrate (dosed as KNO_3) to ensure anoxic conditions; (ii) operational conditions (total operating volume of 6 L, hydraulic residence time of 8.9 h, carrier filling ratio of 33%) and (iii) sparging nitrogen gas to ensure mixing of the carriers and minimize DO concentration in the system. During the continuous-flow operation, two batches were performed, at 100 and 471 days of operation, where both primary substrates and micropollutants were measured to assess removal biokinetics. Notably, in this study, no spike

of micropollutants was performed meaning that only micropollutants present in the pre-clarified wastewater were quantified. Furthermore, samples of attached biomass were taken during the continuous operation at 0 (inoculum sample), 42, 59, 74, 88, 218, 300, 434 and 471 days of operation.

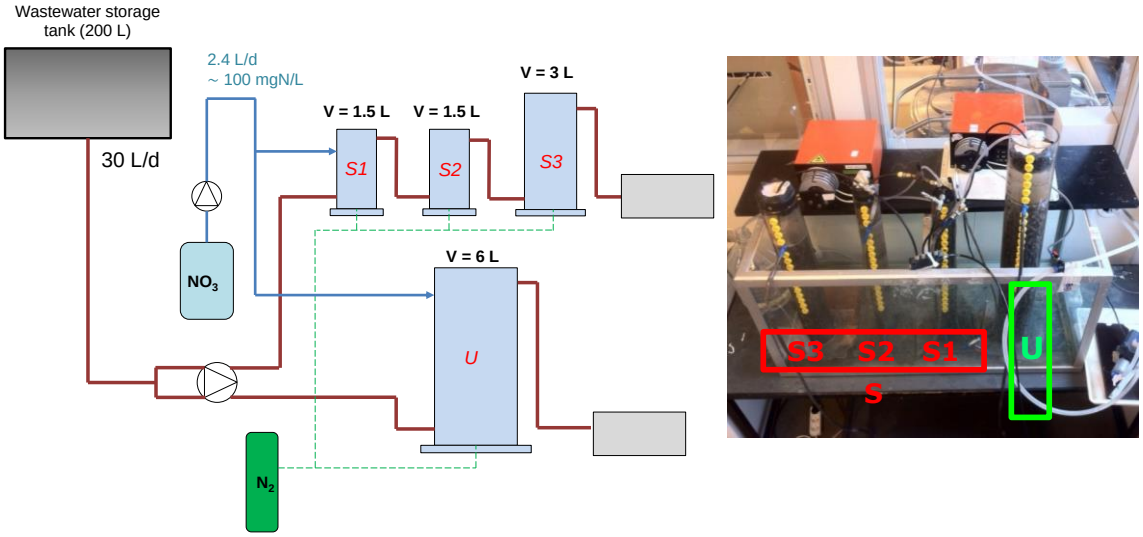


Figure 6.1. Schematic representation (left) and picture (right) of three-stage (S1, S2, S3) and single-stage (U) pre-denitrifying MBBR systems under continuous-flow operation.

6.1.1. Removal of macro- and micropollutants in single and three-staged MBBRs

As a result of the design principles employed to size the 3-stage system, during continuous-flow operation, most (~70% averaged value) of the easily degradable fraction (S_S) of the soluble COD was removed in the first stage of staged systems. This resulted in growth limiting S_S concentrations occurring in S2 and S3 also driven by hydrolysis products derived from the slowly (particulate) biodegradable fraction and biomass decay products (Fig. 1 in **Paper IV**). Batch experimental data of batch 2 suggested a significant different denitrification potential and kinetics among the four MBBRs, where the maximum and the minimum mean specific denitrification rate $\bar{k}_{NOX}$ (mgN gTSS^{-1} d^{-1}, Eq. 2 in **Paper III**) was found in S1 (48.2 mgN gTSS) and S3 (12.4 mgN gTSS^{-1}), respectively. Interestingly, initial lag phases in NO_X reduction were observed during batch experiment in S2, U (1.5 h) and S3 (3 h) (Fig. 2 in **Paper III**), possibly due to carbon storage as a consequence of the prior low exposure of biofilm to readily biodegradable carbon (Beun et al., 2000).

Removal of micropollutants in aqueous phase exhibited patterns described in 3.1, such as biotransformation and retransformation of human metabolites to parent compound (3.1.3) that occurred e.g., for sulfamethoxazole (SMX), sulfadiazine (SDZ), diclofenac (DCF) and metoprolol (MET) during batch 1 (Fig 6.2) due to the possible presence of their human metabolites in the preclarified wastewater. For the case of SDZ, the metabolite acetyl-sulfadiazine was measured. No cometabolic processes were observed in either of the batch experiments. For the case of atenolol (ATN), a tentative approach of enantioselective biotransformation was performed to estimate $k_{bio,1}$ and $k_{bio,2}$ of the two form of enantiomers of atenolol typically present in wastewater (Kasprzyk-Hordern and Baker, 2011). In batch 1, the highest values of k_{Bio} were estimated for S1 and U. Importantly, overall transformation kinetics of U MBBR overall decreased in batch 2, and highest transformation rate constants of all non-recalcitrant micropollutants (k_{Bio}, k_{Dec} > 0.1 L $gTSS^{-1}$ d^{-1}) were found in S1 (except for ATN). When comparing staged MBBRs, the order of k_{Bio} was found to be S1>S2>S3, in agreement with the decreasing concentration of COD available during continuous-flow operation.

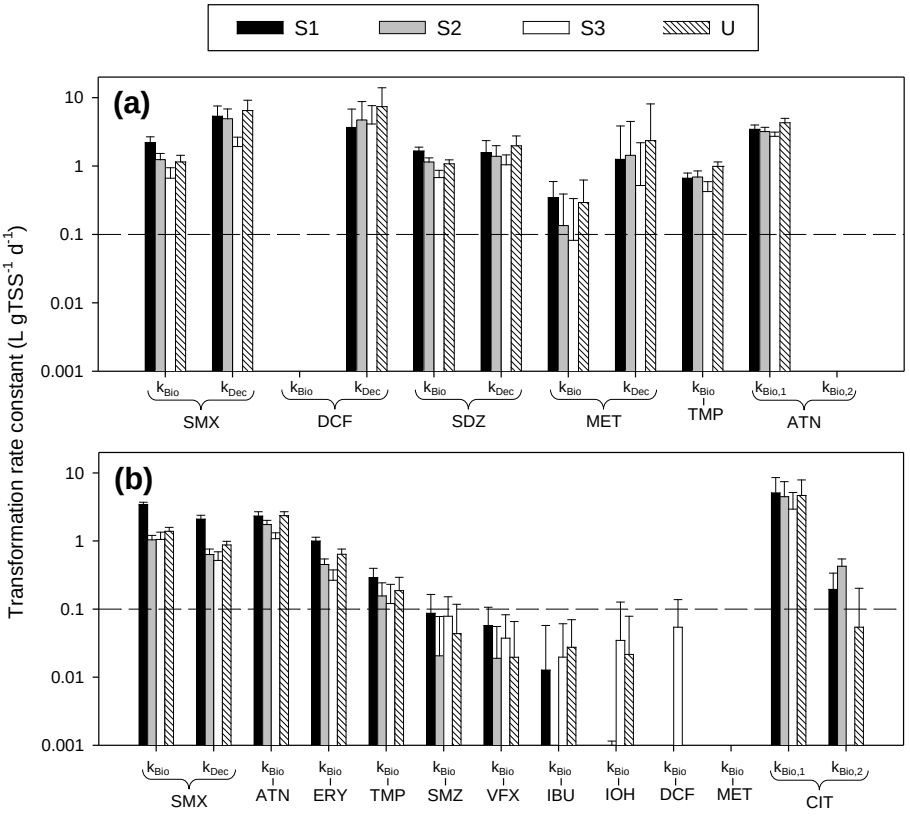


Figure 6.2. Estimated transformation rate constants for pharmaceuticals in batch experiment 1 (a) and batch experiment 2 (b).

During continuous-flow operation, S configuration exhibited a slight improvement of denitrification (up to 20%) compared to single-stage configuration, but not significant differences in the removal of micropollutants (Fig. 1 and S6 in **Paper IV**).

6.1.2. The influence of staging on microbial diversity and composition

The microbial community structure in the pre-denitrifying MBBR biofilms was investigated by 16S rRNA amplicon sequencing at different sequencing similarity i.e., 93%, 95%, 97% and 99%, to maximize the resolution of the alpha-diversity analysis between the four reactors. Alpha- and beta diversity data were obtained in each stage of the S system as well as for the total community in S (by combining OTU libraries of S1, S2 and S3 reactors) to investigate whether staged-configuration can lead to a total higher biodiversity compared to a single stage configuration.

Shannon, richness and evenness indices highly varied over long-time operation with similar trend in S and U configurations (Fig. 2 in **Paper IV**). These similar observed trends were linked to influent organic carbon in the wastewater, observed by positive ($p<0.05$) linear relationship between microbial richness (at 99% similarity) and soluble COD (R^2 of 0.9 and 0.8 in U and S1, respectively).
Moving window analyses (MWA) was implemented using reciprocal of Bray-Curtis indices to assess the time needed for the two microbial communities to reach a stable microbial composition that was dissimilar from the initial biofilm inoculum. MWA revealed that after 200 days of operation the two systems reached a stable microbial composition until the end of the experiment (Fig. 3 in **Paper IV**). Thus, biodiversity indices collected in 4 sampling days after 200 days of operation were averaged to assess statistical difference between the two systems and for each stage (Fig. 6.3). For all four tested sequencing similarities, no significant difference in terms of Shannon diversity and evenness was observed in the biofilms in S and U MBBR. On the other hand, microbial richness (ACE and Chao) was higher ($p<0.05$) in S compared to U at 99% sequence similarity (Fig. 4 D). Additionally, although not significant, Shannon diversity, evenness and richness were found higher in S3 compared to S1 and S2 at 99% of sequences similarity. During continuous operation most of the easily degradable carbon was consumed in S1 (Fig. 1 in **Paper IV**), likely favouring microbial groups that dominate the microbial

community due to their higher metabolic activity. Conversely, the more refractory and slowly degradable carbon available for S2 and in S3 possibly led to the co-existence of a more diverse and even microbial community. This hypothesis is in agreement with a study in full-scale managed aquifer recharge systems (MAR), in which higher community diversity was observed in the more oligotrophic depths of MAR compared to the surface levels where more easily degradable carbon was available (Li et al., 2013, 2012).

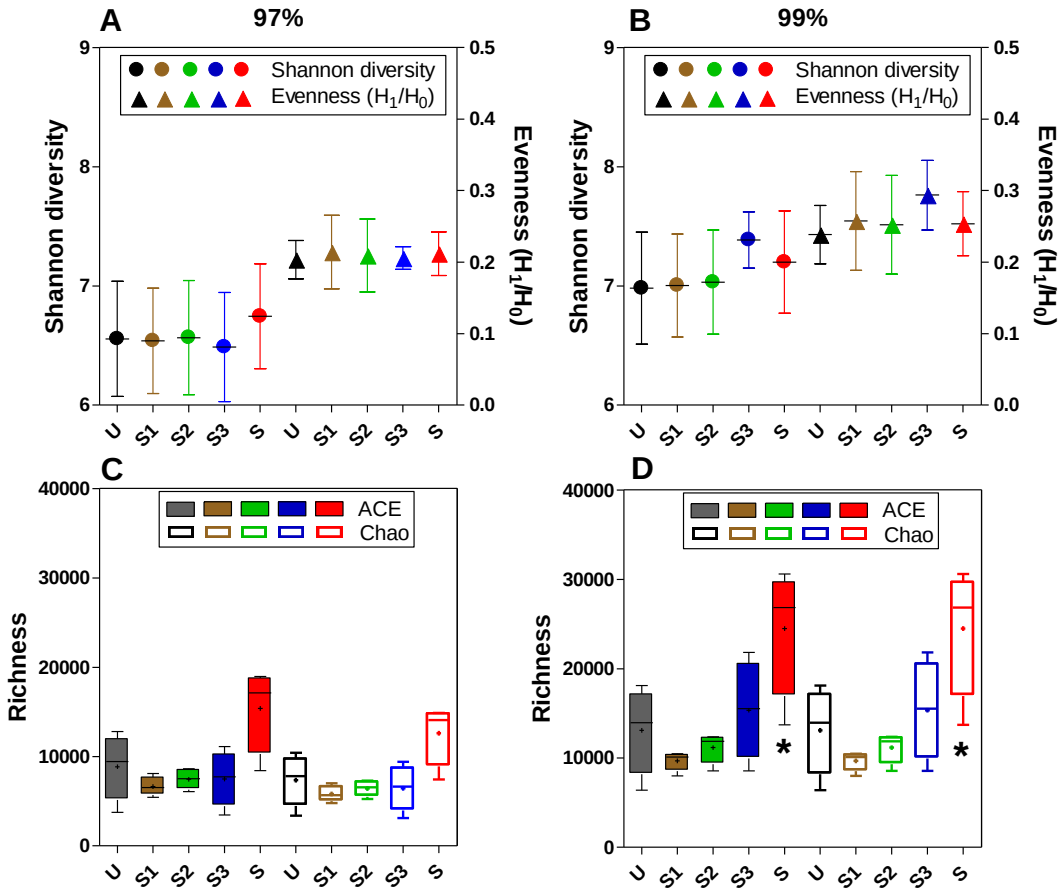


Figure 6.3. Averaged values of Shannon diversity, evenness, ACE and Chao indices after 200 days of operation (n=4) at 97% (left) and 99% (right) sequence similarity for the three stages (S1, S2, S3), the staged system as a total (S) and the un-staged system (U). Asterisks in Fig. d indicates significance difference. Mean is shown as +.

The composition and the time-dependent variation of the microbial structure in the two systems was also studied by creating heatmaps of the 100 most abundant OTUs at order level sorted by the most abundant OTUs after 200 days of operation (200, 300, 434, 471 days) (Fig. 6.4). Here, we reported heatmaps of (i) the ratio of the log sequences abundance of S over U and (ii)

of S3 over S1. Notably, compared to the single stage, S selected for the OTU *Candidate division WS6* and *Deinococcales* after day 200 and in specific in S3 (Fig. 6.4b), suggesting a correlation of these OTUs with low readily biodegradable carbon availability. *Acidobacteria subgroup 4* was selected in U as well as in S3 operated to the lower carbon concentration compared to S1 during continuous operation. This taxa has been previously observed to negatively associate with organic carbon availability and C-to-N ratio in grassland soils (Naether et al., 2012; Will et al., 2010).

Taken together, staging of MBBR and the consequent gradient in carbon available selected for a different microbial community, generally with higher richness compared to a single stage configuration.

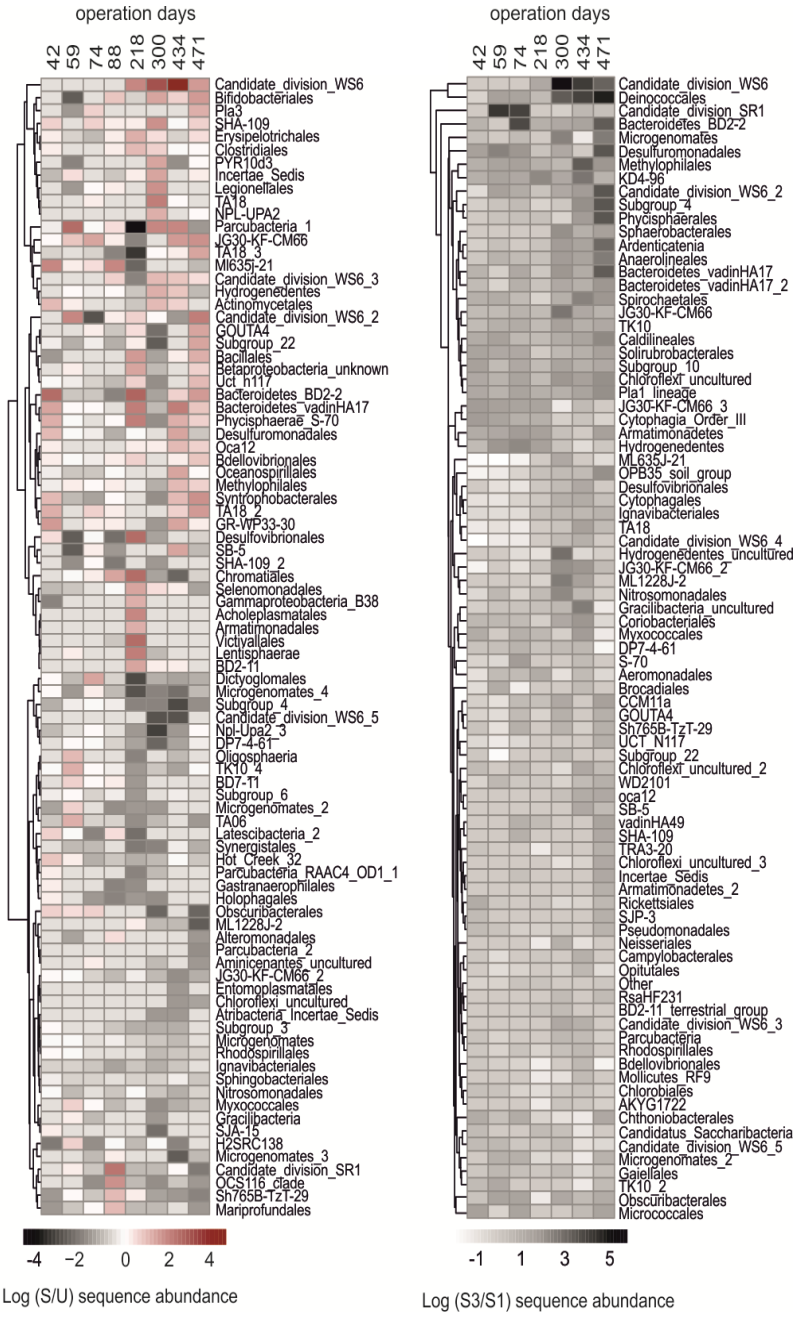


Figure 6.4. Heatmaps of the 100 most abundant order level taxa for the ratio of log sequence abundance of S/U (left) and of S3/S1 (right). On the left side, in red the taxa mostly enriched in S, and on the right side, in black the taxa mostly enriched in S3.

6.2. Post-denitrifying MBBR

As mentioned in section 2.1, in post-denitrifying systems denitrification is enhanced by the addition of an external carbon source. While external carbon source has been widely studied to understand the impact on denitrification performance (Mokhayeri et al., 2009; Peng et al., 2007), no evidence currently exists regarding the fate of micropollutants in post-denitrification systems and the influence of external carbon dosing on their removal. This is of particular interest due to the importance of primary substrate on the removal of micropollutants (3.2.2). In **Paper V**, we evaluated the effect of dosing two different external carbon sources commonly used in post-denitrification, methanol (MeOH) and ethanol (EtOH) on the removal of the 23 targeted micropollutants. Additionally, we evaluated the influence of the carbon availability (of both MeOH and EtOH) by variation of one of the key parameters in designing post denitrifying system, i.e., $COD_{added}/NO_3\text{-}N_{influent}$ ratio (2.1) during continuous-flow experiment.

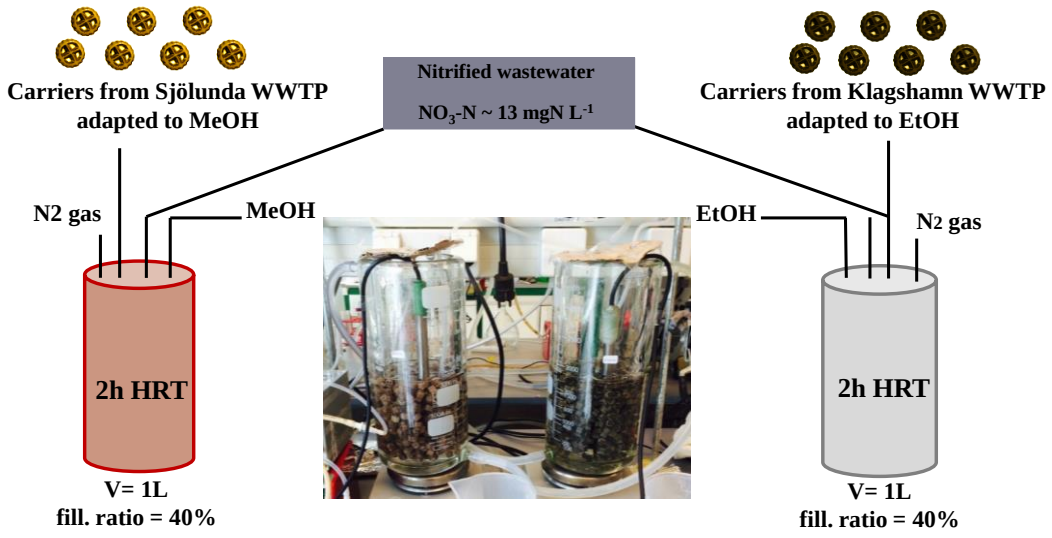


Figure 6.5. Schematic representation and picture of the post-denitrifying MBBR systems dosed with methanol and ethanol used in Paper V.

Two laboratory-scale MBBRs, containing AnoxKaldnes K1 carriers were operated in continuous-flow using wastewater dosed with methanol and ethanol, respectively (Fig. 6.5). Biofilm inoculum on carriers was taken from post-denitrifying full-scale MBBR where currently MeOH and EtOH dosage occur. Wastewater effluent from nitrifying tricking filter was used as feed to

both systems, where only native concentration of nitrate (~ 12 mgN L^{-1}) was present. The two MBBR systems were thus operated with identical operational conditions (T= 15 °C, nitrogen sparging, HRT, and filling ratio of carries) during continuous-flow operation.

6.2.1. Influence of dosed carbon source on removal of micropollutants

Batch experiments were performed separately for the two systems to assess biotransformation kinetics of micropollutants with excess of carbon (MeOH and EtOH, respectively) and nitrate to ensure anoxic conditions. During batch experiments, the EtOH-dosed MBBR presented higher denitrification rate surface normalized (r_{NOx-N}, gN m^{-2} d^{-1}) and comparable denitrification rate biomass normalized (k_{NOx-N}, gN g^{-1} d^{-1}) to the MeOH-dosed MBBR.

Most of the micropollutants were biotransformed following biotransformation kinetics (Eq. 3.4). However, the removal of carbamazepine, venlafaxine, sulfamethoxazole and sulfamethizole was better predicted (R^2>0.9) with the cometabolic biotransformation kinetics (Eq. 3.5) in both MBBRs. This suggested that the removal of the abovementioned micropollutants was enhanced in the presence of organic carbon at the beginning of the experiment, followed by lower biotransformation kinetics when the organic carbon was depleted (approximately after 3 h), Fig. 6.6.

Biotransformation kinetics were estimated for both systems and compared in Fig. 6.7. For 9 compounds the estimated values of k_{Bio} and q_{Bio} (L g^{-1} d^{-1}) for the MeOH-dosed MBBR were higher (1.5-2.5fold) than those for the EtOH-dosed reactor (namely atenolol, citalopram, trimethoprim, ibuprofen, iopromide, metoprolol, iohexol, iomeprol, sotalol, venlafaxine). Conversely, the sulfonamides acetyl-sulfadiazine, sulfamethoxazole, sulfamethizole and sulfadiazine were transformed at higher rate constants (up to 2.8-fold) in the EtOH-dosed reactor. The remaining targeted compounds (n=10) exhibited similar biotransformation in the two MBBRs.

For the compounds classified as easily degradable (q_{bio} and k_{bio} > 2), the highest biotransformation kinetics possibly derived by from the normalization to a higher amount of biomass of the EtOH-dosed reactor. On the other hand, several moderately degradable chemicals (0.2≤ k_{bio} and q_{bio} ≥2) presented biotransformation kinetics approximately 2 times higher in MeOH-dosed MBBR compared to the EtOH-dosed MBBR, suggesting that biotransformation of these chemicals was likely affected by two different microbial

communities structures. 16S rRNA amplicon sequences data revealed higher microbial richness in the MeOH-dosed MBBR compared to EtOH-dosed MBBR (ACE of 999 ± 103 and 781 ± 87 OTUs respectively).

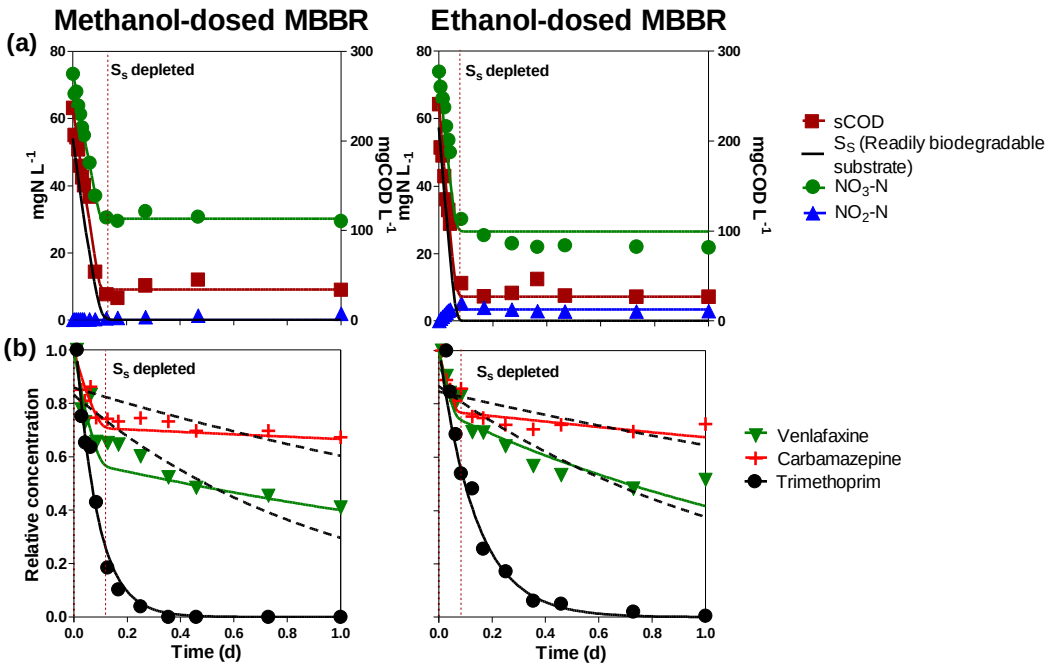


Figure 6.6. Concentration of measured soluble COD (sCOD), nitrate, nitrite and simulated readily biodegradable COD (S_S), a; measured aqueous concentration of selected micropollutants (symbols) and simulated concentration assuming biotransformation (dotted line for venlafaxine carbamazepine, and trimethoprim) and cometabolism (dashed lines), b.

The results of the continuous-flow experiment performed at different carbon loading of methanol and ethanol (Fig. S8 in **Paper V**) in the two MBBR systems, respectively, showed that the removal efficiency of micropollutants did not present any correlation with the tested $COD_{added}/NO_{3\text{-}Ninfluent}$ ratios and did not significantly differ between the two types of carbon sources (with exception of trimethoprim). The short HRT used in the study (2 h, typically operated in denitrification stages in full scale WWTPs) might have been too short to observe differences in the removal of micropollutants. Accordingly, the increase of HRT has been found to enhance the removal of a number of micropollutants in activated sludge (Maurer et al., 2007; Petrie et al., 2014) and MBBR (Mazioti et al., 2015).

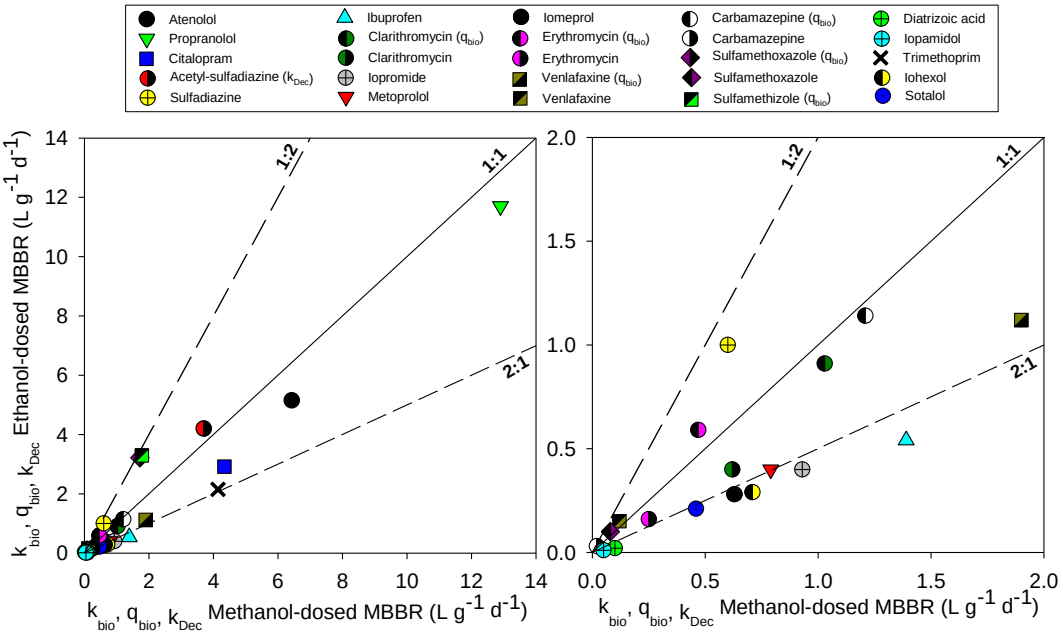


Figure 3. Estimated biotransformation rate (k_{Bio}), cometabolic biotransformation rate (q_{Bio}), retransformation rate (k_{Dec}) during batch experiments in the methanol- and ethanol-dosed MBBRs.

7. Elucidating the impact of microbial structure on micropollutant biotransformation in MBBR

As discussed in 3.2.4, more and more evidences suggest a positive relationship between biodiversity and general ecosystem functionalities (Cardinale et al., 2006; Emmett Duffy, 2009) and even in specific functionality such as micropollutant biotransformations in WWTP (Helbling et al., 2015a, 2010b, Johnson et al., 2015a, 2015b; Stadler and Love, 2016).
By understanding these possible associations, opportunities for enhancing biotransformation of micropollutants during biological treatment could be exploited. However, while the abovementioned studies focus exclusively on activated sludge suspended biomass and aerobic systems, no evidence is currently available for this relationship in biofilms and anoxic systems.

In this PhD thesis, the possible relationships between taxonomic diversity (assessed by amplicon sequencing of the 16S rRNA gene) and micropollutant biotransformation rates were investigated in different MBBR systems. Microbial diversity was experimentally manipulated by varying operational conditions in biofilm systems, such as biofilm thickness (**Paper I**) and carbon source availability (**Paper IV**). We recognize that stronger associations may be captured by looking at rRNA gene transcript instead of rRNA genes, as the transcript pool can better reflect the active microbial community (Hallin et al., 2009), yet such was not feasible within the project constraints. Further discussion on the latter is reported in Chapter 10.

Results from Pearson´s correlation analysis (1.1.5) in the nitrifying MBBR system (**Paper I**) between k_{Bio} (Fig.4.3) with biodiversity indices, nitrification rate and *amoA* abundance suggested that 14 over 22 of the estimated micropollutant k_{Bio} were positively associated with Shannon taxonomic diversity and evenness indices ($r > 0.8$) (Fig.7.1). To support this statement, nonparametric rank correlations (section 1.1.5) and permutation tests were performed, predicting true associations between biotransformation and microbial diversity for at least 8 compounds out of 14. On the contrary, also negative association ($r > -0.9$) between micropollutant k_{Bio} and diversity were observed for a specific group of targeted compounds, i.e., the sulfonamides and diclofenac. For these micropollutants a positive relation with nitrification ($r > 0.9$) and *amoA* abundance at lower extent ($r > 0.7$) was observed. This suggests

that the biotransformation of the latter compounds correlated to ammonium removal, however not necessarily catalysed by the ammonia monooxygenase but maybe by other enzymes involved in nitrification, as suggested elsewhere (Helbling et al., 2012).

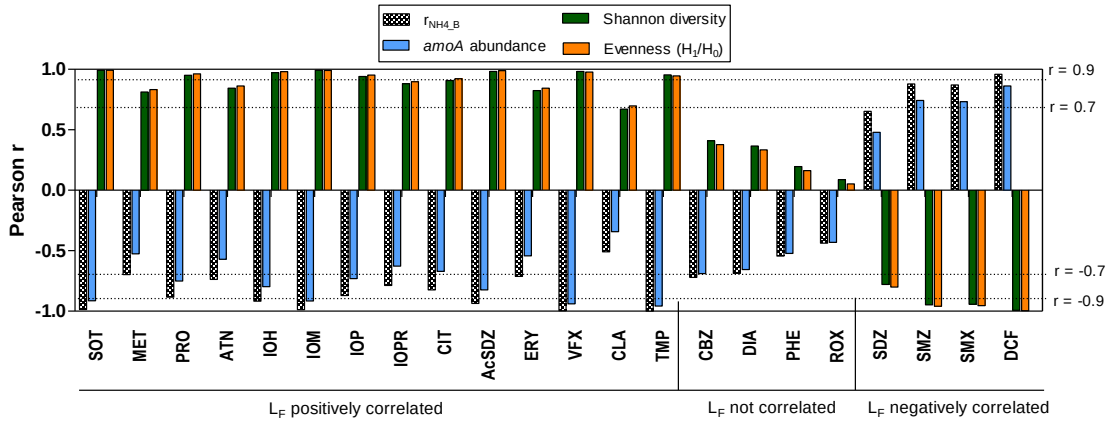


Figure 7.1. Correlation between estimated biotransformation rate constants k_{bio} with nitrification rate r_{NH4_B}, *amoA* abundance, Shannon diversity, and evenness indices. L_F indicates biofilm thickness.

In the pre-denitrifying MBBR study (**Paper IV**), correlations were investigate between biodiversity, k_{Bio} and k_{Dec} of specific compounds, as well as for collective k_{Bio} and k_{Dec} of all targeted micropollutants, which were calculated by averaging the scaled biotransformation rates (Johnson et al., 2015a; Zavaleta et al., 2010). As discussed in 6.1.2, alpha-diversity of the biofilms in the staged and un-staged MBBR configuration varied over the long-time operation, reaching a more stable microbial community after 200 days of operation when batch 2 was performed (471 day of operation).

Pearson´s analysis results showed that k_{Bio} and k_{Dec} estimated in batch 2 did not correlate significantly with microbial richness for the targeted compounds but positive significant correlations with denitrification rates were observed for several micropollutants (Fig. 7.2). Positively correlations ($p<0.05$) between k_{Bio} (for ERY and TMP) and collective k_{Bio}, with abundance of genes *narG*, *nirS* (but not nirK), and atypical *nosZ*, all genes coding for enzymes in the denitrification respiratory chain, supporting the hypothesis that the biotransformation of these targeted micropollutants is likely related to denitrification.

As described in sections 3.2.4, a positive relationship between diversity (or richness) would occur if the microbial community consists of a number of strains which exploit unique niches (Cardinale, 2011).

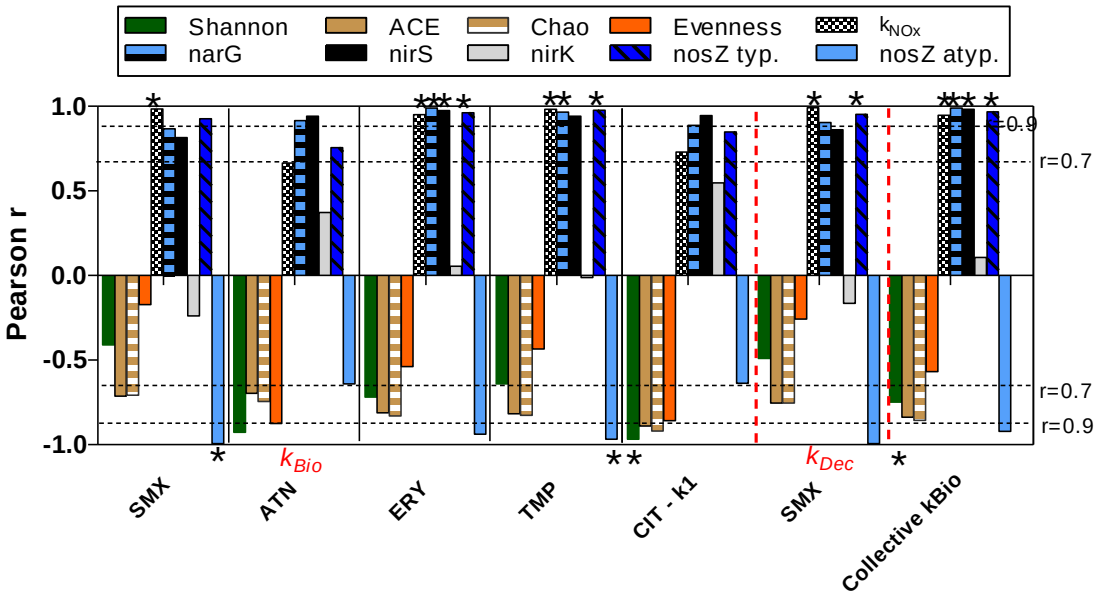


Figure 7.2. Correlation between estimated biotransformation and retransformation rate constants k_{Bio} and k_{Dec} (L g^{-1} d^{-1}) with rate specific denitrification rate (k_{NOx}), denitrifying genes abundance (cells $ngDNA^{-1}$), Shannon diversity, evenness and richness (ACE and Chao) indices. Asterisks indicate significant differences ($p<0.05$).

However, functional redundancy, the notion that many different taxa exist to perform the same function, may challenge this positive association. In fact, if a process (i.e., the biotransformation of a specific compound) is performed by a large number of different taxa (thus defined as a broad process), the increase in diversity (e.g., richness) would not necessary positively impact the processes as it is not limited by the number of taxa which can perform it (Helbling et al., 2015b). Additionally, a negative association may derive whenever the increase of biodiversity is at the expenses of the taxa from the redundant population. Conversely, if relatively few taxa are able to perform the biotransformation of that specific micropollutant (narrow processes) differences in abundance of those taxa and total biodiversity may positively impact the biotransformation, due to the fact that communities with more taxa are likely to have more functional traits (Johnson et al., 2015b).

Denitrification is a process widespread across microorganisms (functional redundant process). The low or negative correlation between biotransformation rates and biodiversity, combined with the positive correlation with primary metabolism observed in this study, suggests that the targeted com-

pounds could be possibly catalysed by broader processes rather than narrow processes under denitrifying conditions.

Given (i) the wide range of chemical structure of the targeted micropollutants and (ii) the lack of knowledge of their transformation pathway (and thus transformation products), it may be difficult to conclude if an increase of biodiversity can positively enhance the removal of micropollutants in biological wastewater treatment. Due to the unexpected absence of correlation between biotransformation of micropollutants and microbial diversity under denitrifying condition, it is likely that under highly redundant processes (such as denitrification), the biotransformation is catalysed by broad metabolic pathway and biodiversity may not have an effect on the removal of a broad range of micropollutant. On the contrary, under nitrifying conditions it is more likely that hydroxylation (the main removal pathway under nitrification, 3.2.1) may not be carried out by most taxa and not for all micropollutants, and maximizing biodiversity and thus increasing the likelihood of enriching the microbial community of the taxa performing specific functionalities, may positively impact the biotransformation of micropollutants.

8. Engineering significance and application

8.1. Comparison between MBBR configurations and conventional activated sludge

Recent studies have claimed the high potential of biofilm systems such as MBBR in enhancing the removal of several micropollutants (Escolà Casas et al., 2015; Falås et al., 2012; Hapeshi et al., 2013; Mazioti et al., 2015) compared to activated sludge. In Fig.8.1, biotransformation kinetics of the targeted micropollutants of the three studies—MBBR1 (denitrifying system), MBBR2 (nitrifying system) and MBBR3 (post-denitrifying denitrifying), Table 1.2 —were compared with the range of biotransformation kinetics reported in the relevant studies in activated sludge and MBBR. For each study, bar graphs were made with the range of values of k_{Bio} obtained for each micropollutant under the conditions tested (e.g., of the different biofilm thicknesses in MBBR1 study or the 3 stages of S in MBBR2 study). For a number of compounds, the comparison is reported only between MBBR2 and MBBR3 study, as only micropollutants naturally found in pre-clarified wastewater were studied in MBBR1 (asterisks in Fig. 8.1).

A general trend of enhanced biotransformation rate constant k_{Bio} in MBBR3 was observed for a major number of micropollutants (~60%). One of the possible reasons for this observation can be found in the carbon source used in MBBR3 study, consisting of high readily degradable carbon sources (ethanol and methanol) generally added to boost denitrification in post-denitrifying systems. Conversely, in MBBR1 only carbon present in influent pre-clarified wastewater, generally with less amount of biodegradability and with a broader spectrum of carbon, was used for micropollutant biotransformation. For the nitrifying MBBR2, the organic carbon content was very low with ammonium as main primary substrate for biofilm growth. Taken together, this finding suggest that the addition of an easily carbon source (such as methanol or ethanol) could be beneficial the removal of a number of micropollutants, possibly due the cometabolic effect of the primary substrate on their removal.

Accordingly in MBBR3 study, the effect of cometabolic biotransformation in the presence of primary substrate was observed in removal of compounds such as sulfamethoxazole, sulfamethizole, carbamazepine and venlafaxine during batch experiments (6.2). Notably carbamazepine, a compound generally considered recalcitrant in wastewater (Clara et al., 2004), presented enhanced cometabolic biotransformation rate in MBBR3 in the presence of the primary substrate during batch experiments. This would indicate that low re-

moval of this and other compounds in WWTP may be attributed to the lack of primary substrate for cometabolic biotransformation. However, the additional costs given by the dosing of external carbon sources in post-denitrifying systems should be evaluated with respects to other biological treatment processes presenting comparable efficiency (i.e., nitrification).

When comparing k_{Bio} estimated in the three MBBRs with values from relevant literature for conventional activated sludge, an improvement of several (but not all) targeted micropollutants was shown, i.e., for atenolol, carbamazepine, citalopram, clarithromycin, diclofenac, propranolol, sulfamethoxazole, trimethoprim and venlafaxine. Notably, the removal of diclofenac, one of the compounds of the watch list in the Directive 2013/39/EU, have been reported to be enhanced in previous studies in aerobic MBBR (Falås et al., 2013; Zupanc et al., 2013), suggesting that aerobic biofilm systems could be a key treatment process for the removal of this specific compound. Conversely, compounds such as X-ray contrast media presented significant lower k_{Bio} compared to aerobic activated sludge (Joss et al., 2006).

8.2. Biotransformation of micropollutants based on use-category

In this paragraph, the pharmaceuticals targeted in this research were grouped in six categories according to their use to provide possible suggestions for optimization of existing WWTP for the removal of the targeted compounds. Notably, compounds exhibiting k_{Bio} lower than 0.1 L g^{-1} d^{-1} were considered recalcitrant (<25% in WWTP, Falås et al., (2016)).

Beta-blockers. Although, compounds of this category (atenolol, metoprolol, propranolol and sotalol) present a similar chemical structure (Table S1 and S2 in **Paper II**), atenolol and propranolol resulted in higher removal compared to the other beta-blockers, in agreement with previous studies (Escolà Casas et al., 2015; Maurer et al., 2007). Their removal was also observed under both aerobic and anoxic conditions. Atenolol have been previously reported to be removed under aerobic (Stadler et al., 2015) and anoxic conditions (Carucci et al. 2006, Stadler et al., 2015). Notably, the removal of propranolol was significantly enhanced in MBBR3 (> 11 L g^{-1} d^{-1}) under anoxic conditions, and no evidence was previously reported under this condition. Furthermore (with exception of sotalol), beta-blockers presented relatively high sorption in nitrifying biofilms. Hence, it is optimal to take into account

for the sorption coefficient K_d when estimating biokinetics parameters for this group of compounds.

Iodinated X-ray Contrast Media. Diatrizoic acid, iohexol iomeprol, iopamidol and iopromide are usually detected in high concentration in WWTP (Margot et al., 2015), thus generally considered persistent (Hapeshi et al., 2013). In specific, diatrizoic acid and iopamidol are more persistent as previously reported (Joss et al., 2006). For this group of compounds, differences biotransformation between aerobic and anoxic conditions were not observed, concluding that possibly other treatment technologies than biological treatment may facilitate their removal.

Sulfonamides. The targeted sulfonamides (sulfamethoxazole, sulfamethizole, sulfadiazine and the human metabolite acetyl-sulfadiazine) were removed in all the three MBBRs in both anoxic and anaerobic conditions. Few interestingly findings were observed for the sulfonamides: (i) under both anoxic conditions (in MBBR1 and 3), their removal seemed to be related to the cometabolic activity of the biofilm, being enhanced by the higher metabolism (denitrification) in the first staged of the staged pre-anoxic MBBR and by the presence of readily carbon source in the post-denitrifying MBBR. Accordingly, cometabolic biotransformation kinetics were used to predict their removal in MBBR3 for sulfamethizole and sulfamethoxazole; (ii) furthermore in MBBR3, sulfonamides were the only targeted compounds removed at higher extent by using ethanol instead of methanol as carbon source. Ethanol present a higher growth yield compared to methanol, which also resulted to higher denitrification rates during batch experiments; (iii) under nitrifying conditions, the biotransformation of the sulfonamides (with exception of acetyl-sulfadiazine) was positively correlated with nitrification, being higher in the thinnest biofilm presenting the faster metabolism. Taken together, the three studies suggest a relation between the removal of this group of compounds with a fast metabolism, possibly due to cometabolic biotransformation. Finally, significant retransformation processes of human metabolites present in the wastewater were observed in batch experiments, with k_{Dec} comparable to estimated k_{Bio}, indicating a possible increase in concentration of sulfonamides at full-scale WWTP.

Macrolides and other antibiotics. In this work, biotransformation of the targeted macrolides (erythromycin, clarithromycin and roxithromycin) was not enhanced compared to previous studies on activated sludge or MBBR (Fig. 8.1). Thus these compounds were confirmed to be generally moderately recalcitrant (Joss et al., 2006) under both aerobic and anoxic conditions. On the

contrary, biotransformation of trimethoprim instead occurred in all the three studies, although enhanced with addition of external readily carbon sources.

Antidepressants/antiepileptic. The biotransformation of citalopram, carbamazepine and venlafaxine was significantly enhanced by using MBBR compared to conventional activated sludge (Fig.8.1). Additionally, higher biotransformation was also observed under post-anoxic MBBR than aerobic conditions, suggesting that the addition of an easily carbon source could enhance their removal. Thus, post-denitrifying MBBR systems may be highly beneficial for the polishing of this group of compounds.

Analgesics. Ibuprofen is generally considered an easily degradable micropollutant under aerobic conditions, with k_{Bio} higher than 10 g L^{-1} d^{-1}. Accordingly, in the nitrifying MBBR study, ibuprofen was removed in the first 15 minutes of the batch experiment, not allowing estimation of k_{Bio} (reported in Fig. 8.1, higher significantly than the one under anoxic conditions). However, ibuprofen removal have been mainly associated to aerobic heterotrophic activity (Falås et al., 2012; Tran et al., 2009), rather than autotropic activity. Lower biotransformation was reported under anoxic conditions, although enhanced by the addition of the external carbon source in MBBR3. Phenazone was persistent under both aerobic and anaerobic conditions. As previously discussed, diclofenac biotransformation was highly enhanced in the nitrifying MBBR2 in this study, compared to activated sludge. The positive correlation between diclofenac and nitrifying activity found in MBBR2 was also previously observed in activated sludge fed with synthetic wastewater (Suarez et al., 2010) and in nitrifying culture (Tran et al., 2009). However, the relative contribution between aerobic autotrophic and heterotrophic activity is still under discussion (Falås et al., 2016). Nonetheless, diclofenac was not biotransformed in both anoxic MBBR in this study, supporting the hypothesis that its removal is mainly occurring under aerobic conditions (Suarez et al., 2010). As diclofenac is one of the compounds of the European watching list (1.1), optimization of existing aerobic biological treatment stages or addition of polishing aerobic technologies are thus suggested to comply with the directive. At this regards, MBBR carriers have been observed in this study and in previous studies to enhance the removal of diclofenac compered to activated sludge (Falås et al., 2013; Zupanc et al., 2013), possibly due to the development of more diverse nitrifying community on the MBBR carriers. Retrofitting of existing activated sludge systems by addition of carriers can be thus considered for the removal of this compound.

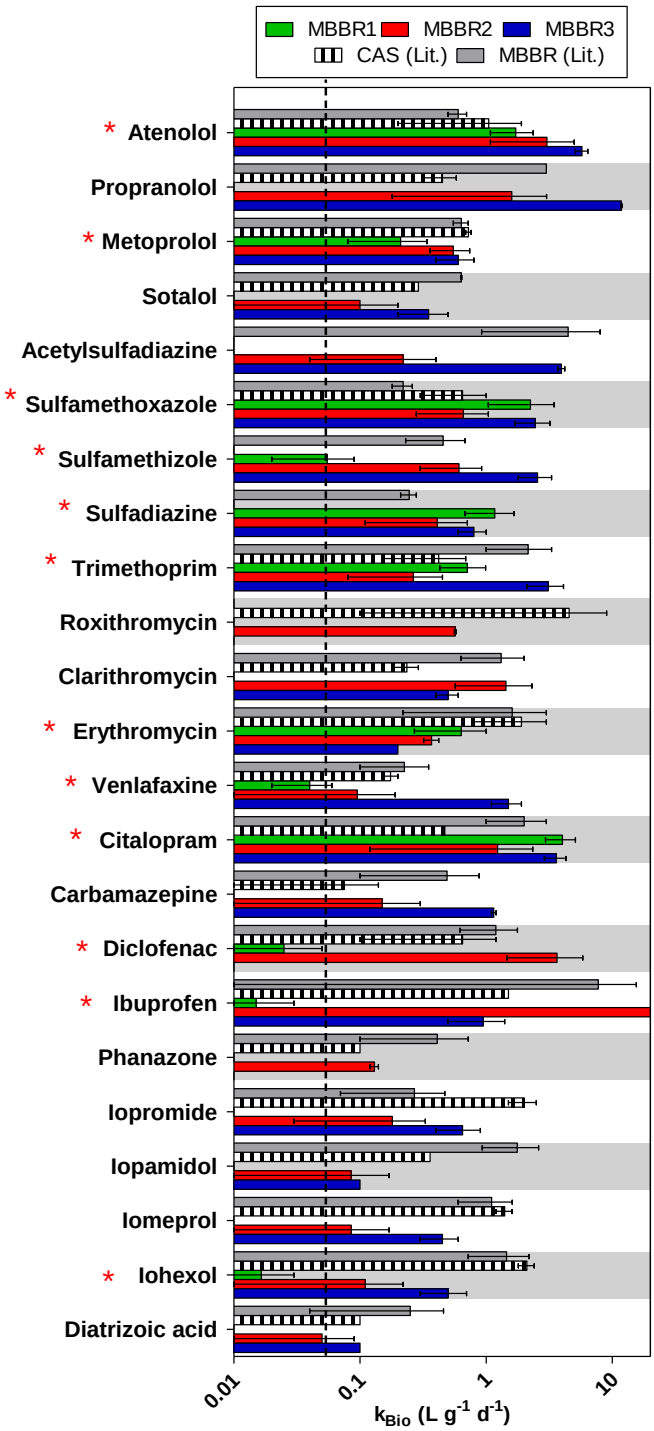


Figure 8.1. k_{Bio} estimated in pre-denitrifying (MBBR1), nitrifying (MBBR2) and post-denitrifying (MBBR3). Literature values of k_{Bio} found for conventional activated sludge (CAS) (Fernandez-Fontaina et al., 2014, 2012; Joss et al., 2006; Maurer et al., 2007; Plósz et al., 2012; Sathyamoorthy et al., 2013; Suarez et al., 2010; Suárez et al., 2012, 2005; Tran et al., 2009; Vieno and Sillanpää, 2014; Wick et al., 2009) and MBBR (Escolà Casas et al., 2015; Falås et al., 2013, 2012). Bar graphs were made with minimum and maximum values of k_{Bio} reported in the 3 studies and in literature. Asterisk indicates compounds found in pre-clarified wastewater in MBBR1 study.

9. Conclusions

The work presented in this thesis aimed at elucidating the potential of biofilm systems, specifically MBBRs, as biological treatment technology for the removal of micropollutants in municipal WWTP. By means of targeted long- and short-term experiments performed in laboratory-scale reactors, (i) the fate of 23 selected micropollutants and (ii) the effect of different operational strategies on the microbial community and on the removal of these micropollutants in MBBRs were investigated. The major findings of this research are summarized below.

I. Operational strategies of MBBR for removal of micropollutants

(i) *Influence of biofilm thickness on nitrification and biotransformation of selected micropollutants in nitrifying MBBR* (**Paper I**).

The use of thinner (~ 50 µm) rather than thicker biofilms (>200 µm) was shown to have a positive effect on nitrification in MBBR. Additionally, thinner biofilms exhibited higher biotransformation of compounds such as diclofenac and the three targeted sulfonamide antibiotics. However, such biofilms were also incapable of biotransform micropollutants such as X-ray contrast media, while the increase of biofilm thickness was beneficial for more than 60% of targeted compounds. Accordingly, biofilm technologies based on thicker biofilms can likely improve the biotransformation of a broader range of micropollutants, however resulting in lower specific and surface normalized nitrifications rates.

(ii) *Influence of a three-stage MBBR (S=S1+S2+S3) configuration on the denitrification and on the biotransformation of micropollutants under pre-denitrifying conditions* (**Paper III**).

The three-stage configuration in continuous-flow operation effectively created a gradient in organic substrate loading and availability in staged pre-denitrifying MBBR, with the first stage (S1) exposed to highest organic carbon loading and the last stage (S3) to lowest loading. During batch experiment, the highest and lowest denitrification and micropollutants kinetics were found in the S1 and S3, respectively (up to 4-fold decrease for selected compounds). This suggested that biotransformation of pharmaceuticals is likely a cometabolic process, enhanced by the higher microbial activity (i.e., denitrification) in S1. Results from continuous-flow operation showed a slight improvement of denitrification in S (up to 20%) compared to single-stage configuration, but not significant differences in the removal of micropollutants.

(iii) *Impact of type and availability of additional carbon sources (methanol and ethanol) on the biotransformation of micropollutants in post-denitrifying MBBR* (**Paper V**).

Under post-denitrifying conditions, only four micropollutants were recalcitrant and most of the targeted compounds were classified as easily or moderately degradable ($0.2<k_{bio}<2$ L $g_{biomass}^{-1}$ d^{-1}). The methanol-dosed MBBR showed higher biotransformation kinetics than the ethanol-dosed MBBR for 19 over 23 micropollutants, equal for 10 compounds, while three compounds (i.e., sulfonamides) were biotransformed faster in the ethanol-dosed reactor. The higher denitrification rates exhibited by the ethanol-dosed MBBR during batch experiments likely influenced the biotransformation of the sulfonamides antibiotics. The removal of a number of compounds was associated to cometabolic biotransformation, with enhanced micropollutant biotransformation in the presence of organic carbon, and a cometabolic model was used to estimate biokinetics. The increased loading of both carbon sources in the two MBBRs during continuous-flow experiment, however, did not influence the removal of the targeted micropollutants. Longer HRT was thus suggested to possibly improve the removal of several micropollutants in full-scale post-denitrifying systems.

II. Fate processes of micropollutants in MBBR

Microbially-mediated transformation was the major process occurring in MBBR for the targeted compounds with negligible contribution of abiotic removal (<10%). Results from batch experiments revealed the possible influence of retransformation (e.g., from conjugated metabolites) and enantioselectivity on the removal of selected pharmaceuticals. In the nitrifying MBBR, sorption was significant only for eight out of the 23 targeted compounds in the nitrifying study, all positively charged at experimental pH. Sorption tendency increased with increasing of biofilm thickness, with the antibiotic macrolides showing the highest sorption coefficient in the thickest biofilm. Calibration of a diffusion-sorption model (and thus estimation of effective diffusivity coefficients *f*) showed higher diffusion limitation of micropollutants compared to other organic and inorganic compounds with lower molecular volume previously investigated in literature. Diffusion in biofilms of the sorptive micropollutants increased with increasing biofilm thickness, thus decreased with increased biofilm density.

III. Relationship between microbial structure and function

Microbial diversity (alpha-diversity) was investigated in MBBR1 and 2 by Illumina sequencing of the 16S rRNA gene. Increasing microbial diversity and evenness was observed with increasing biofilm thickness in nitrifying biofilms (MBBR1). Diversity-function relationships (Pearson's analysis) revealed a positive influence of biodiversity for most of the targeted compounds (60%). For few compounds (diclofenac and sulfonamides) biotransformation was positively associated to microbial activity rather than biodiversity. In the pre-denitrifying MBBR1, an increasing gradient of biodiversity in the three stages was observed after 200 days of operation of the MBBR staged configuration, with the highest microbial diversity in the biofilms adapted to lower loading of easily degradable carbon (S3). Diversity-function relationships revealed positive correlations ($p<0.05$) between k_{bio} of most of the detected pharmaceuticals with the specific denitrification rate and denitrifying functional genes, while negatively or no association with biodiversity was shown. The relationship between biodiversity and micropollutant biotransformation may depend on whether a biotransformation reaction is catalysed by a narrow (i.e., performed by few species) or broad processes. Under denitrifying conditions, micropollutants biotransformation that is possibly catalysed by broad metabolic pathway is not necessarily affected by biodiversity as it is not limited by the number of species performing the biotransformation. On the contrary, under nitrifying and aerobic heterotrophic conditions maximizing biodiversity may increase the likelihood of enriching the microbial community of the taxa performing specific functionality which likely catalyse the biotransformation of micropollutants.

Comparing the three investigated MBBR systems under anoxic and aerobic conditions, biotransformation of a number of micropollutants (~60% of the targeted compounds) was enhanced in the anoxic MBBR when dosing of methanol and ethanol as carbon sources. This suggests the positive impact of easily degradable organic carbon on the cometabolic biotransformation of several micropollutants. Finally, when compared with conventional activated sludge systems, MBBR was beneficial for the removal of compounds such as propranolol atenolol, citalopram, venlafaxine (under post-denitrifying conditions) and diclofenac (under aerobic conditions), known to be recalcitrant and (diclofenac) targeted in the European watch list.

10. Future research and perspectives

This research investigated on the fate in biofilm systems of micropollutants, intended as parent compound and/or conjugated forms excreted by human metabolism. In fact, existing directives focus on the parent compounds, rather than transformation products (1.1). However, as biotransformation pathways are characterized by the use of specific substrates or active enzymes (still unknown), the analytic identification of **transformation products** is becoming necessary of an in-depth elucidation of the biotransformation processes (Fischer and Majewsky, 2014). At this regards, effort on identification of transformation products of several organic micropollutants (Boix et al., 2016; Helbling et al., 2010a,b) has been recently made as well as on their associated risks in environmental compartments (Escher and Fenner, 2011). Given the higher abundance of transformation products in the environment compared to the parent compounds (Boxall et al., 2004), as well as their possible toxic action and synergistic effects (Neuwoehner et al., 2010), future research should also include transformation products when studying fate of micropollutants in WWTP.

This study suggested both through experimental and modelling investigations, a cometabolic biotransformation of several micropollutants. Criddle, (1993) stated that "*cometabolism results from the lack of specificity of enzymes and cofactors* ". However despite this statement, few studies focused on the **identification of enzymes** possibly responsible for micropollutant biotransformation (reviewed by Fischer and Majewsky, (2014)). Inhibition of AMO enzyme (Sathyamoorthy et al., 2013; Shi et al., 2004) suggested an involvement of this enzyme in biotransformation of specific micropollutants; however, additional research is needed to further identify enzyme under different redox condition and for broader spectrum of micropollutants.

As mentioned in 3.2.4, diversity-function relationship study is an upcoming field of research, given the emergence of next generation sequencing tools. In this research, diversity-function relationships were based on taxonomic biodiversity rather than functional biodiversity. It has been observed that **taxonomic and functional diversity** associate each other in wastewater (Johnson et al., 2015b), although inconsistent associations were found also in aquatic and soil microbial community, possibly due to functional redundancy (Fierer et al., 2012; Yergeau et al., 2012). Both taxonomic and functional diversity-functions relationships showed a positive relation in full-scale wastewater treatment plant (Johnson et al., 2015a), and stronger relationships based on

functional diversity than taxonomic diversity were found in activated sludge by dilution-to-extinction approach (Stadler et al., 2016). This suggests that in when based on taxonomic biodiversity, some of the associations may be underestimated. Using taxonomic diversity for assessing associations may be advantageous, as it is more affordable, less computational demanding and it could be a sufficient measure of biodiversity. However, this might not be true for highly redundant microbial community as taxonomic and functional diversity may not associated, as previously mentioned. Thus, studies which aim at understanding the important of biodiversity on ecological functions should be based on both taxonomic and functionality diversity.

Finally, this research aimed at understanding the mechanisms behind the removal of micropollutants in order to optimize biological systems in their removal and thus to limit the use of advanced treatment technologies. However, further effort should we put on the use **biological systems as polishing technology** at municipal WWTP. In fact, due to the limited concentration of carbon and nutrient in the effluent wastewater, it is difficult to sustain high microbial biomass and thus activity towards the removal of the more recalcitrant compounds. Further research is on-going to create an MBBR polishing technology which will allow supporting biomass activity even at the effluent stage of the WWTP, without further requirement of external carbon, nitrogen sources or of advanced physical treatment technology (e.g., ozone or activated carbon).

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Papers

I **Elena Torresi**, S. Jane Fowler, Fabio Polesel, Kai Bester, Henrik R. Andersen, Barth F. Smets, Benedek Gy. Plósz, Magnus Christensson. Biofilm thickness influences biodiversity in nitrifying MBBRs – Implications on micropollutant removal. Environmental Science & Technology (2016), 50, 9279-9288.

II **Elena Torresi**, Fabio Polesel, Kai Bester, Stefan Trapp, Barth F. Smets, Magnus Christensson, Henrik R. Andersen, Benedek Gy. Plósz. Sorption and diffusion of micropollutants in nitrifying MBBRs- The influence of biofilm thickness. Submitted.

III Fabio Polesel, **Elena Torresi**, Luca Loreggian, Monica Escolá Casas, Magnus Christensson, Kai Bester, Benedek Gy. Plósz. Elimination of pharmaceuticals in pre-denitrifying MBBR – Influence of organic substrate availability in single-stage and three-stage configurations. Submitted.

IV **Elena Torresi**, Arda Gulay, Fabio Polesel, Marlene M. Jensen, Magnus Christensson, Barth F. Smets, Benedek Gy. Plósz. Microbial composition and diversity of staged Moving Bed Biofilm Reactor for removal of micropollutants. Manuscript.

V **Elena Torresi**, Monica Escolá Casas, Fabio Polesel, Benedek Gy. Plósz, Magnus Christensson, Kai Bester. Impact of external carbon dose on the removal of micropollutants using methanol and ethanol in post-denitrifying Moving Bed Biofilm Reactors. Water Research (2017), 108, 95-105

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